ABSTRACT

CHEN, FANG. Monitoring and Manipulating Motions of Single Molecules/Nanoparticles. (Under the direction of Dr. Gufeng Wang).

This dissertation has two main research components: 1. the study of mass transport in confined environments; 2. the effort toward driving a molecular car on a solid surface.

Understanding mass transport processes, e.g., diffusion, migration, and adsorption/desorption in confined space is important not only to fundamental sciences but also to advanced applications. So far, they are poorly understood because of technical challenges: insufficient spatial and/or temporal resolutions. In this dissertation, we made efforts toward understanding molecular/particular dynamics in confined space by combining a recently developed super resolution technique, stimulated depletion emission microscopy (STED), with the high temporal resolution technique, fluorescence correlation spectroscopy (FCS).

We first explored the feasibility of using conventional FCS to study diffusion in a model confined space: cylindrical pores. Since there is no analytical solution to solve the autocorrelation function (ACF) in confined space, we simulated single particle diffusion in hundred-nanometer pores using Monte Carlo simulation. We found that confined 2D diffusion and unconfined 1D diffusion dynamics are separated in both intensity traces and autocorrelation functions, which gives a new opportunity to extract the axial diffusion coefficient in cylindrical pores. We then experimentally studied 45 nm particles diffusing in 300 nm alumina pores. The acquired axial diffusion coefficient is consistent with the expected value.

Conventional confocal FCS is insufficient to resolve lateral diffusion in confined space because of the diffraction limit in spatial resolution. To pave the way of using STED
microscopy to study the anisotropic diffusion in confined space, we theoretically investigated STED-FCS in cylindrical pores. It showed that by reducing the spatial resolution from 250 nm to 50 nm in STED microscopy, we would be able to determine both lateral and axial diffusion coefficients in hundred-nanometer pores in theory.

We then experimentally studied nanoparticles diffusing on membrane filters containing 200 nm polyethyleneglycol- or C18-modified pores. Using STED microscopy, we resolved for the first time how small particles are retained by the pores. Trapping by the pore entrances rather than adsorption is responsible for the retention. Further studies on C18-modified pores showed consistency in Gibbs free energy about the retention process.

In addition, in order to understand how nanoparticles interact with the surface when they are forced to be on, or very close to, the surface, we studied nanosecond rotation dynamics of gold nanorods with one end attached on the surface. We found that the nanorod motion is dominated by van der Waals interaction-induced immobilization rather Brownian rotational diffusion as previously thought. The actual rotation, during which the nanorod transits from one immobilized state to the other, slows down by ~ 50 times.

The second part of the research is the collaboration with Tour’s group in Rice University. The ultimate goal is to use light to drive a motorized nanocar at ambient conditions. To fulfill this goal, we first studied the moving kinetics of adamantane-wheeled nanocars on hydroxylated and PEG-modified surfaces using single molecule fluorescence microscopy. We found that nanocars’ diffusion slows down on solid surface over time, which is possibly caused by the increased hydrophobicity of the substrate surface due to the adsorbates from the air. A sticky-spots model was proposed to explain the observed slowing down.
To find out whether a light-activatable motor works when it is incorporated into a nanocar, we carefully designed a series of molecules containing a regular motor, a slow motor, a non-unidirectional motor, and no motor. We found that a fast unidirectional rotating motor enhanced the diffusion of the molecule in solution upon UV-illumination. Detailed analysis suggested that the unimolecular submersible nanomachine (USN) will give 9-nm step upon each motor actuation. This is the first nanomachine that gives mechanical motion at small molecular scale.
Monitoring and Manipulating Motions of Single Molecules/Nanoparticles

by
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Fang Chen was born in Lanzhou, Gansu Province in China. His hometown was one of the important cities along the ancient Silk Road and now is famous for beef noodle soup all over the country. In Lanzhou, eating up a large bowl of beef noodle soup covered with spicy chili sauce in morning is a good start of the daytime, especially in chilly winters. No matter how hard the life is, a bowl of beef noodle soup is enough to get rid of fear, worry, and sadness. That is the lifestyle of people in Lanzhou: facing tough life with optimism and enjoying every moment in life. This is one of the most important spirits in his soul given by his hometown. After graduating from the high school, he was enrolled in Chemistry Department at University of Science and Technology of China (USTC), one of the top universities in China. Coming across so many smart and outstanding people sometimes makes him feeling stressful. But he is lucky and happy to have the chance to make friends with those people and learnt a lot from them. Inspired by his research experience in USTC, he attended Department of Chemistry at North Carolina State University and started his research career with the direction under Dr. Gufeng Wang. In here, he has had a very meaningful 5 years, which is an invaluable collection in his memory.
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Chapter 1 Background and Significance

1.1 Molecular Transport in Nanoconfined Environment

1.1.1 Motivation and Goals

Understanding interfacial phenomena between a liquid and a solid substrate is important to not only fundamental sciences but also emerging applications in renewable energy and human health. Especially of interests are nanoconfined environments, e.g., voids, pores, planar, channels, etc., where molecules are confined by interface(s) within a length scale of nanometers. Understanding mass transport such as sorption and diffusion in nanoconfined environments is an important step for many applications such as separations, micro to nanofabricated tools, heterogeneous catalysis, controlled drug release, enzymatic reactions, and trans-cell membrane biological processes, etc.\textsuperscript{1-8}

Molecular transport processes, e.g., diffusion, migration, and adsorption/desorption, in nanoconfined environments can be significantly different from those in the bulk solution. For example, extraordinarily slow diffusion and transport have been reported in porous materials with nanometer pore sizes in multiple studies.\textsuperscript{9-18} However, there is no consensus of abnormally slow molecular diffusion in confined space on two essential questions: first, how far the anomaly extends to the bulk. A number of studies consistently show that the interface only affects water molecules that are in direct contact with, or within a couple of water molecule layers from the substrate (~1 nm length scale).\textsuperscript{10,19} These observations suggest that the interfacial effects are short-ranged. Thus, it is reasonable that slow diffusion happens in small pores with a dimension on the 1 nm length scale.\textsuperscript{13,16-18} However, there are a number of reports from multiple research groups showing that slow diffusion occurs in nanopores with a
diameter on the scale of or larger than 20 nm. These macroscopic phenomena suggest that the interfacial effect may be long-ranged, e.g., as far as several tens to hundreds of nanometers into the solution. Second, what causes this anomaly in nanoconfined environment? One answer to this question is that the macroscopic slow diffusion is caused by chromatographic process – microscopically frequent adsorption/desorption events on the interface. However, there are multiple studies suggesting that other unknown factors also contribute to the slow diffusion significantly. For example, a recent study shows that in polyethylene glycol (PEG)-coated 100 nm polycarbonate nanopores, the diffusion of protein molecules becomes 2 orders of magnitude faster than that in untreated nanopores. Unexpectedly, the diffusion is still 2 orders of magnitude slower than that in the bulk solution. This observation suggests that adsorption is indeed one of the major reasons but there are other factors that may contribute to the slow diffusion in nanoconfined environments.

These questions are unanswered because of technical challenges: insufficient spatial and temporal resolutions. Wide-field optical microscopy, e.g., epi-fluorescence or total internal reflection fluorescence (TIRF) microscopy is a sensitive technique that allows us to make observations at single molecule level. However, wide-field optical microscopy usually has a low time resolution (~30 frames per second) that is limited by the camera readout speed. Confocal fluorescence microscopy has been proved to be a powerful tool that has single molecule sensitivity and microsecond to nanosecond time resolution. Confocal fluorescence correlation spectroscopy (FCS) is frequently used to measure dynamics of molecular processes in complicated systems, e.g. translational and rotational diffusion coefficients, chemical rate constants, association and dissociation constants, and photodynamic in vitro as well as in vivo. It becomes a routine tool to study fluid-liquid and solid-liquid interface. However,
the spatial resolution of confocal fluorescence microscopy is limited to ~ 200 nm by the optical diffraction. It is incomplete to study molecular dynamics in confined spaces, which has a similar or smaller dimension to the diffraction limit.

The ultimate goal of this dissertation is to take the advantage of recently developed super-resolution technique, stimulated depletion emission microscopy (STED), to study molecular dynamics in confined space. Anodized aluminum oxide membrane filters is chosen as a model system because they contain uniform straight pores aligned vertically to the membrane plane; little tortuosity contributes to the molecular processes in the confined space. Along this line, we conducted a series of studies in both theory and experiments toward the understanding how particle/molecule transport in nanoconfined space.

1.1.2 Investigating Axial Diffusion in Cylindrical Pores Using Confocal Fluorescence Correlation Spectroscopy

In Chapter 2, we first explored the feasibility of using the high temporal resolution technique - confocal fluorescence correlation spectroscopy - to study small nanoparticle diffusion in hundred-nanometer-sized cylindrical pores. It has been recognized that FCS measurement based on conventional confocal fluorescence microscopy can NOT be applied in confined space.25,26

To overcome this problem, we modeled single particle diffusion in tube-like confined 3D space aligned parallel to the confocal optical axis. We showed that two diffusion dynamics can be observed in both original intensity traces and the autocorrelation functions (ACFs): the confined two-dimensional (2D) lateral diffusion and the unconfined one-dimensional (1D) axial diffusion. The separation of the axial and confined lateral diffusion dynamics provides
an opportunity to study diffusions in different dimensions separately. We further experimentally studied 45 nm carboxylated polystyrene particles diffusing in 300 nm alumina pores. The experimental data showed consistency with the simulation. To extract the accurate axial diffusion coefficient, we found that a 1D diffusion model with a Lorentzian axial collection profile needs to be used to analyze the experimental ACFs. The diffusion of the 45 nm nanoparticles in polyethyleneglycol-passivated 300 nm pores slowed down by a factor of ~2, which can be satisfactorily explained by hydrodynamic frictions.

This study shows that confocal fluorescence microscopy is a powerful technique and has sufficient time resolution to resolve the axial diffusion coefficient in the model system: cylindrical pores. However, due to the limited spatial resolution, it is in sufficient to resolve the lateral diffusion coefficient in the hundred nanometer cylindrical pores.

1.1.3 Feasibility of using STED-FCS to study Anisotropic Diffusion in Hundred-nanometer Pores

In pores, there are at least two conditions that shall make molecular diffusion different than that in the bulk solution. The first is hydrodynamic friction. The axial diffusion will experience a large friction because of the enforced boundary, which leads to a larger frictional force due to a larger velocity gradient. In addition, the hindered mobility will be a function of the solute size and the distance from the solute to the surface. Second, because of the direction of the molecule-surface interactions, the axial and lateral motions will be affected differently. Both shall point to anisotropic diffusion in pores. However, there is little experimental data in the literature showing how molecules/particles diffuse in nano- to micrometer sized pores because of technical challenges. Knowing how particles diffuse in pores will give a whole picture
of mass transport in confined environments and help understand the molecule/particle surface interactions.

In Chapter 2, we discussed using confocal – based FCS to separate lateral and axial diffusion and extract axial diffusion coefficient accurately. However, because of diffraction limited spatial resolution of confocal fluorescence microscopy, the lateral diffusion coefficient cannot be resolved.\textsuperscript{26,36} Recently developed stimulated emission depletion microscopy (STED) microscopy can provide a spatial resolution as small as \textasciitilde 20 nm.\textsuperscript{37} By tuning the depletion beam power, differently sized collection spot, or the spatial resolution, can be tuned. Compared to other super resolution techniques, the biggest advantage of STED microscopy is that it offers a very short pixel integration time, or an excellent time resolution. STED microscopy has been combined with FCS technique to study diffusion dynamics on bilipid membranes.\textsuperscript{37,38}

In Chapter 3, we explored the feasibility of combing STED and FCS to study anisotropic diffusion in cylindrical pores in theory. We performed systematic Monte Carlo simulations of particle diffusion in cylindrical pores. By varying the beam size, beam position, pore size, pore shape, etc., we theoretically studied how to extract lateral and axial diffusion coefficients in cylindrical pores.

1.1.4 Particle Retention on Membrane Surface

We then used STED-FCS to study single particle diffusing in the presence of porous materials. How molecules/particles enter the pores involves many interactions such as electrostatic, hydrodynamic, and van der Waals interactions, etc. To fully understand and manipulate particle transport, different effects have been discussed both in theory and in experiments.\textsuperscript{39-45} For example, Lin studied how micron-sized particles deposit on a microsieve,
and observed that particles are preferred to be captured on the edge of the pores before they enter the pores from experiments.\textsuperscript{44} Similarly, Belfort and colleagues evaluated particle trajectories during cross-flow microfiltration and concluded that small particles tend to deposit on the membrane surface.\textsuperscript{46-48} As a contrast, Kim et al. theoretically studied the effect of Brownian forces and concluded that with the help of Brownian forces, particles could overcome electrostatic repulsion and enters pores directly in low filtration flow.\textsuperscript{43} Bowen et al. concluded that electrostatic interactions and hydrodynamic interactions could control the rejection of the charged spherical particles. They quantitatively investigated how entrance shape, zeta-potential of particle and membrane, and electrolyte concentration, etc. affect that the force balance and found the critical velocity to avoid adsorption during separation process.\textsuperscript{40-42} It is unclear that the adsorption of the particle on the pore entrance affects favorably or unfavorably the entering of the particle to the pore.

So far, there is little evidence of how small particles are retained by the pores. Current techniques do not have sufficient spatial or temporal resolution to resolve this complicated process.\textsuperscript{49-51,52,53} For example, confocal fluorescence correlation spectroscopy (FCS), is a powerful technique that provides high temporal resolutions up to several microseconds. However, due to the diffraction limit of light microscopy, the applications of confocal-FCS for systems smaller than 200 nm are limited.\textsuperscript{36-26} It is impossible to differentiate confined diffusion or adsorption within an area that is smaller than 200 nm. Therefore, super resolution techniques are required.

In Chapter 4, we studied 45 nm particles diffusion near membrane filter surface containing 200 nm pores using STED and confocal-FCS. The high spatial and temporal resolutions provided by STED-FCS helps us distinguish confined diffusion from adsorption and confirms
that particles are microscopically diffusion in lateral direction while it is trapped by the pore entrance. Such trapping is also valid even when the pore wall is modified with C18, which significantly retains the particle in the pore for longer time. With qualitative understanding of particle motion near the pore surface, a 2D diffusion with trapping model has been proposed to quantitatively analyze the ACFs data. The relationship of Gibbs free energy and the fraction of C18 modification on the pore wall has been investigated.\(^{54}\) Our study shows an active trapping model for particles being retained by pores.

1.1.5 Nanorod Rotation when Forced to Attach on the Surface

In Chapters 2-4, we studied the molecular diffusion in confined space but the nanoparticles still have the freedom to move in all directions. In Chapter 5, we forced the gold nanorods to attach onto the surface and studied their motions using the nanosecond time resolution resonance light scattering technique.

Based on earlier slow wide-field optical microscopic studies, gold nanorods with one end attached to the surface were thought to continuously rotate following their initial attachment.\(^ {55}\) However, in Chapter 5, the nanosecond time resolution study revealed that the apparent continuous rotation actually consists of numerous fast, intermittent transitions or rotations between a small numbers of weakly immobilized states, with the particle resting at the immobilized states for the most of the time. The actual rotation, during which the anchored nanorod transits from one immobilized state to the other, happens at a 1 ms time scale, ~50 times slower than in the bulk solution. The high temporal resolution is of vital importance to disclose how attach nanorods move because all the fast dynamics are averaged using a slow imaging technique.
This study shows that when a nanoparticle is forced to be on or near the surface within a distance <1 nm, the nanorod rotation will slow down significantly, and its motion will be dominated by the van der Waals interaction-induced immobilization.

1.2 Techniques Used in This Study

1.2.1 Fluorescence Correlation Spectroscopy (FCS)

Fluorescence correlation spectroscopy (FCS) is a powerful tool used to measure rates of molecular processes in the detection volume, e.g. translational and rotational diffusion coefficients, chemical rate constants, association and dissociation constants, and photodynamics in vitro as well as in vivo. Advantages of FCS include: 1. Steady state measurement. Kinetic parameters are measured in equilibrium, which is especially important for biological samples. 2. Spectroscopic selectivity and high sensitivity. Spectroscopic selectivity allows the discrimination of compounds that are not in the detection spectral range. Thus, the biological processes or the chemical reaction kinetics of interest can be detected by the small fluorescence changes. The high sensitivity of the fluorescence detection makes FCS suitable for characterizing systems with very low concentrations up to single molecule levels, for example, in live biological cells. 3. High spatial resolution and conservation of materials. In confocal fluorescence microscopy, FCS detection volume is in the femtoliter regime. Therefore, different regions in biological cells or in the sample chambers can be measured separately. In addition, it is a very economic technique due to the low amount requirement of samples and non-destructive nature of measurements. 4. Wide time range. The low limit of the time range can reach nanosecond, which can be used in the study of rotational diffusion,
photophysics of the excited states, etc. The up limit is only set by the stability of the optical system. A characteristic time of 1-10 s can be frequently found in the literature.

The principle of FCS is to obtain kinetic rate constants through the analysis of the dynamic fluorescence intensity fluctuations in a small detection volume. That is, any process that shall introduce the fluorescence intensity change will be reflected in the autocorrelation function (ACF) of the fluorescence intensity trace. Correspondingly, the kinetic constants can be recovered from the ACF. To increase the signal to noise ratio, a confocal fluorescence microscope is frequently used to minimize the background (Figure 1.1).

The simplest case is the fluorescence fluctuation caused by diffusion. When fluorophores diffuse in and out of the detection volume, they give fluorescence signals. The fluctuating signal is collected by the microscope objective and imaged on a fast and sensitive detector, for example, an avalanche photo diode (APD). The fluorescent intensity trace, $F(t)$, is recorded and analyzed. The corresponding autocorrelation function is:

$$G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2}$$  \hspace{1cm} (1.1)

Where $\langle \rangle$ denotes the time average, $\delta F(t) = F(t) - \langle F(t) \rangle$. $\tau$ is the lag time. For a system where the only fluctuation is caused by Brownian motion of the molecules, the ACF follows:

$$G(\tau) = \frac{1}{N} \left( 1 + \frac{\tau}{\tau_D} \right)^{-1} \left( 1 + \frac{\tau}{S^2 \tau_D} \right)^{-1/2}$$ \hspace{1cm} (1.2)
where $N$ is the average number of fluorescent species in the observation volume, $S$ is the ratio of axial to radial dimensions of $V_{obs}$. $\tau_D$ is the characteristic diffusion time, which is related to their diffusion coefficient $D$:

$$\tau_D = \frac{r_{xy}^2}{4D}$$  \hspace{1cm} (1.3)

where $r_{xy}$ and $r_z$ are the distances from the center to where the emission intensity drops to $1/e^2$ in the lateral and axial directions of the probe volume, respectively.

In practice, translational diffusion is not the only cause of the intensity fluctuation. In solutions containing rod-like nanoparticles, rotational motion also contributes to the signal variation.\textsuperscript{59,60} Using the statistical fluorescence correlation spectroscopic (FCS) method, one can extract both translational and rotational diffusion coefficients. Specifically, the total autocorrelation function (ACF) $G(\tau)$, can be described as the product of two components:

$$G(\tau) = G_{Tr}(\tau)G_{Rot}(\tau).$$  \hspace{1cm} (1.4)

$G_{Tr}$ follows the three dimensional (3D) translational diffusion model discussed above in equation 1-4. $G_{Rot}$ follows the free rotational model:\textsuperscript{59-61}

$$G_{Rot}(\tau) = 1 + A \times \exp(-\tau / \tau_{Rot})$$  \hspace{1cm} (1.5)

where $A$ is a constant that collectively accounts for rod anisotropy and experimental geometry of the detection system; $\tau_{Rot}$ is the characteristic rotational diffusion time:

$$\tau_{Rot} = \frac{1}{6D_{Rot}}$$  \hspace{1cm} (1.6)

The rotational diffusion coefficient $D_{Rot}$ is defined as the mean square angular displacement divided by 2 times of the observation interval $t$. 


In the last two decades, FCS has been widely applied in colloid and interface science, e.g., measuring the hydrodynamic radii of molecules and particles, characterizing their interactions, studying diffusion in inhomogeneous media such as polymer, gels, and porous structures, quantifying the formation kinetics and the size of micelles in surfactants and block copolymers, etc. In this study, we take the advantage of the fast time resolution of FCS in our studies of diffusion in confined environments and fast gold nanorod rotation on surface.

1.2.2 Super Resolution Microscopy

As mentioned earlier, confocal fluorescence microscopy has a diffraction limited spatial resolution of ~ 200 nm, which is not sufficient to resolve diffusion in confined environments with a dimension of tens to hundreds of nanometers. To overcome the diffraction limit problem of optical microscopy, several techniques have been introduced to achieve sub-diffraction limited resolution in far-field optical microscopy.63-68

In principle, to overcome the diffraction limit, spatially and/or temporally modulating the transition between two molecular states of a fluorophore are necessary. According to this, two approaches are usually used. The first one relies on the stochastic single molecule switching followed by super localization, i.e. localizing the fluorophore with a precision below the diffraction limit. This type of technique includes stochastic optical reconstruction microscopy (STORM)69/ photoactivated localization microscopy (PALM)66. The basic idea is that through the multiple rounds of stochastic turning on few fluorophores and super localizing their positions, an image with fine structures smaller than the diffraction limit can be obtained. Due to the multiple turn-on and turn-off cycles, the temporal resolution is usually very low.
The other approach depends on illumination light pattern to spatially address the modulation. One early technique to achieve super resolution is structured illumination microscopy (SIM). SIM uses a spatially modulated illumination pattern to form interference fringes. Through complicated computation using multiple raw images, a super resolution image with half of the normal spatial resolution can be obtained. The resolution of SIM is limited because of the modulated light pattern is also diffraction limited. The other frequently used technique is stimulated emission depletion microscopy (STED). Instead of adding extra excitation patterns, STED used another light beam (depletion beam) to suppress fluorescence emission at designated locations. STED, together with the single molecule localization-based microscopy (STORM/PALM), won Nobel Prize in Chemistry in 2014.

The basic principle of STED is using of a doughnut-shape depletion beam to overlap with the excitation spot to turn off the excited molecules at the outer ring of the excitation spot, achieving a collection spot smaller than the diffraction limit. The turn off process is called stimulated emission depletion. In normal fluorescence microscopy, fluorophore is excited from ground state \( S_0 \) to single excited state \( S_1 \) (Figure 1.2). After initial internal conversion and/or vibrational relaxation, fluorescence will be spontaneously emitted and the fluorophore goes back to the ground state \( S_0 \). In addition to the spontaneous emission, an incident photon can also cause the emission of the excited state, namely, stimulated emission. The efficiency of stimulated emission process is unnoticeable under low illumination power. However, when enough numbers of photons are incident to excited fluorophores, the stimulated emission overwhelms the natural emission process, reaching an excited fluorophore depleted state. STED microscopy takes advantage of this process to achieve the super resolution. Apparently, the quality of the donut beam is of vital importance to the spatial resolution. To form a donut
shape, usually a high-aperture vortex phase plate is used. For example, 0-2\pi vortex phase plane (VPP) is designed in a way that electric field of the transmitted beam around the optical axis changes continuously from 0 to 2\pi. The electric field cancels out completely on the axis, yielding a donut-shaped beam.

Compared to STORM/PALM and SIM, STEP microscopy has the advantage of both high spatial resolution and high temporal resolution for data collection at a single pixel (~\mu s). It has been shown that STED imaging of fixed cells could achieve a spatial resolution of ~20 nm.\textsuperscript{72,73} Thus, it is ideal for our study of the mass transport in confined environment within a dimension < 200 nm. We constructed a CW-STED microscope, which has a spatial resolution of ~70 nm,\textsuperscript{74} to study the diffusion and adsorption in cylindrical pores using STED-FCS.\textsuperscript{37,38,75}

1.2.3 Monte Carlo Simulation

In our study, the mass transport dynamics without a closed-form expression is encountered. In fact, when arbitrary boundary conditions representing the confined environments are enforced, there is generally no analytical solution available to describe the diffusion dynamics. To have a better understanding of our experimental data, we also performed Monte Carlo simulations in the same system.

Monte Carlo (MC) experiments refer to a class of computational algorithms that are used to obtain numerical results. The basic idea is repeating random sampling and calculating the probabilities just as practical playing and recording. They are mainly used in simulating systems with numbers of degrees of freedom in three usual directions: optimization, numerical integration, and generating samples from probability distribution. The basic process follows four steps: 1. Define possible input domain. 2. Generate random inputs from probability
distribution. 3. Determine computation inputs. 4. Aggregate the results. With the help of Monte Carlo method, the real dynamics without a closed-form expression can be simulated. In our studies, to understand how nanoconfinement affects particle diffusion, we used Monte Carlo simulation to generate particle diffusion trajectories in pores. Elastic or inelastic reflection can be assumed based on the boundary conditions. With sufficient steps generated and reasonable parameters used, the practical situation could be replicated and studied in theory, which helps us make approximations and revise existing models to fit the experimental data. The details of how to simulate Brownian diffusion in confined space is discussed in Chapter 2 and Chapter 3. The typical Matlab programs are shown in Appendix I.

1.3 Monitoring and Controlling Single Molecule Nanomachines

1.3.1 Overview of Nanomachines

In the talk entitled “There’s Plenty of Room at the Bottom”, Dr. Richard Feynman first expressed ideas and concepts of nanotechnology, where scientists would be able to manipulate and control individual atoms and molecules. Inspired by his talk, as well as the request for miniaturized machinery in science and technological fields currently, a variety of organic molecules that can generate controlled mechanical motions, e.g., motors, switches, shuttles, turnstiles, gears, gyroscopes, and elevators, etc., have been produced as members of nanomachines.76-83,84,85

The term machine is defined as a tool containing parts that could use input energy to perform intended actions. Similarly, the term nanomachine is mainly used to name the machines at the molecular scale, where a stimulus triggers the controlled motion of a molecule or a submolecular component relative to another that can potentially result in a net task being
performed. Before mankind could design and synthesize artificial nanomachines, natural molecular machines have already played vital roles in biological processes. They participate in almost every major biological process and assist in accomplishing a variety of chemical and mechanical tasks such as transporting cargo in cells, powering the movement of organisms, separating strands of DNAs, and synthesizing proteins, etc. Take the most familiar enzyme as an example, transmembrane ATPases hydrolyze ATPs and release energy to generate directional transport of charged and polar species across the cell membrane.

Although natural biomolecular machines could perform a series of complex and intricate functions which are at much higher level than current artificial nanomachines, they are restricted in applications ex vivo because of the requirement of physiological environments. That is, they are only applicable in aqueous environments with specific buffer conditions, temperatures, pHs, etc. In addition, due to their complex nature and poorly understood working mechanism, it is challenging to modify or redesign these biological nanomachines for specific needs. Thus, there is a need to design and synthesize wholly synthetic systems which could not only tolerate a more diverse range of conditions, but also provide simpler functions in the nanoscopic world.

1.3.2 Motivation and Goals

This is a collaboration work with Dr. James Tour’s group in Rice University. The ultimate goal is to use light to drive a motorized nanocar at ambient conditions. “Nanocars” refers to molecules resembling cars in the macroscopic world, which has chassis, axles, and wheels. They were first proposed and synthesized by Dr. James Tour’s group and have been demonstrated to “roll” on a solid surface. The most recent work includes incorporating a
light activatable motor into the car and the attempts of driving the nanocar using light. A series of efforts have been made toward this goal.

1.3.3 Review of Nanocar Structures and Developments

So far, a series of nanocar molecules that could translate on solid surface have been synthesized (Figure 1.3). They consist of three basic molecular mechanical parts: a chassis made of fused aromatic rings or oligo(phenylene ethynylene)s (OPEs), alkynyl axles, and wheels made of rotatable molecules like fullerene. Several different nanocar structures have been reported and their motilities on solid substrates have been characterized. For example, differently shaped nanocars such as, 3-wheeled, 4-wheeled, 6-wheeled, and nanocars with an angled chassis, etc., have been designed and studied. Nanocars with a “Z shaped” oligo(phenylene ethynylene) (OPE) chassis has been synthesized. The better flexibility of the OPE chassis combined with increased numbers of alkoxy units (OC\textsubscript{10}H\textsubscript{21}) brings more flexibility to the chassis and gives the nanocar degrees of freedom orthogonal to the surface plane. These studies extend our understanding of how chassis shapes affect nanocar movement patterns.

The wheel-surface interaction is another important factor that affects nanocar mobility on surface. Three generations of wheels: carbon-based C\textsubscript{60} wheels, p-carborane wheels, and adamantane wheels, respectively, were applied to nanocars (Figure 1.4). As the first generation wheels, C\textsubscript{60} fullerene has been proven to be a good choice for rolling motions observed using scanning tunneling microscopy (STM). However, because of the electronic structure of fullerenes, rapid energy transfer from a motor to the fullerenes occurs and limits their further development when incorporating a motor to the car. In the second generation, \(p-\)
carboranes are used to substitute C₆₀ because of its spherical shape and non-conjugated molecular structure. However, these cars show low mobility on a hydrophilic substrate, e.g., glass, because strong hydrogen bonds formed between the wheels and the surface.⁹⁸ Therefore, the third generation, adamantane wheels are currently being used.⁹¹

1.3.4 Monitoring Nanocar Movement at Ambient Conditions

One of the goals of developing these car-like molecular machines is to drive them at ambient conditions. To achieve this goal, the first important step is to understand how the molecular structure and the molecule-surface interaction affect the nanocars’ mobility at an air-solid interface. In Chapter 6, we studied the moving kinetics of adamantane-wheeled nanocars on differently modified surfaces. Single molecule fluorescence imaging was used to track the nanocar movement so that the molecules were minimally perturbed. We found that on freshly cleaned, hydroxylated glass surfaces, nanocars with hydrophobic adamantane wheels can diffuse with a relatively large diffusion coefficient of 7.6 ×10⁻¹⁶ m²/s. Both the number of moving molecules and the mobility of the moving molecules decreased over time when the sample was exposed in the air. Similar declinations in movement were observed on a polyethyleneglycol (PEG)-modified glass surface, but the declination rate was lowered. The slowing of molecular surface diffusion is correlated to the hydrophobicity of the surface and is likely caused by the adsorption of hydrophobic molecules from the air. A proposed sticky-spots model explains the decreasing apparent diffusion coefficient of the hydrophobic-wheeled nanocars.
1.3.5 Light Driven Motors and Unimolecular Submersible Nanomachines.

The ultimate goal is to drive the nanocars using light. We have synthesized these motorized nanocars and they have shown some indication that their motion will respond to light illumination. However, due to the complexity of the molecule-surface as well as the dye-motor interactions, we have not achieved convincing results that we are “driving” the nanocars.

To systematically approach this problem, we changed the working media from a heterogeneous solid surface to a solution system. In Chapter 7, we studied the light-actuation of unimolecular submersible nanomachines (USNs) bearing light-driven motors in solution system. Through careful design of control molecules with no motor and with a slow motor, we found using single molecule fluorescence correlation spectroscopy that only the molecules with fast rotating speed (MHz range) show an enhancement in diffusion by 26% when the motor is fully activated by UV light. This suggests that the USN molecules give ~9-nm steps upon each motor actuation. A non-unidirectional rotating motor also results in a smaller, 10%, increase in diffusion. The results give new insight into how photoactivatable motors at molecular size respond to activation and possibly, how to better design molecular motors in the future.

1.3.6 Further Efforts

With our conducted studies, we have better understand of nanocar mobility on surface, however to fulfill the ultimate goal, more works still need to be done to fundamentally understand nanocar systems. For example, how temperature is going to affect nanocar diffusion on surface. How motorized nanocar could be activated on surface by UV light. This requires better designs which could effectively convert external energy to molecular kinetics
energy. In addition, it is also important to set up experimental conditions used for testing motorized nanocar mobility on surface, especially for UV light activation. The study of USN diffusion enhancement in solution by UV light provides important information of how to use motor molecule to expedite nanomachines. With its unique structures and further understanding of its properties, we might apply them in practical applications in future. In Chapter 8, we briefly discussed what we are going to do in future.
FIGURES

Figure 1.1 Principle of Fluorescence Correlation Spectroscopy.\textsuperscript{57}
Figure 1.2 Simplified Jablonski diagram of the molecular states involved in STED imaging. After initial excitation from the $S_0$ to the $S_1$ state and vibrational relaxation, fluorophores can emit fluorescence photons. Alternatively, the fluorescence can be silenced by stimulated emission into a higher vibrational state of the electronic ground state $S_0$. The red-shifted stimulated emission light can be spectrally separated during detection. The bleaching pathways indicated in the diagram are addressed in the text.\cite{71}
Figure 1.3 A series of nanocars designed.⁹⁷
Figure 1.4 Models of C\textsubscript{60}, p-carborane, and adamantane wheels (from left to right).\textsuperscript{90}
Chapter 2 Investigating Axial Diffusion in Cylindrical Pores Using Confocal Single-Particle Fluorescence Correlation Spectroscopy

2.1 Introduction

Understanding interfacial phenomena between a liquid and a solid substrate is important to not only fundamental sciences but also emerging applications in renewable energy and human health. Especially of interests are nanoconfined environments, e.g., voids, pores, planar channels, etc., where molecules are confined by interface(s) within a length scale of nanometers. Understanding mass transport such as sorption and diffusion in nanoconfined environments is an important step for advanced applications in separations, micro to nanofabricated tools, heterogeneous catalysis, controlled drug release, enzymatic reactions, and trans-cell membrane biological processes, etc.\textsuperscript{1-8}

Mass transport processes in nanoconfined environments can be significantly different from those in the bulk solution. For example, slow diffusion and transport have been reported in porous materials with nanometer-sized pores in multiple studies.\textsuperscript{9-14,16-18,99,100} Some observations suggest that slow diffusion can occur in pores larger than 20 nm, in which most of the water molecules (> 90\%) are no longer affected by the interface.\textsuperscript{9-12,14,99,100} The reasons for the slow diffusion are under investigation. While adsorption on the pore wall is deemed as one of the main reasons, there are studies suggesting that other unknown factors also contribute to the slow diffusion significantly.\textsuperscript{10,14}

Most of previous studies are based on bulk measurements, where the gross diffusion/migration speed is measured for a dye passing through a porous material with a known thickness. The mass transport of individual molecules/small particles, e.g., adsorption and
translational movements are not disclosed in the bulk experiments. Especially in larger pores (~100 nm), how axial and lateral diffusion is affected by the interface is largely unknown.

In this study, we investigated the feasibility of using confocal fluorescence correlation spectroscopy to study diffusion of small nanoparticles (i.e. 45 nm carboxylated polystyrene nanoparticles) in hundred-nanometer sized cylindrical alumina pores (pore diameter 300 nm). Anodized aluminum oxide membrane filters were introduced as a model system because they contain uniform straight pores aligned vertically to the membrane plane and tortuosity contributes little to the diffusion. Confocal fluorescence correlation spectroscopy (FCS) was employed because of its high spatial resolution and sub-millisecond time resolution even in highly scattering medium. The optical axis of the confocal microscope was aligned parallel to the long axis of the cylindrical pores. Thus, the particle diffusion was confined in the $x$-$y$ plane but unconfined in the $z$-direction. Using a home-built confocal fluorescence microscope with single molecule sensitivity, we were able to track the fluorescence intensity traces of individual particles diffusing in individual pores.

It has been recognized that FCS measurement in confined space can NOT be analyzed with conventional bulk diffusion models. For example, Gennerich and Schild derived confined diffusion model of molecules in long neuron cell dendrites lying perpendicular to the optical axis.\textsuperscript{25} Sanguigno et al. studied molecular diffusion in thin, flat microchannels with rectangular cross-sections.\textsuperscript{26} To the best of our knowledge, confined diffusion in a vertical tube with a considerable diameter (several hundred nm) has not been systematically studied. While the mathematical models in the literature provide valuable information about molecular diffusion in confined space, they require ideal experimental geometries, e.g., the laser beam aligned to the center of the confined space. Practically, such requirements are not trivial, and artifacts
may be introduced. Thus, a systematic approach is needed to comprehend, and extract diffusion information from, the original data and corresponding statistical analysis.

In this study, we first performed a Monte Carlo simulation to systematically investigate the relationship between the confined diffusion as a function of the pore size, the pore geometry, and beam positions in the absence of inelastic particle-pore interaction. The results showed that in hundred-nanometer sized pores, two types of dynamics: axial diffusion and confined lateral diffusion can be observed in both original fluorescence intensity traces and statistical FCS analysis. This provides an opportunity to study diffusion in different dimensions separately. Although there is no analytical solution for the statistical FCS model for this kind of diffusion, the original data and the autocorrelation function (ACF) provide rich information about diffusion in different dimensions. Especially, accurate axial diffusion coefficient can be extracted by averaging fast lateral diffusion using a proper binning time.

Then, based on the knowledge acquired from the simulations, we experimentally studied 45 nm particles diffusing in 300 nm alumina pores: unmodified and PEG-modified. The experimentally collected fluorescence intensity traces showed consistent characteristics with the simulated data. We further found that to extract the correct axial diffusion coefficient, a Lorentzian axial collection profile-based 1D diffusion model should be used to analyze the autocorrelation functions. The diffusion of 45 nm particles slowed down by ~2 times in PEG modified pores, which can be satisfactorily explained by hydrodynamic frictions.
2.2 Experimental

2.2.1 Chemicals and Materials

Carboxylated polystyrene 45 nm fluorescent nanoparticles were acquired from Thermo/Duke Scientific. The absorption and fluorescence emission maxima of aqueous suspension of the particles were 505 and 515 nm, respectively. The original aqueous solution containing 1-2% particles (w/v) was diluted in 25 mM phosphate buffer to a concentration of $1.0\sim4.0 \times 10^{12}$ particle/mL. Alumina membrane filters with a thickness of 60 µm were purchased from Whatman International (Maidstone, U.K.). The vendor reported pore size was 200 nm. However, we found that the pores are asymmetric at either side of the membrane filter, with pore size at one side consistent with the reported value while the other side significantly larger. The pore size on the second side varies from batch to batch (batches are identified by the lot number). In this study, we used the larger pores, which are measured to be $300 \pm 50$ nm. Since the membrane filter is 60 µm in thickness, the pore size can be viewed as uniform within the objective focal plane.

2.2.2 PEG Modification of the Pore Surface

To chemically modify the surfaces, the alumina membrane filters were boiled first in 30% hydrogen peroxide and then in deionized water for 15 min each. Then, they were dried in purified air flow before further modification. Hydroxy(polyethyleneoxy) propyltriethoxysilane (PEG-silane) (Gelest, MW:350-750) was dissolved in 20 mL of anhydrous toluene to make a 1% PEG solution. The membrane filters were incubated in the modification solution for 1 h, followed by rinsing with toluene, acetone and DI water. All membrane filters were soaked in 25 mM phosphate buffer (pH ~10) for 0.5 hours before being exposed to the particle
suspension. At this pH and buffer concentration, both the particle and the membrane surface were negatively charged. No significant adsorption was observed.

2.2.3 Confocal Microscopy

The confocal microscope was modified from a home-built continuous-wave stimulated emission depletion microscope (Figure 2.5A). The construction of the microscope was discussed in detail previously. Briefly, The 488 nm excitation light was provided by an air-cooled, wavelength-tunable Ar⁺ laser (Model 35-LAP-431-240, CVI/Melles Griot, CA) and expanded to slightly overfill the back aperture of the focusing microscope objective (CFI Plan Apo VC 100X oil, NA=1.4, Nikon Inc.) after being circularly polarized using a quarter-wave plate (ACWP-400-700-06-4, CVI/Melles Griot, CA). Fluorescence signal was collected using the same objective and imaged onto a 50 µm, multimode fiberoptic (M16L01, Thorlabs, NJ) serving as the confocal pinhole. The signals were detected by an avalanche photo diode (SPCM-AQRH-15-FC, Perkin Elmer), and counted by a computer board.

The PSF of the confocal system was characterized by measuring immobilized 45 nm particles on cover glass surface. In the FCS measurement, the membrane filter (60 µm thickness) was sandwiched between a glass slide and a standard coverglass. The thickness of the solution layer between the membrane filter and the coverglass was ~ 5 µm. The laser beam was focused into the membrane filter by 3 µm from the coverglass side (Figure 2.5B). The total distance from the surface of the coverglass to the focal plane was ~ 8 µm. There is minimal distortion of the PSF in this collection geometry, which is consistent with the literature reports. The acquired data were analyzed using MATLAB and Origin (OriginLab, Northhampton, MA).
2.2.4 SEM

The alumina porous membrane and polystyrene nanoparticles were imaged with a JEOL JSM-6400F field emission scanning electron microscope. For polystyrene nanoparticles, 10 μL of concentrated aqueous suspension of polystyrene nanoparticles was dispensed on the coverslip and air dried for 30 minutes at ambient temperature. Before SEM measurement, all samples were vacuum dried and then a thin layer of Pd-Au (<5 nm) was coated to make the sample conductive.

2.2.5 Random Walk Simulation in Pore

In the simulation, the lateral diffusion and axial diffusion were treated separately because they are independent of each other. The axial and lateral particle trajectories were then combined to create the 3D diffusion trajectory. The diffusion coefficient was assumed to be identical for lateral and axial diffusion. The theoretical diffusion coefficient of a particle was estimated from Stokes-Einstein equation:

\[
D = \frac{k_B T}{6\pi \eta R}
\]

(2.1)

where \( k_B \) is Boltzmann constant; \( T \) is the temperature in Kelvin; \( \eta \) is the viscosity of water (0.001002 Pa·s); \( R \) is the hydrodynamic radius of the particle. With eq 2.1, 45 nm nanoparticles have a diffusion coefficient of \( 9.7 \times 10^{-12} \text{ m}^2/\text{s} \).

For the axial diffusion, the particle stepped away from its original position with a step size \( L \) after each dwell time \( \Delta t \). The step size \( L \) for each step was randomly generated using Matlab program so that it has a Gaussian distribution with a standard deviation of:
\[ \sigma^2 = 2nD\Delta t \] (2.2)

where \( n \) is the number of dimensions. One particle was assumed to be in or near the probe volume. In order to prevent the particle from diffusing away, two boundaries, 60 \( \mu m \) away from either side of the center of the probe volume, were enforced so that the particle would be reflected back toward the probe volume when it surpasses the boundary.

For the lateral diffusion, since the diffusion in the \( xy \) plane is confined, boundaries were set representing the pore wall. Elastic reflections were assumed in the simulation when the particle meets the pore wall. Simulations were performed differently for rectangular pores and circular pores. For a rectangular (or square) pore, since the reflection on the \( x \)-boundary does not change its \( y \) coordinate, and similarly for the reflection on the \( y \)-boundary, the random walk in the \( x \)- and \( y \)-directions can be generated independently using eq 2.2 and combined together to create a 2D trajectory. If after 1 step, the particle falls out of the pore wall, reflection(s) will be carried out accordingly.

For circularly pores, two random variables were generated to describe the 2D random walk: a normally distributed step size \( L \) with a standard deviation \( \sigma \) as given in eq 2.2, and a uniformly distributed angle \( \phi \) between 0 and \( \pi \). After each step, the new location of the particle was:

\[ x(t+1) = x(t) + L(t) \cdot \cos\{\phi(t)\} \] (2.3)

and

\[ y(t+1) = y(t) + L(t) \cdot \sin\{\phi(t)\} \] (2.4)
When the pore was assumed to be circular, a circular boundary was enforced so that the particle would be bounced back to be within the boundary. Specifically, if after one step, the particle position was outside the boundary, a crossing point between the particle trajectory and the boundary was calculated and the particle would be reflected elastically by the tangent of the circle at the crossing point. If the particle was still outside the circle, another reflection would be executed. We carefully selected the dwell time $\Delta t$ so that the average step size is smaller than the smallest pore size used in the simulation. The simulation program allowed 5 times of reflection. If after 5 reflections, the particle was still outside the circle, we deemed that this was an event with very small probability. Current pair of $L$ and $\phi$ was discarded and a new pair was generated. When the total diffusion time was provided long enough, the particle would show up everywhere in the circle with a uniform distribution. Figure 2.6A shows an example of 2D lateral diffusion of particle in a circular nanopore.

In calculating the fluorescence intensity traces and corresponding ACFs, the 3D diffusion trajectories were generated by combining the lateral and axial trajectories. The Gaussian beam was then assumed to park at the designated location in the pore. Figure 2.6B shows an example that the laser beam was parked in the center of the pore. The particle was treated as a point object in the intensity calculation. In comparing the pore size effect, we used the same set of axial diffusion trajectory but different lateral trajectories representing different pore sizes. The reason for using the same set of axial diffusion trajectory is that we can directly compare the fluorescence intensity variation caused by the different extent of the lateral confinement. In comparing the effect of the laser parking spot, the same set of 3D trajectory was used but the laser spot was varied.
Note that the signal-to-noise ratio of the ACF is dependent on the total number of diffusion events. In order that the ACF is smooth at the long time end, the trajectory must be sufficiently long so there are sufficient axial diffusion events. However, since in small pores, a very small time interval has to be employed so that the particle lateral step is smaller than the pore size. It is not realistic to simulate long trajectories. The simulated ACFs are usually noisy at the long time end. To ensure that the dynamics reflected in the ACFs are accurate, we tested multiple simulation runs using different time intervals. Figure 2.6C shows one example that multiple simulation runs using different time intervals are practically identical except noises at the long time end. Both sets of ACFs show two different dynamics no matter a 10-µs or a 50-µs time interval was used in the simulation.

2.2.6 Fluorescence Intensity Traces and Diffusion model in the 3D Space.

The autocorrelation function (ACF) is defined as:

$$ G(\tau) = \frac{\langle \delta I(t) \delta I(t + \tau) \rangle}{\langle I \rangle^2} $$

(2.5)

where $\tau$ is the correlation time; $\delta$ is the operator indicating the quantity deviation from its mean; $I$ is the fluorescence intensity. Given that the fluorescence emission is induced by a Gaussian beam and collected through a confocal pinhole, the detectable fluorescence emission intensity distribution, or the collection profile, is described by a 3D Gaussian function:\textsuperscript{105}

$$ I = I_0 \exp \left( -\frac{2(x^2 + y^2)}{r_{xy}^2} \right) \exp \left( -\frac{2z^2}{r_z^2} \right) $$

(2.6)

where $I_0$ is the maximum emission intensity when the emitter is placed at the center of the beam; the beam waists $r_{xy}$ and $r_z$ are the distances from the center to where the emission
intensity drops to $1/e^2$ in the lateral and axial directions, respectively. In the simulation, $r_{xy}$ and $r_z$ were assumed to be 250 nm and 1200 nm, respectively, which were similar to the experimentally obtained values.

Under this condition, the translational diffusion in unbound three dimensional (3D) space is: 

$$G(\tau) = \frac{1}{<N>} \frac{1}{1 + \tau/\tau_{diff}} \frac{1}{\sqrt{1 + \tau / S^2 \tau_{diff}}} \tag{2.7}$$

where $<N>$ is the average number of emitters in the probe volume; $S$ is the aspect ratio of the collection volume $r_z/r_{xy}$; $\tau_{diff}$ is the characteristic diffusion time assuming the emitter has an isotropic diffusion coefficient $D$:

$$\tau_{diff} = \frac{r_{xy}^2}{4D} \tag{2.8}$$

When diffusions in each direction are independent from each other, the autocorrelation function can be expressed separately:

$$G_{xyz}(\tau) = \frac{1}{<N>} g_x(\tau)g_y(\tau)g_z(\tau) \tag{2.9}$$

where

$$g_k(\tau) = \frac{1}{\sqrt{1 + \tau / S_k^2 \tau_{diff}}}, \quad (S_k = 1 \text{ for } k = x \text{ or } y; \ S_k = S \text{ for } k = z) \tag{2.10}$$
2.3 Results and Discussion

2.3.1 Two Diffusion Dynamics in Hundred-nanometer Sized Circular Pores

To model particle diffusion in nanopores, we first assumed that the pores have a circular shape and the laser beam is focused on the center of the pore. We simulated the fluorescence intensity traces for particle diffusing in differently sized pores. Figures 2.1A-C show 3 selected such traces assuming the $D$ to be $9.7 \times 10^{-12}$ m$^2$/s (corresponding to 45 nm particles). The ACFs calculated from the simulated fluorescence intensity traces are shown in Figure 2.1D. Starting from the simulated diffusion in 100 nm pores, the ACF decay becomes faster as the pore size increases. In fact, the ACF decay resembles that of 1D axial diffusion for narrow pores and 3D axial diffusion for wide pores. For clarity, the theoretical ACFs for 1D axial, 2D lateral, and 3D bulk diffusion, calculated from eqs 2.9 and 2.10, are plotted in Figure 2.7. The ACF of the 1D axial diffusion decays much slower than that of the 2D lateral diffusion. It is not because axial diffusion is slower but that the collection volume is an elongated ellipsoid, for which it takes much longer time for a particle to diffuse across the probe volume axially than laterally. The ACF of the 3D diffusion is very similar to that of the 2D lateral diffusion because the lateral diffusion is sampled more that the 3D diffusion ACF is dominated by the 2D lateral diffusion.

For particles diffusing in narrow pores, the ACF decay half time, which is defined as the time that ACF decays to half of its maximum, is ~ 100 ms, consistent with the theoretical ACF for the 1D axial diffusion in Figure 2.7. This shows that in narrow pores, the diffusion is dominated by the axial diffusion. As the pore size becomes larger, we can clearly identify a second diffusion dynamics with an ACF decay half time of a few ms in addition to the 100 ms
slow diffusion dynamics. The fast diffusion dynamics is a reflection of the particle’s confined lateral diffusion. When the pore size becomes even larger, the 2D lateral diffusion becomes more significant and eventually dominates the ACF. In sufficiently large pores, e.g., 1.5 \( \mu \)m in diameter, the ACF is no longer distinguishable as that of the 3D diffusion in the bulk solution.

The simultaneous detection of the axial and confined lateral diffusion dynamics can also be readily recognized from the original simulated fluorescence intensity traces. Figures 2.1A-C show selected portions of the simulated fluorescence intensity traces. (For an example of a whole trace that lasts for 400 s with a pixel time of 500 \( \mu \)s, see Figure 2.8). Figure 2.1A shows the trace of a point object diffusing in an infinitely small tube (radius equals to 0). The fluorescence intensity trace contains many peaks with widths on the order of magnitude of 100 ms. The peaks are originated from particles diffusing across the axial direction of the detection volume, whose time scale is \( \sim 100 \) ms.

When the pore size increases to 400 nm, many sharp peaks emerge on top of those \( \sim 100 \) ms broad peaks (Figure 2.1B). This is because the particles now have the freedom to move laterally. As they move in the laser beam with different intensities, the fluorescence intensity fluctuates. Since the time for a particle diffusing across the pore laterally is much shorter, these lateral diffusion-induced peaks are much sharper, with a time of a few ms. When the pore size becomes as large as 800 nm, the fluorescence intensity trace is dominated by these sharp, lateral diffusion-induced peaks (Figure 2.1C), similar to that of the free diffusion in the bulk.

2.3.2 Laser Beam off Pore Center

Above discussion is based on perfect alignment, where the laser is focused on the center of the pore. A practical issue is that it is nearly impossible to achieve perfect alignment. In
addition, the anodisc membranes used in the experiment contain many parallel pores. The particles may not diffuse in the pore that the laser beam is focused on. To make the simulation representing experimental conditions, we simulated the fluorescence intensity traces when the laser beam is off the pore center and even out of the pores.

Figure 2.2A shows the ACFs for simulated particle \((D = 9.7 \times 10^{-12} \text{ m}^2/\text{s})\) diffusing in 200 nm pores when the laser beam is off the pore center to different extent. Since the pore size is relatively small, we see mainly the axial diffusion dynamics in the ACF when the laser beam is in the center. Interestingly, when the laser beam is off, the fast dynamics becomes more obvious. This trend can be clearly seen in the simulated fluorescence traces in Figures 2.2B-D, which show the laser beam is on the center, off center by 50 nm, and 100 nm (on the pore wall), respectively. Again, we see sharp peaks representing the confined 2D diffusion on top of the broader peaks representing axial diffusion. The further off the laser beam is, the more pronounced the sharp spikes are in Figures 2.2B-D. The reason is that in the FCS experiments, the fluorescence intensity fluctuation is detected when the particle is diffusing around. The Gaussian-distributed laser beam has a flatter profile in the center as compared to the flanks of the beam. The result is that the particle shows small fluorescence intensity fluctuation when the laser beam is centered and large intensity variation when the laser beam is off. Thus, the fast lateral diffusion becomes more significant in both the fluorescence trace and the ACF when the laser beam is off center. However, the axial diffusion dynamics is not affected (Figure 2.2A).

When the laser beam is completely off the pore that the particle is diffusing in, the fluorescence intensity drops significantly to a level close to the background noise (Figure
We deem that particles diffusing in other pores have negligible contribution to the ACF in the laser targeted pore.

2.3.3 Pores with Other Geometries Show Two Diffusion Dynamics

The ideal alumina membranes have uniform, circular nanopores aligned in a 2D hexagonal lattice. However, the actual pore shapes in commercial anodic membranes are not uniform, including irregular shapes resembling all sorts of polygons.

To better understand the diffusion in actual pores, we also tested pores with other simple geometries. For example, we show in Figure 2.9 the simulated ACFs of particle diffusion in square pores. Similarly, the ACFs for intermediate pores also show 2 diffusion dynamics: one for the confined 2D diffusion, and the other for the unconfined axial diffusion. In fact, we found that the ACFs of particle diffusion in different pore geometries all give similar 1D to 3D transition trend as the pore size is varied. A more relevant parameter to characterize the 1D to 3D transition is possibly the pore area, for which the ACFs are similar when the pore areas are nearly identical, regardless of the pore geometry.

2.3.4 Extracting Axial Diffusion Coefficient using FCS Analysis

The simultaneous detection of the axial and confined lateral diffusions yields new information and also brings new challenges. First, for diffusion in medium-sized pores (pore diameter between 200 nm and 1000 nm), two dynamics are observed. Conventional 3D or 1D diffusion model only produces 1 transition in the ACF curve and can no longer be used in the ACF analysis. Forced fitting using conventional 1D or 3D diffusion model only results in poor fitting and an erroneous $D$. 

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Second, with the caution in mind, we can still estimate the lateral or axial diffusion coefficient in a medium sized pore, given certain conditions are met. For example, when the pore size is \(~8\) times larger than the beam size, the lateral confinement effect can be ignored\(^{25}\) and the diffusion can be analyzed directly using the 3D diffusion model. However, considering the pore sizes, this condition can only be met when super-resolution optical microscopy, e.g., stimulated emission depletion (STED) microscopy, with a beam size of \(10\sim50\) nm, is used. In the other limit when the pore size is sufficiently small, only the axial diffusion dynamics is visible thus the axial diffusion coefficient can be recovered. In a medium sized pore (200 nm - \(1\) µm), the lateral and axial diffusion dynamics are well separated in the ACF. However, using a proper temporal resolution, we can average the fast confined lateral diffusion and focus only on the slow axial diffusion. For example, Figure 2.10 shows the simulated ACFs in differently sized pores binned to 20 ms resolution. Only the axial diffusion dynamics is visible. The decay speed for ACFs in 100-1000 nm pores are practically identical to that of 1D diffusion, which shows that the axial diffusion coefficient can be accurately estimated.

2.3.5 Experimental Collection Profile and Implications on the FCS Analysis

With the knowledge acquired from above random walk simulation, we studied particle diffusion in the bulk solution and in 300 nm pores using our home-constructed confocal fluorescence microscope. The microscope is sufficiently sensitive to detect single diffusing FITC dyes (Figure 2.11).

To analyze the ACF and obtain the diffusion coefficient, we need to know the confocal microscope collection profile in the xy- and z-directions. We scanned immobilized 45 nm fluorescent nanoparticles in the xy plane and in the axial direction (Figure 2.12). In the focal
plane, the beam profile can be satisfactorily fitted with a Gaussian distribution function with a full width at half maximum (FWHM) of 355 nm. Equivalently, the Gaussian beam waist $r_{xy}$ is 300 nm because $FWHM = r_z \sqrt{2 \ln 2}$ (Figure 2.12A).

However, in the axial direction, the collection profile is better fitted with a Lorentz distribution function than a Gaussian distribution function (Figure 2.12B vs. 2.12C). The Lorentz distribution function used was:

$$I = I_0 \frac{1}{\pi} \left[ \gamma \left( Z - Z_0 \right) + \frac{\gamma^2}{(Z - Z_0)^2 + \gamma^2} \right]$$  \hspace{1cm} (2.11)

where $\gamma$ denotes the half of the FWHM. The fitting gives a FWHM of 1115 nm.

Note that the Lorentzian collection profile in the z-direction is consistent with the literature reports but inconsistent with the assumption in FCS ACF analysis, where a Gaussian distribution function is assumed. We point out here that using a Gaussian function to approximate the actual z-profile is reasonable in conventional 3D diffusion model in the bulk solution because the lateral diffusion dominates the experimentally collected ACFs. However, when we analyze the 1D axial diffusion, using a Gaussian axial profile as the substitute will introduce >100% error as will be discussed below.

For example, Figure 2.13A shows the 45 nm fluorescent nanoparticles diffusion in aqueous solution (laser power 2 $\mu$W). When using the conventional 3D diffusion model and the experimentally measured collection profiles to fit the ACF, we obtain a good fitting (Figure 2.13B) with a diffusion coefficient of $6.6 \pm 0.5 \times 10^{-12}$ m$^2$/s from 10 measurements, which is consistent with the theoretical value ($9.7 \times 10^{-12}$ m$^2$/s). This shows that in the bulk diffusion model, the ACF is dominated by $r_{xy}$ while $r_z$ has little effect (see Figure 2.7).\textsuperscript{105}
However, in the 1D diffusion, $r_z$ has a huge impact on the recovered diffusion coefficient thus a correct diffusion model is of vital importance. To show this, we used again Monte Carlo simulation to generate fluorescence intensity traces for a particle freely diffusing in the 1D space with the collection profile following Lorentzian distribution. The particle diffusion coefficient was assumed to be $9.7 \times 10^{-12}$ m$^2$/s and the FWHM of the Lorentzian distribution was 900 nm. Assuming we use a Gaussian distribution function to approximate the Lorentzian profile, the axial collection profile $r_z$ can be estimated to be 764 nm from the FWHM of the Lorentzian distribution. Forced fitting using the conventional 1D diffusion model with a Gaussian axial collection profile is shown in Figure 2.14: (1) the fitting was poor; and (2) the recovered diffusion coefficient was $4.6 \times 10^{-12}$ m$^2$/s, which is 110% different from the expected value ($9.7 \times 10^{-12}$ m$^2$/s). The huge deviation indicates that using conventional 1D diffusion model with a Gaussian collection profile is no longer appropriate in this situation.

To overcome this problem, we used the Lorentzian collection profile-based 1D diffusion model derived by Blom to fit the simulated ACF:

\[
g(\tau) = A \cdot \exp\left(\frac{\gamma^2}{(D\tau)}\right)(\pi D\tau)^{1/2} \frac{1 - \text{erf}[\gamma/(D\tau)^{1/2}]}{D\tau/\gamma}
\]  

(2.12)

where \(\text{erf}\) is the error function; \(A\) is the pre-exponential factor reflecting the molecule number and the decay caused by lateral diffusion. Using this equation, a better fitting could be achieved (Figure 2.14) and the recovered diffusion coefficient was $1.1 \times 10^{-11}$ m$^2$/s, closer to the theoretical value. Thus, eq 2.12 was used in the analysis of experimentally collected data.
2.3.6 Experimental Particle Diffusion in 300 nm Porous Membrane

We then studied 45 nm particles diffusing in unmodified and PEG modified 300 nm alumina cylindrical pores. The anodisc membranes have a very high background, possibly caused by impurities in the inorganic material, and also the strong scattering by the porous media. The loss of signal due to the scattering media is also enormous. To guarantee that (1) we are observing single particles inside the pore, and (2) that the particle diffusion is not affected by the opening of the pore, we set the focal plane 3.0 \( \mu \)m below the membrane surface. In the experiment, 45 nm particles were selected so that they give enough signals in the pores and can enter 300 nm pores in the membrane by free diffusion. Under our experimental condition, the Debye length was \( \sim 1 \) nm. Thus, the thin electrical double layer approximation can be applied.

We successfully captured multiple fluorescence traces at different locations in several different anodisc membranes with a temporal resolution of 500 \( \mu \)s or 1.0 ms (75 for unmodified pores and 22 for PEG-modified pores). The laser power was 20 \( \mu \)W. Figure 2.3A shows an example of recorded fluorescence intensity traces lasting for 65.5 s with an integration time of 1.0 ms. Typically, we see two types of signal peaks: sharp spikes in ms time range on top of broader peaks that span several hundred ms (for expanded view, see Figures 2.3B). The two diffusion dynamics are also reflected in the corresponding ACFs (Figure 2.3D). The 2 diffusion dynamics are common for all collected traces (e.g., Figure 2.15 shows 35 arbitrarily selected ACFs).

These observations are consistent with the simulations. Thus, the slow dynamics is assigned to the axial diffusion of the particles in the pores and the fast dynamics is assigned to the
confined lateral diffusion. It should be noted that this fast dynamics is not caused by the noise of the background. Figure 2.3E shows a typical intensity trace of the background in the absence of the particles. The intensity fluctuation level was ~ 20 counts/0.5 ms as compared to 200 counts/0.5 ms in the presence of the particle. The ACF of the background shows that the decay of noise goes to zero in the first few data points (Figure 2.3F).

As discussed earlier, with a time resolution of ~20 ms, the fast confined lateral diffusion dynamics will be averaged while the ACF curves still contains sufficient information to recover axial diffusion coefficient. To study the axial diffusion of 45 nm particles in 300 nm pores, we binned the data to have a 20 ms resolution and fit the ACFs using the Lorentzian collection profile-based 1D diffusion model. Figure 2.4A shows a typical fitting of the particle in unmodified pores. To reduce the amount of calculations in eq 2.12, we reduced the data density by using data points that are equally spaced on a log scale. The fitting shows that the data matches the model at both the short and the long time ends very well. Through studying all 22 traces for the PEG-modified pores, the axial diffusion coefficient was $3.1 \pm 0.6 \times 10^{-12}$ m²/s, ~2.1 times smaller than that measured in the bulk measurement (Figure 2.4B). Similarly, in unmodified pores, the diffusion coefficient from the 75 traces was $2.1 \pm 0.8 \times 10^{-12}$ m²/s, ~3.1 times slower than that in the bulk solution (Figure 2.4C).

What causes the slow axial diffusion? In PEG-modified pores, if we considered the hydrodynamic effect, the hindered diffusivity $\xi$ could be estimated from:

$$\xi = \frac{D_{\text{pore}}}{D_{\text{bulk}}} = (1 - \lambda)^2 K^{-1} (\lambda, 0)$$  \hspace{1cm} (2.13)
where $D$ is diffusion coefficient; $\lambda$ denotes the particle/pore size ratio. When $\lambda < 0.4$, the enhanced friction $K$ has a numerical value:

$$K^{-1}(\lambda, 0) \approx 1 - 2.1044\lambda + 2.089\lambda^3 - 0.948\lambda^5$$  \hspace{1cm} (2.14)

Based on eqs 2.13 and 2.14, the 45 nm particles diffusing in 300 nm pores will be slowed down by a factor of 2.0 times, which is consistent with our experimental observation.

For unmodified pores, the particle is further slowed down, ~3 times slower than in the bulk solution. The additional slowing down could be caused by the particle – pore wall interactions. Millisecond time scale adsorptions have been reported in porous media in chromatography and deemed to be responsible for the peak tailing in high performance liquid chromatography (HPLC).\textsuperscript{52,110,111} However, we carefully examined all traces and found that millisecond time scale adsorption events, characterized by long-lasting, constant fluorescence intensities, are generally not observed (Figure 2.4C). Thus, we can conclude that long time adsorption events (ms-time scale) are not the dominant reason in our case. However, we were unable to exclude the possibility of short time (microsecond time scale) adsorption, or transient trapping of the particles by the pore wall. How the particles are slowed down in unmodified pores needs to be further investigated.

\textbf{2.4 Conclusions}

In this study, we systematically investigated small particles diffusing in membrane filters containing cylindrical nanopores using confocal fluorescence correlation spectroscopy. Our modeling shows that for particle diffusion in cylindrical pores, the autocorrelation function for conventional 3D or 1D model is no longer applicable. In medium sized pores (few hundred nanometers), we can see two diffusion dynamics: the axial diffusion and the confined lateral
diffusion, in both original intensity traces and in statistical FCS analysis. The separation of axial and confined lateral diffusion dynamics provides an opportunity to study diffusions in different dimensions separately. Further, we found that to extract the accurate axial diffusion coefficient, 1D diffusion model with a Lorentzian axial collection profile needs to be used. We then experimentally studied 45 nm carboxylated polystyrene particles diffusing in 300 nm alumina pores. In PEG modified pores, the diffusion of the 45 nm nanoparticles slowed down by ~ 2 times, which can be satisfactorily explained by hydrodynamic frictions.
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Figures

Figure 2.1 Modeling autocorrelation functions of point objects diffusion in circular pores with different sizes. (A)-(C) Portions of simulated fluorescence intensity traces in cylindrical pores with different sizes. (A) The point object is assumed to diffuse in an infinitely narrow pore; (B) in a 400 nm pore; (C) in a 800 nm pore. The diffusion coefficient of the point object was assumed to be $9.7 \times 10^{-12}$ m$^2$/s (45 nm particle). The laser beam is assumed to be focused on the center of the pore. The temporal resolution was 0.5 ms. Normally distributed errors with a standard deviation of 5% of the maximum intensity value was imposed at each time pixel. (D) Autocorrelation functions calculated from simulated intensity traces.
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Figure 2.15 35 arbitrarily selected ACFs of 45 nm particles diffusion in unmodified 300 nm pores collected with a temporal resolution of 1.0 ms.
Chapter 3 Theoretically Investigating the Feasibility of Probing Anisotropic Diffusion in Nanopores using Stimulated Emission Depletion Microscopy-Based Fluorescence Correlation Spectroscopy

3.1 Introduction

Micro- to nano-porous materials are of great interest because they are relevant to a variety of industrial processes. In the pores, the fluids are under extreme conditions of non-uniformity as a result of the molecule-interface and molecule-molecule interactions. Mass transport in pores involves many processes and is poorly understood. For example, slow diffusion and non-Brownian motion of solute molecules in nanometer- to micrometer-sized pores have been reported but a satisfactory interpretation is still missing. In addition, many questions are unresolved. For example, how are molecules distributed in the pores/cavities? Do they spend more time in the center or close to the wall? Do the molecules have preference in diffusing directions, i.e., anisotropic diffusion? Understanding mass transport e.g. diffusion, migration and adsorption/desorption, in these porous materials is important to both fundamental sciences and search frontiers, e.g. drug delivery, enzyme reactions, heterogeneous catalysis and chromatography, etc.

In the pores, there are at least two conditions that shall make molecular diffusion different than that in the bulk solution. The first is hydrodynamic friction. The axial diffusion will experience a large friction because of the enforced boundary, which leads to a larger frictional force due to a larger velocity gradient. In addition, the hindered mobility will be a function of the solute size and the distance from the solute to the surface. Second, because of the direction of the molecule-surface interactions, the axial and lateral motions will be affected
differently. Both point to anisotropic diffusion in pores. However, there is little knowledge in the literature about how molecules diffuse in nano-to micro-pores because of technical challenges.\textsuperscript{29-35} Knowing how particles diffuse in pores will give a whole picture of mass transport in confined environments and help understand the molecule/particle surface interactions.

In this chapter, we theoretically explore the possibility of resolving anisotropic diffusion in cylindrical pores as a model system. Cylindrical pores can be found in anodic alumina membrane filters.\textsuperscript{121} In our earlier study, we investigated the feasibility of using confocal fluorescence correlation spectroscopy to study diffusion of small nanoparticles (i.e. 45 nm carboxylated polystyrene nanoparticles) in hundred-nanometer sized cylindrical alumina pores (pore diameter 300 nm) both in theory and in experiments.\textsuperscript{122} We found that in hundred-nanometer sized pores, confined lateral diffusion and axial diffusion are separated in both original fluorescence intensity traces and statistical autocorrelation functions (ACF). It gives us an opportunity to extract the axial diffusion coefficient, which was further confirmed in experiments of 45 nm particles diffusing in 300 nm alumina pores. However, because of diffraction limited spatial resolution of confocal fluorescence microscopy, the lateral diffusion coefficient cannot be resolved.\textsuperscript{26,36}

Recently developed stimulated emission depletion microscopy (STED) microscopy can provide a spatial resolution as small as 20 nm.\textsuperscript{37} STED microscopy is similar to confocal fluorescence microscopy in experiments. The difference is that it imposes an additional donut-shaped depletion beam on the diffraction-limited excitation spot. The depletion beam depletes excited fluorophores at the outer-rim of the excitation spot, leading to a much smaller fluorescence spot. The resolution is thus improved. By tuning the depletion beam power,
different sized collection spot, or the spatial resolution, can be tuned. Compared to other super resolution techniques, the biggest advantage is that it offers a very short pixel integration time, or an excellent time resolution. STED microscopy has been combined with FCS technique to study diffusion dynamics on bilipid membranes.37,38,38

In this study, we explore the feasibility of combing STED and FCS to study anisotropic diffusion in cylindrical pores in theory. We performed systematic Monte Carlo simulations of particle diffusion in cylindrical pores. With the understanding how beam size, beam position, pore size, pore shape, we theoretically provide the approach to extract lateral and axial diffusion coefficients in cylindrical pores.

3.2 Experimental

3.2.1 Monte Carlo Simulation

The trajectories of particle diffusion in pores were generated the same way as reported in Chapter 2. Briefly, the lateral and axial diffusion were separately simulated because they are independent to each other. 3D diffusion trajectory was obtained by combining lateral and axial trajectories. The diffusion coefficient used for isotropic diffusion was estimated from Stokes-Einstein equation. For 45 nm particles, the diffusion coefficient of $9.7 \times 10^{-12}$ m$^2$/s was used.

For isotropic diffusion, both lateral and axial diffusion coefficients used the same value; for anisotropic diffusion, different Ds were applied. One particle was assumed to be in the probe volume. The axial diffusion of the particle was allowed to freely diffuse but with boundaries of 72 μm away from either side of the center of the probe volume to prevent it diffusing away. The lateral diffusing area is confined by pore sizes. To keep the particle in the pore, reflections were applied when the particle stepped out of the boundary. 5-times of elastic reflections were
allowed. If after 5 reflections, the particle was still outside the circle, we deemed that this was an event with very small probability and was discarded. Another step was generated instead. Because of the confinement size is small, a small time interval of 10-µs or a 5-µs were used to ensure particle lateral step is smaller than pore size. To obtain the ACF with good signal-to-noise ratio, large total numbers of diffusion events is required. Therefore, a total steps of 6 million were generated for each direction. Although more steps will lead to smoother ACFs, it is not realistic and necessary.

3.2.2 Fluorescence Intensity Trace

Given that the emitted fluorescence signal of each point is decided by the intensity distribution of laser beam, the fluorescence intensity trace could be calculated. From our studies in Chapter 2, the practical intensity profile could be described as a 2D Gaussian function laterally and a Lorentzian function axially:

\[
I = I_0 \exp \left( \frac{-2(x^2 + y^2)}{r_{xy}^2} \right) \left[ \frac{1}{\pi} \frac{\gamma}{(Z - Z_0)^2 + \gamma^2} \right]
\]

where \(I_0\) is the maximum emission intensity when the emitter is placed at the center of the beam in the focal plane; the beam waists \(r_{xy}\) and \(r_z\) are the distances from the center to where the emission intensity drops to \(1/e^2\) in the lateral and axial directions, respectively. In the simulation, \(\gamma\) denotes the half of the FWHM, which is assumed to be 450 nm to be consistent with our measurements; \(r_{xy}\) is assumed to be 250 nm, 100 nm, 50 nm, and 20 nm, respectively, simulating the spatial resolution of STED microscopy; \(x, y,\) and \(z\) are positions of the particle, which was treated as a point object in Monte Carlo simulations.
In comparing the pore size effect, we used the same set of axial diffusion trajectory but different lateral trajectories representing different pore sizes. The reason for using the same set of axial diffusion trajectory is that we can directly compare the fluorescence intensity variation caused by the different extent of the lateral confinement. In comparing the effect of the laser parking spot and the beam size effect, the same set of 3D trajectory was used but the laser spot position was varied. In all simulations, a shot noise-limited error, which is ~5% of the maximum intensity of the trace, was added.

3.2.3 Diffusion Models in the 3D Space

The autocorrelation function (ACF) based on the 3D Gaussian beam is defined as:

\[ G(\tau) = \frac{\langle \delta I(t)\delta I(t+\tau) \rangle}{\langle I \rangle^2} \]  \hspace{1cm} (3.2)

where \( \tau \) is the correlation time; \( \langle > \) is the average operator; \( \delta \) means the deviation from its mean; \( I \) is the fluorescence intensity. When diffusions in each direction are independent from each other, the autocorrelation function can be expressed separately:

\[ G_{xyz}(\tau) = \frac{1}{\langle N \rangle} g_x(\tau) g_y(\tau) g_z(\tau) \]  \hspace{1cm} (3.3)

where

\[ g_k(\tau) = \frac{1}{\sqrt{1 + \tau / S_k^2 \tau_{diff}}} \], \( (S_k = 1 \text{ for } k = x \text{ or } y; S_k = S \text{ for } k = z) \]  \hspace{1cm} (3.4)

where \( \langle N \rangle \) is the average number of emitters in the probe volume; \( S \) is the aspect ratio of the collection volume \( r_z/r_{xy} \); \( \tau_{diff} \) is the characteristic diffusion time assuming the emitter has an isotropic diffusion coefficient \( D \):
For the free 3D diffusion model, the ACF is dominated by the lateral diffusion.\textsuperscript{105}

When particles diffuse only in the axial direction, the ACF should be analyzed using:\textsuperscript{108}

\[
g_z(\tau) = \frac{A \cdot \exp(\gamma^2/(D\tau))(\pi D \tau)^{1/2} \left(1 - \text{erf}\left[\frac{\gamma}{\sqrt{D\tau}}\right]\right)}{D \tau / \gamma}
\]

where \(\text{erf}\) is the error function; \(A\) is the pre-exponential factor reflecting the molecule number and the decay caused by lateral diffusion. Note above equations can only be applied to free diffusion situations where particles are not confined by a boundary, or the confined volume is much larger than the confocal probe volume (i.e., a ratio > 10).

### 3.3 Results and Discussion

#### 3.3.1 Effect of Beam Size on Observed Diffusion Dynamics

As discussed in Chapter 2, when a 45 nm particle diffuse in a pore with infinitely small size, the diffusion could be considered as pure axial diffusion. When pore size increases but is still much smaller that the diffraction limited laser beam, although lateral movement occurs, the intensity change in lateral direction is too small to be reflected in both intensity trace and ACFs. With the pore size continually increasing to the medium size (i.e., similar to the beam waist), the lateral diffusion will be observed. Thus, two dynamics are shown in both intensity traces and in ACFs: one is the fast dynamic with a half decay time of several ms; the other is the slow dynamic with a decay time of several hundred ms. These two dynamics have been carefully studied and assigned to the confined lateral diffusion and the unconfined axial diffusion, respectively. Finally, when pore size is large enough (>750 nm in radius), the diffusion can be
modeled as free 3D diffusion. These conclusions were obtained from the confocal-FCS analysis when with the beam size is ~250 nm. The study shows that lateral and axial diffusions separate in medium sized pores, from which we can determine the axial diffusion coefficient. However, because of the limited confocal spatial resolution: (1) there is no analytical solution for confined lateral diffusion. Therefore, we could not obtain lateral diffusion coefficient using FCS analysis. (2). Only a limited range of pore sizes are applicable, from which we can measure the axial diffusion coefficient.

Note that the dynamics shown in ACFs are relevant to both beam size and pore size. Changing the beam size would be ideal to solve these two problems mentioned above. To achieve this, we studied the beam size effect on the ACFs using Monte Carlo simulation. In the simulation, we generated a set of trajectories of 45 nm particles diffusion in 150 nm (radius) pores. The same set of trajectories were applied to different laser beam profiles, i.e. 250 nm, 100 nm, 50 nm, and 20 nm, respectively, to obtain intensity traces. The typical intensity traces in different situations are shown from Figures 3.1A - 3.1D. In the observation with 250 nm beam size, the diffusion intensity trace showed a group of wide peaks, which were characterized in ACF as 1D diffusion dynamics (Figure 3.1E). The characteristic time is ~ 100 ms, which is consistent with the 1D axial diffusion. With laser beam decreasing to 100 nm, more and more sharp peaks were observed within those wide peaks, which reflected the intensity changes caused by lateral diffusion. When the beam size decreased to be similar to the pore size, the intensity changes in lateral direction became obvious. The intensity trace contains both narrow lateral diffusion peaks and broad axial diffusion peaks. The same dynamics are shown in the corresponding ACFs, too. With a laser beam of 100 nm, two distinct dynamics are clearly displayed, with the characteristic times of ~ 0.2 ms and ~ 100 ms,
respectively. When 50 nm beam size is applied, the intensity traces show exclusively sharp peaks. Correspondingly, the ACFs showed the dominated lateral diffusion dynamics with a characteristic time of ~ 70 µs, which is consistent with the diffusion time (64.4 µs) for a particle transverses the laser beam with a size of 50 nm. These three situations reflect how different beam sizes affect intensity traces and ACFs of particle diffusion in pores.

From above study, we can observe and estimate both the lateral and axial diffusion coefficients of a point object diffusing in a 150 nm pore by changing the laser beam size. With beam size close to or larger than pore size, the axial diffusion coefficient could be either directly extracted (with a beam size of 250 nm) or using the method discussed (with a beam size of 100 nm) in Chapter 2. When beam size is decreased to 50 nm or smaller (e.g., 20 nm), the lateral diffusion coefficient could be obtained from fitting the ACF to conventional 3D diffusion model. In theory, it’s the lateral resolution of STED microscopy could be changed from 250 nm down to 20 nm by changing the power of depletion beam. Therefore, combining STED microscopy with the FCS technique, we could vary the depletion beam power to acquire different beam sizes to obtain lateral and axial diffusion coefficient, respectively.

A practical consideration in addition to the limit of spatial resolution for STED microscopy is the temporal resolution. Reduced probe volume also means reduced signal level. For example, when beam size is as small as 20 nm, the characteristic diffusion time would be ~ 10 µs. An integration time at least 2/3 times smaller than the characteristic diffusion time is required in the measurement. The practical limit is that we may not be able to obtain sufficient signals within such a short time.
3.3.2 Extracting Lateral Diffusion Coefficient in ‘Larger’ Pores

It is clearly shown that for different beams, the size range in which the diffusion dynamics transitions from 1D to 3D diffusion is different. This indicates that the beam/pore size ratio is a more useful parameter to predict and design experiments to obtain lateral and axial diffusion coefficients. To clarify this, we will use beam/pore size ratio to categorize the three different size ranges where different diffusion dynamics are shown in the ACFs. The beam size is defined by beam waist of laser, which is the distances from the center to where the emission intensity drops to $1/e^2$ in the lateral direction. The radius of pore is used to characterize pore size. With the reference to the categorization criteria, we could better design and change laser beam to measure both lateral and axial diffusion coefficients.

In Chapter 2, we showed that when the size of the pore was larger than 750 nm (radius), only one dynamics is shown in the ACF collected using the 250 nm beam, which is free 3D diffusion (data shown in Figure 2.1). Also, when beam size is smaller than 100 nm, there is only one dynamics shown, which represents the 1D axial diffusion. For example, the ACF simulated from 50 nm pore and 100 nm pore are overlapping to each other (Figure 3.2A), which indicates that in this size range, the ACF is dominated by the axial diffusion. Between 100 nm and 750 nm, the transition between 1D and 3D diffusion happens and two dynamics are shown. With our previous study, we know that if we carefully discard the fast dynamics by binning the data to a low time resolution, the axial diffusion coefficient still could be calculated with 1D diffusion model. Therefore, for 250 nm pores, we could preliminary define large pores for the beam/pore size ratio ($\alpha$) < 0.33, medium pores for 0.33 < $\alpha$ < 2.5, and small pores for $\alpha$ > 2.5.
After decreasing the laser beam to 50 nm, the pore size range with a 1D to 3D transition changed as expected (Shown in Figure 3.2B). Since 50 nm is a practically achievable spatial resolution for STED, we used the same trajectories but applied a beam size of 50 nm. Two dynamics could be observed starting from 30 nm to 150 nm pores. To confirm α still works for the 50 nm beam, we first fit the ACF of 20 nm pores with the 1D free diffusion model (Figure 3.2D). The perfect fitting with a recovered D of $9.56 \times 10^{-12} \text{ m}^2/\text{s}$ indicated that 1D free diffusion is a valid assumption. We then used 3D free diffusion model to fit the ACF in 150 nm pores (fitting not shown), however the 3D free diffusion model could not fit the ACF well after 1 ms, which gives a D of $8.76 \times 10^{-12} \text{ m}^2/\text{s}$. The deviation after 1 ms indicated that the ACF still has a combined contribution from the lateral and axial diffusion dynamics. Increasing pore size to 200 nm, the 3D free diffusion model could perfectly fit the ACF and gives a D of $9.78 \times 10^{-12} \text{ m}^2/\text{s}$ (Figure 3.2C). Thus, when $\alpha < 0.33$, the ACF could be mainly considered as dominated by the lateral diffusion. Especially, when $\alpha < 0.25$, the ACF could be fitted using the free 3D diffusion model. Also, the transition is shown for $0.33 < \alpha < 2.5$ for 50 nm beam size. Thus, the beam/pore ratio, $\alpha$, could help us design and set up lateral resolution to acquire lateral diffusion coefficient. In theory, with the confocal setup (beam size of ~ 250 nm), we could always obtain axial diffusion coefficient from pore size smaller than 750 nm. When increasing spatial resolution from 250 nm to 50 nm, we could use 3D diffusion model to estimate the lateral diffusion coefficient in the pores with a size larger than 200 nm. By measuring diffusion with confocal and STED microscopy, the lateral diffusion and axial diffusion coefficient could be accurately estimated within the pore size range from 200 nm to 750 nm.
3.3.3 Pore Shape and Position Effect

In previous discussions, all the simulations were based on the ideal condition, where the pore was perfectly circular in shape and the laser beam was focused on the center of pore. However, in practice, the pore shapes are not regular; the laser beam might be focused off the center. To consider the effect of these non-ideal situations, we simulated two cases: (1) particle diffusion in rectangular pores and (2) beam off the pore center. In this part of the study, we fixed laser beam size as 50 nm. Changing from circular pores to rectangular pores (Figure 3.3A), we could still see the transitions when pore size is from 25~200 nm. This observation is consistent with that in circular pores.

Then, we moved laser beam away from pore center in a circular pore. Figure 3.3B showed the simulation of 45 nm particles diffusion in 50 nm pores with a beam size of 50 nm. At the beginning, the laser beam center was overlapping with pore center. Since the pore size is close to beam size (0.33<\alpha<2.5), two dynamics could be observed in ACF. While gradually moving the beam size toward the outside of the pore, two dynamics become more and more obvious. Checking the intensity traces from Figure 3.4, we could see the trends, too. For the original intensity trace, we could see very wide peaks with a width of ~70 ms, which denotes the axial diffusion. The wide peaks are full of sharp peaks inside, which represent the confined lateral diffusion. With moving laser beam away from the center for 25 nm, the wide peaks are decomposed into groups of sharp peaks. When the beam center is parked on the edge of the pore, the trace is composed of purely sharp peaks. The trend that lateral diffusion becomes more dominant in intensity traces is also reflected in the ACFs as described earlier. The reason has been explained in Chapter 2: The Gaussian-distributed laser beam has a flatter profile in the center as compared to the flanks of the beam. When the laser beam is off the center, more
fluctuations in the intensity will be observed. When the beam is completely off (Figure 3.4D), the intensity collected is like the background. Thus, the diffusion in other pores will not affect diffusion in studied pore.

How off-beam effects on obtaining lateral and axial diffusion coefficient? For axial direction, the intensity profile in axial direction has not changed and the diffusion dynamic is not affected. For pore size smaller than 750 nm, the axial diffusion could always be separated using relevant larger beam from lateral diffusion and fitted with 1D model. In addition, when tuning the laser beam size accordingly, for pore size larger than 200 nm, the diffusion is dominated by the lateral diffusion. The characteristic diffusion time is decided only by the laser beam size. It still could be analyzed with the conventional model.

3.3.4 The Effect of Anisotropic Diffusion in Pores

All discussions above are based on the isotropic diffusion in lateral and axial directions. The STED-FCS and confocal-FCS provide the approach to separate diffusion in lateral direction and axial direction, allowing their diffusion coefficients to be measured, respectively. The reason why this approach is applicable is because the length scales of the probe volume is different in lateral and axial directions, leading to the different time scales for the particle to diffuse across the probe volume laterally and axially. Especially, the lateral confinement in small pores will make the ACF decays faster in the short time scale, further separating the two dynamics.

When considering anisotropic diffusion coefficients, the analysis can be more complicated. To have a better understanding of this, we first simulated 45 nm particles diffusing in different pores with 10 times faster/slower lateral diffusion coefficients. The ACFs have been calculated
and shown in Figure 3.5A and 3.5B. For faster lateral diffusion coefficient, since the lateral diffusion time becomes even smaller, the lateral dynamics in ACF shifts to short time, leading to better separation of lateral and axial diffusion dynamics. Similarly, with slower lateral diffusion coefficient, the lateral diffusion dynamics will be shifted toward the slow axial diffusion dynamics. This raises the questions: under what situations, the lateral and axial diffusion could not be separated? If so, how to calculated the diffusion coefficients? Special interests are in systems with $0.33 < \alpha < 2.5$, and $\alpha < 0.33$, since when $\alpha > 2.5$, the intensity trace will be dominated by the lateral diffusion and the axial diffusion cannot be resolved using FCS measurements.

As a typical example, we simulated 45 nm particles diffusing in 50 nm and 150 nm pores with a beam waist of 50 nm. The ratios of lateral to axial diffusion coefficient of: 1, 10, 20, 1/10, 1/20, and 1/50 have been studied (Figure 3.5C-3.5D). For 50 nm pores, the transitions between lateral and axial diffusion are shown in ACFs. With increased lateral diffusion coefficient, the lateral diffusion dynamics decays faster and is shifted to the short time scale. It further separates the two diffusion dynamics and makes it easier to extract the axial diffusion coefficient. Conversely, lateral diffusion coefficient will shift the lateral diffusion dynamics to the longer time scale. When the lateral diffusion coefficient is smaller than axial diffusion coefficient for less than 50 times, the separation of the two dynamics could still be observed. Thus, if we carefully bin the data to a lower time resolution, we could still estimate the axial diffusion coefficient. However, when the lateral diffusion coefficient is 50 times smaller than the axial diffusion coefficient, the two dynamics overlaps with each other completely. This means that there is no way to analyze the lateral diffusion coefficient. However, using a laser beam with larger size (or, poorer resolution), we can still analyze the axial diffusion coefficient.
As a contrast, in 150 nm pores, the ACF is dominated by the lateral diffusion dynamics. Correspondingly, the lateral diffusion coefficient can be analyzed.

Thus, the change of the lateral diffusion coefficient causes the shift of lateral diffusion dynamics in the ACF as expected in Equation 3.5. Whether they can be separated and analyzed needs to be studied case by case.

3.4 Conclusion

We used Monte Carlo simulation to generate particle diffusing trajectories in pores with sizes ranging from 20 nm to several hundred nanometers. Different excitation intensity profiles with different spatial resolutions have been applied to generate fluorescence intensity traces. With different beam sizes, there were always three types of ACFs observed: ACFs dominated by the axial diffusion, by the lateral diffusion, and having a mixed contribution from both lateral and axial diffusions, respectively. The dynamics shown in ACFs is determined by the size ratio of the beam and the pore ($\alpha$). We carefully categorized those three situations with the criterion of $\alpha$. When $\alpha < 0.25$, the ACFs could be simply fitted with the 3D diffusion model; while $\alpha > 2.5$, the ACFs could be characterized by 1D diffusion model. When $0.33 < \alpha < 2.5$, the ACFs are mainly the combination of lateral and axial dynamics. With carefully binning data, the axial diffusion coefficient could be extracted from fitting the 1D model to the low resolution data.

Because the lateral resolution could be tuned by changing the depletion laser power in STED microscopy, STED-FCS provides a more flexible way to separate lateral and axial diffusions in pores and resolves lateral and axial diffusion coefficients, respectively. As commonly acquired spatial resolution of STED is ranging from 50 nm to 250 nm, in theory, it
is expected that for the pore size ranging from 200 nm to 750 nm, both the lateral and axial diffusion coefficient could be measured.

In addition, we studied how anisotropic diffusion will impact the diffusion coefficient measurement. Simulating trajectories with changing the lateral diffusion coefficient, we found that the lateral diffusion dynamics shifts as expected in Equation 3.4. Correspondingly, the axial and lateral diffusion coefficients can or cannot be resolved, depending on the overlap of the two dynamics. This systematically study provides theoretical understanding and the guide for using STED-FCS to study particle diffusion in cylindrical pore
FIGURES
Figure 3.1 Simulated intensity traces using differently sized beams for particle diffusion in 150 nm pores: A. 250 nm beam. B. 100 nm beam. C. 50 nm beam. D. 20 nm beam. E. Corresponding ACFs.
Figure 3.2 Simulated ACFs collected from differently sized pores using typical confocal and STED beam sizes: A. confocal beam (beam size 250nm). B. STED beam (beam size 50 nm). C. Free 3D diffusion model fitting of particle diffusing in 200 nm pores using the STED beam. D. Free 1D diffusion model fitting of particle diffusion in 20 nm pore using the STED beam.
Figure 3.3 Simulated ACFs. A. ACFs calculated from the simulation of particle diffusion in rectangular pores. B. Relative STED beam (radius 50 nm) position effect on ACFs of particle diffusing in 50 nm pore (radius).
Figure 3.4 Simulated intensity traces of particle diffusion in 50 nm pores with a 50 nm laser beam: A. The beam right on the center. B. 25 nm off from the pore center. C. 50 nm off from the pore center. D. 100 nm off from the pore center.
**Figure 3.5** Anisotropic diffusion of particle in different pores (A-B): A. $D_{xy}/D_z = 10$. B. $D_{xy}/D_z = 1/10$. Different lateral diffusion coefficients used in anisotropic diffusion in: C. in 50 nm (radius) pore. D. in 150 nm (radius) pore.
Chapter 4 Retention by Porous Materials Studied with STED Microscopy

4.1 Introduction

Porous materials are important in a variety of research and industrial applications such as drug-delivery, separations, heterogeneous catalysis, and enzymatic reactions, etc.\textsuperscript{1,112-120} Mass transport in the presence of porous materials is complicated. First, molecules or small particles behave differently when they are near an interface than in the bulk solution. Since the high area/volume ratio of porous materials, there will be a significant amount of time that the solutes will be in the pores, during which the solutes are surrounded by a liquid-solid interface. Multiple studies have shown that in porous materials with nanometer sized pores, diffusion and migration slow down significantly.\textsuperscript{9-13,16-18,118,124} Although the underlying mechanism is not well understood, it is generally believed that the solvents in the pores are under extreme conditions of non-uniformity as a result of the molecule-interface interactions, which contributes to the anomaly diffusion in nanopores. In addition, hydrodynamic friction becomes significant in narrow pores, which further complicates the mass transport.

Second, adsorption may happen. Adsorption is a fundamental process and may contribute positively or negatively to the applications. It can happen at any time scale. While long-time adsorption are frequently observed,\textsuperscript{49-51,52} short-time adsorption is more illusive and poorly understood.

Third, how molecules/particles enter the pores involves many interactions such as electrostatic, hydrodynamic, and van der Waals interactions, etc. To fully understand and manipulate particle transport, different effects have been discussed both in theory and in
experiments.\textsuperscript{39-45} For example, Lin studied how micron-sized particles deposit on a microsieve, and observed that particles are preferred to be captured on the edge of the pores before they enter the pores from experiments.\textsuperscript{44} Similarly, Belfort and colleagues evaluated particle trajectories during cross-flow microfiltration and concluded that small particles tend to deposit on the membrane surface.\textsuperscript{46-48} As a contrast, Kim et al. theoretically studied the effect of Brownian forces and concluded that with the help of Brownian forces, particles could overcome electrostatic repulsion and enters pores directly in low filtration flow.\textsuperscript{43} Bowen et al. concluded that electrostatic interactions and hydrodynamic interactions could control the rejection of the charged spherical particles. They quantitatively investigated how entrance shape, zeta-potential of particle and membrane, and electrolyte concentration, etc. affect that the force balance and found the critical velocity to avoid adsorption during separation process.\textsuperscript{40-42} It is unclearly that the adsorption of the particle on the pore entrance affects favorably or unfavorably the entering of the particle to the pore.

So far, there is little evidence of how small particles are being retained by the pores. Current techniques do not have sufficient spatial or temporal resolution to resolve this complicated process.\textsuperscript{49-51,52,53} For example, confocal fluorescence correlation spectroscopy (FCS), is a powerful technique that provides high temporal resolutions up to several microseconds. However, due to the diffraction limit of light microscopy, the applications of confocal-FCS for systems smaller than 200 nm are limited.\textsuperscript{36-26} It is impossible to differentiate confined diffusion or adsorption within an area that is smaller than 200 nm. Therefore, super resolution techniques are required.

Stimulated Emission Depletion (STED) Microscopy could provide spatial resolution down to $\sim$ 20 nm in lateral direction.\textsuperscript{72,73} Compared to other techniques, the biggest
advantage of this technique is that it offers both super spatial resolution and high temporal resolution. With those, STED-FCS has been proven to be a powerful tool to directly reveal nanoscopic dynamics, e.g., on lipid membranes.\textsuperscript{37,38} For example, Eggeling et al. used STED-FCS to detect single diffusion molecules in the plasma membrane of living cells. They successfully distinguished transient trapping dynamics in 20 nm diameter areas from slow diffusion.\textsuperscript{38}

In this Chapter, we studied 45 nm particles diffusion near membrane filter surface containing 200 nm pores using STED and confocal-FCS. The high spatial and temporal resolutions provided by STED-FCS helps us distinguish confined diffusion from adsorption and confirms that particles are microscopically diffusion in lateral direction while it is trapped by the pore entrance. Such trapping is also valid even when the pore wall is modified with C18, which significantly retains the particle in the pore for longer time. With qualitative understanding of particle motion near the pore surface, a 2D with trapping model has been proposed to quantitatively analyze the ACFs data. The relationship of Gibbs free energy and the fraction of C18 modification on the pore wall has been investigated.\textsuperscript{54} Our study shows an active trapping model for particles being retained by pores.

4.2 Experimental

4.2.1 Chemical and Materials

Carboxylated polystyrene 45 nm fluorescent nanoparticles were acquired from Thermo/Duke Scientific. The absorption and fluorescence emission maxima of aqueous suspension of the particles were 505 and 515 nm, respectively. The original aqueous
solution containing 1-2% particles (w/v) was diluted to a proper concentration (1.0~4.0 × 10^{12} particle/mL) with corresponding buffer made from 18.2-MΩ Milli-Q water. Porous alumina membranes with pore diameters of 20 nm and 200 nm with a thickness of 60 μm were purchased from Whatman International (Maidstone, U.K.).

4.2.2 C18/PEG Modification of Pore Surface

To modify surface with C18 and PEG, the similar silane reaction reported has been used.\textsuperscript{102,103} Alumina membranes have been firstly hydroxylated and cleaned by boiling in 30% hydrogen peroxide and deionized water for 15 min, respectively. To make 10% of C18/PEG surface, 200 μL of Hydroxy(polyethyleneoxy)propyltriethoxysilane (PEG) (Gelest, MW:350-750, 50% in Ethanol) with total PEG of ~ 0.18 mmol was measured. 6.25 mg of Chloro(dimethyl)octadecylsilane (C18) (Sigma-Aldrich, MW:347.09) was dissolved into 200 μL of anhydrous toluene, making total C18 moles of 0.018 mmol. The final solution was prepared by mixing up those two solutions and diluting to final volume to 20 mL. By mixing 200 μL PEG solution with different volumes of C18 solution, different ratios of C18/PEG solutions could be prepared for further modification. Then the hydroxylated filter has been soaked in prepared solution for 1h, followed by rinsing with toluene, acetone and DI water. Before each measurement, the modified membrane filters were soaked in 25 mM phosphate buffer (pH~10) for 0.5 hours. At this pH and buffer concentration, both the particle and the membrane surface were negatively charged. No significant adsorption was observed.
4.2.3 Confocal and STED Microscopy

Our home-built continuous-wave stimulated emission depletion microscope has been discussed in details in previous publication. Briefly, air-cooled Ar ion laser (35-LAP-431-240, CVI/Melles Griot) provided excitation laser beam with wavelength of 488 nm. The excitation beam was circularly polarized by a quarter-wave plate (QWP) (CVI/Melles Griot, ACWP-400-700-06-4) and collimated to overfill the back aperture of a microscope objective (Nikon, Plan Apo, 100 × /1.40–0.7, Oil). The depletion laser of 592 nm (1.0 W, MPB communication, VFL-P-1000-592-OEM1) first passed through a 0-2π vortex phase plate (RPC photonics, VPP1a) to generate a donut-profiled beam and together with excitation beam, was guided to microscope objective back aperture by the combination of a 505 nm long-pass and a 570 nm short-pass dichroic mirrors. The fluorescence signals were imaged into a piece of multimode fiberoptics (diameter of 50 µm) and detected by an avalanche photodiode (Perkin Elmer, SPCM-AQRH-15-FC). A programmable counting board was used for photon counting. The confocal microscope is the same setup of STED with depletion light off.

4.3 Results and Discussion

4.3.1 Adsorption-like Phenomenon Observed when Particle Diffusion Close to the Membrane Filter Surface with Open Holes

When particle diffuses close to the surface of a solid substrate, there is a chance that it is retained by the surface. This is one of the most important processes in chemistry, for example, in heterogeneous catalysis. Things become more complicated for surface with opening holes where particle has a chance to enter the pores. We have observed 100 nm
particles diffusing above the surface of membrane filters with 200 nm pores with epi-fluorescence microscopy (shown in supplementary movie 1, 20 frames per second, or fps). Two basic motion modes could be recognized from the movie: permanent adsorption, and free 3D Brownian diffusion. However, there is another motion which is very difficult to characterize: the particle motion is temporarily delayed when they are in contact with the surface, giving a discontinuous motion. As the membrane filter surface is modified with hydrophobic C18, the discontinuous motion becomes more apparent. Here comes the question: is this process simply adsorption/desorption, or the particle diffuses into the pores and being trapped in the pore briefly, or both? Just like mentioned earlier, there are different opinions regarding how the particles being retained and going into the pores. To distinguish these two different motions, the key is that we have sufficient temporal and spatial resolutions so that we can observe whether the particle is moving in the lateral direction.

Here, epi-fluorescence microscopy does not have either resolutions. In order to solve this problem, we need a spatial resolution smaller than the pore size. Considering the integration time: the particle is confined within the 200 nm pores, the lateral diffusion time across the pore could be estimated by:

\[
< L^2 > = 2nD\Delta t
\]  

(4.1)

where \( < L^2 > \) denotes to the mean squared displacement, which is assumed to be 100 nm – the average step size a particle can make when it rests on the pore wall, \( n \) is the number of dimensions, \( D \) is the diffusion coefficient, and \( \Delta t \) is the time interval between observations. For 100 nm particles with a diffusion coefficient of \( \sim 4.4 \times 10^{-12} \text{ m}^2/\text{s} \) confined in a 200 nm
pore, the diffusion time of less than 1 ms is expected. Apparently, wide-field fluorescence microscopy cannot achieve this goal.

4.3.2 Particle Diffusion Dynamics on Different Surface

To investigate particle motion near surface with open-holes, we used confocal-FCS because of high temporal resolution and optical sectioning property, which could exclude the signal beyond 1 μm out of focal plane. By changing focal plane, we could observe particle motion in different axial positions. In experiments, we mainly investigated 45 nm carboxylated polystyrene particle to keep sufficiently high signal to noise ratio and minimize the size effect in pore. We first characterized how particle diffusion on solid surface with small holes (i.e., particles could not enter).

With the excitation laser power of 2.0 μW and integration time of 50 μs, each 20 intensity traces of particle diffusion in the bulk (3 μm away from cover slide surface) and on glass surface have been collected, separately. The autocorrelation function (ACF) curves have been calculated and plotted in Figure 4.1A. The typical ACFs collected in bulk solution and on glass surface could be fitted with 3D free diffusion model and give a diffusion coefficient of $6.9 \times 10^{-12}$ m$^2$/s and $5.9 \times 10^{-12}$ m$^2$/s (Figure 4.6), which is consistent with the theoretical estimation using Einstein-Stokes equation and our measurements in previous study. The perfect overlapping of the ACFs from above glass surface with those from the bulk solution indicated that the same diffusion dynamics in both situations. Although there is a chance that particle could be adsorbed on the surface, there is no adsorption events observed in the experiments. Besides, the same dynamics of diffusion in the bulk solution and on glass surface also proved that the hydrodynamics effect is
negligible. The same phenomenon has also been observed when particle diffusion on surface with 20 nm pores. In the experiments, we parked the laser beam in the bulk solution and right on the surface, respectively, and collected 20 intensity traces for each situation. The corresponding ACF curves were calculated (Figure 4.1B). The same dynamics have been shown by the complete overlapping of the 3 different batches of ACFs from different positions. Thus, we conclude that the particle diffusion follows the 3D free diffusion model on solid glass surface and on small pore surface. The same dynamics shown in the bulk solution, on glass surface, and on membrane surface with 20 nm pores indicated that there is very little hydrodynamic effect or adsorption events which could hinder particle diffusion near surface.

We then studied 45 nm particles diffusing on unmodified membrane surface containing 200 nm pores. In experiments, to maintain high temporal resolution with good signal to noise ratio, we first used the laser power of 20 μW and the integration time of 500 µs. Differently, the half decay time increased to ~ 10-20 ms, which is ~ 3-4 times slower than in the bulk solution. To analyze the data, we used conventional 1D and 3D diffusion models. However, both failed to fit the data or give unreasonable diffusion coefficients. This indicates that on surface with 200 nm pores, complicated dynamics happen.

4.3.3 Slow Dynamics Observed when Particle Diffusion on 200 nm Membrane Surface

What actually caused the slowing down of the diffusion on 200 nm membrane surface? From the discussion above, it is less possible that particles adsorb on the solid surface with our experimental condition. In addition, there is very little hydrodynamic effect that might slow down the particle diffusion. Aware of that, we carefully checked intensity traces
collected from the 200 nm membrane surface. Different from diffusion in the bulk, the
typical intensity trace (Figure 4.2A) showed two different dynamics: a lot of sharp peaks
with a width of ~ ms, and wide peaks with a width of several tens to even hundred ms.

It is easy to recognize the sharp peaks as the reflection of free diffusion in solution due
to the same time scale of the 3D diffusion time. However what are those wide peaks? Two
characteristics of those peaks could be seen: 1. plateau peaks. These peaks have intensity
that varied little. 2. Sharp spikes could be observed in those wide peaks, which means that
although macroscopically the intensity changed very little, but there is still active intensity
change included in the slow dynamics. Since fluorescence emission of particle is induced
by Gaussian profile of the laser beam, the fluorescence emission intensity follows:

$$I = I_0 \exp\left(-\frac{2(x^2 + y^2)}{r_{xy}^2}\right)\exp\left(-\frac{2z^2}{r_z^2}\right)$$  \hspace{1cm} (4.2)

where $I_0$ is the maximum emission intensity when the emitter is placed at the center of the
beam; $r_{xy}$ and $r_z$ are the distances from the center to where the emission intensity drops to
$1/e^2$ in the lateral and axial directions, respectively. Therefore, very little change in
intensity indicated that particle has little mobility. These wide peaks with little intensity
change could lead to the conclusion that they are caused by adsorption events on surface.

However, there are two important concerns: one is the dynamics observed on solid glass
surface and on membrane surface with 20 nm pores. They have the same diffusion
dynamics as in the bulk solution, which suggests that in our experiment condition, the
adsorption and hydrodynamic effect could be ignored. The other one is that the artifacts
caused by the low spatial resolution of conventional confocal fluorescence microscopy.
The typical lateral beam radius used in confocal-FCS is 250~300 nm, which is larger than the pore size (radius of 100 nm). Therefore, even when particles diffuse in the lateral direction, the intensity change is less than 20% based on equation 4.2. Little fluctuation in intensity is expected, which makes it to look like adsorption events. In this case, it is too reckless to attribute these wide peaks as caused by adsorption. To solve this problem, higher spatial resolution techniques are required.

4.3.4 Lateral Diffusion Confirmed by STED-FCS

As reported earlier, we set up a home-built STED microscope. With the depletion beam power of 800 mW, we scanned 45 nm particles immobilized on glass surface and obtained the full width at half maximum (FWHM) resolution of 75 ± 10 nm. By changing the depletion beam power, different spatial resolution could be acquired. To make sure STED is applicable in FCS, we used both confocal-FCS and STED-FCS to measure 45 nm particle diffusion in solution. With the integration time of 50 μs, STED microscopy with full depletion power showed the same high signal to noise ratio as compared to confocal microscopy (Figure 4.7A). Then, we used STED-FCS to study particle diffusion on 200 nm membrane surface. In our experiments, to minimize the scattering of the depletion beam from the membrane filter, we used a depletion power as ~100 mW. The intensity profiles of 45 nm particles were measured and fitted with Lorentzian distribution. It gives a FWHM resolution of 166 nm (Figure 4.7B), which has a ~2 times improvement in spatial resolution compared to confocal microscopy. With this higher spatial resolution, the lateral diffusion confined in 200 nm pores showed more obvious variance in intensity change. The typical intensity trace with an integration time of 500 μs has been shown in Figure 4.2B. Similar to the intensity traces from confocal collection, two types of peaks have been
shown: Many narrow peaks which are attributed to free lateral diffusion outside the pore. Besides, there are also many wide peaks, which are assigned as the particle being trapped in the pores. These wide peaks show fluctuations in intensity, indicating that the particle is actively diffusing when they are in the pores. The same information has been shown in ACF curves (Figure 4.2C). The decreased diffusion time indicated that the dynamics is relevant to the diffusion rather than adsorption since only the laser beam decrease could not affect the adsorption time. Therefore, with the help of high spatial and temporal resolution provided by STED microscopy, we could distinguish confined lateral diffusion from adsorption.

Now we have a clearer image when particles diffuse close to surface: particles could first stay free diffusion in solution outside the pore; when it comes to the opening of the hole, they might enter the pore and diffuse in the axial direction with confined lateral diffusion for briefly and then diffuse out. However, there are still several questions remain unanswered: 1. slowing down dynamics in the pore. Although the ACF of STED indicated that the dynamics shown here is relevant to diffusion, it is still ~ 10 times slower than expected free diffusion. What does this slow dynamics represent? 2. When particles diffuse into the pore, how long will they diffuse in pore? To answer those two questions, The ACFs need to be analyzed in detail.

4.3.5 Trapping Time Acquired from ACF Curves

To answer these questions, we modified pore surface with PEG, which further eliminates the possible unspecific adsorption. With the integration time of 300 μs, we collect 25 intensity traces from different positions on the same modified pore and different
pores using confocal microscopy. The ACFs have been calculated and the similar slow
dynamics could be observed (data not shown). Although confocal-FCS is insufficient to
distinguish confined lateral diffusion from adsorption, the ACFs provides sufficient
information to understand the dynamics of diffusion on membrane surface. To characterize
these dynamics, we first fit the ACFs using different conventional models (Figure 4.8A).
However, none of the 3D, 1D or adsorption and diffusion models (models have been
introduced in details in supporting information) were appropriate here, which indicated that
the dynamics shown in ACF is not a simple process. Based on our observations from the
STED-FCS, the diffusion dynamics should be the mixture of free diffusion outside the pore
(a characteristic time of ~ ms) and confined axial diffusion in the pore (a characteristic time
from several tens to hundreds ms). As from our earlier simulation studies, when particles
freely diffuse in 200 nm pores, the ACF is dominated by the 1D diffusion; while for free
diffusion in the bulk solution, it is dominated by the 2D diffusion events. Thus, to simplify
the model, we used 2D diffusion model to characterize the particle diffusion outside the
pores and adsorption-like model to character particle’s confined diffusion inside the pore.
The model used is similar to Wirth’s diffusion-adsorption model, and can be expressed
as:

$$ G(\tau) = \frac{1}{N} \left( A_1 \frac{1}{1 + 4Dt/r_x^2} + A_2 \exp(-\tau/t_{in}) \right) $$

where \( <N> \) is the average number of emitters in the probe volume; \( D \) is diffusion
coefficient outside pore, and \( t_{in} \) is axial trapping time inside the pore. Here, we point out
that instead of the ‘adsorption time’, we used the term of ‘trapping time’ to describe \( t_{in} \).
because particles are still actively moving inside the pore. $A_1$, $A_2$ are fraction coefficients and $A_1 + A_2 = 1$.

The perfect fitting with this model has been shown (Figure 4.3A). When fixing $r_{xy}$ as 300 nm and floating both $D$ and $t_{in}$, it gives a diffusion coefficient of $6.0 \times 10^{-12}$ m$^2$/s and a trapping time of 77 ms. With the same fitting process for 5 ACFs, the average diffusion coefficient of $6.6 \pm 1.1 \times 10^{-12}$ m$^2$/s could be obtained, which is consistent with the $D$ value recovered from the bulk solution measurements. Therefore, to make fitting more accurate, we fixed the $D$ to be $6.6 \times 10^{-12}$ m$^2$/s and calculated the retention time for all 25 collected ACFs (shown in Figure 4.3B). The median trapping time was 67 ± 12 ms. Once we know the particle’s trapping time in the pore, the diffusion distance in the pore could be estimated using equation 4.1. Due to the hydrodynamic effect, the measured axial diffusion coefficient in PEG-modified pore was $\sim 3.1 \times 10^{-12}$ m$^2$/s in previous study. Thus, a depth of $\sim 322$ nm for particle diffusion in the pore could be estimated. This shows that most of the particles only diffuse very briefly into the pore before they leave the pore. Now with the ACFs analysis, the particle diffusion on surface could be described quantitatively.

4.3.6 Longer Trapping Time on C18 Modified Surface

Furthermore, we investigated how hydrophobicity affects particle retention by the membrane filter. By increasing the percentage of C18, membrane surface becomes more hydrophobic. Stronger van der Waals interactions between the surface and the particle could be expected. We modified the pore surface by changing the mole percentage of C18 in a mixture of thiols containing C18 and PEG (1%, 5%, and 10%). 56, 30, and 25 intensity traces of 45 nm particles from different positions on modified surface were collected using
confocal microscopy, respectively. The ACFs in each condition were obtained and used to calculate the trapping time with our proposed model. The perfect fitting of 10% C18 data (Figure 4.8B) has been shown and proved that our model still works well in this study. The trapping time distributions on different surfaces have been plotted (Figure 4.4) and the median trapping times have been summarized (Table 4.1). That trapping time increases with more C18% indicated that stronger van der Waals interaction between the surface and the particle. The stronger interaction might lead to surface withholding particle on surface, therefore the longer ‘trapping time’ is no more simply axial diffusion time in pore but also includes the possible adsorption time between the pore surface and the particle. In our earlier study of 45 nm particle diffusion in PEG modified pore,\textsuperscript{122} the adsorption in pores has been excluded. Thus, here we define the trapping time calculated from PEG surface as diffusion time ($t_d$), and adsorption time, which is the time spent when particle desorbed from the surface, $t_a$, could be calculated as:

\begin{equation}
\begin{aligned}
t_a &= t_{C18} - t_{PEG} \\
&= \text{diffusion time (4.4)}
\end{aligned}
\end{equation}

where $t_{C18}$ and $t_{PEG}$ are the trapping times on C18 surface and on PEG surface, respectively. In our observation time scale in each collection (~ 20s), the Gibbs free energy of desorption process could be calculated:

\begin{equation}
\begin{aligned}
\Delta G &= -RT \ln K \\
&\text{(4.5)}
\end{aligned}
\end{equation}

where $K$ is the equilibrium withholding constant; $R$ is the gas constant; and $T$ is temperature. The equilibrium constant is determined as:

\begin{equation}
\begin{aligned}
K &= \frac{k_d}{k_a} = \frac{1}{t_a} = \frac{t_d}{t_a} \\
&\text{(4.6)}
\end{aligned}
\end{equation}
where $k_a$ is the rate constant for the adsorption process; $k_d$ is the rate constant for the desorption process. Assuming that Gibbs free energy is proportional to C18 fraction, there is:

$$\Delta G = \Delta G_0 + E_d = \Delta G_0 + E_d^0 \times f_{C18}$$  \hspace{1cm} (4.7)

where $\Delta G_0$ is the Gibbs free energy for desorption on surface without C18, $E_d^0$ is a constant. Combining Equations 5, 6, and 7, we have:

$$\Delta G = RT \ln\left(\frac{t_a}{t_d}\right) = \Delta G_0 + E_d^0 \times f_{C18}$$  \hspace{1cm} (4.8)

Then, we calculated the Gibbs free energy from 1% to 10% surface and fitted with Equation 4.8. A good linear fitting could be acquired (Figure 4.5), which gives $E_d^0 = 60$ kJ/mol, and $\Delta G_0 = -6.8$ kJ/mol. Negative values are obtained up to 10% C18, indicating that desorption could spontaneously happen at room temperature. Thus, it is rare to see long time adsorption. Our discussion here is consistent with our earlier study using 3D tracking microscopy to investigate particle diffusion in 200 pore,\(^5^4\) where no mobility could be observed in pores if C18 is beyond 20%.

\textbf{4.4 Conclusion}

In summary, we studied 45 nm particles diffusion on 200 nm membrane surface using confocal-FCS and STED-FCS. In both situations, ACFs showed ~ 10 times (~ 20 ms) slowing down dynamics, which indicates complicated particle diffusion dynamics near the membrane surface containing pores. Carefully checking intensity traces, two different
dynamics have been observed: the free diffusion outside the pore and the axial and confined lateral diffusion inside the pore. With the help of high spatial resolution provided by STED, we could distinguish confined lateral diffusion events from adsorption. Thus, the active lateral diffusion has been confirmed when particles are trapped and diffusing in pores.

In addition, useful quantitative information has been extracted from analyzing the ACFs collected using Confocal-FCS with our proposed 2D plus trapping model. The diffusion coefficient recovered from PEG modified 200 nm pores is consistent with that measured in the bulk and on solid surface. This supports our assumption that normal free diffusion happens outside the pore. Besides, the particle trapping time in pores could be obtained, which showed that particle mostly diffused into pore for ~ 300 nm. By increasing the hydrophobicity using C18 to modify the surface, longer trapping time has been observed, which is possibly caused by the surface withholding of the particles due to stronger van der Waals interaction. Overall, this study provides us detailed scenario of how particles diffusion close to the opening of holes both qualitatively and quantitatively.

4.5 Supporting Information

4.5.1 Conventional Models in Diffusion:

Based on the Gaussian distribution of fluorescence emission, the translational diffusion in unbound three dimensional (3D) space is:

\[
G(\tau) = \frac{1}{<N>} \frac{1}{1 + \tau / \tau_{\text{diff}}} \frac{1}{\sqrt{1 + \tau / S^2 \tau_{\text{diff}}}}
\]
where \(<N>\) is the average number of emitters in the probe volume; \(S\) is the aspect ratio of the probe volume \(r_z/r_{xy}\); \(\tau_{\text{diff}}\) is the characteristic diffusion time assuming the emitter has an isotropic diffusion coefficient \(D\):

\[
\tau_{\text{diff}} = \frac{r_{xy}^2}{4D} \tag{s4.2}
\]

When molecules diffusion only in axial direction, it could be described as:

\[
G(\tau) = \frac{1}{<N>} \left( \frac{1}{\sqrt{1 + \tau / S^2 \tau_{\text{diff}}}} \right) \tag{s4.3}
\]

For the adsorption/desorption process, the model could be described as:

\[
G(\tau) = A \cdot \exp(-\tau / t_{\text{ad}}) \tag{s4.4}
\]

Where \(t_{\text{ad}}\) is the adsorption time.
Table 4.1 Particle trapping time on different modified 200 nm pore surface.

<table>
<thead>
<tr>
<th>Pore Modification (Mole % of C18)</th>
<th>Trapping Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>67 ± 12</td>
</tr>
<tr>
<td>1</td>
<td>73 ± 8</td>
</tr>
<tr>
<td>5</td>
<td>80 ± 10</td>
</tr>
<tr>
<td>10</td>
<td>120 ± 10</td>
</tr>
</tbody>
</table>
Figure 4.1 45 nm particle diffusion ACF curves in the bulk, on solid surface, and on surface with different pore sizes: A. Collection in the bulk (red) and on solid glass surface (blue); B. Collection on 20 nm membrane surface; C. Collection on 200 nm membrane surface.
Figure 4.2 45 nm particle diffusion on 200 nm surface collected using confocal and STED microscopy: A. An intensity trace collected using confocal microscopy; B. Intensity trace collected using STED microscopy; C. Comparison of ACF curves collected using confocal setup and STED setup.
Figure 4.3 45 nm particle diffusion on PEG-modified surface: A. ACF curves fitted with 2D with retention model; B. Trapping time distribution.
Figure 4.4 Trapping time on different percentages of C18 modified surfaces: A. 1% C18 surface; B. 5% C18 surface; C. 10% C18 surface.
Figure 4.5 Gibbs energy change with different percentage of C18 surface.
Figure 4.6 Typical ACFs collected from the bulk and on glass surface fitted with 3D diffusion model: A. Collected from the bulk, B. Collected on glass surface.
Figure 4.7 A. A typical intensity trace of 45 nm particle diffusion in the bulk solution collected using confocal and STED setups with an integration time of 50 μs. B. Lateral intensity profile of STED microscopy measured by line scanning of 45 nm particles immobilized on glass surface. The fitting was using the Lorentzian distribution function, which gives a FWHM resolution of 166 nm.
Figure 4.8  A. ACF of 45 nm particle diffusion on 200 nm PEG-modified membrane surface fitted with 1D free diffusion model, 3D free diffusion model, and pure adsorption model, respectively.  B. ACF of 45 nm particle diffusion on 10% C18 modified 200 nm membrane surface fitted using 2D with retention model. Both ACFs are collected using the confocal microscope.
MOVIES

Movie 4.1 100 nm particle diffusion on 200 nm membrane surface with integration time of 50 ms and is played at rate of 20 frames per second.
Chapter 5 Nanosecond Time Resolution Study of Gold Nanorod Rotation on Liquid-Solid Interface

5.1 Introduction

How macromolecules or functionalized nanoparticles (NPs) interact with a liquid/solid interface is an important question for fundamental sciences and nanotechnology applications. Measuring the rotational motion of nanoparticles provides additional dimensions of information for understanding this interaction. Conventional single particle/molecule tracking techniques only provide information about translational motions. Recently developed methods have been reported to track the rotational motion\(^3,125-130\) of individual rod-like nanoparticles, e.g., gold nanorods, on various surfaces including glass,\(^128,129\) C18-modified silica,\(^131\) synthetic lipid bilayer membranes,\(^132\) and live cell membranes.\(^55,133\)

In our past studies, we found that, upon initial contact with a surface, a significant number of gold nanorods go through a loosely bound state during which the particles lose translational motion but retain rotational freedom.\(^55\) Here, we use the term “anchored” to specify this initial stage of “adsorption”. Note that adsorption does not necessarily mean that the motion of the adsorbate will be frozen. In this study, the latter state where the motion of the adsorbate becomes non-detectable is defined as “immobilization”. According to German physicist Heinrich Kayser, who coined the word in 1881,\(^134\) “adsorption” refers to the adhesion of molecules to a surface. According to this definition, the anchored state describes an early stage of adsorption, which is followed by firmer attachment(s), leading to eventually complete immobilization.\(^135,136\)
How does an anchored nanorod rotate in the 3D space? A general perspective is that with one end of the nanorod attached to the surface, the other end would continuously wave in the solution randomly. In our early studies, we found that a freshly anchored nanorod can rotate very fast and on a time scale that could not be resolved using conventional 2D array detectors. For a nanorod with a transverse diameter of 25 nm and an aspect ratio of ~1:3, the characteristic rotational time $\tau_{\text{Rot}}$, which is the time that the nanorod rotates by 1 radian due to rotational diffusion, is on the $\mu$s time scale in the bulk solution, several orders of magnitude faster than the temporal resolution of most cameras.

To study the fast dynamics of the anchored nanorods on surfaces, we constructed a confocal polarization-dependent scattering light microscope with ns time resolution (Figure 5.). Gold nanorods (25 × 92 nm) were used as the model because of their excellent optical properties and wide applications. When the gold nanorods were excited at their longitudinal surface plasmon resonance (SPR) wavelength of 780 nm, we were able to monitor the fast rotation dynamics at ns time resolution for individual nanorods. The gold nanorods were capped with cetyl trimethylammonium bromide (CTAB) or modified with TAT peptide to give positive charges on their surface, allowing them to readily attach onto negatively charged glass surfaces. Only non-specific adsorption is considered here. Interestingly, we found that the apparent “continuous rotation” observed in low time resolution imaging for anchored gold nanorods actually consists of numerous fast, intermittent transitions between a small numbers of weakly immobilized states. Additionally, the actual rotation, during which the anchored nanorod transits from one immobilized state to the other, happens at 1 ms time scale, ~50 times slower than that in the bulk solution.
5.2 Experimental

5.2.1 DIC/Dark Field Imaging

An upright Nikon Eclipse 80i microscope was used in this study. This microscope can be switched between DIC mode (100× Plan Apo/1.40 oil immersion objective) and dark-field mode (100×, NA 0.7-1.25 oil immersion objective). The DIC images at a selected wavelength were collected by inserting the corresponding bandpass filter into the light path in the microscope. An Andor iXon EM+ camera (512×512 imaging array, 16×16-μm pixel size) or a Hamamatsu camera (Orca 2.8, 1920×1440 imaging array, 3.63×3.63-μm pixel size) was used to record the dynamic nanorod images. MATLAB and NIH ImageJ were used to analyze the collected images and videos.

5.2.2 Confocal Resonance Light Scattering Microscopy

A simple schematic of the microscope used in this study is shown in Figure 5.5.138 Excitation wavelength (780 nm) was provided from a Ti-sapphire laser (~200 fs, 80 MHz, vertically polarized). The laser beam was fully expanded and directed through an objective (NA 1.4, 100×, Plan Apo/oil immersion) for illumination. The laser power was attenuated by using a set of neutral density filters so that the power at the objective back aperture was ~ 0.5 μW to 5 μW, orders of magnitude lower than the power required for multiphoton excitation. The back scattered signal was collected by the same objective and directed through a neutral beam splitter. Resonance light scattering signals passed through the dichroic mirror and were focused onto a fiberoptics pinhole (50 μm). The signal was then read out using an avalanche photodiode (Perkin Elmer, SPCM-AQRH-15-FC) and counted using a photon counting board. The spatial resolution of this setup was characterized by scanning an immobilized gold nanorod
in the XY and XZ directions, respectively, and monitoring for consistency on a daily basis. The XY and Z collection profiles were 355 ± 10 nm and 1040 ± 30 nm in width (full width at half maximum), respectively, over the entire period of the study (for an example, see Figure 5.6). These two values were used as $r_{xy}$ and $r_z$ in NLLS fitting for diffusion coefficients.

5.2.3 Gold Nanorod Modification

Cetyl trimethylammonium bromide (CTAB)-capped gold nanorods with different aspect ratios were purchased from Nanopartz (Salt Lake City, UT) and used directly. The size distribution and geometric profile were evaluated with scanning electron microscopy and agreed well with the manufacturer’s data. The absorption spectrum of the particles in bulk solution was measured with a Cary 300 UV-VIS spectrophotometer (Varian, Palo Alto, CA). In DIC or dark field microscopy, shorter rods with a SPR wavelength of 700 nm ($25\times73$ nm, $2.0\times10^{11}$ particles/mL) were used. In resonance light scattering experiments, longer rods with a SPR wavelength of 780 nm ($25\times92$ nm, $1.3\times10^{11}$ particles/mL) were used.

To functionalize the surface of CTAB-coated gold nanorods with TAT 47-57 peptide (sequence: YGRKKRRQRRR; AnaSpec, San Jose, CA), a NHS-PEG disulfide linker (Sigma-Aldrich) was used by following a published protocol. The NHS-PEG disulfide linker has both disulfide and succinimidyl functionalities for respective chemisorption onto gold and facile covalent coupling of TAT peptide. Briefly, excessive surfactant was first removed from 1.0 mL gold nanorod solution by centrifugation at 3000 g for 10 minutes and the particles were resuspended in 1.0 mL 2 mM borate buffer. A proper amount of fresh NHS-PEG disulfide solution (in dimethyl sulfoxide) was added to reach a final thiol concentration of 0.2 mM and reacted with gold nanorods for 2 hours. The solution was then cleaned up by centrifugation.
and resuspended in 2 mM borate buffer. For TAT modified gold nanorods, 2.0 µg TAT peptide was added to the gold colloidal solution and reacted for 8 hours. Before use, the colloidal gold nanorod probes were centrifuged and re-suspended 2mM borate buffer to a desired concentration.

Both CTAB- and TAT-capped gold nanorods are positively charged. The CTAB-capped nanorods have a zeta potential of 30 mV (manufacturer data). The TAT-modified nanorods have a zeta potential of 22.3 ± 0.3 mV in DI water (Nano-ZS90 Zetasizer, Malvern Instruments, United Kingdom).

5.2.4 Imaging of Anchored Gold Nanorods on Glass Surface

CTAB-coated or TAT-modified gold nanorods (6 µL of the diluted solution) was added onto a freshly cleaned glass slide and covered with a 22×22 mm No.1.5 coverslip (Corning, NY). Most of the positively charged gold nanorods will quickly anchor onto the negatively charged surface of the glass slide.

5.3 Results and Discussion

5.3.1 Nanorod Rotation in Solution

To know how fast the 25 × 92 nm nanorods rotate, we first measured their rotational diffusion coefficient in the bulk solution using fluctuation correlation spectroscopy (FCS). A Ti:Sa laser beam (780 nm, 0.50~5.0 µW at the back aperture of the microscope objective) was focused to the tightest (full width at half maximum of ~350 nm laterally, Figure 5.6) and parked in the solution. When a CTAB-coated nanorod diffused through the focal volume, photon bursts were generated and collected (Figure 5.1A). The photon bursts contain both translation and rotation information of the rod as the scattering intensity adopts a cos² relationship with
respect to the angle between the nanorod’s long axis and the laser polarization direction (Figure 5.7). By fitting the autocorrelation function (ACF) $G(\tau)$ of over 20 traces collected in the solution (2 µm from the surface),$^{59,137,142}$ we recovered the characteristic translation diffusion time $\tau_{Tr}$ and rotational diffusion time $\tau_{Rot}$ to be $2.4 \pm 0.2$ ms and $33 \pm 3$ µs, respectively (mean ± standard deviation, Supporting Information 5.5.1, 1.2, and Figure 5.1B), where $\tau_{Tr}$ is the time for the nanorod to diffuse across the laser beam. The translational and rotational diffusion coefficients are thus $9.4 \pm 0.8 \times 10^{-12}$ m$^2$s$^{-1}$ and $5.1 \pm 0.5 \times 10^{3}$ s$^{-1}$, respectively. These values are consistent with the theoretical values of the gold nanorods ($D_{Tr}$ and $D_{Rot}$ are $8.2 \times 10^{-12}$ m$^2$s$^{-1}$ and $4.4 \times 10^{3}$ s$^{-1}$, respectively).$^{59,137,142}$

Next, we measured $D_{Rot}$ as a function of the distance of the nanorods from the liquid-solid interface by parking the laser focal point at different depths from the glass surface. From the ACF (Figure 5.1C) and the non-linear least squares (NLLS) analysis (Figure 5.1D and Table 5.1), it is clear that the $D_{Tr}$ and $D_{Rot}$ did not change significantly as the focal plane was dialed from 2.0 µm above the surface to right on the glass surface. Thus, the nanorods are still rotating actively when they diffuse very close to the surface (within a distance of ~500 nm, which represents the axial waist of the confocal collection volume, Figure 5.6B).

5.3.2 Apparent “Rotation” of Anchored Nanorods

By introducing the positively charged gold nanorods to a freshly prepared, negatively charged glass surface in 2 mM borate buffer at pH 8.5, adsorption started to happen immediately. Under a differential interference contrast (DIC) microscope, we were able to find that a significant amount of the adsorbed nanorods (e.g., for CTAB-rod ~30%) went through the anchored state by showing flickering bright/dark images. Movie 1 gives one example of
the DIC images of an anchored CTAB-coated nanorod on the solvent-glass interface. In this study, we tested two types of gold nanorods: CTAB- and TAT-coated gold nanorods. We found that anchoring is common for both types of nanorods. Most of the anchored particles stayed in motion on the surface for much longer time than the observation window (~10 minutes) although desorption or permanent immobilization did occur for some of the particles. Eventually, all the particles will be completely immobilized on the surface after prolonged exposure, e.g., overnight. Thus, we conclude that both anchored CTAB- and TAT-nanorods have a stochastic length of active time that is much longer than our observation window (10 minutes).

We then studied the fast rotation dynamics of the freshly anchored gold nanorods on the glass surface by moving the laser beam to a “just anchored” nanorod and starting collection of the scattering intensity traces within a few seconds of anchoring. Surprisingly, the freshly anchored nanorods showed nearly “quantized” scattering intensities. Figure 5.2A shows one example trace from a freshly anchored CTAB-nanorod. The trace shows a two-state model: one state having higher scattering intensity and the other lower intensity. Within a time scale of seconds, the particle stayed in one of the states for the most of the time and then switched rapidly to the other state. During the long durations of analysis, the particle moved actively, producing numerous peaks/dips in the intensity trace. In the transition periods, the nanorod motion became more active (Figures 5.2BC), showing more frequent peaks/dips. Notably, many of these peaks/dips show flat top/bottom, indicating the temporary halt of the motion, or immobilization, of the nanorods by the surface-particle interaction (Figure 5.2C). In addition, there are also many Gaussian-profiled peaks/dips in the trace, indicating rotational diffusion. Most of these peaks/dips have a width of a few to a few tens of ms. For example, the median
times of the bright and dark bright states for the trace in Figure 5.2B are 22.4 ms (2~200 ms) and 13.8 ms (2-69 ms), respectively.

Similarly, anchored TAT-modified particles also showed few preferred states as indicated by the “quantized” scattering intensities (Figures 5.8 and 5.9). The majority of these nanorods showed a two-state model (~70% for >40 observations), with only a few of them having 3 or more states (e.g., Figure 5.9 shows an example with 3 “quantized” states).

What are these “quantized” states? Since the nanorods stayed in all of these states for milliseconds to seconds, the nanorods would be expected to be in brief immobilized states with different orientations with respect to the polarization direction of the laser beam. Consistent with this expectation, the anchored nanorods produced different scattering intensities for these immobilized states. The nanorods usually transitioned back and forth and did not move away from the observation field (~ 350 × 350 nm²). Thus, it is reasonable to believe that these anchored states share a common permanent attachment point. It is very likely that the immobilized states correspond to nanorod conformations lying flat on the surface, which would create a local potential energy surface (PES) minimum due to the strong, short-ranged van der Waals interaction.

Our experiments clearly suggest a pattern of motion for anchored nanorods that includes rapid rotation between temporary immobilization states. The fast temporal resolution data shows that the apparent nanorod “rotation” observed in low temporal resolution imaging of nanorods in the anchored state is actually numerous fast, intermittent transitions of the gold nanorod between few weakly immobilized states, with the particle in the immobilized states for the most of the time. This scenario is in stark contrast to our initial perspective that the free
end of the anchored nanorod will randomly traverse a virtual hemisphere surface for the most of the time (Figure 5.10A). Simulation showed that such a continuous random rotation model will generate a completely different scattering intensity pattern with Gaussian-profiled peaks at arbitrary peak heights (Figure 5.10B and Supporting Information 5.5.3).

Notably, the high temporal resolution (up to ns) in this study is of critical importance in understanding the immobilization-and-transition dominated behavior of anchored nanorods. Using lower time resolution will average signals so that the whole process appears as gradual transitions. Figures 5.2C and 5.2D show such an example when the data collected at μs time resolution are binned to 30 ms per frame (typical frame time for array detectors), the multiple fast transitions between the two immobilization states at 17.4~ 17.8 seconds are wiped out, providing misleading evidence for a slow, gradual rotation of the nanorod.

5.3.3 Time Scale for the Nanorod Transition between Immobilized States

The transition of an anchored nanorod from one immobilization state to another involves an erratic rotation about the fixed point on the nanorod. How fast do these transitions, or erratic rotation, happen? This information can be obtained by (1) statistical FCS analysis, and (2) closer analysis of the fast events in the original scattering intensity traces.

(1) We analyzed the ACF statistics measured in the scattering intensity traces. All of the ACFs show complicated pattern that contains multiple transition dynamics (e.g., Figures 5.2E, 5.8C, and 5.5D). For example, the ACF for the trace in Figure 5.2 can be fitted with two transition dynamics: one with a characteristic time in the ms-time scale (1.4 ms) and the other in the second-time scale (530 ms), respectively. Apparently, the slow dynamics represents the long residence times of the particle staying in one of the immobilized states that lasts for
seconds. The fast dynamics reflects the sharp peaks/dips, which could originate from the erratic rotation or the short-time immobilization. The analysis of 5 CTAB-nanorod traces shows that the fast transition has a characteristic time at ms time scale (median 1.4 ms with a range of 0.8-7.5 ms) and the slow transition at second time scale (median 0.53 s with a range of 0.3-1.5 s). Note that the short time dynamics is not caused by other interference factors such as laser instability or microscope stage vibration. Figure 5.11 shows the scattering intensity trace and the corresponding ACF of a permanently immobilized CTAB-nanorod on glass surface. The autocorrelation function drops to the background at the second data point, indicating that in the absence of the nanorod rotation, the fluctuation in the scattering intensity is random noise. Thus, the short time dynamics recovered from the ACF function is a real reflection of the fast nanorod movements.

(2) To have a better understanding of the fast events, we increased the temporal resolution and focused on these fast transition events in the scattering intensity traces. Figure 5.3A shows a typical scattering intensity trace of an anchored TAT-nanorod with an integration time of 2.0 µs. The signal appeared to be noisy at this integration time. A statistical analysis discloses that the signal fluctuation scales with the square root of the total photons counted in each unit time, indicating that the signal fluctuation is limited by shot noise. Binning the data to 200 µs time resolution (Figure 5.3B), we can identify square peaks/dips representing immobilization behavior with a width of a few ms to a few tens of ms. Thus, the short-time immobilization is reflected in the fast dynamics recovered from the ACF.

On top of the square peaks, there are many Gaussian peaks with a width of a few ms. This fluctuation may have mixed contributions from the wiggling of the nanorod in these weakly immobilized states, as well as photon shot noise. The transition times from the dark to the
bright states, e.g., 30-35 ms, and the bright to the dark states, e.g., 50-55 ms in the trace, are also on the scale of ~ms. Occasionally, a nanorod rotating back and forth can be observed during the transitions, giving Gaussian peaks during the transition (e.g., at ~20 ms and ~70 ms in the trace). Thus, we conclude that both the short-time immobilization and the fast rotational motion between the immobilization states contribute to the recovered fast dynamics in the ACF. Since their characteristic times are close to each other, we are not able to resolve them from the ACF. However, the two kinds of motions can be identified from the original traces.

More importantly, we did not observe the fast, 30 µs rotation dynamics measured in the bulk solution. A statistical ACF analysis of the anchored nanorods with a temporal resolution of 2.0 µs shows only one transition in the ACF at ms (e.g., a characteristic transition time of 7.5 ms for the trace in Figure 5.3C), which should have mixed contributions from both the short-time immobilization events and the rotation events as disclosed in Figure 5.3B. The 30 µs rotation dynamics was not observed in Figure 5.3C, confirming that: (1) the signal fluctuation in Figure 5.3A is mainly contributed by noise, and (2) the rotation of the nanorod anchored on the surface is slower than that in the bulk solution. Considering the 1.4 ms characteristic transition time from the analysis of multiple traces, the rotation of the anchored nanorods slowed down by a factor of ~50 times compared to the nanorods in free solution (30 µs).

5.3.4 Transient Adsorption/Desorption Events

For all the anchored nanorods, the ms-time scale and the second-time scale dynamics for the apparent rotation can be observed. The rotational activity varies from particle to particle, and from time to time for the same particle. Generally, the particle will lose activity gradually
over time (from minutes to hours) after being anchored on the surface. It is also noted that for some particles, the activity will increase before they desorb from the surface. Figure 5.12 shows an example of a complete transient sorption event of a CTAB-nanorod on glass surface.

In order to observe the highly dynamic sorption processes, we parked the laser spot on the solvent-glass interface and waited for the adsorption to happen. Since the adsorption event is very rare at a spot with the size of the optical diffraction limit, we used a higher concentration (1.0 × 10^{10} particles/mL) of the nanorods so that there were an increased number of encounters between particles with the surface. There were numerous sharp spikes in the scattering intensity trace due to the diffusion of the nanorods in the solution across the confocal detection volume. Occasionally, we measured transient adsorption events as in Figure 5.12. It is interesting to note that before the nanorod was stably immobilized on the surface at ~ 3.6-4.4 s, the nanorod was anchored on the surface, with active rotational motion at 3.3-3.6 s. The nanorod then experienced another anchored state from 4.4-4.7 s before it desorbed from the surface. This shows that not all anchored states progress to complete immobilization.

5.3.5 Rotation Model for Anchored Nanorods

Now we have a new and clearer picture of how anchored nanorods rotate. Unexpectedly, the apparent “continuous rotation” observed under low temporal resolution is actually numerous fast, intermittent transitions between a limited numbers of weakly immobilized states, with the nanorods spending more time in the immobilized states (Figure 5.4A). There are at least two types of “apparent rotational” dynamics of the anchored nanorods. The slower one has a time scale of seconds and reflects the residence times that the nanorod stays in any of the immobilization states. The faster dynamics are on the ms scale and include mixed
contributions from the short-time immobilization states, the wiggling of the nanorod in the
immobilization states, and the fast transitions between the immobilization states.

This view is in stark contrast to initial speculations about rotation of nanoparticles anchored
to surfaces and gives two pieces of new information: (1) Unlike that of a macroscopic object,
the movement of the anchored nanorod is very susceptible to the particle-surface interactions.
That is, the “anchored” nanorods are immobilized on the solvent-glass interface for the most
of the time. The immobilization happens at few fixed positions rather than random locations,
possibly because of the heterogeneity of the surface. For example, in Figure 5.2, the nanorod
jumps back and forth between only two weak immobilization sites. By analyzing the median
residence times of the nanorod in each of these two states, which are 13.8 and 22.4 ms
respectively, we can estimate the Gibbs free energy $\Delta G$ to be $2.0 \times 10^{-21}$ J, smaller than the
thermal energy $k_B T$ at room temperature (Figure 5.4B). If the transitions between the
immobilized states were frequent, the activation energy for the transitions between these two
states would be expected to be in the same order of magnitude. The fact that the anchored
nanorod conformations “hop” between two or few immobilization sites suggests a steep
activation energy barrier between the immobilization states. Short-range van der Waals
attractions may explain this behavior: with attractive forces of a few nm, a “lying” nanorod is
more energy-favorable than a “standing” nanorod. Thus, the nanorod spends most of the time
in the immobilized states rather than rotating in the solution when it initially anchors to a
surface. In this study, both CTAB and TAT-modified nanorods showed the immobilization-
and-transition dominated movement for the anchored particles. The CTAB-capped nanorods
have a zeta potential of 30 mV (manufacturer data), slightly higher that of TAT-modified
nanorods ($22.3 \pm 0.3$ mV). The glass surface is weakly charged under the experimental
conditions employed (pH 8.5). This possibly suggests that charge-charge interaction initiates the attachment of the nanorod to the surface. However, if there is not another charge center nearby, the interaction between the nanorod and the surface will be dominated by van der Waals force, to reduce the rotational movement of the nanorods to multiple intermittent transition patterns.

(2) The adsorbed nanorods do rotate when transitioning from one immobilization site to the other, or wiggle even when they are in a single immobilization state. However, the characteristic rotation time on the surface is significantly slower than that in the bulk solution. It increased from 30 µs in the solution to 1.4 ms on the surface, a factor of ~ 50 times slower. The possible contributing factors may include the extraordinarily large hydrodynamic friction near the surface, steric interference or other forces.

5.4 Conclusions

We used a ns-time resolution technique to study rotation of anchored nanorods on a solid-liquid interface. The results revealed the immobilization-and-transition motion for the nanorod “rotation” previously observed using slower time resolution techniques. The results also defined the time scales for two distinct states of motion, most likely correlated with the position of the nanorod relative to the surface. The data also suggest that the dominant forces controlling the motion of the nanorods anchored to a surface may be influenced by the nanoscale topography of the surface and interacting nanoparticle.
5.5 Supporting Information

5.5.1 Theoretical Rotational and Translational Diffusion Coefficient of Gold Nanorods in Solution

The theoretical rotational and translational diffusion coefficients can be estimated using equation in the literature:\textsuperscript{59,137}

\begin{equation}
D_{\text{Rot}} = \frac{3k_B T}{\pi \eta L^3} \left[ \ln \left( \frac{L}{d} \right) + \sigma \right] \tag{S5.1}
\end{equation}

\begin{equation}
D_{\text{Tr}} = \frac{k_B T}{3\pi \eta L} \left[ \ln \left( \frac{L}{d} \right) + \nu \right] \tag{S5.2}
\end{equation}

where \( L \) is the length of the rod; \( d \) is the transverse diameter of the rod; \( \eta \) is the viscosity of solvent; \( k_B \) is Boltzmann constant; \( T \) is the temperature; \( \sigma = -0.662 + 0.917(d / L) - 0.05(d / L)^2 \); \( \nu = 0.312 + 0.565(d / L) - 0.1(d / L)^2 \). For 25 × 92 nm nanorods at 298 K in aqueous solution, the estimated values for \( D_{\text{Tr}} \) and \( D_{\text{Rot}} \) are \( 8.2 \times 10^{-12} \) m\(^2\)s\(^{-1}\) and \( 4.4 \times 10^3 \) s\(^{-1}\), respectively.

5.5.2 FCS Analysis – Nanorods in Solution

Rotational and translational diffusion dynamics of gold nanorods and other rod-like particles has been studied using luminescence or scattering.\textsuperscript{59,137} Using the statistical fluctuation correlation spectroscopic (FCS) method, one can extract both translational and rotational diffusion coefficients from the scattering intensity traces. Specifically, the total autocorrelation function (ACF) \( G(\tau) \), which is defined as:

\begin{equation}
G(\tau) = \frac{<\delta I(t)\delta I(t+\tau)>}{<I>^2}, \tag{S5.3}
\end{equation}

can be described as the product of two components:
\[ G(r) = G_{Tr}(r)G_{Rot}(r). \]  

(S5.4)

\( G_{Tr} \) follows the three dimensional (3D) translational diffusion model:

\[ G_{Tr}(r) = \frac{1}{N_{Eff}} \frac{1}{\left(1 + \frac{r}{\tau_{Tr}}\right)} \frac{1}{\left(1 + \left(\frac{r_{xy}}{r_{z}}\right)^2 \left(\frac{r}{\tau_{Tr}}\right)^2\right)} \]  

(S5.5)

where \( N_{Eff} \) is the effective concentration of the diffusing entities in the focal volume; \( r_{xy} \) and \( r_{z} \) are the distances from the center to where the confocal signal drops to \( 1/e^2 \) in the lateral and axial directions, respectively; and \( \tau_{Tr} \) is the characteristic translational diffusion time:

\[ \tau_{Tr} = \frac{r_{xy}^2}{4D_{Tr}} \]  

(S5.6)

where \( D_{Tr} \) is the translational diffusion coefficient. \( G_{Rot} \) follows the free rotational model:\textsuperscript{59,137,142}

\[ G_{Rot}(r) = 1 + A \times \exp(-r / \tau_{Rot}) \]  

(S5.7)

where \( A \) is a constant that collectively accounts for rod anisotropy and experimental geometry of the detection system; \( \tau_{Rot} \) is the characteristic rotational diffusion time:

\[ \tau_{Rot} = \frac{1}{6D_{Rot}} \]  

(S5.8)

\( D_{Rot} \) is the rotational diffusion coefficient.

Figure 5.1B shows the ACF of the gold nanorod’s rotation in the bulk solution. There are two transitions in the ACF, representing the rotational and translational diffusion dynamics, respectively. This indicates that the translational and rotational diffusion dynamics are well
separated in the time scale. Using non-linear least squares (NLLS) fittings, we were able to recover characteristic translation and rotational diffusion times of 2.4 ms and 33 μs, respectively (Figure 5.1B). This corresponds to the translational and rotational diffusion coefficients of $9.4 \times 10^{-12} \text{ m}^2\text{s}^{-1}$ and $5.1 \times 10^{3} \text{ s}^{-1}$, respectively. These values agree well with the theoretical values.

5.5.3 Simulation of the Scattering Intensity of a Rotating Nanorod with One End Attached to the Surface

To validate the adsorption/rotation models, we simulated the scattering intensity traces under different models using Matlab program. Essentially, the scattering intensity was assumed to be proportional to the square of the projection of the nanorod dipole onto the polarization direction of the illumination light:

$$I_s \propto I_0 \cos^2 \phi$$  \hspace{1cm} (S5.9)

where $I_s$ is the scattering light intensity; $I_0$ is the illumination light intensity; $\phi$ is the angle between the long axis of the nanorod and the polarization direction of the laser beam.

5.5.4 FCS Analysis – Nanorods on Interface

The apparent nanorod “rotation” during the anchored state is actually numerous fast, intermittent transitions of the gold nanorod between few weakly immobilized states. To quantitate the immobilization and transition dynamics, we analyzed the ACFs of the anchored nanorods. There is no translational diffusion. So the $G_{Tr}$ terms can be removed from the total ACF term $G(\tau)$. According to the characteristics of the scattering intensity trace, we can assume that the nanorod trace is the result of multiple rotational movements and adsorption/desorption events.$^{144,145,147-149}$
\[ G(\tau) = \prod G_{\text{Rot}} \prod G_{\text{Ads}} \]  

(S5.10)

where:

\[ G_{\text{Ads}} = 1 + C \times \exp(-t / \tau_{\text{Ads}}) \]  

(S5.11)

\( C \) is the time fraction of the adsorption events and \( \tau_{\text{Ads}} \) is the reverse of the desorption rate constant.

Realizing that the autocorrelation functions of both the rotation model \( G_{\text{Rot}} \) (Equation S5.7) and the adsorption model \( G_{\text{Ads}} \) (Equation S5.10) can be described by exponentials, we can use the following equation to fit the apparent multiple dynamics irrespective of their origins:

\[ G = 1 / N_{\text{Eff}} \times \prod [1 + A_i \times \exp(-t / \tau_i)] + B \]  

(S5.12)

where \( A_i \) is an empirical pre-exponential factor\(^{59} \) and \( B \) is a constant that is used to reflect \( g(\tau) \) as \( \tau \to \infty \).\(^{143} \)

Generally, two transitions can be identified for most of the ACFs of anchored nanorod rotations, with one characteristic time in the ms time scale and the other in the second time scale (e.g., Figures 5.2E, 5.8C and S5.5D). The result is that they can be satisfactorily fitted with a two-component \( (i = 2) \) ACF model. For example, the ACF shown in Figure 5.2A can be satisfactorily fitted with Equation S5.11 with a two-dynamics model (Figure 5.2E). There may be more dynamic transitions involved but their characteristic times are too close to be resolved by current FCS analysis.

Two characteristic transition times recovered in Figure 5.2E are 1.4 ms and 530 ms, respectively. Apparently, the slow dynamics represents the long residence times of the particle.
staying in one of the immobilized states that last for ~1 s, as indicated by the trace in Figure 5.2A. The fast dynamics should have mixed contributions from the short immobilization events and the fast rotational diffusion. However, their characteristic transition times are too close to be resolved individually.
PUBLICATION CLAIM:


In this publication, my work mainly focused on data analysis and model development.
**Table 5.1.** Rotational and translational diffusion coefficients of gold nanorods in the bulk solution as a function of the distance from glass surface. The diffusion coefficients are reported as the mean plus/minus the standard deviation.

<table>
<thead>
<tr>
<th>Distance from surface (µm)</th>
<th>Characteristic rotation time (µs)</th>
<th>Rotational diffusion coefficient (rad/s)</th>
<th>Characteristic translational time (ms)</th>
<th>Translational diffusion coefficient (m²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>26 ± 7</td>
<td>6.4 ± 1.2 × 10³</td>
<td>1.6 ± 0.3</td>
<td>1.4 ± 0.3 × 10⁻¹¹</td>
</tr>
<tr>
<td>0.5</td>
<td>26 ± 7</td>
<td>6.4 ± 1.2 × 10³</td>
<td>2.7 ± 0.4</td>
<td>8.4 ± 1.2 × 10⁻¹²</td>
</tr>
<tr>
<td>1</td>
<td>26 ± 10</td>
<td>6.4 ± 1.7 × 10³</td>
<td>1.9 ± 0.3</td>
<td>1.2 ± 0.2 × 10⁻¹¹</td>
</tr>
<tr>
<td>2</td>
<td>33 ± 8</td>
<td>5.1 ± 1.3 × 10³</td>
<td>2.4 ± 0.4</td>
<td>9.4 ± 1.6 × 10⁻¹²</td>
</tr>
</tbody>
</table>
Figure 5.1 Gold nanorod diffusion and rotation in the bulk solution. (A) Resonant light scattering photon bursts of CTAB-gold nanorods with an integration time of 500 ns. (B) ACF and NLLS fitting of a gold nanorod diffusing in the bulk solution. (C) ACFs of gold nanorods diffusing in the solution at different depths from the surface. (D) Recovered characteristic rotation times of gold nanorods at different depths from the surface.
Figure 5.2 Rotation of a freshly anchored CTAB-nanorod on glass surface. (A) Scattering intensity trace collected with an integration time of 500 µs. (B) and (C) Expanded view. (D) The same trace as in (C) but binned to have an integration time of 30 ms. (E) ACF of the intensity trace and NLLS fitting.
Figure 5.3 Fast rotation dynamics of an anchored TAT-nanorod on glass surface. (A) Scattering intensity trace collected with an integration time of 2 µs. (B) Scattering intensity of the same trace binned to have an integration time of 200 µs. (C) ACF shows there is one type of dynamics at the ms-time scale.
Figure 5.4 Illustration of the immobilization-dominant movement of an anchored nanorod. (A) Schematic. The red arrow represents the polarization direction of the excitation laser beam. (B) Schematic of the PES of the nanorod-surface interaction.
**Figure 5.5** Experimental setup.
Figure 5.6 Typical confocal resonance light scattering collection profiles. (A) XY profile. Dots: experimental data. Line: non-linear least squares (NLLS) fitting with a Gaussian distribution function. The fitting shows that the standard deviation of the Gaussian distribution function is 151 nm (a beam radius of 301 nm, or a FWHM of 355 nm). (B) Z profile. Gaussian fitting shows the standard deviation to be 443 nm, (a beam waist of 885 nm, or a FWHM of 1042 nm). The scattering was from an immobilized gold nanorod.
Figure 5.7 Gold nanorod scattering intensity as a function of the orientation. (A) Dark field image of a nanorod being rotated under a microscope. (B) Maximum intensity of the nanorod as a function of the orientation angle $\phi$. (C) Definition of orientation angle $\phi$. 
Figure 5.8 Rotation of an anchored TAT-nanorod on glass surface. (A) Scattering intensity trace collected with an integration time of 500 µs. (B) Expanded views. (C) ACF.
Figure 5.9 Rotation of an anchored TAT-nanorod on glass surface. (A) Scattering intensity trace collected with an integration time of 500 µs. (B) and (C) Expanded views. (D) ACF shows two transitions: one at ~10 ms and one at several hundred ms to seconds.
Figure 5.10 Simulated gold nanorod rotation. (A) The model: one end of the gold nanorod attached to the surface while the other end randomly walks on the surface of a hemi-sphere. (B) Simulated scattering intensity change. (C) ACF calculated from the simulated intensity trace, which only shows one transition.
Figure 5.11 A scattering intensity trace and the corresponding ACF of a permanently immobilized CTAB-nanorod on glass surface. (A) Scattering intensity trace collected with an integration time of 500 µs. (B) ACF shows that the autocorrelation function drops to the background at the second data point, indicating the fluctuation in the scattering intensity is random noise.
Figure 5.12 A transient adsorption/desorption event. (A) Scattering intensity trace collected with an integration time of 500 µs. (B) and (C) Expanded views.
MOVIES

Movie 5.1. An anchored CTAB-nanorod rotating on glass surface. The video was collected using a DIC microscope with a frame rate of 30 ms and played at real time.
Chapter 6 Moving Kinetics of Nanocars with Hydrophobic Wheels on Solid Surfaces at Ambient Conditions

6.1 Introduction

Current requests for miniaturized machinery in science and technological fields have inspired the design and synthesis of molecular-sized machines. A variety of organic molecules that can generate controlled mechanical motions, e.g., motors, switches, shuttles, turnstiles, gears, gyroscopes, and elevators, have been successfully produced. Among them, a series of molecules that are able to translate on a solid surface have been synthesized and named as nanocars. Just as their names imply, the structures of the molecular family consist of chassis, axles, and wheels. Several different nanocar structures have been reported and their mobilities on solid substrates characterized. For example, three generations of nanocar molecules attached with different wheels: carbon-based C\textsubscript{60} wheels, p-carborane wheels, and adamantane wheels, respectively, were reported. Differently shaped nanocars have been designed and studied, e.g., 3-wheeled, 4-wheeled, 6-wheeled nanocars, and nanocars with an angled chassis, etc. Current research interests include upgrading the nanocar with a “motor”, a molecule (or a part of the molecule) that can convert external energy, such as electricity, heat, or light, to mechanical motions thereby fulfilling translational demands. For instances, Kudernac et al. recently designed a 4-rotary-motored nanocar that can be activated and driven directionally with a scanning tunneling microscopic tip. We have synthesized a unimolecular submersible nanomachine molecule that shows enhanced diffusion in solution upon the exposure to UV light.
The ultimate goal of developing these car-like molecular machines is to drive them at ambient conditions. To achieve this goal, the first important step is to understand how the molecular structure and the molecule-surface interaction affect the nanocars’ mobility at an air-solid interface. For example, Khatua et al. studied differently treated and chemically modified glass surfaces and found that both the surface roughness and the molecule-surface interaction affect the mobility of $p$-carborane-wheeled nanocars. It is found that the $p$-carborane wheeled cars show relatively low diffusion coefficients because of the relatively strong hydrogen bonds formed between the wheels and the glass surface. When the carborane wheels were replaced with adamantane wheels, both the percentage of the moving molecules and the diffusion coefficient increased significantly on hydrophilic glass surface.

For the new generation of nanocars with the hydrophobic adamantane wheels, the optimal driving surface has not been systematically studied. Thus, there is little understanding regarding the factors that affect the movement of these nanocars. More importantly, long-duration tracking of nanocars on a solid surface under ambient conditions has never been reported in the literature to the best of our knowledge. The time factor is important especially under the ambient conditions where there are plenty of reactive molecules, both organic and inorganic, in the air that will adsorb to and change the substrate surface properties. These factors are directly related to practical problems such as how to keep the nanocar molecules active for longer time scales. The nanocars here are thermally active and diffuse spontaneously on solid surfaces at room temperature. We study the factors that retard the movement of the nanocars on surfaces, and the inputs needed to reactivate them. A kinetics study of nanocar movement will help us understand the change of nanocar-surface interaction potential energy.
surface (PES) over time and provide useful information in re-activation of nanocars with external energy.

Due to the small size of the nanocars, scanning tunneling microscopy (STM) is the most commonly used technique in observing these single molecules on solid surfaces and for studying their moving dynamics.\textsuperscript{160-163} STM is an excellent tool that provides atomic resolution and offers topological information of substrate as well.\textsuperscript{154,162} It has been applied in studying the movement of thermally or electrically agitated nanocars on solid substrates.\textsuperscript{154,157,164} However, there are two disadvantages. (1) STM generally can only be operated in vacuum and the surface must be electrically conductive.\textsuperscript{93} (2) Due to the potential bias on the tip and the tunneling electron beam, their movements could be perturbed and efforts are required to exclude such possible influence. For example, C\textsubscript{60} on a gold surface has been observed to deform strongly near the tip.\textsuperscript{165} Several studies have reported that STM perturbs adsorbate activities and different mechanisms have been proposed.\textsuperscript{166,167} Tunneling and field emitted electrons can excite the adsorbate vibrational states, leading to desorption.\textsuperscript{167} Rezaei et al. showed that instead of simple thermal effects, the desorption rate of the adsorbates has a quartic dependence on the applied electric field.\textsuperscript{168} Interestingly, the applied electric filed on the STM tip is frequently used to manipulate the movement of the adsorbates.\textsuperscript{169}

In this study, we used confocal single molecule fluorescence microscopy (SMFM) to study the diffusing kinetics of adamantane-wheeled nanocars on different surfaces (Scheme 6.1). It has been shown that this type of nanocar can adopt a wheel-like rolling translation on the surface, similar to those C\textsubscript{60}- and carborane-wheeled nanocars.\textsuperscript{91,170} We are especially interested in understanding how the molecule-surface interaction PES changes over time after exposing the sample to the air. This can provide new information on the de-activation and
potential re-activation of nanocars on surfaces and lead to a better design of the nanocars and
the corresponding substrates.

6.2 Experimental

6.2.1 Chemicals and Nanocar Synthesis

The synthesis of four-wheeled adamantane nanocars has been reported.91 The structure of
molecule is shown in Scheme 6.1. The bodipy dyes were embedded in the nanocar chassis.
The UV-VIS and fluorescence emission spectra are shown in Figure 6.6.

6.2.2 Glass Surface Cleaning and Modification

High quality glass coverslips were purchased from Corning and used as the substrates. The
root mean square roughness of the glass surface is 0.4 ± 0.2 nm as characterized using atomic
force microscopy (AFM). To clean coverslip surfaces, they were sonicated in soap water once,
DI water 3 times, and ethanol once, each for 15 min.141 Two types of surfaces were used.

Hydrophilic glass surface: To achieve a high hydrophilicity of the glass surface, the cleaned
coverglasses were boiled in 30% hydrogen peroxide for 15 min. They were then washed with
DI water and dried in nitrogen flow. The hydroxylated surfaces were used as hydrophilic
surfaces or further modified.

PEG-modified surface: The modification of PEG on glass surface followed Papra’s
method.171 Briefly, the hydroxylated coverglasses were soaked in a solution of 1.5% (v/v%) of
[hydroxy(polyethyleneoxy) propyl] triethoxysilane (8-12 units, 50% in ethanol from Gelest.
Inc) in toluene with 0.1% (v/v%) of concentrated HCl. After reaction at room temperature for
18 h (Scheme 6.2), the glass surface was washed once in toluene, twice in ethanol, twice in DI
water, and then sonicated in DI water for 2 min to remove non-grafted materials. The modified surfaces were then dried in nitrogen flow before use.

6.2.3 Sample Slide Preparation

The synthesized nanocars were dissolved in methanol as the stock solution (~ 65 µM). Before the diffusion experiments, 7.0 µL of a freshly diluted solution in methanol (~ 0.4 nM) were drop cast onto a pre-cleaned coverglass. The solution spreads out and dries immediately. The sample slide was then thoroughly dried in nitrogen flow for ~ 1 min. In studying the diffusion kinetics, the sample slides loaded with nanocars were stored in a dark petri dish covered with aluminum foil at room temperature. The relative humidity of the environment was maintained by first cooling the ventilated air to 12 °C and then heating back to 25 °C. The measured relative humidity was 37 ± 3% during these experiments.

The freshly prepared hydroxylated glass surface is hydrophilic. Water can be observed to spread and form a thin film on the surface (negligible contact angle). After 24 hours exposure of the glass surface to the air, water droplet will form with a contact angle of 30° ± 5°. As a comparison, the uncleaned glass surface has a contact angle of 55° ± 5°.

6.2.4 Confocal Single Molecule Fluorescence Microscopy

The schematic of the homebuilt confocal microscope is shown in Figure 6.5. The 514 nm excitation light was provided by an air-cooled, wavelength-tunable Ar⁺ laser (Model 35-LAP-431-240, CVI/Melles Griot, CA). The excitation beam was circularly polarized using a quarter-wave plate (ACWP-400-700-06-4, CVI/Melles Griot, CA) and then expanded to slightly overfill the back aperture of the focusing microscope objective (CFI Plan Apo VC 100X oil, NA=1.4, Nikon Inc.). Fluorescence signal was collected using the same objective and imaged
onto a 50 µm, multimode fiberoptic (*M16L01, Thorlabs, NJ*) serving as the confocal pinhole. The signals were detected by an avalanche photo diode (*SPCM-AQRH-15-FC, Perkin Elmer*), and counted by a computer board. Sample images were obtained by scanning the sample in the XY plane using a piezo-stage (*PI Nano, Physik Instrumente*) mounted on a manual XY translational stage. The precision of the piezo-stage was 1.0 nm. Images with the dimension of 20 µm × 20 µm (or otherwise specified) with the pixel size of 100 nm were acquired with an integration time of ~1 ms/pixel. Images were acquired every 60 s until most of the molecules were bleached. The excitation laser beam was ~3 µw before the back port of the microscope. Under this excitation level, typically 20~30 frames can be acquired before most of the dyes were bleached.

### 6.2.5 Image Data Analysis

The acquired scanned images from the same area were converted to a movie using a home-written NIH ImageJ program. To track the molecule locations, the *video spot tracker software* provided by Center for Computer Integrated Systems for Microscopy and Manipulation (CISMM) from UNC Chapel Hill was used. The software reports single molecule positions up to ~1/256 pixel (or 0.4 nm for our data set). To eliminate the stage drifting effect, 3-5 immobilized molecules were used as references in the localization. The practical localization precision was 28 nm in our experiment determined by repeatedly measuring the position of the same immobilized molecule multiple times. 172
6.3 Results and Discussion

6.3.1 Single Molecule Imaging

In order to observe and track the movement of individual nanocar molecules on solid substrate surface, several important factors must be carefully controlled: (1) The solid substrate surface needs to be cleaned thoroughly to remove significant residual organics left on the surface. Interestingly, we were only able to observe these nanocars moving on thoroughly cleaned surfaces. In addition, cleaning also improves the signal-to-noise ratio (S/N) of the single molecule fluorescence images by reducing the background. (2) The laser beam needs to be focused to the diffraction limited spot so that the background can be minimized. Observing single molecules on air-solid interfaces is more challenging than on liquid-glass interface because the practical numerical aperture of the objective is smaller. To achieve this, the laser beam was expanded to slightly overfill the back aperture of the objective. The measured full width at half maximum (FWHM) resolution was ~260 nm, approaching the theoretical limit (Figure 6.5B). (3) We need to spatially separate these individual molecules so that we are observing these molecules individually. In practice, a small amount of solution (~7 µL) of very low concentration nanocar (~0.4 nM) was drop-casted onto the clean glass surface. The solvent was methanol so that the solution well-wets the surface and dries immediately. The sample was then further dried in nitrogen flow for 1 min. The expected number of nanocar molecules was ~2 molecules/µm², which is consistent with the observed molecular surface concentration in the field of the view.¹³⁸

Single molecules images can be acquired continuously from all dried surfaces with similar signal-to-noise (S/N) ratios. For example, Figure 6.1 shows the nanocar single molecule
images on a glass surface acquired continuously at 1 frame/min (for more examples, see Movies 6.1-6.6). In general, during the single molecule observation, we can notice: (1) a slight lateral drifting of the image due to the microscope stage drifting during the long-time observation (~20 min). The drifting effect on the nanocar movement can be eliminated by using the reference system as described in the Experimental section. (2) The total number of molecules decreased due to photobleaching of the dye molecules. Soaking the microscope slide in nitrogen flow reduces the bleaching rate by a factor of ~2 (data not shown) but not completely eliminating the bleaching. Generally, a series of 20 consecutive images from the same area can be obtained before most of the molecules were bleached. (3) Blinking of the nanocar molecules was also observed. For example, during the line-scanning of the image, some molecules gave a dark line in the middle of the single molecule image, indicating that they became “quiet” for milliseconds, or “blinked” in during the recorded time. These effects pose challenges in observing single molecules at the air-glass interface.

Note that not all molecules can diffuse freely on glass surface at room temperature. As will be discussed below, only when the interaction strength between the molecule and surface falls in a proper range can we observe their diffusion at our observation time scale. For example, we also imaged bodipy dye molecules on hydroxylated glass surface. The majority (>99%) of the bodipy dye molecules were permanently immobilized on glass surface, consistent with the literature reports.\textsuperscript{91,153-155} It was ascribed to the strong hydrophilic interactions between the dye and surface that hinder the molecules from translational motion at room temperature.
6.3.2 Nanocar Diffusion Coefficient on Solid Surface

Despite of the difficulties mentioned above, we were able to track the trajectories of single nanocars under all experimental conditions. To quantitatively study the mobility of nanocars, we analyzed the trajectories of ~20 nanocars in each experiment (each slide). A threshold of 100 nm displacement for the entire trajectory was applied to discriminate the immobilized molecules from moving molecules. Note that most of the immobilized molecules (>90%) show no significant movement over a period of up to 6 hours (data not shown), indicating that they belong to a different group of molecules that reflect the properties of special spots on the surface, e.g., strong surface adsorption sites. Thus, we treat them as a different group of “inactive” molecules. They are not used in estimating the surface diffusion coefficient or the diffusion activation energy of the “active” molecules.

The conventional method to estimate the diffusion coefficient was to use the mean squared displacement (MSD) method. According to the Einstein equation, the MSD $<L^2>$ of a molecule scales with the observation time interval $\Delta t$:

$$\langle L^2 \rangle = 2nD\Delta t$$

(6.1)

where $n$ is the number of dimensions (here, $n = 2$); $D$ is the diffusion coefficient. Plotting MSD as a function of $\Delta t$, $D$ can be obtained from the slope. Usually, the MSD up to ~20% of the total trajectory collection time is needed to estimate the $D$. Figure 6.2A shows one example of the MSD plot of a nanocar on a fresh glass surface, whose diffusion coefficient was $5.0 \times 10^{-16}$ m$^2$/s. The mean diffusion coefficient from 5 nanocars was $5.3 \pm 1.3 \times 10^{-16}$ m$^2$/s.

However, due to the high bleaching rate, the lengths of the trajectories were usually very limited so that the diffusion coefficient could not be accurately determined. Thus, we estimated...
the diffusion coefficient using the pooled individual steps of the molecules. Brownian diffusion of molecules can be modeled as random walks, where the molecule step size $L$ after each $\Delta t$ is normally distributed with a standard deviation of $\sigma_L$:

$$\sigma^2_L = 2nD\Delta t \quad (6.2)$$

In practice, we collected the trajectories for ~20 moving molecules for each sample slide and pooled their displacements $L$ together (100 ~ 200 individual steps). Ideally, the distribution of the absolute displacement should follow the positive half of Gaussian distribution. By fitting the distribution with the half Gaussian function, we can obtain the diffusion coefficient using Equation 6.2. To avoid the impact of the outliers, we estimated the variance $\sigma^2_L$ using the median square displacement following the relationship below for half Gaussian distribution:

$$m_{L^2} = m^2_L = [\sqrt{2erf^{-1}(1/2)}]^2 \sigma^2_L = 0.4547\sigma^2_L \quad (6.3)$$

where $m$ stands for median. Figure 6.2B shows a typical squared displacement distribution of 4-wheeled nanocars on a freshly prepared glass slide. The diffusion coefficient was $7.6 \pm 1.5 \times 10^{-16}$ m$^2$/s, where the standard deviation was generally obtained from 3 to 5 different glass slides (a total of >50 molecules analyzed for each reported D).

The two $D$s determined using the two methods are consistent in the order of magnitude. Since the individual steps were pooled from more molecules, we deem that the $D$s from the individual step method more heavily reflect the average mobilities of the nanocars on a surface. Thus, all the discussions below are based on the $D$s from individual steps.
6.3.3 Nanocar Diffusion Kinetics on Hydrophilic Glass Surface

Interestingly, we observed that nanocar movement on surfaces slowed down significantly over time. For example, Movies 6.1, 6.2 and 6.3 show that the nanocar molecules 0, 3, and 24 h, respectively, after being deposited on the hydroxylated glass surface. It is apparent that the nanocars are less active by showing more fractions of immobilized nanocars and smaller steps of the nanocar movements. Thus, as surface aging time increases, the molecular mobility decreases. After 24 h, almost all of the molecules ceased moving. To quantitatively study this decreased mobility, we counted the fraction of the moving molecules (Figure 6.2C); for the nanocars still active, we measured their diffusion coefficients (Figure 6.2D). The fraction of the moving nanocars dropped from ~50% to ~5% within the first 24 h. For the moving nanocars, the diffusion coefficient dropped from $7.6 \pm 1.5 \times 10^{-16}$ m$^2$/s to $1.8 \pm 0.4 \times 10^{-16}$ m$^2$/s. The typical trajectories of molecular diffusion at different aging times are in Figure 6.3. It is clear that the molecular steps became smaller over time. The corresponding MSD plots for 3 h and 24 h are in Figure 6.7. They deviated from a straight line possibly because the diffusion became non-Brownian as the molecular-surface interaction became stronger.

6.3.4 Activation Energy for Surface Diffusion

Why does the diffusion of the nanocars decline on an aged glass surface? To understand nanocar diffusion kinetics on surfaces, we shall first address how a nanocar diffuses on a surface. As demonstrated in previous studies, four-wheeled nanocars prefer a wheel-rolling diffusion mode rather than a hopping mode on surfaces. Generally, the surface diffusion coefficient $D$ can be estimated by:\textsuperscript{173}

$$D = D_0 \cdot \exp(-E / RT) \quad (6.4)$$
where $E$ is the activation energy for translational motion; $T$ is the temperature; $R$ is the gas constant; $D_0$ is the two-dimensional diffusion coefficient in air, which follows the Einstein-Stokes equation:

$$D_0 = \frac{kT}{4\pi \eta a}$$  \hspace{1cm} (6.5)

where $\eta$ is the viscosity of air ($1.983 \times 10^{-5}$ Pa·s) and $a$ is the radius of the nanocar (~2.5 nm). Thus, $D_0$ is $6.6 \times 10^{-9}$ m$^2$/s. Based on the measured diffusion coefficient ($2.7 \times 10^{-16}$ m$^2$/s), Khatua et al. found that the diffusion activation energy for carborane-wheeled nanocars on a glass surface was ~42 kJ/mol.\textsuperscript{93,155} Considering the wheel rotation energy barrier (4.2 kJ/mol per wheel),\textsuperscript{93,155,174} and the hydrogen bond energy between the carborane wheel and the glass surface (4.8 kJ/mol per H-bond), they concluded that the hopping mode diffusion is unlikely because it needs to break all H-bonds and has a theoretical activation energy > 65 kJ/mol.\textsuperscript{24} Thus, the wheel rolling mode is more likely, which requires lower activation energy and is more consistent with the observed diffusion coefficient. Further, increasing the interaction between $p$-carborane wheels and the surface by increasing the polarity of the surface will hinder the diffusion of the nanocars.\textsuperscript{159}

For adamantane-wheeled nanocars, it is expected that the total interaction energy between the surface and wheels will decrease because of the negligible polarity of adamantane C-H bonds.\textsuperscript{90} It was indeed observed that both the percentage of the moving molecules and the diffusion coefficient of these adamantane-wheeled nanocars increased significantly on oxygen plasma cleaned glass surface ($4.7 \pm 0.8 \times 10^{-16}$ m$^2$/s). In this study, we used hydrogen peroxide to hydroxylate the glass surface and found that the diffusion coefficient further increased to
7.6 ± 1.5 × 10⁻¹⁶ m²/s. Correspondingly, we estimated that the activation energy of nanocar diffusion reduced to ~ 39.5 kJ/mol on a freshly prepared glass surface.

This slowing trend is consistent with the repeated report that the hydrophobicity of the air-exposed silica or glass surface will increase overtime. The contact angle of a freshly prepared silica surface will change from 0 degree to 20°~40° after prolonged exposure to the air (≥24 h). The same phenomenon was observed in our lab, where water formed a uniform layer on the fresh glass surface (~0° contact angle). The surface became “dirty” after aging at ambient conditions. After 24 h of exposure of the glass surface to the air, a water droplet formed with a contact angle of 30° ± 5°. As a comparison, the uncleaned glass surface has a contact angle of 55° ± 5°. Storing the cleaned glass slide in a sealed, thus relative cleaner environment, e.g., a desiccator, extends the lifetime of the clean glass surface.

The increased hydrophobicity was ascribed to the adsorption of hydrocarbon-like contaminants from the air, which is consistent with our observation. The increased hydrophobicity might also come from the condensation of the surface silanol groups, forming more hydrophobic siloxane groups. However, under our experimental conditions of room temperature and a relative humidity of 37 ± 3 %, such reaction is unlikely to occur. In addition, it is reported that the equilibrium between the vapor molecules and the exposed silica surface is established immediately. Thus, the organic adsorbates are likely the main reason for the observed hydrophobicity increase of the surface. It further supports our hypothesis that a hydrophobic surface increases the molecule-surface interaction and hinders the movement of nanocars with hydrophobic wheels.
6.3.5 Sticky-spots Model vs. Uniform Friction Model for the Diffusion Declination

How do the nanocars slow down? There are two possible mechanisms that could be
dominant for the observed slowing down of nanocars. (1) In the first model, the slowing down
of the diffusion can be caused by the increased frictional force everywhere across the surface
due to the structural and/or chemical changes on the molecule or surface. The friction force
increases in a gradual manner so that the molecular diffusion slows down continuously. We
name this the uniform friction model (UFM). (2) In the second model, we can view that slowing
down as caused by a few special spots on the surface, e.g., organic molecules adsorbed from
the air to the surface and to form “sticky” spots that will transiently or even permanently retain
the nanocars. Outside of these sticky spots, the molecular diffusion is not affected. These
temporary retention events are short compared to the observation time resolution (60 s) so they
cannot be observed in current experimental settings. But the apparent diffusion is slowed down
because of multiple retention events during the observation window. Over time, there is an
increasing number of sticky spots forming on the glass surface so that the apparent diffusion
coefficient become gradually smaller. We name this model the “sticky-spots” model (SSM).

We do not know which model is dominant for the decline in the diffusion rate. It is
challenging to resolve these two models with current imaging temporal resolution. However,
further considering Equation 6.4, we can find out that $D$ is very sensitive to the activation
energy change. For example, on flat surfaces, when the activation energy increases by 3.6
kJ/mol (which is the typical van der Waals interaction energy level between organic
molecules),\footnote{The typical van der Waals interaction energy level between organic
molecules.} the diffusion coefficient decreases by a factor of 420\%. That is, a small change
in the surface, e.g., even a single adsorbed organic molecule, would drastically reduce the
molecular diffusion. It is unlikely that the gradual decrease of the apparent diffusion coefficient
is caused by a gradual increase of the molecule-surface interaction energy everywhere. Therefore, the SSM is more reasonable in describing what happens on the glass surface: multiple retention events of nanocars on these stick spots make them appear to diffuse more slowly, giving a smaller apparent diffusion coefficient that also decreases over time. In addition, the increased fraction of immobilized molecules also suggests that the stickiness of the surface does not increase uniformly but rather heterogeneously, i.e., some adsorbates will interact with the nanocar strongly so that the nanocar will be permanently immobilized as observed in our movies. Further, our calculation shows that under the SSM model, the surface adsorption rate of hydrocarbon-like molecules on the surface is reasonable and consistent with the literature reported values (Supplementary Information). All these suggest that the SSM is more likely responsible for the slowing down of nanocars surfaces.

6.3.6 Nanocar Diffusion Kinetics on PEG-modified Glass Surface

As a comparison, we also studied nanocar diffusion on PEG-modified glass surfaces to assess the interactions between the nanocars and the surface movement effects. PEG modification is frequently used to reduce non-specific adsorption on surfaces. As has been reported, the surface modified with PEG molecules, each containing 8-12 (CH₂CH₂O) repeat units, showed excellent monolayer formation without polymerized structures. The reported root-mean-square roughness of the surface was 0.3 nm. Therefore, the modification of the surface adds little roughness to the surface. In addition, PEG-coated surface is more hydrophilic. Thus, weaker interactions between PEG-surface and the adamantane wheels are expected.
In this study, we followed the same protocols to modify the glass surface. Movies 6.4 to 6.6 show the freshly prepared sample, the sample aged for 12 h, and 72 h, respectively. It is apparent that the nanocars were more active on PEG-modified surfaces for the fresh sample by showing a greater fraction of moving nanocars and more active movements for the moving nanocars. In addition, the nanocars on PEG surface remained active for longer time periods. Even after 3 days, diffusing nanocars on the PEG-modified glass surface were observed.

To quantitatively characterize the nanocar diffusion behavior on a PEG surface, we also counted the fraction of moving molecules (Figure 6.4A) and estimated their diffusion coefficients from the nanocar individual steps (Figure 6.4B). The diffusion coefficient of nanocars was $8.4 \pm 1.1 \times 10^{-16}$ m$^2$/s for the fresh PEG-modified glass surface, which is slightly more than that of nanocars on a bare glass surface. Correspondingly, the activation energy for adamantane-wheeled nanocars was 39.3 kJ/mol, slightly smaller than that on the hydroxylated glass surface. Interestingly, ~90% of the nanocars showed active mobility, which is ~2× higher than on a hydroxylated glass surfaces. This indicates that adamantane-wheeled nanocars tend to be more active on hydrophilic surfaces, possibility due to lower interactions between the nanocar wheels and the surface. Both the diffusion rate and the fraction of the diffusing molecules decreased similarly, with a decay time ~ 3 times longer than that on the hydroxylated glass surface.

6.4 Conclusion

We studied the kinetics of adamantane-wheeled nanocar diffusion on hydroxylated and PEG-modified glass surfaces. The apparent nanocar diffusion rate lowers gradually on both surfaces over time. The declination of nanocar surface diffusion is correlated to the increased hydrophobicity of the surface and is likely caused by the adsorption of hydrophobic molecules.
from the air. A sticky-spots model was used to explain the decreasing apparent diffusion coefficient of the hydrophobic wheeled nanocars.

6.5 Supporting Information

6.5.1 Hydrocarbon-like Molecule Deposition Rate on Clean Glass Surface in Sticky-spots Model

In the sticky spots model, it is assumed that the organic molecules in the air will adsorb on the glass surface and form “sticky” sites. There are many “sticky” sites distributed randomly on the glass surface. The nanocar molecule will diffuse slower on these sites because of higher activation energy needed. Thus, whenever a nanocar molecule encounters such a sticky site, it will be retained on the site for a much longer time, just like a macroscopic car met a “sticky spot”. The nanocar will have “on time” $t_{on}$ when it is on the sticky site, and “free time” $t_{free}$ when it is freely diffusing on glass surface. In each “on” and “free” cycle, the molecule moving distance could be described as:

$$L^2 = 4D(0)t_{free} \quad (S6.1)$$

where $L$ is the step size within a “on” and “free” cycle; $D(0)$ denotes the diffusion coefficient of a nanocar on a clean glass surface assuming no adsorbate on the surface. Generally, we deem that the diffusion coefficient measured at the fresh surface can be viewed as $D(0)$.

 Practically, the apparent diffusion coefficient $D(t)$ after aging time $t$ is determined:

$$L^2 = 4D(t)t_{total} \quad (S6.2)$$

where $t_{total}$ is the total cycle time. Since in each cycle, the nanocar will meet a sticky site once, the sticky site density ($n$) can be estimated:
\[ L^2 \cdot n = 1 \]  

(S6.3)

Thus, from S1 and S3, we obtain:

\[ t_{\text{free}} = 1/4nD(0) \]  

(S6.4)

From S1 and S2, the ratio of real diffusion coefficient and apparent diffusion coefficient could be estimated as:

\[
\frac{D(0)}{D(t)} = \frac{t_{\text{total}}}{t_{\text{free}}} = 1 + \frac{t_{\text{on}}}{t_{\text{free}}} = 1 + 4nD(0)t_{\text{on}}
\]  

(S6.5)

With time increasing, more and more organic molecules will be adsorbed on the surface and form more sticky sites. Assuming that the number of sticky sites increases linearly with respect to the aging time:

\[ n = kt \]  

(S6.6)

where \( k \) is the adsorption rate of organic molecules; \( t \) is aging time. The nanocar diffusion kinetics can be described as a linear equation:

\[
\frac{D(0)}{D(t)} = 1 + 4D(0)t_{\text{on}}k \cdot t
\]  

(S6.7)

The slope contains information about \( D(0) \), the on time \( t_{\text{on}} \), and the adsorption rate \( k \).

(1) This relationship is qualitatively consistent with the experimental data both for hydroxylated glass surface and the PEG-modified surface (Figure 6.8). Importantly, the slope for the glass surface is \(~4\) times that of the PEG modified surface, indicating the adsorption rate is much faster on the glass surface than on the PEG modified surface.
(2) More importantly, the adsorption rate of the hydrocarbon-like molecules can be estimated. From the slope for the glass surface (0.11 l/h) and the \( D(0) \times 10^{-16} \text{ m}^2/\text{s} \), we can estimate the \( \tau_{on} \cdot k \) to be \( \sim 36 \text{ (s)(#/\mu m^2/h)} \). If we further assume \( \tau_{on} \) to be on a time scale from ms to second (1 ms~1s), we can find that \( k \) to be \( 36 \sim 36000 \text{ molecules/\mu m^2/h} \). This value is consistent in the order of magnitude to the values reported in the literature. For example, Chasse et al. reported 16% of carbon coverage on Ar-implanted silicon surface with 200 h aging time in air, which gives an adsorption rate of \( \sim 0.08\% \) per hour.\(^{179}\)

These estimates may not necessarily prove that our SSM model is correct but it shows that our model is self-consistent and possible.
PUBLICATION CLAIM:

Scheme 6.1 The structure of BODIPY-embedded, adamantane-wheeled nanocar.
Scheme 6.2 Schematic of glass surface modification.
Figure 6.1 A selected series of images showing the nanocar molecules diffusing on glass surface. (A)-(D) shows the sequential images with a time delay of 1 min between images. Green circles: immobilized nanocar molecules or aggregates. The trajectories of three arbitrarily selected molecules were given as orange lines. Note most of the other molecules are moving but unlabeled.
Figure 6.2 Nanocar diffusion kinetics on hydroxylated glass surface. (A) MSD of a selected nanocar molecule diffusing on a freshly prepared glass surface. (B) Histogram of squared displacement /s of multiple nanocar molecules. (C) Fraction of moving molecules and (D) Diffusion coefficient of moving nanocar molecules as a function of surface aging time.
Figure 6.3 Typical molecular trajectories of nanocars on glass surface. (A) Freshly prepared surface. (B) Nanocars deposited on glass surface and aged for 3 hours. (C) 24 hours.
Figure 6.4 Nanocar moving kinetics on PEG-modified surface. (A) Fraction of moving molecules and (B) Diffusion coefficient of moving nanocar molecules as a function of surface aging time.
Figure 6.5 Schematic of the home-built confocal single molecule fluorescence microscope. (A) Schematic of the confocal setup. (B) Intensity profile of one selected molecule. The FWHM peak maximum is 260 nm.
Figure 6.6 UV-VIS and fluorescence spectra of 4-wheeled adamantane nanocars. (A) UV-Vis. (B) Fluorescence. Spectra were taken in 2.0 µM solution in acetonitrile.
Figure 6.7 MSD as a function of time. (A) Sample aged for 3 hours. (B) 12 hours. The non-linearity of the MSD curve possibly suggests that the surface diffusion became non-Brownian.
Figure 6.8 Ratio between $D(0)$ and $D(t)$. (A) Hydroxylated glass surface. (B) PEG-modified glass surface.
MOVIES

All movies collected at 1 frame/min and played at 15 frames/second (900 times faster). The image areas in all movies are all 20 × 20 µm².

**Movie 6.1** Freshly prepared nanocar molecules diffusing on hydroxylated glass surface.

**Movie 6.2** Nanocar molecule diffusion on glass surface after 3 h.

**Movie 6.3** Nanocar molecule diffusion on glass surface after 24 h.

**Movie 6.4** Freshly prepared nanocar molecules diffusing on PEG-modified glass surface.

**Movie 6.5** Nanocar molecule diffusion on PEG-glass surface after 12 h.

**Movie 6.6** Nanocar molecule diffusion on PEG-glass surface after 72 h.
Chapter 7 Unimolecular Submersible Nanomachines: Synthesis, Actuation and Monitoring

7.1 Introduction

Inspired by the “bottom up” approach used by nature to build functional macroscopic entities using nanoscopic buildings blocks, synthetic chemists have designed a variety of molecular machines and nanovehicles such as nanoscale motors, switches, turnstiles, barrows, shuttles and nanocars. Specifically, we have used scanning tunneling microscopy (STM) and single molecule fluorescence microscopy (SMFM) to track nanocars on surfaces. However, these imaging methods cannot be directly applied to unimolecular nanomachines in solution because they drift quickly out of focus in 3-dimensional (3D) environments, thus producing trajectories that are too short to determine accurate diffusion coefficients.

As biological processes take place in solution, the development of nanomachines that are able to enhance their diffusion and perform work in that phase is of great interest. This has led to the development of self-propelled nanowires, microrockets, Janus-particle motors, enzymatic motors and mineral micropumps powered by chemical reactions through self-electrophoretic mechanisms, bubble propulsion or difusioosmosis. However most of those micromachines use or generate toxic chemicals that are inappropriate for \textit{in vivo} applications. To address the disadvantage of using toxic chemicals, cleaner systems that convert photonic energy to translational motion have been developed. Silver chloride particles and TiO$_2$ micromotors are some examples of micromachines able to move in solution under UV light illumination via a self-diffusiophoresis mechanism.
All of the micromachines mentioned above range from hundreds of nanometers to micrometers in size. At present, there are only two examples of catalytically driven unimolecular nanomachines (< 10 nm in size) reported in the literature. These unimolecular motors consist of a ruthenium-based Grubbs’s catalyst and are powered by a ring-opening or a ring-closing metathesis polymerization. Though there are many examples of synthetic light-driven rotary molecular motors, particularly as developed by Feringa, their potential to promote solution-phase locomotion at the molecular scale remains unreported. Therefore, the development of truly molecular-sized light-driven nanomachines capable of directed motion, or promoted diffusion over a relatively long time scale (microseconds) in solution has not been reported. The main hurdle in the development of actuated unimolecular nanomachines is the smallness in size of the propelled entity. At this scale, not only are monitoring and tracking difficult tasks, but the influence of Brownian motion can be overwhelming.

Microscopic and even nanoscopic “swimmers,” residing in the domain of ultra-low Reynolds numbers, have been extensively studied by theorists: in the 1950s (Taylor and Ludwig) through the 1970s and 1980s (Purcell and Brenner) and more recently (Nelson, Zhang, Peyer and Powers); the results are now summarized in a recent book. Since inertia has no influence at these scales, macroscale swimming dynamics are inapplicable. Movement is generally accomplished by mitigating time-reversibility and escaping from the so-called “scallop” effect. Actuated diffusional increases of molecular-sized entities are predicted to be possible by some mechanical mechanisms, such as propagation of sinusoidal traveling waves along the small-sized body, or by screw-like or flexible oar-like movements.
In this study, we used single molecule fluorescence correlation spectroscopy (FCS) to monitor promoted motion of single-molecule nanomachines in solutions when being activated by UV light. As we shall see in the later discussion, in free solution, the movement of single nanomachine molecules is always under the influence of rotational and translational Brownian motions. For example, the molecule can diffuse ~ 17 nm within the shortest motor cycle time (~500 ns), assuming the nanomachine molecule has a diffusion coefficient of $10^{-10}$ m$^2$s$^{-1}$. However, when we excited the motor at a rate approaching its maximum cycling speed, we observed that the apparent diffusion coefficient significantly increased, indicating a directed motion, at least for some periods of time, when the molecular machines were activated by light. These molecules bear unidirectional rotating motors and fluorophores for optical tracking. We name these systems unimolecular submersible nanomachines (USNs). The design includes a light-driven motor functionalized at the stator with aliphatic chains that work as spacers between the motor and the fluorophores (Figure 7.1). But when the molecular motors are activated by UV light, USN-1 showed expedited diffusion by a factor of 1.26 (26%). We carefully designed and studied the diffusion of control molecules with no rotor (CM-2), a slow motor (USN-3), or a non-unidirectional spinning motor (USN-4). We found that a fast rotating motor with its 2 to 3 MHz rotational rate is critical for enhanced UV light-activated diffusion, while the non-unidirectional spinning motor (USN-4) also shows enhanced diffusion, albeit smaller. The enhancement of 26% in diffusion suggests that upon each motor actuation, the USN molecules will give a ~9-nm step, a length several times larger than its molecular size! The mechanism by which motor actuation drives the molecule in solution is still under study, but our results give new insight into the design of solution-based motorized nanomachines.
7.2 Experimental

7.2.1 Sample Synthesis and Bulk Characterization

The structures of USN-1, CM-2, USN-3, and USN-4 shown in Figure 7.1 have been synthesized and kindly provided by Dr. James Tour. The details of synthesis process and bulk characterization using NMR could be found in our recent publication. All the molecules have been attached with Cy5 dyes as a fluorophore for two reasons: it has near zero absorption at the 350-370 nm activation region of the motor and its maximum absorption region (640 nm) is optically well-separated from the activation region of the motor, minimizing the possibility of energy transfer (Figure 7.2).

To verify that no quenching of the motor was induced by the cy5, half of the rotation of the slow motor without cy5 (32) and with cy5 (USN-3) was monitored by $^1$H-NMR (Figure 7.3). Due its fast rotation, USN-1 cannot be monitored by NMR. After 1 h of UV irradiation, the unstable isomer was formed with 88% yield for motor 32 and 86% yield for USN-3. This demonstrates that the cy5 does not interfere with the photoisomerization of the motor. Then, the samples were heated at 60 °C for 1 h to facilitate the thermal helix inversion and to obtain the stable isomers. The chemical shifts returned to the original values indicating that no photo- or thermal-decomposition occurs during UV irradiation and heating.

7.2.2 Sample Preparation for Microscopic Measurements.

Cy-5 attached-USN molecules were first dissolved in ACN (Fisher Scientific Inc.) as a stock solution with a concentration of ~ 50 µM. In single molecule fluorescence correlation spectroscopy (FCS) experiments, the solution was serially diluted in ACN to a final concentration of 2.0 nM. The solution was then sandwiched between a piece of Corning No. 1.5 coverglass and a piece of glass slide using two pieces of double sided Scotch tape (~ 90
µm) as the spacers. Finger nail polish was used to seal the solution in the chamber. To study the viscosity effect on the increased diffusion by UV-light, 2,2’-thiodiethanol (TDE, Sigma Aldrich) was used to form a binary mixture with ACN at different compositions. All solutions were prepared fresh daily.

7.2.3 Confocal Single Molecule Fluorescence Correlation Spectroscopy with UV Activation

The excitation was provided by an unpolarized 633 nm HeNe laser focused to the diffraction limited spot with an output power of (~3.0 MW/cm²) (Uniphase) unless otherwise specified. The excitation beam was collimated to overfill the back aperture of a microscope objective (Nikon, 100x Plan Apo/1.40–0.7 oil-immersed). The fluorescence signal was filtered through a 655 long-pass dichroic mirror and a 684 ± 24 nm band pass filter and imaged into a piece of multimode fiberoptics (Thorlabs) and detected by an avalanche photodiode (Perkin Elmer, SPCM-AQRH-15-FC). The diameter of the fiberoptics was 50 µm (~ 0.8 AU). A programmable counting board was used for photon counting.

In the UV activation experiments, a gallium indium nitride UV LED emitting at 365 nm was used. The LED emission was filtered using a 350 ± 25 nm optical filter and focused by an oil immersion objective (NA 1.4) from the opposite side of the microscope objective. The total power of the UV light was ~10 mW after optical filter cleaning. The UV spot size was estimated to ~ 10 µm. The UV activation and no activation experiments were always collected in pairs using the same solution and at the same collection spot. The sequence of collection has no observable effect on the diffusion coefficient measurements.

The integration time was 30 ~ 100 µs, depending on the diffusion speed of the USN molecules. The acquired data were analyzed using MATLAB and Origin software.
7.2.4 Data Analysis

When a molecule diffuses into the detection volume of a confocal fluorescence microscope, a photon burst will be generated and recorded. A typical fluorescence intensity trace for USN molecules diffusing in ACN is shown in Figure 7.7. The autocorrelation function (ACF) of the intensity trace follows a three-dimensional model eq 7.1:

\[
G(\tau) = \frac{1}{\langle N \rangle} \frac{1}{1 + \tau/\tau_{\text{diff}}} \frac{1}{\sqrt{1 + \tau/S^2 \tau_{\text{diff}}}}
\]  \hspace{1cm} (7.1)

where \(\langle N \rangle\) is the average number of emitters in the probe volume; \(S\) is the aspect ratio of the probe volume \(r_z/r_{xy}\); \(\tau_{\text{diff}}\) is the characteristic diffusion time assuming that the emitter has an isotropic diffusion coefficient \(D\), eq 7.2:

\[
\tau_{\text{diff}} = \frac{r_{xy}^2}{4D}
\]  \hspace{1cm} (7.2)

where \(r_{xy}\) and \(r_z\) are the distances from the center to where the emission intensity drops to \(1/e^2\) in the lateral and axial directions. The \(r_{xy}\) and \(r_z\) were estimated to be \(\sim 300\) nm and \(\sim 900\) nm, respectively. The apparent diffusion coefficient \(D\) in the absence and presence of UV activation was obtained through NLLS fitting of the experimentally acquired data.

7.3 Results and Discussion

7.3.1 USN-1 Enhanced Diffusion Measured Using FCS

To study the natural and activated diffusion of the USNs in acetonitrile (ACN), a home-built confocal fluorescence microscope system was used (Figure 7.6).\textsuperscript{74} The cy5 dye excitation was performed at 633 nm and for the motor activation a UV LED emitting at 365 nm was used. In single molecule FCS experiments, determination of the absolute diffusion coefficient of molecules depends on experimentally adjustable parameters such as laser beam waist-size. Such parameters may vary slightly from time to time, introducing errors to the measurements.\textsuperscript{52}
To minimize these systematic errors, the FCS experiments with and without UV excitation were always collected in pairs using the same solution and at the same collection spot. Hence, the only contrast was with or without UV light illumination. The sequence of collection has no observable effect on the diffusion coefficient measurements.

In the absence of UV light activation, USN-1 diffuses freely in bulk solutions. The autocorrelation function (ACF) can be satisfactorily fitted with the 3D diffusion model (Figures S7.7 and 7.8). The diffusion coefficient \( D \) of USN-1 was \( 0.92 \pm 0.07 \times 10^{-10} \text{m}^2\text{s}^{-1} \) (95% confidence interval from Student’s t-test) from repeated measurements on different days and for different samples. This \( D \) is on the same order of magnitude for other small molecules in ACN.\(^{214}\)

When the UV light was turned on, the diffusion of USN-1 becomes faster. This can be viewed from the ACF decays. Figure 7.4A shows the normalized ACFs of 20 measurements each in the absence and the presence of UV light. It is apparent that the ACFs are bundled into two groups, with the ACFs in the presence of UV light decaying faster, indicating a faster diffusion. Figure 7.4B displays the recovered \( D \) distributions, which shows that the \( Ds \) of USN-1 in the presence of UV light are significantly larger than those in the absence of UV light. The mean and 95% confidence intervals are reported in Table 7.1. The diffusion coefficient was enhanced by a factor of 1.26 (26%). A Student’s t-test shows that with a confidence level >99.95%, the diffusions in the presence and absence of UV excitation are different.

### 7.3.2 Motor Structure Effect on Enhanced Diffusion

As a contrast, we also measured the \( D \) of a control molecule, CM-2, in the presence and absence of UV light. The only difference between USN-1 and CM-2 is that there is no rotor
moiety in CM-2. Figure 7.4C and 7.4D show the corresponding ACFs and their recovered $D$ distributions, respectively. The two bundles of ACFs completely overlap, indicating that there is no observable diffusional difference with or without UV light activation. The recovered $D$ of CM-2 shows a similar $D$ in ACN than USN-1 (Table 7.1). The lack of difference in the recovered $D$s of CM-2 indicates that UV light does not increase the diffusion of that rotorless control molecule.

To further study the relationship between the enhanced molecular diffusion and the motor activation, we studied two USNs with varied structures. USN-3 has a motor with a 6-membered ring, reducing the rotation speed to ~ 2 revolutions per hour. Figure 7.4E and 7.4F show the observed ACFs and diffusion coefficient distributions in the presence and absence of the UV excitation. Therefore, no enhanced diffusion was observed when the motor is rotating at slow speed.

USN-4 is designed without the methyl group as seen in USN-1. This structural change causes the loss of unidirectionality and subsequently the rotor randomly inverts its rotational direction. As shown in Figure 7.4G and 7.4H, the mean of the diffusion coefficients of UV-activated USN-4 was marginally enhanced by a factor of 1.10 (10%). A Student’s t-test confidence level is > 99.8%, suggesting that the diffusion of USN-4 is enhanced in the presence of UV activation.

7.3.3 Heating Effects from Light

The enhanced diffusion is not caused by the local heating effect of the excitation laser or the UV light. First, both USN-3 and CM-2 serve as excellent control molecules since they have a similar mother-ring structure and the same amount of fluorophores (cy5) as USN-1. However, their diffusion does not increase with UV excitation. Second, we further designed
control experiments to exclude the possibility of a heating effect. There are three possible sources for the heating effect: (1) solvent absorption of the excitation laser; (2) fluorophore absorption of the excitation laser; (3) motor absorption of the UV light.

(1) The heating effect caused by the solvent absorption of the excitation laser. It has been well-documented and generally accepted that a mW level laser beam will not cause significant temperature change in the solvent due to solvent absorption; this has been extensively studied by Hell.\textsuperscript{215}

(2) The heating effect caused by the fluorophore absorption of the 633 nm laser. It is generally accepted in single molecule FCS that the heating caused by fluorophore absorption at the 1.0-mW laser excitation level has a negligible effect upon diffusion. We further confirmed this by varying the 633 nm excitation laser power by a factor of 2.5 (1.2 mW). Note the window for the excitation laser power is very narrow as too much laser power photobleaches the molecules, we do not obtain sufficient signal for too low laser power.\textsuperscript{44} The corresponding ACF curves and their statistical analyses are shown in Figure 7.9. The recovered diffusion coefficients using a 3D diffusion model are $0.91 \pm 0.11 \times 10^{-10}$ m$^2$ s$^{-1}$ and $0.93 \pm 0.10 \times 10^{-10}$ m$^2$ s$^{-1}$ for the 3.0 mW and 1.2 mW excitation laser powers, respectively. Overlapping of the corresponding ACF curves and their statistical analyses show that there is no significant difference in diffusion, indicating that there is negligible local heating effect from the 633 nm laser.

(3) The heating effect caused by motor absorption of the UV light. The UV light power (10 kW/cm$^2$) is two orders of magnitude smaller than that of the 633 nm laser (3.0 MW/cm$^2$). It is reasonable to infer that the heating caused by the absorption of the UV light is also negligible. However, we noticed that there is a small difference in the UV-VIS spectra for USN-1 and its
control molecules (Figures 7.10, 7.11, 7.12, 7.13 and Table 7.3). Interestingly, the molar absorptivity of the fast rotating motors: USN-1 and USN-4 at 360 nm (15,400 M⁻¹cm⁻¹ and 14,700 M⁻¹cm⁻¹, respectively) are larger than those of CM-2 and USN-3 (6,400 M⁻¹cm⁻¹ and 7,500 M⁻¹cm⁻¹, respectively). It is likely that this difference in UV absorption is related to the excitation and subsequent rotation of the motors. To further exclude the possibility of the heating effect of the UV light due to this difference in molar absorptivity, we did another control experiment using a previously synthesized nanocar 33. Nanocar 33 has four adamantane-wheels and two-BODIPY dyes whose extinction coefficient is 64,900 M⁻¹cm⁻¹ at 360 nm (Figure 7.14). The diffusion coefficient of nanocar 33 was measured in the presence and absence of the UV light illumination on the same confocal fluorescence microscope with a 514 nm laser excitation (0.3 mW, or 0.5 MW/cm²). The corresponding ACF curves and their nonlinear least squares (NLLS) analyses are show in Figure 7.15. The recovered diffusion coefficients are 1.11 ± 0.04 (×10⁻¹⁰ m²·s⁻¹) and 1.10 ± 0.05 (×10⁻¹⁰ m²·s⁻¹) in the absence and presence of the UV light, respectively. There is no significant difference in diffusion, indicating that the heating effect due to the absorption of the UV light is negligible even for molecules with an absorption coefficient 4× larger at 360 nm. Based on these arguments, we conclude that the observed enhanced diffusion is not due to the heating effect of the excitation UV light or the laser beam. The enhanced diffusion is due to the motor actuation by UV light.

7.3.4 Motor Increased Steps in Diffusion

The enhanced diffusion for USN-1 and USN-4 molecules can only be observed when the UV photon flux is sufficiently high as our early attempts using low illumination power all failed. At the specified excitation level, the molecule should diffuse by a distance L ~ 17 nm
in the 3D space in each motor excitation cycle (~ 500 ns) according to Einstein eq 7.3 ($D \sim 1 \times 10^{-10} \text{ m}^2\text{s}^{-1}$):

$$L^2 = 2nD_0t,$$  \hspace{1cm} (7.3)

where $L^2$ is the mean square displacement; $n$ is number of dimensions; $D_0$ is the diffusion coefficient; $t$ is time interval between two motor excitations. When the UV excitation is close to or over the motor saturation level, $t$ can be approximated as the limiting cycle time of the motor. Under UV activation, eq 7.3 becomes:

$$L^2 + r^2 = 2nD't,$$  \hspace{1cm} (7.4)

where $r$ is the displacement of the USN after each actuation; $D'$ is the apparent diffusion coefficient. Note that, $r$ is randomly oriented with respect to $L$. Thus, an increased $D'$ by 1.26 times indicates that $r$, the displacement of the nanomachine under each motor stroke is ~8.6 nm, a length several times larger than its molecular size!

### 7.3.5 Viscosity Effect on Enhanced Diffusion

To investigate how the motor responds to UV light in viscous environments, the diffusion of USNs in a more viscous solution was also investigated. A viscous solvent, 2,2’-thiodiethanol (TDE, S(CH$_2$CH$_2$OH)$_2$) was used to mix with ACN to form a binary mixture. 10% of TDE was added so the dynamic viscosity of the solvent was nearly doubled (1.9 ×) while the viscosity was still low (0.65 mPa s). The diffusion coefficient of USN-1 becomes smaller in the viscous solvent by a factor of 1.7 (Table 7.2 and Figure 7.5), qualitatively consistent with Einstein-Stokes equation:

$$D = \frac{k_BT}{6\pi
u R_m}$$  \hspace{1cm} (7.5)
while USN-1 diffusion was enhanced when the UV excitation was turned on in the viscous solvent, the ratio of the enhancement in the diffusion is approximately constant. As the relative viscosity increased by a factor of 1.9, the diffusion enhancement only changed from 1.26 to 1.23. This shows that the viscosity of the solvent will not significantly affect the diffusion enhancement.

7.4 Conclusion

In conclusion, we observed that USNs bearing fast light-driven motors show increased diffusion in the solution phase when the motor is activated by UV light. We demonstrated that the motor rotation is not affected by the fluorophores. Through careful design of control molecules with no motor, a slow motor, or a non-unidirectionally rotating motor, we found that a fast unidirectional rotating motor at the MHz range is crucial for increased diffusion, but a non-unidirectional motor can also work, albeit less effectively. No significant change in the diffusion enhancement ratio with increased solvent viscosity was observed. The enhancement of 26% in diffusion suggests that the USN molecules will give ~9-nm step upon each motor actuation. While the mechanism of movement is still under study, the activated motion of the molecular-sized entities is possible in spite of Brownian motion in solution. This study provides insight in molecular designs for submersible nanomachines.
PUBLICATION CLAIM:


In this publication, the sample synthesis and sample characterization of UV-Vis, NMR are done by Victor. All FCS experiment, data analysis are done by me.
**Table 7.2.** Apparent diffusion coefficients of the USN series in the absence and presence of UV light activation. The diffusion coefficients are reported with 95% confidence intervals using Student’s t-test.

<table>
<thead>
<tr>
<th></th>
<th>$D$ (no activation) ($\times 10^{-10}$ m²·s⁻¹)</th>
<th>$D$ (UV activation) ($\times 10^{-10}$ m²·s⁻¹)</th>
<th>Diffusion coefficient ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>USN-1</td>
<td>0.92 ± 0.07</td>
<td>1.16 ± 0.10</td>
<td>1.26</td>
</tr>
<tr>
<td>CM-2</td>
<td>0.92 ± 0.07</td>
<td>0.93 ± 0.06</td>
<td>1.01</td>
</tr>
<tr>
<td>USN-3</td>
<td>0.90 ± 0.06</td>
<td>0.93 ± 0.08</td>
<td>1.03</td>
</tr>
<tr>
<td>USN-4</td>
<td>0.89 ± 0.04</td>
<td>0.98 ± 0.04</td>
<td>1.10</td>
</tr>
</tbody>
</table>
Table 7.2 Apparent diffusion coefficients of USN-1 in viscous solutions in the absence and presence of UV light activation. The diffusion coefficients are reported with 95% confidence intervals using Student’s t-test.

<table>
<thead>
<tr>
<th>TDE %</th>
<th>Viscosity (mPa·s)</th>
<th>( D ) (no activation) ( \times 10^{-10} \text{ m}^2\cdot\text{s}^{-1} )</th>
<th>( D ) (UV activation) ( \times 10^{-10} \text{ m}^2\cdot\text{s}^{-1} )</th>
<th>Diffusion Enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.34</td>
<td>0.92 ± 0.07</td>
<td>1.16 ± 0.10</td>
<td>1.26</td>
</tr>
<tr>
<td>10</td>
<td>0.65</td>
<td>0.53 ± 0.02</td>
<td>0.65 ± 0.01</td>
<td>1.23</td>
</tr>
</tbody>
</table>
Table 7.3 Molar absorptivity of USNs at 360 nm and 641 nm

<table>
<thead>
<tr>
<th></th>
<th>USN-1</th>
<th>CM-2</th>
<th>USN-3</th>
<th>USN-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>ε_{360 \text{ nm}} (M^{-1}\text{cm}^{-1})</td>
<td>15,400</td>
<td>6,400</td>
<td>7,500</td>
<td>14,700</td>
</tr>
<tr>
<td>ε_{641 \text{ nm}} (M^{-1}\text{cm}^{-1})</td>
<td>322,000</td>
<td>330,000</td>
<td>291,000</td>
<td>299,000</td>
</tr>
</tbody>
</table>
**Figure 7.1** Unimolecular submersible nanomachines (USNs) and a control molecule. (a) USN-1 with a 2 to 3 MHz unidirectional rotating motor,\(^{26}\) (b) control molecule CM-2 without a rotor, (c) USN-3 with a slow motor which operates at 2 rotations per hour\(^{27,39}\) and (d) USN-4 with a non-unidirectional preference for motor rotation. The rotor portions are shown in red, the stator portions in black, and the fluorophores (part of the stator) in blue. In this and the following figures and schemes, the four structures are drawn in conformations to underscore the motor operation. However, in reality, they will certainly have many randomly oriented conformations in solution.
Figure 7.2 Absorption spectra of USN-1, CM-2, USN-3, and USN-4 in acetonitrile (ACN).
Figure 7.3 Partial $^1$H-NMR (CD$_3$CN) spectra of half-rotation of the slow motor in 32 and USN-3. (A) Schematic representation of half rotation of the slow motor. (B) Partial $^1$H-NMR spectra of half-rotation of slow motor 32 showing 88% photoisomerization conversion and 99% thermal helix inversion. (C) Partial $^1$H-NMR spectra half-rotation of USN-3 showing 86% photoisomerization conversion and 99% thermal helix inversion. The yields of the conversion were calculated using the integration values of the methyl group (Me).
Figure 7.4 Comparison of diffusion coefficients of USNs in ACN in the presence and absence of UV light activation. (A, B) USN-1, (C, D) CM-2, (E, F) USN-3, (G, H) USN-4. (A, C, E, G) are the normalized ACFs of 20 measurements each in the presence and absence of UV light. Red: without UV activation. Blue curves: with UV. (B, D, F, H) are the histograms of recovered diffusion coefficient using nonlinear least squares fitting from the ACFS. For USN-1 and USN-4, the ACFs are bundled into separate groups in the presence and absence of the UV light, respectively, indicating their diffusion behaviors are significantly different with or without UV light illumination. Using NLLS fitting, the recovered diffusion coefficient $D_s$ of USN-1 and USN-4 in the presence of UV light are significantly larger than those in the absence of UV light (Table 7.1). The UV light was provided by a gallium indium nitride 365 nm UV LED with an intensity of ~10 mW. The UV light was optically filtered and tightly focused by a high numerical aperture objective (NA 1.4) to a spot with an estimated diameter of ~10 µm. The excitation level was ~ $1.0 \times 10^4$ Wcm$^{-2}$.
Figure 7.5 UV light - enhanced diffusion coefficient of USN-1 molecule in a more viscous solvent (ACN:TDE 9:1). (A) The normalized ACFs in the presence and absence of UV light. Red curves: without UV. Blue curves: with UV activation. (B) Recovered diffusion coefficient distributions.
Figure 7.6 Schematic of the confocal single molecule fluorescence microscope with UV illumination. The excitation can be provided by a HeNe laser (633 nm) or an Ar+ laser (514 nm) focused to the diffraction limited spot. BPF: bandpass filter; DCLP: dichroic mirror (long pass); PH: pinhole; APD: avalanche photodiode detector.
Figure 7.7 Selected fluorescence intensity trace of USN-1 molecules diffusing in bulk solution. (A) Without UV activation, (B) with UV activation. Integration time: 60 µs
Figure 7.8 Selected NLLS fitting of the ACFs of the USN molecules using the 3D diffusion model. (A) USN-1 without UV. (B) CM-2 without UV. (C) USN-3 without UV. (D) USN-4 without UV. (E) USN-1 with UV. (F) USN-4 with UV.
Figure 7.9 Heating effect of the excitation laser beam at 633 nm. A) Typical ACF curves of USN-1 molecule diffusion collected at 3.0 mW (red lines) and 1.2 mW (cyan lines) laser powers, respectively. B) A selected ACF curve and corresponding NLLS fitting with 3D diffusion model in the presence of 3.0 mW laser power; and C) in the presence of 1.2 mW laser power. The statistical diffusion coefficient is $0.91 \pm 0.11 \times 10^{-10}$ $\text{m}^2\cdot\text{s}^{-1}$ and $0.93 \pm 0.10 \times 10^{-10}$ $\text{m}^2\cdot\text{s}^{-1}$, respectively, from the fitting of 10 measurements each. There is no significant difference with respect to the excitation laser power, indicating the heating effect of the excitation laser power is negligible.
Figure 7.10 A) UV-vis spectra of USN-1. B) UV-vis spectra of USN-1 in the motor region. C) Calibration curve at 360 nm. D) Calibration curve at 641 nm.
Figure 7.11 A) UV-vis spectra of CM-2. B) UV-vis spectra of CM-2 in the motor region. C) Calibration curve at 360 nm. D) Calibration curve at 641 nm.
Figure 7.12 A) UV-vis spectra of USN-3. B) UV-vis spectra of USN-3 in the motor region. C) Calibration curve at 360 nm. D) Calibration curve at 641 nm.
Figure 7.13 A) UV-vis spectra of USN-4. B) UV-vis spectra of USN-4 in the motor region. C) Calibration curve at 360 nm. D) Calibration curve at 641 nm.
**Figure 7.14** UV-Vis spectrum of 2.0 μM of nanocar 33 in ACN. Nanocar 33 has 4 adamantane wheels and two BODIPY dyes.
Figure 7.15 Heating effect of the UV excitation light at 365 nm. The selected molecule is nanocar 33 in ACN with an extinction coefficient of 64,900 M⁻¹ cm⁻¹ at 360 nm, more than 4× larger than that of the USN-1. A) Typical ACF curves of 33 diffusion in the presence (red) and absence (blue) of UV light excitation, respectively. B) A selected ACF curve and corresponding NLLS fitting with a 3D diffusion model without UV light excitation; and (C) with UV light excitation. The statistical diffusion coefficient is $1.11 \pm 0.04 \times 10^{-10} \text{ m}^2 \cdot \text{s}^{-1}$ and $1.10 \pm 0.05 \times 10^{-10} \text{ m}^2 \cdot \text{s}^{-1}$, respectively, from the fitting of 10 measurements each. There is no significant difference between with and without UV light excitation, indicating the heating effect of the UV excitation is negligible.
Chapter 8 Conclusions and Outlook

8.1 Conclusions

In summary, we studied single particle diffusion in nanoconfined environments using anodized aluminum oxide membrane filters as the model system. To investigate nanoconfinement effect on mass transport, we constructed a super resolution STED microscope and combine it with FCS to achieve both high spatial and temporal resolutions. We conduct a series of studies toward the understanding the system both theoretically and experimentally.

We first systematically investigated small particles diffusing in membrane filters containing cylindrical nanopores using confocal fluorescence correlation spectroscopy. Our modeling shows that for particle diffusing in cylindrical pores, the autocorrelation function for conventional 3D or 1D model is no longer applicable. In medium sized pores (few hundred nanometers), we can see two diffusion dynamics: the axial diffusion and the confined lateral diffusion, in both original intensity traces and in statistical FCS analysis. The separation of axial and confined lateral diffusion dynamics provides an opportunity to study diffusions in different dimensions separately. Further, we found that to extract the accurate axial diffusion coefficient, 1D diffusion model with a Lorentzian axial collection profile needs to be used. We then experimentally studied 45 nm carboxylated polystyrene particles diffusing in 300 nm alumina pores. In PEG modified pores, the diffusion of the 45 nm nanoparticles slowed down by ~ 2 times, which can be satisfactorily explained by hydrodynamic frictions. However, because of limitation of lateral spatial resolution, the lateral diffusion coefficient could not be accurately obtained.
To obtain lateral diffusion coefficient, we built a super resolution STED microscope, which improved the spatial resolution from 250 nm up to ~75 nm. To pave the way of applying STED-FCS to investigating molecular/particle diffusion in pores, we did a series of Monte Carlo simulation to understand the system. In the study, we carefully investigated how beam/pore size ratio ($\alpha$) affects diffusion dynamics shown in ACFs. Based on our understanding, we categorized three different situations: with $\alpha < 0.25$, the ACFs could be simply fitted by the 3D diffusion model; with $\alpha > 2.5$, the ACFs could be characterized by simply the 1D diffusion model; when $0.33 < \alpha < 2.5$, the ACFs are mainly the combination of the lateral and the axial dynamics. However, with carefully binning data, the axial diffusion coefficient still could be extracted from fitting the 1D model. Based on above discussions and experimental achievable spatial resolution, the lateral and axial diffusion coefficient in the pores with a size ranging from 200 nm to 750 nm could be determined. This systematic study provides theoretical understanding for using STED-FCS to study particle/molecule diffusion in cylindrical pores.

With the high spatial and temporal resolution provided by STED-FCS, we experimentally studied 45 nm particles diffusing on membrane filters containing 200 nm pores. In this study, STED-FCS disclosed that particle keeps active lateral diffusion in the entrance of modified pores instead of adsorption as conceived in previous studies. It helps us quantitatively study how hydrophobicity of surface affects particle diffusion in the pores.

From these studies, we had a better understanding of molecular diffusion in confined space but with the freedom to move in all directions. Then, we studied particle motion with one end attached on the surface. We used a ns-time resolution technique to study rotation of anchored nanorods on a solid-liquid interface. The results revealed the immobilization-and-transition
motion for the nanorod “rotation”, which was previously observed using slower time resolution techniques and thought to be continuous rotation.

Collaborating with Tour’s group in Rice University, we conducted a series studies on monitoring and manipulating molecular nanomachies with light. The ultimate goal of this project is to use light to drive a motorized nanocar at ambient conditions. We studied the kinetics of adamantane-wheeled nanocar diffusion on hydroxylated and PEG-modified glass surfaces. The apparent nanocar diffusion rate lowers gradually on both surfaces over time. The declination of nanocar surface diffusion rate is correlated to the increased hydrophobicity of the surface, which is likely caused by the adsorption of hydrophobic molecules from the air. A sticky-spots model was used to explain the decreasing apparent diffusion coefficient of the hydrophobic wheeled nanocars. This study provides us new information on the de-activation and potential re-activation of nanocars on surfaces and lead to a better design of the nanocars and the corresponding substrates.

Then, we observed that USNs bearing fast light-driven motors show increased diffusion in the solution phase when the motor is activated by UV light. We demonstrated that the motor rotation is not affected by the fluorophores. Through careful design of control molecules with no motor, a slow motor, and a non-unidirectionally rotating motor, we found that a fast unidirectional rotating motor at the MHz range is crucial for increased diffusion; a non-unidirectional motor can also work, albeit less effectively. No significant change in the diffusion enhancement ratio with increased solvent viscosity was observed. The 26% enhancement of the diffusion coefficient suggests that the USN molecules will give ~9-nm steps upon each motor actuation. While the mechanism of movement is still under study, the
activated motion of the molecular-sized entities is possible despite of Brownian motion in solution. This study provides insight in molecular designs for submersible nanomachines.

8.2 Future Work

8.2.1 Thermal Effect on Nanocar Mobility

Our previous study in Chapter 6 of adamantane-wheeled nanocar diffusion kinetics on glass has disclosed the effect of adsorbate from air on nanocar mobility effect. More effects need to be done in controlling nanocar mobility. Among them, one important parameter is temperature. By modifying our SMFM setup with a heating system, we could heat our sample and monitor nanocar mobility. With this study, we could understand how thermal energy affects nanocar diffusion, which could provide more information to help us better control and activate nanocar in the future.

8.2.2 UV Light Driven Nanocar with Motor on Surface

Our ultimate goal is to activate and control nanocar diffusion on surface with converting UV light using motor molecules. The important step is to design nanocar structures which could efficiently absorb UV light and expedite nanocar mobility. To fulfill this goal, it is vital to test nanocar mobility under UV light illumination. Inspired by our previous study, we could first deposit nanocar molecules on modified surface, age for a while to make sure all molecules are immobilized on surface, and then use UV light to re-activate the molecules on surface. By controlling UV light exposure time, UV light power, and surface properties, we could estimate the activation energy needed to re-activate molecule on surface. This study will help us develop better structure to converting UV light to molecular kinetics energy and helps us better control molecule mobility in future.
8.2.3 USN Perturbation of Liposome Membrane

With UV light exposure, the light driven rotary molecules could rotate up to 3 MHz. The unimolecular submarine nanomachine molecules’ diffusion coefficient showed increasing to ~26% in solution in Chapter 7. Note the unique structure of the USNs: where the rotary part is hydrophobic and the two claws of molecules are hydrophilic. It is expected that the motor part would insert into the bilipid membrane while the two claws would stay on the surface of the bi-lipid layer. By encapsulating reference dyes and USN in vesicles, we could use our SMFM to monitor UV light activating rotor in USN and perturb the lipid bilayer, leading to the leak of dyes from the liposomes. We believe this study could provide the first molecular-scale mechanical (molechanical) opening of cell membranes and have the potential application in smart drug delivery, where the drug release could be triggered by UV light.
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%%%
Lateral Confined Diffusion:

g=1
while g<=15
dt=1e-5;
nstep=6000000;
D=9.67e-12;
sL=sqrt(4*D*dt);
phi=180*rand(nstep,1)/180*pi();
R0=400*1e9;xx=zeros(nstep,1);yy=zeros(nstep,1)
if g>1
    xx(1)=px;yy(1)=py;
end
k=1;Lm=zeros(nstep,1);
cps(1,1)=0;cpr(1,1)=sqrt(xx(1)*xx(1)+yy(1)*yy(1));
cps(1,2)=100*exp(-2*cpr(1,1).^2/6.25e-14);
while k<=nstep-1;
    L=sL*randn(1);
    x0=xx(k);y0=yy(k);
    x1=x0+L*cos(phi(k));
    y1=y0+L*sin(phi(k));
    r1=sqrt(x1*x1+y1*y1);
    LL=abs(L);
    for k1=1:5
        if abs(r1)<=R0
            break;
        end
        a1=(y1-y0)/(x1-x0);
        b1=-a1*x0+y0;
        a2=1+a1*a1;
        b2=2*a1*b1;
        c2=b1*b1-R0*R0;
        xxx=(-b2+sqrt(b2*b2-4*a2*c2))/2/a2;
        if (x1-xxx)*(xxx-x0)<0
            xxx=(-b2-sqrt(b2*b2-4*a2*c2))/2/a2;
        end
        x3=xxx;
        y3=a1*x3+b1;
        L1=sqrt((x3-x0)*(x3-x0)+(y3-y0)*(y3-y0));
        L2=LL-L1;
        a1=y3/x3;
        b1=-a1*x1+y1;
        a2=1+a1*a1;
        b2=2*a1*(b1-y3)-2*x3;
end


\[
c2 = (b1 - y3)(b1 - y3) + x3x3 - L2L2;
\]
\[
xxx = \frac{-b2 + \sqrt{b2b2 - 4a2c2}}{2a2} / 2a2;
\]
\[
xxx1 = \frac{-b2 - \sqrt{b2b2 - 4a2c2}}{2a2} / 2a2;
\]
\[
\text{if } \text{abs}(xxx - x1) < \text{abs}(xxx1 - x1)
\]
\[
\quad \text{xxx} = \text{xxx1};
\]
\[
\text{end}
\]
\[
x2 = xxx; y2 = a1x2 + b1;
\]
\[
x0 = x3; y0 = y3;
\]
\[
x1 = x2; y1 = y2;
\]
\[
LL = L2;
\]
\[
\text{r1} = \sqrt{x1x1 + y1y1};
\]
\[
\text{end}
\]
\[
\text{if } \text{abs}(r1) > R0
\]
\[
\quad \text{continue;}
\]
\[
\text{end}
\]
\[
\text{if } \text{isreal}(y1) < 1 || \text{isreal}(x1) < 1
\]
\[
\quad \text{continue}
\]
\[
\text{end}
\]
\[
xx(k+1) = x1; yy(k+1) = y1;
\]
\[
cps(k+1, 1) = k\times dt; cpr(k+1, 1) = \text{abs}(r1);
\]
\[
cps(k+1, 2) = 100\times \exp(-2*(x1.\times^2 + y1.\times^2) / 6.25e-14);
\]
\[
Lm(k+1) = L;
\]
\[
k = k + 1;
\]
\[
\text{end}
\]
\[
\text{if } g == 1
\]
\[
\quad da = \text{cps}(:, 2);
\]
\[
\quad cprx = xx;
\]
\[
\quad cpry = yy;
\]
\[
\text{else}
\]
\[
\quad da = [da; cps(2:k, 2)];
\]
\[
\quad cprx = [cprx; xx(2:k, 1)];
\]
\[
\quad cpry = [cpry; yy(2:k, 1)];
\]
\[
\text{end}
\]
\[
g = g + 1;
\]
\[
px = x1;
\]
\[
py = y1;
\]
\[
\text{save temppnew cprx cpry}
\]
\[
\text{end}
\]

%%%% Axial Diffusion without Boundary:

\[
\text{count} = 1;
\]
\[
\text{for cou = 1:count}
\]
\[
\text{dt} = 1e-6;
\]
\[
\text{nstep} = 6000000;
\]
D=9.67e-12;
sZ=sqrt(2*D*dt);
Z=sZ*randn(nstep,1);
rz=1.2e-6;cri=60*rz;
mm=rand(nstep,1);
if rand(1)>0.5
    sym=1;
else
    sym=-1;
end
Za(1,1)=rand(1)*rz*sym;dataz(1,1)=0;k1=2;
for k1=2:nstep
    if mm(k1,1)>0.5
        Za(k1,1)=Za(k1-1,1)+Z(k1,1);
    else
        Za(k1,1)=Za(k1-1,1)-Z(k1,1);
    end
    dataz(k1,1)=(k1-1)*dt;
    m=1;
    while m<2
        if Za(k1,1)>cri
            Za(k1,1)=cri-(Za(k1,1)-cri);
        end
        if Za(k1,1)<-cri
            Za(k1,1)=-cri-(Za(k1,1)+cri);
        end
        if Za(k1,1)>cri || Za(k1,1)<-cri
            continue
        end
        m=m+1;
    end
end
if cou==1
    Zz=Za;
else
    Zz=[Zz;Za];
end
dataz(:,1)=(k1-1)*dt;
I=1./(Zz.^2+450e-9^2);
100*exp(-2*Za.^2/rz^2);
eval(['save dataz', int2str(cou),', Za']);
end