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Identifying Mechanisms of Interfacial Dynamics Using Single-Molecule Tracking

Mark Kastantin⁺, Robert Walder⁺, and Daniel K. Schwartz^{*}

Department of Chemical and Biological Engineering, University of Colorado Boulder, Boulder, CO 80309

Abstract

The “soft” (i.e. non-covalent) interactions between molecules and surfaces are complex and highly-varied (e.g. hydrophobic, hydrogen bonding, ionic) often leading to heterogeneous interfacial behavior. Heterogeneity can arise either from spatial variation of the surface/interface itself or from molecular configurations (i.e. conformation, orientation, aggregation state, etc.). By observing adsorption, diffusion, and desorption of individual fluorescent molecules, single-molecule tracking can characterize these types of heterogeneous interfacial behavior in ways that are inaccessible to traditional ensemble-averaged methods. Moreover, the fluorescence intensity or emission wavelength (in resonance energy transfer experiments) can be used to simultaneously track molecular configuration and directly relate this to the resulting interfacial mobility or affinity. In this feature article, we review recent advances involving the use of single-molecule tracking to characterize heterogeneous molecule-surface interactions including: multiple modes of diffusion and desorption associated with both internal and external molecular configuration, Arrhenius activated interfacial transport, spatially dependent interactions, and many more.

1. Introduction

Characterizing the dynamic behavior of individual molecules at interfaces is critical for understanding such processes as colloidal stabilization, detergency, heterogeneous catalysis, affinity-based chromatographic separations, biosensors and DNA microarrays, protein aggregation and layer formation, biological membrane structure and organization, and many others. Towards this goal, single-molecule tracking allows direct observation of heterogeneous interfacial molecular behavior, often leading to new mechanistic insights. By observing molecules one-by-one, fundamental physical interactions between molecules and surfaces (e.g. hydrogen bonding versus ionic interactions) can be assessed individually, rather than as an average behavior with ambiguity regarding the relative contributions of each type of interaction.

Recent research in our group aims to understand the interfacial behavior of fluorescently labeled single-molecules (SM) using total internal reflection fluorescence microscopy (TIRFM) to restrict the excitation volume of fluorophores to the near-surface environment. Several experimental setups for TIRFM have been described in the literature along with a more extensive discussion of theoretical and practical considerations than will be discussed here.^{1,2} The small penetration depth (~100 nm) of the evanescent field used in TIRFM excitation minimizes background emission from fluorophores in solution such that individual fluorophores on a surface can be detected in the presence of fluorescent molecules in the bulk. Tracking involves the continuous spatial localization of individual

^{*}To whom communication should be addressed (daniel.schwartz@colorado.edu).

⁺These authors contributed equally to this work

molecules in the interfacial plane from adsorption to desorption. Physical properties, such as interfacial diffusion coefficients or characteristic residence times, are subsequently extracted from these trajectories and provide information about the nature of molecular interactions with the surface. An alternative approach to acquiring single-molecule data is fluorescence correlation spectroscopy (FCS),^{3,4} which is capable of much faster time resolution than particle tracking with TIRFM. However, it is often difficult for FCS to separate heterogeneous behaviors that occur on comparable time scales, because FCS requires modeling to interpret the fluorescence autocorrelation function. Also, because FCS observes a fixed volume in space, it is impossible to measure diffusion and surface residence time simultaneously as mobile molecules will diffuse out of the observation volume and this process can be confused with desorption. TIRFM particle tracking is less susceptible to these issues as it is a direct observation of molecular position as a function of time.

In SM-TIRFM, characteristic residence times or desorption probabilities are related to the energy landscape experienced by a molecule in the surface-normal direction, while diffusion coefficients yield information on barriers to movement within the interfacial plane. These two types of barriers may stem from similar molecule-surface interactions, but this is not always the case. In addition to spatial localization, one can also measure the object's fluorescence intensity in one or more wavelength ranges. Such information can identify aggregation phenomena^{5,6} (e.g. by observing discrete increments in fluorescence intensity) or macromolecular conformation as measured by resonance energy transfer (RET) efficiency between two strategically located fluorophores.⁷⁻⁹

Heterogeneous behavior can stem from differences in internal molecular conformation or orientation relative to the surface and other molecules. These distinct configurations may be characteristic to individual molecules, or a given molecule may sample multiple configurations as a function of time. Heterogeneity in the underlying surface, due to defects, surface patterning, or self-assembly processes, may also affect the dynamic molecular behavior as a function of spatial position. This feature article highlights our recent efforts to characterize heterogeneous interfacial phenomena in a variety of different systems using combinations of the basic measurable properties of molecular trajectories: adsorption and desorption, diffusion, and fluorescence intensity or emission spectra. Our results have led to new perspectives regarding the fundamental interactions between molecules and surfaces.

2. A High Throughput Approach To Single-Molecule Tracking

Compared to techniques that average molecular properties over a large ensemble (i.e. populations comparable in size to Avogadro's number) SM tracking experiments are typically limited to small sample sizes due to the fact that significant computational effort must be expended to identify and track each molecule. This is fundamentally at odds with the SM goal of characterizing heterogeneous phenomena, because the relative prevalence of each type of behavior often cannot be known a priori. If the probability of observing one type of behavior among several in a heterogeneous population is p , then $N \gg 1/p$ observations must be made in order to characterize that behavior with statistical significance. For typical SM tracking experiments, N is typically 10^2 – 10^3 , making it difficult to characterize behaviors for which $p \sim 0.01$, despite the fact that relatively rare populations may be disproportionately important to the macroscopic behavior of the system. In select cases, experiments can be designed to naturally focus on a small subset of behaviors, but this is not a good general strategy. For example, if one wishes to focus on long-lived objects, time-lapse movies with large gaps between image acquisitions will inherently ignore populations of short-lived objects.¹⁰ However, not only will this experimental design fail to characterize behaviors of long-lived objects that occur on short time scales (e.g. fast diffusion or conformational changes), but one must also have a priori

knowledge that only long-lived objects are of interest. A more robust strategy is to track and analyze all observable objects and this leads to the need for high throughput tracking methods.

Although heterogeneous behavior may stem from differences in internal and external molecular configuration, the surface itself may be heterogeneous on a variety of length scales due to variation in surface topography or chemistry and this variation may be a result of controlled patterning or uncontrolled variation in the quality of surface treatment. In order to fully understand the role of the surface in the apparent heterogeneous behavior, one cannot ignore spatial variation in molecular behavior. A recent approach to address surface heterogeneity has been to “map” a surface by collecting molecular trajectories and grouping them spatially. This technique will be discussed in detail in the following section, but we introduce it here as further motivation for high-throughput tracking methods. Mapping the physical interactions between molecules and a surface with high spatial resolution requires a high surface density of molecular trajectories.

High throughput SM tracking essentially automates the tracking process with minimal human oversight of individual trajectories that are generated from the data. Details of the algorithms we use have been provided previously and will not be repeated here.^{6,11} However, we will briefly discuss two aspects of our data collection and analysis methods that increase sensitivity to rare populations.

The first aspect is the determination of object position with super-resolution precision by calculating the center of intensity of the object’s point spread function. The positional uncertainty introduced at this stage is a function of both the signal-to-noise ratio^{12,13} as well as the specific algorithm used to calculate position.^{14,15} In this work, we use a centroid of intensity formula to calculate an object’s position, in which the (x,y) position is given by:

$$\left(\frac{\sum_i x_i F_i}{\sum_i F_i}, \frac{\sum_i y_i F_i}{\sum_i F_i} \right)$$

where (x_i, y_i) is the position of the i^{th} pixel with intensity F_i , and the sum is over all the pixels identified as associated with a single object. We have found that this localizes static objects with positional uncertainty of ~50 nm under typical conditions.¹⁶ In contrast, a Gaussian fit to the two-dimensional intensity profile of an immobilized single-molecule can yield positional uncertainty near 1 nm at the expense of significantly more computational time to perform the fitting routine.¹⁴ The added computational expense for increased spatial resolution is worthwhile when testing a molecular-level hypothesis that depends on small displacements of molecules. For example, Yildiz et al. saw that kinesin motion on microtubules proceeded in increments of ~16 nm and concluded that it moved using a “hand-over-hand” mechanism rather than an “inchworm” mechanism that predicts ~8 nm steps.¹⁷ Towards our general goal of characterizing heterogeneous (and potentially rare) molecule-surface interactions, we have found that a larger positional uncertainty of ~50 nm still allows one to distinguish between diffusive modes that stem from different molecule-surface interactions and computational effort is better used to acquire larger numbers of molecular trajectories.

Positional uncertainty leads to systematic elevation of the apparent diffusion coefficients that are extracted from single-molecule trajectories. For systems exhibiting a single diffusive mode, straightforward corrections for this error have been demonstrated.^{12,18,19} While

positional uncertainty also affects apparent diffusion coefficients in systems with multiple diffusive modes, this situation is often treated incorrectly in the literature. For example, in systems with both mobile and immobile populations of molecules, the effect of positional uncertainty is accounted for in the immobile population while no correction is made for the mobile population.^{20,21} Our recent treatment of this situation has shown that positional uncertainty systematically elevates the apparent diffusion coefficient of *each* diffusive mode in the system.¹⁶ As in the single-mode case, a straightforward correction procedure yields quantitatively accurate values of multimodal diffusion coefficients regardless of the value of positional uncertainty.

A second aspect of our data analysis involves the preferential use of integrated, or cumulative, probability distributions to analyze surface residence time and squared-displacement data for the purpose of determining the multiple characteristic residence times and multimodal diffusion coefficients of various sub-populations. In general, integrated distributions (C) are given by Equation 1 where $p(x') dx'$ is the probability of observing the value x' . Thus, the integrated distribution gives the probability of observing a value that is equal to or greater than x .

$$C(x) = \int_x^{\infty} p(x') dx' \quad (1)$$

Two examples of integrated probability distributions are shown in Figure 1. An integrated residence time distribution is shown in Figure 1a for the protein fibrinogen (Fg) on bare and functionalized fused silica (FS).⁵ To a good approximation, the integrated distribution is

expected to obey $C(t) = \sum_{i=1}^N f_i e^{-t/\tau_i}$ where t_i is the mean residence time, f_i is the fraction of population i and there are N such populations. On log-linear axes, such a distribution would appear as a straight line if there were only one population (i.e. $N=1$). This is clearly not the case for these data, and four populations are required for an adequate fit. Some of the populations contained in these distributions are very rare, with f_i as low as 0.004 for the longest-lived population on hydrophobic trimethylsilane (TMS) modified FS. As will be discussed in Section 5, these particular heterogeneous populations are believed to result from Fg aggregation in bulk solution, prior to adsorption.

Figure 1b shows integrated squared-displacement distributions for BODIPY-labeled hexadecanoic acid on a layer of TMS-modified FS as a function of temperature. As with the integrated residence time distribution, the expected integrated squared-displacement distribution may exhibit multiple exponential decays due to different diffusive modes. The

integrated distribution is given by $C(r^2, t) = \sum_{j=1}^M g_j e^{-r^2/(4D_j t)}$ where D_j is the diffusion coefficient, g_j is the relative fraction of mode j and there are M such modes. The time interval between observations of position (a.k.a. lag time) is denoted as t . As will be discussed in Section 4, these data allow us not only to identify two distinct interspersed diffusive modes, but also to independently determine the temperature dependence and corresponding apparent activation energy of each mode.

While both raw and integrated probability distributions are sensitive to heterogeneous behavior, integrated distributions can be constructed directly from experimental data while raw distributions require the choice of an arbitrary histogram bin size that could introduce artifacts into the data. However, the greater advantage of integrated distributions for characterizing potentially rare heterogeneous behaviors is that the natural “bin size” is

theoretically infinite (i.e. between x and ∞) but practically lies between x and the largest observed value of the quantity in question. Therefore, each data point in an integrated distribution represents many more observations and can be treated with greater certainty. This advantage has been demonstrated to increase sensitivity to rare populations in heterogeneous systems by several orders of magnitude.¹¹

We conclude this section with a discussion regarding dye labeling and photophysical effects like blinking and photobleaching that can potentially cause anomalous results in single-molecule fluorescence experiments. Blinking has the potential to cause objects to disappear and then reappear, affecting the perceived residence time distributions. Blinking in individual fluorophores often occurs on time scales of a few tens of ms²² and objects are unlikely to disappear anomalously if single-frame acquisition times are used that are much longer than this time scale. Photobleaching can cause objects to permanently disappear, and this is a first order process that depends on the intensity of the excitation source, among other things. High excitation intensity is also important for achieving faster acquisition times to track fast dynamics, so excitation intensity and acquisition time must be balanced between these competing factors.

In this work, several factors are used to minimize anomalies due to blinking and photobleaching. In our work on proteins, many fluorophores are attached to a single protein making it unlikely that all fluorophores will simultaneously blink to an 'off' state. Multiple labeling and the use of relatively photostable dyes (e.g. Alexa 488) also minimize the effects of photobleaching relative to the dynamics of interest. In experiments with single fluorophores, acquisition times of 200–400 ms typically eliminate blinking effects. Photobleaching in these single fluorophore systems typically occurs with time scales of tens of seconds, but the relevant dynamics in these systems occur over time scales ~ 1 s. So long as photobleaching does not coincide with physically relevant surface residence times, single-molecule methods are adept at separating a photobleaching population as these methods are sensitive to multiple populations with different characteristic surface residence times. A photobleaching population, which is generally independent of experimental conditions, can be separated from the rest and subsequently ignored.

Finally, the addition of dye to a molecule has the potential to affect the surface behavior of that molecule due to the potential addition of a variety of intermolecular forces. Due to the potential influence of labeling, we typically do not present our data as a quantity that should apply to the unlabeled species as well. Rather, we prefer to make relative comparisons within the same class of labeled molecules or to use the entire labeled molecule as a probe of its local environment. When results are presented as an absolute quantity, such as an activation energy for protein desorption, we interpret such energies relative to thermal energy or the energy of a hydrogen bond while leaving the precise molecule-surface interactions that lead to this value open to speculation.

3. MAPT

In most SM tracking experiments, one of the unstated underlying assumptions is that the surface is homogeneous. However, most solid surfaces are heterogeneous on some length scale, and this poses a major challenge to the characterization and understanding of molecular interactions with surfaces. To characterize and account for this sort of spatial heterogeneity, we have developed a new chemical imaging technique that we call Mapping using Accumulated Probe Trajectories (MAPT), in which large numbers of molecular trajectories are spatially distributed to create surface maps of fundamental physical quantities that stem from molecule-surface interactions. The resulting surface maps can be

used either to characterize the surface itself²³ or to group trajectories based on different types of spatially-dependent behavior for separate analysis of each group.²⁴

The basis of MAPT imaging is to divide the microscope viewing area into a spatial grid with bins of user-determined size. The different observable properties/events of each molecular trajectory (adsorption, desorption, occupancy, number and size of diffusive steps) are then sorted into each bin based on the spatial position at which the observation occurred. Relevant physical quantities are then calculated as a function of spatial position from the accumulated data for every bin. For example, the local adsorption rate is calculated by counting the number of molecular trajectories that start in a given bin. The raw number of adsorptions is then normalized by bin area, total time of observation, and the concentration of the molecules in solution. This normalized adsorption rate is a fundamental physical quantity measured on an absolute scale associated with the interactions between the probe molecule and the surface. We have also developed MAPT imaging algorithms to map desorption probabilities, average surface coverage (a.k.a. occupancy), mean diffusion coefficient, mean diffusion direction, and quantities associated with fluorescence intensity or emission wavelength (such as single channel intensity and multichannel resonance energy transfer data).

In MAPT, it is important to minimize drift in the sample and we have done this by mechanically fixing our flow cells to the microscope stage. Our measured rate of drift is less than 2 microns per hour, which corresponds to displacements of less than 0.2 nm per 400 ms acquisition time per frame, for example. Such a slow drift rate has a minimal effect on the localization accuracy as it is much less than the uncertainty introduced in the calculation of super-resolution object position (50 nm).¹⁶

In order to validate this chemical imaging methodology, we applied MAPT imaging to a UV photo-patterned hydrophobically-modified surface²⁵ as a model system, using a fluorescently-labeled fatty acid (BODIPY-dodecanoic acid) as a probe molecule. The photopatterned TMS surface created chemically-contrasting hydrophobic (“lines” protected by the photomask) and hydrophilic (“squares” partially degraded by UV light) spatial areas. We previously used similarly patterned TMS surfaces to demonstrate the hydrophobic affinity of fatty acid probes to hydrophobic surfaces²⁵ and connected gradients in hydrophobicity to novel nanoparticle dynamics.²⁶ MAPT images of the hydrophobic BODIPY-labeled dodecanoic acid dynamics on photopatterned TMS surfaces (Figure 2) exhibited significant contrast associated with adsorption rate, diffusion, desorption probability, and surface coverage.

MAPT images are not associated with arbitrary relative contrast mechanisms, such as point counts of individual objects used in similar single-molecule methods.²⁷ Instead, MAPT images are local spatial averages of physical properties on an absolute scale that are direct consequences of molecular interactions between a probe molecule and a surface. These physical properties formed the basis of a chemical identification strategy, allowing us to perform a set of calibration experiments on uniform surfaces with varying hydrophobicity created by homogeneous photodegradation. When we compared the results of these calibration measurements to a series of regions of interest on a surface photopatterned for 180 s (see Figure 3), we were able to correctly identify the surface chemistry within experimental error. In particular, Figure 3b shows that as one moves from region 1 to region 5, both the adsorption rate and the surface coverage systematically increase from values consistent with a homogeneous TMS surface that has been photodegraded for 180 s to values consistent with an un-degraded TMS surface.

In SM imaging, the position of each molecule can be determined with a spatial resolution smaller than that associated with the optical diffraction limit. Since MAPT imaging relies on SM measurements, it is intrinsically a super-resolution imaging technique. Indeed, we have shown the resolution limits of this technique are currently near 100 nm.²³ This spatial resolution limit is due to a number of factors, including the number of trajectories observed, mechanical drift, signal-to-noise ratio, and the mobility of the molecules on the surface. In particular, without the ability to track at least 10^4 molecules in our typical viewing areas ($130\text{ }\mu\text{m} \times 65\text{ }\mu\text{m}$), the density of observations would be too small to generate high-resolution MAPT images with sufficient statistical certainty.¹¹

MAPT also allows us to quantitatively analyze and compare dynamic behavior in distinct spatial regions. For example, we have visualized heterogeneous spatial adsorption of proteins at the oil-water interface and directly connected this to the formation of an interfacial protein layer.²⁸ We have also used heterogeneous MAPT images to investigate novel modes of desorption-mediated diffusion at the solid-liquid interface;²⁹ these examples will be revisited in Section 4 of this paper.

In another example of the utility of MAPT, the ability to perform spatially selective analyses of molecular trajectories provided important insight into the dynamics of a fibrinogen (Fg) film forming on nanostructured melt-drawn high density polyethylene (HDPE).²⁴ A previous AFM study of Fg layer formation on ultra high molecular weight polyethylene (UHMWPE), with similar nanostructure properties to HDPE, demonstrated that a well-organized Fg layer formed at high surface coverage in which Fg molecules were aligned with rectangular crystalline domains that protruded out of the interconnecting amorphous region by a few nm.³⁰ These organized and aligned Fg layers did not form at either low surface coverage of Fg, or when UHMWPE was treated with Ar plasma to render it hydrophilic. MAPT was used to complement the AFM study³⁰ of Fg behavior on nanostructured polyethylene by providing dynamic information at both high and low protein surface coverage, under conditions that were nominally identical to the previous AFM study. While low Fg surface coverage is naturally suited to single-molecule microscopy, SM resolution in the high coverage situation was obtained by using a very small fraction of labeled Fg within a larger population of unlabeled Fg. This strategy allowed us to sample the behavior of Fg in a dense protein layer using the labeled Fg molecules as representative probes.

On hydrophobic HDPE at high Fg surface coverage, a surface occupancy map revealed elongated areas of high Fg occupancy (Figure 4) whose orientation corresponded to the drawing direction of the film. While the elongated areas had particularly high values for surface occupancy, the majority of the surface was sampled by Fg molecules but had low mean surface occupancy. Having identified these distinctive spatial regions using MAPT, it was then possible to separately characterize the dynamic molecular behavior in regions of high versus low occupancy. Interestingly, Fg was found to have extremely long surface residence times in the elongated high occupancy areas (greater than a 100-fold increase over low occupancy areas). Furthermore, diffusion was observed to be anisotropic in these areas as Fg was confined to move up and down individual columns of crystalline domains but could not jump between adjacent columns. These behaviors were not observed in areas of low occupancy. Furthermore, there was no observation of long residence times or anisotropic diffusion under conditions of low Fg coverage on hydrophobic HDPE or on hydrophilic HDPE even at high Fg coverage. These conditions were previously demonstrated via AFM not to induce Fg alignment.³⁰ This suggested that Fg alignment by the underlying hydrophobic HDPE film greatly enhanced protein-protein interactions and led to increased film stability, a result that may be important for the design of artificial joint surfaces in which stable Fg layers could be used as a natural lubricant to reduce joint wear.

These insights would not have been possible without spatially sensitive analyses because the vast majority of Fg that interacted with HDPE did not exhibit long residence times and anisotropic diffusion. The rare population that exhibited these behaviors could easily have gone unnoticed in an analysis of all observed molecules without regard to spatial position. For example, many molecular observations represented transient interactions involving protein that was blocked from film incorporation by existing, unlabeled protein, and never exhibited behaviors representative of incorporation into the aligned film. Thus although long residence times and anisotropic diffusion are expected to be a common feature of all Fg molecules in an aligned protein film, single-molecule observations of these behaviors will be rare within the larger population of all molecules that interact with the surface; MAPT increases sensitivity to these rare observations.

4. Interfacial Dynamics of Surfactants Illustrate Heterogeneous Interactions

Interfacial diffusion is fundamentally different from diffusion in bulk aqueous environments. In the bulk, diffusion is driven by isotropic, random collisions between molecules. However, interfacial transport (adsorption, diffusion and desorption) is primarily driven by molecule-surface interactions that provide barriers to molecular relocation. In heterogeneous systems, these different transport mechanisms can be easily confused, making it difficult to test mechanistic hypotheses. For example, ensemble-averaged measurements of surface adsorption, acquired with quartz crystal microbalance or surface plasmon resonance techniques, measure the net accumulation of mass on a surface rather than adsorption and desorption independently. However, these techniques are advantageous in that they do not require the use of fluorescently labeled molecules. Fluorescence recovery after photobleaching (FRAP) measures a mean interfacial diffusion coefficient, which may represent contributions from different diffusive modes and can be affected by molecular desorption and re-adsorption. This section illustrates the use of SM tracking to measure each of these interfacial transport processes independently and directly, and how these measurements can be used to understand heterogeneous interactions between molecules and surfaces. Adsorption and diffusion are the primary focus of this section whereas desorption is considered extensively in subsequent sections.

In our research, fatty acid adsorption at the solid-liquid interface has served as a valuable model system for investigating dynamics associated with molecule-surface interactions. Hexadecanoic acid adsorption at the fused silica-water interface was found to be an Arrhenius activated process,³¹ in which the adsorption rate (k_{ads}) varied with temperature (T) according to $k_{ads} = A e^{-E/(RT)}$ where E is the activation energy, R is the ideal gas constant, and A is an Arrhenius prefactor. The activation energy of adsorption was calculated by measuring the adsorption rate at many temperatures and fitting a line to a plot of $\ln(k_{ads})$ versus $1/T$. The activation energy of $19 \pm 2 \text{ kJ mol}^{-1}$ was similar to the bond energy associated with an OH---O hydrogen bond, leading to the hypothesis that the activation energy for adsorption is directly associated with the strength of the solvent-surface interaction, i.e. the energy required to remove a solvent molecule from a potential adsorption site. Given the high polarity of the fused silica surface, this hypothesis was tested by using solvents with different polarities.³² Water, which was the most polar solvent used, had the highest activation energy for adsorption. Tetrahydrofuran (intermediate polarity) had a lower activation energy of $10 \pm 2 \text{ kJ mol}^{-1}$, and hexadecane (lowest polarity) had the lowest activation energy at $5 \pm 2 \text{ kJ mol}^{-1}$. The systematic increase in these activation energies with solvent polarity and, therefore, with the expected strength of solvent-surface interactions suggested that adsorption of fatty acids from solution can be viewed as a competitive exchange between amphiphilic solutes and surface-bound solvent in which the activation energy for adsorption is related to the strength of solvent-surface interactions.

Diffusion of fatty acids on solid-liquid interfaces was also extensively studied using SM tracking.^{23,25,29,33,34} Interestingly, we typically observe that fatty acid molecules exhibit diffusive trajectories that are consistent with multiple interspersed diffusive modes. For example, a given molecule will often undergo diffusion associated with a particular diffusive mode for a period of time, then switch to a different mode, and perhaps back again. In one example, BODIPY-labeled hexadecanoic acid at the hexadecane-FS interface was shown to have multiple modes of diffusion, where one diffusive mode involved slow confined motion within surface compartments and the other was associated with jumps between these compartments.³³ Arrhenius analysis can also be applied to interfacial diffusion. To calculate the activation energy of a diffusion mode, we measured the diffusion coefficient of different modes as a function of temperature, then followed the previously described Arrhenius analysis of the adsorption rate. The Arrhenius analysis of these diffusion modes revealed that the jumping mode of diffusion had an activation energy of $\sim 50 \text{ kJ mol}^{-1}$ and the confined diffusive mode had an upper limit of $\sim 10 \text{ kJ mol}^{-1}$.

These two diffusive modes were investigated further with a series of fatty acids (with alkyl tail lengths of 2, 4, 10 and 15 carbons) on a hydrophobic ($\sim 90^\circ$ water contact angle) TMS surface.³⁴ By increasing hydrocarbon chain length, the hydrophobic interaction between the fatty acid and the surface was systematically controlled. Diffusion data from this series of fatty acids on TMS once again showed two modes of diffusion, with each mode being an Arrhenius activated process (see Figure 1b). Whereas the activation energy of the slow mode was independent of tail length, the fast “jumping” mode of diffusion exhibited an apparent activation energy that systematically increased with increasing hydrocarbon chain length for probe molecules with lengths between 2 and 10 carbons. These activation energies, representing interactions between probe molecule and surface, were consistently equal to $\sim 60\%$ of the total hydrophobic interaction energy required to solubilize the alkyl tail in water.³⁵ This suggested that the activation barrier associated with the jumping mode involved partial removal of the extended hydrophobic tail from the hydrophobic surface. Interestingly, the activation energy for a 15-carbon tail was less than either the 4 or 10 carbon tails, consistent with the expected hydrophobic collapse of long alkane chains that can reduce tail-surface interactions.

Collectively, these results suggest that most of the transport processes associated with molecule-surface interactions are Arrhenius activated processes. Many of the measured activation energies correspond to identifiable physical processes, such as the energy required to displace a solvent molecule or the strength of the hydrophobic interaction. The ability to extract activation energies and connect them to real physical processes is a powerful application of the SM approach. In contrast, ensemble averaged measurements of adsorption, desorption, or diffusion would average over all modes of molecule-surface interactions simultaneously. The averaged *apparent* activation energy derived from these measurements would provide only minimal physical insight because of ambiguity in the relative contributions from each fundamental physical process that contributed to the average behavior.

MAPT imaging has given us tools beyond Arrhenius analysis to investigate the mechanisms of transport on surfaces. In one example of this, we used MAPT to investigate the mechanisms of BODIPY-labeled dodecanoic acid diffusion on photo-degraded TMS surfaces.²⁹ Specifically, we used the heterogeneity of the surface itself as a way to separate different modes of diffusion. In Figure 5, MAPT images of (a) surface coverage, (b) mean diffusion, and (c) diffusion direction are shown for dodecanoic acid on a photodegraded TMS surface. The surface coverage MAPT image (Figure 5a) shows three small regions of high surface coverage separated by gaps of negligible coverage. These three regions likely represent hydrophobic “patches” that survived the partial photo-degradation. The mean

diffusion MAPT image (Figure 5b) shows that the areas of high surface coverage exhibit slow diffusion, while the empty regions between the regions of high surface coverage exhibit fast diffusion coefficients. In the MAPT image depicting diffusion direction (Figure 5c), the diffusive steps between the upper and lower regions are in the vertical direction (blue colors) while diffusion between the two upper islands is horizontal (yellow/orange). By convention, diffusive steps are mapped at the midpoint of their initial and final positions causing steps from one region to another to appear *between* these regions in Figure 5c. The combination of fast, directed diffusion in areas where there is no surface coverage led us to the conclusion that these were desorption-mediated diffusion steps between regions of high surface coverage. In other words, adsorbed molecules diffused slowly within a given hydrophobic patch and sometimes jumped from one patch to another through the adjacent solvent phase.

One important experimental consideration was the possibility that these apparent desorption-mediated diffusion events might have been artifacts due to the coincidental desorption of one probe molecule and the near-simultaneous adsorption of another. Using independently measured adsorption and desorption rates, we calculated that this would account for only ~1% of the observed displacements. Therefore, we concluded from these MAPT images that two modes of diffusion were occurring on surfaces. The slow “crawling” mode of diffusion ($0.01 \mu\text{m}^2 \text{s}^{-1}$) occurred on atomic/molecular length scales and likely involved continuous motion with at most partial detachment from the surface. The desorption-mediated “flying” mode ($0.18 \mu\text{m}^2 \text{s}^{-1}$) of diffusion occurred when the molecule completely detached from the surface, diffused in the local aqueous subphase and then reattached to the surface.

Comparing the diffusion coefficients on the heterogeneously photo-degraded, “patchy” surface to more homogeneous fused silica and TMS surfaces revealed that these two modes of diffusion also occur on chemically uniform, solid-liquid surfaces rather than being a unique feature of patchy surfaces. Interestingly, the “flying” mode of diffusion was the dominant mode of transport on all of these surfaces, typically accounting for >85% of diffusion steps.

We had previously investigated surfactant diffusion where this type of desorption mediated diffusion was measured indirectly. Using FRAP, we investigated the mobility of phospholipids at the oil-water interface.³⁶ In that case, we systematically changed both the fractional area coverage of the phospholipids and the viscosity of the oil phase. For viscosities below 10^4 cP, we found the diffusion followed the expected Stokes-Einstein scaling for “disks” immersed in both the oil and water phases simultaneously. However, above 10^4 cP we found the diffusion coefficient gave a constant value that was the same order of magnitude as the flying mode that was observed at solid-liquid interfaces. It seems plausible that the flying mode also occurs at liquid-liquid interfaces. Indeed, these FRAP results indicated the flying mode and Stokes-Einstein diffusion were occurring simultaneously. However, the flying mode is the dominant mode of surfactant diffusion at high oil viscosities (when the fluid interface is analogous to a solid-liquid interface), while the Stokes-Einstein mode is dominant at lower viscosities. Furthermore, we have directly observed this same behavior using SM tracking of single BSA proteins at the oil-water interface.³⁷ It is important to emphasize that these two modes of diffusion are always occurring simultaneously at all oil viscosities. While the magnitude of the flying diffusion is constant at all viscosities, the Stokes-Einstein diffusion decreases with increasing oil viscosity. Therefore, the flying diffusion dominates at high viscosities because the Stokes-Einstein mode is significantly slower than the flying mode at oil viscosities greater than 10^4 cP. However, at oil viscosities below 10^4 cP, the Stokes-Einstein mode is significantly faster than the flying mode.

An important application of surface transport of surfactants is in understanding the mechanisms of the nucleation and growth of self-assembled monolayers. In previous studies of the growth of self-assembled monolayers, a surface diffusion coefficient was calculated from a kinetic population balance model by using the growth rates of self-assembled monolayer patches measured from AFM images.³⁸ Until the surfactant (octadecylphosphonic acid) attaches to a small, self-assembled island of adsorbate molecules, the interactions between the phosphonic acid and the surface will be of primary importance. Therefore, the dynamics of the octadecylphosphonic acid in the beginning of self-assembly and the fatty acids observed in our SM tracking experiments should be similar (with modest variations due to different experimental conditions). Given that the crawling mode is nearly 30 times slower than the diffusion coefficient from the kinetic population balance model for octadecylphosphonic acid ($0.29 \mu\text{m}^2 \text{s}^{-1}$), it seems unlikely that crawling could be the mechanism of diffusion for these self-assembled monolayers. The diffusion coefficient from the kinetic population balance model used in AFM studies was actually somewhat faster but within a factor of 2 of the flying mode diffusion coefficient that we measured using SM tracking. The similarity between the numerical value of the flying mode diffusion coefficient from SM studies and the ensemble-averaged surface diffusion coefficient led us to conclude that the flying mode is the primary transport mode for the formation of self-assembled monolayers.

Surfactants are a useful model system for understanding fundamental surface transport, and their transport to, on, and from interfaces has proven to have far richer and more diverse dynamics than previously thought. SM tracking has revealed many modes of surfactant diffusion, each with distinct mechanisms. Analysis of this multimodal diffusion behavior has given new insights into important processes, such as the formation of self-assembled monolayers. These model systems also provide a basis for understanding the interfacial behavior of more complicated macromolecular adsorbates, such as proteins or DNA.

5. Oligomer Populations in Adsorbing Proteins

We have also observed interesting heterogeneous behavior in proteins adsorbing at solid-water and oil-water interfaces.^{5,6} Models of protein adsorption, and subsequent film formation, at interfaces often assume protein monomers adsorb in random locations and remain fixed in place thereafter, neither desorbing nor diffusing.³⁹ Our SM studies of adsorbed proteins both at solid-water and oil-water interfaces universally show that these assumptions are unwarranted, and that protein molecules are generally highly-mobile and often relatively short-lived on surfaces.^{5,6,28,40} Moreover, we have found that protein oligomerization/aggregation in solution, prior to adsorption, is an important factor that affects post-adsorption behavior.

At the silica-water interface, the presence of Fg aggregates/oligomers was detected by the observation of protein objects with different fluorescence intensities that were roughly integer multiples of the lowest observed intensity. Thus dimers were twice as bright as monomers, trimers were three times as bright, and so on. Rather than forming on the surface, we observed these aggregates adsorbing from solution. The presence of aggregates in solution was confirmed by ultracentrifugation, size exclusion chromatography (for both labeled and unlabeled protein) and dynamic light scattering although the exact distribution of aggregation numbers was difficult to infer from these measurements.^{5,41,42}

SM techniques allowed the separation of molecular trajectories by their fluorescence intensities for separate analysis and identification of the effect of aggregation on Fg behavior. As seen in Figure 6, increasing aggregation number caused an exponential increase in the mean residence time and an exponential decrease in the mean diffusion

coefficient on hydrophilic FS, FS hydrophobically modified with TMS, and FS modified with a polyethylene glycol brush (PEG, MW = 5000 Da).⁵ That is, bigger aggregates remained on surfaces longer and diffused more slowly. In related experiments at the oil-water interface, the hydrodynamic radii (calculated from a modified Stokes-Einstein relation) of bovine serum albumin (BSA) explicitly showed the presence of monomers, dimer and trimers. Similar trends were seen in BSA on FS surfaces,^{43,44} suggesting that one must generally consider population heterogeneity due to oligomers in solution when studying these types of systems.

When studying proteins with multiple fluorescent labels, there is a statistical distribution of labels on any given protein, which along with photobleaching and blinking, causes the fluorescence intensity to vary somewhat within a given oligomer population. However, this variation is not so large as to preclude identification of monomers, dimers, trimers, etc. For example, using the expected Poisson statistics for labeling free amines on each protein, Fg was labeled with 15 ± 4 fluorophores; dimers and trimers are expected to have 30 ± 5 and 45 ± 7 fluorophores, respectively, permitting robust resolution of these species. There is less separation between oligomer species for BSA, which was labeled with 5 ± 2 fluorophores, dimers with 10 ± 3 fluorophores and trimers with 15 ± 4 fluorophores. Nevertheless, ranges of intensity corresponding to each species could still be identified. Furthermore, it is important to note that there is an unmistakable correlation of both residence time and diffusion with nearly integer multiples of fluorescence intensity. These trends cannot be comprehensively explained by photobleaching, blinking, or variation in labeling.

Although both Fg at the solid-water interface (for all different surface chemistries) and BSA at the oil-water interface exhibited longer residence times for larger oligomers, the mean residence times for the monomer population were relatively short at about 1 s. More recent temperature-dependent single-molecule studies of BSA and Fg on hydrophilic FS and hydrophobic TMS surfaces identified apparent activation energies for desorption of the different oligomers.⁴⁰ The apparent activation energy for desorption increased with aggregation number, from $1\text{--}2 k_B T$ for monomers to $3\text{--}5 k_B T$ for trimers. Notably, these energies (as well as the apparent activation energies associated with surface diffusion) were small in absolute terms, suggesting that direct interactions between the surface and individual isolated proteins are relatively weak.

Despite the fact that the vast majority of all protein species are short-lived at the interface, with low activation barriers for desorption and diffusion on a variety of surface chemistries, there are clear differences in ultimate protein coverage for different surfaces in the literature. For example, PEG is often able to resist high protein coverage when exposed to blood or serum^{45–47} while hydrophobic surfaces tend to foul more easily.^{48–50} Certainly the picture surrounding PEG-protein interactions is complicated; while lower grafting densities may facilitate protein penetration into the PEG layer,⁵¹ higher grafting densities may force the exposure of the hydrophobic, methoxy-terminated PEG ends in a fully stretched brush.⁴⁷ Both effects could explain increased PEG-protein attractions at the single-protein level. Nevertheless, the PEG grafting density of 31 monomer units nm^{-2} used in this work has been shown to confer protein resistance in human blood serum.⁵² These apparently conflicting perspectives on protein-surface interactions may be reconciled by the view that the interface can play an indirect role in mediating the protein-protein interactions that may be the major determinant in the behavior of the protein layer. For example, surfaces that promote higher quasi steady-state surface coverage (as determined by considering only the adsorption and desorption rate of individual proteins) and fast diffusion will lead to more protein-protein collisions and potentially more layer-stabilizing interactions. It may also be true that protein-protein interactions can affect the way that each individual protein interacts

with the surface. Thus an exciting direction for future studies is to use SM techniques to probe protein-protein interactions in the near-surface environment.

6. Protein Aggregation at the Oil-Water Interface

At the dilute protein concentrations (10^{-12} – 10^{-15} M) typically used in SM tracking experiments, protein-protein interactions are negligibly rare events because of the large average separation (~ 10 μm) between single-molecules on the surface. SM tracking becomes problematic at higher fluorophore surface concentrations; when the fluorophores are too dense, it becomes impossible to uniquely identify one molecule from another between adjacent image acquisitions. Therefore, we often use unlabeled proteins mixed with a small fraction of fluorescently-labeled proteins to probe protein-protein interactions at higher surface coverage. In particular, we use the same dilute concentrations of fluorescently labeled protein as in standard SM tracking experiments mixed with orders of magnitude higher concentrations of unlabeled protein, controlling the frequency of protein-protein interactions by varying the bulk aqueous concentration of unlabeled protein. This allows us to investigate interfacial phenomena that occur at much higher surface concentrations than are typically used in SM tracking experiments.

Tracking fluorescently labeled tracer molecules is qualitatively similar to particle tracking microrheology (PTM).^{53,54} In PTM, the positions of micron-scale beads are tracked over time and their diffusion coefficients are used to determine the local viscoelastic properties of the region surrounding the bead. Using PTM at interfaces, the formation of a protein layer can be *indirectly* monitored through the change in viscoelastic properties of the interface. In our SM tracking approach, the individual proteins that we track are constituents of the interfacial protein layer. Therefore, the changes in dynamics we observe are a *direct* measure of the molecular changes to the proteins themselves during protein layer formation. While these two approaches are qualitatively similar, the direct quantitative information about the proteins using our SM approach has allowed us to investigate the mechanisms of protein layer formation in ways that PTM and other ensemble averaged measurements cannot.

We used this approach to investigate the formation of an interfacial protein layer at the oil-water interface.²⁸ In this case, we mixed fluorescently labeled BSA at 10^{-5} $\mu\text{g mL}^{-1}$ with unlabeled BSA at concentrations ranging from 0.5 $\mu\text{g mL}^{-1}$ to 25 $\mu\text{g mL}^{-1}$. We then tracked fluorescently labeled BSA using single-molecule TIRFM. The mean diffusion coefficient of BSA objects as a function of time (Figure 7A) revealed a multistep process of protein layer formation. First, there was an initial induction period in which the diffusion coefficient did not change from its initial value (in the range of 0.19–0.23 $\mu\text{m}^2 \text{s}^{-1}$). The diffusion coefficient then showed a steady decrease and eventually leveled off at approximately 0.05 $\mu\text{m}^2 \text{s}^{-1}$.

This multi-step behavior is qualitatively similar to measurements of dynamic interfacial tension during protein layer formation.^{55–57} Direct comparison to our data reveals that changes in diffusion occur six times faster than any change in interfacial tension and orders of magnitude faster than any changes in interfacial rheology. Thus, SM tracking is significantly more sensitive to the earliest stages of the formation of an interfacial protein layer than interfacial tension or interfacial rheology methods. This enhanced sensitivity to protein layer formation is likely due to the sensitivity of the dynamics of a single-molecule to its local environment and oligomeric state. In contrast, a macroscopic change of interfacial tension from its baseline value typically occurs only after fairly dense surface crowding over the entire interface.

Two types of models have been proposed to explain the mechanisms by which interfacial protein layers are formed. Homogeneous models predict a formation of a “soft glassy”

protein layer consisting mostly of monomers in which molecular crowding slows diffusion. Heterogeneous models predict the formation of interfacial protein aggregates that diffuse more slowly because they experience greater drag due to their increased hydrodynamic radius. SM tracking allowed us to directly distinguish between these two models.

We examined the distribution of hydrodynamic radii (calculated from individual protein object diffusion coefficients) of the observed labeled proteins at various stages of protein layer formation. It should be noted that the oil viscosity, which is the dominant viscosity in determining hydrodynamic radius, is approximately 350 times greater than that of water. Any changes in the local viscosity from high protein concentrations in the aqueous phase would be negligible compared to the oil viscosity. In Figure 8, histograms of BSA objects' hydrodynamic radii are presented at times (A) 1 s, (B) 600 s, and (C) 1800 s for a bulk concentration of $2.5 \mu\text{g mL}^{-1}$. In Figure 8A, the distribution is narrow and represents mostly monomers, dimers and trimers. As the protein layer begins to form, the distribution of hydrodynamic radii begins to broaden, with some hydrodynamic radii near 180 nm. The homogeneous, "soft glassy" models would predict a narrow distribution at all times, with the peak position shifting over time. The data in Figure 8 matches the predictions of a heterogeneous, aggregation model in which the distribution of hydrodynamic radii broadens over time.

While these results implied an aggregation model, the layer formation kinetics exhibited a dependence on bulk protein concentration, c , that was inconsistent with typical models. For example, the lag time associated with the induction period decreased approximately as c^{-2} instead of c^{-1} as naively expected. We determined that, in order to explain these results, a model must include the process of desorption, which is generally omitted from such models. Moreover, in these²⁸ and previous results,⁶ we explicitly observed desorption rates that correlated with aggregate state. Thus, we created a new kinetic population balance model that included adsorption, monomer addition to aggregates, dimer addition to aggregates, and desorption. Interestingly, we found this model can be described by one free parameter $R = kF/b^2$, where k is the rate of aggregation, F is adsorption rate of monomers, and b is the monomer desorption rate. Thus, R is a dimensionless indicator of the net aggregation rate. This model (Figure 7B) correctly matched the quantitative features (an initial induction period, followed by a steady decrease in diffusion) that we observed in our average diffusion data (Figure 7A). A schematic of this kinetic population balance model is depicted in Figure 9. Furthermore, the model predicted the correct dependence of layer formation kinetics on the bulk aqueous concentration of protein.

7. Relating Macromolecular Conformation to Dynamic Interfacial Behavior

Although many SM experiments focus extensively on the measurable properties of trajectories (e.g. adsorption, diffusion, desorption, etc.), the vast majority examine the properties independently. For example, multiple populations with unique characteristic surface residence times or diffusion coefficients can identify different modes of molecule-surface interaction, even in systems as simple as peptides interacting with a supported lipid bilayer.^{20,58} One can also observe time-dependent conformational changes to study protein or DNA folding pathways.^{9,59} In these experiments, important information was obtained from the variation of the measurable quantity with experimental conditions. Such studies have made interesting discoveries regarding molecule-surface interactions but do not fully exploit the information available using SM tracking. Increasingly we have focused on the relationship between measurable dynamic properties (on a molecule-by-molecule basis) to answer more sophisticated questions about interfacial phenomena. For example, do certain conformational states lead to faster diffusion or increased desorption probability? Do molecules that desorb less readily diffuse more slowly? Put in terms of fundamental physical

properties, this latter question can assess the relationship between the energy landscapes for movement in the perpendicular and parallel directions relative to the interface as the two may stem from similar or different physicochemical interactions.

In order to assess these relationships between dynamic behaviors, it is helpful to adopt a “segmented trajectory” approach to data analysis. This approach recognizes that a molecule may change its interactions with the surface during its residence time due to conformational changes, or orientation relative to the surface. Consequently, properties derived from an entire trajectory are not necessarily the most accurate way to characterize molecular behavior. For example, if a molecule switches between a conformation with strong surface affinity and one with weak affinity, it will likely desorb from the latter. The surface residence time, therefore, will roughly correspond to the fraction of time spent in each state but will not be useful in independently assessing the behavior of each conformational state. To address the above scenario using the segmented trajectory approach, we would align each trajectory at its desorption point and observe the distributions of molecular conformations as the molecules approach desorption.

This approach was used to examine the behavior of single-stranded DNA (ssDNA) on an amine-modified FS surface with the goal of determining the relationship between ssDNA conformation and its interactions with the surface.⁷ A fluorescence energy donor (D) was attached to one end of ssDNA while an energy acceptor (A) was attached to the other end for RET studies. Relative end-to-end distance (d) was used to assess molecular conformation, and this quantity was measured by as $(F_D/F_A)^{1/6}$ where F_D and F_A are the fluorescence intensities in the donor and acceptor channels, respectively. The relative end-to-end distance is proportional to the absolute end-to-end distance (r) such that $r = \mu R_o d$, where R_o is the Förster radius and μ is an instrument constant that depends on the properties of both fluorophore as well as the optical detection system. Both R_o and μ are difficult to determine experimentally or theoretically for energy transfer in near-interfacial environments interfaces and so results are presented in terms of d . Nevertheless, with reasonable assumptions that R_o is near its value in bulk solution (5 nm for the fluorescein-rhodamine pair used in this work) and that μ is near unity, the absolute end-to-end distance can be approximated.

This study identified a unique conformational state that was both highly extended and had weak surface affinity. For example, we observed that the fraction of ssDNA in the distinctive highly extended state ($d \approx 1.2$) doubled in the frame immediately before desorption (Figure 10a). Thus, once ssDNA entered the highly extended state, it was significantly more likely to desorb than when it was less extended. In this analysis, only trajectories with surface residence times > 2.7 s were included to avoid losing trajectories from the analysis as time increased before desorption or after adsorption. This left $> 10^4$ molecules for analysis, with roughly 2–4% of these in the highly extended state at any given moment.

In a partial desorption model of diffusion on a solid surface, a molecule must desorb at least partially in order to change its interfacial position. Thus, weaker surface interactions should result in faster diffusion. Again, the segmented trajectory analysis was used to test this theory by observing the mean diffusion coefficient of ssDNA as a function of both molecular extension and time before desorption. The mean diffusion coefficient ($\bar{D}$) is given by $\bar{D} = \langle R^2 \rangle / (4\Delta t)$ where R^2 is the squared displacement and Δt is the lag time between observations of position. As shown in Figure 10b, highly extended molecules diffused more rapidly immediately before desorption than at times well before desorption, providing support for the partial desorption model of diffusion for this experimental system. Relative to less extended molecules, highly extended molecules diffused much faster, further

supporting the claim that highly extended ssDNA experiences weaker surface attraction. Again, it is important to note that the independent dynamic correlations between conformation and adsorption, desorption, and diffusion cannot be explained by photophysical phenomena such as blinking or photobleaching, and we refer the reader to the original work for further discussion of this topic.⁷

The finding that highly extended ssDNA has weak surface interactions is unexpected if one assumes that individual nucleobase-surface interactions are energetically favorable (e.g. ionic attraction between negatively charged ssDNA phosphate backbone and positively-charged primary amines on the surface). If this were the case, an extended molecule would “lie down” on the surface in order to maximize favorable surface interactions; this would result in both low desorption probability and slow diffusion for highly extended ssDNA. This disconnect between observation and expectation suggests that highly extended ssDNA can exist in a non-equilibrium conformational state that is different from a random coil with attractive monomer-surface interactions. As shown in Figure 11, we postulate that the weakly-bound, highly-extended state involves extension perpendicular to the surface and that there is some energy barrier separating this state from a random coil state. This motivates further exploration of the mechanisms leading to this unique conformational state, the influence of the surface in its prevalence, and its role in interfacial DNA hybridization. One might hypothesize, for example, that a molecule that extends away from the surface might hybridize more readily with a complementary strand adsorbing from solution.

More broadly, this work provides direct evidence that molecular conformation can directly affect dynamic interfacial behavior. This concept was invoked in Section 4 to explain a maximum in activation energy for diffusion as a function of surfactant tail length³⁴ and conformational arguments are often hypothesized in literature to rationalize different molecular behaviors observed in a macroscopically heterogeneous sample. Using the methodology established in this work, it is now possible to test some of these hypotheses experimentally.

8. Summary

High throughput SM tracking is useful for independently and directly measuring adsorption, desorption, diffusion, and configuration of fluorescently labeled molecules. It is particularly well-suited to study systems in which molecules can interact with a surface in different ways, and the MAPT approach greatly increases sensitivity to heterogeneous behavior by resolving both spatial and temporal heterogeneity. In heterogeneous systems, it is now possible to study how each mode of surface interaction changes under different experimental conditions. Another unique approach to SM data analysis involves the determination of how each of the measurable properties of a trajectory relate to each other in real time. For example, one can observe how molecular configuration affects adsorption and/or desorption. This provides an exciting new way to test mechanistic hypotheses regarding molecule-surface interactions.

While the methods described above provide a powerful toolset for investigating interfacial phenomena, future work is necessary to improve the ways in which molecular configuration is coupled to the observable fluorescence signal. Recent advances in chemical biology now allow for site-specific incorporation of non-native amino acids into proteins that could be used as sites for fluorophore labeling. This level of control over fluorophore placement into complicated biomolecules could lead to far superior information on specific types of intramolecular conformational changes than is currently available using standard conjugation methods. Additionally, information about molecular orientation can be obtained by measuring the polarization of emitted photons. Different molecular orientations on a

surface can then be identified by shifts in the polarization angle of the emission light. These new methods will only further enhance this emerging field that is providing many new insights into interfacial phenomena.

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Figure 1.

Integrated probability distributions on log-linear axes. (a) Integrated residence time distribution of Fg on bare and functionalized FS shows multiple populations with unique characteristic residence times. The solid lines represent fits corresponding to four residence time populations. Modified from reference 5. (b) Integrated squared-displacement distributions of fluorescent hexadecanoic acid on TMS surfaces at different temperatures. The solid lines represent fits corresponding to two interspersed diffusive modes. The dotted lines on the highest temperature data indicate slopes corresponding to the slow and fast modes, respectively. Modified from reference 34.

Figure 2.

MAPT images of a photopatterned TMS surface exhibiting a pattern associated with the electron microscope grid photomask.²³ (a) adsorption rate ($10^{13} \mu\text{m}^{-2} \text{s}^{-1} \text{M}^{-1}$) (b) average lateral diffusion coefficient ($\mu\text{m}^2 \text{s}^{-1}$) (c) desorption probability per 400 ms and (d) surface coverage/occupancy ($10^{13} \mu\text{m}^{-2} \text{s}^{-1} \text{M}^{-1}$).

Figure 3.

MAPT image regions compared to data for homogenous TMS calibration surfaces.²³ (a) Spatial map of adsorption of BODIPY-labeled dodecanoic acid on a TMS surface patterned by UV light for 180 s with the regions of interest marked by yellow squares and labeled with corresponding identification numbers. (b) Surface coverage versus adsorption rate from homogenous calibration surface measurements of BODIPY-labeled dodecanoic acid on TMS surfaces of varying degradation (plotted with symbols representing the duration of photodegradation) and the regions of interest (plotted with numbers corresponding to the region of interest in (a)).

Figure 4. MAPT image of Fg surface occupancy of an HDPE film at 10 mg L^{-1} Fg concentration.²⁴ (a) Several features are observed with high surface occupancy; the features (indicated by arrows) are elongated in the film drawing direction and exhibit a range of aspect ratios. (b) One of the elongated features in (a) is magnified 2.5x. (c)–(e) Representative structures from areas of the film that are not shown in (a) are shown on the same scale as (b).

Figure 5. MAPT images of (a) surface coverage ($10^{-12} \mu\text{m}^{-2} \text{s}^{-1} \text{M}^{-1}$) (b) diffusion magnitude ($\mu\text{m}^2 \text{s}^{-1}$) and (c) diffusion direction for a selected region of the degraded TMS surface. Copyright 2011 by The American Physical Society.²⁹

Figure 6.

Effect of aggregation number on Fg surface mobility and affinity.⁵ The mean residence time and mean diffusion coefficient are shown on logarithmic axes as a function of aggregation number on a bare FS surface. With increasing aggregation number, mean residence time increases while the mean diffusion coefficient decreases.

Figure 7.

(a) Normalized mean diffusion coefficient of BSA proteins at the oil water interface vs time for BSA concentrations of 0.5, 1.25, 2.5, 10, and 25 $\mu\text{g mL}^{-1}$ in aqueous solution. (b) A quantity proportional to the mean diffusion coefficient predicted from the kinetic population balance model using R values of 0.0032, 0.008, 0.016, 0.064, and 0.16. Reproduced by permission of The Royal Society of Chemistry.²⁸

Figure 8.

The distribution of hydrodynamic radii for BSA protein objects at times (A) 1 s, (B) 600 s, and (C) 1800 s for a bulk concentration of $2.5 \mu\text{g mL}^{-1}$. Reproduced by permission of The Royal Society of Chemistry.²⁸

Figure 9.

A graphic illustration of the aggregation process with the mixture of fluorescently labeled BSA (green dots) and unlabeled BSA (black dots). At early times, a few monomers and dimers are at the oil-water interface. As time progresses, interfacial aggregation begins. Larger oligomers begin to form and the interfacial protein concentration at the oil-water interface increases. Reproduced by permission of The Royal Society of Chemistry.²⁸

Figure 10.

Molecular behavior changes before desorption and after adsorption.⁷ (a) The highly extended fraction of ssDNA with $d \geq 1.2$ is shown as a function of time either after adsorption or before desorption. The fraction of highly extended conformations nearly doubles immediately before desorption and also decreases following adsorption. (b) The mean diffusion coefficient ($\bar{D}$) is shown as a function of time either before desorption or after adsorption, for ssDNA conformations meeting the stated criteria for d . Compact conformations ($d < 1.2$) exhibit modestly increasing $\bar{D}$ prior to desorption and decreasing $\bar{D}$ after adsorption. This effect is greatly magnified in highly extended conformations ($d \geq 1.2$), particularly before desorption.

Figure 11.

Physical interpretation of the coil and weakly bound states.⁷ One hypothesis is that the weakly bound state extends away from the surface, and its fewer surface contact points lead to fast diffusion and increased likelihood of desorption. The coil state can extend in the plane of the surface but has many favorable surface contacts.