FEATURE ARTICLE

Adsorption and Diffusion of Single Molecules at Chromatographic Interfaces

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Received: April 16, 2003

This article details the contributions of single-molecule spectroscopy to the current understanding of an important problem in chromatography. Tailing is a long-standing problem in pharmaceutical and protein separations, and it is believed to be caused by unwanted strong adsorption sites on the substrate, which is typically silica. We probed the strong adsorption phenomenon directly by using single-molecule spectroscopy. Single molecules of a fluorophor, 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI), were monitored as they were in dynamic equilibrium at a chromatographic interface composed of chemically modified fused silica in contact with water or acetonitrile. The experiments revealed that most molecules laterally diffused at the interface, but occasionally, a molecule would stop diffusing temporarily and then resume. This was interpreted to be the manifestation of strong adsorption. An analysis of large data sets revealed three different strong adsorption processes occurring with desorption times on distinctly different time scales: 70 ms, 7 s, and >2 min. These events exhibited no pH dependence, and the two longer events were directly observed at nanometer-scale topographical indentations. The desorption kinetics from commercial chromatographic silica gel are in agreement with the single-molecule experiments on fused silica, indicating that the phenomena being probed are chromatographically relevant. These single-molecule experiments provide important new information about the so-called "active silanols" that cause tailing to persist at low pH, revealing that these sites are neutral and that surface topography plays a role.

Introduction

Liquid chromatography is a widely used technique for separating mixtures containing dozens of nonvolatile components. Its use includes diverse applications in chemical manufacturing, pharmacy, clinical chemistry, the petroleum industry, and environmental science. Despite its success, a persistent problem is that the zones for organic bases are broad, and tailing occurs when silica-based materials are used. Tailing causes an untold loss of productivity in the pharmaceutical industry by necessitating additional time for both column selection and methods development to satisfy regulatory requirements. Tailing is more severe for proteins, and the advent of proteomics has made the improved separation of proteins critically important. Human cells contain tens of thousands of proteins, but today's separation technology, which includes electrophoresis as well as chromatography, falls more than an order of magnitude short in resolving such mixtures. Achieving advances and breakthroughs in separations for pharmaceuticals and proteins will require creative basic research to understand chemical and physical interactions between molecules and surfaces to enable the design of new materials.

Silica is the most widely used support material in liquid chromatography and capillary electrophoresis. The many advantages are that silica is inexpensive, it can be drawn into capillaries or made into spherical particles of high surface area, its surface chemistry allows for covalent bonding of a large assortment of functional groups at high coverage, and its mechanical rigidity allows high inlet pressures required for efficient separations in liquid chromatography. Advances in manufacturing now provide silica gel of controlled pore size and high purity, where metal impurities are on the order of only ppm. Today's silica can often perform efficient separations that were impossible on silica-based columns 20 years ago.[1]

Despite advances in the manufacturing of silica, the problem of tailing and broadening of chromatographic zones has not been completely eliminated. Tailing of organic bases and proteins is attributed to the presence of strong adsorption sites on the silica substrate. There is ample evidence in the literature that these sites are composed of residual silanols, −SiOH, which are acidic. Several excellent reviews document the evidence that the resulting $-SiO^-$ groups cause tailing through ion-exchange interactions.[2,3] As an illustration, Figure 1 shows the chromatograms of a cationic dye molecule, DiI (1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate) at neutral pH where the surface is charged and at low pH where the surface is uncharged. Tailing and broadening are evident at low pH, which is achieved by the addition of 0.1% trifluoroacetic acid to a mobile phase of 90% acetonitrile/water by volume. When the mobile phase is switched to 90% acetonitrile/water without trifluoroacetic acid, the retention time increases, and the zone significantly broadens. The broader chromatogram at high pH is due to stronger interaction with the stationary phase, and the pH dependence indicates that this stronger interaction arises from a Coulombic interaction. If ion exchange were responsible for strong adsorption and if the adsorbate were to remain in contact with the hydrocarbon while undergoing ion exchange, then a

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10.1021/jp030449p CCC: $25.00
Published on Web 06/10/2003

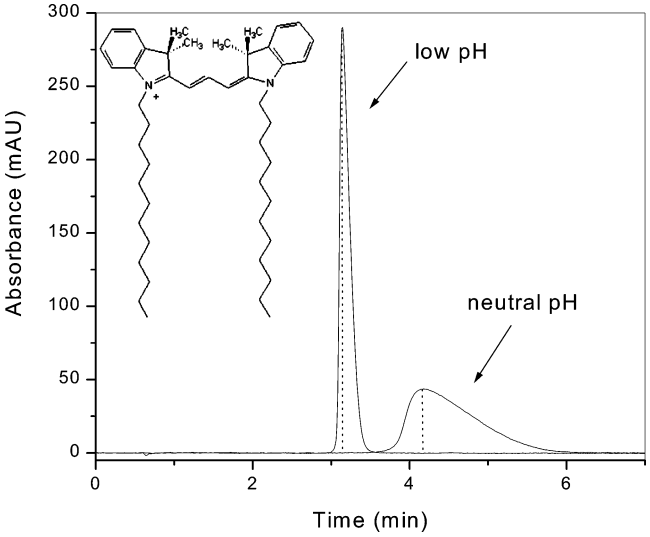


Figure 1. Chromatograms of DiI at low and high pH. An absorbance detector was used at the absorbance maximum of DiI. The stationary phase was a high-quality silica gel, Zorbax-RX, which was silylated with diisobutyloctadecyl (C_8) chains. The mobile phase was 90% acetonitrile/water V/V at a flow rate of 1 mL/min, and low pH was achieved by using 0.1% trifluoroacetic acid. The injected concentration was 10 μM. The structure of DiI is shown in the inset with shorter hydrocarbon chains for compactness; the actual hydrocarbon chain lengths were dodecyl chains.

Coulombic interaction would add another term to the free energy of adsorption beyond that from the hydrophobic interaction with the stationary phase.

$$\Delta G_{ads} = \Delta G_{hyd} + \Delta G_{coul} \quad (1)$$

A much larger adsorption coefficient would result, thus constituting a "strong" adsorption site. It is believed that tailing persists at low pH because there is a very small population of unusually acidic silanols, often called "active silanols", that impart a local charge when the silica substrate is nominally uncharged.[2,3] This tailing at low pH is evident in Figure 1. Other oxide supports (e.g., titania and zirconia), which are charged at all pH values, give worse tailing than silica. Avoiding a solid oxide support (e.g., using a polymeric resin) gives broader zones due to hindered diffusion through the polymer. For silica, a recent review describes many empirical testing procedures that have been devised to evaluate the silanol activities of commercial stationary phases, with the results of these tests often contradicting one another.[4] Two tests rely on the behaviors of polar but neutral compounds, phenol[5] and 1-nitronathphalene,[6] which casts doubt on the simple interpretation that ion exchange entirely explains tailing. The phenomenon of tailing is not well understood.

Zhuravlev has shown that amorphous silica, regardless of its origin, has a hydrophilic surface whose silanol coverage is 8 μmol/m^2.[7] In the field of chromatography, this maximum coverage of silanols is called a fully hydroxylated surface. Commercial manufacture of silica gel involves a heat treatment that dehydrates the surface, condensing silanols into siloxane bonds, and the recovery to the equilibrium, fully hydroxylated surface at room temperature is slow. Köhler et al. showed that the procedure for making chromatographic silica must result in a fully hydroxylated surface or else tailing is increased.[8,9] They showed from FTIR that isolated silanols appear as silanol coverage is reduced and that the abundance of these isolated silanols correlates with an increase in tailing. They propose that isolated silanols are the "active silanols" that cause tailing at low pH. They explain that isolated silanols would be more acidic than silanols in proximity to one another because the hydrogen bonding among the silanols would lower the acidity. This is contradicted by analogies to dicarboxylic acids. For example, oxalic acid has $pK_{a1} = 1.2$, making it much more acidic than acetic acid, for which $pK_a = 4$. One could argue with many other examples that isolated silanols would be less acidic than hydrogen-bonded silanols. Whether the explanation is correct or not, the fact remains that experimental steps taken to maximize the number of surface silanols give a reduction in tailing. It is possible that these experimental steps, which involve the base-catalyzed hydrolysis of the silica, eliminate an undetected group that causes tailing and that the isolated silanol is only a spectator. Alternatively, it could be that the isolated silanol is strongly adsorptive through hydrogen bonding rather than ion exchange. It would be insightful to know the explanation for why commercial silica gels that are fully hydroxylated invariably give the best performance for pharmaceutical and protein separations.

At first glance, one might think that there should not be tailing in chromatography because of these interactions with silica because the silane monolayer ought to be able to occupy the surface area to steric completion, allowing no exposure of the silanols to the adsorbates. The reagent used to make the C_{18} phase, which is the most commonly used stationary phase, is chlorodimethyloctadecylsilane. This reacts with surface silanols to form a covalent siloxane (Si−O−Si) bond with the surface, as depicted in Scheme 1. The reaction is reversible: acidic conditions in separations cleave the siloxane bond to remove the monolayer. The role of the dimethyl side groups is to prevent the self-assembly of the alkyl chains, sterically limiting the density of hydrocarbon chains to about 3.5 μmol/m^2 of chains. This density is comparable to that of the liquid-expanded phase of a Langmuir film, which is the optimal density for solubilizing adsorbates in a monolayer of alkyl chains.[10] The density is close-packed from the point of view of the reagent dimethylsilane groups, although there could be small gaps due to the spatially random arrival of reagent groups at the surface. A process called endcapping is used to reduce tailing by filling any gaps with a smaller reagent, most typically chlorotrimethylsilane. Even at steric completion, these silylation reactions leave half or more of the original 8 μmol/m^2 of surface silanols unreacted, which is the origin of the term "residual silanols". One might think that the high density in the plane of reagent dimethylsilane groups would prevent pharmaceuticals and proteins from contacting the residual silanols because these adsorbates are often larger than the C_{18} reagent and usually larger than the endcapping reagent. There is evidence that the surface reorganizes after the silylation to expose fresh substrate, which would explain why large molecules can contact the silica substrate. Specifically, Wang and Harris showed that covalently attached pyrenyl groups migrate to cluster together, apparently becoming mobile by the hydrolysis of the siloxane bonds because of adsorbed water on the silica substrate.[11] Further evidence is that double or even triple endcapping is often employed for commercial silica gels, where endcapping to completion is performed, the sample is brought back to room temperature and rinsed, and then the process is repeated. If the surface did not rearrange, then there would be no observed practical advantage to multiple endcapping. Our group had success with the mixed self-assembly of trichlorosilanes having C_{18} and C_1 alkyl chains, where the siloxane bonds among the reagents conferred close-packed density in the plane of the reagent silicon atoms.[12,13] This mixed self-assembled monolayer provided a surface that

SCHEME 1

was acid-stable and had reduced tailing compared to conventional materials. Nonetheless, tailing was not completely eliminated because of inevitable defects in the self-assembled monolayer. Despite decades of efforts, the silica substrate cannot be 100% blocked from adsorbates. This is why advances in the preparation of silica gel has had such a large impact on high-performance chromatography of pharmaceuticals and proteins, and it is why an improved understanding of the strong sites on the silica substrate might have significant impact on this field in the future.

The accessibility of the residual silanols to the mobile phases for commercial dimethylsiloxane stationary phases was studied by Fóti and Kováts, who measured H−D exchange.[14] Their results showed that about 15% of the residual silanols were immediately accessible to exchange, and the remaining silanols were accessible on a longer time scale. These bonded stationary phases thus block the accessibility of most, but not all, silanols at any one time. The interpretation is that the chains of the stationary phases are dynamic, blocking the accessibility of most of the residual silanols on fast time scales but then slowly moving to allow eventually all residual silanols to contact the mobile phase.

One of the unexplained paradoxes in the chromatographic literature regards endcapping. The silanes are known to be hydrolyzed slowly from the silica surface, which gradually increases the area of exposed silica. Glajch et al. studied the effect of acid hydrolysis on the hydrocarbon coverage of an endcapped C_{18} surface, along with the tailing behavior.[15] They observed the surprising behavior that the tailing did not increase significantly even though the carbon coverage dropped with hydrolysis time. The surface was degraded all the way to half coverage, which would normally give pronounced tailing if this were the original coverage, yet there was little tailing. Because the large number of exposed silanols did not cause tailing, this indicates that the story is more complicated. McCalley obtained chromatographic data showing that tailing can be sterically hindered.[16] It is possible that endcapping is most important at sterically hindered sites, where its hydrolytic cleavage would be impeded.

Most tailing in reversed-phase liquid chromatography can be explained in general terms by nonlinearity in the adsorption isotherm. Intuitively, the zone tails because the saturation of sites prevents further adsorption, causing a sharp front in the mobile phase, and the subsequently desorbing material shapes the tail. Lenhoff was the first to suggest that a bi-Langmuir of eq 2, rather than a single Langmuir, is required to describe tailing quantitatively.[17]

$$\Gamma = \Gamma_{\text{sat,weak}} \frac{K_{\text{weak}} a}{1 + K_{\text{weak}} a} + \Gamma_{\text{sat,strong}} \frac{K_{\text{strong}} a}{1 + K_{\text{strong}} a} \qquad (2)$$

We explain this by noting that a weak, prevalent type of site does not saturate at the injected concentration, but a strong, rare site does saturate. The rarity of the strong site allows early elution, and its high adsorption coefficient gives rise to the nonlinear tailing. Guiochon et al. have since studied tailing and multicomponent adsorption isotherms in more detail, and they have also showed that, in addition to nonlinear tailing, one can also have kinetic tailing due to slow desorption from rare sites.[18−20] Kinetic tailing would be manifested by an increase in tailing with flow rate. Kinetic and nonlinear tailing can both contribute, and studies of both flow rate and concentration dependence are needed to determine the contributions of each.

There is recent information about multicomponent adsorption isotherms for tailing organic amines, where two published studies provide mathematical models of the zone profiles through the adsorption isotherms. In one study, the chromatogram of *N*,*N*-dimethylaniline was used to calculate its adsorption isotherm, where methanol/water at pH 5 was used as the mobile phase.[21] The results showed three types of adsorption sites: a prevalent weak site ($K_{\text{weak}} < 0.1$), a rare strong site ($K_{\text{strong,1}} = 250$), and another rarer but stronger site ($K_{\text{strong,2}} > 5000$). The data fit best to a distribution of adsorption coefficients for the site having intermediate strength, but only limiting values could be determined for the other two sites. The site of intermediate energy had a saturated coverage that was more than 4 orders of magnitude lower than that of the weak site, but the coverage of the strongest site could not be determined uniquely. The chromatogram reconstructed from these parameters resembled, but did not fit well, to the experimental chromatogram. In the other study, the chromatograms of three basic drugs were used to calculate the adsorption isotherms, where acetonitrile/water at pH 3 was used as the mobile phase.[22] The drugs behaved similarly, exhibiting bi-Langmuir behavior, with $K_{\text{strong}}/K_{\text{weak}} \approx 500$. The weaker site had a saturated coverage that was a factor of 20 greater than that of the strong sites. The chromatogram reconstructed from these parameters fit well to the experimental chromatogram. Published studies thus support the notion that multiple adsorption sites explain tailing, with tailing caused by nonlinearity and with fast elution allowed by the low abundance of the strong site. Specifics such as the choice of adsorbate, mobile phase, pH, and silica gel could be the cause of the differing results.

Fluorescent adsorbates are sensitive to the heterogeneity of chromatographic surfaces. Lochmüller et al. showed in 1981 that the electronic spectrum of dansylamine groups covalently attached to a chromatographic surface had a broad and inhomogeneous electronic spectrum that they explained by three general types of environments: nonpolar, aprotic polar, and hydrogen bonding.[23] To relate this information to a chromatographic zone for an untethered adsorbate, one would need to know the adsorption coefficient and the capacity for each of these environments as well as any distributions of these parameters. The spectroscopic and chromatographic pictures have only slowly begun to merge over the ensuing 20 years since the paper by Lochmüller et al. because heterogeneous systems are difficult to study spectroscopically. New experimental techniques with a higher information content are required to unravel and separately characterize the multiple interactions that are occurring at these heterogeneous surfaces.

Single-molecule spectroscopy is uniquely suited to probing heterogeneous systems. This article describes our studies of the interactions between chromatographic silica surfaces and small

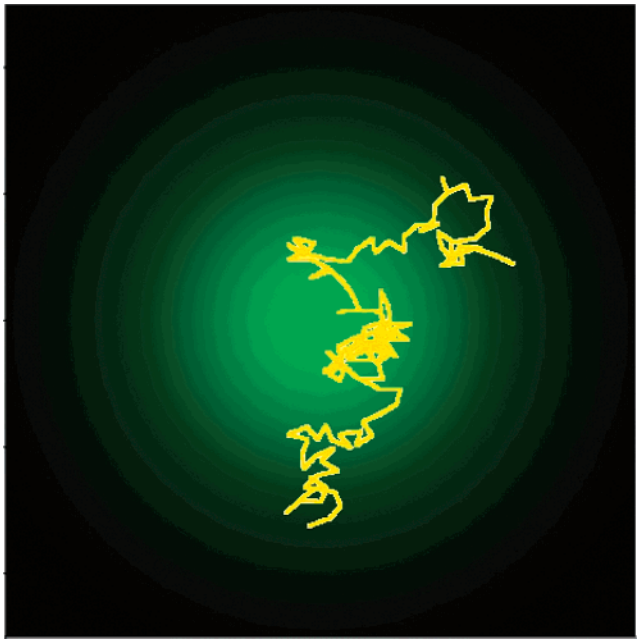


Figure 2. Principle behind distinguishing moving and stationary adsorbates. A green Gaussian beam is focused on the surface. A path for the Brownian motion of a diffusing fluorophor is depicted in yellow, illustrating that the intensity of excitation would change as the molecule changed its radial position in the beam, giving fluctuating fluorescence. A stationary molecule, not shown, would remain at one radial position in the beam to give constant fluorescence.

molecules that exhibit chromatographic tailing. The interactions of small molecules with surfaces can be used to mimic segments of proteins interacting with surfaces; therefore, an understanding of tailing for small molecules will advance the understanding of tailing for proteins. The fundamental questions are many: (1) How many types of these strong sites are there? (2) What is their number density? (3) What is the free energy of adsorption for important functional groups, and how do the interactions combine to comprise the total free energy? (4) If there are multiple types of sites, are they discrete sites, or is there a continuous distribution of adsorption energies? (5) What are these sites chemically? (6) How can we reduce their abundances or their effects? To address these questions, we employ single-molecule spectroscopy, fluorescence correlation spectroscopy, fluorescence imaging, and atomic force microscopy.

Experimental Considerations

Our idea is to use single-molecule spectroscopy to probe strong adsorption events directly by observing single molecules in dynamic equilibrium at the chromatographic interface. We chose DiI as the spectroscopic probe of tailing behavior for two reasons. First, DiI has a high fluorescence quantum efficiency to allow for the spectroscopic detection of single molecules, which was previously demonstrated for DiI in a poly(methyl methacrylate) film.[24] Second, DiI exhibits pH-dependent chromatographic tailing, as is shown in Figure 1. A single molecule of DiI interacting with the weak sites (i.e., the hydrocarbon) would be expected to undergo rapid lateral diffusion on the basis of our previous studies of the amphiphile acridine orange.[25] If DiI were to stop at specific sites, then this should be easy to observe for single molecules, provided that the time scales for moving and stopping can be resolved by the detection technology. The thought was that if we could observe strong adsorption events directly then we would have an experimental handle to learn what causes strong adsorption.

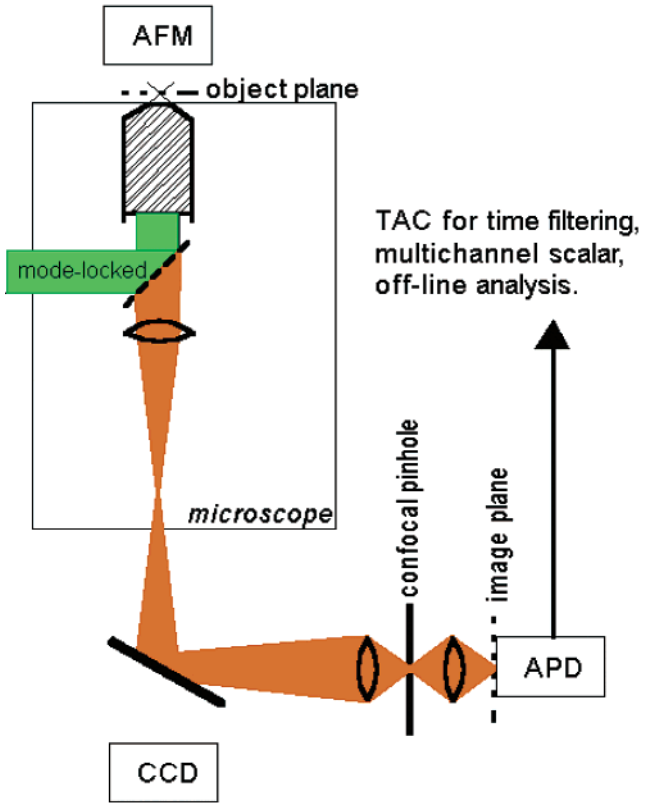


Figure 3. Schematic for single-molecule experiments. A mode-locked argon ion laser at 514.5 nm is reflected at a dichroic mirror for excitation with a 100× oil-immersion objective of an inverted microscope. Fluorescence is collected by the objective and transmitted through the dichroic mirror. The fluorescence is focused onto an avalanche photodiode (APD) using a lens pair and confocal pinhole that are external to the microscope. A movable mirror allows for imaging with a CCD camera. The AFM scans the topography of the same surface being investigated spectroscopically.

Figure 2 illustrates the principle behind the single-molecule experiment. Moving and stationary molecules in a Gaussian beam can be distinguished on the basis of whether the fluorescence fluctuates. Figure 2 depicts a cross section of a Gaussian beam focused at the chemical interface, where a diffusing molecule enters and undergoes a random walk. The diffusing molecule samples many radial positions in the beam profile during its transit; therefore, its fluorescence fluctuates because of the continually changing excitation power. If this molecule were to stop, then it would reside at a fixed radial position, and the fixed excitation power would make the fluorescence constant within the shot noise. Moving and stationary molecules should thus be distinguishable by the level of fluctuation of the fluorescence, provided there is a sufficient ratio of signal to shot noise on the time scale of the detected event.

The main source of shot noise when studying a molecule of high quantum efficiency is Raman emission from the solvent. Two advances in the field of single-molecule spectroscopy have helped to reduce this problem. Wilkerson et al. implemented time filtering to reject solvent Raman emission,[26] and Zare and Nie introduced the confocal microscopy for spatially rejecting Raman emission.[27] We employ both time filtering and spatial filtering to minimize the contribution from Raman emission. The apparatus is illustrated in Figure 3. A Zeiss Axiovert-100 is the basis for the instrument. An imaging lens is set up external to the microscope to direct the emission to a confocal pinhole on an *xyz* translation stage to achieve spatial filtering. The pinhole allows light over $\pm 2\sigma$ of the Gaussian excitation beam

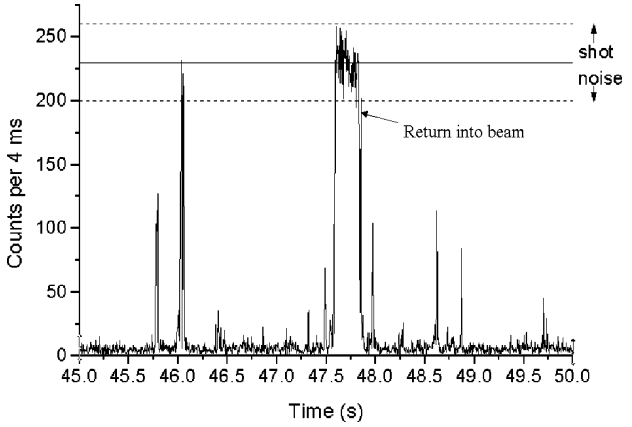


Figure 4. Photon counts vs time from the avalanche photodiode. The dwell time was 4 ms, and a 5-s subset of a 65-s data set is shown. The bursts of fluorescence are due to single molecules passing through beam. The event from 47.6 to 47.8 s is due to reversible strong adsorption, and the 95% confidence level for the shot noise is indicated by the dotted lines. A spike shortly after the strong adsorption event is consistent with the molecule returning to the beam briefly.

to reach the avalanche photodiode (APD). The APD is internally wired for photon counting, and its output TTL pulses are sent to a time-to-amplitude converter, which is synchronized to the mode-locked laser pulses and windowed to accept counts only after most of the Raman emission has decayed away. The time-to-amplitude converter also allows periodic checks of the fluorescence lifetime to confirm that the single molecules being detected are actually DiI. Photon counts from the avalanche photodiode are acquired using a multichannel scalar for which we typically set the dwell to be 4 ms. Using a 14-bit multichannel buffer allows data to be collected for 65 s in each run. The apparatus also shows that a CCD camera can be used to acquire fluorescence images of the surface, and a tip-scanning AFM can be used to probe the topography of the same region of the surface being investigated by fluorescence.

Results and Discussion

Photon counts were monitored at the avalanche photodiode as a function of time for a concentration of DiI that was maintained to be less than 0.1 molecule in the beam, on the average. Figure 4 shows a sample of single-molecule data that reveals a strong adsorption event. Two large, brief bursts of fluorescence occur at 45.7 and 46.0 s, which we attribute to molecules diffusing through the beam. Several more bursts occur with count rates of less than 50, which is attributable to the diffusing molecules that did not approach the center of the Gaussian beam. A molecule becomes strongly adsorbed at 47.6 s. The fact that it is a strong adsorption event is indicated by the fluorescence remaining constant within the shot noise, which is shown in the Figure by the dotted lines that bound the 95% probability distribution for the shot noise. The burst is also much longer than the typical burst due to a diffusing molecule. In this case, the molecule remained strongly adsorbed for 200 ms. The arrow points to a brief burst occurring after the desorption, which is consistent with the same molecule returning to the beam before diffusing out again. Of course, it could be a different molecule, but it is statistically more likely to be the same molecule. It is less probable to have a return spike than for the molecule simply to diffuse away, but the presence of occasional return spikes is affirmation that the adsorbed molecules are desorbing rather than photobleaching. The longer the molecules stay adsorbed, the greater the danger of photobleaching. In practice, the excitation power must be set to a level that minimizes photobleaching for the time scale of adsorption under study. The data of Figure 4 show that high levels of signal to background are achievable by combining spatial filtering and time filtering and that the time scales of diffusion and strong adsorption are apparently separated.

Blinking of single molecules is a common phenomenon that can potentially interfere with the detection of strong adsorption.[28−30] Blinking is a temporary loss of fluorescence from a single molecule, and it is due to a molecule passing into a triplet state, where it cannot be detected by fluorescence. The presence of oxygen enhances intersystem crossing to return the molecule from the triplet state to the singlet ground state, where it can once again be excited for fluorescence detection. We have made no efforts to remove oxygen, thereby promoting intersystem crossing so that the time scale for blinking is short. We observed blinking of DiI only when the dwell time was set to 1 ms.[31] Even in the presence of blinking, a strongly adsorbed molecule returns to the same initial intensity level because it remains in the same radial position in the beam profile. The blinking thus slightly complicates the detection of strong adsorption on the time scale of 1 ms and shorter, but it does not pose a significant impediment.

The fluorescence from an adsorbed molecule would remain constant only if the molecule could not change its orientation or have a shift in its fluorescence spectrum during adsorption. Trautman and Macklin showed that DiI in a poly(methyl methacrylate) film exhibits spectral diffusion, where the emission spectrum of a single molecule shifts, apparently due to a change in environment.[24] Different emission spectra could arise from different adsorption sites on silica, in light of the findings of Mei et al., who observed different emission spectra from different single molecules of rhodamine B in sol−gel-derived silica films.[32] In our case, for most strong adsorption events, the intensity of a given DiI molecule did remain constant within the shot noise. On a few occasions, we observed a change in intensity during the strong adsorption event.[31] The occasional intensity change could be due either to spectral shifting, as in the polymer film, or to a change in the orientation of the molecule. Either requires the motion of the molecule, and we did not explore the origin of the intensity changes. The fact that intensity can change during strong adsorption indicates that the potential well is sufficiently shallow along the surface to allow the adsorbate to move sometimes without desorbing. Studies of these events might eventually provide details about how molecules desorb from these sites.

For DiI, we observed strong adsorption events for 1% of the molecules that entered a beam of 10-μm diameter.[31] The average duration of these adsorption events was estimated to be 100 ms on the basis of the average of 22 observed events. Calculating the areas under the bursts for diffusing and strongly adsorbed molecules revealed that the population of strongly adsorbed molecules was $^1/_3$ of the total population of adsorbates. These strong adsorption events contribute substantially to the adsorbed population, despite their rarity, because the residence time is long once a molecule does adsorb. The concentration ratio of immobile to mobile adsorbates, $\Gamma_{strong}/\Gamma_{weak}$, is $^1/_3$ for this region of this surface. This ratio is proportional to the ratio of adsorption coefficients of eq 2 through the saturated coverage for each type of adsorption site. Equation 3 is obtained from eq 2 in the limit of low solute activity, *a*.

$$\left(\frac{\Gamma_{strong}}{\Gamma_{weak}}\right)_{\lim a\to 0} = \frac{K_{strong}\Gamma_{strong,sat}}{K_{weak}\Gamma_{weak,sat}} \qquad (3)$$

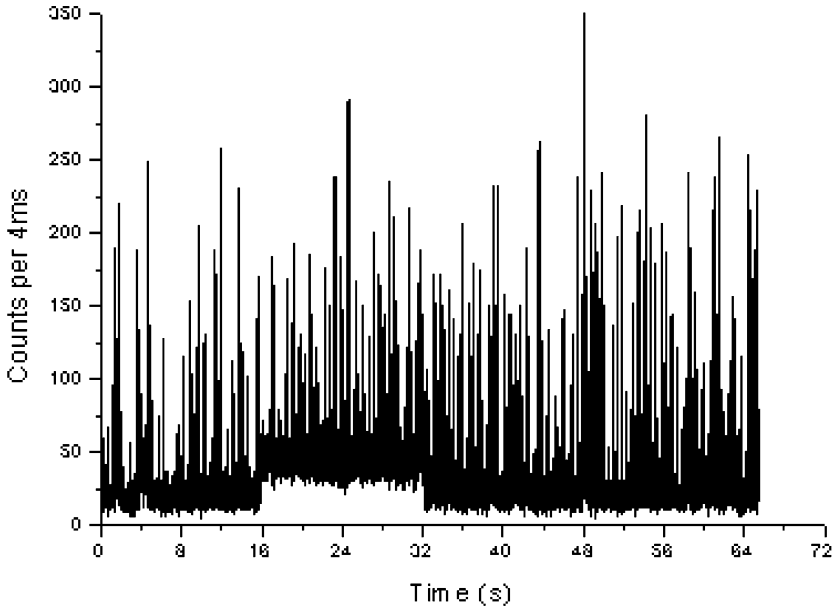


Figure 5. Photon counts vs time from the avalanche photodiode. A strong adsorption event started at 16 s and ended at 32 s.

The low abundance of the strong sites limits the adsorbed population on the strong site. On the basis of unpublished simulations of chromatographic zones, if $\Gamma_{strong} \approx \Gamma_{weak}$, then the zone elutes early and tails. For $\Gamma_{strong} \ll \Gamma_{weak}$, the zone elutes early and is symmetric. Because $\Gamma_{strong}/\Gamma_{weak} = {}^1/_3$ for fused silica, these strong sites would cause tailing. However, it would be a leap of faith at this stage to assume that the distribution of sites on this fused silica surface is representative of that of chromatographic silica gel.

As we studied more surfaces to get better statistics on rare strong adsorption, we made several interesting observations. First, endcapping of the surfaces reduced the frequency of strong adsorption. This sensitivity to endcapping correlates with chromatographic observations, where endcapping reduces tailing. This supports the interpretation that the single-molecule experiment is sensing adsorption to the exposed silica substrate. Second, we observed considerable variation in the frequency of strong adsorption events from one surface to the next, even though the average coverage of hydrocarbon was the same, as probed by FTIR. This is also consistent with chromatographic observation, where the variation in chromatographic tailing from one manufacturer to the next and even from one lot to the next for a given manufacturer despite the same carbon coverage, has been one of the long-standing puzzles in the practice of chromatography. This variation in tailing has especially been a problem in the pharmaceutical industry, where FDA approval rests on reliable chemical separation to assay drug purity. The parallel between the behaviors in single-molecule spectroscopy and chromatography and the importance of tailing motivated us to investigate strong adsorption further in the hopes of answering why tailing has been so variable. Third, in the course of acquiring much more single-molecule data, we occasionally observed very long adsorption events. Figure 5 shows one unusually long event, where a molecule adsorbed at time = 16 s and remained adsorbed until time = 32 s. During this exceptionally long time that the molecule remained adsorbed, many other molecules entered and exited the beam, thus the 16-s burst has a hairy appearance due to the many diffusing molecules. Events longer than 1 s in duration were seen so rarely that good counting statistics could not be readily achieved. Longer desorption times and rarer adsorption might be relevant to reproducibility problems in HPLC. Consequently, we redoubled our efforts to detect rarer, longer events.

Two experimental factors aided in detecting a greater number of single-molecule events having desorption times longer than 1 s. First, we observed that using acetonitrile as the solvent gave more frequent strong adsorption events. Because acetonitrile is a commonly used solvent in chromatographic mobile phases, we changed from using water to using acetonitrile without fear of the results being irrelevant. Second, by increasing the surface concentration of DiI by 10-fold, rare events would become 10-fold more frequent, giving us better statistics. At higher concentration, there would often be multiple molecules in the beam, hampering single-molecule spectroscopy. Under these conditions of few but multiple molecules in the beam, one can use fluorescence correlation spectroscopy, where autocorrelation of the burst data is analyzed to detect slow desorption processes in the presence of diffusion. Because there is now a considerable separation of time scales between diffusion and the events of interest, fluorescence correlation spectroscopy becomes feasible for probing rare adsorption.

Elson and Magde showed that one can probe binding equilibria in the presence of diffusion using fluorescence correlation spectroscopy,[33] and this has become a widely used method of probing protein binding equilibria. Their binding model can be applied to strong adsorption at surfaces. Consider a diffusing dye, DiI_{mob}, that binds at a strong adsorption site to form the adsorbed dye molecule, DiI_{imm}.

$$\text{site} + \text{DiI}_{\text{mob}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{DiI}_{\text{imm}} \tag{4}$$

Statistical fluctuations on the average concentration, C_i, of any of these three species, i, occur because of the small number of molecules in dynamic equilibrium. Elson and Magde showed elegantly that a frequency-domain representation of the concentration fluctuations, $\tilde{C}(\nu, \tau)$, is solvable.

$$\frac{\partial \tilde{C}_i}{\partial \tau} = \sum_j \mathbf{M}_{ij} \delta \tilde{C}_j \tag{5}$$

M is the matrix of kinetic parameters, where ν is the spatial

frequency, s is the standard deviation of the Gaussian beam profile, and D_i is the diffusion coefficient of each species i, where i corresponds to mob, imm, or the site. Under their conditions of frequent binding, Elson and Magde found simple solutions to eq 5 to describe the autocorrelation.

$$\mathbf{M} = \begin{vmatrix} -(v^2 D_{\text{site}} + k_1 \tilde{C}_{\text{mob}}) & -k_1 \tilde{C}_{\text{site}} & k_{-1} \\ -k_1 \tilde{C}_{\text{mob}} & -(v^2 D_{\text{mob}} + k_1 \tilde{C}_{\text{site}}) & k_{-1} \\ k_1 \tilde{C}_{\text{site}} & k_1 \tilde{C}_{\text{site}} & -(v^2 D_{\text{imm}} + k_{-1}) \end{vmatrix} \quad (6)$$

In our case, the binding between DiI and a strong adsorption site occurs rarely; therefore, their solution does not apply to our system. We showed that eq 5 can be solved under the condition of rare binding, yielding a different but simple function.[34] Under this condition of rare adsorption, where $D^{\text{mob}}/s^2 \gg k_1 C_{\text{site}}$ and $k_{-1} \gg k_1 C_{\text{site}}$, the autocorrelation, $G(\tau)$, is the sum of two types of terms, as shown in eq 7.

$$G(\tau) = \frac{\epsilon_B Q_B f_B}{1 + \tau \dfrac{D}{s^2}} + \sum_i \epsilon_{C,i} Q_{C,i} f_{C,i} \exp(-k_{-1,i}\tau) \quad (7)$$

The first term is due to the diffusion of mobile DiI molecules through the beam, according to their lateral diffusion coefficient, D, and the second term is a sum of exponential decays for i desorption processes. The desorption rate constants for the i processes, $k_{-1,i}$, can be determined experimentally by fitting the autocorrelation decay to eq 7. The fractional amount of each species is f. The terms ϵ and Q are the molar absorptivities and quantum efficiencies of each species, which we assume to be the same for adsorbed and diffusing species. The ability to detect desorption due to rare strong adsorption events relies on a large diffusion coefficient combined with a small beam variance to make the first term decay much faster than the second term in eq 7.

We performed fluorescence correlation spectroscopy for DiI at the interface of acetonitrile in contact with a C_{18} monolayer on silica. We had separately determined the diffusion coefficient using a wider beam to accentuate the contribution of the first term in eq 7, and this revealed a diffusion coefficient of 6.5×10^{-6} cm^2/s. We then subtracted the decay component due to diffusion from the experimental autocorrelation that we measured with the narrower beam, and the residual autocorrelation decay is shown in Figure 6 on a semilog scale. The experimental data fit well to two distinct exponential decays, where one desorption time is 68 ms, in agreement with the previous estimate of 100 ms, and the other is 2.6 s. There is some danger of the 2.6-s decay constant being shortened by photobleaching because the laser power had been optimized for the 100-ms time scale. Assuming no change in molar absorptivity or quantum efficiency upon strong adsorption, the relative equilibrium concentrations of the three species are 75% diffusing, 11% adsorbed at the 68-ms sites, and 14% adsorbed at the 2.6-s sites. Fluorescence correlation spectroscopy thus reveals three distinct types of adsorption sites for DiI: diffusing molecules and molecules adsorbed at two different types of strong adsorption sites as well as the desorption rates and relative populations at these sites.

If we turn our attention to identifying what constitutes these strong adsorption sites chemically, then one question is whether these are at localized hot spots or homogeneously distributed

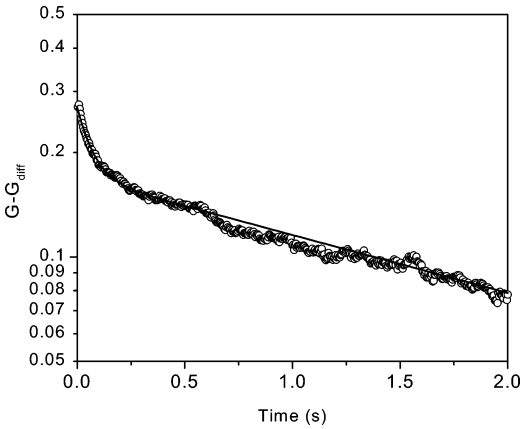


Figure 6. Semilog plot of experimental (○) autocorrelation decay from confocal microscopy after subtracting the contribution from diffusion and the best fit (−) to a double-exponential decay.

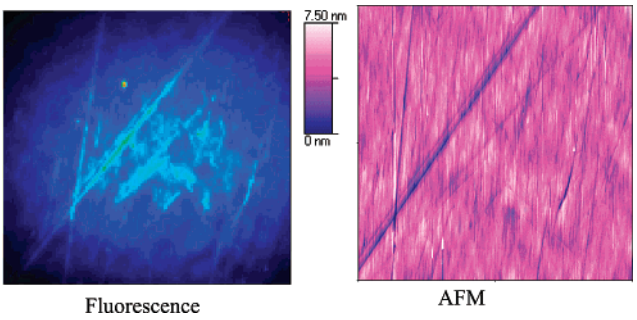


Figure 7. Fluorescence image for DiI on a C_{18} surface at high concentration, measured using a CCD camera (left). AFM image of the same region (20 μm × 20 μm) (right). Images from ref 35.

over the surface. It has been assumed that these sites are homogeneously distributed because of the exposure of the silica substrate by random statistical fluctuations in the density of hydrocarbon coverage. To test homogeneity, we studied fluorescence images of chromatographic interfaces on silica coverslips, and we found that the images were never homogeneous.[35] Figure 7a shows a fluorescence micrograph for DiI adsorbed to C_{18} on silica in contact with water. The image shows a pattern of stripes and spots that are reminiscent of marks left by the chemical−mechanical polishing process. Figure 7b shows an atomic force micrograph of the same region, which confirms that the marks are due to nanometer-scale topographical indentations. Lines are impressed on the fused silica surface in the chemical−mechanical polishing process by the presence of particles that are larger than the average particle size, which imparts a greater force under the particle. Pits are caused by strong adhesion between polishing particles and the surface, where rinsing the particles away causes the removal of some silica. Polished fused silica surfaces tend to have more polishing spots than polishing lines, but here we chose a region of the surface that had more polishing lines to make it easier to compare patterns between the two images. The AFM height scale indicates that these marks are on the scale of 7.5 nm. Images with sharpened tips (diameter ≈ 5 nm) reveal pits as deep as 10 nm that are hundreds of nanometers wide. Overall, accounting for the distortion of the AFM image due to hysteresis of the piezoelectrics, the fluorescence and AFM images agree remarkably, showing that adsorptivity correlates with topography on the nanometer scale.

The agreement between the fluorescence and AFM images in Figure 7 establishes that more DiI is adsorbed at these polishing marks. However, it does not necessarily mean that

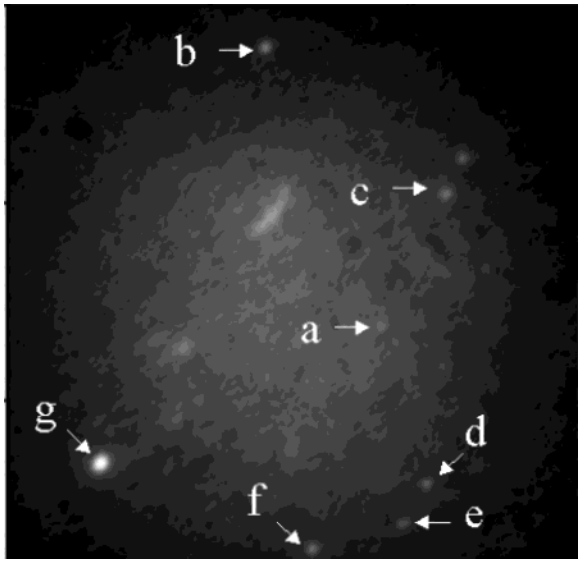


Figure 8. Fluorescence image for DiI on a different C_{18} surface at low concentration, measured using an ICCD camera. The bright spots, denoted a−g, are discussed in the text. Reprinted from ref 38.

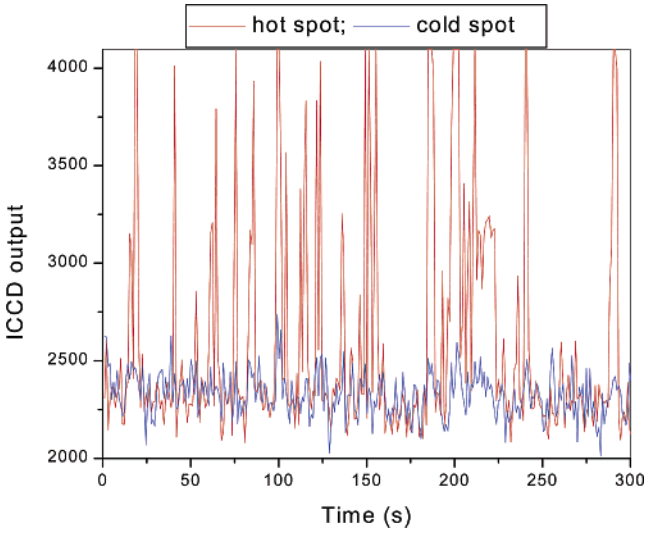


Figure 9. Signal from ICCD camera vs time for spot a of Figure 8 (red) and a nearby pixel (blue).

the polishing marks are where the strong adsorption sites are because one could argue that the greater brightness is due to the surface area being higher at the polishing marks. The surface area calculated from the AFM data even for sharpened tips is not high enough at the polishing marks to explain the increased brightness, but the AFM tip is so much larger than a molecule that the AFM measurement might underestimate roughness on the molecular scale. What is needed is to employ spatially resolved single-molecule spectroscopy to study the adsorption kinetics at polishing marks compared to that of flat regions of the surface. Xu and Yeung, for example, demonstrated the use of an intensified CCD (denoted as ICCD) camera in single-molecule spectroscopy, where the pixels in the camera are used to spatially resolve fluorescence from single molecules as a function of time.[36,37] The ICCD acts as an array of avalanche photodiodes, although the quantum efficiency of the ICCD camera is lower and the time response is slower due to data readout time. If adequate signal-to-noise can be achieved, then the ICCD camera can probe the adsorption and desorption kinetics at polishing marks compared to those of flat regions.

We used an ICCD camera to combine the spatial and temporal resolution of single molecules in dynamic equilibrium at the acetonitrile/C_{18} interface.[38] Figure 8 shows a fluorescence image over a 20-μm × 20-μm region, which was obtained using the ICCD camera. A region was chosen that had many spots due to polishing pits, and the image shows a variety of these spots that give brighter-than-average fluorescence. Spots that we studied are denoted in the Figure as a−g. This image is an average of 300 frames taken sequentially in time, where each frame was collected over a 1-s interval of time. The transfer time between frames was 0.022 s. The averaging over 1.0 s loses all information about the strong adsorption events that we had observed on the 100-ms time scale, but it retains information about sites that have desorption times on the scale of seconds. Consider point *a*, which is a rather dim spot close to the center of the beam. The fluorescence intensity at spot *a* as a function of time is shown in red in Figure 9, which reveals that the fluorescence fluctuates enormously on the time scale of seconds. When a single molecule is adsorbed at that spot, the fluorescence intensity causes saturation of the 12-bit analog-to-digital converter, registering its maximum output of 4095. The fluorescence only two pixels away is shown in blue in the same Figure, revealing that there are no significant excursions in the fluorescence. Single molecules adsorb to position *a* but not to any positions nearby on the scale of 1 s or longer. This experiment indicates that strong adsorption takes place at localized hot spots, and point *a* is a hot spot for strong adsorption.

Other spots in the average image of Figure 8 are further from the center of the beam, yet they are brighter. We found that the fluctuations in intensity at each of these spots near the periphery of the Gaussian beam are due to multiple molecules. These are concluded to be due to clusters of strong adsorption sites that are close enough together that they fall within one pixel, which is approximately 200 nm across. In each case, the fluorescence significantly fluctuates because of the statistically small numbers of molecules.[38] Fluorescence at points a few pixels from these hot spots did not fluctuate beyond random noise. There are a few pixels in the image where no spot is visible in the average image, but strong adsorption occurs once. For the vast majority of pixels, strong adsorption is never observed on the scale of 1 s or longer. For point g, the intensity always registered 4095, indicating that there are so many molecules adsorbed at this point that fluctuations cannot be observed because of the constant saturation of the analog-to-digital converter. The difference in intensity fluctuations among the spots a−g indicates that polishing pits have a widely varying number of strong adsorption sites.

This collection of hot spots, except for point g, allows one to obtain good statistics on strong adsorption to compare the desorption decay time of 2.6 s obtained from confocal microscopy. The intensity fluctuations for each individual spot, a−f, in Figure 8 were autocorrelated separately; then the individual autocorrelations were added together. Figure 10 shows the average of autocorrelations from the hot spots and the best fit to a sum of exponential decays.[38] The exponential decay of 2.6 s agrees with the autocorrelation decay of confocal microscopy, and the spatial resolution establishes that these events occur at only polishing marks.

The autocorrelation of Figure 10 reveals a slower desorption process that also occurs at these polishing marks. The very long time scale of this decay was down in the noise in the autocorrelation decay obtained by confocal microscopy. The very long desorption time is likely to be shortened by photobleaching, suggesting that the desorption time is even longer. To test this, we repeated these experiments at much lower

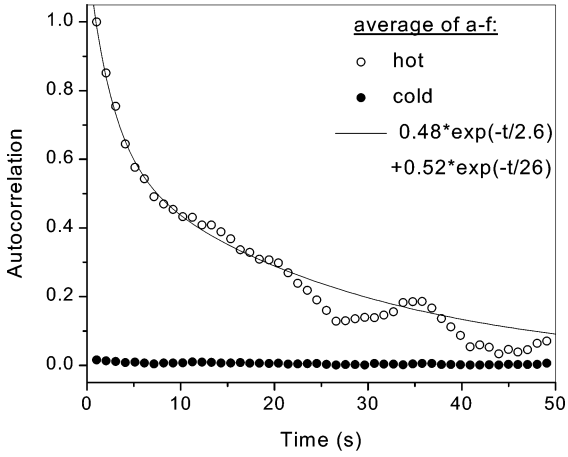


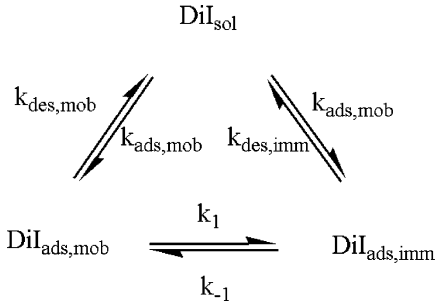
Figure 10. Average of autocorrelations for spots a−f of Figure 8: experimental (○) and best fit (−) to a double exponential. Reprinted from ref 38.

excitation power such that the fluctuations were not far above the noise, which prohibited any direct observation of single molecules but allowed the autocorrelation decay to be pulled out of the noise. We also acquired an order of magnitude more frames to enhance the signal-to-noise ratio. The lower excitation power caused both decay constants to lengthen in time: the 2.6-s decay became an 8-s decay, and the noisy 26-s decay becomes a less noisy 2-min decay.[39] The latter might still suffer shortening from photobleaching. The two decay components are about equal in their preexponential factors.

Combining all results, there are apparently four states of being adsorbed for DiI at this interface: (1) mobile adsorbates, (2) adsorbates that are immobile on the time scale of >2 min, and (3, 4) adsorbates transiently stopped for two different times, 68 ms and 8 s. The two adsorption processes of 8 s and >2 min are known to occur at polishing marks. It is not known yet whether the 68-ms transient adsorption process occurs at polishing marks. The mobile adsorbates are virtually everywhere on the surface and diffuse as though they constitute the intended and desirable interfacial adsorption.

One can only speculate about the chemical nature of the strong adsorption sites at this time. Rivera and Harris used infrared spectroscopy to probe pyridine and a sol−gel silica surface simultaneously.[40] They found that pyridine exhibited a bi-Langmuir adsorption isotherm, with adsorption coefficients an order of magnitude apart. The weaker adsorption sites were found to be −OH groups of bound water, and the stronger adsorption sites were −OH groups of isolated or weakly hydrogen bonded silanols. Interestingly, neither of these sites is charged. In relation to the single-molecule work, it is possible that water and isolated silanols correspond to our sites having 68-ms and 8-s desorption times, respectively, and that these are responsible for chromatographic broadening and tailing. Further evidence is needed to support this idea. Another speculation is that the >2-min desorption process is caused by DiI first being adsorbed to the type of site associated with either the 68-ms or the 8-s desorption time, but then the dye is buried by the C_{18} chains, allowing its release at a much later time. This could be tested by studies of monolayers having shorter chain lengths. The motion of the C_{18} chains could also be the cause of the rare shifts in fluorescence intensity, although this has not been studied yet. This slow process having a desorption time of >2 min would contribute, in practice, negligibly to the chromatogram because its time scale is too long. Further experiments are required to relate the single-molecule findings to the sites identified by Rivera and Harris.

SCHEME 2



We have assumed thus far that fused silica, sol gel films, and silica gel have similar types of adsorption sites because the Zhuravlev model[7] holds that all amorphous silicas have comparable surfaces. However, chromatographic silica gel is not polished, raising the obvious question of whether our results showing the unique role of the polishing marks has anything at all to do with chromatography as it is practiced. It is plausible that the same types of sites can be on silica gel because silica gel is probably not flat. A topographical origin of strong adsorption would explain quite a few quandaries about tailing. It would explain why there are different amounts of tailing from lot to lot even though the composition of the silica is the same, its nominal BET surface area is the same, and its hydrocarbon coverage is the same. A small percentage of niches could contribute to rare strong adsorption while negligibly affecting the average surface area. A topographical origin might explain why endcapping has the effect of persistently reducing tailing even after acid hydrolysis of the surface: the endcapping reagent makes its way into the nanometer indentations, where it is needed the most, which is also where subsequent hydrolysis would be slowed sterically. Proving that strong adsorption for chromatographic silica has a topographical origin will require direct evidence of these same desorption rates from chromatographic silica.

We studied the desorption behavior of DiI from chromatographic silica gel.[39] To show that desorption from strong to weak sites can be rate-limiting for the desorption of DiI from particles, Scheme 2 depicts the relevant equilibria, depicting only one type of strong site for conciseness. This model assumes that desorption from strong sites to weak sites along the surface is the main path for depopulating the strong sites, which is reasonable. Under these conditions, the desorption time constant for DiI from the particles would approach $1/k_{-1}$. For fused silica, there are three possible values for $1/k_{-1}$, and a multiple-component decay would be observed for chromatographic silica if it had the same three types of strong adsorption sites. To probe the chromatographic particles experimentally, we placed individual silica gel particles inside the channel of a microfluidic chip and equilibrated them with DiI in acetonitrile. We made use of the fact that the flow can be switched rapidly by the use of high electric fields to control electroosmotic flow.[39] We switched the flow stream from being DiI in acetonitrile to being pure acetonitrile, and we monitored the decay of fluorescence simultaneously from the particles and from the mobile phase by using an ICCD camera. The time resolution was 0.1 s, limited apparently by the inertia of the fluid and precluding the study of the 68-ms component. The results are shown in Figure 11, where there is a distinct lag between the drop in fluorescence of the solution compared to that of the particles. The decay fits well to an exponential with a decay constant of 7.1 ± 0.5 s, where the error is estimated from replicate experiments. The decay constant agrees with the 8.0 ± 2 s time constant we

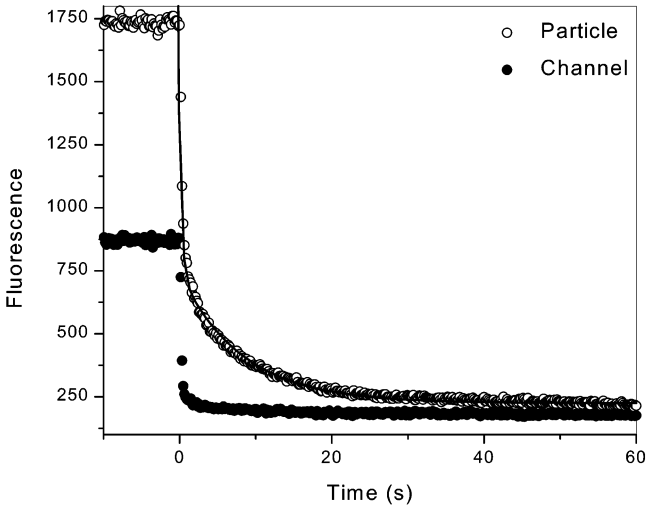


Figure 11. Fluorescence vs time as DiI desorbs from a silica gel particle (○) and the open channel (●). The solid line is an exponential decay of 8 s whose amplitude is approximately $^1/_3$ of the initial fluorescence.

observed for desorption from strong sites on the fused silica. We observed this decay when the particles equilibrated with DiI solution for >1 min; equilibration times of 10 s or less showed no decay. The slow desorption kinetics are thus associated with very slow adsorption kinetics. In this same work, we also investigated the presence of a longer decay component of >2 min for the silica gel.[39] The results revealed an exponential decay component of 4.0 min, and the adsorption kinetics were also slow. Overall, the agreement in desorption times between fused silica and chromatographic silica gel for these two desorption processes supports the notion that the strong adsorption sites on fused silica, which have a topographical origin, are the same as those on chromatographic silica.

The difficulty in rapidly switching flowing streams makes it more challenging to determine directly whether the 68-ms desorption process of fused silica also corresponds to sites on silica. We are presently addressing this question by studying chromatograms of DiI quantitatively using simulations,[18−20] in combination with experimental studies of the flow-rate dependence of the zone shape, to determine the time scales of the processes that cause tailing of DiI on commercial chromatographic silica gel. Preliminary results show that the primary contributor to the tailing zone is a strong adsorption site having desorption somewhere in the range of 45−90 ms. This again points to the same types of strong adsorption sites being on both fused silica and silica gel. The surprise is that the slower two processes (8 s and >2 min) do not contribute significantly to the zone. Perhaps this owes to their adsorption kinetics being too slow. These sites might be important for peptides and proteins, which tail more severely than DiI, and this is currently under investigation.

Summary and Future Work

The single-molecule results, in combination with the body of literature in chromatography, suggest that the nature of the so-called active silanols that cause tailing in chromatography at low pH is that of neutral, isolated silanols. The spectroscopic results show that the two extremely strong adsorption sites, having desorption times 8 s and >2 min, are prevalent at nanoscale topographical features on fused silica. Efforts to obtain vibrational spectra with nanoscale resolution would help to meet this goal, and an example is nonlinear chemical imaging nanomicroscopy.[41] The most frequent strong-adsorption process for fused silica exhibits a desorption time of 68 ms, and it is not yet known whether these sites are located at topographical indentations. Preliminary results in our lab indicate that, on the basis of desorption time, fused silica and silica gel have the same three strong adsorption sites. The significance of this correspondence of sites is that fused silica allows complementary tools for study such as AFM and chemical force microscopy.

A remaining quandary is the role of pH. A qualitative glance at the chromatogram of Figure 1 leads one to expect strong adsorption to decrease at low pH. We varied pH to "titrate" these strong adsorption sites by fluorescence imaging, hoping to learn the pK_a values. What we observed instead was at first surprising: there is no pH dependence. Fluorescence images are independent of pH down to pH = 1.[35] Furthermore, the autocorrelations of single-molecule events also show no dependence on pH. It does not matter whether the solvent is pure water, pure acetonitrile, or a mixture of the two solvents; there is simply no pH dependence. The strong adsorption processes observed by single-molecule spectroscopy are also unaffected by changes in KCl concentration; therefore, these cannot involve ion exchange. The change in the chromatogram with pH that is so evident in Figure 1 is unrelated to the strong adsorption processes that we observe. The single-molecule studies apparently report only the adsorption that leads to chromatographic tailing at low pH, and the results indicate that this tailing is not caused by ion exchange. This agrees qualitatively with the results of Rivera and Harris for pyridine adsorption, which pointed to neutral adsorption sites. The single-molecule results indicate that the same neutral adsorption sites existing at low pH are in the same abundance at high pH; however, high pH has an additional contribution to tailing that is not sensed by the single-molecule measurements. We speculate that Coulombic interactions at higher pH explain the increased broadening.

This would require a revised interpretation of tailing in chromatography at low pH. The single-molecule studies indicate that the acidity of the isolated silanols is not what causes tailing at low pH; instead, the isolated silanols are neutral. In addition to being consistent with the results of Rivera and Harris,[40] the idea of neutral strong adsorption sites is also consistent with the papers that have demonstrated the value in probing silanophilic activity with polar but uncharged adsorbates.[5,6] Furthermore, we observed the strong adsorption of single molecules of oligonucleotides,[42] which are negatively charged at neutral pH. These indeed adsorb to polishing marks.[43] We observed two types of transient adsorption sites for oligonucleotides, with the decay constants being shorter than for DiI: 16 and 220 ms.[44] These studies used homooligonucleotides, showing the prevalence of strong adsorption correlated with the ability of the base to act as a hydrogen bond acceptor, consistent with its adsorbing to neutral, isolated silanols. Although these ideas are consistent with observations, further experiments using a wider variety of adsorbates are needed to test the generality of these ideas.

An interesting question is why topography and strong adsorption would be related. There are two possibilities. First, nonplanar topography might make it easy to isolate a silanol from its neighbors, allowing strong hydrogen bonding to DiI. Second, molecular-scale niches might be places at which complete coverage is sterically difficult to achieve. These are two very different physical origins, one invoking a unique substrate site and the other a unique opening in the hydrocarbon. Further experiments are needed to distinguish these two possibilities.

The discussion of tailing has been motivated by the need for improving protein separations. Protein adsorption is more

complicated to understand because proteins can denature and they can have multiple points of contact due to their greater size, in addition to having the various interactions that small molecules have with these heterogeneous surfaces. We have observed that proteins adsorb much more strongly to polishing marks than do DiI and oligonucleotides.[45] Some polishing marks had different adsorptivities for proteins than do others, which was indicated by differences in the ease of desorbing protein by high ionic strength. It could be that multiple interactions with silanols and SiO^- groups are responsible for the stronger adsorption. Protein separations are often performed at neutral pH (e.g., size-exclusion chromatography and capillary electrophoresis). The sensitivity to ionic strength for protein adsorption to polishing marks underscores the importance of understanding ion-exchange interactions in the long run.

It is not at first obvious why the Coulombic interaction, which has the most marked effect on the chromatogram, has no affect on desorption times in the single-molecule experiment. One possibility is that Coulombic interactions, being long-range, exert the same force regardless of whether molecules are adsorbed at silica sites or diffuse across hydrocarbon sites. We have studied the lateral diffusion behavior of DiI extensively at neutral pH using confocal microscopy.[46] Over the course of these studies, we noticed that low pH and higher ionic strength gave a 25% enhancement in the apparent lateral diffusion coefficient. The Coulombic interactions could be manifesting themselves by pulling the charged dye closer to the surface, giving rise to increased friction from the tethered hydrocarbon chains. The single-molecule experiment, as it is designed, does not sense equilibrium between the surface and the solution directly; instead it senses equilibrium within the surface plane, where the Coulombic potential is constant. This enabled the single-molecule experiment to reveal that the strong adsorption sites are neutral.

To use the kinetic and thermodynamic information for designing new materials, more details are needed. The sites having the slowest desorption times (8 s, 4 min) might not necessarily have the largest partition coefficients because the adsorption kinetics are slow. It is speculated that these sites have larger interaction strengths with DiI to give slow desorption, but they have an entropic barrier to slow adsorption. This speculation is consistent with McCalley's observation of an apparent steric hindrance of strong adsorption.[16] Sterically inaccessible strong adsorption sites would also explain why they are so hard to cover by endcapping. An unusual entropic barrier to explain slow kinetics for strong adsorption would be revealed from a study of the temperature dependence of the adsorption rate. The hydrogen-bonding moiety of lysine, which adsorbs strongly to silica, is much more sterically accessible than that for DiI. Therefore, single-molecule studies of rhodamine-labeled synthetic peptides at the same sites, as compared with DiI, will be illuminating. Tailing will be understood much better once research can sort out the entropic barriers to adsorption and energetic barriers to desorption, identify the functional groups involved in the strong adsorption, and reveal how nanoscale morphology contributes to these adsorption properties. New tools are needed.

Acknowledgment. This work was initiated by a grant from the Petroleum Research Foundation (29660-AC6,7) and has been supported by grants from the National Science Foundation (CHE-9610446 and CHE-0078847). M.D.L. and D.J.S. are grateful to the University of Delaware for fellowship support.

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