Effect of Coulombic Interactions on Rotational Mobility of Guests in Sol−Gel Silicate Thin Films

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We investigate the effect of Coulombic interactions on the mobility of rhodamine 6G (R6G) and Oregon Green 514 (ORG) in sol−gel silicates by measuring their mobility distributions using single molecule polarization measurements. While R6G is a cationic dye, ORG ($pK_a = 3.69 \pm 0.08$) is an anionic dye at neutral pH. The presence of more tumbling ORG molecules in sol−gel silicates indicates that R6G and ORG experience opposite Coulombic interactions with a predominately anionic silica sol−gel surface. On the other hand, the fact that tumbling ORG only represents a minor portion of ORG investigated, even at pH 7, clearly illustrates that Coulombic interaction alone does not control the mobility of an encapsulated guest molecule in sol−gel silicates.

Introduction

Surface adsorption can alter the physical and chemical properties of an adsorbate. This has been advantageously exploited in silica sol−gel chemistry to synthesize new optical and biocomposite materials. For example, organic dyes encapsulated inside a sol−gel silicate were reported to exhibit a higher photostability.[1−6] It has also been demonstrated that a higher temperature is required to denature a silica sol−gel bioencapsulate.[7−10] In these materials, a guest molecule entrapped inside a mesoporous silica sol−gel matrix is expected to experience a substantial amount of surface interaction, which has a direct impact on the mobility of the guest molecule. As a result, a wide range of molecular mobility originating from different extents of surface interaction has been observed.[11−15] Such a broad distribution of molecular mobility will inevitably lead to diverse physical and chemical properties of the entrapped guest molecules. Electrostatic interaction is an important driving force for molecular adsorption on a silica surface. Under physiological conditions, a negatively charged silica surface (isoelectric point $= 2.0 \pm 0.2$) can facilitate the immobilization of small organic dye, DNA, RNA, and large protein molecules through adsorption. To facilitate the development of silica sol−gel composite materials and to gain control of molecular dynamics inside sol−gel silicates, we examine the significance of Coulombic interaction on the mobility of a guest molecule entrapped inside a silica sol−gel matrix. Owing to the extensive structural and chemical heterogeneities found inside sol−gel silicates, we employ single molecule spectroscopy to examine how Coulombic interaction is manifested at the molecular level.[16,17] The mobility of a molecule can be examined by monitoring the changes of its orientation in real time. This can be accomplished at levels ranging from simple two-dimensional projection to sophisticated three-dimensional orientation determinations.[18−24] In a previous report, we have successfully demonstrated how single molecules inside a silica sol−gel matrix can be separated into different

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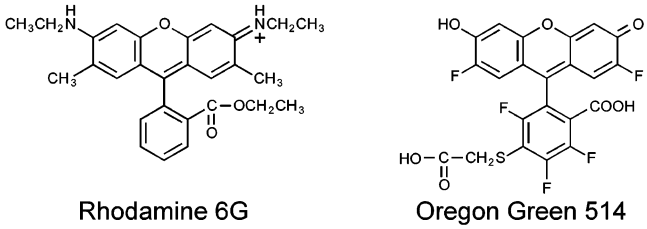

Figure 1. Molecular structures of rhodamine 6G (R6G) and Oregon Green 514 (ORG).

mobility classes using fluorescence polarization measurement.[25] In the present study, we employ molecular mobility to monitor the extent of guest−host interaction that is specifically contributed by Coulombic interaction. Here, we assume that guest−host interaction will hinder the rotational motion of a guest molecule, with weak guest−host interaction favoring the encapsulation of freely tumbling guest molecules whereas strong anisotropic guest−host interaction at a liquid/surface interface will lead to efficient immobilization of the guest molecules.

To examine the effect of Coulombic interaction on guest−host interaction, we report the mobility measurement of rhodamine 6G (R6G) and Oregon Green 514 (ORG) encapsulated in silica sol−gel thin films. The molecular structures of R6G and ORG are shown in Figure 1. R6G contains an iminium ion and is regarded as a positively charged probe molecule. Since the positive charge in R6G delocalizes throughout the entire xanthene moiety, the iminium proton normally does not participate in acid−base equilibria. R6G fluorescence is therefore quite insensitive to external pH variations at physiological conditions. At high pH, however, say pH 12, the ester group of R6G will undergo base-catalyzed hydrolysis (p$K \sim 11$) and turn into a zwitterion.[26] For the choice of negatively charged probe molecule, we chose ORG in favor of fluorescein for our investigation in view of its higher photostability. Similar to fluorescein, the fluorescence properties of ORG are also pH sensitive. Depending on its surrounding pH, an ORG molecule can be either neutral (weakly fluorescent) or negatively charged (strongly fluorescent). Since the carboxylic acid groups in ORG have low pK_a values (via infra), its fluorescence gradually becomes insensitive to pH greater than 6.0. In this study, ORG is regarded as a negatively charged probe molecule because, by default, it is more difficult to observe the weakly fluorescent neutral ORG molecules at a single molecule level.

The effect of Coulombic interaction was readily observed when more tumbling ORG than R6G molecules were found in sol−gel silicates. Presumably, charge−charge repulsion between ORG and a silica surface promotes ORG mobility by preventing it from adhering strongly to the surface of the inorganic matrix. On the other hand, Coulombic attraction between R6G and the silica matrix favors surface adsorption and substantially restricts R6G mobility. Surprisingly, we also found that tumbling R6G and ORG only account for a small portion of single molecules that we investigated. The majority of the molecules we examined were either completely fixed or loosely bound to a surface and capable of changing orientation occasionally. Our results therefore indicate that long-range Coulombic interaction influence molecular mobility inside a sol−gel silicate only to a limited extent. The nanoscopic confinement of a highly polarizable guest molecule imposed by a silica sol−gel matrix may favor guest−host interactions contributed by shorter range electrostatic interactions such as van der Waals interactions and H-bonding. Alternatively, it is also quite possible that guest−host interaction is dominated by mere physical confinement as a result of molecular templating.

Experimental Section

Materials. 99.9+% Tetraethyl orthosilicate (TEOS), spectrophotometric grade 95% ethanol, and 85 wt % phosphoric acid were purchased from Sigma-Aldrich. Oregon Green 514 and rhodamine 6G were purchased from Molecular Probes. All chemicals were used without further purification. A phosphoric acid solution to catalyze TEOS hydrolysis was prepared by diluting the 85 wt % stock solution 100 times using deionized water. Microscope cover glasses (Fisher Premium) were purchased from Fisher Scientific and were thoroughly cleaned before use.

Preparations of Silica Sol−Gel Thin Films. Sample preparation, polarization measurements, and the scheme for mobility classification are employed as previously described.[25] Briefly, tetraethyl orthosilicate (TEOS) sol solution was prepared from the acid hydrolysis of TEOS in an ethanol−water solution mixture in 1:8:7 molar ratios. Thus, neglecting the ~5% water in ethanol, our sol solution consisted of 177 μL, 358 μL, and 100 μL of TEOS, ethanol, and water, respectively. To facilitate hydrolysis, the solution mixture was sonicated for 2 h, during which time the temperature of the sonication bath was kept at 0 °C to minimize condensation of the sol. To prepare samples for single molecule measurements, the sol solution was slightly doped to 1.7 nM final dye concentration right after the sonication process. On the other hand, to record bulk emission spectra of ORG in silicate films, samples were prepared from sol solutions that contained a higher dye concentration of 30.7 nM. Regardless of the final dye concentration, the dye-doped sol solution was allowed to age at ambient temperature in the dark for 16−20 h before it was spun cast onto a cleaned coverglass at 6100 rpm for 70 s. Samples prepared as such were considered dry films and were examined at their nascent pH. To examine the mobility of ORG at different pHs, a dry film coated coverglass was used to seal one end of a plastic tube and make a sample cuvette that could be used to hold buffer solutions, thereby allowing a buffer-wetted silica sol−gel film to be examined in situ.

Single Molecule Fluorescence Measurements. Fluorescence images of single ORG and R6G molecules were acquired by a homebuilt sample-scanning confocal microscope, which was based on an inverted microscope (Nikon, TE−200) and a nanopositioning stage with positional feedback electronics (Melles Griot, NanoBlock). Fluorescence images and kinetics traces from single molecules were obtained under continuous laser excitation delivered to the epi-illumination port of the microscope by a single-mode optical fiber, which also served as a 3.3-μm diameter spatial filter. The excitation laser that came out from the other end of the fiber was first collimated by a 10× objective, filtered through an interference filter, and then reflected up to a 100× microscope objective (Nikon, CFI Achromat oil immersion 1.25 N. A.) by a dichroic beam splitter (Chroma Technology). To ensure that randomly oriented single molecules were equally selected, all samples were studied with circularly polarized laser excitation. Single molecule fluorescence collected by the same microscope objective was directed to exit from the side port of the microscope and clear a 100-μm aperture positioned at the first image plane. Fluorescence

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diverging from the aperture was then collimated with an achromatic lens, passed through a long-pass filter (Omega Optical, ALPHA Technology) to eliminate scattered laser excitation before it was directed to a polarizing beam splitter cube. The beam splitter cube resolved the fluorescence into two orthogonally polarized components which we arbitrarily designated as parallel ($I_{\parallel}(t)$) and perpendicular ($I_{\perp}(t)$). The polarization-resolved fluorescence was subsequently detected by two separate avalanche photodiode (APD) detectors (Perkin-Elmer, SPCM-AQR).

Upon raster scanning of a sol−gel silicate sample, each APD detector would produce a fluorescence image from the same sample area with the respective fluorescence polarization. The spot size of the dyes were mostly diffraction limited and were measured at ∼280 nm. The typical size of a fluorescence image was fixed at 10 μm × 10 μm. To examine the photophysical properties of single molecules, a molecule was first chosen from a fluorescence image and then transported to the laser focus by the nanopositioning stage. Afterward, fluorescence from the chosen molecule was monitored by two separate APDs where $I_{\parallel}(t)$ and $I_{\perp}(t)$ were simultaneously recorded by the APDs at 50-ms dwell time upon continuous laser excitation. To record bulk emission spectra of ORG-doped silica films, collimated fluorescence from thin-film samples was directed to a spectrometer that is made from a polychromator (Acton, SpectraPro 150) and a liquid-nitrogen cooled CCD camera (Roper Scientific, SpectruMM).

Polarization Measurements and Mobility Classification. To perform polarization measurements, single molecule fluorescence polarization ($P(t)$) was calculated according to eq 1.

$$P(t) = (I_{\parallel}(t) - I_{\perp}(t))/(I_{\parallel}(t) + I_{\perp}(t)) \quad (1)$$

In addition, the statistical average of $P(t)$ for the entire course of measurement performed on each molecule was also calculated as the average polarization ($\bar{P}$). This $\bar{P}$ was compared with the average standard deviation (σ_{iso}) of several $\bar{P}$ measurements made daily on concentrated dye solutions. Assuming that the dye solution emitted isotropic fluorescence ($\bar{P} = 0$), a molecule would be classified as "tumbling" if its $\bar{P}$ was within $\pm\sigma_{iso}$ from zero polarization. Otherwise, it would be assigned as "fixed". The exact value of σ_{iso} varied slightly on a daily basis and depended strongly on fluorescence intensity. Using dye solutions to produce fluorescence that was of similar intensity to single molecule emission, σ_{iso} of 0.11 ± 0.02 and 0.066 ± 0.015 were obtained and used for the classification of ORG and R6G, respectively. This classification scheme is unable to differentiate a tumbling molecule from a fixed molecule that lies exactly 45° between the parallel and perpendicular polarization axes. Thus, the number of tumbling molecules reported in this study should be regarded as an upper limit.

Results and Discussion

A total of 193 R6G and 241 ORG molecules in dry films were examined and their mobility distributions are compared in Figure 2. The opposite charge between R6G and ORG results in a 7-fold difference in the population of freely tumbling molecules, with 2% in R6G and 14% in ORG. Since it is possible that some molecules are erroneously assigned as tumbling (via supra), the actual percentage of tumbling molecule is expected to be less in both samples. This should lead to an even bigger contrast in the percentage tumbling molecules between R6G and ORG. The low number of tumbling R6G molecules suggests that they interact strongly with the silicate matrix when trapped, presumably through Coulombic attraction. In addition to fixed and tumbling molecules, there is also a sizable portion of "intermediate" molecules in both samples. Intermediate molecules

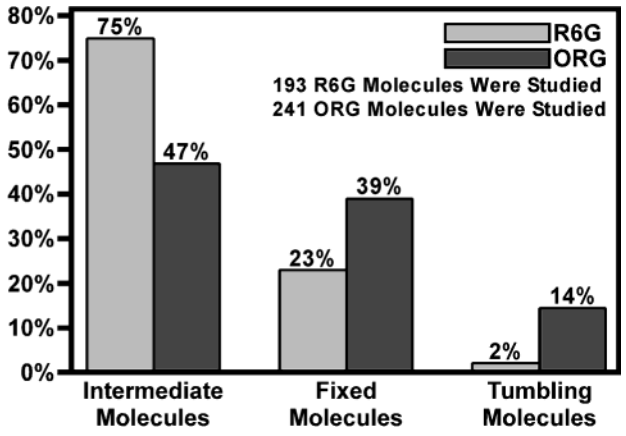

Figure 2. Mobility distributions of R6G and ORG encapsulated in dry sol−gel silicate films.

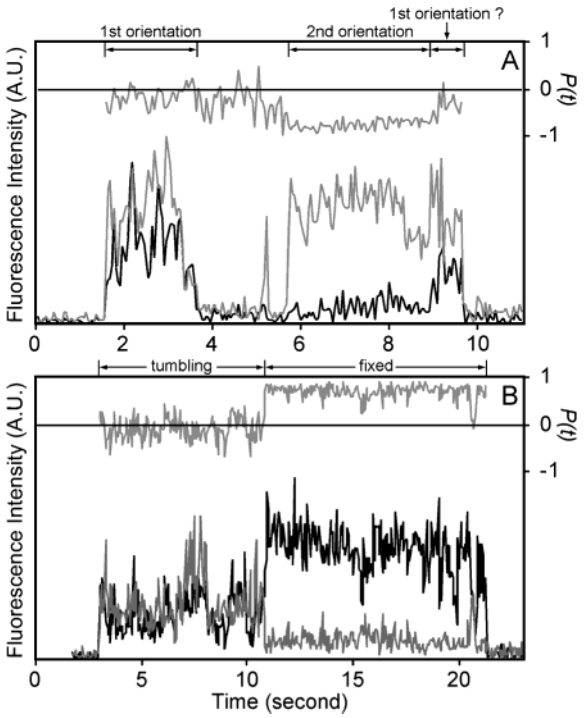

Figure 3. Examples of intermediate ORG molecule fluorescence time traces. Panel A illustrates a molecule that switches between two fixed orientations, with $\bar{P}$ for both orientations below zero. The molecule in panel B is probably best described as a tumbling molecule that is suddenly immobilized at a fixed orientation. Photobleaching of a single molecule is indicated by the abrupt drop of fluorescence intensity at the end of both panels. The black line and gray line in each panel represent $I_{\parallel}(t)$ and $I_{\perp}(t)$, respectively. An additional gray line that is drawn close to the top of each panel represents $P(t)$ of the respective molecule.

are molecules that switch polarization occasionally because of a weaker interaction with the silica surface. These molecules tend to jump between different orientations or they may temporarily dissociate from a silica surface and tumble into the solvent medium during the course of a measurement, resulting in a change in emission polarization ($P(t)$) as shown in Figure 3.

The substantial amount of immobilization observed in R6G can be partially explained by Coulombic attraction between the cationic imino group and the anionic silica surface, resulting in very few tumbling R6G molecules. In view of the structural similarity between

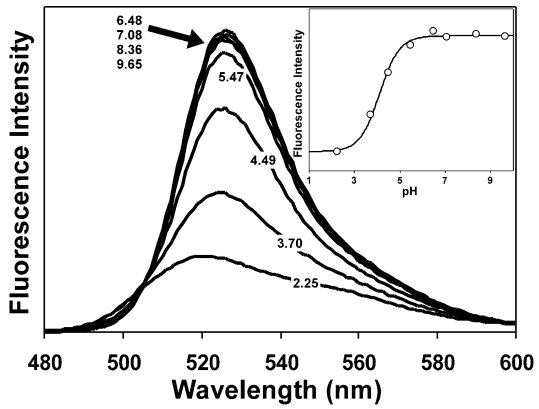

Figure 4. Emission spectra of ORG dissolved in different pH buffers. All spectra were recorded by a Shimadzu RF-5310PC fluorometer using 457-nm excitation. Inset: Data points were obtained by integrating the fluorescence intensity between 465 and 600 nm from each emission spectrum. The solid line is a fit to the Henderson−Hasselbalch equation, which gives an estimated pK_a value of 3.69 ± 0.08 for ORG.

R6G and ORG, however, the noticeable increase in the amount of tumbling ORG molecules is most likely attributed to Coulombic repulsion between ORG and the silica surface. Nevertheless, the relatively small portion of tumbling ORG molecules found may imply that (i) Coulombic interaction alone does not determine molecular mobility in sol−gel silicate and (ii) the acidic hydrolysis of TEOS during sample preparation led to low internal sample pH and rendered both ORG and the silica surface neutral (isoelectric point = 2.0 ± 0.2).[27] This would eliminate Coulombic interaction between ORG and the silica surface and produce fewer tumbling molecules. Figure 4 shows the emission spectra of ORG dissolved in a series of pH buffer solutions when excited at 457 nm. As pH increases, the fluorescence intensity of ORG also increases while anionic ORG becomes the dominating species. According to a fit to the Henderson−Hasselbalch equation depicted in the inset, the pK_a value of ORG was 3.69 ± 0.08. Thus, it is expected that the effect of Coulombic repulsion between ORG and a silica surface would diminish if the internal pH of a silica sol−gel film drops below 3.69.

To estimate the internal pH of a silica sol−gel film, we compare in Figure 5 the normalized bulk fluorescence spectra of ORG when excited at 488 nm in a dry film, ORG dissolved in different pH buffers, and ORG in films that were in contact with various pH buffers. It is apparent from Figure 5 that the emission spectra of ORG in solutions are considerably narrower than those obtained from ORG in the wet films. The solution spectra also exhibit a systematic blue-shift of the peak maximum from 535 to 526 nm with increasing pH, whereas the peak maxima of the film spectra are relatively unchanged at 526 ± 1 nm, regardless of the external pH. The broadening of ORG emission from the wet films clearly reflects the heterogeneous nature of sol−gel silicates. Surprisingly, the fluorescence spectrum of ORG in a dry film is characterized by a narrow peak maximum located at 531 nm and it strongly resembles the solution spectrum of ORG at pH 4.49. While it is quite unlikely that ORG in a dry film would

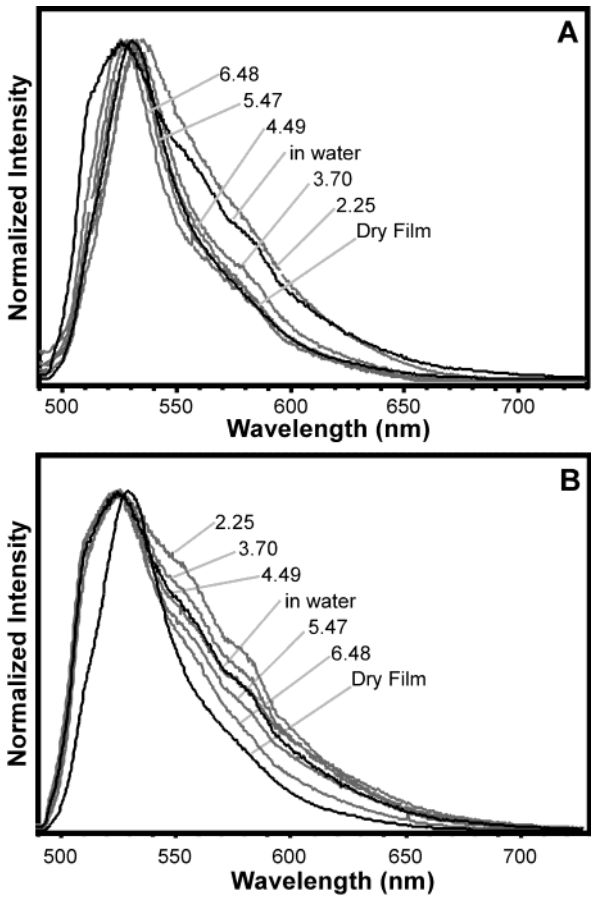

Figure 5. Panel A: Gray lines, emission spectra of ORG dissolved in different pH buffers. Black lines, emission spectra of ORG-doped dry silica sol−gel films before and after it is in contact with water. Panel B: Gray lines, emission spectra of ORG-doped silica sol−gel films in contact with different pH buffers. The emission spectra of ORG-doped dry silica sol−gel film before and after it is in contact with water are reproduced as the black lines. All spectra in panel A and B are collected from a homebuilt confocal microscope equipped with a polychromator and a liquid nitrogen cooled CCD camera using 488 nm excitation.

experience a more solution-like environment than in a wet film, an alternative explanation which suggests that the narrow peak reflects a more homogeneous internal environment in dry films is truly intriguing. The exact reason that leads to this interesting phenomenon is unclear and is currently being investigated. If the internal pH of a dry film was indeed close to 4.49, then both ORG and the silica surface should be negatively charged and Coulombic repulsion between them should be operational. That would explain why silica sol−gel encapsulated ORG displays more tumbling molecules than R6G. Meanwhile, the emission spectra of ORG are significantly broadened when the films are in contact either with water or with different pH buffers, indicating that the encapsulated ORG molecules were exposed to a much more heterogeneous and dynamic environment once the film was wet. The broadening of ORG emission spectrum also confirmed that despite being encapsulated, the ORG molecules were still capable of interacting with external reagents, in this case, water. Since a dramatic reduction of ORG emission in a wet film was also observed, we believe that the dynamic interaction between an encapsulated ORG and water

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Table 1. Mobility Distributions of Rhodamine 6G and Oregon Green 514 in Different Sol−Gel Silicate Thin Films

	fix	tumbling	intermediate	total number of molecules
dry R6G	45 (23 ± 3%)	4 (2 ± 1%)	144 (75 ± 3%)	193
R6G in water	131 (69 ± 3%)	8 (4 ± 1%)	51 (27 ± 3%)	190
dry ORG	93 (39 ± 3%)	35 (14 ± 2%)	113 (47 ± 3%)	241
ORG in water	82 (43 ± 4%)	22 (12 ± 2%)	86 (45 ± 4%)	190
ORG in pH 7.0	54 (41 ± 4%)	31 (23 ± 4%)	48 (36 ± 4%)	133

is responsible for both spectral broadening and the quenching of ORG fluorescence. Interestingly, the emission spectrum of a water-wetted ORG film indicates that the internal pH was between 4.49 and 5.47, similar to its internal pH when it was dry. This suggests that water alone did not alter the internal pH of the silicate film significantly and that the internal pH remained close to 5.0 ± 0.5.

To explore if external pH can alter the mobility of silica sol−gel encapsulated ORG, we measured the mobility distributions of ORG in wet films that were in contact with water and with a pH 7.0 buffer. The results are summarized in Table 1. Despite a significant broadening of the emission spectrum of ORG as indicated in Figure 5, exposing the film to water apparently causes no noticeable change to the mobility of ORG. This further confirms that water alone did not alter the internal pH of the silicate film. On the other hand, when the internal pH of the film was brought to 7.0 by the buffer, the percentage of tumbling ORG molecules almost doubled. This demonstrates that the effect of Coulombic repulsion between anionic ORG and anionic silica surface is considerably enhanced at high pH. Despite the Coulombic repulsion, tumbling molecules only account for 23% of all ORG examined at pH 7.0. This suggests that molecular mobility inside sol−gel silicates is not determined by Coulombic interaction alone. Table 1 also reveals an interesting fact that the percentages of fixed ORG in dry and wet films are insensitive to pH and they remain constant at ∼40%. This could be due to ORG entrapped inside tight silica sol−gel pockets that were inaccessible to external reagents, hence contributing to additional heterogeneity observed in wet films. Alternatively, this could be a strong indication of the existence of a non-Coulombic guest−host interaction that effectively suppressed molecular mobility inside sol−gel silicates. Our attempts to investigate how leaching distorted mobility distributions were inconclusive as the number densities of ORG were observed to both increase and decrease by as much as 15% upon wetting. Since it is unlikely that wetting would add more ORG into a silica sol−gel film, the increase in ORG number density was most likely the result of either inhomogeneous redistribution of dye or increasing ORG fluorescence intensity with higher pH. Nevertheless, we believed that leaching did not skew our observations in any significant way.

Unlike ORG, the permanent cationic imino group in R6G continues to experience strong attractions toward the silica surface in a wet film and produced mostly surface-bound fixed molecules. Biexponential fluorescence anisotropy decay and high residual fluorescence anisotropy have been reported from R6G and oxazine dyes encapsulated inside alcogels.[28−31] These previous observations were, respectively, attributed to different R6G local environments and strong R6G adsorption to the silica surface of the alcogels, which restricts rotational motions. Our results directly demonstrate that global reorientation of R6G is mostly prohibited in both dry and wet film.

Assuming that the overall structural and chemical environments of the sol−gel films are identical in the ORG and R6G samples, any difference between the guest−host interaction in ORG and R6G should originate directly from the different physical attributes of the two dyes. Under such circumstances, Coulombic repulsion between the two anionic carboxylic groups in ORG and the silica surface will weaken guest−host interactions to such an extent that surface-bound ORG has a higher tendency than R6G to dissociate from the silica surface. A similar argument has been proposed on a recent time-resolved fluorescence anisotropy study on sulforhodamine B in xerogels.[31]

The low number of tumbling R6G and ORG molecules in our samples is in sharp contrast to Geddes and Birch's observation that a probe molecule can remain bound to primary silica particles that reorient in different microviscosity environments long after gelation set in. On the basis of time-resolved fluorescence anisotropy measurements, they were able to estimate both the sol microviscosity and the radius of the primary silica particles in their monolith samples simultaneously.[32−34] Unlike sol−gel monoliths, our samples generally contain much less solvent because of rapid evaporation associated with the spin-coating process, thereby leading to a substantial loss of molecular mobility.[35] Very high steady-state fluorescence polarization has also been reported from probe molecules embedded in dip-coated thin films. Similar to a spin-coated sample, solvent evaporation and the rapid collapse of sol−gel cavities in a dip-coated film were believed to cause the loss of molecular mobility.[36]

In view of the minor percentage of tumbling ORG molecules found in both dry and wet films, we conclude that Coulombic interaction is unlikely to be the major force for surface immobilization inside sol−gel silicates. Other workers have suggested that in addition to Coulombic interactions, an immobilized molecule may

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also be subjected to H-bonding, hydrophobic interactions, and other nonspecific short-range interactions simultaneously. For example, hydrophobic interactions enhance the interaction between organic dyes and organically modified silicates in many occasions.[17,30,37,38] Thus, using organically modified precursor silicates to increase the internal hydrophobicity of silica nanoparticles, highly fluorescent organic dye-doped silica nanoparticles have been synthesized recently.[39] Finally, it is also quite possible that a probe molecule can act as a template for silica sol−gel matrix formation, thereby encapsulating the probe molecule inside a pore with a similar shape and physical dimension.[40] If it is indeed a major factor, this templating effect is expected to contribute to effective immobilization. Thus, immobilization should depend more on the conditions for sample preparation and less on any postsynthesis experimental condition. This may help explain the significant portion of fixed molecules that are insensitive to external pH changes that we observed.

Conclusion

For the first time, single molecule spectroscopy has allowed us to monitor directly the influence of Coulombic interaction on the global rotational mobility of a silica sol−gel encapsulated guest molecule at a molecular level. By directly observing the global rotational motion of a probe molecule at 50-ms intervals, our mobility classification is immune from the fluorescence depolarization effect of rapid segmental motions originating from immobilized molecules. Such fluorescence depolarization effect can be mistakenly identified with tumbling molecules in an ensemble measurement and tends to overestimate their overall population. The ability to observe rotational motion from single molecules also allowed for the detection of subtle changes in mobility distribution, as in dry and wet ORG films. Moreover, the presence of intermediate molecules revealed only by single molecule spectroscopy will provide new insights into slow molecular dynamics that are unlikely to be captured by both conventional steady-state and time-resolved ensemble measurements. Even though no silica particle bound R6G and ORG molecules were detected, our experiments reveal the existence of different classes of mobility that should provide an alternative to the interpretation of biexponential decay components and high residual fluorescence anisotropy commonly found in ensemble measurements.

Using R6G and ORG mobility to probe guest−host interaction, this study clearly demonstrates that Coulombic interaction alone only has a minor effect on the immobilization of a probe molecule in sol−gel silicates. It is therefore highly likely that guest−host interaction is mostly dominated by the effect of molecular templating or other short-range interactions such as van der Waals interaction and H-bonding. Our ongoing investigations on the mobility distributions of neutral and multiply charged probes is expected to provide a deeper understanding on the significance of electrostatic interaction as well as the effects of hydrophilicity and hydrophobicity on molecular mobility inside sol−gel silicate matrices.

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