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Single Molecules

Single Molecules as Optical Nanoprobes for Soft and Complex Matter

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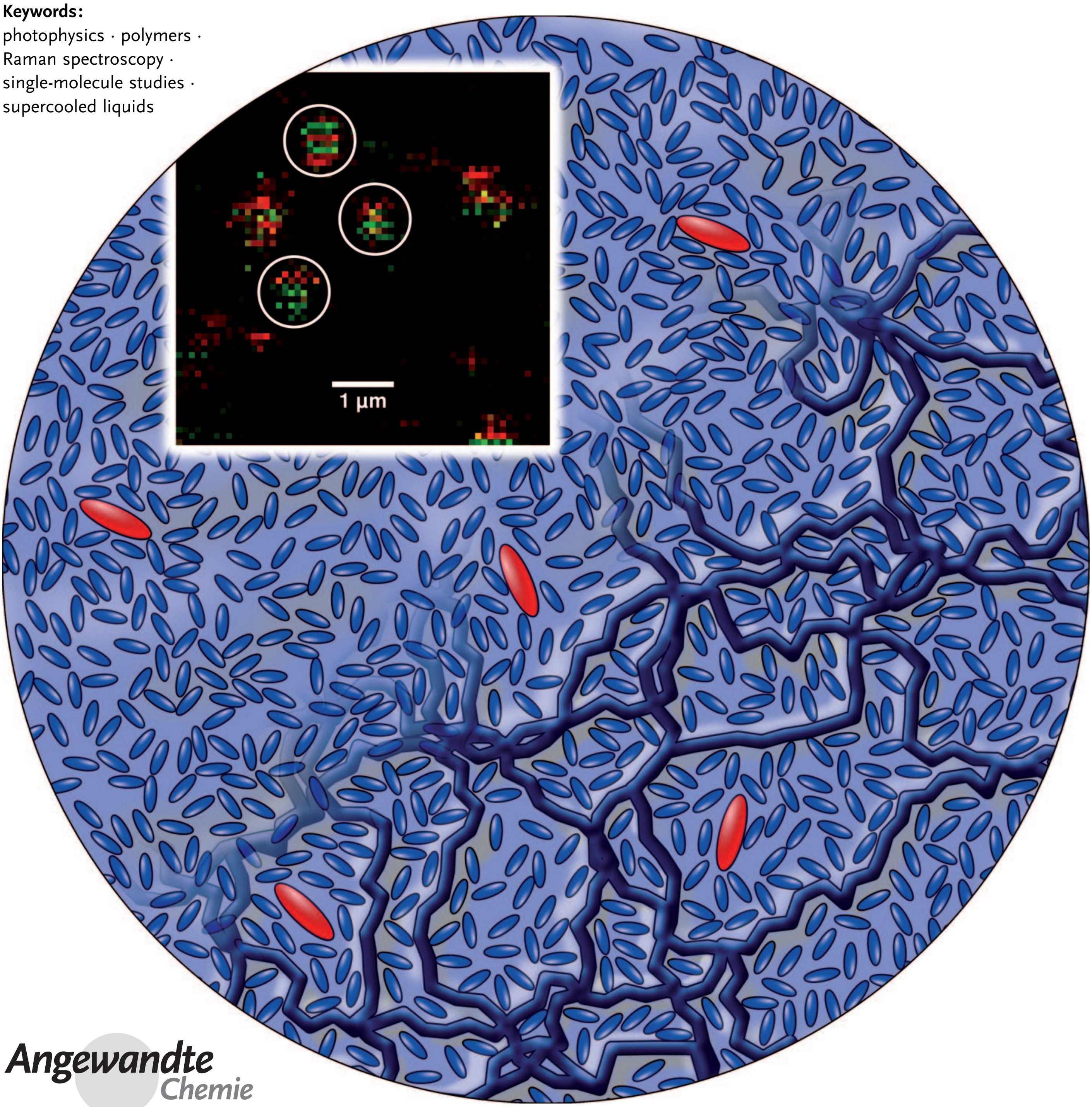

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The optical signals of single molecules provide information about structure and dynamics of their nanoscale environment, free from space and time averaging. These new data are particularly useful whenever complex structures or dynamics are present, as in polymers or in porous oxides, but also in many other classes of materials, where heterogeneity is less obvious. We review the main uses of single molecules in studies of condensed matter at nanometer scales, especially in the fields of soft matter and materials science. We discuss several examples, including the orientation distribution of molecules in crystals, rotational diffusion in glass-forming molecular liquids, polymer studies with probes and labeled chains, porous and heterogeneous oxide materials, blinking of single molecules and nanocrystals, and the potential of surface-enhanced Raman scattering for local chemical analysis. All these examples show that static and dynamic heterogeneities and the spread of molecular parameters are much larger than previously imagined.

From the Contents

1. Introduction

Nanoscience is the branch of the physical sciences dealing with the structure, dynamics, and properties of condensed matter at nanometer scales, that is, at length scales ranging from 1 to about 100 nm. Is there a real need for this new concept? A skeptic might argue that quantum mechanics, on the one hand, is the only proper description of matter at the scale of electrons, atoms, and molecules. On the other hand, many concepts drawn from continuum physics often appear to describe matter at microscopic scales quite accurately, at least for scales still significantly larger than molecules. It turns out, however, that the introduction of an intermediate, mesoscopic scale is not only convenient, but necessary for two distinct reasons. First, quantum mechanics, though irreplaceable as the conceptual framework of nanoscale physics, is notoriously difficult to apply practically to extended systems. This difficulty arises mainly from the exponential growth of accessible quantum states with size. Incidentally, this complexity is the very source of the powerful algorithms for the quantum treatment of information. The wealth of exotic phenomena displayed by assemblies of correlated fermions is a good illustration of quantum complexity. Many of these effects would have been very hard to predict theoretically. As stated by Anderson in a well known column,[1] "*more is different*", that is, complexity can lead to the emergence of qualitatively new properties. Second, the direct observation of matter at nanometer scales, which has only recently become possible, has already provided us with many unexpected, often counterintuitive observations, which challenge world views based only on macroscopic concepts. Matter at nanometer scales appears much more complex, rich, and surprising than was thought previously. As was found time and again in many scientific fields, new observation windows lead to fresh insights.

Herein the well-known advantages of molecular-scale observations are briefly summarized. In single-molecule studies, ensemble averaging is removed completely, and the full variety of microenvironments is revealed, together with the full extent of the distributions of molecular or local parameters. This direct observation of static or spatial heterogeneity is particularly useful in complex systems, such as porous materials, where disorder is intrinsic to the structure of the materials. In addition to structural information, single-molecule measurements form a unique approach to dynamics, because the details of individual fluctuations are no longer drowned in an unchanging average, and because no synchronization step is required to observe them. As a consequence, the comparison to theoretical models is direct and no longer involves adjustable distribution functions. It therefore becomes much more reliable and relevant.

In this Review, we discuss only optical single-molecule methods. More established methods, such as electron microscopy, and in particular scanning-probe microscopies (scanning tunneling microscopy, STM, and atomic force microscopy, AFM) and their different variants remain indispensable doorways to nanoscience. Powerful as they are, however, these methods have their limitations. They are often restricted to surfaces and thin samples, and to a narrow range of experimental conditions. They require a great stability, and their field of view is often limited.

Optical single-molecule spectroscopy is based on a "light" technique, far-field optical microscopy. Because it relies on the optical signal of a single molecule, or of a single emitter or absorber of light, it has all the advantages of optical

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techniques. It is generally non-invasive, it is easier to implement than scanning-probe techniques, and complements them in many ways, enabling transparent media to be probed in three dimensions, far below their surface, or a parallel recording of data on many molecules simultaneously. Moreover, a versatile toolbox of spectroscopic techniques offers time- or frequency-resolved information about the sample, which may provide spatially resolved chemical information, down to the level of one single molecule or of a few molecules.[2]

After the invention of scanning probe microscopies in the 1980s,[3,4] single-molecule spectroscopy started in the early 1990s,[5,6] first with experiments at low temperatures. An important advance was the imaging of the fluorescence of immobilized single molecules with near-field optics,[7–10] which was soon followed by similar experiments with confocal microscopes.[11–14] Thanks to steady progress in confocal microscopy and in solid-state avalanche photodiode detectors, the method quickly extended to many different systems in the late 1990s, in particular because of its great potential for the study of biophysical and biochemical phenomena. The applications to physical chemistry, although present from the start, have been somewhat eclipsed by the spectacular boom of biophysical work, but have steadily expanded. The aim of this Review is to illustrate the potential of single-molecule optical methods in physical chemistry and in materials sciences, in particular for soft and complex matter, stressing some of the surprises given by single-molecule observations. We shall review the different techniques used in single-molecule measurements to-date, and mention a few which could become of interest in the future. We shall then illustrate these techniques with recent results in the study of complex systems and materials, and we shall speculate on possible applications to new areas where single-molecule optical probing could become useful.

2. Concepts and Techniques in Single-Molecule Optics

In this section we introduce the main concepts and techniques of single-molecule optics to help the reader follow the topics discussed in the remainder of the article. In-depth Reviews of single-molecule optics, beyond the scope of the present article, can be found in Refs. [15–19].

In general, optical single-molecule techniques rely on the combination of two strategies to achieve the selective detection of a single molecule: The first is the spatial selectivity of optical microscopy, under the condition that the average separation of the investigated species in a diluted sample is above the resolution limit of the employed microscopy technique. The traditional far-field microscopy approaches are subject to the diffraction limit, which, roughly speaking, restricts the resolution to several hundreds of nanometers, while the experimentally more demanding near-field and other scanning-probe techniques can achieve higher resolution (tens of nanometers). Spatial selection by itself, however, cannot achieve molecular-scale (sub-nanometer) resolution. For this, a higher-order nonlinearity is needed, which can be seen as a correlation of several photons.[20] Such a correlation can be realized in a number of different ways. One of them is spectral selection, which is achieved by ensuring that only the targeted molecule interacts strongly with the excitation light. All the other molecules that will unavoidably be present in the optical detection volume—as part of the solvent or matrix that surrounds the species, as well as of the substrate that supports the sample—have to be “transparent” at the wavelength of the excitation light, that is, they have to have a very low efficiency for absorption or scattering. At low temperatures, spectral selection can be pushed so far that thousands of individual molecules of the same chemical species can be distinguished within the same diffraction-limited focal spot.

The most common practical implementation of these general principles is based on fluorescence microscopy: Fluorescent molecules or nanoparticles are excited by a laser with a suitable wavelength; a significant part of the emitted fluorescence occurs at longer wavelengths as a result of the Stokes shift and can therefore be separated from scattered excitation light by efficient spectral filters. Two important techniques are confocal microscopy and wide-field epifluorescence imaging. In confocal fluorescence microscopy, the excitation laser is focused to a (nearly) diffraction-limited volume and the light collected from the sample passes a spatial filter to reject background contributions arising from outside the excitation volume; larger areas of a sample have

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Ted Xia was born in Dalian, China, in 1977. He obtained his bachelor degree in medicine from Beijing College of Acupuncture and Orthopedics in 2000 and his master degree in biomolecular sciences from Utrecht University in the Netherlands in 2005. He has been a PhD student with Michel Orrit at the Leiden Institute of Physics since September 2005. His research interests include single-biomolecule dynamics at variable temperatures and heterogeneities in supercooled liquids.

to be imaged point-by-point by scanning the focus over the sample and recording the position-dependent fluorescence intensity. In wide-field imaging, on the other hand, an extended area is imaged onto a pixelated charge-coupled device (CCD) detector, which makes it possible to observe a number of individual chromophores simultaneously. The previously discussed limits for the fluorophore concentration relative to the optical resolution apply to both these approaches.

Many different types of molecules can be utilized as fluorophores in single-molecule experiments; usually chromophores are chosen with an extended π-electron system that absorbs in or close to the visible spectral range, for example xanthene or cyanine dyes. In addition to these organic fluorophores, semiconductor nanocrystals have become an attractive new class of fluorescent nano-objects.[21] The important photophysical properties for all single-molecule chromophores are 1) a short enough fluorescence lifetime to ensure that a sufficient photon emission rate can be achieved, 2) a low propensity to enter "dark" states (triplet states, radicals, charge-separated states), 3) little tendency to undergo irreversible photochemical modifications that destroy the fluorescent moiety.

Other methods besides fluorescence have been proposed for the optical study of individual nano-objects. They have not reached the ultimate goal of single-molecule detection yet, but they are very successful for larger nanoparticles whose optical response differs sufficiently from those of the surrounding medium, in particular metal nanoparticles. Several of these methods have been reviewed in Ref. [22], including various nonlinear signals, for example second-[23] and third-harmonic generation.[24] Two-photon excited photoluminescence[25] also has a nonlinear origin, but is more closely related to fluorescence. Detection methods based on linear optics mostly rest on scattering, and have been reviewed in Ref. [26]. Briefly, dark-field scattering[27,28] has the advantage that it is easy to measure and offers low background. The background, however, is never completely nil, and dark-field scattering does not work well for very small particles because the signal decreases as the square of the volume. For example, the scattering signal of small objects, such as 30 nm gold particles, cannot be distinguished against the residual roughness of an ordinary glass cover slip; the method therefore requires very smooth substrates.[28] For absorbing objects, this problem is solved by photothermal detection,[29–31] where the field scattered by the variable thermal lens around the absorbing object is detected against a dark background. Photothermal detection and tracking has been applied mainly to noble-metal nanoparticles,[30–32] but also to semiconductor nanocrystals[30] and to carbon nanotubes.[33] It can be applied to immobilized objects[29,30,34] or to freely diffusing ones,[35–37] and offers new possibilities to probe complex matter in a complementary way to fluorescence, free from blinking and bleaching.

Michel Orrit was born in Toulouse, France, in 1956. He studied physics at Ecole Normale Supérieure in Paris, joined C.N.R.S in Bordeaux, France, in 1979, and presented his Thèse d'État in 1984 on excitons in molecular crystals. In 1985–1986 he worked with H. Kuhn and D. Möbius at the MPI for biophysical Chemistry in Göttingen, Germany. Back in Bordeaux, in 1990, he began single-molecule fluorescence experiments. Since 2001, he is Professor at Leiden University, the Netherlands. His current research interests concern the optical spectroscopy and microscopy of single molecules and semiconductors or metal nanoparticles, and their application to probing soft and biological matter.

In this Review, we discuss the potential of single fluorophores and chromophores as nanoprobes of the structure and dynamics of disordered environments. Every material of sufficient optical quality can be investigated in principle, as long as chemically compatible fluorescent probes can be found that can be excited in a spectral range where the investigated material itself is transparent. The chromophore serves as a nanoprobe if the properties of the photons it emits can be traced back to the structure of its environment: Determining the position of a single fluorophore, for example, which can be achieved with an accuracy that is higher than the diffraction-limited resolution, makes it possible to follow molecular motion over time and thus, for example, measure local viscosities. Similar information can be obtained from the rotational diffusion of a probe molecule, which is accessible through analysis of the fluctuations in the polarization of the emitted fluorescence. Other phenomena whose effects can be manifest in the spectral properties of single-molecule fluorescence and/or its temporal emission characteristics, include local electric and magnetic fields, strain, effective refractive index, energy transfer, and variations in the local density of states. We will discuss illustrative examples for some of these effects in Section 3.

3. Examples of Single-Molecule Approaches

In the past ten years, hundreds of publications have reported the application of single-molecule methods in investigations of complex and soft matter. Our aim herein is not to review this body of literature exhaustively, but rather to discuss a few important examples in some detail, referring to more complete Reviews of the different domains where possible. We hope the following examples illustrate the main concepts and prepare the reader for future applications of single-molecule methods in original situations.

3.1. Static Disorder in Crystals and in Shpol'skii Matrices

An organic guest chromophore can substitute one or more host molecules in a molecular crystal, provided that a structurally compatible host/guest combination can be found. If such samples are investigated at low temperatures, generally a small number of insertion geometries are encountered, leading to spectrally distinct insertion sites. Each of those insertions presents an absorption band centered at an average frequency and broadened by defects. The spread of absorption frequencies for the same chemical species in what might naively be assumed to be an identical, well-defined environment is called inhomogeneous broadening. This phenomenon is present even in the most carefully prepared samples and, at low-enough concentrations, it can be used to spectrally distinguish individual fluorophores, even when they cannot be spatially resolved in the microscope focal volume. However, the origin and nature of inhomogeneous broadening is an old and difficult question in the optical spectroscopy of molecular crystals. Crystal defects and stress fields induced by other impurities will clearly perturb intermolecular distances slightly, and thereby shift in a complicated way the spectral position of the electronic transition of an absorbing impurity. Whereas it is easy to measure the spectral positions of single molecules in mixed molecular crystals at low temperatures, it would be interesting to correlate these spectral shifts with other molecular parameters, such as the orientation of the transition dipole moments of individual impurities, to get new insight into the origin and extent of spectral inhomogeneity and into the nature of microscopic disorder in a mixed crystal.

The orientation distribution of single impurity molecules by itself contains valuable information. Such distributions have been measured in a broad variety of crystalline hosts: aromatic mixed crystals,[38,39] Shpol'skii matrices (shock-frozen solutions of organic fluorophores in *n*-alkanes of suitable length, which are thought to exhibit microscopic crystalline domains),[40,41] and potassium hydrogen phthalate.[42] An example of such a measurement in a Shpol'skii system can be seen in Figure 1.

The angular distributions are generally found to be significantly broader (10–30°) than might have been expected from the quality of the crystals. Although this observation is not completely understood yet, we propose that the fluctuations of the transition dipole vectors are actually much larger than those of the molecular axes. Small fluctuations in the relative positions of the molecules can lead to large variations of van der Waals interactions between host and guest, which are mixing guest electronic levels and the associated transition dipole moments. Similar small transient variations of intermolecular distances were estimated to induce fluctuations in the magnitude of the transition dipole moment of single fluorophores in polymers hosts close to the glass transition, leading to fluorescence lifetime fluctuations.[43,44] Such fluctuations of the magnitude of the dipole should clearly be accompanied by fluctuations of its direction. The correlation between the inhomogeneous width and the static dipoles responsible for the Stark effect of single molecules was discussed in Refs. [39,45]. Although both the positional order and the orientational order can be very high in the host single crystal, small deviations in intermolecular distances can be amplified by the sensitivity of the electronic wavefunctions, and therefore of the spectroscopic observables, to the molecular positions. This situation confirms optical spectroscopy as a sensitive but indirect indicator of the structure of the insertion site on the molecular scale, a precise description of which is still elusive.

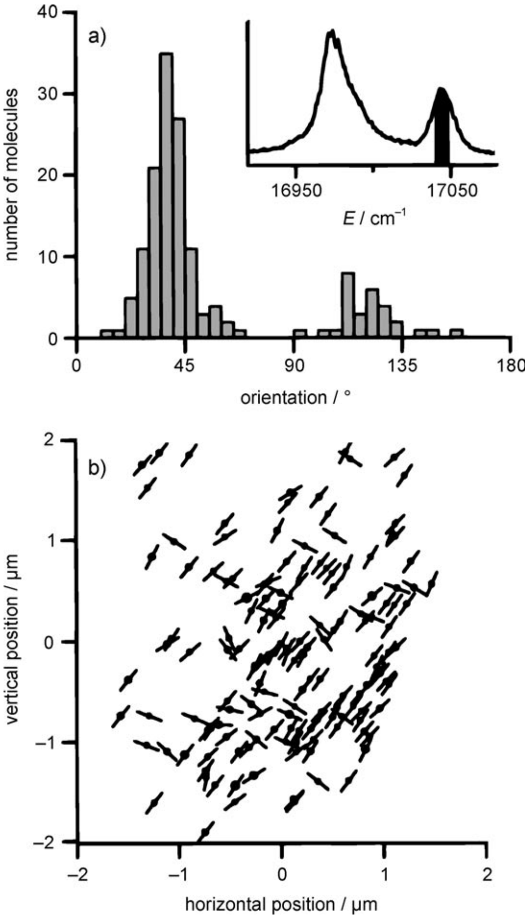

Figure 1. Orientation and lateral position (determined with an accuracy of 23 nm) of 2.3,8.9-dibenzanthanthrene (DBATT) molecules in *n*-tetradecane. The molecules were frequency-selected in a confocal volume of 10 µm^3. a) Histogram of the orientation of 151 DBATT molecules that were chosen from the high-energy band as indicated in the ensemble spectrum in the inset. b) Relative lateral positions (dots) together with the orientations (bars) of the same 151 DBATT molecules. The radii of the dots correspond to the accuracy of each position. Reprinted with permission from Ref. [41]. Copyright 2002, American Institute of Physics.

3.2. Orientational Dynamics and Local Mobility in Supercooled Molecular Liquids

The mobility of embedded chromophores can be used to probe the local viscosity of the host material, unveiling the degree of structural inhomogeneity and the presence of memory and aging effects. By following the orientations of individual probe molecules as functions of time, a rotational correlation time (τ_R) can be determined, which is related to the hydrodynamic volume of the probe (V_H) approximated as a sphere) and to the local viscosity η. By application of the Stokes–Einstein–Debye relation [Eq. (1), τ_R = rotation time] the local viscosity of the host is obtained.

$$\tau_R = \frac{\eta V_H}{k_B T} \qquad (1)$$

This local probing is particularly interesting when the medium is neither solid nor liquid, or when it is close to, but above, the glass transition. Below the viscosimetric glass point, the solution is usually completely frozen and most probes are immobile. In other words, the large-amplitude motions necessary to reorient a sizeable probe molecule become improbable. A number of glass-forming systems have been studied to date, ranging from supposedly simple supercooled molecular liquids, such as *o*-terphenyl and glycerol, to polymers, which we discuss separately in Section 3.3.

In the glass-forming systems investigated to date, all the single-molecule experiments agree and point to the existence of a large degree of dynamic heterogeneity at the molecular length scale. In a typical single-molecule measurement, individual probe molecules are separated by a distance of several microns, and their orientation is monitored by recording the time-varying linear dichroism of the emitted fluorescence, or sometimes by 3D-polarization tracking. Although each individual molecule displays a well-defined tumbling rate, at least over periods corresponding to several full reorientations, the tumbling rates are found to differ markedly from molecule to molecule, that is, at different locations in the sample. This observation contradicts the idea that a supercooled liquid loses memory of local fluctuations on the rotational timescale of a liquid molecule, which is usually significantly shorter than that of the probe. The various tumbling rates therefore directly demonstrate the dynamic heterogeneity of the sample, with orientational diffusion rates of the probes varying from point to point in the sample. There also appears to be a general agreement on the extent of the heterogeneity, and its increase in relative magnitude when the temperature approaches that of the glass transition. Figure 2 illustrates this effect on the rotational correlation times of perylenediimide (PDI) molecules in supercooled glycerol near its glass transition temperature.

On a longer timescale, each probe molecule is expected to sample different local viscosities, either because the probe diffuses to a different spot, or because the local conditions change upon fluctuations of the sample around its equilibrium state, or because of aging. The corresponding events are currently called environmental exchanges. There is a stark disagreement between different experiments about the timescale of environmental exchanges, which appears to depend strongly on the sample and on its thermal history. Vanden Bout and Deschenes found exchange times of the order of some tens of tumbling times in *o*-terphenyl,[48] but in their study of aged glycerol, Zondervan et al.[47,49] hardly found any exchanges, which led them to estimate the exchange times to at least a million times longer than the tumbling time of the glycerol molecule (alpha relaxation time). Single molecules thus point to an intrinsically heterogeneous picture of a glass-forming supercooled liquid, with a coexistence of solid-like and liquid-like fractions. These can be pictured as "curds and whey" or perhaps "curd and wheys", depending on which phase first percolates and how it coarsens with time. Clearly, rearrangements of solid-like and liquid-like parts could be kinetically or geometrically frustrated, making local aging very dependent on the local structure and conditions.

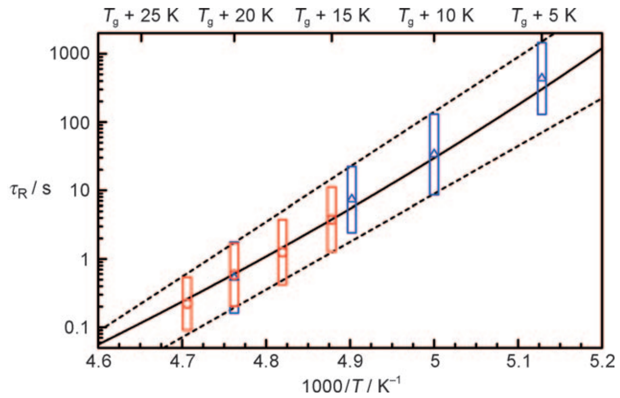

Figure 2. Arrhenius-like plot of the rotational correlation times τ_R of perylenediimide (PDI) molecules in supercooled glycerol. The heterogeneity of the supercooled liquid is reflected in broad distributions of tumbling times, because the individual PDI molecules experience different local viscosities. The triangles and circles mark the center values of log-normal distributions fitted to the rotation times observed in two measurement runs; the bars around the center values indicate the full-width at half maximum (FWHM) of the respective distribution. The solid curve shows that the center values are consistent with the known temperature dependence of the viscosity of glycerol[46] and a molecular hydrodynamic volume of 0.45 nm^3 used in Equation (1). The two dashed lines represent fits of Arrhenius behavior ($\log(\tau_R) \propto 1/T$) to the upper and lower bounds of the FWHM ranges and are meant to serve as guides to the eye to illustrate the relative broadening of the distributions as one approaches the glass transition temperature ($T_g = 190$ K). The results at 195 K are skewed by the effect of photobleaching because of the long observation time required and have therefore been disregarded in the fitting procedures. This Figure is based on data that was published in Ref. [47].

Besides revealing heterogeneity close to the glass transition, single-molecule techniques could help clarify or answer many of the open questions left about the glass transition itself. What are the correlation times and lengths of the dynamic inhomogeneities? Single-molecule tracking with sub-wavelength resolution[50] would help map out the regions explored by diffusing single molecules in different parts of the sample. What are the structures of the solid-like and liquid-like regions? For simple liquids, such as glycerol or *o*-terphenyl, it could be expected that the solid-like fraction is composed of micro- or nanocrystals, with many quenched defects and boundaries which could not be annealed on experimentally accessible timescales. More complex systems, such as polymers, cannot form crystals, but the coexistence of denser and more fluid regions could lead to strongly heterogeneous dynamics of the same kind. How would such a composite microstructure affect large-scale properties of the supercooled liquid, in particular its rheology? Would it be possible to monitor the aging of the material and its possible fragmentation into liquid-like and solid-like regions, for example by using adapted probes that are designed to segregate into one or the other fraction? For all these questions, a single-molecule approach may provide first-hand data, or even lead to complete reformulation of the problem.

3.3. Single-Molecule Studies of Polymers

The structure and dynamics of polymers present a number of challenging questions, which are not only important for the many applications of polymers as materials, but also because of their fundamental role in physical chemistry and in molecular biology. Single-molecule methods provide unique opportunities to probe polymer physics at nanometer scales. Wöll et al. have recently published a Review of single-molecule measurements in these materials,[51] which we recommend to readers whose interest goes beyond the overview that we give herein.

One of the earliest uses of polymers in single-molecule studies was as matrices to immobilize individual dye emitters, at low temperature[52] as well as at ambient temperature.[7,9,53] Most polymers are amorphous materials presenting all the structural and dynamic features of glasses, from the absence of long-range order to the exponential slow-down of relaxation on approaching the glass transition.[54] In cryogenic experiments, the disordered structure of a polymer gives rise to a small number of randomly distributed regions in which iso-energetic rearrangements may take place. Thermally induced jumps between different conformations mainly occurs by tunneling and can be modeled as jumps of two-level systems.[55] These low-energy excitations lead to spectral jumps of single-molecule optical lines,[52,56,57] a process known as spectral diffusion. At higher temperatures, but still below the glass transition, spectral diffusion affects the fluorescence and absorption spectra of molecules[14,58] and their photophysics (see Section 3.5). The experience gained from molecular crystals suggests that the angular distribution of single-molecule absorption axes is not a faithful image of the molecular orientation distribution, particularly in such disordered systems as polymers.[59–63]

The local dynamics of polymers has also been studied with single-molecule fluorescent probes. Observed fluctuations of the fluorescence lifetimes of the probes have been attributed to local rearrangements of free volume in the polymer in the vicinity of the probe,[44,64] which lead to changes of the transition dipole moment.[43] Local rearrangements can arise from the two types of relaxations in polymers, the α-relaxation which requires correlated motions of a large number of molecules, and gives rise to the divergence of the macroscopic viscosity at the glass transition, and the β-relaxation, more characteristic of the segmental motions of the polymers and of vibrations of the chains in their cages.

Rotational diffusion of dye molecules was studied in polymers[65,66] by fluorescence polarization. Schob et al.[66] investigated translational diffusion, too, and found that the heterogeneity seen close to the glass transition (at temperature T_g) did not persist at temperatures higher than 1.2 T_g. A full 3D orientational tracking method by defocused imaging was applied by Uji-i et al.[67] to perylenediimide (PDI) dye molecules in a thin film of poly(methyl acrylate), as is illustrated in Figure 3.

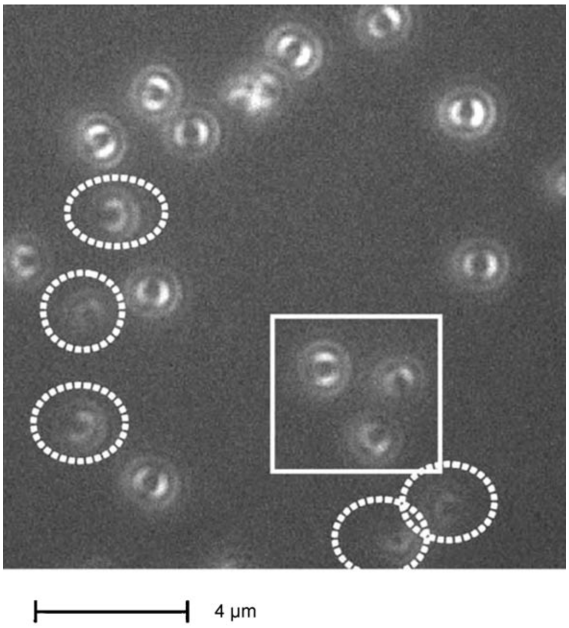

Figure 3. A defocused image of perylenediimide (PDI) molecules embedded in a 50 nm film of poly(methyl acrylate), recorded with 1 μm defocusing toward the sample. The analysis of each pattern yields the 3D orientation of the transition dipole moment of the respective PDI chromophore. The white, dashed circles indicate molecules that are substantially oriented out-of-plane. In the original work, the highlighted molecules were further analyzed as examples. Reprinted with permission from Ref. [67], copyright 2006 Elsevier Ltd.

These authors found a broad range of dynamic behaviors, ranging from isotropic diffusion to molecules orientationally trapped, alternations of different regimes and environmental exchanges between slow and fast diffusion. All of this demonstrates a complex heterogeneity in space and time, which gives rise to the stretched-exponential angular correlation functions found in ensemble experiments on other glass formers. It would be interesting in future studies to combine rotational diffusion with translational diffusion studied by superresolution techniques, in particular in view of clarifying the translational–rotational paradox.[68a] The translation–rotational paradox is thought to arise close to the glass transition. Some measurements indicate a faster translational diffusion of probes in a glass-forming material than would be deduced from their rotational diffusion.[68b]

Besides probing polymers with small dye molecules, it is particularly interesting to monitor the motion of a single fluorescent polymer chain in a concentrated solution or in a melt. Studies of a single fluorescent polymer chain were first demonstrated with labeled DNA,[69] which remains a benchmark object,[70] and with conjugated polymers (see Ref. [71] for a Review), the emitting polymer being included in a matrix of a saturated polymer. More recent studies have focused on labeled polymers dispersed in a matrix of unlabeled polymer chains, by fluorescence correlation spectroscopy (FCS)[72] or by the imaging of very long chains.[73] The dynamics of the labeled chain can then be compared to the theoretical models of Zimm, Rouse, and the reptation model.[74]

The case of the dynamic of proteins must be considered separately, as these polymers are neither random nor homogeneous. Single-molecule measurements of various proteins[14,75,76] have provided overwhelming evidence of the strong time-heterogeneity of protein dynamics.[77,78] The many conformations of a protein are arranged in a complex potential-energy landscape,[79] where minima are separated by activation barriers with widely different heights. Because of the exponential dependence of the hopping rate with barrier height, the distribution of protein relaxation times is broad on a logarithmic scale, leading to stretched kinetics which result in long relaxation tails in ensemble experiments.[80]

3.4. Translational and Rotational Diffusions in Heterogeneous Media

Liquids close to interfaces or strongly confined in small solid pores may display complex dynamics, which can be explored on nanometer scales with single molecules. Von Borczyskowski, et al. have studied single molecules in thin films of silicone oil that wet a solid surface.[81–83] They found evidence for layering, for an extended precursor film, and for differential diffusion of the probe in the layer (or interlayer space) with slower jumps from layer to layer. These investigations were more recently extended by Cichos and Schob to mechanical measurements with an apparatus for determining surface force.[84]

Single molecules are particularly well suited to study inhomogeneous materials, for example the various silica-based materials obtained from sol–gel processes. A large number of papers, including single-molecule studies, have been devoted to this important field, and have been excellently reviewed by Ye et al.[85] The first studies of molecular mobility found it to depend on the type of silica, on the presence of organic groups, of residual surfactant molecules, as well as on water content. Different subpopulations with different diffusion coefficients can be observed, sometimes with a clear distinction between diffusing and trapped molecules. Dead ends in channels or corrals in two or three dimensions can lead to anomalous diffusion (subdiffusion). Particularly detailed studies of the motion of single probe molecules in thin films of mesoporous and nanoporous silica-based materials were conducted by Bräuchle's group;[86–92] examples of single-molecule diffusion tracks in these materials can be seen in Figure 4. Diffusion paths could be correlated with electron-microscope images, confirming the one-dimensional diffusion of probes in nanochannels, and their two-dimensional diffusion at the surface of the film.

Another interesting way of probing the environment is to use the spectral sensitivity of certain dyes. Nile Red, for example, is very sensitive to polarity and provides information about local static and dynamic polarity fluctuations through the shifts and breadths of its absorption and emission spectra.[93] Similar studies have concentrated on the fluctuations of acidity in the matrix by means of single molecules as indicators of local pH value.[94] The studies often show wide distributions of the local pKa values depending on the local environment. The main conclusion of these studies is that they underscore the large extent of heterogeneity in these materials, which becomes immediately apparent at the scale of single molecules.

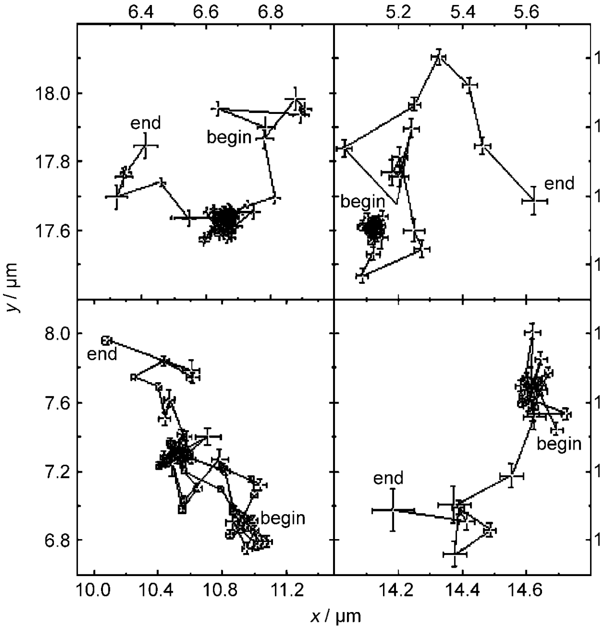

Figure 4. Four representative tracks of streptocyanine molecules diffusing in a nanoporous silica gel which has an average pore size of 3 nm. The tracks show alternating periods of free diffusion and trapping (dense clouds of data points), each lasting for several seconds. Reprinted with permission from Ref. [86], copyright 2004 American Chemical Society.

3.5. Photophysics and Blinking

Excited molecules may undergo many photochemical and photophysical processes, ranging from regular photoreactions, such as additions and photooxidations, to photoinduced charge transfer involving less extensive molecular rearrangements. Intramolecular processes such as isomerization (and even intersystem crossing) often depend to a large extent on the molecular environment, in particular on its polarity and its rigidity. Therefore, distinguishing between intrinsic (proper to the chromophore) from extrinsic (proper to the environment) processes, as proposed in Ref. [58], will in general be difficult. The class of reactions involving electron transfer from or to the environment is important for the durability of fluorescent labels.[95,96] Their study may lead to better dyes with improved resistance to photobleaching, which currently limits the uses of fluorophores for confocal and superresolution imaging.[50]

The latter class of reversible photoreactions or photorearrangements is the origin of blinking, i.e., processes in which the fluorescence intensity of a single emitter switches back and forth between high and low (often completely zero) levels. These transitions limit the brightness of ensembles of fluorophores, and produces characteristic on- and off-periods in the intensity traces of individual emitters of many different kinds. One obvious source of blinking, recognized in the first single-molecule experiments,[97] are the intersystem crossing transitions to and from the triplet manifold of an aromatic molecule. This mechanism, however, leads to single-exponential distributions of on- and off-times. (The off-times can follow a bi-exponential distribution at temperatures that are low enough to suppress thermal mixing of the triplet sublevels.) The observations of extremely long off-times in the case of molecules,[98,99] and of an enormous spread of off- and on-times in the case of semiconductor nanocrystals,[100–102] which is illustrated in Figure 5, therefore came as a big surprise.

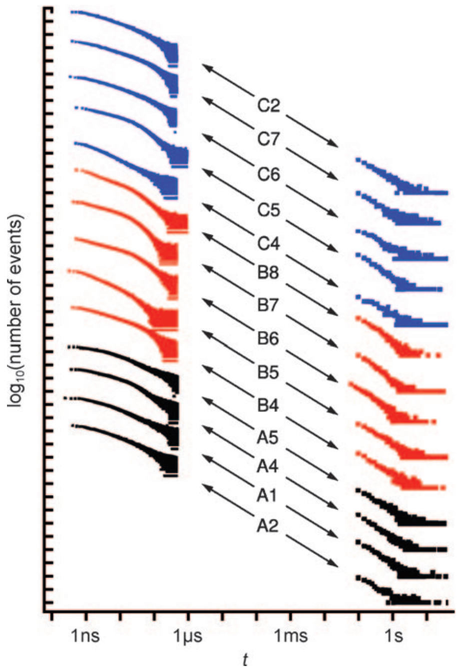

Figure 5. Time-resolved luminescence (nanosecond to microsecond range) and blinking data histograms (milliseconds to minutes) on common logarithmic axes for 14 different single nanocrystals (type A: bare CdSe nanocrystals; type B: CdSe/ZnS core–shell structures; type C: $Zn_{0.5}Cd_{0.5}S$/CdSe/ZnS triple-layer particles). The increments on both axes correspond to factors of 10. Reprinted with permission from Ref. [102]. Copyright 2008, American Institute of Physics.

In both cases,[103,104] the weak temperature dependence of the blinking suggested that charge separation and transfer was the central mechanism in blinking, rather than regular photochemistry. For molecules, charge separation was demonstrated by ensemble magnetic resonance experiments.[104] Moreover, experiments on nanocrystals,[100,101] and later on molecules[105–108] indicated that on- and off-time distributions often follow power laws from microseconds to minutes. The mechanism behind this striking and nearly universal behavior is still hotly debated.[109–111] Herein, we will concentrate on a peculiar aspect of power-law blinking: It is observed only in materials presenting at least some degree of disorder: surfaces,[107] glasses and polymers,[105,106,108] disordered crystals.[42,112,113] It is not observed in ordered molecular crystals,[114] nor in diamond,[115] nor for the self-assembled dots of heteroepitaxial structures,[116,117] nor in nanocrystals when their shell is thick enough.[118,119] When the confinement potential is soft, Auger processes are reduced and the charged crystal still fluoresces.[120] Blinking therefore appears to be intimately connected to the structure of the environment, and therefore could be exploited to probe it. If, as we believe, blinking in rigid environments is caused by charge transfer followed by self-trapping[*] in the close environment of the emitter,[121,122] and the eventual back-transfer restores luminescence, then blinking statistics may give insight into the potential-energy landscape for an ejected electron in this neighborhood. This information is very difficult to access by ensemble methods, because of the strong heterogeneity of disordered local environments.

As we have seen, blinking of a single nanocrystal or molecule in a disordered solid is a complex phenomenon. At room temperature, in presence of a liquid phase where many reactive species can diffuse, the situation is further confused by a plethora of possible redox and other secondary reactions. For example, blinking of quantum dots at an interface between a solid and a solution is clearly influenced by changes of composition or of the redox potential of the solution,[122–125] or by the presence of another dye in a bichromophoric construct. The bichromophoric systems exhibit a switching behavior which can be driven by light.[126] This switching reaction is already exploited for superresolution imaging.[127,128] Charge transfer may occur over longer ranges than usual, either because the large excitation intensities required for fluorescence excitation lead to highly excited ionizing states, or because charges are shuttled by redox active species, such as oxygen.[104,129] Blinking provides information about the dynamics of the matrix, as was found for rhodamine 6G in glycerol.[130] Charge recombination may be assisted by the activated molecular motion above the glass-transition temperature, or by tunneling at still lower temperatures. A particularly important subject is the interfacial charge transfer between an excited molecule and a semiconductor, in a film or nanoparticle. These are the transfer processes at work in certain solar cells. Single-molecule studies of the transfer and recombination rates[131–133] demonstrate the broad distribution of these rates, leading to power-law kinetics and distributions. Such studies will lead to a better understanding and control of the photochemical processes active in solar cells.

3.6. Surface-Enhanced Raman Scattering (SERS)

The ideal tool for an optical analysis of soft and complex matter at nanometer scales would be a chemically sensitive technique such as Raman scattering, confined to very small volumes. Surface-enhanced Raman scattering (SERS) is therefore very appealing. SERS exploits an increase of the Raman scattering cross section of certain molecules deposited on a metal surface (often silver), particularly a rough or patterned surface, or aggregates of colloidal metal particles.[134,135] This enhancement can reach more than ten orders of magnitude, making even minute amounts of the compound detectable in a Raman spectrometer. More than ten years

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[*] Self-trapping of a charge (for example, an electron) in an otherwise homogeneous material (for example, a crystal) arises from a local deformation of the lattice under the electron's field. The ensuing trapping potential localizes the electron, somewhat as a heavy ball sinks when dropped onto a flat rubber membrane.

ago, microscopic experiments demonstrated that the sensitivity of SERS can reach the single-molecule limit.[136,137] The main observations were the sudden spectral jumps of Raman line frequencies or intensities,[136,138] and the large population of short-lived vibrational levels, compatible with a small number of heavily excited emitters.[137]

There are several possible sources for the large enhancement values.[139,140] First, SERS appears to require an electronic interaction between molecule and metal. This partial electronic hybridization leads to a so-called chemical enhancement, which may be further reinforced by resonance conditions. Additional pre-resonance between excitation laser and molecule may additionally boost the Raman signal. Most of the effect, however, appears to be due to the concentration of the optical electric fields by metal structures, possibly helped by plasmon resonances. The latter effect, known as the electromagnetic enhancement, can be calculated from finite-element simulations of Maxwell's equations. The results, insightfully reviewed in Ref. [140], show how exceedingly sensitive the enhancement can be to the exact position of a molecule with respect to tips or gaps, and to the exact structure of the metallic system, as is illustrated in Figure 6.

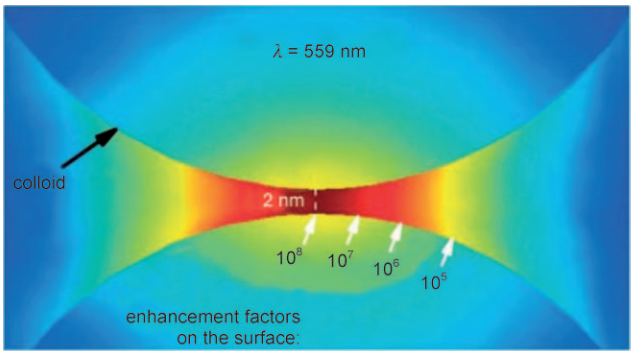

Figure 6. Distribution of the electromagnetic enhancement factor F in a 2 nm gap between two gold colloids with radii of 30 nm. The enhancement factor was calculated in the electrostatic approximation with finite-element modeling for polarization along the vertical axis of the dimer. The calculation was performed for $\lambda = 559$ nm, where F exhibits its maximum value of about 10^8 at the surface of one of the colloids. Several values of F along the surface at different distances from the vertical axis are shown; they can vary substantially over length scales comparable to a characteristic molecular size. Reprinted with permission from Ref. [140], copyright 2008 Royal Society of Chemistry.

A shift of only a few nanometers can lead to variations of the electromagnetic enhancement by orders of magnitude. This sensitivity is clearly compounded by the molecular-scale details of the chemical enhancement. The resulting extreme dispersion of signal strengths makes a quantitative exploitation of SERS signals challenging, because the single-molecule quantification is hidden by logarithmic distributions. Thus in addition to the dwell-time distributions in blinking, we find another example of an exceedingly broad distribution of a single-molecule property, for which averaging loses its familiar physical meaning: One or a few isolated spots may completely dominate, even in an ensemble average.

In view of the difficulty of a quantitative assignment of single-molecule SERS signals, we may wonder how far SERS can bring us toward a chemically sensitive analysis of matter at nanometer scales? It is clear that the electronic interactions and the short distances required by SERS can bring about dramatic molecular distortions, and, unavoidably, denaturation in the case of proteins. The dream of a universal single-molecule Raman spectroscopy therefore remains an open challenge.

4. Outlook and Conclusion

As we have shown, the optical detection and study of single molecules and single nanoparticles now finds many applications in the exploration of complex matter, not only intricately organized biological matter, but also the disordered and changing structures found in soft matter or in elaborate materials. As a rule, the molecular-scale, average-free picture conveyed by such studies is surprisingly heterogeneous and complex, both in space and time. The concept of an "average molecule" (or pore, environment, value, reaction rate, etc.), is far from being a reliable guide, and should often make room for more sophisticated descriptions.[141] This new insight may be crucial in many areas of physical chemistry and materials science, for example in the following cases.

The contact area between two solids is a complex space,[142] which determines such important properties as adhesion and friction. Nominally flat surfaces often touch on a few points only, with accordingly strong stress and strain, distributed in a highly heterogeneous fashion. Whereas atomic-force microscopy yields the parameters of molecular contact in a small area, there is urgent need for complementary investigations of the distribution of these contact points and of the local deformations in a real, extended contact between two solid bodies. This information could be accessed from single molecules dispersed on the surfaces, whose reorientations or spectral shifts would probe local stress and strain. Similarly, in this way, the interaction of a solid surface with a liquid upon wetting and drying, or the influence of mechanical action could be explored, extending the first pioneering studies discussed above. The applications of such studies could be numerous in fields such as the plastic deformation of solids and polymers, the dynamics of fractures in solids or composite materials, the contacts between grains in concentrated suspensions, pastes, and slurries.

Besides the glass transition discussed above, first-order phase transitions often lead to strong heterogeneity in the spatial distribution of the phases. For example, domain walls appear upon cooling a *p*-terphenyl crystal beneath the temperature of the ferro-elastic transition. Their distribution and dynamics can be studied at low temperature by fine spectroscopy of the zero-phonon lines of single probe molecules.[143,144] Several other studies of molecular crystals have appeared recently, for example, of the incommensurate phase of biphenyl,[145,146] or of the disorder and molecular reorientations in dimethylnaphthtalene in a broad temperature range.[147] For a Review of the recent advances in the physics and chemistry of solids at low temperatures, including the dynamic aspects of crystals and more disordered systems, we refer to a book chapter in press.[148]

For all these problems, it will be necessary to find relevant and convenient optical observables, which will report on the local conditions. Almost all characteristics of a fluorescence signal (polarization, spectrum, lifetime, position, etc.) can be exploited for this purpose.

In conclusion, single molecules provide a detailed picture of condensed matter, often revealing surprisingly large inhomogeneity and deviations from the average. A recurring theme in this Review has been the broad distribution of certain molecular parameters, for example, reaction rates which give rise to stretched protein dynamics and power-laws in blinking or interfacial charge transfer. The logarithmic distribution of field enhancements in SERS is another striking illustration of this rule. Far from generally resulting from an average over many molecules with largely different properties, these broad distributions often already appear at the single-molecule level, through conformational and/or dynamic averaging. In this sense, stretched kinetics and broad distributions[149] appear to lie at the very core of the chemical physics of soft matter.

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