Probing spatial heterogeneity in supercooled glycerol and temporal heterogeneity with single-molecule FRET in polyprolines

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1 Introduction

The work presented in this thesis contains two lines of research. On the one hand, we investigate heterogeneity in supercooled glycerol by means of rheometry, fluorescence imaging, and small-angle neutron scattering. This study was triggered by earlier single-molecule work on supercooled glycerol by Zondervan et al. [1]. On the other hand, we study the conformational dynamics of polyprolines by single-molecule FRET (Förster resonance energy transfer) combined with temperature-cycle microscopy, a novel technique developed in our group, and demonstrate the potential of this new method to address complex molecular dynamics, for example the dynamics of protein-folding, at the single-molecule level.

1.1 Glass transition and heterogeneity

A liquid below its standard freezing point will generally crystallize in the presence of a seed crystal or nucleus around which a crystal structure can form. However, lacking any such nucleus, the liquid phase can be maintained all the way down to the temperature at which the system becomes so viscous that it freezes on the accessible experimental time scales, forming an amorphous (non-crystalline) solid, a continuous transition known as the glass transition. Such a liquid-glass transition is not a transition between states at thermodynamic equilibrium. The true equilibrium state is widely believed to be always crystalline. Therefore, the glass transition is considered as a dynamic phenomenon only.

Although glass has been produced for about 3500 years, the full structural description of how a liquid cools down to a glass is still missing. Early models assume the existence of cooperatively rearranging regions (CRR) in glass-forming systems below their critical temperature [2], a temperature at which "normal" and "complex" liquid behaviors are distinctly separated. A "normal" liquid behavior means that the time dependence of the relaxation dynamics can be described by an exponential function, whereas for a "complex" liquid behavior a stretched exponential function is typically employed. Within the CRRs the molecules do not relax independently of one another. Due to a small free volume at low temperatures, close to T_g, the motion of a particular

molecule depends to a large degree on that of its neighbors [3]. The length scale of CRRs is generally estimated to be a few nanometers [4] and is assumed to increase for decreasing temperatures. Such CRRs are thought to be the origin of the observed dynamic heterogeneities in glass-forming systems [4, 5]. The system is dynamically heterogeneous if it is possible to select a dynamically distinguishable subensemble by experiments or computer simulation [6]. Although such heterogeneities have been reported both experimentally and numerically, the associated length and time scales are still debated. Indirect experiments, mainly from NMR [7, 8] and dielectric relaxation [9, 10], suggest that the length scale of dynamical heterogeneity is on the order of a few nanometers and the time scale comparable to molecular reorientation times (α-relaxation). However, optical experiments, notably light and X-ray scattering [11, 12], polarized hole-burning [13, 14] and single-molecule spectroscopy [15, 16], reveal much slower relaxations than α-relaxation and longer length scales, typically 100 nm.
Our group recently investigated the local relaxation of supercooled glycerol at temperatures up to 30 K above its glass transition (190 K) by following the rotational diffusion of individual (fluorescent) probe molecules embedded in the host matrix [1]. These experiments showed that each individual probe molecule rotates at different rates in the supercooled glycerol and that the breadth of rotation times spans nearly one decade, indicating a spatially dynamic heterogeneity. Most strikingly, upon following the rotation of the same molecules as a function of temperature, a long-term memory effect was revealed, i.e., the molecules that rotate faster than the average remain faster and the molecules that rotate slower remain slower than the average, irrespective of the changes of temperature. The associated lifetime is on the order of days, about a million times longer than the α-relaxation time of the host molecule. Such long-lived heterogeneity strongly suggests that some nearly static structures already develop at temperatures well above the glass transition. Motivated by this surprising finding, we further explored the heterogeneity in supercooled glycerol by different techniques, such as rheometry, fluorescence imaging, and small-angle neutron scattering. This study constitutes the first line of research in this thesis.

1.2 Single-molecule fluorescence and temperature-cycle microscopy

Pioneered by Moerner and Orrit [17–19], single-molecule spectroscopy has evolved from a specialized optical spectroscopy at low temperatures in the

early nineties of last century into a versatile and powerful tool to address problems in physics, chemistry, biology, and materials science at present. Because they are inherently free from ensemble averaging, single-molecule approaches can reveal a full variety of nanoscale environments in the system of interest, together with the full extent of the distributions of molecular or local parameters. This makes the techniques highly suitable to study systems in which inhomogeneity is intrinsic to the structure or dynamics of the materials, for example glassy materials and polymers. Examples of such applications in soft and complex matter can be found in a recent review article [20]. Another advantage of single-molecule studies is that since individual molecules can be addressed independently, a synchronization step is no longer required, which makes the design of the single-molecule measurements less troublesome than conventional ensemble experiments.

The application of single-molecule spectroscopy to biological problems is currently expanding rapidly. Hereafter, the discussion will mainly focus on single-molecule fluorescence spectroscopy. Other optical approaches not based on fluorescence such as the detection of photothermal [21–24] or interferometric scattering [25–27] signals from nano-objects (nanogold particles, quantum dots, or even molecules), which play an equally important role in biological applications, are outside the scope of this thesis. Fluorescence has been used for more than one century in many disciplines, ranging from analytical chemistry, biochemistry to medicine. The nearly non-invasive nature of fluorescence is particularly appreciated by cell biologists, who use fluorescence to image labeled biomolecules in a living cell in their daily work. Thanks to the green fluorescent protein and its variants [28, 29] and to advances in genetic engineering, it is now virtually possible to fluorescently label any protein in any organism, which further advances the understanding of some important biological processes such as cell division, gene expression and regulation, and cell signaling at the molecular level. Protein molecules are involved in virtually every biological process. They typically engage in complex and dynamic interactions with other proteins or even other species like DNA/RNA molecules and lipids to fulfill their function. Protein conformations are highly dynamic rather than static, and subtle conformational changes play a crucial role in protein function. Therefore understanding their dynamic behaviors is important for the full description of their functions. However, it is extremely difficult to characterize such heterogeneous dynamics in an ensemble-average experiment, especially when the proteins are involved in multiple-step, multiple-conformation complex chemical interactions and transformations, such as enzymatic reactions, protein-protein interactions and ion-channel membrane pro-

tein processes. Alternatively, single-molecule fluorescence spectroscopy is a powerful approach to probing and analyzing protein conformational dynamics in real time [30–32]. In the design of such experiments, a single dye molecule can be attached to the host biomolecule. By following the temporal fluctuation of the fluorescence intensity, together with the polarization response of the fluorophore, one can obtain the information about the motion and activity of the individual host biomolecule. Probably, the most general approach is to use two fluorophores instead of one, in the form of Förster resonance energy transfer (FRET). The FRET method has proven to be a powerful spectroscopic technique for measuring distances in the range $10 - 75$Å [33, 34]. Since the first demonstration of single-molecule FRET by Ha et al. in 1996 [35], there have been many experiments designed for biological applications [36–44]. In the process of FRET, excitation energy of the donor is transferred to the acceptor via dipole-dipole interaction. The efficiency of energy transfer, E, is given by

$$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6} \quad ,$$

where R is the distance between the donor and acceptor. The distance at which 50 % of the energy is transferred, R_0, is a function of the properties of the dyes. The distance $\mathrm{R_0}$ contains a contribution from the relative orientation between the two dyes, called κ^2, which may vary from 0 to 4. If the dipole moments of donor and acceptor are free to sample all the possible orientations on a time scale much faster than their radiative lifetimes, a geometric averaging of all the angles will give rise to the value of 2/3 for κ^2. The FRET efficiency, E, determined through the fluorescence intensities from both the donor and acceptor or the fluorescence lifetime of the donor in the presence and absence of the acceptor, can then be directly related to the inter-dye distance, R. However, the host molecule may interact with the attached fluorophores, thus restricting their motion. Therefore, one should be cautious when using $\kappa^2 = 2/3$ for the calculation of R.

In single-molecule FRET experiments, doubly labeled molecules are either freely diffusing in a solution or immobilized on a surface through a linker. In the former scheme, a confocal microscope is employed to detect photon bursts when the molecule diffuses through the detection volume. The FRET efficiencies from each burst are calculated and the associated distribution can be constructed. However, due to the limited dwell time of the molecules in the detection volume, dynamics on time scales much slower than the characteristic diffusion time of the molecule are not accessible. For this reason, an immobilized scheme is chosen for probing slow dynamics. In this case, indi-

vidual molecules can be addressed one at a time in a confocal configuration or many at the same time via a wide-field imaging setup. Since the molecule is illuminated all the time during the measurements, one limiting factor is the photophysical behavior of the dyes, namely, photoblinking and photobleaching. Photoblinking is the reversible transition of the fluorophore between a fluorescing state and a non- or less-fluorescing state, a "dark" state, which is induced by the excitation light [45]. Photobleaching is the irreversible conversion of a fluorescent molecule into a non-fluorescent entity, which will limit the observation time of the molecules. In addition, since a single molecule can only emit a limited number of photons per second, a certain minimum integration time has to be set in order to detect a signal above the shot noise, which in return restricts the accessible dynamics on the short times. To achieve longer observation windows and access fast dynamics, our group has developed a new type of temperature-cycle microscopy [46]. The method relies on rapid thermal cycling of a microscopic sample to separate the room-temperature conformational evolution of, for example, a single biomolecule from the optical probing of the fluorophore(s), which takes place under cryogenic conditions. That way we want to probe a frozen "snapshot" of the biomolecule at a temperature where the photophysical parameters of the label(s) are more favorable than at room-temperature. The temporal resolution in this case is no longer limited by photon statistics and, instead, is determined by the time it takes to undergo the temperature jump. The second line of research in this thesis involves the first application of this method to study the conformational dynamics of single-molecule FRET-labeled polyprolines.

1.3 Outline of the thesis

Chapter 2 describes our first rheological measurements on supercooled glycerol and *ortho*-terphenyl with a home-built rheometer, which is based on a Couette cell (see Figure 2.7). In the experiments, we applied very weak and constant stresses (on the order of 100 Pa) to the sample and the mechanical response was monitored as a function of time. We have identified a solid-like behavior in both materials in the supercooled state, i.e., above the glass transition, suggesting the gradual emergence of an extended solid-like network in the liquid bath. This network stiffens as it ages and can break and melt upon application of large shears, producing all well-known features of soft glassy rheology (yield-stress, shear melting and aging) in the material. The rheological results here are consistent with the earlier single-molecule observation on supercooled glycerol that some nearly static structures already develop at

temperatures well above the glass transition.

Chapters 3 extends the work in Chapter 2 by employing a commercial rheometer to further explore the solidification of supercooled glycerol. The commercial rheometer enables us to perform small strain amplitude oscillatory measurements to avoid the previously observed "breaking event" of the fragile network while measuring its response. We first tried to reproduce the solidification in a Couette cell with similar dimensions as in Chapter 2. We found that an initial slow cooling period was crucial for the formation of the solid-like state. However, as the apparatus compliance, i.e., the deformation of the measurement tools, may limit the maximum measurable rigidity of the sample, we switched to a plate-plate geometry, where gap and plate size were chosen such that the tool compliance is negligible. Another advantage of using the plate-plate configuration is that the sample can be visualized during the measurements. Surprisingly, we could not reliably reproduce the solid-like state in the plate-plate geometry with the thermal profile, which reliably led to the solidification in the Couette cell. Nevertheless, we observed once a slush-like phase that grew from the top plate at the growth speed of the crystal phase. The shear modulus of this slushy phase, however, was two orders of magnitude lower than that of the crystal phase.

Chapter 4 reports on micrometer-sized structures in a thin glycerol film observed by imaging fluorescent probes doped in the host matrix at temperatures close to but above the glass transition temperature. We observed two distinct heterogeneous patterns of the fluorescence intensity, depending on how fast the sample was cooled down. A Swiss-cheese-like pattern in which many micrometer-sized dark spots were nucleated in a bright background was detected in a slowly cooled sample. In contrast, a quickly cooled sample showed a spinodal decomposition pattern where many bright island-like features of micrometer sizes were dispersed in a dark matrix. Those heterogeneous patterns, which are ascribed to differential dye distributions in the glycerol film, can persist for days unless they are heated considerably, pointing to long-lived and micrometer-scale density fluctuations in supercooled glycerol.

Chapter 5 presents the preliminary results of small-angle neutron scattering experiments on solidified glycerol. We have performed two series of experiments on two glycerol samples respectively with thermal histories similar to the one used in Chapter 2. We observed the growth of solid-like structures in one sample only, evidenced by both direct visual inspection and by the scattering spectra. A new peak, centered around $0.1\,\text{Å}^{-1}$, is a hallmark of this solid-like state. It is clearly absent from the pure liquid state and from the crystal at the same temperature.

Chapter 6 reports on the first application of temperature-cycle microscopy in the study of single-molecule FRET in polyprolines. We measured static FRET efficiencies of individual constructs frozen in a thin glycerol film at low temperature, which revealed a broad distribution of FRET efficiency. We tried temperature-cycle measurements on polyproline 6 and we observed the change of FRET efficiency due to the conformational changes induced by the temperature jump. These preliminary results demonstrate that our temperature-cycle microscopy combined with single-molecule FRET labeling has potential for the study of protein-folding dynamics at the single-molecule level.