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

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## 7 Summary

### 7.1 Heterogeneity in supercooled liquids

Understanding how a liquid becomes a glass is the central topic in glassy studies. Early models postulate the existence of cooperatively rearranging regions (CRR) in glass-forming systems at temperatures close to the glass transition. Such CRRs are believed to be intimately connected to heterogeneity and characteristic of the glass transition. Understanding heterogeneity is, therefore, a key step to the full structural description of the glass transition. Motivated by earlier single-molecule studies on supercooled glycerol, which uncovered a long-lived heterogeneity at temperatures well above the glass transition, we further investigated the heterogeneity in glycerol by rheometry, fluorescence imaging, and small-angle neutron scattering.

In Chapter 2, we described our first rheological measurements on supercooled glycerol and *ortho*-terphenyl, respectively. The experimental setup was a home-built rheometer based on a Couette cell. It allowed us to apply small and constant shear stresses (on the order of 100 Pa) to the sample and to monitor the mechanical response as a function of time. We have detected a solid-like response in both materials at temperatures well above their respective glass transitions. We found that a proper thermal treatment was necessary to observe such a solid-like behavior. The thermal treatment involved an initial cooling to a temperature just a few Kelvin above the glass transition, followed by annealing at that temperature for a few hours, and aging at a higher temperature. We also realized that the thermal history of earlier temperature-dependent single-molecule rotation measurements in glycerol was very similar to those used for the rheological measurements here. Consistent with the single-molecule observation, supercooled glycerol already develops a solid-like network percolating in the liquid bath at temperatures well above the glass transition. The network becomes stiff with time, responds elastically for a small shear, and breaks with large shears, conferring to the material all well known features of soft glassy rheology, such as aging, yield-stress, and shear thinning.

In Chapter 3, we expanded the work on supercooled glycerol discussed in Chapter 2 by employing a commercial rheometer, which allowed us to per-

form oscillatory measurements with very small strains to avoid disturbing the fragile solid-like network as much as possible during the experiments. We first reproduced the solidification of glycerol in a Couette cell as was observed in Chapter 2. We found that an initial slow cooling (5 K/h) prior to annealing at a higher temperature was crucial for the onset of the solid-like behavior. The associated waiting time for such onset decreased with increasing annealing temperature. Upon heating, the solid glycerol melted at the melting point of crystalline glycerol. However, due to the apparatus compliance, i.e., the deformation of the measurement tools, the maximum measurable rigidity of glycerol in the Couette cell was only on the order of  $10^7$  Pa. We therefore switched to a plate-plate geometry, where gap and plate size were chosen such that the tool compliance was negligible. Additionally, this configuration allowed us to access the sample optically during the measurements. To our surprise, the thermal profile which led to the solidification in the Couette cell could not reliably reproduce the solid-like state of glycerol in the plate-plate geometry. We detected the solid-like behavior only in one run (out of six). In that run, a slush-like phase 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 smaller than that of the crystal phase, which we measured independently from the seeded sample.

In Chapter 4, we studied the thin film of glycerol doped with fluorescent probes by fluorescence imaging. We detected two distinct heterogeneous patterns of the fluorescence intensity, depending on the cooling rate applied to the sample. A slowly cooled sample showed a Swiss-cheese-like pattern in which many micrometer-sized dark spots were nucleated in a bright background, whereas a quickly cooled sample resulted in a spinodal decomposition pattern where many bright island-like features of micrometer sizes were dispersed in a dark matrix. These two heterogeneous patterns, due to inhomogeneous dye distributions in the glycerol films, could persist for days if they were not heated considerably, suggesting long-lived and micrometer-sized density fluctuations in supercooled glycerol.

The results from Chapters 2 and 3 raise one important question: what is the structural origin of the solid-like state of glycerol. The observed viscoelastic behavior seems to be related to a crystal growth in supercooled glycerol, since the solid-like structures grew at more or less the same speed as the crystal and melted upon heating to the melting point of crystalline glycerol. On the other hand, the rigidity of the solidified glycerol is two orders of magnitude smaller than that of the crystal. Moreover, the measurements in Chapter 4 suggested that the glycerol film was free from large microcrystallites no matter how

slowly it was cooled.

In Chapter 5, we tried to understand the structural origin of the solid-like state of glycerol by performing small-angle neutron scattering experiments. We did two series of measurements on two glycerol samples, respectively, with thermal histories similar to the one used in Chapter 2. We saw the growth of solid-like structures in one sample only, evidenced by both direct eye visualization and by the scattering spectra. A new peak, centered around  $0.1 \text{ \AA}^{-1}$ , in the spectra is a hallmark of this solid-like state, since it is clearly absent from the pure liquid state and from the crystal at the same temperature. However, as the Q range ( $0.008$  to  $0.431 \text{ \AA}^{-1}$ ) chosen for the measurements did not cover the range ( $0.5$  to  $5 \text{ \AA}^{-1}$ ) where the Bragg peaks are normally seen, we could not confirm whether the solidified glycerol has a crystalline identity or not. Future measurements in the large Q range should give more insight into the nature of this solid-like state in supercooled glycerol.

## 7.2 Temperature-cycle microscopy of single-molecule FRET in polyprolines

Observing a single dye molecule for an infinitely long time and probing fast dynamics from single molecules without accumulating photon events from different individual molecules are the dreams of experimentalists working with single-molecule fluorescence spectroscopy. Although it is now possible to observe an immobilized dye molecule for minutes at room temperature in a solution by adding triplet quenchers and oxygen scavengers, the time resolution is still limited to the range of a few milliseconds in order to have a reasonable signal to background ratio. To achieve a longer observation time without immobilizing single molecules and to probe fast dynamics from these molecules in the submillisecond or even microsecond range, we have recently proposed a new type of temperature-cycle microscopy. In Chapter 6, we demonstrated how to use this new method to study the conformational dynamics of polyprolines with single-molecule FRET. We first measured the static FRET efficiency of frozen polyproline 6 and polyproline 20 molecules at low temperature in glycerol, respectively. Both of the measurements revealed a broad distribution of FRET efficiencies. The broadening originated from the wide distribution of the orientation factor and the fluctuation of the interdye distance. In the fluorescence time traces of individual constructs, we studied the variations of the donor and acceptor signals and observed 3 types of events. (1) A correlation between the two signals, i.e., the acceptor intensity was positively correlated with the donor intensity, was observed when the donor was in a dark state

or bleached. (2) A noncorrelation, i.e., the change of the acceptor intensity is independent of the donor intensity, was seen when the acceptor was in non-fluorescing states and it could still quench the donor emission. This is probably because the absorption spectrum associated with such non-fluorescing form(s) is very similar to or even identical with that of the fluorescing state of the acceptor although the fluorescence is extinguished. The energy transfer between the two molecules therefore remains unchanged. (3) An anticorrelation between the two signals was detected if a non-fluorescing form of the acceptor did not accept the excitation energy from the donor. We performed our first temperature-cycle measurements on polyproline 6 and we could indeed detect the conformational changes induced by temperature cycles by following the FRET efficiency. These preliminary results show that temperature-cycle microscopy combined with single-molecule FRET has potential for studies of protein-folding dynamics at the single-molecule level.