Single molecules in soft matter : a study of biomolecular conformation, heterogeneity and plasmon enhanced fluorescence

Yuan, H.

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Author: Yuan, Haifeng
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1 Introduction

Soft matter, such as liquids, polymers and bio-materials, are sensitive to weak mechanical action and thermal fluctuations. Widely spread in fields of physics, chemistry and biology, soft matter draw much attention owing to their fundamental importance in both science and technology. In particular, we are interested in bio-molecules and molecular glass-forming liquids. This thesis thus consists of two distinct areas of research. On the one hand, we study fast conformational dynamics of fluorescent labeled bio-molecules in glycerol using a temperature-cycle method. On the other hand, we study heterogeneity in supercooled glycerol by neutron scattering/diffraction and by following the dynamics of guest nanoparticles. Despite the differences in the experimental systems, far-field single-molecule optical microscopy is the main method applied in our research. In this chapter, we motivate our research and present the outline of this thesis.

1.1 Conformational dynamics of single bio-molecules

Soft and complex biological molecules such as DNA, RNA and proteins often require certain conformations to realize their functions. For instance, proteins, which comprise of chains of amino acids, have to fold into correct three-dimensional structures to perform their functions. Moreover, many enzymatic interactions and dynamical functions can only be achieved by precise conformational reactions. The folding process, however, is complex and may involve different reaction pathways and intermediates [1–3]. The folding process can be represented by a potential-energy landscape which describes the energy barriers between different states or intermediates [4]. When the energy barriers to other states are high, a protein's conformation can thus be relatively stable. At the opposite, transitions between different states can take place when the energy barrier between them is low. The reaction rates are determined by the heights of energy barriers. Hence, understanding such reaction dynamics requires insight into the whole landscape and intermediates.

Although much is known about crystallized proteins' structures, there is much to be learned on the conformations exhibited by the proteins along

their folding pathways. Altering local environments such as temperature and chemical concentrations, the kinetics of the protein folding process can be studied [5–9]. Many such experiments are performed in bulk, averaging over ensembles of molecules and smearing out many details. In particular, parallel or alternative pathways, non-accumulative intermediates and heterogeneous reactions can hardly be resolved. Therefore, the underlying mechanisms that ensure correctly folded structures are best examined at the single-molecule level.

1.2 Single-molecule fluorescence microscopy and temperature-cycle method

Developments in the past decades have made it possible for scientists to reach the ultimate resolution of a single molecule in the optical far field. [10] The far-field optical contrast is often provided by means of emission [11–13], absorption [14–18] and enhanced Raman scattering [19–23]. Watching one molecule at a time, optical microscopy on individual molecules is free from ensemble averaging. It provides unique access to rare species, heterogeneous structures and dynamics [24–31]. Among different single-molecule techniques, fluorescence-based detection is the most commonly employed method for its fast detection, high contrast against background and ease in instrumentation. The key requirement is that the objects under investigation must have sufficiently high emission rates. Many organic dyes, fluorescent proteins of good photostability and high quantum yields are often used as probes or labels to study molecular dynamics, interactions and material properties. Following single-molecule fluorescence observables, such as intensity, lifetime, polarization and energy transfer, many new insights have emerged in various fields and systems [28, 32–36].

Proceeding via dipole-dipole interaction, fluorescence resonance energy transfer (FRET) between fluorophores is sensitive to distances of several nanometers [37]. It allows conformational dynamics on molecular scales to be probed [2, 38]. Implemented in the single-molecule regime, FRET reports the time-dependent distance between two molecules or two positions within one molecule. Therefore, FRET of single-molecules is an ideal fluorescence observable for studying heterogeneous conformational dynamics and for probing folding energy landscapes [1, 24, 38–41].

Nevertheless, limitations on both slow and fast temporal scales keep single-molecule FRET studies challenging in many cases. On the one hand, it is difficult to follow FRET signals on the same molecule for long times. Of-

ten carried out on freely diffusing molecules in solution, the observation time on each single molecule is determined by the molecule's dwell time in the detection volume (about one millisecond). Thus, real-time observation on single-molecule folding and unfolding process for longer times requires some form of immobilization, such as attaching onto surfaces [42, 43], optical tweezers [44] or encapsulation in surface-tethered lipid vesicles [1, 45]. However, such immobilization is often complex and may influence the dynamical system under investigation. Moreover, photobleaching, an irreversible photochemical reaction, universally takes place in fluorophores. This limits the observation time on each molecule to tens of seconds at best. On the other hand, the emission rates of single emitters limit the temporal resolution to millisecond-scales [24, 46–48]. This comes from two factors. (i) The fluorescence emission process takes place on several nanoseconds. (ii) Instrumental collection and detection efficiencies are low. The above mentioned two factors limit fluorescence intensity of single molecules to be a few thousand photons per second. Therefore, it is difficult to resolve dynamics that happens in less than 1 ms. However, conformational dynamics of bio-molecules occurs at a broad range of timescales. For instance, protein folding covers 13 orders of magnitude in timescale, ranging from nanoseconds to hours [38, 49, 50]. In particular, those intermediates or transitions on timescales faster than a millisecond are often difficult to measure [41, 43].

To extend the timescales accessible to single-molecule fluorescence microscopy, a temperature-cycle method was proposed and demonstrated [51]. This method, closely resembling the temperature-jump method [5, 52], relies on rapid thermal cycles between two temperatures. Extreme temperatures are selected so that molecular conformation can evolve at the higher temperature but are frozen at the lower temperature. Once frozen, the conformation state can be probed for arbitrary long times, as needed. Therefore, the temporal resolution is no longer limited by photon's statistics but by the time for freeze and thaw to take place. Repeating such "thaw-freeze-probe" cycles, consecutive series of snapshots of frozen states can be recorded. We thus hope to reconstruct conformational dynamics on different timescales or at different temperatures from the recorded snapshots on individual molecules.

1.3 Heterogeneity in supercooled liquids

Many liquids fail to crystallize upon cooling below their melting point (T_m). Instead, they turn into a supercooled state in which they remain amorphous but their dynamics slows dramatically. Accompanied by the slow dynamics,

supercooled liquids become heterogeneous close to the glass transition (T_g) [33, 53–56]. Often appearing around 1.1-1.2 T_g [53], dynamics in one region in a supercooled liquid can be one order of magnitude different from that in another region a few micrometers away. Moreover, supercooled liquids exhibit temporal heterogeneity. Dynamics in the same region can appear different with time [33]. Such phenomena, without any obvious structural origins, have been observed in various supercooled liquids by different experimental methods [33, 53–55]. Free from ensemble averaging, dynamics of individual fluorescent molecules reflects properties of their local surroundings. Following rotational motions of single molecules embedded in supercooled liquids, spatial heterogeneity near the glass transition has been reported by different groups [57–59]. In these experiments, the spatial heterogeneity was found to be on length scales comparable to the sizes of the probe molecules. However, temporal heterogeneity appeared differently. In supercooled glycerol, Zondervan *et al.* observed surprisingly long-lived heterogeneity which is almost static [58]. In the same material, Mackowiak *et al.* found temporal heterogeneity about 10-100 times longer than the structural relaxation time of glycerol molecules [59]. Although differences in thermal history of samples are suspected for differences in temporal heterogeneity [33], the underlying mechanism is not yet clear. Without an agreement on these basic physical measures, deeper mechanistic questions will remain elusive.

Structural heterogeneity, which can be a natural source of heterogeneous dynamics, was also reported in supercooled liquids [60–64]. It often relates to the thermodynamic properties of materials. Recent rheology studies reported formation of solid-like networks in supercooled glycerol [65, 66]. Moreover, the formation of solid-like structures strongly depends on the thermal history of the sample. Nevertheless, the influence of structural heterogeneity and thermal history on experimentally observed dynamical heterogeneity is not well documented.

The other essential element for a full picture of the dynamical heterogeneity is its evolution with temperature. The crossover temperature (T_c), below which the supercooled liquids become heterogeneous, is predicted to be around 1.2 T_g by the mode coupling theory (MCT) [53, 67, 68]. In the work of Zondervan *et al.*, extrapolation of single molecules' tumbling rates as functions of temperature implies the heterogeneous dynamics converges at approximately 230 K for supercooled glycerol, shown in figure 1.1 [58]. Single-molecule fluorescence microscopy, however, has not been able to directly report on T_c. The main challenges come from the following factors. (i) Limited by the temporal resolution (1 ms) of single-molecule fluorescence microscopy, rotational

dynamics of individual fluorophores becomes difficult to follow in real-time at temperatures above $1.2\,T_g$. (ii) Translational motion of fluorophores become more pronounced at higher temperatures. Although translational dynamics can also report heterogeneity, it is difficult to follow the same molecule for a long time. (iii) Photoblinking and photobleaching of organic fluorophores often influence the accuracy of measurements.

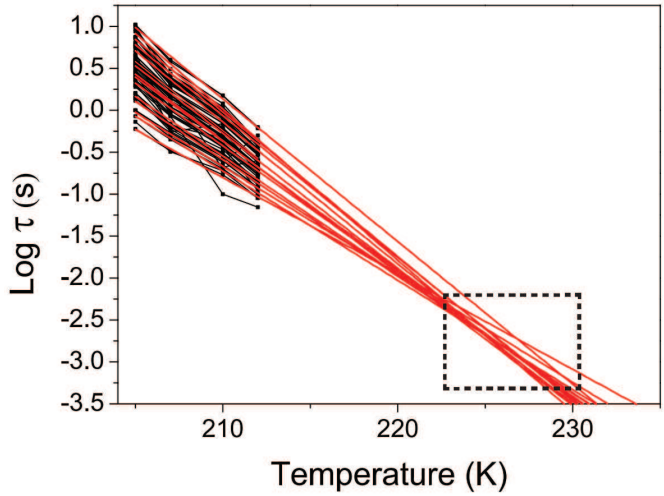

Figure 1.1: Extrapolation of single molecules' rotational times as functions of temperature. The black squares ad black lines represent rotational times measured on individual PDI dye molecules (data from Zondervan *et al.* [58]). The red lines show the extrapolation of the single-molecule data. The dashed box shows the region where the extrapolation converges.

Given all above unresolved problems, dynamical heterogeneity has to be studied in extended spatial/temporal scales and temperature ranges with careful consideration of thermal history. Therefore, other nanoprobes are required to supplement currently used fluorescent molecules.

1.4 Gold nanorods for probing dynamical heterogeneity

Gold nanorods (GNRs), which can be detected by their absorption, scattering and photoluminescence contrasts [69], provide polarization-dependent optical response due to their anisotropic shape. Therefore, their orientational

dynamics can be tracked optically. Despite its low quantum yield [70], photoluminescence from GNRs can be easily detected on the single-particle level. Moreover, already established single-molecule fluorescence methods can be directly applied to individual GNRs. GNRs possess many advantages for probing heterogeneity in supercooled liquids. (i) GNRs have superb photostability. They neither blink nor bleach in their photoluminescence. (ii) Dimensions of GNRs can be tuned by chemical synthesis [69]. Therefore, they can be used for probing heterogeneity of 10 nm-100 nm. (iii) GNRs can be chemically modified on their surface for various environments and solvents.

Besides directly following rotational dynamics of GNRs, one can also probe local dynamics with dye molecules diffusing near a GNR. Plasmonic nanostructures, such as GNRs, are known to enhance fluorescence of molecules nearby [71]. Such fluorescence enhancement comes from two factors. On the one hand, plasmonic nanostructures can concentrate the optical field in their vicinity. Therefore, dye molecules in the near-field volume experience much higher excitation intensity. On the other hand, plasmonic nanostructures modify the spontaneous emission rates of nearby dye molecules via the antenna effect. When dye molecules diffuse through the near-field volume (approximately $1000\,\mathrm{nm}^3$) of a GNR, sudden increases in fluorescence intensity can be observed [72, 73]. Other dye molecules in the focal volume contribute only to the background. The signal-to-background ratio therefore depends on the enhancement of fluorescence signals and on the number of dye molecules present in the focal volume. By carefully selecting dyes and concentrations, single molecules diffusing near a GNR can be detected. Local single-molecule diffusion dynamics can then be reflected by correlation of the fluorescence signals. Although detected in the optical far field, the correlation volume is determined by the near field. This method therefore provides a spatial resolution of $\sim$10 nm and can cover broad temperature ranges.

1.5 Outline of the thesis

Chapter 2 demonstrates the application of single-molecule temperature-cycle microscopy on FRET-labeled polyprolines. We report broad FRET distributions in steady state when conformations of FRET constructs are frozen. These results differ from the prediction for isotropically oriented fluorescent labels by Förster theory. Applying temperature cycles of a few microseconds on single FRET constructs, we observed large FRET changes. Following long series of temperature cycles on the same molecule, reversible transitions between a low-FRET state and a high-FRET state were observed. These two

FRET states indicate different conformations occupied by the single molecule. Although the origin of such conformational reactions was not determined, the FRET histogram measured on a single molecule under temperature cycles closely resembles that measured on single molecules in steady state.

Chapter 3 presents a single-molecule FRET study on both polyprolines and double-stranded DNA (dsDNA) using the temperature-cycle method. By means of a solid immersion lens and a single-component reflecting objective, the spatial resolution and collection efficiency are improved. Although being similar in inter-dye distances, polyproline and dsDNA samples showed apparently different FRET distributions in steady state. Applying temperature cycles of different parameters than those used in chapter 2, we revealed another dark state besides the low-FRET and high-FRET states on single polyproline molecules. Moreover, we observed different dynamics on dsDNA molecules. The possible reasons for such differences in FRET measurements lies in different linker dynamics and dye-dye interactions, as suggested in molecular dynamics simulations. Our results open the possibility to access fast dynamics of complex molecules by the temperature-cycle method.

Chapter 4 reports on crystallite nucleation in supercooled glycerol near the glass transition. Structural heterogeneity is often found in supercooled liquids at temperatures near the glass transition. However, it strongly depends on the thermal history of the sample. By means of neutron scattering, we examined different thermal histories for the solid-like structures to appear in supercooled glycerol. The result shows that solid-like structures appear only after at least 30 hours aging at temperatures very close to the glass transition. Furthermore, we determined the crystalline nature of such structures using the neutron diffraction method. The observation indicates nanocrystallite formation in supercooled glycerol near its glass transition, which was recently suggested in random first-order transition (RFOT) theory [74].

Chapter 5 describes the first application of gold nanorods to study dynamical heterogeneity in supercooled glycerol. Following rotational motions of individual GNRs in glycerol at 238 K, we estimated volumes of GNRs using the bulk viscosity. The estimate agrees nicely with the volumes measured in scanning electron microscopy (SEM). Rotational dynamics was then studied as function of temperature. As the temperature went below 225 K, we observed broadening in volume-normalized tumbling rates of the same GNRs. This observation directly shows dynamical heterogeneity appearing on length scales of at least 30 nm and on strikingly long time scales. Moreover, the indicated crossover temperature of about 225 K is in good agreement with that predicted by MCT [53, 67, 68] and previous single-molecule studies [58].

Chapter 6 reports on large fluorescence enhancement on single dyes near individual GNRs. Dyes with low quantum yields provide low fluorescence backgrounds at high concentrations. Moreover, their fluorescence can be largely enhanced by plasmonic nanostructures. Therefore, they are suitable for fluorescence correlation spectroscopy in the near-field volume. In this chapter, we demonstrate individual GNRs for enhancing single-molecule fluorescence. We report more than a thousand times fluorescence enhancement on crystal violet molecules.