Single Molecule Measurements of Stimuli-Responsive Polymers

Junya Uchida[1], Yuichi Kondo[2]

[1]Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo

[2]Department of physics, School of Science, The University of Tokyo

Authors

Junya Uchida: His research interests include organic chemistry, polymer chemistry, and supramolecular chemistry. In this study, he designed and synthesized dye-functionalized temperature-responsive polymers. He also contributed to discussion on the results of fluorescent resonance energy transfer (FRET) in response to thermal stimuli.

Yuichi Kondo: Specialty is single molecule measurements of molecular motor. In this study, he conceived experiment of single molecule polymer, constructed polymer-coated bead, carried out FRET measurement and spectroscopic measurement and analyzed the data.

Abstract

Clarification of dynamic motion of stimuli-responsive polymers in a molecular level is important not only for the basic understanding of molecular behavior but also for the design of functional polymer materials. Here we report single molecule measurements of thermo-responsive polymers based on approaches that have been used for analyses of biomolecular motors. FRET measurements suggest that macroscopic aggregation of the responsive polymers in aqueous solution is originated from the single molecule conformational change. Furthermore, we have designed experimental systems for single molecule optical trapping to clarify the relationship between structure changes and forces, and tried to optimize the experimental conditions.

1. Introduction

Stimuli-responsive polymers that show dynamic structural changes have attracted growing attention.[1] For example, poly(*N*-isopropylacrylamide) (PNIPAM) is one of the representative thermo-responsive polymers exhibiting large macroscopic deformation in the film or gel states in response to environmental stimuli due to the lower critical solution temperature (LCST) behavior.[2] These responsive polymers have great potential in the field of materials science. Although the macroscopic motion of such class

of polymers has been widely studied, fundamental research on their behavior in a molecular level is still limited. Our aim here is to analyze single molecule behavior of stimuli-responsive polymers. Clarification of responsive behavior of these polymers will provide the new design strategy of stimuli-responsive polymer materials for further functionalization, as well as new insights in the fundamental polymer science. In the present study, we report initial attempts toward single molecule measurements of PNIPAM based on fluorescent measurements and optical trapping.[3,4]

2. Experimental Section

2.1. Design and synthesis of dye-functionalized polymers

Temperature-responsive polymer with carboxylic groups at chain terminals **PNIPAM-COOH** was synthesized by reversible addition–fragmentation chain transfer (RAFT) polymerization.[5] Nitrobenzoxadiazole-ethylenediamine derivative **NBD-NH$_2$** and rhodamine B-ethylenediamine derivative **RhB-NH$_2$** were synthesized as reported.[6] Condensation of **PNIPAM-COOH** with **NBD-NH$_2$** and **RhB-NH$_2$** yielded dye-functionalized PNIPAM, which were purified by gel permeation chromatography (GPC) to remove unreacted dyes.

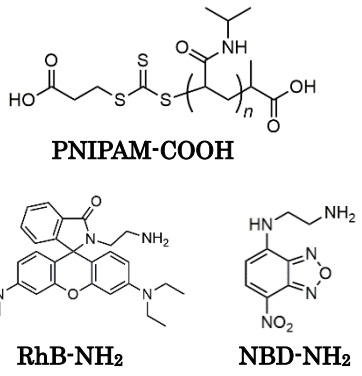

Figure 1. Molecular structures of **PNIPAM-COOH**, **RhB-NH$_2$**, and **NBD-NH$_2$**.

2.2. Experimental

In order to perform single molecule measurement of the polymer, a method used in a biomolecular motors was applied. Biotinylated glass and polymer-coated fluorescent beads are prepared. Biotinylated glass was prepared by nonspecifically adsorbing biotinylated BSA on the glass surface.

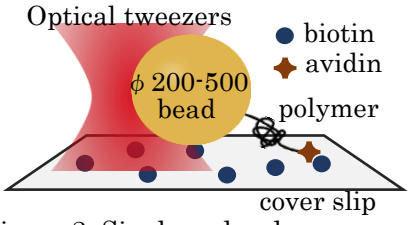

Figure 2. Single molecule assay

The polymer-coated beads are prepared by crosslinking the fluorescent bead surface and one end of the polymer. The other end of polymer was attached to avidin. Polymer-coated beads are trapped with optical tweezers in a chamber made of biotinylated glass and interact with biotin on the glass surface and load is applied. The response of the polymer is measured via positional change of the bead at some temperature.

2.3. Methods

2.3.1. Polymer modification of bead surface

The amine-modified polystyrene beads and the carboxylic acid at polymer terminal were crosslinked using EDC (FIG. 3). 100 µl of 50 mM MES (pH 5) and 50 µl of 50 mM EDC were added to 50 µl of 10 mg / ml polymer and allowed to stand at room temperature for 20 minutes. Next, 50 µl of 4 nM amine-modified polystyrene beads (diameter 200 nm) was added and stirred with a turntable for 90 minutes. This was centrifuged at 15 krpm for 10 minutes to remove unreacted EDC and dispersed in water to collect polymer-modified beads.

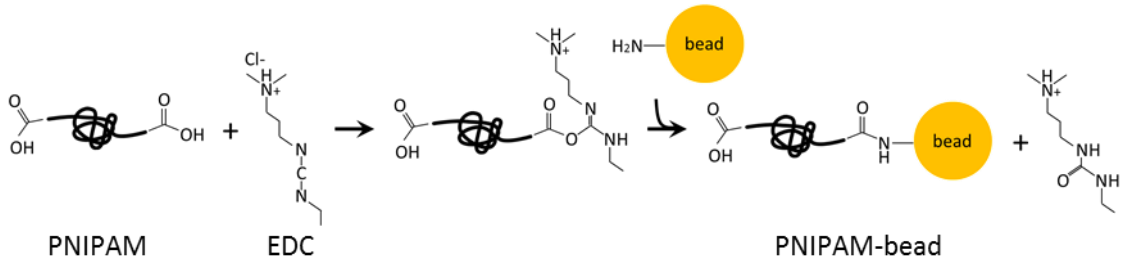

Figure 3. Polymer modification of bead

2.3.2. Measurements of transmitted light intensities

Transmitted light intensities at wavelengths of 300 nm and 600 nm were measured using a spectrophotometer. 500 µl of each concentration polymer prepared by dissolving in water was placed in a block incubator at 25 ° C. or 60 ° C. for more than 10 minutes, and measurement was carried out immediately after taking out the samples.

2.3.3. FRET measurements

The fluorescence spectra of the dye-modified polymer dissolved in water or 10 mM HCl (pH 2) were measured using a spectrofluorophotometer. 100 µl of the polymer dissolved in each solvent was placed in a block incubator at 25 ° C. and 80 ° C. for 3 minutes. Measurements were carried out immediately after the incubation. Measurements conditions are shown in Table 1.

Table1. Conditions of FRET measurements

Ex. Band	5nm
Em. Band	6nm
Response	0.2sec
Sensitivity	medium
Ex. Wavelength	470
Em. Wavelength	480-750
wavelength interval	2nm
Scan speed	200nm/min
Concentration	0.02mg/ml

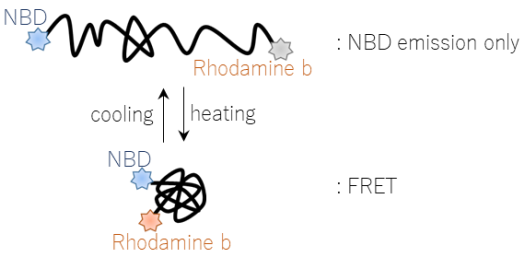

Fig4. Schematic illustration of FRET

3. Results and Discussion

3.1. Characterization of synthesized molecules

The structures of target polymers and dyes were confirmed by nuclear magnetic resonance (NMR) measurements. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) revealed the molecular weight of **PNIPAM-COOH** to be about 8,000 Da.

3.2. LCST behavior of the polymer

3.2.1. Measurements of transmitted light intensity

When the temperature of the solution of polymer concentration of 4mg/ml was raised to 37 ° C, it turned white turbid (FIG. 5). However, when the concentration was lowered to 0.2mg/ml, turbidity was not observed by eye. Therefore, the turbidity of this polymer solution was quantified from the change in transmitted light intensity. As a result, the transmitted light intensity decreased with temperature rise (Fig. 6). This result seems to be due to the aggregation of molecules and the increase of scattered light intensity. Even with the concentration of 0.2 mg/ml , the change in transmitted light intensity was detected (20-40%), indicating that aggregation occurred. The transmitted light intensity decreases when measured at a wavelength of 300 nm and 25 degrees, which is derived from the fact that the polymer absorbs light at the wavelength. Fig. 7 shows the result of eliminating the influence of absorption by using the curve at 25 degrees as the baseline. The reason that the transmitted light intensity decreases at low wavelength is considered to be because Rayleigh scattering is inversely proportional to the fourth

power of wavelength (in the case of a sphere).

Then, when the concentration was sufficiently lowered, significant turbidity change was not observed even with the spectrophotometer. It is considered that the size when molecules are aggregated is small and the change of scattered light intensity becomes small. When the concentration was 0.02 mg/ml, the change in transmitted light intensity was less than 10%. From the above results, it was found that aggregation of the polymer occurred at high concentration. However, from this result, it cannot be judged at the low concentration that whether the structure of the polymer has changed due to temperature change in single molecule level.

Figure 5. LCST behavior of 4mg/ml PNIPAM

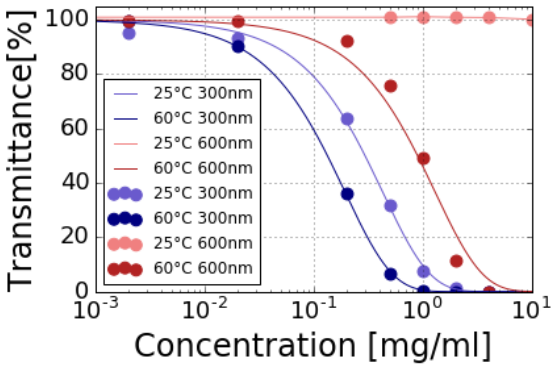

Figure 6. transmitted light

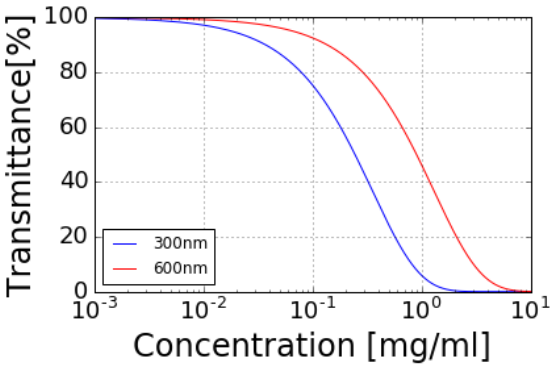

Figure 7. transmitted light intensity (eliminated the influence of absorption)

3.2.2. FRET measurements

FRET measurements were carried out to examine single molecule conformational changes of PNIPAM in response to thermal stimuli. The emission intensity of

rhodamine b was maximum when 10mM HCl (pH 2) aqueous solution was used as solvent. Therefore, fluorescence spectra were recorded for 0.02 mg/ml aqueous solution (pH 2) of dye-functionalized PNIPAM. Excitation wavelength is chosen as 470 nm, which excites NBD dyes efficiently. The detailed conditions of the FRET measurements are shown in Table 1.

Figure 8 shows fluorescence spectra of aqueous solution of dye-functionalized PNIPAM at 25 °C and 80 °C. Only one broad peak at 540 nm corresponding to the emission of NBD was observed at 25 °C, indicating that no FRET process occurred at 25 °C. On the other hand, two peaks at 540 nm and 578 nm were observed at 80 °C, which are assigned to the emission of NBD and rhodamine b, respectively. These results suggest that the distance between terminal dyes is decreased at higher temperature in a single molecule level due to LCST behavior, leading to the occurrence of FRET process.

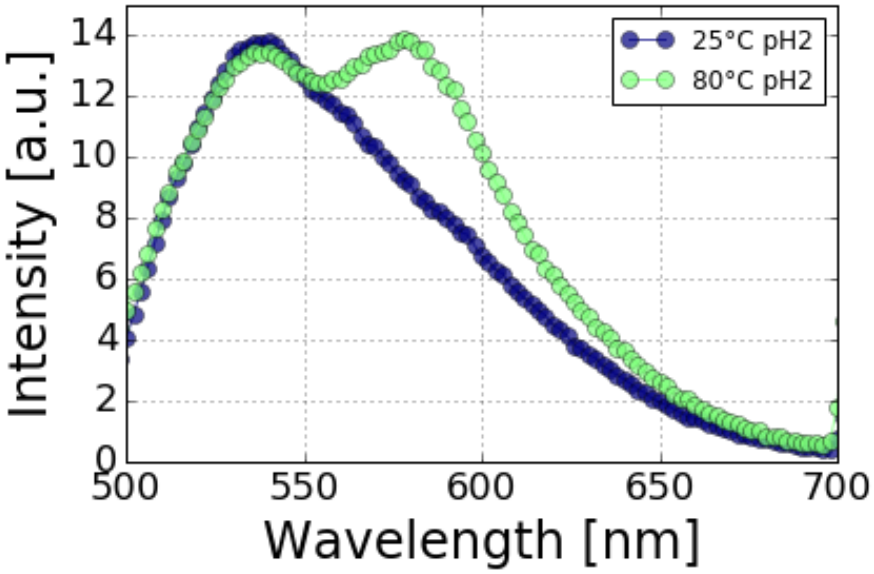

Figure 8. Fluorescence spectra of aqueous solution of dye-functionalized PNIPAM.

3.3. Polymer modification of bead surface

The polymer was coated on the amine modified bead surface with EDC. The obtained bead concentration was approximately 1 nM. When the beads were placed at room temperature or 60 ° C. for 10 minutes or more, no significant difference was observed in fluorescent microscopy. Reasons for no aggregation of the polymer with temperature rise are as follows: (1) polymer modification is not successful, (2) polymer concentration is low. For single molecular mechanics measurement, it is necessary to evaluate that the

bead surface is exactly modified by polymer. In the future we want to clarify the surface state of beads by coating the glass surface with polymer and observing the interaction between the beads and the glass surface at some temperature under a microscope.

4. Conclusion

Temperature-dependent aggregation behavior of PNIPAM was examined by single molecule measurements. FRET measurements indicate that macroscopic aggregation of PNIPAM in aqueous solution is originated from the single molecule conformational change. Although the experimental condition using optical trapping has not yet been optimized at this stage, some insights obtained in the present study will be useful toward single molecule optical trapping experiments in the future.

Acknowledgements

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References

[1] J. M. Abendroth, O. S. Bushuyev, P. S. Weiss, C. J. Barrett, *ACS Nano,* **2015**, *9*, 7746.

[2] H. G. Schild, *Prog. Polym. Sci.,* **1992**, *17*, 163.

[3] S. M. Block, L. S. B. Goldstein, B. J. Schnapp, *Nature*, **1990**, 348, 348.

[4] T. Ha, T. Enderle, D. F. Ogletree, D. S. Chemla, P. R. Selvin, S. Weiss, *Proc. Natl. Acad. Sci. USA.,* **1996**, 93, 6264.

[5] A. J. Convertine, N. Ayres, C. W. Scales, A. B. Lowe, C. L. McCormick, *Biomacromolecules,* **2004**, *5*, 1177.

[6] X. Wan, S. Liu, *J. Mater. Chem.,* **2011**, *21*, 10321.