

First Observation of Two-Stage Collapsing Kinetics of a Single Synthetic Polymer Chain

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Using a narrowly distributed poly(*N*-isopropylacrylamide) (PNIPAM) chain with a degree of polymerization (N) of 3100, randomly labeled with pyrene, we have, for the first time, observed the two-stage kinetics of the coil-to-globule transition. Two characteristic relaxation times, τ_{fast} for the crumpling of a random coil (~ 12 ms) and τ_{slow} for the collapsing of the crumpled chain to a compact globule (~ 270 ms), were measured. To our knowledge, this is the first experimental evidence supporting the two-stage collapsing kinetics of single synthetic polymer chain previously proposed by de Gennes, Dawson, and Grosberg.

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The protein folding, more precisely the dynamics of a protein as it collapses from a denatured random coil to its native globule state, is still not well understood. At a much simpler level, the understanding of the collapsing of a homopolymer chain from an extended random coil to a compact globule is relevant to the initial stage of the folding of protein chains from a denatured state to a bioactive form [1–6]. The possibility of obtaining kinetic laws of the collapsing of homopolymer chains in dilute solutions has long been considered as a prerequisite to a better understanding of the protein folding.

However, it should be noted that such a much simpler collapsing problem of homopolymer chains in a poor solvent has not been fully understood [6]. Theoretically, different models have been proposed and numerically tested for kinetic paths of the coil-to-globule transition of a homopolymer chain [7–18]. de Gennes [7,8] stated that the collapsing of a random-coil linear chain leads to the formation of crumples on a minimal scale along the chain backbone, which thickens and shortens under diffusion of the monomers and then form new crumples of growing scales until a compact globule is reached. Dawson *et al.* [9] consider a different two-step mechanism, namely, fast formation of a series of “pearls” along the chain followed by a slower compaction. They expected that the first step would take many microseconds and the second one may occur over a period of 1 s. In a refined model, Grosberg *et al.* [10,11] included the self-entanglement in the de Gennes model and considered a two-stage mechanism. Namely, the crumpling has a characteristic time (τ_{crum}) scaled to the number of statistic segments (N) as $\tau_{\text{crum}} \sim N^2$ followed by a chain knotting via the chain reptation with a longer characteristic time of $\tau_{\text{eq}} \sim N^3$ [10,11].

Experimentally, it is rather difficult if not impossible to study such a kinetic process. The only example of an attempted study was reported by Chu *et al.* [19–21], in which they observed two steps in the coil-to-globule transition of long linear polystyrene chains in cyclohexane by using a capillary tube with a thin wall (0.01 mm) in

dynamic laser light scattering. They attributed the two steps to those proposed by Grosberg with $\tau_{\text{crum}} = 357$ s and $\tau_{\text{eq}} = 323$ s, respectively, which are much longer (4–5 orders) than those predicted by de Gennes. In our opinion, this study had the following problems. First, although such a thin wall scattering cell was used, it still took at least 120 s for the temperature jump from 35 °C to 28 °C to reach its equilibrium, which made the light-scattering results in this time window less certain. Second, the interchain association occurred several seconds after the temperature jump so that in the solution the collapsed single-chain globules coexisted with the multichain aggregates that become more with time. Third, the depth of the temperature jump is not sufficient enough to make the chain reach its fully collapsed globule state [6]. Later, Raos and Allegra [22] argued theoretically that the intermediate “crumpled” state in Chu’s experiment might correspond to the single-chain globular state and the second stage could be related to the chain aggregation. So far, there has been no convincing experimental evidence to support previously predicted two-stage collapsing kinetics. How fast a synthetic polymer chain can collapse from its random-coil conformation to its globular state and whether such a collapsing is a two-stage process are unsolved problems in polymer physics.

On the other hand, using poly(*N*-isopropylacrylamide) (PNIPAM) in water with a lower critical solution temperature near 32 °C and laser light scattering, Wu *et al.* [23,24] observed thermodynamically stable globules only in a narrow temperature range without the formation of any interchain aggregates. Later, they also observed the coil-to-globule transition of long PNIPAM homopolymer chains in a mixture of methanol and water with a proper ratio although both pure methanol and pure water are good solvents for PNIPAM [25]. Excess addition of water or methanol can redissolve or “melt” compacted single-chain globules into extended random coils.

Recently, Martinho *et al.* [26–30] employed equilibrium excimer fluorescence to study the coil-to-globule transition

of individual chains. Using fluorescence technique enables them to study short chains when the fluorescence dye was properly labeled. On the basis of their results, we thought that by using a combination of the fluorescence and stopped-flow techniques we might be able to study the kinetics of the coil-to-globule transition of linear PNIPAM chains by addressing two questions: whether the coil-to-globule transition has two steps, and if yes, how fast each step is.

Free-radical copolymerization of *N*-isopropylacrylamide (NIPAM) with 4-(1-pyrenyl)butyl acrylate (PyBA) in benzene at 55 °C using 4,4'-azobis(isobutyronitrile) (AIBN) as the initiator led to linear PNIPAM chains randomly labeled with pyrene. The resultant PNIPAM-Py copolymer was fractionated by successive dissolution/precipitation cycles at 30 °C in a mixture of extremely dried acetone and *n*-hexane. The third fraction denoted PNIPAM-Py- F_3 was selected for the current study. Gel permeation chromatography analysis of PNIPAM-Py- F_3 in *N,N*-dimethylformamide in the presence of 1.0 g/L LiBr at 60 °C revealed that $M_n = 3.64 \times 10^5$ g/mol and $M_w/M_n = 1.17$ (using polystyrene standards). The average molar ratio of NIPAM units to PyBA units for PNIPAM-Py- F_3 is ~ 46 . On average, there are 65 pyrene groups per PNIPAM chain. Note that here the pyrene content on PNIPAM is only 2 mol % determined by using the method described before [31]. Therefore, it is expected that PNIPAM-Py- F_3 will exhibit a similar reentrant coil-to-globule-to-coil transition in a proper mixture of methanol and water (40–68 v/v% water content) [25,32–34].

Stopped-flow studies are conducted on a Bio-Logic SFM300/S device. The stopped-flow device is attached to the MOS-250 spectrometer through optical fiber connection. Thirty shots were done successively for each mixing ratio and an average dynamic curve was obtained. Kinetic data were fitted using the Biokine software (Bio-Logic). For the light-scattering intensity detection, both the excitation and emission wavelengths were adjusted to 335 nm. While for the fluorescence detection, the excitation wavelength was set at 330 nm, the emission wavelength was, respectively, set at 378 and 480 nm to record the time dependence of fluorescence monomer and excimer emission intensity. Slits of 5 nm are always used for both the excitation and emission monochromator. Using FC-08 or FC-15 flow cell, the typical dead times of the stopped flow are about 1.1 and 2.6 ms, respectively. Temperature was maintained at 25 °C by circulating water around the syringe chamber and the observation head. We focused on the kinetics of the coil-to-globule transition after mixing equal volumes of PNIPAM-Py solution in methanol with water by recording the time dependence of the ratio of excimer to monomer emission intensities (I_E/I_M).

First, we use the time-dependent scattered light intensity in the stopped-flow device to determine the concentration range in which the chains can collapse into single-chain globules without any interference of interchain aggregation. Figure 1 shows that when the final polymer concen-

tration in 1:1 v/v methanol/water mixture is lower than 2×10^{-6} g/mL, the scattering intensity remain a constant, indicating that there is no interchain aggregation within the first 5 s. This is because the scattered light intensity is proportional to the square of the mass of the scattering objects. For higher polymer concentrations ($> 5 \times 10^{-6}$ g/mL), the scattering intensity increases quickly with time even within 1 s, revealing the interchain aggregation. To be safe, in the following stopped-flow experiments, we kept the PNIPAM concentrations lower than 2×10^{-6} g/mL and studied only the kinetics within the first 5 s to eliminate any interference from possible interchain aggregation.

It has been known that pyrene forms a cofacial excimer when two pyrene molecules are close (< 4 Å) and excited by a light of ~ 330 nm [35,36]. The pyrene excimer shows characteristic fluorescence distinct from an isolated pyrene molecule. The excimer is stable only in its excited state and affected by the collision of its adjacent pyrene molecules. Therefore, the pyrene excimer band at 480 nm is usually accompanied by monomer fluorescence at 380 and 400 nm. Pyrene excimer fluorescence depends on the ability of two pyrene molecules coming together, which is determined by the chain mobility and the local concentration of pyrene [26–31,35,36].

Since PNIPAM-Py- F_3 in pure methanol exists as individual random-coil chains, it is expected that its fluorescence spectrum shows an emission related to locally excited pyrene chromophores (intensity I_M , “monomer emission”) with the [0, 0] band located at 378 nm and a broad structureless emission centered at 480 nm due to the excimer emission (intensity I_E). Identical excitation spectra were obtained for the emission monitored at 378 and 480 nm and I_E/I_M in methanol remained a constant over a wide concentration range (5×10^{-7} – 5×10^{-5} g/ml), implying that in methanol the pyrene molecules form some intrachain excimers due to dynamic encounter of an excited-state and a ground-state pyrene molecule [31,36].

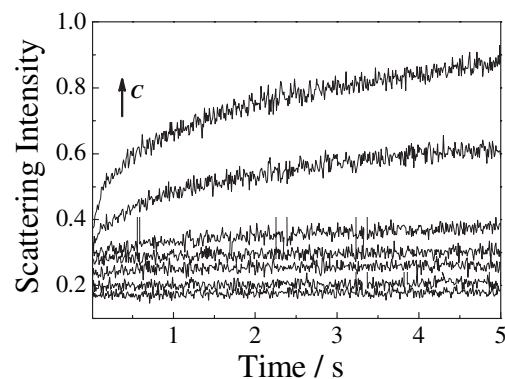


FIG. 1. Time dependence of scattering intensity after mixing PNIPAM-Py- F_3 methanol solution with an equal volume of water in a stopped-flow device at 25 °C. From bottom to top, the final polymer concentrations in 1:1 v/v methanol/water are 5.0×10^{-7} , 1.0×10^{-6} , 2.0×10^{-6} , 5.0×10^{-6} , 1.0×10^{-5} , 1.5×10^{-5} , and 2.5×10^{-5} g/ml, respectively.

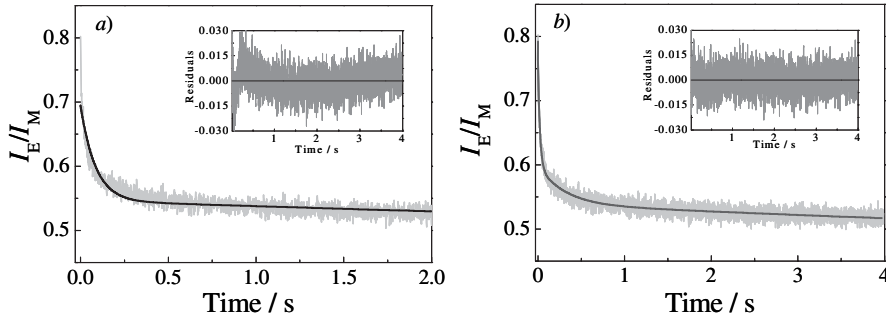


FIG. 2. Time dependence of ratio of excimer to monomer emission intensities (I_E/I_M) after mixing methanol solution of PNIPAM-Py- F_3 (2.0×10^{-6} g/ml) with an equal volume of water in a stopped-flow device at 25 °C. The solid lines represent (a) single exponential and (b) double exponential fitting curves. Insets are plots of fitting residuals. The dynamic curve is averaged from 30 successive runs.

Figure 2 shows a dramatic decrease of I_E/I_M immediately after the stopped-flow mixing, followed by a much slower and gradual decrease. We expected an increase of I_E/I_M since the collapse of a PNIPAM chain would increase the local concentration of pyrene and shorten their average spatial distance. Taken PNIPAM-Py- F_3 as an example, its average hydrodynamic radius $\langle R_h \rangle$ in pure methanol is 18.2 nm, but becomes ~ 5 nm in its collapsed globular state [25]. Assuming a uniform distribution of pyrene groups, we can estimate the average spatial distances between two pyrene groups. They are ~ 7.3 and 2.1 nm, respectively, for the random coil and globular state. It is clear that the average spatial distance between two neighboring pyrene molecules is still much larger than the characteristic distance (0.4 nm) [36]. Therefore, it is the mobility of PNIPAM chain to which pyrene molecules are covalently attached that determines I_E/I_M . The decrease of I_E/I_M reflects the decrease of chain mobility, hindering the rotation and diffusion of pyrene molecules on the chain so that pyrene molecules in the excited and ground state have less chance to form excimers. Martinho *et al.* [26–30] always observed a decrease of I_E/I_M during the coil-to-globule transition. Therefore, we can use the decrease of I_E/I_M to monitor the kinetic of the coil-to-globule transition.

The time-dependent I_E/I_M (defined as I_t) can be converted to a normalized function, namely, $(I_t - I_\infty)/I_\infty$ vs t , where I_∞ is I_t after an infinitely long time. The fitting of the relaxation curve by a single exponential function is not good [Fig. 2(a)], especially for the first 0.1 s, which is the most interesting to us since the measured kinetic data are the most accurate in this initial stage. Empirically, we found that such a dynamic curve could be well fitted by a double exponential function [Fig. 2(b)]:

$$(I_t - I_\infty)/I_\infty = c_{\text{fast}} e^{-t/\tau_{\text{fast}}} + c_{\text{slow}} e^{-t/\tau_{\text{slow}}}, \quad (1)$$

where c_{fast} and c_{slow} are the normalized amplitudes ($c_{\text{fast}} = 1 - c_{\text{slow}}$), and τ_{fast} and τ_{slow} are the characteristic relaxation times of the initial and later stages, respectively. The mean collapsing time of the whole coil-to-globule process, τ_{collapse} , can be calculated as

$$\tau_{\text{collapse}} = c_{\text{fast}} \tau_{\text{fast}} + c_{\text{slow}} \tau_{\text{slow}}. \quad (2)$$

The existence of two separate processes can be better

viewed from the semilogarithmic plot of I_E/I_M vs t , as shown in Fig. 3. We can clearly discern an inflection point around 0.1 s. This explains why the double exponential fitting [Fig. 2(b)] is very good. For PNIPAM-Py- F_3 , τ_{fast} and τ_{slow} are experimentally determined to be 11.6 ± 0.1 ms and 257.3 ± 1.8 ms, respectively, and $\tau_{\text{collapse}} = 74.5 \pm 0.7$ ms.

We can attribute τ_{fast} and τ_{slow} to the predicted two processes, namely, the fast crumpling of a random coil and the slow collapsing of the crumpled chain to the compact globule, respectively. Previously, it was observed that during the folding of a PNIPAM chain attached with hydrophobic short polystyrene segments, the copolymer chain underwent a crumpling “ordered-coil” state before it finally collapsed into a globular state [37]. Namely, during the crumpling process, the hydrophobic polystyrene segments flipped into the center and the chain became more ordered in comparison with its random-coil conformation. The energy barrier involved in such a flipping was very low, which might explain why here τ_{fast} is much smaller than τ_{slow} because the crumpling process is much easier than the collapsing process in which topological constraints are expected to become dominant.

de Gennes [8] stated that $\tau_{\text{fast}} \approx \eta a^3 \Delta T N^2 / k_B \theta^2$, where η is the solvent viscosity (1.62×10^{-2} p for 1:1 v/v methanol/water mixture), a is the monomer size (estimated to be 0.15 nm), k_B is the Boltzmann constant, N is the

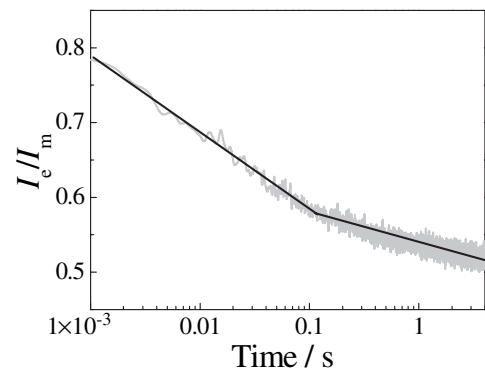


FIG. 3. Semilogarithmic plot of time dependence of ratio of excimer to monomer emission intensities (I_E/I_M) after mixing methanol solution of PNIPAM-Py- F_3 (2.0×10^{-6}) with an equal volume of water in a stopped-flow device at 25 °C. The solid line is just a guide for the eye.

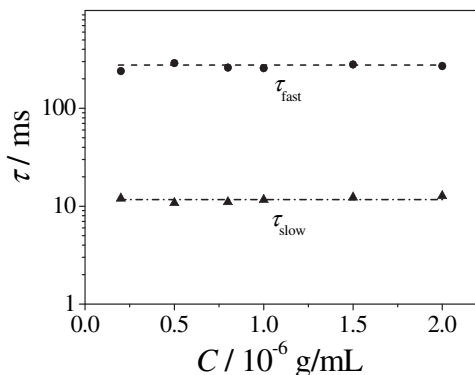


FIG. 4. Polymer concentration dependence of characteristic relaxation times (τ_{fast} and τ_{slow}) of fast and slow processes during the coil-to-globule transition of PNIPAM-Py- F_3 chains in 1:1 v/v methanol/water mixture, where the temperature was kept at 25 °C.

number of repeat units per chain, ΔT can be estimated from the difference between experimental temperature (298 K) and θ temperature of PNIPAM in for 1:1 v/v methanol/water mixture (~ 268 K) if we could transfer the solvent composition change to an equivalent temperature quench. The estimated τ_{fast} for PNIPAM-Py- F_3 is a few milliseconds, which is quite comparable to the measured 11.6 ms. Dawson *et al.* [9] also estimated that the first and second stages would take many microseconds and 1 s, respectively, in their two-step collapsing mechanism. Therefore, our observed τ_{fast} is in a reasonable agreement with the estimated value.

Figure 4 shows that both τ_{fast} and τ_{slow} are independent of the polymer concentration as long as it is lower than 2.0×10^{-6} g/ml. This ensures us that what we have observed involves on the coil-to-globule transition of individual chains. Otherwise, we would observe strong concentration dependence of τ_{fast} and τ_{slow} if the interchain association were involved.

In summary, we have, for the first time, observed that the coil-to-globule transition of individual long linear PNIPAM chains ($N = 3100$) labeled with pyrene molecules is a two-stage process, namely, the fast crumpling of a random coil and the slow collapsing of the crumpled chain to the compact globule, with two distinct characteristic relaxation times (~ 12 and ~ 270 ms). It agrees well with previous theoretical predications. Further studies of the chain length dependences of these two separate collapsing processes are underway in our group.

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