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Time-resolved fluorescence studies of the interactions between the thermoresponsive polymer host, poly(*N*-isopropylacrylamide), and a hydrophobic guest, pyrene

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Abstract

The time-resolved fluorescence anisotropy behaviour of pyrene solubilized (10^{-6} M) in ultra-dilute (10^{-3} wt%) aqueous solutions of the thermoresponsive polymer poly(*N*-isopropylacrylamide), PNIPAM, shows unusual characteristics. Rather than decay to zero (as expected of a freely rotating species in a homogeneous fluid environment) the anisotropy of the emission from the probe attains a minimum but finite value, some 40 ns after excitation of the pyrene. Thereafter, over a timescale of hundreds of nanoseconds, the anisotropy increases: the fluorescence from the solubized pyrene guest becomes more polarized with time! These deviations from "expectation" are significant both in terms of the aqueous solution behaviour of the polymer and basic photophysics.

The anisotropy data confirm that, above the lower critical solution temperature (LCST) of PNIPAM, the polymer host (in the form of single, "collapsed" macromolecules) is capable of solubilizing hydrophobic guests. The complexity of the time dependence of anisotropy of fluorescence from pyrene, above the LCST of the system, reflects the heterogeneous nature of the medium in which the probe is dispersed. At one extreme, we have pyrene molecules located within the bulk aqueous phase. At the other extreme, pyrene species are occluded within the hydrophobic interiors of the collapsed PNIPAM globules. The initial rapid loss in emission anisotropy reflects the contribution from shorter lived and rapidly tumbling solutes dispersed largely in the aqueous phase. The subsequent enhancement in the anisotropy at longer times, results from the ever-increasing importance of fluorescence from the longer lived, slowly rotating probes solubilized within the highly viscous cores of the globular conformation of the macromolecule.

Below the polymer's LCST, most of the pyrene guest is released to the bulk aqueous phase but a proportion of the organic guests remains associated with the (otherwise) open coils of the polymer.

In photophysical terms, the data presented constitute the first example of a study in which the motion of a fluorescent guest, mediated by the influence of its interactions with a synthetic polymer, *appears* to become more restricted as the time of sampling of its emission, following excitation, increases.

Keywords: Anisotropy; Fluorescence; *N*-isopropylacrylamide

1. Introduction

Poly(*N*-isopropylacrylamide), PNIPAM, exhibits thermoreversible phase-separation in aqueous solution [1]. This behaviour is characterized by a lower critical solution temperature (LCST) of ca. 31–32°C [1,2]. At moderate polymer concentrations, ca. $\geq 10^{-2}$ wt% in polymer, the presence of this LCST is evident in cloud-point measurements (see, for example, Refs. [3–5] and references therein).

Winnik et al. [2,6–11] have used fluorescence spectroscopy to study the thermoresponsive characteristics of PNIPAM and certain hydrophobic modifications of the polymer in aqueous media. It was proposed, largely on the basis of energy-transfer measurements, that the mechanism of phase separation comprised two distinct steps. First, the polymer coil collapses into a shrunken state. This is then followed by aggregation of individual chains to produce the light-scattering conglomerates which are evident in cloud-point observations. Recently, we have confirmed [12] the occurrence of the first stage of this dual-step mechanism viz. that of coil collapse at the LCST, using time-resolved (fluorescence) anisotropy measurements (TRAMS) upon fluorescently labelled PNIPAM in ultra-dilute aqueous solutions (10^{-3} wt%) to minimize the occurrence of inter-polymer aggregation. (In a further study, we have confirmed that

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such ultra-dilute conditions are sufficient to ensure that aggregation between PNIPAM (and some of its hydrophobic modifications) is obviated [13].)

'Smart' thermoresponsive polymers, based upon *N*-isopropylacrylamide (NIPAM), are of obvious technological significance [14]. In this context, the capacity of the polymer to solubilize organic guest species above its LCST has attracted particular attention with a view to adopting PNIPAM as a 'carrier' species (above its LCST) which will allow (controlled) release at lower temperatures (see, for example, Ref. [3] and references therein).

In the current work, we have used the TRAMS approach to study the solubilization of pyrene (as a model, hydrophobic guest) in the individual collapsed PNIPAM coils which exist in ultra-dilute aqueous solutions, above the LCST of the polymeric host. In addition, the TRAMS technique has allowed examination of the interactions which occur between the macromolecular host and the hydrophobic solute at temperatures below the LCST of the polymer. The TRAMS experiments reveal that pyrene interacts (at a much reduced level compared to that above the LCST of the PNIPAM) with the polymer below its LCST. Furthermore, the TRAMS data show 'unusual' trends which can be rationalized in terms of the complex range of environments accessible by the organic guest within the 'collapsed' and 'open' forms of the polymer which exist, respectively, above and below its LCST.

2. Experimental

All materials were supplied by Aldrich, unless otherwise stated. NIPAM was used as received. Dioxane (99.9%; HPLC grade) was used as received. Diethylether (May and Barker) was fractionally distilled. Water was doubly distilled. Pyrene (99%) was purified by multiple recrystallization from ethanol (spectroscopic grade). NIPAM was polymerized, under high vacuum in dioxane, using AIBN as initiator. Polymerization was terminated at ca. 5% conversion to polymer. The polymer, PNIPAM, was purified by multiple reprecipitation from dioxane into diethylether. All spectroscopic samples contained 10^{-3} wt% polymer in an aqueous solution containing 10^{-6} M pyrene.

2.1. Characterization

Estimates of the molar mass of the labelled polymer were obtained by using the MALDI-TOF technique. These estimates were kindly performed by Thermobioanalysis Limited. Values of $\bar{M}_n = 40$K and $\bar{M}_w = 62$K were obtained for the PNIPAM sample. The molar mass was also estimated using a size-exclusion chromatography system comprising Waters Associates 510 pump, a Rheodyne injector and a PSS "HEMA" column, thermostatted at 35°C with a Hewlett–Packard HP 1047 A r.i. detector. The eluent was DMF, containing 0.1% ammonium acetate (flow rate 0.5 $cm^3\,min^{-1}$) and poly(ethylene glycol) standards were used to calibrate the columns. The values of $\bar{M}_n$ and $\bar{M}_w$, recovered by SEC, were concordant with those obtained by MALDI-TOF.

2.2. Instrumentation

Fluorescence decays were collected using time-correlated single photon counting (TCSPC) detection. Excitation was effected using the frequency-doubled output of a cavity-dumped picosecond dye laser (Spectra-Physics model 3500, running DCM) synchronously pumped by a mode-locked argon ion laser (Spectra-Physics model 2030). Fluorescence from the pyrene solute was excited at 338 nm and sampled (at 380 nm) in a conventional "right-angle geometry" with respect to the incident excitation. In anisotropy measurements, the fluorescence was collected in a computer-controlled "toggling procedure" in which the emission was sampled, sequentially, in planes parallel and perpendicular, respectively, to that of the (vertically) polarized excitation. Fluorescence was isolated via a holographic grating monochromator (Jobin-Yvon H-20) after being passed through a "scrambling plate" (Oriel) to minimize polarization bias of the monochromator. (The anisotropy data (see below) were subjected to a "*G* factor" correction [15] to compensate for any anomalies which persisted, following these precautions.) A Hamamatsu R1564U01 microchannel plate detector was used to sample the emission from the fluorescent probe. A Tracor-Northern NS-512 MCA was used to record the single photon fluorescence and excitation events. Details of the time-resolved spectrometer and its use for TRAMS have been described elsewhere [16].

2.3. Data analysis

Details of the rigorous and systematic procedures generally adopted in the analysis of TCSPC data have been described elsewhere. (An excellent account appears in Ref. [17]).

Experimentally observed intensities of fluorescence $I_{\parallel}(t)$ and $I_{\perp}(t)$, analysed in planes parallel and perpendicular to that of the vertically polarized excitation pulse of the laser (as described above) were transformed into a "raw" time-dependent anisotropy, $R(t)$ via Eq. (1),

$$R(t) = \frac{GI_{\parallel}(t) - I_{\perp}(t)}{GI_{\parallel}(t) + 2I_{\perp}(t)} = \frac{D(t)}{S(t)} \quad (1)$$

where $D(t)$ is the "difference function" which contains both fluorescence and (chromophore) reorientational information and $S(t)$ is the "sum function" which contains purely fluorescence information. Both $D(t)$ and $S(t)$ are distorted by the finite pulse width induced by the excitation source and the associated detection system. (This is a negligible effect in the current experiments.) G is a factor introduced [15] to compensate for the "polarization bias" which might be introduced by the photon detection system and instrumental geometry.

In the current experiments, G was estimated by the use of

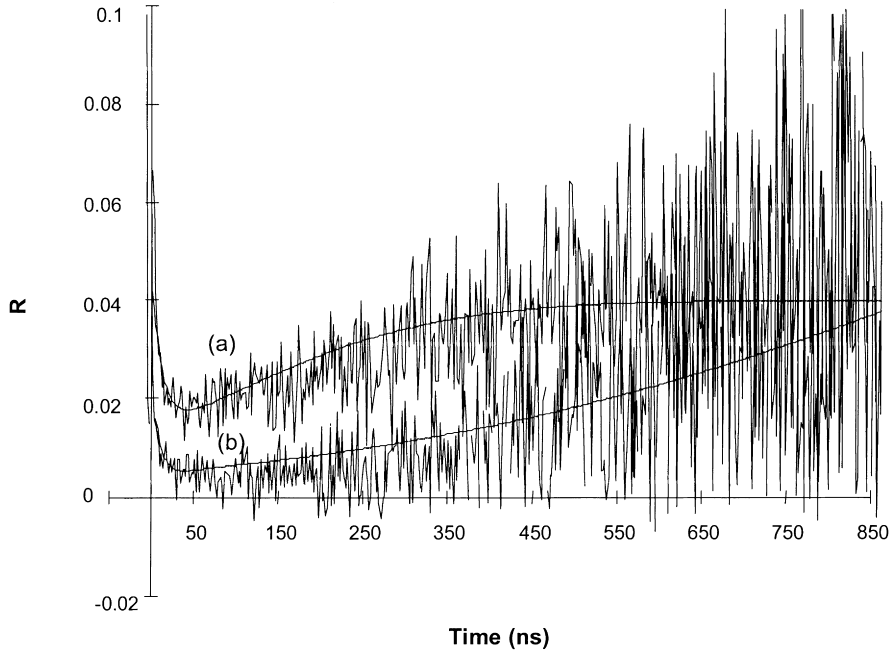

Fig. 1. Time-resolved fluorescence anisotropy behaviour of pyrene (10^{-6} M) dispersed in aqueous PNIPAM solution (10^{-3} wt%) at: (a) 310 K; and (b) 297 K with associated "best fit" curves based upon: (a) two-site; and (b) three-site models (see text), respectively.

"tail matching" of $I_{\|}(t)$ and $I_{\perp}(t)$ under conditions where rotation of the pyrene probe should be sufficiently fast to ensure complete loss of emission anisotropy (i.e. in the fluid environments afforded by pure aqueous pyrene solutions). A G factor of 0.981 was found to apply in these experiments (where $G = I_{\perp}(t)/I_{\|}(t)$).

In analysis of the TCSPC data, functions representative of the "expected" forms of the true anisotropy, $r(t)$, and the total fluorescence ("pure sum function") $s(t)$, were chosen to model $R(t)$ and $S(t)$, as described below.

3. Results and discussion

Fig. 1(a) shows the time-resolved anisotropy, $R(t)$, obtained upon analysis of the fluorescence from a 10^{-6} M dispersion of pyrene within an aqueous solution of PNIPAM (10^{-3} wt%) at 37°C (i.e. above the LCST of the polymeric host).

The data are 'unusual': in a relatively homogeneous dispersion of fluorophores in a fluid solvent matrix, $R(t)$ would be expected to decay in a continuous fashion (with a complexity dictated by the distribution of environments presented to the solute probe) from an initial value, R_0, determined by the point, in time, from which analysis is initiated, to zero anisotropy, at long times. In contrast, the current data exhibit an initial, rapid decay to a *finite* minimum in $R(t)$, followed by a gradual increase in the polarization of the emission from the pyrene probe.

The time-resolved fluorescence behaviour of a chromophore dispersed (either as a molecular solute or as a label, covalently bound to a macromolecular solute) in an aqueous polymer solution, tends to be complex. This results from the heterogeneous variety of environments which are experienced by the fluorescent species. (This is discussed, to some extent, in Ref. [18] and references therein.). To a degree, this can be rationalized in terms of two extreme conditions in which the luminescent 'reporter' molecule is located within the bulk aqueous phase and the hydrophobic domains afforded by the 'contracted conformation' of a water-soluble polymer, respectively [19–21]. This interpretation, as we have observed previously [18,22], should not be extended too far, but proves convenient in the present case, in seeking to obtain a *qualitative* explanation for the TRAMS behaviour observed above the LCST and exemplified in Fig. 1.

Modelling the time-resolved fluorescence of pyrene, dispersed at 10^{-6} M both in aqueous solution and in 10^{-3} wt% solutions of PNIPAM, required the use of three exponential terms to achieve 'statistically justified' fits to the observed decay profiles. 'Force-fitting' to a dual exponential decay function (although inadequate upon strict statistical criteria) afforded fluorescence lifetimes of ca. 82 and 218 ns for pyrene dispersed in a solution of PNIPAM at 37°C (i.e. above its LCST). Within the (considerable) limitations of a "dual site" model for the distribution of pyrene excited states, the former value can be loosely associated with pyrenes located within the bulk aqueous phase. The longer-lived species are therefore representative, under this crude analysis, with excited states of the solute/probe solubilized within the intramolecular hydrophobic domains created within the PNIPAM above its LCST. Within such a simplistic framework, the existence of a fluorescence component leading to enhanced anisotropy at longer times

of analysis, in TRAMS, can be rationalized, as discussed below.

If a fluorescent probe were to be partitioned between two environments of markedly differing microviscosity in which its excited state lifetime remained constant, its anisotropy would display a dual exponential decay law, described by Eq. (2).

$$r(t) = a_1 \exp(-t/\tau_{c1}) + a_2 \exp(-t/\tau_{c2}) \quad (2)$$

τ_{c1} and τ_{c2} would represent the correlation times characteristic of rotational relaxation of the probe within the two distinct environments. The intrinsic anisotropy, r_0, would be given by

$$r_0 = a_1 + a_2 \quad (3)$$

where $a_i = f_i r_0$, f_i being the fraction of the total fluorescence emitted by the probe located in a given environment, provided r_0, itself, did not vary with environment. If, as in the present case, the excited state lifetimes, τ_{fi}, of the probe, also depend upon the probe's microenvironment, the time-dependent anisotropy of its fluorescence would reflect this additional complexity and would be described [23] by Eq. (4).

$$r(t) = \frac{b_1 \exp(-t/\tau_{f1}) r_0 \exp(-t/\tau_{c1}) + b_2 \exp(-t/\tau_{f2}) r_0 \exp(-t/\tau_{c2})}{b_1 \exp(-t/\tau_{f1}) + b_2 \exp(-t/\tau_{f2})} \quad (4)$$

b_1 and b_2 are the pre-exponential terms which would characterize the dual exponential decay of the total fluorescence emitted by the probe, $s(t)$ (corresponding to the denominator in Eq. (4)). Again it is assumed that r_0 does not vary according to the nature of the probe's microenvironment.

Eq. (4) can be simplified to Eq. (5),

$$r(t) = c_1 \exp(-t/\tau_1) + c_2 \exp(-t/\tau_2) \quad (5)$$

in which $c_i = f_i(t) r_0$ and $\tau_i^{-1} = \tau_{fi}^{-1} + \tau_{ci}^{-1}$. Clearly, the functional forms of $r(t)$ expressed in Eqs. (2) and (5) are similar. However, the time-dependence of the anisotropy is made much more complex by a dependence of the fluorophore's excited state lifetime upon the environment in which it is located:

1. the decay times, τ_1 and τ_2, implicated in Eq. (5) contain fluorescence information in addition to that of probe reorientation (characterized by τ_{c1} and τ_{c2}).
2. the fractional contributions, $f_1(t)$ and $f_2(t)$, to the overall time-dependence of $r(t)$ are themselves time-dependent. It is this latter observation that allows us to rationalize the time-dependent fluorescence anisotropy data presented (Fig. 1(a)) for pyrene solubilized in PNIPAM above its LCST.

If the fluorescence lifetime, τ_{fl}, of a probe molecule in a fluid environment, the motion of which is characterized by a short correlation time, τ_{c1}, is significantly reduced relative to that, τ_{f2}, exhibited by the probe in a medium of higher microviscosity (for which $\tau_{c2} \gg \tau_{c1}$) 'anomalous' anisotropy decays, of the type shown in Fig. 1, would be expected [16,23,24]. At short times of analysis, the time-dependence of the fluorescence anisotropy (cf. Eqs. (4) and (5)) would be dominated by the shorter-lived, and faster rotating, pyrene probes, located within the bulk aqueous phase and/or more polar regions of the water-permeated coils of the PNIPAM. At longer times, fluorescence from pyrene species solubilized within the highly viscous and 'protective' hydrophobic domains created within the 'collapsed' PNIPAM host, would dominate the observed anisotropy: $R(t)$ would increase at longer sampling times, as exhibited in Fig. 1(a).

Analysis of the anisotropy decay data shown in Fig. 1(a) using a functional form of the type represented by Eq. (2) was, not surprisingly, unsuccessful: meaningless values of τ_{c1} and τ_{c2} were obtained. In particular, a negative value was obtained for τ_{c2}. Consequently, a functional form of the type represented in Eqs. (4) and (5) was adopted to model $r(t)$. In order to help the fitting procedure to converge to a 'unique solution' (given the 'cross-correlation' which would exist in analyses in which all variables might be allowed free variance) values of b_1 and b_2 and τ_{f1} and τ_{f2} were 'fixed' to those resultant upon dual exponential modelling of the total fluorescence, $S(t)$, as described above. This resulted in a plausible fit to the anisotropy data, as shown in Fig. 1(a).

The analytical approach resulted in an estimate of τ_{c1} of ca. 12.5 ns. The second component of the overall decay of $R(t)$, characterized by τ_{c2} in Eqs. (4) and (5), could not be recovered, with precision. (This is due, in part, to the extremely slow rate at which guest molecules, such as pyrene, reorient within the hydrophobic host domains, created within the PNIPAM globule, relative to the timescale imposed by the pyrene probe's excited state (ca. 200 ns) in these domains.) It suffices to say that τ_{c2} is of the order of some tens of microseconds.

Direct analyses of $R(t)$ are always limited [25]. First, it is not possible to make allowance for the distorting influence exerted by the finite width of the excitation pulse upon the observed fluorescences, $I_{\parallel}(t)$ and $I_{\perp}(t)$. This, in turn, means that the intrinsic anisotropy, r_0, cannot be recovered, with confidence. Give the brevity of the excitation pulse delivered by the laser excitation source employed in these investigations, relative to the time ranges over which fluorescence from the pyrene guest was sampled, such considerations are of negligible importance in this instance.

The main limitation placed upon our analysis of the anisotropy of the pyrene's emission is the simplicity of the "dual site" model that we have (perforce) adopted. The two correlation times τ_{c1} and τ_{c2} which result from such modelling have limited physical significance. They do not characterize the tumbling of pyrene solutes located in two distinct environments such as "the aqueous phase" and "within the collapsed PNIPAM globule". The value of

τ_{c1}, for instance, is about two orders of magnitude greater than that which might be expected [26] for a molecule of pyrene's molar volume, in water. τ_{c1} and τ_{c2} serve merely, at best, to characterize the broad ranges of environments available to the pyrene in both the more polar regions and the largely hydrophobic domains, respectively, of the system.

The fact that τ_{c2} is of the order of tens of microseconds shows that there are some very slowly relaxing components within the pyrene's reorientational spectrum. Indeed given that τ_{c2} is at least associated with the presence of species in the long-time tail of the overall distribution of rotors, it is much longer than might have been anticipated. It is at least two orders of magnitude greater than the value of τ_c afforded in TRAMS estimates [12] of the rate of segmental relaxation within the PNIPAM globule.

Eq. (6), applicable to spherical rotors,

$$\tau_c = \frac{\eta V}{RT} \tag{6}$$

(where η = solvent viscosity, V = molar volume of a spherical rotor and T = temperature (K)) allows us to estimate the apparent dimensions of the tumbling moiety. A value of τ_{c2} between 10 and 50 μs would implicate a rotating particle of radius lying between 30 and 55 nm. Using dynamic laser light scattering, Wu and Zhou [27] have estimated the hydrodynamic radius of a single, kinetically stabilized PNIPAM globule (of $\bar{M}_w = 1.08 \times 10^7$ and low polydispersity (1.06)) as ca. 30 nm above the LCST. Our sample is polydisperse and of much lower molar mass than that of Wu and Zhou [27]. Nevertheless, given the naievety of the "dual-site" model adopted in characterizing "solubilized" vs "free" fluorescent probes our estimate of rotor dimension (and thence of τ_{c2}) might not appear unreasonable. Within such a framework, it would appear that large, hydrophobic solutes, such as pyrene, interact with the matrix constituting the interiors of the PNIPAM globule, creating a microenvironment of high local viscosity such that rotational motion of the probe, independent of the globule, is not possible.

Fig. 1(b) shows the time-resolved anisotropy, $R(t)$, obtained upon analysis of the fluorescence from a 10^{-6} M dispersion of pyrene within the PNIPAM solution (10^{-3} wt%) at 297 K (i.e. below the LCST of the system). The decay of fluorescence from the dispersed pyrene is complex. Dual exponential fitting to $S(t)$ (as dictated by the "two-microenvironments" model) resulted in estimates of ca. 28 and 135 ns, respectively, for the excited state lifetimes characterizing the distribution of the pyrene solute species in the aqueous dispersion. The latter value is close to that of ca. 130 ns obtained as the "average fluorescence lifetime" of pyrene, (10^{-6} M) in water, at this temperature [22]. The former lifetime of ca. 30 ns is frequently recovered [22,28] in multi-exponential analyses of aqueous dispersions containing pyrene at high dilutions. In the absence of fluorescence anisotropy data, these observations would be interpreted as indicating that the pyrene is located within the bulk aqueous phase, having been "ejected" from the coils of its polymeric host as they expand during the conformational change, which results upon cooling through the LCST. Such an interpretation would be reinforced by the fact that the vibronic structure of the probe's fluorescence spectrum (which is sensitive to the polarity of its microenvironment [29,30]) is very similar to that of pyrene in water [22,30]. However, the anisotropy data show that, at long sampling times, a slight increase in $R(t)$ is observed. This indicates that a small proportion of the pyrene probes exhibit a relatively long-lived fluorescence and that this population of excited-states is constrained in its rotational freedom relative to species dispersed in the bulk aqueous phase. Clearly, a small fraction of the pyrene guests remains associated with the polymer in the open coil conformation which it adopts below its LCST. This observation, based upon visual inspection of the TRAMS data, is supported by analysis of the anisotropy decay, as described below.

Below the LCST, the TRAMS data are even more complex than above it. $R(t)$ could not be modelled satisfactorily using Eq. 4. Adequate description of the time-profile of the anisotropy could be achieved only when a three-state model was adopted as the scheme of minimum complexity required for fitting the data. The resultant function is given in Eq. (7)

$$r(t) = \frac{r_0 \sum_{i=1}^{i=3} b_i \exp\left(\frac{-t}{\tau_i}\right)}{\sum_{i=1}^{i=3} b_i \exp\left(\frac{-t}{\tau_{fi}}\right)} \tag{7}$$

where $\tau_i^{-1} = \tau_{fi}^{-1} + \tau_{ci}^{-1}$. Values of the b_i and τ_{fi} terms were constrained, in the fitting procedure, to those obtained in modelling the fluorescence decay, $S(t)$, in order to aid convergence to a unique solution.

At 297 K, such three-component modelling produced τ_c values of <1 ns, 220 ns and >20 μs. These serve not only to parameterize the anisotropy decay but also demonstrate the broad range of environments inhabited by the excited state probes and show that a small, but finite, proportion of the overall pyrene population, reorients very slowly. These pyrenes are closely associated with the polymer, presumably through hydrophobic interaction, and their motion seems to reflect whole molecule tumbling of the PNIPAM in solution. Detection of the existence of these polymer–pyrene hydrophobic aggregates has been made possible only by the use of careful, high-resolution anisotropy measurements and by operating close to the solubility limit of pyrene (ca. 10^{-6} M) [31] in water at 297 K. (This maximizes the partitioning of pyrene as "polymer-associated" and "aqueously-dispersed" species, at a fixed, low concentration of polymer).

4. Conclusions

1. TRAMS have shown that, above its LCST, PNIPAM can

solubilize hydrophobic guests, such as pyrene, within its intramolecular microdomains. In addition, it has been shown that the microviscosity of the domains in which the (pyrene) guest is located is very high: the guest's mobility is determined largely by that of its macromolecular host, rotating as an entire globular entity.

2. Below PNIPAM's LCST, most of the solubilizing capacity of the polymer is lost. However, a small proportion of the overall population of an aromatic solute, such as pyrene, will interact closely with the polymer and the motion of such species will become associated with that of the polymeric host.
3. The "anomalous" TRAMS behaviour revealed in the fluorescence behaviour of pyrene solubilized in PNIPAM is probably part of a previously unrecognized, but general, phenomenon. Indeed, as previously observed [32,33], reports of "residual anisotropies" extant at long times in TRAMS investigations of fluorescently labelled water-soluble polymers, probably reflect the influence of this "partitioning effect" rather than exposing some less plausible feature of polymer dynamics.

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