# Fluorescent Labeling of a Bisurea-Based Supramolecular Polymer

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Ⓢ Supporting Information

ABSTRACT: Bisurea-based supramolecular polymer 2-ethyl-hexyl-3-[3-(3-(2-ethylhexyl)ureido)-4-methyl-phenyl]urea (EHUT) has been shown previously to self-assemble through hydrogen bonding into high-molecular-weight structures. The present publication reports the study of the thermodynamics of these tubular structures by time-resolved fluorescence spectroscopy, with the help of a tetrazine labeled monomer. Results of calorimetry and time-resolved fluorescence spectroscopy show that the as-modified monomer EHUTz does not interfere with the formation of the supramolecular assembly. When incorporated, these labeled monomers exhibit a longer fluorescence lifetime due to the electron-rich tolyl group buried in the structure. Dilution experiments allowed us to measure their partition coefficient, and to compare it with the critical aggregation concentration of EHUT. Measurements at higher dye loads, where interactions between neighboring tetrazines occur, show that EHUTz is uniformly dissolved in the supramolecular polymer. Tetrazine dye is a good reporter of events occurring in solution, such as disruption of the assembly upon heating. Our results confirm the pseudophase diagram for EHUT solution in toluene obtained previously with other techniques such as infrared spectroscopy and calorimetry.
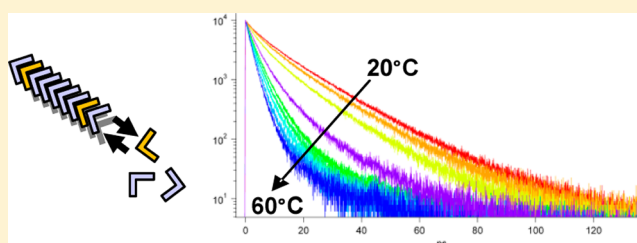
INTRODUCTION

Supramolecular polymers are linear chains of low molar mass monomers held together by reversible and directional non-covalent interactions.[1−6] In suitable experimental conditions, they can display polymer-like rheological or mechanical properties, because of their macromolecular architecture. However, because of the noncovalent nature of the interactions involved, the assemblies can be reversibly broken, which brings about additional features compared to usual polymers, and can potentially lead to new properties, such as improved processing, self-healing behavior, or stimuli responsiveness.

In particular, we have shown that the bisurea-based supramolecular polymer 2-ethylhexyl-3-[3-(3-(2-ethylhexyl)-ureido)-4-methyl-phenyl]urea (EHUT) forms interesting vis-coelastic gels,[7−10] due to the entanglement of long and rigid nanotubes at concentrations larger than 1 wt % in nonpolar solvents, or drag reducing agents at much lower concentrations.[11] These nanotubes are also useful precursors for microporous materials.[12] More recently, we have shown that these nanostructures can be used as a supramolecular platform to study weak interactions between the monomers[13] or between encapsulated solvent molecules.[14] Indeed, the cooperative nature of these nanostructures makes them sensitive to extremely weak perturbations, which allows measurement of the effect of interactions as low as 60 J/mol. In order to fully exploit this property, it is important to improve

our knowledge of the structure of these nanotubes. Up to now, they have been characterized by the combination of scattering (small-angle neutron scattering (SANS)) and spectroscopic techniques (Fourier transform infrared (FTIR), dielectric, circular dichroism (CD)).[10,15−18] Moreover, their thermodynamic stability has been investigated by isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC).[19,20] However, up to now their dynamics has not been satisfactorily understood.

Fluorescence techniques (time-resolved, anisotropy, fluorescence correlation spectroscopy, etc.) are ideally suited to studying the dynamics of supramolecular assemblies.[21] Therefore, we have started a project to study the dynamics of EHUT supramolecular polymer with the help of a suitably labeled fluorescent monomer. O-monosubstituted tetrazine is chosen as the labeling moiety because of its unusual spectroscopic properties: long lifetime (almost 160 ns in dichloromethane) and high luminescence quantum yield (38%). Moreover, the tetrazine luminescent lifetime can be modulated by interaction with aromatic groups such as the toluene moiety of EHUT. Thus, the lifetime can be a relevant parameter to probe the formation of supramolecular assemblies of EHUT. Before
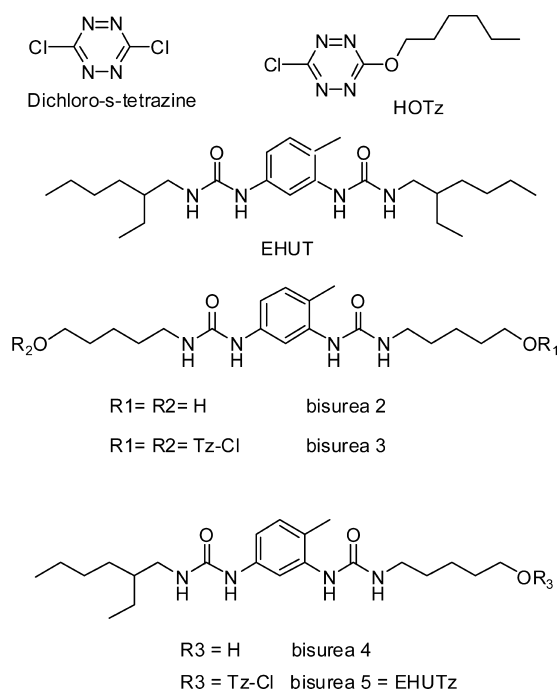
actually studying the dynamics of the assemblies, the present report focuses on the thermodynamics of the labeled monomer in order to assess the possible interference between the label and the supramolecular assembly. We show that the introduction of EHUT molecules labeled by tetrazine does not modify the structure of the supramolecular polymers, and that the probe does uniformly dissolve in the supramolecular polymer. When incorporated in the supramolecular polymer, the labeled molecules exhibit a longer fluorescence lifetime due to the electron-rich tolyl group buried in the structure. This allows us to measure their partition coefficient and to compare it with the critical aggregation concentration of EHUT.

■ RESULTS AND DISCUSSION

EHUT (see Scheme 1) has been shown previously to self-assemble through hydrogen bonding into high-molecular-weight structures, respectively thin filaments and tubes, depending on the experimental conditions.[10,15−17,20] The grafting of a dye to a bisurea monomer would allow the use of this labeled monomer as a fluorescent reporter of the supramolecular assembly, supposing that the physicochemical properties of the dye depend on its local environment.

Several conditions were required in the choice of an appropriate dye, in order to avoid any destabilization of the supramolecular assembly and thus any modification of the system which is being explored. First of all, the dye has to be small. Moreover, its functionalities have to be compatible with the presence of hydrogen bonds, as any competitive interaction between the dye and bisurea monomers would interfere with the formation of the assembly. Strong fluorescence properties (high fluorescent quantum yield) are also required, as it is preferable to use the dye at reduced concentrations in the supramolecular polymer.

Tetrazine dye (Scheme 1) was chosen because it fulfills all of these requirements.[22] The parent dichloro-s-tetrazine is one of the smallest organic fluorophores known with a relatively high quantum yield (14%) and a long fluorescent decay time (about 58 ns in dichloromethane).[23] Tetrazine molecules are fluorescent and electroactive molecules that may be quenched within a given time scale; they are thus potentially good reporters of any changes in their local environment. These properties have already led to a successful incorporation of the dye in spectroelectroactive polymers,[24−26] and its potential use as a probe of electron-rich rings has been investigated.[27]

Synthesis. The fluorescence of heteroatom-substituted tetrazines has already been studied:[28] whereas the N-substituted tetrazines or sulfur-substituted tetrazines[29] have only poor quantum yields, O-monosubstituted tetrazines are strongly fluorescent,[27,30] with an even higher quantum yield and a longer fluorescence decay time than the dichlorotetrazine (respectively 38% and about 160 ns for the 1-methoxy-4chloroterazine). The grafting of the dichloro-s-tetrazine dye on a bisurea via nucleophilic substitution on a pendant alcohol group at one end was thus envisaged.

The dichloro-s-tetrazine was synthesized according to a procedure described by Hiskey et al.[31,32] We first envisaged the synthesis in two steps: the grafting of the dichlorotetrazine on the alcohol group of 2-aminobutanol, with its amino group protected by a carbamate, and then coupling with toluenediisocyanate after deprotection (Scheme 2, pathway A). However,

Scheme 1. Structures of Compounds Synthesized for This Study

Scheme 2. Synthesis of Labeled Monomer
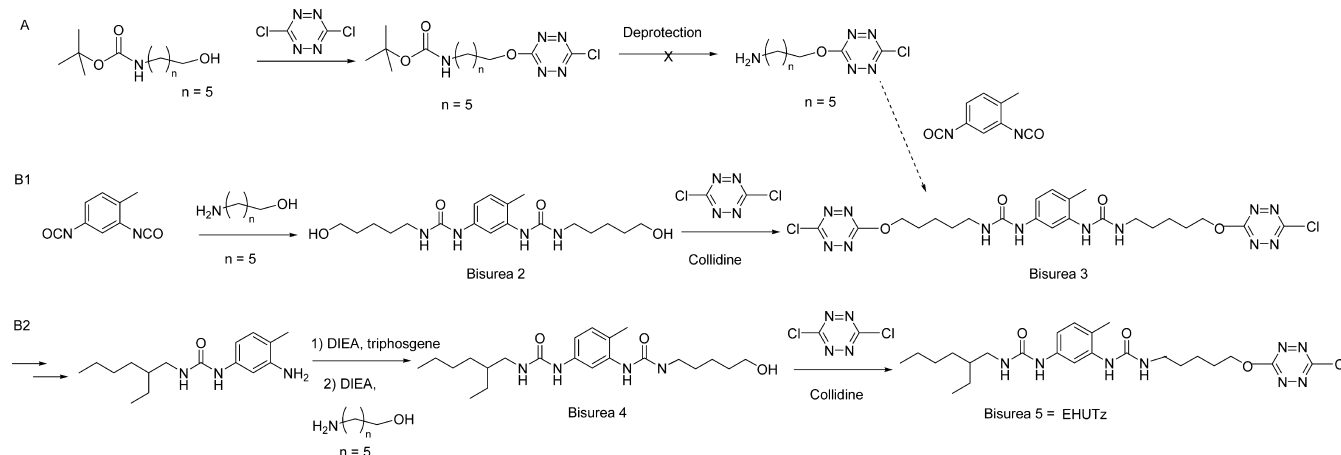
the deprotection of the amino group in acidic medium resulted in the degradation of tetrazine, leading to nonfluorescent adducts. As tetrazine is also sensitive to basic conditions and hydrogenation, we did not envisage another deprotection method or any other protective group. We thus chose to perform the grafting of dichlorotetrazine directly on bisurea **2** (Scheme 1), bearing an alcohol group at both ends of its two hexyl pendant arms. The coupling between the bisurea and the dye was then performed by nucleophilic substitution in the presence of an organic base, collidine (Scheme 2, pathway B1). The reaction occurs with low yields (21% for the disubstituted bisurea **3** and 23% for the monosubstituted derivative), but other attempts in changing the nature of the base (stronger or inorganic base) resulted in higher quantities of byproducts, probably coming from side reactions involving the urea groups.

Unfortunately, both the mono- and disubstituted bisureas were found to be insoluble in toluene or other solvents in which the EHUT supramolecular assembly is observed, certainly due to the too-high polarity of the alcohol or ether moiety and to the symmetry of the molecule. In order to improve the solubility, we synthesized the nonsymmetric bisurea **4** (Scheme 1), bearing only one alcohol group, in a four-step reaction, and then we performed the grafting of the tetrazine (Scheme 2, pathway B2). The solubility of the corresponding tetrazine-substituted bisurea **5** (EHUTz), obtained with 40% yield, was found to be improved compared to the previous compounds.

Finally, a model compound, i.e., hexyloxytetrazine (HOTz, see Scheme 1) was also synthesized, starting from hexanol and using the same protocol. Its structure mimics the structure of the dye grafted to a bisurea, and this compound was used for blank experiments in order to check that the presence of the tetrazine does not modify, or at least does not inhibit, the formation of the supramolecular assembly.

Preliminary Experiments. After controlling the static fluorescence behavior of HOTz (see Supporting Information), we then performed the blank dynamic experiments (Table 1,

Table 1. Decay Constants (τ), Relative Pre-exponential Factors (A), and χ^2 Parameter for the Fits of the Fluorescence Decays of 10^{-4} mol·L^{-1} HOTz and 10^{-4} mol·L^{-1} HOTz + 10^{-2} mol·L^{-1} EHUT, in Toluene ($t° = 20$ °C)

	τ (ns)	A (%)	χ^2
HOTz	71	100	1.01
	70[a]	100	1.05
HOTz + EHUT	69/4.9	72/28	1.04
	70[a]/5.3	72/28	1.06

[a]Constrained value.

first row). The value of 70 ns for HOTz decay in toluene is shorter than the time constant of other O-monosubstituted tetrazines already reported in the literature, although these values were measured in other solvents.[27] We thus checked, for both O-monosubstituted tetrazine and dichloro-s-tetrazine, if the solvent could have any effect on fluorescence decay time constants. We found that the time constants of dynamic fluorescence spectroscopy of tetrazine were deeply dependent on the solvent used (Table S1 in the Supporting Information): from 58 ns in dichloromethane to 0.83 ns in toluene for the dichlorotetrazine. For the O-monosubstituted tetrazine, the change is also important, from 160 ns in dichloromethane, to

70 ns in toluene. It can be suggested that the redox potential of the solvent influences the fluorescence time constant of the tetrazine (see the Supporting Information). This photoreactivity of tetrazine with aromatics has been used for the detection of benzene vapor.[33]

HOTz with or without EHUT has the same maximum of absorption (523 nm) and emission fluorescence spectra (563 nm, see Supporting Information). The fluorescence of HOTz was then studied by time-resolved fluorescence spectroscopy in the presence and absence of EHUT. Tetrazine dye keeps its fluorescence spectra, but the decays are not identical. In the presence of EHUT, the monoexponential behavior is lost for a biexponential one. The 70 ns major component (72%) can be attributed to the characteristic fluorescence decay time of HOTz at 10^{-4} M alone in toluene (70 ns at 20 °C). The second decay time observed is shorter (about 5 ns) and contributes 28% of the decay. It can be attributed to HOTz interacting with EHUT molecules (see Table 1, second row). This interaction might be due to photoinduced electron transfer (PET) between the toluene moiety in EHUT and the excited state of tetrazine (Table S2 in the Supporting Information). This seems to indicate that HOTz partitions between the supramolecular assemblies and the solvent.

Therefore, various quantities of HOTz were added to an EHUT solution in toluene, in order to check that the tetrazine dye does not destabilize the assembly. The viscosities of the solutions remained qualitatively unchanged in the presence of the additive. Nano-DSC experiments confirmed that even large amounts of tetrazine (ratio HOTz/EHUT > 1) did not modify the transition temperature from tube to filament, i.e., T^{**}, around 42 °C (Table 2). These results show that the presence of the tetrazine dye is compatible with the supramolecular assembly.

Table 2. T^{**} Values for 9.7×10^{-3} mol·L^{-1} EHUT Solution in Toluene, with Different Amounts of HOTz

[HOTz] ($\times 10^3$ mol)	T^{**} (°C)	ΔH (kJ·mol^{-1})
−	42.9	4.4
10.1	42.2	4.3
19.9	42.1	4.3

After having shown the compatibility between EHUT supramolecular assembly and HOTz, and the possible influence of bisurea monomers on the fluorescence behavior of the dye, we studied the incorporation of EHUTz labeled bisurea into the supramolecular assembly.

Incorporation of the Labeled Monomer in the Assembly. The solubility of EHUTz is reduced compared to that of EHUT monomer in solvents in which supramolecular structures are observed. For example, after heating at 80 °C a 10^{-2} M EHUTz solution in toluene, a viscous solution is obtained when going back to room temperature without stirring. However, after a few minutes, a precipitate is formed and the solution loses its viscosity. This suggests a competition between a kinetic supramolecular assembly and a thermodynamic solid. EHUTz monomer alone is thus clearly not able to form stable high-molecular-weight structures; however, it can be cosolubilized with EHUT.

Nano-DSC studies show that T^{**} is unchanged for EHUT solutions containing EHUTz (EHUTz = 2×10^{-6}–5×10^{-4} mol·L^{-1}, with a constant global concentration of monomers at 10^{-3} mol·L^{-1}). This confirms that the modified monomer does

not inhibit the assembly of EHUT monomers (see Supporting Information). However, when the amount of EHUTz increases, the endothermic peak at $T^{**} = 40$ °C broadens and a new peak appears at lower temperature (around 15 °C). The broadening indicates that EHUTz is at least partly incorporated into the supramolecular assembly, and modifies slightly its stability. The presence of a new small peak at lower temperature indicates that the situation is more complex below 15 °C, with a possible segregation of EHUTz.

Fluorescence Characterization of the Assembly. As will be developed in this section, fluorescence experiments allowed us to demonstrate that (i) the EHUTz monomer is homogeneously incorporated in the assembly and (ii) the fluorescence properties of the monomer report changes of the assembly, for example, when the assembly is disrupted upon heating.

Modification of the Fluorescence Properties upon Incorporation into the Assembly. The absorption spectrum of the EHUTz monomer is not influenced by the incorporation into the supramolecular assembly: $\lambda_{max} = 327$ and 523 nm and $\varepsilon = 570$ L·mol^{-1}·cm^{-1} are unchanged for EHUTz alone or in the presence of increasing concentrations of EHUT (see the Supporting Information for the spectra). Such characteristics are moreover similar to those of chloroalkoxytetrazines previously studied. Stationary fluorescence experiments show that the maximum of emission of EHUTz ($\lambda_{max} = 563$ nm) is identical for EHUTz with or without EHUT, but the fluorescence intensity is higher when EHUT concentration is increasing, i.e., when EHUTz is incorporated into the supramolecular structure. This would suggest a "protective" effect of the assembly on the tetrazine dye.

In order to understand the influence of the incorporation into the supramolecular assembly on the fluorescence of the dye, time-resolved fluorescence experiments were then realized. Several mixtures of EHUTz/EHUT were prepared: increasing concentrations of EHUT were added to diluted solutions of EHUTz in toluene, at two constant concentrations of 2×10^{-6} and 10^{-4} mol·L^{-1}. The concentration of EHUTz at 10^{-6} mol·L^{-1} was chosen as low as possible in order to avoid precipitation of the labeled monomer, and the higher one was chosen with the aim to explore interprobe interactions in the supramolecular structure. The fluorescence decay curves were globally fitted with a sum of three exponentials of constrained lifetimes (Figure 1, Table 3). In all cases, an exponential with a long time constant (about 50 ns) is observed. The relative importance of this term never exceeded 2%. It was then attributed to impurities (we observed that tetrazine-containing solutions degrade slowly with time) and its relative importance has been omitted in the following tables for clarity. The two other exponentials are much more meaningful, and the ratio of their contributions varies considerably with the total amount of monomer.

The fluorescence decay of EHUTz alone at 2×10^{-6} mol·L^{-1} fits to a monoexponential, with a characteristic time (4.7 ns; Table 3, first row) much shorter than that for HOTz in the same conditions (70 ns). This short decay time is very similar to the one obtained in preliminary experiments, for HOTz in the presence of EHUT (4.9 ns). This result suggests that intramolecular interaction in the O-monosubstituted tetrazine occurs in EHUTz and leads to fast extinction of the fluorescence of the dye.

For the next mixture, EHUTz in the presence of 10^{-5} mol·L^{-1} EHUT (Table 3, second row), no supramolecular
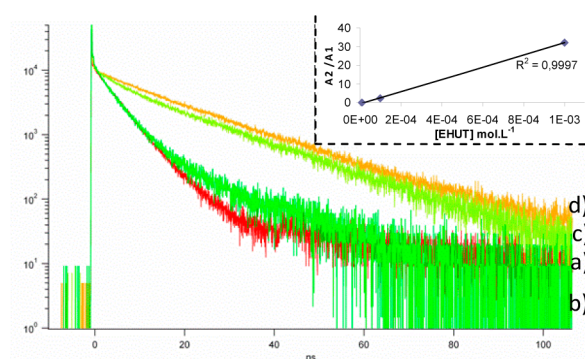
Figure 1. Fluorescence decays of different solutions in toluene of EHUTz/EHUT at room temperature. [EHUTz] = 2×10^{-6} mol·L^{-1} (a, red) without EHUT or with (b, green) [EHUT] = 10^{-5} mol·L^{-1}, (c, yellow) [EHUT] = 10^{-4} mol·L^{-1}, and (d, orange) [EHUT] = 10^{-3} mol·L^{-1}. Inset: Ratio of the pre-exponential factors deduced from the fit of the decays (see Table 2) for increasing amounts of EHUT.

Table 3. Relative Pre-exponential Factors for Triexponential Fits of the Fluorescence Decays of EHUTz/EHUT Solutions in Toluene Based on Constrained Decay Constants of 4.7, 17, and 52 ns

EHUTz/EHUT (mol·L^{-1})	A_1 (%) (4.7 ns)	A_2 (%) (17 ns)	χ^2
2×10^{-6}/–	98	2	0.97
2×10^{-6}/10^{-5}	95	5	1.29
2×10^{-6}/10^{-4}	30	70	1.08
2×10^{-6}/10^{-3}	4	96	0.95

assembly is expected in solution, according to the EHUT phase diagram.[20] In fact, one characteristic decay time is obtained, and the decay fits to a monoexponential and has a value similar to that with EHUTz alone (4.7 ns). In the presence of higher amounts of EHUT (higher than 10^{-5} mol·L^{-1}; Table 3, third and fourth rows), a second characteristic decay time (17 ns) becomes significant, and its contribution becomes very important (96%) for 10^{-3} mol·L^{-1} EHUT. This new decay time can be attributed to EHUTz incorporated into the supramolecular assembly. Longer decay time (17 ns compared to 4.7 ns) may come from a loss of intramolecular interactions (in EHUTz) because of the incorporation of the dye into the assembly.

The lifetimes measured for EHUTz in solution (4.7 ns) and dispersed in the tubes (17 ns) are different. The slow decay obtained for EHUTz incorporated into the assembly shows, as was observed for the stationary experiments, a protective effect of the assembly on the fluorescence of the dye. If one considers, as we suggested before, that the interaction between the bisurea and the O-substituted tetrazine is responsible for the quenching of the dye fluorescence, then this result could be explained by a reduced mobility, and thus very low interactions allowed in the supramolecular assembly, because of steric hindrance (see Figure 2).

According to our interpretation, the pre-exponential factors in the decay decomposition are respectively proportional to the amount of EHUTz molecules free in solution (EHUTz$_f$) and in the supramolecular assembly (EHUTz$_a$). The ratio of these pre-exponential factors is thus related to the partition coefficient of EHUTz (K_{EHUTz}) as a monomer between the two phases. Let us express this partition coefficient as a function of the "volume" of the aggregated phase. Using the infinite dilution as the reference state for the free phase and the pure compound
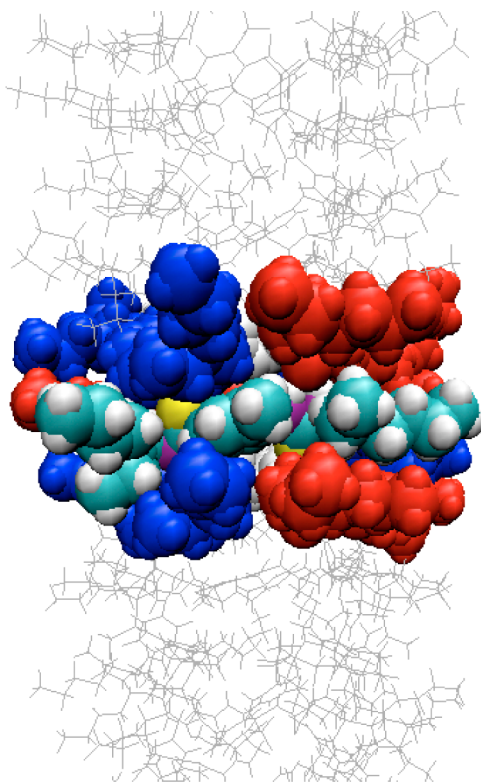
Figure 2. Modeling of EHUT supramolecular assembly, "tube" structure.[10,18] For the molecule in the center of the figure, the following color code is used: H, white; C, blue; N, pink; O, yellow. The tolyl spacer (between the two urea groups) is tightly packed in the structure.

limit for the aggregated phase, we can express the chemical potential of EHUTz in both phases as

$$\mu_{\text{EHUTz}_f} = \mu^0_{\text{EHUTz}_f} + RT \ln([\text{EHUTz}_f]/C^0)$$

$$\mu_{\text{EHUTz}_a} = \mu^0_{\text{EHUTz}_a} + RT \ln(X_{\text{EHUTz}_a})$$

where $[\text{EHUTz}_f]$ is the concentration of EHUTz in the solution. C^0 is the conventional reference concentration for infinite dilution. X_{EHUTz_a} is the molar fraction of EHUTz in the aggregated phase.

We can express the partition coefficient K_{EHUTz} as a function of the concentration $[\text{EHUTz}_f]$:

$$\text{EHUTz}_f \overset{K_{\text{EHUTz}}}{\rightleftarrows} \text{EHUTz}_a$$

$$K_{\text{EHUTz}} = \frac{X_{\text{EHUTz}_a}}{[\text{EHUTz}_f]/C^0}$$

In order to get the dependence as a function of [EHUT], the total concentration of EHUT in the samples, we make the following approximations: $n_{\text{EHUTz}_a} \ll n_{\text{EHUT}_a}$ and $n_{\text{EHUTf}} \ll n_{\text{EHUT}}$, where n_{EHUT_a} and n_{EHUT_f}, n_{EHUTz_a}, and n_{EHUTz_f} are the numbers of moles of EHUT and EHUTz respectively in the aggregated and free phases.

We can thus approximate

$$X_{\text{EHUTz}_a} = \frac{n_{\text{EHUTz}_a}}{n_{\text{EHUTz}_a} + n_{\text{EHUT}_a}} \approx \frac{n_{\text{EHUTz}_a}}{n_{\text{EHUT}_a} + n_{\text{EHUT}_f}}$$

and

$$K_{\text{EHUTz}} = \frac{X_{\text{EHUTz}_a}}{[\text{EHUTz}_f]/C^0} = \frac{n_{\text{EHUTz}_a}}{n_{\text{EHUTz}_f}} \frac{C^0}{[\text{EHUT}]}$$

Finally, the ratio of the pre-exponential factors is given by

$$\frac{A_2}{A_1} = \frac{n_{\text{EHUTz}_a}}{n_{\text{EHUTz}_f}} = [\text{EHUT}]\tilde{K}_{\text{EHUTz}}$$

where

$$\tilde{K}_{\text{EHUTz}} = \frac{K_{\text{EHUTz}}}{C^0}$$

This ratio is plotted as a function of the EHUT concentration (Figure 1, inset). From the plot, a partition coefficient of approximately 30 000 per mol·L^{-1} EHUT can be deduced.

This partition coefficient of labeled EHUTz can be compared to that of unlabeled EHUT (K_{EHUT}) between free (EHUT$_f$) and aggregated phases (EHUT$_a$).

$$\text{EHUT}_f \overset{K_{\text{EHUT}}}{\rightleftarrows} \text{EHUT}_a$$
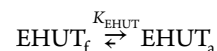
$$K_{\text{EHUT}} = C^1/\text{CAC}$$

where CAC stands for the critical aggregation concentration. C^1 is taken equal to 1 mol·L^{-1}.

Both equilibria are governed by the chemical potentials of the molecules in both phases. Let us use the infinite dilution as the reference state for the free phase and the pure compound limit for the aggregated phase. Both equilibria imply the equality of the chemical potentials:

for EHUTz

$$\mu^0_{\text{EHUTz}_a} + RT \ln(X_{\text{EHUTz}_a})$$
$$= \mu^0_{\text{EHUTz}_f} + RT \ln([\text{EHUTz}_f]/C^0)$$

$$RT \ln(K_{\text{EHUTz}}) = \mu^0_{\text{EHUTz}_f} - \mu^0_{\text{EHUTz}_a}$$

for EHUT

$$\mu^0_{\text{EHUT}_a} + RT \ln(1) = \mu^0_{\text{EHUT}_f} + RT \ln(\text{CAC}/C^1)$$

$$RT \ln(\text{CAC}/C^1) = \mu^0_{\text{EHUT}_a} - \mu^0_{\text{EHUT}_f}$$

Thus $K_{\text{EHUTz}} = C^1/\text{CAC} = K_{\text{EHUT}}$. The partition coefficient of the labeled molecule equals that of the neat molecule that is given by the CAC.

The critical aggregation concentration (CAC) of EHUT (about 2×10^{-5} mol·L^{-1} at room temperature)[20] is close but inferior to the inverse of the partition coefficient of EHUTz ($1/\tilde{K}_{\text{EHUTz}} \approx 3 \times 10^{-5}$ mol·L^{-1}) found in this experiment. This confirms that the presence of the tetrazine labels slightly destabilizes the supramolecular assembly.

In order to study the interactions between two tetrazine dyes in the assembly, the measurements were repeated with a higher constant concentration of EHUTz, at 10^{-4} mol·L^{-1} (Figure 3, Table 4).

First, whereas EHUTz alone at 2×10^{-6} mol·L^{-1} fits to a monoexponential, at 10^{-4} mol·L^{-1} the solution presents a mixture of dissolved and precipitated phases, and indeed decays can be fitted by a multiexponential function (Table 4, first row). As it was done above, the fluorescent decays of EHUTz/EHUT mixtures were fitted as the sum of three exponentials: a long
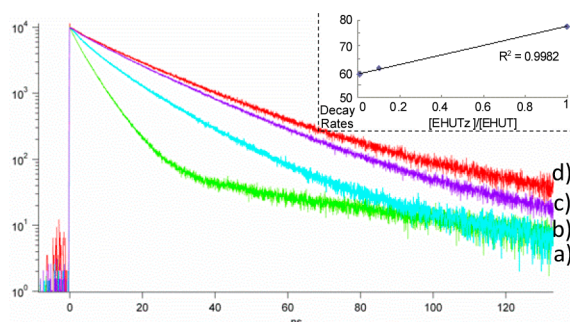
Figure 3. Fluorescence decays of different toluene solutions of EHUTz/EHUT at room temperature. [EHUTz] = 10^{-4} mol·L^{-1} (a, green) without EHUT or with (b, blue) [EHUT] = 10^{-4} mol·L^{-1}, (c, purple) [EHUT] = 10^{-3} mol·L^{-1}, and (d, red) [EHUT] = 10^{-2} mol·L^{-1}. Inset: Decay rates ($\times 10^{-6}$ s^{-1}) as a function of the molar fraction ([EHUTz]/[EHUT]).

Table 4. Relative Pre-exponential Factors for Triexponential Fits of the Fluorescence Decays of EHUT/EHUTz Solutions in Toluene Based on Two Constrained Decay Constants of 4.7 and 52 ns and One Free

EHUTz/EHUT (mol·L^{-1})	A_1 (%)	A_2 (%)	χ^2
10^{-4}/−	−	−	−
10^{-4}/10^{-4}	43 (4.7 ns)	55 (12.9 ns)	1.15
10^{-4}/10^{-3}	14 (4.7 ns)	83 (16.3 ns)	1.10
10^{-4}/10^{-2}	11 (4.7 ns)	84 (17.0 ns)	1.03

time constant (52 ns) of minor intensity attributed to impurities and an intermediate time constant (13−17 ns) and a short time constant (4.7 ns) characteristic of free EHUTz (Table 4). At a ratio of EHUTz/EHUT equal to 1%, one expects the presence of EHUTz dispersed within the EHUT assemblies, with no interaction possible between EHUTz molecules. Indeed, the value for the intermediate time constant (17 ns) is the same as in the previous experiment (Table 4, fourth row). Furthermore, it can be observed that decreasing the amount of EHUT monomer favors the fast component (4.7 ns) compared to the intermediate component (13−17 ns). This is again interpreted as the progressive release of the labeled monomer from the assembly. When the molar fraction of EHUTz reaches 10% or higher proportions, the lifetime of the intermediate component becomes shorter: this is an indication that interactions between the dyes are possible (Table 4, second and third rows). The interactions between neighboring tetrazines in the assembly must thus be considered in order to account for these results. Indeed, studies on tetrazine crystals show that tetrazine reacts in its excited state with neighboring tetrazine leading to a reduction of the fluorescence lifetime (20 ns).[27] Therefore, in mixtures of EHUTz/EHUT with high concentration of EHUTz, one can expect interactions between the dyes in the assembly, which would in a similar manner induce a diminution of the time decay constant.

The model we chose to apply to describe such interactions, called the "Poisson model", has already been developed and applied for the study of fluorescence quenching of an excited probe in aqueous micelles.[34] It considers the presence of several types of neighboring bisureas, depending on whether they carry the dye. A probability study reveals a perturbation of the exponential term by two factors: the amount of dyes in the neighborhood (C), directly linked to the molecular ratio of labeled monomer, and the subsequent quenching constant of

the fluorophores ($1/t_C$). The fluorescence decay can then be written as

$$F(t) = A_1 \exp(-t/\tau_1) + A_2$$
$$\exp(-t/\tau_2 - C(1 - \exp(-t/t_C))) + A_3$$
$$\exp(-t/\tau_3) \tag{1}$$

where τ_1 is the fluorescence lifetime of free fluorophores, τ_2 is the fluorescence lifetime of the incorporated dyes, and τ_3 is the fluorescence lifetime of impurity.

The observation time t is limited by the fluorescence lifetime (τ_2 = 17 ns), which appears to be small compared to t_C (quenching time). In the Taylor series at the first order in t/t_C, the Poisson distribution simplifies to

$$F(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2 - Dt) + A_3$$
$$\exp(-t/\tau_3) \tag{2}$$

The influence of the two parameters C and t_C is described by their product ($D = C/t_C$). This model can be used in our case and provides the following results (Table 5).

Table 5. Relative Pre-exponential Factors and First Order Poisson Parameter of the Fluorescence Decays of EHUTz/EHUT Solutions in Toluene, Based on Constrained Decay Constants of 4.7, 17, and 52 ns

EHUTz/EHUT (mol·L^{-1})	A_1 (%) (4.7 ns)	A_2 (%) (17 ns)	D (s^{-1})	χ^2
10^{-4}/10^{-4}	43	56	1.9×10^7	1.12
10^{-4}/10^{-3}	15	82	2.2×10^6	1.09
10^{-4}/10^{-2}	11	84	0	1.03

The χ^2 values obtained are satisfying. The Poisson model fits well for these curves and allows us to constrain time constants. Tables 4 and 5 show that the two models give similar values and similar fit quality, but the Poisson model gives a more rich interpretation. If one considers the ratio EHUTz/EHUT 1:1 (Table 5, first row), at 10^{-4} mol·L^{-1}, in this mixture the tubular supramolecular assembly is formed. Each monomer has several neighbors below, above, and in the same plane (see Figure 2). A minimum value for the number of interacting neighbors C for EHUTz is thus 2. With D equal to 1.9×10^7 s^{-1} in this case, it gives a minimum value of 100 ns for t_C. t_C is indeed larger than the fluorescence time t, which is the condition that must be fulfilled to make the simplification (eq 2). For the next ratio (Table 5, second row), 10^{-4} mol·L^{-1} EHUTz/10^{-3} mol·L^{-1} EHUT, the number of neighbors C should be 10 times less, and t_C is not supposed to vary. This is in agreement with the observed value of $D = C/t_C$, which is approximately 10 times smaller (2.2×10^6 s^{-1}). This shows that the approximation made for the Poisson model is coherent and that the labeled molecules are uniformly distributed in the supramolecular structure. The values of 17 ns for the incorporated dye and 4.7 ns for the free dye, which were determined at small dilutions, are confirmed in this experiment.

Fluorescence as a Good Reporter of Supramolecular Events. The stability of the supramolecular assembly is a function of the temperature, and the tubular structure, even at high concentration in monomer, is known to be unstable for temperatures above 45 °C. In order to show if the labeled monomer can be a good reporter of the events occurring in solution, fluorescent decays of a solution of EHUT/EHUTz

$(10^{-4}$ mol·L^{-1}/10^{-4} mol·L^{-1}) were recorded every 5 °C between room temperature and 60 °C (Figure 4, Table 6).
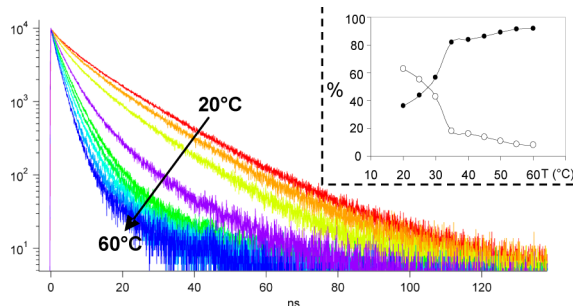
Figure 4. Fluorescence decays of a solution of EHUTz/EHUT $(10^{-4}$ M/10^{-4} M) in toluene at different temperatures from 20 (red) to 60 °C (blue), every 5 °C. Inset: Percentages of the two different decay constants (● = τ_1, fast component; ○ = τ_2, slow component) for the fits of the fluorescence decays.

Visually, a jump occurs between 30 and 40 °C and divides the results into two groups of fast and slow decays. To fit these curves, the Poisson model was used. The Poisson term D was constrained at the value of 1.9×10^7 ns^{-1}, which was previously established (Table 5).

The two main components of the decay can be assigned to the two possible environments of EHUTz, depending on whether the labeled monomer is incorporated in the assembly or free in solution. At high temperature, as the assembly is destabilized, the faster component (τ_1) becomes preponderant. The observed change in the percentages of the two populations (Figure 4, inset) between 30 and 35 °C is consistent with $T**$ observed by nano-DSC ($T** = 35$ °C for EHUT at 2×10^{-4} mol·L^{-1}).

As they depend on the temperature, we draw the Arrhenius plots for the fast and slow decay constants, $\ln(\tau) = f(1/T)$ (Figure 5). As linear relations are obtained, this shows that our system follows the Arrhenius law.

It can be first observed from these curves that the rate of the slow component exhibits a small decrease between 35 and 40 °C. We established that the slow component corresponds to the signature of the supramolecular assembly, where the dye is preserved from interactions with the environment. This inflection of the curve could then be due to a change in the structure of the assembly. This interpretation is in perfect agreement with the fact that, above $T**$, EHUT assembles into filaments with a single molecule in the cross section, i.e., a structure in which the monomers must be less constrained than in the tube structure.[16]

From the Arrhenius plot, activation energies and frequency factors were deduced (Table 7). Graphically, it can be observed that the slopes of the two curves (even if two parts are observed
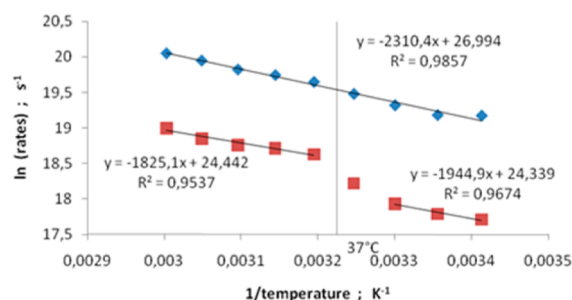
Figure 5. Arrhenius plot of fast (◆) and slow (■) components of fluorescence decays of EHUTz $(10^{-4}$ mol·L^{-1})/EHUT $(10^{-4}$ mol·L^{-1}) in toluene at different temperatures.

Table 7. Activation Energy and Frequency Factor for the Three Different States of Aggregation of EHUTz

	activation energy (kJ/mol)	freq factor (s^{-1})
fast component (free monomer)	19.2	5.27×10^{11}
slow component, $T > 40$ °C (filament conformation)	15.2	4.11×10^{10}
slow component, $T < 40$ °C (tube conformation)	16.2	3.72×10^{10}

for the slow component) seem similar, and that the values extrapolated at the origin differ.

The activation energies are thus similar, which is expected since, in all three cases, the limiting step responsible for the observable decay is the electron transfer. The frequency factors, which in a first approximation do not depend on the temperature, are related to collision frequencies and steric effects. As their values differ, this reflects the change in the accessibility of the EHUT core.

Therefore, the quenching of the tetrazine by EHUT is an activated process. It has the same activation energy but different pre-exponential factors depending on the molecular organization corresponding to the different accessibilities.

The activation energy is positive as expected from the redox potential of the excited tetrazine and toluene. The frequency factor of the quenching in the free molecule is 10 times higher than in the assemblies, as expected for a steric protection of the core by the supramolecular structure.

■ EXPERIMENTAL SECTION

Chemicals and solvents (technical grade) were purchased from Acros Organics, Fluka, or Aldrich and used as received unless otherwise stated. Anhydrous tetrahydrofuran was obtained by distillation over sodium/benzophenone, and anhydrous dichloromethane was obtained by distillation over calcium hydride. NMR spectra were recorded on a Brüker DRX200 (^{1}H, 200 MHz; ^{13}C, 50 MHz) spectrometer at room temperature. Chemical shifts are given in parts per million (ppm) relative to TMS or residual undeuterated solvent as

Table 6. Decay Constants (τ) and Relative Pre-exponential Factors (A) for Fits of Fluorescence Decays of EHUTz $(10^{-4}$ mol·L^{-1})/EHUT $(10^{-4}$ mol·L^{-1}) in Toluene at Different Temperatures

|	temperature (°C)								
	20	25	30	35	40	45	50	55	60
τ_1/τ_2 (ns)	4.7/20.3	4.66/18.8	4.06/16.3	3.46/12.2	2.92/8.12	2.65/7.48	2.45/7.14	2.16/6.51	1.95/5.61
A_1/A_2 (%)	37/62	45/54	57/43	82/18	84/16	85/14	89/11	91/09	91/08
χ^2	1.10	1.13	1.11	1.18	1.17	1.10	1.13	0.90	0.89

internal reference. Thin layer chromatography was performed by using silica gel 60 F_{254} (0.2 mm) on aluminum plates. For column chromatography, a silica gel of 60−200 Å was used.

3,6-Dichloro-1,2,4,5-tetrazine was obtained by the method recently described by Hiskey,[32] and EHUT was synthesized as previously reported.[35] The syntheses and NMR spectra of all other compounds are detailed in the Supporting Information.

UV−visible absorption spectra were measured with a Varian CARY 500 spectrophotometer. Excitation and emission spectra were measured on a SPEX Fluorolog-3 (Jobin-Yvon). The solvent used was toluene (spectroscopic grade) from Sigma-Aldrich. Right-angle configuration was used and concentrations were adjusted in order to ensure an optical density below 0.1, thus avoiding reabsorption artifacts. Fluorescence decay curves were obtained with a time-correlated single-photon-counting method using a titanium−sapphire laser pumped by an argon ion laser (82 MHz, 1 ps pulse width, repetition rate lowered to 4 MHz due to a pulse peaker; a doubling crystal was used to reach 495 nm excitation).[36] The decays were measured at the maximum of emission. The Levenberg−Marquardt algorithm was used for nonlinear least squares fit.[37] In some cases, constrained time constants were used. If all the constants remain free, similar fits are obtained, but the χ^2 values obtained are less satisfying.

CONCLUSION

We showed that the labeling of EHUT monomers by tetrazine dyes does not disturb the supramolecular assembly. Both dilution and temperature studies allowed us to determine the fluorescence behavior of the labeled monomer in its different aggregation states. Fluorescence confirms the transition phase diagram obtained previously by calorimetry and infrared spectroscopy (see Figure 6).[20] The free EHUTz has a lifetime
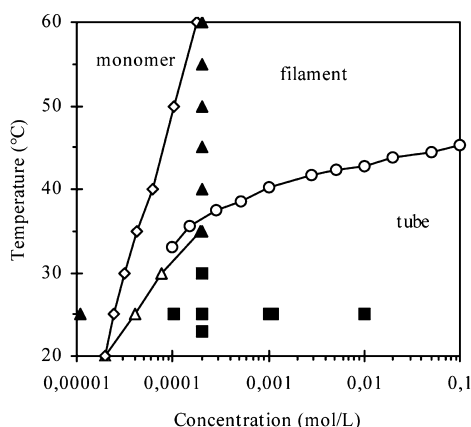
Figure 6. Pseudophase diagram for EHUT solutions in toluene. Transition between monomers and supramolecular filaments determined by ITC (◇). Transition between filaments and tubes determined by ITC (△) or DSC (○).[20] The results of this fluorescence study are included: fluorescence decays of EHUTz/EHUT solutions in toluene; preponderance of the slow component (■); preponderance of the fast component (▲).

of 4.7 ns because of the collision of the excited tetrazine with the aromatic core that leads to a quenching by electron transfer. When incorporated into the assembly the labeled molecules exhibit a longer lifetime, since the fluorescent label cannot interact with the aromatic core, due to steric hindrance. This allows us to measure the partition coefficient of the label in the

supramolecular structure. The value of the partition coefficient of EHUTz ($1/\tilde{K}_{EHUTz} \approx 3 \times 10^{-5}$ mol·L^{-1}) is very close to the CAC of EHUT (around 2×10^{-5} mol·L^{-1}). This confirms that the presence of the tetrazine labels affects only weakly the EHUT supramolecular assembly.

At high loading ratio, intermolecular quenching occurs between neighboring tetrazines. The effect is proportional to the loading ratio, which shows that the probes are uniformly incorporated into the supramolecular polymer. The experiments at different temperatures show that tetrazine is a good reporter of the events occurring in solution such as the disruption of the assembly. The Arrhenius plot confirms the idea that the insertion of the EHUTz molecule in the polymer prevents collisions of the excited fluorescent label with the aromatic cores. It also suggests that it is possible to distinguish the tubular form and the filament assembly.

ASSOCIATED CONTENT

S **Supporting Information**

Synthesis and NMR spectra of the different molecules, absorption and static fluorescence spectra of HOTz and EHUTz with or without EHUT, effect of the solvent on the fluorescence time constant of HOTz, nano-DSC experiments, and dynamic fluorescence behavior of solid EHUTz. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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