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Polymerization of tetrazine-substituted diacetylenes as aggregates in suspension

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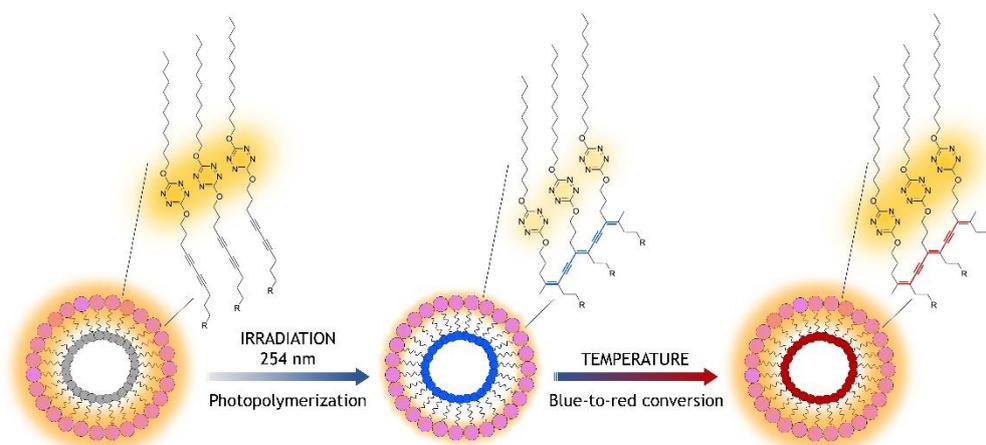
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Abstract

Polydiacetylenes (PDAs) are conjugated polymers that have been widely exploited for their chromogenic and fluorogenic transitions upon exposure to external stimuli and biomolecules of interest. Herein, we propose a comparative study of the polymerization dynamics of two diacetylene derivatives, **TzDA1** and **TzDA2**, in the form of aggregates in suspension prepared by reprecipitation method from organic solvents in water, varying the diacetylene concentration and solvent proportions, and sonication in water, varying the time and temperature. Both derivatives bear a tetrazine fluorophore, which serves both to increase the fluorescence quantum yield of the system and to track the polymerization by fluorescence quenching exclusively by the blue-PDA, and differ by the chain termination. It was shown that adding a butyl ester function in **TzDA2** to a simple urethane (**TzDA1**) influences the polymerizability and kinetics of polymerization of the aggregates in suspension. In addition, we showed that also the preparation method and preparation conditions do have an influence on the polymerization dynamics, suggesting that a careful study of these properties should be carried out before investigating the applications of such objects.

Graphical abstract



1 Introduction

Since their discovery by Wegner *et al.* in 1969 [1] PDAs have attracted a lot of attention over the years because of their unique photophysical properties that lead to numerous applications for sensing various analytes [2]. PDAs are prepared by topochemical polymerization of ordered diacetylenes, to form long π -conjugated chains. Polymerization usually occurs via 1,4-addition of diacetylene monomers triggered by UV (254 nm), γ light irradiation, X-rays or e-beams, that results in alternated triple and double bonds (ene-yne) polymer chains. The first studies on the polymerization properties of this family of molecules appeared in the 1970s and have developed over the years. [3] Unlike the vast majority of polymeric materials, diacetylene (DA) polymerization can occur in the solid state or in ordered aggregates in suspension, as long as the monomer units are self-

assembled in a specific geometrical and packing order to favor reaction between two diacetylenes. [4, 5]. As it was shown in recent studies by Schott *et al.* on thin films of two urethane-substituted derivatives, [6] the polymerization quantum yield is lower than 100% and the reaction kinetics is significantly slowed down by the formation of new polymer chains. UV irradiation, compared to for example γ -rays, leads to lower polymerization extents in the solid state. [7]

Thanks to the alternation of ene-yne motifs, electrons in the main backbone are highly delocalized, giving the polymer interesting optical characteristics. The crystalline powder, film or aggregate shows an intense blue color appearing when the sample is polymerized (blue-PDA), with a characteristic absorption band centered at ~ 640 nm. [8, 9] More interestingly, another chromogenic transition occurs when the blue-PDA is exposed to external stimuli: the absorption spectrum shifts towards lower wavelengths, with a characteristic absorption band centered at 540 nm (red-PDA). This transition is visible to the naked eye. However, the most promising feature of these molecules in the context of sensing is that the blue phase is non-fluorescent, while the red phase of the polymer exhibits fluorescence with minimal bleaching. [10–13] The exact mechanism behind the electronic transition between blue and red phase is still not well understood and under debate. However, it is presumed to be connected with the supramolecular arrangement of the polymer that goes from strictly planar (blue-phase) to non-planar (red-phase), causing the mentioned blue shift in the absorption spectrum. [12, 14] Polymerization of DAs, however, is a complicated process that often in literature is reduced to the formation of two distinguishable and spectroscopically different phases, blue and red. Lifshitz *et al.* showed for the same compound that irradiation in the UV of differently prepared samples (Langmuir films polymerized in water; Langmuir films polymerized on solid support; spincoated films) is a much more dynamic process than it is usually assumed to be. In fact, polymerization in films indeed induces deformations in the sample, which are responsible for progressive shifts of the polymer absorption bands, usually towards higher energy. Therefore, it is incorrect to speak dichotomically of blue and red PDA phases since intermediated states in between the two do exist. The intermediate phase that absorbs around 600 nm can also be called purple phase (in between blue and red) and depending on the monomer structure and side chains, it can be obtained at the very beginning of the polymerization, by progressive irradiation or not witnessed at all. [15]

DA amphiphiles self-assemble in water solution forming nanoparticles of bilayers with an enclosed volume, which in the literature are called indistinguishably vesicles or liposomes. Vesicles prepared by self-assembly in water found extensive use as sensors, based on the chromatic blue-to-red transition upon exposure to stimuli and on the fluorescence signature of the red-PDA. The first report on polydiacetylene-based sensors by Charych *et al.* in 1993 on sialic-modified PDA bilayers [16] and later in 1995 on liposomes [17] for selective detection of influenza virus, exploiting the blue-to-red chromatic transition, opened the lead on PDA aggregates sensing applications. Amongst them we find bio- or chemo-specific binding for microorganisms and biomolecules of interest [18–25], temperature, [26–28] pH, [29, 30] metal ions, [31–33] solvent detection [34] and mechanical forces. [35]

However, the majority of the publications focus on the application of such systems as solution-based sensors or substrate-immobilized responsive aggregates. The polymerization dynamics and how the efficiency of the polymerization process is affected by the preparation method and preparation conditions were rarely assessed until very recently. [36, 37] Therefore, we propose in this paper a comparative study on two DA derivatives, **TzDA1** and **TzDA2** (in these compounds [38, 39] which structures are shown in Fig. 1, the acronym TzDA stands for Tetrazine-Diacetylene) with different chain terminations, which were used to prepare aggregate suspensions by reprecipitation and sonication methods. Both derivatives present a 1,2,4,5-tetrazine fluorophore on one side of the chain. Fluorophore substitution is a common way to increase the fluorescence quantum yield of the red phase of the polymer. [40, 41] The use of tetrazine also allows to track the polymerization process by fluorescence since it is quenched by energy transfer exclusively by the PDA in its blue form. [38, 42] Polymerization to the blue form is thus expected to lower progressively the emission of the system, which is restored upon thermal stimulus when the blue-PDA is converted to the red form, as depicted in the schematics in Fig. 1b. The preparation parameters like DA concentration, solvent and solvent proportions for reprecipitation and time and temperature for sonication were varied. The polymerization process was studied by steady-state spectroscopy, acquiring a UV–Vis and fluorescence spectrum after well-defined UV irradiations at 254 nm.

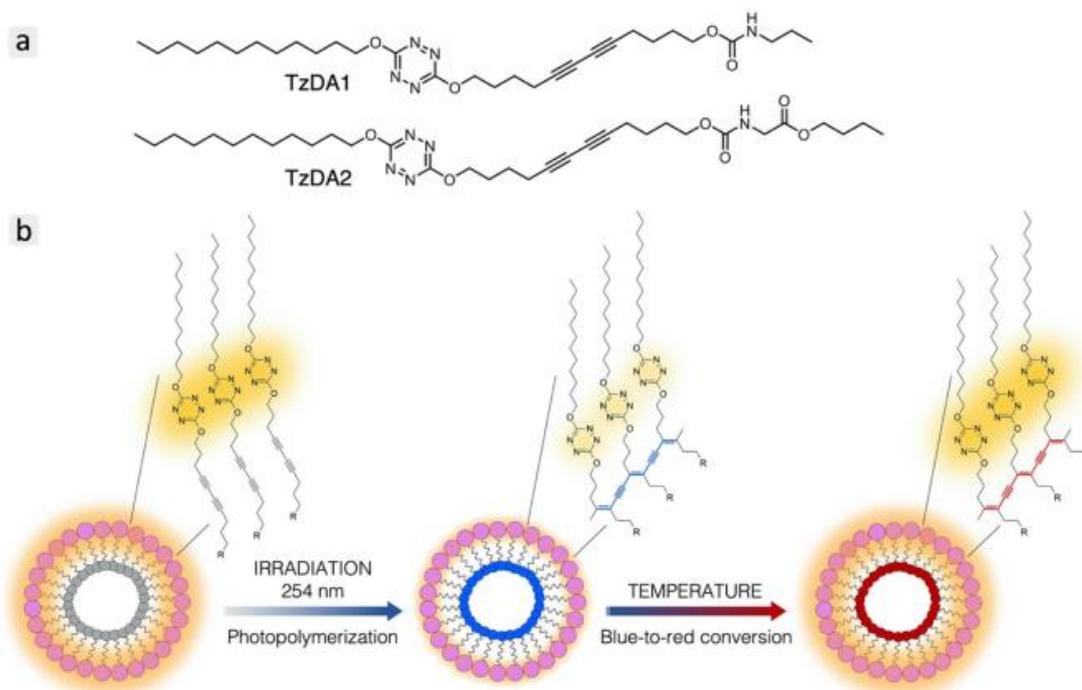


Fig. 1 Structure of the diacetylene derivatives under study. **a** Schematics of the fluorescence evolution in monomeric and polymerized aggregates of tetrazine substituted DAs. **b**

2 Experimental

2.1 Suspension preparation methods

2.1.1 Reprecipitation

Reprecipitation is by far the simplest and most direct method to obtain aggregates in suspension. It consists in dissolving the monomeric diacetylene in a “good” solvent (organic), which is then injected in a stirring volume of “bad” solvent (usually water) in which the compound is not soluble and the “good” solvent is miscible with. Different parameters can be adjusted to potentially achieve different shapes and morphologies of aggregates in solution [43–45]: the nature of the organic solvent, especially its polarity and its partial miscibility with water; the concentration of the diacetylene in the starting solution; the proportion of the initial solution and the bad solvent. Temperature and stirring rate also play an important role, and they were kept constant throughout the experiments to 25 °C and 2000 rpm.

2.1.2 Sonication

The number of diacetylenes that is soluble in water is pretty limited and those molecules usually bear highly hydrophilic or charged side groups. This, however, does not ensure the solubility of the polymerized diacetylene. [46, 47] The molecules under study are not soluble in water. Nonetheless, it is possible to force aggregation and suspension formation in water by ultrasound under heating, as it was shown for a number of DA molecules in literature. [48, 49] The DA unit is dissolved in a small volume of a volatile solvent, like chloroform, and dried by rotavapor to form a thin film of DA in the flask. Water is added to the flask, which is then sonicated using a S30H Elmasonic sonicator at a constant temperature. The suspension is then let rest overnight at 4 °C for stabilization. The final concentration is calculated directly with the injected volume of water, as if the DA was completely dispersed in water. Sonication time, temperature and DA final concentration are the parameters than can be varied.

2.2 Spectroscopy setup

The UV/Vis absorption spectra were recorded on a double beam spectrophotometer Cary 100 from Agilent Technologies equipped with deuterated/halogen lamps. The emission spectra were recorded on a Fluoromax 3

from Horiba Jobin–Yvon with xenon light source. Irradiation at 254 nm was performed by a Hg/Xe LC8–Lightningcure (200 W) light source from Hamamatsu. An interferential filter from Semrock (FF01-254/8–25) is added to select the 254 nm mercury line. The power of the irradiation was measured with an Ophir powermeter (PD300-UV), using a Schott long pass filter (LP) 385 nm to subtract the residual infrared light that is let through the Semrock filter, considering a 90% transmittance of the LP filter.

3 Results and discussion

The DA suspensions were prepared via reprecipitation or sonication method, changing the parameters like DA concentration or temperature. The evolution of these systems was then observed under controlled irradiation at 254 nm by means of absorption and fluorescence spectra to conclude on how the system polymerizes, in respect to the type of molecule and type of suspension. Absorption and fluorescence spectra for progressively UV-irradiated (photopolymerized) aggregates in solution are expected to evolve because of the formation of polydiacetylene chains inside the aggregate. The formation of blue polymer corresponds to an increase of the absorption band centered around 600–640 nm. When the absorption band is centered around 600 nm, it is defined in the literature as “purple” phase rather than blue. A pure blue form has a strong absorption around 640 nm, while a purple form absorbs at lower wavelengths. To avoid confusion, we will keep using the term blue-PDA to designate any form that is different from red-PDA ($\lambda_{\text{abs}} = 540$ nm). Tetrazine fluorescence is expected to drop concomitantly to blue-PDA formation, because of energy transfer between the fluorophore and the PDA acceptor. [38, 39, 42]

3.1 TzDA1

3.1.1 Reprecipitation

A 0.25 mM **TzDA1** solution in MeCN is mixed with water (proportion of H₂O: MeCN is 8:2) to give aggregates in solution that polymerize with UV irradiation at 254 nm as proven by the absorption and fluorescence spectra shown in Fig. 2a, b, respectively. The scattering contributions and the baseline shifts induced by the polymerization are averaged and taken into account in the absorption spectra by subtracting the extinction at 700 nm, where the absorption is expected to be negligible and the scattering less pronounced. The absorption band at 615 nm corresponding to blue-**polyTzDA1** increases with UV irradiation (black to grey to blue line in Fig. 2a) and the tetrazine fluorescence intensity ($\lambda_{\text{em}} = 560$ nm) decreases consistently when the sample is polymerized (Fig. 2b). We can trace the polymerization kinetics by plotting the absorbance of blue-**poly-TzDA1** and tetrazine fluorescence signal versus the irradiation energy. The curves are shown in Fig. 2c. The system polymerizes efficiently up to 500 mJ·cm⁻². From this point onwards, even long irradiations cause small increases in polymer quantity (represented by the absorbance at 615 nm, black line) and small decreases in tetrazine’s fluorescence signal (red line). The final polymerized suspension is tested to temperature, by placing the cuvette on a heating plate for 3 min at 100 °C. The blue solution is converted to a bright red solution, as confirmed by the absorption spectra before (blue line) and after (red line) heating in Fig. 2d. The fluorescence intensity, as discussed previously, is quenched when polymerization is performed (black to blue line in Fig. 2e). When the blue-**poly-TzDA1** is converted to red-**poly-TzDA1** by heating, the fluorescence signal grows back, as shown by the red line in Fig. 2e. It is worth noticing that the spectral shape of tetrazine fluorescence is affected by heating, resulting in a narrower band at 560 nm, plus a large shoulder at higher wavelength. This is probably due to a rearrangement of the polymer chains coming from blue-to-red conversion and a contribution of the intrinsic fluorescence of red-**poly-TzDA1** in the same range. Figure 2f shows absorption and fluorescence evolution of progressively polymerized aggregates of **TzDA1** prepared by reprecipitation in water starting from a 0.5 mM solution in MeCN. Irradiation at 254 nm produces blue-**polyTzDA1** as confirmed by the growing absorption band at 615 nm (see also Figure S2a) and by the decrease in fluorescence signal intensity at 560 nm with UV irradiation (see also Figure S2b). Blue polymer formation is efficient up to 1.7 J·cm⁻² irradiation dose and progressively slows down. From 1.7 J·cm⁻² to 3.4 J·cm⁻² the increase in absorbance at 615 nm is equal to only 13% of the increase caused by the first 1.7 J·cm⁻² irradiation dose. At both concentrations (0.25 mM and 0.5 mM **TzDA1** solution in MeCN, H₂O: MeCN 8:2), the same blue polymer phase is formed, absorbing at 615 nm which quenches tetrazine’s fluorescence. However, the fluorescence extinction was more efficient for the 0.5 mM suspension, reaching 91% versus 68% for the 0.25 mM, suggesting that in the 0.25 mM solution some **TzDA1** molecules either remain non-

aggregated, or form aggregates that do not polymerize. Colloidal stability of the suspension prepared from the 0.25 mM initial concentration was checked by recording absorption spectra at different times after the photopolymerization (see Figure S3). The absorption spectra showed very little evolution with time, confirming a good stability of the **poly-TzDA1** suspension in the timescale of the experiments.

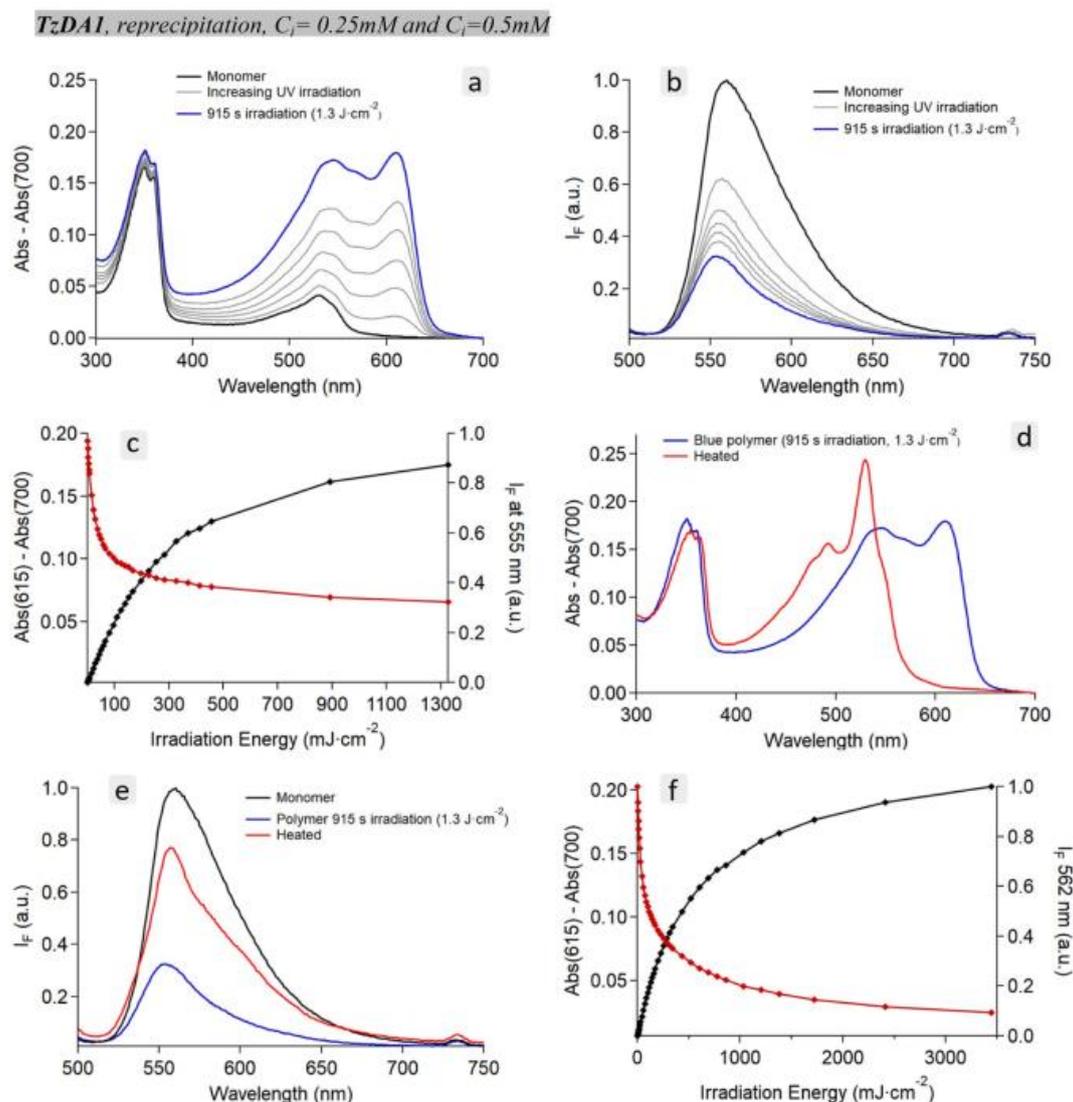


Fig. 2 Absorption **a** and fluorescence **b** spectra of a **TzDA1** suspension progressively irradiated at 254 nm, prepared by reprecipitation method in MeCN at 0.25 mM initial concentration. The proportion of H₂O:MeCN is 8:2. Absorbance (black line) and fluorescence (red line) evolution along the irradiation **c**; absorption **d** and fluorescence **e** spectra of monomer aggregates (black line), polymerized aggregates (blue line) and heated aggregates (red line), λ_{exc} of fluorescence is 488 nm. Absorbance (black line) and fluorescence (red line) evolution along the irradiation **f** of a **TzDA1** suspension progressively irradiated at 254 nm, prepared by reprecipitation method in MeCN at 0.5 mM initial concentration. The proportion of H₂O:MeCN is 8:2, λ_{exc} of fluorescence is 500 nm. Absorption spectra are represented as Abs-Abs(700) to average and suppress the contribution of scattering and baseline shifts of the suspension due to polymerization of **TzDA1**

TzDA1 reprecipitation was tested as well starting from THF solutions. Figure S1 shows the absorption and fluorescence spectra of UV irradiated suspensions of **TzDA1**, prepared by reprecipitation in THF/H₂O for two different initial concentrations (0.1 and 0.5 mM at H₂O: THF 8:2 proportions). As for reprecipitation in MeCN, reprecipitation in THF gives aggregates that polymerize and quench tetrazine's fluorescence signal efficiently. Therefore, both MeCN and THF **TzDA1** initial solutions give aggregates in suspension for a range of different initial concentrations. These aggregates all polymerize efficiently when irradiated at 254 nm. The blue-**poly-TzDA1** absorption band is centered at 615 nm in both cases and in both cases some red-**poly-TzDA1** formation

is observed ($\lambda_{\text{abs}} = 540 \text{ nm}$). In all these systems, tetrazine showed efficient fluorescence quenching under UV irradiation at 254 nm, which is also qualitatively reversible by heating.

3.1.2 Sonication

A 0.5 mM solution of **TzDA1** aggregates in water is prepared by sonication at 60 °C for 15 min. Absorption spectra of the resulting solution exposed to irradiation at 254 nm for photopolymerization are shown in Fig. 3a. The monomeric suspension spectrum (black line) shows the characteristic features of tetrazine (two absorption bands at 350 and 520 nm). When the sample is irradiated in the UV (grey lines), the absorption band between 480 and 560 nm increases progressively. No absorption band corresponding to blue-**poly-TzDA1** is observed. To better visualize the evolution of the absorption spectral shape along polymerization, the monomeric suspension spectrum has been subtracted to the final polymerized suspension absorption spectrum in Fig. 3b (red line). This way tetrazine's contribution to the visible absorption is suppressed, and only the polymer absorption is visible. It is clear that in this specific aggregation state, at these preparation conditions, **TzDA1** polymerizes directly to the red phase with the characteristic spectrum shown by the red line in Fig. 3b, with a maximum absorption band at 541 nm and a vibronic band at 500 nm. Even though blue-**poly-TzDA1** is not formed, tetrazine fluorescence intensity at 560 nm decreases as shown in Fig. 3c, due to the non-negligible spectral overlap between tetrazine fluorescence and red-**poly-TzDA1** absorption. Figure 3d traces the evolution of the red polymer absorption and tetrazine fluorescence intensity as a function of irradiation energy. Short irradiations at the beginning do not affect either the absorption or the fluorescence spectra, causing minimal variations. Longer irradiations, instead, for an energy comprised between 0.7 and 3 J·cm⁻², form red-**poly-TzDA1** and quench tetrazine fluorescence. Therefore, the so-prepared suspensions have no use in the context of sensing, since the red form is not responsive to stimuli and it is back-converted to its blue counterpart only in specific systems. [27, 50–55]

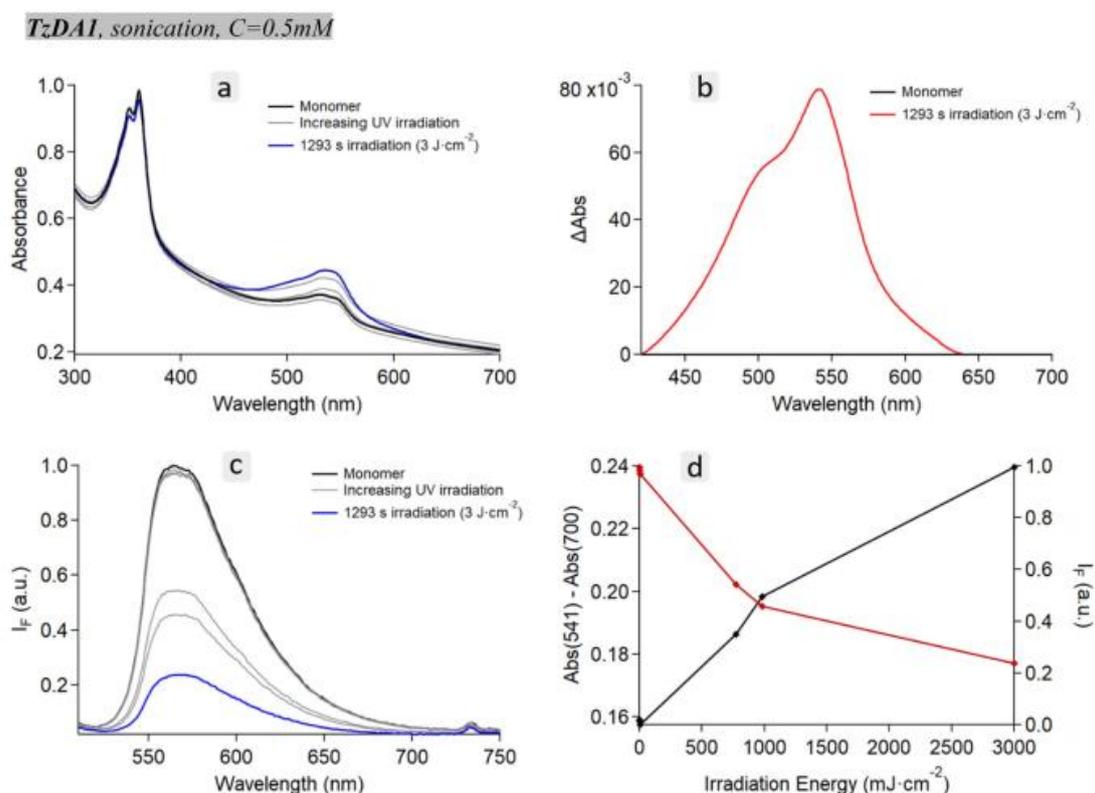


Fig. 3 Absorption spectra of **TzDA1** suspension, progressively irradiated at 254 nm. **a** Absorption spectra of monomeric (black line) and polymerized suspension (red line) after subtraction of monomer suspension absorption. **b** Fluorescence spectra of **TzDA1** suspension, progressively irradiated at 254 nm. **c** Absorbance (black line) and fluorescence (red line) evolution along the irradiation. **d** The suspension is prepared by sonication in water at 60 °C for 15 min. The final concentration is 0.5 mM. λ_{exc} of fluorescence is 488 nm.

Polymerization of **TzDA1** in suspension can be compared with polymerization of the same molecule in the form of thin films. [39] Solid-state films form a blue phase that absorbs at 600 nm (defined as “purple” phase in the literature) [12, 14, 51], while the aggregates prepared by reprecipitation polymerize in a more classical blue phase, absorbing at 615 nm and the ones prepared by sonication form the red phase: indeed, the preparation method of **TzDA1** suspensions has probably an influence on the geometrical disposition of the self-assembled DAs, which is responsible for the formation of either blue or red PDA form.

3.2 TzDA2

3.2.1 Reprecipitation

Figure 4 shows the spectroscopic study of photopolymerization of a **TzDA2** suspension prepared starting from a 0.25 mM solution of **TzDA2** in MeCN reprecipitated in water. Irradiation at 254 nm of the suspension produces an absorption band at 605 nm corresponding to blue-**polyTzDA2** (Fig. 4a). Subtracting the monomer spectrum to the irradiated ones allows to see more clearly the absorption bands corresponding to the PDA (Fig. 4b). The band at 605 nm increases progressively along the photopolymerization process and, as depicted in Fig. 4d, the increase is very steep at the beginning (0–1000 mJ·cm⁻²) until the maximum polymer quantity is obtained around 1500 mJ·cm⁻² and the curve reaches a plateau. Blue-**poly-TzDA2** absorbance seems to decrease in the last irradiations, at irradiation doses higher than 4000 mJ·cm⁻². The maximum of absorbance is at first centered at 605 nm and shifts progressively towards lower wavelengths until 585 nm (25 nm shift) in the last steps of the photopolymerization (Fig. 4a, b). It is impossible to determine by steady-state measurements if from this point different forms of **poly-TzDA2** coexist or if the initial blue-**poly-TzDA2** is converted to another phase. Consistently with the energy transfer mechanism, the production of blue-**poly-TzDA2** in suspension causes a quenching of tetrazine’s fluorescence intensity, as shown in Fig. 4c. The decrease reflects the blue polymer formation, being very efficient between 0 and 1000 mJ·cm⁻² and reaching a plateau around 1500 mJ·cm⁻² (Fig. 4d). It is also worth noticing that the fluorescence intensity at the final step of the polymerization has been quenched by 70% of the initial value, against 94% in **TzDA2** thin films. [39] The residual fluorescence signal at the end of the photopolymerization process may be due to the presence of non-aggregated **TzDA2** molecules that do not take part in the polymerization process; therefore, their emissive unit is not quenched by the formation of blue-**polyTzDA2**. As for **poly-TzDA1**, **poly-TzDA2** suspension is stable for several hours at room temperature (see Figure S4).

TzDA2, reprecipitation, $C_i = 0.25\text{mM}$

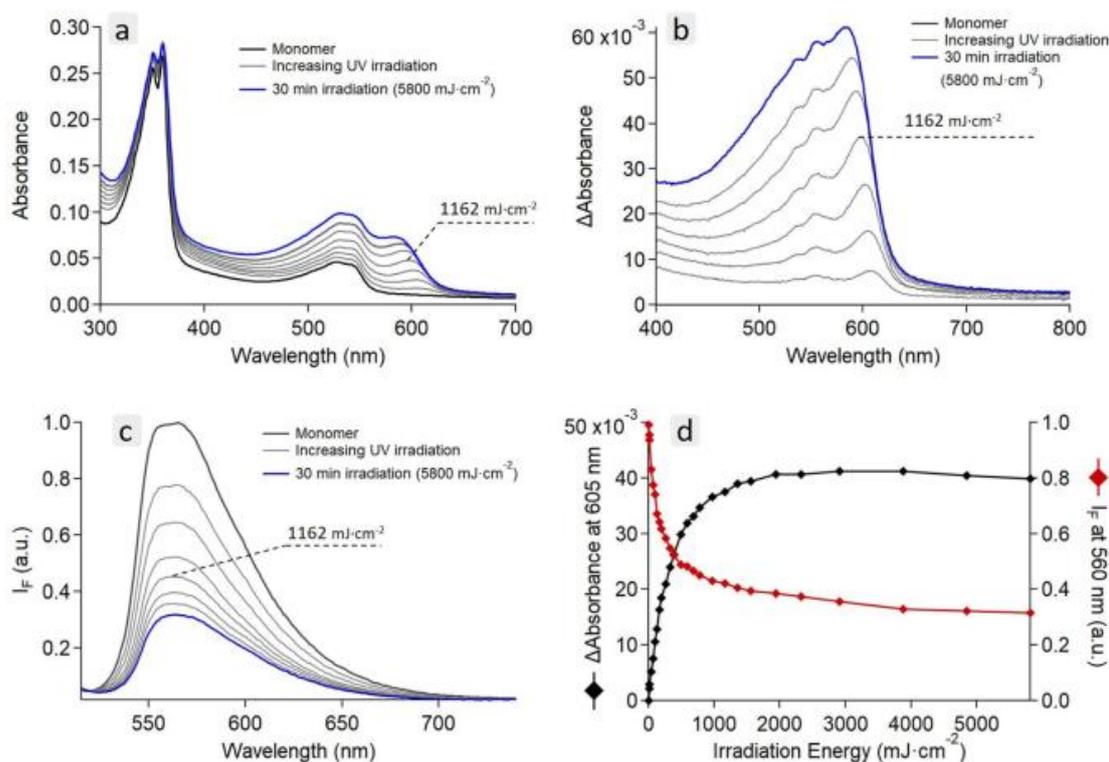


Fig. 4 Absorption **a**, Δ absorption **b** and fluorescence **c** spectra of progressively irradiated at 254 nm **TzDA2** suspensions, prepared by reprecipitation method in MeCN at 0.25 mM initial concentration. The proportion of H₂O : MeCN is 8:2. Increase of blue polymer absorption at 605 nm and decrease of tetrazine emission intensity at 560 nm. **d** λ exc of fluorescence is 500 nm.

To investigate the influence of **TzDA2** concentration, a suspension prepared from a 0.5 mM **TzDA2** solution in MeCN (H₂O : MeCN 8:2) reprecipitated in water is subjected to consecutive irradiations at 254 nm and for each irradiation an absorption and fluorescence spectrum are recorded (shown in Fig. 5). The same blue-**poly-TzDA2** absorption band at 605 nm that is formed for lower concentrations is observed when the suspension is irradiated in the UV, as can be seen in Fig. 5a. To visualize better this increasing band, the monomer spectrum is subtracted to the consecutive spectra, as shown in Fig. 5b. Along the polymerization process, the band shifts towards the shorter wavelengths, as in the 0.25 mM suspension and the thin films. The blue-**poly-TzDA2** band is first centered at 605 nm and is shifted to 590 nm. Compared to the 0.25 mM suspension, the total band shift is smaller and equal to 15 nm, which is not surprising knowing that the irradiation dose for the 0.5 mM suspension was lower ($\approx 1600\text{ mJ}\cdot\text{cm}^{-2}$ versus $6000\text{ mJ}\cdot\text{cm}^{-2}$). If the aggregates formed from a 0.25 mM solution show no sign of degradation up to $6000\text{ mJ}\cdot\text{cm}^{-2}$, the tetrazine in 0.5 mM suspensions seems to undergo photobleaching, represented by a decrease in the band at 350 nm in Fig. 5a. Blue-**poly-TzDA2** increases progressively with irradiation and reaches a plateau around $800\text{ mJ}\cdot\text{cm}^{-2}$ (Fig. 5d), while it was almost the double for 0.25 mM suspensions ($1600\text{ mJ}\cdot\text{cm}^{-2}$). Concerning tetrazine fluorescence evolution with polymerization, the intensity at 565 nm is quenched progressively as expected (Fig. 5c). The total fluorescence quenching is equal to 95% at the end of the photopolymerization process (Fig. 5d), and it is difficult to say how much tetrazine photobleaching takes part in this reduced fluorescence signal. In conclusion, the polymerization process approaches the solid-state case in terms of blue polymer formation (similar irradiation energy to reach blue-**poly-TzDA2** maximal quantity) and tetrazine quenching (95% in suspension versus 94% in solid films). This polymer form has an absorption band centered at 605 nm, which resembles the one that is formed in **TzDA2** thin films ($\lambda_{\text{abs}} = 600\text{ nm}$), and exhibits an extremely efficient energy transfer from the tetrazine donors to the PDA chains acceptors. [39]

TzDA2, reprecipitation, $C_i=0.5\text{mM}$

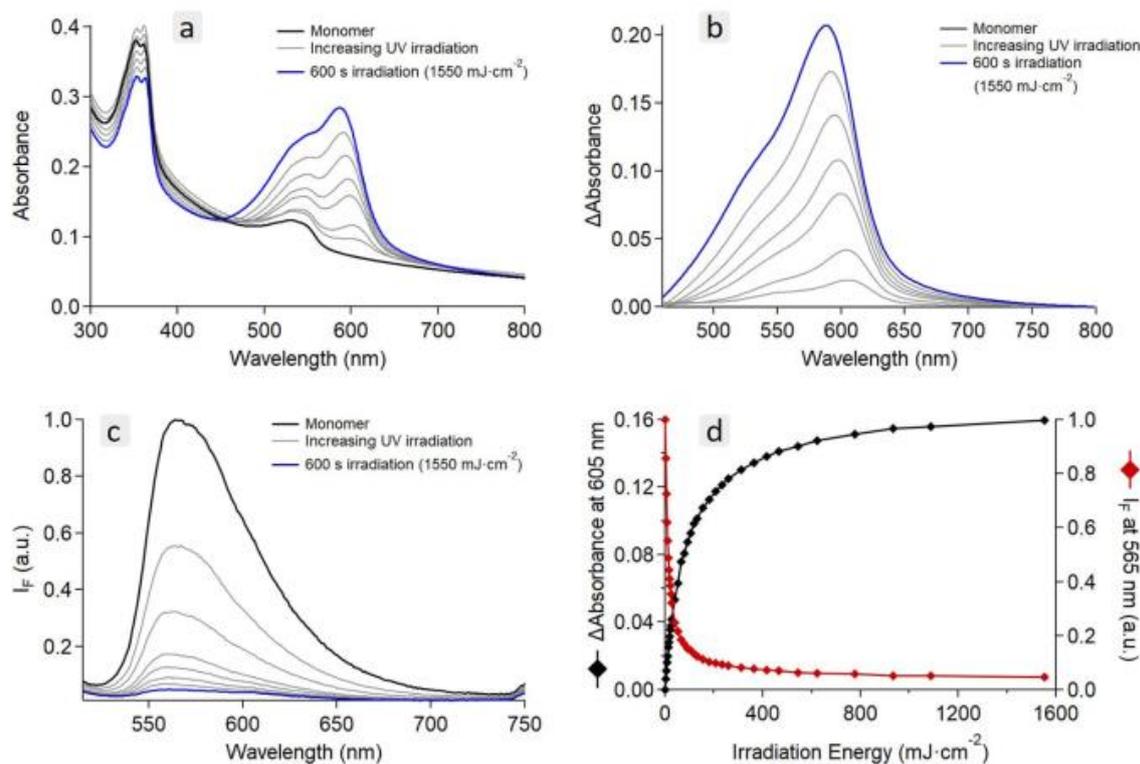


Fig. 5 Absorption **a**, Δ absorption **b** and fluorescence **c** spectra of progressively irradiated at 254 nm **TzDA2** suspensions, prepared by reprecipitation method in MeCN at 0.5 mM initial concentration. The proportion of H₂O : MeCN is 8 :2. Increase of blue polymer absorption at 605 nm and decrease of tetrazine emission intensity at 565 nm. **d** λ_{exc} of fluorescence is 500 nm

TzDA2 aggregates were also precipitated from solutions in MeCN at 0.1 and 1 mM concentrations (H₂O : MeCN 8:2 proportions). Their spectroscopic features are shown in Figure S5 and S6, respectively. UV irradiation on the suspension prepared from the 0.1 mM solution produce initially blue-**poly-TzDA2** (band at 605 nm in Figure S5a) and a decrease of the tetrazine emission intensity (Figure S5b). From 350 s of irradiation, the absorbance at 605 nm decreases (orange-blue arrow) while a new band at 540 nm and a small vibronic band at 480 nm appear and further irradiations reduce the band at 605 nm and increase the one at 540 nm, suggesting a progressive blue-to-red polymer conversion. An ulterior proof of such a conversion is represented by the increase in the emission intensity (Figure S5b, blue line), which can be explained by the reduced energy transfer efficiency of the red-**poly-TzDA2** compared to the blue one. Such a conversion for long irradiation times is common in systems that have a fast polymerization kinetics. [15] When increasing the initial concentration of **TzDA2** in MeCN to 1 mM and keeping the water to solvent proportion at 8 :2, polymerization still occurs upon 5 s irradiation (at 2.6 mW·cm⁻¹) as shown in Figure S6a. This suspension is also scattering like those prepared at lower concentrations.

To investigate the influence of water to solvent proportion, a suspension 9 :1 H₂O: MeCN from a 1 mM **TzDA2** solution is prepared and exposed to UV light at 254 nm. Photopolymerization at these conditions does not occur as shown in Figure S6b, suggesting that the solvent proportion has an influence on the diacetylene aggregation.

Indeed, the initial concentration has an interesting influence on the polymerization properties of the aggregates. When preparing them from 0.1 mM solution, the UV irradiation produces initially an absorption band at 605 nm that suddenly decreases at long irradiation times to leave space to the red-**poly-TzDA2** absorption band at 540 nm. The same absorption band at 605 nm is recorded for suspensions prepared from more concentrated solutions of **TzDA2** (0.25 and 0.5 mM). With UV irradiation, the band increases and progressively shifts towards shorter wavelengths. However, no clear blue-to-red conversion is achieved by irradiation only despite the long irradiation energies supplied to the sample ($\approx 1600\text{ mJ}\cdot\text{cm}^{-2}$ and $6000\text{ mJ}\cdot\text{cm}^{-2}$, respectively). When looking at

tetrazine fluorescence of the three samples (1 mM excluded), the intensity is decreased by the formation of **poly-TzDA2**. It is found that aggregates prepared from a 0.5 mM solution approach the quenching efficiency achieved in the solid state (95% in suspension versus 94% in solid films), which is 20% higher than for aggregates prepared from a 0.25 mM **TzDA2** solution.

No polymerization was recorded when the **TzDA2** is reprecipitated from THF solutions, at different concentrations (0.05, 0.1 and 0.5 mM). At all concentrations, no clear sign of polymerization is detected, as shown in Figure S7. The same experimental conditions of preparation were used for **TzDA1**, which self-assembled properly to give UVpolymerized aggregates in suspension. If no blue or red **poly-TzDA2** absorption band is formed upon irradiation, it means that most probably **TzDA2** does not self-assemble properly to polymerize, which could be due to the polarity of the solvent. It is concluded that THF is not the suitable solvent to produce **TzDA2** aggregates in suspension.

3.2.2 Sonication

A 0.5 mM suspension obtained by sonication at 70 °C for 15 min is exposed to subsequent UV irradiation at 254 nm. The absorption spectra after each irradiation are shown in Fig. 6a. In Fig. 6b, the monomer spectrum has been subtracted to cancel out the contribution of tetrazine thus to highlight the progressive increase of blue-**poly-TzDA2** absorption. The absorbance maximum starts at 605 nm, increases along the irradiation and shifts progressively to 595 nm at end of the polymerization process, as already witnessed in the previous experiments. When the solution is heated on a heating plate, the suspension turns from purple-blue (blue spectrum in Fig. 6c) to deep red (red spectrum in Fig. 6c), demonstrating that also PDA aggregates in suspension respond to external stimuli. Tetrazine emissive state switches from an ON state in the monomeric form (black line), to an OFF state in the polymerized sample (blue line) and back to the ON state when blue-**poly-TzDA2** is converted by heating to red **poly-TzDA2** (red line), as shown in Fig. 6d. Contrarily to **TzDA1** that formed only the red phase, irradiation in UV of these samples produced blue-**poly-TzDA2** with the usual absorption band centered at 605 nm (like in thin films and reprecipitated aggregates). The blue-to-red conversion was achieved by thermal stimulus and the tetrazine fluorescence was also restored. Photopolymerizable aggregates are also obtained at milder temperature conditions (40 °C) but for longer exposure time to sonication (30 min) as shown in Figure S8. Upon UV irradiation, blue-**poly-TzDA2** forms, with an absorption band initially centered at 612 nm and progressively shifting to 600 nm as witnessed for other preparation methods (Figure S8a and b). Fluorescence is progressively and efficiently switched off as shown in Figure S8c. The versatility of DAs to form photopolymerizable aggregates in water at different temperatures and sonication time is a great advantage for application-oriented preparations, which may require specific conditions.

To investigate the concentration effect, a suspension at 0.1 mM was prepared with the same process and photopolymerized. The aggregates polymerize efficiently producing blue-**poly-TzDA2** with an absorption band centered at 605 nm (Fig. S9a). Tetrazine fluorescence is efficiently quenched as shown in Fig. S9b. The quenching efficiency of a 0.5 mM and 0.1 mM suspensions of **poly-TzDA2** at the same irradiation energy are both around 70%. Therefore, the quenching properties are less affected by concentration when the aggregates are prepared by sonication compared to reprecipitation in MeCN, where reducing the concentration from 0.5 to 0.25 mM entailed a decrease in quenching efficiency of 25%.

TzDA2, sonication, C=0.5mM

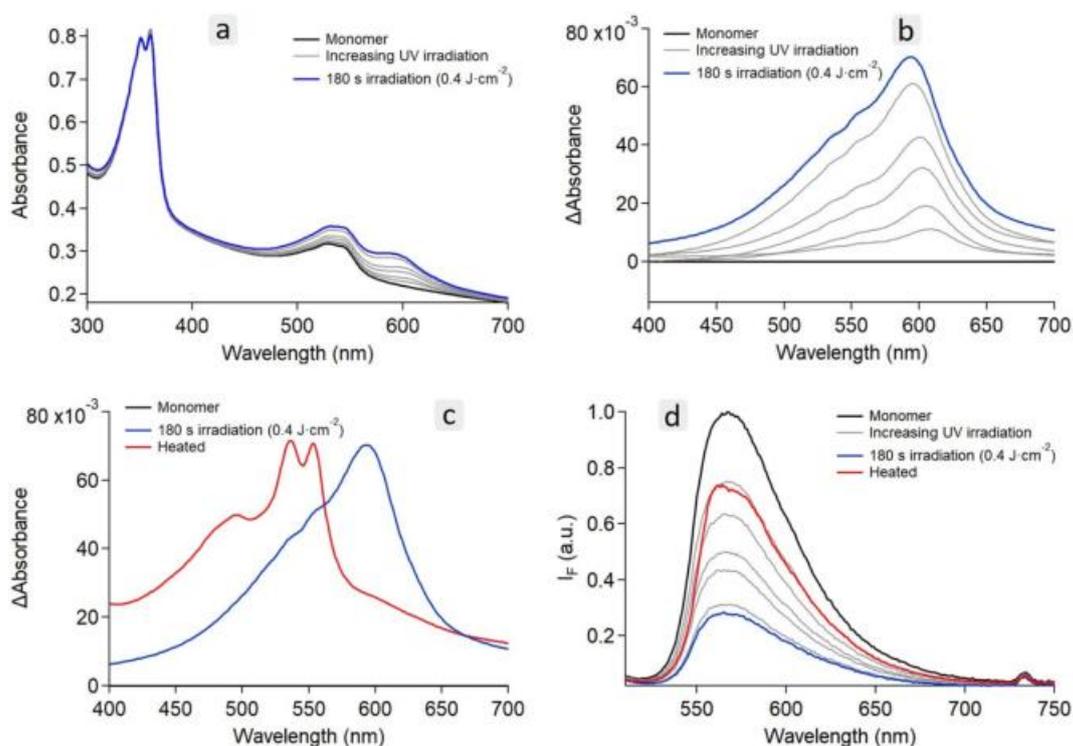


Fig. 6 Absorption **a**, Δ absorption **b** and fluorescence **d** spectra of progressively irradiated at 254 nm **TzDA2** suspensions, prepared by sonication in water at 70 °C for 15 min. Spectra of polymerized aggregates (blue line) and heated aggregates (red line) **c**, the final concentration is 0.5 mM. λ exc of fluorescence is 488 nm

4 Conclusions

Suspensions of **TzDA1** and **TzDA2** were prepared by different methods: reprecipitation in water from differently concentrated DA solutions in either THF or MeCN and sonication in water. For both compounds, we tested different parameters in the preparations and studied how they affected the polymerization by UV irradiation in suspension. **TzDA1** efficiently polymerizes in suspensions prepared from both THF and MeCN solutions. Absorption spectra upon irradiation of these suspensions have all the same spectral features that evolve consistently upon photopolymerization. An absorption band at 615 nm, corresponding to blue-**polyTzDA1**, is formed and evolves with increasing irradiation. For all suspensions, red-**poly-TzDA1** is also formed along polymerization, with a characteristic absorption band at 540 nm. Accordingly with the energy transfer mechanism, blue-**poly-TzDA1** formation quenches tetrazine fluorescence signal. In addition, when heating a suspension prepared from a 0.5 mM **TzDA1** solution (H₂O : MeCN 8:2), the blue phase is converted to the red phase of the polymer, restoring the quenched tetrazine fluorescence due to a reduced energy transfer efficiency between red-PDA and tetrazine. Sonication of **TzDA1** in water for 15 min at 60 °C successfully produced aggregates in suspension that polymerize upon irradiation at 254 nm. However, the polymer phase that is formed is red-**poly-TzDA1**, highlighting that the preparation method strongly influences the photophysical properties of the aggregates. **TzDA2** suspensions were prepared with the same methods. Contrarily to **TzDA1**, reprecipitated suspension in THF did not give UV-polymerizable aggregates for the three tested initial concentrations of 0.05, 0.1 and 0.5 mM. We believe that the reason must be connected with the polarity of the solvent. In fact, when using a more polar solvent for reprecipitation like MeCN, polymerization efficiently occur, but yield to polymers that behave differently under prolonged irradiation, and display different tetrazine fluorescence quenching efficiencies. Therefore, we believe that the initial concentration has an influence on the arrangement of self-assembled DAs that behave differently to 254 nm irradiation. Sonication of **TzDA2** in water at 70 °C for 15 min gave aggregates in solution that polymerize upon irradiation at 254 nm. Contrarily to **TzDA1**, which polymerizes in the red form, **TzDA2** sonicated suspensions form blue-**poly-TzDA2**, absorbing at 605 nm as thin films and the

other suspensions. Tetrazine fluorescence is also quenched as expected, and thermal stimulus recovers the fluorescence signal by blue-to-red conversion of **poly-TzDA2**. We can conclude that the preparation method and parameters (solvent and concentration for the reprecipitation method) have an influence on the arrangement of DAs in suspension that eventually have different polymerization behaviors. The sensing properties of these PDAs aggregates should also depend on their preparation method, and will be investigated in the future.

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Data availability Data will be made available from the authors upon reasonable request.

Conflict of interest There are no conflicts to declare.

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