Aggregation behavior of a temperature- and pH-responsive diblock copolymer in aqueous solution

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Abstract

Orthogonally switchable diblock copolymers show rich phase behavior in aqueous solution, in
dependence on external stimuli. This behavior can be utilized for the preparation of 'smart' drug
delivery systems, which are tuned to respond in accordance to a specific physiological environment.
We investigate the aggregation behavior in water of a dual-stimuli responsive diblock copolymer with
a pH-responsive block composed by (diisopropylamino) ethyl methacrylate (DPA), a basic tertiary
amine-based monomer, and a temperature-responsive block composed by 2-(2-methoxyethoxy) ethyl
methacrylate (MEO₂MA), a polyethylene glycol (PEG) analogue monomer, featuring a lower critical
solution temperature (LCST). In the present work, the temperature- and pH-responsive PDPA-b-
PMEO₂MA diblock copolymer was successfully synthesized by reversible addition-fragmentation chain
transfer (RAFT) polymerization, as confirmed by ¹H NMR spectroscopy. The cloud-point temperature
(T_{cp}) was determined through turbidimetry for different pH values, and the polymer hydrodynamic
radii dependence on pH at room temperature was evaluated by fluorescence correlation spectroscopy
(FCS), and near T_{cp} by temperature-resolved fluorescence correlation spectroscopy (T-FCS). The
characteristic length of the aggregates formed was also assessed below and above T_{cp}, under acidic and
alkaline conditions, by small-angle X-ray scattering (SAXS). Below T_{cp}, our experiments have shown
the formation of small aggregates with different densities depending on the pH in solution, mainly
attributed to the presence/absence of electrostatic repulsions arising from protonated/deprotonated
amine groups and to the increased hydrophilicity of the temperature-responsive block under neutral
and alkaline conditions. Additional large aggregates were formed above T_{cp}, which was found to
increase above acidic pH values.

Keywords: pH/temperature-responsive polymers, amphiphilic diblock copolymer, (diisopropyl
amino)ethyl methacrylate (DPA), 2-(2-methoxy ethoxy)ethyl methacrylate (MEO₂MA), fluorescence
correlation spectroscopy (FCS), small-angle X-ray scattering (SAXS)

1. Introduction

Over the past decade, amphphilic block copolymers have received much attention in many fields,
due to their ability to self-assemble into organized structures in selective solvents above the critical mi
celle concentration (CMC), which makes this class of polymers good candidates to be employed as drug
delivery systems (DDS). These polymeric systems are of particular importance by providing the ad
vantages of efficient stabilization and delivery of poorly water-soluble and/or highly toxic anticancer
drugs, thus improving the pharmacokinetics release profile [1]. Among the systems developed, the most
interesting ones are stimuli- responsive polymers, which may change their physical parameters (solu
bility, shape, size, etc.) in response to external stim
uli, such as pH, ionic strength, temperature, specific ligands or light. Among them, pH and temperature
are the most commonly used external triggers. Such stimuli-responsive polymers are attractive candi
dates when considering DDS based on micelles, nanoparticles, dendrimers, vesicles and others [2, 3].
For instance, pH variations have been exploited to control the delivery of drugs in specific organs or
intracellular compartments (pH 4.5-6.5) as well as to trigger the release of the drug when subtle envi
ronmental changes are associated with pathological situations, such as cancer or inflammation (pH 6.5-
7.2) [4, 5]. One of the strategies is to use polymers (polyacids or polybases) with ionizable groups that
undergo reversible conformational and/or solubility changes in response to environmental pH vari
ation. These include typically, anionic or cationic moieties, such as sulfonates, carboxylic acids or
amines, respectively. Temperature-responsive poly
mers are amongst the most widely investigated
polymers due to their potential biomedical applications and the easy to control stimulus, that can be applied externally or be inherent of the disease condition [6]. Temperature-responsive polymers respond reversibly to small temperature stimuli by strong changes in their solubility and chain conformation, commonly showing lower critical solution temperature (LCST) behavior. Thus, when the temperature surpasses the cloud-point temperature \((T_{cp})\), hydrogen bonding interactions between the polymer and water molecules are broken, and the polymer chains undergo coil-to-globule transition and precipitate in the medium. However, the detailed reasons of the underlying collapse mechanism are still unclear [7]. Among the most studied temperature-responsive polymers is Poly(N-isopropylacrylamide) (PNIPAAm), which a lower critical solution temperature, LCST of around 32°C, a very useful temperature for biomedical applications. Due to its questionable biocompatibility, alternative polymers with oxyethylene (or ethylene glycol), hydroxy and amide groups have been suggested, among others [8]. Sensitivity to more than one stimulus can even further improve the targeting specificity and therapeutic efficacy of polymer drugs. For example, temperature and pH dual-responsive block polymers have been designed to improve drug release caused by the disassembling and precipitation of polymeric DDS [9, 10].

Taking into account the above considerations, in the present work focus on the study of the aggregation behavior of the temperature- and pH-responsive PDPA-b-PMEO\(_2\)MA diblock copolymer. The temperature sensitivity of this polymer is given by the short PEG methacrylate derivatives side chains, namely 2-(2-methoxyethoxy)ethyl methacrylate (MEO\(_2\)MA), an uncharged, water-soluble, non-toxic and LCST type monomer [11]. On the other hand, the sensitivity to pH arises from the biodegradable (diisopropylamino)ethyl methacrylate (DPA) monomer with a pKa of around 6.4, (close to the acidic environment of tumors) soluble in acidic solution as a cationic polyelectrolyte by protonation of its amine groups but becomes less hydrophilic at neutral pH [12]. This dual-stimuli responsive polymer was synthesized using a straightforward controlled/living radical polymerization technique such as reversible addition-fragmentation chain-transfer polymerization (RAFT), achieved through the reversible chain transfer of growing radicals to thio-carbonylthio compounds. This is a metal-free process that avoids product contamination, which is important for biomedical applications [13].

For the investigation of the aggregation behavior in water of the polymer under study, fluorescence correlation spectroscopy (FCS) provided more details at low concentrations, where molecularly dissolved chains (i.e. unimers) are of interest. By adding a hydrophobic fluorescent dye to the solution, it was possible to correlate the observed diffusing time of the unimers/aggregates with physical parameters (e.g. hydrodynamic radius) and extrapolate their aggregation behavior [14]. For higher concentrations, methods such as turbidimetry and scattering experiments such as small-angle X-ray scattering (SAXS) can be employed. The latter is sensitive to the internal structure of the scattering objects, with respect to the solvent. Through modeling of the resulting scattering curves, information about the structural properties of the polymer, such as size and conformation, allowed for the investigation of the aggregates at the local scale, thus verifying their aggregation behavior. With these techniques, we focused on the investigation of the mechanism of aggregation of the temperature- and pH-responsive PDPA-b-PMEO\(_2\)MA diblock copolymer in aqueous solution, for temperature and pH values near its \(T_{cp}\) and pKa.

2. Experimental

2.1. Polymer synthesis

Materials

2-(N,N-diisopropylamino) ethanol (Sigma-Aldrich, 98%), methacryloyl chloride (Fisher Scientific, 97%), triethylamine (TEA), tetrahydrofuran (THF) (Scharlau, 99%), hydroquinone (Sigma-aldrich, 99%), 1,4-dioxane (Scharlau, 99.5%, stabilized with 2.5 ppm of 2,6-di-tert-butyl-4-methylphenol), dichloromethane (Sigma-Aldrich, 96%), 2,2-azobis(2-methyl-propionitrile) (AIBN) (Sigma-Aldrich, 99%), dimethylformamide (DMF)(Scharlau, 99.8%), trifluoroacetic acid (TFA) (Sigma-Aldrich, 99%), ethanol (Sigma-Aldrich, 99.8%) and sodium sulfate (Panreac, 99%). 3-(benzylsulfonylthiocarbonylsulfonyl)- propionic acid (CTA/RAFT agent) was synthesized according to the literature procedures [15].

\(^1\)H NMR spectroscopy

All the polymerization reaction products were analyzed directly by \(^1\)H NMR spectroscopy (Bruker Avance II 400 MHz, UltraShield Magnet - Lisbon, Portugal) at room temperature (RT), to determine the reaction conversions and the theoretical molecular weight through processing of the resulting \(^1\)H NMR spectra with MestReNova software [16]. The requisite peak areas were obtained by the numerical integration of the \(^1\)H NMR spectra, after the assignment of each spectrum and identification of the corresponding peaks. The spectra were calibrated to the signals of residual protonated solvent signals (CDCl\(_3\), 7.26 ppm)
2.2. Synthesis

Monomer synthesis

DPA monomer was synthesized by adding hydroquinone (0.20 g) and anhydrous THF (100 mL) to a double necked flask, which was closed afterwards with rubber septa to avoid intake of atmospheric oxygen and humidity that terminates/hinders the reaction. After the flask was put under argon atmosphere, a solution of 2-(N,N-diisopropylamino)ethanol (2.4 mL, 14 mmol, 1 eq), and anhydrous TEA (2 mL, 0.014 mmol, 1 eq) were added. After cooling the reaction to 0°C, methacryloyl chloride (1.4 mL, 14 mmol, 1 eq) was introduced dropwise. The resulting solution was refluxed in an oil bath (80°C) for 2 h, and then filtered to remove the precipitated TEA-hydrochloric salt. The resulting residue was diluted with dichloromethane and washed with water, 10 wt% potassium carbonate solution, saturated NaCl solution, and water again. The organic layer was dried, filtered, and concentrated under vacuum to give pure DPA as a transparent oil (1.8 g, 8.3 mmol, 59% conversion).

RAFT polymerization

For the preparation of DPA homopolymer (PDPA), which served as a macro-CTA for the subsequent block copolymerization, CTA (24 mg, 0.087 mmol), AIBN (1.45 mg, 0.009 mmol), DPA (0.82 g, 3.77 mmol, 1eq), TFA (0.48 mg, 3.77 mmol) and ethanol (3 mL) were introduced into a Schlenk tube. The flask was sealed with a rubber septum, subsequently degassed through freeze pump cycles with liquid nitrogen, and left un-ber septum, subsequently degassed through freeze pump cycles with liquid nitrogen, and left un-

2.3. Methods for the characterization of the aggregation behavior

Fluorescence correlation spectroscopy (FCS)

FCS measurements were performed using a ConfoCor2 spectrometer (Carl Zeiss Jena GmbH). As a light source, an Ar+ laser operated at 488 nm, a motorized pinhole of diameter 80 μm, a C-Apochromat 40x/1.2 water immersion objective, a BP (band-pass) 530-600 emission filter and an HFT 488 plate beam splitter. A Lab-tek 8-well chambered coverglass from Nalge Nunc International was used as a sample chamber. Each measurement (run) time was 60 s, and the measurements were repeated 10-15 times, and the correlation functions were averaged. The laser power was attenuated to 0.1-1.0% of maximum power (200 mW) to avoid bleaching of the dye probe. The count rate was maintained between 5-15 kcounts/s. The resulting autocorrelation curves were normalized with respect to the lowest diffusion time value (τ = 0.004 ms), after being fitted using the following equation [17]:

\[
G(τ) = 1 + \frac{1}{N} \left[ 1 + \frac{T_T}{1 - T_T} \exp \left( \frac{τ}{T_T} \right) \right] \times \sum_{i=1}^{n} \left( 1 + \frac{τ}{τ_{D,i}} \right)^{-\frac{1}{2}} \left( \tau_{D,i} + \frac{1}{(w_0/2)^2} \tau_{D,i} \right) \]

where \( N \) is the average total number of fluorescent particles in the detection volume, \( i \) the number of different fluorescent species, the decay time, \( τ_{D,i} \) the characteristic diffusion time of component \( i \), \( ρ_i \) the relative amplitude of the fluorescent species (i.e., the fraction of component \( i \)), and \( w_0 \) and \( w_{0,h} \) the half-height and half-width of the observation volume, respectively. \( T_T \) and \( τ_{D} \) are the triplet fraction and time, respectively. From the fit, the values were found to be in the range \( T_T = 0.1-0.2 \) and \( τ_{D} = 1-3 \mu s \). \( w_0 \) was determined before by measuring the diffusion time of the fluorescent dye Rhodamine 6G (Rh6G) (Sigma-Aldrich, \( D_{Rh6G} = 2.8 \times 10^{-10} \text{m}^2\text{s}^{-1} [18] \)), \( τ_{D,Rh6G} \), and by using \( w_0 = \sqrt{4D_{Rh6G}τ_{D,Rh6G}} \). Values \( w_0 \) ≥0.2 μm was typically obtained. The ratio \( z_0/w_0 \) (i.e. structure factor \( S \)) determined from the fit to the Rhodamine 6G correlation functions was typically 6. The diffusion coefficient of the unimers or aggregates, \( D \), was calculated
using
\[
D = D_{Rh6G} \times \frac{T_{D,Rh6G}}{T_D}
\]

The hydrodynamic radii of the unimers or aggregates, \(r_H\), were determined using the Stokes-Einstein equation,
\[
r_H = \frac{k_B T}{6\eta Q D}
\]

with \(\eta\) being the viscosity of water, \(T\) the absolute temperature, and \(k_B\) being the Boltzmann constant.

To study the influence of the pH at room temperature on the aggregation behavior of the polymer under study with FCS experiments, aqueous solutions of 1.5 mL with polymer concentration ranging from 0.01 to 1.2 mg/mL were prepared. Rh6G was added to the prepared solutions and to the control sample (deionized water with Rh6G), in such way that the final concentration of Rh6G was \(5 \times 10^{-7}\) mg/mL (10\(^{-9}\) M). After being briefly agitated, the pH of the solutions was adjusted dropwise, by adding a strong acidic/alkaline aqueous solutions (10 \(\mu\)L of 0.1 M solution of either HCl or NaOH). The deionized water, HCl and NaOH solutions were filtered (Rotilabo syringe filter; pore diameter, 0.22 \(\mu\)m) to remove dust particles, before the addition of the polymer and the Rh6G dye. Using this sample preparation routine, 4 series of polymer solutions were prepared having concentrations between 0.01 to 1.2 mg/mL and pH 3, 6, 7 and 10, taking into account that the pKa of the pH-responsive block monomer DPA is \(\sim 6.4\). The solutions were agitated overnight and their pH measured afterwards for confirmation.

Temperature-resolved fluorescence correlation spectroscopy (T-FCS)

T-FCS allowed for characterization of the aggregation behavior of the polymer under study around the \(T_p\) (~24°C) of the temperature-responsive PMEO\(_2\)MA block, for different pH values (3, 6, 7 and 10). The same technical parameters (such as run time, laser power, etc) used in the FCS measurements at room temperature were employed in this case. The resulting curves were fitted with Equation 1 and normalized in the same manner. For the T-FCS measurements, instead of the usual 8-well sample holder chamber used at room temperature, a custom made sample holder (Figure 1) able to accommodate a electrical conducting indium-tin-oxide (ITO) coated glass cover slips (8-12\(\Omega\), Spi Supplies) was used. The temperature of the sample was installed and controlled using a voltage generator coupled with a temperature control system (thermostat) [19]. Briefly, a voltage was imposed onto the ITO cover slip, forcing a current flow through it, causing an increase of its temperature. The temperature was measured by a Pt100 platinum resistance thermometer and controlled in the same fashion as a thermostat. The temperature was set by the user and fluctuated by \(\pm0.2^\circ\)C around the mean value. Several other steps were performed to optimize the process: (i) placing of thermopaste onto the contact point Pt100 sensor-ITO slip to improve heat conduction, (ii) gold coating of the edges of the ITO slip for better electrical conduction, (iii) design of a PVC frame for the ITO slip to minimize optical issues arising from slip bending, (iv) filling of the laser protection PVC box with styrofoam for better thermal insulation and (v) design of a sample lid to minimize sample evaporation. To avoid heat losses, the C-Apochromat 40x/1.3 oil objective was additionally heated by an objective heater (Carl Zeiss Jena GmbH) with its own temperature control, in combination with the immersion oil Zeiss Immersol\textsuperscript{TM} 518F.

To ensure that the temperature shown on the thermostat display was representative of the temperature of the ITO slip, and to assess possible heat losses between the latter and the sample droplet, a temperature calibration was carried out prior to T-FCS experiments, by measuring and comparing the resulting resistance values of Pt100 sensors when put in direct contact with each component (ITO slip and sample droplet). After converting the resistance values to temperature, by using a standard Pt100 resistance vs temperature table (\(R[\Omega]=0.387 T[^\circ\text{C}]+100.058\)), it was found a temperature offset up to 1\(^\circ\)C between the temperature shown by the thermostat display and the one featured by ITO slip, and a temperature difference of less than 0.5\(^\circ\)C between the latter and the sample droplet, which was considered to be negligible.

T-FCS measurements were performed on the polymer sample with a concentration of 0.8 mg/mL of each pH value (3, 6, 7 and 10) used in the room temperature FCS experiments, as it was the concentration above which the fraction of the slow decay reached a plateau. Afterwards, T-FCS measurements were done in steps of 0.5\(^\circ\)C starting at room temperature. After the desired temperature of the ITO slip was reached, an equilibration time of at least 5 min was applied before each run. Furthermore, to minimize evaporation, the analyzed sample droplet was replaced by a fresh one, from the same batch, after every two measurements (i.e., every 1\(^\circ\)C steps).

Small-angle X-ray scattering (SAXS)
SAXS measurements were performed on a GANEXHSA 300XL SAXS system (SAXSLAB Aps, Copenhagen/Denmark) equipped with a GENIX 3D micro-focus X-ray source and optics together with a three slit collimation system. Both the sample chamber and the beam path were under vacuum. A two-dimensional (2D) Pilatus 300K detector was used, positioned at the desired SDD. The X-ray source was operated at 50 kV/0.6 mA with a Cu anode (\(\lambda_{\text{Cu}} = 1.54 \text{ Å} \)). A SDD of 406.2 mm was chosen with 3600 s of acquisition time. A pin diode was used to measure the transmission of each sample taking as reference the intensity of the empty beam. 2D images were masked (to remove parasitic scattering) and azimuthally averaged using the Fit2D software [20]. The intensity profiles were plotted with the scattering intensity, \(I(q)\), as a function the scattering vector, \(q = (4\pi/\lambda)\sin(\theta/2)\) (\(\theta = \) scattering angle).

All curves normalized with respect to the measurement time, and the transmission (\(T_r\))-corrected background. The background corresponding to the intensity of the empty capillary (\(I_{\text{bg}}\)) was subtracted (Equation 4).

\[
I(q) = \frac{I_{\text{sample}}(q)}{T_r \times \text{time}\text{sample}} - \frac{I_{\text{bg}}(q)}{\text{time}\text{bg}} \times T_{\text{bg}}(q)
\] (4)

Polymer solutions with a concentration of 50 mg/mL were prepared in deionized water. Two samples were prepared, having pH 6 and pH 10, i.e. below and above the pKa of the pH-responsive PDPA block (pKa \(\sim 6.4\)), respectively. The pH of the sample solutions was achieved by adding aqueous solutions of HCl (0.1 M) and NaOH (0.1 M) dropwise to the polymer solutions and leaving them in agitation overnight. Both samples were mounted into a quartz capillary (\(\varphi = 2 \text{ mm}\)), and were measured at 23°C and 28°C, i.e. below and above the \(T_{cp} (\sim 24^\circ)\), respectively. The temperature of the samples was controlled using a water circulation bath Julabo F12-MC thermostat system. Due to heat loss inherent of the bath systems, the temperature of the sample inside the capillary was \(\sim 1^\circ\)C lower compared to the value given by the thermostat.

Model fitting of the SAXS curves

Model fitting was carried out using the NIST software implemented in Igor Pro [21]. Regarding the temperature and pH conditions used in SAXS experiments, it was expected to observe the collapse of the temperature-responsive block at \(T = 28^\circ\)C, and its dissolution at \(T = 23^\circ\)C, as well as the same behavior for the pH-responsive block at pH 10 and pH 6, respectively. Consequently, due to the high concentration of the polymer in solution (50 mg/mL), it was plausible to consider the formation of self-assembled structures, such as core-shell micelles, at \(T = 23^\circ\)C and pH 10, as well as at \(T = 28^\circ\)C and pH 6. Dissolved chains and aggregates were expected to be present in solution at \(T = 23^\circ\)C and pH 3, and at \(T = 28^\circ\)C and pH 10, respectively. Preliminary fits conducted using the model describing core-shell micelles, have shown not to be appropriate for the characterization of the scattering curves obtained for the conditions which core-shell micelles could be formed. Moreover, T-FCS results (discussed further on in Section 3) showed a direct transition from unimers to large aggregates for temperatures higher than the \(T_{cp}\), regardless of the pH. Thus, we suspect that the polymer is likely to be present as single chains or as small aggregates, rather than to self-assemble into organized structures. Therefore, the curves were fitted using the following function:

\[
I(q) = I_{PL}(q) + I_{OZ}(q) + I_{bg}
\] (5)

where \(I_{PL}\) denotes the forward scattering modeled by a power law (see below), \(I_{OZ}\) is the scattering from small aggregates modeled by the Ornstein-Zernicke (OZ) structure factor, and \(I_{bg}\) the background. The power law model characterizes the scattering intensity contributions of large polymer-rich aggregates above the \(T_{cp}\). In this characteristic type of scattering a strong forward scattering dominates the signal and the direct estimation of the radius is not possible. In our case, this behavior is described by a particular case of the general power law when \(\alpha=4\) (otherwise known as Porod law [22]), and reads

\[
I_{PL}(q) = \frac{I_0^{PL}}{q^\alpha} \implies I_{PL}(q) = \frac{2\pi(\Delta\rho)^2(S/V)}{q^4}
\] (6)

which give the characteristic \(q^{-4}\) behavior at low q-values. \(\Delta\rho\) is the contrast between the two phases (i.e. polymer aggregates and water) and S/V is the specific surface of the particles, i.e. surface to volume ratio. The scaling factor \(I_0^{PL}\) is proportional to the specific surface, which means that an increase in the particle size results in a decrease of the S/V. On the other hand, the contributions from the density fluctuations of small aggregates (or other structures, such as in polymer meshes, swollen micelles, etc) are described by the OZ structure factor [23] and reads,

\[
I_{OZ}(q) = \frac{I_0^{OZ}}{1 + (q\xi)^2}
\] (7)

where \(I_0^{OZ}\) is the amplitude of the scattering contribution, and \(\xi\) is the correlation length of the density fluctuations, in the present case it is the characteristic length between the chains compositing the small aggregates. Thus, in order to describe our system for temperatures below the \(T_{cp}\), where no large aggregates are present, the function \(I(q) = I_{PL}(q) + I_{OZ}(q) + I_{bg}\) was used, whereas for temperatures above the \(T_{cp}\) it was employed \(I(q) = I_{PL}(q) + I_{OZ}(q) + I_{bg}\) to account for the contributions of the small and large aggregates simultaneously.

3. Results and discussion

3.1. Polymer characterization after synthesis

1H NMR analysis was carried out on the DPA monomer, the PDPA homopolymer and on the PDPA-b-PME042MA diblock copolymer, in order to confirm the presence of those as reaction products of the synthesis (DPA) and RAFT polymerizations (PDPA and PDPA-b-PME042MA). In the spectra regarding the PDPA homopolymerization, the peaks from the protons of the methacrylate group of unreacted DPA monomer

\[
\text{Equation 4).
\]
were expected to appear, namely, from the vinyl (CH=CH(CH=CH2)) and the methyl (C=CH(CH3)) groups, as well as the peaks from the protons of the ethyl (-CH2CH2)- (a, b) and the tertiary amine diisopropyl (-CH(CH3)2) (c, d) groups. After polymerization, only the peaks from the protons of the vinyl group (v) of unreacted DPA monomers were expected to feature a lower intensity, by comparison to the peaks of the side groups (a, b, c and d), as that group is converted into bonds between newly polymerized monomers. As for the diblock copolymer spectra, the peaks of the PDPA homopolymer (a, b, c and d) should appear, with the addition of the peaks from the protons of the ethoxy groups (-(OCH2CH22)x)(f) and of the methoxy groups (-(OCH3)y). Once more, the peaks from the vinyl group (v') of the unreacted MEO2MA monomers should appear, featuring a low intensity relatively to the peaks of the side group (e, f).

Figure 2: 1H NMR spectra used for the characterization of the polymer. (a) PDPA homopolymer after the polymerization. (b) PDPA-b-MEO2MA copolymer at the end of polymerization. Peak integration values used to calculate the conversion of the PDPA and MEO2MA homopolymerization are shown under the selected peaks ((v, c) and (v', f), respectively). CDCl3 - deuterated chloroform (reference - 7.260 ppm); THF - trifluoroacetic acid; Diox - Dioxane.

In Figure 2 (a), the peaks corresponding to the PDPA homopolymer side groups (b, c and d) were located at 2.61, 2.97, and 3.37 ppm, respectively. As expected, the peaks of the vinyl group (v) from unreacted DPA monomers feature a reduced peak intensity, located at 6.11 and 5.55 ppm. Moreover, the peaks of the protons of the CTA aromatic ring (x and z) occur at 7.4-7.7 ppm, indicating that the CTA moiety remained at one end of the homopolymer. In addition, the presence of the used solvents (THF and dichloromethane DMC) does not appear to strongly affect the overall signal of the PDPA homopolymer. 1H NMR (400 MHz, CDCl3) δ: 6.11 (v, s, 1H), 5.55 (v, s, 1H), 3.54-3.7 (a, s, 2H), 2.97 (c, s, 2H), 2.61 (b, s, 2H), 0.98 (d, s, 12H).

Figure 2 (b) depicts the spectrum of the diblock copolymer where the typical peaks of the PDPA homopolymer (a, b, c and d) appear once more, in addition to the peak of the ethoxy protons (f) of the MEO2MA homopolymer side group (3.37 ppm) and of the vinyl protons from unreacted MEO2MA monomer (v'). In this case, the peak of the methoxy groups (e) overlapped with the one of dioxane (Diox) and therefore could not be assigned rigorously. 1H NMR (400 MHz, CDCl3) δ: 6.11 (v', s, 1H), 5.55 (s, 1H), 4.12 (a, s, 2H), 3.37 (f, s, 3H), 2.97 (c, s, 2H), 2.61 (b, s, 2H), 0.98 (d, s, 12H). Finally, the conversion values of each synthesis, as well as the DP and the Mn are compiled in Table 1.

3.2. Determination of the cloud-point temperature
The transmittance of the PDPA-b-MEO2MA diblock copolymer in water was evaluated as a function of temperature at pH 3, 6, 7 and 10, using polymer solutions with a concentration of 2 mg/mL. As we will see in Section 3.3, this concentration is high enough to verify the collapse of the polymer. Figure 3 shows the resulting transmittance curves. Both samples with a pH below the polymer pKa (~6.4), namely pH 3 and 6, behave similarly, presenting the same Tcp (22°C). As for the remaining samples, interestingly the Tcp increases with increasing pH, being 23°C for pH 7 and 24°C for pH 10. Overall, the phase transitions appear to be sharp, with the polymer in solution at pH 10 featuring a slightly broader one. Furthermore, phase separation was verified for all samples, which were indeed in the one-phase state, whereas visual inspection indicated its reversibility. Although there is no strong difference in the Tcp for the different pH values, these results are counterintuitive.

Figure 3: Normalized transmittance curves for the pH values given on the graph for a polymer concentration of 2 mg/mL. The considered Tcp for each sample is indicated.
Table 1: Characteristics of DPA monomer synthesis and RAFT polymerization of the PDPA homopolymer and PDPA-\(b\)-PMEO\(_2\)MA obtained from the PDPA as the macro-CTA.

<table>
<thead>
<tr>
<th>Reaction time (h)</th>
<th>Peak Label</th>
<th>PAR(^a) (%)</th>
<th>Conv.(^b)</th>
<th>[CTA]_0/([\text{Monomer}]_0)</th>
<th>[CTA]_0/([\text{Monomer}]_0)</th>
<th>DP(^c)</th>
<th>(M_n) (g/mol)</th>
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</thead>
<tbody>
<tr>
<td>DPA</td>
<td>2</td>
<td>—</td>
<td>59(^d)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PDPA</td>
<td>24</td>
<td>v/c</td>
<td>1.0/82.2</td>
<td>98</td>
<td>10</td>
<td>50</td>
<td>10 725(^e)</td>
</tr>
<tr>
<td>PDPA-(b)-PMEO(_2)MA</td>
<td>24</td>
<td>v/f</td>
<td>1.0/7.2</td>
<td>62</td>
<td>10</td>
<td>100</td>
<td>50-62 22 018(^f)</td>
</tr>
<tr>
<td>PMEO(_2)MA</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>62</td>
<td>11 293</td>
</tr>
</tbody>
</table>

\(^a\) PAR - Peak area ratio
\(^b\) Calculated from the areas under the peaks of the characteristic signals from monomer and polymer in \(^1\)H NMR spectra:
Conversion (%) = 1 - PAR
\(^c\) DP = ([Monomer]_0 / [CTA]_0) \times conv.(
\(^d\) DPA monomer synthesis: Conversion (%) = ([Reagents]_0 / [DPA]) \times 100
\(^e\) \(M_n\) \(=\) DP \(\times\) M(PDPA monomer) + M(CTA) (M(CTA) = 272.22 g/mol)
\(^f\) \(M_n,\text{total}\) \(=\) DP \(\times\) M(PMEO\(_2\)MA monomer) + M(PDPA)

assuming that this behavior is related to an increase in solubility, which may not be given by the pH-responsive PDPA, we should consider the effect of the high pH in solution on the temperature-responsive PMEO\(_2\)MA block. In fact, an increase of the \(T_m\) of PMEO\(_2\)MA homopolymers (\(M_w = 6 280 \text{ g/mol} \) and \(20 380 \text{ g/mol} \)) from neutral (pH 7) to alkaline (pH 10) conditions in solution of 1-2\(^\circ\)C has been reported [24]. The authors stated that this effect was most likely due to slightly stronger interactions between the ethylene glycol (i.e. methoxymethoxy) chains of the polymer and the hydrogen ions (OH\(^-\)) present in solution, when compared to water molecules. This phenomenon was also verified by another group [25], who investigated the long-ranged electrostatic forces that may be related to the known anti-fouling properties of ethylene glycol-terminated self-assembled monolayers (SAMs). They concluded that these forces can also be explained by the negative charges originating from preferential hydroxide ion adsorption to the methoxy group of the ethylene glycol, which leads to the penetration of water molecules into the SAM in order to stabilize the adsorbed hydroxide ions (arising from water autoionization or added to the solution). Conversely, we can speculate that at higher pH values, there might be an increased adsorption of hydroxide ions on the methoxy end groups of the chains of the PMEO\(_2\)MA block, which acquire a negative charge that may be keeping them hydrated (i.e. hydrophilic) at higher temperatures, thus causing an increase in the \(T_m\). Nevertheless, the effect of the pH and temperature on the polymer under study will be further evaluated in the next experiments.

3.3. pH dependence of the aggregation behavior at room temperature

FCS experiments allowed for an insight of the effect of the pH in solution on the aggregation behavior at room temperature. Because the measurements were performed below the \(T_m\) of the temperature-responsive PMEO\(_2\)MA block, it was expected to be hydrophilic for all pH values. The pH-responsive PDPA block is protonated (i.e. positively charged), and thus hydrophilic at pH values below its pKa (~6.4), whereas above it is deprotonated, being less hydrophilic. Thus, at pH values above the pKa of the PDPA and for polymer concentrations above the CMC, the formation of core-shell micelles may be detected, having a deprotonated and, therefore, collapsed PDPA core and a hydrophilic PMEO\(_2\)MA shell. As depicted in Figure 4 (a), for polymer concentration up to 0.1 mg/mL, a single, fast decay component is present in the FCS autocorrelation functions, except for the polymer solutions at pH 10, where a single decay component was sufficient up to 0.4 mg/mL.

The mean hydrodynamic radii for all pH values, derived using Equations 2 and 3, is found to be \(\bar{r}_H = 0.77 \pm 0.07 \text{ nm} \), which is close to the size of pure Rh6G alone (0 mg/mL of polymer concentration), and thus it was assigned to correspond to freely diffusing Rh6G molecules. As the polymer concentration is increased (typically above 0.1 mg/mL), a second, slower decay component is observed at higher correlation times, indicating additional slower diffusional process, which was attributed to a slower diffusional process corresponding to larger structures with incorporated Rh6G dye. In this case, the resulting mean hydrodynamic radii, for each pH value, were \(\bar{r}_H = 2.61 \pm 0.06 \text{ nm} \) (pH 3), 2.43 \(\pm\)0.06 nm (pH 6), 2.45 \(\pm\)0.03 nm (pH 7) and 2.25 \(\pm\)0.17 nm (pH 10). Because these are comparable with the sizes of unimers in similar temperature- and pH-responsive diblock copolymers [12], they were attributed to unimers (single dissolved polymer chains). Thus, for low polymer concentrations only freely diffusing Rh6G molecules are mainly detected, which above 0.1 mg/mL start to interact in a greater extent with the unimers in solution. Consequently, although a transition is present at polymer concentrations between 0.1 and 0.4 mg/mL, there is no indication of micelle formation. Furthermore, it is observed that, at the highest polymer concentrations (0.8-1.2 mg/mL), the hydrodynamic radii in the acidic solutions (pH 3) are slightly higher than the ones at the higher pH values (pH 6-10). This may be a consequence of the electrostatic repulsions arising from the fully protonated tertiary amine group at pH 3. On the other hand, Rh6G requires higher polymer concentrations to...
block at pH 10 should favor the adsorption of the Rh6G dye molecules to it, by comparison at pH 3 where both blocks are hydrophilic. This may be explained by recalling the hypothesis proposed in the previous turbidimetry results - an increase on the hydroxide ions concentration may hydrate the chains of the PMEO$_2$MA block, increasing the overall solubility of the polymer. This could also explain a lower affinity of the water-insoluble Rh6G dye molecules to the unimer at pH 10, as a solvation layer in the PMEO$_2$MA block may be restricting the assess of Rh6G to the deprotonated PDPA block, which in turn may be enclosed within the former. Thus, it is likely that the hydrodynamic radii obtained for the unimers at pH 10 are simply a consequence of the lack of proper interaction between the probe and the polymer, and therefore may not represent its true dimensions, by defect. Thus great care has to be taken on the analysis of the results from FCS. To put in perspective, it seems that the transition from acidic to alkaline pH alone, at room temperature, is causing the blocks to change their properties mutually, i.e. the same conditions that make the PDPA block undergo deprotonation give charge to the PMEO$_2$MA, and vice-versa.

3.4. Temperature dependence of the aggregation behavior

T-FCS was attempted to evaluate how the polymer behaves, for different pH conditions, when the temperature is increased above the $T_{c_p}$ of the temperature-responsive PMEO$_2$MA block, when it becomes hydrophobic. Generally speaking, all samples show two regimes where, below a certain temperature, the autocorrelation curves could be reasonably fitted with a double decay, showing nearly identical behavior. As soon as the $T_{c_p}$ is crossed for each pH value, the autocorrelation functions featured an additional slow and broad decay component which were attributed to the temperature-induced large aggregates. These perturbed substantially the data quality. The autocorrelation curves measured by the temperature-resolved FCS at room temperature yielded higher values of the hydrodynamic radii and lower fractions of the slow decay component compared to the ones obtained in the room temperature FCS experiments (crossed markers in Figure 5). The values of the latter seem to be more reliable because they were measured in the usual way using the water immersion objective, water as an immersion fluid and the dedicated NUNC chambers having a glass slide as the support. Whereas for temperature-resolved FCS measurements, an oil immersion objective, immersion oil and ITO-coated glass slides were used which tend to bend under their own weight. All these may lead to distortions of the light path and on the detection volume, thus causing erroneous results. Nevertheless, useful information could still be extracted by a general analysis of the resulting hydrodynamic radii and the fraction of the slow decay component (Figure 5).

The $T_{c_p}$ was considered to be the temperature corresponding to the last curve allowing for acceptable fitting, before the formation of aggregates - namely, 25°C (pH 3 and 6), 25°C (pH 7) and 25.5°C (pH 10). These $T_{c_p}$ values were in the same range of the ones

![Diagram](image-url)

Figure 4: Results from FCS measurements of the PDPA-b-PMEO$_2$MA solutions at pH 3 (green squares), 6 (blue diamonds), 7 (red circles) and 10 (black triangles) at room temperature. Fixed values of free Rh6G, used as the fast decay when two diffusional decays were employed, are shown (open triangles). (a) Hydrodynamic radii. Magenta arrows indicate the lowest concentration for which two diffusional decays were needed for proper fitting of the autocorrelation curves - of the solutions with pH 3, 6 and 7 (left arrow) and pH 10 (right arrow). (b) Relative amplitude of the slow decay. (In some cases, error bars are smaller than the symbol size.)

attach to unimers at pH 10, whereas the fraction of the slow decay component (Figure 4 (b)) is considerably lower than for the solutions at lower pH values. This means that the equilibrium between the freely diffusing Rh6G molecules and the ones adsorbed to the unimers may be biased towards the former, and may be reverted only at higher unimer concentrations. Thus, the poorly water soluble Rh6G molecules will not be always bound to the unimer, featuring periods of free diffusion, which decreases on average the time that the labeled unimer was recorded to diffuse through the detection volume. This may be the reason why the hydrodynamic values at pH 10 for polymer concentrations above 0.4 mg/mL have considerably larger errors associated (Figure 4 (a)). Consequently, there is strong evidence that the interaction between the Rh6G dye molecules and the polymer chain is impaired at pH 10. Since only the pH of the solution was changed, the deprotonation of the PDPA...
are guides to the eye.

Results from temperature-resolved FCS measurements of a 0.8 mg/mL polymer solutions at pH 3 (green squares), 6 (blue diamonds), 7 (red circles) and 10 (black triangles). Open markers correspond to the last autocorrelation curve to be acceptably fitted before the respective transition temperature is reached. Crossed markers show the hydrodynamic radii/fraction of the slow decay of the 0.8 mg/mL solution from the FCS measurements at room temperature, for comparison. Dashed lines between markers are guides to the eye. (a) Hydrodynamic radii. (b) Relative amplitude of the slow decay. (In some cases, error bars are smaller than the symbol size).

Figure 5: Results from temperature-resolved FCS measurements of a 0.8 mg/mL polymer solutions at pH 3 (green squares), 6 (blue diamonds), 7 (red circles) and 10 (black triangles). Open markers correspond to the last autocorrelation curve to be acceptably fitted before the respective transition temperature is reached. Crossed markers show the hydrodynamic radii/fraction of the slow decay of the 0.8 mg/mL solution from the FCS measurements at room temperature, for comparison. Dashed lines between markers are guides to the eye. (a) Hydrodynamic radii. (b) Relative amplitude of the slow decay. (In some cases, error bars are smaller than the symbol size).

obtained in turbidimetry measurements. In addition, despite the small differences, the $T_{cp}$ increased with the increasing of the pH in solution (open markers in Figure 5). Furthermore, larger aggregates are found in more acidic solutions, where the largest are found at pH 3, due to the electrostatic repulsions arising from the protonated PDPA block. The smallest aggregates are found at pH 10, however, contrary to the room temperature FCS measurements (Section 3.3), there should be no adsorption of hydroxide ions onto the temperature-responsive PMEO$_2$MA block, likely due the release of the adsorbed hydroxide ions, and the surrounding water molecules, upon the collapse of the temperature-responsive PMEO$_2$MA block at temperatures above the correspondent $T_{cp}$. Thus, at pH 10 above $T_{cp}$, both blocks are collapsed leading to the formation of smaller aggregates. In addition, as depicted in Figure 5 (b), at temperatures higher than 24°C, the fraction of the slow decay component is comparable across the different pH values, hence we can rule out interaction issues between the Rh6G dye molecules and the aggregates, which confirms that, for high temperatures, the size of the formed aggregates depends mainly on the presence or absence of electrostatic repulsions from the pH-responsive PDPA block.

3.5. Structure dependence on temperature and pH

SAXS experiments allowed a deeper insight into the mechanism of aggregation and the structure of the polymer under study at the local scale, for temperatures around the $T_{cp}$ and the pKa value. The resulting scattering curves are shown in Figure 6. From model fitting analysis it was observed that below the $T_{cp}$, the polymer associates into small aggregates described by the OZ structure factor which characterized them with a correlation length of 3.22±0.04 nm and 3.83±0.04 nm for pH 6 and pH 10, respectively. Furthermore, OZ structure factor was sufficient to characterize the scattering curves of the polymer under alkaline conditions for both temperatures, meaning that 28°C was not enough...
to cause the complete collapse of the temperature-responsive PMEO2MA blocks at pH 10 (i.e. its $T_{cp}$ was not crossed), whose small aggregates featured a correlation length of 3.05±0.07 nm. Overall, the correlation length of the small aggregates was mainly influenced by the electrostatic repulsions of the protonated pH-responsive PDPA block under acidic conditions, and by the increased hydrophilicity of the PMEO2MA under alkaline conditions, which we indicate as the underlying reason for the difference in the correlation length of the small aggregates between pH 6 (3.22±0.04 nm) and 10 (3.82±0.04 nm). On the other hand, the difference in the correlation length obtained under alkaline conditions at 23°C (3.82±0.04 nm) and 28°C (3.05±0.07 nm) was attributed to the greater extent of the collapse of the temperature-responsive PMEO2MA blocks, in the latter case. Above the $T_{cp}$, reached only at pH 6, an additional power law was necessary to describe the higher forward scattering, as a result of temperature-induced large aggregates, whereas the co-existing small aggregates yielded a correlation length of 2.61±0.41 nm, being the lowest value of all the tested conditions. We argue that this was due to fewer water molecules interacting with the polymer chains, i.e. increased hydrophobic domains, as a result of the collapse of the temperature-responsive PMEO2MA blocks, which through hydrophobic interactions lead, ultimately, to the formation of large aggregates. However, detailed information about them was not extracted due to the limited q-range of the scattering curves. Furthermore, we did not found evidences of the influence of the electrostatic interactions in the size of the small aggregates above the $T_{cp}$, as it was not crossed at pH 10. Nevertheless, the results from the SAXS experiments confirmed the effects of the pH in solution on both the pH-responsive PDPA and temperature-responsive PMEO2MA block on the size of the small aggregates, with the latter contributing in a greater extent for the overall solubility of the polymer chains. At high temperatures, the size of the aggregates was mainly determined by the temperature-responsive PMEO2MA block collapse, which did not occur under alkaline conditions.

4. Conclusions
RAFT polymerization proved to be a suitable method to synthesize diblock copolymers from DPA and MEO2MA monomers, using 3-(benzylsulfanylthiocarbonyl) pro-pionic acid as CTA. The $T_{cp}$ of the temperature-responsive PMEO2MA block was firstly assessed via turbidimetry analysis. We found that its $T_{cp}$ increased for neutral and alkaline conditions. We attributed this behavior to an increased hydrophilic degree of the temperature-responsive PMEO2MA block. Thus, the influence of the pH in solution was found to be a key point on the aggregation behavior of our polymer for temperatures near the $T_{cp}$. From the FCS measurements, under different pH conditions at room temperature, we found that only unimers were detected within the range of polymer concentration tested. Furthermore, the influence of the pH in solution also influenced the aggregation behavior of our polymer, which was manifested by the hydrodynamic radii obtained. While electrostatic repulsions seem to have slightly influenced the hydrodynamic radii of the unimers at low pH, the weak interaction between the fluorescent dye and the unimers, under alkaline conditions, were considered to be an issue that impaired the proper determination of the hydrodynamic radii of the unimers. Furthermore, using a custom-made sample holder based on heatable ITO glass slips, the temperature influence on the aggregation behavior of our polymer near the $T_{cp}$ was evaluated for different pH conditions with temperature-resolved FCS measurements. We observed the formation of large aggregates for temperatures above the $T_{cp}$, manifested through the reduced quality of the FCS signal. Nevertheless, we observed the formation of the large aggregates above the $T_{cp}$, whose dimensions increased with decreasing pH in solutions due to the electrostatic repulsions. In addition, there was no indication of lack of Rh6G dye molecules adsorption with the formed aggregates above the $T_{cp}$. Moreover, we verified the increase of the $T_{cp}$ for neutral and alkaline conditions, as observed with the results from the turbidimetry measurements, thus confirming a successful proof of concept of the temperature-resolved FCS setup. SAXS measurements have shown that the polymer chains associate into small aggregates, regardless of the temperature or pH conditions. For temperatures above the $T_{cp}$, large polymer aggregates were formed, which were not subject of characterization due to the limited q-range. However, the $T_{cp}$ was only crossed for the polymer under acidic conditions, whereas for the remaining cases, the characteristic length of small aggregates was mainly dependent on the pH in solution, whether due to the electrostatic repulsions under acidic conditions or to the increased hydration of the temperature-responsive PMEO2MA block under alkaline conditions. With this work, we conclude that the temperature- and pH-responsive PDPA-b-PMEO2MA diblock copolymer featured sensitivity to both stimuli, and that its $T_{cp}$ is susceptible to modulation with the pH, which may be further used as an additional variable for the design of novel DDS. The completion of this investigation was a worthy and important goal to pursue, and it is the source of much motivation for future studies.

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