Understanding the temperature-responsive self-assemblies of amphiphilic random copolymers by SANS in D_2O solution

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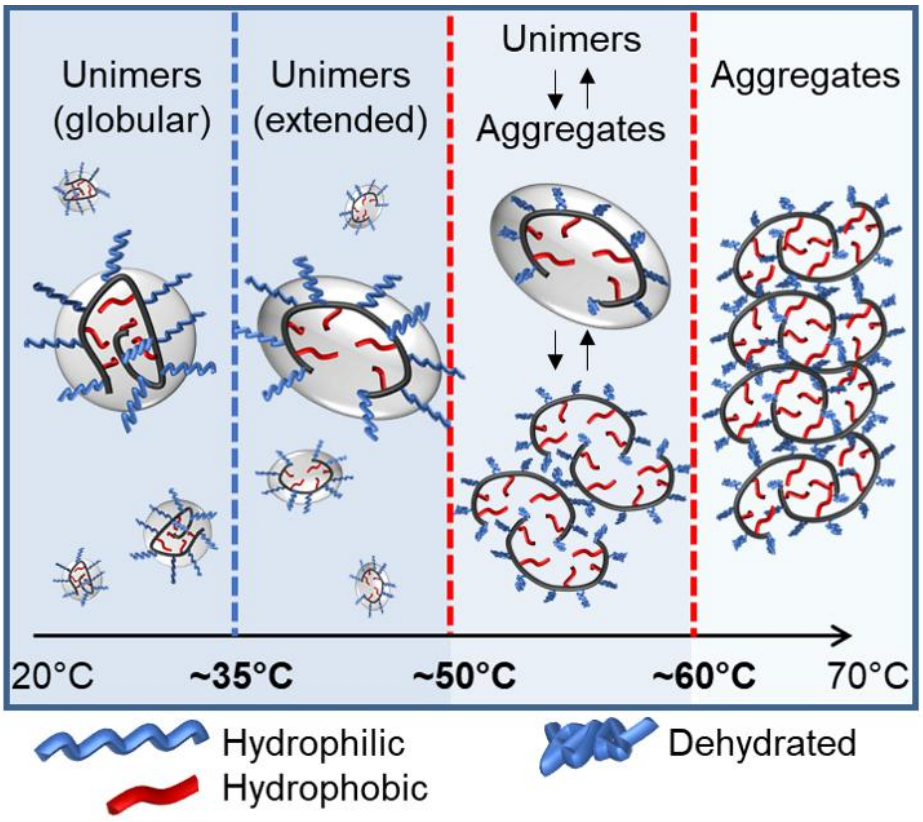
Abstract

The self-assembly behavior, in aqueous solution, of amphiphilic methacrylic random copolymers with perfluoroalkyl and polyoxyethylene side chains and the morphology of the resulting nanostructures were investigated by small angle neutron scattering (SANS). The results allowed to clarify the effect of temperature on the self-assembly features of copolymers and the structural morphology of both unimer single chain entities and larger multi-chain aggregates of unimers. Shape, flexibility, and size of the aggregates were proved to be modulated by temperature and copolymer composition. The existence of three distinct phases of self-assembled structures was pointed out as a function of increasing temperature: i) globular unimers; ii) co-existence of extended unimers and aggregates; iii) aggregates, that overall showed full reversibility in a wide range of copolymer concentrations.



Focus on supramolecular, polymeric structures leading to complex architectures has been directed from both the physico-chemical and biophysical stand points. Hydrophilic/hydrophobic interactions at the base of self-folding and self-assembling processes are enabled in synthetic polymers, in a way of mimicking the behavior of natural polymers.[1-5]

Amphiphilic polymers self-assemble in water as a result of a delicate balance of hydrophobic and hydrophilic interactions determining hierarchical structures on different length scales, from unimer single-chain entities to larger globular aggregates.[2] Exploiting the influence of composition and temperature on the size, shape and conformation of these self-assembled architectures in the water phase may pave the way for a novel "green nanochemistry" with huge potential, in e.g. environmental chemistry to combat biofouling, industrial catalysis of fine chemicals, selective drug delivery to specific targets.[3,4,6]

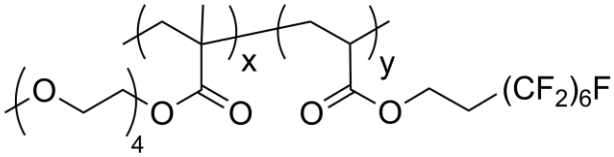
Structural motifs of polymer backbones with different degrees of hydrophobicity have been reported,[7-10] such as those including alkyl, fluoroalkyl and siloxane side chains. In particular, the inclusion of low surface energy fluoroalkyl side chains into a polymer chain is known to drastically enhance the hydrophobic and lipophobic nature of the entire system, which results in a strong capability to self-assemble in solution, in bulk and at the surface of thin films.[7-10] Hydrophobic monomers can be randomly copolymerized in the presence of a large excess of hydrophilic monomers, mainly consisting of poly(ethylene glycol) chain segments. In such arrangements self-assembled nanostructures from the association and/or self-folding of the amphiphilic copolymer chains have recently been predicted[9,10] due to the presence of perfluoroalkyl hydrophobic sequences.

In this study, the amphiphilic behavior of two random copolymers from hydrophobic 2-(perfluorohexyl)ethyl acrylate (FA) and hydrophilic poly(ethylene glycol) methyl ether methacrylate (PEGMA) in PEGMA/FA mole ratios of 70/30 and 90/10 was explored. The ability of these random copolymers PEGMA-*co*-FA to self-fold into single-chain nanoassemblies in water was predicted by molecular dynamics (MD) simulations and experimentally supported by dynamic

light scattering and fluorescence emission investigations.[11] The MD simulations showed that self-folding of the copolymer in water leads to nanostructures where the hydrophobic units become more buried in the core of the globule causing the collapse of the copolymer. However, the structural and environmental constraints, such as counit sequencing, chain length, temperature and solvent, allow for different arrangements of PEGMA-*co*-FA to be adopted. Moreover, alongside the self-folding process of single copolymer chains or unimers, recent dynamic light scattering (DLS) studies evidenced the presence of micron-sized structures associated with the aggregation of unimers.[12] The intermolecular interaction among PEGMA-*co*-FA unimers might be triggered at a critical temperature (T_c), reminiscent of the lower critical solution temperature (LCST) in water of the corresponding PEGMA homopolymers.[12] According to DLS findings, beyond T_c the unimers coalesce into micrometric scale, narrowly dispersed aggregates leading to thermoresponsive and chemical versatile multi-compartment particles with potential for bio-conjugation of proteins and enzymes, for transport and targeting of either hydrophobic or hydrophilic drugs. The dependence on temperature of the suggested formation of aggregates, as well as the possible influence of concentration and other structural constraints of the amphiphilic copolymer have not been fully disclosed and are sometimes conflicting. In this frame, a systematic static small angle scattering study assessing the behavior of amphiphilic copolymer chains in aqueous solution can provide a reliable ground which is lacking to date.

Small angle neutron scattering (SANS) methodology[13–15] offers an opportunity to enrich and eventually clarify the information available in this context. The high contrast and adequate resolution of this technique enables to follow the form factor, the surface, the stability and reversibility features that testify to the conformational change of the PEGMA-*co*-FA self-assemblies when they are either individually self-folded in unimers with a nanometric scale length (so called unimer micelles) or clustered together into aggregates with micro-sized super-structures. The potential of SANS for studying such systems was previously demonstrated.[12] In this work we provide an in-depth SANS study on the copolymers PEGMA70-*co*-FA30 and PEGMA90-*co*-FA10

in a polymer concentration from 1 to 10 mg/mL in D_2O, as a function of temperature in a range from 20°C to 70°C. The explored concentrations and temperatures allowed an investigation to be performed on the unimer, intermediate and aggregate regimes, thus clarifying the influence of temperature on the structural morphology and self-assembly features of PEGMA-*co*-FA unimers.



PEGMAx-*co*-FAy

Scheme 1. Chemical structure of the amphiphilic copolymers PEGMAx-*co*-FAy (x/y = 90/10 and 70/30 mol/mol composition).

The chemical structure of the amphiphilic copolymers PEGMA90-*co*-FA10 (M_n = 34300 g/mol, $Đ$ = 1.89) and PEGMA70-*co*-FA30 (M_n = 28900 g/mol, $Đ$ = 1.43) is shown in Scheme 1. For details on synthesis and characterization of copolymers, see ESI (section S1).

The absolute average molecular weights of the copolymers were previously evaluated by size-exclusion chromatography (SEC) experiments with a triple detection calibration system and universal calibration in different organic solutions.[12] Values of the Mark-Houwink slope α were 0.43–0.46 for copolymer solutions in tetrahydrofuran and chloroform, typical of random coil polymer chains in relatively good solvents; lower values (α = 0.37–0.39) were found for copolymer solutions in dimethylformamide (10 mM LiBr), which suggests adoption of more compact polymer conformations in this solvent. By contrast, reliable values of molecular weights and viscometry parameters (α and log$K$) could not be obtained for the two copolymers in either water (0.02 wt% NaN_3) or water/methanol (90/10 v/v) solution. This could be due to a poor solvation in those SEC conditions, owing to the enhanced hydrophobic nature of the self-folded nanostructured copolymers. Nonetheless, combined ^{1}H NMR and SEC measurements enabled reliable

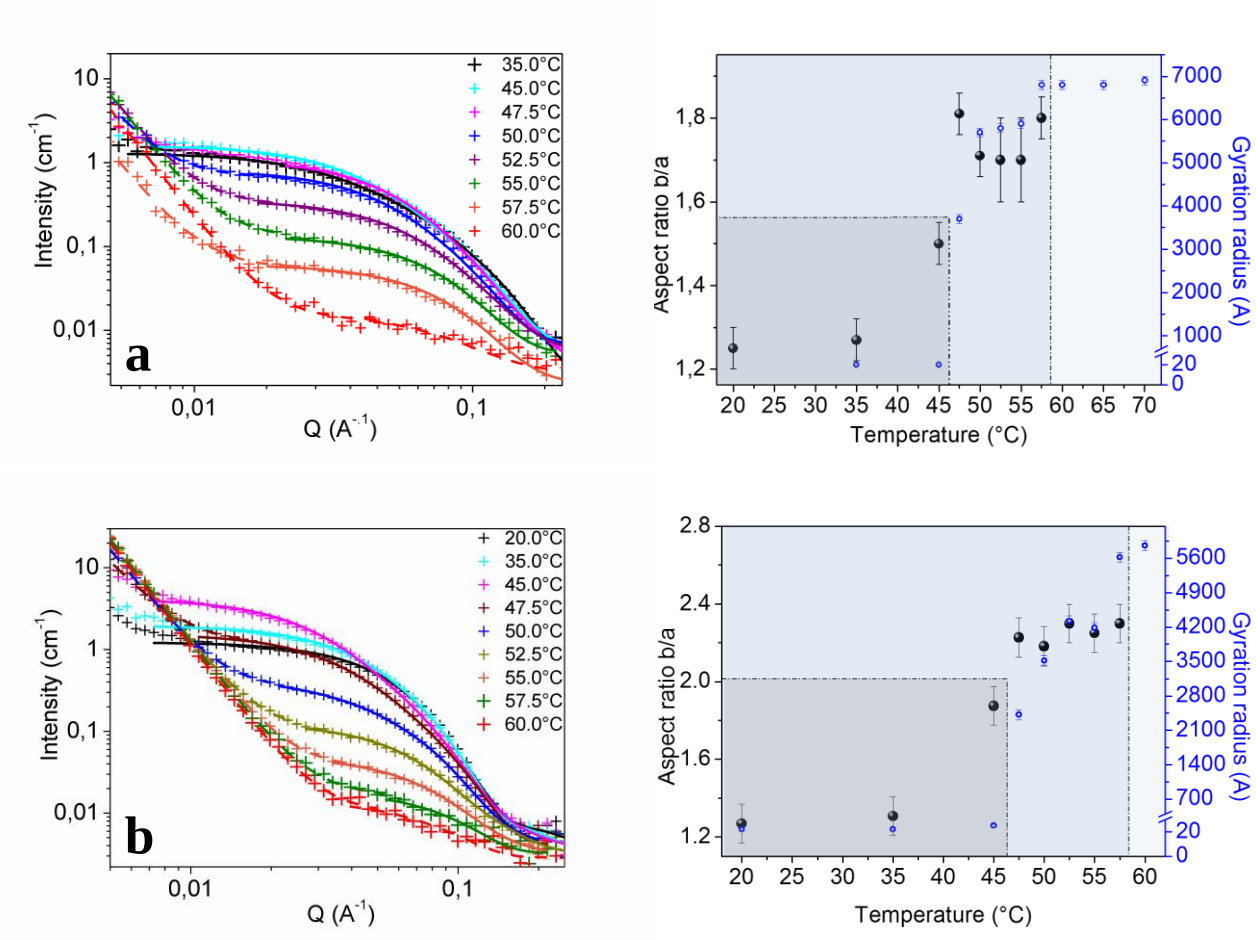
characterization of closely related analogue copolymer PEGMA77-*co*-FA23 in solutions.[11] The values of M_n determined by ^{1}H NMR in water (37,200 g/mol) and in both chloroform and acetone (34,000 g/mol) ($M_{n(chloroform)}/M_{n(water)} \approx 0.9$) confirmed adoption of different conformations by the copolymer in water and organic solutions, consistent with the existence of single-chain folded, unimer nanostructures in the former solvent. Thus, it appears that copolymers PEGMA90-*co*-FA10 and PEGMA70-*co*-FA30 form unimer nanostructures in water solution, as opposed to more conventional random coils in organic solutions.[16]

The complete set of measurements at varying concentration and temperature of copolymer solutions is reported in section S2. Through a first screening (Figures S1 and S2) it was possible to ascertain that SANS profiles of unimers are stable in time and reproducible, with the intensities scaling with polymer concentration (section S2.1), and also showing significant reversible variations with temperature (section S2.2). For both copolymers, the overall changes were observed to fall in the temperature range of 20–60°C, independent of the copolymer composition and concentration. Specifically, the SANS profiles show that the shape and size of both PEGMA90-*co*-FA10 and PEGMA70-*co*-FA30 change gradually with temperature, including early fine shape changes, overall reflecting the entire process of reversible unimer association into aggregates. A decrease in scattering intensity is observed in the SANS profiles recorded at the highest sample concentrations (≥ 7 mg/mL) when kept for a long time at 65°C and 70°C (and consequently, in the corresponding profiles obtained at 35°C after the cooling cycle), because of a loss of the aggregates solubility (Figure S1 and Figure S2). The quantitative analysis was thus performed on profiles recorded for copolymer solutions up to 5 mg/mL and temperatures up to 60°C where the polymer conformational changes are completed in solution while preserving their full reversibility.

Kratky plots, shown in section S2.3, confirm that the conformation of the copolymers in solution is influenced by temperature and copolymer composition rather than copolymer concentration. According to the plots of Figure S6, the bell-like shaped curves are consistent with the presence of unimers exhibiting self-folded, compact (low flexible), globular conformation with size (i.e.,

pseudo-Guinier radius, R_{pg}) of ~3 nm. On raising temperature up to ~50°C, the shifts of the plots only indicate an apparent increase in size of the unimers, this effect being more evident in the copolymer with higher FA content. Above 50°C, the intensity of Kratky plots associated with the unimers decreases progressively and the changes in SANS profiles of both copolymers clearly also involve the low-Q region (where the SANS intensity instead increases), reflecting a co-existence of free unimers and aggregates (i.e., unimers connected to each other) in D_2O solution. We then detected a structural T_c value above which both copolymers were found to be in an aggregate conformation only, whose shape and size were unaffected by copolymer concentration in the investigated range.

In order to assess accurately the influence of temperature on size and shape of the copolymers in the single unimer and self-aggregation states, a comparative analysis of the SANS profiles according to ellipsoid and Guinier-Porod models was performed. The representative SANS profiles of PEGMA90-*co*-FA10 and PEGMA70-*co*-FA30 at varying temperature together with the best fit are superimposed in Figure 1a and 1b. The extracted information is reported in Table 1 and Table 2,



respectively.

Figure 1. Left: SANS profiles of PEGMA90-*co*-FA10 (a) and PEGMA70-*co*-FA30 (b), dissolved in D_2O at 5 mg/mL concentration, together with best fits according to ellipsoid and Guinier-Porod models (solid and dashed line, respectively); Right: values of the semi-major to semi-minor axis ratio b/a and radii of gyration as a function of temperature in double y plots. A sequence of three distinct states is disclosed depending on temperature: unimers only ( ); equilibrium of unimers and aggregates ( ); aggregates only ( ).

Table 1. Values of the fit parameters from SANS profiles of PEGMA90-*co*-FA10 relating to unimers from ellipsoidal model (Ell. Param.: a and b ± fit tolerance), and to the aggregates from Guinier-Porod model (GP. Param.: R_g, m, Dim ± fit tolerance)). Reduced χ^2 are in the range 0.06-0.09. "c" label indicates the cooling cycle temperatures. See also section S1.

T (°C) / Ell. Param.	20.0	35.0	45.0	47.5	50.0	52.5	55.0	57.5	60	55 c	45 c	35 c
Radius a (±1 Å)	20	18	18	16	17	18	18	16	-	17	18	18
Radius b (±1 Å)	25	23	27	29	29	30	30	29	-	30	28	25
Rg (±1 Å)*	18	17	19	20	20	21	21	20	-	20	19	18

Scattering length density of the copolymer: $1.366 \cdot 10^{-6}$ Å^{-2}; *from an oblate spheroid

T (°C) / GP. Param.	47.5	50.0	52.5	55.0°C	57.5	60.0	65.0	70.0	55 c
Rg (±100Å)	3700	5700	5800	5900	6800	6800	6800	6900	6000
m (±0.2)	3.7	4.0	4.0	4.1	4.3	4.2	4.2	4.0	3.9
Dim (±0.1)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Table 2. Values of the fit parameters from SANS profiles of PEGMA70-*co*-FA30 relating to unimers from ellipsoidal model (Ell. Param: a and b ± fit tolerance;), and to the aggregates from Guinier-Porod model (GP. Param.: R_g, m and Dim ± fit tolerance). Reduced χ^2 are in the range 0.06-0.09. "c" label indicates the cooling cycle temperatures. See also section S1.

T (°C) / Ell. Param.	20.0	35.0	45.0	47.5	50.0	52.5	55.0	57.5	60	55 c	45 c	35 c
Radius a (±1 Å)	26	26	24	22	22	20	20	20	-	20	24	26
Radius b (±1 Å)	33	34	45	49	48	46	45	46	-	46	45	34
Rg (±1 Å)*	22	22	25	26	26	24	24	24	-	20	19	22

Scattering length density of the copolymer: $1.718 \cdot 10^{-6}$ Å^{-2}; *from an oblate spheroid

T (°C) / GP. Param.	47.5	50.0	52.5	55.0	57.5	60.0	55 c
Rg (±100 Å)	2400	3500	4300	4200	5600	5900	4200
m (±0.2 Å)	3.7	4.0	4.0	4.0	4.2	4.2	3.9
Dim (±0.1)	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Within their existence domain, unimers exhibit slightly oblate spheroid shape that we named "globular folding" whose major to minor semi-axes aspect ratio, b/a, depends on their composition and temperature. The formation of globular folded structures in aqueous solution was in agreement with the computational analysis of asphericity and solvent-accessible surface area.[11] According to those simulations, the globular folding of the unimer micelles in water is predicted to be driven by the intra-polymer interactions among perfluorohexylethyl side chains of PEGMA-*co*-FA copolymers.

In Figure 1, the b/a ratio is plotted as a function of temperature for both copolymers. At the lower temperatures, both copolymers present a quite compact structure. Noteworthy, the values of the aspect ratio b/a parameter gradually increased with increasing temperature and were higher for the

copolymer containing the higher amount of FA, consistent with the Kratky analysis (Figure S6). Such an increase suggests that a change in the folding of unimers occurs. More in detail, on increasing temperature from 20°C to 47.5°C, unimers of both PEGMA70-*co*-FA30 and PEGMA90-*co*-FA10 adopt a progressively less compact, slightly oblate shape due to a weakening of the intra-polymer interactions among the perfluorohexylethyl side chains. Above the latter temperature, according to the Guinier-Porod analysis the formation of aggregates takes place, thought to be triggered by the breaking of hydrogen bonds between water and hydrophilic oxyethylene side chains.[11] This suggests that b/a is a valuable indicator to trace the precursor changes of copolymer folding in favor of an interacting multi-unimer assembly.

Additional information on the chain intrinsic flexibility of the two copolymers at temperatures where they are stable in aqueous solutions as unimers can also be derived from the Kratky plot shown in Figure 2, providing the Q value, Q*, where the scattering law changes from that of an overall globule chain to a rigid rod segment.[17]

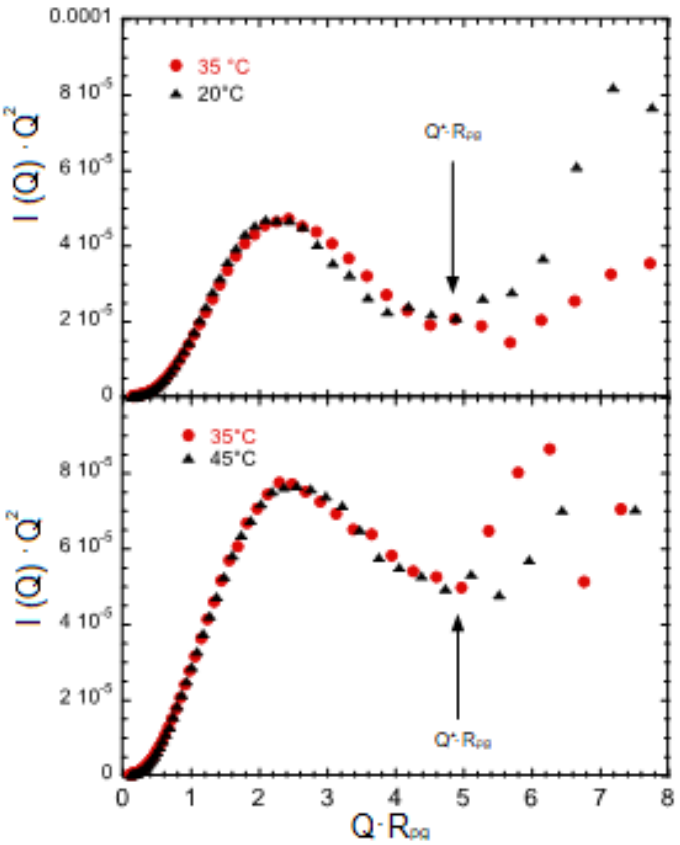


Figure 2. Kratky plots for PEGMA70-co-FA30 (upper plot) and PEGMA90-*co*-FA10 (lower plot) copolymers. Arrows indicate the Q* values of intersect between the profile of a globular chain structure and that of a rigid rod segment linked to the Kuhn length.

This threshold is linked to the length of the Kuhn segment, l_k, according to $Q^*=12/\pi l_k$.[17] From the contour length, L_c, determined from the average number of PEGMA and FA counits in the polymer chain, with a length of the PEGMA-*co*-FA average repeating unit of 2.4 Å, the number of Kuhn elements, N_k, can be determined. Table 3 summarizes the semiflexible coil parameters of the two copolymers.

Table 3. Structural parameters of the copolymers in solution as unimers

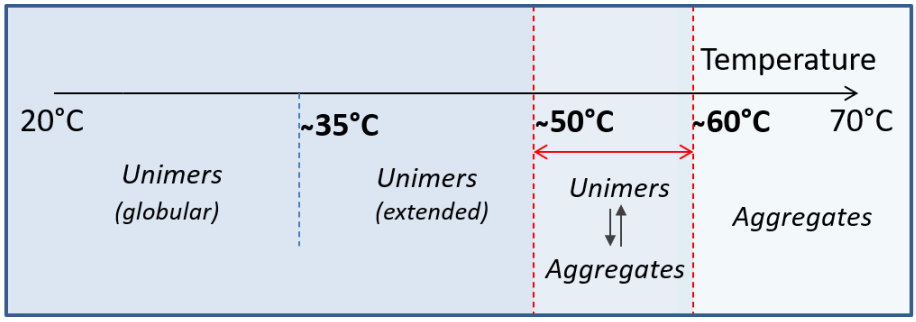
Copolymer	Q* ($Å^{-1}$)	L_c (Å)	l_k (Å)	N_k
PEGMA70-*co*-FA30	0.150	264	25.5	10.4
PEGMA90-*co*-FA10	0.175	330	22.0	15.0

As summarized in Table 3, PEGMA70-co-FA30 shows a slightly larger Kuhn length than PEGMA90-co-FA10, indicating a comparatively higher backbone stiffness, in agreement with the pseudo-Guinier radius, R_{pg}, evaluated from the Q_{max} of the Kratky plots (see also section S2.3).

As reported in more detail in section S2, the empirical Guinier-Porod model can be employed for simple particle shapes like spheres, cylinders or lamellae, and it was herein employed to derive information on the supramolecular assembly of unimers.

The overall analysis (Figure 1, Table 1 and Table 2) indicates that multi-chain aggregates are formed by the packing of unimers in extended (i.e., less compact) conformation and resulting in a rod-like shaped structure exhibiting a smooth surface according to the Porod coefficient (m ~4). R_g strongly depends on the temperature of the solution, whereas it does not show a dependence on concentration (in the range 1–5 mg/mL). This highlights that the aggregation process can be considered as a nearly closed assembling of unimers.

The values of R_g at varying temperature of both copolymers are plotted in Figure 1 together with the b/a ratio**.** The double y-plot clearly indicates that the transition from unimers to aggregates for both copolymers in D_2O occurs in two main stages: i) within a wide and well-defined range of intermediate temperatures, an equilibrium stage between unimers and aggregates is established, and the respective percentage weights estimated by SANS fit modeling are reported in Table S3 of ESI; ii) above this interval, stable colloidal aggregates are formed. The steps of these assemblies are sketched in Scheme 2.



Scheme 2. A sketch of the three distinct phases detected by SANS as a function of temperature for both copolymers.

The phenomenon of aggregation with temperature of the two copolymers in D_2O was also evaluated by DLS in terms of change in the hydrodynamic radius distribution, and by turbidimetry (see section S3 of ESI). As reported in section 2 of ESI, the accessible Q-range of SANS is not wide enough to provide high sensitivity of detection of large scale-length particles and these related methodologies may offer more sensitivity in this respect.

The latter measurements indicate that above 35°C significant events of unimer association can already take place, and became substantial above 45°C. The latter results corroborate the hypothesis emerged by SANS that a perturbation of the globular folding of the unimer micelles occurs, thus promoting their mutual interaction. Both DLS and turbidimetry analyses also confirm that the aggregates result stable in D_2O solution, well-tunable by temperature, and reversible. Above 55°C the number and the size of aggregates do not change significantly meaning that the “thermal conversion” from unimers to aggregates of unimers is completed.

The measure of the heat associated with the structural rearrangements of the copolymers in solution, from the unimer folding to the aggregation, was investigated by differential scanning calorimetry (DSC). In section S4, Figures S9 a and b, two representative DSC thermograms of PEGMA70-*co*-FA30 and PEGMA90-*co*-FA10 solutions are shown, both revealing the presence of a significant, broad endothermic transition ranging from ~48°C to ~60°C. The enthalpy values for PEGMA70-*co*-FA30 and PEGMA90-*co*-FA10 are ~6.26 J/g and ~1.76 J/g, respectively. The peak of the thermograms falls around 50 °C, which corresponds to the appearance of the aggregates of unimers, following the net heat of redistribution of non-covalent bonds (e.g., hydrophobic interactions, polar interactions, hydrogen bonding) occurring in the same temperature range identified by SANS. The broadness of the DSC profiles shows roughly two thermal stages (see in particular Figure S9 b). We hypothesize a concurrence of contributions of hydrophobic FA and hydrogen bonding PEGMA counits in triggering a perturbation in the folding of the unimers and LCST-like behavior of PEGMA, thereby inducing the formation of aggregates. The comparatively low value (i.e., three times lower) of the unimer-to-aggregate transition enthalpy of PEGMA90-*co*-FA10 may be traced back to the higher amount of PEGMA. This result is consistent with those reported for amphiphilic poly(oligoethylene glycol) methyl ether acrylate copolymer grafted cellulose nanocrystals.[18] The net absorbed heat of the unimer-to-aggregate transition is in accordance with the heat denaturation of reversible or partially reversible, multi-step folding-unfolding equilibrium of small monomeric globular proteins.[19]

Our results point out a temperature-sensitive mechanism for complex aggregation of PEGMA-*co*-FA unimers, involving self-aggregation of the perturbed, nano-globular unimer structures with increasing temperature into stable micron-sized, rod-like shaped amphiphilic architectures. In previous work,[11,12] we followed by DLS the effect of temperature on the hydrodynamic size distribution of copolymers PEGMA-*co*-FA, with the awareness that changes in unimer shape, concentration, sample polydispersity and solvent viscosity may artifact or hidden information on the copolymer behavior.[21] The SANS results offset this uncertainty, providing reliable detailed

information on the unimer conformation in solution and the structural variation of the copolymers which gives rise to the formation of stable temperature-driven assemblies in water solution. The present more detailed study, integrating SANS with DLS and DSC methodologies, reveals that the threshold temperature to trigger the structural transition from free self-folded unimers into aggregates is not significantly influenced by the difference in copolymer PEGMA/FA composition of the investigated copolymers.

In conclusion, the shape, the flexibility, as well as the aggregate size of copolymers PEGMAx-*co*-FAy are modulated by temperature and copolymer composition rather than by concentration of unimers in solution. More importantly, the structural and phase modifications enlightened herein show full reversibility with temperature over a wide range of copolymer concentrations. The present findings may help to deepen understanding and to facilitate manipulating the assembly of synthetic amphiphilic polymers in the quest for analogous structures to biopolymers (e.g., celluloses and proteins) in strings of microfibrils and "thermophilic" gels.

Experimental Section

The amphiphilic random copolymers PEGMAx-co-FAy with x/y mol/mol ratios 70/30 (Mn = 28900 g/mol, Đ = 1.43) and 90/10 (Mn = 34300 g/mol, Đ = 1.89) were prepared as previously reported [12] and briefly summarized in S1 of ESI.

For SANS analysis, the copolymers were dissolved in D_2O at ten different concentrations ranging from 1 to 10 mg/mL. After dissolution and overnight equilibration, neither aggregation processes nor appearance of precipitate was detected by visual inspection. Dry weight tests were performed in order to ascertain the effective polymer concentration. SANS measurements were conducted at varying temperature from 20°C to 70°C using the fixed geometry, time-of-flight, small angle neutron scattering Larmor instrument of the ISIS Spallation Source at the Rutherford Appleton Laboratory, U.K. A scattering vector $Q = (4\pi/\lambda) \sin(\theta/2)$ (where λ is the neutron wavelength and θ is the scattering angle) in the range between ~0.004 $Å^{-1}$ and ~0.68 $Å^{-1}$ was obtained by using

neutron wavelengths spanning from 0.9 Å to 13.3 Å, with sample-to-detector distance of 4 meters. The samples were contained in 2 mm path length and 0.6 mL volume quartz cuvettes (Hellma, GmbH), tightly closed and mounted on aluminum holders on top of an enclosed, computer-controlled, sample rack. Temperature was controlled using a circulating thermostat, achieving a temperature stability of the samples within ± 0.1°C. The SANS measurements were repeated twice to ensure that the system reached the equilibrium, mediating the two ones that had the same shape.

All scattering data were (a) normalized for the sample transmission, (b) background corrected with a quartz cell containing only the solvent (D_2O), and (c) corrected for the linearity and efficiency of the detector response (component of the instrumental smearing) using the instrument specific software package.[20] The experimental time of a single measurement was 20 min. The experimental data were analyzed using the ellipsoid morphology and Guinier-Porod model, in the fitting routine SASView 4.2.2 to monitor shape and size with temperature of both self-folded unimers and aggregates. In the range of temperature where an equilibrium stage between unimers and aggregates is established the respective fractions were estimated as the weight coefficients of the unimer and the aggregate fit models matching the SANS profile. Details on fitting are reported in ESI (section S2). Comparative DLS and cloud point measurements at varying temperature are shown in section S3 of ESI.

The enthalpy change involved in the assembling of the copolymers was analyzed using Differential Scanning calorimetry and the experimental details are shown in section S4 of ESI.

Acknowledgements

Experiments at the ISIS Pulsed Neutron and Muon Source were supported by a beamtime allocation (RB1720256, DOI: 10.5286/ISIS.E.RB1720256) from the Science and Technology Facilities Council. This work benefitted from SasView software, originally developed by the DANSE project under NSF award DMR-0520547.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

Amphiphilic copolymers, PEGMA-FA, nanoparticles, SANS

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