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ORIGINAL CONTRIBUTION

Role of the tracer in characterizing the aggregation behavior of aqueous block copolymer solutions using fluorescence correlation spectroscopy

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Abstract The hydrodynamic radii of micelles formed by amphiphilic poly(2-alkyl-2-oxazoline) diblock copolymers in aqueous solution determined using fluorescence correlation spectroscopy (FCS) depend on the nature of the fluorescent tracer used. We have compared the values of the hydrodynamic radii of the unimers and the micelles as well as the critical micelle concentrations (CMC), using as tracers (1) the identical diblock copolymers being fluorescence-labeled at the hydrophilic or the hydrophobic block terminus [Bonné et al. Colloid Polym Sci (2004) 282:833–843], and (2) a low molar mass fluorescence dye, rhodamine 6G. Whereas similar values for the CMC were found for both probes, the hydrodynamic radius of micelles is significantly underestimated using a free dye as a tracer in FCS, especially near the CMC. We attribute this discrepancy to the fast exchange of the dye between micelles and solution.

Keywords Polymer physics · Amphiphilic block copolymers · Aggregation behavior · Fluorescence correlation spectroscopy

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Introduction

Fluorescence correlation spectroscopy (FCS) allows the determination of diffusion coefficients of fluorescent molecules in solution [1–5] by measuring their average diffusion times through the detection volume, which is as small as ~1 μm^3. By means of the Stokes-Einstein equation, the hydrodynamic radii of the molecules or their aggregates can be inferred. To maximize the relative intensity fluctuations, low concentrations of the fluorescent molecules (below ~1 μM) are required. In solutions of amphiphilic copolymers, on the other hand, a concentration range over several orders of magnitude needs to be investigated to characterize their complex phase behavior (e.g. the unimer-to-micelle transition and gel formation). This can be realized by carrying out tracer experiments with fluorescence-labeled molecules as tracers in a solution of non-labeled polymers.

In this way, we have previously studied the unimer-to-micelle transition of two amphiphilic poly(2-alkyl-2-oxazoline) diblock copolymers in aqueous solution, which consisted of a short, hydrophobic poly(2-*n*-nonyl-2-oxazoline) [P(NOx)] block and a longer, hydrophilic poly(2-methyl-2-oxazoline) [P(MOx)] block [6]. Poly(2-oxazoline)s constitute a very versatile system to study the aggregation behavior as a function of the polymeric architecture: The nature of the substitution in the 2-position of the monomer unit determines the solubility of the respective polymer segment. For instance, a methyl group in the 2-position results in a hydrophilic, a 2-*n*-nonyl substitution in a hydrophobic segment. In contrast to the popular poly [(ethylene oxide)-*b*-(propylene oxide)] {P[(EO)(PO)]} or other polymeric amphiphiles, an amphiphilic contrast is given not only between the two poly(2-oxazoline) blocks, but also within each monomer, i.e., a non-ionic polysoap is

formed. Furthermore, the cationic living polymerization guarantees a good control of the block lengths at narrow molar mass distributions, and the chain termini are easy to functionalize via the initiation and/or termination reactions [7], which, in the present case, was exploited to attach the fluorescence dye.

In our recent study of the aggregation behavior, [6] we have used as tracers the identical diblock copolymers with the fluorescence label tetramethylrhodamine isothiocyanate (TRITC) attached to the terminus of either the hydrophilic {P[$(NOx)_{10}(MOx)_{32}$]-TRITC} or the hydrophobic block {P[$(MOx)_{40}(NOx)_7$]-TRITC}: The subscripts denote the average degrees of polymerization. These fluorescence-labeled copolymers were added at low concentration ($<6\times10^{-8}$ M) to solutions of the identical batch of the non-labeled copolymers. In this way, we covered a concentration range between $\sim10^{-8}$ and $\sim10^{-2}$ M and could determine the critical micelle concentrations (CMC) as well as the hydrodynamic radii of the unimers and the micelles, r_H^{uni} and r_H^{mic}. The values of r_H^{mic} were verified using photon correlation spectroscopy (PCS) on solutions of non-labeled polymers [6].

However, as an identical batch of a fluorescence-labeled copolymer is not always available, one may ask the question whether the same results for the CMC and for r_H^{mic} can be obtained by FCS using commercially available, low molar mass fluorescence dyes. As for other fluorescence techniques to determine the CMC, [8, 9] a dye of poor water-solubility should be chosen to maximize its affinity to the hydrophobic micellar core. Moreover, it should be small compared to the dimensions of the micelle to minimize the influence on the polymer aggregation.

A number of FCS studies reported in the literature suggest that the choice of the tracers is of importance, both in low molar mass and in polymeric amphiphiles. Hink et al. [10] determined the hydrodynamic radii of the micelles formed by various detergents in buffer solutions with FCS, using as tracers phospholipids being labeled by Bodipy at the hydrophilic or the hydrophobic end. They observed that the hydrodynamic radii measured in this way were in general higher than the previously reported values determined without tracers. Furthermore, when the tracer was labeled on the hydrophobic end, the discrepancies in hydrodynamic radius were larger than when labeling on the hydrophilic end. PCS experiments confirmed these changes in hydrodynamic radius induced by the tracers, presumably due to the relatively large size of the tracers compared to the micelles. Zettl et al. [11] have recently studied the micelle formation of hexadecyltrimethylammonium chloride (CTAC), a cationic surfactant, using negatively or positively charged dyes as tracers. In a range of several decades of surfactant concentration around the CMC (determined by other means), a gradual increase of the micellar diffusion time was observed.

Also in polymeric amphiphiles, a similar increase of the micellar hydrodynamic radius has been found. Schuch et al. [12] studied the unimer-to-micelle transition of poly[(isobutylene)-*b*-(methacrylic acid)] diblock copolymers with different degrees of polymerization in aqueous solution. As a tracer, they used a C_{16} fatty acid being fluorescence-labeled with Bodipy. Above the CMC, the micellar diameters of the copolymers were found to increase with concentration over a broad concentration range (two decades for one of the copolymers) and only reached a plateau value at higher concentrations. Assigning the beginning of the increase to the CMC leads to values of 1×10^{-8}–4×10^{-8} M, thus in the range expected. Similar behavior was observed by Loos et al. [13] who have measured the CMC of poly [(amylose)-*b*-(styrene)] diblock copolymers in tetrahydrofurane (THF), a selective solvent for polystyrene with Rhodamine B (which is hardly soluble in THF) as a tracer.

In the present contribution, we report on the results from FCS tracer measurements using polymer-bound TRITC or a free fluorescent probe (Rhodamine 6G; Rh6G) to study the aggregation behavior of defined amphiphilic, non-ionic block copolymers in aqueous solution.

Experimental

Materials and methods The characteristics of the diblock copolymers and the tracers are compiled in Table 1. The synthesis and fluorescence labeling of the polymers and the preparation of their aqueous solutions have been previously

Table 1 Results from the FCS experiments of the diblock copolymer solutions

	Tracer	CMC (10^{-6} M)	r_H^{uni} (nm)	r_H^{mic} (nm)
$P[(MOx)_{40}(NOx)_7]$	$P[(MOx)_{40}(NOx)_7]$-TRITC	7.4±1.6	1.4±0.4	13±2
$P[(NOx)_{10}(MOx)_{32}]$	$P[(NOx)_{10}(MOx)_{32}]$-TRITC	22±7	1.3±0.2	11.3±0.9
	Rh6G	20±7		4.9−9.3[a]

$(MOx)_n$ and $(NOx)_m$ denote the hydrophilic methyl-2-oxazoline and the hydrophobic 2-nonyl-2-oxazoline monomer units of the copolymer block with average degrees of polymerization n and m, respectively.
[a]The values depend on the copolymer concentration.

described [6]. Rhodamine 6G was purchased from Sigma-Aldrich. In solutions of non-labeled $P[(NOx)_{10}(MOx)_{32}]$ and Rh6G, the concentration of Rh6G was kept constant at 1×10^{-8} M.

FCS FCS experiments were performed using ConfoCor 2 instruments from Carl Zeiss Jena GmbH together with a HeNe laser (λ=543 nm), a pinhole with a diameter of 80 μm, a BP 560-615 emission filter and an HFT 543 plate beam splitter. Measurements were conducted at room temperature. The autocorrelation functions of the fluctuations of the fluorescence intensity, $G(\tau)$, were analyzed by fitting the following expression [14]:

$$G(\tau) = 1 + \frac{1}{N}\left[\frac{T_T}{1-T_T}\exp\left(-\frac{\tau}{\tau_T}\right)\right] \times \sum_{i=1}^{n}\frac{\rho_i}{\left(1+\frac{\tau}{\tau_{D,i}}\right)\sqrt{1+\frac{\tau}{(z_0/w_0)^2\tau_{D,i}}}} \quad (1)$$

where N is the total number of fluorescent particles in the observation volume, n is the number of different fluorescent species, $\tau_{D,i}$ is the diffusion time of the ith species, ρ_i is the relative fluorescence intensity of the ith species, and z_0 and w_0 are the half-height and half-width of the observation volume, respectively. T_T and τ_T are the triplet fraction and time, respectively. From the fit, the values were found to be in the range T_T=0.05–0.15 and τ_T= 0.005–0.01 ms. w_0 was determined before each session by measuring the diffusion time of Rhodamine 6G (Sigma-Aldrich, $D_{Rh6G}=2.8\times10^{-10}$ m^2s^{-1}; [3]), $\tau_{D,Rh6G}$, and by using $w_0 = \sqrt{4D_{Rh6G}\tau_{D,Rh6G}}$. Values $w_0\cong$0.2–0.3 μm have typically been obtained. The ratio z_0/w_0 determined from the fit to the Rhodamine 6G correlation functions was typically $z_0/w_0\cong$5–6. Using the Stokes-Einstein relation together with the viscosity of water at room temperature, 10^{-3} Pa, the hydrodynamic radii $r_{H,i}$ corresponding to $\tau_{D,i}$ were calculated.

Results

We have previously reported on FCS measurements on aqueous solutions of labeled and non-labeled diblock copolymers with the compositions $P[(NOx)_{10}(MOx)_{32}]$ and $P[(MOx)_{40}(NOx)_7]$ [6]. In all measurements, the identical copolymers labeled with TRITC were used as tracers. In this paper, we present more detailed results of the $P[(MOx)_{40}(NOx)_7]$ aggregation behavior over the relevant concentration range around the expected CMC. Moreover, experiments with the same copolymer system but with Rh6G as a free fluorescent tracer are presented.

FCS experiments with polymer-bound fluorescence tracers In Figs. 1 and 2, the results of the FCS experiments on $P[(MOx)_{40}(NOx)_7]$ and $P[(NOx)_{10}(MOx)_{32}]$ with the respective fluorescence-labeled copolymers as tracers are summarized. At low polymer concentrations, a single diffusional decay is present in the FCS correlation curves, which has been attributed to the diffusion of the individually solubilized labeled copolymers (unimers) in water. Measurements carried out with purely fluorescence-labeled copolymer

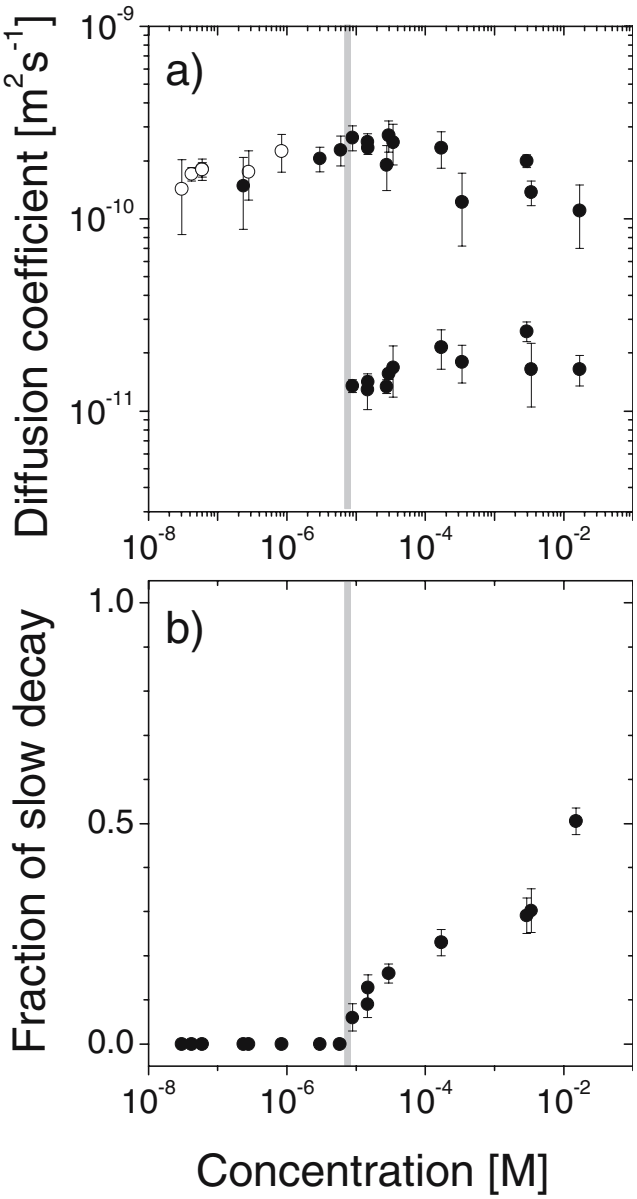

Fig. 1 FCS results from $P[(MOx)_{40}(NOx)_7]$ with the identical fluorescence-labeled copolymers as tracers in dependence on the overall copolymer concentration. **a** The concentration-dependent diffusion coefficients. *Open circles*, solutions containing only labeled copolymers. *Filled circles*, solutions containing both fluorescence-labeled and non-labeled copolymers; the concentration of labeled copolymers was kept below 2×10^{-7} M. **b** The fraction of the slow decay in the FCS correlation curves

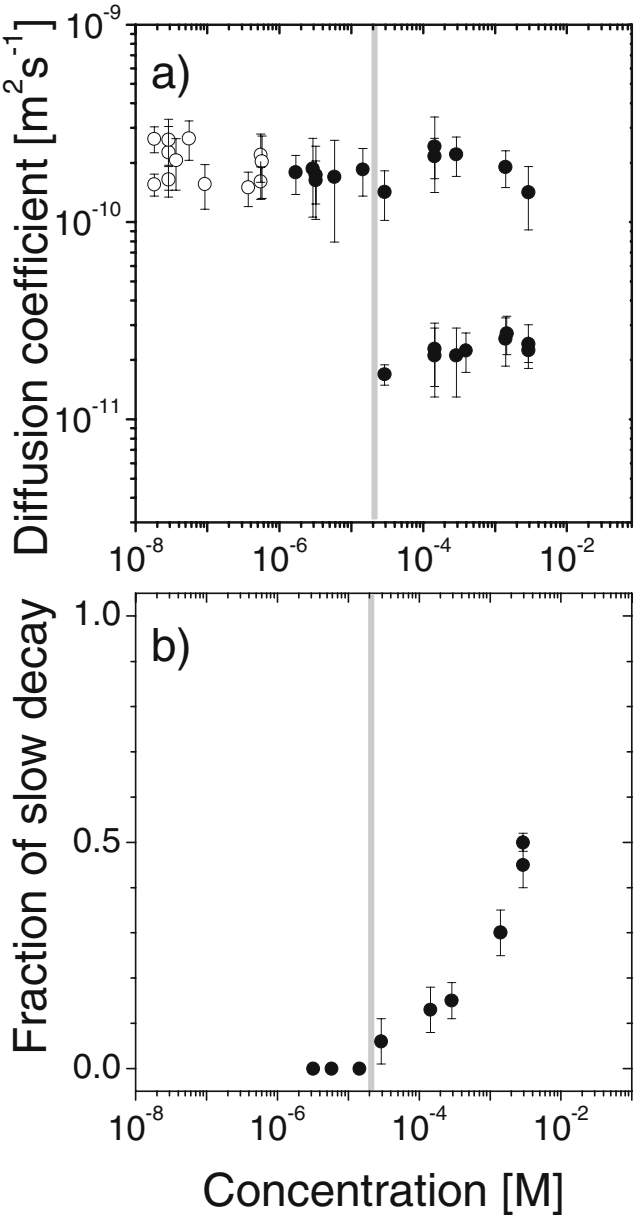

Fig. 2 FCS results from P[(NOx)$_{10}$(MOx)$_{32}$] with the identical fluorescence-labeled copolymers as tracers in dependence on the total copolymer concentration. **a** The concentration-dependent diffusion coefficients. *Open circles*, solutions containing only labeled copolymers. *Filled circles*, solutions containing both fluorescence-labeled and non-labeled copolymers; the concentration of labeled copolymers was kept below 6×10^{-8} M.[6] **b** The fraction of the slow decay in the FCS correlation curves

batches or with solutions of both labeled and non-labeled copolymers result in the same diffusion coefficients for the respective copolymer systems. The hydrodynamic radii of the unimers, r_H^{uni}, can be calculated using the Stokes-Einstein relation. With $r_H^{uni} = 1.4 \pm 0.4\ nm$ for the P[(MOx)$_{40}$(NOx)$_7$]/P[(MOx)$_{40}$(NOx)$_7$]-TRITC and 1.3±0.2 nm for the P[(NOx)$_{10}$(MOx)$_{32}$]/P[(NOx)$_{10}$ (MOx)$_{32}$]-TRITC system, very similar values of r_H^{uni} are found for the two copolymers. This is expected from the fact that the total degrees of polymerization are similar, the length of the hydrophilic and hydrophobic blocks are comparable, and the polydispersity indices are low $\left(\overline{M}_w/\overline{M}_n = 1.02 \text{ and } 1.07\right)$. We conclude that the location of the fluorescent dye seems to have no impact on r_H^{uni}.

As the polymer concentration is increased, a second decay in the FCS correlation function is observed at higher correlation times, indicating an additional, slower diffusional process of the polymeric tracers. It is assigned to the diffusion of micelles with incorporated fluorescence-labeled polymeric tracers, i.e., the CMC has been reached. As in both cases, the concentration of the tracers is much lower than the CMC, every detected micelle carries at most one fluorescence-labeled copolymer. With increasing copolymer concentration, the amplitude of the second decay increases, reflecting the increasing concentration of micelles (Figs. 1b and 2b). The CMCs as well as the micellar hydrodynamic radii, r_H^{mic}, of the two copolymers are similar as expected: $(7.4\pm1.6)\times10^{-6}$ M and 13±2 nm for the P[(MOx)$_{40}$(NOx)$_7$] copolymer and $(22\pm7)\times10^{-6}$ M and 11.3±0.9 nm for P[(NOx)$_{10}$(MOx)$_{32}$], respectively.

Experiments using PCS on solutions of the non-labeled copolymers gave equal values for r_H^{mic} [6]. This means that in the solutions studied using FCS, every detected micelle carries a labeled copolymer over the entire detection time or, in other words, the average residence time of the labeled copolymers in a micelle is longer than the average diffusion time of the micelle through the detection volume (~300–500 μs, as estimated from the micellar diffusion coefficient and the Einstein equation).

It can be concluded that neither the hydrodynamic radii of the unimers and the micelles nor the values of the CMC as determined by FCS depend on the position of the fluorescence label. The polymer-bound TRITC itself did not induce aggregation, which is reasonable as TRITC is water-soluble and is present at low concentration. The aggregation, and thus, the unimer-to-micelle transition is entirely due to the hydrophobicity of the 2-*n*-nonyl-2-oxazoline block. Furthermore, the influence of the attached probe on the micelle formation is minimized by attaching it to the terminus of the polar polymer backbone and not directly to the end of the hydrophobic *n*-nonyl-tail, for instance.

FCS experiments with a free, low molar mass fluorescence tracer (Rh6G) As the aggregation of amphiphilic copolymers in water is a fundamental issue and not always a suitable copolymer-bound fluorescent tracer is available, the simplest alternative would be to add a small amount of a low molar mass dye to the polymer solutions. If the water solubility of the dye is sufficiently low, it is expected to enrich in the hydrophobic domains as the CMC or the critical micelle temperature (CMT) is crossed and the

polymers aggregate. In FCS, the resulting slowing-down of the dye diffusion can easily be traced. To answer the question whether reliable results for the CMC and for r_H^{mic} can be obtained by FCS using small (non-bound) dyes, our system is most suitable, as the poly(2-oxazoline) copolymer system is well defined and has already been studied by PCS and FCS with polymer-bound fluorescent dyes, and the results can be directly compared. From the large number of low molar mass dyes that are commercially available and suitable for FCS experiments, rhodamine 6G (Rh6G) was selected due to its finite solubility in water: It is present as a monomer up to concentrations of ~10^{-5} M; above this concentration, it forms dimers, bridged by a water molecule, which results in fluorescence quenching [15, 16]. The solubility of Rh6G monomers in protic as well as non-polar organic solvents is good [15] (e.g., in methanol or ethanol ~0.1 M), thus upon appearance of hydrophobic compartments from copolymer micelles above the polymers' CMC or CMT, Rh6G monomers will preferentially solubilize in the micellar cores and diffuse along with the entire micelle. Dimer formation within the micelle is unlikely as water is needed to form this aggregate: Recently, Vogel et al. [17] found a dimer-to-monomer transformation of Rh6G, which solubilized in the micelles formed in aqueous solutions of P[(EO)(PO)(EO)] block copolymers. Moreover, the excitation/emission characteristics of Rh6G are suitable for standard FCS instruments, and finally, the diffusion coefficient of Rh6G is well known [3].

FCS experiments were carried out using the same P[$(NOx)_{10}(MOx)_{32}$] copolymer batch as before and adding Rh6G at a concentration 10^{-8} M, i.e., far below the concentration at which Rh6G dimer formation in water occurs. In Fig. 3, the FCS correlation functions are shown as a function of copolymer concentration. Below 2×10^{-5} M (closed symbols), good fits of the correlation functions are obtained with a single diffusional process with the diffusion coefficients being equal to the one of Rh6G monomers in water (Fig. 4) [3]. At polymer concentrations above 2×10^{-5} M (open symbols), the correlation functions contain an additional, slower decay. This indicates that the CMC has been reached and that a fraction of the Rh6G molecules are solubilized within the micelles. The concentration of 2×10^{-5} M coincides well with the CMC determined with fluorescence-labeled copolymers as tracers (Table 1). Moreover, the increase of the fraction of the second slow decay with increasing polymer concentration is similar. Apparently, more and more dyes are solubilized in the micellar cores (Fig. 4b). Above 2.6×10^{-4} M, the fast decay attributed to the diffusion of free Rh6G is no longer observed, i.e., all Rh6G molecules are preferentially located within the micelles. However, in contrast to the FCS experiments using a polymer-bound dye as a tracer, this slower decay in the correlation functions is steadily shifted to longer correlation times (Fig. 3, curves with open symbols), thus the apparent diffusion coefficient of the micelles decreases with increasing copolymer concentration and does not reach a constant value even two decades above the CMC (Fig. 4a). Normally, this would indicate that the micelles grow with increasing polymer concentration. This was, however, neither observed in the PCS experiments nor in the FCS experiment with the fluorescence-labeled polymers (Fig. 5).

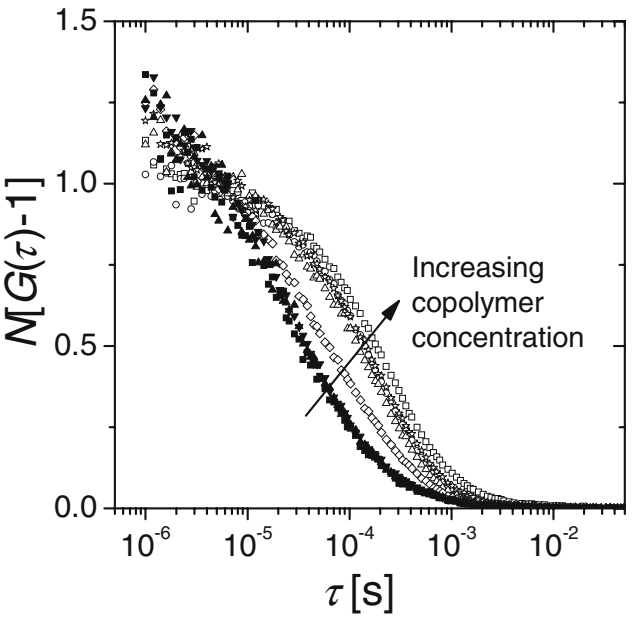

Fig. 3 FCS correlation functions of solutions of Rh6G and P[$(NOx)_{10}(MOx)_{32}$] at the following copolymer concentrations: (*filled inverted triangle*) 1.3×10^{-6} M, (*filled triangle*) 2.6×10^{-6} M, (*filled square*) 1.3×10^{-5} M, (*open diamond*) 2.6×10^{-5} M, (*open triangle*) 1.3×10^{-4} M, (*open circle*) 2.6×10^{-4} M, (*asterisk*) 1.3×10^{-3} M and (*open square*) 2.6×10^{-3} M. The *arrow* denotes the increase in copolymer concentration. The curves were normalized by the inverse average number of fluorescent molecules in the detection volume, $1/N$, as determined from the fit of Eq. (1) to the experimental curves

In addition to their difference in molar mass, the two tracer systems differ by their 'strength' of attachment of the tracers to the micelle: Whereas the polymer-bound fluorescence dye is incorporated into the micelle together with the entire amphiphilic block copolymer, Rh6G has a much higher solubility in water. A possible scenario is therefore that Rh6G exchanges rapidly between the micelle and the solution. If this exchange happens many times during the diffusion time of the micelle through the FCS detection volume (which is in the range 300–500 μs), the average diffusion coefficient, $\overline{D}$, is an average of the diffusion coefficients of free Rh6G molecules, D_{sol}, and of Rh6G molecules attached to micelles, D_{mic} [18]:

$$\overline{D} = (1-p)D_{sol} + pD_{mic} \quad (2)$$

where p denotes the probability of the Rh6G tracer being attached to micelles and may also be regarded as the fraction

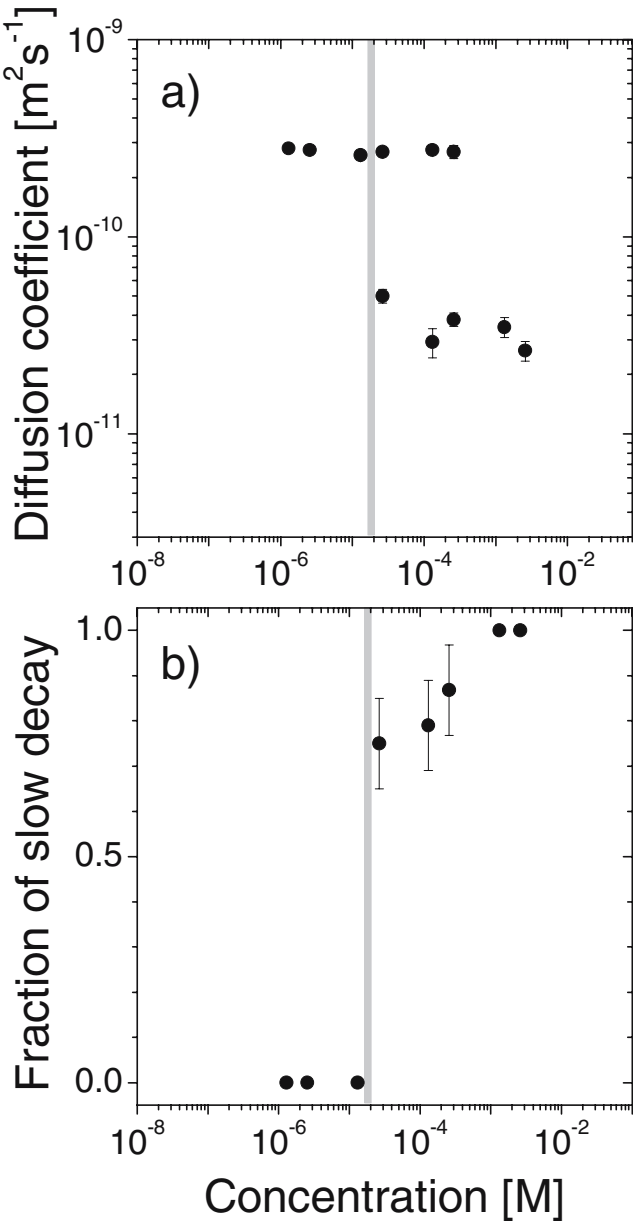

Fig. 4 FCS results from P[$(NOx)_{10}(MOx)_{32}$] with Rh6G as tracers as a function of the copolymer concentration. The concentration of Rh6G labeled copolymers was kept at 10^{-8} M. **a** The concentration-dependent diffusion coefficient. **b** The fraction of the slow decay in the FCS correlation curves

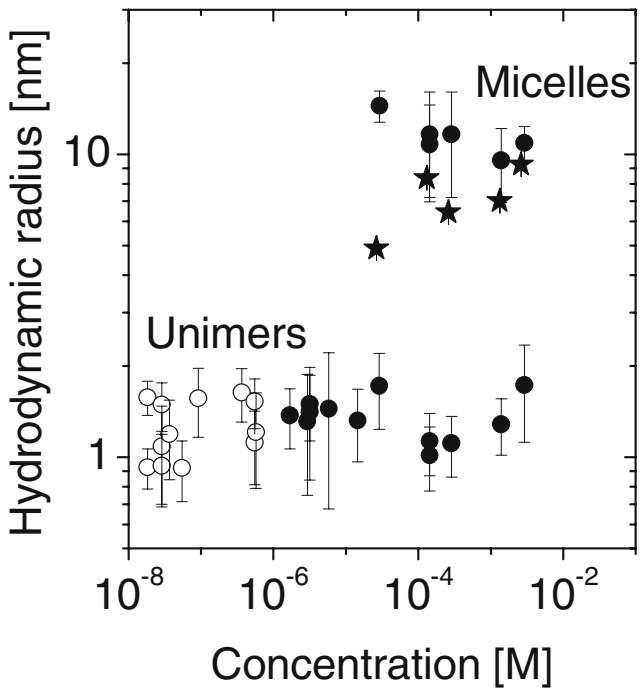

Fig. 5 (Apparent) hydrodynamic radii as determined by FCS on P[$(NOx)_{10}(MOx)_{32}$] with different tracers: *open circles*, solutions containing only labeled copolymers. *Filled circles*, solutions containing both fluorescence-labeled and non-labeled copolymers. *Stars*, solutions containing non-labeled polymers and Rh6G

of time that the dye is attached to micelles. We assumed that the properties of the dye do not change when it joins a micelle. As the diffusion coefficients of the fluorescence dye Rh6G in water as well as the one of the micelles are known, p can be calculated as a function of copolymer concentration. We find that, with increasing copolymer concentration above the CMC, p increases from 0.85±0.02 to 0.98±0.01. Increasing the copolymer concentration and thereby also the concentration of micelles leads to an increase of the time fraction that Rh6G is attached to the micelles.

This effect is not observed with the polymer-bound dye because its average residence time is presumably much longer than the diffusion time of the micelles through the FCS detection volume. The average diffusion time of the micelles can be estimated from the Einstein relation

$$w_0^2 = 4D_{\mathrm{mic}}t \tag{3}$$

with $D_{\mathrm{mic}}=2\times10^{-11}$ m^2/s (Figs. 1a and 2a); t is found to be ~0.5 ms. For comparison, the residence time for Pluronics systems (ABA triblock copolymers of EO and PO) has been estimated to be similar or below 1 ms [18, 19]. For the P[(MOx)(NOx)] system, we expect longer residence times because of the long side chains of P(NOx) leading to high friction and effective van der Waals interactions within the core of the micelle.

Our experiments thus show that using a commercially available, low molar mass fluorescence dye, it is possible to determine the CMC of amphiphilic diblock copolymers in aqueous solution reliably. In contrast, the hydrodynamic radii of the micelles suffer from fast exchange of the dye between the micelles and the solution, especially near the CMC, in agreement with previous observations [11–13]. We attribute this discrepancy to the low number of micelles in a certain concentration range above the CMC.

Conclusion

We conclude that using polymer-bound fluorescence dyes as tracers, precise studies of the aggregation behaviour of amphiphilic block copolymers over a broad concentration range can be carried out using FCS. When the dyes are bound to polymers which are identical to the aggregating polymers under study, the most reliable results both for the CMC and the micellar hydrodynamic radius are obtained. The location of the preferably small and water-soluble dye in the polymer does not have any influence on these values.

Using a low molar mass dye as a tracer in FCS experiments has the advantage that they are readily available and no additional synthesis is needed. Care needs to be taken that the solubility of the tracer in the solvent is as low as possible and that it is small compared to the micelles/aggregates under study. The CMC can be reliably determined from the appearance of an additional slower decay in the FCS correlation function, which is due to the dye being solubilized in micelle core. The micellar hydrodynamic radius, however, may suffer from a fast exchange of the dye between the micelle and the solvent. More experiments on different systems are necessary to find out whether this phenomenon is due to the characteristics of the polymer itself or to the solubility or finite residence time of the fluorescent dye in the micellar core.

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