Fluorescence Correlation Spectroscopy of Single Dye-Labeled Polymers in Organic Solvents

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ABSTRACT: We discuss the use of fluorescence correlation spectroscopy (FCS) to study the diffusion of single dye-labeled polymer chains in organic solvents. Monodisperse batches of polystyrenes labeled with a single Rhodamine B molecule have been synthesized via anionic polymerization of styrene and ethylene oxide end-capping followed by a polymer analogous coupling reaction. MALDI-ToF mass spectrometry is used to characterize the resulting material. A commercial FCS system has been modified to permit FCS measurements in volatile organic solvents. FCS was used to determine the molecular weight dependence of the diffusion coefficient of 10 nM solutions of end-labeled polystyrenes in toluene. The data are utilized to establish a calibration procedure for FCS measurements in organic solvents.

Introduction

Experimental techniques based on the detection of single molecule fluorescence have gained increasing interest throughout the past decade. They offer the unique opportunity to study the structural and dynamical properties of single molecular entities and circumvent the loss of information inherent of ensemble averaging typical for most of the classical experimental techniques, requiring the detection of a large number of molecules. Prominent examples of this class of techniques are single molecule microscopy, single molecule spectroscopy, and fluorescence correlation spectroscopy (FCS). Despite their scientific beauty and their high potential, however, the use of single molecule fluorescence techniques in polymer science has almost entirely been limited to the area of biological macromolecules and aqueous media.[1−3] This is even more astonishing, as a variety of potential applications in polymer materials science can be envisaged: the ultrahigh sensitivity of FCS experiments could be utilized to study molecular self-assembly in solution in systems exhibiting extremely low critical aggregation concentrations (e.g., block copolymers, nanoparticles, etc.), and single molecule microscopy could be used to study the elementary processes of polymer crystallization or the movement of single chains in confined environment (polymer networks, ordered polymeric mesophases, thin films, etc.). Finally, single molecule spectroscopy could be used to follow the segmental dynamics of polymer chains via the time-resolved detection of changes in their immediate environment. Very recently, first applications of the type mentioned above have been reported, including the self-aggregation of complex block copolymer aggregates[4−6] via FCS, the visualization of single dye-labeled polymer chains by single molecule microscopy[7] and FCS in water,[8,9] and the combination of FCS and surface forces measurements for the study of dye molecules confined to ultrathin films.[10]

For single molecule fluorescence experiments to be used on synthetic polymers in organic solvents, a variety of problems have to be solved. At first, well-defined test polymer chains containing a single entity of a suitable dye have to be synthesized. Quite importantly, the resulting dye-labeled polymer must not contain any measurable amount of free dye molecules, so careful separation steps are crucial. Moreover, the issue of photostability of the dye has to be addressed both in view of the organic environment and in view of the fact that the time scales of molecular motion in concentrated polymer solutions and melts are considerably larger than in most aqueous systems studied so far. In addition, most organic solvents have a considerably higher vapor pressure than water. Therefore, solvent-tight sample chambers need to be developed. The beam path of the optical setup has to be adapted to the index of refraction of the solvent, which proves difficult for most commercial experimental setups, which happen to be optimized for an aqueous environment.

The above considerations hold for any kind of optical single molecule experiment in organic environment. For fluorescence correlation spectroscopy (FCS) in particular, an additional problem occurs. Changes in the optical setup typically come along with changes in the dimensions of the detection volume. Therefore, suitable calibration procedures have to be devised. FCS calibration in aqueous environment typically relies on the straightforward measurement of the characteristic diffusion time of a dye molecule with known diffusion coefficient. In organic media, however, no such simple means is available. Most dye molecules tend to aggregate in organic solvents, and in contrast to aqueous systems the diffusion coefficients are barely known. This problem can be circumvented if well-soluble objects of known hydrodynamic radius are labeled with a single dye molecule.[11] Alternatively, luminescent semiconductor nanoparticles of narrow size distribution and known hydrodynamic radius could be used.[12] Finally, single dye molecules may be linked to polymer chains of known molecular weight and narrow molecular weight distribution. In these cases, either the diffusion constant will be known or it can easily be determined from classical experiments like dynamic light scattering.

In the present article, we describe first experiments along these lines. We have synthesized monodisperse

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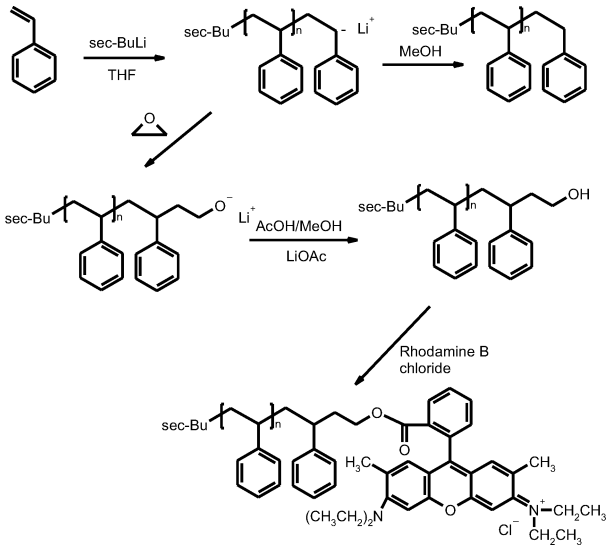

Figure 1. Scheme of the polymer synthesis and the subsequent polymer analogous coupling reaction.

batches of polystyrene (PS) labeled with a single Rhodamine B dye molecule via anionic polymerization of styrene and ethylene oxide end-capping followed by a polymer analogous coupling reaction. MALDI-ToF mass spectrometry is used to characterize all intermediate materials during the synthesis and the resulting material. The molecular weights M_w range between 10 and 1000 kDa, and the polydispersity M_w/M_n is as low as 1.05. Preparative gel permeation chromatography was used to separate labeled polymer and free dye molecules. We have modified a commercial FCS setup (Confocor II, Carl Zeiss GmbH, Germany) such that experiments in volatile solvents with an index of refraction different from water become possible. As an example, we have followed the diffusion of the labeled PS molecules at a concentration of 10^{-8} M in toluene as a function of molecular weight. Finally, our data are discussed in view of their potential use to calibrate the FCS setup for measurements in organic solvents.

Experimental Section

Hydroxyl-Terminated Polystyrene (PS-OH). The synthesis was performed using standard anionic polymerization techniques (Figure 1). 1 L of freshly distilled THF was cooled to −78 °C. 2.3 mL of *sec*-BuLi (1.3 M solution in *n*-hexane/cyclohexane) was injected before 20.31 g of styrene was added. The polymerization was allowed to proceed for 60 min, before the styryl anions were capped with 5 mL of ethylene oxide (freshly distilled from CaH_2). On the following day the reaction was terminated with a 5 mL mixture of degassed AcOH/MeOH (5:1 v:v). The polymer was precipitated in 5 L of methanol, redissolved in THF, and reprecipitated two more times. The successful end-capping reaction was verified using MALDI-ToF mass spectrometry (see Figure 2).

Rhodamine B-Labeled Polystyrene (PS-RhB). 2 g of the PS−OH was dissolved in 20 mL of THF under a nitrogen atmosphere. A 2 M excess of the acid chloride of Rhodamine B was added before 0.5 mL of anhydrous pyridine was injected into the reaction mixture. The reaction was allowed to proceed for 3 days at 40 °C, before terminating with 1−2 mL of methanol. The resulting polymer was precipitated into 500 mL of methanol.

MALDI-ToF Mass Spectrometry. The MALDI measurements were performed on a Bruker Reflex III spectrometer equipped with a nitrogen laser (337 nm) in reflector mode. An acceleration voltage of 20 kV and a reflector voltage of 23 kV together with matrix/low-mass suppression up to 3000 g/mol were used. 1000 laser shots for all spectra were coadded. Polymer sample (10 mg/mL), matrix (dithranol, 20 mg/mL), and salt (10 mg/mL) were dissolved in THF and mixed by volume at a ratio of 20:5:1 (matrix:sample:salt). 1 mL of this mixture was placed onto the target and allowed to dry.Polystyrene is usually measured by using dithranol as matrix and silver trifluoroacetate as cationizing agent. Thus, the quantitative conversion of PS to ethylene oxide functionalized polystyrene (PS−OH) can be visualized as the signal of the PS repeating units is shifted to higher mass by the C_2H_4O unit (44 g/mol). The same should be possible with the dye labeled PS. However, two series of signals were obtained when silver triflate as cationizing agent was used. The mass of the most intense signal does not correspond to the theoretical mass of the target molecule PS-RhB carrying one Ag^+ attached to the main chain but shows the same mass signal as the precursor.

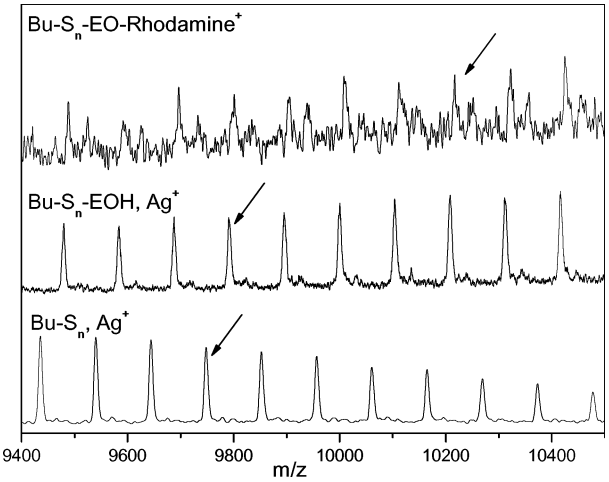

Figure 2. MALDI-ToF mass spectra of the PS precursor (bottom), the OH-end functionalized PS (middle), and the dye-labeled PS-chains (top). The arrows indicate a polymer chain with 93 styrene units ($n = 93$).

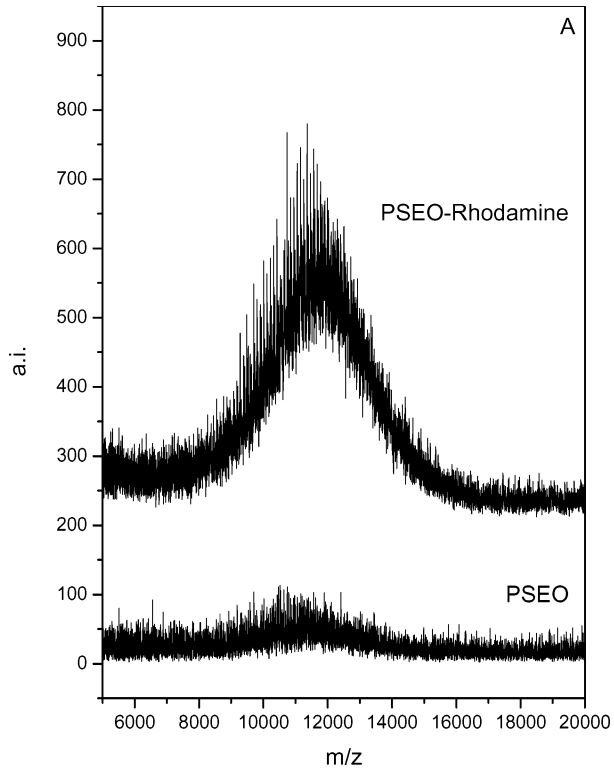

Figure 3. MALDI-ToF mass spectra of the dye-labeled PS-chains (top) and OH-end functionalized PS (bottom) measured without addition of Ag salt.

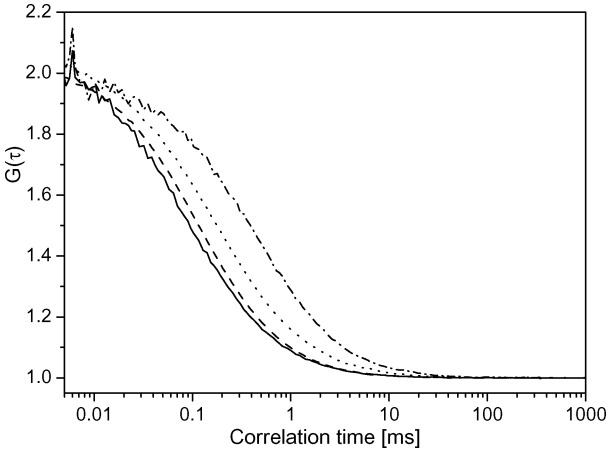

Figure 4. Normalized FCS autocorrelation curves for 10 nM solutions of different labeled polystyrenes in toluene. The molecular weight of the polystyrenes is from left to right: 11.5K, 19K, 63K, and 285K.

The second series also does not exactly correspond to the mass of the target molecule but shows a mass that is slightly higher than expected. However, a comparison of the MALDI-ToF spectra of the precursor molecule (PS−OH) and the dye-labeled polymer (PS-RhB) without adding salt to enhance cationization shows 8 times higher intensity for the dye-labeled product (Figure 3). Under these conditions of measurement no or very weak signal is expected for the precursor and the product in the case of an unsuccessful synthesis. Since Rhodamine B is an organic cationic salt, the result of the measurement leads to the conclusion that Rhodamine B-labeled polymer chains are intrinsically cationized. This notion is corroborated by the fact that the experimentally observed mass signals are in agreement with the expected mass signals for such species (Figure 2).

FCS Measurements. For the FCS measurements we modified the commercially avialable ConfoCor II. To adopt the optics for the use in organic solvents, we use a 40× Plan Neofluar objective with a numerical aperture of 0.9 instead of the 40× C-Apochromat objective which is used for experiments in aqueous solutions. The correction ring of this multiimmersion objective allows to adjust the optics for a large range of refractive indices, yielding a well-defined observation volume for organic solvents. The Rhodamine B-labeled PS chains were excited by an Ar ion laser at 514 nm. The autocorrelation function is analyzed by fitting the data with a homemade software using the Levenberg−Marquardt algorithm. For more detailed information see refs 13−15. To avoid evaporation of the organic solvent, we designed a well-sealed sample chamber with a 0.14 mm cover glass at the bottom. The polymer solutions were freshly prepared with toluene p.a. grade and investigated immediately. For each molecular weight three measurements of 5 min each were performed.

Results and Discussion

In Figure 4, we show normalized FCS autocorrelation curves for 10^{-8} M dye-labeled polystyrenes in toluene. The data clearly show an increase of the diffusion time with increasing molecular weight M. From a fit to the data we obtain the single molecule diffusion time τ as a function of molecular weight (Figure 5). These times are correlated to the waist radius of the observation volume, ω, and the diffusion coefficient, D, via the relation

$$\tau(M) = \frac{\omega^2}{4D(M)} \qquad (1)$$

For a known waist radius one could immediately extract the diffusion coefficients $D(M)$. In the case of toluene, however, the waist radius is not a priori known, and

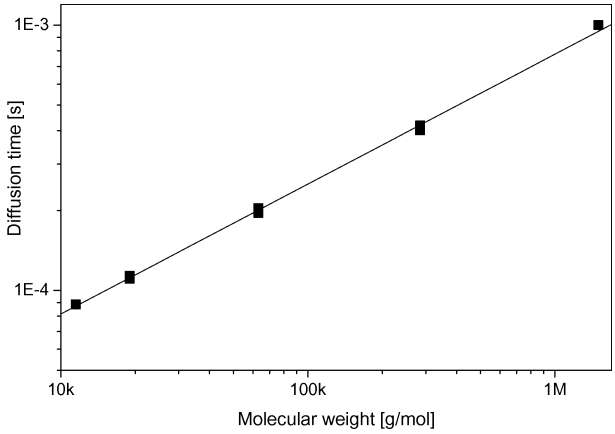

Figure 5. Single molecule diffusion times as a function of molecular weight as determined from least-squares fits to the data. The straight line shows a linear fit to the data.

proper calibration of the FCS setup is required. Since the relationship between diffusion coefficent and molecular weight is well established for the polystyrene/toluene system[16]

$$D(M) = (3.0 \pm 0.4) \times 10^{-4} M^{-0.549 \pm 0.013} \qquad (2)$$

we can in turn use the data shown in Figure 5 to calibrate the waist radius. A linear fit to the data shown in Figure 5 yields a slope $a = 0.498 \pm 0.004$, in fair agreement with the literature value.[16] Combining eqs 1 and 2, we can directly calculate the waist radius ω_{toluene}. The analysis yields a value of $\omega_{\text{toluene}} = 335 \pm 16$ nm. This value differs significantly from the value obtained from Rhodamine 6G measurements in water ($\omega_{\text{water}} = 212 \pm 4$ nm) determined with the same optical setup operated at the same excitation wavelength. This rather large difference corroborates the need for proper calibration procedures when FCS is operated in organic solvents.

One may be tempted to explain the different values for ω by the different indices of refraction between water and toluene. However, to optimize the optical setup for FCS measurements in organic solvent, the spherical and chromatic aberrations have to be minimized in the presence of the solvent. As this minimization involves changes in the distances between the lenses, the size of the excitation volume changes as well. Technical details of this procedure are beyond the scope of this paper. Here we only note that in consequence of this optimization the difference between ω_{toluene} and ω_{water} *cannot* simply be calculated assuming Gaussian optics and taking into account the different indices of refraction of the two solvents. We note in passing that for the particular experimental setup used by the Granick group,[10] who combine FCS with a surface forces apparatus, the issue of calibration is different from the situation described here. Since these studies deal with molecularly thin liquid films confined between two plates kept at known distance, the dimension of the excitation volume along the optical axis is trivially known. Therefore, calibration as described above is not needed.

In summary, we have synthesized monodisperse batches of polystyrenes labeled with a single dye

molecule enabling FCS measurements at ultralow polystyrene concentrations. We have shown that the polymers can be used for FCS calibration in toluene. Since the molecular weight dependence of the diffusion coefficient of PS solutions is well established for most organic solvents, we can use the same procedure to calibrate the setup for other solvents and solvent mixtures with very different refractive indices. For the calibration procedure it is not necessary to use the same solvent or solvent mixture which is used for the measurement. Only the refractive indices of the solutions used for calibration and experiment should be nearly the same. Thereby we expand the potential of this technique to a wide variety of issues in polymer materials science.

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