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Comparison Between Fluorescence Correlation Spectroscopy and Time-Resolved Fluorescence Anisotropy as Illustrated with a Fluorescent Dextran Conjugate

Nina V. Visser,[1] Mark A. Hink,[1] Arie van Hoek,[1] and Antonie J. W. G. Visser[1,2]

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The motional properties of rhodamine green alone and conjugated to 10-kDa dextran have been studied by fluorescence correlation spectroscopy (FCS) and time-resolved fluorescence anisotropy (TRFA). With FCS the translational diffusion times of the fluorescent particles can be determined, which are directly proportional to the shear viscosity as shown in aqueous solutions of different sucrose concentrations. With TRFA the rotational correlation times of the fluorescent particles can be determined. TRFA experiments in the case of fluorescent dextran reveal a distinct restricted internal motion of the fluorescent probe independent of the slower overall rotation of the polysaccharide. The fast depolarization is most likely due to internal motion and not to energy transfer between different rhodamine green molecules in the same dextran, since a higher viscosity of the solvent increases the correlation time for internal motion proportionally. FCS and TRFA yield complementary information in the sense that the correlation time for overall dextran rotation can be accurately determined from the translational diffusion coefficient.

KEY WORDS: Fluorescence correlation spectroscopy; fluorescence anisotropy; rotational correlation time; translational diffusion; rotational diffusion; fluorescent dextrans.

INTRODUCTION

Fluorescence correlation spectroscopy (FCS) has emerged as an ultrasensitive technique operating at the level of single fluorescent molecules diffusing in and out of the confocal volume created by a focused laser beam [1,2]. At these low molecular concentrations the fluorescence intensity shows fluctuations which are on-line autocorrelated. Analysis of the autocorrelation function yields parameters such as the number of molecules in the detection volume and the translational diffusion constant. In time-resolved fluorescence anisotropy (TRFA) experiments, the time-correlation function of the emission transition moment is determined. The combination of FCS and TRFA yields complementary information and provides insight into the molecular dynamics of the system under investigation.

Because of their water solubility and biological stability, fluorescent dextrans have found numerous applications in cell biology as markers for membrane permeability, endocytosis, intracellular ion concentration, intercellular communication, and fluidity in the cytoplasm (for a survey of applications see Ref. 3). We present a comparative study of a dextran which has been conjugated with rhodamine green using both FCS and TRFA. FCS yields a translational diffusion coefficient. Assuming that the polysaccharide rotates as a spherical particle, the

[1] MicroSpectroscopy Centre, Department of Biomolecular Sciences, Laboratory of Biochemistry, Wageningen Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands.
[2] To whom correspondence should be addressed.

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overall rotational correlation time is obtained from TRFA. Both experiments could lead to the same hydrodynamic radius of the dextran by applying classical Stokes–Einstein relationships. However, TRFA gave an average correlation time too short for the fluorescent dextran. In principle, there are two additional causes of depolarization which can account for this observation: rapid restricted motion of the dye independent of the overall dextran rotation and energy transfer between multiple dyes in one dextran molecule. The strategy to distinguish between the two depolarization mechanisms is to alter the viscosity of the medium [4]. In the case of energy transfer the rate of depolarization would not yield a systematic dependence on the shear viscosity of the solvent, while both restricted motion and overall rotation should exhibit a viscosity dependence.

EXPERIMENTAL

Rhodamine green (the succinimidyl ester) and the dextran (10-kDa) conjugate of rhodamine green were purchased from Molecular Probes, Inc. (Leiden, The Netherlands). Solutions were made in water, 30% (by weight) sucrose in water and 50% sucrose in water.

FCS measurements were performed with a Zeiss ConfoCor inverted confocal microscope (Jena, Germany). The 488-nm excitation light from an argon ion laser was focused by a water-immersible objective into the sample of investigation. The fluorescent light was collected by the same objective, passed through the dichroic mirror and the appropriate band-pass filter and through a pinhole in the image plane (to reject out-of-focus light), and, finally, impinged upon an avalanche photodiode in the single-photon counting mode whose signal was processed by a correlator. Data analysis was performed with the FCS ACCESS software package (developed by EVOTEC Inc., Hamburg, Germany) using a one-component fit model. This model determines the average number of fluorescent molecules in the detection volume as well as their characteristic diffusion times (τ_{dif}). The listed diffusion times are the averages of 10 experiments. Typical dye concentrations were 10 n*M*.

Polarized time-resolved fluorescence experiments were determined using a setup extensively detailed elsewhere [5]. The excitation wavelength was 450 nm and the fluorescence was detected through a band-pass filter with maximum transmission at 540 nm. Decay analysis was performed using global analysis software obtained from Globals Unlimited (Urbana, IL). The fluorescence anisotropy decay of rhodamine green could be analyzed with one correlation time, while that of rhodamine green conjugated to dextran required two correlation times for an optimal fit. Typical dye concentrations were 100 n*M*.

The temperature of the experiments was always 20°C.

RESULTS AND DISCUSSION

Fluorescence Correlation Spectroscopy

Normalized autocorrelation traces (experimental and fitted) of rhodamine green and of rhodamine green-conjugated dextran are presented in Fig. 1. The fitted curves yield the diffusion time τ_{dif} (s), which is the time needed for the fluorescent particle to diffuse over a distance ω_1 (m). The diffusion time is related to the translational diffusion constant D (m^2/s) via

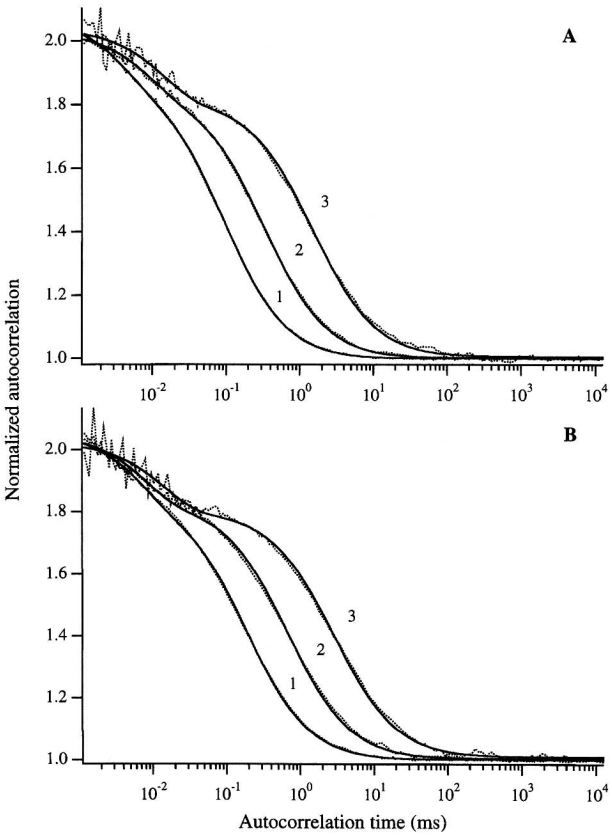

Fig. 1. Normalized autocorrelation traces (experimental, dotted lines; fitted, solid lines) of rhodamine green (A) and of rhodamine green–dextran conjugate (B). (1) In water; (2) in an aqueous 30% sucrose solution; (3) in an aqueous 50% sucrose solution. The diffusion times τ_{dif} obtained from the fits are listed in Table I.

$$\tau_{dif} = \frac{\omega_1^2}{4D} \quad (1)$$

The translational diffusion constant is related to the hydrodynamic radius of the fluorescent particle r_h (m):

$$r_h = \frac{kT}{6\pi D\eta} \quad (2)$$

where k is the Boltzmann constant (J · K^{-1}), T the temperature (K), and η the viscosity (Pa · s). The τ_{dif}, D, and r_h values of all samples are collected in Table I. Note that by combining Eqs. (1) and (2), the diffusion time is proportional to the viscosity. The viscosity of the 30% sucrose solution is a factor of 3.2 higher than that of water; for the 50% sucrose solution this factor is 15.4 [6]. The diffusion times of both free and dextran-conjugated rhodamine green indeed show the proportionality with viscosity (see Table I). It can also be seen that the dextran samples have retarded diffusion, in agreement with the larger molecular mass (for dextran, 10 kDa; for rhodamine green alone, 621 Da), which is also reflected in the hydrodynamic radius r_h. In addition, the hydrodynamic radius of the dextran tends to be somewhat larger in sucrose solution.

Time-Resolved Fluorescence Anisotropy

Fluorescence anisotropy decays (experimental and fitted) of rhodamine green and of rhodamine green-conjugated dextran are presented in Fig. 2. TRFA of free rhodamine green can be adequately described by a single rotational correlation time. TRFA of rhodamine green-conjugated dextran, however, cannot be fitted with a single correlation time, indicating that additional depolarization processes are present during the 25-ns duration of the fluorescence anisotropy decay experiment. Generally, the fluorescence anisotropy decay $r(t)$ can be described by a Soleillet product of correlation functions, provided that the time scales of these independent processes are different:

$$r(t) = r_0 \cdot C_{rot}(t) \cdot C_{other}(t) \quad (3)$$

where r_0 is the fundamental anisotropy, C_{rot} the correlation function describing the overall dextran rotation, and C_{other} the correlation function accounting for other depolarization processes. There are two possibilities for the other sources of depolarization: restricted internal motion and energy transfer between different fluorescent molecules in the same dextran. One can distinguish between the two modes of depolarization by performing measurements of fluorescent dextran in solutions of increasing viscosity. The internal restricted rotation of a fluorescent probe

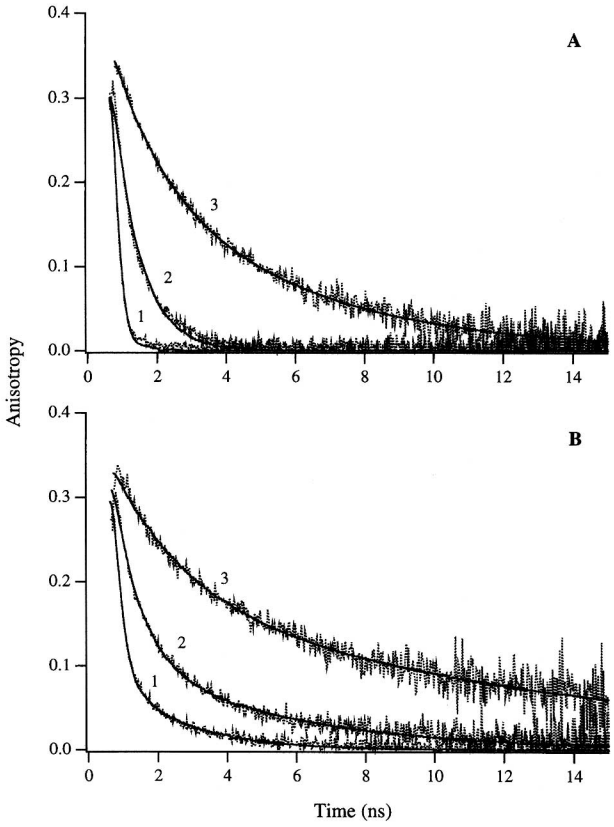

Fig. 2. Fluorescence anisotropy decay curves (experimental, dotted lines; fitted, solid lines) of rhodamine green (A) and of rhodamine green–dextran conjugate (B). (1) In water; (2) in an aqueous 30% sucrose solution; (3) in an aqueous 50% sucrose solution. The correlation times ϕ (and ϕ_1) and preexponential factors β_i obtained from the fits are listed in Table II.

Table I. Translational Diffusion Times (τ_{dif}) and Coefficients (D) and Hydrodynamic Radii (r_h) of Rhodamine Green and Rhodamine Green-Conjugated Dextran[a]

Sample	τ_{dif} (μs)	D (m^2 s^{-1}) · 10^{11}	r_h (nm)
Rhodamine green			
Water	89 ± 1	23.3 ± 0.3	0.92 ± 0.01
30% sucrose	315 ± 5	6.58 ± 0.10	1.02 ± 0.02
50% sucrose	1572 ± 91	1.32 ± 0.08	1.06 ± 0.06
Rhodamine green–dextran conjugate			
Water	180 ± 7	11.5 ± 0.4	1.87 ± 0.07
30% sucrose	657 ± 20	3.16 ± 0.10	2.14 ± 0.06
50% sucrose	3151 ± 192	0.66 ± 0.04	2.12 ± 0.13

[a] The standard deviation is based on 10 determinations of τ_{dif}. ω_1 [see Eq. (1)] was determined separately and is equal to 288 nm.

attached at the surface of a dextran molecule would certainly be influenced by the shear viscosity of the solvent. Energy transfer, on the other hand, would not have such a significant effect on the correlation time responsible for this process [4]. However, some cautious remarks should be made. In Ref. 4 the situation has been described for a dimeric protein, which has both fluorophores rigidly bound in the protein matrix. Energy transfer would then occur between more or less fixed transition dipoles. When the fluorophores are flexibly bound to the macromolecular support, energy transfer may occur between very rapidly rotating dipoles (implying the dynamically averaged limit of the orientation factor [7]). When the viscosity is increased, the slower motion of the transition dipoles is not averaged out any more. The rate constant of energy transfer may then depend on the orientation factor, which then becomes undetermined (the orientation factor will approach the anisotropic static limit [7]). In any case, a systematic effect of the transfer rate constant (or the transfer correlation time) on viscosity cannot be expected.

In Table II the rotational correlation times recovered after global analysis are collected for all samples. The average fluorescence lifetimes are also listed in Table II. In the case of fluorescent dextran it can be clearly observed that a short correlation time (ϕ_1) is predominantly present. This short correlation time becomes longer when the solvent viscosity increases. This is evidence that the rapid depolarization process is due to internal, restricted rotation of the dye molecule. This observation is in agreement with information provided by the manufacturer: the degree of substitution of the dextran conjugate is typically one to two dyes per dextran in the 10-kDa range.

Because of the high amplitude of the internal motion, the long correlation time assigned to overall rotation is not well determined. Let us assume in the first approximation that the polysaccharide rotates as a spherical particle. The overall rotational correlation time ϕ obtained from fluorescence anisotropy decay would then lead to the same hydrodynamic radius as obtained from FCS via the Stokes–Einstein relationship:

$$\phi = \frac{4}{3}\pi r_h^3 \eta / kT \tag{4}$$

The overall dextran rotational correlation time ϕ can therefore be obtained from FCS and can be fixed during the global analysis. For a probe in a spherical macromolecule exhibiting both rapid reorientation and overall tumbling, the following simplified expression for the anisotropy holds true [8]:

$$r(t) = \{\beta_1 \exp(-t/\phi_1) + \beta_2\} \exp(-t/\phi) \tag{5}$$

where ϕ_1 is the time constant for rapid, internal motion and $\beta_1 + \beta_2$ is equal to the fundamental anisotropy r_0. We can also define a second-rank order parameter S for the fluorophore in the dextran:

$$S^2 = \frac{\beta_2}{\beta_1 + \beta_2} = \frac{1}{2}\cos\psi(\cos\psi + 1) \tag{6}$$

where ψ is the angular displacement of the fluorophore due to internal motion. The rate of reorientation is given by the diffusion coefficient $D_\perp$ of internal motion [8]:

$$D_\perp = \frac{1 - S^2}{6\phi_1} \tag{7}$$

The results of this analysis for rhodamine green-conjugated dextran are presented in Table III.

The TRFA data have been reanalyzed according to Eq. (5), in which the overall rotational correlation time of the dextran (ϕ) has been fixed to values obtained from FCS. The only parameters which must be optimized are then β_1, β_2, and ϕ_1. Interestingly, the rapid correlation time ϕ_1 scales with the viscosity just like the overall rotational correlation time ϕ. The order parameter S is

Table II. Average Fluorescence Lifetimes ($\langle\tau\rangle$) and Fluorescence Anisotropy Decay Parameters (β_1, β_2, ϕ_1, ϕ) of Rhodamine Green and Rhodamine Green-Conjugated Dextran

Sample	$\langle\tau\rangle$ (ns)	β_1	ϕ_1 (ns)	β_2	ϕ (ns)
Rhodamine Green					
Water	3.94	—	—	0.317	0.167
30% sucrose	3.85	—	—	0.303	0.721
50% sucrose	3.70	—	—	0.328	3.44
Rhodamine green–dextran conjugate					
Water	3.85	0.226	0.239	0.081	1.99
30% sucrose	3.68	0.201	0.698	0.114	4.50
50% sucrose	3.50	0.140	1.94	0.192	12.0

Table III. Fluorescence Anisotropy Parameters (β_1, ϕ_1, β_2, S, ψ, $D_\perp$) of Rhodamine Green-Conjugated Dextran[a]

Sample	β_1	ϕ_1 (ns)	β_2	ϕ^b (ns)	S^2	ψ (deg)	$D_\perp$ (ns^{-1})
Water	0.250	0.39	0.030	6.8	0.108	79	0.383
30% sucrose	0.258	1.23	0.034	32	0.115	79	0.120
50% sucrose	0.252	3.50	0.075	152	0.228	70	0.037

[a] See text for explanation of symbols used.
[b] Calculated from FCS experiments and fixed in the global analysis of TFRA data.

small in all three cases and apparently seems a little higher at the highest sucrose concentration. This may be due to the higher fundamental anisotropy, implying that some ultrarapid depolarizing processes in the fluorescent dextran escaped observation at lower viscosities. The angular displacement ψ is quite large and similar in all samples, indicating that the covalently bound dye molecule moves with high amplitude. The rate of reorientation $D_\perp$ is inversely proportional to the internal correlation time and thus to the viscosity.

From the data in Table III it can be concluded that the dye is attached to the surface of the dextran and sticks to the solvent surrounding the polysaccharide. The probe can move independently about the linking axis to the dextran.

CONCLUSIONS

From both FCS and TRFA we can draw the following conclusions on the physical properties of dextran. From the translation diffusion measurements we have obtained the hydrodynamic radii for the polysaccharide under different solution conditions. If the dextran is assumed to be a sphere, then the hydrodynamic radii can be converted into molecular mass via

$$\frac{4}{3}\pi r_h^3 \cdot 6.02 \cdot 10^{23} \cdot 1.038 \cdot 10^6 = 2.5216 \cdot 10^{30} \cdot r_h^3 \tag{8}$$

where Avogadro's number and the density appear and r_h is in nanometers. In this way a mass of 16.5 kDa can be calculated for dextran in water and 24.0 kDa for dextran in an aqueous solution of 50% sucrose. Both mass values are distinctly larger than the 10.6-kDa mass supplied by the manufacturer (dextran with one dye molecule attached). Our conclusion is that the polysaccharide is highly hydrated and that sucrose is in some way taken up by the dextran. In the case that the rhodamine green–dextran conjugate is nonhydrated, then $r_h = 1.61$ nm instead of $r_h = 1.87$ nm (Table I).

From the TRFA results we can conclude that the dye has almost-unrestricted motional freedom, is located at the surface of the dextran, and senses the shear viscosity of the solvent.

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