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ORIGINAL PAPER

Diffusion Coefficients of Several Rhodamine Derivatives as Determined by Pulsed Field Gradient–Nuclear Magnetic Resonance and Fluorescence Correlation Spectroscopy

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Abstract Rhodamine derivatives are popular, photostable fluorophores that are used in a number of fluorescent based techniques, including fluorescence correlation spectroscopy (FCS). Indeed, in FCS, both rhodamine 6G (R6G) and rhodamine 110 (R110) are used as calibration standards to determine the dimensions of the instrument confocal volume. In spite of a requirement for precise values of the diffusion coefficients, literature values are scarce and vary over an order of magnitude. In this paper, the diffusion coefficients of four rhodamine fluorophores (rhodamine 6G (R6G), rhodamine B (RB), rhodamine 123 (R123), rhodamine 110 (R110)) were determined by pulsed field gradient nuclear magnetic resonance (PFG-NMR) spectrometry and then validated by comparison with fluorescence correlation spectroscopy. With the objective of validating the FCS calibration, diffusion coefficients of several dextrans and a polystyrene nanoparticle were also determined and compared with literature values or theoretical values that were based upon the Stoke–Einstein equation. The work presented here lead us to conclude that the diffusion coefficients for R6G and R110 have generally been underestimated in the literature. We propose revised values of 4.4×10^{-10} m^2 s^{-1} for R110 and 4.0×10^{-10} m^2 s^{-1} for R6G. Using the revised D value for R110 to calibrate the FCS instrument, diffusion coefficients have then been systematically determined for different conditions of pH, ionic strength and concentration. To correct for differences due to solvent effects (D_2O vs. H_2O), an isotopic correction factor, D_{D_2O}/D_{H_2O} of 1.23, was determined from both FCS and from the solvent auto-diffusion coefficients obtained by NMR.

Keywords FCS · NMR · Rhodamine · Diffusion coefficient

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Introduction

Due to their photostability, their large extinction coefficients and high fluorescence quantum yield [1], rhodamine derivatives are widely used for diffusion measurements or as labels in environmental [2–6] biochemical [7–11] and interfacial studies [12]. Among other uses, they are employed to calibrate fluorescence correlation spectroscopy (FCS), a technique now widely employed to determine molecular sizes and to characterize translational (Brownian) diffusion [13, 14] in complex media. Nonetheless, despite their use as calibration standards, their translational diffusion coefficients in water have not been precisely characterized, due in part to the inherent difficulty in measuring D precisely and to the fact that values of the diffusion coefficients will depend upon ionic strength, pH and concentration effects due to dimerization [15–17] and/or aggregation. Indeed, literature values of D, where available, vary by nearly an order of magnitude (Tables 1 and 2).

Since large errors in the diffusion coefficients will introduce systematic errors of similar magnitude on the final FCS measurements, the aim of the present work was to determine accurate and precise values of the translational diffusion coefficients of several rhodamine derivatives

Table 1 Summary of literature values of diffusion coefficients for rhodamine 6G

Conditions	D (10^{-10} m^2 s^{-1})	Technique	Reference
Water	2.8±0.3	FCS	[18]
Water, 20 °C	3	FCS	[29]
Water/D_2O	2.9±0.7[a]	NMR	[30]
Water	4.0	–	[31]
0.2 M KCl, pH 7.0	4.0 (D_{Ox})	Voltammetry	[32]
	4.2 (D_{Red})	Ox and Red species not specified	
20 mM Boric acid, 100 mM Tris, 25 °C	4.59	E-Field method capillary/microchannel electrophoresis static imaging	[33]
20 mM Boric acid, 100 mM Tris, 25 °C	4.14±0.01		[33]
50:50 water/methanol	2.71±0.09	Capillary/microchannel electrophoresis, various detection techniques	[33]
	2.684±0.005		
	2.69±0.02		
	2.88±0.17		
50:50 water/methanol	2.7±0.1	Capillary/microchannel electrophoresis	[34]
Various combinations of water and alcohol	2.9±0.3 (ethanol)	Patterned FCS	[35]
	2.5±0.3 (60:40 H_2O/methanol)		
	2.5±0.2 (50:50 H_2O/methanol)		
	2.8±0.3 (40: 60 H_2O/methanol)		
	3.4±0.4 (20: 80 H_2O/methanol)		
	4.5±0.6 (5: 95 H_2O/methanol)		

[a] Values were not corrected for isotopic effect. Corrected values would be *ca*. 3.5×10^{-10} m^2 s^{-1}

(rhodamine 6G (R6G), rhodamine B (RB), rhodamine 123 (R123), rhodamine 110 (R110), Fig. 1) under relevant conditions of pH, ionic strength and concentration. In FCS, values of 2.8×10^{-10} m^2 s^{-1} (R6G) [18] and 3.0×10^{-10} m^2 s^{-1} (R110) [19] are commonly employed to calibrate the instrument. Therefore, a main objective of the study was to verify the accuracy of these values. The study was performed initially using pulsed field gradient nuclear magnetic resonance (PFG-NMR) [20] then validated using FCS [18, 21].

Theory

In FCS, translational diffusion coefficients are determined by the measuring diffusion times of a fluorescent probe inside a carefully calibrated small (confocal) volume (typically ~0.2 μm^3). Variations of the fluorescence intensity inside the confocal volume are attributed to the translational diffusion of a small number of fluorophores. For a 1-component system, in the absence of phosphorescence, diffusion times are determined from an autocorrelation function, $G(t)$:

$$G(t) = a + \frac{1}{n}\left(1+\frac{t}{\tau}\right)^{-1}\left(1+\frac{t}{p^2\tau}\right)^{-0.5} \quad (1)$$

where a is the limiting value of $G(t)$ for $t \to \infty$ (usually a= 1), n is the average number of fluorescent particles diffusing in the confocal volume, τ is the diffusion time of the fluorescent species inside the confocal volume, p is the structure parameter (which is the ratio of the transversal ω_{xy} to the longitudinal ω_z radius of the confocal volume $p=\omega_z/\omega_{xy}$). Diffusion coefficients of the unknown molecules or particles are calculated from their diffusion times following calibration of the dimensions of the confocal volume, generally using R6G ($D = 2.8 \times 10^{-10}$ m^2 s^{-1}) or R110 ($D = 3.0 \times 10^{-10}$ m^2 s^{-1}).

In a PFG-NMR experiment, magnetic pulsed-field gradients with a echo sequence [22], are applied to the sample in addition to the instrument's static magnetic field.

Table 2 Summary of diffusion coefficients determined from the literature for rhodamine B

Conditions	D (10^{-10} m^2 s^{-1})	Technique	Reference
20 mM Boric acid, 100 mM Tris, 25 °C	4.27±0.04	Capillary/microchannel electrophoresis	[33]
0.05 M H_2SO_4, 9.6 10^{-4} M rhodamine B	3.2±0.2	Rotating disk voltammetry	[36]
Water, 21.5 °C	3.6	Time-lapse microscopy	[7]

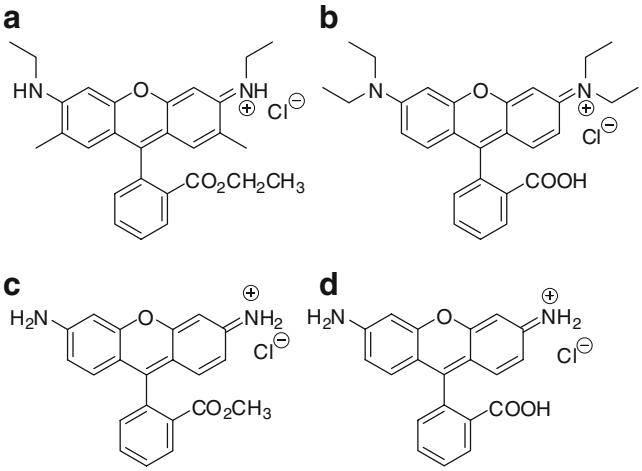

Fig. 1 Structures of **a** rhodamine 6G, **b** rhodamine B, **c** rhodamine 123 and **d** rhodamine 110

The gradient pulse labels the nuclear spin based upon the molecule's position in solution. After diffusing a short distance through solution, a decoding gradient pulse is applied. The intensity, I, of the resonances of the molecule in the NMR spectrum will be attenuated in proportion to the magnetic gradient pulse amplitude G and the diffusion coefficient D:

$$I = I_0 \exp\left[-D(G\delta\gamma)^2\left(\Delta - \frac{\delta}{3}\right)\right] \tag{2}$$

where I_0 is the signal intensity or integral in the absence of the gradient pulse, δ is the duration of the pulse, γ is the gyro-magnetic ratio of the nucleus under investigation and Δ is the diffusion delay time. This equation holds only for simple PFG-NMR experiments and is slightly modified for more sophisticated pulse sequences [23]. The optimization of PFG-NMR experiments to achieve precise measurements of the diffusion coefficient has been addressed by Antalek [24].

For hard spheres, diffusion coefficients can be determined from their radius using the Stokes–Einstein equation:

$$D = \frac{kT}{6\pi\eta r} \tag{3}$$

where k is Boltzmann's constant, T is the temperature, η is the viscosity and r the hydrated radius of the particle.

Since NMR experiments are more easily performed in D_2O, it is necessary to correct for the isotopic effect on the translational auto-diffusion coefficients. The dependency of the diffusion coefficient on temperature, T, is strong and different for the two isotopes and can been taken into account using the following equation [25]:

$$\frac{D_{H_2O}}{D_{D_2O}} = 0.9194\frac{\left(\frac{T-209.443}{209.443}\right)^{2.191}}{\left(\frac{T-222.54}{222.54}\right)^{2.011}} \tag{4}$$

The above constants are auto-diffusion coefficients that reflect the friction of the solvent on itself. Assuming that rhodamine–rhodamine interactions are negligible due to the high dilution used in the experiments (for FCS, concentrations are $<10^{-8}$ M), a solvent friction similar to that of the water should be determined:

$$\frac{D_{H_2O}}{D_{D_2O}} \approx \frac{D(\text{rhodamine})_{H_2O}}{D(\text{rhodamine})_{D_2O}} = \frac{\tau(\text{rhodamine})_{D_2O}}{\tau(\text{rhodamine})_{H_2O}} \tag{5}$$

Since diffusion times, τ, can be measured directly without calibration of the confocal volume, no mathematical assumptions are required in order to determine the isotopic effects of solvent deuteration on the diffusion coefficients (Eq. 5) when determining D_{H2O}/D_{D2O} by FCS.

Materials and methods

Compounds

R6G, R110 (laser grade, 99%,) and three dextrans labeled with tetramethylrhodamine (molar masses of 10,000, 40,000 and 70,000) were purchased from Molecular Probes. R123 (>99%) and RB (>99%) were obtained from Sigma-Aldrich. The polystyrene nanoparticles (57 nm diameter, green dragon label) were purchased from Bangs Laboratory. Solutions of the four fluorophores (R6G, R123, R110, RB) were prepared in Milli-Q water or in D_2O (Sigma Aldrich) at final concentrations of <0.1 μM (FCS) or 0.016, 0.08, 0.4 and 2.0 mM (NMR experiments). For experiments examining pH (pD) or ionic strength effects using NMR, KOD or DCl was used to adjust the pD (2.9, 7.3, 10.3) and KCl was added to adjust I (2, 10 and 100 mM). Due to the limited solubility of some of the fluorophores, some experiments were performed in aqueous solutions of MeOH or MeOD that were purchased from Sigma-Aldrich. Water was Milli-Q grade (R>18 MΩ cm, total organic carbon concentrations <2 μg C L^{-1}). High purity potassium chloride, potassium hydroxyde and nitric acid were employed for pH and ionic strength adjustments.

FCS

Two FCS devices were employed in this study. The first (Geneva) was a Confocor Axiovert 135TV on a Carl Zeiss platform while the second was a system that was mounted on a Leica TCS SP5 laser scanning microscope (Montreal). Excitation of the rhodamine derivatives was performed using an Ar ion laser at 488 or 514 nm. Fluorescence intensity fluctuations were quantified with an avalanche photodiode detector. Three experiments, each with ten replicate measurements, were performed for each experi-

mental condition. Due to the temperature dependence of the diffusion coefficients, temperature was carefully measured and controlled (22.5 °C). On the Confocor instrument, data were acquired with a control program from Carl Zeiss Jena GmbH (version 1.2.1) and interpreted using FCS Access Fit Software (version 1.0.12, Evotec BioSystems GmbH). The Leica SP5 used ISS Vista FCS software (version 3.6_37). The FCS confocal volume was initially calibrated using five known concentrations of R110 with an assumed initial diffusion coefficient of 3.0×10^{-10} m^2 s^{-1} or with R6G ($D = 2.8\times 10^{-10}$ m^2 s^{-1}). FCS was also used to determine the isotope effect (D_2O/H_2O) on the diffusion coefficients of the rhodamine derivatives. In that case, diffusion times were measured in D_2O or H_2O without calibration of the confocal volume.

PFG-NMR

Nuclear magnetic resonance (NMR) experiments were performed on a Bruker Avance 400 or 600 MHz instrument using a simulated echo sequence (Fig. 2). In order to compare diffusion coefficients acquired by NMR with those obtained using FCS, the temperature of the NMR measurements was matched to that of the FCS measurements (22.5 °C). Finally, in order to ascertain that the measurements were reproducible, three to 12 different peaks were used for D determinations, depending on concentration and signal quality. Under these conditions, 7,168 scans were required to acquire data with an acceptable signal to noise ratio for the least concentrated solutions (16 μM) of R6G and RB while 256 scans were performed for the other conditions. Eight to 16 different gradient strengths from 7 to 200 G were used for each PFG-NMR experiment to fit for the diffusion coefficients.

Results and discussion

Determination of water isotopic effect on D

Measurements of NMR D values are greatly facilitated by performing the measurements in D_2O. For comparison with values obtained in H_2O, it was thus necessary to quantify the isotopic effect. As expected, diffusion times of the rhodamine derivatives obtained in D_2O were significantly slower than those obtained in H_2O, corresponding to smaller D values in D_2O. Indeed, for R6G, R123 and RB, the ratio of the diffusion coefficients was 1.23 at 22.5 °C, which compares well with values obtained previously by experimentation [26] (1.26) or by simulation [27] (1.20).

Determination of D for R110 and R6G

Following correction for the isotopic effect ($D_{H_2O}/D_{D_2O} = 1.23$), a D value of 4.8×10^{-10} m^2 s^{-1} was determined by NMR for R110. Nonetheless, due to the fluorophore's limited solubility and the small number of non aromatic protons (fewer signals), the uncertainty on the value was quite large, *ca*. 21%. For R6G, a value of $(4.0\pm0.3)\times10^{-10}$ m^2 s^{-1} was determined by NMR for R6G. Both D values were significantly higher than the generally accepted literature values but were somewhat less precise. Two complementary strategies were therefore employed to reduce uncertainty and increase accuracy of the D values: (a) *for several model fluorophores*, diffusion coefficients determined by FCS (using standard calibrations of the confocal volume) were compared to values determined by NMR and (b) *for several model size standards*, D values determined by FCS were compared to theoretical values calculated from the Stokes Einstein equation.

Based upon a calibration using R110, diffusion coefficients were obtained for a number of standard solutions using NMR and FCS (Fig. 2): (1) R6G in 50% MeOH: 50% H_2O; (2) R110 in 5% MeOD: 95% D_2O; (3) R110 in 10% MeOD, 90% D_2O; (4) R6G; (5) RB. When plotted together, FCS values were on average 41% lower than values obtained by NMR following correction for the D_2O isotopic effect. For example, in addition to the larger D values that were observed above for R110 and R6G, a value of 4.2×10^{-10} m^2 s^{-1} was obtained by NMR for RB (H_2O).

Literature values of the diffusion coefficients of three dextran standards [28] and that of a polystyrene particle standard determined from the Stokes Einstein equation (Eq. 3) were also plotted (Fig. 4) against values of D obtained from FCS. By calibrating the FCS with a D value of R110 $= 3.0\times10^{-10}$ m^2 s^{-1}, FCS determinations were once again significantly smaller than the expected values for the particles (slope=1.33, R^2=0.913). The value of the

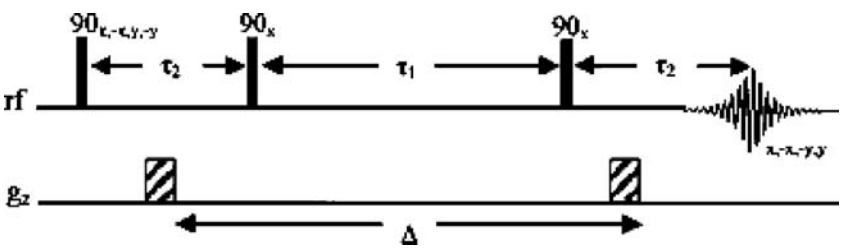

Fig. 2 The pulse gradient stimulated echo pulse sequence[24], where Δ is between 10 and 100 ms depending on the concentration and τ is the pulse time

slope suggests that D for R110 should be on the order of 4.0×10^{-10} m^2 s^{-1}.

Both the NMR determinations and the above calibrations suggested that the FCS calibration values ($D_{R6G} = 2.8\times10^{-10}$ m^2 s^{-1};$D_{R110} = 3.0\times10^{-10}$ m^2 s^{-1}) were too low. Therefore, FCS experiments were recalibrated using an intermediate D value of 4.4×10^{-10} m^2 s^{-1} for R110 determined from the NMR value (4.8×10^{-10} m^2 s^{-1}) and the calibration slopes (4.0×10^{-10} m^2 s^{-1}). By recalibrating the FCS experiments with the revised R110 D value of 4.4×10^{-10} m^2 s^{-1}, revised slopes of 1.01 were obtained for Fig. 3 (R^2=0.90) and 1.01 for Fig. 4 (R^2=0.999). The above experiments also imply a significantly higher value of D for R6G of 3.7×10^{-10} m^2 s^{-1} (also see below).

Critical evaluation of the revised D values

Both the NMR results and the FCS measurement of the standard compounds indicated a significant underestimation of the generally accepted values of the diffusion coefficients for R110 and R6G. Furthermore, in spite of hundreds of citations that refer to the low D values for R6G, other values are available in the literature for R6G (and RB) that are more in line with the values determined here (Tables 1 and 2). For example, our values are consistent with those determined previously in aqueous solutions by capillary/microchannel electrophoresis and voltammetry: (4.0–4.6)$\times10^{-10}$ m^2 s^{-1} for R6G (Table 1), and 4.3×10^{-10} m^2 s^{-1} for RB (Table 2). In fact, most of the lower values of D in the literature were previously determined by FCS. Nonetheless, some of the variability in the literature values may have resulted from a lack of a control over temperature, from variable fluorophore concentrations or to the absence of a correction of the isotope effect for experiments performed in D_2O.

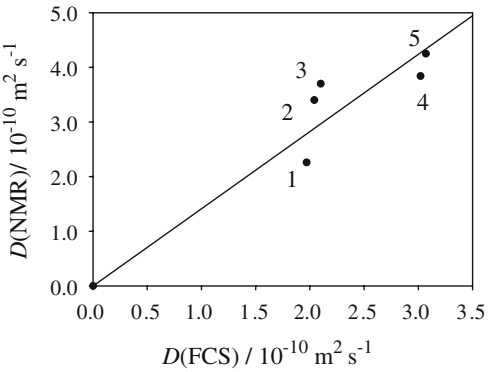

Fig. 3 Experimental diffusion coefficients determined by NMR as a function of those determined by FCS (obtained by assuming a value of 3.0×10^{-10} m^2 s^{-1} during the calibration of the confocal volume with R110). Several fluorophores/conditions were examined: *1* R6G in 50% MeOH, 50% H_2O; *2* R110 in 5% MeOD, 95% D_2O; *3* R110 in 10% MeOD, 90% D_2O *4* R6G in H_2O *5* RB in H_2O

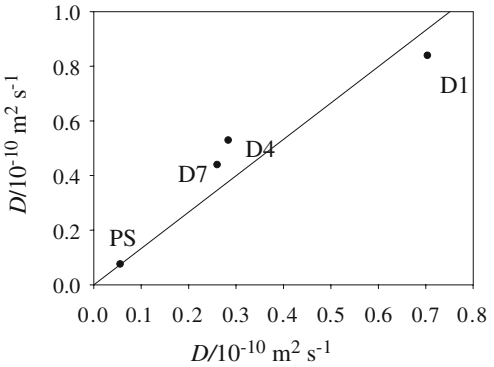

Fig. 4 Theoretical diffusion coefficients of particle standards determined as a function of those determined by FCS (obtained by assuming a value of 3.0×10^{-10} m^2 s^{-1} during the calibration of the confocal volume with R110). Several particle standards were examined: 10,000 molar mass dextran (*D1*); 40,000 molar mass dextran (*D4*), 70,000 molar mass dextran (*D7*) and a polystyrene particle standard (*PS*) with a radius of 57 nm

It is notable that few fluorescent compounds are currently available with precisely known values of their diffusion coefficients. Furthermore, orthogonal methods are rarely used to confirm D values. For the above FCS evaluations of the particle standards, diffusion coefficients were outside the calibration range of the rhodamine derivatives and thus should be interpreted with care. With respect to diffusion coefficient determinations made using NMR, the measurements are much more accurate when performed at fairly high concentrations in D_2O. The correction for the isotopic effect will, however, add some uncertainty to values presented for H_2O. Most importantly, some of the compounds that were examined have a tendency to dimerize or aggregate at high concentrations [15, 16] which could result in an apparent decrease in D. The concentration effect might partially explain why such a large variation of diffusion coefficients has been observed among the different literature sources since each technique has its own analytical window. The effect of fluorophore concentration was examined in detail in the next section.

Effect of concentration on D

For R110, it was not possible to perform NMR experiments in the same concentration range as R6G and RB due to its limited solubility and the scarcity of non-aromatic protons. Indeed, only a single peak could be used to determine the diffusion coefficient for R110 after 48 h of instrument time for a nominal concentration of 0.05 mM. Furthermore, due to the sensitivity differences of the NMR and FCS techniques, the examined concentration ranges did not overlap. For both R6G (Fig. 5a) and RB (Fig. 5b), diffusion coefficients decreased significantly (ANOVA, $p<0.05$) as a function of fluorophore concentration for concentrations down to 16 μM. Such results are consistent with the work

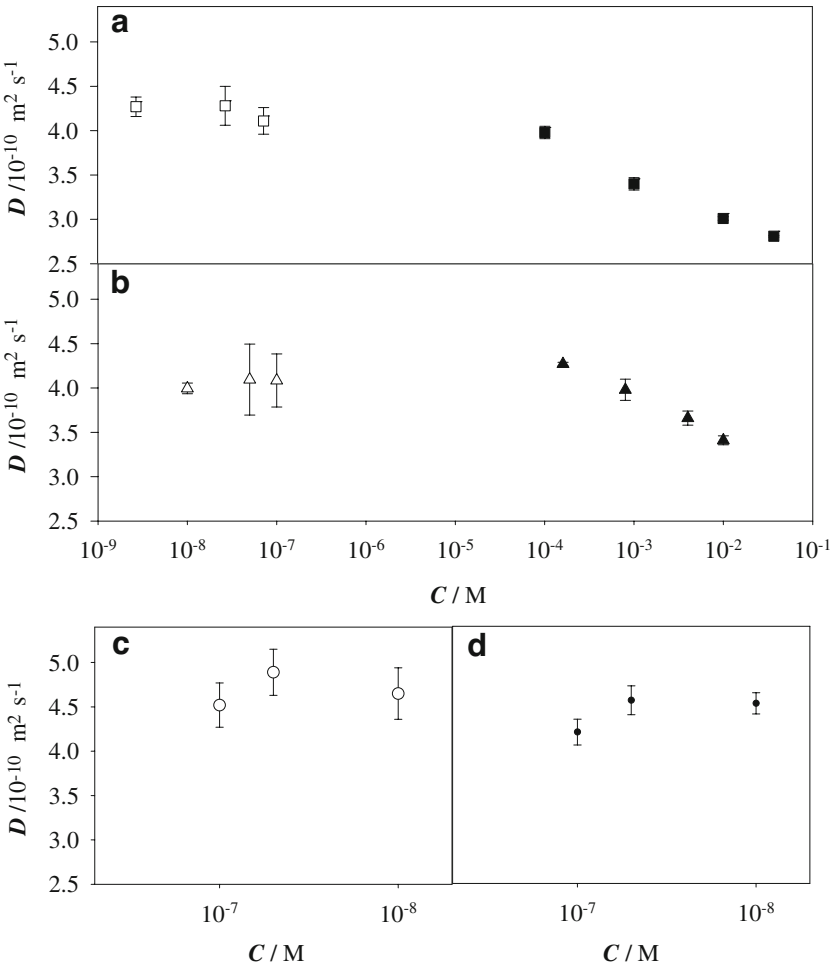

Fig. 5 Influence of the concentration on the diffusion coefficient of four rhodamine derivatives, as determined by FCS (*white symbols*) and NMR (*full symbols*). Figures correspond to: **a** R6G, **b** RB, **c** R123, **d** R110. NMR experiments were performed in D_2O at pH 6.5 at an ionic strength of 2 mM. FCS experiments were performed in H_2O at pH 5.8 at the same ionic strength

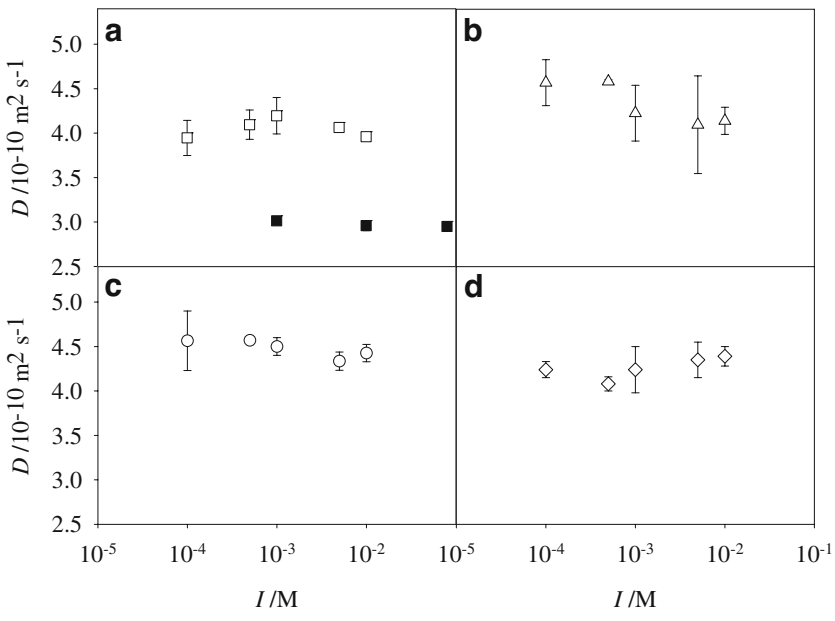

Fig. 6 Influence of the ionic strength on the diffusion coefficient of **a** R6G, **b** RB, **c** R123 and **d** R110 as determined by FCS (*white symbols*) and NMR (*full symbols*). FCS experiments were performed using 100 nM of each fluorophore at pH=6.3 whereas 2 mM of fluorophore was used in the NMR experiments (pH=5.5)

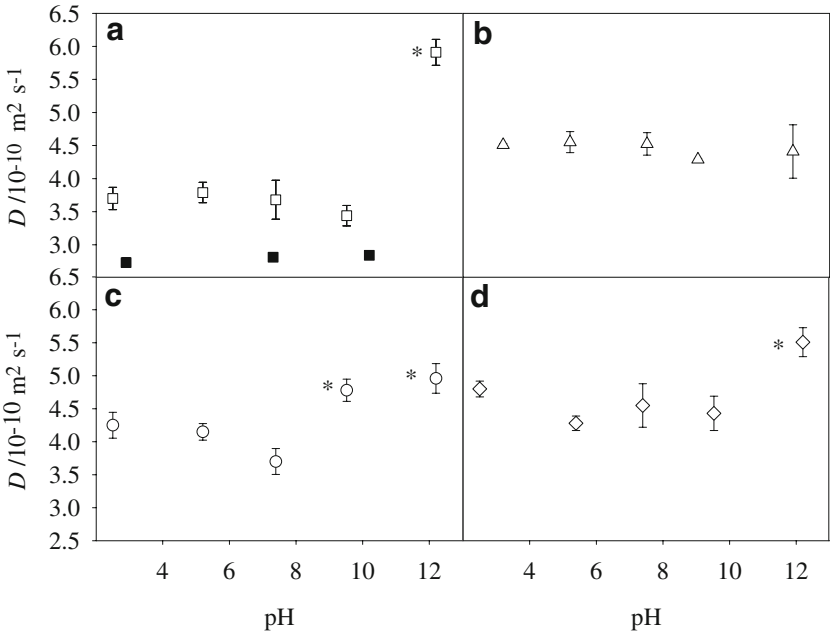

Fig. 7 Influence of the pH on the diffusion coefficient of: **a** R6G, **b** RB, **c** R123, **d** R110 as determined by FCS (*white symbols*) and NMR (*full symbols*). FCS experiments were performed using 100 nM of the fluorophores while 2 mM of fluorophore was used in the NMR experiments (I=2 mM). The *asterisks* indicate significant differences among values (one-way ANOVA, $p<0.001$, Student t-test)

of Dare-Doyen et al. who have shown that in the millimole to micromole range, R6G has a strong tendency to dimerize in water. No such decrease was observed for R110 or R123, although this was mainly due to our inability to perform systematic NMR experiments at the higher concentrations necessary for NMR. For all of the fluorophores that were examined, no apparent aggregation was observed for concentrations below 100 nM (Fig. 5). Note that some controversy exists in the literature on the importance of the R6G dimers. Sariri et al. have shown that the monomer: dimer abundance was invariant with concentration, while Daré-Doyen et al. used molecular modeling studies to show that the dimerization was thermodynamically favorable. In this study, the observed reduction in D with increasing concentration indicated that dimerization/aggregation was indeed favored at the higher concentrations of R6G, suggesting that the thermodynamic explanation is the most appropriate. It would appear that the rhodamine monomers are stable only at the lowest concentrations in aqueous solution. Given the important role of concentration on the dimerization or aggregation of these partially charged fluorophores, ionic strength and pH effects were also examined.

Effect of ionic strength and pH on D

For charged molecules, increased ionic strength is expected to result in increased charge screening leading to decreased electrostatic repulsion and increased aggregation (smaller D). For ionic strengths varying between 10^{-4} and 10^{-2} M (10^{-1} M for R6G), no significant changes in the diffusion coefficient were observed for any of the four fluorophores (Fig. 6, ANOVA, $p>0.05$). All observed variations were within the analytical precision of the two techniques that were used. For R6G, the large observed difference in the results obtained by FCS and NMR were attributed to the very different concentrations that were employed for the studies (FCS: 0.1 μM; NMR: 2 mM).

Given the protonable functional groups in all of the fluorophores, a decrease in pH could be expected to result in a decreased intermolecular electrostatic repulsion resulting in a potentially large increase in aggregation. Nonetheless, for the conditions that were examined here (Fig. 7), a significant pH effect was observed only at very high pH values corresponding to the deprotonation of the amine protons on the R6G, RB and R110. No significant changes were observed for any of the fluorophores below pH 9.5. Furthermore, for the higher concentrations that were employed in the NMR experiments, no changes in D were observed for R6G. Nonetheless, it should be noted that due to solubility concerns, pH could not be manipulated over the same range as for the FCS experiments. As above, the NMR determined diffusion coefficients were significantly lower than those determined by FCS.

Table 3 Values of diffusion coefficients proposed for the rhodamine fluorophores for moderate pH values (2–9.5), ionic strengths <0.1 M and concentrations <10 μM

	Rhodamine 6G	Rhodamine B	Rhodamine 123	Rhodamine 110
D 10^{-10} [m^2 s^{-1}]	4.0±0.3	4.2±0.3	4.4±0.3	4.3±0.3

Comparison of the four fluorophores

Due to the relative invariability of D with pH, I and concentration, diffusion coefficients that are valid for a fairly broad range of experimental conditions (pH 2–9.5, $I < 10^{-1}$ M, fluorophore concentration <10 μM) could be provided (Table 3). In both D_2O and H_2O, diffusion coefficients decreased in the order R110<R123<RB<R6G. Most of the effect can be attributed to differences in the molar masses: R110 (366 g/mol) and R123 (380 g/mol) have smaller molar masses than R6G and RB (480 g/mol) and thus the observed differences are, for the most part, consistent with a scaling of D with $M_w^{1/3}$. The small difference between the values of D for R6G and RB can be explained by two effects: (a) hydrogen bonding should increase in the order RB<R6G with a resulting increase in diffusion time, corresponding to a smaller diffusion coefficient for R6G with respect to RB; (b) increased photodecomposition might lead to shorter diffusion times (if photodecomposition occurs for times that are of similar or shorter duration to diffusion across the confocal volume). The second explanation is unlikely here since, under the conditions of the experiments, R6G and RB had very similar measured triplet fractions. In fact, of the four fluorophores that were evaluated, only R110 had a systematically smaller triplet fraction when laser excitation (Ar ion) of 488 or 514 nm was employed (data not shown). Indeed, the smaller value of measured triplet fraction for R110, suggests that the R110 is likely to be the most appropriate calibration standard for FCS when using a D value of 4.4×10^{-10} m^2 s^{-1} (as compared to 3.0×10^{-10} m^2 s^{-1} used previously).

Conclusion

Translational diffusion coefficients have been determined for four rhodamine derivatives: R6G, RB, R123 and R110, of which two are commonly used as calibration standards for fluorescence correlation spectroscopy. A diffusion coefficient of 4.0×10^{-10} m^2 s^{-1} was found for R6G and a value of 4.4×10^{-10} m^2 s^{-1} was determined for R110. These values are much higher than the values that are frequently employed to calibrate the FCS technique but in good agreement with a number of other analytical techniques (capillary/microchannel electrophoresis, E-Field method electrophoresis or voltammetry). The combination of PFG-NMR and FCS proved to be a powerful characterization tool to probe diffusion coefficients in aqueous solutions once the observed large isotopic effect ($D_{D_2O}/D_{H_2O} = 1.23$) was taken into consideration. Ionic strength and pH had relatively little effect on the diffusion coefficients of the rhodamine derivatives, while concentration had a significant effect for >10 μM concentrations.

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References

1. Krichevsky O, Bonnet G (2002) Fluorescence correlation spectroscopy: the technique and its applications. Rep Prog Phys 65:251–297
2. Leng X, Starchev K, Buffle J (2001) Applications of fluorescence correlation spectroscopy: measurement of size–mass relationship of native and denatured schizophyllan. Biopolymers 59:290–299
3. Meunier F, Wilkinson KJ (2002) Non-perturbing fluorescent labeling of polysaccharides. Biomacromolecules 3:857–864
4. Leng X, Startchev K, Buffle J (2002) Application of fluorescence correlation spectroscopy: a study of flocculation of rigid rod-like biopolymer (schizophyllan) and colloidal particles. J Colloid Interface Sci 251:64–72
5. Avena MJ, Wilkinson KJ (2002) Disaggregation kinetics of a peat humic acid: mechanism and pH effects. Environ Sci Technol 36:5100–5105
6. Fatin-Rouge N, Buffle J (2007) Study of environmental systems by means of fluorescence correlation spectroscopy. In: Wilkinson KJ, Lead JR (eds) IUPAC series on analytical and physical chemistry of environmental systems. Wiley, Chichester, pp 507–554
7. Rani SA, Pitts B, Stewart PS (2005) Rapid diffusion of fluorescent tracers into *Staphylococcus epidermidis* biofilms visualized by time lapse microscopy. Antimicrob Agents Chemother 49:728–732
8. Stroh M, Zipfel WR, Williams RM, Webb WW, Saltzman WM (2003) Diffusion of nerve growth factor in rat striatum as determined by multiphoton microscopy. Biophys J 85:581–588
9. Roumi M, Kwong E, Deghenghi R, Locatelli V, Marleau S, Du Souich P, Beliveau R, Ong H (2001) Permeability of the peptidic GH secretagogues hexarelin and EP 51389, across rat jejunum. Peptides (New York, NY, United States) 22:1129–1138
10. Dos Santos Silva AL, Joekes I (2005) Rhodamine B diffusion in hair as a probe for structural integrity. Colloids Surf, B Biointerfaces 40:19–24
11. Stroh M, Saltzman WM (2003) Diffusion of neurotrophins and conjugates in brain tissue as determined by multiphoton microscopy. AIChE Annual Meeting, Conference Proceedings, San Francisco, CA, United States, Nov. 16–21:2003:103–111
12. Mukhopadhyay A, Zhao J, Bae SC, Granick S (2002) Contrasting friction and diffusion in molecularly thin confined films. Phys Rev Lett 89:136103/136101–136103/136104
13. Thompson NL (1991) Fluorescence correlation spectroscopy. Topics in Fluorescence Spectroscopy 1:337
14. Fatin-Rouge N, Milon A, Buffle J, Goulet RR, Tessier A (2003) Diffusion and partitioning of solutes in agarose hydrogels: the relative influence of electrostatic and specific interactions. J Phys Chemistry B, 107:12126–12137
15. Dare-Doyen S, Doizi D, Guilbaud P, Djedaieni-Pilard F, Perly B, Millie P (2003) Dimerization of xanthene dyes in water: experimental studies and molecular dynamic simulations. J Phys Chemistry B, 107:13803–13812
16. Ilich P, Mishra PK, Macura S, Burghardt TP (1996) Direct observation of rhodamine dimer structures in water. Spectrochim Acta Part A Mol Biomol Spectrosc 52A:1323–1330
17. Sariri R, Zakerhamidi MS, Baharpaima K, Ghanadzadeh A (2004) The anion effect and molecular association of rhodamine dyes in isotropic and anisotropic solvents. J Mol Liq 115:55–61

18. Magde D, Elson EL, Webb WW (1974) Fluorescence correlation spectroscopy. I. Conceptual basis and theory. Fluorescence correlation spectroscopy conceptual basis and theory. II. Biopolymers 13:29–61
19. Mueller JD, Gratton E (2003) High-pressure fluorescence correlation spectroscopy. Biophys J 85:2711–2719
20. Price KE, Lucas LH, Larive CK (2004) Analytical applications of NMR diffusion measurements. Anal Bioanalytical Chemistry 378:1405–1407
21. Magde D, Elson EL, Webb WW (1972) Thermodynamic fluctuations in a reacting system—measurement by fluorescence correlation spectroscopy. Phys Rev Let 29:705
22. Stejskal EO, Tanner JE (1965) Spin diffusion measurements: spin echoes in the presence of a time-dependent field gradient. J Chem Phys 42:288–292
23. Price WS (1997) Pulsed-field gradient nuclear magnetic resonance as a tool for studying translational diffusion: part I. Concepts Magn Reson 9:299–336
24. Antalek B (2002) Using pulsed gradient spin echo NMR for chemical mixture analysis: how to obtain optimum results. Concepts Magn Reson 14:225–258
25. Hardy EH, Zygar A, Zeidler MD, Holz M, Sacher FD (2001) Isotope effect on the translational and rotational motion in liquid water and ammonia. J Chem Phys 114:3174–3181
26. Svishchev IM, Kusalik PG (1994) Dynamics in liquid water, water-d2, and water-t2: a comparative simulation study. J Phys Chem 98:728–733
27. Hernandez de la Pena L, Kusalik PG (2004) Quantum effects in light and heavy liquid water: a rigid-body centroid molecular dynamics study. J Chem Phys 121:5992–6002
28. Pluen A, Netti PA, Jain RK, Berk DA (1999) Diffusion of macromolecules in agarose gels: comparison of linear and globular configurations. Biophys J 77:542–552
29. Rigler R, Grasselli P, Ehrenberg M (1979) Fluorescence correlation spectroscopy and application to the study of Brownian motion of biopolymers. Phys Scr 19:486–490
30. Gell C, Brockwell DJ, Beddard GS, Radford SE, Kalverda AP, Smith DA (2001) Accurate use of single molecule fluorescence correlation spectroscopy to determine molecular diffusion times. Single Molecules 2:177–181
31. Jones MC, Nassimbene RD, Wolfe JD (1996) Mixing and dispersion measurements on packed bed flows using a fiber-optic probe array. Eng Science 51:1009
32. Yu J-S, Zhou T-Y (2001) The electrochemistry and thin-layer luminescence spectroelectrochemistry of rhodamine 6G at a 4,4¢-bipyridine-modified gold electrode. J Electroanal Chem 504: 89–95
33. Culbertson CT, Jacobson SC, Ramsey JM (2002) Diffusion coefficient measurements in microfluidic devices. Talanta 56:365–373
34. Fister JC III, Jacobson SC, Davis LM, Ramsey JM (1998) Counting single chromophore molecules for ultrasensitive analysis and separations on microchip devices. Anal Chem 70:431–437
35. Hansen RL, Zhu XR, Harris JM (1998) Fluorescence correlation spectroscopy with patterned photoexcitation for measuring solution diffusion coefficients of robust fluorophores. Anal Chem 70:1281–1287
36. Austin JM, Harrison IR, Quickenden TI (1986) Electrochemical and photoelectrochemical properties of rhodamine B. J Phys Chem 90:1839–1843