Determination of equilibrium and rate constants for complex formation by fluorescence correlation spectroscopy supplemented by dynamic light scattering and Taylor dispersion analysis†

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The equilibrium and rate constants of molecular complex formation are of great interest both in the field of chemistry and biology. Here, we use fluorescence correlation spectroscopy (FCS), supplemented by dynamic light scattering (DLS) and Taylor dispersion analysis (TDA), to study the complex formation in model systems of dye–micelle interactions. In our case, dyes rhodamine 110 and ATTO-488 interact with three differently charged surfactant micelles: octaethylene glycol monododecyl ether \( \text{C}_{12}\text{E}_{8} \) (neutral), cetyltrimethylammonium chloride \( \text{CTAC} \) (positive) and sodium dodecyl sulfate \( \text{SDS} \) (negative). To determine the rate constants for the dye–micelle complex formation we fit the experimental data obtained by FCS with a new form of the autocorrelation function, derived in the accompanying paper. Our results show that the association rate constants for the model systems are roughly two orders of magnitude smaller than those measured by Giustini. Therefore, further development of advanced techniques and theories to study quantitatively the formation of molecular complexes is still needed.

Fluorescence correlation spectroscopy (FCS) was first introduced by Magde and Elson in the early 1970s to determine the chemical kinetic constants of the interaction between DNA and ethidium bromide. Since the advent of confocal microscopy illumination, FCS experienced a renaissance in the 1990s. In FCS, we monitor the fluctuations of the fluorescence signals originating from molecules diffusing through the focal volume. The autocorrelation analysis of these fluctuations provides information on the diffusion coefficients of the molecules, their concentrations and structures, single–triplet dynamics, etc. Recent advances in theoretical studies make it possible to determine the equilibrium and rate constants of noncovalent interactions by fitting the experimental autocorrelation function of FCS to a theoretical model. For example, McNally et al. proposed a new model of the autocorrelation function for analyzing the association and dissociation rates of DNA binding in live cells. Al-Soufi et al. introduced a general correlation...
function to investigate the supermolecular dynamics and dye–micelle exchanging dynamics. However, the values of the equilibrium and rate constants obtained from these models usually disagree with the results of other works even by a few orders of magnitude. These huge discrepancies may come from unproven assumptions in the theoretical models or too many free parameters in the fitting procedures. In general, the existing models of the autocorrelation function focus on fast reactions, which may be the reason that the rate constants obtained from FCS are inconsistent with those obtained from other techniques. Therefore, an analytical theory for the FCS autocorrelation function is demanded for probing the interaction dynamics of diffusants in soft matter and biology.

In this work, we study the interactions between fluorescent dyes and surfactant micelles by FCS, in particular, the kinetics of the dye–micelle complex formation. We use three surfactants with different charges: C12E8 (neutral), SDS (negative) and CTAC (positive), which have been extensively studied previously. Their physicochemical properties regarding the micelle formation and diffusion are well-established. As fluorescent probes we choose two zwitterionic dyes: rhodamine 110 and ATTO-488 (see Fig. 1). Their diffusional properties in the three surfactant solutions of various concentrations are obtained by FCS. To determine the rate of the dye–micelle complex formation, we apply an approximate form of the autocorrelation function, which is derived in the accompanying theoretical paper on the basis of Magde’s theory. A sketch of the deviation is presented in the ESI†. This new formula works for both slow and fast reactions and can be useful in the studies of chemical kinetics of noncovalent reactions, which we demonstrate here. In the case of a slow reaction, equilibrium is established outside the focal volume since the characteristic time of the reaction is much longer than the typical time of diffusion through the focal volume. Therefore, we observe two diffusing species with different diffusion times: the fluorescent reactant and the complex. In contrast, the characteristic time of a fast reaction is much shorter than the diffusion time through the focal volume and only a single effective diffusion is observed. For fast reactions, our autocorrelation function reduces to the known expression for single-component effective diffusion.

Materials and methods

Materials

The cationic surfactant CTAC (purity: 99%) and anionic surfactant SDS (purity: 99.9%) were purchased from TCI and Roth, respectively. The non-ionic surfactant C12E8 (purity: 99%) was purchased from Fluka. Fluorescent dyes rhodamine 110 and ATTO-488 were purchased from Sigma-Aldrich and ATTO-TEC.

![Fig. 1](image-url) (A–E) The chemical structures of rhodamine 110, ATTO-488, C12E8, SDS and CTAC. (F) FCS setup. The light from an argon-ion laser (488 nm) passes through a water immersion microscope objective to excite the fluorescent dye in the micellar solutions. The emitted fluorescence signals are collected by the detector and further fed to a TCSPC unit. Kinetic information such as the equilibrium and rate constants for the dye–micelle systems can be derived from our approximate autocorrelation function.
GmbH, respectively. All chemicals were used without further purification.

**Formation of dye–micelle complexes**

Surfactant molecules in an aqueous solution aggregate to form micelles denoted as A, when their concentration is above the critical micelle concentration (CMC). Above the CMC, the concentration of micelles [A] can be determined from the surfactant concentration [S] as follows:

\[
[A] = \frac{[S] - \text{CMC}}{N_{ag}}
\] (1)

where \(N_{ag}\) is the mean number of aggregated surfactant molecules. In dilute solution \(N_{ag}\) is constant since the shape and size of micelles are fixed. The values of \(N_{ag}\) and CMC for all surfactants used in this work are listed in Table 1.

When dye molecules diffuse in a micellar solution the noncovalent bonding between a dye and micelle is treated as a chemical reaction with the equilibrium constant \(K\):

\[
A + B \xrightleftharpoons[k_\text{+}]{k_\text{-}} C
\] (2)

where B and C denote the dyes and dye–micelle complexes, respectively. The equilibrium constant is related to the association, \(k_\text{+}\), and dissociation, \(k_\text{-}\), rate constants and to the equilibrium concentrations of the components, \([A]^{eq}\), \([B]^{eq}\), and \([C]^{eq}\), as follows:

\[
K = \frac{k_\text{+}}{k_\text{-}} = \frac{[C]^{eq}}{[A]^{eq}[B]^{eq}}
\] (3)

The relaxation rate \(R\) of the pseudo-first-order reaction, which describe the rate of a reaction's return to equilibrium, is defined as:

\[
R = k_\text{-}([A]^{eq} + [B]^{eq}) + k_\text{+}
\] (4)

Because the micelle concentration is always much higher than the dye concentration in our FCS experiments, \(i.e., [A]^{eq} \gg [B]^{eq}\), \(\approx 10^{-9} \text{ M}\), we use the approximation:

\[
R = k_\text{-}[A]^{eq} + k_\text{+}
\] (5)

Thus, we can control the relaxation rate to some extent by changing the micelle concentration. Combining eqn (4) and (5), we get

\[
R = k_\text{-}[A]^{eq} + k_\text{+}/K
\] (6)

**Table 1** Diffusion coefficients, hydrodynamic radii, electric charges, critical micelle concentrations and the number of aggregated surfactant molecules of the studied samples. All values are obtained from the references or DLS measurements at 25 °C

| D (× 10^{-10} m^2 s^{-1}) | R₀ (nm) | Charge | CMC (mM) | N_{ag} |
|-----------------------------|--------|--------|----------|--------|
| Rh110 (4.7 ± 0.4)²³ | 0.52 ± 0.05 | Zwitterionic | 0.082²⁰ 95²⁹ | |
| ATTO-488 (4.0 ± 0.1)²⁰ | 0.62 ± 0.02 | Zwitterionic | 0.082²⁰ 95²⁹ | |
| CTAC (0.80 ± 0.01)²⁰ | 3.1 ± 0.04 | Positive | 1.1²⁵ 80²² | |
| SDS (0.92 ± 0.02)²⁰ | 2.7 ± 0.06 | Negative | 8.2²⁹ 60²¹ | |
| C₁₂E₈ (0.34 ± 0.01)²⁰ | 7.2 ± 0.02 | Neutral | 0.082²⁰ 95²⁹ | |

² Values obtained from our DLS measurements at 25 °C.

**FCS: experiment and theory**

The FCS setup used in our experiments was a commercial inverted NIKON EZ-C1 confocal microscope. The focal setup was additionally equipped with a PicoHarp 300 FCS setup made by PicoQuant. The experiments were conducted at 25 °C using a 488 nm argon-ion laser for illumination. A water immersion objective with a numerical aperture of 1.2 and magnification of 60 was used in FCS measurements. Before each measurement a drop of filtered, de-ionized water was used as the immersion medium between the objective and sample container (an 8-chambered coverglass, Lab-Tek²²). During measurements the laser power was set at a constant level and the focal volume was at a constant distance of 10 μm from the edge of the coverglass. An avalanche photo diode was used for detection. All surfactant solutions were prepared with a probe concentration of \(\approx 10^{-9}\) M. 200 μl of the solution was transported into the sample container and analyzed by FCS. Each measurement (duration 60 s) was repeated at least ten times and the autocorrelation function curves were further analyzed by the SymPhoTime program and Gnuplot version 4.5.

In FCS experiments, the distribution of the laser light intensity, \(I\), in the focal volume is often approximated by a three-dimensional Gaussian: \(I(x, y, z) = I_0 \exp[-2(x^2 + y^2)l^2 - 2z^2(H^2)]\), where \(L\) is the transverse radius of the focal volume and \(H\) is its height. Fluctuations of the fluorescence intensity, \(\delta I(t)\), are analyzed by means of the autocorrelation function: \(G(\tau) = \langle \delta I(0)\delta I(\tau + \tau)\rangle/I_0^2\), where \(\delta I(t) = I(t) - \langle I(t) \rangle\) and \(\langle \rangle\) denotes the average over time \(t\). In the case of a three-dimensional isotropic single-component diffusion with the triplet-state correction, \(G(\tau)\) is given by

\[
G(\tau) = \left(1 + p \frac{\tau}{1 - \tau/\tau_1}\right)\left(1 + \frac{\tau}{\tau_3}\right)^{-\frac{1}{2}} \left(1 + \frac{\tau}{\tau_5}\right)^{-\frac{1}{2}}
\] (7)

where \(p\) is the fraction of dye molecules in the triplet state, \(\tau_1\) is the triplet lifetime, \(N\) is the average number of molecules in the focal volume, and \(\omega = H/L\). The characteristic diffusion time through the focal volume is defined as \(\tau_4 = L^2/4D\), where \(D\) denotes the diffusion coefficient. The transverse radius \(L\) was obtained from the calibration measurement before each experiment. The free diffusion of rhodamine 110 (standard sample) in water was measured at 25 °C for calibration and we obtained the value \(D_{\text{Rh110}} = 4.7 \times 10^{-10}\) m² s⁻¹ for the diffusion coefficient.³⁷ The typical diffusion time through the focal volume, \(\tau_{\text{Rh110}}\), is around 20 μs. The calculated value of \(L\) is around 0.2 μm and the value of \(\omega\) is about 5 in our FCS setup.

**Approximate form of the autocorrelation function**

Due to the reaction of dyes with micelles there are three diffusional components in the system: micelles (A), dyes (B) and dye–micelle complexes (C), whose diffusion coefficients are denoted as \(D_A\), \(D_B\) and \(D_C\), respectively. To investigate the kinetics of the dye–micelle interaction, which is characterized by the equilibrium constant \(K\) and relaxation rate \(R\), we use an approximate form of the FCS autocorrelation function, \(G_d(\tau)\), derived in the framework of Magde’s theory.¹⁴–¹⁶ The full expression for \(G_d(\tau)\) is presented in the accompanying paper.³³
but here we use a slightly simplified form (see formula (8)). The derivation of \( G_a(t) \) is based on the following two assumptions. (1) The diffusion coefficient of the micelle is constant and much smaller than that of the dye, hence the diffusion coefficient of the dye–micelle complex can be approximated by \( D_C = D_A \ll D_B \). (2) The quantum yield of the dye fluorescence, \( Q_B \), does not change after binding to the micelle, i.e., \( Q_B = Q_C = Q \), which has been confirmed by the quantum yield measurements (ESI†). Taking into account the intrinsic triplet states of fluorescent dyes, we obtain the following approximate formula for the autocorrelation function:

\[
G_a(t) = \frac{1}{N} \left( 1 + \frac{p}{1 - p} \int \frac{1 - e^{-Rt \tau_A}}{\tau_A} \right) \int \left( 1 - e^{-Rt \tau_A} \right)
+ \beta h \left( \frac{t}{\tau_A} \right) e^{-t/R \tau_A} + (1 - \beta) h \left( \frac{t}{\tau_B} \right) e^{-t/R \tau_B} - \frac{R^2 \tau_A}{t^2} - \frac{R^2 \tau_B}{t^2}
\]

where \( \tau_A = L^2/4D_A \), \( \tau_B = L^2/4D_B \), \( \tau_S = L^2/4D_A \), \( \tau_s = L^2/4A \), and \( A = D_A - D_S < 0 \). The parameter \( \beta = k[A]^m[R] = K[A]^n(1 + K[A]^m) \) and \( D_D = D_A \beta + D_B(1 - \beta), D_C = D_A(1 - \beta) + D_B \beta \) are the effective diffusion coefficients. The function \( h(t) = (1 + t)^{1/2} (1 + t/\tau)^{1/2} \) is the normalized autocorrelation function for the diffusion of one component and \( t \) denotes the dimensionless lag time. The parameters characterizing the focal volume: \( H, L, \omega \) are known from the calibration measurements of reference fluorescent dyes with known diffusion coefficient \( D_B \). \( D_A \) is the diffusion coefficient of micelles determined by DLS. The equilibrium constant can be determined by an independent technique, e.g., TDA. The rest of the parameters \( \tau_A, \tau_B, \tau_S, \tau_s, \) and \( \beta \) whose values depend on the structure of the focal volume and the micellar concentration in formula (8) can be exactly calculated and then fixed during the fitting procedure. The target quantity \( R \) is the only free model parameter in \( G_a(t) \) to fit, besides the triplet-state parameters \( p \) and \( \tau_s \). In the case of highly concentrated micellar solutions, in which the dye–micelle association is extremely fast, the limit of fast reaction \( (R \to \infty) \) can be applied in formula (8). Then, \( G_a(t) \) reduces to the known formula for the single-component diffusion (cf. eqn (7)):

\[
G_a(t) = \frac{1}{N} \left( 1 + \frac{p}{1 - p} e^{-t/\tau} \right) h(t/\tau_s)
\]

In the limit of fast diffusion \( (R = 0) \), formula (8) yields

\[
G_0(t) = \frac{1}{N} \left( 1 + \frac{p}{1 - p} e^{-t/\tau} \right) \beta h(t/\tau_A) + (1 - \beta) h(t/\tau_B)
\]

which is the autocorrelation function for the two-component model of diffusion.\(^{39}\)

Results and discussion

A dye molecule in a micellar solution can be either in the free-motion state or in the bound-motion state, where the latter is due to the molecular interaction with micelles.\(^{23,24,40}\) In our experimental studies, we record the diffusion of rhodamine 110 and ATTO-488 in different surfactant solutions of various concentrations by FCS. In the case of rhodamine 110 in the CTAC solution, we observe a gradual shift of the autocorrelation curves towards longer diffusion times when the surfactant concentration increases (see Fig. 2). However, the viscosity of a very dilute solution should not change noticeably with the surfactant concentration. Thus, the increase in the diffusion time of rhodamine 110 implies that the free diffusion of the dye is gradually hindered due to the formation of dye–micelle complexes. The more dye–micelle complexes are formed in the solution, the longer the diffusion time observed. Such a hindered diffusion was also reported in the work of Zettl et al.,\(^{41}\) in which they focused on the investigation of micelle formation. They analyzed their experimental autocorrelation curves by a two-component model for FCS with the assumption that the relaxation rate of the dye–micelle reaction was much longer than the typical diffusion time of the focal volume. However, without a proper analysis of the dynamics of the reaction that occur in the system, the two-component model may give meaningless values of the diffusion time and fraction of the second component, i.e., micelles. As a result, the size of CTAC micelles probed by different tracers was not equal if the curves were fitted by the two-component model.\(^{42}\) The two-component model could only be applied to bound and freely moving tracers, but not for the analysis of dynamics of molecular interaction.

Analysis of FCS data using \( G_a(t) \)

The autocorrelation function, given by formula (8), is suitable to study the kinetics of intermediate interaction (i.e., Rh110–CTAC system) whose relaxation rate is smaller than the diffusion time through the focal volume. In the Rh110–CTAC system, the size and concentration of rhodamine 110 \((r \approx 0.5 \text{ nm}, \text{at } 5 \text{ nM concentration})\) are too small to be observed by DLS, compared to the much bigger micelles at much higher concentrations \((R \approx 3 \text{ nm}, \text{in the } \mu \text{M concentration range})\). Therefore, we only measured the collective diffusion coefficient \( D_C \) of the CTAC micelles in aqueous solution as a function of the micellar concentration at 25 °C. \( D_C \) measured in DLS reduces to \( D_0 \)
Table 2  Calculated values of the parameters appearing in formula (8) for the CTAC–Rh110 system, based on the results obtained from DLS and TDA measurements. See the Materials and method section and ESI for more details.

| Conc. (µM) | β      | τ_0 (µs) | D_0 (×10^{-10} m^2 s^{-1}) | D_x (×10^{-10} m^2 s^{-1}) | D_A (×10^{-10} m^2 s^{-1}) |
|------------|--------|----------|-------------------------------|-----------------------------|------------------------------|
| 1.88       | 0.07   | 17.3     | 4.3                           | 70.1                        | 1.1                          |
| 11.6       | 0.33   | 22.4     | 3.4                           | 36.4                        | 2.1                          |
| 25.3       | 0.52   | 28.6     | 2.6                           | 27.0                        | 2.8                          |
| 40.9       | 0.64   | 34.4     | 2.2                           | 23.2                        | 3.2                          |
| 60.5       | 0.72   | 40.4     | 1.9                           | 21.1                        | 3.6                          |

Fig. 3 Upper panel: Experimental autocorrelation curves for rhodamine 110 diffusing in diluted CTAC micellar solutions and the fitting curves of formula (8). The plotted residuals correspond to the fits of experimental data using formula (8). Lower panel: The fitted values of $R$ plotted versus the CTAC micellar concentration according to eqn (6) with $K$ known from TDA measurements. The association rate constant $k_A$ is obtained from the slope of the red line, and then the dissociation rate $k_A$ is calculated according to eqn (3).

from the dependence of the effective diffusion coefficient $D_e$ on the concentration of micelles according to the definition of $D_e$ and $β$ (in the Materials and method section):

$$\frac{D_e - D_B}{D_A - D_e} = K[A]^{eq}$$

where $D_e$ is calculated from the relation $D_e = L^2/4τ_+$, and $τ_+$ is the effective diffusion time of dyes and dye–micelle complexes through the focal volume obtained from the fitting of experimental curves by $G_∞(t)$.

To check this possibility, we study rhodamine 110 in a series of CTAC micellar solutions at relatively high concentrations of micelles. Fig. 4 shows that the experimental curves are well fitted by $G_∞(t)$. Then, by substituting $D_e$, obtained from FCS and the diffusion coefficient of micelles, $D_A$, from DLS into eqn (11), we obtain the equilibrium constant $K$ from the slope of the linear fit shown in Fig. 4. For the Rh110–CTAC system the equilibrium constant determined using eqn (9) is $4.32 \times 10^4$ M$^{-1}$.
The coefficient of TAMRA also does not change in the C\textsubscript{12}E\textsubscript{6} solution and rhodamine 110 in the CTAC solution experience a sudden increase when the surfactant concentration is just above the CMC (see Fig. 5(C) and (D)). The dyes are supposed to bind to micelles immediately and then move together in the solution all the time. Dosche's and Ghosh's groups also observed such an attachment of hydrophobic dyes to various surfactant micelles by using FCS.\textsuperscript{46,47} The formation of complexes between dyes and micelles depends mainly on hydrophobic effects and electrostatic forces. A higher affinity of the zwitterionic dye ATTO-488 to cationic CTAC micelles compared to anionic SDS and neutral C\textsubscript{12}E\textsubscript{8} micelles can be attributed to the strong hydrophobic effect of the CTAC micelles which possess four additional alky groups in their chemical structure compared to SDS and C\textsubscript{12}E\textsubscript{8} do. Rhodamine 110 can interact with SDS, CTAC and C\textsubscript{12}E\textsubscript{8} micelles but its affinities decline due to the differences in molecular charges.

**Kinetics of dye–micelle interactions**

We categorize the dye–micelle interactions as: weak, intermediate or strong, according to the dependence of \(D\), on the surfactant concentration. For weak interactions, \(i.e.,\) in the case of ATTO–C\textsubscript{12}E\textsubscript{8} and ATTO–SDS systems, \(D\) changes marginally as a function of the surfactant concentration, which indicates the free diffusion of ATTO-488 in these solutions (inset plot in Fig. 5(A) and (B)). For strong interactions, namely in Rh110–SDS and ATTO–CTAC systems, \(D\) drops sharply when the surfactant concentration exceeds the CMC (inset plots in Fig. 5(C) and (D)), which suggests that almost all dye molecules bind strongly to the micelles. For intermediate interactions, \(i.e.,\) in the case of the Rh110–CTAC system, \(D\) decreases moderately when the surfactant concentration increases. In the absence of analytical theory, these three concentration-related behaviours of \(D\), in FCS were also reported by molecular dynamics simulation of a generic bead–spring polymer model and spherical tracers with no attraction (single diffusion), weak attraction (single slow diffusion), and strong attraction (double diffusion) to the polymer in the work of Vagias’s group.\textsuperscript{27}

Since FCS has showed a good accuracy in evaluating the equilibrium constant of the Rh110–CTAC system at relatively high concentrations of micelles, we use this method for other dye–micelle systems as well. As in the case of CTAC, the diffusion coefficients of SDS and C\textsubscript{12}E\textsubscript{8} are determined by DLS at 25 °C and they amount to \(0.92 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}\) and \(0.34 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}\), respectively (see Fig. S3 and S4 in the ESI\textsuperscript{†} for details). These values are consistent with the published results.\textsuperscript{32,48} In Table 3, we show the values of \(K\) for the studied dye–micelle systems. In the case of strong interactions (Rh110–SDS and ATTO–CTAC systems), \(K\) is roughly one order of magnitude larger than that in the case of intermediate interactions. The reported value of \(K\) for the interaction between rhodamine 123 and C\textsubscript{12}E\textsubscript{8} is \((1.6 \pm 0.2) \times 10^4 \text{ M}^{-1}\),\textsuperscript{25} close to the value for the Rh110–C\textsubscript{12}E\textsubscript{8} system studied in this work.

Next, we use the equilibrium constant obtained by FCS at high concentrations of micelles to determine the relaxation rates of the same dye–micelle system, which is achieved by fitting the FCS autocorrelation function in the low concentration regime.
In other words, we apply the same procedure as in the case of the aforementioned Rh110–CTAC system. The fitted values of \( k_+ \) and \( k_\mp/C_0 \) for intermediate and strong interactions are collected in Table 3 together with the diffusion-controlled association rate constant \( k_{dc} \). The reported values of the association rate constant for the dye–micelle interactions vary from \( 10^6 \text{ M}^{-1}\text{s}^{-1}/C_0 \) to the diffusion controlled limit, i.e., \( 10^1 \text{ M}^{-1}\text{s}^{-1}/C_0 \). The values of \( k_+ \) in our dye–micelle systems are roughly two orders of magnitude smaller than \( k_{dc} \) (see Table 3). The latter can be estimated from the Smoluchowski equation:

\[
k_{dc} = 4\pi D_{dm} R_{dm} N_A
\]

where \( D_{dm} \) and \( R_{dm} \) are the sums of the diffusion coefficients and the hydrodynamic radii of the dyes and micelles, respectively, and \( N_A \) is Avogadro’s constant.

|          | \( K \times 10^4 \text{ M}^{-1} \) | \( -\Delta G \text{ (kJ mol}^{-1} \) | \( k_+ \times 10^4 \text{ M}^{-1}\text{s}^{-1} \) | \( k_- \times 10^3 \text{ s}^{-1} \) | \( k_{dc} \times 10^{10} \text{ M}^{-1}\text{s}^{-1} \) |
|----------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Rh110–C12E8 | 2.32 ± 0.21                     | 24.90                           | 12.16 ± 0.07                    | 52.4 ± 26.2                     | 2.9                             |
| Rh110–CTAC  | 4.32 ± 0.32                     | 26.44                           | 2.14 ± 0.01                     | 6.58 ± 0.06                     | 1.50                            |
| Rh110–SDS   | 15.8 ± 0.09                     | 29.66                           | 0.51 ± 0.08                     | 0.32 ± 0.05                     | 1.36                            |
| ATTO–CTAC   | 46.7 ± 7.8                      | 32.34                           | 0.74 ± 0.01                     | 0.16 ± 0.01                     | 1.35                            |

**Fig. 5** (A–D) Normalized experimental autocorrelation curves for ATTO-488 and rhodamine 110 in the solutions of C12E8, SDS and CTAC of various surfactant concentrations, respectively. Inset: The effective diffusion coefficients \( D_{eff} \) obtained from the fit of experimental curves by eqn (9). The overlapping sets of data points in (A) and (B) indicate that the diffusion time of ATTO-488 in the C12E8 and SDS solutions is independent of the surfactant concentration. In (C) and (D), a sudden increase in the diffusion time is observed in the Rh110–SDS and ATTO–CTAC systems at a concentration just above the CMC. The dyes are believed to bind to large micelles immediately once they encounter.
We describe the dye–micelle reaction as a two-step process: the intermediate formation and complex formation

$$A + B \xrightleftharpoons[k_{dc}]{k_r} [A \cdot B] \xrightleftharpoons[k_r]{k_r} C$$

(13)

In the first step, one micelle and one dye molecule get into contact to form an intermediate [A·B] driven by free diffusion under the diffusion-controlled rate $k_{dc}$. Next, the dye–micelle complex is formed through hydrophobic and electrostatic interactions with a reaction rate $k_r$. The value of $k_r$ can be simply estimated via

$$\frac{1}{k_r} = \frac{1}{k_{dc}} + \frac{1}{k_r}$$

(14)

The calculated value of $k_r$ is roughly equal to $k_r$ in our dye–micelle systems, indicating that complex formation is a reaction-controlled process.

The micelles behave as “soft cages” which hinder the entrance and exit of dyes when they collide driven by Brownian motion. The activation energies, labelled as $\Delta G^+$ and $\Delta G^-$, for the studied dye–micelle interactions are shown in Fig. 6. In thermodynamics we can only determine the Gibbs free energy difference but not the absolute values. The free energy differences ($\Delta G$) between the bound and unbound states for various dye–micelle systems are calculated on the basis of the expression: $\Delta G = -RT\ln K$, and the values of the rate constants are shown in Table 3. The energy barriers of intermediate interactions are much lower than those of strong interactions. The stability of the dye–micelle complex is determined by its residence time expressed as $\tau_R = 1/k_r$.\(^{46}\) The values of the dissociation rate constant for strong interactions are at least one order of magnitude smaller than those for the intermediate interactions, suggesting longer times of the bound state.

Conclusions

In this work, we use FCS to study the reaction kinetics of selected dye–micelle systems. The interactions between dyes and micelles are categorized into three types: weak, intermediate and strong, in terms of the equilibrium constant $K$. To fit the FCS experimental data, we apply a new approximate form of the FCS autocorrelation function, $G_2(t)$, derived on the basis of Magde’s formalism. Our results show that $G_2(t)$ can be used to determine the association and dissociation rate constants by studying diluted micellar solutions, in which the relaxation rate can be reduced below the fast-reaction limit. Then, the rate constants follow from the dependence of the relaxation rate on the micelle concentration. On the other hand, FCS studies in the region of high micelle concentrations, in which the fast-reaction limit usually applies, provide information about $K$ for the dye–micelle system. The values of $K$ obtained by FCS are consistent with the results from an independent technique, i.e., TDA and with the data obtained from references. Our results show that the association rates of the dye–micelle interactions are much smaller than the diffusion-controlled rate. Therefore, a two-step reaction mechanism is used to explain the complex formation between dyes and micelles. Generally, our method can potentially facilitate kinetic studies of molecular interactions involving drugs, proteins and DNA.

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Notes and references

1. A. Michelman-Ribeiro, D. Mazza, T. Rosales, T. J. Stasevich, H. Boukari, V. Rishi, C. Vinson, J. R. Knutson and J. G. McNally, *Biophys. J.*, 2009, **97**, 337–346.
2. R. N. Day and F. Schaufele, *Mol. Endocrinol.*, 2005, **19**, 1675–1686.
3. C. C. Govern, M. K. Paczosa, A. K. Chakraborty and E. S. Husebyb, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 8724–8729.
4. L. M. Berezhkovskiy, *Expert Opin. Drug Metab. Toxicol.*, 2008, **4**, 1479–1498.
5. M. Goyal, M. Rizzo, F. Schumacher and C. F. Wong, *J. Med. Chem.*, 2009, **52**, 5582–5585.
6. G. G. Mironov, V. Okkonin, S. I. Gorelsky and M. V. Berezovski, *Anal. Chem.*, 2011, **83**, 2364–2370.
7. A. S. M. Dyuk, U. Kisiel and B. Garcia, *J. Phys. Chem. B*, 2003, **107**, 11652–11659.
8. P. Thordarson, *Chem. Soc. Rev.*, 2011, **40**, 1305–1323.
9. W. Al-Soufi, B. Reija, M. Novo, S. Felekyan, R. Kuhinemuth and C. A. M. Seidel, *J. Am. Chem. Soc.*, 2005, **127**, 8775–8784.
10. W. Bujalowski, *Chem. Rev.*, 2006, **106**, 556–606.
11. P. Thordarson, *Chem. Soc. Rev.*, 2011, **40**, 1305–1323.
12. C. Perez-Arnaiz, N. Busto, J. M. Leal and B. Garcia, *J. Phys. Chem. B*, 2014, **118**, 1288–1295.
13. M. Airoldi, G. Barone, G. Gennaro, A. M. Giuliani and M. Giustini, *Biochemistry*, 2014, **53**, 2197–2207.
14. E. L. Elson and D. Magde, *Biopolymers*, 1974, **13**, 1–27.
15. D. Magde, E. L. Elson and W. W. Webb, *Biopolymers*, 1974, **13**, 29–61.
16. D. Magde, W. W. Webb and E. Elson, *Phys. Rev. Lett.*, 1972, **29**, 705–708.
17. R. Rigler, U. Mets, J. Widengren and P. Kask, *Eur. Biophys. J.*, 1993, **22**, 169–175.
18. H. Qian and E. L. Elson, *Appl. Opt.*, 1991, **30**, 1185–1195.
19. M. Eigen and R. Rigler, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 5740–5747.
20. X. Z. Zhang, A. Poniewierski, S. Hou, K. Sozanski, A. Wisniewska, S. A. Wieczorek, T. Kalwarczyk, L. L. Sun and R. Holyst, *Soft Matter*, 2015, **11**, 2512–2518.
21. K. Koynov and H. J. Butt, *Curr. Opin. Colloid Interface Sci.*, 2012, **17**, 377–387.
22. D. Granadero, J. Bordello, M. J. Perez-Alvite, M. Novo and W. Al-Soufi, *Int. J. Mol. Sci.*, 2010, **11**, 173–188.
23. M. Novo, S. Felekyan, C. A. M. Seidel and W. Al-Soufi, *J. Phys. Chem. B*, 2007, **111**, 3614–3624.
24. J. Bordello, M. Novo and W. Al-Soufi, *J. Colloid Interface Sci.*, 2010, **345**, 369–376.
25. S. Freire, J. Bordello, D. Granadero, W. Al-Soufi and M. Novo, *Photochem. Photobiol. Sci.*, 2010, **9**, 687–696.
26. V. C. Reinsborough and J. F. Holzwarth, *Can. J. Chem.*, 1986, **64**, 955–959.
27. A. Vagias, R. Raccis, K. Koynov, U. Jonas, H. J. Butt, G. Fytas, P. Kosovan, O. Lenz and C. Holm, *Phys. Rev. Lett.*, 2013, **111**, 088301.
28. E. Feitosa, W. Brown, K. Wang and P. C. A. Barreiro, *Macromolecules*, 2002, **35**, 201–207.
29. M. Zulauf, K. Weckstrom, J. B. Hayter, V. Degiorgio and M. Corti, *J. Phys. Chem.*, 1985, **89**, 3411–3417.
30. G. Olofsson, *J. Phys. Chem.*, 1985, **89**, 1473–1477.
31. E. Dukiewicz and A. Jakubowska, *Colloid Polym. Sci.*, 2002, **280**, 1009–1014.
32. L. T. Okano, F. H. Quina and O. A. El Seoud, *Langmuir*, 2000, **16**, 3119–3123.
33. R. Holyst, A. Poniewierski and X. Zhang, *Soft Matter*, 2016, accompanying paper.
34. A. Bielejewska, A. Bylina, K. Duszczyk, M. Fialkowski and R. Holyst, *Anal. Chem.*, 2010, **82**, 5463–5469.
35. A. Majcher, A. Lewandrowska, F. Herold, J. Stefanowicz, T. Słowiński, A. P. Mazurek, S. A. Wieczorek and R. Holyst, *Anal. Chim. Acta*, 2015, **855**, 51–59.
36. A. Lewandrowska, A. Majcher, A. Ochab-Marcinek, M. Tabaka and R. Holyst, *Anal. Chem.*, 2013, **85**, 4051–4056.
37. P. O. Gendron, F. Avaltroni and K. J. Wilkinson, *J. Fluoresc.*, 2008, **18**, 1093–1101.
38. T. Derfing, A. Loman, B. Ewers, C. B. Muller, B. Kramer and J. Enderlein, *Opt. Express*, 2008, **16**, 14353–14368.
39. O. Krichesy and G. Bonnet, *Rep. Prog. Phys.*, 2002, **65**, 251–297.
40. H. Zettl, Y. Portnoy, M. Gottlieb and G. Krausch, *J. Phys. Chem. B*, 2005, **109**, 13397–13401.
41. B. A. Scalettar, J. E. Hearst and M. P. Klein, *Macromolecules*, 1989, **22**, 4550–4559.
42. J. Gapinski, J. Szymanski, A. Wilk, J. Kohlbrecher, A. Patkowski and R. Holyst, *Langmuir*, 2010, **26**, 9304–9314.
43. H. U. Kim and K. H. Lim, *Bull. Korean Chem. Soc.*, 2004, **25**, 382–388.
44. R. Holyst, A. Bielejewska, J. Szymanski, A. Wilk, A. Patkowski, J. Gapinski, A. Zywocinski, T. Kalwarczyk, E. Kalwarczyk, M. Tabaka, N. Ziebacz and S. A. Wieczorek, *Phys. Chem. Chem. Phys.*, 2009, **11**, 9025–9032.
45. J. Szymanski, A. Patkowski, A. Wilk, P. Garstecki and R. Holyst, *J. Phys. Chem. B*, 2006, **110**, 25593–25597.
46. F. Luschtinetz and C. Dosche, *J. Colloid Interface Sci.*, 2009, **338**, 312–315.
47. S. Ghosh, U. Mandal, A. Adhikari and K. Bhattacharyya, *Chem. – Asian J.*, 2009, **4**, 948–954.
48. R. B. Dorshow, C. A. Bunton and D. F. Nicoli, *J. Phys. Chem.*, 1983, **87**, 1409–1416.
49. A. J. Elliot, D. R. Mccracken, G. V. Buxton and N. D. Wood, *J. Chem. Soc., Faraday Trans.*, 1990, **86**, 1539–1547.
50. A. C. Pan, D. W. Borhani, R. O. Dror and D. E. Shaw, *Drug Discovery Today*, 2013, **18**, 667–673.
51. H. Blom, A. Chmyrov, K. Hassler, L. M. Davis and J. Widengren, *J. Phys. Chem. A*, 2009, **113**, 5554–5566.