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SHORT COMMUNICATION

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Time-resolved fluorescence anisotropy measurements of the adsorption of Rhodamine-B and a labelled polyelectrolyte onto colloidal silica

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Abstract The application of time-resolved fluorescence anisotropy measurements (TRAMS) to the investigation of the adsorption of the dye Rhodamine B and a Rhodamine B-labelled cationic polyelectrolyte onto colloidal silica (Ludox) is described. For Rhodamine B the time-resolved fluorescence anisotropy behavior observed can be interpreted using a model consisting of fluorophores with two distinct fluorescence decay lifetimes and two rotational correlation times corresponding to the fluorophore free in solution and bound to the Ludox. Details of the binding obtained from a global analysis of the data are reported. Restricted motion of the fluorescently labelled polyelectrolyte is also observed on adsorption. The considerations for the general application of TRAMS for monitoring adsorption behavior are discussed.

Key words Time-resolved fluorescence anisotropy – dye adsorption – polymer adsorption – colloidal silica

Introduction

The adsorption of molecules to particulate matter is of importance in many areas of colloid science. In water clarification, for example, cationic salts and polyelectrolytes are often used as flocculants of negatively charged contaminants which can be subsequently removed. The adsorption of molecules to particulate matter is accompanied by changes in the mobility of the adsorbate and techniques which monitor molecular motion should be useful for investigating molecule–surface interactions. Time-resolved fluorescence anisotropy measurements (TRAMS) have the capability of detecting changes in the rotational behavior of molecules on a nanosecond and sub-nanosecond time scales with high sensitivity, and can provide information in addition to that available from steady-state fluorescence methods [1].

In TRAMS, the fluorescence decay profile from a fluorophore is monitored through polarizers set parallel and perpendicular to the polarization of the excitation light. The time-resolved fluorescence anisotropy function, $r(t)$, is then generated which contains information on the motion of the fluorophores on the time scale of the lifetime of the excited state. Fitting the anisotropy data with functions derived from particular models for motion can lead to a description of the dynamics of the system under study.

As part of an ongoing program of work investigating the application of TRAMS for monitoring the adsorption of various fluorescently labelled polyelectrolytes and other adsorbates to colloidal particles, we have examined the adsorption of the fluorescent dye Rhodamine B onto colloidal silica as a model system. We report here the results of a successful interpretation of the time-resolved anisotropy function from a dye/silica particle system, and discuss the potential complexities of interpreting TRAMS data. We also illustrate the application of TRAMS to the study of the adsorption behavior of a fluorescently labelled cationic polyelectrolyte.

Experimental section

The particulate matter used in this work was a commercially available 1 wt% aqueous suspension of colloidal silica particles (Ludox HS-30 (DuPont)) which contains 30% SiO_2 and 0.32% Na_2O stabilizer, and has a high negative charge density at pH 6.5 [2]. The average particle diameter, determined from transmission electron microscopy and dynamic light scattering was 18 nm ($\pm$4 nm). Rhodamine B (TCI) (RhB) was used as supplied.

A copolymer of diallyldimethyl ammonium chloride (DADMAC) and *N*-(3-aminopropyl)methylacrylamide hydrochloride (APMAA) was prepared by aqueous-free radical polymerization using azobis(2-amidino propane) dihydrochloride as an initiator. Free amine groups on the APMAA were labelled with RhB isothiocyanate (Polysciences) [3]. Unreacted RhB isothiocyanate and monomers were removed by dialysis against distilled water using Sigma D-0655 dialysis tubing. ^{1}H NMR spectra of the copolymer indicated that the mole ratio of DADMAC to APMAA is 68.8–31.2, and the degree of fluorescent probe labelling was determined by absorption spectroscopy to be 0.27 wt%.

Fluorescence decays were collected by time correlated single photon counting (TCSPC) detection [4] using as the excitation source the 514.5 nm output of a mode-locked argon ion laser (Coherent) which was pulse picked to $\sim$4 MHz. The fluorescence photons were collected at right angles to the direction of the vertically polarized excitation beam, passed through a rotatable polarization analyzer and polarization scrambler and focussed onto the entrance slit of a 0.1 m single grating monochromator (Jobin Yvon H-10). The emission (monitored at $\sim$600 nm) was detected with a Hamamatsu model R2809U-01 microchannel plate photomultiplier tube. The total response function of the system is $\sim$45 ps FWHM. For emission anisotropy measurements, the rotatable emission polarization analyzer was aligned at 0° and 90° relative to the vertically polarized excitation to measure parallel, $I_{\parallel}(t)$, and perpendicular, $I_{\perp}(t)$, fluorescence decays, respectively. Data acquisition and the emission polarizer direction were under computer control, with the $I_{\parallel}(t)$ and $I_{\perp}(t)$ decays being collected in two 512 channel memories of a multichannel analyzer (MCA). The emission polarizer direction and MCA memories were switched with dwell times of 20 s for 50–60 cycles to account for any fluctuations in the laser intensity. Further details of the instrumentation can be found elsewhere [5, 6].

Data were transferred from the MCA to a computer where subsequent analysis of the decays was performed using non-linear least-squares iterative fitting [4]. In most cases, convolution was deemed unnecessary due to the narrow width and lack of sub-structure in the instrument response function. The experimentally obtained $I_{\parallel}(t)$ and $I_{\perp}(t)$ decays were used to generate the sum, $S(t)$, difference, $D(t)$, and time-resolved anisotropy, $r(t)$, functions which are defined in Eq. (1). The $S(t)$ profile corresponds to the decay in the absence of rotational effects equivalent to the decay which is recorded with the emission polarizer set at the magic angle (54.7°) [7, 8] relative to the vertically polarized excitation.

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} = \frac{D(t)}{S(t)} \,. \tag{1}$$

Decays were fitted globally using sums of exponentials in the cases of $S(t)$ and $D(t)$, and using the function described in Section 3 for $r(t)$ in which the fluorescence lifetimes, τ_f, and rotational correlation times, τ_c, were linked across all data sets and adjusted in the global analysis. The goodness of fit in each case was assessed by considering the randomness of the weighted residuals and autocorrelation function, the reduced chi square (χ^2) value, and the Durbin–Watson (DW) parameter [4].

Results and discussion

Time-resolved fluorescence decays were collected from solutions of RhB in water as a function of Ludox concentration with the emission polarizer set parallel, $I_{\parallel}(t)$, and perpendicular, $I_{\perp}(t)$, to the polarization of the excitation beam, and the $S(t)$ decay curves generated as discussed above. In the absence of Ludox, a single exponential decay function is adequate to describe the decay, with the lifetime of 1.7 ns consistent with previous values for the lifetime of free RhB in aqueous solution [9]. In the presence of Ludox, however, a two component fluorescence decay function of the form $S(t) = a_1 \exp[-t/\tau_{f_1}] + a_2 \exp[-t/\tau_{f_2}]$ is required. Analysis of the $S(t)$ curves collected from RhB solutions (0.5 μM) as a function of added Ludox (concentrations in the range 0.04–0.27 wt%) were analyzed globally which resulted in fluorescence lifetimes, τ_{f_1} and τ_{f_2}, of 1.6 and 3.65 ns, respectively. The shorter component corresponds to the fluorescence lifetime of RhB in the absence of Ludox reported above. The pre-exponential factors (a_1 and a_2) obtained from this analysis (Table 1) indicate an increasing contribution from the component with the longer fluorescence lifetime as the amount of added Ludox increases. These observations suggest that the RhB molecules are present in two environments: the shorter-lived species being free RhB in solution and the longer-lived component corresponding to the RhB adsorbed onto the Ludox particles. Previous photophysical studies have shown that restrictions on intramolecular dynamics for RhB, such as might occur on adsorption, can

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Table 1 Fitting parameters resulting from global analysis of decays from RhB/Ludox solutions as a function of Ludox concentration

wt% Ludox	$S(t)$[a] ($\tau_{f_1} = 1.60$ ns, $\tau_{f_2} = 3.65$ ns)		$r(t)$[b] Eqs. (2) and (3)
	a_1	a_2	Mole fraction: x_b
0.04	88	12	0.10
0.08	82	18	0.18
0.27	72	28	0.30

[a] $S(t)$ (double exponential).
[b] $r(t)$ (Eqs. (2) and (3), as shown in Fig. 1: $\tau_{f_u} = 1.64$ ns, $\tau_{f_b} = 3.42$ ns, $\tau_{c_u} = 0.304$ ns, $\tau_{c_b} = 685$ ns (fixed), $r_{o_u} = 0.32$ and $r_{o_b} = 0.35$).

reduce non-radiative processes and result in a longer fluorescence lifetime [9].

The presence of RhB in bound and free forms should be confirmed by the rotational behavior of the fluorophore as provided by TRAMS since we might expect that the two forms of the RhB will exhibit different rotational correlation times. In principle, analysis of any of the $I_{\parallel}(t)$, $I_{\perp}(t)$, $D(t)$ or $r(t)$ decay profiles can provide rotational information, and the advantages and disadvantages of the various methods have been discussed in detail elsewhere [10, 11]. Methods in which the $S(t)$ and $D(t)$ functions are analyzed simultaneously are often found to be the most reliable. In the work reported here, we have found it illustrative to calculate and analyze the $r(t)$ function directly, in addition to analysis of the $S(t)$ and $D(t)$ functions, as discussed below.

Fluorescence anisotropy decay curves of RhB in water in the absence and presence of varying amounts of added Ludox HS-30 are presented in Fig. 1. In the absence of Ludox, the anisotropy decays rapidly to zero. A single exponential fit of this decay gave a rotational time of $\sim$300 ps which corresponds simply to the fast depolarizing rotation of free RhB. In the presence of Ludox, however, the anisotropy does not decay completely to zero, but instead regains intensity with time. This unusual behavior can be explained as follows. In the case under discussion here, in addition to the two fluorescence decay lifetimes corresponding to the two environments for the fluorophore, we can also expect two rotational correlation times corresponding to rotation of the free fluorophore in solution and the fluorophore adsorbed to the colloidal silica particle. In such a case, an analytical form for $r(t)$ can be derived [5]:

$$r(t) = f_u(t) r_u(t) + f_b(t) r_b(t) \,, \tag{2}$$

where $f_u(t)$ is the fraction of fluorescence at time t due to unbound RhB, $r_u(t)$ describes the anisotropy decay of these free probes, and $f_b(t)$ and $r_b(t)$ are the corresponding quantities for RhB bound to the colloid particle [12]. The fraction term for the unbound dye is given by Eq. (3) and

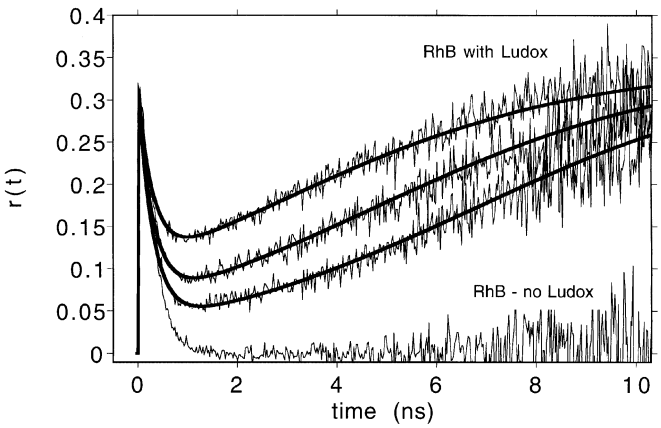

Fig. 1 Time-resolved fluorescence anisotropy decay curves from aqueous solutions of RhB (0.5 μM) in the absence and presence of added Ludox HS-30 (concentrations of 0.27, 0.08 and 0.04 wt%; upper-to-lower curve). The fits resulting from the global analysis of the curves using Eq. (2) with the parameters quoted in Table 1 are also shown

an analogous expression describes the fraction term for the bound form:

$$f_u(t) = \frac{x_u \sum_{j=1}^{N} \alpha_{ju} \exp(-t/\tau_{ju})}{x_u \sum_{j=1}^{N} \alpha_{ju} \exp(-t/\tau_{ju}) + x_b \sum_{j=1}^{N} \alpha_{jb} \exp(-t/\tau_{jb})} \,, \tag{3}$$

where τ_{ju}, α_{ju}, and τ_{jb}, α_{jb} are the corresponding fluorescence lifetimes and amplitudes for the two (unbound and bound) populations of probes. Immediately after excitation ($t = 0$), $f_u(t)$ and $f_b(t)$ are the actual fractions of the bound and unbound probe molecules, x_u and x_b, respectively, assuming that the extinction coefficient and radiative rate constant are the same for both populations. In the present case, we are dealing with a single rotational correlation time and a single fluorescence decay time for each of the two forms of the probe, so $N = 1$ in Eq. (3).

$r_u(t)$ and $r_b(t)$ in Eq. (2) are given by single exponential functions: $r_u(t) = r_{0_u}\exp(-t/\tau_{c_u})$, $r_b(t) = r_{0_b}\exp(-t/\tau_{c_b})$ where r_{0_u} and r_{0_b} are the initial anisotropy values for the free and bound dye (which can provide information about the angle between the absorption and emission dipoles); τ_{c_u} and τ_{c_b} are the rotational correlation times of free RhB and RhB bound to Ludox. It is apparent from Eq. (2) that if the larger fluorescence lifetime component is associated with a slower rotation time, then at long times after excitation $r(t)$ can also show a growth in intensity due to the increasing contribution of this species relative to the shorter lived, faster rotating free dye molecules.

The anisotropy data shown in Fig. 1 were fitted globally using Eq. (2) [5] with the fits and the results presented in the figure and Table 1. In this analysis the two fluorescence decay times, the two r_o values and the shorter rotational correlation time, τ_{c_u}, were all adjustable and linked across the three data sets. The long rotational correlation time, τ_{c_b}, was fixed at 685 ns in order to restrain the χ^2 minimization routine. Due to the short time scales of these measurements the results of the analysis were found to be quite insensitive to the magnitude of this parameter. The value of 685 ns was calculated using the molar volume, V (from the known average size of the Ludox particles, 18 nm, and assuming a spherical particle) and the Stokes–Einstein relation (Eq. (4)), and using a viscosity, η, of 0.00894 P (the viscosity of water at 20 °C).

$$\tau_{c_b} = \frac{V\eta}{RT}\,. \tag{4}$$

The excellent global fits of the data using Eq. (2) and the agreement between the expected and recovered magnitudes of the various parameters confirm the adequacy of the two state model proposed. The τ_{c_u} value of ~ 300 ps agrees well with the value obtained for free RhB in solution. The fluorescence lifetimes, τ_{f_u} and τ_{f_b} extracted are consistent with those obtained from analysis of the magic angle (or $S(t)$) fluorescence decays. The initial anisotropy values, r_{o_u} and r_{o_b}, are close to the theoretical limit of 0.4, expected if the absorption and emission transition moments of the fluorophore are parallel. These might be expected to differ slightly from one another due to the different environments proposed for the probe. The global fitting also gives the mole fraction of bound and free dye, x_u and x_b (cf. Table 1), at various Ludox concentrations providing information on the adsorbed amount. The pre-exponential factors resulting from the global analysis of the double exponential fluorescence decay curves, $S(t)$, (Table 1) also agree very well with the terms for the mole fractions of dye molecules bound to the Ludox, x_b, and unbound, x_u, obtained from the analysis of $r(t)$.

As mentioned above, analysis of any of the $I_\parallel(t)$, $I_\perp(t)$, $D(t)$ or $r(t)$ decay profiles can, in principle, provide the required rotational information. The difference function ($D(t)$) for a system such as that described above requires a double exponential function of the form given by Eq. (5) [13–15].

$$D(t) = a_1 r_{0_u}\exp[-t/\tau_{f_u} - t/\tau_{c_u}] + a_2 r_{0_b}\exp[-t/\tau_{f_b} - t/\tau_{c_b}]\,. \tag{5}$$

While we have successfully fitted the $D(t)$ curves using equations of this type and extracted values for the binding fractions quite similar to those obtained from the $r(t)$ analysis, we consider that the generation and analysis of the $r(t)$ function serves several useful purposes. Firstly, it is invaluable for visualizing the most appropriate model for a given system, in particular, the long correlation time due to adsorption. The "dip and rise" $r(t)$ profiles obtained here immediately suggest that the system is not best described by a more simple model as might be the case if the $I_\parallel(t)$, $I_\perp(t)$ or $D(t)$ decay profiles were viewed or analyzed in isolation. Secondly, analysis of $r(t)$ functions using the approach taken here, that is, to globally fit a series of decays as a function of the degree of adsorption, allows for independent determination of the various parameters without pre-associating particular correlation times with particular fluorescence decay times. Finally, it has been found empirically, that analysis using the anisotropy function rather than just the difference function facilitates the measurement of very small amounts of bound probe populations [6]. This analysis is, however, more sensitive to the mole fraction terms than the lifetime terms, which are obtained more reliably from a complementary analysis of the $S(t)$ decays.

The RhB serves as a model system for our studies of the adsorption of polyelectrolytes to particulate matter. As an example of the application of TRAMS to polymer/particle adsorption, the cationic polymer, poly[DADMAC/APMAA], labelled with a RhB probe as outlined in the Experimental Section, was studied by TRAMS both in the absence and presence of Ludox. Binding profiles determined from optical absorption measurements of the supernatent liquid following centrifugation of the equilibrated polymer/colloid dispersions, indicated total adsorption of the polymer under the conditions outlined in the caption to Fig. 2. The $r(t)$ profiles obtained are shown in Fig. 2. In contrast with the free RhB in solution (Fig. 1), the $r(t)$ profile for the labelled polymer in the absence of Ludox does not decay exponentially to zero on the time scale of the experiment. Analysis of this decay using Eq. (2) failed to provide a satisfactory fit. Instead, a more simple, double exponential analysis of the $S(t)$ and $r(t)$ data returned two fluorescence lifetimes (600 ps and 2.04 ns), and two decay components for $r(t)$ (600 ps and 12.3 ns – fit also shown in Fig. 2). While we cannot definitively ascribe a particular

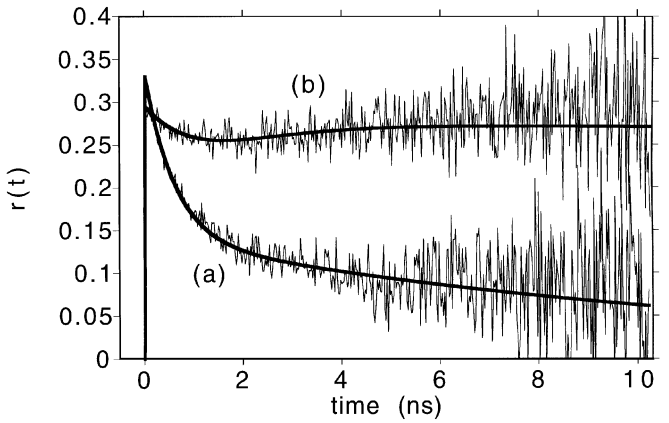

Fig. 2 Time-resolved fluorescence anisotropy decay curves from aqueous solution of RhB-labelled DADMAC/APMAA in the absence (a) and presence (b) of Ludox HS-30 (0.75 wt%). DADMAC/APMAA concentration was 0.02 wt% in each case (corresponding to 100% polymer adsorption in (b)). The solid lines are empirical fits to the data using (a) a double exponential function and (b) Eq. (2) with values for the parameters given in the text

model to this anisotropy behavior, the results are suggestive of a homogeneous population of dye sites on the polymer in which the fluorophores' fluorescence decay is non-single exponential, and each fluorophore experiences two degrees of rotational mobility; these possibly being related to restricted rotation of the probe (or probe/polymer segments) compared to the free dye and the slower rotation of a portion of the polymer coil in solution.

In contrast to the behavior exhibited by the RhB-labelled DADMAC/APMAA polymer in the absence of Ludox, when Ludox is present we again observe a decay and rise in the intensity of the $r(t)$ profile. This "dip and rise" behavior is less marked than for the non-polymer RhB case but is suggestive that the model described by Eqs. (2) and (3) is applicable. An empirical fit of these equations of this $r(t)$ profile is presented in Fig. 2 using values for τ_{c_1} and τ_{c_2} of 2.8 and 685 ns, r_{o_1} and r_{o_2} of 0.31 and 0.275, τ_{f_1} and τ_{f_2} of 0.6 and 2.6 ns, and mole fractions of the two sites, x_1 and x_2 of 0.49 and 0.51, respectively. Such a model could arise if the adsorbed probe/polymer is considered to consist of looped segments which have considerable mobility (and containing probes with a short decay time) and polymer segments which are firmly bound to the colloid particle where the probe has a longer fluorescence decay and displays the rotational dynamics of the particle. However, at this stage we wish to simply indicate, in a qualitative manner, that the polymer binding to the particle can be readily detected by the changes in the fluorescence anisotropy behavior. The use of fluorescence probes with longer lifetimes would be desirable to provide a more complete description of the slower rotational dynamics of the system [16].

The overall results discussed here also have important implications in the analysis of time-resolved fluorescence anisotropy data using the method of impulse reconvolution [17]. In this method, the $S(t)$ profile is deconvoluted (often using an arbitrary sums-of-exponentials function) and a function (often of the form: $r(t) = (r_o - r_\infty)\exp(-t/\tau_r) + r_\infty = D(t)/S(t)$) is assumed for $r(t)$. The initial fitting parameters making up this function are estimated, and the product $r(t)\cdot S(t)$ calculated. This product is then convoluted with the appropriate instrument response function, and the result is compared to $D(t)$. The fitting parameters of $r(t)$ are then adjusted iteratively by non-linear least-squares minimization to obtain the best fit of $S(t)\cdot r(t)$ to $D(t)$. This method is being used increasingly for analysis of time-resolved fluorescence anisotropy data, since in this method a single, unique fluorescence lifetime is not required to extract a rotational correlation time. However, in the impulse reconvolution method, it may be tempting to underestimate the importance of the multi-exponential behavior of the $S(t)$ profile and use too simple a functional form for $r(t)$. Impulse reconvolution of the data analyzed in this paper using more typical forms for $r(t)$ would have produced incorrect interpretations.

Conclusions

This work illustrates the power of inspection of the fluorescence anisotropy function, $r(t)$, even though direct analysis of $r(t)$'s is inherently complex. Analysis of just the parallel ($I_\parallel(t)$), perpendicular ($I_\perp(t)$) or difference ($D(t)$) functions would have been less likely to have enabled identification of the most appropriate model for these systems. It is obvious from these results that a far longer lived fluorophore is needed to fully characterize the system, and work to this end is in progress. The RhB probe used here is useful for elucidating the short time behavior and indicating the most appropriate model for this system. The results demonstrate that TRAMS can be applied successfully to monitor adsorption behavior, although care is required in the interpretation of the data obtained. These results are also important in view of the consequences of analysis of time-resolved fluorescence anisotropy data using the method of impulse reconvolution.

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