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Fluorescence Studies of Solubilization and Dye Binding in Hypercoiled Poly(methacrylic Acid): A Connected Cluster Model

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ABSTRACT

The compact conformation of poly(methacrylic acid) which is observed in the unneutralized polymer has been studied using the fluorescence probes 9-methyl anthracene and Rhodamine B. Steady-state and time-resolved fluorescence anisotropy measurements have been made. The results show that this conformation contains a number of binding sites which have molecular weights in the range 9,000-10,000 and which can rotate independently of the remainder of the chain.

INTRODUCTION

The solubilization of aliphatic and aromatic hydrocarbons and fatty acids within proteins is well known [1]. The nature of the binding site in the protein appears to be a "hydrophobic patch" of a volume just sufficient to contain one or two hydrocarbon molecules of suitable size [2]. Some polyelectrolytes also display this solubilizing property. Poly(methacrylic acid) (PMA), at a concentration of repeating units of 0.1 M, increases the solubility of anthracene in water from

4.47×10^{-7} to 0.31×10^{-4} M [3]. PMA is known to exist as a compact conformation at a degree of neutralization (α) less than 0.2 as a result of the operation of the hydrophobic effect whereby entropically unfavorable water-methyl side chain contacts are minimized. It has been suggested that hydrocarbons are bound within hydrophobic cores or structures in the compact form. A number of highly colored, water-soluble dye molecules also interact with polyelectrolytes [4, 5]. Here the binding can involve both electrostatic and nonelectrostatic factors.

In this work we report experiments made on both types of interaction, solubilization and binding, using fluorescence decay and steady-state and time-resolved fluorescence anisotropy methods in an attempt to investigate the nature and physical size of the binding sites in each case. We have used 9-methyl anthracene as the water-insoluble compound and Rhodamine B as the water-soluble dye.

Considerable theoretical work has been aimed at describing the viscoelastic properties of polymer solutions. This approach is centered on the development of mathematically tractable models which permit derivation of the diffusion equation. One such is the "bead and spring" model discussed by Bixon [6]. The beads of this model are not to be thought of as permanent structures but rather are statistical segments which can be treated as single units (with respect to frictional properties) for the sake of mathematical simplicity [7]. It is possible, however, that in certain systems such as hypercoiled PMA, actual aggregates may exist, at least on the time scale of fluorescence lifetimes. Again, dynamic fluorescence depolarization measurements offer the possibility of elucidating the situation.

EXPERIMENTAL

Atactic PMA was prepared by free radical polymerization using AIBN as initiator in 2-butanone solution at 60°C; the polymer precipitated from solution during the reaction. The polymer was filtered, redissolved in methanol, and then precipitated by adding it to diethyl ether, filtered, and dried. After dialysis in water it was freeze-dried. Atactic PMA with 9-methyl anthracene end groups was prepared by free radical polymerization in the presence of 9-methyl anthracene (9-MeA) as a transfer agent. It was separated from unreacted 9-MeA by gel permeation chromatography in methanol solution on a Sephadex LH20 column. Polymer molecular weights were determined by viscometry in 0.001 M aqueous HCl [8]. The 9-methyl anthracene was recrystallized from methanol. Rhodamine B was a laser-grade sample and was shown by thin-layer chromatography to have negligible impurities.

The 9-methyl anthracene was dissolved in atactic PMA, determined by titration to be 0.1 M in the repeating units, to give a 10^{-5} M solution. The ratio of 9-MeA molecules to PMA molecules was 0.25,

thus making the presence of more than one 9-MeA molecule per polymer chain unlikely. Rhodamine B concentrations were always less than 10^{-5} M. Dimerization of Rhodamine B is unimportant at concentrations less than 10^{-3} M.

All measurements were made at an ambient temperature of 20°C. Fluorescence intensities were recorded with a Perkin-Elmer MPF-44A spectrofluorimeter. Steady-state fluorescence anisotropy measurements were made on an instrument constructed according to the design of Bashford et al. [9].

Fluorescence decay measurements were made using the time-correlated single photon counting technique. The light source was a nitrogen-filled spark lamp operating at 15 kHz. Vertically polarized exciting light of 365 nm wavelength was used and emission observed at 90° to the excitation direction through a cut-off filter and a polarizer set parallel or perpendicular to the vertical plane. The experimental decay curves $I_{\parallel}'(t)$ and $I_{\perp}'(t)$ were transferred to an on-line NOVA 2-10 computer and were used to generate $D'(t) = I_{\parallel}'(t) - I_{\perp}'(t)$ and $S'(t) = I_{\parallel}'(t) + 2\ I_{\perp}'(t)$. These were then deconvolved from the exciting lamp intensity profile (collected using a Ludox scattering solution), using a nonlinear least squares procedure. The exciting lamp suffers intensity fluctuations over the long counting times necessary for these measurements, and the two intensity profiles were set in the correct proportions by making measurements of the total $I_{\parallel}$ and $I_{\perp}$ over short time periods and using their ratio to normalize the ratio of the total number of counts in $I_{\parallel}'(t)$ and $I_{\perp}'(t)$.

For Rhodamine B, the 365 nm absorption band had negative polarization, so that $(I_{\parallel} - I_{\perp})$ is negative, whereas for 9-methyl anthracene 365 nm is within the lowest energy band and so has positive polarization. In both cases $(I_{\parallel} - I_{\perp})$ should decay to zero.

The validity of combining data convolved with the instrument response function was confirmed by showing that the decay times extracted from S'(t) so produced were the same as those obtained from the decay curve collected with the emission polarizer set at 54.7° $(3\cos^2\theta - 1 = 0)$ to the vertical plane [10].

Rotational correlation times, τ_c, were obtained by generating the anisotropy decay function $A(t) = D(t)/S(t)$ from the deconvolved data and fitting this to single or double exponential functions. For isotropic rotation of a spherical molecule, $A(t) = A_0 \exp(-t/\tau_c)$ and $\tau_c = 1/6D$, where D is the rotational diffusion coefficient [11]. Double exponential decays may result if there is more than one type of rotating group present or if the molecules are not of an appropriate symmetry [12]. Since the D'(t) data have a low signal-to-noise ratio as expected, being a difference in two decay curves, the uncertainty in the rotational correlation times is much larger than for the fluorescence decay times.

TABLE 1. Fluorescence Lifetimes and Rotational Correlation Times for 9-Methyl Anthracene in PMA Solutions

Solution	Molecular weight	Fluorescence lifetimes		Fraction of (1)	Rotational correlation times	
		$\tau_{F(1)}$/ns	$\tau_{F(2)}$/ns		$\tau_{C(1)}$	$\tau_{C(2)}$
PMA/9-MeA end group in water. $\alpha = 0$	120,000	15.2	3.4	0.33	48	4
Atactic PMA and solubilized 9-MeA. $\alpha = 0$	216,000	12.9	5.0	0.88	21	5
9-MeA/hexane		4.1				
9-MeA/methanol		5.0				
9-MeA/paraffin oil (Shell Ondina Oil 33)		7.7				
9-MeA/propanediol		9.1				

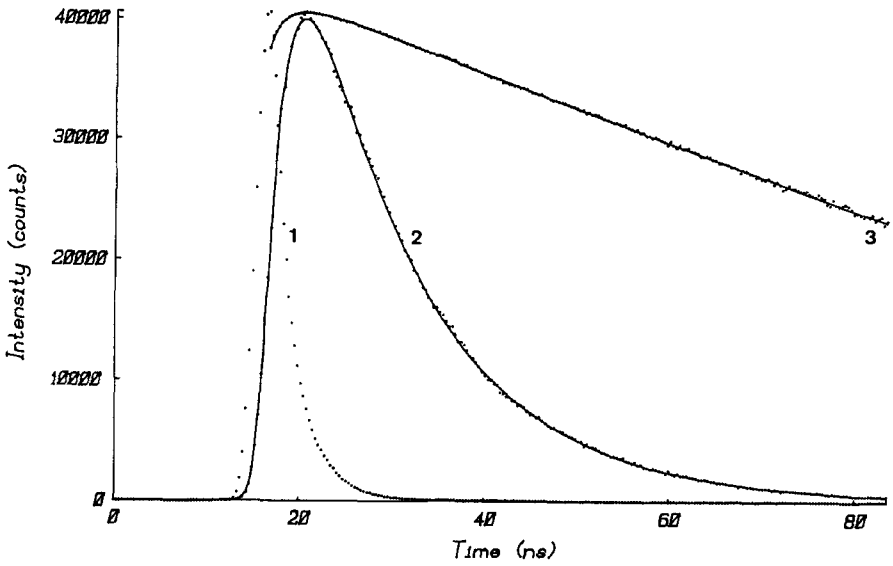

FIG. 1. Fluorescence decay of 9-methyl anthracene solubilized by atactic poly(methacrylic acid). Dotted curves (representing experimental points) from left: (1) lamp response function; (2) fluorescence decay convolved with lamp response function; (3) natural logarithm of (2). Solid lines represent biexponential fitted curves with parameters given in Table 1.

RESULTS AND DISCUSSION

PMA with Solubilized 9-Methyl Anthracene

The fluorescence decay of solubilized hydrocarbons is biexponential. Two lifetimes were extracted by fitting the fluorescence decay data to a function of the form

$$S(t) = S_0[\, f \exp(-t/\tau_{F(1)}) + (1 - f) \exp(-t/\tau_{F(2)})]$$

convolved with the instrument response function, where f is the fraction of the component having lifetime $\tau_{f(1)}$ (Table 1). The data and the analysis are shown in Fig. 1. The longer lifetime component of about 13 ns approaches the theoretical maximum radiative lifetime of 9-MeA (15.4 ns) [13]. Since the only nonradiative process in 9-MeA is intersystem crossing, which we have shown [14] to be strongly

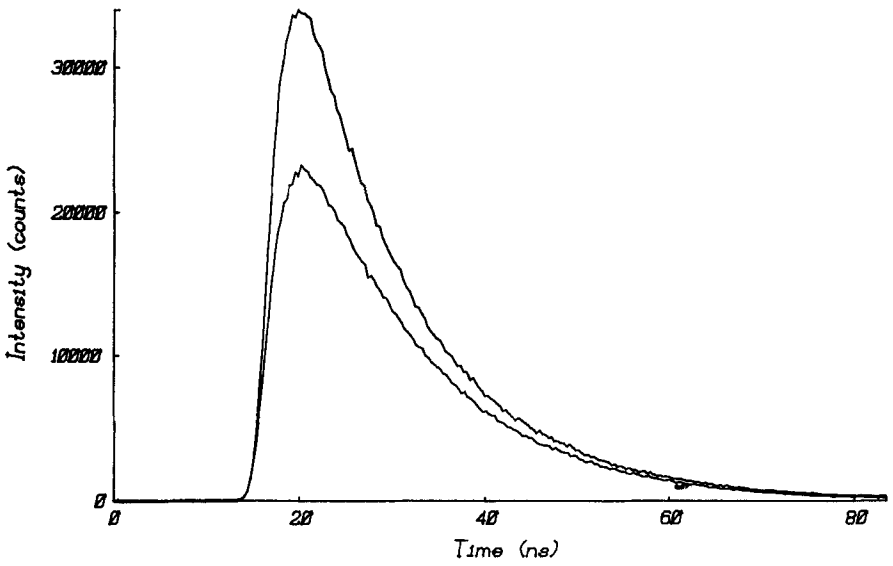

FIG. 2. Polarized components, $I_{\parallel}'(t)$ and $I_{\perp}'(t)$, of fluorescence decay of 9-methyl anthracene solubilized by atactic poly(methacrylic acid). $I_{\parallel}'(t)$ has the greater intensity.

dependent on viscosity at constant temperature, this result implies that the solubilized 9-MeA is rigidly held in the polymer matrix; the fraction of 0.88 shows that most of the 9-MeA is in this state. The short lifetime component of 5 ns is close to the value found in hexane or other low viscosity solvents, and indicates that some solubilization occurs in rather loose binding sites partially accessible to the solvent.

The rotational correlation times for this system shed light on the nature of these two different binding sites. Figure 2 shows the $I_{\parallel}'(t)$ and $I_{\perp}'(t)$ data from which the anisotropy decay function, A(t), is generated and fitted to an expression of the form [15]

$$A(t) = A_0[f \exp(-t/\tau_{c(1)}) + (1 - f) \exp(-t/\tau_{c(2)})]$$

For the purposes of the following discussion, it is reasonable to assume that the larger rotating units associated with the longer correlation time of 21 ns would provide a more viscous environment for the probe. The probes in these larger structural units would thus be expected to have a longer fluorescence lifetime as discussed above. Similarly, the microviscosity experienced by the dye molecules in the smaller cluster sites is likely to be comparatively lower and may be associated with the shorter fluorescence lifetime component.

The close correspondence between the long τ_c component in this case and that for PMA of molecular weight 10,000 with solubilized perylene suggests that one of the most probable size clusters in an atactic chain in water has a molecular weight 10,000 [5]. As the polymer molecular weight is 216,000, there must be more than one such cluster in a single chain. In the absence of the hydrocarbon, the chain cannot have the form of a tight single sphere since the addition of hydrocarbon should strengthen rather than weaken a hydrophobic interaction. We conclude then that when the molecular weight of PMA exceeds the value of 10,000, the hypercoiled form does not have the form of a tight single sphere but contains a number of clusters. Furthermore, on the basis of the present interpretation, the clusters must have sufficient length of chain separating them to permit rotation to be isotropic.

The short lifetime component has a rotational correlation time of 5 ns; this corresponds to the rotation of a spherical particle of radius 1.7 nm. We have constructed a section of an atactic PMA chain of 20 monomer units with space-filling molecular models and find that this section is just sufficient to enclose a 9-MeA molecule. It has a nearly spherical form and a radius of 1.5 nm. The majority of methyl groups in the model are directed toward the 9-MeA, while the carboxylic acid groups face the exterior. As a consequence, this structure could arise in response to unfavorable entropic interactions between a 9-MeA molecule and water molecules. There is presumably a delicate balance between the unfavorable entropy reduction, due to coiling of a short length of the chain, and the increase of entropy on removing methyl groups from water [2]. This can be pushed in the direction of coiling by the added presence of a 9-MeA molecule which can be wholly removed from its contacts with water.

PMA with 9-MeA End Groups

Time-resolved measurements gave double exponential behavior for both fluorescence lifetimes and fluorescence anisotropy behavior. The two rotational correlation times are 48 ns and 4 ns. The value of 4 ns corresponds to a fluorescence lifetime of 3.4 ns and is explained as a terminal section of the chain enclosing the end group as in the solubilized case. The value of 48 ns corresponds to a molecular weight of 17,000. These results have been discussed elsewhere [15] and are quoted here for the light they throw on steady-state fluorescence anisotropy results. These are given in Fig. 3. The results are presented in terms of fluorescence anisotropy which is connected to the more familiar fluorescence polarization, p, by the relationship

$$(p^{-1} - \frac{1}{3}) = \frac{2}{3} A^{-1}$$

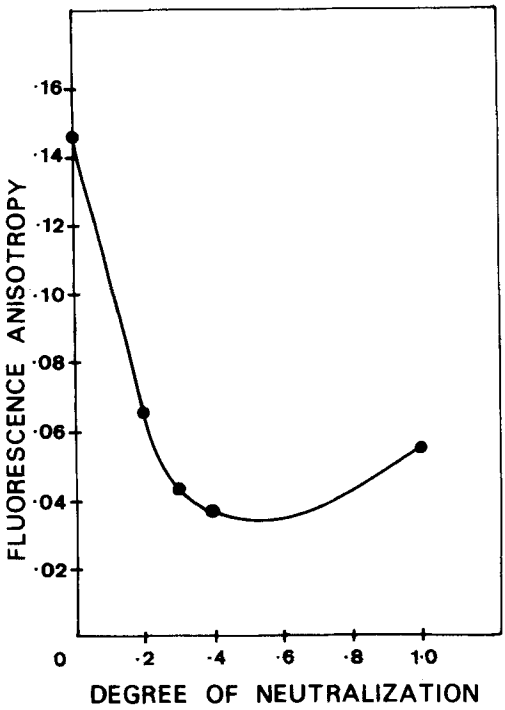

FIG. 3. Steady-state fluorescence anisotropy of 9-methyl anthracene end group labeled poly(methacrylic acid) in water as a function of degree of neutralization.

These results can be used to calculate the rotational correlation time using the Perrin relation,

$$A^{-1} = A_0^{-1}(1 + \tau_F/\tau_c)$$

provided that both the fluorescence decay time, τ_F, and τ_c, are represented by single exponentials and that the value of the limiting anisotropy A_0 is known. This is the value of the anisotropy for a random immobile assembly of molecules. For a substituted anthracene excited in the lowest energy band, this value theoretically should be equal to 0.4. For example, A_0 for anthracene in rigid solution is 0.38 [16]. However, values of A_0 obtained from the limiting intercept of plots of A^{-1} (in fact p^{-1}) against T/η in solvents of increasing viscosity vary greatly with polymer type [17].

A value of A_0 of 0.148 has been obtained for 9-MeA end groups on polystyrene [18]. This reduction in the value of A_0 from the free molecule value is most reliably explained in terms of librational motions of the fluorescent molecule attached to the polymer chain; these motions are independent of the viscosity of the medium and only depend on the mode and geometry of attachment to the polymer. In the present case this will vary as the charge on the PMA varies, since the chain will expand. If we assume that the long τ_F and τ_c component for the PMA/9-MeA end group system contributes most to the steady-state fluorescence anisotropy value, the above equation can be used to calculate a value of A_0 of 0.19. This is much greater than that found for 9-MeA end groups on polystyrene quoted above and agrees with our suggestion that the end group in PMA is deeply buried in a tight polymer coil with little freedom of movement. There are no results available at present for time-resolved measurements in the partially neutralized PMA solutions to permit an approximate calculation of A_0 for these.

PMA with Bound Rhodamine B

The fluorescence lifetime of Rhodamine B depends on solvent polarity and viscosity. Measurements in several solvents are given in Table 2. The results in butanol compared to those in water suggest that the solvent polarity as measured by dielectric constant influences τ_F. However, the result in PVA film indicates that rigidity of the environment is a major factor in determining a long value for τ_F of Rhodamine B. The effects of solvent on the wavelengths of maximum absorption and emission are small but sufficiently characteristic to be useful in assigning the state of the dye bound to polyelectrolytes. Table 2 also lists data for Rhodamine B bound to PMA and for comparison purposes to poly(acrylic acid) (PAA), which does not have a hypercoiled conformation at $\alpha = 0$.

Rhodamine B is very strongly bound to PMA at $\alpha = 0$, but can be dialyzed away, and so is only weakly bound, at $\alpha = 1$ (pH = 9.5). The fluorescence decay data are plotted in Fig. 4 and analyzed as a single exponential. The τ_F value of 4.0 ns quoted is an average value so obtained. For such short lifetimes, a two-exponential analysis did not give reliable values but if a second exponential is present it is a minor component. The magnitude of the average lifetime indicates that the binding site is rigid and the maximum absorption wavelength suggests that the binding site is more polar than water itself. We propose that the binding site consists of a carboxylic acid group and is also highly viscous in character. Since the binding decreases as the negative charge on the chain increases, the binding is not purely ionic but

TABLE 2. Fluorescence Properties of Rhodamine B at 20°C

Solvent	λ_{max} (absorption)/nm	λ_{max} (emission)/nm	τ_F/ns
H_2O (acid)[a]	558	579	1.4
(base)[b]	553	576	1.5
n-Butanol (acid)[a]	555	575	2.4
(base)[b]	544	566	3.0
Glycerol (base)[b]	556	577	3.2
PVA film	-	573	4.3
PMA solution, 0.03 M, $\alpha = 0$	563	578	4.0
PMA solution, 0.03 M, $\alpha = 1$	553	576	2.0
PAA solution, 0.03 M, $\alpha = 0$	560	581	3.0
PAA solution, 0.03 M, $\alpha = 1$	553	576	1.7

[a]Rhodamine B with the carboxyl group protonated.
[b]Rhodamine B zwitterion [19].

probably involves hydrogen bonding interactions with the carboxylic acid group on the phenyl ring pendant on the xanthene chromophore.

Comparison of the results for the PMA system with those found for PAA shows considerable differences. The wavelength of maximum absorption for Rhodamine B bound to PAA is closer to the value for Rhodamine B in water, although still indicating a polar binding site. However, the fluorescence lifetime suggests that some freedom of motion of the dye still remains, similar to that in the viscous solvent of glycerol. We suggest that the binding site for Rhodamine B in PAA at $\alpha = 0$ is also a carboxylic group within the relatively compact polyelectrolyte domain but has a lower rigidity or viscosity than the PMA binding site, which consists of a carboxylic acid group within the hypercoiled structure. The cluster arises as a result of hydrophobic interactions involving methyl groups on the chain.

Measurements of the decay of fluorescence anisotropy support this conclusion. Figure 5 shows that $(I_{\parallel}'(t) - I_{\perp}'(t))$ has a decay time of 3.3 ns. This value, taken with $\tau_F = 4.0$ ns, leads to a rotational correlation time of 19 ns. This corresponds to the isotropic rotation of a compact sphere of molecular weight approximately 9,000. As we argued in the case of PMA with solubilized 9-MeA, it is unlikely that

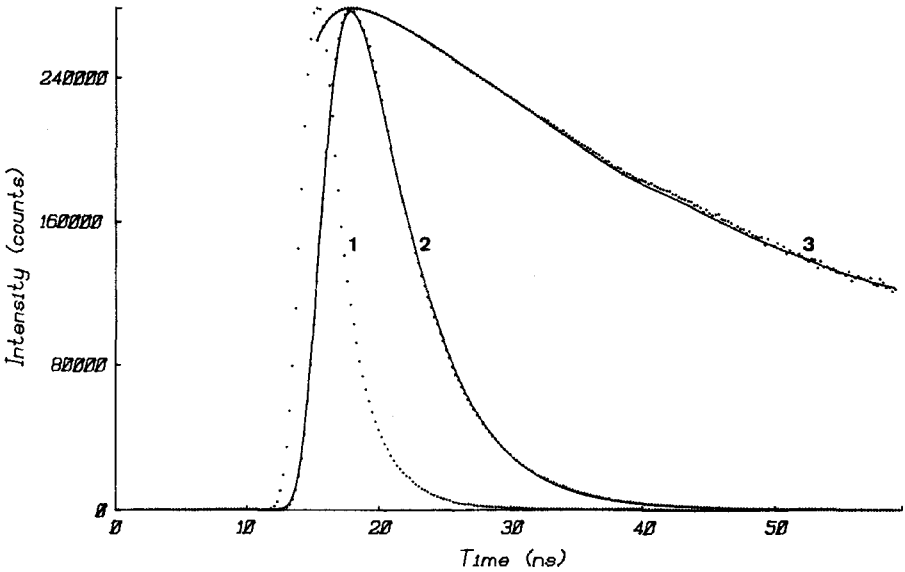

FIG. 4. Fluorescence decay of Rhodamine B bound by atactic poly-(methacrylic acid). Significance of curves as for Fig. 1. Solid lines represent single exponential fitted curves with parameters given in Table 2.

a cluster of this size would arise in response to a single dye molecule and so it must be present as a normal feature of the "native" poly-electrolyte.

The results for PMA and PAA at $\alpha = 1$ are included in Table 2 to demonstrate that no binding of Rhodamine B occurs when the carboxylic acid groups have all been converted to anions. The values of λ_{max} of absorption and emission are indistinguishable from the values for free Rhodamine B in basic solution. The values of τ_F are slightly greater than the free dye values; this may be a residual viscosity effect.

CONCLUSION

We conclude that the hypercoiled form of PMA cannot be considered as a single compact coil. The rotational motion can be analyzed as an isotropic rotation of clusters of molecular weight 9,000-10,000, connected by lengths of more randomly coiled chain. A second component

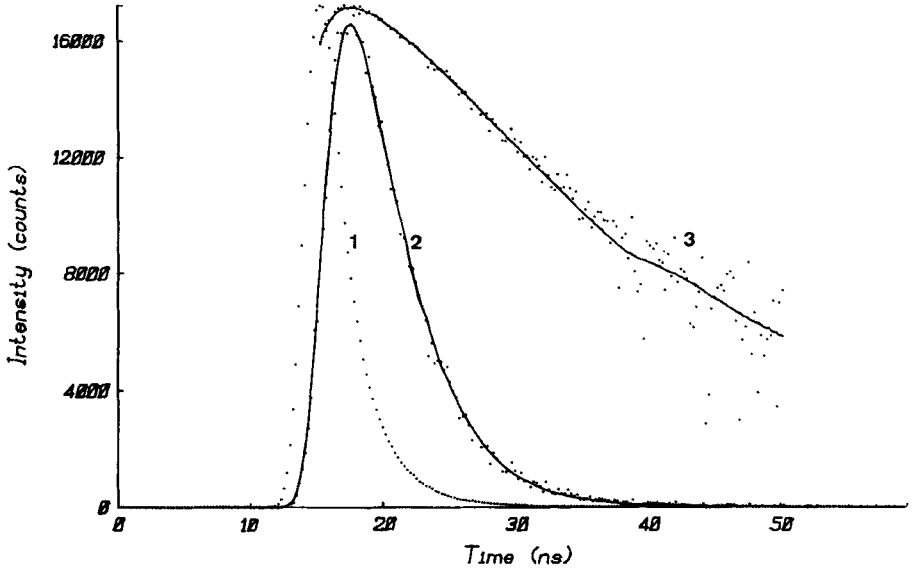

FIG. 5. Decay of $(I_{\parallel}'(t) - I_{\perp}'(t))$ for Rhodamine B bound by atactic poly(methacrylic acid). Significance of curves as for Fig. 1. Solid lines represent single exponential fitted curves with a decay time of 3.3 ns.

is detected from the decay characteristics of the 9-MeA probe molecule when it is solubilized in atactic PMA. This has a rotational correlation time corresponding to the rotation of a spherical cluster of molecular weight 3300 and approaches the limiting structure where the chain just encloses the hydrocarbon.

Fluorescence lifetime and rotational correlation time measurements indicate that the probe molecules are rigidly held in the polymer matrix to the extent that the transition dipole is fixed in each of the clusters. The similarity between the estimated cluster size determined from rotation of hydrophobic and hydrophilic probes, located in different regions of the polymer coil, supports the conclusion that the correlation times correspond to rotations of the natural clusters in the PMA, rather than to independent movements of the probe molecules.

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