Luminescence studies of polyelectrolyte behaviour in solution: 2. The application of steady-state fluorescence anisotropy measurements to the study of the conformational behaviour of poly(methacrylic acid) in dilute aqueous solution

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Steady-state anisotropy and fluorescence-quenching measurements have been used to study the segmental relaxation of poly(methacrylic acid) (PMAA) in dilute aqueous solution. None of the quenchers (CH_3NO_2, Tl^+ and I^-) employed in this work exhibited truly dynamic quenching of the excited states of the fluorescent species (0.5 mol% copolymerized acenaphthylene and 1-vinylnaphthalene) used to label the PMAA. As a consequence, the dependence of the measured anisotropy r upon the relative, quenched fluorescence intensity could not be used as a means of determination of the intrinsic anisotropy r_0. Furthermore, the influence of a static component in the quenching process rendered plots of r^{-1} as a function of fluorescence lifetime less reliable for estimation of r_0 than would be the case with a purely dynamic quencher. (This limitation is a direct consequence of the difficulties encountered in the measurement of r itself in the presence of significant static quenching.) Consequently, under these conditions, steady-state fluorescence anisotropy measurements have been shown to be of limited value in studying the conformational behaviour of PMAA. In contrast, time-resolved measurements have allowed the determination of rotational correlation times which are internally consistent and independent of the choice of fluorescent label at the pH values studied.

(Keywords: luminescence; fluorescence; polyelectrolyte)

INTRODUCTION

Luminescence techniques have featured prominently in studies of the conformational behaviour of polyelectrolytes. In many instances, fluorescent probes, dispersed within the medium, have been used to report upon the nature of their microenvironments through the resultant effects upon fluorescence intensity or lifetime[1–6]. For certain probes, notably pyrene[6], details of the fluorescence spectral structure can be used to gauge the relative polarities of media in which they are dispersed[6–10]. In addition, the use of fluorescent labels incorporating pyrene[7,11], dimethylanthracene[12], diphenylanthracene[13] or 5-dimethylamino-1-naphthalenesulphonate[14,15], being covalently attached to the polymer chain, gives direct information on the intracoil environment, regardless of the nature of the coil conformation (i.e. whether it is capable of solubilizing guest probes or not).

Studies of the pH-controlled conformational behaviour of polyelectrolytes such as poly(acrylic acid) (PAA) and poly(methacrylic acid) (PMAA) have been considerably enhanced through the use of mobile quenchers of the fluorescence of labels and/or solubilized guests. The ability of a given quencher to access a particular label or guest will depend *inter alia* upon the nature of the quencher itself, the polymer chain mobility and the intracoil microviscosity and hydrophobicity. A variety of quenchers have been used in such studies including ionic species, such as Tl^+ (refs 1, 7, 11), Cu^{2+} (refs 11, 13) and I^- (refs 1, 7, 11, 13), and neutral molecules (notably nitromethane[1,7,11,13]. More recently, advantage has been taken of the ability of water-soluble polymers to sustain room-temperature phosphorescence from covalently bound chromophores[16–18] in novel investigations of the conformational behaviour of PMAA[1] and PAA[18] in aqueous media.

Studies of the spectroscopic properties and susceptibility to quenching of luminescent labels and sequestered probes provide information regarding the changes in the local environment of the chromophore resultant upon conformational changes and thereby the transitions of the water-soluble polymeric hosts. Fluorescence anisotropy can, in principle, yield information upon changes in macromolecular dynamics concomitant with conformational transitions. In this way, such studies offer a different perspective in the investigation of the physical behaviour of polyelectrolytes in aqueous media to that

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gained from measurements of luminescence intensity and excited-state lifetime alone.

Despite the attraction of anisotropy measurements, relatively few reports of their application in the study of water-borne polymers have appeared to date[12,19–23]. In this paper we investigate the potential of steady-state fluorescence anisotropy measurements in the study of the conformational behaviour of PMAA in aqueous solution. Despite the limitations of the steady-state approach in the study of macromolecular dynamics[24–26] relative to time-resolved measurements, the technique has the advantage of providing information regarding polymer dynamics in a very straightforward manner in situations in which characterization of the macromolecular relaxation processes may be approximated by simple first-order kinetics. Time-resolved anisotropy measurements only realize their full potential in the resolution of macromolecular relaxation dynamics if high repetition rate excitation sources (such as lasers or synchrotrons) are used. Consequently it is desirable that the applicability of steady-state anisotropy measurements in the study of water-soluble polymers be assessed.

EXPERIMENTAL

Materials

Fluorescently labelled samples of poly(methacrylic acid) (PMAA) containing small amounts (ca. 0.5 mol%) of copolymerized residues of 1-vinylnaphthalene (1VN-MAA) or acenaphthylene (ACE-MAA) were prepared by free radical solution copolymerization in benzene, as described previously[27]. Unlabelled PMAA was prepared in a manner similar to that employed in the synthesis of the fluorescently labelled samples. Thallium nitrate (Aldrich, 99.999%), potassium iodide (BDH) and nitromethane (Aldrich, Gold label) were used without further purification. Water (solvent) was doubly distilled.

Solutions for spectroscopic analyses contained 10^{-3} wt% of polymer. The pH was adjusted by addition of sodium hydroxide, sulphuric acid (May and Baker) or hydrochloric acid (Aldrich, Spectrosol) as required.

Instrumentation and techniques

Steady-state fluorescence spectra and anisotropy data were recorded on a Perkin–Elmer MPF-3 spectrometer. Pure, unlabelled PMAA solutions were used as corrective 'scatter blanks' in all anisotropy determinations.

Estimates of fluorescence 'lifetimes' were obtained from time-resolved data recorded on an Edinburgh Instruments 199 spectrometer operating on the time-correlated single photon counting (TCSPC) principle. A nanosecond, thyratron-gated flashlamp (with H_2 as the discharge medium) was used as the excitation source. Fluorescence was detected using a fast photomultiplier tube (Philips XP2020Q). Under these conditions, an instrument response function (*FWHM*) of ca. 1.2 ns resulted. Corroborative lifetime data were determined by analyses of fluorescence decays obtained using TCSPC following magic angle (54.7° with respect to the plane of vertically polarized excitation) observation of the emission generated by synchrotron excitation using the Synchrotron Radiation Source (SRS), SERC Daresbury Laboratory, Warrington, UK. Fluorescence was detected using a fast photomultiplier tube (Philips XP2020Q). Under these conditions, an instrument response function (*FWHM*) of ca. 600 ps resulted. Time-resolved fluorescence anisotropy measurements of macromolecular relaxation were performed using the SRS for excitation. The SRS and associated TCSPC analysis system have been described previously[27].

RESULTS AND DISCUSSION

Steady-state fluorescence anisotropy experiments, which seek to quantify the rate of rotational reorientation of a chromophore, usually employ vertically polarized excitation and measure the intensities of emission analysed in planes oriented parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the plane of excitation. The extent to which fluorescence polarization is retained, within the average lifetime of the fluorescent excited state, may be expressed in terms of the anisotropy r

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \tag{1}$$

The anisotropy may, in turn, be related to the correlation time τ_c, characteristic of the rotational motion of the chromophore by the Perrin relationship

$$r^{-1} = r_0^{-1}(1 + \tau/\tau_c) \tag{2}$$

Estimation of τ_c from empirical r data requires that both τ, the lifetime of the fluorescent excited state, and r_0, the intrinsic anisotropy of the chromophore, be determined. The value of τ may be determined directly (e.g. by TCSPC). However, estimation of r_0 is potentially problematic.

Examination of equation (2) reveals that r_0 might be determined, in principle, by extrapolation of empirical anisotropy data either to zero τ or to infinite τ_c. Extrapolation to infinite τ_c is conventionally accomplished using a Perrin plot, wherein it is assumed that τ_c is proportional to the ratio of viscosity η to temperature T. In this way, r_0 is estimated as the intercept of a plot of r^{-1} against T/η. However, despite its popularity, the conventional Perrin plot is subject to considerable limitations in applications concerning macromolecular relaxation[24,26]. Even if such restrictions could be ignored for polymers dissolved in organic media, the sensitivity of the conformational behaviour of polyelectrolytes in aqueous media to environmental conditions would preclude variation of T/η over a sufficient range for meaningful data analysis. Consequently, if steady-state anisotropy measurements are to be applied to the study of the relaxation behaviour of polyacids in aqueous media, r_0 must be determined from r data estimated at various values of τ as τ_c is held constant.

Reduction of τ without essentially altering the nature of the solvent (and thence τ_c) may be accomplished[28] by the addition of a dynamic quenching agent. Clearly, for polyelectrolytes in aqueous media, the assumption that the addition of quenching species exercises a minimal perturbation effect upon the macromolecular behaviour must be treated with caution. Indeed, the testing of this assumption is one of the aims of this investigation.

Fluorescence lifetime and quenching data

In a previous publication[1] we reported upon the use of fluorescence lifetime and quenching measurements as a means of investigating the pH-dependent conformational transition of PMAA from its compact, 'hypercoiled' form, extant at low pH, to the open coil structure adopted

by the polysalt form at high pH. The salient features of this earlier work[1] are summarized below inasmuch as they affect the considerations of the choice of quencher and experimental procedure to be adopted in steady-state anisotropy studies of the relaxation behaviour of PMAA in aqueous media.

In the instance where interactions between the fluorescent excited state of the label and the mobile quencher result in a truly dynamic form of quenching, the relative fluorescence intensity I/I^0 and lifetime τ/τ^0 data are equivalent as expressed in the Stern–Volmer equation

$$I^0/I = \tau^0/\tau = 1 + k_q\tau^0[Q] \quad (3)$$

where k_q is the bimolecular rate constant governing the collisional deactivation of the excited state and F^0 represents the value of the appropriate fluorescence parameter ($F = I$ or τ) in the absence of quencher Q. Under such ideal conditions, the combination of steady-state emission anisotropy determinations with quenching information achieves maximum simplicity of application in the study of macromolecular relaxation. Determination of r over a range of [Q] values and the corresponding fluorescence intensity data allow r_0 to be estimated, according to equation (2), from a plot of r^{-1} as a function of I/I^0 (since extrapolation to $I = 0$ effects extrapolation to $\tau = 0$). Consequently, determination of τ_c, for a given system from the Perrin equation simply requires that τ be determined at a single, convenient value of [Q] (e.g. zero [Q]). Unfortunately, neither in the case of 1VN-MAA nor ACE-MAA was truly dynamic quenching behaviour achieved with any of the quenchers employed (CH_3NO_2, Tl^+ and I^-)[1]. The consequences of this observation for the determination of τ_c for PMAA in aqueous solution are discussed in the section dealing with the steady-state fluorescence anisotropy data.

When iodide (as KI(aq)) was used as the water-borne quencher at high pH, values of I^0/I and τ^0/τ which approached equivalence at any given [I^-] were obtained[1]. However, a slight curvature in the intensity data was apparent when plotted in Stern–Volmer form[1]. As[I^-] was increased, the fluorescence intensity ratio I^0/I was increasingly enhanced relative to the corresponding value of τ°/τ. The consequence of this effect upon the resultant 'Perrin' plots of r^{-1} against I/I^0 and τ/τ^0 is shown for the ACE-MAA system in *Figure 1* and discussed in the section which follows. At low pH ($\leqslant 5$), quenching by I^- resulted both in marked deviations from dynamic quenching and Stern–Volmer plots showing considerable deviations from linearity (both in terms of intensity and lifetime dependences upon [I^-]). Reasons for these effects have been discussed[1].

Nitromethane acts as an efficient quencher of the intensity of fluorescence observed from either naphthalene-based label of PMAA at low degrees of neutralization of the polyacid. Unfortunately, a large component of 'static quenching' was observed in the comparison of the lifetime and intensity data[1]. Not only does this render the intensity data inappropriate for use in the determination of r_0 via equation (2), it poses considerable problems in the estimation of r_0 from lifetime data (as discussed below). Worse yet, nitromethane proves to be totally unusable as a quencher in anisotropy studies of the relaxation behaviour of naphthyl-labelled PMAA in its fully neutralized form. At high pH, some form of specific interaction between the CH_3NO_2 and the naphthalene

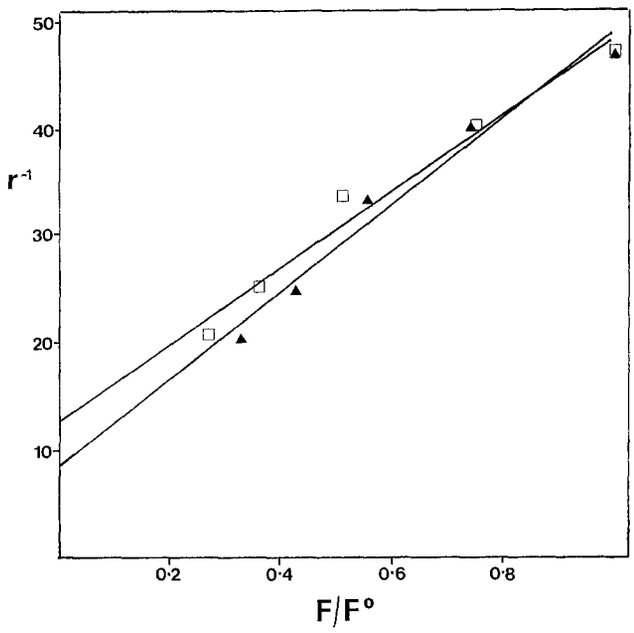

Figure 1 Perrin plots of r^{-1} as a function of relative fluorescence parameter F/F^0 for ACE-MAA at pH = 12.7 using I^- as the quencher, where F = intensity (□) and lifetime (▲)

chromophores occurs which produces a continuous reduction in the observed fluorescence intensity of either label during attempted photophysical characterization of the labelled polysalts[1].

Recently, we have used the thallium (I) ion (Tl^+) as a means of generating room temperature stabilized phosphorescence (RTSP) both in chromophores covalently bound to polyacids[1,16,17,29,30] and in luminescent guest molecules sequestered within the coils of the polyacid[17,29]. A major contributor to the induction of RTSP in these systems is the ability of the 'heavy-ion' species Tl^+ to promote population of the triplet state of the appropriate chromophore through enhancement of the process of intersystem crossing from the fluorescent excited state. The resultant quenching of the fluorescence of the chromophore is of potential interest in the current context. Unfortunately, the quenching action of Tl^+ upon the fluorescence of either 1VN-MAA or ACE-MAA is complicated. At low pH, Tl^+ experiences difficulty in accessing the fluorescent excited state of the naphthalene-based labels since it is effectively excluded from the hydrophobic domains created within the acidic form of the PMAA[1]. (Such exclusion of Tl^+ from the hydrophobic interior of the polymer is evident from conjunct studies of the lifetime of the phosphorescent (T_1) state of the label and the resultant phosphorescence intensity[1,29].) Furthermore, high degrees of 'static quenching' are observed at all pH values[1]. In view of these complications, Tl^+ was excluded from subsequent studies of the use of quenching data in combination with steady-state fluorescence anisotropy investigations of the relaxation of PMAA.

Finally, we note that the transient fluorescence behaviour of both 1VN-MAA and ACE-MAA is pH dependent. At high pH, the fluorescence decay of either species approaches that expected from first-order kinetic behaviour[1]. At low pH, the fluorescence decay of either label shows significant deviations from single exponentiality[1]. Such deviations reflect the heterogeneity of the

environments experienced by a given fluorescent label of the acid form of PMAA. This, in itself, does not present a problem in respect of the use of steady-state anisotropy data in the evaluation of τ_c for the polyacid form of PMAA. The lifetime of the excited state of the fluorescent label may be described, perfectly adequately, by means of an average lifetime $\langle\tau\rangle$ defined by

$$\langle\tau\rangle = \sum_i A_i\tau_i^2 \Big/ \sum_i A_i\tau_i \qquad (4)$$

where A_i and τ_i represent the pre-exponential factors and 'lifetimes', respectively, of the parameters derived following multiexponential fitting (usually, double-exponential 'fits' supply statistically 'adequate' descriptions) to the fluorescence decays obtained from the naphthyl labels of the acid forms of PMAA.

Steady-state fluorescence anisotropy data

Figure 2 shows the variation of r^{-1} with I/I^0 for ACE-MAA and 1VN-MAA at high pH, using iodide as the quencher. In the absence of other information, these plots would appear to satisfy the requirements of the steady-state anisotropy method as applied to the study of macromolecular relaxation in fluid media. The plots are reasonably linear and the range over which extrapolation is required, in the estimation of r_0, is not excessive. Indeed, the resultant estimates of r_0^{-1} (see *Table 1*) at 8.5 (1VN-MAA) and 12.6 (ACE-MAA) lie within the range of values which might be expected on the basis of previous studies of these labels in a variety of polymer/solvent combinations[20,26,31–33]. Consequently, these estimates might be regarded as being reasonable. However, as reference to *Figure 1* and *Table 1* reveals, the relatively small deviations from strict equivalence of the intensity and lifetime data, upon quenching with I^-, result in a fair degree of variance between the estimates of r_0 from 'Perrin' plots based upon lifetime and intensity data, respectively. As is shown in *Figure 3* (and *Table 1*), the situation is exacerbated in acidic media with CH_3NO_2 as the quencher.

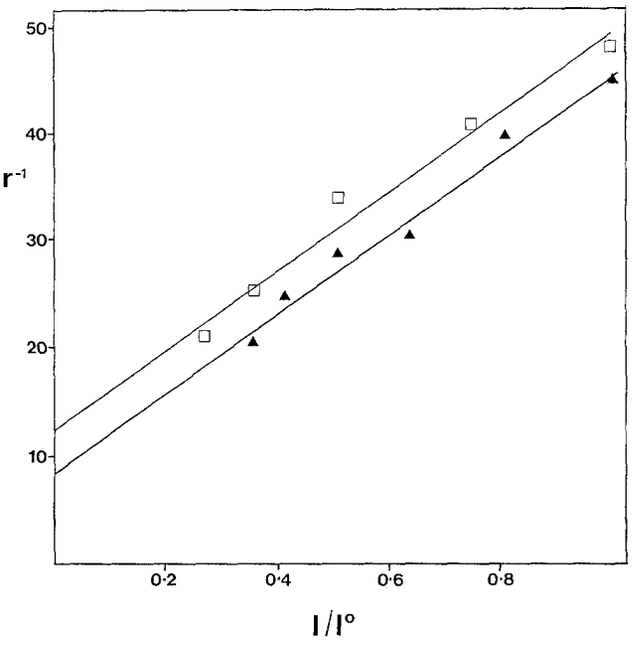

Figure 2 Comparison of Perrin plots of r^{-1} as a function of relative fluorescence intensity for ACE-MAA (□) and 1VN-MAA (▲) using I^- as the quencher at high pH

Table 1 Intrinsic anisotropies as estimated from Perrin plots

Label	pH	r_0^{-1}
1VN	11.6[a]	8.5[c]
		10.2[d]
1VN	3.6[b]	13.5[c]
		8.2[d]
ACE	12.7[a]	12.6[c]
		8.7[d]
ACE	4.6[b]	13.7[c]
		9.3[d]

[a] Using I^- as quencher
[b] Using CH_3NO_2 as quencher
[c] Derived using intensity data
[d] Derived using lifetime data

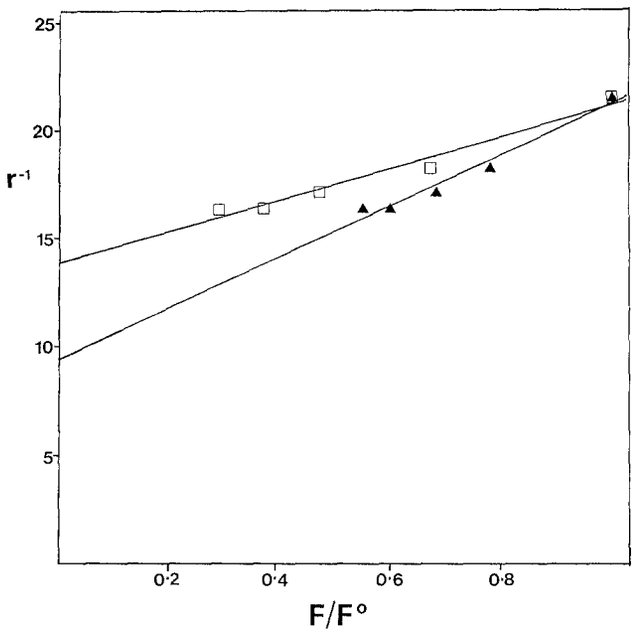

Figure 3 Perrin plots of r^{-1} as a function of relative fluorescence parameter F/F^0 for ACE-MAA at pH = 4.6 using CH_3NO_2 as the quencher, where F = intensity (□) and lifetime (▲)

Estimates of τ_c are very sensitive to r_0. (Taking the ACE-MAA system at pH = 4.6 as an example, a variation in the estimate of r_0^{-1} from 13.7 to 9.3 results in a variation in τ_c from 57 to 25 ns.) Consequently, it is important that both the correct extrapolative procedure be adopted and the range over which the extrapolation is to be performed be kept as short as possible. Clearly, consideration of equation (2) establishes that the correct extrapolative procedure to be implemented in this instance involves the use of lifetime rather than intensity data. However, examination of *Figure 3* reveals a further problem. The existence of a significant static component to the quenching of the ACE-MAA fluorescence by CH_3NO_2 in acidic media means that the intensity of fluorescence to be sampled decreases markedly upon successive additions of quencher. This, in turn, imposes severe restrictions upon the attempts to estimate r, with confidence, at high quenching ratios: the true values of the individual, diminishing, intensity components $I_\parallel$ and $I_\perp$ become increasingly difficult to determine in the presence of the constant scattered 'background' interference.

Table 2 Rotational correlation times for the segmental relaxation of PMMA

Label	pH	τ_c^a (ns)	τ_c^b (ns)	τ_c^c (ns)
1VN	11.6	7.3	6.0	8.7
	3.6	26.3	23.9	21.2
ACE	12.7	4.1	3.7	7.9
	4.6	24.6	19.3	23.0

[a] Obtained via equation (2) using r_0 derived by extrapolation of the relevant plot of r^{-1} against τ/τ^0

[b] Obtained via equation (2) using $\langle r_0 \rangle$ determined from TRAMS data

[c] Derived from TRAMS data via analyses, in terms of single-exponential models to the anisotropy decay, using the impulse reconvolution method[34]

Consequently, the range of effectively accessible lifetime ratios τ/τ^0 is considerably reduced (relative to that attainable in the absence of a large static contribution to the quenching). As a result, the lifetime range over which extrapolation must be performed is larger than would be desirable, leading to enhanced uncertainties in the estimates of r_0 (and thence of τ_c).

Table 2 lists the values of τ_c obtained, via equation (2), using the r_0^{-1} values derived by extrapolation to $\tau = 0$, as listed in *Table 1*. Considerable variation is to be noted between the values of τ_c obtained in basic media using the two different fluorescent labels 1VN and ACE. The labels have the potential to report differently upon the motions of the macromolecule: the transition vector for fluorescence is not perfectly aligned along the short axis of the naphthalene ring. Consequently, a contribution to the overall emission depolarization could result from motion, independent of that of the polymer backbone, around the bond of attachment of the chain to the naphthalene. However, if such motion was to occur (even at the high frequencies corresponding to sampling in the nanosecond time domain), it should result in a reduction of the apparent value of τ_c for 1VN-MAA relative to that for ACE-MAA. Hence, in the absence of either label exerting some form of perturbing influence upon the macromolecular behaviour, it must be assumed that the variation between the values of τ_c reflects the uncertainty in the estimation of the relaxation parameters using the steady-state fluorescence anisotropy approach.

Interestingly, closer agreement is found between the values of τ_c obtained for 1VN-MAA and ACE-MAA in acidic media. (The potential for variance might be expected to be greater in this instance since the degrees of static quenching encountered at low pH are greater than those in alkaline conditions (see *Figures 1* and *3*), resulting in a reduction in the range of τ values over which reliable estimates of r can be made (see above) and thence in extended extrapolation lengths.) The situation is complicated in acidic media by the fact that the excited state decays exhibit marked deviations from single-exponential behaviour. Consequently, 'average lifetimes', defined according to equation (4), were used in the corresponding 'Perrin' plots as noted previously for the associated Stern–Volmer analyses[1] (see above).

Time-resolved anisotropy measurements (TRAMS)

TRAMS were performed on the 1VN-MAA and ACE-MAA systems using synchrotron radiation (SRS, Daresbury) for excitation. Full details will be published in the near future[34]. TRAMS data were analysed (using single-exponential functions to model the decay of fluorescence anisotropy) using the impulse reconvolution[35] method. Representative data are shown in *Table 2*. Much better agreement is achieved between the τ_c values obtained for the 1VN-MMA and ACE-MAA systems than was apparent on the basis of steady-state anisotropy measurements. Clearly, the two labels appear, as expected, to report upon the same macromolecular relaxations, and the discrepancy between the τ_c values for the different systems apparent in steady-state studies merely reflects the limitations of the latter in the study of polyelectrolytes in aqueous media. (It should be noted that, at partial degrees of neutralization of the PMAA, the 1VN label does appear to move independent of the polymer chain. Its anisotropy, in highly acidic media, is not purely reflective of the segmental motion of the macromolecule, as we shall report at a later date[34].)

Impulse reconvolution not only allows τ_c to be determined but also furnishes an estimate of r_0. Data from TRAMS have been collected over a range of pH values[34]. Little variation in r_0 is apparent with pH (and thence with the local environment of the label). Values of r_0^{-1} averaged across the pH range are in reasonable agreement with those (see *Table 1*) obtained in steady-state extrapolation to zero fluorescence lifetime. A value for $\langle r_0^{-1} \rangle$ of 8.7 (obtained from analyses of TRAMS data for the 1VN-MAA system) is to be compared with those of 8.2 and 10.2 resultant upon analysis of steady-state data measured at pH values of 3.6 and 11.6, respectively. For the ACE-MAA system, the value of 8.0 for $\langle r_0^{-1} \rangle$ is to be compared with those of 9.3 and 8.7 derived for pH values of 4.6 and 12.7, respectively. The consistency between the steady-state and time-resolved data is particularly gratifying in the case of ACE-MAA. It has been reported[36], erroneously[37], that the fluorescence from the ACE chromophore (i.e. that incorporated into a macromolecule by copolymerization of acenaphthylene) is subject to such high degrees of intrinsic depolarization as to be of little value for anisotropy studies. The current work clearly demonstrates that this is not the case. It is inconceivable that both steady-state and time-resolved experiments could be subject to similar extents of distortion by a polarizing perturbing influence (such as scattered radiation) and in a manner not immediately apparent in the time-resolved data.

Given that the TRAMS data seem to indicate that r_0 is not dramatically affected by the local environment of the label, we have recalculated the values of τ_c, based upon steady-state estimates of r (derived from 'least-squares best fits' to 'Perrin' plots) at $\tau/\tau^0 = 1$, using values of $\langle r_0^{-1} \rangle$ determined from impulse reconvolution fitting of the time resolved data (see *Table 2*). It is evident that these estimates of τ_c do not show an improvement in concordance between the 1VN-MAA and ACE-MAA systems relative to that achieved using 'Perrin' extrapolations to $\tau/\tau^0 = 0$. Consequently, it would appear that, even with the degree of 'hindsight' afforded by the TRAMS experiments, steady-state fluorescence anisotropy experiments suffer considerable limitations in applications concerning the conformational behaviour of polyelectrolytes.

CONCLUSIONS

1. Difficulties have been encountered in attempts to apply the steady-state fluorescence anisotropy tech-

nique in studies of the conformational behaviour of PMAA. The problems lay in our inability to find truly dynamic quenchers of fluorescence. An immediate consequence is the fact that the simplest approach to the estimation of the intrinsic anisotropy (that of plotting r^{-1} against I/I^0) is invalid in this instance.

2. Steady-state anisotropy measurements can detect the effects of the pH-controlled conformational transition of PMAA upon the macromolecular relaxation characteristics. However, considerable uncertainties will exist, in the derivation of rotational correlation times, in experiments in which significant degrees of static quenching are encountered.
3. Time-resolved anisotropy measurements are required (preferably using high repetition rate excitation sources) for accurate estimates of the relaxation parameters of PMAA in aqueous media. The results of such investigations will be published in due course[34].

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