Polymer-bound flavins: 5. Characterization by static and time-resolved fluorescence techniques

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(Received 24 September 1984)

The composition of several flavin-containing cationic polyelectrolytes is found to affect static and dynamic fluorescence properties of the pendant flavin. The relative fluorescence quantum efficiencies and average lifetimes decrease with increasing content of ammonium ions in the polymers. The fluorescence decay of the polymer-bound flavins is highly heterogeneous and can be described with 3 life-time components. The fluorescence anisotropy decay can be resolved into 2 relaxation times. The longer correlation time component can be considered to result from polymer chain motions. The shorter one is slightly longer than the rotational correlation time of the free isoalloxazine (~80 ps). The results demonstrate a decrease of polymer mobility with increasing hydrophobic character of the polyelectrolytes. Addition of 2-propanol to an aqueous solution results in polymer coil expansion and in an increase in polymer chain mobility, i.e. shorter correlation times.

(Keywords: flavin; microenvironment; polymer chain mobility; fluorescence decay; anisotropy decay)

INTRODUCTION

In recent years the use of luminescence measurements for the study of a diversity of phenomena occurring in biological and synthetic macromolecules has become widespread. For instance, dyes whose fluorescence properties are sensitive to the polarity of the medium can be used as 'reporters' to characterize the polarity of their microenvironment, when adsorbed or covalently bound to specific protein sites[1] or synthetic polymer chains[2,3]. Depolarization of the fluorescence of chromophores that are bound to globular proteins may be used to estimate the rotational diffusion constants of the proteins, or to follow the association of two proteins[4].

Time-resolved fluorescence techniques such as excimer formation and decay and fluorescence anisotropy decay, may yield considerably more information on molecular motion occurring in macromolecules than static measurements[4-6]. With modern equipment it is possible to study the decay characteristics of fluorescence on a nanosecond or even picosecond time-scale. This time-scale coincides with that for various motions in macromolecular systems, of both biological and synthetic origin. This permits us to use the fluorescence decay characteristics of probes incorporated in the polymer back-bone or bound to the back-bone as a pendant group, for studying conformational mobility of flexible chains, helix–coil transition phenomena, amorphous phase orientation, or polymer compatibility[4-8].

Flavins and flavoproteins have been extensively investigated both with steady-state and with time-resolved fluorescence techniques[9-14]. Spectroscopic properties of flavins embedded in (model) membranes, vesicles, or reversed micelles have also been studied[15-17]. In this contribution we report on the luminescence characteristics of flavin bound to several water-soluble cationic polymers. In previous publications we have discussed the influence of polymer composition (varying content of hydrophilic and hydrophobic monomer units) on the activity of the bound flavin as a redox-catalyst[18-20]. The present work gives an account of the spectroscopic behaviour of the polymer-bound flavins, such as fluorescence spectra and dynamic properties as derived from fluorescence and anisotropy decay. The spectral results point to an altered microenvironment of the bound-flavin as compared to plain water dependent on the composition of the copolymers. The results of anisotropy decay analyses can qualitatively be explained in terms of polymer chain mobility. The influence of polymer composition and the solvent (water/2-propanol mixtures) on the calculated correlation times is also discussed.

EXPERIMENTAL

The syntheses of the polymer-bound flavins **1a–e**, **2** and **3** have been described in previous papers[18,19] (see *Figure 1* for structures and *Table 1* for data on compositions).

Absorption spectra were recorded on a Pye-Unicam SP8-200 UV/VIS spectrophotometer. Fluorescence spectra were run on an Aminco SPF-500 fluorimeter. The excitation wavelength employed was 445 nm, the emission and excitation band widths were 4 nm. The flavin concentration was in all (static and dynamic) experiments about 1×10^{-5} M, consequently the polymer concentration varied between 0.2 and 0.5 g.dm^{-3}, depending on its flavin content. All aqueous solutions (aqua bidest.) were buffered at pH 8.0 with tris(hydroxymethyl)amino-

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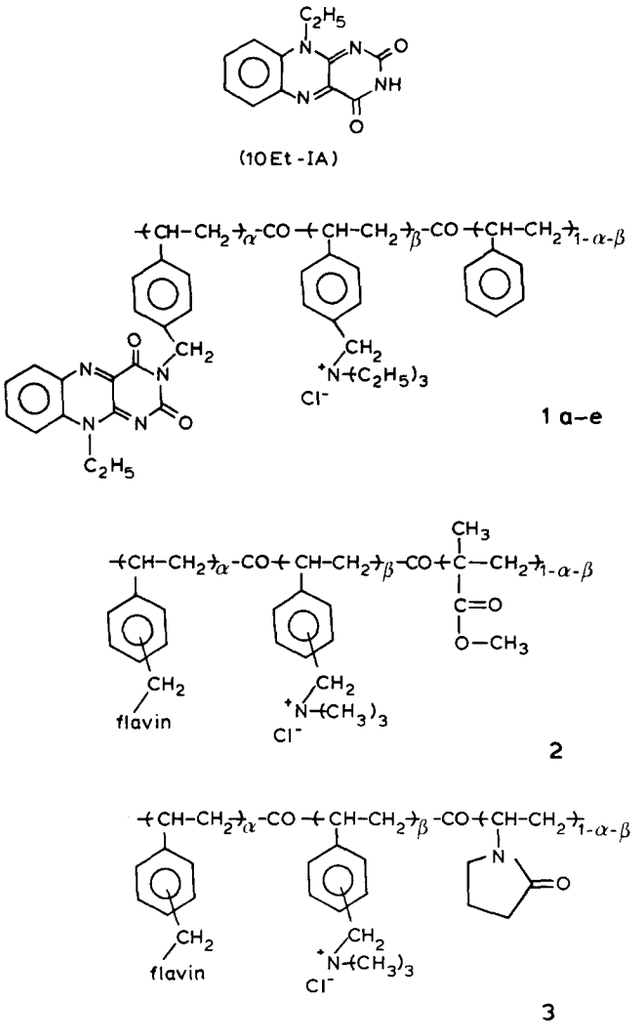

Figure 1 Structures of the flavins investigated

Table 1 Composition and absorption maxima of flavin-containing polymers

Sample	α (mol fraction)	β (mol fraction)	λ_{max} (nm)	λ_{max} (nm)
Isoalloxazine	–	–	341	433
1a	0.004	0.19	336	440(s)
1b	0.013	0.45	340	440(s)
1c	0.017	0.54	341	440(s)
1d	0.013	0.76	341	440(s)
1e	0.014	0.95	341	439(s)
2	0.008	0.23	341	437
3	0.006	0.14	341	436

(s) indicates that shoulders appear in the absorption band

methane and hydrochloric acid, with ionic strength $I = 0.05$ M (KCl).

Fluorescence life-times and anisotropies were determined on a time-resolved single photon counting system with the 457.9 nm line of a mode-locked Ar ion laser as excitation source. Fluorescence was measured via a Balzers 531 nm interference filter. This system, the tests with single life-time standards, and the non-linear least-squares fluorescence decay analysis have been extensively detailed elsewhere[16,21–23]. Recently, the determination of rotational correlation times from deconvoluted fluorescence anisotropy decay curves has been critically appraised[24].

RESULTS AND DISCUSSION

Light absorption and emission spectra

In *Table 1* the maxima of the visible light absorption spectra of the different polymer-bound flavins are listed and compared with the values found for the free flavin (isoalloxazine). The spectrum of the flavin is markedly changed when the flavin is bound to the copolymers.

For all polystyrene based polyelectrolytes the lowest energy absorption band is shifted to somewhat longer wavelengths and shows a distinct vibronic structure, but for the second electronic transition a significant blue-shift is observed only for **1a** (the polystyrene derivative with only about 20 mol% of quaternized monomer units). The spectral changes for **2** and **3** are less pronounced. It is well known that the absorption spectrum of flavin is sensitive towards solvent polarity changes and the formation of hydrogen bonds[9–12]. The spectral results indicate that the flavin group bound to a polymer chain is situated in a somewhat apolar or hydrophobic environment. The altered interaction with solvent molecules may be caused by an ordering in the surrounding water induced by the hydrophobic polymer back-bone. It is worthy to note that addition of an analoguous polyelectrolyte without flavin groups to a solution of the free isoalloxazine has no influence on absorption (and emission) spectra.

The fluorescence spectra of all flavins are characterized by broad structureless bands, and only small shifts in emission maxima are observed for the polymer-bound flavins, see *Table 2* and *Figure 2*. This can be an indication that water molecules are able to diffuse to the isoalloxazine during the life-time of the excited state[17].

The spectral differences are not large, so flavin, in general, is not extremely suitable as a polarity probe, as compared to other reporter molecules that are more sensitive to microenvironmental changes[2,7,25,26]. The quantum efficiencies of fluorescence are significantly lowered for the polymer-bound flavins, and vary with copolymer composition (see *Table 2*). There appears to be a correlation between the lowering of quantum efficiency and the amount of quaternary ammoniumchloride ions present in the copolymers. Possibly the fluorescence is quenched by collisional contacts with these ions that are present at a relatively high concentration within the macromolecular domains. This also suggests that the flavin is not completely immobilized and shielded by binding to the polymer chains. Short-range interaction between two flavin molecules leading to mutual quenching, is not likely to occur, because (i) the flavin content is always very low and (ii) the polymer concentration is well below the concentration for mutual coil overlap.

Fluorescence life-times

Free flavins in water can very suitably be used as single life-time standards[16,17]. This is illustrated in *Figure 3a* for isoalloxazine showing the graphical presentation of experimental decay and the decay calculated with a single life-time of 4.04 ns. It can be observed that the decay is single exponential over four decades of intensity. On the other hand the fluorescence decay of polymer-bound flavins can be best described with a sum of three exponential terms (see *Figure 3b*). The fluorescence decay of the flavin-

Table 2 Fluorescence characteristics of polymer-bound flavins

Sample	λ_{max} (nm)	Relative quantum efficiencies	Fluorescence life-times						
			α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	$\langle\tau\rangle^a$ (ns)
Isoalloxazine	514	1.0	1.0	4.04					
1a	507	0.80	0.22	0.65	0.57	3.27	0.21	5.87	4.13
1b	510	0.61	0.41	0.13	0.39	2.42	0.20	4.96	3.63
1c	510	0.69	0.25	0.39	0.45	2.51	0.30	4.89	3.73
1d	510	0.47	0.27	0.51	0.43	2.00	0.29	4.15	3.09
1e	511	0.42	0.30	0.36	0.47	1.88	0.22	3.96	2.77
2	509	0.90	0.18	0.41	0.48	3.06	0.34	5.34	4.23
3	512	0.68	0.27	0.47	0.44	2.40	0.29	4.39	3.32

[a] Average life-time defined as $\langle\tau\rangle = \sum_{i=1}^{3} \alpha_i\tau_i^2 / \sum_{i=1}^{3} \alpha_i\tau_i$

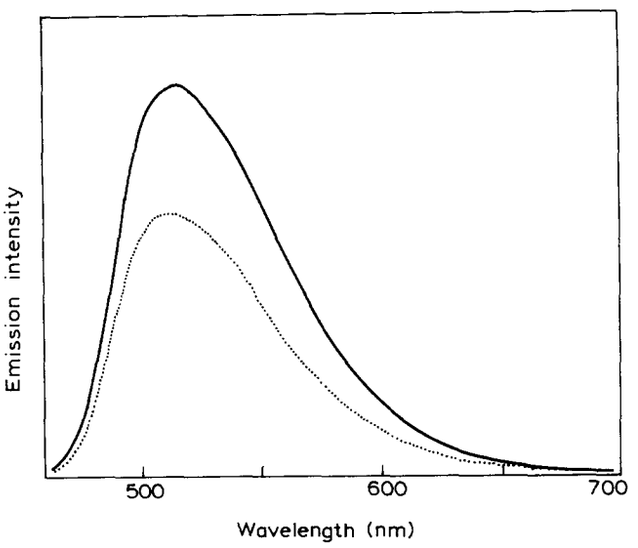

Figure 2 Fluorescence spectra of free isoalloxazine (—) in the presence of a styrene/vinylbenzylammoniumchloride copolymer (44 mol% quaternized units, $c = 0.5\,g \times dm^{-3}$, $[flavin] = 1.0 \times 10^{-5}$ M) and (.....) isoalloxazine covalently attached to this polymer, sample **1b** ($[flavin] = 1.1 \times 10^{-5}$ M)

copolymers is systematically analysed in 3 life-time components and relative amplitudes. The results are collected in *Table 2*. The life-time heterogeneity should be ascribed to excited state processes occurring on the same time scale as the excited state life-time. When the free flavin is dissolved in water reorientation of water molecules around the chromophore is much faster than the fluorescence, and fluorescence originates from a solvent relaxed state characterized by a single life-time. In the polymer coils the relaxation of solvent molecules is hindered causing a continuous change in microenvironment during the life-time of the flavin in the excited state. In this case analysis of the fluorescence decay into three exponential terms is an approximation for a life-time distribution. The average life-times of polymer-bound flavins, as given in *Table 2*, are qualitatively in line with the relative quantum efficiencies.

Fluorescence anisotropy decay

When absorbing a suitable wavelength, a molecule will behave like an electric dipole oscillator with a fixed orientation with respect to the geometry of the molecule ('absorption transition moment'). In the same way, the fluorescence emission of the molecule can be described as originating from an 'emission transition moment'. If a chromophore is excited with polarized light, the fluorescence is usually polarized parallel to the absorption, because normally the absorption and emission transition moments have the same direction. For a fixed single molecule the fluorescence intensity component parallel to the plane of polarization of the incident radiation (reference direction), $I_\parallel$ and that perpendicular to the reference direction, $I_\perp$, will vary with the angle between the reference direction and the direction of the optical transition moment. In a solid, fixed system the degree of polarization $P = \dfrac{I_\parallel - I_\perp}{I_\parallel + I_\perp}$ depends on the orientational distribution of the structural units, but not on time. If during the life-time of the excited state the fluorescent molecules undergo fast motions, all anisotropy introduced in the sample upon excitation is lost through the rotational relaxation prior to emission. If the fluorescence life-time and relaxation times are comparable, then partial relaxation will occur on the same time scale as emission of fluorescence, and thus analysis of the time-dependence of the fluorescence anisotropy can yield information about the rotational motion. If the $I_\parallel$ and $I_\perp$ are recorded as functions of time, it is possible to follow the decay of the emission anisotropy, as defined by

$$r(t) = \frac{I_\parallel(t) - I_\perp(t)}{I_\parallel(t) + 2I_\perp(t)}$$

From the experimental data rotational correlation times can be recovered after deconvoluting the anisotropy decay curves. Free isoalloxazine in water rotates very rapidly, characteristically in the picosecond time range, and a correlation time of about 60 ps was found (see *Figure 4a* and *Table 3*). This value is the lowest that can be resolved by the apparatus. To illustrate the latter point the laser pulse response function and the experimental fluorescence have been incorporated in *Figure 4a*.

The time-dependence of the fluorescence anisotropy of the polymer-bound flavins could not adequately be described with a single exponential function. Decay analysis with two correlation times yields much better results, as is demonstrated by an example in *Figure 4b*. The resulting correlation times from such analyses are shown in *Table 3*. For all samples the fitting procedure leads to a short (30–100 ps) and a long correlation time (1–10 ns). Before drawing conclusions, however, one has to realise that this mathematical analysis is just one way of describing the undoubtedly complex motions of the bound chromophore. The flavin can, for instance, rotate or oscillate around the methylene link to the polymer

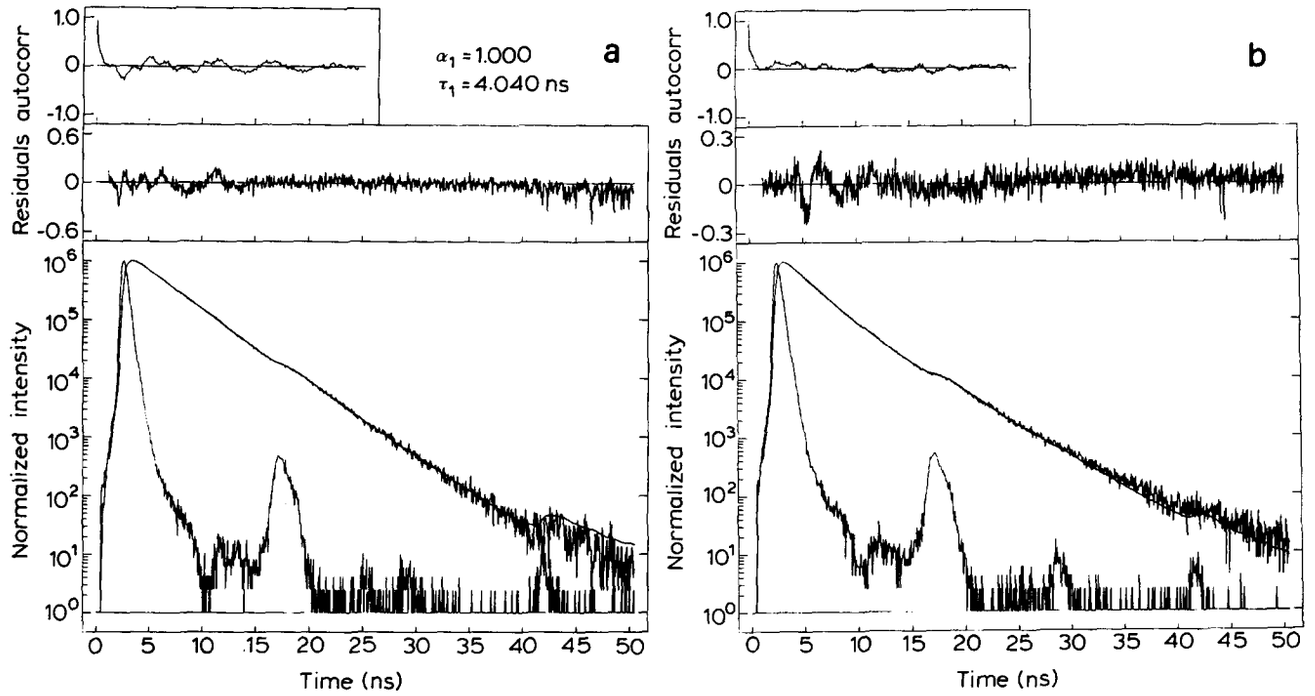

Figure 3 Fluorescence decay of free isoalloxazine (a) and sample **1b** (b). Shown are the laser excitation profile, the experimental $F(t)$ and the fitted fluorescence $F_c(t)$ composed of the convolution product of the laser pulse with either a single- (a) or triple-exponential function (b). The weighted residual values in each channel i are given by $[F_c(i)-F(i)] \times w(i)$, wherein the weighing factors $w(i)=1/F(i)$. Also shown is the autocorrelation function of the residuals. A good fit implies that residuals and autocorrelation function fluctuate around the base line. Life-times are collected in *Table 2*

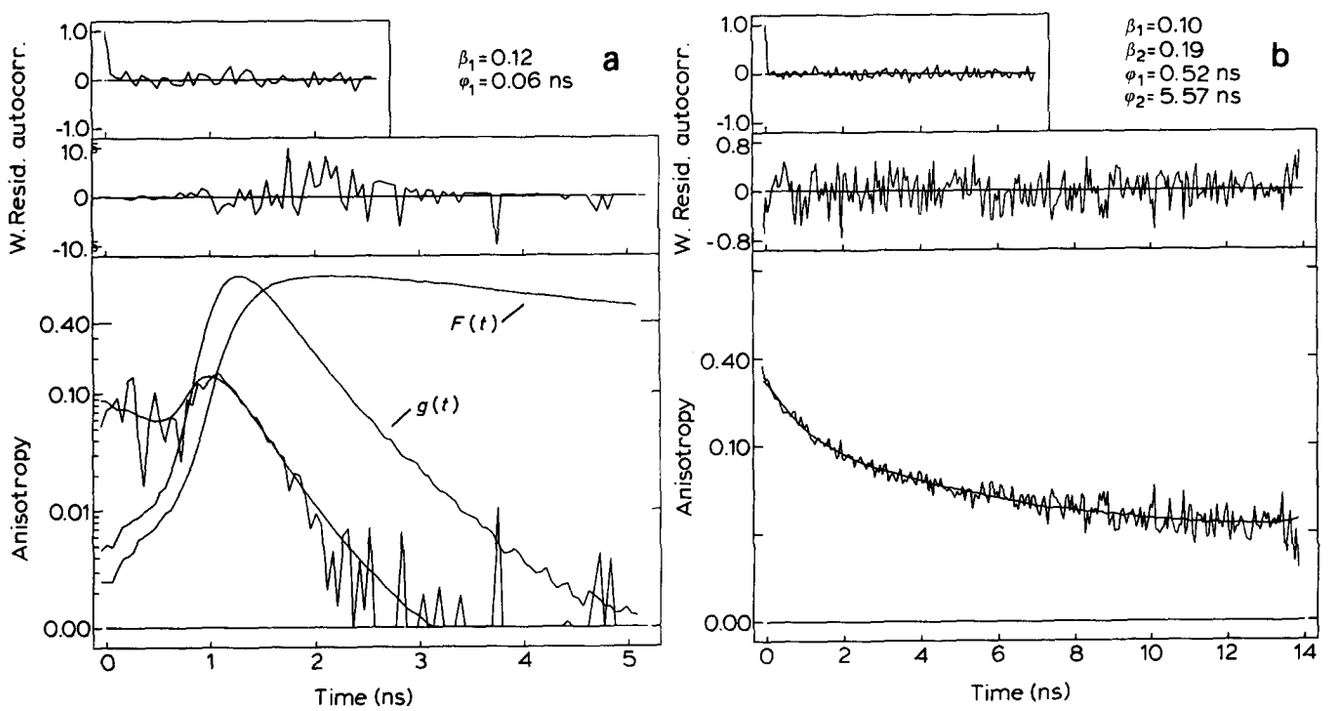

Figure 4 Anisotropy decay of free isoalloxazine (a) and sample **1b** (b). Shown are the experimental anisotropy $r(t)$ and the fit to a single- (a) or double-exponential function (b). Quality of the fits are determined by the course of weighted residuals and autocorrelation of the residuals (cf legend to *Figure 3*). Correlation times are collected in *Table 3*. In *Figure 4a* the exitation pulse $g(t)$ and the fluorescence $F(t)$ have also been incorporated

independent of the polymer chain. But on the other hand, the flavin will also take part in the continuous movements and relaxation processes of polymer chain segments and in rotations of whole polymer coils. All these processes will contribute to the overall motion of the flavin, so considerable cross-correlations will exist between the correlation times derived from the experimental curves.

At this time, we do not intend to give a quantitative explanation of the data in terms of polymer chain mobility and molecular dynamics[6,27,28]. Nevertheless, some qualitative conclusions can be drawn. The short correlation time may be an indication of fast movements of the flavin independent of the polymer. The longer correlation time should be associated with relaxation processes of polymer chain segments. The variation of this component Φ_2 with the composition of the copolymers

must then be interpreted as a variation in polymer chain mobility. The highest correlation time was found for **1a**, which is the most hydrophobic and least water-soluble polymer. Probably, the apolar parts in separate coils of this polyelectrolyte are aggregated in aqueous medium, while the ionic groups are exposed to the medium. By increasing the amount of charged monomer units the hydrophilicity increases and the polymer will be more solvated and less aggregated. In this way, the mobility of the polymer chain will, on the average, also increase. The decrease in the values of the correlation times Φ_2 going from the polymers **1a** to **1e**, i.e. an increase in mobility, is in accordance with this explanation.

Sample **3**, which is a terpolymer that consists for more than 85% of *N*-vinylpyrrolidone monomer units, has the highest chain mobility as shown by the smallest correlation time. This is understandable, since poly(vinylpyrrolidone) itself is a water-soluble polymer and will have less tendency to form intramolecular aggregates. The copolymer with methylmethacrylate monomer units (**2**) takes an intermediate position.

From the solubilization power for the water-insoluble dye Orange OT, and from the kinetics of 1-benzyl-1,4-dihydronicotinamide oxidation catalysed by this series of flavin-containing polymers, the same conclusion regarding the hydrophobicity of the polymers was reached[18].

Influence of medium composition

In previous publications we have reported on the strong influence of the composition of water/2-propanol mixed solvent on the rates of dihydronicotinamide oxidation catalysed by our copolymers **1**[18–20]. We showed that addition of 2-propanol diminishes hydrophobic interactions which are responsible for substrate enrichment and stabilization of a compact coil structure of the partially hydrophobic polyelectrolytes in a highly aqueous medium[19]. When the hydrophobic interactions are broken, electrostatic repulsion of the quaternary ammonium ions results in an expansion of the polyelectrolyte coils. This increase in polymer coil dimensions has been visualized by measuring the reduced viscosity of dilute solutions of a copolymer of styrene and vinylbenzyltriethylammoniumchloride, that has almost the same content of ionic groups (45 mol%) as **1b**, but that has no flavin groups and is of higher molar mass[19]. A relevant example of this type of experiments, performed under the same conditions as the measurements in *Tables 4* and *5*, is presented in *Figure 5*. The changes in coil dimensions can also be expected to result in variations of the mobility of the polymer chain, and to affect the fluorescence and anisotropy decay characteristics of the bound flavin[5,28].

The data in *Table 4* indicate that the composition of water/2-propanol mixtures has practically no effect on the quantum efficiencies of polymer **1b**. The average life-time decreases slightly for higher 2-propanol contents, whereas the single life-time of the free isoalloxazine of 4.04 ns in water was found to be 4.14 ns in a 50/50 water/2-propanol mixture.

The influence of medium composition on anisotropy decay, however, is more pronounced. The rotational correlation time of isoalloxazine increases from 60 ps in water to 130 ps in 50/50 water/2-propanol, which is in accordance with the higher viscosity of the mixture compared to water[19]. The long correlation time Φ_2 found for **1b** is significantly lowered upon addition of 2-propanol, which indicates faster movements of the flavin (see *Table 5*). In *Figure 6* we have plotted the reciprocal values of Φ_2 as a function of the medium composition. *Figure 6* clearly shows that the variation in Φ_2^{-1} parallels the changes in polymer coil dimensions depicted in *Figure 5*. These results support the idea that Φ_2 gives an indication of the mobility of the polymer chain (or part of the chain) to which the flavin chromophore has been attached.

Table 3 Correlation times of polymer-bound flavins from fluorescence anisotropy decay

Sample	β_1[a]	Φ_1 (ns)	β_2[a]	Φ_2 (ns)
Isoalloxazine	0.12	0.060	–	–
1a	0.042	0.07	0.27	8.30
1b	0.10	0.52	0.19	5.57
1c	0.04	0.38	0.24	3.69
1d	0.10	0.11	0.19	4.20
1e	0.09	0.12	0.20	3.76
2	0.27	0.03	0.03	3.04
3	0.09	0.06	0.15	1.33

[a] Pre-exponential factors

Table 5 Influence of medium composition on anisotropy characteristics of **1b**

Water/2-propanol v/v)	Correlation times					
	β_1	Φ_1 (ns)	β_2	Φ_2 (ns)		
100/0	0.10	0.52	0.19	5.57	(0.12	0.060)[a]
90/10	0.10	0.63	0.19	6.85		
80/20	0.06	0.43	0.22	4.39		
70/30	0.09	0.40	0.19	3.22		
60/40	0.07	0.45	0.22	2.34		
50/50	0.012	0.51	0.26	1.69	(0.18	0.130)[a]
30/70	0.024	0.89	0.26	1.82		

[a] Values for β and Φ observed for free isoalloxazine

Table 4 Influence of medium composition on fluorescence characteristics of **1b**

Water/2-propanol (v/v)	Relative quantum efficiencies	Fluorescence life-times						
		α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	$\langle\tau\rangle$ (ns)
100/0	0.61	0.41	0.13	0.39	2.42	0.20	4.96	3.63
90/10	0.66	0.33	0.16	0.45	2.57	0.22	5.06	3.71
80/20	0.65	0.26	0.21	0.48	2.52	0.26	4.89	3.66
70/30	0.67	0.39	0.13	0.38	2.48	0.23	4.55	3.50
60/40	0.67	0.27	0.15	0.43	2.31	0.30	4.26	3.35
50/50	0.65	0.37	0.12	0.44	2.47	0.19	4.42	3.24
30/70	0.71	0.17	0.19	0.63	2.49	0.20	4.49	3.17

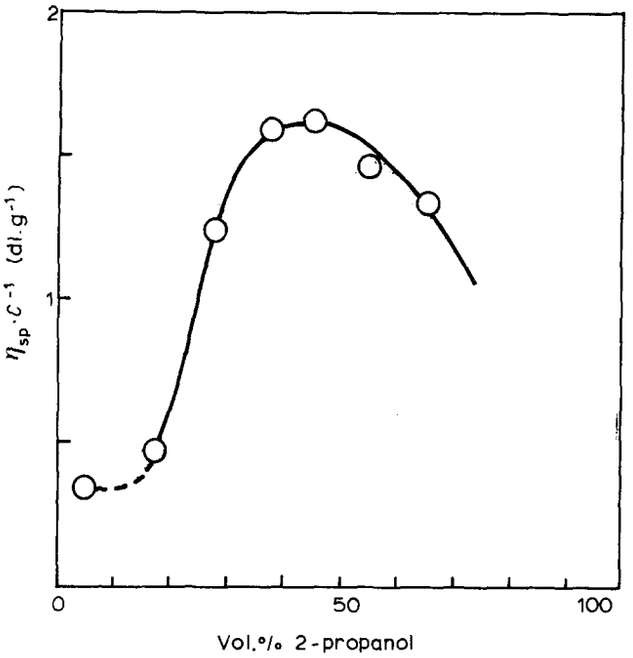

Figure 5 Effect of medium composition on the reduced viscosity of poly(styrene-co-vinylbenzyltriethylammoniumchloride) with 44 mol% quaternized units (polymer concentration 0.5 g dm^{-3}, pH 8.0, $I = 0.05$ M at 25.0°C)

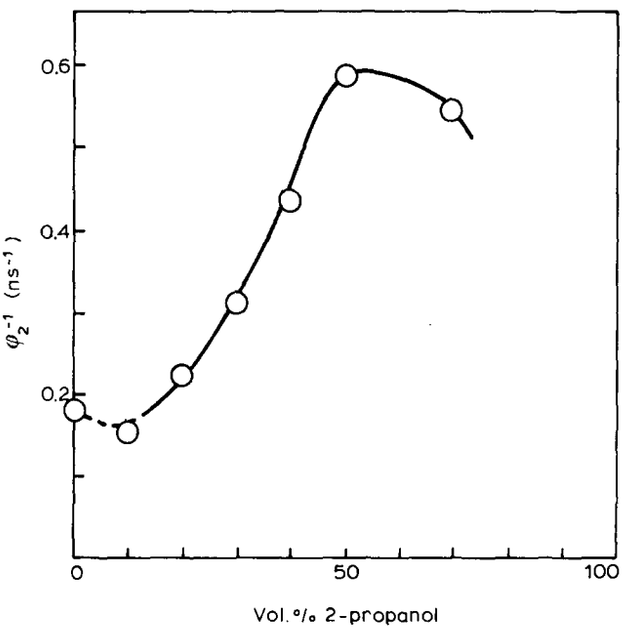

Figure 6 Variation of the reciprocal values of the long correlation time component (Φ_2^{-1}) of a two exponential fit of the fluorescence anisotropy decay observed for polymer **1b** as a function of the medium composition

CONCLUSIONS

The light absorption and emission spectra of flavin covalently attached to synthetic linear polymers gave an indication of the microenvironment of the flavin. From time-resolved fluorescence measurements, and more specifically fluorescence anisotropy decay characteristics, (qualitative) information could be obtained on the mobility of the probe and the polymer chain. In this way, we gained more insight into the effects of polymer composition on the mobility of the chain of some cationic polyelectrolytes. Also changes in polymer coil dimensions and mobility as a function of medium composition could be monitored with this technique.

ACKNOWLEDGEMENT

The authors would like to thank Mr A. van Hoek for his assistance to performing the fluorescence decay experiments.

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