Triazinylaniline (TA) Derivatives as Fluorescence Probes. Part 2.[1] Steady-state and Time-resolved Fluorescence Anisotropy Studies of TA Probes in Alkanols and in Small Unilamellar Vesicles of Phospholipids

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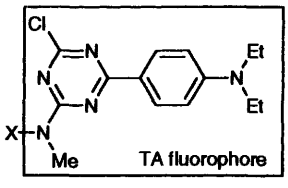
The fluorescence behaviour is reported of three functionalized triazinylaniline, TA, dye probes *p*-$Et_2C_6H_4C_3N_3(Cl)N(Me)X$, where X = C_4H_7, $C_{18}H_{37}$ and $CH_2[CHOH]_4CH_2OH$, solubilized in small unilamellar vesicles of dimyristoyl- and dipalmitoyl-L-α-phosphatidylcholines, DMPC and DPPC respectively, and of DMPC–DPPC binary mixtures.

Steady-state fluorescence yields (*ca.* 0.15) and anisotropies monitor the phospholipid phase transitions; the glucamine dye probe sensitively reflects the rearrangement of phospholipid head groups in the pre-transition region and probably resides in the vicinity of the glycerol backbone. Fluorescence decays of the probes are complex (mean lifetimes *ca.* 1.2 ns), but are not influenced strongly by the nature of the lipid phase.

For the vesicle-bound probes the fluorescence anisotropy decays rapidly (lifetime *ca.* 1 ns) from an initial high value (r = 0.30–0.32) to reach a plateau value (range 0.0–0.24) indicative of a restricted angular rotation of the probes which has been related to the nature of the probe tail; in alkanols the anistropic decay is monoexponential and the pseudo-isotropic rotation rate varies linearly with solvent viscosity.

The structural organization of biomembranes has been extensively studied by a plethora of techniques.[2–8] The relative importance of the various (inter)molecular interactions controlling the precise location, orientation and the dynamics of individual components in bilayer systems, continue to be explored in an effort to understand the functional properties of membranes *per se* and with their embedded proteins. Spectroscopic methods (*e.g.* NMR, EPR and optical fluorescence) applied to membranes, doped with suitably constructed probe molecules as necessary, have revealed useful details of the structural and diffusional–rotational constraints.[3–10]

We report here on the fluorescence characteristics in phospholipid unilamellar vesicles of three members of a new series of membrane probes [designated TAN(Me)Bu, TANMOD and TANGLUC] which are based on a common triazinylaniline fluorophore[1] to which a tail composed of a variety of other functionalities can be readily attached by easy synthetic steps. Steady-state and time-resolved fluorescence intensity and anisotropy measurements reveal the constraints on the location and mobility of the probes imposed by the lipid organization and the lipid-probe interactions.



X = Butyl	TAN(Me)Bu
X = Octadecyl	TANMOD
X = $CH_2[CH(OH)]_4CH_2OH$	TANGLUC

Experimental

Materials.—The triazinylaniline probes, prepared by reaction of *N*,*N*-diethyl-4-(4,6-dichloro-1,3,5-triazin-2-yl)aniline with the appropriate aliphatic amine, were purified and characterized as described previously.[1] The L-α-phosphatidylcholines, DMPC, DPPC and DSPC (99+% purity by TLC), were obtained from Sigma Chemicals and used as received.

Methods.—Preparation of small unilamellar vesicles (SUV).[7] A fresh solution of the required phospholipid (3 mg) dissolved in *ca.* 500 μl (1 μl = 1 mm^3) chloroform was evaporated under a stream of N_2 gas to form a film on the walls of a Pyrex test-tube; the gas flow was continued for a further 30 min to remove entrapped solvent. Aqueous Tris buffer (3 cm^3, typically at pH 8 and containing 150 mmol dm^{-3} KCl) was added and the solution purged with N_2 before vortexing briefly and sonicating in a Semat Ultrasonicator bath for 15 min at a temperature 10 °C above the appropriate gel–liquid crystalline transition temperature T_c. The vesicle solution was brought into contact with a film of probe dye (obtained by evaporation of a solution of the dye in acetonitrile of known concentration) and sonication was continued for a further 5 min. The dye-loaded SUV solution, after centrifuging and filtering, was thermally recycled several times between T_c ± 10 °C before use in the fluorescence studies. The molar ratio of probe to phospholipid was kept below 1:500 *i.e.* < six dye molecules per small unilamellar vesicle.

The background fluorescence of TA dye solutions prepared as above, but lacking phospholipid, was < 0.5% of that found for solutions of dye-loaded SUVs, thus confirming the high incorporation of the dyes in the lipid membranes.

Steady-state Fluorescence.—Spectra were measured on a Perkin-Elmer MPF-44B fluorometer for solutions in thermostatted 10 × 10 mm quartz cuvettes of absorbance < 0.06 at the excitation wavelength (360 nm) with excitation and emission bandwidths of 4 nm. For quantum yield determinations, emission spectra of the probes were corrected by, and referenced to, quinine bisulfate emission (Φ_F = 0.54).

The fluorescence intensity at a fixed wavelength, *e.g.* the peak at *ca.* 405 nm, and the fluorescence polarization were monitored

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as a function of increasing or decreasing temperature using a custom-built accessory to the MPF-44B fluorometer controlled by a BBC model B microcomputer. The sample was excited by plane-polarized UV light using a Glan–Thompson prism polarizer and the emission intensities for parallel and perpendicular orientations of an analysing polarizer were measured each over 10–20 s in a sequence including a dark (baseline) period. The mean temperature of the sample was simultaneously recorded using a calibrated thermocouple; by a minor interpolation procedure the fluorescence polarization at a given temperature was computed. A temperature range of typically 30 °C was scanned over a 2 h period to allow adequate time for the equilibria to be established. No thermal hysteresis effects were observed.

Fluorescence Lifetimes and Anistropy Decays.—All data were obtained using the facilities at the HA12 port of the synchrotron radiation source at SERC, Daresbury.[11] The highly polarized excitation pulse (intrinsic half width *ca.* 200 ps) in the single bunch mode (repetition every 320 ns) was passed through a Spex 1500 SP Czerny-Turner monochromator with a band pass of 2 nm and through a filter polarizer. For sample excitation a wavelength of 360 nm was chosen; since the synchrotron radiation profile is independent of wavelength the excitation pulse profile, including the detection system response, was obtained at a wavelength of 410 nm (probe dye fluorescence maxima) using a ground glass scatterer, thus avoiding the errors due to wavelength-dependent transit time spread in the photomultiplier tube.[12]

Filtered emission after passage through an analysing polarizer and an 8 nm bandpass or cut-off filter (see tables), was detected by a Mullard XP2020 photomultiplier tube with conventional single photon counting electronics. Counting was performed for 30 s periods alternately for each polarizer position; sample peak counts of 100 000 were accumulated and buffer blank solutions were counted for equal times. The data were analysed using the in-house deconvolution programs FLUORFIT and FLUORROT without recourse to time shift corrections.[13]

Results

TAN(Me)Bu in Alkanol Solvents.—This triazinylaniline dye is representative of the electronic character of the series of fluorescent TA probes considered here. In the primary alcohol solvents at 20 °C for excitation at 360 nm, the fluorescence of this probe dye decays monoexponentially; the lifetimes (Table 1) correlate linearly with the macroscopic relative permittivity of the alcohol, following the trend previously established for such TA dyes in a variety of solvents.[1]

The fluorescence anisotropy decay is also found to be monoexponential (Fig. 1) under these conditions. The rotational correlation times are directly proportional to the solvent viscosity (Table 1; the appropriate plot has a gradient of 115 ps cP^{-1} from which an effective volume for isotropic rotation of the dye of 0.473 nm^3 can be deduced).

Thus the molecular tumbling of TAN(Me)Bu in these homogeneous protic solvents is pseudo-isotropic. The fluorescence anisotropy $r(t)$ decays towards zero from an initial measured value of 0.30–0.32; the steady-state fluorescence anistropy of the dye in glycerol solvent, approximating closely to the limiting anisotropy r_0 in the absence of molecular rotation, was determined to be 0.34. The angle α between the absorption and emission transition dipoles is thus small (18°),[14] as expected from the intramolecular charge-transfer (ICT) nature of the electronic transition and the relative location of electron donor and acceptor moieties in the molecular framework.

Table 1 Fluorescence and rotational correlation times for TAN(Me)Bu in alkanols at 293 K

Solvent	η^a/cP	K_r^b	Φ_F/ns	Φ_R/ns	r_0
Butan-1-ol	2.72	17.5	0.31	0.28	0.320
Hexan-1-ol	4.75	13.2	0.70	0.57	0.314
Octan-1-ol	9.30	10.2	0.91	1.04	0.303
Decan-1-ol	12.2	8.1	1.10	1.46	0.296

[a] η is the solvent macroviscosity. [b] K_r is the relative permittivity of the solvent.

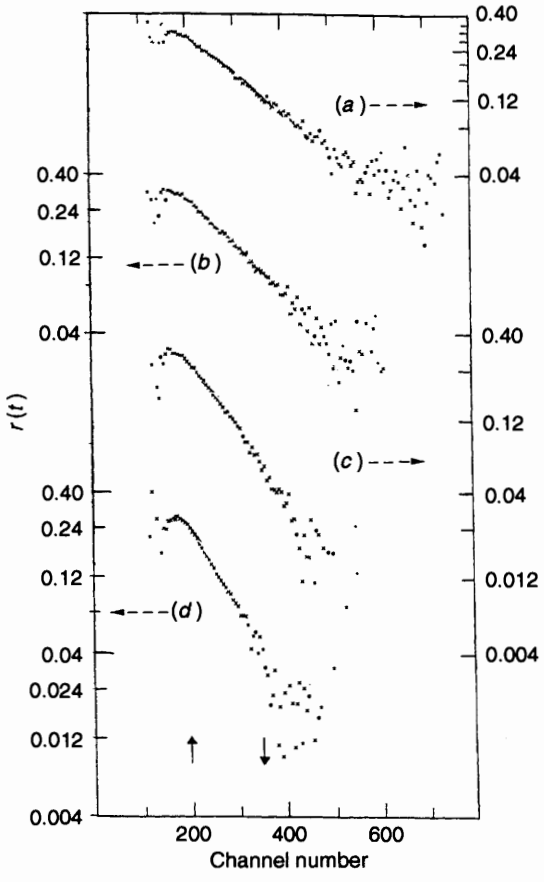

Fig. 1 Fluorescence anisotropy decays for TAN(Me)Bu in (*a*) decan-1-ol, (*b*) octan-1-ol, (*c*) hexan-1-ol and (*d*) butan-1-ol, at 20 °C. Time-scale = 10.1 ps $channel^{-1}$. The upward and downward arrows indicate the time points of rise and decay, respectively, at which the excitation pulse was 1% of its maximum intensity.

TA Probe Dyes in DPPC, DMPC and mixed DPPC–DMPC Small Unilamellar Vesicles.—Steady-state fluorescence. (*a*) Fluorescence intensity (I_F) as a function of temperature. The dyes TAN(Me)Bu, TANMOD and TANGLUC solubilized in the bilayer of small unilamellar vesicles (SUVs) of DMPC and of DPPC show strong fluorescence with emission maxima in the range 398–405 nm (Table 2) indicative of a polarity of a microenvironment akin to that of hexan-1-ol.[1] The variations in the fluorescence yields of the dye probes in DPPC vesicles with temperature show significant features (Fig. 2) related to the functionality of the 'tail' attached to the common TA fluorophore.

For TAN(Me)Bu and TANMOD, which possess alkyl chains of four and 18 carbon atoms, respectively, a general decline in the fluorescence intensity with increasing temperature occurs which becomes more pronounced above the main gel-to-liquid-crystalline phase-transition temperature (T_c) of the phospholipid bilayer.[15,16] A very small rise in intensity precedes this transition temperature for these two dyes. This latter feature

Table 2 Emission properties of triazinylaniline dye probes in small unilamellar vesicles of phosphatidylcholines

Probe	Phospholipid	T/K	λ_{em}/nm	Φ_F
TAN(Me)Bu	DMPC	283	400	0.155
		323	399	
	DPPC	293	405	
		323	404	
TANMOD	DMPC	283	399	0.16
		323	398	
	DPPC	293	402	
		323	401	
	DSPC	293	401	
		343	400	
TANGLUC	DMPC	283	405	0.14
		323	403	
	DPPC	293	407	
		323	405	

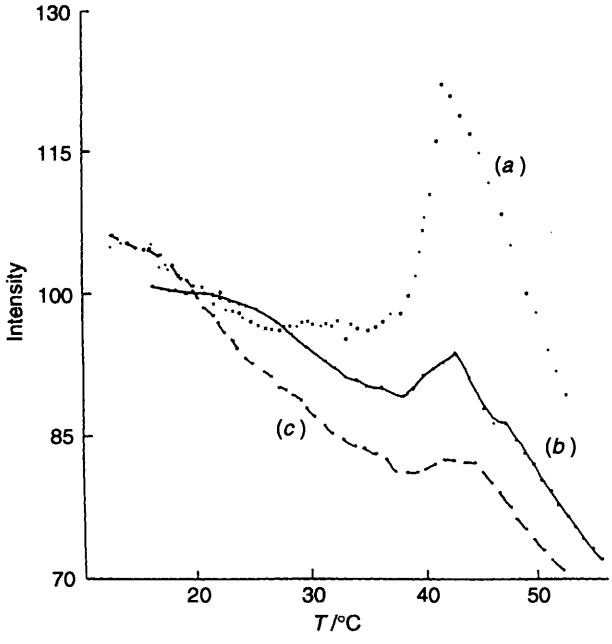

Fig. 2 Relative fluorescence intensities, individually normalized to 20 °C, of (*a*) TANGLUC, (*b*) TAN(Me)Bu, (*c*) TANMOD in small unilamellar vesicles of DPPC in aqueous Tris buffer, pH 8, as a function of temperature

is greatly enhanced in the case of TANGLUC, which possesses an hydrophilic glucosamine tail. A significant rise in the fluorescence intensity commences in the region of the expected pre-transition temperature (T_p) at which a rearrangement of the phosphatidyl head groups without a change in the hydrocarbon acyl chain packing is believed to occur.[17]

For the purpose of data analysis, as given in Table 3, T_p was taken to be the temperature at the intersection point of the line following the general decline in I_F and the line of steepest slope in the ascent immediately prior to the (local) maximum I_F (Fig. 2). The intersection of this latter line and the line of steepest decline in I_F of the dye in the liquid-crystalline phase region of the phospholipid bilayer is believed to give T_c.

The observed variations in I_F were reproducible and reversible, *i.e.* no hysteresis effects on heating or cooling were found, and thus may not be ascribed to alterations in the size distribution or aggregation of the SUVs or to a redistribution of the dye between different sets of vesicles.[7] The general patterns of behaviour found for DPPC vesicles were repeated in the cases of DMPC and mixed DMPC–DPPC vesicles for TANGLUC and TAN(Me)Bu; some estimated transition temperatures are given in Table 3.

Table 3 Characteristic transition temperatures of phospholipid bilayers (SUV) as monitored by the steady-state fluorescence intensity (I_F) and anisotropy (r) profiles of TA dye probes

		Transition temperature[a]			
		[T_p/K]		[T_c/K]	
Probe	Phospholipid	I_F	r	I_F	r
TAN(Me)Bu	DMPC	291.0	291.2	295.4	298.1
TANMOD		289.5	290.1	296.0	296.7
TANGLUC		287.2	290.6	298.2	299.0
$TAN(Me)_2$	DPPC	310.2	311.0	315.2	315.7
TAN(Me)Bu		310.2	309.9	316.0	316.2
TANMOD		309.8	309.7	315.7	316.7
TANGLUC		310.3	310.2	315.2	315.2
TANMOD	DSPC	323.2		328.2	

[a] Calorimetric measurements of multi-compartment PC vesicles in dilute aqueous media (summarized in ref. 7, p. 136) gave values for T_p, T_c of 287.4, 297.1 [DMPC]; 308.2, 315.0 [DPPC]; 322.3, 328.1 K [DSPC], respectively. Raman spectroscopic studies (ref. 16) yielded T_c values of 297.0, 314.5, and 326.7 K for DMPC, DPPC and DSPC, respectively. T_c values of 296.4, 313.7 and 327.2 K for DMPC, DPPC and DSPC, respectively, were obtained from EPR spin probe partitioning data (E. J. Shimshick and H. M. McConnell, *Biochemistry*, 1973, **12**, 2351).

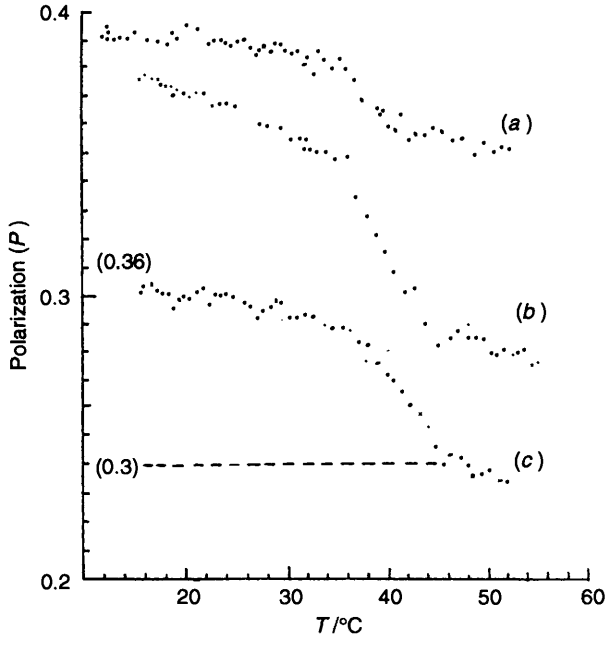

Fig. 3 Fluorescence polarization P of (*a*) TANGLUC, (*b*) TAN(Me)-Bu, (*c*) TANMOD in small unilamellar vesicles of DPPC in aqueous Tris buffer, pH 8, as a function of temperature. Curve (*c*) has been displaced 0.06 units for clarity.

(*b*) Fluorescence anisotropy as a function of temperature. In DPPC vesicles the three probe dyes exhibited high polarization of fluorescence (P 0.39–0.29, equivalent to anisotropy r values of 0.30–0.21) which varied with temperature in a sigmoidal manner (Fig. 3). A major part of the drop in polarization occurs between the expected pre-transition and main gel–liquid crystalline transition temperatures of the DPPC vesicle bilayer; the fall was less for TANGLUC than for TANMOD or TAN(Me)Bu. T_p and T_c values reported in Table 3 were estimated from the intersections of the appropriate tangents to the curve of fluorescence polarization (anisotropy) *vs.* temperature. Apparent transition (inflexion) temperatures for TAN(Me)Bu in SUVs prepared from mixtures of DMPC and DPPC are given in Table 4.

Table 4 Phase transition temperatures of DMPC–DPPC mixed bilayers (SUV) obtained from fluorescence anisotropy profiles

Mol% DMPC	Transition temperature [T_p/K] TAN(Me)$_2$	[T_p/K] TANGLUC	[T_c/K] TAN(Me)$_2$	[T_c/K] TANGLUC
100	291.2	290.6	298.1	299.0
75	299.1	299.1	302.7	302.8
50	299.9	300.7	309.2	308.5
25	301.4	302.2	311.4	311.2
0	309.9	310.2	316.2	315.2

Table 5 Fluorescence lifetime data of TA probes in DPPC and mixed DPPC–DMPC vesicle bilayers

System	T/K	τ_1/ns	W_1(%)[a]	τ_2/ns	W_2(%)[a]	Mean τ/ns
A[b]	293.9	0.83	64	2.07	36	1.28
	297.2	0.67	38	1.73	61	1.32
	309.8	0.30	65	1.57	35	0.94
	323.6	0.78	43	1.33	57	1.09
B[c]	293.0	0.51	18	1.71	82	1.49
	303.2			1.42	>99	1.42
	322.8	0.20	7	1.02	93	0.96
C[d]	293.0	0.43	22	1.67	78	1.40
	303.2	1.09	15	2.00	85	1.86
	312.0	0.94	54	1.56	46	1.22
	322.8	0.40	17	1.10	83	0.98
D380[e]	298.6	0.49	39	1.71	61	1.23
	309.8	0.78	58	1.78	42	1.20
	323.6	0.43	46	1.24	54	0.93
D405[e]	293.9	0.51	38	1.60	62	1.19
	313.7	0.63	44	1.42	56	1.07
D436[e]	293.9	0.65	53	1.78	47	1.18
	310.7	0.95	81	2.19	19	1.17
	313.7	0.92	89	2.07	11	1.05
	323.2	0.78	89	1.84	11	0.90
E[f]	298.6	0.56	28	1.88	72	1.51
	309.8	1.05	57	2.10	43	1.52
	323.6	0.13	13	1.20	87	1.06

[a] W_1 and W_2 are fractional intensities for lifetimes τ_1 and τ_2. [b] A = TAN(Me)Bu in DPPC unilamellar vesicles. [c] B = TAN(Me)Bu in DMPC. [d] C = TAN(Me)Bu in 1:1 DMPC–DPPC. [e] D380, D405 and D436 = TANGLUC in DPPC with the probe emission filtered by a 380 nm cutoff filter, a 405 nm interference filter (8 nm bandpass), and a 436 nm interference filter (8 nm bandpass), respectively. [f] E = TANMOD in DPPC.

For TANGLUC in such mixed lipid vesicles (data not shown) a small ill resolved decline in polarization with increasing temperature was found; a gradual shift of the curve along the temperature axis as the proportion of DPPC increased (while maintaining an approximately constant slope) was observed, similar to that observed for TAN(Me)Bu. These polarization data were not utilized since the variation in the fluorescence intensity with temperature provided a clearer guide in this instance.

Time-resolved fluorescence. (*a*) Fluorescence decay times. The fluorescence decays of the three dye probes in DPPC, DMPC and mixed DMPC–DPPC vesicle bilayers were measured over the temperature range 20–50 °C (Fig. 4 provides typical excitation and decay curves). In all cases the decay, derived from the appropriate sum of the parallel and perpendicular polarized intensities, could not be adequately fitted by a single exponential, in contrast to the dye TAN(Me)Bu in homogeneous alcohol solvents. Table 5 records the results of data analysis on the basis of two lifetime components. The chi squared values for monoexponential fits were at least three times larger than those for two exponential fits. Inclusion of a third lifetime component gave a marginal improvement in the data fits, mainly by way of a very short lifetime (< 100 ps), low weighted (< 5%) fractional contribution which may represent a small scattered light contribution. Since the data had been acquired under collection conditions chosen to optimize the error statistics for the anisotropy decay measurements, the two lifetime fits for the fluorescence decays were deemed to be adequate for that task.

The mean fluorescence lifetimes (see Table 5) were similar for all the dyes; the decrease in mean lifetimes with rise in temperature was relatively small. For TANGLUC probe dye in DPPC vesicles no systematic trends in the fluorescence lifetimes were discernible using a limited selection of cut-off and narrow band pass filters (entries D380, D405 and D436 of Table 5).

(*b*) Fluorescence anisotropy decays. Good anisotropy decay profiles were obtained for most samples from a time corresponding to the rising edge of the excitation pulse (*e.g.* 1% of the maximum pulse counts) to 15 ns after the pulse (representing *ca.* 10 mean fluorescence lifetimes). Figs. 5 and 6 are representative of the range of decay type.

The anisotropy decays were fitted very satisfactorily by a single rotational correlation time Φ_R with residual anisotropy r_∞ at infinite time according to eqn. (1). Experimentally (see

$$r_t = r_\infty + a\exp(-t/\Phi_R) \quad (1)$$

Table 6), the limiting anisotropy at zero time, r_0', was *ca.* 0.30 ± 0.02, being slightly below the value of 0.34 found from steady-state fluorescence measurements on the dyes in glycerol solvent. The r_∞ values range from 0–0.24 and are much more sensitive to temperature than the recovered rotational correlation times,[18] which are typically *ca.* 1 ns.

The anisotropy decay parameters r_∞ and Φ_R were interpreted on the basis of a wobbling-in-the-cone model.[19,20] The derived maximum cone angle of wobble from an axis perpendicular to the bilayer surface, θ_{max}, and the diffusional coefficient, D_w, were evaluated for the bilayer membrane-bound triazinylaniline probes as a function of temperature (Table 6). Apparent activation energies for diffusional wobbling in the cone were obtained from the gradients of log D_w *vs.* T^{-1} plots (Fig. 7).

Discussion

TA Probes in Homogeneous Solvents.—The general photophysical characteristics of the TA dye probes in homogeneous solutions have been discussed previously.[1] The present study on TAN(Me)Bu in neat alkanol solvents confirms the single exponential decay of fluorescence and reveals also the single exponential decay time of the fluorescence anisotropy, proportional to solvent viscosity, which is indicative of pseudo-isotropic rotation of the probe in such media. The high r_0 value (> 0.32) of the TA fluorophore is a useful feature of these TA probes.

By contrast, in vesicle bilayers the form of the anisotropy decay indicates major restrictions on the rotational freedom of all the probes examined, the fluorescence decay is multi-exponential and the fluorescence parameters are very responsive to changes in the physical state of the phospholipid bilayer.

Location of TA Probes in the Lipid Bilayers.—The nature and range of location of the TA probes in the bilayer of the small unilamellar phospholipid vesicles can be circumscribed from several lines of evidence.

The observed quantum yields of fluorescence ($\Phi_F \approx 0.15$, at 293 K) and the wavelength of the emission maximum (*ca.* 400 nm) reveal clearly the moderately low polarity of the average microenvironment around the probe dyes which, by comparison with the results for homogeneous solvents,[1] is 'hexanol-like'

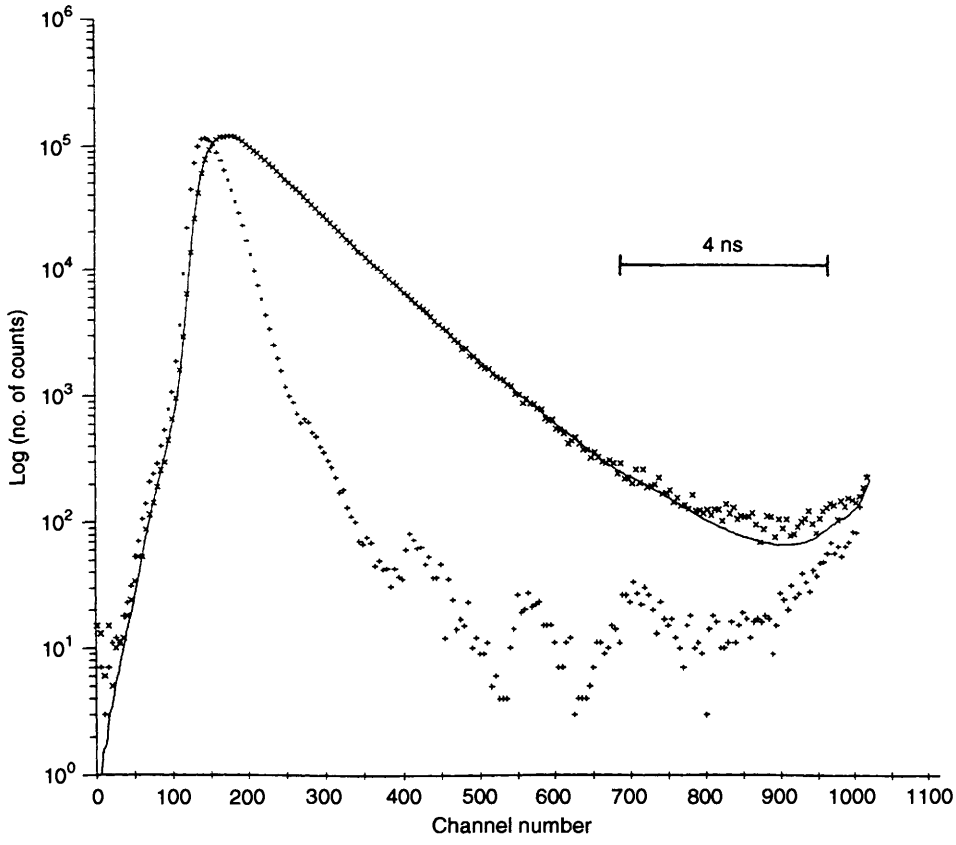

Fig. 4 Excitation pulse and emission curve of TAN(Me)Bu in DMPC small unilamellar vesicles at 50 °C (Tris buffer pH 8). Timescale = 14.4 ps channel^{-1}. The solid curve indicates a two lifetime fit.

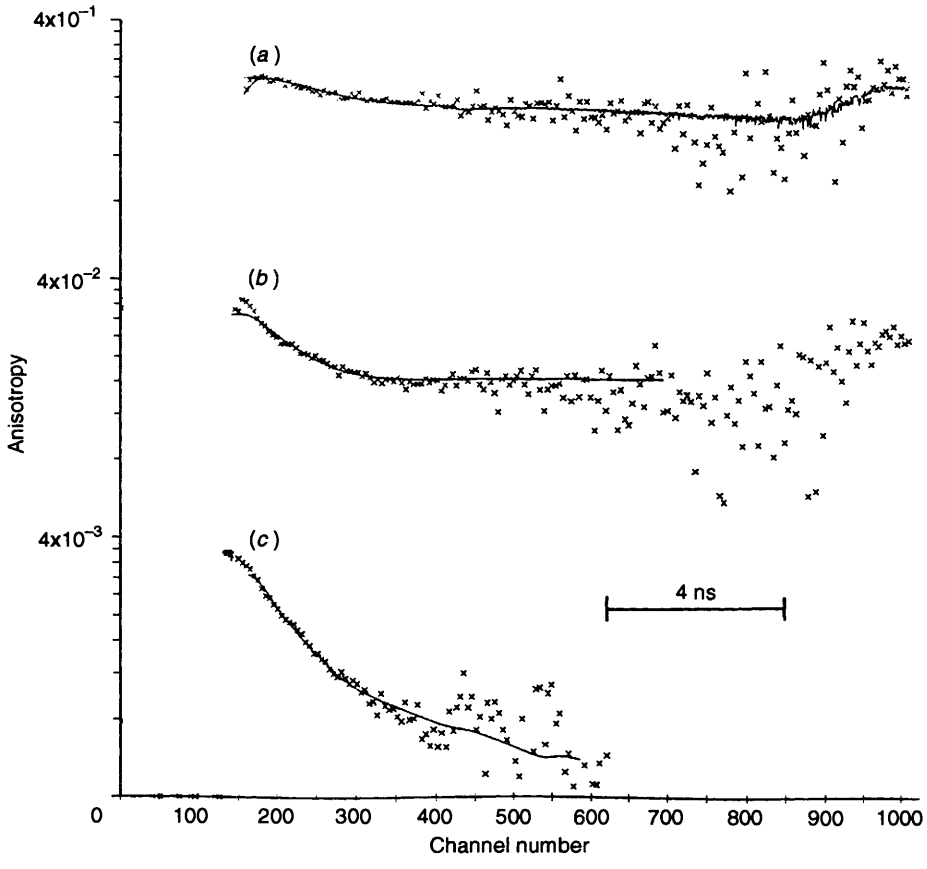

Fig. 5 Fluorescence anisotropy decay curves of TANMOD in DPPC small unilamellar vesicles at (*a*) 25, (*b*) 36.6 and (*c*) 50.0 °C (Tris buffer, pH 8). Timescale = 16.1 ps channel^{-1}.

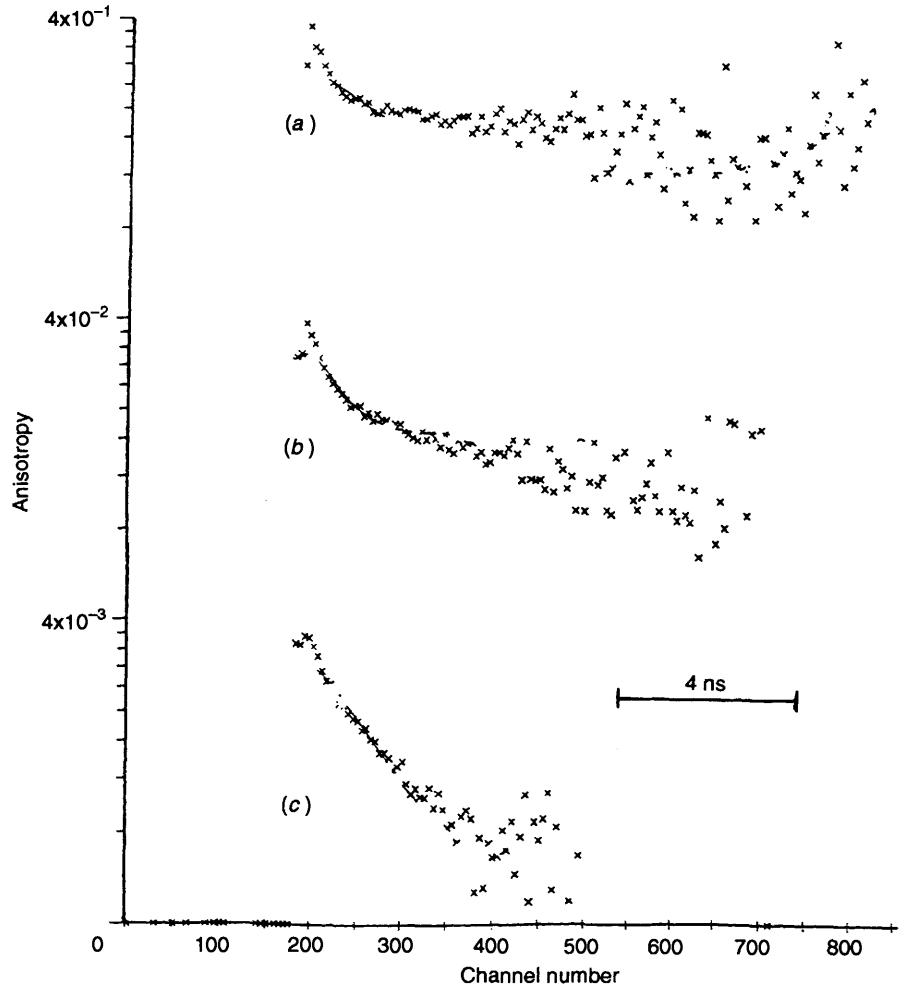

Fig. 6 Fluorescence anisotropy decay curves of TANGLUC in DPPC small unilamellar vesicles at (*a*) 20.7, (*b*) 37.5 and (*c*) 50.0 °C (Tris buffer, pH 8). Timescale = 20.1 ps channel^{-1}.

Table 6 Fluorescence anisotropy decay data for TA probes in phospholipid unilamellar vesicles and derived parameters using the wobbling-in-a-cone model

		Anistropy decay			Cone model	
System	*T*/K	r_0' [a]	r_∞	Φ_R/ns	φ_{max}	D_w/ns^{-1}
A [b]	293.9	0.30	0.123	1.32	46	0.116
	298.6	0.31	0.165	1.14	40	0.107
	309.8	0.33	0.115	0.76	48	0.220
	323.6	0.32	0.028	0.54	66	0.441
B [c]	293.0	0.30	0.104	1.07	49	0.155
	303.2	0.28	0.059	1.03	58	0.200
	322.8	0.29	0.004	0.96	80	0.270
C [d]	293.0	0.28	0.120	1.05	47	0.146
	303.2	0.29	0.078	1.02	54	0.186
	312.0	0.30	0.057	0.81	59	0.257
	322.8	0.28	0.017	0.82	71	0.299
D380 [e]	298.6	— [g]	0.240	1.21	30	0.066
	309.8	— [g]	0.168	1.16	40	0.107
	323.6	— [g]	0.091	0.90	52	0.202
D405 [e]	293.9	— [g]	0.190		37	
	313.7	— [g]	0.120	0.99	47	0.159
D436 [e]	293.9	— [g]	0.185	1.04	37	0.106
	310.7	— [g]	0.120		47	
	313.7	— [g]	0.110	1.26	48	0.129
	323.2	— [g]	0.080	1.04	53	0.179
E [f]	298.6	0.26	0.192	1.24	36	0.085
	309.8	0.33	0.162	0.64	41	0.197
	323.6	0.34	0.060	0.39	58	0.526

[a] r_0' is the fluorescence anisotropy at the start of the excitation pulse, estimated by back-extrapolation of the log $r(t)$ data curve. [b–f] See Table 5. [g] Values in the range 0.32 to 0.36 but some problems in extrapolation due to light scattering effects at very short times.

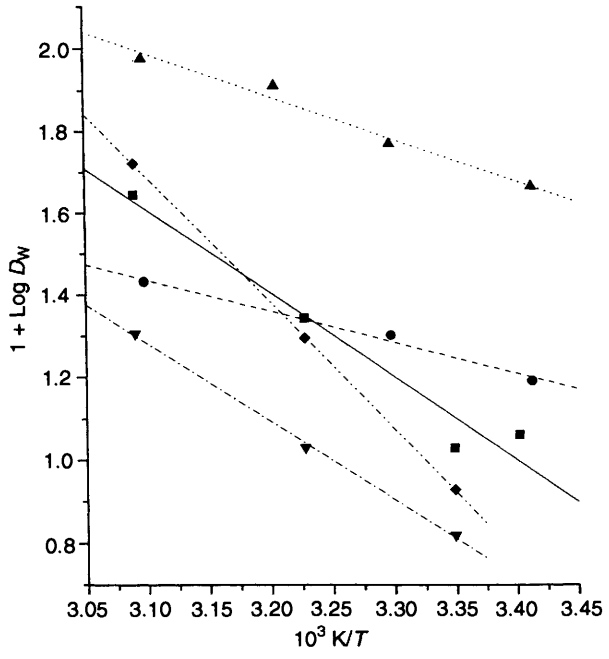

Fig. 7 Arrhenius plot for wobbling diffusion coefficient D_w of TA probes in phospholipid small unilamellar vesicles in Tris buffer, pH 8. (▼) TANGLUC in DPPC [D380]; (●) TAN(Me)Bu in DMPC [B]; (■) TAN(Me)Bu in DPPC [A]; (◆) TANMOD in DPPC [E]; (▲) TAN(Me)Bu in 1:1 mol ratio DMPC:DPPC [C]. (Symbols in [ ] give the corresponding entry in Table 6.)

(K_r = 13.2 at 293 K). The quantum yield of fluorescence of the dyes in aqueous buffer, pH 4–11, is very low (< 0.005) and can be neglected here.

The steady-state fluorescence anisotropy, and to a lesser degree the fluorescence intensity (dependent on the probe tail), undergo significant alterations in the region of the pre-transition temperature[6] of the phospholipid bilayer. Thus these TA probes sense changes in packing of the phosphatidylcholine head groups which lie roughly parallel[21,22] to the undulating[23] membrane surface of the P phase. An average location of the TA fluorophore close to the glyceryl section of the phospholipid molecules is indicated, as found for *N*-dansylhexadecylamine,[23] which is neither highly exposed to the aqueous phase nor buried deep among the hydrocarbon chains of the lipids. The depth in the bilayer and the precise situation is dependent on the functionality of the probe and the temperature, as discussed later.

The uniqueness of site location and depth in the bilayer of various probe fluorophores and the relationship of fluorescence lifetime heterogeneity to a spectrum of environments has been examined by several workers.[24–26] The evidence from limited fluorescence lifetime analysis for the TA probes is equivocal. The fitting of the fluorescence decays required the use of at least two exponentials, indicating a distribution of probe environments, or a single site with time-varying incursions of aqueous phase. However, the fluorescence decay of TA dyes in homogeneous solutions is very sensitive to solvent polarity and further, in aqueous solutions, an equilibrium has been deduced to exist between the free dye and the dye directly hydrogen-bonded at its anilino nitrogen centre to water.[1] A complex distribution of fluorescence lifetimes is to be expected, therefore, on the basis of these factors, for a TA probe residing inside a lipid membrane but still partially accessible to water. Efforts were made in the representative case of TANGLUC to compare the fluorescence decays as measured for the whole emission band (380 nm cut-off filter), the central portion of the emission (405 nm interference filter) and the red edge of the band (436 nm interference filter). At a given temperature no significant differences in mean fluorescence lifetimes were found, but the weighting of the shorter lifetime component, for two lifetime fits, was generally higher for the red-edge emission data. This latter trend may be an artefact, however, and reflect the instabilities in fitting complex decays with only two lifetimes. The mean lifetime analyses should be much more reliable in reflecting real trends.

The anisotropy decay data is more useful in delineating applicable models for the probe milieu. The observed anisotropy $r(t)$ decay curve for a variety of conditions (of temperature, nature of probe, vesicle lipid composition) consists of a rapid initial depolarization over 1–2 ns followed by a plateau of residual polarization persisting for at least 15–30 ns as far as can be ascertained. Consider a simple two-site model[9] for the TA probe dye in the vesicle bilayer in which species A and B are distinguishable by their limiting anisotropies, f_A and f_B, at time zero, their mean fluorescence decay times, τ_A and τ_B, and their individual rotational relaxational decay times, Φ_A and Φ_B, respectively. The experimentally observed functional form of $r(t)$ is achievable only in the following cases, as verified by simulations (numerical data not given here): (*i*) $\tau_A = \tau_B$, with $\Phi_B = \infty$, *i.e.* species B is immobile on any relevant timescale; (*ii*) A and B differ in fluorescence lifetime, but are equally constrained and have the same time course of rotational relaxation; (*iii*) only one site exists, *i.e.* species A only, with either a sharp value for fluorescence lifetime or a range of lifetimes and the probe undergoes constrained, angularly-restricted rotation.

Since the observed fluorescence lifetime decay is not mono-exponential, case (*i*) for the anisotropy behaviour does not apply and is excluded from the discussion. On the basis of our data we are unable to distinguish between cases (*ii*) and (*iii*). These represent at the molecular membrane level situations where either the probe resides at two or more specific sites with different exposures to water, but have similar constraints on the rotational movement orthogonal to the fluorophore dipole axis or the probe occupies a single depth-invariant site which experiences fluctuating incursions of water. Either choice provides a reasonable working hypothesis for interpretation of the rotational anisotropy decay data.

TA Probe Behaviour during Lipid Phase Transitions.—The probe TANGLUC, possessing several hydroxy groups in its functional tail capable of hydrogen bonding to the carbonyl and phosphate groups of the lipid head[27] and to water (and closely related to *N*-acetyl-D-glucosamine which is counter-transported in lysosomal membrane vesicles,[28] shows the highest sensitivity to the repacking of the lipid head groups in the pre-transition corrugated phase.

Significantly, the rise in fluorescence intensity at the onset of this pre-transition phase, which runs counter to the general mild decline expected on temperature increase, indicates a displacement of at least the TA fluorophoric head of the probe into a more hydrocarbon-like, lower polarity region of the bilayer when the phospholipid head groups rearrange. Above the main gel-to-liquid-crystalline phase transition the fluorescence intensity of the TANGLUC (and the other TA dyes) falls at a greater rate with increase in temperature than below T_c, and this fall is comparable to that seen for TA dyes in fluid homogeneous solvents. Thus the lipid bilayer forces appear to control the probe location in this instance rather than the probe seriously perturbing the bilayer packing and dynamics. Ekland *et al.*[29] have suggested that the conformation of phosphatidylcholine in the liquid-crystalline state is determined largely by intramolecular, rather than intermolecular, interactions even in the presence of other rigid and bulky components such as cholesterol.

The probes TAN(Me)Bu and TANMOD, having simple hydrocarbon tails of four and 18 carbon atoms attached to the TA fluorophore, must reside deeper in the bilayer than TANGLUC since (*i*) their fluorescence peaks are blue-shifted relative to that of TANGLUC (Table 2) and (*ii*) they signal the repacking of the PC head groups to a much lesser extent. Nevertheless, the temperatures at the bilayer transitions are the same as judged by all three probes (Table 3) and they each find reduced restriction on molecular rotation above the pre-transition temperature.[30]

Motion of the TA Probes in the Lipid Bilayer.—The dynamic behaviour of the probes can be compared using the wobbling-in-a-cone model for interpretation of the fluorescence anisotropy decay data (see Table 6). The appropriate defining equations[19] are eqn. (2) where φ_{max} is the maximum cone-angle-of-wobble, and eqn. (3). The single exponential anisotropy decay time is

$$A_\infty = r_\infty/r_0 = [(1/2)\cos\varphi_{max}(1 + \cos\varphi_{max})]^2 \quad (2)$$

$$D_w = \langle\sigma\rangle/\Phi_R = \text{wobbling diffusion constant} \quad (3)$$

equated here with Φ_R. The constant $\langle\sigma\rangle$ is a function of φ_{max} and A_∞ [values were estimated from Fig. 3(*b*) of the paper by Kinosita *et al.* (ref. 19)].

TAN(Me)Bu is less restricted in angular range and is more mobile in DMPC than in DPPC bilayers as expected. The apparent activation energies for diffusion-in-the-cone, E_a(diff.) are 20 and 45 kJ mol^{-1}, respectively. At 323 K, in the DMPC bilayer this probe undergoes nearly isotropic motion. The anchoring effect of the longer alkyl chain of TANMOD increases E_a(diff.) to 57 kJ mol^{-1} for the DPPC bilayer and the angular range is reduced at a given temperature compared with TAN(Me)Bu. Interestingly, while the angular confinement for TANGLUC in the DPPC bilayer is appreciably stronger than for the other probes, the diffusional motion exhibits a lower E_a(diff.) of 35 kJ mol^{-1}. By comparison, the well known fluorescent probe DPH exhibits values[31] of 29–33 kJ mol^{-1}.

TA Probes in Mixed Phospholipid Bilayers.—As monitored by the changes in the steady-state fluorescence intensities and polarizations with temperature of TAN(Me)Bu and TANGLUC, the transition points T_p and T_c (see Table 4) are almost linear functions of the mole fraction of DPPC in the mixed lipid DMPC–DPPC vesicles. Thus the two phospholipids form near ideal mixtures, being perfectly miscible in all proportions as expected on chemical grounds and demonstrated by other methods.[2,3]

The anisotropy decay parameters of TAN(Me)Bu in a 1:1 mole ratio bilayer of DMPC and DPPC (Table 6, entry C) are intermediate as expected in relation to those of the probe in pure DMPC and pure DPPC bilayers, when compared at equivalent temperatures, for the r_∞, cone angle φ_{max}, and to a lesser extent, D_w, variables. Surprisingly, the diffusional activation energy of 22 kJ mol^{-1} for in-cone-wobbling is, within experimental error, the same as that for pure DMPC bilayers suggesting that the probe itself is surrounded preferentially by DMPC molecules, *i.e.* a very local clustering effect around the probe. This could be rationalized on the grounds that, at a given temperature, the acyl chain regions of DMPC are conformationally more mobile and can accommodate more readily the packing demands of the bulky probe impurity. However, this may not be taken to imply the separate existence of DMPC and DPPC domains in the bulk of the bilayer, *i.e.* immiscibility of the phospholipids, contrary to all the other static and dynamic fluorescence evidence and the results using other methodologies. The above attempted explanation must remain tentative at present.

Conclusions

The new series of triazinylaniline fluorescent probes are sensitive reliable reporters of the state of phosphatidylcholine vesicle bilayers and their rotational anisotropies on a nanosecond timescale reveal some of the subtle preferential interactions which decide the location and constraints on movement of foreign molecules within lipid bilayer systems.

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