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SHORT COMMUNICATION

Using Time-Resolved Fluorescence Anisotropy of di-4-ANEPPDHQ and F2N12S to Analyze Lipid Packing Dynamics in Model Systems

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Abstract

The fluorescence probes di-4-ANEPPDHQ and F2N12S have solvochromatic emission spectra and fluorescence lifetimes that are sensitive to order within the environment of lipid membranes. We show in this communication that the time-resolved fluorescence anisotropy of these probes, analyzed either by the wobble-in-a-cone model or by the model-independent order parameter S^2, provides complementary information about dynamics and lipid packing in a variety of homogeneous lipid membranes systems.

Keywords di-4-ANEPPDHQ · F2N12S · Wobble-in-a-cone · Order parameter · Lipid packing · Lipid dynamics · Fluorescence anisotropy

Introduction

Our laboratory has been using the dye Di-4-ANEPPDHQ, developed by Loew and coworkers [1, 2], and the dye F2N12S, developed by Klymchenko, Mely and coworkers [3] (structures shown in Fig. 1) to investigate how cell membrane dynamics are affected by interaction with various small, lipophilic molecules and nanomaterials. Both probes are solvochromatic and tend to localize in the outer leaflet of lipid membranes [3–5].

Model membranes can exist in various states of order (Lo) or disorder (Ld); Di-4-ANEPPDHQ prefers the Ld phase, whereas F2N12S will partition into both lipid phases. Di-4-ANEPPDHQ has been used for neuronal activity work, because of its large fluorescence changes during action and synaptic potentials [4]. It has been shown that the emission spectra of Di-4-ANEPPDHQ will shift to longer wavelength as the environment in which the probe is located moves from Lo to Ld [6]. Similar to F2N12S, Di-4-ANEPPDHQ has been shown to have high-fluorescence quantum efficiency when incorporated in a membrane [7].

F2N12S is a 3-hydroxyflavone that can undergo excited-state intramolecular proton transfer (ESIPT) [5]. A ratiometric analysis of the F2N12S emission spectrum, performed by calculating a ratio of the short and the long wavelength intensities (I_{short}/I_{long}), has been previously used for imaging applications [3, 8]. This ratiometric analysis is usually performed on the usually observed dual peak emission, which is dominated by the normal (N*) and tautomer (T*) excited-state forms [5, 9]. A third excited-state form, the H-bonded normal form (H-N*) can exist when the probe is near the membrane surface, where it can form a hydrogen bonds with a water molecule in the surrounding aqueous environment [5]. This hydrogen bonding has been hypothesized to prevent the ESIPT reaction [10]. Fluorescence-detected linear dichroism data and a combination of quantum chemical calculations and molecular dynamics simulations together show that the dye has a single absorption transition dipole moment that is approximately parallel to the long axis of the aromatic system of the probe; in giant unilamellar vesicles the mean tilt angle of the transition dipole is nearly 50° with respect to the membrane normal [11, 12].

Cell membrane dynamics are regulated, in part, by lipid motions and lipid packing, and probes in the Lo phase are

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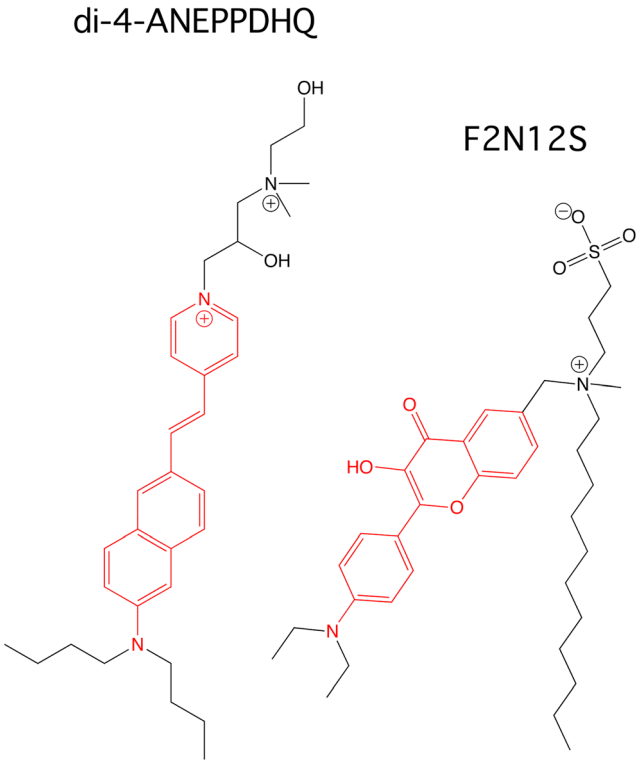

Fig. 1 Structure of Di-4-ANEPPDHQ and F2N12S. Structures were generated using ChemDraw Ultra (CambridgeSoft)

highly constrained compared to probes in the Ld phase. Thus, a probe in the Ld phase has greater freedom of motion. It has been shown that both Di-4-ANEPPDHQ and F2N12S are sensitive to local motions in membranes [6, 13, 14], and a direct way to determine local order or disorder is to measure the fluorescence anisotropy of the probe [15]. We show in this communication that analysis of the time-resolved fluorescence anisotropy of these two probes in terms of the wobble-in-a-cone model [16–18], or alternatively the order parameter (S^2) [19], provides a direct way to assess the degree of motional freedom of these probes in their local environment.

Theoretical Background

As discussed by Lakowicz [20], the time-resolved fluorescence anisotropy, $r(t)$, is given by

$$r(t) = \frac{I_{VV}(t) - I_{VH}(t)}{I_{VV}(t) + 2I_{VH}(t)} \quad (1)$$

where $I_{VV}(t)$ represents the vertical decay and $I_{VH}(t)$ represents the horizontal decay. Both emission decays are obtained from vertical excitation. The denominator of the above equation is the total intensity decay, $I(t)$,

$$I(t) = I_{VV}(t) + 2I_{VH}(t) = \sum_{i=1}^{n} \alpha_i e^{-t/\tau_i} \quad (2)$$

where τ_i is the lifetime and α_i is the amplitude of the ith component. Thus

$$r(t) = \frac{I_{VV}(t) + I_{VH}(t)}{I(t)} = \sum_{j=1}^{5} \beta_j e^{-t/\phi_j} \quad (3)$$

where β_j and ϕ_j are the amplitude and correlation time of the jth rotational decay component. The sum of β_j for all anisotropy components is equal to the time-zero limiting anisotropy, r_0.

As discussed in detail elsewhere [19, 21, 22], the average rotational correlation time, $\langle\phi\rangle$, is a weighted harmonic mean,

$$\phi = \sum_j \beta_j \left(\sum_j \left[\beta_j / \phi_j \right] \right)^{-1} \quad (4)$$

and $r(t)$ of a probe in a membrane will not decay to zero due to rotational constraints within the lipid bilayer that inhibit the motion of the probe during the lifetime of its excited state. Instead, $r(t)$ will reach a non-zero, limiting value called the residual anisotropy, r_∞.

The wobble-in-a-cone or "degree of orientational constraint" model [16–18] establishes that the relationship r_∞/r_o describes the probes motion inside of a cone-shaped volume with an angle of θ. The angle θ can be used as a measure of the constraint of the probe. The r_∞/r_o ratio can be determined from the anisotropy decay, and

$$S^2 = \frac{r_\infty}{r_o} = \left[\frac{1}{2} \cos(\theta)(1 + \cos(\theta)) \right]^2 \quad (5)$$

The smaller the cone angle, the tighter the lipid packing and the greater the restriction of the movement of the probe. At 0°, a probe is considered frozen in position and $S^2 = 1$. The more the cone angle diverges from 0°, the more movement a reporter experiences; in the limit of unrestricted motion, $S^2 = 0$.

Material and Methods

The following lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, Al): L-α-phosphatidylcholine (Chicken Egg PC) (Cat#840051); 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0 PC (DMPC)) (Cat#850345); 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18:1 (Δ9-Cis) PC (DOPC)) (Cat#850375); 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (16:0 PC (DPPC)) (Cat#850355); 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (18:1 PS (DOPS)) (Cat#840035); 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0–18:1 PC (POPC)) (Cat#850457). Di-4-ANEPPDHQ (Cat#D36802) and F2N12S (Cat#A35137) were purchased from ThermoFisher.

Liposome Preparation

All lipids were dissolved in HPLC grade B&J Chloroform, containing amylene and 1% ethanol preservatives. Appropriate aliquots were dried for 1–2 h under a steady stream of nitrogen gas. Lipids were then rehydrated in a 50 mM Tris (pH 7.4), 150 mM NaCl buffer. Following a ten-minute cycle of sonication and vortexing, 100-nm diameter liposomes were generated using a mini extruder (Avanti Polar Lipids, Alabaster AL) heated 10 °C above the lipid transition temperature. Lyophilized dyes were dissolved in spectral grade DMSO and then added to the liposomal samples at 400 nM and 100 nM for Di-4-ANEPPDHQ and F2N12S, respectively (final DMSO concentration of less than 1%). Dyes were incubated with the liposomes for a minimum of 15 min before data collection to mimic incorporation used in in vivo cellular systems [2, 3, 5, 23, 24]. In order to increase dye incorporation in these model membrane systems, liposomes were heated to 70 °C during incorporation. Then samples were equilibrated to experimental temperature. In experiments performed by our lab (data not shown), heating during dye incorporation does not seem to affect incorporation of Di-4-ANEPPDHQ, but it does affect incorporation of F2N12S.

Emission Spectra

Emission spectra were acquired using a SpectraMax M4 (ROM v3.0.22 16Feb11) and SoftMax Pro 6.4.2 (Molecular Devices, Downingtown PA) with the sample cuvette at 24.5 ± 0 .3°C. Di-4-ANEPPDHQ samples were excited at 470-nm and emission spectra collected with a 495-nm long-pass dichroic filter. F2N12S samples were excited at 420-nm and emission collected with a 435-nm long-pass dichroic filter. Emission spectra were measured at 1 nm increments. The bandwidths were 9 nm for excitation and 15 nm for emission. Full-width half-maximum, emission maximum, and center-of-gravity were calculated using MATLAB vR2018a (9.4.0.813653). Area normalization of spectra was performed using a|e - UV-Vis-IR Spectral Software (v1.2) (FluorTools.com).

Fluorescence Lifetime and Time-Resolved Anisotropy Decay Measurements

TCSPC data was collected using a custom fluorimeter (Quantum Northwest, Liberty Lake, WA) as described previously [25]. Di-4-ANEPPDHQ was excited using a 470-nm pulsed-diode laser (LDH-P-C-470; PicoQuant, Berlin GmbH). F2N12S was excited using a 421-nm pulsed-diode laser (LDH-P-C-425; PicoQuant, Berlin GmbH). The emission was isolated using a 500-nm long-pass filter (Chroma, Bellows Falls VT) and detected using a red-sensitive Edinburgh Instruments (Livingston, UK) photomultiplier tube (H10720–01). Data was collected at 20 ± 0.02 °C. TCSPC data were analyzed using FluoFit v4.6.6 (PicoQuant Berlin, Germany). The one-way ANOVA with Tukey HSD post-hoc test results comparing individual variables can be found in the supplemental information were performed using Statistica (TIBCO Software, Inc.).

Results and Discussion

As shown in Fig. 2, the emission spectra of Di-4-ANEPPDHQ in the lipids studied have similar emission profiles. The emission curves have similar half-widths (Table 1), and the emission peaks are all within 12 nm near 600 nm. The emission curve of Egg PC indicates an ordered membrane similar to the other heterogeneous membranes. In fact, the Egg PC curve is almost identical to DOPC, which represents a third of lipid composition of Egg PC.

The emission spectrum of F2N12S, as previously described [3, 8], is comprised of three emission bands (Fig. 3). The relative intensity of the bands depends on the excited-state kinetics for ESIPT and hydrogen bond formation. As detailed in Table 2, two dominant emission bands are observed with peaks near 515 and 580 nm. These emission peaks can be fit using the I_{short}/I_{long} ratiometric previously discussed. DOPS appears to be the only lipid studied that shows evidence of H-N* excited-state form. This would indicate that the probe sits closer to the membrane bilayer. The presence of the H-N* excited-state form appears to be associated with the negatively charged headgroup of DOPS.

Table 3 shows the time-resolved parameters for both dyes (results from Tukey's HSD tests comparing parameters are in

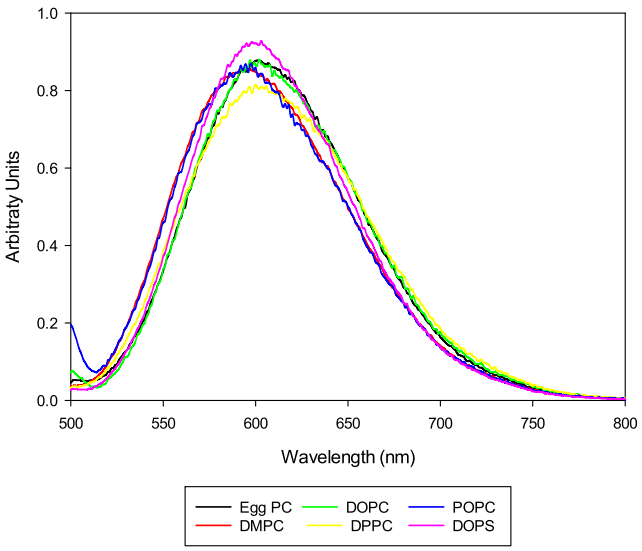

Fig. 2 Area-Normalized Technical Emission Spectra of Di-4-ANEPPDHQ. 400 nM dye was incubated with 100 nm liposomes for a minimum of 15 min. Emissions were collected in 50 mM Tris (pH 7.4), 150 mM NaCl buffer at 24.5 ± 0 .3°C, excitation using a 470 nm pulsed diode laser

Table 1 Di-4-ANEPPDHQ emission details

Lipid	Peak Emission (nm)	Full-Width Half-Max (nm)	Center of Gravity (nm)
DMPC	590	114.03	610.89
DOPC	602	108.83	619.67
DPPC	600	116.74	618.86
POPC	597	114.06	612.69
DOPS	597	100.48	614.27
Egg PC	601	100.35	617.57

Table 2 F2N12S emission details

Lipid	Emission Peaks (nm)		Center of Gravity (nm)	Ratiometric Comparison (I_{short}/I_{long})
	I_{short}	I_{long}		
DMPC	513	576	550.06	0.6262
DOPC	512	572	537.78	0.9973
DPPC	520	574	551.68	0.8238
POPC	514	575	544.33	0.8425
DOPS	526	572	542.62	1.317
Egg PC	513	574	542.34	0.9166

the supplemental material). As previously observed by Owen et al. [2], the fluorescence lifetime of Di-4-ANEPPDHQ was independent of intensity and concentration. The overall variation in average lifetimes of both probes was 1 ns or less. The limiting anisotropy (r_0) for F2N12S in all lipid environments was close to 0.4, indicating the absorption and emission transition dipole moments are essentially colinear. This again supports the hypothesis that despite having multiple excited-state forms, F2N12S can be used as an anisotropy probe.

Both Di-4-ANEPPDHQ and F2N12S showed similar lipid-dependent cone angles and order parameters in DMPC, DOPC, DPPC and POPC liposomes. Egg PC showed the larges difference in cone angle between the two probes (~12°). This most likely indicates that each probe is interacting with a different lipid constituent in heterogeneous Egg PC. The differences in cone angles would support the preference Di-4-ANEPPDHQ has for the Ld phase and suggest that F2N12S prefers the Lo phase.

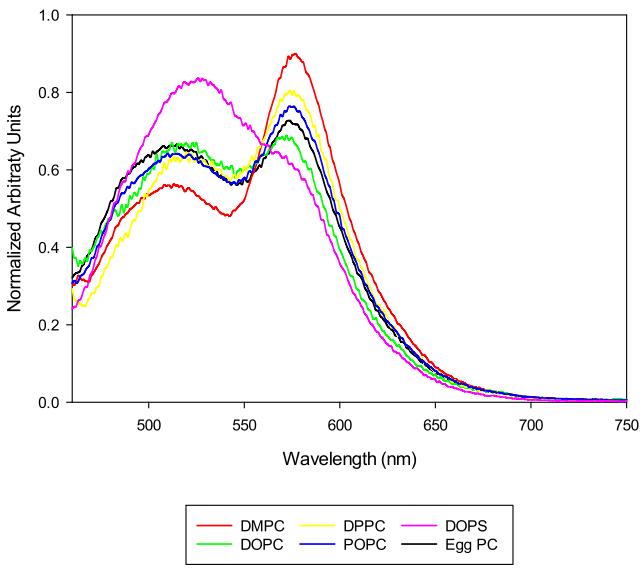

Fig. 3 Area-Normalized Technical Emission Spectra of F2N12S. 100 nM dye was incubated with 100 nm liposomes for a minimum of 15 min. Emissions were collected in 50 mM Tris (pH 7.4), 150 mM NaCl buffer at 24.5 ± 0 .3°C, excitation using a 420 nm pulsed diode laser

The transition temperature (Tm) defines the phase transition between Lo or Ld in a membrane [26]. Increasing the experimental temperature through the Tm will result in an increase in the freedom of the probe and an increase in the cone angle or a decrease in the order parameter. To characterize the response of the probes to different local acyl chain environments (i.e., dynamics in different phases), neutral lipids were selected with Tm values above, below and near the anisotropy measurement temperature (20 °C). As expected, both probes showed small cone angles in DPPC, which has a Tm of 41 °C, and the cone angles were significantly larger in DOPC, POPC and egg PC, which all have Tm values below 20 °C [27]. In DMPC, which has a Tm of 24 °C [27], the dyes behaved similarly and in related studies (not published here) we confirmed that the cone angles increase as the temperature is rises through the Tm.

The cone angles of the two probes were also measured in DOPS, which has a Tm of −11 °C [27], to examine possible effects due to introduction of net negative charge in the lipid head group. F2N12S showed a significant decrease in cone angle in DOPS compared with DOPC, while the cone angles of Di-4-ANEPPDHDQ were indistinguishable in the two lipids. Thus, Di-4-ANEPPDHQ appears to little or no interaction with the head groups, whereas F2N12S, which has a positive charge in the ground state, appears to interact with the negatively charged PS head group and thereby lose mobility.

Conclusions

The results of this study show that both Di-4-ANEPPDHQ and F2N12S can be used as reliable fluorescence anisotropy probes, and when combined with time-resolved measurements can be compelling reporters of the dynamics of membrane environments. Based on the characteristics of lipid environment being examined (transition temperature, head group charge, order phase), one dye might be more or less ideal for probing the system. In a future manuscript, we will show how these probes can be used to study the effects that

Table 3 Time-resolved parameters describing the dynamics of fluorophores inside indicated lipid environment

Lipid	Intensity-Weighted Lifetime (ns) [a]	Time-resolved Anisotropy					
		Correlation Time (ns)	R_{inf}	Limiting Anisotropy (r_0)	S^2	Cone Angle (deg) [b]	
Di-4-ANEPPDHQ							
DMPC [c]	2.58 ± 0.07	3.58 ± 1.28	0.149 ± 0.008	0.238 ± 0.010	0.63 ± 0.01	31.45 ± 0.67	*
DOPC [c]	2.22 ± 0.03	3.32 ± 0.16	0.120 ± 0.011	0.276 ± 0.016	0.44 ± 0.03	41.19 ± 1.82	
DOPS [d]	2.68 ± 0.02	5.07 ± 0.26	0.132 ± 0.013	0.302 ± 0.012	0.44 ± 0.03	40.91 ± 1.46	*
DPPC [c]	2.34 ± 0.16	1.49 ± 0.57	0.254 ± 0.021	0.317 ± 0.014	0.80 ± 0.03	21.81 ± 1.87	*
POPC [c]	2.27 ± 0.06	3.53 ± 0.14	0.122 ± 0.012	0.266 ± 0.005	0.46 ± 0.04	39.95 ± 1.88	*
Egg PC [c]	2.51 ± 0.03	3.92 ± 0.10	0.103 ± 0.015	0.285 ± 0.015	0.36 ± 0.03	45.24 ± 1.91	
F2N12S							
DMPC [d]	4.04 ± 0.14	13.23 ± 5.87	0.209 ± 0.003	0.351 ± 0.021	0.60 ± 0.02	32.82 ± 1.38	
DOPC [c]	3.28 ± 0.44	7.26 ± 4.88	0.136 ± 0.017	0.330 ± 0.086	0.44 ± 0.13	39.98 ± 5.14	*
DOPS [c]	3.79 ± 0.11	4.83 ± 0.34	0.217 ± 0.015	0.343 ± 0.010	0.63 ± 0.03	31.12 ± 1.46	
DPPC [d]	3.87 ± 0.07	8.72 ± 1.21	0.291 ± 0.008	0.355 ± 0.017	0.82 ± 0.03	20.55 ± 2.20	*
POPC [c]	3.97 ± 0.03	7.09 ± 2.93	0.143 ± 0.018	0.317 ± 0.064	0.46 ± 0.05	39.99 ± 2.46	*
Egg PC [d]	3.38 ± 0.10	11.13 ± 3.10	0.227 ± 0.007	0.400 ± 0.004	0.57 ± 0.02	33.22 ± 1.21	

[a] The lifetime is defined as $\langle \tau \rangle = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i}$

[b] ANOVA significance compared against Egg PC was performed using Tukey's multiple comparisons test with and Alpha of <0.05. Full results of the Tukey analysis can be found in the supplemental information

[c] Error represents standard deviation based on $n = 4$

[d] Error represents standard deviation based on $n = 3$

various small, lipophilic molecules and nanomaterials have on individual lipid molecules.

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