

Lipid assemblies mediating the fusion of photosystem-II enriched membranes characterized by DPH (1,6-diphenyl-1,3,5-hexatriene) fluorescence and fluorescence anisotropy lifetimes

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ABSTRACT

The lipid composition of thylakoid membranes (TMs), the site of light reactions in oxygenic photosynthetic organisms, is dominated by the non-bilayer lipid species monogalactosyldiacylglycerol. It has been documented that plant TMs display strong lipid polymorphisms (Garab et al. 2022 *Prog Lipid Res* 86:101163). Recently, we have also shown that BBY membrane sheets, large, laterally fused photosystem-II (PSII) enriched membrane pairs, beside the lamellar phase, contain an intense isotropic phase, the lipid molecules of which mediate the fusion of membranes (Böde et al. 2024 *Photosynth Res* 161:127–140). To demonstrate the composite nature of BBY, we employed confocal laser scanning microscopy, using the lipid-label BODIPY-phosphatidylcholine and chlorophyll-a fluorescence emissions. To characterize the physico-chemical microenvironments of lipid molecules, we stained BBY membranes with the hydrophobic fluorescent dye DPH (1,6-diphenyl-1,3,5-hexatriene). DPH emission spectra from face- and edge-aligned BBY membranes indicated the existence of at least two distinct microenvironments. Fluorescence lifetime analyses revealed three components; the fastest one was sensitive to the enzymatic treatment with wheat germ lipase (WGL), which had earlier been shown to selectively eliminate the isotropic lipid phase of BBY and to disassemble the large sheets into its constituent membrane pairs of grana. Although the anisotropic fluorescence decay kinetics discerned no lifetime heterogeneity of the untreated DPH-stained BBY, WGL treatment led to the appearance of a second lifetime component. These data provide independent experimental evidence on the lipid polymorphism of BBY membranes and reveal that the bilayer lipids and the non-bilayer lipid arrays mediating the fusion of TMs possess distinct physico-chemical environments.

1. Introduction

The light reactions of the photosynthetic energy conversion occur in the thylakoid membranes (TMs), highly specialized protein-rich flattened membrane vesicles. In the chloroplasts of vascular plants, the main ultrastructural domains of TMs are the granum, a unit composed of stacked discs of 300–500 nm diameter, piled on top of each other, and the unstacked stroma lamellae, which are helically wound around the

grana cylinders and are connected to them through narrow slits [1]. The self-assembly of this ultrastructure depends on the lateral segregation of the two photosystems (PSs) – PSII and its main light-harvesting antenna complexes (LHCII) are enriched in the granum while PSI, as well as the ATP synthase, are found in the stroma lamellae; the granal ultrastructure is stabilized by the stacking of the PSII-enriched membranes [2].

The generation of the proton-motive force (pmf) by the photochemical activity of the two PSs and the consecutive electron and proton

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transport and the utilization of pmf for ATP synthesis [3], requires the bulk lipid molecules to be arranged into bilayer structures. Bilayer membranes warrant the electrical insulation of the inner and outer aqueous phases via securing the impermeability of membranes to ions and protons, in particular. However, about half of the TM lipids is comprised of monogalactosyldiacylglycerol (MGDG) [4], which is a non-bilayer-forming lipid species [5]. MGDG molecules possess a small polar headgroup and large hydrophobic fatty acid tails and thus exhibit strong intrinsic propensity to form non-lamellar assemblies such as inverted hexagonal (H_{II}) or cubic structures [5–8]. The bilayer lipid species of TMs are the digalactosyldiacylglycerol (DGDG, 25–30%), the sulfoquinovosyldiacylglycerol (SQDG, 5–12%) and the phosphatidylglycerol (PG, 5–12%). It is important to point out that the relative distribution of the four lipid classes is statistically identical in all subchloroplast particles and in whole thylakoids [9].

In the past years, it has been thoroughly documented that functional plant TMs and sub-chloroplast particles display marked lipid polymorphisms – a large fraction (>50%) of the bulk lipid molecules have been shown to assemble into non-lamellar structures; these non-bilayer lipid phases coexist in close proximity with the bilayer domains [10,11]. Molecular dynamic studies have shown that, due to the presence of MGDG, TM lipids are prone to form non-bilayer lipid assemblies, and the lipid membranes readily fuse with each other [12,13]. The coexistence of bilayer and non-bilayer phases is conceptualized within the Dynamic Exchange Model (DEM), which posits a dynamic equilibrium of lipid molecules exchanging between these phases [10,14,15]. The model proposes that because of the high abundance of MGDG ‘excess’ lipids are expelled from the bilayer – a mechanism that safeguards the high protein density of TMs [14]. Hence, the bilayers are confined to discrete lipid ‘patches’ or inclusions between the photosynthetic supercomplexes, an organization that has been proposed to play key role in the generation and utilization of pmf based on the contiguity of protein networks [11,16].

Regarding the structural entities and roles of the non-bilayer lipid phases, it has been clarified that they are located in regions distinct from the lamellar phase. The bilayer domains embed the main protein complexes of the photosynthetic machinery. Non-bilayer lipid assemblies have been found in association with the activity of the water-soluble, luminal photoprotective enzyme, violaxanthin de-epoxidase [17,18]. H_{II} phase has been shown to originate from lipids surrounding or encapsulating stromal-side proteins [19]. The phase behaviour of the curvature region of TMs, containing the CURT1 proteins, have been found not to contain lamellar phase but to be dominated by isotropic (I) lipid phases [20]. It has also been proposed that the self-assembly of the highly interwoven complex ultrastructure of TMs, containing fusions and fissions, require the presence of non-bilayer lipids. In particular, it has been hypothesized that a substantial part of the I phase(s) of TMs arise from the fusion of TMs [10].

Experimental evidence for I-phase-mediated membrane fusion has been provided by using BBY particles, large (occasionally >10 μm in diameter) sheets of laterally fused stacked PSII membrane pairs of grana [21]. ^{31}P NMR spectroscopy has revealed that about 60% of the bulk lipid molecules of BBY particles assume lamellar (L) phase; these are small inclusions in between PSII supercomplexes; and about 14% appears to form I phases with sharp peaks, indicating their origin from rapidly moving lipid molecules; the remaining fractions, displaying broad-band resonances, B_1 and B_2 bands, most probably originate from partially detached shell lipids of the supercomplexes, a side effect of the detergent used in the isolation procedure. (For comparison, in TMs, the L and I phases, with similar spectroscopic features as in BBY, account, respectively, for about 40 and 16% of the bulk lipids, and the remaining fraction originates from stromal side protein:lipid assemblies giving rise to H_{II} phase; TMs contain no B_1 or B_2 phases.). By using a range of biochemical and biophysical techniques it has been shown that the large sheets of BBY membranes can be disassembled into its constituent stacked membrane pairs by wheat germ lipase (WGL), a substrate

nonspecific general tri-, di-, and monoglyceride hydrolase. WGL selectively destroys the sharp I phases, without noticeable effect on the molecular organization and functional activity of PSII membranes [21]. Hence, these data have shown that BBY membranes can be used as a suitable system to study the role of non-bilayer lipids in the fusion of biological membranes, a purely lipid mediated process.

The fusion of biological membranes is of utmost importance in the self-assembly and structural dynamics of cells and cellular organelles as well as in a range of fundamental physiological processes [22,23]. Despite intense research on these areas, our understanding of the molecular and physical mechanisms of membrane fusion, and of the role of lipids, in particular, is far from being complete [24–28].

To gain further insights into the nature of lipid-mediated fusion of TMs, and to obtain information on the physico-chemical environment of lipid molecules that participate in the lateral fusion of PSII membranes, we studied the steady-state fluorescence spectroscopy and fluorescence lifetime and fluorescence anisotropy decay kinetics of DPH-stained BBY particles (DPH, 1,6-diphenyl-1,3,5-hexatriene). DPH is one of the most widely used lipophilic fluorescent probes for investigating the dynamic and structural properties of natural and artificial lipid membranes [29–32]. DPH has been shown to be sensitive to the physico-chemical environment of lipid molecules, which depend on the polarity, local rotational mobility, and lateral packing in the membrane [33–35], and on the lipid phase behaviour, in particular [36,37].

2. Materials & methods

2.1. Isolation of PSII-enriched membrane pairs (BBY particles)

BBY isolation was performed according to the protocol described by Yamamoto et al. [38], with minor modifications. Approximately 100–150 g of spinach leaves was homogenized in 300 mL of buffer containing 20 mM Tricine – NaOH (pH 8.0), 0.4 M NaCl, 0.2% BSA, 2 mM MgCl_2). The homogenate was filtered and centrifuged at 6000 $\times g$ for 10 min. The supernatant was discarded, and the pellet was carefully resuspended in 80 mL of the same buffer using a soft brush. This suspension was then centrifuged at 400 $\times g$ for 30 s.

The resulting supernatant was centrifuged again at 35,000 $\times g$ for 7 min. The pellet was resuspended in 40 mL of a buffer containing 20 mM Tricine–NaOH (pH 8.0), 0.15 M NaCl, 0.2% (w/w) BSA, 5 mM MgCl_2) and centrifuged again under the same conditions. The final pellet was resuspended in approximately 7 mL of buffer containing 40 mM MES–NaOH (pH 6.5), 0.4 M sucrose, 10 mM NaCl, 5 mM MgCl_2 , and the total volume (a mL) was recorded. The chlorophyll concentration (b mg Chl mL^{-1}) of the suspension was determined and adjusted to 2.5 mg Chl mL^{-1} by adding ($a \times b/2.5 - a$) mL of the same buffer. Subsequently, one-fourth of the suspension volume of buffer containing 40 mM MES–NaOH (pH 6.5), 0.4 M sucrose, 10 mM NaCl, 5 mM MgCl_2 , 14% (w/v) Triton X-100) was added carefully to avoid foaming, yielding a final concentration of 2 mg Chl mL^{-1} and establishing a Triton X-100 to chlorophyll mass ratio of 14:1.

The mixture was gently stirred for 10 min using a magnetic stirrer, followed by centrifugation at 35,000 $\times g$ for 15 min. The pellet was resuspended in 40 mL of buffer containing 40 mM MES–NaOH (pH 6.5), 0.4 M sucrose, 10 mM NaCl, 5 mM MgCl_2) and centrifuged at 3000 $\times g$ for 3 min. The supernatant was collected and centrifuged again at 35,000 $\times g$ for 20 min. Finally, the pellet was resuspended in a small volume of the same buffer and the chlorophyll concentration was determined.

Freshly isolated BBY samples were either kept on ice for same-day measurements or stored at -80°C for several days or weeks. In some cases, freshly prepared samples were subjected to freeze-thaw cycles. Regardless of the storage conditions, the samples yielded consistent results across all experiments.

2.2. Enzymatic treatment of BBY membranes with WGL

The isolated BBY membranes were subjected to digestion with WGL, a substrate-nonspecific general tri-, di-, and monoglyceride hydrolase/lipase [39]. WGL (Sigma-Aldrich, Burlington, MA, USA) was applied at concentrations of 20 U mL⁻¹. The treatments were performed at room temperature. It has been previously demonstrated by thin-layer chromatography that WGL hydrolyzes the main thylakoid membrane lipid, monogalactosyldiacylglycerol (MGDG) [19]. WGL is widely used in food industry [40,41].

2.3. Confocal laser-scanning microscopy (CLSM) of BODIPY-stained BBY membranes

BODIPY (1,3,5,7-Tetramethyl-8-phenyl-4,4-difluoroboradiazaindane) (Sigma-Aldrich, Burlington, MA, USA) was dissolved in DMSO to prepare a 100 μ M stock solution. Isolated BBY membranes were adjusted to a chlorophyll concentration of 300 μ g Chl mL⁻¹ and incubated with 0.1 μ M BODIPY for 15–20 min. Microscopy coverslips were coated with 0.1% (w/v) poly-L-lysine for 15–20 min, rinsed with Milli-Q water, air-dried and loaded with the stained membranes. The membranes were allowed to adhere for 20–30 min and the excess material was removed by gently washing the coverslip with Milli-Q water.

Imaging was performed on an INFINITY confocal laser-scanning microscope (Abberior Instruments GmbH, Göttingen, Germany) equipped with two MATRIX array detectors. BODIPY and Chl fluorescence was excited using a 488 nm pulsed (40 MHz) diode laser, and emission was collected between 498 and 608 nm for BODIPY and 648–758 nm for Chl fluorescence using the MATRIX array detectors. All images were acquired using a 60 $\times$ /1.42 NA oil-immersion objective with identical acquisition parameters for all samples (pinhole size 10.1 AU at 574 nm, pixel size 40 nm, z-step size 100 nm, 37 planes, pixel dwell time 20 μ s for BODIPY and 5 μ s for Chl, average laser power at sample plane 0.5 μ W for BODIPY and 0.25 μ W for Chl). The acquisition settings were optimized for subsequent deconvolution in Huygens Professional (SVI, Hilversum, The Netherlands) using the array detector confocal module. Deconvolved images were further processed and analysed in Fiji/ImageJ 1.54r [42].

2.4. Steady-state and time-correlated fluorescence lifetime measurements with DPH

DPH was purchased from Sigma-Aldrich (Burlington, MA, USA). To assess the mobility of DPH in different microenvironments and upon WGL treatment, fluorescence lifetime measurements were performed using a Fluorolog-3 (FL3–222) spectrofluorometer (Horiba, Jobin Yvon, Kyoto, Japan) equipped with time-correlated single photon counting (TCSPC) option, the double emission arms ("T" configuration) and excitation and emission polarizers. A 0.2 mM DPH stock solution was prepared in tetrahydrofuran (THF; Sigma-Aldrich, Burlington, MA, USA) and stored at –20 °C in the dark. Prior to the measurements, DPH was added to BBY (at a final chlorophyll concentration of 10 μ g Chl mL⁻¹) to a final DPH concentration of 3 μ M and incubated for 15 min at room temperature. This procedure is similar to that applied by [43] for isolated cyanobacterial TMs. The fluorescence anisotropy values obtained on four different batches of isolated spinach TMs were found at 0.19 ± 0.03 and on BBY at 0.23 ± 0.01 ; these values compare well with that, 0.25, obtained at 25 °C on TMs isolated from *Synechocystis* PCC 6803 cells grown at 22 °C [43].

For recording the DPH emission spectra, the samples were excited at $\lambda_{\text{exc}} = 370$ nm (excitation slit width, 5 nm), and the fluorescence emission was detected between 385 and 625 nm (emission slit width, 7 nm).

Single decay fluorescence lifetime measurements were performed using NanoLED-370 pulsed laser source. The fluorescence decay curves (with a peak count of 3000) were recorded by TCSPC at 440 nm with the

slit width of 5 nm using a DataStation v2.2 software package.

The instrument response function (P) was recorded using 0.01% Ludox AS30 (Sigma-Aldrich, Burlington, MA, USA) aqueous suspension as the scattering medium.

Anisotropy lifetime measurements were performed using a NanoLED-370 pulsed laser source (peak wavelength 371 nm; pulse width 1.2 ns). The excitation beam passed through a polarizing beamsplitter cube (Thorlabs, CCM5-PBS201/M) that was manually adjusted either vertically or horizontally, while the emission side polariser was software-controlled. The detection was at 440 nm with the slit width of 12 nm. The measurement was carried out in two ways with the following parameters: a) In direct anisotropy mode, in which polarization components are alternated rapidly during acquisition: peak difference 3000, polariser dwell time 30 s, for G factor: polarizer dwell time 30 s, cycles 12. b) Sequential acquisition (single decay measurements) of parallel (I_{VV}) and perpendicular (I_{VH}) components, followed by post-acquisition anisotropy reconstruction. In VV position recording was made until 3000 peak counts were reached, and in VH position recording was made for the same time. In both cases, 180 counts were collected for G-factor determination for both HV and HH positions. Decay curves were fitted by the Decay Analysis Software (DAS6) from HORIBA (Horiba Ltd., Kyoto, Japan) [44], whereas multi-exponential fits and additional analysis were done using Igor Pro, version 8.02 (Wave Metrics, Lake Oswego, OR, USA).

It should be noted, that according to our experience, the above measurement conditions provided the best balance between sample stability, fluorescence intensity, spectral- and temporal resolution and sensitivity to different decay components. In addition, the sample stability (such as any loss of signal intensity) was monitored during all experiments.

3. Results and discussion

3.1. Domain structure of BBY membrane sheets as revealed by CLSM

It has earlier been shown by freeze-fracture electron microscopy [45] and scanning electron microscopy (SEM) [21] that isolated stacked PSII-enriched membrane pairs of 300–500 nm diameter, obtained by cleaving granal TMs with mild detergent (Triton X-100), tend to fuse laterally and to form large sheets with diameters of up to ~ 10 μ m or more. To visualize the domain structure of the membrane sheets and the role of lipids, we employed CLSM imaging on BBY membranes stained with the bright, stable fluorescence dye BODIPY-phosphatidylcholine; these dye molecules preferentially partition in lipid-rich assemblies. By imaging the Chl-a fluorescence of PSII, the domain structure of these membrane sheets could clearly be recognized as being composed of ~ 500 nm diameter patches (Fig. 1A, domains dominated by Chl-a fluorescence). Indeed, the quasi-periodic pattern of these domains can also be recognized in the 1-dimensional intensity plot shown in Fig. 1B (Chl trace). With regards to the fluorescence emission of BODIPY-phosphatidylcholine, significant intensities can be discerned in regions between the domains with intense Chl-a fluorescence: small green spots in areas with weak Chl-a fluorescence (Fig. 1A) and an intensity pattern that appears not to correlate with that of Chl-a fluorescence (Fig. 1B). Qualitatively, these findings are in harmony with our data published earlier revealing that lipids, in addition to the bilayer, are also found in isotropic phases, which mediate the fusion of the patches of stacked PSII membrane-pairs [21]. It is to be noted here, that the dimensions of the domains in BBY do not permit quantitative investigations. Indeed, our attempts to characterize the different lipid phases, e.g. by fluorescence lifetime imaging of BBY membranes stained with lipophilic dyes, such as Merocyanine 540 (cf. [15]), proved inconclusive (data not shown). The nature of the different lipid phases in BBY were, thus, investigated by spectroscopic tools – using DPH, a fluorescent probe which carries information on the dynamic and structural properties of lipid-enriched assemblies (see Introduction).

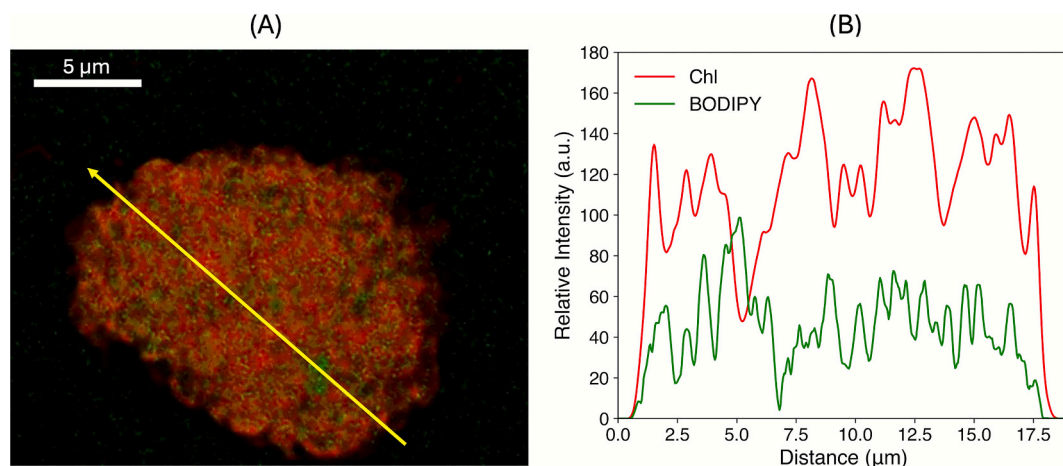


Fig. 1. Typical CLSM image of a BBY membrane sheet stained with BODIPY-phosphatidylcholine (green) and overlapped with Chl autofluorescence (red) (A). Fluorescence intensity profile was extracted along the indicated line (yellow arrow) and smoothed using a moving average of 9 pixels. The corresponding red and green channel intensities are shown in panel B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. DPH subpopulations revealed by fluorescence emission spectra of magnetically aligned BBY membranes

It has been shown that the spectral distribution of DPH fluorescence emission, and the intensity of different vibrational bands in particular, depend on the polarity and microviscosity of the solvent [46–48]. This opens the possibility to detect the presence of different lipid phases based on fluorescence emission spectroscopy, provided that we can

discriminate between the emissions from lipid assemblies of different phases. Bilayer organization is characterized by weakly polarizable, low dielectric constant hydrocarbon interior due to their tightly packed acyl chains and limited water penetration into the hydrophobic core. In contrast, because of the different packing of lipid molecules in non-bilayer phases the exposure of the acyl chains to water can differ substantially from that in the bilayer [49,50]. For example, cubic phases, which appear as I phases in ^{31}P NMR might trap water molecules inside

BBY membrane sheets align according to the magnetic field

Excitation of probes occurs in distinct microenvironments depending on the membrane orientation

Emission spectra under different magnetic orientation

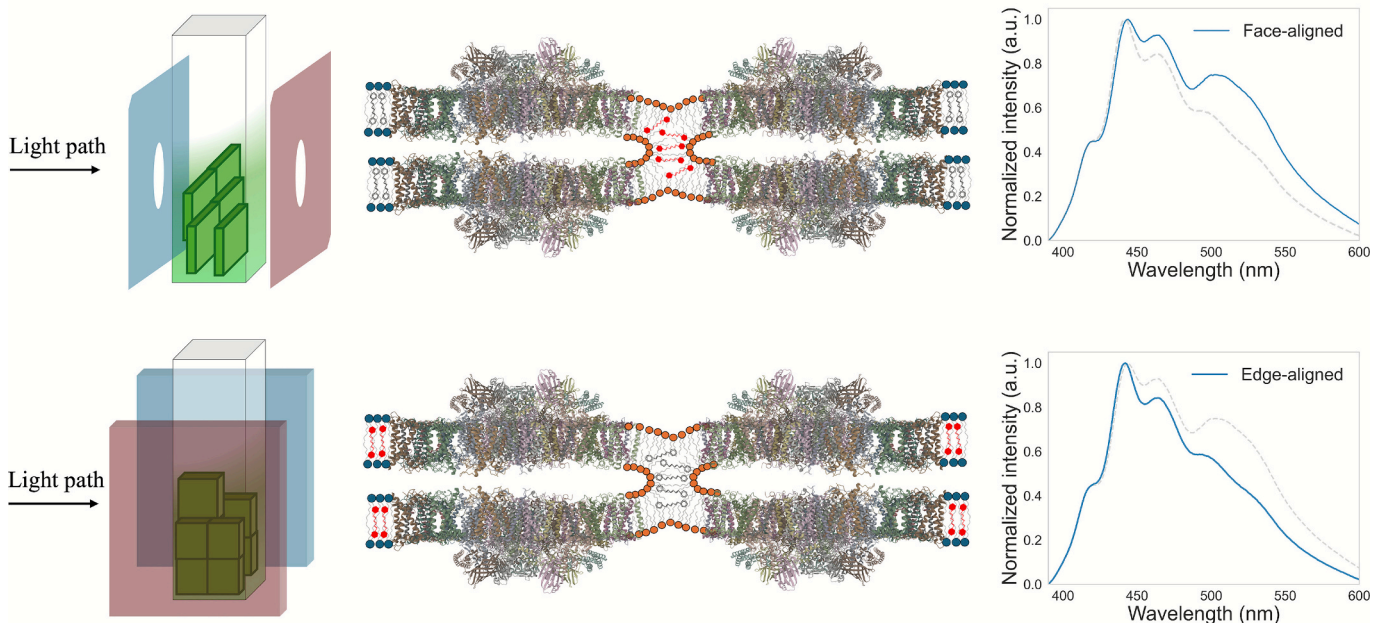


Fig. 2. Magnetic field alignment of BBY membranes. Schematic representation of the fluorescence emission spectroscopy experiment using face-aligned (upper panel) and edge-aligned (lower panel) DPH-stained BBY membranes. Three biological replicates were recorded for each condition which yielded similar spectral features; the figure shows a representative spectrum (baseline-corrected at 390 nm and normalized to the maximum at 440 nm). The magnetic alignment alters the orientation of membrane domains relative to the excitation beam, leading to selective excitation of DPH in the bilayer (L) and in the isotropic (I) lipid phases. In face-aligned samples, DPH molecules residing in the I phase are preferentially excited, at the expense of the dye molecules in the L phase. In contrast, in edge-aligned samples, the absorbance of the excitation beam is maximized for the molecules in the L phase. The excited probes are indicated by red colour. (For easier comparison, in each panel, both the face- and edge-aligned spectra are plotted but only one of them is highlighted.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

micelles [49].

As shown in Fig. 2, the fluorescence emission spectra of DPH stained, magnetically aligned BBY membranes, reveal the existence of at least two distinct populations of the fluorescent dye molecules. This is explained by the preferential excitation of the probe in face- and edge-aligned orientations of the membranes and using that the transition dipole moment of DPH aligns preferentially parallel to the lipid acyl chains. In face-aligned orientation of BBY (Fig. 2 upper panel) with respect to the excitation beam, DPH residing in the isotropic lipid assemblies (I phase) are preferentially excited. In contrast, the edge-aligned geometry (Fig. 2 lower panel) enhances the excitation of the dye molecules found in the bilayer (L phase). These data are consistent with the polarity dependence of fluorescence emission of DPH [51]: the spectral distributions in the face- and edge-aligned orientations, preferentially originating from the isotropic (polar) and the bilayer (apolar) phases, respectively, resembling those observed in ethanol and hexane. It is to be noted here that the observed heterogeneity of the fluorescence emission spectra of DPH cannot be explained by the positional heterogeneity [36] of the probe with respect to the lipid headgroups – because of its marked orientation dependence.

When two or more species exhibit overlapping emission profiles, time-resolved spectroscopy provides more detailed information about the subpopulations of the dye molecules and their dynamic behaviour. To this end, we employed time-resolved fluorescence lifetime measurements.

3.3. Multi-exponential fluorescence lifetime analysis indicates distinct DPH populations in BBY membranes

As shown in Fig. 3A, the time-resolved decay curves of DPH fluorescence emission display multi-exponential features. The observed decay kinetics (Y_i) could be fitted with the sum of three exponentials. This was obtained from the analysis using the reconvolution of the

instrument response function ($P(i)$) and the sum of three exponential functions ($I(i)$):

$$Y(i) = P(i) \otimes I(i) \quad (1)$$

where $I(i)$ is the undistorted fluorescence intensity (Eq. 2).

$$I(i) = A_1 \exp\left(-\frac{i}{\tau_1}\right) + A_2 \exp\left(-\frac{i}{\tau_2}\right) + A_3 \exp\left(-\frac{i}{\tau_3}\right) \quad (2)$$

where A_i is the contribution of fluorescent species to the fluorescence decay and τ_i is the fluorescence lifetime ($i = 1, 2, 3$). The quality of the fits was evaluated based on the χ^2 values.

The decay components in the control samples (Table 1), most likely represent DPH molecules residing in distinct membrane regions. Upon treatment with WGL, which specifically disrupts the non-bilayer, isotropic lipid phase of fastest motional characteristics, the shortest lifetime component ($\tau_1 = 0.3$ ns) decreased markedly in amplitude (A_1),

Table 1

Fluorescence lifetime components (τ) and their corresponding amplitudes (A) of control and WGL-treated BBY ($n = 3$) and TM samples ($n = 3$). Mean values $\pm$ SD.

	Control		WGL-treated	
	τ_1 τ_3 (ns) $\pm$ SD	A_1 - $A_3 \pm$ SD	τ_1 τ_3 (ns) $\pm$ SD	A_1 - $A_3 \pm$ SD
BBY	0.31 $\pm$ 0.02	63.4 $\pm$ 5.3	0.37 $\pm$ 0.03	38.2 $\pm$ 3.6
	3.49 $\pm$ 0.44	26.1 $\pm$ 4.8	3.42 $\pm$ 0.17	40.7 $\pm$ 0.9
	12.53 $\pm$ 0.37	10.4 $\pm$ 0.6	10.28 $\pm$ 0.65	21.1 $\pm$ 3.7
TM	τ_1 τ_3 (ns) $\pm$ SD	A_1 - $A_3 \pm$ SD	τ_1 τ_3 (ns) $\pm$ SD	A_1 - $A_3 \pm$ SD
	0.34 $\pm$ 0.01	60.1 $\pm$ 8.5	0.50 $\pm$ 0.06	26.8 $\pm$ 2.7
	3.14 $\pm$ 0.11	29.0 $\pm$ 4.6	3.16 $\pm$ 0.31	42.8 $\pm$ 3.5
	12.20 $\pm$ 3.09	10.9 $\pm$ 4.4	8.65 $\pm$ 0.42	30.7 $\pm$ 2.3

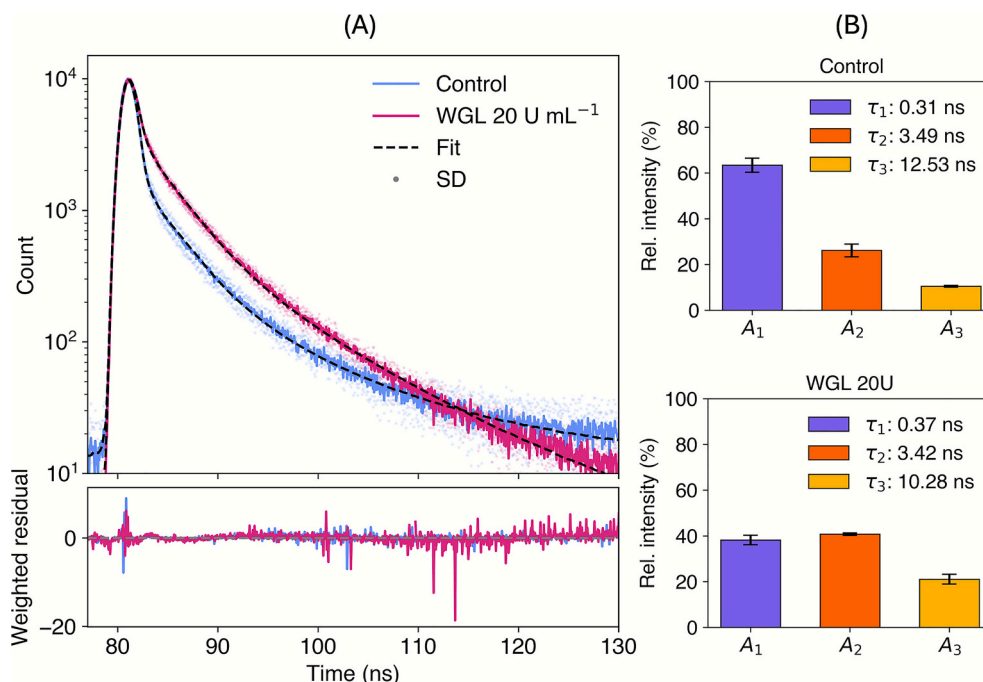


Fig. 3. Fluorescence lifetime analysis of BBY particles before and after WGL treatment (20 U mL⁻¹) ($n = 3$). (A) Time-resolved fluorescence decay curves for control (blue) and WGL-treated (magenta) samples, plotted as mean $\pm$ SD on a semi-logarithmic scale, together with the multiexponential fits (dashed lines). Weighted residuals ($(y_{\text{data}} - y_{\text{fit}})/\sigma$, where σ is the pointwise standard deviation across the three biological replicates, are shown in the lower panel. Each of the three biological replicates were fitted independently; the goodness-of-fit values ranged between 1.15 and 1.27. (B) Mean relative amplitude contributions $\pm$ SD (A_1 , A_2 , A_3) of the three lifetime components (τ_1 , τ_2 , τ_3 ; purple, orange, yellow) obtained from the fits. The mean lifetimes corresponding to each amplitude group are indicated in the panels for the control (upper) and WGL-treated (lower) samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

while the relative contributions of τ_2 and τ_3 increased (Fig. 2B). This shows that the shortest lifetime component can be assigned to originate mainly from DPH molecules located in the isotropic lipid phase; as shown by ^{31}P NMR spectroscopy, this phase was specifically diminished upon WGL treatment of the particles [19,21]. It is interesting to note that heterogeneity of the physico-chemical environment of lipid molecules could also be discerned in intact TMs, with similar decay components in BBY and in TMs (Table 1). These data strongly suggest that time-resolved spectroscopy of DPH is a suitable technique to test the lipid polymorphism in different biological membranes.

3.4. Anisotropy lifetime measurements

To gain further information on the dynamics of the different subpopulations of DPH in BBY and thus on the dynamic features of the different lipid phases, we performed time-resolved anisotropy measurements. The anisotropy decay depends on the lifetime of DPH and the micro-viscosity of the molecular environment, and thus indirectly reports on the phase behaviour of the lipid assemblies.

We used Impulse Reconvolution Anisotropy Analysis. The time dependence of anisotropy ($r(i)$) is based on (Eq. 3):

$$r(i) = \frac{I_{VV}(i) - I_{VH}(i)}{I_{VV}(i) + 2I_{VH}(i)} = \frac{I_d(i)}{I_s(i)} \quad (3)$$

where I_{VV} is parallel (vertical-vertical), I_{VH} (vertical-horizontal) is perpendicular component, I_d is the difference and I_s is the summarised undistorted fluorescence intensity. First, the sum of the observed decay curves, $I_s(i) = Y_{VV} + 2GY_{VH}$ (parallel observations (Y_{VV} , Y_{HH}), perpendicular observations (Y_{VH} , Y_{HV}), G-factor ($G=Y_{HV}/Y_{HH}=1$) was fitted by the reconvolution of the instrument response function ($P(i)$) and $I_s(i)$, resulted in parameters free of anisotropy effects (Eq. 2). Fitting was performed using either fixed fluorescence lifetimes, obtained from independent, unpolarised lifetime measurements (see previous section) or allowing the fluorescence lifetimes to vary freely during the fit. Next, $Y_d(i) = Y_{VV} - GY_{VH}$, the difference decay curve was analysed, that is both fluorescence and anisotropy dependent. Based on Eq. 3

$$I_s(i)I_r(i) = I_d(i) \quad (4)$$

where $I_r(i)$ is the undistorted anisotropy impulse response function.

Convolution of both sides of (4) with $P(i)$, we get the function of $Y_d(i)$.

$$P(i) \otimes [I_s(i)I_r(i)] = P(i) \otimes I_d(i) = Y_d(i) \quad (5)$$

Parameters for anisotropy impulse response function (with two rotational components) can be calculated from (6):

$$I_r(i) = r_\infty + B_1 \exp\left(-\frac{i}{\tau_{R1}}\right) + B_2 \exp\left(-\frac{i}{\tau_{R2}}\right) \quad (6)$$

where $r_0 = r_\infty + B_1 + B_2$, τ_{R1} and τ_{R2} are the anisotropy lifetimes, r_0 is the anisotropy at $i = 0$, r_∞ is the anisotropy at $i = \infty$.

Evaluation for the two acquisition strategies, i.e. the direct anisotropy mode and the sequential acquisition of parallel (I_{VV}) and

perpendicular (I_{VH}) components, followed by post-acquisition anisotropy reconstruction, yielded consistent results (Table 2), confirming that photobleaching and the time delay between sequential acquisitions did not significantly influence the extracted anisotropy decay parameters. Surprisingly, however, as shown in Table 2, the anisotropy decay kinetics discerned no lifetime heterogeneity. The control BBY samples consistently showed a single rotational correlation time in the range of ~ 0.17 – 0.2 ns. In contrast, WGL-treated BBY membranes exhibited two well-separated τ_R components: a shorter component between 0.046 and 0.066 ns and a slower component in the range of 2.57 – 5.5 ns range (Table 3). This confirms the presence of DPH subpopulations in the sample – despite the absence of a well-distinguishable heterogeneity of the anisotropy decay in the control.

There might appear a contradiction between the low number of rotational states: one in the control samples and two in the WG-treated samples vs the three fluorescence lifetimes in both cases; hence apparently questioning a multi-phase model. Therefore, we have done further analysis of the fluorescence and anisotropy decays to compare two different models. Model 1: fluorescence relaxation and rotational dynamics processes are independent; in this model each fluorescence lifetime is present in each rotational dynamic subpopulation. Model 2: each subpopulation has their distinct characteristic lifetime and rotational correlations times. The fits with DAS6, which uses only Model 1, gave three fluorescent lifetimes – both for the non-polarized experiment and also for $I_{VV} + 2I_{VH}$ sum of the polarized experiment, and one or two rotational correlation times from the lifetime anisotropy data. To test the two models, we have fitted the decays obtained for the I_{VV} and I_{VH} separately to multi-exponentials (not shown). The reason was that if the fit provides a sufficient number of significant exponentials, then the comparison of the observed effective time constants with the known fluorescent lifetimes could determine whether model 1 or model 2 are more compatible with the decays (because the effective lifetimes of exponentials are a different combination of the fluorescent lifetimes and rotational correlation times in the two models). Unfortunately, the resolution and the signal-to-noise ratio of the experimental data was not good enough for separating sufficient numbers of exponentials. This is due to unavoidable technical limitations determined by the relationship between sample concentration, stability and bleaching of DPH, and the spectrometer settings.

Within the constraints of the technical limitation mentioned above, we made two important observations: (i) Whereas the control samples yielded 3 fluorescent lifetimes and a single rotational correlation time, the WGL-treated samples also gave 3 fluorescent lifetimes and identified an additional (second) rotational correlation time. This observation could not be explained if the fluorescent relaxations were independent of the rotational dynamics. (There could be several reasons for not seeing a second rotational correlational time in the control samples, e.g., the rotational correlation time of the second rotational dynamics could have either been outside the detectable range or hidden under a different subpopulation but with the same rotational correlation time as the detected one). (ii) WGL treatment affected the 3 fluorescence lifetimes differently: two lifetimes were changed, and one was left unchanged. Considering how WGL affects BBY membranes, this would be not possible if the fluorescence lifetimes were the same in the subpopulations with different rotational dynamics. Considering all the

Table 2

Anisotropy lifetimes (τ_R) of DPH embedded in BBY particles ($n = 3$), obtained from two measurement modes. The fluorescence lifetimes were fixed at the values determined in the corresponding unpolarized lifetime measurements.

Analysis mode	Sample	τ_{R1} (ns) $\pm$ SD
Direct anisotropy	BBY1	0.17 ± 0.09
	BBY2	0.17 ± 0.10
	BBY3	0.20 ± 0.02
Sequential acquisition	BBY1	0.19 ± 0.11
	BBY2	0.17 ± 0.10
	BBY3	0.15 ± 0.08

Table 3

Rotational correlation times (τ_R) of DPH embedded in untreated (control) and WGL-treated BBY particles ($n = 3$).

Condition	Sample	τ_{R1} (ns) $\pm$ SD	τ_{R2} (ns) $\pm$ SD
Control	BBY1	0.17 ± 0.09	–
	BBY2	0.17 ± 0.09	–
	BBY3	0.20 ± 0.02	–
WGL-treated	BBY1	0.07 ± 0.02	2.60 ± 0.44
	BBY2	0.06 ± 0.02	5.50 ± 1.47
	BBY3	0.05 ± 0.02	3.35 ± 0.71

above observations, we conclude that DPH is located in at least two lipid environments with different rotational dynamics and fluorescent lifetimes.

4. Conclusion

The major aim of the present study was to characterize the spectroscopic and dynamic features of the lipid molecules that mediate the lateral fusion of PSII-enriched membranes into extended membrane sheets. By using the lipophilic fluorescence dye DPH, we confirmed that the lipid molecules are found in different physico-chemical environments. This is in harmony with our earlier findings showing that, in addition to the bilayer, TM lipids in their native membranes assume different non-bilayer phases [10]. Further, we have shown that – similar to our earlier conclusion [21] – a distinct (WGL-sensitive) population of the TM lipids play key role in the fusion of PSII (BBY) membranes. It is proposed that time-resolved DPH spectroscopy might be suitable to monitor the fusion of different biological membranes and/or to detect their lipid polymorphisms. It is also worth noting that DPH might be used in combination with WGL in distant areas, such as in food industry.

CRediT authorship contribution statement

Kinga Bode: Writing – original draft, Visualization, Investigation, Formal analysis. **Krisztina Sebok-Nagy:** Writing – original draft, Visualization, Investigation, Formal analysis. **Ondrej Dlouhy:** Writing – review & editing, Visualization. **Gabor Steinbach:** Resources. **Ales Benda:** Methodology, Supervision. **Gyozo Garab:** Writing – review & editing, Supervision, Conceptualization. **Tibor Pali:** Writing – review & editing, Supervision, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Research data will be sent upon request.

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