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**Title:** Exploring fast proton transfer events associated with lateral proton diffusion on the surface of membranes

**Year:** 2019

**Version:** Published version

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**Please cite the original version:**

Amdursky, N., Lin, Y., Aho, N., & Groenhof, G. (2019). Exploring fast proton transfer events associated with lateral proton diffusion on the surface of membranes. *Proceedings of the National Academy of Sciences*, 116(7), 2443-2451. <https://doi.org/10.1073/pnas.1812351116>



# Exploring fast proton transfer events associated with lateral proton diffusion on the surface of membranes

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Edited by Harry B. Gray, California Institute of Technology, Pasadena, CA, and approved December 19, 2018 (received for review July 18, 2018)

**Proton diffusion (PD) across biological membranes is a fundamental process in many biological systems, and much experimental and theoretical effort has been employed for deciphering it. Here, we report on a spectroscopic probe, which can be tightly tethered to the membrane, for following fast (nanosecond) proton transfer events on the surface of membranes. Our probe is composed of a photoacid that serves as our light-induced proton source for the initiation of the PD process. We use our probe to follow PD, and its pH dependence, on the surface of lipid vesicles composed of a zwitterionic headgroup, a negative headgroup, a headgroup that is composed only from the negative phosphate group, or a positive headgroup without the phosphate group. We reveal that the PD kinetic parameters are highly sensitive to the nature of the lipid headgroup, ranging from a fast lateral diffusion at some membranes to the escape of protons from surface to bulk (and vice versa) at others. By referring to existing theoretical models for membrane PD, we found that while some of our results confirm the quasi-equilibrium model, other results are in line with the nonequilibrium model.**

proton diffusion | excited-state proton transfer | lipid vesicles | photoacid | molecular dynamics

The translocation of protons across the two sides of biological membranes by transmembrane proteins is a fundamental biochemical process, such as the transport of protons in the aerobic respiration system in the mitochondria of eukaryotes or in the chloroplast of plants and prokaryotes during photosynthesis (1, 2). In this proton translocation process, protons arrive at the entrance of the protein-based proton pump either from the membrane or from the bulk solution. The diffusion of protons in bulk aqueous solution is highly efficient, with a diffusion coefficient ( $D_{H^+}$ ) of  $D_{H^+} = 9.3 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$ , as commonly explained by the Grotthuss mechanism, which describes a hydrogen bond-assisted proton hopping between water molecules (3, 4). However, the diffusion of protons along the surface of the membrane toward the entrance of the pump is still under investigation. The protein structure of proton pumps suggests that a protein structural motif in the form of ionizable side chains of amino acids is responsible for shuttling the protons from the surface of the membrane to the protein pump (1, 2, 5). It was established more than 20 years ago that proton transfer (PT) from the surface of membranes to bulk water (and vice versa) is different from PT events in bulk water (6–10). Proton diffusion (PD) along a membrane surface is more complicated than bulk PD since the structure of the lipid molecule, the distance between adjacent lipid molecules, and the water molecules in and around the lipid membrane all affect PD. Due to advances in fast spectroscopy experiments and molecular dynamics (MD) simulations, significant progress in our understanding of this type of PD has been made in the past decade (11–23).

In a pioneering type of experiment, the groups of Pohl and coworkers (11–14) and Widengren and coworkers (15, 16) have explored the role of the lipid and bulk proton concentration on PD along membranes. Widengren and coworkers (15, 16) have used a fluorescent chromophore that was tethered to the membrane surface; by fluorescence correlation spectroscopy, they followed the protonation of the chromophore. Their main conclusion was that

the lipids act as proton-collecting antennas from the bulk solution. They further found that (i) incorporating phosphatidic acid (PA) in vesicles of phosphatidylcholine (PC) or phosphatidylglycerol (PG) caused an increase in PD (16) and (ii) both the ionic strength of the solution and the mode of protonation of the proton-collecting antennas influence the PD parameters (15). The lateral PD coefficient on the membrane was indirectly estimated by the authors while assuming a proton hopping between adjacent lipid molecules (with a distance of 1 nm), and resulted in a diffusion coefficient of  $D_{H^+} = 2 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ . Pohl and coworkers used a similar chromophore as in the latter experiments, but the release of protons was either photoinitiated from a proton release chromophore that was situated dozens of micrometers from the probed chromophore (11, 13) or by proton injection a few micrometers from the detection site (12, 23). This experimental approach can only be used with planar membranes (instead of vesicles), but it enables the direct calculation of the diffusion coefficient from the kinetic curves. The authors' main conclusions were (i) PD along a PC membrane was similar to the one along a phosphatidylethanolamine (PE) membrane sharing a diffusion coefficient of  $\sim 4 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$ ; (ii) surprisingly, the diffusion along a glycerol monooleate (GMO) membrane was similar to the PC and PE membranes; and (iii) PD is most probably due to protons in the second layer of water around the membrane.

Here, we explore PD along the surface of lipid vesicles by following the light-initiated proton release from a membrane-anchored photoacid, which dissociates upon light excitation due to the large difference in  $pK_a$  value between its ground and excited states. Using time-resolved fluorescence enables us to extract the PT efficiency to and from the photoacid, the PD coefficient, and

## Significance

**Proton diffusion (PD) across biological membranes is a fundamental process in many biological systems, such as in aerobic respiration or in the photosynthesis process. Even though this diffusion process has been studied for many decades, it is still highly enigmatic. Here, we suggest an experimental technique to study the very fast PD events taking place upon the photoinduced release of a proton on the surface of the membrane. We show that the diffusion, as well as the interaction between protons on the surface and protons in bulk media can be influenced by the nature of the membrane's lipids. Our results shed some light on this unique PD process and help to overcome previous experimental contradictions in the literature.**

Author contributions: N. Amdursky, Y.L., and G.G. designed research; N. Amdursky, Y.L., and N. Aho performed research; N. Amdursky, Y.L., N. Aho, and G.G. contributed new reagents/analytic tools; N. Amdursky, N. Aho, and G.G. analyzed data; and N. Amdursky wrote the paper.

The authors declare no conflict of interest.

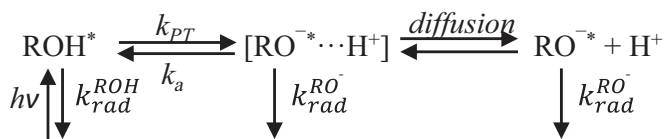
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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1812351116/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1812351116/-DCSupplemental).

Published online January 24, 2019.



**Scheme 1.** ESPT photoprotolytic cycle of photoacids.  $h\nu$ , photon absorption;  $rad$ , radiative decay.

the dimensionality of the diffusion. We explore four types of membranes with different phospholipid headgroups: a zwitterionic headgroup (PC), a negative headgroup (PG), a headgroup that is composed only of the negative phosphate group (PA), and a positive headgroup without the phosphate group [trimethylammonium-propane (TAP)] (24). The use of photoacids to follow proton dynamics on the surface of lipid membranes started in the pioneering works of Nachliel and Gutman (25) nearly 30 years ago. In contrast to that study, in which the photoacid was solvated in solution, we have tightly tethered the photoacid to the surface of the lipid, and thus follow only protons that have been released on the surface.

### Theoretical Model

Photoacids (Brønsted type) are aryl-OH molecules, whose  $pK_a$  values differ between ground and excited states. For instance, the common photoacid of 8-hydroxy-1,3,6-pyrenetrisulfonate (HPTS) has a  $pK_a$  value of 7.4 in the ground state and a  $pK_a^*$  value of 1.3 in the excited state (26). Accordingly, the molecule dissociates during the excited-state lifetime, and the excited-state proton transfer (ESPT) photoprotolytic cycle can be described as follows.

Following light excitation of the protonated species (ROH), the excited molecule (ROH\*) deprotonates with a PT rate of  $k_{PT}$ . The proton can then either recombine with the anion (RO<sup>-\*</sup>), with a recombination rate of  $k_a$ , or diffuse along the media, which can be described by the Debye–Smoluchowski equation (27). The arrows pointing down in Scheme 1 represent the radiative decay of the ROH\* and RO<sup>-\*</sup>, which share a similar radiative lifetime,  $\tau$ , of  $\sim 5.4$  ns, which enables following the PD for at least 30 ns using time-resolved experimental methods. The fluorescence intensity and decay of the ROH\* and RO<sup>-\*</sup> forms can be easily followed individually since each form emits at a different wavelength. Photoacids have been used to follow the hydration of biological surfaces of proteins (26, 28–30) and polysaccharides (26, 31, 32). The photoprotolytic cycle of photoacids (Scheme 1) has been theoretically modeled by Agmon and coworkers (27, 33–35), who suggested the following expression for the ROH\* fluorescence decay:

$$I_f^{ROH^*} \exp(-t/\tau) \cong \frac{\pi a^2 k_a \exp[-R_D/a]}{2k_{PT}(\pi D_{H^+} t)^{d/2}} \quad [1]$$

This model describes an ESPT process occurring on a surface of a reaction sphere with a radius of  $a$ , where  $d$  is the dimensionality

of the PD and  $R_D$  is the Debye radius. In their model,  $R_D$  represents the distance at which the coulombic attraction between the negative excited-state anion (RO<sup>-\*</sup>) and the positive proton equals the thermal energy ( $k_B T$ ):

$$R_D = \frac{|Z_1 Z_2| e^2}{4\pi \epsilon_0 \epsilon_r k_B T} \quad [2]$$

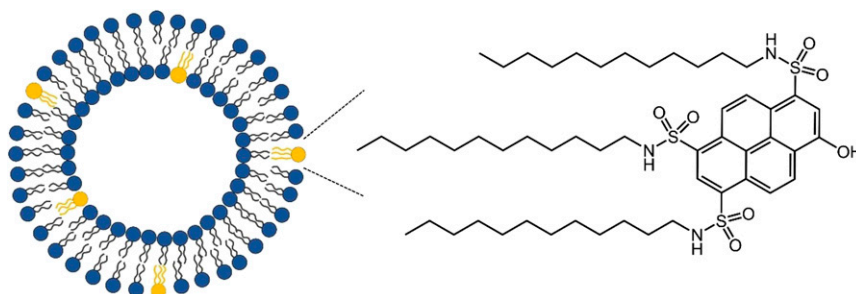
where  $Z_1$  and  $Z_2$  are the electron charge ( $e$ ) units of RO<sup>-\*</sup> and the dissociated proton,  $\epsilon_0$  is the permittivity of free space, and  $\epsilon_r$  is the relative dielectric permittivity of the medium. The relative dielectric permittivity of the water layer surrounding the lipid vesicle is hard to estimate, and we have used an averaged value of 20–27 for the first nanometers around the lipid vesicles as was found experimentally and theoretically (36, 37). Accordingly, the calculated Debye radius for our membrane-bound modified photoacid (Scheme 2) is  $R_D = 20$ –28 Å. HPTS in solution has a fourfold higher  $Z_1$  value (its RO<sup>-\*</sup> form has four electron charges), but due to the high relative dielectric permittivity of water ( $\epsilon_r = 80$ ), HPTS has a value of  $R_D = 28$  Å.

### Results

For tethering the photoacid to the surface of a lipid vesicle, we synthesized a hydrophobic photoacid where a dodecyl group has been attached to each of the sulfonate groups of HPTS (C<sub>12</sub>-HPTS; Scheme 2). Due to the hydrophobic nature of our newly synthesized photoacid, it could not be dissolved in water. Comparing the steady-state emission of HPTS and C<sub>12</sub>-HPTS in ethanol and methanol has allowed us to conclude that C<sub>12</sub>-HPTS can be considered as a stronger photoacid than HPTS (*SI Appendix, Fig. S1* and further discussion). A stronger photoacid is more likely to undergo ESPT. Our finding is in line with the works of Jung and coworkers (38, 39), who observed an increase in the photoacid strength upon modifications of the HPTS sulfonic groups.

C<sub>12</sub>-HPTS was tethered to the membrane (at a ratio of 1:100 probe/lipid; *Materials and Methods*), which was composed of the following lipids: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-1'-*rac*-glycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA), or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). We have used dynamic light scattering to quantify the size distribution of the different formed vesicles, showing similar distribution of our different vesicles (*SI Appendix, Fig. S2*).

**MD Study.** Our working hypothesis here is that our newly designed probe can integrate into the membrane while exposing its hydroxy group toward the membrane surface (i.e., in the water membrane interface) and release a proton upon light irradiation. To test this hypothesis, we performed atomistic MD simulations of the C<sub>12</sub>-HPTS incorporated in lipid bilayers of POPC, POPG, and POPA. The incorporation in DOTAP vesicles was not simulated since, as will be discussed below, ESPT from C<sub>12</sub>-HPTS in



**Scheme 2.** Molecular scheme of C<sub>12</sub>-HPTS incorporated into a lipid vesicle.

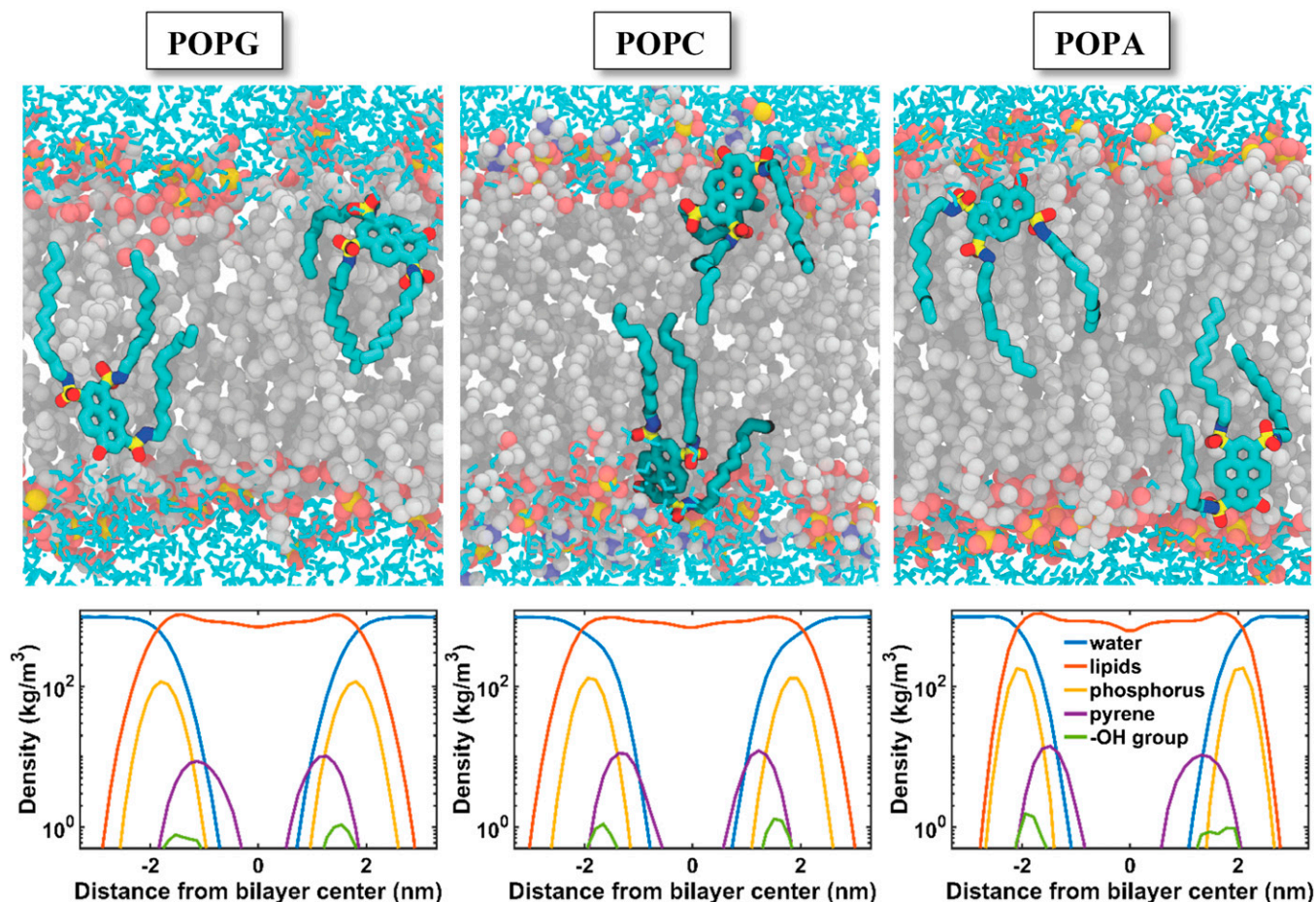


Fig. 1. (Top) Snapshots from atomistic MD trajectories showing the position of the C<sub>12</sub>-HPTS dye in the upper and lower leaflets of the three phospholipid bilayers (water molecules are shown as cyan sticks). (Bottom) Density distributions along the bilayer normal of the water (blue), lipids (red), phosphorus atoms of the lipid headgroups (yellow), C<sub>12</sub>-HPTS pyrene moieties (violet), and C<sub>12</sub>-HPTS hydroxy groups (green).

DOTAP could not be followed in physiological aqueous conditions. The molecular snapshots of the MD simulations (Fig. 1, Top) show that C<sub>12</sub>-HPTS is well integrated within the membrane, exposing its –OH toward the water–membrane interface, in close proximity to the location of the phosphate groups, as can be observed in the density plots (Fig. 1, Bottom). The MD simulations also allowed us to estimate whether the –OH of the photoacid can release a proton by following the ability of the photoacid's hydroxy group to form hydrogen bonds with nearby water molecules or phosphate groups (Table 1). The table shows that the –OH can form hydrogen bonds with nearby moieties for the vast majority of the simulation time. Furthermore, the ratio between the number of hydrogen bonds to water molecules and the number of hydrogen bonds to nearby phosphate groups depends on lipid, with the maximum number of hydrogen bonds to

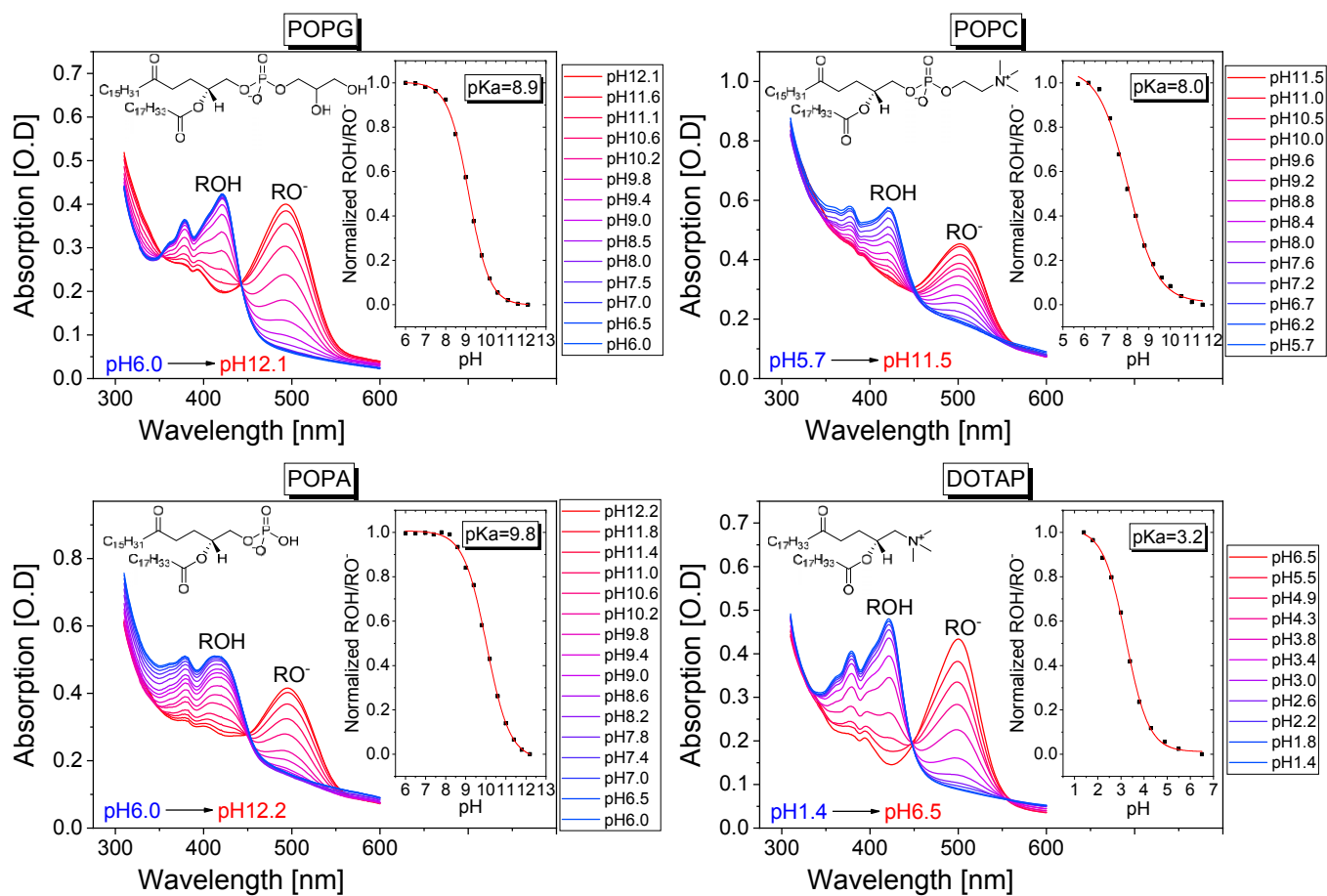
water for the POPA membrane. As will be discussed later (see Discussion), these results support our spectroscopic measurements.

**Steady-State Spectroscopy.** Steady-state absorption (Fig. 2) studies of the photoacid-containing lipid vesicles allowed us to verify that C<sub>12</sub>-HPTS has been tethered to the membrane and to determine the pK<sub>a</sub> of the photoacid by ratiometric titration with a strong acid/base. Fig. 2 and Table 2 show that the pK<sub>a</sub> of the photoacid is highly sensitive to the charge of the lipid headgroup (molecular schemes in Fig. 2). We found that the pK<sub>a</sub> in the negatively charged headgroup vesicles (POPG and POPA) is higher than in the zwitterionic headgroup vesicle (POPC), while the pK<sub>a</sub> in the positively charged headgroup vesicles (DOTAP) is extremely low. It has been shown that the pK<sub>a</sub> of HPTS in aqueous solutions can be changed as a function of salt concentration (40) at different water/organic solvent ratios (41) or next to a protein surface (30),

Table 1. Percentage of time that a hydrogen bond is formed between the –OH group of C<sub>12</sub>-HPTS and the surrounding water molecules or lipid phosphate groups

Lipid system	Hydrogen bonds with water molecules, %	Hydrogen bonds with lipid phosphates, %	Total hydrogen bonds with everything, %
POPG	31.9	65.9	97.8
POPC	27.1	71.3	98.4
POPA	37.4	56.2	93.6

The values are averaged between the upper and lower membrane leaflets in the MD simulations.



**Fig. 2.** Absorption spectrum of C<sub>12</sub>-HPTS incorporated in the different lipid vesicles at different pH values. (Insets) pK<sub>a</sub> calculations and the molecular schemes of the lipid. O.D., optical density.

but, as far as we are aware, such drastic change in the pK<sub>a</sub> value of a given photoacid has never been observed before. Our latter finding further suggests that the photoacid is in tight proximity to the headgroup of the lipid, in line with the results from our MD simulation. In a similar manner, Widengren and coworkers (15) also observed an increase in the pK<sub>a</sub> values of fluorescein and Oregon green when these photoacids were tethered to PG and PC vesicles. We have used the absorption and emission maxima (Table 2) to calculate the ΔpK<sub>a</sub> values (ΔpK<sub>a</sub> = pK<sub>a</sub> - pK<sub>a</sub><sup>\*</sup>) of the photoacid at different lipid vesicles using the Förster cycle (42):

$$\Delta pK_a = \frac{\Delta E}{\ln(10)RT}, \quad [3]$$

where ΔE is the energy difference between the protonated (ROH) and deprotonated (RO<sup>-</sup>) forms of the photoacid, R is the gas constant, and T is temperature. To calculate ΔE, we have used the averaged values for the absorption (E<sub>Ab</sub>) and emission (E<sub>Em</sub>) maxima to compensate the solvent contribution to the Stokes' shifts (43):

$$\Delta E = (E_{Ab}^{ROH} + E_{Em}^{ROH})/2 - (E_{Ab}^{RO^-} + E_{Em}^{RO^-})/2. \quad [4]$$

For following the light-induced proton release from photoacids, they have to be excited in their ROH form. Accordingly, the above-calculated pK<sub>a</sub> values limit the pH range of the measurement, in which only low pH values could be used for DOTAP vesicles. Fig. 3 shows the normalized emission spectrum with different lipid vesicles and at different pH values, where several important observations can be made. The first one is that for all of

our measurements, the main form of the molecule is RO<sup>-</sup>, meaning that the photoacid can dissociate and release a proton. This is a further validation that the photoacid is on the surface of the lipid vesicle, since if it were buried within the hydrophobic membrane, we would expect to see the ROH peak as the main one, as was previously shown for HPTS within hydrophobic binding sites (26).

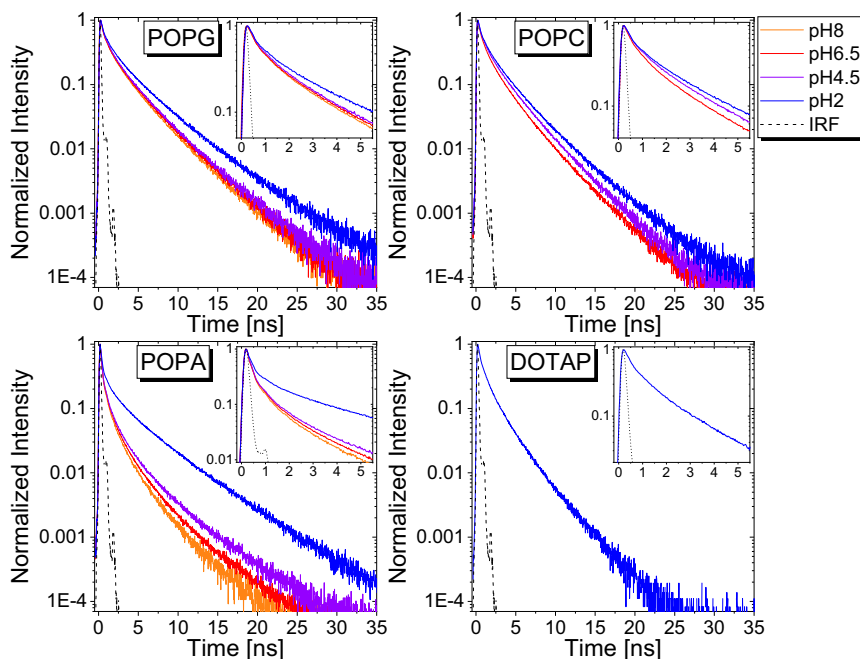
The second important observation is that the emission profile is different for different lipid vesicles, which indicates that the lipid headgroup has a major role in determining the ESPT parameters of the photoprotonic cycle of C<sub>12</sub>-HPTS (Scheme 1). We can use the RO<sup>-</sup>/ROH ratio of the emission spectrum at different pH values (SI Appendix, Table S1) as a first indication for the role of the lipid headgroup in ESPT to/from the photoacid and for PD. In general, high RO<sup>-</sup>/ROH ratios (low-intensity ROH peak; Fig. 3) can be a result of a fast PT rate, a slow recombination rate, or a fast PD

**Table 2.** Absorption and emission maxima of ROH and RO<sup>-</sup>, together with the calculated pK<sub>a</sub>, ΔpK<sub>a</sub>, and pK<sub>a</sub><sup>\*</sup> values

System	POPG	POPC	POPA	DOTAP
λ <sub>abs</sub> <sup>ROH</sup> , nm	419	420	411	422
λ <sub>abs</sub> <sup>RO-</sup> , nm	501	502	500	501
λ <sub>em</sub> <sup>ROH</sup> , nm	475	476	473	459
λ <sub>em</sub> <sup>RO-</sup> , nm	543	543	541	544
pK <sub>a</sub>	8.9	8.0	9.8	3.2
ΔpK <sub>a</sub>	7.0	6.9	7.5	7.6
pK <sub>a</sub> <sup>*†</sup>	1.9	1.1	2.3	-4.4

<sup>†</sup>The values were estimated by pK<sub>a</sub><sup>\*</sup> = pK<sub>a</sub> - ΔpK<sub>a</sub>.





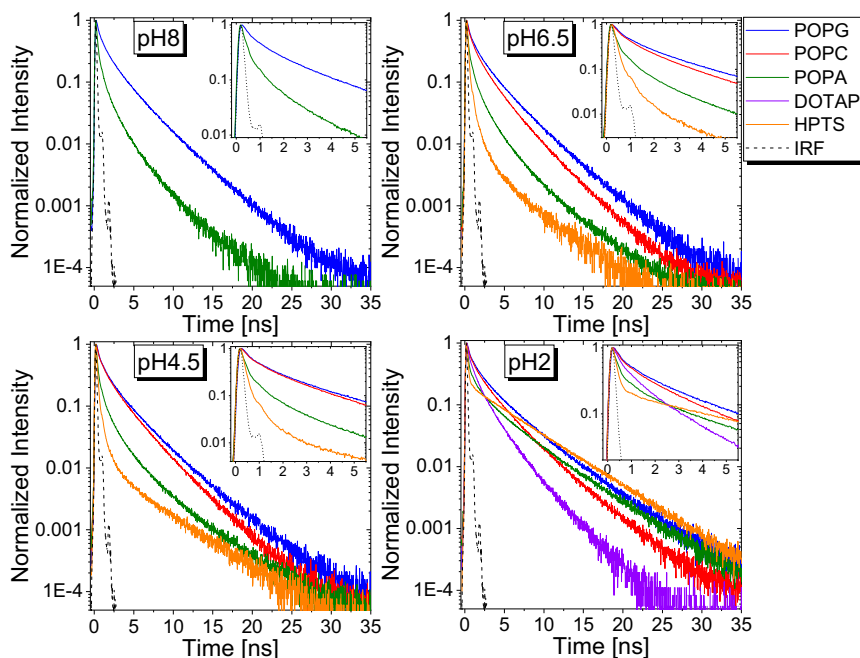
**Fig. 4.** Time-resolved fluorescence decay of the ROH\* form in the four lipid vesicles as a function of pH value. (Insets) Zoomed-in views of the first nanoseconds. IRF, instrument response function.

- iv) The dimensionality ( $d$ ) of the PD from the photoacid on the lipid vesicles is reduced from the dimensionality value of 3 of HPTS in bulk water. POPC and POPG have a dimensionality value of 2, and POPA has a higher dimensionality value of 2.5.
- v) The  $R_D$  values differ between different lipid vesicles, indicating that the dielectric constant next to the lipid vesicle changes for different lipid headgroups.

#### Discussion

According to the time-resolved fluorescence measurements and their fitting, we can divide the PD behavior into three different types of diffusion patterns.

The first one relates to the surfaces of POPC and POPG, which, despite a different surface charge, share the same PT and PD properties. They both exhibit lateral (2D) PD on the surface of the membrane, with a coefficient of  $3.5 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$ , nearly



**Fig. 5.** Time-resolved fluorescence decay of the ROH\* form at each of the pH values as a function of the lipid vesicle, in comparison to the decay of HPTS. (Insets) Zoomed-in views of the first nanoseconds. IRF, instrument response function.





fast-exchange regimes, which refer to the rate of proton exchange between surface and bulk. They suggest that the fast regime is the most probable one, and that long-distance PD processes that take place on the millisecond-second time scale can be explained by numerous  $k_{\text{on}}/k_{\text{off}}$  events that lead to a quasi-equilibrium state between surface and bulk protons. The second mechanism proposed by Agmon and coworkers (13) and Agmon and Gutman (46) suggests that protons on the surface are not in equilibrium with bulk protons. They further propose that the membrane is not acting as a proton-collecting antenna and that protons do not escape from surface to bulk (i.e., protons have very low  $k_{\text{off}}$  values). They suggest that protons on the surface diffuse via proton wires in what they envision as a propagation of an advancing front of protons, where protons at the front protonate all possible titratable sites, and that the protons following that front diffuse in an unhindered fashion. Both theories have been developed to explain results observed in the (sub)second regime. Our measurements follow only diffusion events taking place in the excited state and, accordingly, are limited to the nanosecond regime. Nevertheless, we believe that our results can shed insights on the possible fast kinetics of this diffusion process.

As discussed, our results with POPA vesicles are very different from the ones of POPC and POPG. The strong influence of the solution pH on the kinetic parameters observed for the POPA vesicles suggests that protons released on the surface of the membrane can escape to bulk, and that during the excited-state lifetime, protons from the bulk can go to the surface, which supports the quasi-equilibrium model. It is important, however, to mention that PA lipids are frequently referred to as “buffered lipids” (i.e., due to their bare phosphate group, they act as buffer molecules in solution, and can therefore be easily titratable). On the other hand, our POPC and POPG results suggest PD with limited surface-to-bulk and bulk-to-surface PT events, which, in combination with a relatively high PD coefficient, support the nonequilibrium model, as well as disproving the possibility of diffusion by proton hopping between titratable groups (11). Nevertheless, since we observed some pH dependency of the kinetic parameters, the protons cannot be completely nonequilibrated with the solution, and there is probably some bulk-to-surface PT. Thus, while our results seem to support the nonequilibrium model, we cannot ascribe all of our results to a single suggested PD mechanism, but hope that our results will ignite new theoretical efforts toward a unifying approach for PD on the surface of membranes.

## Conclusions

In summary, we have developed a photoacid-based probe that can be tethered to membranes for studying light-induced PD processes on the surface of membranes. While the photoacid enables the exploration of fast PT events within the PD process, the time interval in which PD can be studied is limited to the excited-state lifetime of the probe, which is on the order of a few dozen nanoseconds. As a consequence, the accessible length scales are also limited to few dozen nanometers (48). We used our photoacid probe to explore PD across phospholipid mem-

branes with different lipid headgroups, namely, PA, PC, PG, and TAP, as well as the effect of pH on the PD process. We found that protons diffuse laterally on the surface of PC and PG vesicles with a diffusion coefficient of  $3.5 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$ , which cannot be explained by proton hopping between titratable groups, with only a moderate pH dependence on the kinetic parameters. For PA vesicles, we found that protons could escape to bulk, and vice versa (the antenna effect), which also leads to a more prominent effect of the solution pH on the kinetic parameters. For TAP vesicles, we found a slower PT from the photoacid with little influence of bulk protons, which resembles the behavior of hydrophobic pockets.

## Materials and Methods

**Synthesis of C<sub>12</sub>-HPTS Derivative.** To a mixture of dodecanamine (0.5 mmol) and triethanolamine (0.5 mmol) in 10 mL of dichloromethane (0 °C), 0.1 mmol of 8-acetoxypyrene-1,3,6-trisulfonyl chloride (38) in 10 mL of dichloromethane was added dropwise. After warming to room temperature, the reaction mixture was stirred for another 48 h. The organic phase was washed with sodium bicarbonate and saturated sodium chloride solution before being dried over sodium sulfate. After evaporation, the crude product was purified via column chromatography (3:7 ethyl acetate/petrol ether). The product was confirmed by MALDI-TOF.

**Preparation of Lipid Vesicles.** All lipids (POPA, POPC, POPG, and DOTAP) were purchased from Avanti Polar Lipids, Inc. The lipid was mixed with the C<sub>12</sub>-HPTS probe in chloroform at a 100:1 (lipid/probe) molar ratio. The mixture was dried under a stream of N<sub>2</sub> and placed under vacuum for at least 2 h to remove any residual solvent. The dried lipid film was then rehydrated in 5.0 mM phosphate buffer and vortexed for 30 s. The resulting solution was extruded 31 times through a polycarbonate membrane with 200-nm pores (0.2 μm; Nucleopore). The liposome solution was diluted to a lipid concentration of 1.0 mg/mL and stored at 4 °C. Before later use, the pH of the solution was adjusted using either HCl or NaOH.

**Spectroscopic Measurements.** The above-mentioned lipid vesicle solutions or a 35-μM HPTS solution was used for the spectroscopic measurements. Dynamic light scattering measurements were recorded with a Malvern Zetasizer using a 1-cm-pathlength cuvette. The UV-visible absorption measurements were recorded with a PerkinElmer spectrometer using a 1-cm-pathlength quartz cuvette. The steady-state fluorescence measurements were recorded with a Horiba Fluorolog system using an excitation wavelength of 395 nm with 1-nm bandpass slits for both entrance (excitation) and exit (emission). The time-resolved fluorescence measurements were acquired with a Horiba Deltaflex system, using a 405-nm diode laser with a pulse duration of <100 ps as the light excitation source. The ROH fluorescent decay was collected at 445 nm and 455 nm for HPTS and C<sub>12</sub>-HPTS, respectively, where at least 30,000 counts (at peak) were collected. The instrument response function was collected at the excitation wavelength using a diluted Ludox solution in water.

**MD Simulations.** Details of MD experiments are given in *SI Appendix*.

**ACKNOWLEDGMENTS.** We thank D. Huppert and N. Agmon for fruitful discussions. G.G. and N. Aho thank the CSC – IT Center for Science (Espoo, Finland) for computational resources. N. Amdursky received financial support from the Chaya Career Advancement Chair, the Russel Berrie Nanotechnology Institute, and the Grand Technion Energy Program, and G.G. and N. Aho were supported by the Academy of Finland (Grant 311031).

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