White-Fluorescent Dual-Emission Mechanosensitive Membrane Probes that Function by Bending Rather than Twisting

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Abstract: Bent N,N'-diphenyl-dihydrodibenzo[a,c]phenazine amphiphilic mechanosensitive membrane probes that operate by an unprecedented mechanism, namely, unbending in the excited state as opposed to the previously reported untwisting in the ground and twisted in the excited state. Their dual emission from bent or “closed” and planarized or “open” excited states is shown to discriminate between micelles in water and monomers in solid-ordered (S_{0}) and liquid-disordered (L_{o}) bulk membranes. The dual-emission spectra cover enough of the visible range to produce vesicles that emit white light with ratiometrically encoded information. Strategies to improve the bent mechanophores with expanded π systems and auxochromes are reported, and compatibility with imaging of membrane domains in giant unilamellar vesicles by two-photon excitation fluorescence (TPEF) microscopy is demonstrated.

The bending of polycyclic aromatic planes has attracted much scientific attention owing to the emergence of unique spectroscopic, electrochemical, chiroptical, and functional supramolecular properties.[1,2] Many inspiring examples exist for spheres, tubes, helices, bowls, saddles, rings, and other curved motifs.[1] However, unlike twisting and untwisting, the spectroscopic consequences of bending and unbending of polycyclic aromatic planes in the ground state (GS) and the first excited state (ES) have been met so far with surprisingly little success in sensing applications and have been completely ignored with regard to fluorescent membrane probes.[3–13] In 2015, Tian, Chou, and co-workers reported that the molecular distortion of N,N'-diphenyl-dihydrodibenzo[a,c]phenazines in the GS provides access to coupled conformational changes toward planarity in the ES, a process reminiscent of a butterfly or “papillon” opening its wings (Figure 1).[2]

This brilliant breakthrough was inspiring for the design of fluorescent membrane probes. Today, three main classes of mechanosensitive probes can be distinguished. Molecular rotors are best developed.[1,7] These push–pull fluorophores operate by off-equilibrium ES vibrational deactivation in response to decreasing viscosity. A unique inverted molecular rotor has been created recently that reports by untwisting rather than twisting in the ES.[15] We have introduced a family of mechanosensitive flipper probes that operate by untwisting of push–pull fluorophores in the GS.[13] Their mechanical planarization under equilibrium conditions reports increasing membrane order as a large red shift in excitation rather than emission. Herein, we introduce mechanosensitive membrane probes that function by bending rather than twisting.

Scheme 1. a) NaIO_{4}, RuCl_{3}, 3 H_{2}O, NMI, THF, CH_{2}Cl_{2}, H_{2}O, RT, 3 h, 32%; b) aniline, pyridine, TiCl_{3}, CH_{2}Cl_{2}, RT, overnight, 56%; c) hydrazine hydrate, Pd/C, THF, RT, 2 h, 74%; d) K_{3}CO_{3}, Cu(OTf)_{2}, 1,2,4-trichlorobenzene, 210°C, overnight, 22%; e) NaOH, H_{2}O, THF, reflux, 3 h, 76%.

Figure 1. General structure, emission maxima, and intensity ratios i_{b2}/i_{b1} of papillon probe 1 during increasing ES planarization in, from left to right, micelles in water and monomers in solid-ordered (S_{0}) and liquid-disordered (L_{o}) bulk membranes. See Scheme 1 and Figure 3; b/p = bent/planar; dashed bonds indicate expansion sites explored in this study.

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https://doi.org/10.1002/anie.201804662.
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Table 1: Characteristics of papillon probes.

| Cpd[a] | ETOAc | 25 °C | DPPC | 25 °C | H₂O | 25 °C |
|--------|--------|-------|-------|-------|------|-------|
|        | λtw [nm] | Φex [%][b] | σ[0] | Φex [%][b] | σ[0] | Φex [%][b] |
| 1      | 354 (6.7) | 700 | 14.4 | 4.0 | 465/592 | 0.2 | 459/- | > 20 | 467/587 | 3.4 (0.5) | 0.7 (6.4) | 1.7 (10.1) | 0.7 (12.8) |
| 2      | 378 (4.9) | 750 | 5.0 | 1.7 | 510/614 | 0.1 | 502/- | > 20 | 517/595 | 2.2 (0.3) | 0.6 (3.3) | 1.0 (10.6) | 0.5 (15.4) |
| 3      | 334 (11.7) | 692 | 4.2 | 2.5 | 435/580 | 0.3 | 449/- | > 20 | 495/570 | 3.1 (3.5) | 0.4 (21.2) | 0.7 (4.9) | 0.3 (7.6) |

[a] For compounds see Scheme 1. [b] Wavelength of one-photon absorption maxima [nm] (extinction coefficient [m⁻¹ cm⁻¹]). [c] Wavelength of two-photon absorption maxima [nm]. [d] Fluorescence quantum yield, determined using Coumarin 1a as a reference. [e] Two-photon cross-section, in Goeppert–Mayer (10⁻⁴ cm²·photon⁻¹); Supporting Information, Equation (S3). [f] Wavelength of the first (e1) and second emission maxima (e2), λem at the maximum of the lowest energy band (from uncorrected spectra). [g] Fluorescence intensity ratio Iₐ/Iₐ₂. [h] Partition coefficient x1⁰. DPPC = dipalmitoylphosphatidylcholine, DOPC = dioleoylphosphatidylcholine.

Amphiphiles 1-3 were readily accessible by adapting the reported procedures (Scheme 1 and Supporting Information, Schemes S1–S4). The new pyrene papillon 2, for instance, was prepared in only five steps from pyrene 4 (Scheme 1). Reaction of the resulting diketone 5 with aniline in the presence of pyridine and TiCl₄ gave diamine 6 in 56 % yield. Reduction to diamine 7 was achieved in 74 % yield. Papillon 8 was obtained by a copper-catalyzed domino reaction proceeding through intramolecular C–Hamination with 9 and subsequent Ullmann coupling. Basic ester hydrolysis afforded the target amphiphile 2.

In ETOAc, papillon 1 absorbed at λtw = 354 nm and emitted red light at λem = 592 nm (without correction, Table 1, Figures 2a, 3a; spectra with correction: Supporting Information, Figure S15a). Insensitivity to solvent polarity was as reported and confirmed that the large red shift in emission originates from ES planarization and not from solvatochromism (Figure 3a; Supporting Information, Figures S14, S15). A weaker emission at λe₂ = 465 nm from bent ES was detected with an intensity ratio Iₐ/Iₐ₂ = 0.2 (b/p = bent/planar; Table 1, Figures 2a, 3a).

Core expansion in the pyrene papillon 2 shifted the absorption in ETOAc to λtw = 378 nm, with a broad plateau extending toward λem = 400 nm (Figure 2b). The red fluorescence from planar ES was at λe₂ = 614 nm also slightly red shifted. A weaker ratio Iₐ/Iₐ₂ = 0.1 without a distinct peak for emission from bent ES intermediates around λe₁ = 510 nm indicated that core expansion in papillon 2 facilitates planarization in the ES. Fluorescence quantum yields dropped from Φex = 14.4 % for 1 to Φex = 5.0 % for 2 (Table 1), the photostability under continuous illumination was comparable to Nile Red (Supporting Information, Figure S16). The introduction of methoxy donors in papillon 3 shifted absorption and emission maxima in ETOAc to the blue and reduced photostability and fluorescence quantum yield (Table 1, Figure 2c; Supporting Information, Figure S16).

In Tris buffer, probe 1 emitted blue light at λe₁ = 459 nm (Figures 2a, 3g). The superlinear concentration dependence, increasing with decreasing temperature, revealed the occurrence of aggregation-induced emission (AIE)[14] (Figure 3b; Supporting Information, Figures S17, S18). This strongly blue-shifted AIE at λe₁ suggested that amphiphile 1 forms micelles in water that pack the bent mechanophores in a manner that prevents planarization in either GS or ES but enhances fluorescence emission intensity (cmc < 300 nm, Figure 1). Following a previously optimized procedure,[15] small spherical particles could be observed by confocal fluorescence microscopy (Figure 3c; Supporting Information, Figures S19, S20).
The excitation maxima of papillon probes in aqueous micelles, solid-ordered (S₃) DPPC membrane at 25°C,[13] liquid-disordered (Lₐ) DPPC membranes at 55°C[13] and ETOAc were indistinguishable, thus confirming that GS unbending does not occur. Compared to AIE from micellar probes in water, the emission of monomers in S₃ DPPC large unilamellar vesicles (LUVs) was much stronger (Figure S3; Supporting Information, Figure S8). For 1 in S₃ DPPC compared to water, the λₒ, from bent ES red-shifted only slightly (Δλₒ = +8 nm), and the λₐ₂ from planarized ES became detectable at λₐ₂ = 587 nm (Figure 2a; Supporting Information, Figure S8). Their Iᵦₒ/ᵦₐ = 3.4 confirmed that ES planarization is hindered in these highly viscous membranes (Table 1).

For 1 in DPPC LUVs at 55°C, intensity ratios inverted to Iᵦₒ/ᵦₐ ≈ 0.7 (Table 1, Figures 2a, 3d, red, solid). Contributions from thermochromism,[16] thermal fluctuations in viscosity[18] and the transition from S₃ into Lₐ membranes to this inversion were dissected using DOPC LUVs, which are in Lₐ phase at both temperatures (Tₐ (DPPC) = 41°C, Tₐ (DOPC) = −18°C).[17] At 55°C, the emission spectra of 1 in Lₐ DPPC and DOPC membranes were superimposable (Figure 3d, red). At 25°C, however, the Iᵦₒ/ᵦₐ in DOPC was half the Iᵦₒ/ᵦₐ in DPPC (Table 1, Figures 3d, blue, 2a, magenta, dashed). This remaining difference at 25°C originated from differences between S₃ and Lₐ membranes and thus demonstrated the compatibility of papillon 1 with ratiometric imaging in biomembranes.

The emission peaks from bent and planar ES in S₃ and Lₐ membranes were separated by λₒ ≈ +120 nm (4380 cm⁻¹, Figure 2a). With probes that operate by GS untwisting, the maximal red shift in excitation achieved so far is around λₒ ≈ 70 nm.[19] The resulting dual-emission spectra of 1 cover enough of the visible range that fluorescence photographs taken of the vesicles show the emission of white light, with the encoded ratiometric information beautifully visible in the reflections, blue for S₃, pink for Lₐ membranes (Figure 3g).

With core-expanded probes 2 and 3, the red-shift in emission from bent and planar ES in S₃ and Lₐ membranes decreased to Δλₒ = +78 nm and +75 nm, respectively (2540 cm⁻¹ and 2660 cm⁻¹, Figure 2). This loss was accompanied by an increased red shift from AIE in micelles to monomer emission in S₃ DPPC membranes of up to Δλₒ = +46 nm (Figures 2b,c, black dotted vs. blue solid). Importantly, the residual bent ES in Lₐ DOPC at 25°C decreased from Iᵦₒ/ᵦₐ = 1.7 for 1 to Iᵦₒ/ᵦₐ = 1.0 for 2 and Iᵦₒ/ᵦₐ = 0.7 for 3 (Figure 2, magenta). More distinct spectral signatures were the consequence, culminating in a full inversion from Iᵦₒ/ᵦₐ = 3.1 in S₃ to Iᵦₒ/ᵦₐ = 0.7 in Lₐ membranes for papillon 3 (Figure 2c). These trends supported implications from bulk solvent that ES planarization in core-expanded papillons 2 and 3 is facilitated compared to original 1.

The partitioning of amphiphiles 1–3 into lipid bilayers was almost instantaneous (Supporting Information, Figure S1). Partition coefficients Kₓ revealed an up to 10-fold preference for Lₐ over S₃ membranes (Table 1, Figure 3c,f; Supporting Information, Figures S6–S13). For ratiometric sensing, this preference was desirable because emission from S₃ is more intense than from Lₐ membranes (Figure 3d). Independence of Iᵦₒ/ᵦₐ on probe concentration confirmed that they emit as monomers from S₃ and Lₐ membranes and excluded probe aggregation as origin of the change in ratios (Supporting Information, Figures S3, S4).

Ratiometric sensing is interesting for imaging because the response is concentration-independent. This has been achieved previously by coupling responsive and unresponsive fluorophores,[4,6] dissecting shifts of single-emission maxima,[9] and creating probes with two maxima with different response.[5–12] 3-Hydroxyflavones stand out among the rare examples for single ground states with dual emission realized so far.[19] Operating by a completely different mechanism (membrane hydration sensitive),[11] these ratiometric probes, like papillon 1, produce overall white fluorescence and can discriminate membranes of different order.[13]

Because their excitation maximum occurs at rather short wavelength for confocal laser scanning microscopy, two-photon excitation fluorescence microscopy (TPFM)[18–20] was considered for imaging. Two-photon absorption spectra...
and roughly identical with those from S, DPPC (Figure 2b, blue) and Lᵣ, SM/CL LUVs (Supporting Information, Figure S5). Similarity and concentration independence were also confirmed for emission spectra of 2 in Lᵣ, DOPC GUVs (Figure 4a,d) and LUVs (Figure 2b, magenta).

The emission spectra recorded for TPEFM-imaged L₀ (SM/CL 7:3) and Lᵣ (DOPC) GUVs (Figure 4d,e) were used to deconvolute the TPEFM images of SM/DOPC/CL 58:25:17 GUVs. Comparable to the white fluorescence of LUVs (Figure 3g), non-deconvoluted global TPEFM images of papillon 2 produced almost uniform white emission without clearly visible domains (Figure 4c). After spectral deconvolution,[30] Lᵣ and L₀, domains became visible (Figure 4f). The emission spectra in these domains were identical with emission spectra of pure L₀ and Lᵣ GUVs.

In summary, papillon probes 1–3 allow to distinguish micelle and membrane environments with fluorescence spectroscopy, ratiometric TPEF microscopy (Figure 4), and with the naked eye (Figure 3g). With increasingly ordered membranes, the decreasing planarization found for papillon probes is contrary to increasing planarization for flipper probes.[13] This difference is interesting: Whereas flippers operate in the GS under equilibrium conditions and respond exclusively to mechanical confinement in space, papillons operate off-equilibrium. Our results reveal that their ES unbending reports on kinetics exclusively, that is, viscosity, also against expectations from steric.

In biomembranes, fluorescent probes report on complex lipid mixtures that are exposed to chemical and physical stimulation such as tension or voltage. Their response depends on many parameters such as partitioning between different domains, positioning and repositioning in the membrane, disturbance of the local environment, and so on. In this context, the introduction of mechanosensitive membranes probes with different shape that operate with fundamentally different modes of action is of general importance because acting differently, they can be expected to respond differently and, at best, reveal characteristics of biological relevance that pass unnoticed by other probes.

Acknowledgements
We thank N. Chuard, K. Straková, J. López-Andarias, A. Goujon, and A. Colom for assistance, the NMR, the MS, and the bioimaging platforms for services, and the University of Geneva, the Swiss National Centre of Competence in Research (NCCR) Chemical Biology, the NCCR Molecular Systems Engineering and the Swiss NSF for financial support. H.V.H. is a fellow of the Alfred Werner Foundation.

Conflict of interest
The authors declare no conflict of interest.

Keywords: bent aromatics · fluorescent probes · lipid bilayer membranes · mechanochemoactivity · white fluorescence

How to cite: Angew. Chem. Int. Ed. 2018, 57, 10559–10563
Angew. Chem. 2018, 130, 10719–10723

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were measured for all probes in EtOAc (Figure 2; Supporting Information, Figure S20) and cross-sections [31] were determined (Table 1). Giant unilamellar vesicles (GUVs)[32] composed of sphingomyelin (SM), DOPC and cholesterol (CL) are the gold standard to probe for the imaging of co-existing liquid-ordered (Lₒ) SM/CL and Lᵣ DOPC domains, also for ratiometric detection with two-photon excitation fluorescence microscopy (TPEFM).[13,12–18] In LUVs, the DPPC membranes were preferable to characterize papillon probes because Lₒ-S phase transition can be studied in the presence of the probes (Tₒ = 41 °C, Figure 3d). To assess the comparability of these two standard conditions, emission spectra of 2 in TPEFM-imaged Lₒ, SM/CL 7:3 GUVs were recorded at 10 nm resolution, using 25 independent detection channels to cover the range from 400 nm to 650 nm. The obtained spectra were concentration independent up to 2 µM 2 (Figure 4b,c).
