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Abstract

In this report, “fluorescent flippers” are introduced to create planarizable push–pull probes with the mechanosensitivity and fluorescence lifetime needed for practical use in biology. Twisted push–pull scaffolds with large and bright dithienothiophenes and their S,S-dioxides as the first “fluorescent flippers” are shown to report on the lateral organization of lipid bilayers with quantum yields above 80% and lifetimes above 4 ns. Their planarization in liquid-ordered (Lo) and solid-ordered (So) membranes results in red shifts in excitation of up to +80 nm that can be transcribed into red shifts in emission of up to +140 nm by Förster resonance energy transfer (FRET). These unique properties are compatible with multidomain imaging in giant unilamellar vesicles (GUVs) and cells by confocal laser scanning or fluorescence lifetime imaging microscopy. Controls indicate that strong push–pull macrodipoles are important, operational probes do not relocate in response to lateral membrane reorganization, and two flippers are indeed needed to “really swim,” i.e., achieve high mechanosensitivity.

Reference


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Fluorescent Flippers for Mechanosensitive Membrane Probes

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Supporting Information

ABSTRACT: In this report, “fluorescent flippers” are introduced to create planarizable push–pull probes with the mechanosensitivity and fluorescence lifetime needed for practical use in biology. Twisted push–pull scaffolds with large and bright dithienothiophenes and their S,S-dioxides as the first “fluorescent flippers” are shown to report on the lateral organization of lipid bilayers with quantum yields above 80% and lifetimes above 4 ns. Their planarization in liquid-ordered (Lo) and solid-ordered (So) membranes results in red shifts in excitation of up to +80 nm that can be transcribed into red shifts in emission of up to +140 nm by Förster resonance energy transfer (FRET). These unique properties are compatible with multidomain imaging in giant unilamellar vesicles (GUVs) and cells by confocal laser scanning or fluorescence lifetime imaging microscopy. Controls indicate that strong push–pull macromolecules are important, operational probes do not relocate in response to lateral membrane reorganization, and two flippers are indeed needed to “really swim,” i.e., achieve high mechanosensitivity.

T
o uncover the secrets of biological membranes, that is, their order, homogeneity, tension, potential, and so on, many inspired approaches to fluorescent probes have been conceived over the years.1 They explore the usefulness of excited-state polarization (intramolecular charge transfer in push–pull chromophores, solvatochromism, electrochromism),2 twisted intramolecular charge transfer (TICT),3 excited-state intramolecular proton transfer (ESIPT),4 Förster resonance energy transfer (FRET),5 photoinduced electron transfer (PET),6 two-photon absorption (TPA),7 second harmonic generation (SHG),8 fluorescence lifetime imaging microscopy (FLIM),9 and 8-ratiometry.10 Recently, we have introduced the concept of planarizable push–pull probes (Figure 1A).11 This combination of polarization1,2,12 and planarization13 is interesting because it applies lessons from nature11,12 and promises access to the imaging not only of the lateral organization of biomembranes13,5–8 but also of membrane potentials1,3–5,7,8,12 and the poorly detectable but biologically important membrane tension.14 Focusing on ground-state planarization and thus changes in the excitation spectrum,11 planarizable push–pull probes can be envisioned as complementary to molecular rotors that operate with excited-state deplanarization and, thus, as most other fluorescent probes, with changes in emission or quantum yield.3,9

The concept of planarizable push–pull probes has been elaborated with oligothiophenes.11 Meticulous sculpting with regard to length, donors, acceptors as well as a comprehensive coverage of the quaterthiophene twistome gave probe 1 that could visualise the fluidity of lipid bilayer membranes for the “naked eye” (Figure 1A). Most importantly, it was found that intermediate twisting was ideal, whereas weak twists gave poor shifts because there is little to planarize and strong twists gave poor shifts because planarization became too hard. However, with oligothiophenes,11 red shifts obtained upon planarization in So lipid bilayers could reach only up to Δλex = +44 nm, and fluorescence became very weak with increasing twisting. These limitations were attributed to the poor mechanosensitivity of the small thiophene ring and the negligible fluorescence of isolated thiophene monomers in twisted oligomers. To overcome these two limitations, monomers with large surface area and high intrinsic quantum yield would have to be incorporated into the twisted oligomers. In the following, the term “fluorescent flippers” (or swimfins) is used as a “symbol” for such large and bright monomers in twisted oligomers because, although not fully fitting with regard to all aspects, they provide a helpful memorizer that associates correctly with the characteristics of interest (Figure 1B). The previously explored thiophene monomers11 failed to perform as fluorescent flippers because they do not fluoresce and their surface area is too small to feel the environment well. Here, we introduce dithienothiophenes and their S,S-dioxides16 as the first fluorescent molecular flippers and show that the new push–pull mecanophore 2 provides access to membrane probes with high mechanosensitivity and long fluorescence lifetime.

Flipper probe 2 was designed as follows (Figure 1B). The twist between the dithienothiophene and the dithienothiophene S,S-dioxide flipper was induced with two proximal methyl groups. To enhance the push–pull system, the electron-deficient dithienothiophene S,S-dioxide was terminated with an aldehyde acceptor, whereas the electron-rich dithienothiophene was terminated with a methylene donor. A negative charge was added at the donor terminus to ensure delivery to and oriented partitioning into lipid bilayer membranes. Fluorophores 4–6 were designed as controls with either reduced macrodipole or one flipper only.

The synthesis of mecanophore 2 is outlined in Scheme 1. Bromothiophene 7 was converted into the dithienothiophene monomer 8 following reported procedures.16 Vilsmeier formulation afforded the key intermediate 9. Bromination of
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aldehyde 9 and oxidation of product 10 with mCPBA gave S,S-
dioxide 11. Reduction of aldehyde 9 followed by silyl protection
of the obtained alcohol gave dithienothiophene 12. Stille
coupling of 11 and 12 gave the double flipper 13, deprotection
and esterification with the cyclic anhydride 14 the target probe 2.
Double-flipper control 4 was accessible in a few steps from 13,
single flipper 6 from intermediate 12, donor 3 and control 5 were
pre pared similarly (Schemes S2–S4).

The absorption maximum of the push–pull system in flipper
probe 2 in chloroform was at \( \lambda_{\text{abs}} = 435 \) nm (Figure S5). The
emission maximum showed significant solvatochromism from
S30 to almost 700 nm (Figure S5). Lippert analysis \(^{34}\) of the
dependence of the Stokes shift on solvent polarity gave a variation
of permanent dipole moment upon excitation of \( \Delta \mu = 14 \) D
(Table S1). In comparison, blue-shifted absorption in chloroform
at 418 nm and with \( \Delta \mu = 11 \) D weaker solvatochromism
confirmed that the push–pull system of 2 is weaker than that of 2.
The fluorescence quantum yield of the push–pull system of 2 in
chloroform was \( \phi = 83\% \) (control 4, \( \phi = 66\% \); 5, \( \phi = 32\% \); cf, 1, \( \phi = 20\% \))\(^{34}\). This excellent value compared very well
with the reported quantum yields of dithienothiophene S,S-dioxides \(^{35}\) and
thus confirmed that chromophore twisting does not reduce
fluorescence.

Dependent on the nature of the phospholipids used, bilayer
membranes undergo a sharp transition from liquid-disordered
(\( \text{L}_d \)) or fluid phase to solid-ordered (\( \text{S}_o \)) or gel phase at a

characteristic temperature, the chain-melting temperature
\( T_{\text{m}} \). With increasing concentration of cholesterol, this
phase transition disappears, and the mixed membranes exist
permanently in the liquid-ordered (\( \text{L}_o \)) phase, which is
characterized by short-range orientational order and long-range
translational disorder. \(^{36}\) In multicomponent bilayers, binary
mixtures of lipids with cholesterol and beyond, \( \text{L}_o \) and \( \text{L}_d \)
membranes can coexist as immiscible microdomains. \(^{37,38}\)
Viscosity and elastic modulus increase from \( \text{L}_d \) over \( \text{L}_o \) to \( \text{S}_o \),
whereas lateral diffusion decreases. For example, diffusion
coefficients in \( \text{L}_o \) are about 3 to 5 times smaller than in \( \text{L}_d \) phase. \(^{39}\)
Viscosity increases from 100–300 cP in \( \text{L}_d \) to up to 1300 cP in \( \text{S}_o \)
phase.

The mechanosensitivity of flipper probe 2 was evaluated in
large unilamellar vesicles (LUVs). LUVs composed of DPPC
(dipalmitoyl-sn-glycero-3-phosphocholine) have \( \text{S}_o \, \text{–} \text{L}_d \) transition at 41 °C. The excitation spectrum of 1.0 \( \mu \text{M} \) (1.3 mol %)
added to \( \text{L}_d \) DPPC LUVs at 55 °C showed two maxima at \( \lambda_{\text{ex}} = 453 \) nm and \( \lambda_{\text{em}} = 329 \) nm of equal intensity (\( \Delta F_{\text{ex}} / \Delta F_{\text{em}} = 0.97 \),
Figure 2A, red, solid). The \( \Delta \lambda_{\text{ex}} = +18 \) nm from chloroform
suggested that flipper probe 2 could already be partially
planarized in \( \text{L}_d \) DPPC. Cooled down to 25 °C, an intense
peak with a flat maximum \( \lambda_{\text{ex}} = 498–533 \) nm emerged,
accompanied by a sharper but weaker band at \( \lambda_{\text{ex}} = 352 \) nm
(\( \Delta F_{\text{ex}} / \Delta F_{\text{em}} = 1.48 \), Figure 2A, blue, solid). A red shift of up to
\( \Delta \lambda_{\text{ex}} = +80 \) nm in response to \( \text{L}_d \rightarrow \text{S}_o \) transition, obtained with the
first unoptimized flippers, clearly exceeded \( \Delta \lambda_{\text{ex}} = +44 \) nm of the
best flipper-free probe 1. This finding supported that increasing
surface area in twisted push–pull probes increases mechanosensitivity,
as expected from “fluorescent flipper.”

Several control experiments were conceived to probe the
validity of these important conclusions. The temperature
independence of the excitation maxima of 2 in DOPC
(dioleoyl-sn-glycero-3-phosphocholine) was important because
DOPC membranes are always in \( \text{L}_d \) phase (Figure 2A, dotted).
Excluding thermostochromism, this finding supported that the up to
\( \Delta \lambda_{\text{ex}} = +80 \) nm found in \( \text{S}_o \, \text{DPPC} \) originates indeed from ground-
state planarization of the twisted flipper. The concentration
independence of the spectroscopic properties, most importantly,
excluded contributions from aggregation \(^{34}\) to the mechanosensitivity
of 2 (Figure S4). The insensitivity of the emission maxima of 2 at \( \lambda_{\text{em}} = 600 \) nm to temperature, concentration, lipid
composition and lateral organization (Figure 2B) supported

Figure 1. (A) Planarizable push–pull probes are conjugated oligomers with electron donors (D) and acceptors (A) at their termini and bulky twist
inducers along the scaffold (red circles); their planarization in lipid bilayers is expected to report on membrane order, potential and tension (horizontal
gray arrows). (B) Fluorescent flipper, i.e., monomers in twisted push–pull probes with high surface area and fluorescence, are introduced to maximize
mechanosensitivity and fluorescence lifetime. Double-flipper probe 2 is shown together with FRET donor 3 (B), push–pull control 4, single-flipper
controls 5 and 6 (C) and original oligothiophene 1 (A).

Scheme 1a

(a) 1. S(SnBu3)2, Pd(PPh3)4, toluene, 130 °C; 2. BuLi, CuCl2, Et,O,
0 °C to rt, 64%; (b) POCl3, DMF, 50 °C, 75%; (c) NBS, DMF, 80 °C,
80%; (d) 1. NaBH4, DMF, 2. imidazole, TBDPSCI, DMF, 70%;
(e) mCPBA, CH2Cl2, 40 °C, 65%; (f) 1. LDA, Bu3SnCl, THF; 2. 11,
Pd(PPh3)4, DMF, 46%; (g) 1. TBAF, AcOH, THF, rt, 2. 14, TEA,
THF, 60 °C, 38%; (h) several steps, see Scheme S1.

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the first relaxed excited state is fully planarized in all environments, and excluded contributions from solvatocolchism. Clearly, the twisted flipper 2 act very differently from ordinary push–pull membrane probes.1–3

Double-flipper control 4 with reduced macrodipole gave not only an overall blue-shifted excitation maximum ($\lambda_{\text{ex}} = 486$ nm; 2: $\lambda_{\text{ex}} = 498–533$ nm) but also an incomplete planarization in $S_m$ membranes ($\lambda_{\text{em}} = 420$ nm observed in $L_d$ membranes was partially preserved, $\Delta \lambda_{\text{emp}} = +66$ nm of 4 in response to planarization remained significant (2: $\Delta \lambda_{\text{emp}} = +45$ to +80 nm). All single-flipper controls prepared and tested were useless because of poor partitioning (6, Figure S2) or mechanosensitivity (5, Figure S3).

Fluorescence depth quenching was used to explore probe repositioning upon membrane reorganization.18 DPPC vesicles were labeled with DOXYL-PC probes carrying the quencher either in position 5 or 12 of the lipid tails (Figure 1B). Parallax analysis of the different quenching efficiencies of 5- and 12-DOXYL-PC gave $z_q$ that is the traverse distance $z$ from the plane of the bilayer center to the plane containing the fluorophore (Figures S8, S9). The $z_q = 15.1$ Å obtained for 2 exceeded $z_q = 9.6$ Å of 4 and $z_q = 1.6$ Å of 3 clearly (Figure 1). Assuming that positioning is determined by external charges and coaxial alignment with the lipid tails (Figure 1), $z_q$ decreasing with $2 > 4 > 3$ suggested that quenching occurs preferably near the donor terminus of push–pull fluorophores. Coaxial probe alignment with lipid tails was in agreement with preliminary results from fluorescence anisotropy measurements in GUVs, depth quenching by hole transfer was consistent with the literature.19 Cooling down from $L_d$ into $S_m$ DPPC, the $z_q$ of flipper 2 decreased from $z_q = 15.1$ Å to $z_q = 12.3$ Å, i.e., $\Delta z_q = -2.8$ Å (4, $\Delta z_q = 0$ Å; 3, $\Delta z_q = +2.1$ Å). This very minor probe repositioning upon membrane reorganization is quite remarkable. Mismatched objects are not often tolerated in crystalline $S_m$ membranes and usually simply ejected.22 Depth quenching experiments thus provided powerful experimental support that the up to $\Delta \lambda_{\text{emp}} = +80$ nm observed upon $L_d$ to $S_m$ phase change originates indeed from planarization of the twisted push–pull mechanophore 2 and not a change in location of the fluorophore.

Fluorescence lifetimes of 2 increased from 2.2 ns in $L_d$ DOPC LUVs to 4.3 ns in $S_m$ DPPC LUVs (Figure 2D, circles). Clearly better than those of the flipper-free original 1 (DOPC, 0.47 ns; DPPC, 0.76 ns, Figure 2D, squares), these lifetimes are in the range of established bioprobes,3a and thus demonstrate compatibility of fluorescent flipper 2 with FLIM. Preliminary results with GUVs fully support this conclusion. In agreement with the high $\phi = 83\%$ found in CHCl$_3$, these findings provide corroborative evidence for the validity of the concept of fluorescent flipper.

Partition coefficients measured21 for flipper 2 indicated a weak preference for $L_d$ membranes over $S_m$ membranes at 25 °C (DPPC, $K_p = 7.7 \times 10^4$; DOPC, $K_p = 1.3 \times 10^5$, Figure S10). Confoocal laser scanning microscopy (CLSM) of GUVs composed of SM/DOPC/CL 58:25:17 (SM: sphingomyelin, CL: cholesterol) and labeled with flipper 2 showed two domains, which light up by exciting at different wavelengths (Figure 3A, $\lambda_{\text{ex}} = 480$ nm, green; Figure 3B, $\lambda_{\text{ex}} = 560$ nm, red; Figure 3C, merged). Co-labeling experiments with a commercial probe for the $L_d$ phase (cyan, $\lambda_{\text{ex}} = 630$ nm, Figure 3E) confirmed that the emission observed upon excitation at longer wavelength (red, $\lambda_{\text{ex}} = 551$ nm, Figure 3E) arises from flipper 2 in the $L_d$ phase (red, Figure 3B).

Twisted push–pull flipper report on their environment with shifts of their excitation maxima. Although unproblematic for fluorescence imaging (Figure 3), shifts in emission rather than
excitation are preferable for biological probes. To transcribe shifts in excitation to shifts in emission, terthiophene 3 was considered as FRET donor for mechanophore 2 (Figure 1). Donor 3 exhibited a mechanosensitive hypsochromic \( \Delta \lambda_{em} = 400 \text{ nm} \) in DOPC and DPPC membranes (Figure 2E, dashed, cyan and purple). In this region, the excitation of flipper 2 is weak and mechanosensitive (Figure 2E, dotted, blue, red). Flipper 2 will thus not interfere significantly with the excitation of donor 3 under varied conditions.

The emission maximum of donor 3 at \( \lambda_{em} = 460 \text{ nm} \) (Figure 2F, dashed, cyan) coincided roughly with the excitation maxima of flipper 2 in DOPC (Figure 2F, dotted, black) and DPPC (Figure 2F, dotted, gray). In DOPC LUVs colabeled with donor 3 and acceptor 2 at equal concentrations, excitation of donor 3 at 405 nm gave an emission spectrum with dominant donor emission at \( \lambda_{em} = 460 \text{ nm} \) (Figure 2F, dashed, red). In DPPC LUVs under identical conditions, a significant new maximum appeared at \( \lambda_{em} = 600 \text{ nm} \) (Figure 2F, solid, blue). This difference was consistent with the occurrence of significant FRET to the planarized, more fluorescent flipper 2 in \( s_m \) membranes and negligible FRET to the deplanarized, less fluorescent flipper 2 in \( L_m \) membranes. Control experiments under identical conditions in DPPC with original probe 1 in place of flipper 2 did not afford significant FRET because of insufficient fluorescence of acceptor 1. These results demonstrate that the transcription of the red shift up to \( \Delta \lambda_{em} = +80 \text{ nm} \) achieved by ground-state planarization of push–pull flipper 2 to \( \Delta \lambda_{em} = +140 \text{ nm} \) in emission is possible with FRET.

Taken together, these results describe the first mechanosensitive push–pull probe that (a) operates, with all likelihood, by planarization of single isolated mechanophores in the ground state (rather than the formation or rearrangement of aggregates) and (b) offers properties that are sufficient for use in practice. This breakthrough was achieved with the introduction of “fluorescent flipper” (large, bright monomers). They provide access to the mechanosensitivity and fluorescence lifetime needed for biological applications (i.e., \( \Delta \lambda_{em} \uparrow \) up to \( +80 \text{ nm} \) from ground-state planarization), \( \Delta \lambda_{em} = +140 \text{ nm} \) (transcribed by FRET), \( \Delta \tau = 2.1 \text{ ns}, \phi > 80\% \). Current efforts focus on the polishing of the operational flippers with regard to twist, macrodipole and terminal charges, on covalent FRET probes, and on applications toward biophysical and biological questions related to membrane order, potential and tension.

**REFERENCES**


**Notes**

The authors declare no competing financial interest.

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