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Headgroup engineering in mechanosensitive membrane probes†

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Systematic headgroup engineering yields planarizable push-pull flipper probes that are ready for use in biology – stable, accessible, modifiable –, and affords non-trivial insights into chalcogen-bond mediated mechanophore degradation and fluorescence enhancement.

The need to image forces in biological systems calls for the invention of mechanosensitive fluorescent probes.[1] To contribute to solutions for this most important challenge in the life sciences, planarizable push-pull probes have been introduced.[2] Applying lessons from nature and supramolecular chemistry,[3] the best mechanophore realized so far is the twisted dithienothiophene (DTT)[4] dimer **1** (Figure 1).[5] In **1**, the DTTs are considered as "fluorescent flippers" that are bright enough to keep on shining when twisted out of conjugation in dimer **1** and offer a large enough surface to feel the environment really well. In **1**, the two flippers are twisted out of planarity with two methyl groups that are repelled by the σ hole on the endocyclic sulfur on the other side of the twistable bond, an arrangement that can be referred to as chalcogen-bond repulsion or chalcogen anti-bond[6-7] (Figure 1*). Initial polarization of mechanophore **1** is introduced by combining an electron-rich DTT flipper with an electron-poor, highly fluorescent DTT S,S-dioxide with donating "sulfides" and withdrawing "sulfones" as bridges between the external thiophenes. The acceptor side is further enhanced with an aldehyde acceptor that is prevented from rotational quenching (but also weakened) by an intramolecular 1,4 S-O chalcogen bond (Figure 1**).[7] The donor terminus continues with a "thenyl" ester that might act as non-covalent donor via a 1,6 S-O chalcogen bond (Figure 1***).

Fluorescent flipper **1** was designed to serve as fluorescent membrane probe.[8-10] The specific objective is to tackle one of the key challenges in the field, that is the imaging of membrane tension.[1] These high expectations were supported by a red shift of the excitation maximum in response to the transition from liquid-disordered (L_d) to solid-ordered (S_o) lipid bilayer membranes.[5] However, flipper probe **1** is chemically unstable, and thus, cannot be derivatized. In the following, we describe the chemically not trivial headgroup engineering that was needed to find the stable, operational and easily derivatizable fluorescent membrane probe **2**.

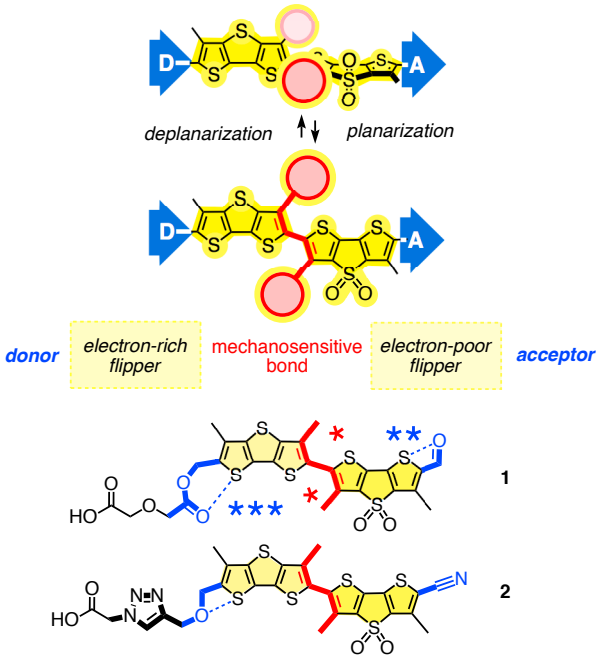

Fig. 1. The concept of planarizable push-pull probes together with the structure of the unstable probe **1** and the stable probe **2**. □: σ-hole repulsion for deplanarization. □□: Possible 1,4 S-O chalcogen bond to prevent rotational quenching. □□□: Possible 1,6 S-O chalcogen-bonding long-distance donor.

We ascribed the instability of **1** to the aldehyde and the thenyl ester groups. Replacement of the former by a cyano acceptor, also to prevent eventual imine formation in cells, was unproblematic. The latter, activated thenyl ester likely suffers from acid- or base-catalyzed hydrolysis, and thus its substitution with a more stable carbamate was considered first. Using the elegant method from the Bochet group,[11] this carbamate was easily accessible from alcohol **3**. However, acidic conditions applied to cleave the *tert*-butyl ester in **4** resulted in degradation of the mechanophore into a complex mixture.

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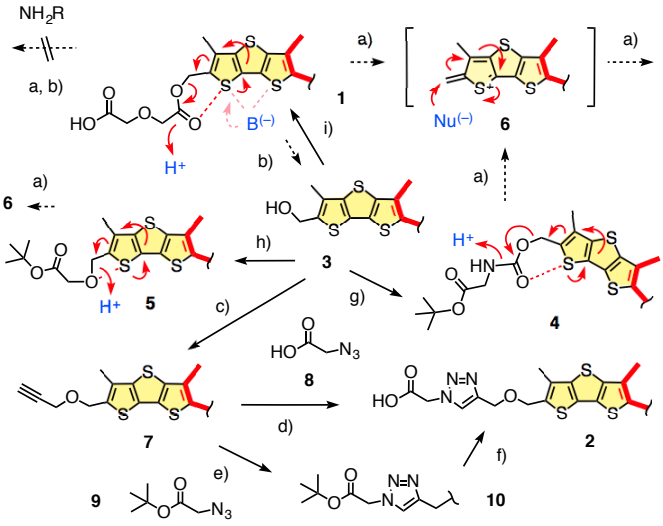

Scheme 1. Synthesis of probe **2** (c-f) together with failed alternatives (g, h) and possible degradation pathways (dashed arrows). a) possible, acid (H^+), base ($B^{(-)}$) and chalcogen-bond catalyzed probe decapitation; b) possible ester hydrolysis; c) 1. NaH, THF, –20 °C, 1 h, 2. propargyl bromide, THF, rt, 2 h, 80%; d) sodium ascorbate, $CuSO_4 \bullet 5\ H_2O$, TBTA, CH_2Cl_2, rt, 10 min, 90%; e) idem, 30 min, 90%; f) TFA, CH_2Cl_2, rt, 1 h, 50%; g) 1. CDI, CH_2Cl_2, 0 °C to rt, 2 h, 2. *tert*-butyl glycinate, CH_2Cl_2, rt, 1 h, 63%; h) 1. NaH, THF, –20 °C, 1 h, 2. *tert*-butyl bromoacetate, THF, rt, 2 h, 75%.

More stable thenyl ethers were tested next. With cyano (but not aldehyde) acceptors at the other side of the probe, Williamson ether synthesis of **5** from alcohol **3** with activated alkylhalides was tolerated, but the *tert*-butyl ester in **5** could not be hydrolyzed either without decomposition of the probe.

According to ^{1}H NMR spectra of the reaction mixture, the structure of the DTTs seems affected under deprotection conditions (Figure S14). The most convincing explanation was the elimination of the headgroup to yield the reactive intermediate **6** (Scheme 1a), an intriguing process that can be triggered by acid, base as well as intramolecular chalcogen bonds (below). The cleanly guillotinylated intermediate **6** was generally detectable as main peak in ESI mass spectra (Figure S16). In solution, the strong electrophile **6** will react with nucleophiles of free choice to produce the observed complex mixtures (Figure S14).[12]

These synthetic problems were solved with copper-catalyzed alkyne-azide cycloaddition (CuAAC).[13] Alkyne **7** was accessible without problems in 80% yield from thenyl alcohol **3** and the reactive propargyl bromide. CuAAC with azide **8** and thenyl ether **7** gave flipper probe **2** under mildest conditions in excellent 90% yield (10 min, rt, Scheme 1d). CuAAC with *tert*-butyl ester **9** in place of azide **8** afforded triazole **10** in similarly good yield. Ironically, when it was not needed anymore to access the target amphiphile **2**, hydrolysis of *tert*-butyl ester **10** proceeded without problems. The stability of mechanophores **2** and **10** but neither **1** nor **4** nor **5** under acidic conditions was in excellent agreement with the proposed elimination mechanism. Namely, the triazole (conjugate acid: pK_a ~1.3)[14] is a stronger base than the thenyl ether. The triazolium cation obtained first in the presence of acid destabilizes the conjugate acid of the thenyl ether by intramolecular charge repulsion, i.e., further weakens the basicity of the thenyl ether. This most general proximity effect[15] prevents the protonation of the thenyl ether. The resulting inhibition of acid-catalyzed activation of the leaving group for headgroup elimination (compare **5** in Scheme 1) then explained why the presence of the triazole is essential for probe stability.[16]

Flipper probe **2** was evaluated first under routine conditions in DPPC LUVs (large unilamellar vesicles composed of dipalmitoyl-*sn*-glycero-3-phosphocholine).[5] Upon cooling from L_d membranes at 55 °C into S_o DPPC membranes at 25 °C, the excitation maximum of **2** shifted from λ_{ex}^{Ld} = 435 nm to a broadened maximum at λ_{ex}^{So} = 485 nm but extending until λ_{ex}^{So} = ~525 nm (Figure 2). This calculated to a red shift of $\Delta\lambda_{ex}$ = 50–90 nm. The absence of such shift in dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) membranes, which stay in L_d state at both 55 and 25 °C, demonstrated that membrane order and not temperature accounts for this change (Figure 2). Compared to the unstable probe **1**, the spectroscopic properties of the stable **2** were similar but overall blue shifted by ~50 nm.[5]

The $\Delta\lambda_{ex}$ = 50–90 nm of **2** upon L_d-S_o transition coincided with an increase in fluorescence intensity by a factor of I_{So}/I_{Ld} = 2.7 (**1**: I_{So}/I_{Ld} = 2.1). In FLIM images of GUVs, the fluorescence lifetimes increased from τ^{Ld} = 2.7 ns in L_d (DOPC) to τ^{Lo} = 5.8 ns in L_o membranes (SM/CL 7:3, Table S3; FLIM: Fluorescence lifetime imaging microscopy, GUVs: Giant unilamellar vesicles, SM: Spingomyelin, CL: Cholesterol). Consistent with increasing fluorescence intensity, this increase of the fluorescence lifetime of probe **2** exceeded even that of **1** slightly (τ^{Lo}/τ^{Ld} = 2.1; **1**: τ^{Lo}/τ^{Ld} = 2.0; τ^{Ld} = 2.8 ns, τ^{Lo} = 5.7 ns, Table S3). As with probe **1** at λ_{em} = 600 nm, identical emission maxima of **2** in S_o and L_d membranes at λ_{em} = 570 nm (Figure 2) supported that the changes in excitation originate from ground-state planarization of the twisted flipper probe with increasing membrane order (Figure 1) and not from solvatochromism,[9] excited-state deplanarization of molecular rotors[10] and other known processes.[8] Fluorescence kinetics under constant irradiation in EtOAc revealed that probe **2** is clearly more photostable than **1**, which in turn is much more photostable than fluorescein (Figure S3).

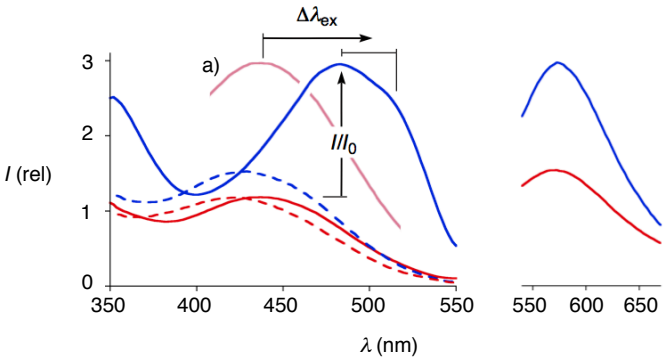

Fig. 2. Excitation and emission spectra of **2** in DPPC LUVs (solid) and DOPC LUVs (dotted) at 25 °C (blue) and 55 °C (red). a) Excitation of **2** in DPPC LUVs at 55 °C normalized to DPPC LUVs 25 °C (solid blue).

The main disadvantage of probe **2** compared to **1** is a reduced fluorescence quantum yield. In EtOAc, a ϕ_{fl} = 25% was measured for **2** against rhodamine 6G (Table S2). This remains satisfactory for use in practice considering that a) fluorescence intensity increases significantly upon planarization in membranes and b) the increase in fluorescence lifetime of **2** upon planarization (τ^{Lo}/τ^{Ld} = 2.1) even exceeds that of **1**

slightly (τ^{Lo}/τ^{Ld} = 2.0, Table S3). These changes in fluorescence lifetime are used for quantitative measurements by FLIM in living systems.[17] Simple substitution of the aldehyde in **1** by a cyano acceptor gave spectroscopic properties similar to **1** rather than **2**.[18] This control confirmed that it is the thenyl ester that accounts mostly for the two main advantages of **1** compared to **2**, i.e. high fluorescence quantum yield in EtOAc and a global red shift of ~50 nm. This result could surprise because the only way for remote thenyl esters to connect with the mechanophore is *via* an intramolecular 1,6 S-O chalcogen bond (Figure 1***). The resulting global red shift suggested that this chalcogen bond could function as a non-covalent electron donor for the push-pull chromophore. This indication for spectral tuning with chalcogen bonds was most intriguing and deserves full consideration in future probe design. In the present probes, however, the long-distance chalcogen-bond donors also activate the destruction of the mechanophores (Scheme 1). The best fluorescent properties coincide with the worst stability, and the two properties cannot be decoupled. Thus, flipper **2**, with thenyl ether (as potential 1,4 S-O donor), triazole (to prevent headgroup elimination) and carboxylate (to deliver and orient in membranes) as essential triad, emerged as best for practical applications in cells.

In general, flipper probes can be added to cells without washing them because they fluoresce only when they are inserted within membranes, and not when dissolved in aqueous solution. The original flipper probe **1** became visible inside MDCK cells almost instantaneously (Figure 3a). Maybe because of its higher chemical instability, probe **1** was also quite cytotoxic (not shown).

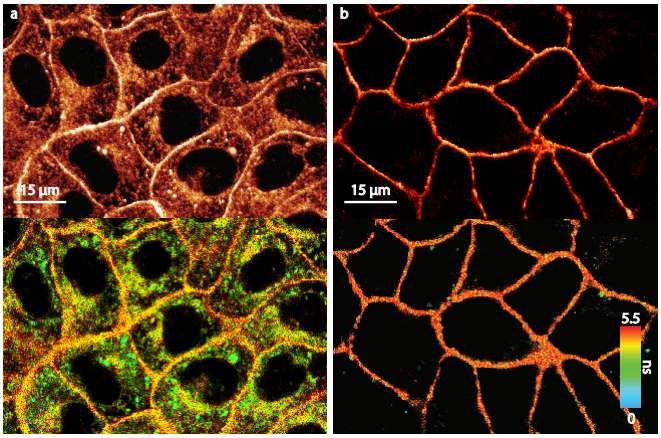

Fig. 3. Confocal (top) and FLIM images (bottom) of MDCK cells 2 min after the addition of flipper probes **1** (a) and **2** (b).

In clear contrast, the chemically stable probe **2** stained exclusively the plasma membrane during the first minutes (Figure 3b). Afterward, fluorescence started to appear inside the cell, cleanly imaging the normal recycling process of the membrane (not shown). Moreover, cell death after 3 days did not exceed that in control populations without flipper probes. Differences in lifetime observed with **1** may reflect that membrane ordering in organelles to be less than in the plasma membrane.[10] Local differences in lifetime could be used in the future to probe differences in lipid composition and membrane properties between organelles. Comprehensive studies in cells are ongoing, focusing on the possibility to image forces in biological systems with the new probe **2**. Preliminary results are encouraging and will be reported in due course.

These findings identify flipper probe **2** as ready for use in model membranes and cells: chemically stable, easily to make, easy to derivatize, less toxic, cleanly localized in cells and detectable by FLIM. The stunning difference identified between the dysfunctional original **1** and the operational flipper **2** nicely illustrates that the chemistry of new fluorescent probes has to be clarified before initiating studies in cells, particularly if they are meant to address significant challenges in biology. The involved chemistry turns out to be most intriguing, providing conceptually innovative examples for intramolecular chalcogen bonds in catalysis and fluorescent probes.[6,7]

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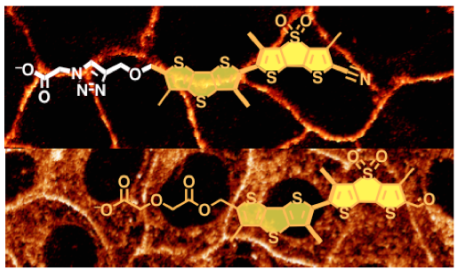

We describe the development of an unstable, toxic mechanosensitive membrane probe into a stable mechanophore that is ready for use in cells, and discover chalcogen-bond mediated reactions and fluorescence on the way.