pKa tuning in quadrupolar-type two-photon ratiometric fluorescent membrane probes

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Two bolaamphiphilic quadrupoles built from a fluorene core conjugated with azine endgroups were designed and successfully used to stain GUV membranes. Their quadrupolar character induces both a shift of the pKa values close to physiological pH and large two-photon absorption responses (i.e. over 1000 GM for acidic forms). As such they hold promise as ratiometric two-photon pH probes for monitoring slight variations of pH near cell membranes using two-photon excitation in the NIR region.

Multiphotonic excitation techniques (in particular two-photon laser scanning microscopy or TPLSM)[1, 2] have gained increased popularity in the biology community since the pioneering work by W.W. Webb and coll., due to the many advantages it provides in microscopic imaging (intrinsic 3D resolution, increased penetration depth…). This has triggered the search for dedicated biphotonic fluorescent probes having large two-photon absorption (2PA) cross-sections (σ_2) in the biological spectral range (i.e. 700-1100 nm) for improved sensitivity.[3-13] This requirement is particularly relevant for detection of species in very low concentrations (such as heavy metals,[14-18] metabolites…) or for real-time imaging of fast biological events which requires fast scanning speed. In that general framework, our goal is to design highly sensitive biphotonic pH probes for imaging little pH changes close to physiological pH (7.2-7.4) in cell membranes. Such probes should allow recognition of cancer cells (pH=6.4-6.8)[19-21] at early stages. Several water-soluble biphotonic pH probes have been developed in recent years, mainly based on dipolar (push-pull) architectures, which either have reasonable but not considerable 2PA cross-sections,[22-25] and/or are not highly sensitive in the targeted pH range (i.e. showing with pKa values between 4.5 and 5.5)[26-29] Quadrupolar-type biphotonic probes combining large 2PA responses and high sensitivity to pH changes were also reported. Yet they were well suited only for pH conditions far away from physiological conditions (i.e. very acidic or very basic pH), with pKa of 3.5 and 9.5, respectively.[30]

In the present paper, we describe our approach towards biphotonic ratiometric membrane probes that combine large 2PA responses in the NIR region, as well as high pH sensitivity in the targeted pH range (pH= 6-7). Our strategy is based on the exploitation of the *quadrupolar effect to shift pKa* by using symmetrical bolaamphiphilic probes having pH sensitive acceptor end-groups (Figure 1). Based on our earlier work on molecular optimization of quadrupolar chromophore for 2PA, we selected derivatives built from a fluorene core and having phenylene-ethynylene connectors.[31] Butyl aliphatic chains were grafted on the core fluorene moiety to improve the lipophilicity of the rod part or the probes, while hydrophilic terminal moieties were used to provide the bolaamphiphilic character. The chromophores length (l~3.4 nm) matches the thickness of the lipidic bilayer to facilitate incorporation in cell membranes in a preferential transmembrane fashion. We focused on pyridine (pKa=5.2) and quinoline (pKa=4.85) moieties because we posited that the quadrupolar effect would shift the electronic distribution in such a way that the basicity of the end moieties would be reinforced (Figure 1) thus shifting the pKa value towards pH 7.

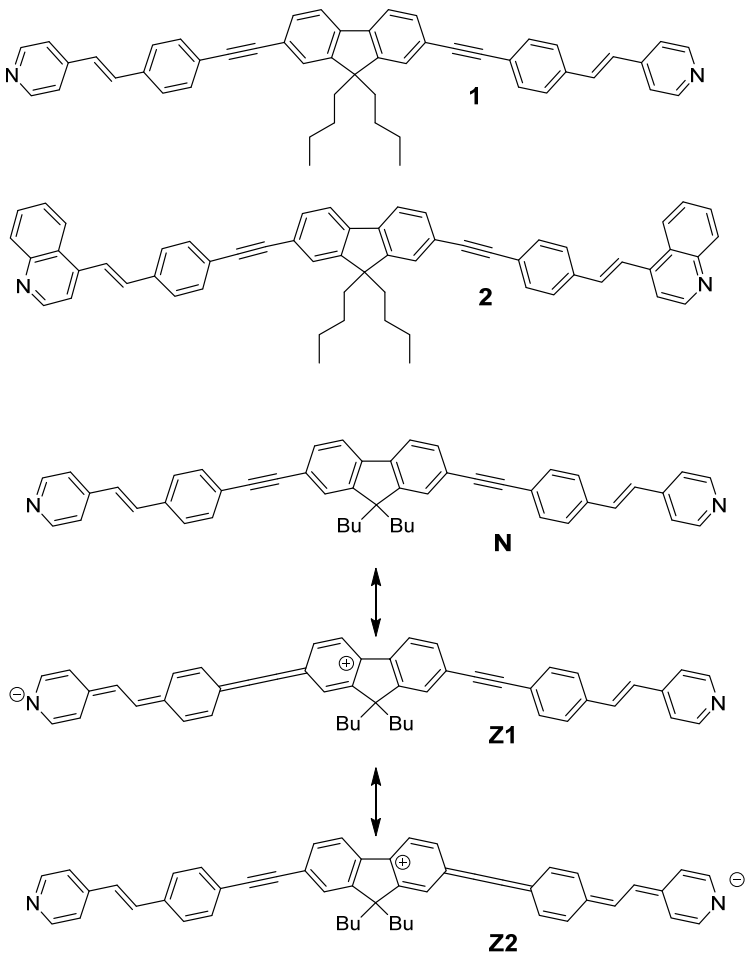


Fig. 1 Bolaamphiphilic chromophores **1** and **2**. Limiting-resonance forms for chromophore **1** are shown to illustrate the quadrupolar effect that is expected to promote basicity enhancement.

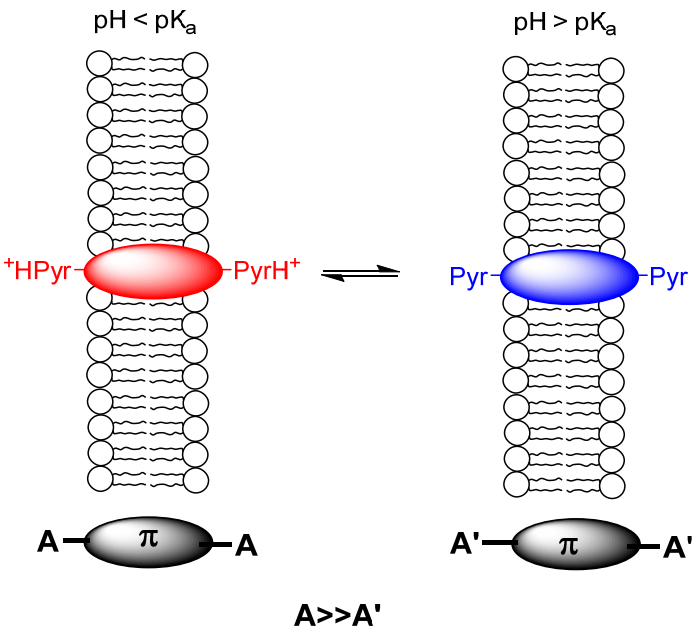
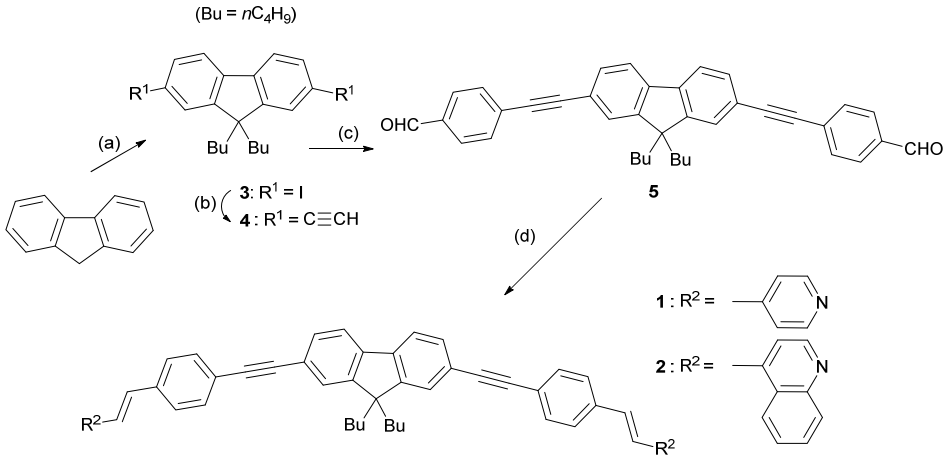


Fig. 2 Design of fluorescent membrane probes for pH sensing of slight variation of pH close to physiological pH.

The synthesis of chromophores **1** and **2** is described in Scheme 1. The fluorene core **4** was synthesized via a double Sonogashira coupling of 9,9-dibutyl-2,7-diiodo-9*H*-fluorene[32] (**3**) with ethynyltrimethylsilane, followed by base-promoted deprotection of the resulting intermediate. A twofold Pd(II)-catalyzed cross-coupling of **4** with 4-bromobenzaldehyde gave bisaldehyde **5**.[32] The Wittig-Horner condensation of the latter product with 4-[(diphenylphosphinyl)methyl]pyridine and the corresponding quinoline analogue led to **1** and **2**, respectively, which were obtained as pure *E*,*E* stereoisomers as shown by ^{1}H, ^{13}C, HRMS and elemental analyses.



Scheme 1 Reagents and conditions: (a) (i) I_2, HIO_4, H_2SO_4, AcOH, 75 °C, 2 h, 80%; (ii) tetrabutylammonium bromide, KOH, *n*-butyl bromide, PhMe/H_2O (2 : 1), 65 °C, 1 h, 90%; (b) (i) ethynyltrimethylsilane, CuI, $Pd(PPh_3)_2Cl_2$, PhMe/Et_3N (1:1), 40 °C, overnight, 79%; (ii) 1N KOH, THF/MeOH (3 : 1), rt, 30 min, 84%; (c) 4-bromobenzaldehyde, CuI, $Pd(PPh_3)_2Cl_2$, PhMe/Et_3N (4 : 1), 40 °C, overnight, 75%; (d) R^2-CH_2-$P(O)Ph_2$, NaH, THF, rt, 24 h, 81% (**1**), 79% (**2**).

As a result of their bolaamphiphilic character, the molecules were found to be well soluble in water in the presence of surfactants. In these conditions, both chromophores show intense absorption in the near UV region, and fluorescence emission in the violet (**1**) or green (**2**) visible (Table 1). In comparison to absorption, the emission spectrum has a defined vibronic structure indicative of increased rigidity in the emitting excited state (Figure 3 and Figure S1). Upon protonation, both absorption and emission are red-shifted to a significant extent, indicating that these molecules can be used as pH ratiometric probes (see Fig. S2-S3).

The pKa values of molecules **1** and **2** were determined in water solution, taking advantage of the marked red-shift of the emission bands upon protonation (Figure 4). In both cases, a marked increase of the pKa values is observed as compared to isolated pyridine or quinoline (ΔpKa=1.8 for the pyridine and 1.4 for the quinoline moieties) leading to pKa values close to physiological conditions (6.2 and 7.0 respectively).

Table 1 Absorption and emission properties of bolaamphiphilic chromophores **1** and **2** in micellar water

	λ_{abs}^{max} (nm)	ε^{max} (M^{-1} cm^{-1})	λ_{em}^{max} (nm)	Φ_f	λ_{2PA}^{max} (nm)	σ_2^{max} [c] (GM)	$\sigma_2^{max}\Phi_f$ [d] (GM)
1 [a]	380	103000	417	0.67	700	260	174
1 [b]	401	94000	544	0.26	750	1070	276
2 [a]	386	95000	436	0.52	700	700	364
2 [b]	420	67000	590	0.12	730	1260	151

Spectroscopic data were collected using diluted solutions of chromophores in micellar water (SDS/butanol/water, 6 :9 :89% wt) in [a] basic (pH = 11, 10^{-3} M NaOH) or [b] acidic (pH = 3, 10^{-3} M HCl) conditions. [c] two-photon absorption cross section given in GM (1 GM = 10^{-50} cm^4 s $photon^{-1}$). [d] two-photon brightness defined as the product of the maximum 2PA cross-section and of the fluorescence quantum yield (Φ_f).

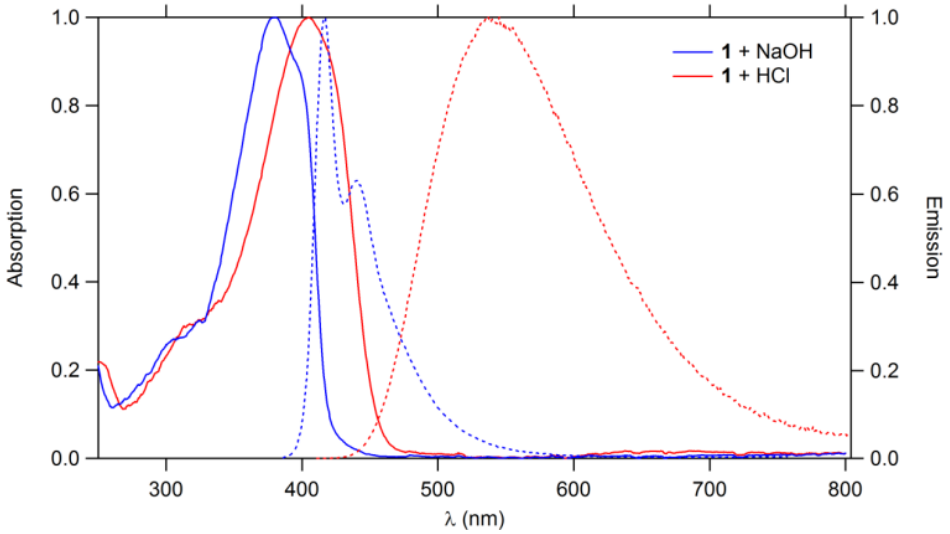


Fig. 3 Absorption (solid line) and emission (dotted line) spectra of compound **1** in basic (blue) and acidic (red) conditions.

This provides evidence that the quadrupolar character of the probes does indeed allow to shift the pKa values to larger values than that of the isolated terminal moieties, getting them closer to physiological pH.

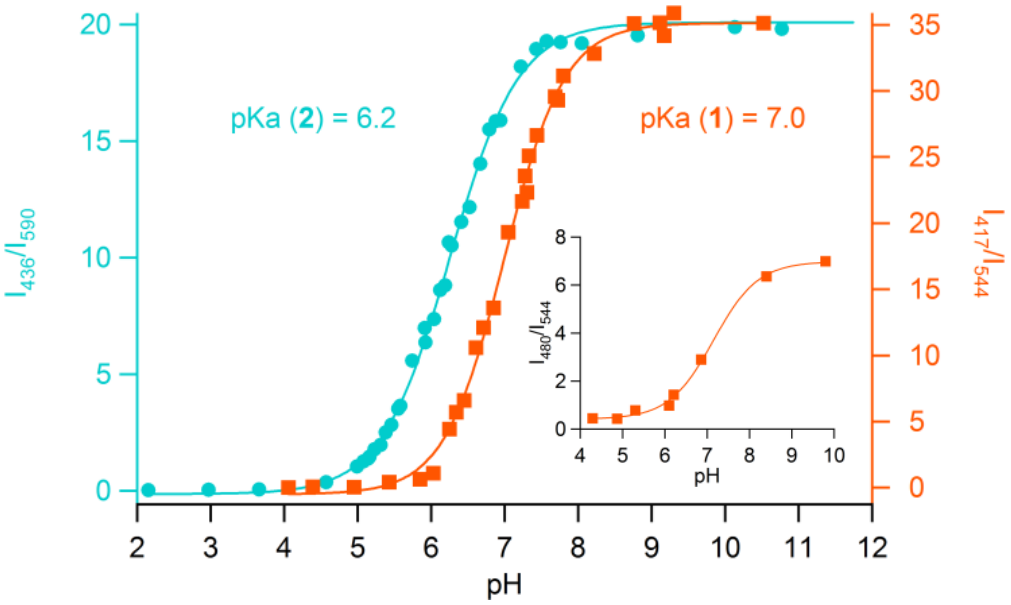


Fig. 4 Determination of pKa values of compound **1** (orange) and **2** (cyan) under 1P excitation at 380 nm and 386 nm, respectively. Inset: ratiometric behavior of **1** under 2P excitation at 750 nm.

Following this, two-photon induced fluorescence measurements were conducted to determine the 2PA response of the protonated and non-protonated forms. A significant modulation of the maximum 2PA response in acidic and neutral conditions is achieved, the bis-protonated forms leading to enhanced and red-shifted 2PA bands (Table 1) as a result of the increase of the EW strength of the terminal moieties (Fig. S4-S5). We note that both probes show large 2PA responses (typically 3 times larger than the best 2P pH probes[25, 29]) and large brightness (150-360 GM). This allowed determining the pKa values using 2PE in the NIR (Inset of Fig. 4 and Fig. S6) leading to very close values to that determined upon standard 1P excitation conditions. This demonstrates that probe **1** can indeed be used as a biphotonic pH probe with pKa close to 7.

In order to demonstrate the ability of these bolaamphiphilic probes to label lipid membranes, we first tested their incorporation into phospholipidic giant unilamellar vesicles (GUVs) which were grown in presence of compound **1** under basic (8.1) and neutral (7.2) pH buffer conditions (see SI). Confocal fluorescence microscopy reveals a strong blue emission (415-500 nm) delimited to the membrane (Figure 5) at both pH, while emission at higher wavelengths (550-700) corresponding to the acidic form of **1** is also observed at neutral pH. The confinement of the fluorescence at membranes location shows that chromophore **1** is able to stain phospholipidic membranes and is stable enough for imaging purposes. We thus tested the use of this probe in two-photon imaging of living cells. COS cells were incubated with compound **1** then imaged under two-photon excitation at 700 nm (Figure 5). Probe **1** was found to stain specific part of cells membranes as well as intracellular organelles. Thanks to the large two-photon brightness of probe **1** and to its ratiometic behaviour, we were able to estimate an overall pH for these specific environments of 6.8.

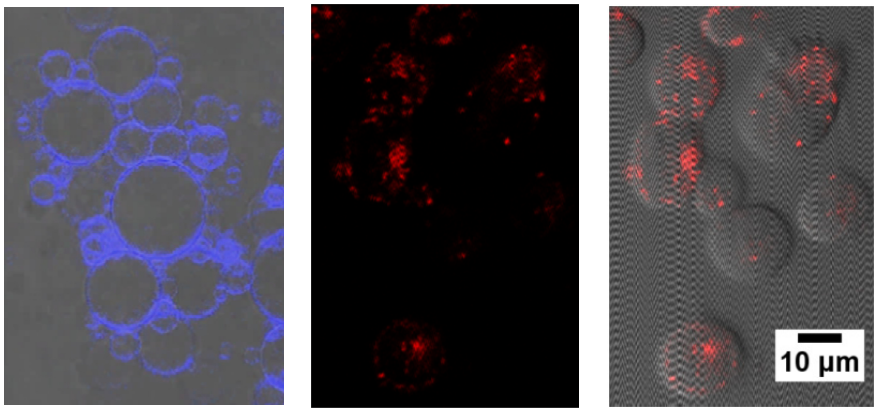


Fig. 5 Left: Incorporation of **1** in GUVs at basic pH, images are the overlap of bright field and fluorescence (λ_{ex} = 405 nm) detections to compare the membrane and chromophore localisations. Middle and right: Two-photon imaging of COS7 cells (middle) incubated with 1µM of for **1** (λ_{ex} = 700 nm) and overlap of bright field (right).

In conclusion, we reported the synthesis of two bolaamphiphilic quadrupoles built from a lipophilic fluorene core, which exhibit large 2PA response in NIR region. By integration of pyridine or quinoline end-groups linked to the π-system, we tuned their pKa close to physiological pH. We further demonstrated that these probes are able to stain efficiently lipidic membranes and have suitable photostability to allow monitoring of pH in living COS cells. The large shift of emission between protonated and basic forms and their high 2PA cross-sections make these bolaamphiphilic chromophores promising as ratiometric two-photon pH probes for bioimaging and monitoring of slight pH variations close to physiological pH. As such they hold promise for detection of acidic pH in specific cellular compartiments.

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Notes and references

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Electronic Supplementary Information (ESI) available: synthetic procedures, photophysical methods, absorption and emission spectra of **2**, determination of pKa values, two-photon absorption experimental details, preparation of GUVs, confocal and two-photon cell imaging. See DOI:

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