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Strong Modulation of Two-Photon Excited Fluorescence of Quadripolar Dyes by (De)Protonation

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Multiphoton microscopies are increasingly important tools for the study of cellular processes.[1] The utility of these microscopic techniques is greatly enhanced in combination with suitable nonlinear optical molecular probes[2] that can generate types and levels of contrast unattainable otherwise. In the case of laser-scanning two-photon excited fluorescence microscopy, it is important to use fluorescent dyes with high two-photon excited fluorescence (TPEF) action cross sections.[3] Conventional fluorescent dyes, such as fluorescein, the rhodamines, and so forth, have action cross sections $\sigma_2\Phi$ that are on the order of 1−100 GM.[4] Recent work has shown that the action cross section $\sigma_2\Phi$ can be increased by several orders of magnitude through intelligent optimization of the molecular structure.[5] Higher values of $\sigma_2\Phi$ allow detection of lower dye concentrations and diminish the laser power needed for recording images, resulting in less background fluorescence from endogeneous chromophores and less photodamage. This makes such chromophores interesting candidates for molecular TPEF labels and probes.

For the development of TPEF-optimized molecular probes, it is important to have a significant modulation of the photophysical properties of the chromophore in response to external stimuli, such as polarity, pH, ion concentration, and temperature. At the same time, the chromophore should retain a significant TPEF action cross section in a suitable excitation window for biological imaging (700−1000 nm, corresponding to an optimum combination of reduced scattering and absorption in biological samples). Here we report on two quadripolar dyes that display large changes in both one- (OPA) and two-photon absorption (TPA) characteristics as well as in their fluorescence emission upon passing from their neutral to their doubly (de)protonated state in ethanol, which makes them interesting prototypes of TPEF pH probes and potential building blocks for other types of molecular probes.[6]

From previous work, it is known that quadripolar push−pull−push chromophores have large $\sigma_2\Phi$ values, especially when strong electron donors, such as *N*,*N*′-dialkylamino groups, are present at the extremities.[5b,c,e] A phenolic hydroxyl group is not a very strong donor but becomes one upon deprotonation. We might therefore expect large pH-induced changes in the photophysical behavior of quadripolar TPEF dyes bearing phenolic hydroxyl groups at their extremities. Likewise, terminal primary amino groups may also lead to protonation-modulated optical properties.

As prototypes of pH-sensitive TPEF dyes, we have synthesized compounds **1** and **2** by double Heck couplings (see Supporting Information). Their photophysical behavior in neutral and basic (0.1 M NaOH) or acidic (0.1 M trifluoroacetic acid, TFA) ethanolic solutions was studied using UV−vis spectrometry, fluorescence spectroscopy, and two-photon excitation spectroscopy.

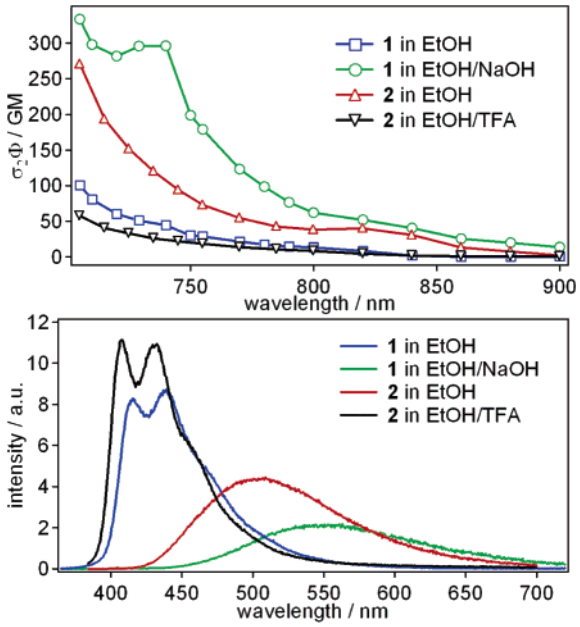
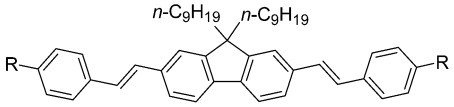


Figure 1. Two-photon excited fluorescence action cross section spectra (top, Ti:sapphire femtosecond pulses) and fluorescence emission spectra (bottom, recorded using one-photon excitation) of **1** and **2** in ethanol (with and without acid or base).



1: R = OH
2: R = NH_2

Upon deprotonation, **1** shows drastic changes in its one- (OPA) and two-photon absorption (TPA) and in its fluorescence emission. The one-photon absorption maximum undergoes a bathochromic shift. The fluorescence spectrum changes from a structured band in the blue to a featureless broad band in the green. The two-photon absorption cross section is greatly enhanced in the 700−900 nm range. This results not only from a red shift of the two-photon absorption maximum but also from the intrinsic enhancement of the two-photon transition probability related to the stronger donating capacity of O^- versus OH. The σ_2 of the bisphenolate form of **1** reaches a maximum of 955 GM at 740 nm, which is similar to that of the bis(*N*,*N*′-dibutylamino) derivative (1260 GM at 740 nm).

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Table 1. Photophysical Data for **1** and **2**

		One-Photon Absorption		Fluorescence			Two-Photon Absorption	
		λ_{max} (nm)	ϵ_{max} (M^{-1} cm^{-1})	λ_{max} (nm)	Φ^b	τ^c (ns)	λ_{max} (nm)	σ_2 (GM)
1	EtOH	378, 397[a]	7.8×10^4	415, 439	0.64	1.00	≤705[d]	≥155
	EtOH + NaOH	409, 430[a]	7.7×10^4	550	0.31	1.23	740, ≤705[d]	955, ≥1070
2	EtOH	390	7.2×10^4	505	0.55	1.37	≤705[d]	≥490
	EtOH + TFA	372, 391[a]	7.1×10^4	408, 431	0.75	0.96	≤705[d]	≥77

[a] Shoulder. [b] Quantum yield. [c] Lifetime. [d] Maximum not yet reached.

The neutral protonated form of **1** still attains respectable values, especially at the short wavelength side of the excitation window. For comparison, fluorescein has a two-photon absorption maximum at 780 nm of 38 GM.[4] Analogously, protonation of **2** by trifluoroacetic acid (TFA) leads to important photophysical changes in this compound. The electron-donating capacity of the aromatic amino group is canceled by protonation (actually reversed into electron-accepting), which leads to the observed blue shifts in OPA and fluorescence and to a decrease in the TPA cross section. The protonation has an effect on **2** opposite to that of the deprotonation on **1**. Finally, both compounds have their green fluorescent form at the basic side and their blue fluorescent form at the acidic side of their pK_a. The pK_a of **1** and **2** were spectrophotometrically determined to be 9.5 and 3.5, respectively (ethanol/water 3:1, 25 °C). Not surprisingly, these values are far apart, indicating that there is ample room for tuning the pK_a via the appropriate choice of end groups. It would be particularly useful to shift the present pK_a values into the range of more "usual" biological interest.

A salient feature of the photophysical properties of both dyes is that the neutral forms as well as the conjugate acid or base display appreciable fluorescence at markedly different wavelengths. This makes these chromophores interesting as the basis of ratiometric probes. In such probes, the signal is the intensity ratio of the two emission bands and is therefore independent of probe concentration. This is a useful property as controlling local probe concentration in biological samples can be difficult.

Although clearly intended as prototypes with emphasis on the photophysical behavior of the chromophore, we tested the behavior of **1** and **2** in multiphoton scanning fluorescence microscopy on artificial 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) phospholipid giant unilamellar vesicles (GUVs), using a previously described setup.[7] As seen in Figure 2, the dyes stain the membranes and appear to be aligned parallel to the membrane lipids. This may be understood considering their bolaamphiphilic nature, the elongated conjugated system being rendered highly hydrophobic by the nonyl chains on the fluorenyl core, and the polar end groups pointing toward the membrane surface (the region of high dielectric constant gradient). Unfortunately, we did not observe any significant modulation of the two-photon-induced fluorescence upon microinjection of small amounts of acid or base near the stained objects. The dyes may be buried deeply inside the membrane, isolated from the outside hydroxylic world. Nonetheless, we did observe differences in fluorescence emission spectra of membranes labeled with **1** under neutral conditions and membranes labeled under basic conditions. This indicates that both protonated and deprotonated forms are able to reside in the membrane, but that their protonation state becomes insensitive to external changes once incorporated inside the membrane. An interesting way to circumvent this might be to increase the length of the conjugated system such that the extremities reach the membrane surface while adding charged substituents to the end groups.

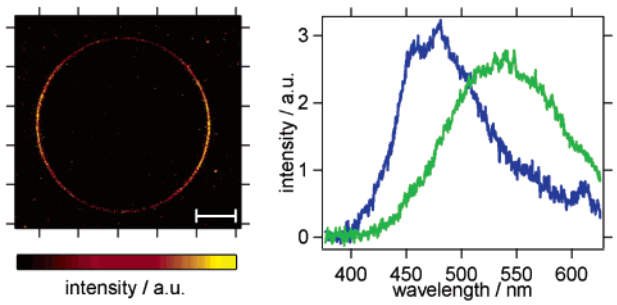

Figure 2. Left: TPEF image of a GUV labeled with **1**. The excitation light (λ = 780 nm, 1−3 mW/μm^2) is polarized parallel to the horizontal axis of the image. The scale bar equals 10 μm. Right: Microspectroscopy. TPEF emission spectra from GUV membranes, stained under neutral (pH 7) and basic (pH 12) conditions with **1**.

In conclusion, the fluorescence of quadripolar dyes can be made to respond strongly to changes in pH in solution while maintaining large cross sections for two-photon excitation. This is achieved through the introduction of auxochromic groups at the extremities of the chromophores that change their electron-donating strength upon (de)protonation.

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Supporting Information Available: Preparation of compounds **1** and **2** and experimental details of spectroscopic measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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