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Amphiphilic Porphyrins for Second Harmonic Generation Imaging

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Second harmonic generation (SHG) microscopy is a rapidly growing technique in the fields of neurophysiology[1] and cardiology.[2] SHG is a nonlinear optical (NLO) effect whereby incident light is converted to light of exactly half the wavelength, propagating in the forward direction. It is only exhibited by noncentrosymmetric dyes in noncentrosymmetric environments, such as surfaces and interfaces, so it provides a way of probing membranes without a background signal from the isotropic solution.[3] As with two-photon excited fluorescence (TPF), the intensity of SHG depends on the square of the incident light intensity, leading to excellent spatial resolution, but in contrast to TPF, SHG does not require the formation of excited states, so in principle problems of photobleaching and phototoxicity can be eliminated. A dye exhibiting a large voltage-sensitive SHG signal from cellular membranes would be extremely valuable for the optical recording of electrical activity in excitable tissue such as neurons and myocytes. SHG imaging is used to probe membrane potential both *in vitro*[2] and *ex vivo*,[4] but the technology is currently limited by the poor signal-to-noise ratios resulting from the low SHG efficiency of available dyes.

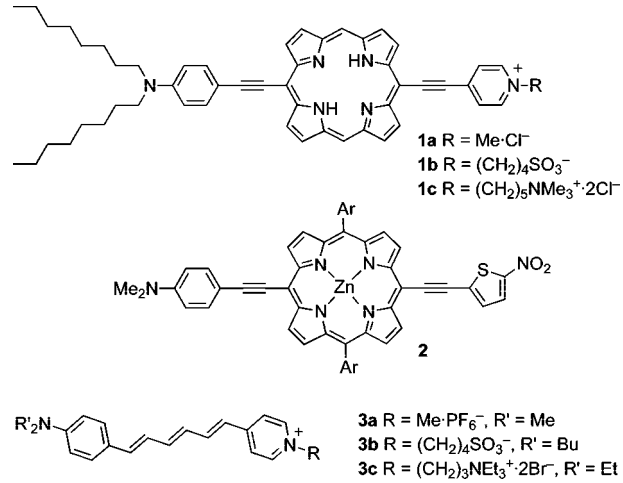
Here we present a new family of amphiphilic porphyrin dyes **1a**−**c** which exhibit strong SHG signals in lipid membranes. Previous work by Therien and co-workers has shown that related donor−acceptor *meso*-ethynyl porphyrins such as **2** exhibit strong second-order NLO activity.[5,6] We have modified the structure of these dyes by using polar pyridinium electron acceptors and omitting the orthogonal *meso*-aryl substituents, to create molecules with high affinities for biological membranes. Our SHG dyes can be viewed as hybrids between Therien's donor−acceptor porphyrins and commercially available membrane-binding dyes such as **3b** (RH237) and **3c** (FM4-64), which have been used to probe potential changes via SHG despite their weak NLO activity.[7,8]

Hyper-Rayleigh scattering (HRS) is a convenient technique for measuring the molecular first hyperpolarizability (β) of a dye, and thus for evaluating its second-order NLO activity and potential for SHG imaging. We used a high amplitude-modulation frequency-resolved HRS technique[8,9] to measure the fluorescence-free first hyperpolarizabilities of compound **1a** (in chloroform solution) at wavelengths of 800, 840, 1064, and 1300 nm. The results at 800 nm are compared with published parameters for compounds **2** and **3a** in Table 1.

The free-base porphyrin **1a** has a high β_{zzz} value at 800 nm, which is further increased to (5800 ± 200) × 10^{-30} esu at 840 nm due to resonance with the Soret band (λ_{max} 448 nm). The HRS depolarization ratios at 800, 840, and 1300 nm are 1.95 ± 0.15, 2.00 ± 0.20, and 1.75 ± 0.25, respectively, indicating a significant off-diagonal contribution to the dipolar response in **1a**. The NLO activity of porphyrin **1a** is enhanced relative to other *meso*-ethynyl donor−acceptor porphyrins such as **2**,[5,6] and it is significantly higher than those of conventional membrane probes such as **3a**−**c**. The high first hyperpolarizability of **1a** at 840 nm is ideal for SHG imaging because light of this wavelength penetrates deeply into biological tissues and is available from commercial Ti:sapphire lasers.

Table 1. Linear and Nonlinear Optical Parameters for **1a**, **2**, and **3a**

compound	absorption λ_{max} (nm)	emission λ_{max} (nm)	$\beta_{800,zzz}$ (10^{-30} esu)	reference
1a	448, 643, 723	803	2300 ± 150	this work
2	465, 685	714	860	6
3a	500	–	1085 ± 35	7



Initial SHG imaging experiments were carried out using water droplets, coated with lipid monolayers, in dodecane. This protocell model[10] provided a stable asymmetric interface for optimizing imaging protocols. Dyes **1a**−**c** and **3b** were introduced into the oil layer and rapidly adsorbed onto the surfaces of the water droplets. Confocal laser-scanning microscopy (840 nm; Ti:sapphire laser; 100 fs pulse width) revealed a TPF signal from the isotropic dye dissolved in the oil and a SHG signal from dye molecules oriented in the surfaces of the water droplets (Figure 1a). As expected, the TPF signal is observed in both the reflection and transmission detection channels, whereas the SHG signal is only detected in the transmission channel (i.e., in the forward direction of the incident light). The SHG signal (λ_{SHG} 420 nm) was collected with a wide aperture condenser and passed through a 350−450 nm bandpass filter to remove the TPF component (λ_{em} 803 nm). Under these

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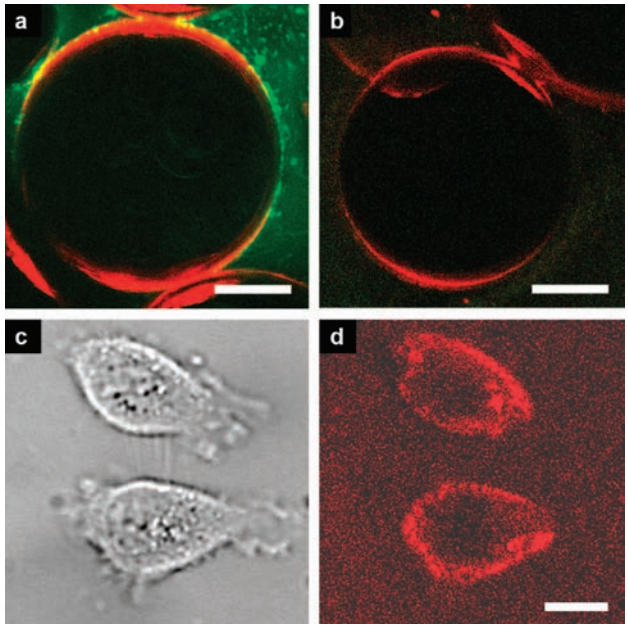

Figure 1. (a) SHG (red) and TPF (green) images of a droplet of aqueous potassium chloride (0.5 M) in a solution of palmitoyl oleoyl phosphatidyl choline (POPC, 10 μM) in dodecane containing **1b** (scale bar: 75 μm). (b) SHG/TPF image of a sample identical to that of (a) except using **Cu-1b** rather than the free-base porphyrin. (c and d): Transmission and SHG images of SK-OV-3 cells stained with porphyrin **1c**, 10 μM concentration, 10 min incubation time (numerical aperture: 0.3; scale bar: 10 μm).

conditions, the SHG signal from **1b** is ~30 times brighter than that from **3b**, and the SHG from these porphyrin dyes can be clearly observed using a dwell time of 4.9 μs per pixel which is much less than the time span of an action potential.[4] The SHG signal is not uniform around the circumference of the water droplets: a significant signal is only generated in those regions where the molecular β_{zzz} tensor is aligned with the polarization of the incident laser light.[1b]

Dye photobleaching and biological photodamage are common limitations with optical imaging. SHG promises to evade these problems, because, unlike fluorescence, it does not depend on the population of excited states. One- and two-photon excitation may occur in parallel with SHG, but any effect which rapidly relaxes the excited state competes with photochemical degradation, and quenches fluorescence, without directly reducing the SHG intensity. Metalation of porphyrin-based SHG dyes may enable the photochemical behavior to be modified, without strongly affecting the SHG. Open-shell metalloporphyrins, such as copper(II) and nickel(II) complexes, have extremely short excited-state lifetimes under normal conditions, resulting in quenched fluorescence, low singlet oxygen yields, and low phototoxicity,[11] so we decided to test these metal complexes. HRS experiments at 840 nm on free-base **1a**, **Cu-1a**, and **Ni-1a** in DMF gave β_{zzz} values of (8000 $\pm$ 250) $\times$ 10^{-30}, (4000 $\pm$ 100) $\times$ 10^{-30}, and (650 $\pm$ 25) $\times$ 10^{-30} esu, respectively.[12] Metalation changes the absorption spectra, reducing the resonance enhancement of the NLO activity at 840 nm; however microscopy experiments with **Cu-1b** and **Ni-1b** in water/dodecane/POPC gave clear SHG images in both cases, with no detectable TPF signal, reflecting the quenched fluorescence (**Cu-1b**: Figure 1b; **Ni-1b**: Figure S19, Supporting Information).

The intracellular accumulation and localization of **1a**−**c** were studied using adherent human epithelial bladder (T24) and ovarian (SK-OV-3) adenocarcinoma cells. Fluorescence imaging showed that dye **1a** exhibits poor *in vitro* membrane localization, probably due to its low amphiphilicity. However **1b** and **1c** localize rapidly in the plasma membrane, giving similar distributions to the commercially available dyes RH237 **3b** and FM4-64 **3c**.[2] Over longer time scales (>2 h), the porphyrins are taken up by lysosomes, resulting in punctate localization near the nucleus (Figure S23, Supporting Information). A strong SHG signal is observed from **1c** in the plasma membrane of SK-OV-3 cells, as shown in Figure 1c and 1d. This compound was also used to obtain SHG images of live neurons (in slices of *ex vivo* rat neocortex), using both extracellular and intracellular patch-clamp delivery. During the patch-clamp experiments, we observed an SHG signal from the porphyrin adsorbed onto the surface of the glass micropipette, as well as in the neuronal membrane (Figure S25, Supporting Information).

In summary, we have shown that amphiphilic donor−acceptor *meso*-ethynyl porphyrins with polar pyridinium acceptor head groups and hydrophobic dialkyl-aniline donors have strong second-order NLO activity and high affinities for biological membranes, making them promising probes for SHG imaging. To the best of our knowledge this is the first time that porphyrins have been used as dyes for SHG imaging. An attractive feature of these porphyrin-based SHG dyes is that their photochemistry can be modified by complexation of different metals. This should be a valuable approach toward eliminating photochemical damage.

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Supporting Information Available: Synthesis, characterization, SHG images, and details of HRS experiments. This information is available free of charge via the Internet at http://pubs.acs.org/.

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(12) The higher hyperpolarizability of **1a** in DMF, compared to chloroform, is an expected consequence of the greater solvent polarity.[9]

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