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(Physicochimie/*Physical chemistry*)

DOSSIER

PHOTONIQUE MOLECULAIRE : MATÉRIAUX, PHYSIQUE ET COMPOSANTS

MOLECULAR PHOTONICS: MATERIALS, PHYSICS AND DEVICES

Molecular engineering of NLO-phores for new NLO microscopies

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Abstract Novel microscopies based on nonlinear optical (NLO) phenomena such as two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG) have gained overwhelming popularity in the biology community owing to the many advantages they provide in biological imaging. Examples of molecular engineering approaches toward NLO-probes specifically designed for SHG and/or TPEF imaging of lipid membranes and biological cells are given here, providing an illustration of their intriguing potential in the area of real-time, non-damaging imaging of biological structures. Optimized NLO-markers open new routes for improved monitoring and better understanding of fundamental dynamic processes. ***To cite this article: M. Blanchard-Desce, C. R. Physique 3 (2002) 439–448.*** © 2002 Académie des sciences/Éditions scientifiques et médicales Elsevier SAS

NLO-phores / fluorophores / two-photon absorption / TPEF / SHG / molecular probes / cell membranes

Nouvelles techniques d'imagerie multiphonique : vers une nouvelle génération de marqueurs moléculaires

Résumé Les nouvelles techniques d'imagerie multiphotonique telles que la microscopie de génération de deuxième harmonique (SHG) et ou de fluorescence induite par excitation à deux photons (TPEF) connaissent à l'heure actuelle un véritable essor, de part les nombreux multiples avantages qu'elles offrent pour l'imagerie du vivant. Le développement de ces techniques passe par l'ingénierie de marqueurs moléculaires adaptés. Cette approche est illustrée par le design de chromophores et fluorophores membranaires optimisés pour l'imagerie SHG et/ou TPEF des membranes cellulaires. Au-delà d'une imagerie structurale, de tels marqueurs ouvrent également la voie à une véritable imagerie fonctionnelle. ***Pour citer cet article : M. Blanchard-Desce, C. R. Physique 3 (2002) 439–448.*** © 2002 Académie des sciences/Éditions scientifiques et médicales Elsevier SAS

chromophores / fluorophores / absorption biphotonique / TPEF / SHG / sondes moléculaires / membranes cellulaires

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1. Introduction

The field of molecular nonlinear optics (NLO) has attracted increasing interest over the past twenty years (see, for instance, the special issue of *Chemical Physics* devoted to molecular nonlinear optics [1]), owing to its connection with fundamental issues such as electronic conjugation and intramolecular charge transfer, hyperpolarizabilites, electron–phonon coupling, as well as to the numerous applications it offers in various areas such as telecommunications [2,3], optical data storage [4] and information processing, optical power limiting [5,6], microfabrication [7], etc. Concomitantly to such developments related to material science issues, novel techniques of microscopic imaging based on of NLO phenomena have recently been recognized as innovating and superior tools for imaging of biological structures and processes [8]. For instance, in contrast with standard fluorescence microscopy where molecular excitation is caused by the absorption of a single photon, the molecular excitation by the simultaneous absorption of two or more photons can often be advantageous for specific imaging applications. This has given rise to the techniques of two-photon [9] and three-photon [10] microscopy – or more generally, multi-photon excited fluorescence (MPEF) microscopy [11]. Among these, two-photon-excited fluorescence (TPEF) has gained overwhelming popularity in the biology community owing to the many advantages it provides in biological imaging. These include a capacity for a highly confined excitation and intrinsic three-dimensional resolution when used in microscopic imaging, as well as the ability to image at an increased penetration depth in tissue with reduced photodamage and background fluorescence by operating with excitation radiation in the visible red-NIR region (as opposed to typical one-photon excitation in the UV-visible blue region).

Nonlinear microscopy has also been extended to the use of harmonic light generation, wherein the energy of incident photons, instead of being absorbed by a molecule, is scattered via a process of harmonic up-conversion [12]. As in the case of fluorescence, the use of second [13–16] or third harmonic generation [17,18], opens the field to multi-harmonic generation (MHG) microscopy. Both second-harmonic generation (SHG) and TPEF are nonlinear optical phenomena which scale with excitation intensity squared, and hence give rise to the same intrinsic three-dimensional resolution when used in microscopic imaging. However, since SHG is a coherent phenomenon involving radiative scattering while TPEF is an incoherent phenomenon involving radiative absorption and re-emission, the two provide intrinsically different contrasts [19,20]. SHG produces radiation patterns that are highly sensitive to phase. As a consequence, it provides a unique window into molecular spatial organization that is inaccessible to fluorescence [20]. In particular, because of its sensitivity to local asymmetry, SHG microscopy holds promises as a powerful tool for the study of membrane dynamics [19–21].

NLO imaging of biological systems actually is an emerging field whose current and future developments will benefit not only from the fast developments of NLO microscopy but also on the design of NLO-phores tailored for defined purposes, that are imaging of specific biological structures and, eventually of processes/dynamics. This relies on the design of novel NLO molecular markers that combine optimized NLO molecular responses and affinity for the biological targets. Additional issues such as (photo)stability and (photo)toxicity also have to be taken into account.

The objective of this paper is to illustrate, via specific examples, the implementation and the potential of such approaches, which involve a sequential, multiparameter molecular optimization. Molecular engineering routes towards optimized NLO-probes specifically engineered for TPEF and/or SHG microscopies will be presented. Their applications in cellular imaging will be illustrated, focusing on several applications related to structural and functional imaging of biological membranes.

2. Quadrupolar fluorophores for TPEF

Initially, TPEF was developed using conventional fluorophores whose two-photon absorption (TPA) characteristics were not optimized and thus led to the use of either high laser intensity and/or high fluorophore/label concentration, which can be detrimental for biological media. It was thus soon realized that molecules specifically engineered for TPEF may significantly outperform standard fluorophores

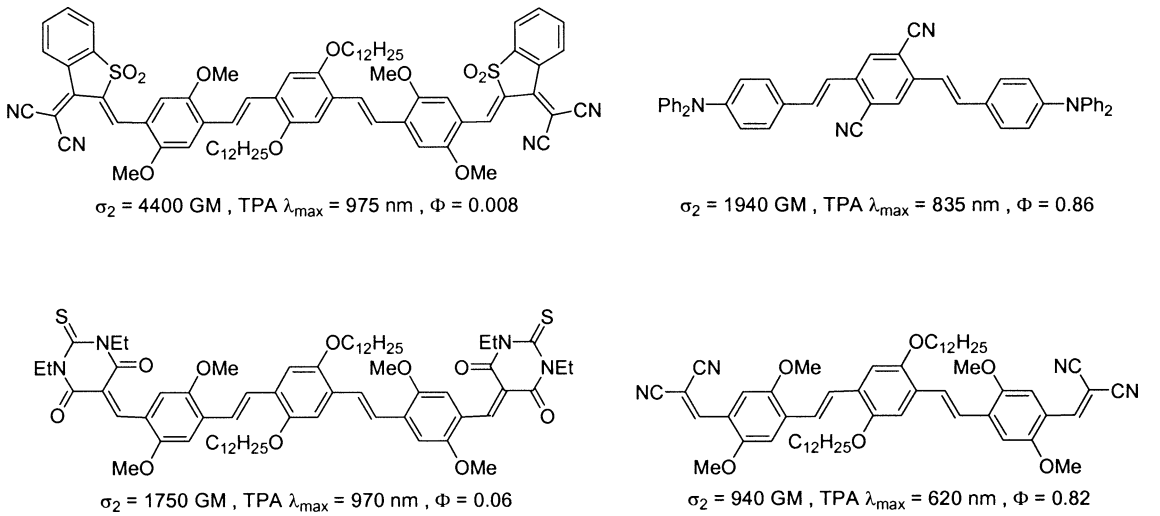


Figure 1. Quadrupolar molecules with high TPA cross section, 1 GM $= 10^{-50}$ cm^4·s·photon^{-1} (from [27,28]).

optimized for one-photon excitation. This realization triggered the search for novel fluorophores specifically designed for TPEF. Key ingredients for such purpose are a high fluorescence quantum yield (Φ) and a high TPA cross section (σ_2) in the red-NIR region, which is the spectral range of interest for imaging of biological structures. The relevant figure of merit is thus the TPEF action cross section $\sigma_2\Phi$.

2.1. Quadrupolar chromophores: a route towards enhanced TPA cross section

In recent years, there has been two main routes for the optimization of the TPA cross section (though there has been recently some effort devoted to increased dimensionality or branched structures [22–24]). The first one relies on chromophores basically 'push–pull' in nature (i.e. combining electron-releasing and electron-withdrawing groups interacting via a conjugated linker) [25,26]. Recently Marder, Perry, Brédas and coworkers have opened an innovating route based on quadrupolar systems [27], (i.e. symmetrical conjugated molecules bearing two electron-releasing (D) or electron-withdrawing (A) end-groups). Such derivatives are liable to display very high TPA cross sections in relation to an intramolecular quadrupolar charge redistribution taking place between the periphery and the center of the molecule [27–30]. Giant σ_2 values can be obtained with DAAD or ADDA in DAAD or ADDA systems bearing strong D and A end and middle groups but often at the expense of a significant reduction of the fluorescence quantum yield and/or a red-shift of the TPA maximum to the NIR-IR region (Fig. 1).

2.2. Design of nanoscale rod-like fluorophores for enhanced TPEF in the visible red-NIR region

Based on these limitations, the objective was the design of quadrupolar systems displaying giant σ_2 in the red-NIR region while maintaining a high fluorescence yield. A successful approach, supported by a 3-state 3-level description of quadrupolar systems [29,30], was implemented. The strategy is based on the push–push or pull–pull functionalization of a rigid and/or semi-rigid nanoscale conjugated system [31–35]. The structure was built from the symmetrical grafting, onto a conjugated core, of two elongated conjugated rods bearing either D or A end groups (Fig. 2).

Bridged biphenyl (dihydrophenanthrene [31,32,34] and fluorene [35]) or free biphenyl [33,35] moieties as well as fused bithiophene moiety (dithienothiophene) [31,32] have been considered as central units that may assist quadrupolar intramolecular charge transfer by acting either as a (weak) donor or acceptor core. Conjugated rods built from arylene–vinylene [31–34] and/or phenylene–ethynylene [35] oligomers were

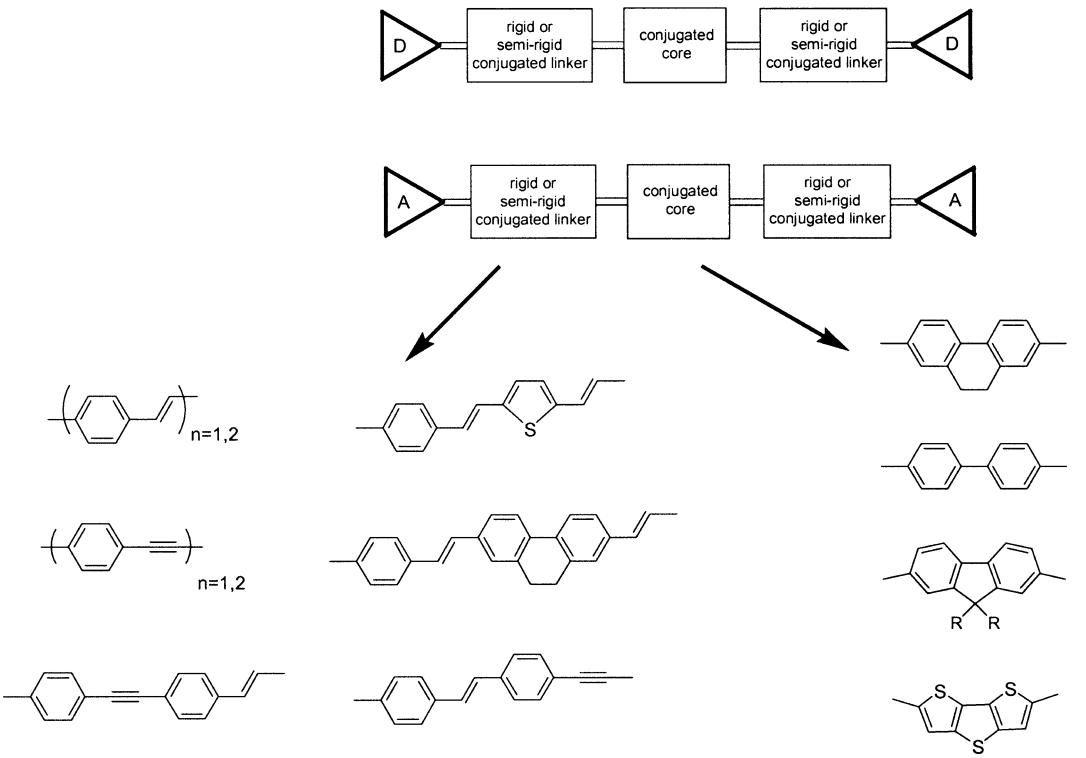


Figure 2. Molecular engineering of nanoscale fluorophores for enhanced TPEF.

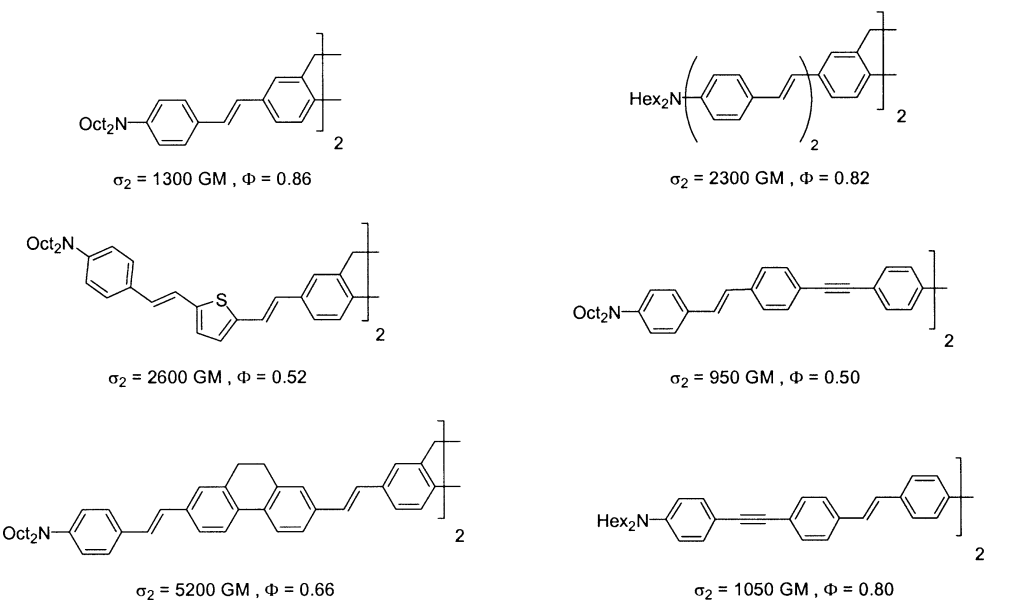


Figure 3. Nanometric flurophores with enhanced TPEF action cross section (σ_2 are given at 740 nm) [32–35].

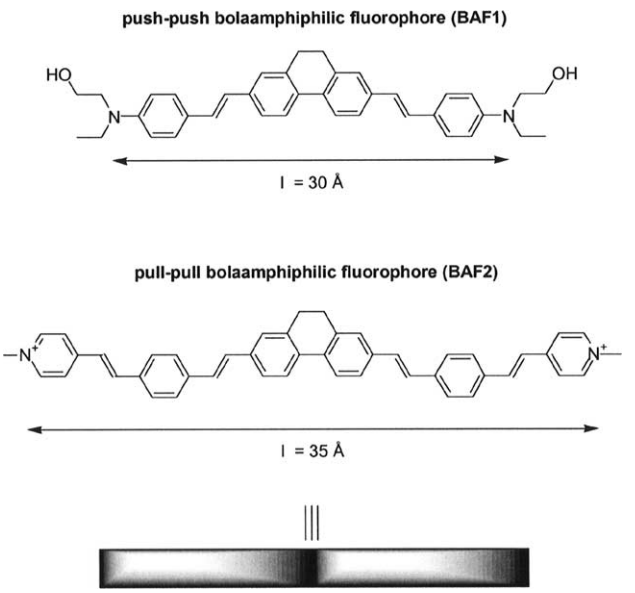
investigated in order to preserve fluorescence and modulate the electronic communication between the ends and the center of the molecules.

This empirical approach actually led to nanoscale fluorophores combining giant TPA cross sections in the red region (1000–5000 GM) and high fluorescence quantum yield (Fig. 3). Their TPA spectra were determined by investigating the two-photon-excited fluorescence properties in solution and comparing to that of the standard fluorophore: fluorescein [36].

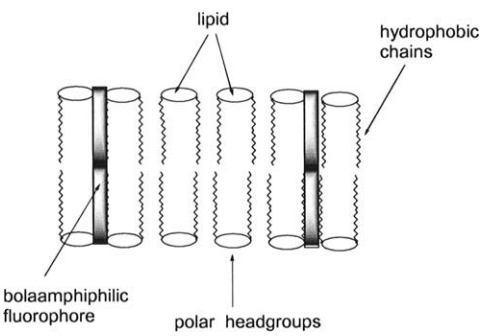
Both the nature of the end groups and of the core moiety play an important role in determining the TPA spectra [31–35]. In addition, by adjusting the length and nature of the conjugated extensors, both amplification and spectral tuning of TPA cross sections can be achieved [34,35]. As a result, push–push fluorophores showing TPA cross section two orders of magnitude larger than standard fluorophores like fluorescein in the visible red have been obtained [34].

2.3. Bolaamphiphilic quadrupolar fluorophores for membrane imaging

Besides the attractive flexibility in terms of engineering of the linear and nonlinear optical responses (i.e. amplification and spectral tuning of the TPA spectra), these modular structures also offer wide possibilities in terms of controlling their solubility in various media. In particular, different appendices can be added onto the end groups and on the central block in order to obtain highly soluble derivatives in either apolar or highly polar environments. Furthermore, given the modular nature (and synthesis) of such compounds, a lipophilic/hydrophilic gradient can be easily imposed along the rod-like structure. Such strategy can be used to favor anisotropic organization within organized molecular assemblies. For instance, the molecular structure can be engineered so as to confer specific affinity for cellular membranes: *bolaamphiphilic derivatives* can be designed by taking advantage of the elongated lipophilic central conjugated system and by adding hydrophilic terminal moieties (Fig. 4). The total thickness of such derivatives (BAF1 and BAF2) approximately matches the thickness of the lipidic bilayer which is the basic component of biological membranes. Such bolaamphiphilic fluorophores are thus expected to display particular affinity for lipidic membranes, via notably favorable (i) hydrophilic interactions between the polar end groups of



(a)



(b)

Figure 4. (a) Bolaamphiphilic fluorophores for TPEF imaging of cell membranes.
(b) Incorporation into a model membrane (lipidic bilayer).

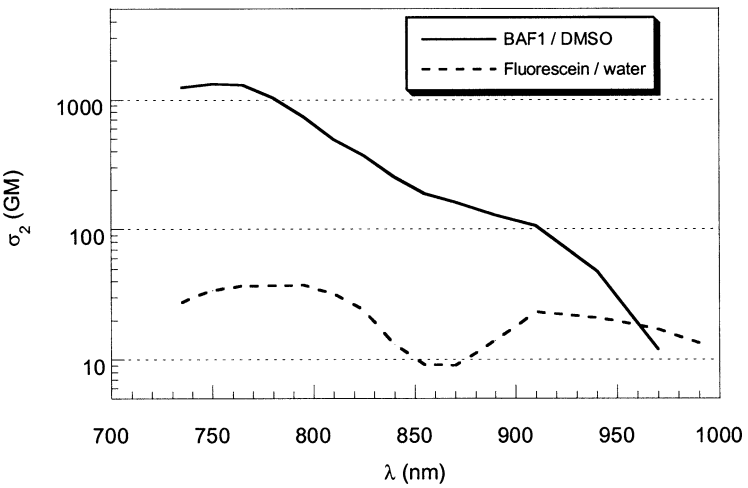


Figure 5. Two-photon excitation spectra of bolaamphiphilic fluorophore BAF1.

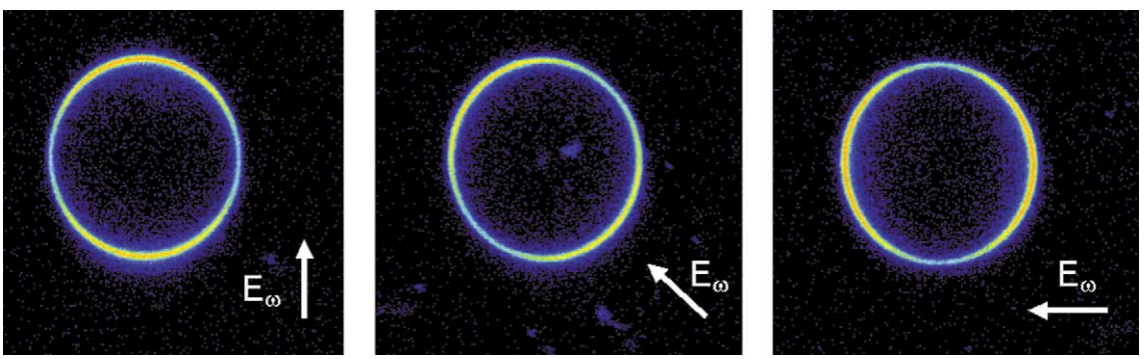


Figure 6. TPEF image of a DOPC giant unilamellar vesicle labeled with fluorophore BAF1, excited at 750 nm. A linearly polarized beam is used (directed along white arrow) and the corresponding parallel component of the TPEF radiation is selected.

the fluorophores and the lipid hydrophilic headgroups and (ii) hydrophobic interactions between the lipid hydrophobic chains and the central conjugated moieties of the fluorophores (Fig. 4).

As illustrated in Fig. 5, quadrupolar bolamphiphilic derivatives can show large TPA cross sections in the visible red region. In particular the bis-donor neutral derivative combine a high fluorescence yield and a very high TPA cross section, outperforming the standard fluorophore fluorescein by nearly two orders of magnitude [31,32].

The potential of the bolaamphiphilic quadrupolar fluorophores for the imaging of biological membranes has been investigated by testing their incorporation in model lipid membranes. Fluorophore BAF1 was successfully incorporated into model lipid membranes [34]. Giant unilamellar vesicles (GUV) made of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were prepared and externally perfused with fluorophore BAF1 [34]. By exciting at 750 nm with a linearly polarized beam, a TPEF anisotropy is observed (Fig. 6), providing evidence of a preferential transmembrane incorporation of flurorophore BAF1 (whose total length approximately matches the width of the DOPC bilayer at room temperature) [34]. Such molecules can actually be used to image cell membranes [37].

3. Molecular design of NLO-phores for combined TPEF and SHG imaging

3.1. Design of push–pull chromophores for NLO membrane imaging

The strategy towards NLO-phores for combined TPEF and SHG imaging of biological membranes is based on the design of nanoscale amphiphilic push–pull chromophores. Molecular engineering of 'push–pull' compounds has been a very active area in the past 15 years and already lead to molecules displaying giant first-order hyperpolarizability β [38,39], which is of particular interest in the elaboration of electro-optical materials [40,41]. The push–pull structure provides the molecular asymmetry. However, to favor asymmetrical/anisotropic interactions of the push–pull NLO-phore with the lipid membrane, further molecular engineering is required in order to ensure asymmetrical incorporation and thus generate the non-centrosymmetry required for SHG. This can be done by enforcing a lipophilic/hydrophilic gradient along the push–pull axis, for instance by grafting hydrophobic chains onto the electron-releasing part and by conferring hydrophilic character to the electron-withdrawing part (Fig. 7).

Following this route, amphiphilic push–pull polyenic chromophores (APPPC) programmed to interact in an anisotropic way with lipidic membranes were designed (Fig. 8). Such derivatives show very large β which can be enhanced by increasing the polyenic chain [42]. These amphiphilic NLO-phores do interact in an asymmetric way with lipidic membrane and label preferentially the outer leaflet of the membrane of a GUV perfused with external molecular NLO probes (Fig. 8).

In addition their hydrophilicity/lipophilicity balance can be tuned by varying the hydrophilic character of the head group (via modulation of R') as well as the lipophilicity of the hydrophobic chains (via modulation of R). Interestingly these derivatives also show very large TPA cross sections in the NIR region as exemplified in Fig. 9. Although the fluorescence quantum yield of such chromophores are usually small in polar enviroments, a significant increase in the fluorescence quantum yield is often observed upon incorporation in lipidic membranes as opposed to a dramatic decrease in water [43]. This pronounced

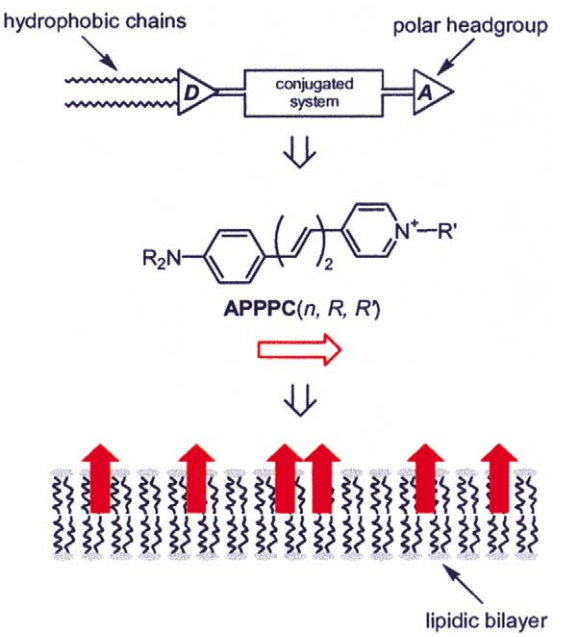


Figure 7. Molecular design of amphiphilic push–pull chromophores for asymmetrical interaction with lipid membranes.

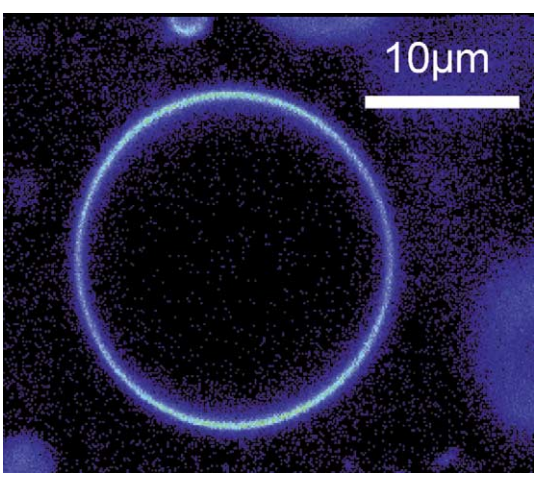


Figure 8. SHG image (equatorial slice) of a DOPC giant unilamellar vesicle externally labeled with APPPC (1, Hex, $(CH_2)_4SO_3^-$), excited at 880 nm.

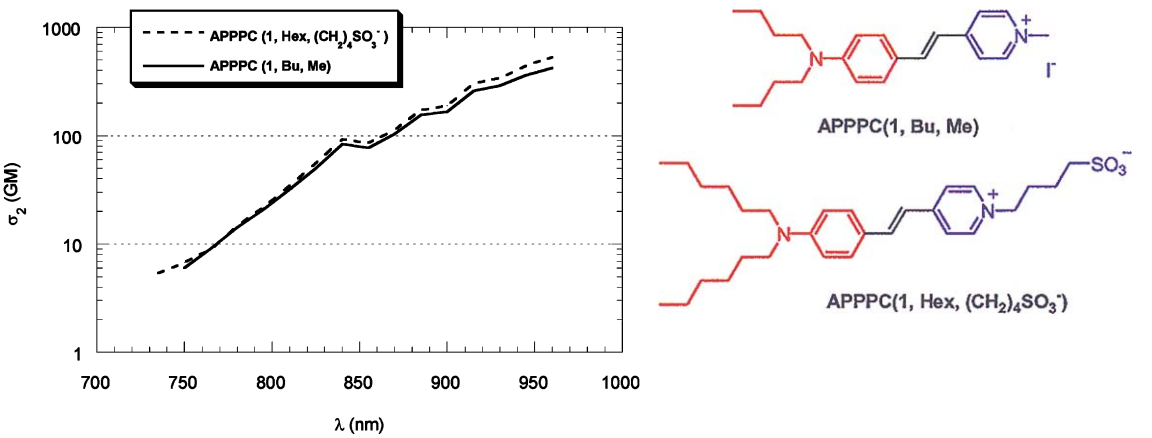


Figure 9. Two-photon excitation spectrum of amphiphilic push–pull chromophores APPPC (1, Bu, Me) and APPPC (1, Hex, $(CH_2)_4SO_3^-$) [37].

difference in fluorescence yields will result in a enhanced contrast when using such amphiphilic dyes for specific imaging of membranes via TPEF.

Such amphiphilic push–pull chromophores combining (i) large TPA cross section and significant fluorescence quantum yield when incorporated in lipidic membranes, and (ii) high first-order hyperpolarizabilities as well as tunable affinity for lipidic membrane and ability for asymmetrical incorporation in lipid bilayer, were hence particularly attractive candidates for combined SHG and TPEF imaging of membrane dynamics.

3.2. Combined SHG and TPEF imaging of biological membranes

Amphiphilic polyenic push–pull chromophores can indeed be used for combined TPEF and SHG imaging of both model [19,20] and of biological membranes [20,21] as illustrated in Fig. 10. We observe from the TPEF image that APPPC(3,Bu,$(CH_2)_4SO_3^-$) has manifestly entered the cell body, presumably through endocytosis or internalization mechanisms [37]. In contrast, the same image acquired with SHG reveals only the cell membrane. This illustrates the fact that SHG requires a non-centrosymmetric radiating source: in the cell membrane the amphiphilic NLO-phores are preferentially aligned in the same direction perpendicular to the membrane surface, whereas in the cytoplasm they become randomly oriented and therefore centrosymmetric.

The sensitivity of SHG to centrosymmetry stems directly from its coherent nature, and may be used to reveal information on molecular dynamics that is inaccessible to fluorescence [8,19–21]. In particular, as seen above, amphiphilic push–pull chromophores that label only the outer membrane leaflet generate efficient SHG. With time, however, these molecules will undergo thermally induced flip–flop to the internal membrane leaflet where they will point in the opposing direction. The SHG signal will therefore eventually disappear, indicating that the molecular populations in the outer and inner leaflets have equilibrated [8,20,21]. At opposite, the TPEF signal will remain relatively unchanged since it is insensitive to symmetry. As such, SHG provides a unique tool for the direct visualization of molecular flip–flop dynamics that would be impossible with fluorescence alone.

4. Towards future developments

The theory of phased-emission in SHG may be applied to more complicated geometries than a single membrane plane. For example, when two membranes approach one another to within a proximity smaller than the microscopic resolution, then SHG can serve as an accurate measure of the separation between

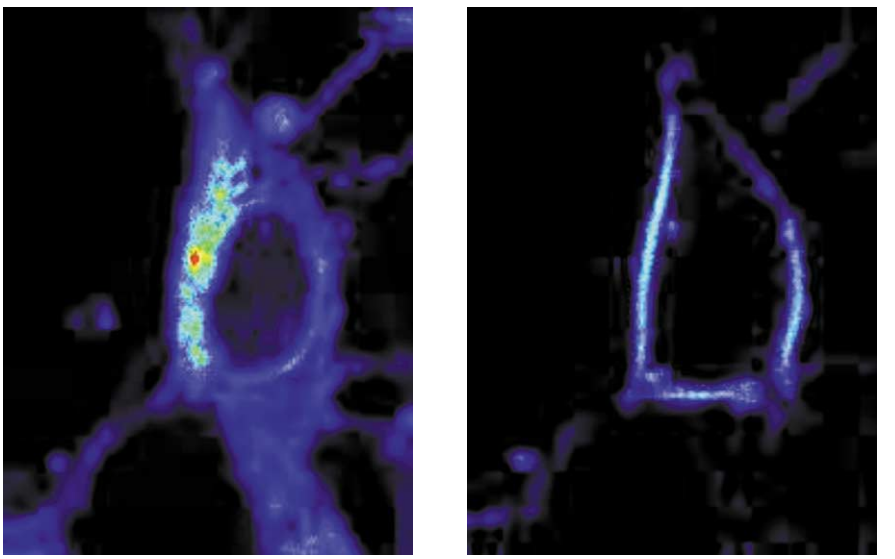


Figure 10. Simultaneous TPEF (left) and SHG (right) images of cultured dissociated neurons labeled with APPPC (3, Bu, $(CH_2)_4SO_3^-$) (equatorial slice), excited at 830 nm [37].

membranes with sub-wavelength distance resolution [21]. The range of separation distances that can be measured by SHG is of particular interest since it is inaccessible to ordinary diffraction limited imaging, as well as to measurements involving fluorescence resonant energy transfer (FRET) which are short ranged. As a result, SHG provides an interesting tool bridging the gap between these techniques [8]. In addition, a generalized theory of SHG based on phased-array emission [20] suggests that the SHG power and radiation pattern are remarkably sensitive to the molecular concentration distribution in the membrane plane, and can thus provide signatures of concentration fluctuations and aggregation dynamics that are also inaccessible to fluorescence imaging [37].

Finally, optimized amphiphilic push–pull chromophores hold great promises as NLO-probes for real-time imaging of fundamental biological processes such as adhesion [19–21] or reporting of membrane electric potentials [8,44,45]. In particular a significant effort is presently directed towards the design of *both fast and sensitive reporters* of membrane potentials. Again, a specific molecular strategy aiming at designing novel electro-NLO-chromic probes generating electrically dependent SHG signals when incorporated into lipidic membranes is of crucial importance. Promising results have already been obtained [8,46], opening attractive routes towards real-time imaging of fundamental processes involving membrane electrical potentials changes (including neuronal activity, . . .).

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