Contrast Agents

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Water-Soluble Dendrimeric Two-Photon Tracers for In Vivo Imaging**

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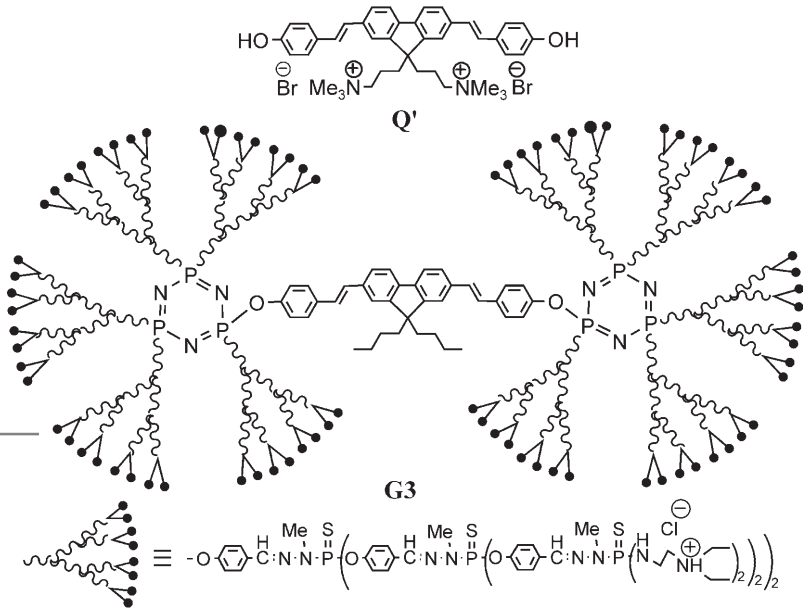
Novel microscopies based on nonlinear optical (NLO) phenomena such as two-photon excited fluorescence (TPEF)[1] are rapidly gaining popularity in biological imaging owing to the many advantages they provide. These include a capacity for a highly spatially confined excitation and intrinsic three-dimensional resolution, 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 near-infrared (NIR) region. At first, TPEF was developed using conventional fluorophores whose two-photon absorption (TPA) characteristics were not optimized,[2] but it was soon realized that molecules specifically engineered for TPEF can significantly outperform standard fluorophores optimized for one-photon excitation. This realization triggered the search for fluorophores combining a high fluorescence quantum yield (Φ) and a high TPA cross-section (σ_2) in the spectral region of interest for bioimaging (typically 700–1000 nm, corresponding to the combination of reduced absorption and scattering). Molecular engineering of optimized chromophores with large TPEF cross-sections has been particularly active in the last decade[3–5] and has led, for example, to bolamphiphilic TP markers of interest for imaging of biological membranes and cells or pH sensing.[4–6] However, water-soluble TP fluorophores that maintain both a high fluorescence quantum yield and a large TPA cross-section in the spectral range of interest are still rare. In addition, fluorophores with a high TPA cross-section in toluene (that is, in a lipophilic environment) have been reported to undergo a significant decrease of their TPA characteristics in water as a result of molecular aggregation.[7]

Herein, we report a general route for the design of water-soluble TP markers from lipophilic TP fluorophores that could be applied to a variety of TP chromophores. To prevent aggregation while ensuring water solubility, we included a TP-active lipophilic chromophoric unit in the core of a dendrimer whose periphery is covered with cationic groups[8] (**G3** in Scheme 1). The dendritic sheaths should, in principle, provide isolation of the central chromophore from the outer environment, thus preventing fluorescence quantum yield decrease by nonradiative processes mediated by water molecules, as well as aggregation processes that are detrimental to the TPA response. An alternative (and simpler) route consisting in grafting water-solubilizing cationic groups directly onto the TP-active lipophilic chromophore (chromophore **Q′** in Scheme 1) was also tested to evaluate the relevance of the dendrimer approach.



Scheme 1. Chromophore **Q′** and a schematized structure of dendrimer **G3**.

We will show herein that the strategy to incorporate a lipophilic chromophore in a protecting dendrimer shell is indeed successful and leads to nanometric water-soluble fluorescent TP-active tracers. These molecules were built from the core reagent **C** by successive repetition of nucleophilic substitution and condensation reactions. The water solubility is ensured by the ammonium groups that are linked to the surface of **G1**–**G3** in the last step by reaction with *N*,*N*-diethylethylenediamine (Scheme 2).

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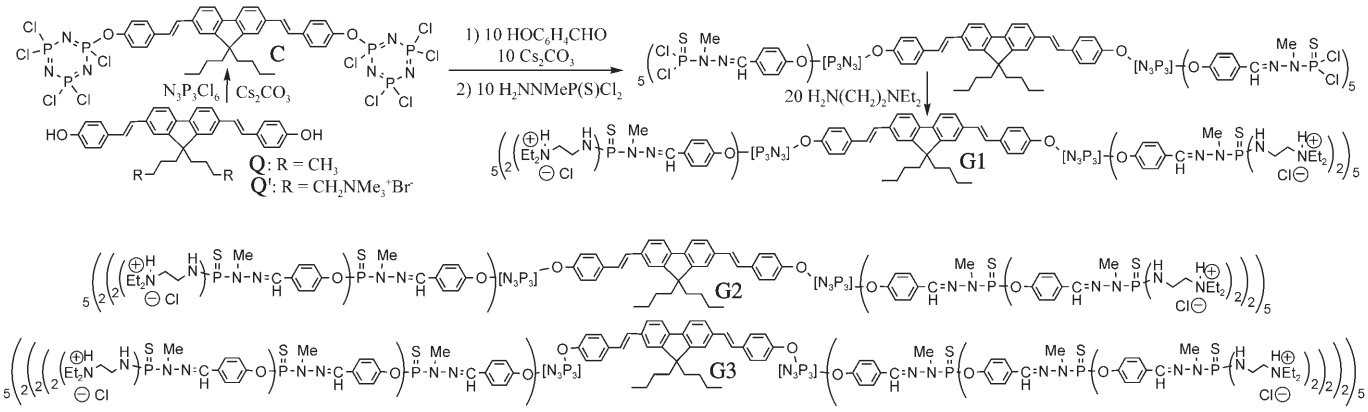
Supporting Information for this article (synthesis of chromophores **Q** and **Q′**; preparation of dendrimers **G1**–**G3**; experimental details of spectroscopic measurements; comparison of emission spectra of **G1**–**G3** in water and ethanol (Figure S1); fluorescence anisotropy results) is available on the WWW under http://www.angewandte.org or from the author.



Scheme 2. Synthesis of **G1** from **Q**, and structures of **G2** and **G3**. The core reagent **C** was prepared from the bisphenol quadrupolar chromophore **Q** by treatment with $N_3P_3Cl_6$ under basic conditions.

Dendrimers **G1**–**G3** show strong one-photon absorption in the near UV and strong emission in the blue-visible region (Figure 1). Interestingly, their photoluminescence (PL) efficiencies are similar in water and ethanol. This indicates that the dendrimeric approach is efficient in providing water solubility while maintaining high PL efficiency. In contrast, adding water-solubilizing ammonium groups directly to the aliphatic chain of the quadrupolar chromophore **Q** (to give the water-soluble chromophore **Q′**) does not lead to retention of the PL efficiency in water (Table 1). This decrease in PL efficiency is most probably related to molecular aggregation phenomena. Indeed, the PL is restored upon addition of a surfactant (sodium dodecylsulfate) to a water solution of **Q′**.

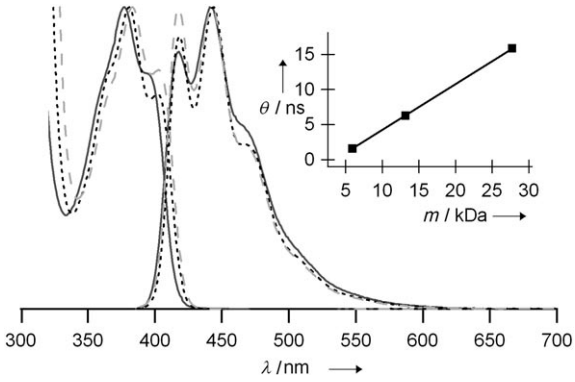


Figure 1. Absorption and emission spectra of **G1** (——), **G2** (-----), and **G3** (– – –) in water. Inset: rotational correlation time, θ, as a function of molecular weight m for dendrimers **G1**–**G3** in water ($T = 298$ K), calculated from the steady-state fluorescence anisotropy and the fluorescence lifetime using the Perrin equation. These anisotropy data indicate that **G1**–**G3** behave as isolated globular objects as their rotational correlation times increase linearly with the molecular weight. The hydrodynamic diameters of **G1**–**G3** were estimated from the rotational correlation times to be approximately 2.4, 3.8, and 5.2 nm, respectively.

Interestingly, the differences in relative vibronic intensities in the emission spectra of dendrimers **G1**–**G3** in ethanol and water tend to disappear with increasing generation number (see the Supporting Information). As a result, the emission spectra of the highest-generation dendrimer **G3** show similar vibronic intensities in ethanol and water, thereby indicating that the dendritic branches provide shielding layers that isolate the core chromophore from interactions with the external environment.

Table 1: Photophysical properties of compounds **Q**, **Q′**, and **G1**–**G3**.

	Solvent	$\lambda_{abs,max}$ [nm]	ε_{max} [10^4 M^{-1} cm^{-1}]	λ_{em} [nm]	Φ[a]	σ_2 [GM][b]
Q	EtOH	379	7.80	435	0.79	155[5]
Q′	water	349	–	354	0.22	8
G1	water	377	7.27	442	0.53	104
G2	water	381	6.24	444	0.71	119
G3	water	383	6.31	443	0.66	127

[a] Fluorescence quantum yield determined relative to fluorescein in 0.1 N NaOH. [b] At 705 nm; 1 GM = 10^{-50} cm^4 s photon^{-1}.

TPA measurements were conducted by investigating the TPEF of dendrimers **G1**–**G3** as well as model chromophores **Q** and **Q′** in solution. TPEF measurements allow for direct determination of the TPEF action cross-section $\sigma_2\Phi$, the relevant figure of merit for imaging applications and from which the TPA cross-sections can be derived.[9,10] In addition, the TPEF method has been recognized to be more reliable than nonlinear transmission measurements.[11] The quadratic dependence of the TPEF signal on the excitation intensity was checked for each data point and indicated that no photodegradation or saturation occurs. We should stress that the reported values, although moderate, are non-one-photon resonant values, which means that these chromophores could actually allow for the 3D resolution offered by selective two-photon excitation in the NIR region. This is not the case when even a small one-photon absorption is present at the excitation wavelength, which is the case in a number of chromophores with a giant resonant TPA reported recently.[12,13]

Strikingly, the water-soluble quadrupole **Q′** undergoes a marked decrease of its TPA characteristics (Table 1) in water as compared to **Q** in ethanol. As a result, although being water-soluble and based on a quadrupolar chromophore that

shows reasonable TPA efficiency in nonaqueous protic media, **Q′** has a low TPEF cross-section in water. In contrast, dendrimers **G1**–**G3** maintain a significant TPA cross-section in water, thereby confirming that the dendritic branches indeed isolate the chromophoric core, thus preventing aggregation and PL quenching. Interestingly, the TPA characteristics appear to increase slightly with increasing generation. This effect could be related to the slight increase of local polarity at the core with increasing generation due to the nature of the dendritic branches, which provide a somewhat polar environment.[14] Recent calculations and experiments[15] have shown that increasing solvent polarity, in a certain range, can lead to TPA enhancement.

Our results demonstrate that water-soluble, luminescent two-photon markers can be obtained by incorporating a lipophilic two-photon chromophoric unit within shielding layers built by a dendritic approach. This covalent layer-by-layer approach allows us to modulate the solubility by varying the nature of the peripheral groups while isolating the core TP chromophore from deleterious effects. This route allows us to overcome the drawbacks encountered when solubilizing groups are grafted directly onto a TP chromophore and provides a unique modular approach. In particular, replacing the core unit by more efficient TP chromophores[3] and taking advantage of the polar environment provided by the dendritic environment should lead to more efficient TP tracers, while further surface functionalization opens up a route for further functionality (such as recognition or targeting). This strategy can, in principle, be applied to various lipophilic chromophores that show high TPA cross-sections and large fluorescence quantum yields as long as phenol moieties can be grafted onto them, thus allowing for the building of the dendritic sheath.

This is of particular interest for biological applications due to the low toxicity detected for phosphorus-based dendrimers.[16] This is a major advantage—in addition to the accessibility for both inner and surface functionalization using covalent chemistry—over semiconductor quantum dots (QD). These inorganic nano-objects have gained a lot of popularity as photonic imaging agents[17] but their toxicity is still a main concern. Taking into account potential risks is one of the most important issues in nanoscience. With this aim in mind, we have demonstrated that the organic-nanodots route can provide a promising alternative in terms of two-photon brilliance,[18] and that it allows us to maintain excellent PL properties while conveying water solubility, which leads to water-soluble tracers that can indeed be used for in vivo imaging (Figure 2). This is an important step that establishes that organic nanodots definitely merit consideration as an alternative to semiconductor QDs for two-photon imaging, although further studies focusing on toxicity and (photo)-stability are needed. In this respect, it is important to add that in preliminary experiments we have found that polycationic phosphorus dendrimers bearing the same ammonium end groups are stable for months towards hydrolysis and that the photostability of dendrimers **G1** and **G2** is significantly higher than that of chromophore **Q′** in water, thus indicating that this strategy is also of interest in terms of photostability improvement.

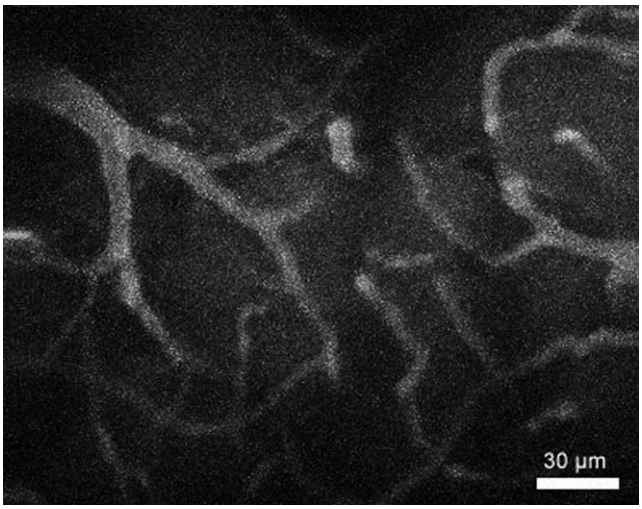


Figure 2. Two-photon imaging of the vascular network in the dorsal part of the rat olfactory bulb. Vessels were labeled after injecting intravenously a small bolus of 500 μM of dendrimer **G2** in water. The fluorescence was excited at 710 nm, epi-collected, and band-pass filtered (440/40 nm). The image was taken at a depth of about 200 μm. No obvious toxic effects were observed during the experiment (for technical details, see reference [19]).

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