Communication

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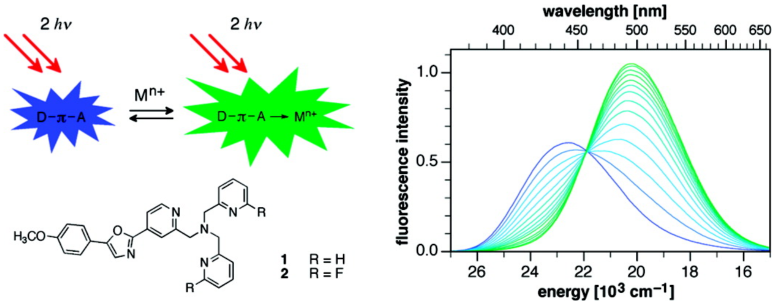

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Design of Emission Ratiometric Metal-Ion Sensors with Enhanced Two-Photon Cross Section and Brightness

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Two-photon excitation fluorescence microscopy (TPEM) has rapidly evolved into a widely used tool in biological and biomedical research.[1] Compared to traditional fluorescence microscopy, TPEM offers intrinsic 3D resolution combined with reduced phototoxicity, increased specimen penetration, and negligible background fluorescence. At present, most fluorophores used as labels or sensor platforms in TPEM have been adopted from linear microscopy and are not optimized for two-photon excitation.[2] Notably, the fluorescence brightness ($\eta\delta$), defined by the product of TPA cross section (δ) and emission quantum yield (η), is typically low due to a modest δ.[3] The development of new TPEM-optimized fluorophores is particularly vital in the context of biological metal-ion sensing since most of currently available ratiometric sensors, including the widely used dyes fura-2 and indo-1,[4] exhibit a low brightness that decreases even further upon cation binding. In addition, the majority of ratiometric metal-ion sensors offer only a large shift of the excitation peak but not emission energy. If only a single two-photon excitation source is on hand, such sensors are not suitable for dynamic ratiometric TPEM imaging with temporal resolution. In this communication, we address these problems with a molecular design approach that yields both an increase in δ and a shift of the peak emission energy upon metal-ion binding in a polar environment.

Molecular design strategies of organic molecules with large δ are well-established.[5,6] In general, the magnitude of δ increases with an increasing degree of intramolecular charge transfer (ICT) upon excitation. For example, centrosymmetric fluorophore architectures consisting of a conjugated linear π-system with an acceptor moiety sandwiched between two electron donors (D−π−A−π−D) exhibit exceptionally large δ values.[6] However, in water, the highly polarized excited state gives rise to enhanced solvent−solute interactions, which in turn leads to a reduced δ, more efficient nonradiative deactivation, and thus to a drastically reduced brightness.[7] The centrosymmetric architecture poses additional challenges for the design of metal-ion sensors: (1) the interpretation of the sensor response is complicated due to the presence of two metal binding sites (Scheme 1a); (2) metal binding induces a reduction of ICT which in turn results in a smaller δ accompanied by a strongly blue-shifted peak excitation energy; and (3) partial decomplexation or ejection of the metal cation in the excited state is presumably responsible for typically small emission shifts that are not suitable for ratiometric sensing.[8] Here, we propose to overcome these problems by using a *simplified D−A*[9] *motif where the metal-ion binds to the acceptor rather than donor site* (Scheme 1b). Besides eliminating the second binding site, such an arrangement should yield an increase rather than decrease of ICT upon excitation. As a consequence, metal-ion binding is expected to result in an increased δ, enhanced fluorescence brightness, and a red-shifted peak emission suitable for ratiometric measurements.

Scheme 1

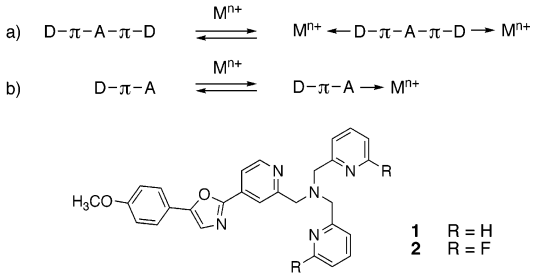

Table 1. Computed One and Two-Photon Photophysical Properties of the Free and Zn(II) Complexes of Fluorophores **1** and **2**[a]

	1	[**1**-Zn]SO$_4$	**2**	[**2**-Zn]SO$_4$
E_{ge} (eV), λ (nm)	3.54, 350	3.30, 376	3.53, 351	3.32, 373
$\hbar\omega_{max}$ (eV), λ_{max}(nm)	1.77, 701	1.65, 752	1.76, 703	1.66, 748
δ_{max} (GM)[b]	38	62	41	84
M_{ge} (D)	7.6	7.9	7.5	7.7

[a] Based on geometries optimized at the B3LYP/6-31G* level of theory (see Supporting Information for details). [b] Unit Göppert-Mayer (1 GM = 10^{-50} cm^{4}s/photon).

Quantum chemical calculations have proven to be a reliable tool for rationalizing structure−property relationships of two-photon absorbing fluorophores.[5,6,10] To evaluate the proposed design, we calculated δ based on the well-established methodology combining the sum-over-states (SOS) perturbative approach with highly correlated MRD-CI (multireference determinant−configuration interaction) calculations of the excited states based on the INDO Hamiltonian.[5,10] Given our interest in Zn(II)-selective fluorescent probes,[11] we combined a tris(picolyl) metal-chelating unit[12] with an anisole donor linked through a rigid oxazole core[13] (fluorophore **1**). The calculations indicate that Zn(II) coordination to the acceptor site should increase the gas-phase TPA cross section of **1** by almost a factor of 2, provide a significant red shift of the two-photon peak absorption energy from 1.77 to 1.65 eV, and enhance the oscillator strengths (Table 1).

Encouraged by these results, we synthesized **1** and studied its properties. Spectrochemical titrations in methanol or water as solvent revealed that **1** coordinated to Zn(II) with a 2:1 rather than 1:1 stoichiometry, an unexpected finding that was further confirmed by Job's method of continuous variation (Supporting Information). To avoid misinterpretations of the photophysical properties due to the presence of multiple coordination equilibria, we investigated fluorophore **2** with a modified chelating moiety containing two weaker coordinating 6-fluoropyridine ligands. As evident from Table 1, the photophysical properties of **1** were predicted to remain essentially unaltered upon fluorine substitution. Consistent with a single, well-defined coordination equilibrium, UV−vis titration of

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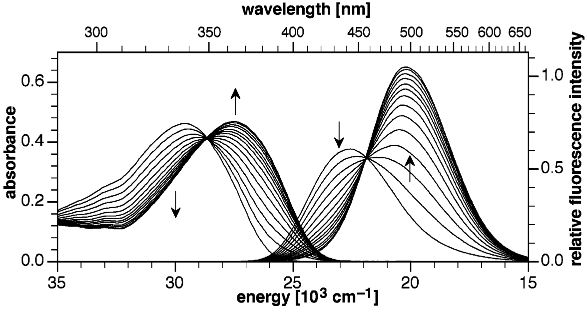

Figure 1. Absorption (left) and fluorescence emission (right) spectra for the titration of fluorophore **2** (21 μM) with $Zn(OTf)_2$ in MeOH.

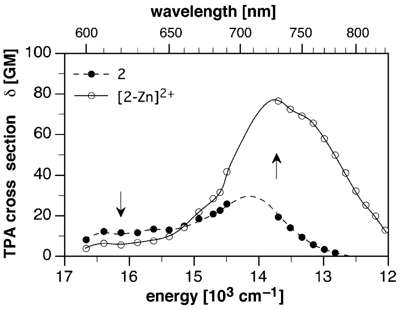

Figure 2. Two-photon excitation spectra of fluorophore **2** (100 μM solution in MeOH) in the absence (●) and presence (○) of Zn(II).

Table 2. Photophysical Data^a for Fluorophore **2** and [**2**-Zn]$^{2+}$

	2	[**2**-Zn]$^{2+}$
absorption λ_{max} (nm)	338 (2.15)^b	362 (2.27)^b
emission λ_{max} (nm)	441^c	497^c
quantum yield^d	0.35	0.71
$\delta\eta$ (GM)^e	11	55
δ (GM) (λ_{max}, nm)^f	31 (690)	77 (730)

[a] MeOH, 25 °C. [b] Molar extinction coefficient in parentheses [10^4 cm^{-1} M^{-1}]. [c] Excitation at isosbestic point. [d] Quinine sulfate as reference. [e] Two-photon action cross section. [f] Two-photon absorption cross section.

2 with Zn(II) in MeOH revealed a sharp isosbestic point and an overall bathochromic shift of the peak absorption energy (Figure 1 and Table 2). Nonlinear least-squares fit analysis of the absorption traces was consistent with a 1:1 ligand−Zn(II) binding mode and yielded a dissociation constant of $K_d = 2.4 \pm 0.1$ μM. We additionally confirmed the 1:1 stoichiometry by Job's method and ^{1}H NMR titration (Supporting Information).

In concurrence with the calculated photophysical properties (Table 1), the fluorescence emission maximum of **2** was also shifted to lower energy concomitant with an increase in quantum yield upon saturation with Zn(II) (Figure 1 and Table 2). Nonlinear least-squares fit analysis of the emission traces yielded within experimental error an identical dissociation constant. Furthermore, analysis of the ratio of emission intensities at 520 and 420 nm in the presence of various biologically important cations revealed good selectivity toward Zn(II) combined with a large dynamic range ($R_{max}/R_{min} = 46$) suitable for sensitive ratiometric measurements (Supporting Information).

In agreement with the theoretical predictions, δ_{max} of **2** increased from 31 to 77 GM upon saturation with Zn(II) in methanolic solution. Similar to the one-photon absorption spectra, the peak wavelength was also shifted to lower energy (Figure 2). Two-photon excitation at 730 nm produced similar emission spectra compared to one-photon excitation (Supporting Information). Given the considerable emissive quantum yield of both the free and Zn(II)-bound fluorophores (Table 2), the fluorescence brightness $\delta\eta$ increased 5-fold from 11 to 55 GM, thus exceeding the brightness of many fluorophores widely used in biology, including fluorescein, BODIPY, DAPI, or GFP.[3,4] Compared to fluorescein, **2** showed also improved photostability (Supporting Information). Most importantly, unlike fluorophores with a D−π−A−π−D architecture, fluorescence emission of **2** is not reduced but substantially enhanced upon Zn(II) coordination. It is worthwhile noting that at wavelengths above 800 nm δ of **2** is negligible in the absence but strongly enhanced in the presence of Zn(II), thus offering a strong turn-on response (>10 000-fold increase) of the fluorescence emission in this spectral range.

In conclusion, we have demonstrated that metal-ion coordination to the acceptor site of fluorophores with a D−A architecture results not only in a significant increase of the TPA cross section and brightness but also a substantial shift of the peak emission energy suitable for ratiometric sensing. This approach is not limited to the design of Zn(II) sensors but should be applicable to a broad range of donor−acceptor fluorophores modified with tailored chelating sites for selective metal-ion sensing.

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Supporting Information Available: Procedures for the synthesis of **1** and **2**, complexation studies, ^{1}H NMR titrations, and a summary of the computational results (Cartesian atomic coordinates for the optimized geometries and the photophysical data). This material is available free of charge via the Internet at http://pubs.acs.org.

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