Three-Photon Probes

A Three-Photon Active Organic Fluorophore for Deep Tissue Ratiometric Imaging of Intracellular Divalent Zinc


Abstract: Deep tissue bioimaging with three-photon (3P) excitation using near-infrared (NIR) light in the second IR window (1.0–1.4 μm) could provide high resolution images with an improved signal-to-noise ratio. Herein, we report a photostable and nontoxic 3P excitable donor-π-acceptor system (GMP) having 3P cross-section ($\sigma_3$) of 1.78 × 10^−40 cm^6 s^2 photon^−2 and action cross-section ($\sigma_2$) of 2.31 × 10^−41 cm^6 s^2 photon^−2, which provides ratiometric fluorescence response with divalent zinc ions in aqueous conditions. The probe signals the Zn^{2+} binding at 530 and 600 nm, respectively, upon 1150 nm excitation with enhanced $\sigma_3$ of 1.85 × 10^−39 cm^6 s^2 photon^−2 and $\sigma_2$ of 3.33 × 10^−40 cm^6 s^2 photon^−2. The application of this probe is demonstrated for ratiometric 3P imaging of Zn^{2+} in vitro using HuH-7 cell lines. Furthermore, the Zn^{2+} concentration in rat hippocampal slices was imaged at 1150 nm excitation after incubation with GMP, illustrating its potential as a 3P ratiometric probe for deep tissue Zn^{2+} ion imaging.

In-depth imaging of heterogeneous biological tissues, where scattering from various biological components restricts the imaging process, has been a significant challenge to scientists. In this context, recent progress in super-resolution fluorescence imaging and development of multiphoton microscopy have been identified as promising candidates in medical diagnostics. However, the bottleneck in deep tissue imaging is the need for stable and nontoxic multiphoton active fluorophores. Most of the reported 3P active fluorophores are based on inorganic semiconductor crystals, several of which are highly toxic to biological tissues, with the exception of doped ZnS nanocrystals. Therefore, the design of organic fluorophores that allow excitation in the second IR window is of much interest to chemists and biologists. A potential class of molecules towards this end are donor-π-acceptor (D-π-A) systems that are active in the second IR window. Such fluorophores may allow high spatial resolution imaging of biological specimen up to several micrometers in depth.

Even though a few multiphoton-active fluorophores are known for deep tissue imaging, the design of analyte-specific 3P active fluorophores in a heterogeneous medium is more challenging. In such cases, a multiphoton probe with the possibility of single wavelength excitation in the second IR window, allowing the ratiometric determination of the emission maximum at two distinct wavelength positions, would be ideal.

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Such an internally calibrated ratiometric sensor with 3P excitation is of great contemporary relevance in the context of clinical bioimaging applications. The cubic dependence of 3P process on the input intensity renders stronger spatial confinement, and therefore yields high contrast and high resolution images.[15-18]

Zinc(II), the second most abundant element in human body after iron, is vital for many biological processes.[19,20] More importantly, it has been found that concentration of free zinc ions is associated with certain neurological disorders such as Alzheimer’s disease, Parkinson’s disease, and epilepsy.[21] Several groups have attempted the development of efficient probes for imaging Zn$^{2+}$ in living systems.[22] An interesting recent report demonstrates the development of a fluorescent probe for Zn$^{2+}$ that could monitor how zinc fluxes regulate fertilization events in cells.[22] We had earlier reported intramolecular charge transfer (ICT) based linear donor-acceptor-donor systems that show enhanced single-photon[24-26] and 2P responses to Zn$^{2+}$ in vivo.[22] Further need for the development of multi-photon active fluorescent probes has prompted us to design a 3P active fluorescent probe, GMP, with excellent 3P action cross section and ratiometric signal response upon Zn$^{2+}$ binding, that facilitates deep tissue fluorescence imaging. As suggested earlier, D-$\pi$-A systems form the ideal choice for designing multi-photon active molecules. Our molecular design carries a bipyrindine acceptor attached to a pyrrole donor through a vinyl linkage. Binding of Zn$^{2+}$ at the bipyrindine unit not only enhances the extent of charge separation but also rigidifies the chromophore, making it a better D-$\pi$-A system that can possibly enhance the nonlinear optical properties. We also attached an oxyethylene chain to achieve better solubility and biocompatibility.

GMP was synthesized according to previous reports[27,28] and characterized by NMR spectroscopy and high-resolution mass spectroscopy. The photophysical properties of GMP were studied in 3% DMSO/HEPES buffer (pH 7.2; DMSO = dimethylsulfoxide, HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) solution. GMP ($c = 12 \mu M$) exhibited an absorption maximum at 370 nm ($\varepsilon = 7300 M^{-1} cm^{-1}$) and an emission maximum at 530 nm ($\lambda_{em} = 370$ nm, Figure 2a) with a Stokes shift of 8159 cm$^{-1}$. The fluorescence characteristics of GMP and the resulting changes upon Zn$^{2+}$ binding under 3P excitation are shown in Figure 1. The change in solvent polarity did not have any obvious influence on the original absorption spectrum of the molecule. However, the emission spectrum showed solvatochromism, suggesting the formation of a polar excited state that is stabilized with increasing solvent polarity (Figure S1 in the Supporting Information). The fluorescence quantum yield of GMP in 3% DMSO/HEPES buffer (pH 7.2) was determined as 0.13 using quinine hemisulfate as the standard ($\Phi_f = 0.54$ in 1 M H$_2$SO$_4$).[29] The fluorescence lifetime was measured by excitation at 370 nm and monitoring the emission at 530 nm. The decay profile exhibited a monoexponential decay, suggesting the presence of a single species in the excited state with a lifetime of approximately 1 ns (Figure S2).

The initial screening of metal binding with GMP was conducted in acetonitrile and was found to interact with Zn$^{2+}$ and Cd$^{2+}$ (Figure S3). Since Cd$^{2+}$ is not a biological cation, this probe can be used for the selective sensing of Zn$^{2+}$ in biological samples. GMP, upon binding with Zn$^{2+}$, induces attenuation in the absorption intensity at 370 nm with the concomitant formation of a new band at 400 nm (Figure S4). Upon Zn$^{2+}$ complexation, the emission spectrum of GMP exhibited a ratiometric response with a quenching of the native fluorescence intensity at 530 nm along with the formation of a red-shifted band at 600 nm (Figure 2). The fluorescence quantum yield of the GMP-Zn$^{2+}$ complex in DMSO was 0.18 using Nile red ($\Phi_f = 0.7$ in 1,4-dioxane)[33] as the reference. The binding stoichiometry as well as the binding constant were estimated using the Benesi–Hildebrand (B-H) plot and equation, respectively. The B-H plot of $1/\Phi_f$ vs. $1/[Zn]^{2+}$ shows a straight line confirming 1:1 binding, and the binding constant, $K_b$ obtained upon dividing the intercept by the slope was found to be 7.50×10$^3$ M$^{-1}$ (Figure S5). The value is relatively low when compared to other similar fluorophores.[24,27] The fluorescence decay curves monitored at 530 nm and 600 nm revealed a biexponential decay process, indicating the presence of two excited-state species (Figure S2). The low binding constant explains the existence of both the free and bound species in the solution, which exist in dynamic equilibrium with each other. A summary of the photophysical parameters of GMP and

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**Figure 1.** Zn$^{2+}$ chelation induced emission changes from GMP upon 3P excitation at 1150 nm.

**Figure 2.** a) Changes in the emission spectra of GMP (12 $\mu M$, 3% DMSO/HEPES buffer, pH 7.2) upon the addition of Zn(ClO$_4$)$_2$. b) Corresponding ratiometric plot showing the response of GMP with Zn$^{2+}$. Inset shows the fluorescence color change of GMP in the absence and presence of Zn$^{2+}$ ($\lambda_{ex} = 365$ nm, UV light).
GMP-Zn\(^{2+}\) is presented in Table S1. Structural optimization of GMP and its Zn\(^{2+}\) complex, GMP-Zn\(^{2+}\), computed at the B3LYP/6-311G(d,p) level and their absorption properties evaluated at TDDFT-PBE0/6-311G(d,p) level, are shown in Figure S6 and Table S2.

The fifth-order nonlinear optical behavior corresponding to the 3P excitation properties of GMP and GMP-Zn\(^{2+}\) were then investigated in detail. We carried out the 3P excitation studies in the 1100–1400 nm range using a Ti: Sapphire tunable femtosecond laser. Upon excitation at 1150 nm, GMP exhibited a broad emission with the maximum at 533 nm (Figure 3a).

Similarly, the excitation of GMP-Zn\(^{2+}\) complex at 1150 nm exhibited a redshift of the emission from 533 to 600 nm (Δλ = 67 nm, Figure 3a). Changes in the 3P absorption cross-section (α\(_{3}\)) of GMP in the absence and presence of Zn\(^{2+}\) are shown in Figure 3b. The logarithmic plot of fluorescence intensity as a function of incident laser power with a slope of approximately three confirmed the direct 3P excitation of GMP and GMP-Zn\(^{2+}\) (Figure 3c). Photographs of GMP and GMP-Zn\(^{2+}\) upon excitation with 1150 nm light visually demonstrate the fluorescence changes (Figure 3d,e). The multieponential excitation wavelength of 1150 nm corresponds to nearly three times that of the one-photon absorption maximum of GMP (370 nm). Therefore, the observed 3P excited fluorescence is the result of the simultaneous 3P resonant absorption of the 1150 nm photons by GMP.

The 3P cross-section (α\(_{3}\)) of GMP and GMP-Zn\(^{2+}\) were determined to be 1.78×10\(^{-90}\) cm\(^2\)s\(^{-1}\) photon\(^{-1}\) and 1.85×10\(^{-46}\) cm\(^2\)s\(^{-1}\) photon\(^{-1}\), respectively. The 3P action cross-section (α\(_{3},g_{3}\)) which is calculated as the product of fluorescence quantum yield, φ, and α\(_{3}\), was determined to be 2.31×10\(^{-20}\) cm\(^2\)s\(^{-1}\) photon\(^{-1}\) for GMP and 3.33×10\(^{-20}\) cm\(^2\)s\(^{-1}\) photon\(^{-1}\) for GMP-Zn\(^{2+}\). These values are one to two orders of magnitude higher than those of known 3P active fluorescent dyes.\(^{[15,34]}\) The increase in 3P absorption upon Zn\(^{2+}\) binding may be attributed to the planarization and rigidification of the chromophore, making it a better D-π-A system. A decrease in the emission intensity at 530 nm with a concomitant increase in 3P excited emission at 600 nm allowed the ratiometric detection of Zn\(^{2+}\). 3P parameters of GMP and the corresponding Zn\(^{2+}\) complex are shown in Table 1.

![Figure 3](image-url) 3P properties of GMP and GMP-Zn\(^{2+}\): a) 3P excited (1150 nm) emission spectra of GMP (2×10\(^{-10}\)) before and after addition of Zn\(^{2+}\) (0–2×10\(^{-10}\) M), b) 3P action spectra of GMP in the absence (red) and presence (black) of Zn\(^{2+}\), c) Logarithmic plot of fluorescence output with incident power intensity of GMP and GMP-Zn\(^{2+}\), d,e) Photographs showing 3P (1150 nm) excited fluorescence from GMP (d) and GMP-Zn\(^{2+}\) (e).

The photostability of GMP and GMP-Zn\(^{2+}\) under the imaging conditions was then determined. Both GMP and GMP-Zn\(^{2+}\) were found to be photostable upon irradiation at 1150 nm over a period of 15 min (Figure S7). To examine the use of GMP for bioimaging, we first evaluated the inherent cytotoxicity of GMP using PrestoBlue assay in HuH-7 (hepato cellular carcinoma) cell line (Figure S8). GMP exhibited good cell viability with low to moderate cytotoxicity at relatively high probe concentrations. In the in vitro 3P imaging capability of GMP was investigated by incubating GMP (6 μM) in HuH-7 cells for 6 h by following reported procedures.\(^{[28]}\) A schematic illustration of the microscopy setup adopted for the 3P imaging is depicted in Figure S9. Screening of excitation wavelength was carried out from 1100 to 1250 nm, which showed high fluorescence intensities upon excitation between 1150 and 1250 nm. We chose an excitation wavelength of 1150 nm for the 3P bioimaging studies. The internalization of the probe and its homogeneous distribution in the cytoplasm is evident from the in vitro studies (Figure S10a–d). Another set of HuH-7 cell cultures was incubated with Zn\(^{2+}\) ions prior to GMP incubation and then imaged using the same excitation wavelength (Figure S10e–h). The yellow fluorescence corresponding to GMP-Zn\(^{2+}\) inside the cells demonstrates the potential of GMP to image Zn\(^{2+}\) ions in vitro. The false-color pixel intensity map clearly shows internal distribution of GMP (Figure S10c) as well as GMP-Zn\(^{2+}\) (Figure S10g) in the cells.

The deep tissue 3P ratiometric imaging of Zn\(^{2+}\) was demonstrated using hippocampal slices of 20 month old rats. Hippocampus is a critical structure involved in synaptic plasticity and memory.\(^{[29]}\) Surgical operations were carried out and hippocampi were collected from male Wistar rats and immediately preserved at −80°C. Transverse hippocampal slices were prepared by following reported procedures (see the Experimental Section for more details).\(^{[30]}\) GMP was then incubated with hip-
pocampal slices in Dulbecco’s modified Eagle’s medium (DMEM). After 4 h, the slices were washed and imaged pristine-ly. Figure 4a–c show the 3P microscopy images of hippocampal slices after GMP incubation followed by excitation at 1150 nm and collection of the emission at 530 and 600 nm. Figure 4a shows the 3P excited fluorescence images corresponding to the unbound GMP, indicating effective localization of GMP in the hippocampal slices. Figure 4b shows yellow fluorescence with same pixel intensities, revealing the presence of the bound GMP (i.e., GMP·Zn$^{2+}$). The overlay image (Figure 4c) further reveals the separate position of the bound and the unbound GMP in the CA3 area of the selected hippocampal slice under analysis. Figure 4d shows fluorescence intensity profiles through dashed lines in Figure 4a,b. The profile plot clearly indicates that the mean ratio of fluorescence intensity of GMP·Zn$^{2+}$/GMP is ca. 1.9:1. As evident from the average intensity profile comparison, a relatively high amount of Zn$^{2+}$ was observed in CA3 area of the hippocampal slices of 20 month old rats.

In conclusion, we have developed a 3P active donor-π-acceptor type fluorophore (GMP) that can effectively signal and image Zn$^{2+}$ in biological specimens. The GMP probe exhibited selective ratiometric response to Zn$^{2+}$ in the visible region upon excitation at 1150 nm. The observed $\sigma_3$ and $\sigma_3\sigma_3$ values are among the highest of reported 3P active fluorophores. Good cell viability, high fluorescence quantum yield, large 3P cross-section and high 3P brightness of GMP facilitate the 3P imaging of free zinc ions in live cells. The demonstration of the 3P hippocampal slice imaging further indicates the viability of GMP as an efficient ratiometric 3P fluorescent probe for deep tissue Zn$^{2+}$ ion imaging.

Experimental Section

Measurements of three-photon action (3PA) cross-section: 3PA spectra of GMP and GMP·Zn$^{2+}$ were obtained by using the multi-photon luminescence method with Rhodamine B in water as reference. A Ti:Sapphire system that produces 110 fs (HW1/e) pulse in the wavelength range of 260–2600 nm with a repetition of 1000 Hz was used as the excitation source. Self-reabsorption during 3PA cross-section measurements was avoided by focusing the excitation beam close to a quartz cell, and photoluminescence was measured in back-scattering. The cubic dependence of 3P excited luminescence ($F_2$) and square dependence of two-photon excited luminescence ($F_3$) to incident power intensity are represented as below [Eq. (1)]:

$$F_2 \sim \eta_2 \sigma_2 N_2 I_{ex}$$
$$F_3 \sim \eta_3 \sigma_3 N_3 I_{ex}^2$$

(1)

where $F_2$ and $F_3$ are the three-photon and two-photon excited luminescence intensity respectively, $\eta$ is the luminescence quantum efficiency, $\sigma_2$ represents 3PA cross section of samples, $\sigma_3$ represents the two-photon absorption cross-section of Rhodamine B, $N_2$ and $N_3$ indicate the numbers of luminescent molecules in the focal volume, and $I_{ex}$ indicates the incident intensity at the focus. 3PA cross-section values were calculated by comparing the three-photon excited luminescence of the samples with the two-photon excited luminescence intensity of Rhodamine B.

Hippocampal slice preparation: Hippocampal slices prepared from male Wistar rats (20 months old) were used for this study. All procedures were approved by the guidelines from the Animal Committee on Ethics in the Care and Use of Laboratory animals of the National University of Singapore. Briefly, after anaesthetization using CO$_2$, the rats were decapitated and the brains were quickly removed and placed in artificial cerebrospinal fluid (ACSF) at 4 °C. Transverse hippocampal slices of 400 µm were prepared from the right hippocampus using a manual tissue chopper (Stoelting) and snap frozen in liquid nitrogen for further studies. The ACSF contains the following species (in mM): 124 NaCl, 4.9 KCl, 1.2 KH$_2$PO$_4$, 2.0 MgSO$_4$, 2.0 CaCl$_2$, 24.6 NaHCO$_3$, and 10D-glucose. Carbogen consumption was 20 L h$^{-1}$.

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