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6-Substituted quinoline-based ratiometric two-photon fluorescent probes for biological Zn^{2+} detection†

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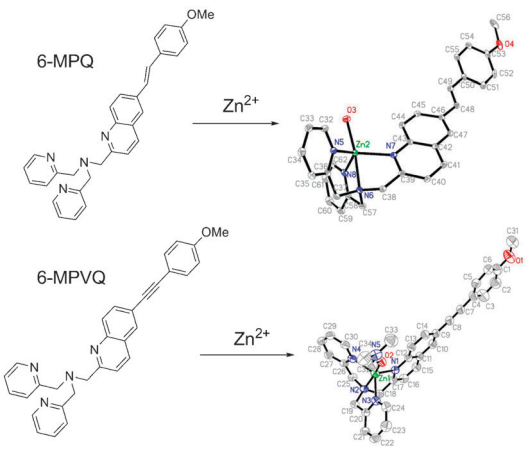
New ratiometric two-photon fluorescent probes are developed from 6-substituted quinolines for biological Zn^{2+} detection. They show large red shifts and good ratiometric responses upon Zn^{2+} binding. They also exhibit high ion selectivities and large two-photon absorption cross sections at nearly 720 nm. Because the new probes are cell-permeable, they can be used to detect intracellular zinc flux under two-photon excitation.

As the second most abundant transition metal ion in the human body, zinc is involved in many biochemical processes including neurotransmission, enzymatic regulation and cell apoptosis.[1] However, the biological roles of Zn^{2+} have been difficult to accurately understand because of its colourlessness and magnetic silence.[2] At present, fluorescent probes provide the optimal choice to detect Zn^{2+} ions in biological samples due to their high sensitivity and selectivity. Earlier Zn^{2+} probes are mostly designed on the basis of the technology of single-photon fluorescence.[3] Recently, as two-photon microscopy (TPM) has evolved to become a widely used tool in biology with low phototoxicity, good spatial resolution, and increased specimen penetration,[4] considerable attention has been directed to the development of two-photon fluorescent probes for detection of Zn^{2+}.

Some previous studies in this regard have led to the development of several two-photon Zn^{2+} probes based on fluorescein and coumarin.[5] However, most of these two-photon Zn^{2+} probes were designed to exhibit enhancement of the fluorescence intensity at only one wavelength. This design may cause difficulty in quantitative determination and quantitative bio-imaging due to the background interference.[6] By comparison, ratiometric probes are better choices that can overcome this particular limitation, because they allow quantitative detection of the analyte by measuring the ratio of emission at two different wavelengths.[7] Up to now, very few two-photon yet ratiometric Zn^{2+} probes have been successfully developed, and their properties still need to be improved to meet the requirements of the applications.[7,8] Thus the search for new ratiometric two-photon Zn^{2+} fluorescent probes, in particular those with new skeleton structures, remains an important task in the field.

In the present study, we design and synthesize new two-photon ratiometric Zn^{2+} probes based on the quinoline skeleton. The rationale for using quinoline as the basic structure is that quinoline-based Zn^{2+} probes showed stable fluorescent properties, good water solubility, and lower cell toxicity. To achieve ratiometric detection, we consider the intramolecular charge transfer (ICT) mechanism in our design.[4,9] This mechanism involves a charge transfer process from an electron donor to an electron acceptor within a fluorophore. To promote ICT in quinolines, we propose to incorporate an electron-donating substituent at the 6-position. To improve the two-photon cross section, we also propose to enlarge the conjugation system. Because biphenyl does not give good conjugation due to its intrinsic steric repulsion, we put a vinyl or alkynyl moiety into the system ending up with probes **6-MPVQ** and **6-MPQ**.

As described in the ESI†, **6-MPVQ** and **6-MPQ** can be easily synthesized in four steps with good yields. With the two probes in hand, we then make their complexes with Zn^{2+}. The X-ray crystal structures of the two complexes (Scheme 1) show that Zn^{2+} is coordinated by four nitrogen atoms. An additional H_2O- or DMF-oxygen coordinates to Zn^{2+} to complete the five-coordination geometry. Moreover, the ^1H NMR analysis confirms that the coordination of the probes with Zn^{2+} can take place in solution (ESI†). At this point, it is interesting to



Scheme 1 **6-MPVQ** and **6-MPQ** for Zn^{2+} detection.

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note that the conjugated system in **6-MPQ** is not fully planar as compared to that in **6-MPVQ** (Scheme 1). This means that the alkynyl connected system may enjoy a better conjugation than the vinyl one. As shown below, such difference may cause **6-MPVQ** to be advantageous over **6-MPQ** for a number of physicochemical properties.

To measure the spectroscopic properties of the two probes, we first examine their responses to Zn^{2+} in methanol–water solution (1 : 1, v/v, 50 mM HEPES, pH = 7.4). As shown in Fig. 1 (and also ESI†), when excited at 320 nm **6-MPQ** and **6-MPVQ** show maximum emission at 412 and 443 nm, respectively. The addition of Zn^{2+} causes the maximum emission of **6-MPVQ** to shift from 412 to 493 nm with a significant red shift of 81 nm. Such a large red shift has been rarely achieved for the Zn^{2+} probes reported to date.[10] This red shift also indicates that the nitrogen atom of the quinoline platform is coordinated with Zn^{2+} causing an enhanced ICT. Note that for **6-MPQ**, the maximum emission shifts from 443 to 515 nm with a red shift of 72 nm. These large red shifts provide a good opportunity to achieve the ratiometric detection. As shown in Fig. 1, upon titration with Zn^{2+} the ratio of emission intensity ($I_{493\ nm}/I_{419\ nm}$) of **6-MPVQ** increases dramatically with *ca.* 14-fold enhancement. A similar behavior is also observed for **6-MPQ** but the enhancement is lower (7-fold).

Note that the maximum ratio of fluorescence emission intensity is obtained when the Zn^{2+} concentration equals that of the probes. Job's plot analysis also indicates that when the Zn^{2+} molar fraction reaches 0.5, the maximum fluorescence intensity is obtained indicating a 1 : 1 binding model between the probe and Zn^{2+}. As to the quantum yield, we find that **6-MPVQ** exhibits a quantum yield enhancement of *ca.* 7-fold (from $\Phi_{free} = 0.036$ to $\Phi_{Zn^{2+}} = 0.266$) upon binding to Zn^{2+}. By comparison, the quantum yield of **6-MPQ** increases by *ca.* 5-fold. The apparent dissociation constants (K_d) of **6-MPVQ** and **6-MPQ** with Zn^{2+} are measured to be 0.45 and 0.58 nM respectively. Thus, both the probes can detect free Zn^{2+} in the nanomolar range, which affords sufficient sensitivity for Zn^{2+} in living cells.

The two-photon cross sections of the two probes and their Zn^{2+} complexes are determined by using the two-photon induced fluorescence measurement technique. As shown in Fig. 2, **6-MPVQ** and **6-MPQ** show the δ_{max} values of 335 GM at 710 nm and 135 GM at 725 nm, respectively. Upon saturating

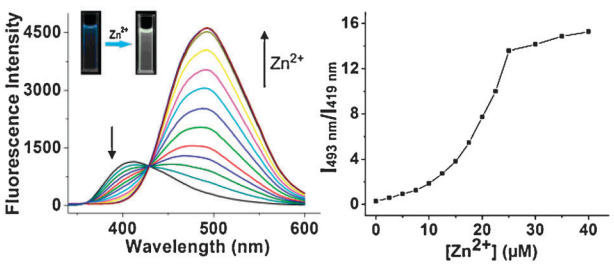

Fig. 1 (a) Emission spectra of **6-MPVQ** (25 μM) with the excitation at 356 nm upon titration with Zn^{2+} (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4 and 1.6 equiv.) in the methanol–water solutions (1 : 1, v/v, 50 mM HEPES buffer, pH = 7.4); (b) ratiometric calibration curve between 419 and 493 nm ($I_{493\ nm}/I_{419\ nm}$) as a function of Zn^{2+} concentration.

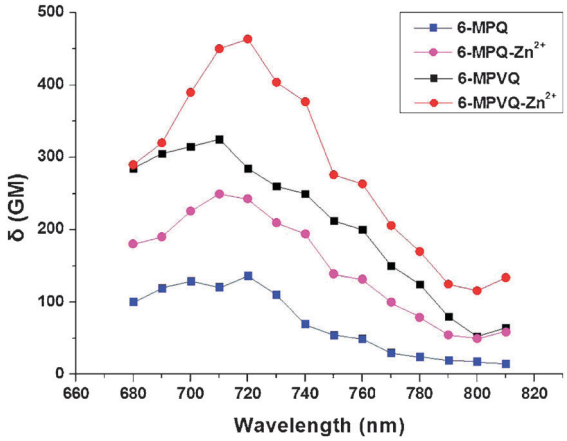

Fig. 2 Two-photon excitation spectra of **6-MPVQ** and **6-MPQ** with and without Zn^{2+}.

with Zn^{2+}, the δ_{max} values increase to 470 and 250 GM, respectively. It is evident that **6-MPVQ** shows a much better two-photon activity than **6-MPQ**, presumably because the former corresponds to a better conjugation system. Nonetheless, it is important to point out that the two-photon cross-section values of both probes are significantly larger than the previously reported ones.[11]

Ion selectivity is another important property of the fluorescence probes. As shown in Fig. 3, high concentrations of Na^+, K^+, and Ca^{2+} (1 mM) show negligible effects on the fluorescence of **6-MPVQ** (also for **6-MPQ** as shown in the ESI†). Besides, our tests show that both the probes are pH-insensitive in the biologically relevant pH range (ESI†). Moreover, other metal ions including Co^{2+}, Cr^{3+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Ni^{2+} do not interfere with the probe. Nonetheless, Cd^{2+} exhibits some enhancement of the fluorescence, which is a phenomenon observed for many previously developed Zn^{2+} probes.[12] Fortunately, the interference of Cd^{2+} is negligible in living cells. As a result, we believe that the present probes should have good selectivity for Zn^{2+} in the biological studies.

To investigate the use of **6-MPVQ** and **6-MPQ** *in vivo* under two photon excitation, HeLa cells are cultured and stained with the two probes within 30 min respectively. The cells are then washed once and bathed in Dulbecco's modified Eagle medium (DMEM) containing no fetal calf serum (FCS) prior

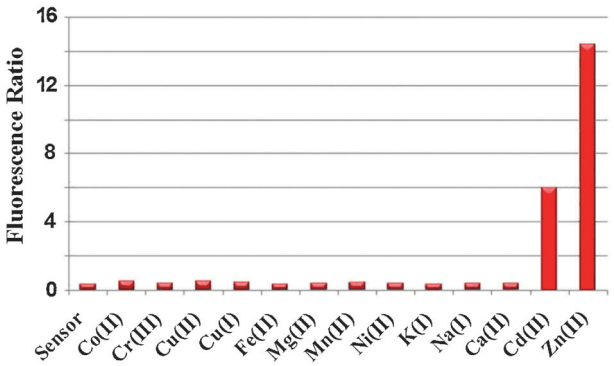

Fig. 3 Fluorescence ratio (493 nm/412 nm) of **6-MPVQ** upon addition of various metal ions. Experimental conditions: 25 μM **6-MPVQ**, 1.0 mM for Na^+, K^+, and Ca^{2+}, and 25 μM for Co^{2+}, Cr^{3+}, Cu^+, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+}, $\lambda_{ex} = 320$ nm.

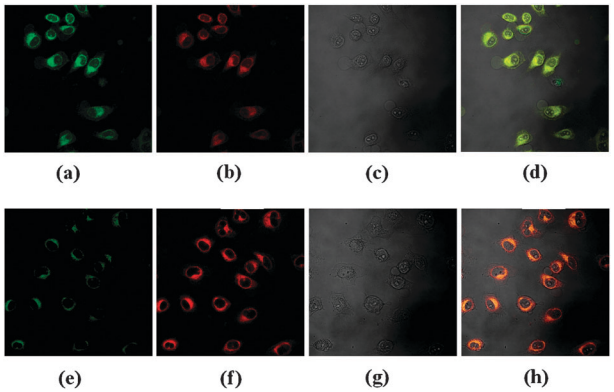

Fig. 4 (a) Two-photon image of HeLa cells incubated with 15 μM **6-MPVQ** after 30 min of incubation, washed with PBS buffer. λ_{ex} = 800 nm (emission wavelength from 390 to 465 nm). (b) Emission wavelength from 500 to 550 nm. (c) Bright-field image of HeLa cells. (d) The overlay of (a), (b) and (c). (e) Two-photon image following a 30 min treatment with Zn^{2+}/pyrithione (30 μM, 1 : 1 ratio). Emission wavelength from 390 to 465 nm. (f) Emission wavelength from 500 to 550 nm. (g) Bright-field image of HeLa cells. (h) The overlay of (e), (f) and (g).

to imaging and/or Zn^{2+} addition. TPM images are then obtained by exciting the probes with a mode-locked titanium–sapphire laser source set at wavelength 800 nm. According to the fluorescent properties of the probes, the optical windows at 390–465 and 500–530 nm are chosen for confocal imaging of **6-MPVQ**. As shown in Fig. 4, before addition of Zn^{2+} the optical window at 390–465 nm shows moderate fluorescence, whereas the optical window at 500–530 nm exhibits weak fluorescence. This experiment indicates that the new probes are cell permeable. After Zn^{2+} is added to the cells, the fluorescence intensity of the optical window at 500–530 nm increases dramatically, whereas that at 390–465 nm significantly decreases. Similar behaviors are also observed with **6-MPQ** under TPM excitation (ESI†). Thus the ratiometric fluorescence images generated from the above optical windows demonstrate that the new probes can reveal the variation of the intracellular zinc flux *in vivo* system under two-photon excitation.[13] Moreover, the 5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide (MTT) assay demonstrates that the probes show essentially no cytotoxicity after a long period of incubation.

In summary, we have developed two 6-substituted quinoline-based ratiometric two-photon zinc probes (**6-MPVQ** and **6-MPQ**) and investigated their applications *in vitro* and *in vivo*. Among the two probes, **6-MPVQ** shows a large red shift upon Zn^{2+} binding with 14-fold emission enhancement and a significant increase of the two-photon cross section. Moreover, **6-MPVQ** exhibits high ion selectivity and sensitivity for Zn^{2+} in a neutral aqueous solution. The *in vivo* two-photon microscopy imaging experiments show that the new probes are cell permeable and can be used for imaging Zn^{2+} in living cells in a ratiometric fashion. The good two-photon properties of the 6-substituted quinolines also hint the potential of their applications to the design of probes for other metal ions.

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