Two-photon excited fluorescent probes for calcium based on internal charge transfer†

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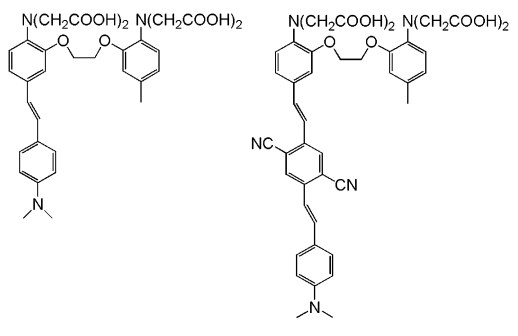
Two-photon excited (TPE) calcium fluorescent probes are designed and synthesized based on internal charge transfer (ICT) with high Ca^{2+} affinity and large two-photon action cross section, which can be used in living cells and detected with two-photon microscopy (TPM).

Calcium fluorescent probes have been the focus of biological and chemical researches for a long time owing to the vital role of calcium in various cellular processes. Since the Ca^{2+} chelator BAPTA was prepared by Tsien in 1980,[1] numerous BAPTA derivates have been synthesized and used as calcium probes. Early calcium probes, such as Indo-1, Quin-2 and Fura-2, worked under ultraviolet light excitation.[2] It has been commonly recognized that there are some disadvantages when these molecules are applied in biological samples, *i.e.*, the UV light-caused damage to living cells/tissue, the shallow penetration depth, the autofluorescence of samples and photobleaching of fluorophores.[3] Although some visible light-excited probes including Fluo-, Rhod- and Ca Green-series[4] are able to partly resolve the above problems, new molecules with the potential to circumvent such shortcomings are still desired. Recently, two-photon (TP) excitation has attracted much attention in biological applications due to its anti-Stokes photoluminescence nature. TPE molecules can be excited in the NIR region to give emission in the visible region, therefore one can reasonably expect lowered photodamage to biological samples and photobleaching of fluorophores, eliminated autofluorescence and enhanced penetration depth in microscopic imaging. In TP excitation applications, the TP cross section is undoubtedly one of the most important and crucial properties of TPE molecules. Some Ca^{2+} indicators, for instance Oregon Green BAPTA-1,[5] were utilized in TPM, however, the TP cross sections were rather poor, which required impractically high probe concentrations and/or a high laser power. Most recently, Kim and co-workers reported a two-photon calcium probe, ACa1, with enlarged TP cross section, *i.e.*, 110 GM at 780 nm.[6] They have subsequently developed a series of interesting ACa1 analogs, ACa2 and ACa3.[7] Like most fluorescent probes for metal ions, these calcium indicators are designed on basis of the photoinduced electron transfer (PET) principle, which presents enhanced fluorescence upon chelation of metal ions.[8–10] An example is the probe ACa1, containing the calcium chelator (BAPTA) and the chromophore derived from 2-acetyl-6-(dimethylamino)-naphthalene which are functionally independent and were structurally linked with an amide bond so as to enable the signaling process.

Aiming at further developing TPE calcium fluorescent probes with improved performance, and according to our previous experiences in preparing TPE molecules as fluorescence donors,[11] we hereby designed and prepared two TPE calcium probes on basis of the ICT principle with BAPTA as chelator (namely TP-BAPTA and TP-CN-BAPTA, Scheme 1) in the present work. The TP chromophores were derived from 1,2-diphenylethene, which is an efficient TPE fluorescent motif. Two types of scaffold, *i.e.*, D–π–D for TP-BAPTA and D–π–A–π–D for TP-CN-BAPTA (where A refers to an electron acceptor and D an electron donator),[12] which have different length of conjugate chain, different electronic effect and consequently different TP cross section and quantum yield, were constructed and compared. In the as-designed molecules, part of the chelating domain (BAPTA) also belongs to the fluorescence domain, which essentially differs from PET-based probes. The ICT process occurs because of the structural integration of the fluorophore and the chelating domain.[13] In such a process, fluorescence is quenched upon metal ion chelation. One of the remarkable advantages of fluorescence quenching is that it can overcome the background signal and afford high sensitivity appropriate for quantitative



TP-BAPTA TP-CN-BAPTA

Scheme 1 The structures of TP-BAPTA and TP-CN-BAPTA.

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analysis, and that is why quenching-based chemosensors have been extensively developed for analytical and biochemical applications.[14] With such molecular design, both the TP cross section and the calcium affinity of the probe are significantly increased, as compared to existing Ca^{2+} probes.

Both TP-BAPTA and TP-CN-BAPTA showed two-photon induced fluorescence under 800 nm excitation (Fig. 1), which could be easily confirmed with the fact that neither of the two molecules has linear absorption around 800 nm. TP-BAPTA has a maximum absorption at 370 nm and a maximum emission at 440 nm. As a result of the enlarged conjugation plane and facilitated flow of the electronic cloud, the maximal absorption and fluorescence emission of TP-CN-BAPTA were red-shifted to 434 and 564 nm, respectively, relative to TP-BAPTA. The two-photon fluorescence of the molecules matched exactly with their one-photon fluorescence, which suggested that the excitation mode did not affect the excited state of the molecules.

The Ca^{2+} ion-binding ability of the as-prepared probes were tested in MOPS buffer (30 mM, pH = 7.2). With TP-BAPTA as an example, the binding of metal ions resulted in a decrease of fluorescence intensity (Fig. 2(a)), which is explained by the fact that the aromatic amine lone pairs of BAPTA are involved in both the chelation and fluorescence emission. After binding to Ca^{2+} ions, the charge of the aromatic amine electron-donator was transferred to the cation, thus the electron donating ability of the nitrogen atom was weakened, which resulted in the decrease of the electron density on the conjugate plane and caused fluorescence quenching. As is mentioned above, quenching-based assays can always afford high sensitivity in quantitative analysis. The

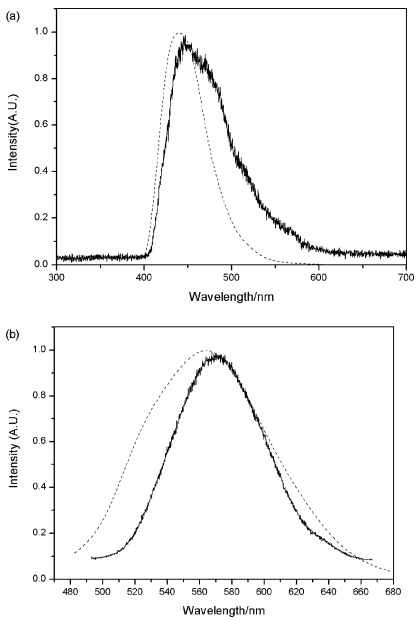

Fig. 1 (a) Fluorescence emission of TP-BAPTA under one-photon excitation (dotted line, excited at 370 nm) and two-photon excitation (solid line, excited at 800 nm). (b) fluorescence emission of TP-CN-BAPTA under one-photon excitation (dotted line, excited at 434 nm) and two-photon excitation (solid line, excited at 800 nm). Spectra were recorded in DMF, and the fluorescence intensities are given in normalized form.

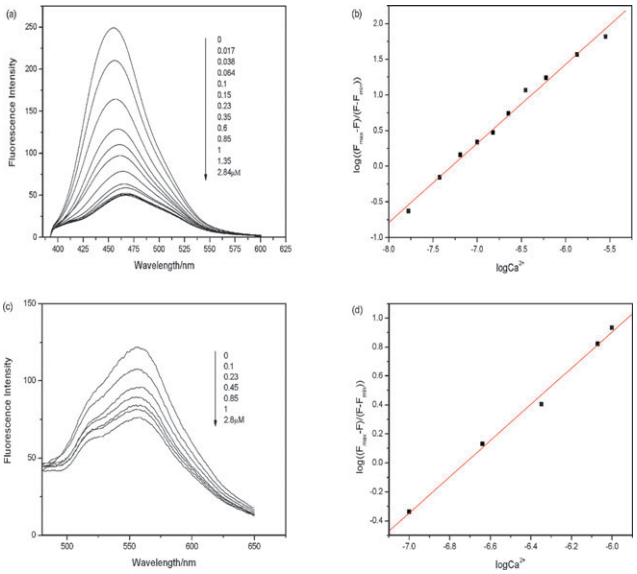

Fig. 2 (a) Fluorescence titration of 1 μM TP-BAPTA (100 mM KCl, 30 mM MOPS, 10 mM EGTA, pH = 7.2) with free Ca^{2+} from 0 to 2.84 μM. (b) Hill plot for the complexation of TP-BAPTA with free Ca^{2+} (0–2.84 μM). (c) fluorescence titration of 3×10^{-5} M TP-CN-BAPTA (100 mM KCl, 30 mM MOPS, 10 mM EGTA, pH = 7.2) with free Ca^{2+} from 0 to 2.8 μM. (d) Hill plot for the complexation of TP-CN-BAPTA with free Ca^{2+} (0–2.8 μM).

apparent dissociation constant (K_d) of TP-BAPTA determined with fluorescence titration confirmed this hypothesis (Fig. 2(b)). The Hill plot gave a straight line where the slope indicated the combining ratio between TP-BAPTA and Ca^{2+}, and the *x*-axis intercept represented the dissociation constant. The result showed a 1 : 1 complex and a K_d value of 51 nM. Compared to the K_d values of other calcium probes such as Fura-2 (145 nM), Fluo-3 (390 nM), Ca Green-1 (190 nM) and ACa1 (270 nM), the Ca^{2+} affinity of TP-BAPTA was notably improved. It should be noted, however, that high Ca^{2+} affinity is not always an advantage. Although it could be promising in terms of quantitative determination of Ca^{2+} concentration, it may make the probe unsuitable for measuring the calcium wave in living cells because of irreversible binding (*vide infra*).

The interference of other metal ions was investigated through measuring the fluorescence quenching of TP-BAPTA with the co-existence of Ca^{2+} and various ions (Fig. S1, ESI†). Mg^{2+}, Zn^{2+}, Mn^{2+} and Cd^{2+} led to little interference, but Co^{2+} and Ni^{2+} caused a rather serious fluorescence decrease. The influence of pH change was also tested. In the biologically relevant pH range, TP-BAPTA was pH-insensitive (Fig. S2, ESI†), which could be favorable in biological applications. Generally, the TP action cross section ($\Phi \times \delta$), which is the product of the TP-cross section (δ) and quantum yield (Φ), is regarded as the most important characteristic of potential TPE fluorophores. TP-BAPTA had a satisfying quantum yield in both DMF (Φ = 0.35) and MOPS buffer (Φ = 0.27, for determination details see ESI†). However the TP cross section was rather poor, *i.e.*, δ = 36 GM under 800 nm excitation (1 GM = 1×10^{-50} cm^4 s $photon^{-1}$, ESI†). Considering to improve the TP action cross section, TP-CN-BAPTA was designed and synthesized. In addition

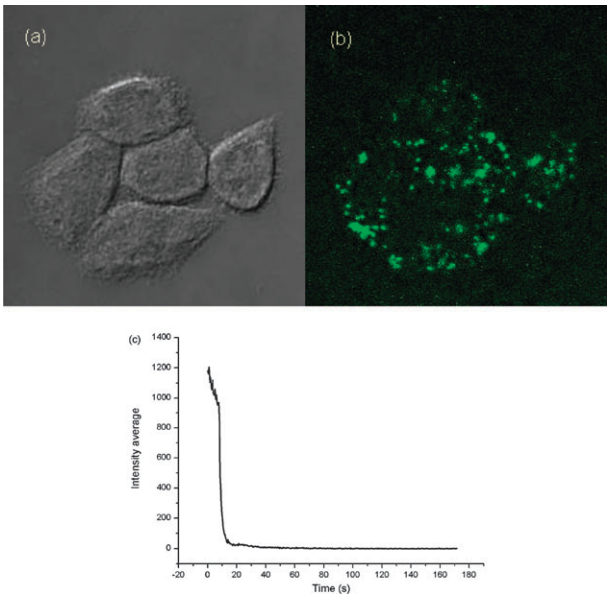

Fig. 3 (a) Bright field and (b) fluorescent images of HeLa cells loaded with 5 μM TP-CN-BAPTA-ESTER under two-photon microscopy, (c) quenching of intracellular fluorescence after stimulating the probe-loaded cells with 20 μM ATP. Excitation wavelength = 800 nm.

to a larger conjugated plane, cyano groups were added to the conjugated system to make a quadrupole molecule with a D–π–A–π–D scaffold. As expected, the TP cross section of TP-CN-BAPTA was dramatically increased to 917 GM. Compared to TP-BAPTA, although the introduction of cyano groups reduced the probe's quantum yield to 0.12 (in DMF) (still acceptable in fluorescence assays), the TP action cross section was significantly enhanced. The K_d value of TP-CN-BAPTA was calculated as 39 nM (Fig. 2(c) and (d)). The interference of other metal ions on this probe was also studied, which showed similar results to TP-BAPTA (Fig. S3, ESI†).

The cytotoxicity of both TP-BAPTA and TP-CN-BAPTA was investigated by MTT assay, which revealed that the probes were nontoxic to HeLa cells at a concentration as high as 24 μM (Fig. S4, ESI†). TP-CN-BAPTA was then chosen for intracellular experiments owing to its satisfactory TP action cross section. After incubating HeLa cells with TP-CN-BAPTA tetraethyl ester (TP-CN-BAPTA-ESTER) and exhaustive washing, uniform and bright green fluorescence was clearly seen with TP fluorescence microscopy in living cells (Fig. 3), which showed that TP-CN-BAPTA-ESTER was membrane-permeable. After stimulating HeLa cells with ATP to release free Ca^{2+}, the fluorescence of the probe was completely quenched (Fig. 3(c)), which demonstrated that TP-CN-BAPTA-ESTER was successfully hydrolyzed by intracellular esterase and could combine with free cytoplasmic calcium. As mentioned above, the binding, unfortunately, was nearly irreversible due to the inappropriately high Ca^{2+} affinity. As a consequence, the calcium wave could not be detected, although varying levels of ATP stimulation were tried. Nonetheless, the overall results still strongly suggested the feasibility of designing ICT-based TPE probes for calcium, which could be applicable in biological imaging or determination.

In summary, we prepared two TPE calcium probes on the basis of ICT, with D–π–D and D–π–A–π–D scaffolds, respectively. Large TP cross section and high Ca^{2+} affinity were obtained, and the TP action cross section was tunable through adjusting the structure of the molecules. The probe could transfer across the cell membrane, hydrolyze in the cytoplasm, and combine with free cytoplasmic calcium. The very high Ca^{2+} affinity together with the TP excitation nature make such probes suitable in quantitative determination of Ca^{2+} in biological samples. However, when the calcium wave in living cells/tissue is to be measured, further modification to the molecular structure would be needed so as to achieve moderated Ca^{2+} affinity.

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