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Two-photon fluorescence probes for imaging of mitochondria and lysosomes†

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Novel biocompatible cyanines show not only a very large two-photon cross-section of up to 5130 GM at 910 nm in aqueous medium for high-contrast and -brightness two-photon fluorescence live cell imaging but also highly selective subcellular localization properties including localization of mitochondria and lysosomes.

Among various conventional bioimaging techniques, two-photon excited fluorescence microscopy is a very attractive and non-invasive tool for living cell and tissue imaging.[1] Since the two-photon absorption (TPA) process is nonlinearly intensity-dependent, it can provide higher spatial resolution in imaging when compared with that of the one-photon counterparts. In addition, the use of the low-energy near infrared excitation would enable deeper tissue penetration and reduce the photobleaching and photodamage of the tissue during imaging which is particularly important for prolonged studies.[2] However, most of the currently used biological fluorophores by one-photon excitation show only low TPA cross-sections. Furthermore, TPA fluorophores often show substantially lower TPA cross-sections and fluorescence quantum yield in aqueous medium than those measured in organic solvents.[3] On the other hand, most of the highly active TPA molecules are not designed and suitable for biological applications as they often are either water insoluble or incompatible with the biological systems or form non-fluorescence aggregates in aqueous medium. To be useful for two-photon excited fluorescence microscopic applications, fluorophores need to possess large TPA cross-sections (σ_2). Besides, other desirable physical and biological properties including a high fluorescence quantum yield (Φ_{FL}), good water-solubility, high photostability and low cytotoxicity would play a crucial role in the realization of practical applications.[4] As a result, developing useful biological TPA fluorophores with a large two-photon excited fluorescence ($\Phi_{FL}\sigma_2$) and good solubility in aqueous medium without aggregation as well as low cytotoxicity still remains a great challenge to the scientific community.[5]

Fluorescence probes that can selectively target specific subcellular compartments and organelles are particularly useful tools for the study of localization, movements and intracellular concentration of a species of interest in living cells as well as for the study of cellular activities and processes. For instance, lysosomes are membrane-bound acidic organelles responsible for the breakdown of unwanted macromolecules. Two-photon probes for such lysosomes are still limited.[6] Mitochondria produce ATP, the energy currency of the cell, through a chain of electron transport and oxidative phosphorylation pathways and mediate apoptosis which are of great interest to study and monitor. As a result, the development of novel TPA biological fluorescence probes that show a specific targeting property is of practical significance.

It was previously demonstrated that methylpyridinium derived cyanines showed no specific subcellular localization properties although these dyes could afford high brightness for two photon excited fluorescence (TPEF) images of the HeLa cells.[7] However, by modifying the functional substituent(s) attached onto the heteroaromatic ring(s), we have successfully developed novel carbazole-based cyanine fluorophores that exhibit highly selective subcellular localization properties. In this contribution, we report herein the synthesis and investigation of biocompatible, mitochondrial and lysosomal probes with high two-photon cross-sections in aqueous medium based on the carbazole-based cyanine framework for TPEF imaging of live cells. The molecular structures of TPA carbazole-based cyanines are shown in Fig. 1. It is worth mentioning that the R substituent not only acts as a guiding moiety to selectively localize at a specific subcellular compartment but also affects the ultimate physical and biological properties of the probe.

Synthesis of carbazole-based cyanines is shown in Scheme 1. By adapting the convergent approach established previously,[1,2] the Knoevenagel reaction of the corresponding carbazolyl-3-aldehyde or carbazolyl-3,6-dialdehyde and functionalized 4-methyl-pyridinium

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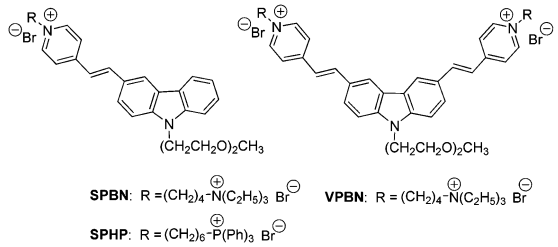
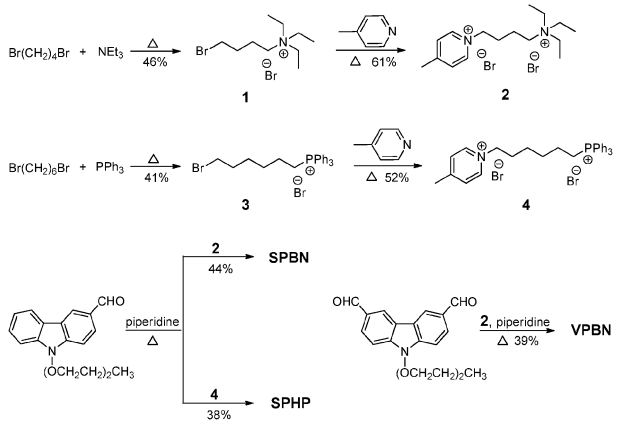


Fig. 1 Molecular structure of carbazole-based cyanines.



Scheme 1 Synthesis of carbazole-based cyanines.

halide was used as the key step to synthesize functionalized carbazole-based cyanines. 9-(2-(2-Methoxy-ethoxy)ethyl)-9*H*-carbazole-3-carbaldehyde and 9-(2-(2-methoxyethoxy)ethyl)-9*H*-carbazole-3,6-dicarbaldehyde were synthesized according to the published literature.[1,2] 1,4-Dibromobutane was reacted with triethylamine to afford ammonium salt **1** followed by alkylation with 4-methylpyridine to afford functionalized 4-methylpyridinium halide **2**. On the other hand, 1,6-dibromohexane was refluxed with triphenylphosphine to afford phosphonium salt **3** followed by heating with 4-methylpyridine to afford functionalized 4-methylpyridinium halide **4**. The Knoevenagel reaction of 9-(2-(2-methoxyethoxy)-ethyl)-9*H*-carbazole-3-carbaldehyde with methylpyridinium halide **2** and **4** afforded the desired mono-cyanines **SPBN** and **SPHP**, respectively. Furthermore, the Knoevenagel reaction of 9-(2-(2-methoxyethoxy)ethyl)-9*H*-carbazole-3,6-dicarbaldehyde and halide **2** gave the bis-cyanine **VPBN**. All of the new cyanines were fully characterized using ^{1}H and ^{13}C NMR, and HRMS. The data obtained are in good agreement with the proposed structures.

All of the cyanines are highly soluble in common organic solvents and water. Both of the mono-cyanines show very similar absorption characteristics with a broad and structureless charge-transfer band at ~424 nm in phosphate buffer. On the other hand, the bis-cyanine shows a distinct overlapping of two absorptions at the long wavelength region which is relatively red-shifted (*i.e.* λ_{max} = 443 nm) as compared to that of the mono-cyanines (Fig. S1, ESI†). This suggests that the two conjugated arms of the bis-cyanine are structurally and spectroscopically non-equivalent. Upon excitation at the absorption maximum, these cyanines exhibit relatively strong fluorescence at ~600 nm in organic solvents such as CH_3CN and DMSO but very weak and blue-shifted emission in aqueous medium (Table 1) due to the strong intermolecular interaction between the cyanines and water molecules.[8] Nevertheless, upon binding to a host such as ct-DNA and protein, these cyanines would exhibit a strong fluorescence enhancement. In addition, these cyanines are found to be stable to the change in the pH value from 4.0 to 10.6 in the buffer medium as reflected in the absorption and emission spectra of these cyanines in various pH buffered solutions (Fig. S2, ESI†). This property is especially beneficial to the application of imaging in a cell environment, in particular for acidic lysosomal probes.

Table 1 Summary of photophysical measurements of carbazole-based fluorophores measured in pH 7 phosphate buffer solution and DMSO

	$\lambda_{max}^{abs\ a}$/ nm (ε)	$\lambda^{em\ a}$/ nm	$\Phi_{FL}{}^{a,b}$	$\sigma_{max}{}^{c}$/ GM	$\lambda_{max}^{abs\ d}$/ nm (ε)	$\lambda^{em\ d}$/ nm	$\Phi_{FL}{}^{b,d}$
SPBN	424 (1.99)	600	0.02	243	435 (1.80)	603	0.37
SPBP	423 (1.20)	595	0.07	109	435 (1.53)	600	0.45
VPBN	443 (4.26)	582	0.003	5130	453 (3.92)	618	0.03

[a] Measured in buffer, $\varepsilon \times 10^4$ M^{-1} cm^{-1}. [b] Using rhodamine 6G (Φ_{488} = 0.95) as a standard and average of two independent measurements. [c] Maximum TPA cross-section. [d] Measured in DMSO, $\varepsilon \times 10^4$ M^{-1} cm^{-1}.

Upon excitation at 800 nm by femtosecond laser pulses in phosphate buffer, moderate fluorescence at around 572–590 nm is observed. The power-squared dependence of two-photon excited fluorescence of these cyanines measured at 800 nm fs laser pulses was followed with the slope of 1.8–1.9 indicating that these cyanines are two-photon excitation active (Fig. S3, ESI†). The TPEF spectra as shown in Fig. 2a are very similar to those of the one-photon excited counterparts suggesting that these cyanines have the same emissive states for the two-photon excitation process. The standard two-photon induced fluorescence method was used to determine the TPA cross-sections and two-photon excited spectra of the cyanines in the range of 620–1100 nm using a femtosecond pulsed laser in buffered solution.[9] Fig. 2b shows the two-photon excited spectra of the three newly synthesized cyanines in which the bis-cyanine, **VPBN**, shows a maximum TPA cross-section (σ_{max}) of 5130 GM at 910 nm which is among the highest σ_{max} reported in aqueous medium. Such a large value highlights the merit of using multi-dimensional structure motifs to enhance σ_2.[10] Furthermore, the mono-cyanines **SPBN** and **SPBP** afford a σ_{max} of 243 and 109 GM at 890 nm in buffered solution, respectively. Despite the identical donor–acceptor π-conjugated backbone for **SPBN** and **SPBP**, the discrepancy in the TPA cross-sections could arise from the difference in the solute–solvent interaction. Nevertheless, these cyanines with such high TPA cross-sections in aqueous medium have potential for TPA live cell imaging.

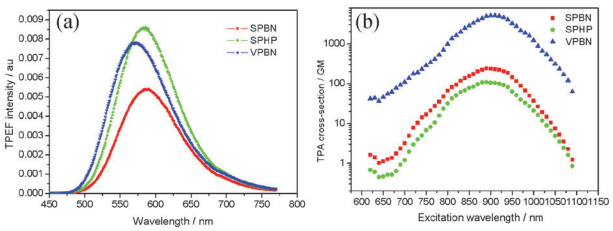


Fig. 2 (a) The two-photon fluorescence spectra excited at 800 nm fs laser pulses and (b) two-photon excited spectra of the cyanines in phosphate buffer solution.

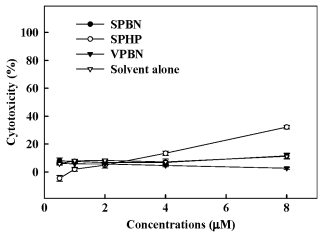


Fig. 3 Cytotoxicity of **SPBN**, **SPHP** and **VPBN** on HK-1 cells. Cells were treated with various concentrations of the compounds (0.5–8 μM) for 24 hours. MTT reduction assay was carried out to determine the cell viability. Data are expressed as mean value ± standard derivation of three separate trials.

To ascertain the potential of these cyanines for cellular imaging, the cytotoxicity of the cyanines towards HK-1 cells was assessed by MTT cell viability assay. Fig. 3 shows the cytotoxicity data of HK-1 cells after treatment with various concentrations (0.5–8 μM) of the cyanines for 24 h. The cytotoxicities of these cyanines are well below 20% at most of the dye concentrations indicating no significant cytotoxicity of these cyanines. Such low cytotoxicity is beneficial to prolonged cellular imaging.

To assess the ability of these newly developed cyanines as probes for live cell imaging and determine the location of the probes in the cells, the colocalization of these cyanines with various one-photon fluorescence organelle trackers in HK-1 cells was carried out. The one-photon fluorescence images showed that all the cyanines were efficiently taken up by the HK-1 cells at a concentration of 5 μM affording bright images. The one-photon fluorescence images of HK-1 cells co-stained with the cyanines and Mito Tracker or Lyso Tracker are shown in Fig. S4 and S5 (ESI†). Interestingly, the fluorescence image of **SPBN** greatly overlapped with that of the Mito Tracker indicating that **SPBN** is localized to the mitochondria; in contrast, the fluorescence image of **VPBN** largely colocalized with that of Lyso Tracker suggesting that the intracellular localization of **VPBN** is at the lysosomes (Fig. S5, ESI†) despite the same targeting triethylamino moiety being incorporated. In addition, the fluorescence images of **SPHP** and Mito Tracker are almost identical indicating that **SPHP** can exclusively stain mitochondria (Fig. S5, ESI†).

Fig. 4 and 5 depict the two-photon excited fluorescence (TPEF) images of HK-1 cells with **SPHP** and **VPBN** excited at 850 nm and 910 nm, respectively, under the same incubation conditions (2 μM, 24 h). The selective staining properties of **SPHP** to mitochondria and **VPBN** to lysosomes were clear and further confirmed with the one-photon tracker dyes. Consistent with the very large 2PA cross-section, **VPBN** affords excellent

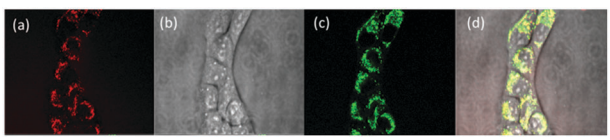


Fig. 4 (a) TPEF and (b) transmission images of HK-1 cells upon incubation with **SPHP** for 24 h at a concentration of 2 μM. (c) One-photon confocal microscopic images of the same sample after being co-stained with Mito Tracker (100 nM) for 10–20 minutes in the dark, (d) overlapped images of (a)–(c).

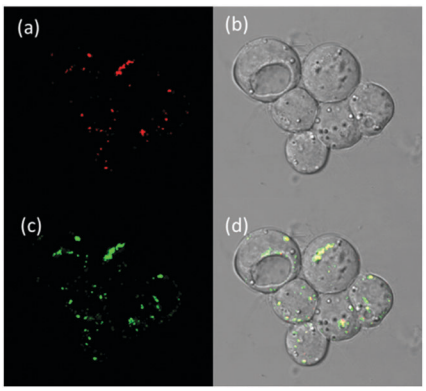


Fig. 5 (a) TPEF and (b) transmission images of HK-1 cells upon incubation with **VPBN** for 24 h at a concentration of 2 μM. (c) One-photon confocal microscopic images of the same sample after being co-stained with Lyso Tracker (100 nM) for 10–20 minutes in the dark, (d) overlapped images of (a)–(c).

brightness for TPEF images with high resolution. It is also clearly shown that the **SPHP** probe shows better contrast and brightness than that of **SPBN** (Fig. S6, ESI†), which is attributed to a larger two-photon excited fluorescence ($\Phi_{FL}\sigma_2$) of **SPHP**.

In summary, three new carbazole-based cyanine fluorophores have been developed as highly active two-photon fluorescence probes for live cell imaging. These probes not only show no significant cytotoxicity but also exhibit highly selective subcellular localization properties including localization of mitochondria and lysosomes. These cyanines have been found to show large two-photon cross-sections in aqueous medium in which a record high two-photon cross-section of 5130 GM at 910 nm has been obtained for **VPBN**. The TPEF images of HK-1 cells with these cyanines show good to excellent contrast and brightness. This study demonstrates that subtle structure modifications provide a means to optimize the functional properties of carbazole-based fluorophores for bio-imaging applications.

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