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Reaction-based two-photon probes for *in vitro* analysis and cellular imaging of monoamine oxidase activity†

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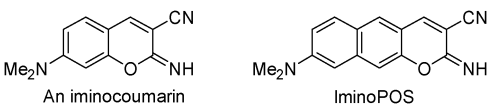
Reaction-based fluorescent probes for monoamine oxidases A and B are developed based on a new two-photon absorbing compound and its precursor. The probes show turn-on fluorescence response to the enzymes owing to the two-photon absorbing compound produced by the enzymatic activity, as monitored by one- as well as two-photon microscopy for the first time.

Tools for the detection and imaging of biological processes are essential for modern chemical biology. For example, our understanding of a given enzymatic process can benefit from fluorescent probes that provide an optical signal from the enzymatic process.[1] As a result, many fluorescent probes have been developed for various target analytes, most of which are based on one-photon absorbing dyes and thus can be analyzed by one-photon microscopy (OPM). Along with these efforts, the development of two-photon (TP) absorbing materials and the corresponding TP probes has received considerable attention during the last decade for their potential applications in optical imaging by two-photon microscopy (TPM). TPM utilizes two photons of lower energy to obtain the excited state of a fluorophore, and thus has several advantageous features over OPM, such as increased penetration depth, lower tissue auto-fluorescence and self-absorption, and very high resolution, in addition to the reduced photodamage and photo-bleaching.[2]

Recently several TP probes for metal cations in the biological systems have been developed based on acedan (2-acyl-6-dimethylamino-naphthalene), which gives bright TPM images in live cells and tissues.[3] Acedan is a naphthalene-based D–π–A system with the 2-dimethylamino group as an electron donor (D) and the 6-acetyl group as an electron acceptor (A), both of which induce the formation of the intramolecular charge transfer (ICT) state that dictates its photophysical properties.[4] Alternatively, the coumarin derivatives that have the D–π–A feature have also been widely used for the development of fluorescent probes; however, their applications in the development of TP probes are limited.[5]

Recently, the reaction-based or "reactive" fluorescent probes have attracted revived interest for their specificity toward given analytes. Various reactive fluorescent probes have been developed for metal cations, organic and inorganic anions, neutral and biological targets.[6] Most of these probes are based on one-photon absorbing compounds, and the corresponding TP probes remain unexplored. The advantageous features of TP probes prompted us to initiate the development of reaction-based TP probes. To this end, first we have synthesized a conjugatively extended coumarin analogue, which exhibits desired TP absorbing property. On the basis of this compound, next we have prepared reactive TP probes that provide the TP compound upon reaction with enzymes, in this study, monoamine oxidases A and B (MAOs A and B). With these probes, we were able to fluorescently monitor the enzymes' activity *in vitro* and to image the enzymes' activity in live cells by OPM as well as TPM for the first time.

The new coumarin analogue is named as IminoPOS, which has a hybrid feature of an iminocoumarin and acedan.



IminoPOS showed TP absorbing properties distinguishable from those of acedan and a typical coumarin (Table 1). IminoPOS emits at longer wavelength (λ_{em} = 585 nm) compared to the conventional coumarin when excited at λ_{max} = 446 nm. The quantum yield of IminoPOS (Φ_F = 0.63) is higher than those of acedan (Φ_F = 0.52) and coumarin 153 (Φ_F = 0.50). Also, the maximum two photon absorption cross-section values (GM) among those determined at three different wavelengths (780, 800, 820 nm) show that IminoPOS (GM = 180) has a comparable value to those of acedan (GM = 151) and coumarin 153 (GM = 141) (Table S2, ESI†), given than GM values show large fluctuations depending on measurements.

Given that IminoPOS has the desired optical properties, we may develop different types of reaction-based TP probes from

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† Electronic supplementary information (ESI) available: Characterization data for IminoPOS, probes **1a** and **1b**, and their absorption and emission data; enzyme activity assays, one-photon cell imaging data, and the probes' cell viability data. See DOI: 10.1039/c2cc32424e

Table 1 Photophysical properties of IminoPOS compared with acedan and coumarin 153

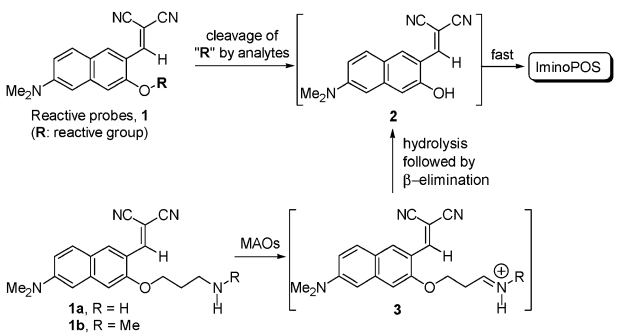
	λ_{max}; λ_{em} [b] (nm)	Φ_F [c]	σ (GM) [d]
IminoPOS	446; 585	0.63	180
Acedan	365; 501	0.52	151
Coumarin 153	422; 548	0.50	141

[a] All data were obtained for a 10 μM solution of the fluorophore dissolved in EtOH. [b] Measured at each absorption λ_{max}. [c] Quantum yields were determined by exciting at 410 nm, using rhodamine 6G (Φ_F = 0.6) as standard in EtOH.[16] [d] σ (GM) values were determined at three different excitation wavelengths (780, 800, and 820 nm) in EtOH, and the maximum one among the three values is given. Rhodamine 6G was used as a standard.[17]

its precursor. As the first example of such applications, we have designed MAO probes **1**. Both aminopropyl (**1a**) and N-methylaminopropyl (**1b**) were introduced as the reactive groups R, as these are known to be MAO substrates (Scheme 1).[9–11] MAOs would transform the aminopropyl moieties into the corresponding iminium ions, which, upon hydrolysis followed by β-elimination, would generate the hydroxyl group that would subsequently condense with one of the nitrile groups to produce IminoPOS as the final product.[7] As a result, probes **1a** and **1b** would show significant fluorescence changes.

MAOs play an important role in regulating tissue levels of amine neurotransmitters and dietary amines. Much efforts have been made to search for MAO inhibitors for the treatment of neurological disorders, such as Parkinson's disease and schizophrenia.[8] Although there are several fluorescent probes for MAOs, for example, luciferin–luciferase,[9] resorufin[10] and quinazolinone[11] derivatives, these are based on one-photon fluorophores and no TP probe has been reported so far.

First, photophysical properties of probes **1** and IminoPOS were compared. Probes **1a** and **1b** exhibited almost the same absorption and emission behaviours as expected. Both **1a** and **1b** showed broad absorption bands with λ_{max} at 448 nm. Interestingly, both the probes themselves showed very weak fluorescence, probably owing to the photo-induced electron transfer from the amine functionality to the fluorophore. IminoPOS showed a higher absorbance than those of **1a** and **1b**, with the same λ_{max} at 448 nm (ε = 19 230 L mol^{-1} cm^{-1}); it emitted a strong fluorescence with λ_{em} at 600 nm (Fig. 1a, Fig. S4, ESI†). Therefore, probes **1a** and **1b** are expected to behave as turn-on probes for MAOs if the reaction pathway in Scheme 1 is fulfilled.



Scheme 1 Structure of reactive probes **1a** and **1b** for MAOs and the sensing mechanism.

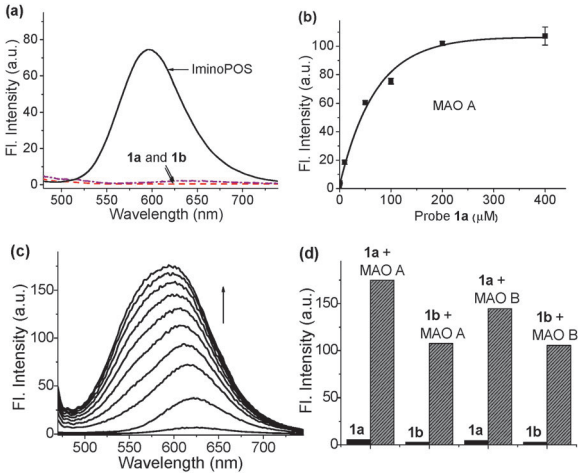


Fig. 1 (a) Fluorescence spectra of **1a**, **1b**, and IminoPOS (10 μM, each) in pH 7.4 buffer (100 mM HEPES containing 5% glycerol and 1% DMSO). (b) Fluorescence response of **1a** toward MAO A (10 μg mL^{-1}) in the buffer at 37 °C. (c) Time-dependent fluorescence changes of **1a** (70 μM) upon treatment with MAO A (10 μg mL^{-1}). Each of the data was recorded during 0–90 min at 10-min intervals. λ_{ex} = 448 nm. (d) Fluorescence intensity changes of **1a** and **1b** (at the K_m concentration) upon reaction with MAOs A and B (10 μg mL^{-1}), after 90 min. λ_{ex} = 448 nm.

The sensing ability of probes **1** toward MAOs was evaluated *in vitro* by following the fluorescence changes. Both the probes are found to be good substrates of MAO A and MAO B, two major isoforms of the MAO enzymes, as evidenced by the fluorescence enhancement owing to the formation of IminoPOS through the enzymatic reaction. The emission spectra obtained for probes **1** treated with the enzymes overlapped with that of IminoPOS but with a broader shape; the "enzyme-bound" IminoPOS might cause the broadening (Fig. S4, ESI†).

Next, the enzyme activity was assayed over a wide concentration range of the probes (0–400 μM) at a fixed concentration of MAOs (10 μg mL^{-1}) in pH 7.4 buffer (100 mM HEPES containing 5% glycerol and 1% DMSO) at 37 °C. The assay data for MAOs are given in Fig. 1b and Fig. S7 (ESI†). Probe **1a** is more sensitive toward MAOs A and B than probe **1b**. From the data, the Michaelis–Menten constants (K_m) were obtained, which are listed in Table S2 (ESI†). The K_m values were lower than those observed for the reactions of MAOs A and B with natural amine substrates, which are in the μM range.[10]

At the K_m concentration of the probe (**1a**/MAO A; 70 μM, **1b**/MAO A; 252 μM, **1a**/MAO B; 75 μM, **1b**/MAO B; 210 μM), we measured fluorescence enhancement using MAOs (Fig. 1c and d and Fig, S5 and S6, ESI†). The results show turn-on responses in the fluorescence titrations of probes **1a** and **1b** against both the MAOs.

Monoamine oxidase inhibitors (MAOIs) such as benmoxin, caroxazone, nialamide, pirlindole, moclobemide, and pargyline are a class of antidepressant agents prescribed for the treatment of depression. By inhibiting the activity of monoamine oxidase, MAOIs prevent the breakdown of monoamine neurotransmitters and thereby increase their availability. MAOIs such as moclobemide and pargyline are useful for detecting MAOs' activities.[12] In this study, we used these inhibitors for the *in vitro* enzyme inhibition assay. Thus, MAOs A and B were separately treated

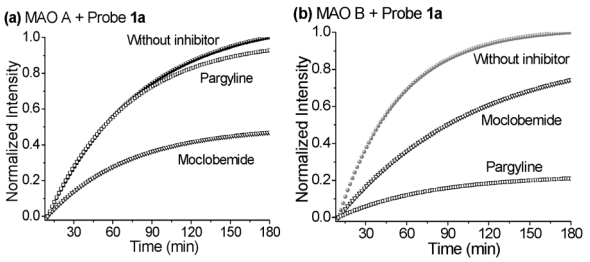


Fig. 2 (a) Enzyme inhibition assays. Fluorescence response of probe **1a** (70 μM) toward (a) MAO A or (b) MAO B (the final concentration: 10 μg mL^{-1}) in the absence and presence of inhibitors, moclobemide or pargyline (70 μM for each). Fluorescence intensity was measured during 0–180 min at 10 min intervals and 37 °C.

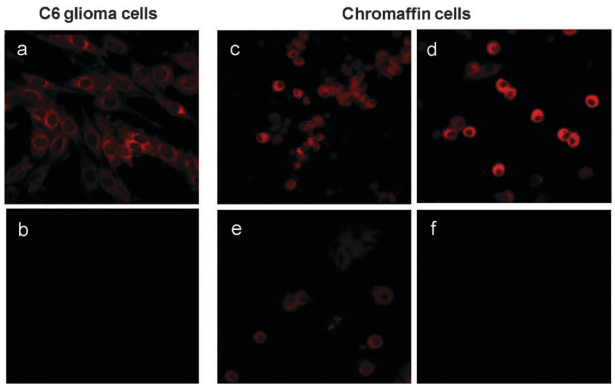


Fig. 3 Representative two-photon fluorescence images of C6 glioma cells and chromaffin cells that were treated with (a) IminoPOS (control), (b) probe **1a**, (c) IminoPOS (control), (d) probe **1a**, (e) moclobemide followed by probe **1a**, (f) pargyline followed by probe **1a**.

with moclobemide and pargyline, respectively, for 2 h at 37 °C, and then incubated with probe **1a** (70 μM) at the same temperature while the fluorescence increase was followed by fluorimetry at 10-min intervals during 3 h (the final concentration of each enzyme was 10 μg mL^{-1}) (Fig. 2 and Fig. S8, ESI†). The normalized fluorescence intensity data at each of the K_m values show that moclobemide inhibits 50% of the MAO A activity and 20% of MAO B activity, which are similar to the reported data (80% inhibition toward MAO A and 30% inhibition toward MAO B).[13] In contrast, pargyline shows slight inhibition toward MAO A activity but 80% inhibition toward MAO B activity, because it is a highly selective MAO B inhibitor.[14] The results from the spectroscopic and *in vitro* assays demonstrate that probes **1a** and **1b** are useful for the detection of MAOs' activity.

We further investigated an application of probe **1a**, which is more reactive than **1b**, toward MAOs for the fluorescent imaging of MAOs' activity in live cells. In this study, the chromaffin cell line was chosen as our main target for its high expression of endogenous MAO B.[15] As reference, we used the C6 glioma cell line that does not express MAOs.[11] Thus, MAOs' activity in chromaffin cells was fluorescently imaged with probe **1a** by OPM (Fig. S9, ESI†) as well as TPM (λ_{ex} = 900 nm, 10 mW laser power, and 0.382 frames per second imaging speed) (Fig. 3). Chromaffin cells treated with IminoPOS (Fig. 3c) and probe **1a** (Fig. 3d) show fluorescence, whereas the cells pretreated with the inhibitors show weak fluorescence (Fig. 3e and f), especially in the case of pargyline.

These results also confirm that MAOs recognize probe **1a** as a substrate and thus produce IminoPOS, a two-photon fluorophore, through the enzymatic oxidation followed by the hydrolysis and cyclization processes. The cellular fluorescent imaging experiments also indicate that probe **1a** penetrates the cells readily. As probes **1** have good cell-viability (Fig. S10, ESI†), they can be potentially useful for imaging MAOs' activity in living systems.

In summary, we have developed reactive fluorescent probes for MAOs based on a novel two-photon absorbing material, IminoPOS. The probes, precursors of IminoPOS, showed turn-on fluorescence changes upon reaction with the enzymes owing to the IminoPOS produced through the enzymatic process. The enzymatic activity in cells was also fluorescently imaged in the presence and absence of inhibitors, using one of the probes by OPM as well as TPM for the first time.

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