ChemComm

Cite this: *Chem. Commun.*, 2011, **47**, 12640–12642

www.rsc.org/chemcomm

COMMUNICATION

Visualization of tyrosinase activity in melanoma cells by a BODIPY-based fluorescent probe†

Tae-Il Kim,[a] Jihye Park,[a] Seonhwa Park,[b] Yongdoo Choi*[b] and Youngmi Kim*[a]

Received 15th August 2011, Accepted 30th September 2011
DOI: 10.1039/c1cc15061h

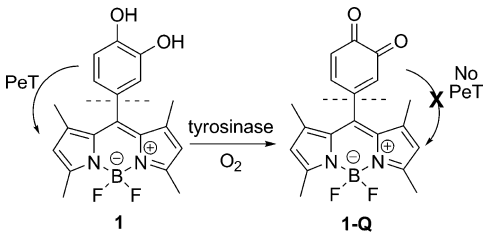
We have presented a fluorescent probe 1 that exhibits a fluorescence turn-on signal upon reaction with tyrosinase, and we show that it is readily employed for the assessment of tyrosinase activity and tyrosinase inhibitor activity in buffered aqueous solution, and further utilized for the visualization of endogenous tyrosinase activity in living melanoma cells.

Tyrosinase, which is widespread in plants and animal tissues, is a copper-containing enzyme that catalyzes the hydroxylation of phenol derivatives, such as tyrosine or tyramine, to the respective catechol derivatives (*i.e.*, L-DOPA or dopamine, respectively), followed by further oxidation to the corresponding *o*-quinone products.[1] The enzyme, specifically found in melanocytes, plays an essential role in the biosynthesis of melanin, the pigment that gives skin color, protects DNA in skin cells from ultraviolet (UV) radiation, and removes reactive oxygen species (ROS).[1] In humans, disruption of tyrosinase is responsible for severe skin diseases such as oculocutaneous albinism type I,[2] and elevated expression of this enzyme is found in melanoma cells.[3] In addition, tyrosinase might contribute to dopamine neurotoxicity and neurodegeneration associated with Parkinson's disease.[4] Consequently, a sensitive and selective assessment of this biochemical marker would be highly valuable not only for understanding its role in biological and pathological processes but also for providing effective diagnostic approaches and/or therapeutic targets in biomedical research.

Fluorescent probes have recently attracted considerable attention owing to their simple and rapid implementation, as well as their high sensitivity and high spatial resolution. However, there are only a few fluorescent probes that can detect catalytic activity of tyrosinase, with the exception of quantum dots (QDs)-, cyanine dye-, and oligo(phenylenevinylene)-based fluorescent probes.[5] Although these probes may be used to monitor tyrosinase activity, a general problem is that they all function in a turn-off mode in which the tyrosinase-catalyzed oxidation product, the quinone moiety, quenches fluorescence of the fluorophores or QDs *via* electron transfer. For practical applications, however, it is preferable to perform a bioassay in the turn-on mode because this mode is much more sensitive and better suited for bioimaging intracellular tyrosinase activity in living systems.

4,4-Difluoro-4-borata-3*a*-azonia-4*a*-aza-*s*-indacene (BODIPY) derivatives are well-known fluorophores with many valuable properties, including elevated (photo)chemical stability, relatively high fluorescence quantum yields and absorption coefficients, and versatility in terms of chemical derivatization.[6] In addition, the fluorescence properties of BODIPY derivatives can be systematically modulated by controlling the relative free energy change of an intramolecular photoinduced electron transfer (PeT) process (ΔG_{eT}).[7] Several research groups have used the PeT-dependent fluorescence off/on switching mechanism to successfully design *meso*-modified BODIPY dyes as fluorescence probes for redox active molecules and conditions (*e.g.*, nitric oxide and nitrative stress),[8] pH,[9] and various metal ions (*e.g.*, Fe^{3+}).[10] In these, fluorescence enhancement is achieved by perturbing the highest occupied molecular orbital (HOMO) energy levels of the *meso*-substituents upon interaction with target analytes. We utilized this finding to construct a tyrosinase-selective fluorescence "turn-on" probe, which functions despite the strong quenching of fluorescence by the tyrosinase-generated oxidation product. Herein, we report a PeT-controlled fluorescence turn-on probe for monitoring tyrosinase activity in aqueous media and in living cells.

Scheme 1 illustrates our design for tyrosinase assays, in which we consider substrate specificity of the enzyme and the turn-on signaling mechanism. Studies have reported that tyrosinase is able to use mono-, di-, and trihydroxyphenols



Scheme 1 Proposed fluorescence turn-on assay for tyrosinase by probe **1**.

[a] *Department of Chemistry, 126 Jukjeon-dong, Yongin-si, Gyeonggi-do, 448-701, Korea. E-mail: youngmi@dankook.ac.kr; Fax: +82 31-8005-3148; Tel: +82 31-8005-3156*
[b] *Molecular Imaging & Therapy Branch, National Cancer Center, 323 Ilsan-ro, Goyang-si, Gyeonggi-do 410-769, Korea. E-mail: ydchoi@ncc.re.kr; Fax: +82 31-920-2529; Tel: +82 31-920-2512*

† Electronic supplementary information (ESI) available: Experimental details, additional characterization studies, and additional information about experiments performed in living cells. See DOI: 10.1039/c1cc15061h

as substrates but has greater affinity for dihydroxyphenols, especially for catechol;[11] therefore, we incorporated a catechol moiety into the BODIPY chromophore to provide the fluorogenic substrate **1**. For the turn-on signaling mechanism, we harnessed the PeT process from the pendant catechol moiety to the excited fluorophore to quench the emission of the probe in aqueous media. Before the reaction of probe **1** with tyrosinase, the HOMO energy level of the catechol moiety is higher than that of the BODIPY unit in polar aqueous solution; hence, the fluorescence of probe **1** is quenched through a reductive PeT process.[7] The tyrosinase-catalyzed reaction will trigger the oxidation of the catechol unit of probe **1** to the corresponding benzoquinone; the HOMO energy level of the benzoquinone moiety is lower than that of the BODIPY skeleton. Such a transformation will suppress the PeT process and result in enhanced fluorescence intensity. This provides a method to selectively monitor tyrosinase activity, wherein fluorescence intensity depends upon the enzyme activity.

Probe **1** was prepared according to Scheme S1 (see ESI†). UV-Vis absorption and emission spectra of **1** were measured in an aqueous buffered solution (50 mM PBS, pH 6.3) containing 0.2% DMSO as a co-solvent. Probe **1** shows an absorption maximum at 496 nm ($\varepsilon = 8.8 \times 10^4$ $M^{-1}\cdot cm^{-1}$) and an emission maximum at 512 nm with a low fluorescence quantum yield (Φ_F) of approximately 0.001. This is similar to the observations by Burdette *et al.*, and the low quantum yield is attributed to an efficient PeT process in aqueous solution.[10] To confirm our hypothesis, the expected quinone product **1-Q** was synthesized independently, purified, and fully characterized. Conversion to the quinone is accompanied with slight bathochromic shifts in the absorption (λ_{abs} of 498 nm) and emission (λ_{em} of 515 nm) maxima. Moreover, **1-Q** exhibits an enhanced fluorescence quantum yield (Φ_F of 0.018) in an aqueous buffered solution, as anticipated. This result suggests that the PeT process of probe **1** will be attenuated upon tyrosinase-catalyzed oxidation, resulting in enhanced fluorescence intensity.

To examine whether probe **1** can be used to measure tyrosinase activity, we conducted enzymatic assays with probe **1** as the tyrosinase substrate in the presence of O_2 under optimized assay conditions (50 mM PBS buffer solution, 0.2% DMSO, pH 6.3, 37 °C).[12] Fig. 1 shows the time-dependent changes in absorption and fluorescence spectra of probe **1** upon the reaction with tyrosinase/O_2. When probe **1**

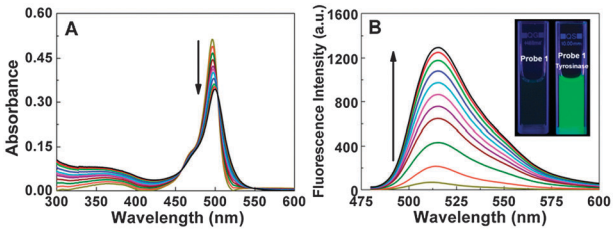


Fig. 1 Time-dependent absorption (A) and emission (B) spectra of probe **1** (5 μM) upon treatment with tyrosinase (90 nM)/O_2 at 37 °C in a PBS buffer solution (50 mM, pH 6.3, 0.2% DMSO). The spectra were obtained every 30 min (0–300 min). The emission spectra were obtained after excitation at 465 nm. Inset shows photographs, taken under UV light illumination (365 nm), of probe **1** (5 μM) before (left) and after (right) addition of tyrosinase (90 nM)/O_2 and incubation at 37 °C for 300 min.

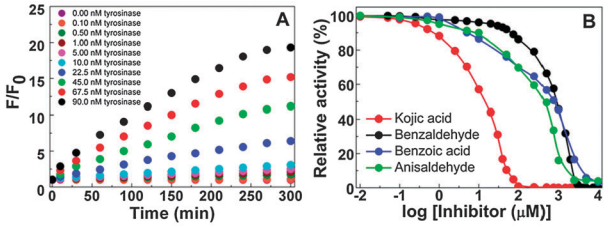


Fig. 2 (A) Increase in fluorescence intensity of probe **1** (1 μM) at 515 nm (λ_{ex} 465 nm) upon incubation with different amounts of tyrosinase (from bottom to top: 0 to 90 nM) in the presence of O_2. The reaction was conducted in PBS buffer (50 mM, pH 6.3, 0.2% DMSO, 37 °C). F_0 and F correspond to the fluorescence intensity of probe **1** in the absence and presence of tyrosinase, respectively. (B) Inhibition assay of tyrosinase activity by kojic acid, benzaldehyde, anisaldehyde, and benzoic acid: relative fluorescence intensity of the reaction with probe **1** as a function of concentration of inhibitors. Tyrosinase (90 nM) was incubated with an inhibitor for 20 min at 25 °C before addition of probe **1** (1 μM). After 300 min of incubation at 37 °C, the emission intensity at 515 nm was recorded (λ_{ex} 465 nm). Each point represents the average value of three measurements.

(5 μM) was incubated with tyrosinase (90 nM), the fluorescence signal at 515 nm increased with the incubation time (Fig. 1B). As shown in Fig. 2A, the results of kinetic analysis clearly indicate that the enzyme-catalyzed reaction is time-dependent. In addition, the change in fluorescence intensity is directly proportional to the amount of enzyme added over the range tested (see ESI†); higher concentrations of tyrosinase result in a greater signal-to-noise ratio. Tyrosinase could be assayed with a sensitivity limit as low as 0.5 nM of tyrosinase. Control experiments revealed little change in the fluorescence intensity of probe **1** in the absence of tyrosinase or O_2 from the reaction mixture during the same period of time (see ESI†). These results indicate that the tyrosinase-catalyzed oxidation of the catechol unit leads to the increase in fluorescence intensity of probe **1**. Complementary LC-MS analysis of the assay solution confirmed that the tyrosinase-induced reaction generated the corresponding quinone product, **1-Q** ([M + H] = 355.1, see ESI†). The nonfluorescent nature of probe **1** and the significant fluorescence turn-on response of tyrosinase-treated probe **1** are clearly visualized (Fig. 1B inset). We then determined kinetic parameters for the enzymatic oxidation reaction of probe **1** using Lineweaver–Burk analysis. The k_{cat} and K_m values of probe **1** ($k_{cat} = 0.01$ s^{-1}, $K_m = 5.3$ μM) were higher than those of L-DOPA, a commercially available tyrosinase substrate ($k_{cat} = 0.007$ s^{-1}, $K_m = 1.7$ μM). These data indicate that enzymatic efficiency for probe **1**, as estimated by a k_{cat}/K_m of 2.0 $mM^{-1}\cdot s^{-1}$, is lower than that of L-DOPA ($k_{cat}/K_m = 4.0$ $mM^{-1}\cdot s^{-1}$).

Next, to investigate the utility of probe **1** for screening tyrosinase inhibitors, tyrosinase activity was tested in the presence of common tyrosinase inhibitors, kojic acid, benzaldehyde, anisaldehyde, and benzoic acid at pH 6.3 (Fig. 2B).[11] Tyrosinase was pre-incubated with each inhibitor before being added to probe **1**, and the enzyme reaction was monitored by measuring fluorescence intensity at 515 nm. The enhancement of fluorescence intensity of probe **1** was inhibited in a dose-dependent manner by each inhibitor. In particular, addition of 100 μM kojic acid entirely blocked the fluorescence increase,

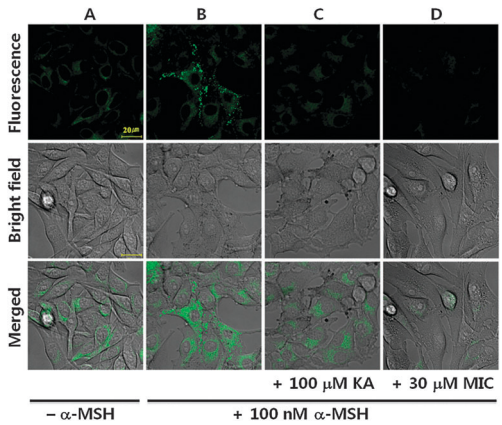


Fig. 3 Relative confocal fluorescence images of B16F10 melanoma cells treated under different conditions with probe **1**. (A) Melanoma cells were incubated with 2.5 μM probe **1** for 30 min at 37 °C and then imaged. (B) Melanoma cells were stimulated with 100 nM α-melanocyte-stimulating hormone (MSH) for 48 h and then incubated with 2.5 μM probe **1** for 30 min at 37 °C followed by imaging. Either 100 μM kojic acid (KA) (C) or 30 μM miconazole (MIC) (D) was co-incubated with cells during α-MSH stimulation, while all other conditions were the same. Top row of panels, fluorescence images; middle, bright field images; bottom, merged images. λ_{ex} = 488 nm; λ_{em} = 505–550 nm.

confirming that enzymatic activity of tyrosinase is required to oxidize probe **1**. The IC_{50} was calculated to be 18 μM for kojic acid, 850 μM for benzaldehyde, 460 μM for anisaldehyde, and 750 μM for benzoic acid; these values are in good agreement with previously reported values.[11,13] These results highlight the use of probe **1** for tyrosinase activity assays as well as for screening potential tyrosinase inhibitors.

To evaluate the practical utility of probe **1** for the detection of tyrosinase activity, photo- and chemical stabilities of **1** and **1-Q** were investigated under the assay conditions employed. Photostability studies of **1** and **1-Q** were performed by continuous irradiation of **1** and **1-Q** using a 150 W steady-state Xe lamp as the light source. The photoinduced bleaching was quantified by monitoring fluorescence intensity as a function of irradiation time (see ESI†). The fluorescence intensity of **1** and **1-Q** remained stable after irradiation at 465 nm for 2.5 h. In addition to photostability, the chemical stability of **1** and **1-Q** was studied under aerobic conditions, and the results indicate negligible changes in fluorescence spectra of both compounds in aqueous buffers for periods as long as 5 h (see ESI†). These properties suggest that probe **1** may be suitable for the investigation of such biological events.

We then evaluated the applicability of probe **1** to living cells. Bioimaging of tyrosinase activity was carried out in B16F10 cells, a melanoma cell line that expresses tyrosinase activity, with and without treatment with α-melanocyte-stimulating hormone (α-MSH), which upregulates tyrosinase expression in these cells.[14] Confocal fluorescence microscopic imaging of α-MSH-stimulated B16F10 cells that were incubated with probe **1** showed a significant increase in green fluorescence, indicative of tyrosinase activity (Fig. 3B); cells that were not treated with α-MSH exhibited less intense intracellular fluorescence (Fig. 3A). In particular, substantial fluorescent responses in α-MSH-stimulated cells were acquired at peripheral cell regions in a spotted pattern. These are in good agreement with the results of Ballotti *et al.* that α-MSH induces the accumulation of melanosomes at the dendrite tips of melanocytes.[14] Treatment of B16F10 melanoma cells with the tyrosinase inhibitor kojic acid (KA; 100 μM) during α-MSH stimulation resulted in a reduced fluorescent signal in the cells (Fig. 3C). In addition, we investigated tyrosinase activity in melanoma cells treated with miconazole (MIC), which significantly suppresses tyrosinase activity and tyrosinase protein expression in the cells (see ESI†).[15] Following addition of 30 μM MIC to the α-MSH-stimulated B16F10 cells, negligible fluorescence intensity at both peripheral and overall intracellular regions was observed (Fig. 3D). These results clearly demonstrate that probe **1** can be used to visualize the tyrosinase-induced oxidation in melanoma cells. Cell viability assays confirm that 5 μM probe **1** shows low cytotoxicity to B16F10 cells even upon treatment of cells for as long as 24 h (see ESI†).

In summary, we have shown that our BODIPY-based fluorescent probe **1** can be used for the selective detection of tyrosinase activity in buffered aqueous solution. Probe **1** is also suitable for screening potential inhibitors of tyrosinase, as well as for bioimaging intracellular tyrosinase activity in living cells.

This research was supported by a grant from the Fundamental R&D Program for Core Technology of Materials that was funded by the Ministry of Knowledge Economy, Republic of Korea, and by a Korea Research Foundation (KRF) grant funded by the Korean government (MEST) (No. 2010-0011160).

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