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# A Two-photon Fluorescent Probe for Intracellular Detection of Tyrosinase Activity

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Tyrosinase is a copper-containing enzyme and it can promote the hydroxylation of relevant catechol derivatives. These products could be further synthesized to the orthoquinone oxidation products.[1] Tyrosinase is widespread in biological tissues and can be obtained from commercially available mushrooms, *Agaricus bisporus*.[2] Melanoma is a malignant tumor originating in melanocytes, in which tyrosinase mediates the biosynthesis of the pigment melanin.[3] Owing to its overexpression level, tyrosinase is regarded as a biomarker for melanoma.[4] Consequently, the detection of tyrosinase with high sensitivity is of significant importance not only for observing its activities in biological and pathological processes but also for providing efficient diagnosis and treatment in biomedical and clinical research.[5]

Recently, several methods for the detection of tyrosinase have been developed on the basis of colorimetry, electrochemistry, and gold nanoparticles.[6] Nevertheless, fluorometry still attracts much attention due its accessibility and high sensitivity. Until now, only a few fluorescent probes were utilized for tyrosinase analysis, including quantum dots, conjugate polymers, and near-ultraviolet stimulated dyes.[7] Besides these probes, however, the use of a two-photon fluorescent group as signal output has been recognized because of its lower excitation energy, increased penetration depth (>500 nm), and greatly reduced autofluorescence in tissue.[8]

Previous studies illustrated that tyrosinase can be used to mediate the release of a cytotoxic agent from urea prodrugs in melanocyte-directed enzyme prodrug therapy (MDEPT).[9] This MDEPT mechanism can be employed to release a latent fluorophore for visualization of tyrosinase activity in a turn-on mode. Herein, we design a novel tyrosinase-detecting system that combines a phenol group as a substrate for the enzyme and a naphthylamine group (such a group is highly fluorescent due to its typical one-photon and two-photon absorption properties) as activatable signal reporter through a urea linkage.[10] In this approach, compounds NHU (1-(6-acetylnaphthalen-2-yl)-3-(4-hydroxyphenyl)-1-methylurea) and NDU (1-(6-acetylnaphthalen-2-yl)-3-(3,4-dihydroxyphenethyl)-1-methylurea) were newly synthesized with satisfactory yield (Figure 1a; the synthetic steps are described in the Supporting Information). As detailed below, their fluorescent emission mainly depends on the activity of the enzyme, which provides a method to selectively monitor tyrosinase activity in vitro and in cancer cells.

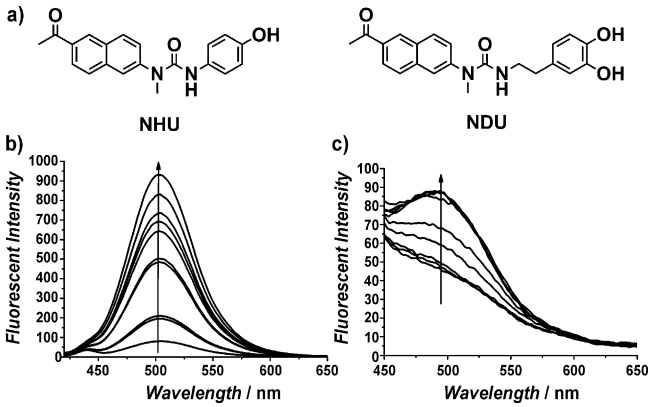


Figure 1. a) Structures of NHU and NDU. b,c) Dose-dependent one-photon fluorescent emission spectra of NHU and NDU (5 μM) upon incubation with tyrosinase for 24 h at 37 °C in 10 mM potassium phosphate buffer (pH 6.4) containing 0.5% DMSO. The concentrations of tyrosinase are 0 (control), 0.05, 0.1, 0.5, 1, 5, 10, 20, 40, 100 U mL$^{-1}$, respectively. The spectra were obtained after excitation at 382 nm.

First, to evaluate whether NHU and NDU can be used to measure tyrosinase activity, one-photon fluorescent titration experiments were conducted with these two compounds as substrates (Figure 1b and 1c). Since the optimum pH value for tyrosinase activity has been reported to lie between pH 6–7, a potassium phosphate buffer of pH 6.4 was chosen as simulated physiological conditions. In our experiments, NHU and NDU (5 μM) were incubated in the absence and

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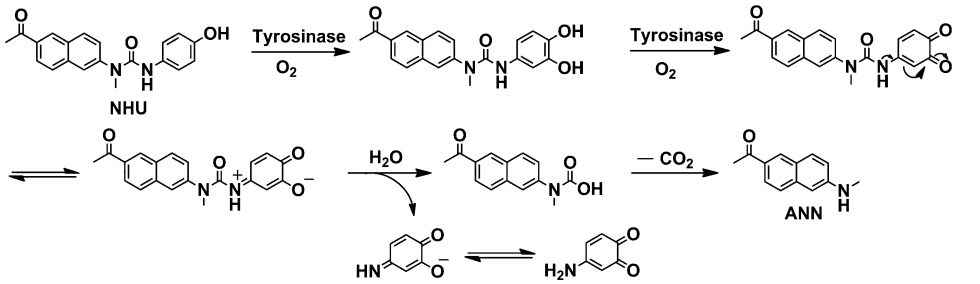
presence of tyrosinase (from 0.05 to 40 U mL$^{-1}$) for 24 hours at 37 °C. At the excitation wavelength of 382 nm, the emission peak of NHU was centered at 503 nm; it was weak initially and then gradually increased with increasing concentration of tyrosinase, displaying a nearly 12-fold enhancement when 40 U mL$^{-1}$ tyrosinase were used. By contrast, NDU exhibited a small change in fluorescence, with only a about two-fold increase in the presence of 40 U mL$^{-1}$ tyrosinase.

Hence, these results suggest dose-dependent changes in emission spectra of NHU and NDU upon the addition of tyrosinase. With regard to sensitivity, NHU is more suitable for tyrosinase detection in comparison with NDU. Furthermore, the nonfluorescent nature of NHU and the distinctive fluorescence turn-on response of NHU can clearly be visualized by the naked eye (Scheme 1 and Scheme S1 in the Supporting Information). A similar increase in fluorescence intensity was observed in the two-photon fluorescent spectrum of NHU upon addition of tyrosinase (Figure S1 in the Supporting Information, $\lambda_{Ex} = 770$ nm).




Scheme 1. Proposed mechanism for turn-on fluorescent detection of tyrosinase by NHU.

To confirm that the enzyme-catalyzed oxidation reaction is time-dependent, kinetic analysis was undertaken within a fixed time of 12 hours. As shown in Figure S2 in the Supporting Information, the change in one-photon fluorescent intensity at 503 nm, which was recorded once every hour, is directly proportional to the amount of enzyme added. Control experiments revealed a small increase in the emission of NHU in the absence of tyrosinase during the same period of time. These results indicate that the oxidation of NHU can be accelerated by tyrosinase catalysis, through which the fluorescent response was significantly enhanced.

To determine the optimal pH conditions for the detecting system, tyrosinase-mediated oxidation of NHU was investigated in 10 mM potassium phosphate buffer at pH values of 6.0, 6.4, and 7.4. The most rapid fluorescent response was observed at pH 6.4 (Figure S3 in the Supporting Information), in agreement with the optimal pH value reported in the literature.[3] Moreover, any interference of the biologically essential metal ions (e.g., $Na^+$, $K^+$, $Mg^{2+}$, $Fe^{3+}$, and $Ca^{2+}$) with the tyrosinase-catalyzed oxidation should be excluded. The results demonstrate that NHU has high selectivity toward tyrosinase even in the presence of these potentially interfering ions (Figure S4 in the Supporting Information). Next, we evaluated the selectivity of the probe NHU with other oxidizing enzymes. Alcohol dehydrogenase (ADH), which is widely distributed in human and animal liver, and known to facilitate the interconversion between alcohols and aldehydes or ketones, was chosen for the comparison (Figure S5 in the Supporting Information). As expected, NHU responded only slightly toward ADH at the concentration of 40 U mL$^{-1}$, thereby confirming that this probe is highly selective toward the target enzyme tyrosinase rather than other oxidizing enzymes.

The mechanism by which the tyrosinase-catalyzed reaction is postulated to occur is illustrated in Scheme 1. The 4-aminophenol group that was attached to the fluorescent group 6-acyl-*N*-methyl-2-naphthylamine (AAN) through a urea linkage supposedly serves as the substate for tyrosinase. It is hypothesized that tyrosinase-triggered two-step oxidation produces an orthoquinone intermediate that is unstable under aqueous conditions and, consequently, undergoes a rapid intramolecular cyclization to release the fluorescent AAN. In support of the proposed mechanism, the 3-methyl-2- benzothiazolinone hydrazone (MBTH) color test was performed to trap the formed orthoquinone intermediate in our current study (Figure 2a).[11] Upon exposure to tyrosinase, the 4-aminophenol group was oxidized to orthoquinone which reacted with MBTH through a Michael reaction to form a dark-pink product. Furthermore, complementary HPLC analysis of a sample of NHU incubated with tyrosinase validated the generation of the final fluorescent product AAN. As shown in Figure 2b, the peak at the retention time of 5.87 min, which corresponded to NHU, disappeared after incubation with tyrosinase. Instead, a new peak with a retention time identical to ANN ($t = 7.37$ min) was observed. Taken together, the results confirm that tyrosinase triggers the structural conversion of the 4-aminophenol group in NHU into orthoquinone and ultimately releases the product AAN.

Next, we assessed the feasibility of NHU as a two-photon probe for tyrosinase detection in living cells. To this end, imaging of tyrosinase activity by confocal microscopy was carried out in the murine melanoma cell line B16-F1 (which highly expresses tyrosinase) and in HeLa cells (a tyrosinase-deficient cell line). As shown in Figure 3, under irradiation at 770 nm, B16-F1 cells that were incubated with NHU (10 μM) for 12 hours displayed a bright green fluorescence in the cytoplasm, whereas analogously treated HeLa cells elicited a relatively weak response. These data imply that the oxidation reaction of NHU can be activiated by the overexpressed tyrosinase in the melanoma cells. Brightfield imaging confirmed cell viability during the imaging experiment.

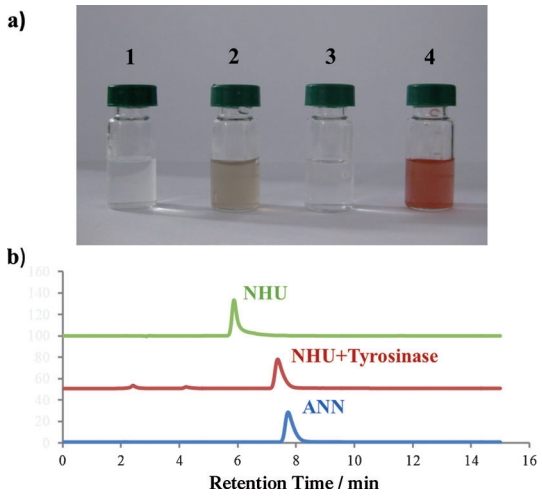



Figure 2. a) MBTH color test based to trap the formed intermediate orthoquinone during the tyrosinase oxidation. The concentrations of NHU, MBTH, and tyrosinase were fixed at 500 μM, 1 mM, and 40 U mL$^{-1}$, respectively. a) Images of vials containing NHU and MBTH (1), NHU and tyrosinase (2), MBTH and tyrosinase (3), and NHU, tyrosinase, and MBTH (4). b) HPLC analysis of NHU alone (top), NHU incubated for 24 h at 37 °C with tyrosinase (middle), and fluorescent AAN only (bottom). The retention time at 5.87 min corresponds to NHU, while that at 7.37 min corresponds to the fluorescent product AAN. The signals were monitored at 503 nm under irradiation at 382 nm.

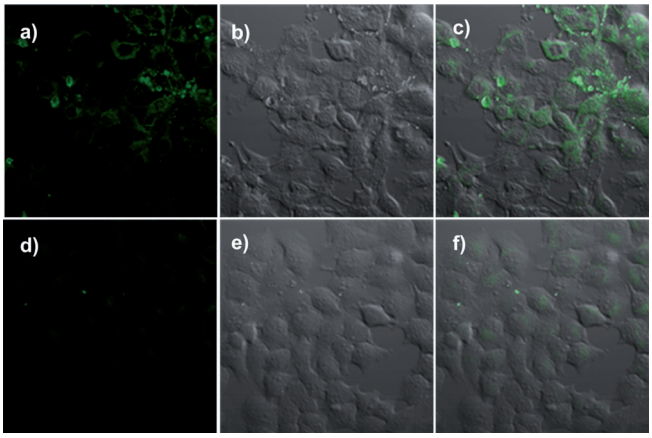



Figure 3. Imaging of B16-F1 cells (a–c) and HeLa cells (d–f) after treatment with NHU (10 μM) for 24 h. a,d) Two-photon microscopic images collected at 460–550 nm upon excitation at 770 nm. b,e) Brightfield images. c,f) Merged images. Cells shown are representative images from replicate experiments ($n=4$).

Therefore, the probe NHU can be utilized for the visualization of endogenous tyrosinase activity.

In summary, we have developed the first two-photon turn-on fluorescent probe, NHU, for monitoring tyrosinase activity in aqueous buffer solution as well as in living cells. Taking advantage of two-photon fluorescence, we believe that this probe holds promise for tyrosinase detection in clinical applications.

## Experimental Section

*Optical Properties Study*

One-photon fluorescent emission spectra were collected in the range 420–650 nm on a PerkinElmer LS 55 fluorescence spectrometer with an excitation wavelength of 382 nm using excitation and emission slit widths of 10 and 12 nm, respectively. A quartz cuvette with a capacity of 600 μL was used for emission measurements. For two-photon excitation experiments, all samples were excited at 770 nm by a mode-locked Ti:Sapphire femtosecond pulsed laser (Chameleon Ultra I, Coherent Inc.) with a pulse width of 140 fs at a repetition rate of 80 MHz. Photoluminescence was recorded on a DCS200PC Photon Counting with single-photon sensitivity through an Omni-λ5008 monochromator (Beijing Zolix Instruments Co., Ltd). Unless otherwise specified, all spectra were taken at 37 °C in 10 mM sodium phosphate buffer.

*Cell Culture and Confocal Imaging*

B16-F1 and HeLa cell lines were purchased from the China Center for Type Culture Collection (CCTCC) and cultured in Modified Eagle Medium (MEM) supplemented with 10% FBS, penicillin (100 units mL$^{-1}$), and streptomycin (100 μg mL$^{-1}$). Cells were maintained in a humidified atmosphere of 5% $CO_2$ at 37 °C. One day before imaging, the cells were harvested using trypsin and plated on glassbottomed dishes (Hyclone). Subsequently, both kinds of cells were incubated with 10 μM NHU for 12 h at 37 °C. Two-photon fluorescence microscopy images of NHU-labeled cells were obtained with spectral confocal and multiphoton microscopes with a ×40 objective lens. Two-photon microscopic images were collected at 460–550 nm upon excitation at 770 nm. Cells are shown representative images from replicate experiments ($n=4$).

*MBTH Assay*

Compound NHU (500 μM) was incubated with 40 U mL$^{-1}$ of mushroom tyrosinase (this enzyme and alcohol dehydrogenase (ADH) were obtained from Sigma–Aldrich) and 1 mM MBTH for 1 h in 10 mM sodium phosphate buffer. MBTH solutions were freshly prepared before use.

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[1] a) S. Briganti, E. Camera, M. Picardo, *Pigm. Cell Res.* **2003**, *16*, 101; b) G. Battaini, E. Monzani, L. Casella, E. Lonardi, A. W. J. W. Tepper, G. W. Canters, L. Bubacco, *J. Biol. Chem.* **2002**, *277*, 44606; c) T. I. Kim, J. Park, S. Park, Y. Choi, Y. Kim, *Chem. Commun.* **2011**, *47*, 12640.

[2] a) H. M. I. Osborn, N. A. O. Williams, *Org.Lett.* **2004**, *6*, 3111; b) G. H. Müller, A. Lang, D. R. Seithel, H. Waldmann, *Chem. Eur. J.* **1998**, *4*, 2513.

[3] M. Bai, J. Huang, X. Zheng, Z. Song, M. Tang, W. Mao, L. Yuan, J. Wu, X. Weng, X. Zhou, *J. Am. Chem. Soc.* **2010**, *132*, 15321–15327.

[4] a) X. Li, W. Shi, S. Chen, J. Jia, H. Ma, O. S. Wolfbeis, *Chem. Commun.* **2010**, *46*, 2560; b) M. J. Perry, E. Mendes, A. L. Simplício,

  

A. Coelho, R. V. Soares, J. Iley, R. Moreira, A. P. Francisco, *Eur. J. Med. Chem.* **2009**, *44*, 3228.

[5] I. Tessari, M. Bisaglia, F. Valle, B. Samorì, E. Bergantino, S. Mammi, L. Bubacco, *J. Biol. Chem.* **2008**, *283*, 16808.

[6] a) D. Li, R. Gill, R. Freeman, I. Willner, *Chem. Commun.* **2006**, 5027; b) H. B. Yildiz, R. Freeman, R. Gill, I. Willner, *Anal. Chem.* **2008**, *80*, 2811.

[7] a) X. Feng, F. Feng, M. Yu, F. He, Q. Xu, H. Tang, S. Wang, Y. Li, D. Zhu, *Org. Lett.* **2008**, *10*, 5369; b) Q. Xu, J. Yoon, *Chem. Commun.* **2011**, *47*, 12497; c) E. Jang, K. J. Son, B. Kim, W. G. Koh, *Analyst* **2010**, *135*, 2871; d) R. Gill, R. Freeman, J. P. Xu, I. Willner, S. Winograd, I. Shweky, U. Banin, *J. Am. Chem. Soc.* **2006**, *128*, 15376.

[8] a) R. Huang, X. Zheng, C. Wang, R. Wu, S. Yan, J. Yuan, X. Weng, X. Zhou, *Chem. Asian J.* **2012**, *7*, 915; b) J. H. Lee, C. S. Lim, Y. S. Tian, J. H. Han, B. R. Cho, *J. Am. Chem. Soc.* **2010**, *132*, 1216.

[9] a) S. Knaggs, H. Malkin, H. M. I. Osborn, N. A. O. Williams, P. Yaqoob, *Org. Biomol. Chem.* **2005**, *3*, 4002; b) A. M. Jordan, T. H. Khan, H. Malkin, H. M. I. Osborn, A. Photiou, P. A. Riley, *Bioorg. Med. Chem.* **2001**, *9*, 1549; c) A. M. Jordan, T. H. Khan, H. Malkin, H. M. I. Osborn, *Bioorg. Med. Chem.* **2002**, *10*, 2625.

[10] C. Su Lim, D. W. Kang, Y. S. Tian, J. H. Han, H. L. Hwang, B. R. Cho, *Chem. Commun.* **2010**, *46*, 2388.

[11] a) A. M. McMahon, E. M. Doyle, K. E. O Connor, *Enzyme Microb. Technol.* **2005**, *36*, 808; b) A. J. Winder, H. Harris, *Eur. J. Biochem.* **1991**, *198*, 317.

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