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A cyanine-modified upconversion nanoprobe for NIR-excited imaging of endogenous hydrogen peroxide signaling *in vivo*

Yi Zhou [a], Wenbo Pei [b], Xiao Zhang [a], Wangqiao Chen [a], Jiansheng Wu [a], Cheng Yao [c], Ling Huang [b], Hua Zhang [a], Wei Huang [b], Joachim Say Chye Loo [a, d, **], Qichun Zhang [a, e, *]

[a] School of Materials Science and Engineering, Nanyang Technological University, Singapore 639798, Singapore
[b] Institute of Advanced Materials (IAM) and Jiangsu-Singapore Joint Research Center for Organic/Bio-Electronics & Information Displays, Nanjing Tech University (NanjingTech), Nanjing 211816, China
[c] College of Science, Nanjing Tech University (NanjingTech), Nanjing 211816, China
[d] Singapore Centre on Environmental Life Sciences Engineering (SCELSE), Nanyang Technological University, 637551, Singapore
[e] Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, Singapore 637371, Singapore

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ABSTRACT

Endogenous hydrogen peroxide is an important parameter associated with cellular signal transduction and homeostasis. However, abnormal H_2O_2 regulation in live systems has been implicated in many pathological conditions. Monitoring this signal in live systems is essential but challenging because current H_2O_2 probes are impractical for efficient bio-imaging due to UV/visible light as the excitation source. We herein present a novel design based on an organic fluorophore-attached lanthanide-doped upconversion nanoprobe (CYD1-UCNPs) for selective UCL detection of H_2O_2. This nanoprobe represents the next-generation imaging tool that features a robust UCL "turn-on" response to H_2O_2 with NIR-excited ratiometric signals and has potential applications in ratiometric UCL imaging of endogenous H_2O_2 generating in living cells and whole-body animals.

1. Introduction

Hydrogen peroxide (H_2O_2) is one of important reactive oxygen species (ROS) in living organisms because its homeostasis plays a key role in mediating various physiological processes [1,2]. For example, H_2O_2 is believed to serve as a second messenger in regulation of cellular signal transduction pathways [3,4], and as a killing agent generated by immune cells to combat microbial invasion [5]. However, abnormal H_2O_2 regulation has been implicated in many pathological conditions such as DNA damage [6], cardiovascular disorders [7], and genetic instability [8], as well as some neurodegenerative diseases including Alzheimer's [9], Huntington's [10], and Parkinson's [11] diseases. Thus, it is highly desirable to develop a real-time imaging method, which could allow for the precise visualization of *in situ* generated H_2O_2 signal in living systems and understand its physiological role in pathological states. Conventional methods for both detection and imaging of H_2O_2 to date are heavily based on H_2O_2-sensitive organic fluorophores [12–16]. Although some organic probes have already shown some promising results in imaging the endogenous H_2O_2 in living samples, the usage of visible lights as sources has become a big obstacle in practical applications, which is mainly because visible light sources can not perform in-depth imaging due to their poor ability for deep tissue penetration and their photobleaching behaviors unsuitable for long-term detection. To address these problems, developing NIR-excited systems for both *in vitro* and *in vivo* detection and imaging of H_2O_2 is highly desirable.

Lanthanide-doped upconverting nanoparticles (UCNPs) can convert a longer wavelength radiation (typically 980 nm) into shorter wavelength emissions (UV, visible and/or NIR light) via a multiphoton process [17], which has emerged as an attractive platform for the construction of upconversion luminescence (UCL) imaging probes [18–22]. In contrast to traditional organic-dye-

* Corresponding author. School of Materials Science and Engineering, Nanyang Technological University, Singapore 639798, Singapore.
** Corresponding author. Singapore Centre on Environmental Life Sciences Engineering (SCELSE), Nanyang Technological University, 637551, Singapore.
E-mail addresses: JoachimLoo@ntu.edu.sg (J.S. Chye Loo), qczhang@ntu.edu.sg (Q. Zhang).

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based detection systems, this unique UCL sensing mechanism has several innate advantages including no autofluorescence from biosamples, higher light penetration depth, less damage to biosamples, and no photobleaching [23,24]. Herein, we present the design and biological evaluation of an organic fluorophore-attached UCNP probe for selective UCL detection of H_2O_2. We believe that this type of nanoprobes might represent the next-generation detection tool that features a robust UCL "turn-on" response to H_2O_2 with NIR-excited ratiometric signals that can be used to image endogenous H_2O_2 generated in living cells and whole-body animals.

Our design strategy for UCL imaging of H_2O_2 is based on the modulating luminescent resonance energy transfer (LRET) from the UCL emission of UCNPs to the H_2O_2-triggered absorbance change in a cyanine dye CYD1. This dye juxtaposes a neurotransmitter dopamine (DPA) sensing site, which could be oxidized into DPA-*o*-quinone in the presence of H_2O_2 [25], with bipodal carboxylic-terminated alkyl arms to match the hydrophobicity of the OA ligands on the surfaces of UCNPs. It is worthy to note that nanoprobes made through physically attaching organic chromophore are not stable (dissociation) and are not suitable for *in vivo* UCL imaging due to the attack of some anions (e.g. HPO_4^{2-}, $H_2PO_4^-$, HCO_3^-, and CO_3^{2-}) to UCNPs in complex biological environments [26]. In order to avoid this problem, α-cyclodextrin (CD) has been integrated onto the surface of nanoprobes, and then, the OA ligands can be easily inserted into this lipophilic cavity structure, which not only creates a water-soluble three layer nano-architecture but also prevents CYD1 from dissociation in the harsh environment [27]. As illustrated in Fig. 1, the CYD1 quenching red UCL emission are hybridized with UCNPs emitting UCL emission at 541 nm and 654 nm as UCL H_2O_2 responsive nanoprobe CYD1-UCNPs, in which UCNPs (doped with 0.5% Tm^{3+}) served as built-in NIR reference signals for providing the correction to avoid disturbance from environmental factors.

2. Experimental section

2.1. Characterization

^1H NMR and ^{13}C NMR were measured on a BrukerAV-400 spectrometer with chemical shifts reported in ppm (in $CDCl_3$ or DMSO-d_6; TMS as internal standard). Electrospray ionization mass spectra (ESI-MS) were measured on a Micromass LCTTM system. Powder X-ray diffraction measurements were performed on a Shimadzu XRD-6000 diffractometer at a scanning rate of 1°/min with the 2θ range from 10 to 90°(Cu Kα radiation, λ = 1.54056 Å). CYD1-UCNPs were dispersed in HEPES buffer, which was further dropped on the TEM copper grid for characterization. HR-TEM were measured on a JEOL JEM-2100F transmission electron microscope with an accelerating voltage of 200 kV. UV–Vis spectra was recorded using a Shimadzu UV-2501 spectrometer. The upconversion luminescence spectra were measured on a Horiba Jobin yvon FluoroMax-4 spectrofluorometer using an external 0–2 W adjustable CW laser (980 nm, Connect Fiber Optics, China) as the excitation source instead of the xenon source. FTIR were obtained using a Perkin–Elmer Lambda 783 spectroscopy with KBr pellets.

2.2. Chemicals and materials

Deionized water used in this study was the triple-distilled water which was further treated by ion exchange columns and a Milli-Q water purification system. TLC analysis was performed on silica gel plates. Column chromatography was conducted over silica gel (mesh 200–300), and both were obtained from ACS Chemicals. OA, 1-octadecane (ODE 90%), 11-bromoundecanoic acid, 2,3,3-Trimethylindolenine, 3-Hydroxytyramine hydrochloride, NH_4F, and α-cyclodextrin were purchased from Sigma–Aldrich. Rare chloride hexahydrate $YCl_3{\cdot}6H_2O$ (99.9%), $YbCl_3{\cdot}6H_2O$ (99.9%), $ErCl_3{\cdot}6H_2O$ (99.9%), and $TmCl_3{\cdot}6H_2O$ (99.9%) were purchased from Alfa Aesar. Absolute ethanol, cyclohexane, dimethyl sulfoxide, chloroform, and methylene chloride were of analytical grade. All of the chemicals were used as received without further purification. HOCl was prepared from the source of NaOCl at room temperature and the concentration of HOCl was determined by titration with $Na_2S_2O_3$. NO_2^- was prepared from $NaNO_2$ at 25 °C. The synthesis of peroxynitrite involved the nitrosation of H_2O_2 at pH ≥ 12.0 by isoamyl nitrite. The peroxynitrite concentration was determined by using an extinction coefficieny of 1670 ± 50 cm^{-1} $(mol/L)^{-1}$ at 302 nm [44]. Hydroxyl radicals was generated by the reaction between Fe^{2+} (200 μM) and H_2O_2 (200 μM) at 25 °C and the mixture was then stirred for 30 min. Superoxide was prepared from the source of KO_2 at 25 °C. Nitric oxide was prepared from a saturated NO aqueous solution (2 mM) at room temperature. Nitroxyl donor (HNO) was generated from sodium trioxodinitrate ($Na_2N_2O_3$, Angeli's salt) [45]. ROO• was generated from 2,2′-azobis(2-amidinopropane)dihydrochloride, which was dissolved in deionized water first and then added into testing solutions at 25 °C in HEPES buffer.

2.3. Synthesis of Ethyl 11-bromoundecanoate (1)

11-Bromoundecanoic acid (5.30 g, 20.0 mmol) was dissolved in EtOH (100 mL) and 8 mL of H_2SO_4 were added dropwise to the solution. The mixture solution was refluxing for 12 h, allowed to cool naturely, and the solvents were evaporated. The resulted residue was dissolved in ethyl acetate and washed with brine. The organic phase was dried with $MgSO_4$, and evaporated to give the compound 1 as a colorless oil (5.39 g, yield: 92%). ^1H NMR (400 MHz, $CDCl_3$): δ = 4.12 (t, J = 7.0 Hz, 2H, $-CH_2-$), 3.41 (t, J = 6.9 Hz, 2H, $-CH_2-$), 2.28 (t, J = 7.1 Hz, 2H, $-CH_2-$), 1.90 (m, 2H, $-CH_2-$), 1.60 (m, 2H, $-CH_2-$), 1.41 (m, 2H, $-CH_2-$), 1.19–1.34 (m, 13H, $-CH_2-$, $-CH_3$).

2.4. Synthesis of 1-(11-ethoxy-11-oxoundecyl)-2,3,3-trimethyl-3H-indol-1-ium bromide (2)

2,3,3-Trimethylindolenine (1.59 g, 10.0 mmol) and Ethyl 11-bromoundecanoate (2.94 g, 10.0 mmol) were dissolved in acetonitrile (10 mL) under argon atmosphere, and then, the mixed solution was heated to reflux for 48 h. After cool down, the mixture was treated with diethylether (50.0 mL) for ~24 h, and the solvents were decanted. The final product was repeatedly dissolved in a small amount of ethanol, following precipitation with diethylethe. Finally, the product 2 was obtained in 79% (3.57 g) as a pinkish-grey solid by vacuum filtration. ^1H NMR (400 MHz, $CDCl_3$ + CD_2Cl_2): δ = 8.17 (m, 1H, Ar–H), 7.95 (m, 1H, Ar–H), 7.61 (m, 2H, Ar–H), 4.78 (t, J = 7.1 Hz, 2H, $-CH_2-$), 4.13 (q, J = 7.6 Hz, 2H, $-CH_2-$), 3.20 (s, 3H,$-CH_3$), 2.39 (t, J = 7.3 Hz, 2H, $-CH_2-$), 1.95 (m, H, $-CH_2-$), 1.67 (s, 6H, $-CH_3$), 1.55 (m, 2H, $-CH_2-$),1.45 (m, 2H, $-CH_2-$), 1.20–1.36 (m, 15H, $-CH_2-$, $-CH_3$). *ESI*-MS: *m/z* 372.5 $[M-Br]^+$.

2.5. Synthesis of 2-chloro-1-formyl-3-(hydroxymethylene) cyclohex-1-ene (3)

Compound **3** was prepared according to a literature method [46]. $POCl_3$ phosphorus oxychloride (37.0 mL, ~400 mmol) in DCM (40 mL) was added dropwise to N,N-dimethylformamide (40.0 mL, ~510 mmol) at 0 °C, and the reaction mixture stirred for 30 min. The resulted solution was added into cyclohexanone (10 g, 100 mmol) and refluxed for 4 h. After the mixture cooled to R.T. and was