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Project Details

Project Number :	706410
Project Title(English) :	Design, Synthesis and Biological Applications of Fluorescent Probes for Peroxynitrite and Hypochlorous Acid
Project Title(Chinese) :	用于检测过氧亚硝酸根和次氯酸的荧光探针的设计、合成及生物应用
Principal Investigator(English) :	Prof Yang, Dan
Principal Investigator(Chinese) :	
Department :	Department of Chemistry
Institution :	The University of Hong Kong
E-mail Address :	yangdan@hku.hk
Tel :	2859 2159
Co - Investigator(s) :	
Panel :	Physical Sciences
Subject Area :	Physical Sciences
Exercise Year :	2010 / 11
Fund Approved :	755,700
Project Status :	Completed
Completion Date :	30-9-2013
Project Objectives :	Develop red fluorescent and ratiometric probes for detecting peroxynitrite Develop new-generation green fluorescent and ratiometric probes for detecting hypochlorous acid Apply our fluorescent probes to investigate the roles of peroxynitrite and/or hypochlorous acid in several biological processes
Abstract as per original application (English/Chinese):	View View
Realisation of objectives:	We have developed a a rhodol-based probe named HKYellow and its cell-permeable form HKYellow-AM with fluorescence excitation and emission wavelengths at 547 nm and 570 nm, respectively, for sensitive and selective detection of peroxynitrite in multiple cell lines and primary cells. An impressive 93-fold fluorescence turn-on response of HKYellow was observed toward 1 equiv peroxynitrite treatment, much higher response than that toward potentially interfering hydroxyl radical or hypochlorous acid. Moreover, a ratiometric fluorescent probe, FRET-1, comprising a coumarin and rhodol as the fluorescence donor and acceptor, respectively, has been found to

exhibit dual fluorescence responses at both 470 nm and 535 nm upon peroxynitrite addition, and therefore can be exploited for ratiometric detection and imaging of peroxynitrite. Based on this design principle, we then generated three new FRET probes (HJ-2-118, HJ-2-124, HJ-3-163) which attempt to address FRET-1's limitations in cellular retention, photo-transfer efficiency, and quenching of donor fluorophore (coumarin). To our delight, in chemical characterization, the probes showed improved sensitivity over FRET-1, and ratiometric properties that can be further validated in cellular systems. To overcome the drawbacks of our previously published BODIPY probe HKOCl-1, we have developed new-generation green fluorescent probes, HKOCl-2 series. HKOCl-2 shows excellent sensitivity and selectivity for detecting HOCl, and more importantly, the fluorescent product of HKOCl-2 is much more stable than that of HKOCl-1. Green fluorescent probe HKOCl-2b has been reliably exploited in detection of endogenous hypochlorous acid produced in human and mouse macrophages stimulated with PMA or yeast zymosan. More recently, we have discovered a new switch motif for HOCl detection and developed ultrasensitive green fluorescent probe HKOCl-3 and yellow fluorescent probe HKOCl-yellow. Those new probes are being validated in cellular systems. We used HKGreen-4A, our fourth-generation peroxynitrite probe, to establish methodologies for peroxynitrite detection and imaging in live cells and tissues. The roles of peroxynitrite in host-pathogen interactions in immune defense and free radical-mediated tissue injury in metabolic dysfunction were scrutinized in the contexts of bacterial infection and atherosclerosis, respectively. So far, in sensors literature, the kinetics of peroxynitrite induction following bacterial infection remains unknown. We address this by using a mouse macrophage cellular model (RAW264.7 cells) of Gram-negative bacterial infection to characterize the time course of peroxynitrite formation in the immune cells challenged with different pharmacological and bacterial stimulants.

Summary of objectives addressed:

	Objectives	Addressed	Percentage achieved
1.	Develop red fluorescent and ratiometric probes for detecting peroxynitrite	Yes	100%
2.	Develop new-generation green fluorescent and ratiometric probes for detecting hypochlorous acid	Yes	100%
3.	Apply our fluorescent probes to investigate the roles of peroxynitrite and/or hypochlorous acid in several biological	Yes	100%

	processes

Research Outcome	
Major findings and research outcome:	HKGreen-4A, our fourth-generation green peroxynitrite probe, has recently been successfully concluded as a manuscript submitted to J. Am. Chem. Soc. It is currently under minor revisions and the revised version will be submitted before July 5, 2014. The current manuscript reports on our findings on the probe's high selectivity and sensitivity in chemical characterization, the kinetics of endogenous peroxynitrite formation induced by bacterial infection (E. coli), and the enzymatic pathways involved. In addition, we report the newly established methodology of live tissue staining with HKGreen-4A in an ApoE-KO mouse model of atherosclerosis. Using HKGreen-4 as the probe, we have established a cell based assay system for the detection of peroxynitrite with flow cytometry and 96-well plates. Several manuscripts on HKGreen-4 and its derivatives for improved performance in cell imaging are in preparation. HKOCl-2, a series of second-generation BODIPY hypochlorous acid probes designed to address the limitations of a previous probe HKOCl-1, has been published Organic Letters (June 2014). In this report, we demonstrated that HKOCl-2 probes are superior to HKOCl-1 in three aspects of comparison: selectivity, sensitivity and chemo-stability. More recently, we have discovered a new switch motif for HOCl detection and developed ultrasensitive green fluorescent probe HKOCl-3 and yellow fluorescent probe HKOCl-yellow. Manuscripts summarizing those two new probes are in preparation. We have collaborated with Prof. Hidehiko Nakagawa (Graduate School of Pharmaceutical Sciences, Nagoya City University, Japan) on the use of our fluorescent probe HKGreen-3 in development of two peroxynitrite donors. The results have been published in Chem. Commun. (2011) and J. Am. Chem. Soc. (2012). We have collaborated with Prof. Massimo Delledonne (Dipartimento di Biotecnologie, Università degli Studi di Verona, Italy) on the use of our fluorescent probe HKGreen-2 in the first detection of peroxynitrite in plants. The result has been published in Nitric Oxide (2011). We have been collaborating with Dr. Jian-Gang Shen (School of Chinese Medicine, The University of Hong Kong) on the use of our probes HKYellow-AM in detection of peroxynitrite in neuronal cells under hypoxia condition. A manuscript on this work is in preparation.
Potential for further development of the research and the proposed course of action:	Based on our success of developing green and red fluorescent probes for detection of peroxynitrite and hypochlorous acid in cells and tissues, we plan to focus on the design and synthesis of fluorescent probes that can be used for in vivo detection of peroxynitrite and hypochlorous acid in living animals. In addition, we will also explore further applications of our probes in elucidating the biological roles of peroxynitrite and hypochlorous acid in development

	using zebrafish models.
Layman's Summary of Completion Report:	Peroxynitrite (ONOO–) is a potent oxidizing and nitrating species implicated in numerous diseases including diabetes, stroke, cancer, and neurodegenerative disorders. Another important ROS (reactive oxygen species), hypochlorous acid (HOCl), figures prominently in innate immune response against microbial infections, but potentially contributes to pathological conditions such as atherosclerosis and Alzheimer's disease. Rapid and accurate detection of ONOO– and HOCl has been challenging due to their transient nature and difficulty of achieving high selectivity and sensitivity for single ROS in probe design. Thus far, many existing probes for these species are either employing green-emission fluorophores or possessing insufficient selectivity and/or sensitivity for single ROS detection in live cell or tissues. We have developed the highly selective and sensitive HKRed-1 and HKYellow as red and yellow probes for cellular ONOO– detection, respectively. The ratiometric probe FRET-1 and analogs have been successfully developed for exploration in quantitative ONOO– detection. A HKOCl-2 series of green HOCl probes has been designed to address practical limitations of our previously published HKOCl-1. In biological applications, we used a green ONOO– probe HKGreen-4A to define the kinetics of ONOO– formation in mouse macrophages during bacterial infection, and establish a method for live tissue ONOO– staining in atherosclerotic mice

Research Output

Peer-reviewed journal publication(s) arising directly from this research project : (* denotes the corresponding author)

Year of Publication	Author(s)	Title and Journal/Book
2010	T. Peng, D. Yang*	HKGreen-3: A Rhodol-Based Fluorescent Probe for Peroxynitrite. Org. Lett., 2010, 12, 4932–4935.
2011	N. Ieda, H. Nakagawa*, T. Horinouchi, T. Peng, D. Yang, H. Tsumoto, T. Suzuki, K. Fukuhara, N. Miyata*.	Peroxynitrite Generation from NO-releasing Nitrobenzene Derivative in Response to Photoirradiation. Chem. Comm. 2011, 47, 6449–6451.
2011	F. Gaupels, E. Spiazzi-Vandelle, D. Yang, M. Delledonne*	Detection of Peroxynitrite Accumulation in Arabidopsis thaliana During the Hypersensitive Defense Response. Nitric Oxide 2011, 25, 222–228.

2012	N. Ieda, H. Nakagawa*, T. Peng, D. Yang, T. Suzuki, N. Miyata*	Photo-controllable Peroxynitrite Generator Based on N-Methyl-N-nitrosoaminophenol for Cellular Application. J. Am. Chem. Soc. 2012, 134, 2563–2568.
2014	Jun Jacob Hu, Nai-Kei Wong, Qiangshuai Gu, Xiaoyu Bai, Sen Ye, Dan Yang*	HKOCl-2 Series of Green BODIPY-based Fluorescent Probes for Hypochlorous Acid Detection and Imaging in Live Cells. Organic Letters. ASAP Articles Publication Date (Web): June 20, 2014 (Letter) DOI: 10.1021/ol501496n
	Tao Peng, Nai-Kei Wong, Xingmiao Chen, Yee-Kwan Chan, Derek Hoi-Hang Ho, Zhenning Sun, Jiangang Shen, Hani El Nezami, and Dan Yang*	Molecular Imaging of Peroxynitrite with HKGreen-4 in Live Cells and Tissues. Journal of The American Chemical Society. Under minor revision.

Recognized international conference(s) in which paper(s) related to this research project was/were delivered :

Month/Year/City	Title	Conference Name
06/2011/Seoul, South Korea	Using Synthetic Organic Chemistry to Probe Biological Mechanisms	the 1st Korea Forum on Organic Chemistry
12/2010 /Honolulu, Hawaii, USA	Developing Highly Selective Fluorescent Sensors for the Detection of Peroxynitrite and Hypochlorite in Cells	Pacifichem 2010 symposium “Molecular Probes and Fluorophores for Cellular Imaging”
02/2012/Hanoi, Vietnam	Using Synthetic Organic Chemistry to Probe Biological Mechanisms	Asian Chemical Biology Initiative, Hanoi Meeting
09/2011/Trieste, Italy	Inspiration from Triptolide: New Synthetic Methods and	TWAS 22nd GENERAL MEETING

		Probes	
	08/2012 /Lanzhou, China	A Highly Selective Fluorescent Sensor for Peroxynitrite Detection in a Mouse Macrophage Model of Bacterial Infection	The 1st International Chinese Symposium on Free Radical Biology and Free Radical Medicine Research
	03/2014/Kyoto, Japan	Design, Synthesis and Applications of Fluorescent Probes for Molecular Imaging of Superoxide, Peroxynitrite, and Hypochlorous Acid	The 17th Biennial Meeting of Society for Free Radical Research International
	12/2013/Hong Kong	Fluorescent Probes for Molecular Imaging of ROS and RNS	Croucher Advanced Study Institute on Chemical Biology
	03/2014/Umeå, Sweden	Inspiration from Natural Products — New Synthetic Methods, Probes and Protein Targets	Trends in Organic Chemistry Symposium on Natural Product Inspired Chemistry
	03/2014/Kyoto, Japan	Detection of Hypochlorous Acid in Activated Phagocytes with a Highly Selective and Sensitive Fluorescent Probe	the 17th Biennial Meeting of Society for Free Radical Research International
	03/2014/Kyoto, Japan	A Highly Selective Fluorescent Probe for Peroxynitrite Detection and Imaging in Live Cells and Tissues	The 17th Biennial Meeting of Society for Free Radical Research International
Other impact (e.g. award of patents or prizes, collaboration with other research institutions,	Several patents on our fluorescent probes have been awarded: 1. D. Yang, H.-L. Wang, Z.-N. Sun, J.-G. Shen. Reagents for Highly Specific Detection of		

technology transfer, etc.):	Peroxynitrite. U.S. Patent 7,705,040, 2010. 2. D. Yang, Z.-N. Sun, Y. Chen, F.-Q. Liu. Reagents for Detection of Hypochlorous Acid. U.S. Patent 7,858,598, 2010. 3. D. Yang, Z.-N. Sun, Y. Chen, F.-Q. Liu. Reagents for Detection of Hypochlorous Acid. European Patent EP2134724B1, 2011. 4. T. Peng, D. Yang. Luminescence Quenchers and Fluorogenic Probes for Detection of Reactive Species. U.S. Patent No. 8,114,904, 2012. 5. T. Peng, D. Yang. Fluorophore Compounds. U.S. Patent No. 8,148,423 B2, 2012. 6. D. Yang, H.-L. Wang, Z.-N. Sun, J.-G. Shen. Reagents for Highly Specific Detection of Peroxynitrite. Chinese Patent No. ZL200680045462.9, 2012. 7. T. Peng, D. Yang. Fluorophore Compounds. China Patent Application No. 200980112496.9 filed on 30 September 2010 was approved on 28 May, 2014. Several patent applications on our new fluorescent probes have been filed: T. Peng, D. Yang. Diarylamine-Based Fluorogenic Probes for Detection of Peroxynitrite. U.S. Provisional Application (No. 61/592,122), filed on Jan. 30, 2012. T. Peng, D. Yang, J.-G. Shen, X.-M. Chen. Diarylamine-Based Fluorogenic Probes for Detection of Peroxynitrite. U.S. Application (No. 13/754,499), filed on 30 January 2013. T. Peng, D. Yang, J.-G. Shen, X.-M. Chen. Diarylamine-Based Fluorogenic Probes for Detection of Peroxynitrite. PCT Application, filed on 30 January 2013. In addition, the patent on HKGreen-4 has been licensed to Invitrogen for commercialization. Dan Yang has won the following awards: Chinese Young Women in Science Fellowship, 2011 TWAS Prize in Chemistry, 2010 Dan Yang has been invited to deliver lectures at the following international conferences or institutions for the work on fluorescent probes: Keynote lecture at the 2012 Dorothy Crowfoot-Hodgkin (DCH) Symposium in University of Zurich (Oct., 2012; Zurich, Switzerland) Invited lecture at the 8th Pong Ding Yuen International Symposium on Traditional Chinese Medicine cum The 2nd International Chinese Symposium on Free Radical Research & The 6th Symposium for Three Districts of Cross-straits on Free Radical Research (Nov. 2014; Hong Kong) Keynote lecture at the IUPAC 2015 Pusan (Aug. 2015; Pusan, South Korea) Invited lecture at University of Toronto (Dec. 2013; Toronto, Canada) Invited lecture at Fudan University (Oct. 2013; Shanghai, China) Invited lecture at the ABCI Seminar Series of Hong Kong Polytechnic University (Jan. 2013; Hong Kong) Nankai University Lectureship in Organic Chemistry (Jun. 2013; Tianjin, China)

Return

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Abstract of 706410

As two kinds of highly reactive species generated in biological systems, peroxynitrite and hypochlorous acid are important mediators of a variety of physiological and pathological events. The abnormal production of peroxynitrite and hypochlorous acid have been implicated in a series of human diseases such as vascular diseases, ischemia–reperfusion injury, circulatory shock, inflammation, cancer, and neurodegenerative disorders. Unfortunately, further investigations of the biological actions of peroxynitrite and hypochlorous acid have been greatly hampered by the difficulty of direct and unambiguous detection of them. To facilitate the direct studies of these species in biological systems, it is of ultimate importance to develop methods, especially fluorescent probes providing high sensitivity, real-time detection and spatial imaging, for the detection of peroxynitrite and hypochlorous acid.

In our previous research funded by RGC, we have developed several efficient green fluorescent probes, the **HKGreen** series and **HKOCl-1**, for the sensitive and selective detection of peroxynitrite and hypochlorous acid, respectively. In this proposal, we plan to further expand the repository of our probe family by developing preferable probes, such as red fluorescent probes avoiding the interference from the auto-fluorescence of cells, and ratiometric probes allowing for quantification of peroxynitrite and hypochlorous acid. Another objective of this project is to address certain biological questions related to peroxynitrite and/or hypochlorous acid by utilizing our new probes.

This project is of great significance as it will provide powerful tools to directly visualize and quantify peroxynitrite and hypochlorous acid produced in biological systems, which will be extremely helpful in studying the pathological roles and mechanisms of these two species in related human diseases. This project will also find its merits in elucidating the actions of peroxynitrite and hypochlorous acid in immune response, inflammatory diseases and neuron degeneration.

过氧亚硝酸根和次氯酸是两种在生物体内产生的高活性反应物，它们在很多的生理和病理过程中都起着非常重要的作用。过氧亚硝酸根和次氯酸的非正常水平产生已经跟很多的人类疾病联系在一起，包括心血管疾病，缺血-再灌注损伤，循环休克，炎症，癌症以及神经退行性疾病等等。但不幸的是，对过氧亚硝酸根和次氯酸在生物体内作用的进一步研究却由于难以直接确定地检测它们而受到极大的阻碍。因此，为了促进对它们生物体内作用的直接研究，发展对过氧亚硝酸根和次氯酸的检测方法，尤其是具备高灵敏度，实时检测以及空间成像特点的荧光探针检测方法是非常重要的。

在我们之前获香港研究资助局资助的研究中，我们已经成功地研发出了几种绿色荧光探针**HKGreen**系列和**HKOCl-1**，它们分别实现了对过氧亚硝酸根和次氯酸的高灵敏高选择性检测。在这份研究计划里，我们打算进一步发展更优越的荧光探针成员来丰富我们的探针“家族”，比如说红色荧光探针以及比例检测荧光探针。前者可以有效地避免检测过程中来自细胞自发绿色荧光的干扰，而后者可以实现对过氧亚硝酸根和次氯酸的定量检测。此外，这份研究计划的另一个目的是利用我们的新探针来解决一些与过氧亚硝酸根或者次氯酸相关的生物问题。

这份研究计划的重要性在于它将提供一些强有力的工具来直接地观察和定量检测过氧亚硝酸根和次氯酸在生物体内的产生，这对于研究过氧亚硝酸根和次氯酸在人类疾病中

的病理作用和机制是有很大帮助的。同时，这份研究对于阐明过氧亚硝酸根和次氯酸在免疫反应，炎症以及神经退化中的作用也有相当大的意义。