Biomaterials 58 (2015) 72–81

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

A fast and selective two-photon phosphorescent probe for the imaging of nitric oxide in mitochondria

Xiang Chen, Lingli Sun, Yu Chen, Xiaolin Cheng, Weijun Wu, Liangnian Ji, Hui Chao*

MOE Laboratory of Bioinorganic and Synthetic Chemistry, School of Chemistry and Chemical Engineering, Sun Yat-Sen University, Guangzhou 510275, PR China

ARTICLE INFO

Article history:
Received 11 January 2015
Received in revised form
2 April 2015
Accepted 4 April 2015
Available online 4 May 2015

Keywords:
Iridium complex
Nitric oxide
Mitochondria
Two-photon probe
Zebrafish

ABSTRACT

Nitric oxide (NO) is a vitally important cellular messenger molecule related to numerous physiological events and diseases. It is more than 10 years now that mitochondria are suspected to be sources of nitric oxide. Hence, mitochondrial NO tracking probes play an indispensable role in NO behavior analysis. However, a majority of previously reported NO probes can only be employed under one-photon microscopy, often with several drawbacks during application. In the present study, an iridium(III) complex containing 1,10-phenanthroline-5,6-diamine (Ir-Mito-NO) was synthesized and determined to possess high specificity to NO in mitochondria, low cytotoxicity, and rapid and specific "off-on" two-photon phosphorescence. Thus, this complex was developed to image mitochondrial NO levels in living cells, 3D multicellular spheroids and zebrafish.

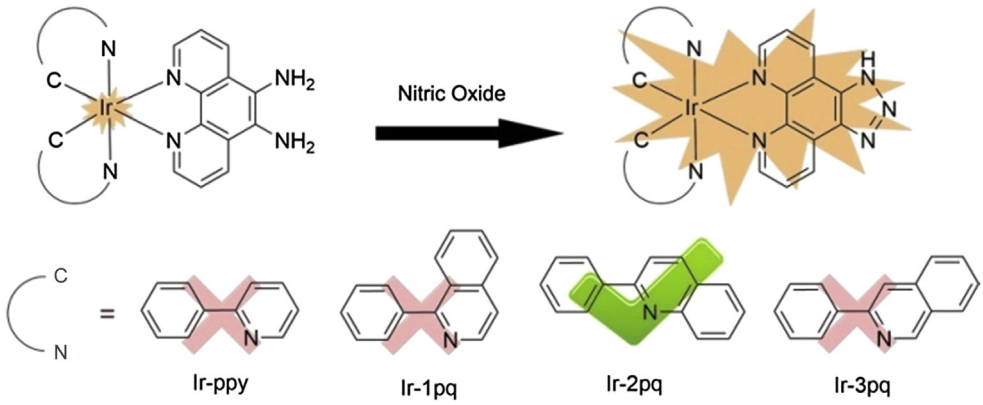
1. Introduction

Endogenous nitric oxide (NO) is an important cellular signaling molecule involved in inflammation [1–3], cardiovascular disease (CVD) [4–6], neurological disease [7–9], cancer [10–14] and various other physiological processes. Endogenous NO is synthesized in mammalian cells by nitric oxide synthase (NOS) via the process of transforming L-arginine into L-citrulline [15–18]. This process is suspected to be carried out in the mitochondria [19], and the free radical property of NO renders it capable of reacting with other intra-mitochondrial radicals, such as reactive nitrogen species (RNS) and reactive oxygen species (ROS) [20–22]. Recent reports indicate that NO is an essential molecule that regulates the mitochondria apoptosis pathway by causing cellular hypoxia and mitochondrial respiration-chain dysfunction [16]. Although NO is widely distributed in organisms and exerts both positive and negative influences on physiological events, the actual mechanisms of NO in mitochondria have yet to be elucidated. Thus, the development of a specific probe for mitochondrial NO will be of benefit for studying the occurrences, therapies and prevention of diseases.

To our knowledge, fluorescent probes for mitochondrial NO can act as powerful tools for tracking and studying the behavior of NO and provide a deeper insight in numerous fields as mentioned above. At present, a variety of NO-sensitive fluorescent molecular probes have been developed for imaging intracellular NO by one-photon microscopy (OPM). The *o*-diaminophenyl group has been used as an effective chemical group that specifically reacts with nitric oxide in commercial NO probes and includes 2,3-diaminonaphthalene, 1,2-diaminoanthra-quinone, DAF-FM and DAR 4M. However, of these four probes, only DAR 4M might has the mitochondria targeting ability due to its cationic property of rhodamine, others are not capable of targeting the mitochondria. Compared with these organic dyes, metal-based emissive probes are promising alternatives because they display several excellent physicochemical properties for bioimaging, such as large Stokes shifts (hundreds of nm) and enhanced photostabilities (lower photobleaching). Lippard and Yuan reported on a few NO probes based on metal complexes containing *o*-diaminophenyl molecules [23–28]. On the one hand, the known NO probes fail to meet the requirements of tracking mitochondrial NO. On the other hand, two-photon fluorescent probes are more suitable for long time *in vivo* observations in living cells than one-photon probes due to the advantages of deeper sectioning ability, smaller photon white area, lower photo-toxicity and stronger transmittance. However, researchers working on monitoring mitochondrial NO face difficult

* Corresponding author. Tel.: +86 20 84110613.
E-mail address: ceschh@mail.sysu.edu.cn (H. Chao).

http://dx.doi.org/10.1016/j.biomaterials.2015.04.012
0142-9612/



Scheme 1. Schematic illustration of the reaction between iridium(III) complexes and NO.

challenges because the existing methods are designed for one-photon fluorescence; few two-photon NO probes have been reported [29–31]. Therefore, it is desirable to devise novel probes with rapid response, mitochondria targeting and two-photon fluorescence imaging capabilities for tracking the fluctuations of NO levels in mitochondria.

To the best of our knowledge, no two-photon phosphorescent probes have been synthesized for mitochondrial NO imaging *in vivo*. Herein, we focus on iridium(III) complexes because of their excellent photochemical properties and mitochondria-targetable properties. A series of four Ir(III) complexes, $[Ir(C \sim N)_2(PDA)]Cl$ (C ~ N = ppy (2-phenylpyridine), 2pq (2-phenylisoquinoline), 1pq (1-phenylisoquinoline), 3pq (3-phenylisoquinoline), PDA = 1,10-phenanthroline-5,6-diamine), have been synthesized (Scheme 1). In order to investigate the two-photon phosphorescence property and photostability of these probes, the triazole final product $[Ir(C \sim N)_2(TAP)]Cl$ (TAP = 1H-[1,2,3]triazolo[4,5-f] [1,10]phenanthroline) were also synthesized.

2. Materials and methods

2.1. Materials and general instruments

All chemicals were directly purchased from commercial sources and were of analytical reagent grade unless otherwise indicated. Ultrapure water was used throughout all experiments. The stock solutions of all complexes were prepared at 5 mM in DMSO. ROS and NOS solutions were freshly prepared and promptly used as described below. The synthesis of peroxynitrite ($ONOO^-$) involved the nitrosation of H_2O_2 at pH $\geq$ 12.5 by isoamyl nitrite [32]. Nitric oxide (NO) was provided by the NO gas-saturated aqueous solution. Superoxide radical ($O_2^{\bullet -}$) was generated by dissolving KO_2 in dry DMSO. ClO^- solution was prepared from NaClO at room temperature in PBS buffer (pH = 7.2). The ClO^- concentration was determined based on the molar extinction coefficient at 292 nm (350 M^{-1} cm^{-1}). •OH was generated by the Fenton reaction between $FeSO_4$ and H_2O_2, and the concentrations of $FeSO_4$ represented the concentrations of •OH [33]. Mito-tracker Green (MTG) was purchased from Invitrogen. HeLa cells were purchased from the Committee on Type Culture Collection of the Chinese Academy of Sciences.

Electrospray mass spectra were recorded with an LCQ system (Finnigan MAT, USA). Emission spectra were recorded with a Perkin–Elmer LS 55 Luminescence Spectrometer. Electronic absorption spectra were recorded with a Perkin–Elmer Lambda850 UV/Vis Spectrometer. ^1H NMR spectra were recorded on a Nuclear Magnetic Resonance Spectrometer (Varian, Mercury-Plus 300) at 300 MHz. The two-photon fluorescence data were acquired using an Opolette™ 355II + UV I & II (pulse width $\leq$ 100 fs, 80 MHz repetition rate, tuning range 210–355, 410–2400 nm, OPOTEK Inc., Carlsbad, CA, USA).

2.2. Synthesis

The following compounds were synthesized according to methods in the literature [34–36]. The abbreviation mentioned below are as follow: ppy (2-phenylpyridine), 2pq (2-phenylisoquinoline), 1pq (1-phenylisoquinoline), 3pq (3-phenylisoquinoline), PDA (1,10-phenanthroline-5,6-diamine) and TAP (1H-[1,2,3]triazolo[4,5-f] [1,10]phenanthroline).

2.2.1. Synthesis of $[Ir(2pq)_2(PDA)]Cl$ (Ir-Mito-NO)

A solution of $[Ir(2pq)Cl]_2$ (63 mg, 0.05 mmol) in methanol (15 mL) was added dropwise to a refluxing suspension of 5-nitro-6-amine-1,10- phenanthroline (12 mg, 0.05 mmol) in 10 mL of a chloroform/methanol (1:1) mixture. After a 12 h reflux, the mixture was cooled to R.T., and a solution of 80% hydrazine hydrate (0.5 mL) and Pd/C catalyst (0.1 g) was added to the reaction solution with care. After an additional 2 h reflux to cool to R.T., the mixture was filtered and the solvent was removed under reduced pressure to obtain the crude product. Additional purification was performed by column chromatography using neutral alumina with chloroform/methanol (30/1, v/v) as the eluant. The resultant yield was 58 mg; 77%. Anal. Calcd. for $C_{42}H_{30}N_6ClIr$ (%): C, 59.60; H, 3.57; N, 9.93. Found (%): C, 59.58; H, 3.56; N, 9.95. ES-MS [CH_3OH, *m/z*]: 811.5 ($[M]^+$). ^1H NMR (300 MHz, DMSO) δ 8.64 (d, J = 8.6 Hz, 2H), 8.56 (d, J = 8.9 Hz, 2H), 8.46 (d, J = 8.8 Hz, 2H), 8.31 (dd, J = 4.6, 3.3 Hz, 2H), 8.10 (d, J = 4.8 Hz, 2H), 7.79 (dd, J = 8.5, 5.0 Hz, 4H), 7.24 (t, J = 7.3 Hz, 2H), 7.15 (dd, J = 13.8, 8.1 Hz, 4H), 6.81 (dd, J = 14.2, 7.0 Hz, 4H), 6.48 (d, J = 7.6 Hz, 2H), 5.73 (s, 4H).

2.2.2. Synthesis of $[Ir(2pq)_2(TAP)]Cl$ (Ir-Mito-TAP)

A solution of $[Ir(2pq)Cl]_2$ (63 mg, 0.05 mmol) and 1,2,3-triazolo-1,10-phenanthroline (11 mg, 0.05 mmol) in 20 mL methanol was refluxed for 12 h. The solution was filtered and the solvent was removed under reduced pressure after the mixture was cooled to R.T. to form the precipitate. The crude product was purified by neutral alumina with chloroform/methanol (25/1, v/v) as the eluant. The yield was 47 mg; 63%. Anal. Calcd. for $C_{42}H_{27}N_7ClIr$ (%): C, 58.84; H, 3.17; N, 11.44. Found (%): C, 58.81; H, 3.18; N, 11.46. ES-MS [CH_3OH, *m/z*]: 822.4 ($[M]^+$). ^1H NMR (300 MHz, DMSO) δ 9.09 (d, J = 8.1 Hz, 2H), 8.59 (d, J = 6.7 Hz, 2H), 8.47 (dd, J = 10.3, 6.3 Hz,