Biosensors and Bioelectronics 91 (2017) 115–121

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Multicolor imaging of hydrogen peroxide level in living and apoptotic cells by a single fluorescent probe

Ying Wen[a,1], Fengfeng Xue[a], Haichuang Lan[a,b], Zhenhua Li[a], Shuzhang Xiao[b,*], Tao Yi[a,*]

[a] Department of Chemistry and Collaborative Innovation Center of Chemistry for Energy Materials, Fudan University, Shanghai 200433, China
[b] College of Biological and Pharmaceutical Sciences, China Three Gorges University, Yichang 443002, Hubei, China

ARTICLE INFO

Keywords:
Fluorescent probe
Hydrogen peroxide
Caspase 3
Living cells
Apoptotic cells
Multicolor imaging

ABSTRACT

To understand the entangled relationship between reactive oxygen species (ROS) and apoptosis, there is urgent need for simultaneous dynamic monitoring of these two important biological events. In this study, we have developed a fluorescent probe, **pep4-NP1**, which can simultaneously detect H_2O_2 and caspase 3, the respective markers of ROS and apoptosis. The probe contains a H_2O_2 fluorescence reporter (**NP1**) and Cy5 fluorescent chromophore connected by a caspase 3 specific recognition peptide. The detecting strategy was realized through a controllable fluorescence resonance energy transfer (FRET) process between **NP1** and Cy5 of **pep4-NP1**, after reaction with H_2O_2, which was verified by molecular calculation and *in vitro* spectral studies. In the absent of caspase 3, the accumulation of H_2O_2 induces red fluorescence of **pep4-NP1** centered at 663 nm in living cells due to the existence of FRET. In contrast, FRET is inhibited in apoptotic cells due to cleavage of the peptide spacer of **pep4-NP1** by over-expressed caspase 3. Consequently, green fluorescence (555 nm) predominated when labelling production of H_2O_2 in apoptotic cells. Moreover, **Pep4-NP1** shows excellent selectivity towards H_2O_2 and caspase 3 on their respective reaction sites. Therefore, **pep4-NP1** can distinguish endogenously generated H_2O_2 between living cells and apoptotic cells with different fluorescence wavelengths, providing additional information on the ROS production pathways.

1. Introduction

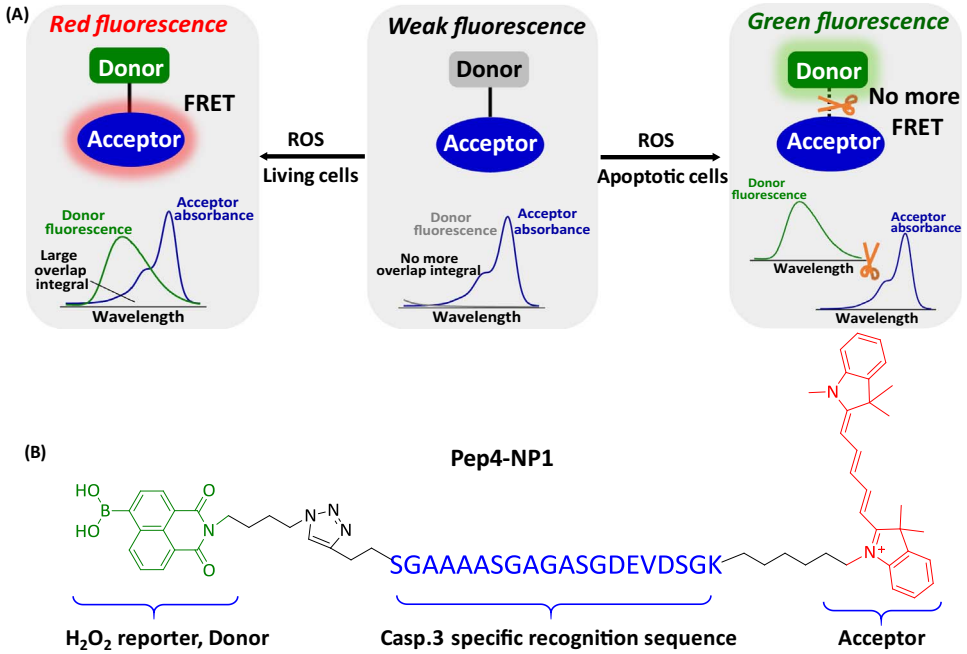
Reactive oxygen species (ROS), natural byproducts of normal oxygen metabolism, play vital roles in cellular signal transduction and homeostasis. However, under environmental stress or stimulation by exogenous chemicals, aberrant production of ROS may result in oxidative stress. The excess ROS could attack cellular biomolecules, such as protein, lipid and DNA, leading to cancer, diabetes, and neurodegenerative disorders (Kawagishi and Finkel, 2014; Lin and Beal, 2006). It is thus critical to monitor dynamic changes of intracellular ROS. Hydrogen peroxide (H_2O_2) is the most important marker for ROS. Numerous fluorescent probes have been designed and synthesized for detection of H_2O_2 at physiological levels *in vitro* and *in vivo* (Liu et al., 2016; Carroll et al., 2014; Xu et al., 2013; Karton-Lifshin et al., 2011; Abo et al., 2011). Especially, Chang group have done excellent work on the development of new H_2O_2 probes (Lippert et al., 2011). Considering that oxidative DNA damage plays a critical role in the induction of tumors (Noble et al., 2016), we have contributed to the development of fluorescent sensors to monitor H_2O_2 levels in close proximity to nuclear (Wen et al., 2014) and mitochondrial DNA recently (Wen et al., 2015).

The DNA damage elicited by ROS, if not repaired in a timely manner, promotes activation of programmed cell death (apoptosis), a gene-controlled cell suicide process. Apoptosis can be initiated by two mechanisms: ligation of cell-surface death receptors (the “extrinsic” pathway) and activation of the mitochondrial pathway (the “intrinsic” pathway). The two pathways both activate initiator cysteine proteases (caspases), which then activate executioner caspases that indiscriminately degrade proteins and inevitably lead to cell death. Caspase 3 (Casp.3), one of the most important executioner caspases, is well established as a biomarker of apoptosis (Marino et al., 2014).

Oxidative stress is closely associated with apoptosis. However, during unusual accumulation of ROS, the synergetic association with apoptosis is complex and uncertain (Xiao et al., 2016). There is not only a concentration or time correlation. For instance, H_2O_2 has been reported to induce apoptosis *via* the mitochondrial apoptotic pathway (Rai et al., 2015). However, H_2O_2 has also been reported to be generated as an early major mediator in apoptosis (Kim et al., 2006). It seems that the initiation of apoptosis is an integrated mechanism not only related to changes of the level, but also associated with the

* Corresponding authors.
E-mail addresses: shuzhangxiao@ctgu.edu.cn (S. Xiao), yitao@fudan.edu.cn (T. Yi).
[1] Present Addresses: Institute of Molecular Science, Shanxi University, Taiyuan 030006, China.

http://dx.doi.org/10.1016/j.bios.2016.12.027
Received 28 October 2016; Received in revised form 12 December 2016; Accepted 12 December 2016
Available online 13 December 2016



Scheme 1. (A) Design strategy and the FRET schematic diagram; (B) chemical structure of **pep4-NP1**. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

generation pathways and subcellular distribution of ROS (Danial and Korsmeyer, 2004; Douda et al., 2015). Therefore, to dissect the complex relationship between abnormal production of ROS and initiation of apoptosis in cells or living systems, there is urgent need for simultaneous dynamic monitoring of these two important biological processes.

Current methods monitor oxidative stress and apoptosis separately using, for example, multiple fluorescence probes. However, the combination of several sensors complicates the process and causes greater invasive damage because of the different localization and metabolism of these probes (Srikun et al., 2011; Yuan et al., 2012; Van de Bittner et al., 2013). Thus, a new general strategy for simultaneous *in situ* visualization of the two biological responses is needed.

In this study, we set out to establish a versatile strategy to develop a fluorescent probe that could simultaneously monitor ROS levels and determine the apoptotic status of cells. The idea was to expand the utility of a ROS probe by building a new composite probe based on fluorescence resonance energy transfer (FRET), which was expected to emit fluorescence at different wavelengths in living and apoptotic cells (Scheme 1A). According to the strategy, **pep4-NP1** was designed based on our reported H_2O_2 dye **NP1** (as a donor) (Wen et al., 2014) and Cy5 cyanine dye (as an acceptor) linked by the SGDEVDSG peptide as a spacer (Scheme 1B). The Cy5 cyanine dye emits red fluorescence, with the absorption band partially overlapping the emission of **NP1** (after reaction with H_2O_2). The SGDEVDSG sequence is an extended specific recognition substrate for Casp.3 (Prasuhn et al., 2010; Boeneman et al., 2009; Vickers et al., 2013). FRET occurs in living cells, but is inhibited in apoptotic cells because the peptide spacer is cleaved by specific proteases. Thus, when monitoring changes of H_2O_2 level, the single probe successfully produced fluorescence at markedly different wavelengths in living (red fluorescence) and apoptotic (green fluorescence) cells. In addition, the simultaneous multicolor fluorescence imaging strategy revealed that endogenous generation of ROS was not always accompanied by apoptosis.

2. Experiment

Details of the experimental part including materials, apparatus, cell culture, confocal laser microscopy, Casp.3 activity assay and Annexin V-FITC/PI staining assay can be found in the Supplementary Information.

3. Results and discussion

3.1. Synthesis of pep4-NP1

The synthetic details of **pep4-NP1** are provided in the Supplementary Information (Scheme S1). The peptide was custom-synthesized by a commercial supplier. Two terminuses of the peptide were functionalized with Cy5 and 4-pentine acid to produce **pep4** (HRMS calc. for $C_{101}H_{148}N_{23}O_{33}^{2+}$ $[M+2H]^{2+}$ 1105.5305; found 1105.5318; calc. for $C_{101}H_{149}N_{23}O_{33}^{3+}$ $[M+3H]^{3+}$ 737.3563; found 737.3577). Click reaction between **pep4** and **NP1** provided **pep4-NP1**, which was purified by HPLC (HRMS calc. for $C_{117}H_{159}N_{27}O_{35}B^{2+}$ $[M-2H_2O+2H]^{2+}$ 1256.5793; found 1256.5816; calc. for $C_{117}H_{160}N_{27}O_{35}B^{2+}$ $[M-2H_2O+3H]^{3+}$ 838.0554; found 838.0584).

3.2. FRET process of pep4-NP1 with responding to H_2O_2

The FRET process of **pep4-NP1** was determined by spectroscopic study and quantum molecular calculation. Quantum mechanical calculations were performed for the optimized structures of the **pep4-NP1** molecule (Fig. S1). Consideration of both efficiency and accuracy, calculations were performed with the B97D density functional method (Grimme, 2006) with the 6–31G basis set (McLean and Chandler, 1980) using the Gaussian 09 software package (Frisch et al., 2009). The empirical long-range correction which has been proven to be reliable in describing π-π interactions was used for the B97D functional (Grimme et al., 2010). From the results of the calculations, the closest distance between **NP1** and Cy5 of **pep4-NP1** was 3.36 Å, indicating that the folded form was the more stable conformation because of intramolecular interactions between donor and acceptor, increasing FRET efficiency. The distance was appropriate for the occurrence of FRET (less than 10 Å) (Hohng et al., 2014; Ray et al., 2014).

The basic spectroscopic properties of **pep4-NP1** were assessed *in vitro* (PBS, pH 7.2). The probe (5 μM) showed two absorption bands at 352 nm and 643 nm (ε=318,894 M^{-1} cm^{-1}, Fig. 1a), which were attributed to the 1,8-naphthalimide and cyanine dye, respectively, by