Peroxy-caged luciferin (PCL-1) probe for peroxynitrite detection

Application of Triple Quad LC-MS for detection of luciferin-6'-p-nitrobenzyl ether – a footprint of peroxynitrite

Members of the Dye Technology team, part of the Institute of Polymer and Dye Technology, Lodz University of Technology

Dye Technology Team at the Institute of Polymer and Dye Technology, Lodz University of Technology

The team's current research interests are focused on the application of fluorescent and bioluminescent dyes in the detection of reactive oxygen, nitrogen and sulfur species. The research involves design and synthesis of fluorescent dyes; characterization of novel fluorescent compounds; application of optical probes in cell-free systems as well as in intracellular milieu.

The main research goals are achieved by using organic synthesis in combination with chemical and spectroscopic characterization of synthesized probes applying fluorescence and HPLC techniques. In addition, the research works focus on the design and synthesis of novel dyes engineered for functionalization of textile materials and plastics, and development of new photosensitizers of polymerization.

The Institute educates students in the field of "Dyes and Household Chemicals Technology." The program prepares students for solving of practical tasks which they may encounter in their professional activity.

Free Radical Research Laboratory in the Department of Biophysics, Medical College of Wisconsin, USA

The Free Radical Research Laboratory led by Dr. Jacek Zielonka, focuses on understanding the role of cellular oxidants and redox signaling pathways in cancer proliferation and anticancer treatments. This includes the de-

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velopment of new, rapid methods for detection of reactive oxygen and nitrogen species, and application of high-throughput screening for development of new inhibitors of NADPH oxidases as possible cancer therapeutics. Another research topic being explored is exploitation of the differences in bioenergetic profiles and redox status between normal and cancer cells for development of novel strategies to selectively target cancer cells. Specifically, the scientists develop mitochondria-targeted redox modulating agents to be used alone or in combination with standard-of-care drugs to inhibit the growth of cancer cells *in vitro* and *in vivo*.

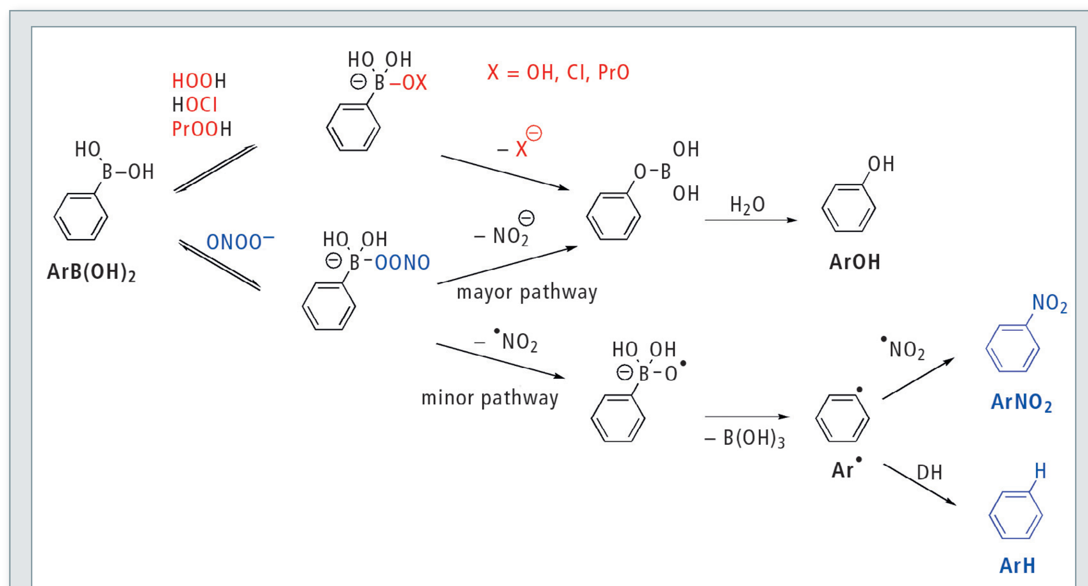

Figure 1: Oxidative conversion of arylboronic acid ($ArB(OH)_2$) induced by different oxidants (hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), protein hydroperoxides (PrOOH), peroxynitrite ($ONOO^-$)). Reaction between peroxynitrite and arylboronic acid leads to the major phenolic product ArOH and the minor peroxynitrite-specific products ($ArNO_2$, ArH).

Peroxynitrite

Peroxynitrite ($ONOO^-$), a highly reactive oxidizing and nitrating agent is generated via a spontaneous, diffusion-controlled reaction between nitric oxide ($^{\bullet}NO$) and superoxide radical anion ($O_2^{\bullet -}$). Peroxynitrite has been implicated as a key pathophysiological intermediate in various diseases, including acute and chronic inflammatory processes, diabetes, sepsis, ischemia-reperfusion, atherosclerosis and neurodegenerative disorders as well as chemotherapy-induced nephrotoxicity. Low steady state concentrations and unfavorable spectroscopic properties make direct detection of peroxynitrite in cells practically impossible. One of the methods to detect $ONOO^-$ is to use a chemical probe which is selective and/or forms a footprint product after reaction with this oxidant.

Oxidation of arylboronic acid

Over the last decade [1], it has been demonstrated that several oxidants such as hydrogen peroxide, hypochlorous acid, protein hydroperoxides and peroxynitrite convert arylboronic acids **($ArB(OH)_2$)** to phenolic products **ArOH** (figure 1). However, the reactivity of boronates with peroxynitrite ($ONOO^-$) is of special interest. Not only the rate constant of the reaction of boronate probes with $ONOO^-$ is the highest of all tested biological oxidants, but the reaction also typically leads to the formation of minor but $ONOO^-$-specific products **($ArNO_2$, ArH)** in addition to the major phenolic product **(ArOH)**. Since the minor pathway is specific only for the reaction of boronates with peroxynitrite, formation of these products **($ArNO_2$, ArH)** may serve as a unique footprint providing a diagnostic marker for peroxynitrite formation in cells-free and cellular systems.

Peroxy-caged luciferin (PCL-1) as probe for peroxynitrite detection

Bioluminescent imaging (BLI) is commonly used for sensitive monitoring of various biomolecular processes in cells and living animals. Generally, BLI employs firefly luciferase and its highly specific substrate luciferin **(Luc-OH)** to produce light in the presence of ATP, O_2 and Mg^{2+}. One of the first luciferin-based bioluminogenic probes designed for imaging of hydrogen peroxide in living systems was peroxy-caged luciferin, **PCL-1** (figure 2A) [2]. The hydroxyl group of **Luc-OH** is alkylated by the boronobenzyl moiety in **PCL-1** which prevents recognition of the probe by luciferase enzyme. Upon reaction of **PCL-1** with H_2O_2, the boronate moiety is replaced by the hydroxyl group, leading to the elimination of quinone methide and formation of the luciferase substrate **Luc-OH**.

However, peroxynitrite oxidizes arylboronic acid nearly one million times faster than does H_2O_2. The high rate constant of the reaction of **PCL-1** probe with $ONOO^-$ ($k \sim 10^6\ M^{-1}\ s^{-1}$[3]) made it possible to detect peroxynitrite in the presence of its physiological scavengers (e.g., CO_2, glutathione, albumin) in cell-free and cellular systems. Recently, we characterized in detail the reaction intermediates and stable products formed in the reaction between **PCL-1** probe and selected inflammatory oxidants (H_2O_2, HOCl, $ONOO^-$) [4]. In that study, a protocol was proposed based on profiling all products formed during peroxynitrite-induced oxidation of **PCL-1**. The scientists detected: (i) major primary phenolic intermediate **Luc-Bz-OH**; (ii) stable phenolic product, **Luc-OH**, formed after elimination of quinomethide **QM**, and (iii) minor products: luciferin-6'-p-nitrobenzyl ether **Luc-Bz-NO_2** and luciferin-6'-benzyl ether **Luc-Bz-H** (figure 2A).

Prof. Jolanta Sokołowska has supervised seven PhD theses. She was Vice-Dean for Student Affairs (1999 - 2005), Vice-Director of the Institute of Polymer and Dye Technology (2004 - 2019), and since 2004 Head of the Dye Technology team.

The scientific work of the Prof. Sokołowska's team has been focused on the photochemical stability of disperse azo dyes, the usage of photoelectrochemical methods to remove dyes from wastewater, also with the application of photocatalysts, and the synthesis of fluorescent dyes for special applications such as sensitizers in visible initiator systems of radical, cationic and hybrid polymerization or chemosensors for biothiols.

Her scientific achievements comprise of 91 publications, including 77 papers published in journals in the JCR list, an academic textbook and four chapters in scientific books. She is a co-author of 26 patents and patent applications.

Formation of **Luc-Bz-NO$_2$** in the reaction of **PCL-1** with ONOO$^-$ includes the incorporation of part of the oxidant into the product and provides an opportunity to specifically detect and identify peroxynitrite in biological systems.

LC-MS protocol

LC-MS analyses of **PCL-1** and its oxidation products were performed using Shimadzu Triple Quad LCMS coupled to Shimadzu Nexera X2 UHPLC system. Analysis was done on Cortecs C_{18} column (Waters, 50 mm x 2 mm, 1.6 µm) equilibrated with 10 % of acetonitrile in water containing 0.1 % of formic acid. The compound was eluted by increasing the acetonitrile concentration in the mobile phase from 10 to 80 % over 4 minutes. The flow rate was set at 0.5 mL/min and the flow was diverted to waste during the first minute and after 4 minutes, counting from the time of injection. **PCL-1**, **Luc-OH**, **Luc-Bz-NO2** and **Luc-Bz-H** were detected as positive ions using multiple reaction monitoring (MRM) mode, with primary/fragment ion pairs of 415 > 135, 281 > 235, 416 > 234 and 371 > 91, respectively. **Luc-Bz-OH** was detected in positive mode using single ion monitoring (SIM) mode, set at the m/z value of 387 [4].

Observations

Oxidation of **PCL-1** by excess H_2O_2 leads to the formation of the primary phenol **Luc-Bz-OH**, detectable when sample was analyzed immediately (< 2 min) after mixing (figure 2B, C). After one hour incubation, no **Luc-Bz-OH** was observed and the probe had been completely converted into luciferin **(Luc-OH)**. Similar behavior was noticed when peroxynitrite was used instead of H_2O_2, but in addition to the phenolic products, **Luc-Bz-NO$_2$** formation was observed. Furthermore, when the reaction was performed in the presence of 2-propanol (2-PrOH), applied as a hydrogen atom donor to the phenyl radical, additional product **Luc-Bz-H** formed in the reaction of **Luc-Bz$^•$** with 2-PrOH was detected (figure 2C). Application of the probe to the RAW 264.7 macrophages activated to produce both $^•$NO and $O_2^{•-}$ led to increased probe oxidation and appearance of the peroxynitrite-specific product **Luc-Bz-NO$_2$** (figure 2D).

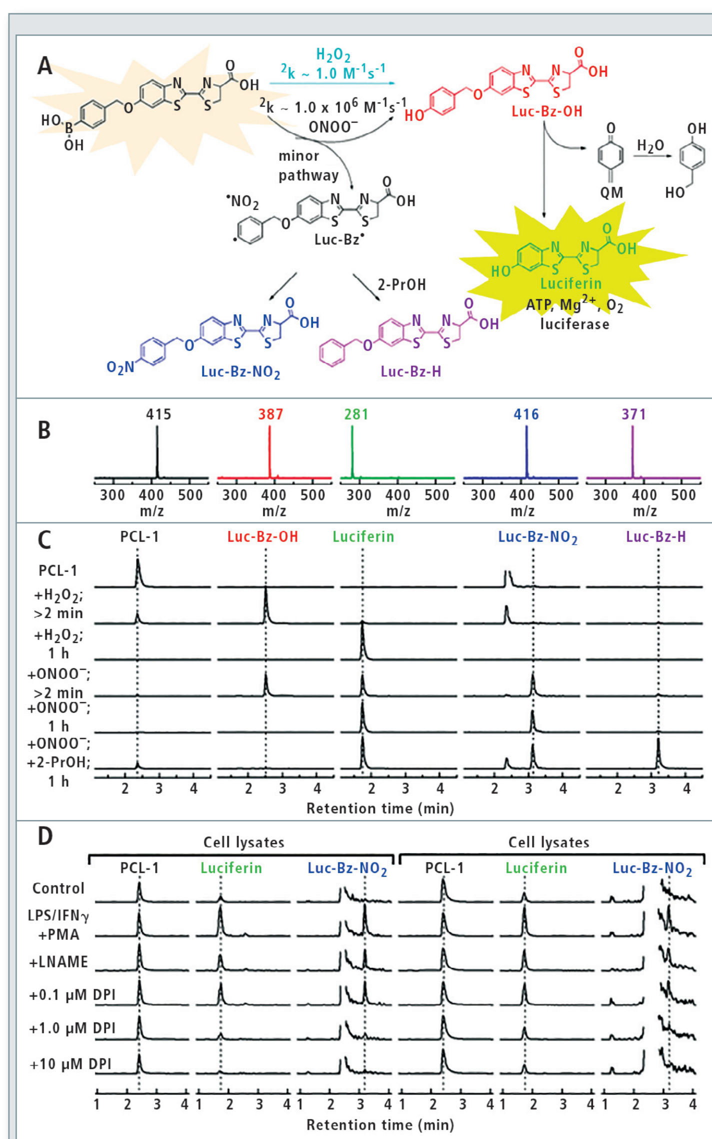

Figure 2: LC-MS analyses of the products of PCL-1 oxidation. (A) Scheme of peroxynitrite-induced transformation of PCL-1 probe leading to firefly luciferin and peroxynitrite-specific products: luciferin-6'-p-nitrobenzyl ether (Luc-Bz-NO$_2$), and luciferin-6'-benzyl ether (Luc-Bz-H). (B) Online mass spectra recorded for each product and (C) LC-MS traces of the reaction mixtures of PCL-1 (100 µM) alone or after addition of H_2O_2 (10 µM) or ONOO$^-$ (80 µM). (D) LC-MS/MS traces of PCL-1, luciferin and Luc-Bz-NO$_2$ detected in RAW 264.7 macrophages activated to produce ONOO$^-$ [modified from ref. 4]

Conclusions

The protocol allows LC-MS-based profiling of different oxidation products formed from the **PCL-1** probe, including the ONOO$^-$-specific product, luciferin-6'-p-nitrobenzyl ether **Luc-Bz-NO2**. Using this method, it was demonstrated that **Luc-Bz-NO2** is formed by activated RAW 264.7 macrophages incubated in the presence of the **PCL-1** probe, proving the formation of ONOO$^-$ upon co-production of $^•$NO and $O_2^{•-}$ in cellular systems [4]. **PCL-1**-based bioimaging of ONOO$^-$, in combination with LC-MS-based detection of the **Luc-Bz-NO2** provides an opportunity to detect, localize and identify this inflammatory oxidant *in vitro* and *in vivo*.

Acknowledgement

The methodology for the detection of peroxynitrite-specific products was developed within the research project "Fluorogenic and luminogenic probes for in vivo biophotonic imaging of peroxynitrite – from synthesis to footprint detection", which was supported by the Polish National Science Centre (NCN) within the SONATA BIS 6 program (Grant no. 2016/22/E/ST4/00549). LC-MS analyses were developed and performed in the Cancer Center Redox & Bioenergetics Shared Resource in the Medical College of Wisconsin.

Authors

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