ChemComm

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Journal:	*ChemComm*
Manuscript ID:	CC-COM-12-2014-010345.R1
Article Type:	Communication
Date Submitted by the Author:	31-Jan-2015
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Journal Name

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COMMUNICATION

Real Time Detection of Live Microbes with a Highly Sensitive Bioluminescent Nitroreductase Probe

Cite this: DOI: 10.1039/xoxxoooooox

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Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

A highly sensitive and selective nitroreductase probe, with a rapid and strong bioluminescence enhancement (>100 fold in 5 minutes), and its initial application in the real time detection of both Gram positive and Gram negative live bacteria and monitoring of their growth is reported.

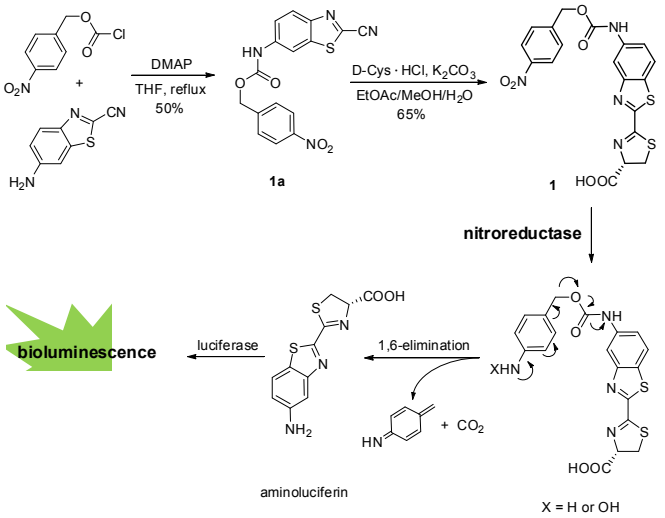
Nitroreductase is a class of flavin-containing enzymes that catalyses the reduction of nitroaromatics to the corresponding amines.[1] The enzyme plays important roles in the bioremediation and toxicity of organic nitro compounds mediated by bacteria.[2] Nitroreductase activity has also been exploited as a strategy for prodrug activation in cancer gene therapy in recent years.[3] Therefore, methods that can sensitively, selectively, rapidly, and efficiently monitor nitroreductase activity will be valuable bioanalytical tools for tracing the activity of the enzyme in both environmental and biomedical perspectives. Recently a few fluorescence-based sensors that can detect nitroreductase activity have been reported.[4] In view of the correlation between hypoxic condition and reductase activity, preliminary applications of these fluorescent probes are largely focused on hypoxia and tumor imaging.[4b,c,e]

It is well recognized that nitroreductase is abundant among the bacterial kingdom[5] and such activity could be a universal target for detecting pathogenic microbial contamination. It is anticipated that a luminescence-based sensor selective to nitroreductase activity coupled with a simple plate-reader assay will allow rapid and efficient real time monitoring of microbes in various specimens, like food and environmental samples, for sterile control and various monitoring purposes. To develop a probe for the sensitive and selective detection of nitroreductase activity, we turned our attention to bioluminescence-based detection from the catalytic conversion of firefly luciferin by luciferase. Compared with the more common fluorescence-based methods,[4] the enzymatically produced bioluminescence features high photon production efficiency and low background, therefore resulting in good signal-to-noise contrast and high sensitivity. Bioluminescence-based methods also avoid the problems of photobleaching and background autofluorescence. In addition, the small and diffusible luciferin enables good cell penetration and mobilization throughout the organism, rendering it as a desirable modality for whole organism imaging. To the best of our knowledge, no bioluminescence-based nitroreductase probe has yet been reported.

Chemical modification of luciferin is an easy and versatile strategy to the selective detection of small molecules and enzyme activity in live biological samples. The luciferin is modified in a way that only upon selective chemical transformation by the target analyte, the luciferin is uncagged and bioluminescence is produced upon luciferase activity. In this regard, several luciferin-based bioluminescent probes for *in vitro* enzyme activity assays or *in vivo* imaging have been reported.[6] The required luciferase enzyme for bioluminescence production is either produced by the genetically transfected cells/animals in the *in vivo* experiments, or is added to cell/tissue lysates or enzyme solutions in the *in vitro* assays.[6,7] Here we present a new *bioluminescent nitroreductase probe* that is highly selective and applicable in both aqueous buffer and for the detection of live bacteria. In contrary to measure ATP production[8] as an indirect reflection of viable bacteria, our probe is designed to detect specific nitroreductase activity which is widespread in bacteria and is targeted for live microbial detection. We also demonstrate that transfecting or lysing the cells, as oppose to most other bioluminescent imaging experiments, are not necessary due to the high sensitivity of the probe, which greatly simplifies the detection procedures and renders it a high potential for practical application.

Our strategy to bioluminescent nitroreductase detection is shown in Scheme 1. Aminoluciferin was caged by the 4-nitrobenzyl carbamate group, which is a commonly used caging group that targets reductase

activity for sensor and prodrug development.[4b,9] Upon enzymatic reduction of the nitro group to the corresponding amine and 1,6-elimination, the active luciferin substrate is released and bioluminescence is produced upon luciferase activity. The synthesis is composed of two straight-forward steps starting from commercially available materials. The luciferin core was constructed by reaction of **1a** with D-cysteine. Analytically pure probe **1** was obtained as a yellow solid by simple filtration, washing with solvents and drying under reduced pressure.



Scheme 1. Synthesis of **1** and its reaction with nitroreductase.

Nitroreductase-dependent luminescence production from **1** was first evaluated in aqueous buffer (50 mM Tris at pH 7.4). Solutions of **1** at 5 μM were incubated with 1 unit of nitroreductase (0.01 mg/ml) with 0.5 mM NADH as electron source for various period of time. Bioluminescence was initiated by addition of luciferase and ATP, and the produced luminescent signal was monitored by a plate reader. As shown in Figure 1, a robust luminescence signal with over 100 folds turn-on was resulted from a 5-minute incubation of **1** with nitroreductase, showing the probe can rapidly detect nitroreductase activity with high sensitivity. Stronger luminescent signals were observed when **1** was incubated with the enzyme for a longer period of time (e.g. 250-fold increase for 60 minutes incubation). Incubation of probe **1** with only NADH, or other biological and inorganic reducing agents such as thiols, ascorbate and Fe^{2+} did not result in any luminescence enhancement, demonstrating the bioluminescence from **1** is highly selective to nitroreductase.

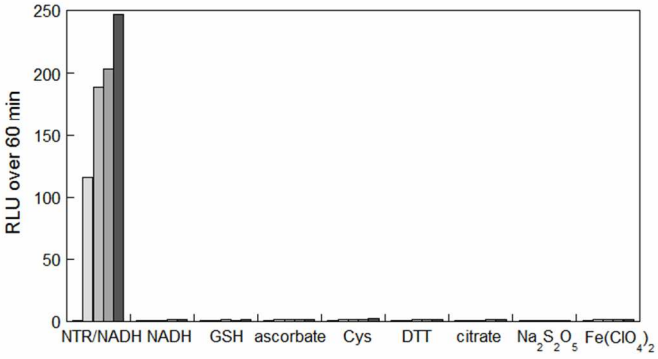


Figure 1. Relative luminescent intensity from incubation of **1** (5 μM) with 1 unit of nitroreductase (NTR) in the presence of 0.5 mM NADH for 5, 20, 40 and 60 min. Also shown are the bioluminescent response of **1** (5 μM) incubated with NADH (0.5 mM) or other reducing agents (20 eq., 0.1 mM).

Next, we examined whether the nitroreductase probe **1** can detect microbial nitroreductase activity *in vivo* by measuring luminescence intensity from both Gram positive and negative types of bacteria. Grown bacterial cultures were treated with 10 μM of **1** and subjected to subsequent lysis. Luminescence was generated by further incubation with luciferase. As shown in Figure 2a, luminescence intensity significantly increased to *ca.* 5 folds in live *E. coli* as compared to the buffer control. No induction of luminescence intensity was detected by incubating the bacterial samples at 85°C before treatment with **1**, showing the luminescence enhancement is due to the growing, live bacteria, in which the heat kill treatment abolished their growth and the nitroreductase activity. Addition of dicoumarin (15 μM), a common inhibitor of reductase enzymes, to the bacterial culture before treatment with the probe significantly reduced the luminescence intensity from the live *E. coli* (Figure S3), further demonstrating the specificity of the probe towards nitroreductase.[10] Similar to Gram negative *E. coli*, luminescence intensity increased to *ca.* 4 folds in live Gram positive *Bacillus subtilis* as compared to the buffer control (Figure 2b). Heat kill treatment and addition of dicoumarin also resulted in a reduction in the luminescence intensity (Figure S4), demonstrating the capability of our probe to detect nitroreductase activity from both representative of Gram negative and positive bacteria with distinct cell wall structure and thickness, suggesting the versatility of **1** to detect nitroreductase from a wide range of bacterial species.

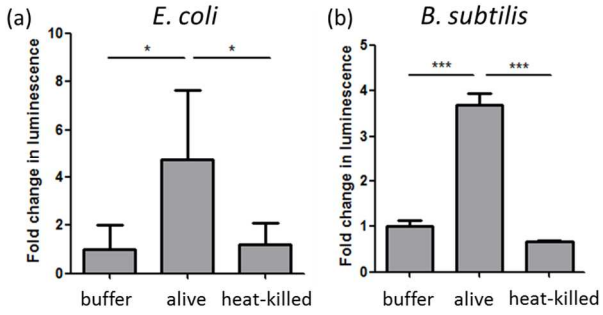


Figure 2. Luminescence intensity from clarified cell lysate of (a) *E. coli* (in LB medium); and (b) *B. subtilis* (in BHI medium) after growing for two hours and followed with or without heat-kill treatment prior to treatment with 10 μM of **1**. Luminescence was generated by addition of luciferase. Error bars indicate the s.e.m. of three samples,*: p <0.05; ***: p <0.0001.

To simplify the detection protocol and investigate whether our probe can be utilized for detection of nitroreductase activity in live and intact bacteria without any subsequent manipulation, we attempted to measure luminescence produced in an extracellular manner. Surprisingly, nitroreductase activity can be detected from the culture supernatant of live bacteria, where intact bacteria without lysis treatment were incubated with luciferase (Figure S3). The increase in luminescent intensity clearly indicated that both probe **1** and free luciferin can freely diffuse across the cell wall and membrane, allowing the nitroreductase and luciferase transformations to occur in the intracellular bacterial cytoplasm and the extracellular environments respectively. Comparing with other common

microbial detection methods like PCR-based nucleotide extraction and detection or sulfide precipitation, our protocol greatly simplifies the sample processing procedures and offers high selectivity towards bacteria. The "no-lysis" protocol can also be easily adapted to high-throughput screening platforms, giving it a high potential to be applied in microbial detection with minimum sample processing and manipulation.[11]

The time-course experiment further demonstrates the applicability of **1** in the real time detection of bacteria and their growth monitoring. Increasing luminescence intensity was detected with live *E. coli* growing for an increasing time period (Figure 3). The sigmoid curve of luminescence intensity correlated well with the growth curve of *E. coli* as determined by the OD_{600} value, which reflects the growth status of the bacterial cells. It is noted that there was a sharp increase at early time point as compared to other fluorescence based nitroreductase probes.[12] A 2-fold enhancement in luminescence was detected in the bacterial sample after just one hour incubation, showing the detection limit of the probe is well below the cell concentration at that time point which is 3.7 x 10^7 cells/ml (or 0.01 CFU/ml, see ESI). The ability of our probe to detect bacterial growth at lag phase indicates its potential for rapid detection of microbial.[13]

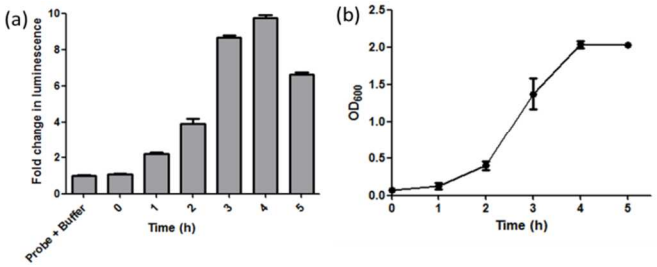


Figure 3. Comparison of (a) luminescence intensity from clarified cell lysate of *E. coli* grown in LB medium at 37°C for various length of time and (b) time-series measurement of absorbance at 600 nm of *E. coli* growing in LB medium at 37°C. Error bars indicate the s.e.m. of three samples.

In summary, a new bioluminescent probe that is specific and selective to nitroreductase activity was developed. The probe features a rapid and robust bioluminescent turn-on, and is applicable for both whole cell and lysate samples, demonstrating its potential for quick, real-time and quantitative detection of nitroreductase in microbes and use as a microbial growth indicator. In addition, the possibility of *ex vivo* light measurement not only simplifies the protocol that requires minimum manipulation and has a high potential to translate into practical applications, but also suggests that luciferin-based luminescence detection could be applied to other organisms without the need of genetic modification.

Financial supports from The University of Hong Kong and the Croucher Foundation were gratefully acknowledged. This work was partly supported by HKU Seed grant 201311159079 to W.H.F.R. We also thank Prof. Christopher J. Chang, Dr. Genevieve C. Van de Bittner and Miss Teera Chantarojsiri for their helpful assistance.

Notes and references

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† Electronic Supplementary Information (ESI) available: Details of synthesis and compound characterization, *in vitro* bioluminescent assays and bacterial experiments. See DOI: 10.1039/c000000x/

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