A novel luciferin-based bright chemiluminescent probe for the detection of reactive oxygen species†

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Received (in Cambridge, UK) 23rd February 2009, Accepted 16th March 2009
First published as an Advance Article on the web 9th April 2009
DOI: 10.1039/b903751a

This communication reports the synthesis, chemiluminescence properties, and biological application of KEIO-BODIPY–imidazopyrazine (KBI), a yellow-green chemiluminescent probe for the detection of reactive oxygen species (ROS) generated from living cells.

Chemiluminescence analysis is a highly sensitive, rapid, and extremely useful analysis method, which does not require any excitation light source, because the excitation energy is provided by an oxidation reaction.[1] The features of chemiluminescence analysis are ideally suited to use chemiluminescence probes for the detection of reactive oxygen species (ROS) produced by living cells. On one hand, ROS, such as for example superoxide radical anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and peroxynitrite ($ONOO^-$), play a vital role in living organisms.[2] On the other hand, too high or too low ROS concentrations can cause damage to biological functions, and finally result in ageing or various diseases.[2] However, the mechanisms resulting in damage to living organisms are up to now not fully known.[2] Whereas there is a large variety of fluorescent probes available to detect ROS generated from living cells,[3] there are relatively few chemiluminescent probes suitable for biological applications, because the known chemiluminescent probes have some disadvantages. For example, the common chemiluminescent probe luminol is not able to react efficiently under neutral pH conditions. Moreover, luminol, acridinium esters and 1,2-dioxetane emit light at relatively short wavelengths (*ca.* 400 nm).[4] This issue possibly results in the decrease in emission generated from chemiluminescent probes because of light autoabsorption in the short wavelength range from biomolecules omnipresent in biological samples (*e.g.* NADH, collagen).[5] Therefore, alternative chemiluminescent probes that allow to overcome these disadvantages are still required.

Cypridina luciferin analogs (*e.g.* CLA[6,7] λ_{CL}: 360 nm and MCLA[6,8] λ_{CL}: 465 nm), which show emission under neutral pH conditions, have gained attention and have been developed as new candidates for bioapplicable chemiluminescent probes.[6,9] Additionally, longer-wavelength emitting chemiluminescent probes based on *Cypridina* luciferin (FCLA[6,10] λ_{CL}: 520 nm and Red-CLA[6,11] λ_{CL}: 610 nm) have been developed in order to red-shift the short-wavelength emission of CLA and MCLA.[6] In these probes, MCLA and a fluorophore (fluorescein or sulforhodamine 101) are connected through a linker, and chemiluminescence resonance energy transfer (CRET) occurs based on the Förster theory;[12] the energy of the singlet-excited state generated by the reaction of MCLA with ROS is transferred to the fluorophore moiety, which emits long-wavelength chemiluminescence specific for the respective fluorophore. However, for instance, the superoxide-induced chemiluminescence intensities of CRET-based chemiluminescent probes are sometimes low compared to those directly emitted by MCLA.[13] Recently, Teranishi and co-workers synthesized γ-cyclodextrin-conjugated CRET-based chemiluminescent probes in order to reduce this decrease in chemiluminescence.[6] This also implies that there is still a requirement for further improved chemiluminescent probes.

In recent years, new types of long-wavelength chemiluminescent probes with high optical performance have been developed, which rely on the connection of luminol and fluorophores by an acetylene linker[14] or by a direct bond,[15] respectively. We considered that this molecular design concept will allow the establishment of high-performance chemiluminescent probes based on *Cypridina* luciferin analogs. Therefore, we designed and synthesized the novel luciferin-based chemiluminescent probe KBI (KEIO-BODIPY–imidazopyrazine). The KBI molecule consists of two parts connected through a direct bond: the imidazopyrazine moiety known as a chemiluminescence emitter, and the BODIPY fluorophore. Imidazopyrazine, which is the luminescent site of *Cypridina*, emits chemiluminescence by an oxidation reaction under neutral pH conditions. BODIPY (1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene), a known fluorophore, has many excellent optical characteristics, such as a long fluorescence wavelength (above 500 nm), high fluorescence quantum yield, and insensitivity towards pH or solvent polarity.[16] Additionally, the fluorescence emission wavelength of BODIPY is almost identical to that of fluorescein (emission moiety of FCLA). According to this molecular design concept, it was expected that bright and long-wavelength chemiluminescence derived from the BODIPY unit would be detected under neutral pH conditions when the imidazopyrazine unit is oxidized. KBI was successfully synthesized in seven steps as shown in Scheme 1. First, the BODIPY fluorophore was synthesized in two steps. It was then coupled to a precursor of the imidazopyrazine, and the final compound was obtained by a cyclization step.

The chemiluminescent characteristics of KBI were evaluated and compared with those of MCLA and FCLA. Fig. 1 shows the chemiluminescence spectra of KBI, MCLA, and FCLA (2.5 μM) in MOPS buffer (pH 7.2, containing 5% MeOH in

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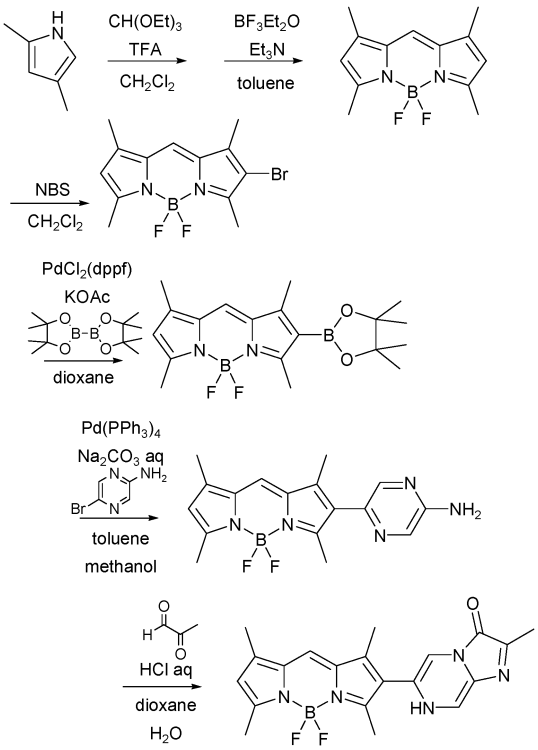
† Electronic supplementary information (ESI) available: Detailed synthetic procedures, measurement conditions, and supplemental data. See DOI: 10.1039/b903751a



Scheme 1 The synthesis scheme for KBI.

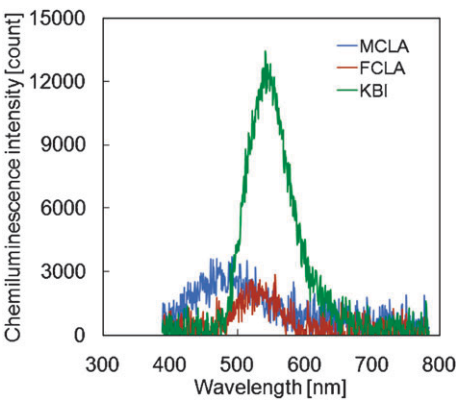


Fig. 1 Chemiluminescence spectra of KBI, MCLA, and FCLA (2.5 μM) oxidized by O_2^- generated by the enzymatic reaction of HPX and XOD.

the case of KBI) after oxidation by O_2^- generated from the enzymatic reaction of hypoxanthine (HPX) and xanthine oxidase (XOD) (detailed experimental conditions are described in the ESI†). The emission maximum of KBI (545 nm; yellow-green color) appeared in a longer wavelength range than that of MCLA, which is attributed to the long emission wavelength of BODIPY. No imidazopyrazine-derived emission peak (400–480 nm) was observed. This can be also seen from Fig. 2, which shows the chemiluminescence colors of KBI and MCLA in the presence of excess H_2O_2 in methanol. Interestingly, the observed chemiluminescence emission maximum of KBI was located at longer wavelength than the fluorescence emission of BODIPY. This suggests that the emitting species is the entire fully conjugated KBI molecule with its oxidized imidazopyrazine unit, rather than the BODIPY moiety alone.

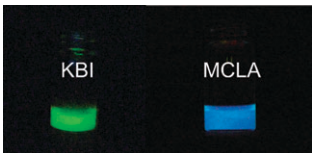


Fig. 2 Chemiluminescence colors of KBI and MCLA oxidized by H_2O_2.

This is an indication against a mechanism where the energy of the excited-state oxidized imidazopyrazine is transferred to the BODIPY fluorophore, which would then result in an emission at the BODIPY fluorescence wavelength. Additionally, it was confirmed that the fluorescence emission of a ring-opened KBI derivative corresponding to the oxidized form of the KBI molecule is essentially identical to the chemiluminescence wavelength of KBI (see Fig. S-1 and S-2 of the ESI†). Therefore, it is assumed that KBI with the imidazopyrazine and fluorophore units linked through a direct bond behaves as a single molecule emitting high-performance chemiluminescence. The integrated chemiluminescence intensity of KBI was much higher than that of MCLA and FCLA as shown in Fig. 1. This is assumed to be due to the high fluorescence quantum yield of BODIPY. An obvious difference between KBI and FCLA in terms of the chemiluminescence intensity was confirmed. Consequently, it is considered that KBI, which emits bright and long-wavelength chemiluminescence, has sufficient potential for bioanalytical application.

The relative chemiluminescence intensity (RCI) of KBI (5.2 μM) in the presence of various ROS (O_2^-, H_2O_2, ClO^-, $ONOO^-$, $^{\bullet}OH$, $^1O_2$) and antioxidants (superoxide dismutase: SOD; and catalase) was measured with an AB-2200 Luminescencer-PSN (ATTO corporation). The results are summarized in Table 1. All RCIs are reported as values normalized to the blank KBI chemiluminescence observed in the absence of any ROS. The response of KBI towards enzymatically generated O_2^- or H_2O_2 was mostly suppressed by addition of SOD or catalase, respectively. The experiments showed that a significantly high RCI of KBI was observed in the presence of O_2^-, while only weak signals were obtained in response to O_2^-–SOD, H_2O_2–catalase, and other ROS. The reason for the relatively strong response towards H_2O_2 is assumed to be due to excessive addition of hydrogen peroxide compared to the rather low amounts of O_2^- generated by the enzymatic reaction. Therefore, it can be considered that KBI shows a highly specific response towards O_2^- under neutral pH conditions in correspondence to the characteristics of *Cypridina* luciferin analogs (detailed experimental conditions

Table 1 Relative chemiluminescence intensity (RCI) observed upon reaction of KBI with various ROS and antioxidants

Compounds	RCI[a]	Compounds	RCI[a]
Blank	1		
O_2^-	620	$^{\bullet}OH$	13
H_2O_2	168	$^1O_2$	6
ClO^-	3	O_2^- + SOD	8
$ONOO^-$	5	H_2O_2 + catalase	21

[a] All data was obtained for two minutes.

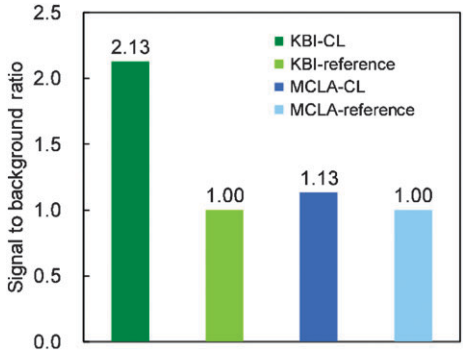


Fig. 3 The signal to background ratio for chemiluminescence generated from stimulated cells and non-stimulated cells (reference) using KBI and MCLA.

are described in the ESI†). In order to demonstrate the applicability of KBI for bioanalytical applications, the detection of O_2^- generated from living cells (the human leukemia cell line: HL-60) was investigated.

HL-60 cells (10^6 cells) were loaded with KBI (HBSS buffer, pH 7.4 containing 1% DMSO: 1 μM, 200 μl) or MCLA (HBSS buffer, pH 7.4: 1 μM, 200 μl) by incubation at 37 °C/CO_2 5% for 40 min. Then, the suspensions containing probe-loaded cells were transferred to a black 96-well microplate, phorbol 12-myristate 13-acetate (PMA; HBSS buffer, pH 7.4 0.1% DMSO: 1 μM, 20 μl) was added as a stimulating agent to each well, and the chemiluminescence was detected on an AB-2350 PHELIOS (ATTO corporation). ROS are known to be generated when HL-60 cells are stimulated with PMA.[17] The chemiluminescence at each well was collected every seven seconds for KBI, and every ten seconds for MCLA, respectively. The averaged chemiluminescence intensities are represented as bars in Fig. 3. The signal to background ratio, that is the chemiluminescence intensity observed for stimulated cells (KBI-CL and MCLA-CL, respectively) compared to the intensity observed for non-stimulated cells (KBI-reference and MCLA-reference, respectively), is higher for KBI than for MCLA. This indicates that KBI responds to ROS generated from HL-60 cells, and that the sensitivity of KBI is higher than that of MCLA. Furthermore, the absolute integrated chemiluminescence of KBI is much larger than that of MCLA (see Fig. S-3 of the ESI†). Significantly, the signal to noise ratio of the emission spectra of KBI is obviously high, whereas that of MCLA is too low to be distinguished as a significant signal from the background (see Fig. S-3 of the ESI†). Thus, KBI enables the sensitive detection of ROS generated from stimulated cells, which is difficult to achieve by using commercially available luciferin-based chemiluminescent probes such as MCLA. Furthermore, it can be assumed with high probability that O_2^- was generated from HL-60 cells, because KBI provides a highly specific chemiluminescence response towards O_2^-.

In conclusion, a novel luciferin-based long-wavelength chemiluminescent probe (KBI), which shows yellow-green emission (545 nm) under neutral pH conditions after oxidation by ROS, was successfully designed and synthesized. The chemiluminescence intensity of KBI was much higher than the intensities of known commercially available *Cypridina* luciferin analogs such as MCLA and FCLA, and at a longer wavelength than the chemiluminescence of MCLA. The direct bonding of the imidazopyrazine moiety to the BODIPY fluorophore evoked high-performance chemiluminescence. The selectivity measurements of KBI towards various ROS revealed that KBI has high specificity for O_2^-. Finally, the highly sensitive detection of ROS generated from HL-60 cells was demonstrated with KBI. Consequently, a new type of functional chemiluminescent probe for bioanalytical application has been developed. In the future, the detection of ROS generated from particular organelles (*e.g.* mitochondria) will be attempted by modifying KBI with organelle specific targeting moieties.

We kindly acknowledge Mr Hidehiro Kubota, Mr Atsushi Asakawa, and Mr Tsutomu Irie from ATTO corporation for granting us access to the chemiluminescence measurement devices.

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