Bioimaging

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# Ultrasensitive Chemiluminescent Detection of Cathepsin B: Insights into the New Frontier of Chemiluminescent Imaging

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Optical analytical techniques for quantitative measurement and imaging in biological specimens have been developed to achieve ever higher sensitivity and accuracy. Methods based on absorbance and light attenuation such as bright-field microscopy are still central to cellular biology and other fields, but fluorescence techniques have exploded and currently occupy a privileged place in the analysis of cellular samples due to the ability to monitor biological molecules with bright fluorescent dyes that can outshine the autofluorescence of endogenous molecules. This autofluorescence, however, still poses a significant limitation by causing a non-zero background in fluorescence microscopy experiments. Combined with light scattering from the excitation source and problems with photobleaching, these issues ultimately represent limitations to the intrinsic sensitivity of fluorescence-based detection and imaging experiments.

Chemiluminescence, the generation of light from a chemical reaction, has emerged as a new imaging technique in cells and animals that displays improved depth penetration due to the elimination of an extraneous light source, and therefore autofluorescence, photobleaching, and much of the light scattering that plagues fluorescence imaging.[1,2] As opposed to bioluminescent imaging, genetic modification of organisms is not needed, thus providing a great deal of flexibility for experimental procedures. This modality requires the synthesis of small-molecule probes to detect specific analytes, and to date, the available tools are still limited. Schaap's spiroadamantane 1,2-dioxetane[3] was used in some of the earliest examples of in vivo chemiluminescent molecular imaging in combination with polymeric "Enhancer" solutions to aid in chemiluminescence efficiency and energy transfer to dyes with red-shifted emission wavelengths. Excitingly, strategies using intramolecular energy transfer[4] and modifications of the molecular structure of the core chemiluminescent scaffold[1,5] have enabled bright and red-shifted chemiluminescence emission without the need for polymeric additives, thereby effectively simplifying experiments. Chemiluminescent imaging agents have been synthesized for the in vivo imaging of a range of analytes, including β-galactosidase,[4,6] nitroreductase and hypoxia,[7] $H_2S$,[8] and $H_2O_2$.[9] Moreover, chemiluminescent probes with emission in the near-infrared window have been demonstrated,[8] thus providing greater capability for in vivo imaging through increased depth penetration and decreased phototoxicity.

In addition to in vivo experiments, instrumentation is now commercially available to perform chemiluminescence microscopy, with a key example of imaging β-galactosidase activity[1,5] in monolayer cell culture. In their recent work,[10] Roth-Konforti and co-workers report a new chemiluminescent probe for the ultrasensitive detection and chemiluminescence microscopy imaging of cathepsin B. Cathepsin B is a member of the cysteine protease family that is expressed in invasive cancer cells and is often associated with increased malignancy.[11] Cathepsin B is found in the focal adhesions and invadopodia of aggressive cancers, where it promotes degradation of the extra-cellular matrix (ECM) by targeting the breakdown of key ECM building blocks such as laminin, fibronectin, and type IV collagen. Cathepsin B can also inactivate cell-adhesion proteins and activate other proteases involved in ECM degradation. Continuous dissolution of the ECM leads to the release of metastatic cells from solid tumors. There are a number of methods for monitoring cathepsin B and other proteases, including traditional molecular biology techniques, fluorogenic substrates for the enzyme, and radiotracers based on irreversible covalent inhibitors, but new methods for sensitive detection and imaging are desired for both clinical and preclinical application.[11]

The authors synthetically link a bright green chemiluminescent scaffold to a dipeptide (Valine-Citrulline) that can serve as a substrate for cathepsin B (Scheme 1). Enzyme-mediated cleavage of the amide bond induces a self-immolative 1,6-elimination reaction that leads to the release of a phenolate, followed by spontaneous decomposition in a light-emitting chemically initiated electron-exchange chemiluminescence (CIEEL) reaction. Several derivatives were prepared and it was found that addition of an acrylate ester to

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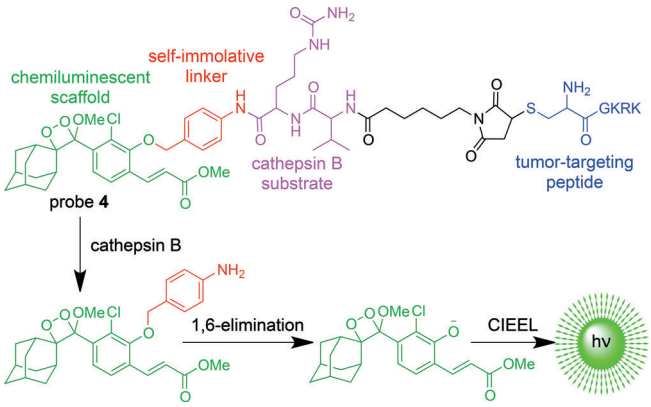


***Scheme 1.*** Design of probe **4** for the chemiluminescence detection of cathepsin B activity.

the chemiluminescent core (probe **2**) increased the chemiluminescent emission by 18-fold. Furthermore, probe **4**, which was synthesized by linking the dipeptide recognition motif to a triply charged tumor-targeting peptide sequence (CGKRK), showed the best performance due to better solubility and cellular uptake, with an additional 4.5-fold improvement in chemiluminescent emission versus probe **2**. These probes have a peak chemiluminescence emission within the first two hours of incubation with the enzyme, and a tailing glow that persists for up to eight hours. An LV200 Olympus microscope was used to acquire chemiluminescence microscopy images of probe **4** in mammalian cells, where a chemiluminescent signal was observed in RAW 264.7 and CT26 cells that express cathepsin B, but not in 3T3 fibroblast cells, which do not. Finally, the authors directly compared their chemiluminescent probe to a known fluorogenic substrate (Z-Val-Cit-PABA-7HC) and show a 16,000-fold improvement in sensitivity.

This highlights the key advance reported in this paper and showcases an important advantage of chemiluminescence over fluorescence: a reduction in background signal caused by autofluorescence and light scattering that leads to drastic improvements in the signal-to-noise ratio. A similar phenomenon can readily be observed with the naked eye by anyone who has ever stared up at the night sky. In a bustling city, the moon and a few bright stars may be visible, but light pollution from cars, buildings and street lamps obscures the finer details. This light pollution is caused by scattering of the light from the city off of the atmosphere, which generates a high background and drowns out much of the information coming at us from the deepest reaches of the cosmos. Far from the city, by contrast, the light of the cosmos paints a more vivid picture, with uncountable stars adorning the night sky and the disk of our home galaxy stretched overhead. Reducing the background caused by the city lights allows our eyes to see a truer and more detailed picture of the universe. This same idea holds true for optical imaging of biological systems, and chemiluminescence provides great opportunities for clarity since the background "light pollution" is largely eliminated. Each chemiluminescent molecule is its own source of light, and shows good contrast against a dark background, just like the brilliant picture of a clear night sky.

While this dramatic increase in in vitro sensitivity versus a fluorogenic cathepsin B substrate is certainly a significant advance, questions remain and more work is needed to validate the reported probe **4** as a reliable tool for measuring protease activity in living cells. How selective is probe **4** for cathepsin B? Is it possible that the signal is due solely to a difference in cellular uptake between cancerous and non-cancerous cells? Could there be other factors that cause the chemiluminescence signal in cells? How robust and reproducible are these results? Can cathepsin B activity be absolutely or relatively quantified? In order to fully address these questions and establish probe **4** as a useful tool, future experiments with a higher number of biological replicates, quantification, and careful controls need to be performed. These experiments will help to fully validate the significance of the results, which are critical for the wide-spread adoption of any analytical method.

Nevertheless, the chemical advances reported here[10] and in previous work[4,5,9] represent exciting developments in the field and will surely enable productive future research. Bright chemiluminescent scaffolds with emission wavelengths that span the visible spectrum could enable multiplexed chemiluminescence imaging of complex systems, and the number of fully validated chemiluminescent probes to detect biologically relevant species will surely expand. As more laboratories begin to use these probes to address biological questions, a greater understanding of the factors needed to provide robust and reliable data will emerge to help scientists navigate this new frontier of chemiluminescence imaging.

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## Conflict of interest

A.R.L. declares a financial stake in Biolum Sciences, LLC, a company aiming to develop a device for home monitoring of asthma.

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# Highlights

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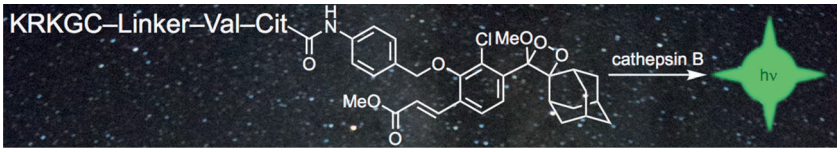


**Let there be light**: Chemiluminescence provides a bright detection signal against a dark background and offers an excellent signal-to-noise ratio for analysis. Now, a chemiluminescent probe for cathepsin B has been developed that provides a 16,000-fold improvement in sensitivity for detecting protease activity.