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In Vivo Bioluminescence Imaging of Cobalt Accumulation in a Mouse Model

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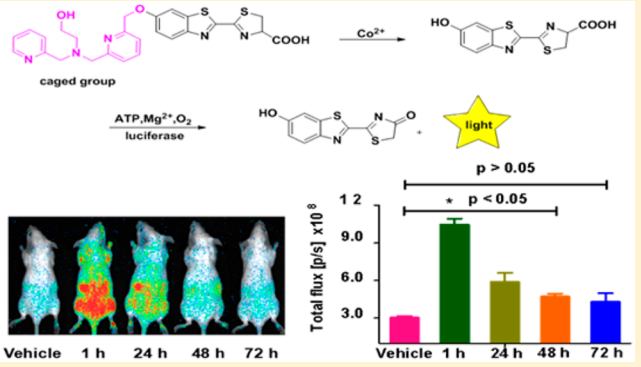
Supporting Information

ABSTRACT: As a trace element nutrient, cobalt is critical for both prokaryotes and eukaryotes. In the current study, a turn-on Cobalt Bioluminescent Probe 1 (**CBP-1**) for the detection of cobalt has been successfully developed based on oxidative C−O bond cleavage. This probe exhibited high selectivity and sensitivity toward cobalt over other analytes. By using **CBP-1**, the successful *in vivo* imaging of cobalt accumulation was carried out in a mouse model. Such an ability to determine cobalt in living animals provides a powerful technology for studying the system distribution, toxic potency, and biological effect of Co^{2+}.



As an essential trace element, cobalt is well known for its critical roles in biological processes not only in prokaryotes but also in eukaryotes.[1,2] Basically, cobalt is essential to the metabolism of all animals as a key constituent and the active center of cobalamins, several chemical forms of vitamin B_{12}, which performs various physiological functions in the normal functioning of nervous system, the formation of red blood cells, DNA synthesis, and amino acid metabolism as well.[3−5] It has been demonstrated that the lack of cobalt results in cardiovascular, anemia, osteomyelitis, glaucoma and other diseases. The benefit or detriment of cobalt depends on its level or consumption. This excessive redox-active metal ions can catalyze the production of reactive oxygen species (ROS) and compete with other essential metal ions to inhibit the biological function of enzymes, which result in the high toxicity of cobalt ions toward living cells.[6] As such, long-term environmental exposure to cobalt or acute intake of cobalt may lead to a variety of diseases, including contact dermatitis, pneumonia, allergic asthma, and lung cancer.[7] Briefly, this element is closely associated with human health.

Since cobalt contributes to physiological and pathological processes, the underlying mechanism of the correlation between its biological occurrence/trafficking and healthy and disease status is still unclear. Therefore, the detection of cobalt in the environmental system and living organs has drawn intensive attention. In this content, a number of techniques are known to be widely applied in cobalt analysis, such as HPLC, flame atomic absorption spectroscopy (FAAS), spectrophotography, and adsorptive voltammetric and inductively coupled plasma atomic emission spectrometry (ICP - AES).[8−12] However, the bulk traditional analysis methods typically require sophisticated sample preparation procedures, highly trained individuals, and costly instruments. More importantly, none of them are compatible with living organisms. Among these collections, the optical probes, due to good sensitivity and bioavailability, construct a great figure as a valuable chemical tool to effectively monitor and track analytes of interest in the biological specimens/living organisms and therefore are extremely beneficial for the broader scientific community.[13] To date, some fluorescent and colorimetric probes have been reported for the detection of cobalt,[14−21] and none of them has been applied to *in vivo* imaging. Therefore, there is an urgent demand on developing novel strategies for monitoring cobalt flux, in particular, in the whole animal.

To meet the stringent criteria of *in vivo* imaging of cobalt in the whole animal, we sought to utilize bioluminescence imaging (BLI) as a noninvasive strategy to establish detection platform. Different from fluorescence, bioluminescence generates a photon signal catalyzed by an enzyme in the absence of external light as a natural phenomenon widely spread among marine and terrestrial species.[22,23] As a result, it obviates the intrinsic limitations of fluorescence and affords high sensitivity and low background which render it suitable for imaging in

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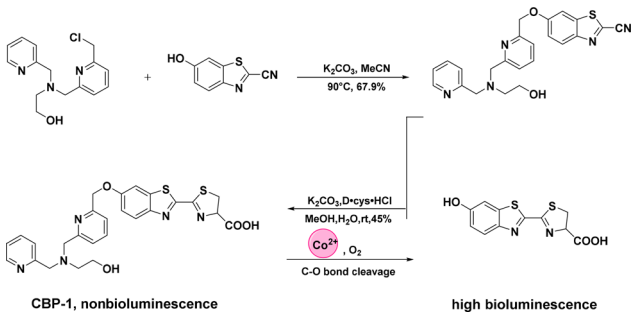
deep tissue and long-term studies of living animal. In the past decade, diverse bioluminescent probes have been developed based on the luciferin/luciferase system.[24−28] Using a caging strategy, appropriate protective groups are employed to cage the reactivity of the substrate toward the enzyme and to block the luminescence production. Analytes can selectively uncage bioluminescent probes and release luciferin substrates. Subsequent oxidation of luciferin can transfer the chemical energy of ATP to a light signal for analyte detection. It should be noted that recently, along this line, several bioluminescent probes were developed for detecting accumulation of metal cations *in vivo*, including Cu^{2+} and Fe^{2+}.[29,30] Herein, we described the development of the first bioluminescent cobalt probe, as well as its initial applications in *in vivo* imaging of cobalt accumulation in a mouse model.

Our design strategy in establishing such a reaction-based bioluminescent probe is to introduce a cobalt reporter into a 6′-hydroxyl group of luciferin. The modification of luciferin at this position normally affords the blockade of identifiability of the substrate toward luciferase. The tetradentate ligand N_3O was chosen as an appropriate trigger for cobalt since it has been demonstrated to be feasible for turn-on detection of Co^{2+}.[31,32] To validate this proof of concept, we designed and synthesized a cobalt bioluminescent probe (**CBP-1**), a new turn-on chemosensor for selective detection of Co^{2+} (Scheme 1). We

Scheme 1. Design and Synthesis of Cobalt Bioluminescent Probe 1 (CBP-1), a Selective Co^{2+}-Mediated Release of the D-Luciferin Substrate

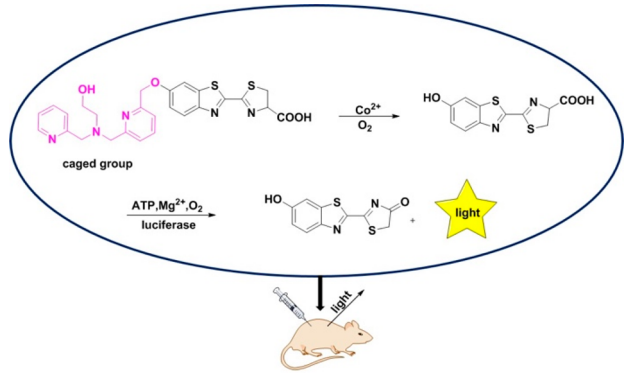


envisioned that, taking advantage of the cobalt-mediated C−O bond cleavage, the bioluminescent probe can release the luciferin substrate in the presence of Co^{2+}, thus generating a bioluminescent readout upon reacting with luciferase (Scheme 2).

By utilizing the N_3O ligand with a C−O benzyl ether bond, we successfully prepared **CBP-1** based on D-luciferin via three straightforward steps (Scheme 1). More synthetic details can be found in the Supporting Information (Scheme S1).

Initial experiments evaluated the efficiency of **CBP-1** to detect Co^{2+} in an aqueous buffer system (Tris buffer at pH 7.4, with 10 mM $MgCl_2$). Consistent with our expectation, **CBP-1** was no longer a light-emitting substrate for firefly luciferase, so that produced a weak signal in the absence of analyte. After pretreatment with 30 μM $CoCl_2$ at 37 °C for 60 min followed by addition of luciferase, **CBP-1** (15 μM) displayed a 40-fold bioluminescence emission (Figure S1). We subsequently evaluated the capability of this probe to detect Co^{2+} in a concentration-dependent manner. Such a probe (15 μM) was incubated with various concentrations of Co^{2+} in a Tris-HCl buffer at 37 °C for 60 min in a 96-well plate. After adding luciferase into the plate, clear bioluminescent signals were

Scheme 2. Proposed Mechanism of CBP-1 for Detection of Co^{2+}



observed and recorded. Bioluminescence signals generated from such a probe consistently increased with gradual concentrations of $CoCl_2$ before Co^{2+} concentration reached 30 μM. Then, the bioluminescence intensity began to decline. All these results indicated that **CBP-1** possesses the considerable ability for detecting Co^{2+} at diverse concentrations in the aqueous environment.

Having determined that **CBP-1** has the excellent ability to react with Co^{2+}, we turn our attention to examine the selectivity of the probe toward Co^{2+} over other cations. A solution of **CBP-1** (15 μM) was treated with a variety of relevant metal ions (30 μM), including Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Cu^+, Cu^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, Cr^{3+}, Ag^+, Pd^{2+}, Cd^{2+}, and Vitamin B12 (Vit. B12), at 37 °C for 60 min, respectively (Figure 1). In the presence of ATP and luciferase, there was

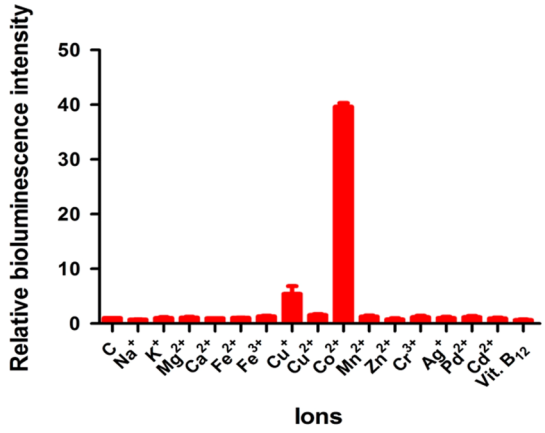


Figure 1. Relative bioluminescence responses of 15 μM **CBP-1** toward 30 μM different cations (Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Cu^+, Cu^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, Cr^{3+}, Ag^+, Pd^{2+}, Cd^{2+}, and Vit. B12). All assays were performed in triplicate and represented as the mean ± SEM.

little to no bioluminescence response to other cations except Cu^+ and Co^{2+}. Furthermore, the intensity of the bioluminescence signal generated from the reaction of the probe with Co^{2+} is 40-fold higher than with other ions. The concern was also addressed as to whether copper levels would interfere with the probe in a biologically relevant setting. **CBP-1** with Co^{2+} treatment exhibited 8-fold higher bioluminescent signal intensity than that with Cu^+ ($1.42\ e^{10}$ vs $1.86\ e^9$), even at low concentration (30 μM). This evidence suggested the high selectivity of **CBP-1** toward Co^{2+} under the same condition,

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which confirms this probe as a promising chemical toolkit for the detection of cobalt in the aqueous environment.

With these impressive data in hand, we next sought to apply **CBP-1** to the detection of Co^{2+} in living cells by taking advantage of its high selectivity and fast response ability. ES-2-Luc cells (luciferase transfected cell line) were employed in the cell-based bioluminescence imaging. We first evaluated the cytotoxicity of the probe and Co^{2+} on ES-2-Luc cell by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure S2). **CBP-1** exhibited low toxicity (IC_{50} > 1000 μM) to cell lines in the experiment, which suggested this probe possesses high biocompatibility. The effect of Co (II) on living cells has also been investigated. Loss of cell viability was observed when ES-2-Luc cells were exposed to increasing Co (II) concentrations for 24 h. IC_{50} value of cobalt was 731 μM in the MTT assay. To examine whether **CBP-1** was interacting with dynamic Co^{2+} in intracellular space, ES-2-Luc cells were pretreated with different concentrations of $CoCl_2$ solution ranging from 0 to 750 μM at 37 °C for 30 min. After the removal of the medium, cells were washed with phosphate buffered saline and then incubated with 50 μM probe for an additional 5 min. As shown in (Figure 2), a

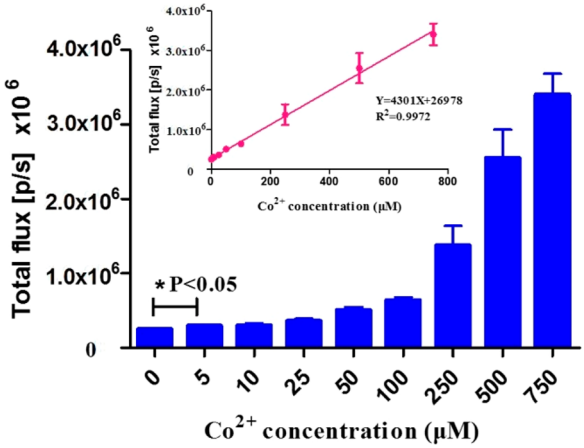

Figure 2. Bioluminescence intensities of ES-2-Luc cells incubated with **CBP-1** with various concentrations of a $CoCl_2$ solution and the linearity between bioluminescence intensity and Co^{2+} concentrations. All assays were performed in triplicate and represented as the mean ± SEM.

gradual increasing bioluminescence signal generated from living cells was recorded upon addition of **CBP-1**. Moreover, there is a good linearity (R^2 = 0.997) between the bioluminescence intensities and Co^{2+} levels. These data demonstrated that **CBP-1** is capable of detecting fluctuation in Co^{2+} levels in living cells.

After confirming that **CBP-1** has distinct advantages in aqueous solution and in cell culture, we continued to examine whether the performance of this probe was translated to the whole animal and whether its favorable properties could be kept in an *in vivo* study. Evaluation of **CBP-1** for *in vivo* imaging was performed with the transgenic FVB-luc+ mice, ubiquitously expressing firefly luciferase. A D-luciferin control study was performed for *in vivo* assays as it is not clear what effects cobalt has on overall enzyme activity and viability. In control studies, we mimic the conditions for the experimental studies to test whether cobalt at different levels would affect the bioluminescence system in FVB-luc+ mice. *In vivo* imaging results showed that even high levels (200 μL, 10 mM) of Co^{2+} did not interfere the luminescent signals from animals in this experiment (Figure S3), which indicated that Co^{2+} did not disturb the expression of luciferase and the level of cofactors (ATP, O_2, and Mg^{2+}) under the experimental condition. To account for the ability of the probe to detect Co^{2+}, normal saline (200 μL) and a sublethal dose of $CoCl_2$ (200 μL, 10 mM) was injected into FVB-luc+ mice from a tail vein. At 60 min after intravenous (i.v.) injection of Co^{2+}, 200 μL **CBP-1** (1 mM) was injected into the intraperitoneal (i.p.) cavity in transgenic mice. The bioluminescence signals produced from transgenic FVB-luc+ animal models were monitored in real time using a CCD camera per 3 min. During the extension of the tracking time, a robust increase in bioluminescence signals generated from pretreating FVB-luc+ mice was observed in a time-dependent manner, and the highest bioluminescence intensity was detected (Figure 3A and B). Moreover, a 4-fold

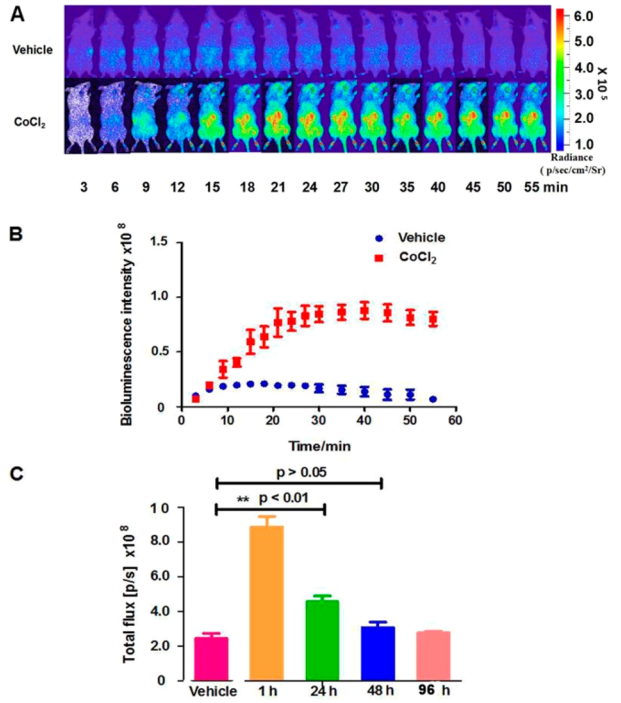

Figure 3. (A) Bioluminescence imaging of FVB-luc+ mice injected i.p. with 200 μL **CBP-1** (1 mM) after i.v. injection of $CoCl_2$ solution (200 μL, 10 mM). (B) Bioluminescent intensities measured at various time points (3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 35, 40, 45, 50, 55 min), in the presence or absence of Co^{2+}. (C) Quantification of the total flux (photon/s) from the whole-body area except the tail in the presence or absence of Co^{2+} (200 μL, 10 mM) at 1, 24, 48, and 96 h, respectively. All assays were performed in triplicate and represented as the mean ± SEM **: $P < 0.01$.

enhancement in bioluminescence intensity was clearly measured compared with the vehicle-treated control animal, which strongly indicated the practicability of **CBP-1** for BLI *in vivo*. Under the experimental conditions, mice still showed a significant bioluminescence signal within 24 h, indicating that there is still a fairly high cobalt ion level. However, compared with the animals in the control group, almost no bioluminescence signal increases were observed at 48 and 96 h, respectively (Figure 3C). It suggested that exogenetic Co^{2+} could be metabolized or excreted within 48 h, and there is no excessive accumulation of Co^{2+} in mice. Notably, **CBP-1** produced a weak but measurable background in the vehicle-

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treated control animal. Luciferase recognition of the caged substrate, the endogenous degredation of the probe, or the response to Cu^{+} and Co^{2+} from the diet may be a reasonable cause.

Generally, Co^{2+} in nature is taken into the organism orally; subsequently, we next turned our attention to performing **CBP-1** to monitor labile cobalt dynamics in living animals with Co^{2+} intragastric (i.g.) administration. FVB-luc^{+} mice were i.g. administrated with several concentrations (0, 10, 25, 50, and 100 mM) of 200 μL $CoCl_2$ solutions for 1 h, before i.p. injection of **CBP-1** (200 μL, 1 mM). The bioluminescent imaging and signal (Figure 4A and B) produced from FVB-luc^{+}

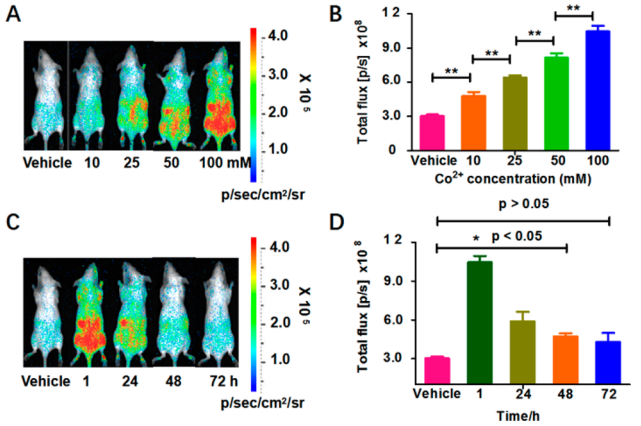

Figure 4. CBP-1 can monitor labile cobalt dynamics in luciferin-expressing mice administrated i.g. with $CoCl_2$. (A) Representative imaging of FVB-luc+ mice injected i.p. with **CBP-1** (200 μL, 1 mM) after i.g. administration of various concentrations (0, 10, 25, 50, and 100 mM) of 200 μL $CoCl_2$ solutions. (B) Photon intensities from above these mice. (C) Bioluminescence imaging and (D) total flux (photon/s) output of FVB-luc+ mice injected i.p with **CBP-1** after i.g. administration of Co^{2+} (200 μL, 100 mM) at 1, 24, 48, and 72 h, respectively. All assays were performed in triplicate and represented as the mean ± SEM **: $P < 0.01$, *: $P < 0.05$.

mice were recorded in real time using a cooled CCD camera. In this experiment, differences in bioluminescent signals produced from these mice were measured within 60 min following injection. The total photon flux integrated over 1 h for each mouse exhibited a significant dose-dependent growth. To evaluate the accumulation of Co^{2+} in the living animal, FVB-luc^{+} mice were i.g. administrated with a sublethal dose of $CoCl_2$ solution (200 μL, 100 mM). Cobalt enrichment in the blood, kidney, and liver of Kun Ming (KM) mice was independently confirmed by GFAAS analysis (Figure S4). Within 24, 48, and 72 h after the administration of Co^{2+}, **CBP-1** (200 μL, 1 mM) was injected into the intraperitoneal cavity of these mice, and subsequently, signals generated from mice were measured. A robust increasing bioluminescence signal was detected in a real-time manner. The total photon flux integrated over 1 h indicated that Co^{2+} can metabolize gradually in living animal, which provided evidence that mice can excrete most of the Co^{2+} within 24 h (Figure 4C and D).

In this study, a new bioluminescent probe was designed and developed for the detection of cobalt. By using this probe, we successfully carried out the first *in vivo* imaging of cobalt. Our work represents the first attempt to monitor fluctuation of cobalt level in real time in an intact, living animal model. Such information may be correlated with other analyses to provide a valuable opportunity for studying system distribution, toxic potency, and biological effect of Co^{2+}, as well as to understand the complicated molecular processes of cobalt in the biological system.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b00391.

Full experimental procedure, NMR, MS, fluorescence, and cell imaging data. (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Author Contributions

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Notes

The authors declare no competing financial interest.

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