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Mix-and-Read Bioluminescent Copper Detection Platform Using a Caged Coelenterazine Analogue

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Serum copper levels are biomarkers for copper-related diseases. Quantification of levels of free copper (not bound to proteins) in serum is important for diagnosing Wilson's disease, in which the free copper concentration is elevated. Bioluminescence is commonly used in point-of-care diagnostics, but these assays require genetically engineered luciferase. Here, we developed a luciferase-independent copper detection platform. A luminogenic caged coelenterazine analogue (**TPA-H1**) was designed and synthesized to detect copper ions in human serum. **TPA-H1** was developed by introducing a tris[(2-pyridyl)-methyl]amine (TPA) ligand, which is a Cu^{+} cleavable caging group, to the carbonyl group at the C-3 position of the imidazopyrazinone scaffold. The luciferin, named HuLumino**1,** is the product of the cleavage reaction of **TPA-H1** with a copper ion and displays "turn-on" bioluminescence signals specifically with human serum albumin, which can be used to quantitatively analyse copper ions. **TPA-H1** exhibited a fast cleavage of the protective group, high specificity, and high sensitivity for copper over other metal ions. This novel caged coelenterazine derivative, **TPA-H1**, can detect free copper ions in serum in a simple "mix-and-read" manner.

Introduction

Copper, an essential trace element in the human body, is involved in several biophysical processes, including acting as a cofactor for many enzymes. It plays an important role in biochemical processes, such as erythropoiesis, cellular respiration, and hormone biosynthesis, and is required for proper development and function of the central nervous system.[1, 2] As copper homeostasis is critical to human health, its imbalance contributes directly or indirectly to the onset of diseases. In humans, copper deficiency can lead to disorders such as Menkes disease and occipital horn syndrome,[2] whereas excessive amounts of copper cause high toxicity because of its oxidative potential and are correlated with diseases such as Alzheimer's, Parkinson's, and Wilson's diseases.[2] The use of copper chelators, such as D-penicillamine and tetrathiomolybdate, has been recognized as the most promising approach for maintaining copper homeostasis.[3, 4] However, excessive addition of these copper chelators may induce adverse health effects that interfere with the native physiological functions of copper.[1]

Serum copper levels are biomarkers for copper-related diseases. Particularly, it has been reported that in Wilson's disease, the concentration of free copper (copper not bound to proteins) in serum is more than six-fold higher than that in healthy adults (normal level is 0–1.6 μM).[5] Wilson's disease is caused by the accumulation of excess copper in tissues due to a defect in the gene *ATP7B*, which encodes the major transporter responsible for copper excretion. This disease affects both liver and neurological functions, but is treatable. Leaving it untreated, however, can be fatal; therefore, early diagnosis and analytical techniques to monitor serum copper levels before and after treatment are critical.[5] Currently, flame atomic absorption spectroscopy, inductively coupled plasma mass spectroscopy (ICP-MS), and plasma atomic emission spectroscopy are commonly used for sensitive analysis of copper in human serum.[6-8] However, these techniques require costly equipment and highly skilled, well-trained personnel and are time-consuming, limiting their wide application. Several alternative methods have recently been reported, including colorimetric assays based on localised surface plasmon resonance technology and fluorescent assays based on materials such as AuAg nanoclusters or hairpin DNA, which achieve sensitive nano to micromolar detection of copper in human serum but require sample pre-treatment and hours-long incubation with copper.[9-11]
Bioluminescence (BL) is a standard analytical tool commonly used to detect molecular events in complex biological systems, with a wide range of applications, from molecular imaging to point-of-care diagnostics.[12-14] The generation of BL is mainly due to enzymatic reactions involving bioluminescent substrates

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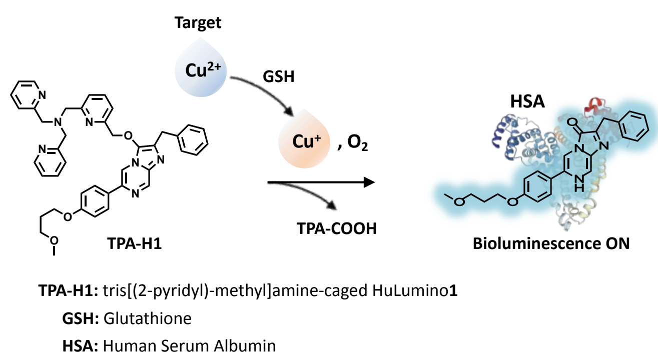
† Electronic Supplementary Information (ESI) available: Detailed synthetic procedures of novel compounds, and additional information on luminescent properties are available as supporting information. See DOI: 10.1039/x0xx00000x



TPA-H1: tris[(2-pyridyl)-methyl]amine-caged HuLumino**1**
GSH: Glutathione
HSA: Human Serum Albumin

Scheme 1 Schematic illustration of copper assay based on a probe comprising a luminophore with a specific reaction for HSA

(luciferin) and enzymes (luciferase), which can detect biological analytes specifically, rapidly, and non-invasively without the use of external light sources.[15, 16] In particular, coelenterazine, an imidazopyrazinone-type luciferin produced by marine organisms, coupled with marine luciferases is often utilized as a versatile optical reporter because the simple luminescence reaction requires only an oxygen molecule.[17, 18] However, luciferase-dependent BL assays generally entail complex bioanalytical protocols, requiring the addition of genetically engineered luciferases to biological samples. As an alternative BL assay, we recently designed a "luciferase-independent" BL assay for albumin in human serum by developing a novel coelenterazine analogue, named HuLumino**1**. HuLumino**1** emits light in the presence of human serum albumin (HSA), but not with luciferases from luminous organisms or any other human proteins. The high specificity is due to the mechanism by which HuLumino**1** recognizes and emits light from the drug-binding site 2 of HSA, allowing simple quantitative analysis of albumin in human serum without any sample pre-treatments.[19] In addition, this simple "mix-and-read" assay enables testing within 1 min, greatly reducing the assay time compared to that for conventional enzyme-linked immunosorbent assays (assay time is approximately 3 h).

The caging of luciferin is an attractive approach for use *in vitro* and *in vivo*, as it takes advantage of its low background signal (auto-luminescence) and light emission control.[17, 20, 21] Numerous caged-luciferins, which are enzyme-inactive luciferin analogues that have been chemically uncaged to yield bare luciferin in the presence of an analyte biomolecule or molecular event of interest for following enzymatic oxidation of luciferin to emit light have been reported.[22, 23] We also monitored biothiols, both *in vitro* and *in vivo*, by combining a caged coelenterazine, named AMCM, with a mutant of *Renilla* luciferase that is genetically expressed in living cells.[24] In this study, to achieve a simple and accurate "mix-and-read" BL analysis of copper in serum, we developed a copper-mediated uncaged luciferin that emits light in the presence of excessive HSA in human serum (Scheme 1), inspired by a reported fluorescence-based assay for butyrylcholinesterase based on the dansyl analogue that specifically binds to HSA.[25]

Here, we designed a novel sensing platform with a turn-on-type BL probe for "mix-and-read" detection of copper in human serum based on the HuLumino**1**/HSA luminescent pair. HuLumino**1**, whose BL signal is significantly elevated upon adding HSA, allows HSA to be directly used as a part of the signal read for detecting a biomarker in human serum (Scheme 1). Caging of HuLumino**1** inhibits enzymatic oxidation from HSA and reduces its BL signal. Caged HuLumino**1** can be irreversibly uncaged by a selective reaction with a given biomarker, after which it can be oxidized by HSA, resulting in enhancement of the BL signal. The type of protective ligand used depends on the biomarker of interest.[26] In this study, we designed and synthesised a novel caged luciferin, named **TPA-H1**, which contains a tris[(2-pyridyl)-methyl] amine (TPA) ligand conjugated to the carbonyl group at the C-3 position of the imidazopyrazinone scaffold via a benzyl ether linkage. Upon oxidative cleavage by Cu^{+}, HuLumino**1** is formed, generating a BL signal with HSA (Scheme 1). We demonstrate a novel sensing strategy for the simple and rapid semi-quantitative detection of free (unbound) copper both in buffer aqueous solution and in human serum without incubation and modification processes.

Experimental

Materials

All reagents and solvents were purchased from commercial suppliers (Tokyo Kasei, Sigma Aldrich, FUJIFILM Wako Pure Chemical) and used without further purification. All organic synthesis reactions were carried out in an argon atmosphere. The composition of mixed solvents is given as the volume ratio (v/v). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on an Avance III-500 (Bruker, Ltd., Billerica, MA, USA) spectrometer at room temperature (RT). ^{1}H-NMR and ^{13}C-NMR measurements were performed at 500 and 125 MHz, respectively. All chemical shifts are relative to an internal standard of tetramethylsilane (δ = 0.0 ppm) or solvent residual peaks ($CDCl_3$: δ = 7.26 ppm for ^{1}H; $CDCl_3$: δ = 77.16 ppm for ^{13}C), and coupling constants are given in Hz. High-resolution mass spectra of compounds dissolved in chloroform were recorded on a Thermo Fisher Scientific Exactive spectrometer (Waltham, MA, USA), with methanol as the eluent.

Equipment for BL measurements

BL intensities were measured on a GloMax Explorer (Promega, Madison, WI, USA), with a measurement time of 1 min. BL spectra were recorded using a Cytation5 Imaging Reader (BioTek Instruments, Winooski, VT, USA), with a step size of 5 nm from 390 to 550 nm and an integration time of 30 s. All data were analysed with the GraphPad Prism 9.0 software (GraphPad, Inc., La Jolla, CA, USA).

BL assay for copper detection

Fatty acid-free HSA (20 μM) (A3782, Sigma-Aldrich, St. Louis, MO, USA) was added to the buffer solution (10 mM HEPES at pH7.4) containing copper ($Cu(MeCN)_4(PF_6)$ or $CuCl_2$ (100 μM) in the presence or absence of glutathione (GSH) (2 mM). BL signals were rapidly recorded after mixing with buffer solution

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containing **TPA-H1** (20 μM) in a well of a white 96-well plate (Thermo Fisher Scientific). For the luminescence characterisation of **TPA-H1**, fatty-acid-free HSA was used for signal reading unless otherwise stated. This is because the binding of fatty acids to drug-binding site 2 and other binding sites of HSA may block the luminescence reaction site of HuLumino**1** or induce protein conformational changes,[27] resulting in a decrease in the luminescence signal of the probe.

Metal ion selectivity

The BL response of **TPA-H1** (20 μM) to various metal ions $CuCl_2$, $Zn(NO_3)_2$, $Ni(NO_3)_2$, $Co(NO_3)_2$, $Al(NO_3)_2$, $Ca(NO_3)_2$, $Mg(NO_3)_2$, $Mn(NO_3)_2$, $NaNO_3$, KNO_3, $Fe(NO_3)_3$) (100 μM) was measured in 10 mM HEPES at pH 7.4, with fatty acid-free HSA (20 μM) and GSH (2 mM).

Free copper sensing in aqueous buffer and human serum

For assays in aqueous buffer solution, various concentrations of $CuCl_2$ (0.25–50 μM) were added to the buffer (10 mM HEPES at pH 7.4) containing GSH (2 mM) and non-defatted HSA (20 μM) (Sigma-Aldrich, A9511). The BL signals were rapidly recorded after mixing with buffer solution containing **TPA-H1** (20 μM) in a well of a white 96-well plate (Thermo Fisher Scientific). Most Cu^{2+} in the serum is bound to proteins, including ceruloplasmin. To remove copper-binding proteins, human serum (Sigma-Aldrich, H4522) was subjected to ultrafiltration using a 30-kDa Millipore centrifugal filter and centrifuged for 30 min at 4000 ×*g,* as described previously.[5] The ultrafiltered serum was spiked with an appropriate concentration of $CuCl_2$ (0.25-5 μM) in the buffer (10 mM HEPES at pH7.4) containing GSH (2 mM) and non-defatted HSA (20 μM). The serum solution and **TPA-H1** (20 μM) were mixed in the wells of a white 96-well plate, after which the BL signal was immediately recorded. The commercial copper assay based on absorption (Metallogenics, Chiba, Japan, copper assay kit, CU04M) was conducted using the ultrafiltered serum spiked with copper according to the manufacturer's protocol.

Results and discussion

We designed and synthesised TPA ligand-caging HuLumino**1** (**TPA-H1**), which utilises HSA, the most abundant protein in human serum, for signal output rather than using a luciferase sourced from luminous organisms. First, the ability of **TPA-H1** to detect copper in aqueous buffer (10 mM HEPES at pH7.4) in the presence of HSA was investigated (Figs. 1 and S1). Additionally, as most cellular copper in the human body is in the form of Cu^{2+},[28] in the proposed BL assay, GSH was used to reduce all copper to Cu^+ according to copper assays based on TPA-caging probes[22, 29] (Scheme 1). As a result, **TPA-H1** did not significantly change its BL intensity in the presence of HSA, copper ions, or GSH alone, whereas the BL signal of **TPA-H1** tremendously increased in the presence of HSA, copper ions, and GSH (Fig. 1). This indicates that **TPA-H1** reacts highly selectively with Cu^+ in aqueous solution and exhibits light emission in combination with HSA. In the presence of another common reducing agent, ascorbic acid, we found that **TPA-H1** displayed an autoluminescent signal with copper in the absence of HSA (Fig. S1) but not in the presence of GSH (Fig. 1a). Ascorbic acid acts as a prooxidant in the presence of heavy metals, particularly copper, resulting in the generation of reactive oxygen species,[30] which may induce the degradation of **TPA-H1**.

We then plotted the BL spectra, which showed an emission peak of **TPA-H1** in the presence of HSA, copper, and GSH at around 450 nm, which is similar to that of the HuLumino**1**/HSA pair (Figs. 1b and S2). In addition, the luminescence of **TPA-H1** (20 μM) was rapidly enhanced after mixing with one equivalent of copper (20 μM), and its luminescence intensity reached about 36% of that of the HuLumino**1**/HSA pair (Fig. 2a), indicating the possibility of the quantitative analysis of copper without any pre-incubation with copper. **TPA-H1** pre-incubated with copper for 30 min exhibited a similar total BL signal and signal decay to that of the HuLumino**1**/HSA pair (Fig. 2a and b). Decay of the luminescence signal indicates that flash luminescence occurred, which is commonly observed with imidazopyrazinone-type compounds. These results indicate that **TPA-H1** was uncaged by copper ions to form HuLumino**1**, allowing it to be oxidatively catalysed by HSA. The uncaging reaction of **TPA-H1** was completed in 30 min in the presence of copper.

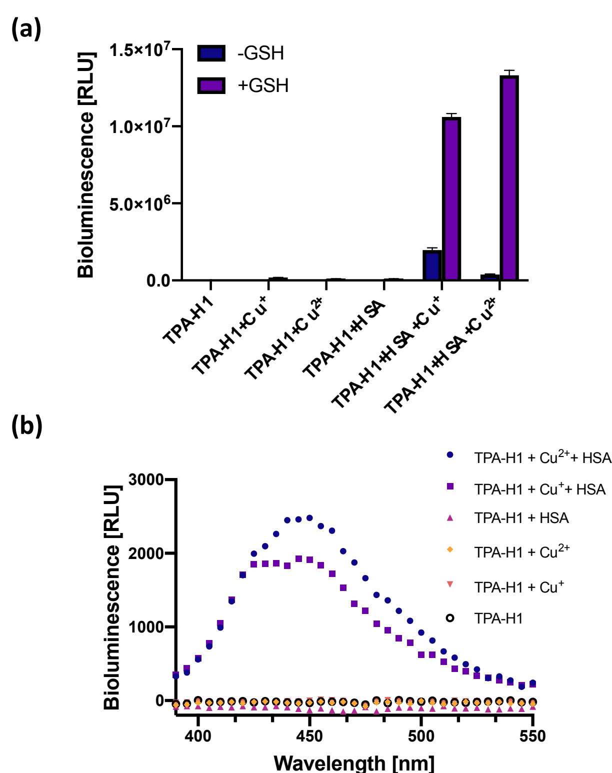

Fig. 1 (a) Response of **TPA-H1** (20 μM) to fatty acid-free HSA (20 μM) and copper ions (100 μM) in the presence or absence of GSH (2 mM). Error bars represent the standard deviation of four measurements. (b) Bioluminescent spectra of **TPA-H1** (20 μM) in the presence or absence of fatty acid-free HSA (20 μM), copper ions (100 μM), and GSH (2 mM).

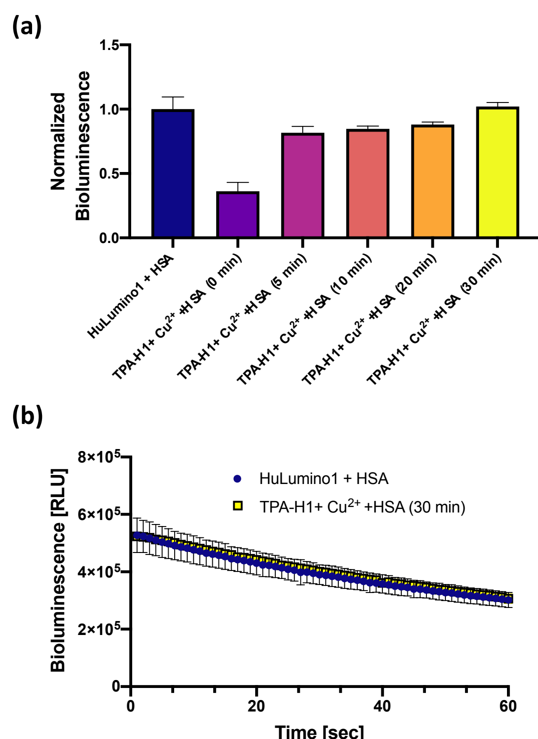

Fig. 2 (a) Normalized BL intensities of **TPA-H1** (20 μM) containing fatty acid-free HSA (20 μM) at various incubation times with $CuCl_2$ (20 μM) in buffer solution (HEPES, 10 mM, pH7.4) including 2 mM GSH. The BL intensity of the HuLumino**1**/HSA pair was normalized to 1.0. (b) BL decay of HuLumino**1**/HSA pair and **TPA-H1** pre-incubated with $CuCl_2$ for 30 min/HSA pair in buffer solution (HEPES, 10 mM, pH7.4) including 2 mM GSH. Error bars represent the standard deviation of four measurements.

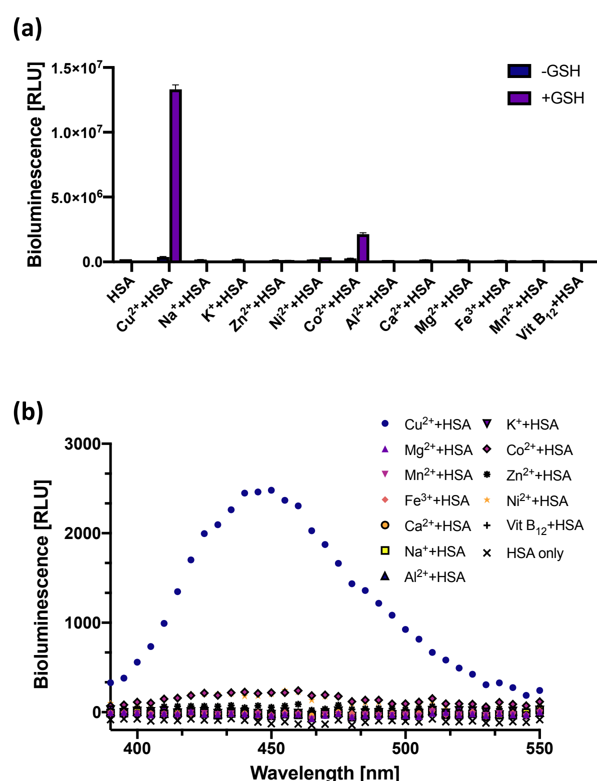

Fig. 3 (a) Variation of the luminescence of **TPA-H1** (20 μM) in the presence of metal ions (counteranion: Cl^- or NO_3^-) (100 μM) and fatty acid-free HSA (20 μM). Error bars represent the standard deviation of four measurements. (b) BL spectra of **TPA-H1** (20 μM) in the presence of metal ions and HSA.

Furthermore, the BL intensity of **TPA-H1** (20 μM) in the presence of copper (100 μM) increased with increasing HSA or GSH concentrations, and reached saturation at 20 μM and 1 mM, respectively (Figs. S3 and S4). In contrast, in the absence of copper ions, **TPA-H1** exhibited no auto-luminescence signal, even in the presence of excess HSA or GSH, and was stable in aqueous solution (Figs. 1a and S3).

Hence, the experiments were conducted under the following optimised conditions: pH 7.4 buffer solution (HEPES, 10 mM), 2 mM GSH, 20 μM HSA, and 20 μM **TPA-H1**. To demonstrate the selectivity of the **TPA-H1**-based copper assay, the response of **TPA-H1** to other common metal ions (Na^+, K^+, Zn^{2+}, Ni^{2+}, Co^{2+}, Al^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, and Mn^{2+}) was investigated. As shown in Fig. 3, addition of these metal ions (100 μM) to the **TPA-H1** solution caused no significant enhancement in BL intensity compared to that in the control in the presence of Cu^{2+} because these metal ions could not trigger the uncaging of **TPA-H1**. However, free Co^{2+} slightly de-graduated **TPA-H1** and induced non-specific luminescence signals, which has also been observed in other TPA-based luminogenic caged compounds.[22, 29] As approximately 96% of Co^{2+} in serum is loosely bound to proteins,[22, 31] we examined the effect of vitamin B_{12}, a metal complex of ring-contracted modified tetrapyrrole containing Co^{2+}, using our sensing platform. This measurement allowed us to identify the state of the metal (free or bound to a ligand) with which **TPA-H1** can react. [22] As expected, **TPA-H1** exhibited no response to vitamin B_{12} (100 μM) (Fig. 3a and b), indicating that **TPA-H1** can be used to quantitatively analyse free metals that are not bound to ligands and proteins.

Next, to investigate the sensitivity of **TPA-H1** for Cu^{2+} detection in aqueous solution, the luminescence intensities of **TPA-H1** in the presence of non-defatted HSA and various concentrations of copper were examined. The BL response of **TPA-H1** was enhanced in a copper concentration-dependent manner and exhibited a constant response at copper concentrations above 10 μM (Fig. 4). The BL response increased with the concentration of copper from 0.25 to 5 μM, showing a linear relationship (R^2 = 0.996) (Fig. 4), and the detection limit was calculated to be 0.11 μM based on the 3σ rule.

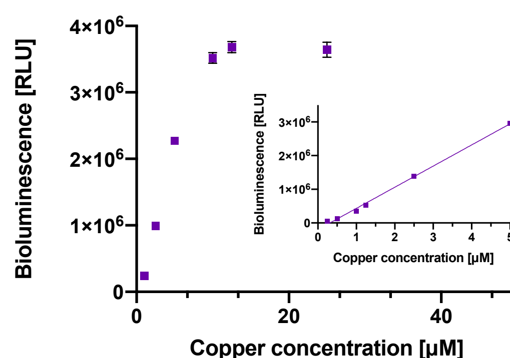

Fig. 4 BL response of **TPA-H1** (20 μM) to buffer solution (HEPES, 10 mM, pH 7.4) containing various concentrations of Cu^{2+} (0.25–50 μM) in the presence of HSA (20 μM) and GSH (2 mM). The inset indicates the linear relationship between the BL response of **TPA-H1** and the concentration of Cu^{2+} (0.25–5 μM) in the presence of HSA (20 μM) and GSH (2 mM), Y = 627983X – 195478, R^2 = 0.996. Each data point represents at least four independent measurements.

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Unbound copper levels in human serum range from 0 to 1.6 μM.[5] As **TPA-H1** can monitor the changes in copper concentrations near the free copper threshold (1.6 μM),[4] it shows potential for use as a diagnostic tool for copper-related diseases such as Wilson's disease, in which free copper levels are approximately 6-fold higher than normal. Finally, to investigate the capacity of **TPA-H1** to detect free copper levels present in the human serum, we compared the sensitivity of **TPA-H1** for Cu^{2+} in human serum using a commercial kit (detection range: 0.47–62.8 μM), an absorption-based method that correlates well with the ICP method[32] (Table 1). In human serum, approximately 90% of Cu^{2+} is present in ceruloplasmin; as such, accurate levels of Cu^{2+} in human serum cannot be directly measured.[33] In addition, as shown by the results of the luminescence activity test using vitamin B_{12}, **TPA-H1** cannot use metal ions bound to a ligand or protein for the de-caging reaction (Fig. 3). Then, we tested serum in which proteins that may bind copper, such as ceruloplasmin and albumin, were removed by ultrafiltration using a molecular weight cut-off value of 30 kDa. The ultrafiltrate should contain only "free" copper,[5] but its concentration was too low to be measured with commercial kits (data not shown). Therefore, we confirmed the usability of the luminescence method by using ultrafiltrate spiked with copper (0.5–*5 μM*). In the BL method, copper and HSA (final concentration: 20 μM) were added to ultrafiltered human serum and mixed with an aqueous solution of **TPA-H1** to confirm the BL reaction, which is the same procedure used to evaluate samples in buffer solution (Fig. S5). As shown in Fig. S5, the response of **TPA-H1** to copper in human serum increased linearly in the concentration range of 0.25–5 μM, similar to the results obtained with buffer solution (R^2 = 0.994). Furthermore, analytical recoveries from human serum samples ranged from 94.3% to 96.4% in the range of abnormally low to high levels (0.5–5 μM) based on the results obtained using commercial kits (Tables 1 and S1). HSA, which binds free copper ions[34], was found to have no effect on this assay range. Moreover, the addition of physiological levels of ascorbic acid had little effect on the response of **TPA-H1** to free copper in human serum (Fig. S6). These results indicate that **TPA-H1** can be used for quantitative analysis of copper in a mix-and-read fashion without the need for sample pre-treatment and long-incubation.

Table 1 Assay of copper in human serum

$CuCl_2$ added (μM)	Detected by developed method[a]	Detected by copper assay kit from Metallogenics Co., Ltd.	Recovery
0.5	0.549 ± 0.001	0.582 ± 0.007	94.3
2.5	2.498 ± 0.053	2.607 ± 0.417	95.8
5	4.829 ± 0.052	5.007 ± 0.625	96.4

[a]Copper concentration spiked into human serum was determined based on the calibration curve obtained in Fig. 4 (Y = 627983X – 195478, R^2 = 0.996.). Each data point shown is the mean of at least three independent measurements.

Conclusions

Caged luciferin (**TPA-H1**) is a precursor of HuLumino**1**, which irreversibly sheds its cage upon selective reaction with copper to form HuLumino**1**, which is oxidized to emit light in the presence of HSA. **TPA-H1** detected copper within 1 min by simple mixing with the sample and showed good linearity in the range of 0.25–5 μM of copper. The selectivity of the assay was excellent, and the results for detecting copper in human serum agreed well with those of absorption-based copper assay kits that correlate well with ICP-based assays. Particularly, the developed luminescence assay did not require any sample pre-treatment or incubation, and the assay time was significantly reduced (from hours to around 1 min) compared with that for the previous copper assay methods using localised surface plasmon resonance and fluorescence. Therefore, this assay is a simple, rapid, and highly sensitive diagnostic method for medical conditions such as Wilson's disease, in which it is important to determine the free copper concentration in the blood. However, the luminescence intensity of HuLumino**1**/HSA, the signal-reading component of this assay, was significantly lower than that of the versatile luciferase system (e.g., luminescence quantum yield is approximately 1/167 that of the NanoLuc/furimazine pair). Therefore, to apply the BL method using caged luciferin/HSA pairs to point-of-care diagnostics using versatile mobile devices as detectors, novel luciferins that emit stronger luminescence signals in combination with HSA must be developed.

Author Contributions

Ryo Nishihara: Conceptualization, Methodology, Investigation, Validation, Resources, Writing – original draft, Supervision. Ryoji Kurita: Resources, Writing – review & editing, Supervision.

Conflicts of interest

There are no conflicts to declare.

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