Chapter

Design of Coelenterazine Analogue to Reveal Bioluminescent Reaction of Human Serum Albumin

Ryo Nishihara, Kazuki Niwa, Tatsunosuke Tomita and Ryoji Kurita

Abstract

This chapter describes the design of an imidazopyrazinone-type luciferin named as HuLumino**1** by us and investigation of its luminescence properties. This luciferin was designed to generate bioluminescence by human serum albumin (HSA) rather than by luciferase derived from luminous organisms. HuLumino**1** was developed by modifying a methoxy-terminated alkyl chain to the C-6 position and eliminating a benzyl group at the C-8 position of coelenterazine. To clarify the basis of light emission by HSA, the detailed kinetic properties of the HuLumino**1**/HSA pair were investigated using a calibrated luminometer. The enzymatic oxidation of HuLumino**1** was observed only in the presence of HSA. Results of HSA quantification experiments using HuLumino**1** agreed with less than 5% differences with those of enzyme-linked immunosorbent assays, suggesting HuLumino**1** could be used for quantitative analysis of HSA levels in serum samples without any pretreatments. These results demonstrate the advantages of the coelenterazine analogue as a bioluminescence reagent to detect non-labeled proteins, which generally do not function as enzymes.

Keywords: Bioluminescence, Coelenterazine, Luciferin, Luciferase, Human serum albumin, Quantum yield, Enzyme-linked immunosorbent assay

1. Introduction

Luminous organisms, such as *fireflies*, *Gaussia princeps*, *Oplophorus gracilirostris*, and *sea pansy Renilla reniformis*, generate bioluminescence (BL), which is the light emission in the absence of external energy sources [1, 2]. In the core of BL, an enzymatic reaction occurs involving a bioluminescent substrate (luciferin) and enzyme (luciferase). In general, the enzymatic luminescence reaction proceeds between a specific luciferin-luciferase pair to allow for highly sensitive and specific detection/imaging of diverse molecular events in living subjects [2–4].

However, in some cases, bioluminescent or chemiluminescent substrates may induce enzymatic luminescence activity of non-bioluminescence enzymes. For example, CycLuc2, a synthetic analog of firefly luciferin, can be catalyzed to emit light by long-chain fatty acid acyl-CoA synthetase found in non-luminous insects

[5–7]. In addition, the heme-containing enzyme myeloperoxidase, which is abundantly expressed in neutrophils and monocytes, can catalyze the luminescence reaction of xenobiotic luminol [8, 9]. These reports suggested that the introduction of appropriate exogenous luminescent substrates reveals luminous activity of non-bioluminescence enzymes, which can significantly differ from the conventional function of the enzyme and has potential for use in quantitative analysis of enzymes without any labeling procedures, including transgene introduction of luciferase from luminous organisms. Here, we describe the design and bioluminescence characterization of a luciferin analogue which was selectively catalyzed to exhibit bioluminescence by human serum albumin (HSA) [10]. Serum albumins perform various physiological functions; they maintain colloid osmotic blood pressure and transport several exogenous and endogenous molecules. However, they are not categorized in the list of EC number, indicating they are not considered typical enzymes. The bioluminescence system of HSA with the luciferin analogue synthesized by us is novel and different from the conventional luciferin-luciferase reaction systems.

2. Design of coelenterazine analogue

2.1 Coelenterazine analogue with HSA-specific bioluminescence

Most luciferases from luminous marine organisms use coelenterazine (CTZ) as their luciferin to form coelenteramide in an excited state, with emission ranging from blue to green at approximately 400–500 nm (**Figure 1**) [11, 12]. CTZ is oxidized by bovine serum albumin (BSA) in addition to luciferase, and this has been considered as nonspecific reaction mainly occurs because of a simple luminescence reaction that requires only an oxygen molecule [1] (**Figure 1**).

The emission ability of CTZ is derived from the imidazopyrazinone ring, and the chemical structure of sidechains at the C-2, C-6, and C-8 position of the imidazopyrazinone core significantly affect enzyme recognition. For example, *Cypridina* luciferase oxidizes only *Cypridina* luciferin, which contains a basic guanidine moiety at the C-8 position of the imidazopyrazinone ring, not CTZ (**Figure 2**) [13]. A mutant *Oplophorus* luciferase (NanoLuc) uses furimazine rather than CTZ, and is known as a versatile reporter of BL (**Figure 2**) [14]. Thus, each bioluminescence probe has been individually developed with an imidazopyrazinone analogue that is suitable for the geometry of the active site in the pocket of mutant luciferase. We also reported that RLuc8.6-535SG, a mutant *R. reniformis* luciferase, utilizes BottleBlue2.3 (BBlue2.3), a CTZA that can permeate the cell membrane and emits bright visible luminescence suitable for deep-tissue imaging of cancer cells in vivo (**Figure 3a**) [15].

To clarify the potential enzymatic luminescence activity of human proteins, we focused on HSA, which accounts for approximately 65% of serum proteins in the human body [16]. This abundant protein is involved in a wide variety of

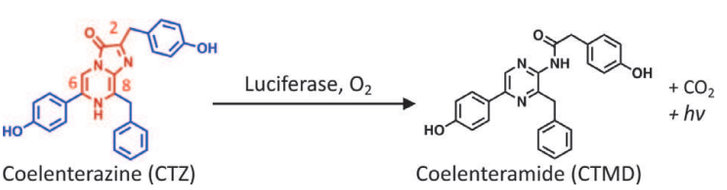

Figure 1.
Chemical reaction of coelenterazine (CTZ)-dependent bioluminescence. The imidazopyrazinone structure and modifiable substituent are highlighted in red and blue, respectively.

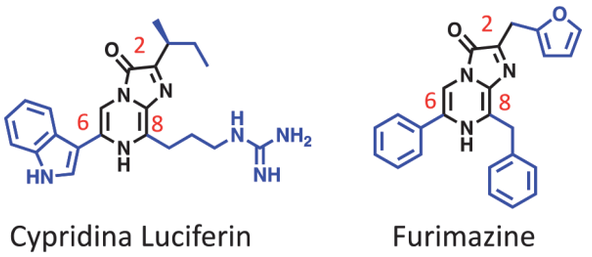

Figure 2.
Chemical structures of imidazopyrazinone-based analogues.

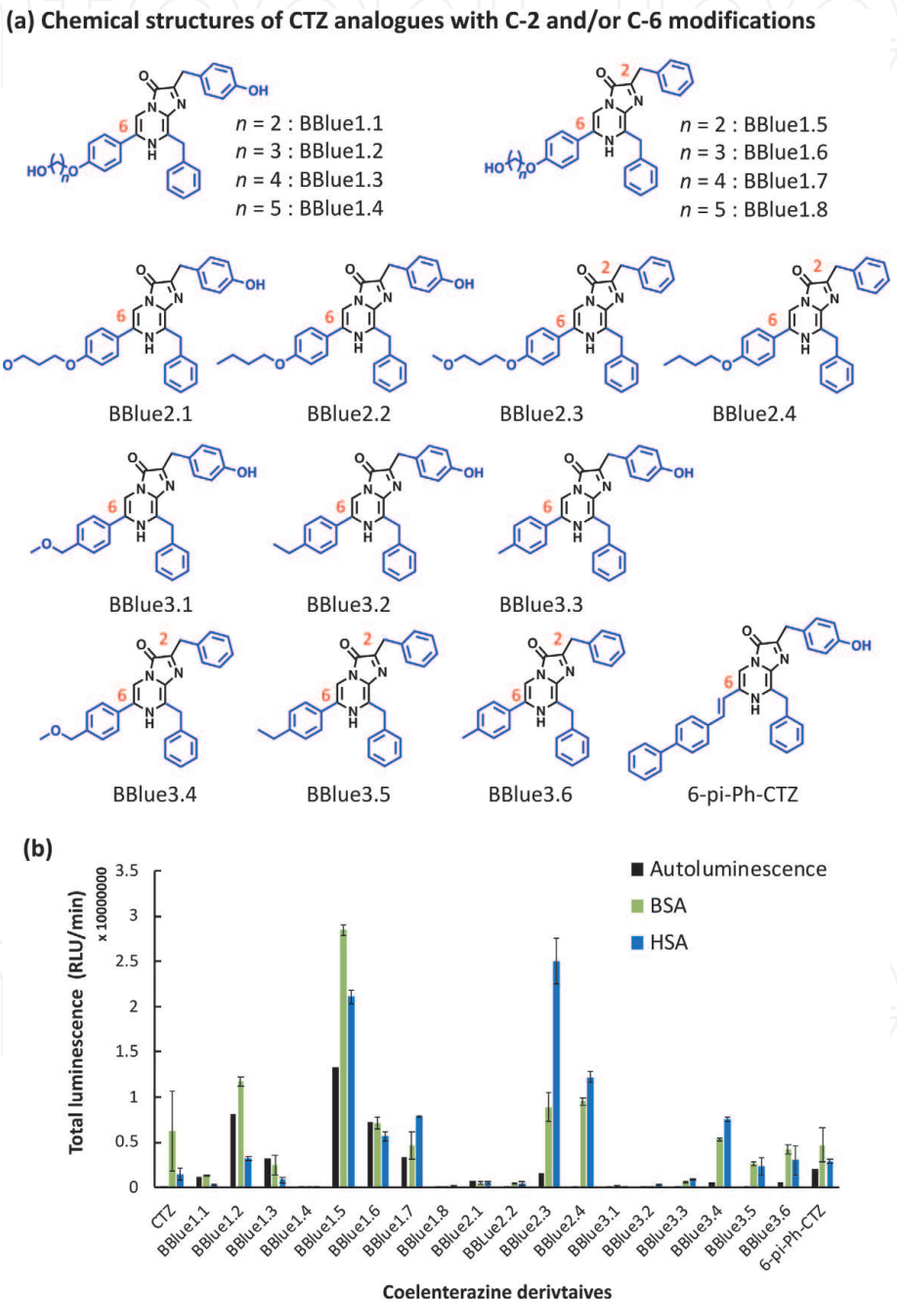

Figure 3.
Chemical structures of coelenterazine analogues (CTZAs) upon (a) C-2 and/or C-6 substitution. (b) Luminescence intensities obtained with serum albumin (0.1 mg/mL).

physiological functions, such as maintaining osmotic pressure, buffering blood pH levels, and carrying ligands including hormones, amino acids, and fatty acids [17, 18]. In addition, HSA and some ligand complexes often possess enzymatic activities, such as Kemp elimination and hydrolysis of esters because of their unique

ability to bind small hydrophobic molecules in some cavities; however, the potential enzymatic activities remain unclear [17, 19].

First, to obtain a rational luciferin with an imidazopyrazinone core for HSA-specific BL, we assayed CTZ and previously reported 18 CTZAs named as Bottle Blue (BBlue), where the *p*-hydroxy phenyl group at the C-6 position of CTZ was modified by alkylation (**Figure 3**), with serum albumins (fatty acid free HSA and BSA). In this chapter, except for in Section 2.2, luminescence measurements were performed using luminometers (GloMax20/20 Luminometer or GloMax Explorer Multimode Microplate Reader) manufactured by Promega (Madison, WI, USA). Next, BBlue2.3, a CTZA with a methoxy-terminated alkyl linker chain of three methylene units at the C-6 position, exhibited the brightest emission, which produced 16.6-fold higher luminescence when combined with HSA (i.e. BBlue2.3/HSA pair) compared to that of the CTZ/HSA pair (**Figure 3**).

Based on these results, we predicted that elimination of the benzyl group at the C-8 position of BBlue2.3 would relieve its steric hindrance with key amino acids in the substrate binding site of HSA and enhance the enzymatic luminescence reaction of HSA. We then designed and synthesized a novel CTZA, named as Human Luminophore **1** (HuLumino**1**) based on the synthetic procedures of BBlue2.3 [15] and an array of 5 CTZAs containing known analogues [20] to investigate the effect of substitution at the C-2, C-6, and C-8 positions of CTZ on serum albumin-dependent luminescence (**Figure 4b–d**). Moreover, the luminescence of

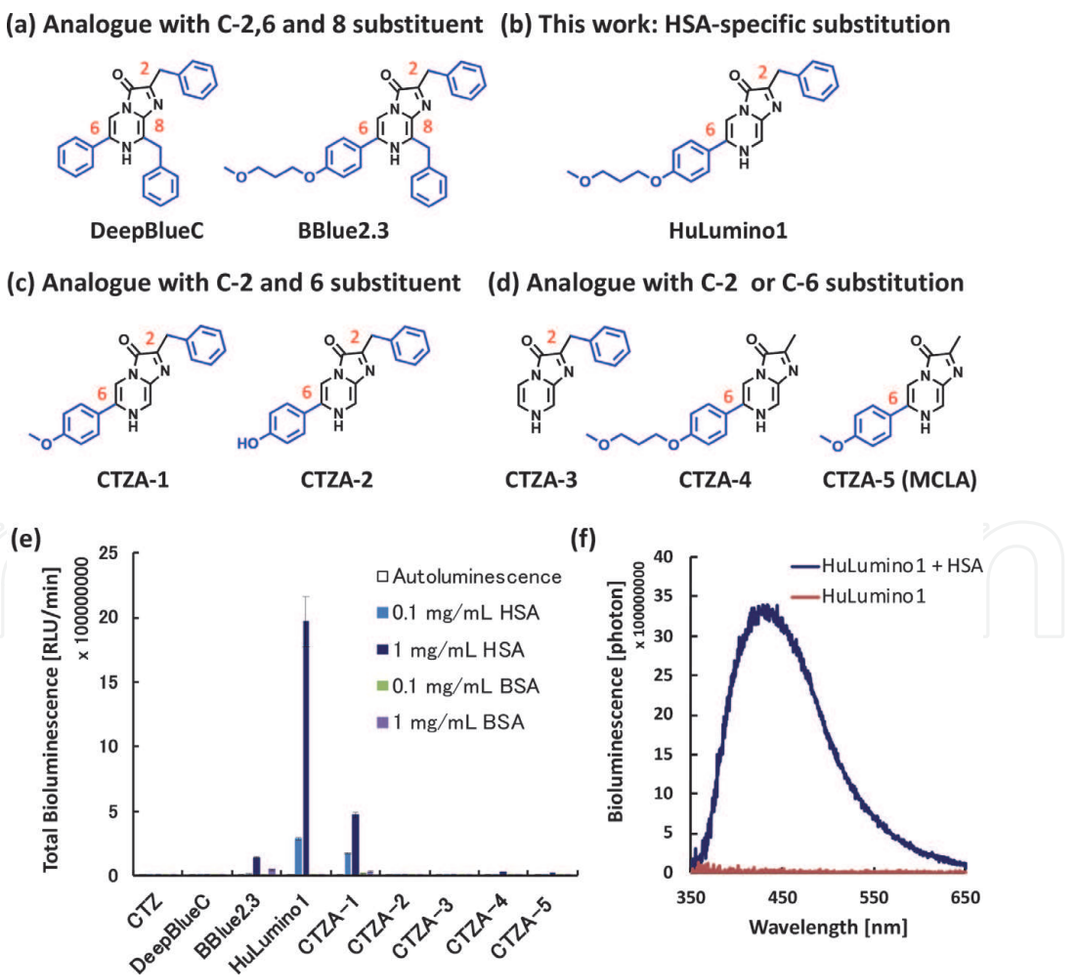

Figure 4.
*Chemical structures of coelenterazine analogues (CTZAs) upon (a) C-2, C-6, and C-8 substitutions, (b) HSA-specific substitution, (c) C-2 and C-6 substitution, and (d) C-2 or C-6 substitution. (e) Luminescence from serum albumins (0.1 or 1 mg/mL) treated with the indicated substrate (10 μM); error bars represent the standard deviations of three measurements. (f) Bioluminescence spectra of HuLumino**1** in the presence or absence of HSA.*

commercially available CTZAs (DeepBlueC and MCLA) was compared with the synthesized CTZAs when fatty acid free HSA and BSA were added (**Figure 4e**). Although the autoluminescence levels in PB buffer of HuLumino**1** was similar to that of CTZ, only HuLumino**1** displayed significantly enhanced luminescence dependent on HSA (but not BSA containing fatty acid) concentrations, as indicated by the 14.1-fold higher emission compared with that of the BBlue2.3/HSA pair. Furthermore, the luminescence intensity of the HuLumino**1**/HSA pair was found to be 718-fold higher than that of the CTZ/BSA pair, which has been reported to produce luminescence [11, 12]. The HuLumino**1**/HSA pair exhibited flash-type luminescence with a peak wavelength of 432 nm. (**Figure 4f**). Unexpectedly, HuLumino**1** selectively activated HSA by recognizing the subtle conformational difference in the substrate binding site, although the overall sequence homology between HSA and BSA is 75.6%.

These results suggest "luciferase" activity of HSA catalyzes the enzymatic luminescence reaction of CTZAs to produce "bioluminescence".

2.2 Quantitative evaluation of luminescence intensity

To characterize the enzymatic luminescence reaction with HSA, the bioluminescence intensity of CTZAs and HSA pairs was quantitatively evaluated. Bioluminescence intensity is generally determined by several reaction factors including the bioluminescence quantum yield (φ_{BL}) of luciferin, turnover number (k_{cat}), and active luciferase concentration. Kinetic parameters were determined using a custom-built luminometer with a photomultiplier tube (PMT) (H11890–01; Hamamatsu Photonics, Japan), and its absolute responsibility for total number of emitted photons in the instrument was calibrated for luminescence spectrum of each luciferin-luciferase pair [21, 22]. The absolute responsibility of the luminometer for the CTZ-utilizing bioluminescent system was determined as described previously [21]. The φ_{BL} values were calculated from the total number of emitted photons and total number of reacted luciferin molecules. To integrate all photons derived from the enzymatic reaction, the number of photons was monitored using the luminometer from before initiating the reaction to until the reaction was completed. The reaction was initiating by injection of fatty acid-free HSA PB solution (100 μg/mL or 10 mg/mL) into the preinstalled luciferin PB solution (20 nM) in the luminometer.

The Michaelis–Menten constant (K_m) of luciferin was calculated from Lineweaver-Burk plots constructed using a standard method. The catalytic constant (k_{cat}), which is the turnover number of the reaction for luciferin by a single luciferase molecule per second, was calculated from the φ_{BL} value and maximum velocity (V_{max}) determined from the Lineweaver-Burk plots.

The apparent K_m of the HuLumino**1**/HSA pair was 4.3 μM, which was comparable to that of the NanoLuc system [23]. In contrast, the k_{cat} value of the NanoLuc system was 294-fold higher than that of the HuLumino**1**/HSA pair, and the catalytic efficiency of the luminescence reaction by HSA was lower than that of conventional luciferase [24]. However, in the luminescence system with HSA, HuLumino**1** displayed a slightly higher K_m value than CTZA-1, but its k_{cat} value was approximately 3-fold higher (**Table 1**), resulting in 4.2-fold stronger light emission than that of CTZA-1 (**Figure 4e**). For luciferin with high enzyme affinity (e.g., CTZA −1), oxyluciferin, a product of the BL reaction, appears to competitively inhibit the luminescence reaction [25]. Moreover, HuLumino**1** showed an enzyme affinity and bioluminescence quantum yield (φ_{BL}) of more than 5- and 100-fold higher than those of CTZ, respectively. The detailed luminescent profiles suggest that the structural properties of the alkyl linker chain modified at the C-6 position of the

Pair	φ_{BL} [a] [$\times 10^{-5}$]	K_m [b] [μM]	k_{cat} [c] [s^{-1}]
CTZ/HSA	0.32 ± 0.03	25.3 ± 5.2	2.75 ± 0.3
HuLumino**1**/HSA	30.9 ± 3.1	4.28 ± 1.24	0.30 ± 0.06
CTZA-4/HSA	42.2 ± 9.1	2.46 ± 0.40	0.11 ± 0.09

[a–c]Errors represent standard error of the mean values for triplicate experiments.
[b]Michaelis-Menten constant (K_m) values were determined by Lineweaver-Burk plots via measurements of initial rates of light emission over a range 0.5 to 20 μM.
[c]Turnover rate (k_{cat}) values were calculated by dividing maximum velocity (V_{max}) by the φ_{BL}. The V_{max} were determined by Lineweaver-Burk plots.

Table 1.
Luminescent profiles of CTZ and its analogues with HSA.

imidazopyrazinone core contribute to the efficient HSA-catalyzed emission reaction. Although the catalytic efficiency of HuLumino**1**/HSA is much lower than that of the NanoLuc system, HuLumino**1** is a luciferin that is relatively more suitable for HSA than other existing luminescent substrates.

2.3 Enzymatic reaction site of HSA

The crystal structure of HSA, with binding to a variety of drugs, clarified the two principal drug binding sites in different subdomains (site 1 in subdomain IIA and site 2 in subdomain IIIA) [26, 27]. To investigate the luminescent reaction site between HSA and HuLumino**1**, a competitive assay was conducted with two site-specific HSA drugs (warfarin-site1 and ibuprofen-site2) [27]. Fatty acid free HSA PB solution was pre-treated with the drugs (0–100 μM) to fill binding site 1 or 2 before adding HuLumino**1**. The luminescence of HuLumino**1** was negligibly low in the presence of HSA-ibuprofen complex (**Figure 5a**). In contrast, HuLumino**1** exhibited efficient luminescence with the HSA-warfarin complex, indicating that HuLumino**1** selectivity binds to the site 2 cavity of HSA. In detailed analysis of the inhibitory kinetics, Lineweaver-Burk plots displayed that ibuprofen competitively inhibited the binding of HuLumino**1** to HSA (K_i of ibuprofen was 6.3 nM) (**Figure 5b**). Therefore, the enzymatic reaction site of HuLumino**1** was experimentally determined to be binding site 2. Next, docking simulation with the Molecular Operating Environment software package was carried out to predict the binding poses. The simulation displayed the specific binding of HuLumino**1** to the hydrophobic cavity of site 2 by interacting with several amino acids including R410, K414, and L453 (**Figure 5c–e**). Particularly, R410, a key amino acid residue in the esterase activity of HSA [17], is also involved in the luminescence reaction (**Figure 5e**).

Next, to investigate the effect of the steric structure of HSA on luminescence, HSA pretreated with 10 M guanidine hydrochloride, a reagent commonly used to induce denaturation of the α-helix structure of proteins [28], was prepared, and the luminescence of HuLumino**1** was extremely low in the presence of denatured HSA (data not shown). Therefore, the enzymatic reaction of HuLumino **1** depends on the microenvironment and steric structure such as binding site 2 constructed by the folding structure of HSA. These results suggest that emission of HuLumino**1**/HSA is not a non-specific chemiluminescence commonly found in other imidazopyrazinone compounds.

2.4 Bioluminescent assay for HSA

Low levels of HSA in the serum (<35 mg/mL) are biomarkers of several diseases such as malnutrition, cirrhosis, and chronic hepatitis [29]. In hospitals, HSA is

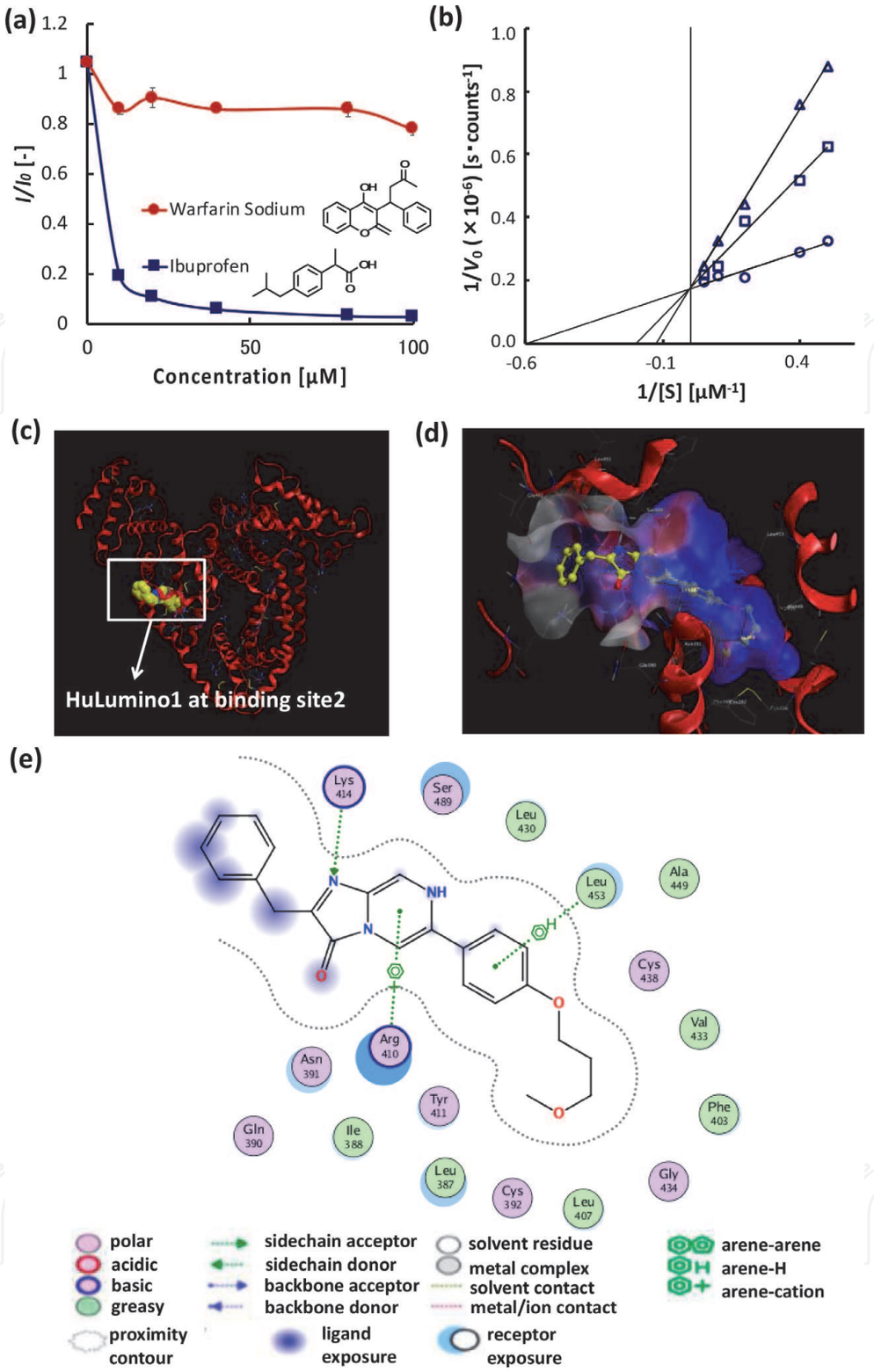

Figure 5.
(a) Luminescence response in the presence (I) vs. absence (I_o) of the binding drug concentration (0–100 μM). (b) Lineweaver-Burk plot indicating competitive inhibition by ibuprofen. V_o is defined as the luminescence intensity over the initial 30 s and [S] is the substrate concentration. The concentrations of ibuprofen were 1 nM (square) and 5 nM (triangle). Open circles indicate negative controls with no ibuprofen. (c) Ligand-binding site 2 of HSA with HuLumino1 as posed by the molecular operating environment software (d) magnified view of binding site 2. The ligand binding site in the blue region indicates the presence of the hydrophobic environment. (e) Predicted interaction between HuLumino1 and HSA.

evaluated using the colorimetric bromocresol green assay or ELISA. Both can provide a reliable assessment of albumin but require sample preparation and processing time (e.g. 3 h for ELISA) [30]. Therefore, an assay for the simple, accurate and

rapid detection of HSA in the serum should be developed for clinical diagnosis. We demonstrated that the BL assay can be used to evaluate HSA based on the enzymatic luminescence reaction of HuLumino**1**. Regarding the selectivity of the reaction of HuLumino**1**, no reactivity with other proteins (BSA, *β*-galactosidase,*β*-lactoglobulin, catalase, *α*-chymotrypsinogen, hemoglobin, human IgG, porcine lipase, papain, pepsin, trypsin, *γ*-globulin, carbonic anhydrase, concanavalin A, glucosidase, histone, myoglobin, and RNase, 0.1 mg/mL) was observed, and only HSA led to distinct luminescence enhancement (**Figure 6a**). Although the coexistence of most proteins did not affect the enzymatic reaction of the HSA/Hulumino**1** pair, a slight decrease in luminescence was observed in the presence of some proteins (data not shown). This indicates that HuLumino**1** nonspecifically binds to other proteins but does not exhibit BL. Hence, HuLumino**1** can be used to detect HSA without interference from other proteins, as it exhibited excellent selectivity for HSA even in a complicated biological system. The luminescence of HuLumino**1** was enhanced in an HSA concentration-dependent manner and exhibited a constant intensity at HSA concentrations above 10 mg/mL (**Figure 6b**). A linear increase in luminescence, within the spiked HSA concentration range of 0–0.1 mg/mL in PBS-diluted serum, resulted in a detection limit of 8.6 μg/mL for HSA, which was comparable to the standard detection limit of HSA in physiological systems (**Figure 6c**) [31].

Finally, two HSA assays, including our developed BL-based assay and ELISA, were performed to evaluate human serum from male AB plasma. The HSA levels calculated with HuLumino**1** agreed well with those estimated by ELISA within 5% error. The spike and recovery tests also showed results within the margin of 7%

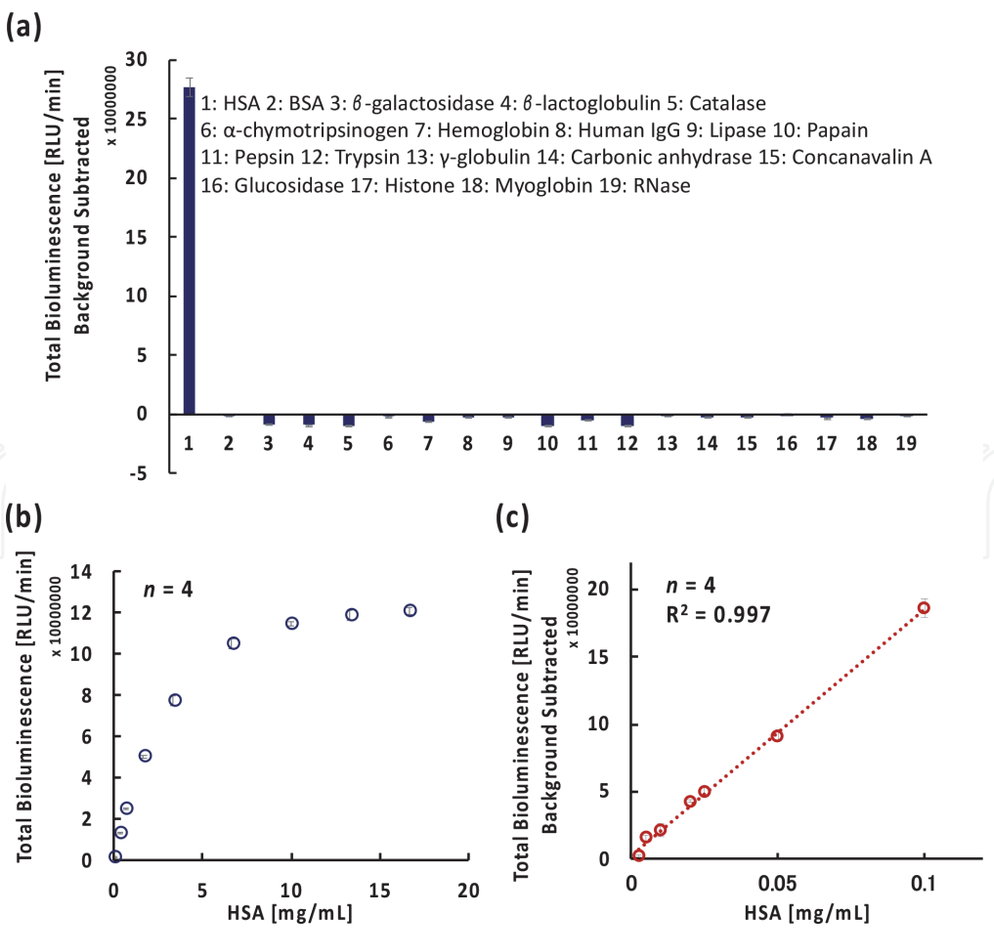

Figure 6.
*(a) Variation in luminescence of HuLumino**1** (10 μM) in the presence of proteins (0.1 mg/mL); error bars represent the standard deviations of three measurements. (b) Luminescence intensity of HuLumino**1** (20 μM) containing various concentrations of HSA (0–17 mg/mL) in PBS and (c) HSA (0–17 mg/mL) in PBS-diluted plasma (100-fold, pH 7.4).*

Amount of HSA added (mg/mL)	HSA (mg/mL) determined by developed method[a]	HSA (mg/mL) determined by ELISA	Recovery
0	39.0 ± 3.1	41.0 ± 3.6	95.2
1	44.5 ± 0.5	ND	106.1
2.5	45.2 ± 0.5	ND	104.1

ND: Not Determined.
[a]*Conditions: HuLumino**1** (20 μM) in PBS-diluted serum (1000-fold, 10 mM, pH 7.4).*

Table 2.
Assay of HSA in human serum.

error (**Table 2**). Therefore, the BL-based HSA assay showed analytical capability with high sensitivity and could detect HSA within 10 min including the sample preparation and measurement times. In addition, we detected the expression of recombinant HSA in living COS-1 cells (data not shown), indicating that HuLumino**1** can be used in molecular biology studies and in biomedical applications.

We designed and synthesized the first luciferin (HuLumino**1**), an analogue with C-6 and C-8 modification of CTZ, which exhibited bioluminescence with HSA. HuLumino**1** rapidly detected HSA with high sensitivity and specificity, even in real human plasma containing various interfering biomolecules. Detailed kinetic investigation of the enzymatic reaction clarified the enzyme recognition of HuLumino**1** from HSA drug binding site 2, resulting a highly selective reaction and revealing a reaction with both native HSA and recombinant HSA expressed in COS-1 cells. Therefore, the BL-based assay with HuLumino**1**, either used alone or coupled with ELISA, can be used for the early diagnosis of HSA-related diseases, enabling accurate and rapid detection of HSA in serum samples without pre-treatment. The information obtained through detailed investigation of the HuLumino**1**/HSA pair may be extended to protein assays based on a luminescent reaction without genetically engineered luciferases.

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Conflict of interest

The authors declare no conflict of interest.

Notes/thanks/other declarations

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