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Communication

Powerful Chemiluminescence Probe for Rapid Detection of Prostate Specific Antigen Proteolytic Activity: Forensic Identification of Human Semen

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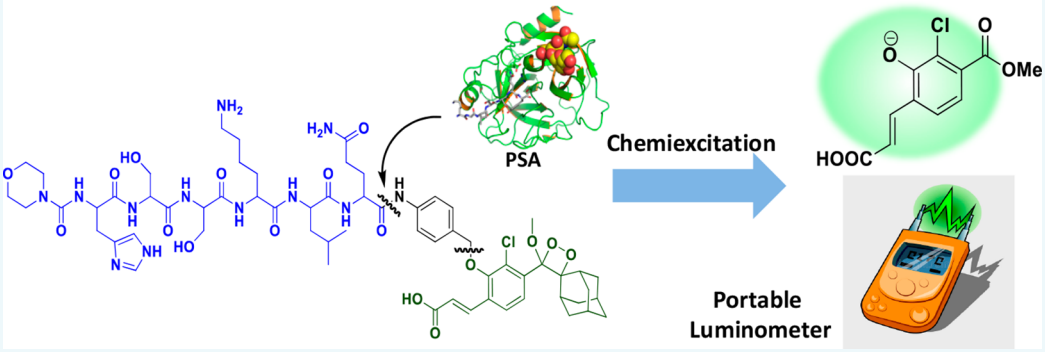

ABSTRACT: The prostate specific antigen (PSA), a serine protease with chymotrypsin-like activity, is predominantly expressed in the prostate and is considered as the most common marker in use to identify and follow the progress of prostate cancer. In addition, it is also now accepted as a marker for detecting semen in criminal cases. Here, we describe the design, synthesis, and evaluation of the first chemiluminescence probe for detection of PSA enzymatic activity. The probe activation mechanism is based on a catalytic cleavage of a specific peptidyl substrate, followed by a release of a phenoxy-dioxetane luminophore, that then undergoes efficient chemiexcitation to emit a green photon. The probe exhibits a significant turn-on response upon reaction with PSA and produces strong light emission signal with an extremely high signal-to-noise ratio. Comparison of the chemiluminescence probe with an analogous fluorescence probe showed superior detection capability in terms of response time and sensitivity. In addition, the probe was able to efficiently detect and image human semen traces on fabric, even after 3 days from sample preparation. The advantageous sensitivity and simplicity of a chemiluminescence assay to detect seminal fluid was effectively demonstrated by on-site measurements using a small portable luminometer. It is expected that the new chemiluminescence probe would be broadly useful for numerous applications in which PSA detection or imaging is required.

Chemiluminescence is considered as one of the most efficient diagnostic tools for biosensing and bioimaging.[1,2] In contrast to fluorescence, chemiluminescence does not require irradiation by an external light source, and as a result, the background signal is extremely low and the obtained sensitivity is considerably enhanced. Among the known chemiluminescent compounds, the triggerable phenoxy-dioxetanes, discovered by Paul Schaap in 1987, are of particular interest, as the light emission of these compounds can be generally initiated by deprotection of various substrates.[3] Unfortunately, the extremely weak chemiluminescence emission of these compounds in aqueous conditions prevents their use in bioassays without additives. Several years ago, our lab explored new approaches for amplifying chemiluminescence intensity under physiological conditions.[4] We have developed a novel methodology that significantly improves the light emission efficiency of phenoxy-dioxetanes in water.[5,6] Introduction of an electron-withdrawing substituent at the ortho position of the phenol group generated new phenoxy-dioxetane luminophores with up to 3000-fold increase of chemiluminescence quantum yield (ΦCL) in aqueous media.[7] Masking of these luminophores with different substrate groups led to the development of several new efficient chemiluminescence molecular probes for various enzymes and bioanalytes.[8−11] The most effective probes, in terms of signal-to-noise ratio, were obtained by equipping our phenoxy-dioxetane luminophores with enzymatic responsive groups composed of peptide substrates.[12] Such compounds are

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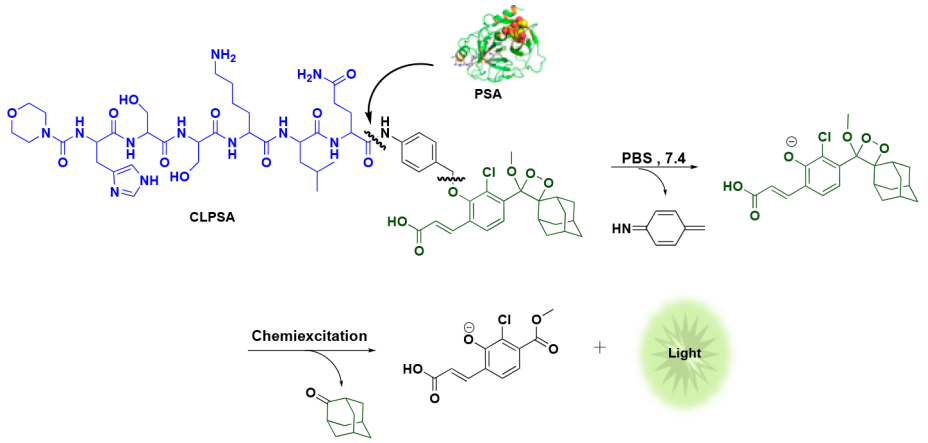

Figure 1. Molecular structure of the **CLPSA** probe and its chemiluminescence activation pathway.

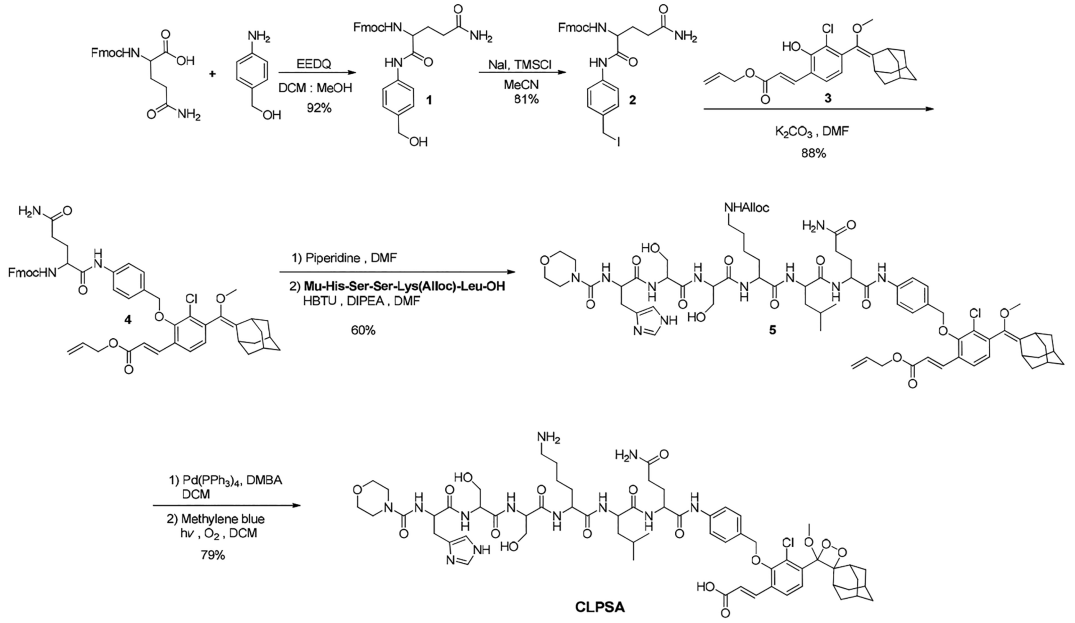

Figure 2. Synthetic pathway used for preparation of probe **CLPSA**.

highly stable to spontaneous hydrolysis and, therefore, do not produce any background signal. This observation has promoted us to design and study a new chemiluminescence probe for the enzymatic activity of the protease: prostate specific antigen (PSA).

PSA, the most common marker in use to identify and follow prostate cancer, is a serine protease with chymotrypsin-like activity and is predominantly expressed in the prostate, although it is present in other normal and tumor tissues at low concentrations.[13,14] It is also now accepted as a marker for detecting semen in criminal cases including vasectomized or azoospermic males.[13,15] The main physiological function of PSA in the human male is to liquefy the seminal fluid by cleaving seminogelin, thus allowing a sufficient motility of the sperm cells. PSA exhibits high substrate specificity, being the only human endopeptidase known to cleave peptide substrates at the amide bond after glutamine. Thus, the attachment of PSA-specific peptide substrates to a dye molecule is applied as a common approach to prepare turn-on fluorescent molecular probes.[16−18] Similarly, masking of a drug molecule, by a PSA-specific peptide substrate, is used to generate a prodrug that can be activated by PSA.[19−21] In this study, we chose to focus on detection of seminal PSA rather than serum PSA, since PSA, secreted to serum, is kept catalytically inactive. Thus, a probe designed to detect catalytic activity will not be able to sense such form of inactive PSA.

The particular peptidyl substrate Mu-HSSKLQ has shown the highest specificity for PSA, in comparison to similar proteolytic enzymes found within body fluids.[22] We therefore sought to evaluate a new chemiluminescence probe for detection of PSA by masking our phenoxy-dioxetane luminophore with the Mu-HSSKLQ as a triggering substrate. Here, we report the design, synthesis, and evaluation of the first chemiluminescence probe for detection of PSA enzymatic activity.

The molecular structure of our **CLPSA** probe and its chemiluminescence activation pathway are depicted in Figure 1. The probe is composed of a phenoxy-dioxetane luminophore with an ortho acrylic acid substituent, masked by Mu-HSSKLQ peptide, through para-amino-benzylalcohol self-immolative linker. Proteolytic cleavage of the Mu-HSSKLQ substrate by PSA, followed by 1,6-elimination of

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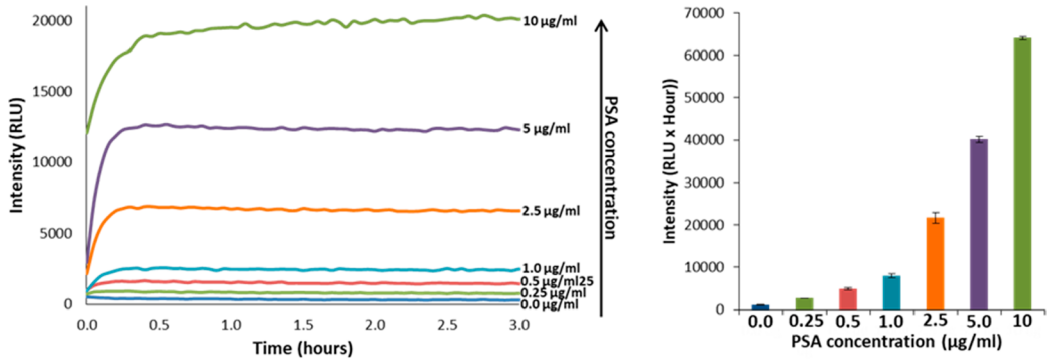

Figure 3. Chemiluminescence kinetic profiles (left) and total light emission (right) of **CLPSA** [10 μM] in PBS, pH 7.4, DMSO 10%, with different concentrations of purified PSA [0, 0.25, 0.5, 1.0, 2.5, 5.0, 10 μg/mL]. Error bars represent the mean of three different replicate measurements.

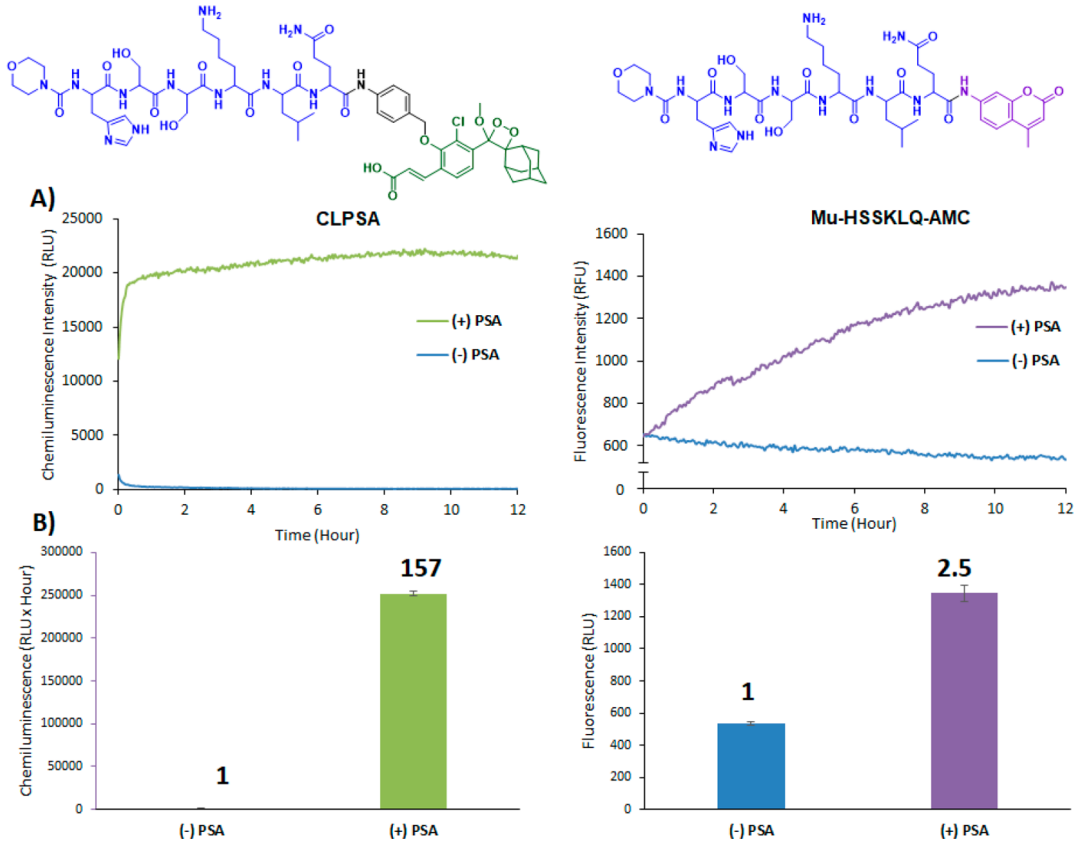

Figure 4. (A) Chemiluminescence (left) and fluorescence (right) kinetic profiles of **CLPSA** and **Mu-HSSKLQ-AMC** [10 μM] in PBS, pH 7.4, DMSO 10%, with and without of PSA [10 μg/mL]. (B) Chemiluminescence (left) and fluorescence (right) total light emission and signal intensity ratios of **CLPSA** and **Mu-HSSKLQ-AMC** [10 μM] in PBS, pH 7.4, DMSO 10%, with and without PSA [10 μg/mL]. Error bars represent the mean of three different replicate measurements.

azaquinone-methide, leads to formation of the phenoxy-dioxetane luminophore that then undergoes efficient chem-iexcitation disassembly to produce a green photon.

The synthesis of the **CLPSA** probe was achieved as described in Figure 2. Fmoc-Gln-OH was coupled with 4-aminobenzyl-alcohol to form the amide **1**. The latter was converted to benzyl-iodide **2** by treatment with sodium iodide and trimethylsilyl chloride. Nucleophilic substitution of benzyl-iodide **2** by the previously synthesized phenol enol-ether **3** afforded ether **4**. Next, the Fmoc protecting group of compound **4** was removed by piperidine, and the obtained amine was coupled with the peptide Mu-His-Ser-Ser-Lys-(Alloc)-Leu-OH (prepared by standard solid-phase synthesis) to generate amide **5**. The Alloc and the allyl ester protecting groups of compound **5** were removed by a Pd(0) complex, and the crude product was subsequently oxidized by singlet oxygen to afford probe **CLPSA**.

To evaluate the ability of probe **CLPSA** to detect proteolytic activity PSA, we used commercially available purified PSA from human semen. The chemiluminescence emission of the probe as a function of time was measured in the presence and in the absence of PSA. The kinetic profiles of the chemiluminescence signals and the relative total-photon emissions are shown in Figure 3. Probe **CLPSA** exhibits a typical chemiluminescence kinetic profile upon incubation with PSA, under physiological conditions, with an initial rapid increase in signal to a

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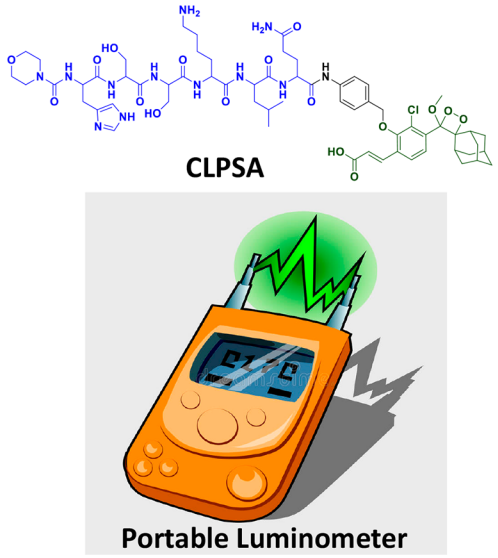

SF dilution	End point measurement [RLU]	Signal to noise ratio
1:10	521664.7 ± 18915.1	2255.0 ± 82.8
1:50	104601.3 ± 5844.4	452.2 ± 25.3
1:250	21219.0 ± 1287.1	91.7 ± 5.6
1:1250	5185.7 ± 329.5	22.4 ± 1.4
1:6250	1035.7 ± 41.1	4.5 ± 0.2
1:31250	488.7 ± 28.7	2.1 ± 0.1
1:156250	293.7 ± 16.0	1.3 ± 0.1
Control	231.3 ± 16.0	1.0

Figure 5. Determination of the minimum detectable concertation of seminal fluid (SF) by **CLPSA** [100 μM] in PBS, pH 7.4, DMSO 10% with various dilution of SF. Light emission measurements of the sample were recorded over 5 min by the portable chemiluminescence Lu-mini instrument. Error values represent the mean of three different replicate measurements.

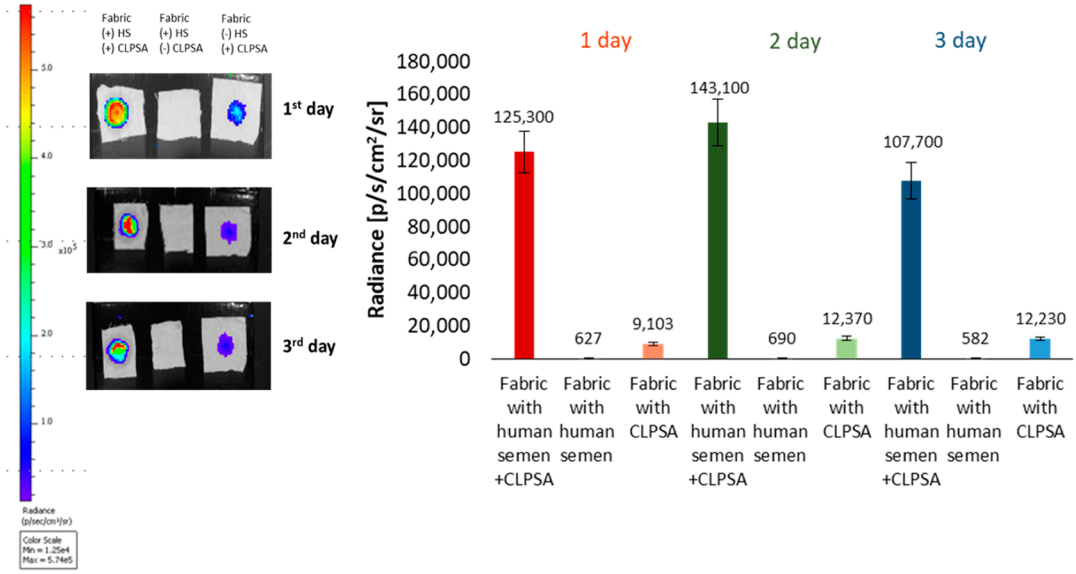

Figure 6. (Left) Chemiluminescence images of human semen (HS) [10 μL] by **CLPSA** [100 μM] in PBS 7.4, 10% DMSO over 3 days vs proper controls, after deposition of semen on fabric surface. (Right) Chemiluminescence total light emission of **CLPSA** in PBS 7.4, 10% DMSO, with or without deposition of human semen on fabric surface (signal was measured after 10 min with two min exposure time). Negative control was performed by measuring the signal emitted from human semen in the absence of the probe. Error bars represent the mean of three different replicate measurements.

maximum, followed by steady-state signal. The intensity of the chemiluminescence signal and the total light emission proportionally correspond to the incremental doses of PSA. Almost no light emission was observed by the probe in the absence of PSA.

We next sought to compare the detection capability of our **CLPSA** probe toward PSA, vs that of commercially available fluorescent probe. **Mu-HSSKLQ-AMC** is an analogous fluorescent probe,[23] composed of aminomethy-coumarin (AMC) masked by the same peptidyl substrate used in our chemiluminescence probe. Both probes were evaluated for their ability to detect PSA under identical conditions (Figure 4). The probes exhibited a significant turn-on response upon incubation with PSA with a stable background signal. However, the signal intensity exhibited by the **CLPSA** probe with PSA was 157-fold higher than the signal intensity without PSA. In contrast, probe **Mu-HSSKLQ-AMC** exhibited only a 2.5-fold increase of the signal-to-background ratio. The 63-fold higher ratio in signal-to-noise, obtained for **CLPSA** vs **Mu-HSSKLQ-AMC**, clearly demonstrates the superior PSA-detection capability of a chemiluminescence probe vs a fluorescence one. Remarkably, **CLPSA** displays a much faster response with an increased signal-to-noise ratio in comparison with a corresponding fluorescence analogue and, thus, allows the probe a unique mode of rapid detection for PSA.

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As mentioned above, PSA is a marker that indicates the presence of seminal fluid. Thus, a probe designed to react with PSA can usefully be applied for semen detection. The superb sensitivity and fast turn-on response exhibited by **CLPSA** toward detection of PSA has encouraged us to further evaluate the ability of the probe to detect seminal fluid. In order to demonstrate a rapid, simple, field method for semen detection, we measured the light emission signal by using the small portable luminometer, Lu-mini (see Figure 5). A sample of seminal fluid was diluted in PBS 7.4 (using 5-fold serial dilution protocol), starting from ratio of 1:10 down to 1:156250. Probe **CLPSA** was added to the seminal fluid solutions and the light emission signal was recorded over 5 min using the Lu-mini luminometer. The data presented in the table of Figure 5 clearly indicate that a simple, portable luminometer can be used to detect traces of seminal fluid with the **CLPSA** probe. Seminal fluid could be detected even when a sample was diluted by a factor of 1:31250, with a signal-to-noise ratio of 2:1.

Finally, we evaluated the ability of the CLPSA probe to detect traces of human semen deposited on a piece of fabric for a possible use in a crime scene. Untreated human semen samples were deposited on a fabric surface and allowed to dry during 1, 2, and 3 days. Then, a solution of the CLPSA probe in PBS 7.4 was added dropwise onto the fabric. After 10 min incubation time, the chemiluminescence signal emitted from the samples was recorded by IVIS imager. The obtained imaging data are presented in Figure 6.

Overall, probe **CPLSA** was able to efficiently detect and image human semen traces on fabric after 1, 2, or 3 days since the deposition event. The intensity of the light emission signal of semen samples with the probe was about 14-fold higher than that of a sample without semen after 1 day; 12-fold larger after 2 days; and 9-fold larger after 3 days. Control semen samples in the absence of the probe show only negligible light emission signal.

Serum PSA is kept catalytically inactive in a variety of different forms.[23] Therefore, optical probes that are turned on through a catalytic mechanism are not suitable for detection of PSA in serum. Accordingly, blood tests for PSA detection are usually based on classic immunoassays.

The enzymatic properties of PSA have been comprehensively studied and reported in numerous publications.[24,25] This protein is considered as a very poor protease with low catalytic activity. Therefore, optical probes with turn-on response toward PSA activity produce only a slow increase of signal over time. In addition, the requirement for an excitation light source, for fluorescent probes, results in a relatively high background signal. The combination of the high background signal and the PSA low enzymatic activity leads to a significant reduction in the signal-to-noise ratio produced by fluorescent probes. Indeed, commercially available fluorescent probe **Mu-HSSKLQ-AMC** generates only 2.5 signal-to-background value. On the contrary, chemiluminescence probes almost do not produce any background signal. This advantage was effectively reflected with the 157 signal-to-background value obtained by probe **CLPSA** upon incubation with PSA.

As can be seen in Figure 4, probe **CLPSA** generates a rapid increase response of light-emission signal in the presence of PSA, followed by a steady-state kinetic signal. This phenomenon is attributed to the relatively low enzymatic activity of PSA that enables saturation kinetics. The release rate of the phenoxy-dioxetane luminophore and its concentration remain constant over time. As a result, the signal-to-background ratio obtained by the chemiluminescence probe, vs that of the fluorescence one, is significantly higher even after 2 min since the beginning of the measurement.

In summary, we have developed the first chemiluminescence probe for direct detection of proteolytic activity of PSA. The probe activation mechanism is based on catalytic cleavage of a specific peptidyl substrate, followed by release of the phenoxy-dioxetane luminophore that then undergoes efficient chemiexcitation to emit a green photon. The probe exhibits significant turn-on response upon reaction with PSA and produces strong light emission signal with a very high signal-to-noise ratio. Comparison of the chemiluminescence probe with an analogous fluorescence probe showed superior detection capability in terms of response time and sensitivity. In addition, the probe was able to efficiently detect and image human semen traces on fabric, even after 3 days from sample preparation. The advantage and simplicity of a chemiluminescence assay to detect seminal fluid was effectively demonstrated by on-site measurements using a small portable luminometer. We expect that our new chemiluminescence probe would be broadly useful for numerous applications in which PSA detection is required.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.0c00500.

Synthetic procedures, characterization data (NMR and MS) for all new compounds, and chemiluminescence control experiments (PDF)

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Notes

The authors declare no competing financial interest.

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