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Cyclic Regulation of the Sulfilimine Bond in Peptides and NC1 Hexamers via the HOBr/H_2Se Conjugated System

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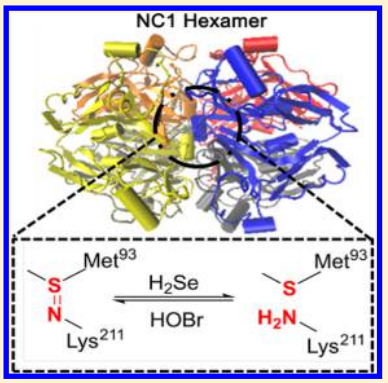
Supporting Information

ABSTRACT: The sulfilimine bond (−S═N−), found in the collagen IV scaffold, significantly stabilizes the architecture via the formation of sulfilimine cross-links. However, precisely governing the formation and breakup process of the sulfilimine bond in living organisms for better life functions still remains a challenge. Hence, we established a new way to regulate the breaking and formation of the sulfilimine bond through hydrogen selenide (H_2Se) and hypobromous acid (HOBr), which can be easily controlled at simulated physiological conditions. This novel strategy provides a circulation regulation system to modulate the sulfilimine bond in peptides and NC1 hexamers, which can offer a substantial system for further study of the physiological function of collagen IV.



Collagen IV scaffolds, as important tissue generation factors for fibrosis, are crucial for the formation and function of basement membranes (BMs).[1,2] Deficiency or disruption in the collagen IV scaffold causes BM destabilization and tissue dysfunction in humans.[3−5] Meanwhile, excessive cross-linking of collagen IV networks leads to collagen IV accumulation in BMs, which may contribute to pathological states such as hepatic[6−9] and renal[10−15] fibrosis. Therefore, study of the structure and function of type IV collagen[1,16−20] has been of great interest for controlling its in vivo activity. In 2009, Hudson's group discovered the sulfilimine bond (−S═N−) in collagen IV, which stabilizes the scaffold via the formation of sulfilimine cross-links between the C-terminal noncollagenous (NC1) domains[21] of two residues, methionine 93 (Met^{93}) and hydroxylysine 211 (Hyl^{211}) or lysine 211 (lys^{211}).[1,16] They further found that hypobromous acid (HOBr) catalyzes the formation of sulfilimine bonds through tissue genesis.[1] However, to date, controllable regulation of sulfilimine bonds and thus enhancement of the optimization of collagen IV are still in their preliminary steps. Hence, development of methods that can interrupt the sulfilimine bond is urgently needed in the regulation of collagen IV, thereby treating hepatic fibrosis and renal fibrosis.

Previously, we established a sulfilimine-bond-based fluorescence probe (BPP) to detect native HOBr in living cells and zebra fish[22] and a mitochondria-targeting near-infrared probe for imaging HOBr[23] as well. In addition, we also developed fluorescence probes for detecting hydrogen selenide (H_2Se) in organisms.[24,25] Since H_2Se is an important sodium selenium (Na_2SeO_3) metabolism intermediate involved in many physiological and pathological processes with highly reactive and reductive properties,[26−29] in this study, we found that H_2Se exhibits excessive specificity to break the sulfilimine bond. Hence, H_2Se serves as an ideal regulator for the "off mode" of the sulfilimine bond in dipeptide Met-Lys and NC1 domains of the bovine glomerular basement membrane (GBM), while HOBr works as a controllable "on mode" regulator. In this regard, we hypothesized that a HOBr/H_2Se conjugated system will be available to mediate the sulfilimine cross-link[30−33] in vitro as an "on−off" cyclic regulation control,[34,35] which will support further in vivo study of the physiological function of the sulfilimine bond in collagen IV and treatment of collagen IV related diseases.

EXPERIMENTAL SECTION

Materials and Instruments. Aluminum selenide (Al_2Se_3), DL-2-amino-4-(methylthio)butyric acid (DL-Met), and L-2,6-diaminohexanoic acid (L-Lys) were purchased from Aladdin (China). Dipeptide Met-Lys was obtained from KareBay (China). The NC1 fraction from the bovine glomerular basement membrane (GBM) was purchased from Chondrex (United States). All other chemicals and solvents used were local products of analytical grade. Ultrapure water (18.2 MΩ·

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cm) was used throughout the experiments. HepG2 cells were purchased from the Committee on Type Culture Collection of the Chinese Academy of Sciences. The high-resolution mass spectrometry (HRMS) was performed with a Bruker maXis ultra-high-resolution TOF MS system. Fluorescence spectra were obtained with a fluorescence spectrometer (Edinburgh, Scotland). The fluorescence images were obtained with a confocal fluorescence spectrometer (Leica, Germany). Centrifugation was performed on an Eppendorf 5417R centrifuge. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) images were acquired by the ChemiDoc touch imaging system (Bio-Rad).

Preparation of HOBr and H_2Se. Preparation of HOBr followed the procedure in previous literature.[22] Typically, 0.50 g of $AgNO_3$ was dissolved in 20 mL of ultrapure water. A 100 μL volume of brown-red liquid bromine was dissolved in 15 mL of ultrapure water, titrated with $AgNO_3$ solution in an ice bath to the colorless end point, and then filtered with a 0.22 μm microfiltration membrane. The concentration of HOBr was calculated through Lambert−Beer's law (ε_{260} = 160 L/mol·cm).

H_2Se was freshly prepared by the reaction of Al_2Se_3 with H_2O in a N_2 atmosphere before each use.[25,36,37] First, 3 mL of ultrapure water was bubbled with N_2 for 30 min at room temperature to remove all O_2. Then 0.0017 g of Al_2Se_3 was accurately weighed and dissolved in 3 mL of the above ultrapure water in a sealable glass bottle. The concentration of H_2Se stock solution was calculated to be 5×10^{-3} M.

$$Al_2Se_3 + 6H_2O \rightarrow 2Al(OH)_3 + 3H_2Se$$

Cell Culture. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS under 10% CO_2, 1% penicillin, and 1% streptomycin at 37 °C (w/v) in a 5% CO_2/95% air incubator.

Preparation of the BPP Coupling Reaction Production (cBPP) Solution. BPP was synthesized according to the previous method.[22] cBPP was obtained by the reaction of 5 μM BPP and 20 μM HOBr for characterization and 25 μM BPP and 50 μM HOBr for the next applications.

Kinetics Experiment. The time course for the fluorescence intensity changes was obtained with the fluorescence spectrometer. A 5 μM concentration of BPP was treated with 20 μM HOBr, and the fluorescent responses of the resulting mixture were analyzed via the fluorescence spectrometer. A 100 μM concentration of H_2Se was added to the reaction mixture after 15 min, and then the fluorescent responses were continually collected. All data were acquired in 10 mM PBS at pH 7.4 (λ_{ex} = 480 nm, λ_{em} = 525 nm) at room temperature and atmospheric pressure.

pH Dependence of the cBPP Reaction. The selectivity toward H_2Se at various pH values was determined to demonstrate the potential behaviors of cBPP via the fluorescence spectrometer. Typically, BPP was dissolved in ultrapure water to prepare a 5 μM solution, and the fluorescent responses were obtained under different pH values. Then 5 μM BPP was reacted with 20 μM HOBr, and the fluorescent responses of the reaction mixture were obtained at various pH values. Next, 100 μM H_2Se was added to the reaction mixture, and then the fluorescent responses were collected. All data were acquired at room temperature and atmospheric pressure with $\lambda_{ex}/\lambda_{em}$ = 480 nm/525 nm.

Selectivity toward Metal Ions and Amino Acids. The selectivity of the probe toward metal ions and amino acids was determined via the fluorescence spectrometer. A mixture of 5 μM BPP and 20 μM HOBr was prepared, and different metal ions [Zn^{2+}, Fe^{2+}, and Fe^{3+} (200 μM); K^+, Na^+, Mg^{2+}, Ca^{2+}, and Cu^{2+} (1 mM)] and amino acids [phenylalanine (Phe), methionine (Met), serine (Ser), histidine (His), glycine (Gly), tyrosine (Thr), tryptophan (Trp), glutamic acid (Glu), proline (Pro), arginine (Arg), lysine (Lys), and aspartic acid (Asp) (1 mM)] were added to this reaction mixture. Then the fluorescent responses were acquired. Subsequently, 100 μM H_2Se was added to the above reaction mixture until well blended, respectively. Then the fluorescent responses were again obtained. All data were acquired in 10 mM PBS at pH 7.4 (λ_{ex} = 480 nm, λ_{em} = 525 nm) at room temperature and atmospheric pressure.

Fluorescence Imaging. Fluorescence images of the living cells were obtained via the confocal fluorescence spectrometer. HepG2 cells were grown on confocal dishes and washed three times with PBS buffer (pH 7.4) before imaging with an objective lens (40×). Excitation of probe-loaded cells at 488 nm was performed using an argon laser, and the emitted light was collected with a META detector between 500 and 600 nm.

Solution Preparations for HRMS Analysis. A 0.0149 g portion of DL-2-amino-4-(methylthio)butyric acid (DL-Met), 0.0146 g of L-2,6-diaminohexanoic acid (L-Lys), and 0.0278 g of dipeptide Met-Lys were accurately weighed and dissolved in 10 mL of ultrapure water via three separate volumetric flasks to obtain a 10 mM stock solution of each.

Cross-Linking of Amino Acids. First, 1 mM DL-Met solution and 1 mM L-Lys solution were analyzed by HRMS. Next, DL-Met solution (50 μL, 10 mM) and L-Lys solution (50 μL, 10 mM) were combined in a 2 mL plastic tube and dissolved in 400 μL of acetonitrile. The data were obtained by HRMS. Afterward, the mixture was titrated with 2 mM HOBr until a white haze was obtained. Then the reaction mixture was centrifuged, and 100 μL supernatant solutions were added to 100 μL of acetonitrile for HRMS analysis. H_2Se (5 mM, 1 mL) was added to the above mixture in a vortex and the resulting mixture analyzed by HRMS.

Cross-Linking of the Dipeptide. The dipeptide Met-Lys solution (100 μL, 10 mM) in acetonitrile/water (1:1 by volume) was analyzed by HRMS. First, the dipeptide Met-Lys solution was titrated with 2 mM HOBr at room temperature until a white haze was obtained. The resulting mixture was then centrifuged, and the supernatant solution (100 μL) was combined with 100 μL of acetonitrile for HRMS analysis. In addition, the mixture was also treated with H_2Se (5 mM, 1 mL) before the final HRMS analysis.

SDS−PAGE Analysis of the NC1 Fraction. A stock solution of the NC1 fraction was prepared. A 1 mg portion of the lyophilized powder of the NC1 fraction was dissolved in 1 mL of ultrapure water with a final concentration of 1 μg/μL. The NC1 fraction, NC1 fraction with 100 μM H_2Se, and NC1 fraction with 100 μM H_2Se and 200 μM HOBr were incubated with a nonreducing loading buffer (5×) at 100 °C for 5 min and analyzed by nonreducing SDS−PAGE in 12% (w/v) bisacrylamide minicells with Tris−glycine−SDS running buffer. After the frations were stained with Coomassie blue (Solarbio, China), gel images were acquired and analyzed in a ChemiDoc touch imaging system using Image Lab 5.2 software.

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RESULTS AND DISCUSSION

Following the previous method,[22] BPP was prepared to examine its sensing mechanism toward cBPP for H_2Se (Figure 1a) via high-resolution mass spectrometry (HRMS). The

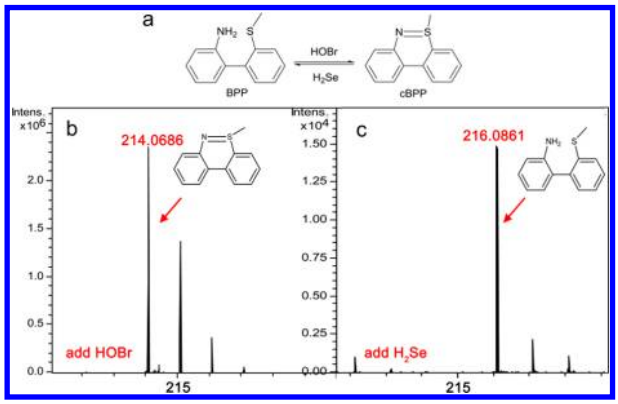

Figure 1. (a) Proposed reaction mechanism of BPP and cBPP for the HOBr/H_2Se-induced cycle. (b, c) HRMS spectra for (b) the reaction solution by adding 5 μM BPP and 20 μM HOBr and (c) the following mixture by adding 100 μM H_2Se to the mixture in (b).

HRMS spectrum of the reaction between BPP and HOBr was obtained in 1:1 acetonitrile/water at room temperature and atmospheric pressure, which yields a characteristic peak at m/z 214.0686 ($[M + H]^+$) (Figure 1b) that matches the theoretical mass of the reaction product cBPP (214.0684 $[M + H]^+$). Subsequently, by adding H_2Se to the above mixture, a dominant peak at 216.0861 ($[M + H]^+$) that corresponds to BPP (theoretical mass 216.0841 $[M + H]^+$) emerged (Figure 1c), indicating that H_2Se can break the sulfilimine bond of cBPP rapidly.

The change in the fluorescence intensities of cBPP ($\lambda_{ex}/\lambda_{em}$ = 480/525 nm) with the presence of H_2Se was studied.[22] A gradually decreasing fluorescence intensity was observed with increasing H_2Se concentrations from 0 to 100 μM, and a well-correlated linearity was obtained (Figure 2a). The regression equation was $F = 401.1294 - 3.3512[H_2Se]$ μM with a linear coefficient of 0.9914, while the detection limit (3 S/m) was 8 μM in 10 mM phosphate-buffered saline (PBS) at pH 7.4 (Figure 2b). Afterward, the response time of cBPP to H_2Se was evaluated via a kinetic experiment (Figure S1, Supporting Information). As cBPP was prepared with HOBr and BPP, the fluorescence intensity immediately increased to its maximum. After 15 min, while H_2Se was added to the above reaction solution, an instant decrease in fluorescence intensity proved that cBPP has a fast response to H_2Se.

Generally, the pH has a significant impact on the probes' performance in living systems; thus, the pH influence on the fluorescence responses of BPP to HOBr and cBPP to H_2Se were investigated at different pH values (Figure S2, Supporting Information). Both fluorescent responses exhibit no significant change at various pH values, indicating that pH varying from 2.0 to 12.0 hardly affects the fluorescence properties. Hence, BPP and cBPP have promising sensing abilities to HOBr and H_2Se under stimulated physiological conditions, respectively.

Additionally, to further investigate the specificity of cBPP toward H_2Se, cBPP was tested against various interfering agents under stimulated physiological conditions (10 mM PBS, pH 7.4). Considering H_2Se's high reductivity, herein different kinds of reductants as comparison groups were used. Typically,

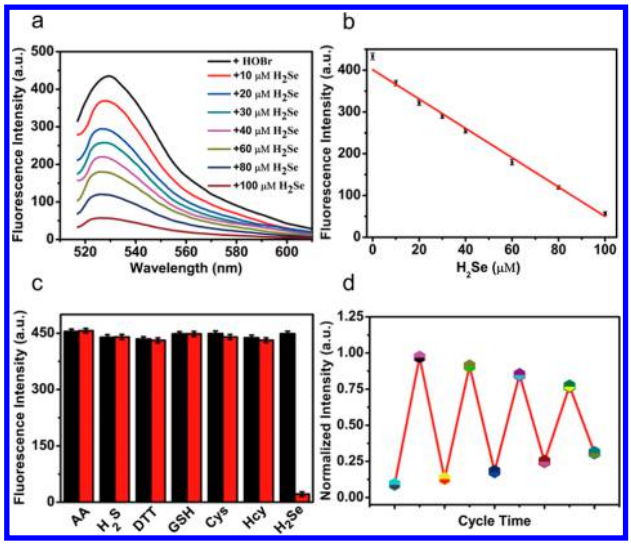

Figure 2. (a) Fluorescence responses of cBPP with H_2Se under different concentrations (0–100 μM). (b) Linear relationship between fluorescence intensities and H_2Se concentrations with a range from 0 to 100 μM. (c) Fluorescence intensities of cBPP (black bars) with different additional reductants (red bars). (d) Normalized fluorescence intensities of BPP (5 μM) and cBPP reversible cycles. All data of (a)–(d) were acquired in 10 mM PBS at pH 7.4 (λ_{ex} = 480 nm, λ_{em} = 525 nm).

cBPP was first obtained by the reaction between BPP and HOBr and then treated with H_2S, ascorbic acid (AA), dithiothreitol (DTT), glutathione (GSH), cysteine (Cys), homocysteine (Hcy), and H_2Se, respectively. In Figure 2c, the fluorescence intensities of cBPP decreased immediately after treatment with H_2Se, but none of the other reductants triggered fluorescence weakening. Furthermore, the response of cBPP toward metal ions and amino acids was examined (Figure S3, Supporting Information), and none of them triggered obvious fluorescence weakening, indicating no influence on the fluorescence interferences. These results strongly confirmed that cBPP exhibited extraordinary selectivity toward H_2Se over possible in vivo interfering species derived from living cells. Thus, H_2Se can break the sulfilimine bond of cBPP specifically.

The reversibility of the reaction between BPP and cBPP in solution was also explored. BPP was first oxidized by 20 μM HOBr, followed by an addition of 100 μM H_2Se. When the fluorescence signals recovered back to the original levels, another 20 μM dose of HOBr was added to the reaction mixture (Figure 2d) to test the repeatability, and at least four cycles with a modest fluorescence decrement were acquired (F_{480nm}/F_{525nm}). Therefore, H_2Se is suitable for cyclic mediation of the sulfilimine cross-link via HOBr reaction with BPP under stimulated physiological conditions.

To better evaluate the regulatory effect of HOBr/H_2Se toward the sulfilimine bond, the bioimaging applications of BPP and cBPP for detecting the HOBr/H_2Se cycle in HepG2 cells were studied.[22] Meanwhile, our probe can be used in the following living cell experiment due to its low cytotoxicity via MTT assay (Figure S4, Supporting Information). The cells were incubated at 37 °C for 30 min in Dulbecco's modified Eagle's medium (DMEM) containing 25 μM BPP (Figure 3a) and treated with 50 μM HOBr for 30 min (Figure 3b). Then 100 μM H_2Se was added to these cells for 20 min (Figure 3c), and the cells were treated with another 50 μM HOBr for 30

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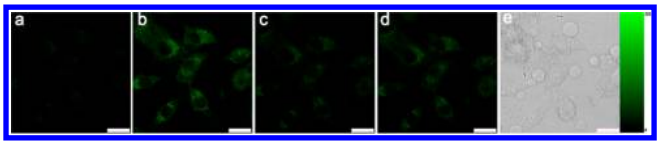

Figure 3. (a–d) Fluorescence confocal microscopic images of HepG2 cells loaded with BPP and exposed to cycles between HOBr and H_2Se. (e) Bright-field images of (a)–(d). All images were acquired with 488 nm excitation and an emission collection range of 500–600 nm. Scale bars = 25 μm.

min again (Figure 3d). The fluorescence intensity changed as follows: from the blank with probe only, it was first significantly increased upon the HOBr addition and then expressively decreased with H_2Se addition, but again reappeared stronger with another dose of HOBr. The further cell imaging experiment exhibited similar results for the quantitative fluorescence intensities under different visual fields (Figure S5, Supporting Information). In summary, these results reveal that BPP and cBPP allow the reversible detection of HOBr and H_2Se with high sensitivity and reversibility in living cells, which suggested that a HOBr/H_2Se conjugated system may have great potential in cyclically regulating the breakup and formation of the sulfilimine bond in biomolecules, such as amino acids, peptides, and proteins.

To further apply the HOBr/H_2Se system to amino acids, DL-2-amino-4-(methylthio)butyric acid (DL-Met) and L-2,6-diaminohexanoic acid (L-Lys) were chosen, and HRMS with high mass accuracy[38] was performed for analyzing the reaction process in methanol/water (1:1 by volume) at room temperature and atmospheric pressure. DL-Met and L-Lys first display signature peaks at m/z 150.0589 and 147.1148, respectively (Figures S6 and S7, Supporting Information), and their mixture shows identical peaks at 150.0586 and 147.1130 (Figure S8, Supporting Information), which correlates the theoretical total masses of DL-Met ($[M + H]^+$, 150.0583) and L-Lys ($[M + H]^+$, 147.1128). Afterward, HOBr was added to the above mixture, and a new intense peak signal was clearly found at 294.1456 ($[M + H]^+$) (Figure 4a),

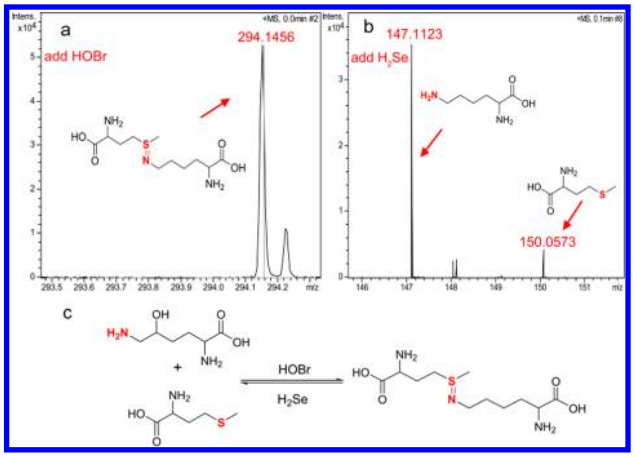

Figure 4. HRMS spectra for the cyclic reaction mediated by HOBr/H_2Se for the peptide. (a) Reaction product by adding 2 mM HOBr to the mixture of 1 mM DL-Met and 1 mM L-Lys. (b) Following reaction product by adding 5 mM H_2Se to the mixture in (a). (c) Proposed chemical reaction mechanism: structures of DL-Met and L-Lys and sulfilimine-cross-linked peptide formation by HOBr and breaking by H_2Se.

attributed to the sulfilimine-cross-linked peptide ($C_{11}H_{23}N_3O_4S$, 294.1482 ($[M + H]^+$) in vitro. Subsequently, the new sulfilimine-cross-linked peptide solution was treated with H_2Se, and two peaks of DL-Met ($[M + H]^+$, 150.0573) and L-Lys ($[M + H]^+$, 147.1123) were observed again (Figure 4b), indicating the new sulfilimine-cross-linked peptide was partially reduced to DL-Met and L-Lys by H_2Se. Eventually, HOBr was added to the above reaction mixture again to see the expected reappearance of the peak at 294.1454 ($[M + H]^+$) (Figure S9, Supporting Information). The results reveal that the HOBr/H_2Se conjugated system can promote the formation and breakup of the sulfilimine cross-link between the amino acids DL-Met and L-Lys with repeatability (Figure 4c).

Besides amino acids, the dipeptide Met-Lys was also applied for further investigation. The structure of dipeptide Met-Lys was first characterized by high-performance liquid chromatography and electrospray ionization mass spectrometry (Figures S10 and S11, Supporting Information), and then the signature peak at 278.1500 corresponding to the theoretical total mass (278.1533, $[M + H]^+$) was obtained via HRMS (Figure S12, Supporting Information). With HOBr addition to the dipeptide solution, a new peak agreeing with the theoretical total mass ($[M + H]^+$, 276.1376, $C_{11}H_{21}N_3O_3S$) appears at 276.1337 ($[M + H]^+$) following the disappearance of the primary dipeptide Met-Lys (Figure 5a). However, the process

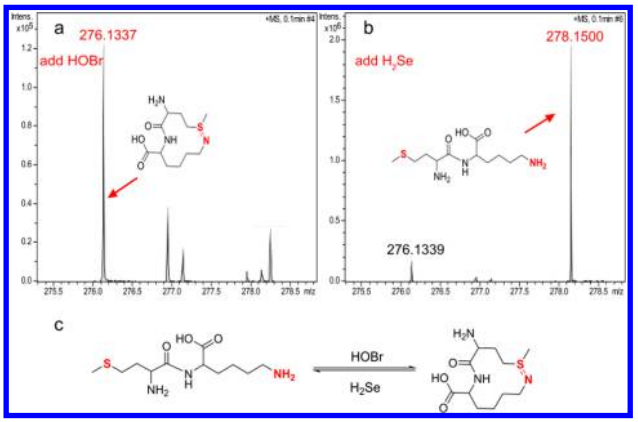

Figure 5. HRMS spectra for the cyclic reaction mediated by HOBr/H_2Se for the dipeptide. (a) Reaction product by adding 2 mM HOBr to the dipeptide Met-Lys solution. (b) Following product by adding 5 mM H_2Se to the solution in (a). (c) Proposed chemical reaction mechanism: structure of the dipeptide Met-Lys and sulfilimine-cross-linked dipeptide Met-Lys breaking by H_2Se and formation by HOBr.

can be reversed by adding H_2Se to the solution, indicating that the sulfilimine-cross-linked dipeptide Met-Lys could be broken in the presence of H_2Se (Figure 5b). Additionally, this reaction progression could be cycled by adding HOBr again (Figure S13, Supporting Information). Therefore, these results provide further evidence for the reaction mechanism (Figure 5c) that the HOBr/H_2Se system can mediate the sulfilimine cross-link as on–off circulation control.

Finally, the NC1 domain, known as a signature structural feature indicative of sulfilimine cross-links and governing the collagen IV network assembly,[16,39,40] was chosen to test the conjugated system for proteins. Typically, the NC1 fraction from bovine GBM as a protein model was examined via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Figure 6a and Figure S14 (Supporting Information) reveal that the NC1 fractions dissociated into

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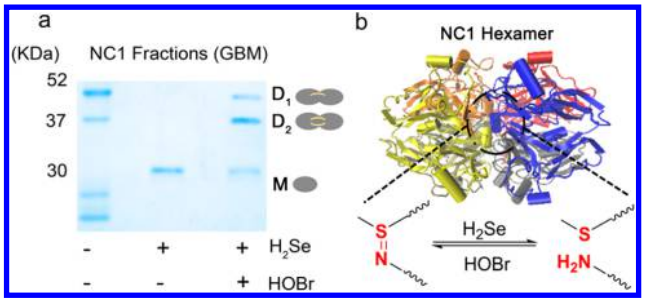

Figure 6. (a) SDS−PAGE comparison of NC1 banding patterns from bovine GBM. (b) Proposed reaction mechanism of the breaking and formation in NC1 hexamer via H_2Se/HOBr.

sulfilimine-cross-linked dimeric subunits (D_1 and D_2 represent the single- and double-cross-linked forms, respectively) and un-cross-linked monomers (M). After treatment with H_2Se, the single- and double-cross-linked binding patterns of the NC1 fractions disappeared in the gel with the concomitant generation of un-cross-linked monomers, showing H_2Se can effectively break the sulfilimine cross-link in NC1 hexamers. In parallel experiments, the NC1 fractions were treated with 100 μM H_2Se for 5 min and then reacted with 200 μM freshly prepared HOBr for another 5 min at room temperature. The D_1 and D_2 binding patterns reappeared in the gel, which further confirms that H_2Se can break the sulfilimine bond, while HOBr can effectively promote cross-link formation in a protein (Figure 6b). Thus, an ideal cyclic regulator to control the sulfilimine bond via H_2Se/HOBr was established.

CONCLUSIONS

In conclusion, H_2Se was found to specifically break the sulfilimine bond, and the HOBr/H_2Se system serving as an on−off cyclic regulation control for the sulfilimine bond was developed. Both in the dipeptide and NC1 hexamers, the system works well in regulating the formation and breakup of the sulfilimine bond. This HOBr/H_2Se-mediated conjugated system will lead to a new direction for describing the role of HOBr and H_2Se in living organism and provide a significant tool for further study of the physiological function of collagen IV. Moreover, it offers a new way for prediction and treatment of collagen IV related diseases, which also inspires us with great potential applications of the small molecules in modulating the activity and function of biomolecules and proteins in living systems.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b02228.

Experimental procedures and additional data (PDF)

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Notes

The authors declare no competing financial interest.

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