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

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ABSTRACT: The sulfilimine bond (-S=N-), found in Collagen IV scaffold, significantly stabilizes the architecture *via* the formation of sulfilimine crosslinks. However, to precisely govern the formation and break-up process of 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 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 circulate regulation system to modulate the sulfilimine bond in peptides and NC1 hexamers, which can offers a substantial system for further study in the physiological function of collagen IV.

INTRODUCTION

Collagen IV scaffolds, as an important tissue generation factor for fibrosis, is crucial for the formation and function of basement membranes (BMs).[1,2] Deficiency or disruption in the collagen IV scaffold causes BMs' destabilization and tissue dysfunction in humans.[3-5] Meanwhile, excessive crosslinking of collagen IV networks leads to its accumulation in BMs, which may contribute to pathological states such as hepatic[6-9] and renal fibrosis[10-15]. Therefore, study of the structure and function of type IV collagen[1,16-20] have been of great interest for controlling its *in vivo* activity. In 2009, Hudson's group discovered sulfilimine bond (-S=N-) in collagen IV, which stabilizes the scaffold *via* the formation of sulfilimine crosslinks 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] Afterwards, they further found out hypobromous acids (HOBr) catalyzes the formation of sulfilimine bonds through tissue genesis.[1] But up to date, controllable regulation on sulfilimine bonds and thus enhances the optimization of collagen IV is still in its preliminary step. Hence, to develop methods that can interrupt the sulfilimine bond is an urgently needed in the regulation of the 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. Besides, we also developed fluorescence probes for detecting hydrogen selenide (H_2Se) in organism.[24,25] Since H_2Se is 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 out that H_2Se exhibits excessive specificity to break sulfilimine bond. Hence, H_2Se serves as an ideal regulator for the "off mode" of sulfilimine bond in dipeptide Met-Lys and NC1 domains of 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 crosslink[30-33] *in vitro* as an "on-off" cyclic regulation control,[34,35] which will support further *in vivo* study in the physiological function of sulfilimine bond in collagen IV and treatment towards collagen IV related disease.

EXPERIMENTAL SECTION

Materials & 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). NC1 fraction from bovine glomerular basement membrane (GBM) was purchased from Chondrex (U.S.A). All other chemicals and solvents used were local products of analytical grades. Ultrapure water (18.2 MΩ·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 spectra (HRMS) were obtained *via*

Bruker maXis ultra-high resolution TOF MS system. Fluorescence spectra were obtained with a fluorescence spectrometer (Edinburgh). The fluorescence imaging was obtained with a confocal fluorescence spectrometer (Leica, Germany). Centrifugation was performed on an Eppendorf 5417R Centrifuge. SDS-PAGE images were acquired by the ChemiDoc™ Touch Imaging System (Bio-Red).

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

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

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

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 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 next applications, respectively.

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

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

Selectivity towards Metal Ions and Amino Acids. The selectivity of probe toward metal ions and amino acids were collected *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 into the above reaction mixture and the fluorescent responses was acquired. Subsequently, 100 μM H_2Se were added into the above reaction mixture until well blended, respectively. Then the fluorescent responses were again obtained. All data was acquired in 10 mM PBS at pH 7.4 (λ_{ex} = 480 nm, λ_{em} = 525 nm) at room temperature and atmosphere pressure.

Fluorescence Imaging. Fluorescence images of the living cells were obtained *via* 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. Accurately weighed 0.0149 g 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 dissolved in 10 mL of ultrapure water *via* three separated volumetric flasks respectively to obtained 10 mM stock solution of each.

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

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

SDS-PAGE Analysis of NC1 Fraction. The stock solution of NC1 fraction was prepared. 1 mg lyophilized powder of NC1 fraction was dissolved in 1 mL of ultrapure water with final concentration of 1 μg/μL. NC1 fraction, NC1 fraction with 100 μM H_2Se, NC1 fraction with 100 μM H_2Se and 200 μM HOBr were both incubated with a non-reducing loading buffer (5×) at 100 °C for 5 min and analyzed by non-reducing SDS-PAGE in 12% (wt/vol) bis-

acrylamideminicells with Tris-Glycine-SDS running buffer. After staining with Coomassie blue (Solarbio, China), gel images were acquired and analyzed in a ChemiDoc™ Touch Imaging System + imaging system using Image Lab 5.2 software.

RESULTS AND DISCUSSION

Following previous method,[22] BPP was prepared to examine its sensing mechanism towards cBPP for H_2Se (Figure 1a) *via* high-resolution mass spectroscopy (HRMS). HRMS spectrum of the reaction between BPP and HOBr was obtained in 1:1 acetonitrile/water at room temperature and atmosphere pressure, which yields a characteristic peak of 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), representing H_2Se can break the sulfilimine bond of cBPP rapidly.

The change on the fluorescence intensities of cBPP ($\lambda_{ex}/\lambda_{em}$ = 480/525 nm) with the presence of H_2Se was studied.[22] Gradual decreased 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 is 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). Afterwards, the response time of cBPP to H_2Se was evaluated *via* a kinetics experiment (Figure S1). As cBPP prepared through HOBr and BPP, the fluorescence intensities immediately increased to its maximum. After 15 min, while H_2Se added to the above reaction solution, an instant decrease in fluorescence intensities proves that cBPP owns fast response to H_2Se.

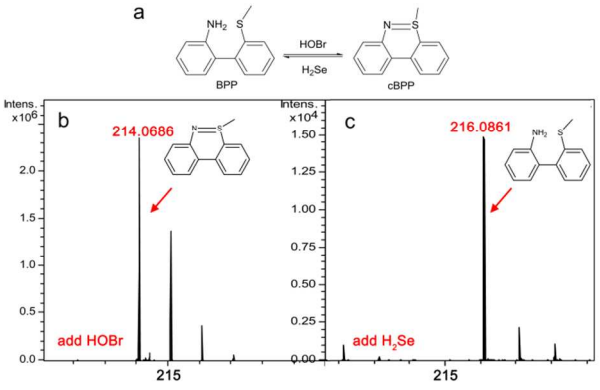

Figure 1. a) Proposed reaction mechanism of BPP and cBPP for the HOBr/H_2Se induced cycle. HRMS spectra for b) the reaction solution by adding 5 μM BPP and 20 μM HOBr and c) the following mixture by adding 100 μM H_2Se to b).

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

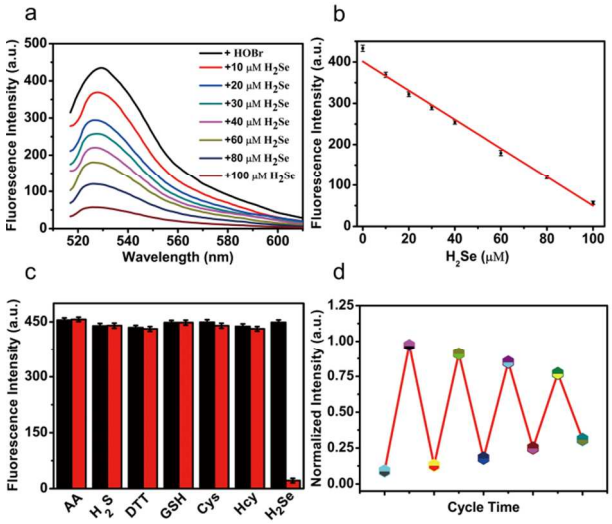

Figure 2. a) Fluorescence responses of cBPP with H_2Se under different concentrations (0~100 μM). b) The liner relationship between fluorescence intensities and H_2Se concentrations with a range from 0 to 100 μM. c) Fluorescence intensities of cBPP (black bars) with different additional reductants (red 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).

Additionally, to further investigate the specificity of cBPP towards H_2Se, cBPP was tested against various interferents 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, 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 treating with H_2Se but none of other reductants can trigger fluorescence weakening. Furthermore, the response of cBPP towards metal ions and amino acids were examined (Figure S3), and none of them triggered obvious fluorescence weakening, representing no influences on the fluorescence interferences. These results strongly confirmed that cBPP exhibited extraordinary selectivity towards 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 firstly oxidized by 20 μM HOBr, followed by an addition of 100 μM H_2Se. When fluorescence signals recovered back to the original levels, another 20 μM dose of HOBr was added to the reaction mixture (Figure 2d) to test repeatability, and at least four times with a modest fluorescence decrement

was acquired (F_{480nm}/F_{525nm}). Therefore, H_2Se is suitable for cyclic mediating the sulfilimine crosslink *via* HOBr reaction with BPP under stimulated physiological conditions.

To better evaluate the regulatory effect of HOBr/H_2Se toward sulfilimine bond, the bio-imaging applications of BPP and cBPP for detecting the HOBr/H_2Se cycle in HepG2 cells was studied.[22] Meanwhile, our probe can be used in the following living cells experiment due to its low cytotoxicity *via* MTT assay (Figure S4). The cells were incubated under 37 °C for 30 min in Dulbecco's Modified Eagle Medium (DMEM) containing 25 μM BPP (Figure 3a), and treated with 50 μM HOBr for 30 min (Figure 3b). Secondly, 100 μM H_2Se were added into these cells for 20 min (Figure 3c), and then treated with another 50 μM HOBr for 30 min again (Figure 3d). The fluorescence intensities changed as follows: from blank with probe only, it was first significantly increased upon the HOBr addition; then expressively decreased with H_2Se addition; but again reappeared stronger with another dose of HOBr. The further cells imaging experiment exhibited the similar results by the quantitative fluorescence intensities under different visual fields (Figure S5). In summary, these results reveal that BPP and cBPP allow the reversible detection of HOBr and H_2Se with highly sensitivity and reversibility in living cells, which suggested that a HOBr/H_2Se conjugated system may have great potential in cyclically regulating of the break-up and formation of sulfilimine bond in bio-molecular, such as amino acids, peptides and proteins.

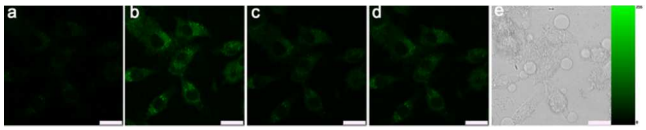

Figure 3. a)-d) Fluorescence confocal microscopic images of HepG2 cells loaded with BPP and exposed to cycles between HOBr and H_2Se. e) Bright field images of a)-d). All images were acquired with 488 nm excitation and emission collection range of 500–600 nm. Scale bars = 25 μm.

To further apply the HOBr/H_2Se system into 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 applied for analyzing the reaction process in methanol/water (1:1 by volume) at room temperature and atmosphere pressure. DL-Met and L-Lys first display signature peaks at m/z 150.0589 and 147.1148 respectively (Figure S6 and S7), and their mixture shows identical peaks at 150.0586 and 147.1130 (Figure S8) which correlates the theoretical total mass of DL-Met ($[M+H]^+$: 150.0583) and L-Lys ($[M+H]^+$: 147.1128). Afterwards, HOBr was added into the above mixture and a new intensive peak signal was clearly found at 294.1456 ($[M+H]^+$) (Figure 4a), attributing 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) was 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). The results reveal that HOBr/H_2Se conjugated system can promote the formation and break-up of sulfilimine crosslink between the amino acids DL-Met and L-Lys with repeatability (Figure 4c).

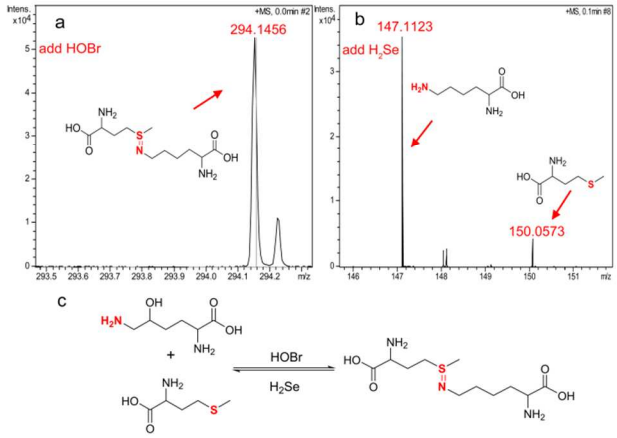

Figure 4. HRMS spectra for the cyclic reaction mediated by HOBr/H_2Se for peptide. a) The reaction product by adding 2 mM HOBr to the mixture of 1 mM DL-Met and 1 mM L-Lys. b) The following reaction product by adding 5 mM H_2Se to a). c) Proposed chemical reaction mechanism: structure of DL-Met, L-Lys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

Besides amino acids, the dipeptide Met-Lys was also applied for the further investigation. The structure of dipeptide Met-Lys were first characterized by high performance liquid chromatography and electrospray ionization mass spectrometry (Figure S10 and S11), and then the signature peak at 278.1500 corresponding to the theoretical total mass (278.1533 $[M+H]^+$) was obtained *via* HRMS (Figure S12). With HOBr addition into the dipeptide solution, a new peak agrees the theoretical total mass ($[M+H]^+$: 276.1376, $C_{11}H_{21}N_3O_3S$) shows up at 276.1337 ($[M+H]^+$) following the vanish of the primary dipeptide Met-Lys (Figure 5a). However, the process can be reversed by adding H_2Se into the solution, representing 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). Therefore, these results provide further evidence for the reaction mechanism (Figure 5c) that HOBr/H_2Se system can mediate the sulfilimine-cross-linked as "on-off" circulate control.

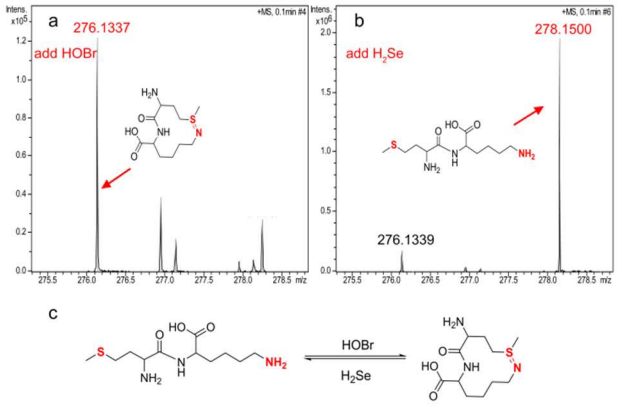

Figure 5. HRMS spectra for the cyclic reaction mediated by HOBr/H_2Se for dipeptide. a) The reaction product by adding 2 mM HOBr to the dipeptide Met-Lys solution. b) The following product by adding 5 mM H_2Se to a). c) Proposed chemical reaction mechanism: structure of the dipeptide Met-Lys and the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

Finally, NC1 domain, known as a signature structural feature indicative of sulfilimine crosslinks and governs the collagen IV network assembly,[16, 39, 40] was chosen to test the conjugated system for proteins. Typically, 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 reveal that NC1 fractions dissociated into sulfilimine cross-linked dimeric subunits (D_1 and D_2 represent single and double cross-linked forms, respectively) D_1 and uncrosslinked monomers (M). After treating with H_2Se, the single and double cross-linked binding patterns of NC1 fractions were disappeared in gel with the concomitant generation of uncrosslinked monomers, showing H_2Se can effectively break the sulfilimine crosslink in NC1 hexamers. In parallel experiments, 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 gel, which further confirms that H_2Se can break the sulfilimine bond, while HOBr can effectively promote crosslink formation in a protein (Figure 6b). Thus, an ideal cyclic regulator to control the sulfilimine bond *via* H_2Se/HOBr was established.

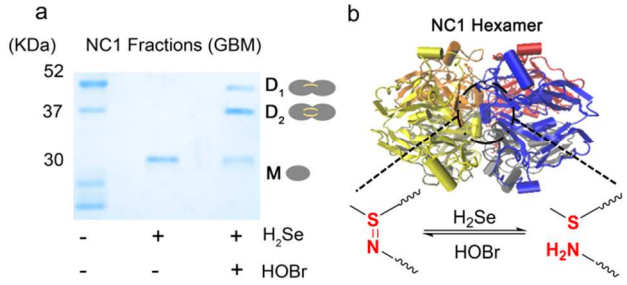

Figure 6. a) SDS-PAGE comparison of NC1 banding patterns from bovine GBM. b) Proposed reaction mechanism of the breaking and formation in NC1 hexamer *via* H_2Se/HOBr.

CONCLUSIONS

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

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and additional data. The Supporting Information is available free of charge *via* the Internet at http://pubs.acs.org.

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Author Contributions

‡These authors contributed equally.

Notes

The authors declare no competing financial interest.

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