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Supplementary Information

Photo-crosslinkable Elastomeric Protein-derived Supramolecular Peptide Hydrogel with Controlled Therapeutic CO-release

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Materials and Instruments Rink Amide MBHA resin (100–200 mesh), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetra-methyluronium hexafluorophosphate (HBTU), fluorenlymethoxycarbonyl (Fmoc)-protected amino acids such as Fmoc-Phe-OH, Fmoc-Glu(Otbu)-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Gly-OH, Fmoc-Tyr(tBu)-OH, and *N*-ethyldiisopropylamine (DIPEA, 99%) were purchased from Merck (Darmstadt, Germany). Boc-Asp-OtBu was purchased from BeadTech (Seoul, Republic of Korea). Lyophilized myoglobin from equine heart, tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$), sodium methoxide (NaOMe) solution (0.5 M), trifluoroacetic acid (TFA, 99%), calcium chloride ($CaCl_2$) anhydrous powder (99.99%), hydrogen peroxide (H_2O_2, 30%), and tricarbonylchloro(glycinato)ruthenium(II) (CORM-3, $Ru(CO)_3Cl(glycinate)$) were purchased from Sigma-Aldrich (St Louis, MO, USA). Sodium dithionite ($Na_2S_2O_4$) was obtained from Junsei Chemicals Co., Ltd (Tokyo, Japan). Ammonium persulfate (APS), triisopropylsilane (TIS), and tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate ($Ru(bpy)_3Cl_2$) were purchased from TCI Chemicals CO., Ltd (Tokyo, Japan). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP, 99+%) and *N,N*-dimethylformamide (DMF, 99.5%) were obtained from Thermo Fisher Scientific (San Jose, CA, USA). Dicholoromethane (DCM, 99.5%), *N*-methylpyrrolidone (NMP, 99.7%), piperidine (99%), diethyl ether (Et_2O, 99%), and dimethyl sulfoxide (DMSO, 99%) from Daejung Chemicals (Siheung, Republic of Korea) were used as received. Cell Counting Kit-8 (CCK-8) assays kit was obtained from Dojindo Laboratories (Kumamoto, Japan).

Techniques Peptides were sequenced using CEM Focused Microwave™ Synthesis System, Discover (Matthews, NC, USA) and purified using YL9100 high pressure liquid chromatography (HPLC, Anyang, Republic of Korea) equipped with a C18 reverse phase chromatographic column. Mass spectrometry was performed on a Bruker Ultraflextreme matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) using a matrix, α-cyano-4-hydroxycinnamic acid dissolved in acetonitrile:water = 1:1 mixed solution (0.1% TFA). Ultraviolet-visible (UV-vis) absorption spectra were recorded from a NEOSYS-2000 spectrometer (Scinco, Seoul, Republic of Korea). Fluorescence spectra were obtained from FS-2 fluorescence spectrometer (Scinco, Seoul, Republic of Korea). Fourier transform infrared (FTIR) spectra were obtained from a FTS-175C (Bio-Rad Laboratories, Inc., Cambridge, MA, USA). Circular dichroism (CD) measurement was carried out with a Jasco J-810 Spectropolarimeter (Jasco Inc., Tokyo, Japan). Transmission electron microscopy (TEM) micrographs were taken from 120 kV TEM (JEM-1400, JEOL, Tokyo, Japan). Inductively coupled plasma mass spectrometry (ICP-MS) was performed on a PerkinElmer OPTIMA 7300DV (Waltham, USA). Rheology was measured from Rheometer (MARS 40, Haake, Germany). HITACHI DT00771 projector lamp 285W (Tokyo, Japan) was used as a light source for photo-induced crosslinking.

1. Synthesis of peptides

i-KD **i-KD** (Fmoc-FFEEK(D)GGY, F; phenylalanine, E; glutamic acid, K; lysine, D; aspartic acid, G; glycine, and Y; tyrosine) was synthesized according to the conventional solid-phase peptide synthesis (SPPS) protocol[1] on CEM Focused Microwave™ Synthesis System. First, the Rink amide MBHA resin was washed with DCM and swollen in 1:1 mixed solution of DMF and DCM for over 30 min in shaking incubator. The Fmoc group of resin was removed by 20% piperidine in DMF over 3 min

twice on microwave and the resins were thoroughly washed with DCM and DMF. Then, the mixed solution of NMP with Fmoc-Tyr(OtBu)-OH (5.0 equiv.), HBTU (5.0 equiv.), and DIPEA (10.0 equiv.) was added to the resins and treated under microwave over 15 min. Then, Fmoc-Gly-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Glu(OtBu)-OH, and Fmoc-Phe-OH were coupled to the *N*-terminus of the peptide on resin in a sequence of FFEEKGGY-resin. The mtt group from the amine of the Lys(mtt) was removed with 4% TFA and 4% TIS in DCM over 30 min in shaking incubator. Then, the Boc-Asp-OtBu was added to the peptide solution. For each coupling and deprotecting procedure, kaiser test was conducted for confirming the presence of free amino group. The resin was treated with a cleavage solution (TFA:TIS:H_2O = 95:2.5:2.5) for over 3 h in shaking incubator. The excess TFA was removed by argon (Ar) gas blowing and the crude oily product was precipitated in cold Et_2O followed by centrifugation at 4000 rpm for 5 min. **i-KD** was purified by reverse-phase HPLC on C18 column (SUPELCO, Discovery® BIO Wide Pore C18, 5 μm, 10 x 250 mm) using linear gradient of water (0.1% TFA) and acetonitrile (0.1% TFA) at 2 mL min^{-1}, and the column eluents were monitored by UV absorbance at 230 and 254 nm (Figure S1a). The molecular weight of **i-KD** was confirmed by MALDI-TOF/TOF mass spectrometry: *m/z* 1312.6 (1312.5 calc'd m/z for $[M+H]^+$), *m/z* 1334.6 (1334.5 calc'd m/z for $[M+Na]^+$) (Figure S1c).

o-KD **o-KD** (Fmoc-FFEEGGK(D)Y) was synthesized on Rink Amide MBHA resin and purified using the same protocol described for **i-KD** (Figure S1b). The molecular weight of **o-KD** was confirmed by MALDI-TOF/TOF mass spectrometry: *m/z* 1312.5 (1312.5 calc'd m/z for $[M+H]^+$), *m/z* 1334.5 (1334.5 calc'd m/z for $[M+Na]^+$) (Figure S1d).

i-CORM **i-CORM** was synthesized by functionalization of purified **i-KD** with $[Ru(CO)_3Cl_2]_2$ in the presence of NaOMe.[2] **i-KD** (10 mg) was dissolved in HFIP (500 μL) and the solvent was removed by vacuum evaporation. **i-KD** was taken up in dry MeOH (1842 μL) and 0.5 M NaOMe solution (112 μL). And then, suspension was formed. A solution of $[Ru(CO)_3Cl_2]_2$ (2.1 mg) in dry MeOH (210 μL) was added to the suspension. The mixed solution was shaken for 36 h while protected from light as the release of CO from $[Ru(CO)_3Cl_2]_2$ can be triggered by light with ligand substitution.[3] The product was recovered by vacuum evaporation. The yellow solid compound was taken up in a solution of 0.05% ammonium hydroxide (3 mL). The solution was then lyophilized to yield a fluffy pale yellow powder. The molecular weight of **i-CORM** was confirmed by MALDI-TOF/TOF mass spectrometry (Figure S2a).

o-CORM **o-CORM** was synthesized by functionalization of purified **o-KD** with $[Ru(CO)_3Cl_2]_2$ with the same protocol described for **i-CORM**. The molecular weight of **o-CORM** was confirmed by MALDI-TOF/TOF mass spectrometry (Figure S2b).

2. Preparation of hydrogel

i- and o-KD hydrogel The stock solution of **i-KD** and **o-KD** was prepared in 10 mM NaOH, respectively before use. To make a 300 μL of hydrogel, 270 μL of peptide solution at a desired concentration was transferred to 1.5 mL microtube. And then, 30 μL of $CaCl_2$ solution (1.0 M) was slowly added on the top of the peptide solution. The solution was quickly gelled upon addition of $CaCl_2$.

i- and o-CO-releasing hydrogel (CORH) The co-assembled peptide solution of **i-KD** (or **o-KD**) with **i-CORM** or (**o-CORM**) in a 1:1 molar ratio was prepared in 10 mM NaOH before use. To make a 300 μL of hydrogel, 270 μL of peptide solution at a desired concentration was transferred to 1.5 mL microtube. And then, 30 μL of $CaCl_2$ solution (1.0 M) was slowly added on the top of the peptide solution. The solution was quickly gelled upon addition of $CaCl_2$.

3. Preparation of photo-crosslinked hydrogel

Visible light-induced crosslinking of hydrogel The stock solution of peptide was prepared in 10 mM NaOH before use. To make a 300 μL of hydrogel, 270 μL peptide solution at a desired concentration was transferred to 1.5 mL microtube, followed by addition of $Ru(bpy)_3Cl_2$ (8 μL, 18.9 mM) and APS (16 μL, 273.9 mM). The sample was vortexed and then 30 μL of $CaCl_2$ solution (1.0 M) was slowly added on the top of the peptide solution. To initiate the photo-crosslinking dityrosine formation, the resulting solution was irradiated with visible light.

4. Water stability of crosslinked hydrogel

A 900 μL of deionized water was added to each of 300 μL crosslinked and non-crosslinked peptide hydrogel in 1.5 mL microtube. And then, the hydrogel was stored with water in it. The hydrogels had been immersed up to 22 days.

5. Characterization

TEM A drop of each peptide in aqueous solution was placed on a formvar/carbon-coated copper grid and allowed to evaporate under ambient conditions. When sample was stained, a drop of uranyl acetate solution (2 wt %) placed onto the surface of the sample-loaded grid. The staining agent solution deposited about 3 min at least, and excess solution was wicked off by filter paper. The specimen was observed with a JEM-1400 operating at 120 kV and the electron micrographs were taken by a side-mounted 2k x 2k Veleta CCD camera (Olympus Soft Imaging Solutions, Münster, Germany). The data were analyzed with imaging software RADIUS (Olympus Soft Imaging Solutions, Münster, Germany).

FTIR spectroscopy FTIR measurements were performed using ZnSe pellet. The spectra of **i-CORM** and **o-CORM** in acidic or basic condition were monitored in the range of 1800–2200 cm^{-1}. FTIR spectra indicated the successful ligation of the metal carbonyl fragment to peptides and the CO-releasing capability (Figure 1b and Figure S2c).

CD spectroscopy An aqueous solution of peptides (0.1 mM, **i-KD**, **o-KD**, **i-CORM**, and **o-CORM**) was prepared in a quartz cuvette (1.0 cm light path length). CD spectra of each peptide solution were recorded in the wavelength range of 260−190 nm at room temperature. All reported spectra were baseline corrected.

Rheology measurements The experiments were carried out in frequency-sweep mode on a rheometer with parallel-plate geometry (20 mm) with 1.5 mm gap. An integrated temperature controller was used to maintain the temperature of the sample stage at 20 °C. The controlled strain was fixed at

0.05% and the oscillation frequency was from 0.1 to 10 Hz. The hydrogels were directly formed on the rheometer plate prior to the measurements.

UV-vis spectroscopy The peptide solution (**i-KD**, **o-KD**, **i-CORM**, and **o-CORM**) containing 140 μg mL^{-1}, APS (0.2 mM), and $Ru(bpy)_3Cl_2$ (14 μM) in a mixed solution of water:acetonitrile = 50:50 (v/v) were prepared in a quartz cuvette (1.0 cm light path length). The UV-vis spectra were recorded using NEOSYS-2000 spectrometer with different light-irradiation times.

Fluorescence spectroscopy The peptide solution (**i-KD**, **o-KD**, **i-CORM**, and **o-CORM**) containing 140 μg mL^{-1}, APS (0.2 mM), and $Ru(bpy)_3Cl_2$ (14 μM) in a mixed solution of water:acetonitrile = 50:50 (v/v) were prepared in a quartz cuvette (1.0 cm light path length). The Fluorescence spectra were obtained using FS-2 fluorescence spectrometer with different light-irradiation times. Emission spectra were obtained in a range of 290 to 500 nm (λ_{ex} = 265 nm).

Myoglobin (Mb) kinetics assay For kinetic assays of CO-releasing materials including CORM-3, **i-CORM**, **o-CORM**, **i-CORH**, **o-CORH**, crosslinked **i-CORH** and **o-CORH** were prepared in 0.1 M phosphate buffer at pH 7.4. A Mb solution (2 mg·mL^{-1}, 66 μL) was freshly prepared by dissolving Mb in 0.1 M phosphate buffer and was degassed by bubbling with Ar gas for at least 15 min. The reason for addition of Ar is to saturate with protective gas prior to and after reduction with $Na_2S_2O_4$. A freshly prepared solution of $Na_2S_2O_4$ (24 mg·mL^{-1}) was added to the degassed Mb solution for conversion of Mb to deoxy-Mb. The volume ratio of $Na_2S_2O_4$: deoxy-Mb is 1:10 (v/v), providing a 0.1 mM deoxy-Mb solution. The deoxy-Mb solution was added to an appropriate amount of CO-releasing peptide solution to yield 80 μM solution. The solution was quickly transferred to a cuvette and absorption spectra were taken at room temperature at predetermined time points using a UV-vis spectrophotometer measuring between 500 and 600 nm wavelengths. All experiments were carried out in quintuplicate. Quantification of CO release was calculated from the obtained spectra according to the equation below as previously reported.[4]

$$[MbCO] / ([Mb] + [MbCO]) = (\varepsilon_{d542}/\varepsilon_{iso} - A_{542}/A_{iso})\cdot(\varepsilon_{iso}/(\varepsilon_{d542} - \varepsilon_{CO542}))$$

Where A_{542} and A_{iso} are the absorbance at each time point at 542 and 550 nm, respectively. ε_{d542}, ε_{CO542}, and ε_{iso} are the extinction coefficient of deoxy-Mb and MbCO at 542 nm, and of the isosbestic point at 550 nm, respectively. $\varepsilon_{d542}/\varepsilon_{iso}$ and $\varepsilon_{CO542}/\varepsilon_{iso}$ are 0.836 and 1.227, respectively.[4]

6. Cell viability of CO-releasing materials

Cell culture Rat embryonic cardiomyoblast-derived H9C2 cells were obtained from the Korean cell line bank (Seoul, Republic of Korea). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Logan, Utah, USA) supplemented with 10% FBS. All cell lines were routinely cultured at 37 °C in humidified atmosphere with 5% CO_2.

Cytotoxicity assay Cells were seeded in 96-well plates (100 μL per well) and treated with various concentrations of CORM-3, **i-CORM**, **o-CORM**, **i-CORH**, **o-CORH**, crosslinked **i-CORH** and **o-CORH** for a desired time. Cell viability and cytotoxicity were measured by CCK-8 assay according to the guidelines recommended. 10 μL of CCK-8 solution was added into each well and incubated

with cells for the desired time. The absorbance was measured on a microplate reader (Spectra Max M5, Molecular Device Co.) at 450 nm. Cell viability was obtained by comparing the absorbance of treated cells to that of control cells. All experiments were repeated three times independently.

Intracellular reactive oxygen species (ROS) detection Generation of intracellular ROS in H9C2 cells was measured using a 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) probe. CM-H_2DCFDA penetrates a cell membrane and enters the cells where intracellular ROS converts it to 2',7'-dichlorofluorescein diacetate (DCFDA) that trapped inside the cells. H9C2 cells were plated onto clear-bottom black side 96-well plates at 1×10^4 cells/well to measure ROS by fluorometric analysis. Cells were treated with CM-H_2DCFDA for 30 min and then 200 μM H_2O_2 for 30 min, followed by treatment and incubation of either CORM-3, **i-CORM**, **o-CORM**, **i-CORH**, **o-CORH**, crosslinked **i-CORH** or crosslinked **o-CORH** for 2 h. Fluorescence was measured with a microplate reader (Spectra Max M5, Molecular Device Co., λ_{ex} = 495 nm and λ_{em} = 527 nm).

7. Quantification of ruthenium complexed to the CORHs.[5]

Samples for quantitative analysis were prepared by taking 200 μL from the stock solution of **i-CORH** and **o-CORH**, respectively, and placed in a vial filled with nitric acid and hydrochloric acid with a volume ratio of 3:1. The solution was ultrasonicated overnight and then digested at 110 °C for 2 h. Samples were diluted in deionized water (10 mL) for ICP-MS analysis.

For **i-CORH**, calculated Ru content: 6.6 wt%, found: 6.7 wt%

For **o-CORH**, calculated Ru content: 6.6 wt%, found: 7.0 wt%

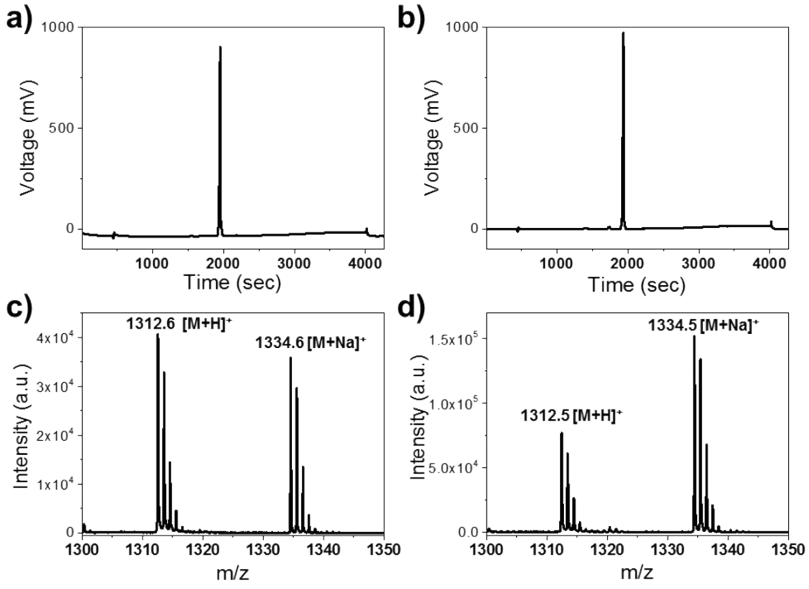

Figure S1. (a,b) HPLC and (c,d) MALDI-TOF/TOF mass spectra of (a,c) **i-KD** and (b,d) **o-KD**.

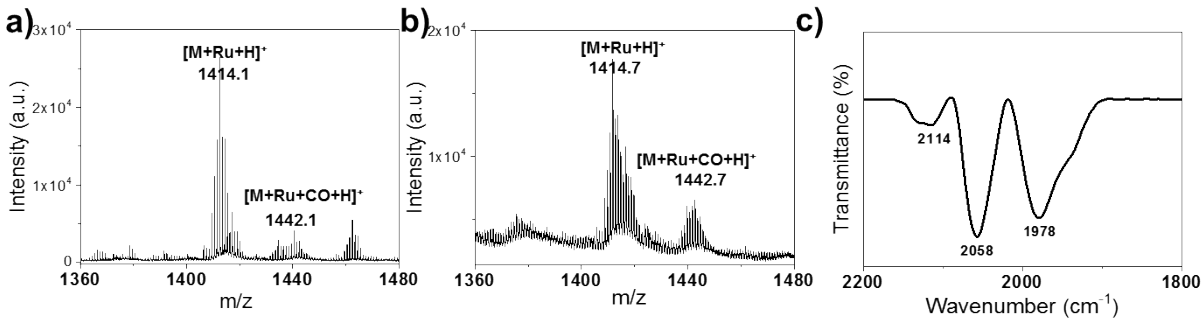

Figure S2. MALDI-TOF/TOF mass spectra of a) **i-CORM** and b) **o-CORM**. c) FTIR spectrum of **o-CORM** measured in acidic condition (pH = 3) indicating the successful attachment of CORM into **o-KD**.

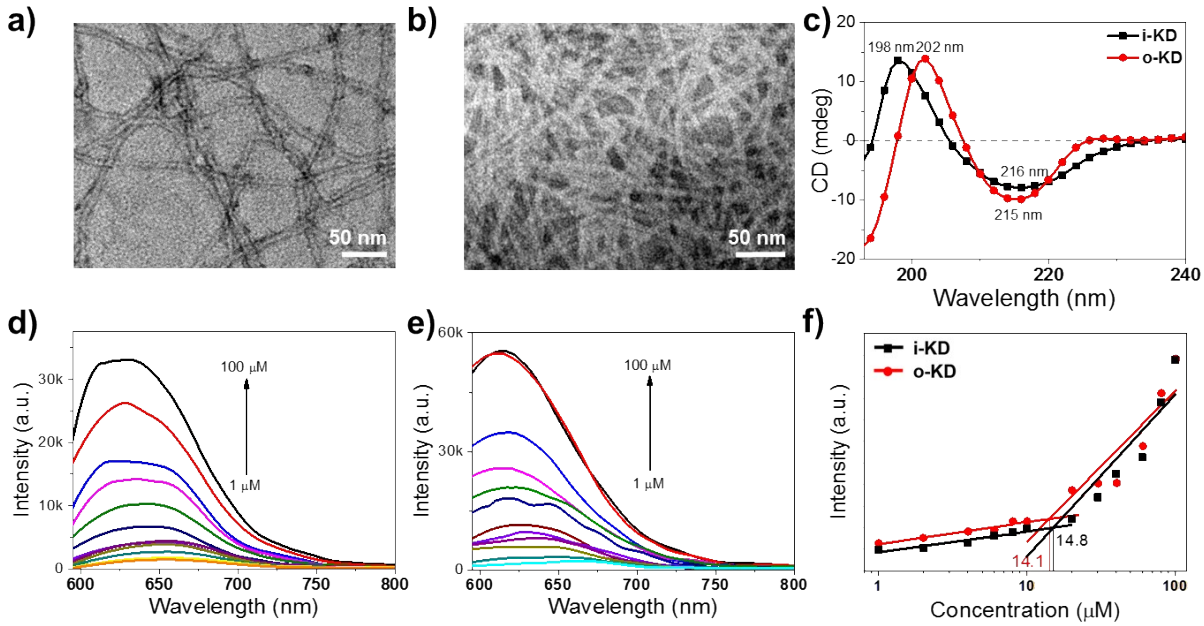

Figure S3. Negatively stained TEM images of self-assembled a) **i-KD** and b) **o-KD** (0.1 mM) with 2 wt% uranyl acetate. c) CD spectra of **i-KD** and **o-KD** in water. Emission spectra of Nile red-encapsulated self-assembled NF of d) **i-KD** and e) **o-KD** as a function of peptide concentration (λ_{ex} = 550 nm). f) The critical micellar concentrations (CMCs) of **i-KD** and **o-KD**, respectively, measured by using Nile red. The emission intensities of Nile red were measured at 614 nm.

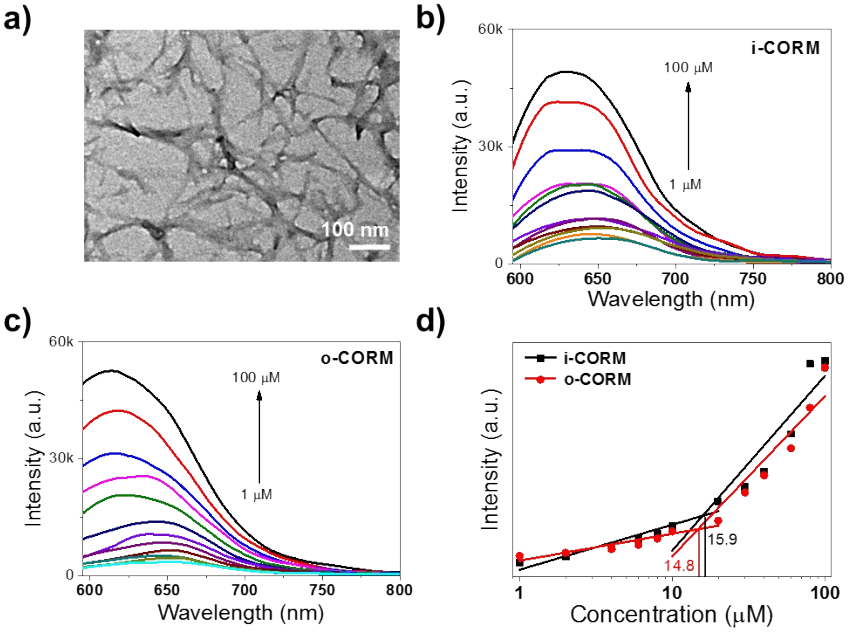

Figure S4. a) Negatively stained TEM image of self-assembled **o-CORM** (0.1 mM) with 2 wt% uranyl acetate. Emission spectra of Nile red-encapsulated NF of b) **i-CORM** and c) **o-CORM** as a function of peptide concentration (λ_{ex} = 550 nm). d) CMCs of **i-CORM** and **o-CORM**, respectively, measured by using Nile red. The emission intensities of Nile red were measured at 614 nm.

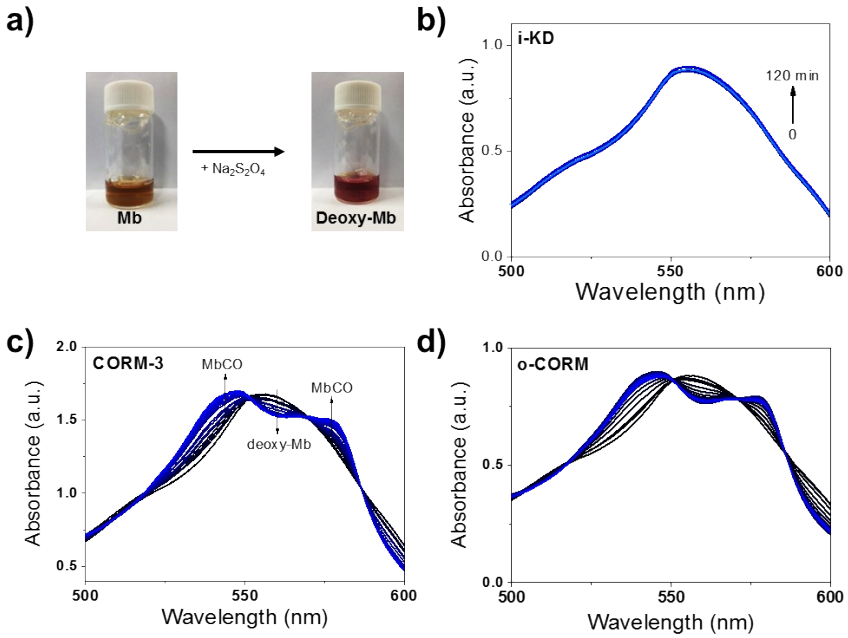

Figure S5. a) Formation of deoxymyoglobin (deoxy-Mb) by addition of $Na_2S_2O_4$ to myoglobin (Mb) solution. b) Time-course absorption spectra of the myoglobin Q-band for **i-KD** showing the no conversion of deoxy-Mb to carbonmonoxy myoglobin (MbCO) due to the absence of CORM-3 within molecular structure (40 µM in phosphate buffer, pH 7.4 at 37 °C for 2 h). Time-dependent absorption spectra of c) CORM-3 and d) **o-CORM** showing the conversion of deoxy-Mb to MbCO with CO-release determined by myoglobin assay (40 µM in phosphate buffer, pH 7.4 at 37 °C for 1 h).

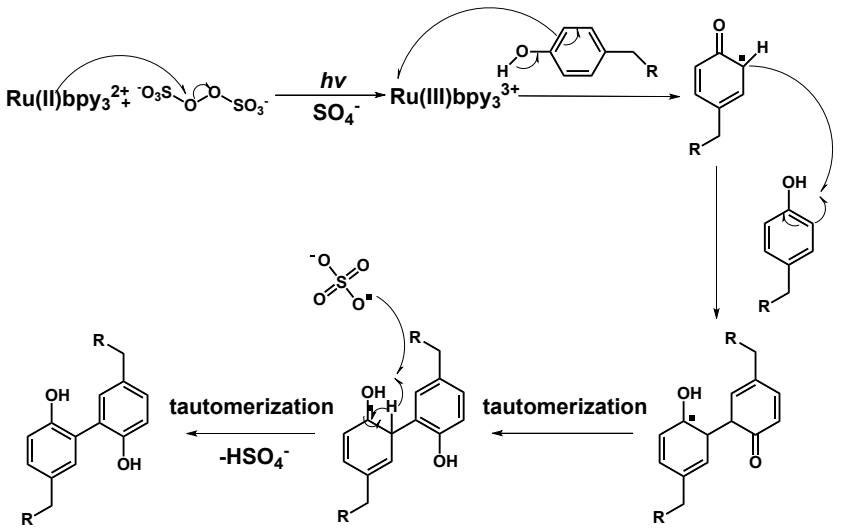

Figure S6. Proposed mechanism of photo-induced tyrosine-tyrosine dimerization.[6]

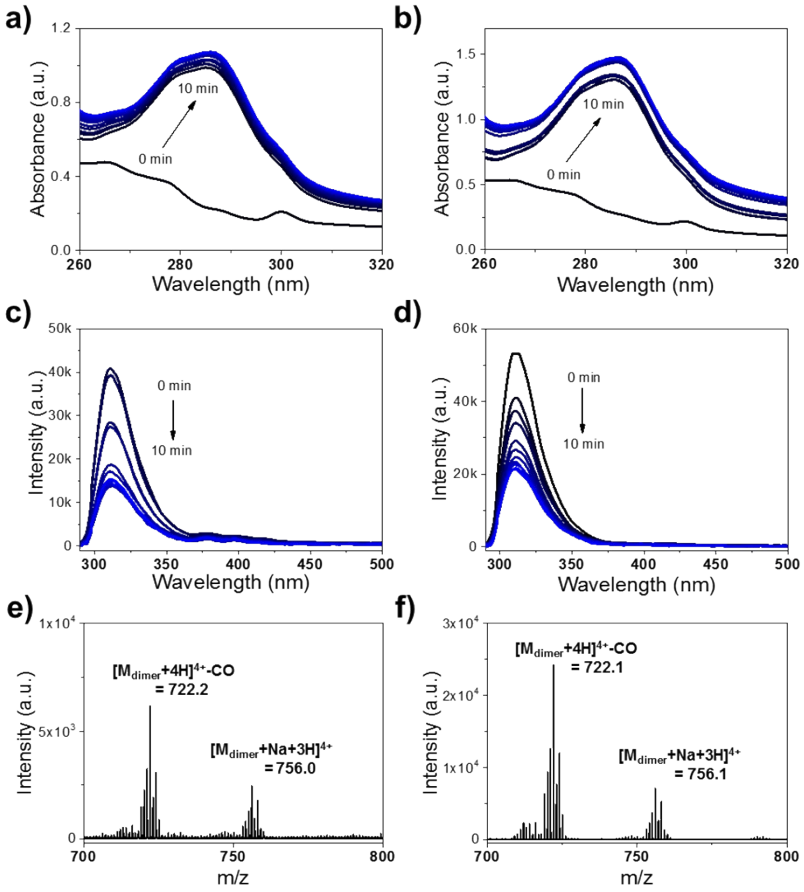

Figure S7. Spectroscopic evidence of the visible light-induced tyrosine-tyrosine dimerization of **i-CORM** and **o-CORM**. UV-vis spectra of a) **i-CORM** and b) **o-CORM** and emission spectra of c) **i-CORM** and d) **o-CORM** (λ_{ex} = 265 nm). MALDI-TOF/TOF mass spectra of e) **i-CORM** and f) **o-CORM** confirming the visible light-induced dimerization.

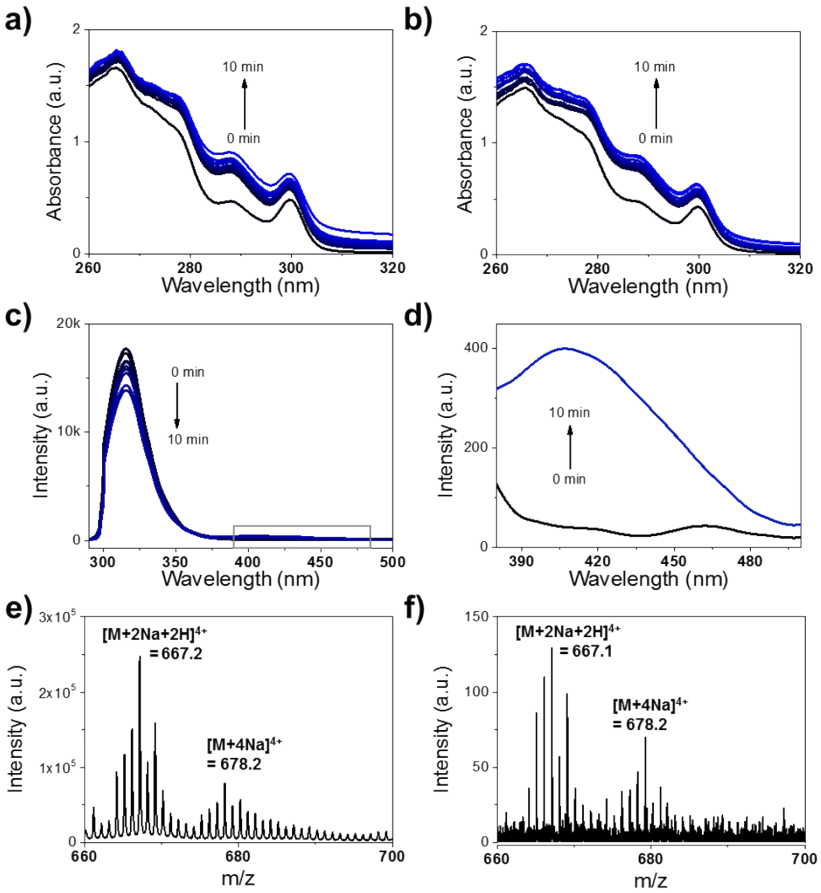

Figure S8. Spectroscopic evidence of the visible light-induced tyrosine-tyrosine dimerization. UV-vis spectra of a) **i-KD** and b) **o-KD** and c) emission spectra of **o-KD** (λ_{ex} = 265 nm). d) The magnified emission spectra of **o-KD** in grey solid line of c) to clarify the emergence of characteristic emission peak of dityrosine near 407 nm. MALDI-TOF/TOF mass spectra of e) **i-KD** and f) **o-KD** confirming the visible light-induced dimerization.

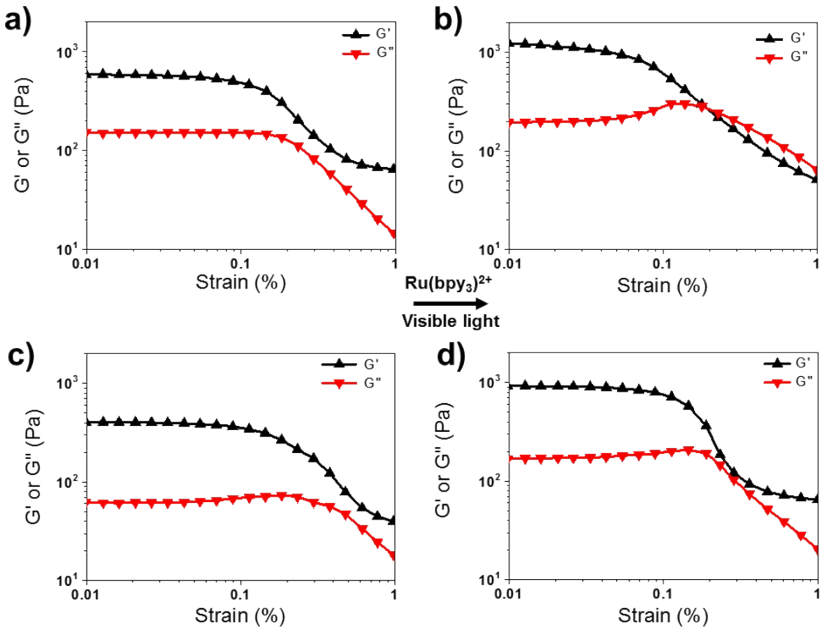

Figure S9. Strain sweep rheological measurement at a fixed frequency of 1.0 Hz of a,c) non-crosslinked and b,d) photo-crosslinked a,b) **i-CORH** and b,d) **o-CORH**.

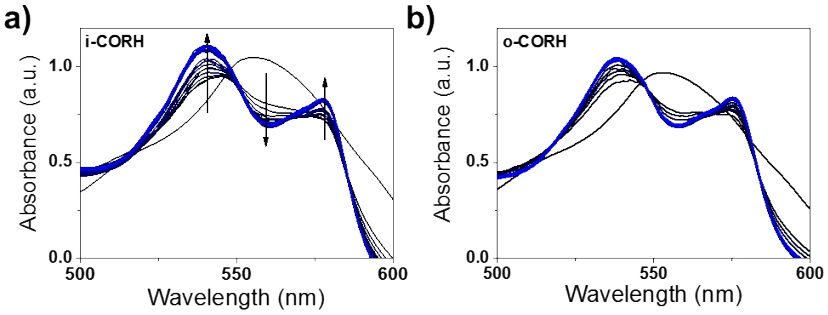

Figure S10. Absorption spectra of the myoglobin a) **i-CORH** and b) **o-CORH** showing the conversion of deoxy-Mb to MbCO with CO-release determined by myoglobin assay (80 μM in phosphate buffer, pH 7.4 at 37 °C for 2 h).

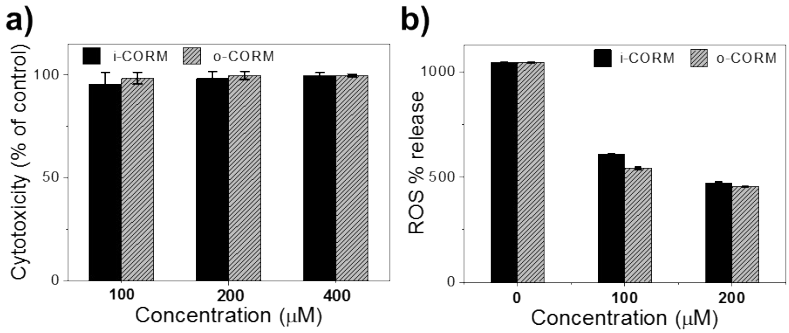

Figure S11. a) Cytotoxicity of **i-CORM** and **o-CORM** on H9C2 cells evaluated after 24 h incubation using CCK-8 assay as a function of the peptide concentration. b) Effect of the concentration-dependent treatment of **i-CORM** and **o-CORM** on H_2O_2-mediated ROS synthesis in H9C2 cells. Cells were treated with CM-H_2DCFDA for 30 min and then 200 μM H_2O_2 for 30 min, followed by treatment and incubation of either **i-CORM** or **o-CORM** for 2 h.

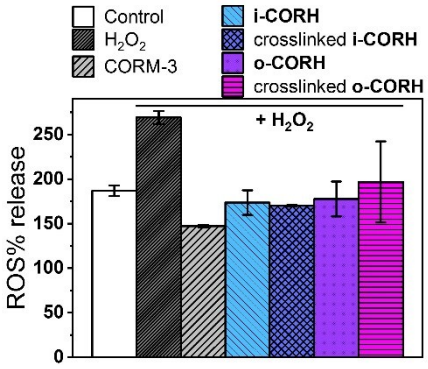

Figure S12. ROS release of non-crosslinked and crosslinked **i-CORH** and **o-CORH** in H9C2 cells. Cells were treated with CM-H_2DCFDA for 30 min and then 200 μM H_2O_2 for 30 min, followed by treatment and incubation of either CORM-3, **i-CORH**, **o-CORH**, crosslinked **i-CORH**, or crosslinked **o-CORH** for 24 h.

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