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Electronic Supporting Information

Polymer-Protein Hybrid Scaffolds as Carriers for CORM-3: Platforms for the Delivery of Carbon Monoxide (CO)

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Experimental methods

Materials: All chemicals were used as received unless otherwise indicated: Hexane (Ajax Chemical), acetone (Ajax Chemical), *N,N*-dimethylformamide (Sigma-Aldrich), Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich), 2,2′-dipyridyl disulfide (Sigma-Aldrich), 2-mercaptoethanol (Sigma-Aldrich), 1,3-dicyclohexylcarbodiimide (DCC, Sigma-Aldrich), 4-(dimethylamino)pyridine (DMAP, Sigma-Aldrich), tricarbonylchloro(glycinato)ruthenium (II) (CORM-3, Sigma-Aldrich), 2-Hydroxyethyl acrylate (HEA, Sigma-Aldrich), bovine serum albumin (BSA, Sigma-Aldrich). Dialysis membrane (MWCO 3500, MWCO 50000) were obtained from Spectrum Laboratories. 2,2-Azobisisobutylronitrile (162 g/mol, AIBN) which was purchased from Sigma-Aldrich was recrystallised from methanol before use. 5,5'-dithiol-bis-(nitrobenzoic acid) (Ellman's reagent, Sigma-Aldrich), was recrystallised from aqueous ethanol. RAFT chain transfer agent, 2-(((butylsulfanyl)carbothioyl)sulfanyl)propanoic acid, was synthesised according to the literature.[1] Deionised (DI) water was produced by a Milli-Q water purification and had a resistivity of 17.9 mΩ/cm. For the measurement of carbon monoxide release, myoglobin from equine skeletal muscle was purchased from Sigma-Aldrich.

Characterisation methods

NMR Spectroscopy*:* ^{1}H-NMR spectroscopy was conducted using Bruker DPX-300 (300MHz) and DPX-400 (400MHz) spectrometers. Chloroform-d3 ($CDCl_3$), Methanol-d4 (MeOD), Deuterium oxide (D_2O) was used as the solvent. All chemical shifts are quoted in parts per million (ppm) relative to tetramethylsilane (δ = 0 ppm), referenced to residual solvent frequencies (^{1}H NMR: $CDCl_3$ = 7.26; MeOD = 4.78; D_2O = 4.80).

Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy: ATR-FTIR spectra of polymer samples were obtained using a Bruker Spectrum BX FTIR system using diffuse reflectance sampling accessories. The spectrophotometer was equipped with a tungsten halogen lamp and Si/Ca beam splitter. Spectra were obtained at regular time intervals in the MIR region of 4000 – 500 cm^{-1} at a resolution of 4 cm^{-1} (64 scans) and analysed using OPUS software.

UV-Vis Spectroscopy*:* UV-vis measurements were performed on a CARY 300 spectrophotometer (Bruker) using a quartz cuvette.

Size exclusion chromatography *(SEC):* SEC was performed using dimethylacetamide (DMAc) as the eluent. The SEC system was a Shimadzu modular system comprising an auto injector, a Phenomenex 5.0 μm beadsize guard column (50 × 7.5 mm) followed by three Phenomenex 5.0 μm bead-size columns (10^5, 10^4, and 10^3 Å), and a differential refractive-index detector. The system was calibrated with narrow molecular weight distribution methyl methacrylate (MMA) standards with molecular weights ranging from 200 to 10^6 g mol^{-1}.

Dynamic light scattering (DLS)*:* DLS was carried out on a Malvern Zetasizer Nano Serries running DTS software (He-Ne laser, 4 mW, λ = 633 nm, angle 173^0). Polymer samples were dissolved in Milli-Q grade water (1mg/mL) and filtered through a 0.45 μm pore size filter to remove dust prior to analysis. The samples were transferred to disposable cuvettes for analysis.

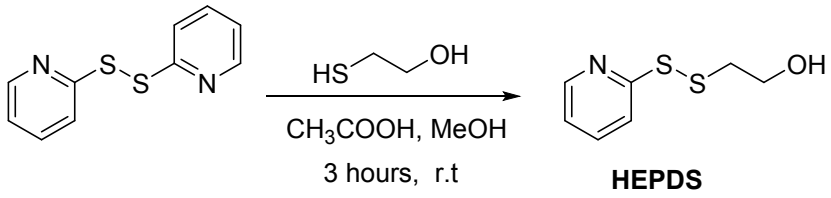
Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES): The percentage of ruthenium in the micelles was determined by the ICP-OES technique using a Perkin Elmer OPTIMA 7300 ICP

optical emission spectrometers. Briefly, 10 mg of sample was dissolved in 10 mL of DI water, and the solution was then analysed by ICP-OES using a detection wavelength of 240.272 nm.

Synthetic Procedures

Synthesis of pyridyl disulfide containing RAFT agent (PDS-RAFT)

Synthesis of hydroxyethyl pyridyl disulfide (HEPDS): 2,2′-dipyridyl disulfide (7.3 g, 33 mmol) was dissolved in 50 mL of methanol and then 0.85 mL of glacial acetic acid was added as a catalyst. To this mixture, a solution of 2-mercaptoethanol (3.2 g, 41 mmol) in methanol (15 mL) was added drop-wise at room temperature while vigorously stirring. The reaction was continued at room temperature for an additional 3 h. The resulting solution was evaporated under vacuum to obtain the crude product as a yellow oil, which was then purified by column chromatography on silica gel with a ethylacetate/hexane mixture as eluent. The second fraction was collected and the solvent was removed under reduced pressure to obtain the product as a colourless oil (yield: 69%).



Scheme S1. Schematic representation of synthetic approach to HEPDS

^{1}H-NMR ($CDCl_3$, 298K, 400 MHz, ppm) (see structure below for proton assignment):

δ 2.84-2.87 (t, 2H, H_e); 3.68-3.71 (t, 2H, H_f); 7.11-7.15 (ddd, 1H, H_d); 7.67-7.75 (m, 2H, H_{b+c}); 8.30-8.33 (m, 1H, H_a).

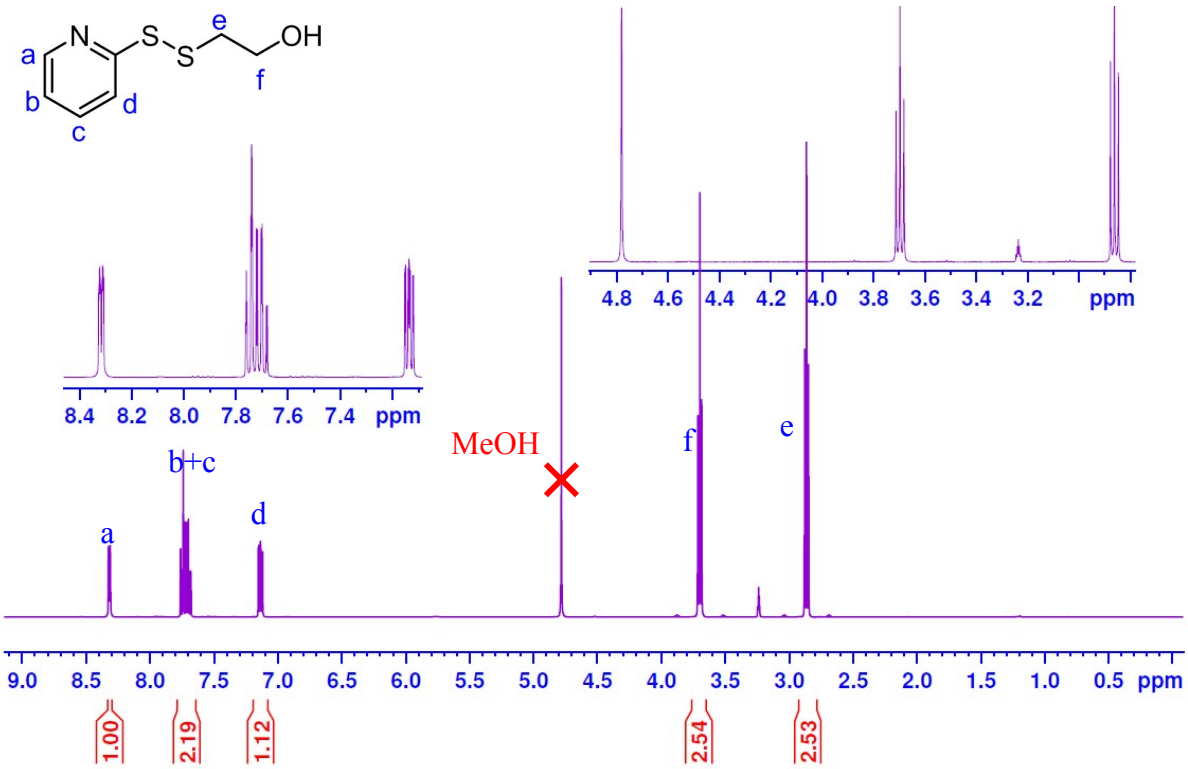
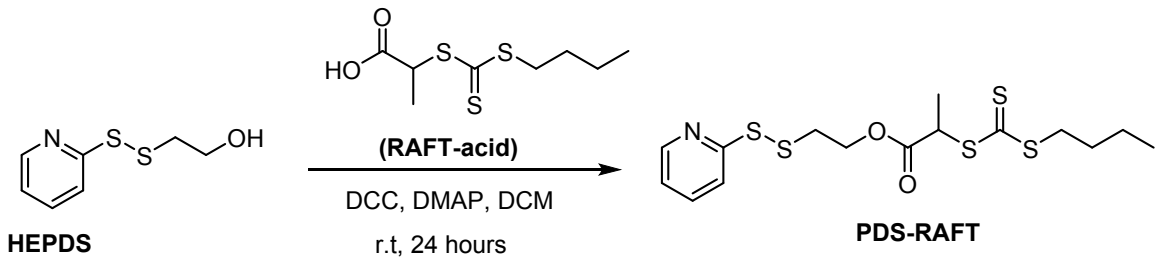


Figure S1. ^{1}H-NMR spectra of hydroxyethyl pyridyl disulfide (HEPDS)

Synthesis of pyridyl disulfide containing RAFT agent (PDS-RAFT)

2-(((butylsulfanyl)carbothioyl)sulfanyl)propanoic acid (664 mg, 2.8 mmol) and HEPDS (600 mg, 3.2 mmol) were introduced into a round bottom flask and dissolved in anhydrous dichloromethane (DCM, 10 mL). The reaction mixture was cooled to 0°C in an ice bath while vigorously stirring. DCC (660 mg, 3.2 mmol) and DMAP (34 mg, 0.28 mmol) were then added. After 1 h at 0°C and 24 h at room temperature, the mixture was then filtered and the solvent was evaporated under vacuum. The crude product was purified by silica column chromatography (petroleum spirit/ethyl acetate, yield: 61%).



Scheme S2. Schematic representation of synthetic approach to RAFT agent functionalised pyridyl disulfide PDS-RAFT.

^{1}H-NMR ($CDCl_3$, 298K, 400 MHz, ppm) (see structure below for proton assignment):

δ 0.89-0.93 (t, 3H, H_l); 1.36-1.46 (m, 2H, H_k); 1.57-1.59 (d, 3H, H_h); 1.63-1.70 (m, 2H, H_j); 3.02-3.05 (t, 2H, H_e); 3.33-3.36 (t, 2H, H_i); 4.36-4.39 (t, 2H, H_f); 4.76-4.82 (q, 1H, H_g); 7.07-7.10 (ddd, 1H, H_d); 7.61-7.69 (m, 2H, H_{b+c}); 8.44-8.46 (m, 1H, H_a).

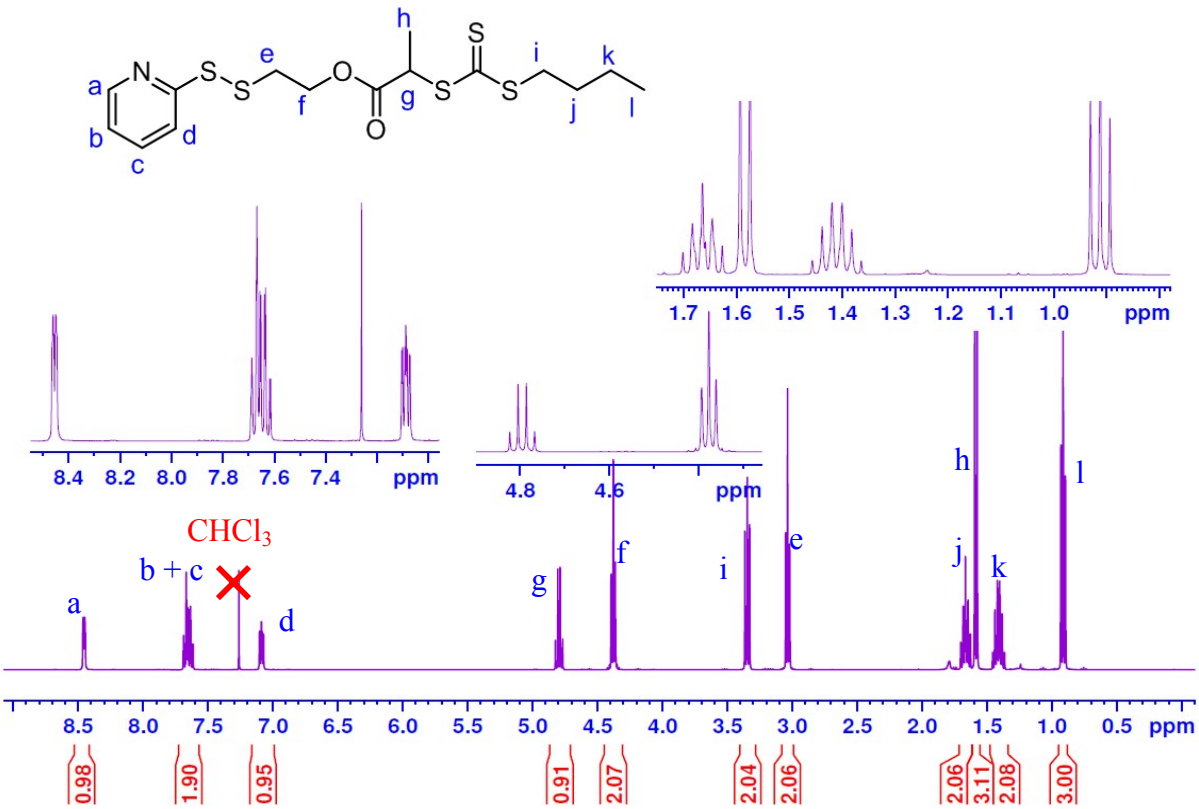
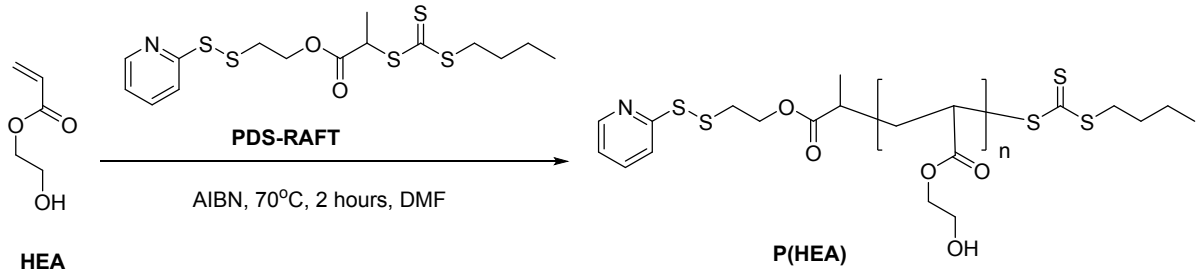


Figure S2. ^{1}H-NMR spectra of pyridyl disulfide containing RAFT agent (PDS-RAFT)

Synthesis of P(HEA)

[HEA]: [PDS-RAFT]: [AIBN] = 200 : 1.0 : 0.1 HEA (5.34 g, 46 mmol), PDS-RAFT (94 mg, 0.23mmol), AIBN (3.8 mg, 0.023 mmol) and DMF (16 mL) were added to a 50 mL round bottom flask equipped with a magnetic stirrer bar. The reaction mixture was deoxygenated with nitrogen for 30 min. The reaction mixture was then placed into an oil bath preheated to 70°C and the polymerisation was run for 2 h. Upon completion, the reaction was quenched in an ice bath for 15 min and two aliquots were collected for GPC and ^{1}H-NMR analysis. The monomer conversion was determined by ^{1}H-NMR analysis. The reaction solutions were diluted with acetone and the polymeric product was purified via

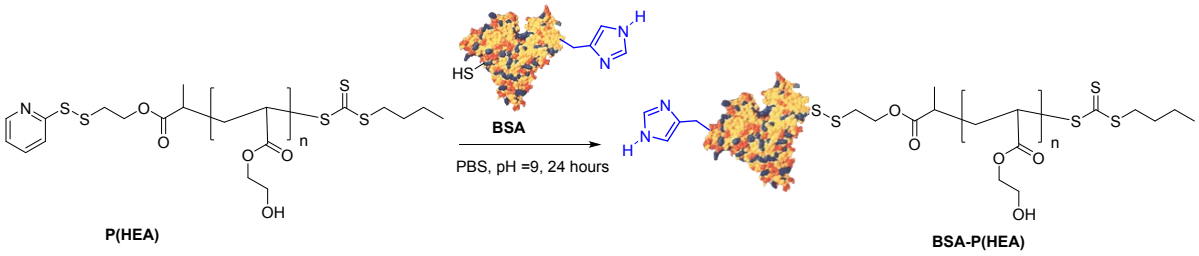
three precipitations with hexane and centrifuging (5 min, 8000 rpm). After removal of solvent and drying under vacuum, polymer P(HEA) with $M_{n,NMR}$ = 20 900 g/mol, $M_{n,\ SEC}$ = 23 300 g/mol and Đ = 1.20 was obtained.



Scheme S3. Schematic representation of synthetic approach to poly(2-hyroxyl athlacrylate) via RAFT polymerisation using RAFT agent functionalised pyridyl disulfide as RAFT agent.

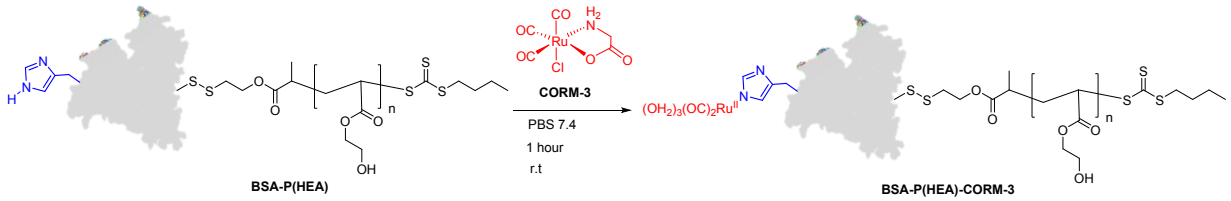
Conjugation of protein BSA to reactive polymer P(HEA)

Polymer P(HEA) (520 mg, 0.025 mmol) containing pyridyl disulfide was dissolved in 2 mL of phosphate buffer solution (PBS pH = 8.2) and added dropwise to a solution of BSA (55 mg, 8.33×10^{-4} mmol) in 5 mL of PBS. The reaction mixture was stirred at room temperature. After 24 h, the mixture was dialysed (MWCO 50 000 Da) against DI water for 2 days to remove excess polymer and then freeze dried. Quantification of polymer conjugated protein was evaluated via Ellman's assay.



Scheme S4. Schematic representation of synthetic approach to protein-polymer hybrid BSA-P(HEA).

Conjugation of CORM-3 to polymer-protein hybrid*:* A PBS (pH = 7.4) solution of CORM-3 (3 mg, 0.01 mmol)) was added to a PBS solution of protein-polymer BSA-P(HEA) (17 mg, 1.96×10^{-4} mmol). The reaction mixture was stirred for 1 h at room temperature in the absence of light. The reaction medium was dialysed (MWCO 3500 Da) against DI water and then freeze dried.



Scheme S5. Schematic representation of synthetic approach to CORM-3 conjugated to protein-polymer hybrid BSA-P(HEA)-CORM-3

Studies of CO release by myoglobin assay

Myoglobin assay procedure: The amount of CO liberated from BSA-P(HEA)-CORM-3, was quantified by myoglobin assay. The myoglobin assay procedure was similar to as previously published by our group[2] with slight modification. A stock solution of myoglobin (Mb) from equine skeletal muscle (2 mg/mL) was freshly prepared in 0.1 M phosphate buffer solution (PPS, pH = 7.4) and deoxygenated by bubbling with nitrogen for at least 15 min. To this degassed solution was added a freshly prepared solution of sodium dithionite (24 mg/mL) at 1:10 dithionite/Mb (v/v) to convert met-Mb to deoxy-Mb. UV-Vis spectra were recorded before and after addition of reducing agent to confirm complete reduction of myoglobin. Two controls were performed in duplicate, the negative control (0% CO-Mb), a deoxy-Mb solution and the positive control (100% CO-Mb), obtained by bubbling pure CO gas into deoxy-Mb solution. An aliquot of BSA-P(HEA)-CORM-3 and PBS were then added to the deoxy-Mb solution to produce final concentrations of 5 µM while keeping $A_{557} < 1$. This solution was quickly transferred to a cuvette and then overlaid with 500 µL light mineral oil (Sigma) to prevent CO escaping and myoglobin being oxygenated. The absorption was recorded at room temperature at predetermined time points using a CARY 300 spectrophotometer, measuring between 500 nm and 600 nm with a step of 2 nm.

The amount of MbCO formed was calculated utilising the formula as previously reported.

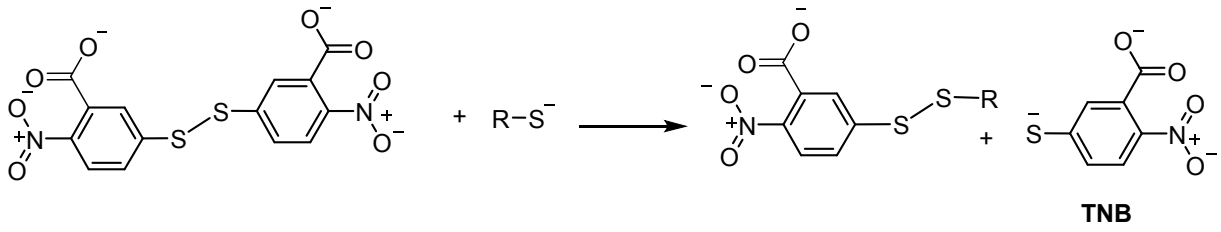
$$\frac{[COMb]}{[Mb]+[COMb]} = \left(\frac{\varepsilon_{d542}}{\varepsilon_{iso}} - \frac{A_{542}}{A_{iso}}\right).\left(\frac{\varepsilon_{iso}}{\varepsilon_{d542} - \varepsilon_{CO542}}\right)$$

Where: A_{542} and A_{iso} are the measured absorbance at each time point at 542 and 552 nm, respectively. ε_{d542}, ε_{CO542} and ε_{iso} are the extinction coefficients of deoxyMb at 542 nm, COMb at 542 and of the isosbestic point at 552 nm, respectively.

The concentration of MbCO was calculated and plotted against different time points. By applying a suitable fit function, the plateau level of the MbCO concentration was determined and subsequent division by the initial concentration of BSA-P(HEA)-CORM-3 gave equivalents of CO liberated. The half-life of CO release from different pH was then estimated from the graph.

Ellman's assay

The amount of free thiols present in the protein-polymer conjugate was investigated by Ellman's assay. It is known that a water-soluble colourimetric reagent, 5,5'-dithiol-bis-(nitrobenzoic acid), reacted with free thiol moieties yields a yellow-coloured product, 2-nitro-5-thio benzoic acid (TNB), which can be measured at 412 nm by UV-Vis spectroscopy.



Scheme S6. Schematic representation of colour-forming reaction between 5,5'-dithiol-bis-(nitrobenzoic acid) and free thiol moiety.

The Ellman's reagent solution was prepared by dissolving 4 mg of 5,5'-dithiol-bis-(nitrobenzoic acid) in 1 mL of buffer solution (0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA). 250 µL of BSA or BSA-P(HEA) (0.28 mM) was added to 2.5 mL buffer mixed with the 50 µL of Ellman's reagent and incubated at room temperature for 15 min. The absorbance at 412 nm was measured by a UV-Vis spectrometer. The amount and concentration of the thiol residue was determined from the Beer-Lambert law.

$$c = \frac{a}{b \times E} \; where \; a = Absorbance; b = path \; length \; in \; centimetres; E = \; 14{,}150$$

(The reported molar extinction coefficient of TNB in this buffer system is 14,150 $M^{-1}cm^{-1}$ at 412 nm).

According to the Ellman's assay, 59 mol % of the Cys-34 was oxidised leaving 41 mol % of the Cys-34 available for conjugation. After conjugation with P(HEA), 1.5 mol % of free thiol was still present due to steric hindrance from the functional polymer P(HEA).

Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

PAGE was performed with polyacrylamide gradient gel using 12%Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) at 120 V, 400 mA current for 75 min. Samples was dissolved in DI water ([BSA] = 0.5 mg/mL), mixed with 2× Laemmli loading buffer and heated to 95-100°C before loading under reducing and non-reducing conditions (Heating was applied for reduced samples only). Staining was accomplished with Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad). Under reducing conditions (in the presence of 2-mercaptoethanol), the disulfide bond formed between the protein and the pyridyl disulfide polymer was cleaved. In contrast, the disulfide bond from the resulting polymer-protein conjugate was preserved in non-reducing conditions (in the absence of 2-mercaptoethanol).

Additional Figures

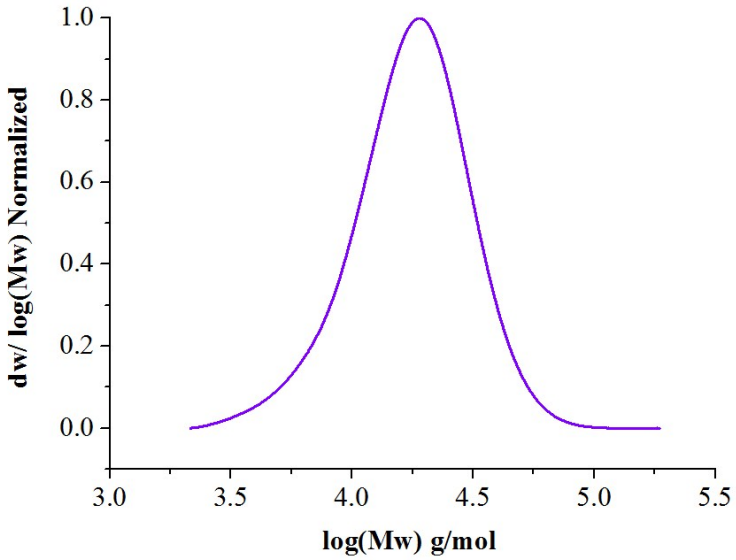


Figure S3. Molecular weight distributions of P(HEA) using PDS-RAFT as RAFT agent.

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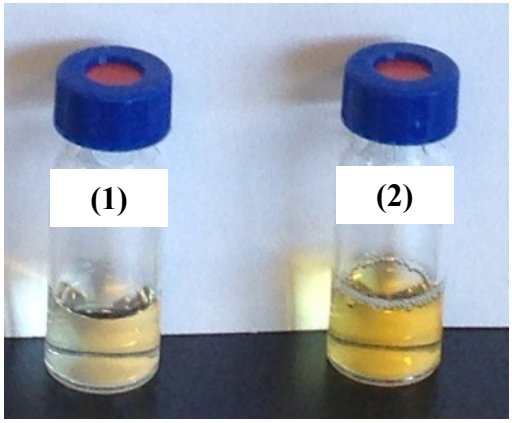

Figure S4. Digital photo of BSA-P(HEA) **(1)** and BSA-P(HEA)-CORM-3 **(2)** in DI water.

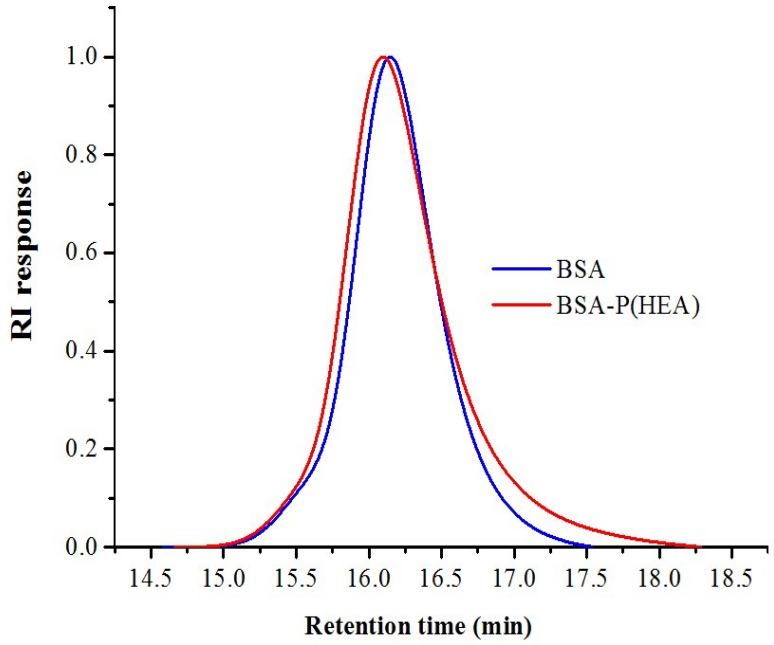

Figure S5. Aqueous GPC traces of BSA before and after conjugation with polymer P(HEA)

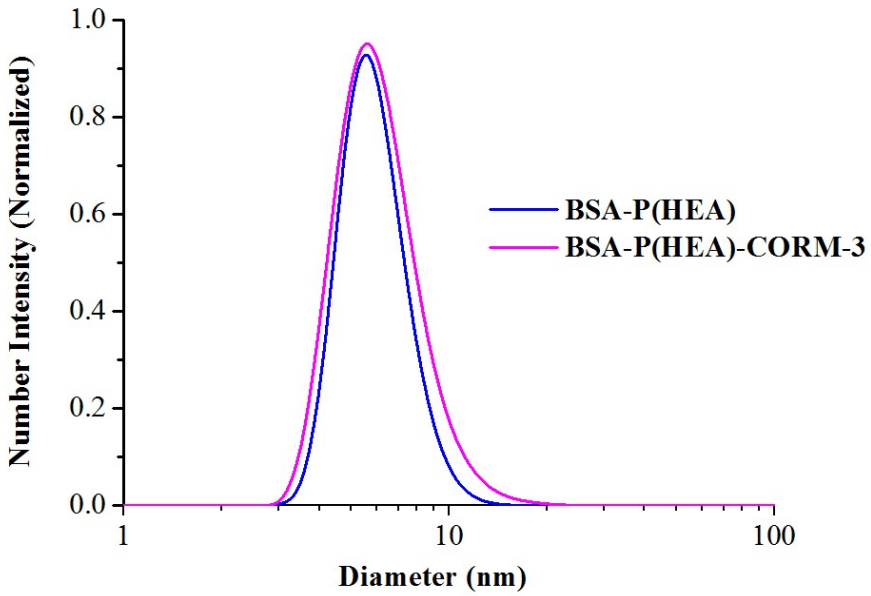

Figure S6. Hydrodynamic diameter analysis via DLS of polymer-protein hybrid before after conjugation with CORM-3: BSA-P(HEA) and BSA-P(HEA)-CORM-3.

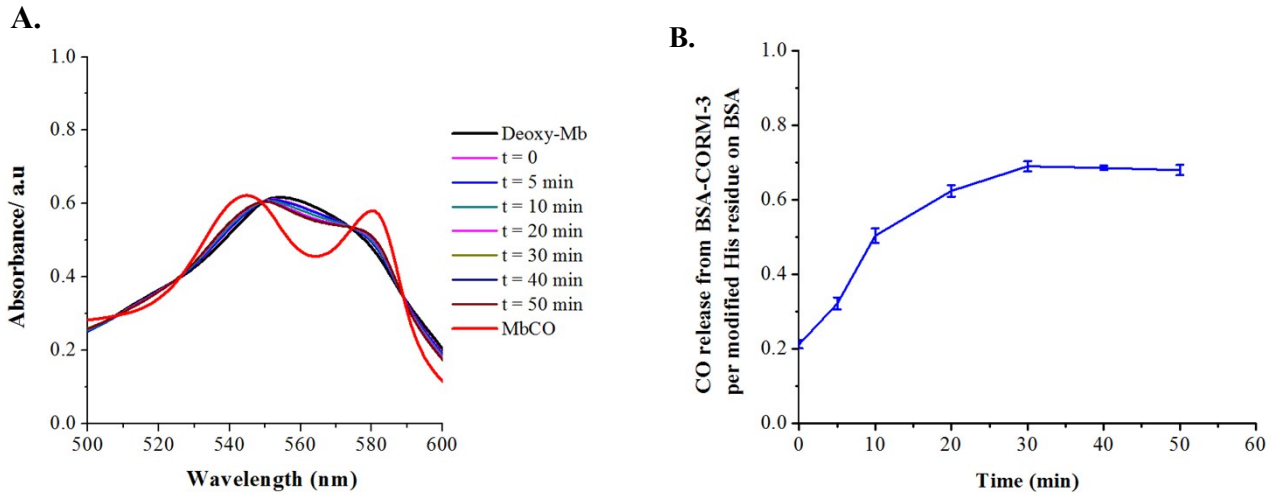

Figure S7. A. UV-Vis spectra change of a solution of reduced horse skeletal muscle myoglobin in the presence of BSA- CORM-3 (3.5 μM) in 0.1 M phosphate buffer solution **B.** CO release from BSA-CORM-3 (3.5 μM) versus time in reduced myoglobin solution.

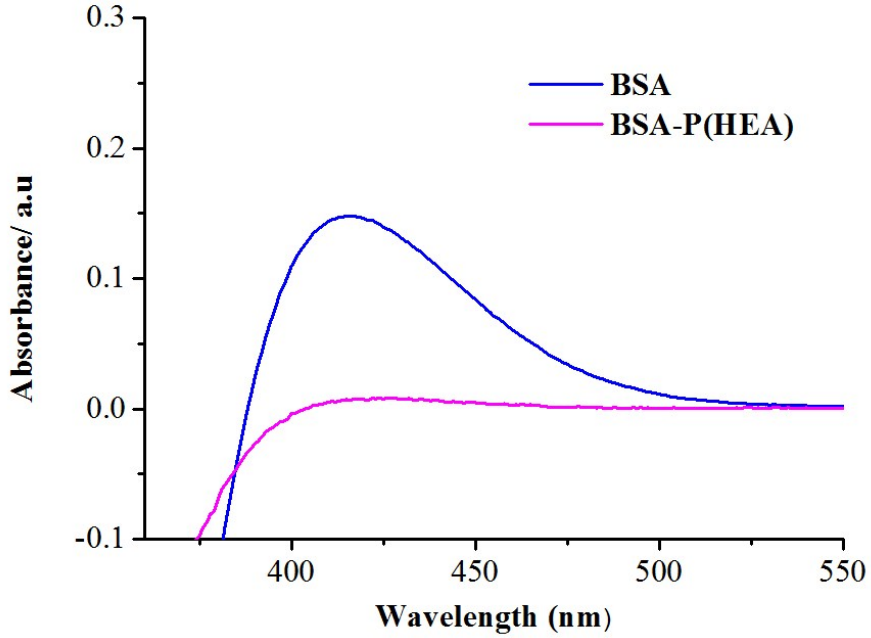

Figure S8. Ellman's result of BSA before and after conjugation with P(HEA). Absorbance at 412 of BSA and BSA-P(HEA): 0.14583 and 0.00531 respectively.

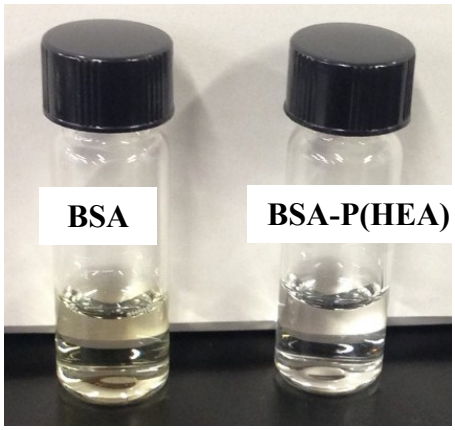

Figure S9. Digital photo of BSA and BSA-P(HEA) in Ellman's reagent solution.

Reference List

(1) Ferguson, C. J.; Hughes, R. J.; Nguyen, D.; Pham, B. T. T.; Gilbert, R. G.; Serelis, A. K.; Such, C. H.; Hawkett, B. S. Ab Initio Emulsion Polymerization by RAFT-Controlled Self-Assembly§. *Macromolecules* **2005**, *38*, 2191-2204.

(2) Nguyen, D.; Nguyen, T.-K.; Rice, S. A.; Boyer, C. CO-Releasing Polymers Exert Antimicrobial Activity. *Biomacromolecules* **2015**, *16*, 2776-2786.