Supporting Information

for

Biomimetic Pheomelanin to Unravel the Electronic, Molecular and Supramolecular Structure of the Natural Product

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Materials and Methods

Materials

L-3,4-dihydroxyphenylalanine (L-DOPA), tris(2-carboxyethyl) phosphine hydrochloride (TCEP·HCl), cysteine, cystine and Dulbecco's phosphate buffered saline (DPBS) were purchased from Fisher Scientific. Hydrogen peroxide, horseradish peroxidase (HRP), Triton™ X-100, Papain from papaya latex was purchased from Sigma-Aldrich. Proteinase-K was purchased from Gold Biotechnology. Human red hair was donated by a Caucasian male (red hair I) and female (red hair II) affiliated with Northwestern University. All reagents and materials were used as received unless otherwise stated.

Instrumentation

Solution Nuclear Magnetic Resonance (NMR) characterization. ^{1}H NMR spectra and ^{19}F NMR spectra were recorded on a Bruker Avance III HD system equipped with a TXO Prodigy probe (500 MHz) in D_2O. ^{13}C NMR spectra were recorded on a Bruker Avance III 500 MHz system equipped with DCH CryoProbe in D_2O.

Electrospray Ionization Mass Spectrometry (ESI-MS). ESI-MS spectra were collected on a Bruker Amazon-SL Mass-spectrometer configured with an ESI source in both negative and positive ionization mode.

Dynamic Light Scattering (DLS) measurements were performed on a DynaPro Nanostar (Wyatt Technology Corp, 633 nm laser) at room temperature in ultrapure water using disposable cuvettes.

Zeta potential was measured on a Malvern Zetasizer in ultrapure water at room temperature.

UV-Vis spectra were collected on a Cary Series 100 UV-vis spectrophotometer in a quartz cuvette.

Fourier Transform Infrared (FTIR) spectra were collected under transmission mode on a Nexus 870 spectrometer (Thermo Nicolet) in NU Atomic and Nanoscale Characterization Experimental Center (NUANCE) at Northwestern University.

Scanning Electron Microscope (SEM) images were acquired on a Hitachi SU8030 at an accelerating voltage of 10 kV and an emission current of 15 μA. For nanoparticle samples, silicon

chips were mounted onto aluminum SEM stubs with carbon tape. 2 μL of the sample dispersion in water was drop-casted onto the silicon and left to dry overnight, followed by coating with 10 nm of osmium prior to imaging. For pheomelanin films on glass substrates, the dry films were mounted onto aluminum SEM stubs using carbon tape and then coated with 10 nm osmium prior to imaging.

Scanning Transmission Electron Microscopy (STEM) and **Energy Dispersive Spectroscopy** (STEM-EDS) images were obtained on a Hitachi HD2300 STEM operating at 200 kV. 400 mesh TEM grids were surface plasma treated using a PELCO easiGlow glow discharge cleaning system prior to use. 2 μL of the pheomelanin sample suspended in water was dropcasted onto a TEM grid and left to dry. EDS images were obtained using a frame time of 10.0 s and a dwell time of 200 s, and the acquisition stopped after 100 frames.

Methods

Synthesis of 5-cysteinyl-DOPA (5-CD)

5-CD was synthesized according to a previous literature preparative procedure.[1] Briefly, the Michael addition of cysteine to oxidized L-DOPA was performed in strong acidic conditions to inhibit further oxidation and polymerization. Afterward the crude product was purified twice by DOWEX 50wx8 column to get rid of the $_{L}$-DOPA, 2-cysteinyl-DOPA and 2,5-dicysteinyl-DOPA. Then a reverse-phase HPLC was used to purify the compound. The pure compound was obtained as white powder. For detailed characterization of 5-CD, see **Figure S2**.

^{1}H NMR (500 MHz, Deuterium Oxide, ppm) δ 6.98 (d, J = 2.1 Hz, 1H), 6.86 (d, J = 2.1 Hz, 1H), 4.18 (dd, J = 7.6, 5.5 Hz, 1H), 4.03 (t, J = 5.7 Hz, 1H), 3.44 (d, J = 5.7 Hz, 2H), 3.19 (dd, J = 14.6, 5.5 Hz, 1H), 3.07 (dd, J = 14.7, 7.7 Hz, 1H).

^{13}C NMR (126 MHz, Deuterium Oxide, ppm) δ 171.75, 170.64, 144.90, 144.80, 126.83, 126.71, 118.48, 117.65, 54.32, 52.35, 34.88, 33.89.

^{19}F NMR (470 MHz, Deuterium Oxide, ppm) δ -75.6.

ESI-MS: calculated for $C_{12}H_{17}N_2O_6S$ $[M+H]^+$ 317.08, found 317.06.

Polymerization of 5-CD to obtain the synthetic pheomelanin

Pheomelanin was synthesized following a previously reported procedure.[2] In a typical experiment, 24 mg 5-CD was dissolved in 6 mL 100 mM DPBS in a 15 mL falcon tube. To this solution, 4.8 mg of HRP was added to afford a final concentration of 100 U/mL. Then, 45 mg $ZnSO_4 \cdot 7H_2O$ and 54 µL hydrogen peroxide (concentration 30% v/v) was added to the reaction system. The mixture was stirred at room temperature for 24 h. To work up the reaction, it was centrifuged at 11000 rpm for 10 min, treated with 1% acetic acid and then washed with ultrapure water. The pheomelanin pigment was obtained as reddish dispersion with yield ~17% (the yield was based on the mass ratio of the final pheomelanin powder to the starting material 5-CD). We observed that the synthetic pheomelanin after lyophilization was difficult to redisperse in water, similar to PDA type eumelanin. Note that we specifically avoid using Tris buffer because Tris can react with the quinone structure, thus becoming covalently incorporated in the melanin structure.[3]

UV-vis spectroscopy monitoring of the reaction

To monitor the reaction using UV-vis spectrometry, a 20 µL aliquot was taken from the reaction at desired intervals and diluted in 400 µL deionized water before measurement to meet the detection limit of the UV-vis spectrometry. The absorption spectrum was measured immediately to minimize the reaction lag after dilution since no quenching agent is used for this experiment.

High-Performance Liquid Chromatography (HPLC) and MS analysis

For HPLC monitoring, a 100 µL aliquot was diluted with 300 µL deionized water at the same stages of the reaction as the UV-vis measurements. 400 µL $NaBH_4$ aqueous solution (2 mg/mL, 0.05 mol/L) was added to quench the aliquoted reaction. Analytical HPLC analysis of peptides was performed on a Jupiter 4 Proteo 90Å Phenomenex column (150 × 4.60 mm) using a Hitachi-Elite LaChrom L-2130 pump equipped with UV-vis detector (Hitachi-Elite LaChrom L2420). The solvent gradient for HPLC was 0-60% acetonitrile in 30 min. To analyze the different species, fractions were collected manually and analyzed on a Bruker Amazon-SL Mass-spectrometer.

Pheomelanin extraction from the covert feathers of a Rhode Island red rooster or human red hair

We isolated intact melanosomes from rooster feathers and human red hair following the enzymatic (Proteinase-K based) extraction method of Liu et al (2004).[4] Compared to other harsher methods

traditionally employed in melanin extraction (e.g. acid-base extraction), this method is expected to retain the integrity of the chemical composition of melanin. Taking the human red hair I as an example, briefly, 35 g of hair was washed sequentially with organic solvents, including acetone, dichloromethane, and ether, and then with ultrapure water. Then the hair was treated with Dithiothreitol (DTT, 1 g) and Proteinase-K (90 mg, 2700 U) in DPBS under nitrogen atmosphere for 48 h at 40 ℃. After centrifugation, the hair was treated with papain (50 mg) and DTT (1 g) and incubated with continuous nitrogen for 72 h at 60-70 ℃. The sample was collected by centrifuge and washed 6 times with deionized water. Afterward, the pellet was redispersed in DPBS containing proteinase-K (30 mg, 900 U) and DTT (100 mg) under nitrogen atmosphere for 48 h at 40 ℃. The sample was stirred at room temperature in 2% Triton™ X-100 for 4 h and then washed with 2 x water, 2 x methanol and 2 x water. The sample was then treated with proteinase-K (80 mg) and DTT (400 mg) at 40 ℃ with continuous nitrogen flow. The pheomelanin from human red hair was obtained as a red to brown dispersion in water and lyophilized overnight for ssNMR. The total amount of pheomelanin obtained from the human red hair I was more than 200 mg.

The preparation of PDA eumelanin control

PDA nanoparticles were synthesized by the auto-oxidation of dopamine hydrochloride by air under alkaline conditions as reported before.[5] After reacting overnight, the samples were centrifuged at 11,000 rpm for 10 min followed by washing with ultrapure water for 3 times.

Cross-polarization magic-angle spinning ^{13}C solid-state NMR

20-80 mg of lyophilized melanin sample was packed into a H14355 4.0 mm magic-angle spinning (MAS) rotor from Bruker. The temperature was set to 298 K. Each 1D ^{13}C cross polarization (CP) was acquired using 3k-13k scans (depending on the amount of sample) and a recycle delay of 5.0 s on a 400 MHz Bruker spectrometer. The CP contact time was optimized to 3 ms. All ^{13}C NMR spectra were recorded with complete proton decoupling of 83 kHz. The spinning speed was 10 kHz. ^{13}C chemical shifts were referenced to adamantane external reference at 38.3 ppm and reported in parts per million (ppm). FID files were processed using MestRenova 7 software (Mestrelab Research).

Note: the intrinsic heterogeneous structure of polymeric melanin results in broad linewidth in the NMR spectra.

UVA irradiation of 5-CD pheomelanin

The powder sample of 5-CD pheomelanin was irradiated with UV lamp ($\lambda \sim 365$ nm, 7.0 mW/cm^2) for 30 h. The experimental condition was chosen to match the previous report by Wakamatsu (UVA dose 4.0 mW/cm^2, radiation time: 56 h).[6] Then ssNMR were collected on a 400 MHz Bruker spectrometer.

XPS experiment

X-ray photoelectron spectroscopy (XPS) spectra were collected on a Thermo Scientific ESCALAB 250Xi. For nanoparticles, samples were drop-casted onto a silicon substrate. Film samples on glass substrate were used as prepared. All XPS spectra were calibrated with reference to the carbon C1s peak at 284.8 eV.

EPR experiment

Continuous wave EPR measurements (CW EPR) were performed at X-band (9.5 GHz) fields using a Bruker Elexsys E680 spectrometer equipped with a 4122SHQE resonator. Scans were performed with a magnetic field modulation amplitude of 2 G and non-saturating microwave power of 1.544 mW. The results are the average of 32 scans. Dispersion samples were contained in quartz tubes with I.D. 1.50 mm and O.D. 1.80 mm and measured at room temperature. For quantification, 4-amino-TEMPO was dissolved in ultrapure water as the spin standard. EPR spectra for solution samples were taken under identical conditions as the standard. To quantify the spin concentrations, the EPR spectra were double integrated and then the double-integration areas were plotted against the spin concentration.

Solid samples and the pulse EPR measurements were also contained in quartz tubes with I.D. 1.50 mm and O.D. 1.80 mm and measured at room temperature on an E680 X/W EPR spectrometer with a split ring resonator (ER4118X-MS3). A 1 kW TWT amplifier (Applied Systems Engineering 117X) was used to generate high-power microwave pulses resulting in pulses, $\pi/2$ = 16 ns and π = 32 ns. The resonator was partially over-coupled to maximize echo intensity and minimize ringing following microwave pulses. Spin-lattice relaxation times (T_1) of the PDA-type eumelanin and pheomelanin samples (synthetic and natural ones extracted from bird feathers) were determined using the saturation recovery technique (**Figure 5**). The spin ensemble of interest was

saturated with a series of eight 24 ns pulses spaced 2 μs apart and the recovery was monitored at logarithmically spaced delays T starting with 100 ns and a pulse $\pi/2 - \tau - \pi - \tau$ - echo detection sequence, in which $\pi/2$ = 16 ns, π = 32 ns and τ = 200 ns. The signals recovered in exponential fashion and were fit using a well-established relaxation model.[7]

For the power saturation curve, X axis is the square root of incident power *P*. Y axis is the intensity values of the EPR maximum peak. *P* is calculated from the following equations:

$$\frac{P}{P_0} = 10^{-\frac{dB}{10}}$$

In which P_0 is 196.2 mW.

The preparation of pheomelanin film

For pheomelanin film preparation, a glass slide was cleaned with deionized water and immersed in the polymerization reaction of 5-CD for 24 h. Afterward, the glass was taken out, rinsed with ultrapure water for 3 times and then immersed in 1% acetic acid for 20 min followed by another 3 rounds of washing with ultrapure water. We note here that glass slides cleaned by piranha solution have much lower absorbance after pheomelanin film deposition than untreated glass slides.

The film from natural pheomelanin was prepared by drop casting of a drop of natural pheomelanin dispersion onto a clean glass slide.

The preparation of eumelanin film control

To prepare PDA-type eumelanin film, 2 mg/mL solution of dopamine was prepared. Around 180 μL of 0.2 M NaOH solution were added to adjust to pH 8.8. Glass slides were submerged in the solution for 24 h to allow the eumelanin film deposition. The oxidative polymerization was trigger by ambient oxygen under the alkali condition.

The coating of 3D-printed objects

The 3D-printed poly-methacrylate birds were printed with a Formlabs Form2 printer using Formlabs Clear V4 resin. The bird design was downloaded from https://free3d.com/3d-model/bird-v1--875504.html, and used under the non-commercial personal use license as an example part. After printing, the birds were rinsed in two successive isopropyl alcohol baths for

10 minutes each, then allowed to dry overnight before the pheomelanin coating using the as-described method.

To prepare PDA-type eumelanin coated birds, we use 2 mg/mL solution of dopamine was prepared in a pH 8.5 Tris buffer solution.

Reflectance Spectrophotometry

We used UV-vis spectrophotometry to characterize the reflectance of pheomelanin films. We measured specular reflectance between 300-700 nm using an Avantes (Avantes Inc., Boulder, CO, USA) AvaSpec-2048 spectrometer and an AvaLight-XE pulsed xenon light source, relative to a glass slide as reference. The spectral data were collected at a 90° angle of incidence for both the light and probe using AvaSoft v7.2.

Film disassembly studies

The as-prepared film on glass substrate was cut to around 3 mm or 5 mm squares by a diamond knife. The films were put in 96-well plate and submerged with 200 μL salt solution. Solutions used included water, pH 10 KOH solution, and pH 10 solution with KCl, NaCl, KBr, and K_2SO_4. The 96-well plated was placed under darkness for 24 h. Then each film was washed three times by ultrapure water. The absorbance mappings of the film samples were recorded using a Perkin Elmer EnSpire multimode Plate Reader. The absorbance at 400 nm was plot by MATLAB using a blank well to subtract the background.

Supporting figures

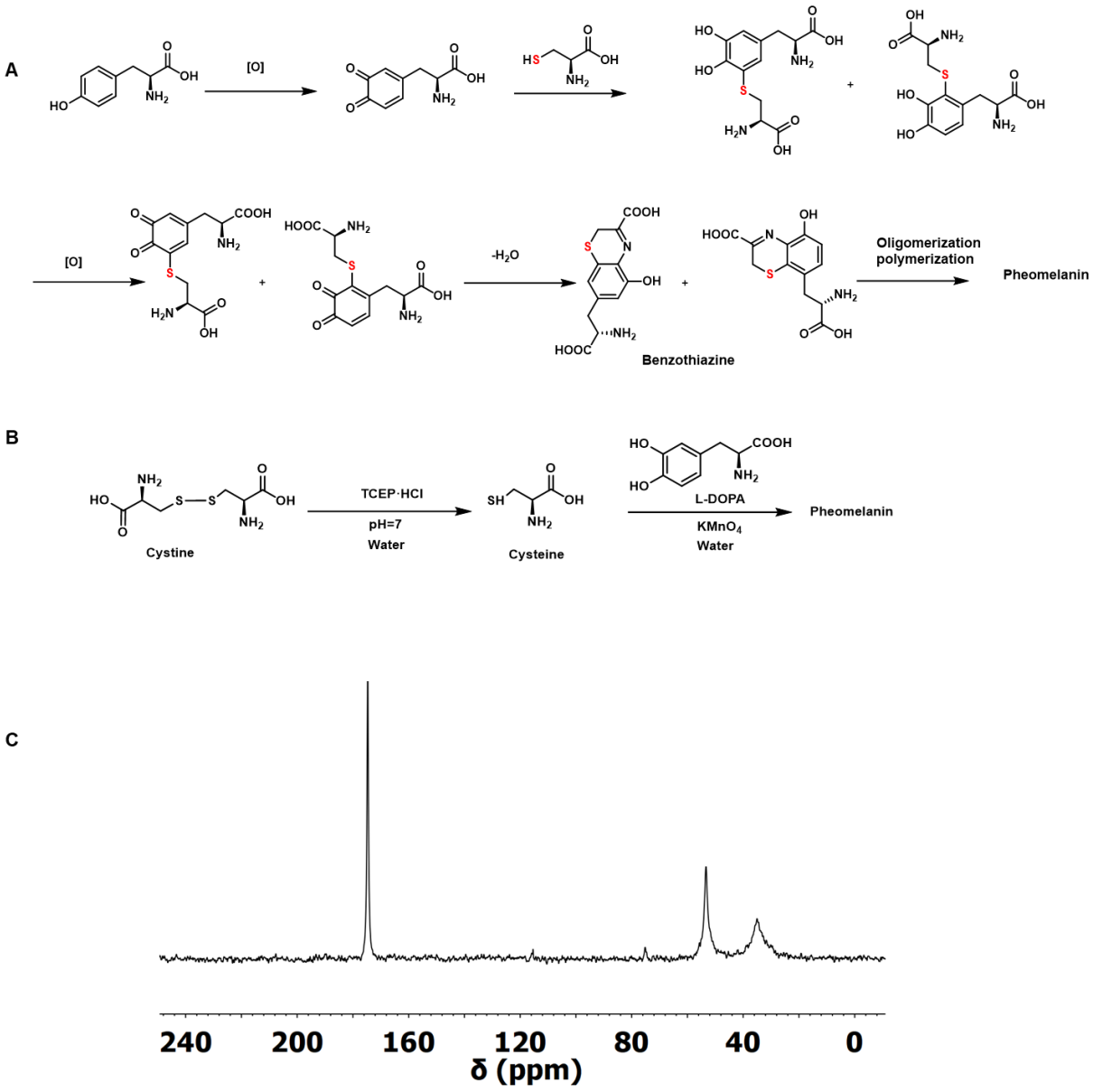

Figure S 1. Mechanism of oxidation polymerization to generate pheomelanin and the synthetic attempts to generate pheomelanin using L-DOPA, and cystine as the starting materials. A. Mechanism of oxidation polymerization to generate pheomelanin. B. To circumvent the cysteine dimerization in air, $KMnO_4$ was used to oxidize a solution phase mixture of cysteine and L-DOPA in water at pH 7, following the in situ reduction of cystine. C. ^{13}C ssNMR of the black powders from this method showed similar sharp peaks to the $KMnO_4$ and tyrosinase method. The aromatic peaks were barely observed in this case.

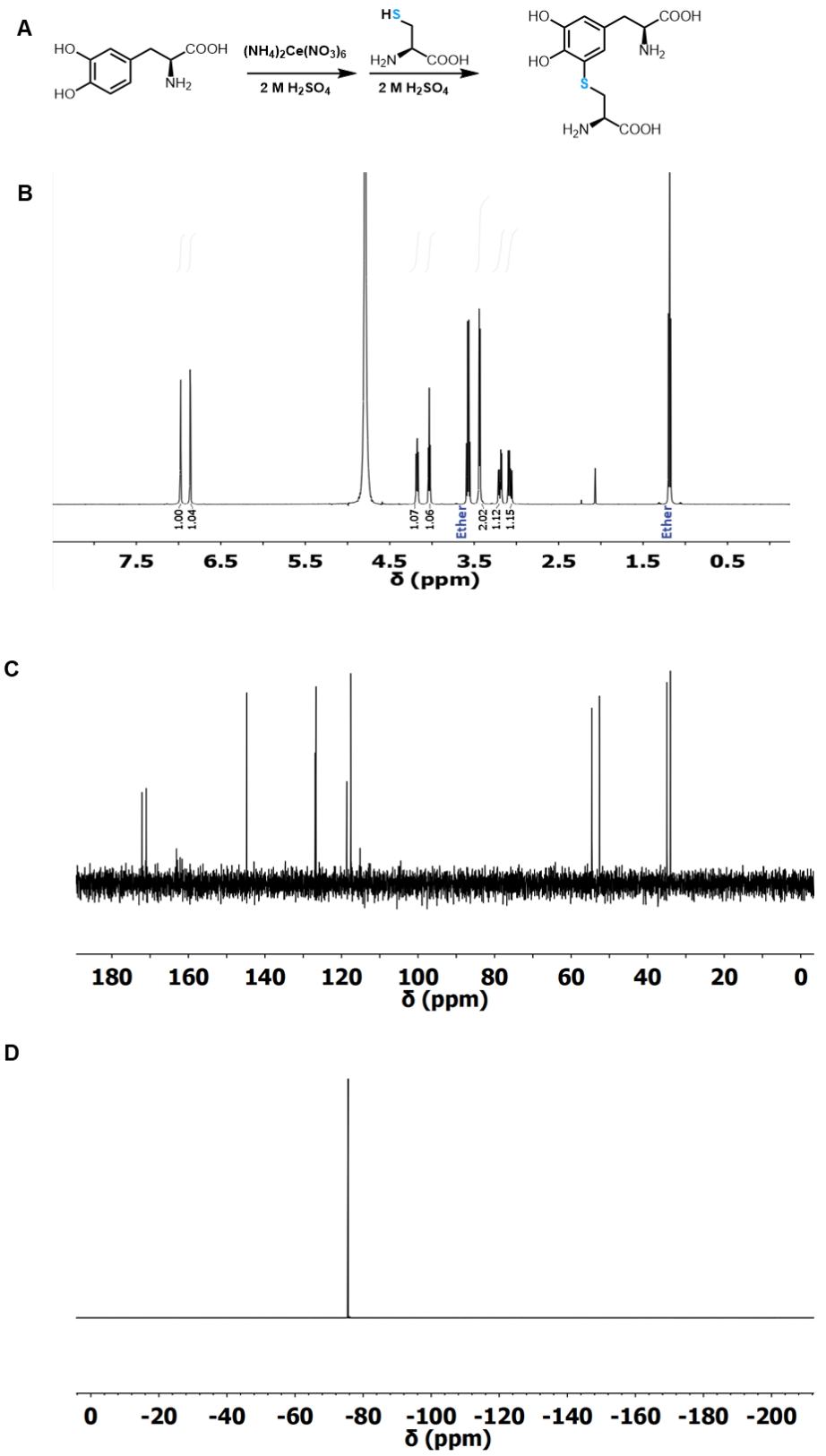

Figure S 2. Synthetic route and solution NMR characterization of 5-CD monomer. A. Synthesis of 5-CD. B. ^{1}H NMR, C. ^{13}C NMR, and D. ^{19}F NMR spectra of 5-CD in D_2O.

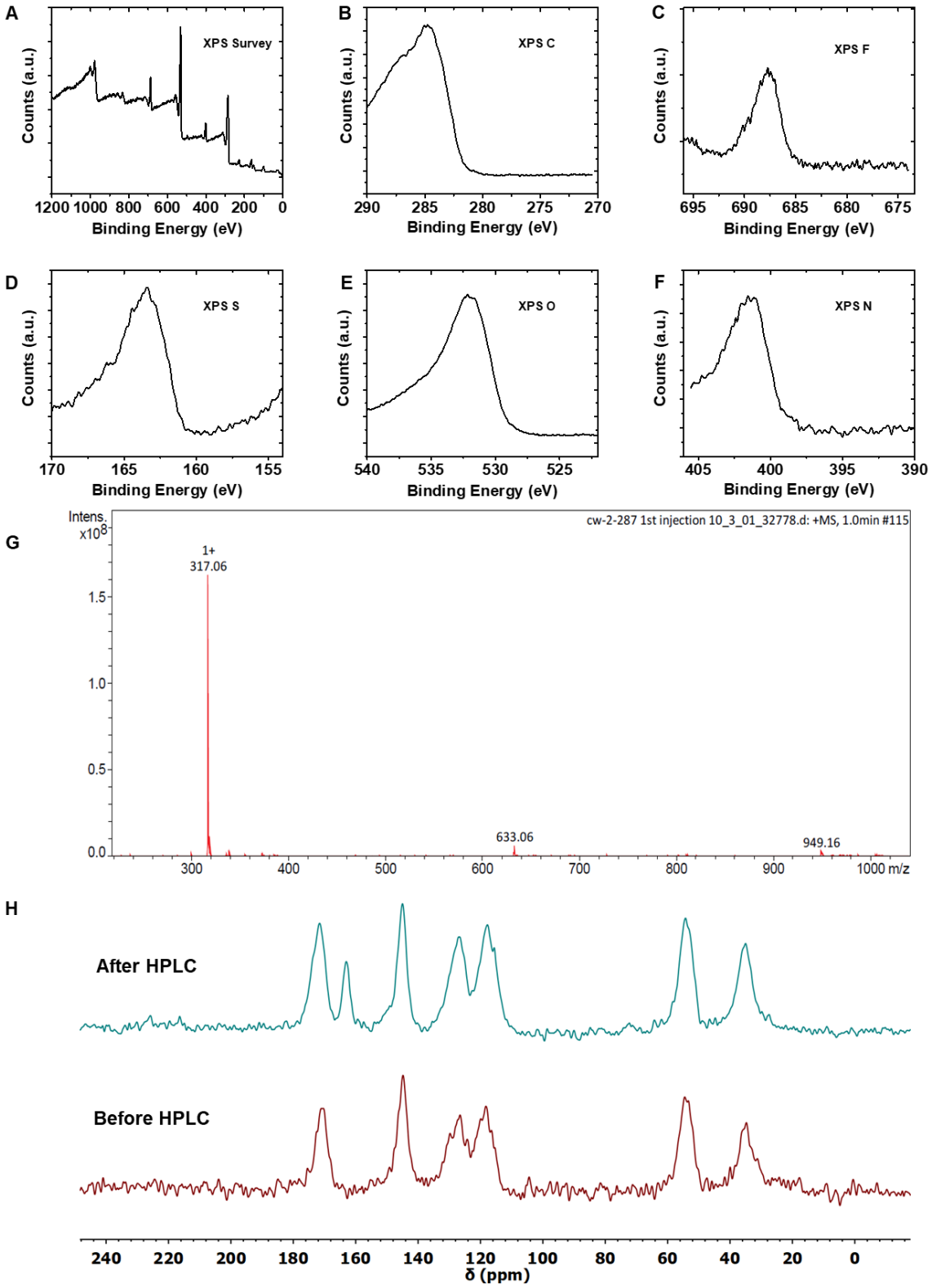

Figure S 3. XPS, ESI-MS, and ssNMR characterization of 5-CD monomer. A-F. XPS spectra of 5-CD. A. Wide-scan XPS survey spectrum. B. Deconvoluted C1s spectrum. C. F1s spectrum. D.

S2p spectrum. D. O1s spectrum, E. N1s spectrum. G. ESI-MS spectra of 5-CD. Solvent: water. $[M+H]^+$ calculated 317.08, found 317.06, $[2M+H]^+$ calculated 633.15, found 633.06, $[3M+H]^+$ calculated 949.23, found 949.16. H. ssNMR spectra of 5-CD before (lower) and after (upper) HPLC purification. The extra peak at 163.0 ppm after HPLC purification is assigned to the trifluoroacetate counterion, coresponding to the ^{19}F NMR in Figure S 2 and the XPS F 1s signal in Figure S 3 C.

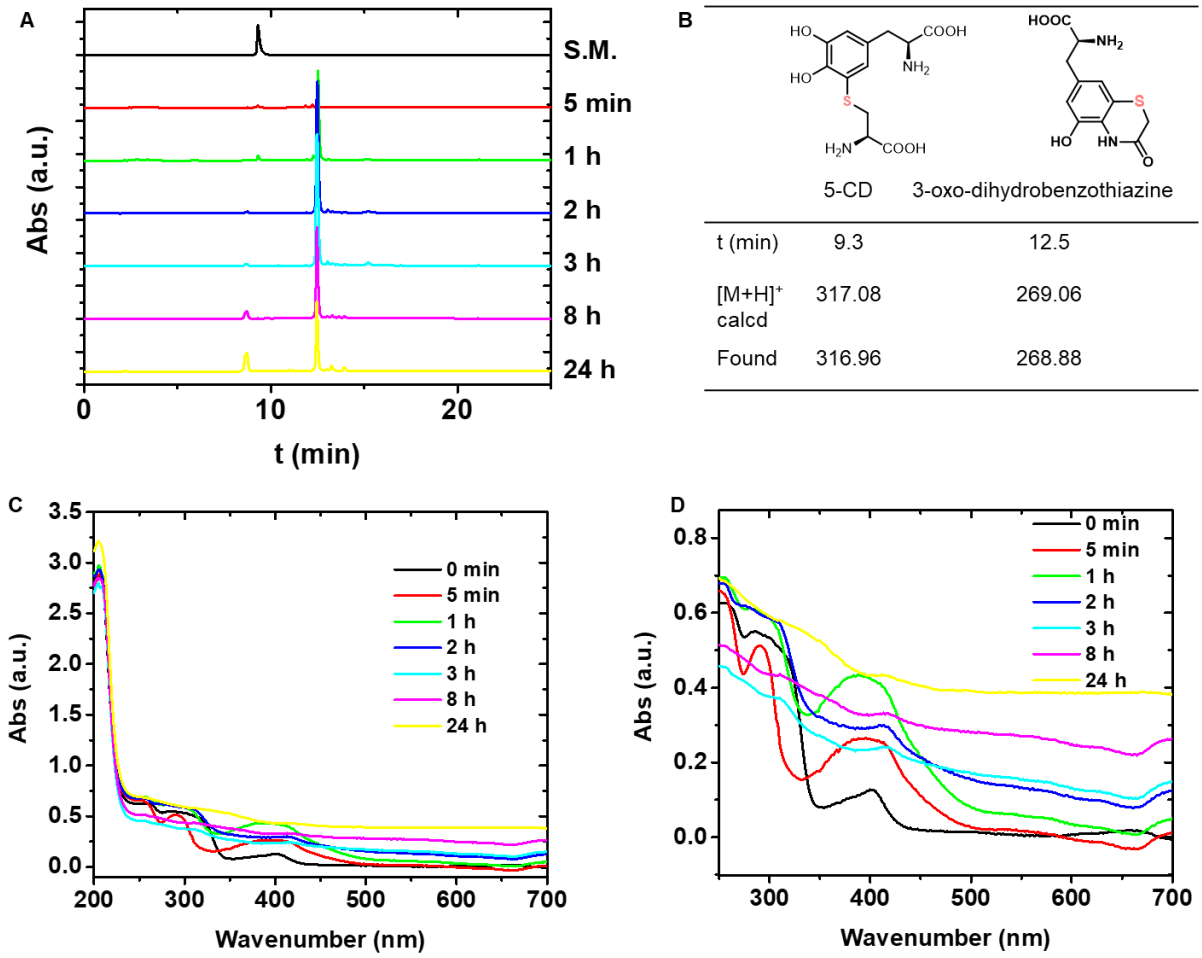

Figure S 4. Polymerization reaction of 5-CD to prepare synthetic pheomelanin. A. HPLC analysis of the reaction at various stages. B. The reaction intermediates identified by ESI-MS spectra of the fractions from the HPLC. C. UV-vis spectroscopy monitoring of the reaction. D. Zoom of UV-vis spectroscopy from 250 nm to 700 nm.

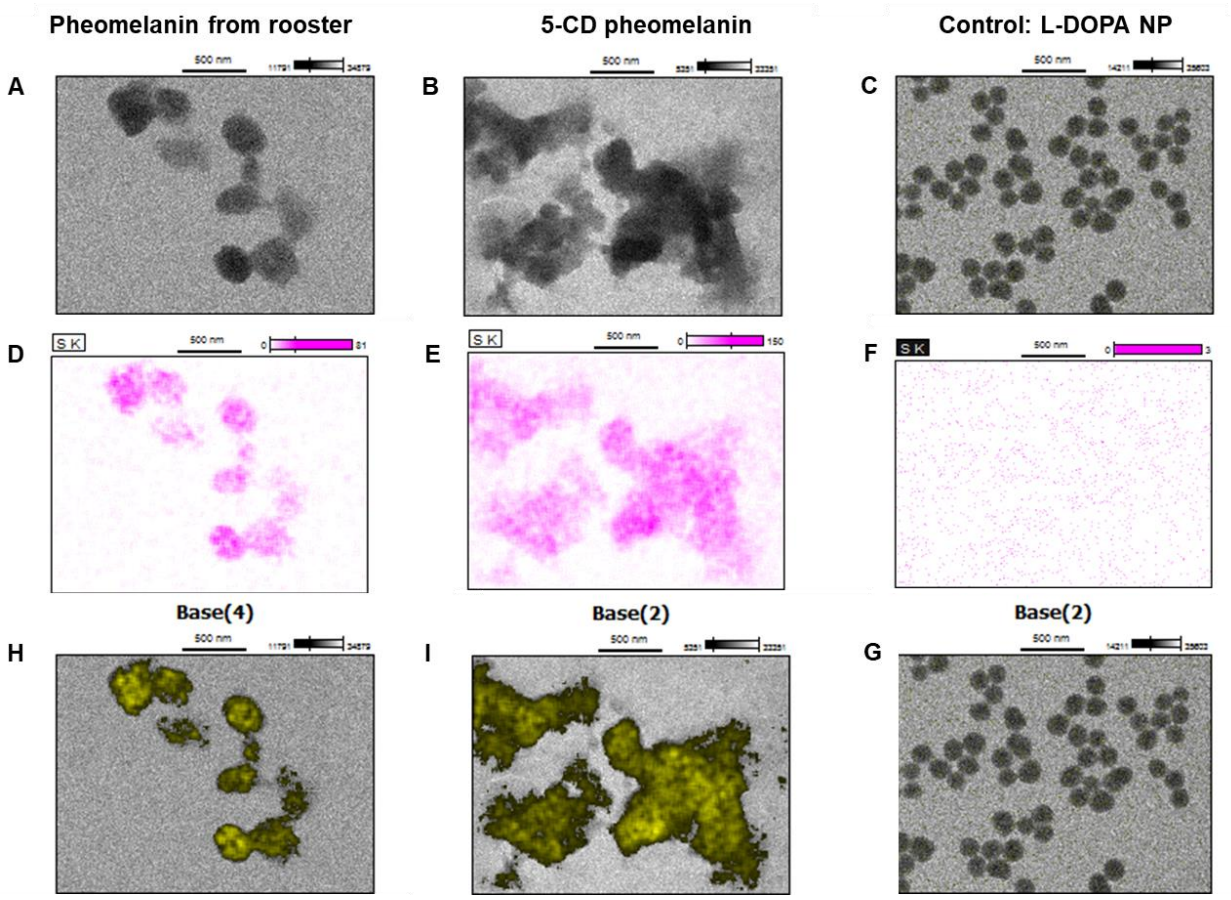

Figure S 5. STEM images with sulfur element EDS mapping of the feather pheomelanin (A, D, H), synthetic pheomelanin (B, E, I) and control L-DOPA NP (C, F, G). Sulfur-free L-DOPA NP control was made by polymerization of L-DOPA using $KMnO_4$ as the oxidative agent. A, B, C, TEM images, D, E, F, sulfur mapping, H, I, G, the overlay of mapping and TEM. Sulfur signal is false colored with yellow.

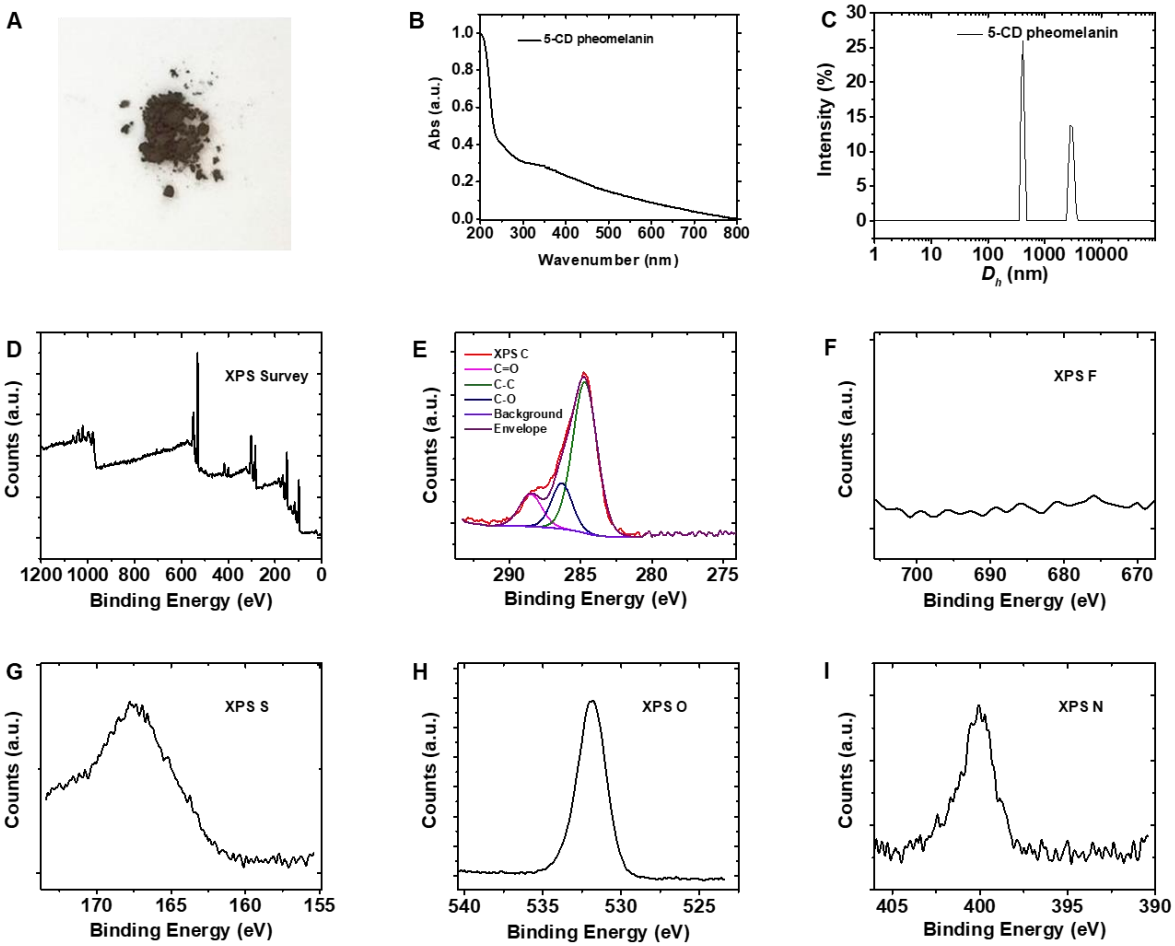

Figure S 6. Characterization of the 5-CD synthetic pheomelanin. A. Optical photograph of the synthetic pheomelanin powder. B. Normalized UV-vis plot of the synthetic pheomelanin suspension. C. DLS plot of the synthetic pheomelanin sample. D-I. XPS spectra of synthetic pheomelanin. D. Wide-scan XPS survey spectrum. E. Deconvoluted C1s spectrum. F. F1s spectrum shows that the TFA counterion diminished after the polymerization, corresponding to the intramolecular cyclization of amine to form the benzothiazine structure. G. S2p spectrum. H. O1s spectrum. I. N1s spectrum.

A

Pheomelanin Source	R_h (nm)	Zeta potential (mV)
Synthetic sample	Multimodal	-27.3±1.8
Rooster feathers	174	-44.4±1.05
Human red hair I	1015	-39.3±1.27
Human red hair II	227	-34.0±3.21

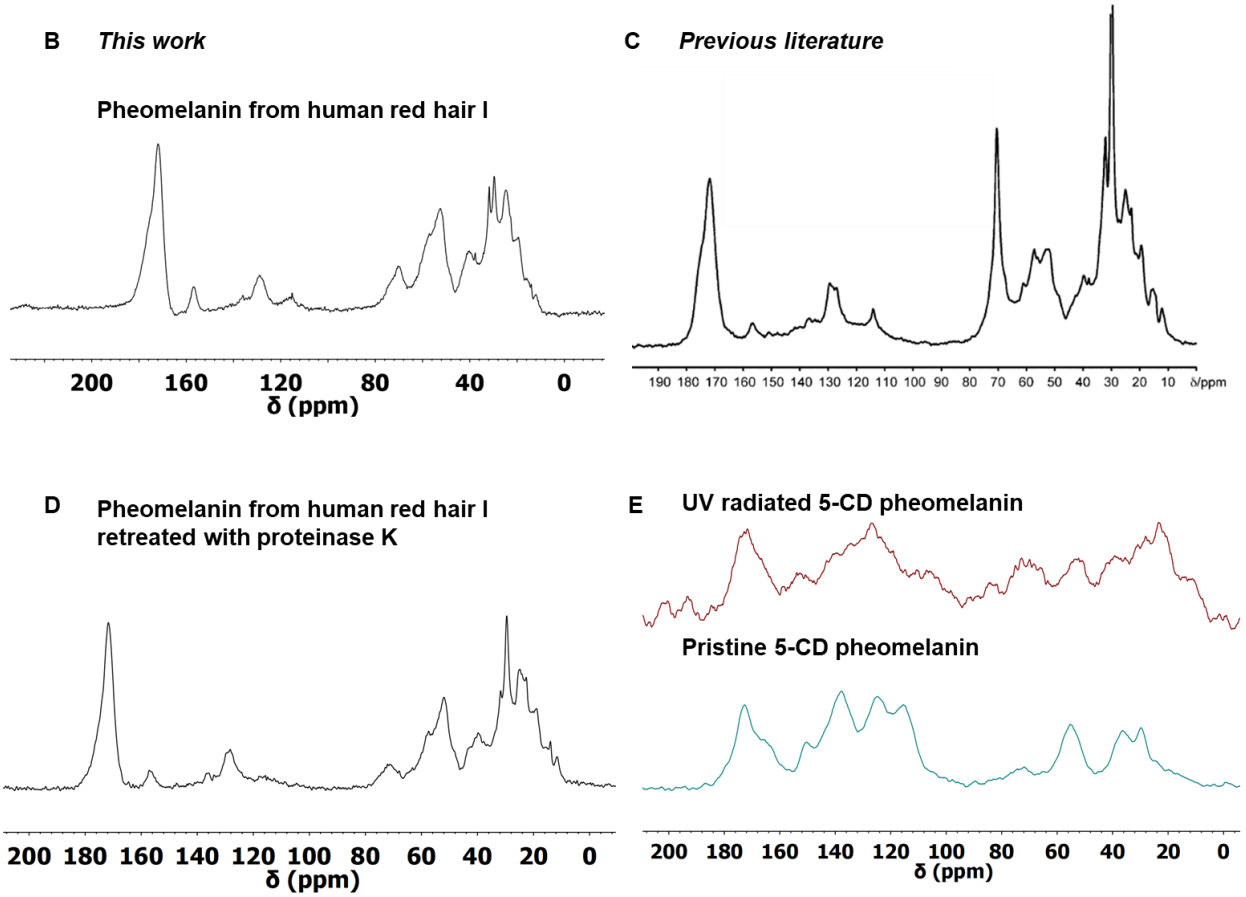

Figure S 7. DLS and ssNMR comparison of the synthetic and natural pheomelanin samples. A. Hydrodynamic radii and Zeta potentials of the synthetic pheomelanin and natural pheomelanin. B. The ssNMR spectrum of pheomelanin from human hair sample I. C. Reproduced from previous literature result of enzymatically extracted pheomelanin from human hair.[8] D. The ssNMR spectrum of pheomelanin from human red hair I after retreatment with proteinase K. E. The ssNMR spectrum of 5-CD pheomelanin irradiated with UVA. The pristine 5-CD pheomelanin was plotted here for reference.

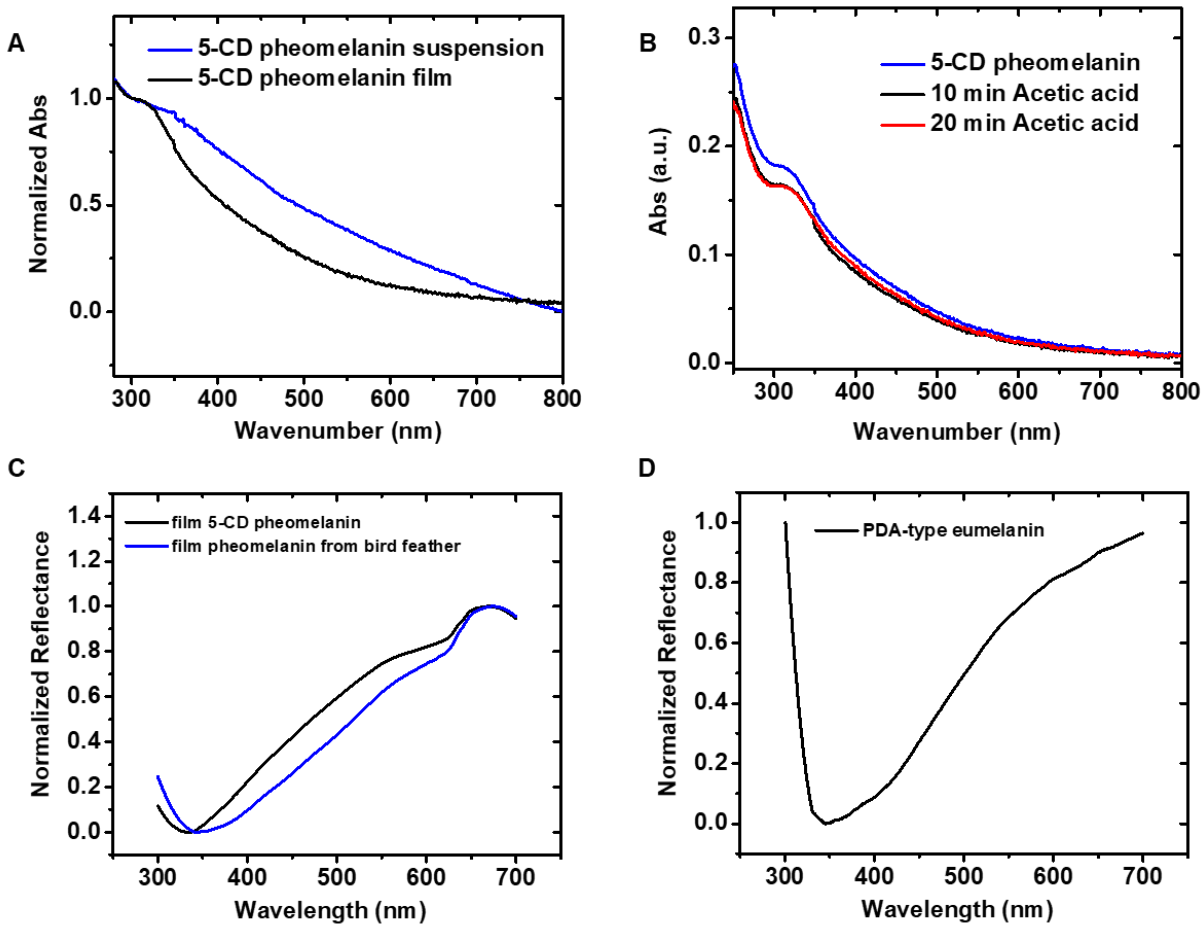

Figure S 8. Absorption and reflectance spectra of the melanin films. A. UV-vis absorption spectra comparision of 5-CD pheomelanin suspension and film. B. UV-vis absorption spectra of 5-CD pheomelanin film before and after treatment with 1% acetic acid. C. Reflectance spectrum for the films made using 5-CD pheomelanin, and pheomelanin from bird feathers. D. Reflectance spectrum for the PDA type eumelanin film.

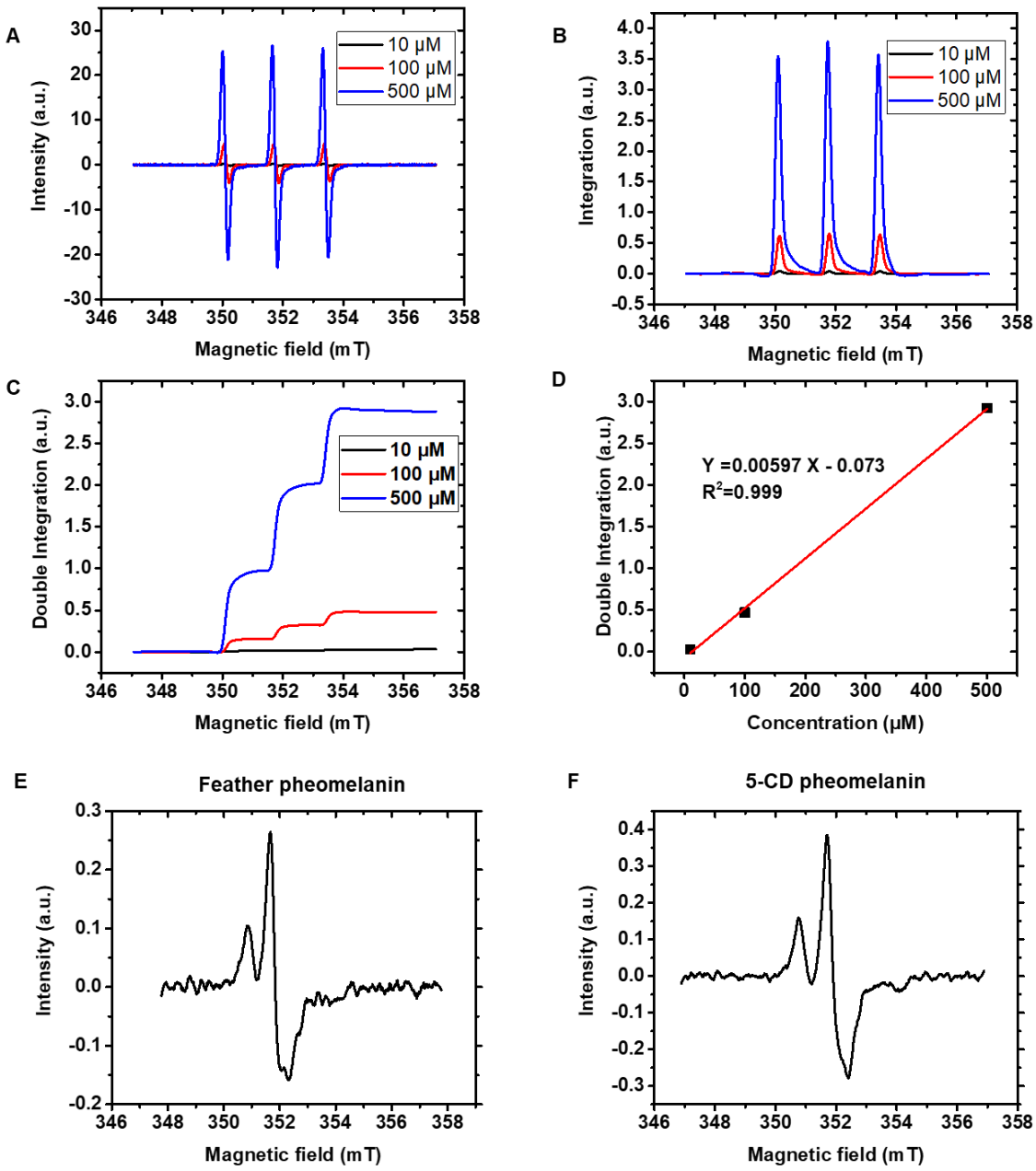

Figure S 9. EPR quantification of the radical content in pheomelanin. A. EPR spectra of 4-amino-TEMPO standard solutions of 500, 100 and 10 μM. B. Integration plots of EPR spectra in A. Plots were baseline corrected in Origin software. C. Integrated EPR spectra of B. D. The EPR calibration curve of double integration area *vs* spin concentration. E-F. EPR spectrum of feather pheomelanin (E) and 5-CD pheomelanin (F) in aqueous dispersion.

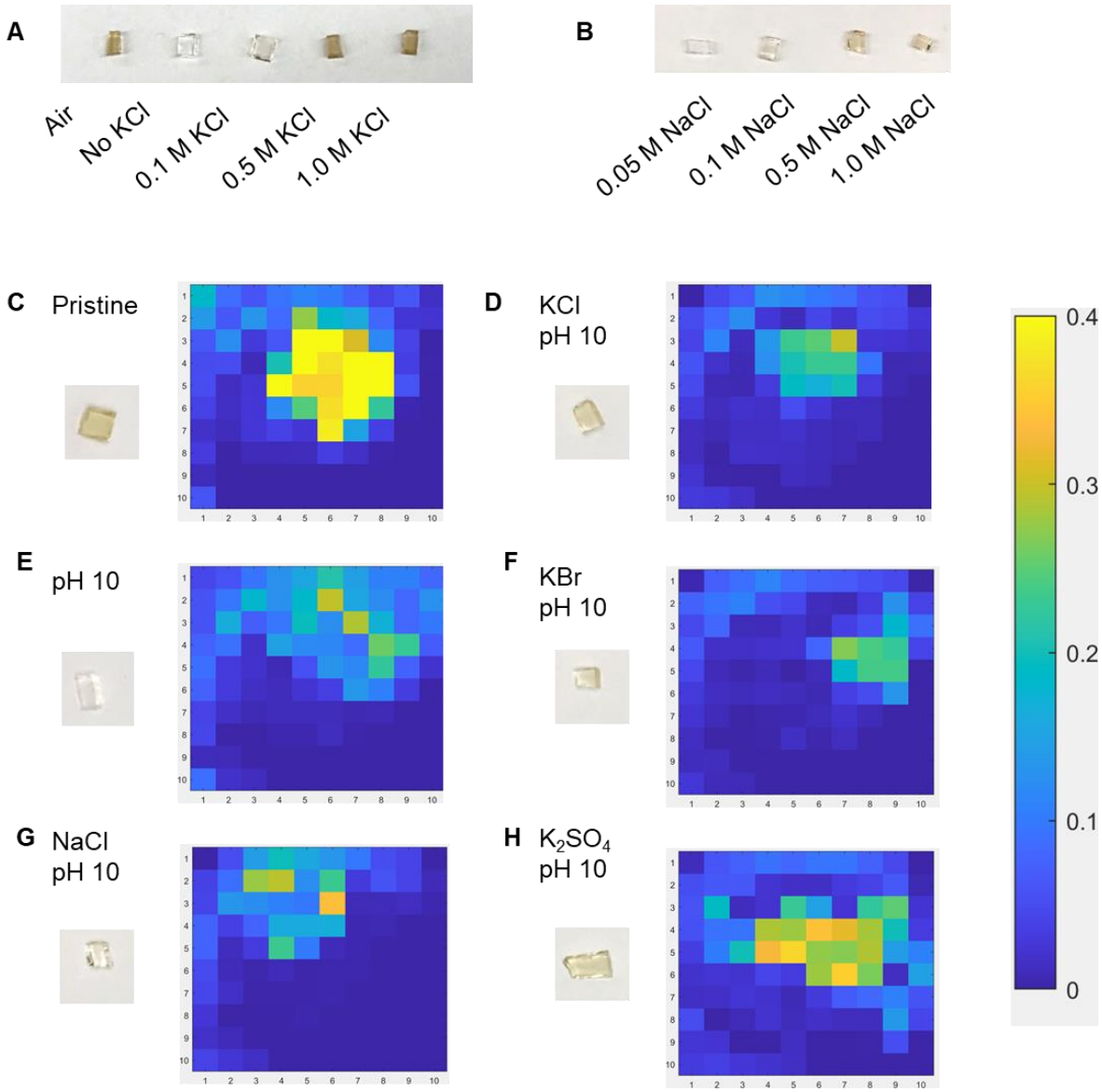

Figure S 10. 5-CD Pheomelanin films after treatment with different solutions at pH 10. Optical images and absorbance mapping at 400 nm on glass slide substrates are shown. Here substrates with small areas are used to facilitate screening. A. Optical images of the pheomelanin film treated with KCl solution (pH 10) at various concentrations, including 0 M, 0.1 M, 0.5 M and 1.0 M. B. Optical images of the pheomelanin film treated with NaCl solution (pH 10) at various concentrations, including 0 M, 0.1 M, 0.5 M and 1.0 M. C-H are the optical images and the corresponding absorbance mapping at 400 nm. C. The pristine pheomelanin film. D. Film treated with 0.5 M KCl solution (pH 10). E. Film treated with pH 10 KOH solution. F. Film treated with 0.5 M KBr solution (pH 10). G. Film treated with 0.5 M NaCl solution (pH 10). H. Film treated with 0.5 M K_2SO_4 solution (pH 10).

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