Supporting Information

A Melanin-Inspired Pro-oxidant System for Dopa(mine) Polymerization: Mimicking the Natural Casing Process

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General Methods

Materials. L-DOPA, L-cysteine, dopamine hydrochloride (DA), L-epinephrine (EN), L-phenylalanine hydrogen peroxide (30% v/v), horseradish peroxidase (EC 1.11.1.7), mushroom tyrosinase (EC 1.14.18.1), bovine erythrocytes superoxide dismutase (EC 1.15.1.1), bovine liver catalase (EC 1.11.1.6) were commercially available and were used as obtained. 5-*S*-cysteinyldopa (CD),[1] 6-(2-amino-2-carboxyethyl)-2-carboxy-4-hydroxybenzothiazole (BTCA),[2] pyrrole-2,3,5-tricarboxylic acid (PTCA)[3] were prepared as described.

Methods. Analytical HPLC was carried out on an apparatus equipped with a UV detector set at 254 or 280 nm using a Sphereclone ODS (5 micron, 4.6 × 250 mm) column, and a solution of 0.5% TFA/methanol (90:10, vol/vol) at flow rate of 0.8 mL/min as eluant. Analytical HPLC of the degradation mixtures was carried out as reported.[6]

Morphological analyses of DOPA-melanin, CD-melanin, polymer obtained by CD-melanin oxidation of DOPA (DOPA/CD-melanin 1:1 or 10:1, w/w) were performed using a FEI Quanta 200 FEG environmental scanning electron microscope (ESEM) (Eindhoven, The Netherlands) in low vacuum mode, using a large-field detector (LFD) and an accelerating voltage ranging between 15 and 20 kV. Before the analysis, samples were air-dried at 25°C and 50% relative humidity and mounted on aluminium stubs by means of carbon adhesive disks.

Synthetic pigment preparation. Eumelanin from L-DOPA was prepared by tyrosinase/O_2 oxidation as reported.[4] Pheomelanins from CD were prepared by peroxidase/H_2O_2 oxidation as previously described.[5] The polymer from CD-melanin induced oxidation of DOPA (DOPA/CD-melanin 10:1, w/w) was prepared according to the following procedure. A solution of DOPA (35.0 mg, 0.17 mmol) in 0.1 M phosphate buffer (pH 7.4; 175 mL) in the presence of CD-melanin (3.5 mg) was allowed to stand at room temperature under vigorous stirring. The mixture was then acidified to pH 3, the melanin precipitate was collected by centrifugation (6793 *g* × min, 4°C, 30 min) and washed with 1% acetic acid (3 × 10 mL). The polymer from CD-melanin induced oxidation of DOPA (DOPA/CD-melanin 1:1, w/w) was prepared according to the same procedure described above. A solution of DOPA (20.0 mg, 0.10 mmol) in 0.1 M phosphate buffer (pH 7.4; 100 mL) in the presence of CD-melanin (20.0 mg) was allowed to stand at room temperature under vigorous stirring. The mixture was then acidified to pH 3, and the melanin precipitate was collected as above. The polymer from CD-melanin induced oxidation of DOPA (DOPA/CD-melanin 5:1, w/w) was prepared according to the same procedure described above. A solution of DOPA (5.0 mg, 0.025 mmol) in 0.1 M phosphate buffer (pH 7.4; 25 mL) in presence of CD-melanin (1.0 mg) was allowed to stand at room temperature under vigorous stirring. The mixture was then acidified to pH 3, and the melanin precipitate was collected as above and lyophilized (3.0 mg).

Catecholamine oxidation

A solution of DOPA (10.0 mg, 0.05 mmol) in phosphate buffer (pH 7.4, 50 mL) was allowed to stand at room temperature under vigorous stirring. When required this reaction was carried out in the presence of CD-melanin (2.0 mg) or DOPA-melanin (2.0 mg). In other experiments the reaction was repeated in the presence of CD-melanin as above and i) superoxide dismutase (4 U/mL final concentration), or ii) catalase (25 U/mL final concentration), or iii) superoxide dismutase (4 U/mL final concentration) and catalase (25 U/mL final concentration), or iv) under an Ar atmosphere, or v) in the dark.

In other experiments a solution of DOPA (15.0 mg, 0.08 mmol) in phosphate buffer (pH 7.4, 75 mL) was allowed to stand at room temperature under vigorous stirring in the presence of variable amounts of CD-melanin (1.0 mg, 3.0 mg, 15.0 mg or 75 mg).

A solution of DA or NE or phenylalanine (10.0 mg) in phosphate buffer (pH 7.4, 50 mL) was allowed to stand at room temperature under vigorous stirring in the presence or in the absence of CD-melanin (2.0 mg).

In all cases, aliquots of the reaction mixtures were periodically withdrawn and analyzed by HPLC/UV.

Oxidative degradation procedure

CD-melanin or the polymer from CD-melanin induced oxidation of DOPA (DOPA/CD-melanin 5:1, w/w) (1.0 mg) was degraded with 1 M NaOH containing 1.5% H_2O_2 and was analyzed by HPLC after workup as reported.[6]

In separate experiments each polymer (2.0 mg) was loaded on a nylon filter (Millex-GV, 0.22 mm) and treated with solutions at increasing pHs (4 × 200 μL phosphate buffer 0.1 M pH 8; 4 × 200 μL phosphate buffer 0.1 M pH 9). Each eluate was then treated with NaOH (100 μL) and 30% v/v H_2O_2 (15 μL) and allowed to stand at room temperature under vigorous stirring. After 24 h the reaction mixtures were treated with 5% $Na_2S_2O_5$ (40 μL), taken to pH 3 with 85% H_3PO_4 and analyzed by HPLC as reported.[6]

The insoluble material was treated with NaOH (300 μL) and 30% v/v H_2O_2 (15 μL) and analyzed by HPLC after workup as reported.[6]

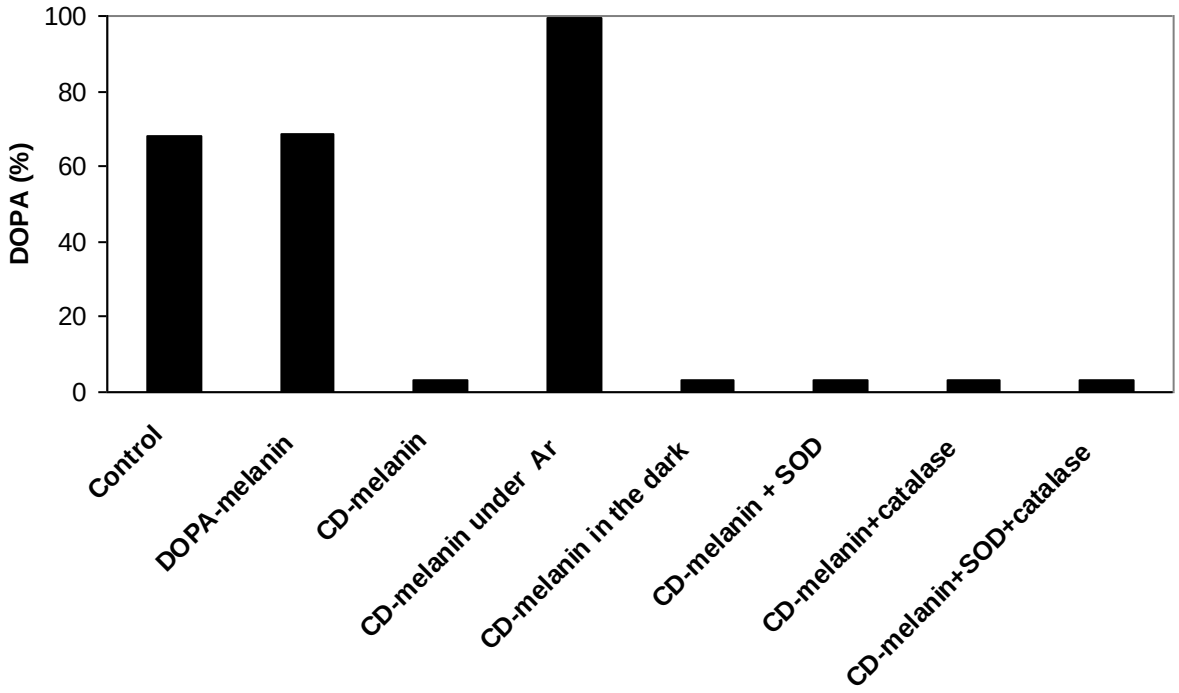

Figure S1 Decay of DOPA after 24 h in the presence of CD-melanin under different reaction conditions and in presence of varying additives as indicated *vs* control run in the absence of CD-melanin. For reaction conditions see exp procedure above.

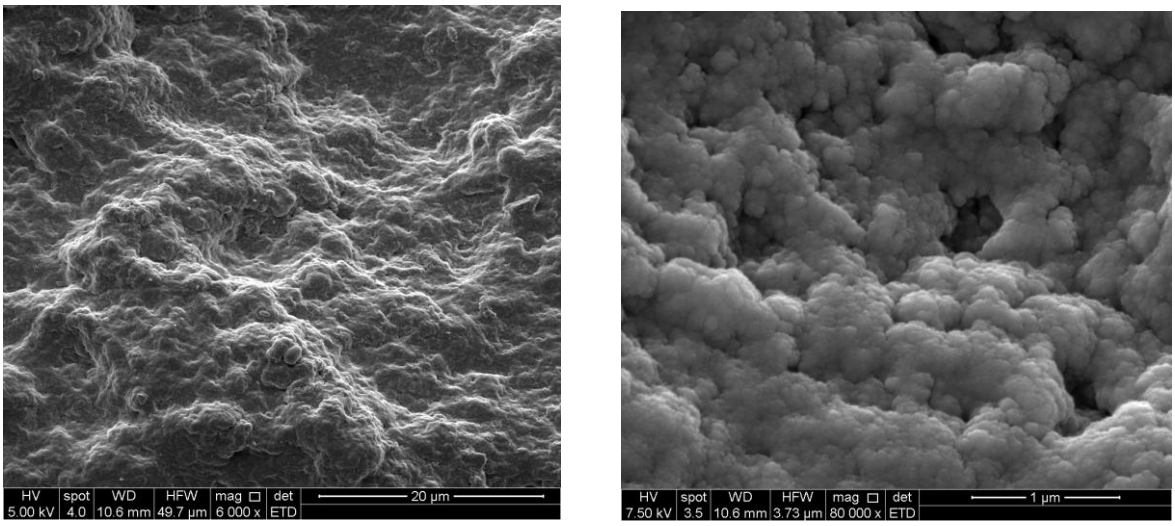

Figure S2. SEM images of DOPA-melanin.

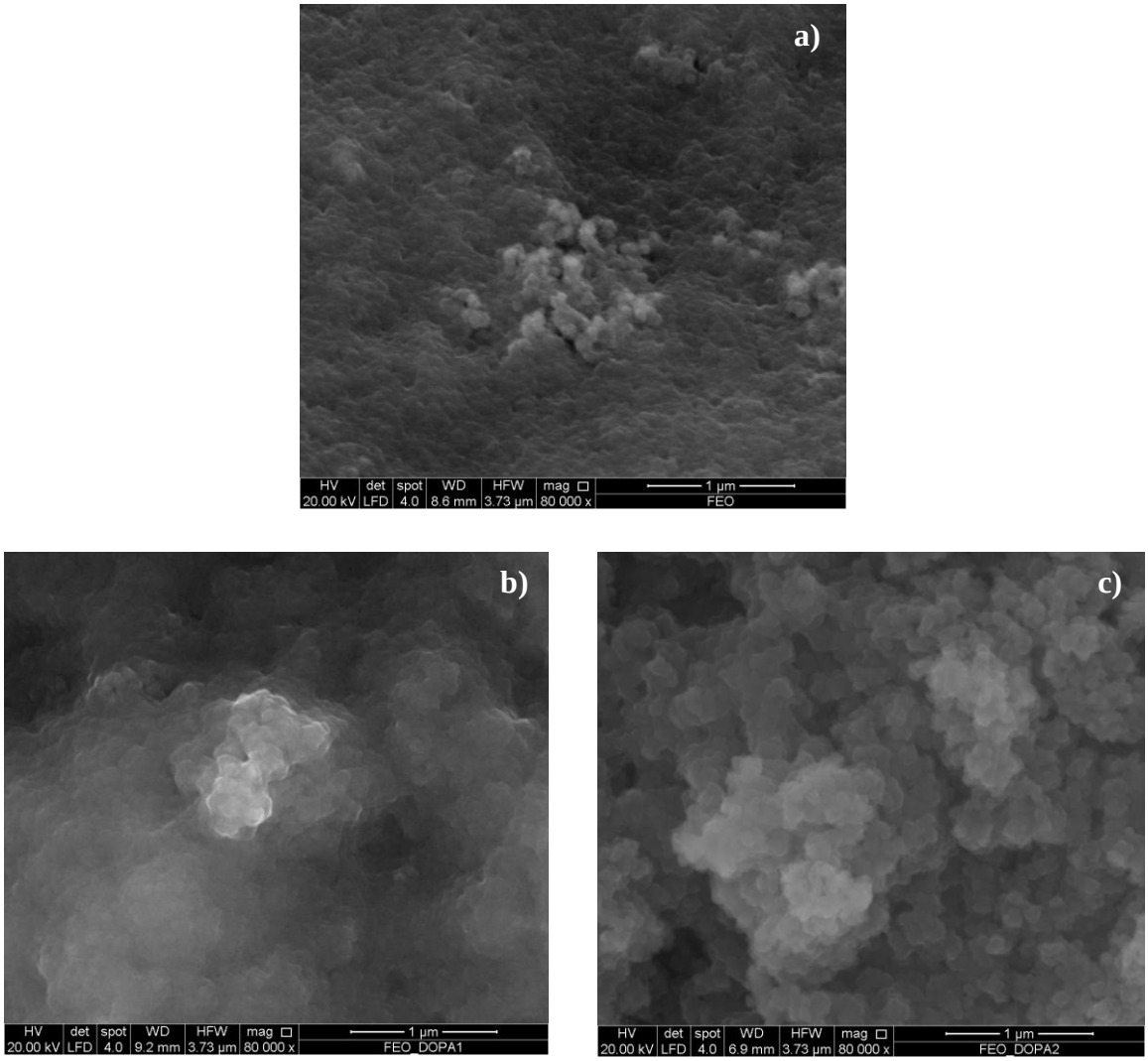

Figure S3. a) SEM image of CD-melanin; b) as a) but 24 h after addition of an equal weight amount of DOPA; c) as a) but 24 h after addition of a 10-fold weight excess of DOPA.

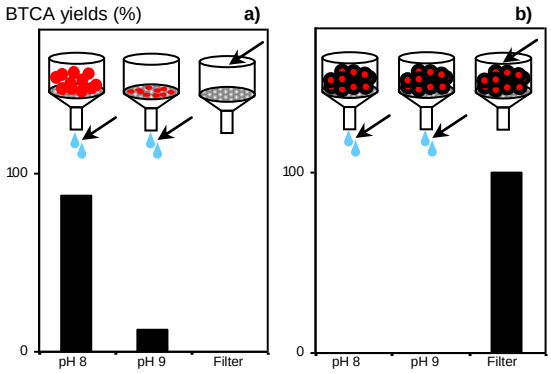

Figure S4. CD-melanin determination by BTCA quantitation in sequentially washed polymer samples. a) Pure CD-melanin. b) CD-melanin encased into DOPA-melanin. Arrows indicate the fraction (eluate or insoluble polymer) subjected to analysis. Shown is the sum of the yields obtained from 4 sequential washings at the pH indicated, expressed as % of the overall yields (mean values for three separate experiments with SD≤10%).

Chemical degradation experiments: the polymer obtained by CD-melanin induced oxidation of DOPA (DOPA/CD-melanin 5:1 w/w) was sequentially washed on a filter with solutions of phosphate buffer pH 8 and pH 9 (4 times each), and the washings and the insoluble material were separately analyzed for CD-melanin content by an established alkaline H_2O_2 degradation protocol, based on HPLC determination of the pheomelanin marker 6-(2-amino-2-carboxyethyl)-2-carboxy-4-hydroxybenzothiazole, BTCA. The same washing sequence was applied to a pure CD-melanin for comparison. BTCA determination (panel a) showed that most of CD-melanin was readily solubilized and released from the filter since the initial washings with phosphate buffer at pH 8. By contrast, no CD-melanin was detected in the washings of the DOPA-melanin coated sample after each step of the polymer washing sequence (panel b). In a control experiment direct analysis of the untreated coated sample afforded consistent BTCA yields. As expected this sample afforded also significant yields of the eumelanin marker pyrrole-2,3,5-tricarboxylic acid (PTCA) [6] (*cf.* Table S1).

Table S1. Yields of pyrrole-2,3,5-tricarboxylic acid (PTCA) and 6-(2-amino-2-carboxyethyl)-2-carboxy-4-hydroxybenzothiazole (BTCA) from alkaline oxidative degradation[6] of the eluates obtained by sequential washings with phosphate buffer pH 8 and pH 9 and of the insoluble fraction of CD-melanin, and of the polymer obtained by CD-melanin induced oxidation of DOPA. The yields of the melanin markers from degradation of the untreated pigments are also reported.

	CD-melanin Yields (μg/mg pigment)		Polymer by CD-melanin induced oxidation of DOPA Yields (μg/mg pigment)	
	PTCA	BTCA	PTCA	BTCA
pH 8	22.39	55.25	/	/
	5.29	16.79	/	/
	4.84	15.36	/	/
	4.55	13.31	/	/
pH 9	3.89	13.50	/	/
	1.47	5.49	/	/
	1.15	3.64	/	/
	0.24	0.95	/	/
Insoluble material	/	/	102.93	38.51
Untreated Pigment	56.22	104.61	99.25	43.53

Shown are the mean values for three separate experiments with SD ≤ 10%.

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