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ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

Pee-dots: Biocompatible Fluorescent Carbon Dots Derived from the Upcycling of Urine

Jeremy B. Essner,[†] Charles H. Laber,[†] Sudhir Ravula,[†] Luis Polo-Parada,[‡] and Gary A. Baker*,[†]

[†]Department of Chemistry, University of Missouri-Columbia, Columbia, MO 65211

[‡]Department of Medical Pharmacology and Physiology, University of Missouri-Columbia, Columbia, MO 65211

Experimental

Materials and Reagents

All experiments were carried out using Ultrapure Millipore water (18.2 MΩ cm). All human urine used was collected from a single source (i.e. one person). The vitamin C tablets and asparagus were both purchased from a local grocery store. Mice embryonic fibroblast (MEF) cells were obtained from colleagues within the Dalton Cardiovascular Center and the BT-474 human mammary gland, breast/duct carcinoma cells were obtained from the ATCC. The 96 well plates and the chloride salts of Cu^{2+} (lab grade), Fe^{3+} (ACS >98%), Sr^{2+} (99+% ACS), Ba^{2+} (>99%), and Ca^{2+} (99.999%), were obtained from Fisher Scientific (Pittsburg, PA). Sulforhodamine B (SRB), fetal bovine serum, cell culture media, phosphate buffer solution (PBS), trichloroacetic acid, acetic acid, ethylenediaminetetraacetic acid (EDTA), the chloride salts of Ni^{2+} (98%), Hg^{2+} (ACS >99.5%), Sn^{2+} (≥99.995% trace metal basis) and Mn^{2+} (99.99% trace metals basis), and the sulfate salt of Cu^{2+} (≥98.0%) were purchased from Sigma-Aldrich (St. Louis, MO). The acetate salt of Pd^{2+} (99.95+%-Pd) and the anhydrous chloride salt of Zn^{2+} (99.95% metals basis) were acquired from Strem Chemicals (Newburyport, MA) and Alfa Aesar (Ward Hill, MA), respectively. All chemicals were used as received. Dialysis tubing (132105, Spectra/Por 7, 1k MWCO) was purchased from Spectrum Labs. Cells were observed and photographed using an inverted microscope Olympus X-71 with Normanski and fluorescence optics using a 4, 10, 20 and 40× lenses and a black and white (Qimaging Retiga EXi) or a color (Axiocam MRc5, Carl Zeiss) camera. Confocal images were obtained using an Olympus Fluoview 1200 laser scanning (Olympus America) with a IX83 microscope with appropriate lenses.

Experimental procedures

Cell Imaging

For the fluorescent imaging studies, freshly isolated MEF cells were cultured in a 35 mm glass culture dish in the presence of each type of PD (0.2 mg/mL). The cells were fixed with a 70% ethanol solution for 10 min and mounted with Prolong-Gold Antifade Mountant (Invitrogen) for imaging. Fluorescence micrographs shown in Fig. 5 combine the signal from DAPI staining of the nuclear material with APD-derived signal collected through (A) FITC (Ex/Em: 479 nm/524 nm) or (B) TRITC (Ex/Em: 543 nm/593 nm) filter cubes (Semrock, Inc.; Rochester, NY).

Cell Viability by Sulforhodamine B Assay

Cell viabilities were evaluated based on the sulforhodamine B (SRB) assay, because it produces more consistent, less varying (standard error of measurement, SEM, or coefficient of variation, CV) results for adherent cell cultures over the common MTT assay. After the addition of PDs, cell viability was quantitated using the SRB assay as described previously.[1, 2] This cell protein dye-binding assay is based on the measurement of protein content of surviving cells as an index to determine cell growth, inhibition, and cell viability. Briefly, 8×10^3 cell/well (BT-474) in 100 µL culture media were seeded into each well of a 96-well plate and incubated overnight at 37 °C with 10% CO_2. Media was removed, cells were washed with 100 µL of serum-free medium (DMEM/F12) once, and cells were then treated in 5% FBS culture medium for 48 h in the presence of 1, 3, 10, and 30 µL of a 5 mg/mL stock solution, with the final volume per well being 100 µl. Following treatment, the medium was removed, surviving or adherent cells were fixed in situ by adding 100 µl of PBS and 100 µl of 50% cold tricholoacetic acid (TCA) and then incubating at 4 °C for 1 h. Cells were then washed with ice-cold water 5 times and dried. Cells were stained using 50 µl of 4% SRB (in 1 vol% acetic acid solution) for 8 min at room temperature. Unbound dye was removed by washing five times with cold 1% acetic acid and drying. The bound stain was then solubilized with 150 µl of 10 mM Tris buffer (pH 7.4). The absorbance of the samples was read at 560 nm with a microplate reader. Three wells per assay for each concentration and each sample were measured in triplicate.

Metal Quenching and Fluorescence Recovery Studies

Stock solutions of the various metal salts with concentrations of at least 5 mM were generated, which were then diluted to 3.1 mM. The C-dot concentration was kept constant at 0.05 mg/mL throughout all of the proceeding studies. For the metal screening tests, 100 µL of the 3.1 mM metal salt solutions was added to a cuvette containing the 0.05 mg/mL C-dot solution. Fluorescence and UV-Vis spectra were collected before and after the metal additions. For the metal titration quenching studies, stock solutions of the Hg^{2+} and Cu^{2+} salts with concentrations of at least 10 mM were generated. These stocks were then diluted to 2.7 mM followed by two serial 10-fold dilutions to generate metal salt concentrations of 270 µM and 27 µM. Starting with the lowest concentration (27 µM) and working up to the higher concentration (2.7 mM), additions were made to the cuvette, collecting fluorescence after each aliquot was added. For the fluorescence recovery studies, three separate cuvettes per sample were treated in the following manner:

Cuvette 1: 3× 100 µL water

Cuvette 2: 1× 100 µL water followed by 2× 100 µL 6.4 mM EDTA

Cuvette 3: 1× 100 µL 3.1 mM $HgCl_2$ followed by 2× 100 µL 6.4 mM EDTA

Fluorescence and UV-Vis spectra were collected after each 100 µL addition. After the EDTA additions, the cuvette was vigorously shaken prior to collecting the spectra. This process of shaking then collecting was repeated until the fluorescence emission stabilized.

For all the studies discussed in this section, all data were blank subtracted and all fluorescence emission were dilution corrected. The fluorescence emission data for the metal screening and recovery studies were also corrected for inner filter effects through the approximate correction factor below:

$$F_{corr} = F_{obs}(10^{\frac{A_{ex}+A_{em}}{2}})$$

where F_{corr} is the corrected fluorescence values, F_{obs} is the observed (blank subtracted and dilution corrected) fluorescence, A_{ex} is the absorbance of the sample at the excitation wavelength (450 nm), and A_{em} is the absorbance at each wavelength over the emission range collected (460–800 nm). The fluorescence data for the titration quenching studies was not inner filter corrected as the metals used for these studies showed no measureable absorbance over 460–800 nm even at concentrations much higher (1 mM) than those used in the titrations (maximum concentration of 100 μM).

Characterization techniques

Absorbance and fluorescence data were collected on a Cary Bio 50 UV-Vis spectrophotometer and Varian Cary Eclipse Fluorometer, respectively. Quantum yield values were calculated using the equation listed below with quinine sulfate, coumarine 153, fluorescein, rhodamine B, and cresyl violet as reference fluorophores (fluorophore and fluorescence measurement information is provided below in Table S1).

$$QY_S = QY_R\left[\frac{F_S}{F_R}\right]\left[\frac{OD_R}{OD_S}\right]\left[\frac{n_S^{\,2}}{n_R^{\,2}}\right]$$

where R and S stand for reference and sample respectively, F stands for integrated fluorescence intensity (calculated over the wavelength range of interest), OD stands for optical density (at the excitation wavelength used in the fluorescent measurements), and *n* stands for refractive index.

Table S1. Reference fluorophores used to determine quantum yield (*QY*) values.

Fluorophore	**Excitation Wavelength (nm)**	**Solvent**	***n*** [a]	***QY* (%)**	**Ref.**
Quinine sulfate	350	0.1 M H_2SO_4	1.343	58	[3]
Coumarin 153	421	EtOH	1.366	38	[4]
Fluorescein	470	0.1 M NaOH	1.336	91	[5]
Rhodamine B	514	Water	1.334	31	[6]
Cresyl violet	580	MeOH	1.332	53	[3]

[a] refractive index.

Raman spectra were collected on a Renishaw Raman spectrometer employing a laser, operating at an incident wavelength of 514.5 nm. The samples were drop-casted on a clean Si wafer and allowed to air–dry at room temperature. The X-ray diffraction patterns were recorded at 25 °C by a Rigaku PXRD system using Cu Kα radiation (λ = 1.5418 Å) at 40 kV and 44 mA with the spectral range of 2θ from 10° to 80°. The samples were dropped on a glass slide and allowed to air–dry at room temperature. Transmission electron microscopy (TEM) studies were conducted on carbon coated copper grids (Ted Pella, Inc. 01822-F, support films, ultrathin carbon type-A, 400 mesh copper grid) using a FEI Tecnai (F30 G2, Twin) microscope operated at a 300 keV accelerating electron voltage.

Supporting Figures

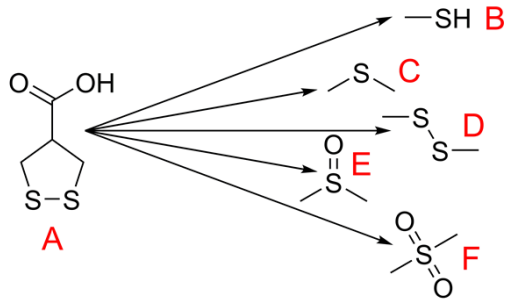

Fig. S1 Key suspected products of asparagusic acid (A) metabolism: (B) methanethiol, (C) dimethyl sulfide, (D) dimethyl disulfide, (E) dimethyl sulfoxide, and (F) dimethyl sulfone.

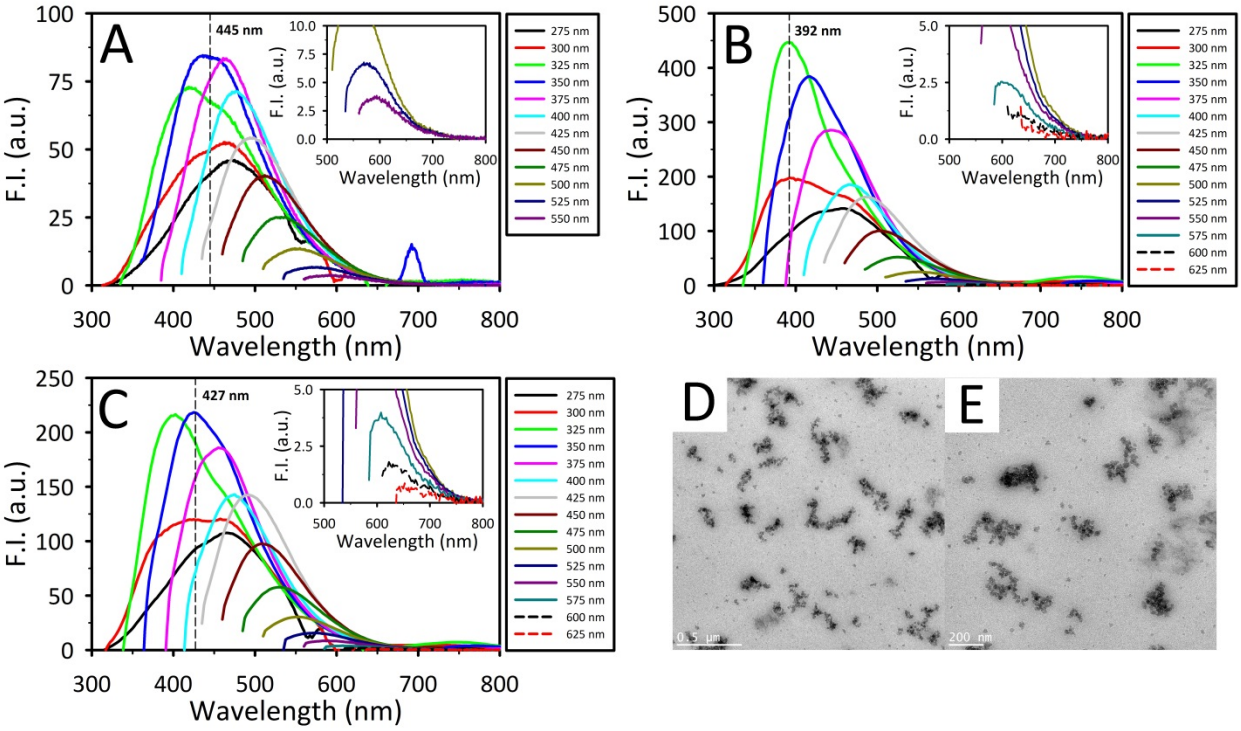

Fig. S2 Longer pyrolysis time (24 h) of the un-supplemented urine appears to generate a red-shift in the peak emission (A) over the shorter time of 12 h (B). For the 12 h treatment, the peak emission occurred at 392 nm under 325 nm excitation but by doubling the treatment time the peak emission shifted to 445 under 350 nm excitation. The TEM images (D and E) confirm that small nanocrystals are still generated under the extended pyrolysis conditions. The CPDs (C) display a slight red-shift over the UPDs but do not show as large of a red-shift as the APDs (Fig. 2A) or the extended treatment time sample above. This loosely implies that a longer treatment time of the other urine samples could lead to an even further red-shift in the fluorescent emission.

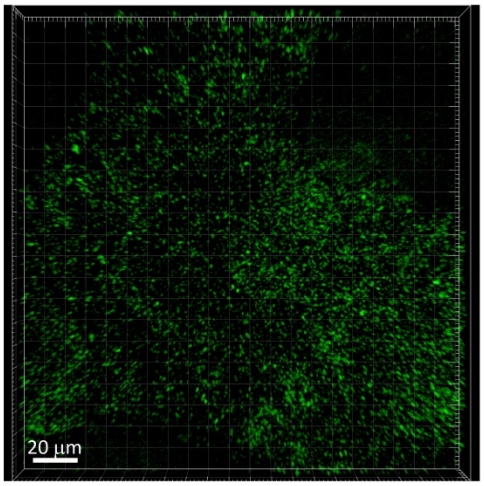

Fig. S3 Confocal fluorescence microscopy image of APD labelled BT-474 cells (human mammary gland, breast/duct carcinoma) adhered to a 96-well plate.

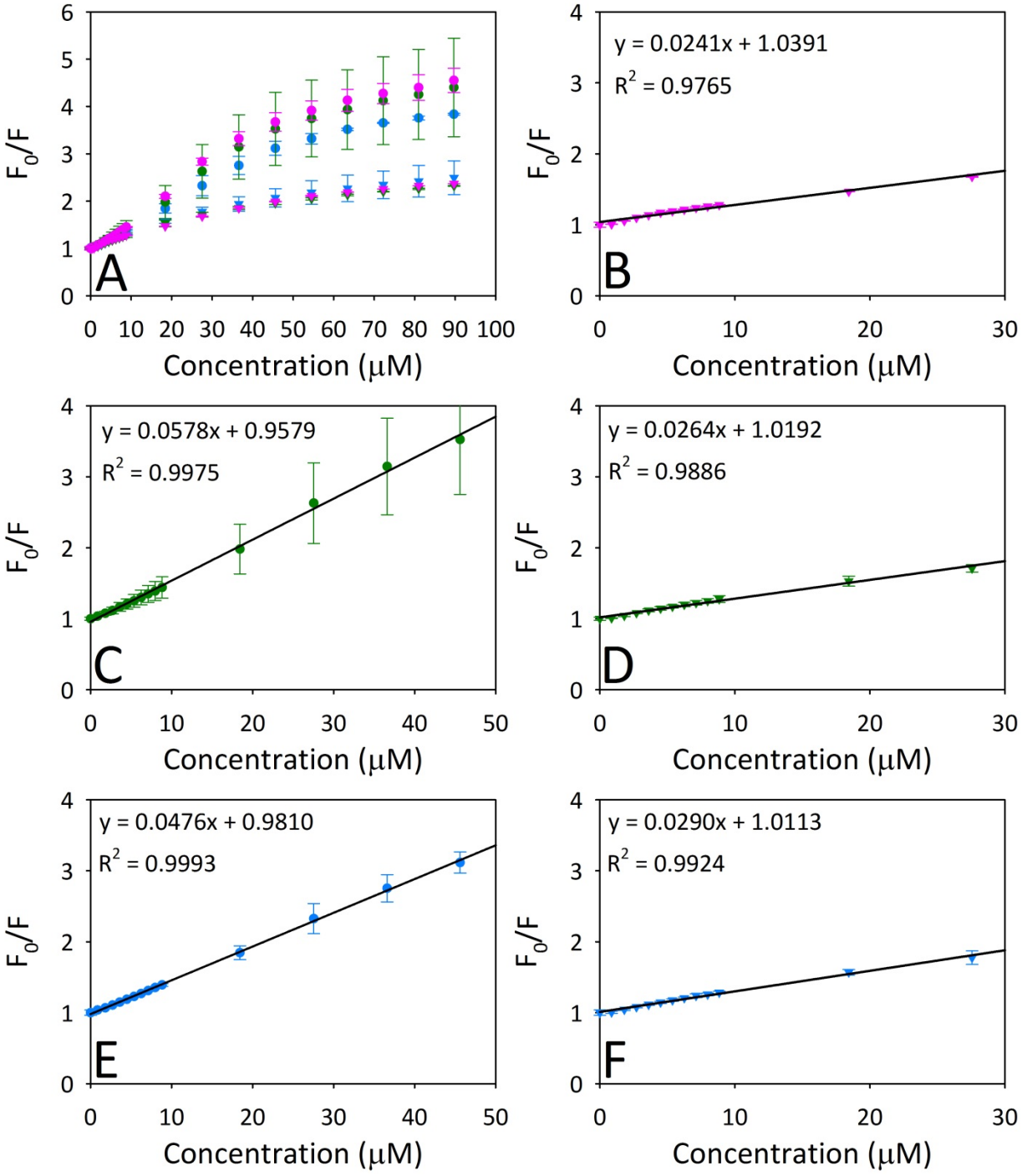

Fig. S4 (A) Quenching curves for UPDs (blue), CPDs (green) and APDs (pink) in the presence of Hg^{2+} (circles) and Cu^{2+} (triangles). Both metals showed fluorescence quenching with Hg^{2+} displaying stronger quenching over Cu^{2+}. Each respective metal quenched all three samples in a similar manner. (B)–(F) Quenching curves used for quenching constants and limit of detection (L.O.D) calculations. Color and shape schemes are the same in (B)–(F) as is in (A). The APD Hg^{2+} quenching curve used for this calculation was provided in Fig. 6 of the paper.

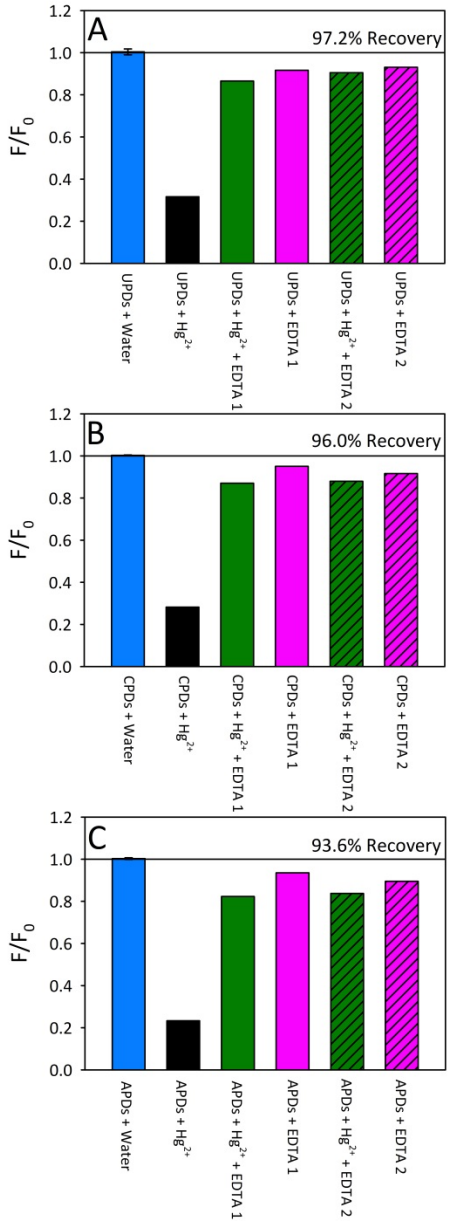

Fig. S5 PD fluorescent recovery using EDTA to preferentially chelate Hg^{2+}, restoring the previously quenched fluorescent site. All three samples showed >90% signal recovery with the (A) UPDs, (B) CPDs, and (C) APDs displaying 97.2%, 96.0%, and 93.6% recovery, respectively.

References

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