Supporting Information

Surface moieties drive the superior protection of curcumin-derived carbon quantum dots against retinal ischemia-reperfusion injury

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Supplementary experimental section

Preparation and characterization of Cur-CQDs

Cur-CQDs were synthesized by directly pyrolyzing curcumin (0.5 g) at 120, 150, or 180 °C in a muffle furnace (DH 300, Dengyng, Taipei, Taiwan) for 2 h as described previously.[1] The as-obtained brown-black residues were cooled down to room temperature and dissolved in 50 mL of sodium phosphate buffer (200 mM, pH 12.0). Solutions were sonicated (DC200H, Honeywell, Charlotte, NC, USA) for 2 h and then centrifuged with a relative centrifugal force (RCF) of 35,000 g for 1 h at 25 ^{o}C to remove larger-sized particles. Supernatants were further purified by dialysis in sodium chloride solution (10 mM) for 5 h using a dialysis membrane (MWCO = 0.5–1.0 kDa; Float-A-Lyzer G2, Spectrum Laboratories, Rancho Dominguez, CA, USA), with the surrounding solution being changed every hour. After 5 h, the sodium chloride solution was replaced with deionized water and dialyzed for another 18 h. Finally, the purified CQDs solution was stored at 4 °C, and the concentration of the purified CQDs solution was determined by placing the completely frozen sample under a vacuum to remove water and measure the weight of the residues (freeze-drying method).

Antioxidant capacity analysis

We utilized the Cupric ion-based total antioxidant capacity (CuTAC) assay, which had been previously developed,[2] to evaluate the antioxidant capacity of different Cur-CQDs. The bicinchoninic acid (BCA) and $CuSO_4$ used in the assay were procured from ThermoFisher Scientific (included in the PierceTM BCA Protein Assay Kit, Catalog number: 23225, Waltham, MA, USA). Ascorbic acid was obtained from Fisher Scientific (B581-05, JT Baker, Phillipsburg, USA), while trolox was purchased from Sigma-Aldrich (Inc.St. Louis, MO, U.S.A). To compare their antioxidant capacity with established standards, we employed trolox as a widely used antioxidant in antioxidant capacity assays and ascorbic acid, known to be the most abundant and significant antioxidant in the human eye.

Each of the examined solutions was serial diluted ranging from 4.6875 to 300 mg/L. In brief, 10 μL of the serially diluted sample was applied to the wells of a 96-well microplate, followed by the addition of 200 μL of a 0.08% $CuSO_4$ solution, diluted with BCA, and incubated for 20 minutes, away from light. Various antioxidant compounds facilitated the reduction of Cu^{2+} to Cu^{1+}, and the Cu^{1+} would further form a violet chelate complex through interaction with BCA. The resulting colorimetric measurements were obtained at 570 nm using an absorbance microplate reader (Sunrise™ Tecan, Switzerland). The accuracy and stability of the CuTAC assay were previously validated in our research study.[3]

In situ TUNEL labeling

Eyeballs were harvested 24 h after IR injury and were sectioned along the vertical meridian to include the optic nerve head. For each rat, two 3-μm thick retinal sections that included the ora serrata and the optic nerve were stained by TdT-mediated dUTP nick-end labeling (TUNEL)–based kit (TdT FragEL; Oncogene, Darmstadt, Germany). The number of TUNEL-positive cells for each retinal section was counted in 6 selected superior and inferior retinal areas, each 0.425 mm in length. The two first chosen segments were 0.425 mm superior and inferior to the optic nerve head, the two-second segments were 0.425 mm away from the first segments, and the other two third segments were 0.425 mm away from the second segments. The total number of TUNEL-positive cells in these 12 retina areas was averaged as a representative of TUNEL-positive cells per one eye sample.

Immunofluorescence staining

At 24 h after IR injury, immunofluorescence was performed to examine the localization of the hypoxia-induced factor (HIF)-α, the cluster of differentiation (CD) 68 molecule, B-cell lymphoma (BCL)-2, and glucose regulatory protein (Grp) 78. Briefly, rats were euthanized 24 h after IR injury, the eyeballs were embedded in paraffin or Optimal Cutting Temperature

(OCT) compound. The tissue specimens were then incubated with one of the following primary antibodies: rabbit polyclonal antibody against rat HIF-α (1:50, Bioss, US), mouse polyclonal antibody against rat CD68 (1: 1000, Abcam, Cambridge, UK), mouse monoclonal antibody against rat BCL-2 subunit protein (1:50, Santa Cruz, US), and mouse monoclonal antibody against rat glucose-related protein (Grp78, 1: 100, Proteintech, Germany). Immunoreactivity was detected by a fluorescein isothiocyanate (FITC)-labeled or rhodamine-labeled secondary antibody (1: 200, Abcam, Cambridge, U.K.), cell nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI).

Western blot analysis

Twenty-four hours following IR injury, rats were euthanized in a CO_2-saturated chamber, anterior segments were removed, and retina wholemounts were isolated and shock frozen at –80°C within 2 minutes after enucleation. Retinas were later ultrasonically homogenized into 300 μL of a Ripa buffer containing 50mM Tris (pH 7.4), 150 mM NaCl, 10 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EGTA, 1 mM PMSF and proteinase inhibitor at 4 °C. The protein extracts (20 μg of protein in each lane) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred to a nitrocellulose membrane. The membranes were then blocked and probed with rabbit polyclonal anti-heme oxygenase (HO)-1 (Abcam, Cambridge, U.K.), anti-GRP78 (Proteintech), anti-CCAAT (C)/Enhancer binding protein (EBP)-homologous protein (CHOP) (Santa Cruz), anti-caspase 3 (Abcam), anti- nuclear factor erythroid 2-related factor (Nrf) 2 (Thermo Fisher Scientific) and anti-GAPDH (Santa Cruz) antibody at different dilution. A peroxidase-conjugated anti-rabbit secondary antibody (PerkinElmer, USA) was used at a dilution of 1:15000.

Oxidative Stress and antioxidant marker analysis

Twenty-four hours following IR injury, rats were euthanized in a CO_2-saturated chamber, and retinal tissues were immediately isolated and shock-frozen at −80°C within 2 minutes post-enucleation. Retinas were ultrasonically homogenized in 150 µL of RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 10 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EGTA, 1 mM PMSF, and protease inhibitors at 4°C. The homogenates were centrifuged at 12,000 × g for 10 minutes, and the supernatants were collected for protein normalization using the bicinchoninic acid (BCA) assay. Oxidative stress was assessed by measuring malondialdehyde (MDA) levels using the Reed Biotech MDA Assay Kit (RBC0016), based on the thiobarbituric acid (TBA) method. Antioxidant activities were evaluated using commercial kits from Reed Biotech, including the SOD Assay Kit (RBC0017) for superoxide dismutase activity and the GSH-PX Assay Kit (RBC0022) for glutathione peroxidase activity.

Inflammatory cytokine levels analysis

Retinal inflammatory cytokine levels were quantified using ELISA. Twenty-four hours following IR injury, rats were euthanized in a CO_2-saturated chamber, and retinal tissues were immediately isolated and shock-frozen at −80°C within 2 minutes post-enucleation. Retinas were ultrasonically homogenized in 150 µL of RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 10 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EGTA, 1 mM PMSF, and protease inhibitors at 4°C. The homogenates were centrifuged at 12,000 × g for 10 minutes. The supernatants were collected to estimate the concentrations of IL-6 (DY406), IL-18 (DY7625-05), IL-1β (DY401) and TNF-α (DY410) using ELISA kits from R&D Systems (Minneapolis, MN, USA) and following the manufacturer's protocols. All samples were assayed in triplicates.

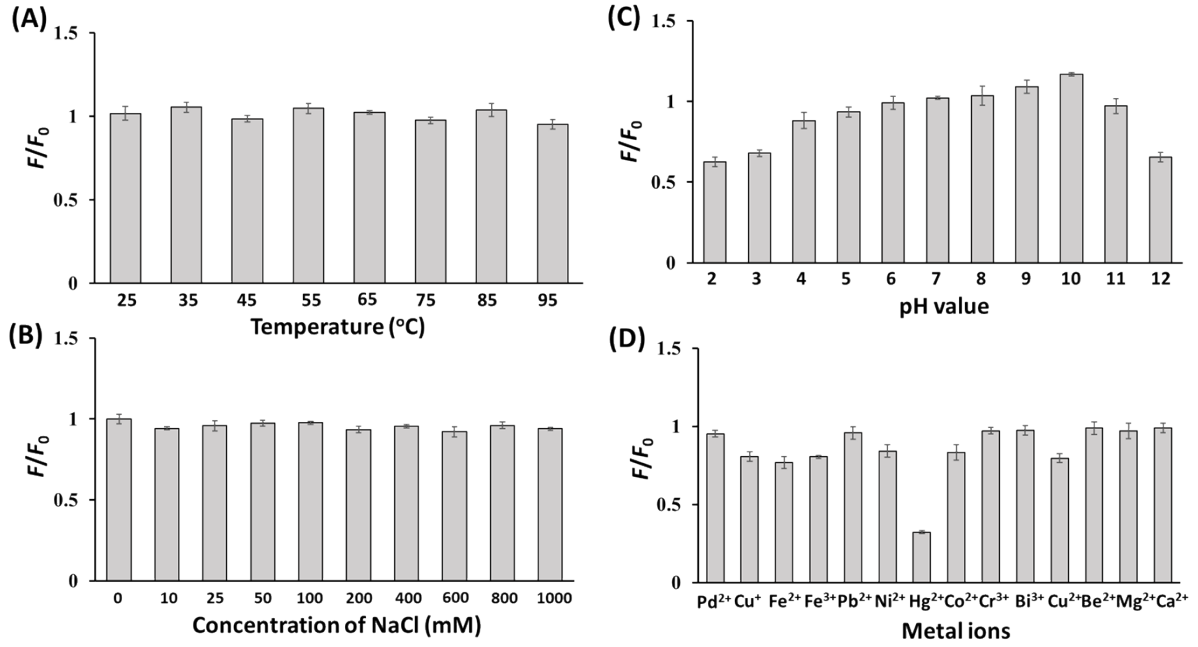

Fig. S1. Stability assessment of Cur-CQDs-150 under various environmental conditions. (a) Thermal stability was evaluated by measuring the fluorescence intensity ratio (F/F_0) of Cur-CQDs-150 (100 μg mL^{-1}) after incubation in deionized (DI) water at temperatures ranging from 25°C to 95°C for 2 h. F and F_0 represent the fluorescence intensity of Cur-CQDs-150 at the specified temperature and at 25°C, respectively. (b) pH stability was examined by incubating Cur-CQDs-150 in 20 mM sodium phosphate buffer across a pH range of 2–12 for 2 h. F and F_0 denote the fluorescence intensity of Cur-CQDs-150 at the tested pH and at pH 7, respectively. (c) Salt tolerance was assessed by monitoring the fluorescence intensity of Cur-CQDs-150 in 20 mM sodium phosphate buffer (pH 7.0) with NaCl concentrations ranging from 0 to 1000 mM. F and F_0 indicate the fluorescence intensity in the presence and absence of NaCl, respectively. (d) Metal ion stability was analyzed by incubating Cur-CQDs-150 in 20 mM sodium phosphate buffer (pH 7.0) with 100 μM concentrations of Pd^{2+}, Cu^{+}, Fe^{2+}, Fe^{3+}, Pb^{2+}, Ni^{2+}, Hg^{2+}, Co^{2+}, Cr^{3+}, Bi^{3+}, Cu^{2+}, Be^{2+}, Mg^{2+}, and Ca^{2+} for 2 h. F and F_0 represent the fluorescence intensity in the presence and absence of the tested metal ions, respectively.

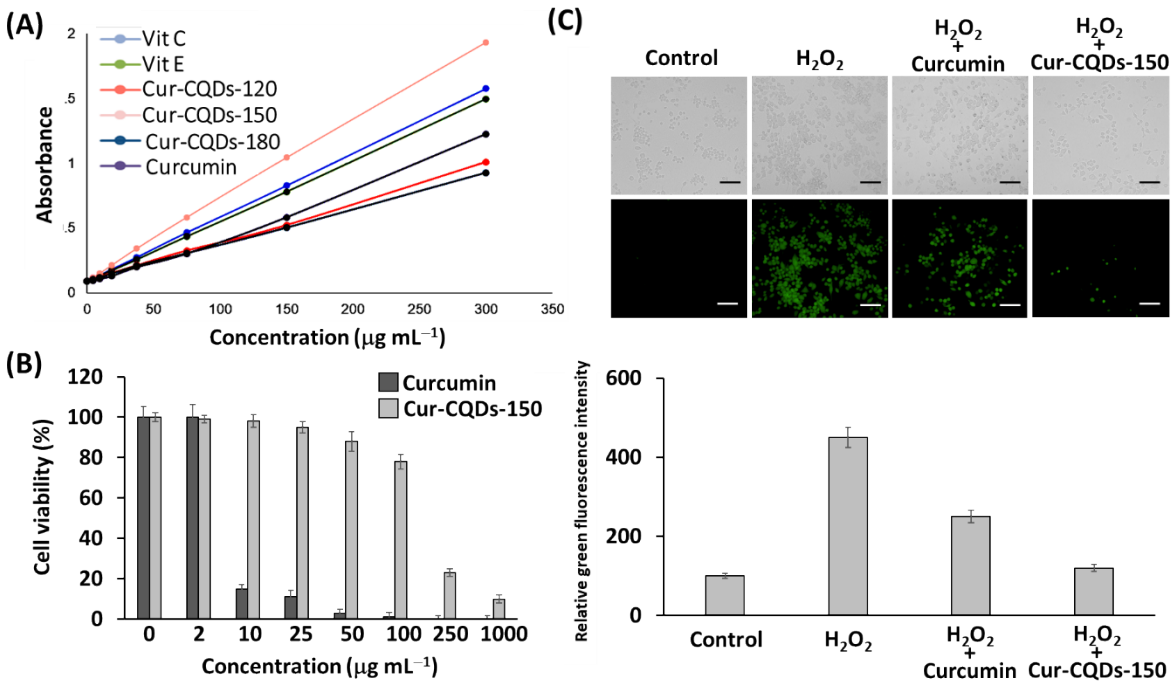

Fig. S2. (A) Comparison of anti-oxidative capacities of vitamins (Vit), curcumin, and Cur-CQDs, (B) cell viability of RGC-5 cells after being treated with curcumin or Cur-CQDs, and (C) intracellular ROS analysis of RGC-5 cells after being treated with H_2O_2 followed by curcumin or Cur-CQDs treatment. Data are the means ± SD of four separate experiments.
(A) The principle total antioxidant capacity (TAC) assay involves the reduction of copper (II) ions to copper (I) ions by antioxidants present in the sample. The reduced copper (I) ions can then form a complex with bicinchoninic acid violet color that can be measured by the absorption at 570 nm. (B) Relative cell viability of RGC-5 cells treated with curcumin or Cur-CQDs with concentrations from 1 to 1000 μg mL^{-1}. RGC-5 cells (1×10^6 cells per well) were exposed to curcumin or Cur-CQDs for 48 h at 37 °C and then assessed cell viability using an MTT assay. (C) Bright-field and fluorescence images (a) and green fluorescence intensity (b) (500–550 nm) of RGC-5 cell cells after being treated with H_2O_2 (100 μM) for 1 h and subsequent exposure to curcumin or Cur-CQDs (10 μg/mL) for 6 h. Intracellular ROS levels were evaluated using 2'-7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) staining, which undergoes deacetylation to non-fluorescent dichlorodihydrofluorescein (H_2DCF) by cellular esterases and oxidation to fluorescent 2',7'-dichlorofluorescein (DCF) under ROS presence, with excitation and emission peaks at 480 nm and 520 nm, respectively.

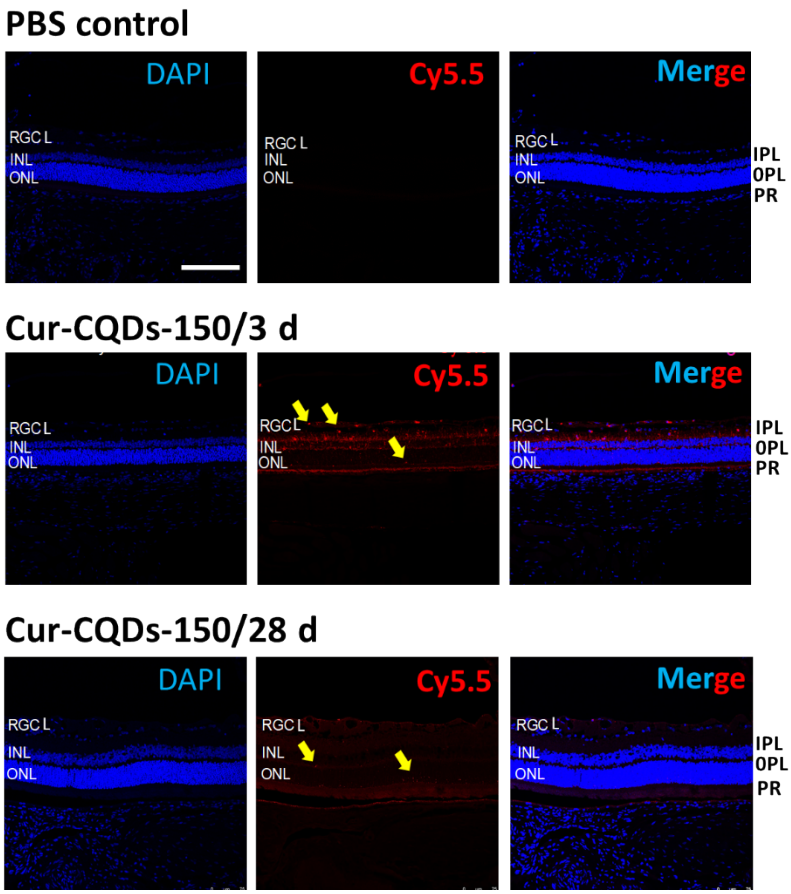

Fig. S3. Longitudinal distribution of Cur-CQDs-150 in ocular tissues at 3 and 28 days post-intravitreal injection. The layers of the retina are labeled as follows: RGCL (retinal ganglion cells layer), IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), ONL (outer nuclear layer), and photoreceptors (PR). Scale bar = 500 μm.
This figure illustrates the penetration and spread of Cy5.5-labeled Cur-CQDs-150 within ocular tissues at 3 and 28 days post-intravitreal injection. Fluorescent signals (red), highlighted by yellow arrows, demonstrate the presence of Cur-CQDs in distinct retinal layers.

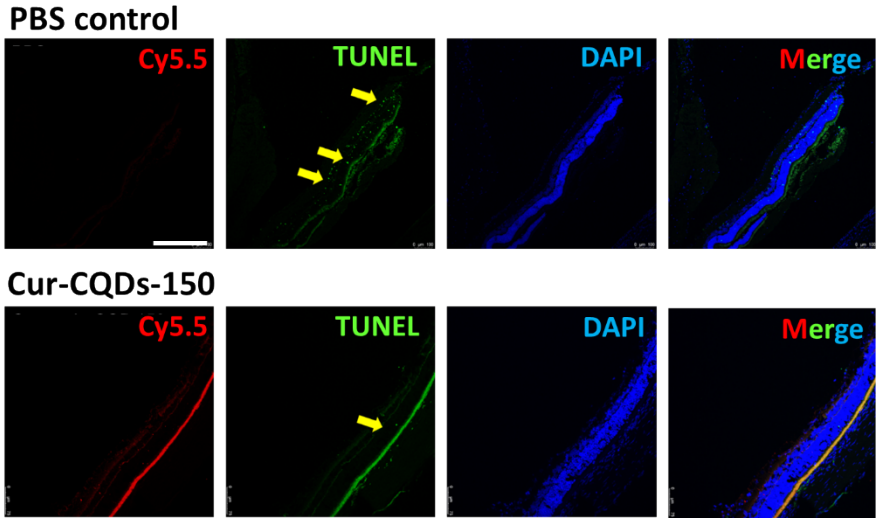

Fig. S4. Dual Staining of Cy5.5-labeled Cur-CQDs-150 and TUNEL in retinal tissues post-ischemia-reperfusion (IR) injury. Scale bar = 400 μm.

This result demonstrates the co-labeling of Cy5.5-labeled Cur-CQDs-150 fluorescence (red) and TUNEL staining (green) in retinal tissues following IR injury. The Cy5.5-labeled Cur-CQDs are indicated by red fluorescence, which correlates with fewer apoptotic signals, highlighted by yellow arrows. The reduced green fluorescence signifies decreased apoptotic cell presence, suggesting a protective effect of Cur-CQDs against IR-induced apoptosis.

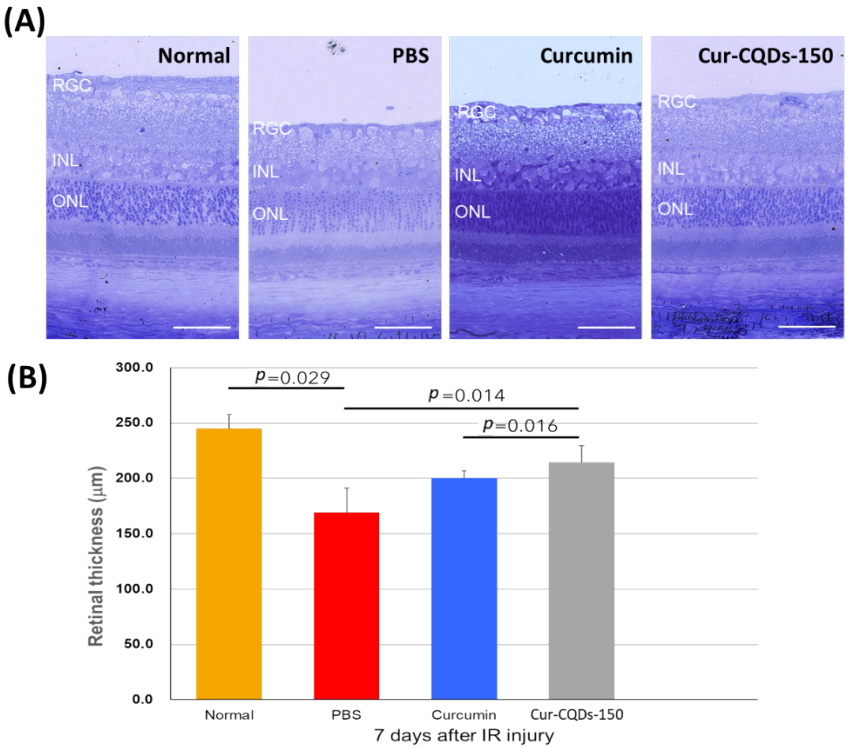

Fig. S5. (A) Representative histological images of retinal sections and (B) quantification of retinal layer thickness in normal mice (negative control group) and IR injury mice 7 days after intravitreal injection of PBS, curcumin, or Cur-CQDs-150. Error bars in (B) indicate the standard deviation from 4 repeated measurements. RGCL: retinal ganglion cell layer, INL: inner nuclear layer, ONL: outer nuclear layer. Scale bar: 75 μm.

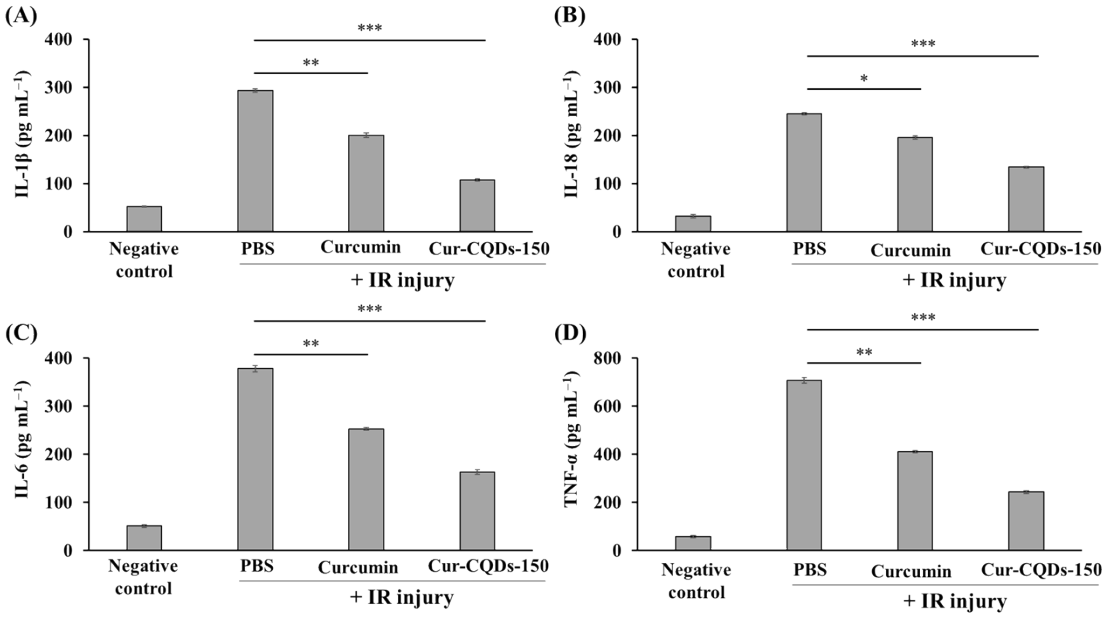

Fig. S6. Analysis of inflammatory cytokines in retinal tissues following IR injury. ELISA quantification of inflammatory cytokines, including (A) IL-1β, (B) IL-18, (C) IL-6, and (D) TNF-α. Retinal tissues were collected from rats subjected to 60 min of ischemia followed by 24 h of reperfusion. Prior to IR induction (negative control), intravitreal injections of PBS, curcumin, or Cur-CQDs-150 were administered. Retinas were homogenized in 150 μL of RIPA buffer for sample preparation. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

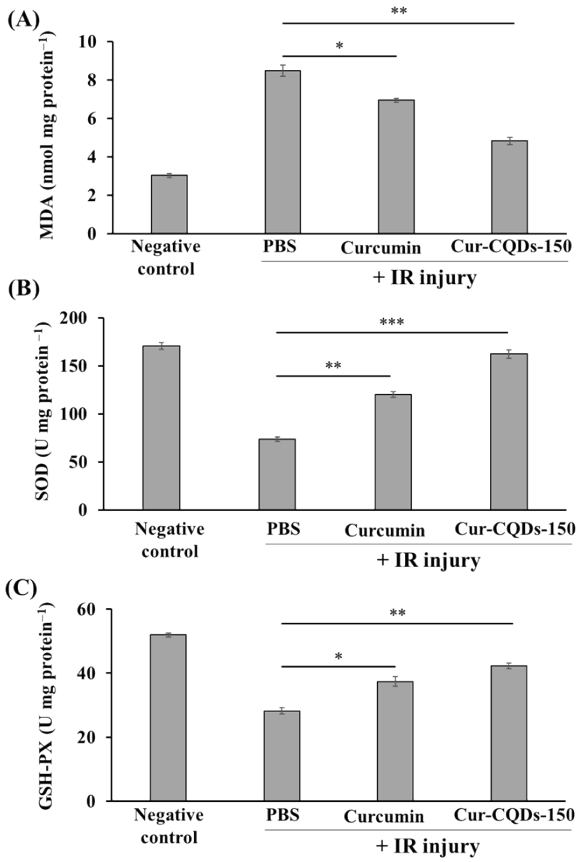

Fig. S7. Oxidative stress and antioxidant marker analysis in retinal tissues following IR injury. (A) Measurement of oxidative stress levels *via* malondialdehyde (MDA) content. (B, C) Assessment of antioxidant markers, including superoxide dismutase (SOD) activity (B) and glutathione peroxidase (GSH-PX) activity (C). Retinal tissues were collected from rats subjected to 60 min of ischemia followed by 24 h of reperfusion. Prior to IR induction (negative control), intravitreal injections of PBS, curcumin, or Cur-CQDs-150 were administered. Retinas were homogenized in 150 μL of RIPA buffer for sample preparation, and analyte levels were normalized to protein content. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

References

1. C.-J. Lin, T.-L. Hwang, R.Y.L. Wang, A. Nain, R.-H. Shih, L. Chang, H.-J. Lin, S.G. Harroun, H.-T. Chang and C.-C. Huang, *Small*, 2024, **20**, e2307210.
2. Y.-T. Tsao, W.-C. Wu, K.-J. Chen, L.-K. Yeh, Y.-S. Hwang, Y.-J. Hsueh, H.-C. Chen and C.-M. Cheng, *Bioeng. Transl. Med.*, 2021, **6**, e10199.
3. Y.-T. Tsao, W.-C. Wu, K.-J. Chen, C.-F. Liu, Y.-J. Hsueh, C.-M. Cheng and H.-C. Chen, *Antioxidants (Basel)*, 2022, **11**, 397.