Supporting Information

Synergistically dual-functional nano eye-drops for simultaneously anti-inflammatory and anti-oxidative treatment of dry eye disease

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Experimental section:

Chemicals. Tetrachloroauric(III) acid was purchased from Acros (Geel, Belgium). Sodium phosphate tribasic, sodium phosphate dibasic, Tris(hydroxymethyl)aminomethane (Tris), acetic acid, potassium chloride, magnesium chloride, calcium chloride, isopropanol, acetic acid, calcium chloride and benzalkonium chloride (BAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and the antibiotic/antimycotic (A/A) solution (10000 U mL^{-1} penicillin, 10 mg mL^{-1} streptomycin and 25 mg mL^{-1} amphotericin B) were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). MEM, GlutaMAX™ Supplement cell culture media was purchased from Thermo Fisher Scientific (Cramlington, UK). SIRC cells, a Statens Seruminstitut rabbit cornea cell line (ATCC No. CCL-60), were purchased from the American Type Culture Collection (Manassas, VA, USA). The nucleolin antibody [C23 (D-6)] was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). MUC5AC antibody was purchased from Abcam (Cambridge, MA, USA). Annexin V-fluorescein isothiocyanate and terminal dUTP nick-end labeling (TUNEL) were purchased from Roche Diagnostics (Sydney, Australia). DAPI (4',6-diamidino-2-phenylindole) was purchased from Vector Laboratories (Orton Southgate, Peterborough, U.K.). Schirmer test paper strips was purchased from EagleVision (Memphis, TN, USA). Nitrocellulose filter paper was obtained from Pall (New York, NY, USA). Quantikine enzyme-linked immunosorbent assay (ELISA) kit of PGE_2 was purchased from Cayman Chemicals (Ann Arbor, MI, USA). The ELISA kit of TNF-α, IL-6 and vascular endothelial growth factor (VEGF) were purchased from R&D Systems (Minneapolis, MN, USA). Phosphate buffered saline (PBS; containing 137 mM NaCl, 5.0 mM KCl, 0.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM Na_2HPO_4, and 2.0 mM KH_2PO_4; pH 7.4) was used to mimic physiological conditions. Amicon Ultra 3K device was purchased from EMD Millipore (Billerica, MA, USA). Milli-Q ultrapure water (18.2 MΩ·cm; EMD Millipore, Billerica, MA, USA) was used in all experiments. New Zealand white rabbits (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan, ROC), weighing 3.0−3.5 kg and 16−20 weeks of age, were used for this study.

Synthesis of Poly-CH. The Au@Poly-CH NPs were synthesized and purified three times *via* centrifugation [relative centrifugal force (RCF), 10,000 g] for 20 min. The purified pellet was collected and resuspended in KI/I_2 solution for 12 h for etching of the Au NPs from the Au@Poly-CH NPs to obtain Poly-CH. After three-times of purification with DI water by Amicon Ultra 3K spin tube, the purified Poly-CH solution was stored at 4 °C until in use.

Characterization of core-shell AF/Au@Poly-CH NPs. The mean diameter of the as-prepared AF/Au@Poly-CH NPs was determined by transmission electron microscopy (TEM) (Tecnai 20 G2 S-Twin TEM, Philips/FEI, Hillsboro, OR, USA). A Synergy H1 multi-mode monochromatic microplate

spectrophotometer (Biotek Instruments, Winooski, VT, USA) was used to measure the absorbance of the AF/Au@Poly-CH NP solution. The X-ray diffraction (XRD) pattern of the AF/Au@Poly-CH NP prepared on Si substrates was recorded using a PANalytical X'Pert PRO diffractometer (PANalytical B.V., Almelo, Netherlands) with Cu-Kα radiation (λ = 0.15418 nm). The particle concentration of the as-prepared AF/Au@Poly-CH NPs was determined assuming the presence of ideal spherical particles and by using equation 1.

$$n = 3\ m/4\pi r^3 s \quad (1)$$

Where, n is the number of Au particles per milliliter, m is the concentration of Au in the substance (g mL^{-1}), r is the particle radius (cm), and s is the specific gravity of colloidal gold (19.3 g cm^{-3}). The m and r values were determined via inductively coupled plasma mass spectroscopy (ICP–MS; PerkinElmer ELAN 6000, Waltham, MA, USA) and TEM measurements, respectively. In order to determine the final molar concentration of Au NPs, the concentration obtained was converted into the number of Au particles per liter, and divided by Avogadro's number. AF concentration was quantitatively determined using a Waters Alliance e2695 HPLC system (Waters Corporation, Milford, MA, USA). Further characterizations of AF/Au@Poly-CH NPs were conducted by Fourier transform infrared spectroscopy (FT-IR; Santa Clara, CA, USA), and dynamic light scattering (DLS; Nano ZS, Malvern Instruments, Worcestershire, UK). The thermal characteristics of CH, AF, Au@Poly-CH NPs, and AF/Au@Poly-CH NPs were studied by thermogravimetric analysis (TA Instruments, Inc., New Castle, DE, USA) over a temperature range of 25 to 700 °C at a heating rate of 10 °C min^{-1} under nitrogen gas flow.

Drug release studies. Drug release tests were performed with AF/Au@Poly-CH NPs ([Au NPs] = 7.57 pM) in 5 mL PBS (pH 7.4) and incubated at 25 °C. Samples were collected at predetermined time intervals and analyzed by HPLC, Waters ACQUITY series LC system (Waters Alliance e2695). The chromatographic separation was achieved using a Waters Symmetry C18 reverse phase column (4.6 mm I.D. × 150 mm, 5 µm, 100 Å). Waters® 2998 Photodiode Array (PDA) Detector is designed to complement our LC-UV systems, and the detector was set at a wavelength of 238 nm. The solvent system consisted of DI water (solvent A) and methanol (solvent B). The chromatographic separation of AF was performed at 35 °C at a flow rate of 1.0 mL min^{-1} using a linear gradient with ratio of methanol to DI water from 15%:85% to 80%:20% over 15 min. The sample volume for injection was 50 µL and the total separation time was 20 min. The cumulative release percentage is obtained based on the released and total amounts of AF at each time interval, and multiplied by a factor of 100.

Folin-Ciocalteu assays. The total phenolic content in the of the AF/Au@Poly-CH NPs was measured by using Folin-Ciocalteu reagent (Sigma Chemical Co., St. Louis, MO, USA) and CH as a standard. Briefly,

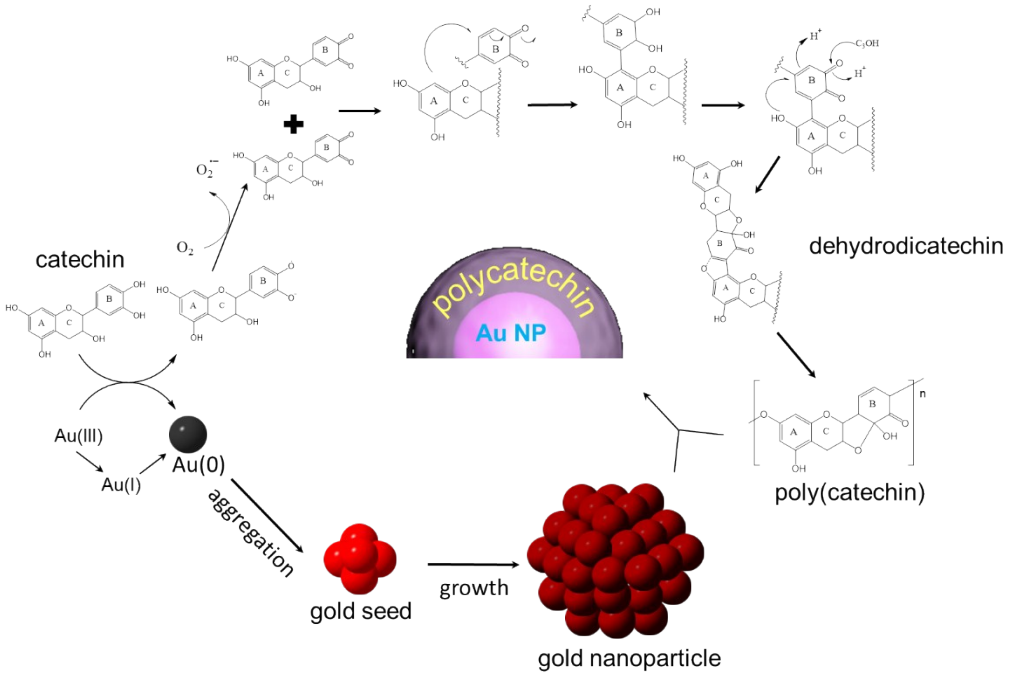
the Au@Poly-CH NPs ([Au NPs] = 7.57 pM) or AF/Au@Poly-CH NPs ([Au NPs] =7.57 pM) samples were mixed with Folin-Ciocalteu reagent (1.0 mL) and sodium carbonate (1.5 mL, 20%). After incubation at room temperature for 2 h, the absorbance of mixture was measured at 760 nm using a UV-vis absorption spectrometer (Synergy 4 Multi-Mode).

Cellular ROS assays. The SIRC cells were seeded at a density of 2.5×10^4 cells $well^{-1}$ in 24-well plates and with regular growth medium containing MEM, 10% FBS, and 1% A/A solution overnight for cell attachment. Then, the SIRC cells in the culture wells were incubated with Au@poly-CH NPs and AF/Au@poly-CH NPs samples for 24 h. After incubation, the cell cultures were treated with a further incubation of 6 h in medium containing 200 μM hydrogen peroxide. For the purpose of comparison, the cells were exposed to hydrogen peroxide (H_2O_2) of 0 μM (Ctrl group) and 200 μM (H_2O_2 group) for 6 h following 24 h of incubation in the absence of the test samples. Intracellular accumulation of reactive oxygen species (ROS) was measured by oxidative conversion of cell-permeable 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes) to fluorescent 2',7'-dichlorofluorescein (DCF). The SIRC cells in the culture wells were incubated with 10 μM DCFH-DA solutions at 37°C for 1 h. The DCF fluorescence imaging (Ex. 488 nm; Em. 525 nm) was acquired with a fluorescence microscope (Axiovert 200M; Carl Zeiss, Oberkochen, Germany). Furthermore, the fluorescence reading was done with a multi-mode microplate reader (BioTek Instruments, Winooski, VT, USA) to detect the difference in the fluorescence intensity. All experiments were conducted in quadruplicate.

Xanthine oxidase (XO) inhibition assays. Xanthine oxidase activity was measured by uric acid formation of the absorbance at 295 nm. The reaction contained 0.1mM xanthine and 0.001 unit/mL XO in 50 mM phosphate buffer (pH 7). The mixtures of different samples [CH (0−250 μM), AF (0−624.52 μM), Au@Poly-CH NPs ([Au NPs] = 0–266.44 pM) or AF/Au@Poly-CH NPs ([Au NPs] = 0−87.31 pM)] were incubated for 15 min. The inhibition percentage of XO assay was calculated to the formulation = $(A_{control} - A_{inhibition}) / A_{control} \times 100$ %.

PGE_2, TNF-α, IL-6 and VEGF assays. The SIRC cells were seeded at a density of 1×10^5 cells/well in a 24-well plate and incubated in MEM medium overnight for cell attachment. Lipopolysaccharide (LPS) with the concentration of 1.0 μg mL^{-1} was used to induce inflammation of SIRC cells. After 1 h incubation, we added AF (41.94 μM), CH (7.10 μM), Au@Poly-CH NPs ([Au NPs] = 7.57 pM) or AF/Au@Poly-CH NPs ([Au NPs] = 7.57 pM) into the wells and then collected the supernatant after 24 h. Each of these solutions was validated and the samples were reconstituted in ELISA buffer and assayed for PGE_2 by the PGE_2 competitive ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA). The TNF-α,

IL-6 and VEGF assay was performed similar to the PGE_2 assay. Following the same protocol as mentioned in the PGE_2 assay, solution was collected after incubation with LPS and assayed in duplicate using the TNF-α, IL-6 and VEGF ELISA Quantikine kit (R&D Systems, Minneapolis, MN, USA).



Scheme S1. Proposed reaction mechanism of $HAuCl_4$ and catechin (CH) to synthesize core-shell Au@Poly-CH NPs.

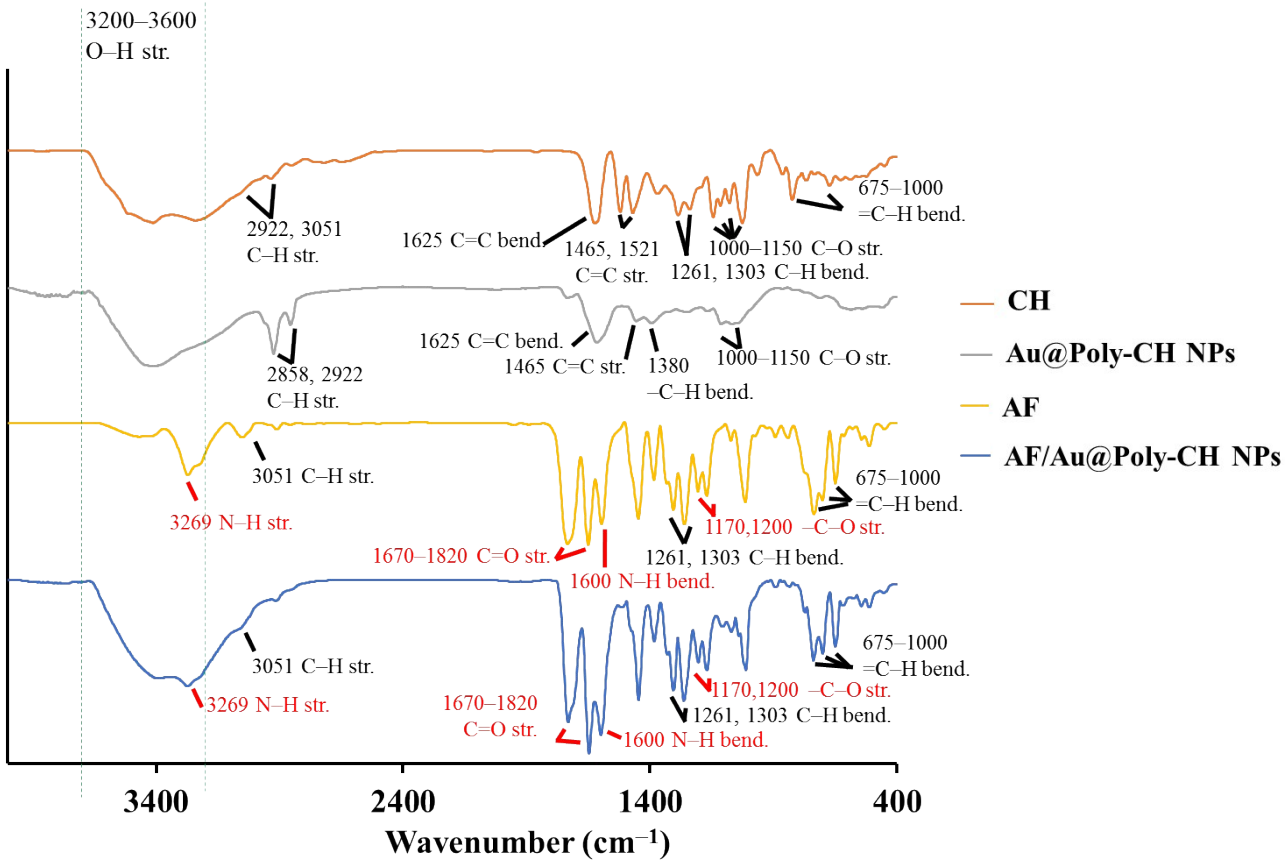


Figure S1. FT-IR spectra of CH, Au@Poly-CH NPs, AF, and AF/Au@Poly-CH NPs.

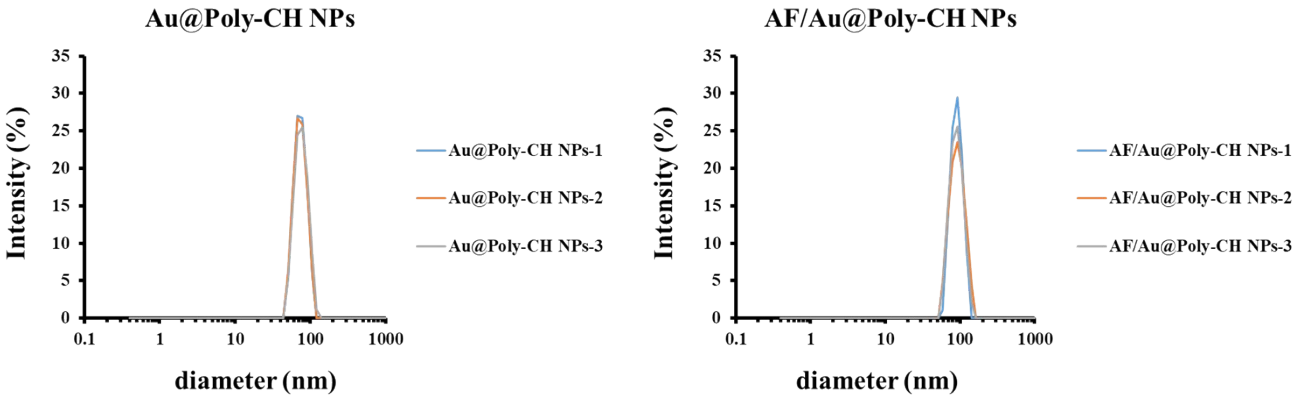


Figure S2. DLS spectra of Au@Poly-CH NPs and AF/Au@Poly-CH NPs dispersions in ATS. Each sample was analyzed in triplicate.

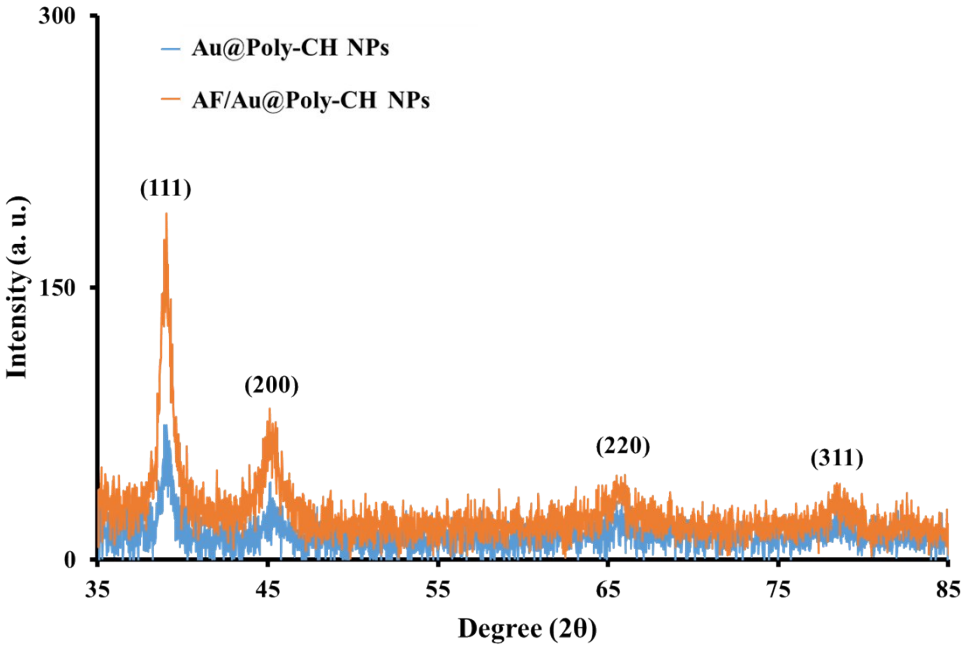


Figure S3. XRD spectra of Au@Poly-CH NPs and AF/Au@Poly-CH NPs.

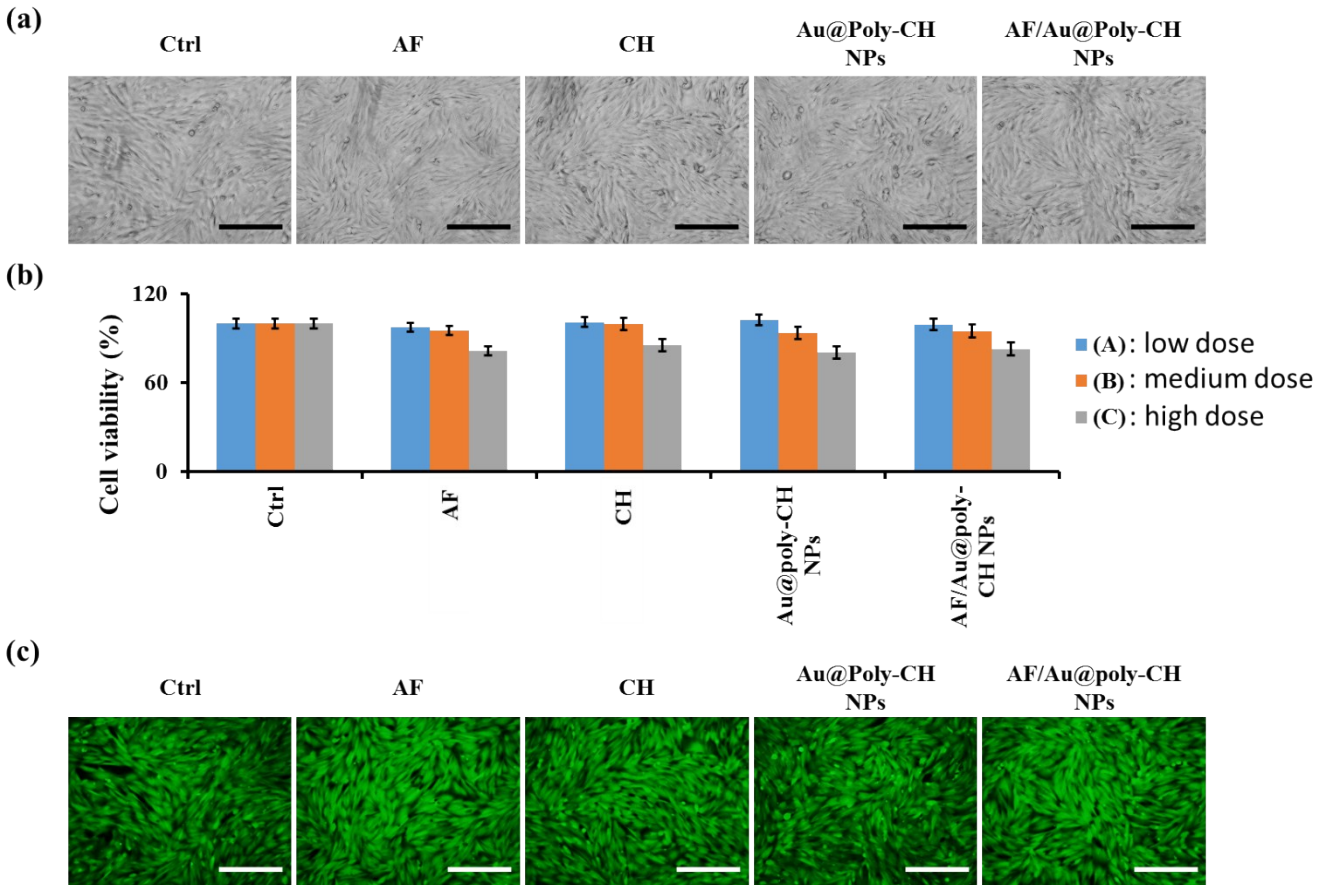


Figure S4. (a) Cell viability of SIRC cells without any drug (Ctrl) and treated with AF (191.97 μM), CH (35.51 μM), Au@Poly-CH NPs ([Au NPs] = 37.85 pM; 35.51 μM CH equivalents), or AF/Au@Poly-CH NPs ([AF] = 209.72 μM; [Au NPs] = 37.85 pM; 108.36 μM CH equivalents) in culture medium at 37 °C for 24 h. (b) Quantitated cell viabilities for each group mentioned in (a) by MTT assay. The cells without any drug treatment is the Ctrl. The groups treated with (A) is low dose: CH (7.10 μM), AF (38.39 μM), Au@Poly-CH NPs ([Au NPs] = 7.57 pM; 7.10 μM CH equivalents), or AF/Au@Poly-CH NPs ([AF] = 41.94 μM; [Au NPs] = 7.57 pM; 21.67 μM CH equivalents); (B) is medium dose: CH (35.51 μM), AF (191.97 μM), Au@Poly-CH NPs ([Au NPs] = 37.85 pM; 35.51 μM CH equivalents), or AF/Au@Poly-CH NPs ([AF] = 209.72 μM; [Au NPs] = 37.85 pM; 108.36 μM CH equivalents); and (C) is high dose: CH (71.02 μM), AF (383.94 μM), Au@Poly-CH NPs ([Au NPs] = 75.69 pM; 71.02 μM CH equivalents), or AF/Au@Poly-CH NPs ([AF] = 419.43 μM; [Au NPs] = 75.69 pM; 216.72 μM CH equivalents). (c) Live/Dead cell staining of Calcein AM/EthD-1 to SIRC cells treated with the same concentrations as in (a). Calcein AM is a non-fluorescent dye that easily permeates live mammalian cells with an intact cell membrane. The hydrolysis of Calcein AM by intracellular esterases produces Calcein, which can be well-retained in the cell cytoplasm. Calcein exhibits strong green fluorescence at 520 nm upon excitation at 480–500 nm. Scale bars in (a) and (c) are 200 μm.

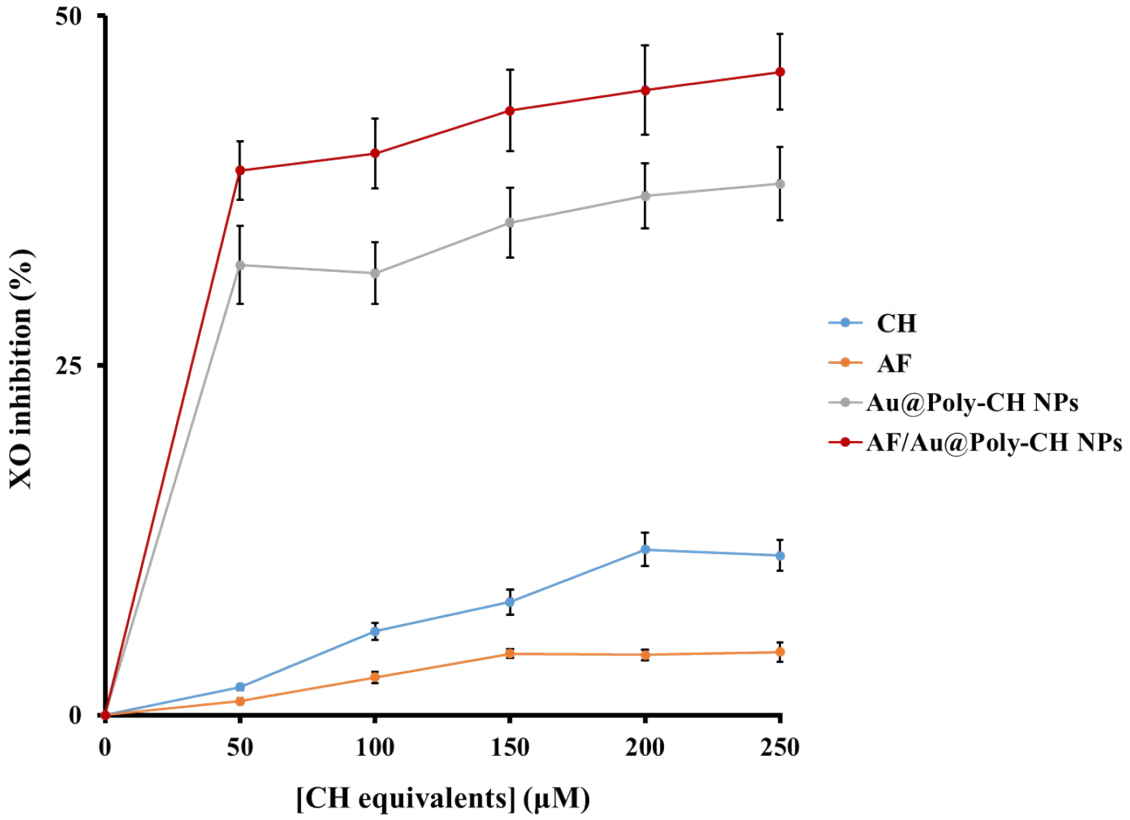


Figure S5. XO inhibition activities of CH (0−250 μM), AF (0−624.52 μM), Au@Poly-CH NPs ([Au NPs] = 0–266.44 pM) or AF/Au@Poly-CH NPs ([Au NPs] = 0−87.31 pM). Error bars represent the standard deviation of four repeated measurements.

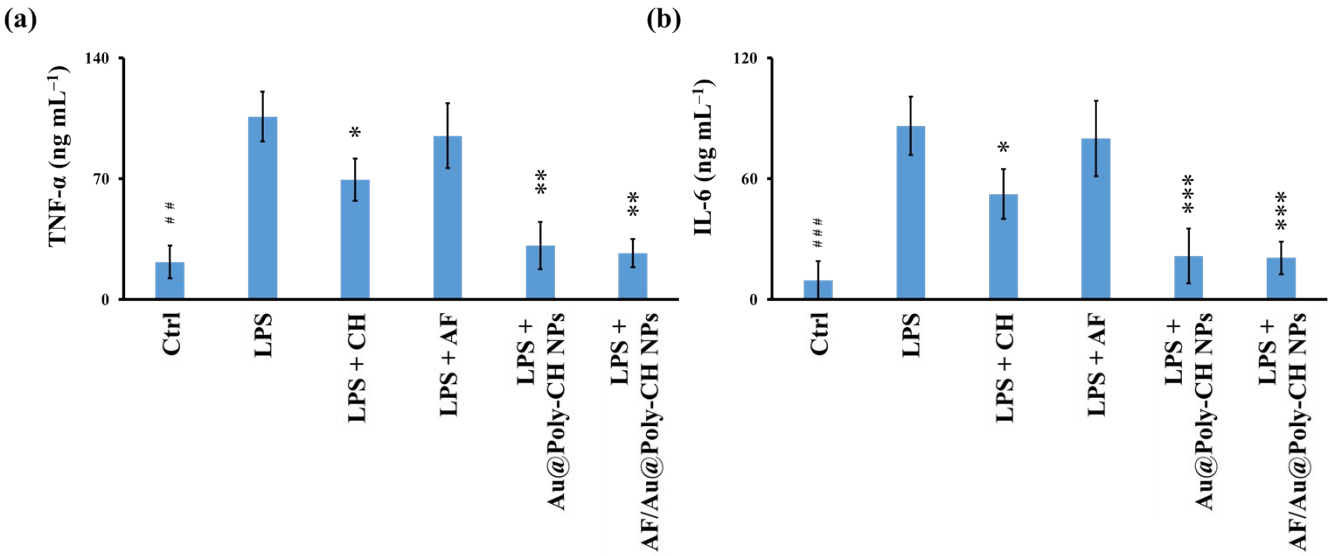


Figure S6. Level of (a) TNF-α and (b) IL-6 released from SIRC cultures after LPS-induced inflammation for 1 h, and followed by treatment with CH (7.10 μM), AF (41.94 μM), Au@Poly-CH NPs ([Au NPs] = 7.57 pM; 7.10 μM CH equivalents), or AF/Au@Poly-CH NPs ([Au NPs] = 7.57 pM; [AF] = 41.94 μM; 21.67 μM CH equivalents) for 12 h. The cells without treatment with LPS and receiving PBS without nanomaterial serve as a control (Ctrl) group. Values are mean ± standard deviation (n = 4). Asterisks indicate statistically significant differences as compared treated groups to the LPS group (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$) and LPS group to Ctrl group (##$p < 0.005$, ###$p < 0.001$).

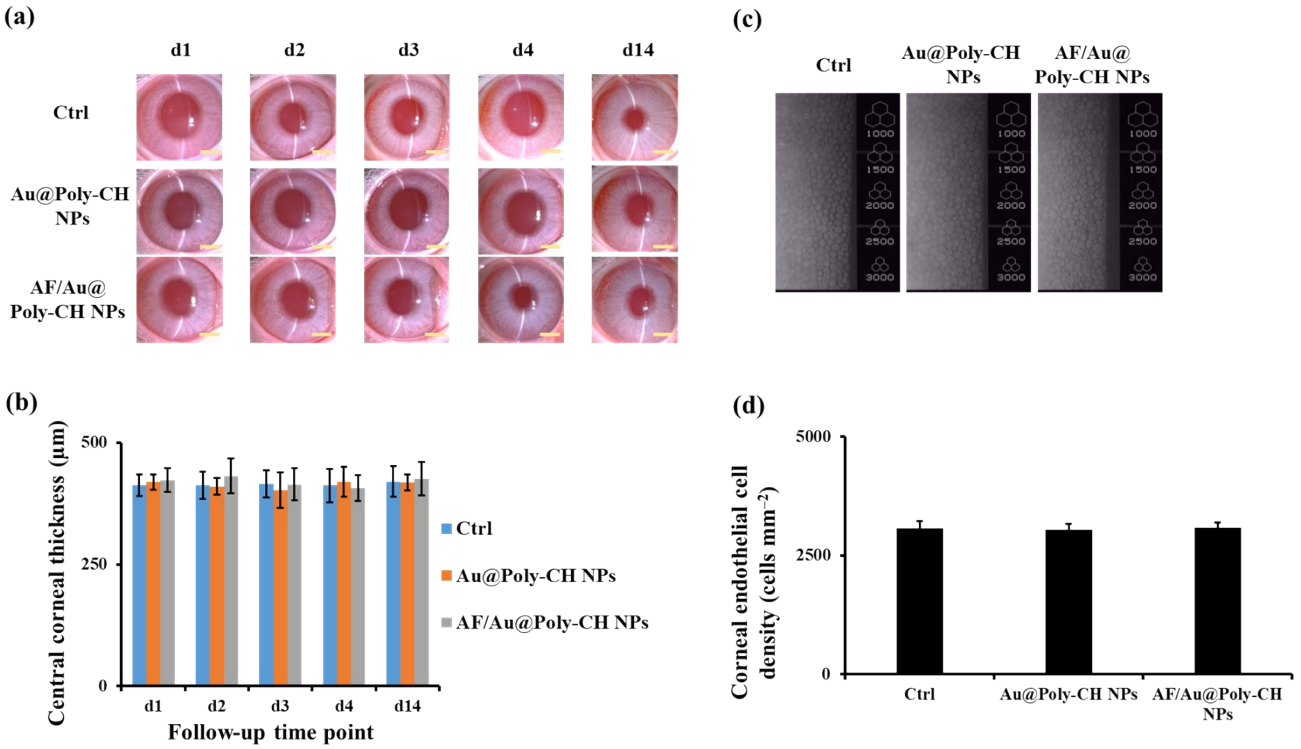


Figure S7. *In vivo* ocular biocompatibility of Au@Poly-CH NPs ([Au NPs] = 37.85 pM) and AF/Au@Poly-CH NPs ([Au NPs] =37.85 pM) after topical administration in rabbit eyes. The rabbits receiving artificial tear solution (ATS) without nanomaterial serve as control (Ctrl) group. (a) Time-course slit-lamp biomicroscopic images, (b) corneal thickness values during the follow up time, (c) morphology and end-point specular microscopic analysis of corneal endothelial cell, and (d) corneal endothelial cell density after 14 days of treatment. Values are mean ± standard deviation (n = 6). Follow-up time point: day (d). Scale bars in (a): 5 mm.

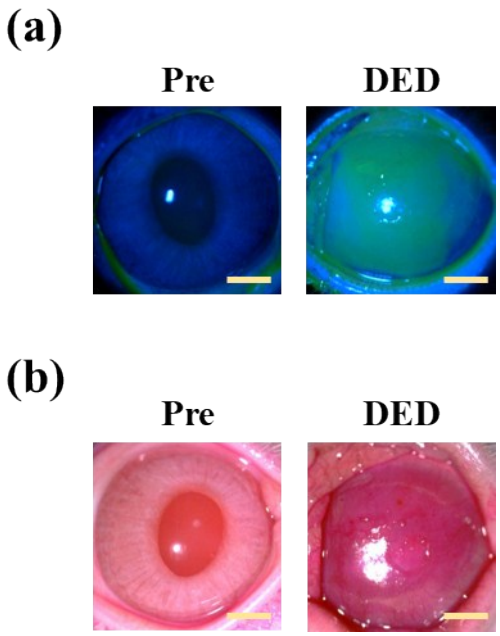


Figure S8. Corneal (a) fluorescein and (b) rose bengal staining images of preoperative (Pre) and experimentally induced DED rabbit eyes. Scale bars: 5 mm.

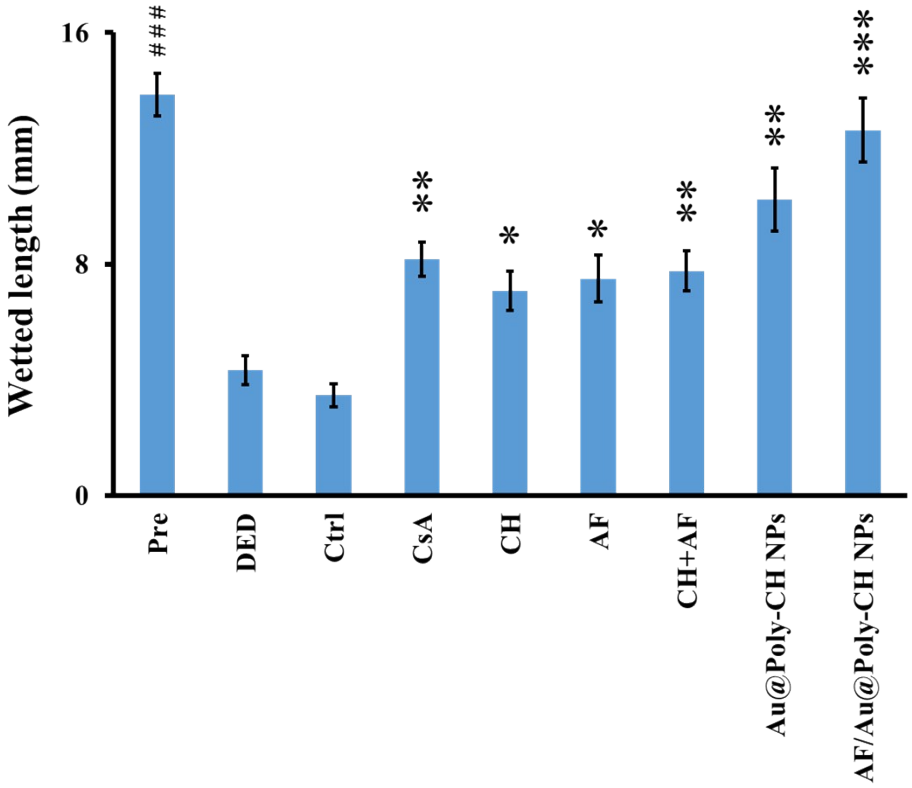


Figure S9. The wetted length of the Schirmer paper strip for the rabbit eyes before drug administration (Pre) and those with experimentally induced DED 4 days after topical administration of CH, AF, a mixture of CH and AF, Au@Poly-CH NPs or AF/Au@Poly-CH NPs. DED animals receiving artificial tear solution (ATS) without nanomaterial and drug serve as control groups (Ctrl). Values are mean ± standard deviation ($n = 6$). Asterisks indicate statistically significant differences as compared treated groups to Ctrl group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) and DED group to Pre group ($^{###}p < 0.001$).

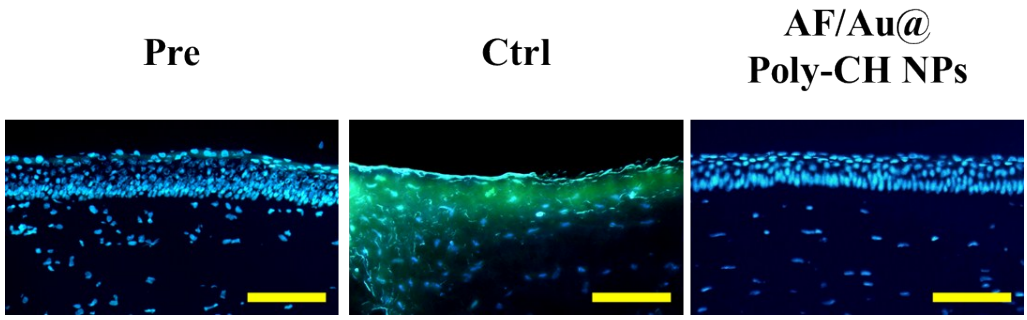


Figure S10. Fluorescence images of corneal epithelium in rabbit eyes and those with experimentally induced DED 4 days after AF/Au@Poly-CH NPs administration. DED animals receiving ATS without nanomaterials and drug served as control groups (Ctrl). Scale bars in 50 μm. Green fluorescence is DCFH-DA-positive staining.