Supplementary Information

High-safety tacrolimus nanoemulsion for dry eye disease treatment through anti-oxidant and anti-inflammatory effects

Meixing Shi[1], Xuemeng Guo[1], Kedong Sun[1], Litong Wang[1], Yanyan Zhou[1], Huiqi Wang[5], Weigen Qiu[1], Yingying Shi[1], Zeliang Lou[1], Yuena Zhang[1], Lihua Luo[1,4*], Jian You[1,2,3,4*]

Authors' Affiliations:

[1]College of Pharmaceutical Sciences, Zhejiang University, 866 Yuhangtang Road, Hangzhou, Zhejiang 310058, P. R. China

[2]State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, 79 Qingchun Road, Shangcheng District, Hangzhou, Zhejiang 310006, P. R. China

[3]The First Affiliated Hospital, College of Medicine, Zhejiang University, 79 QingChun Road, Hangzhou, Zhejiang 310000, P. R. China

[4]Jinhua Institute of Zhejiang University, Jinyi New District, Jinhua, Zhejiang 321015, P. R. China

[5]College of Pharmaceutical Science & Collaborative Innovation Centre of Yangtze River Delta Region Green Pharmaceuticals, Zhejiang University of Technology, Hangzhou 310014, P. R. China

*** Corresponding Authors**

Lihua Luo: luolihua@zju.edu.cn

Jian You: youjiandoc@zju.edu.cn, 086-571-88208439

1. Experimental

Reagents

Phosphatidylcholine (PC) and triglyceride (TG) were purchased from Lipoid Co. (Ludwigshafen, Germany). Vitamin E (VE) was from Sigma-Aldrich (Merk, Darmstadt, Germany). Tacrolimus (FK506, CAS: 104987-11-3) was from Belka Pharmaceutical Co., Ltd. (Wuhan, China). Tacrolimus eye drops (Talymus®) were from Senju Pharmaceutical Co., Ltd. (Japan). High-Glucose Dulbecco's modified Eagle's medium (DMEM), Trypsin, and the powder of phosphate buffered saline (PBS) were acquired from Jinno Biotechnology Co., Ltd. (Zhejiang, China). The fetal bovine serum was acquired from Tianhang Biotechnology Co., Ltd. (Zhejiang, China). 2'-(4-ethoxyphenyl)-6-(4-methylpiperazin-1-yl)-1H,3'H-2,5'bibenzo[d]imidazole trihydrochloride (Hoechst 33342, CAS: 23491-52-3) was from Beyotime Institute of Biotechnology (Jiangsu, China). ROS detection kit (2',7'-Dichlorodihydrofluorescein diacetate, DCFH-DA, CAS: 4091-99-0) and cell counting kit8 (CCK-8) were from MedChemExpress (Monmouth Junction, NJ, USA). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD, CAS: 127274-91-3), 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyaine iodide (DiR, CAS: 100068-60-8), and Annexin V-FITC/PI apoptosis detection kit were from Meilunbio Co. Ltd. (Dalian, China). Indocyanine green (ICG) was from Tokyo Chemical Industry (TCI, Tokyo, Japan). Benzalkonium chloride (BAC, CAS: 63449-41-2) was from Yuan Ye Biotechnology Co., Ltd. (Shanghai, China). Lissamine green (CAS: 3087-16-9) was from Mclin Biochemical Technology Co., Ltd. (Shanghai, China). The deionized water utilized in the experiment was produced using a Milli-Q system (Millipore, Boston).

Cell lines

The HCECs were purchased from BeNa Culture Collection (Henan, China, BNCC337876). The cells were cultured in high-glucose DMEM medium supplemented with 10% fetal bovine serum. As adherent cells, the expanded HCECs were enzymatically digested using a solution of 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) for passaging or seeding in subsequent experiments. These cells were maintained at a temperature of 37°C within a humidified incubator (Heraeus, Germany) infused with

5% CO2.

Animals

The C57BL/6 female mice utilized in this study (aged 6-8 weeks, weighing 16-20g) were procured from Hangzhou Hangsi Biological Technology Co., Ltd. The mice were housed in a controlled experimental environment, with the temperature maintained 21- 25 ℃, and a light cycle established at 12 hours (with illumination from 9 am to 9 pm and darkness from 9 pm to 9 am the following day). Mice had access to adequate sterilized feed and drinking water within a pathogen-free setting. About a one-week acclimatization period was allowed prior to the commencement of the animal experiments. All procedures involving animal experimentation adhered to the approved protocol by the Institutional Animal Care and Use Committee of Zhejiang University (Approval no. 32168).

Statistical analysis

The data were presented in graphical form as mean ± standard deviation. Comparisons between two or more groups were conducted using unpaired t-test or one-way ANOVA (Tukey's multiple comparisons test or Dunn's multiple comparisons test). Data analysis was performed using GraphPad Prism 9 software. The significance levels were indicated as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, “ns” denoted no significant difference (P > 0.05).

2. Supplementary Figures

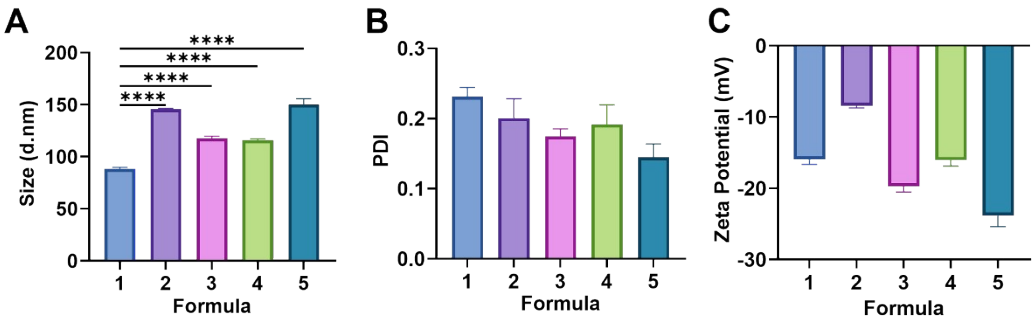

Fig. S1 Assessment of various prescription nanoemulsions. A probe-type ultrasonic generator was employed at a power setting of 40% and operational durations of 6 minutes to assess the (A) hydrodynamic size, (B) polydispersity index (PDI) and (C) zeta potential of different formulations, n=3. All data above are presented as mean ± standard deviation, ****P < 0.0001.

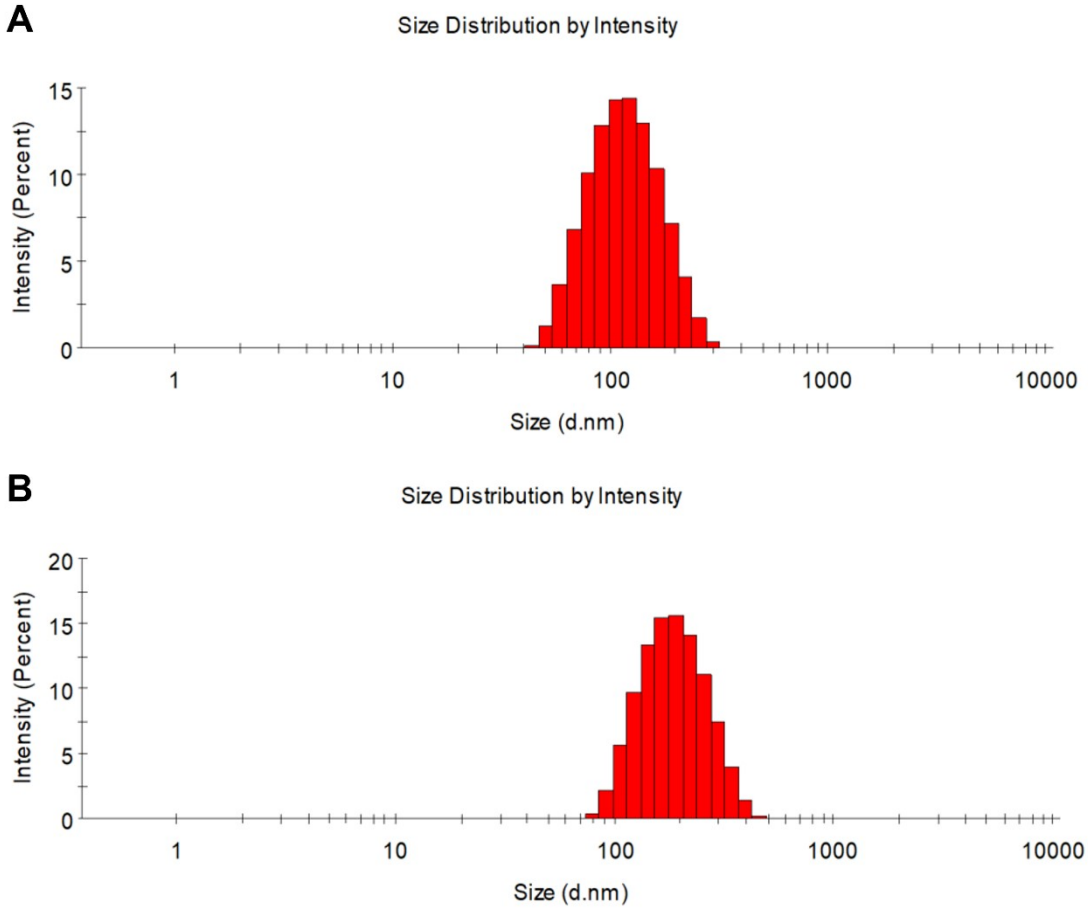

Fig. S2 Size distribution of (A) BNE and (B) FK@NE measured by dynamic light scattering (DLS).

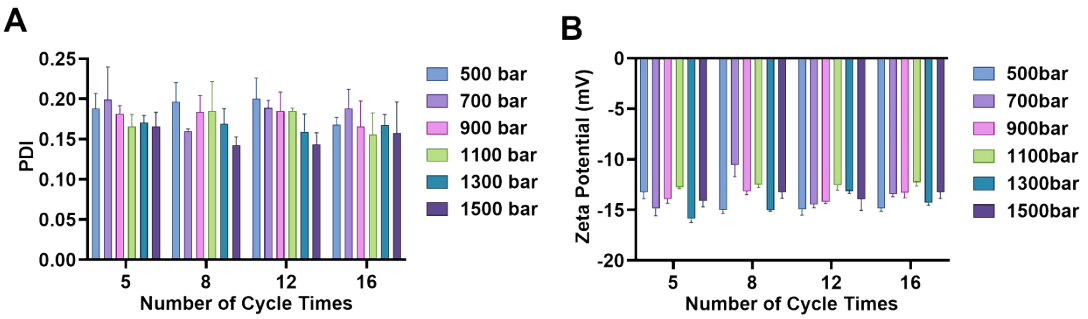

Fig. S3 The (A) PDI and (B) zeta potential of BNE under varying homogenization pressures and cycle numbers (temperature: 1°C), n=3. All data above are presented as mean ± standard deviation.

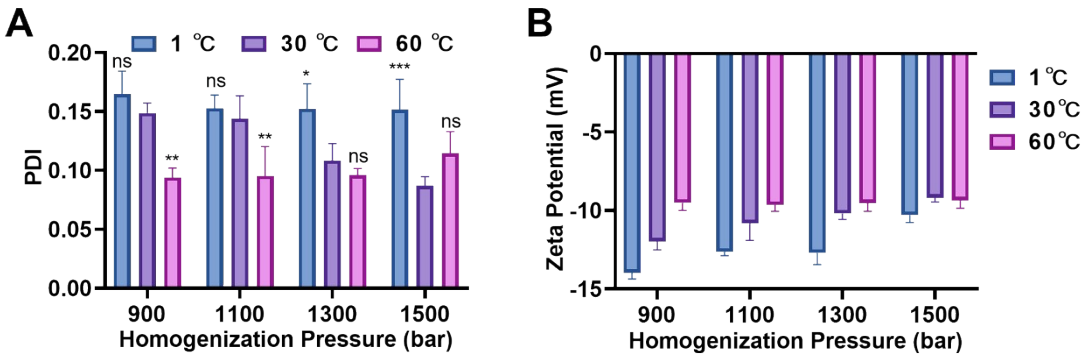

Fig. S4 The (A) PDI and (B) zeta potential of BNE at different homogenization temperatures and pressures (cycle number: 12), n=3. All data above are presented as mean ± standard deviation, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, “ns” denoted no significant difference (P > 0.05).

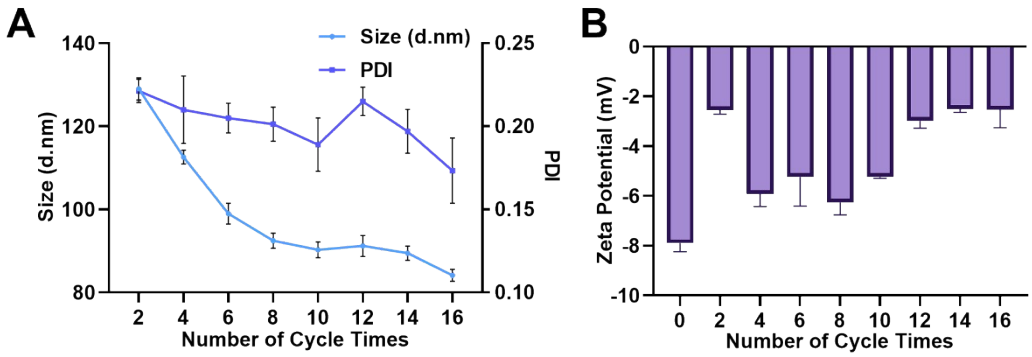

Fig. S5 At a homogenization pressure of 1300 bar and a temperature of 30°C, the (A) size, PDI and (B) zeta potential of BNE at were evaluated after varying numbers of cycles, n=3. All data above are presented as mean ± standard deviation.

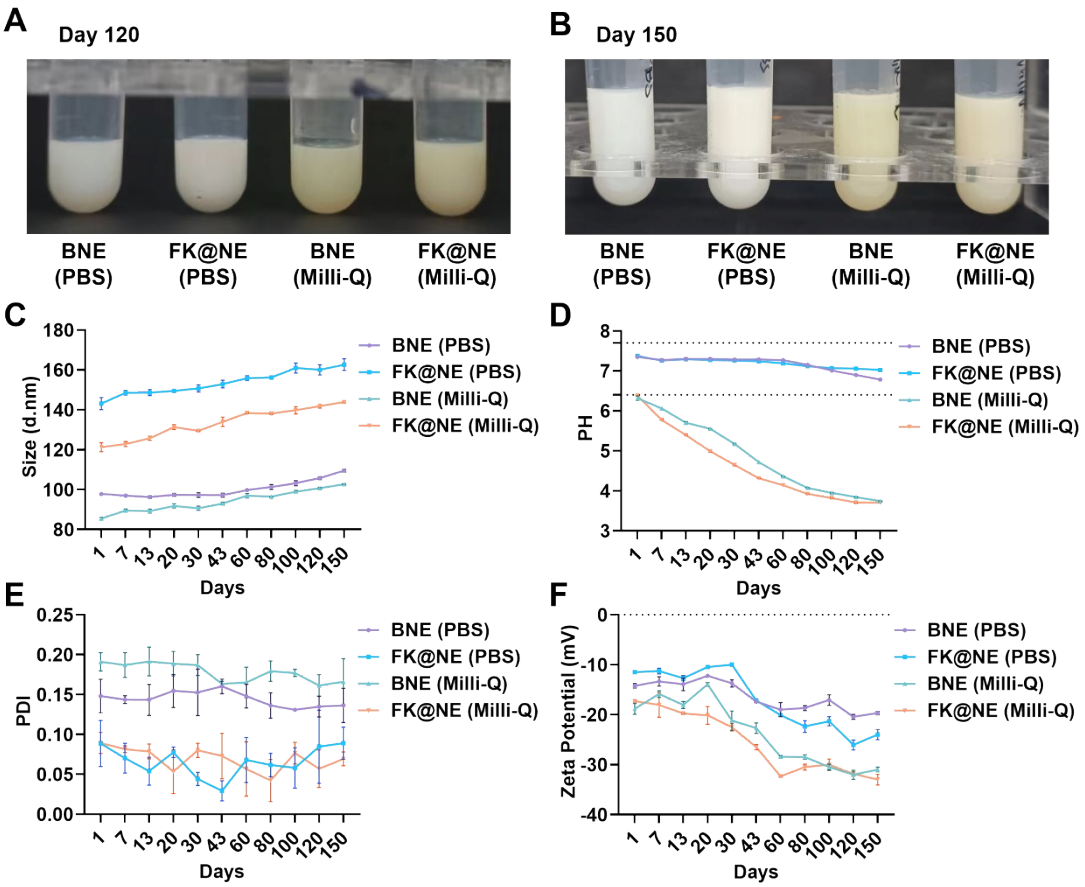

Fig. S6 Stability assessment of nanoemulsions under long-term storage conditions. The appearance of BNE (PBS), FK@NE (PBS), BNE (Milli-Q), and FK@NE (Milli-Q) on the (A) 120th day and (B) 150th day of storage. Over the storage period, the (C) particle size, (D) pH value, (E) PDI, and (F) zeta potential of each sample was systematically evaluated. All data above are presented as mean ± standard deviation.

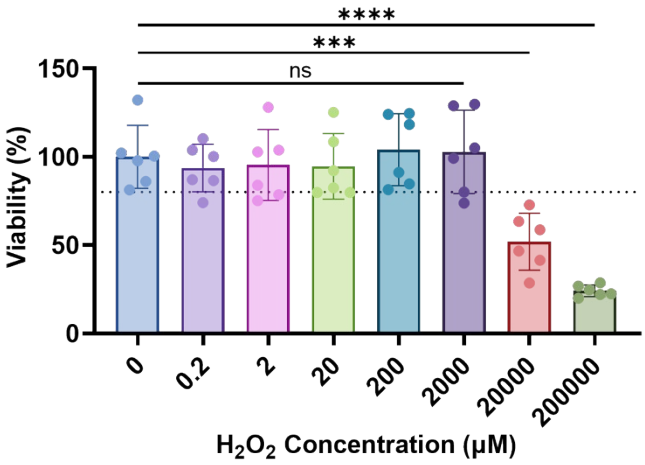

Fig. S7 Cell survival of HCECs induced by various concentrations (0, 0.2, 2, 20, 200, 2000, 20000, and 200000 μM) of H_2O_2, n=6. All data above are presented as mean ± standard deviation, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, “ns” denoted no significant difference (P > 0.05).

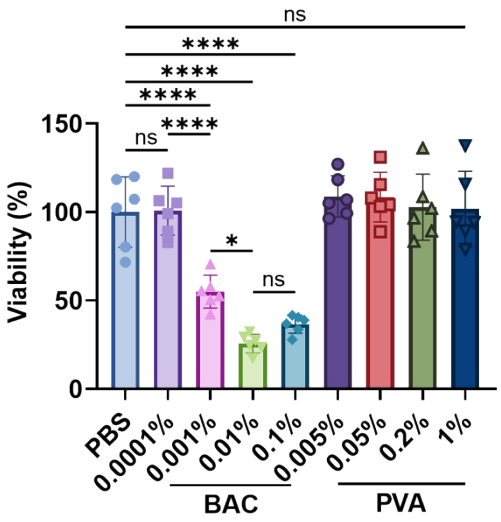

Fig. S8 Cell viability of HCECs was assessed using the CCK-8 assay under varying concentrations of benzalkonium chloride (BAC) and polyvinyl alcohol (PVA) treatments, n=6. All data above are presented as mean ± standard deviation, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, “ns” denoted no significant difference (P > 0.05).

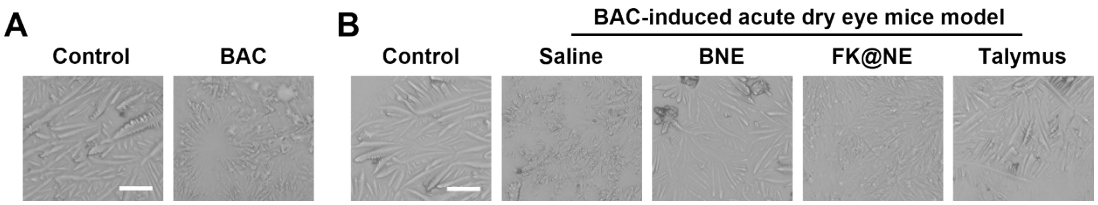

Fig. S9 The morphology of tear ferns. (A) Tear ferns morphology in the Control group and modeling group on Day 0. (B) Tear ferns morphology observed in each group on Day 7.

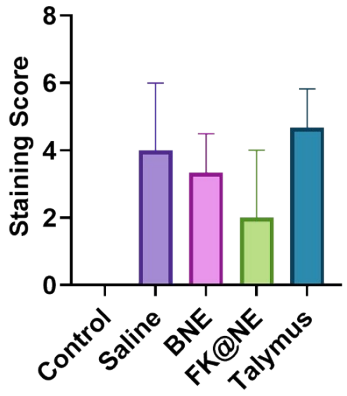

Fig. S10 The score of corneal staining outcomes for each group on Day 8, n=3. All data above are presented as mean ± standard deviation.

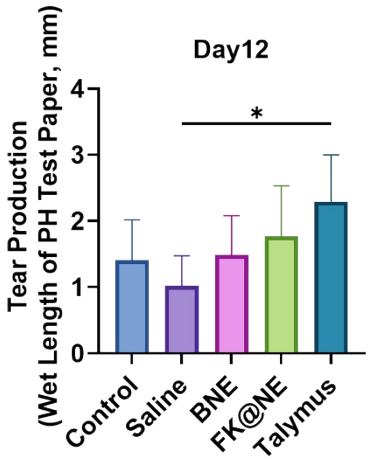

Fig. S11 Tear volume of mice on Day 12 was measured using 1mm-wide pH strips under the Control, Saline, BNE, FK@NE, and Talymus treatments, n=6. All data above are presented as mean ± standard deviation, *P < 0.05.

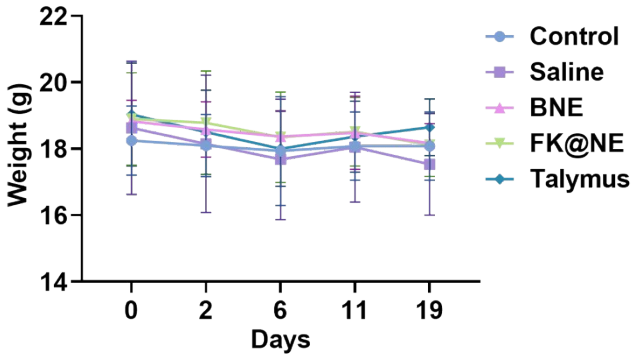

Fig. S12 Changes in body weight of mice during the modeling and treatment phases of sustained dry eye, n=6. All data above are presented as mean ± standard deviation.

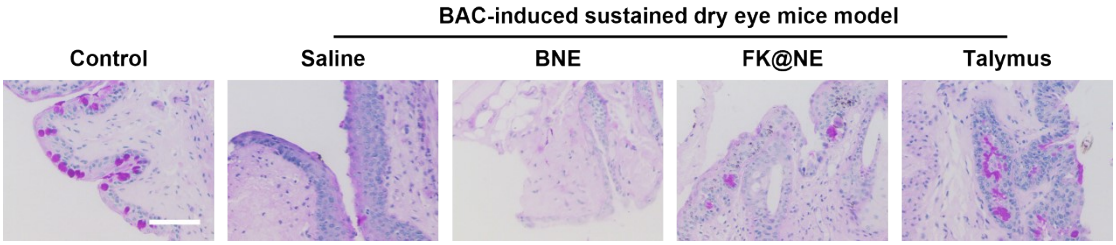

Fig. S13 PAS staining of conjunctival goblet cells. Scale bar: 50 μm.

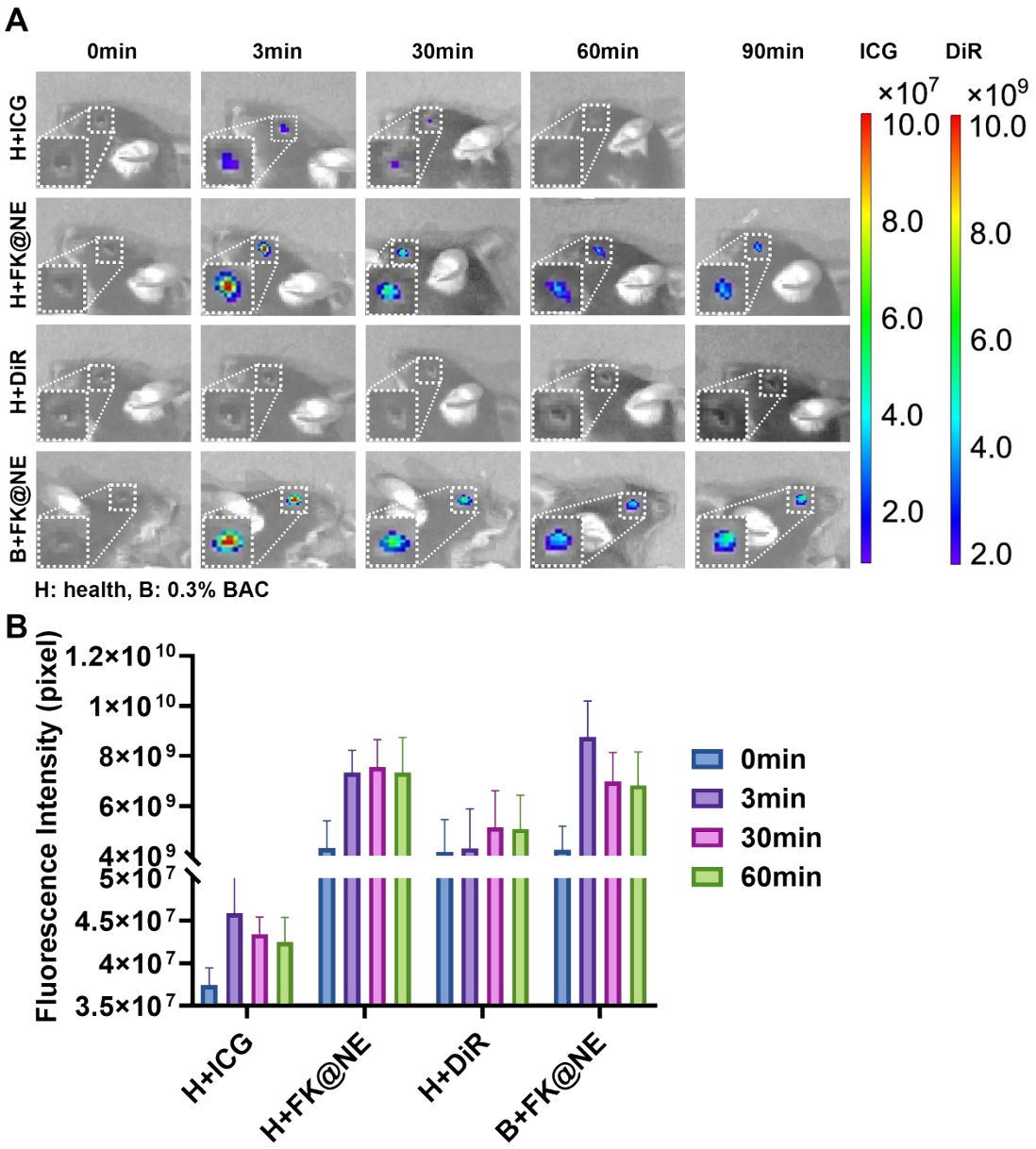

Fig. S14 Ocular retention of FK@NE. (A) Retention of free fluorescent dye and DiR-labeled FK@NE in the murine eye. (B) Quantitative results from the ocular retention assay, n=5. H: health, B: 0.3% BAC. All data above are presented as mean ± standard deviation.