

# **ROS-responsive nanozyme hydrogel reprograms repair signaling networks in radiation-induced skin injury**

## **Authors**

**Zining Tan<sup>a</sup>, Wei Liu<sup>a</sup>, Jincal Lv<sup>a, b</sup>, Jinlong Wei<sup>b</sup>, Yuwei Huang<sup>a</sup>, Jinwen Zhao<sup>a</sup>, Ying Xin<sup>a\*</sup>, Xin Jiang<sup>b\*</sup>**

## **Affiliations**

<sup>a</sup> Key Laboratory of Pathobiology, Ministry of Education, and Medical Basic Research Innovation Center of Airway Disease in North China, College of Basic Medical Sciences, Jilin University, Changchun, 130021, China

<sup>b</sup> Jilin Provincial Key Laboratory of Radiation Oncology and Therapy, Department of Radiation Oncology, The First Hospital of Jilin University, Changchun, China

\* Corresponding author:

Prof. Ying Xin, Ph.D.

E-mail: xiny@jlu.edu.cn, Tel.: +86 13504310452

## **Supplementary Text**

### **● Preparation and characterization of Cu<sub>5.4</sub>O USNPs**

Cu<sub>5.4</sub>O USNPs were synthesized according to a previously reported method. Briefly, CuCl<sub>2</sub> (10 mM) was dissolved in 50 mL of double-distilled water, transferred to a 250 mL Erlenmeyer flask, and heated at 80 °C under magnetic stirring for 10 min. L-ascorbic acid (AA, 100 mM) was separately dissolved in 50 mL of deionized water and fully dispersed by ultrasonication. The AA solution was then slowly added dropwise to the CuCl<sub>2</sub> solution under continuous stirring. Subsequently, the pH of the reaction mixture was adjusted to 8.0-9.0 using 1 M NaOH. The mixture was maintained at 80 °C with constant stirring for 12-14 h to complete the reaction. After the above solution was dialyzed for 48 h, the Cu<sub>5.4</sub>O USNPs were obtained by centrifugation. The as-prepared Cu<sub>5.4</sub>O USNPs were characterized by UV-visible spectroscopy. As shown by the absorption spectrum, the absorbance of Cu<sub>5.4</sub>O gradually increased with increasing wavelength. It exhibited a distinct absorption peak at approximately 580 nm, followed by a gradual decrease at longer wavelengths ([Supplementary](#)

Fig. S1C). This characteristic absorption peak was therefore used as a reference for the subsequent quantitative analysis of the ROS-responsive release behavior of  $\text{Cu}_{5.4}\text{O}$  from the  $\text{Cu}_{5.4}\text{O} @\text{PVA/B/Gel}$  hydrogel.

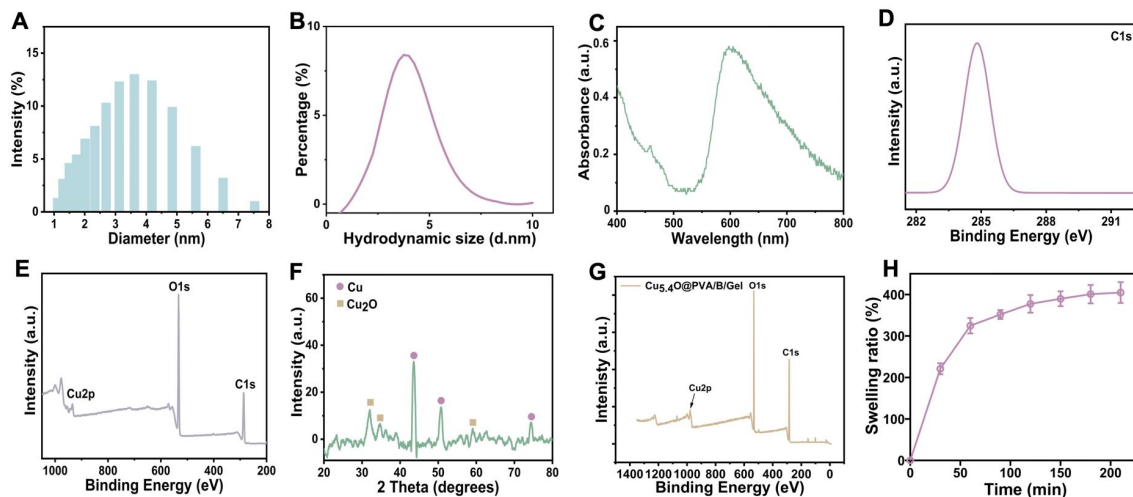


**Figure S1.** Characterization of  $\text{Cu}_{5.4}\text{O} @\text{PVA/B/Gel}$  hydrogels. (A) Particle size distribution of  $\text{Cu}_{5.4}\text{O}$  USNPs, showing a uniform size profile with an average diameter of approximately 3.8 nm. (B) DLS size distribution of  $\text{Cu}_{5.4}\text{O}$  USNPs. (C) UV-Vis absorption spectrum of  $\text{Cu}_{5.4}\text{O}$  USNPs, displaying a distinct characteristic peak around 580 nm, indicative of their electronic structure. (D) High-resolution XPS spectrum of the C1s peak, revealing clearly defined bonding states. (E) XPS survey spectrum confirming the presence of Cu, O, and C elements. (F) XRD pattern demonstrating the crystalline structure of  $\text{Cu}_{5.4}\text{O}$ , with diffraction peaks corresponding to metallic Cu and  $\text{Cu}_2\text{O}$  phases. (G) XPS survey spectrum of  $\text{Cu}_{5.4}\text{O} @\text{PVA/B/Gel}$  hydrogel, showing the presence of Cu 2p, C 1s, and O 1s signals. (H) Swelling behavior of the  $\text{Cu}_{5.4}\text{O} @\text{PVA/B/Gel}$  hydrogel measured in PBS (pH 7.4) at 37 °C.

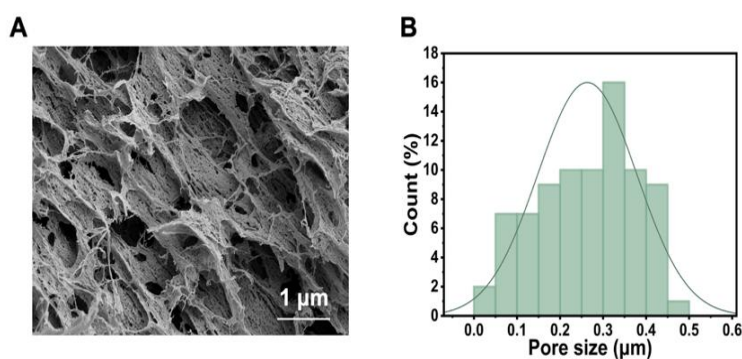


**Figure S2.** ROS-responsive properties of  $\text{Cu}_{5.4}\text{O}@PVA/B/\text{Gel}$  hydrogels. (A) SEM images of  $\text{Cu}_{5.4}\text{O}@PVA/B/\text{Gel}$  hydrogels after exposure to PBS for 24 h. (B) Pore size distribution after 24 h PBS treatment.

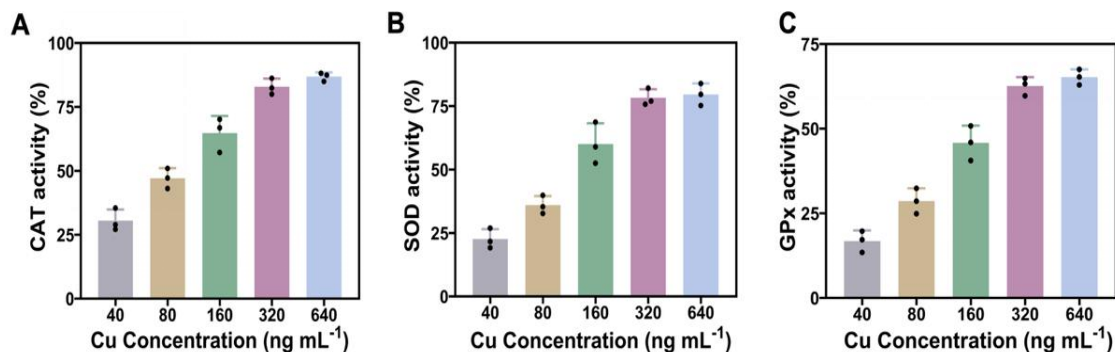


**Figure S3.** Enzyme-mimetic activities of  $\text{Cu}_{5.4}\text{O}$  nanoparticles (NPs). (A) Catalase (CAT)-like activity of  $\text{Cu}_{5.4}\text{O}$  NPs at different concentrations. (B) Superoxide dismutase (SOD)-like activity of  $\text{Cu}_{5.4}\text{O}$  NPs at different concentrations. (C) Glutathione peroxidase (GPx)-like activity of  $\text{Cu}_{5.4}\text{O}$  NPs at different concentrations. Data are presented as mean  $\pm$  SD ( $n = 3$ ).

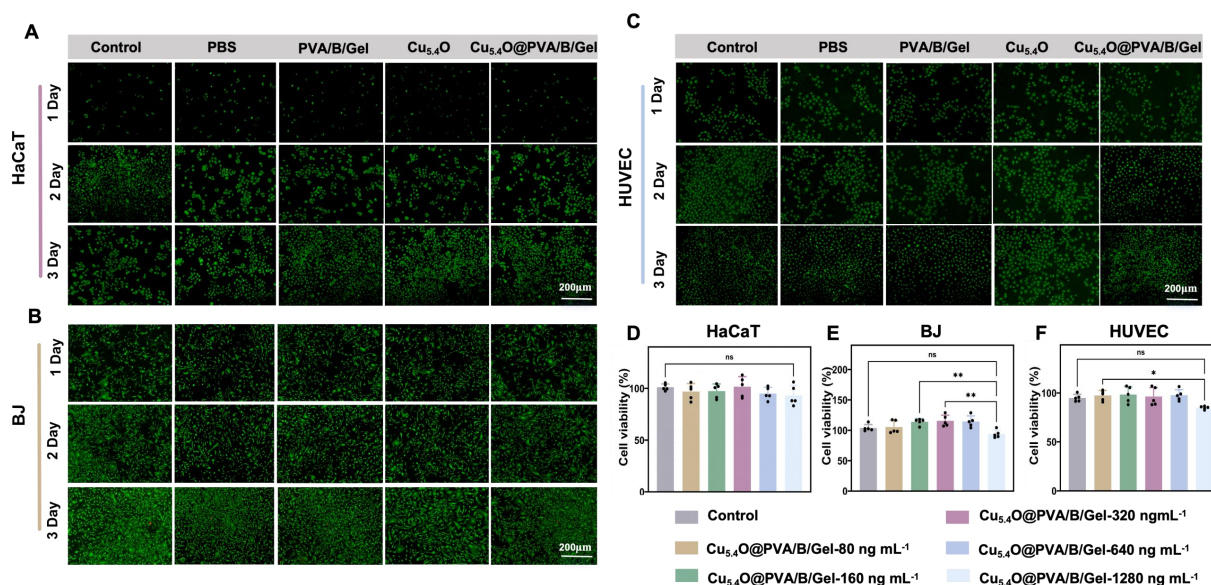


**Figure S4.** In vitro cytocompatibility evaluation of  $\text{Cu}_{5.4}\text{O}@PVA/B/\text{Gel}$  and its components. (A-C) Live/dead fluorescence images of HaCaT, BJ and HUVEC cells under different treatment conditions, including the control, PBS, PVA/B/Gel,  $\text{Cu}_{5.4}\text{O}$ , and  $\text{Cu}_{5.4}\text{O}@PVA/B/\text{Gel}$  groups,  $n = 3$ . Green fluorescence indicates live cells stained with Calcein-AM, while red fluorescence represents dead cells stained with PI. Scale bar: 200  $\mu\text{m}$ .

(D-F) Cell viability of HaCaT (D), BJ (E) and HUVEC (F) cells after exposure to different composite hydrogels for 72 h, showing good cytocompatibility within the concentration range used for subsequent experiments ( $n = 5$ ). Data are presented as mean  $\pm$  SD. Statistical significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , ns = not significant.

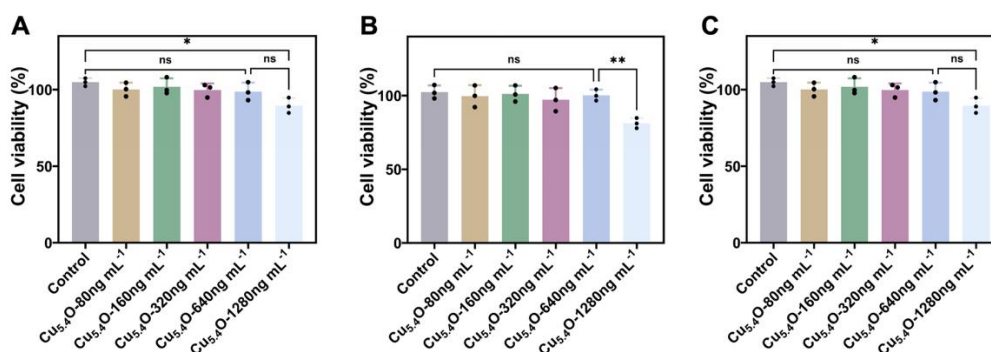


**Figure S5.** In vitro cytocompatibility evaluation of Cu<sub>5.4</sub>O USNPs. (A-C) Cell viability of HaCaT (A), BJ (B) and HUVEC (C) cells after exposure to different concentrations of Cu<sub>5.4</sub>O USNPs for 72 h, showing good cytocompatibility within the concentration range used for subsequent experiments ( $n = 3$ ). Data are presented as mean  $\pm$  SD. Statistical significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , ns = not significant.

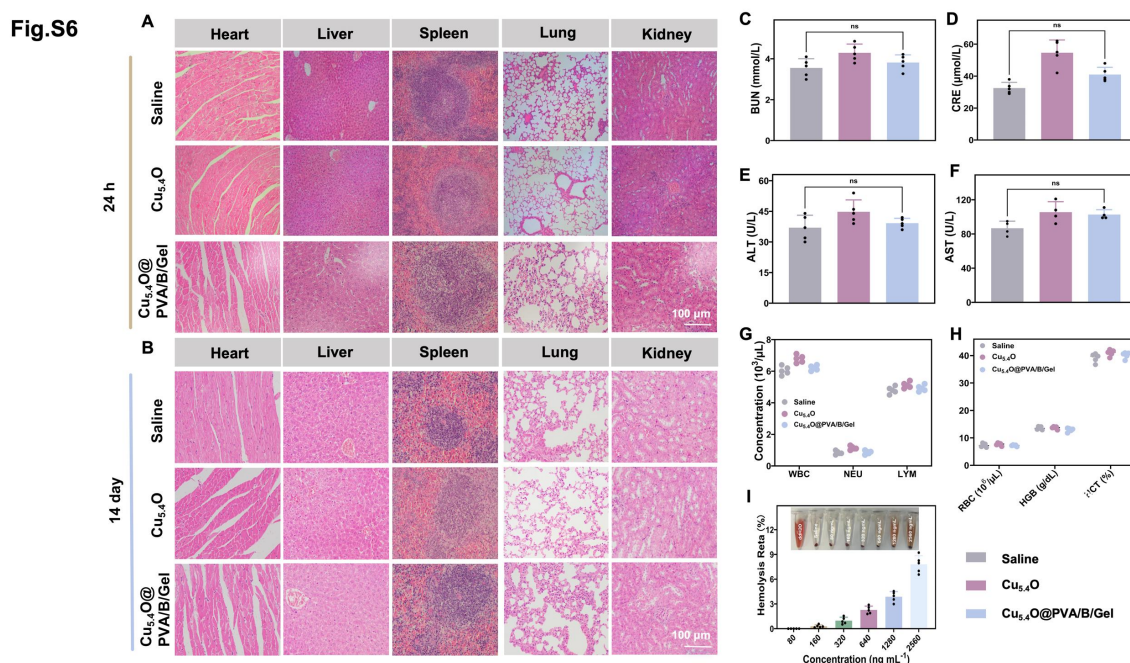


**Figure S6.** Systemic biosafety evaluation of Cu<sub>5.4</sub>O USNPs and Cu<sub>5.4</sub>O@PVA/B/Gel hydrogel extract. (A-B) Representative H&E-stained images of major organs (heart, liver, spleen, lung, and kidney) collected 24 h and 14 days after a single tail-vein injection of saline,

Cu<sub>5.4</sub>O USNPs, or Cu<sub>5.4</sub>O@PVA/B/Gel hydrogel extract, showing no apparent histopathological abnormalities. Scale bar = 100  $\mu$ m. (C-F) Serum biochemical markers of liver and kidney function, including BUN, CRE, ALT and AST. (G-H) Hematological analysis, including WBC, NEU, LYM, RBC, HGB, and HCT, showing no significant differences among groups, further supporting the in vivo biosafety of the tested formulations. (I) Hemolysis rate of red blood cells treated with different concentrations of Cu<sub>5.4</sub>O USNPs. Data are presented as mean  $\pm$  SD (n = 3). Statistical significance: \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, \*\*\*\* $p$  < 0.0001, ns = not significant.

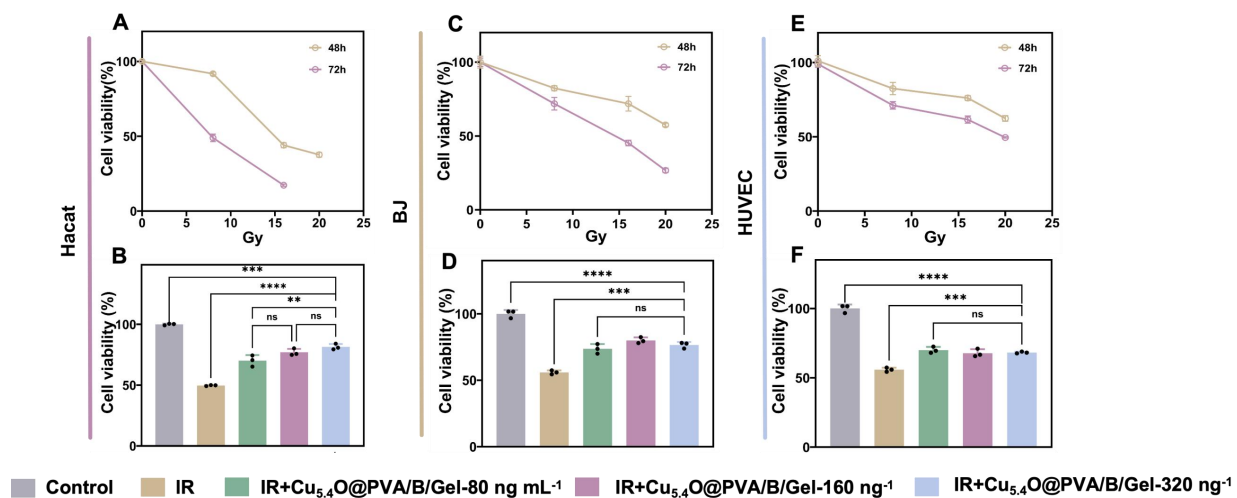


**Figure S7.** (A, C, E) Cell viability of HaCaT keratinocytes, BJ fibroblasts, and HUVEC endothelial cells at 48 and 72 h after irradiation, assessed by CCK-8 assays. HaCaT cells were irradiated at 0, 8, and 16 Gy for the 48 h assay and at 0, 8, 16, and 20 Gy for the 72 h assay; BJ and HUVEC cells were irradiated at 0, 8, 16, and 20 Gy at both time points. IC<sub>50</sub> values were calculated using CCK-8 assays and used to inform subsequent experimental design. (B, D, F) Protective effects of Cu<sub>5.4</sub>O@PVA/B/Gel hydrogel on HaCaT (B), BJ (D) and HUVEC (F) cells against radiation-induced oxidative injury. Compared with the IR group, the hydrogel significantly enhanced cell survival in all three cell types in a dose-dependent manner. Data are presented as mean  $\pm$  SD (n = 3). Statistical significance: \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, \*\*\*\* $p$  < 0.0001, ns= not significant.

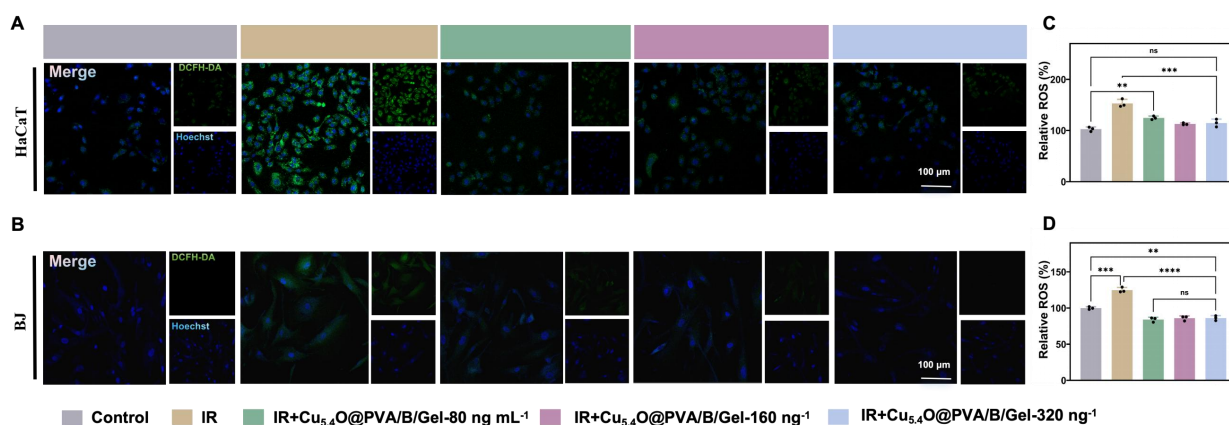


**Figure S8.** (A-B) Confocal laser scanning microscopy (CLSM) images showing the effects of different concentrations of Cu<sub>5.4</sub>O@PVA/B/Gel hydrogel (80, 160, and 320 ng mL<sup>-1</sup>) on intracellular ROS levels in HaCaT and BJ cells following IR treatment. Green fluorescence represents ROS labeled by the DCFH-DA probe, and blue fluorescence indicates nuclear staining with Hoechst 33342. Scale bar = 100 μm. (C-D) Quantitative analysis of intracellular ROS levels in HaCaT (C) and BJ (D) cells measured by a fluorescence microplate reader, showing results consistent with the imaging trends. Data are presented as mean ± SD (n = 3). Statistical significance: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001, ns = not significant.

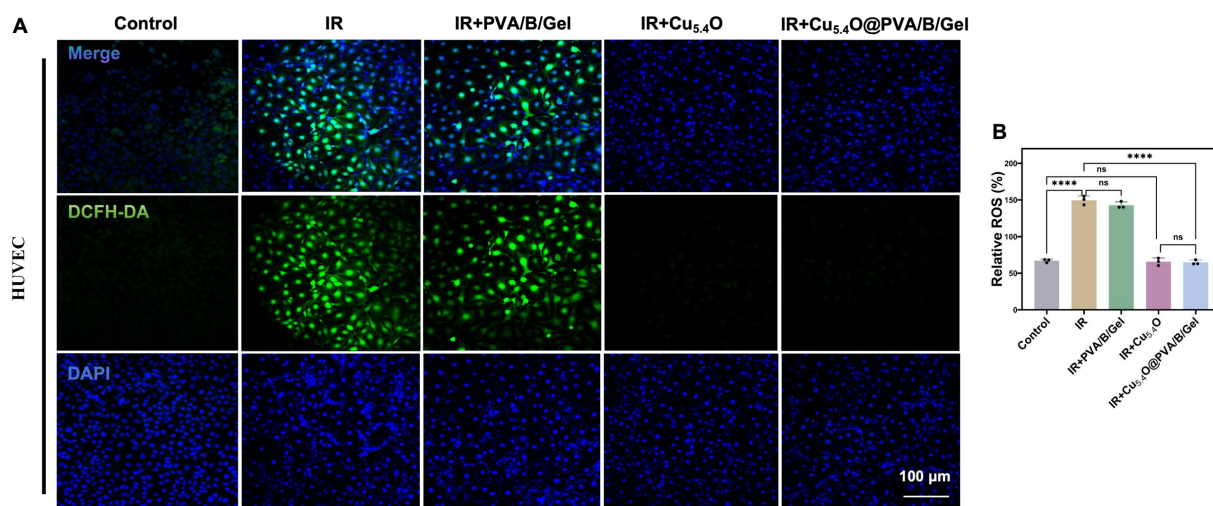


**Figure S9.** Intracellular ROS-scavenging effects in irradiated HUVECs. (A) Representative fluorescence images of intracellular ROS levels in HUVECs after 72 h of treatment, as detected by DCFH-DA staining (green). Nuclei were counterstained with DAPI (blue). Scale bar, 100 μm. (B) Quantification of relative intracellular ROS levels based on DCFH-DA fluorescence intensity. Data are presented as mean ± SD (n = 3). \*\*\*\**p* < 0.0001; ns, not significant.

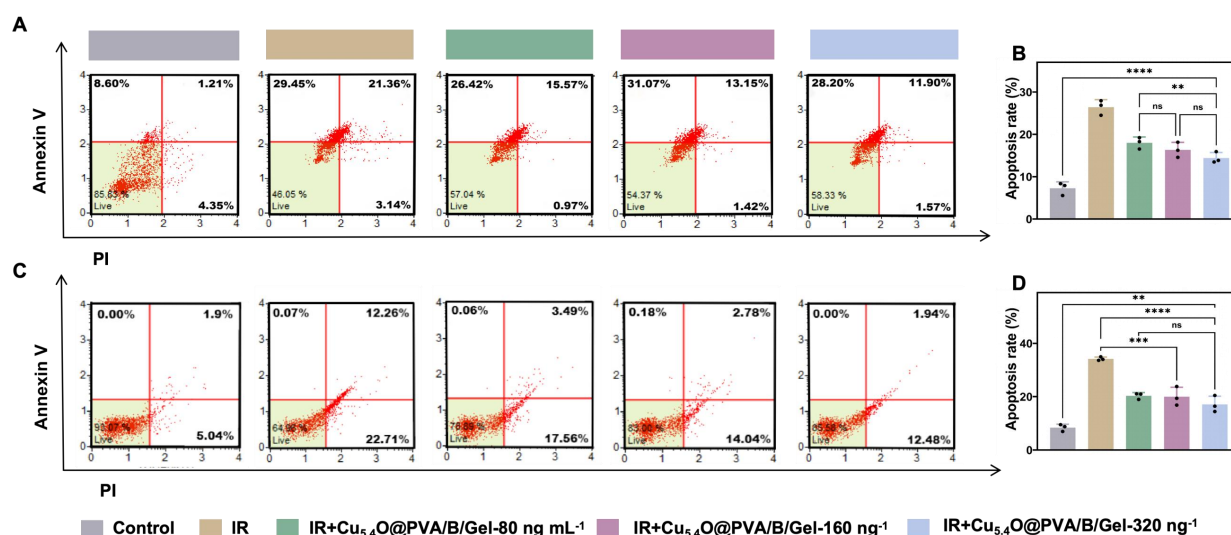


**Figure S10.** (A, B) Representative Annexin V/PI dual-staining flow cytometry plots showing apoptosis of HaCaT cells under different treatment conditions. (C, D) Representative Annexin V /PI dual-staining flow cytometry plots showing apoptosis of BJ cells under different treatment conditions. The quadrants represent live cells (lower left), early apoptotic cells (lower right), late apoptotic cells (upper right), and necrotic cells (upper left). Quantitative analysis of total apoptosis rates based on flow cytometry demonstrates that Cu<sub>5.4</sub>O@PVA/B/Gel treatment significantly attenuated IR-induced apoptosis in both HaCaT and BJ cells in a concentration-dependent manner, with a more pronounced protective effect observed at Cu<sub>5.4</sub>O@PVA/B/Gel-320 ng mL<sup>-1</sup>. Data are presented as mean  $\pm$  SD (n = 3). Statistical significance: \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, \*\*\*\* $p$  < 0.0001, ns = not significant.

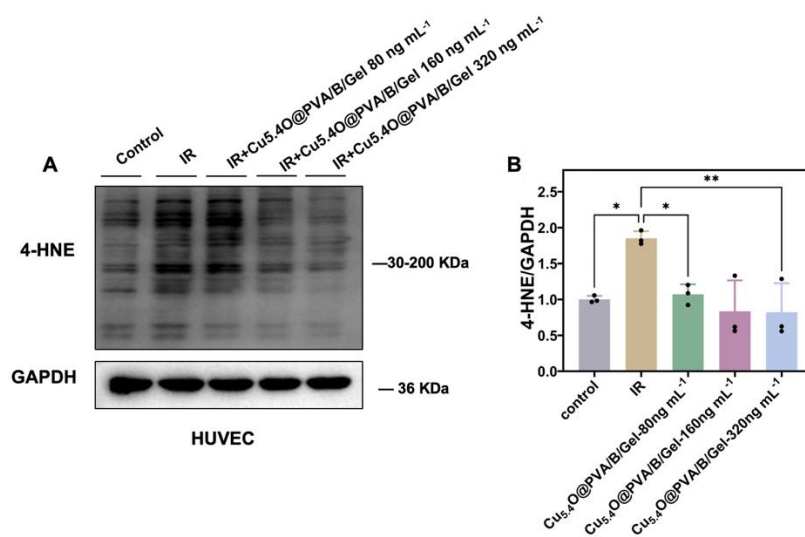


**Figure S11.** (A) Western blot analysis of 4-HNE protein expression in HUVEC cells exposed to 20 Gy IR and subsequently treated with different concentrations of Cu<sub>5.4</sub>O@PVA/B/Gel hydrogel (80, 160, and 320 ng mL<sup>-1</sup>). (B) Densitometric quantification of 4-HNE/GAPDH expression ratios. Data are presented as mean ± SD (n = 3). Statistical significance: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001, ns = not significant.

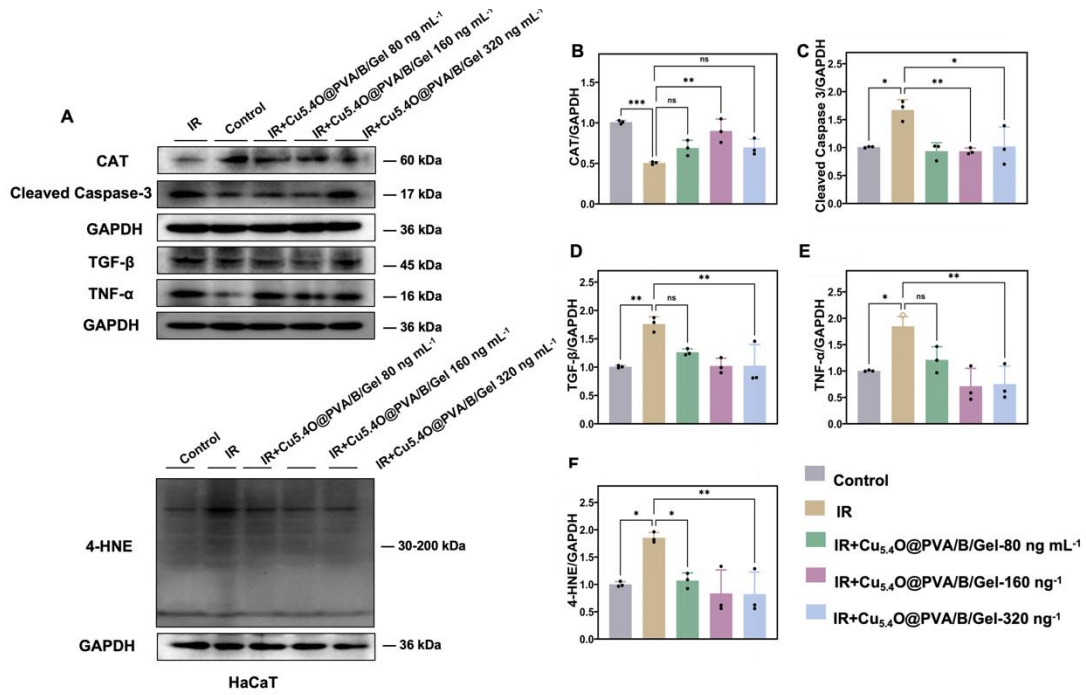


**Figure S12.** (A) Western blot analysis of CAT, cleaved caspase-3, TNF-α, TGF-β, and 4-HNE protein expression in HaCaT cells exposed to 8 Gy IR and subsequently treated with different concentrations of Cu<sub>5.4</sub>O@PVA/B/Gel hydrogel (80, 160, and 320 ng mL<sup>-1</sup>). (B–F) Quantitative analysis of protein expression levels. IR treatment significantly downregulated the antioxidant enzyme CAT while markedly upregulating cleaved caspase-3, TGF-β, TNF-α, and 4-HNE, indicating synergistic activation of oxidative stress and inflammatory responses. Treatment with Cu<sub>5.4</sub>O@PVA/B/Gel hydrogel effectively restored antioxidant capacity, inhibited apoptosis, and reduced the expression of pro-inflammatory and pro-fibrotic proteins in a concentration- dependent manner. Data are presented as mean ± SD (n = 3). Statistical significance: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001, ns = not significant.

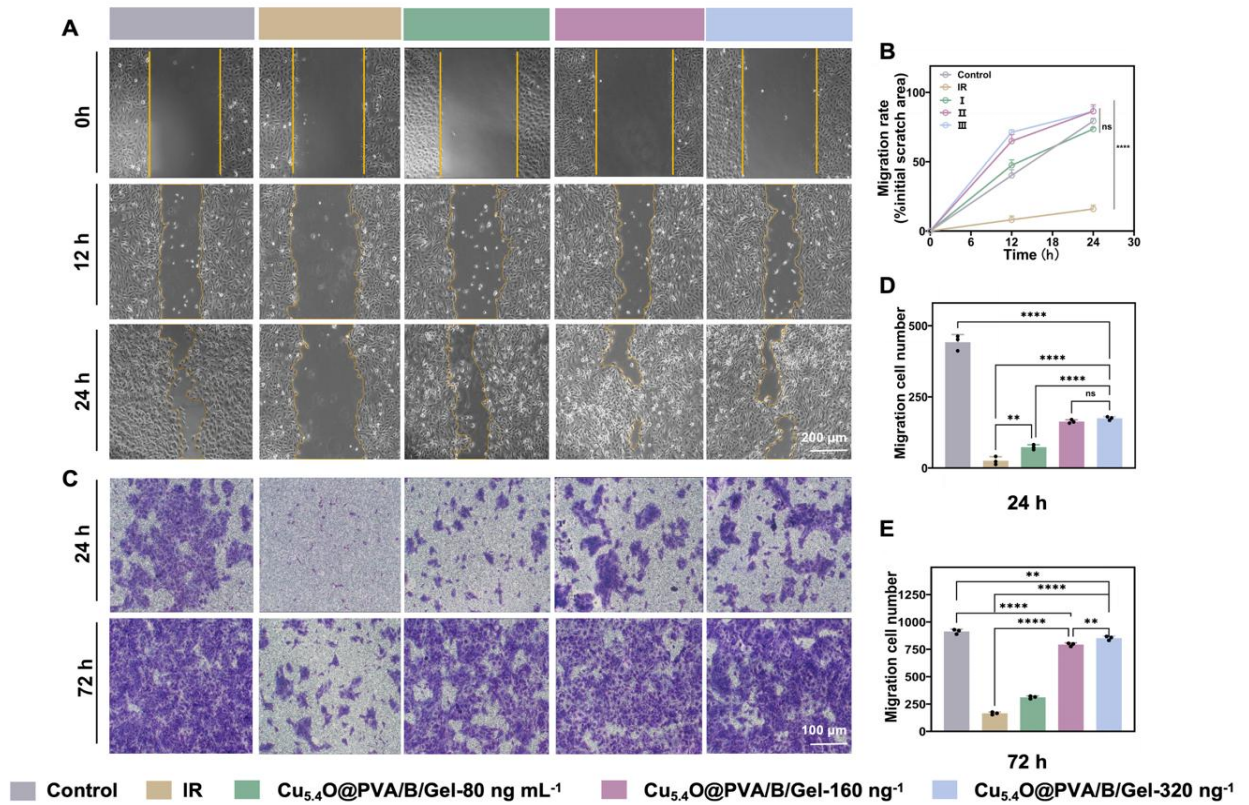


**Figure S13.** (A) Representative images of scratch wound closure in HaCaT cells at 0, 12, and 24 h after treatment with different concentrations of hydrogel. (B) Quantitative analysis of wound closure rates in HaCaT cells at various time points in the scratch assay. (C) Representative images of migrated HaCaT cells in the Transwell assay after 24h and 72 h under different treatment conditions. (D-E) Quantitative analysis of HaCaT cells after 24 h (D) and 72h (E) under different treatment conditions. Representative crystal violet-stained images show migrated HaCaT cells in the control, IR, and Cu<sub>5.4</sub>O@PVA/B/Gel hydrogel treatment groups at varying concentrations (80, 160, and 320 ng mL<sup>-1</sup>). Quantitative analysis indicates that irradiation markedly suppressed HaCaT cell migration, whereas treatment with Cu<sub>5.4</sub>O@PVA/B/Gel hydrogel partially restored migration capacity in a dose-dependent manner. Data are presented as mean  $\pm$  SD (n = 3). Statistical significance: \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, \*\*\*\* $p$  < 0.0001, ns = not significant.

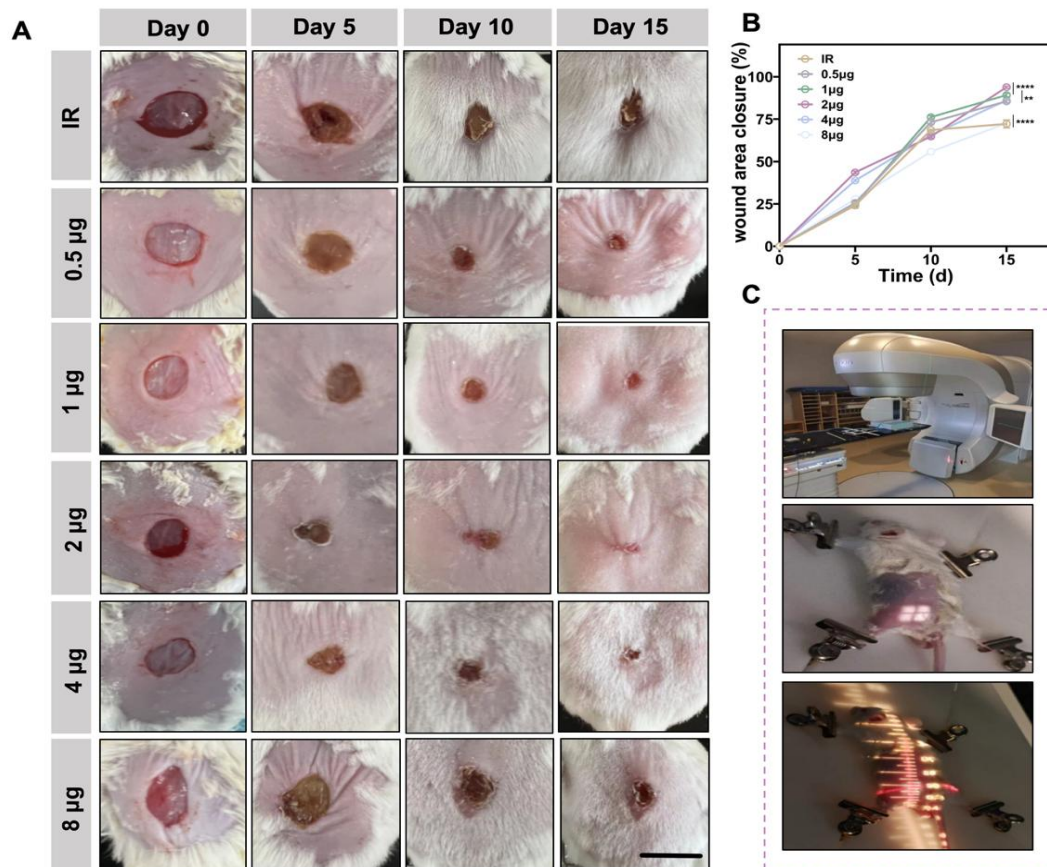


**Figure S14.** (A) Representative macroscopic images showing the wound healing progression of mouse irradiated skin following a single localized 15 Gy irradiation, treated with different doses of  $\text{Cu}_{5.4}\text{O}@\text{PVA/B/Gel}$  hydrogel (0.5, 1, 2, 4, and 8  $\mu\text{g}$  per wound) on days 0, 5, 10 and 15. (B) Quantitative analysis of wound closure rates over 15 days shows that  $\text{Cu}_{5.4}\text{O}@\text{PVA/B/Gel}$  hydrogel significantly accelerated repair of radiation-induced skin injury, with the 2  $\mu\text{g}$  group showing the most pronounced therapeutic effect. By day 15, wounds in the 2  $\mu\text{g}$  group were nearly fully healed, showing a statistically significant improvement compared to other dosage groups ( $p < 0.0001$ ). (C) Schematic illustration of the linear accelerator and the precise buttock-targeted irradiation setup used to establish the mouse model of radiation-induced skin injury. Data are presented as mean  $\pm$  SD ( $n = 6$ ). Statistical significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , ns = not significant.

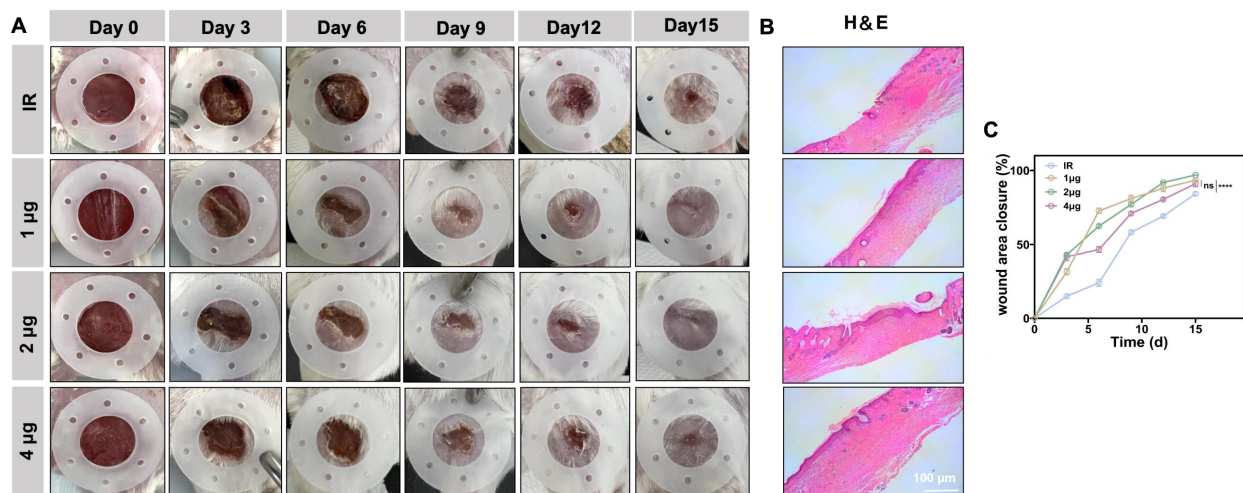


**Figure S15.** (A) Representative macroscopic images of wound healing in mouse from day 0 to day 15 post-irradiation across different treatment groups, including the IR control, 1 µg, 2 µg, and 4 µg groups (each hydrogel containing 1 µg, 2 µg, or 4 µg of Cu<sub>5.4</sub>O, respectively). (B) Representative H&E staining images of irradiated skin tissues harvested on day 15 after treatment. Compared with the IR group, Cu<sub>5.4</sub>O@PVA/B/Gel treatment at different doses promoted epidermal re-epithelialization and improved dermal tissue organization, with reduced inflammatory cell infiltration and more continuous epidermal structure. Scale bar: 100 µm. (C) Quantitative analysis of wound closure rates based on wound area measurements demonstrates that both the 1 µg and 2 µg groups significantly accelerated wound healing compared with the IR control (\*\*\*\* $p < 0.0001$ ), with the 2 µg group showing the most favorable overall healing trend. No statistically significant difference was observed between the 1 µg and 2 µg groups (ns). Data are presented as mean  $\pm$  SD (n = 6). Data are presented as mean  $\pm$  SD. Statistical significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , ns = not significant.

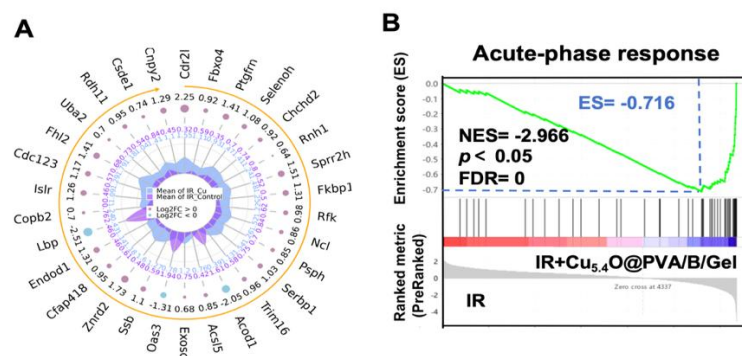


**Figure S16.** (A) Radar plot of representative differentially expressed proteins between the IR+Cu<sub>5.4</sub>O@PVA/B/Gel group and the IR group. (B) Gene set enrichment analysis

(GSEA) plots illustrating significantly downregulated pathway in the IR+Cu<sub>5.4</sub>O@PVA/B/Gel group compared with the IR group. Enrichment scores (ES), normalized enrichment scores (NES), nominal p values, and false discovery rates (FDR) are indicated within each plot.

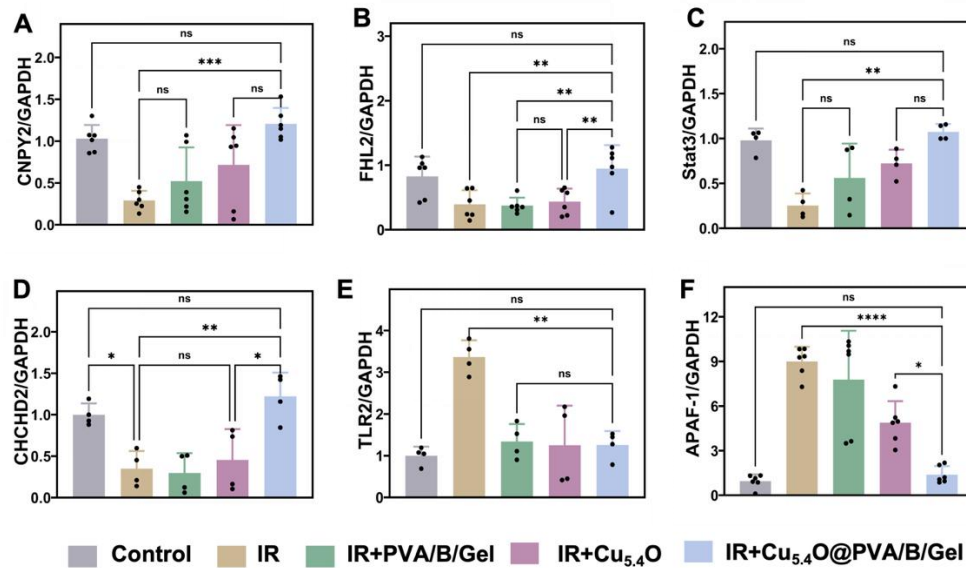


**Figure S17.** Western blot validation and quantitative analysis of representative differentially expressed proteins identified by proteomics in mouse skin tissues. (A) CNPY2, (B) FHL2, (C) STAT3, (D) CHCHD2, (E) TLR2, and (F) APAF1 expression levels were analyzed across the Control, IR, IR+PVA/B/Gel, IR+Cu<sub>5.4</sub>O, and IR+Cu<sub>5.4</sub>O@PVA/B/Gel groups. Compared with the IR group, IR+Cu<sub>5.4</sub>O @PVA/B/Gel treatment modulated the expression of multiple proteins, showing increased levels of CNPY2, FHL2, STAT3, and CHCHD2, along with reduced expression of TLR2 and APAF1, consistent with the proteomics results. Data are presented as mean  $\pm$  SD ( $n = 6$  for CNPY2/FHL2/STAT3/CHCHD2 and  $n = 4$  for TLR2/APAF1). Statistical significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , ns = not significant.

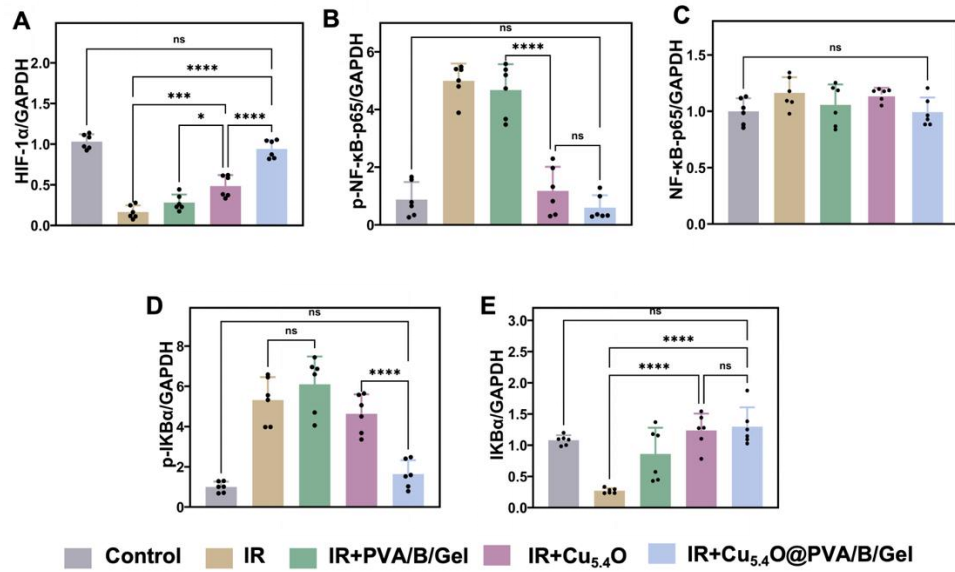


**Figure S18.** Regulation of HIF-1 and NF-κB signaling-related proteins in irradiated skin tissues. (A) Relative expression of HIF-1α normalized to GAPDH. (B) Relative expression of phosphorylated NF-κB p65 (p-NF-κB p65) normalized to GAPDH. (C) Relative expression of total NF-κB p65 normalized to GAPDH. (D) Relative expression of phosphorylated IκBα (p-IκBα) normalized to GAPDH. (E) Relative expression of total IκBα normalized to GAPDH. Data are presented as mean ± SD (n = 6). Statistical significance is indicated as  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ , and  $p < 0.0001$ ; ns, not significant.

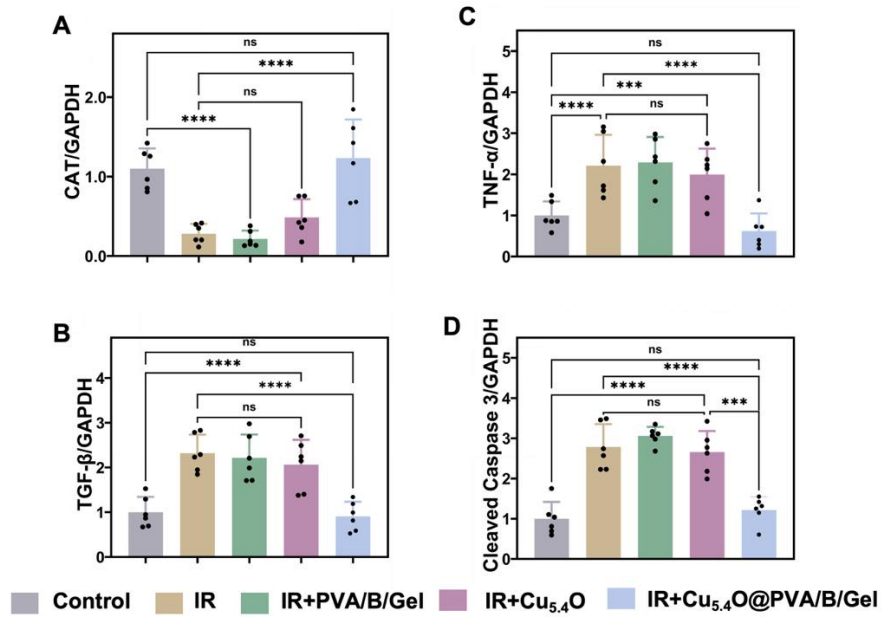


**Figure S19.** (A-D) Quantitative Western blot analysis of representative proteins associated with oxidative stress, inflammation, apoptosis, and fibrosis in peri-wound tissues, including CAT (A), TGF-β (B), TNF-α (C) and cleaved caspase-3 (D). Compared with the IR group, treatment with Cu<sub>5.4</sub>O@PVA/B/Gel hydrogel increased the expression of the antioxidant enzyme CAT, while reducing the levels of the pro-inflammatory cytokine TNF-α, the fibrosis-related factor TGF-β, and the apoptosis-associated protein cleaved caspase-3. Data are presented as mean ± SD (n = 6). Statistical analysis was performed using two-way ANOVA. Statistical significance: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001, ns= not significant.

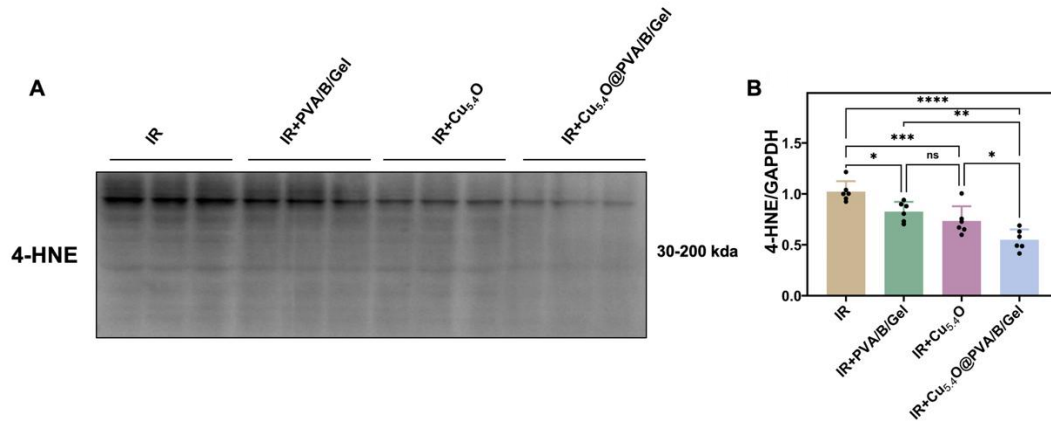


**Figure S20.** (A) Western blot analysis of 4-HNE expression in wound tissues,  $n = 6$ . GAPDH serves as the loading control. (B) Relative expression of 4-HNE normalized to GAPDH. Data are presented as mean  $\pm$  SD ( $n = 6$ ). Statistical analysis was performed using two-way ANOVA. Statistical significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , ns= not significant.

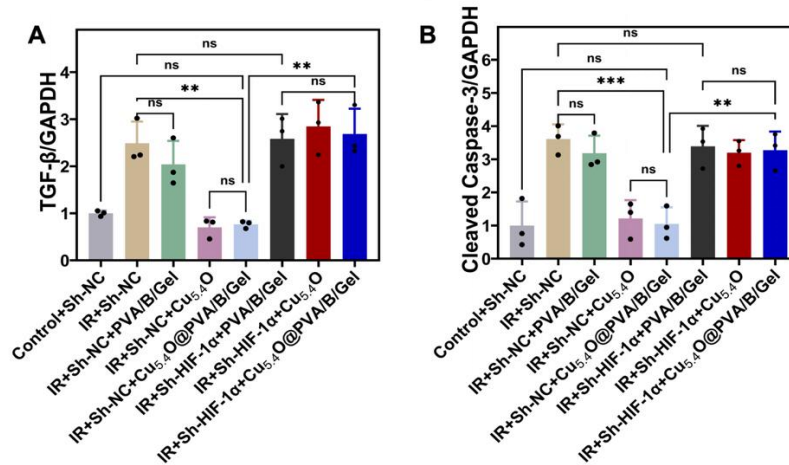


**Figure S21.** (A–B) Quantitative analysis of TGF- $\beta$  (A) and cleaved caspase-3 (B) protein expression normalized to GAPDH in HUVECs subjected to the indicated treatments and shRNA-mediated HIF-1 $\alpha$  knockdown. Data are presented as mean  $\pm$  SD ( $n = 3$ ). Statistical significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , ns= not significant.

**Table S1.** Antibodies for western blot, immunohistochemistry, and immunofluorescence.

| <b>Antibodies</b>                   | <b>Dilution, supplier, and catalogue number</b>       |
|-------------------------------------|-------------------------------------------------------|
| Anti-CAT                            | 1:1000, MedChemExpress, Cat#YA6310                    |
| Anti-4-HNE                          | 1:1000, Alpha Diagnostic International, Cat# HNE 11-S |
| Anti-cleaved Caspase 3              | 1:1000, Selleck, Cat# F0135                           |
| Anti-TNF- $\alpha$                  | 1:1000, Proteintech, Cat# 17590-1-AP                  |
| Anti-TGF- $\beta$                   | 1:1000, Proteintech, Cat# 81746-2-RR                  |
| Anti-CHCHD2                         | 1: 1000, Proteintech, Cat# 19424-1-AP                 |
| Anti-APAF1                          | 1:1000, Cell Signaling Technology, Cat# 8969          |
| Anti-TLR2                           | 1:1000, Cell Signaling Technology, Cat# 13744         |
| Anti-STAT3                          | 1:1000, Cell Signaling Technology, Cat# 9139          |
| Anti-CNPY2                          | 1:1000, Proteintech, Cat# 66173-1-lg                  |
| Anti-FHL2                           | 1:1000, Proteintech, Cat# 21619-1-AP                  |
| Anti-HIF-1 $\alpha$                 | 1:1000, Proteintech, Cat# 9139                        |
| Anti-VEGFA                          | 1:1000, Zenbio, Cat# 251622                           |
| Anti-NF- $\kappa$ B p65             | 1:1000, Zenbio, Cat# YA4176                           |
| Anti-p-NF- $\kappa$ B-p65           | 1:1000, Cell Signaling Technology, Cat# 3033          |
| Anti-I $\kappa$ B $\alpha$          | 1:1000, Jingjie Bio, Cat# PTM-5020                    |
| Anti-p-I $\kappa$ B $\alpha$        | 1:1000, Proteintech, Cat# 82349-1-RR                  |
| Anti-IL-1 $\beta$                   | 1:1000, Proteintech, Cat# 82696-15-RR                 |
| Anti-IL-10                          | 1:1000, Proteintech, Cat# 60269-1-lg                  |
| Anti-CD31                           | 1:200, Proteintech, Cat# 11265-1-AP                   |
| Anti- $\alpha$ -SMA                 | 1:2000, Proteintech, Cat# 14395-1-AP                  |
| Anti-CD86                           | 1:200, Proteintech, Cat# 83523-4-RR                   |
| Anti-CD206                          | 1:200, Proteintech, Cat# 18704-1-AP                   |
| Anti- F4/80                         | 1:200, Proteintech, Cat# 28463-1-AP                   |
| Recombinant Anti-GAPDH              | 1:3000, Servicebio, Cat# ZB15004-HRP-100              |
| HRP-conjugated goat anti-rabbit IgG | 1:3000, Proteintech, Cat# RGAM701                     |
| HRP-conjugated goat anti-mouse IgG  | 1:3000, Proteintech, Cat# RGAR301                     |