Supplementary Material

Cyclodextrin-Based Deep Eutectic Solvent-Constructed Chitosan Eutectogel for Therapeutic Delivery of Glabridin in Diabetic Wound Management

Running title: Supramolecular Eutectogel Platform for Diabetic Wound Healing

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1.1 Reagents

Sulfobutyl ether-β-cyclodextrin (SBE-β-CD, average degree of substitution ~7.0), levulinic acid (Lev, purity ≥99%), glabridin (GLD, purity ≥98%, CAS: 59870-68-7), and chitosan (CS, degree of deacetylation ≥95%, molecular weight ~100 kDa) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. The Cell Counting Kit-8 (CCK-8), Matrigel basement membrane matrix, lipopolysaccharide (LPS, from *Escherichia coli* O55:B5), H&E staining kit, and Masson's trichrome staining kit were obtained from Beyotime Biotechnology. Fluorescence-labelled antibodies (APC-conjugated anti-mouse CD86 and PE-conjugated anti-mouse CD206) were purchased from Cell Signaling Technology. Enzyme-linked immunosorbent assay (ELISA) kits for mouse tumor necrosis factor-alpha (TNF-α), interleukin-10 (IL-10), and vascular endothelial growth factor (VEGF) were obtained from Lanpai Bioscience. Primary antibodies (anti-CD31, anti-Ki-67, and anti-CK5) were purchased from Abcam. *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* (ATCC 25922) were obtained from the American Type Culture Collection (ATCC). The murine macrophage cell line Raw 264.7 (ATCC TIB-71) and human umbilical vein endothelial cells (HUVECs) were purchased from Shanghai Fuheng Biotechnology Co., Ltd. DMEM culture medium and fetal bovine serum (FBS) were purchased from Gibco. Endothelial Cell Medium (ECM) was obtained from ScienCell Research Laboratories. Streptozotocin (STZ) was purchased from Sigma. Isoflurane was obtained from RWD. 4% Paraformaldehyde was purchased from Beyotime. Lysozyme (activity ≥50,000 U/mg) was obtained from Sigma. The alkaline phosphatase (AKP) assay kit was purchased from Beyotime. The potassium ion assay kit was obtained from the Nanjing Jiancheng Bioengineering Institute. Phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), methanol, ethanol, and other common solvents were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. Ultrapure water used in the experiments was prepared using a Milli-Q water purification system (18.2 MΩ·cm).

1.2 ^{1}H NMR Spectroscopy

^{1}H NMR spectra were recorded on a Bruker AVANCE III 400 MHz spectrometer at 25°C. Samples were dissolved in D_2O at a concentration of 10 mg/mL. Chemical shifts were referenced to the residual solvent signal (δ 4.79 ppm). Data were processed using MestReNova software.

1.3 Preparation of GLD/CS/DES gel

Exactly 1.4 g of the DES solution was weighed into a 20 mL glass vial, and 0.6 g of deionized

water was added. The mixture was magnetically stirred at 800 rpm for 10 minutes to obtain a homogeneous DES/water mixed solvent. This stirring speed was chosen to ensure uniform mixing while avoiding the introduction of air bubbles. Subsequently, 0.08 g of GLD was added. The vial was placed in a 60°C water bath and stirred at 800 rpm in the dark for 2 hours until a uniform, transparent solution with no visible particles was obtained, indicating complete dissolution of GLD in the DES medium. The temperature of 60°C was chosen to promote GLD dissolution while preventing thermal degradation. After cooling the solution to room temperature (approximately 25°C), 0.08 g of CS powder was slowly added under continuous stirring at 800 rpm. Stirring was continued for 4 hours to obtain a viscous, homogeneous pre-gel solution. The pH of the pre-gel solution was carefully adjusted to 6.0 ± 0.1 with acetic acid. Subsequently, the mixture was transferred into a mold and statically crosslinked in a refrigerator at 4°C for 12 hours to form the complete GLD/CS/DES eutectogel. These conditions were optimized based on preliminary crosslinking studies to achieve optimal gel mechanical properties while maintaining drug stability. To confirm complete dissolution, pre-gel samples were analyzed by HPLC (conditions described in Section 1.5) to quantify GLD content. Recovery of >98% of the added GLD was considered to indicate complete solubilization. As a control, a blank CS/DES gel without GLD was prepared using the same procedure. The specific concentrations and ratios were determined based on preliminary optimization studies evaluating gel formation, mechanical properties, and drug-loading capacity.

1.4 Structural Characterization

The prepared GLD/CS/DES eutectogel and blank CS/DES gel were freeze-dried. Samples were dried using a freeze dryer at -50°C and 0.1 mbar for 48 hours to obtain porous xerogels. The lyophilized samples were characterized using the following techniques:

Fourier Transform Infrared Spectroscopy (FT-IR): FT-IR spectra were recorded on a Nicolet iS50 spectrometer (Thermo Fisher Scientific, USA) using the potassium bromide (KBr) pellet method, over a wavenumber range of 4000–400 cm^{-1}, with a resolution of 4 cm^{-1} and 32 scans per sample.

Scanning Electron Microscopy (SEM): The morphology of the lyophilised samples was observed using a scanning electron microscope (SU8010, Hitachi, Japan). Fresh cross-sections of each sample were adhered to conductive carbon tape, sputter-coated with gold for 60 seconds, and then imaged at an accelerating voltage of 5.0 kV.

X-ray Diffraction (XRD): X-ray diffraction (XRD) patterns were obtained using a Bruker D8 Advance diffractometer (Bruker, Germany) with Cu Kα radiation (λ = 1.5418 Å) at 40 kV and 40 mA. Samples were scanned over a 2θ range from 5° to 60° at a scanning rate of 4°/min.

1.5 High-Performance Liquid Chromatography (HPLC) Analysis

Quantitative analysis of GLD in this study was performed under the following HPLC conditions. Chromatographic system: Essentia LC-16 system (Shimadzu, Japan); Column: XSelect® CSH™ C18 column (4.6 × 150 mm, 3.5 μm); Column temperature: 30°C; Mobile phase: Acetonitrile: 0.1% aqueous formic acid solution = 45:55 (v/v); Flow rate: 1.0 mL/min; Detection wavelength: 303 nm; Injection volume: 10 μL. Data acquisition and processing were performed using the EMPOWER 3.0 chromatography workstation. All samples were filtered through a 0.22 μm microporous membrane prior to injection.

The HPLC method was validated in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines. The calibration curve exhibited excellent linearity ($R^2 > 0.999$) over the concentration range of 0.5–100 μg/mL. The recoveries determined at low (1 μg/mL), medium (20 μg/mL), and high (80 μg/mL) concentrations ranged from 98.2% to 101.5%. Intra-day precision (relative standard deviation, RSD) was below 2.5%, and inter-day precision over three consecutive days was below 3.8%. These validation parameters meet the acceptance criteria for quantitative analysis of biological samples.

1.6 Steady-State Shear Rheology

Steady-state shear measurements were performed using an Anton Paar MCR 302e rheometer with a 20 mm parallel plate geometry at 25°C. The shear rate was increased logarithmically from 0.1 to 100 s^{-1}, and apparent viscosity was recorded. A solvent trap was used to minimize evaporation.

1.7 Adhesive Property Testing

Freshly obtained porcine skin was trimmed to remove subcutaneous fat, then cut into rectangular test pieces measuring approximately 1 mm in thickness and 4 × 4 cm in area. The surface was moistened with PBS to simulate physiological conditions before use. Approximately 0.2 g of the test gel was uniformly applied to a central 4 × 4 mm area on one piece of porcine skin. Another piece of porcine skin was quickly aligned and placed on top, and a pressure of 5 kPa was applied for 10 seconds to form a "sandwich" structure. The assembled construct was placed in a constant-temperature, constant-humidity chamber (37°C, 90% relative humidity) and incubated for 30

minutes. Immediately after incubation, a 180° peel test was performed using a WDW-5E electronic universal testing machine at a tensile rate of 5 mm/min until the two porcine skin pieces were completely separated. The maximum force during peeling was recorded, and the adhesive strength (kPa) was calculated as $\sigma = F_{max}/A$, where A is the adhesion area (16 mm^2). Each sample group was tested 5 times.

To simulate wet adhesion performance in a tissue fluid environment, wet testing was performed according to the above procedure: a dry blank gel sheet (4 × 2 mm) was attached to the surface of one piece of porcine skin, then covered with another piece of porcine skin, and pressed for 20 seconds. Subsequently, 20 μL of deionized water was added to the adhesion interface to simulate body fluid. After standing for 1 minute to allow full hydration, the 180° peel test was performed under the same conditions.

1.8 Surface Wettability

Approximately 1 mL of the liquid pre-gel solution was cast into a silicone mold, crosslinked at 4°C, and dried at room temperature to form a uniform film. Using a contact angle analyzer (DataPhysics OCA20), 3 μL of deionized water was deposited onto the film surface at room temperature. The shape change of the droplet over 120 seconds was automatically recorded by a high-speed camera. The static contact angle was calculated using the accompanying Drop Shape Analysis software. At least three different positions on each sample were tested.

1.9 Swelling Behavior

The gravimetric method was used. Accurately weighed lyophilized samples (W_0, approximately 20 mg) were completely immersed in an excess volume (50 mL) of deionized water and allowed to swell at 25°C. At predetermined time points (0.5, 1, 2, 4, 6, 8, 12, and 24 hours), the samples were removed, gently blotted with filter paper to remove excess surface water, and immediately weighed (W_s). The swelling ratio (SR) was calculated as: $SR = (W_s - W_0) / W_0$. Results were expressed as a curve of swelling ratio over time. Experiments were performed in triplicate.

1.10 Hemocompatibility Determination

Determined according to GB/T 16886.4-2003 standard. Freshly collected heparinized mouse whole blood (heparin concentration, 10 U/mL) was used, and the blood was washed three times with normal saline to prepare a 5% (v/v) red blood cell (RBC) suspension. The GLD/CS/DES eutectogel was dissolved in normal saline to prepare a 10% (w/v) extract stock solution, which was

then incubated at 37°C for 24 hours. The stock solution was diluted with normal saline to obtain extracts at 100%, 50%, 10%, and 5% (v/v).

In 2 mL centrifuge tubes, 600 μL of each concentration extract, 1200 μL of a 5% RBC suspension, and 1200 μL of normal saline were mixed, respectively, and incubated at 37°C for 12 hours. After incubation, the sample was centrifuged at 1000 × g for 3 minutes. 200 μL of supernatant was aspirated and transferred to a 96-well plate, and absorbance (A_{sample}) was measured at 540 nm using a microplate reader. Normal saline was used as the negative control (A_{neg}), and a 1% Triton X-100 solution was used as the positive control (A_{pos}). Hemolysis rate (%) was calculated as:

Hemolysis rate = $(A_{sample} - A_{neg}) / (A_{pos} - A_{neg}) \times 100\%$.

According to the standard, a hemolysis rate less than 5% is considered acceptable.

1.11 *In Vitro* Hemostatic Capacity Determination

Using freshly collected heparinized mouse whole blood (heparin concentration 10 U/mL). In a 1.5 mL centrifuge tube, 200 μL of the pre-gel solution of CS/DES or GLD/CS/DES eutectogel (i.e., the viscous liquid before pH adjustment) was added. Then, 200 μL of heparinised whole blood was added, and the mixture was vortexed for 5 seconds. Adding 200 μL normal saline instead of gel served as a negative control. The mixture was allowed to stand at 37°C, and the time to blood coagulation and the cessation of flow (clotting time) were repeatedly observed and recorded by inverting the tube. The experiment was repeated five times.

1.12 Intracellular ROS Scavenging Assay

RAW 264.7 macrophages in the logarithmic growth phase were seeded into 24-well plates at a density of 8×10^4 cells per well. Cells were cultured in high-glucose DMEM complete medium containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a 5% CO_2 incubator for 24 hours until reaching approximately 80% confluence. The supernatant was aspirated, and the cells were gently washed once or twice with warm sterile PBS (pH 7.4). Treatments were then applied according to the following groups (with 3 replicate wells per group): the **Control group** was cultured in fresh, serum-free, high-glucose DMEM for 8 h. The **H_2O_2 group** was cultured in serum-free DMEM medium containing 600 μmol/L H_2O_2 for 4 h. The **CS/DES group** was first pretreated with serum-free DMEM containing CS/DES extract (100% stock solution) for 4 h, followed by the addition of H_2O_2 (final concentration, 600 μmol/L) and further culture for 4 h. The **GLD/CS/DES group** was first pretreated with serum-free DMEM containing GLD/CS/DES extract

(100% stock solution) for 4 h, followed by the addition of H_2O_2 (final concentration, 600 μmol/L) and further culture for 4 h.

After treatment, the supernatant was aspirated, and cells were gently washed 2-3 times with PBS. Then, 200 μL of DCFH-DA fluorescent probe working solution (final concentration 5 μmol/L), diluted in serum-free DMEM, was added to each well. Cells were incubated at 37°C in the dark for 30 minutes, with gentle agitation 2-3 times during incubation to ensure adequate probe contact with cells. After incubation, the probe solution was aspirated, and cells were washed 2-3 times with PBS to remove unentered free probe. A small amount of PBS was added to each well to keep cells moist. Observations and image acquisition were performed immediately using an inverted fluorescence microscope (excitation wavelength 488 nm, emission wavelength 525 nm). For each well, 3-5 random fields of view were photographed, and the mean fluorescence intensity of each field was quantified using ImageJ software to reflect intracellular ROS levels. All operations were performed in a protected environment from light.

1.13 Flow Cytometry

Flow cytometry using surface markers CD86 (M1) and CD206 (M2) was employed to assess the polarization state of RAW 264.7 macrophages. Cells were seeded in 6-well plates and treated for 24 hours. After treatment, cells were collected, washed, and stained with APC-labelled anti-CD86 antibody and PE-labeled anti-CD206 antibody at 4°C for 30 minutes. Gating was performed using corresponding isotype controls. Data were acquired on a flow cytometer and analyzed using FlowJo software. The median fluorescence intensity for each marker was quantified. Experiments were performed in triplicate and independently repeated three times.

1.14 Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were extracted using a nuclear extraction kit according to the manufacturer's protocol. Protein concentrations were determined by BCA assay. For each reaction, 5 μg of nuclear protein was incubated with 20 fmol of biotin-labeled NF-κB probe (5'-AGTTGAGGGGACTTTCCCAGGC-3') in binding buffer for 20 min at room temperature. DNA-protein complexes were resolved on 6% native polyacrylamide gels in 0.5× TBE buffer at 100 V for 1 h, transferred to nylon membranes, and detected by chemiluminescence.

1.15 Animal Husbandry

ARRIVE guidelines statement: This study was conducted and reported in accordance with the

ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines 2.0 to ensure comprehensive and transparent reporting.

Eight-week-old male BALB/c mice were purchased. Mice were housed under standard laboratory conditions with a 12-hour light/12-hour dark cycle, an ambient temperature of 23°C ± 2°C, and a relative humidity of 55% ± 10%. All animals had free access to standard rodent chow and sterilized drinking water. All experimental procedures were approved by the Animal Experiment Ethics Committee of Yueyang Central Hospital and were performed in accordance with the relevant guidelines and regulations for the care and use of laboratory animals.

Euthanasia method: At the designated experimental endpoints, mice were humanely euthanized. Deep anesthesia was first induced by inhalation of isoflurane (5% for induction in an induction chamber, maintained at 1.5–2% via a nose cone, in 100% oxygen). Once the pedal reflex was absent and respiration was deeply suppressed, cervical dislocation was immediately performed as a secondary physical method to ensure death. Death was confirmed by cessation of respiration and heartbeat, and absence of corneal reflex. All procedures were performed by experienced personnel to minimize suffering.

1.16 Immunohistochemical Analysis

Skin tissues were fixed in 4% paraformaldehyde overnight, dehydrated through a graded ethanol series, and then embedded in paraffin. Sections were incubated in 10 mmol/L citrate buffer (pH 6.0) at 94°C for 15 minutes for antigen retrieval, cooled to room temperature, and blocked with 1% bovine serum albumin for 30 minutes. Subsequently, sections were incubated overnight at 4 °C with primary antibodies against Ki-67, CK5, and CD31, followed by incubation with the corresponding HRP-labeled secondary antibodies. DAB was used for color development, and nuclei were counterstained with hematoxylin. Stained sections were examined under a light microscope, and quantitative analysis was performed by counting positive cells in selected fields of view using ImageJ. Immunofluorescence staining for CD206 was performed on day 21 post-modeling to observe macrophage differentiation at the wound site.

1.17 Cytokine Measurement in Wound Tissue

Wound tissue samples (approximately 50 mg) were homogenized in 500 μL of ice-cold PBS containing protease inhibitor cocktail. Homogenates were centrifuged at 12,000 × g for 15 min at 4°C, and supernatants were collected. TNF-α, IL-10, and VEGF levels were quantified using ELISA

kits (LanpaiBIO) according to the manufacturer's instructions. Cytokine concentrations were normalized to total protein content determined by BCA assay.

1.18 *In Vivo* Hemostatic Effect Evaluation

A mouse tail amputation model was used to evaluate the hemostatic effect of the eutectogel. Briefly, mice were anesthetized and immobilized, and the tail was amputated approximately 1 cm from the tip using sterile surgical scissors. After allowing the wound to bleed freely for 5 seconds, a pre-weighed sterile filter paper (W_1) was used to absorb the initial blood droplets. Subsequently, an adequate amount of CS/DES eutectogel or GLD/CS/DES eutectogel was immediately and evenly applied to the wound site, and gently pressed for 10 seconds to ensure tight adhesion. After hemostasis, another filter paper (W_2) was used to collect any residual blood. Total blood loss was calculated as the weight difference between the two filter papers (W_2 - W_1). The experiment was repeated six times.

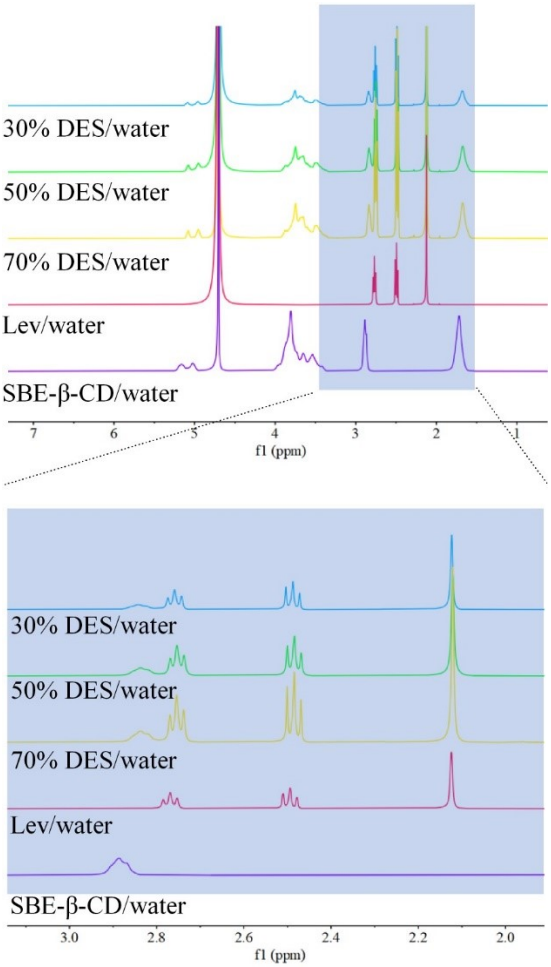

Figure S1. Full ^{1}H NMR spectra of DES with different water contents.

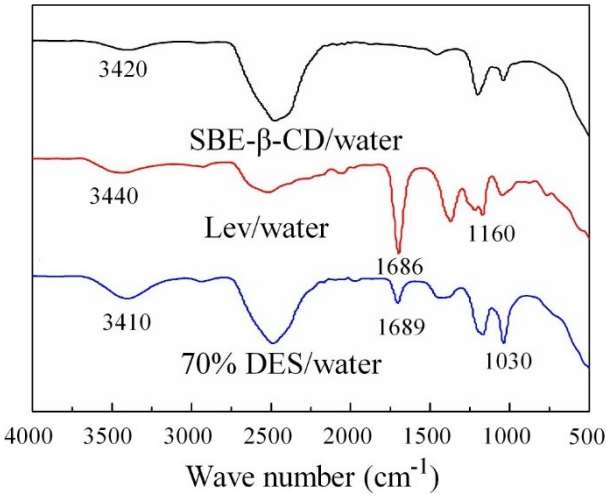

Figure S2. Hydrated FT-IR spectra of DES components.

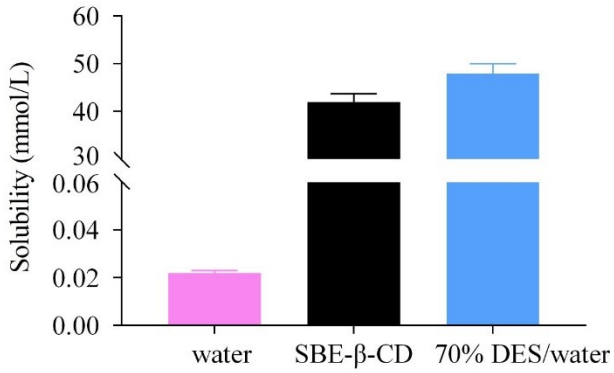

Figure S3. Solubility of GLD in different solvent systems.

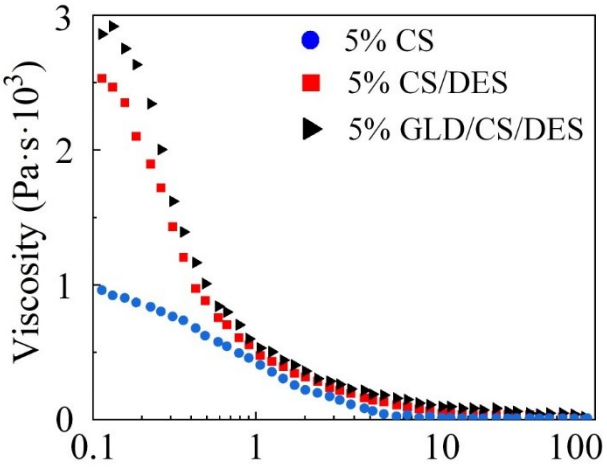

Figure S4. Steady-state shear rheology of hydrogels.

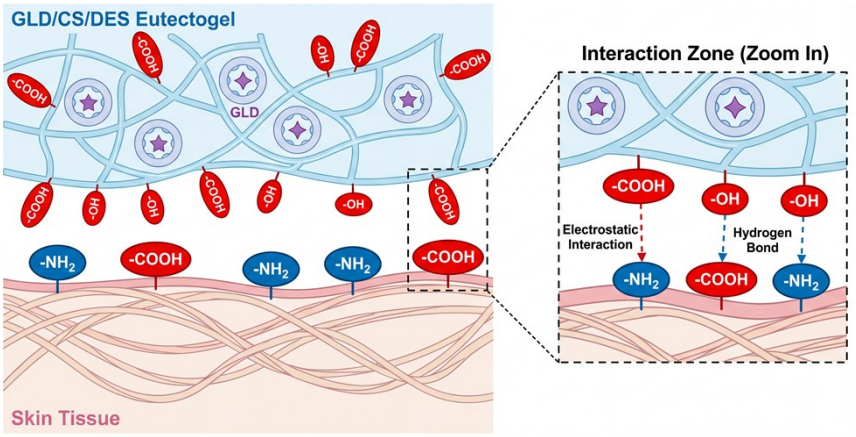

Figure S5. Schematic diagram of the proposed adhesion mechanism.

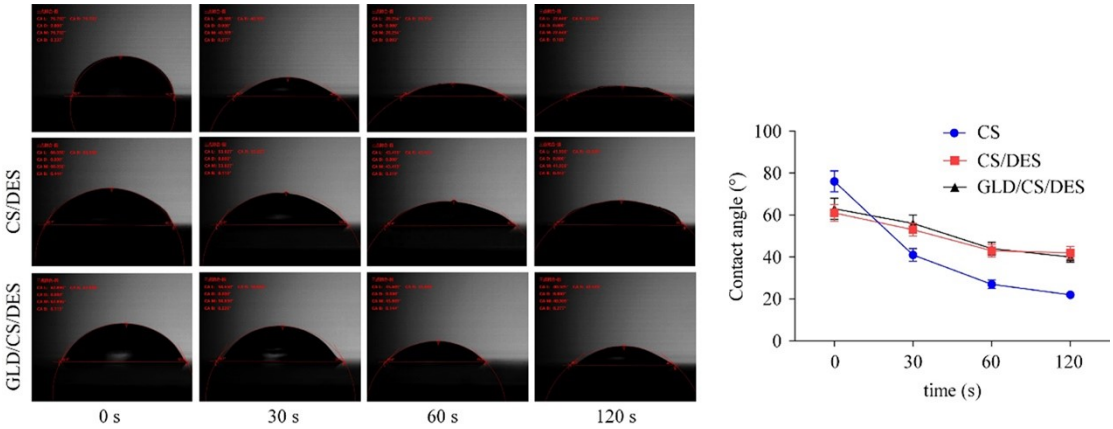

Figure S6. Water contact angle measurements.

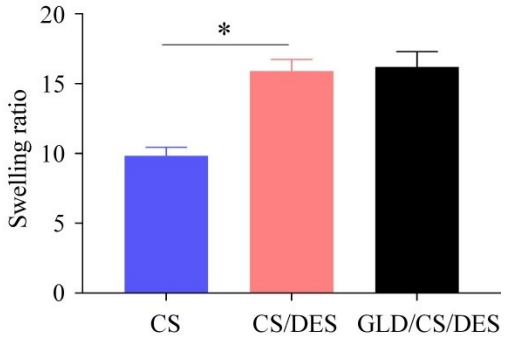

Figure S7. Equilibrium swelling ratios of CS, CS/DES, and GLD/CS/DES. *$P < 0.05$。

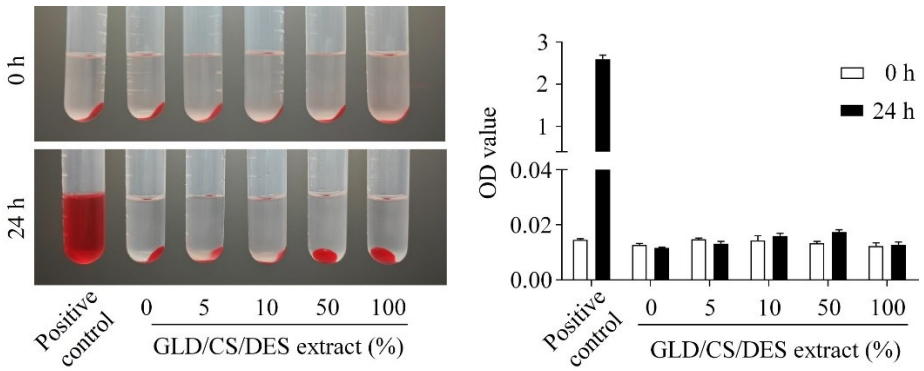

Figure S8. Hemocompatibility of GLD/CS/DES eutectogel.

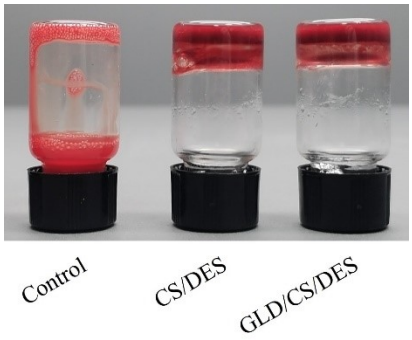

Figure S9. *In vitro* coagulation assay.

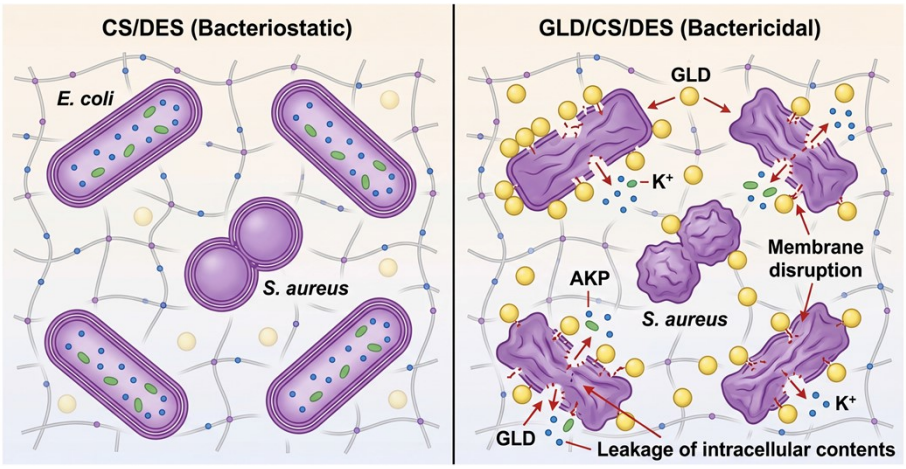

Figure S10. Schematic diagram of the proposed antibacterial mechanism.

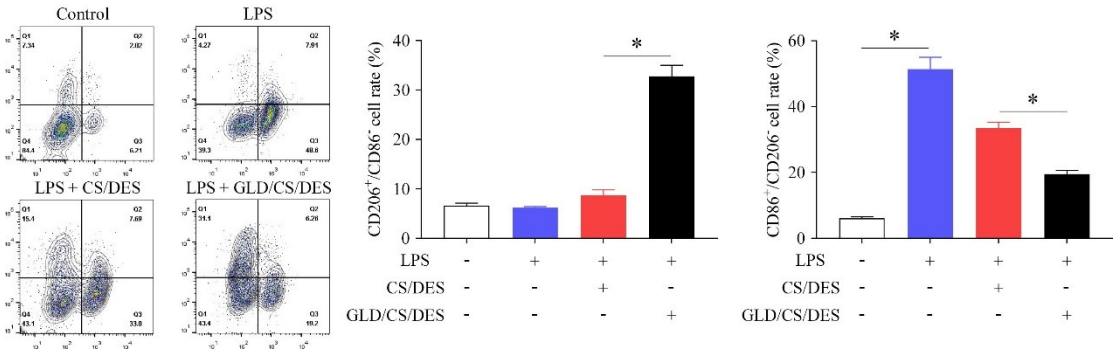

Figure S11. Flow cytometry analysis of macrophage polarization. *$P < 0.05$.

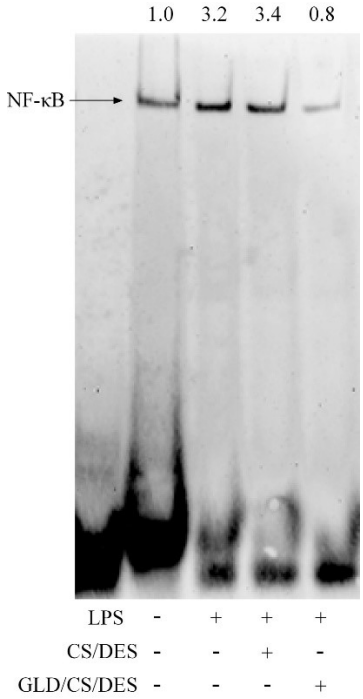

Figure S12. EMSA analysis of NF-κB activation.

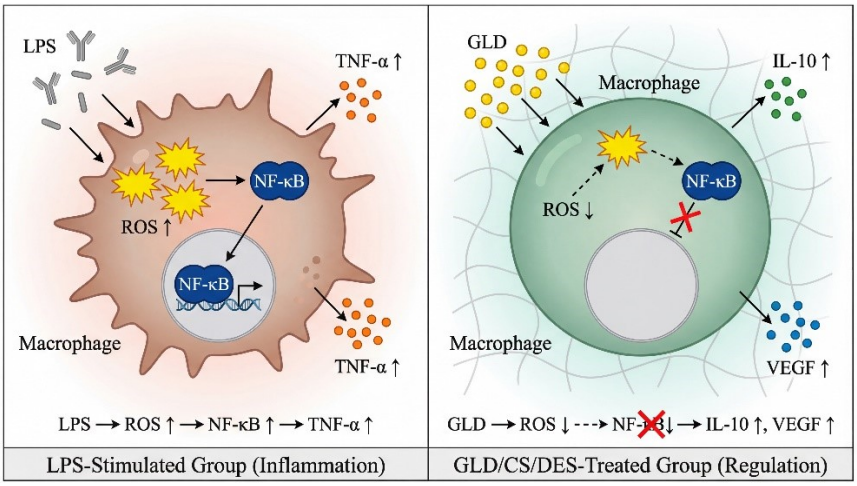

Figure S13. Schematic diagram of the proposed immunomodulatory mechanism.

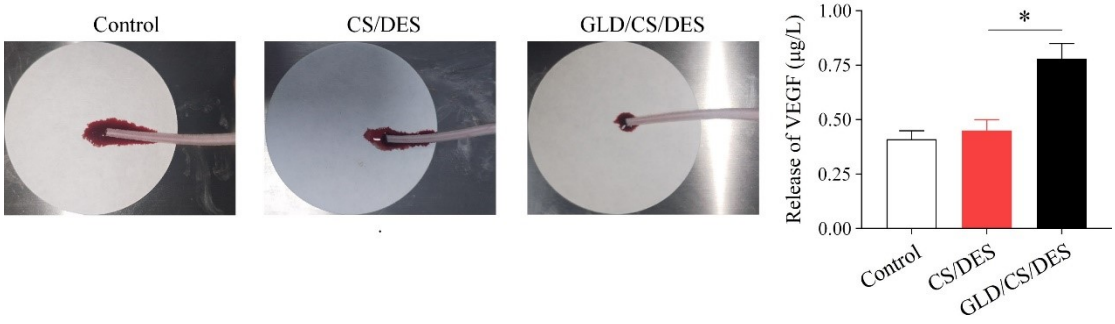

Figure S14. *In vivo* hemostatic effect of GLD/CS/DES eutectogel.

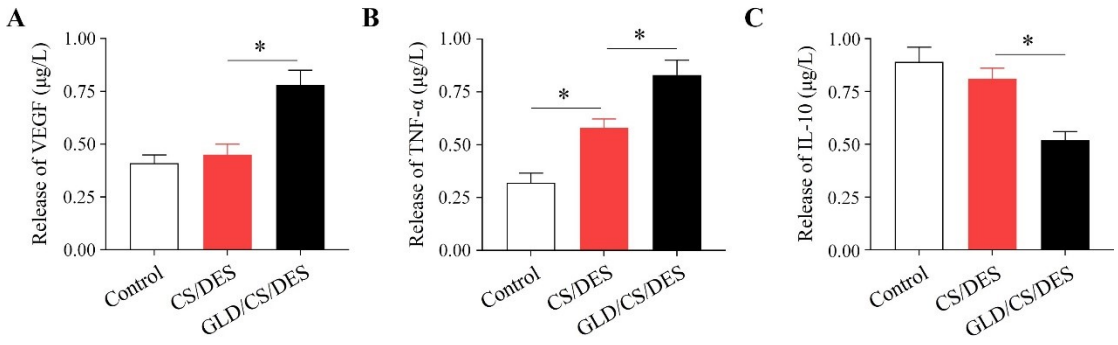

Figure S15. Cytokine levels in wound tissue. (A) Quantitative analysis of VEGF expression level in wound tissue. (B) Determination of pro-inflammatory cytokine TNF-α content in wound tissue. (C) Determination of anti-inflammatory cytokine IL-10 content in wound tissue. *$P < 0.05$.

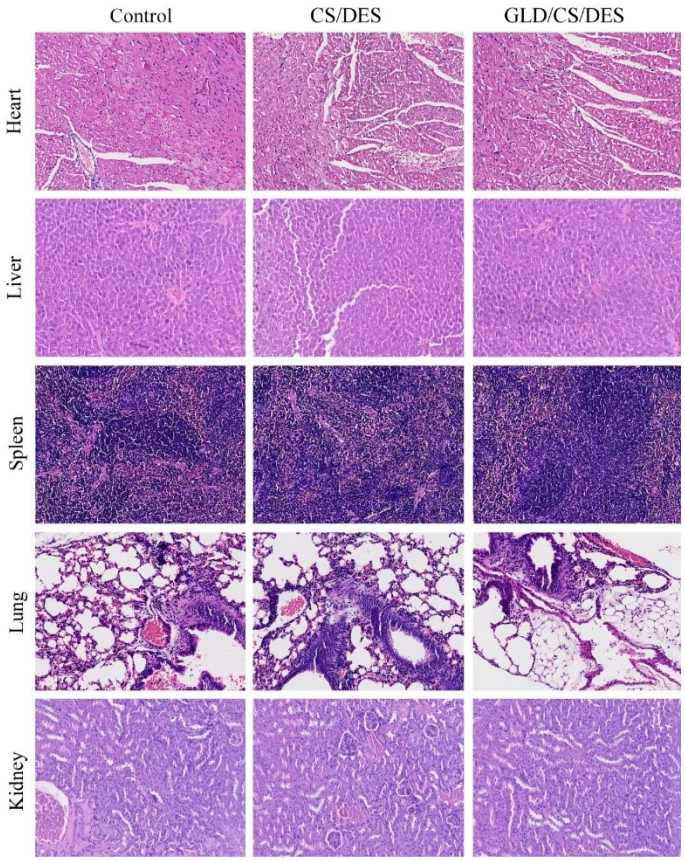

Figure S16. Representative H&E-stained images of cardiac, hepatic, splenic, pulmonary, and renal tissues from each group.

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