

Supplementary Information

Dual-functional gold nanoparticles with antimicrobial and proangiogenic activities improve the healing of multidrug-resistant bacteria-infected wounds in diabetic mice

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Experimental section

Materials. VasucLife® EnGS endothelial cell growth (ECG) medium was purchased from Lifeline Cell Technology (Frederick, MD, USA). Fetal bovine serum (FBS) and all other cell culture media were purchased from Gibco BRL (Grand Island, NY, USA). Heparin (sodium salt) was purchased from Merck (Darmstadt, Germany). Antibiotic–antimycotic, L-glutamine and nonessential amino acids (NEAA) were obtained from Biowest (Lewes, UK). The alamarBlue reagent was purchased from BioSource (Camarillo, CA, USA). The human umbilical vein endothelial cells (HUVECs, PCS-100-013) and Methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33592) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The recombinant human VEGF-A₁₆₅ (rhVEGF-A₁₆₅) and the ELISA Kits for human VEGF-A₁₆₅ and mouse cytokine (IL-1 β , TNF- α , IL-10, and TGF- β 1) were purchased from R&D systems (Minneapolis, MN, USA). Culture–Inserts and μ -Slide angiogenesis microscopy chambers were purchased from ibidi GmbH (Martinsried, Germany). The Matrigel matrix was purchased from Corning Inc. (Corning, NY, USA). Calcium chloride, hydrochloric acid, magnesium chloride, potassium chloride, sodium chloride, and tris(hydroxymethyl)aminomethane (Tris) were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). The (11-mercaptoundecyl)-*N,N,N*-trimethylammonium bromide, bovine serum albumin (BSA), trisodium citrate, phosphoric acid, and trisodium phosphate used in this study were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Hydrogen tetrachloroaurate(III) trihydrate was purchased from Acros (Geel, Belgium). The LIVE/DEAD® BacLight™ bacterial viability kit, BacLight membrane potential kit, B-PER bacterial protein extraction reagent (bacteria lysis agent), calcein acetoxymethyl ester (AM) and ethidium homodimer-1 (EthD-1; LIVE/DEAD® viability/cytotoxicity kit) were purchased from Molecular Probes (Thermo Fisher Scientific Inc., Eugene, OR, USA). Primary

antibodies against the M1 marker CD86 (anti-mouse) and the M2 marker CD206 (anti-rat) were purchased from Proteintech (Proteintech Group Inc., Illinois, USA). HRP-conjugated IgG secondary antibodies was purchased from Enzo (Enzo Life Sciences, Inc, New York, USA).

Preparation and Characterization of VEGF–Au NPs and 11-MTA/VEGF₆₀–Au NPs. We prepared 15 nM Au NPs (diameter ca. 13 nm) according to a reported method.¹ VEGF–Au NPs were prepared by sequentially adding 6 mL of DI water, 1 mL of sodium phosphate buffer (50 mM, pH 9.0), 1 mL VEGF-A₁₆₅ (100–800 nM), and 1 mL of Au NPs (10 nM) into a 20-mL sample vial. After incubation for 1 h, the VEGF-A₁₆₅-absorbed Au NPs were mixed with 1 mL BSA solution (1.0 mM) for 2 h. The mixture was centrifuged at 30,000 g for 20 min to remove excess VEGF-A₁₆₅. The pellet was then washed three times with sodium phosphate buffer (5.0 mM, pH 7.4) containing 100 μM BSA and the purified VEGF–Au NPs were stored at 4 °C.

For preparation of 11-MTA/VEGF₆₀–Au NPs, 100 μL of 11-MTA solution (200 μM) is mixed with 9.9 mL of VEGF₆₀–Au NPs ([Au NPs] = 1.0 nM) solution and the mixture was incubated for 30 min. The mixture was centrifuged at 30,000 g for 20 min to remove excess 11-MTA. After the supernatant was removed, the pellet was washed with sodium phosphate buffer (5.0 mM, pH 7.4) containing 100 μM BSA. After three centrifuge/wash cycles, the purified 11-MTA/VEGF₆₀–Au NPs were stored at 4 °C. Their absorption spectra were recorded using a Synergy 4 Multi-Mode monochromatic microplate spectrophotometer (Biotek Instruments, Winooski, VT, USA). The amount of VEGF-A₁₆₅ in the supernatant was measured using a Human VEGF Quantikine ELISA Kit (R&D Systems, Minneapolis, USA) to determine the number of VEGF-A₁₆₅ molecules on each Au NP and the release of VEGF-A₁₆₅ in the cell medium. Dynamic light scattering (DLS) and zeta potential experiments were conducted using a Zeta sizer

Nano-ZS90 analyzer (Malvern Instruments Ltd., Malvern, UK). VEGF–Au NPs solution was dropped and dried on a ZnSe window prior to recording the Fourier transform infrared (FT-IR) spectra (TENSOR II Fourier transform infrared spectrometer, Bruker, Rheinstetten, Germany) in transmission mode in a range of 500 to 4,000 cm^{-1} with 32 scans.

Cell Proliferation Assay. Human umbilical vein endothelial cells (HUVECs) were routinely cultured in tissue culture flasks in vasucLife EnGS ECG medium under a humidified atmosphere (37 °C) containing 95% air and 5% CO_2 . In this study, all HUVECs were used between passages 2 and 5. The proliferation of HUVECs in the presence of various VEGF- A_{165} , VEGF–Au NPs or 11-MTA/VEGF $_{60}$ –Au NPs was measured by conducting the alamarBlue assay. In brief, the HUVECs were seeded into 48-well plates in triplicate at a density of 6.0×10^3 cells in 200 μL of ECG medium per well and were incubated overnight at 37 °C. Next, the ECG medium was replaced with M199 medium (200 μL) containing 2% FBS and VEGF- A_{165} , VEGF–Au NPs or 11-MTA/VEGF $_{60}$ –Au NPs at the same concentration as VEGF- A_{165} (125 pM) and then incubated for 72 h. The culture medium was then discarded, and a mixture of 180 μL of M199 medium with the alamarBlue reagent (20 μL) was added to each well. After incubation for 4 h, fluorescence intensities at 590 nm (I_{F590}) with excitation at 560 nm were measured using a Synergy 4 Multi-Mode spectrophotometer.

Antimicrobial Activity. The minimal inhibitory concentrations (MICs) of the 11-MTA, VEGF $_{60}$ –Au NPs and 11-MTA/VEGF $_{60}$ –Au NPs against different bacterial strains were determined using the broth microdilution method.² *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Salmonella enterica serovar Enteritidis* (*S. enteritidis*), *Staphylococcus aureus* (*S. aureus*), *Bacillus subtilis* (*B. subtilis*), and methicillin-resistant *Staphylococcus aureus* (MRSA) [10^4 colony-forming unit (CFU)

mL⁻¹] were incubated separately with 11-MTA, VEGF₆₀-Au NPs or 11-MTA/VEGF₆₀-Au NPs at various concentrations in sodium phosphate buffer (5.0 mM, pH 7.4) at 37 °C while shaking at a speed of 250 rpm for 1 h. Then, aliquots (100 µL) of diluted inoculum from the bacteria suspensions were spread on the surface of the LB agar plates and allowed to solidify. The MIC values were reported as the lowest concentration of antibacterial agents capable of completely (>99%) inhibiting the growth of each bacterial strain that was tested.

***In Vitro* Cell Migration.** An *in vitro* wound-healing assay was performed to measure the induction effect of free VEGF-A₁₆₅, VEGF-Au NPs or 11-MTA/VEGF₆₀-Au NPs after inducing HUVEC migration in ibidi Culture-Inserts according to the manufacturer's instructions. HUVECs were seeded at a density of 1.0×10^4 cells in both wells of the Culture-Insert in a 12-well plate and incubated in ECG medium overnight for cell attachment. Next, the Culture-Insert was gently removed to create a cell-free gap of approximately 180 µm. The medium was then removed, and the cells were washed once with phosphate buffered saline (PBS; pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2.0 mM KH₂PO₄) at room temperature. Then, the cells were treated with free VEGF-A₁₆₅, VEGF-Au NPs or 11-MTA/VEGF₆₀-Au NPs, each containing same concentration of VEGF-A₁₆₅ (125 pM), in M199 medium containing 2% FBS. Cell migration was examined with an inverted phase-contrast microscope (Olympus BX 51, Tokyo, Japan) at 0 h and 18 h. Four independent experiments were performed. The width of migration by each group was determined with the ImageJ computer program (National Institutes of Health, Bethesda, MD).

Matrigel-based Tube-Formation Assay. A Matrigel-based tube-formation assay was conducted to assess the induction effects of free VEGF-A₁₆₅, VEGF-Au NPs, and 11-

MTA/VEGF₆₀-Au NPs on tube formation by HUVECs. Matrigel, which is a hydrogel mainly containing extracellular matrix proteins (laminin, collagen IV and entactin), was dissolved at 4 °C. Then, using cooled pipette tips, 10 µL of Matrigel (10 mg mL⁻¹) was added to each well of a prechilled µ-slide chamber, which was incubated at 37 °C for 30 min to promote gelation prior to the assay. Next, HUVECs were seeded at a density of 2.5×10^4 cells per Matrigel-coated well and were subsequently treated with free VEGF-A₁₆₅, VEGF-Au NPs or 11-MTA/VEGF₆₀-Au NPs, each containing same concentrations of VEGF-A₁₆₅ (125 pM) in M199 medium containing 2% FBS for 18 h. After incubation for 18 h, images of the formation of capillary-like structures were captured with computer-assisted microscopy (Olympus BX51, Tokyo, Japan) at 40X magnification. Tube formation was assessed by manually counting the number of branches formed by the connected cells in randomly selected fields and was expressed as a percentage, using cells untreated groups as 100%.

Viability of Bacteria after Treatment with 11-MTA/VEGF₆₀-Au NPs. The bacterial suspensions of MRSA (1.0×10^8 CFU mL⁻¹) were treated with 11-MTA/VEGF₆₀-Au NPs (5.0 nM) in 5.0 mM sodium phosphate solution (pH 7.4) at 37 °C with orbital shaking for different time durations (15, 30 and 60 min). The mixtures (1.0 mL) were then centrifuged (RCF 3,500 g, 10 min, 25 °C) and the pellets were washed twice with PBS (pH 7.4, 1.0 mL) for fluorescence measurement and quantification of live and dead bacteria. The bacteria viability was determined by a LIVE/DEAD BacLight bacterial viability kit. Briefly, for each 1 mL of the bacterial suspension, 3 µL of the dye mixture (SYTO 9:PI = 1:1) were added, and the dye-suspension mixtures were incubated at room temperature for 15 min in the dark. The mixtures were then centrifuged (RCF 3,500 g, 10 min, 25 °C) and the pellets were washed twice with PBS (pH 7.4; 1.0 mL), then dropped onto the glass slide for fluorescence measurement. The live/dead ratios of

bacteria untreated and treated with the nanomaterials at different time points were calculated from the intensities of green (excitation with blue light 460–480 nm) and red (excitation with green light 510–530 nm) fluorescence using an Olympus IX71 microscope (Tokyo, Japan).

Permeability of Bacterium Membrane. The bacterial suspensions of MRSA (1.0×10^7 CFU mL⁻¹) were treated with solutions of 11-MTA, VEGF–Au NPs or 11-MTA/VEGF₆₀–Au NPs at the same concentrations of 11-MTA (1.7 μM) or Au NPs (1.0 nM) in 5.0 mM sodium phosphate solution (pH 7.4, 1.0 mL) containing 1.0 mM MgCl₂ and 10 mM KCl at 37 °C with orbital shaking for 1 h. Untreated and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) treated MRSA were used as negative and positive controls, respectively. The samples were centrifuged at an RCF of 3,500 g for 10 min; then the pellets were retrieved for use in the membrane potential assay. The pellets were diluted ten-fold in PBS solution (495 μL), and then 5 μL-aliquots of 3.0 mM DiOC₂ (3) were added to the mixture and allowed to react at room temperature for 10 min. Then, 200 μL of each sample was transferred to a 96-well flat bottom microplate, and the fluorescence of each sample was measured using a Synergy 4 Multi-Mode microplate spectrophotometer at an excitation wavelength of 450 nm, and emission from 490 nm to 750 nm.

***In Vitro* cytotoxicity tests.** HUVECs were routinely cultured in tissue culture flasks in vasucLife EnGS ECG medium in a 37 °C humidified atmosphere containing 95% air and 5% CO₂. The cell number was determined with the trypan blue exclusion method. Cell viability was determined using an alamarBlue assay. Following the incubation of HUVECs (approximately 1.0×10^4 cells per well) in culture media for 24 h at 37 °C containing 5% CO₂, each of the culture media were replaced with 200 μL of medium containing 11-MTA/VEGF₆₀–Au NPs ([Au NPs] = 0–5.0 nM). The cells were carefully

rinsed three times with PBS and treated with the alamarBlue assay (10-fold dilution, 200 μ L per well) for 4 h. Then, the fluorescence intensities at 590 nm (I_{F590}) with excitation at 560 nm were measured using a Synergy 4 Multi-Mode microplate spectrophotometer. Since the fluorescence is proportional to the number of metabolically active cells, the relative viability of cell cultures was calculated by assuming 100% viability in the control set (medium only).

Live/Dead cell staining of Calcein AM/EthD-1. The HUVECs were seeded onto a 48-well tissue plate in vasucLife EnGS ECG medium (approximately 1.0×10^4 cells per well) and incubated for 12 h. The cells were then treated with 11-MTA/VEGF₆₀-Au NPs at different concentrations ($[\text{Au NPs}] = 0\text{--}5.0$ nM) in serum-free M199 medium for 24 h. After incubation, the medium was discarded, and the cells were washed with Dulbecco's PBS. The cytotoxic effect of 11-MTA/VEGF₆₀-Au NPs was assayed by staining the cells with two vital dyes that simultaneously detected the intracellular esterase activity and plasma membrane integrity. The cells were incubated with a freshly prepared mixture of 2 μ M calcein AM and 4 μ M EthD-1 in Dulbecco's PBS for 30 min at room temperature. Calcein AM is a membrane-permeable dye that can be cleaved by intracellular esterases to produce a green fluorescent fluorophore in living cells, whereas EthD-1 is a red fluorescent fluorophore that selectively permeates the broken cell membranes of dead cells and stains their nuclei. After incubation, the cells were examined in an Olympus IX-71 inverted fluorescence microscope with two emission band filters (522 nm and 605 nm).

Hemolysis assays. Fresh blood samples from a healthy volunteer (female, 23 years old) were drawn from the vein into tubes containing EDTA and were immediately (within 30 min of collection) centrifuged (RCF 3000 g, 10 min, 4 $^{\circ}$ C) to remove the serum. All experiments were performed in accordance with the Code of Ethics of the World

Medical Association (Declaration of Helsinki), and experiments were approved by the ethics committee at National Taiwan University. Informed consents were obtained from human participants of this study. The red blood cell (RBCs) collection procedures were performed in compliance with relevant laws and institutional guidelines. Fresh RBCs were then washed three times with sterile isotonic physiological buffer (500 μ L). Following the last wash, the RBCs were diluted with sterile isotonic PBS to obtain an RBC stock suspension (8 vol % blood cells). The RBC stock suspension (250 μ L, 8% blood cells stock) was added to each 11-MTA/VEGF₆₀-Au NPs solution (250 μ L with a final concentration of Au NPs ranging from 0 to 5.0 nM) in vials. After 1 h of incubation at 37 °C, each of the mixtures was centrifuged at an RCF of 1000 g for 10 min. Hemolysis activity was determined by measuring hemoglobin absorption at 576 nm (OD₅₇₆) in the supernatant (200 μ L). The sterile isotonic physiological buffer was used as a reference for 0% hemolysis (OD_{576 blank}). One hundred percent of hemolysis for the control was measured by adding ultrapure water to the RBC suspension (OD_{576 ultrapure water}). The hemolysis activity was calculated as follows:

$$\text{Hemolysis (\%)} = \frac{[(\text{OD}_{576 \text{ 11-MTA/VEGF}_{60}\text{-Au NPs}} - \text{OD}_{576 \text{ blank}})]}{(\text{OD}_{576 \text{ ultrapure water}} - \text{OD}_{576 \text{ blank}})} \times 100$$

Wound Healing. The wound healing efficacy of the 11-MTA/VEGF₆₀-Au NPs was evaluated using a mouse model.³ All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of National Taiwan University and approved by the Institutional Animal Care and Use Committee of the National Laboratory Animal Center (NLAC; Permit No. IACUC2012-037). BKS.Cg-7m^{+/+} Lepr^{db}/J (db/db) male mice weighing ca. 30 g were anesthetized with a mixture containing Zoletil and Xylazine with doses of 5 mg kg⁻¹ and 117 mg kg⁻¹ of body weight, respectively. The dorsal skin of the mouse was shaved and then disinfected

using 70% ethanol for aseptic surgery. The skin was removed from circular skin wounds of diameter 1 cm, made by incision of the dorsal skin of each mouse with stainless-steel scissors. Then, an MRSA suspension (10 μ L) containing 1×10^9 CFU in presterilized saline was applied on the wound site and covered with a sterilized cotton gauze moistened with phosphate buffered saline (PBS; pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2.0 mM KH₂PO₄) to keep the bacteria growing. 2 days postsurgery, the wound area was sampled using a sterile swab, and plated on LB agar and incubated for 8 h at 37 °C to confirm MRSA infection. The samples were taken after 5, 9, and 12 days in a similar manner and cultured to test for antibacterial activity. A dressing was prepared by dipping 11-MTA (8.5 μ M), VEGF₆₀-Au NPs ([Au NPs] = 5.0 nM) or 11-MTA/VEGF₆₀-Au NPs ([Au NPs] = 5.0 nM, [11-MTA] = 8.5 μ M; 10 μ L) in gauze (1.0 cm \times 1.0 cm). The wounded areas were covered with 11-MTA/gauze, VEGF₆₀-Au NPs/gauze or 11-MTA/VEGF₆₀-Au NPs/gauze, and each piece of gauze was fixed with an elastic adhesive bandage. Wounds covered with gauze without any drug loading were the untreated control. Wound closure in each mouse was assessed by a digital camera on day 2, 5, 9, and 12 after the wounding surgery. The relative wound size is expressed by the following equation.

$$\text{Relative wound size (\%)} = A_t / A_0 \times 100$$

where A_0 is the original wound area, and A_t is the wound area at a specified time.

For skin histology, specimen containing the entire wound with adjacent normal skin (including dermis and subcutaneous tissue) was excised and fixed with 10% formaldehyde solution. The tissue samples were analyzed by H&E staining for

histological testing and by Masson's trichrome staining for examining the collagen formation.

Immunohistochemical Analysis. After fixation with 4% paraformaldehyde, punched with 0.3% Triton X-100 and washing in 0.6% H₂O₂ in methanol, cutaneous excised tissues were blocked with 5% BSA in PBS, and then incubated with the desired primary antibodies against the M1 marker CD86 (anti-mouse 1:100) and the M2 marker CD206 (anti-rat 1:200) overnight at 4°C, following by HRP-conjugated IgG secondary antibodies (anti-rabbit 1:100) for 30 min at room temperature. After each binding step, the tissue sections were washed three times with PBS solution (ca. 0.5 mL) for 5 min. Then cover tissue sections with diaminobenzidine tetrahydrochloride (DAB; containing 2.5 ml H₂O, 250 µl substrate buffer, 2 drops liquid DAB chromogen, and 1 drop of H₂O₂) solution and incubate for 5 min. After washing with H₂O for 5 min, dip in Harris's modified Hematoxylin with acetic acid for ~90 s. Finally, wash slides in water after counterstaining. The staining slides were observed by fluorescent inverted microscope Olympus X70 (Olympus, Japan).

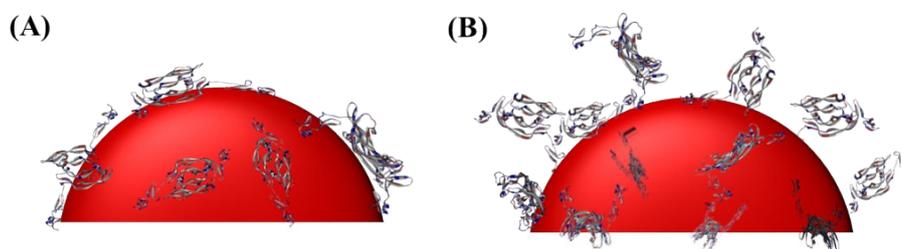
ELISA of IL-1β, TNF-α, IL-10, and TGF-β1. For ELISA analysis, animals were euthanized by an overdose of anesthetic and the wounds and surrounding skin area were removed and snap frozen in liquid nitrogen. On thawing and processing, the tissue was homogenized in extraction solution (100 mg tissue mL⁻¹), containing 0.4 M NaCl, 0.05% Tween 20, 0.5% BSA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzethoniumchloride, 10 mM EDTA, and 20 KI (~33 µg mL⁻¹) aprotinin, using bath sonication for 30 min. The suspension was then centrifugation at an RCF of 10,000 g for 10 min at 4°C. The supernatants were collected and the concentrations of IL-1β, TNF-α, IL-10, and TGF-β1 were separately quantitated by ELISA kits according to the manufacturer's instructions. All samples were assayed in four replicate.

The results of statistical analysis were expressed as the mean \pm standard deviation (SD). Comparative studies of the means were carried out using one way analysis of variance (ANOVA). Significance was accepted with $P < 0.05$.

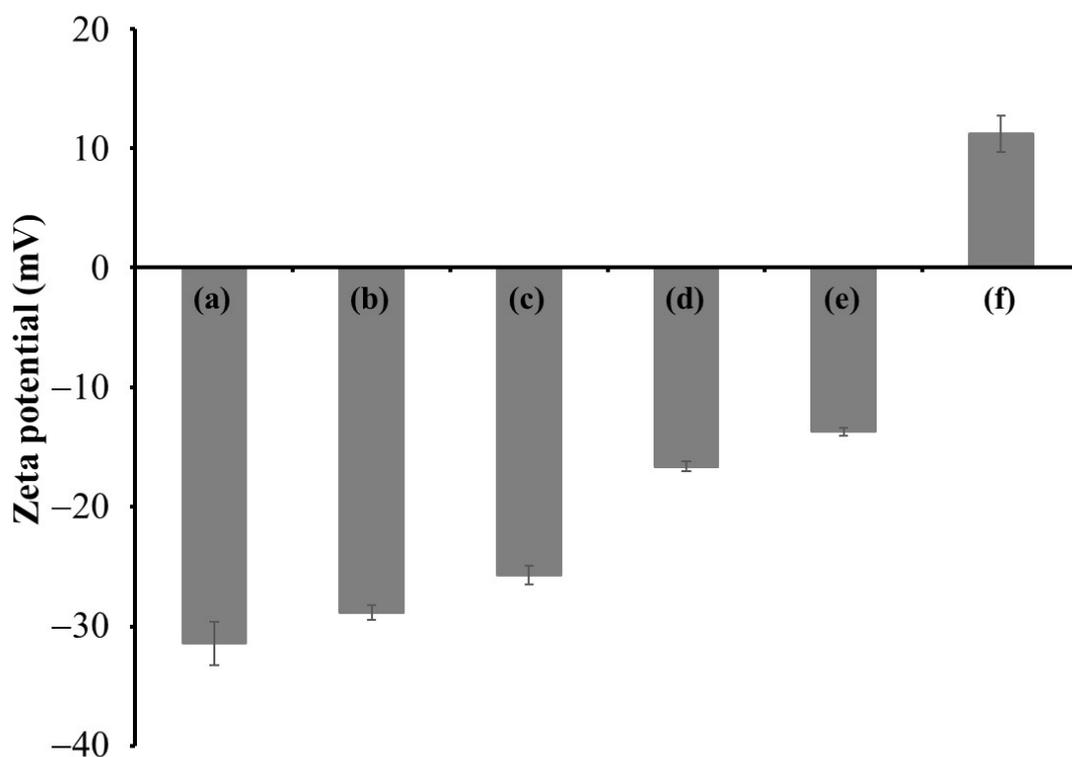
References:

- (1) Enüstün, B. V.; Turkevich, J. Coagulation of Colloidal Gold, *J. Am. Chem. Soc.* **1963**, *85*, 3317–3328.
- (2) Larrisey, M. P. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; approved standard, ninth ed., Clinical and Laboratory Standards Institute, USA, 2012.
- (3) Shi, C.-M.; Nakao, H.; Yamazaki, M.; Tsuboi, R.; Ogawa, H. Mixture of Sugar and Povidone-Iodine Stimulates Healing of *MRSA*-Infected Skin Ulcers on db/db Mice. *Arch. Dermatol. Res.* **2007**, *299*, 449–456.

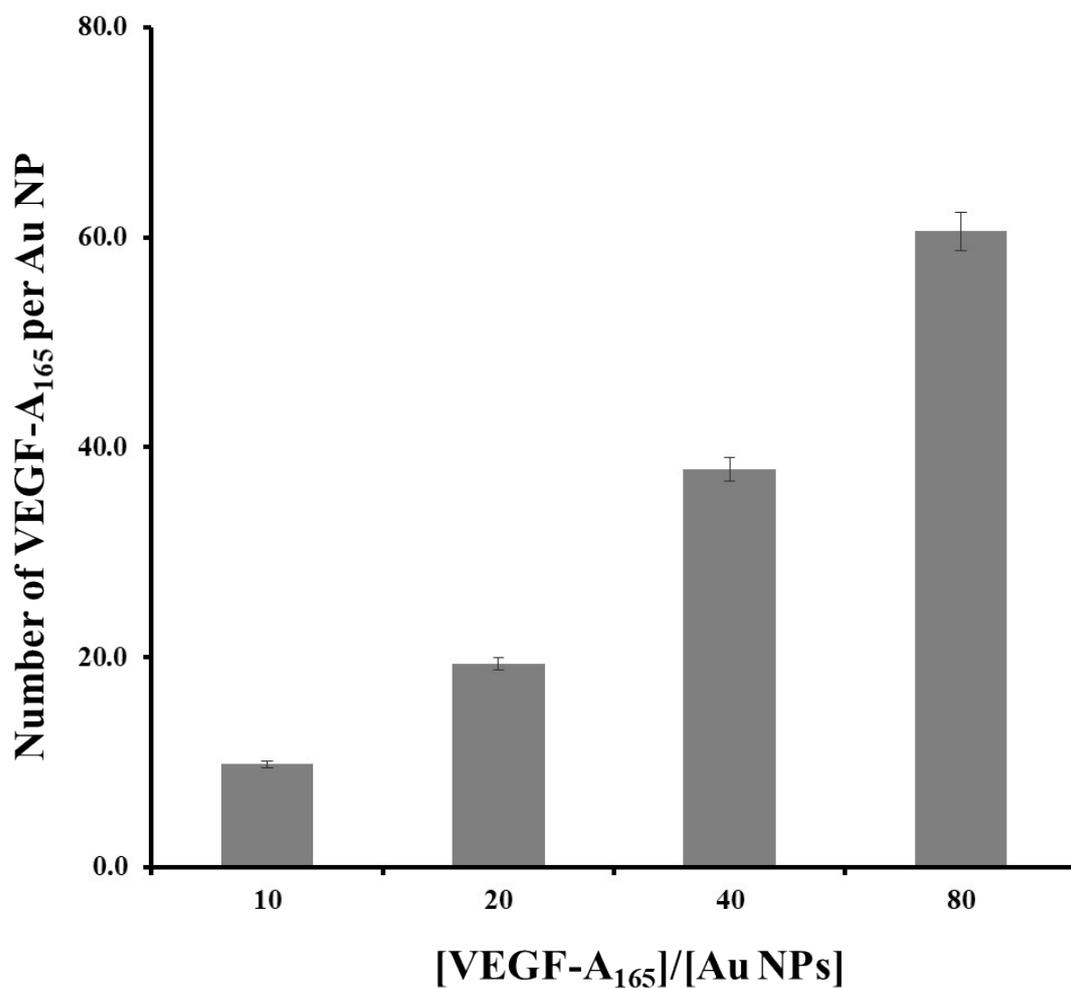
Supporting scheme and figures:



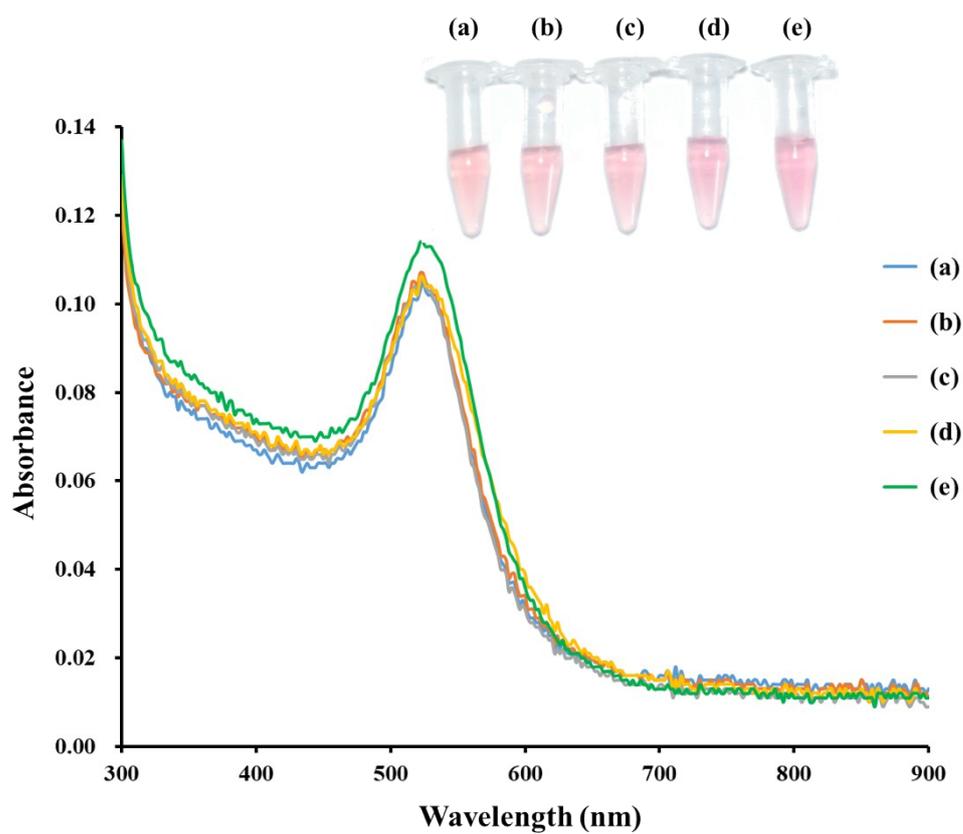
Scheme S1. Schematic representations of the adsorption of VEGF-A₁₆₅ on Au NP with (A) low density and (B) high density.



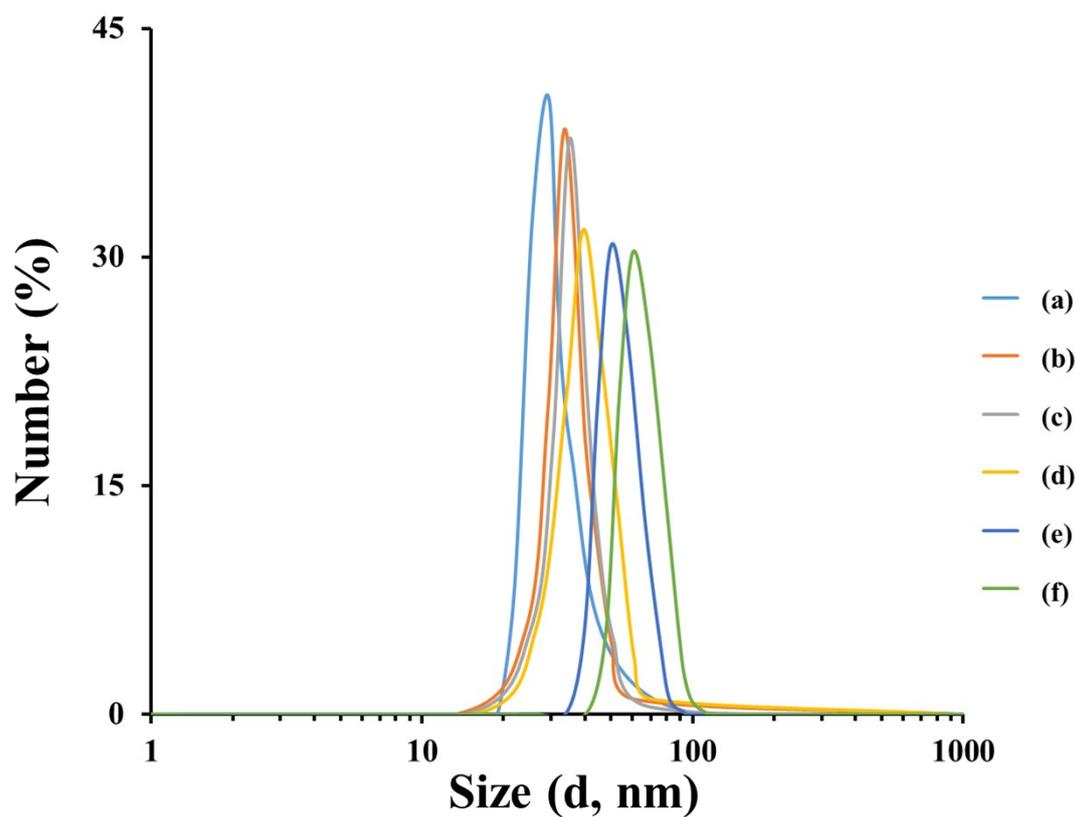
Figures S1. Zeta potentials of (a) unmodified Au NPs, (b–e) VEGF–Au NPs prepared by the molar ratio of [VEGF- A_{165}]/[Au NPs] in (b) 10, (c) 20, (d) 40, and (e) 80, and (f) 11-MTA/VEGF₆₀–Au NPs. The Au NPs and VEGF–Au NPs were prepared in 5.0 mM sodium phosphate (pH 7.4) for zeta potential measurements. The concentration of the Au NPs was 0.5 nM. The error bars represent the standard deviation of three repeated measurements.



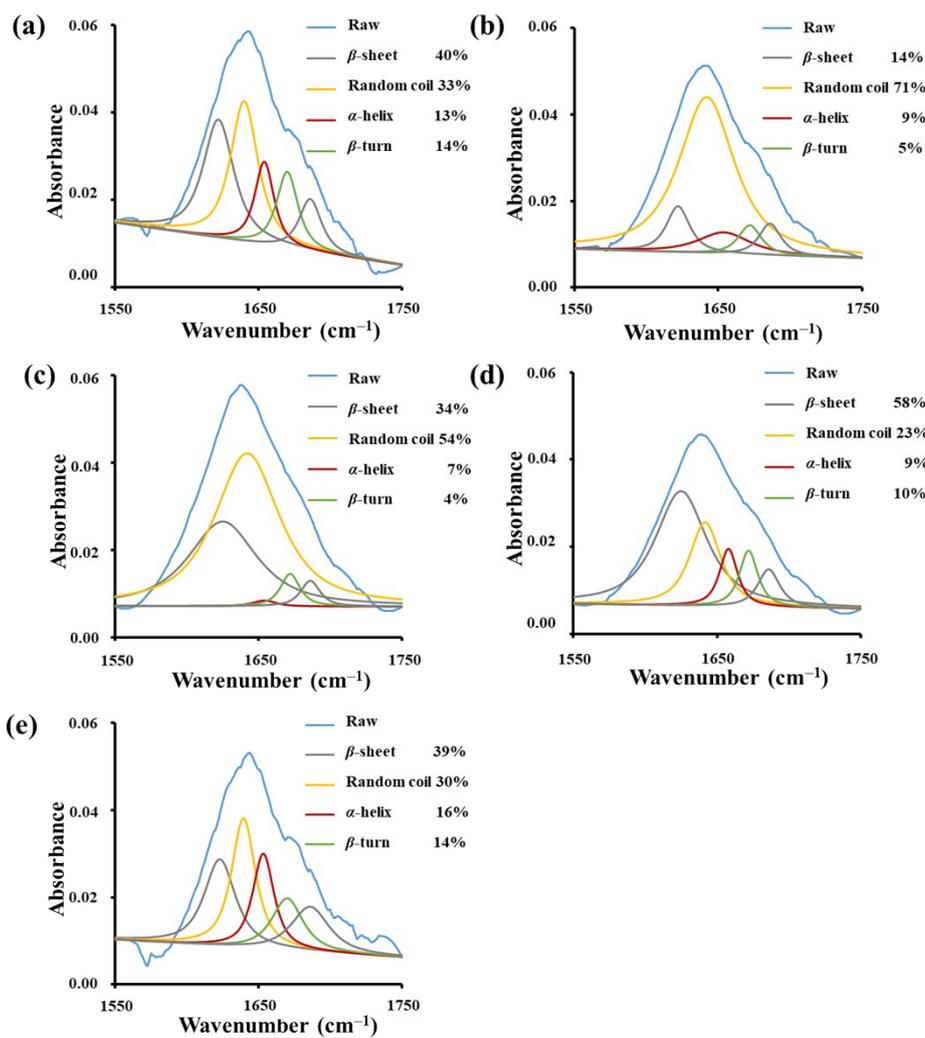
Figures S2. Quantification of VEGF-A₁₆₅ bound to Au NPs, which were prepared using various molar ratios of [VEGF-A₁₆₅]/[Au NPs] ranging from 10 to 80 in sodium phosphate buffer (5.0 mM, pH 9.0). The error bars represent the standard deviation of four repeated measurements.



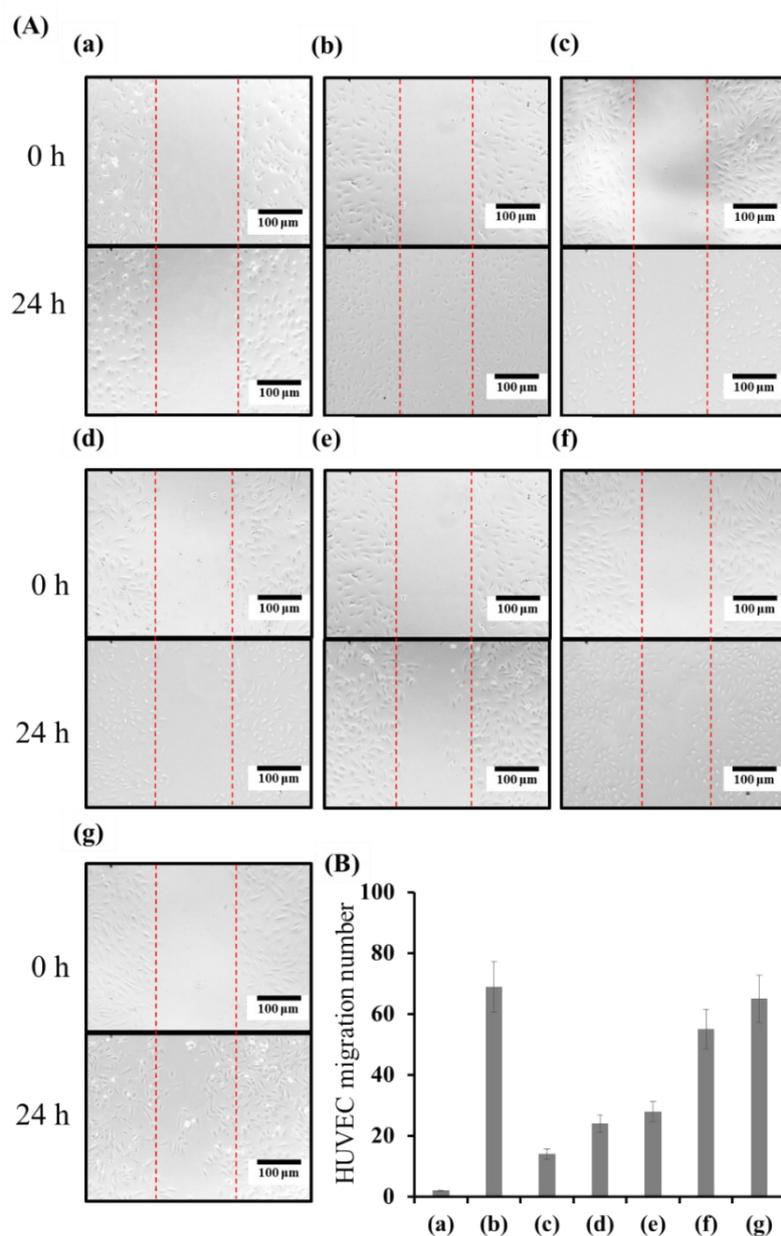
Figures S3. UV-Vis absorption spectra of (a) Au NPs, (b) VEGF₁₀-Au NPs, (c) VEGF₂₀-Au NPs, (d) VEGF₄₀-Au NPs, and (e) VEGF₆₀-Au NPs in 5.0 mM sodium phosphate (pH 7.4). The Au NP concentration is 0.5 nM. Insets: Photographic image of the Au NP solutions.



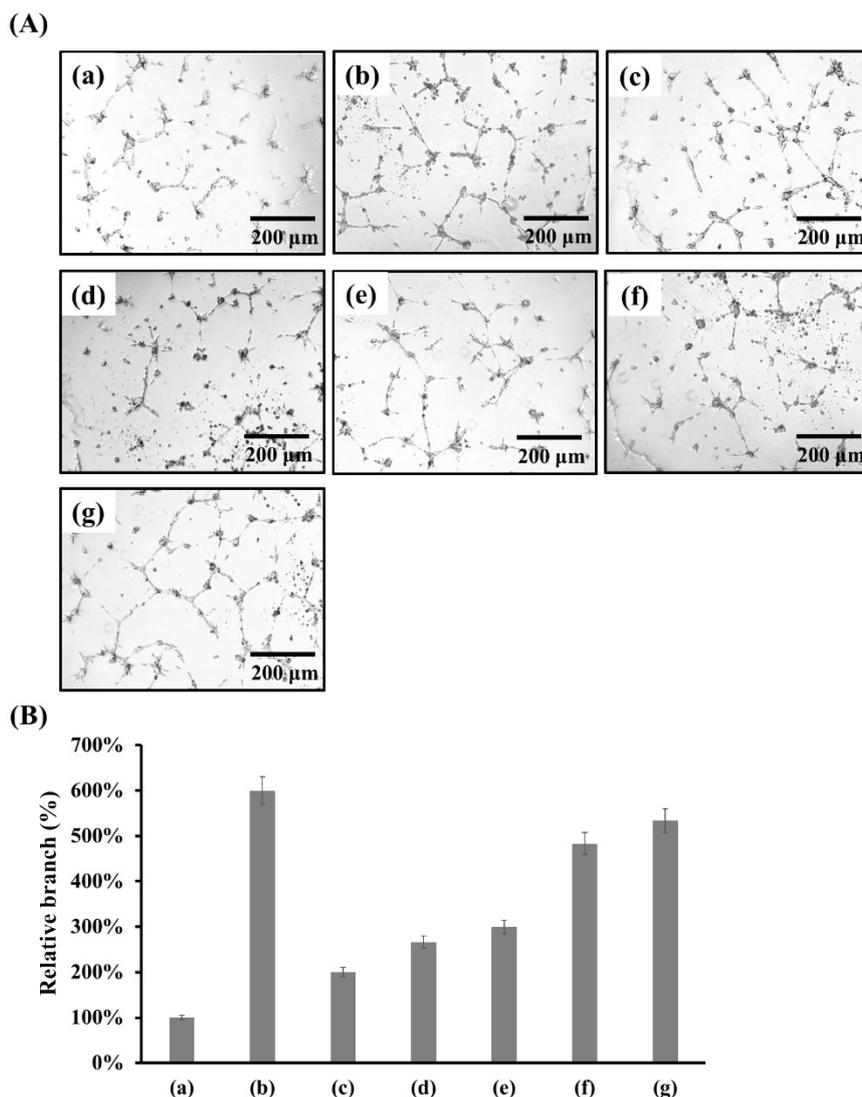
Figures S4. Dynamic light scattering (DLS) spectra of (a) unmodified Au NPs, (b) VEGF₁₀-Au NPs, (c) VEGF₂₀-Au NPs, (d) VEGF₄₀-Au NPs, (e) VEGF₆₀-Au NPs, and (f) 11-MTA/VEGF₆₀-Au NPs in sodium phosphate buffer solution (5.0 mM, pH 7.4).



Figures S5. FT-IR spectra of (a) free VEGF-A₁₆₅, (b) VEGF₁₀-Au NPs, (c) VEGF₂₀-Au NPs, (d) VEGF₄₀-Au NPs, and (e) VEGF₆₀-Au NPs in 5.0 mM sodium phosphate buffer (pH 7.4).



Figures S6. (A) Representative microscopic images of HUVECs (a) without treatment and (b–g) treated with (b) free VEGF-A₁₆₅, (c) VEGF₁₀-Au NPs, (d) VEGF₂₀-Au NPs, (e) VEGF₄₀-Au NPs, (f) VEGF₆₀-Au NPs, and (g) 11-MTA/VEGF₆₀-Au NPs for 24 h. The concentration of VEGF-A₁₆₅ in (b–g) is 125 pM. The red lines indicate the initial wound edge. (B) Average migration number of HUVECs to the original wound after treatment with free VEGF-A₁₆₅, VEGF-Au NPs, or 11-MTA/VEGF₆₀-Au NPs. The error bars in (B) represent the standard deviations of four replicate experiments. The other conditions are the same as those described in Figure 1A.



Figures S7. Representative microscopic images of HUVECs inoculated on Matrigel and (a) without treatment and (b–g) treated with (b) free VEGF- A_{165} , (c) VEGF $_{10}$ -Au NPs, (d) VEGF $_{20}$ -Au NPs, (e) VEGF $_{40}$ -Au NPs, (f) VEGF $_{60}$ -Au NPs, and (g) 11-MTA/VEGF $_{60}$ -Au NPs for 18 h. The concentration of VEGF- A_{165} in (b–g) is 125 pM. (B) Relative numbers of branches formed by the corresponding HUVECs are plotted. The error bars in (B) represent the standard deviations from four replicate experiments. The other conditions are the same as those described in Figure 1A.

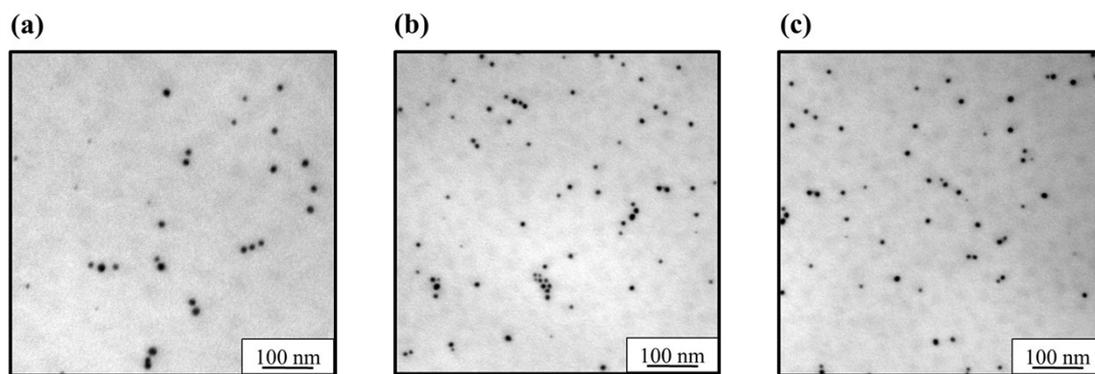
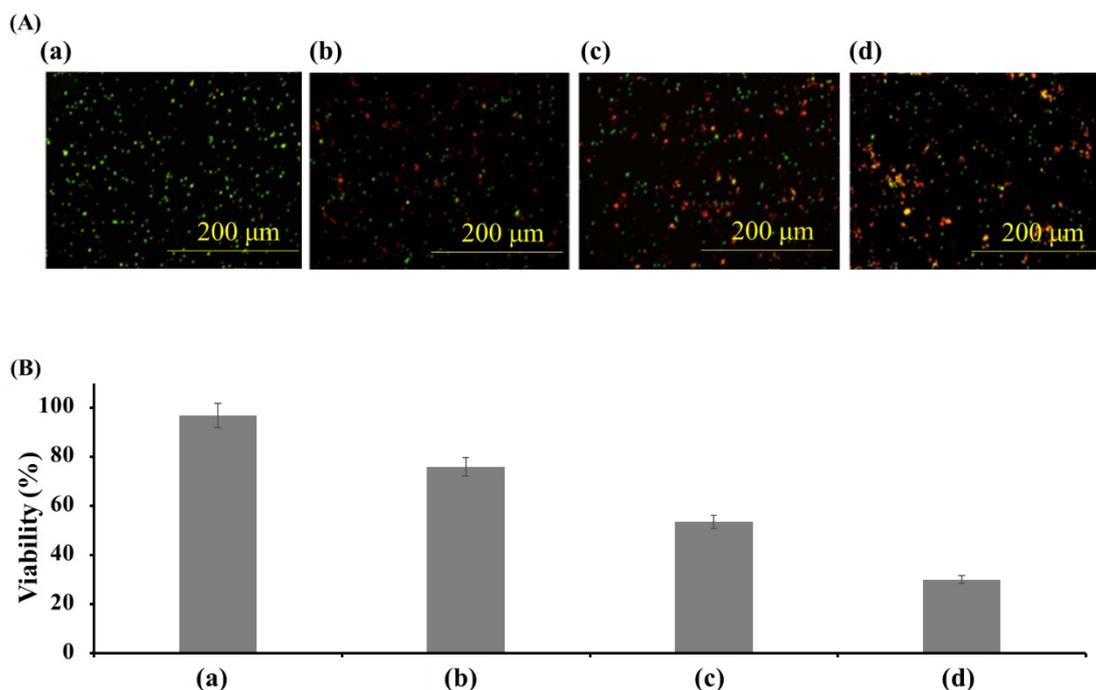
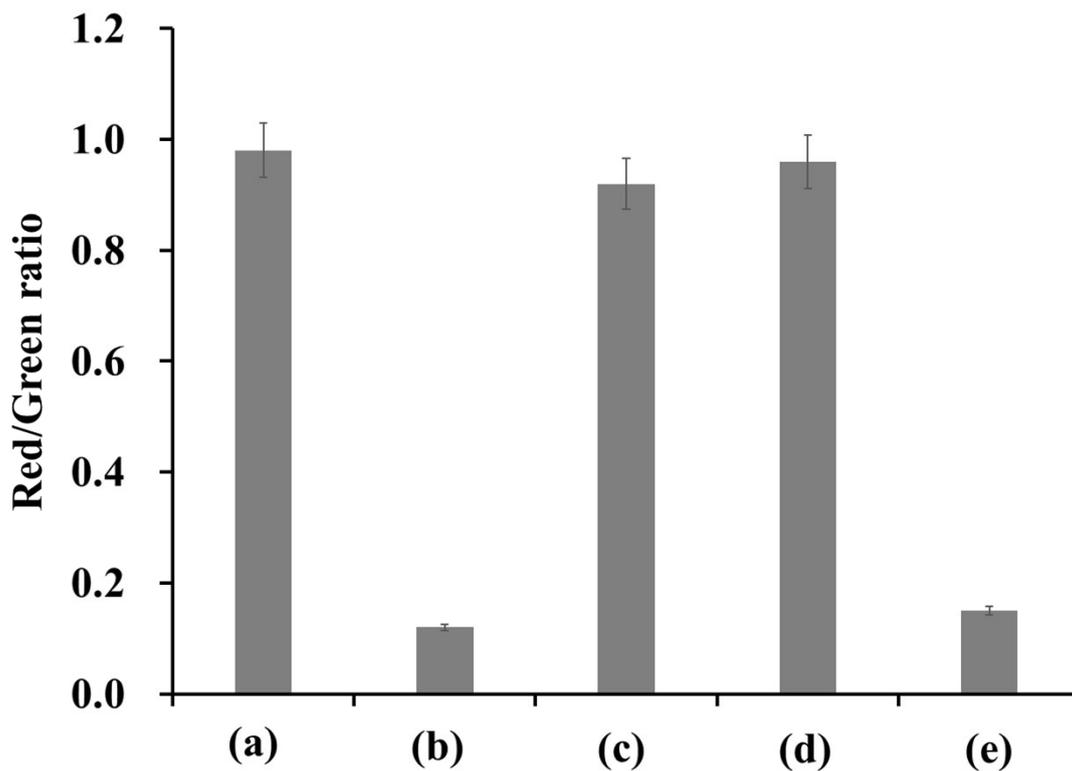


Figure S8. TEM images of (a) bare Au NPs, (b) VEGF₆₀-Au NPs, (c) 11-MTA/VEGF₆₀-Au NPs.



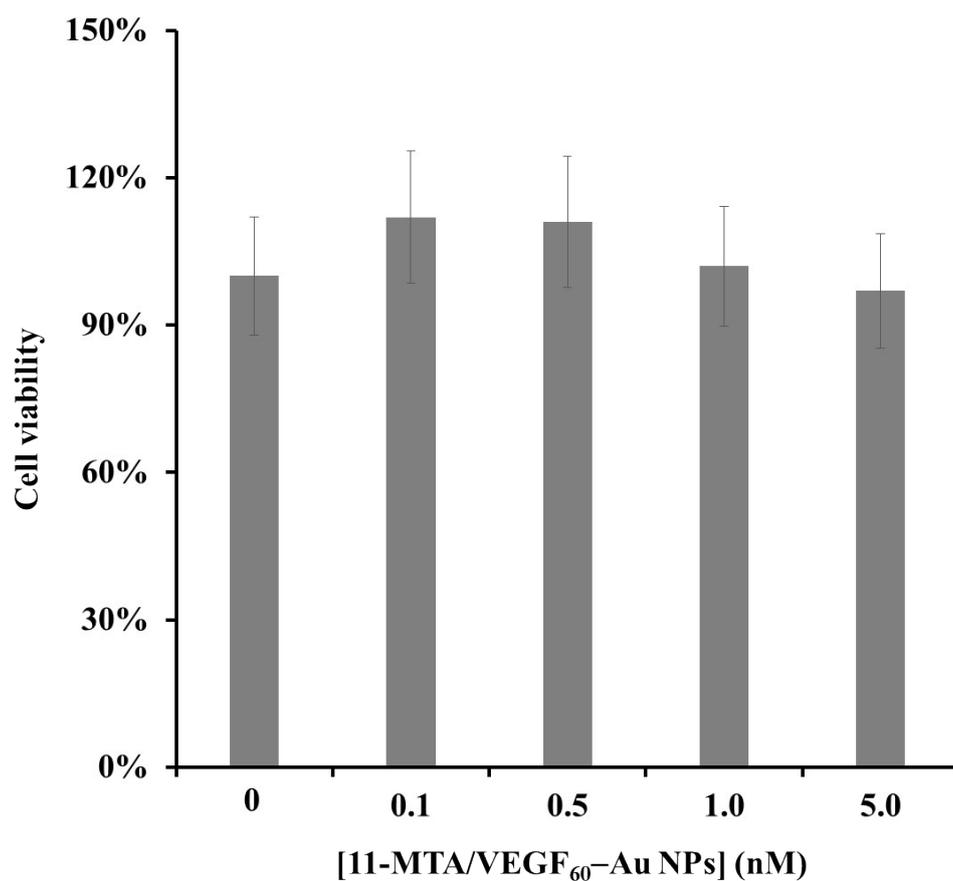
Figures S9. (A) Fluorescence images and (B) viability of MRSA (10^8 CFU mL^{-1}) in 5.0 mM sodium phosphate solution (pH 7.4) that were (a) untreated or (b–d) treated with 11-MTA/VEGF₆₀-Au NPs ($[\text{Au NPs}] = 5.0$ nM) for (b) 15, (c) 30, and (d) 60 min. The cells are stained with SYTO 9 and PI. The green fluorescent stains represent live cells, and the red fluorescent stains represent dead or compromised cells. The error bars in (B) represent the standard deviation of three repeated measurements.

Green-fluorescent SYTO 9 could permeate the intact cell membrane and is used for nuclear counterstaining. The PI (nucleic acids staining dye) could readily enter cells when the cell membranes are compromised or disrupted but is generally excluded from live cells, which have intact plasma membranes. Therefore, it is commonly used as a fluorescent probe for distinguishing dead cells from healthy cells.

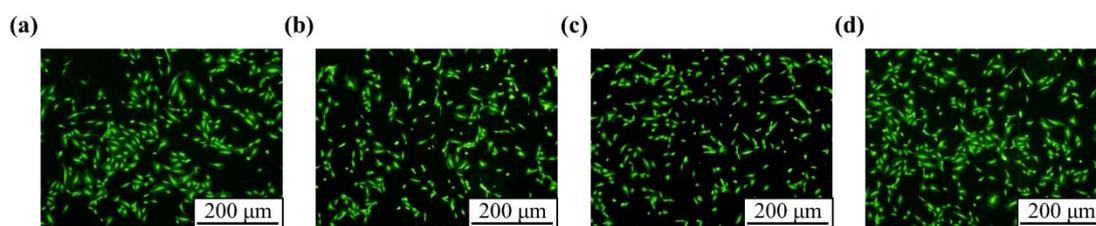


Figures S10. Fluorescence intensity ratio of DiOC₂(3) at 590 and 520 nm (Red/Green ratio) of the MRSA (1.0×10^7 CFU mL⁻¹) solution incubated (a) without or with (b) protonophore CCCP (as a control), (c) 11-MTA (1.7 μ M), (d) VEGF₆₀-Au NPs ([Au NPs] = 1.0 nM), and (e) 11-MTA/VEGF₆₀-Au NPs ([Au NPs] = 1.0 nM, [11-MTA] = 1.7 μ M). The reaction was carried out in 5.0 mM sodium phosphate solution (pH 7.4) containing 1.0 mM MgCl₂ and 10 mM KCl for 10 min. The error bars represent the standard deviation of three repeated measurements.

The DiOC₂(3) loaded cells fluoresce red (emission 590 nm), which is consistent with the presence of DiOC₂(3) aggregates and an intact membrane potential. Cells treated with the protonophore CCCP fluoresce green (emission 520 nm) consistent with the presence of monomeric DiOC₂(3) and the dissipation of membrane potential.

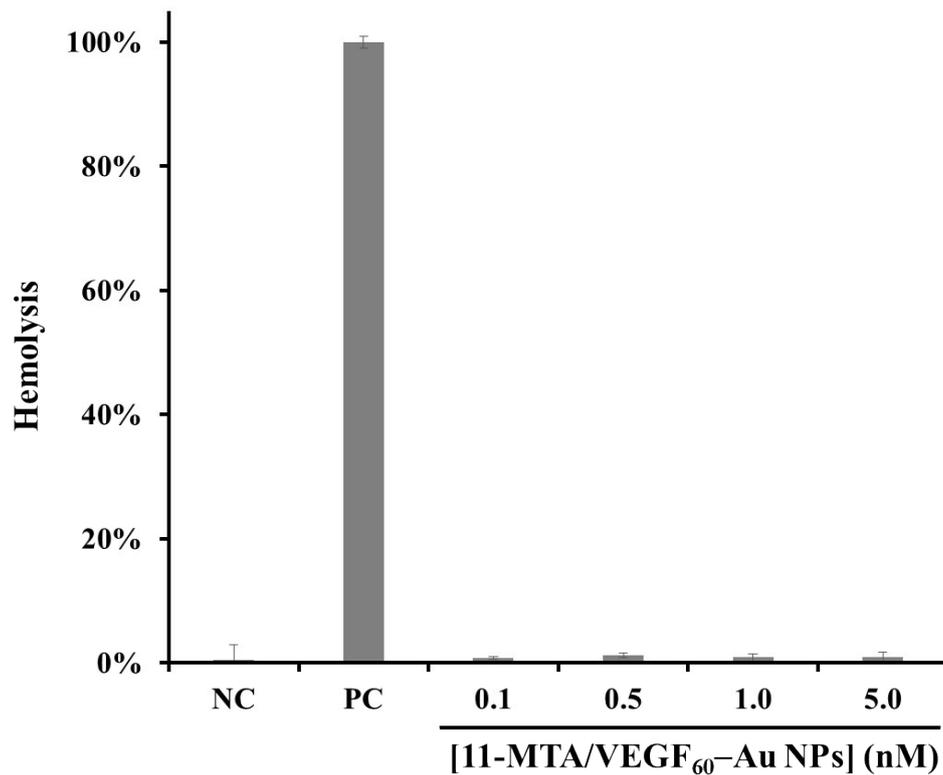


Figures S11. Viability of HUVECs (1.0×10^4 cells per well) after treatment with 11-MTA/VEGF₆₀-Au NPs with different concentrations ($[Au\ NPs] = 0-5.0\ nM$) in culture media at 37 °C for 24 h. The error bars represent the standard deviation of five repeated measurements.



Figures S12. Live/Dead cell staining with Calcein AM/EthD-1 in HUVECs (a) without treatment or (b–d) treated with 11-MTA/VEGF₆₀-Au NPs with concentrations of (b) 0.1, (c) 1.0 and (d) 5.0 nM (in term of Au NPs) in culture medium at 37 °C for 24 h.

Calcein AM is a nonfluorescent 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 retained in the cell cytoplasm. Calcein exhibits strong green fluorescence at 520 nm upon excitation at 480–500 nm. EthD-1 is excluded by the intact plasma membrane of live cells. EthD-1 enters cells with damaged cell membranes and undergoes enhancement of fluorescence (~40-fold) upon binding to nucleic acids, which produces a strong red fluorescence (~635 nm) in dead cells upon excitation at 480–500 nm.



Figures S13. Hemolytic activities of 11-MTA/VEGF₆₀-Au NPs ([Au NPs] = 0–5.0 nM) solutions in RBCs in PBS. The hemolysis assays with PBS and DI water served as the negative control (NC) and positive control (PC) groups, respectively.

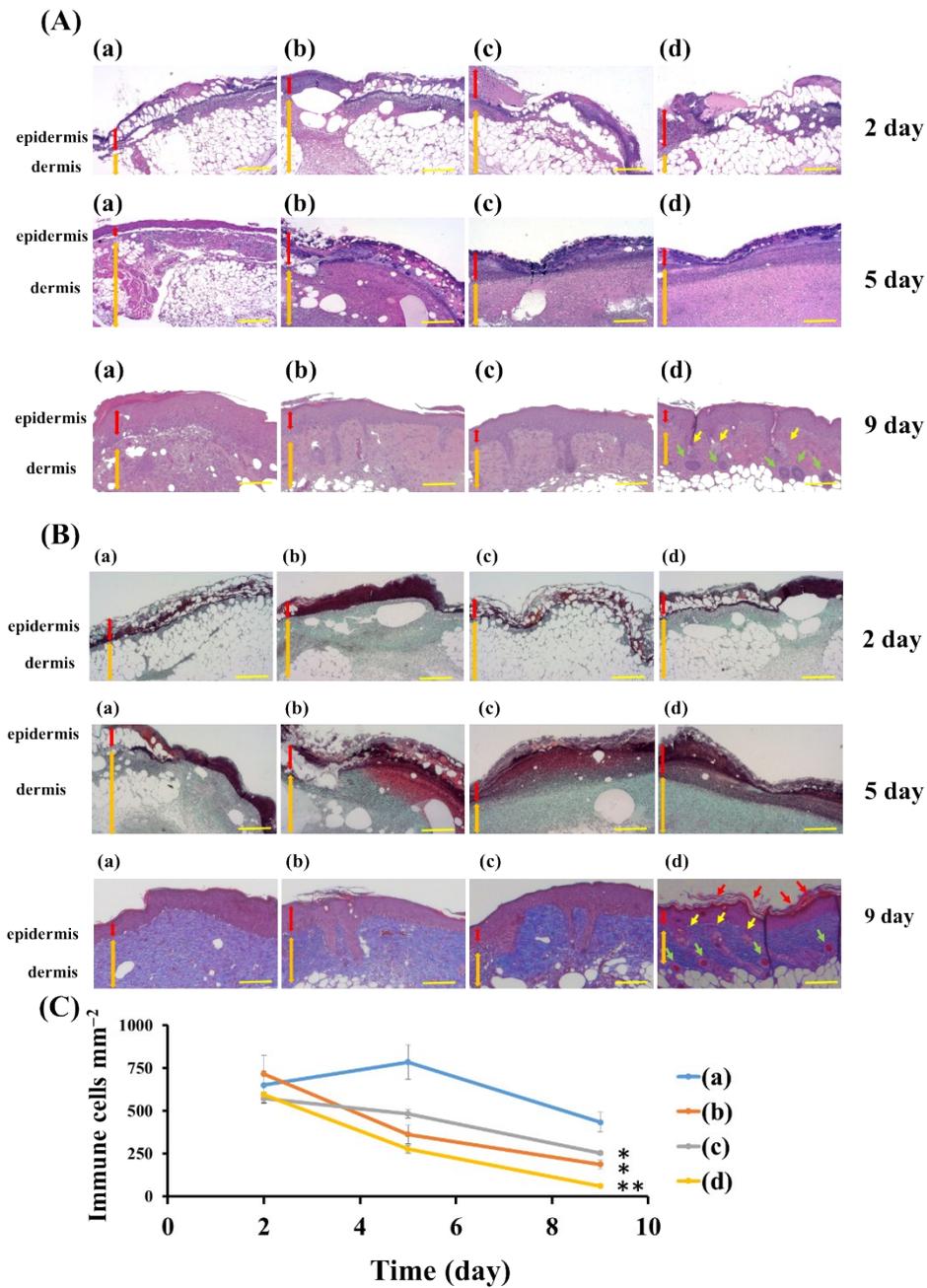


Figure S14. Histological analysis of wound tissue from (a) untreated mice or (b–d) mice treated with (b) 11-MTA (8.5 μM), (c) VEGF₆₀-Au NPs ([Au NPs] = 5.0 nM), and (d) 11-MTA/VEGF₆₀-Au NPs ([Au NPs] = 5.0 nM, [11-MTA] = 8.5 μM) on the 9th day using (A) H&E and (B) Masson's trichrome staining. (C) Average number of immune cells in the graph of H&E staining. The yellow and green arrows indicate hair follicles and blood vessels, respectively. Scale bar: 100 μm . Error bars in (C) represent the standard deviations of four replicate experiments. Asterisks indicate statistically significant differences between treated groups and untreated group on day 9 (* $p < 0.005$, ** $p < 0.001$).

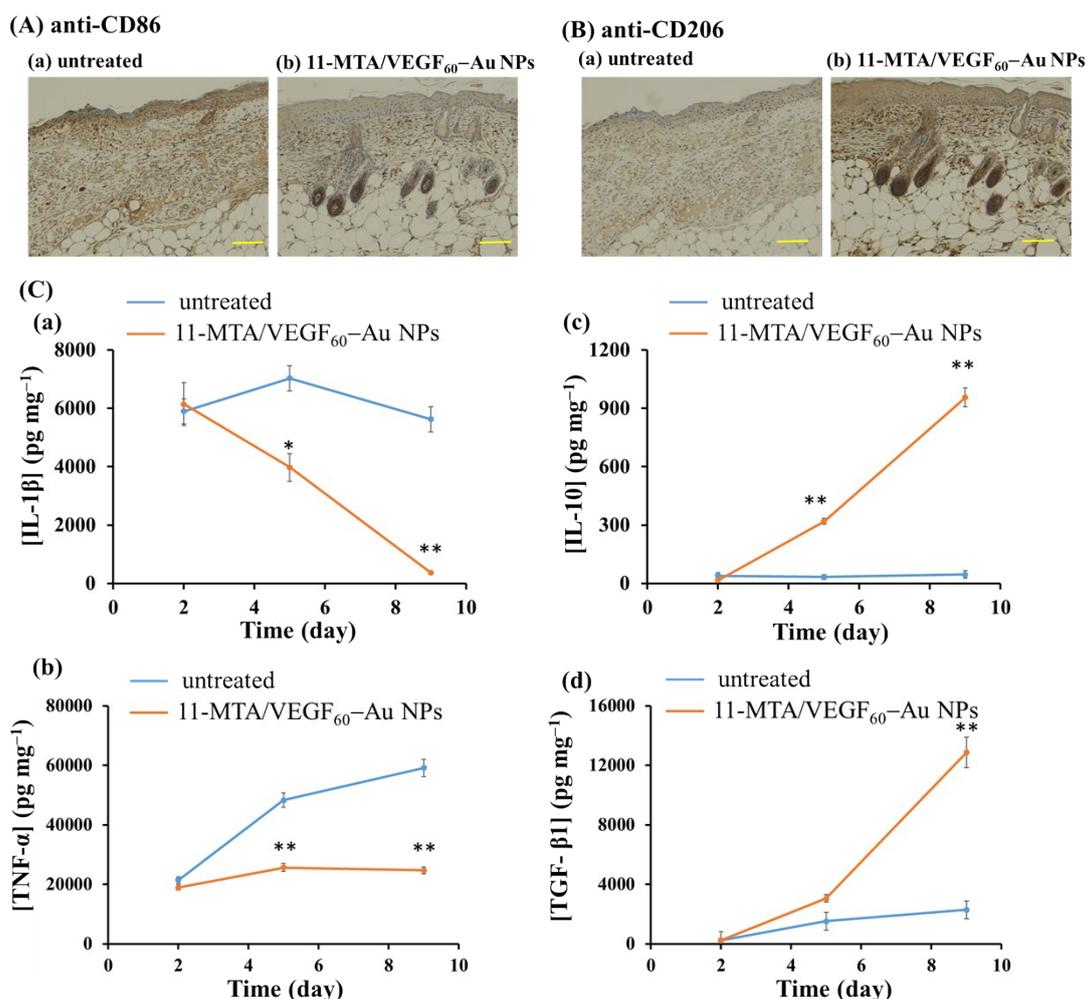


Figure S15. IHC analysis of wound tissue from (a) untreated mice and (b) mice treated with 11-MTA/VEGF₆₀-Au NPs ([Au NPs] = 5.0 nM, [11-MTA] = 8.5 μM) on the 5th day using (A) anti-CD86 and (B) anti-CD206 staining. (C) Levels of cytokines in wound homogenates measured using ELISA, including proinflammatory cytokines (a) IL-1β, and (b) TNF-α and healing-associated cytokines (c) IL-10, and (d) TGF-β1. Scale bar in (A) and (B): 100 μm. Error bars in (C) represent the standard deviations of four replicate experiments. Asterisks indicate statistically significant differences between treated groups and untreated group on day 5 and 9 (* $p < 0.005$, ** $p < 0.001$).