Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Liquid Metal Dressing for Anti-inflammation and Anti-infections to Treat Diabetic Wound

Shunchao Bi, Jie Qi, Wei Zhang, Xingyu Jiang**

S Bi, J Qi, Prof. W Zhang
CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, CAS Center for Excellence in Nanoscience, National Center for NanoScience and Technology, No. 11 Zhongguancun Beiyitiao, Beijing 100190, P. R. China
E-mail: zhangw@nanoctr.cn
S Bi, J Qi
University of Chinese Academy of Sciences, No. 19A Yuquan Road, 100049 Beijing, P. R. China
Prof. X. Jiang
Guangdong Provincial Key Laboratory of Advanced Biomaterials Department of Biomedical Engineering, Southern University of Science and Technology, No. 1088, Xueyuan Rd., Xili, Nanshan District, Shenzhen, Guangdong 518055, P. R. China.
E-mail: jiang@sustech.edu.cn

Experimental Section/Methods

Materials

Ampicillin sodium, sulbactam sodium, sulbactam/ampicillin sodium, Pt nanoparticles, methylene blue, WO_3, and streptozocin are purchased from Aladdin, China. Liquid metal (EGaIn) is purchased from Beijing Hawk, China. Lipopolysaccharide is purchased from Solarbio, China. Antibody CD86, CD 206 and F4/80, CD31 are purchased from Biolegend, USA. DPPH antioxidant kit, 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) probes, 2-[6-(4′-hydroxy) phenoxy-3Hxanthen-3-on-9-yl] benzoate (HPF) probes, dihydroethidium (DHE) probes and CCK-8 kit are purchased from Beyotime Biotechnology, China. Bacterial viability kit is purchased from Invitrogen, USA. All cell culture reagents are purchased from Gibco. The deionized water is purified by the Milli-Q (Millipore, USA). The others reagents are purchased from Sinopharm Chemical Reagent Co., Ltd., China. All reagents are used without further purification.

Fabrication of liquid metal-antibiotic particles (LA)

The antibiotic-modified liquid metal particles are prepared with an ultrasonic method. Add 50 mg of liquid metal, 1.8 mL of deionized water and 200 μl sulbactam/ampicillin sodium solution (5 mg ml^{-1}) into a 5 mL centrifuge tube, and sonicated at 20% amplitude for 2 minutes (with 5 seconds intervals) (Scientz-IID, Scientz, China), followed by 3 times of centrifugation and washing with deionized water to obtain LA. For pure liquid metal particles (LM), the preparation process is the same as above, only 2 ml deionized water needs to be added instead of the antibiotic solution.

Characterization of LA

The Scanning Electron Microscope (SEM) images of LA were taken at 10kV using SU8220 (HITACHI, Japan). The Transmission Electron Microscope (TEM) images and X-ray Energy Dispersive Spectroscopy (EDS) were obtained using Talos F200 G2 TEM (Thermal Fisher, USA) with an accelerating voltage of 200kV. Ultraviolet (UV)-visible spectroscopy is acquired by UV2600 (Shimadzu, Japan). The X-ray photoelectron spectroscopy of LA are tested by EscaLab Xi^+ (ThermalFisher, USA). The DLS and ZETA potential of LA are analyzed by Zetasizer Nano ZS (Malvern, UK). Thermogravimetric analysis of LA are tested by TGA 55 (Discovery, USA) under N_2 atmosphere.

We also use TEM to observe the morphology change under different pH. Firstly, we preserve LA in different pH PBS buffers (pH=7.4 and 8.5). After 24 hours, we drop LA on the support film to observe the morphology.

Oxygen consumption of LA

The oxygen concentration of LA solution is acquired by a Dissolved oxygen analyzer (PreSens, Germany). Dilute the LA stock solution to 5, 10, 20,50 and 100 mg ml^{-1} with deionized water and measure the oxygen concentration of the solution.

H_2 generation capacity and antioxidant performance of LA

We measured the H_2 yield of LA by redox titration. Specifically, the LA solution was titrated with a mixture of methylene blue (MB) and Pt nanoparticles (MB-PtNPs solution), and H_2 caused the MB to fade under the catalytic effect of Pt. When the titration was to the point where the solution was no longer blue, the endpoint was reached. MB can react with equimolar H_2 according to the following equation:

$$MB(blue) + 2H^+ + 2e^- \xrightarrow{Pt} leucoMB\ (colorless)$$

Preparing MB-PtNPs solution: Add 0.15 g methylene blue and 0.4 g 2 % PtNPs aqueous solution into 49.45 g anhydrous ethanol to obtain 0.3 wt% MB solution. Different concentrations of LA solution (2.5, 5, 10, 20, 50, 100 mg mL^{-1}) are titrated by MB-Pt solution to measure the H_2 production.

We use WO_3 colorimetric reaction and DPPH kit to conduct a double antioxidant analysis, which indirectly proves the generation of H_2. For WO_3 colorimetric reaction, 100 mg ml^{-1} LA aqueous solution and 100 mg ml^{-1} LA ethanol solution are prepared, followed by adding 5 mg WO_3 powder. The control group is deionized water mixed with 5 mg WO_3 powder. After reacting for 24 hours, we take pictures of each solution. The reaction equation of H_2 and WO_3 is as follows:

$$WO_3 + 3H_2 \rightarrow W\downarrow + 3H_2O$$

Cell culture

HUVECs are cultured in DEME medium containing 10% fetal bovine serum (FBS) and RAW 264.7 are cultured in RPMI 1640 medium containing 10% FBS. All two lines are cultured under 5% CO_2 and 37 °C in the incubator.

Antioxidant capacity of LA in vitro

The antioxidant ability of LA is measured by testing its ROS scavenge performance. We use two ROS fluorescent probes, DCFH-DA and HPF, to visualize the ROS intensity of cells. We seed 10^4 second-generation HUVECs and RAW 264.7 cells into the lower chamber of 24-well transwell, respectively. We add 100 μM H_2O_2 into wells to stimulate cells for 1 hour. The control group is incubated with basal DMEM or RPMI 1640 medium. Then, we add LA or PBS into the upper chamber of H_2O_2 groups and control groups. After incubation for 24 hours, we stain all groups of cells with DCFH-DA (1 μM) and HPF (1 μM) for 30 mins in dark,

respectively. The results are observed by a confocal laser scanning microscope (SP8, Leica, Germany) and quantified the fluorescence intensity by ImageJ.

Anti-inflammation capacity of LA in vitro

To evaluate the inflammation regulation ability of H_2 generated from LA, we add lipopolysaccharide (LPS) as an exogenous antigen to stimulate immune cells RAW 264.7 cells and activate its polarization. We seed 10^4 second-generation RAW 264.7 cells in the lower chamber of 24-well transwell. We divide cells into two 2 main groups, the LPS group which are stimulated by 100 μm LPS and the control group which is cultured by FBS-free RPMI 1640 medium. After 4 hours, we add LA and PBS into the upper chamber of the LPS contain/free well, respectively. 24 hours later, we resin all RAW 264.7 cells with PBS and block cells with 5% BSA solution for 2 hours. Next, we stained all raw 264.7 cells by antibody F4/80 and CD206 at 4 ℃ for 24 hours. Then all cells are washed with PBS and stained by DAPI for 10 min. We use the confocal laser scanning microscope and ImageJ to observe and quantify the results.

We also performed ELISA assays on all the above RAW 264.7 cells to quantify the inflammatory cytokines IL-6 and IL-10. According to the instruction of IL-6 and IL-10 ELISA kit, we test the ELISA assay of IL-6, IL-10 and get the corresponding ELISA data by a microplate reader.

Cell viability test of LA

We seeded HUVEC and RAW 264.7 cells on 96-well plates and culture them with the corresponding medium for 3 days. Next, we replaced the fresh culture medium and added LA in the well plate, incubated for 24 hours, and then stained with CCK-8 reagent according to the instructions for 4 hours, and used the microplate reader to confirm the absorbance at 650 nm. We also use live/dead kit to stain HUVEC and RAW 264.7 cells above (about 30 mins). After they were washed with PBS, we observed cells with a confocal microscope.

The releasing of antibiotics from LA

The release of antibiotics is measured by UV-vis spectrophotometer. We use 1000 Da dialysis bag to preserve LA water solution and dialysis LA in PBS (pH=8.0 or pH=7.4). We collect PBS solution several times in different time points and test the UV spectra of the solution. The releasing of antibiotic from LA are calculated through TGA results and concentration-absorbance standard curve of antibiotics.

Antibacterial performance of LA

Clinical isolates of multidrug-resistant *P. aeruginosa* and *A. baumannii* were generously donated by Shenzhen University General Hospital. We used Luria-Bertani medium to culture

these two bacteria for subsequent antibacterial experiments. Bacteria are cultured at 37 ℃ for 4 hours on a shaker incubator before use.

We diluted the bacteria to 50 mg ml^{-1} LA PBS solution. The bacterial population was 10^{4-5}. After 4 hours, the bacterial solution was spread on LB agar plates and incubated. 24 hours later, a ChemiDoc MP Imaging System (Bio-Rad, USA) was used to take pictures. The colonies are counted with ImageJ. For the control group, we directly diluted the bacteria in PBS and then cultured and counted the plates according to the same steps.

We also use bacteria live/dead kit to stain the LA-treated bacteria to test its permeability change. Briefly, after 4 hours of incubation, LA-treated bacteria and control group bacteria are washed and collected. Next, we used Syto-9 (2 μM) and PI (1μM) to stain bacteria in the dark for 20 min. The results are observed by the laser confocal microscope.

LA therapy for diabetic mouse wounds

Balb/C mice (male, 4 weeks) purchased from Vitalriver. Co., China. are fed with high fat and sugar food. All mice have free access to water and food without any limitations. All operations of animal experiments are approved by the Institutional Animal Care and Use Committee, Shenzhen Advanced Animal Study Service Centre.

The preparation of diabetic mice is as follows: Mice with diabetes are induced by injection of streptozotocin (STZ), which can lead to pancreatic island injury. We use citrate buffer (pH=4.5, 0.1 M) to prepare a 10 mg ml^{-1} STZ solution, which must be freshly prepared before use and preserved in an ice bath and dark environment. All mice are divided into 4 groups LA (diabetic mice treated with LA), AS (diabetic mice treated with ampicillin/sulbactam), DL (diabetic mice treated with liquid metal particles), DC (diabetic mice set as control). We also set a group of mice who do not suffer from diabetes as an acute wound control group. For all diabetic groups, we injected STZ at a dose of 40 mg kg^{-1} into each mouse for 4 days, followed by a high-sugar and high-fat diet for 2 weeks. After the establishment of the diabetic animal model, we create an 8 mm circular wound on each mouse (including non-diabetic mice), followed by inoculating bacteria on the wound site. After 30 mins, we drop 100 μL of corresponding drugs (The concentration of each drug is: LA 50 mg ml^{-1}, liquid metal particles 50 mg ml^{-1}, ampicillin/sulbactam 1 mg ml^{-1}. PBS is dropped directly without further treatment.) into the wound of each group of mice. We take pictures of each wound of diabetic group mice at days 0, 3, 6 and 12. The area of wounds is calculated by ImageJ. We also collect the tissue of wounds of diabetic mice for bacterial counting and H&E stain. The wound tissue soaked in 1ml PBS was ground with a tissue grinder (Solarbio, China) for 10 minutes. The tissue solution was spread on LB agar plates for bacterial counting.

Immunofluorescence staining and ELISA assay of wound tissue

We collect all wound tissue and embed them in paraffin wax for cryosection. Then, the 8 μm thick sections are deparaffinized by cold acetone, washed with PBS and blocked with 5% BSA for 2 hours. We use antibody CD31, CD206, CD86, and F4/80 to stain the slice at 4 ℃ for 24 hours. Then, we rinsed the slice with PBS three times. Next, we use DAPI and DHE to stain nuclei and ROS for 15 min. We use a laser confocal microscope to observe the results. The quantitative analysis is carried out by ImageJ.

The collected wound tissues of mice are ground by a tissue grinder for 10 min. After configuration at 10000 rpm, we collected the supernatant to carry out the ELISA test. We carried out the subsequent operation according to the instruction of IL-6, IL-8, and IL-10 ELISA kits. Each group of tests is repeated 6 times.

Statistics and Software.

The graphs are generated by originPro 2017. All data are analyzed by originPro 2017 to calculate Mean and Standard Deviation (S.D.). “n” means the repeats of biological independent experiments. The significance level of each group of data is analyzed by Student’s T-test. p values< 0.05 are considered statistically significant.

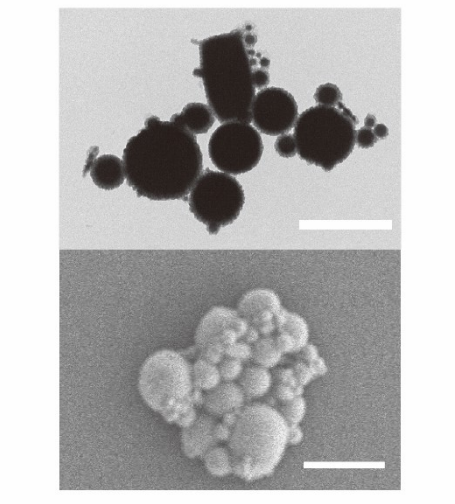

Fig. S1 Visuallization of LA. (top is TEM image and bottom is SEM image) Scale bar=1 μm.

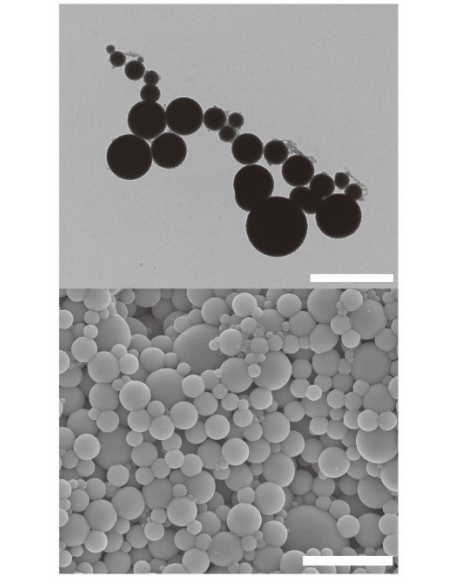

Fig. S2 Visuallization of pure liquid metal particles. (top is TEM image and bottom is SEM image) Scale bar=1 μm.

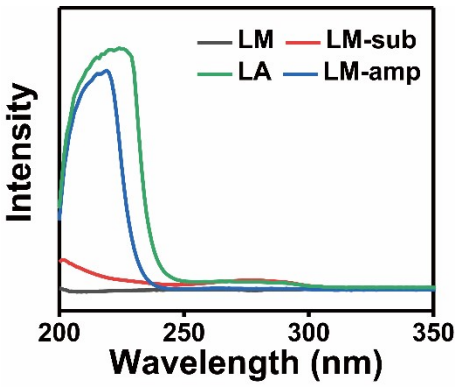

Fig. S3 UV-vis spectra of LM, LM modified with sulbactam, LM modified with ampicillin and LA.

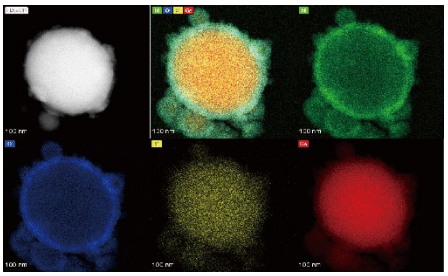

Fig. S4 HAADF-STEM mapping of LA with N, O, S, Ga elements. Scale bar=100 nm.

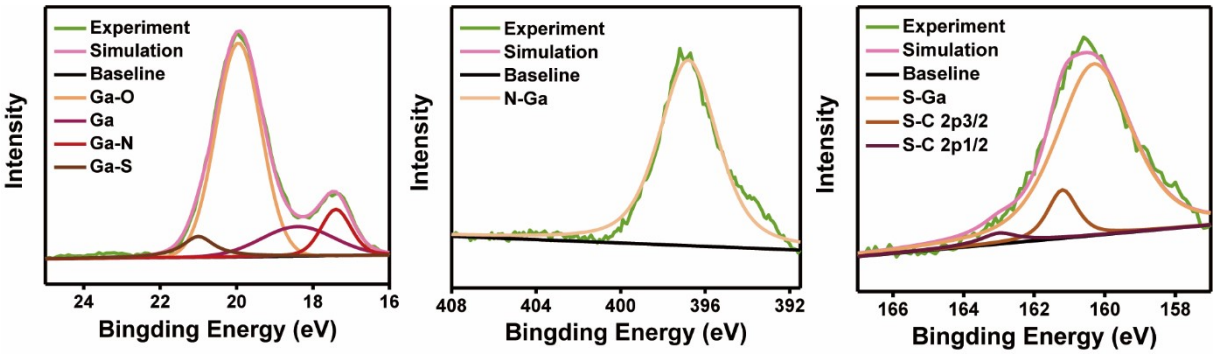

Fig. S5 XPS spectra of LA in Ga3d, N1s and S2p region.

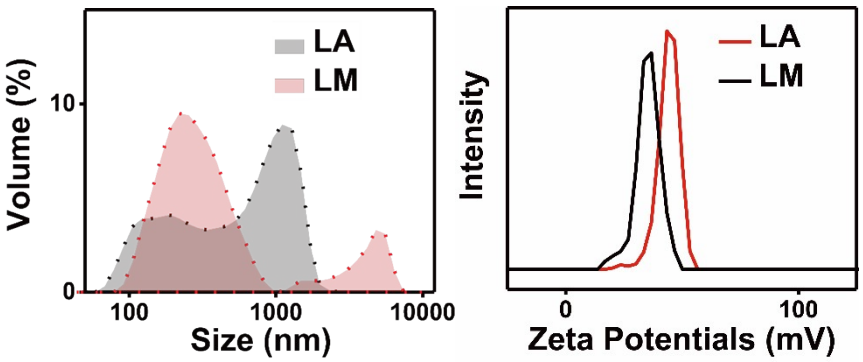

Fig. S6 Size distruibution and zeta potential of LA and LM.

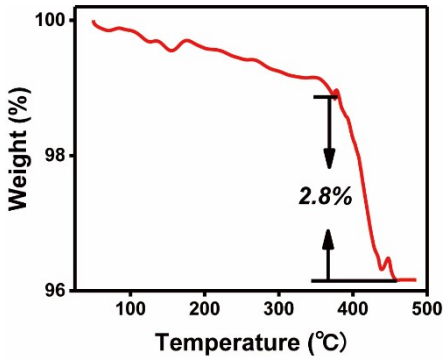

Fig. S7 Thermogravimetric curve of LA.

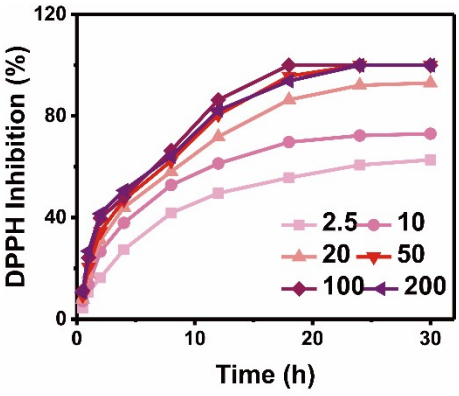

Fig. S8 The antioxidant performance of LA tested by DPPH antioxidant kit.

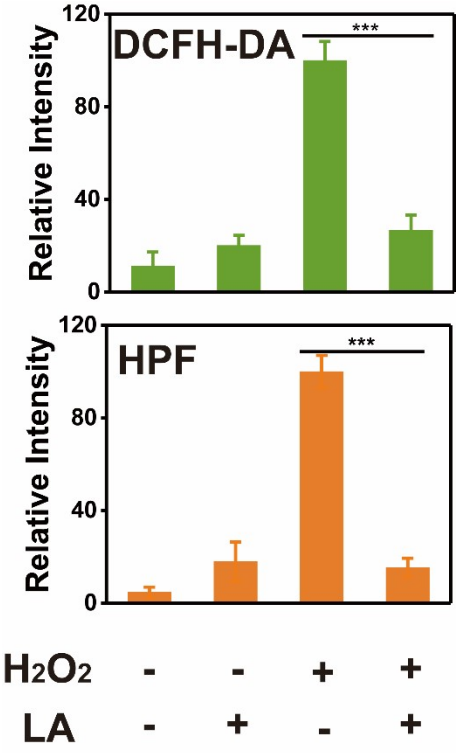

Fig. S9 Quantification of the fluorescence intensity of PBS, LA, H_2O_2, LA+ H_2O_2-treated HUVECs stained with ROS probes. (n=6)

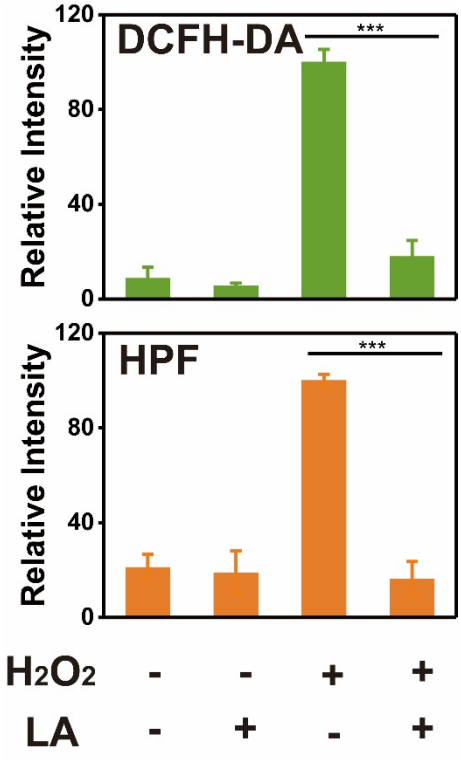

Fig. S10 Quantification of the fluorescence intensity of PBS, LA, H_2O_2, LA+ H_2O_2-treated HUVECs stained with ROS probes. (n=6)

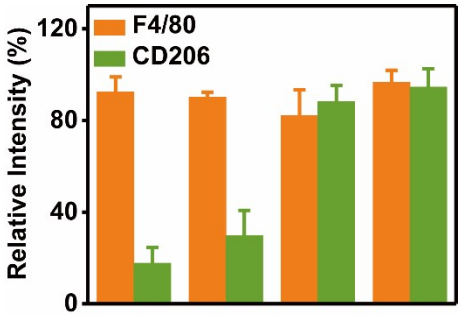

Fig. S11 Quantification of fluorescence intensity of the CD206 and F4/80 probes in different groups of RAW 264.7 cells.

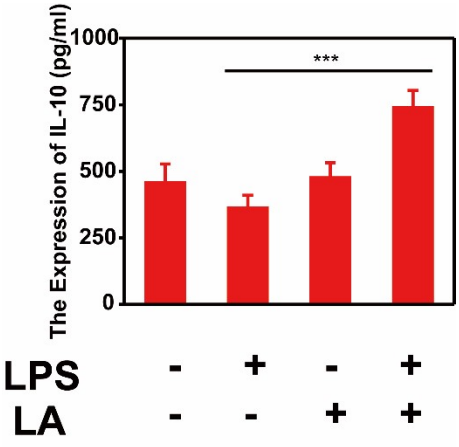

Fig. S12 Expression of IL-10 of RAW 264.7 cells treated with PBS, LPS, LA, LPS+LA. (n=6)

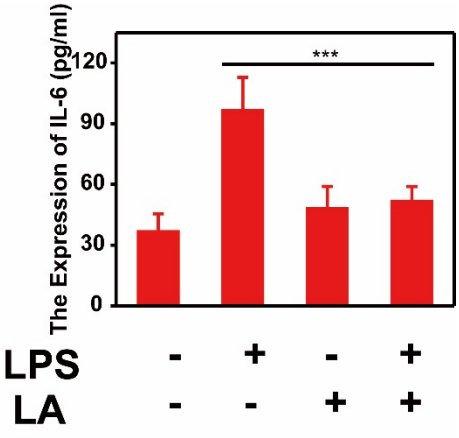

Fig. S13 Expression of IL-6 of RAW 264.7 cells treated with PBS, LPS, LA, LPS+LA.

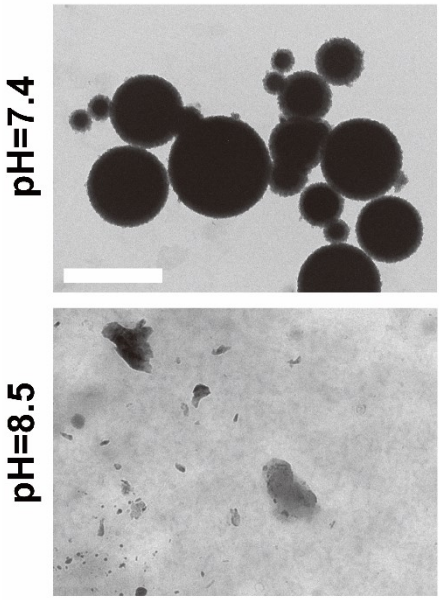

Fig. S14 The morphology of LA preserved at different pH for 24 hours. Sacle bar=1 μm.

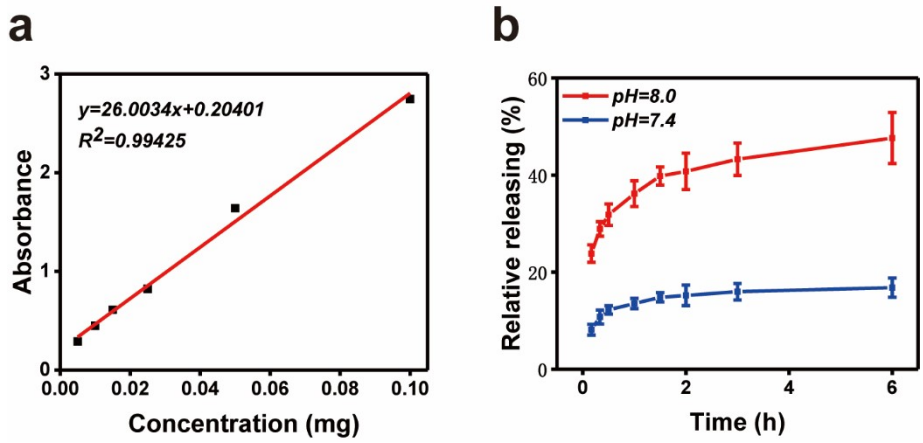

Fig. S15 (a)Standard curve of ampicillin sulbactam sodium, (b)release curve ampicillin sulbactam sodium on LA at different pH. n=6

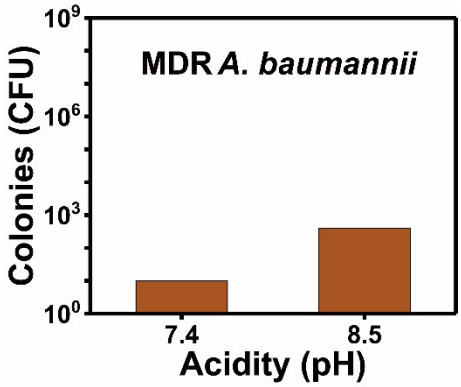

Fig. S16 The bacterial counts of 50 mg ml^{-1} LA treated MDR *A. baumannii.* at different pH conditions.

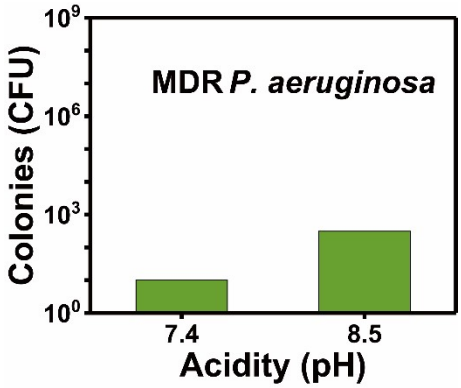

Fig. S17 The bacterial counts of 50 mg ml^{-1} LA treated MDR *P. aeruginosa.* at different pH conditions.

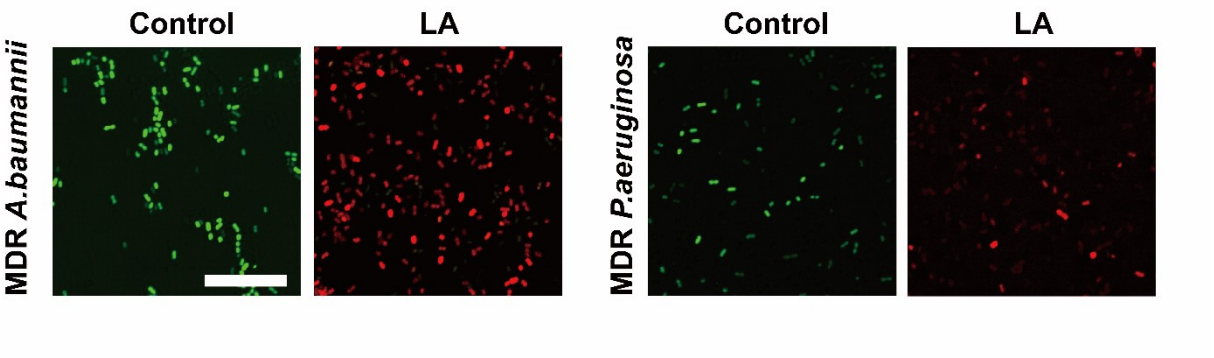

Figure S18. Live-dead staining of MDR *A. baumannii* and MDR *P. aeruginosa* treated with/without 50 mg ml^{-1} LA.

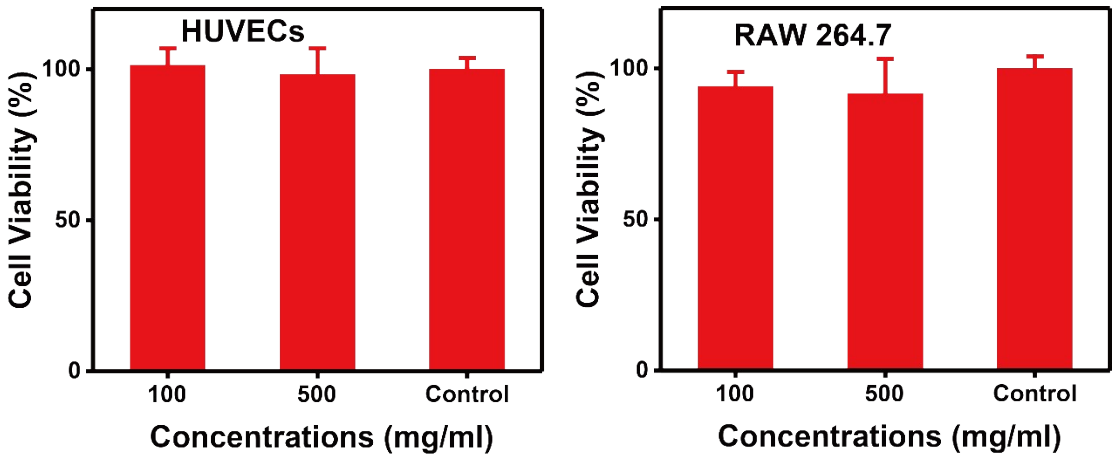

Fig. S19 Cell viability of HUVECs and RAW 264.7 cells exposed to different concentrations of LA.

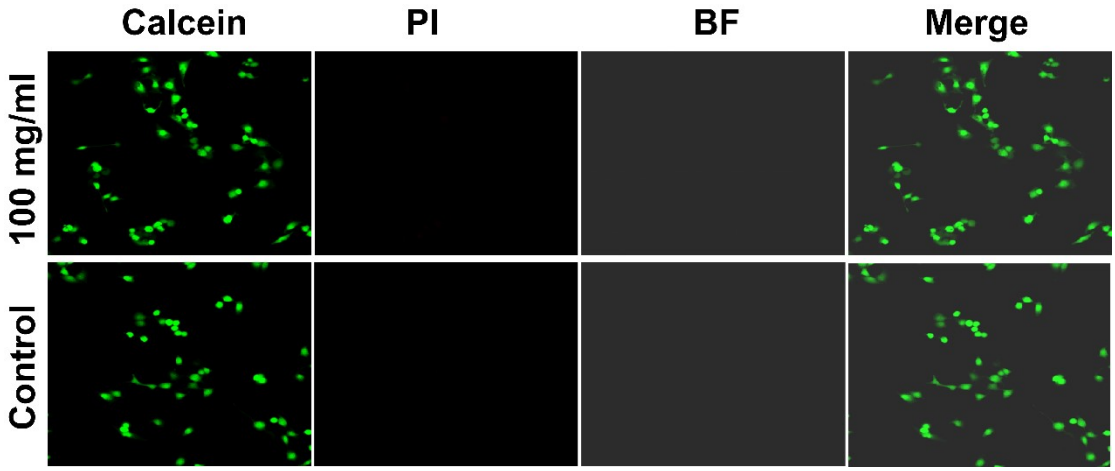

Fig. S20 Live-dead staining of HUVECs exposed to 100 mg ml^{-1} LA and PBS (Control).

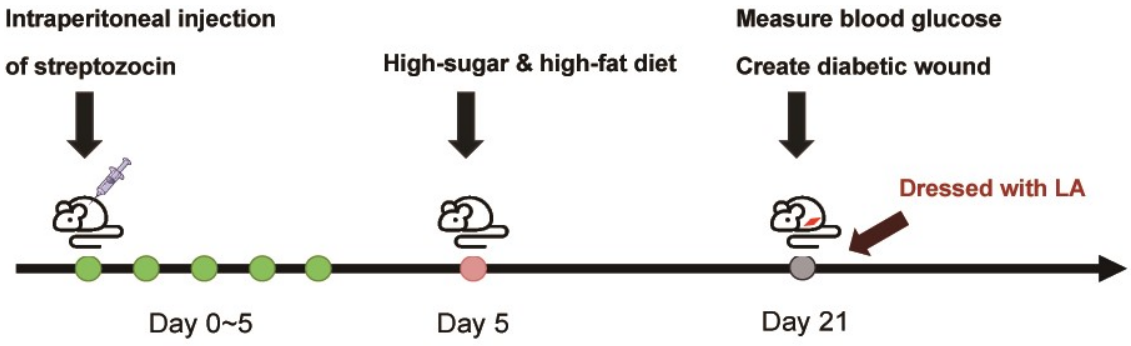

Fig. S21 The construction of diabetic mice with infected-chronic wounds and treatment plan.

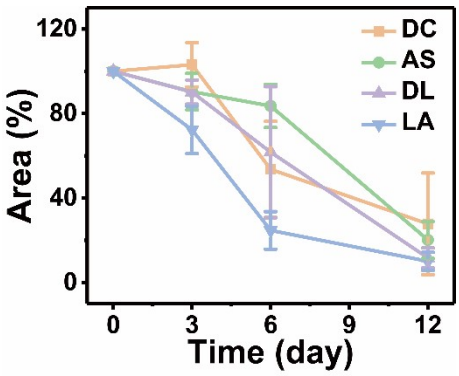

Fig. S22 the size of diabetic wounds in different groups mice.

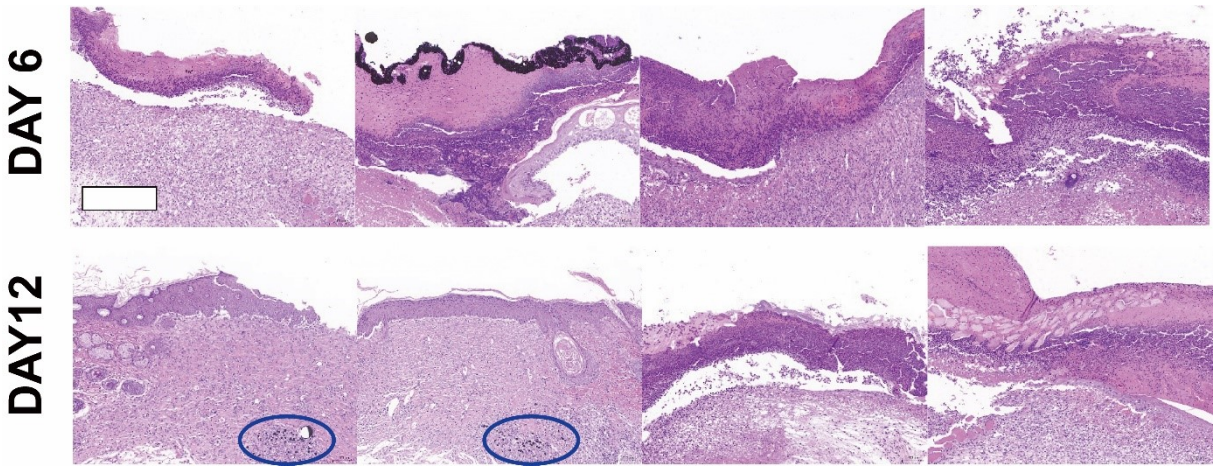

Fig. S23 The H&E staining of the tissue at wound site.

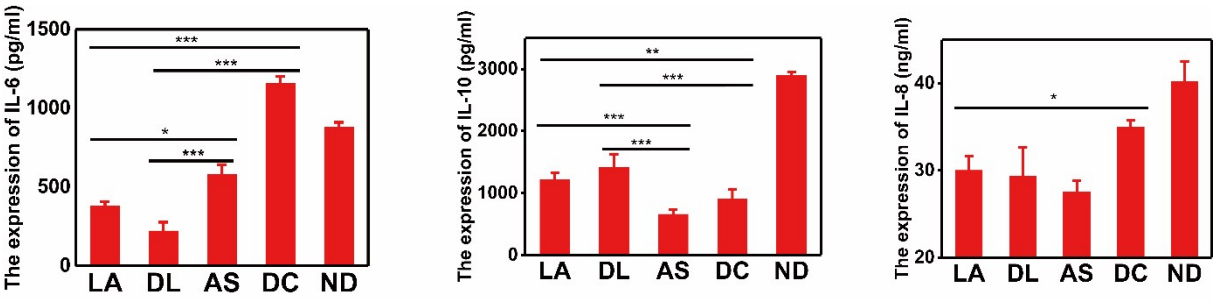

Fig. S24 The expression of IL-6 (C), IL-10 (D), and IL-8 (E) in vivo for different groups of mice. (n=6)

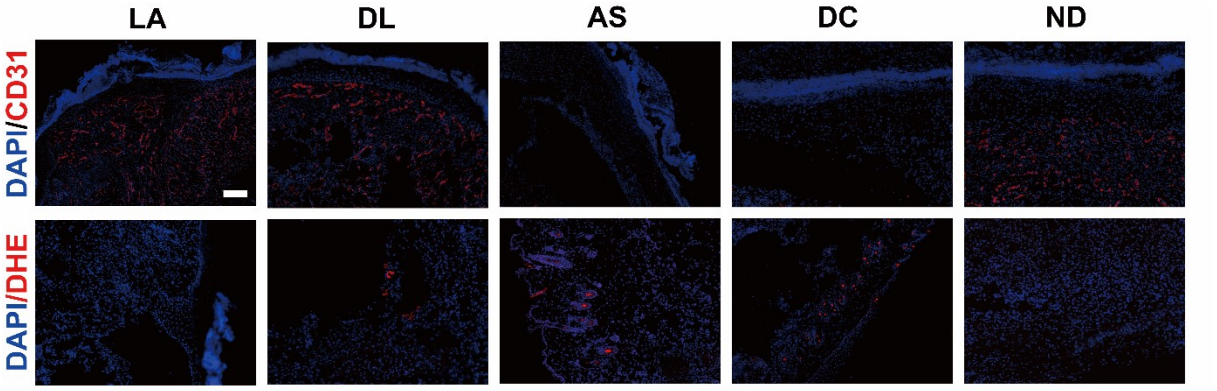

Fig. S25 The angiogenesis (labeled by CD31) and oxidant levels (labeled by DHE) in wounds for different groups of mice. Scale bar=100 μm.

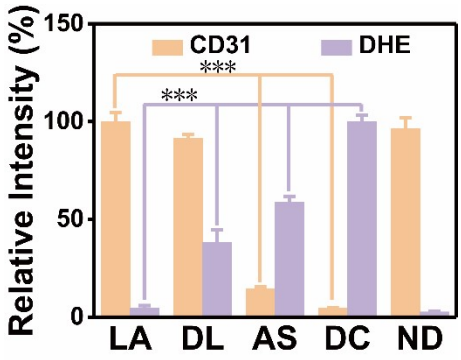

Fig. S26 Quantification of fluorescence intensity CD31 and DHE for different groups of mice. Mean±SD, n=6, ***p<0.001