HEALTH AND MEDICINE

Ångstrom-scale silver particle–embedded carboxer gel promotes wound healing by inhibiting bacterial colonization and inflammation

Chun-Yuan Chen1,2,3, Hao Yin1,2,3, Xia Chen4, Tuan-Hui Chen1, Hao-Ming Liu1, Shan-Shan Rao1,5, Yi-Juan Tan1,2,3, Yu-Xuan Qian1, Yi-Wei Liu1,6, Xiong-Ke Hu1, Ming-Jie Luo5, Zhen-Xing Wang1,2,3, Zheng-Zhao Liu1,6,7, Jia Cao1, Ze-Hui He3, Ben Wu1, Tao Yue1, Yi-Yi Wang1, Kun Xia1, Zhong-Wei Luo1, Yang Wang2,3,8, Wei-Yi Situ2,3, Wen-En Liu4, Si-Yuan Tang2, Hui Xie1,2,3,6,7,9,10* 

Poor wound healing after diabetes or extensive burn remains a challenging problem. Recently, we presented a physical approach to fabricate ultrasmall silver particles from Ångstrom scale to nanoscale and determined the antitumor efficacy of Ångstrom-scale silver particles (AgÅPs) in the smallest size range. Here we used the medium-sized AgÅPs (65.9 ± 31.6 Å) to prepare carboxer gel incorporated with these larger AgÅPs (L-AgÅPs-gel) and demonstrated the potent broad-spectrum antibacterial activity of L-AgÅPs-gel without obvious toxicity on wound healing–related cells. Induction of reactive oxygen species contributed to L-AgÅPs-gel–induced bacterial death. Topical application of L-AgÅPs-gel to mouse skin triggered much stronger effects than the commercial silver nanoparticles (AgNPs)–gel to prevent bacterial colonization, reduce inflammation, and accelerate diabetic and burn wound healing. L-AgÅPs were distributed locally in skin without inducing systemic toxicities. This study suggests that L-AgÅPs-gel represents an effective and safe antibacterial and anti-inflammatory material for wound therapy.

INTRODUCTION

The healthy skin serves as an effective barrier to protect the internal organs against pathogen invasion, ultraviolet radiation, and other external threats (1). Poor wound healing after extensive burn, severe traumatic injury, surgery, or chronic diseases such as diabetes remains a challenging clinical problem that leads to low quality of life, substantial healthy cost, and high mortality (2, 3). Open, moist, and exuding wound provides a perfect environment for bacterial colonization, and bacterial infection is recognized as a major factor that delays the healing process of wound (4, 5). The host’s inflammatory response is a local protective reaction in response to injury and essential for infection control in the wound sites (3). However, the presence of bacteria and their endotoxins induces excessive production of inflammatory mediators and renders the wound into a persistent inflammatory phase, thus disturbing the normal repair process of wound (6). Hence, strategies to prevent bacterial colonization/infection and inhibit inflammation will facilitate rapid wound healing.

The notable increase in bacterial resistance to current available antibiotics has led to a need to develop alternative antibacterial agents (7). Nanoparticles (NPs) have unique physicochemical properties (ultrasmall size, high surface reactivity, large surface area–to–volume ratio, etc.) and have emerged as alternative tools to control bacterial infection and overcome antibiotic resistance (7). Silver NPs (AgNPs) exhibit efficient antimicrobial, anti-inflammatory, and anticancer activities and have been widely used in food and medical industry (8, 9). Dressings and preparations containing AgNPs have been used extensively to reduce the risk of wound infection and to kill bacteria in the infected wounds, thereby accelerating the wound healing process (8, 9). Smaller AgNPs have a superior ability to bind to the surface of bacteria and then penetrate bacterial membranes to kill bacteria (10, 11). Thus, it will be optimal to obtain AgNPs with the smallest possible particle size for the reduction of bioburden and the promotion of wound healing.

Different routes have been adopted to prepare AgNPs, including chemical, physical, and biological synthesis methods (12). Recently, we used a highly efficient physical approach to fabricate silver particles reaching the Ångstrom (1/10 of a nanometer) scale using a self-designed automatic evaporation-condensation system, which did not involve the use of any toxic chemicals and had no potential risks of biohazard (13). We demonstrated the antitumor efficiency of these Ångstrom-scale silver particles (AgÅPs) and the low toxicity of AgÅPs toward healthy cells and tissues (13, 14). During the preparation of AgÅPs, we also obtained the larger silver particles with diameters predominately at the nanoscale through the hierarchical particle collection system. These larger silver particles were graded by two different particle collectors with diameters generally <20 or >20 nm. Since a certain proportion of AgÅPs still existed in silver particles below 20 nm, but not in those >20 nm, we called the particle population less than 20 nm “large AgÅPs (L-AgÅPs),” but not “AgNPs.”

Here, we prepared and characterized the L-AgÅP–embedded carboxer gel (L-AgÅPs-gel) and evaluated their antibacterial efficacy against both Gram-positive and Gram-negative bacteria. As the
induction of reactive oxygen species (ROS) production has been shown to contribute to the cytotoxicity of AgNPs against bacteria (15, 16), we next explored whether ROS was also involved in L-AgÅPs-gel–induced bacterial death. In vivo, we compared the effects of L-AgÅPs-gel with the commercial AgNP-embedded gel (AgNPs-gel) on wound closure, bacterial colonization, and inflammatory responses in streptozotocin-induced diabetic mice and in mouse models of acute burn injury. Moreover, we assessed the toxicities and tissue distribution of L-AgÅPs after topical application of L-AgÅPs-gel. Our study aimed to demonstrate whether L-AgÅPs-gel could be used as an efficient and safe approach for the acceleration of chronic and acute wound healing and to determine the underlying mechanism.

RESULTS
Characterization of L-AgÅPs and L-AgÅPs-gel
Figure 1A shows the x-ray diffraction (XRD) pattern of the prepared L-AgÅPs. The diffraction peaks appeared at \( \theta = 38.1^\circ, 44.3^\circ, \) and \( 64.4^\circ \), which are indexed to the (111), (200), and (220) crystallographic planes of face-centered cubic silver crystals, respectively (Joint Committee on Powder Diffraction Standards, file No. 04-0783), suggesting that L-AgÅPs are well crystallized and consist of a pure silver phase. Ultraviolet–visible–near-infrared (UV-vis-NIR) spectrum revealed that L-AgÅPs in deionized water had a localized surface plasmon resonance peak centered around 322 nm (Fig. 1B), which displayed a blue shift compared with the UV-vis-NIR spectra of those chemically synthesized AgNPs (>400 nm) (17).

Figure 1C shows the digital photos of the blank carbomer gel (blank-gel) and L-AgÅPs-gel, both of which had a homogeneous, smooth, transparent, and colorless appearance. Scanning electron microscope (SEM) images showed a smooth surface of blank-gel. After doping with L-AgÅPs, the gel surface became rough and granular-like structures were uniformly distributed in carbomer gel (Fig. 1D), suggesting the incorporation of L-AgÅPs. The chemical composition of blank-gel and L-AgÅPs-gel was measured by energy-dispersive x-ray spectrometry (EDS). The elemental silver was only detected in
L-AgÅPs-gel but not in blank-gel (Fig. 1E), which further confirmed the successful embedding of L-AgÅPs into carbomer gel. Transmission electron microscope (TEM) image revealed that L-AgÅPs in carbomer gel had a sphere shape with diameters ranging from 7.2 Å to 16.8 nm (65.9 ± 31.6 Å; Fig. 1, F and G).

The blank-gel and L-AgÅPs-gel were dissolved in deionized water and subjected to dynamic light scattering (DLS) analysis. The results showed that the zeta potential values of blank-gel and L-AgÅPs-gel ranged from −49.24 to −70.69 mV and −62.01 to −78.83 mV, respectively (Fig. 1H), indicating that L-AgÅPs-gel is much better dispersed and more stable than blank-gel in aqueous solution.

Fourier-transform infrared (FTIR) spectra revealed that blank-gel and L-AgÅPs-gel displayed the same peak shape, position (3357 and 1639 cm⁻¹), and intensity of absorbance bands attributed to the NH stretching vibrations and the vibrations of carbonyl bonds (C=O) (18), respectively (Fig. 1I), suggesting that the incorporation of L-AgÅPs does not cause any structural changes of carbomer gel.

Thermogravimetric analysis was conducted to estimate the amount of L-AgÅPs doped into carbomer gel. As shown in Fig. 1J, three stages of weight loss were observed in both blank-gel and L-AgÅPs-gel. The first weight loss (for blank-gel, 30° to 188°C; for L-AgÅPs-gel, 30° to 190°C) about 9% was possibly due to water evaporation. The second weight loss (about 46.5% for blank-gel, 188° to 280°C; about 22.5% for L-AgÅPs-gel, 190° to 351°C) might be due to the depolymerization of carbomer gels. The third weight loss (about 41.7% for blank-gel, 280° to 800°C; about 50.5% for L-AgÅPs-gel, 351° to 800°C) was probably attributed to the further degradation of polymeric chains of carbomer gels. The results revealed that L-AgÅPs incorporated in carbomer gel were about 15.2% of total L-AgÅPs-gel weight (0.152 g g⁻¹) and that doping with L-AgÅPs enhanced the thermal stability of carbomer gel.

Rheological properties of L-AgÅPs-gel were then assessed. As indicated by the amplitude sweep test at a fixed frequency of 1 Hz, the linear viscoelastic region of L-AgÅPs-gel was determined to be below 3.033% strain (Fig. 1K). In the deformation range <38.1% strain, the higher elastic moduli (G’) than viscous moduli (G″) revealed an elastic solid-like viscoelastic behavior of L-AgÅPs-gel (Fig. 1K). Frequency sweep test at a constant strain of 1.0% also showed that L-AgÅPs-gel displayed a dominant elastic character (G’ > G″) with a weak frequency–dependent feature in the range of 0.1 to 5 Hz (Fig. 1L). The complex viscosity (|η*|) continued to decline with the increase of frequency (Fig. 1L), indicating a shear thinning behavior of L-AgÅPs-gel.

**Antibacterial efficacy of L-AgÅPs-gel in vitro**

*Staphylococcus aureus* (S. aureus; Gram-positive) and *Pseudomonas aeruginosa* (P. aeruginosa; Gram-negative) are two of the most widely implicated bacteria in wound infection (19). Thus, methicillin-sensitive *S. aureus* American Type Culture Collection (ATCC) 25923, methicillin-resistant *S. aureus* ATCC29213, and *P. aeruginosa* ATCC27853 were chosen to evaluate the antibacterial efficacy of L-AgÅPs-gel. Filter paper disks infiltrated with blank-gel, L-AgÅPs-gel, or the commercially available AgNPs-gel were prepared, and agar disk diffusion test was conducted to test the susceptibility of the above bacteria against these materials. As shown in Fig. 2 (A and B), the inhibition haloes were clearly observed around the paper disks infiltrated with L-AgÅPs-gel or AgNPs-gel, but the inhibition zone diameters of L-AgÅPs-gel (for *S. aureus* ATCC25923, 10.37 ± 0.71 mm; for *S. aureus* ATCC29213, 9.40 ± 0.85 mm; and for *P. aeruginosa* ATCC27853, 12.28 ± 0.85 mm) were much larger than that of AgNPs-gel (for *S. aureus* ATCC25923, 7.40 ± 0.50 mm; for *S. aureus* ATCC29213, 7.63 ± 0.45 mm; and for *P. aeruginosa* ATCC27853, 7.80 ± 0.44 mm), suggesting a much stronger antibacterial ability of L-AgÅPs-gel than AgNPs-gel. The blank-gel, L-AgÅPs-gel, and AgNPs-gel were subjected to serial dilutions using Mueller-Hinton (MH) broth and added to the culture media of the tested bacteria to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The much lower MIC and MBC values further confirmed the much more potent antibacterial activity of L-AgÅPs-gel compared with AgNPs-gel (Table 1).

L-AgÅPs-gel and AgNPs-gel were diluted to the concentrations of MIC or MBC values of L-AgÅPs-gel against the above-described bacteria and then incubated with these bacteria. Bacterial colony counting assay on agar plates showed the fewest bacterial colonies formed by the L-AgÅPs-gel–treated bacteria (Fig. 2, C and D). AgNPs-gel also decreased the number of the survived bacteria that were able to grow and form colonies on agar plates, but the effect was much lower than that of L-AgÅPs-gel (Fig. 2, C and D). The live/dead cell assay by calcein-AM/propidium iodide (PI) staining revealed the vast majority of calcein-AM–negative and PI-positive (calcein-AM PI⁺) dead bacteria in the L-AgÅPs-gel group, but not in the AgNPs-gel and especially in blank-gel groups, indicating the potent bacterial killing effect of L-AgÅPs-gel (Fig. 2, E and F). Alamar blue is a cell viability indicator based metabolic activity and has been widely used for evaluating the susceptibility of pathogens to antimicrobial compounds (20). The much lower relative fluorescence intensity of the reduced alamar blue (indicating live cells) in the L-AgÅPs-gel group compared with the AgNPs-gel group further confirmed the stronger inhibitory effect of L-AgÅPs-gel on bacterial viability (Fig. 2G). Biofilm is a community of bacteria embedded in self-secreted extracellular matrix. Crystal violet staining assay was conducted to compare the effects of L-AgÅPs-gel, AgNPs-gel, and blank-gel on biofilm formation and survival based on the optical density (OD) values at 570 nm. The significantly decreased OD values in the L-AgÅPs-gel and AgNPs-gel groups indicated that treatment with L-AgÅPs-gel or AgNPs-gel at the early stage markedly inhibited biofilm formation by *S. aureus* ATCC25923, *S. aureus* ATCC29213, and *P. aeruginosa* ATCC27853 (Fig. 2H), and the addition of L-AgÅPs-gel or AgNPs-gel to these bacteria at the later phase significantly destructed the biofilm that had been formed (Fig. 2I). However, the inhibitory effects of L-AgÅPs-gel on biofilm formation and survival were much higher than that of AgNPs-gel (Fig. 2, H and I). TEM showed that all the blank-gel–treated bacteria had normal round- or rod-shaped morphologies with intact cell wall and membrane (Fig. 2J). After exposure to L-AgÅPs-gel, the cell wall and membrane of the bacteria were damaged, leading to the leakage of cytoplasm and the collapse of the bacteria (Fig. 2J). The bacteria exposed to AgNPs-gel also exhibited different extents of distortion of the intracellular structures, but the changes were much weaker than that of the bacteria treated with L-AgÅPs-gel (Fig. 2J). Together, these findings suggest the promising prospect of L-AgÅPs-gel as a highly potent antibacterial material superior to AgNPs-gel.

**ROS generation contributes to L-AgÅPs-gel–induced bacterial death**

We next asked whether ROS induction contributed to L-AgÅPs-gel–induced bacterial death. Staining signals for dichloro-dihydro-fluorescein diacetate (DCFH-DA; a ROS-sensitive probe) measured...
**Fig. 2. Antibacterial efficacy of L-AgÅPs-gel in vitro.** (A) Zones of inhibition surrounding the blank-gel–, L-AgÅPs-gel–, or AgNPs-gel–infused paper disks against *S. aureus* ATCC25923, *S. aureus* ATCC29213, and *P. aeruginosa* ATCC27853 on agar plates. (B) Quantification of the average diameters of inhibition zones. *n* = 3 per group. 1 − β = 1. (C) Digital photos of bacterial colonies grown on agar plates in different treatment groups. (D) Quantification of the numbers of bacterial colonies in (C). *n* = 3 per group. 1 − β = 1. CFU, colony-forming units. (E and F) Calcein-AM/PI staining images of the above bacteria receiving different treatments for 3 hours (E) and quantification of the ratios of live bacteria [calcein-AM + PI − ; (F)]. Scale bar, 10 μm. *n* = 3 per group. 1 − β = 1. (G) Cell viability analysis of bacteria in presence of blank-gel, L-AgÅPs-gel, or AgNPs-gel for 3 hours by alamar blue assay. *n* = 3 per group. 1 − β = 1. (H) Absorbance of the crystal violet–stained biofilms formed by bacteria treated with blank-gel, L-AgÅPs-gel, or AgNPs-gel for 36 hours. *n* = 3 per group. 1 − β = 1. (I) Absorbance of the crystal violet–stained survived biofilms exposed to blank-gel, L-AgÅPs-gel, or AgNPs-gel for 36 hours. *n* = 3 per group. 1 − β = 1. (J) TEM images of ultrathin sections of different bacteria receiving different treatments for 3 hours. Scale bar, 500 nm. *P* < 0.05, **P** < 0.01, and ***P** < 0.001. Photo credit: Chun-Yuan Chen and Hao Yin, Central South University.
by a fluorescence microplate reader indicated that L-AgÅPs-gel caused significant increases in the production of ROS in S. aureus ATCC25923, S. aureus ATCC29213, and P. aeruginosa ATCC27853 (Fig. 3A). When these bacteria were additionally treated with the antioxidant N-acetylcysteine (NAC), the L-AgÅPs-gel–triggered increase of DCFH-DA signals was significantly inhibited (Fig. 3A), indicating that NAC can suppress ROS overproduction induced by L-AgÅPs-gel. Cotreatment with NAC significantly rescued the L-AgÅPs-gel–induced inhibition of bacterial colony formation and reduction of the percentages of live bacteria, as revealed by bacterial colony counting assay on agar plates (Fig. 3, B and C), alamar blue assay (Fig. 3D), and calcein-AM/PI double staining (Fig. 3, E and F), indicating that the blockage of ROS production attenuates the inhibitory effects of L-AgÅPs-gel on bacterial growth and survival. However, lower levels of bacterial colony formation and ratios of live bacteria were detected in the NAC + L-AgÅPs-gel group compared to the NAC group (Fig. 3, B to F). These findings suggest that ROS mediates, but not completely, the L-AgÅPs-gel–induced inhibitory effects on bacterial growth and survival.

L-AgÅPs-gel accelerates the healing of diabetic and burn wounds

To explore the therapeutic benefits of L-AgÅPs-gel in wound healing, we firstly used a dialysis membrane to mimic the skin tissues and packaged L-AgÅPs-gel in the dialysis membrane to assess the release behavior of silver from L-AgÅPs-gel for 15 consecutive days by using inductively coupled plasma mass spectrometry (ICP-MS). As shown in Fig. 4A, a sustained release of silver was detected over a period of 15 days with a cumulative release amount up to 94.38 ± 2.01% of the total dose, indicating that silver can be slowly and stably released from L-AgÅPs-gel to outside.

Subsequently, we evaluated the effects of L-AgÅPs-gel on wound repair in streptozotocin-induced diabetic mice with full-thickness excisional wounds. The digital photos of the wounds and quantification of the closure rates at days 5, 9, and 11 after wounding revealed that the topical application of L-AgÅPs-gel markedly accelerated the closure of diabetic wounds in both female and male mice (Fig. 4, B and C, and fig. S1, A and B). The AgNPs-gel–treated mice also showed faster wound closure rates compared with the sex-matched blank-gel–treated mice, but their closure rates were much lower than that of the sex-matched L-AgÅPs-gel–treated mice (Fig. 4, B and C, and fig. S1, A and B). It required 14.50 ± 0.97 days, 11.20 ± 0.79 days, and 13.10 ± 0.88 days to fully close the diabetic wounds in the blank-gel–, L-AgÅPs-gel–, and AgNPs-gel–treated male mice, respectively (Fig. 4D), which further demonstrated the much higher ability of L-AgÅPs-gel than AgNPs-gel to stimulate wound closure. The wound samples from female mice at day 11 after wounding were then processed for further analyses. Hematoxylin and eosin (H&E) staining showed that treatment with L-AgÅPs-gel led to the highest levels of re-epithelialization and dermis regeneration and the lowest degree of scar formation among the groups (Fig. 4, E to G). The wounds treated with L-AgÅPs-gel also had markedly higher extents of collagen deposition and skin cell proliferation compared with the wounds receiving blank-gel or AgNPs-gel treatments, as indicated by Masson’s trichrome staining (Fig. 4, H and I) and immunohistochemical staining for the proliferative marker ki67 (Fig. 4, J and K), respectively. We also established the mouse models of skin burns and compared the effects of blank-gel, L-AgÅPs-gel, and AgNPs-gel on burn wound healing. Consistently, the L-AgÅPs-gel–treated wounds exhibited the fastest closure rates at days 3, 5, 7, and 13 after wounding in female mice (fig. S2, A and B), and at days 7, 11, and 13 after wounding in male mice (fig. S2, C and D), as compared with the sex-matched mice treated with blank-gel or AgNPs-gel. In male mice, it took 17.80 ± 0.79 days, 14.70 ± 0.67 days, and 16.40 ± 0.52 days to achieve complete closure of the burn wounds treated with blank-gel, L-AgÅPs-gel, and AgNPs-gel, respectively (fig. S2E). H&E, Masson’s trichrome, and ki67 staining of the burn wounds from female mice revealed that the wounds treated with L-AgÅPs-gel had much longer regenerated epidermis and dermis with narrower scars (fig. S2, F to H), higher intensities of Masson-stained collagen fibers (fig. S2, I and J), and larger numbers of proliferative skin cells (fig. S2, K and L), respectively, as compared to the other two groups. These results demonstrate the excellent pro–wound healing properties of L-AgÅPs-gel.

L-AgÅPs-gel suppresses wound bacterial colonization and inhibits inflammation in vivo and in vitro

We next asked the impact of L-AgÅPs-gel on bacterial colonization and inflammatory responses in wound areas. The diabetic wound samples were homogenized, and the extracts were subjected to bacterial colony counting assay on agar plates. As shown in Fig. 5 (A and B), large numbers of bacterial colonies were observed in extracts of the blank-gel–treated diabetic wounds. Both L-AgÅPs-gel and AgNPs-gel were able to effectively reduce the number of bacteria in the wound sites, but the inhibitory effect of L-AgÅPs-gel on bacterial colonization was much stronger than that of AgNPs-gel (Fig. 5, A and B). Immunohistochemical staining revealed that L-AgÅPs-gel was more competent than AgNPs-gel to suppress the expression of proinflammatory cytokines including interleukin-1β (IL-1β), IL-6, and tumor necrosis factor–α (TNF-α) (Fig. 5, C and D). CD86 and CD206 have been widely used as markers for proinflammatory M1 macrophages and anti-inflammatory M2 macrophages, respectively (21, 22). M2 macrophages can be assessed more precisely by the coexpression of CD68 (the pan-macrophage marker) and CD206.
Fig. 3. ROS generation contributes to L-AgÅPs-gel–induced bacterial death. (A) Intracellular ROS measured by DCFH-DA staining using a fluorescence microplate reader in *S. aureus* ATCC25923, *S. aureus* ATCC29213, and *P. aeruginosa* ATCC27853 treated with blank-gel, L-AgÅPs-gel, NAC, or NAC + L-AgÅPs-gel for 3 hours. *n* = 3 per group. 1 − β = 1. (B) Digital photos of bacterial colonies on agar plates in blank-gel, L-AgÅPs-gel, NAC, and NAC + L-AgÅPs-gel groups. (C) Bacterial colonies on agar plates were calculated. *n* = 3 per group. 1 − β = 1. (D) Cell viability of bacteria in different treatment groups measured by alamar blue assay. *n* = 4 per group. 1 − β = 1. (E and F) Calcein-AM/PI staining images of the above bacteria in different treatment groups (E) and quantification of the ratios of live bacteria [calcein-AM⁺ PI⁻; (F)]. Scale bar, 10 µm. *n* = 3 per group. 1 − β = 1. **P < 0.01 and ***P < 0.001. Photo credit: Chun-Yuan Chen and Hao Yin, Central South University.
with the M2 macrophage marker CD206 (22). As shown in Fig. 5 (E and F), immunohistochemical staining for CD86 and immunofluorescence double staining for CD68/CD206 showed the lowest number of CD86⁺ proinflammatory M1 macrophages and the largest number of CD68⁺CD206⁺ anti-inflammatory M2 macrophages in the wounds treated with L-AgÅPs-gel among all the treatment groups. The much higher ability of L-AgÅPs-gel to promote M2 macrophage generation in the wound sites was further confirmed by the greatest staining intensity for CD163 (Fig. 5, E and F), which is also a typical cell surface marker for M2 macrophages (23). In a skin burn injury model, the L-AgÅPs-gel–treated wounds also displayed the lowest level of bacterial colonization (fig. S3, A and B), the lowest staining intensities for the above-described proinflammatory factors (fig. S3C), the lowest number of CD86⁺ M1 macrophages (fig. S3, D and E), and the highest number of CD68⁺CD206⁺ and CD163⁺ M2 macrophages (fig. S3, D and E), as compared to the wounds treated with blank-gel or AgNPs-gel. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis showed that incubation with L-AgÅPs-gel did not obviously affect the expression of Il-1β, Il-6, and Tnf-α in RAW264.7 macrophages under normal condition but markedly abolished the lipopolysaccharide (LPS)–induced increases in mRNA levels of these proinflammatory factors in RAW264.7.
Fig. 5. L-AgÅPs-gel reduces bacterial colonization in diabetic wounds and inhibits inflammation in vivo and in vitro. (A and B) Homogenates of diabetic wounds treated with blank-gel, L-AgÅPs-gel, or AgNPs-gel were spread onto agar plates and bacterial colonies grown on agar plates were photographed (A) and counted (B). n = 3 per group. 1 − β = 0.99. (C and D) Immunohistochemical staining images of proinflammatory factors including IL-1β, IL-6, and TNF-α in diabetic wounds (C) and quantification of the mean intensities for the areas positive for these proinflammatory factors (D). Scale bar, 50 μm. n = 5 per group. 1 − β = 0.99 (for IL-1β), 0.84 (for IL-6), or 0.96 (for TNF-α). (E) Immunohistochemical or immunofluorescence staining images of M1 (CD86+, brown) and M2 (CD68+/CD206+, yellow; CD163+, brown) macrophage markers in diabetic wounds. Scale bar, 50 μm. (F) Quantification of the numbers of CD86+, CD68+/CD206+, and CD163+ cells. n = 5 per group. 1 − β = 0.98 (for CD86), 1 (for CD68+/CD206), or 0.99 (for CD163). (G) qRT-PCR analysis of IL-1β, IL-6, and Tnf-α in RAW264.7 macrophages receiving different treatments. n = 3 per group. 1 − β = 1. *P < 0.05, **P < 0.01, ***P < 0.001. Photo credit: Hao Yin and Chun-Yuan Chen, Central South University.
cells (Fig. 5G), indicating that L-AgÅPs-gel is able to directly block the inflammatory responses of the activated macrophages. These findings suggest that the potent inhibitory effects on bacterial colonization and inflammation are likely important contributing factors to the pro–wound healing action of L-AgÅPs-gel.

**Biocompatibility, safety, and tissue distribution of L-AgÅPs-gel**

During wound healing, keratinocytes, fibroblasts, and endothelial cells are responsible for wound re-epithelialization, collagen synthesis, and angiogenesis, respectively (24, 25). The biocompatibility of L-AgÅPs-gel was tested in human skin keratinocytes (HaCaT), human skin fibroblasts (HSFs), and human microvascular endothelial cells (HMECs). Tetramethyl rhodamine isothiocyanate (TRITC) phalloidin staining showed no obvious changes in the actin cytoskeleton architecture of HaCaT, HSFs, and HMECs after exposure to L-AgÅPs-gel at the dose of 64 parts per million (ppm) (MIC value of L-AgÅPs-gel against S. aureus ATCC29213 and P. aeruginosa ATCC27853) (Fig. 6A). Calcein-AM/PI staining revealed that both the blank-gel– and L-AgÅPs-gel–treated cells exhibited an excellent survival rate, as revealed by nearly 100% of cells negative for red PI and positive for green calcein-AM (live cells) (Fig. 6, B and C). These results indicate that L-AgÅPs-gel has a good biocompatibility.

We then tested whether the topical application of L-AgÅPs-gel in diabetic wounds could induce toxicities in mice. Routine blood test (Fig. 6D and table S1) and hepatic/renal function tests including assessments of the serum levels of alanine transaminase (ALT), aspartate aminotransferase (AST), creatinine (Cre/SCr), and blood urea nitrogen (BUN) (Fig. 6E) revealed that L-AgÅPs-gel treatment did not induce any significant alterations in the levels of blood routine parameters and function indicators of liver and kidney. H&E staining of the major organs (brain, heart, liver, spleen, kidney, and lung) did not indicate any abnormal changes after use of L-AgÅPs-gel (Fig. 6F). These results suggest that L-AgÅPs-gel has a good safety at the therapeutically efficacious dose. AgNPs-gel also did not cause marked changes in the levels of blood routine parameters (Fig. 6D and table S1), serum concentrations of hepatic and renal function indicators (Fig. 6E), and histological structures of the major organs (Fig. 6F). ICP-MS analysis revealed that silver was primarily accumulated in the wound tissues after L-AgÅPs-gel treatment, and only very small amounts of silver were detected in the healthy organs including liver, spleen, kidney, and lung (Fig. 6G). All these findings suggest that L-AgÅPs-gel has a good safety for wound therapy.

**DISCUSSION**

Currently, AgNP-based gels, ointments, creams, and wound dressings are commercially available for different medical applications owing to the antimicrobial and anti-inflammatory properties of silver (8, 9). Chemical reduction is the most widely used approach for the preparation of AgNPs (9, 12). Biological method represents an expedient alternative, as this route is environment friendly and less toxic (12). Evidences have shown the pro–wound healing efficiency of the chemically or biologically synthesized AgNPs (26–28). Unlike the biological and chemical approaches that produce AgNPs by reduction of silver salts using microorganisms, plant extracts, or some toxic chemical agents such as citrate and borohydride, the physical methods avoid the use of hazardous reducing agents and have no solvent contamination and biohazard risks (12, 29, 30). The physical approaches can quickly produce AgNPs with high purity (31). As they are generally generated at a high temperature, the physically fabricated AgNPs have a good thermal stability (31). However, complex equipment requirement and high energy consumption limit the use of physical routes to fabricate AgNPs (12, 29, 30).

Our group has developed a pure physical approach with an enclosed, automatic, and stable evaporation-condensation system that can efficiently generate ultrasmall pure silver particles from Ångstrom scale to nanoscale (13). The method does not involve the use of any toxic reducing agents or other solvents and thus has no requirement for additional purification steps. Smaller NPs tend to easily aggregate because of their large surface area–to–volume ratio, thus resulting in a large particle size (32). For this reason, we have equipped the system with a high-intensity ultrasonic dispersing device and a demagnetization device to prevent the aggregation of silver particles (13). Under certain gas flow rates in the hierarchical particle collection system, silver particles with well-defined size ranges (AgÅPs, L-AgÅPs, and AgNPs) can be obtained because of their different sedimentation velocities. Recently, we have reported the promising potential of AgÅPs as an efficient agent for cancer treatments (13, 14). In this study, we incorporated L-AgÅPs into carbomer gel and demonstrated the much stronger in vitro bacteriostatic and bactericidal activities of L-AgÅPs-gel than the commercially available AgNPs-gel at the same dosage. We also found the much higher ability of L-AgÅPs-gel to inhibit biofilm formation and survival of various pathogens compared to AgNPs-gel. Topical application of L-AgÅPs-gel to mouse skin triggered much more potent effects than AgNPs-gel to prevent bacterial colonization, reduce inflammation, and accelerate the healing process of both diabetic and burn wounds. L-AgÅPs-gel at the bacteria-sensitive dose was well tolerated in wound healing–related cells, and no signs of systemic toxicity occurred in mice after daily topical application of L-AgÅPs-gel. Our study suggests the promising prospect of L-AgÅPs-gel as a highly effective and safe antibacterial and anti-inflammatory material for the treatment of diabetic wounds and burn injuries.

The exact mechanism of the toxicity of AgNPs toward bacteria is not fully elucidated yet. Various theories have been proposed regarding how AgNPs exert antibacterial action. AgNPs have the ability to interact with the bacteria cell wall and penetrate cells, thus leading to cellular structure damage, disruption of cell function, and bacterial death (9). The induction of ROS is considered to be another mechanism that mediates AgNP-induced bacteria death (15, 16). ROS can oxidatively modify nucleic acids, proteins, lipids, and other cellular components, which results in genomic damage, disturbance of enzyme functions, alteration of membrane fluidity, and ultimate bacterial death (33). In this study, we found that incubation of two different S. aureus strains and P. aeruginosa with L-AgÅPs-gel led to the distortion of the cellular structures, disintegration of the cell wall and membrane, cytoplasm leakage, and collapse of the bacteria, all of which were much more drastic than that observed in the commercial AgNPs-gel–treated bacteria. This was most likely due to the much smaller sizes of L-AgÅPs than AgNPs, as smaller silver particles have a stronger bacterial killing activity (10, 11). Other differences, such as the methods for the preparation of silver particles and silver particle–embedded gel, may be also the potential contributing factors to the higher antibacterial efficacy of L-AgÅPs-gel than AgNPs-gel. In the L-AgÅPs-gel–induced antibacterial effect, ROS played an important role, as incubation with L-AgÅPs-gel markedly augmented the production of ROS and the antioxidant agent was...
able to significantly reverse the L-AgÅPs-gel–induced inhibitions of growth and survival of bacteria. Silver particles have the ability to interact with membrane and intracellular proteins and influence the structure and function of these proteins (34). In our recent study, we have demonstrated that fructose-coated AgÅPs can induce osteosarcoma cell apoptotic death via the alteration of glucose metabolic phenotype by inhibiting pyruvate dehydrogenase kinase (14). In this study, the promotion of ROS overproduction in bacteria

Fig. 6. Biocompatibility, safety, and tissue distribution of L-AgÅPs-gel. (A) TRITC phalloidin staining of the actin cytoskeleton architecture in normal cells treated with blank-gel or L-AgÅPs-gel for 12 hours. Scale bar, 20 μm. (B) Calcein-AM/PI staining images of HaCaT, HSFs, and HMECs treated with blank-gel or L-AgÅPs-gel for 12 hours. Scale bar, 100 μm. (C) Quantification of the ratios of live cells (calcein-AM+’PI−’) in (B). n = 3 per group. 1 − β = 0.16 (for HaCaT), 0.42 (for HSFs), or 0.22 (for HMECs). (D) Total numbers of red blood cells (RBC), white blood cells (WBC), and platelets (PLT) and total levels of hemoglobin (HGB) in diabetic mice treated with blank-gel, L-AgÅPs-gel or AgNPs-gel for 11 days. n = 5 per group. 1 − β = 0.49 (for RBC), 0.08 (for WBC), 0.05 (for PLT), or 0.19 (for HGB). (E) The serum levels of liver and kidney functional indicators in mice with diabetic wounds. SCr: serum creatinine. n = 4 to 5 per group. 1 − β = 0.08 (for ALT), 0.07 (for AST), 0.19 (for SCr), or 0.25 (for BUN). (F) H&E staining images of brain, heart, liver, spleen, kidney, and lung sections from mice with diabetic wounds. Scale bar, 50 μm. (G) ICP-MS analysis of silver contents in skin wound, brain, heart, liver, spleen, kidney, and lung from mice with diabetic wounds. n = 4 per group.
implies that L-AgAP-gel may affect the function of some proteins related to the metabolic processes, which still needs future investigation.

Wound healing involves the activation of macrophages, keratinocytes, fibroblasts, endothelial cells, etc. Macrophages participate in all stages of wound healing (35, 36). Classically activated M1 macrophages induced by bacterial LPS, peptidoglycan, or a variety of intracellular stimuli are proinflammatory and contribute to host defense and clearance of damaged tissue (35, 36). Alternatively activated M2 macrophages (induced by ILs, glucocorticoids, etc.) are anti-inflammatory and can secrete various molecules to promote wound repair and tissue remodeling (35, 36). Incomplete switch from M1 macrophages toward M2 macrophages represents a common feature of diabetic wounds (36). Here, we found that treatment with L-AgAPs-gel led to a shift of the macrophage phenotype from proinflammatory M1 to anti-inflammatory M2 in both diabetic and burn wounds. Considering the important role of bacteria and their endotoxins in triggering inflammation in the wound sites (6), we hypothesized that the bacterial killing activity of L-AgAPs-gel may contribute importantly to the anti-inflammatory effects of L-AgAPs-gel. Biofilm formation protects bacteria against environmental threats such as antibiotics and host immune defenses (37) and has been considered as an important reason for prolonged inflammation and impaired wound healing (38). L-AgAPs-gel caused much higher levels of inhibition and destruction of biofilm compared with AgNPs-gel, which may be another critical factor that leads to the stronger anti-inflammatory and pro–wound healing activities of L-AgAPs-gel. However, it should be noted that incubation with L-AgAPs-gel directly blocked the expression of inflammatory factors in the LPS-activated macrophages, suggesting that L-AgAPs-gel can exert anti-inflammatory effect independent of its antibacterial action.

During wound healing, the proliferation and migration of keratinocytes are critical to the formation of a new epidermal layer (re-epithelialization) and restoration of tissue integrity (25). Fibroblasts play a central role in wound contraction, collagen production, and tissue remodeling (24, 39). Angiogenesis by endothelial cells is essential for supplying oxygen and nutrients for wound tissues, thus sustaining the activities of keratinocytes and fibroblasts and facilitating rapid wound healing (25). Our study showed that L-AgAPs-gel at the bacteria-sensitive dose was well tolerated in these wound healing–related cells. Whether L-AgAPs-gel is able to directly modulate the functional properties of these wound healing–related cells still needs future exploration. In vivo, no signs of systemic toxicity occurred in mice after daily topical application of L-AgAPs-gel. These findings suggest that L-AgAPs-gel has a promising prospect as a safe material for wound healing. Nevertheless, it should be noted that the achieved power (1 − β) of the generated results for analyses of the levels of blood routine parameters and function indicators of liver and kidney was just from 0.05 to 0.49 with α = 0.05 and n = 5 mice per group, suggesting that it requires larger sample sizes for these assays to further determine the non-toxicity of L-AgAPs-gel on hematological, liver, and renal function in mice.

**MATERIALS AND METHODS**

Experiments described in our study were approved by the Ethics Committee of Xiangya Hospital of Central South University.

**Production of L-AgAPs and L-AgAPs-gel**

The procedures for fabrication of silver particles using our self-designed automatic evaporation-condensation system had been described in our recently published study (13). Briefly, after removal of air within the entire system by the vacuum pump, the protective gas argon at the pressure of 1 × 10^5 to 1 × 10^6 Pa was filled and circulated in the system using an inert gas blower. The pure silver wire of 8 to 12 cm in length and 0.1 to 0.8 mm in diameter was continuously fed toward the explosion chamber. When the silver wire approached and contacted the high-voltage electrode plate in the chamber, a high voltage (25 to 45 kV) accompanied by arc lightning was generated and the metal wire was exploded and gasified. The silver vapor with the shielding gas was then entered into the cooling chamber (0° to 4°C), where the silver atoms formed nuclei and particle growth occurred by coagulation. Silver particles from Angstrom scale to nanoscale were formed and then dispersed by the ultrasonic waves at 15 kV and 15 kHz using a high-intensity ultrasonic dispersing device, followed by demagnetization at a current of 1000 mA, a voltage of 24 to 36 V, and a frequency of 23 Hz, all of which effectively prevented the aggregation of silver particles. The treated silver particles were then transferred to the particle collection system equipped with three series-connected collectors. Under a gas flow rate of 1.2 m s^−1, the largest (AgNPs; > 20 nm), medium-sized (L-AgAPs; with diameters less than 20 nm and centered at 50 to 70 Å), and smallest (AgAPs; with diameters ranging from 1 to 50 Å) silver particles orderly fell into the collectors owing to their different sedimentation velocities.

For preparation of L-AgAPs-gel, L-AgAPs (1.0 g liter^−1) were added to the high-purity distilled water and dispersed by the ultrasonic waves for 60 s at an ultrasonic power of 5 kW and a frequency of 40 kHz. The suspension was mixed with carboxerm 934P (9.8 g liter^−1) with a high-speed mechanical agitator at a mixing rate of 2000 rpm. When the solution became clear, triethanolamine (5 g liter^−1) was added to the solution and then stirred at 2000 rpm until a homogeneous gel (L-AgAPs) formed. The blank carboxerm gel (blank-gel) that was not doped with L-AgAPs served as a control. L-AgAPs-gel and blank-gel were photographed and subjected to further analyses.

**Characterization of AgAPs and L-AgAPs-gel**

The crystal structure and phase purity of L-AgAPs were measured with XRD analysis using a PANalytical X’Pert Pro MPD diffractometer (Holland) with Cu Kα radiation at 40 mA and 40 kV. UV–vis–NIR spectrum of L-AgAPs was tested using a LAMBDA 750 spectrophotometer (PerkinElmer, USA). The surface morphologies of L-AgAPs-gel and blank-gel were examined by a Sigma HD field emission SEM (Carl Zeiss, Germany), followed by EDS analysis to assess their elemental constitutions. The morphology and size distribution of L-AgAPs in carboxerm gel were detected under a Hitachi HT7700 TEM (Tokyo, Japan). DLS analysis using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) was conducted to examine the zeta potentials of L-AgAPs-gel and blank-gel. FTIR spectra of L-AgAPs-gel and blank-gel were measured with a Nicolet iS5 FT-IR spectrometer (Thermo Fisher Scientific, USA). The thermal stabilities of L-AgAPs-gel and blank-gel were tested by thermogravimetric analysis with a Q500 thermogravimetric analyzer (TA Instruments, USA) from 30° to 800°C at a heating rate of 20°C min^−1. The rheological properties of L-AgAPs-gel and blank-gel were analyzed by amplitude and frequency sweep tests using a HAAKE MARS 60 rheometer (Thermo Haake, Germany). The strain amplitude sweep test was conducted at a constant frequency of 1 Hz with strain amplitude ranging from 0.1 to 100%. Frequency sweeps were taken at a constant strain of 1% from 0.1 to 5 Hz.
Bacterial strains and culture
S. aureus ATCC25923, S. aureus ATCC29213, and P. aeruginosa ATCC27853 were provided by the Department of Clinical Laboratory in Xiangya Hospital of Central South University. The bacteria were cultured in nutrient broth at 37°C before experiments.

Agar disk diffusion assay
Antimicrobial susceptibility testing was conducted using the agar disk diffusion assay. Briefly, the filter paper disks of 6-mm diameter were soaked with blank-gel, L-AgÅPs-gel, or the commercial AgNPs-gel (Shenzhen Yuanxing Pharmaceutical Co. Ltd., Shenzhen, China) for 12 hours. MH agar was prepared and poured into petri dishes. The bacterial suspensions [1 × 10^8 colony-forming units (CFU) ml⁻¹; 150 µl per dish] were swabbed onto petri dishes. The blank disks and the disks impregnated with blank-gel, L-AgÅPs-gel, or AgNPs-gel were placed onto the inoculated MH agar plates and incubated at 37°C. Eighteen hours later, the plates were photographed and the inhibition zones around the disks were measured.

MIC and MBC measurements
L-AgÅPs-gel and AgNPs-gel were serially diluted to the concentrations of 512, 256, 128, 64, 32, and 16 ppm by MH broth and mixed with bacterial suspension to a final concentration of 5 × 10^6 CFU ml⁻¹. The bacteria receiving no treatments or treated with blank-gel served as the controls. The cultures were incubated for 18 hours at 37°C and 300 rpm. The lowest dose where there was no visible bacterial growth (no marked changes in turbidity at 595 nm compared with the blank samples) was defined as MIC. For MBC testing, the bacterial suspensions were swabbed on Luria-Bertani (LB) agar plates and incubated for 24 hours at 37°C. The lowest dose at which bacteria failed to grow was recorded as MBC.

Morphological analysis
The bacteria (1 × 10^8 CFU ml⁻¹) were treated with L-AgÅPs-gel or AgNPs-gel at the doses of MIC values of L-AgÅPs-gel against the tested bacteria, or with an equal volume of blank-gel for 3 hours, followed by centrifugation for 5 min at 2000 rpm. The supernatant was removed and the bacteria were processed for TEM observation.

Bacterial colony counting assay
For comparing the antibacterial efficacy of L-AgÅPs-gel and AgNPs-gel, they were diluted to the concentrations of MIC values of L-AgÅPs-gel against the tested bacteria and then mixed with bacterial suspensions (5 × 10^6 CFU ml⁻¹). The blank-gel–treated bacteria served as the control. For testing the role of ROS in the L-AgÅPs-gel–induced antibacterial effect, 5 × 10^6 CFU ml⁻¹ bacteria were treated with L-AgÅPs-gel (MIC doses against the tested bacteria), NAC (5 mM; Sigma-Aldrich, St. Louis, USA), L-AgÅPs-gel + NAC, or blank-gel. After incubation for 8 hours at 37°C and 300 rpm, the mixture was diluted by 5000-fold with LB broth, and 200 µl of the diluted bacterial solution was spread onto an LB agar plate. After incubation at 37°C for 12 hours, the numbers of bacterial colonies in the plate were quantified, and the bacterial counts per milliliter of bacterial solution were calculated.

Calcein-AM/PI staining
The bacteria (5 × 10^5 CFU per well) in 24-well plates were treated with blank-gel, MIC doses of L-AgÅPs-gel or AgNPs-gel, 5 mM NAC, or L-AgÅPs-gel + NAC for 3 hours. After washing with assay buffer (Yeasen Biotech, Shanghai, China), the bacteria were incubated with calcein-AM (4 µM; Yeasen Biotech) and PI solution (9 µM; Yeasen Biotech) for 30 min at 37°C and 300 rpm. After that, the bacteria were washed with phosphate-buffered saline (PBS), resuspended in assay buffer, and placed on glass slides. Images were obtained with a Zeiss fluorescence microscope (Jena, Germany). The dead (PI-positive) and live (calcein-AM–positive) bacteria were counted.

Alamar blue assay
The tested bacteria (8 × 10^1 CFU per well) in 96-well plates were treated with blank-gel, MIC doses of L-AgÅPs-gel or AgNPs-gel, 5 mM NAC, or L-AgÅPs-gel + NAC in LB medium containing alamar blue (10 µl per well; Yeasen Biotech) for 3 hours. The fluorescence intensity was then measured at 545-nm excitation and 590-nm emission with a Varioskan LUX Multimode fluorescence microplate reader (Thermo Fisher Scientific, USA). The viability of cells (%) was evaluated by the ratio of fluorescence intensity values between the treated cells and the control cells.

Biofilm formation and destruction assay
To assess the formation of bacterial biofilm, the tested bacteria were diluted to the concentration of 1.5 × 10^7 CFU ml⁻¹ using RPMI 1640 medium (Gibco), and the diluted bacterial suspension (1 ml per well) was plated in 24-well plates. After incubation at 37°C for 6 hours, the medium was then replaced with fresh RPMI 1640 medium containing L-AgÅPs-gel or AgNPs-gel at the doses of MIC values of L-AgÅPs-gel against the tested bacteria or an equal volume of blank-gel. Thirty-six hours later, the planktonic bacteria were removed by washing three times with normal saline. The biofilm adhering to the culture plates was stained for 15 min with 1 g liter⁻¹ crystal violet (100 µl per well; Solarbio, Beijing, China). After three times of washing, the biofilm was allowed to air-dry, followed by dissolving the bounded crystal violet with 95% ethanol. The resulting solution was transferred to 96-well plates, and the OD values were read at 570 nm with a microplate reader. To test the destruction of bacterial biofilm, the tested bacteria (1.5 × 10^7 CFU per well) were cultured in RPMI 1640 medium for 6 hours and then allowed to form biofilm in fresh medium for another 36 hours. The biofilm was treated with L-AgÅPs-gel or AgNPs-gel at the doses of MBC values of L-AgÅPs-gel against the tested bacteria or with an equal volume of blank-gel in fresh RPMI 1640 medium for 36 hours. The residual biofilm was then assessed using crystal violet staining assay with procedures described above.

ROS measurement
After treatment of the bacteria (5 × 10^5 CFU per well) in 24-well plates with blank-gel, MIC doses of L-AgÅPs-gel, 5 mM NAC, or L-AgÅPs-gel + NAC for 3 hours, the bacteria were incubated at 37°C for 20 min with DCFH-DA (10 µM; Beyotime, Jiangsu, China) and analyzed by a fluorescent microplate reader (Thermo Fisher Scientific, USA).

Release behavior of silver by L-AgÅPs-gel
L-AgÅPs-gel (5 g) was packaged into a commercially available dialysis membrane (2-kDa molecular weight cutoff; Millipore, Billerica, USA). PBS (40 ml) was added to the membrane. Supernatant (500 µl) was collected every day during a 15-day period and subjected to ICP-MS analysis of silver. The cumulative amounts of released silver from days 0 to 15 were calculated.
Quantitative real-time polymerase chain reaction
RAW264.7 macrophages were incubated in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, USA) containing 10% fetal bovine serum (FBS) and supplemented with blank-gel, L-AgÅPs-gel (64 ppm), LPS (100 ng ml⁻¹; Sigma-Aldrich), or LPS + L-AgÅPs-gel for 24 hours. Total RNA form RAW264.7 cells in different treatment groups was extracted and processed for complementary DNA synthesis and then qRT-PCR analysis as described previously (40). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene for normalization. The primers were shown as follows: IL-1β, 5′-GGAATGCACCTTGTACGTACCGAGAAG-3′ (forward) and 5′-TTGGTTCTCTGCTACCGAGACCAGG-3′ (reverse); IL-6, 5′-TAGTCCTTCCCTACCCCCAATTCCC-3′ (forward) and 5′-TTGGTTCTTACGCCACTCCTTC-3′ (reverse); TNF-α, 5′-GAACCTCGGGGTATCGGTCGACG-3′ (forward) and 5′-CAACCAGTGGAGGCCGCGG-3′ (forward) and 5′-GACGGGACACATTGCAGG-GGTTAG-3′ (reverse).

Animals and treatments
Three-month-old female or male C57BL/6 mice were used. The procedures for the generation of streptozotocin-induced diabetic mice and establishment of full-thickness excisional cutaneous wounds (6 mm in diameter) in diabetic mice and skin burn wounds (1 cm in diameter) in normal mice were detailed in our recently published studies (24, 25). Then, L-AgÅPs-gel, AgNPs-gel, or blank-gel were smeared daily around and on the surfaces of diabetic and burn wounds (10 mice for each group). Images of wounds at the indicated times. The time for complete wound closure represented the original size of wound and t represented the diameter of wound. Wound closure rate (%) = (A₀ − At)/A₀ × 100, where A₀ indicated the original size of wound and At represented the diameter of wounds at the indicated times. The time for complete wound closure was recorded in male mice with diabetic or burn wounds. At day 11 (for female mice with diabetic wounds) or day 13 (for female mice with burn wounds) after wounding, three mice in each group were killed, and the wound bed with adjacent healthy skin tissues (1 cm by 1 cm square areas centered at the wound) were collected for counting bacteria. The remaining mice were killed after collecting blood by eyeball enucleation. For diabetic mice, 200-μl blood sample for each mouse was subjected to routine blood test. Serum was separated from the remaining blood by centrifugation for 15 min at 1000g and subjected to hepatic and renal function tests. These tests were conducted on automated instruments in the Department of Clinical Laboratory in Xiangya Hospital. The diabetic and burn wounds tissues were harvested as described above and processed for histological, immunohistochemical, and immunofluorescent analyses.

Bacteria counting of wound extracts
The homogenates of the diabetic and burn wounds were prepared with a tissue homogenizer in a biological safety cabinet and then diluted 100 times using PBS. The diluted homogenates (50 μl per plate) were spread onto LB agar plates and incubated at 37°C. Twenty hours later, the numbers of bacterial colonies were counted.

Histological, immunohistochemical, or immunofluorescent staining
After fixing for 24 hours using 4% paraformaldehyde, the paraffin-embedded tissues were prepared. Sections (4 μm thick) were made and subjected to H&E staining using reagents from Servicebio (Wuhan, China). For skin tissues, the width of scar and the rate of re-epithelialization were measured. The skin sections were also stained with Masson’s trichrome (Servicebio) to test the extent of collagen deposition. Immunohistochemical staining for ki67 was conducted to assess cell proliferation. The inflammatory activities were tested by immunohistochemical staining for IL-1β, IL-6, and TNF-α. Macrophages were detected by immunohistochemical staining for CD86 and CD163, and also by immunofluorescence double staining for CD86 and CD163. The procedures for H&E staining, Masson’s trichrome staining, and immunostaining had been described in detail in our previous studies (21, 24, 25). The sections were observed with an Olympus CX31 optical microscope (Tokyo, Japan) or a Zeiss fluorescence microscope (Jena, Germany). Anti-CD86, anti-CD163, and anti-CD206 were purchased from Abcam (Cambridge, Britain). Anti-CD86 and anti-TNF-α were obtained from Novus Biologicals (Littleton, USA) and MultiSciences Biotech Co. Ltd. (Hangzhou, China), respectively. Other antibodies were purchased from Servicebio.

Tissue distribution of silver
The diabetic mice with excisional cutaneous wounds were treated topically with L-AgÅPs-gel, AgNPs-gel, or blank-gel once a day. Eleven days later, the mice were euthanized. The skin wound, brain, heart, liver, spleen, kidney, and lung tissues were harvested, cut into small pieces, and digested in aqua regia. The digestion liquid was filtered with a 0.22-μm filter (Millipore) and subjected to ICP-MS.

Biocompatibility of L-AgÅPs-gel
HaCaT and HSFs were cultured in high-glucose DMEM (Gibco) containing 10% FBS (Gibco) and added with 64-ppm L-AgÅPs-gel or an equal volume of blank-gel. HMECs were incubated in MCDB131 medium (Gibco) added with 1% GlutaMAX (Gibco), 10% FBS (Gibco), and 64-ppm L-AgÅPs-gel or an equivalent volume of blank-gel. Twelve hours later, calcein-AM/PI staining was performed to measure the percentages of live cells (calcein-AM+/PI⁻). TRITC phalloidin staining was conducted to detect the changes in the structure of actin cytoskeleton.

Statistical analysis
A Student’s t test (unpaired, two tailed) was used for two-group comparison and one- or two-way analysis of variance (ANOVA) with Bonferroni post hoc test was conducted for multiple comparisons. Data were presented as means ± SD. P < 0.05 was regarded as statistically significant. The power (1 − β) of each test was calculated using the freely downloadable software G Power.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/43/eaba0942/DC1
View/request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES
1. T. Dainichi, A. Kitoh, A. Otsuka, S. Nakajima, T. Nomura, D. H. Kaplan, K. Kabashima, The epithelial immune microenvironment (EIME) in atopic dermatitis and psoriasis. Nat. Immunol. 19, 1286–1298 (2018).
2. S. O. Blacklow, J. Li, B. R. Freedman, M. Zeidi, C. Chen, D. J. Moorey, Bioinspired mechanically active adhesive dressings to accelerate wound closure. Sci. Adv. 5, eaaw3963 (2019).
A. Gupta, S. Mumtaz, C.-H. Li, I. Hussain, V. M. Rotello, Combatting antibiotic-resistant bacteria using nanomaterials. Chem. Soc. Rev. 48, 415–427 (2019).

J. S. Möhler, W. Sim, M. A. T. Blaskovich, M. A. Cooper, Z. M. Ziora, Silver bullets: A new lustre on an old antimicrobial agent. Biotechnol. Adv. 36, 1391–1411 (2018).

A. C. Burdese, O. Gerasim, A. Muromecz, L. Mogoanta, A. Fica, E. Andronescu, Biomedical applications of silver nanoparticles: An up-to-date overview. Nanomaterials 8, 681 (2018).

J. Kang, M. J. Dzieci, K. Hughes, M. Xing, L. Li, Silver nanoparticles present high intracellular and extracellular killing against Staphylococcus aureus. J. Antimicrob. Chemother. 74, 1578–1585 (2019).

G. Franci, A. Falanga, S. Galdiero, L. Palomba, M. Jai, M. Morelli, Silver nanoparticles as potential antibacterial agents. Molecules 20, 8856–8874 (2015).

P. Mathur, S. Jha, S. Ramteke, N. K. Jain, Pharmaceutical aspects of silver nanoparticles. Acta. Cell. Sinomol. Biotechnol. 46, 115–126 (2016).

Z.-X. Wang, C.-Y. Chen, Y. Wang, F.-X.-Z. Li, J. Huang, Z.-W. Luo, S.-S. Rao, Y.-J. Tan, Y.-W. Liu, H. Yin, Y.-Y. Wang, Z.-H. He, K. Xiu, B. Wu, X.-K. Hu, M.-J. Luo, H. Liu, T.-H. Chen, C.-G. Hong, J. Cao, Z.-Z. Liu, Z. Long, P.-P. Gan, W.-Y. Situ, R. Fan, L.-Q. Yuan, H. Xie, Silver Angstrophotons: Angstrophome silver salt particles as a promising agent for low-toxicity broad-spectrum potent anticancer therapy. Adv. Funct. Mater. 29, 1808556 (2019).

X.-K. Hu, S.-S. Rao, Y.-C. Tan, Y.-J. Tan, H. Yin, M.-J. Luo, J.-H. Zhou, C.-G. Hong, Z.-W. Luo, W. Du, B. Wu, Z.-Q. Yan, Z.-H. He, Z.-Z. Cao, J. Liu, W.-Y. Situ, H.-M. Liu, J. Huang, Y.-Y. Wang, K. Xia, Y.-Q. Qian, Y. Zhang, T. Yue, Y.-W. Liu, H.-Q. Zhang, S.-Y. Tang, C.-Y. Chen, H. Xie, Fructose-coated Angstrophome silver inhibits osteosarcoma growth and metastasis via promoting ROS-dependent apoptosis through the alteration of glucose metabolism by inhibiting PDK. Theranostics 10, 7710–7729 (2020).

S. Panda, T. K. Rout, A. D. Prusty, P. M. Ajayan, S. Nayak, Electron transfer directed antibacterial properties of graphene oxide on metals. Adv. Mater. 30, 1702149 (2018).

G. Wang, W. Jin, A. M. Qasim, A. Gao, X. Peng, W. Li, H. Feng, P. K. Chu, Antibacterial effects of titanium embedded with silver nanoparticles based on electron-transfer-induced reactive oxygen species. Biomaterials 124, 35–34 (2017).

L. Nahar, R. J. Esteves, S. Hafiz, Ü. Özgür, I. U. Arachchige, Metal–semiconductor hybrid aerogels: Evolution of optoelectronic properties in a low-dimensional CdSe/Ag nanocrystals impregnated with silver nanoparticles. Adv. Funct. Mater. 29, 385–406 (2019).

Q.-Z. Zhang, W.-R. Su, H.-S. Shi, P. Wilder-Smith, A. P. Xiang, A. Wong, A. L. Nguyen, C. W. Kwon, A. D. Le, Human gingiva-derived mesenchymal stem cells elicit polarization of m2 macrophages and enhance cutaneous wound healing. Stem Cells 28, 1856–1868 (2010).

J. Yan, G. Tie, S. Wang, A. Tutto, N. DeMarco, L. T. Fazzio, L. M. Messina, Diabetes impairs wound healing by Drnmt1-dependent dysregulation of hematopoietic stem cell differentiation towards macrophages. Nat. Commun. 9, 33 (2018).

Y. Wang, U. Kadiyala, Z. Qu, P. Elvati, C. Altheim, A. N. Kotov, A. Violii, J. S. VanEpps, Anti-biofilm activity of graphene quantum dots via self-assembly with bacterial amyloid proteins. ACS Nano 13, 4278–4289 (2019).

Y.-K. Wu, N.-C. Cheng, C.-M. Cheng, Biofilms in chronic wounds: Pathogenesis and potential therapeutic targets. Theranostics 7, 505–517 (2018).

Y. Hu, S.-S. Rao, Z.-X. Wang, J. Cao, Y.-Y. Jan, T. Luo, H.-M. Li, W.-S. Zhang, C.-Y. Chen, H. Xie, Exosomes from human umbilical cord blood accelerates cutaneous wound healing through miR-21-3p-mediated promotion of angiogenesis and fibroblast function. Theranostics 8, 169–184 (2018).

C.-Y. Chen, C.-S. Rao, Y.-J. Tan, M.-J. Luo, X.-K. Hu, H. Yin, J. Huang, Y. Hu, Z.-W. Luo, Z.-Z. Liu, Z.-X. Wang, J. Cao, Y.-W. Liu, H.-M. Li, Y. Chen, W. Du, J.-H. Liu, Y. Zhang, T.-H. Chen, M.-J. Liu, B. Wu, T. Yue, Y.-W. Wang, K. Xia, P.-F. Lei, S.-T. Tang, H. Xie, Extracellular vesicles from human urine-derived stem cells prevent osteoporosis by transferring CHC1RC and DPG. Bone Res. 7, 18 (2019).

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