Supporting Information

Graphene oxide-silver nanocomposites modulate the biofilm formation and extracellular polymeric substances (EPS) production

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Experimental Section

Materials

In this study, sodium nitrate, silver nitrate ($AgNO_3$), Luria Bertani (LB) and Luria-Bertani no-salt (LBNS) media were purchased from Sinopharm Chemical Reagent (China). Graphite powder, sulfuric acid, potassium permanganate, crystal violet (CV) and other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). LIVE/DEAD Baclight Bacterial Viability Kit (L13152, Molecular Probes) and concanavalin-A Alexa Fluor 647 conjugate were purchased from Invitrogen (USA). All aqueous solutions were prepared with deionized (DI) water from Milli-Q-Water (Millipore Corp, 18.2 MΩ/cm at 25 °C).

P. aeruginosa was purchased from China General Microbiological Culture Collection Center.

The Preparation of GO and GO-AgNPs

GO was synthesized according to our previous studies.[1, 2] The GO stock solution (2 mg/mL) was stored at 4 °C for further experiments. The preparation of GO-AgNPs was based on the proposal reported by Zhou et al.[3] Briefly, GO solution (30 mg, 0.5 mg/mL) was obtained by diluting GO stock solution with sodium citrate aqueous solution (0.12

M), followed by ultrasonic treatment for 2 h. Then $AgNO_3$ aqueous solution (3.6 mM, 30 mL) was added into the GO solution under vigorous stirring. After ultrasonic treatment at room temperature for 30 min, the GO solution with $AgNO_3$ and sodium citrate was added slowly into fresh $NaBH_4$ solution (0.22 mM, 60 mL) under vigorous stirring. The reaction mixture was adjusted to pH 11 with NaOH solution (0.5 M) under magnetic stirring for 75 min at room temperature. The product was centrifuged, washed with DI water several times, and dialyzed for 3 days to remove any impurities.

Characterizations

The morphology of the GO-AgNPs composite and AgNPs were characterized by transmission electron microscopy (TEM, Tecnai G2F20S-TWIN, FEI, USA). Their structure was measured by UV-vis spectrophotometer (U-3010, Hitachi, Tokyo, Japan) and X-ray diffraction spectra (XRD Bruker D8 Advance). The content of Ag element in GO-AgNPs was measured by inductively coupled plasma mass spectrometry (ICP-MS; Thermo Elemental X Series).

Bacterial Culture

The gram-negative bacteria *P. aeruginosa* was as a model microorganism used in this study. Bacterial Cells were grown on LB plates (1.5% agar) at 37 °C for 24 h. A single colony was inoculated in LB medium and grown in a shaker incubator at 220 rpm at 37 °C. After incubation overnight, *P. aeruginosa* cells were harvested in the mid-exponential growth phase by centrifugation at 10,000 rpm for 1 min and suspended in phosphate buffered saline (PBS) for further bacterial experiment.

Total Cell Growth and Biofilm Formation

The sterile coverslips (diameter 12 mm) were first placed in each well of a 24-well microtiter plates. Bacteria with concentration of 10^7 CFU/mL in 0.9 mL LBNS medium was added to each well. Then, 0.1 mL of different concentrations of GO-AgNPs solution were added to wells. The final concentration of GO-AgNPs was 0, 10, 15, 20, and 25 μg/mL, respectively. The cells were grown statically at 37 °C. After 24 h, total cell growth and suspended cell growth were measured by recording the optical density at 600 nm (OD_{600}) using a microplate reader (Bio-Rad 680, USA). Total cell growth represents the cells in the supernatant and the biofilm attached to the bottom. Suspended cell growth represents the cells in the supernatant. After removing the supernatant on the well, the biofilm indwelling the coverslips were investigated.

Crystal Violet Staining Assay (CV Staining)

After 24 h incubation, the biofilm colonizing on the coverslip was gently rinsed three times with 1 mL PBS and air-dried for several minutes. Then the biofilm was stained by 300 μL of 0.1% (w/v) CV at room temperature for 15 min. After removing the excess stain, the coverslips were gently washed three times with PBS. Digital camera

(Canon EOS 750D) was adopted to record the stained biofilm. The biomass was quantified by measuring the OD_{595} using a microtiter plate reader (Bio-Rad 680, USA) after biofilms dissolved in 1 mL of 95% ethanol. All measurements were carried out at least three times.

Scanning Electron Microscopy (SEM)

The morphology of biofilm was observed by SEM. After 24 h incubation, the biofilm on the coverslips were washed gently three times using PBS, then fixed with 2% glutaraldehyde for 2 h at 4 °C. The fixed samples were dehydrated via freeze-drying for 24h. The samples were coated with gold and observed by SEM (FEI, Magellan 400, USA) with a TLD detector in SE mode and accelerating voltage of 10 kV.

Confocal Laser Scanning Microscopy (**CLSM)**

The coverslips with biofilm were rinsed gently three times using PBS and stained with 300 µL of SYTO9 (6 µg/mL, green fluorescence, labeled bacterial cells) for 15 min in the dark at room temperature. After gently washing residual SYTO9 with PBS, the samples were stained with 300 µL of concanavalin A-Alexa Fluor 647 conjugate solution (50 µg/mL, red fluorescence, labeled EPS) for 15 min. The bacteria and EPS production were observed by CLSM. [4, 5] The excitation/emission of SYTO9 and Alexa Fluor 647 were 480/500 nm and 650/668 nm, respectively. Stack images were obtained by scanning the biofilm along the Z-axis at 1.8 µm intervals. The confocal images were analyzed using COMSTAT software for simultaneous visualization and quantification of EPS and bacterial cells within intact biofilms [6]. Biofilm thickness was determined by multiplying the number of slices taken in the biofilm by the thickness of each slice. COMSTAT was then used to threshold the images to reduce background noise. For each image stack, cell biomass ($\mu m^3/\mu m^2$) is defined as the percentage of area occupied by cells labelled by SYTO9 (green fluorescence); EPS biomass ($\mu m^3/\mu m^2$) is defined as the percentage of area occupied by EPS labelled by concanavalin A-Alexa Fluor 647 (red fluorescence); Total biomass ($\mu m^3/\mu m^2$) is cell biomass plus EPS biomass. The average and the roughness coefficient, as an indicator of biofilm heterogeneity, were calculated by COMSTAT from the threshold images[7, 8].

Electron Spin Resonance Spectroscopy (ESR)

The generation of reactive oxidative species (ROS) induced by nanomaterials was measured by electron spin resonance (ESR, JES-FA200) at room temperature. All samples (GO, GO-AgNPs, AgNPs) were centrifuged at 12000 rpm for 15 min followed by removing supernatant. The precipitation was re-suspended in absolute ethanol. 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO, 0.1 M) was used as a spin trap to detect singlet oxygen. The spin traps were ESR silent but formed stable radicals with an ESR signal after donating electrons. Spectra was obtained at center field of 329 G; sweep width 150 G; microwave power 1 mW; sweep time 2 min.

Glutathione (GSH) Oxidation Test

GSH oxidation test was used to analyze the generation of ROS that can trigger the oxidation of thiol groups in GSH.[9, 10] The concentration of thiols in GSH was quantified by the Ellman's assay. Briefly, 0.4 mM GSH solution was prepared by dissolving GSH in 50 mM bicarbonate buffer (pH 8.2). The concentration of GSH was added to 20 µL nanomaterial solution in 48-well microtiter plates and incubated at 37 °C under the dark condition. After 2 h, 348 µL of Tris-HCl (pH 8.2) and 6.7 µL of 100 mM Ellman's reagent (5, 5'-dithio-bis-2-nitrobenzoic acid, DTNB, Invitrogen) were added into the mixtures, generating a yellow product with the absorbance at 412 nm. OD_{412} was measured by a microtiter plate reader. The negative and positive control were bicarbonate buffer and 1 mM H_2O_2, respectively. All samples were prepared in triplicate. Remain of GSH was calculated by the following formula:

$$Remain\ of\ GSH\ (\%) = \frac{OD\ samp}{OD\ ctrl} x\ 100$$

Statistical Analysis

Data were expressed as mean±standard deviation. The values of the experimental groups were compared with control groups. Statistical significance was analyzed by Student's T-test to detect the presence of statistically significant differences ($p < 0.05$). All tests were repeated at least three times.

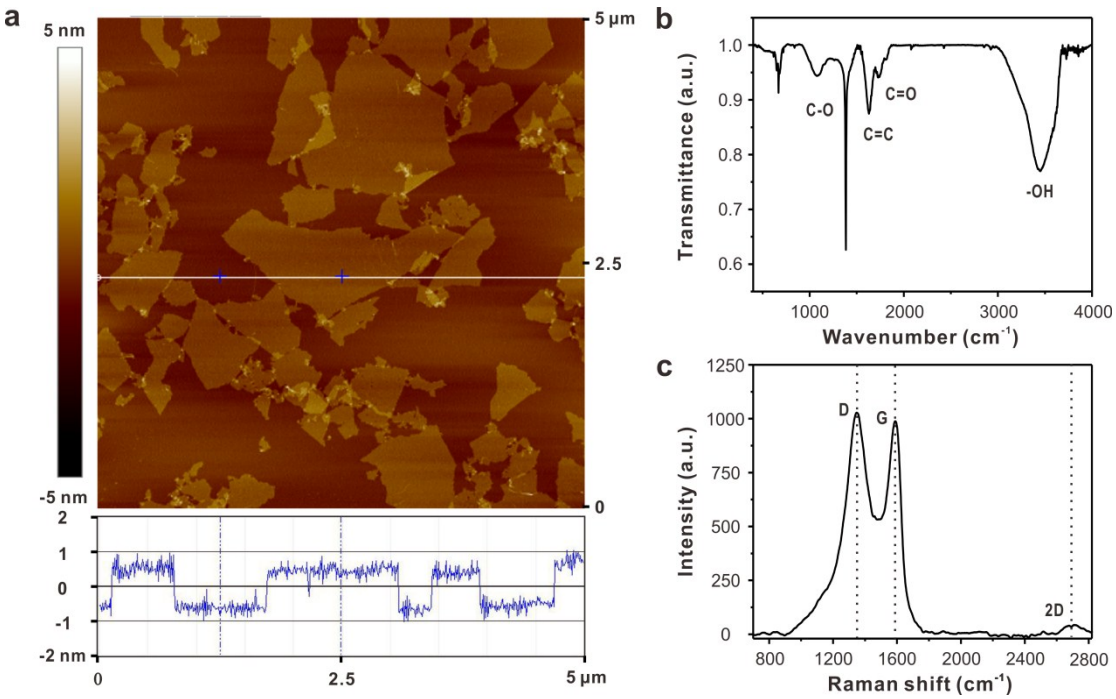

Figure S1 The characterization of GO. (a) AFM image of GO (b) The FT-IR spectrum and (c) Raman spectrum of GO.

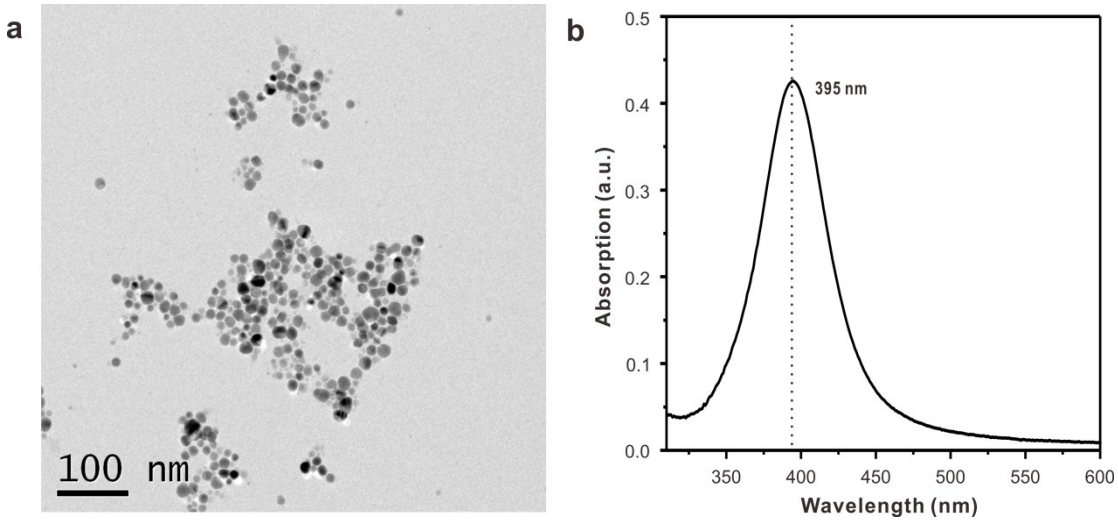

Figure S2 The characterization of AgNPs. (a) TEM image of AgNPs (b) The UV-vis spectrum of AgNPs.

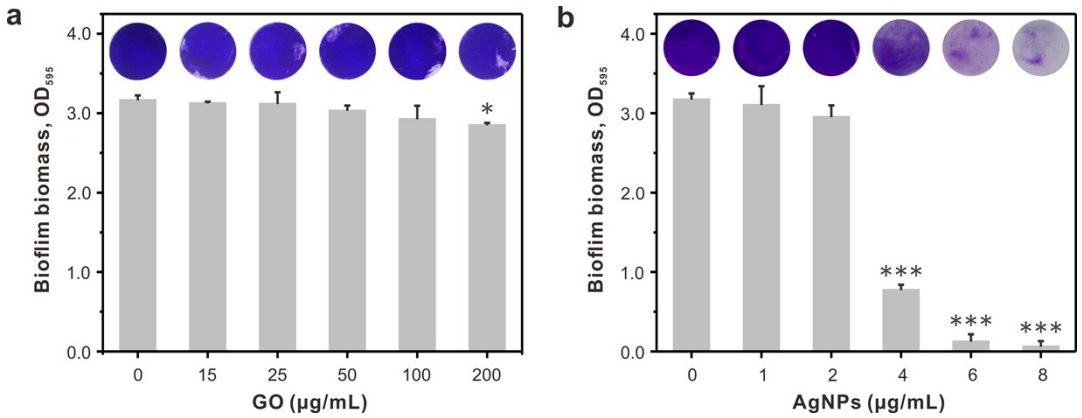

Figure S3. Biofilm formation treated with (a) GO and (b) AgNPs. All bacteria were incubated in LBNS media with nanomaterials for 12 h at 37 °C under static condition. The insert pictures are the digital images of biofilm staining with CV. * indicates significant difference at $p<0.05$ compared with control. *** $p<0.001$

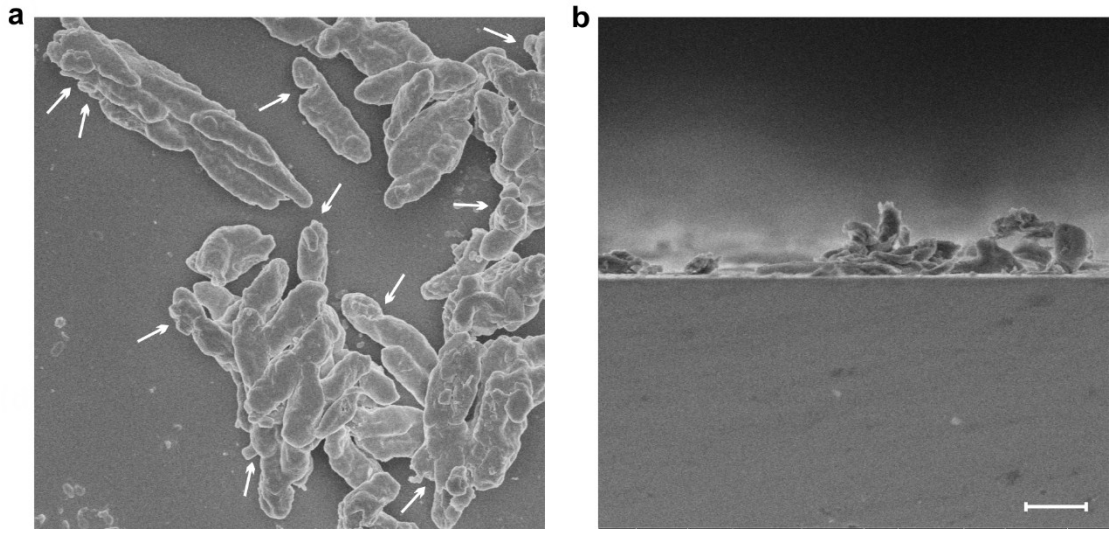

Figure S4. SEM images of biofilm exposed to 25 μg/mL GO-AgNPs. (a) Top and (b) Side view of biofilm for 24 h. Scale bar is 1 μm.

Table S1 Compared to mature biofilm untreated and treated with 15 μg/mL GO-AgNPs

Analyses	Control	15 μg/mL
Total biomass ($\mu m^3/\mu m^2$)	27.88±4.68	32.26±2.62
Cell biomass ($\mu m^3/\mu m^2$)	26.28±4.13	18.52±1.97
EPS biomass ($\mu m^3/\mu m^2$)	1.46±0.45	13.74±2.20
Substratum coverage (%)	99.20±1.60	98.47±2.40
average thickness (μm)	35.42±1.53	32.30±5.08
roughness coefficient	0.12±0.043	0.11±0.007

Data were shown as mean±s.d.

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