Electronic Supplementary Information

Silver carbonate nanoparticles stabilised over alumina nanoneedles exhibiting potent antibacterial properties.

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1. Material synthesis:

The mesoporous alumina support was synthesised by an aluminium hydroxide suspension which was obtained by hydrolysis of 43.8 g of aluminium tri-sec-butoxide with 10.3 g of deionised water and 275 g of 1-propanol. This was stirred for 1 hour at room temperature before 10.8 g of lauric acid was added. The mixture was aged for 24 h at room temperature and then heated under static air conditions at 110 °C for 2 days. The solid was then filtered, washed with ethanol and dried at room temperature. Finally the template was removed by calcined in air with samples heated to 500 °C for 3 hours with a ramp rate of 0.5 °C/ minute.

A series of mesoporous alumina supported silver particles of varying weight percents (wt %) were prepared by wet impregnation using $AgNO_3$ (>99% SigmaUltra). The relevant masses of precursor and support were added to 50 ml deionised water in 250 ml round bottomed flasks and stirred for 24 hours (300 rpm) in air at room temperature. They were then heated to 100 °C to remove the water for 24 h in an oven, and calcined at 500 °C in air for 3 h with a ramp rate of 20 °C/ minute. Final bulk Ag loadings were determined by elemental analysis (AA) on HF digested samples.

2. Material Characterisation:

a. Porosimetry/Surface Areas

Porosity and surface areas were determined on a Quantasorb Nova instrument by N_2 adsorption. Surface areas were calculated using the BET equation over the pressure range P/P_0 = 0.02–0.2, where a linear relationship was maintained, while pore-size distributions were calculated using the BJH model up to P/P_0 = 0.6. Surface areas of processed Ag-meso Al_2O_3 were 300 ± 20 m^2g^{-1} with an average pore diameter of 3.5 nm (**Figure S1**).

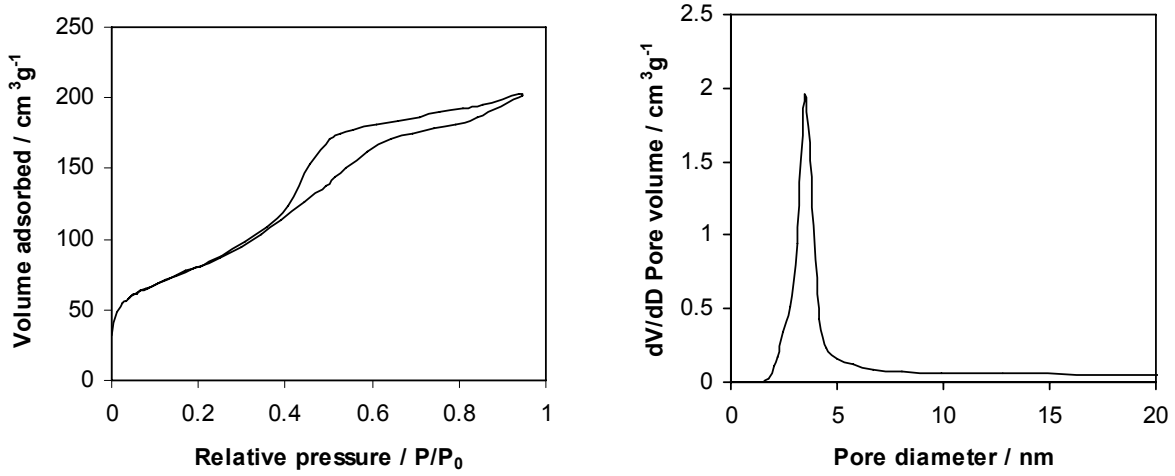

Figure S1: N_2 porosimetry on mesoporous alumina support.

b. Powder X-ray Diffraction (XRD)

Powder X-ray patterns were recorded using a Bruker AXS D8 diffractometer equipped with a Cu K_α X-ray source and LynxEye™ detector. Data were collected over a 2θ range of 10 – 70 ° with a step size of 0.02 ° and dwell time of 0.5 s per point. Low angle patterns were recorded between 2θ = 0.1 - 5°, with a step size of 0.01 ° and dwell time of 2 s per point. The low angle region in **Figure S2a** shows a peak at 2θ = 1.06 ° indicative of a crystalline matrix with pore repeats around 8.7 nm. In combination with the pore size distribution in **Figure S1** this indicates a wall thickness of 2.6 nm. The only peaks visible in **Figure S2b** by wide-angle XRD were at 46.6, 67.1 and 60.9 °, and characteristic of γ-Al_2O_3 (JCPDS 29-63) nanoneedles. Identical patterns were observed following Ag incorporation, with the absence of any silver phases indicating high silver dispersions with nanocrystal dimensions below 2-3 nm.

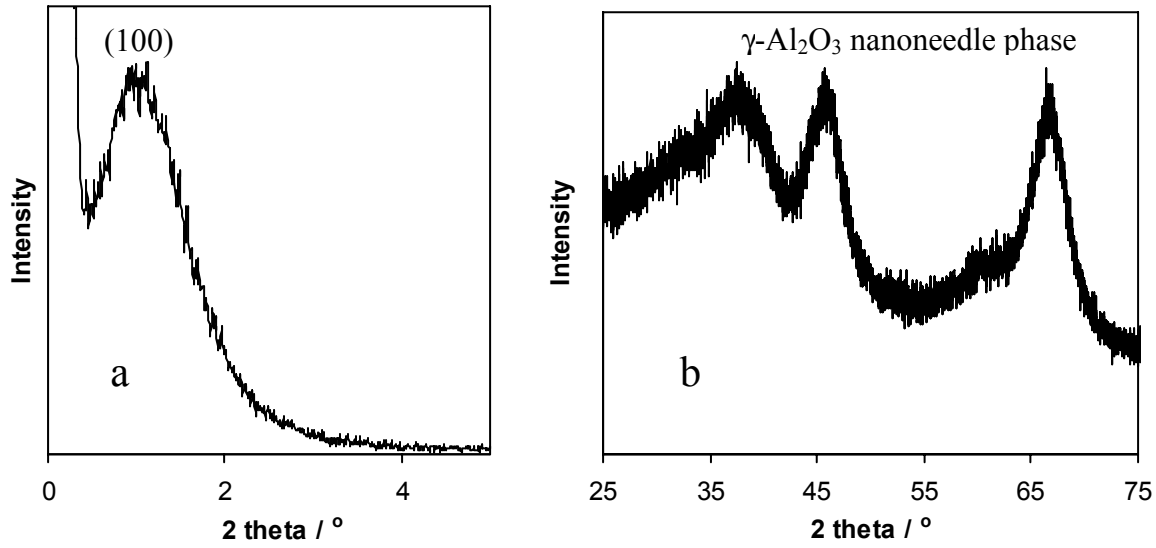

Figure S2: (**a**) low and (**b**) wide angle powder XRD of mesoporous alumina support.

c. X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectra were acquired at normal emission on a Kratos AXIS HSi spectrometer equipped with a charge neutralizer and Mg K_α excitation source (1253.6 eV). Binding energy referencing was employed using the adventitious carbon peak (285 eV) and valence band. Wide scans were recorded for surface elemental analysis (pass energy 160 eV), with high resolution spectra recorded at 40 eV pass energy. For the lowest loading sample, 50 scans were necessary to obtain good signal:noise statistics. Spectral fitting was performed using CasaXPS Version 2.3.5

Auger parameters (APs) were calculated from the following equation using the spectra shown in **Figure S3:**

AP = Kinetic Energy (Ag M_5NN Auger) + Binding Energy (Ag $3d_{5/2}$)

These values are tabluated in **Table S1**.

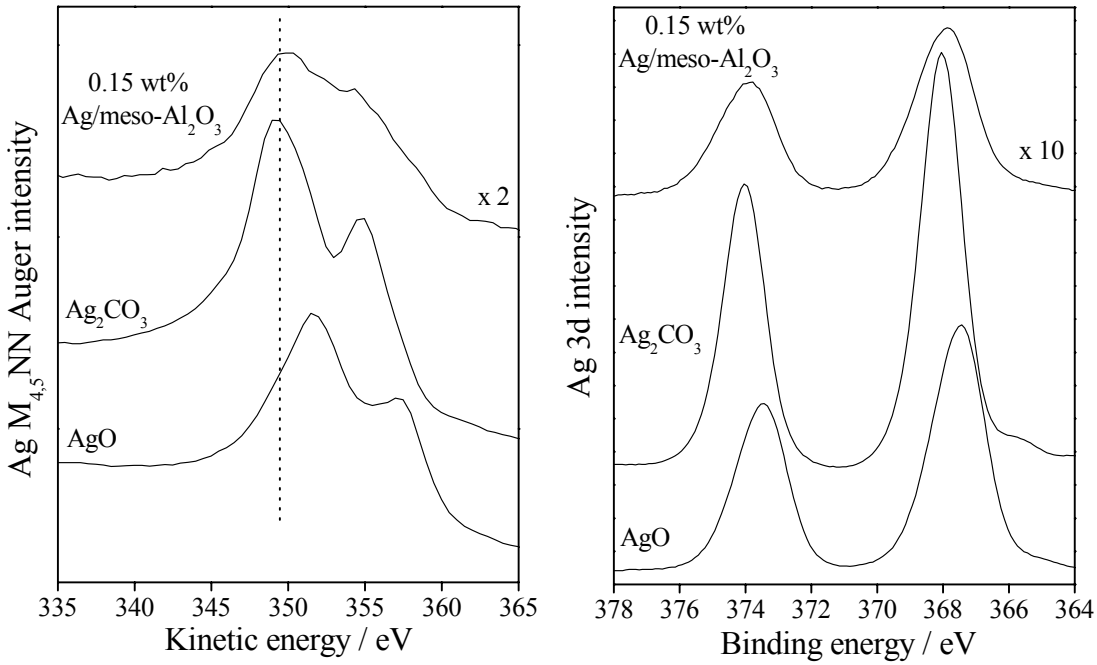

Figure S3: Ag $M_{4,5}$NN Auger and 3d XP spectra.

Table S1: Auger parameters for Ag meso-Al_2O_3 and standards calculated from Ag M_5NN Auger kinetic energy and Ag $3d_{5/2}$ binding energies.

Sample	**Kinetic energy Ag M_5NN Auger / eV**	**Binding energy Ag $3d_{5/2}$ / eV**	**Auger Parameter / eV**
AgO	354.76	364.46	719.22
Ag_2CO_3	352.21	365.04	717.25
0.15% Ag meso Al_2O_3	352.16	365.63	717.79

d. X-ray Absorption Near Edge Spectroscopy (XANES)

Ag K-edge XANES measurements were made on the XAFS beamline of the Elettra synchrotron facility, using a Si(111) double-crystal monochromator with a beam current/energy of 250 mA / 2 GeV. Transmission Ag K-edge spectra (25.5 keV) were acquired using powdered samples mounted in a stainless steel cell washers. Silver standards were diluted with BN to achieve absorbances around unity. Linear combination fitting of the Ag/meso-Al_2O_3 XANES data to Ag_2O, AgO , Ag_2CO_3 and Ag foil standards was undertaken using the ATHENA 0.8.050 application from the IFEFFIT Open Source software suite (http://cars9.uchicago.edu/iffwiki/Ifeffit). The resultant best fit shown in **Figure S4**, which reveals 85 % of all the silver within the ultra-dilute Ag/meso-Al_2O_3 material exists as the carbonate, with the remainder present in reduced form. Neither silver (I) or (II) oxide environments could be fitted. The invariance of nanoparticle composition with total silver loading for our mesoporous alumina supported materials is seen in **Figure S5**.

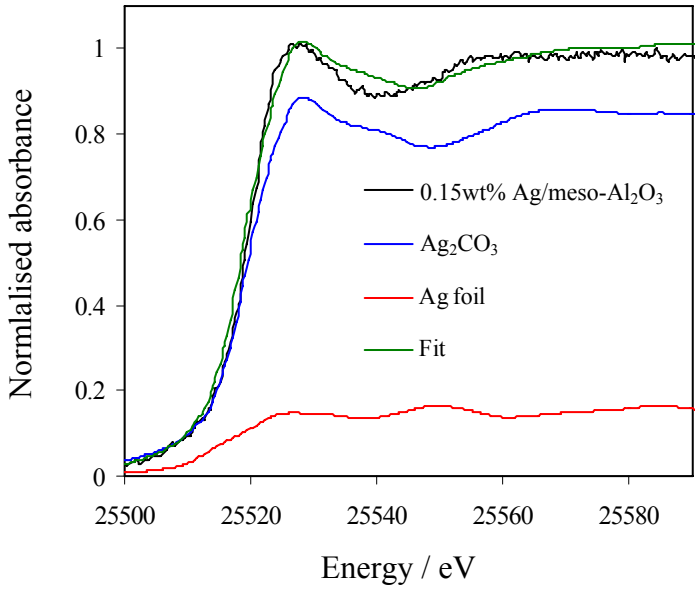

Figure S4: Normalised Ag K-edge XANES spectra for the 0.15 wt% Ag/meso-Al_2O_3 material showing best fit to silver standards.

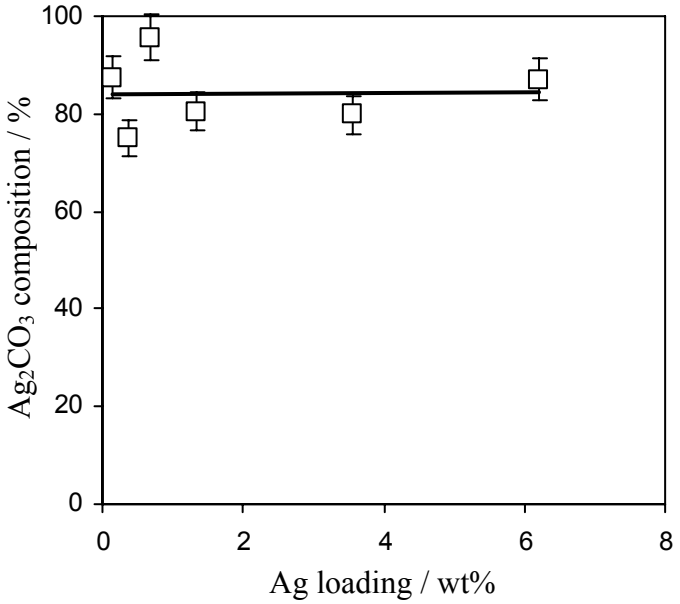

Figure S5: Fitted Ag K-edge XANES compositions across Ag/meso-Al_2O_3 series.

e. Electron microscopy

High-resolution transmission electron microscopy (TEM) was undertaken on the double-aberration corrected JEOL JEM 2200FS TEM/STEM within the York JEOL Nanocentre at 200 kV. Aberration correction gave improved resolution demonstrable down to 0.12 nm, and reduced image delocalisation, and was achieved through tableau alignment. Samples were supported on holey-carbon filmed copper grids. **Figure S6a** shows a standard bright field low-maginfication image of the alumina nanoneedles. **Figure S6b** shows a corresponding

scanning electron micrograph recorded on a JEOL JSM7000 Fabrika SEM, for which samples were sprinkled onto a 10 mm carbon self-adhesive pad placed onto 10 mm brass studs.

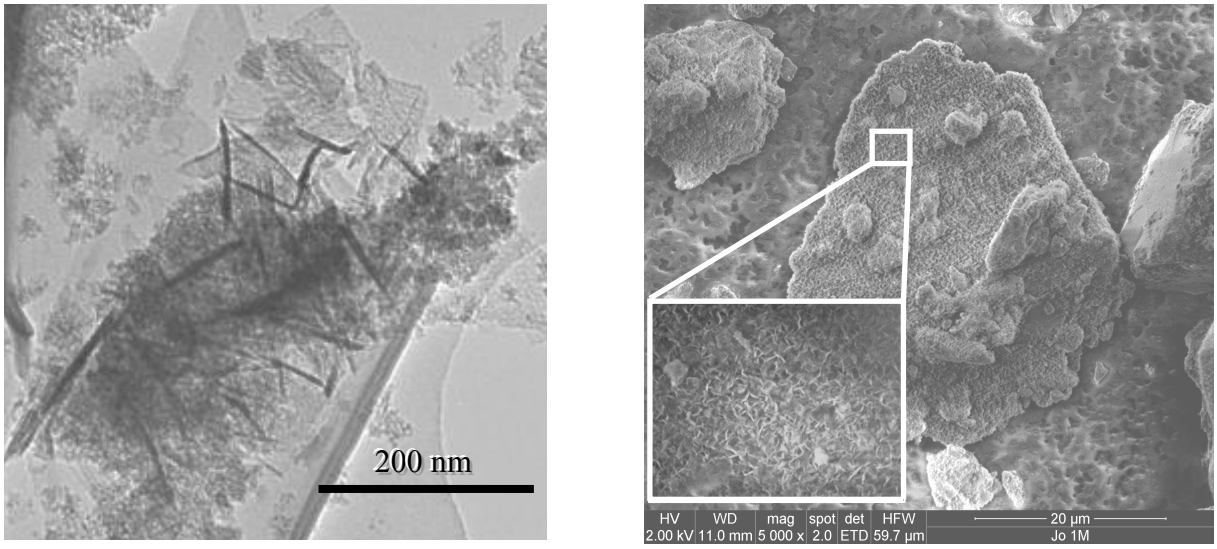

Figure S6: (**a**) High resolution TEM and (**b**) SEM images of 0.15 wt% Ag/meso-Al_2O_3.

3. Antibacterial testing:

a. Zone of Inhibition

Tests were performed against two bacterial strains, *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIMB 8626 - examples of Gram-positive and Gram-negative bacteria typically found in chronic wounds. Limited by diffusion, this test generates a zone of no growth around the sample if it inhibits bacteria.

The test organisms were harvested from overnight cultures grown at 37 °C on fresh triptone soya agar (TSA - soyabean casein digest agar formulation, widely used to cultivate microorganisms) slopes. Test organisms were harvested into 9 ml Ringers solution (containing sodium, iron and potassium) and washed twice. The test suspensions were adjusted to give a bacterial inoculum containing 10^8 colony forming units/ml (cfu/ml). The number of cfu/ml was confirmed by performing a 10-fold serial of the test suspension to 10^{-8} and plating the last three dilutions using the pourplate method. In the pourplate method, 1 ml of the required dilutions are pipetted into Petri dishes and ~20 ml TSA added. The plates are incubated at 37 °C for 24 h to allow growth of colonies, which can be manually counted and the original bacterial concentration deduced.

Zone plates were prepared by pouring 140 ml of Mueller Hinton agar (MHA - containing infusion of beef and acid hydrolysate of casein) into a 23x23cm assay plate. Once set, a second 140 ml of MHA was seeded with 1.4 ml of the 10^8 cfu/ml suspension of the corresponding test organism (final concentration 10^6 cfu/ml) which was mixed gently and poured over the previous layer. Once this layer has set, the plates were dried at 37 °C for 30 minutes with the lid removed. Using an 8 mm bore, corresponding wells were cut into the agar. The bore was dipped in 70 % ethanol before being heated to a high temperature to minimise contamination and the agar disks taken out with a sterile micro-spatula. 200 µl of sterile water was added to each 10 mg sample to be tested and pipetted into the wells. Assay plates were sealed with lab film and incubated for 24 h at 37 °C. **Figures S7a** and **S7b** show

the zones of inhibition observed with Ag standards and the Ag/meso-Al_2O_3 when tested with *Pseudomonas aeruginosa* NCIMB 8626 and *Staphylococcus aureus* NCTC 10788. Where the antimicrobial sample had diffused into the agar and inhibited bacterial growth, clear zones were observed and measured. **Figure S8** shows the zone (normalised to the mass of Ag in each sample to provide a fair comparison of their activity) as a function of Ag loading. The corresponding values obtained for bulk silver standards are given in **Table S2**. These results show the potency of the ultra-dilute Ag_2CO_3/meso-Al_2O_3 sample for bacterial inhibition.

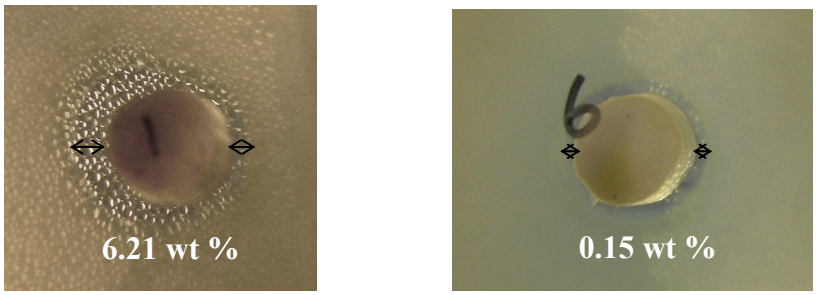

Figure S7a: Zones of inhibition for Ag/meso-Al_2O_3 with *Pseudomonas aeruginosa* NCIMB 8626. Zones shown for two different silver loadings.

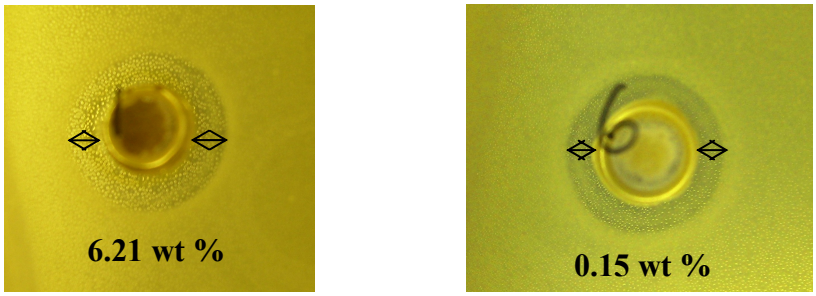

Figure S7b: Zones of inhibition for Ag/meso-Al_2O_3 with *Staphylococcus aureus* NCTC 10788. Zones shown for two different silver loadings.

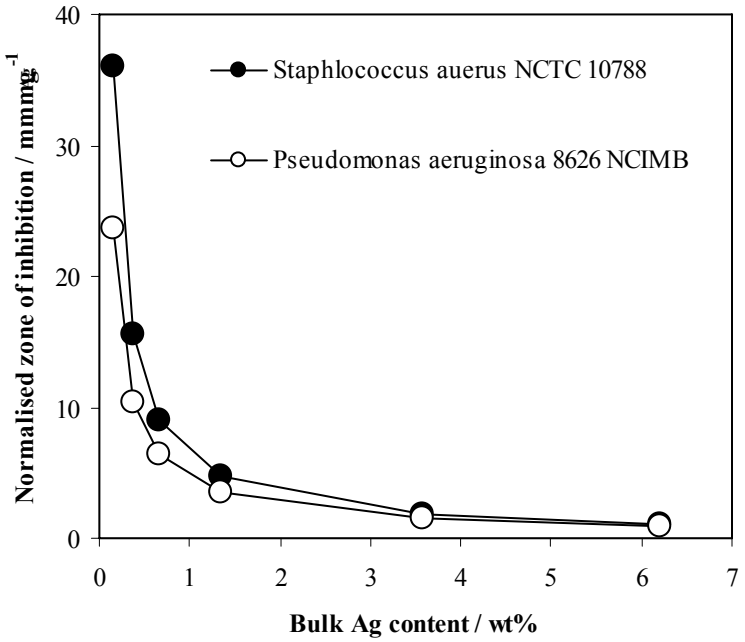

Figure S8: Normalised zone of inhibition for Ag/meso-Al_2O_3

Table S2: Normalised zones of inhibition for bulk silver standards

Standard	Bulk Ag content / wt%	Normalised Z of I / mm.mg^{-1}
Ag_2O	93	0.072
AgO	87	0.18
Ag_2CO_3	73	0.11

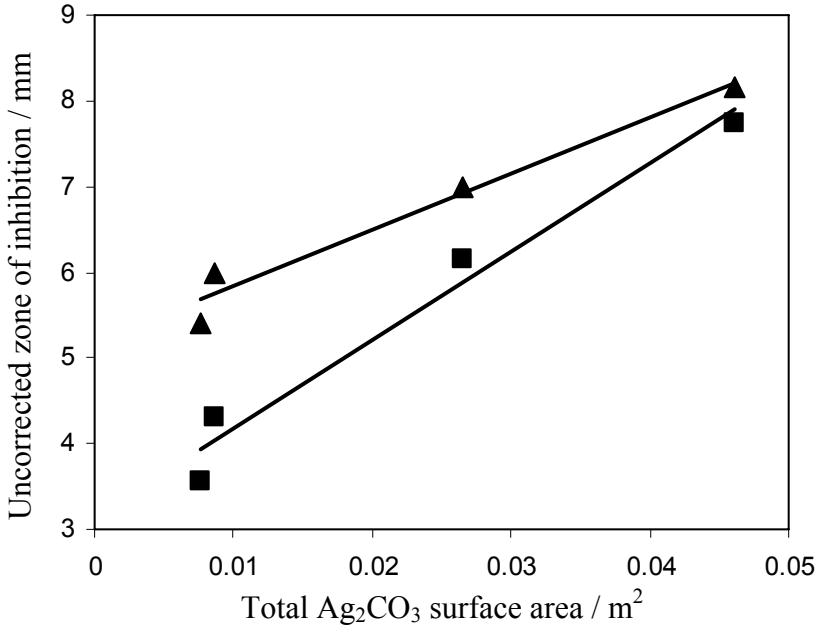

Figure S9: Linear dependence of zone of inhibition and total Ag_2CO_3 nanoparticle surface area for Ag/meso-Al_2O_3 samples and form a bulk Ag_2CO_3 standard: surface areas derived from mean TEM particle sizes and volume-averaged XRD particle sizes respectively.

b. Log reduction kill tests

S. aureus NCTC 10788 was harvested using the same method detailed above. The principal behind the microbial kill test involves adding a known number of microorganisms (the inoculum) to the product to be tested, and then recovering those microorganisms still present after a set period of time. The $\log_{10}$ number of microorganisms recovered is subtracted from that of the inoculum, and the ability of the product to kill microorganisms is thereby determined.

The *S. aureus* inoculum was prepared to contain 10^7 cfu/ml. In duplicate, 10 mg of the product was inoculated with 0.5 ml of the inoculum in sterile universals. These samples were incubated at 32 ° C for the length of time required (24, 72, 168 hours). At these set time points a neutralising agent was added- sodium thiosulfate (STS), (0.4% Sodium thioglycollate, 0.85 % Sodium chloride and 1 % Tween 20) , to instantly end the activity of the silver (validation of neutralisation was carried out previously, in order to ensure that the antimicrobial activity of the product is fully inhibited). The resulting solution was serially diluted to obtain a range of countable dilutions. A baseline time 0 h count is always carried out immediately following inoculation and is used to calculate the log reduction.

Following incubation, colonies are counted, multiplied back up and the original count present in the neat dilution deduced. The log count of microorganisms present at each time point can be subtracted from the log count present at time 0. This value represents the log reduction in numbers.

One log reduction is a 90 % decrease in the number of bacteria so it follows that the industry standard is a 3 log (99.9%) reduction in numbers of viable bacteria which is what is required to be classed as bactericidal. A value of approximately 0 indicates that the product is bacteriostatic whilst a negative log reduction value indicates that growth is actually occurring in the presence of the product. The bare support does not contribute to any of the microbial kill, as this was used as a control and generates a slightly negative log reduction.

c. Ag dissolution rates

A Nico 2000 Silver Ion-Selective Electrode (ISE) was employed for measuring silver ion concentrations in solution over various time points. Before use, the electrode was calibrated against a series of known standard solutions over the range 5, 10, 50, 100 and 1000 ppm made by serial dilution of a 1000 ppm standard solution.

Measurements were performed in a 0.1 M buffer solution of $NaNO_3$. In a typical experiment 200 mg of Ag-meso/Al_2O_3 was stirred in 25 cm^3 of deinionised water. 5 cm^3 aliquots of this sample was then periodically added to 25 cm^3 of the 0.1 M $NaNO_3$ solution and the Ag ppm concentration in the starting sample determined. A similar protocol was adopted for standards using the masses below tailored to offer the same maximal 50 mg silver in solution.

<u>Standards - 50 mg of Ag</u>
$AgNO_3$ = 78.74 mg
AgO = 57.47 mg
Ag_2O = 53.76 mg
Ag_2CO_3 = 64.10 mg
SSD = 151.5 mg