Supplementary Information

Silver Nanocoatings at Large Length Scales: Influence of the AgNPs Morphology and Capping Agents on the Coating Chemical Stability and Antimicrobial Effect

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Methods

AgNPs characterization

The light absorption spectra acquired for all AgNPs suspensions were recorded in a UV-visible light spectrophotometer (Thermo Scientific GENESYS™ 10s), by scanning the absorbance in 200-700 nm. The nanoparticles size and zeta (ζ) potential were calculated from the measured diffusion coefficient introduced in equations (for size and electrical mobility) derived from the Einstein-Smoluchowski relation. The diffusion coefficients were measured with the AgNPs in suspension in deionized water (1.0 mmol L^{-1} of Ag) by using dynamic light scattering (DLS), in a Zetasizer Nano ZS (Malvern Instruments, UK).

Image processing

A contrast value in an X-ray image pixel is originated from an element peak area in the EDS spectrum associated with that particular pixel. When downgrading the image definition in Mathematica (i.e. binning process in an image resize step), the resulting pixel contrast value corresponds to the averaged contrast values from adjacent pixels present in the raw-image. This binning process increases the precision of the comparative analysis performed, since it is used an averaged signal (i.e. contrast) between adjacent pixels. Through this approach, the quantification of spurious signal is avoided.

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In order to obtain precise quantitative morphological information at scales below 1.0 μm, it must be used high magnifications and long beam dwell times for obtaining individual scans. The resolution can be also increased in high-vacuum mode (as the Si substrate does not have a high electron conductivity, the LF scan in this work was performed in the low-vacuum mode).

For localizing the AgNPs and their agglomerates (Figure 2a), the X-ray images (i.e. Ag elemental maps) were rescaled in grayscale, i.e., the pixel in the map that presents the largest contrast value is converted to value "1"; the pixel in the map that presents the smallest contrast value is converted to value "0"; and all other pixels are converted to proportional and intermediate values between 0 and 1. Further, the elemental map was binarized from a threshold value of 0.15. Thus, smaller contrast values are converted to value "0" (a black pixel, indicating the absence of the element in that pixel), and contrast values similar to or larger than 0.15 are converted to value "1" (a yellow pixel, indicating the presence of the element in that pixel; Figure 2a). The choice for this threshold value (0.15) was made based on a qualitative evaluation that compared electron micrographs with X-ray images in order to precisely identify a numerical value that minimizes artifacts that might come from fluctuations in the contrast function (an example of this threshold evaluation was previously provided by our group).[1]

In the concentration analysis (relative amount of AgNPs; Figure 2b), the X-ray image matrix numerical values (i.e. contrast values given in grayscale, with values varying from 0 to 1 for each pixel; 8-bits/channel; 2465 × 2465 pixels) were summed (i.e. integrated) and the result is given as intensity counts values. All Ag elemental maps had their image contrast values normalized in regard to Si, which is the standardized element in all samples. This calculation was performed for at least three elemental maps acquired from at least three different samples, thus resulting in the mean and standard deviations values shown in Figure 2b.

For determining the agglomerate sizes (Figure 2c), all element clusters equal to and larger than 1 pixel (ca. 2 μm^2) were identified in the binarized LF Ag image (Figure S6), and their equivalent areas were determined. Histograms were further generated simply by organizing these area values for each sample. This calculation was performed for at least three elemental maps acquired from at least three different samples, thus resulting in the mean and standard deviations values shown in Figure 2c.

X-ray photoelectron spectroscopy of the SiO_2/Si substrate

The analysis of the surface microchemical environment of the Si substrate used in the formation of AgNPs coating was carried out in a K-Alpha X-ray photoelectron spectrometer (Thermo Fisher Scientific, United Kingdom) equipped with a hemispherical electron analyzer and an aluminum anode (Kα = 1486.6 eV) X-ray source. The high-resolution spectrum was recorded using pass energies of 200 and 50 eV.

Adherence and antibacterial assays

Silicon substrates containing the AgNPs coatings were sterilized by UV light for 30 min (15 min each side). For bacterial growth, 125 μL of a *Staphylococcus aureus* (ATCC 25913)

suspension matching 7 on McFarland nephelometric scale were placed into 875 µL of BHI broth in a 24-well cell culture plate, resulting in a bacterial concentration of approximately 2.6×10^8 bacteria mL^{-1}. The substrates were then placed at the bottom of individual wells, immersed in the bacteria suspension for 24 h at 35 °C. After incubation, each silicon substrate was washed 3 times by PBS-1x solution. Assays were performed at least in triplicate.

Confocal laser scanning microscopy (CLSM) analyses were performed in order to evaluate the cell viability (after 24 h at 35 °C) on AgNPs coated-substrates. BacLight Live/Dead viability kit (Molecular Probes, USA) was used to access the bacterial distribution and viability. After the incubation and washing processes, 2 drops of the live/dead staining solution were placed onto each silicon substrate, and they were incubated for 15 min in the dark. Each stained silicon substrate was then washed once with a 0.9% (m/v) saline solution, in order to remove loosely attached bacteria. The lasers wavelengths used for excitation were 488 nm (for SYTO9) and 543 nm (for propidium iodide), and the emission was monitored at 485-515 nm for SYTO9 and 620-650 nm for propidium iodide. Images were acquired in a Zeiss LM710 microscope using a 50× objective. A large-field (LF) scanning (tile scanning mode) was performed in order to examine a 1 × 1 mm substrate area, which is equivalent to 36 adjacent individual scans. Resulting images had 6144 × 6144 pixels (12-bits/channel). Image processing for these images were the same used to identify and count AgNPs in LF-SEM.

Results

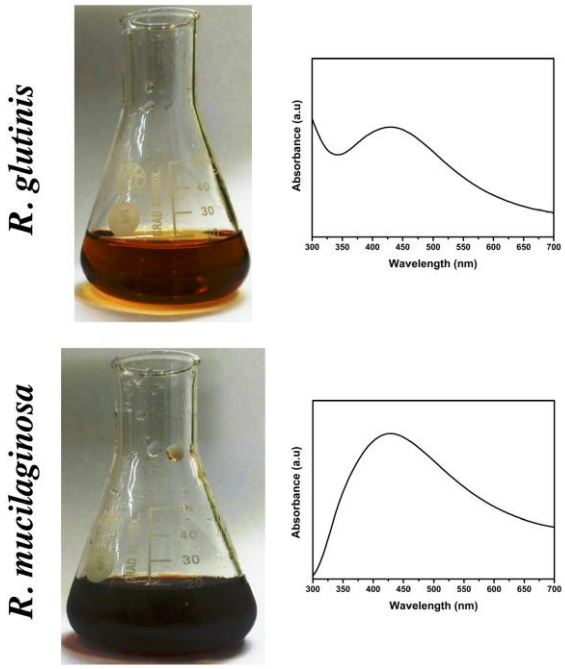


Figure S1. Fungi extracts used in the AgNPs synthesis (first column), and UV-Vis absorption spectra of the AgNPs formed in the fungi extract after 168 hours of synthesis (second column).

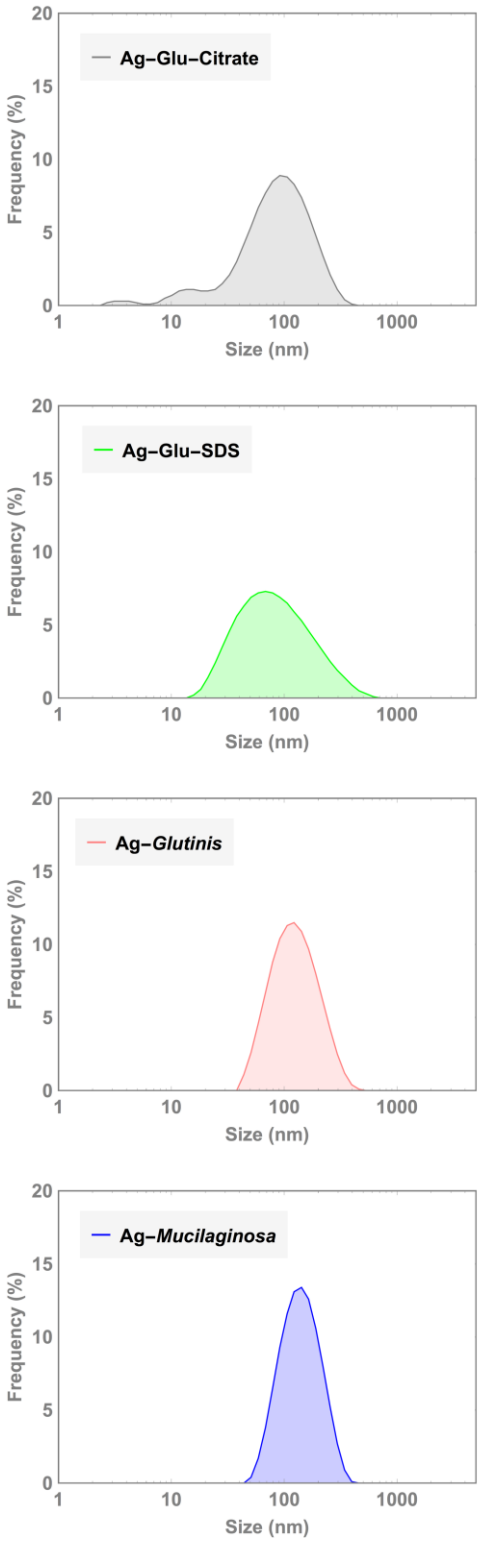


Figure S2. Nanoparticle size distribution determined by dynamic light scattering (DLS) for synthetic AgNPs produced from glucose and capped with citrate (Ag-Glu-Citrate) and sodium dodecyl sulfate (Ag-Glu-SDS), and for biogenic AgNPs produced from the extract of *R. glutinis* (Ag-Glutinis) and *R. mucilaginosa* (Ag-Mucilaginosa).

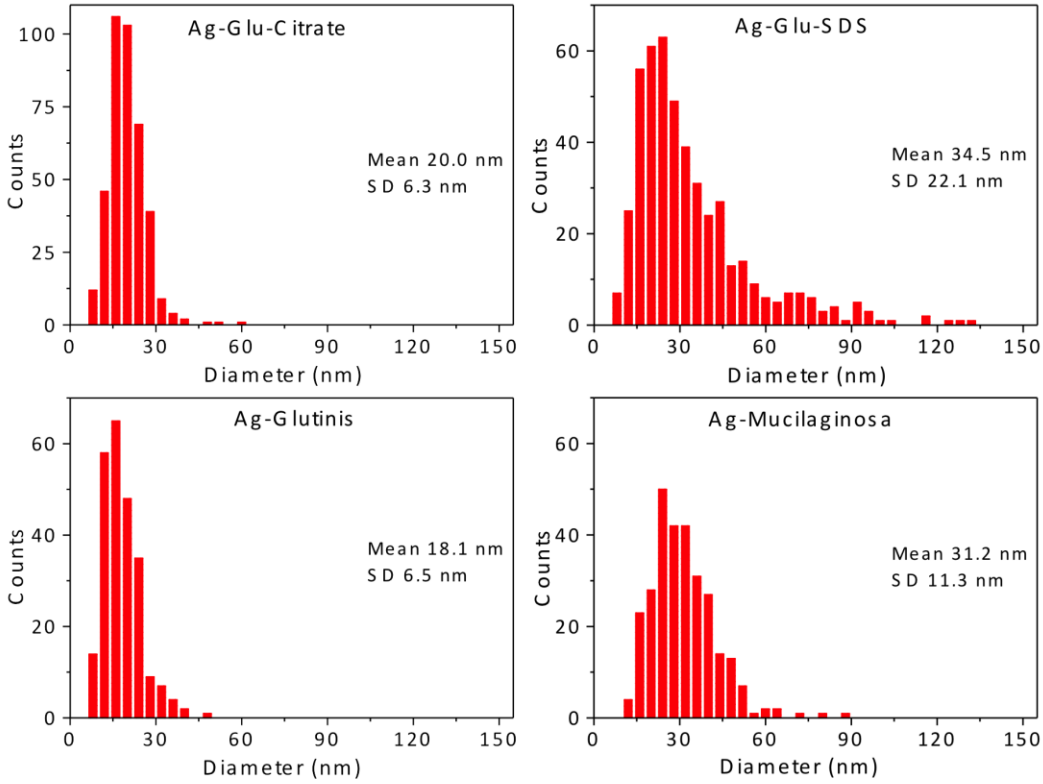


Figure S3. Histograms (distribution) of the AgNPs sizes measured for more than 200 particles in SEM images.

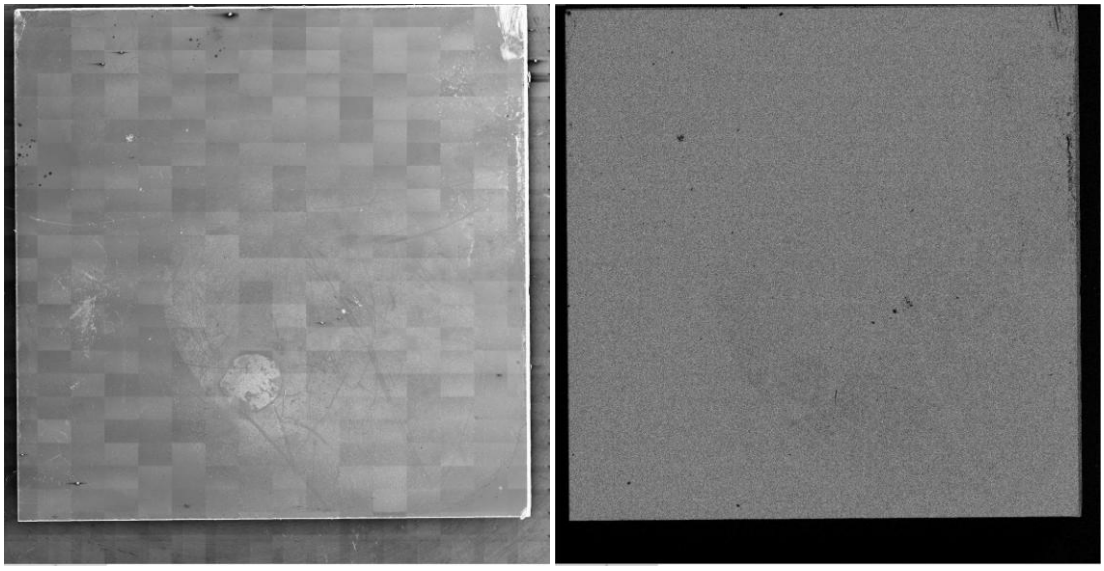


Figure S4. An example of a raw large-field (LF) micrograph from secondary electrons (left column) acquired after scanning the whole silicon substrate (5 × 5 mm). Right column: LF Si map (LF X-ray image) assembled from EDS signal after the large field scan.

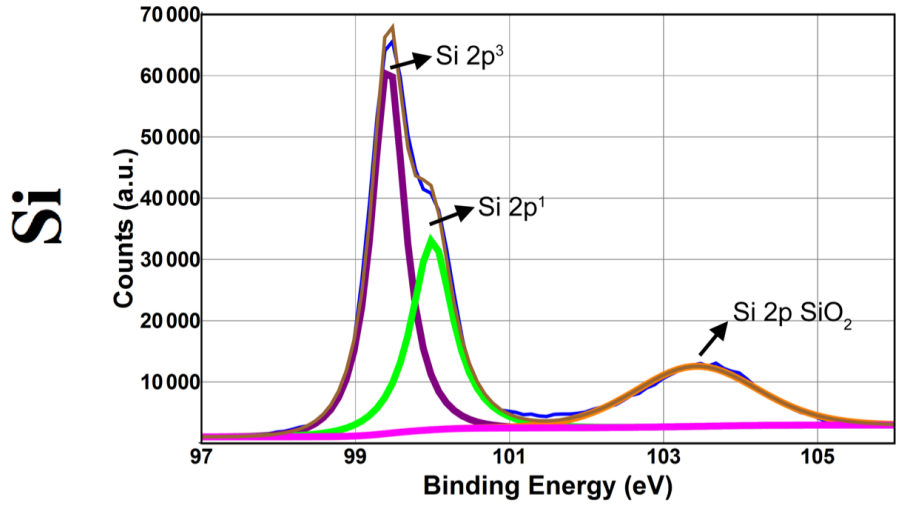


Figure S5. X-ray photoelectron spectrum (XPS) of the Si substrate covered with a SiO_2 layer (< 5 nm).

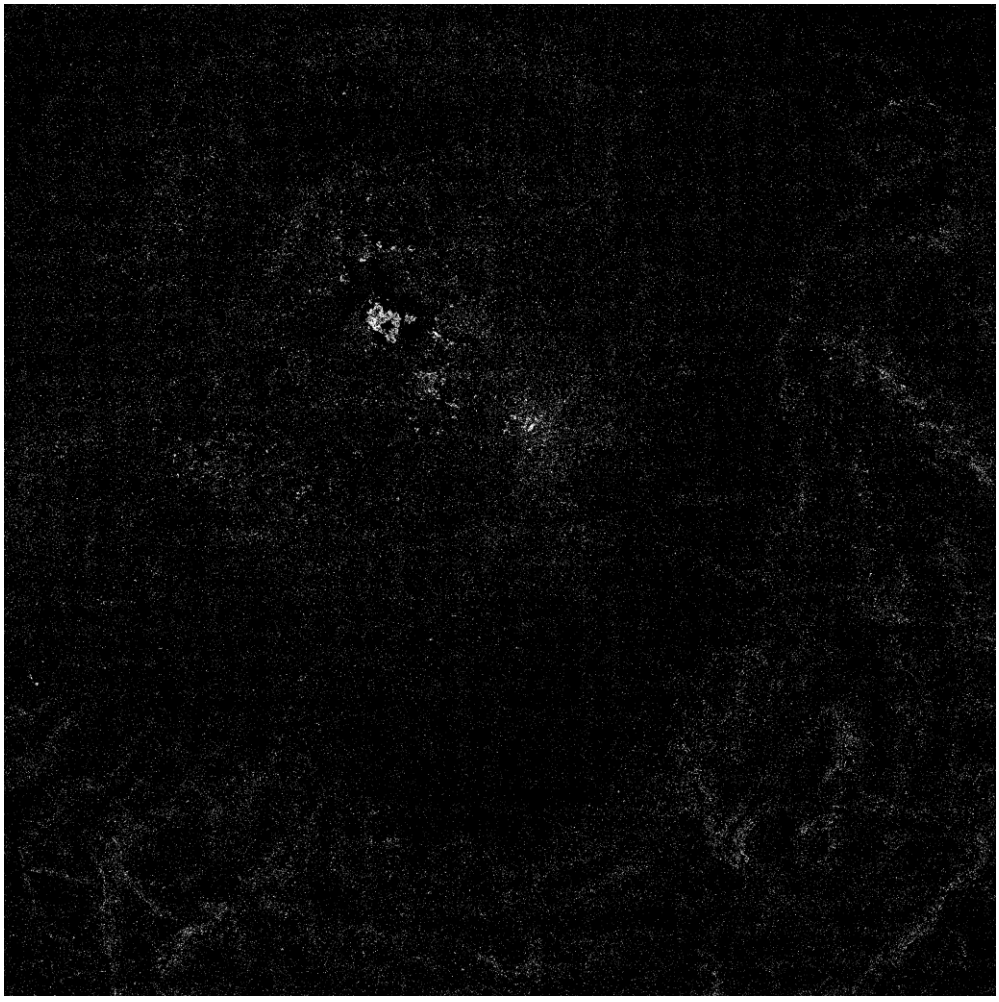
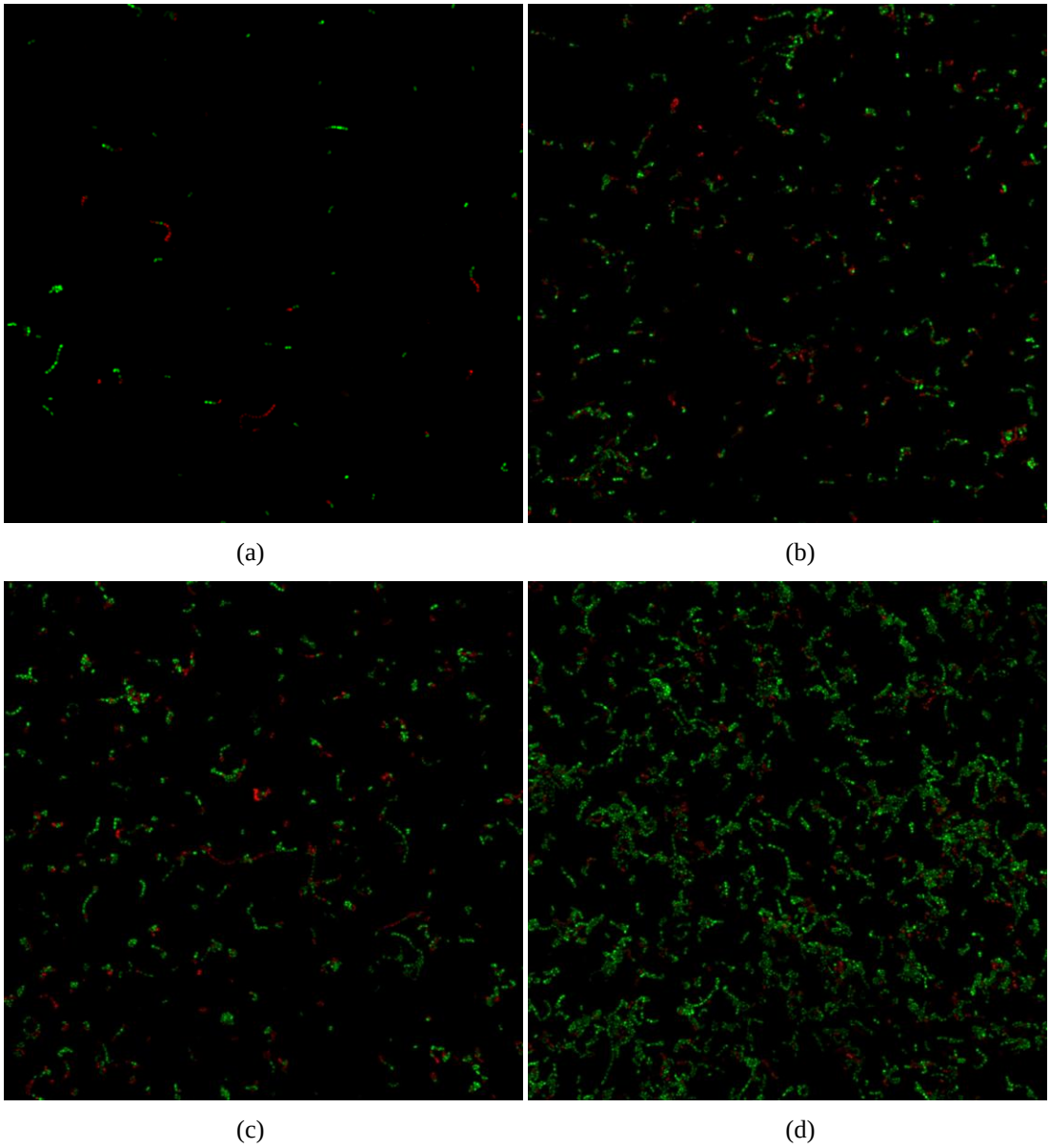


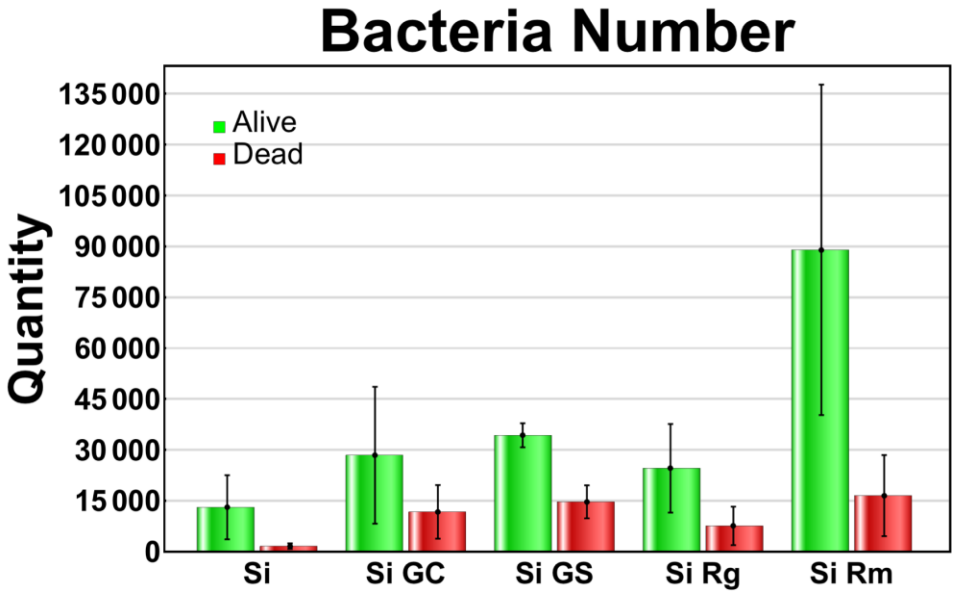
Figure S6. An example of a binarized LF Ag map in which AgNPs agglomerates were identified (3.6 × 3.6 mm; definition of 2465 × 2465 pixels).



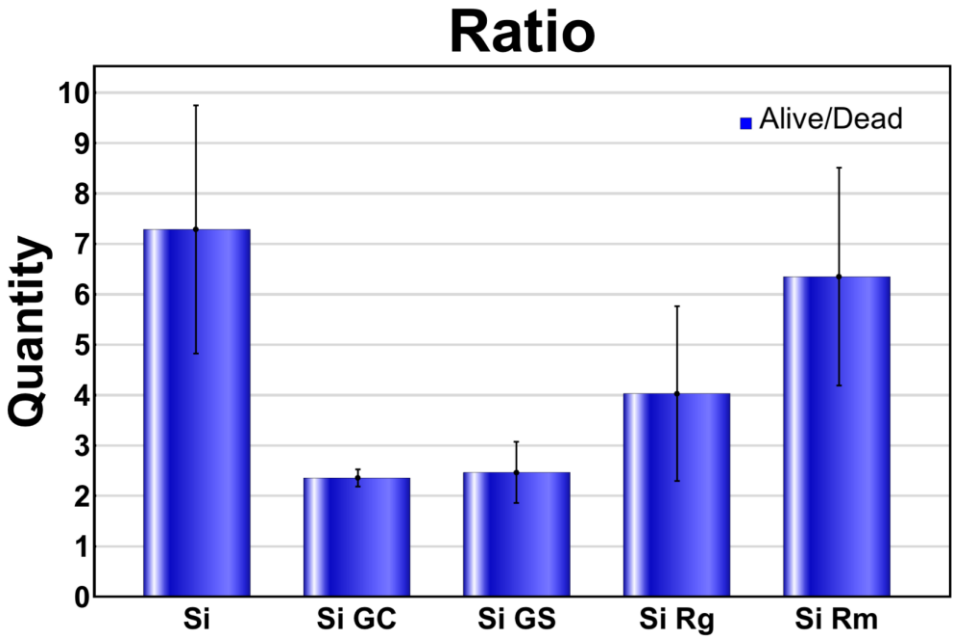
(a) (b)

(c) (d)

Figure S7. Example of single scans acquired through confocal laser scanning microscopy (CLSM) for live (in green) and dead (in red) bacterial cells of *Staphylococcus aureus* adhered on coatings formed on SiO_2/Si substrates. Coatings were produced by using synthetic ((a) Ag-Glu-Citrate and (b) Ag-Glu-SDS) and biogenic ((c) Ag-Glutinis and (d) Ag-Mucilaginosa) silver nanoparticles.



(a)



(b)

Figure S8. (a) Number of live and dead bacteria, and (b) live/dead ratio for bacterial cells of *Staphylococcus aureus* adhered on raw SiO_2/Si substrates (Si), and adhered on coatings formed on SiO_2/Si substrates by using synthetic (Ag-Glu-Citrate [Si GC] and Ag-Glu-SDS [Si GS]) and biogenic (Ag-Glutinis [Si Rg] and Ag-Mucilaginosa [Si RM]) silver nanoparticles. This calculation was performed for bacterial cells identified in large-field confocal laser scanning microscopy (LF-CLSM) images, which comprised a substrate area of 1 × 1 mm (36 adjacent fields assembled in a single image; in Figure S7 there are examples of single fields for each sample).

Reference

1. Oliveira, N. C.; Silva, J. H.; Barros, O. A.; Pinheiro, A. P.; Santana, W.; Saraiva, A. A. F.; Ferreira, O. P.; Freire, P. T. C.; Paula, A. J.; *Anal. Chem.* **2015**, *87*, 10088.