Electronic Supplementary Material (ESI) for Chemical Communications

Self-Assembled Monolayers of Gold Nanostars: a convenient tool for Near-IR Photothermal Biofilm Eradication

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ELECTRONIC SUPPLEMENTARY INFORMATION

S1) MATERIALS AND METHODS

Reagents and solvents. N-Dodecyl-N,N-dimethyl-3-ammonium-1-propanesulfonate (LSB), Tetrachloroauric acis (99%), Sodium Boronhydride, L-ascorbic acid, Silver nitrate (>99%), 3-mercaptopropyl-trimethoxy silane (MPTS), dimethylformamide, n-heptane were purchased from Sigma-Aldrich and used as such. Toluene, ethanol (99%), n-butanol, acetonitrile, ethyl acetate were purchased from Carlo Erba.
Glass slides. FORLAB glass coverslides (rectangular, 21x26 mm ; circular Ø = 10 mm) were purchased from Carlo Erba. Indium-Tin oxide (ITO) coated glass slides (24x24 mm, thickness 1 mm, surface resistivity 70/100 Ω/sq and 8-12 Ω/sq) were purchased from Sigma-Aldrich

GNS synthesis. Synthesis was carried out according to a described method (ref 5a, main text). In brief, a seed solution (sol **A**) is prepared in a 20 mL vial by adding 5.0 mL of 0.20M LSB solution in waterand 5.0 mL of $HAuCl_4$ $5x10^{-4}$M in water (the pale yellow color of diluted $AuCl_4^-$ is obtained). Then 600 μL of 0.01 M ice-cooled $NaBH_4$ solution in water are added. The vial is gently hand-shaken for 10 seconds and the solution assumes the brown-orange color of Au nanoseeds. This solution is used in a 20 min < t < 180 min interval from preparation. Growth solution (sol **B**) is prepared in 20 mL vial by using 5.0 mL of 0.2 M LSB solution in waterand 180 μL $AgNO_3$ 0.004M in water. To this solution, 5.0 mL of aqueous 0.001M $HAuCl_4$ are added (obtaining a pale yellow color) after gentle mixing. A volume of 0.0788 M aqueous ascorbic acid solution is then added (volume in the 70μL – 300 μL range: the concentration of ascorbic acid regulates the aspect ration of the GNS branches and consequently their LSPR position). The solution becomes colorless after few seconds of gentle mixing, and is immediately used for the next step.
A 12 μL volume of sol **A** is added to the whole sol **B** and the vial is gently hand-shaken for few seconds. A grey-blue colour immediatly appears, whose intensity rapidly increases. Solutions are allowed to react in the still vials for 1 h, yielding the colloidal solutions **C**, containing GNS.

Glass|MPTS slides preparation (and analogous surfaces). We described and discussed in detail the preparation in P. Pallavicini et al. J Colloid Interf. Sci, 2009, 332, 432-438. Briefly: prior to MPTS grafting, glass slides (or ITO slides, or the internal walls of a glass cuvette) were treated with piranha solutions (3:1 v/v H_2SO_4 96% and H_2O_2 30%) for 30 minutes. Then, the slides were dipped in bidistilled water and sonicated for 3 minutes (the water is then discarded and the sonication cycle is repeated 2 more times). The washed slides were dried in an oven (140 °C) for 1 h, then allowed to cool to room temperature in air. Then, inside a 8-position staining jar for microscopy (if using rectangular slides, see Figure S1, right), or inside single-position conical test tubes (if using circular slides, see Figure S1, left), the slides are fully immersed in a 5% (v/v) solution of MPTS in toluene and allowed to react for 4 hours on a reciprocating shaker thermostated at 40 °C. After this time the toluene solution is removed and a 3-cycles washing procedure is carried out by sonicating for three minutes the jar (or the test tube) with i) pure toluene; ii) 1:1 v/v toluene/ethanol; iii) pure ethanol. Finally, the slided into the jar (or test tube) are dried for 30 minutes in a N_2 flux.
Random samples (~1 out of 20) were checked with contact angle measurements and with the colorimetric method described in the J. Colloid Interf. Sci. paper of 2009 to determine the –SH surface density, finding the expected values for regular preparations (c.a. 61°-64°; surface density 1.1-1.4x 10^{14}/cm^2 –SH units)

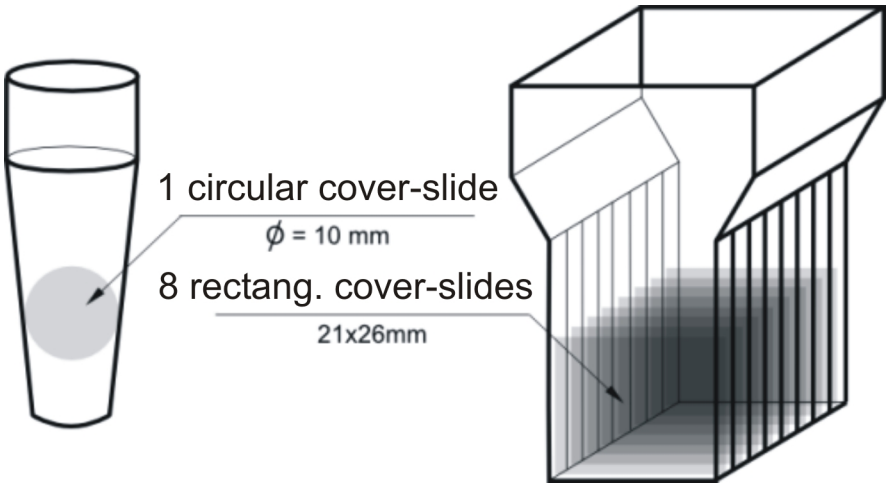


Figure S1. Sketch of the glassware setup used to prepare coated surfaces

Glass|MPTS|GNS slides preparation (and analogous surfaces). The glass|MPTS surfaces prepared from the previous step are placed standing in a 8-position jar or 1-position test-tube (previously washed with piranha solution as already described) and fully immersed in the chosen GNS solution (sol **C**) and allowed to stand still for the chosen coating time.
The typical coating time for a standard preparation is 18 hours. When carrying out kinetical experiments, 8 slides were used and taken off one by one from the coating solution at the chosen time, and then analyzed (ICP, absorption spectra).
The coated samples were washed three times in bidistilled water, sonicating for 3 minutes.
The washed samples were kept in air, in the jar were they were prepared, before any other use.
Their absorption spectra did not change in three months.

Total gold analysis (by ICP OES). Each analyzed slide was placed on the bottom of a 50 ml becker and fully coated with 3.0 mL of aqua regia (diluted with bidistilled water 4:25 v/v). The becker was closed with parafilm and allowed to react overnight on a reciprocating shaker, at room temperature. The blue color of the GNS completely disappeared after few minutes, due to oxidation. The Au content was then analyzed by stadard techinques by ICP-OES, as we have already described (ref 10a, main text). Analysis was carried out both on Au and Ag. We have already found for the GNS colloidal solutions (ref 5a) that some Ag is randomly distributed in the GNS lattice, with an atomic percentage of 7-8%. The same % value was found for the grafted GNS in glass|MPTS|GNS materials.

TEM samples preparation. 10 mL of the GNS colloidal solutions (sol **C**) were ultracentrifuged for 12 min at 13000 rpm. The supernatant was discarded and the GNS cake easily redissolved in 10.0 ml bidistilled water. The solution was further diluted (1:10 v/v) with bidistilled water and 10 µL were dropped on a Nickel grid (300 mesh) coated with a 139 Parlodion membrane. The samples were vacuum dried for 2 hours before TEM imaging.

SEM samples preparation. Glass|MPTS|GNS samples were prepared on a ITO slide (24x24 mm, thickness 1 mm, surface resistivity 8-12 Ω/sq) with the usual procedure. The morphologies of Gold nanostar slides were observed under Tescan Mira XMU variable

pressure Field Emission Scanning Electron Microscope – FEG SEM (Tescan USA Inc., USA). Slides were mounted onto Aluminum stubs using double sided carbon adhesive tape and were then made electrically conductive by coating in vacuum with a thin layer of gold (5 nm). Observations were made in backscattered electrons mode (BSE) at 30 kV and with InBeam se condary electron detector for higher spatial resolution.

Bacterial strain and culture condition. The microorganism used in this study was a methicillin-resistant *S. aureus* LP strain (ref 8c main text) which was showN to be a good biofilm producer. *S. aureus* LP was a clinical isolate provided by the Department of Clinical Surgical, Diagnostic And Pediatric, University of Pavia, Pavia, Italy. The strain was routinely grown overnight in tryptic soy broth (TSB) (Difco, Detroit, MI, USA) under aerobic conditions at 37°C using a shaker incubator (New Brunswick Scientific Co., Edison, NJ, USA).

Biofilm growth and cell viability. For biofilm growth (ref 8c main text), an overnight culture of *S. aureus* LP was diluted at 1:50 in TSB containing 0.25% glucose. Aliquots (500 µL) of the diluted bacterial suspension were inoculated into 24-well microplates (Costar; Corning, Inc., NY) containing plain glass slides or GNS-coated glass slides (specimens) and incubated for 16 hours at 37°C. The formed biofilms on the glass slides or on the specimens were untreated or laser-treated for the indicated times. Then, the biofilms were scraped carefully, sonicated, and then vortexed for 20 seconds to homogenize the samples. The samples were serially diluted, plated on the TSB agar plates, and incubated for 24 hours at 37°C. For each set of measurements, control was consisting of biofilms grown on glass slides and unexposed to laser. Cell survival was expressed as the percent of the CFU from bacteria grown on glass exposed to laser, specimens unexposed and laser exposed to the CFU from bacteria grown on unexposed glass (set as 100%). Noticeably, similar experiments were carried on specimens prepared 3 months earlier, obtaining similar results.

CLSM studies. CLSM studies aimed at detecting the viability of bacteria were performed on biofilm grown for 24 hours on each type of samples. Briefly, 500 µL of diluted cell suspensions were dispersed into 48-well microplates (Costar) containing glass coverslips and glass coverslips specimens and incubated for 24 hours at 37°C. Then, the biofilms were untreated or laser treated for indicated times. To determine the viability of bacteria within the biofilms, a BacLight Live/Dead viability kit (Molecular Probes, Eugene, OR, USA) was used as previously described (ref 13, main text). The kit includes two fluorescent nucleic acid stains: SYTO9 that penetrates both viable and nonviable bacteria, and propidium iodide that penetrates bacteria with damaged membranes and quenches SYTO9 fluorescence. Dead cells, which take up propidium iodide, fluoresce red, and cells fluorescing green are deemed viable. For assessing viability, 1 µL of stock solution of each stain was added to 3 mL of PBS and, after mixing, 500 µL of the solution was dispensed into 24-well microplates containing biofilms and incubated at 22°C for 15 minutes in the dark. Stained biofilms were examined under a Leica CLSM (model TCS SPII; Leica, Heidelberg, Germany) using a 40x oil immersion objective. The excitation and emission wavelengths used for detecting SYTO9 were 488 and 525 nm, respectively. Propidium iodide was excited at 520 nm, and its emission was monitored at 620 nm. Optical sections of 0.9 µm were collected from the complete thickness of the biofilm, and for each sample, images from three randomly selected positions were acquired. The resulting stacks of images were analyzed using Leica confocal software.

Statistical methods for biological experiments. Continuous data were expressed as

means and SD. Two group comparisons were performed with Student's t-test. All analyses were performed using GraphPad Prism 4.0 (Graph Pad Software Inc., San Diego, CA, USA). Two-tailed p values <0.05 were considered statistically significant.

Laser irradiation. The light source used for the samples' irradiation was based on the use of a multimode AlGaAs laser diode (model L808P200, by Thorlabs GmbH), characterized by a maximum output power of 200 mW, and emitting light at the wavelength of about 808 nm. A common feature of the laser beams emitted by semiconductor lasers is to have a quite large beam divergence and a significant astigmatism [*Orazio Svelto, Principles of Lasers, Springer*]; in the considered case the beam divergence in the two principal transverse directions is 10° and 30°. In order to reduce beam divergence, and to correct for the astigmatism, it is sometime possible to combine a cylindrical and a spherical lens; however, as the used source was multimode (i.e. it is not possible to describe the light-intensity distribution as a Gaussian curve) this solution is not very effective, and in addition the presence of two lenses along the optical path would introduce an unavoidable loss of the 15% of the optical power, which is generally not desirable.
Given the multimode light-intensity distribution, and the high beam divergence, we decided to irradiate the samples from the bottom of the culture plate and not from the top (see Figure), as done in other experiments [*M.S. Sbarra, A. Di Poto, E. Saino, L. Visai, P. Minzioni, F. Bragheri, I. Cristiani, "Merocyanine-540 Mediated Photodynamic Effects on Staphylococcus epidermidis Biofilms" European Conference on Biomedical Optics (ECBO), Munich, Germany, May 2009; N. Bloise, E. Saino, F. Bragheri, P. Minzioni, I. Cristiani, M. Imbriani, L.Visai "In vitro analysis of low - level laser irradiation on human osteoblast-like cells proliferation" European Conference on Biomedical Optics (ECBO), Munich, Germany, May 2011*].

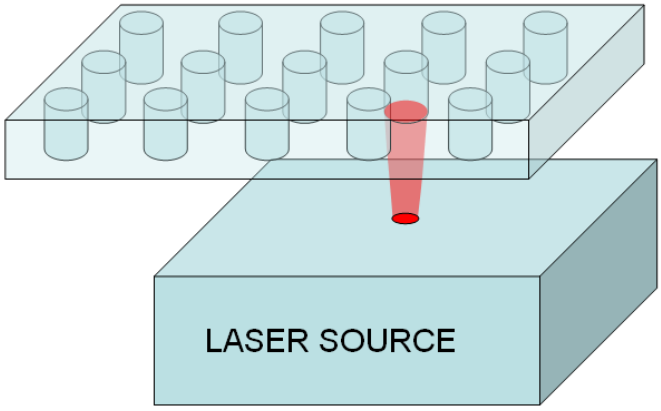


Figure S2: Schematic setup showing the laser light irradiation Figure . In order to irradiate only one sample at a time a black screen with a calibrated hole (not shown in the figure) was positioned between the laser source and the culture plate.

This solution has the drawback that setting up the laser source and the irradiation setup is a bit more complicate, but yields some important advantage: the first one is that the glass sample is in contact with the plate bottom, and thus the radiation impinges on the glass slide without having to pass through several millimeters of fluid medium (which could absorb/scatter the radiation), while the second one is that a good control of the irradiated area can be achieved even if the beam is highly divergent.
With the described setup we have been able to obtain a sufficiently homogeneous intensity distribution and to irradiate the sample with an optical intensity of 0.090 W/cm^2 (also taking into account the ≈ 24% loss introduced by the plate bottom). Before

starting the experiments on the biofilms-coated glass samples a preliminary analysis has been carried out to assess the heating effect induced by the irradiation on 0.5 ml of water under different conditions. Irradiating for 10 minutes the fluid only a very small temperature growth was measured ($\Delta T \approx 0.7^{\circ}C$); on the other side, when a glass coverslip covered with Au-nanoparticles a significantly larger increase ($\Delta T \approx 2.3^{\circ}C$) was observed (this result inly highlights that the GNS significantly increase the laser beam absorption; these data, obtained by inserting a thermocouple directly in the water are not sufficient to assess the system performance, as they give only the overall temperature increase of the whole fluid, heaten from the glass slide, and not the local temperature increase on the bacteria-covered glass surface).

Measurement of temperature increase on laser irradiation on glass|MPTS|GNS slides. We employed a ThermaCAM SC 3000 (FLIR Systems; Italy) camera (320x240 microbolometers array) operating in the spectral range of 8-9 microm and a numerical aperture NA = 0.46 x 0.34. The acquisition frequency is 9 Hz with a sensitivity of $0.1^{\circ}C$ and an accuracy of $\pm 0.2^{\circ}C$. For NIR irradiation we employed a collimated 4 mm (waist) tunable NIR laser (MaiTai Spectra Physics, CA), working in continuous wave. The thermocamera, placed at a distance of 0.5 m from the sample, measured the heating until a plateau level was reached (typically in 120s aquisition). The temperature as a function of time was obtained from a ROI selected on the thermal image.

2) INSTRUMENTATION

UV-Vis absorption spectra on colloidal soutions were taken on HP8453 and Varian Cary 50 instruments, in 1 cm glass or quartz cuvettes. UV-Vis-NIR absorption spectra on glass slides were taken on Varian Cary 100 or Varian Cary 6000 spectrophotometers, equipped with a dedicated solid samples holder.
Ultracentrifugation was carried out with a Hermle Z366 ultracentrifuge using dedicated plastic test tubes (10 or 40 ml volume)
ICP-OES were carried out with an Optima 3300 DW Perkin Elmer instrument.
Thermograms (T vs time) were collected with a ThermaCam SC3000 Flir instrument.
TEM images were taken with a Joel JEM-1200 EX II 140 instrument

3) PHOTOGRAPHIC IMAGES OF GLASS | MPTS | GNS SLIDES.

Glass slides shown here (A-D in figure, real dimensions 2.1x2.6 cm) were prepared with GNS colloidal solutions with different LSPR. A blue shift of ~ 52 nm was observed in the UV-vis-NIR absorption spectra in all cases, on passing from solution to glass slides. In the case of glass A, GNS were poorly developed on purpose in the seed-growth synthesis, and have a spherical shape.

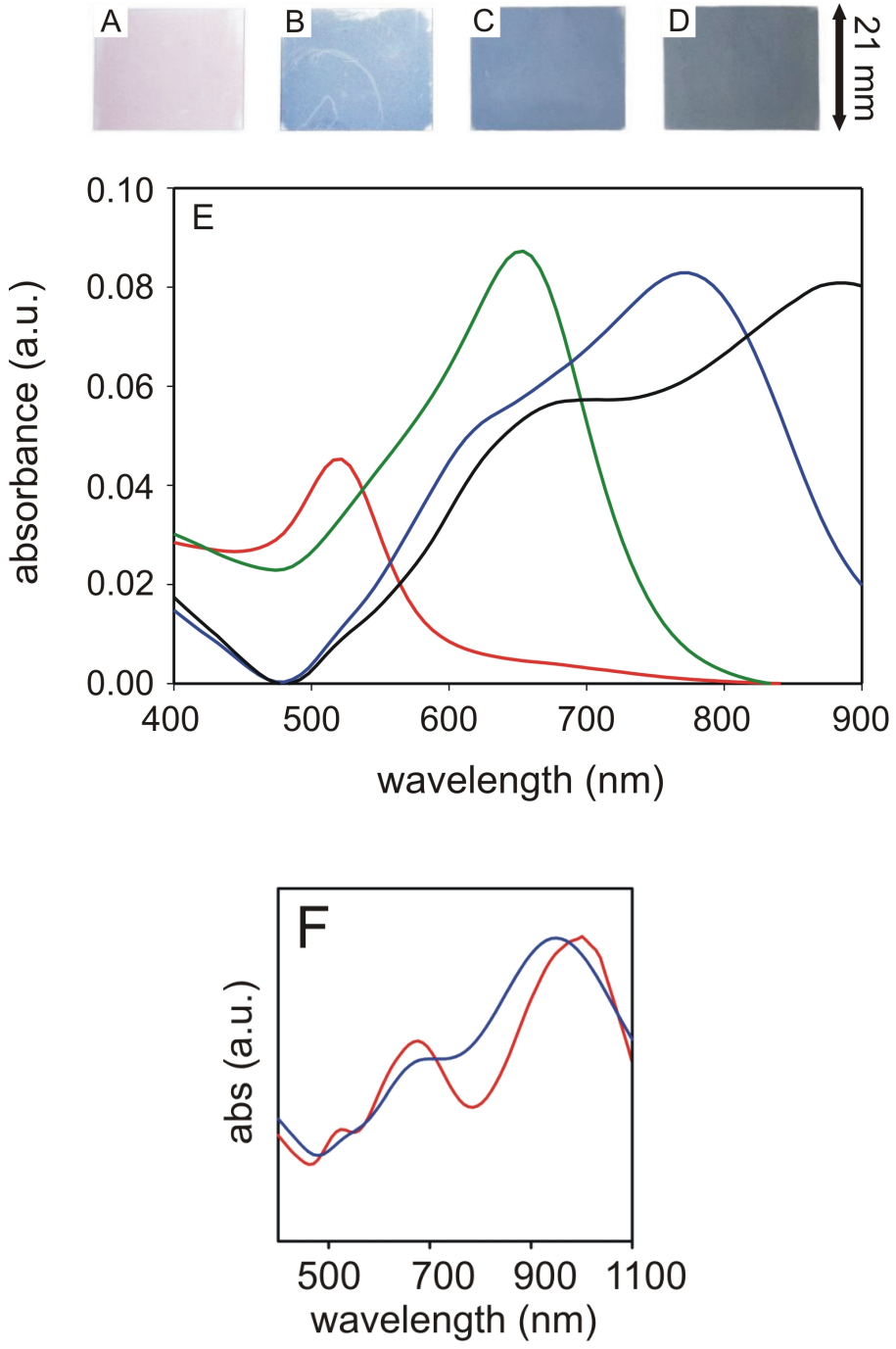


Figure S3. **A-D**: photographs of representative coated glass slides. **E**: absorption spectra taken on the A-D glass slides (red spectrum, slide A; green, slide B; blue, slide C; black, slide D). **F**: GNS absorption spectrum as colloidal solution (red) and as monolayer on dry glass (blue), obtained from the same colloidal solution.

4) SEM IMAGES.

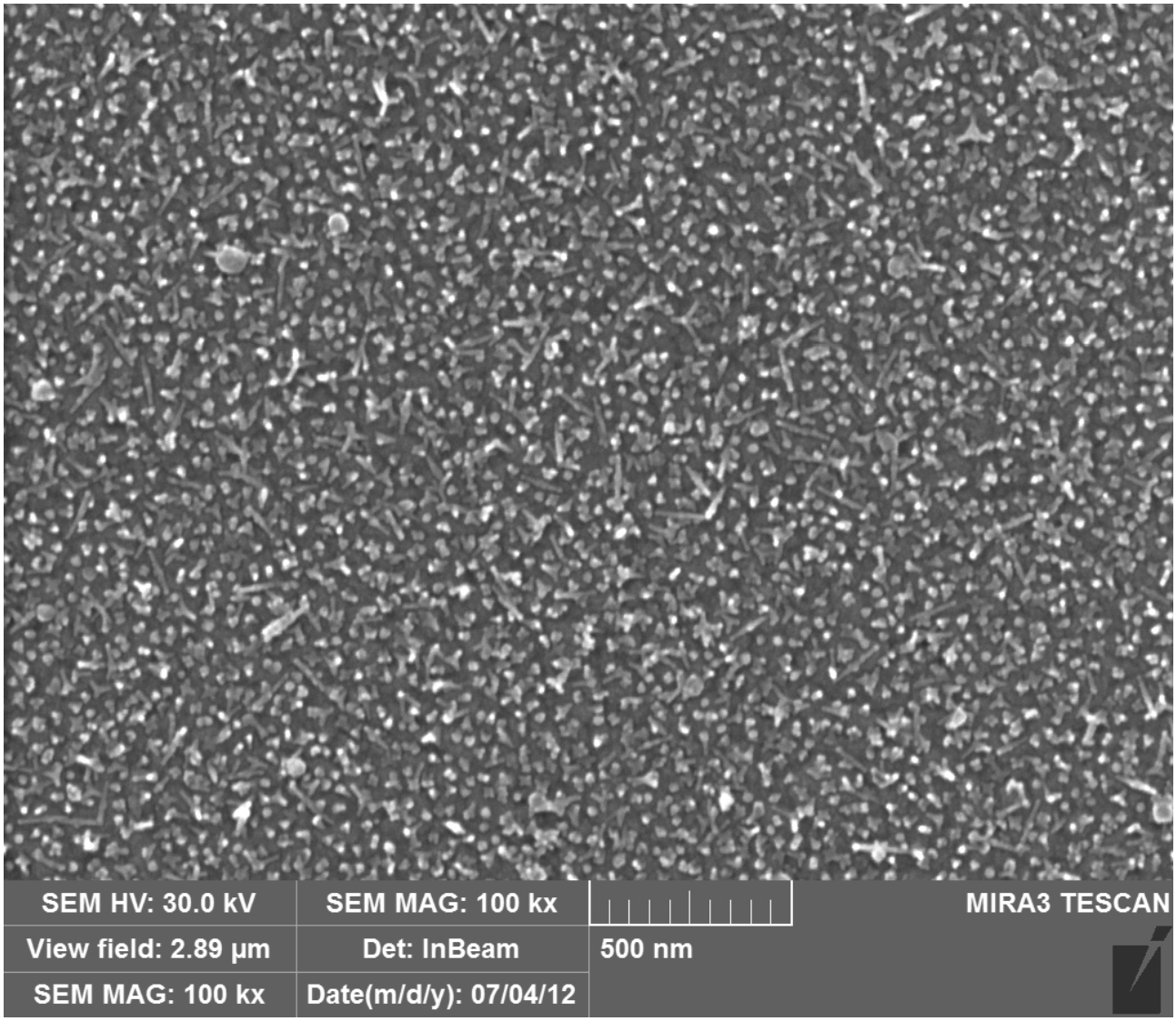


Figure S4 A – same as Figure 2A, main text, full picture

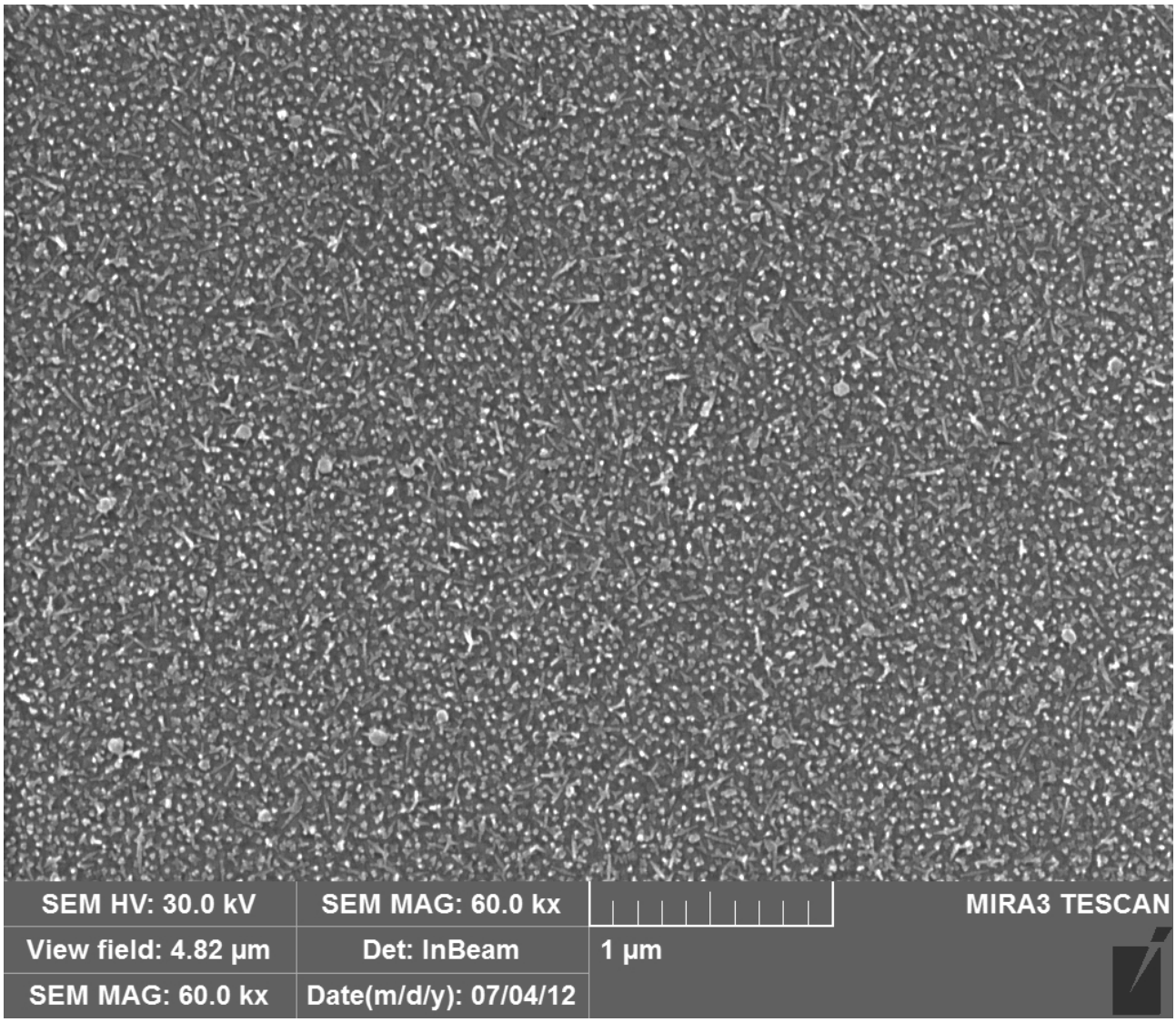


Figure S4 B – Same slide, lower magnification

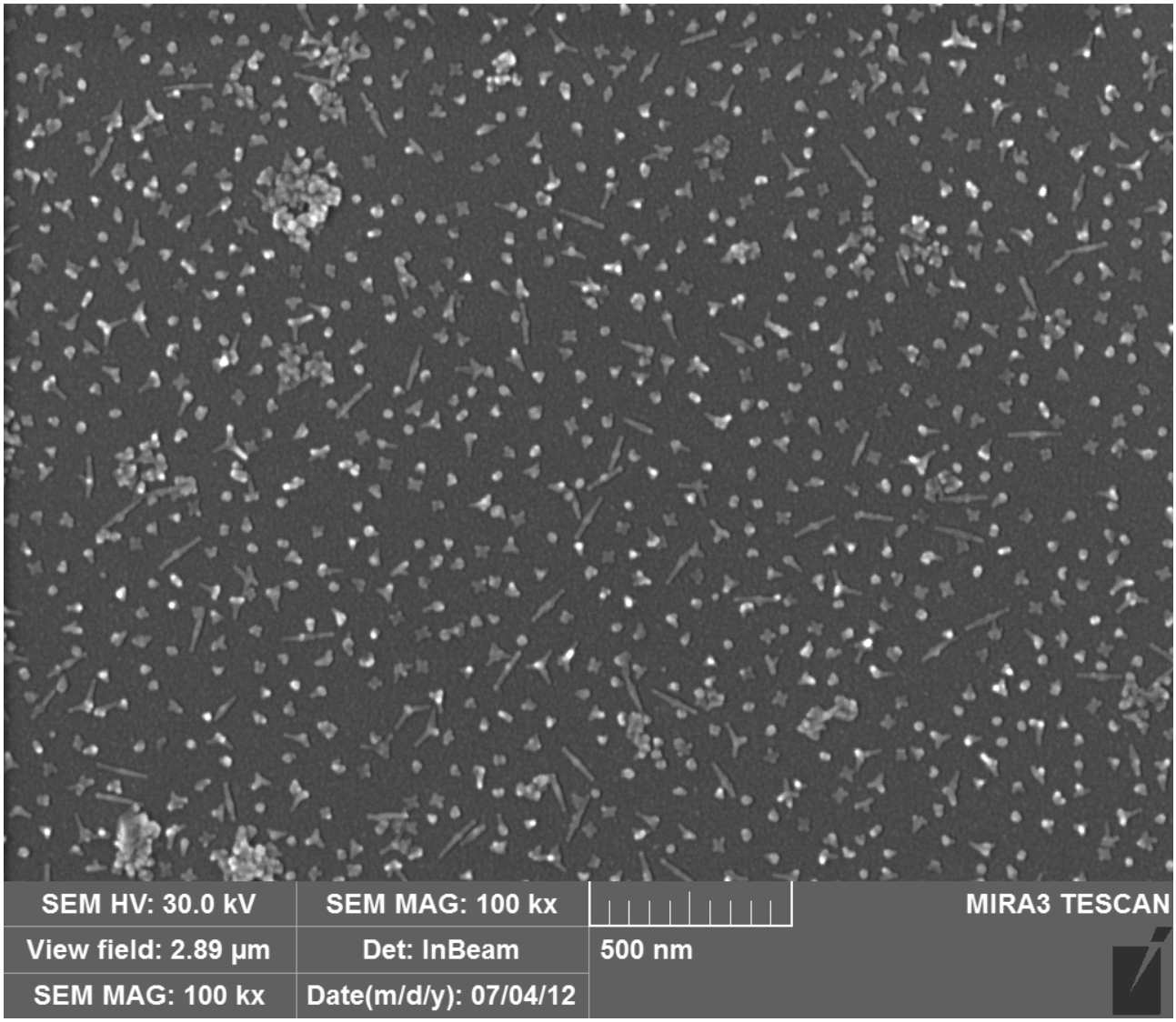


Figure S4 C. Shorter dipping time (4 hours) for the preparation of glass|MPTS|GNS

S5) Absorption spectra obtained in a glass cuvette, with internal walls functionalized as glass|MPTS|GNS, when filled with air or solvent with different refractive index (*n*)

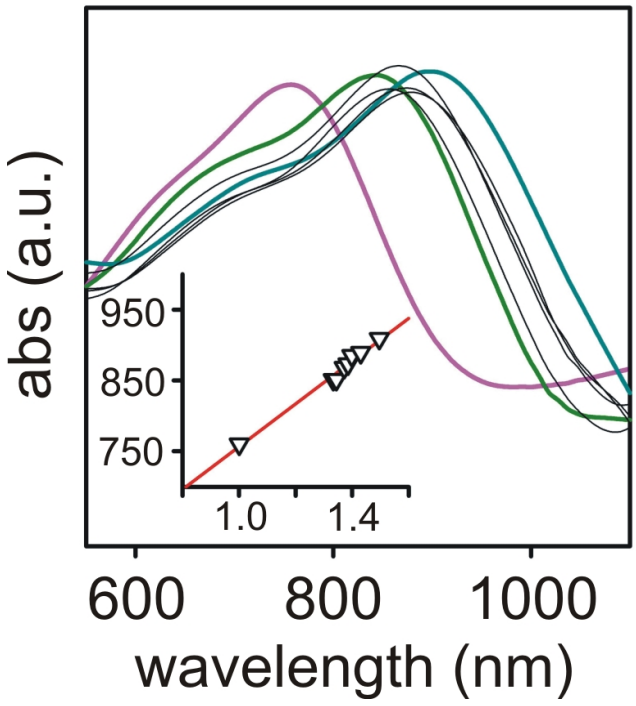


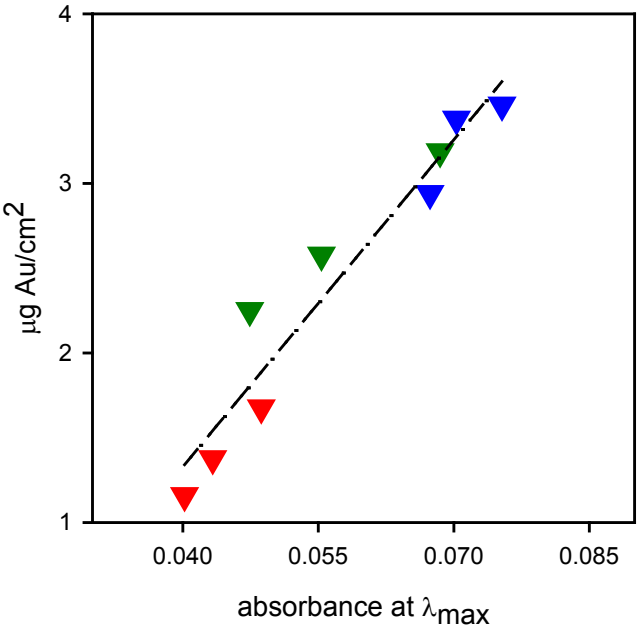
Figure S5. Spectra, left to right: air (violet), water (green), CH_3CN, ethyl acetate, n-eptane, n-butanol, DMF, toluen (azure). The inset reports the λ_{max} position vs *n*.

Experiments were carried with this sequence: air, water, acetonitrile, DMF, n-butanol, ethyl acetate, toluen, n-heptane. After each measurement, the solvent was discarded, and the cuvette washed with 3x3,5 mL of the next solvent (that is alsways miscible with the preceding one). Then, the solvent was left to equilibrate with the functionalized cuvette walls for one hour before taking the absorption spectrum. The sequence of spectra of figure S5 shows shift in the position but no significant change in shape or intensity of the absorption spectra. This indicates that at least for the esposition time (> 1 hour) the grafted GNS monolayers are stable toward the whole set of solvents.
Additional experiments were carried our on glass|MPTS|GNS slides, left in ethanol and toluen for 24 hours, after which time they were dried and an absorption spectrum measured: the spectra did not change after the exposition to the solvent.

S6) ABSORPTION SPECTRA OF GLASS SLIDES FROM DIFFERENT PREPARATIONS (18 H DIPPING TIME).

The data of the μg Au vs Abs on the NIR LSPR maximum were obtained from three different preparative jars, using 18 hours dipping time of glass|MPTS slides, and three different colloidal solutions (ie with LSPR in different positions).

Points of a given colour corresponds to the absorption spectra of the same colour (FigureS6B-D). After recording the spectra, the glass slides were treated with aqua regia and analyzed by ICP-OES, as described..



FigureS6A

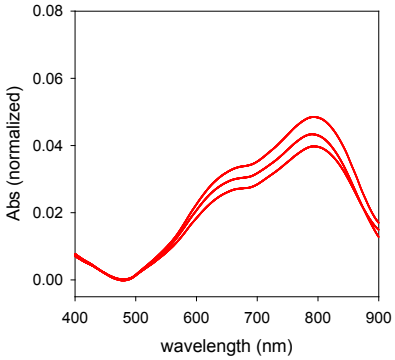


Figure S6B

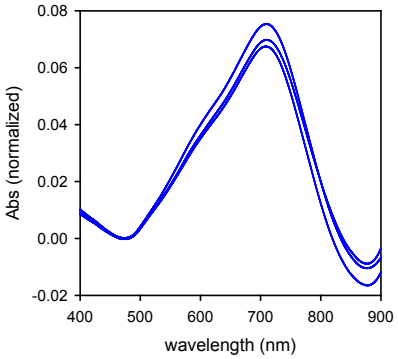


Figure S6C

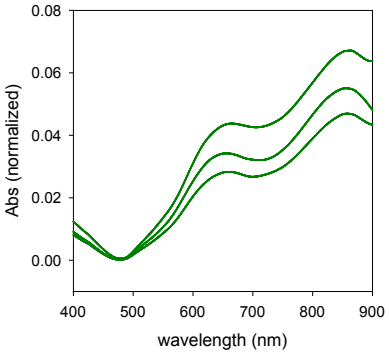


Figure S6D

7) Absorption spectra of the round glass|MPTS|GNS slides (Ø = 10 mm) used for the experiments on biofilms

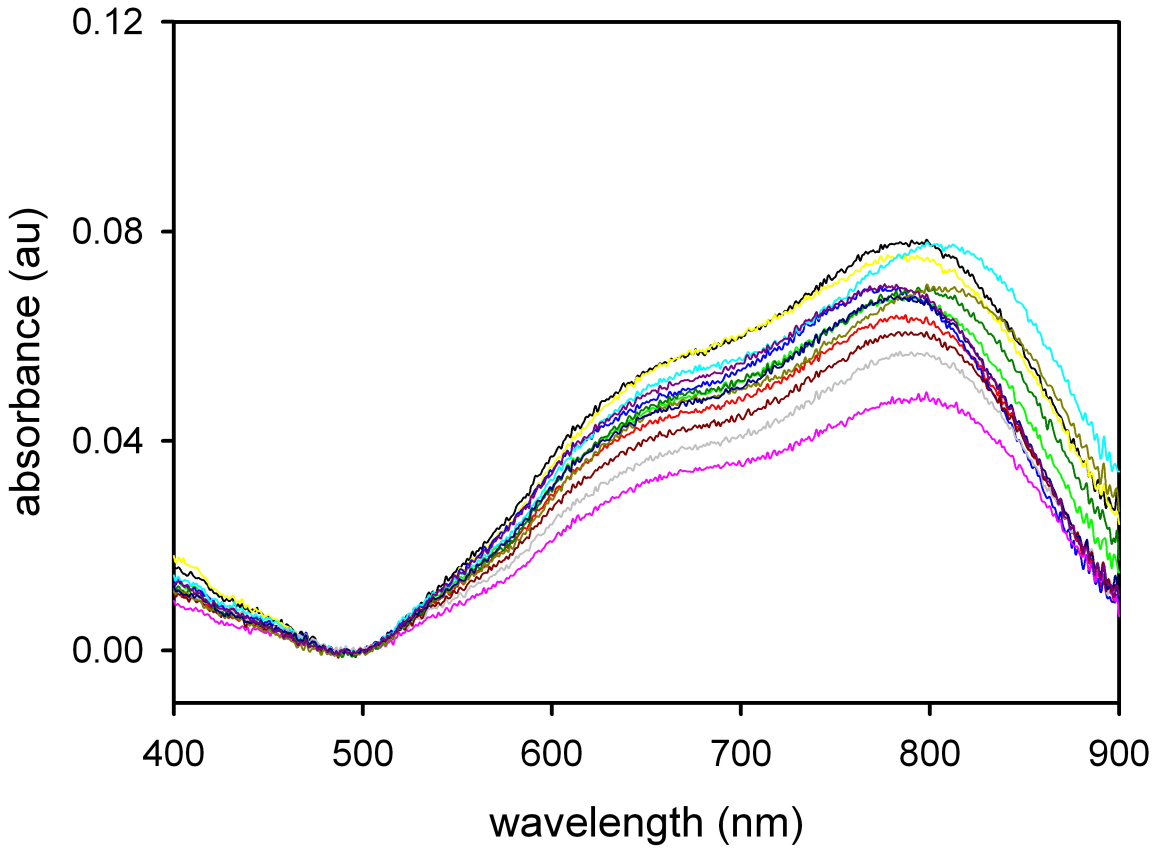


Figure S7. Series of 12 representative absorption spectra, on 12 out of 32 slides (the remaimin slides displayed comparable spectra)

8) FIGURE 3 MAIN TEXT – GRAPHICALLY ENHANCED VERSIONS

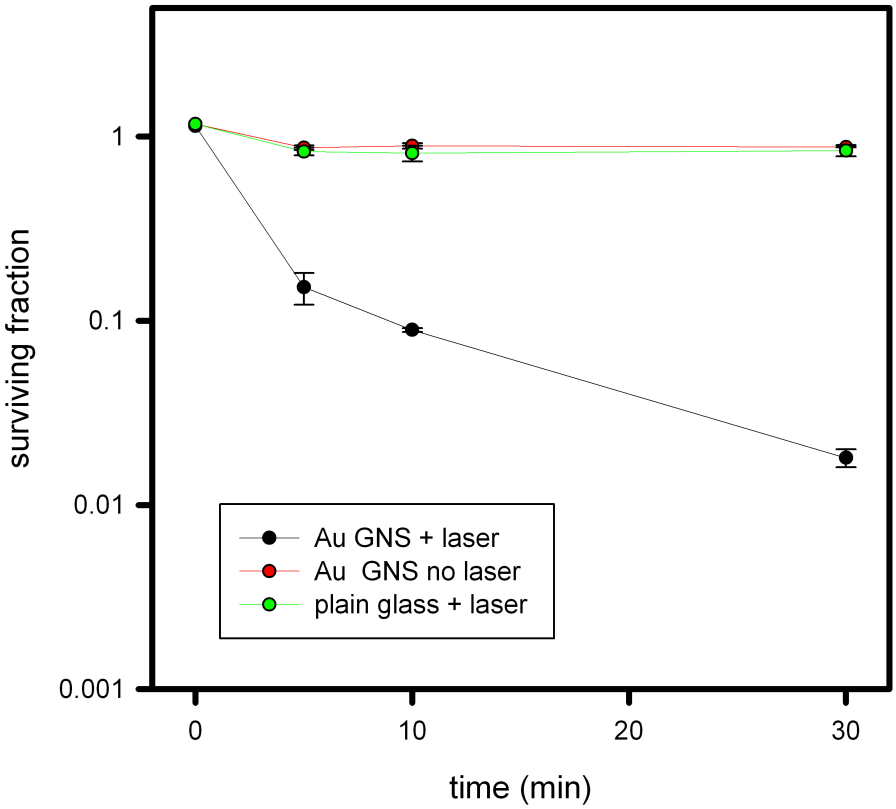


Figure S8a - Figure 3A main text, with smaller symbols to evidence error bars

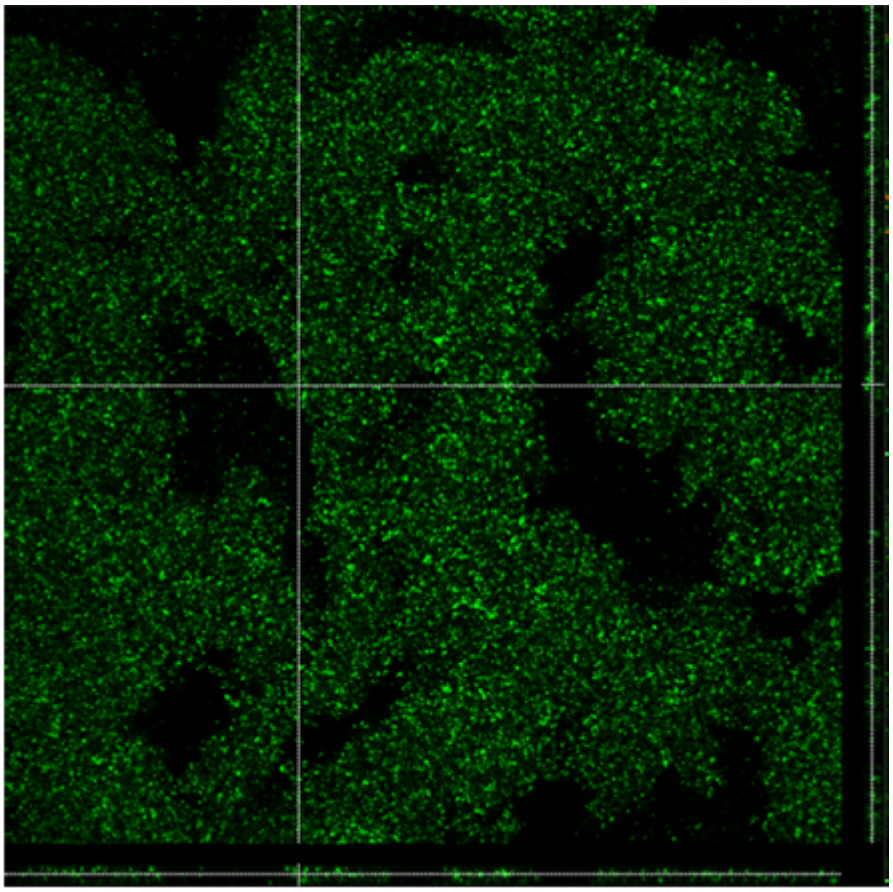


Figure S8b: larger version of Figure 3B in the main text (CLSM images of *S. aureus* biofilm grown on plain glass and irradiated with laser)

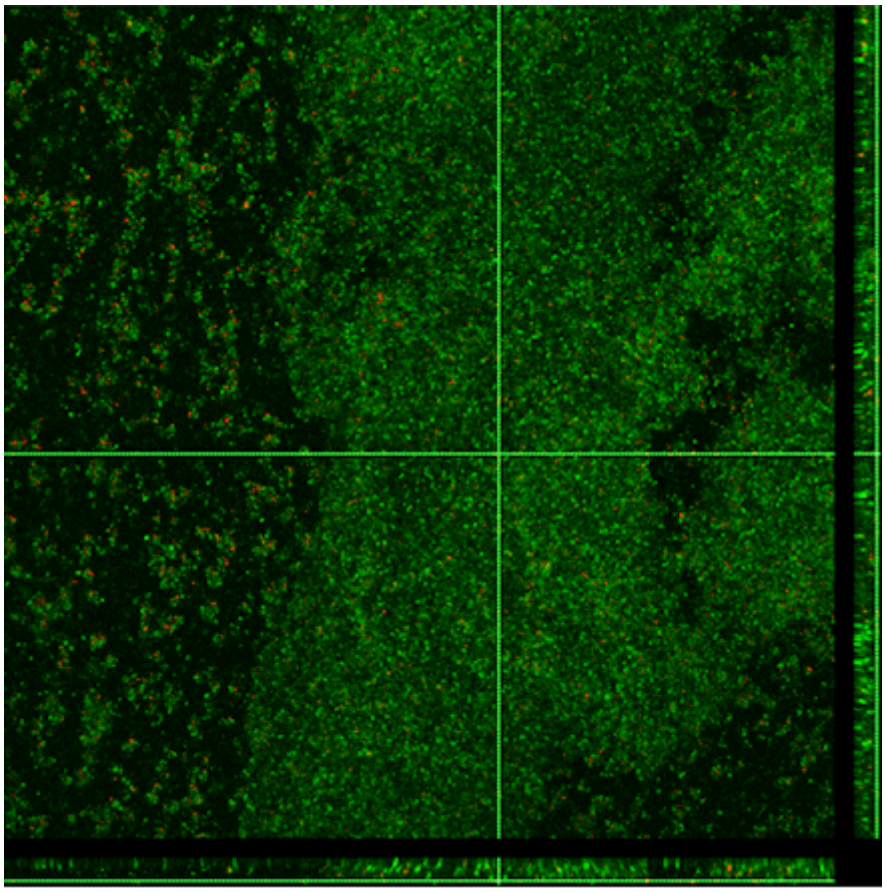


Figure S8c: larger version of Figure 3C in the main text (CLSM images of *S. aureus* biofilm grown on on glass|MPTS|GNS unexposed to laser)

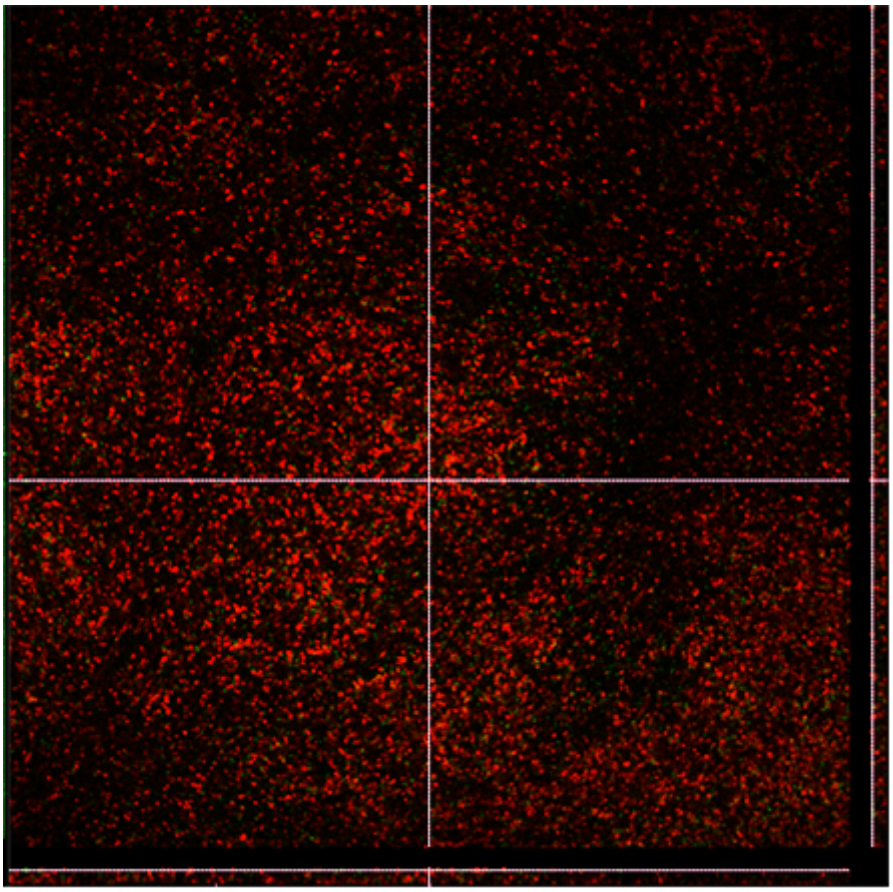
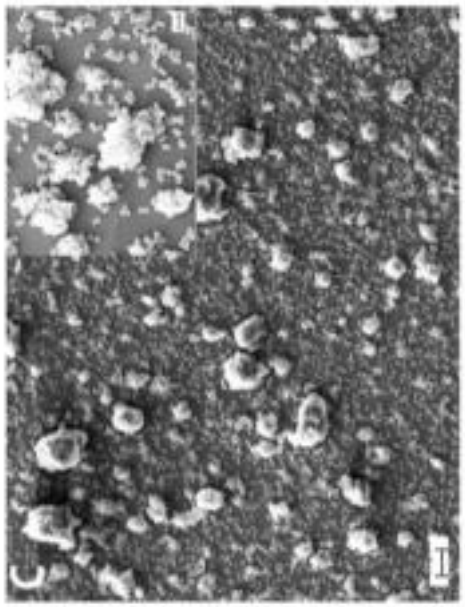
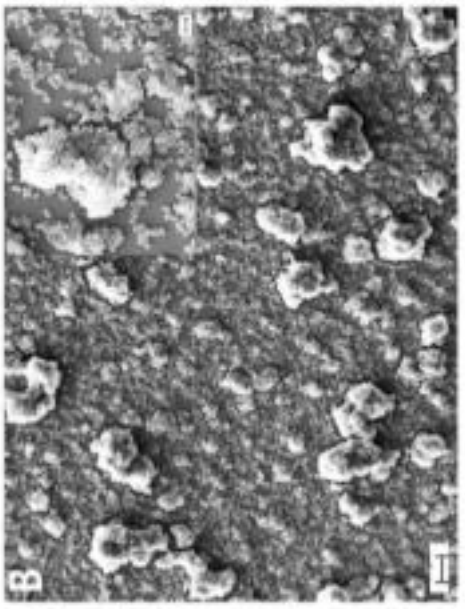


Figure S8d: larger version of Figure 3D in the main text (CLSM images of *S. aureus* biofilm grown on on glass|MPTS|GNS and exposed to laser)

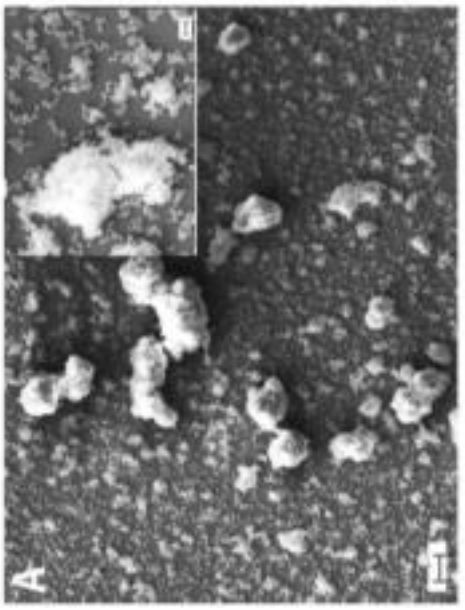
9) SEM IMAGES



FigureS9C - SEM images of *S. aureus* biofilm grown on glass|MPTS|GNS and irradiated with laser (wavelength 808 nm, irradiance 0.090 W/cm^2 for 30 min). Scale bar = 10 μm. Inset is a SEM image at higher magnification: scale bar = 2 μm.



FigureS9B - SEM images of *S. aureus* biofilm grown on glass|MPTS|GNS unexposed to laser. Scale bar = 10 μm. Inset is a SEM image at higher magnification: scale bar = 2 μm.



FigureS9A - SEM images of *S. aureus* biofilm grown on plain glass and irradiated with laser (wavelength 808 nm, irradiance 0.090 W/cm^2 for 30 min). Scale bar = 10 μm. Inset is a SEM image at higher magnification: scale bar = 2 μm.

Due to the setup of our experiments (SI, figure S2), the laser beam hits the glass slides on the lower face, through glass. Biofilms are thick (> 3 μm in this case) and SEM allows instead to take an image of the upper face of the biofilm (the most distant from the GNS monolayer). This leads to similar observed morphologies between biofilms on plain glass, and on laser-treated or not treated glass/MPTS/GNS slides, as it can be clearly observed by comparing Figure S9A-C.
However, we also stress that CLSM experiments (main text Figure 3B-D) sharply show that biofilms, untreated or laser treated, are not very much different morphologically, but also definitely different as regards cell viability: red cells are dead whereas green cells are alive. To furthere evidence this, we address the reader to Figures S8B-D, that are graphically enhanced CLSM images