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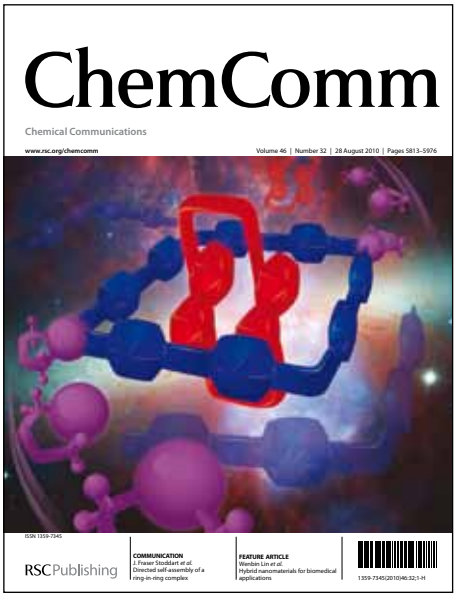


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ARTICLE TYPE

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

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**Monolayers of gold nanostars (GNS) are grafted on Mercaptopropyltrimethoxysilane-coated glass slides. In the formed monolayers the GNS's localized surface plasmon resonance can be tuned in the 700-1100nm range. Under laser excitation of the nearIR LSPR an efficient photothermal response is observed, inducing local hyperthermia and efficient killing of *Staphylococcus aureus* biofilms**

Biofilms are sessile microbial communities, usually constituted by the Gram+ *Staphylococcus aureus* and *Staphylococcus epidermidis*, embedded in a self-produced extracellular polymer matrix. They may develop on the surface of prostheses, catheters and implants, leading to severe infections in an impressive number of hospitalized patients.[1] Conventional antibiotics do not eradicate biofilms from the surfaces where they have formed and often surgical removal of the implant is the only affordable solution. To avoid biofilm formation, it has been proposed to coat medical devices with Ag nanoparticles but with controversial results,[2a-b] as Ag nanoparticles are effective towards Gram- bacterial strains but much less towards Gram+.[2c-d]. We propose here a new approach, based on gold nanostars (GNS). Gold is not intrinsically antibacterial but GNS feature two or more localized surface plasmon resonances (LSPR) that undergo thermal relaxation when irradiated. Moreover, at least one LSPR falls in the near-IR range (NIR, 750-1100 nm)[3] where tissues and blood are semi-transparent. Following some examples of glass surfaces coated with gold nanoparticles monolayers,[4] we have now prepared bulk glass materials coated with a GNS monolayer, that demonstrated very efficient in photothermal biofilm laser-treatment against *S.aureus* biofilms, suggesting the possibility to fabricate medical devices with the same coating: once internalized, they would not need to be removed if a biofilm is formed on their surface but may be treated *in situ*, i.e. through-tissues.

GNS are prepared in water according to a seed-growth method developed by us, with laurylsulfobetaine (LSB) as the directing and coating agent.[5a] The obtained colloidal solutions are mixtures containing mainly asymmetric branched gold nanoparticles (70-80%) and a lower percentage of symmetric 6-branched nanostars, see **Figure 1B** for a representative TEM image. Their absorption spectrum in solution is dominated by the LSPR of the main component, whose maximum can be positioned in the 750-1150 nm range as a function on the reaction

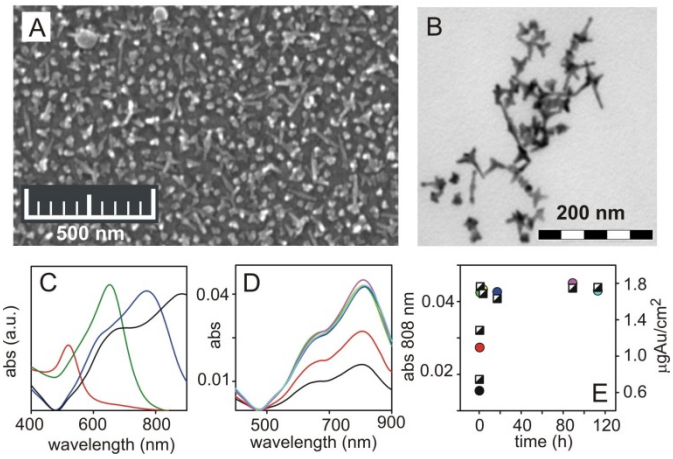


Figure 1. A: SEM image of a glass|MPTS|GNS slide. B: TEM image of GNS from the colloidal solution used to prepare the slide imaged in A. C: representative absorption spectra taken on glass slides prepared from colloidal solutions of GNS with different LSPRs positions. D: absorption spectra (normalised at 480 nm) obtained for glass|MPTS slides dipped for different times in a GNS solution. E: colored circles are the normalized absorbance values at 808 nm (left vertical axis) from panel D, as a function of time; colors correspond between E and D). Half-filled squares are the Au µg/cm2 values obtained by ICP-OES for the same slides (right vertical axis).

conditions. Adhesion of a monolayer of GNS on glass is obtained by forming first a monolayer of MPTS on the surface, according to an established procedure.[6] The obtained glass|MPTS surfaces are then dipped into a colloidal solution of GNS (Au = 0.06-0.07 g/L), typically for 18 hours, yielding glass|MPTS|GNS surfaces, with the formation thiolate-gold bonds (all experimental details in E.S.I., section S1-S2)

The weak nature of the LSB-Au interaction[5a] favours the facile LSB surface displacement by the MPTS thiol groups and efficient GNS adhesion. The slides are sonicated in water 3 times for 3 min before use and dried with a $N_2$ flux. They can be kept in air at room temperature, under light exposure, with no degradation after 3 month (as checked with UV-vis spectra). Control experiments on plain glass gave no coating. GNS-bearing slides are intensely colored but transparent (photographs in SI Figure S3A-E) and absorption spectra are directly measured on dry slides using a common UV-Vis spectrophotometer. The position

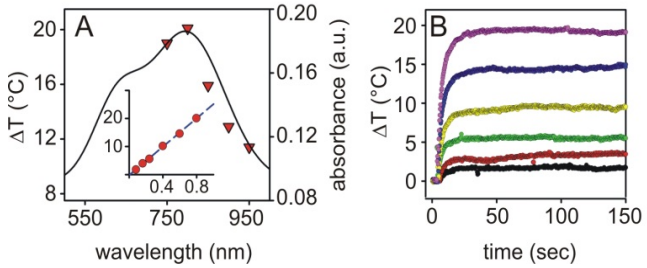


Figure 2. A, black line: absorption spectrum of the slide for laser irradiation. Red triangles are ΔT values (left vertical axis) found using the same irradiance (0.8 W/cm2) with laser sources of different wavelength. Inset: ΔT vs irradiance at 800 nm. B, ΔT vs time, laser at 800 nm, irradiance 0.08 (black), 0.16 (red), 0.24 (green), 0.40 (yellow), 0.60 (blue) and 0.8 W/cm$^2$ (pink)

of the LSPR maximum can be placed at the wanted wavelength inside a large interval (550-1100 nm), see *e.g.* Figure 1C, depending on the LSPR of the chosen coating solution. The surface is densely populated by GNS, as shown by Scanning Electron Microscope (SEM) images, Figure 1A (SI Figure S4A-B for larger images). The grafted GNS maintain the shape they have in the coating colloidal solution. A LSPR blue shift of 52 nm (average on 70 preparations, σ = 10 nm) is observed on passing from the colloidal solution in water to the monolayer on glass in air (SI, Figure S3F), due to change of local refractive index (*n*). Glass|MPTS|GNS surfaces prepared on the internal wall of an optical glass cuvette, then filled with different solvents (SI, Figure S5), show a linear trend for $\lambda_{max}$ vs *n* with a 300 nm/*n* unit variation, and when water is the solvent $\lambda_{max}$ reaches a similar value as in colloidal solution. Glass|MPTS|GNS slides prepared with dipping times shorter than 18 hours show a less dense coating than in Figure 1A, to which corresponds a less intense UV-Vis-NIR absorption (SI, Figure S4C for SEM images). This prompted us to study the kinetics of coating. Using colloidal solutions with $\lambda_{max}$ = 850 nm ($\lambda_{max}$ on glass = 808 nm), glass|MPTS slides are taken off at different times along a 140 hours period. Absorption spectra are recorded on each slide and the total Au content determined by fully oxidizing the GNS monolayer in a small measured volume of aqua regia (3.0 mL). This is finally analysed with standard techniques for diluted cations in solution (ICP-OES, inductively coupled plasma optical emission spectroscopy). Spectra are normalized by setting the absorbance to zero at 480 nm (Figure 1D) and a sharp ascending/plateau trend is obtained plotting by Abs at $\lambda_{max}$ vs time (Figure 1E, coloured circles). The trend parallels the quantity of Au/cm$^2$ found by oxidation/ICP-OES, Figure 1E (half-filled squares). Full coating is reached in <18 hours, confirming that the routine time for preparations is suitable. Many slides were prepared with 18 hours coating time in GNS solutions from different syntheses, and the total Au/cm$^2$ calculated, finding variations from preparation to preparation, albeit in a limited range (1.5-3.5 μg/cm$^2$). By normalizing the absorption spectra at 480 nm and determining by ICP-OES the total gold quantity on the slide, a linear plot for Abs at LSPR maximum vs Au/cm$^2$ is found (SI, Figure S6A). We used this as a calibration plot in all further preparations, in order to quickly calculate the Au/cm$^2$ surface concentration on each freshly prepared slide. For sake of uniformity, in all the following experiments slides are used with Au content only in the 2.0-3.0

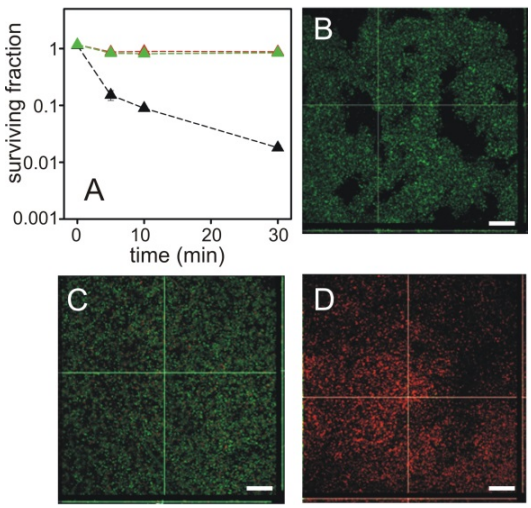


Figure 3. A: S. aureus viability vs time (surviving fraction as CFU/CFU found on glass unexposed to laser); red triangles for plain glass irradiated with laser, green for glass|MPTS|GNS not irradiated, black for glass|MPTS|GNS irradiated with laser. The values are the means of the results from duplicate biofilms. Data are representative of three replicate experiments with similar results (see SI for error bars). B-D: CLSM images of *S. aureus* biofilm grown on plain glass and irradiated with laser (B), on glass|MPTS|GNS unexposed (C), on glass|MPTS|GNS laser exposed (D), wavelength 808 nm, irradiance 0.090 W/cm2 for 30 min. Sagittal sections of the biofilms are shown below and to the right of each panel. Scale bar = 50 μm.

μg/cm$^2$ range. The efficient photothermal properties of the GNS used in this paper have been already demonstrated on their colloidal solutions.[7] Now we use dry glass|MPTS|GNS slides with LSPR maximum at ~800 nm (Figure 2A). The slides were irradiated with continuous laser sources at 750, 800, 850, 900 and 950 nm, with irradiance 0.80 W/cm$^2$. The T increase is measured as a function of time with a thermocamera (see ESI S1).

The highest ΔT is found when using a laser source exactly matching the $\lambda_{max}$ of LSPR (800nm), while the ΔT trend smoothly follows the LSPR band profile (red triangles, Figure 2A). When using the 800 nm soruce, power was also varied, finding that ΔT linearly increases from 2 to 20 °C when irradiance increases from 0.08 to 0.8 W/cm$^2$ (Figure 2A, inset). Figure 2B reports the ΔT vs time trends at each irradiance (laser at 800 nm), showing steep ascending-plateau profiles with a rapidly reached maximum equilibrium temperature (<20 sec). It is worth mentioning that significant T increase is observed using a laser beam intensity (0.08 W/cm$^2$) four times lower than the maximum permitted exposure for skin, as established by the American National Standards Institute (ANSI) Laser Safety Standards.[3c-d,8]

For the antibiofilm studies a series of 32 round glass slides (diameter 1.0 cm) were prepared, with LSPR centred at 802 (σ=12) nm (SI, Figure S7) and a gold surface concentration of 3.0 (σ=0.4) μg/cm$^2$. The microorganism used is a methicillin-resistant *S. aureus* LP strain, which was shown to be an efficient biofilm producer.[1c] For biofilm growth an overnight culture of *S. aureus* LP was diluted at 1:50 in tryptone soy broth (TSB) containing 0.25% glucose, and aliquots (0.500 mL) of the diluted bacterial suspension were inoculated into 24-well microplates containing either plain glass slides or glass|MPTS|GNS slides, and incubated for 16 hours at 37°C. Some of the wells were laser-

treated with a AlGaAs laser diode with emitting light at 808 nm, irradiance of 0.090W/cm$^2$ (also taking into account the ≈ 24% loss introduced by the plate bottom), and a waist with a diameter matching that of the glass slides (SI: details and experimental set up). Exposure time to laser was 5, 10 and 30 minutes. Then, the biofilms were carefully scraped, 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, the control samples were biofilms grown on glass slides and unexposed to laser: the colony forming units (CFU) from bacteria grown on control samples are considered as 100%. Cell survival is expressed as the ratio of the CFU from bacteria grown on the other samples to that of the control. On biofilms grown on glass|MPTS|GNS slides a dramatic reduction of the CFU was observed with laser treatment (**Figure 3A**, black triangles) with a decrease of one order of magnitude of the surviving fraction after 5 minutes irradiation and of two orders of magnitude after 30 min. On the other hand, no reduction of the CFU was observed both on plain glass slides irradiated with laser (Figure 3A, green triangles) and on glass|MPTS|GNS slides with no laser irradiation (Figure 3A, red triangles). Biofilms were also studied by confocal laser scanning microscopy (CLSM), using a BacLight Live/Dead viability kit, according to an established procedure.[9] Biofilms grown overnight on plain glass and exposed for 30 min to laser (Figure 3B) presented a green fluorescence (viable cells) and appeared organized as multilayered aggregates. A similar result was observed for the *S. aureus* biofilm grown on glass|MPTS|GNS slides but not irradiated with laser (Figure 3C). Biofilms grown on glass|MPTS|GNS slides and irradiated at 808 nm for 30 min appeared uniformly red (dead cells) and more dispersed than the controls (Figure 3D), see also SI section SI8 for images of enhanced graphical quality.

Photothermal therapy is a potentially powerful tool against biofilms.[10] In this communication we demonstrate that bulk glass surfaces can be prepared bearing controlled microgram quantities of gold (up to 3.0 μg/cm$^2$) as firmly grafted, stable monolayers of nanostars. Laser irradiation of the GNS monolayer on their NIR LSPR results in efficient photothermal conversion. Using irradiances values significantly lower than the maximum permissible exposure of skin, with a laser source at 808 nm we observed that the photothermal action of the GNS monolayer efficiently induces cell death by hyperthermia in *S. aureus* biofilms. This coating approach may be in principle applied also on steel, ITO, silica, *i.e.* any surface that is prone to MPTS functionalization. Working with a laser source inside the biological "transparent" NIR window, our findings could lead to useful coatings for internalized medical devices and implants: biofilms that eventually form on their surface may be laser-treated in-situ, avoiding surgical removal.

## Notes and references

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† Electronic Supplementary Information (ESI) available: Detailed experimental procedures; reactants and material list; slides photographs; SEM images; normalized UV/Vis spectra and Au μg/cm$^2$ calibration curve; UV-Vis-NIR spectra of round slides used for biofilms.. See DOI: 10.1039/c000000x/

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