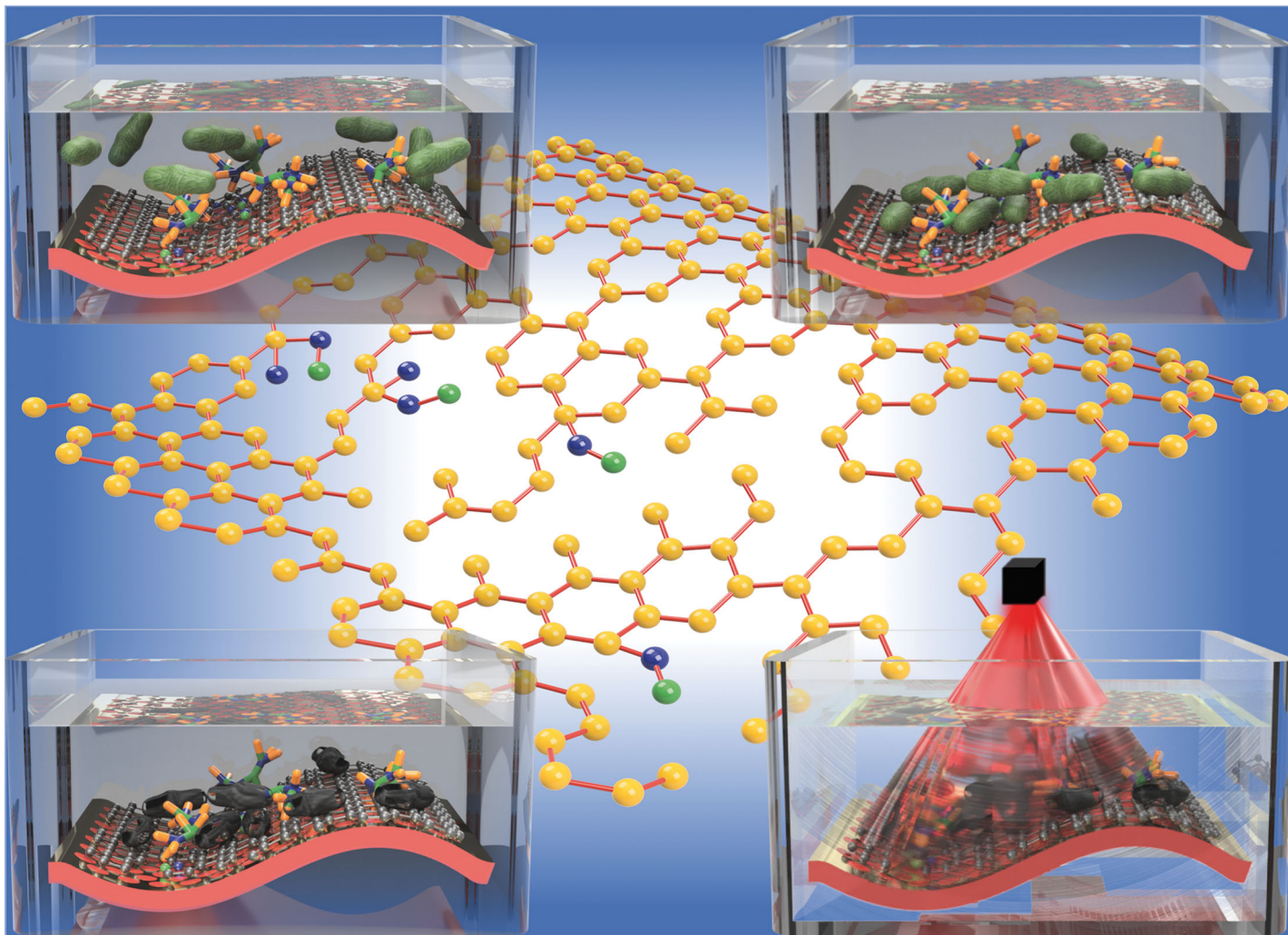


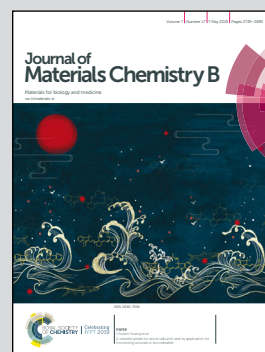


Showcasing joint research from the laboratories of Professor Boukherroub and Professor Melinte at the University of Lille, France, and Université catholique de Louvain, Belgium.

Efficient capture and photothermal ablation of planktonic bacteria and biofilms using reduced graphene oxide–polyethyleneimine flexible nanoheaters

Reduced graphene oxide nanoheaters processed via colloidal lithography on Kapton® foil have been endowed by polyethyleneimine coating as effective substrata to capture and kill planktonic bacteria with potential applications in eradication of pathogen biofilms.

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Efficient capture and photothermal ablation of planktonic bacteria and biofilms using reduced graphene oxide–polyethyleneimine flexible nanoheaters†

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Bacterial infections are one of the leading causes of disease worldwide. Conventional antibiotics are becoming less efficient, due to antibiotic-resistant bacterial strains. Therefore, the development of novel antibacterial materials and advanced treatment strategies are becoming increasingly important. In the present work, we developed a simple and efficient strategy for effective bacterial capture and their subsequent eradication through photothermal killing. The developed device consists of a flexible nanoheater, comprising a Kapton/Au nanoholes substrate, coated with reduced graphene oxide–polyethyleneimine (K/Au NH/rGO–PEI) thin films. The Au NH plasmonic structure was tailored to feature strong absorption in the near-infrared (NIR) region, where most biological matter has limited absorption, while PEI was investigated for its strong binding with bacteria through electrostatic interactions. The K/Au NH/rGO–PEI device was demonstrated to capture and eliminate effectively both planktonic Gram-positive *Staphylococcus aureus* (*S. aureus*) and Gram-negative *Escherichia coli* (*E. coli*) bacteria after 10 min of NIR (980 nm) irradiation and, to destroy and eradicate *Staphylococcus epidermidis* (*S. epidermidis*) biofilms after 30 min irradiation. The technique developed herein is simple and universal with potential applications for eradication of different micro-organisms.

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1. Introduction

Bacterial infections and microbial contamination are among the world's biggest problems with a negative impact on human health, the healthcare system but also water quality, food storage, etc. The main concern nowadays is due to the fact that traditional antibacterial therapies are becoming less efficient, because of the misuse or overuse of antibiotics which has caused mutation in bacterial strains, resulting in antibiotic resistant pathogenic strains.¹

Additionally, multilayered bacterial biofilms are not only extremely resistant to host defense mechanisms, but act as physical barriers to inhibit the penetration of antibiotic agents into the biofilms. Overcoming biofilm-associated infections has consequently become a very difficult task. Therefore, the development of novel antimicrobial agents or coatings to disinfect drinking water or surfaces that will effectively prevent infection due to both Gram-positive and Gram-negative bacteria is urgently needed.²

Nanostructured materials (usually ranging from 1 to 100 nm) have emerged over the years as antibiotic alternatives, revealing in selected cases to have relatively high antibacterial activity, good stability and limited mammalian cytotoxicity.^{1,3–9} These antibacterial materials have shown their capability to prevent biofilm formation, and to solve problems associated with the use of conventional antimicrobial agents, such as residual toxicity, short-term antimicrobial activity, and development of resistance in micro-organisms.¹

Among nanostructured materials, graphene-based nanostructures hold particular promise for combating microbial infections.⁶ Numerous studies investigated the interaction of bacteria with graphene oxide (GO) and reduced graphene oxide

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(rGO) suspensions and demonstrated the loss of bacterial cell viability in a concentration, time and pathogen dependent manner.⁶ To enhance the antimicrobial effects further, polymer containing graphene nanosheets have been considered,^{10–12} including chitosan,^{13,14} poly(vinyl alcohol),^{14,15} polyamide¹⁶ and polyethyleneimine (PEI).¹⁷ Electrostatic interaction between the negatively charged rGO and inherently anti-bacterial polymers such as those containing amine or quaternary ammonium groups has proven to be a particularly easy way to increase the anti-bacterial properties of graphene materials. Lee and co-workers reported on the integration of poly(L-lysine) (PLL) onto GO and rGO to design materials with good biocompatibility and anti-bacterial activity.¹⁸ Flexible polyurethane composites, prepared by incorporation of polyethyleneimine-modified rGO, was proposed recently by Tang *et al.* to enhance bacterial anti-adhesive properties when compared to pure polyurethane and GO–polyurethane, due to the high density of amine groups in the PEI chains.¹¹

The additional strong near-infrared (NIR) absorption ability of rGO nanocomposites that can efficiently convert NIR light into heat,^{19–21} has allowed highly efficient photothermal ablation of pathogens.^{22–28} Following the demonstration of NIR (808 nm) inactivation of *E. coli*,²⁹ many research groups investigated different graphene-based materials for bacteria eradication. Wang *et al.* used rGO functionalized with anti-*S. aureus* polyclonal antibody, through physisorption, for efficient capture and NIR (808 nm) killing of *Staphylococcus aureus* (*S. aureus*).³⁰ We have recently investigated the synergistic effect of gold nanorods and rGO for enhanced photothermal killing of *E. coli*¹⁹ and demonstrated that targeted killing of uropathogenic *E. coli* UTI89 can be achieved through functionalization of the graphene coating with multimeric heptyl- α -D-mannoside ligands. Jia *et al.* applied chitosan-modified magnetic graphene nanocomposite for efficient capture and destruction of Gram-positive *S. aureus* and Gram-negative *E. coli*, and their biofilms with NIR irradiation.³¹ Wu and co-workers designed a graphene-based photothermal agent, consisting of magnetic reduced graphene oxide functionalized with glutaraldehyde (MRGOGA). The composite material possessed excellent cross-linking properties with proteins in bacteria and thus was applied as a capturing agent for both Gram-positive and Gram-negative bacteria.³² Xiao *et al.* observed excellent bactericidal activity of rGO sheets modified with water-soluble polythiophenes in order to obtain rGO-g-P3TOPA and rGO-g-P3TOPS positively and negatively charged sheets, respectively. The positively charged rGO-g-P3TOPA exhibited excellent bactericidal activity towards *E. coli*, due to the combination of photothermal effect and electrostatic interactions.³³ Recently, Hui and co-workers reported an antibacterial surface based on a polyelectrolyte-stabilized rGO sheet that kills airborne bacteria on contact within minutes of solar NIR irradiation. The observed activity was reported to be retained even when the film was placed underneath a piece of pork tissue, indicating that solar light in the near-infrared region plays a dominant role in the observed activity.³⁴

Herein, we report on the antimicrobial and anti-biofilm forming activity of reduced graphene oxide–polyethyleneimine

coated nanoheaters. This work builds on our previous results on surface plasmon resonance demonstrating the high adsorption capacity of *E. coli* UTI89 on PEI–graphene modified Au surfaces,³⁵ and a recent report using PEI-functionalized magnetic nanoparticles for bacteria capture.³⁶ The nanoheater consists of a flexible Kapton interface modified with an array of gold nanoholes (Au NH) supporting localized surface plasmons in the NIR. We assessed the capture efficiency and NIR photo-thermal antibacterial activity of the above-mentioned nanoheater. To that aim, we used as targets, *S. aureus* and *S. epidermidis*, Gram-positive bacteria causing serious healthcare-associated infections, and *E. coli*, a Gram-negative pathogenic strain, able to cause meningitis or urinary and gastrointestinal tract infections.

2. Experimental

2.1 Chemicals

Hydrazine monohydrate, absolute ethanol and branched polyethyleneimine (PEI, $M_w \sim 25\,000$) were purchased from Sigma-Aldrich, and used as received. Graphene oxide (GO) was purchased from Graphenea. Kapton[®] HN polyimide foils with a thickness of 125 μm were obtained from DuPont[™].

2.2 Synthesis of reduced graphene oxide (rGO)

Reduced graphene oxide, prepared by GO reduction with hydrazine monohydrate, was used only as a reference for comparison with rGO–PEI nanocomposite. First, a dispersion of GO in water (3 mg mL^{−1}) was prepared by exfoliation through ultrasonication for 3 h to form a homogeneous brownish solution. Hydrazine monohydrate was added immediately to the GO suspension (1 μL for every 3 mg of GO). Further, the suspension was heated in an oil bath at 80 °C. After 12 h, the reduced GO precipitated out of the solution. The solid was isolated by filtration over an anodisc membrane with a pore size of 0.1 μm . It was washed 2–3 times with water, until the pH was between 6 and 7. After washing, the resulting black powder was collected and dried under vacuum using a mechanical pump.

2.3 Preparation of reduced graphene oxide/polyethyleneimine (rGO–PEI) nanocomposite

Firstly, a dispersion of GO in water (2 mg mL^{−1}) was prepared by exfoliation through ultrasonication for 3 h. A dispersion of PEI in water at a concentration of 10 mg mL^{−1} was prepared separately. The aqueous suspensions were mixed in a round bottomed flask (1:1 weight ratio), and heated in an oil bath under stirring at 80 °C for 12 h. After 12 h, the color of the suspension changed from brown to dark grey, suggesting that GO was partially reduced by PEI. The obtained suspension was centrifuged for 15 min at 13 500 rpm, in order to separate rGO–PEI nanocomposite from the supernatant, and then washed several times with deionized water. After drying, the product was redispersed in ethanol.

We also prepared rGO–PEI composites with different weight ratios (1/2, 2/1 and 1/1), but 1/1 ratio turned out to have the best stability and dispersibility in ethanol.

2.4 Preparation of gold nanoholes modified Kapton (K/Au NH)

Kapton foils ($10 \times 10 \text{ mm}^2$) were cleaned with acetone in an ultrasonic water bath for 30 min, followed with isopropanol for 10 min and then dried under a nitrogen flow. The cleaned Kapton foils were modified with gold nanoholes (K/Au NH). Briefly, a monolayer of 980 nm polystyrene beads was first deposited on the surface of Kapton by self-assembly. To reduce the size of the particles and isolate them, SF_6 and oxygen plasma etching for 11 min was employed (5 mTorr). The samples were coated with 2 nm Ti followed by 40 nm Au at a constant deposition rate of $2 \text{ \AA s}^{-1}$ using physical vapor deposition. The beads on top of the Kapton were removed by dissolution in chloroform (overnight). The arrays display holes of an average size of 630 nm and center-to-center spacing of 980 nm.³⁷

2.5 Preparation of reduced graphene oxide–polyethyleneimine coated K/Au NH (K/Au NH/rGO–PEI)

K/Au NH foils ($10 \times 10 \text{ mm}^2$) were cleaned in ethanol in an ultrasonic water bath for 10 min, and then dried under a nitrogen flow. The K/Au NH foils were modified with rGO–PEI by drop-casting ($50 \text{ }\mu\text{L}$, 2 mg mL^{-1}) four times, each time followed by drying at room temperature.

2.6 Measurement of the photothermal effect

A continuous wave laser (Gbox model, Fournier Medical Solution) with an output light at 980 nm and power density of 2 W cm^{-2} was used for the photothermal experiments. This laser was injected into a 400 μm -core fiber and placed about 3 cm away from the bottom of the wells. The temperature changes were captured by an Infrared Camera (Thermovision A40) and treated using ThermoCam Researcher Pro 2.9 software.

2.7 Cell culture and cytotoxicity assay

NG108-15 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco[®]) containing 10% fetal-bovine serum (FBS, Gibco[®]) and 1% penicillin/streptomycin mix (Gibco[®]) at 37°C in a 5% CO_2 incubator.

Direct cytotoxicity testing. Cells were seeded onto 1 cm^2 square glass, Kapton and K/Au NH/rGO–PEI matrix. Each well was loaded with approximately 1×10^5 NG108-15 cells in 1 mL of medium and cultured for 24 h.

Indirect cytotoxicity testing. NG108-15 cells were seeded into 96-well plates (5×10^3 cells/ $100 \text{ }\mu\text{L}$ media per well) 24 h before experiments. Then, the medium was replaced with $100 \text{ }\mu\text{L}$ of DMEM/10% FBS in which K/Au NH/rGO–PEI matrix was previously immersed for 24 h.

Cytotoxicity assay. The cell viability was assessed by using resazurin cell viability assay. Briefly, for direct cytotoxicity testing, after 24 h of incubation in a 5% CO_2 atmosphere at 37°C , the medium was aspirated and the cells were washed with $500 \text{ }\mu\text{L}$ of PBS to remove the dead cell debris. The incubated surfaces were transferred into a new 24-well culture plate containing $500 \text{ }\mu\text{L}$ of medium and the cells were exposed to resazurin solution ($11 \text{ }\mu\text{g mL}^{-1}$) in DMEM/10% FBS for 4 h at

37°C . Afterwards, the fluorescence with excitation/emission at 554/593 nm (18 nm/20 nm bandwidth) was recorded by using a fluorescence microplate reader (BioTeK[™] Cytation[™] 5 Cell Imaging Multi-Mode Reader). The results were expressed as a percentage compared with controls. Each condition was replicated five times and data are presented as means $\pm$ standard deviation. For indirect cytotoxicity testing, the experimental procedure was similar to that of the previous one.

2.8 Bacterial culture and preparation

The bacteria used in this study were *S. aureus* and *S. epidermidis* (Gram positive bacteria) as well as *E. coli* K-12 MG1655 (Gram-negative) strains. A single *E. coli* K-12 MG1655, *S. aureus* or *S. epidermidis* colony from LB/BHI agar plate was inoculated overnight in LB/BHI medium at 37°C with moderate shaking. The pre-culture was diluted 50-fold and allowed to continue for another 3–4 h, until the optical density at 600 nm (OD_{600}) had reached 0.6–1. The *E. coli* K-12 MG 1655 and *S. aureus* cells were re-suspended in sterile Milli-Q water and adjusted to the required concentration.

2.9 Antibacterial activity of the K/Au NH/rGO–PEI matrix

To assess the antibacterial properties of K/Au NH/rGO–PEI matrix, each substrate was immersed in the bacterial suspension at a concentration of 10^8 cfu mL^{-1} for 2 h at 37°C . Both, the bacteria cell viability and morphological changes in bacteria were investigated. After incubation, $200 \text{ }\mu\text{L}$ of 10^3 -fold diluted planktonic bacteria were spread onto LB/BHI agar plates and the plates were incubated at 37°C overnight. Then, the samples were gently rinsed with Milli-Q water to remove the planktonic cells and prepared for scanning electron microscopy (SEM) measurements.

2.10 Photothermal antibacterial activity of the K/Au NH/rGO–PEI matrix

The interfaces were transferred to a new 24-well plate and irradiated with the laser set at 980 nm illumination for 10 min at 2 W cm^{-2} laser power density. Following this treatment, both the control and NIR exposed samples were prepared for SEM imaging.

2.11 Bacterial biofilm formation

S. epidermidis was grown on a BHI agar plate, upon inoculation overnight in a BHI broth at 37°C with moderate shaking (150 rpm). Upon 50-fold dilution, incubation was prolonged until the OD_{600} had reached 0.5–1. Then $50 \text{ }\mu\text{L}$ of bacteria culture was plated onto each sample and incubated at 37°C . After 30 min, 1 mL of BHI medium was added to each 24-well microtiter plate and incubated at 37°C without shaking for up to 30 h. After incubation, all samples were gently washed with Milli-Q water to remove any non-adherent bacteria.

2.12 Biofilm destruction by K/Au NH/rGO–PEI matrix under NIR irradiation

The *S. epidermidis* biofilms grown on glass slides and K/Au NH/rGO–PEI substrates were irradiated with a 980 nm-continuous

wave laser for 10, 20 min or 30 min at 2 W cm^{-2} laser power density. The NIR-induced bacterial killing of the flexible nano-heater was then studied by agar plate counting (for planktonic cells), by the OD_{600} method, contact plate assay and SEM measurements (for biofilms). Briefly, the viability of *S. epidermidis* cells detached in the aqueous medium from the surface during the exposure (planktonic cells) was estimated by comparing the colony forming units of the samples at different irradiation times, with the initial bacterial suspension. Then, the effects of K/Au NH/rGO-PEI substrates after NIR irradiation against *S. epidermidis* cells adherent on the surface (biofilm) were assessed by cell growth measurements based on the absorbance at 600 nm, by their direct contact with the surface of BHI agar plates and SEM measurements. For OD_{600} method, the samples with the control were transferred into a new 24-well culture plate containing 1 mL of BHI medium and incubated at 37°C until the OD_{600} had reached 0.3. The results were expressed as a percentage compared with the control. In addition, for each type of surface, the killing efficiency was calculated from visual images of bacterial colonies on BHI agar plates formed after application of the test surface and incubation at 37°C for 20 h and from SEM images. Statistical analyses were performed using the ImageJ open-source image treatment program. The results were estimated by comparing the colony forming units for the samples with the control.

2.13 Characterization

Contact angle measurements were performed by a remote-computer controlled goniometer system (DIGIDROP by GBX). The contact angle was measured using $2 \mu\text{L}$ of deionized water. The accuracy is $\pm 2^\circ$, and all the measurements were performed in ambient atmosphere at room temperature.

Fourier transform infrared (FTIR) spectra were recorded using a ThermoScientific FTIR instrument (Nicolet 8700) in the $650\text{--}4000 \text{ cm}^{-1}$ range at a spectral resolution of 6 cm^{-1} . 1 mg of dried sample was mixed with 200 mg of KBr powder in an agar mortar. The mixture was pressed into a pellet under 7 tons of load for 2–4 min, and the spectrum was recorded immediately. A total of 64 accumulative scans were collected. The signal from a pure KBr pellet was subtracted as a background.

UV-Vis spectroscopic measurements were carried out using a PerkinElmer Lambda UV/Vis 950 dual-beam spectrophotometer operating at a resolution of 1 nm. The UV-Vis spectra were recorded in quartz cuvettes of 1 cm path length between 200 and 1100 nm.

X-ray photoelectron spectroscopy (XPS) was recorded using an ESCALAB 220 XL spectrometer from Vacuum Generators featuring a monochromatic Al K α X-ray source (1486.6 eV) and a spherical energy analyzer operated in the CAE (constant analyzer energy) mode (CAE = 100 eV for survey spectra and CAE = 40 eV for high-resolution spectra), using the electromagnetic lens mode. The angle between the incident X-rays and the analyzer is 58° and the detection angle of the photoelectrons is 30° .

Raman spectroscopy measurements were performed on a LabRam HR Micro-Raman system (Horiba Jobin Yvon) using a 473 nm laser diode as excitation source. Visible light is focused by a $100\times$ objective. The scattered light is collected by the same

objective in backscattering configuration, dispersed by a 1800 mm focal length monochromator and detected by a CCD camera.

SEM images of pathogens were recorded using a Zeiss Compact Merlin instrument with a secondary electron detector at 2 kV under high vacuum. The biological samples were fixed with 1% glutaraldehyde solution for 30 min in the dark at room temperature. Then the samples were dehydrated in a graded ethanol series of 25%, 50%, 75%, 95% and 100% (v/v) at 10-min intervals, followed by drying using a vacuum desiccator. Each sample was mounted on stubs and sputter-coated with a 5-nm platinum layer.

3. Results and discussion

3.1 Preparation and characterization of reduced graphene oxide-polyethyleneimine (rGO-PEI)

In this study, we took advantage of the cationic properties of PEI along with the other various interactions (hydrogen, aromatic, electrostatic, hydrophobic) that rGO can form with different molecules and biological matter to design a photo-thermal heating platform, capable of efficiently capturing bacteria and subsequently killing them under NIR irradiation. To this aim, we prepared rGO-PEI nanocomposites at different ratios (1/1, 1/2, 2/1) by heating a mixture of GO and PEI at 80°C for 12 h. The rGO-PEI composites were dispersed in ethanol by ultrasonication for 1 h at room temperature (Fig. 1). The 1/1 ratio displayed better dispersibility and stability in ethanol without compromising its photothermal properties. This composite was further characterized in this study to establish its chemical structure.

FTIR spectroscopy is a useful tool to assess the presence of different functional groups in carbon-based nanomaterials (Fig. 2A). The FTIR spectrum of GO comprises a strong and broad absorption at 3264 cm^{-1} attributed to O–H vibration mode, and the C=O stretching mode of COOH groups situated at the edges of GO sheets at 1731 cm^{-1} . The peak at 1633 cm^{-1} is ascribed to C=C stretching vibration as part of the ring breathing mode in the GO skeleton. The vibration mode at 1422 cm^{-1} is most likely due to a bending vibration of tertiary C–OH groups. The peaks at 1227 and 1057 cm^{-1} are assigned to O–H stretching vibration in carboxylic acid and C–O vibration in epoxy groups, respectively.³⁸ Compared to GO, the FTIR spectrum of rGO, obtained through hydrazine reduction, shows the disappearance of most oxygen-containing groups with a concomitant shift of the C=C stretching vibration to 1568 cm^{-1} , revealing the higher reduction degree of GO and restoration of the sp^2 network.

After reaction with PEI at 80°C for 12 h, the FTIR spectrum of rGO-PEI comprises clearly all of the PEI absorption features. The peak at 1731 cm^{-1} completely disappeared, along with a significant weakening of the peak at 1057 cm^{-1} (Fig. 2A). The strong band at 1666 cm^{-1} in rGO-PEI is related to the formation of amide bonds,³⁹ but could be also due to C=C stretching bonds. This band provides a proof that covalent

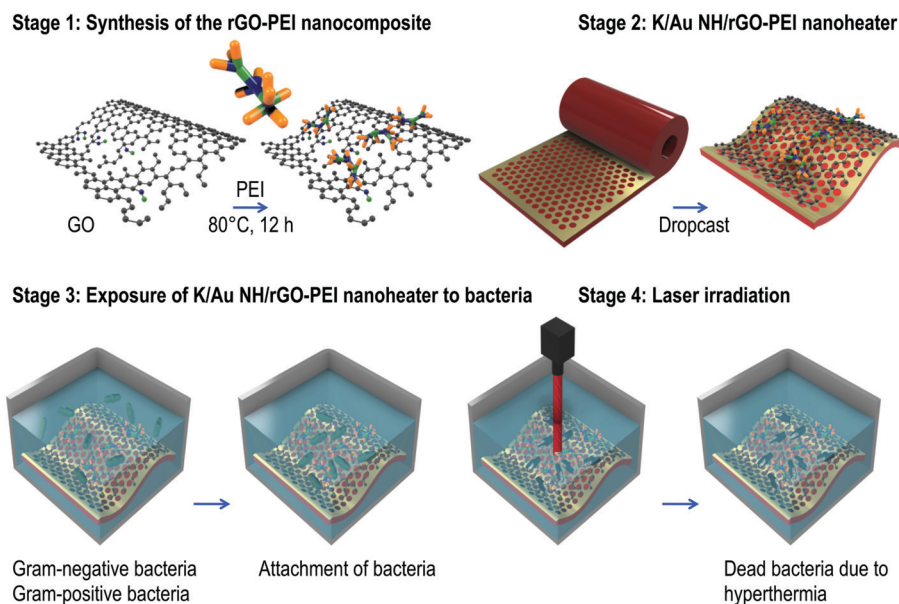


Fig. 1 A sketch illustrating the formation of reduced graphene oxide–polyethyleneimine coated nanoheaters (K/Au NH/rGO–PEI) and their application in bacteria capture and photothermal killing.

bonds are created between GO and PEI under the described experimental conditions, suggesting that PEI was grafted onto the rGO nanosheet surface.

The absorption spectra of the prepared rGO–PEI and starting materials were analyzed through UV–Vis spectrophotometry in the 200–1000 nm range (Fig. 2B). PEI absorbs below 230 nm as expected for a polymer without any aromatic structure in its backbone. The UV–Vis spectrum of GO exhibits a peak at 237 nm and a shoulder at 290 nm, which can be attributed to π – π^* transition due to C=C bonds and n – π^* transition due to C=O bonds, respectively. In contrast, the absorption spectrum of rGO shows a red shifted broad absorption band with a peak at 273 nm after 12 h of reduction with hydrazine. This red-shift is accompanied by an absorption tail at $\lambda > 400$ nm, indicating that the conjugated structure (sp^2) was restored upon reduction of GO. The red shifted absorption band to 247 nm of rGO–PEI nanocomposite suggests that GO was partially reduced under our experimental conditions. The absorption of the rGO–PEI sample in the NIR region (700–1000 nm) slightly decreased compared to rGO due to the presence of PEI in the sample even though it did not influence its photothermal properties, which were still excellent due to the underlying Au NH layer on Kapton (as discussed later).

Raman spectroscopy is a commonly used technique to provide a structural fingerprint by which molecules can be identified. The Raman spectrum of the rGO–PEI matrix (Fig. 2C) displays the main features of graphene-based materials with a D-band at 1360 cm^{-1} and a G-band at 1588 cm^{-1} .⁴⁰ The G peak appears due to the bond stretching of all pairs of sp^2 atoms in both rings and chains. The D peak is due to the breathing modes of sp^2 atoms in rings, and it is caused by the disordered structure of graphene. The degree of the disorder of the sample can be determined based on the intensity ratio of D

and G bands (I_D/I_G).⁴⁰ The I_D/I_G ratio for rGO–PEI was determined to be 1.17, smaller than that of rGO obtained upon reduction with hydrazine ($I_D/I_G = 1.22$), but higher than that of the initial used GO ($I_D/I_G = 1.1$). The increase in the I_D/I_G ratio could be attributed to a decrease in the average size of the sp^2 domains.⁴¹

XPS analysis was in addition carried out to explore the surface elemental composition of the rGO–PEI nanocomposite. The XPS elemental survey spectrum revealed that this nanocomposite was composed of the following elements: C, O, N. This result was confirmed by the specific characteristic signals at 530, 398 and 284 eV, which correspond to O_{1s} , N_{1s} and C_{1s} , respectively (Fig. 3A). The appearance of the N_{1s} peak in the XPS survey spectrum for rGO–PEI indicates that PEI chains are adsorbed or grafted onto the rGO structure.

The high resolution XPS spectrum of the N_{1s} (Fig. 3B) exhibits a dissymmetrical peak that can be fitted with two components at 399.5 and 400.8 eV attributed to $-\text{NH}_2$ and $-\text{NH}-$, respectively.⁴² The high resolution spectrum of the C_{1s} (Fig. 3C) can be decomposed into several bands at 284.2, 285.0, 286.2, 287.4 and 288.8 eV due to Csp^2 , C–H/C–C, C–O/C–N, C=O and OH–C=O , respectively, in accordance with the chemical composition of the material.

3.2 Preparation and characterization of Kapton/Au nanoholes (K/Au NH)

Advancement in healthcare electronic devices is enabled by nanostructuring, since no other combined bottom-up and top-down approaches offer so many degrees of freedom that allow man-made systems to fit the small structures of life. Here, we take advantage of colloidal lithography using polystyrene spheres of 980 nm in diameter to create large-area, self-assembled hexagonally-packed monolayers on Kapton substrates. The size of the self-assembled polystyrene beads is

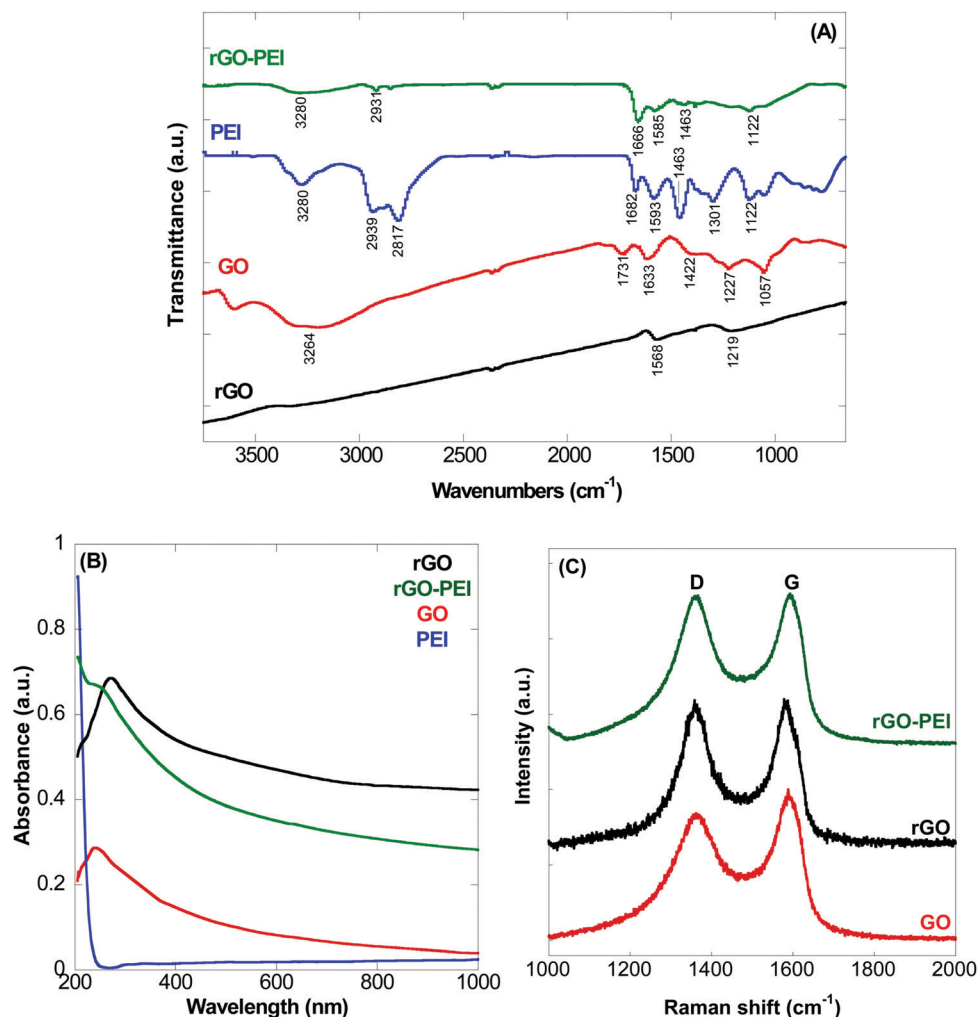


Fig. 2 (A) FTIR spectra of rGO (black), GO (red), PEI (blue) and rGO-PEI (green); (B) UV/Vis absorption spectra of PEI (blue), GO (red), rGO-PEI (green) and rGO (black); (C) Raman spectra of GO, rGO and rGO-PEI.

easily tuned by conventional SF₆ and oxygen plasma etching. Next, for the definition of Kapton-based nanoheaters, physical vapor deposition grown electrical layers (40 nm thick Au) complete the devices, providing the required K/Au NH configuration upon removal of the polystyrene particles for PTT. Fig. 4A depicts representative SEM images of a homogeneous gold nanoholes array with an average diameter of 630 ± 10 nm and a center-to-center spacing of 980 nm. The long-range ordered gold nanohole array supports both propagating and localized surface plasmon resonance (LSPR) modes, with the hole diameter and periodicity being important parameters in tuning the absorption band of the interface.^{43–46} The absorption spectrum of the resulting K/Au NH array is between 500–1100 nm with a maximum absorption at around 950 nm (Fig. 4B). The absorption maximum is close to the incident excitation wavelength of 980 nm used for photothermal heating.

3.3 Preparation and characterization of reduced graphene oxide-polyethyleneimine coated nanoheaters (K/Au NH/rGO-PEI)

The schematic representation of the preparation of the K/Au NH/rGO-PEI nanoheater is displayed in Fig. 1. The K/Au NH

interface was coated with rGO-PEI by drop casting 4 times 50 μ L of an ethanolic solution of rGO-PEI (1/1, 2 mg mL⁻¹) (stage 2). The SEM of the K/Au NH/rGO-PEI shows a homogeneous coating all over the surface (Fig. 5A).

The wetting properties of the prepared interfaces were examined by water contact angle (WCA) measurements. As can be observed from Fig. 5B, Kapton covered with a continuous layer of Au displayed a hydrophilic character with a WCA of 80°. This is very different from the K/Au NH interface, which exhibited a hydrophobic character with a WCA of 105°. The increase of the WCA upon nanohole deposition is most likely connected with an increase of surface roughness. Coating the K/Au NH interface with rGO-PEI led to a decrease of the WCA to 54°, conferring a hydrophilic character to the surface. This property is advantageous as it will enhance the contact between bacteria and the surface of the nanoheater.

The photothermal properties of the different supports were assessed through irradiation with a 980 nm-continuous wave laser, while the samples were placed in wells with 1 mL of water for 10 min; the diameter of the laser beam was adjusted directly

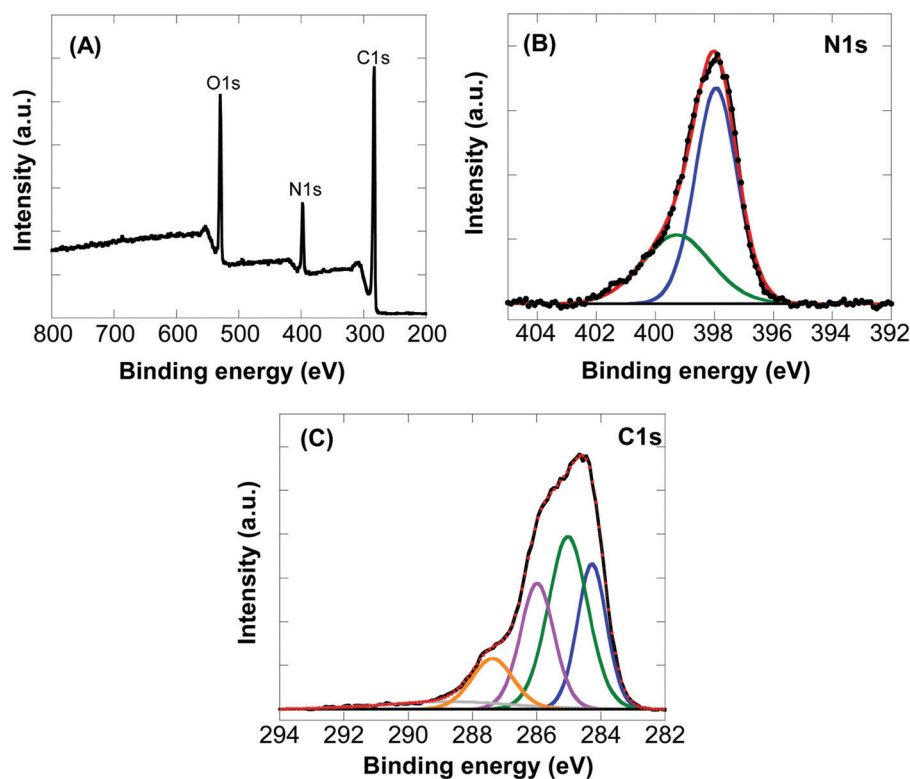


Fig. 3 XPS analysis of rGO-PEI. Wide scan survey spectrum (A), high resolution spectra of the N_{1s} (B) and C_{1s} (C).

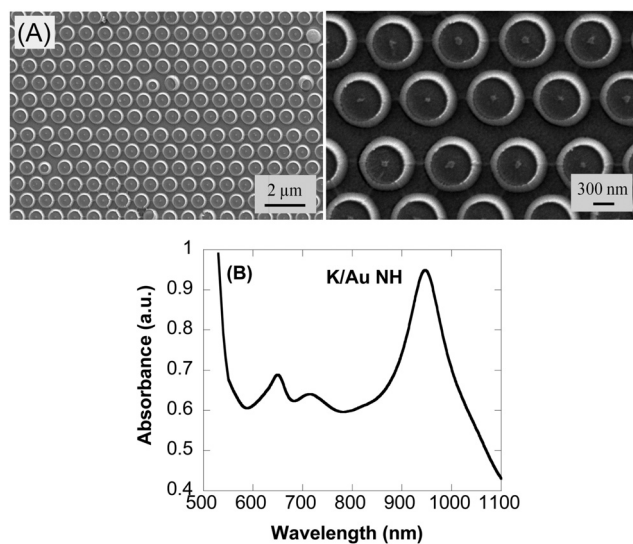


Fig. 4 (A) SEM images at low and high magnifications of K/Au NH formed by using colloidal lithography. (B) Typical absorption spectrum for K/Au NH sample.

to the well (Fig. 5C). The heating properties of Kapton foil and Kapton coated with a continuous Au film are also evaluated for comparison. While Kapton and K/Au film did not show photothermal heating ability under our experimental conditions (600 s of irradiation at 980 nm), K/Au NH displayed a steady increase of the temperature up to 500 s to reach about 42 ± 1 °C,

after 600 s of irradiation. Upon coating with rGO-PEI, the temperature of the K/Au NH/rGO-PEI increased to 70 °C using the same photothermal irradiation conditions, indicating its enhanced photothermal heating ability.

3.4 Bacteria capture and photothermal killing

To assess the capture efficiency of the K/Au NH/rGO-PEI surface, the following experiment was performed. Kapton, K/Au NH/rGO and K/Au NH/rGO-PEI samples were immersed in the bacterial suspension (1 mL), previously diluted to the desired concentration (10^3 cfu mL⁻¹). After 1 h of incubation, an aliquot of 300 μL was taken from each well and used for plating on agar plates. After overnight incubation of the plates at 37 °C, we performed counting and comparison of colony forming units of the samples with the control, in order to calculate the percentage of the bacteria remaining in the solution and the bacteria attached to the surface. The capture efficiency of the K/Au NH/rGO-PEI nanoheater was 42% and 47% towards *E. coli* and *S. aureus* bacteria, respectively (Table S1, ESI†).

The ability of the K/Au NH/rGO-PEI nanoheater to capture and kill bacteria was assessed for both Gram-positive *S. aureus* and Gram-negative *E. coli* bacteria, under NIR (980 nm) laser continuous irradiation for 10 min. The morphology of the nanoheater immersed in a bacterial solution (10^8 cfu mL⁻¹) for 2 h before and after laser irradiation was visualized using scanning electron microscopy (SEM) (Fig. 6 and 7).

The SEM images of *E. coli* bacteria after 2 h of incubation before laser irradiation are shown in Fig. 6A. It can be clearly

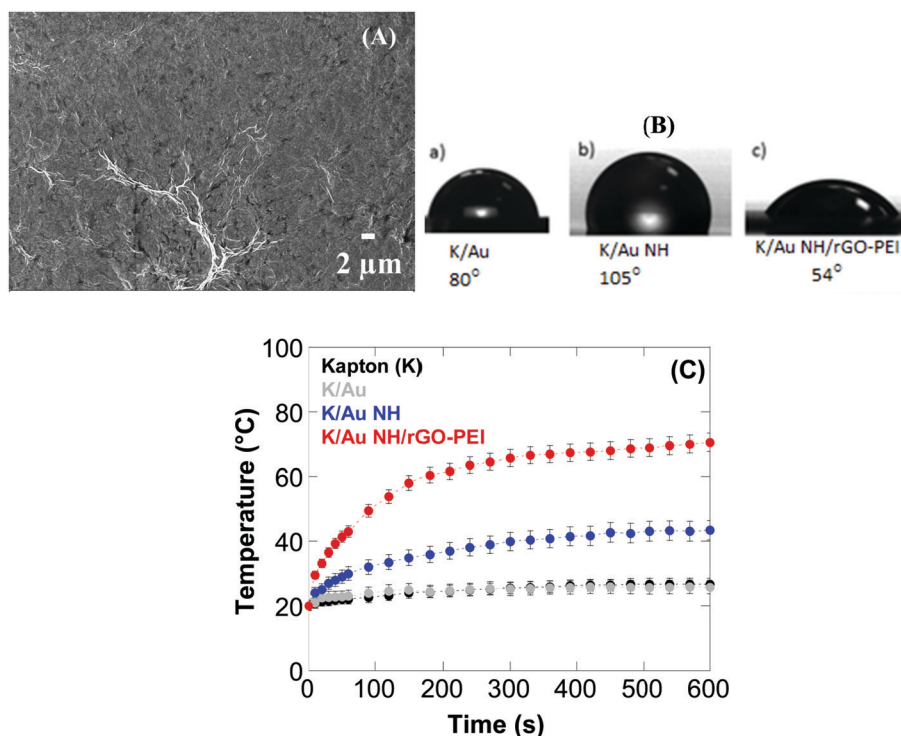


Fig. 5 (A) SEM image of the K/Au NH/rGO-PEI coating. (B) Water contact angles of (a) Kapton covered with a continuous Au film (K/Au), (b) Kapton/Au nanoholes (K/Au NH), (c) Kapton/Au nanoholes coated with rGO-PEI (K/Au NH/rGO-PEI). (C) Temperature changes of different samples after 10 min of NIR irradiation (980 nm) at 2 W cm⁻².

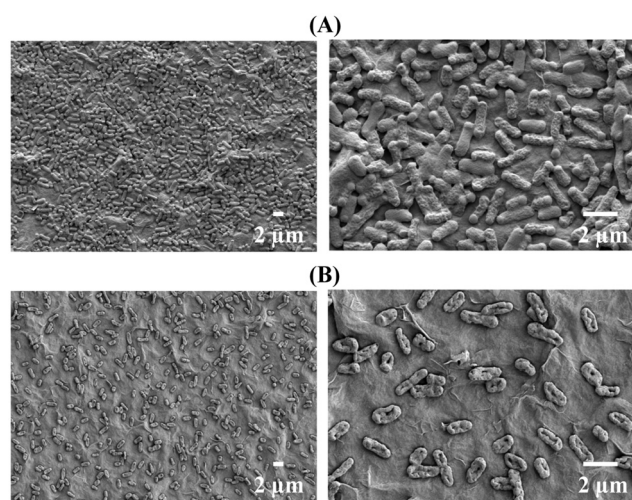


Fig. 6 SEM images of *E. coli* K-12 MG1655 after 2 h of incubation before (A) and after (B) laser irradiation at 980 nm for 10 min.

seen that the nanoheater surface is covered with a compact and intact monolayer of bacteria with a rod-shaped morphology. In contrast, after laser irradiation for 10 min, a decrease in the number of attached *E. coli* bacteria was clear, but also the cells appeared to be damaged with a pronounced rupture of their outer membrane (Fig. 6B).

A similar observation can be made for *S. aureus* bacteria (Fig. 7). The typical near-spherical shape of cocci completely

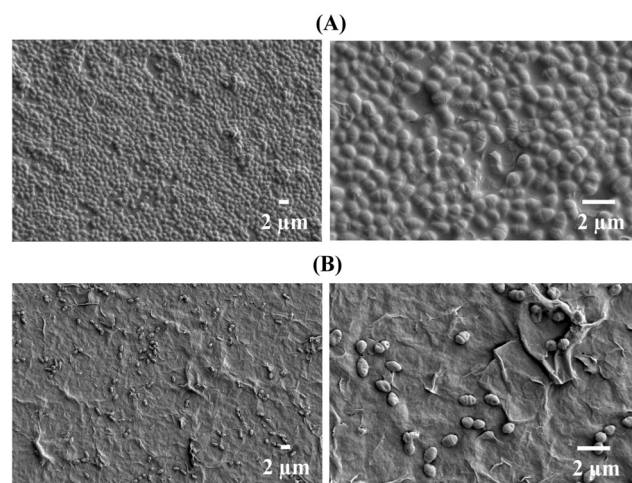


Fig. 7 SEM images of *S. aureus* after 2 h of incubation before (A) and after (B) laser irradiation at 980 nm for 10 min.

covered the surface of the K/Au NH/rGO-PEI nanoheater after 2 h of incubation before laser irradiation (Fig. 7A). After 10 min of laser irradiation, the number of bacteria significantly decreased, and the cells showed different forms of damage on the cell surface (Fig. 7B).

SEM images confirmed both *E. coli* and *S. aureus* adhesion to the rGO-PEI surface, and bacteria killing caused by hyperthermia. Furthermore, SEM imaging and the cell viability evaluation (Fig. 8) showed that the rGO-PEI

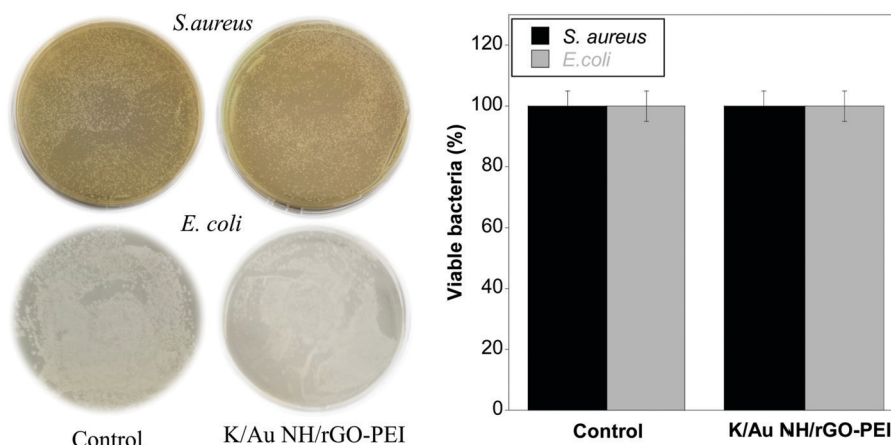


Fig. 8 Typical photographs of *S. aureus* and *E. coli* colonies and the corresponding statistical histograms after 2 h of incubation with K/Au NH/rGO-PEI.

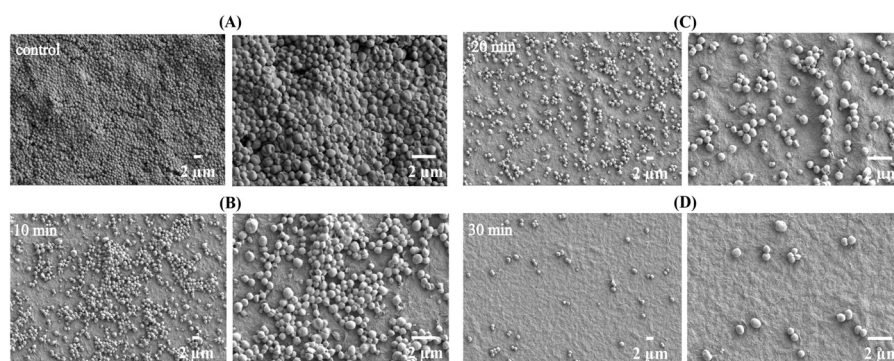


Fig. 9 SEM images of *S. epidermidis* biofilm before (A) and after laser irradiation at 980 nm for 10 min (B), 20 min (C) and 30 min (D).

coating itself was not toxic to bacteria without laser irradiation.

As can be seen in Fig. 8, the amount of viable *E. coli* and *S. aureus* cells after 2 h of incubation on the nanoheater surface is almost the same as the control group. Another aspect of this work is the non-specificity of bacteria capture by PEI. Indeed, the presence of amino groups in the PEI backbone confers an overall positive charge to the surface in water. Under these conditions, the surface is capable of interacting with any chemical or biological matter bearing a negative charge through electrostatic interactions. This property is of high importance for water cleaning applications.

3.5 Bacteria biofilm destruction

It is well known that in contrast to their free-floating planktonic cell counterparts, the pathogenic bacteria grown on a surface in the form of a biofilm is more difficult to eradicate. Living as a structured community of microbial cells, embedded in their self-produced extracellular polymeric matrix, significantly increases their tolerance and resistance to the inhibitory effects of anti-bacterial agents.⁴⁷ In the present study, we investigated the capacity of K/Au NH/rGO-PEI device to destroy *S. epidermidis* biofilms after NIR irradiation for a given time period by SEM and by using a plate-counting method. Before laser irradiation, a uniform and thick biofilm structure consisting of multi-layered

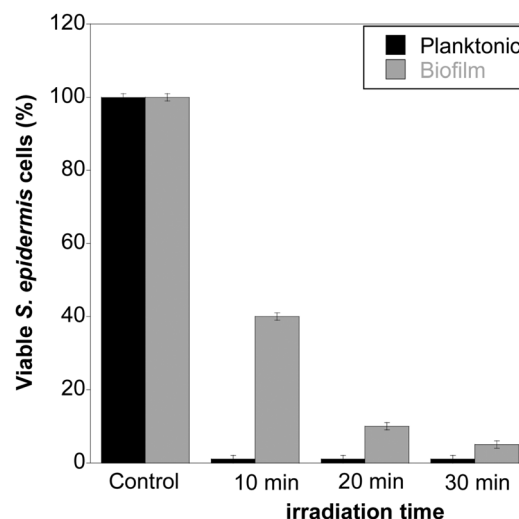


Fig. 10 Planktonic and biofilm cell viability evaluation of *S. epidermidis* at different laser irradiation times.

spherical microbial cells can be observed (Fig. 9A) on the surface of the K/Au NH/rGO-PEI nanoheater. After 10 min of irradiation, the SEM images show a significant decrease in the biofilm mass, as well as morphological damage and collapse of the bacterial cell membrane in the biofilm (Fig. 9B).

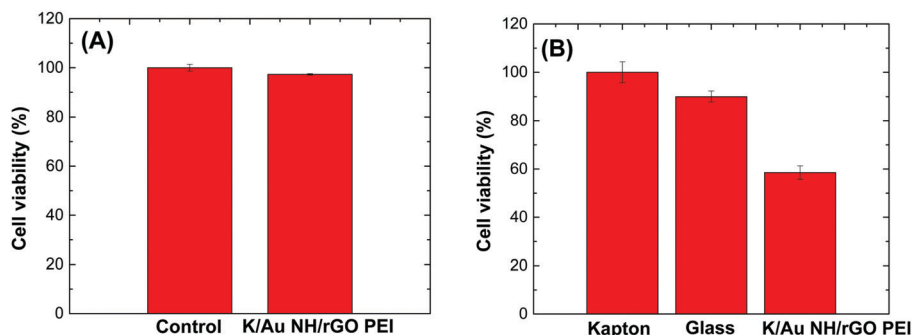


Fig. 11 Relative cell viability of NG108-15 cells after 24 h of culture in DMEM (control) and DMEM in which the K/Au NH/rGO–PEI matrix was previously immersed for 24 h (indirect cytotoxicity test) (A) and after 24 h of culture on 1 cm² Kapton, glass and K/Au NH/rGO–PEI substrates (direct cytotoxicity test) (B).

It has to be highlighted that the NIR irradiation alone, in the absence of the nanoheater, had no visible effect on the biofilm integrity (Fig. S1, ESI[†]). After 20 min of irradiation, a significant decrease in the biofilm mass can be observed (Fig. 9C) and the biofilm was almost completely destroyed after 30 min of treatment (Fig. 9D).

As indicated by analysis of the survival rate and biofilm destruction, the mortality rate of the bacteria from the biofilm grown on the surface of the nanoheater was increasing with the irradiation time, while the supernatant was sterile even after 10 min of irradiation (Fig. 10).

3.6 Cytotoxicity assay

The indirect *in vitro* cytotoxicity test of the K/Au NH/rGO–PEI nanoheater revealed the absence of release of cytotoxic components from the nanocomposite matrix during the 24 h of immersion in DMEM. As can be seen in Fig. 11A, the relative viability of NG108-15 cells remained above 95%. Our results indicate that the nanocomposite is stable. In contrast, the direct *in vitro* cytotoxicity demonstrated that the cells are not able to attach and grow on the sample. After 24 h of incubation, compared with glass and Kapton, a significant decrease in cell viability was measured for the K/Au NH/rGO–PEI nanoheater (Fig. 11B).

3.7 Reusability of K/Au NH/rGO–PEI nanoheater

An ideal antibacterial nanocomposite should, besides its excellent antibacterial efficiency, be reusable in order to lower the costs of production. After the capture and heating experiments, the K/Au NH/rGO–PEI nanoheater was rinsed in ethanol and recovered by dropcasting 50 μ L of previously prepared PEI solution (1 mg mL^{−1}). This modification did not compromise the heating ability of the K/Au NH/rGO–PEI nanoheater, nor did it influence its cytotoxicity. After drying, the sample was rinsed and then immersed in Milli-Q water for 3 h to release excess PEI molecules and finally washed with ethanol again, before the experiment. The capture efficiency of the reused and recovered K/Au NH/rGO–PEI nanoheater was 95% for the *S. aureus* bacteria, and 67% for *E. coli*. The same sample was reused without additional PEI modification and the capture rate after the second reutilization was above 40% for both strains.

4. Conclusion

In this study, we developed a Kapton/Au nanoholes/reduced graphene oxide–polyethyleneimine (K/Au NH/rGO–PEI) nanoheater by using a simple and straightforward approach for efficient capture and photothermal killing of bacteria under NIR irradiation. The nanoheater takes advantage of the enhanced photothermal properties of the Kapton/Au NH upon coating with rGO–PEI. Additionally, the presence of PEI, a branched polymer containing a large number of electron-rich amino groups, enables efficient capture of both Gram-positive and Gram-negative bacteria through electrostatic interactions. We demonstrated that the K/Au NH/rGO–PEI nanoheater was an effective photothermal agent toward both Gram-positive *S. aureus* and Gram-negative *E. coli* bacteria, under low-power NIR (980 nm) laser irradiation with complete bacteria eradication within 10 min. Additionally, the nanoheater was revealed to be very efficient for *S. epidermidis* biofilm destruction upon 30 min irradiation at 980 nm. Nanoheaters with efficient antibacterial properties, that are non-toxic for the environment have great potential for water purification systems, biomedical and industrial applications. The rapid and effective antibacterial activity as well as UV to NIR region absorption property might make K/Au NH/rGO–PEI antibacterial nanocomposites work even under normal solar light.

Conflicts of interest

There are no conflicts of interest to declare.

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