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One-pot synthesis of a new generation of hybrid bisphosphonate polyoxometalate gold nanoparticles as antibiofilm agents

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SUPPORTING INFORMATION

Materials. Sodium metavanadate, sodium citrate, gold(III) chloride trihydrate and rubidium chloride were purchased from Sigma-Aldrich. Triethylamine was obtained from Acros Organics. Alendronic acid $H_2O_3PC(C_3H_6NH_2)(OH)PO_3H_2$ (Ale) was synthesized as previously described.[1]

Milli-Q water (18 MΩ, Millipore, France) was used for the preparation of gold nanoparticles. All glassware used was cleaned with *aqua regia*, then carefully rinced with Milli-Q water before use.

Bacterial strains *Pseudomonas aeruginosa* (ATCC® 27853™) were obtained from American type culture collection and *Staphylococcus epidermidis* (CIP 105 777) were obtained from the Collection of Institut Pasteur. Media were purchased from Conda Laboratory (Spain).

Synthesis

- **Polyoxovanadate :** $Na_3[V_3O_3(H_2O)(O_3PC(C_3H_6NH_3)(OH)PO_3)_3]\cdot 10H_2O$ (V_3Ale_3 or **POV_{red}**)

To a solution of $NaVO_3\cdot H_2O$ (0.067 g, 0.55 mmol) in 5 mL of water was added alendronic acid (0.124 g, 0.50 mmol). The solution was stirred for 1 h at room temperature, and triethyamine was added dropwise to pH = 6. Then, the pH was adjusted to 4.7 with 1M HCl. The solution was stirred for 2 h at reflux, then left to evaporate at room temperature. Green crystals were collected after 2 days and washed with 2M NaCl. Yield 0.105 g (52% based on V). Anal. Calc. (found) for $C_{12}H_{49}Na_3N_3O_{35}P_6V_3$ (1202.41 g mol^{-1}): C 11.97 (12.25), H 4.10 (3.70), N 3.49 (3.53). IR (FTIR): ν (cm^{-1}): 1629 (s), 1508 (w), 1086 (s), 1051(m), 1039(m), 999 (s), 966 (s), 929 (m).

- **Gold nanoparticles using POV_{red} as reducing and stabilizing agent (NPs@POV)**

2.5 mL of an aqueous solution of POV_{red} (1.7 mM) were added at once to 2.5 mL of an aqueous solution of $HAuCl_4$ (0.5 mM) at pH = 6 adjusted with 0.5 M K_2CO_3 aqueous solution. The reaction was heated to 50°C for 1 hour. Then, the nanoparticles were centrifuged at 4000 rpm for 25 min. The colorless supernatant was removed and the nanoparticles were dispersed in 5mL of Milli-Q water.

- **Citrate-protected gold nanoparticles (CitNPs)**

The 15 nm colloidal gold nanoparticles were prepared following the method introduced by Turkevich.[2] 95 mL of a chlorauric acid $HAuCl_4$ (263 µM) solution were refluxed for 30 min and 5 mL of a sodium citrate (34 mM) solution added. The reduction of the gold ions by the citrate was completed after 5 min. The solution was further refluxed for 30 min and then left to cool to room temperature. Then, the colloidal solution was centrifuged at 14500 rpm for 15 min, and the colorless supernatant removed. The nanoparticles were dispersed in Milli-Q water, affording spherical nanoparticles with an average diameter of ca. 15 nm.

The 47 nm citrate-protected gold nanoparticles were synthesized mixing a solution A (20 mL of a chlorauric acid $HAuCl_4$ (360 µM)) and solution B (5mL of sodium citrate (2 µM)). Solution A and B were heated to 60°C under stirring then mixed. As the solution turned red, the mixture was heated up to 95◦C for few minutes and finally cooled on ice. Then, the colloidal solution was washed as the 15 m citrate-protect gold nanoparticles.

- **Functionalization of citrate-protected gold nanoparticles with POV_{red} (CitNPs@POV_{red})**

1.5 mL of an aqueous solution of POV_{red} (2.5 mM) were added with stirring to a vial containing 1.5 mL of an aqueous suspension of citrate-protected gold nanoparticles (CitNPs) prepared as described above. The solution was stirred for 20 h. Afterwards, the nanoparticles were centrifuged at 14500 rpm for 15 min and the colorless supernatant was removed. The colloidal solution was dispersed in 5 mL of Milli-Q water.

Characterization Techniques

Thermal Gravimetric Analysis were performed, in air atmosphere, on a Mettler Toledo-TGA equipment. The ramp rate was 5°C.min^{-1} and the sample was placed in an aluminum pan.

ATR-FTIR spectroscopy measurements were performed on a Nicolet 30 ATR 6700 FT spectrometer with scan range from 4000 to 400 cm^{-1}.

UV-Visible spectroscopy spectra were recorded on a PerkinElmer Lambda 750.

Inductively Coupled Plasma Optical Emission Spectrometry : P, V, and Au contents were determined on previously acid digested samples in aqueous solution on an Agilent

Technologies 700 Series ICP-OES instrument. The digestion procedure was carried out by the addition of a 0.5 mL 10% HNO_3:HCl (v/v) solution to 0.5 mL of each nanoparticle solution. After the digestion process (90 min), the samples were diluted with 4 mL of 2.5% HNO_3/HCl (v/v) and 2 mL of MilliQ water.

Transmission Electron Microscopy : Transmission electron microscopy (TEM) measurements were performed using a JEOL JEM 1011 microscope operating at an accelerating voltage of 100 kV. The TEM grids were prepared by depositing 10 µL of a particle suspension on a carbon-coated copper grid, and dried at room temperature. The nanoparticles size distribution was calculated by measuring a minimum of 300 particles using the ImageJ software.

Dynamic Light Scattering : Batch mode hydrodynamic size (diameter) measurements were performed on a Malvern Zetasizer Nano ZS. A minimum of three measurements per sample were made.

Zeta Potential Measurements : A Malvern Zetasizer Nano ZS instrument was used to measure zeta potential at 25°C for all samples. Samples prepared for the DLS measurements were loaded into a pre-rinsed folded capillary cell for the zeta potential measurements. A minimum of three measurements per sample were made.

Calculation method of the coverage ratio :

The POM/Au^0 nanoparticle coverage ratio σ was calculated considering the following formula:

$$\sigma = \frac{N_{POM}}{N_{AuNps}\, S_{AuNps}}$$

where N_{POM} is the total number of POM molecules by liter, N_{AuNps} the total number of gold nanoparticles by liter and S_{AuNps} the surface of one gold nanoparticle, with:

$N_{POM} = \frac{w_V N_A}{M_{(V)} \times 3}$, w_V being the vanadium mass concentration.

N_{AuNps} is equal to the number of Au atoms by liter divided by the number of Au atoms in a particle and can be written:

$N_{AuNps}= \frac{w_{Au}N_A}{V_{AuNps}/V_{Au}}$, $V_{AuNps} = \frac{\pi d^3}{6}$ (d = nanoparticle average diameter (nm)) being the volume of one gold nanoparticle and $V_{Au} = \frac{M_{Au}}{N_A\,\rho_{Au}}$ the volume of a gold atom.

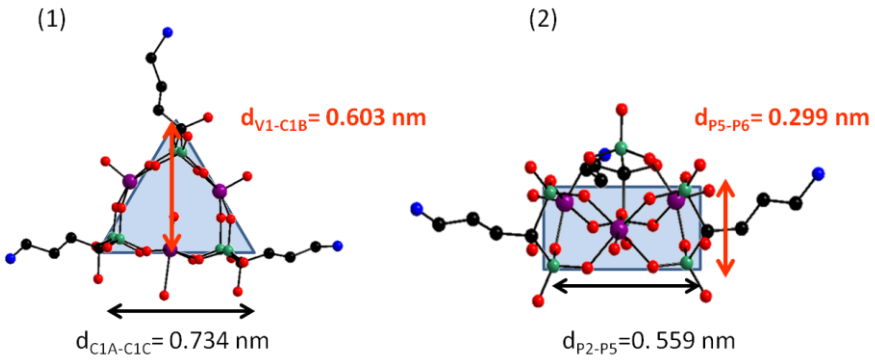
The equation can thus be written:

$$\sigma = \frac{N_{POM}}{N_{AuNps}\,S_{AuNps}} = \frac{w_V\ d\ \ N_A\,\rho_{Au}}{18\,M_V \times w_{Au}}$$

With $S_{AuNps} = \pi d^2$ and w_V and w_{Au} being measured by ICP.

Estimation of coverage ratio for one monolayer :

We can estimate the coverage ratio for a POV's monolayer. The POV can adopt two positions on the gold nanoparticles surface.



	(1)	(2)
Surface of POV (nm²)	0.122	0.168
Surface of 30.8 nm gold nanoparticle (nm²)	2980.24	2980.24
Number of POV for 1 gold nanoparticle	13425	17739
Coverage ratio for a monolayer (POV/nm²)	5	6

Crystallographic data

The data were measured at the XALOC beamline of the ALBA synchrotron (λ = 0.72931 Å) at 100 K using a MD2M single axis diffractometer (Maatel, France) and a Pilatus 6M detector (Dectris, Switzerland).

The crystal was taken directly from its solution, mounted with a drop of Paratone-N oil and immediately put into the cold stream of dry N_2 on the goniometer and kept at 100 K with a CryoStream 700 (Oxford Cryosystems, UK). X-ray diffraction data were collected in rotation steps of 1° over a total range of 360°. The X-ray diffraction images were indexed, integrated and scaled using the XDS package[4,5] and scaled with SCALA from the CCP4 suite.[6]

The structure was solved with the SHELXT[7] structure solution program, using the Intrinsic Phasing solution method. The model was refined with version 2018/3 of SHELXL[8] using Least Squares minimisation.

Empirical formula: $C_{12}H_{27}N_3Na_3O_{32.75}P_6V_3$, formula weight (*g*): 1144.97, crystal system: triclinic, space group: *P*-1, *a* (Å) = 13.241(3) , *b* (Å) = 18.055(4), *c* (Å) = 21.996(4), α (°) = 99.43(3), β (°) = 90.12(3), γ (°) = 90.58(3), *V* (Å^3) = 5187.2, *Z* = 4, ρ_{calc} (g cm^{-3}) = 1.466, μ (mm^{-1}) = 0.88, data (parameters): 27527 (1066) , R_{int} = 0.106, GOF = 1.02, $R_1(>2\sigma(I))$ = 0.134 , wR_2 = 0.341.

The complete data can be found in the cif file (CCDC 1926180).

There are two independent molecules in the unit cell (Fig. S1), which differ by the position of the alkyl chain of one of the alendronate ligand. In the first molecule, the ligands are not disordered while in the second molecule the ligand is disordered over two positions with equal occupancy factors.

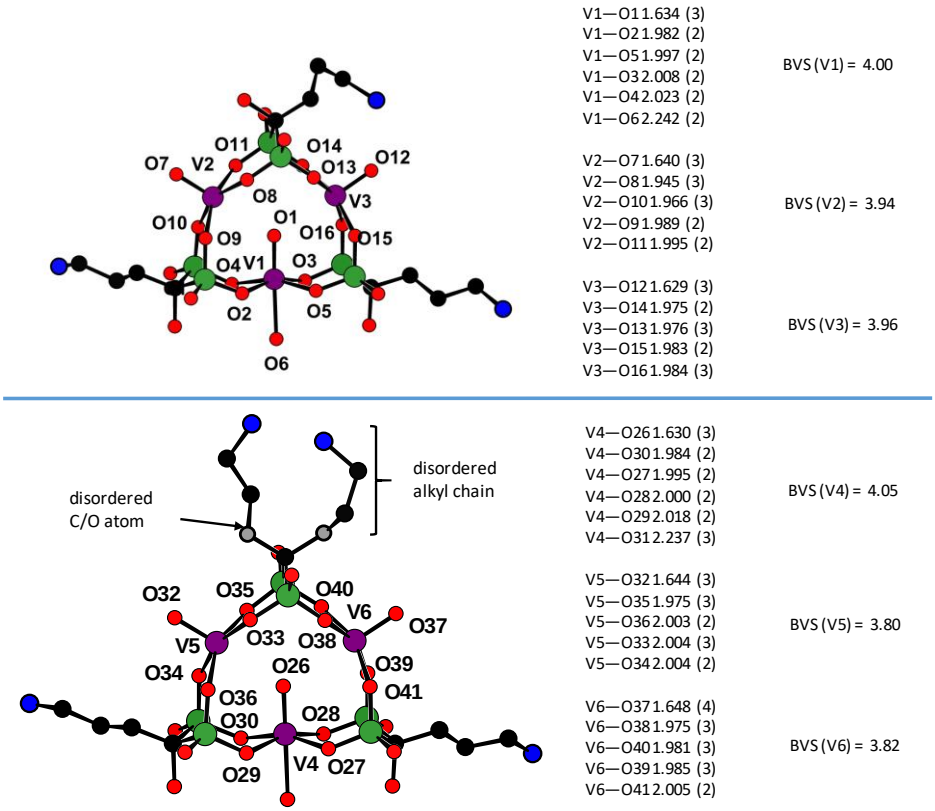


Fig. S1 : Ball-and-stick representations of the two independent molecules in the structure of the polyoxovanadate $V_3(Ale)_3$, V-O bond distances and bond valence sums for the vanadium ions. Purple spheres = V, green spheres = P, red spheres = O, black spheres = C, blue spheres = N, H atoms have been omitted for clarity.

Antibacterial activity

The gram-negative bacteria *Pseudomonas aeruginosa* (ATCC® 27853™) and the gram-positive bacteria *Staphylococcus Epidermidis* (CIP 105 777) were grown on Lysogeny Broth (LB) plates and Mueller-Hinton (MH) plates, respectively, at 37°C for 24 h. A single colony was inoculated in LB or MH medium and grown in shaker incubator at 150 rpm at 37°C. After incubation overnight, bacteria cells were suspended in media.

Biofilm assays were carried out following the procedure described by H. Mu et al.[3] 100µL (10^6 CFU) of *P. aeruginosa* and *S. epidermidis* solution were added to individual wells of a sterile 96-well microtiter plates. The plates were incubated at 37 °C for 24 h to allow biofilm formation.

Then, the supernatant containing non-adhered cells was removed and the wells were washed three times using 0.9% (w/v) NaCl solutions. Afterwards, existing biofilms were

incubated at 37 °C in 90 µL of culture medium and 10 µL of each nanoparticle solution for 24 h and 48 h. Bacteria incubated with medium only were used as control.

After different incubation times the media from each well were removed with a pipette and the plates were gently washed three times with 0.9% NaCl solutions. The plates were air dried at room temperature. Then the biofilm was stained by adding 100 µL of crystal violet (1%, w/v) for 20 min. Excess crystal violet was gently removed by washing. The biofilm material was then solubilized in 150 µL of acetic acid[4] and quantified by measuring the OD_{620} using a microtiter plate reader (Multiskan ^{TM} FC, ThermoFisher Scientific). All assays were performed at least 10 times.

Data were expressed as mean ± standard deviation. The values of the experimental groups were compared with the control group. Statistical significance was analyzed by Students's T-test to detect the presence of statistically significant differences (*** $p<0.001$, ** $p<0.01$, * $p<0.05$). All tests were repeated at least 10 times.

Observation of bacteria morphology by Microscopy

Bacterial morphology analyses were carried out by SEM-FEG imaging, in order to visualize the potential effect of functionalized gold nanoparticles (NPS@POV) upon contact with bacteria.

Since SEM-FEG observations is a high vacuum technique, gold surfaces were prepared by fixing bacterial state using glutaraldehyde reticulation.

Thus, surfaces were immersed 2 h in 2.5% glutaraldehyde (v/v in PBS) solution. After PBS rinsing, surfaces were gently dried using ethanol solutions of increasing concentrations (25, 50, 75, 96 and 100%) before being dried.

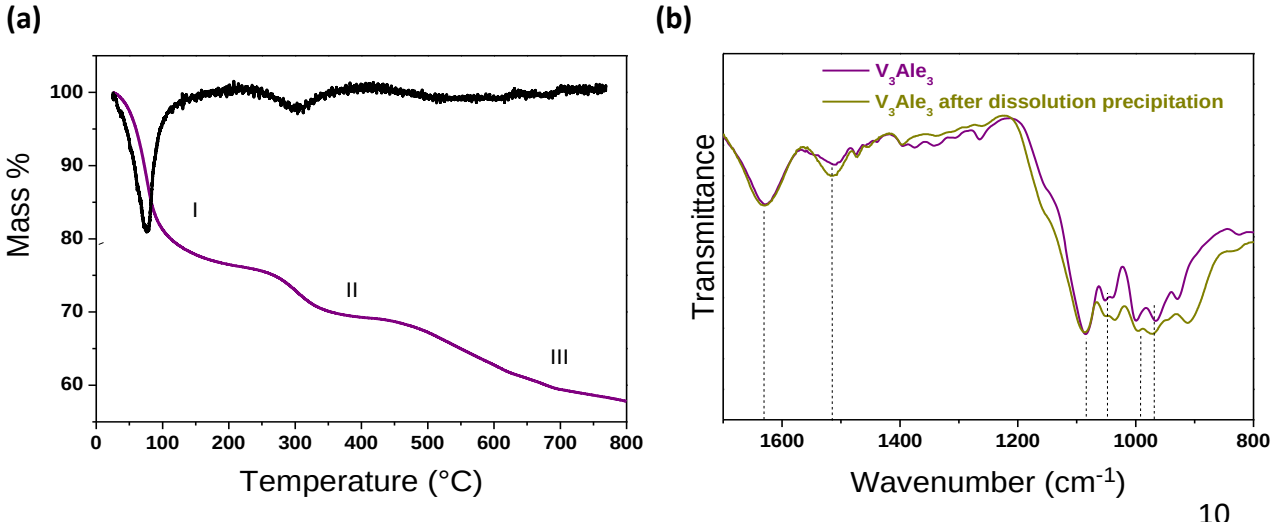


Fig. S2 (a) TGA curve and Derivative weight and (b) ATR-FTIR spectrum of the polyoxovanadate $V_3(Ale)_3$ as synthesized and after dissolution–reprecipitation.

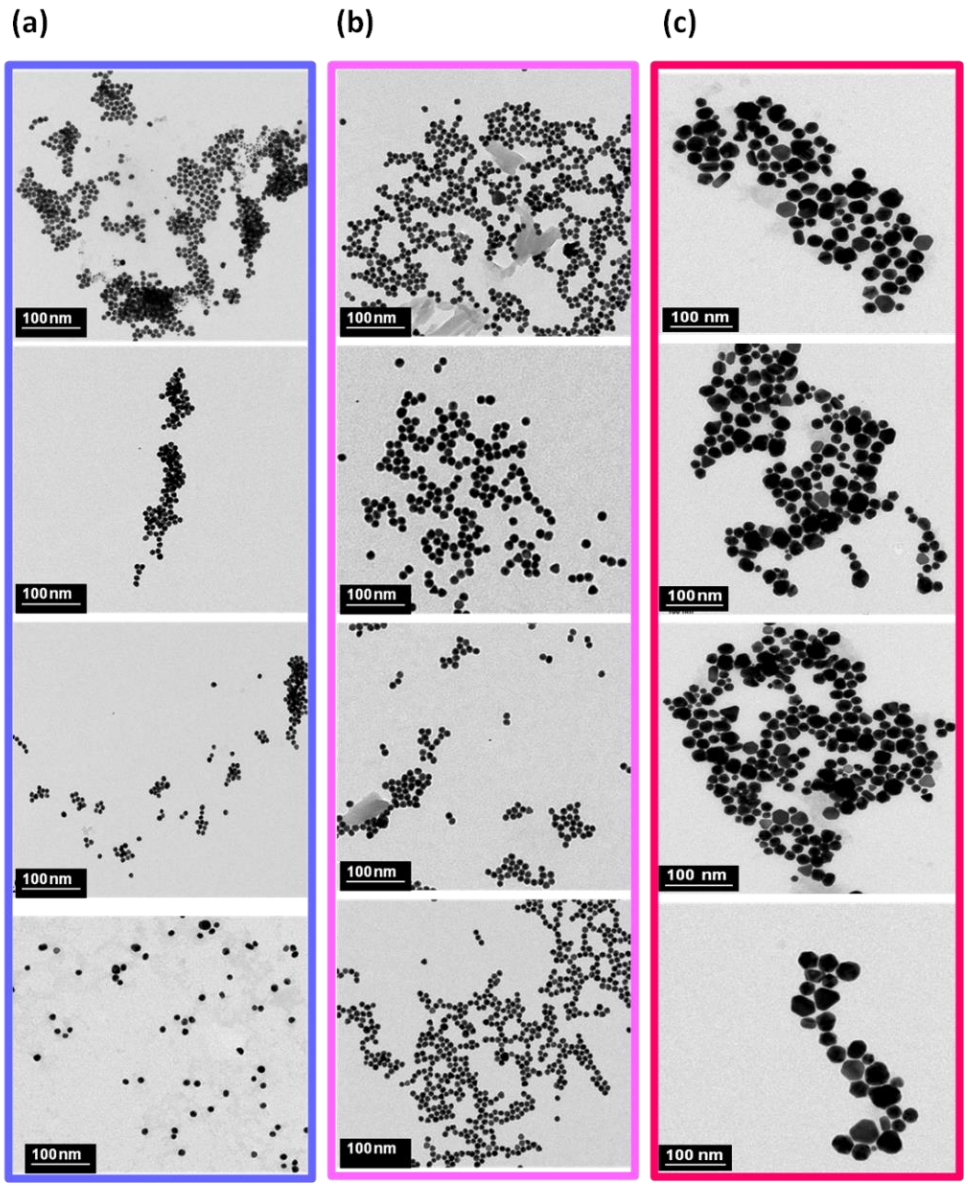


Fig. S3 TEM images used to obtain the size histograms of (a) CitNPs, (b) CitNPs@POV$_{red}$ and (c) NPs@POV.

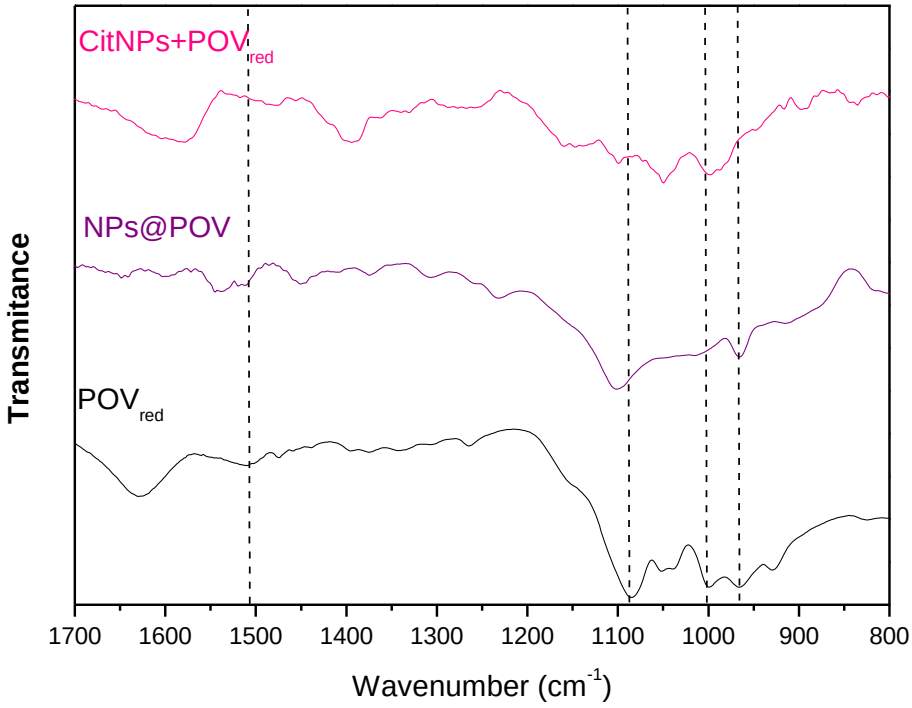


Fig. S4 ATR-IR spectra of POV_{red} (black), NPs@POV (purple) and CitNPs@POV_{red} (red).

Table S1 Infrared Frequencies and Vibrational Assignments for POV, NPs@POVox and CitNPs@POV

Assignment	POV_{red}	NPs@POV	CitNPs@POV_{red}
δs (NH_2)		1598	1577
δs (NH_3^+)	1508	1515	
ν (P-O), ν (P=O)	1086	1099	1097
	1051	1045	1049
	1039	1039	1029
ν (V=O) and ν (V-O),	999	999	999
	966	953	964
	929	916	

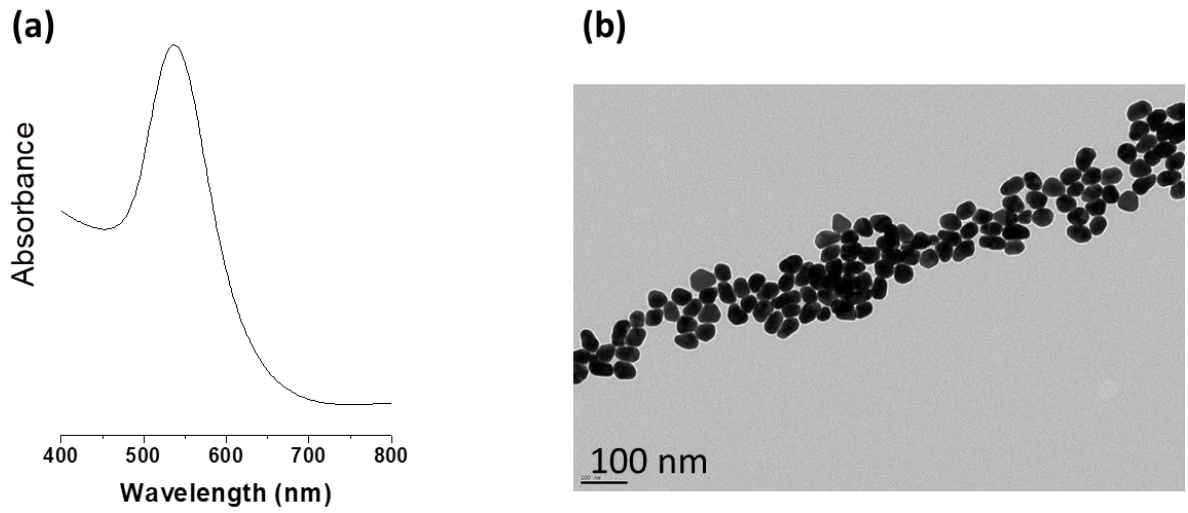


Fig. S5 Characterization of 47 nm CitNPs@POV_{red}. UV-vis spectrum (a), TEM image (b)

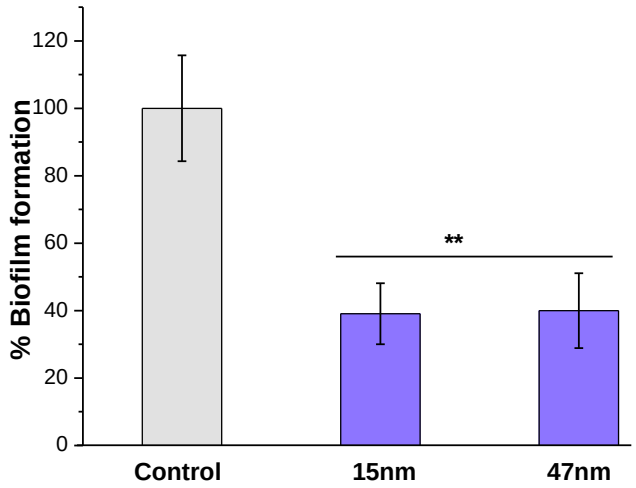


Fig. S6 Effect of the size of CitNPs@POV_{red} on biofilm formation for *P. aeruginosa* ATCC® 27853TM after incubation for 24h in 96-well plates. Statistical significance (Student's T-test):*** p<0.001, **p<0.01, *p<0.05 versus control.

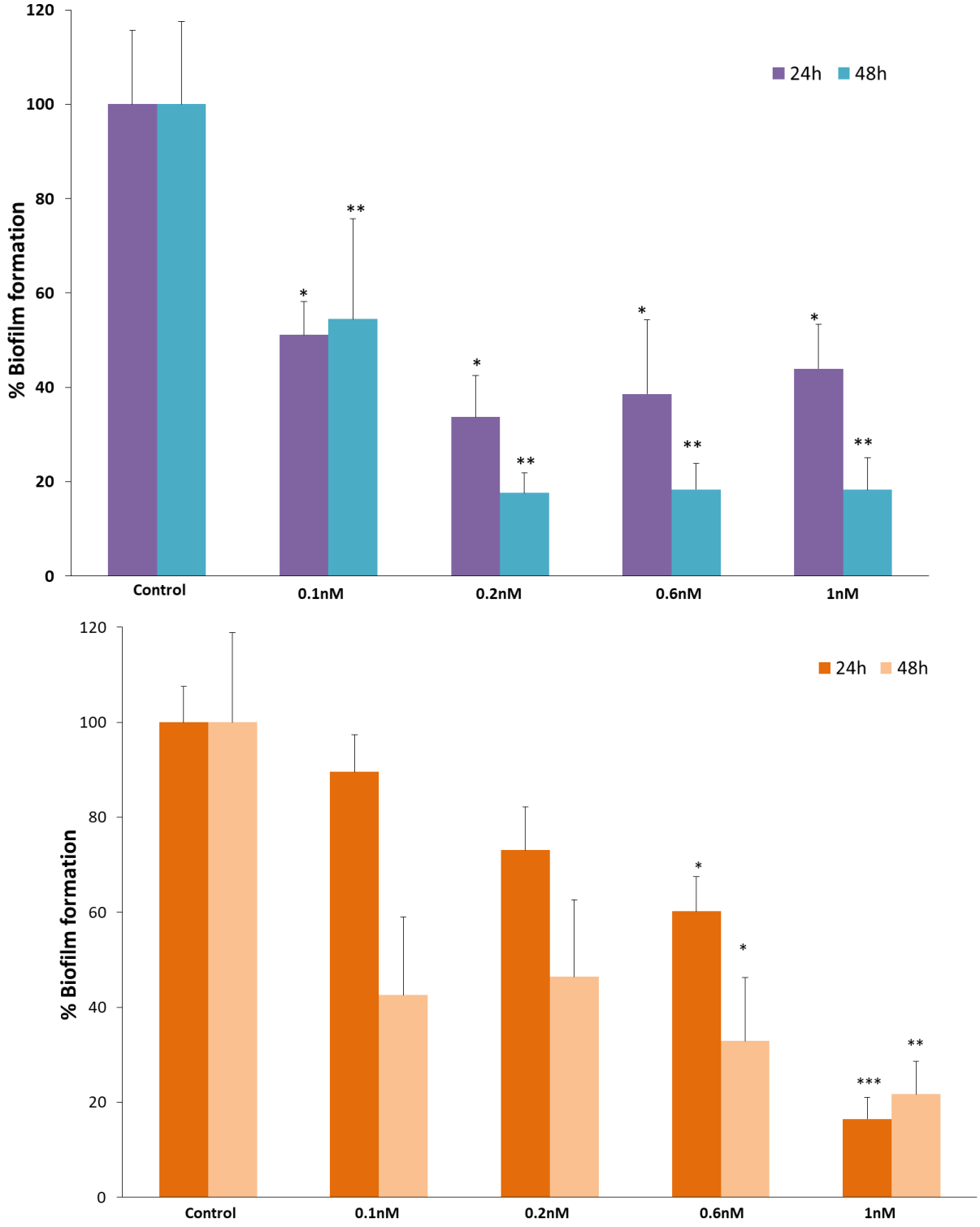


Fig. S7 Effect of the concentration of NPs@POV on biofilm formation for *P. aeruginosa* ATCC® 27853TM (top) and *S. epidermidis* CIP 105 777 (bottom) after incubation for 24 and 48h in 96-well plates. Statistical significance (Student's T-test):*** p<0.001, **p<0.0

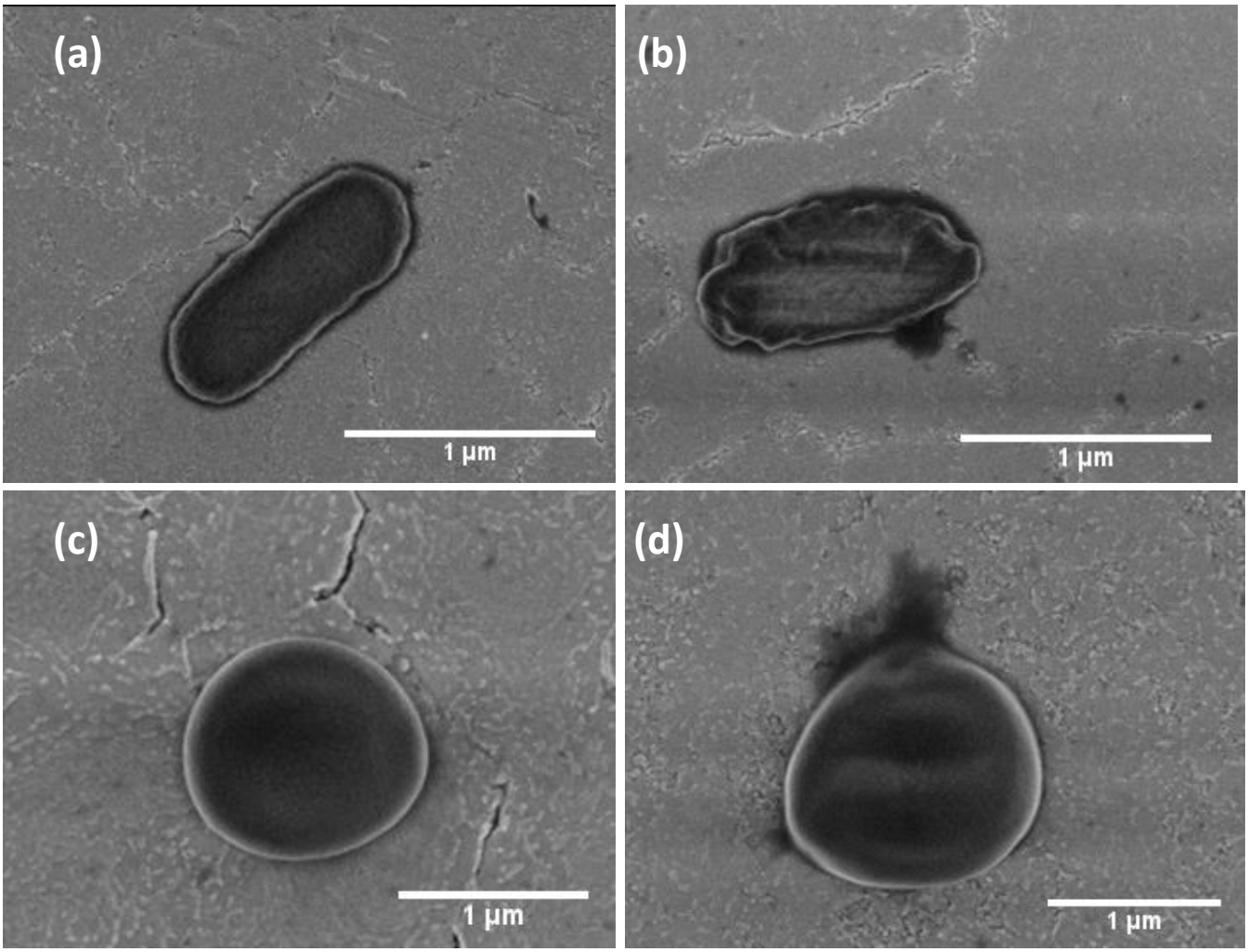


Fig. S8 SEM micrographs of untreated cells of *P. aeruginosa* (a) and after interaction with NPs@POV (b) and *S. epidermidis* (c) and after interaction with NPs@POV (d).

References

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