# Bioinspired antibacterial nanostructured surfaces based on oriented calcium phosphate nanocrystals arrays against carbapenem-resistant *P. aeruginosa* and methicillin-resistant *S. aureus*

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## Introduction

Mitigating antimicrobial resistance requires evidence-based public health measures to prevent the projected O'Neill scenario by 2050 (*The Review on Antimicrobial Resistance chaired by Jim O'Neill, December 2014*). Action should go beyond creating new antibiotics and should focus on agents with resistance-avoidance bactericidal mechanisms. *Neotibicen Canicularis*, a cicada species, possesses a unique nanostructured surface on its wings, which is antimicrobial through a contact-based mechanism.

This finding has prompt the development of synthetic bioinspired antibacterial nanostructured surfaces (ANS) which can counteract antibiotic resistance. Calcium phosphates (CaP) are bioactive materials with excellent biocompatibility, making them promising materials for developing ANS as coatings for orthopedic devices to prevent infections. This study aims to fabricate and characterize organized bioinspired CaP nanocrystals arrays for potential medical use as coating in orthopedic devices.

## Materials and Methods

CaP nanocrystals arrays were grown through a novel bottom-up biomineralization approach onto amorphous calcium phosphate (ACP) substrates. Tested substrates were ACP (**ACP.GS**) or citrate-stabilized ACP (**Cit-ACP.GS**). Flat control surfaces without nanostructuration **ACP.H2O** and **Cit-ACP.H2O** were prepared as references.

Physico-chemical analysis were performed by FEG-SEM and Powder X-ray diffraction (PXRD) to assess ANS morphological features.

Tested bacterial strains were clinically-isolated carbapenem-resistant *P. aeruginosa* and methicillin-resistant *S. aureus*, according to EUCAST breakpoint table for bacteria v13.1 .VERO E6 fibroblast-like kidney cells (ATCC No. CRL-1586) were used for biocompatibility evaluation.

Antimicrobial and biocompatibility assessments encompassed LIVE/DEAD bacterial and mammalian fluorescent assays, SEM imaging, and intracellular ROS quantification (DCFDA / $H_2$DCFDA cellular ROS assay kit).

## Results: Physico-chemical characterization

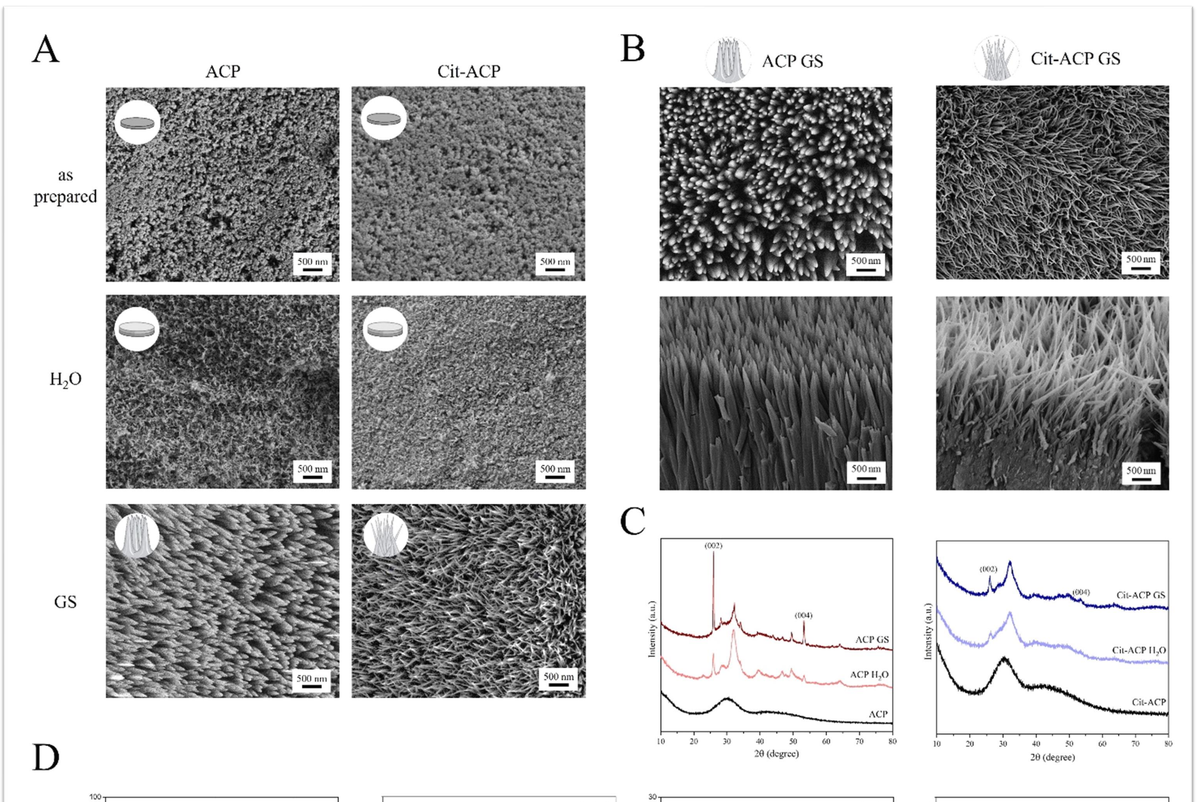


Figure 1 – (**A**) Top-view SEM micrographs of ACP and Cit-ACP substrates surface after molding at 196 MPa (as prepared), after 24 hours of incubation in water at 37 °C (**H2O**), or after 24 hours of incubation in GS at 37 °C (**GS**). (**B**) SEM micrographs of the ANS of ACP GS and Cit-ACP GS (top row) and the respective cross-sections (bottom row). (**C**) PXRD patterns of ACP (left) and Cit-ACP (right) before and after incubation in water or in GS for 24 hours at 37 °C. (**D**) Gaussian fitting of morphological features of ACP GS and Cit-ACP GS topographies measured by FEG-SEM. From left to right: pillar diameter, pillar tip diameter, pillar height, pillar angle with respect to the surface.

## Results: Antimicrobial and biocompatibility characterization

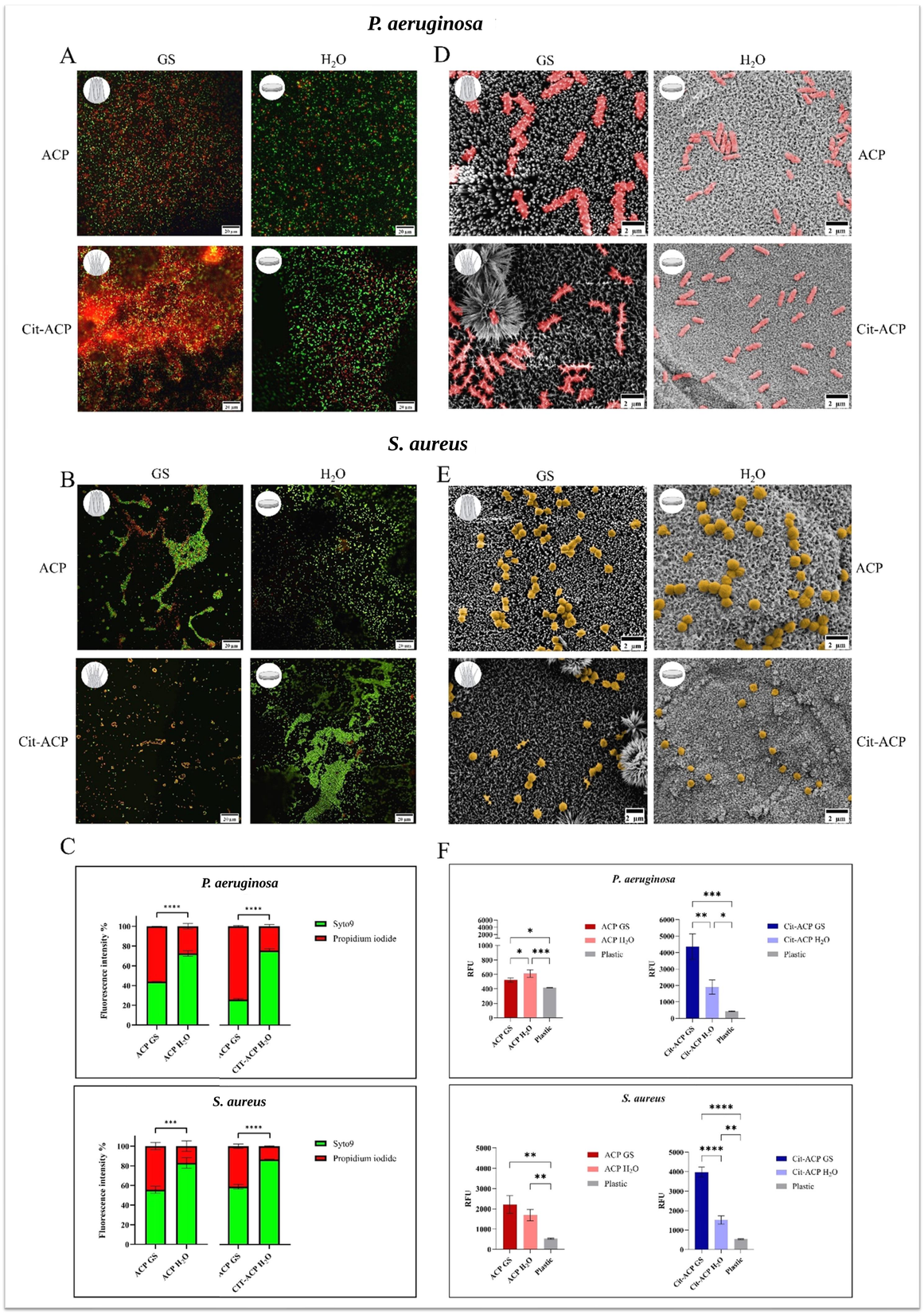


Figure 2 – (**A**,**B**) LIVE/DEAD representative images of *P. aeruginosa* (**A**) and *S. aureus* (**B**) after 4 hours on nanostructured ACP surfaces and relative controls. (**C**) Fluorescence emission quantification of LIVE/DEAD assay. (**D**,**E**) Scanning electron microscopy images of *P. aeruginosa* (**D**) and *S. aureus* (**E**) after 4 hours on tested surfaces. (**F**) Reactive Oxygen Species generated by *P. aeruginosa* and *S. aureus*, quantified after 4 hours of incubation with tested surfaces and plastic as secondary control.

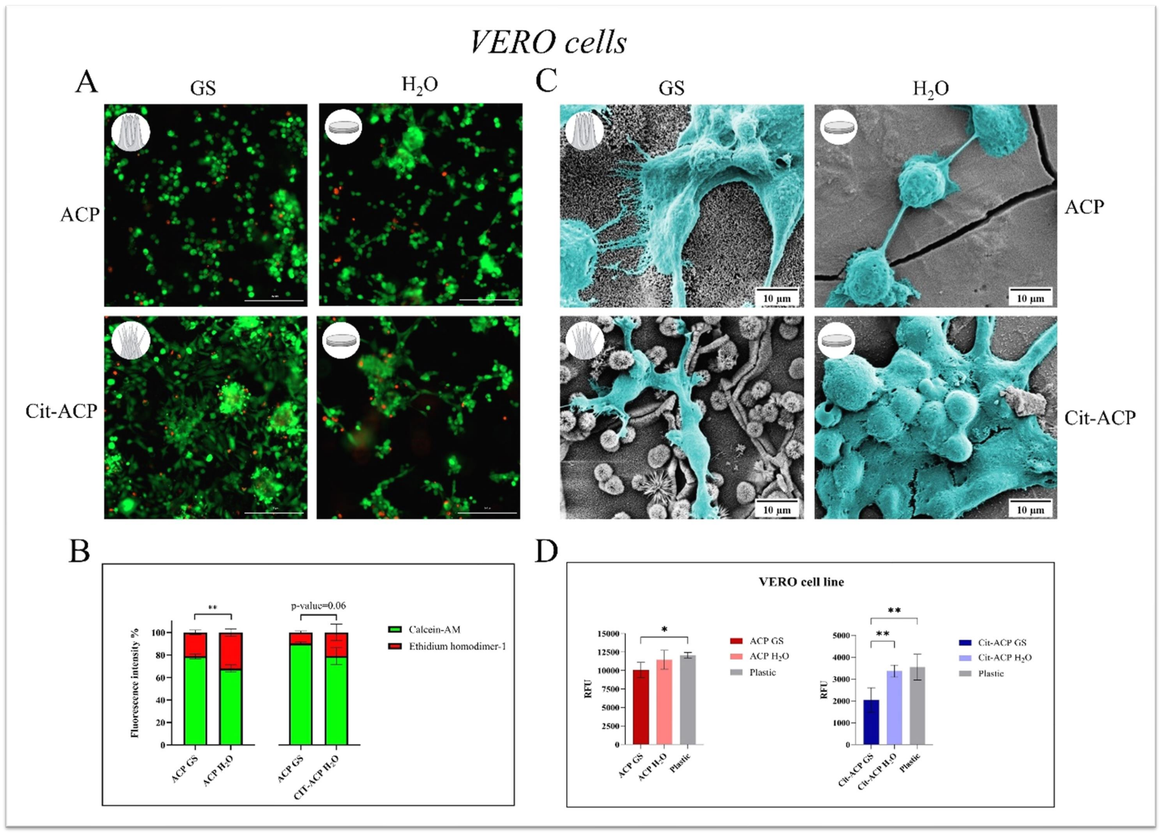


Figure 3 – (**A**) LIVE/DEAD representative images of *VERO* cells after 24 hours on nanostructured ACP surfaces and relative controls. (**B**) Fluorescence emission quantification of LIVE/DEAD assay. (**C**) Scanning electron microscopy images of *VERO* cells after 24 hours on tested surfaces. (**F**) Reactive Oxygen Species generated by *VERO* cells quantified after 24 hours of incubation with tested surfaces and plastic as secondary control.

## Conclusions

Biomimetic ANS based on oriented CaP nanocrystals arrays showed important in vitro potential against MDR pathogens. **Cit-ACP.GS** surface exhibit a major bactericidal tendency for both *P. aeruginosa* and *S. aureus* alongside with an increased generation of ROS, symptoms of induced oxidative stress. *VERO* cells seems to be positively affected by ANS surfaces, due to improved viability compared to flat surfaces, and reduced oxidative stress generated after 24 hours of contact. ANS' contact-based mechanism of action and biocompatibility spectrum need to be further investigated for their potential orthopedic devices manufacturing usage. Currently, our group is investigating transcriptomic differences and regenerative potential on BM-MSC cells.

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