Anti-biofilm surfaces based on the immobilization of a novel recombinant antimicrobial protein using SAMs

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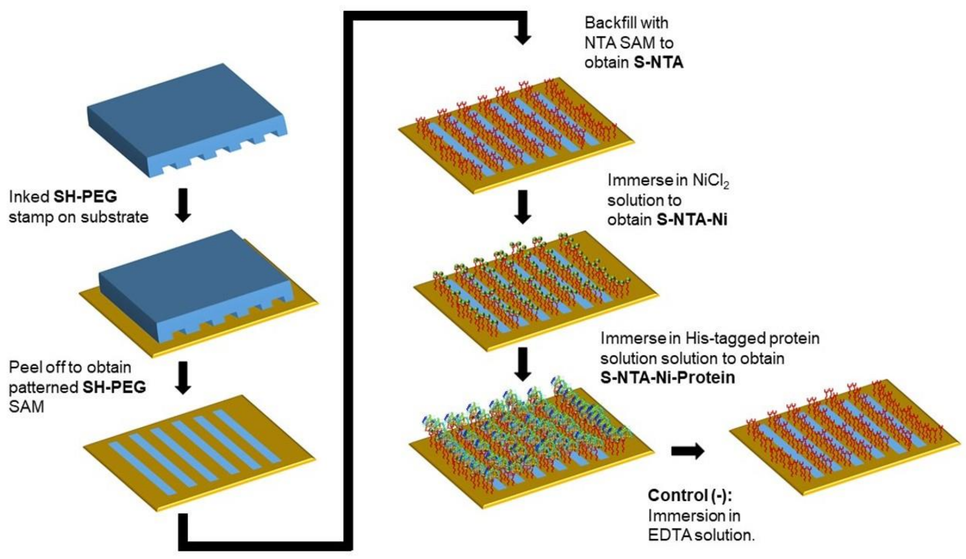
INTRODUCTION

The increasing appearance of resistant (and in many cases multiresistant) bacteria to antibiotics has become a global health emergency [1]. Far from being a phenomenon that will decrease in the coming years, it is estimated that the emergence of new resistances and the number of pan-resistant microorganisms will continue to grow reaching an increase of 67% in 2030 [2]. The antibiotic resistance is even more complicated when bacteria form biofilms. One of the strategies recently used to provide antimicrobial properties to medical devices is the immobilization of antimicrobial peptides (AMPs) on surfaces. The use of self-assembled monolayers (SAM) strategy to anchor AMPs on surfaces has been shown to be one of the best strategies for a controlled design of antibiofilm surfaces to coat medical devices [3]. SAMs are based on well-organized molecules on surfaces which are easy to be prepared and functionalized allowing a fine control at the molecular level [4].

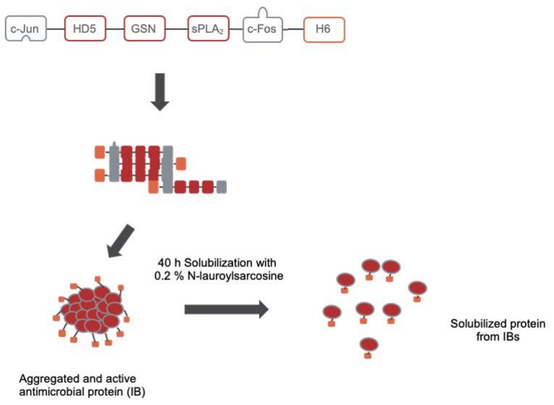
Here we report on the formation of an antimicrobial surface through the immobilization of a novel generation of antimicrobial proteins using a mixed SAM strategy, as a proof of concept for coating medical devices.



Schematic representation of the experimental procedure followed to prepare patterned SH-NTA and SH-PEG mixed SAMs, using the μCP technique. Subsequent protein immobilization via their His-tag termination led to the protein anchoring. Negative controls were prepared by immersing the substrates in an EDTA solution (10 or 100 mM).

ANTIMICROBIAL SURFACE DESIGN

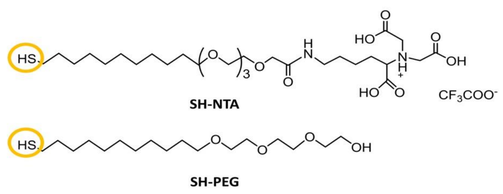
- Here, we report on the use of a new antimicrobial multidomain protein (JAMF1) formed by several AMPs by means of DNA recombinant technology in a soluble and nanoparticulated (inclusion bodies (IBs)) format.



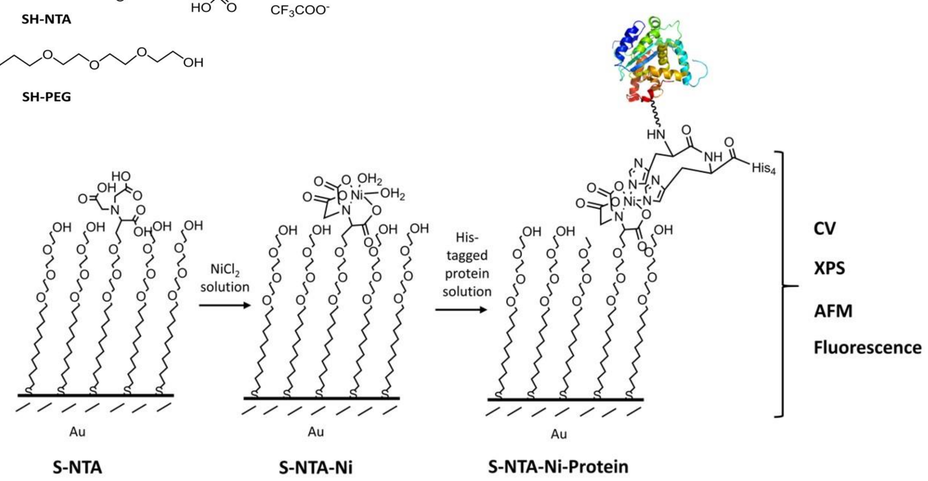
Representation of the antimicrobial protein JAMF1 construct forming IBs and in its soluble form after a solubilization process

- HD5 domain is attracted by electrostatic forces to the negatively-charged lipid bilayer forming the bacterial membrane and this induced a change on the membrane structure.
- $sPLA_2$ domain is an enzyme also from the innate immunity, which effectively hydrolyses the phospholipids components of the bacterial membrane.
- Gelsolin domain also included in JAMF1 is a bacterial binding domain which role is increase the efficiency of JAMF1 molecule binding the pathogen to be treated.

- JAMF1 multidomain polypeptide with recently proved effective antimicrobial activity, [5] will be anchored on a model gold surface using a mixed self-assembled monolayer (SAM) based on ((1-mercapto-11-undecyl)-(tetra(ethylene glycol)) terminated SAM (PEG-SH), and nitriloacetic acid (NTA) terminated EG4-SAM (NTA-PEG-SH).



Molecular structure of the molecules used for NTA SAM formation. The NTA terminated thiol (**SH-NTA**) and the commercial pegylated alkanethiol (**SH-PEG**).

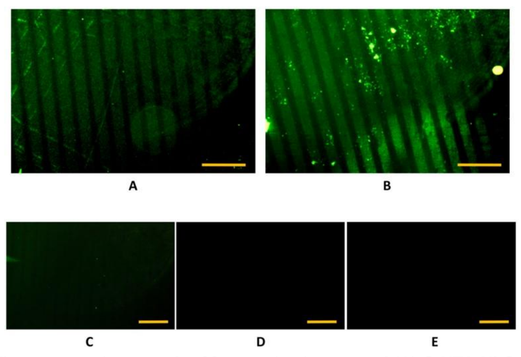


Strategy used for the immobilization of His-tagged JAMF1 protein using the Ni-NTA strategy

RESULTS AND DISCUSSION

Multitechnique surface characterization

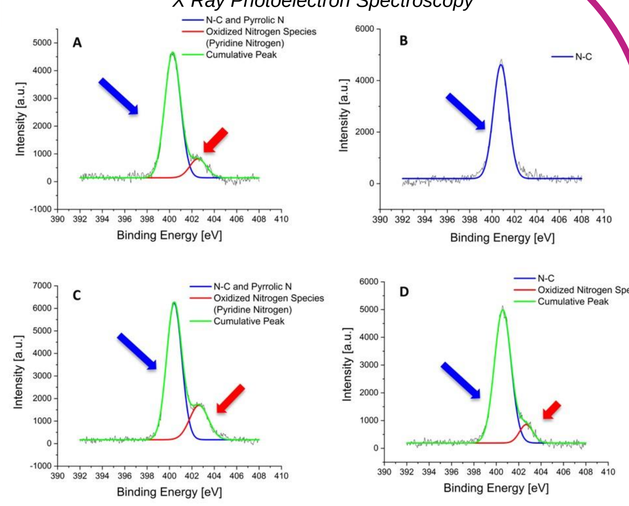
Fluorescence microscopy of Patterned JAMF1 AMP



Fluorescence images of a 20 *μ*m striped pattern of (A) **S-NTA-Ni-Sol** and (B) **S-NTA-Ni-IB**. Negative controls (by immersion in EDTA (10 mM)) of a 20 *μ*m striped pattern of (C) **S-NTA-Ni-Sol**-Ctrl and (D) **S-NTA-Ni-IB**-Ctrl. (E) A generic negative control of the immunostaining technique, which consisted in patterned **S-NTA**. The scale bars correspond to 100 μm.

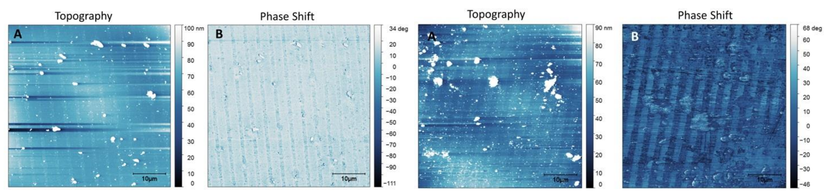
Proteins are depleted of fluorescence, thus, in order to visualize the pattern, an immnunostaining was performed. The fluorescent stripes appear well delimited, and a uniform coverage of the protein is found along the pattern. Moreover, absence of fluorescent pattern in the negative controls, prepared by immersing the substrates in EDTA (10 mM), indicate the reversibility of the union.

X Ray Photoelectron Spectroscopy



XPS deconvolutions of N 1s spectra for (A) S-NTA-Ni-Sol, (B) S-NTA-Ni-Sol- Ctrl (treatment with EDTA 10 mM), (C) S-NTA-Ni-IB and (D) S-NTA-Ni-IB-Ctrl (treatment with EDTA 100 mM). The red arrow indicates the contribution of 'pyridine-like' nitrogen and blue arrow of 'pyrrole-like' nitrogen.

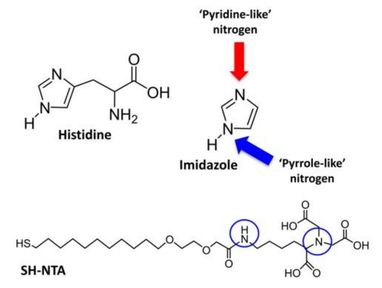
Atomic force microscopy



(A) Topographical and (B) phase shift AFM images of the 2 μm wide striped pattern of **S-NTA-Ni-Sol** (A)(B).

(A) Topographical and (B) phase shift AFM images of the 2 μm wide striped pattern of **S-NTA-Ni-IB** (A)(B).

Both topographical images confirmed the correct protein immobilization and phase shift images show a homogeneous protein coverage in both cases.

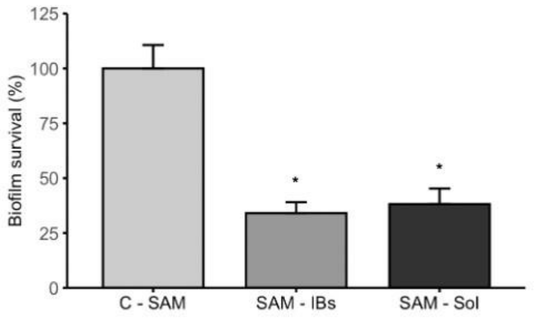


Representation of the different attributions to the nitrogen spectra: 'pyrrolelike' nitrogen, 'pyridine-like', N–C and secondary amides

XPS measurements of N 1s showed peaks, shifts and intensity relations confirming successful protein immobilization.

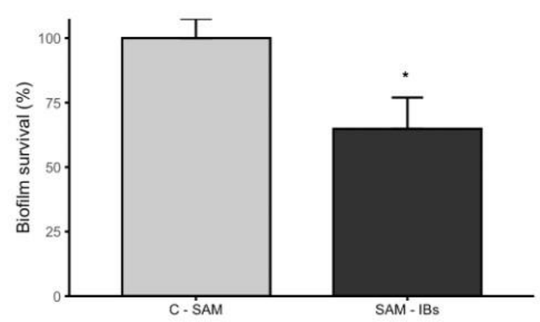
Biofilm Assay

Escherichia coli



Biofilm formation ability (%) of *E. coli* DH5α after treating substrates with niquel and soluble (SAM – Sol) or insoluble (IBs) JAMF1antimicrobial protein. * indicates significant differences ($p \leq 0.05$).

Klebsiella pneumonia carbapenemase



Biofilm formation ability (%) of KPC after treating substrates with nickel and insoluble JAMF1 antimicrobial protein or treating plastic well with JAMF1 IBs. * indicates significant differences ($p \leq 0.05$).

The graphs confirmed the effectiveness of preventing biofilm formation against *E.coli* and *K.pneumoniae* strains

CONCLUSIONS AND PERSPECTIVES

Micropatterns of AMP were successfully formed using the *μ*CP technique through the NTA functionalization of gold surfaces assisted by a thiol group, combined with the his-tagged antimicrobial protein JAMF1, both in soluble form and nanostructured as IB.

- The successful pattern formation not only was verified by fluorescence microscopy, but also by AFM and XPS measurements. This functionalization strategy was then applied to fully coated surfaces, which demonstrated their effectiveness in preventing biofilm formation against *E. coli*.
- The immobilized soluble and IB forms reduced bacteria survival up to a 38% and 34%, respectively
- The IB surfaces also inhibited biofilm formation in *Klebsiella pneumoniae* strains, which are unresponsive to standard antibiotic treatments.

Novel biofunctionalized surfaces with AMP were developed and characterized in response to the need of new antimicrobial agents to overcome the antibiotic crisis, which could be applied to coat medical devices (e.g. catheters) or be incorporated into food packaging materials, among others

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