Diana Filipa Barros Alves **Mussel adhesive-inspired surface modification to design bi-functional antibacterial coatings**

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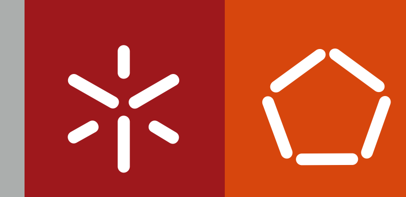

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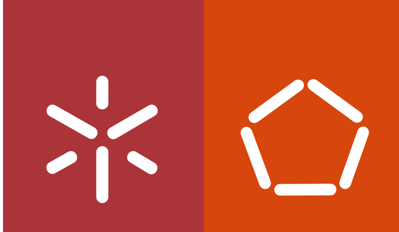

Universidade do Minho
Escola de Engenharia

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Mussel adhesive-inspired surface modification to design bi-functional antibacterial coatings

PhD in Chemical and Biological Engineering

Supervisor:
Professor Maria Olívia Pereira

March 2016

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, 30 de março de 2016

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Signature:

Diana Filipa Barros Alves

The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" but "That's funny..."

(Isaac Asimov)

Aos meus pais.

À minha irmã.

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"It is the long history of humankind (and animal kind, too) those who learned to collaborate and improvise most effectively have prevailed."

Charles Darwin

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Even though the introduction of biomaterials in modern medicine has been crucial in restoring body function and quality of life, all biomaterials are prone to be colonised by microorganisms, representing, therefore, niches for infection *in vivo*. These biomaterial-associated infections (BAI) are often associated to the biofilm mode of growth, in which bacteria encase themselves in a self-produced hydrated matrix of extracellular polymeric substances (EPS), conferring them protection against the host immune system and antibiotic treatment. Since bacterial adhesion to the surface of a biomaterial is a crucial step in BAI pathogenesis, surface modification of biomaterials to impart them with the ability to resist bacterial colonisation represents the most potential approach to fight these infections. Considerable advances in the field of antibacterial coatings have been occurred, but few biomaterials have been designed that effectively reduce the incidence of BAI. Therefore, the key goal of this thesis was to propose an effective coating strategy to impart biomaterials with the ability to prevent bacterial adhesion and simultaneously kill the adherent ones, with low propensity for developing bacterial resistance and with absence of adverse effects on the interaction with mammalian cells. Antimicrobial peptides (AMP) and enzymes targeting different EPS were the compounds chosen as antimicrobials alternatives to be immobilized onto biomaterial surfaces.

Compounds immobilization was performed using a facile mussel-inspired adhesive coating strategy in which materials were immersed in a solution containing dopamine and the compounds together (1-step approach immobilization), or materials were immersed in an alkaline solution of dopamine to form a thin layer of polydopamine (pDA) and then transferred into a solution containing the AMP and/or enzymes (2-step approach immobilization). Mono and bi-functional coatings were physically characterized in what concerns their morphology, wettability, surface composition and roughness. Scanning electron microscopy and atomic force microscopy showed that the presence of pDA increased the surface roughness of both polydimethylsiloxane (PDMS) and polycarbonate materials, while the measuring of water contact angles showed a decrease on the hydrophobicity characteristic of these materials. Further functionalization with AMP or enzymes yielded surfaces with similar morphology or a more homogeneous coating, when a 2-step or 1-step approach immobilization was performed, respectively. Their antimicrobial and anti-adhesive performance as well their cytotoxicity were also evaluated.

A screening with several AMP more traditional and natural such as polymyxins B and E, as well as analogues peptides more active and stable such as Palm and Camel was performed. AMP proved to be good alternatives to antibiotics as they were able to compromise biofilm formation at similar range concentrations to inhibit planktonic growth. Polymyxins B and E were more effective against *Pseudomonas aeruginosa* while Camel and Palm were more promising against *Staphylococcus aureus*. Polymyxin E potential was further demonstrated after its physical adsorption onto polystyrene surfaces as it proved to impair biofilm formation and increase *P. aeruginosa* biofilms susceptibility to antimicrobial treatment.

Peptides immobilization was afterwards optimized using the pDA-based approaches. Immobilization of polymyxins B and E onto PDMS rendered the surfaces with antimicrobial

activity towards the Gram-negative bacteria *P. aeruginosa* and showed great potential to overcome some concerns associated to bacterial resistance and toxicity reported in the past for these compounds when in solution. Palm was, however, the AMP chosen to design bi-functional coatings as its immobilization rendered PDMS with effective antimicrobial activity against both Gram-negative and Gram-positive bacteria, especially against the Gram-positive ones, the most commonly found associated to BAI.

The immobilization of different enzymes (alginate lyase, lysozyme, proteinase K and DNase I) was afterwards optimized and results showed that catechol chemistry allowed their grafting without compromising their catalytic activity. DNase I was the enzyme chosen for further investigations because exhibited the best anti-adhesive features against a wider spectrum of bacterial strains. Once established the AMP and enzyme with most promising features, their co-immobilization was optimized in order to impart PDMS surfaces with potent antimicrobial and anti-adhesive properties against the adhesion of several strains of *P. aeruginosa*, *S. aureus* and *Staphylococcus epidermidis* as single and dual-species, with excellent stability and no cytotoxicity. To better discriminate co-adhesion of both species on modified surfaces, PNA FISH (Fluorescence *in situ* hybridization using peptide nucleic acid probes) was also employed, and results showed that *P. aeruginosa* was the dominant organism, with *S. aureus* adhering afterwards on *P. aeruginosa* agglomerates. The fate of bacteria that managed to adhere to the proposed bi-functional coatings was also investigated and results showed that bacteria were more susceptible to antibiotic treatment and to macrophages phagocytosis, without developing bacterial resistance towards the immobilized AMP.

In conclusion, a facile and non-toxic mussel-inspired adhesive coating strategy was applied to co-immobilize Palm and DNase I onto biomaterial surfaces without compromise their activity and rendering the surfaces with good antimicrobial, anti-adhesive and anti-biofilm features together with no cytotoxicity and no propensity for developing bacterial resistance. This coating strategy holds, therefore, great potential to be further explored in the design of biomaterial implants and devices to combat BAI.

Ainda que a introdução de biomateriais na medicina atual tenha sido fundamental para recuperar funções do corpo humano comprometidas e melhorar a qualidade de vida em geral, todos eles são propensos a ser colonizados por microrganismos, constituindo, desta forma, nichos para infeção *in vivo*. As infeções associadas a biomateriais (BAI) estão frequentemente associadas a biofilmes, estruturas biológicas nas quais as bactérias se envolvem numa matriz hidratada de substâncias poliméricas extracelulares (EPS) por elas produzida, que lhes confere proteção contra o sistema imunitário do hospedeiro e tratamentos com antibióticos. Uma vez que a adesão bacteriana à superfície de um biomaterial desempenha um papel crucial na patogénese de BAI, a modificação de superfícies para as dotar de capacidade de resistir à colonização bacteriana representa a abordagem mais promissora para combater estas infeções. Nos últimos anos tem-se assistido a grandes avanços na área dos revestimentos antibacterianos, contudo, são ainda poucos os biomateriais concebidos que efetivamente reduzem a incidência de BAI. A presente tese teve como objetivo principal propor uma estratégia de revestimento capaz de eficazmente dotar os biomateriais com características de prevenção da adesão bacteriana e, simultaneamente, com capacidade para matar bactérias que eventualmente consigam aderir, e sem potencial para desenvolver resistência bacteriana ou citotoxicidade. Os compostos selecionados como alternativos aos antibióticos para serem imobilizados em biomateriais foram péptidos antimicrobianos (AMP) e enzimas que atuam em diferentes EPS.

A imobilização dos compostos foi efetuada recorrendo a uma estratégia de adesão inspirada em mexilhões, seguindo duas abordagens: numa, os materiais foram colocados numa solução contendo simultaneamente dopamina e os compostos a imobilizar (abordagem de imobilização num passo); noutra, os materiais foram incubados primeiramente numa solução alcalina de dopamina, para formar um filme fino de polidopamina (pDA), e depois transferidos para uma solução contendo AMP e/ou enzimas (abordagem de imobilização em 2 passos). Os revestimentos mono e bi-funcionais foram caracterizados fisicamente no que diz respeito à sua morfologia, molhabilidade, composição atómica da superfície e rugosidade. A microscopia eletrónica de varrimento e microscopia de força atómica demonstraram que a presença de pDA aumentou a rugosidade da superfície do polidimetilsiloxano (PDMS) e do policarbonato, enquanto a medição dos ângulos de contacto da água demonstrou uma diminuição da hidrofobicidade característica destes materiais. A funcionalização posterior com AMP ou enzimas gerou superfícies com morfologia semelhante ou com um revestimento mais homogéneo mediante a realização de uma abordagem de imobilização em 2 ou num passo, respetivamente. Os materiais funcionalizados foram também avaliados em termos do seu desempenho antibacteriano e citotoxicidade.

Um primeiro estudo para averiguar o potencial antimicrobiano de uma série de AMP mais tradicionais e naturais como as polimixinas B e E, bem como péptidos análogos mais estáveis e potentes, como o Palm e Camel, demonstrou que estes constituem uma alternativa aos antibióticos uma vez que foram capazes de comprometer a formação de biofilme quando utilizados em concentrações semelhantes às necessárias para inibir o crescimento planctónico. As polimixinas B e E foram mais eficazes contra *Pseudomonas aeruginosa* enquanto o Camel e o

Palm foram mais promissores contra *Staphylococcus aureus*. O potencial da polimixina E foi, ainda, demonstrada após a sua adsorção física em superfícies de polistireno, ao comprometer a formação de biofilme de *P. aeruginosa* e ainda promover a sua suscetibilidade a tratamentos antimicrobianos posteriores.

A imobilização dos AMP foi posteriormente otimizada usando estratégias baseadas na pDA. A imobilização de polimixinas B e E em PDMS conferiu a esta superfície atividade antimicrobiana contra a bactéria Gram-negativa *P. aeruginosa* e evidenciou o carácter promissor da sua utilização, uma vez que foram superados problemas relacionados com o desenvolvimento de resistência e toxicidade associados a estes AMP quando usados em solução. Palm foi, contudo, o AMP selecionada para manufaturar os revestimentos bi-funcionais dado que a sua imobilização conferiu ao PDMS atividade antimicrobiana contra bactérias Gram-positivas e Gram-negativas, sendo mais relevante contra as Gram-positivas, as mais frequentemente associadas a BAI.

A imobilização de várias enzimas (alginato liase, lisozima, proteinase K e Dnase I) foi também otimizada, tendo-se demonstrado que a imobilização baseada na pDA não comprometeu a sua atividade catalítica. A DNase I foi a enzima que exibiu melhores propriedades anti-adesivas contra um espectro mais alargado de estirpes bacterianas, tendo por isso sido selecionada para a investigação de revestimentos bi-funcionais. Uma vez estabelecido o AMP e a enzima com as caraterísticas mais promissoras, otimizou-se a sua co-imobilização de modo a conferir às superfícies de PDMS atividades antimicrobianas, anti-adesivas e anti-biofilme contra a adesão de várias estirpes de *P. aeruginosa*, *S. aureus* and *Staphylococcus epidermidis*, de forma isolada ou em consórcios de duas espécies, com excelente estabilidade e sem citotoxicidade. A hibridação fluorescente *in situ* combinada com moléculas de ácido péptido-nucléico (PNA FISH) foi ainda utilizada para a discriminação dos microorganismos nos consórcios polimicrobianos. Foi possível observar que a *P. aeruginosa* foi o organismo dominante no cosnsórcio, com *S. aureus* a aderir aos aglomerados de *P. aeruginosa.* A suscetibilidade das bactérias que eventualmente consigam aderir aos revestimentos propostos foi também investigada tendo-se mostrado que estas bactérias foram mais sensíveis ao tratamento com antibióticos e à fagocitose levada a cabo por macrófagos, sem desenvolverem resistência bacteriana em relação ao AMP imobilizado.

Em conclusão, a estratégia de adesão inspirada em mexilhões aplicada para, de forma simples e não-tóxica, co-imobilizar um AMP e uma enzima em biomateriais não comprometeu a sua atividade e dotou as superfícies de PDMS com propriedades antibacterianas relevantes e sem indícios de desenvolvimento de citotoxicidade e de resistência bacteriana. Estes revestimentos apresentam um grande potencial para o desenvolvimento de biomateriais capazes de resistir efetivamente a BAI.

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CONTEXT AND MOTIVATION

Modern medicine has achieved great advances with the introduction of biomaterials to support or restore human body function. It has been estimated that the number of total hip replacements in the world is approximately one million a year, while the number of knee replacements is more than 250 000. A major problem emerging from the increasing use of biomaterial implants and medical devices is BAI. Microorganisms are able to reach the surface of a biomaterial, adhere to it and form a so-called biofilm, a microconsortia of surface adhering cells enclosed in a self-produced matrix of EPS. BAI are extremely difficult to treat, as this biofilm mode of growth offers protection against the host immune system and antibiotic treatment. Surface modification of biomaterials to impart them with the ability to resist or prevent bacterial adhesion represents the most potential approach to fight BAI and several strategies have been proposed in the last few years. However, most of the current strategies presents some important limitations, including the emergence of multi-drug resistant bacteria and toxicity concerns. In addition, most of techniques reported for their formulation require complex, labor and time-consuming techniques as well as the usage of organic solvents which may affect the integrity of biomaterials. Another important aspect to be resolved is the accumulation of dead bacteria on the antimicrobial coatings. These bacteria may allow the adhesion of other bacteria which can promote more bacterial accumulation on the surface, reducing its antimicrobial activity over time. Anti-adhesive coatings, alone, are not able to completely prevent bacterial adhesion. Therefore, an ideal antibacterial coating should combine the strengths of both strategies: to kill bacteria and simultaneously prevent the fouling of proteins and bacteria in the surface. Furthermore, this ideal coating should be prepared using a facile and non-toxic approach. In the search for alternative compounds to be immobilized onto biomaterial surfaces and render them with both antimicrobial and anti-adhesive properties, protein-like antibacterial agents have been recently recognized as promising candidates. This class of compounds includes peptides whose mode of action involves electrostatic interaction with bacterial membranes with subsequent disruption of membrane's structural stability (AMP) and enzymes that target bacterial surface or biofilm matrix components.

RESEARCH AIMS

The ultimate goal of the present thesis was to optimize the co- immobilization of AMP and enzymes, using dopamine chemistry, in order to design a simple and effective coating strategy able to simultaneously prevent bacterial adhesion and kill the adherent ones, with low propensity for developing bacterial resistance and adverse effects towards mammalian cells (Figure 1). It was hypothesized that degradation of biofilm matrix components such as polysaccharides and proteins using matrix-disrupting enzymes, will affect bacterial adhesion to the surfaces, delaying biofilm establishment and enhancing bacterial susceptibility to other antimicrobials such as AMP. The aims of this thesis were:

1. Screening the antimicrobial activity of different AMP in order to establish which immobilization renders biomaterial surfaces with the effective ability to kill adherent bacteria.

2. Screening the anti-adhesive and/or antimicrobial activity of enzymes targeting different bacterial surface components or biofilm EPS in order to establish which immobilization is able to prevent bacterial adhesion.

3. Combine the most promising AMP and enzyme to create a bi-functional coating able to prevent bacterial adhesion and subsequent biofilm establishment, with low propensity for developing bacterial resistance and toxicity.

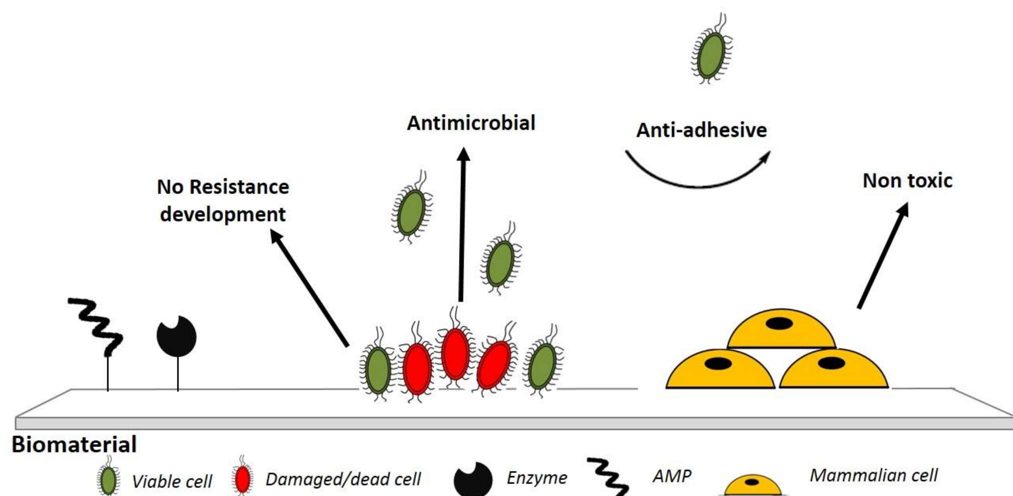

Figure 1. Schematic representation of the main goal of the present thesis: use dopamine chemistry to co-immobilize AMP and enzymes to confer both antimicrobial and anti-adhesive properties to biomaterial surfaces with low propensity for bacterial resistance and toxicity towards mammalian cells (not to scale).

OUTLINE OF THE THESIS

The present thesis reports the research work performed at Centre of Biological Engineering, University of Minho, Braga, Portugal and at Messersmith Research Group, Northwestern University, Evanston, USA, under the supervision of Professor Doctor Maria Olívia Pereira and Professor Doctor Phillip Messersmith, respectively.

This thesis is organized in six chapters that cover the research aims aforementioned. The first chapter summarizes the state-of-the-art on surface modification strategies aimed to control BAI, with a special focus on the potential of two bio-inspired compounds, AMP and enzymes. It is also reviewed the principles of catechol chemistry as a promising approach for materials modification. Chapter 2 describes the microorganisms, culture conditions, materials and techniques used throughout this PhD project as well as the rationale beyond it. Chapter 3 is dedicated to the screen and optimization of AMP immobilization. After evaluating their antimicrobial activity in solution (Chapter 3.1) and when physically adsorbed onto surfaces (Chapter 3.2) the most promising AMP were immobilized onto PDMS (Chapters 3.3 and 3.4). In Chapter 4, enzymes targeting different EPS were immobilized onto surfaces to determine the most efficient one to prevent bacterial adhesion. Chapter 5 describes the co-immobilization of the most promising AMP and enzyme as well as their physical and biological characterization. Finally, the main conclusions are presented in Chapter 6 and clues for future work are also suggested.

A_{450}	Absorbance at 450 nm
A_{490}	Absorbance at 490 nm
A_{570}	Absorbance at 570 nm
A_{640}	Absorbance at 640 nm
AL	Alginate lyase
AFM	Atomic Force Microscopy
AMP	Antimicrobial Peptide(s)
ATCC	American Type Culture Collection
BAI	Biomaterial-associated Infection(s)
BSA	Bovine serum albumin
CIP	Ciprofloxacin
CNS	Coagulase Negative Staphylococci
CFU	Colony Forming Unit
CV	Crystal violet
DAPI	4`, 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
eDNA	extracellular DNA
EPS	Extracellular polymeric substances
et al.	(*et ali*) and others
FBS	Foetal Bovine Serum
FISH	Fluorescence *in situ* hybridization
HAI	Healthcare associated infections
HCl	Hydrogen chloride
kHz	Kilohertz
LbL	Layer-by-layer
log	logarithm with base 10
LPS	Lipopolysaccharides
M	Molar
MBC	Minimum bactericidal concentration
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentration
MTS	(3-(4, 5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium) salt
NaCl	Sodium chloride
nm	Nanometre
nM	Nanomolar
PB	Polymyxin B
PBS	Phosphate buffered saline
PC	Polycarbonate
pDA	Polydopamine
PDMS	Polydimethylsiloxane
PE	Polymyxin E
PEG	poly (ethylene) glycol
pH	potential hydrogen

PMA	Phorbol 12-Myristate 13-Acetate
PMMA	poly (methyl methacrylate)
PMS	Phenazine methosulfate
PNA	Peptide nucleic acid
PNAG	poly-N-acetylglucosamine
QAC	Quaternary ammonium compound(s)
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium -1640
RT	Room Temperature
SD	Standard deviation
SEM	Scanning electron microscopy
TAE	Tris-acetate-EDTA buffer
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UP	Ultrapure
UV	Ultra violet
v	Volume
w	Weight
XPS	X-ray photoelectron spectroscopy
XTT	2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt

THESIS PREAMBLE

Figure 1. Schematic representation of the main goal of the present thesis: use dopamine chemistry to co-immobilize AMP and enzymes to confer both antimicrobial and anti-adhesive properties to biomaterial surfaces with low propensity for bacterial resistance and toxicity towards mammalian cells (not to scale). xx

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Chapter 1

INTRODUCTION

This chapter provides a general outline on surface modification methodologies aimed to control BAI, giving examples of desirable surface properties and the approaches that have been used to obtain these features. Two bio-inspired compounds, AMP and enzymes targeting EPS, as a promising and viable alternative to conventional antibiotics, are also presented. Finally, the relevance of catechol chemistry for the design of adhesive coatings that can serve as a platform for further functionalization of biomaterials with different antibacterial agents is mentioned.

BIOMATERIAL-ASSOCIATED INFECTIONS

Modern healthcare is strongly dependent on the use of biomaterial implants and medical devices, such as heart valves, catheters, stents, arthoprostheses and fracture fixation devices. Their introduction into medical practice was responsible not only for a better quality of life but also for patient survival [1]. It is estimated that every person will require some implant procedure during his/her lifespan [2]. There are, however, some drawbacks associated to their use as they constitute a main source of healthcare associated infections (HAI). Just in 2002, the number of nosocomial infections in USA hospitals were approximately 1.7 million, of which almost 100 000 cases resulted in death [3]. The costs associated with HAI were estimated to range from 28-45 billion dollars per year and more than half of these infections are associated with medical devices and implants [4,5].

Upon implantation, the fate of a biomaterial can be described as a race between its integration into the surrounding tissue and bacterial adhesion to its surface [2,6]. For a successful implantation, tissue integration must occur prior to bacterial adhesion, thereby preventing bacterial colonization at the implant. Conversely, if the race is won by bacteria, the implant surface will become rapidly covered by a biofilm [2,7], a microconsortia of surface adhering cells encased in a self-produced matrix of EPS [8]. This extracellular matrix, which is mainly comprised of water, polysaccharides, proteins and extracellular DNA (eDNA), makes biofilms the most successful forms of life on earth as it provides mechanical support, mediates cell-cell and cell-surface interactions and acts as a protective barrier [9].

There are different routes through which bacteria can reach the surface of a biomaterial and cause BAI [2,7]. The most common source of infection (perioperative contamination) is the direct contamination of the biomaterial implant during its insertion by bacteria present in the ambience of the operating room or by bacteria that normally populate the skin [10]. Contamination can also occur after implantation (postoperative contamination), during the period of hospitalization, caused by direct contamination of open wounds or by the use of invasive devices like catheters or drains. A third possible source of infection, but less likely to occur, is late hematogenous contamination that appears months or years after surgery, when bacteria from local infections elsewhere in the body are spread through the blood, reaching a biomaterial surface. Hematogeneous spreading of bacteria may result from skin infections, surgical or dental interventions, pneumonia, abscesses or bacteraemia [11]. Although the levels of bacteria found

in hospital settings have been reduced by the use of aseptic surgical techniques, microorganisms are still found at the site of approximately 90 % of all implants [12,13]. The most common pathogens implicated in BAI include yeasts (*Candida* species), Gram-positive (*S. aureus*, *S. epidermidis*, *Enterococcus faecalis, Streptococcus viridans)* and Gram-negative (*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *P. aeruginosa)* bacteria (Table 1). Many of these microorganisms can be found in the skin of patients and clinicians (*S. epidermidis* and *S. aureus*), in the water (*E. coli* and *P. aeruginosa*) or in equipment that was not properly cleaned and sterilized [14].

The occurrence of BAI is greatly affected by the location of a device in the body. Medical devices can be either totally external to the body and in contact with the surface of epidermis or mucosal membranes, percutaneous or permucosal and, thus, breaching epithelial or mucosal membranes barriers, or totally internal [15]. For instance, devices in contact with the outer part of the body such as urinary tract catheters or endotracheal tubes are readily reached by microorganisms and consequently have a higher incidence of BAI than totally internal implants (Table 1).

Biofilm formation on biomaterial surfaces (Figure 1) is a developmental process which includes the following main steps: i) transport of bacterial cells to the surface and their initial and reversible adhesion, ii) irreversible attachment, iii) microcolony formation, iv) biofilm maturation and differentiation and v) cell detachment with propagation of infection [16]. Once implanted, the biomaterial surface is first covered with a layer mostly composed of proteins (fibronectin, vitronectin, fibrinogen, albumin and immunoglobulins), a so called conditioning film, which play a role on bacteria-surface interactions [17]. In a first stage, bacteria and surface protein interactions are mediated by weak attraction forces, such as Van der Waals and electrostatic charges, being afterwards strengthened by specific interactions involving bacterial adhesion proteins [18,19] and EPS production. Adhering bacteria can grow and divide, forming microcolonies that are considered the basic organizational units of a biofilm. Entrapment of other planktonic bacteria in the extracellular matrix also occurs, resulting in a multi-layered and mature biofilm. Once established, biofilms are less susceptible to antimicrobial treatment and to the host immune system than their planktonic counterparts [20], making BAI extremely difficult to treat. As a consequence, the fate of an infected implant device is often its surgical removal, leading to considerable costs for healthcare system, patient suffering, prolonged hospitalization and even death [21].

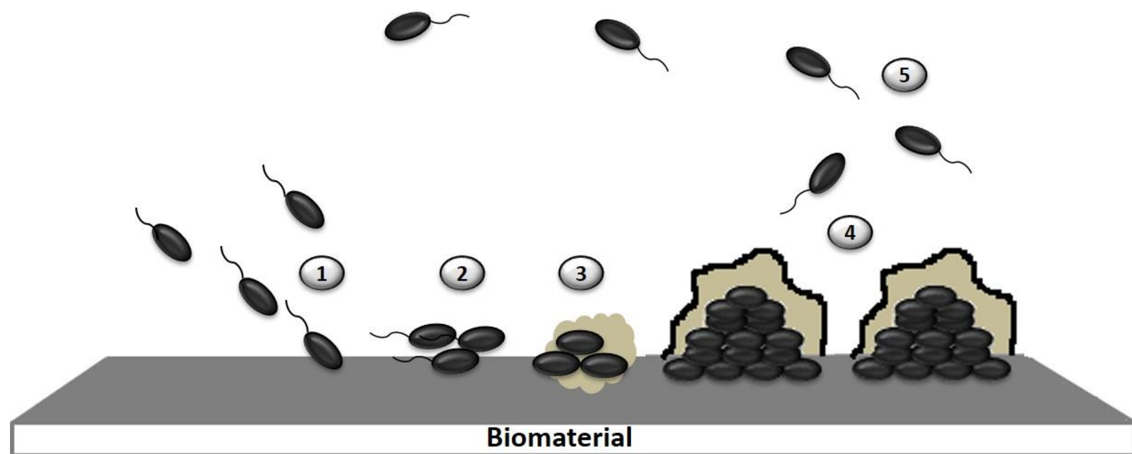

Figure 1. Schematic representation of the steps involved in biofilm formation on a biomaterial surface: transport to the surface and initial and reversible adhesion of cells (1), irreversible attachment (2), microcolony formation (3), maturation and differentiation of biofilm (4) and dispersal of single cells from the biofilm (5).

Table 1. Incidence and causative agents of infections associated with commonly used medical devices and implants.

Implant/device classification	Examples	Material	Commonly causative microorganism	Associated disease or outcomes	Infection incidence	Ref
Superficial	Wound dressings	Gauzes Hydrogels Alginate Collagen	*Staphylococcus aureus* *Pseudomonas aeruginosa*	Healing complications Chronic infection	1-5%	[6,22, 23]
Intracorporeal	Contact lenses	Silicone hydrogel	*Staphylococcus* spp *P. aeruginosa*	Microbial keratitis Peripheral ulcer	0.6%	[15,24]
	Urinary catheters	Silicone Latex	*Escherichia coli* *Klebsiella* spp *Serratia* spp *Citrobacter* spp *P. aeruginosa* Coagulase Negative Staphylococci (CNS) *Enterococcus* spp	Blockage from thick films Catheter-associated bacteriuria	10-20% 33%	[14,25]
	Dialysis equipment	Polycarbonate Silicone	*Staphylococcus* spp *Pseudomonas* spp	Vascular access-related infections peritonitis endotoxin exposure	24%	[15]
Permucosal	Dental implants	Titanium Zirconia	*Streptococcus* spp *Butyrivibrio* spp, *Campylobacter* spp *Peptococcus* spp, *Actinomyces spp*	Peri-implant mucositis and peri-implantitis	6.9%	[15,26]
Totally internal	Joint prosthesis	Titanium Stainless steel	CNS *S. aureus*	Infection	1% (hip prosthesis) 0.7% (knee replacement)	[27]
	Mammary prosthesis	Silicone	*S. aureus* *Streptococcu*s spp, CNS *Propionibacterium* spp	Infection	1-2.5%	[28]

TRENDS IN SURFACE MODIFICATION TO CONTROL BAI

In the last years, great efforts have been devoted to address the problem of BAI. When a BAI is established the main goals involve to cure the infection, prevent its recurrence, preserve body function and reduce the risk of death. In some cases, these goals can be achieved with prophylaxis in the form of systemic administered antibiotics, mainly vancomycin, often in combination with rifampicin [29]. However, because bacteria in biofilms are more resistant to antimicrobial agents, routine antibiotic treatments are often unsuccessful and may be followed by surgical removal of the primary and insertion of a secondary implant. For many implants, especially those in contact with the circulatory system, removal of the implant is dangerous and a high mortality is associated with these infections. [5,30]. A more desirable option to fight BAI relies, therefore, on the development of materials able to resist microorganisms' colonisation in first place. In the past, basic material parameters, such as material composition, were explored to control device infections which was performed by introducing an antimicrobial agent such as silver throughout the bulk of the material [31,32]. Although this approach ensures the long-term antimicrobial effect of materials, adding the antimicrobial agent may negatively interfere with the fundamental properties, stability and processability of the material. Alternatively, surface modification can be applied to existing biomaterials, with little impact on such bulk properties, often more cost and time effective and some of these strategies are summarized in Figure 2 [33].

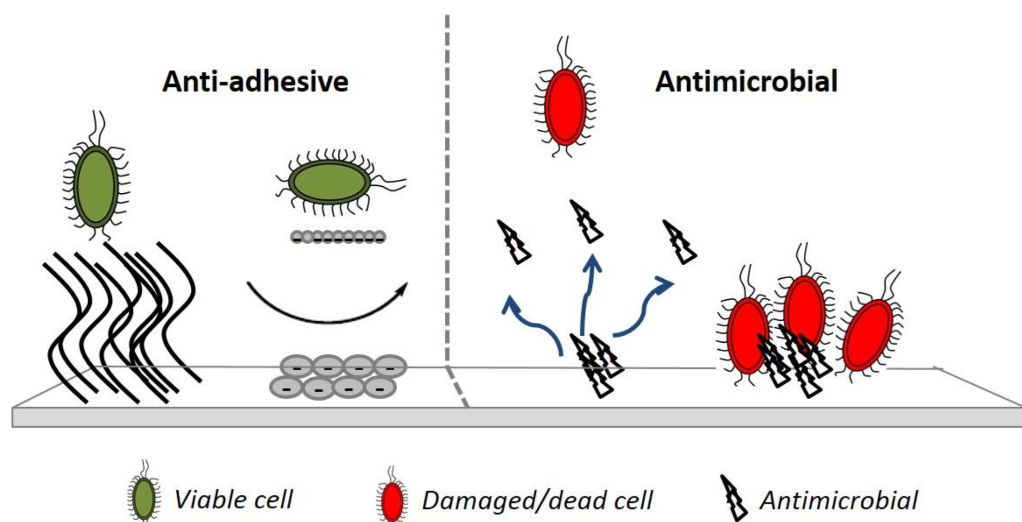

Figure 2. A schematic representation emphasizing different strategies designated to control biomaterial-associated infections (adapted from [6,34]) (not to scale).

ANTI-ADHESIVE COATINGS

As microbial adhesion to the surface of a biomaterial is the first step in biofilm formation and, therefore, a crucial step in BAI pathogenesis, several surface modifications have been developed to prevent bacterial adhesion at first place, the so-called anti-adhesive coatings. It has been found that controlling parameters such as hydrophobicity, surface roughness, electrostatic interactions and surface compliance can reduce bacterial adhesion to a surface [35]. For instance, it was reported that smooth surfaces attract fewer bacteria rather than rough or porous surfaces [36] and also that hydrophilic surfaces decreased bacterial adhesion in contrast to hydrophobic ones [37]. It is also well accepted that bacterial attachment on a biomaterial is facilitated by a layer of adsorbed protein, and thus surfaces that prevent non-specific interactions with the biological environment, namely the adsorption of proteins, also should resist the adhesion of bacteria [38]. Most of these surfaces are based on polymer brushes after their modification with hydrophilic polymers or oligomers [39]. A polymer brush is formed when hydrophilic polymer chains are end-grafted to a surface in a high packing density, forcing the polymer chains to stretch away from the surface. When in an aqueous medium, these hydrophilic polymer brush-coatings form a highly hydrated layer at the surface, which compression upon bacterial attachment results in an osmotic pressure and decreased mobility (conformational entropy) of the polymer chains in the brush. It performs, thus, as repulsive forces to prevent protein adsorption and bacterial adhesion on the surfaces. To prepare an efficient anti-adhesive brush-coating there are two critical parameters, the thickness and density of brush-coatings [40,41]. Polymers or oligomers based on the ethylene glycol repeat unit, such as poly (ethylene) glycol (PEG) are the most commonly used hydrophilic material to fabricate surfaces that resist bacterial adhesion [42]. Another strategy is based on biomimetic zwitterionic polymers which have an equimolar number of homogenously distributed anionic and cationic groups on their polymer chains [43]. For instance, Anagnostou *et al.* demonstrated that functionalized poly (methyl methacrylate) (PMMA) – based polymers inhibited more than 90 % of *S. aureus* adhesion compared to untreated surfaces [44]. Similarly, Cringus-Fundeanu *et al.* found a high reduction (70-92 %) in microbial adhesion to silicon wafers grafted with polyacrylamide brushes [45]. In addition, it was demonstrated that polymer brush-coatings may assist in preventing infection of implant surfaces after revision surgery, by reducing the number of bacteria adhering to a re-implanted biomaterial surface [46].

These anti-adhesive coatings, however, do not completely prevent microbial adhesion and even the few bacteria adhering to a polymer brush have been demonstrated to be able to form a

weakly adhering biofilm [47]. Furthermore, anti-adhesive properties may be compromised after polymer brush-coatings exposure to physiological fluids due to surface overwhelming by continuous protein assault and coating degradation [48]. Traditional approaches to prepare most of these anti-adhesive coatings involve multiple steps and usage of different organic solvents [40]. All together, these issues have limited the clinical application of these strategies.

ANTIMICROBIAL COATINGS

Another strategy to prevent bacterial adhesion is the incorporation of active biocides that promote bacterial killing (Figure 2). These antimicrobial coatings can be further classified as exerting off-surface effects (antimicrobial-releasing coatings), when antimicrobials are released from the surface to target planktonic cells, or on-surface effects (contact-killing surfaces), when the agents are directly immobilized on the surfaces to target attached cells [49].

ANTIMICROBIAL-RELEASING COATINGS

Antimicrobial-releasing coatings are designed to kill bacterial cells before they come into contact with the implant surface. The advantage of these coatings over systemic drug delivery is that a high local dose can be administered without exceeding the systemic toxicity level. Also, as the overall antimicrobial dose in the body can remain low, it is possible to reduce the effects on healthy tissues and beneficial flora which increases patient comfort and simultaneously reduces the costs associated to follow-up care [50]. An important factor to take into account in the design of releasing coatings is the kinetics of the antimicrobial compound. A fast release may provide relatively high doses but short-term action while a slow release may not achieve the required therapeutic level and also induce bacterial resistance. An ideal release coating should provide the release of effective doses of antimicrobial agents over longer periods of time (weeks) at concentrations above minimum inhibitory concentration (MIC) [51].

Among the antimicrobial agents commonly used to design these releasing-coatings, antibiotics and silver stand out for their excellent antibacterial activity. Several antibiotics including vancomycin, ciprofloxacin (CIP), clarithromycin, amoxicillin, tobramycin and gentamicin have been formulated as sustained-release delivery systems [52]. An important example of this design is antibiotic-releasing bone cements in which antibiotics including gentamicin, tobramycin and also vancomycin are loaded into bulk PMMA bone cement formulations intra-operatively, often

placed around total joint arthroplasties [53]. Although these coatings are efficient, there are some drawbacks associated to them. The overuse of antibiotics can induce multi-drug resistant bacteria [54] and if used to treat an infection, a second surgery may be required to remove the cement after complete antibiotic release.

Apart from antibiotics, silver is a potent heavy metal which has been widely incorporated in releasing-coatings formulations. It is considered that silver has active antimicrobial effect only in the ionic form Ag^+, instead of its metallic state, via different mechanisms: inhibiting membrane transport processes, blocking cell replication and disrupt cell metabolism. These effects result from silver ionic ability to bind to DNA and also to key thiol groups of metabolic enzymes of the bacterial electron transport chain, resulting in their inactivation [55]. Clinical development of antimicrobial resistance to silver to date is rare [56] which can be attributed to its multifactorial mode of action. Silver, in its ionic form, has demonstrated antimicrobial efficacy against a broad spectrum of microorganisms commonly found at implant sites such as *P. aeruginosa*, *E. coli*, *S. aureus* and S. *epidermidis* [57]. Although silver-based releasing coatings are effective in killing bacteria, its toxicity to the human body remains a concern. Some studies consider silver to be biocompatible [58,59] but it has also been shown that silver can damage eukaryotic cells and tissues and induce undesirable responses [60,61].

CONTACT-KILLING SURFACES

Given the drawbacks of antimicrobial-releasing coatings, covalent immobilization of antimicrobials offers an alternative approach that avoids exposure to leaching compounds and potentially increases the duration of antimicrobial efficacy [62]. Different compounds such as antibiotics, polymers, metallic and quaternary ammonium compounds (QAC) have been exploited to generate contact-killing antimicrobial coatings [63-66]. Although some of these strategies were appropriate for specific applications, there is a need for wide-spectrum antimicrobials able to prevent bacterial colonisation of biomaterials, with low cytotoxicity and propensity to develop bacterial resistance, and stable for long periods [67].

In the search for compounds meeting the aforementioned criteria, protein-like antibacterial agents have been recognized as promising candidates for the new generation of antibacterial surfaces [68]. This class of compounds include peptides which mode of action involves electrostatic interaction with bacterial membranes and disruption of membrane's structural

stability, called AMP [67,69], and proteins that degrade biofilm matrix components [70], called biofilm-dispersing enzymes or matrix-disruptive enzymes.

AMP AND ENZYMES: PROMISING CANDIDATES AS COATING AGENTS

ANTIMICROBIAL PEPTIDES: AN OVERVIEW

AMP are a key component of the innate immune systems of most living organisms to protect them against invading microorganisms. So far, more than 2680 AMP have been reported in antimicrobial peptide database (http://aps.unmc.edu/AP/main.php) and they have been isolated from a wide variety of sources, including animals (both vertebrates and invertebrates), plants, bacteria, fungi and viruses [71-73]. Despite their structural and functional diversity, these peptides have certain common properties such as containing highly cationic character, having the tendency to adopt amphipathic structures because of their substantial proportion of hydrophobic residues, and being directed to the cell membrane. AMP are classified based on their secondary structure as β-sheet peptides stabilized by two to four disulphide bridges (human α- and β-defensines, plectasin or protegrins), α-helical peptides (LL-37, cecropins or magainis), loop peptides formed from a single disulphide bridge (bactenecin) and extended structures rich in glycine, proline, tryptophan, arginine and/or histidine (indolicidin), with the first two classes being the most common in nature [74,75].

As the first line of defence of animals and plants against invading pathogens, AMP exhibit important features that make them promising candidates for clinical applications and potential alternatives to conventional antibiotics. These features include ability to discriminate between host and microbial cells (cell selectivity); rapid mechanisms of action; activity against a wide spectrum of microorganisms, including resistant and multidrug resistant strains; and low propensity for developing microbial resistance [76]. AMP cell selectivity can be explained by the different composition and topological arrangement of the lipids of cytoplasmic membranes in prokaryotic and eukaryotic cells [77]. The outer leaflet of the membranes of animals and plants is exclusively composed of electrically neutral, zwitterionic phospholipids; most of the lipids with negatively charged head groups are segregated into the inner leaflet, facing the cytoplasm. In contrast, bacterial membranes contain large amounts of negatively charged phospholipid head

groups. The low propensity to develop microbial resistance and the fast killing exhibited by AMP can both be explained by their site of action and the bacterial membrane. A microorganism would have to redesign its membrane, changing the composition and/or organization of its lipids, which probably constitutes a "costly" solution for most of microbial species [78].

The mechanisms of action of AMP have been widely studied and, in spite of being a topic subjected to some controversy [72,79], there is a structural model established by Shai-Matzusaki-Huang [77,78, 80-82] that is generally accepted to explain the activity of most AMP [78]. In this model, it is proposed that cationic AMP are first attracted to bacterial surfaces by electrostatic interactions, followed by displacement of lipids, alteration of membrane structure and, in certain cases, entry of the peptide into the target cell. Once peptides have reached the cytoplasmic membrane they can interact with lipid bilayers. At low peptide/lipids ratios, peptides are bound parallel to the lipid bilayer. After a certain peptide threshold concentration is achieved [83], peptide molecules are oriented perpendicularly to the membrane and inserted into the lipid bilayer, forming transmembrane pores. Several models have been proposed to explain peptide insertion and membrane permeability, namely the "barrel-staves model", the "carpet model" and the "toroidal-pore model". In the first, peptides reorient perpendicular to the membrane and align (like the staves in a barrel) in a manner in which the hydrophobic peptide regions align with the lipid core region of the bilayer and the hydrophilic peptide regions align inward to form transmembrane pores [80,84]. These pores are proposed to allow leakage of cytoplasmic components and also disrupt the membrane potential. In the "carpet model", peptides are electrostatically attracted to the anionic phospholipid head groups covering the surface of the membrane in a carpet-like manner. Once a saturation point is reached, peptides are thought to disrupt the bilayer in a detergent-like manner, eventually leading to the formation of micelles. This local disturbance in membrane stability will cause the formation of cracks, leakage of cytoplasmic components, disruption of the membrane potential and, ultimately, membrane disintegration. Finally, in the "toroidal-pore model", peptides insert into the membrane and then cluster into unstructured bundles that induce the lipid monolayers to bend continuously through the pore so that the water core is lined by the inserted peptides and the lipid head groups. The pores created will be responsible for leakage of ions and possibly larger molecules throughout the membrane. However, not all AMP seem to exert their action on membranes. Actually, an increasing number of peptides have been described as acting on intracellular targets in bacteria altering the

cytoplasm membrane septum formation, inhibiting protein, cell wall or nucleic acid synthesis [85].

As a consequence of host-pathogen interactions during evolution, bacteria have developed some mechanisms to resist peptides, such as efflux pumps, secreted proteases and alterations of the bacterial surface. Efflux pumps, such as QacA of *S. aureus* [86] and MtrCE system of *Neisseria gonorrhoeae* [87], are energy-driven systems that constantly export toxic substances out of the cell wall away from the membrane. Because of their low specificity, these efflux pumps are also able to accept cationic AMP as substrates [88]. Gram-negative bacteria reduce their susceptibility to AMP by the incorporation of positively charged aminoarabinose in lipid A, which reduces the anionic characters of the cell surface and thus the electrostatic interactions with cationic AMP [89]. Likewise, Gram-positive bacteria, which do not have lipid A, achieve the same purpose by transporting D-alanine from the cytoplasm to the surface techoic acids, reducing the net negative surface charges [90]. Since such mechanisms of resistance require considerable levels of energy from bacteria, most of these are subjected to gene regulation, ascertaining that they are only active when needed [91]. Nevertheless, hosts have also invented tricks to circumvent bacterial AMP resistance mechanisms such as the introduction of disulphide bonds or other posttranslational modifications aimed to stabilize AMP against proteolytic inactivation. Furthermore, it takes 30 passages for *P. aeruginosa* in sub-MIC peptide to increase its resistance by 2-to-4-fold [92], whereas under the same conditions, resistance to gentamicin can increase by 190-fold [93].

AMP also present some characteristics that have limited their widespread use in clinical applications. Their potential for toxicity, the cost and complexity of their synthesis constitute the main disadvantages. Furthermore, their susceptibility to be degraded by proteases in the serum, especially AMP that are cationic and show fast degradation due to their arginine and lysine content, limits substantially their use in applications *in vivo* [94]. In fact, to date, only few AMP have proceeded into clinical trials and none of the described peptides has obtained US Food and Drug Administration (FDA) approval for clinical applications. Several approaches have, however, been proposed to address these main disadvantages. For instance, in the last few years, several non-natural mimics of AMP have been developed. These so called *de novo* AMP are different from the natural ones, with simpler but rationally engineered composition, obtained by varying the amino acid content and sequence and overall peptide length to achieve significant resistance

to protease degradation, enhanced activity and very low cytotoxicity properties. The high costs associated to peptides manufacturing has limited both the testing and development of AMP in large quantities. Many attempts have been recently reported to produce them by using biological production systems, such as bacteria, yeast and insect cells [95], as well by random polymerisation of mixtures [96,97].

ANTIMICROBIAL COATINGS BASED ON AMP

Nature provides many examples of successful strategies used by organisms to prevent bacterial colonization on living tissues. For instance, amphibians and fishes have developed an active system of defence as they secrete a dermal chemical slime composed of several AMP and proteins to prevent colonization of their skin by microorganisms [98,99]. Taking this strategy as a source of inspiration, different methods based on physical or chemical immobilization of AMP have been explored to develop antibacterial coatings. Among the physical immobilization methods, layer-by-layer (LbL) has been the most explored technique to immobilize AMP on surfaces. In this approach, which is based on the alternate adsorption of polycations and polyanions on a solid substrate, AMP can be simply embedded in the multilayer architecture to prepare functional films [100]. The amount of AMP bound to the surface can be controlled by the thickness of the LbL coatings, determined by the number of deposited layers. For instance, Etienne *et al.* [101] have explored LbL technique to incorporate the peptide defensin from *Anopheles gambiae* mosquitoes into polyelectrolyte multilayer films. The inhibition of *E. coli* growth at the surface of films functionalized with defensin was found to be 98 % when 10 AMP layers were inserted in the film architecture. This approach is, however, restricted to the use of highly charged and water-soluble AMP, which are not so frequently encountered. Furthermore, the electrostatic interactions between the peptides and the polyelectrolyte matrix may denature the peptide or reduce its motility, which therefore compromises its antimicrobial activity. To overcome these limitations, Guyomard *et al.* [102] proposed an approach in which a non-water soluble AMP, gramicidin A, was conjugated with a non-denaturing amphiphilic polysaccharide (hydrophobically modified carboxymethylpullulan) to obtain a negatively charged complex that was LbL assembled with cationic poly(L-lysine) to form biofunctionalized films. These films exhibited a strong antibacterial activity against *E. feacalis*, resulting from a double mechanism: the slow release of the peptide into the solution surrounding the film and the direct contact

between bacteria and the film surface. The antibacterial activity could be controlled by the number of layers deposited. Another key drawback associated with this strategy is to achieve a sustained release of the AMP entrapped in polyelectrolyte multilayer films into the surrounding bulk. Shukla *et al.* [103] proposed to control AMP release by using hydrolytically degradable LbL assembled films. The AMP Ponericin G1 was successful incorporated into polyelectrolyte assemblies based on a hydrolytically degradable cationic poly (β-amino ester) and an anionic polyanion such as alginic acid. The obtained films were able to inhibit *S. aureus* attachment over 10 days due to the degradation of poly (β-amino ester). Moreover, release profiles could be controlled by changing the film microstructure.

Although the physical entrapment of AMP into polymer layers has been successfully explored to prepare antibacterial coatings, there are some drawbacks associated to these strategies that limit their application into biomaterial implants or medical devices. The gradually decreasing level of released peptide may lead to sub-inhibitory concentrations in the surrounding bulk, which may provide conditions for development of microbial resistance. Moreover, most AMP present local toxicity or haemolytic activity which has limited their applications that require systemic distribution. Examples of AMP with cytotoxic profiles include LL-37 [104], Citropin 1.1, Omiganan, Pexiganan, Protegrin 1 and Temporin A [105]. Another concern inherent to physical immobilization strategies is related to the long-term stability of these assemblies which are still largely not reported. Covalent immobilization of AMP offers an alternative approach that avoids patient exposure to leaching compounds and potentially increases the duration of antimicrobial efficacy and their long-term stability [106]. In covalent immobilization strategies, AMP chemically react with a given surface to form stable and non-leaching antimicrobial coatings [69]. Surfaces that are not reactive toward AMP can undergo some surface treatment to introduce the desired functional groups that will allow the grafting of AMP in a further step [35]. A common method to covalently immobilize AMP involves the use of functionalized resins such as PEG or other polymeric brushes that bear reactive groups suitable for peptide covalent immobilization. For instance, Haynie *et al.* [107] applied standard solid-phase peptide synthesis to immobilize the natural occurring AMP magainin 2 and several idealized synthetic amphipathic peptides onto ethylenediamine-modified polyamide resin (PepsinK). The immobilized peptides proved to retain their lethal activity against several Gram-positive and Gram-negative bacteria. They were also able to demonstrate that the interaction with the outer membrane of the bacteria is sufficient for their

lethal activity, as the immobilized AMP system described consisted of a short spacer (2 or 6 carbon chain linkers) linking the peptide to the support. Recently, Gao *et al.* [108] described the development of infection-resistant coatings based on covalently grafted hydrophilic polymer brushes conjugated with several AMP. The coatings proved to be non-toxic, antimicrobial and biofilm resistant.

Another commonly explored method to covalently immobilize AMP onto surfaces is through their grafting on self-assembly monolayer (SAM). The SAM layer can be functionalized with different reactive groups, enabling the coupling of AMP. Humblot *et al.* [109] used SAMs based on pure 11-mercaptoundecanoic acid (MUA) or a mixture of 11-MUA and 6-mercaptohexanol ($HS(CH_2)6OH$) to immobilize the peptide magainin 1 on gold surfaces. The antibacterial activity of the modified SAMs with maganin 1 was tested against three Gram-positive bacteria and the results revealed that the grafted magainin 1 reduced by more than 50 % the adhesion of bacteria on the surface and killed the adhered ones. No release of the peptide was observed and the activity persisted overtime up to 6 months. The same authors, using a similar approach, were able to immobilize the peptide gramicidin A onto cystamine monolyaer deposited on gold surfaces [110]. Surfaces grafted with this AMP inhibited the adhesion of Gram-positive and Gram-negative bacteria as well as yeast. The formation of a biofilm on these surfaces was delayed for at least 24 h.

As previously mentioned, several non-natural mimics of AMP have been developed in the last years, providing advantages in terms of chemical diversity and significant resistance to protease degradation. For instance, several peptoids (non-natural mimics of polypeptides with the side chains appended to the amide nitrogen instead of the α-carbon) that were designed to mimic helical antimicrobial peptoids, were synthesized by Statz *et al.* [111] with a peptoid spacer chain to allow mobility and an adhesive peptide moiety for easy immobilization onto TiO_2 substrates. Substrates functionalized with these ampetoids and the antifouling polypeptide polymer led to surface coatings composed of both active and passive functionalities. The results showed that the ampetoid retained their antimicrobial activity as the membranes of adhered *E. coli* cells were damaged after 2 h exposure to the modified surfaces.

INFLUENCE OF IMMOBILIZATION PARAMETERS UPON AMP'S ACTIVITY

The retention of antimicrobial activity after AMP immobilization into surfaces is a crucial factor for their potential as antibacterial coatings agents in biomedical applications. In fact, most of the

reported studies about AMP immobilization onto surfaces has found that the activity of bound peptides is lower when compared to that of their soluble counterparts [107,112-114]. Understanding and optimization of immobilization parameters, such as peptide surface concentration, influence of the spacer (length and flexibility) or peptide orientation after immobilization, are essential for developing efficient, safe and long-lasting antibacterial coatings [69]. The amount of peptide bound to the surfaces depends on the immobilization strategy used, as limited accessibility of the peptide reactive groups and different coupling strategies can affect the efficiency of peptide immobilization [67]. For instance, Chen *et al.* [115] observed that the efficacy of antimicrobial activity is related to the attachment method. In their work, the AMP melimine was immobilized on glass substrates by two different bifunctional azides (4-fluoro-3-nitrophenyl azide (FNA) and 4-azidobenzoic acid (ABA)) as cross-linking agents and they found that the 4-fold higher concentration of peptide obtained via ABA immobilization correlated with a higher antimicrobial activity. Hilpert *et al.* [114] have also reported a positive relationship between activity and surface concentration and have shown that increasing the amount of AMP loaded enhanced the antimicrobial activity. Although important, peptide surface concentration does not appear to be the most critical immobilization parameter influencing the antimicrobial activity of tethered AMP. Bagheri *et al.* [112] studied the influence of surface peptide density and spacer lengths on the antibacterial activity and found that an increase in the loading capacity of the resin where the peptides were immobilized was not enough to compensate the decrease in activity due to reduction of the spacer length. They concluded that this last parameter, the spacer length, was the most determinant. Most of the studies reporting AMP immobilization present a spacer attachment step and the antimicrobial activity of some AMP such as LL-37 was completely lost when immobilized on solid supports in the absence of spacers [116]. The presence of a spacer may be important for enabling peptide insertion into the cell and thus membrane permeabilisation, leading to cell death. This working mechanism, known as polymeric spacer effect [117] was first described to explain how surface attached antimicrobial polymers might act as a contact-active surface [118]. The polymeric spacer effect has been the subject of some controversy. In fact, given the usually found active lengths of the grafted polymers, their possibility to reach the inner cell membrane of the attached bacteria would require high stretching of them [119]. Kugler *et al.* [120] have grafted quaternized poly (vinylpyridine) chains on glass surface by two different methods and varied the charge density within the organic layer. They found a sharp transition between biological activity and inertness at a certain partial

alkylation level. The authors proposed a new mechanism based on ion exchange between the bacterial membrane and the functionalized surface: the removal of divalent counterions from the bacteria during adsorption on charged surfaces induces disruption of the bacterial envelope and non-viability. This effect occurs for Gram-positive and Gram-negative bacteria after their adhesion on the functionalized surface.

Both aforementioned mechanisms have been found during AMP immobilization. In fact, although some reports [107,113,114,121] suggested that immobilized peptides demonstrate a similar membrane permeabilisation mode as their soluble counterparts, there are, however, other studies [107,114] where it was found that membrane permeabilisation was not a prerequisite for immobilized AMP induce their lethal activity. These researchers used short spacers of 2 or 6 carbons long to bind the peptides onto polyamide resins and showed that they retained their antibacterial activity. They suggested an electrostatic interference and destabilization rather than a penetrating mechanism.

Another possible mechanism for contact-active antimicrobial surfaces that do not contain a polymeric spacer was proposed by Bieser *et al.* [119]. They investigated a series of cellulose coatings with different quaternary ammonium groups and additional hydrophobic groups and found that the antimicrobial activity of such surfaces against *S. aureus* was mainly controlled by the cationic/hydrophobic balance and not so much by the charge density. These authors proposed the so called phospholipid sponge effect where the antimicrobial action is driven by the attraction between the negatively charged phospholipids in the microbial cell membrane and the surface. This hypothesis was further supported by the fact that all the coatings could be deactivated by treating them with SDS and a negatively charged phospholipid. A recent study conducted by Asri *et al.* [122] also supports this bacterial-killing mechanism. They concluded that whereas the mechanism of QAC in solution is based on adsorption, ion-exchange and membrane damage, immobilized QAC molecules enhance the adhesion forces between a bacterium and a substratum surface to a lethally strong attraction, causing reduced growth, stress de-activation and removal of membrane lipids, leading eventually to cell death.

The flexibility of the spacer is another parameter to be considered as it is correlated with lateral motility of the peptide immobilized. For instance, Gao *et al.* [123] investigated the influence of polymer brushes properties on the immobilization of the AMP Tet213 and on its antimicrobial activity. They found that the peptide density and graft density (related to conformational flexibility)

of the chains on surface are two important parameters with great influence on the antimicrobial activity. Peptide orientation and flexibility, obtained as a result of peptides binding on different chain positions may also significantly impact the antimicrobial activity of immobilized AMP. Strauss *et al.* [124] have reported differences in the activity of the AMP cecropin P1 when immobilized by different immobilization methods which was attributed to changes in the orientation of the immobilized peptide. In another study, it has been reported that random orientation of immobilized peptide LL-37 led to the loss of its antimicrobial activity even when the peptide was linked to a long and flexible PEG spacer [116]. The antimicrobial activity could be restored through oriented binding of LL-37 through its N-terminus. However, it should be mentioned that proper peptide orientation alone in the absence of a long and flexible spacer was not enough to achieve antimicrobial activity, which highlights the complex interdependence of the different immobilization parameters.

ENZYMES AS ANTIMICROBIAL AND ANTI-BIOFILM AGENTS

Biofilm establishment involves two important stages: bacterial initial adhesion to a surface and the production of EPS such as polysaccharides, proteins and eDNA [70]. EPS are responsible for providing mechanical stability of biofilms, mediating microorganisms' adhesion to surfaces and forming a cohesive, three-dimensional polymer network that interconnects and transiently immobilizes biofilm cells [9]. Thus, another promising anti-biofilm strategy may rely on the use of enzymes that can prevent biofilm infections in different ways. They can directly attack the microorganism causing cell lysis; interfere with biofilm formation and/or destroy the biofilm by degrading the compounds involved in microbial initial adhesion or the polymers of biofilm matrix (proteins, polysaccharides, eDNA) or by impairment of intercellular communication when quorum sensing molecules are the target; and/or catalyse reactions which result in the production of antimicrobial compounds [125-127].

PROTEOLYTIC ENZYMES

Proteins and glycoproteins are the dominant molecules mediating adhesion of many fouling organisms, thus proteases (protein hydrolysing enzymes) are the most tested and most successful enzymes used for the control of marine biofouling [126,128]. For example, the commercial proteases Savinase and Esperase proved to effectively inhibit and disperse

Pseudoalteromonas sp. D41 and multiple biofilms, respectively [129,130]. In medical applications, lysostaphin has been the subject of great interest to fight methicillin-resistant *Staphylococcus aureus* (MRSA) because of its ability to cleave the cross-linking pentaglycine bridges of the cell walls of staphylococci [131]. Other proteolytic enzymes investigated to fight biofilm infections include proteinase K, trypsin and serratiopeptidase [132,133]. For example, proteinase K was able to effectively remove the biofilm formed by a clinical isolate of *S. aureus* [134]. The proteolytic enzyme serratiopeptidase was evaluated for the treatment of BAI, revealing a promising effect by inhibiting biofilm formation and enhancing antibiotic action [135].

POLYSACCHARIDE-DEGRADING ENZYMES

The most commonly exploited polysaccharide-degrading enzymes include lysozyme, alginate lyase and dispersin B. Lysozyme is characterized by an enzymatic and non-enzymatic antibacterial mode of action, especially against Gram-positive bacteria. Lysozyme can damage bacterial cell wall by catalysing the hydrolysis of 1,4-β-linkage between N-acetyl-muramic acid and N-acetyl-D-glucosamine residues in peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins. The non-enzymatic mode of action of lysozyme is based on the cationic and amphiphilic properties of the enzyme which leads to perturbations in the cell membrane and activate the autolytic system of bacteria [127,136]. Alginate is another matrix polysaccharide that contributes to mucoid biofilm structure and plays a role in bacterial virulence and persistent nature of lung infections, which makes this polymer an important target in medical research. The potential of alginate lyase, an enzyme able to degrade this polysaccharide, has been investigated in the last years [137]. Alginate lyase treatment has been shown to detach biofilms from abiotic surfaces [138] and to increase gentamicin and CIP killing of mucoid *P. aeruginosa* biofilm [139]. A recent study [140] showed, however, that alginate lyase dispersion of *P. aeruginosa* biofilms and enzyme synergy with tobramycin is completely decoupled from its catalytic activity as equivalent anti-biofilm effects could be achieved with bovine serum albumin (BSA) or simple amino acids. These conclusions highlight the need for a careful re-examination of the fundamental assumptions underlying the interest in this biofilm-dispersing enzyme.

Another well-studied matrix-disruptive enzyme is dispersin B, a glycoside hydrolase produced by a human periodontal pathogen *A. actinomycetemcomitans* [141]. This enzyme is able to degrade poly-N-acetylglucosamine (PNAG), a biofilm matrix polysaccharide that has been shown to play a role in surface attachment and intercellular adhesion of staphylococcal species and *E. coli*, and it

is believed to mediate several important processes such as biofilm formation and pathogenesis [142]. The presence of this enzyme as a complement of growth medium has been shown to cause almost complete inhibition of biofilm formation of several Gram-negative and Gram-positive PNAG-producing bacteria. Furthermore, some studies showed that dispersin B can efficiently detach biofilms and increase their susceptibility to antimicrobial action [143-146].

EXTRACELLULAR DNA-DEGRADING ENZYME

A promising development in biofilm research has been the finding that eDNA plays an essential role as a component of the biofilm matrix in most bacterial species [147]. Whitchurch *et al.* [148] were the first to show that the presence of DNase I in growth medium could prevent biofilm formation by *P. aeruginosa* and also showed that DNase I could dissolve established biofilms. Extracellular DNA is responsible to bind biopolymers in EPS through attractive, short-range acid-base interactions [149] which makes DNase I a promising alternative to inhibit, disperse or even increase biofilms susceptibility to antimicrobials [147]. In fact, this enzyme has been used in the therapeutics of patients suffering from cystic fibrosis [150].

OXIDATIVE ENZYMES

An indirect enzymatic strategy to control biofilms relies on the use of enzymes to produce biocides that actively interfere with bacterial attachment. Enzymes commonly used in this strategy include glucose oxidase [151], hexose oxidase [151] and haloperoxidases [152]. Oxidases are used because they produce hydrogen peroxide while haloperoxidase catalyses the formation of hypohalogenic acid, which have potential cytotoxic effects [153].

ANTI-QUORUM SENSING ENZYMES

Another enzyme-based strategy that has been explored to control biofilms includes the use of enzymes that degrade quorum sensing signal molecules. Quorum sensing is a bacterial cell-cell communication process based on the production, detection and response to extracellular signalling molecules called autoinducers [154]. Two well-investigated quorum sensing systems are the acyl-homoserine lactone (AHL) signalling system of many Gram-negative species and the peptide-based signalling systems of many Gram-positive species [155]. AHL-acylases (cleaving the amide bond between the acyl chain and the homoserine lactone ring) and AHL-lactonases

and paraoxonases (both cleaving the ester bond of the AHL ring) have been identified as promising quorum sensing enzymes [156, 157].

ANTIBACTERIAL COATINGS BASED ON ENZYMES

The general use of therapeutic enzymes in clinical field has been restricted by the issue of enzymatic instability combined with the high cost associated with their isolation and purification. With the advances achieved in recombinant DNA technology, several enzymes are now extensively characterized and available in industrial quantities at affordable prices [127]. Enzymes immobilization had also contributed for the success of enzyme therapy approaches. In general, immobilized enzymes are more active over a broader range of environmental conditions (pH, temperature) than free enzymes and display higher stability when stored. Furthermore, enzyme immobilization improves their efficacy as it allows enzymes localization at where they are needed, at the coating-target interface [126]. A general method that can be applied to immobilize any enzyme does not exist and, usually, satisfactory methods have been developed based on trial and error. The most frequently used immobilization techniques include non-covalent adsorption, immobilization via ionic interactions, covalent attachment, cross-linking and entrapment in a polymeric gel or capsule [158].

Although immobilization of enzymes has been widely applied in marine antifouling technologies [35, 126], the number of studies reporting the immobilization of enzymes onto surfaces with the purpose of preventing BAI has increased in the last few years. Yuan *et al.* [159], have recently described an environmentally friendly approach to impart stainless steel (SS) surfaces with antifouling and antibacterial functionalities by functionalization of lysozyme into antifouling P(PEGMA) brushes immobilized by a biomimetic inspired by the mussel adhesive protein, dopamine. The so obtained hybrid exhibited antifouling properties and the ability to prevent BSA adsorption as compared to the SS surface unmodified, exhibiting also a high antimicrobial efficiency against both Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*). Muszanska *et al.* [160] have described a strategy for developing coatings with both antifouling and antimicrobial properties by conjugation of lysozyme into brushes of Pluronic. The conjugated lysozyme exhibited antibacterial activity against *Bacillus subtilis* and the coatings with a lower degree of lysozyme coverage proved to be more bactericidal. In another strategy reported by Caro *et al.* [161], two hydrolytic enzymes (lysozyme and trypsin) were covalently immobilized onto SS

surfaces through different strategies: directly by adsorption onto the metal surface, onto amino groups of the polymer poly (ethylene imine) (PEI) layer previously adsorbed on the surface and covalently grafted via the glutaraldehyde cross-linker. The antimicrobial tests performed against *Micrococcus lysodeikticus* bacteria showed that samples prepared with enzymes covalently grafted onto the PEI layer via a glutaraldehyde cross-linker exhibited a higher enzymatic activity compared to the samples where enzymes were directly adsorbed. The activity was also enhanced when enzymes were grafted onto a double PEI-glutaraldehyde-PEI layer, which highlights the importance of a distance between enzymes and the surface to improve the accessibility of the active site.

The bacteriolytic enzyme lyostaphin was adsorbed onto polystyrene and fluorinated ethylene propylene catheters to prevent their colonisation by several *S. aureus* strains. Lysostaphin is an endopeptidase that cleaves the crosslinking pentaglycine bridges of the cell wall of staphylococci. The antibacterial activity of the coating was maintained for at least 4 days and it was not compromised by the presence of serum proteins [162]. More recently, Yeroslavsky *et al.* [163] have demonstrated a simple method that utilizes the adhesive property of polydopamine (pDA) to covalently immobilize lysostaphin in order to generate antibacterial and anti-biofilm surfaces.

Pavlukhina *et al.* [164] have reported the development of a biocompatible surface coating in which the enzyme dispersin B was incorporated through a LbL technique. This enzyme was chosen because of its ability to cleave the polysaccharide PNAG, a component of the biofilm matrix produced by several Gram-positive bacteria such as *S. epidermidis* and *S. aureus*. Dispersin B was loaded into a poly (allylamine hydrochloride) (PAH) hydrogel matrix prepared by electrostatic interactions of PAH and poly(methacrylic acid) (PMAA), followed by chemical crosslinking with gluraraldehyde and pH-triggered removal of PMAA. To assess whether dispersin B retained their activity after being immobilized into the surfaces, dispersin B-loaded coatings were challenged with two bacterial suspensions of the strain *S. epidermidis* for different period times: 6 h and 12 h. After this time points, biofilm formation on the surfaces were quantified by counting the number of viable cells and a significant inhibition of biofilm development on dispersin B-loaded coatings was observed (reduction of at least 98 %) for both time points.

Swartjes *et al.* [165] have also reported a new method to prevent biofilm formation on surfaces exploring the potential of enzymes immobilization. In this work, a DNase I enzyme coating was applied to PMMA, using dopamine as an intermediate. The enzymatic coating strongly reduced

the attachment of S*. aureus* (95 %) and *P. aeruginosa* (99 %) and also prevented biofilm formation up to 14 h, without affecting mammalian cell adhesion and proliferation.

METHODS FOR SURFACE MODIFICATION

The most commonly used materials in the design of biomaterial implants and medical devices, such as silicone rubber, polyurethane and polycarbonate (PC), are very hydrophobic and relatively inert. Their surfaces must undergo some treatment to introduce the desired functional groups that will allow the grafting of the bioactive compound in a further step. Sometimes, an intermediate step is used to create a "spacer" or "linker" between the compound and the surface which can improve its bioactivity by reducing the steric constraints and shielding the compound from hydrophobic surface induced denaturation [106]. Several surface modification methods such as wet chemistry, self-assembled monolayers, ionized gas treatment and ultra violet (UV) radiation have been developed to treat the inert polymeric surfaces before further functionalization. However, these pre-treatments can alter the mechanical properties of treated materials or may require sophisticated instrument. Furthermore, these methods either convert existing surface groups to reactive sites or introduce new functionalities to the surfaces and the functional groups produced depend on the substrate used [166]. In the search for a facile surface modification able to introduce a wide variety of desired properties regardless the type of material, Messersmith and co-workers reported, in 2007, a bio-inspired approach which has revolutionized the world of material science [167].

POLYDOPAMINE: A BIO-INSPIRED POLYMER COATING

The natural world provides many examples of adhesive mechanisms used by living organisms that have been a source of inspiration to develop new adhesive strategies for modifying surfaces. Marine mussels, for example, have a remarkable ability to attach to wet surfaces in the sea. Their adhesion must be fast, strong and tough so they can survive in the ocean's turbulent zone [168] and, in fact, mussels have been shown to attach to virtually all types of organic and inorganic surfaces even classically adhesion resistant ones such as poly (tetrafluoroethylene). These adhesive mechanisms have been best characterized in the common blue mussel, *Mytilus edulis*

which anchors itself to substrates through acellular byssal threads composed of collagen and silk-like proteins as well as unique adhesive proteins (Figure 3A). Understanding mussel's adhesion mechanisms may rely in the amino acid composition of proteins found near the plaque-substrate interface. From the 34 known proteins secreted by the mussel foot, at least five subtypes are known to contain 3-4-dihydroxy-L-phenylalanine (DOPA), a hydroxylated version of the natural amino acid tyrosine, at concentrations ranging from a few mol % to 27 mol %. The highest content of DOPA occurs in *M. edulis* foot protein 3 (Mefp-3, 21 mol %) and Mefp-5 (27 mol %), both of which appear to be in higher relative abundance near interface of the plaque with the substrate (Figure 3B). Additionally, they both have large numbers of DOPA-Lys tandem sequences. Assuming that the coexistence of catechol (DOPA) and amine (lysine) groups may be essential for a successful adhesion to a wide range of materials, Messersmith and co-workers identified a small molecule that combines both functionalities, dopamine (Figure 3C). They reported that dopamine, as a simple structural mimic of Mefp-5, could be deposited as a thin adherent polymer film (polydopamine, pDA) on different material surfaces, including metals, polymers and inorganic materials (Figure 3D). Furthermore, the pDA-coated surfaces proved to be versatile substrates for further ad-layer deposition of several compounds.

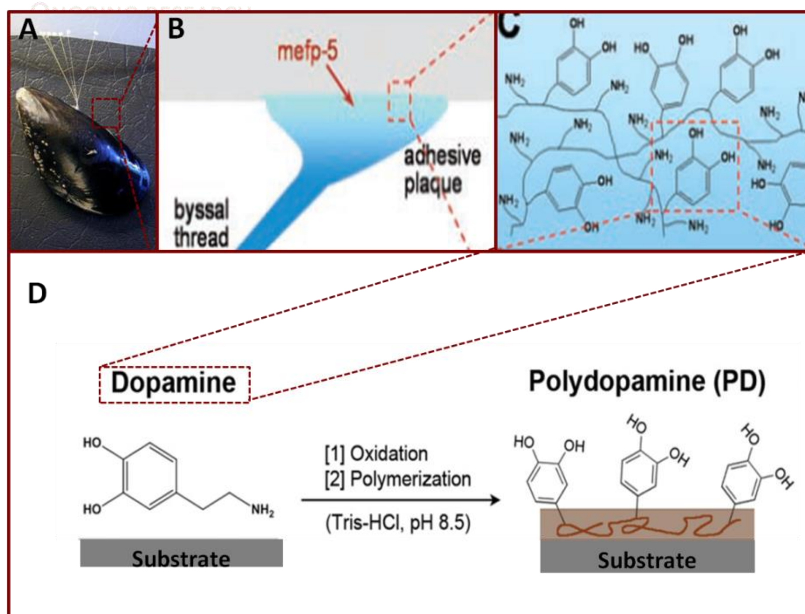

Figure 3. (A) Photograph of a mussel attached to commercial PTFE; (B and C) Schematic illustrations of the interfacial location of Mefp-5 and a simplified molecular representation of characteristic amine and catechol groups; (D) The pDA dip-coating treatment process. (Adapted from [168,169])

The most commonly used protocol for the production of pDA-based materials involves their immersion in aqueous solution of dopamine, buffered to a pH typical of marine environments (usually 2 mg of dopamine per mL of 10 mM Tris buffer, pH 8.5). Dopamine can be oxidized and spontaneously self-polymerize under alkaline conditions with oxygen as the oxidant. This self-polymerization reaction takes place immediately and is associated with a colour change from colourless to pale brown turning deep brown with passing time. The thickness of the pDA film can be controlled by changing the concentration of dopamine monomers and the polymerization time until a maximum value of 50 nm [170].

In spite of the widespread use of this surface modification method, the molecular mechanisms behind pDA formation has not been fully understood. In the early stages of this research field, it was believed that pDA formation shared many characteristics with melanin biosynthesis pathways. Under oxidative (e.g. alkaline pH) conditions, dihydroxyl group protons in dopamine are deprotonated becoming dopamine-quinone which subsequently rearranges via intramolecular cyclization to leokodopaminechrom. Further oxidation and rearrangement leads to 5, 6 dihydroxyindole, which further oxidation causes intermolecular cross-linking to yield a polymer structurally similar to the bio-pigment melanin. The pDA coated surfaces can subsequently interact covalently with several compounds via Schiff-base reactions (amine containing molecules) or Michael type reactions (amine and thiol containing molecules) [167]. Bielawski and co-workers, on the other hand, proposed a new structural model in which pDA is not a covalent polymer but instead a supramolecular aggregate of monomers that are held together through a combination of charge transfer, π-stacking and hydrogen bonding interactions. The combination of these non-covalent interactions is responsible for the high stability of pDA coatings as well as its insolubility [171]. In the model proposed by Lee and co-workers it was proposed that the formation of pDA was a result of the combination of non-covalent self-assembly and covalent polymerization [172].

One of the most important properties of pDA is its ability to react with a wide range of molecules especially with amine and/or thiol containing compounds. Under basic conditions, the catechol in the pDA matrix can be oxidized into to the corresponding quinone, which can then react with the nucleophilic amine or thiol groups by means of a Schiff base reaction or via a Michael-type addition pathway. Polydopamine functionalization is a very simple procedure solvent-free, which does not require time-consuming synthesis of complex linkers, only requiring agents mixing at room temperature (RT) under basic conditions. Furthermore, it has the advantage of proceed in

aqueous environments and remain quite stable, unlike to N-hydroxysuccunimide or maleimide, two commonly used agents in coupling strategies, which are susceptible to hydrolysis leading often to low efficiency of surface bioconjugation [170]. Bioconjugation reactions on pDA surfaces can be modulated by pH, affording selectivity of reaction with amine or imidazole functional groups of biomolecules. Lee and co-worker reported this pH dependent immobilization onto pDA layer using a compound containing two different nucleophiles, lysine and histidine at opposite ends of the molecule. Compound immobilization occurred in a pH-dependent manner due to the large difference in pKa values of histidine (pKa ≈6) and ε-amines (pKa ≈10) [173].

Biocompatibility is an important property required for materials to be applied in the biomedical field. Taking into consideration the fact that pDA is the major component of naturally occurring melanin widely distributed in the human body, it was expected to exhibit excellent biocompatibility. It has been demonstrated that pDA did not compromise the viability or proliferation of many kinds of mammalian cells such as fibroblasts, osteoblasts, neurons and endothelial cells [174]. Furthermore, several studies have reported that pDA layer even promoted cell adhesion and proliferation of substrates in a material-independent manner, providing further evidence of the negligible cytotoxicity of pDA [175, 176].

The interfacial adhesion property of pDA coatings has been widely exploited to introduce new functionalities to the materials for new applications. For example, immobilization of neurotrophic growth factors and adhesion peptides onto polymer substrates enhances differentiation and proliferation of human foetal brain-derived and human induced pluripotent stem cell-derived [177]. Polydopamine coating has been also used to introduce both antimicrobial and anti-fouling properties into a polymeric substrate by deposition of silver nanoparticles and PEG [65]. Examples of other fields where pDA coatings have been exploited includes biomineralization [178], single-cell encapsulation [169], softlithography [179], biocompatible surface modifications [174, 180], attenuation of intrinsic *in vivo* toxicity of biomaterials [176] and sensors [181].

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Chapter 2

METHODOLOGY

This chapter describes the materials, equipment and the methodologies used throughout this work. Although the description of the experimental procedures is presented throughout the subsequent chapters, the rationale beyond the methodologies employed are explained in greater detail in this chapter.

MICROORGANISMS AND CULTURE CONDITIONS

BACTERIAL STRAINS

Three bacterial species, commonly isolated from BAI, were used throughout this work: the Gram-positive *S. aureus* and *S. epidermidis* and the Gram-negative *P. aeruginosa*:

- The type strains purchased from the American Type Culture Collection, *P. aeruginosa* ATCC 27853, ATCC 39324 and ATCC 10145;
- *P. aeruginosa* clinical isolated strains, PD64.8, PD68.7, PD50.2 and PD 96.4. These strains were kindly provided by Dr. Margarida Martins from 3B's Research Group - Biomaterials, Biodegradables and Biomimetics, University of Minho, Taipas/Guimarães, which were obtained under the scope of the project "Insights into peritoneal dialysis catheter associated biofilms" funded by the Portuguese Society of Nephrology to Dr. Anabela Rodrigues.
- *P. aeruginosa* clinical isolated strain U147016-1, kindly provided by Dr. Alberta Faustino from S. Marcos Hospital in Braga.
- The type strain purchased from ATCC, *S. aureus* ATCC 25923.
- *S. aureus* GB 2/1 isolated from explanted voice prostheses at the University Medical Centre of Groningen (the Netherlands) was used throughout this study as a model strain.
- *S. epidermidis* GB 9/6 also isolated from explanted voice prostheses at the University Medical Centre of Groningen (the Netherlands).

BACTERIA PRESERVATION

Bacterial strains were stored at -80 °C in broth medium supplemented with 20 % (v/v) glycerol. Prior to each experiment, cells were propagated by streaking a loopful of cells onto Tryptic Soy Broth medium (TSB, Merck, Portugal) supplemented with 1.2 % (w/v) agar (Merck, Portugal) plates and incubated at 37 °C for 24 h. These stocks were stored at 4 °C for no longer than one week.

MEDIA AND GROWTH CONDITIONS

TSB, MHB (Mueller Hinton Broth, Merck, Portugal) and TSA (Tryptic Soy Agar) were prepared according to the manufacturer's instructions. For all experiments, batches (20 mL in 50 mL Erlenmeyer) of TSB were inoculated with freshly grown cells in an orbital shaker (120 rpm, OS-20) at 37 °C overnight (16-18 h). Bacterial cells were harvested by centrifugation (9000 g, 5 min, at RT) and washed in sterile saline solution (0.9 % NaCl prepared in distilled water). The cellular suspension was then adjusted by spectrophotometric measurement at 640 nm (calibrations were performed for each bacterial strain to relate the absorbance at 640 nm with the number of colony forming units, CFU).

ANTIBACTERIAL COMPOUNDS

In this work two bio-inspired compounds, specifically AMP and enzymes targeting extracellular polysaccharide substances, were investigated as potential alternatives to antibiotics in the design of antibacterial surfaces. Two antibiotics were also used to perform some treatment therapies.

ANTIMICROBIAL PEPTIDES

In this work, different peptides representing different mechanisms of action and different chemical structures were used (Figure 1).

POLYMYXINS B AND E

Polymyxins are a group of cationic polypeptides that consist of a seven-member cyclic ring of aminoacids with a tripeptide side chain bounded to a fatty acid chain that has been found to be either 6-methyl-octanic acid or 6-methyleptanoic acid. The two polymyxins have the same heptapeptide ring, with the exception of a single aminoacid, which is phenylalanine in polymyxin B (PB) and leucine in polymyxin E (PE), commonly called colistin [1]. Their mechanism of action involves cell membrane's disruption by binding to the anionic part of the lipopolysaccharides (LPS) of Gram-negative bacteria, which results in leakage of intracellular components. The clinical use of these compounds was discontinued in the 1970s due to their nephrotoxicity and neurotoxicity. However, the widespread emergence of multidrug resistant strains has led to the

return of these older antimicrobials with acceptable efficacy and less toxicity than reported in the past [2]. PB was purchased from Biochrom (Germany) and PE (colistin sulphate) from Sigma (Portugal).

LIPOPETIDE PALM-KGK-NH2

Palm-KGK-NH_2 (Palm) belongs to a new group of lipopeptides with potent antifungal and antibacterial activities. These lipopeptides are derived from positively charged peptides containing D- and L- amino acids (diastereomers) that are palmitoylated at their N terminus [3]. As a lipopetide its mechanism of action consists of simple disruption of membrane electric potential [4].

CAMEL

Camel (KWKLFKKIGAVLKVL-NH_2) is 15-residue hybrid peptide with seven amino acids that are derived from the sequence of cecropin A, which comes from the larvae of the silk moth *Hyalophora cecropia* and eight amino acids that are derived from the sequence of melittin, which comes from honey bee venom [5]. It has been found that camel is more active than the native molecules and also lacks the undesirable hemolytic properties of melittin [6,7].

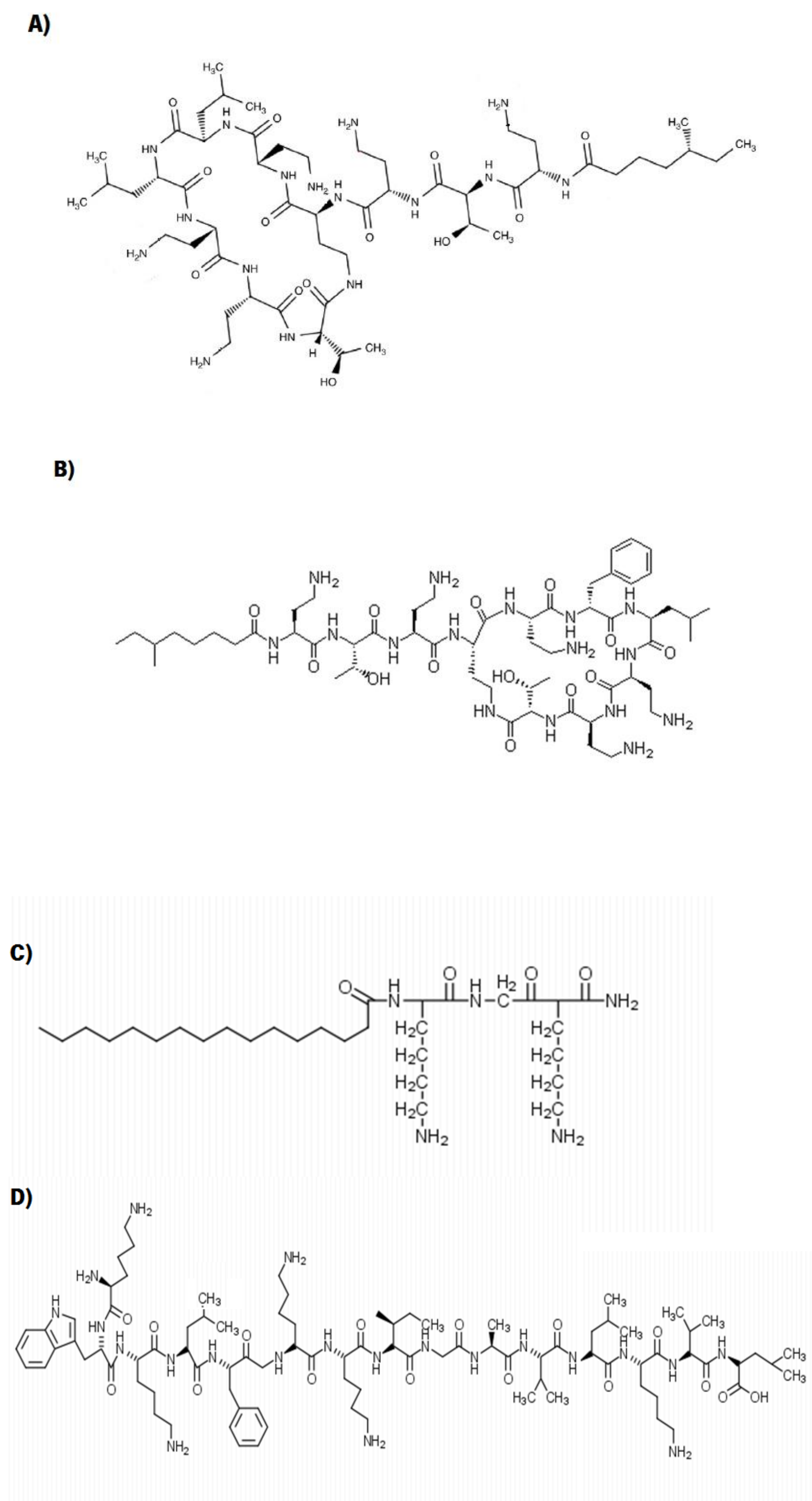

Figure 1. Chemical structures of AMP: polymyxin E (A), polymyxin B (B), Palm-KGK-NH_2 (C) and Camel (D).

PEPTIDE SYNTHESIS

The peptides Palm and Camel were kindly provided by Dr. Wojciech Kamysz, (Faculty of Pharmacy, Medical University of Gdansk, Poland) and Dr. Daria Grzywacz (Lipopharm, Poland). Accordingly, peptides were synthesized manually by solid-phase synthesis method on polystyrene AM-RAM resin, using Fmoc/tButyl strategy [8]. Coupling was performed with HOBt/DIPCDI method, the Fmoc protecting group were removed with 20 % piperidine. Crude peptides were cleaved from resin using a mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIS) and water as scavengers. The final products were purified by reverse-phase high performance liquid chromatography (RP-HPLC) in a mixture of acetonitrile- water with 0.1 % TFA as an eluent. Molecular weights of peptides were determined by matrix–assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF).

PEPTIDES ANTIMICROBIAL ACTIVITY

The antimicrobial activity of AMP was assessed by determining the minimum inhibitory (MIC) and bactericidal (MBC) concentrations by the microdilution method according to Clinical and Laboratory Standards Institute (formerly NCCLS) [9]. Briefly, the wells of a sterile 96-well round-bottom microtiter plates (polystyrene, Orange, USA) were filled with 100 µL of MHB with increasing concentrations of peptide to which were added 100 µL of each bacterium inoculum (adjusted to a final concentration of 5×10^5 CFU/mL). The plates were afterwards incubated at 37 °C for 24 h in an orbital shaker at 120 rpm (OS-20). In this assay, two controls were used, one without bacteria as a negative control and one without peptide as a positive control. Moreover, culture medium with increasing concentrations of peptides without bacteria were also performed in order to avoid misleading results. The MIC of the planktonic fraction was obtained by measuring the absorbance at 640 nm (A_{640nm}), where clear wells (A_{640nm} =0.05 negative control) were evidence of bacterial growth inhibition. MBC determination was performed by adding a droplet of 10 µL from each well with no visible growth on a TSA plate. The lowest concentration that yielded no colony growth after 24 h at 37 °C was identified as the MBC.

ENZYMES

In this work, several enzymes targeting different biofilm matrix or bacterial cell wall components were investigated.

LYSOZYME

Lysozyme is a hydrolytic enzyme able to destruct bacterial cell walls by an enzymatic hydrolysis of 1-4-beta-linkages between N-acetylmuramuc acid and N-acetyl-D-glucosamine residues of peptidoglycan in the bacterial cell wall, especially for Gram-positive bacteria. This enzyme was chosen because of its well-known bactericidal properties [10], physiological abundance (it can be found in several biological fluids and tissues including avian egg, plant and animal secretions), high thermal stability, wide pH activity range and well known structure [11]. It was purchased from Fisher Scientific, USA.

PROTEINASE K

Proteinase K is a serine protease able to cleave peptide bonds of proteins important for bacterial adhesion and/or biofilm establishment. This enzyme is stable in a broad range of conditions such as pH, buffer salts, detergents and temperature [12, 13]. It was purchased from Biochrom, Germany.

DNASE I

DNase I is an enzyme able to non-specifically cleave eDNA by breaking phosphodiester bonds of the phosphate backbone. Extracellular DNA acts as a bridge between the bacterial cell wall and EPS by binding biopolymers in EPS through attractive, short-range acid-base interactions [14] which makes DNase I a promising alternative to inhibit, disperse or even increase biofilms susceptibility to antimicrobials [15]. It was purchased from Applichem, Germany.

ALGINATE LYASE

Alginate lyase is able to degrade the polysaccharide alginate that contributes to mucoid biofilm structure, playing a role in bacterial virulence and persistent nature of lung infections. Alginate lyase treatment has been shown to detach biofilms from abiotic surfaces [16] and to increase gentamicin and CIP killing of mucoid *P. aeruginosa* biofilm [17]. It was purchased from Sigma (Portugal).

DETERMINATION OF ENZYMATIC ACTIVITY

ALGINATE LYASE

Alginate lyase catalyses depolymerisation of alginate through cleavage of the 4-O-glycosidic bond via a β-elimination mechanism which leads to formation of a double bond between C-4 and C-5 and production of 4-deoxy-L-erythohex-4-ebe pyranosyluronate at the non-reducing end of the resulting oligomers which can be detected by measuring absorbance at 235 nm [18]. Briefly, alginate lyase-coated surfaces were covered with 0.6 mL of 0.1 % sodium alginate (Sigma) prepared in 0.2 M phosphate buffer, pH 6.3. After incubation at 37 °C for 30 min, the absorbance of the reaction mixture was measured at 235 nm. As a control, the activity of alginate lyase heat-inactivated before its immobilization was also determined.

DNASE I

DNase I is an endonuclease that acts on phosphodiester bonds adjacent to pyrimidines to produce polynucleotides with terminal 5'-phosphates. Therefore, its activity was determined by analysing the hydrolysis of plasmid DNA which was extracted using the Zyppy Plasmid Miniprep Kit (Zymo Research, USA) according to the manufacturer's instructions. After digestion with restriction enzyme *Hind*III-FH (New England Biolabs, NEB), DNA aliquots of 40 µl were then exposed to immobilized DNase I and analysed by electrophoresis in a 1 % agarose (BIORON, Germany) gel in 1 x Tris-acetate-EDTA (TAE) buffer. The gel was left running for approximately 50 min at a constant potential of 80 V and visualized under UV light using a transilluminator (BioRad).

LYSOZYME

Lysozyme bioactivity was measured using a previously reported based on spectrophotometrically monitoring of *Micrococcus lysodeikticus* (Sigma, Portugal) turbidity as a consequence of cells lysis [19]. Briefly, coupons functionalized with lysozyme were covered with 3 mL of a suspension of *M. lysodeikticus* prepared in 66 mM phosphate buffer, pH 6.24 and adjusted to an optical density of approximately 1.0 at 450 nm. Samples were kept at 37 °C for 9 h and aliquots were withdrawn at different time points to measure their turbidity spectroscopically at 450 nm. As a control, pDA-coated coupons without lysozyme and a bacterial suspension alone were also monitored.

ANTIBIOTICS

Ciprofloxacin, a fluoroquinolone and vancomycin, a glycopeptide antibiotic, were used in this project. Stock solutions were prepared and stored according to the manufactures instructions. Both antibiotics were purchased from Sigma (Portugal).

BIOFILM STUDIES FOR AMP SCREENING

BIOFILM FORMATION

Biofilm formation was based on the microtiter plate test developed by Stepanovic *et al.* [20]. Cells were harvested by centrifugation, washed in sterile saline solution and dilutions were made to prepare standardized cell suspensions in TSB at a cell density of $1x10^6$ CFU/mL. Afterwards, 200 µL/well of the bacterial suspension were transferred to 96-well flat-bottom tissue culture plates (Orange Scientific) that were incubated at 37 °C for 24 h on a horizontal shaker (120 rpm, OS-20).

BIOFILM SUSCEPTIBILITY

Biofilm susceptibility to the antimicrobial compounds was evaluated using different application strategies: preconditioning the adhesion surfaces with antimicrobials prior to biofilm formation, growing biofilms in its presence and treating the biofilms after their establishment.

AFTER CONDITIONING WITH ANTIMICROBIAL AGENTS

Microtiter plates were filled with different concentrations of antimicrobial agent and left at RT for 30 min. Control wells, containing sterilised ultrapure (UP) water only, were treated in the same way. Antimicrobial solutions were, then, removed and the plates air-dried at RT. Biofilms were developed in clean and conditioned wells according to the modified plate procedure developed by Stepanovic *et al.* [20] mentioned above.

IN THE PRESENCE OF ANTIMICROBIALS

Biofilms were formed in microtiter plates as aforementioned but prepared in TSB supplemented with different concentrations of antimicrobial agent.

AFTER TREATMENT WITH ANTIMICROBIAL AGENTS

Biofilms formed on clean and/or pre-conditioned wells were subjected, subsequently, to sudden treatment of antimicrobials at RT for different periods of time. For that, the content of each well was removed and washed with sterilised water and the wells attached biofilms were afterwards treated with antimicrobials. Non-treated wells were filled with sterilised UP water for the same period of time.

BIOFILM CHARACTERIZATION

BIOFILM MASS

Biofilm mass was quantified by the crystal violet (CV) staining method, adapted from Stepanovic *et al.* [20]. CV is a basic dye which binds to negatively charged molecules from the cells surface and the polysaccharides from the biofilm extracellular matrix [21]. After biofilm growth, the content of each well was removed and the wells were washed twice with sterilised water. The plates were then left to dry for 30 min and the remaining bacteria attached were fixed with 200 µL of absolute methanol per well. After 15 min, plates were emptied and left to dry again. The fixed bacteria were then stained with 200 µL of CV (Gram colour-staining, Merck) per well for 5 min and excess staining was rinsed off by washing the wells with distilled water. The plates were air dried and the wells filled with 200 µL of 33 % (v/v) acetic acid (Merck) to solubilise the CV bound to the adherent bacteria. The absorbance of the obtained solution was measured at 570 nm using a microtiter plate reader (Model Sunrise-basic Tecan, Austria). Control experiments to avoid false results were also performed in order to determine whether the tested medium and the material of construction of the plates could interact with biomass quantification.

BIOFILM CELLS METABOLIC ACTIVITY

The metabolic activity of biofilm-encased cells was measured using the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazo-lium-5-carboxanilide sodium salt (XTT, Sigma-Aldrich) colorimetric method as described by Stevens and Olsen [22], with some modifications. This method is based on the reduction of XTT by metabolically active cells to a water-soluble orange formazan. The produced formazan can be quantified by spectrophotometry analysis, being therefore proportional to the bacterial metabolic activity [23]. After biofilm growth and washing procedures

as aforementioned, 200 µL of a combined solution of XTT and phenazine methosulfate (PMS, Sigma) were added to each well in order to obtain a final concentration of 150 µg/mL of XTT and 10 µg/mL of PMS. The plates were afterwards incubated at 37 °C for 3 h and 120 rpm, in the dark. The absorbance of each well was measured at 490 nm using a microtiter plate reader. Control tests, using culture medium and empty wells were also performed in order to avoid misleading results.

BIOFILM-ENTRAPPED CELLS

In order to determine the number of CFU, biofilms were washed as described before and removed by ultrasonic bathe in a Sonicor SC-52 (Sonicor Instruments) operating at 50 kHz, during 6 min (these parameters were previously optimized in order to promote complete removal of biofilm attached without causing lysis). Bacterial suspensions were afterwards collected, gently vortexed to disrupt possible cell aggregates and serially diluted. Serial 10-fold dilutions were performed and plated into TSA plates that were incubated overnight at 37 °C in an aerobic incubator prior enumeration. The number of viable biofilm cells was expressed as CFU per cm^2.

SURFACE MODIFICATION

MATERIALS PREPARATION

Polycarbonate and PDMS were the materials used throughout this work. These materials were chosen because of their use in a wide range of medical devices, such as filters cartridges for dialysis, blood oxygenators, reservoirs and filters, connection components as well as urinary, central venous and peritoneal catheters [24-26]. PC was purchased from McMaster Carr (USA) and was cut into square pieces measuring 1.3 x 1.3 x 0.3 cm^3. It was cleaned by sonication for 20 min in 0.12 M HCl and 20 min in isopropanol, followed by rinsing with UP water and finally air-dried overnight [27]. PDMS was prepared by mixing and curing of two-component kit Sylgard 184 (Dow Corning, USA) at RT. Briefly, base and curing agents in the kit were mixed thoroughly in 10:1 (w/w), cast in a petri dish and kept at RT for 48 h. After curing, the PDMS was cut into circle pieces of 0.9 cm diameter at a thickness of about 0.3 cm. Prior utilization, PDMS coupons were sonicated in a commercial detergent (Sonasol, Henkel Ibérica, Portugal) for about 5 min,

rinsed with distilled water for a few minutes, sonicated in methanol for about 20 min, then rinsed with distilled water and air-dried overnight [28]. Once dried, sterilization was performed by autoclaving coupons for 15 min at 121°C.

POLYDOPAMINE COATING AND FURTHER FUNCTIONALIZATION

Coatings were prepared as illustrated in Figure 2 testing two pDA-based approaches: a two and a one-step immobilization. For compounds immobilization via a 2-step approach (Figure 2B), the first step involved the deposition of a pDA coating on material surfaces which was performed by immersing them in a freshly prepared solution of dopamine (Sigma, Missouri; 1 or 2 mg/mL dopamine-HCl in 10 mM bicine buffer, pH 8.5) for 18 h, at RT and under agitation (70 rpm). Materials were then rinsed with UP water and air-dried. For further functionalization, pDA-coated coupons were immersed in compounds solutions and were incubated for different periods of time and pH, under agitation. A 1-step pDA-based immobilization procedure was also performed (Figure 2A). In this approach, dopamine (2 mg/mL) and compounds were dissolved together in 10 mM bicine buffer solution (pH 8.5) and the coupons were immediately immersed in this solution. After overnight coating at RT and under agitation (70 rpm), the coupons were taken and rinsed with UP water and air-dried.

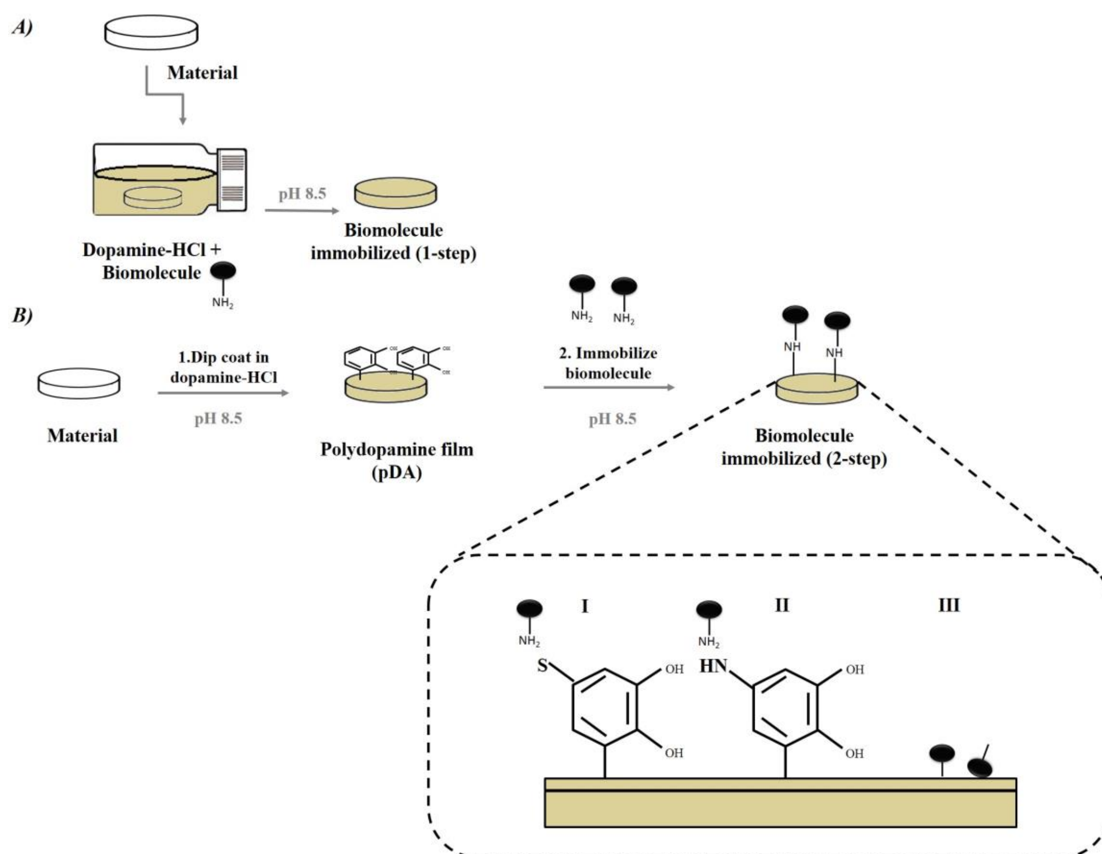

Figure 2. Schematic description of pDA coating developed for AMP and/or enzymes immobilization onto material surfaces. Materials were immersed in a solution containing dopamine and the biomolecule together for 1-ste approach immobilization (A). For the 2-step immobilization approach (B), materials were first functionalized with a layer of pDA, followed by biomolecule(s) immobilization. AMP and enzymes are immobilized to the exposed catechol functionalities on the coated materials via Michael addition/Schiff base reaction of the compounds' inherent thiol (I) and/or amine (II) group, as well via physical adsorption (III).

SURFACE CHARACTERIZATION

SEM

The surface morphology of materials was analysed by scanning electron microscopy (SEM). Prior to observation, samples were sputter coated with gold and observed with an S-360 scanning electron microscope (Leo, Cambridge, MA, USA). SEM imaging was performed with the following parameters: 15 kV accelerating voltage, 22 mm stage distance, 500 x and 5000 x magnification.

XPS

X-ray photoelectron spectroscopy (XPS) (Omicron ESCA Probe; Omicron, Taunusstein, Germany) was used to characterize the chemical composition of substrates. The X-ray source operated at 300 W with a spot size of 1.5 mm and a constant sample deflection angle of 45°. An electron

gun was used to minimize surface charging effects, operating with a beam current of 0.008 mA at 12.5 eV. High-resolution spectra of the C1s region were obtained by averaging 3 separate sweeps between 277.5 and 292.5 eV.

AFM

The surface morphology and roughness were also evaluated using atomic force microscopy (AFM). AFM measurements were performed at RT using a Multimode with a Nanoscope III from Digital Instruments (USA) operating in tapping mode. Scan rates were set at 1 Hz and the scanning area per sample was fixed at 5 µm x 5 µm. Surface morphology and roughness analysis were conducted using NanoScope Analysis 1.10 software.

CONTACT ANGLE MEASUREMENTS

To evaluate the surface wettability of surfaces, the static water contact angle of materials after each deposition step was measured by a sessile drop method using an automated contact angle measurement apparatus (OCA 15 Plus, Dataphysics, Germany) that allows image acquisition and data analysis. Contact angles were measured using 3 µL drops of water.

PHYSICOCHEMICAL CHARACTERIZATION OF SURFACES AND CELLS

Since thermodynamic properties play an essential role in the initial bacterial adhesion to surfaces [29], the physicochemical surface properties of bacterial cells and materials used in this project, were determined by performing contact angle measurements with the sessile drop technique and the method proposed by van Oss approach [30]. Measurements were performed on cleaned and dried materials and on bacterial layers deposited on membrane filters, as previously described [31]. Briefly, a bacterial suspension was adjusted to a concentration of approximately $1x10^9$ CFU/mL in sterile saline solution from an overnight culture and deposited onto a 0.45 µm cellulose membrane filter, previously wetted with 10 mL of distilled water to obtain a thick lawn of cells. The filters with the resultant lawn of cells were afterwards kept on petri dishes containing 1 % (w/v) agar and 10 % (v/v) glycerol for at least 3.5 h, until the so call "dried-plateau" was obtained. All measurements were performed at RT and water, formamide and α-bromonaphtalene were used as reference liquids for standardized contact angles measurements.

According to van Oss approach, the contact angle (θ) formed by a liquid (l) on a solid surface or bacterial cells (s) can be related to surface tension parameters of the liquid and solid surface by the following equation:

$$(1 + cos\theta) \times \Upsilon_l^{TOT} = 2\left(\sqrt{\Upsilon_s^{LW} \times \Upsilon_l^{LW}} + \sqrt{\Upsilon_s^{+} \times \Upsilon_l^{-}} + \sqrt{\Upsilon_s^{-} \times \Upsilon_l^{+}}\right) \text{ (1)},$$

where Υ^{LW} denotes the Lifshitz – van der Waals component of surface free energy, Υ^{+} the electron-acceptor and $\Upsilon^{-}$the electron-donor components of surface free energy. For a non-polar liquid, the polar component of surface tension is null so equation (1) becomes:

$$\Upsilon_s^{LW} = \frac{\Upsilon_l^{TOT}}{4} \times (1 + cos\theta)^2 \text{ (2).}$$

The surface tension components can be then determined by measuring the contact angles formed by three different liquids (θ_w,θ_B andθ_F), for which apolar (Υ^{LW}) and polar components (Υ^{+} Υ^{-}) are knowns (Table 1).

Table 1. Surface tension parameters of the three liquids used in contact angle measurements for the determination of solids surface tension. Data were taken from [32].

	Surface tension (mJ/m²)			
Liquids	Υ_l^{TOT}	Υ_l^{LW}	Υ_l^{+}	Υ_l^{-}
Water	72.8	21.8	25.5	25.5
α-Bromonaphthalene	44.4	44.4	0	0
Formamide	58	39	2.28	39.6

Taking into account the contact angle values obtained with these three liquids and the values from Table 1, three forms of the equation (1) are obtained and simultaneously resolved to calculate the surface tension components, Υ_l^{LW} , Υ_l^{+} and Υ_l^{-}.

$$\Upsilon_s^{LW} = 11.1 \times (1 + cos\theta_B)^2 \text{(3)}$$

$$5.049 \times \sqrt{\Upsilon_s^{+}} + 5.0549 \times \sqrt{\Upsilon_s^{-}} = 36.4 \times (1 + cos\theta_w) - 15.55 \times (1 + cos\theta_B) \text{ (4)}$$

$$6.293 \times \sqrt{\Upsilon_s^{+}} + 1.510 \times \sqrt{\Upsilon_s^{-}} = 29 \times (1 + cos\theta_F) - 20.806 \times (1 + cos\theta_B) \text{ (5)}$$

From the surface tension components it is possible to determine the total surface tension of a surface (Υ_s^{TOT}):

$$\Upsilon_s^{TOT} = \Upsilon_s^{LW} + \Upsilon_s^{AB} \text{ (6)}$$

$$\Upsilon_s^{AB} = 2 \times \sqrt{\Upsilon_s^{+} \times \Upsilon_s^{-}} \ (7)$$

It should be mentioned that negative square roots of surface energy parameters were taken as an indication that the parameter is zero according to van der Mei *et al.* [32].

According to van Oss [30], hydrophobicity can be expressed in the form of the free energy of interfacial interaction (ΔG_{sws}^{TOT}) between the particles of a solid surface (s), in an aqueous environment (w). The free energy of interfacial interaction can be calculated by the sum of polar and apolar components:

$$\Delta G_{sws}^{TOT} = \Delta G_{sws}^{LW} + \Delta G_{sws}^{AB} \ (8)$$

$$\Delta G_{sws}^{LW} = -2 \times \left(\sqrt{\Upsilon_s^{LW} - \Upsilon_w^{LW}}\right)^2 (9)$$

$$\Delta G_{sws}^{AB} = -4 \times \left[\left(\sqrt{\Upsilon_s^{+} \times \Upsilon_s^{-}}\right) + \left(\sqrt{\Upsilon_w^{+} \times \Upsilon_w^{-}}\right) - \left(\sqrt{\Upsilon_s^{+} \times \Upsilon_w^{-}}\right) - \left(\sqrt{\Upsilon_w^{+} \times \Upsilon_s^{-}}\right)\right] (10)$$

According to this model, when $\Delta G_{sws}^{TOT} > 0$, the surfaces are hydrophilic and for $\Delta G_{sws}^{TOT} < 0$, they are hydrophobic.

From the physicochemical parameters of each adhesion entity (bacteria and surface) it was possible to determine the thermodynamic relation between both entities, namely the free energy of adhesion (ΔG_{bsb}^{TOT}) between the bacteria (b) and the surfaces (s). According to the thermodynamic theory, adhesion will be favoured if interaction leads to a decrease on free energy of adhesion.

PEPTIDES IMMOBILIZATION EFFICIENCY AND COATINGS STABILITY

The efficiency of peptide immobilization was determined by quantifying the amount of unattached peptide in the buffer solution retrieved immediately after completing the coating process. The peptide concentration was measured by using a fluorescamine (Sigma) assay [33]. Fluorescamine is a heterocyclic dione that reacts with primary amines to form a fluorescent product. The fluorescence of a solution containing peptides or proteins and fluorescamine will be proportional to the quantity of free amino groups present. Briefly, before and after incubation of peptides onto pDA-coated surfaces, the supernatants containing loaded and unattached peptide, respectively, were retrieved and used as samples to determine the peptide immobilization

efficiency. The amount of peptide lost during washing procedure was also quantified. Fluorescamine assay was performed by mixing fluorescamine solution (Sigma; 3 mg/mL in acetone) and the sample at 1:3 ratio in a 96-black-well plate (Greiner). After 15 min of incubation at RT, the fluorescence intensity of each sample was measured by using a microplate reader (Synergy HT, Biotek). Finally, immobilization efficiency was represented as the percentage ratio of the amount of immobilized peptides to the amount of loaded peptides.

In order to investigate coatings stability, the detachment of immobilized peptides was quantified by measuring the amount of released peptides from the modified surfaces during incubation under a physiologically relevant condition (in phosphate buffered saline, PBS at 37 °C). For that, 500 µL of a fresh PBS (10 mM potassium phosphate, 150 mM NaCl, pH 7.4) solution was added to each well of a 48-well microtiter plate (Orange Scientific, USA) in which coupons functionalized with peptides were placed immediately after peptide immobilization. The coupons were then incubated at 37 °C for 5 days. Every day, supernatant samples (500 µL) were withdrawn to determine the amount of peptide released. The remaining peptides on the surface were quantified by subtracting the released peptides from the total amount of peptides immobilized at the first day.

ANTIBACTERIAL PERFORMANCE OF COATINGS

The antibacterial performance of the functionalized surfaces was investigated using different methods.

BACTERIAL CONTACT KILLING ASSAY

In order to evaluate bacterial contact-killing properties of the modified surfaces, a previously reported method was applied with some modifications [34]. Briefly, bacterial concentration was adjusted in TSB to a final concentration of $1x10^6$ CFU/mL and 20 µL of this solution was added to each well of a microtiter plate (Orange Scientific, USA), in which uncoated and modified coupons were placed. The plate was afterwards incubated at 37 °C, under static conditions for 24 h. After that, materials were placed on a TSA plate, incubated for 24 h at 37 °C and bacterial

growth was checked for all conditions tested and tabulated as "+" for growth and "-"for no visible growth.

QUANTIFICATION OF BACTERIAL ADHESION

Quantification of adherent bacterial cells was performed by the spread plate method as previously described [35]. Briefly, after different incubation periods, the coupons were washed with saline solution and removed from the microtiter plates with sterile forceps into eppendorf tubes to which were added 1 mL of saline solution. Adhered bacteria were then detached by ultrasonic bathe in a Sonicor SC-52 (Sonicor Instruments) operating at 50 kHz, during 6 min followed by rapid vortex mixing for 30 s (these parameters were previously optimized). Serial 10-fold dilutions were performed and plated onto TSA plates that were incubated overnight at 37 °C in an aerobic incubator prior enumeration. The number of viable bacterial cells was expressed as CFU per mL.

BACTERIAL VIABILITY ON MODIFIED SURFACES

The performance of the modified surfaces against bacterial adhesion was evaluated by preparing a bacterial suspension with $1x10^8$ CFU/mL in PBS from an overnight culture at 37 °C. For dual-species adhesion, a combination of 50 % of suspended inoculum of each species was used. Materials were placed into the wells of a tissue culture plate and covered with the bacterial suspension. The samples were kept at 37 °C for 4 h, at 120 rpm (OS-20), washed with saline solution, stained with a live/dead stain (BacLight Bacterial Viability Kit, Invitrogen) and observed in a fluorescent inverted microscope (Leica, DMI 3000B). In this assay, the red-fluorescent nucleic acid staining agent propidium iodide, which only penetrates damaged cell membrane, was used to label dead bacterial cells on the surfaces. In contrast, the SYTO 9 green-fluorescent nucleic acid staining agent, which can penetrate cells both with intact and damaged membranes, was used to label viable cells. ImageJ (Version 1.49m, Wayne Rasband, National Institutes of Health, USA) software was used to subtract the image background and the threshold function was used to render each greyscale image into a binary translation with distinct areas identifying adhered bacteria. The threshold value supplied by ImageJ was used as default but when necessary the threshold value was manually adjusted until all visible cells were included within

the thresholded range. The area measurement function was used to quantify the area of the pixels above the threshold and to thereby quantify the area covered by bacteria discriminating, at the same time, the fraction of live and dead bacteria, depending on the channel being analysed. Values were normalized to unmodified material control as previously performed [27].

SUSCEPTIBILITY PATTERN OF CELLS ADHERED TO MODIFIED SURFACES

In order to evaluate the susceptibility pattern of bacterial cells adhered to PDMS surfaces, cells in contact with modified and unmodified surfaces were recovered and allowed to adhere to new bare and modified samples during a period of 10 days. Briefly, a bacterial suspension with $1x10^7$ CFU/mL was prepared in TSB and 300 µL of this suspension were added to a 48-well microtiter plate in which PDMS, pDA-coated PDMS and PDMS coupons functionalized with AMP were placed. The plate was incubated for 24 h at 37 °C, at 120 rpm. The coupons were subsequently washed 3 times with saline solution to remove free-floating bacteria and transferred to an eppendorf tube with 1 mL of saline solution. The tubes were then sonicated for 6 min, subjected to vortex for 30 s and 300 µL of this suspension were added to a 48-well microtiter plate in which new PDMS coupons were placed. The procedure was repeated for 10 successive days. The MIC and MBC for each condition tested were determined on days 0 and 10 and compared. The number of cells recovered each day was quantified by CFU counting.

LOCALIZATION AND DISTRIBUTION OF BACTERIAL POPULATIONS BY PNA FISH

In order to assess bacterial spatial organization and the species distribution on the coated surfaces, PNA FISH (peptide nucleic acid fluorescence in situ hybridization) method was employed. Before starting the hybridization, co-adhesion of *P. aeruginosa* and *S. aureus* was allowed to occur for 4 h as aforementioned in the sub-section of bacterial viability on modified surfaces. Coupons were then washed with UP sterile water and air-dried for 15 min. Bacteria were fixed with methanol (100 %) for 20 min. This fixation step proved to be crucial to avoid bacterial detachment during hybridization procedure [36]. Fixed bacteria were stored at 4 °C for no longer than 48 h before the multiplex PNA FISH procedure. A specific 16S rRNA PNA probe (Paer565) previously developed [37] was used for *P. aeruginosa* detection and *S. aureus* was identified by counterstaining the samples with 4, 6-diamidino-2-phenylindole (DAPI, Sigma) at the

end of the hybridization procedure. After bacteria fixation with methanol, 20 µL of 4 % (w/v) paraformaldehyde followed by 50 % ethanol (v/v) were applied to cover the entire surface and incubated for 10 min each and allowed to air dry. This step enables the fixation of the cells and increase the permeabilization of the cell membrane to the subsequent hybridization allowing the labeled oligonucleotide probes to diffuse to their intracellular rRNA target molecules [38]. Afterwards, 20 µL of hybridization solution containing the probe at 200 nM was applied on coupons which were covered with coverslips and incubated in the dark for 1 h at 65 °C. After hybridization, coupons were inserted in a 24-well microtiter plate (Orange Scientific) containing a pre-warmed (at 65 °C) washing solution composed of 5 mM Tris Base, 15 mM NaCl and 0.1 % (v/v) Triton X-100 (Sigma). The plate was incubated for 30 min at 65 °C in the dark. Finally, coupons were allowed to air dry in the dark before counterstaining with DAPI (40 µg/mL) for 5 min at RT in the dark and cells were visualized under an epifluorescence microscope. For microscopic visualization, a fluorescence microscope (Olympus BX51, Perafita, Portugal) equipped with the filters sensitive to DAPI (BP 365-370, FT 400, LP 421) and to the signalling molecule of the PNA probe (BP 530-550, FT 570, LP 591, for Alexa 594) was used.

EVALUATION OF BIOFILM FORMATION BY XTT REDUCTION ASSAY

In order to investigate the potential of modified coatings to impair biofilm formation, the metabolic activity of biofilm cells was evaluated using the aforementioned XTT colorimetric method [22], with some modifications. XTT is a tetrazolium that can be reduced by cells in their mitochondria to an orange coloured formazan dye. The amount of formed tetrazolium formazan is thus proportional to biofilm cells metabolic activity. Briefly, a bacterial suspension with $1x10^7$ CFU/mL was prepared in TSB and added to a microtiter plate in which modified surfaces were placed. The plate was incubated for 24 h at 37 °C and 120 rpm (OS-20). The coupons were subsequently washed with saline solution to remove free-floating bacteria and a combined solution of XTT and PMS were added to each well in order to obtain a final concentration of 150 µg/mL of XTT and 10 µg/mL of PMS. The plates were afterwards incubated at 37 °C for 3 h and 120 rpm (OS-20), in the dark. The absorbance of each well was measured at 490 nm using a microtiter plate reader.

CYTOTOXICITY OF MODIFIED SURFACES

DIRECT-CONTACT OF CELLS WITH MODIFIED SURFACES

Cytotoxicity tests were performed using fibroblast cells 3T3 (CCL 163) from ATCC, a cell line commonly used for biomaterial surface compatibility studies [39,40]. Cells were first cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % of foetal bovine serum (FBS) and 1 % penicillin/ streptomycin at 37 °C and 5 % CO_2. After achieving the confluence, cells were detached using trypsin and 500 µL of a cell suspension with $1x10^5$ cells/mL were added to each well of a 48-well microtiter plate in which the modified surfaces were previously inserted. The plates were incubated at 37 °C and 5 % CO_2 for 48 h.

CELL VIABILITY EVALUATION BY MTS

Metabolic activity of cells in contact with modified surfaces was then evaluated by the MTS (3-(4, 5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium), inner salt reduction assay. All the medium was removed and a solution containing 100 µL of MTS (Promega CellTiter 96® AQueous NonRadioactive Cell Proliferation Assay) per each 1 mL of DMEM without phenol red was added to each well. After 1 h of incubation in the dark, at 37°C and 5 % CO_2, the absorbance of the resulting solution was measured at 490 nm.

MACROPHAGE-BACTERIA INTERACTIONS ON MODIFIED SURFACES

CELL CULTURE CONDITIONS AND MACROPHAGES DIFFERENTIATION

A human monocyte line cell (THP-1, ATCC TIB-202) was used to study the role of host immune system once bacteria manage to adhere to bi-functional coatings. Monocytes were routinely cultured in (RPMI-1640) with sodium bicarbonate and L-glutamine, supplemented with 10 % FBS and 1 % penicillin/ streptomycin .The flasks were kept at 37 °C in a humidified atmosphere with 5 % CO_2 and cells were passaged when reached the exponential phase of growth (3 - 8 x 100 000 cells/mL). Cells were harvested by centrifugation (150 *g*, 5 min at RT) and the harvested cells were counted using a Burker–Turk hemocytometer. To induce monocytes differentiation into macrophages, $1x10^6$ cells/mL were diluted in RPMI supplemented with 100 nM of phorbol 12-Myristate 13- (PMA) and cultured for 24 h at 37 °C and 5 % CO_2. Cells in suspension were

afterwards removed by aseptically removing the medium and adhered cells were washed with RPMI-1640 and incubated for more 48 h in medium without PMA.

MACROPHAGES-MEDIATED PHAGOCYTOSIS OF BACTERIA ADHERING TO BI-FUNCTIONAL COATINGS

Differentiated macrophages were detached using trypsin and 300 µL of a cell suspension with $5x10^5$ cells/mL were added to each well of a 48-well microtiter plate in which the modified surfaces with staphylococci ($1x10^8$ CFU/mL in PBS) adhered for 4 h, were previously inserted. The plates were incubated at 37 °C and 5 % CO_2 for 2 h. The coupons were washed with PBS, stained with DAPI (40 µg/mL) and visualized under an epifluorescence microscope.

STATISTICAL ANALYSIS

Results were presented as mean ± standard deviation (SD). The statistical analysis for this project was performed as follows: outliers were identified and removed by applying Grubbs' test and data normality was checked using Kolmogorov-Smirnov test. After this analysis, parametric tests (one way ANOVA followed by Tukey's test) or nonparametric (Kruskal–Wallis test) were used depending on whether the samples were from normally distributed populations or not, respectively. These analysis were formed using Microsoft Excel and Graph Pad Prism 5.0 software.

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Chapter 3

AMP POTENTIAL TO CREATE ANTIMICROBIAL SURFACES

3.1

Screening of the susceptibility patterns of planktonic and sessile cultures towards AMP

AMP have been recognized as excellent candidates as alternatives to antibiotics for the new generation of antimicrobial surfaces. Prior to their immobilization, it was aimed to evaluate their efficacy, when in solution, against planktonic and sessile cultures of both Gram-positive and Gram-negative bacterial strains commonly found in BAI. Different AMP, representing different mechanisms of action, were used in this screening study, namely polymyxins (B and E), Camel and Palm. Results demonstrated the potential use of these AMP in the early stages of biofilm growth to impair its establishment, highlighting their potential as candidates for the development of antimicrobial coatings for medical devices. Polymyxins were more effective against the Gram-negative strain while Camel and Palm against the Gram-positive one.

INTRODUCTION

Nowadays, the major global healthcare problem is the growing number of nosocomial infections associated to the emergence of resistance microorganisms. This problem gets worse when microorganisms switch from planktonic to sessile lifestyle and live in biofilms. Once established, biofilms are less susceptible to antimicrobial treatment and to the host immune system than their planktonic counterparts [1], making nosocomial infections a burden to the public health systems.

In order to prevent bacterial adhesion and subsequent biofilm formation or even kill harmful microbes, a wide range of biocides have been extensively used in healthcare settings. Although most of them show broad spectrum antimicrobial activity, their overuse, inappropriate selection, dosing and deficient time of application may be at the root of microbial resistance development [2]. A potential solution to overcome this problem may lie in the use of AMP.

AMP play a crucial role in the innate immune systems of most living organisms defending them against invading microorganisms. Several studies have focused on designing analogue peptides more active and stable than the natural AMP without causing harm to mammalian cells [3]. A way of optimizing these compounds include the synthesis of hybrid peptides containing portions of the amino acid sequences of two peptides with different mechanisms. Camel is a 15-residue hybrid peptide derived from the sequences of two insect peptides, cecropin A (isolated from the larvae of the silk moth *Hyalophora cecropia*) and melittin (isolated from honey bee venom). This hybrid peptide is more active than the native molecules and also lacks the undesirable haemolytic properties of melittin [4]. Some studies have reported promising *in vitro* activities of Camel and its analogues against anaerobic bacteria [5] and staphylococcal skin infections [6]. Another strategy to obtain effective AMP rely on the attachment of palmitic acid to the N terminus of positively charged short peptides, without activities against microorganisms. These so-called lipopetides are granted with a broad spectrum of potent antimicrobial activities and low levels of haemolytic activity [7, 8]. Another group of cationic antimicrobial lipopeptides that has been used as the last resource to fight multi-drug resistant Gram-negative strains are polymyxins [9]. Only polymyxins B and E (also called colistin) have been used in clinical practice [10]. Structurally, they consist of a seven-member cyclic ring of aminoacids with a tripeptide side chain bounded to a fatty acid chain. The two polymyxins have the same heptapeptide ring, with the exception of a single aminoacid, which is phenylalanine in polymyxin B and leucine in colistin [11]. Although effective, some concerns have been raised about the development of bacterial resistance and

toxicity towards these AMP [9] which may be overcome by their covalent immobilization onto a biomaterial surface.

The main goal of this study was to screen the antimicrobial activity of these aforementioned AMP in order to seek the most promising ones which can be later immobilized onto a biomaterial surface. For that, the susceptibility patterns of planktonic and sessile cultures of both Gram-positive and Gram-negative bacteria were determined.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

In this study, a reference strain of *P. aeruginosa* (ATCC 39324) and a clinical isolate of *S. aureus* were used as representative of Gram-negative and Gram-positive strains commonly associated to BAI. Bacteria were preserved and cultured as described in Chapter 2.

ANTIMICROBIAL PEPTIDES

A number of AMP were investigated in this study: polymyxin B and polymyxin E, Camel and Palm. Stock solutions were prepared in sterile UP water and were stored at -20 °C until being used.

PLANKTONIC SUSCEPTIBILITY PATTERNS

Planktonic susceptibility towards AMP was evaluated by determination of MIC and MBC as described in Chapter 2. Three independent assays with six replicates for each condition were performed.

BIOFILM SUSCEPTIBILITY TO AMP

Susceptibility patterns of sessile cultures towards AMP was evaluated by forming biofilms in the presence of increasing concentrations of AMP as described in Chapter 2. Biofilms were then characterized in terms of biomass through the CV staining method and determination of the

number of viable cells, also described in Chapter 2. Three independent assays with six replicates for each condition were performed.

RESULTS

SUSCEPTIBILITY PATTERNS OF PLANKTONIC CULTURES

The concentrations of peptides able to inhibit planktonic bacterial growth (MIC) and those required to kill planktonic (MBC) bacteria are summarised in Table 1.

Table 1. MIC and MBC of peptides against planktonic cultures of *P. aeruginosa* (ATCC 39324) and *S. aureus* (clinical isolate). MIC and MBC are expressed in µg/mL.

AMP	*P. aeruginosa*		*S. aureus*	
	MIC	MBC	MIC	MBC
PE	2	4	16	> 64
PB	2	4	8	> 64
Palm	64	64	32	64
Camel	16	32	2	8

Results showed that polymyxins were the most effective against *P. aeruginosa*, with lower concentrations required to inhibit its planktonic growth. A lower activity was found against *S. aureus* as polymyxins were not able to kill this strain even for the higher concentration tested. These results may be explained by polymyxins' mechanism of action as it involves cell membrane's disruption mainly by binding to the lipid A portion of LPS of Gram-negative bacteria [12]. The Gram-positive strain was more susceptible to Camel and Palm, especially to Camel, with lower concentrations required to prevent its planktonic growth.

Based on these susceptibility patterns, the potential of polymyxins to prevent the formation of *P. aeruginosa* biofilms and the potential of Camel and Palm against *S. aureus* biofilms were afterwards evaluated.

BIOFILM SUSCEPTIBILITY PATTERNS

In order to assess the antimicrobial effects of polymyxins B and E during biofilm development, biofilms of *P. aeruginosa* were allowed to growth for 24 h in the presence of increasing concentrations of these AMP.

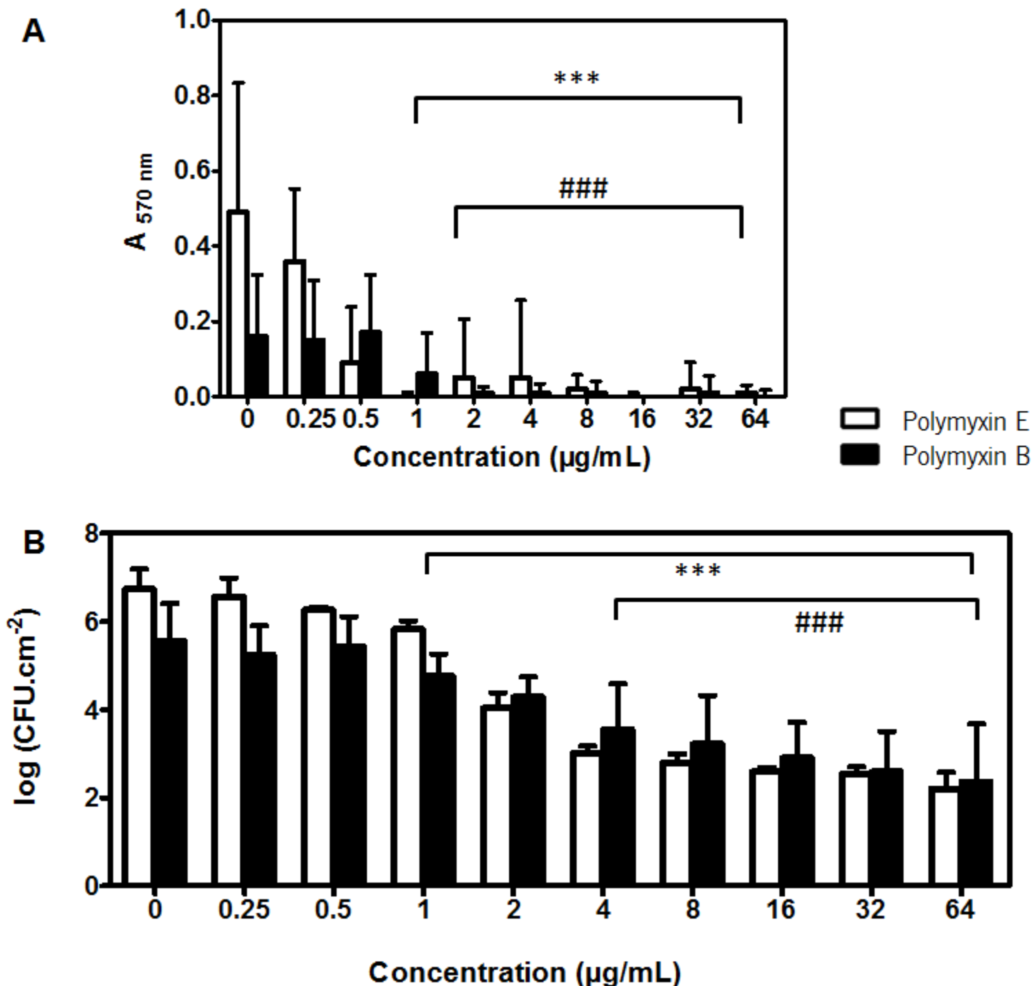

Figure 1. Biomass (A) and number of cultivable cells (B) of *P. aeruginosa* (ATCC 39324) biofilms developed in TSB supplemented with increasing concentrations of polymyxin B (black) or polymyxin E (white). Significant differences were found for PE (***) $p < 0.001$ and PB (###) $p < 0.001$, compared to biofilm formation in the absence of peptides (0 µg/mL).

Figure 1 shows that the presence of 1 µg/mL of both polymyxins (0.5x the MIC value) during biofilm development completely reduced the biofilm mass. Concerning the effect of polymyxins on biofilm entrapped cells, a reduction in a dose-dependent manner and a similar pattern was

observed. Polymyxin E was more efficient than polymyxin B as it was observed a 4.5 and 3.2 log reduction in the number of culturable cells, respectively, after biofilms development in the presence of a concentration of 64 μg/mL (32x the MIC value).

The potential of Camel and Palm to prevent the formation of *S. aureus* biofilms is presented in Figure 2.

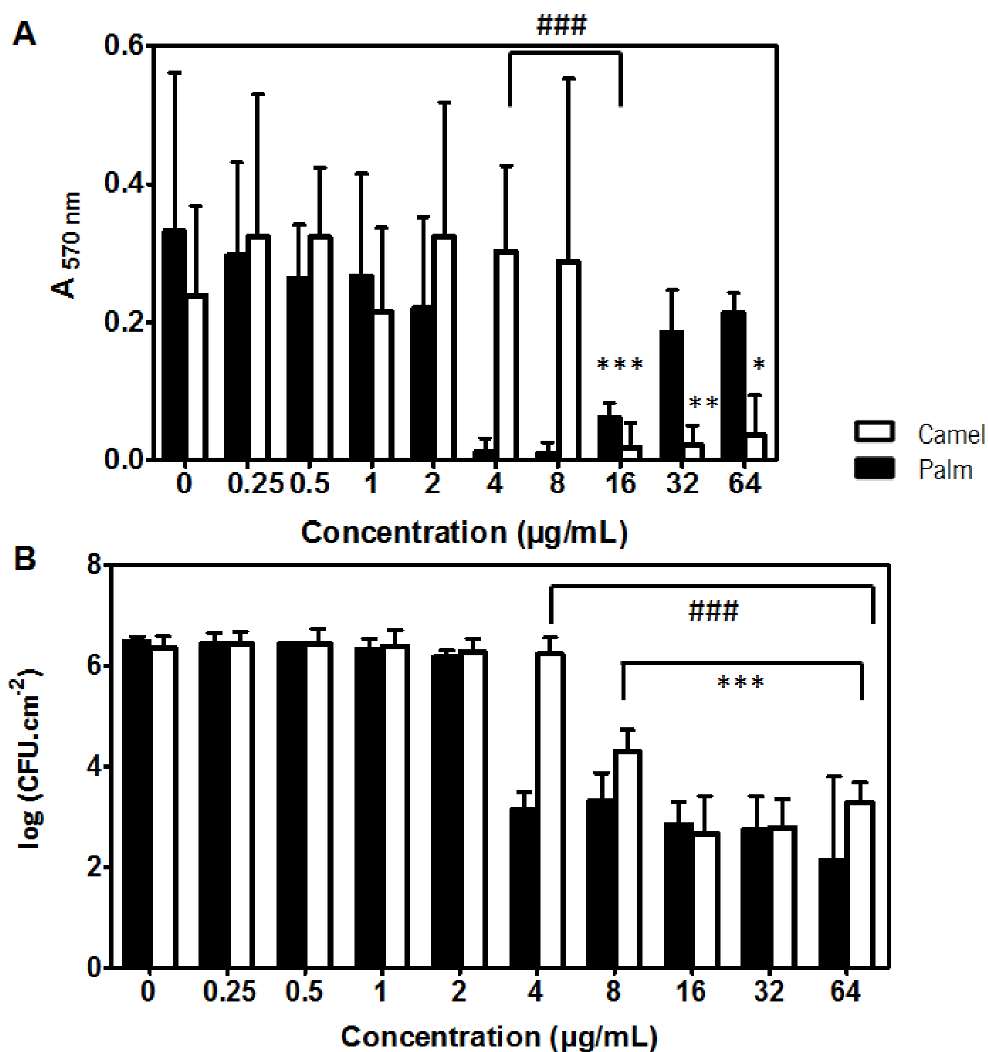

Figure 2. Biomass (A) and number of cultivable cells (B) of *S. aureus* (clinical isolate) biofilms developed in TSB supplemented with increasing concentrations of Camel (white) or Palm (black). Significant differences were found for Camel (***) $p < 0.001$ and Palm (###) $p < 0.001$, compared to biofilm formation in the absence of peptides (0 μg/mL).

Results showed that biofilm mass of *S. aureus* was affected by the presence of Camel from a concentration of 16 μg/mL (8x the MIC value). The presence of Palm in the early stages of biofilm formation, at a range of 4 to 16 μg/mL, reduced biofilm mass but had no significant

effect for higher concentrations. This pattern was not observed, however, on the number of viable cells which may be attributed to the strain phenotype in the absence of peptides. According to Stepanovic *et al.* [13], biofilms formed by this strain can be classified as a moderately adherent and therefore the CV staining method may not the best one to evaluate peptides ability to prevent biofilm formation as the amount of biofilm formed may not cross the limit detection of the method. In fact, no correlation between the number of viable cells and CV staining method has been previously demonstrated [14]. Results showed that biofilm establishment in the presence of Camel and Palm peptides was impaired from a concentration of 8 and 4 μg/mL, respectively, as shown by a decrease in the number of viable cells.

DISCUSSION

The increased prevalence of bacteria with resistance to conventional antibiotics associated to the fact that the number of new antimicrobials is declining, represent a serious worldwide problem [15,16]. In this scenario, AMP have been recognised as promising candidates as alternatives to antibiotics, due to their low toxicity, broad range of activity and unspecific mechanism of action [17]. This work aimed at determining the most promising AMP to afterwards be immobilized onto biomaterial surfaces in order to render them with antimicrobial features. For that, the *in vitro* susceptibility patterns of both planktonic and biofilm cultures involving *P. aeruginosa,* as a Gram-negative representative strain, and, *S. aureus,* as a Gram-positive one, were determined.

Results obtained for planktonic cultures (Table 1) allowed to conclude that polymyxins B and E were the most effective AMP against the Gram-negative strain while Camel and Palm required lower concentrations to inhibit *S. aureus* growth. The ability of these AMP to prevent biofilm formation using a prophylactic approach was then evaluated. Results showed that the same range of concentrations tested in planktonic studies was able to impair biofilm establishment of both strains, which highlights the potential of these antimicrobials as compared to conventional antibiotics. In general, the concentrations of antibiotics required to kill biofilm bacteria are much higher than their MIC values [1]. These effective low concentrations also suggest that toxicity issues should not be raised when using these compounds.

Polymyxins B and E greatly impaired biofilm formation of *P. aeruginosa* (Figure 1) which is in accordance to several studies reporting their potent *in vitro* activity against some multi-resistant Gram-negative pathogens. Most of these studies, however, were performed only with polymyxin E and its efficacy was mostly determined in pre-formed or established biofilms [18-20]. Although effective, some concerns have been raised about polymyxins development of bacterial resistance and toxicity [11,12]. Their immobilization onto a surface, the ultimate goal of the present thesis, may overcome these issues as it avoids patient exposure to sub-inhibitory concentrations.

Palm and Camel's ability to impair biofilm formation of *S. aureus* required higher concentrations than polymyxins against *P. aeruginosa*. However, similar and biological significant log reductions (higher than 3 log) could be achieved for the highest tested concentrations. These results are in accordance to a previous study where these peptides were very effective against staphylococcal strains isolated from skin infections. In that study, the safety of these AMP was also determined and it was concluded that Camel was not toxic at its MIC value, unlike Palm [6].

In conclusion, the overall results demonstrated the potential use of AMP in the early stages of biofilm development to impair its establishment. Unlike antibiotics, these compounds were able to compromise biofilm formation at similar range concentrations able to inhibit planktonic growth, highlighting their potential as candidates for the development of antimicrobial coatings for medical devices.

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3.2

Synergistic antimicrobial effect against P. aeruginosa biofilms: PE preconditioning surfaces plus antimicrobial treatment

Biofilm formation on medical devices is commonly associated with persistent infections. Once established, biofilms are less susceptible to antimicrobial treatment and to the host immune system which often results in implant devices surgical removal. The combination of biofilm preventive measures may be the best option to control such infections. This work aimed to investigate the potential of PE during the early stages of biofilm formation to impair *P. aeruginosa* biofilm establishment. Two strategies were used: pre-conditioning the adhesion surfaces with PE before biofilm formation and growing biofilms in its presence. The effect of treatment with CIP or PE on the 24-h-old *P. aeruginosa* biofilms formed on clean and PE-conditioned surfaces was further assessed. A *P. aeruginosa* reference strain and a clinical isolate were used as biofilm producers and biofilms were characterized in terms of biomass, respiratory activity and number of viable cells. Biofilm formation of both strains was significantly impaired when PE was used either as biofilm growth media complement or to randomly coat the adhesion surfaces before biofilm growth. Furthermore, random deposition of PE on the adhesion surfaces proved to increase biofilm susceptibility to CIP or PE treatment in terms of viable cells. Taken together, these data highlight a promising use of PE as a medical device coating agent and a synergistic effect between PE surface conditioning and antimicrobial treatment.

INTRODUCTION

Bacterial adhesion to surfaces and subsequent biofilm formation remains a serious threat in biomedical field when bacteria are able to reach the surface of medical devices or implants becoming the focus of persistent infections, called BAI [1, 2]. Biofilm formation is a crucial step in the pathogenesis of these infections [3], as bacterial cells within a biofilm encase themselves in a self-produced matrix of EPS [4] which confers them protection against antimicrobial treatments and the host immune system [5].

Biofilm formation on biomaterial surfaces is a developmental process which includes the following main steps: i) transport of bacterial cells to the surface and their initial and reversible adhesion, ii) irreversible attachment, iii) microcolony formation, iv) biofilm maturation and differentiation and v) detachment of individual bacteria or aggregates from the biofilm [6]. Bacterial adhesion [7] is mediated by specific and non-specific interactions between cell surface structures and molecular groups of the surface [8]. Prior to the attachment process, the surface is first covered with a layer of proteins and glycoproteins, the so called conditioning film. The conditioning film on the biomaterial surface changes the physicochemical properties of the surface so the affinity of an organism for a native or a conditioned surface can be greatly different depending on the molecules that constitute the conditioning film [9-11]. After adhesion to biomaterials, biofilm formation takes place by auto-aggregation of the attached cells within a self-produced matrix [4].

P. aeruginosa is the most common Gram-negative bacillus associated with BAI [12] and its emergence as a nosocomial pathogen is a growing concern [13]. Eradication of *P. aeruginosa* infections represents a serious challenge because of its ability to form strong biofilms, its intrinsic resistance to antibiotics [14] and its remarkable ability to develop resistance during antimicrobial treatment [15]. In fact, there has been a recent emergence of *P. aeruginosa* clinical isolates resistant to virtually all antibiotics [16]. The widespread emergence of multidrug-resistant Pseudomonas strains has led to the return of older antimicrobials such as polymyxins with acceptable efficacy and less toxicity than reported in the past [17].

PE, also known as colistin, belongs to an old class of cationic, cyclic AMP with significant *in vitro* activity against some multi-resistant Gram-negative pathogens, representing therefore, a promising treatment option for serious infections caused by *P. aeruginosa* [18]. Although a number of studies have assessed *in vitro* bactericidal activity of PE alone and combined with

other antimicrobials [19,20], PE efficacy was mostly determined in pre-formed or established biofilms [21]. As early bacterial adhesion is a crucial step in BAI pathogenesis, it was hypothesized that PE conditioning of medical devices surfaces could offer an efficient alternative to control *P. aeruginosa* infections. Moreover, its combination with antimicrobial treatment could act synergistically as an effective approach to prevent biofilm formation on medical devices.

The aim of this work was, therefore, to study the effect of PE in the early stages of biofilm formation by two *P. aeruginosa* strains. For that purpose, two different application strategies were first used: pre-conditioning the adhesion surfaces with PE prior to biofilm formation and growing biofilms in its presence. It was also evaluated if the combination of PE conditioning surfaces and antimicrobial treatment could act synergistically as an effective approach to control *P. aeruginosa* biofilms.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

P. aeruginosa reference strain (ATCC 10145) and a *P. aeruginosa* clinical isolate catalogued as U147016-1 were used throughout this study. The strains were preserved and cultures as described in Chapter 2.

ANTIMICROBIAL AGENTS

The AMP PE and the antibiotic CIP were used in this study. Stock solutions were prepared in sterile UP water and were stored at -20 °C until being used.

PLANKTONIC ANTIMICROBIAL SUSCEPTIBILITY

Planktonic susceptibility towards antimicrobials were evaluated by determination of the MIC and MBC as described in Chapter 2. Three independent assays with 4 replicates were performed.

BIOFILM INHIBITION ASSAYS

In order to determine the effect of PE in the early stages of biofilm formation, pre-conditioning and co-incubation experiments were performed as described in Chapter 2. Biofilms were then characterized in terms of biomass, metabolic activity and number of cultivable cells as also described in Chapter 2. Three independent assays with 4 replicates were performed.

BIOFILM TREATMENT WITH CIP OR PE

Biofilms formed on clean and pre-conditioned wells with PE (32 µg/mL) for 30 min were subjected subsequently to sudden treatment of CIP or PE at RT for different periods of time (30 min, 120 min and 240 min). For that, the supernatant content of each well was withdrawn and the wells washed with sterilised UP water. The wells attached biofilms were afterwards treated with CIP at 0.75 µg/mL (MBC) or PE at 4 µg/mL (MBC). Non-treated wells were filled with sterilised UP water for the same period of time. Two or three independent assays with 4-8 replicates were performed.

RESULTS

MIC AND MBC DETERMINATION ON PLANKTONIC CULTURES

The concentrations of PE and CIP able to inhibit planktonic bacteria growth (MIC) and those required to kill planktonic bacteria (MBC) are summarised in Table 1. Both antimicrobials were effective at low concentrations, however, different susceptibility patterns could be observed for the *P. aeruginosa* strains investigated in this study. The reference strain proved to be susceptible to both antimicrobial agents while the clinical isolate was considered resistant to CIP and susceptible to PE, according to CLSI criteria [22].

Table 1. MIC and MBC of CIP and PE against planktonic cultures of *P. aeruginosa* ATCC 10145 and clinical isolate U147016-1. MIC and MBC values are expressed in µg/mL.

P. aeruginosa strain	CIP		PE	
	MIC	MBC	MIC	MBC
ATCC 10145	0.1875	0.75	2	4
U147016-1	16	32	2	4

ANTIMICROBIAL EFFECT OF PE IN BIOFILM DEVELOPMENT

In order to assess the antimicrobial effects of PE during biofilm development, biofilms were allowed to grow for 24 h in the presence of increasing concentrations of PE. Figure 1 shows that the presence of 2 μg/mL of PE (the MIC value) during biofilm development completely reduced the biofilm mass and metabolic activity for both strains. Concerning the effect of PE on biofilm entrapped cells, it was observed a reduction in a dose-dependent manner for both strains investigated. PE proved to be more efficient against the reference strain as it was observed a 5 and 3 log reduction in the number of the reference and isolate culturable cells, respectively, after biofilms development in the presence of 64 μg/mL of PE (32x the MIC value).

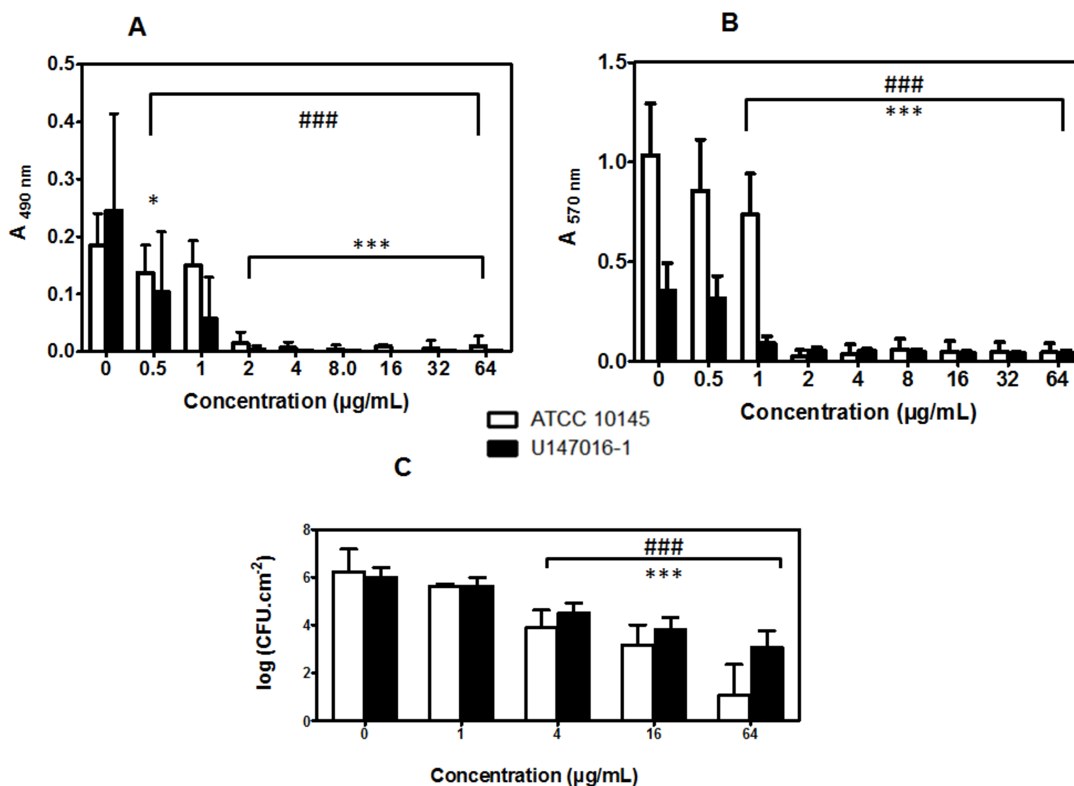

Figure 1. Metabolic activity (A), biomass (B) biofilm and number of cultivable cells (C) of *P. aeruginosa* ATCC 10145 (white) and *P. aeruginosa* clinical isolate U147016-1 (black) biofilms developed in TSB supplemented with increasing concentrations of PE. Significant differences were found for ATCC 10145 (***) $p < 0.001$ and U147016-1 (###) $p < 0.001$, compared to biofilm formation in the absence of peptides (0 μg/mL).

EFFECT OF PE SURFACE PRECONDITIONING BEFORE BIOFILM DEVELOPMENT

The effect of PE surface coating, evaluated at several concentrations, on the biomass, activity and culturable cells of biofilms formed by both *P. aeruginosa* strains are shown in Figure 2.

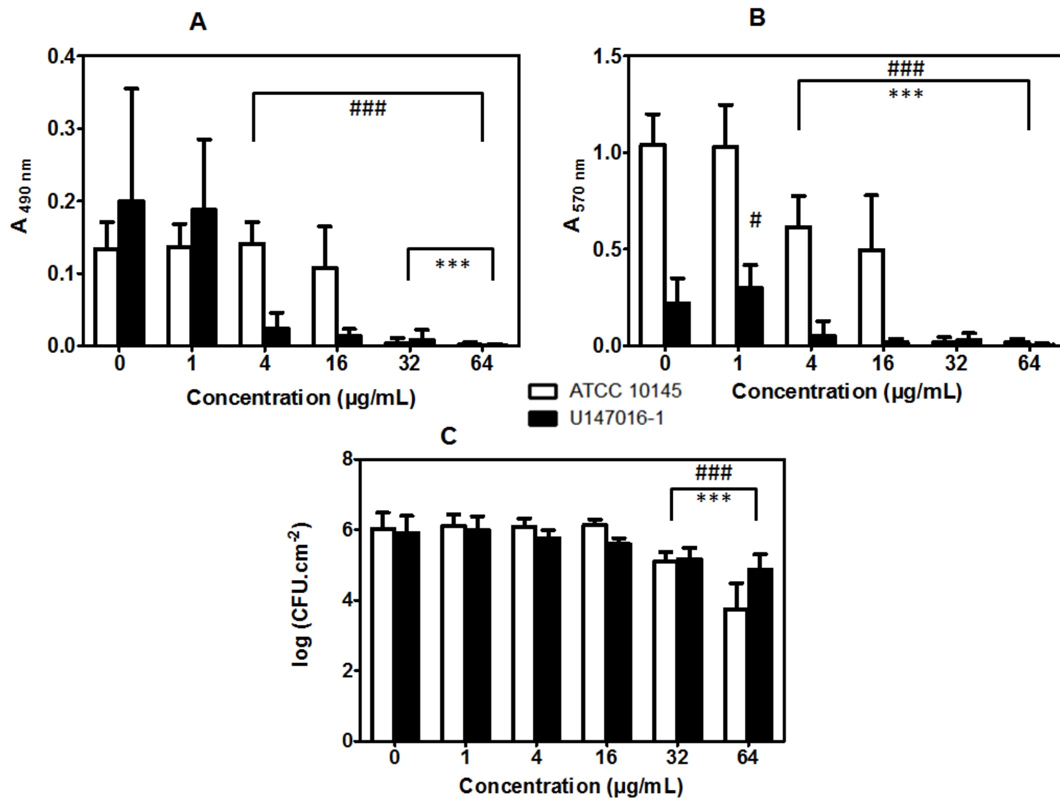

Figure 2. Metabolic activity (A), biomass (B) and number of cultivable cells (C) of *P. aeruginosa* ATCC 10145 (white) and *P. aeruginosa* clinical isolate U147016-1 (black) biofilms developed on surfaces pre-conditioned with increasing concentrations of PE. Significant differences were found for ATCC 10145 (***) $p < 0.001$ and U147016-1 (###) $p < 0.001$, compared to biofilm formation in the absence of peptides (0 μg/mL).

Figures 2 A and B show that, in general, random deposition of PE on the adhesion surfaces reduced biofilm activity and mass accumulated in a dose-dependent manner for both strains except for the activity of biofilms produced by the reference strain which were only inhibited from a PE concentration of 32 μg/mL ($p<0.001$). The presence of a PE conditioning film prepared at a concentration of 32 μg/mL caused a complete reduction of the respiratory activity and mass accumulated of the biofilms developed by both *P. aeruginosa* strains ($p<0.001$). Regarding the effect of PE on biofilm entrapped cells, the presence of the conditioning film only influenced

biofilm formation by both strains when it was prepared at PE concentrations of 32 μg/mL and 64 μg/mL ($p<0.001$), causing 2 log reduction in the reference strain and 1 log in the clinical isolate.

COMBINED EFFECT OF PE SURFACE PRECONDITIONING AND BIOFILM ANTIMICROBIAL TREATMENT

The combined effect of PE conditioning surfaces and antimicrobial treatment with CIP or PE on biofilms formed by the reference and clinical isolated *P. aeruginosa* strains are presented in Figures 3 and 4, respectively. A concentration of 32 μg/mL was chosen for PE conditioning as it proved to cause a reduction in the number of cultivable cells. For the treatment approach, antimicrobials were applied at their MBC.

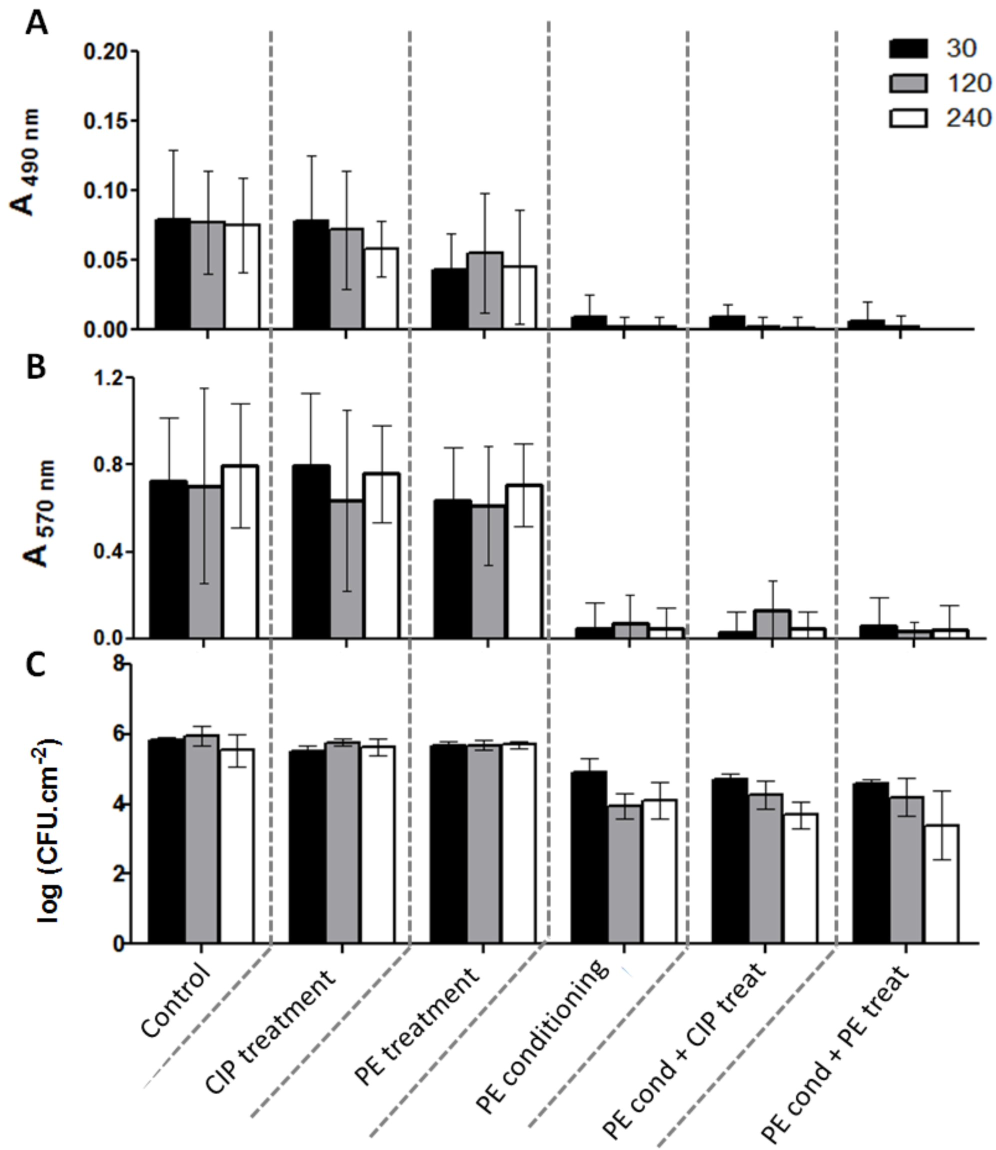

Figure 3. Metabolic activity (A), biomass (B) and number of cultivable cells (C) of *P. aeruginosa* ATCC 10145 24-h-old biofilms developed on PE-conditioned surfaces and treated with CIP or PE for 30 min (black), 120 min (grey) and 240 min (white).

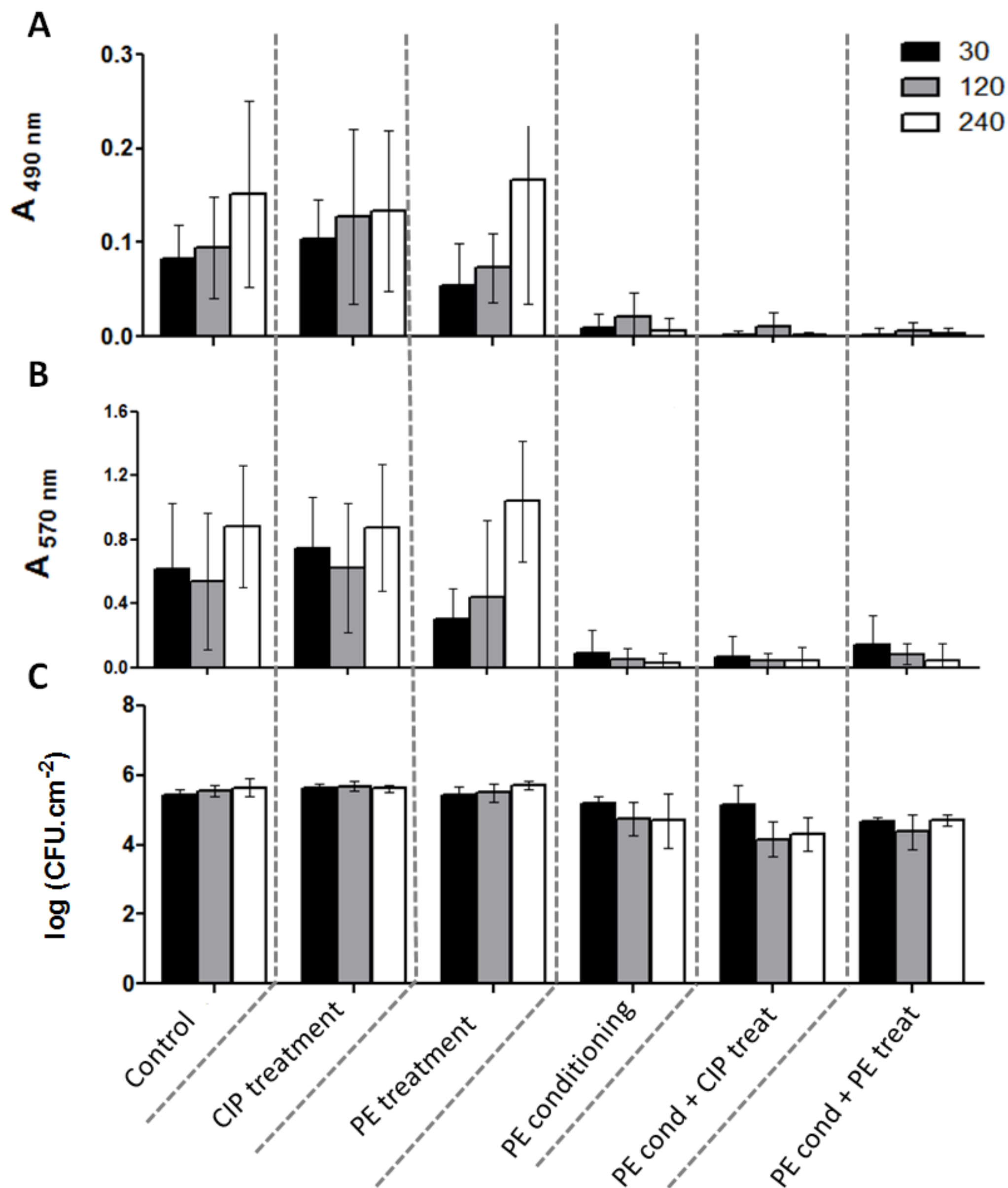

Figure 4. Metabolic activity (A), biomass ((B) and number of cultivable cells (C) of *P. aeruginosa* clinical isolate U147016-1 24-h-old biofilms developed on PE-conditioned surfaces and treated with CIP or PE for 30 min (black), 120 min (grey) and 240 min (white).

Figure 3 shows that, biofilms developed by the reference strain on clean surfaces and subjected to CIP treatment for a period of 30 min, revealed, slightly, a lower number of biofilm cells ($p<0.05$) but similar values of biomass and activity as biofilms not subjected to treatment ($p>0.05$). Treatment with PE, for the same period of time, reduced biofilm activity ($p<0.001$) and mass ($p<0.01$) but had no effect in the number of biofilm cells. Regarding the biofilms developed by the clinical isolate (Figure 4), it was possible to observe that CIP treatment had no influence on biofilm formation, in terms of mass, activity or biofilm entrapped cells. PE treatment reduced

biofilm activity ($p<0.05$) and mass ($p<0.001$) but had no effect in the number of biofilm cells. Surface conditioning with PE before biofilm development was very efficient, as it impaired significantly biofilm formation of both strains, especially in terms of mass and activity ($p<0.001$). It was observed that the reference strain and the clinical isolate had 0.9 log and 0.3 log reduction in the number of biofilm cells, respectively. Concerning the combined application of PE conditioning and biofilm treatment with CIP or PE, it was observed a reduction on biofilm mass and activity similar to the one revealed by biofilms formed on PE conditioned surfaces without being subjected to antimicrobials treatment. The combination of both strategies promoted a similar improvement on CIP and PE efficacy, causing a reduction of approximately 1 log in the cells of the biofilm formed by the reference strain. For the clinical isolate, the combination of both strategies proved to be more effective ($p<0.01$) when PE treatment was performed, causing a 0.7 log reduction.

Biofilms developed by both strains on clean surfaces and subjected to CIP or PE treatment for a higher period of time, 2 h, revealed similar values of mass, activity and biofilm entrapped cells as biofilms formed on clean surfaces and not subjected to the antimicrobial treatment ($p>0.05$). Biofilms of both strains that were formed on surfaces previously conditioned with PE revealed an accentuated reduction in biofilm mass and activity, and a reduction of 2.1 log and 0.8 log in biofilm cells formed by the reference strain and the clinical isolate, respectively. Regarding the combined application of PE conditioning and biofilm treatment with CIP or PE, it was observed, for both strains, the same sharp reduction on biofilm mass and activity as the one revealed by the biofilms formed on PE conditioned surfaces only. The combination of both strategies caused 1.7 log and 1.8 log reduction when biofilms of the reference strain were subjected to CIP and PE treatment, respectively. Regarding the clinical isolate, log reductions of 1.5 and 1.2 were observed when biofilms were subjected to, respectively, CIP and PE treatment.

Increasing the treatment period of biofilms formed on clean surfaces with PE or CIP to 4 h, showed that, in general, neither of the antimicrobials had influence on biofilm formation by both strains. PE conditioning of the surfaces before biofilm formation, on the other hand, caused a marked reduction in biofilm mass and activity and a reduction of 1.4 log and 0.9 log on biofilm cells formed by the reference strain and the clinical isolate, respectively. Biofilms formed, by both strains, on PE conditioned surfaces that were subjected to CIP or PE treatment revealed an accentuated reduction on biofilm mass and activity, similar to the one presented by biofilms formed on conditioned surfaces, only. The combination of both strategies had a significant effect

on biofilm entrapped cells of the reference strain, causing 1.8 and 2.1 log cell reductions when CIP or PE treatment was performed, respectively. As for the clinical isolate, the combination of both strategies had also effect on biofilm entrapped cells, causing a cell reduction of 1.3 log and 0.9 log when CIP or PE treatment was performed, respectively.

Figures 3 and 4 also show that treatment time was a factor that influenced biofilm formation. For the reference strain, no difference in the amount of biofilm mass and activity was observed with the treatment time. However, CIP or PE treatment of biofilms formed on PE conditioned surfaces increased the number of viable cells with the increase of treatment time. Interestingly, the efficacy of PE conditioning film without antimicrobial treatment was increased when biofilms were subjected to sterile water for 2 h or 4 h, when compared to the biofilms subjected for 30 min.

The biofilm formation by the clinical isolate was also affected by treatment time. Unlike the reference strain, increasing the period during which biofilms formed on clean surfaces were subjected to sterile water as a control, proved to enhance biofilm activity and mass for a period of 4 h, being more evident for biofilm activity. Regarding the number of biofilm entrapped cells, treatment time had only effect on biofilms formed on PE conditioned surfaces and subjected to 2 h of CIP treatment h, when compared to a treatment time of 30 min. Application of CIP or PE treatment, for 2 or 4 h, to biofilms formed on PE conditioned surfaces yielded similar values of biofilm entrapped cells. Similarly to the reference strain, the efficacy of PE conditioning film without antimicrobial treatment was increased when biofilms were subjected to sterile water for 2 h or 4 h, when compared to the biofilms subjected to a 30 min treatment.

From the results presented on Figures 3 and 4 it was possible to classify the antimicrobial effect obtained when both strategies, PE conditioning and antimicrobial treatment (with CIP or PE), were combined. The antimicrobial effect was classified as synergistic, additive, indifferent and antagonistic after comparing biofilm inhibitions regarding its biomass, metabolic activity and number of viable cells achieved when both strategies were compared with the theoretical sum that would be achieved taking into account the results obtained when the antimicrobial approaches were applied alone. The results obtained are summarised in Table 2 and an example of how this classification was applied is presented in Figure S1 of Supplemental Material.

Table 2. Antimicrobial effects on 24-h-old *P. aeruginosa* ATCC 10145 and U147016-1 biofilms formed on PE conditioned surfaces after CIP or PE treatment.

Biofilm characterization: biomass							
		CIP treatment			PE treatment		
Treatment time		*30 min*	*2h*	*4h*	*30 min*	*2h*	*4h*
Strain	ATCC10145	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent
	U147016-1	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent
Biofilm characterization: metabolic activity							
		CIP treatment			PE treatment		
Treatment time		*30 min*	*2h*	*4h*	*30 min*	*2h*	*4h*
Strain	ATCC10145	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent
	U147016-1	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent
Biofilm characterization: cell viability							
		CIP treatment			PE treatment		
Treatment time		*30 min*	*2h*	*4h*	*30 min*	*2h*	*4h*
Strain	ATCC10145	Additive	Indifferent	Indifferent	Indifferent	Indifferent	Synergism
	U147016-1	Indifferent	Indifferent	Indifferent	Synergism	Synergism	Synergism

Concerning biofilm mass and metabolic activity, data show that the combination of both strategies yielded the same biofilm reduction achieved by PE conditioning (the most effective approach when applied alone) for all the conditions tested. Regarding the reduction achieved in terms of viable cells, different antimicrobial effects could be observed. For the reference strain, when PE conditioning was combined with 30 min of CIP treatment a similar log reduction was achieved to the theoretical sum of both approaches applied individually (additive effect). Increasing the treatment period, the combination of both strategies yielded similar log reductions achieved by PE conditioning applied alone (indifferent effect). When the antimicrobial treatment was performed with PE instead, a synergistic effect was observed when a 4 h treatment was implemented. Regarding the clinical isolate, the combination of PE conditioning with CIP treatment always had an indifferent effect but when PE was applied, the combination of both strategies was able to achieve higher reductions on the number of viable cells than when the antimicrobial approaches were applied alone, for all the treatment periods.

DISCUSSION

Bacterial colonisation of indwelling devices followed by biofilm formation remains a serious concern in modern healthcare as it is commonly associated to persistent infections [23]. Bacterial adhesion is a crucial step in this colonisation process, representing, therefore, a promising target for the development of biofilm preventive measures. In this work, the presence of PE during the early stages of biofilm formation was able to impair biofilm establishment by a *P. aeruginosa* reference strain and a clinical isolate.

The presence of PE during biofilm growth was able to impair its development and, unlike most antibiotics, PE concentration able to inhibit planktonic bacteria (MIC) was also able to impair biofilms developed by both strains. Generally, antibiotic concentrations required to kill biofilm-encased bacteria are significantly higher when compared with their MIC because, once established, biofilms are often more difficult to eradicate [24]. Moreover, PE's MIC and MBC almost coincide (two-fold difference), which indicates that killing is generally bactericidal, a highly desirable mode of action. This remarkable anti-biofilm activity of PE makes it a promising coating agent for medical devices.

The random deposition of antimicrobials can alter the surface physicochemical properties and, therefore, promote or impair the subsequent bacterial adhesion. In this work, the presence of PE on the surfaces impaired biofilm formation, especially in terms of biofilm activity and mass accumulated. The presence of PE during biofilm formation may have interfered in the transition from reversible and initial adhesion to stable and irreversible interactions [25], disturbing transition from microcolonies to biofilms and thus delaying the mature biofilm development [26]. Other authors [27] have demonstrated the potential of Tachyplesin III as a coating agent to prevent bacterial adhesion to medical surfaces. Coating ureteral stents with this AMP was able to prevent biofilm formation *in vitro* and in a rat model of *P. aeruginosa* ureteral stent infection.

The surface conditioning with PE required higher concentrations to accomplish similar reductions in terms of biofilm mass and activity when this AMP was used as biofilm growth media complement, being also less efficient in the reduction of biofilm entrapped cells. A longer period of time was used to promote surface conditioning (2 h) but there were no statistically differences when compared to surfaces conditioned during 30 min only. In fact, according to Chmielewski and Frank [28], the adsorption of an organic layer onto a substratum can occur within seconds of exposure to an aqueous environment. The slightly decrease in PE efficacy may, thus, be related

to the adsorption process itself which can cause peptide aggregation and also an uneven peptide distribution along the surfaces.

Although surface conditioning with PE proved to impair biofilm activity and mass of both strains, the conditioning film still allowed the adhesion of a considerable number of cells during the 24 h of biofilm growth. Differences found for the different methods may be attributed to a low limit detection of both CV staining and XTT methods as aforementioned in Chapter 3.1. Based on the number of entrapped cells found, it was speculated that these cells could be more susceptible to antimicrobial action. To test this hypothesis, biofilms were allowed to grow for 24 h on PE conditioned surfaces and afterwards subjected to antimicrobial treatments for different periods of time. Antimicrobial treatment was performed with CIP, an antibiotic commonly prescribed to treat *P. aeruginosa* infections, and PE. Several studies have shown synergism between conventional antibiotics and AMP [29,30]. The combined use of these antimicrobials can reduce the dose and side effects, as well to prevent the development of bacterial resistance.

The preventive strategy (PE conditioning) proved to be more efficient than the prophylactic approach (CIP or PE treatment) confirming that biofilms are more difficult to eradicate once established. On the other hand, biofilms established on clean surfaces could not be, in general, impaired by CIP or PE treatment, for any of the periods of time investigated. PE has proved to be less effective on initial stage biofilms (24 h growth) than on mature biofilms [31] which could be explained by the fact that PE preferentially killed cells forming the core/stalk of the *P. aeruginosa* PAO1 mushroom structures, which were less active than the cap forming subpopulations [32]. So, the combination of PE with antibiotics with good bactericidal activity against strains in an active stage, such as CIP, may represent a promising choice to maintain PE efficacy against biofilm-associated infections.

The combined effect of a preventive strategy (PE conditioning) and a prophylactic one (CIP or PE treatment) could only be observed in terms of biofilm encased cells, which may be attributed to a lower limit detection of both CV staining and XTT methods as aforementioned. In general, biofilms formed by the reference strain on PE conditioned surfaces became more exposed to CIP or PE action. Antimicrobial action proved to be more effective for longer periods of time. The clinical isolate proved to be less susceptible than the reference strain which was expected as clinical isolates, frequently exposed to stress conditions in a hospital environment, can suffer a selection process that favours more pathogenic strains [33]. In this study, the effects were

classified based on a statistical analysis. However, it should be highlighted that from a biological point of view, 1.8 log cell reduction (the highest reduction achieved when combing both approaches) may fall short to achieve a successful therapy in clinical practice.

In conclusion, the overall results demonstrated the potential use of PE in the early stages of biofilm growth to impair its establishment. Moreover, and as a consequence of the reduced amount of biofilms attached to PE conditioned surfaces, adhered cells or thin biofilms became more exposed to the subsequent action of CIP or PE. It would be worthwhile to test higher concentrations of CIP and PE during biofilms treatments or even other antimicrobials, in order to achieve a complete eradication of biofilms formed on PE-conditioned surfaces. This study also pointed out that PE is a promising candidate for the development of an antimicrobial coating for medical devices. Although effective, some concerns have been raised about PE development of bacterial resistance and toxicity. PE immobilization onto a biomaterial surface may overcome these drawbacks as it avoids patient exposure to sub-inhibitory concentrations.

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SUPPLEMENTAL MATERIAL

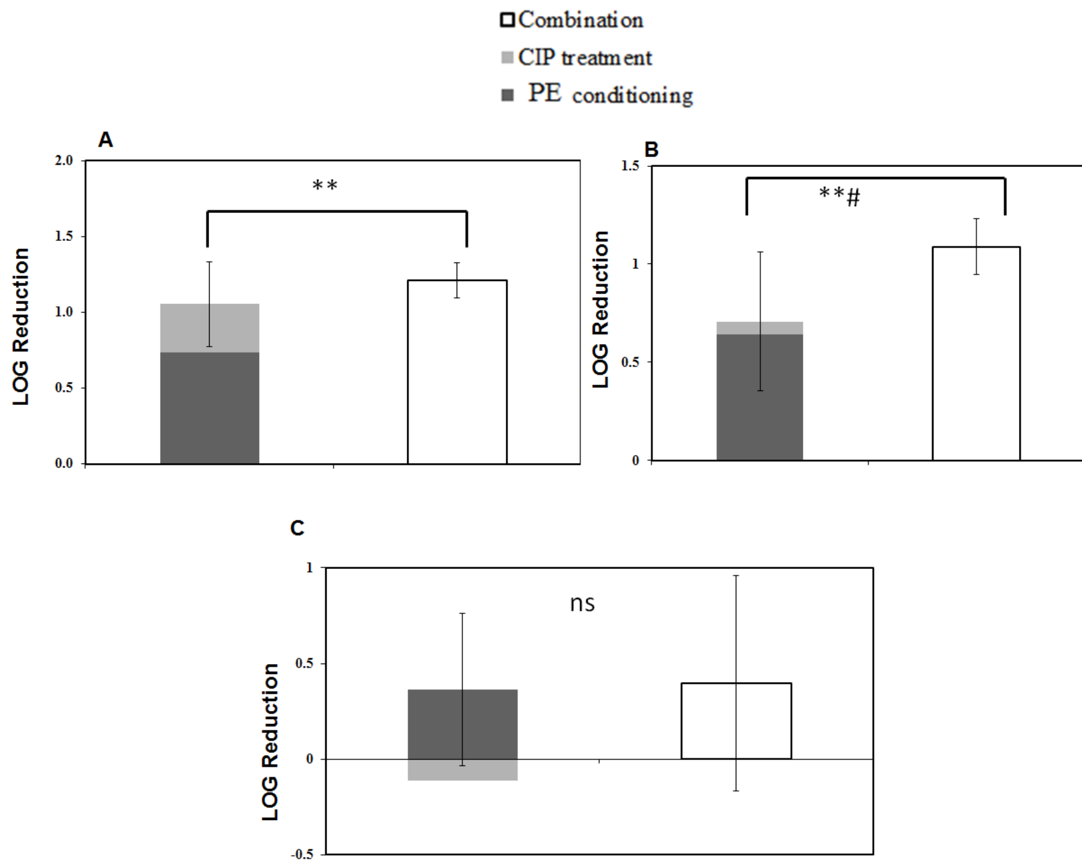

Figure S1. Representative additive (A), synergetic (B) and indifferent (C) effect between PE conditioning and CIP treatment.

3.3

Bio-inspired coating strategies for the immobilization of polymyxins to generate killing-contact surfaces

Microbial colonisation of indwelling devices and subsequent biofilm formation remain a major cause of morbidity and mortality in modern healthcare. The development of novel approaches to prevent BAI are, therefore, in great demand. This study aimed to immobilize two AMP (PB and PE) onto PDMS using two pDA-based approaches: the conventional 2-step method involving first the deposition of a pDA layer to which biomolecules are afterwards immobilized, and a 1-step method where peptides were dissolved together with dopamine before its polymerization. Surface characterization confirmed the immobilization of polymyxins onto PDMS at a non-toxic concentration. Immobilization of polymyxins using a 1-step pDA-based approach was the best method investigated as it was able to prevent *P. aeruginosa* adhesion and kill a significant fraction of the adherent ones, without causing harm to fibroblast cells. PE exhibited a better performance than PB as its immobilization onto PDMS imparted surfaces with antimicrobial properties regardless the immobilization approach used. In addition, cells that managed to adhere to these modified surfaces exhibited the same susceptibility pattern as cells adhered to unmodified surfaces, highlighting that resistance development towards polymyxins did not occur. The overall data suggest that PE functionalization using a 1-sep approach holds great potential as an additional antimicrobial functionality in the development of bi-functional coatings.

INTRODUCTION

Millions of lives are saved, every day in modern healthcare, thanks to the use of biomaterial implants and medical devices. Despite their crucial role in medicine progress, there are some drawbacks associated to their increased use as they all are prone to bacterial colonisation [1,2]. Bacterial adhesion to an indwelling device, followed by biofilm formation, is commonly associated to persistent infections and subsequently to tissue destruction, systemic dissemination of the pathogens and dysfunction of the device, resulting in serious illness and death [3]. BAI are extremely hard to treat because cells within a biofilm encase themselves in the self-produced polymeric matrix which confers them protection against antimicrobial treatment and host immune system [4,5]. The microorganisms most frequently isolated from BAI include the Gram-positive *S. aureus* and *S. epidermidis* and the Gram-negative *P. aeruginosa*. This last one stands out for its ability to form strong biofilms [6,7], intrinsic resistance to antibiotics [8] and remarkable ability to develop resistance during antimicrobial treatment [9].

The development of materials that can resist or prevent bacterial adhesion constitutes the most promising approach to deal with BAI problem and modern biomaterial science has provided several modification and activation strategies to impart biomaterials with antibacterial properties [10]. However, most of the current strategies, which are based on the immobilization of antimicrobial compounds, present some important limitations such as incomplete efficacy, toxicity and the development of bacterial resistance [11,12]. In the search for new compounds that can overcome such drawbacks, AMP have been recognized as promising candidates for the new generation of antimicrobial surfaces [13,14]. Polymyxins are a group of cationic antimicrobial lipopeptides that has been used as the last resort to fight multi-drug resistant *P. aeruginosa* strains [15]. Only polymyxins B and E have been used in clinical practice [16]. Although effective, some concerns have been raised about the development of bacterial resistance and toxicity towards these AMP [15]. The answer for these drawbacks may rely on their stable immobilization onto a biomaterial surface [17].

The aim of the current study was, thus, to immobilize PE and PB onto PDMS, commonly referred as silicone rubber, which has been widely used for implantable biomedical devices such as catheters or voice prostheses [18,19], using dopamine chemistry. Two pDA-based approaches were compared: the conventional 2-step method involving first the deposition of a pDA layer to which biomolecules are afterwards immobilized, and a 1-step method where compounds are

incorporated throughout the full thickness of the pDA film as they are dissolved together with dopamine before its polymerization [20].

MATERIALS AND METHODS

BACTERIAL STRAIN AND GROWTH CONDITIONS

A reference strain of *P. aeruginosa* (ATCC 39324) was used throughout this study. The strain was preserved and cultured as described in Chapter 2.

AMP AND ANTIMICROBIAL SUSCEPTIBILITY

In this work, two AMP were used: polymyxin B and polymyxin E. The MIC and MBC of peptides were determined by the microdilution method as described in Chapter 2.

POLYDOPAMINE COATING AND AMP IMMOBILIZATION

Prior to surface modification, PDMS coupons were cleaned and prepared as described in Chapter 2. Coatings were prepared following two pDA-based approaches as illustrated in Figure 2 of Chapter 2: a two and a 1-step immobilization. For peptides immobilization via the 2-step approach (Figure 2B), the first step involved the deposition of a pDA coating on PDMS coupons which was performed by immersing them in a solution of dopamine (2 mg/mL dopamine-HCl in 10 mM bicine buffer, pH 8.5) for 18 h, at RT and under agitation (70 rpm). Coupons were then rinsed with UP water. For further functionalization with peptides, pDA-coated coupons were immersed in PB or PE solutions (1 mg/mL, 2-step PB[1] or 2-step PE[1] and 5 mg/mL, 2-step PB[5] or 2-step PE[5], in 10 mM bicine buffer supplemented with 600 mM NaCl, pH 8.5) and were incubated for 2 h, at RT, under agitation (70 rpm). For the 1-step pDA-based immobilization (Figure 2A), dopamine (2 mg/mL) and polymyxins (1 mg/mL, 1-step PB[1] and 1-step PE[1]) were dissolved together in 10 mM bicine buffer solution (pH 8.5) and the PDMS coupons were immediately immersed in this solution. After overnight coating at RT and under agitation (70 rpm), the coupons were rinsed with UP water and air-dried for 45 min.

SURFACE CHARACTERIZATION

PDMS prior and after surface modification was characterized by SEM and by measuring water contact angles as described in Chapter 2. Experiments were performed in triplicate.

PEPTIDES IMMOBILIZATION EFFICIENCY AND COATINGS STABILITY

The efficiency of polymyxins immobilization was determined by quantifying the amount of unattached peptide in the buffer solution retrieved immediately after completing the coating process using fluorescamine assay as described in Chapter 2. Three independent assays with three replicates for each condition were performed. For testing coatings stability, the detachment of immobilized peptides was quantified by measuring the amount of release peptides also as described in Chapter 2. These experiments were performed twice with three replicates for each condition tested.

BACTERIAL CONTACT KILLING ASSAY

In order to evaluate bacterial contact-killing properties of PDMS surfaces functionalized with polymyxins, a previously reported method described in Chapter 2 was applied. Two independent assays with three replicates for each condition were performed.

BACTERIAL VIABILITY ON MODIFIED SURFACES

Antibacterial performance of the generated surfaces against adhesion for 4 h was evaluated using the live/dead staining method described in Chapter 2. Three independent assays with three replicates for each condition were performed.

SUSCEPTIBILITY PATTERN OF CELLS ADHERED TO MODIFIED SURFACES

The susceptibility pattern of bacterial cells adhered to PDMS surfaces was evaluated as described in Chapter 2 with some modifications. Briefly, a bacterial suspension with $1x10^8$ CFU/mL was prepared in PBS and 300 µL of this suspension were added to a 48-well microtiter plate in which PDMS, pDA and pDA functionalized with AMP were placed. The plate was incubated for 4 h at 37

°C and 120 rpm. Cells were recovered as described in Chapter 2 and used to determine the MIC and MBC against the same immobilized AMP. Two independent assays with three replicates for each condition were performed.

CYTOTOXICITY ASSAY

Cytotoxicity tests were performed using fibroblast cells 3T3 (CCL 163) obtained from ATCC. Cytotoxicity was evaluated by the MTS reduction assay as described in Chapter 2. Two independent assays with three replicates for each condition were performed.

RESULTS

POLYMYXINS IMMOBILIZATION ON PDMS MATERIAL

In this study, polymyxins B and E were immobilized onto PDMS and a pDA-based surface modification was applied for their immobilization using two different approaches (Figure 2 of Chapter 2). The 2-step approach (Figure 2B of Chapter 2) involved first the deposition of a uniform pDA coating from a dopamine-HCl solution at a slightly alkaline pH. During these incubation period in the dopamine solution, the color of the coupons gradually changed from transparent to dark brown (Figure S1 in Supplemental Material).The pDA coating was then used as a platform for polymyxins' immobilization due to the presence of residual quinones which present convenient sites for covalent grafting of nucleophilic groups such as amino functional groups found in AMP via Michael Addition and/or Shiff reactions. For 1-step pDA-based immobilization procedure (Figure 2A of Chapter 2), the PDMS coupons were immersed in one-pot mixture of dopamine and the polymyxin to be immobilized. Previous work has shown that this procedure not only simplifies immobilization of biomolecules even further but it also increased the total amount of immobilized compounds at surfaces [21]. To quantify the coating efficiency of peptides, the buffer solutions containing the unattached peptides were retrieved immediately after finishing the coating process and results are summarized in Table 1.

Table 1. Efficiency and stability of pDA-mediated immobilization of PE and PB. Stability was evaluated under physiologically relevant conditions (PBS at 37 °C) for 5 days. ND means not determined.

Method/ Polymyxin	Immobilized amount [%]					
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
1-step [PE1]	69.71 ± 4	69.21 ± 0.4	68.75 ± 0.2	68.37 ± 0.1	68.13 ± 0.2	68.06 ± 0.1
2-step [PE1]	39.87 ± 17	39.04 ± 0.5	38.83 ± 0.1	37.84 ± 0.8	37.53 ± 0.3	37.29 ± 0.3
2-step [PE5]	<0	ND				
1-step [PB1]	60.99 ± 4	60.84 ± 0.1	60.64 ± 0.2	60.35 ± 0.1	60.13 ± 0.2	60.13 ± 0.01
2-step [PB1]	30.12 ± 16	29.96 ± 0.1	29.72 ± 0.1	28.75 ± 0.3	28.53 ± 0.2	28.49 ± 0.1
2-step [PB5]	<0	ND				

The percentage of peptide immobilized on PDMS was dependent on the approach used for polymyxins functionalization. Using a 1-step approach, greater amounts of polymyxins B and E were immobilized (70 % and 61 %, respectively). However, taking into account that fluorescamine reacts with the primary amino groups found in the free amines on positively charged diaminobutyric acid (Dab) residue of polymyxins, as well as the amine groups found on dopamine molecule in solution, it is hard to distinguish between the amount of dopamine polymerized and polymyxins immobilized using this 1-step approach. Using the 2-step approach, this limitation was overcame as dopamine polymerization occurred before polymyxins immobilization. For a lower concentration of loading polymyxins B and E (1 mg/mL) results showed a lower immobilization percentage of around 40 % and 30 %, respectively. Increasing the loading concentration for 5 mg/mL, the value of fluorescence measured after coating process was higher than the one obtained for the loading solution, yielding a percentage of immobilization lower than zero. These results suggest that for a higher concentration, polymyxins may have interfered with the pDA coating, so that some amino groups present in this layer were removed from the PDMS surface, increasing, therefore, the content of amino groups detected by fluorescamine assay. In order to assess coatings stability, the detachment of immobilized polymyxins was quantified by measuring the amount of released polymyxin from the functionalized surfaces when incubated in PBS at 37 °C. Results confirmed coatings stability using both strategies as the polymyxins did not significantly detach from the surfaces for up to 5 days.

SURFACE MORPHOLOGY AND SURFACE WETTABILITY DETERMINATION

Surface morphology of unmodified PDMS and pDA-mediated modified surfaces was characterized using SEM analysis and are presented in Figure 1.

The unmodified PDMS exhibited smooth surface morphology compared with the modified ones. Self-polymerized pDA particles could be observed on modified PDMS coupons confirming the pDA coating. Further functionalization with polymyxins B or E yielded surfaces with different morphologies depending on the approach used. Results showed that 1-step approach for immobilization of both polymyxins yielded surfaces with a more homogeneous coating with agglomerates more evenly distributed along the surfaces. When PE was immobilized using the 2-step approach at a lower concentration, a similar morphology to the pDA coating alone was observed with smaller agglomerates. The increase of the loading concentration caused the formation of bigger agglomerates, heterogeneously distributed along the surface. For PB immobilization using the 2-step approach, the same agglomeration formation could be observed. For the lower concentration, PB immobilization seems to slightly increase the surface roughness, as compared to PE at the same concentration.

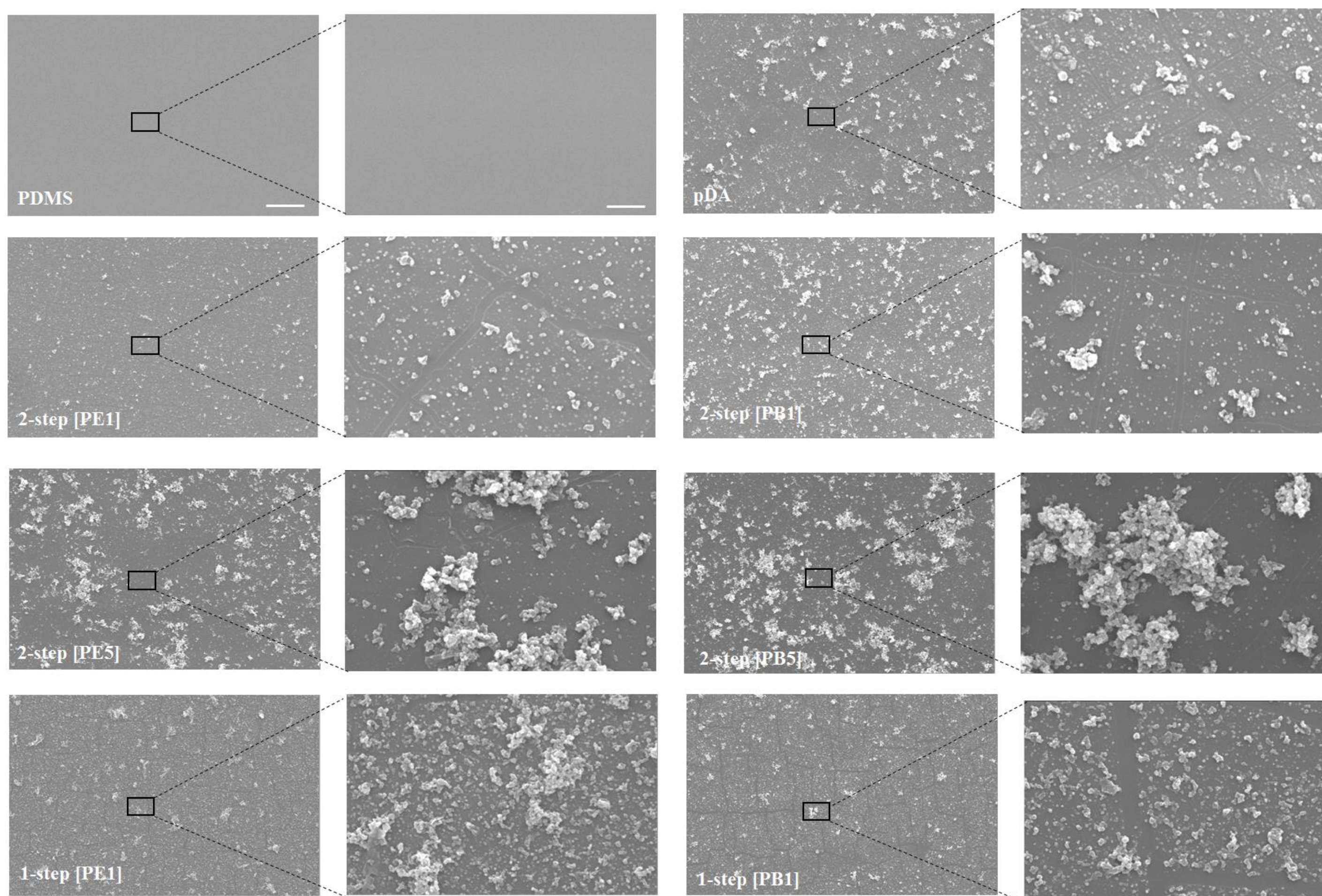

Figure 1. SEM images of unmodified PDMS, pDA-coated PDMS (pDA), pDA-coated PDMS surfaces with immobilized PE and PB via 2-step approach (2-step [PE] or 2-step [PB]) and 1-step approach (1-step [PE] and 1-step [PB]).The scale bars in the first and third column indicate 1 μm and the bar scale in the second and fourth column indicates 10 μm.

To evaluate the surface wettability of the PDMS after surface modification, the static water contact angle of the PDMS after each deposition step was measured (Figure 2).

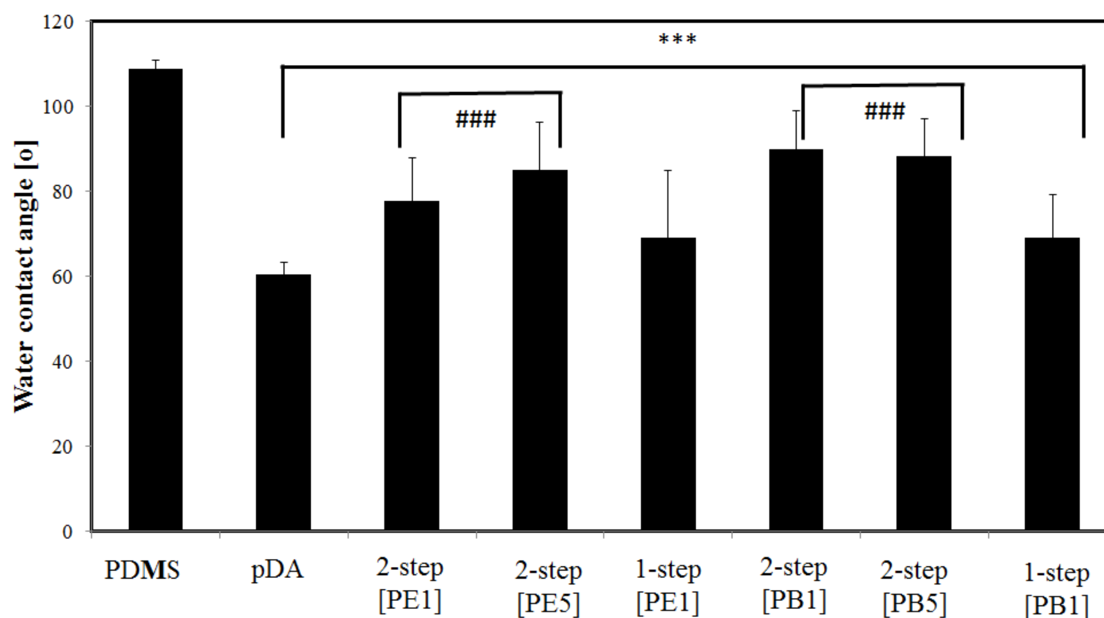

Figure 2. Measurement of the water contact angle of unmodified PDMS, pDA-coated PDMS (pDA), pDA-coated PDMS surfaces with immobilized PE and PB via 2-step approach (2-step [PE] or 2-step [PB]) and 1-step approach (1-step [PE] and 1-step [PB]). Significant differences were found for (***) $p < 0.001$, compared to PDMS control and (###) $p < 0.001$, compared to pDA control.

Bare PDMS surfaces exhibited a water contact angle of 109.9° ± 3.0°. Polydopamine coating decreased the contact angle of PDMS surface (56.6° ± 4.8°) indicating that the surface becomes more hydrophilic after pDA coating. Further immobilization with polymyxins B or E, using the 2-step approach immobilization, increased the water contact angle when compared to the PDMS with pDA coating alone. Increasing the concentration of polymyxins B or E from 1 mg/mL to 5 mg/mL had no significant effect on surface wettability. When polymyxins B or E were immobilized via 1-step approach, no significant interference was introduced to surface wettability, as compared to the pDA coating.

ANTIMICROBIAL AND ANTI-ADHESION PROPERTIES OF PDMS FUNCTIONALIZED WITH POLYMYXINS

Contact-killing of *P. aeruginosa* was evaluated by dropping a small volume of bacterial suspension on the surfaces of PDMS functionalized with polymyxins B or E for 24 h at 37 °C. Representative pictures of antimicrobial growth and contact-killing activity are presented in Figure S2 of Supplemental Material.

Table 2. Contact-killing activity of unmodified PDMS, pDA-coated PDMS (pDA), pDA-coated PDMS surfaces with immobilized PE and PB via 2-step approach (2-step [PE] or 2-step [PB]) and 1-step approach (1-step [PE] and 1-step [PB]). Visible growth was used as an indicator of contact-killing activity and it was tabulated as "+" for growth and "-" for no visible growth.

Condition tested	Visible Bacterial growth
PDMS	+
pDA	+
2-step [PE1]	-
2-step [PE5]	-
1-step [PE1]	-
2-step [PB1]	+
2-step [PB5]	-
1-step [PB1]	-

Table 2 shows that no contact-killing was observed for bare PDMS and after pDA coating. Further functionalization with PB yielded surfaces with bacterial contact-killing activity but only when a higher concentration of this peptide (5 mg/mL) was used during immobilization process. In turn, PDMS functionalized with PE exhibited bacterial contact-killing activity for both concentrations tested. When 1-step immobilization approach was applied, only PE exhibited contact-killing activity.

For further evaluation of the antimicrobial performance of functionalized PDMS surfaces, an attachment assay was also performed in which bacteria were allowed to attach for 4 h and the remaining cells on the PDMS coupons were imaged with fluorescence microscopy. It was possible to measure the remaining cells on the modified surfaces and simultaneously discriminate between live and dead cells, or more, specifically, evaluate bacterial membrane's integrity (Figures 3 and 4).

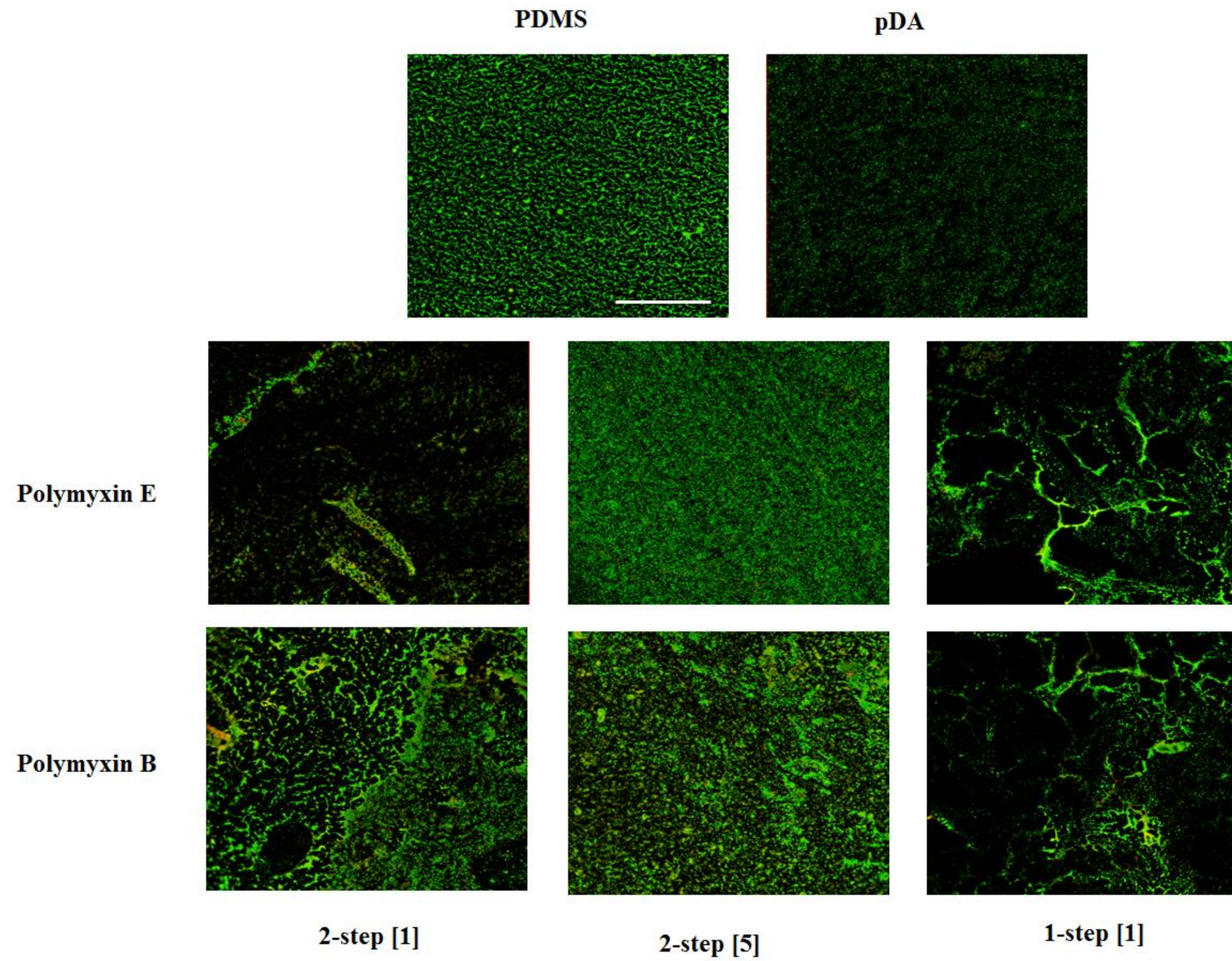

Figure 3. Representative fluorescent live/dead stain images obtained during *P. aeruginosa* attachment assays. The scale bar indicates 100 µm.

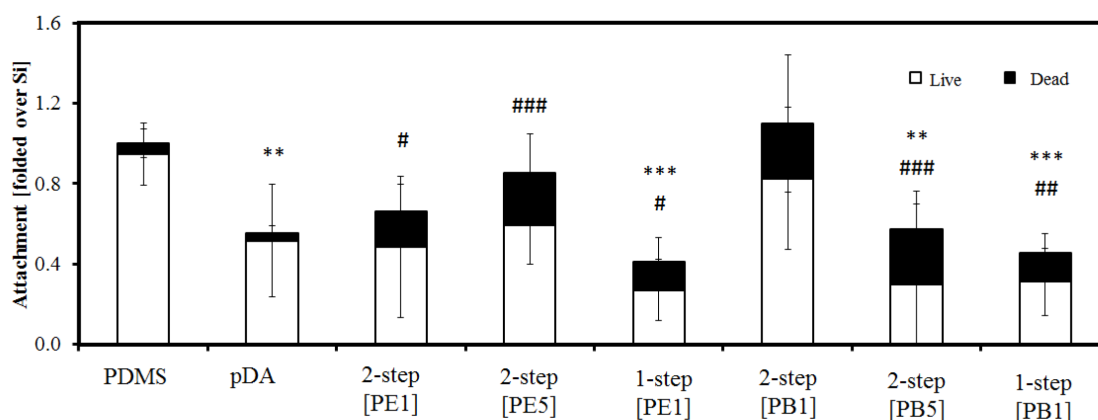

Figure 4. Normalized attachment of *P .aeruginosa* to unmodified PDMS, pDA-coated PDMS (pDA), pDA-coated PDMS surfaces with immobilized PE and PB via 2-step approach (2-step [PE] or 2-step [PB]) and 1-step approach (1-step [PE] and 1-step [PB]). All values were normalized to % coverage on PDMS control. Significant differences were found for (**) $p < 0.01$ and (***) $p < 0.001$, compared to PDMS control attachment and (##) $p < 0.01$ and (###) $p < 0.001$, compared to PDMS fraction of dead cells.

Unmodified PDMS material allowed the adhesion of *P. aeruginosa* cells and most of them remained alive. Polydopamine-coated surfaces slightly decreased the adhesion of this strain as compared to the unmodified PDMS but no significant antimicrobial effect was observed. PE immobilization via 2-step approach had no significant effect on bacterial attachment but was responsible for a higher fraction of dead cells. Increasing the concentration of PE during this 2-step approach immobilization, had no effect on anti-adhesive or antimicrobial properties of the coating. On the other hand, when PE was immobilized during dopamine polymerization (1-step approach), bacterial attachment was decreased to the same levels as the ones achieved by pDA coating alone but a higher fraction of dead cells could be found. For PB immobilization via 2-step approach, it was possible to conclude that the increase of the concentration enhanced the antimicrobial and anti-adhesive properties of the PDMS coupons. PB immobilization via 1-step approach yielded similar results to PE as it led to a reduction of bacterial attachment to the same level as the pDA coating alone and an increase of the fraction of dead cells.

SUSCEPTIBILITY OF CELLS ADHERED TO THE MODIFIED SURFACES

Although the resistance to polymyxins as well as to other AMP has been slower than to antibiotics [22], it has been showed that *P. aeruginosa* exposure to subinhibitory levels of PB and E induces resistance towards higher, and otherwise lethal, levels of these antimicrobials [23]. In order to evaluate if their covalent immobilization could overcame this issue, the potential development of

bacterial resistance toward these modified surfaces was assessed. In this assay, cells in contact with unmodified and modified PDMS surfaces were recovered and used to determine the MIC and MBC of polymyxins B and E (Table 3).

Table 3. Susceptibility (MIC and MBC) of adhered cells to unmodified PDMS pDA-coated PDMS (pDA), pDA-coated PDMS surfaces with immobilized PE and PB via 2-step approach (2-step [PE] or 2-step [PB]) and 1-step approach (1-step [PE] and 1-step [PB]).

Cells recovered from	MIC (µg/mL)	MBC (µg/mL)
PDMS	2	8
pDA	2	8
2-step [PE1]	2	8
2-step [PE5]	1	2
1-step [PE1]	2	4
2-step [PB1]	2	8
2-step [PB5]	2	8
1-step [PB1]	2	8

Results showed that cells adhered to PDMS functionalized with polymyxins B or E, using all the different approaches, exhibited the same or lower susceptibility pattern as cells adhered to PDMS or coated with pDA, suggesting no development of resistance during this period of time. The higher MBC found for adhered cells when compared to planktonic cultures was expected taking into consideration that adhered cells are inherently less susceptible than their planktonic counterparts [24].

EFFECT OF PDMS MODIFIED SURFACES ON FIBROBLAST GROWTH AND ADHESION

Besides the antibacterial performance of coating surfaces, the knowledge of their effect on the human cells is also crucial. Therefore, to predict the effects of the functional coatings developed in this study on mammalian cells, a cytotoxicity assay was performed (Figure 5). Results showed that further functionalization of pDA-coated PDMS surfaces with both polymyxins had no significant effect on 3T3 fibroblast metabolic activity.

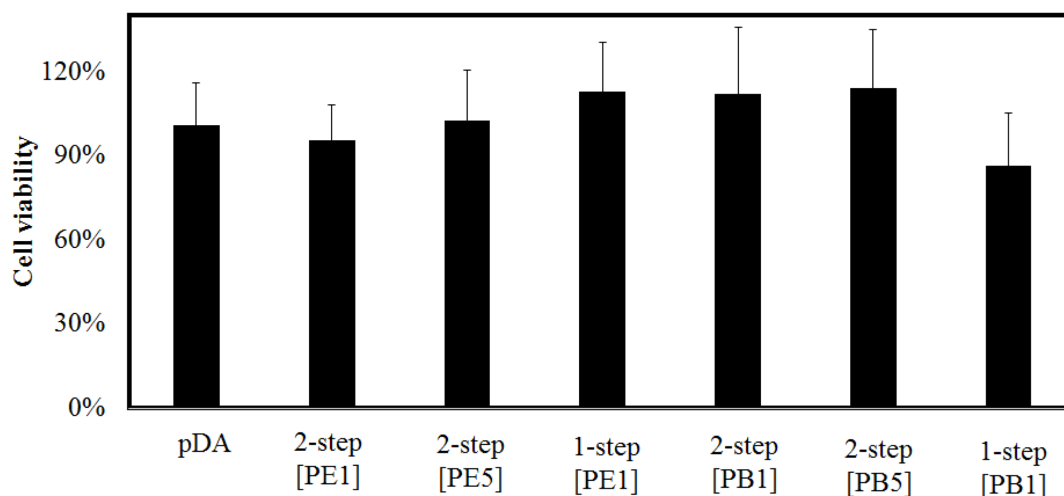

Figure 5. Viability of mammalian cells after 48 h of contact with to unmodified PDMS, pDA-coated PDMS (pDA), pDA-coated PDMS surfaces with immobilized PE and PB via 2-step approach (2-step [PE] or 2-step [PB]) and 1-step approach (1-step [PE] and 1-step [PB]), measured with an MTS assay. Significant differences were not found for $p > 0.05$ compared to pDA-coated PDMS surfaces (pDA).

DISCUSSION

With an ageing society, the problem of BAI is expected to increase in the coming years. When antimicrobial treatment fails, the removal of the infected implant may not completely solve the problem due to the remaining pathogen in the body, which is responsible for recurrent infections [1, 25]. Preventive approaches such as the modification of biomaterials to render them with antibacterial properties appear, therefore, as the best strategy to deal with these infections. In this study, pDA-mediated catechol functionalization was applied to render PDMS surfaces, a widely used biomaterial in clinical applications, with antimicrobial properties through the immobilization of two AMP: polymyxins B and E.

Polymyxins B and E have been used as the last resort to fight multi-drug resistant strains so there should be some caution in their widespread use to avoid the development of resistance which has already been reported [23, 26]. An alternative approach for their use that may minimize the potential development of microbial resistance as well as the toxicity toward mammalian cells relies on their covalent immobilization [17]. Polymyxins B and E share many similarities regarding their mechanism of action, antimicrobial spectrum, clinical uses and toxicity. However, they also differ in several aspects, including chemical structure, formulation, potency, dosage and pharmacokinetic properties [27]. Their mechanism of action involves the disruption of membrane's stability after their binding to the anionic part of the LPS of Gram-negative bacteria, which causes the leakage of intracellular components. Because of its wider global availability, most clinical studies have been focused on PE. However, some studies have suggested that the incidence of nephrotoxic effects is higher with colistinmethate (the inactive form of PE) than with PB [28, 29].

Polydopamine-mediated immobilization of PE onto PDMS generated surfaces able to kill adhering *P. aeruginosa* bacteria upon contact, regardless the immobilization approach applied. Such antimicrobial activity was not, however, as pronounced when bacteria were allowed to adhere to the modified surfaces from a liquid phase, as bacteria were able to adhere and a significant fraction were still alive. Membrane damage, which was confirmed by the fraction of dead cells, was mainly observed when PE was immobilized via a 2-step approach. Increasing the concentration of PE did not improve the antimicrobial properties of the coating and slightly increased bacterial attachment, which may be attributed to the higher surface roughness observed in these surfaces. Moreover, the method used to quantify the amount of PE immobilized suggested that increasing its concentration had some interference with pDA coating. Increasing peptide concentration, increased the amount of amine groups that may have reacted with dopamine aggregates via noncovalent interactions [30]. Therefore, it is reasonable to expect some changes in coating stability under aqueous conditions. However, in this study the PDMS functionalized with polymyxins using a loading concentration of 5 mg/mL retained its antimicrobial functionality, which is a sign that polymyxins were still grafted onto surfaces although some stability disturbance may have occurred.

PE immobilization during dopamine polymerization, on the other hand, caused less membrane damage but was able to prevent bacterial attachment at some extent, which may be attributed to

a more efficient immobilization. The 1-step approach involves self-polymerization of dopamine in the presence of compounds to be immobilized, hence leading to homogeneous mixing of covalently linked compounds throughout the layer of pDA [20] and surface characterization confirmed a more homogeneous coating. Moreover, while the amount of immobilized compounds via 2-step approach is limited as the amount of reactive quinone groups that can react is limited to the surface of the outer surface, it is expected that biomolecules incorporation using 1-step approach occurs throughout the full thickness of the pDA layer than only at its outer surface [21].

PB immobilization onto PDMS yielded antimicrobial coatings less efficient against *P. aeruginosa* when compared to PE. A higher concentration of PB was needed to obtain coatings able to kill adhering bacteria upon contact as well as to prevent bacteria adhering from a liquid phase. The lower efficacy of PB at a concentration of 1 mg/mL as compared to PE may be explained by the presence of more agglomerates which results in a higher surface roughness. Increasing the concentration of PB using the 2-step approach also resulted in the formation of larger agglomerates. Similar results were obtained to PE immobilization, when PB was co-dissolved with dopamine as it caused less membrane damage but was able to prevent bacterial attachment at some extent.

To investigate the anti-adhesive performance of coatings functionalized with polymyxins, cells were allowed to adhere to their surfaces for 4 h. This period of time was chosen because the first 6 h after surgery (the so-called "decisive period") are identified as being critical for preventing bacterial adhesion in order to ensure the long-term success of the implant [31]. During this period of time, there is a competition between integration of the material into the surrounding tissue and adhesion of bacteria to the implant surface [32].

Bacteria are well known for their ability to adapt in response to their environment, and indeed the development of resistance to polymyxins by *P. aeruginosa* strains has already been reported [26]. Results showed that, for all the immobilization approaches investigated, some viable cells could be found on the modified surfaces. To infer if their presence could be attributed to some development of resistance towards polymyxins immobilized on the surface, an assay was performed in which cells in contact with unmodified and modified surfaces were used to determine the MIC and MBC of polymyxins used to functionalize PDMS. Results showed that cells adhered to PDMS functionalized with both polymyxins exhibited the same or lower susceptibility pattern as cells adhered to PDMS unmodified and coated with pDA, suggesting no development

of resistance. This first evidence is very important and promising, however, it should be taken into consideration that 4 h of adhesion may not be a sufficient period of time to conclude about resistance development. To strengthen the non-appearance of resistance, further studies should be performed where cells in contact with modified and unmodified surfaces should be continuously recovered and allowed to adhere to new samples during a longer period of time.

Another important concern associated to the use of polymyxins is their toxicity [16]. It should be emphasized that, although in the present study, a higher concentration of polymyxins (1 mg/mL and 5 mg/mL) was used for their immobilization, a much lower concentration was actually immobilized on the surfaces (about 40 %). Moreover, the effects of coatings functionalized with these peptides were evaluated on fibroblast cells and results showed that their presence caused no harm to these cells which may be attributed to their covalent immobilization without leaching.

The overall results suggested that immobilization of PE using a 1-step pDA-based strategy may be a useful added functionality in the development of bi-functional coatings composed by anti-adhesive compounds, such as the ones developed with polymer brushes [33] or enzymes targeting EPS [34].

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SUPPLEMENTAL MATERIAL

Figure S1. Polydimethylsiloxane samples before and after pDA deposition.

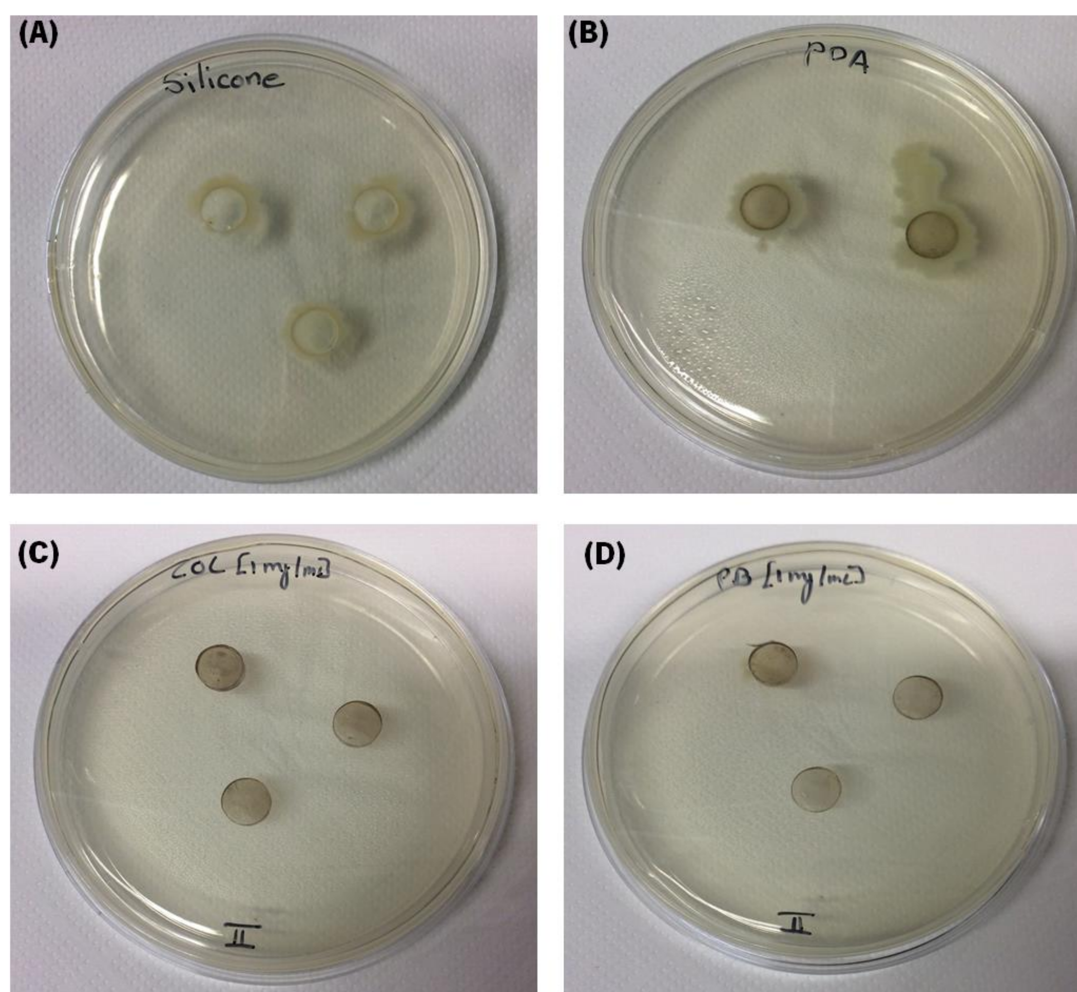

Figure S2. Representative pictures of contact-killing assay. Bacterial growth can be observed on TSA plates containing bare PDMS (A) and pDA (B) and no growth was visible for pDA-coated PDMS further functionalized with colistin or polymyxin E (C) and polymyxin B (D).

3.4

Characterization and biological activity of surface-tethered Palm and Camel

Recent studies have focused on the immobilization of AMP to render the surfaces with antimicrobial properties. A crucial factor for AMP potential as antibacterial-coating agents is the retention of antimicrobial activity after their immobilization. In this chapter, the 2-step approach pDA-mediated was explored to tether the peptides Camel and Palm onto PDMS materials. Different modifications were introduced to these peptides in an attempt to enhance their antimicrobial activities after immobilization. Results showed that only Palm was able to retain its antimicrobial activity once immobilized, causing membrane damages to adhered cells, mainly the Gram-positive strain tested. Camel immobilization was not succeeded even when a linker was introduced which may be attributed to its native mechanism of action in solution which involves the formation of pores. In conclusion, Palm exhibited great potential to be further used in the design of bi-functional coatings and this chapter also highlights the complex interplay of immobilization parameters to assure peptides activity is retained.

INTRODUCTION

In the past years, a number of studies have highlighted the potential of AMP of the innate immune system and their synthetic derivatives as alternatives to conventional antibiotics [1,2]. Although their therapeutic potential as antibiotics has been already established [3], some issues concerning their toxicity and lack of stability *in vivo* have limited their clinical use. Recent studies have focused, therefore, on several approaches for AMP immobilization onto a number of surfaces in an attempt to confine and maintain their activity while minimize their toxicity [4 5].

A crucial factor for AMP potential is the retention of antimicrobial activity after their immobilization. In fact, most of the reported studies on AMP immobilization have found that the activity of bound peptides is lower as compared to that of their soluble counterparts [5-9]. Several parameters such as peptide surface concentration, the spacer (length and flexibility) or peptide orientation should be taken into account for developing efficient and long-lasting antimicrobial coatings [10]. For instance, the presence of a spacer may be important for enabling peptide insertion into the cell and thus membrane permeabilization, leading to cell death. PEG with different lengths has been commonly used as linkers during the preparation of surfaces functionalized with peptides [8,11]. The reason for its wide usage relies on the fact that this polymer puts together a number of promising characteristics for clinical applications, namely its solubility in water, lack of toxicity, excellent biocompatibility and simple elimination from living organisms [12]. Furthermore, it may be an advantage for the coverage of surfaces due to its anti-fouling properties towards proteins and cells [13].

This chapter aimed to optimize the immobilization of the peptides Camel and Palm onto PDMS materials and evaluate the influence of two different spacers on their immobilization.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

A reference strain of *P. aeruginosa* (ATCC 39324) and a clinical isolate of *S. aureus* were used throughout this study. The strains were preserved and cultured as described in Chapter 2.

AMP, PEPTIDE SYNTHESIS AND MODIFICATIONS

In this work, two peptides were used: Palm and Camel. Peptides were synthesized manually by solid-phase synthesis method as described in Chapter 2. The terminal residues of Camel were modified with cysteine (C), lysine (K) and two glycine residues (GG) as previously described [14]. The peptide modified was called Camel-CKGG. The C-terminal of Palm peptide was modified with cysteine (C) to which poly ethylene glycol (PEG) was introduced. The peptide modified was called Palm-PEG.

ANTIMICROBIAL SUSCEPTIBILITY OF PEPTIDES

MIC and MBC of peptides with and without modifications were determined by the microdilution method as described in Chapter 2.

POLYDOPAMINE COATING AND PEPTIDES FUNCTIONALIZATION

Prior to surface modification, PDMS coupons were cleaned and prepared as described in Chapter 2. For pDA coating, materials were immersed in dopamine (2 mg/mL dopamine-HCl in 10 mM bicine buffer, pH 8.5) for 18 h, at RT under agitation. Coupons were then rinsed with UP water and air-dried. For further functionalization, pDA-coated substrates were immersed in peptides solutions (1 mg/mL dissolved in PBS, pH 7.4 or bicine buffer, pH 8.5) for 6 h. After coating at RT, under agitation (70 rpm), the coupons were taken and rinsed with UP water and air-dried.

PEPTIDES IMMOBILIZATION EFFICIENCY

The efficiency of peptides immobilization was determined by quantifying the amount of unattached peptides in the buffer solution retrieved immediately after completing the coating process. The peptide concentration was measured by using a fluorescamine assay as described in Chapter 2. Three independents assays with three replicates for each condition were performed.

BACTERIAL CONTACT KILLING ASSAY

In order to determine whether the AMP retained their antimicrobial activity after their immobilization, a contact-killing assay was performed as described in Chapter 2. Two independent assays with three replicates for each condition were performed.

BACTERIAL VIABILITY ON MODIFIED SURFACES

Antibacterial performance of the generated surfaces against bacterial adhesion was also evaluated by fluorescence microscopy, after live/dead staining as described in Chapter 2. Three independent assays with three replicates for each condition were performed.

RESULTS

OPTIMIZATION OF AMP IMMOBILIZATION

A preliminary optimization of the surface modification method was performed, regarding the buffer pH in which AMP were allowed to immobilize to pDA-coated PDMS surfaces. Two different buffers, PBS (pH 7.4) and bicine (pH 8.5), were tested and AMP immobilization was performed as illustrated in Figure 2B of Chapter 2, using a 2-step immobilization approach.

To confirm peptides antimicrobial activity, a contact-killing assay was performed in which a small volume of bacterial suspension was dropped on the surfaces functionalized with AMP. After 24 h of contact, coupons were transferred to TSA plates and antimicrobial activity was evaluated based on their ability to prevent any bacterial growth. Bacterial growth was tabulated as "+"and no visible growth as "-" (Table 1). As positive controls, growth was observed on bare PDMS and pDA-coated PDMS. The Gram-positive clinical isolate of *S. aureus* was used in this study given its higher antimicrobial susceptibility towards these AMP (Chapter 3.1). Results suggested that only Palm retained its antimicrobial activity when immobilized using a neutral pH of 7.4. For further assays, AMP were then immobilized on PBS.

Table 1. Contact-killing activity of pDA-coated PDMS surfaces with immobilized Palm and Camel using different buffers. Visible growth was used as an indicator of contact-killing activity and it was tabulated as "+" for bacterial growth and "-"for no visible growth.

AMP	Bacterial growth	
	PBS	Bicine
Palm	–	+
Camel	+	+

BACTERIAL VIABILITY ON MODIFIED SURFACES

For further evaluation of the antimicrobial performance of these surfaces, an attachment assay was also performed in which bacteria were allowed to attach for 4 h and the remaining cells on the substrates were imaged with fluorescence microscopy. It was possible to measure the remaining cells on the modified surfaces and simultaneously discriminate between live and dead cells, or more, specifically, evaluate bacterial membrane's integrity.

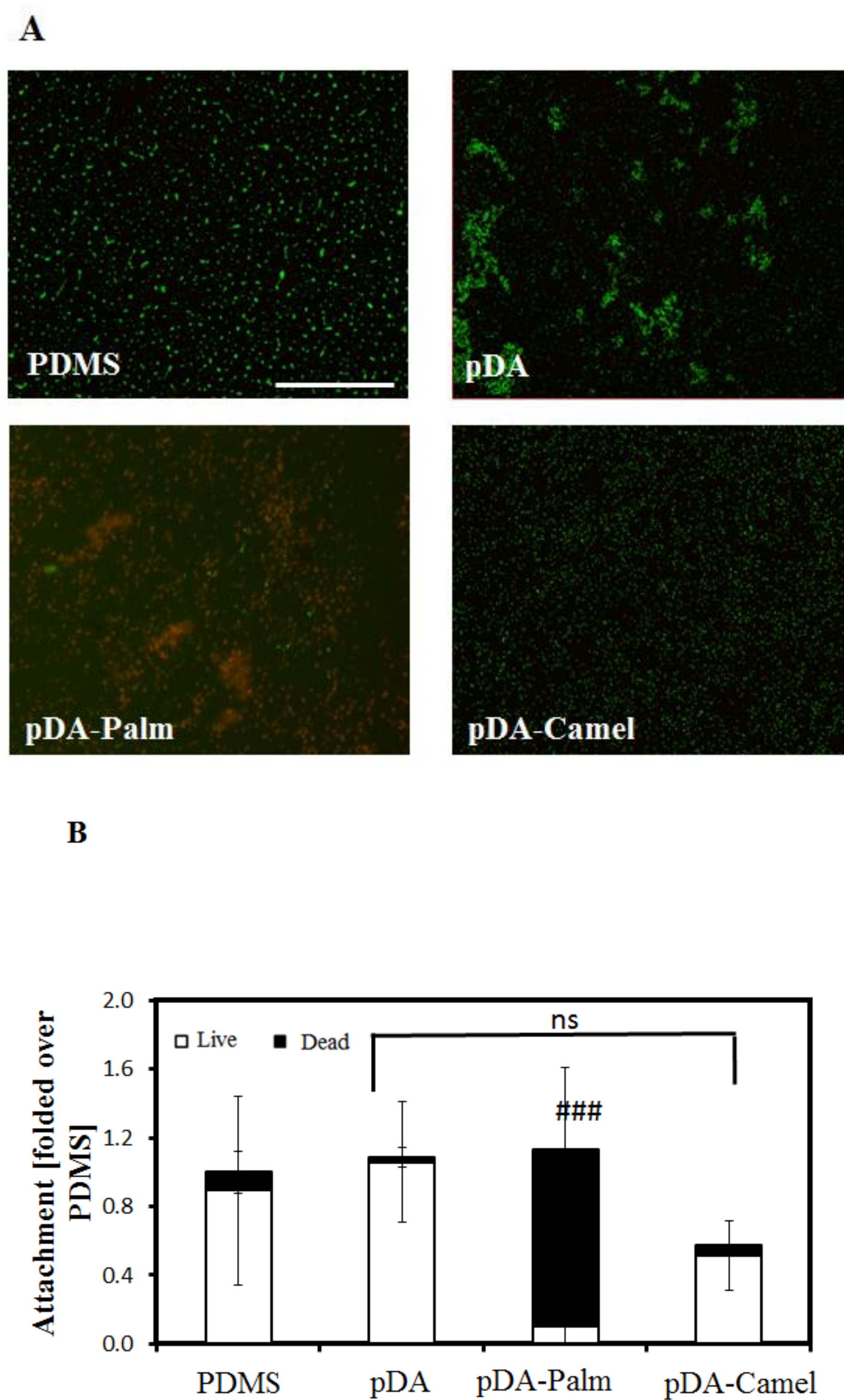

Figure 1. (A) Representative fluorescent live/dead stain images obtained during adhesion assays of *S. aureus.* The scale bar indicates 100 μm. (B) Normalized attachment and viability of cells on unmodified PDMS, pDA-coated PDMS (pDA), and pDA-coated PDMS functionalized with PALM (pDA-Palm) and Camel (pDA-Camel). All values were normalized to PDMS control. No significant differences were found for (*) $p > 0.05$, compared to PDMS control attachment but significant differences were found (###) $p < 0.001$, compared to PDMS fraction of dead cells.

As shown in Figure 1, *S. aureus* was able to adhere to bare PDMS as well as to the pDA coating, without compromising bacterial viability. Further immobilization with both AMP had no significant effect on bacterial attachment. Camel had no significant effect on bacterial viability while Palm functionalization yielded the surfaces with the best antimicrobial performance (approximately 90 % of dead cells). Comparing to the Palm peptide, Camel has a larger sequence, and therefore, a higher chance to undergo folding or self-assembly, which may had interfered with efficient binding of the peptide to the pDA layer. To confirm this hypothesis, the immobilization efficiency of both AMP were determined by retrieving the loaded and unattached peptides during the coating process.

AMP IMMOBILIZATION EFFICIENCY

Results in Table 2 revealed that more than 60 % of loaded peptide Palm was immobilized onto the pDA-coated PDMS surface. On the other hand, for Camel, the value of fluorescence measured after the coating process was higher than the one obtained for the loading solution. These results suggest that Camel may have interfered with the pDA coating, so that some amino groups present in this layer were removed from the PDMS surface, increasing, therefore, the content of amino groups detected by fluorescamine assay.

Table 2. Efficiency of pDA-mediated immobilization of Palm and Camel peptides.

AMP	Immobilization efficiency [%]
Palm	65.91 ± 2.29
Camel	< 0

AMP MODIFICATIONS

In order to improve Camel immobilization mediated by pDA, it was modified with cysteine (C) and lysine (K) to introduce thiol and amino groups and two glycine residues (GG) as a flexible linker. Regarding Palm peptide, since it exhibited great antimicrobial properties against the model strain investigated, PEG was added in order to introduce anti-fouling properties as well. To determine

the effect of AMP modifications on their antimicrobial activity, the MIC and MBC were compared to AMP without modifications (Table 3). Different susceptibility patterns could be observed for the *P. aeruginosa* and *S. aureus* strains investigated in this study. In general, *P. aeruginosa* was less susceptible than the Gram-positive *S. aureus* as higher concentrations of AMP were required to inhibit its growth. Camel was the most promising AMP tested with lower concentrations needed to prevent planktonic growth of both strains. Conjugation of PEG with Palm caused a decrease on antimicrobial activity, not being able to kill any strain for the higher concentration tested. Although the addition of the amino acids CKGG to Camel has affected its antimicrobial activity, MIC and MBC against both strains could be determined.

Table 3. MIC and MBC of peptides with and without modifications against planktonic cultures of *P. aeruginosa* and *S. aureus.* MIC and MBC are expressed in µg/mL.

AMP	*S. aureus*		*P. aeruginosa*	
	MIC	**MBC**	**MIC**	**MBC**
Palm	32	64	64	64
Palm-PEG	64	> 64	64	> 64
Camel	2	8	16	32
Camel-CKGG	16	16	32	64

To determine the success of AMP modifications, an attachment assay was also performed in which bacteria were allowed to attach for 4 h and the remaining cells on the substrates were imaged with fluorescence microscopy.

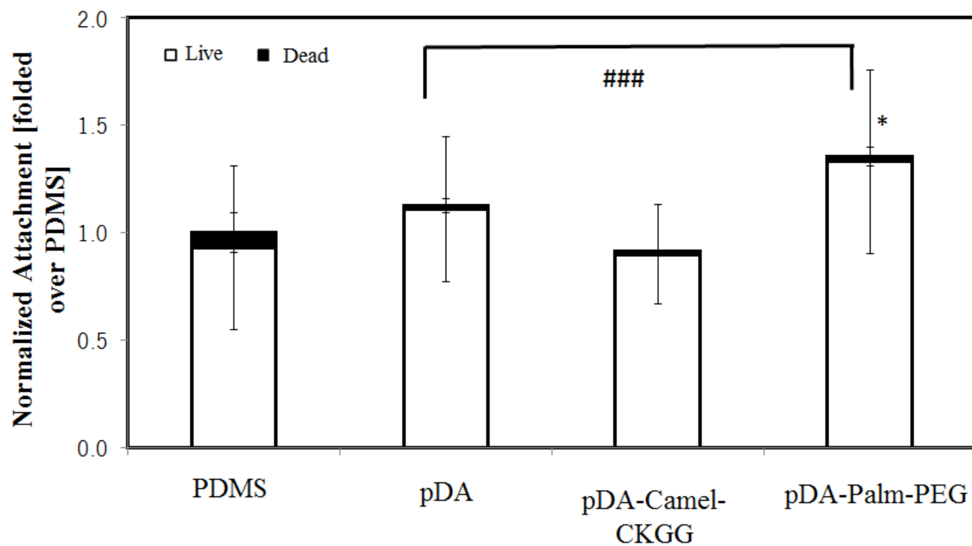

Figure 2. Normalized attachment and viability of cells on unmodified PDMS, pDA-coated PDMS (pDA), and pDA-coated PDMS functionalized with Camel-CKGG (pDA-Camel-CKGG) and Palm-PEG (pDA-Palm-PEG). All values were normalized to PDMS control. Significant differences were found for (*) $p < 0.05$, compared to PDMS control attachment and (###) $p < 0.001$, compared to PDMS fraction of dead cells.

Results in Figure 2 showed that none of the modified peptides were able to impair bacterial attachment or cause damages to membrane cells. In fact, the presence of PEG on Palm actually increased bacterial adhesion to these surfaces, and no antimicrobial activity was observed.

BACTERIAL VIABILITY ON PDA-PALM SURFACES

The aforementioned results highlighted the great potential of Palm without modification to be used in the design of bi-functional coatings to provide the antimicrobial component. Because it is intended to obtain a broad spectrum coating, the antibacterial performance of Palm-modified coatings (pDA-Palm) was also evaluated against the Gram-negative *P. aeruginosa* strain.

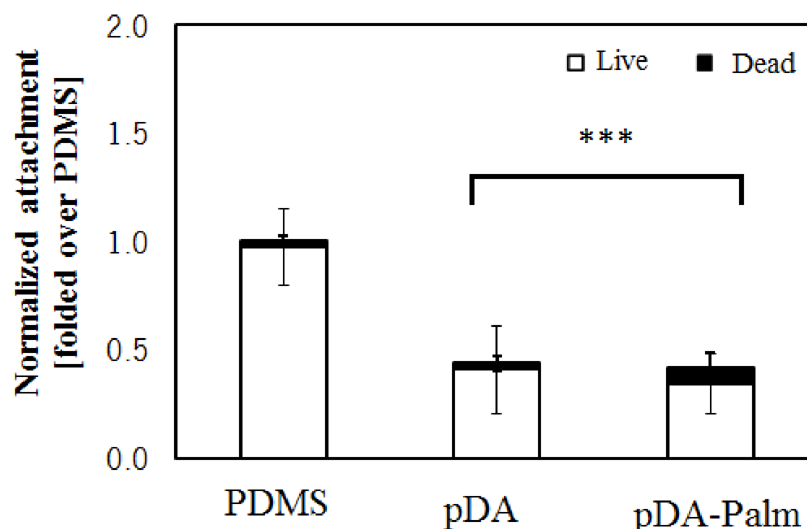

Figure 3. Normalized attachment and viability of cells of *P. aeruginosa* on unmodified PDMS, pDA-coated PDMS (pDA), and pDA-coated PDMS functionalized with PALM (Si-pDA-PALM). All values were normalized to PDMS control. Significant differences were found for (***) $p < 0.001$, compared to PDMS control attachment and no significant differences were found, compared to PDMS fraction of dead cells.

Results showed that unlike for the Gram-positive *S. aureus*, PDMS functionalized with only pDA was able to prevent bacterial adhesion as compared to bare PDMS surfaces. These results may be attributed to differences found on the hydrophobicity parameters of bacteria and surfaces, which were evaluated through contact angle measurements and explained in Chapter 3.3. Further functionalization with PALM had no significant effect on bacterial adhesion, as compared to pDA layer alone but slightly increased the number of cells with damaged membrane cells, confirming the potential broad-spectrum activity of this coating.

DISCUSSION

In Chapter 3.1 the peptides Camel and Palm exhibited great potential to be further immobilized onto surfaces and provide them with antimicrobial properties. Moreover, it has been reported Camel potential for the treatment of bacterial skin infections as it did not cause any toxic effect on human HaCat keratinocytes at their MIC [15]. In the same study, a similar lipopeptide to the one used in this study (Palm-KK-NH_2) was also investigated and, unlike, Camel, this peptide

became toxic at concentrations near MIC. These toxicity issues may, however, be overcame with its immobilization onto a surface.

For their immobilization, the 2-step pDA mediated approach was explored. The bare PDMS surface was first functionalized with a layer of pDA followed by exposure to concentrated peptide solutions, under oxidizing conditions, for their attachment via covalent and/or physical adsorption [16]. In solution, Camel was more active than Palm as it required lower concentrations to impair both planktonic growth and biofilm formation (Chapter 3.1). Its antimicrobial activity, however, was not retained after its immobilization, which may be related to its proposed mechanism. It has been reported that, in solution, the antibacterial activity of cecropins and related peptides such as Camel is due to formation of large pores in bacterial cell membranes [17, 18]. Its immobilization, may have resulted in a deviation from its native mechanism in solution, as it has been proposed for another peptide with a pore forming mechanism of action [19]. Moreover, results concerning peptide immobilization efficiency suggested that Camel may have interfered with the pDA coating because of its higher chance to undergo folding or self-assembly for having a larger sequence, as compared to Palm peptide. In an attempt to enhance Camel immobilization to pDA coating, it was modified with cysteine and lysine to introduce thiol and amino groups to increase the coupling specificity to the reactive catechol groups on the pDA coating. Two glycine residues were also introduced as a flexible linker. This modification, however, was not well succeed as the modified Camel did not cause any significant effect on bacterial adhesion. It was hypothesised that the linker introduced was not long enough to provide a proper AMP orientation to fully penetrate the bacterial membranes and form pores, its native mechanism in solution.

Palm immobilization, on the other hand, yielded promising results as it retained its antimicrobial activity, especially against *S. aureus*. It has been suggested that membrane perturbation is at least one of the targets of these lipopeptides [20]. The stronger tendency of longer lipopeptides such as the one investigated in this study to oligomerize and self-associate in solution may explain its better performance after its immobilization, as it is more difficult for them to transverse the bacterial cell wall and to reach an perturb the cell membrane [21]. Palm antibacterial activity after its immobilization may involve the displacement of positive cations from the bacterial membranes which induces disruption of the bacterial envelope and death. This mechanism was observed in studies where no spacer or short spacers were used for peptides immobilization and the activity was retained [6, 22]. Palm immobilization with PEG as a linker

failed to improve its antimicrobial activity or render any anti-fouling properties which may be attributed to the length of the linker used, that, in a similar way that what happened for the linker tested for Camel, may not provide a proper orientation of Palm.

In conclusion, the overall results highlighted the great potential of Palm peptide in the design of antibacterial coatings, imparting them with antimicrobial activity. This chapter also points out the interplay of several immobilization parameters on the activity of tethered AMP.

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Chapter 4

ENZYMES POTENTIAL TO CREATE ANTIBACTERIAL SURFACES

4.1

Polydopamine-mediated immobilization of alginate lyase to prevent P. aeruginosa adhesion

Given alginate's contribution to *P. aeruginosa* virulence, it has long been considered a promising target for interventional therapies, which have been performed by using the enzyme alginate lyase. In this work, instead of treating pre-established mucoid biofilms, alginate lyase was immobilized onto a surface as a preventative measure against *P. aeruginosa* adhesion. A pDA dip-coating strategy was employed for functionalization of PC surfaces. Enzyme immobilization was confirmed by surface characterization. Surfaces functionalized with alginate lyase exhibited anti-adhesive properties, inhibiting the attachment of the mucoid strain. Moreover, surfaces modified with this enzyme also inhibited the adhesion of the non-mucoid strain. Unexpectedly, treatment with heat-inactivated enzyme also inhibited the attachment of mucoid and non-mucoid *P. aeruginosa* strains. These findings suggest that the antibacterial performance of alginate lyase functional coatings was catalysis-independent, highlighting the importance of further studies to better understand its mechanism of action against *P. aeruginosa* strains.

INTRODUCTION

Modern healthcare is strongly dependent on the use of biomaterials and medical devices to support or restore physiological functions after trauma or disease [1]. There are, however, some drawbacks associated with their extended use, as they constitute a primary avenue for nosocomial infections [2]. Bacteria are able to reach the biomaterial surface, adhere to it and form multicellular aggregates enclosed in a self-produced matrix of EPS, with the resultant structures commonly referred to as biofilms [3]. When biofilms are at the root of a bacterial infection, treatment becomes extremely difficult as bacteria within these sessile communities adopt special features that confer increased resistance to antimicrobial treatments and to the host immune system [3, 4]. Biofilm formation is a cyclic and developmental process, initiated by adhesion of bacteria to the surface of a biomaterial, followed by proliferation, aggregation and maturation [5]. Upon interfacing with a biomaterial surface, replicating adherent bacteria secrete mostly insoluble gelatinous exopolymers, which function as a "glue", holding bacterial cells together [6]. This extracellular matrix, comprising mainly of water, polysaccharides, proteins and eDNA, makes biofilms the most successful living structures on earth, providing mechanical support, mediating cell-cell and cell-surface interactions and acting as a protective barrier [7].

Among the organisms most frequently isolated from infections associated with commonly used medical devices and implants, *P. aeruginosa* stands out for its high incidence and remarkable ability to form strong biofilms in devices such as peritoneal catheters [8], ventricular assist devices [9], endoscopes [10] and cochlear implants [11]. To establish an infection, *P. aeruginosa* relies on a number of unique virulence factors, including its extensive genetic regulatory networks [12,13], secretion of enzymes and exopolysaccharides, as well as ability to adhere to various surfaces and form biofilms [14]. The production of the exopolysaccharide alginate is one of the most extensively studied virulence factors. Alginate is a linear polymer of β-D-mannuronic and α-L-guluronic acid residues and it is mainly associated with mucoid *P. aeruginosa* isolates recovered from the lungs of patients with cystic fibrosis [15]. The role of alginate in *P. aeruginosa* adhesion and subsequent biofilm formation has been the subject of some controversy. Several independent studies have shown that overproduction of this exopolysaccharide yields significant architectural and morphological changes in the biofilm [16-18] and contributes to the persistent nature of lung infections [19]. These findings have led to the assumption of alginate as an attractive target for interventional therapies which can be

accomplished by the use of the enzyme alginate lyase. This enzyme is able to depolymerize alginate through a β-elimination reaction that releases unsaturated polysaccharides with C=C double bonds at their non-reducing terminal urinate residues [20]. In support of this role, alginate lyase has been shown to detach mucoid biofilms from abiotic surfaces [21], to increase antibiotic susceptibility of mucoid *P. aeruginosa* biofilms [22], to reduce viscosity in cultures of clinical isolates and in cystic fibrosis sputum [23] and to enhance phagocytosis and killing of *P. aeruginosa* by human immune cells [24]. However, other studies demonstrated that alginate synthesis is not required for biofilm development [25,26] and it was reported that the exogenously added *A. vinelandii* alginate lyase was not able to remove mucoid *P. aeruginosa* biofilms, despite being active toward alginate surface [27]. The authors suggested that alginate did not contribute to the cohesiveness of biofilms or it was protected from enzymatic degradation in biofilms. In a recent study, it was shown that alginate lyase dispersion of *P. aeruginosa* biofilms and enzyme synergy with tobramycin is completely decoupled from catalytic activity, as equivalent results were obtained with an isogenic non-mucoid strain and the same anti-biofilm effects could be achieved with BSA or simple amino acids [28].

In the present study, a new approach for utilizing alginate lyase was investigated. Instead of using the enzyme for treatment of pre-established mucoid biofilms, the ability of alginate lyase to prevent *P. aeruginosa* adhesion to a surface was investigated. For that purpose, a pDA dip-coating strategy was applied for functionalization of PC with alginate lyase.

MATERIALS AND METHODS

STRAINS AND BACTERIAL CONDITIONS

Two reference strains of *P. aeruginosa*, a mucoid strain (ATCC 39324) and a non-mucoid strain (ATCC 27853) were used throughout this study. Four *P. aeruginosa* clinical isolates (from peritoneal catheters), and internally coded as PD 64.8, 68.7, 50.2 and 96.4, were also used. The strains were preserved and cultured as described in Chapter 2.

ALGINATE LYASE AND BSA COATING ON PC

Alginate lyase and BSA (a protein without catalytic activity used as control) coatings were prepared as illustrated in Figure 2B of Chapter 2, adapted from a 2-step method previously developed for immobilizing enzyme onto magnetic nanoparticles [29]. Prior to surface modification, PC surfaces were cleaned and prepared as described in Chapter 2. PC coupons were then immersed in dopamine solution (1 mg/mL dopamine-HCl in 10 mM bicine buffer, pH 8.5) for 18 h, at RT under agitation (70 rpm). The surfaces were then rinsed with UP water and dried with nitrogen gas. To further coat with active or heat denatured (at 105 °C for 15 min) alginate lyase, pDA-coated PC coupons (pDA) were immersed in 5 mL of alginate lyase solution (1 mg/mL in bicine buffer supplemented with 600 mM NaCl, pH 8.5) for 2 h, at RT under agitation (pDA-AL and pDA-inactive AL). For BSA immobilization, pDA-coated PC surfaces were immersed in 5 mL of BSA solution (1 mg/mL in bicine buffer supplemented with 600 mM NaCl, pH 8.5) for 2 h, at RT, under agitation (pDA-BSA). Alginate lyase simply adsorption without the intermediate layer of pDA was also performed by immersing unmodified PC surfaces in 5 mL of active alginate lyase solution prepared in the same conditions (PC-AL).

ENZYMATIC ACTIVITY OF ALGINATE LYASE-COATED SURFACES

The activity of alginate lyase immobilized onto PC surfaces using the coating procedure aforementioned was determined by measuring the increase in absorbance at 235 nm as described in Chapter 2. Experiments were performed in triplicate for each condition.

PHYSICOCHEMICAL CHARACTERIZATION OF SURFACES AND CELLS

The hydrophobicity parameters of material surfaces and bacteria were determined using the sessile drop contact angle method as described in Chapter 2. Experiments were performed in triplicate for each condition.

SURFACE CHARACTERIZATION

Surfaces were characterized by XPS, SEM and measuring static water contact angle as described in Chapter 2. Experiments were performed in triplicate for each condition.

BACTERIAL VIABILITY ON MODIFIED SURFACES

The performance of the modified surfaces against bacterial adhesion was evaluated as described in Chapter 2 with some modifications. Briefly, a bacterial suspension with $1x10^8$ CFU/mL was prepared in sterile saline solution from an overnight culture of each strain. Materials were placed into the wells of a 12-well tissue culture plate and covered with 2 mL of bacterial suspension. The samples were kept at 37 °C for 24 h with agitation at 120 rpm and stained with a live/dead stain as described in Chapter 2. Two independent assays with three replicates for each condition were performed for the reference strains while for the clinical isolates experiments were performed in triplicate.

RESULTS

ALGINATE LYASE IMMOBILIZATION ONTO PC SURFACES

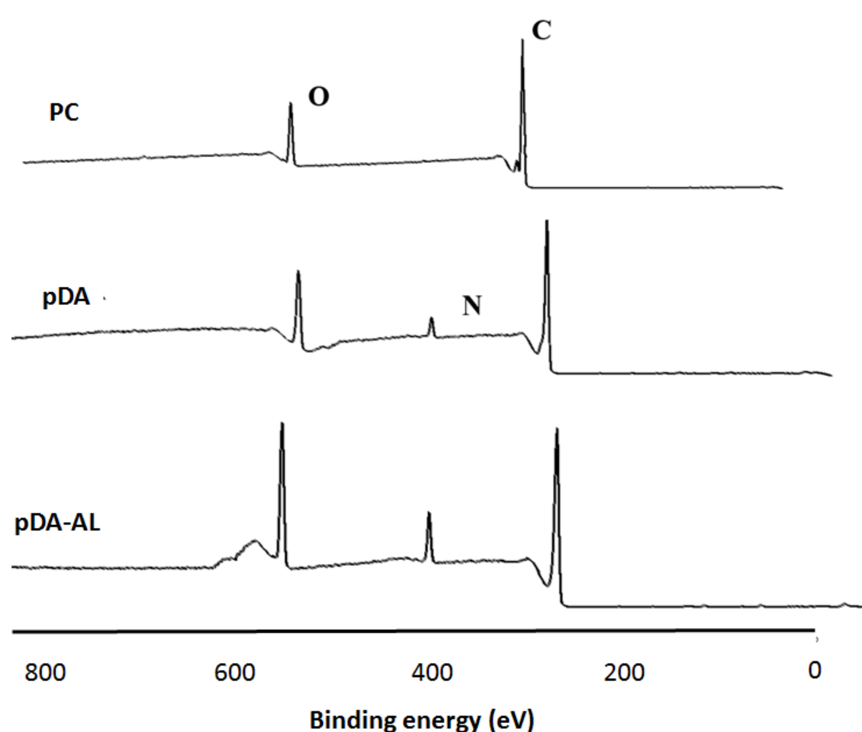
Polydopamine has been the focus of great interest as a surface modification agent to be used in a wide range of applications [30-32]. Messersmith and co-workers [33] have demonstrated that this molecular mimic of marine mussels' adhesion proteins can be deposited as a thin adherent polymer film on surfaces of various materials including metals, polymers and inorganic materials. In this work, the previously demonstrated versatile chemistry of pDA was exploited to functionalize PC surfaces with alginate lyase to impart them with anti-adhesive properties. The first step of the coating strategy involved the deposition of a uniform pDA coating from doplamine-HCl solution at a slightly alkaline pH. The pDA coatings were then used as a platform for enzyme immobilization by immersion in an alginate lyase solution (Figure 2B of Chapter 2). It is likely that the presence of residual quinones within the pDA coating present convenient sites for covalent grafting of nucleophilic groups, such as amino functional groups commonly found in enzymes, via Michael Addition and/or Schiff base reactions [34].

SURFACE CHARACTERIZATION

XPS analysis was employed to confirm each modification step (Figure 1). Polydopamine deposition was established by the presence of the N1s (399 eV) peak in the addition to the C1s (284.7) and O1s (531 eV) peaks present in unmodified PC. Furthermore, pDA-modified surfaces yielded surface chemical compositions similar to the theoretical ones of dopamine (Figure 1B).

Alginate lyase immobilization was suggested by the slight decrease of carbon accompanied by an increase of nitrogen composition. Differences in chemical composition of enzyme powder before and after its immobilization may be attributed to the sampling depth achieved by XPS, which is approximately 10 nm, which means the pDA layer may have contributed to the chemical signature detected by XPS analysis. Sulphur decrease on immobilized enzyme may be attributed to a reduced number of exposed thiol groups on the surface, which were necessary for grafting to the pDA-coated PC.

A)



B)

Sample	C (%)	O (%)	N (%)	S (%)
PC	85.3	14.7	0.0	0.0
pDA	70.13	21.18	8.69	0.0
Dopamine	72.7	18.2	9.1	0.0
pDA-AL	67.39	19.91	12.45	0.25
AL	65.4	26.8	5.7	2.1

Figure 1. XPS analysis of the polymer substrates. (A) XPS peaks of the unmodified PC, pDA-coated PC surfaces (pDA) and pDA-coated PC surfaces with immobilized alginate lyase (pDA-AL). (B) Quantification of atomic compositions on the polymer surfaces, alginate lyase in powder (AL) and the theoretical composition of dopamine based on its molecular composition.

Surface morphology of pDA-mediated surfaces was characterized using SEM analysis. The unmodified PC surfaces exhibited smooth surface morphology compared to the modified surfaces (Figure 2A). Polydopamine particles as a result of dopamine self-polymerization in solution could be observed on both pDA-coated surfaces and after further functionalization with alginate lyase, confirming the presence of pDA coating. For further characterization of the surfaces prepared under these conditions, water static contact angles of the surfaces before and after modification were measured (Figure 2B). After applying the pDA coating, PC surfaces became more hydrophilic with a significantly reduced contact angle (31°) which is in agreement with studies previously reported [33]. Further functionalization with alginate lyase had no significant effect on surface hydrophilicity.

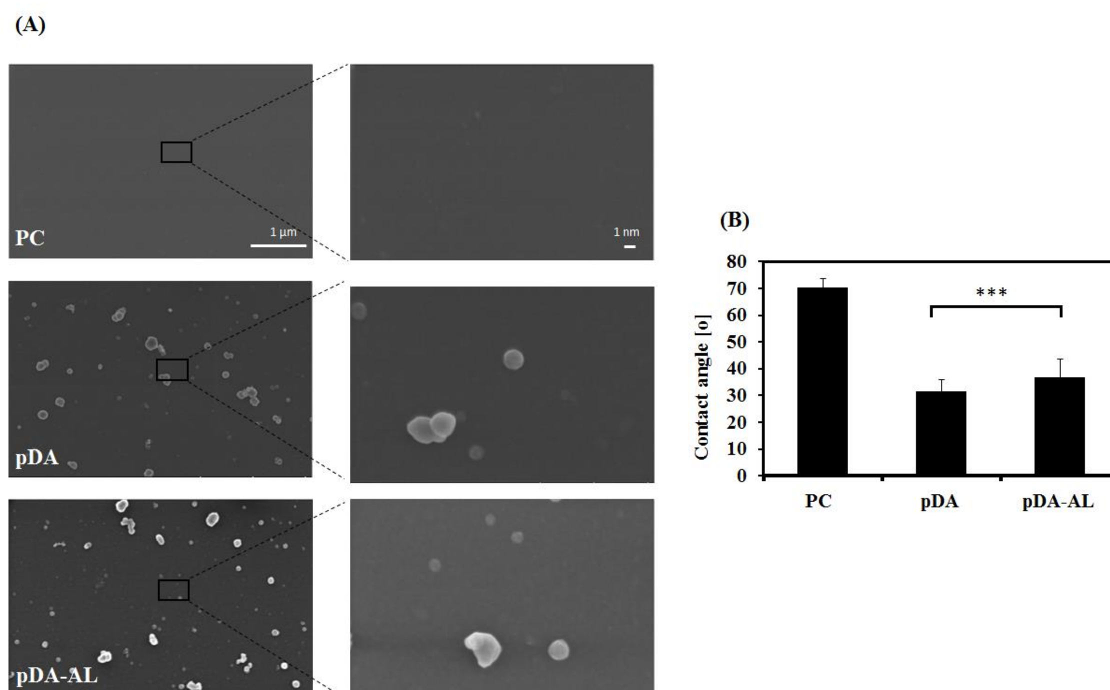


Figure 2. SEM images (A) and measurement of the water contact angle (B) of the unmodified PC, pDA-coated PC surfaces (pDA) and pDA-coated PC with immobilized alginate lyase (pDA-AL).Significant differences were found for (***) $p < 0.001$, compared to PC control.

ENZYMATIC ACTIVITY OF IMMOBILIZED ALGINATE LYASE

The catalytic activity of the immobilized alginate lyase onto PC surfaces by simple adsorption or mediated by dopamine polymerization was assessed by measuring spectrophotometrically its ability to depolymerize sodium alginate. As a control, the activity of alginate lyase heat denatured before its immobilization was also determined. Results in Table 1 showed that alginate lyase

retained its catalytic activity after being immobilized by both coating strategies. A higher activity was, however, obtained when alginate lyase immobilization was mediated by pDA (absorbance values of 0.21 and 0.13, respectively). These results also suggest that immobilization mediated by pDA yielded a better immobilization efficiency although the amount of immobilized enzyme could not be determined. Heating of alginate lyase before its immobilization caused enzyme denaturation as it was not able to act against sodium alginate.

Table 1. Intensities of absorbance measured at 235 nm corresponding to alginate lyase activity against sodium alginate. Values are means ± SD.

Sample	Abs_{235nm}
PC-AL	0.13 ± 0.04
pDA-AL	0.21 ± 0.04
pDA-inactive AL	0.03 ± 0.02

PREDICTION OF ADHESION

The evaluation of the hydrophobicity parameters for the reference strains and the PC surfaces before and after their modification with pDA are shown in Table 2A. Results show that the two reference strains of *P. aeruginosa* showed water contact angles lower than 65° and positive values of free energy of interaction (ΔG_{iwi}), which are indicative of a hydrophilic feature [35, 36]. PC surfaces can be considered hydrophobic since the water contact angle values were higher than 65° and a negative value of free energy of interaction. After modification with pDA, their thermodynamic properties were altered which can be clearly shown in terms of the free energy of interaction (ΔG_{iwi}^{TOT}) for which a positive value was obtained. This means that, theoretically, the affinity of an organism for the unmodified PC surfaces is superior when compared to pDA. From the physico-chemical parameters of each adhesion entity (bacteria and surface), it was possible to determine the thermodynamic relation between both entities, namely the free energy of adhesion (Table 2B). Results suggested that adhesion to both unmodified PC and pDA is more favoured for the non-mucoid strain, as indicated by the lower values of free energy of adhesion.

Table 2. (A) Values of contact angles (°) with water (θ_W), formamide (θ_F), α-bromonaphtalene (θ_B), surface tension parameters (mJ/m²), and free energy of interaction (ΔG_{iwi}^{TOT}) (mJ/m²) between the bacteria and the surfaces (i) when immersed in water (w). (B) Free energy of adhesion between bacteria (b) and the surfaces (s). Values are means ± SD.

(A)

Bacteria/Surface	**Contact angle (°)**			**Surface Tension Parameters (mJ/m²)**			**Free energy of interaction (mJ/m²)**
	θ_W	θ_F	θ_B	γ_i^{LW}	γ_i^{+}	γ_i^{-}	ΔG_{iwi}^{TOT}
P. a ATCC 27853	30.8 ± 9.9	23.0 ± 4.7	53.9 ± 6.7	28.1	4.2	41.7	16.2
P. a ATCC 39324	35.8 ± 11.6	86.5 ± 15.9	26.9 ± 3.4	39.7	0	127.8	121.8
PC	76.2 ± 8.4	63.1 ± 5.7	12.8 ± 2.9	43.3	0	12.5	-37.8
pDA	33.9 ± 5.2	10.2 ± 1.7	25.0 ± 5.3	40.3	2.0	34.7	6.6

(B)

Bacteria	**Free energy of adhesion (mJ/m²)** ΔG_{bsb}^{TOT}	
	PC	PC-pDA
P. a ATCC 27853	2.8	13.3
P. a ATCC 39324	41.7	48.4

ANTIBACTERIAL PERFORMANCE OF SURFACES FUNCTIONALIZED WITH AL

To investigate the antibacterial performance of PC surfaces functionalized with alginate lyase, attachment assays were performed in which bare and treated surfaces were exposed to bacteria and the remaining cells on the surfaces were imaged with fluorescence microscopy. These assays were performed under starvation conditions (saline solution) and for a long period of time, 24 h, in order to enhance alginate production from mucoid strains [37]. Two reference strains of *P. aeruginosa*, one mucoid (ATCC 39324) and the other non-mucoid (ATCC 27853), were first used to assess the antibacterial properties of the modified surfaces (Figures 4 and 5).

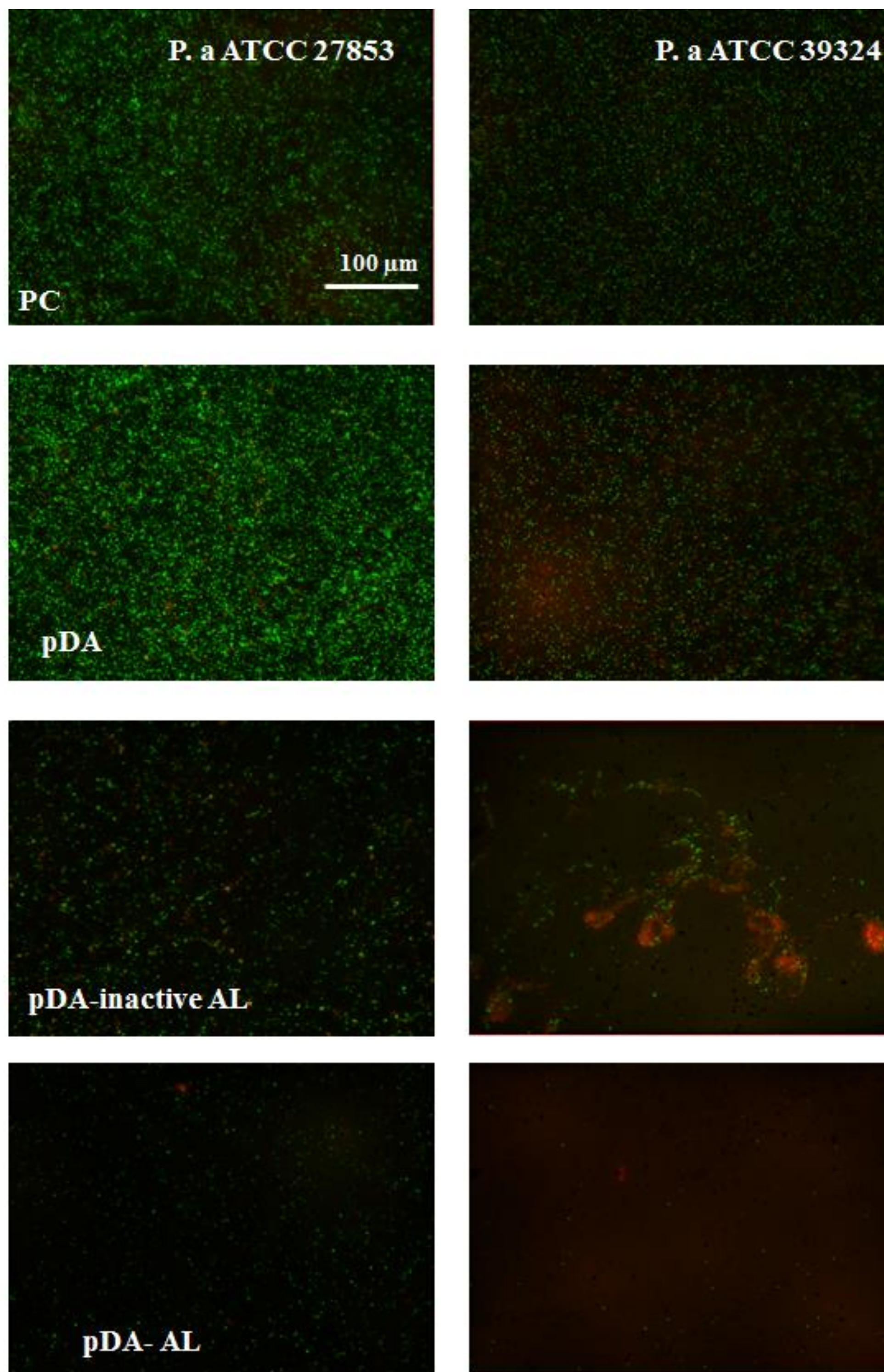


Figure 4. Representative fluorescent live/dead stain images obtained during *P. aeruginosa* ATCC 27853 and ATCC 39324 adhesion for 24 h on unmodified PC, pDA-coated PC surfaces (pDA) and pDA-coated surfaces functionalized with active (pDA-AL) and heat-inactivated alginate lyase (pDA- inactive AL).

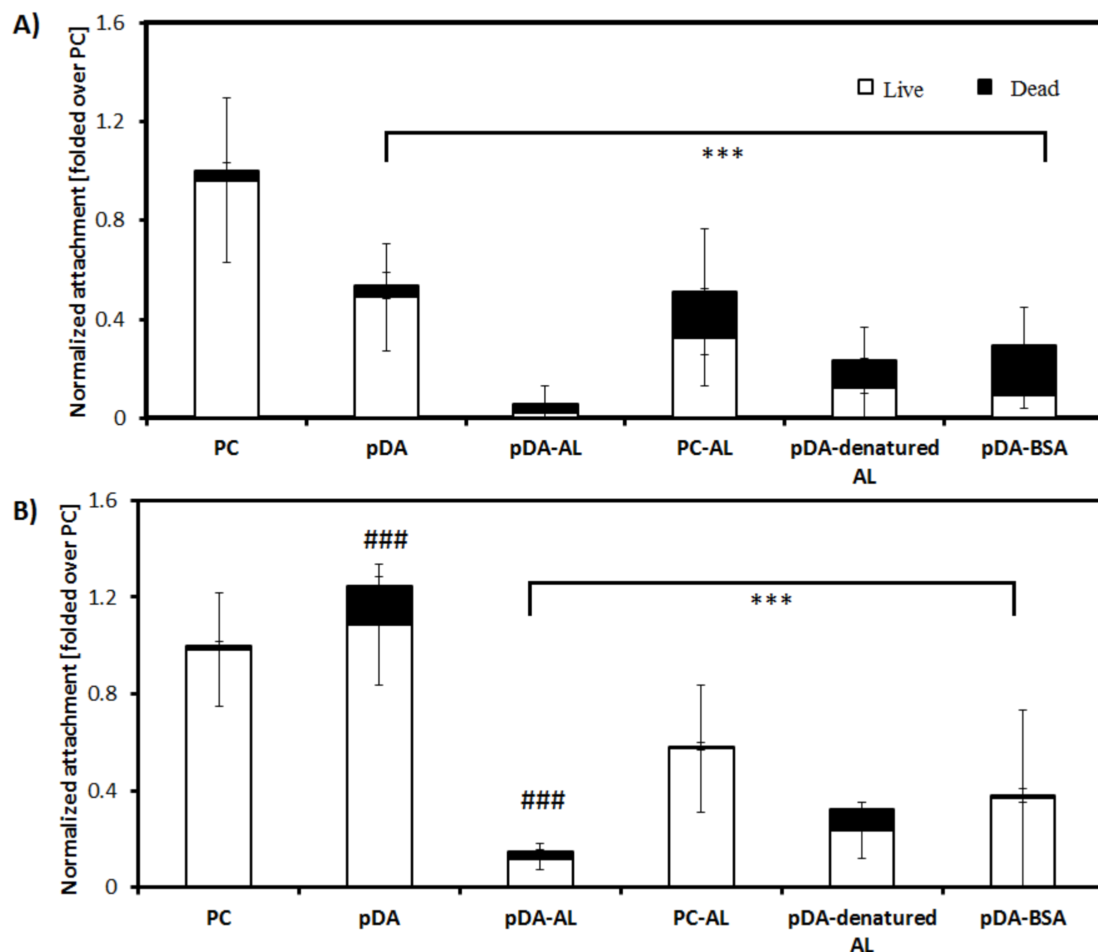


Figure 5. Normalized attachment and fraction of dead cells of *P. aeruginosa* ATCC 39324 (A) and ATCC 27853 (B) on unmodified polycarbonate (PC), pDA-coated PC (pDA), alginate lyase adsorbed onto PC (PC-AL) and pDA-coated PC functionalized with active alginate lyase (pDA-AL), heat-inactivated alginate lyase (pDA- inactive AL) and BSA (pDA-BSA). Attachment values were normalized to PC control. Significant differences were found for (***) $p < 0.001$, compared to PC control attachment and (###) $p < 0.001$, compared to PC fraction of dead cells

Unmodified PC surfaces allowed the adhesion of both bacterial strains and most of them remained alive. The presence of pDA coating decreased mucoid strain attachment and enhanced the adhesion of the non-mucoid strain, as compared to the unmodified surfaces. These results may be attributed to the differences found on the hydrophobicity parameters of bacteria and surfaces aforementioned, as they suggested a higher affinity of non-mucoid strain to PC surfaces before and after their modification with pDA. The fraction of dead cells found on pDA slightly increased after 24 h of incubation, which can be attributed to a decrease in the pH of saline solution in contact with pDA-coated surfaces, as previously reported [31]. The fraction of mucoid bacterial cells found on surfaces functionalized with alginate lyase was significantly lower than on

unmodified PC and pDA. Interestingly, alginate lyase immobilized on pDA-coated surfaces was also able to reduce the number of attached non-mucoid bacterial cells. Regarding cell viability, the presence of alginate lyase resulted, in general, in a higher fraction of dead bacteria, especially for the mucoid strain, which can be attributed to the antibacterial activity of lyase-depolymerized products of alginate previously reported [38]. As a control, alginate lyase was also immobilized onto PC surfaces using simple adsorption and, although it was able to prevent the attachment of both strains, alginate lyase pDA-based immobilization was more efficient in preventing bacterial attachment. As another control, alginate lyase heat-inactivated as well as an irrelevant protein, BSA, were immobilized onto pDA-coated surfaces and their antibacterial performance against the two reference strains evaluated. Results (Figure 5) demonstrated that modified surfaces functionalized with heat-inactivated alginate lyase or BSA were also able to impair bacterial adhesion of all strains investigated, suggesting that alginate lyase effects on bacterial attachment were decoupled from its catalytic activity.

Furthermore, the attachment of four clinical strains of *P. aeruginosa* isolated from peritoneal catheters on alginate lyase functional coatings was evaluated. Although the mucoid phenotype of these clinical isolates was not known, non-mucoid *P. aeruginosa* strains are the predominant clinical and environmental phenotype [26].

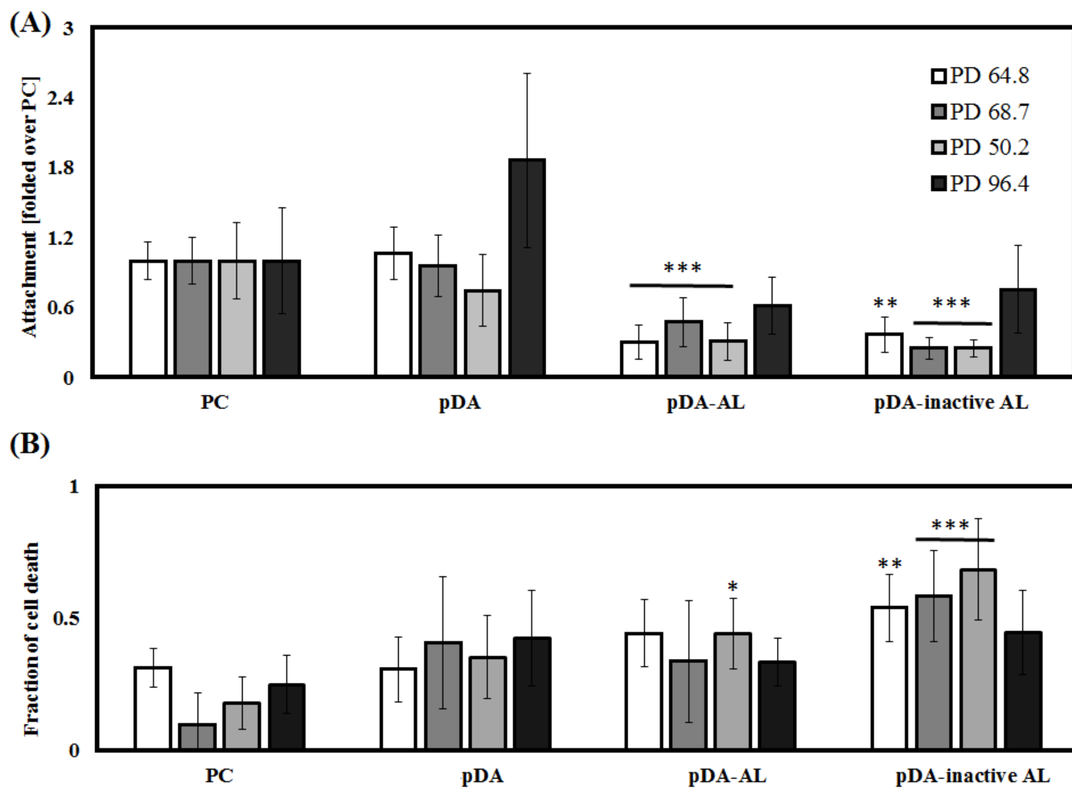


Figure 6. Normalized attachment (A) and fraction of dead cells (B) of clinical isolates of *P. aeruginosa* on unmodified PC, pDA-coated PC (pDA) and pDA-coated PC functionalized active alginate lyase (pDA-AL) and heat-inactivated alginate lyase (pDA-inactive AL). Attachment values were normalized to PC control. Significant differences were found for (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$, compared to PC control.

Results in Figure 6 showed that clinical isolates were allowed to attach to unmodified surfaces and most of them remained alive after a 24 h incubation under non-growing conditions. The presence of a pDA coating did not have a significant effect on bacterial attachment but caused a slight decrease in cell viability, similar to what was seen with the two reference strains. The immobilization of active or heat-denatured alginate lyase on the surfaces, caused a decrease on the attachment of clinical strains with the exception of PD 96.4, as its adhesion to the unmodified PC was already low.

DISCUSSION

The potential of alginate lyase for the treatment of mucoid *P. aeruginosa* biofilms has been the subject of research for the past few decades [18, 19, 22]. Post-treatment of mucoid *P. aeruginosa* biofilms with alginate lyase and subsequent biofilm detachment has led to the assumption that alginate plays an important role on biofilm establishment. In the present work, it was hypothesized that the presence of alginate lyase during the first stages of biofilm establishment, namely bacterial adhesion to a surface, could prevent its establishment. To confirm this hypothesis, alginate lyase was immobilized using a bio-inspired coating strategy. The immobilization platform was performed on PC as it has been reported that there is an annual 6 % demand for its use in medical devices such as filters cartridges for dialysis, blood oxygenators, reservoirs, filters and connection components [39]. The first step of the coating strategy involved the deposition of a uniform pDA coating onto PC surfaces from dopamine-HCl solution at a slightly alkaline pH. The pDA coatings were then used as a platform for enzyme immobilization. Surface characterization studies confirmed alginate lyase immobilization onto pDA-coated PC surfaces. Furthermore, it was possible to confirm that alginate lyase retained its activity after their immobilization and this catalytic activity was enhanced by pDA intermediate functionalization as compared to simple adsorption. As hypothesized, alginate lyase immobilized onto PC was able to prevent the adhesion of the mucoid reference strain of *P. aeruginosa*. However, surfaces modified with this enzyme also inhibited the adhesion of the tested non-mucoid strain. As a control, alginate lyase heat-inactivated as well as an irrelevant protein, BSA, were immobilized onto pDA-coated surfaces and results demonstrated that these modified surfaces were also able to impair bacterial adhesion of all strains investigated, suggesting that alginate lyase effects on bacterial attachment were decoupled from its catalytic activity.

The overall results suggested that alginate lyase immobilized on pDA-coated surfaces is a promising approach to impair *P. aeruginosa* adhesion regardless of its mucoid phenotype, therefore qualifying the strategy to be applied in a different context than just cystic fibrosis, where the mucoid phenotype predominates. For instance, this enzyme could be used to develop functional coatings able to prevent *P. aeruginosa* infections associated with a variety of biomaterials. To confirm this hypothesis, the attachment of four clinical strains of *P. aeruginosa* isolated from peritoneal catheters was also evaluated and similar anti-adhesive properties were observed.

These findings are consistent with a previous study [28] reporting that alginate lyase effects on *P. aeruginosa* biofilms are completely decoupled from its catalytic activity. The authors suggested that the anti-biofilm effects of alginate lyase enzymes could be related to enzyme-mediated changes in cell physiology. They hypothesized that instead of actively degrading the biofilm matrix, alginate lyase enzymes act as a nutrient source, modulating cellular metabolism, and thus inducing biofilm detachment and enhancing antibiotic efficacy. In the present study, however, taking into account the enzyme's immobilization, it may not be available to act as a nutrient source and, therefore, a different mechanism may be at the root of alginate lyase effects on *P. aeruginosa* attachment. The enzyme may prevent nonspecific binding of bacteria in a similar way to BSA, which has been commonly used to inhibit nonspecific biomolecule and bacterial adhesion to surfaces in applications such as bacteria sensors and micro patterning [40, 41]. Accordingly, pDA-based immobilization of BSA on PC was also able to prevent *P. aeruginosa* reference strains attachment. Immobilized alginate lyase proved to be more efficient than BSA to prevent the attachment of non-mucoid strain of *P. aeruginosa*. Such results may be attributed to the physico-chemical properties of the mucoid strain, which makes it more susceptible to the hydrophilic character of both alginate lyase and BSA coatings. Given the similar hydrophilic characters of both alginate lyase and BSA coatings via pDA, results suggest that alginate lyase may have another underlying mechanism for preventing bacterial adhesion, beyond preventing nonspecific adhesion. The combination of this preventive approach with therapeutic therapies, namely, antibiotic therapies may hold great potential to fight BAI, as it is expected that bacterial cells adhered to these modified surfaces will be more susceptible to antibiotic therapy in a similar way to bacteria that adhered more weakly to brush-coated silicone rubber, enhancing their susceptibility to gentamicin treatment [42].

In conclusion, although the mechanism(s) of action of alginate lyase against *P. aeruginosa* strains as well as against other strains commonly associated to BAI such as *Staphylococcus aureus* and *S. epidermidis,* needs to be further explored, this work suggests that alginate lyase immobilization on biomaterials may have potential as a preventive approach to fight BAI.

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4.2

Enzymatic mono-functional coatings to prevent bacterial adhesion onto PDMS

The growing number of BAI has led to the need of developing novel antibacterial coatings for medical devices. The use of enzymes able to degrade biofilm matrix components such as proteins and eDNA represents a promising approach to fight these infections. This study aimed to apply dopamine chemistry for covalent immobilization of different enzymes (lysozyme, proteinase K and DNase I) on PDMS to obtain surfaces able to prevent bacterial adhesion. Results showed that enzymes retained its biological activity after their immobilization using pDA as an intermediate layer. Lysozyme, however, was not able to prevent or cause membrane damage to *S. aureus* which led to its exclusion for further studies. PDMS functionalized with proteinase K or DNase I were able to prevent bacterial adhesion, especially DNase I which exhibited a broader action spectre. The overall results suggested that the use of enzymes for materials functionalization presents a promising strategy for creating antibacterial surfaces to be applied in biomaterials for medical devices and implants.

INTRODUCTION

Polydimethylsiloxane, commonly referred as silicone rubber, is used for a wide variety of biomedical applications due to its excellent biocompatibility and mechanical properties. For instance, it has been used in vascular grafts [1], catheters [2], stents [3], breast implants [4] and voice prostheses [5]. However, PDMS-based biomedical devices are prone to microbial adhesion which can be a prelude for biofilm formation and infection. These infections are extremely difficult to eradicate because cells within a biofilm encase themselves in self-produced matrix which confers them protection against antimicrobial treatment and host immune system [6,7]. Very often, the only solution for an infected implant relies on its surgical removal at the expenses of considerable costs and patient suffering [8].

Several strategies to modify PDMS surface have been reported, in an attempt to overcome this problem. For example, catheters have been impregnated with antibiotics such as minocycline/rifampicine [9], triclosan [10] and nitrofuzane [11]. Although this approach proved to prevent bacterial adhesion and biofilm formation on these surface-coated catheters there are some drawbacks that limit its clinical applications, mainly its short-term antibacterial effect and the potential development of bacterial resistance [12]. An alternative approach relies on the use of silver or silver nanoparticles to coat the surfaces of catheters, but its potential has been compromised by the high cost of the silver coating and the conflicting clinical results [13]. Covalent immobilization of antimicrobials offers an alternative approach that avoids patient exposure to leaching compounds and potentially increases the duration of antimicrobial efficacy [14]. This strategy should be employed with antimicrobial agents working at the level of the cell wall or membrane, since they can only reach the outside of the microbial cells. Quaternary ammonium silane and AMP are two examples of commonly used antimicrobial agents that have been covalently immobilized to PDMS to prevent BAI [15,16].

After bacterial adhesion to a surface, large amounts of EPS such as polysaccharides, proteins and eDNA, are produced. EPS have a crucial role in infection as it binds the biofilm together and to the surface [7]. Therefore, a promising strategy to prevent biofilm formation on the surfaces of biomaterials may rely on the use of enzymes targeting the EPS of biofilms matrix. It is expected that degradation of biofilm matrix or destabilization of their physical integrity can prevent biofilm establishment or promote the detachment of established one. Furthermore, after biofilm dispersion, bacterial cells may become more susceptible to antimicrobial action [14,17].

In this study, a pDA dip-coating strategy was applied for functionalization of PDMS with enzymes targeting different EPS: a polysaccharide degrading enzyme (lysozyme), a protein degrading enzyme (proteinase K) and a DNA degrading enzyme (DNase I).

MATERIALS AND METHODS

BACTERIAL STRAIN AND GROWTH CONDITIONS

A clinical isolate of *S. aureus* was used throughout this study. The strain was preserved and cultured as described in Chapter 2.

ENZYMES

Enzymes targeting different EPS were used in this study: lysozyme, proteinase K and DNase I.

POLYDOPAMINE COATING AND ENZYMES FUNCTIONALIZATION

Prior to surface modification, PDMS coupons were cleaned and prepared as described in Chapter 2. For pDA coating, materials were immersed in dopamine (2 mg/mL dopamine-HCl in 10 mM bicine buffer, pH 8.5) for 18 h, at RT under agitation (70 rpm). Coupons were then rinsed with UP water and air-dried. For further pDA functionalization, dopamine coated coupons were immersed in enzymes solutions at different incubation periods and temperatures. Preliminary optimization studies were performed to identify the conditions used for enzymes immobilization and details can be found in the supporting information (Figure S1 of Supplemental Material). Lysozyme and proteinase K were dissolved in PBS, pH 7.4, and were incubated overnight, at 4 °C under agitation. DNase I (1 mg/mL) was dissolved in PBS (150 mM NaCl, 10 mM potassium phosphate, pH 6.8) supplemented with 10 mM $MgCl_2$ and was incubated for 6 h, at RT, under agitation (70 rpm) as previously described [18].

SURFACE CHARACTERIZATION

Static water contact angle measurements were performed by a sessile drop method as described in Chapter 2. Experiments were performed in triplicate.

BACTERIAL VIABILITY ON MODIFIED SUBSTRATES

Antibacterial performance of the generated surfaces against bacterial adhesion was evaluated by an attachment assay as described in Chapter 2. Two or three independent assays were performed with three replicates for each condition tested.

ENZYMATIC ACTIVITY

The activity of immobilized DNase I and lysozyme was performed as described in Chapter 2. Experiments were performed in triplicate.

RESULTS

ENZYMES IMMOBILIZATION

For enzymes immobilization, the 2-step pDA approach was performed as illustrated in Figure 2B of Chapter 2. Lysozyme and Proteinase K immobilization was optimized (the details can be found on Supplemental Material, Figure S1) while DNase I immobilization was adapted from a study previously performed [18].

ANTIBACTERIAL PERFORMANCE OF ENZYMATIC COATINGS

Since the initial bacterial adhesion onto a biomaterial surface plays a crucial role on biofilm formation and subsequent device infection, it is important to inhibit this initial step. Bacterial attachment was allowed to proceed for 4 h onto PDMS before and after functionalization with enzymes and evaluated by the fluorescence live/dead staining method.

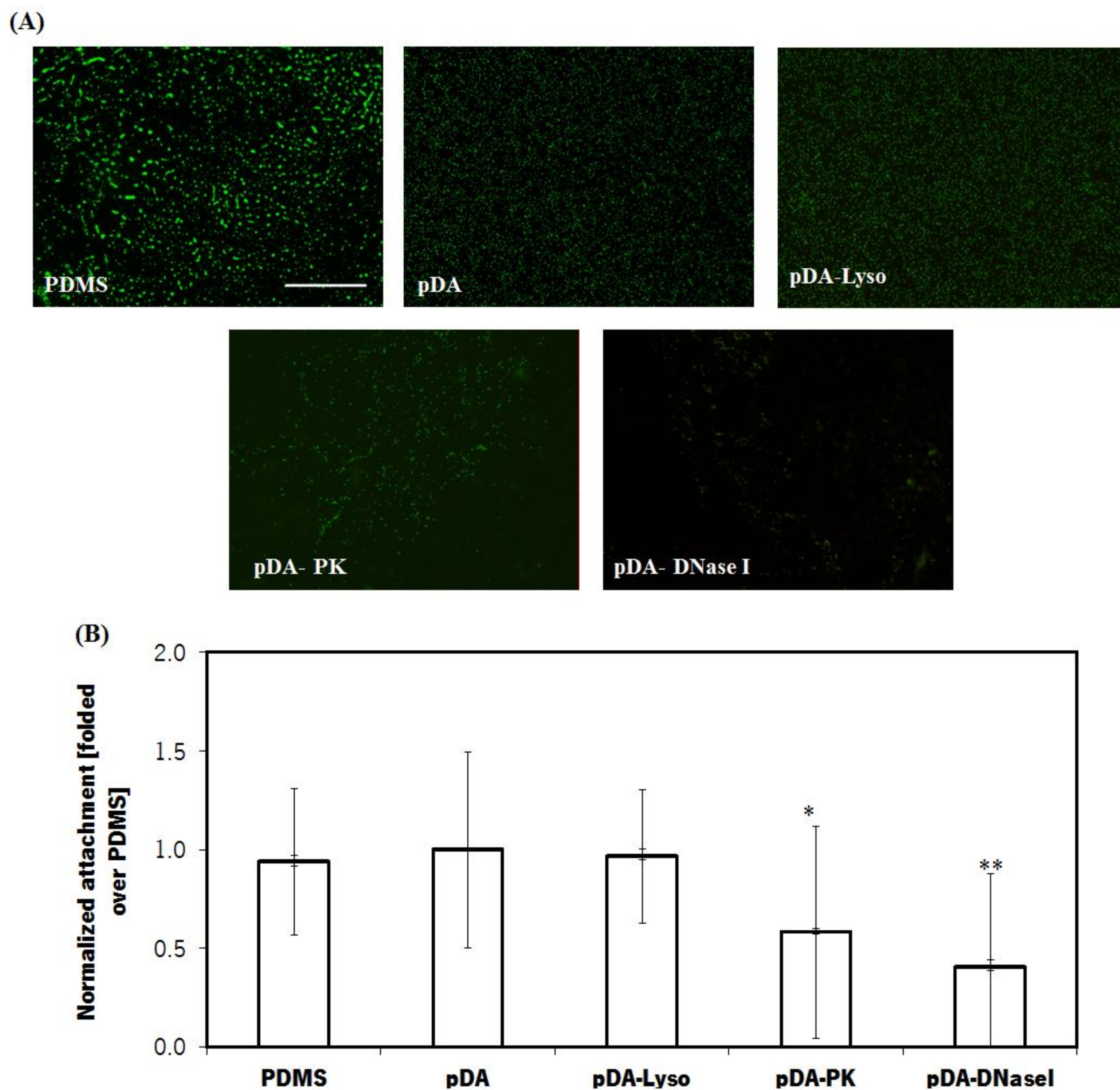

Figure 1. (A) Representative fluorescent live/dead stain images obtained during adhesion of a clinical isolate of *S. aureus*. The scale bar indicates 100 μm. (B) Normalized attachment and viability of cells on unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS functionalized with lysozyme (pDA-Lyso), proteinase K (pDA-PK) or DNase I (pDA-DNase I). Significant differences were found for (*) $p < 0.5$ and (**) $p < 0.01$, compared to PDMS control attachment.

Results showed that unmodified surfaces allowed the adhesion of *S. aureus* and most of them were alive. Further modification with pDA had no effect on bacterial attachment or cell viability. The presence of lysozyme was not able to prevent bacterial attachment or damage bacterial cells membrane. On the other hand, proteinase K immobilized onto pDA-coated PDMS surfaces demonstrated some effect against bacterial adhesion causing some reduction on bacterial attachment. DNase I immobilization had no effect on cell viability as compared to PDMS surfaces but was able to prevent bacterial attachment, confirming, thus, the anti-adhesive properties previously attributed to these DNase I-based coatings. Once confirmed the ability of DNase I

coating to more efficiently prevent Gram-positive *S. aureus* adhesion, two strains (with different mucoid phenotype) of a relevant Gram-negative strain, *P. aeruginosa*, were afterwards evaluated.

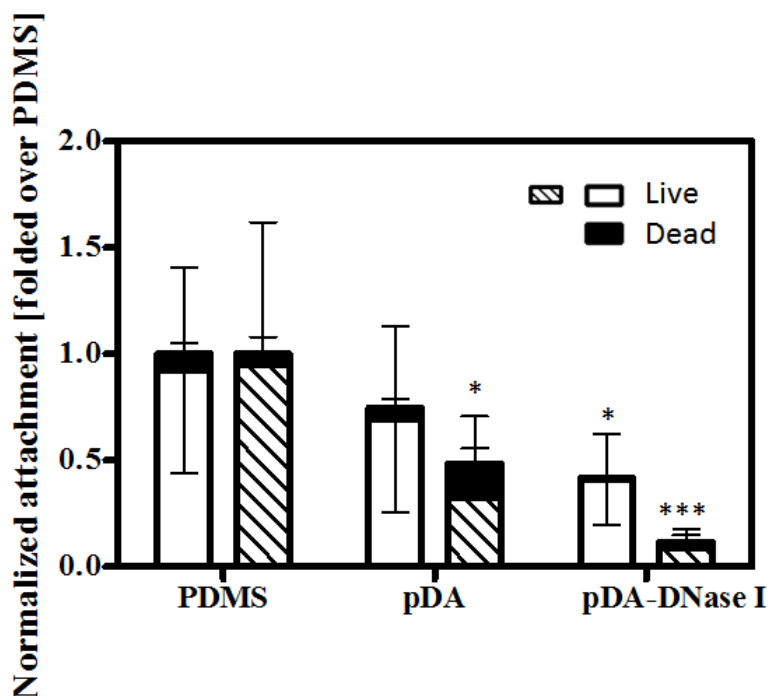

Figure 2. Normalized attachment and viability of cells of *P. aeruginosa* ATCC 27853 (bars without pattern) and ATCC 39324 (bars with pattern) on unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS functionalized with active DNase I (pDA-DNase I). Significant differences were found for (*) $p < 0.5$ and (***) $p < 0.001$, compared to PDMS control attachment.

Results showed that *P. aeruginosa* was also able to colonise bare PDMS surfaces but a higher fraction of cells with compromised membrane was detected, as compared to *S. aureus*. PDMS modification with pDA had no effect on the attachment of non-mucoid strain but prevented in some extent the adhesion of mucoid *P. aeruginosa* strain. Further functionalization of pDA with DNase I prevented the adhesion of both strains, especially the mucoid one.

SURFACE CHARACTERIZATION OF ENZYMATIC COATINGS

To evaluate the surface wettability of the PDMS after surface modification, the static water contact angle of surfaces after each deposition step was measured (Figure 3). Bare PDMS is inherently hydrophobic, with a high contact angle of 108.4° ± 2.5°. Functionalization of PDMS with pDA greatly enhanced the hydrophilicity of the polymer

surface, decreasing the contact angle to 60.2° ± 3.1°, which is a well-established observation in other material surfaces as well [19, 20]. Further immobilization with enzymes lysozyme, proteinase K and DNase I increased the contact angle to 78.3° ± 11.6°, 70.0° ± 11.1°and 83.3°. ± 15.0°, respectively, which may be attributed to the presence of hydrophobic amino acid residues [21].

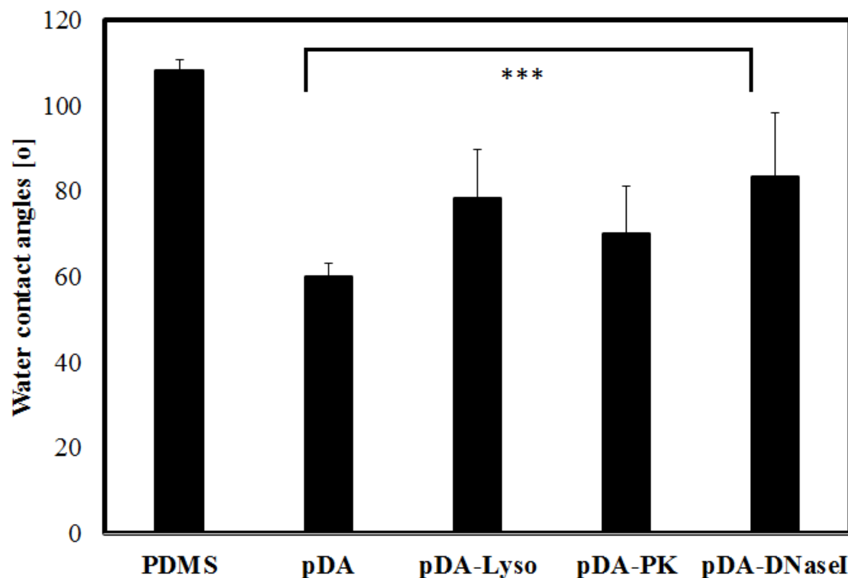

Figure 3. Water contact angle of unmodified PDMS, pDA-coated PDMS (Si-pDA) and pDA-coated PDMS surfaces functionalized with Lysozyme (pDA-Lyso), proteinase K (pDA-PK) or DNase I (Si-pDA-DNase I). Significant differences were found for (***) $p < 0.001$, compared to PDMS control.

ENZYMATIC ACTIVITY AFTER IMMOBILIZATION

The catalytic activity of enzymes after their immobilization was determined so it was possible to conclude about their biological activity. DNase I activity was determined by evaluating the hydrolysis of plasmid DNA in solution droplets placed on the coatings.

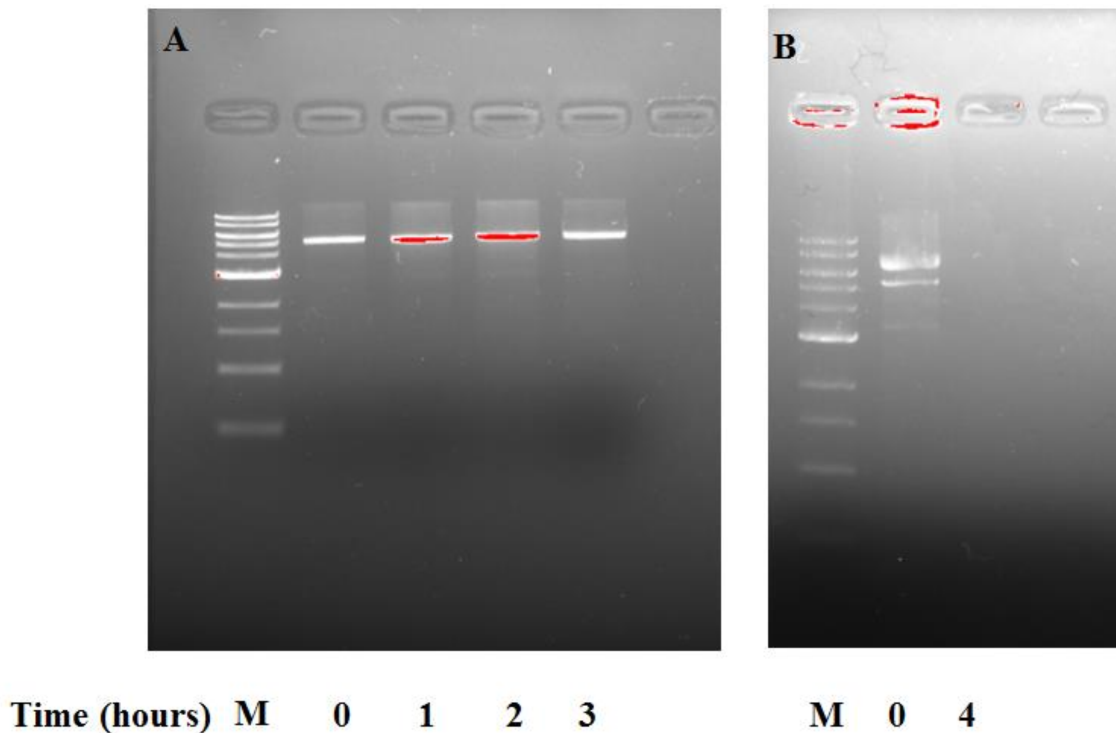

Figure 4. Agarose gel showing the degradation of plasmid DNA in a droplet on PDMS functionalized with DNase I after an incubation period of 1, 3 and 3 h (A) and after a 4 h incubation period (B). M: 1 kb DNA ladder (NEB).

In the first experiment (Figure 4A), samples were withdrawn every hour but after a period of 3 h, no more samples could be taken because the DNA droplet was dried. Since no DNA degradation could be observed during this period of time, a new assay was performed (Figure 4B) in which the first sample was withdrawn only after 4 h. Results showed the degradation of plasmid DNA confirming, therefore, that DNase I retained its biological activity after its immobilization onto PDMS.

Lysozyme is a glycosidase able to cleave the polysaccharidic component of the cell wall of bacteria such as *Micrococcus lysodeikticus*, causing cell lysis. Lysozyme bioactivity after its immobilization was evaluated by immersing PDMS functionalized with this enzyme in a suspension of *M. lysodeikticus* and its turbidity was monitored spectrophotometrically during 9 h.

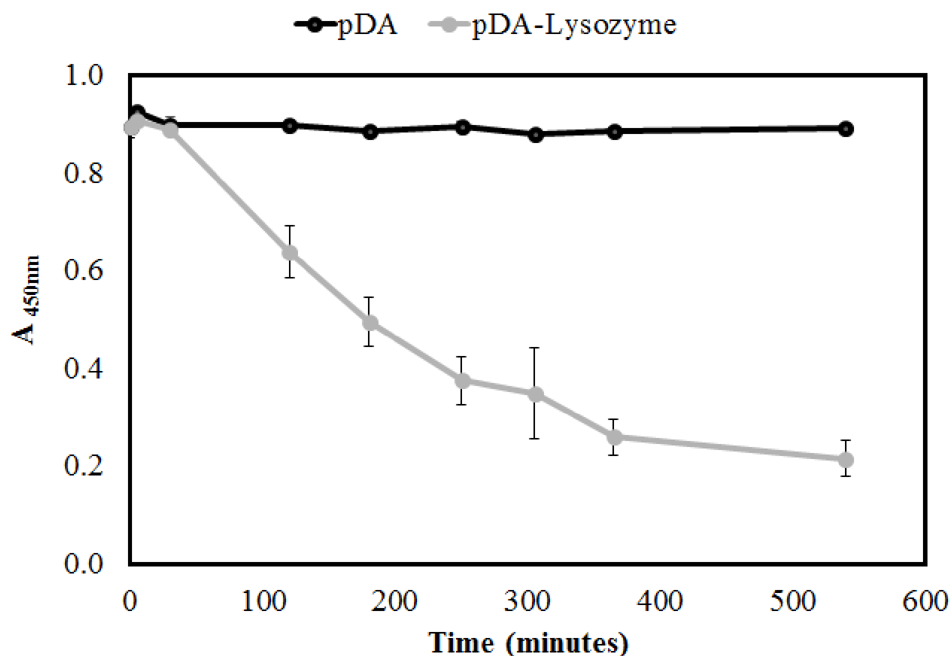

Figure 5. Lysozyme activity after immobilization onto pDA-coated PDMS (pDA-Lysozyme) by measuring the optical density of a suspension of *M. lysodeikticus* in time. As a control pDA-coated PDMS without lysozyme was also monitored.

Results showed that lysozyme immobilization onto PDMS did not compromise its biological activity as indicated by the gradually decrease of suspension turbidity as a consequence of *M. lysodeikticus* lysis. As a control, the suspension in contact with pDA-coated PDMS was also monitored and no lysis was observed during the same period of time.

DISCUSSION

Development of microbial resistance towards antibiotics and BAI are the major concerns faced by modern healthcare. There is, therefore, an urgent need for antibacterial surfaces that can prevent bacterial colonisation and subsequent biofilm formation and do not select for resistant strains. Immobilization of enzymes targeting different components of biofilm matrix or bacterial cells surface has been the focus of great interest in the last years [22-24]. In this sub-chapter, three enzymes with different targets were investigated for immobilization onto PDMS surfaces.

Lysozyme was chosen because it has been used to modify the surface of biomaterials to enhance their antimicrobial properties [25-27]. Its well-known bacteriolytic activity is characterized by an enzymatic and a non-enzymatic mode of action. The enzymatic mode of action depends on its ability to hydrolyse the 1, 4 -β–glycosidic bonds between N-acetyl-muramic acid and N-acetyl-D-glucosamine. These bonds are present in peptidoglycans, which comprise 90 % of the Gram-positive bacteria cell wall, making them very susceptible to lysozyme antimicrobial activity. The non-enzymatic mode of action of lysozyme is based on the cationic and amphiphilic properties of the enzyme which leads to perturbations in the cell membrane and activate the autolytic system of bacteria [28, 29]. Lysozyme is classified as GRAS (generally recognized as safe) by the FDA and as a food additive by the European Union [30]. For lysozyme to retain its enzymatic activity after immobilization, it has to be exposed to the solution rather than being adsorbed to the surface [25]. Results showed that lysozyme immobilization using pDA as an intermediate was not effective to kill or prevent the adhesion of *S. aureus*, although its lytic activity was retained against *M. lysodeikticus.* These results may then be attributed to Staphylococci resistance previously demonstrated. Lysozyme is not able to recognize peptidoglycan modified with O-acetyl groups that enables bacteria such as *S. aureus* to overcome the innate defence system. This modification acts as a steric hindrance and inhibits the binding of lysozyme and is mediated by peptidoglycan-specific, membrane bound O-acetyltransferase widespread only among pathogenic staphylococci, which is the case of the strain used in this study [31,32].

Proteins and glycoproteins are the dominant molecules mediating adhesion of many fouling organisms, thus proteases (protein hydrolysing enzymes) are the most tested and most successful enzymes used for the control of marine biofouling [33,34]. For medical applications, proteinase K may present a promising proteolytic enzyme to fight biofilm infections. This enzyme is stable in a broad range of conditions such as pH, buffer salts, detergents and temperature, and was able to effectively remove the biofilm formed by a clinical isolate of *S. aureus* [35]. When immobilized in this study, on the other hand, proteinase K impaired bacterial attachment at some extent but was not as effective as DNase I. Such results may be attributed to the loss of enzymatic activity after immobilization; this hypothesis needs, however, to be proved in further assays. Another possible explanation may be related to the fact that other polymer, such as the polysaccharide intercellular adhesin (PIA), plays a major role in the adhesion [36]. In fact, it has been reported that proteinase K was more effective in dispersing *S. aureus* biofilm when PIA

content was very less and the biofilm was probably dominated by the presence of proteins [37]. These observations allowed to conclude that immobilization of proteinase K could be a promising approach in controlling infections caused by *S. aureus* strains where biofilms matrix are dominated by proteins but in other strains in which PIA plays a major role in adhesion and biofilm formation, this approach alone may not be useful.

A promising development in biofilm research has been the finding that eDNA plays an essential role as a component of the biofilm matrix in most bacterial species [17, 38]. In fact, this enzyme has been used in the therapeutics of patients suffering from cystic fibrosis [39] and its immobilization has been previously performed with quite promising results [18]. The results in this study endorsed these previous findings as DNase I immobilized onto PDMS material was the most effective enzyme tested in preventing the adhesion of both Gram-positive and Gram-negative bacteria. Results also suggested that its anti-adhesive properties may be attributed to the ability to degrade DNA as enzyme retained its bioactivity after immobilization.

Summarizing, the study conducted in this sub-chapter highlighted the great potential of dopamine chemistry to immobilize enzymes without compromise their biological activity and the great potential of DNase I to create multi-functional coatings and impart them with anti-adhesive properties.

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SUPPLEMENTAL MATERIAL

A)

	GA linker	Incubation time [h]	Temperature [ºC]	Attachment reduction [%]
Lysozyme	No	overnight	4	Ns
	No	3	RT	Ns
	Yes	overnight	4	Ns
Proteinase K	No	overnight	4	54.1
	No	3	RT	39.3
	Yes	overnight	4	Ns

B)

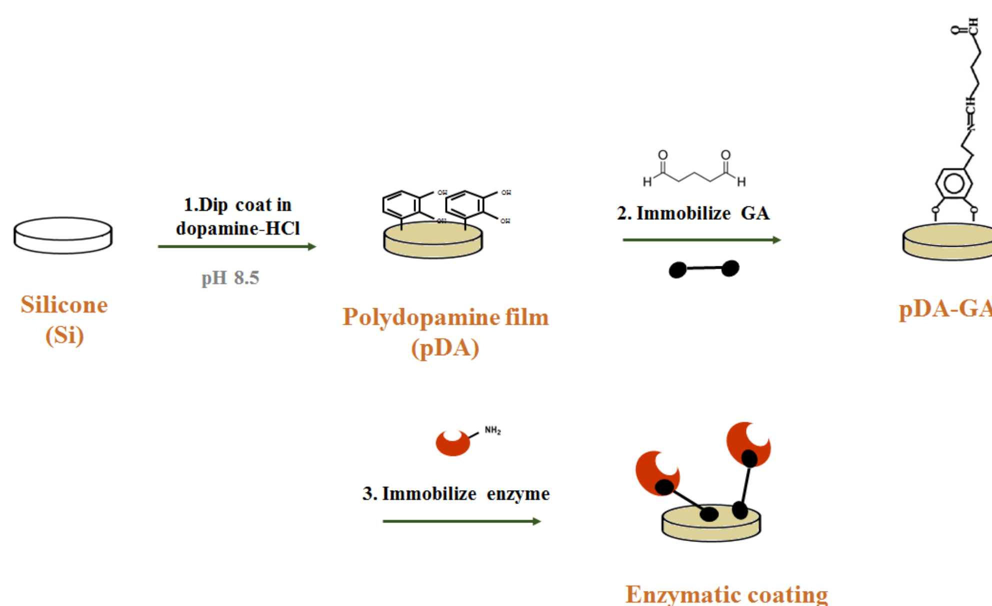


Figure S2. Establishment of key experimental parameters for optimally immobilization of lysozyme and proteinase K onto PDMS material. After pDA functionalization, PDMS coupons were immersed in solutions of lysozyme or proteinase K (1 mg/mL in PBS) and incubated for different periods of time (3 h or overnight, 16-18 h) and different temperatures (4 °C or RT). The influence of a glutaraldehyde linker was also evaluated and immobilization was performed as illustrated in B). PDMS was first covered with a pDA film followed by immobilization of enzymes with GA as a bi-functional linker. Glutaraldehyde provides the reactive aldehyde groups to react with both pDA and different enzyme moieties, mainly involving primary amino groups. As output to determine the effect of experimental parameters, an attachment assay of a clinical isolate of *S. aureus* for 4 h was performed and the percentage of attachment reduction, as compared to bare PDMS, was determined. It was identified the combination of pDA functionalization of PDMS without GA as a linker followed by overnight incubation at 4 °C in enzymes solutions as providing the optimal immobilization parameters.

Chapter 5

FROM MONO TO BI-FUNCTIONAL ANTIBACTERIAL COATINGS

5.1

Co-immobilization of Palm and DNase I to create an antimicrobial and anti-adhesive bi-functional coating

Bacterial colonization of indwelling devices is very often a prelude for biofilm formation and infection. BAI remain a clinical challenge with serious medical and economic consequences, due to their resistance to antimicrobials and to the host immune system. This study aimed to co-immobilize the antimicrobial lipopeptide Palm and the enzyme DNase I to introduce both antimicrobial and anti-adhesive functionalities to PDMS, using dopamine chemistry. Surface characterization confirmed the immobilization of both compounds and that Palm did not detach from the surfaces for up to 5 days. Co-immobilization of both agents resulted in a bi-functional coating able to prevent the single and co-adhesion of *S. aureus* and *P. aeruginosa*, kill the adherent ones, showing no toxicity towards mammalian cells. The overall results highlighted that PDMS functionalization with Palm and DNase I holds great potential to fight BAI if explored in the development of implants or medical devices.

INTRODUCTION

BAI remain the leading cause of failure of biomaterial implants and medical devices. These infections pose a number of clinical and economic challenges due to their resistance to antimicrobials and to the host immune system, and, regardless of the complexity of the implant, all medical devices are prone to microbial colonization and infection [1]. The fate of a biomaterial has been described as a race between its integration into the surrounding tissue and bacterial adhesion to its surface [2, 3]. When the race is won by bacteria, the implant surface will become rapidly covered by a biofilm [2, 4], a microconsortia of surface adhering cells encased in a self-produced matrix of EPS [5]. This extracellular matrix, which is mainly comprised of water, polysaccharides, proteins and eDNA, makes biofilms the most successful forms of life on earth as it provides architectural stability, mechanical support, mediates cell-cell and cell-surface interactions and acts as a protective barrier [6]. Treatment procedures for patients suffering from BAI usually involve intravenous and oral antibiotic therapy in which high doses can be administered for several weeks or months [7]. Emerging microbial resistance to widely prescribed antibiotics compromises the success of this approach and, very often, the treatment fails, and the only solution for the infected implant is its surgical removal, at the expenses of patient suffering and considerable costs [8].

Since bacterial adhesion to the surface of a biomaterial is the first step in biofilm formation, a number of surface modifications have been developed aiming to reduce the contact with approaching bacteria. These anti-adhesive coatings are well known in the literature, mainly the ones using hydrophilic polymer brush coatings, but none of them was able to completely prevent microbial adhesion [9-11]. The performance of anti-adhesive coatings may be improved by adding functionalities that prevent adhering bacteria from growing into a biofilm and to stimulate host tissue cell adhesion, depending on the application intended for the biomaterial.

The aim of the current study was to co-immobilize the AMP Palm and DNase I onto PDMS, using dopamine chemistry. It was intended to obtain a bi-functional coating that combines both anti-adhesive and antimicrobial properties able to prevent bacterial adhesion and subsequent biofilm formation, with low cytotoxicity.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Two reference strains of *P. aeruginosa* (ATCC 39324 and ATCC 27853), a reference strain of *S. aureus* (ATCC 25923) as well as clinical isolates of *S. aureus* and *S. epidermidis* were used throughout this study. The strains were preserved and cultured as described in Chapter 2.

ANTIMICROBIAL PEPTIDE AND ENZYME

The enzyme DNase I and the AMP Palm were used in this study.

POLYDOPAMINE COATING AND FURTHER FUNCTIONALIZATION

Prior to surface modification, PDMS coupons were cleaned and prepared as described in Chapter 2. Coatings were prepared as illustrated in Figure 2B of Chapter 2, using a 2-step immobilization approach. For pDA coating, PDMS coupons were immersed in dopamine (2 mg/mL dopamine-HCl in 10 mM bicine buffer, pH 8.5) for 18 h, at RT and under agitation (70 rpm). Coupons were then rinsed with UP water and air-dried. For further functionalization, pDA-coated coupons were immersed in DNase I solution (1 mg/ml in 150 mM NaCl, 10 mM potassium phosphate buffer supplemented with 10 mM $MgCl_2$, pH 6.8) or in lipopetide Palm solution (1 mg/mL in PBS, pH 7.4) and were incubated for 6 h, at RT under agitation (70 rpm). Co-immobilization was performed by immersing pDA-coated coupons in a mixture solution composed of DNase I and Palm at different proportions.

SURFACE CHARACTERIZATION

The surface morphology and roughness of the materials was analysed by SEM and AFM as described in Chapter 2. Static water contact angle measurements were also performed by a sessile drop method also described in Chapter 2. Experiments were performed in triplicate.

PHYSICOCHEMICAL CHARACTERIZATION OF SURFACES AND CELLS

The hydrophobicity parameters of material surfaces and bacteria were determined using the sessile drop contact angle method as described in Chapter 2. Experiments were performed in triplicate.

PEPTIDE IMMOBILIZATION EFFICIENCY AND COATINGS STABILITY

The efficiency of peptide immobilization and coatings stability was performed as described in Chapter 2. Three and two independent assays with three replicates for each condition tested were performed.

BACTERIAL VIABILITY ON MODIFIED SURFACES

Antibacterial performance of the generated surfaces against bacterial adhesion was evaluated by a live/dead staining as described in Chapter 2. Two or three independent assays with three replicates for each condition tested were performed.

LOCALIZATION AND DISTRIBUTION OF BACTERIAL POPULATIONS BY PNA FISH

In order to assess bacterial spatial organization and the species distribution on the coated surfaces, PNA FISH method was employed as described in Chapter 2. Three independent assays with three replicates for each condition tested were performed.

EVALUATION OF BIOFILM FORMATION BY XTT REDUCTION ASSAY

In order to investigate the potential of modified coatings to impair biofilm formation, the respiratory activity of biofilm cells was evaluated using the XTT colorimetric method as described in Chapter 2. Two independent assays with three replicates for each condition tested were performed.

RESULTS

CO-IMMOBILIZATION OF DNASE I AND PALM ONTO PDMS

In the present study, a pDA-based surface modification was applied to co-immobilize Palm and DNase I onto PDMS to impart it with both antimicrobial and anti-adhesive properties. This AMP and enzyme were the agents selected based on the results reported in Chapters 3 and 4. Surface modification involved the deposition of a uniform coating of pDA from a dopamine-HCl solution at a slightly alkaline pH. The pDA coating was then used as a platform for peptide and/or enzyme's immobilization. For co-immobilization, a mixture solution composed of DNase I and Palm at different proportions were investigated and a proportion of 1:3, respectively, yielded the best combination of both anti-adhesive and antimicrobial properties (details can be found in Figure S1 of Supplemental Material).

SURFACE CHARACTERIZATION

Surface morphology of modified PDMS surfaces was characterized using SEM analysis. The unmodified PDMS surfaces exhibited smooth surface morphology compared with the modified ones (Figure 1). Self-polymerized pDA particles could be observed on modified PDMS coupons confirming the pDA coating. Further functionalization with AMP and/or enzyme yielded surfaces with different morphologies, depending on the compound immobilized. Surfaces functionalized with DNase I present a rougher surface morphology as compared to the ones with Palm, which can be attributed to the presence of more and bigger self-polymerized pDA particles. Co-immobilization of both compounds yields surfaces with an intermediate morphology.

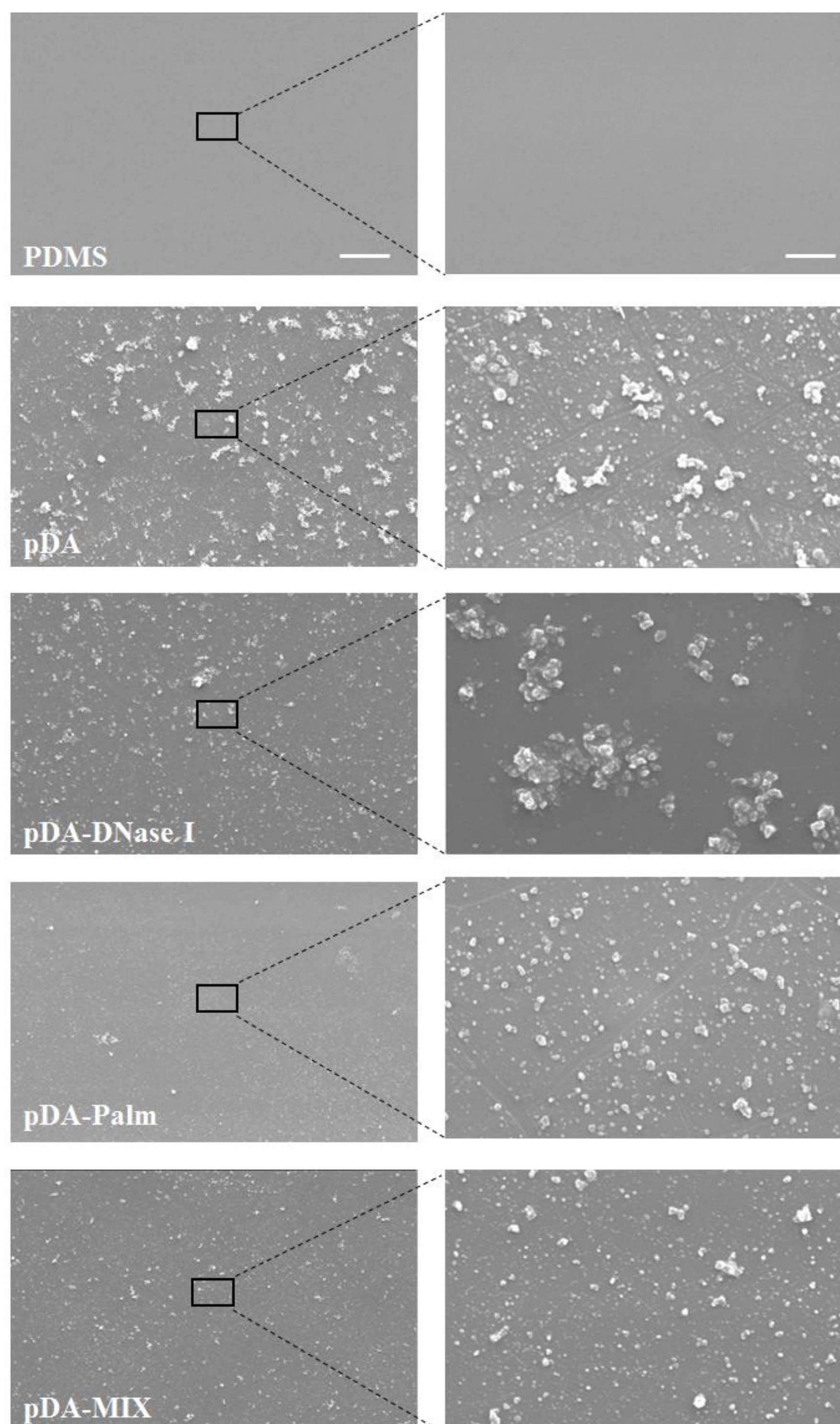
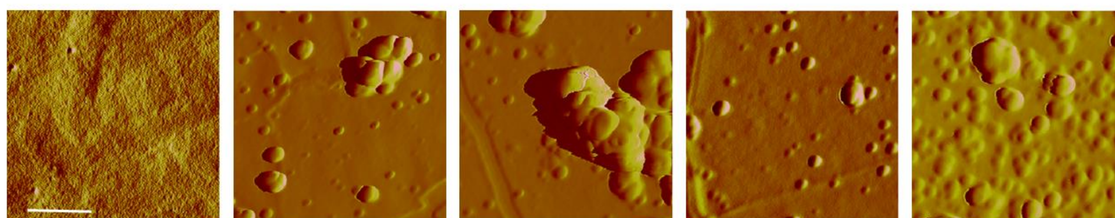


Figure 1. SEM images of unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS surfaces functionalized with DNase I (pDA-DNase I), Palm (pDA-Palm) and both DNase I and Palm [1:3] (pDA-MIX).The scale bars in the left and right column indicate 1 and 10 µm, respectively.

For further surface morphology characterization, samples were also analysed by AFM (Figure 2). AFM results confirmed that bare PDMS possessed a smoother morphology as compared to

modified surfaces. From the AFM images, it was possible to measure the average roughness of surfaces (Figure 2B). Results indicated that the presence of a pDA layer increased the surface roughness of PDMS, which is in agreement with reported studies [12, 13]. Further functionalization with DNase I yielded surfaces with a heterogeneous roughness as the values measured within the same surface presented a large range, suggesting that DNase I immobilization was not successful as Palm's. However, unlike in previous studies reporting other peptides immobilization using pDA as an intermediate layer [12, 14, 15], Palm immobilization decreased surface roughness which correlates with SEM results. When peptide was co-immobilized with enzyme, surface roughness increased which may be attributed to the presence of DNase I.

A)



B)

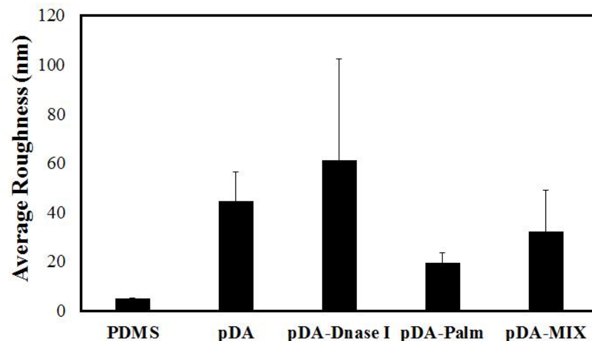


Figure 2. (A) AFM images of unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS surfaces functionalized with DNase (pDA-DNase I), Palm (pDA-Palm) and both DNase I and Palm [1:3] (pDA-MIX). The scale bar indicates 1 μm. (B) Average surface roughness (R_a) of unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS surfaces functionalized with DNase I (pDA-DNase I), Palm (pDA-Palm) and both DNase I and Palm [1:3] (pDA-MIX).

To evaluate the surface wettability of the PDMS after surface modification, the static water contact angle of surfaces after each deposition step was measured (Figure 3). Bare PDMS is inherently hydrophobic, with a high contact angle of 109.9° ± 3.0°. Its functionalization with pDA greatly enhanced the hydrophilicity of the polymer surface, decreasing the contact angle to 56.6°

± 4.8°, which is a well-established observation in other material surfaces as well [14, 16]. Further immobilization with DNase I or Palm slightly increased the contact angle to 75.8° ± 16.7° and 77.3° ± 14.7°, respectively, which may be attributed to the presence of hydrophobic amino acid residues in the enzyme [17] and Palm's hydrophobic lipophilic tail. Co-immobilization of enzyme and peptide yielded surfaces more hydrophobic with a contact angle of 97.9° ± 10.2°, which can be attributed to an addition effect provided by each compound.

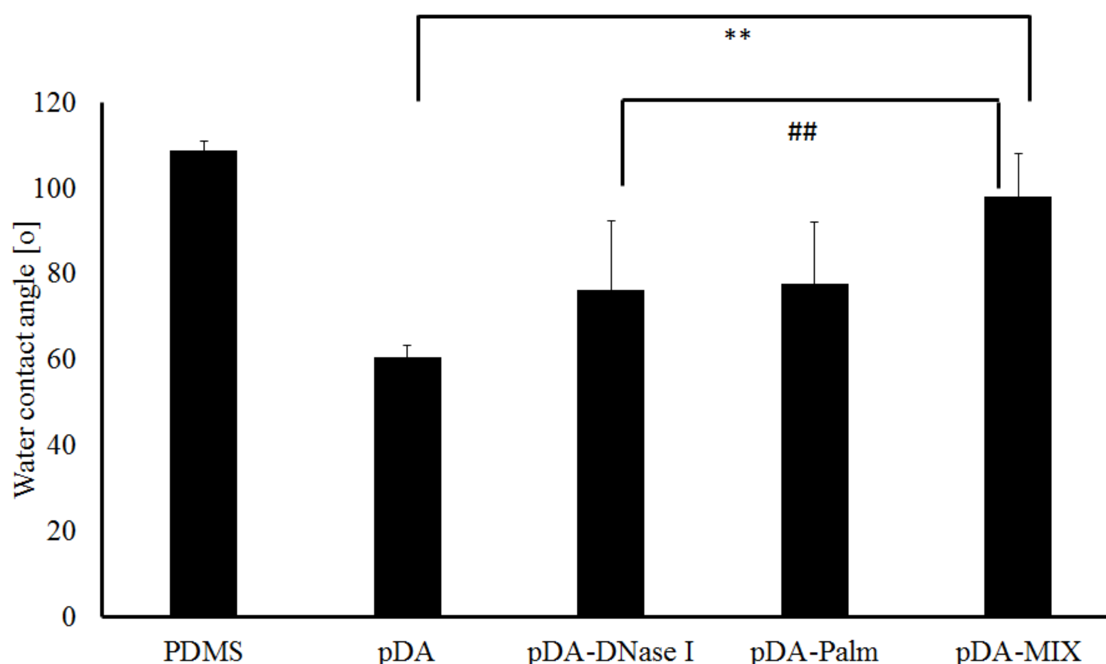


Figure 3. Water contact angles of unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS surfaces functionalized with DNase I (pDA-DNase I), Palm (pDA-Palm) and both DNase and Palm (pDA-MIX).Significant differences were found for (**) $p < 0.01$, compared to PDMS control and (##) $p < 0.01$, compared to pDA control.

PEPTIDE IMMOBILIZATION EFFICIENCY AND STABILITY

To quantify the coating efficiency of Palm, the buffer solutions containing the unattached peptides were retrieved immediately after finishing the coating process. The fluorescamine assay revealed that 65.9 % ± 9.7 % of loaded peptide was immobilized onto the pDA-coated PDMS surface. A similar immobilization efficiency has been previously reported and it proved to be efficient for its application [14]. The fact that most AMP present local toxicity or haemolytic activity has limited

their applications as therapeutics to be administered into the bloodstream [18]. In the present study, this limitation could be overcame by peptide's immobilization as the fluorescamine assay revealed that 56.7 % ± 2.1 % of loaded Palm was still immobilized after being incubated in PBS for 5 days.

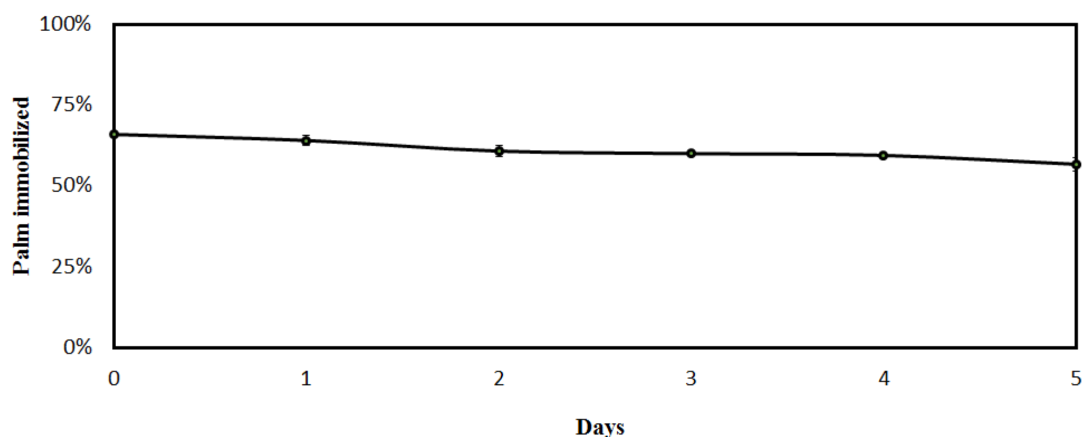


Figure 4. Efficiency of pDA-mediated peptide immobilization. Fluorescamine assay was performed to determine the immobilization efficiency of pDA-coated PDMS functionalized with Palm under physiologically relevant conditions (PBS at 37°C) for 5 days.

ANTIBACTERIAL PERFORMANCE OF MONO AND BI-FUNCTIONAL COATINGS

Prior to co-immobilization of DNase I and Palm, their immobilization was performed alone. The antibacterial performance of these mono-functional coatings was investigated by performing an attachment assay in which bacteria were allowed to attach for 4 h and the remaining cells on the PDMS surfaces were imaged with fluorescence microscopy. For these analysis, a clinical isolate of *S. aureus* was chosen because of its clinical relevance.

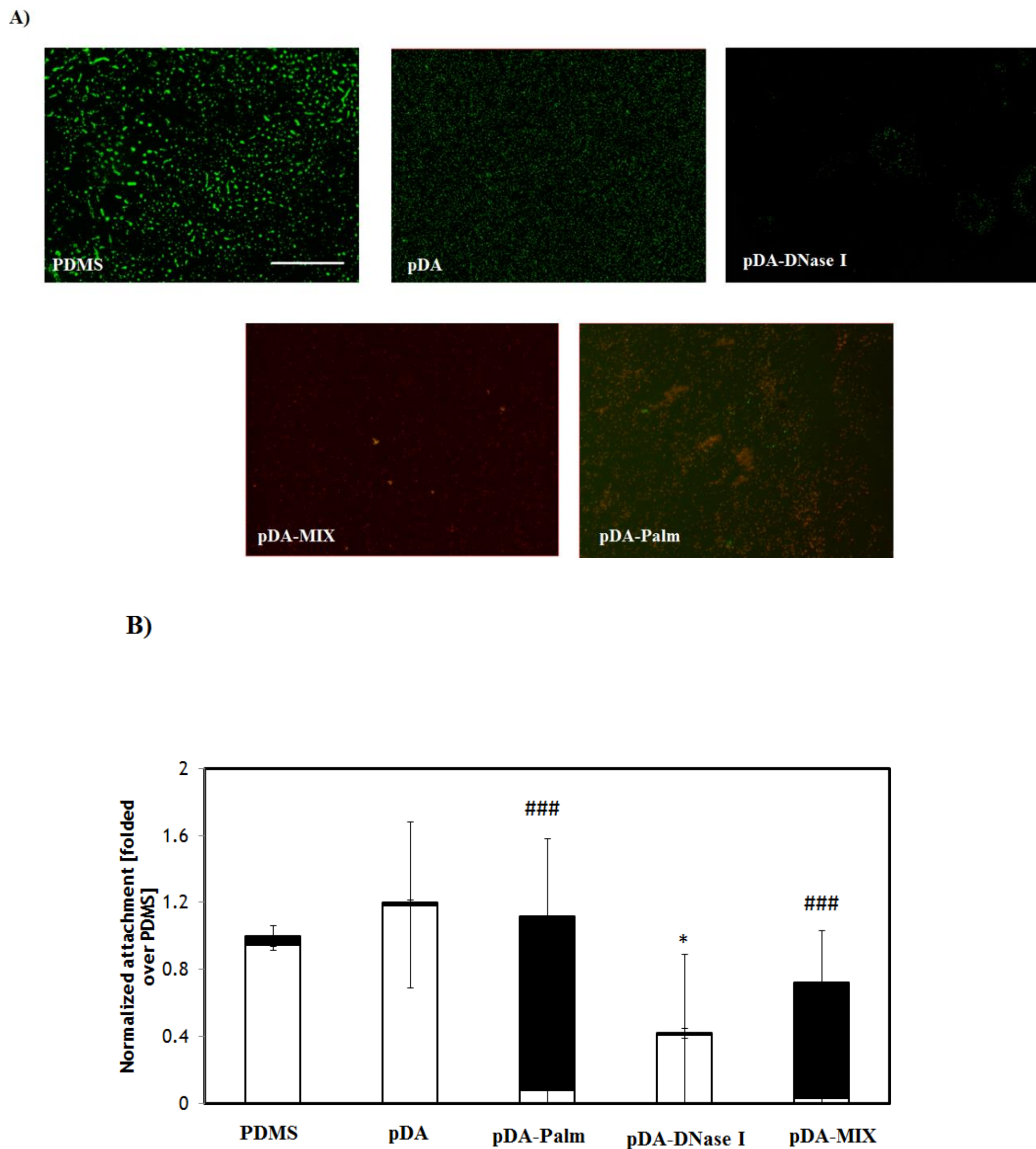


Figure 5. (A) Representative fluorescent live/dead stain images obtained during adhesion assays of a clinical isolate of *S. aureus* .The scale bar indicates 100 μm. (B) Normalized attachment and viability of *S. aureus* on unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS surfaces functionalized with DNase I (pDA-DNase I), Palm (pDA-Palm) and both DNase and Palm (pDA-MIX). All values were normalized to PDMS control. Significant differences were found for (*) $p < 0.05$, compared to PDMS control attachment and (###) $p < 0.001$, compared to PDMS fraction of dead cells.

Bare PDMS surfaces allowed the adhesion of *S. aureus* cells, which have formed agglomerates, and most of them remained alive. Polydopamine-coated surfaces had no significant effect on bacterial attachment or cell viability but cells were more evenly distributed along these surfaces.

Further functionalization with Palm (pDA-Palm), had no effect on bacterial attachment but was responsible for a greater fraction of dead cells. Enzyme mono-functional coating (pDA-DNase I), on the other hand, had no significant effect on cell viability as compared to bare PDMS surfaces but was able to prevent bacterial attachment.

Once confirmed the anti-adhesive and antimicrobial properties conferred by immobilization of DNase I and Palm, respectively, their co-immobilization was performed so that a bi-functional coating integrating both functionalities could be developed. Results showed that with this combination (pDA-MIX), the fraction of dead cells greatly increased as compared to unmodified PDMS and bacterial attachment was slightly reduced as compared to PDMS functionalized with Palm alone, suggesting the additional effect conferred by the presence of DNase I.

ADHESION OF DUAL-SPECIES TO MONO AND BI-FUNCTIONAL COATINGS

In real situations, microorganisms occur in complex ecosystems, where bacteria may present symbiotic relationships and/or distributions that confer best conditions to survive [19]. Most of the coating approaches developed to fight BAI only investigates the antibacterial performance against bacterial strains as single species. The performance of mono and bi-functional coatings proposed in the present study was, therefore, investigated against dual-species adhesion. For that, bacteria were allowed to attach for 4 h and the remaining cells on the PDMS surfaces were imaged with fluorescence microscopy after live/dead staining.

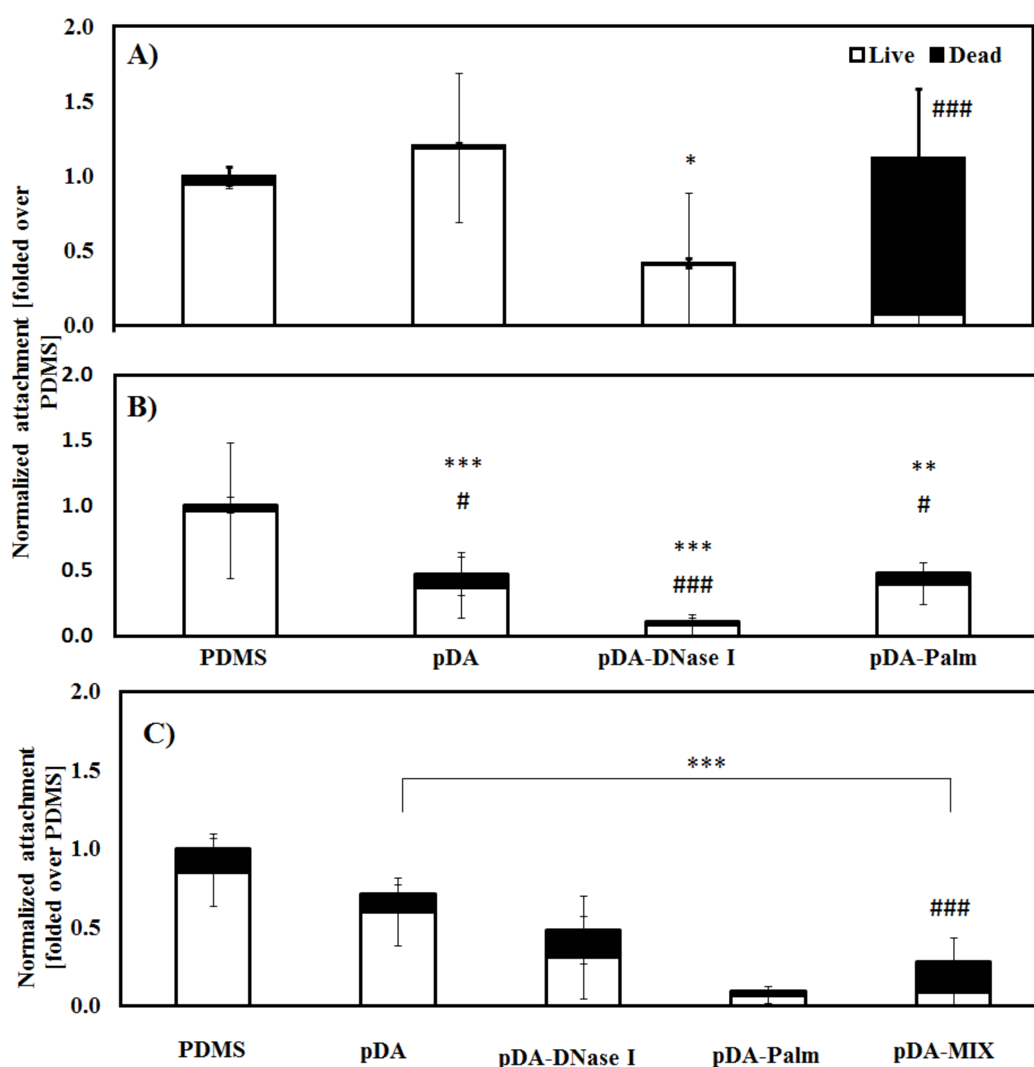


Figure 6. Normalized attachment and viability of cells of a clinical isolate of *S. aureus* (A), *P. aeruginosa* ATCC 39324 (B) single-species and co-adhesion (C) on unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS surfaces functionalized with DNase I (pDA-DNase I), Palm (pDA-Palm) and both DNase and Palm (pDA-MIX). Significant differences were found for (***) $p < 0.001$, compared to PDMS control attachment and (###) $p < 0.001$, compared to PDMS fraction of dead cells.

The fitness of dual-species adhesion (Figure 6C) was compared to single-species adhesion (Figure 6A and B) to mono-functional coatings. The clinical isolate of *S. aureus* and a reference strain of *P. aeruginosa* were used in this assay. As previously established, mono-functional coatings of DNase I (pDA-DNase I) was able to prevent *S. aureus* clinical isolated while Palm mono-functionalization generated surfaces able to kill most of bacteria (Figure 6A). When it comes to *P. aeruginosa* adhesion to these mono-functional coatings, different antibacterial effects were observed. PDMS functionalized with pDA was able to prevent bacterial adhesion and also

affected cell viability as compared to bare PDMS surfaces. These results may be attributed to differences found on the hydrophobicity parameters of bacteria and surfaces, which were evaluated through contact angle measurements, using van Oss approach [20] (Table 1).

The two reference strains showed water contact angles lower than 65° and positive values of free energy of interaction (ΔG_{iwi}), which are indicative of a hydrophilic surface [21, 22]. From the physico-chemical parameters of each adhesion entity (bacteria and surface), it was possible to determine the thermodynamic relation between both entities, namely the free energy of adhesion (Table 1B). Results suggested that adhesion to both unmodified PDMS and pDA-coated PDMS is less favoured for the *P. aeruginosa* strain, as indicated by the higher values of free energy of adhesion.

PDMS surfaces functionalized with DNase I was able to prevent bacterial attachment while mono-functional coatings of Palm yielded similar results to pDA coating alone. Adhesion of *S. aureus* together with *P. aeruginosa* to bare PDMS surfaces yielded a higher fraction of dead cells as compared to their mono-species adhesion, suggesting an antagonistic interaction between bacterial strains. The presence of a pDA layer was responsible for reducing bacterial attachment. As *P. aeruginosa* was affected by the hydrophilic parameters of pDA-coated PDMS surfaces, this results suggested that adhesion on bare PDMS was dominated by this strain. DNase I-based mono-functional coating was able to reduce bacterial attachment without significant effect on cell viability. These results are in accordance with the fitness observed for mono-species adhesion, as PDMS functionalized with DNase I was able to prevent the attachment of both strains, alone. Immobilization of Palm onto PDMS pDA-modified surfaces yielded interesting results as they were able to prevent bacterial attachment in a greater extent as compared to DNase I. These results also suggested that *P. aeruginosa* had some predominant effect on *S. aureus* adhesion. Co-immobilization of both peptide and enzyme at a proportion of 1:3 (pDA-MIX) yielded a bi-functional coating able to prevent bacterial attachment of both strains and increase the fraction of dead cells, as compared to bare PDMS surfaces. These results indicated that bi-functional coatings developed in this study retained their both anti-adhesive and antimicrobial properties also against dual-species adhesion.

Table 1. (A) Values of contact angles (°) with water (θ_W), formamide (θ_F), α-bromonaphtalene (θ_B), surface tension parameters (mJ/m²), and free energy of interaction (ΔG_{iwi}^{TOT}) (mJ/m²) between the bacteria and the surfaces (i) when immersed in water (w). (B) Free energy of adhesion between bacteria (b) and the surfaces (s). Values are means ± SD.

(A)

Bacteria/Surface	**Contact angle (°)**			**Surface Tension Parameters (mJ/m²)**			**Free energy of interaction (mJ/m²)**
	θ_W	θ_F	θ_B	γ_i^{LW}	γ_i^+	γ_i^-	ΔG_{iwi}^{TOT}
S. aureus	12.6 ± 4.7	14.9 ± 7.5	37.6 ±5.3	35.7	1.9	54.1	30.6
P. aeruginosa	35.8 ± 11.6	86.5 ± 15.9	26.9 ± 3.4	39.7	0	127.8	121.0
PDMS	108.6 ± 3.2	104.4 ± 7.6	55.9 ± 6.0	27	0	5.6	-54.6
pDA	59.2 ± 2.4	22.2 ± 10.0	13.6 ± 5.7	43.2	2.5	10.6	-32.2

(B)

Bacteria	**Free energy of adhesion (mJ/m²)** ΔG_{bsb}^{TOT}	
	Si	Si-pDA
S. aureus	2.2	5.1
P. aeruginosa	34.4	38.9

In order to confirm some of the hypothesis raised from aforementioned results, and also directly visualize the location and distribution of bacterial strains within the dual-species community, adhesion of both strains in the same conditions was allowed to proceed on PDMS surfaces that were, afterwards, observed under a fluorescence microscope after applying a multiplex PNA FISH methodology counterstained with DAPI. The strength of each fluorescent signal allowed to distinguish *P. aeruginosa* (red rod-shaped cells) and *S. aureus* (blue cocci) within the bacterial consortia.

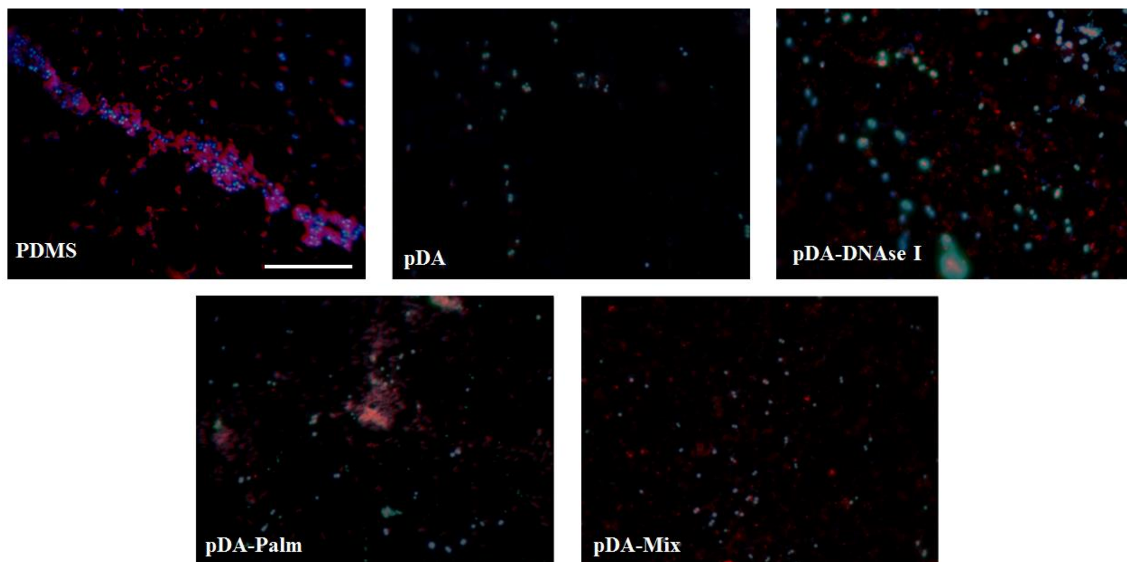


Figure 7. Multiplex PNA-FISH applied to dual-species adhesion onto unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS surfaces functionalized with DNase I (pDA-DNase I), Palm (pDA-Palm) and both DNase I and Palm (pDA-MIX). The scale bar indicates 20 μm.

Results confirmed that *P. aeruginosa* was the dominant organism with *S. aureus* adhering afterwards on *P. aeruginosa* agglomerates. No significant differences could be observed on bacterial distribution on different PDMS modified surfaces.

In order to validate the potential of the bi-functional coating developed in this study, other combinations of bacterial strains was also evaluated, namely two reference strains of *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 27853) (Figure 8A) and two clinical isolates of *S. aureus* and *S. epidermidis* (Figure 8B).

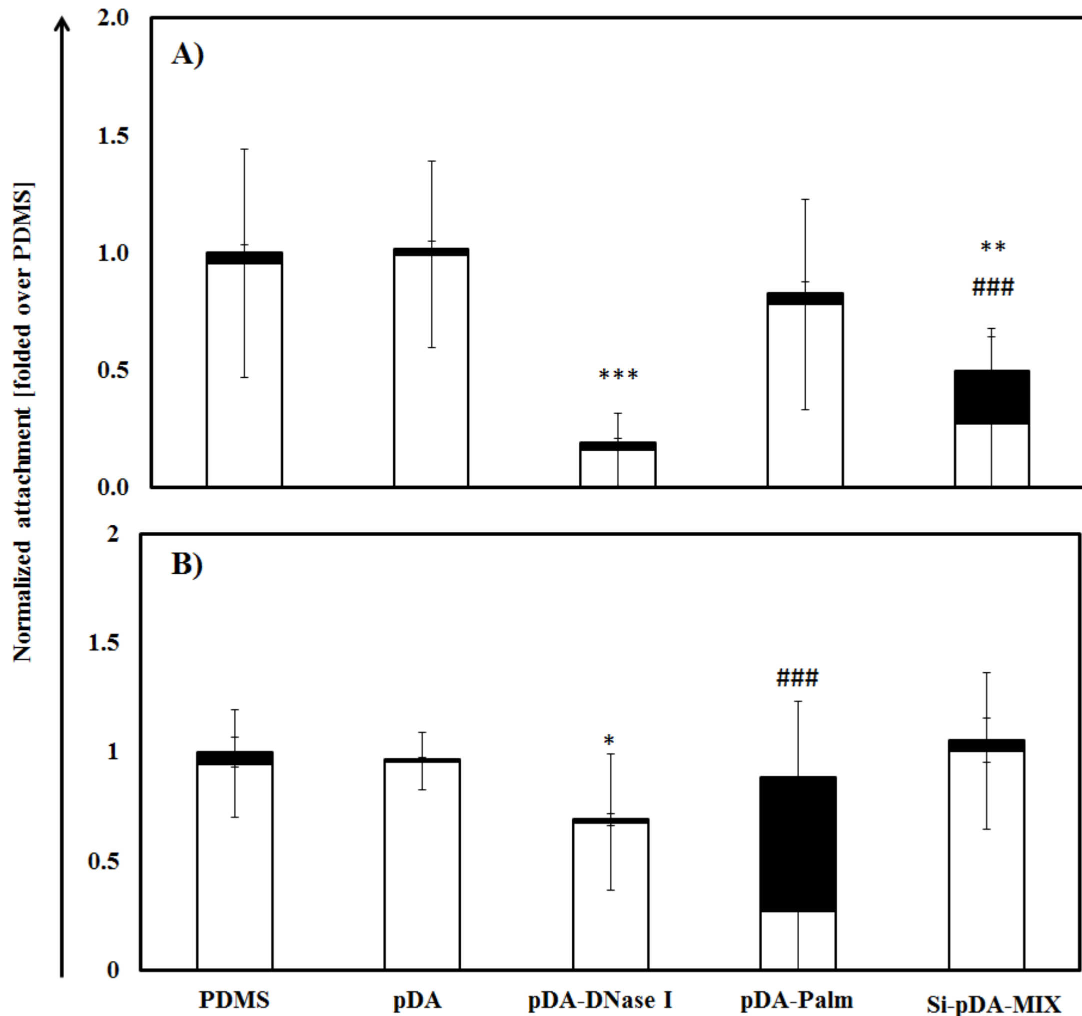


Figure 8. Co-adhesion of *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 (A) and clinical isolates of *S. aureus* and *S. epidermidis* (B) on unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS surfaces functionalized with DNase I (Si-pDA-DNase I), Palm (pDA-Palm) and both DNase I and Palm {1:3] (pDA-MIX). Significant differences were found for (*) $p < 0.5$, (**) $p < 0.01$ and (***) $p < 0.001$, compared to PDMS control attachment and (###) $p < 0.001$, compared to PDMS fraction of dead cells.

Co-adhesion of reference strains of *S. aureus* and *P. aeruginosa* to PDMS surfaces functionalized with pDA had no significant effect on bacterial attachment or cell viability as compared to bare PDMS surfaces. In a similar way to the aforementioned combination of bacterial strains, mono-functional coatings with DNase I (pDA-DNase I) reduced bacterial attachment. On the other hand, surfaces functionalized with Palm (pDA-Palm) had no significant effect on cell viability. These results are in accordance with Palm's antimicrobial activity determined against planktonic cultures of *P. aeruginosa* ATCC 27853 as its MBC could not be detected within the range investigated. Results also suggested that *P. aeruginosa* was again the dominant organism. Co-

immobilization of both peptide and enzyme at a proportion of 1:3 (pDA-MIX) yielded surfaces able to prevent bacterial attachment of both strains and increase the fraction of dead cells, as compared to bare PDMS surfaces. These results indicated that bi-functional coatings developed in this study retained their both anti-adhesive and antimicrobial properties also against the adhesion of these two different strains as well. The other combination of bacterial strains, clinical isolates of *S. aureus* and *S. epidermidis*, were also able to adhere to unmodified PDMS surfaces and the presence of a pDA layer had no significant effect on their attachment or viability. Mono-functional coatings prepared with DNase I (pDA-DNase I) were able to reduce bacterial attachment at a lower extent than from the other combinations investigated. Results suggest that eDNA effect may not be a structural component as important as on *S. aureus* adhesion, and DNase I presence on bi-functional coatings predominated over Palm antimicrobial activity. Palm-based coatings retained their antimicrobial activity against these two species. Bi-functional coatings combining both bioactive compounds (pDA-MIX), however, were not able to significantly impair bacterial attachment or cell viability.

ANTI-BIOFILM PROPERTIES OF MONO AND BI-FUNCTIONAL COATINGS

To investigate the anti-biofilm properties of coatings, the clinical isolate of *S. aureus* was allowed to grow in TSB for 24 h and biofilm cells viability were evaluated using a XTT assay. In this assay, optical density values are proportional to the number of metabolic active cells adhered on the surfaces. As shown in Figure 10 unmodified PDMS exhibited the higher values of optical density, which confirms that PDMS surfaces are prone to *S. aureus* adhesion and subsequent biofilm formation. The presence of a pDA layer slightly decreased cell activity, which may be attributed to the difference adhesion patterns previously observed: *S. aureus* adhered to PDMS surfaces formed agglomerates unlike on pDA where cells were evenly distributed within the surface. Therefore, cell agglomerates on bare PDMS surfaces may have contributed to increase the optical density measured in XTT assay. Mono-functional coatings of DNase I (pDA-DNase I) had no effect on biofilm formation but PDMS functionalized with Palm (pDA-Palm) greatly decreased biofilm cells metabolic activity. Bi-functional coatings (pDA-MIX) had no significant effect on biofilm cells metabolic activity which may be attributed to the presence of DNase I.

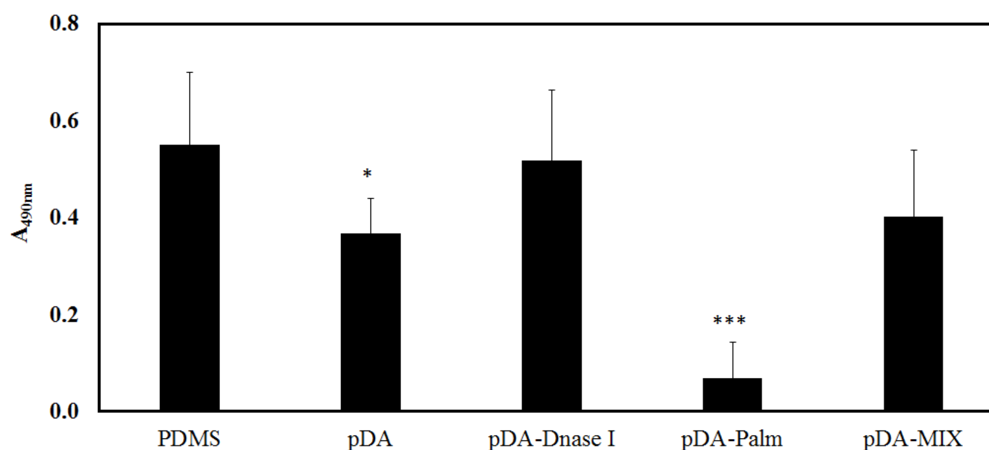


Figure 9. Metabolic activity of biofilm cells adhered unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS surfaces functionalized with DNase I (pDA-DNase I), Palm (pDA-Palm) and both DNase and Palm (pDA-MIX). Significant differences were found for (*) $p < 0.5$ and (***) $p< 0.001$, compared to PDMS control.

EFFECT OF PDMS MODIFIED SURFACES ON 3T3 FIBROBLAST GROWTH AND ADHESION

To predict the effects of mono and bi-functional coatings developed in this study on animal cells, an assay of cytotoxicity was performed (Figure 10). Results showed that further functionalization of pDA-coated PDMS surfaces had no significant effect on 3T3 fibroblast growth and adhesion. Bi-functional coatings (pDA-MIX) slightly increased cell metabolic activity.

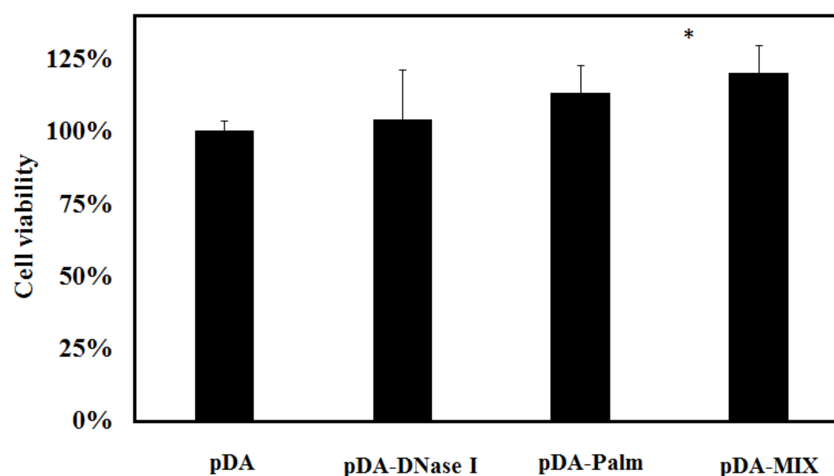


Figure 10. Viability of mammalian cells after 48 h of contact with pDA-coated PDMS (pDA) and pDA-coated PDMS surfaces functionalized with DNase I (pDA-DNase I), Palm (pDA-Palm) and both DNase and Palm (pDA-MIX), measured with an MTS assay. Significant differences were found for (*) $p < 0.5$ compared to pDA control.

DISCUSSION

In the fight to prevent BAI several approaches to impart biomaterial surfaces with antibacterial properties have been developed in the last years, with great emphasis on anti-adhesive and antimicrobial coatings [10, 11]. There are, however, crucial limitations associated to both strategies. The emergence of multi-drug resistant bacteria and toxicity concerns are frequently associated to the usage of antimicrobials such as antibiotics and silver. Another disadvantage associated to antimicrobial contact-killing surfaces is the accumulation of dead bacteria on the antimicrobial coatings. These bacteria may allow the adhesion of other bacteria and this promote more bacterial accumulation on the surface, reducing its antimicrobial activity over time [23]. Anti-adhesive coatings are not able to completely prevent bacterial ahesion and their formulation often requires complex, labour and time-consuming techniques as well as the usage of organic solvents which may affect the integrity of biomaterials [11].

The present study aimed to prepare a bi-functional coating incorporating the strengths of both strategies: to kill bacteria and simultaneously prevent their adhesion to surfaces, using the facile and non-toxic approach developed by Messersmith and co-workers [24]. To confer surfaces with antimicrobial activity, the AMP Palm was chosen. As the first line of defence of animals and plants against invading pathogens, AMP exhibit important features that make them promising candidates for clinical applications and potential alternatives to conventional antibiotics, including a low propensity for developing microbial resistance [25, 26]. Palm belongs to a new group of lipopeptides with potent antifungal and antibacterial activities. These lipopeptides are derived from positively charged peptides containing D- and L- amino acids (diastereomers) that are palmitoylated at their N terminus [27]. As a lipopetide its mechanism of action consists of simple disruption of membrane electric potential [28]. Anti-adhesive component of bi-functional coatings was provided by the enzyme DNase I, targeting eDNA. Because eDNA facilitates the initial stage of bacterial adhesion to biomaterials and, virtually, all bacterial populations produce this structural component, it was hypothesized that it could be a general target [29]. Furthermore, the immobilization of DNase I onto biomaterial surfaces, using a pDA layer as an intermediate, has proved to be effective in preventing bacterial adhesion and biofilm formation up to 14 h [30].

Surface characterization studies confirmed the immobilization of Palm and DNase I onto pDA-coated PDMS surfaces and that peptide did not detach from the surface for up to 5 days. Enzyme immobilization did not compromise its catalytic activity (Chapter 4.2). To assess the

antibacterial performance of developed coatings, adhesion assays were performed in which bacterial cells were allowed to adhere for 4 h. This period of time was chosen because the first 6 h after surgery (the so-called "decisive period") are identified as being critical for preventing bacterial adhesion in order to assure the long-term success of the implant [31]. During this period of time, there is a competition between integration of the material into the surrounding tissue and adhesion of bacteria to the implant surface [2]. Attachment assay confirmed that immobilization of Palm onto pDA-coated PDMS surfaces (pDA-Palm) conferred them with effective antimicrobial properties given its ability to damage most of bacterial cells adhered to these coatings very fast. This behaviour agrees with lipopeptides ability to kill multi-resistant Gram-positive cocci, in solution, previously reported [32]. Enzymatic mono-functional coating (pDA-DNase I) was able to prevent bacterial attachment suggesting its suitability to confer the anti-adhesive properties intended for the bi-functional coating. Once confirmed the anti-adhesive and antimicrobial properties conferred by immobilization of DNase I and Palm, respectively, their co-immobilization was performed so that a bi-functional coating integrating both functionalities could be developed. Surface characterization of this bi-functional coating revealed a surface with intermediate roughness between the one achieved with both compounds, alone. Co-immobilization of DNase I and Palm yielded more hydrophobic surfaces which can be attributed to an addition effect provided by each compound.

Most of the coating approaches developed to fight BAI only investigate the antibacterial performance against bacterial strains as single species. In the present study, the performance of the proposed mono and bi-functional coatings against the adhesion of dual-species was also evaluated. DNase I-based mono-functional coating (pDA-DNase I) had a similar effect against co-adhesion of a clinical isolate of *S. aureus* and *P. aeruginosa* (ATCC 39324) as it was able to prevent the attachment of both strains, alone and together. Immobilization of Palm (pDA-Palm) yielded surfaces able to prevent bacterial attachment in a greater extent as compared to DNase I. Co-adhesion results were closer to the ones obtained with *P. aeruginosa* alone, suggesting that this strain had some predominant effect on *S. aureus* adhesion, which was further confirmed with PNA-FISH analysis. The bi-functional coating was able to prevent bacterial attachment of both strains and increase the fraction of dead cells, as compare to bare PDMS surfaces. The coatings retained, therefore, both anti-adhesive and antimicrobial properties against dual-species adhesion. When a different combination of bacterial strains was investigated, namely co-adhesion of clinical isolates of *S. aureus* and *S. epidermidis*, different performances could be observed.

Mono-functional coatings prepared with DNase I were able to reduce bacterial attachment of both strains but was not effective as it was with *S. aureus* and *P. aeruginosa* strains while PALM-based coatings retained their antimicrobial activity against these two species. Bi-functional coatings combining both bioactive compounds (pDA-MIX), however, were not able to significantly impair bacterial attachment or cell viability. These results may be attributed to the lower efficacy of DNase I against *S. epidermidis*. In fact, it has been reported that eDNA is a structural component of biofilm matrix of *S. aureus* but a minor in *S. epidermidis* matrix as DNase I treatment was not able to detach established biofilms of this last strain [33].

Once established the potential of mono and bi-functional coatings to prevent bacterial attachment and kill bacteria adhered to the surfaces during the first stages of bacterial colonisation process, it is important to evaluate coatings efficacy to impair biofilm formation. For that, *S. aureus* was allowed to grow in a nutrient-rich environment for 24 h and biofilm cells viability were evaluated using a XTT assay. Mono-functional coatings of DNase I had no effect on biofilm formation but PDMS functionalized with Palm greatly decreased biofilm cells metabolic activity. The better results obtained for Palm-based coating surfaces against biofilm formation may be attributed to its lower surface roughness as compared to the heterogeneous immobilization of DNase I. Results suggest, thus, that roughness surface had a more crucial effect on biofilm formation. Another possible explanation for DNase I inefficacy to prevent biofilm formation is the presence of proteases or macromolecules present in established biofilms which may interfered with enzyme activity. It is important to mention, that TSB is a very rich medium, so the worst case scenario was investigated. Bi-functional coatings had no significant effect on biofilm cells metabolic activity which may be attributed to the presence of DNase I. The performance of immobilized Palm in a rich medium also suggests that coating stability under *in vivo* conditions should not be compromised, a concern raised in Chapter 3.3.

In summary, a 2-step pDA-based surface modification strategy was applied to successfully co-immobilize an AMP and an enzyme targeting an important component of biofilm matrix. This immobilization approach imparted PDMS surfaces with both anti-adhesive and antimicrobial properties against the adhesion of relevant bacteria as single and dual-species, with excellent stability and no cytotoxicity, holding, therefore, great potential in the development of materials able to prevent BAI.

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SUPPLEMENTAL MATERIAL

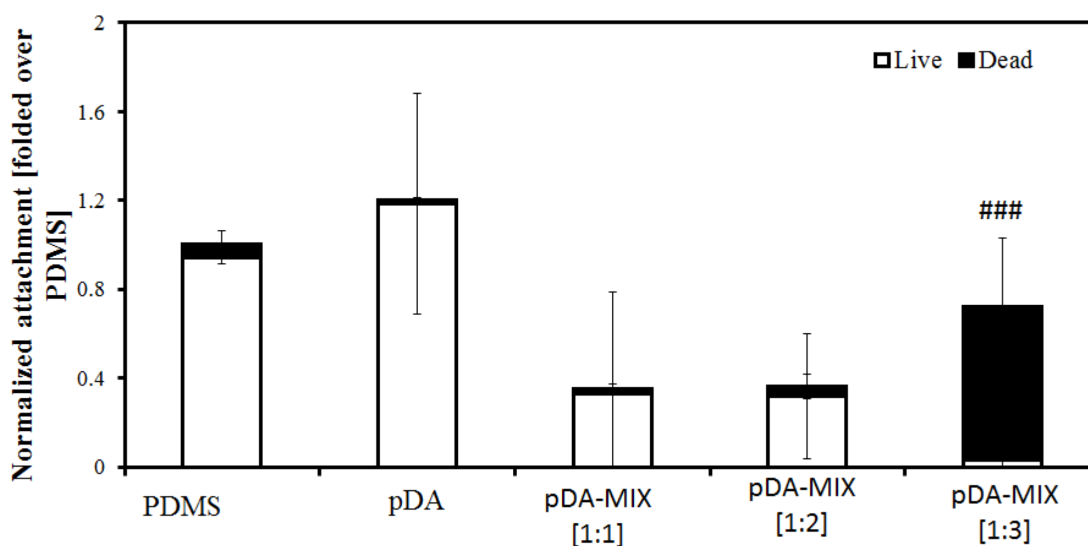

Figure S.1. **Optimization of co-immobilization of DNase I and Palm onto PDMS.** Bi-functional coatings (pDA-MIX) were prepared by mixing different proportions of DNase I and Palm and the antibacterial performance against a clinical isolate of *S. aureus* was evaluated. Significant differences were found for (###) $p < 0.001$, compared to PDMS fraction of dead cells. Co-immobilization of enzyme and peptide at proportions 1:1 and 1:2 yielded surfaces able to prevent bacterial attachment but no significant effect on cell viability, suggesting that DNase I was responsible for the predominant effect. Increasing the peptide proportion for 1:3, however, the fraction of dead cells greatly increased as compared to unmodified PDMS. In addition, bacterial attachment was slightly reduced as compared to PDMS functionalized with PALM alone (Chapter 3.4), suggesting the additional effect conferred by the presence of DNase I. This proportion was, therefore, chosen for further studies to investigate the potential of bi-functional coatings.

5.2

Studying the fate of bacteria adhering to bi-functional coatings

In the previous sub-chapter, Palm and DNase I were successfully co-immobilized onto PDMS to impart it with both anti-adhesive and antimicrobial properties against the adhesion of relevant bacteria as single and dual-species, with excellent stability and non-toxicity. This study aimed to investigate the fate of bacteria that managed to adhere to these modified surfaces in what concerns their susceptibility to antibiotic treatment, potential development of resistance and their clearance mediated by macrophages phagocytosis. Results showed that the bi-functional coating proposed in this thesis holds great potential to fight BAI as it proved to enhance bacterial susceptibility to antibiotic treatment and to macrophages phagocytosis, without developing bacterial resistance towards Palm immobilized.

INTRODUCTION

The development of materials able to resist bacterial colonisation has been proposed as a promising approach to fight BAI [1,2]. Although the results obtained with these strategies have been encouraging, there are some challenges in the field of antibacterial coatings that urgently need to be solved so they can be applied in clinical practice.

An important issue is related to the fact that most of the coatings reported in the literature are not able to completely prevent bacterial adhesion. Therefore, it is crucial to determine if the "few" bacteria that manage to adhere to these coatings are able to grow into a mature biofilm. For instance, Nejadnik *et al.* have demonstrated the ability of few bacteria that adhered on a polymer brush-coating to form a weakly adhering biofilm [3]. The authors, however, identified the slow formation of biofilms on these coatings and their relatively easy detachment as a clinical opportunity for prophylactically administration of antibiotics after implant surgery. Another challenge is the potential development of microbial resistance towards antimicrobials immobilized. Although, antimicrobials permanent immobilization has been described as an alternative approach to minimize this potential because it avoids exposure to sub-inhibitory concentrations [4], there is a lack of studies addressing these issues.

A crucial factor influencing the pathogenesis of BAI, often neglected in the field of antibacterial coatings, is the role of host's immune system. Once a BAI is established, different immune cells are recruited to the infection site but macrophages end up being the prevailing cells responsible to orchestrate the inflammatory process and foreign body reactions [5,6]. Their functions include ingestion of bacteria by phagocytosis, destruction of bacteria within the phagolysosome and recruitment of inflammatory cells to the site of infection, using chemokines and acute-phase proteins. It has been reported, however, that the presence of a biomaterial may compromise the host immune system [7]. Therefore, it is important to better understand how immune cells interact with adhering pathogens.

In the previous sub-chapter, an Palm and DNase I were successfully co-immobilized onto PDMS surfaces to impart them with both anti-adhesive and antimicrobial properties against the adhesion of relevant bacteria as single and dual-species, with excellent stability and non-toxicity. Although quite promising the results obtained, it cannot be overlooked the fact that some bacteria managed to adhere these coatings. The aim of the present study was, therefore, to

determine if bacteria remaining on these surfaces are more susceptible to antimicrobial treatment or developed some kind of resistance towards the AMP immobilized on bi-functional coatings. It was also intended to investigate the role of these bi-functional coatings on bacterial removal and digestion by macrophages.

MATERIALS AND METHODS

BACTERIAL STRAIN AND GROWTH CONDITIONS

A clinical isolate of *S. aureus* was used throughout this study. The strain was preserved and cultured as described in Chapter 2.

ANTIMICROBIAL SUSCEPTIBILITY TO VANCOMYCIN

MIC and MBC of vancomycin were determined by the microdilution method as described in Chapter 2.

PDMS PREPARATION AND FURTHER FUNCTIONALIZATION

PDMS was prepared and functionalized as described in Chapter 2 and 5.1.

BIOFILM SUSCEPTIBILITY TO VANCOMYCIN TREATMENT

Biofilm susceptibility to vancomycin treatment was evaluated by determining cells metabolic activity, using the XTT reduction assay. Briefly, a bacterial suspension with $1x10^7$ CFU/mL was prepared in TSB and 300 µL of this suspension were added to a 48-well microtiter plate in which unmodified and modified PDMS coupons were placed. The plate was then incubated for 24 h at 37 °C and 120 rpm. The coupons were subsequently washed once with saline solution to remove free-floating bacteria and 300 µL of vancomycin (MIC value prepared in TSB) was added to each well. As a control, 300 µL of TSB without antibiotic was also added. The plates were then incubated overnight at 37 °C and 120 rpm and the bacterial metabolic activity was determined

using XTT reduction assay as described in Chapter 2. Two independent assays with three replicates for each condition tested were performed.

SUSCEPTIBILITY OF ADHERED CELLS TO MODIFIED SURFACES

The susceptibility pattern of bacterial cells adhered to PDMS surfaces was evaluated as described in Chapter 2. Experiments were performed in triplicate.

MACROPHAGES ADHESION TO STAPHYLOCOCCI ADHERING TO SURFACES

A human monocyte line cell (THP-1, ATCC TIB-202) was used in this study. Monocytes were routinely cultured as described in Chapter 2. Experiments were performed in triplicate.

RESULTS

SUSCEPTIBILITY OF BIOFILMS TO VANCOMYCIN TREATMENT

In order to evaluate the susceptibility of biofilms formed on mono and bi-functional coatings of Palm and DNase I, after 24 h of biofilm growth they were subjected to antibiotic treatment overnight. Vancomycin is an antibiotic commonly used as a standard therapeutic option against staphylococci infections [8]. The concentrations of vancomycin able to inhibit planktonic bacterial growth (MIC) and those required to kill planktonic bacteria (MBC) of *S. aureus* were both 0.5 µg/mL. Metabolic activity of biofilm cells were afterwards evaluated using XTT reduction assay.

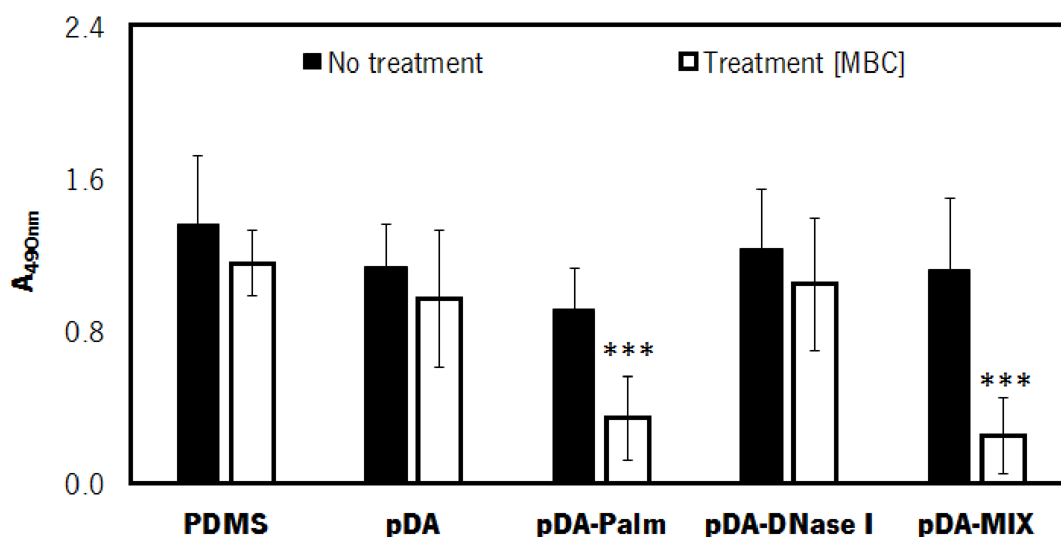

Figure 1. Metabolic activity of biofilm cells adhered to unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS surfaces functionalized with DNase I (pDA-DNase I), Palm (pDA-Palm) and both DNase and Palm (pDA-MIX) after being subjected to no treatment (black) or vancomycin treatment at its MIC (white). Significant differences were found for (***) p< 0.001, compared to No treatment.

Results showed that, in the absence of treatment, the cells that managed to adhere to both unmodified and modified surfaces were able to grow into a biofilm with metabolic active cells. Vancomycin treatment at its MIC had no effect on biofilm cells metabolic activity when biofilms were formed on unmodified or pDA-coated PDMS, as well as PDMS functionalized with DNase I (pDA-DNase I). On the other hand, biofilms formed on PDMS functionalized with Palm alone or combined with DNase I were more susceptible to antibiotic treatment, suggesting a synergistic effect between modified surfaces and antibiotic therapy.

POTENTIAL DEVELOPMENT OF RESISTANCE BY ADHERED CELLS

In order to evaluate the potential of bacterial resistance development toward surfaces modified with Palm, an assay was performed in which cells in contact with unmodified PDMS and modified PDMS surfaces for a period of 10 days, were recovered and used to determine the MIC and MBC of Palm (Table 1). As a control, the antibiotic vancomycin, was immobilized onto PDMS using dopamine chemistry at the same concentration as the AMP and the same assay was performed.

Table 1. Antimicrobial susceptibility of *S. aureus* against Palm and vancomycin: MIC and MBC after10 passages in contact with unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS surfaces functionalized with antimicrobials. MIC and MBC are expressed in μg/mL.

Antimicrobial	MIC			MBC		
	PDMS	pDA	Antimicrobial	PDMS	pDA	Antimicrobial
Palm	64	64	64	>64	>64	>64
Vancomycin	1	1	2	1	2	8

Results showed that cells adhered to PDMS functionalized with Palm exhibited the same susceptibility pattern as cells adhered to PDMS before or after coating with pDA, suggesting no development of resistance. On the other hand, cells adhered to surfaces functionalized with the antibiotic vancomycin were less susceptible to the same antibiotic as indicated by the higher values of MIC and MBC when compared to PDMS unmodified or coated with pDA.

MACROPHAGES PHAGOCYTOSIS OF ADHERED CELLS

Phagocytosis of staphylococci by macrophages on unmodified modified surfaces was evaluated by comparing the adhering cells on the different surfaces before and after the presence of macrophages for a period of 2 h (Figure 2). Results showed that macrophages adhesion to unmodified PDMS tend to cluster which may compromise their mobility and subsequently their phagocytic activity. After pDA coating, macrophages were found more evenly distributed along the surfaces and results suggested a higher number of adhered macrophages on bi-functional coatings (pDA-MIX).

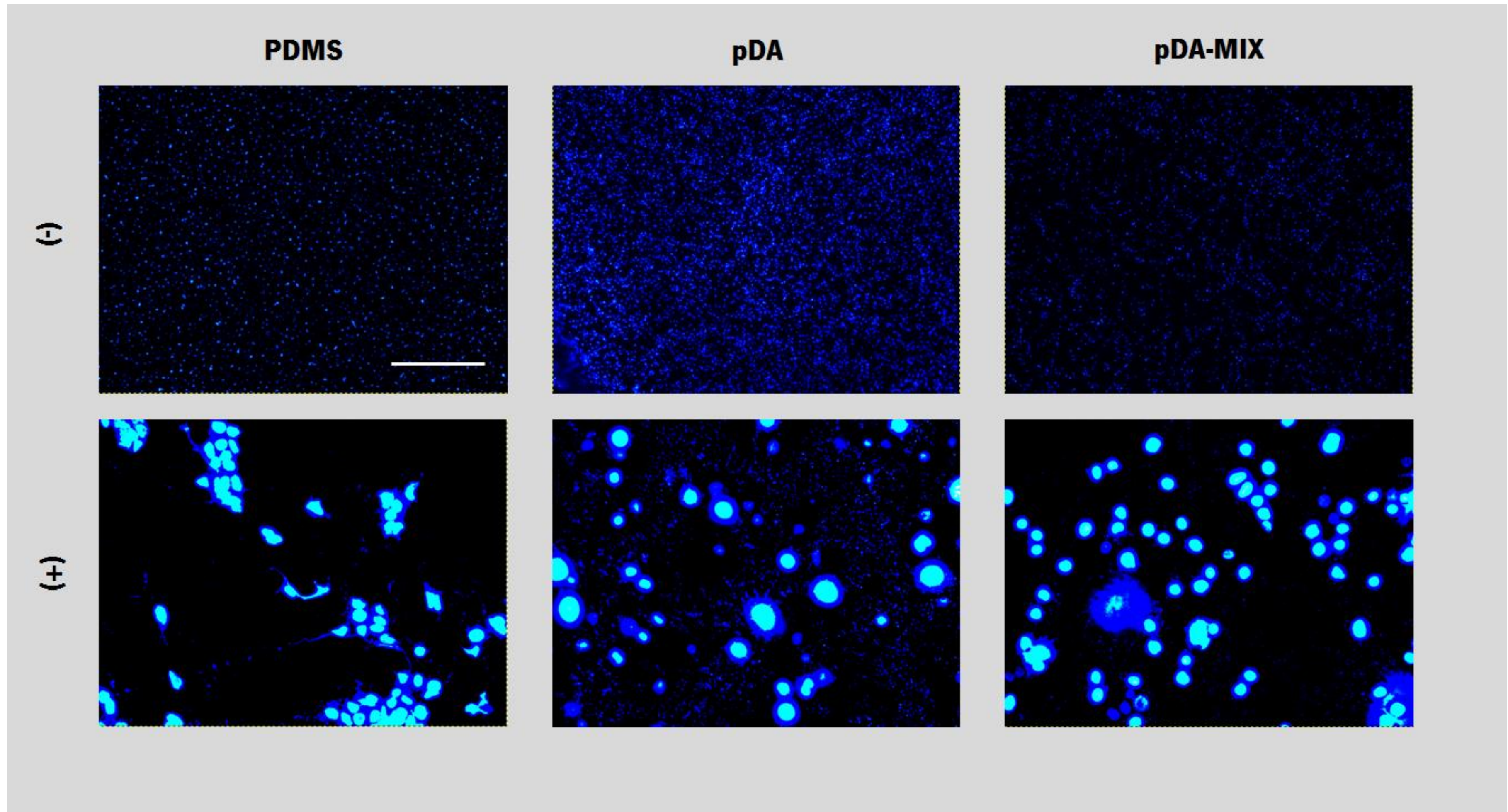

Figure 2. DAPI-stained images of *S. aureus* adhesion to unmodified PDMS, pDA-coated PDMS (pDA) and pDA-coated PDMS surfaces functionalized with Palm and DNase I (pDA-MIX) in the absence (-) of macrophages and after 2 h adhesion of macrophages (+). The scale bar denotes 100 μm.

DISCUSSION

In the fight against BAI, several surface modifications have been proposed to render the biomaterial surfaces with anti-infective properties [9]. Despite the promising results reported in the literature, where reductions on bacterial attachment higher than 90 % are often achieved [3, 10], most of these approaches tend to neglect the fate of the few bacteria that manage to attach to these modified surfaces. In a similar way, the bi-functional coating developed in last sub-chapter, was not able to completely prevent bacterial adhesion to modified PDMS. Therefore, the aim of this study was to investigate the fate of bacteria that managed to adhere to these modified surfaces in what concerns their susceptibility to antibiotic treatment, potential development of resistance and their clearance mediated by macrophages phagocytosis.

Once established a BAI, the most common approach of treatment involves the use of antibiotics, which dose must be higher than their MIC to be able to influence cells in sessile style [11]. Accordingly, in the present study, biofilms formed on unmodified PDMS and pDA were not influenced by vancomycin treatment at its MIC. In the absence of treatment, cells adhered to modified surfaces were able to establish biofilms with similar metabolic activity to those formed on unmodified surfaces. These results suggest that mono and bi-functional coatings alone may not be sufficient to effectively prevent BAI. When, combined with vancomycin treatment, however, biofilm establishment was impaired on PDMS functionalized with Palm as well as with Palm combined with DNase I (pDA-MIX). It also suggests that antibiotic treatment of BAI could be more effective when infections occurs after implantation of a biomaterial with these functional coatings. Palm antimicrobial activity seems to be the main factor enhancing bacterial susceptibility to antibiotic, as vancomycin treatment had no effect on biofilm formed on PDMS functionalized only with DNase I. A similar mechanism of antimicrobial activity for immobilized QAC on a substratum and positively charged surfaces in general, has been proposed [12]. Gottenbos *et al.* [13] demonstrated that the strength of adhesion may be determinant for bacterial growth as they demonstrated that bacteria showing little desorption from surfaces had more difficulty to divide and grow than bacteria adhering more reversibly. Furthermore, a link has been described between strong adhesion forces between bacteria and substratum surfaces yielding membrane stresses and the percentage of dead cells on a surface for which the term “stress deactivation” was coined [14]. Since most of bacterial strains and species exhibit a negative surface charge [15], it is believed that strong adhesion forces can be found on surfaces functionalized with

cationic AMP that has proved to kill bacteria upon contact (Chapter 3.4), in a so called "lethal" regime of strong adhesion forces [16].

Bacteria have a striking ability to adapt in response to their environment, and indeed, some bacterial strains are not susceptible to some AMP, even at high concentrations [17, 18]. Because there is a lack of studies addressing the development of bacterial resistance towards AMP after their immobilization, a study was performed on Palm-based coatings in order to evaluate the potential of bacterial resistance toward these surfaces. Results confirmed that Palm immobilization retained its low propensity to develop bacterial resistance, as opposite to the immobilization of an antibiotic. These results highlighted the risk associated to the immobilization of antibiotics and the promising potential of Palm to be used in the design of materials able to prevent BAI.

Upon the implantation of a biomaterial, tissue trauma and injury trigger a cascade of physiological events that activate the immune system [19]. The interactions between macrophages and bacterially contaminated biomaterials is, therefore, crucial for the establishment of a BAI. Indeed, mature biofilms are less likely to form if macrophages are able to remove and destroy bacteria adhering on a biomaterial surface. For further evaluation of the clinical potential of the bi-functional coating proposed in this thesis, it was also investigated the *in vitro* response of human macrophages to *S. aureus* adhering to these coatings and compared to unmodified surfaces. Results, although preliminary and qualitative, showed that macrophages were better distributed along pDA-coated surfaces which suggests a better mobility to perform their phagocytic activity. Further experiments should be performed to also evaluate, quantitatively, their phagocytic activity [20].

In conclusion, the bi-functional coating proposed in this thesis holds great potential to fight BAI as it proved to enhance bacterial susceptibility to antibiotic treatment and possibly to macrophages phagocytosis, without developing bacterial resistance towards the AMP immobilized.

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Chapter 6

CONLUDING REMARKS AND WORK PERSPECTIVES

This chapter describes the main conclusions drawn from the work performed under the subject of this thesis as well as some suggestions for future research.

GERERAL CONCLUSIONS

With the increase of elderly population as a consequence of the increasing use of biomaterials to support or restore human body function, the problem of BAI may be expected to increase in the coming years. As reviewed in Chapter 1, the field of antibacterial coatings has achieved considerable advances and encouraging results. However, the strategies currently used to prevent BAI, as well as the studies proposed in the literature are still far from perfect. The major gaps identified for these approaches include the emergence of bacterial resistance towards antimicrobials immobilized, toxicity issues and the need for complex, labor and time-consuming techniques for surface modification. The main aim of this thesis was, therefore, to propose a simple and effective coating strategy able to simultaneously prevent bacterial adhesion and kill the adherent ones, with low propensity for developing bacterial resistance and toxicity towards mammalian cells. AMP and enzymes targeting different EPS were the alternatives to antibiotics used to confer anti-adhesive and antimicrobial properties, respectively, to biomaterial surfaces.

Prior to co-immobilization of AMP and enzymes, their single immobilization was first optimized. Chapter 3 describes the studies performed to determine the AMP with most promising antimicrobial effect after immobilization. A preliminary screening was conducted using a group of AMP determining the susceptibility patterns of planktonic and sessile cultures of both *S. aureus* and *P. aeruginosa*. The results obtained in Chapter 3.1 allowed to conclude that AMP are good alternatives to antibiotics as they were able to compromise biofilm formation at similar range concentrations able to inhibit planktonic growth. Polymyxins B and E were more effective against the Gram-negative strain while Camel and Palm were more promising against the Gram-positive one. Based on these results, the potential of polymyxin E during early stages of biofilm formation to impair *P. aeruginosa* was further investigated (Chapter 3.2). Its physical adsorption onto polystyrene surfaces proved to impair biofilm formation and increase biofilm susceptibility to CIP or PE treatment. Likewise, polymyxins E and B immobilization onto PDMS was optimized using two pDA-based approaches (Chapter 3.3). This study pointed out that their immobilization holds great potential to overcome some concerns associated to the use of these compounds, namely, the development of bacterial resistance and toxicity reported in the past. However, taking into account that the spectrum of action of these coatings is directed towards Gram-negative strains, immobilization of other AMP was also optimized against Gram-positive bacteria (Chapter 3.4). Results highlighted the great potential of Palm to impart biomaterial surfaces with potent antimicrobial activity, mainly against Gram-positive bacteria, the most commonly found

associated to BAI. Therefore, Palm was considered the most promising AMP to render the surfaces with antimicrobial functionality.

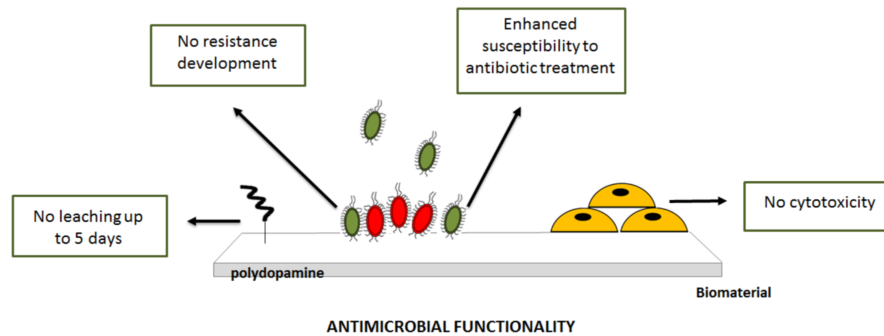
To obtain a bi-functional coating, the immobilization of enzymes targeting different compounds of bacterial structure or biofilm matrix was optimized in Chapter 4. Chapter 4.1 showed that PC surfaces functionalized with alginate lyase (because of alginate's contribution to *P. aeruginosa* virulence) exhibited anti-adhesive properties against mucoid but, unexpectedly, to non-mucoid strains as well. Such results provided important insights about the mechanism of action of alginate lyase against *P. aeruginosa* strains, as enzyme's antibacterial performance was catalysis-independent. To extent the anti-adhesive features of the surfaces, other enzymes were also screened against *S. aureus* in Chapter 4.2. Results found in this study highlighted that DNase I was the most effective in preventing the adhesion of both Gram-positive and Gram-negative bacteria.

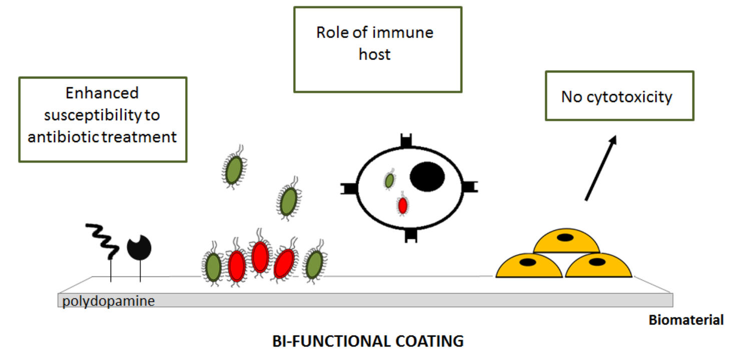
Co-immobilization of Palm and DNase I, the antimicrobials that rendered biomaterial surfaces with the most promising antimicrobial and anti-adhesive properties, is described in Chapter 5. The proposed immobilization approach imparted PDMS surfaces with both anti-adhesive and antimicrobial properties against the adhesion of several relevant bacteria as single and also against the co-adhesion of dual-species, with excellent stability and biocompatible properties. The fate of bacteria that managed to adhere to these bi-functional coatings was also studied in Chapter 5.2. Bacteria were found to be more susceptible to antibiotic treatment and to macrophages phagocytosis, without developing bacterial resistance towards the AMP immobilized, which reinforces the applicability of this co-immobilization strategy to functionalize biomaterials.

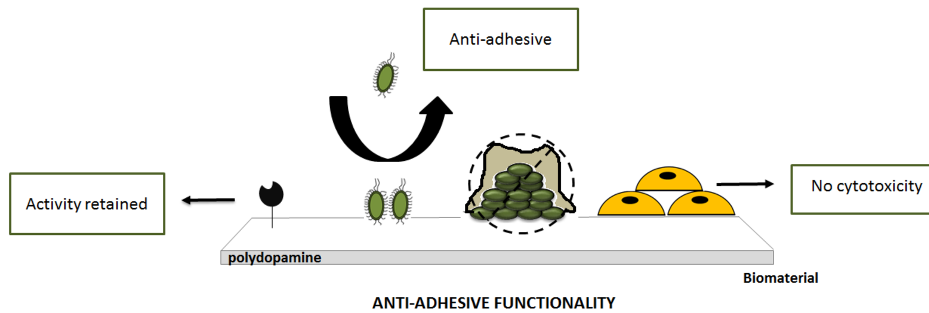
In summary, the work conducted throughout this thesis reassures that **mussel-inspired surface modification** is a **simple approach** that can revolutionise the research of antibacterial surfaces by allowing the co-immobilization of enzymes and AMP to develop a bi-functional coating. PDMS material was, therefore, imparted with both **anti-adhesive** and **antimicrobial** properties against the attachment of several bacteria as **single** and **dual-species**. The coating also exhibited **anti-biofilm properties,** although the role of DNase I was not as evident as in adhesion assays, excellent **stability**, showed **no cytotoxicity** and **development of bacterial resistance** towards the AMP immobilized. The bi-functional coating proposed holds, therefore, great potential to **fight BAI** if explored in the development of implants or medical devices.

FROM MONO-FUNCTIONAL COATINGS

TO BI-FUNCTIONAL COATINGS







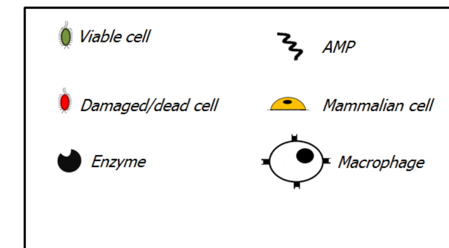


Figure 1. Schematic representation of the work conducted in the present thesis to design a bi-functional coating. Antimicrobial functionality was conferred by AMP immobilization and mono-functional coatings proved to be stable for up to 5 days, with no development of bacterial resistance or cytotoxicity and enhanced susceptibility to antibiotic treatment. Anti-adhesive properties were imparted by enzyme immobilization without compromising their biological activity and no cytotoxicity. These coatings failed, however, in preventing biofilm establishment. Co-immobilization with both compounds yielded a bi-functional coating combining the properties of mono-functional coatings alone. The role of host immune system was also evaluated on these coatings (not to scale).

FUTURE RESEARCH LINES

Although the findings in this thesis have highlighted the great potential of co-immobilization of AMP and enzymes to create bi-functional antibacterial coatings, further investigations should be performed to strengthen the applicability of the aforementioned coatings.

Biomaterial implants come in contact with biological fluids such as blood, urine, tear fluid or saliva, depending on the body place they are inserted. The stability of bi-functional coatings and their effectiveness after exposure to these biological fluids should, then, be performed and compared to PBS. These studies should be conducted using a parallel plated flow chamber, which allows insertion of removable discs, to better mimic flow conditions.

Although *in vitro* studies of both anti-adhesive and anti-biofilm activities have been performed in this thesis, the anti-infective potential of the bi-functional coating needs to be evaluated *in vivo* to confirm their clinical applications. Different animal models have been reported to evaluate the effects of antibacterial coatings, depending on the type of BAI.

The fate of biomaterial has been described as a race between bacterial adhesion and subsequent biofilm growth *versus* tissue integration. Although this concept dates back from 1987, it was only recently that some groups have proposed co-culture experiments to evaluate the simultaneous response of bacteria, mammalian and immune cells on a biomaterial surface. Although the interactions of these three types of cells with the bi-functional coating proposed have been studied in the present thesis, the work would be improved by performing co-cultures experiments and therewith bridge the gap between *in vitro* and *in vivo* studies. The incorporation of another functionality provided by RGD peptide sequence should also be considered. The presence of RGD peptide sequence is expected to promote tissue integration as this peptide is known as one of the major recognition sites of integrin receptors through which mammalian cells connect to their extracellular matrix molecules.

The low propensity for developing microbial resistance is an important feature attributed to AMP which makes them promising alternatives to conventional antibiotics. However, it is well established that bacteria have a remarkable ability to cope with antimicrobials-induced stress and, in fact, there are some resistance mechanisms reported to AMP. In this study, Palm was evaluated for the risk of inducing bacterial resistance after its immobilization. When in solution, resistance towards an antimicrobial is often evaluated either by repeated cultivation of bacteria in

a sub-inhibitory concentration of the agent or by serial passage experiments at progressively increasing concentrations. A similar approach was applied for immobilized antimicrobials and results suggested no development of resistance towards the AMP used in the bi-functional coating proposed in this thesis. These serial passage procedure should, however, be complemented with molecular methods to investigate the presence of resistance genes before and after continuous exposure to these bi-functional coatings.

The work conducted in this thesis greatly focused on evaluating the biological performance of the coatings proposed. A more complete surface characterization could help to better understand this biological performance. Techniques such as ellipsometry or quartz crystal microbalance with dissipation monitoring to determine coatings thickness, surface zeta potential measurements to evaluate surface charge and dynamic mechanical analysis to evaluate mechanical properties should be performed.

The enzyme chosen for the bi-functional coating proposed in this thesis, DNase I, showed some spectrum activity limitations as eDNA may play different roles as a structural component on different strains. A possible way to overcome this issues may rely on the co-immobilization of different enzymes with different targets. The combination of dispersin B, proteinase K and DNase I seems like a potential combination for further investigations.

The characteristically dark color of pDA coatings may compromise some practical applications of the proposed bi-functional coating. A recently proposed bio-inspired approach to the formation of colorless multifunctional coatings, exploiting the versatility and multifunctionality of plant polyphenols and their mimics may be solution for this issues. These coatings retain many of the advantages of pDA and deposit under similar conditions, but are colorless and derived in some cases from reagents less costly than dopamine.