ACKNOWLEDGEMENTS

First of all, I would like to thank to my supervisor Professor Rosário Oliveira for her guidance, great support and kind advice throughout my PhD research studies. It was a real privilege and an honour for me to share of her exceptional scientific knowledge but also of her extraordinary human qualities.

I also would like to thank to my co-supervisor Doctor Pilar Teixeira for her constant support, availability and constructive suggestions, which were determinant for the accomplishment of the work presented in this thesis.

A special thank to Mariana, the first person who taught me what biofilms were and that throughout these years had always encouraging words and was always ready to help me. I am grateful for her cooperation in the work described in chapters 3, 5 and 6.

To Sandro Bordeira and João Fonseca, from the Condensed Matter Physics Centre, University of Lisbon, I want to thank them for their collaboration in the work described in chapter 4.

To the University of Minho and Department of Biological Engineering, I would like to express my sincere gratitude for providing excellent material, logistical and human conditions, helping me in these first steps of scientific demand and, consequently, in the development and conclusion of this research work.

I acknowledge the financial support for my research study of the Portuguese Foundation for Science and Technology through the PhD fellowship SFRH/BD/19265/2004.

To my colleagues and friends of the Applied Microbiology Laboratory in the Department of Biological Engineering, I thank them for their companionship and for providing a so pleasurable and friendly working atmosphere. The moments of leisure shared together helped to overcome some more difficult moments. I am also truly thankful for their always prompt help, whenever I needed it.

To my closer friends, I express my gratitude for their unconditional friendship, support and patience throughout these years.

Last, but not least, I would like to thank to my family, my parents José and Glória and my sister Sandra, and to Fernando, for their unconditional support, encouragement and love, and without which I would not have come this far.

À minha família e ao Fernando

Nothing in life is to be feared. It is only to be understood.

Marie Curie

Staphylococcus epidermidis Adhesion and Biofilm Formation onto Biomaterials

ABSTRACT

Staphylococcus epidermidis is a coagulase-negative *Staphylococcus* (CNS) that often colonizes the skin and mucous membranes of the human body, as part of its normal microflora. However, when a rupture of the cutaneous surface occurs, by any type of trauma or insertion of a medical device, staphylococci can enter the host and become pathogenic. Therefore, *S. epidermidis* has emerged in recent years as a major nosocomial pathogen associated with infections of implanted medical devices, namely prosthetic heart valves and joints, central venous catheters, urinary catheters, contact lenses and hip prostheses. Staphylococci adhere to such devices and have the ability to develop biofilms, which constitutes an important virulence factor and the most relevant pathogenic mechanism of staphylococcal infection.

The work described in this thesis aimed at evaluating the adhesion and biofilm formation capabilities of several *S. epidermidis* strains to biomaterials normally used in the manufacture of indwelling medical devices. The study of the surface properties that affect initial bacterial adhesion as well as of ways to prevent it was also one of the goals of this work. Another objective was to study the properties of a mature biofilm and the phenotypic differences between sessile and planktonic cells. The profiles of cell wall and extracellular matrix proteins were also assessed to evaluate the importance of these proteins on the process of adhesion and biofilm formation.

In order to try to correlate the adhesion ability of *S. epidermidis* strains studied with surface properties of substrata (acrylic and silicone) and cells, hydrophobicity and surface tension components were determined through contact angle measurements. Surface roughness of substrata was also assessed by atomic force microscopy (AFM).

An expedite method to reduce *S. epidermidis* adhesion to acrylic and silicone by heparin and gentian violet surface pre-conditioning was developed. Specific modifications on a polycarbonate surface by gold coating and the subsequent coverage with different self-assembled monolayers (SAMs) were also assayed. Adhesion was performed during two hours and the number of adhered cells was determined by direct enumeration using epifluorescence microscopy.

Concerning biofilm formation on acrylic, the total biomass was quantified by crystal violet staining; the number of cells within the biofilm was determined by colony forming units plating; and the extracellular matrix was extracted by the Dowex resin method. The polysaccharides and proteins content of the matrix was also quantified. These results were correlated with the cellular metabolic activity determined by XTT reduction assay and glucose uptake. Metabolic activity of planktonic cells was as well assessed by both methods.

Protein profiles of cell wall and extracellular matrix of *S. epidermidis* strains under study were analysed by SDS-PAGE.

Considering cell surface properties (surface tension parameters, degree of hydrophobicity), there were no significant differences among the strains assayed, except for strain IE214. No relationship was found between cell surface hydrophobicity and adhesion capability. However, all strains adhered at a higher extent to silicone, more hydrophobic and rougher than acrylic, indicating that substrata surface properties play a role in initial bacterial adhesion.

Both heparin and gentian violet demonstrated to be effective in reducing bacterial adhesion as well as gold covered polycarbonate and methyl terminated SAMs. Thus, these studies have clinical significance, since they point out alternative strategies to the diminishment and prevention of bacterial colonization to biomaterial surfaces.

The analysis of the biofilm formation capability and its composition among *S. epidermidis* strains lead to the confirmation that biofilm formation as well as the production of extracellular polymers are strain dependent and are virulence factors associated to pathogenicity of some *S. epidermidis* clinical strains.

Cell wall and extracellular matrix proteins that are related to the adhesion and biofilm formation processes seem to be present in the proteins patterns analysed, which are potential virulence factors that should be taken into consideration as appropriate targets for the development of novel therapies against staphylococcal infections.

Adesão e Formação de Biofilme de *Staphylococcus epidermidis* em Biomateriais

RESUMO

Staphylococcus epidermidis é um estafilococo coagulase-negativo (ECN) que normalmente coloniza a pele e as mucosas do corpo humano, fazendo parte da sua microflora normal. No entanto, quando ocorre uma ruptura da superfície cutânea, por qualquer tipo de trauma ou inserção de um dispositivo médico, os estafilococos podem penetrar o hospedeiro, tornando-se patogénicos. Deste modo, nos últimos anos, *S. epidermidis* tornou-se um dos principais patogénicos nosocomiais associados a infecções de dispositivos médicos, nomeadamente, válvulas cardíacas, próteses para articulações, cateteres venosos centrais, cateteres urinários, lentes de contacto e próteses da anca. Os estafilococos aderem a esses dispositivos e desenvolvem biofilmes, o que constitui um importante factor de virulência e um dos mais relevantes mecanismos patogénicos de infecção estafilococal.

Este trabalho de investigação teve como objectivo a avaliação da capacidade de adesão e formação de biofilme de várias estirpes de *S. epidermidis* a biomateriais utilizados no fabrico de dispositivos médicos. O estudo das propriedades de superfície que afectam a adesão bacteriana inicial, bem como formas de a prevenir constituíram também um dos objectivos propostos. Outra finalidade foi o estudo detalhado das propriedades de um biofilme maduro e das diferenças fenotípicas entre células sésseis e planctónicas. Os perfis proteicos da parede celular e da matriz extra-celular foram também um dos alvos de estudo, com o intuito de se aferir o papel destas proteínas na adesão e formação de biofilme.

De modo a tentar correlacionar a capacidade de adesão das estirpes de *S. epidermidis* com as propriedades da superfície dos substratos (acrílico e silicone) e das células, a hidrofobicidade e os componentes de tensão superficial foram calculados através da medição de ângulos de contacto. A rugosidade superficial dos substratos foi avaliada por microscopia de força atómica (MFA).

Desenvolveu-se um método expedito com o objectivo de se tentar reduzir a adesão de *S. epidermidis* a acrílico e a silicone por pré-condicionamento da superfície com heparina e violeta de genciana. Foram, também, efectuadas alterações na superfície de policarbonato, através do revestimento com ouro e a subsequente cobertura com diferentes monocamadas auto-organizadas (self-assembled monolayers - SAMs). A adesão foi realizada durante duas horas

e o número de células aderidas foi determinado por enumeração directa, mediante microscopia de epifluorescência.

No que diz respeito à formação de biofilme, testada em acrílico, a biomassa total foi quantificada por coloração com violeta de cristal. O número de células foi determinado por plaqueamento de unidades formadoras de colónias e a matriz extra-celular foi extraída pelo método da resina Dowex. O conteúdo da matriz, em termos de proteínas e polissacáridos, foi também quantificado. Estes resultados foram correlacionados com a actividade metabólica celular determinada pelos métodos de redução de XTT e determinação do consumo de glucose. A actividade metabólica de células planctónicas foi também avaliada pelos dois métodos.

O perfil proteico dos extractos da parede celular e da matriz extra-celular das estirpes em estudo foi analisado por SDS-PAGE.

Relativamente às propriedades da superfície celular (componentes de tensão superficial, grau de hidrofobicidade), não se verificaram diferenças significativas entre as estirpes testadas, excepto para a estirpe IE214, que apresentou um comportamento único de adesão. Não foi encontrada relação entre a hidrofobicidade da superfície celular e a capacidade de adesão. Porém, todas as estirpes aderiram melhor ao silicone, mais hidrofóbico e rugoso do que o acrílico, evidenciando a importância das propriedades de superfície do substrato na adesão inicial.

Tanto a heparina como o violeta de genciana demonstraram ser eficazes na redução da adesão bacteriana, assim como o policarbonato coberto com ouro e as SAMs com grupos terminais metilo. Estes estudos apresentam significado clínico, dado que sugerem possíveis estratégias alternativas para a diminuição e prevenção da colonização bacteriana em superfícies de biomateriais.

A análise da capacidade de formação de biofilme das várias estirpes de *S. epidermidis* estudadas, levou à confirmação de que a formação de biofilme, bem como a produção de polímeros extra-celulares são dependentes da estirpe e são factores de virulência associados à patogenicidade de algumas estirpes clínicas de *S. epidermidis*.

Nos perfis proteicos da parede celular e da matriz extra-celular parecem estar presentes proteínas relacionadas com os processos de adesão e formação de biofilme, as quais são potenciais factores de virulência que devem ser tidos em consideração como alvos para o desenvolvimento de novas terapias contra infecções estafilococais.

SCIENTIFIC OUTPUT

Part of the results presented in this thesis have been published elsewhere:

Papers in peer reviewed journals:

Sousa C, Henriques M, Teixeira P and Oliveira R (2009). Relevance of cell wall and extracellular matrix proteins to *Staphylococcus epidermidis* adhesion and biofilm formation. *Adhesion Sci Technol*. In press. **(Chapter 6)**

Sousa C, Henriques M, Teixeira P and Oliveira R (2009) Influence of surface properties on the adhesion of *Staphylococcus epidermidis* to acrylic and silicone. *Int J Biomater*. In press. **(Chapter 2)**

Sousa C, Henriques M, Teixeira P and Oliveira R (2009) The role of extracellular polymers on *Staphylococcus epidermidis* biofilm biomass and metabolic activity. *J Basic Microbiol*. **49**. **(Chapter 5)**

Sousa C, Henriques M, Teixeira P and Oliveira R (2008) Reduction of *Staphylococcus epidermidis* adhesion to indwelling medical devices: a simple procedure. *Br J Biomed Sci* **65**:184-190. **(Chapter 3)**

Sousa C, Teixeira P, Bordeira S, Fonseca JG and Oliveira R (2008) *Staphylococcus epidermidis* adhesion to modified polycarbonate surfaces: gold and SAMs coated. *J Adhesion Sci Technol* **22**:675-686. **(Chapter 4)**

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Sousa C, Teixeira P and Oliveira R (2008) *Staphylococcus epidermidis* adhesion to modified polycarbonate surfaces: gold and SAMs coated. *Biofilms III, 3rd International Conference*, Munich, Germany, 6-8 October, p. 141.

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Sousa C, Teixeira P and Oliveira R (2006) Heparinization time effect in *Staphylococcus epidermidis* adhesion to silicone. *BIOFILMS II: attachment and detachment in pure and mixed cultures*, Leipzig, Germany, 22-24 March, p. 102.

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NOMENCLATURE

Symbols

P	Significance value
Ra	Average roughness (nm)
Rmax	Maximum roughness (nm)
Rq	Root mean square roughness
T	Temperature (°C)
ΔG_{iwi}^{LW}	Apolar component of the free energy variation between two entities of a given surface (i) immersed in water (w) (mJ/m²)
ΔG_{iwi}^{AB}	Polar component of the free energy variation between two entities of a given surface (i) immersed in water (w) (mJ/m²)
ΔG_{iwi}	Total free energy variation between two entities of a given surface (i) immersed in water (w) (mJ/m²)
γ^-	Electron donor surface tension parameter (mJ/m²)
γ^+	Electron acceptor surface tension parameter (mJ/m²)
γ^{AB}	Polar (Lewis acid-base) surface tension parameter (mJ/m²)
γ^{LW}	Apolar (Lifshitz-van der Waals) surface tension parameter (mJ/m²)
γ^{TOT}	Surface free energy (mJ/m²)
$\theta_{\alpha\text{-B}}$	α-Bromonaphtalene contact angle (°)
θ_F	Formamide contact angle (°)
θ_W	Water contact angle (°)

Abbreviations

Aae	*Staphylococcus epidermidis* autolysin/adhesin
AAP	Accumulation-associated protein
AFM	Atomic force microscopy
Agr	Accessory gene regulator
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
AtlE	*Staphylococcus epidermidis* autolysin
Bap	Biofilm-associated protein
BATH	Bacterial adherence to hydrocarbons

BCA	Bicinchoninic Acid
BSA	Bovine serum albumin
CC	Calix-crown
CFU	Colony forming units
Clf	Clumping factor
CNS	Coagulase negative staphylococci
CRA	Congo Red agar
CRI	Catheter-related infection
CRP	C-Reactive Protein
CVC	Central venous catheter
CW	Cell wall
DAPI	4'-6-Diamidino-2-phenylindole
DNA	Desoxyribonucleic acid
DW	Dry Weight
Ecp	*Staphylococcus epidermidis* cysteine protease
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Extracellular matrix
Embp	*Staphylococcus epidermidis* fibronectin binding protein
EPS	Extracellular polymeric substances
FAME	Fatty-acid modifying enzymes
Fbe	*Staphylococcus epidermidis* fibrinogen-binding protein
Fg	Fibrinogen
Fn	Fibronectin
G	Gold
GehD	*Staphylococcus epidermidis* collagen binding lipase
GV	Gentian violet
HDT	Hexadecanethiol
HIC	Hydrophobic interaction chromatography
HMA	Hydrophobic microsphere assay
IOL	Intraocular lens
IU	International units
MAA	Mercaptoacetic acid
MATH	Microbial adhesion to hydrocarbons
MPA	Mercapto-propionic acid
MRSA	Methicillin resistant *Staphylococcus aureus*

MSCRAMM	Microbial surface component recognizing adhesive matrix molecules
OD	Optical density
OT	Octanethiol
PAA	Poly(acrylic acid)
PAH	Poly(allylamine) hydrochloride
PBS	Buffered saline solution
PC	Polycarbonate
PCR	Polymerase chain reaction
PEM	Polyelectrolyte multilayer
PIA	Polysaccharide intercellular adhesin
PMMA	Poly(methylmetacrylate)
PMPs	Platelet microbicidal proteins
PMS	Phenazine methosulfate
PMSF	Phenylmethylsulfonyl fluoride
PVE	Prosthetic valve endocarditis
SAM	Self-assembled monolayers
SAT	Salt aggregation test
SD	Standard deviation
Sdr	Serine-aspartate repeat protein
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
Ses	*Staphylococcus epidermidis* surface proteins
SI	International system of units
SPSS	Statistical Package for the Social Sciences
SSP	Staphylococcal surface protein
Sub-MIC	Subinhibitory minimal concentration
Tpn	Transferring binding protein
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSST	Toxic shock syndrome toxin
V	Volume
Vn	Vitronectin
W	Weight
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2Htetrazolium hydroxide