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Influence of a Quaternary Ammonium Compound on the Cell Structure of Bacteria using Atomic Force Microscopy

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"Whatever you do will be insignificant, but it is very important that you do it".

Mahatma Gandhi

ABSTRACT

Bacterial adhesion and subsequent biofilm formation remains a serious concern, especially in clinical applications, since bacteria associated with biofilms are more resistant to antibiotic treatment and to the host immune system. A potential approach to deal with this drawback may rely on the use of antimicrobial coatings comprising quaternary ammonium compounds (QACs).

The main purpose of the present thesis was to study the efficacy of a QAC against staphylococci when compared with two other antimicrobial compounds, an antibiotic (Gentamicin) and an antimicrobial peptide (Gramicidin S) using atomic force microscopy (AFM). After assessing the antimicrobial activity of the compounds against planktonic cultures by determining their minimal inhibitory (MIC) and minimal bactericidal concentrations (MBC), adhering staphylococcal cells were exposed to the antimicrobial compounds and their cell surfaces analyzed with AFM. The number of bacteria removed by the AFM tip was determined and taken as an indication of cell surface damage. The antimicrobial action of the compounds on staphylococcal biofilms was evaluated by the determination of the metabolic activity of biofilm through an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Finally, in order to get some insights about QAC mode of action against bacterial cells, its antimicrobial activity in planktonic cultures, as well as their effects on adhering staphylococcal cells by AFM were performed in the presence of calcium ions.

All the antimicrobial compounds proved to exhibit antimicrobial activity against staphylococcal planktonic cultures. AFM measurements allowed the analysis *in situ* of the bacterial cells behaviour when exposed to antimicrobial compounds. Bacterial cell surface wrinkled upon exposure to the antimicrobials until bacteria disappear from the surface. Gentamicin yielded faster wrinkling and removal of bacteria from the surface as compared to QAC and Gramicidin S which yielded similar results. As the staphylococci outside the continuously scanned area did not seem affected by the compounds it was suggested that the pressure of the AFM tip assisted the incorporation of antimicrobials in the membrane, enhancing their bactericidal efficacy. Staphylococci biofilm proved to be susceptible to all the antimicrobials, with QAC being the most effective one, causing a complete loss of metabolic activity at 2xMBC. The antimicrobial action of the QAC was compromised by the presence of calcium ions both in planktonic cultures and in adhering staphylococci which suggests that its mode of action relies on its ability to exchange with calcium ions that are responsible for membrane stability.

RESUMO

A adesão bacteriana e consequente formação de biofilmes constitui um problema sério, sobretudo na área clínica, visto que as bactérias associadas em biofilmes são menos susceptíveis à terapia antibiótica e à acção do sistema imunitário. Uma potencial estratégia para lidar com este problema consiste no uso de revestimentos antimicrobianos constituídos por compostos quaternários de amónio (QACs).

A presente tese teve como principal objectivo o estudo da eficácia de um QAC sobre uma estirpe bacteriana do género estafilococos quando comparado com outros dois compostos antimicrobianos, um antibiótico (Gentamicina) e um péptido antimicrobiano (Gramicidina S). Depois de avaliada a actividade antimicrobiana dos compostos em culturas planctónicas através da determinação da concentração mínima inibitória (MIC) e da concentração mínima bactericida (MBC), as bactérias aderidas a uma superfície foram expostas aos compostos e a sua superfície celular foi analisada por AFM. O número de bactérias removidas pela ponta do AFM foi determinado e considerado como uma indicação dos danos causados na superfície celular. A susceptibilidade dos biofilmes aos compostos foi avaliada pela determinação da actividade metabólica do biofilme através de um ensaio de 3-(4,5-dimetiltiazol-2-il)-2-5 difeniltatrazólio de brometo (MTT). Finalmente, com o intuito de investigar o modo de acção do QAC sobre as células bacterianas, a sua actividade antimicrobiana, bem como os seus efeitos nas células aderidas e analisados por AFM foram realizados na presença de iões de cálcio.

Todos os compostos exibiram actividade antimicrobiana no estado planctónico. O efeito dos compostos sobre as células bacterianas foi analisado *in situ* com o AFM. A superfície celular apresentou-se mais enrugada depois de exposta aos compostos até as bactérias serem removidas da superfície. A Gentamicina resultou numa remoção mais rápida que o QAC e a Gramicidina S que exibiram resultados semelhantes. Como as bactérias localizadas fora da área continuamente analisada pelo AFM não foram igualmente influenciadas pelos compostos foi colocada a hipótese de que a pressão da ponta do AFM auxiliou a incorporação dos compostos na membrana, melhorando assim a sua eficácia bactericida. O biofilme formado pela estirpe estudada na presente tese apresentou-se susceptível a todos os compostos antimicrobianos, sendo o QAC o mais eficaz ao provocar uma redução completa da actividade metabólica do biofilme a 2xMBC. A acção antimicrobiana do QAC foi comprometida pela presença de iões de cálcio o que sugere que o seu modo de acção depende da sua capacidade de substituir estes iões na membrana.

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ABBREVIATIONS

AFM	Atomic force microscopy
ATCC	American type culture collection
BAI	Biomaterial associated infections
CLSM	Confocal laser scanning microscopy
CNC	Coagulase-negative staphylococci
CTAB	Cetyltrimethylammonium bromide
EPS	Extracellular polymeric substances
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NB	Nutrient broth
PEI	Polyethyleneimine
PIA	Polysaccharide intercellular adhesion
PLL	Poly-L-lysine
QAC	Quaternary ammonium compound
QAS	Quaternary ammonium silane
QNM	Quantitative nanomechanical
SEM	Scanning electronic microscopy
TEM	Transmission electronic microscopy
TSB	Tryptone soy broth

SCOPE AND AIMS

Despite considerable research have been conducted in the development of new materials and in the design of innovative devices, infections associated to biomaterial implants and medical devices remain a serious problem. Bacteria are able to reach the biomaterial surface, adhere to it and form multicellular aggregates enclosed in a self-produced matrix of extracellular polymeric substances with increased resistance to antibiotic treatments and to the host immune system. The microbial adhesion and biofilm formation on surfaces of biomedical implants and devices can cause severe problems, often requiring the replacement of the infected device at the expense of considerable costs and patient's suffering. Therefore, big efforts have been conducted to stop and prevent the formation of these microbial biofilms.

The main purpose of the present thesis is to assess the potential of using a QAC (Ethoquad C/25 (Cocoalkyl methyl (polyoxyethylene) ammonium chloride)) in the prevention of microbial adhesion for possible application in antimicrobial coatings of biomedical devices. In order to achieve this goal, the efficiency of the QAC against staphylococci will be studied and results will be compared with two other compounds, an antibiotic (Gentamicin sulphate) and an antimicrobial peptide (Gramicidin S). Initially, the antimicrobial activity of the compounds will be assessed by determining their minimal inhibitory (MIC) and minimal bactericidal concentrations (MBC). Afterwards, adhering staphylococcal cells will be exposed to the compounds and their cell surfaces will be analyzed using AFM. Bacterial detachment during exposure to the target compounds will be followed for 300 min and this parameter will be considered as an indication of cell surface damage. Furthermore, the effect of the compounds will also be investigated against biofilms, and experiments in the presence of calcium cations in the surrounding fluid will be performed in order to prove the role of membrane charge exchange in the integration of QAC molecules in the bacterial cell membrane.

CHAPTER 1

GENERAL INTRODUCTION

1.1|BACTERIAL ADHESION AND BIOFILM FORMATION ONTO BIOMATERIALS

Modern health care is strongly dependent on the use of biomaterials implants and devices such as joint prostheses, heart valves, vascular catheters, contact lenses and dentures (von Eiff *et al*. 2005), to support or restore human body function after trauma or disease. Their introduction in the modern medical practice was responsible not only for a better quality of life, but also for a longer patient's survival (Baveja *et al*. 2004). Despite the remarkable modern advances in medicine and improvements in the materials and design of devices, there are some drawbacks associated to their extended use, mainly the ocurrence of biomaterial associated infections (BAI), when microorganisms are able to reach a biomaterial surface forming a so-called biofilm (Gottenbos *et al*. 2002). Their impact in the medical field is enormous since the rate of BAI for initially inserted implants varies from 1 to 30% with a mortality risk of up to 25%, depending on the type of medical device (Nejadnik 2009). Microorganisms responsible for biofilm formation on indwelling medical devices include yeasts (*Candida* species), gram-positive (*Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans*) and gram-negative (*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa*) bacteria (Table 1.1).

Table 1.1| Incidence and microorganisms most commonly isolated from infections on different biomedical implants and devices. Adapted from Roosjen *et al*. 2006, Maathuis *et al.* 2007.

Implant or device	Incidence	Microorganisms found	Associated disease or consequences
Hip prosthesis	2.6-4.0%	*Enterococcus spp.* *Pseudomonas aeruginosa* *Staphylococcus aureus* *Staphylococcus epidermidis*	Septicemia, prosthesis failure
Voice prosthesis	Every 4 months	*Candida albicans* *Streptococcus spp.* *Escherichia coli* *S. epidermidis*	Prosthesis failure
Central venous catheter	4-12%	*C. albicans* *Enterococcus spp.* *Klebsiella pneumoniae* *P. aeruginosa* *S. aureus* *S. epidermidis*	Septicemia, endocartitis
Intraocular lenses	0.5%	*Pseudomona spp.* *S. epidermidis*	Keratitis
Dental implants	5-10%	*C. albicans* *Streptococcus spp.* *Lactobacillus spp.* *Actinomyces spp*	Periodontitis

The occurrence of BAI is determined by the probability of microorganisms to reach the biomaterial surface. Biomaterials in contact with the outer part of the body, such as contact lenses and intravenous catheters, are colonized as soon as they are placed on tissue surfaces exhibiting therefore a higher incidence of BAI than fully implanted biomaterials, such as hip or knee implants (0.5% - 100% versus 0.1% - 7%) (Gottenbos *et al*. 2000). Microorganisms can reach a biomaterial implant in several ways and at different times post-implantation (Subbiahdoss *et al*. 2009). The most common route of infection is the direct contamination of the biomaterial implant during its insertion (intra-operative contamination) by microorganisms inevitably present in the operating theatre or microorganisms from the skin commensal microflora (Davis *et al*. 1999). Contamination can also occur during hospitalization (post-operative contamination) when the infected material contacts with the wound or after using invasive devices like catheters. Since microorganisms have the ability to stay at a low metabolic state on a biomaterial surface (Gottenbos *et al*. 2000) inside the human body for several years, they can be responsible for the occurrence of an infection (BAI) years after its insertion. A third possible route of infection, but less likely to occur, is late haematogenous contamination when bacteria from local infections elsewhere in the body are spread through blood.

Whenever microorganisms reach the biomaterials surface, initial microbial adhesion which is mediated by physicochemical properties of both bacterium and biomaterial surface can occur. Once adhered, the microorganisms are protected against phagocytosis, as the microorganism and biomaterial together are too large to ingest. After adhesion, most microorganisms start secreting slime and embed themselves in a slime layer, the glycocalix, which is an important virulence factor for BAI (Branda *et al*. 2005). The complex structure of a biofilm on current biomaterials makes it resistant against antibiotic treatment and host immune system (Prakash *et al.* 2003). Even high concentrations of antibiotics have been reported to fail in eradicating mature biofilms (Anwar *et al.* 1992). As a consequence, the only solution to an infected implant device is often its surgical removal at the expense of considerable costs and patient's suffering (Francolini and Donelli 2010). The best approach to overcome this problem is to prevent biofilm formation on the biomaterial surface. In order to accomplish this purpose, a better understanding of mechanisms underlying the biofilm development is required.

1.1.1|BIOFILMS: A SURVIVAL STRATEGY OF MICROORGANISMS

In the history of Microbiology, microorganisms have been primarily characterized as unicellular life forms, living as planktonic, free-suspended cells (Donlan 2002), and their description on the basis of their growth characteristics in nutritionally rich culture medium provided important insights about microbial pathogenesis and physiology (Davey and O'Toole 2000). However, it is now widely recognized that microorganisms have a marked and natural tendency to interact with surfaces and interfaces, where they are found in the form of multicellular aggregates enclosed in a matrix of primarily polysaccharide material, commonly referred as biofilms (Flemming and Wingender 2010).

The discovery of microbial biofilms can be attributed to Antoni van Leeuwenhoek, who first observed microorganisms on tooth surfaces using his simple microscopes, in the 17th century. Afterwards, Hukelekian and Heller (1940) and Zobell (1943) demonstrated that bacterial growth and activity were substantially enhanced by the incorporation of a surface to which these organisms could attach, and the number of bacteria on surfaces was dramatically higher than in the surrounding medium (seawater). The study of Characklis (1973) about microbial slimes in industrial water systems showed that they were not only very tenacious, but also highly resistant to disinfectants such as chlorine. However, it was Costertan *et al.* (1978) that postulated a theory of biofilms explaining the mechanisms by which microorganisms adhere to surfaces and the benefits derived by this mode of growth.

The development of a biofilm is characterized by a series of complex and well-regulated steps (Figure 1.1): adsorption of a conditioning film (1), transport of microbial cells towards the surface followed by reversible adhesion (2), irreversible adhesion (3), biofilm maturation (4) and detachment of individual bacteria or aggregates (5) (Dunne, 2002).

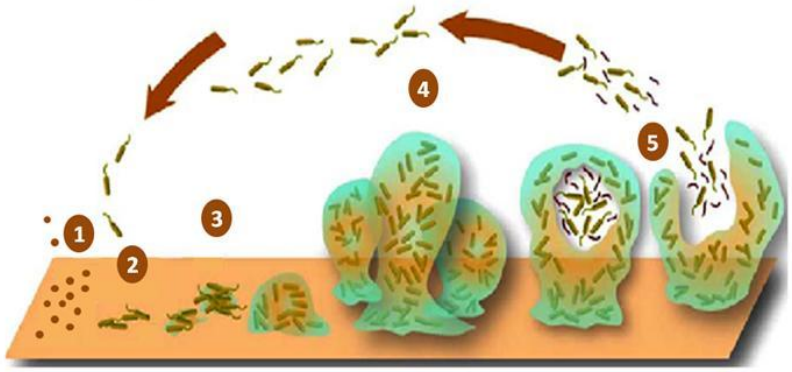

Figure 1.1| Schematic representation of the steps involved in biofilm formation on a surface: conditioning film formation (1), reversible attachment (2), irreversible attachment (3), maturation (4) and detachment (5). Adapted from Stoodley and Dirckz 2003.

Prior to biofilm formation, the surface is first covered with a layer of proteins and glycoproteins such as fibronectin, vitronectin, fibrinogen, albumin and immunoglobulins, which are present in the surrounding aqueous environment. Biofilm formation starts with the transport of bacteria to the surface-liquid interface, which is governed by a combination of transport mechanisms, including Brownian motion, gravity, diffusion, convection or the intrinsic motility of a microorganism (Roosjen *et al*. 2006). The bacterium approaches the surface so closely that its motility is slowed, and it forms a reversible association with the surface and/or other microbes previously attached to the surface (Prakash *et al.* 2003). In this process non-specific interactions which are governed by physicochemical properties are involved, such as surface charge, hydrophobicity and chemical structure of both bacterium and surface. Reversible adhesion of bacteria changes afterwards to irreversible, since the attachment of adhering microorganisms is strengthened through extracellular polymeric substances (EPS) production, unfolding of cell structures and protein-protein interactions. EPS are biopolymers that form hydrogels with water and provide a stable structure to the biofilm. Most of these biopolymers are polysaccharides consisting of sugars such as glucose, galactose, mannose and fructose, but also traces of proteins, lipids and nucleic acids are present. Adhering bacteria grow and divide, forming microcolonies that are considered to be the basic organizational units of a biofilm. Entrapment of other planktonic bacteria in the EPS also occurs, resulting in a multi-layered and mature biofilm. The last step is the detachment of individual bacteria or aggregates caused by occasionally high fluid shear or other detachment forces operative, which enables bacteria to disseminate into other areas for further surface colonization. In the clinical setting, this last step usually leads to severe systemic infections (Katsikogianni and Missirlis 2004).

The final structure and composition of the biofilm are determined by the characteristics of the system where it was developed. Factors such as the type of microorganisms, the hydrodynamic environment, surface roughness, nutrients available, attraction and adhesion to other microorganisms from the surrounding environment regulate biofilm formation (Costerton *et al.* 1987, Hall-Stoodley *et al.* 2004). Biofilm formation is an important survival strategy for bacterial cells. In Nature, more than 99% of bacteria exist as biofilms. The ubiquity of these structures on several and different ecosystems demonstrates the strong survival and selective advantage of sessile communities over planktonic cells (Dunne 2002). In fact, it is estimated that bacterial cells growing as biofilms are up to 1000-fold more resistant to antibiotics and can cope much better with unfavourable external conditions, as the host immune system, than their planktonic counterparts (Falagas *et al*. 2009). This resistance can be attributed to a number of

factors observed in biofilm populations, including restricted penetration, decreased growth rate, a distinct genetic phenotype (Handke *et al.* 2004, Harrison *et al.* 2004, Schierholtz and Beuth 2001), the expression of resistance genes (Maira-Litran *et al.* 2000) and the presence of biofilm persistent cells (Roberts and Stewart 2005).

1.1.2|COAGULASE-NEGATIVE STAPHYLOCOCCI AS NOSOCOMIAL PATHOGENS

Staphylococci are Gram-positive bacteria belonging to the family *Staphylococcaceae* characterized by round cells (coccus or spheroid shaped), with about 1 μm in diameter and found as single cells, in pairs or, more frequently, in clusters that resemble clusters of grapes (Huebner and Goldmann 1999, Fey and Olson 2010). They are clustering, non-motile and non-spore forming cocci, and facultative anaerobes that produce catalase. Although more than 30 species of the genus *Staphylococcus* have been described, *S. aureus* and *S. epidermidis* are the most significant in their interactions with humans (Gotz 2002). Staphylococci are divided into coagulase-positive and coagulase-negative strains depending on its ability or inability to clot blood plasma. Coagulase-negative staphylococci (CNC) have been regarded as harmless skin commensals, but during the past few decades their importance as human (predominantly nosocomial) pathogens has been recognized (Vuong and Otto 2002). From 1990 to 1995, the National Nosocomial Infection Surveillance (NNIS) program reported that CNS were responsible for 11% of all nosocomial infections reported, making this pathogen the third most common nosocomial isolate (Huebner and Goldmann 1999).

S. epidermidis is currently the most significant member of the CNS and constitutes the most widespread and persistent species found on the human skin and mucous membranes, representing an important part of its normal microflora (comprises 65% of the 90% of all staphylococci isolated from these environments) (Vuong and Otto 2002, Otto 2009). In recent years, *S. epidermidis* emerged as one of the most important and frequently causes of nosocomial infection, mainly associated with implanted medical devices (Rupp and Archer 1994). In fact, in what concerns BAI, nearly 80% of the cells involved are *S. epidermidis* (Gotz 2002). The indwelling medical devices mostly colonized by *S. epidermidis* include central venous catheters, cerebrospinal fluid shunts, prosthetic heart valves, ocular lenses implants, prosthetic joints, dialysis devices, hip prostheses and many other invasive biomaterials (Donlan, 2001).

Numerous studies have clearly reported the ability of *S. epidermidis* to form a thick adherent, multi-layered biofilm, strongly resistant to antibiotic treatment which is considered the most important virulence factor involved in its pathogenesis. Biofilm

formation by staphylococci proceeds in two fundamental steps, namely attachment of bacterial cells to the biomaterial surface and cells accumulation to form multilayered cell clusters enclosed in an exopolimeric matrix (Figure 1.2).

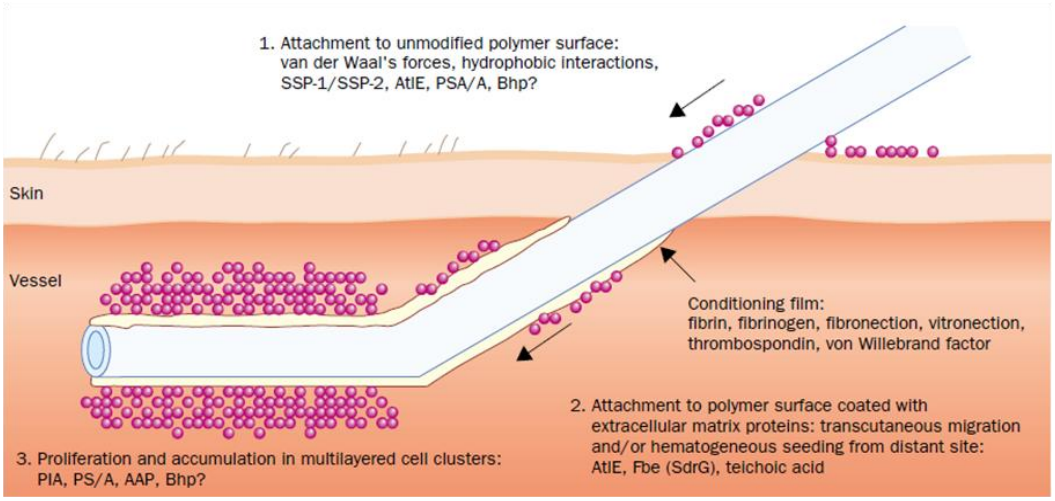

Figure 1.2| Schematic representation of the phases involved in *Staphylococcus epidermidis* biofilm formation and bacterial factors involved. Taken from von Eiff *et al*. 2002.

The first step is influenced by numerous factors, such as hydrophobicity and surface charge (Ziebuhr *et al.* 2006), as well as by means of cell wall teichoid acids and proteins that interfere with matrix proteins, like collagen and fibronectin (McCann *et al*. 2008). *S. epidermidis* adhesion to a foreign body surface is followed by biofilm accumulation which requires the production of factors that mediate intercellular adhesion, such as polysaccharide intercellular adhesion (PIA). PIA is a homoglycan composed of β-1,6-linked 2-amino-2-deoxy-D-glucopyranosyl residues, containing positive charged amino groups as well as negative charges (Rohde *et al*. 2010). Table 1.2 summarizes some of the factors involved in the biofilm formation by staphylococci.

Table 1.2| The major factors contributing to *Staphylococcus epidermidis* biofilm formation and their function. Adapted from von Eiff *et al.* 2002 and Ziebuhr *et al.* 2006.

Factor	Function
Staphylococcal surface proteins: SSP-1, SSP-2	Attachment to uncoated polystyrene
Autolysin E: AtlE	Involved in staphylococcal cell wall synthesis and binding to uncoated polystyrene as well as vitronectin
Capsular polysaccharide/adhesin: PS/A	Attachment to a polymer surface and biofilm accumulation
Biofilm-associated proteins: Bap/Bhp	Surface protein-mediating biofilm formation by bacterial cell-cell contacts
Fibrinogen-binding protein: Fbe	Binding to the β chain of fibrinogen
Polysaccharide intercellular adhesin: PIA	Intercellular adhesion and biofilm accumulation
Accumulation-associated protein: AAP	Surface protein that mediates bacterial aggregation after proteolytic cleavage

1.2|TREATMENT AND PREVENTION OF BIOMATERIAL ASSOCIATED INFECTIONS

Whenever a BAI is detected there are two possible modes of action, namely the removal of the device and/or the initialisation of an antimicrobial treatment. The therapy of choice, especially due to the easiness to change devices such as short term peripheral catheters, is the removal of the infected device and its replacement if still needed (von Eiff *et al.* 2005). However, removing the infected device is not always possible, easy to perform and/or without risk. Therefore, the recovery of the device is sometimes the preferred option. Once a BAI is established it is very difficult to treat it as the minimal inhibitory concentration (MIC) of antimicrobial agents, necessary to kill microorganisms, is significantly higher for microorganisms in a biofilm as compared to their planktonic counterparts (Costerton *et al*. 1999). Although much research has been done to turn biofilms more susceptible to antimicrobial agents, the best approach to overcome this issue is to prevent biofilm formation (Simões *et al*. 2010).

Over the past years several strategies have been used to prevent bacterial adhesion and biofilm formation on biomaterials. In general, these strategies are based in the reduction of the attractive force between microorganisms and a biomaterial surface by optimizing the physicochemical surface properties of the biomaterial (Kazmierska and Ciach 2009). For instance, extremely hydrophobic surfaces (Everaert *et al*. 1999), more negatively charged biomaterials (Hogt *et al.* 1986), biomaterials coated with albumin or heparin (El-Asrar *et al*. 1997), have shown to attract fewer bacteria. Nevertheless, as biomaterial surfaces are often covered with a conditioning film consisting of proteins that can be anchors for microorganisms to adhere to, another approach to prevent biofilm formation can include the application of antimicrobial agents near the biomaterial surface in order to prevent the growth of adhering microorganisms. Gentamicin-loaded bone cements and silver-loaded catheters (Carlsson *et al*. 1978) are two examples in which this approach was implemented. Some drawbacks of such applications are their lifetime (few days to weeks), the limited amount of antibiotic released and the development of antibiotic resistant microbial strains (Gottenbos *et al.* 2002). A better approach to prevent BAI is to render the biomaterial surface antimicrobial properties by functionalizing it with antimicrobial agents covalently attached (Vasilev *et al.* 2009). This strategy can only 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 compounds (QACs) work at the membrane level, and are one of the few known antibacterial molecules that retain their bactericidal properties when covalently bound to a surface (Flemming *et al*. 2000, Kenawy *et al*. 1998). In this approach, no antimicrobial

agents are leaching from the surface, providing long term protection against bacterial colonization, and reducing the risk of developing antimicrobial resistant microbial strains, since the concentration of antimicrobial groups is constantly above the MIC.

1.2.1|THE ROLE OF QUATERNARY AMMONIUM COMPOUNDS ON PREVENTING BAI

QACs constitute a family of surfactants that have been widely used in domestic, agricultural, healthcare, and industrial applications (Garcia *et al.* 1999, Patrauchan and Oriel 2003). Surfactant is an abbreviation for surface active agent that literally means active at a surface, and is characterized by its ability of lowering surface and interfacial tensions of liquids, which comprise the ability to wet surfaces, penetrate soil and solubilise fatty materials (Christofi and Ivshina 2002, Pereira *et al.* 2007). These agents are composed by two distinct structural elements: a hydrophobic (water repellent) group and a hydrophilic (water attractive) group (Figure 1.3).

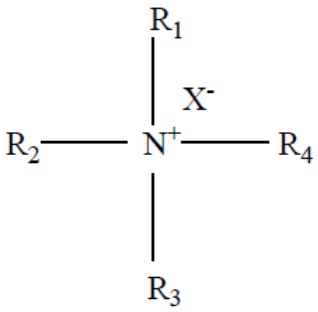

Figure 1.3| General chemical structure of a surfactant (R represents a functional group and X^- represents a counter ion such as Cl^-, Br^- or NO_3^-).

Depending on the basis of the charge or absence of ionization of the hydrophilic group, surfactants can be classified into cationic, anionic, non-ionic, and ampholytic or amphoteric compounds. Of these, the cationic agents, as exemplified by QACs, are the most useful antiseptics and disinfectants. In addition to their detergent properties, QACs also present remarkable antimicrobial properties being used for a variety of clinical purposes, such as pre-operative disinfection of undamaged skin, application to mucous membranes and disinfection of noncritical surfaces (Simões *et al.* 2005b).

1.2.1.1|QAC'S MECHANISMS OF ACTION

Although a wide variety of studies on the interaction between microbial cell surface and QACs have been reported (Ioannou *et al.* 2007, Marcotte *et al*. 2005), the details of their mechanism of action are not completely understood. Two hypotheses have been reported to explain the susceptibility of a broad range of species to these compounds. The most quoted theory hypothesizes that sufficiently long cationic polymers penetrate cells and thereby disrupt the membrane like a needle bursting a balloon (Milovic *et al.* 2005). Several observations indicate that QACs, given their cationic nature, act at the membrane level which is negatively charged at physiological pH and thus, for many decades, such compounds have been designated as membrane active agents. When microorganisms are exposed to cationic agents the following sequence of events occur (Ikeda *et al.* 1984): (i) adsorption and penetration of the agent into the cell wall; (ii) reaction with the cytoplasmic membrane followed by membrane disruption; (iii) leakage of intracellular low-molecular weight material; (iv) degradation of proteins and nucleic acids and (v) cell wall lysis caused by autolytic enzymes. At a molecular level, QAC's action involves an association of the positively charged quaternary nitrogen with the head groups of acidic-phospholipids within the membrane (Figure 1.4 a, b), and the hydrophobic tail is then inserted into the hydrophobic membrane core (Figure 1.4 b, c). At QAC's low concentrations, such interaction is responsible for the increase of surface pressure with a subsequent decrease of membrane fluidity and increase of phase transition temperature. As a consequence, the membrane undergoes a transition from solid to liquid crystalline state losing many of its physiological functions (Figure 1.4 d). For higher concentrations, QACs form micellar aggregates that solubilise hydrophobic membrane compounds, such as lipid A and phospholipids (Figure 1.4 e, f) (Gilbert and Moore 2005).

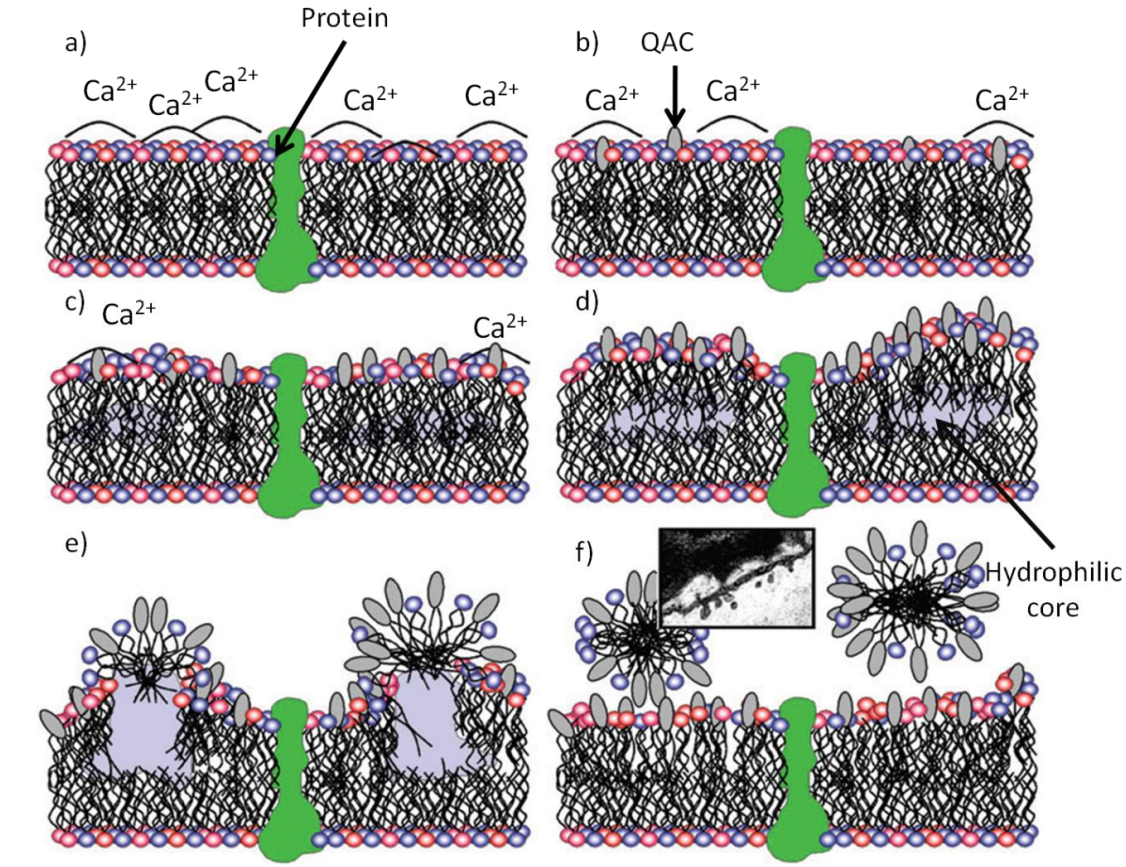

Figure 1.4| Schematic representation showing the mechanism of action for QACs. Progressive adsorption of the quaternary head group to acidic phospholipids in the membrane with increasing QAC exposure/concentration leads to decreased fluidity of the bilayers and the creation of hydrophilic voids in the membrane. Taken from Gilbert and Moore 2005.

The second hypothesis, proposed by Kugler *et al.* (2005), states that a highly charged surface can induce what is essentially an ion exchange between the positive charges on the surface and structurally critical mobile cations within the membrane, such as calcium and magnesium. When approaching to a cationic surface, divalent cations are free to diffuse out of the membrane without performing their role in charge neutralization of the membrane components, which results in a loss of membrane integrity. An attempt to study the contribution of both hypotheses was performed by Murata and co-workers (2007). QACs were used to prepare antimicrobial polymer brushes on inorganic surfaces. By variation of the chain length containing the quaternary ammonium group and the surface density in a gradient manner, the authors could conclude that the density of surface quaternary ammonium groups, and thus the density of cationic surface charges, is a key parameter. However, the authors did not exclude that membrane insertion of alkyl chains may also be a possible mechanism of action.

1.2.1.2| ANTIMICROBIAL AND ANTI-ADHESIVE ACTIVITIES OF QACS

QACs are organic compounds that contain four functional groups attached covalently to a central nitrogen atom (Figure 1.3). These functional groups (R) include at least one long chain alkyl group, and the rest are either methyl or benzyl groups. Their antimicrobial activity depends on their structure and size, but especially on the length of the long-chain alkyl group. In fact, it was reported that there is a parabolic relationship between their antibacterial properties and their hydrophobic character, which means that there is a linear relationship between activity and alkyl chain length with increased carbon number up to a maximum of between 12 an 14, at which region could be observed a decrease in activity (Tomlinson *et al.* 1977).

The antimicrobial activity of several QACs against Gram-positive and Gram-negative bacteria in planktonic cultures, as well as in biofilms, has been reported. For instance, the cationic surfactant cetyltrimethylammonium bromide (CTAB) was investigated by Simões *et al.* (2005a) for its ability to control mature *Pseudomonas fluorescens* biofilms formed under laminar and turbulent flow in flow cell reactors. The authors found that CTAB by itself did not cause the detachment of biofilms, but it reduced the respiratory activity of the biofilm cells. Total respiratory inactivation was not achieved and, in almost all the cases studied, it was observed that the biofilm respiratory activity was recovered over time. However, the same authors, in another study (2005c) reported that the synergistic action of CTAB with the application of high shear stress to mature biofilms (formed in a rotating device) increased its detachment.

In the last decade, continuous effort has been made to develop polymers with antimicrobial properties because polymeric antimicrobials comprise the following advantages: are non volatile, chemically stable, have long-term antimicrobial activity and are hard to permeate through the skin. There are several studies reporting the efficacy of polymers containing quaternary ammonium salt groups against both Gram-positive and Gram-negative bacteria. Kenawy *et al.* (1998) concluded that quaternary ammonium and phosphonium copolymers based on the modified poly(glycildyl methacrylate-co-hydroxyethyl methacrylate) materials could be promising candidates for preventing BAI, after evaluating their antimicrobial activity *in vitro* against Gram-positive (*Bacillus subtilis* and *Bacillus cereus*) and Gram-negative (*E. coli, P. aeruginosa, Shigella sp. and Salmonella typhae*) bacteria.

QACs have also been found to inhibit the adhesion of pathogenic organisms to solid surfaces, thus prior adhesion of these compounds to the surfaces might constitute a new and effective means of avoiding colonization by pathogenic microorganisms. Such

approach was implemented by Tiller *et al.* (2001) that covalently attached by different methods long chains of N-alkylated poly (4-vinylpyridine) (PVP) to a glass slide. The resultant glass slides were able to kill by contact several airborne Gram-negative and Gram-positive bacteria. Such surface modifications can be readily performed with a number of other materials, thus making this approach useful for the coating of various medical devices. The same research group (Lin *et al.* 2002) reported the efficiency of these functionalized surfaces against wild-type and mutant, including antibiotic-resistance, strains of the ubiquitous pathogenic bacterium *S. aureus*. These observations support the assertion of a possible role in preventing microbial adhesion and their potential in developing anti-adhesive biological coatings for implant materials.

Silicone rubber with covalently coupled 3-(trimethoxylsilyl)-propyldimethyloctadecylammonium chloride (a quaternary ammonium silane, QAS) showed antimicrobial properties against adhering bacteria, both *in vitro* and *in vivo* (Gottenbos *et al.* 2002). In another study, reported by Oosterhof *et al.* (2006), two QAS were used to coat silicone rubber tracheoesophageal shunt prostheses in order to evaluate their inhibitory effects against the development of a mixed fungal and bacterial biofilm. This study reported for the first time that both yeasts and bacteria in mixed biofilms are affected by QAS coatings on silicone rubber. Because QAS coatings are non toxic, its clinical use could increase the lifetime of tracheoesophageal shunt prostheses. However, it is important to notice that the relevance of the current findings also extends to all biomedical and environmental applications where mixed biofilms can occur and be detrimental.

1.3| MICROSCOPY TECHNIQUES TO STUDY THE ANTIMICROBIAL EFFECTS

Understanding the interactions between bacterial cells and antimicrobial compounds is crucial to develop solutions that envisage the prevention of infections associated to biomaterials. Over the past, several techniques have been taken into consideration to study the antimicrobial effects of different compounds (Katsikogianni, 2004). Among them, microscopy-based methodologies (Table 1.3) have proved to be particularly useful since visualization of the changes undergone by microorganisms when subjected to antimicrobial treatment can provide new and important insights about the agents' mechanism of action (Hannig *et al*. 2010, Torrent *et al.* 2010).

Table 1.3| Comparison between the most commonly used techniques used to visualize the effect of antimicrobials on bacteria.

Technique	Description	Advantages	Disadvantages	Applications	References
Optical Microscopy	▪ Visible light is used as a source of illumination. ▪ A system of optical lenses is used to magnify the sample.	▪ Measurements performed in liquid, air or vacuum.	▪ Resolution limited by the wavelength of source light.	▪ Evaluation of early stages of oral Streptococci biofilm growth and the effect of antimicrobial agents.	(Cortizo and Lorenzo 2007)
Electron Microscopy (SEM and TEM)	▪ A beam of high-energy electrons is used as a source of illumination. ▪ A system of electromagnetic lenses is used to magnify the sample.	▪ High-resolution. ▪ Higher magnification and larger depth of focus.	▪ Very time consuming and complex technique. ▪ Sample preparation procedure requires freeze drying and gold sputtering. ▪ Measurements performed under vacuum. ▪ Time consuming sample preparation. ▪ Evaluation of different bacterial species not possible.	▪ TEM study of antibiotic action on *Klebsiella pneumonia* biofilm. ▪ SEM study on the effect of doxycylcine and vancomycin on enterococcal induced biofilm.	(Zahller and Stewart 2002, Somayaji *et al*. 2010)
Confocal Laser Scanning Microscopy (CLSM)	▪ A laser beam passes through a light source aperture and then is focused by an objective lens into a small focal volume within or on the surface of the sample.	▪ Visualization of cells *in situ.* ▪ Measurements performed in liquid, air or vacuum. ▪ Ability to control depth of field.	▪ Limited number of excitation wavelengths with common lasers. ▪ Harmful nature of high-intensity laser irradiation on living cells and tissues.	▪ Assess the bactericidal effect of chlorhexidine on dental biofilm.	(Zaura-Arite *et al*. 2001)
Atomic Force Microscopy (AFM)	▪ A sharp tip scans over the surface of a sample while senses the interaction between them.	▪ Measurements performed in liquid, air or vacuum. ▪ High resolution (up-to sub-nanometer). ▪ Minimal sample preparation. ▪ Visualization of cells in real time.	▪ Long image acquisition time.	▪ Action of the antimicrobial peptide CM15 on individual *Escherichia coli* cells.	(Fantner *et al*. 2010)

The atomic force microscopy (AFM) belongs to the broad family of scanning probe microscopes (SPM), in which a probe is used for investigating surface topography and properties with sub-nanometer resolution. This technique, initially developed in 1986 by Binning, Quate and Gerber to overcome the limitations of its ancestor (scanning tunnelling microscope, STM) in imaging non-conducting samples, immediately attracted the attention of the biophysical community (Santos and Castanho 2004). In biological applications, the most appealing advantage of the AFM when compared with other techniques such as SEM and TEM is that it allows the characterization of biological samples regarding their structure and mechanical properties under physiological conditions in real-time with sub-nanometer resolution, avoiding complex sample preparation procedures and the associated artefacts. Since its invention, the significant improvements obtained both at the instrumental and sample preparation levels, as well as recording conditions, led to a revolution of the way in which biologists explore microbial surfaces (Dufrêne 2002).

1.3.1|BASIC PRINCIPLES OF AFM AND AFM IMAGING MODES

The basis of AFM is the measurement of the interaction between the sample surface and a tip located very close to it (Figure 1.5). The sample is mounted on a piezoelectric scanner which can move precisely in three dimensions and the tip is assembled under an extremely flexible cantilever (Liu and Wang 2010). The cantilever can be simple or triangular, usually between 23-300 µm length, 10-30 µm width and 0.5-3 µm thick (Santos and Castanho 2004). The most commonly used are made of silicon nitride (Si_3N_4), with spring constants raging from 0.01 to 100 N/m. Any interaction between the tip and the sample leads to a bending of the cantilever that will be proportional to the interaction force. To detect this bending, AFM uses a small laser focused on the back of the cantilever. The reflection of the laser beam is focused on a photo-detector that measures any minimal bending of the cantilever, and thus the interaction of the tip with the sample (Rajasekaran 2008, Butt *et al*. 2005, Reich *et al*. 2001).

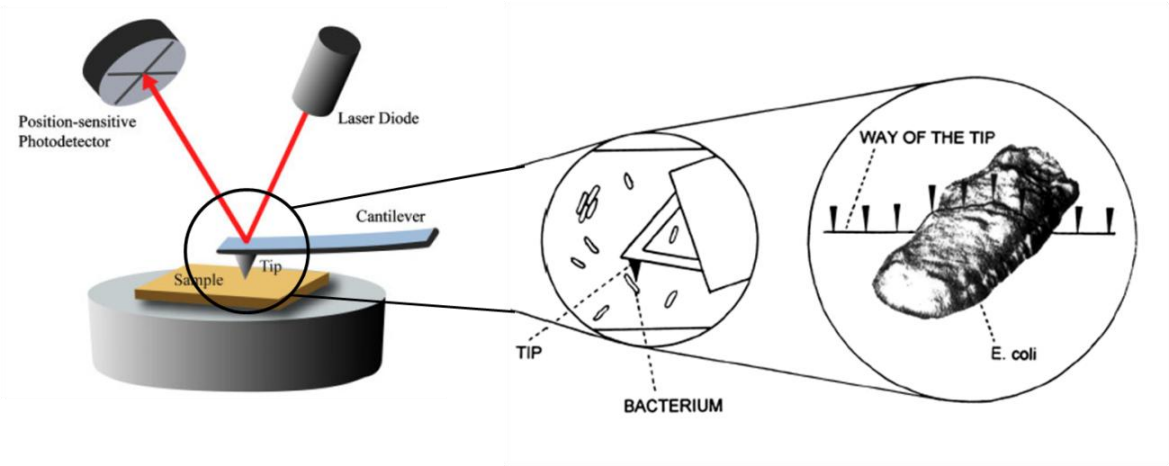

Figure 1.5| Schematic diagram of the AFM working principle. Taken from Liu *et al.* 2010, Braga and Ricci 2000.

Another important advantage of AFM is that it offers the possibility to choose between different imaging modes that differ mainly in the way the tip is moving over the surface sample (Figure 1.6). In contact mode, one of the most widely used, the AFM tip is translated over the sample being in continuously contact with the surface sample while the cantilever deflection and therefore, the force applied to the tip, is kept constant using feedback control. A drawback of this mode is that it is associated with substantial shear forces which can distort soft biological samples (Kasas *et al.* 1997). In dynamic mode, which includes intermittent contact and non contact modes, the cantilever is oscillated near or slightly above its resonance frequency during the scan and therefore, the shear forces between the tip and the sample can be significantly reduced. In non contact mode, the cantilever is vibrated slightly above its resonance frequency at a distance of 1-10 nm above the sample. Since the forces involved in this mode are much lower than those in contact mode, it is possible to image the softest samples without damage. However, since the tip-sample distance is relatively large, a lower resolution is obtained than in contact mode (Dorobantu and Grat 2010). For the intermittent contact or tapping mode, the cantilever is also oscillated above the surface, but in contrast to the non contact mode, the tip periodically touches the surface. It is, therefore, a compromise between the contact and non contact modes. The main advantage of this mode is the reduction of the shear forces which can distort or damage samples.

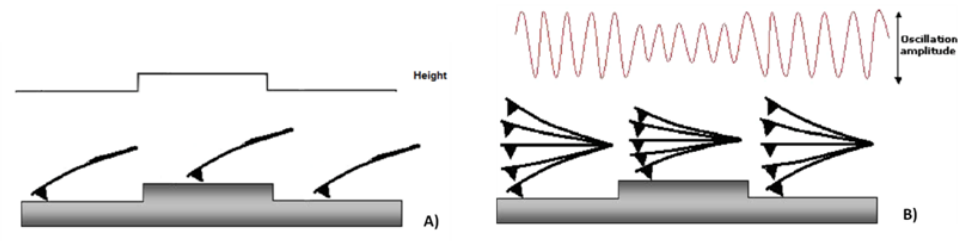

Figure 1.6| Schematic representation of an AFM tip operating in the contact mode (A) and dynamic mode (B). Taken from Alessandrini and Facci 2005.

1.3.2|SAMPLE PREPARATION FOR AFM

An important requirement for successful AFM imaging and force measurements is that the sample must be attached to a solid substrate. For microbiology applications, this is one of the primary challenges encountered when imaging microorganisms, as their size and shape only provide a small contact area between the cell and the underlying substrate, often leading to the cell detachment by the scanning probe. One of the first approaches used to achieve the attachment of microorganisms on a specific substrate required drying the sample. A drop of cell culture was left to dry out on a surface before being re-hydrated and imaged in water or buffer (Kailas *et al.* 2009, Meyer *et al.* 2010). In fact, many studies have dealt with dried bacteria, as sample preparation is easier and the resolution obtained is in general better than when imaging is carried out in liquid environments (Wagner 1998). Another immobilization method can be performed by binding cells covalently to a support by chemical fixation or using cross-linking agents such as glutaraldehyde (Dorubantu and Gray 2010). However, these methods are known to affect cell viability, yielding information that may no longer be representative of their native state and therefore alternative approaches have been proposed. The most commonly used immobilization methods that do not compromise cell viability are physical entrapment in a porous membrane or in an agar gel, or immobilization by electrostatic interactions to surfaces that have been coated with polycations such as polyethyleneimine (PEI), poly-L-lysine (PLL) or gelatine. Mechanical entrapment in membranes is only suitable for imaging and force measurements of spherical cells. It does not allow the analysis of the entire bacterial surface making it difficult to follow active processes such as cell division, and there are some indications of mechanical stress being exerted by the entrapment process (Kailas *et al.* 2009). While electrostatic immobilization does not exert mechanical stress, the strength of the interaction is

weakened at high ionic strength. Meyer *et al.* (2010) have introduced a new method for the immobilization of bacteria based on highly adhesive polyphenolic proteins (Figure 1.7). This method is applicable to cells with different shapes and sizes, and does not require chemical modification or cell deformation. In summary, there is no universal solution to deal with these problems, making it necessary to develop a specific method for each type of system.

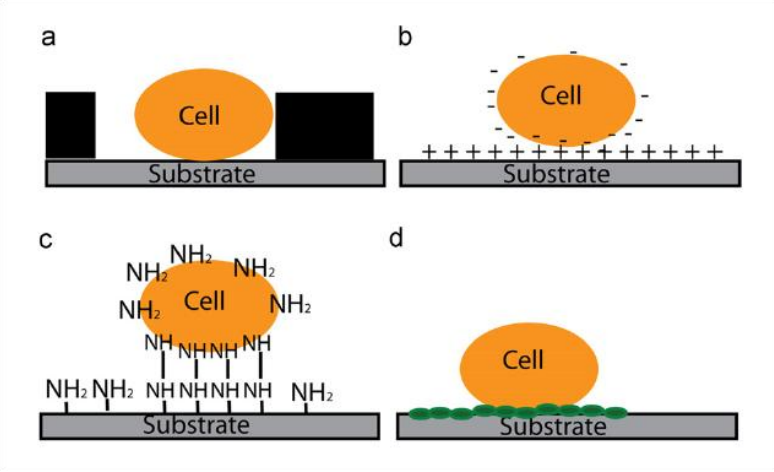

Figure 1.7| Schematic representation of the most commonly immobilization methods for AFM: (a) physical entrapment, (b) attractive electrostatic interactions, (c) covalent binding to amine-functionalized surface by glutaraldehyde and (d) attachment to polyphenolic adhesive proteins. Taken from Meyer *et al.* 2010.

1.3.3|APPLICATIONS OF AFM IN MICROBIOLOGY

One of the most common applications of AFM in microbiology consists in visualizing the antibacterial effects of many agents on the morphology of microorganisms. For example, Eaton *et al*. (2008) studied the morphology changes of *E. coli* and *S. aureus* before and after chitosan treatments. Cell lysis, surface roughening and cell clustering were observed with AFM after such treatments. The observation also revealed that response strategies used by the bacteria as clusters, increased both in amount and size during the treatments to resist the effects of chitosans. The authors concluded that the antimicrobial effect is strongly dependent on the target microorganism and the molecular weight of the chitosan. The damage of *E. coli* cells caused by three different antimicrobial peptides has also been studied by AFM (Meincken *et al.* 2005). In this study, it was found that all peptides caused roughening of the cell surface and lesions in the cell wall, while one of them notably caused large lesions with fluid leaking and vesicles from the interior of the cell. Most of these studies have been conducted under dry conditions. However, as

previously mentioned, one of the most attractive advantages of AFM when compared with other nanoscale microscopy techniques is its capability of being operated under physiological conditions, without sample fixation or other chemical preparation required, and therefore it can be used for the observation of living cells in real time. The resolution obtained in these studies is lower than when AFM is performed in air conditions but they have the great advantage of being conducted in conditions much closer to the natural ones. In order to follow dynamic cellular processes, Kailas *et al.* (2009) mechanically trapped living coccoid cells of the bacterial species *S. aureus* in lithographically patterned substrates under growth media using AFM and a cell division was successfully imaged over time.

Besides morphology imaging, AFM can also be used to measure the physical properties of the sample through a force-distance curve acquired in force spectroscopy mode (Dorubantu and Gray 2010). AFM force-curves are obtained by monitoring the cantilever deflection as a function of the vertical displacement of the piezoelectric scanner when an approach-retract cycle between the tip and the sample is performed. Different parts may be distinguished in a force-curve (Figure 1.8). When the AFM tip is far from the sample, no interaction is detected (region I). As the tip is approaching the sample no interaction is detected until the cantilever deflects towards the sample due to repulsive forces (region II). These forces lead the tip to jump into contact with the sample when the gradient of the interaction force exceeds the cantilever spring constant. After the contact, as the tip approaches further to the sample, repulsive forces are responsible for a positive deflection of the cantilever (region III). The approach portion of the curve-force can be used to measure different surface forces such as van der Waals and electrostatic, solvation, hydration and steric/binding forces (Dufrêne *et al*. 2001). Usually, the cantilever is moved towards the sample until a preset force threshold is reached and afterwards the movement direction is inverted with the cantilever starting to move away from the sample (solid curve). Although initially the behaviour of the cantilever during withdrawal equals the same described for the approach, due to adhesion between the tip and the sample, the cantilever starts to deflect negatively (region IV) until the adhesion force is overcome by the cantilever restoring force and the contact breaks.

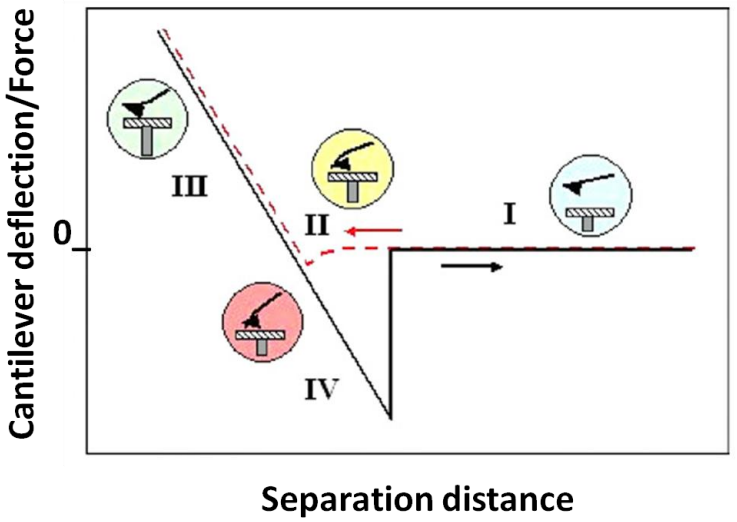

Figure 1.8| Schematic representation of a typical force curve with the different regions of the approach and withdrawal portions. (See text for details). Taken from Alessandriini and Facci 2005.

Understanding microbial physicochemical properties, and how different complexes of biomolecules are assembled under physiological conditions, can provide important insights in processes such as cell adhesion and aggregation (Dorubantu *et al.* 2010). For instance, Dufrêne *et al.* (2001) used AFM to record force-distance curves in aqueous solution for various microbial samples. The authors found strong variations on the characteristics of the force-curve distances according to the type of microorganism (fungal spores and bacterial strains), the physiological state (dormancy versus germination) and the environmental conditions (ionic strength).

CHAPTER 2

MATERIALS AND METHODS

2.1|MICROORGANISMS AND GROWTH CONDITIONS

Staphylococcus epidermidis ATCC 14990, originally isolated from a nose infection, was kindly provided by the University Medical Centre of Groningen (The Netherlands) and used throughout this study. The strain was first streaked on a blood agar plate from a frozen stock solution (7% (v/v) DMSO) and grown overnight at 37^{o}C. The blood agar plates were kept at 4^{o}C for a maximum of two weeks. For each experiment, a colony was inoculated in 10 ml tryptone soy broth (TSB, OXOID, Basingstoke, United Kingdom) and cultured for 24 h at 37^{o}C in ambient air. This culture was then used to inoculate a second culture of 200 ml, which was grown for 16 h under the same conditions. Bacteria were harvested by centrifugation at 6500 g for 5 min at 10^{o}C, washed twice with demineralized water and suspended in 10 ml of demineralized water for further analysis.

2.2| ANTIMICROBIAL COMPOUNDS

In this study, the effects of Ethoquad C/25 (Cocoalkyl methyl (polyoxyethylene) ammonium chloride (QAC) were compared with an antibiotic (Gentamicin) and an antimicrobial peptide (Gramicidin S). The compounds chemical structures are illustrated in Figure 2.1. The QAC was obtained from AKZONobel (Amsterdam, The Netherlands) in the liquid state with a molecular weight of 910 g/mol and a mass density of 1.076 g/cm^{3}. A 20% (v/v) stock solution was prepared by diluting 2 ml QAC in 2 ml methanol and 6 ml of 10 mM potassium phosphate buffer (0.5 M K_2HPO_4/ KH_2PO_4, pH 7.0). This solution was further diluted in potassium phosphate buffer to obtain a 2152 µg/ml solution that was stored in dark at 4^{o}C and from which all the QAC solutions used in this study were prepared. Gentamicin sulphate, provided by Sigma-Aldrich with a molecular weight of 477.596 g/mol was diluted in demineralized water to a concentration of 5 mg/ml and stored at the freezer as stock solution from which all Gentamicin solutions used throughout this study were prepared. Gramicidin S, obtained from Bio-Organic Synthesis, Leiden Institute of Chemistry, University of Leiden, The Netherlands, was provided as a powder with a molecular weight of 1369.5 g/mol. The powder was diluted in 0.1 ml of absolute ethanol and 0.9 ml demineralized water to obtain a stock solution with 890 µg/ml concentration. This stock solution was stored in the freezer and used for preparing adequate dilutions throughout this study.

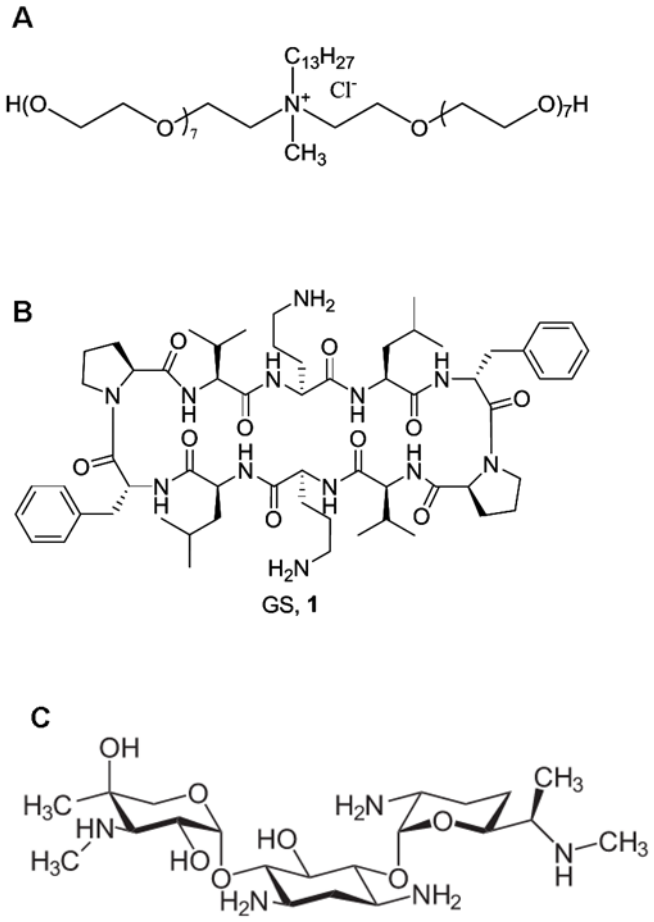

Figure 2.1| Chemical structure of Ethoquad C/25 (Cocoalkyl methyl (polyoxyethylene) ammonium chloride) (A), Gramicidin S (B) and Gentamicin sulphate (C).

2.3| ANTIMICROBIAL ASSAY

The antimicrobial activity of the compounds investigated (QAC, Gentamicin and Gramicidin S) was assessed by determining the MIC and minimal bactericidal concentration (MBC). For MIC determination, the wells of a sterile 96-well tissue culture plate with round bottom (FALCON, USA) were filled with 100 µl antimicrobial compound diluted in TSB with several concentrations being tested (ranging from 440 µg/ml to 1.71 µg/ml for QAC; from 130 µg/ml to 0.5 µg/ml for Gentamicin; from 32 µg/ml to 0.125 µg/ml for Gramicidin S). A pre-culture was diluted in TSB to reach a final concentration of $5x10^4$ bacteria/ml, and 100 µl of this bacterial suspension was added to each well of the microtiter plate. In this assay, two controls were used, one without bacteria as a negative control and one without an antimicrobial compound as a positive control. The plates were

incubated for 24 h at 37^{o}C. The well containing the lowest concentration of the antimicrobial compound that visually did not show any bacterial growth was defined as the MIC. The MBC was determined by adding a droplet of 10 µl from each well with no visible growth on a TSB agar plate that was afterwards incubated at 37^{o}C for 48 h. The MBC was identified as the lowest concentration of compound that prevented any growth of bacteria. All the experiments were done in triplicate.

2.4|ATOMIC FORCE MICROSCOPY

AFM was used to analyze the effect of the compounds investigated (QAC, Gentamicin and Gramicidin S) on the bacterial surface. Bacteria were attached through electrostatic interactions (physical adsorption) to a glass slide made positively charged by the adsorption of poly-L-lysine (PLL, 0.1% solution, SIGMA, United Kingdom) (Bolshakov *et al*. 2001). In order to coat the glass slide surface with PLL, the glass slide was first cleaned by sonication for 3 min in 2% RBS35 (Omnilabo, International BV, The Netherlands), followed by rinsing with tap water, demineralized water, methanol, tap water and finally demineralized water again. A drop of PLL was afterwards added and spread over the dried surface and left to dry at room temperature. After air-drying of the glass slide, a few drops of a bacterial suspension (10^{10} bacteria/ml) diluted in demineralized water were added and left to adhere for 40 min. Subsequently, the bacteria coated slide was rinsed with demineralized water to remove the free floating bacteria and immediately used in the AFM measurements.

AFM measurements were made at room temperature with 10 mM potassium phosphate buffer as a control and in potassium phosphate buffer with determined MBCs of the compounds investigated, using a BioScope Catalyst with a Nanoscope V from Bruker (USA) operating in PeakForce QNM (Quantitative NanoMechanics) mode. V-sharped silicon nitride cantilevers from Bruker with a spring constant of 0.58 N/m and a probe curvature radius of 10 nm, were calibrated for each experiment according to manufacturer specifications. PeakForce images with a scan size of 30 µm x 30 µm were taken at the beginning and in the end of the experiment. Meanwhile, images with a smaller area (scan size of 10 µm x 10 µm) were scanned during 300 minutes, with different applied forces at a scan rate of 0.3 Hz. The number and size of the bacteria were followed during the 300 min of exposure to the potassium phosphate buffer and the antimicrobial compounds. The time necessary to remove each bacterial cell from the

glass slide was followed for each scan and the data obtained was then analyzed with a Kaplan-Meier curve. The experiments were performed in triplicate using independent bacterial suspensions.

2.5|ROUGHNESS ANALYSIS

The roughness (*Ra*) of each cell surface prior and after being exposed to the antimicrobial compounds was calculated using NanoScope Analysis 1.10 software. Images used for roughness determination were acquired in the central part of a cell with a scan size of 345 x 345 nm and were flattened and plane fitted before measurements.

2.6|FLUORESCENCE MICROSCOPY

The evaluation of possible cell surface-damaged of bacteria adhering on the glass slides after being exposed both to potassium phosphate buffer (control) and to the compounds under study was carried out by fluorescence microscopy. Bacterial samples were prepared in the same way as described for the AFM experiments. Some droplets of a bacterial suspension (10^{10} bacteria/ml) diluted in demineralized water were added to the glass slides (previously coated with a solution of PLL) and left to adhere for 40 min. Subsequently, the glass slides were rinsed with demineralized water to remove the free floating bacteria and exposed for 60 min to potassium phosphate buffer and to the antimicrobial compounds at their MBC. After exposure, the glass slides were rinsed with demineralized water, and then 250 µl of LIVE/DEAD BacLight bacterial viability stain (Molecular probes, Leiden, The Netherlands) containing 3.34 mM SYTO 9 dye (fluorescent green) and 20 mM propidium iodide (fluorescent red) was added to each sample to differentiate between undamaged and cell surface-damaged bacteria. After 15 min in the dark, samples were examined on a fluorescence microscope (Leica DM4000 B, Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

2.7|MTT ASSAY

The metabolic activity of the biofilm cells was evaluated using the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma Aldrich St. Louis, USA) assay. In order to grow the biofilm, the wells of a 96-well culture plate with flat bottom (Greiner Bio-One B.V., Alphen a/d Rijn Leiden,The Netherlands) were filled with 196 µl TSB and 4 µl of an overnight bacterial pre-culture and the plate was incubated at 37^{o}C for 24 h. Afterwards, the supernatant from each well was gently removed; the wells were washed twice with 10 mM potassium phosphate buffer and subsequently exposed overnight to different concentrations (0.5, 1 and 2xMBC) of the compounds investigated (diluted in TSB). After this exposure, the supernatant from each well was gently removed; the wells were washed twice with potassium phosphate buffer and then filled with 200 µl MTT solution (0.0050 g MTT, 0.1 g glucose and 100 µl menadion in 10 ml potassium phosphate buffer). The plate was incubated at 37^{o}C for 30 min in the dark. Subsequently, the supernatant from each well was removed and washed once with ultra pure water. Then, 200 µl 5% acid isopropanol (1M) was added to each well and after 15 min the absorbance was read at 560 nm with a Biotech Synergy HT (BioTek Instruments, Inc, Winooski, USA). In this assay, two controls were used, one without bacteria as a negative control and one without compound as a positive control. All the experiments were done in triplicate using independent bacterial cultures.

2.8|CALCIUM EFFECT ON QAC'S MODE OF ACTION

To study the influence of calcium ions on QAC's mode of action against bacterial cells, its antimicrobial activity was determined in the presence or absence of 0.1 M of $CaCl_2$. Antimicrobial activity was assessed in planktonic cultures by determining the MIC and also on bacteria adhering to a glass slide by AFM. In order to prevent calcium precipitation TSB was replaced by nutrient broth (NB, OXOID, Basingstoke, United Kingdom) in these experiments.

To determine the MIC, the wells of a sterile 96-well tissue culture plate with round bottom (FALCON, USA) were filled with 100 µl QAC diluted in $CaCl_2$ or in NB with several concentrations being tested (ranging from 440 µg/ml to 1.71 µg/ml). A pre-culture was diluted in NB to reach a final concentration of $5x10^4$ bacteria/ml. Next, 100 µl of this bacterial suspension was added to each well of the microtiter plate that was incubated for 24 h at 37^{o}C. Absorbance at 575 nm was then recorded with a Biotech Synergy HT

(BioTek Instruments, Inc, Winooski, USA). In order to evaluate possible interferences caused by the addition of the QAC to the medium culture, a negative control without bacteria was used in which 100 µl of NB was added to the wells containing 100 µl of QAC diluted in NB and diluted in $CaCl_2$ for the several concentrations tested. Moreover, a positive control was used in which the bacterial suspension was added to NB and $CaCl_2$ without QAC. The MIC was determined as the lowest concentration of QAC that prevented bacterial growth, which was evaluated by the absorbance recorded (proportional to bacterial growth).

To study the effect of $CaCl_2$ on bacterial adhesion to a glass slide by AFM, some changes were included in the procedure due to the precipitation with calcium ions and the phosphate ions on potassium phosphate buffer. The QAC was diluted in demineralized water instead of potassium phosphate buffer, and therefore two controls were used: bacteria exposed to demineralized water and bacteria exposed to $CaCl_2$. Bacterial samples were prepared in the same way as above described for the AFM experiments. Some droplets of a bacterial suspension (10^{10} bacteria/ml) diluted in demineralized water were added to the glass slides previously coated with a solution of PLL and left to adhere for 40 min. Subsequently, the glass slides were rinsed with demineralized water to remove the free floating bacteria and transferred to AFM. AFM measurements were made at room temperature using demineralized water and 0.1 M $CaCl_2$ as controls and using QAC (MBC) diluted in demineralized water and QAC (MBC) diluted in $CaCl_2$, using a BioScope Catalyst with a Nanoscope V from Bruker (USA) operating in PeakForce QNM (Quantitative NanoMechanics) mode. V”-sharped silicon nitride cantilevers from Bruker with a spring constant of 0.58 N/m and a probe curvature radius of 10 nm, were calibrated for each experiment according to manufacturer specifications. PeakForce images with a scan size of 30 µm x 30 µm were taken at the beginning and in the end of the experiment. Meanwhile, images with a smaller area (scan size of 10 µm x 10 µm) were scanned during 300 minutes, with a force applied of 3 nN at a scan rate of 0.3 Hz. The number and the size of bacteria were followed during the 300 min of exposure to the potassium phosphate buffer and the antimicrobial compounds. The time necessary to remove each bacterial cell from the glass slide was followed for each scan and the data obtained was then analyzed with a Kaplan-Meier curve.

Due to time restrictions for the development of the current work these experiments were conducted in duplicate instead of triplicate.

2.9| STATISTICAL ANALYSIS

Detachment of adhering bacteria exposed to the different antimicrobial compounds solutions was compared to the control (potassium phosphate buffer or demi water only), using Log-rank (Mantel-Cox) of SigmaPlot 11.0 software. The reliability of the differences in roughness measurements was estimated according to non parametric Mann-Whitney test, using Graph Pad Prism 5.01 software. Differences were considered statistically significant for p-values less than 0.05. All the MTT and antimicrobial activity experiments were compared using one-way of variance (ANOVA) by applying the Levene's test of homogeneity of variances, using Microsoft Excel. Student's t-test was applied for rejection of some experimental values, with a confidence level of 95% and 90%.

CHAPTER 3

RESULTS

3.1| ANTIMICROBIAL ACTIVITY

The antimicrobial activities of the compounds used in this study established by the determination of MIC and MBC are summarized in Table 3.1. The three compounds proved to be antimicrobial agents against *S. epidermidis* ATCC 14990, although with different effective concentrations. The highest antimicrobial activity was found for Gentamicin sulphate with the lowest MIC (1 µg/ml) and MBC (2 µg/ml), while the lowest antimicrobial activity was found for QAC (MIC = 55 µg/ml and MBC = 110 µg/ml). Based on the antimicrobial activity results, MBC values of compounds were chosen for the following AFM measurements.

Table 3.1| MIC and MBC values of QAC, Gentamicin sulphate and Gramicidin S obtained for *Staphylococcus epidermidis* ATCC 14990. Data for individual strains were obtained in triplicate yielding identical results.

Compound	MIC (µg/ml)	MBC (µg/ml)
QAC	55	110
Gentamicin sulphate	1	2
Gramicidin S	4	8

3.2| ATOMIC FORCE MICROSCOPY

The effect of the studied compounds on the bacterial cell surfaces was visualized by AFM in PeakForce QNM (Quantitative NanoMechanics) mode, for which it is possible to control the maximum force applied on the tip. The first AFM measurements were performed with a force of 1 nN (*data not shown*). However, from the image analysis it was possible to conclude that a stronger force was needed in order to get better images (i.e. from which the shape of bacteria could be discriminated). The subsequent AFM measurements were thus performed with a 3 nN force and, to assure that this force had no influence on the bacterial morphology, bacteria were first exposed to potassium phosphate buffer and scanned during 300 min (Figure 3.1 A-D).

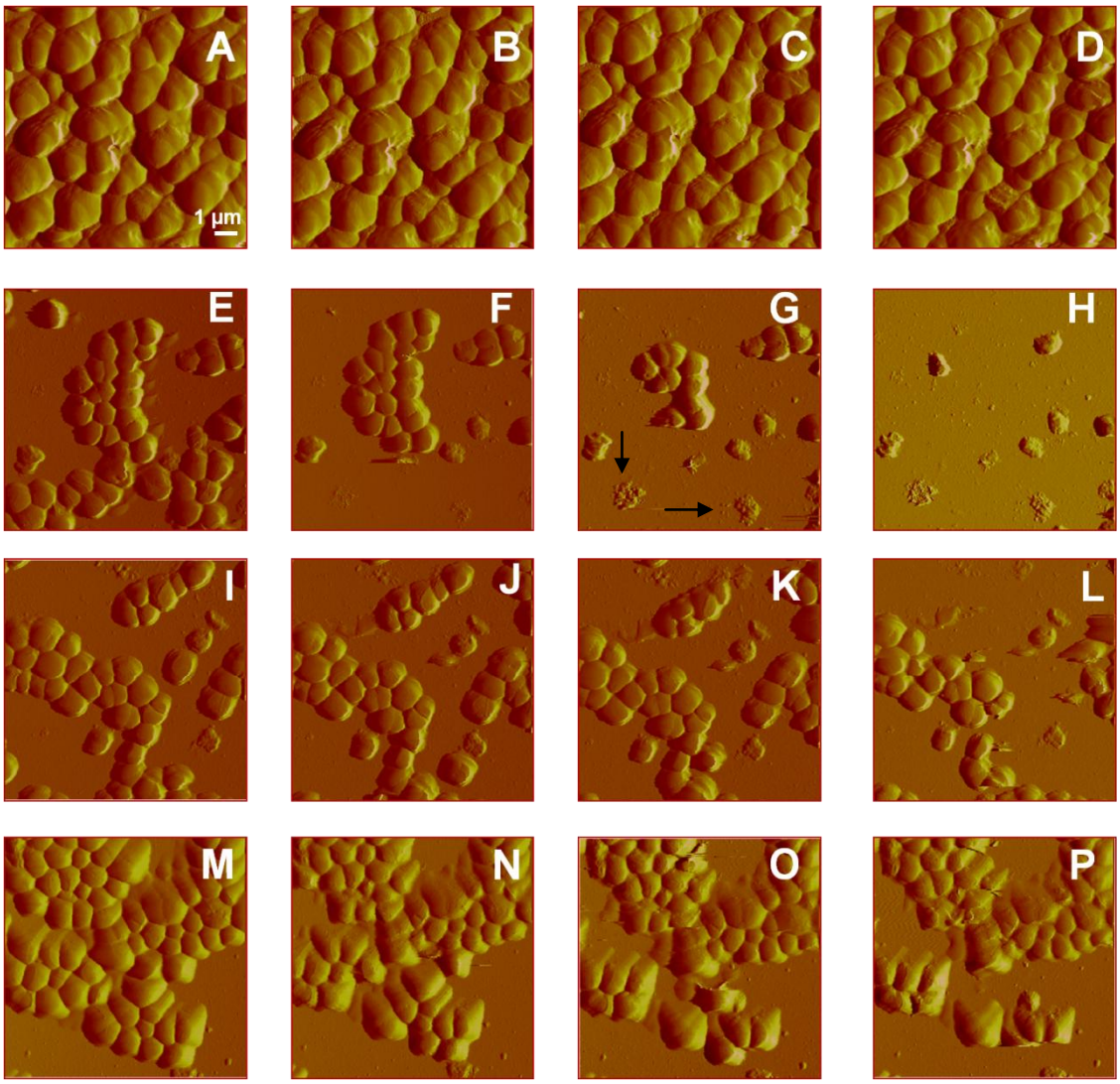

Figure 3.1| AFM Peak Force images of *Staphylococcus epidermidis* ATCC 14990 after being exposed to 10 mM potassium phosphate buffer (control) for 0 min (A), 103 min (B), 161 min (C) and 300 min (D), to 2 µg/ml Gentamicin sulphate for 0 min (E), 182 min (F), 240 min (G) and 284 min (H), to 110 µg/ml QAC for 0 min (I), 90 min (J), 141 min (K) and 264 min (L) and to 8 µg/ml Gramicidin S for 0 min (M), 170 min (N), 259 min (O) and 274 min (P). Experiments were performed with a 3 nN force.

The bacteria that were only exposed to potassium phosphate buffer appeared undamaged and attached to the glass slide during the 300 min scanning, as shown in Figure 3.1 A to D, which suggests that the force applied had no influence on the bacterial morphology and removal from the glass slide. On the other hand, when exposed to the target compounds, bacteria presented some damages and wrinkling on their surfaces and consequently most of them were removed from the glass slide at different time points. Some of the images also suggest the occurrence of leakage of intracellular material (Figure 3.1 G denoted with a black arrow). Gentamicin proved to be the most effective compound being responsible for the removal of almost all bacteria from the surface during the 284 min of exposure. When bacteria were exposed to QAC and

Gramicidin S, the number of bacteria removed from the glass slide was similar between them and smaller comparing to Gentamicin, but it is important to notice that most of the bacteria remaining at the surface shrank or were damaged.

Figure 3.2 shows AFM Peak Force images of *S. epidermidis* ATCC 14990 exposed to potassium phosphate buffer and Gentamicin taken after a single scan over the whole area of 30 µm x 30 µm and after 300 min continuously scanning over the area of 10 µm x 10 µm. When bacteria were exposed to potassium phosphate buffer no damage or removal from the surface was observed. Upon contact with Gentamicin, it was observed that almost all bacterial cells in the smaller scan area which has been continuously scanned for the 300 min were removed from the glass slide, while the bacteria outside the continuously scanned area did not seem to be affected by the compound. Similar results were obtained for QAC and Gramicidin S (*data not shown*).

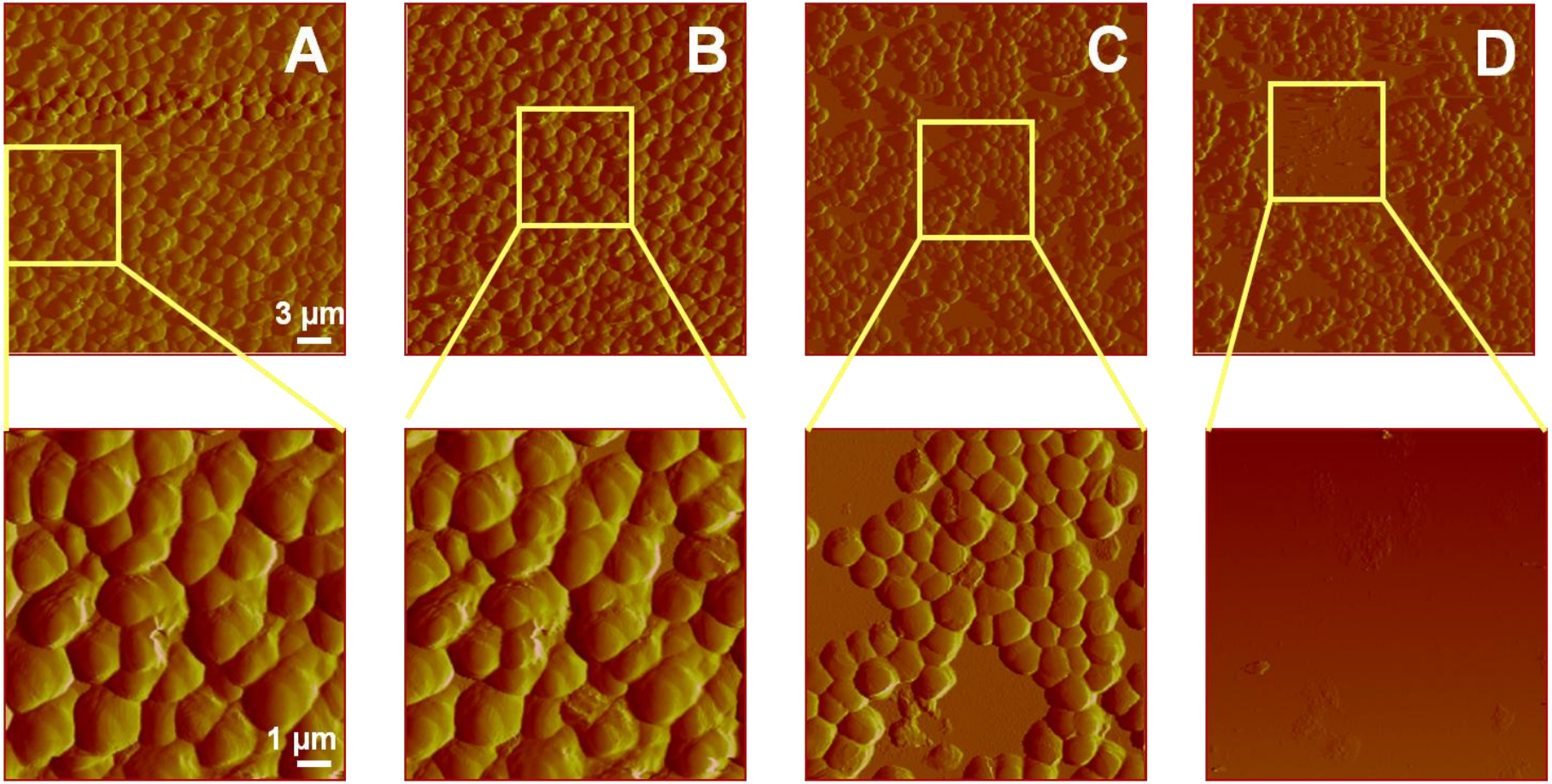

Figure 3.2| AFM Peak Force images of *S. epidermidis* ATCC 14990 when exposed to 10 mM potassium phosphate buffer (A, B) and 2 µg/ml Gentamicin sulphate solution (C, D) after a single scan (30 µm x 30 µm) and after multiple scans in the smaller region (10 µm x 10 µm). Experiments were performed with a 3 nN force

In Figure 3.3, enlarged images with a scan size of 2 µm x 2 µm of *S. epidermidis* after being exposed to potassium phosphate buffer (control), and to QAC solution (MBC concentration) for 60 min, are presented. It was possible to observe a surface wrinkling and hole formation after exposure to QAC, as opposite to the smooth surface expressed by bacteria exposed to potassium phosphate buffer (control).

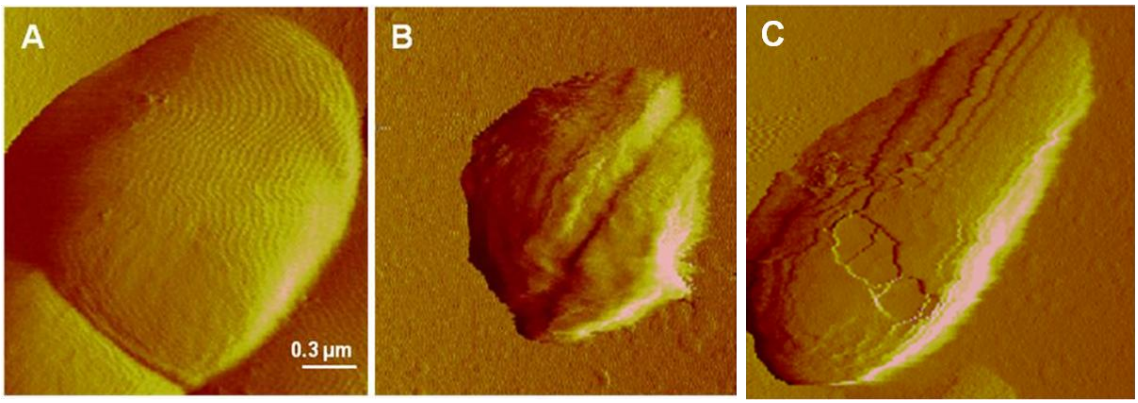

Figure 3.3| AFM Peak Force images of *S. epidermidis* when exposed to 10 mM potassium phosphate buffer (A) and QAC solution for 60 min (B,C). Experiments were performed with a 3 nN force.

Based on the results obtained from the AFM imaging it was possible to build a Kaplan-Meier curve as illustrated in Figure 3.4. To build this curve, the number of bacteria attached to the glass slide in the beginning of the experiment was counted, as well as the time point at which each bacterium was removed from the surface during a 300 min of exposure.

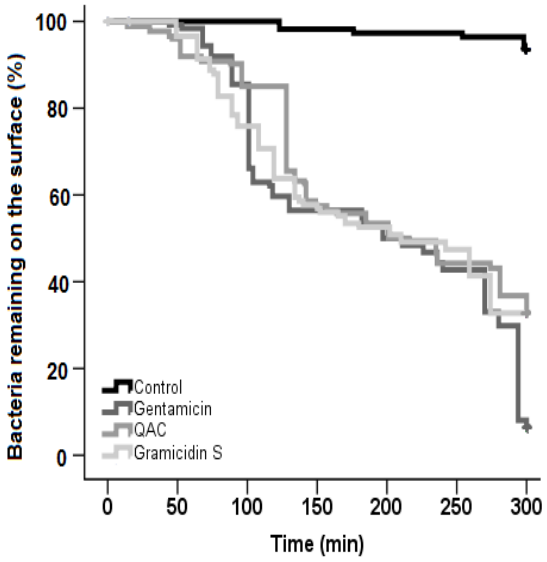

Figure 3.4| Kaplan-Meier curve expressing the percentage of *Staphylococcus epidermidis* ATCC 14990 adhering on a substrate surface during exposure to 10 mM potassium phosphate buffer (control), 2 µg/ml Gentamicin sulphate solution, 110 µg/ml QAC solution and 8 µg/ml Gramicidin S solution while continuously scanning. Experiments were performed in triplicate with a 3 nN force.

As shown in Figure 3.4, in the absence of the target compounds a significant percentage of bacteria (higher than 90%) remained on the surface after the 300 min scanning. On the contrary, exposure of the bacteria to the target compounds conducted to a significant reduction ($p<0.05$) in the number of bacteria that remain attached to the surface. After exposing bacteria to Gentamicin, some bacteria started to be removed from the surface after 60 min, and within 300 min almost all bacteria were removed. Gentamicin proved therefore to be the most effective compound compared to QAC and Gramicidin S. As for QAC, also few bacteria were removed from the surface after 60 min exposure, and a 65% bacterial removal could be achieved within 300 min. Regarding bacteria exposure to Gramicidin S, the same trend was observed being the first bacteria removed after approximately 60 min, and as shown on the Kaplan-Meier curve, bacteria were removed progressively. Gramicidin S showed an effect similar to QAC. From the results obtained, 60 minutes exposure was found to be the appropriate period to initiate bacterial removal from the surface and therefore, that was the exposure time chosen for the following fluorescence microscopy measurements.

To describe the structural changes of cell wall in terms of quantity, the surface roughness analysis prior and after treatment was also performed. As shown in Figure 3.5, cell surface roughness significantly increases ($p<0.05$) after being exposed to the antimicrobial compounds, thus confirming that membrane damage is a significant contributing factor to their bactericidal activities. The roughness results obtained after treatment with different antimicrobial compounds were compared and found to be statistically similar ($p>0.05$).

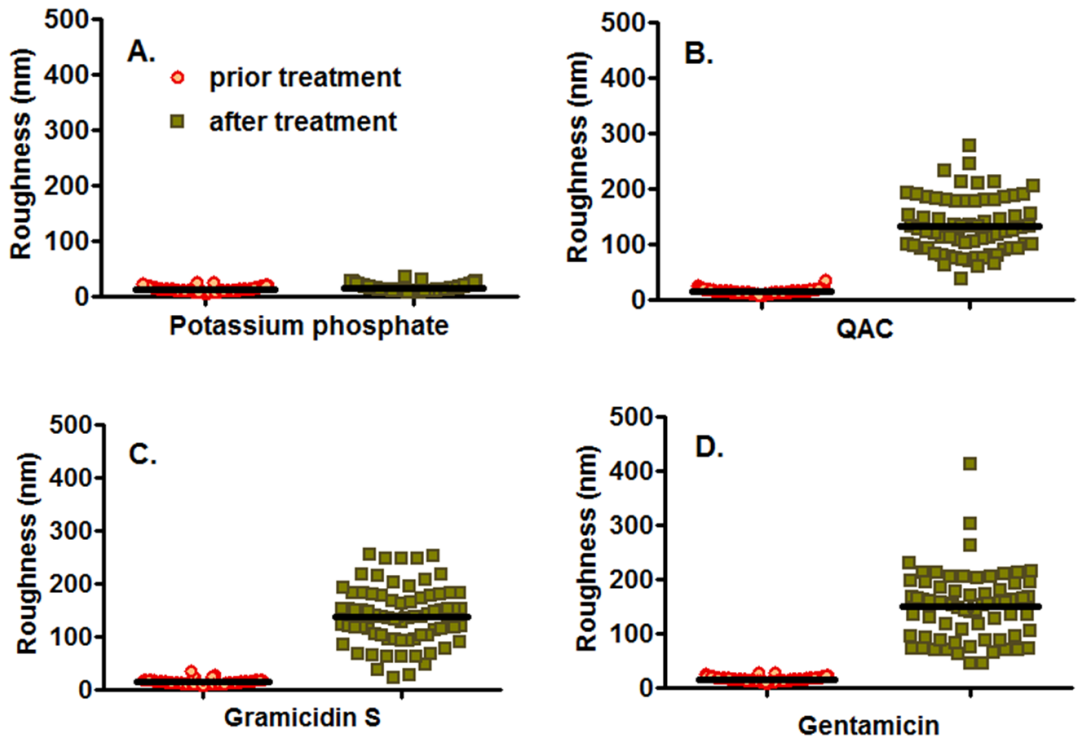

Figure 3.5| Roughness (nm) of *Staphylococcus epidermidis* ATCC 14990 adhering on a substrate surface before and after exposure to 10 mM potassium phosphate buffer (control) (A), 110 μg/ml QAC solution (B), 8 μg/ml Gramicidin S solution (C) and 2 μg/ml Gentamicin sulphate solution (D).

In order to study the effect of the force applied by the tip of AFM, some additional measurements were conducted using higher forces. Due to time restrictions for the development of the current work, these measurements were performed only once and for three conditions (potassium phosphate buffer, Gentamicin and QAC). Therefore, the results are preliminary and should be analyzed with caution. In the first trial experiments, bacteria were exposed to potassium phosphate buffer (control) using a 10 nN force and it was found that all bacteria were immediately removed (*data not shown*). Afterwards, bacteria were exposed to a lower force (6 nN) and, since almost all bacterial cells remained attached to the surface (Figure 3.6 A-C), this force value was established as the control for the study of the effect of higher forces.

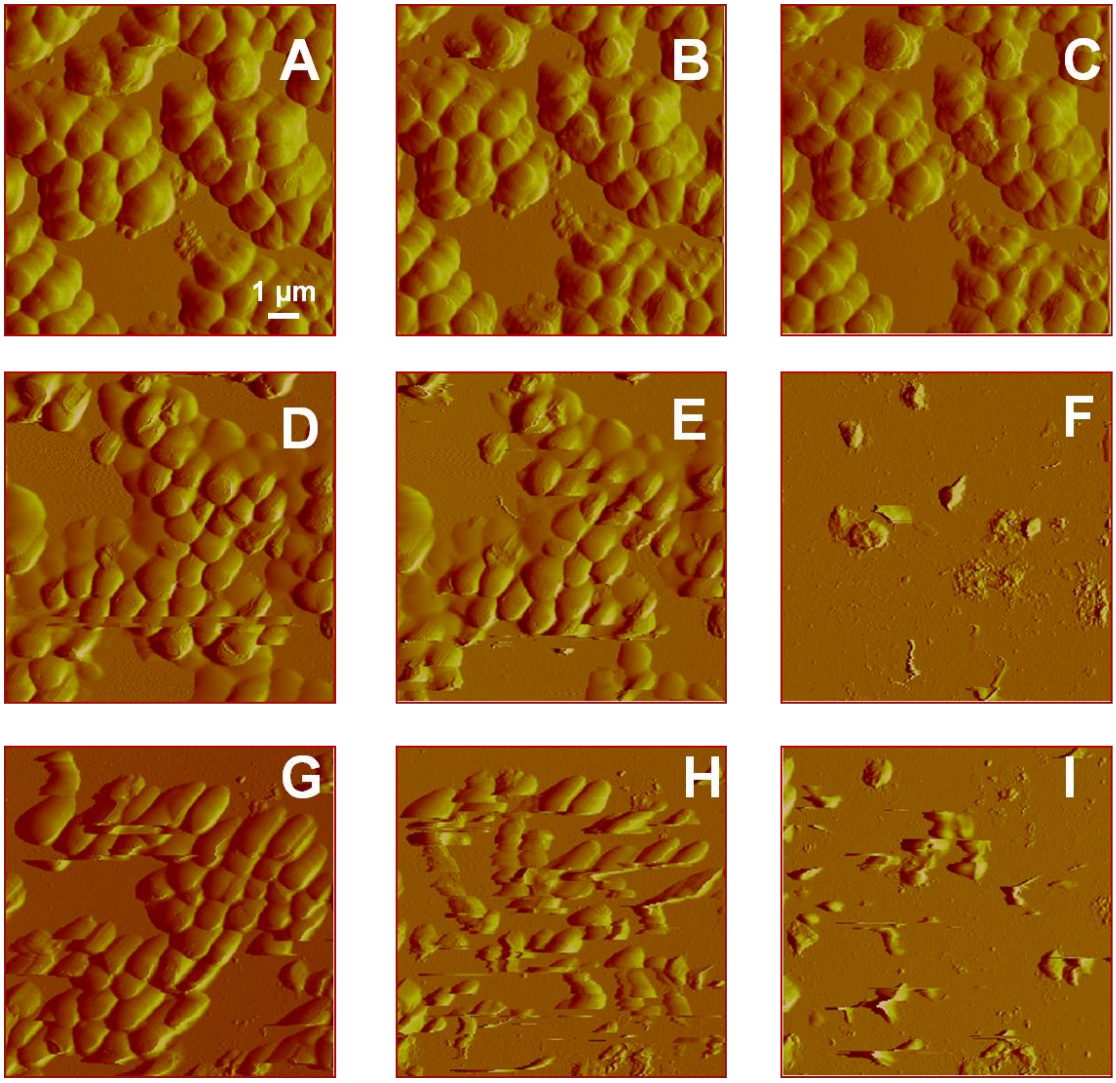

Figure 3.6| AFM Peak Force images of *Staphylococcus epidermidis* ATCC 14990 after being exposed to 10 mM potassium phosphate buffer (control) for 0 min (A), 181 min (B) and 274 min (C), to 2 µg/ml Gentamicin sulphate for 0 min (D), 33 min (E) and 55 min (F) and to 110 µg/ml QAC for 0 min (G), 58 min (H) and 72 min (I). Experiments were performed with a 6 nN force.

The bacteria that were only exposed to the potassium phosphate buffer (control) were not significantly removed from the surface, although some damage on their surface could be observed, which means that the bacteria morphology is affected by increasing forces. Regarding bacteria exposed to Gentamicin and QAC, both compounds proved to be quite effective on removing all bacteria from the surface and much faster than when a lower force of 3 nN was applied.

Bacterial detachment during exposure to the target compounds was followed during 300 min, and the remaining adherent bacteria were plotted in a Kaplan-Meier curve as illustrated in Figure 3.7.

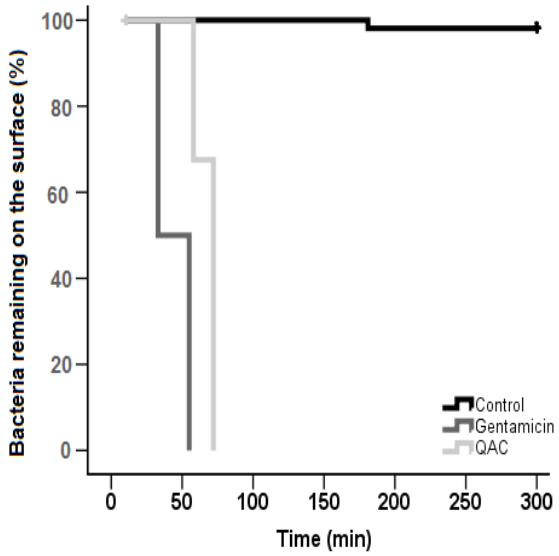

Figure 3.7| Kaplan-Meier curve expressing the percentage of *Staphylococcus epidermidis* ATCC 14990 adhering on a substrate surface during exposure to 10 mM potassium phosphate buffer (control), 2 µg/ml Gentamicin sulphate solution and 110 µg/ml QAC solution while continuously scanning. Experiments were performed once with a 6 nN force.

As previously observed for the 3 nN force experiments, exposure to potassium phosphate buffer did not cause any significant removal, but exposure to Gentamicin and QAC resulted in the complete removal of adhered bacteria, being Gentamicin the compound that produced a faster effect.

3.3|FLUORESCENCE MICROSCOPY

The AFM experiments only allowed the determination of the time point at which each bacterium was removed from the surface. Therefore, to assess the viability of the bacteria adhered onto the glass slides, new samples were prepared in the same manner as for the AFM experiments. After being exposed for 60 min to the target compounds and potassium phosphate buffer (control), the viability was assessed by fluorescence microscopy, as illustrated in Figure 3.8.

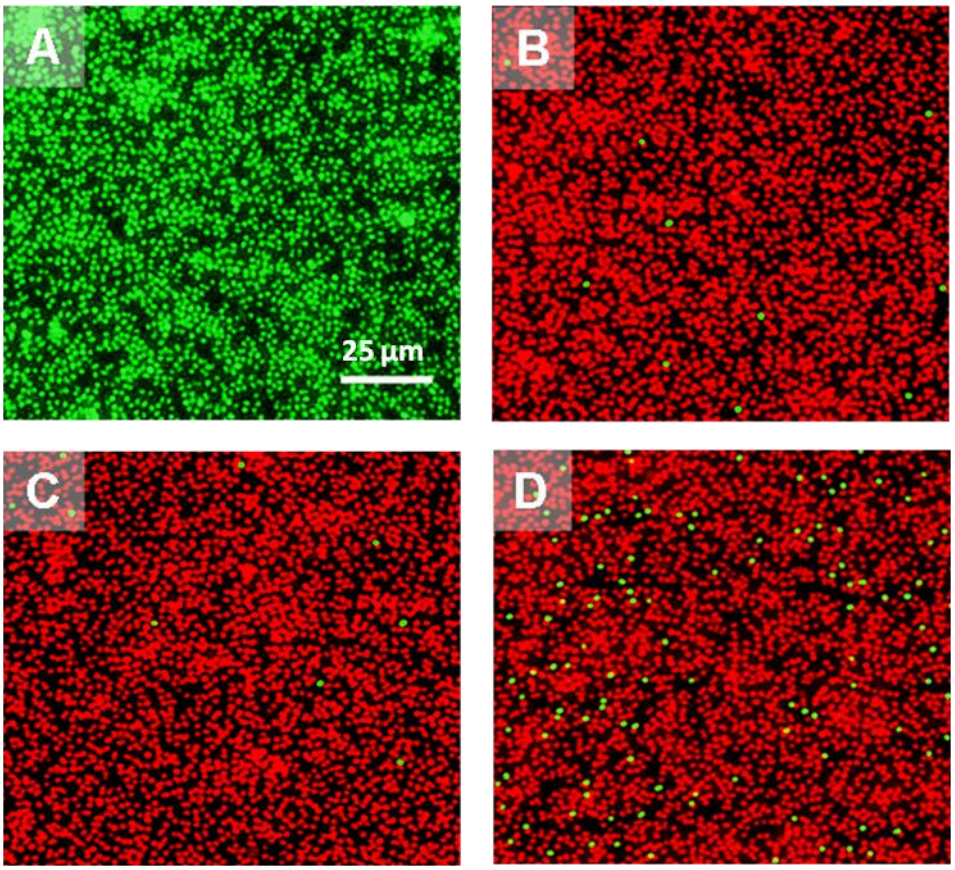

Figure 3.8| Fluorescent live-dead images of *Staphylococcus epidermidis* ATCC 14990 after being exposed for 60 min to 10 mM potassium phosphate buffer as a control (A), to 110 µg/ml QAC solution (B), 8 µg/ml Gramicidin S solution (C) and to 2 µg/ml Gentamicin sulphate solution (D).

Although the viability stain is often reported to distinguish between live and dead bacteria, in fact this stain enables the evaluation of membrane integrity. A functional membrane does not allow accumulation of propidium into bacterium to replace SYTO9 that readily penetrates functional cytoplasmic membranes from the DNA (Stocks 2004). In the control experiment all bacteria showed green fluorescence, which demonstrates that the immobilization technique used in this study for AFM measurements did not damage the cytoplasmic membrane. The exposure to Gentamicin, QAC and Gramicidin S resulted in red fluorescence shown by almost all the bacteria, thus confirming the expected loss of membrane integrity.

3.4|MTT ASSAY

The effect of the target compounds on *S. epidermidis* ATCC 14990 biofilm was also evaluated by the MTT assay, in which the metabolic activity of biofilm cells is assessed. MTT is a tetrazolium salt that can be reduced by cells in their mitochondria to a purple tetrazolium formazan. The amount of formed tetrazolium formazan which can be measured at 560 nm is thus proportional to biofilm metabolic activity. Figure 3.9 shows the percentage of metabolic activity of biofilm cells after being exposed overnight to the target compounds at different concentrations (0.5; 1 and 2xMBC). The percentage is relative to the absorbance value obtained for the biofilm exposed to TSB medium (control) that was established as 100% of metabolic activity.

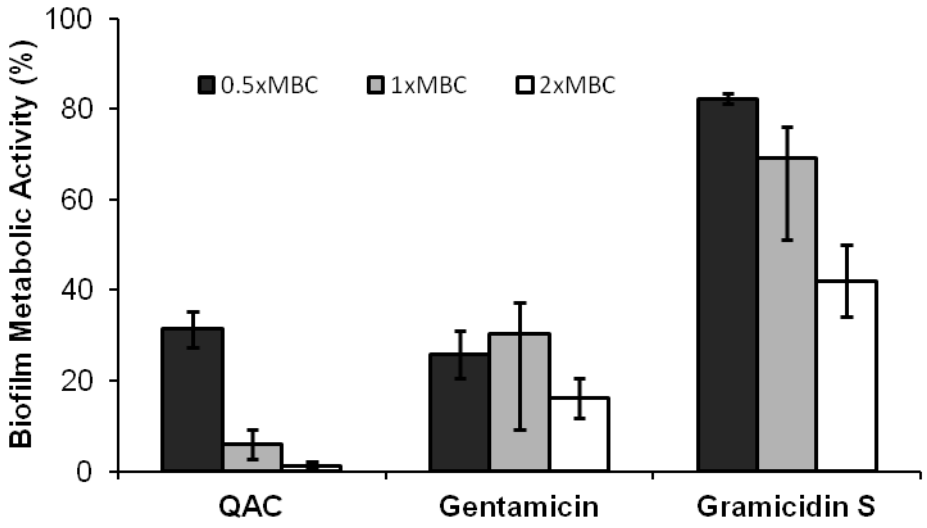

Figure 3.9 |Percentage relative of metabolic activity of *Staphylococcus epidermidis* ATCC 14990 biofilm after being exposed overnight to QAC, Gentamicin sulphate and Gramicidin S solutions at different concentrations (0.5; 1 and 2xMBC). Results correspond to the average of three independent assays.

As shown in Figure 3.9, all the compounds under study proved to have some effect on the biofilm metabolic activity, and their effect increased with increasing concentrations. Comparing the results obtained for each compound, QAC and Gentamicin were found to cause a statistically significant reduction ($p<0.05$) on metabolic activity for all the concentrations tested, while Gramicidin S was found to be effective only at 2xMBC. QAC proved to be the most effective compound resulting in 99% reduction of metabolic activity at 2xMBC.

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3.5 |CALCIUM EFFECT ON QAC'S MODE OF ACTION

To study the influence of calcium ions on QAC's mode of action against bacterial cells, its antimicrobial activity was determined in the presence or absence of 0.1 M $CaCl_2$. Antimicrobial activity was evaluated in planktonic cultures by determining the MIC, and also on bacteria adhering to a glass slide by AFM. In the first pilot experiments performed, it was possible to observe that adding $CaCl_2$ to TSB medium resulted in the formation of a precipitate (calcium ions precipitate with phosphate ions present on TSB growth medium) that hampered the results analysis. Therefore, in these experiments, TSB was replaced by NB, a medium that has no phosphate in its composition, thus avoiding precipitation. The results are illustrated in Figure 3.10.

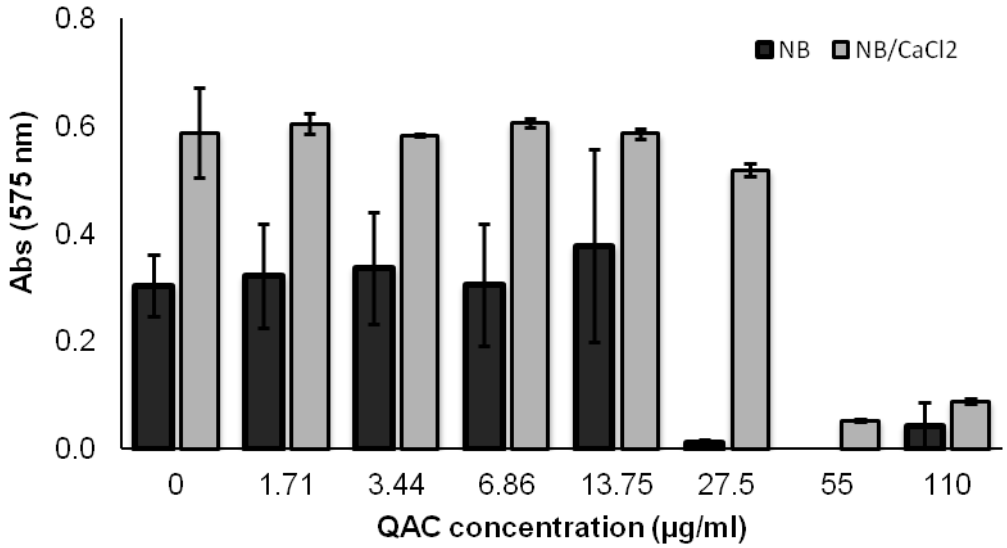

Figure 3.10| *Staphylococcus epidermidis* ATCC 14990 growth in the absence (control) and in the presence of several concentrations of QAC diluted in nutrient broth (NB) and NB supplemented with 0.1 M $CaCl_2$. Results correspond to the average of two independent assays.

In the absence of calcium, bacterial growth was inhibited at a concentration of 27.5 µg/ml; this concentration was defined as the MIC. MIC of the QAC in NB proved to be lower than MIC obtained in TSB (55 µg/ml in Table 3.1), which was expected since TSB is a richer medium. When calcium ions were present, a higher concentration (55 µg/ml) was required to inhibit bacterial growth. The results strongly suggest that the antimicrobial activity of QAC is compromised by high concentrations of calcium ions.

The effect of $CaCl_2$ was also evaluated on bacterial adhesion to a glass slide by AFM. The results obtained are illustrated in Figure 3.11. Bacteria that were only exposed to demineralized water appeared undamaged and attached to the surface during the 300 min scanning. On the other hand, bacteria exposed to 0.1 M $CaCl_2$ solution presented

some surface damages and few bacteria were removed from the glass slide during continuous scanning for 300 min. As expected, bacteria exposure to QAC resulted in a significant bacterial removal and surface damage, and the addition of 0.1 M $CaCl_2$ conducted to a lower removal percentage, as well as to less surface damages. Therefore, the presence of calcium ions was found to reduce the effects of QAC.

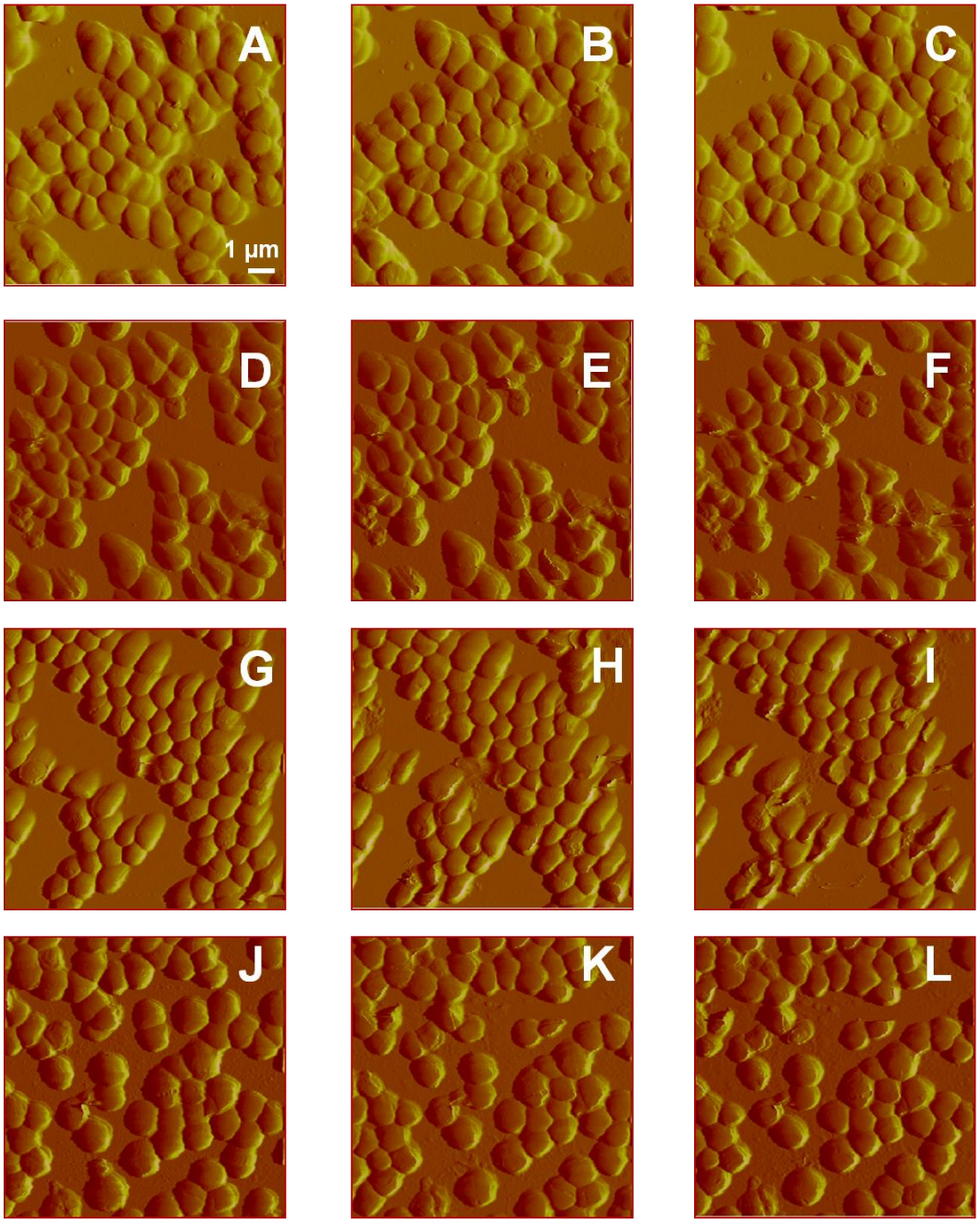

Figure 3.11| AFM Peak Force images of *Staphylococcus epidermidis* ATCC 14990 after being exposed to demineralized water (control) for 0 min (A), 188 min (B) and 300 min (C), to 0.1 M $Cacl_2$ (control) for 0 min (D), 182 min (E) and 300 min (F), to 110 µg/ml QAC in demineralized water for 0 min (G), 191 min (H) and 300 min (I) and to 110 µg/ml QAC in $CaCl_2$ for 0 min (J), 192 min (K) and 300 min (L). Experiments were performed with a 3 nN force.

Bacterial detachment during exposure to the target compounds was followed for 300 min and the bacteria remaining on the surface were plotted in a Kaplan-Meier curve as illustrated in Figure 3.12.

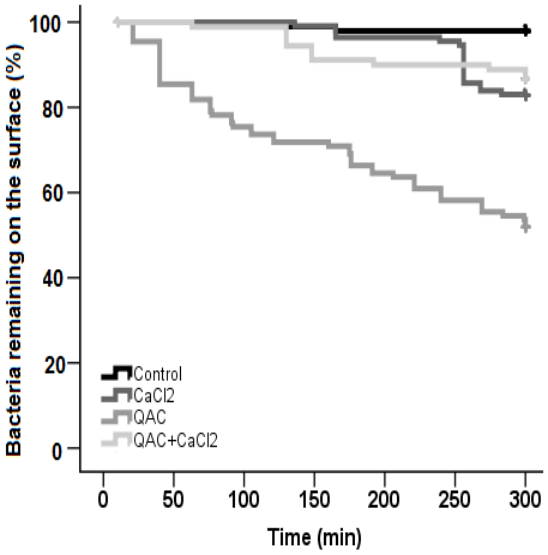

Figure 3.12| Kaplan-Meier curve expressing the percentage of *Staphylococcus epidermidis* ATCC 14990 adhering on a substrate surface during exposure to demineralized water (control), 0.1 M $CaCl_2$ (control), 110 µg/ml QAC in demineralized water and 110 µg/ml QAC in $CaCl_2$ while continuously scanning. Experiments were performed in duplicate with a 3nN force.

Bacterial removal was not significant in demineralized water, but the addition of 0.1 M $CaCl_2$ caused the removal of about 17% of bacteria from the surface. Exposure to QAC was responsible for a significant bacterial removal ($p<0.05$), but the addition of 0.1 M $CaCl_2$ to the solution significantly limited the removal to about 35%.

CHAPTER 4

DISCUSSION

The main purpose of the present thesis was to study the efficiency of a commercially available QAC manufactured from coconut oil (Ethoquad C/25 Cocoalkyl methyl (polyoxyethylene) ammonium chloride) on biofilm and adhering staphylococcal cell surface structure. *S. epidermidis* was the strain investigated throughout this study for being one of the most frequently isolated nosocomial pathogen associated with infections of biomaterial implants and biomedical devices. Atomic force microscopy (AFM) was the central technique used in these studies for being currently the only one that allows the visualization of the surface of living cells with high resolution and in real time.

AFM has been widely used to study antimicrobial effects on bacterial cell surface (Braga and Ricci 1998, Eaton *et al*. 2008), but most of these studies are conducted under dry conditions, as sample preparation is easier and the resolution obtained is general higher than when performed in liquid environments. Furthermore, in these studies, AFM measurements are often performed after bacteria being incubated in the presence of the antimicrobial compound instead of monitoring its effects in real time. So far, only a few studies used the AFM to visualize *in situ* the antimicrobial effects of a given agent (Meincken *et al*. 2005, Shaw *et al*. 2006). Recently, Crismaru *et al*. (2011) investigated the effects of the same QAC on the survival of adhering staphylococci on a surface using AFM. They demonstrated that continuously scanning of adhering staphylococci with AFM in the contact mode was responsible for the wrinkling of the cell surface until bacteria disappeared from the surface. Although contact mode is one of the most widely used for acquisition of AFM images, the continuous direct contact between the tip and surface sample causes significant lateral forces which can distort soft biological samples. Therefore, in the present study AFM measurements were conducted in Peak Force Tapping mode, in which the probe and the sample are intermittently brought together (similar to Tapping mode) to contact the surface for a short period, thus eliminating lateral forces. Unlike Tapping mode, in which the feedback loop keeps the cantilever vibration amplitude constant, the Peak Force Tapping mode controls the maximum force applied on the tip protecting both the tip and the sample from damage.

To complement the study conducted in this thesis, the effects of the QAC on the bacterial cell surface were compared with the ones obtained from an antibiotic (Gentamicin sulphate) and a small antimicrobial peptide (Gramicidin S). Gentamicin sulphate is a broad-spectrum antibiotic widely used in the treatment of staphylococci infections, which works at ribosomal level by inhibiting protein synthesis and also by disrupting the integrity of bacterial cell membrane (Kadurugamuwa *et al*. 1993). Gramicidin S is a cationic cyclic peptide which mode of action is thought to be similar to

the QAC, involving an initial interaction between the anionic phospholipids of bacterial membrane and the cationic amino acid residues of the peptide, followed by its partial penetration into the layer and disruption of membrane's structural integrity.
The MIC and MBC results from this study indicated that the three target compounds exhibit antibacterial activity accordingly to previous reports (Hosseinzadeh *et al.* 2006, Kapoerchan *et al.* 2010). Gentamicin proved to be the most effective with smaller concentrations required to inhibit bacterial growth. After determining antimicrobial activity of the studied compounds in planktonic cultures, their effect on staphylococci adhering on a glass slide using AFM, as well as their effects on biofilm was investigated.

4.1|ANTIMICROBIAL EFFECT ON ADHERING STAPHYLOCOCCAL CELL WALL

The effect of the studied compounds on bacteria adhesion to glass slides could be visualized *in situ* with AFM. The main morphological alterations visualized by bacterial cells under exposure to the three compounds include shrinkage, surface roughness and leakage of intracellular material. Similar morphological effects were observed irrespective of antimicrobial tested which may be explained by the cationic nature of all the compounds investigated. QAC, Gramicidin S and Gentamicin are believed to be "membrane active" agents and therefore, it is expected that attractive electrostatic interactions play a crucial role in their association with negatively charged bacterial cell wall. In fact, AFM and fluorescent images confirmed that removal of adhering staphylococci starts with membrane damage for all the antimicrobials studied and therefore, membrane degradation is a significant contributing factor to their bactericidal activities.

Besides morphology imaging, AFM pictures could be used to investigate the strength of cell adhesion or the ease of removal from the surface. Cell attachment to a surface has been quantified using cell counting methods *in situ* such as radiolabeling of cells or microscopy/image analysis methods, or indirectly by quantifying the number of cells removed from surfaces (Gottenbos et al. 2000). However, these techniques are limited since they only quantify the number of bacteria and not the strength of adhesion. The advantage of using AFM for measuring cell attachment to a surface is that the AFM can image down to the nanometer level, with high resolution, measuring therefore forces applied directly on cell-cell or cell-surface interactions. Whitehead *et al.* (2006) determined how surface roughness could affect the force required to remove differently shaped and sized cells from surfaces with defined topographies and chemistries. Using

AFM, bacterial cells were removed from the surface by application of increasing and defined perpendicular force applied in a lateral/parallel fashion. The authors concluded that the number of cells removed was related to cell shape and size with respect to the shape and size of the surface features. In the present study, after determining the minimal force that could be applied with no influence on the bacterial cell morphology, but high enough to discriminate bacterial shape, this force was continuously and constantly applied during the time of exposure. In this way, it was possible to describe ease of cell removal for each compound that is related to the strength of cell adhesion. Gentamicin proved to be more efficient and faster than the other antimicrobials in removing staphylococci from the surface, which may be explained by its marked antibacterial effect confirmed by its low MIC. Gramicidin S and QAC revealed a similar outcome on bacterial cells which was expected taking into account that both compounds are cationic amphiphiles with similar molecular weight that can interact with cell membranes, and therefore it is likely that both have the same mode of action.

For all the studied compounds, a continuous scanning was required to remove bacteria from the surface since after enlarging the scan area after 300 min of exposure it was possible to observe that most of bacteria outside the continuously scanned area did not seem affected by the compounds. These results are in accordance with the ones from Crismaru *et al*. (2011), thus suggesting that surface damages and consequent removal from the surface induced by the studied compounds were accelerated by an external stress applied in this study by the AFM tip. These results are also supported by the work developed by Liu *et al.* (2008). The authors found a strong correlation between high adhesion forces and the retention of non-viable bacteria. Their observations suggest that certain types of mechanical forces can lead to stress that deactivates bacteria.

In the previous work performed by Crismaru *et al*. (2011), where the effects of the same QAC on the surface integrity of staphylococcal were evaluated using AFM in contact mode, the QAC proved to be more effective, completely removing bacterial cells from a glass slide within 300 min of exposure. A comparison between both works, though different staphylococcal strains have been used, suggests that contact mode has some influence on bacterial removal from the surface and therefore, Peak Force Tapping mode allows studying the influence of the compounds without significant interference from the AFM tip.

In the present study, some preliminary experiments regarding the influence of the force applied by the AFM tip were also conducted. After determining the highest force that could be applied without significant interferences in bacterial cell morphology, it was

observed that increasing the force improved the compounds efficiency as they were able to remove all the bacterial cells much faster than when was applied a lower force. Although preliminary and incomplete, these results confirm that the application of an external stress enhanced the antibacterial efficiency of the studied compounds and this improvement was more pronounced for increasing forces applied.

4.2|ANTIMICROBIAL EFFECT ON BIOFILM

The susceptibility of *S. epidermidis* biofilm to Gentamicin, Gramicidin S and QAC was studied by exposing 24 h-old biofilms to the compounds at different concentrations (0.5, 1 and 2xMBC) overnight. Results support the general observation that staphylococci in biofilms are difficult to completely eradicate with a wide variety of antimicrobial agents, including those used in this study, since no completely reduction in metabolic activity of biofilm could be achieved for any of the compounds studied at their MBC. All the compounds proved to have some antimicrobial activity at different extents which is in accordance with other reports on their antimicrobial activity against clinical isolates (van der Knaap *et al.* 2009, Davison *et al.* 2010, Nuryastuti *et al.* 2010). Gentamicin proved to be the most effective against planktonic cultures, while the highest reduction in metabolic activity of biofilm was achieved by the QAC. Interestingly, the planktonic MBC determined for the QAC proved to be also efficient against biofilm, thus suggesting that this compound has a similar antimicrobial activity against planktonic bacteria and bacteria growing in biofilms. When exposed to Gentamicin, but mostly to Gramicidin S, *S. epidermidis* biofilms did not seem too susceptible to their planktonic MBCs. In biofilms, bacteria embed themselves in self-producer matrix which difficult the penetration of antimicrobial compounds and therefore it is expected that higher concentrations than the ones determined in planktonic cultures will be required to produce a similar effect. Besides, there is some evidence suggesting that binding of the positively charged aminoglycosides to slime retards penetration of these agents (Nuryastuti *et al.* 2011). Gramicidin S and QAC demonstrated different efficiencies when used against biofilms than when used against adhering cells. Such discrepancy may rely on the different molecular structure of the compounds. Gramicidin S is a cyclic peptide which may compromise its penetration in biofilms, while the QAC has a linear structure which may enhance its diffusion through the biofilm.

4.3|INFLUENCE OF CALCIUM CATIONS ON QAC MECHANISM

The present study also confirmed new insights about QAC's mode of action against staphylococcal cells that were recently reported by Crismaru *et al.* (2011). The most quoted theory hypothesizes that sufficiently long cationic polymers interact with bacterial cell surfaces to become integrated in the bacterial cell membrane causing the cytoplasmic membrane instability by creating holes, followed by leakage of intracellular material leading finally to cell death. However, it is believed that the QAC used in this study is not long enough to completely penetrate *S. epidermidis* cells, which are gram-positive bacteria with a thick layer of peptidoglycan surrounding cytoplasmic membrane. Since it was shown that QAC was still able to damage staphylococcal cells it was thought that the role of bacterial membrane charge may have been underestimated in explaining the mechanisms underlying the antimicrobial efficacy of QACs. Gram-positive bacteria have a relatively thicker but porous cell wall made up of inter-connected peptidoglycan layers surrounding a phospholipid cytoplasmic membrane (George *et al.* 2009). Phospholipids comprise two long fatty acids connected via glycerol to phosphoric acid with negative rest charge that is neutralized by divalent cations, such as calcium or magnesium. In the presence of cationic QAC molecules, the replacement of calcium ions by the QAC is expected and may result in a loss of membrane integrity by itself, as confirmed by the fluorescent microscopy results, or in a loss of function of membrane-bound enzymes. For example, divalent cations are required for ATP-ase to exhibit its function, thus removal of these ions would lead to a loss of its activity (Chen and Cooper 2002). Membrane damage is followed by leakage of the intracellular material and loss of turgor pressure evidenced by the wrinkling of the bacterial surfaces and a decrease in bacterial volume (Figure 3.3), as observed after exposure to antimicrobial peptides (Hartmann *et al.* 2010). In the presence of calcium ions, these cations compete with cationic QAC molecules for a place in the membrane, reducing therefore the antimicrobial efficiency of the QACs as demonstrated in the present study. These results are in accordance with the ones from Chen and Cooper (2002) that demonstrated that high concentrations of calcium can limit the efficacy of quaternary ammonium functionalized poly(propylene imine) dendrimers. It is important to mention that the concentration of calcium ions used in this study (0.1 M) is 100-fold higher than the concentration found in blood serum (between 1.1 and 1.4 mM). Therefore, it is not expected that these divalent ions can limit potential applications of the QAC on implant surfaces to be used in the human body.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1| CONCLUSIONS

The main purpose of the present thesis was to assess the potential of using a QAC to develop new strategies for the prevention of adhesion and biofilm formation on biomaterial implant and medical devices' surfaces. To cover this aim, several subjects were studied and different strategies were successfully implemented. With the work developed in the present thesis it was possible to draw the following conclusions:

- AFM represents a promising tool to study the antimicrobial effects of different compounds on bacterial cell surfaces. In this study, AFM allowed the visualization *in situ* of what happens when bacterial cells are exposed to different antimicrobial compounds.
- All the antimicrobial compounds exhibited antimicrobial activity against staphylococcal cells. However, depending on the bacteria modality growth, different efficient rates were obtained. Gentamicin was found to be the most effective compound against planktonic cultures and cells adhering to a glass slide, while QAC showed the highest reduction in metabolic activity of staphylococcal biofilms.
- The application of an external stress on adhering staphylococcal cells may improve the bactericidal properties of different antimicrobial compounds resulting in membrane damage, as well as its disintegration with subsequent removal from a surface. Some preliminary studies also suggested that increasing the external stress applied results in higher removal rates. Stress activation may thus provide an alternative pathway to enhance the efficacy of different antimicrobials.
- The presence of calcium ions reduced the bactericidal efficacy of QAC molecules which suggests that its mode of action relies on its ability to exchange with calcium ions that are responsible for the bacterial membrane stability.
- The QAC investigated in the present study demonstrated promising results regarding its efficiency on biofilm and adhering staphylococcal cell surface structure representing, therefore an interesting alternative that is worth to explore further in the future for the development of antimicrobial coatings of biomedical devices.

5.2|RECOMMENDATIONS

Although the results obtained in this thesis are promising, some work is still required to validate them, as well as to answer some new questions raised:

- The efficacy of the compounds should be tested against more staphylococcal strains and also against Gram-negative strains with clinical relevance, such *as P. aeruginosa* and *E. coli*.
- It would be interesting to take advantage of other AFM applications besides imaging. Physical properties of bacteria when exposed to the antimicrobial compounds could be investigated through a force-distance curve in force spectroscopy mode. Another possibility could be the optimization of AFM measurements using PeakForce QNM mode to also get the information regarding nanoscale material properties, such as modulus, adhesion, deformation and dissipation.
- In the present study, the removal of bacteria from the surface was taken as an indication of membrane damage caused by the antimicrobial compounds. Such consideration was based on fact that it took 15 min to acquire a single image during AFM measurements and therefore, some of the effects immediately before cell removal were missed. The use of high speed AFM that has been developed by some pioneer research groups could be the answer for this drawback since it allows to record movies at high resolution by taking several images per second.
- Enlarged images of single bacteria exposed to the other compounds (Gentamicin and Gramicidin S) should be conducted in order to better discriminate the effects of each one. Besides, similar morphological effects were observed irrespective of antimicrobial tested which may be explained by the cationic nature of all the compounds investigated. Therefore, it would be interesting to investigate the effect of another compound with a different mode of action.
- MTT assay, used in this study to investigate the antimicrobial activity against biofilm, provided an indication of the overall efficacy, in terms of viability reduction but did not consider fundamental phenomena important in the interaction of the biocide with the biofilm. Therefore, confocal scanning laser microscopy should be used to visualize the spatial and temporal patterns of the antimicrobials action in biofilms.

- Finally, the influence of calcium ions should also be studied for Gramicidin S and Gentamicin to confirm that cationic antimicrobials have a similar mode of action. It also should be confirmed the same effect for magnesium ions also responsible for membrane stabilization.

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