CHAPTER 1 - General introduction

This chapter encloses the literature review, presenting in the first sections a brief historical introduction, the current knowledge on the features of biofilm formation and biofilms relevance to medical device-associated infections. Then follows a presentation of *Staphylococcus epidermidis* general characteristics, an overview of the clinical relevance of the bacterium as one of the major nosocomial pathogens responsible for indwelling medical devices infections, and the main properties of *S. epidermidis* biofilms. The experimental methods used to study adhesion and biofilm processes are also addressed as well as biofilm resistance and control.

In the last sections of this chapter the scope and the structure of this thesis are described.

1.1 Microbial biofilms

Microorganisms have primarily been characterized as unicellular life forms, living as planktonic, freely-suspended cells and it is undeniable the importance of the use of bacterial pure cultures growing in liquid medium in the understanding of microbial pathogenesis and physiology (Davey and O'Toole, 2000). However, recent advances in microscopy and molecular technologies have made possible the direct observation of a wide variety of natural habitats, establishing that the majority of bacteria persist attached to surfaces within a structured biofilm ecosystem and not as free-floating organisms (Costerton *et al.*, 1995).

From an historical point of view, the discovery of microbial biofilms can be attributed to Antonie van Leeuwenhoek, who first observed microorganisms in the plaque on his own teeth, using his simple microscope, in the 17th century. Later, in the 20th century, Heukelekian and Heller (1940) and Zobell (1943) showed that bacterial growth and activity were considerably enhanced by the presence of a surface to which bacteria could attach and that the number of microorganisms on surfaces was significantly higher than in the surrounding medium. The study of Characklis (1973), about microbial slimes in industrial water systems, revealed their high cohesiveness as well as their strong resistance to disinfectants, but it was Costerton *et al.*, in 1978, which postulated the general theory of biofilm predominance.

In natural world, more than 99% of all bacteria exist as biofilms (Costerton *et al.*, 1987) and therefore this ubiquity among diverse ecosystems suggests a strong survival and/or selective advantage for sessile cells over their planktonic counterparts. This advantage arises from the fact that bacteria attached to a surface are in a most favourable environment in terms of nutrients availability, metabolic cooperativity and protection against external factors (Davey and O'Toole, 2000). It is therefore well established that sessile cells are physiologically distinct from bacteria growing in planktonic state (Hall-Stoodley *et al.*, 2004) and that biofilms exhibited a distinct phenotype from their free-floating counterparts. The main phenotypic alterations are related to gene transcription, growth rate, respiration rate, rate of oxygen uptake, electron transport activity,

synthesis of extracellular polymers, substrate uptake rates, rate of substrate breakdown, heat production and ability to resist to antimicrobial treatments (Donlan, 2002; Wilson, 2001). In fact, it is estimated that sessile bacteria within biofilms are up to 1,000-fold more resistant to antibiotics and to the host immune defence system than their planktonic counterparts (Ceri *et al.*, 1999). However, cells growing in biofilms are not only physiologically distinct from planktonic cells, but also differ from each other, both spatially and temporally, as biofilm development proceeds. The metabolic activities of the cells within a biofilm, together with diffusion processes, have as outcome gradients of nutrients concentration, signalling molecules and bacterial waste. Thus, bacteria respond to these gradients, adapting to the local chemical conditions, which can modify over time as biofilms develop and, as a result, biofilms exhibit considerable heterogeneity (Stewart and Franklin, 2008).

A biofilm can therefore be defined as a community of bacteria that is irreversibly attached to a biotic or abiotic surface and that is enclosed in a matrix of exopolymeric products (Costerton *et al.*, 1999; Prakash *et al.*, 2003). This matrix is composed of a mixture of extracellular polymeric substances (EPS), such as polysaccharides, proteins, nucleic acids and other substances (Davey and O'Toole, 2000; McSwain *et al.*, 2005).

1.2 Biofilm formation

Biofilm formation is a process involving two main distinct phases: primary attachment of the bacteria to the surface, and the formation of multi-layered cell clusters with cell-to-cell adhesion depending on the production of an extracellular "slimy" matrix (O'Toole *et al.*, 2000) (Figure 1.1). In the first stage of bacterial colonization, the bacterium approaches the surface so closely that its motility is slowed forming a transient association with the surface. The solid-liquid interface between a surface and an aqueous medium provides an ideal environment for the attachment and growth of microorganisms (Costerton *et al.*, 1999). This initial microbial adherence is mostly dependent on bacterial cell surface characteristics and on the nature of the

material surface (von Eiff *et al.*, 2002). However it is mainly due to the physicochemical interactions that bacteria firmly adhere to the biomaterial surface during the adhesion process (Oliveira *et al.*, 2003). These comprise van der Waals forces, cell surface hydrophobicity (Busscher *et al.*, 1990; Oliveira *et al.*, 2001) and charge (van Loosdrecht *et al.*, 1990) as well as the hydrophobicity, charge, roughness and chemical composition of the biomaterial surface itself (An and Friedman, 1998).

The presence of bacterial surface-associated proteins is also associated with cell surface hydrophobicity and initial adhesion (von Eiff *et al.*, 2002). However, in natural environments, bacteria mostly adhere to the layer of adsorbed molecules that coats the surface, the so called "conditioning film", and not directly to the substratum. In this case, attachment is mainly dependent of specific interactions between bacterial adhesins and their complementary receptors present on molecules on the substratum surface (Whittaker *et al.*, 1996). These adhesins are called MSCRAMMs, i.e., microbial surface components recognizing adhesive matrix molecules, and the *Staphylococcus epidermidis* fibrinogen-binding protein Fbe is one example of this type of cell surface proteins that interact with host-matrix proteins (Vuong and Otto, 2002).

After initial adhesion to the foreign body surface, the bacteria multiply forming microcolonies and accumulate as multilayered cell clusters, a step that involves intercellular adhesion and the synthesis of extracellular matrix molecules, such as proteins and polysaccharides. The bacteria begin to multiply and to release chemical signals that communicate, via quorum sensing mechanisms, among the bacterial cells and that activate genetic mechanisms responsible for exopolysaccharide production (Costerton *et al.*, 1999). Therefore, further growth of the attached microorganisms occurs, leading to the formation of dense bacterial aggregates embedded in the exopolymeric matrix, typical of mature biofilms (Wilson, 2001).

Mature biofilms can then undergo a detachment process, due to the exposure to strong mechanical and hydrodynamic forces and to quorum sensing regulation, releasing

planktonic bacteria that can then colonize another region of the substratum to form new microcolonies (Kong *et al.*, 2006; Prakash *et al.*, 2003).

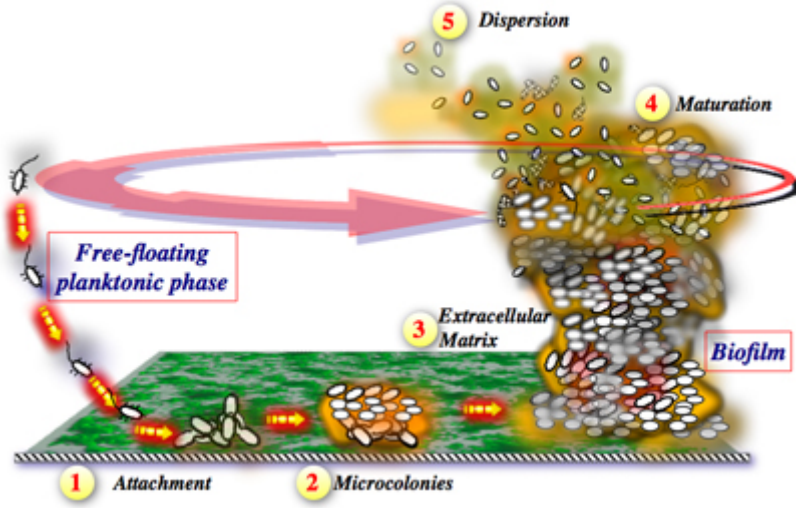


Figure 1.1 Schematic model of the phases involved in bacterial biofilm formation on a surface. **1**- Initial bacterial attachment to the surface. **2**- Bacteria start to multiply, forming microcolonies. **3**- Formation of multicellular clusters, embedded in an extracellular matrix. **4**- Maturation of the biofilm occurs with further growth of the attached microorganisms leading to the formation of dense bacterial aggregates. **5**- Detachment of some biofilm cells can occur, leading to colonization in other parts of the substratum.
Adapted from: http://www.pasteur.fr/recherche/RAR/RAR2006/Ggb-en.html.

1.3 Biofilm structure

The application of advanced microscopy, such as confocal laser scanning microscopy, molecular and electrochemical high-resolution methods has provided insights into the structural organization and function of biofilm communities. Therefore, a mature biofilm is seen as very heterogeneous arrangement, with a basic community structure consisting of microcolonies of bacterial cells encased in EPS matrix separated by water channels (Donlan and Costerton, 2002; Lewandowsky, 2000). But although some structural attributes can generally be considered

universal, every microbial biofilm community is unique (Tolker-Nielsen and Molin, 2000). This is due to the fact that a biofilm structure can be influenced by several conditions, such as surface and interface properties, nutrient availability, the composition of the microbial community, and hydrodynamics (Stoodley *et al.*, 1997), making the exact structure of any biofilm probably a sole feature of the environment in which it develops (Sutherland, 2001). Hence, the structure of a biofilm can range from a dense biofilm model (Wimpenny and Colasanti, 1997), to a heterogeneous mosaic model (Keevil and Walker, 1992) or to one consisting of a more complex organization involving mushroom-like aggregates separated by water channels, normally considered the most typical biofilm architecture (Costerton *et al.*, 1994) (Figure 1.2). This structure is characteristic of biofilms formed under low nutrient concentration, high hydrodynamic shear stress and the absence of mechanical, abrasive and compressive forces (Wilson, 2001).

Concerning the biofilm composition itself, water is considered to be the major component of the biofilm matrix - up to 97% (Zhang *et al.*, 1998) while bacteria occupy only between 10 and 50% of the total volume of the biofilm (Costerton *et al.*, 1995). EPS account for 50 to 90% of the total organic carbon of biofilms (Flemming *et al.*, 2000). Besides polysaccharides, proteins, nucleic acids or phospholipids, non cellular materials such as mineral crystals, corrosion particles or blood components, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix (Donlan, 2002).

The water channels that separate the matrix-enclosed microcolonies are vital to biofilm maintenance, providing a nutrient flow system within it (Donlan and Costerton, 2002) that delivers nutrients deep within the complex community (Stoodley *et al.*, 2002) and allows the exchange of metabolic products with the bulk fluid layer (Costerton, 1995). The hydrodynamic flow of liquid over and through the biofilm can also promote the separation of some fragments with viable organisms away from the surface, which can be carried with the flow and deposited elsewhere for further colonization (Dunne, 2002).

In biofilms, the cells exchange information by signals, denominated "quorum sensing", which are chemical signals, used to regulate cell density-dependent gene expression (De Kievit *et al.*, 2001). The quorum sensing signal molecules are peptides, comparable to pheromones and hormones that control cell division, and thereby the biofilm population density as well as the production of exopolymeric matrix (Fux *et al.*, 2003; Hentzer *et al.*, 2004; Rodney and Costerton, 2002).

Therefore, this complex level of structural organization helps to explain the remarkable metabolic efficiency of microbial biofilms.

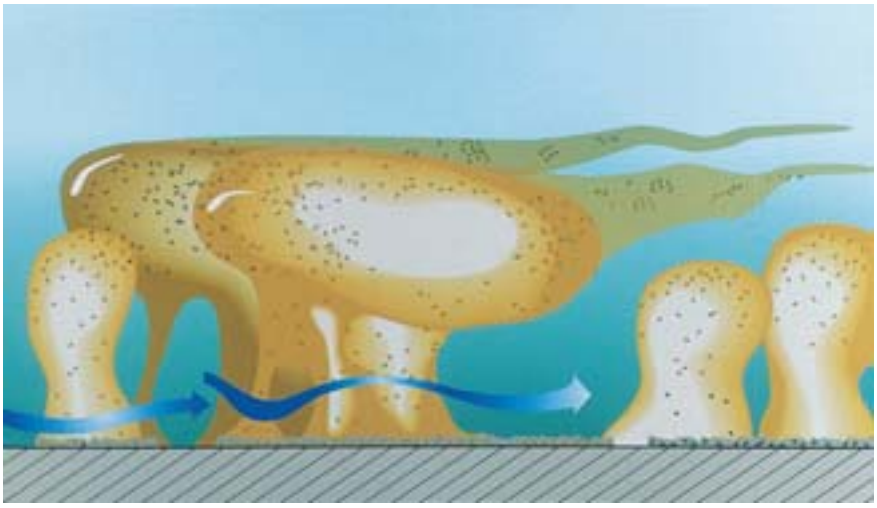


Figure 1.2 Schematic representation of a typical biofilm structure, with mushroom-like stacks containing bacteria embedded in the extracellular matrix. The stacks are separated by water channels and the arrows demonstrate the direction of the fluid flow. Adapted from: Donlan and Costerton, 2002.

1.4 Biofilms and medical device-associated infections

Bacterial adhesion to surfaces of medical devices is considered as the basic pathogenic mechanism of implant infections. The principal medical implants that can be compromised by infections are: intravascular (central venous catheters); cardiovascular (heart

valves, ventricular assist devices, coronary stents); neurosurgical (ventricular shunts, implantable neurological stimulators); orthopaedic (arthro-prostheses, fracture-fixation devices); ophthalmological (mainly intra-ocular lenses) and dental (dental implants) (von Eiff *et al.*, 2005). Also catheters, such as central line, intravenous or urinary catheters, constitute potential surfaces for biofilm formation (Davey and O'Toole, 2000). In fact, infection associated with central venous catheters is a major cause of bacteraemia in hospitalized patients (Elliott, 1993) and its main source of microbial contamination is the skin insertion site, from where bacteria can migrate down the intracutaneous tract on the external surface of the catheter leading to subsequent colonization of the catheter or sepsis (Worthington *et al.*, 2000). The failure of such devices relies on the ability of microorganisms to form biofilms on their surfaces, which are extremely resistant to host defence mechanisms (Costerton *et al.*, 1999) and antibiotic treatment (Donlan and Costerton, 2002). When a biofilm infection occurs, the host establishes an immune response to antigens released from the biofilm. However, not only does the host immune response fail to eradicate the biofilm, but it may also result in damage to surrounding tissues (O'Gara and Humphreys, 2001). The high resistance of biofilm bacteria can arise from factors such as phenotypic changes that result in resistance within the biofilm environment, inactivation of the antibiotics by extracellular polymers or modifying enzymes and nutrient limitation resulting in slowed growth rate (Gilbert *et al.*, 1997).

The main microorganisms responsible for biofilm formation on indwelling medical devices are 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). The sources of contamination of the medical devices by these organisms can be the skin of patients or healthcare workers, tap water to which entry ports are exposed or other sources in the environment (Donlan, 2001).

Table 1.1 Some frequent implant infections caused by biofilms. Adapted from: Davey and O'Toole, 2000.

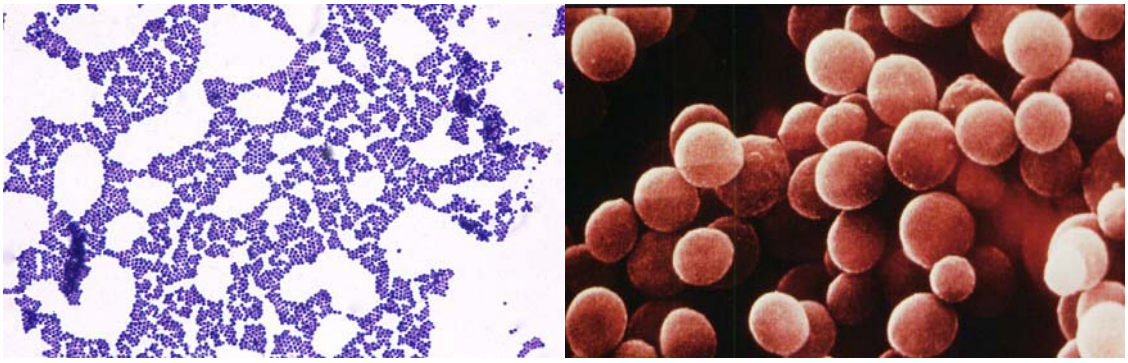
Implant	Organism(s) found	Associated disease or consequences
Prosthetic valve	*S. epidermidis*, *S. sanguis*	Prosthetic valve endocarditis
Contact lenses	*P. aeruginosa*, *S. epidermidis*	Keratitis
Intravascular catheters	*S. epidermidis*, *S. aureus*	Septicemia, endocarditis
Artificial heart	*P. aeruginosa*, *S. epidermidis*, *S. aureus*	Septicemia, device failure
Urinary catheters	*E. coli*, *P. aeruginosa*, *E. faecalis*, *Proteus mirabilis*	Bacteriuria
Joint replacement	*S. epidermidis*, *S. aureus*	Septicemia, device failure
Endotracheal tube	*P. aeruginosa*, *E. coli*, *S. epidermidis*, *S. aureus*	Pneumonia
Voice prostheses	streptococci, staphylococci	Prostheses failure

During the past 20 years it has been reported that between 6 and 14% of patients that enter general hospitals develop a nosocomial infection (Vazquez-Aragon *et al.*, 2003), i.e., an infection that was not present or incubating at the moment of patient admission at a hospital (Garner *et al.*, 1998). Overall, up to of 60% of all nosocomial infections are due to biofilms (Davey and O'Toole, 2000). This makes biofilm-related infections a major cause of morbidity and mortality and frequently the only solution to an infected implanted device is its surgical removal which bears additional economic and health costs (Vinh and Embil, 2005; Wilson, 2001).

1.5 *Staphylococcus* species

Staphylococcus is a bacteria genus characterized by round cells (coccus or spheroid shaped), gram-stain positive [Figure 1.3 (a)], with about 1 micrometer in diameter and found as single cells, in pairs, or more frequently, in clusters that resemble clusters of grapes (Singleton and Sainsbury, 2001) [Figure 1.3 (b)]. The genus name *Staphylococcus* is derived from greek

terms (*staphyle* and *kokkos*) that mean "a cluster of grapes", which is how the bacteria often appear microscopically after gram-staining (Schlegel, 1986).



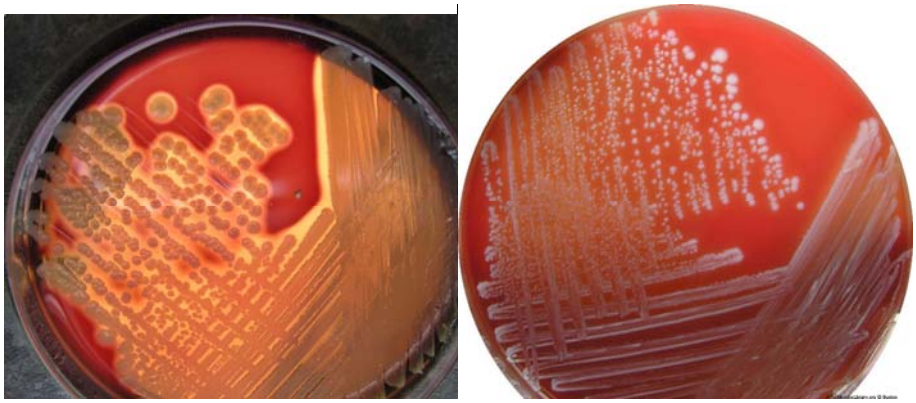
(a) **(b)**

Figure 1.3 *Staphylococcus*. **(a)** Gram-stained staphylococci. **(b)** Staphylococci organized like a cluster of grapes. Adapted from: http://www.portalbiologia.com.br/biologia/principal/conteudo.asp?id=2272 http://www.bact.wisc.edu/themicrobialworld/Staph.html.

Taxonomically, the genus *Staphylococcus* is in the order *Bacillales*, bacterial family *Staphylococcaceae* and they grow in clusters because staphylococci divide in two planes (Prescott *et al.*, 1999). The configuration of the cocci helps to distinguish staphylococci from streptococci (Lim, 1998), which are slightly oblong cells that usually grow in chains (because they divide in one plane only). In 1884, Rosenbach described two pigmented colony types of staphylococci and proposed the appropriate nomenclature: *Staphylococcus aureus* (yellow) and *Staphylococcus albus* (white) (Kloos and Schleifer, 1986; Singleton and Sainsbury, 2001). The latter species is now named *Staphylococcus epidermidis.*

Although more than 30 species of *Staphylococcus* are described, *Staphylococcus aureus* and *Staphylococcus epidermidis* are the most significant in their interactions with humans (Lim, 1998; Pelczar Jr *et al.*, 1988). *Staphylococcus aureus* forms a fairly large yellow, golden colony on rich medium (Pelczar Jr *et al.*, 1988) and is often hemolytic on blood agar while *S.*

epidermidis forms a relatively small white colony and is non hemolytic (Prescott *et al.*, 1999) (Figure 1.4).



(a) **(b)**

Figure 1.4 (a) Large, creamy yellow, hemolytic colonies typical of *Staphylococcus aureus*, on blood agar. **(b)** Non-hemolytic *Staphylococcus epidermidis,* on blood agar. Adapted from: http://www.microbelibrary.org/asmonly/details.asp?id=2037.

Staphylococci are facultative anaerobes that grow by aerobic respiration or by fermentation that yields principally lactic acid (Pelczar Jr *et al.*, 1988). The catalase test is important in distinguishing streptococci (catalase-negative) from staphylococci, which are vigorous catalase-producers. Staphylococci are also oxidase-negative, ferment glucose anaerobically, and have peptidoglycan and teichoic acids in their cell walls (Prescott *et al.*, 1999). They do not produce endospores but are highly resistant to dehydratation (Schlegel, 1986), especially when associated with organic matter such as blood, pus, and other tissue fluids.

Staphylococci are divided into coagulase-positive and coagulase-negative strains. Strains of *S. aureus* and *Staphylococcus intermedius* are coagulase-positive but most species of *Staphylococcus* are coagulase-negative (Devriese *et al.*, 1985). They are normal inhabitants of the upper respiratory tract, skin, intestine, and vagina and they are known as pyogenic cocci, i.e., pus-producing cocci, being among the most important bacteria that cause various suppurative, or

pus-forming diseases (e.g., boils, carbuncles, folliculitis, impetigo contagiosa, scalded-skin syndrome) in humans (Prescott *et al.*, 1999) (Figure 1.5).

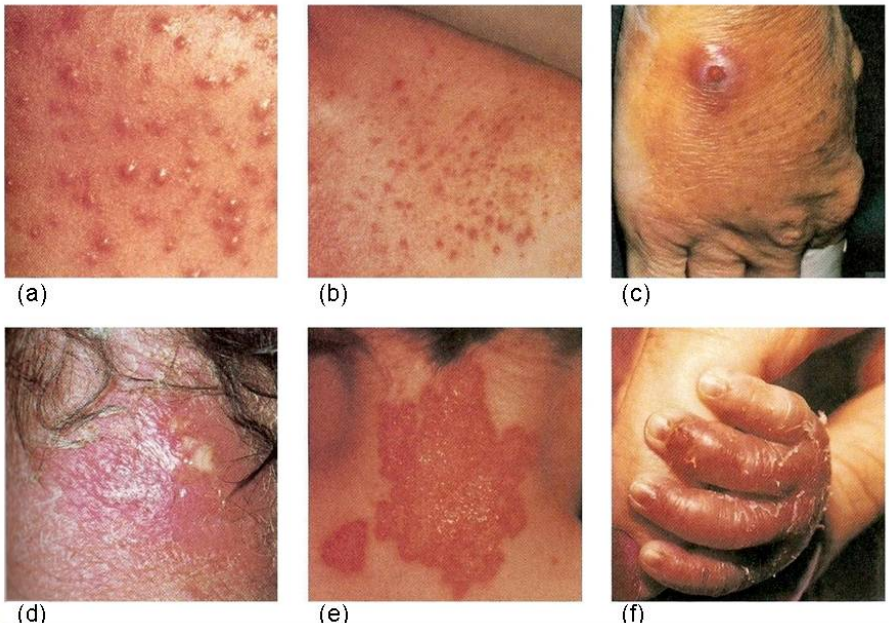


Figure 1.5 Staphylococcal skin infections. **(a)** Superficial folliculitis in which raised, domed pustules form around hair follicles. **(b)** In deep folliculitis the microorganism invades the deep portion of the follicle and dermis. **(c)** A furuncle arises when a large abscess forms around a hair follicle. **(d)** A carbuncle consists of a multilocular abscess around several hair follicles. **(e)** Impetigo on the neck of 2-year-old male. **(f)** Scalded skin syndrome in a 1-week-old premature male infant. Reddened areas of skin peel off, leaving "scalded"-looking moist areas. Adapted from: Prescott *et al.*, 1999, p. 785.

1.5.1 *Staphylococcus epidermidis*

Staphylococcus epidermidis is currently the most significant member of the coagulase negative staphylococci (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 to 90% of all staphylococci isolated from these environments (O'Gara and Humphreys, 2001; Vuong and Otto, 2002). CNS are diagnostically distinguished from *S.*

aureus by its inability to produce coagulase (Vuong and Otto, 2002), an enzyme that converts fibrinogen to fibrin and is responsible for blood plasma clotting (Prescott *et al.*, 1999). Additionally, CNS can be divided into two groups according with its resistance or susceptibility to novobiocin, which is the case of *S. epidermidis* (von Eiff *et al.*, 2002). Infections caused by CNS express clinical symptoms clearly different from those of *S. aureus*: subtle and non-specific clinical signs leading to sub-acute or even chronic infection. Bacteraemia caused by CNS is rarely severe, mainly if treated rapidly and with an adequate approach, despite the fact that sepsis and fatal cases have been reported, particularly in immunocompromised patients and/or if one of more virulent species is involved (Miele *et al.*, 2001; Pagano *et al.*, 1997; Pessoa-Silva *et al.*, 2001).

1.5.2 *Staphylococcus epidermidis* and device-associated infections

In recent years, *S. epidermidis* emerged as one of the most important and frequently causes of nosocomial infection, mainly associated with implanted medical devices (von Eiff *et al.*, 2002; Vuong and Otto, 2002; Wang *et al.*, 2007). Therefore, *S. epidermidis* species subsist both as commensal and as pathogenic, creating strategies in order to transform the hospital environment in a new ecological niche (Ziebuhr *et al.*, 2006). In fact, in what concerns to biomaterial-associated infections, nearly 80% of the cells involved are *S. epidermidis* (Götz, 2002).

S. epidermidis infections are usually associated with immunocompromised individuals such as intravenous drug abusers and AIDS patients (Vuong and Otto, 2002) but also long-term hospitalized and seriously ill patients (individuals receiving immunosuppressive therapy and premature newborns) who represent very susceptible hosts (Ziebuhr, 2001), and therefore this bacterium is considered to be an opportunistic pathogen. While early or acute infections are normally caused by *S. aureus*, *S. epidermidis* are typically responsible for chronic and profound infections, which occur months to years after the prosthesis implantation (Barth *et al.*, 1989;

Bayston *et al.*, 2005). Moreover, *S. epidermidis* infections are often persistent and relapsing (O'Gara and Humphreys, 2001) and result from a rupture of the cutaneous surface, by any type of trauma or surgical insertion of a medical device. Staphylococci can therefore enter the host, becoming pathogenic (Gill *et al.*, 2005; Vadyvaloo and Otto, 2005).

The increasing number of immunocompromised patients, the use of medical devices that provide a suitable habitat for bacterial colonization and the high selective forces to which microorganisms are submitted in the hospital environment due to the extensive use of antibiotics and disinfectants (Ziebuhr *et al.*, 2006) constitute factors that help to explain the success of *S. epidermidis* as a nosocomial pathogenic.

Besides indwelling medical devices infections in immunocompromised patients, *S. epidermidis* is also responsible for native valve endocarditis in immunocompetent individuals, a condition that results from the interaction between the vascular endothelium, generally of the mitral, aortic, tricuspid, and pulmonic valves of the heart, and bacteria or fungi circulating in the bloodstream (von Eiff *et al.*, 2002). In addition, cases of osteomyelitis (acute or chronic inflammatory process of the bone and its structures), wound infection, otitis media (the inflammation of the mucoperiosteal lining, in the middle ear), endophthalmitis (inflammation of the intraocular cavities, i.e., the aqueous or vitreous humor), urinary tract infection, meningitis and pneumonia have also been reported as associated to *S. epidermidis* (Heilmann and Peters, 2001; Huebner and Goldman, 1999; Miele *et al.*, 2001).

The indwelling medical devices mostly affected by *S. epidermidis* persistent infections include, e.g., prosthetic heart valves, central venous catheters, urinary catheters, contact lenses, prosthetic joints, hip prostheses and other orthopaedic devices (Donlan, 2001; Wang *et al.*, 2007) (Figure 1.6), which are described in more detail in the following sections.

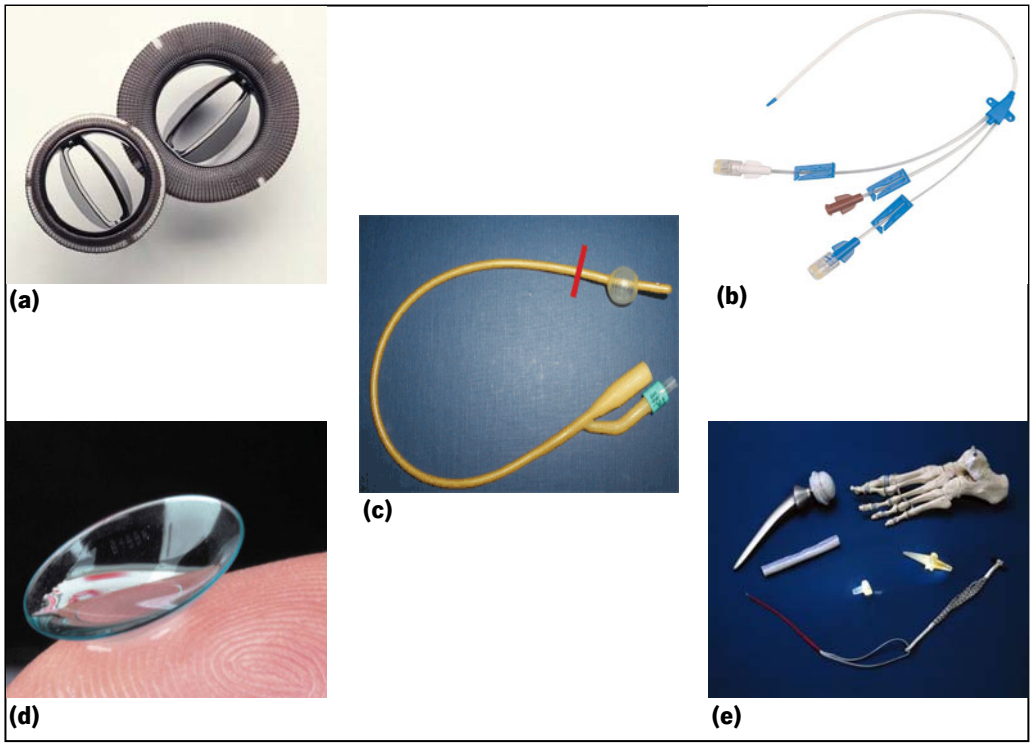


Figure 1.6 Examples of indwelling medical devices affected by *S. epidermidis* infections. **(a)** Prosthetic heart valve. **(b)** Central venous catheter. **(c)** Urinary catheter. **(d)** Contact lens. **(e)** Artificial joints and ligaments and replacement blood vessels.

1.5.2.1 Prosthetic heart valves

Mechanical valves constitute one of the main prosthetic heart valves types that are currently being used (Braunwald, 1997) [Figure 1.6(a)]. When the surgical implantation of the prosthetic valve occurs, bacterial cells can attach and develop biofilms on constituent parts of these mechanical heart valves and on surrounding tissues of the heart (Donlan, 2001). *S. epidermidis* is one of the main early colonizers (Hancock, 1994; Karchmer and Gibbons, 1994), probably due to contamination of the surgical site during the procedure. This normally leads to tissue damage and accumulation of platelets and fibrin at the suture site of the device leading to

a condition known as prosthetic valve endocarditis (PVE) or to microbial infection of the valve and surrounding tissues of the heart (Donlan and Costerton, 2002).

According to Illingworth *et al.* (1998), PVE is predominantly caused by microorganisms colonization of the sewing cuff fabric of the prosthetic valve used to attach the device to the tissue. Bacterial invasion of the valve annulus into which the prosthetic valve is sewn is also possible, which can lead to a division between the valve and the tissue and, consequentially, to serious leakage (Karchmer and Gibbons, 1994). A 3.2 to 5.7% growing incidence of PVE by 5 years after valve implantation was reported in a previous study (Calderwood *et al.*, 1986). In addition, mortality rates of 70% are reached among patients who develop endocarditis (Hyde *et al.*, 1998).

Therapy with antimicrobial agents is applied during valve replacement and whenever the patient has dental work, in order to kill all microorganisms introduced into the bloodstream, thus preventing initial attachment (Donlan, 2001). However, antibiotics efficiency by itself has demonstrated to be scarce (Hancock, 1994). Therefore, new approaches involving sewing cuff silver coating (Illingworth *et al.*, 1998) and sewing cuffs coated with minocycline and rifampin (Darouiche *et al.*, 2002) have been tested, demonstrating a reduction of inflammation levels.

1.5.2.2 Central venous catheters

Central venous catheters (CVCs) comprise 3 to 5% of infection rates, constituting one of the indwelling medical devices with greater risk of device-related infection (Maki, 1994) [(Figure 1.6(b)]. Staphylococci are the most frequently isolated pathogens in catheter-related infection (CRI), particularly CNS, followed by enterococci, *S. aureus* and *Candida* species (Edmond *et al.*, 1999; Haslett *et al.*, 1988; Sherertz *et al.*, 1990). These organisms can arise from patient's skin microflora, health-care personnel microflora, or from contaminated infusates, e.g., nutritional solutions, medications and blood products (Flowers *et al.*, 1989).

Currently, CRIs are a major cause of patient morbidity and mortality, justifying the premature catheter removal and the increase in costs and use of resources (Dimick *et al.*, 2001; Pittet and Wenzel, 1995). The skin insertion site and the catheter hub are the two most important infection sources. Approximately 65% of CRIs originate from the skin flora, 30% from the contaminated hub and 5% from other pathways (Bouza *et al.*, 2002; Reed *et al.*, 1995; Sitges-Serra *et al.*, 1995). In the case of short-term catheters, skin contamination is the most likely mechanism of pathogenesis, whereas for long-term catheters, hub contamination is more frequent (Maki *et al.*, 1997). When skin contamination occurs, bacteria migrate from the skin insertion site along the external surface of the catheter, colonizing the distal intravascular tip of the catheter, and ultimately causing bloodstream infection.

Hub contamination is common in long-term catheters due to the fact that such catheters have to be frequently intercepted and manipulated (Linares *et al.*, 1985) and therefore, bacteria are usually introduced into the hub from the hands of medical personnel. From this contaminated hub, the organisms migrate along the internal surface of the catheter, where they can cause a bloodstream infection (Raad *et al.*, 1994). Given that these devices are in direct contact with the bloodstream, platelets, plasma and host tissue proteins, such as fibrinogen, fibronectin, collagen and laminin, are rapidly adsorbed on the surface of intravenous catheters, forming a conditioning film that enhances bacterial adherence to the foreign material (Pascual, 2002).

Among factors that might explain the frequent colonization of catheters by staphylococci, the microbial production of exopolymeric substances and the presence of receptors to plasma proteins absorbed onto the biomaterial surface have been strongly considered (Ammendolia *et al.*, 1999; Eggimann and Pittet, 2002; Pascual, 2002).

1.5.2.3 Urinary catheters

Urinary catheters are tubular, latex, or silicone devices that are inserted via either urethral or suprapubic placing into the bladder [Figure 1.6(c)]. Their use aims to manage short- or long-term bladder drainage, measuring urine output, collecting urine during surgery, preventing urinary retention, or controlling urinary incontinence (Kaye and Hessen, 1994). The Foley catheter, introduced in the 1920s, remains the most common catheter design with little change except in terms of catheter materials (Warren *et al.*, 2001).

Two types of catheter systems can be considered: open, when the catheter drains into an open collecting container; or closed, when the catheter empties into a firmly secure plastic collecting bag (Kaye and Hessen, 1994). The time extent of catheterization is considered to be the primary risk factor for the development of catheter-associated bacteriuria, i.e., the presence of bacteria in urine not due to contamination from urine sample collection (Platt *et al.*, 1986).

About 10 to 50% of patients undergoing short-term catheterization (up to 14 days) develop infections, whereas basically all patients undergoing long-term catheterization (more than 14 days) will develop urinary tract infections (Niël-Weise and van den Broek, 2005; Stickler, 1996). In fact, there is about 10% of increasing risk of catheter-associated infection for each day of catheter use (McLean *et al.*, 1995). Therefore, catheters should be avoided wherever possible and if a catheter is required, only two principles are commonly recommended for prevention of bacteriuria: maintain the catheter system closed and remove the catheter as soon as possible (Warren *et al.*, 2001).

An additional problem of medical biomaterials in the urinary tract environment is the development of encrustation and consecutive obstruction (Desgrandshamps and Moulinier, 1997). However, this only happens when the drained urinary tract becomes infected by urease-producing bacteria such as *Proteus mirabilis* (Tunney *et al.*, 1999).

1.5.2.4 Contact lenses

Contact lenses are currently classified in two categories: soft contact lenses made of hydrogel or silicone, and polymethylmethacrylate hard contact lenses (Dart, 1996) [Figure 1.6(d)]. Adhesion of bacteria, mainly *Pseudomonas aeruginosa* and staphylococci species, to both types of contact lenses is considered a major risk of severe corneal complications (Buehler *et al.*, 1992; Leitch *et al.*, 1998). This is because contact lenses provide a suitable substratum for bacterial adherence and biofilm formation (Elder *et al.*, 1995), maintaining the bacteria in contact with the cornea for long periods of time. When bacteria adhere to the cornea, microbial keratitis can occur, leading to cornea ulceration (Liesegang, 1997b). Keratitis patients usually have pains, accompanied by muco-purulent discharge, a whitish corneal lesion and in some cases the eyelids become very swollen (Elder and Daniel, 1993).

Recent developments in contact lens materials have led to the introduction of new lenses designed for up to 30 days of continuous use (Holden, 2002) but even though the extended contact lenses wear diminishes the handling frequency and thus the risk of contamination, no protection from infection by regular cleaning and disinfection is provided (Henriques *et al.*, 2005). In fact, biofilms have also been shown to develop on contact lens storage cases, which have been implicated as the bacteria primary source for contaminated lens disinfectant solutions and lenses (Dart, 1996; McLaughlin-Borlace *et al.*, 1998; Wilson *et al.*, 1991). Therefore, particular attention has been given to the design of contact lens materials and lens care products in order to diminish microbial keratitis and other ocular infections rates (Liesegang, 1997a).

S. epidermidis is also the main causative pathogen of acute postoperative endophthalmitis, one of the most serious complications after cataract surgery with intraocular lens (IOL) implantation (Okajima *et al.*, 2006). The disease is still difficult to predict and to diagnose, representing a therapeutic emergency due to its rapid evolution and defective prognostic (Alfonso and Flynn, 1995).

1.5.2.5 Orthopaedic devices

In recent years, hip or knee replacements, fracture fixation, ligament and tendon reconstruction and other surgical implant procedures have become valid and extremely common procedures to repair the function of affected joints, fractured bone segments and damaged members (Campoccia *et al.*, 2006). Artificial joints can be assembled with biocompatible metallic materials, stainless steel and titanium, able to bear load, even as being light [Figure 1.6(e)]. In some cases, the implants are made from more than one material, such as ceramics, metals and metal alloys, and ultra-high molecular weight polyethylene, which requires the production of joints between the potential different materials. These materials should be non-toxic to the patient tissues and, at the same time, they should not have adhesive properties (Verran and Whitehead, 2005). However, the presence of polyethylene, e.g., seems to be very attractive for *S. epidermidis* (Gallo *et al.*, 2003) and it was also suggested that this species is able to metabolize some esters released from bone cement (Gristina, 1994). In fact, according to Arciola *et al.* (2005a), *S. epidermidis* represents about 32% of the species found among orthopaedic isolates of implant associated infections.

In spite of prostheses related infections incidence decreasing to 1% nowadays, mainly due to the introduction of modern standards in the sterility control within the operating room environment and adequate protocols of peri-operative antibiotic prophylaxis, this type of infection remains a research, diagnostic, therapeutic and cost-related problem (Gallo *et al.*, 2003). In orthopaedics, infections have a huge impact in terms of morbidity (Trampuz and Zimmerli, 2005) and medical costs (Hebert *et al.*, 1996; Sculco, 1993). Infection can result in failure of the device, its complete removal, amputation, and possibly death (An and Friedman, 1996).

There are several risk factors that increase the possibility of prosthetic infection, such as psoriasis, steroids, diabetes mellitus and deficiencies in host defences (Salvati *et al.*, 1982). Current strategies for the prevention of prostheses related infections rely on the modification of the chemistry or the micro/nanotopology of the prosthesis material surface, e.g., coating the

device with surfactants (Vacheethasanee and Marchant, 2000), proteins such as albumin (An *et al.*, 1996) and hydrophilic negatively charged polysaccharides such as hyaluronan and heparin (Arciola *et al.*, 1994; Hildebrandt 2002; Pavesio *et al.*, 1997) in order to generate adhesion resistant or even bacteria repellent surfaces (An *et al.*, 2000a).

1.5.3 *Staphylococcus epidermidis* biofilm formation

S. epidermidis device-related infections are triggered by the development of a thick adherent, multi-layered biofilm, strongly resistant to antibiotic treatment and that is considered the most important virulence factor involved in its pathogenesis (Raad *et al.*, 1998; Vadyvaloo and Otto, 2005) (Figure 1.7). *S. epidermidis* biofilm formation comprises two fundamental steps: (i) rapid initial attachment of the bacterial cells to the biomaterial surface; and (ii) cells accumulation to form multilayered cell clusters enclosed in an exopolymeric matrix (Götz, 2002).

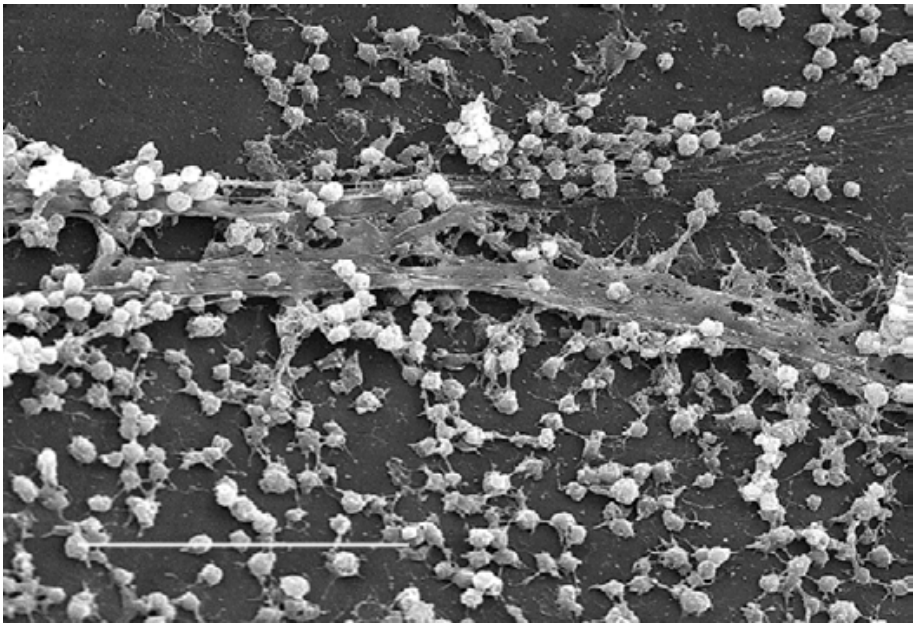


Figure 1.7 Scanning electron micrograph of *Staphylococcus* biofilm on the inner surface of an indwelling medical device. Bar 20 µm. Adapted from: Donlan, 2002.

Initial adhesion of bacteria to the biomaterial surface is thought to be a key step in the colonization of indwelling medical devices and it is a complex process, affected by numerous aspects such as the bacteria and material surface properties and environmental factors (An and Friedman, 1998). This initial step involves non-specific physicochemical (e.g. van der Waals) forces, hydrophobic interactions and polarity changes (Klingenberg and Aarag, 2005; von Eiff *et al.*, 2002). The colonization of the implanted biomaterial may occur by the direct attachment of the cells to the bare polymer surface or by the binding to the host matrix proteins which previously coated the material surface forming the "conditioning film" (Figure 1.8).

The direct binding to the polymer material is mediated by several surface structures of *S. epidermidis* cells, such as the fimbria-like proteins SSP-1 and SSP-2 (Veenstra *et al.*, 1996), the major autolysin AtlE (Heilmann *et al.*, 1996, Heilmann *et al.*, 1997) and teichoic acids (Gross *et al.*, 2001).

Adhesion of bacteria to "conditioning film" occurs through MSCRAMMs, which are considered significant virulence factors, and that include the fibrinogen binding protein Fbe/SdrG (Davis *et al.*, 2001), the fibronectin binding protein Embp (Williams *et al.*, 2002) and the collagen binding GehD lipase (Bowden *et al.*, 2002). AtlE and teichoic acids also play a role in the adhesion to extracellular matrix proteins, since the first has the ability to bind to vitronectin (Heilmann *et al.*, 1997) and teichoic acids enhance adhesion of *S. epidermidis* to immobilized fibronectin (Hussain *et al.* 2001) (Table 1.2).

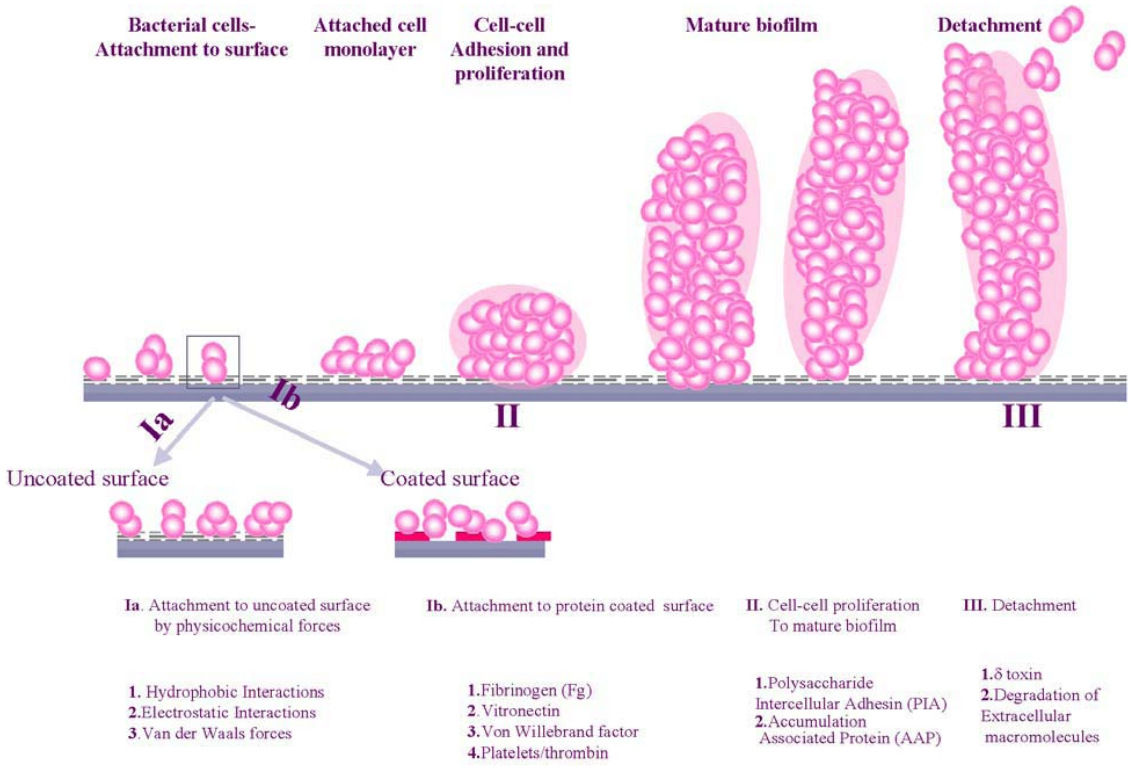


Figure 1.8 Proposed model by Vuong and Otto (2002) for *S. epidermidis* biofilm formation and factors involved in each step. Adapted from: Vuong and Otto, 2002.

After adhesion to the foreign body surface, *S. epidermidis* cells proliferate and accumulate as multilayered cell clusters. This process requires intercellular adhesion, provided by the polysaccharide intercellular adhesin (PIA), which has been described as crucial for the process of cell-to-cell adhesion and biofilm accumulation (Mack *et al.*, 1994; Mack *et al.*, 1992). The PIA molecule is a homoglycan composed of β-1,6-linked *N*-acetylglucosamine residues containing up to 15% de-*N*-acetylated amino groups and substitution with succinate and phosphate residues introducing simultaneously positive and negative charges into the polysaccharide (Mack *et al.*, 1996a; Mack *et al.*, 1996b) (Figure 1.9).

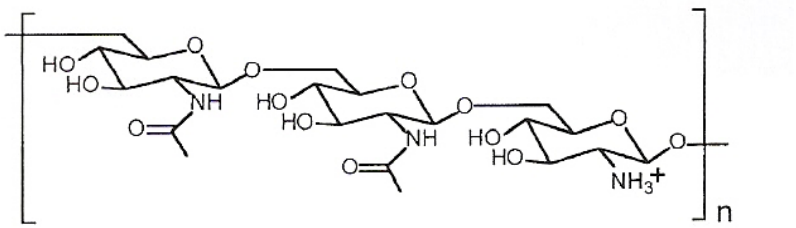


Figure 1.9 Structure of the polysaccharide intercellular adhesin (PIA). The polysaccharide is a linear homoglycan composed of β-1,6-linked *N*-acetylglucosamine residues; up to 15% of the residues are deacetylated and are thus positively charged. Adapted from: Mack *et al.*, 1996a.

Scanning electron microscopy revealed that PIA is located on the surface of *S. epidermidis* as fibrous strands and is a crucial component of the extracellular matrix (Vuong *et al.*, 2004c). Several studies regarding the reduced ability of a PIA-negative mutant to cause infection in animal infection models have emphasized the role of PIA as a virulence factor (Rupp *et al.*, 2001, Rupp *et al.*, 1999a, Rupp *et al.*, 1999b). The genes encoding PIA biosynthesis are organized in the *ica* (intercellular adhesion) operon consisting of *icaR* (regulating gene) and *icaADBC* (biosynthetic) genes (Götz, 2002). Epidemiological studies (Cho *et al.*, 2002; Frebourg *et al.*, 2000; Galdbart *et al.*, 2000; Li *et al.*, 2005; Vandecasteele *et al.*, 2003) demonstrated that the *icaADBC* operon is a typical feature of nosocomial *S. epidermidis* strains obtained from device-associated infections, discriminating these isolates from skin ones, thus corroborating its virulence.

The extracellular 140 kDa protein AAP (accumulation-associated protein), has also been shown to be responsible for accumulative growth on polymer surfaces (Hussain *et al.*, 1997) as well as the cell wall bound surface protein named bhp, homolog of the *S. aureus* bap (Vadyvaloo and Otto, 2005).

Contrary to *S. aureus*, *S. epidermidis* does not produce many toxin and tissue-damaging exoenzymes – the only *S. epidermidis* toxin known is the haemolytic peptide δ-toxin – which contributes to the subacute or chronic character of *S. epidermidis* infections (Otto, 2004; Vuong and Otto, 2002). Some *S. epidermidis* strains also produce lantibiotics, lanthione-

containing extremely stable antibacterial peptides, such as epidermin, epilancib K7, or Pep5, which seem to be important in bacterial competition (Bierbaum *et al.*, 1996). A list of the main *S. epidermidis* virulence factors is summarized in Table 1.2.

Table 1.2 Virulence factors of *S. epidermidis* species. Adapted from: Otto, 2004.

Factor	Function
Attachment to abiotic surfaces	
AtlE	Autolysin/adhesin, attachment to polystyrene, vitronectin binding
SSP-1, SSP-2	Attachment to polystyrene
δ-toxin	Inhibits binding to polystyrene
Attachment to host matrix proteins	
Fbe/SdrG	Fibrinogen binding, inhibition of phagocytosis
Embp	Fibronectin binding
GehD	Collagen binding
Biofilm accumulation	
PIA	Exopolysaccharide, cell-cell adhesion, haemagglutination
AAP	Accumulation
Exoenzymes	
Lipases	Persistence in fatty secretions
Cystein protease	Possibly tissue damage
Metalloprotease Sep-1	Lipase maturation, possibly tissue damage
Serin protease GluSE	Probably biofilm formation, fibrinogen and complement factor C5 degradation
Fatty acid modifying enzyme (FAME)	Detoxification of host-produced bactericidal fatty acids
Regulators of virulence	
agr	Affects lipase and protease production
luxS	Affects biofilm formation
sarA	Probably affects virulence factors production
σ^B	Affects biofilm formation
Others	
Phenol-soluble modulin (PSM)	Several inflammatory effects
Staphyloferrin A, B	Siderophores: iron uptake
SitABC	Probably involved in iron uptake
Lantibiotics (epidermin, Pep5)	Probably bacterial interference
Peptidoglycan/lipoteichoic acid	Stimulation of inflammation

S. epidermidis virulence factors are mainly regulated by the *agr* (accessory gene regulator) locus, a quorum-sensing system that acts according to the cell density (Vuong and Otto, 2002). The *agr* system controls biofilm formation by regulation of biofilm factors, such as AtlE and δ-toxin (Vuong *et al.*, 2003), pro-inflammatory activity, including chemotatic activity on human neutrophils, and virulence in device-related infection (Vuong *et al.*, 2004a, Vuong *et al.*, 2003). The luxS quorum sensing system is also functional in *S. epidermidis* with a significant impact on biofilm formation (Xu *et al.*, 2006). Besides these two main quorum-sensing systems, other two regulatory loci, namely the global stress response alternative sigma factor σ^B (Knobloch *et al.*, 2001; Knobloch *et al.*, 2004), and the global regulator *sarA* (Tormo *et al.*, 2005b), influence biofilm formation of *S. epidermidis* (Vadyvaloo and Otto, 2005).

1.6 Adhesion and biofilm studying methods

Several experimental systems have been developed to study microbial adhesion and biofilm formation on inert surfaces (Donlan and Costerton, 2002).

Concerning bacterial adhesion, static assays are one of the simplest methods available (Bos *et al.*, 1999). In this method, a microbial suspension remains stationary for a determined period of time with respect to an exposed and previously prepared substratum surface and, afterwards, the non-adherent cells are removed by rinsing or centrifugation and the adhered cells on the surface are counted (Katsikogianni and Missirlis, 2004). Enumeration of the adhered cells can be executed by numerous methods which include: microscopic observation (light microscopy, epifluorescence microscopy, scanning electron microscopy, scanning confocal laser microscopy); colony forming units (CFUs) plate counting, radiolabeling and spectophotometry (An and Friedman, 1997).

The parallel-plate flow chamber (Figure 1.10) is a more elaborated method where a pump supplies a steady-state flow, while in the rectangular chamber the fluid enters from one

side and is eliminated from the opposite side (Bakker *et al.*, 2003; Bruinsma *et al.*, 2001). This method has the advantage of providing a more controlled hydrodynamic environment where the general flow within the chamber can be mathematically analysed in a relatively easy way, as well as the initial adhesion rate or the removal rate after passage of an air-liquid interface (Bruinsma *et al.*, 2001).

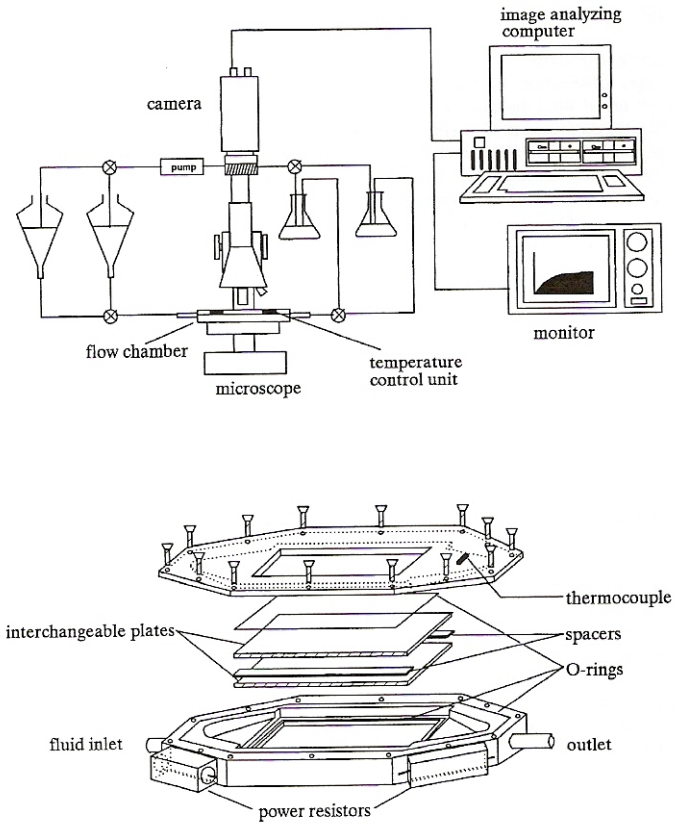


Figure 1.10 Diagram of the parallel plate flow chamber and the chamber itself in more detail. Adapted from: Bos *et al.*, 1999.

Radial flow chamber (Dickinson and Cooper, 1995) and the rotating disc (DeJong *et al.*, 2002) are alternative chambers configurations used to study bacterial adhesion in a dynamic flow.

In order to study biofilms properties in industrial conditions, reactors for open continuous culture are used, e.g., annular reactors (Rototorque) (Chen *et al.*, 1993) and the Robbins device (Gilbert and Allison, 1993).

Biofilm formation to biomaterials can be determined by less intricate methods, such as the tube test (Christensen *et al.*, 1982, 1985), in which the bacterial biofilm lining a culture tube is stained with a cationic dye and visually scaled; or by the microtiter-plate test (Christensen *et al.*, 1985), in which the optical density (OD) of the stained (usually with crystal violet) bacterial film is determined spectrophotometrically. These tests can be applied after biofilm growth in batch or fed-batch systems.

1.7 Control of biofilm biomaterial infections

In order to try to control bacterial adhesion, biofilm formation and the consequent medical device infection, several attempts, including strict hygienic rules during implantation of medical devices, antibiotic prophylaxis in the course of introduction of implants (Wilson, 2001) as well as the development of new materials which could be resistant to microbial adherence and colonization have been tested (von Eiff *et al.*, 2005). Modifications of known biomaterial surfaces have been also developed (Donlan, 2002; Poortinga *et al.*, 2002; Price *et al.*, 2005).

It must be noted that all steps in the pathogenesis of biofilm formation may represent targets against which prevention strategies may be directed. Modification of the foreign body material surface may lead to a change in specific and nonspecific interactions with microorganisms and, thus, to a reduced microbial adherence (von Eiff *et al.*, 2005). For instance, substratum surface charge and hydrophobic properties have been altered in order to produce surfaces with more anti-adherent features (Verran and Whitehead, 2005). Chemical modifications including the incorporation of antimicrobial agents into polymers (antibiotics, antiseptics, metals), physical modifications or the fabrication of very smooth surfaces, have been studied, although none have been independently and totally successful (Fux *et al.*, 2005; Kumon *et al.*, 2001; von

Eiff *et al.*, 2005). Biologic molecules, such as heparin or albumin, have been used, with positive results, for the purpose of interfering in the bacterial adhesive mechanisms (An *et al.*, 1996; Arciola *et al.*, 1994). The use of implants with sensors, which release agents that hamper biofilm development, have been described (Ehrlich *et al.*, 2004) and interference with quorum sensing, the mechanism by which microorganisms communicate in biofilms, provides a potential mechanism for biofilm control (Davies *et al.*, 1998). Treatments that inhibit the transcription of biofilm controlling genes are currently considered one of the most promising strategies to completely inhibit biofilm infections (Costerton *et al.*, 2005).

Nevertheless, it has been extremely difficult to achieve a completely anti-adhesive material mainly due to thermodynamical aspects and to the fact that, *in vivo*, any material surface is rapidly covered by plasma and matrix proteins toward which bacteria display specific adhesins (Arciola *et al.*, 2005c). Therefore, despite the great efforts in implantation techniques, material improvements and antimicrobial therapies, biofilm associated infections are still difficult to eradicate.

1.8 Scope of the work

The main goal of this work was to provide a contribution to a better understanding of the phenomena that involves biomaterials infections caused by *S. epidermidis* and also to provide an insight into their prevention. Therefore, for the accomplishment of these objectives the surface characteristics of the bacterial cells and of biomaterials were studied, as well as their effect in the adhesion phenomenon. Reduction of initial adhesion to inert surfaces was attempted by pre-conditioning with anti-adhesive substances and surface modification. A special focus is given to *S. epidermidis* biofilms and to the properties that distinguish them from planktonic cells.

1.9 Structure of the dissertation

This dissertation is divided in seven chapters. **Chapter one** encloses a general introduction to the work presented here and also a literature review and state of the art. The next five chapters correspond to different parts of the experimental work done.

Thus, **chapter two** regards the study of *S. epidermidis* adhesion to two commonly used biomaterials, acrylic and silicone, and the influence of surface properties on the adhesion process. For this purpose, hydrophobicity and surface tension components of substrata and cells were determined through the contact angle measurement method and surface roughness of substrata was assessed by atomic force microscopy (AFM).

Chapter three describes the study developed towards the diminishment of *S. epidermidis* adhesion by heparin and gentian violet pre-conditioning of the acrylic and silicone surfaces. The influence of pre-incubation time in the reduction of bacterial adhesion is also studied. The goal was to find a simple method to minimize *S. epidermidis* colonization of indwelling medical devices and, in this way, associated infections.

In **chapter four**, *S. epidermidis* adhesion to several polycarbonate modified surfaces is also studied. The effect of gold coating on staphylococcal adhesion was assessed, as well as the coverage with different self assembled monolayers as an alternative method in the reduction of bacterial adhesion to biomedical surfaces.

Chapter five concerns biofilms studies. The differences between cells in suspension and in the sessile form are investigated in terms of glucose uptake ability and metabolic activity. Biofilm composition, concerning number of cells and extracellular polymers is presented and their influence in biofilm characteristics also analysed.

In **chapter six** a descriptive study of proteins pattern of the cell wall and of the extracellular matrix of *S. epidermidis* biofilms is presented in order to relate these protein profiles with *S. epidermidis* adhesion and biofilm forming ability.

Chapter seven regards the major conclusions and suggestions for future work.

CHAPTER 2 - Influence of surface characteristics on the adhesion of *Staphylococcus epidermidis* to acrylic and silicone

Initial adhesion of *Staphylococcus epidermidis* to the biomaterial surface is thought to be a key step in the colonization of indwelling medical devices. In this chapter, the study of the ability of eight *S. epidermidis* strains to adhere to acrylic and silicone, two polymers normally used in medical devices manufacture is described. Furthermore, it is tried to correlate that with the surface properties of substrata and cells. Therefore, hydrophobicity and surface tension components were determined through contact angle measurements and surface roughness of substrata was assessed by atomic force microscopy (AFM).

2.1 Introduction

The main virulence factor associated with *Staphylococcus epidermidis* ability to cause infections is dependant on its adherence to medical devices and formation of a biofilm (Vuong and Otto, 2002). Therefore, microbial adhesion to solid surfaces is a fundamental prerequisite for the formation of biofilms. Bacterial adhesion to an abiotic surface is a complex process, affected by numerous aspects such as surface properties of bacteria and materials, involving physicochemical, protein and polysaccharide factors (Azeredo and Oliveira, 2000; Bruinsma *et al.*, 2001; Busscher and Weerkamp, 1987; Busscher *et al.*, 1997; Cunliffe *et al.*, 1999; Dunne, 2002; Gross *et al.*, 2001) and also environmental factors (An and Friedman, 1998). Therefore, in order to adhesion occur, cell and substratum surfaces must have matching properties, hydrodynamic conditions must be favourable, the aqueous environment must be adequate and time should be sufficient (Doyle, 1991).

The better understanding of all these features is of extreme importance for the development of effective adhesion control mechanisms that will ultimately prevent biofilm formation and thus, the infection of medical devices.

2.1.1 Adhesion process

In its most fundamental form, bacterial adhesion can be divided into two main phases: the primary or docking phase and the secondary or locking phase (An *et al.*, 2000b; Marshall, 1985; Pearce *et al.*, 1995). In some cases, an additional surface conditioning step is added to this process (Boland *et al.*, 2000; Gristina, 1987). Conditioning occurs when the biomaterial is inserted in the human body and interacts with the surrounding environment. As a result, the native surface is modified by the adsorption of host-derived matrix proteins such as albumin, fibronectin or fibrinogen (Vuong and Otto, 2002). When this process occurs, surface properties

become permanently altered, in a way that the affinity of an organism for a conditioned surface can be quite different than for a naked one.

Primary adhesion is a reversible stage that occurs when the bacterial cells first meet the surface, and that is ruled by a number of physiochemical variables that define the interaction between the bacterial cell surface and the conditioned surface of interest (An *et al.*, 2000b; Marshall *et al.*, 1971; Oliveira *et al.*, 2003). First, the organism approaches the surface, either randomly or directed by chemotaxis and motility and, when it reaches a critical proximity to the surface (usually <1 nm), the final determination of adhesion depends on the net sum of attractive or repulsive forces generated between the two surfaces (An *et al.*, 2000b; Carpentier and Cerf, 1993). These forces include electrostatic (van Loosdrecht *et al.*, 1990), hydrophobic (Busscher *et al.*, 1990; Oliveira *et al.*, 2001) interactions, Lifshitz-van der Waals forces (van Oss, 1995a; van Oss, 1994) and acid-base interactions (Azeredo and Oliveira, 2003; van Oss, 1995b). Electrostatic interactions are likely to favour repulsion, because most bacteria and inert surfaces are negatively charged (Carpentier and Cerf, 1993; Jucker *et al.*, 1996).

The physical interactions are further classified as long-range and short-range interactions. The non-specific long-range forces include Lifshitz-van der Waals forces, electrostatic forces, acid-base interactions, and Brownian motion forces (van Oss and Giese, 1995; van Oss, 1994) and are a function of the distance and free energy (distances >50 nm) (Mayer *et al.*, 1999). Short-range specific interactions operate in highly localized regions of the interacting surfaces (distances <5nm) and include hydrogen bonding, ionic and dipole interactions (Mayer *et al.*, 1999; van Oss, 1994).

Bacteria are transported to the surface by the so-called long-range interactions and upon closer contact, short range interactions become more important. In accordance, as reported by Marshall (1985), at separation distances greater than approximately 50 nm, van der Waals forces are the only ones operative since this distance is considered to be too large for the opposing surfaces to recognize specific surface components. However, at separation distances between approximately 10 and 20 nm, only secondary minimum interactions occur as a result of

van der Waals and electrical double layer forces and adhesion is probably reversible. At separation distances of less than about 1.5 nm, a great variety of specific or nonspecific short-range forces can occur, leading to irreversible adhesion. Cell and substratum surfaces hydrophobicity can here play a major role in removing water films from between the interacting surfaces enabling short-range interactions to occur. Roughness and chemical composition of the biomaterial surface itself are also important parameters involved in this initial stage of adhesion (An and Friedman, 1998).

In the case of bacterial adhesion to a previously conditioned surface, it should be pointed out that, depending on the type of molecules of the conditioning film, the hydrophobicity of the surface can be dramatically changed (An *et al.*, 2000b; Boland *et al.*, 2000).

The durability of primary adhesion stage depends on the sum total of the variables described above, but surface chemistry drives the equilibrium in favour of adhesion by predicting that organic substances in solution will concentrate near a surface and that microorganisms tend to congregate in nutrient-rich environs (Carpentier and Cerf, 1993; Costerton *et al.*, 1995).

The second stage of adhesion is the anchoring or locking phase and employs molecularly mediated binding between specific adhesins and the surface (An *et al.*, 2000b). All bacteria produce multiple adhesins, and some are regulated at the transcriptional level, permitting organisms to switch from sessile to planktonic forms under different environmental influences (An *et al.*, 2000b; Ziebuhr *et al.*, 1999). At this point, loosely bound organisms consolidate the adhesion process by producing exopolysaccharides that complex with surface materials and/or receptor-specific ligands located on pili, fimbriae and fibrillae, or both. Such is the case with *S. epidermidis*, which produces the polysaccharide intercellular adhesin (PIA) that is essential for cell-to-cell adhesion and subsequent biofilm formation (Heilmann *et al.*, 1996; Mack *et al.*, 1996b; Mack *et al.*, 1994). At the conclusion of the second stage, adhesion becomes irreversible in the absence of physical or chemical intervention, and the organism is attached firmly to the surface.

As hydrophobicity and roughness are the parameters evaluated in this work, a more detailed discussion of their effect in initial bacterial adhesion will follow.

2.1.1.1 Hydrophobicity

Surface hydrophobicity has been described as one of the most important properties involved in the adhesion phenomenon (Busscher and Weerkamp, 1987; Millsap *et al.*, 1996; Wiencek and Fletcher, 1997). According to van Oss and Giese (1995), in biological systems, hydrophobic interactions are normally the strongest of the long-range non-covalent interactions and can be defined as the attraction among apolar, or slightly polar, cells or other molecules themselves, when immersed in an aqueous solution. Their exclusive driving force is the hydrogen bonding (AB forces or Lewis acid-base) energy of cohesion between the surrounding water molecules (van Oss, 1997). In aqueous medium, adhesion is favoured between hydrophobic surfaces, which can enter in closer contact by squeezing the water layer between them (Oliveira *et al.*, 2003).

Bacterial cells have developed several ways to use the hydrophobic effect in order to adhere to substrata. A microorganism may adhere to a substratum via the hydrophobic effect if the associating sites possess sufficiently high densities of apolar areas (Doyle, 2000). In staphylococcal species, for instance, these hydrophobic areas are provided by proteins that are covalently bound to the cell wall (Meyer and Gattermann, 1994). Besides, some studies correlating hydrophobic qualities of clinical isolates with its virulence have been presented (Christensen *et al.*, 1985; Ness-Greenstein *et al.*, 1995; Reifsteck *et al.*, 1987).

There are several methods to assess cell surface hydrophobicity such as: microbial adhesion to hydrocarbons - MATH, initially called BATH (bacterial adherence to hydrocarbons) and proposed by Rosenberg (1984); salt aggregation test (SAT) (Lindhal *et al.*, 1981); hydrophobic interaction chromatography (HIC) (Hjerten *et al.*, 1974); hydrophobic microsphere assay - HMA (Hazen and Hazen, 1987) and contact angles measurement (van der Mei *et al.*,

1998). However, according to Doyle (2000), the most reliable method is probably by contact angle measurements (Figure 2.1), once it gives an average degree of hydrophobicity, not taking into account cell cycle variations or individual cell contributions. According to this method, hydrophobicity is expressed in terms of the contact angle formed by a sessile drop of water on a lawn of microbial cells, obtained by collecting cells onto a cellulose filter membrane (Busscher *et al.*, 1984). The hydration of the surface is the major problem of this method, requiring a careful preparation of a smooth and dry cell lawn, in a way that the geometry of the droplet will not be distorted.

All the methods have some intrinsic disadvantages, especially those based on the adhesion of cells to some liquid or solid material because they are dependant on factors as temperature, time, pH, ionic strength and relative concentration of interacting species, which can all combine to influence the adhesive event (Ofek and Doyle, 1994). In the case of solid substrata, the most common methods are contact angle measurements for flat surfaces and thin-layer wicking for particulate materials (Chibowski and Holysz, 1992; Teixeira *et al.*, 1998; van Oss, 1991).

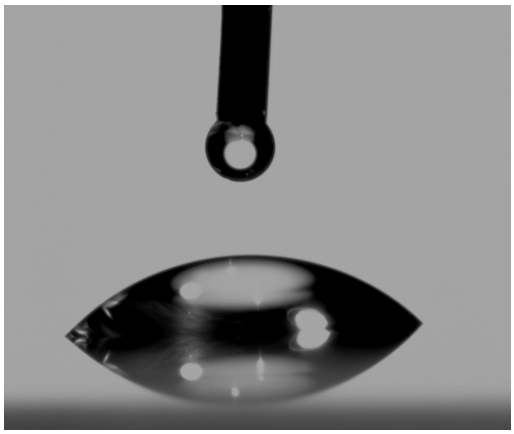


Figure 2.1 Contact angle method. A drop of a reference liquid (e.g., water) is placed in a smooth lawn of dried cells. The contact angle is determined by a series of photographs of the droplet as it equilibrates. If the cells are hydrophilic, the droplet dissipates very quickly, while for hydrophobic cells the droplet will disappear in a slowly way. Adapted from: http://en.wikipedia.org/wiki/Surface_energy.

With the techniques described above it is only possible to assess hydrophobicity in a qualitative way but according to van Oss (1995b), it is possible to determine the absolute degree of hydrophobicity of any given substance (i) compared with water (w), which can be precisely expressed in S.I. units. Thus, using the contact angle measurement method and the approach of van Oss and co-workers (van Oss and Giese, 1995; van Oss *et al.*, 1987; van Oss *et al.*, 1988; van Oss *et al.*, 1989; van Oss, 1995b), it is possible to evaluate surface characteristics such as the surface free energy and hydrophobicity. According to this approach, the absolute degree of hydrophobicity of a given material (i) can be defined in terms of the variation of the free energy of interaction (ΔG) between two moieties of that material immersed in water (w), i.e., ΔG_{iwi}. The free energy comprises a polar (AB) and an apolar (LW) component ($\Delta G_{iwi} = \Delta G_{iwi}^{LW} + \Delta G_{iwi}^{AB}$). ΔG_{iwi} can be calculated through the surface tension components of the interacting entities, according to:

$$\Delta G_{iwi} = -2\left(\sqrt{\gamma_i^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_i^+\gamma_w^-} + \sqrt{\gamma_i^-\gamma_w^+} - \sqrt{\gamma_i^+\gamma_i^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad \textbf{(Eq. 2.1)}$$

where γ^{LW} represents the Lifshitz-van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}), with $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$. According to van Oss and Giese (1995), the boundary between hydrophobicity and hydrophilicity occurs when the difference between the apolar attraction and the polar repulsion between molecules or particles of material immersed in water is equal to the cohesive polar attraction between the water molecules. Thus, when ΔG_{iwi} has a negative value, the free energy of interaction between molecules is attractive and there is less affinity for water than among them and (i) (microbial cell or material surface) is hydrophobic. In an opposite way, (i) is hydrophilic when ΔG_{iwi} is positive.

The surface tension parameters of a solid material are obtained by measuring the contact angles of three pure liquids (one apolar and two polar), with well known surface tension

components (Table 2.1), followed by the simultaneous resolution of three forms of the following Young-Good-Girifalco-Fowkes equation (van Oss *et al.*, 1987):

$$(1+\cos\theta)\gamma_l^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^{+}\gamma_l^{-}} + \sqrt{\gamma_s^{-}\gamma_l^{+}}\right) \textbf{(Eq. 2.2)}$$

where θ is the angle between a liquid with known properties and the solid surface of interest and γ^{TOT} (surface free energy of the solid surface) is the sum of the apolar Lifshitz-van der Waals (γ^{LW}) and polar Lewis acid-base (γ^{AB}) interactions, i.e., $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$. Subscripts s and l mean solid and liquid, respectively. The apolar interactions are mainly due to London dispersion interactions, despite the possible involvement of induction (Debye) and orientation (Keesom) interactions (van Oss *et al.*, 1988). The surface tension of the probe liquids should be higher than that of the solid material in order to avoid the spreading of the liquid on the surface and thus it was established that this parameter should be higher than 40 mJ/m^2 (van Oss *et al.*, 1987). Between the polar components, water and formamide are the most commonly used liquids and α-bromonaftalene and di-iodomethane are the apolar ones.

Table 2.1 Surface tension parameters (mJ/m^2) of liquids often employed in contact angle measurements. Adapted from Bos *et al.*, 1999.

Liquid	γ^{TOT}	γ^{LW}	γ^{+}	γ^{-}
Water	72.8	21.8	25.5	25.5
Glycerol	64.0	34.0	3.9	57.4
Ethylene Glycol	48.0	29.0	1.9	47.0
Formamide	58.0	39.0	2.3	39.6
Dimethylsulfoxide	44.0	36.0	0.5	32.0
α-Bromonaftalene	44.4	44.4	0	0
Di-iodomethane	50.8	50.8	0	0

2.1.1.2 Roughness

Biomaterial surface roughness is another relevant property for the bacterial adhesion process, with the irregularities of the polymeric surfaces normally promoting bacterial adhesion and biofilm accumulation (Scheuerman *et al.*, 1998; Verran *et al.*, 1991). This is due to the increased surface area and depressions, that provide more favourable and additional sites for colonization (Katsikogianni and Missirlis, 2004), as such crevices protect bacterial cells from the shear forces (Fox *et al.*, 1990) (Figure 2.2). However, the accumulation of bacteria in such locations depends largely on their size, cell dimension and division mode (Katainen *et al.*, 2006; Messing and Oppermann, 1979).

According to Katainen *et al.* (2006), surfaces may have roughness in numerous length scales, but due to the short range of the van der Waals interaction, roughness in nanoscale ultimately determines the strength of adhesion. This is corroborated by another work (Mitik-Dineva *et al.*, 2008) where the impact of nanometer-scale roughness on bacterial adherence was tested and according to which, a reduction in the nanoscale roughness lead to a strong increase in the number of adhered bacteria. Thus, besides the already known role of surface roughness in protecting bacteria from environmental factors when crevices have a similar size to that of bacteria, it seems that roughness at a nanoscale, can as well strongly influence initial attachment of bacteria, probably by providing the presence of a greater number of contact points.

There are many different roughness parameters in use, such as *Ra* (arithmetic average height), *Rmax* (maximum height of the profile) or *Rq* (root mean square roughness), among others. *Ra* is the most universally used roughness parameter for generally quality control and it is usually defined as the average absolute deviation of the roughness irregularities from the mean line over one sampling length. This parameter has the advantage of being easy to define and to measure, providing a good general description of height variations. *Rmax* is considered the vertical distance between the highest peak and the lowest valley for each sampling length of the profile. *Rq* it also constitutes a common roughness parameter used and it represents the

standard deviation of the distribution of surface heights, allowing the surface roughness description by statistical methods (Gadelmawala *et al.*, 2002).

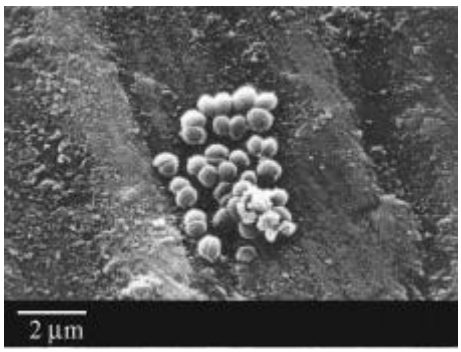


Figure 2.2 Adhesion of streptococci consortia to rough titanium surface with streptococci preferentially attached on the valleys. Adapted from: Díaz *et al.*, 2007.

According to some authors (Boyd *et al.*, 2002; Taylor *et al.*, 1998), a linear relation of bacterial adhesion with surface roughness is not always verified. A small increase in roughness can lead to a significant increase in bacterial adhesion, while a larger increase in roughness can have no significant effect on cellular attachment. Thus, there is some disagreement in the literature as to whether there may be a threshold below which less roughness does not prevent bacterial adhesion or an upper threshold where increased roughness does not promote more attachment (Barnes *et al.*, 1999; Tide *et al.*, 1999). For instance, in the work of Tang *et al.* (2008) surface roughness had a significant effect on bacterial adhesion and colonization only when the root-mean-square roughness (*Rq*) was higher than 200 nm. However, in some cases, the parameters used to evaluate the roughness degree of a material, such as *Rq*, do not correlate well with the levels of bacterial adhesion, even when there is a qualitative evidence of it, i.e., even when there is direct observation of bacteria attached in the surface crevices (Flint *et al.*, 2000). Thus, due to the limitation in terms of quantitative interpretation of the effect of roughness, the design of a material with an anti-adhesive texture for bacteria remains difficult to achieve.

Litzler *et al.* (2007) developed an *in vitro* model and tried to analyse the effect of surface free energy and roughness of different heart valves made of pyrolytic carbon, a material usually incorporated in cardiac and vascular prostheses, in *S. epidermidis* (among other strains) biofilm formation. Their results suggest that *S. epidermidis* adhesion is directly dependant on valves roughness, with the rougher valves exhibiting the greater ability to be colonized. In another study (Lopes *et al.*, 2005), the adhesion ability of *Desulfovibrio desulfuricans* to stainless steel, metallic nickel and polymethylmetacrylate (PMMA) was evaluated and roughness was pointed as one of the factors responsible for the higher number of cells adhered to stainless steel in comparison to PMMA. Also, according to Teughels *et al.* (2006), biofilm formation is facilitated by implant surfaces with a higher surface roughness/surface free energy. Nevertheless, in a recent work (Lichter *et al.*, 2008), polyelectrolyte multilayer (PEM) thin films comprised of poly(allylamine) hydrochloride (PAH) and poly(acrylic acid) (PAA), assembled over a range of conditions, were employed to explore the physicochemical and mechanical characteristics of material surfaces controlling adhesion of *S. epidermidis* bacteria and subsequent colony growth. According to their results, besides interaction energy and charge density of the materials, also roughness had no significant effect on adhesion of viable *S. epidermidis*. Some other authors have also seen no correlation between bacterial adhesion and roughness (Lerebour *et al.*, 2004; Pereni *et al.*, 2006).

Therefore, despite all studies, some of them contradictory, to particular applications, fundamental studies that will lead to a better understanding of surface roughness influence on nanoscale adhesion forces between bacteria and substrata are still missing.

2.1.2 Aims

The aim of the present work was to study the ability of eight strains of *S. epidermidis* to adhere to acrylic and to silicone, two materials commonly used in the manufacture of medical

devices, in relation to the surface properties, such as hydrophobicity and roughness, of these materials. Cell surface properties were also evaluated.

2.2 Materials and Methods

2.2.1 Bacterial strains

Eight *S. epidermidis* strains were studied in this work. *S. epidermidis* 9142 is a known producer of the surface polysaccharide intercellular adhesin (PIA), which was identified as one of the main responsible factors for biofilm formation (Mack *et al.*, 1996b). The strain *S. epidermidis* 9142-M10 is an isogenic mutant with a transposon inserted in the *ica* locus, that encodes the proteins involved in PIA production and thus does not form biofilm. The PIA-positive *S. epidermidis* 1457 was isolated from an infected central venous catheter, while *S. epidermidis* 1457-M10 is a PIA-negative isogenic mutant of *S. epidermidis* 1457, also obtained by insertion of a transposon into the *ica* locus (Rupp *et al.*, 1999b). *S. epidermidis* IE186, *S. epidermidis* IE214 and *S. epidermidis* IE75 were previously isolated from blood of patients with infective endocarditis, while *S. epidermidis* LE7 was isolated from the skin of a healthy individual. All strains were kindly provided by Dr. G. B. Pier, Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston.

2.2.2 Media and growth conditions

For all the assays, cells were firstly grown for ca. 36 h in plates of tryptic soy agar (TSA; Merck, Germany), and then for 24 h in 15 ml of tryptic soy broth (TSB, Merck), at 37 °C under a constant agitation of 120 rpm (SI50; Stuart Scientific, Redhill, UK). TSA and TSB media were prepared according to the manufacturer's instructions. After this period, 50 µl of each

suspension were transferred into 30 ml of fresh TSB and incubated for 18 h, under the same conditions. Then, the cells were centrifuged (Sigma 3-16K, Germany) for 5 min at 10 500 g and 4 °C, washed twice with a saline solution [0.9% NaCl (w/v) (Merck) in distilled water] and sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) during 10 s, with an amplitude of 22% (previously optimized to avoid cell disruption). The cellular suspension was adjusted to a final concentration of approximately 1 x 10^9 cells/ml, determined by optical density at 640 nm, prior to usage in the adhesion assays.

2.2.3 Substrata preparation

2 cm x 2 cm squares of commercial acrylic, specifically Poly(methyl methacrylate) (PMMA) (Repsol, Brønderslev, Denmark) and silicone (Leewood Elastomer AB, Sweden), both with 2 mm in thickness, were used as substrata in the adhesion assays. Prior to use, the coupons were washed several times with sterile distilled water and let to soak overnight. Next, they were transferred to a new container with sterile distilled water and washed for 5 min under agitation, followed by a 30 min immersion period in a 70% ethanol/sterile distilled water solution. Finally, the coupons were aseptically and individually washed with ultra-pure sterile water and let to dry overnight at 60 °C.

2.2.4 Adhesion assays

Adhesion assays were performed as previously described (Cerca *et al.*, 2005b). Briefly, the acrylic and silicone squares were placed in 6-well tissue-culture plates containing 4 ml of bacterial suspension (1x10^9 cells/ml) in saline solution. Initial adhesion to each substrate was allowed to occur for 2 h at 37 °C, in a shaker rotating at 120 rpm. Negative controls were obtained by placing the coupons in a saline solution without bacterial cells. Each coupon was

then carefully removed and washed by immersion, in order to remove loosely attached cells. This procedure was gently undertaken and involved their transference to a glass beaker containing 50 ml of distilled water, where they were kept for about 10 s. Afterwards, a new transfer was made to an additional beaker with 50 ml of distilled water, followed by a third transfer 10 s later. These washing steps were carefully performed in order to remove loosely attached cells (Cerca *et al.*, 2004). The coupons were then let to dry at 37 °C for about 1 h. All experiments were done in triplicate and repeated in four independent assays.

2.2.5 Total cell counts of adhered bacteria

The dried coupons were stained with a 4'-6-diamidino-2-phenylindole (DAPI; Sigma, USA) solution (0.1 g/l) during 30 min. Subsequently, each coupon was rinsed with distilled water in order to remove excess stain and let to air-dry in the dark for 30 min. Adhered cells were visualized under an epifluorescence microscope (Carl Zeiss, Germany) with a filter sensitive to DAPI fluorescence and coupled with a 3CCD video camera. For each coupon, at least 20 images, with an 820 x 560 resolution and 1000x magnification, were taken. Enumeration of adhered cells was performed with automated software (SigmaScan Pro 5, SPSS Inc., Chicago) and the results presented as number of adhered cells/cm^2.

2.2.6 Scanning Electron Microscopy (SEM)

The coupons with adhered bacteria were dehydrated by a 15 min immersion in increasing ethanol concentration solutions: 10, 25, 40, 50, 60, 70, 80, 90 and 100% (v/v), having then been placed in a sealed desiccator. Samples were then mounted on aluminium stubs with carbon tape, sputter-coated with gold and observed with a Leica Cambridge S-360 scanning electron microscope (Leo, Cambridge, UK). In order to assess the extent of bacterial

adhesion in each sample, three fields were used for image analysis. All photographs were taken using a magnification of 3000x.

2.2.7 Substrata and bacteria hydrophobicity

Hydrophobicity parameters of substrata and bacteria surface were determined through the sessile drop contact angle technique (Busscher *et al.*, 1984), using an automated contact angle measurement apparatus (OCA 15 Plus; Dataphysics, Germany). Cleaned and dried substratum surfaces were used for determining the hydrophobicity parameters of acrylic and silicone. In the particular case of bacteria, the measurements were performed on bacterial layers deposited on membrane filters (Busscher *et al.*, 1984). Briefly, a 30 ml suspension of *S. epidermidis* cells, adjusted to a concentration of approximately 1 x 10^9 cells/ml in saline solution, was deposited onto a 0.45 μm cellulose filter (Pall-Life Sciences, USA), previously wetted with 10 ml of distilled water. To standardize the moisture content, the filters with the resultant lawn of cells deposited were then let to dry onto Petri dishes containing 1% (w/v) agar (Merck) and 10% (v/v) glycerol (Sigma), for at least 3.5 h. All measurements (at least 25 determinations for each material and bacterial strain) were performed at room temperature and water, formamide and α-bromonaphtalene, with known surface tension components (Janczuk *et al.*, 1993), were used as reference liquids for standardized contact angles measurements.

Contact angle measurements allowed the calculation of substrata and bacteria hydrophobicity parameters, using the van Oss approach (equation 2.1) (van Oss and Giese, 1995; van Oss *et al.*, 1987; van Oss *et al.*, 1988; van Oss *et al.*, 1989; van Oss, 1995b).

2.2.8 Atomic Force Microscopy (AFM)

Acrylic and silicone surfaces topography was assessed by atomic force microscopy (AFM), using a PicoPlus scanning probe microscope from Molecular Imaging (USA). Surface

imaging was performed in Tapping™ mode and the samples were analysed in air at room temperature. The acrylic surfaces were analysed using a silicon (Si) tip with a Spring Constant ≅ 42 N/m, while for silicone surfaces the Si tip had a Spring Constant ≅ 2.8 N/m. The roughness measurements were performed under a scan range of 2.5 x 2.5 μm, using the SPIP™ version 4.2.2.0 software. Measurements were made in three randomly chosen areas, in all samples.

2.2.9 Statistical analysis

Results from all the assays were compared using one-way analysis of variance (ANOVA) by applying Levene's test of homogeneity of variances and the Tukey multiple comparisons test, using SPSS software (Statistical Package for the Social Sciences Inc., Chicago). All tests were performed with a confidence level of 95%.

2.3 Results

2.3.1 Adhesion to acrylic and silicone

Results of the initial adhesion to acrylic and silicone surfaces are presented in Figure 2.3. As it can be seen, almost all *S. epidermidis* strains adhered at a significantly ($p<0.05$) higher extent to the silicone substrate than to acrylic. The only exceptions were observed for strains 1457-M10, IE75 and LE7, which also adhered more to silicone than to acrylic but in a non-significant way ($p>0.05$). In fact, the extent of adhesion of *S. epidermidis* 9142 to silicone was approximately 2.5 times greater than to acrylic. For strains 9142-M10, IE186 and IE214 this difference ranged between 1.8 and 2.0 times. Concerning acrylic, strains IE214 and IE186 were the ones that most extensively, and significantly ($p<0.05$) adhered to the coupons. In opposition,

strains IE75 and LE7 showed the lowest levels of initial binding to this material, being markedly different from all the other strains ($p<0.05$). Similarly, they also adhered least to silicone coupons ($p<0.05$). *S. epidermidis* IE214 and *S. epidermidis* IE186 were the strains showing the highest number of cells adhered ($p<0.05$) to this substrate, likewise to acrylic.

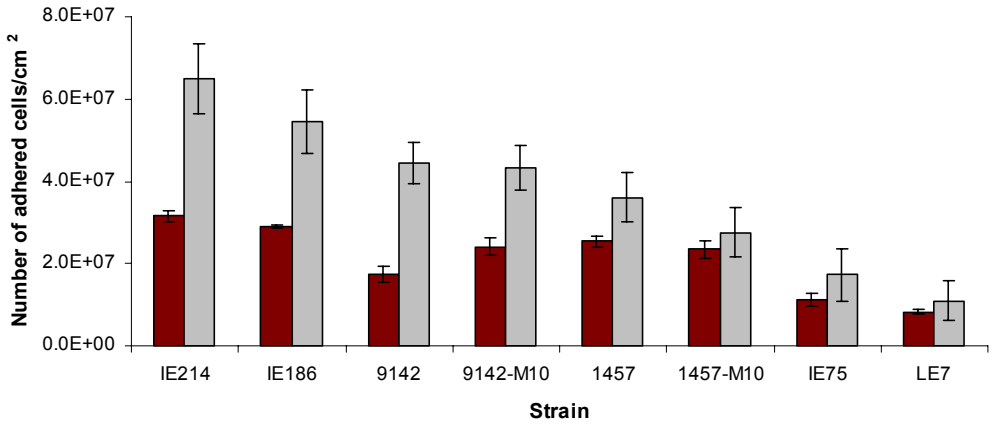


Figure 2.3 Number of adhered cells per cm^2 onto acrylic (■) and silicone (■) coupons, after a 2 h period of contact, for *S. epidermidis* strains IE214, IE186, 9142, 9142-M10, 1457, 1457-M10, IE75 and LE7.

Scanning electron microscope (SEM) images of bacteria adhered to acrylic (a) and silicone (b) squares are presented in Figure 2.4. The images reveal grape-like clusters of variable dimensions. It is also visible the higher extent of adhesion to silicone than to acrylic, especially for strains IE214, IE186, 9142, 9142-M10 and 1457. In addition, it can be seen, both for acrylic and for silicone, how strain IE214 cells [Figure 2.4 (a) - A and Figure 2.4 (b) - A] grew highly aggregated, quite differently from the mode of growth of the remaining strains. In fact, this strain formed flocculent suspensions in liquid medium. In Figure 2.4 (b) – G it must be noted (arrow) how cells adhered to the silicone along the depression on its surface.

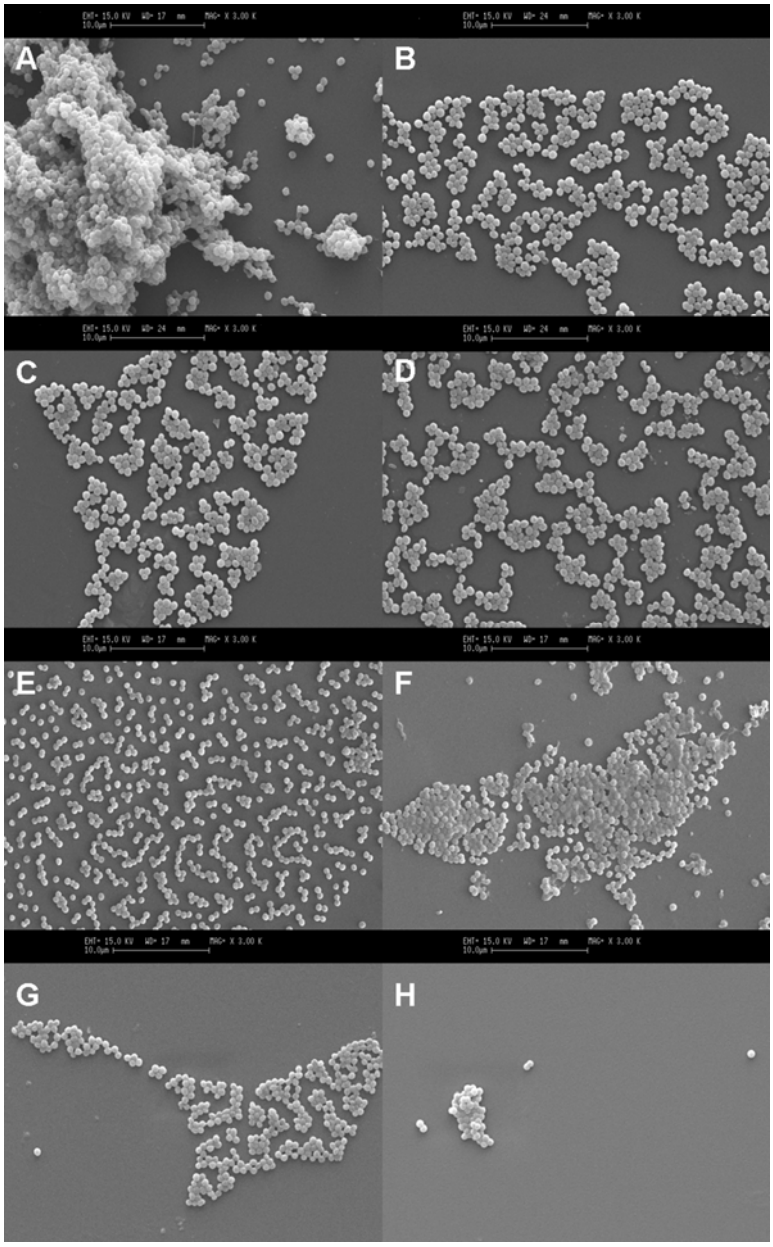


Figure 2.4 (a) SEM photomicrographs of *S. epidermidis* adhered to the acrylic surface. Strains: A – IE214; B – IE186; C – 9142; D – 9142-M10; E – 1457; F – 1457-M10; G – IE75; H – LE7. Magnification ×3000, bar = 10 μm.

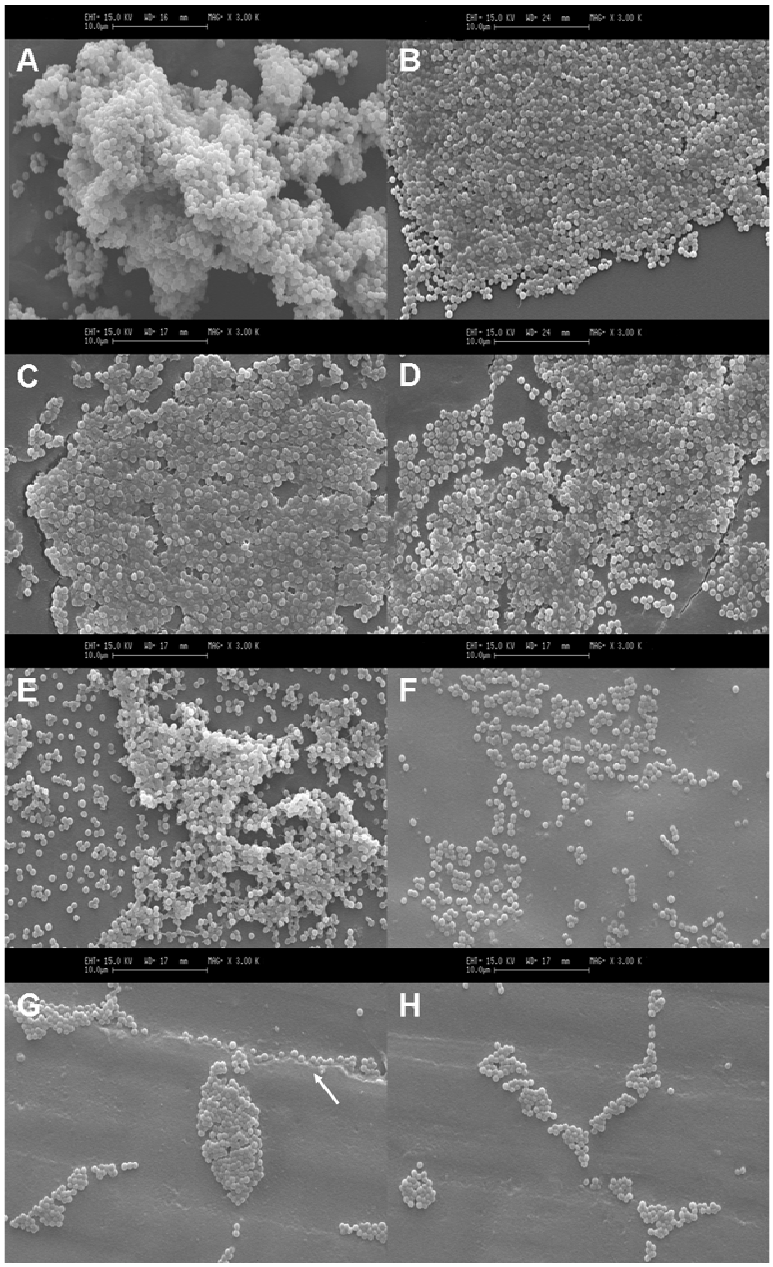


Figure 2.4 (b) SEM photomicrographs of *S. epidermidis* adhered to the silicone surface. Strains: A – IE214; B – IE186; C – 9142; D – 9142-M10; E – 1457; F – 1457-M10; G – IE75; H – LE7. The arrow shows bacterial cells adhered along a depression on silicone's surface. Magnification ×3000, bar = 10 μm.

2.3.2 Substrata and bacteria hydrophobicity

Hydrophobicity of substrata and bacteria was evaluated through contact angle measurements, using the van Oss approach (van Oss and Giese, 1995; van Oss, 1997). Contact angles, surface tension parameters and hydrophobicity of acrylic and silicone are presented in Table 2.2. Water contact angles can be used as a qualitative indication of the surface material hydrophobicity, with values higher than 65° indicating more hydrophobic surfaces (Vogler, 1998). As it can be seen, the water contact angles obtained for both surfaces are high, a fact that is indicative of their hydrophobicity. The values of ΔG_{iwi} also showed that both materials are hydrophobic (ΔG_{iwi}<0), with silicone holding a more hydrophobic character. From Table 2.2 it can also be seen that both acrylic and silicone surfaces are predominantly electron donors (higher values of γ^-), with low electron acceptor parameters (γ^+). In fact, acrylic does not have an electron acceptor parameter (γ^+=0) but is only electron-donor (γ^-).

Table 2.2 Water (θ_W), formamide (θ_F) and α-bromonaphtalene ($\theta_{\alpha\text{-}B}$) contact angles (in degrees), surface tension components and hydrophobicity (in mJ/m²) of the acrylic and silicone coupons surface.

Substratum	Contact angle ± SD (º)			Surface tension components (mJ/m²)			ΔG_{iwi} (mJ/m²)
	θ_W	θ_F	$\theta_{\alpha\text{-}B}$	γ^{LW}	γ^+	γ^-	
Acrylic	85.3±2.2	64.1±1.2	24.5±1.2	40.5	0.0	4.5	-62.5
Silicone	114.5±2.3	104.3 ±2.4	81.4±3.5	14.7	0.4	1.7	-67.1

SD – standard deviation; γ^{LW} – apolar Lifshitz-van der Waals surface free energy component; γ^+ - electron acceptor surface free energy component; γ^- - electron donor surface free energy component; ΔG_{iwi} – degree of hydrophobicity.

Cell surface hydrophobicity parameters of *S. epidermidis* strains, as well as the contact angles obtained using the three liquids tested, are presented in Table 2.3.

Table 2.3 Water (θ_W), formamide (θ_F) and α-bromonaphtalene ($\theta_{\alpha\text{-}B}$) contact angles (in degrees), surface tension components and hydrophobicity (in mJ/m²) of the surface of *S. epidermidis* strains.

S. epidermidis **strain**	**Contact angle ± SD (º)**			**Surface tension components (mJ/m²)**			ΔG_{iwi} **(mJ/m²)**
	θ_W	θ_F	$\theta_{\alpha\text{-}B}$	γ^{LW}	γ^+	γ^-	
IE214	22.3±3.5	21±1.2	59±2.1	20.6	7.9	56.7	20.3
IE186	21.6±1.6	29.9±4.0	54.5±2.0	27.7	2.5	55.5	32.5
9142	25.6±0.9	25.4±2.6	57.0±1.4	26.5	4.0	48.4	22.8
9142-M10	21.8±1.2	19.0±1.9	54.7±1.0	27.6	4.3	48.4	22.0
1457	31.8±1.0	31.4±2.5	53.2±1.5	28.4	2.7	45.9	22.8
1457-M10	24.7±1.8	17.3±0.7	49.6±0.9	30.1	3.8	45.3	19.6
IE75	27.1±1.0	26.5±1.6	50.4±1.3	29.8	2.7	47.9	24.2
LE7	23.7±0.7	9.4±0.6	52.3±1.3	28.8	5.0	43.6	16.5

SD – standard deviation; γ^{LW} – apolar Lifshitz-van der Waals surface free energy component; γ^+ - electron acceptor surface free energy component; γ^- - electron donor surface free energy component; ΔG_{iwi} – degree of hydrophobicity.

The eight strains studied showed similar values of water contact angles, lower than 65°, which is indicative of a hydrophilic surface, ranging from 21.6° (strain IE186) to 31.8° (*S. epidermidis* 1457). These values are quite similar to those obtained for formamide, also polar, with exception of strain LE7 that presented a formamide contact angle much lower than that of water. The contact angles determined by using the apolar liquid, α-bromonaphtalene, showed small variation between strains with values higher than 49.6° (*S. epidermidis* 1457-M10). Also, all strains showed positive values of ΔG_{iwi} and so, can be considered hydrophilic. In what concerns surface tension components, all strains predominantly showed electron donation, with higher values of electron donor parameters (γ^-) comparing to the low values of the electron acceptor parameters (γ^+). Strain IE214 showed the highest values of electron acceptor and electron donor parameters of the acid-base component of the surface tension, while strains IE186 and LE7 revealed the lowest values of electron acceptor and electron donor parameters, respectively.

2.3.3 Substrata roughness

The surface topography of acrylic and silicone was analyzed by AFM in tapping mode (Figure 2.5). Silicone surface displays higher roughness with an average value (*Ra*) of 4.237 nm and a maximum (*Rmax*) of 44.367 nm, in opposition to *Ra* = 0.789 nm and *Rmax* = 15.683 nm for acrylic surface.

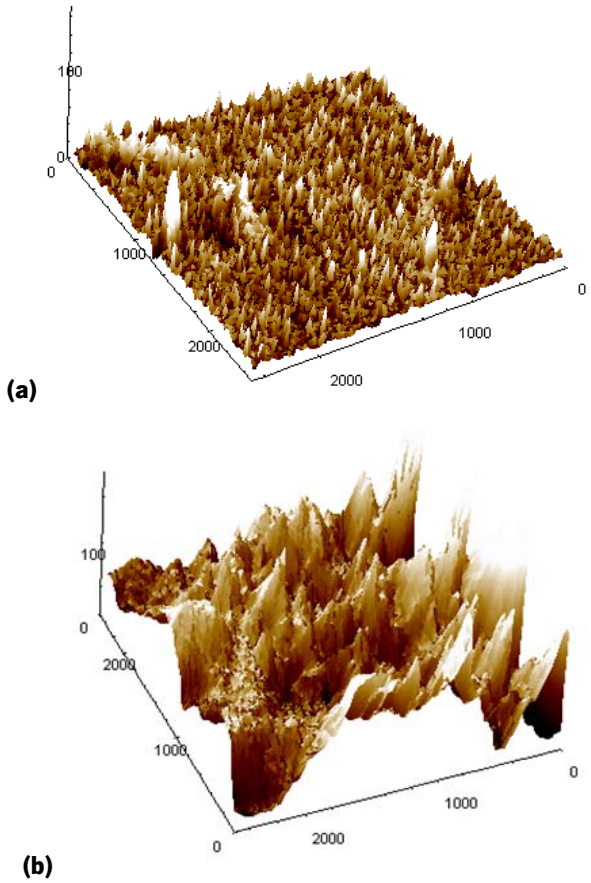


Figure 2.5 AFM images of acrylic **(a)** and silicone **(b)** surfaces with a scan range of 2.5 x 2.5 µm (air Tapping™ mode). Axis x and y – nm; axis z – Å.

2.4 Discussion

S. epidermidis is strongly associated with infections related to implants and medical devices such as joint prostheses, prosthetic heart valves, vascular catheters and contact lenses (Geesey, 2001; Moreillon and Que, 2004; von Eiff *et al.*, 2002). Given the fact that acrylic and silicone are materials normally used in the production of some of these devices, it is of major importance to study the adhesion of *S. epidermidis* to these polymers. Thus, the primary intention of this study was to attempt to correlate the adhesion capability of eight *S. epidermidis* strains with the hydrophobicity parameters of cells and both materials surfaces, or alternatively with materials surface roughness.

As the present results indicated, all *S. epidermidis* strains adhered at a higher extent to silicone substrate than to acrylic (Figure 2.3). These results are in accordance with other studies that refer silicone rubber as being especially prone to being colonized by *Candida*, streptococci *Pseudomonas* species but also by staphylococci and other CNS, depending on the site of implantation (Boswald *et al.*, 1995; Gristina *et al.*, 1988; Salzman and Rubin, 1995). Taking into consideration water contact angle values (114.5±2.3º) and the hydrophobicity degree parameter, ΔG_{iwi} = -67.1 mJ/m², the silicone assayed was found to be more hydrophobic than acrylic (water contact angle = 85.3±2.2°; ΔG_{iwi} = -62.5 mJ/m²) (Table 2.2), despite this difference was more pronounced if only water contact angles values were considered. These results are in accordance with values previously obtained (Cerca *et al.*, 2005b; Oliveira *et al.*, 2001) and clearly demonstrate the importance of the material hydrophobic effect in initial adhesion, since acrylic and silicone have both a hydrophobic character. A higher surface hydrophobicity of silicone is probably responsible for the highest levels of initial binding to this substrate. This fact is corroborated by the work of Oliveira *et al.* (2001) where the attachment of *S. epidermidis* to four polymeric materials (including silicone), commonly used in indwelling medical devices, was assayed. All materials were considered hydrophobic (ΔG_{iwi}<0) and an increase in the degree of

hydrophobicity was linearly correlated with the number of attached cells. A point also to be noted is that acrylic stands solely as an electron-donor (γ^-). Given the fact that a microorganism may adhere to a substratum via the hydrophobic effect, i.e., if hydrophobic areas are available for interactions with hydrophobic sites on substrata (Doyle, 2000), the lower densities of apolar areas in acrylic ($\gamma^+ = 0$) help to justify the preferential adhesion of *S. epidermidis* cells to a more hydrophobic material, such as silicone.

Regarding cell surface hydrophobicity parameters, all strains were considered to be hydrophilic (ΔG_{iwi}>0) (Table 2.3). The most hydrophilic *S. epidermidis* strain, IE186, (ΔG_{iwi} =32.5 mJ/m^2) was the second most adherent strain to both materials, while the least adherent, strain LE7, was the one with the weakest hydrophilic character. Thus, contrary to what was found for materials hydrophobicity, no correlation was found between cell surface hydrophobicity of the *S. epidermidis* strains and their ability to adhere to the two hydrophobic surfaces. This fact is corroborated by previous studies (Cerca *et al.*, 2005b; Oliveira *et al.*, 2007) and suggests that other cell surface factors can as well contribute to the initial attachment to biomaterials surfaces, such as the production of exopolysaccharides like extracellular polysaccharide adhesins and autolysins with adhesive properties like AtlE (Heilmann *et al.*, 1997) and Aae (Heilmann *et al.*, 2003). In fact, some authors attribute cell surface hydrophobicity to covalently bound cell-wall associated proteins (Hogt *et al.*, 1986; Meyer and Gaterman, 1994; Pascual *et al.*, 1986; Timmerman *et al.*, 1991). Furthermore, it was observed that all cell surfaces were predominantly electron donors (higher values of γ^-), with low electron acceptor parameters (γ^+) (Table 2.3). This polar character can be due to the presence of residual water of hydration or polar groups (van Oss, 1994).

However, the high value of the electron acceptor parameter of strain IE214 can justify its highest number of cells adhered to both materials, by increasing the interactions between the electron-donor groups of the substrata and the electron-acceptor groups of cells. These slightly higher values of surface tension parameters comparing to the other strains can also indicate that

acid-base interactions between cells of this strain are more favoured than in the other *S. epidermidis* strains studied. Therefore, when the first *S. epidermidis* IE214 cells approach the surface, they have good conditions to adhere to it but as long as more cells approximate they tend to adhere to another close cell instead of the surface itself. This has as a result the formation of prominent cell aggregates that can be seen in Figure 2.4, which were, still firmly adhered to the surface.

The polysaccharide intercellular adhesin (PIA), a polymer of *N*-acetyl glucosamine (Mack *et al.*, 1996b) synthesized by enzymes encoded by the *ica* operon (Mack, 1999), is crucial to the cell-to-cell adhesion process and biofilm accumulation (McKenney *et al.*, 1998; Ziebuhr *et al.*, 1997). According to the haemagglutination ability (Cerca *et al.*, 2005b), which reflects the level of PIA expression, *S. epidermidis* IE214 is a strong producer of PIA (haemagglutination titer of 1:16). Therefore, the high levels of PIA production by *S. epidermidis* IE214, along with its physicochemical properties, namely surface tension, aid to support the unique behaviour of *S. epidermidis* IE214, comparing to the remaining strains. These specific physicochemical properties are most probably due to IE214 being the only strain capable of producing a 148 kDa cell wall protein (AtlE) (data shown in Chapter 6), which has been described as determinant in the adhesion to unmodified polymer surfaces (Heilmann *et al.*, 1997).

In addition to hydrophobicity and surface tension parameters, the material surface roughness is another factor that has been pointed out as capable of influencing bacterial adhesion to a given material (Katsikogianni *et al.*, 2006).

According to several authors, initial bacterial attachment is directly dependant on the surface roughness of the substratum (Carlen *et al.*, 2001; Kawai *et al.*, 2000). This is probably due to the fact that rough surfaces have greater surface areas and, depressions in the roughened surfaces provide more favourable sites for colonization (Baker and Greenham, 1988). In fact, according to van Hoogmoed *et al.* (1997) there is a microorganism's preference for adherence to scratches or grooves, which could be seen in Figure 2.4 (b) - G. The AFM results obtained (Figure 2.5) showed that the average roughness is higher for silicone, the material where the levels of

bacterial adhesion were higher, than for acrylic, which is in accordance with the results obtained in the work of Litzler *et al.* (2007).

However, it is difficult to ascertain the possible effect of this parameter in cocci adhesion, since for both surfaces the roughness is at a nano-scale, meaning that there are no microcrevices in the surfaces to act as niches for the microbial cells. Nevertheless, according to Katainen *et al.* (2006), while in particles smaller than the surface features the interaction is limited to one contact between the particle and a single asperity, being the strength of adhesion determined by this only contact, particles larger than the surfaces features (which is the present case for silicone) have several contacts with the surface. This thus allows a higher level of interaction leading to a major influence on the adhesion phenomenon.

Therefore, a stable bacterial adhesion to a biomaterial requires a high degree of hydrophobicity, as well as a certain degree of roughness, between other physicochemical properties of the substratum (Oliveira *et al.*, 2003). Silicone is widely used as a biomaterial but it has the disadvantages of being more hydrophobic and rougher than acrylic, thus becoming a more prone material to *S. epidermidis* adherence.

2.5 Conclusions

Bacterial adhesion to the less hydrophobic material (acrylic) was significantly lower than to the more hydrophobic (silicone). These results showed the importance of the hydrophobic effect of the biomaterial surface in initial adhesion. The higher roughness of silicone seems also to exert some effect in bacterial adhesion. On the other hand, bacteria surface physicochemical properties seem to have less effect in their binding to substrata, highlighting the importance of other cell surface factors to the initial adhesion process. Nevertheless, *S. epidermidis* strain IE214 revealed completely distinct adherence behaviour compared to the remaining strains, probably as a consequence of its unique surface features, as displayed by its flocculence ability.

CHAPTER 3 - Reduction of *Staphylococcus epidermidis* adhesion to indwelling medical devices by heparin and gentian violet pre-conditioning

In this chapter, the work performed towards the development of an expedite method to reduce *Staphylococcus epidermidis* adhesion to acrylic and silicone, two materials commonly used in medical devices, by heparin and gentian violet surface pre-conditioning, is described. Different periods of heparin pre-conditioning were studied to evaluate the influence of pre-incubation time in the reduction of bacterial adhesion. A 2-hour period was chosen and thereafter applied in the adhesion assays with either heparin or gentian violet.

3.1 Introduction

It is well known that *Staphylococcus epidermidis* has emerged in the last years as the most frequently isolated pathogen in nosocomial sepsis and it is currently responsible for a larger number of indwelling medical devices-associated infections than any other group of microorganisms (Kogan *et al.*, 2006). Central venous catheters (CVCs) infections, for instance, are a noteworthy cause of morbidity and mortality and a main source of bacteraemia in hospitalized patients (Worthington *et al.*, 2000).

Infections caused by *S. epidermidis* are generally associated with immunocompromised; long-term hospitalized and seriously ill patients, who represent very susceptible hosts (Ziebuhr *et al.*, 2006). These nosocomial infections are chronic rather than acute, and are particularly difficult to treat. If the biofilm is disrupted, then an acute phase infection may occur as cells are released into the bloodstream. Removal of the device is often the option in a severe infection, which constitutes a complex and costly procedure (Verran and Whitehead, 2005).

Moreover, microbial biofilms have become notoriously difficult to treat with antimicrobial compounds (Folkesson *et al.*, 2008). In some extreme cases, the concentrations of antibiotics required to achieve bactericidal activity against adherent organisms can be three to four orders of magnitude higher than for planktonic bacteria, depending on the species-drug combination (Ceri *et al.*, 1999; Schierholz *et al.*, 1999). The failure of antimicrobial agents to treat biofilms has been attributed to a variety of mechanisms such as: binding of the antimicrobial agent to the extracellular matrix of the biofilm, thereby limiting its penetration; inactivation of the antimicrobial agent by enzymes trapped in the biofilm matrix; the reduced growth rate of bacteria in biofilms render them less susceptible to the antimicrobial agent; the altered micro-environment within the biofilm can reduce the activity of the agent; and altered gene expression by bacteria within the biofilm can result in a phenotype with reduced susceptibility to the antimicrobial agent (Costerton, 1999; Donlan, 2001; Reid, 1999).

Concerning antibiotic resistance in staphylococci responsible for nosocomial infections, its onset has been attributed to the positive selective pressure brought about by the widespread clinical use of broad-spectrum antibiotics (Arciola *et al.*, 2005c). Therefore, it is crucial to proceed with practices that minimize the risk of multi-resistant bacteria emergence, and reduce transmission of any microorganisms between patients (Barrett, 1999). Also, given the increased resistance of microorganisms to antibiotics, and to the host defence mechanisms, it is extremely important to find novel alternatives to the use of antibiotics of growing inefficacy.

3.1.1 Infection prevention strategies

The development of a biomaterial infection involves the adhesion of the bacteria to the biomaterial surfaces and subsequent colonization. Thus, initial adhesion of bacteria to the biomaterial surfaces is the critical event in the pathogenesis of foreign body infection (Gristina *et al.*, 1987; Gristina *et al.*, 1991). Prevention of bacterial adhesion or at least significant lowering of the number of viable bacteria seems to be the effective way to minimize foreign body infections, and therefore ways to reduce it have been thoroughly studied in the last decades (Bach, 1999; Costerton *et al.*, 1993).

Great efforts have been made to find materials that prevent adherence and surface colonization but, in practice, there is no synthetic polymer or biocompatible metal onto which *S. epidermidis* does not bind under clinical conditions (Götz and Peters, 2000; Rose *et al.*, 2005). In general, it is aimed to reduce the attractive force between bacteria and biomaterials surface by optimizing the physicochemical surface properties of the biomaterial (Gottenbos *et al.*, 2002).

Current approaches to limit bacterial colonization include surface coating with microbicidal agents (Chang and Merritt, 1992; Tiller *et al.*, 2002; Tiller *et al.*, 2001), surface impregnation with slow-releasing biocides such as gold or silver (Hetrick and Schoenfisch, 2006; Lee *et al.*, 2005; Li *et al.*, 2006; Saygun *et al.*, 2006) and antibiotics (Chang and Merritt, 1992; Kohnen *et al.*, 2003), or surface insertion of specific antimicrobial peptides and polymers

(Etienne *et al.*, 2004; Ignatova *et al.*, 2006; Rudra *et al.*, 2006; Tiller *et al.*, 2001). Hyaluronic acid-based adhesion reduction devices have been shown to reduce adhesion after surgery (Becker *et al.*, 1996; Burns *et al.*, 1995; Seeger *et al.*, 1997). The impregnation of biomedical devices with selective antiseptics has demonstrated to be effective in reducing microbial surface adhesion, thus lowering the risk of nosocomial infection in patients (Edmiston *et al.*, 2004; Gurselt *et al.*, 2002; Shirtliff *et al.*, 2002).

In the specific case of CVCs infections, several strategies for controlling biofilms have been suggested, including using topical antimicrobial ointment, minimizing the length of catheterization, using in-line filtration of intravenous fluids, using a surgically implanted cuff to the catheter or coating the inner lumen with antimicrobial agents. Freeman and Gould (1985) found that 0.05% sodium metabisulfite added with the agents delivered with a catheter to the left atrial system acted as an intravenous antiseptic and eliminated left atrial colonization and endocarditis. The same basic approach was used by Wiernikowski *et al.* (1991), except that sterile saline was used as the locking agent; the time to infection was increased twofold by use of this treatment.

In the case of urinary catheters, control strategies that have been used to inhibit biofilm formation include antimicrobial ointments and lubricants, bladder instillation or irrigation, antimicrobial agents in the collection bags, impregnating the catheter with antimicrobial agents (silver oxide), and using systemic antibiotics for prophylaxis in catheterized patients (Kaye and Hessen, 1994).

More recent alternative approaches are based on molecules able to interfere with quorum-sensing phenomena (Rasmussen and Givsko, 2006; Rice *et al.*, 2005; Waters and Bassler, 2005) or to dissolve biofilms (Banin *et al.*, 2006; Olofsson *et al.*, 2003). A number of laboratories are currently attempting to elucidate the genes that are activated or repressed during initial biofilm formation. In the future, treatments that inhibit the transcription of these genes might be able to completely inhibit biofilms (Costerton *et al.*, 2005). Also, several immunization trials with polysaccharide intercellular adhesin (PIA) have been carried out (Cerca *et al.*, 2007; Maira-Litran *et al.*, 2002; Maira-Litran *et al.*, 2004; Maira-Litran *et al.*, 2005; McKenney *et al.*,

1999) indicating that PIA might be a useful antigen for vaccination to protect against *Staphylococcus aureus* and *S. epidermidis* infection but, to date, no staphylococcal vaccine has found its way into practical application.

Therefore, despite the advancements in the antibacterial treatment field, whether in the development of new biomaterials, antimicrobial surfaces or vaccines, there are still serious difficulties in the treatment of staphylococci infections.

Given that, in this chapter the antimicrobial effect of heparin and gentian violet is studied and a closer look at these substances properties is given below.

3.1.1.1 Heparin

The binding of specific natural substances, such as heparin, to the materials surface has been one of the strategies attempted to try to reduce the extent of bacterial adhesion (Arciola *et al.*, 1994; Keogh and Eaton, 1994; Portolés *et al.*, 1993; Tenke *et al.*, 2004).

Heparin is a proteoglycan with strong anticoagulant activity (Nelson *et al.*, 2000), normally used to minimize thrombus formation (Denizli, 1999), by inhibiting the transformation of fibrinogen into fibrin and promoting low levels of platelet adhesion (Amiji and Park, 1993; Ip *et al.*, 1985; Nojiri *et al.*, 1990). The use of heparin as an antithrombogenic agent in catheters has been widespread for over 20 years allowing expanding the duration of catheter use (Randolph *et al.*, 1998). Heparin and heparin-albumin conjugates have been adsorbed ionically and covalently to several biomaterial surfaces for improvement of blood compability (Langer *et al.*, 1982).

Heparin specific structure consists of a glycosaminoglycan derived from a parent proteoglycan composed of 10-15 polysaccharide chains of 60-100 kDa linked to a 17 kDa core protein. It is an anionic linear polysaccharide consisting of two repeating disaccharide units: D-glucosamine-L-iduronic acid and D-glucosamine-L-glucuronic acid (Tsai *et al.*, 2001) (Figure 3.1).The average molecular weight of most commercial heparin preparations is about 15 kDa (Nelson *et al.*, 2000).

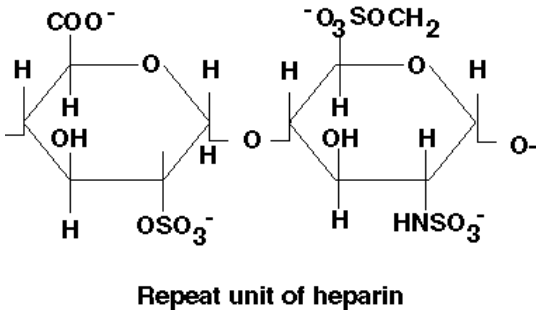


Figure 3.1 Chemical structure of the unfractionated heparin molecule, which is composed of alternating units of sulphated D-glucosamine and D-glucuronic acid. The esterified sulfuric acid component gives unfractionated heparin its acidic property and electronegative charge. Adapted from: Quader *et al.*, 1998.

Heparin can be fixed on the surfaces by physical adsorption, ionic binding and covalent immobilization. Physical adsorption and ionic binding can only maintain haemocompatibility for a few minutes to a few hours, while covalent immobilization is the only means to provide long-term haemocompatibility (Wang *et al.*, 2005). The anti-adhesive activity of heparin is principally correlated to its strong negative charge and hidrophilicity, which render biomaterial surfaces generally repellent for bacteria (Arciola *et al.*, 1994; Fu *et al.*, 2005). Arciola *et al.* (2003) also suggested that heparin can represent a specific inhibitor of the *S. epidermidis* adhesion to biomaterials coated with fibronectin.

There are several studies about the effect of heparin in reducing and preventing initial bacterial adhesion. For instance, Ruggieri *et al.* (1987) showed a 90% reduction in bacterial adhesion on urinary catheter surfaces coated with heparin and Tenke *et al.* (2004) demonstrated that heparin-coated urethral stents protect against encrustation and biofilm formation for 6-12 months, both *in vitro* and *in vivo*. Also Nomura *et al.* (1997) compared the extent of *S. epidermidis* adhesion between heparinized and non-heparinized PVC and silicone, with their results showing lower bacterial adhesion to heparinized surfaces. *S. epidermidis* and other coagulase-negative *Staphylococcus* (CNS) constitute the most common organisms causing post-operative endophtalmitis after cataract extraction and intraocular lens (IOL) implantation (Kattan

et al., 1991). The chemical process of permanent binding of heparin molecules on the IOL surface makes hydrophilic this hydrophobic material and this modification is stable for at least two years after implantation (Larsson *et al.*, 1992). This surface modification has been widely demonstrated to be effective in reducing cellular adhesion to IOL surfaces (Amon *et al.*, 1996; Miyake *et al.*, 1996; Tanaka *et al.*, 2000; Tognetto and Ravalico, 2001).

3.1.1.2 Gentian Violet (GV)

Gentian violet (GV) is a triphenylmethane dye, also called hexamethyl pararosaniline chloride, normally used to colour wood, silk or paper, being also applied as a biological stain and antimicrobial agent (Figure 3.2). GV has been as well used as an inhibitor of mold and fungal growth in poultry feeds (Thompson *et al.*, 1999). Since Nussenzweig *et al.* (1953) demonstrated the activity of GV against the trypomastigote forms of *Trypanosoma cruzi*, this compound has been widely used in blood banks in attempts to eliminate the transmission of Chagas' disease by blood transfusion (Docampo *et al.*, 1983).

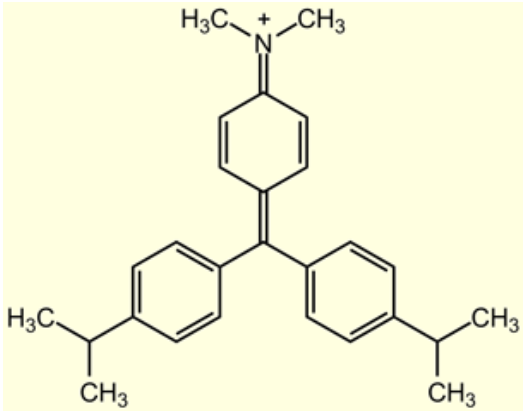


Figure 3.2 Chemical structure of gentian violet. Adapted from: Andersen *et al.*, 2007.

GV has a good bactericidal activity against some Gram-positive cocci, mainly *Staphylococcus* species, and pathogenic yeasts such as *Candida* (Saji *et al.*, 1995), besides having low toxicity and being low cost. It has been used topically to treat skin lesions (Albertini, 2001; Bakker *et al.*, 1992), and infections such as thrush in children and oropharyngeal candidiasis (Chaiban *et al.*, 2005). The good effectiveness of GV against yeasts, especially *Candida* species, was confirmed in the recent study of Camacho *et al.* (2007).

GV has been successfully used as a component of novel antiseptic agent, called gendine, which also contains chlorhexidine, and that has been employed as the impregnating agent of novel antimicrobial catheters (Hanna *et al.*, 2006) as well as of orthopaedic metal devices (Bahna *et al.*, 2007).

According to the work of Cervinka *et al.* (2006), GV exerts an antibiotic effect against *S. epidermidis* species in bone cement and, consequently, against infections in primary total joint arthroplasty. Also, it seems to have a reduced selection pressure for resistant bacteria. The application of GV, among other procedures, demonstrated to be effective in the treatment MRSA (methicillin resistant *S. aureus*) mediastinitis after replacement of the ascending aorta and the aortic valve (Kato *et al.*, 2006). This GV effectiveness against MRSA was already demonstrated in other studies (Kayama *et al.*, 2006; Okano *et al.*, 2000; Saji *et al.*, 1995). The inactivation of influenza A virus by GV was also showed in a recent work (Nagayama, 2006).

All these studies demonstrate the broad-spectrum activity of GV against gram-positive, gram-negative bacteria as well as yeasts and virus and also its prolonged antimicrobial durability.

3.1.2 Aims

The objective of this work was to evaluate how the pre-conditioning of surfaces by heparin or GV can hamper *S. epidermidis* adherence. Accordingly, the ultimate goal was to find a simple method to minimize *S. epidermidis* adhesion and colonization of indwelling medical devices, reducing in this way associated infections.

3.2 Materials and Methods

3.2.1 Bacterial strains

The *S. epidermidis* strains used in this study were: 9142, 9142-M10 and IE186. Information about these strains was already provided in chapter 2, sub-chapter 2.2.1.

3.2.2 Media and growth conditions

The growth conditions were followed according to the procedure described in sub-chapter 2.2.2.

3.2.3 Substrata preparation

Acrylic and silicone were used as substrata in the adhesion assays and prepared as described in sub-chapter 2.2.3.

3.2.4 Heparin and gentian violet pre-contact

The silicone and acrylic coupons were immersed in non fractioned heparin (5 000 IU/ml; Leo Pharmaceutical Products, Ballerup, Denmark) diluted 1:4 in distilled water, or 1% gentian violet (Merck, Darmstadt, Germany) solutions, and left to soak for 2 h. To study the effect of heparin pre-contact time in the extent of bacterial adhesion, silicone coupons were also pre-incubated in heparin for 10 min, 30 min and 24 h, and then aseptically removed and left to dry overnight, at room temperature.

3.2.5 Adhesion assays

Adhesion assays were performed as previously described in sub-chapter 2.2.4.

3.2.6 Total cell counts of adhered bacteria

Image analysis and enumeration of adhered cells was performed as described in sub-chapter 2.2.5.

3.2.7 Scanning Electron Microscopy (SEM)

The coupons with adhered bacteria were prepared for SEM according to the procedure described in sub-chapter 2.2.6.

3.2.8 Statistical analysis

Results obtained were analysed using SPSS software (Statistical Package for the Social Sciences Inc., Chicago) and all tests were performed with a confidence level of 95%. The Kolmogorov-Smirnov test was used to assess the normality of the variables distribution which confirmed a normal distribution of the results.

The effect of pre-conditioning time of silicone coupons with heparin was analysed using a two-way ANOVA (repeated measures) with Wilks` Lambda test. Results of the adhesion assays were compared using one-way ANOVA by applying the Tukey multiple comparisons test. The independent-samples *t*-test was also used to compare the effect of the same conditioning substance in acrylic and in silicone.

3.3 Results

3.3.1 Adhesion of *Staphylococcus epidermidis* to silicone: effect of heparin pre-contact time

The first approach aimed to assess the extent of adhesion of *S. epidermidis* strains to silicone coupons pre-conditioned with heparin throughout: 10 min, 30 min, 2 h and 24 h.

It was possible to observe a statistically significant effect of time on the extent of adhesion of all *S. epidermidis* strains to heparin pre-contacted silicone (Wilks`Lambda = 0.001, $p<0.001$ for strain 9142-M10; Wilks`Lambda = 0.023, $p<0.001$ for strain 9142 and Wilks`Lambda = 0.025, $p=0.007$ in the case of strain IE186). Figure 3.3 shows that there was always a significant difference ($p<0.05$) between the number of cells adhered to silicone control coupons and the extent of bacterial adhesion to all heparin pre-contacted coupons during several pre-conditioning times, and for all strains. Nevertheless, comparing the different times of pre-contact, the smallest adhesion extent was observed for 2 h of heparin pre-contact, for the three strains assayed. Adhesion of strains 9142 and IE186 showed small variations among all the pre-contact periods tested, while strain 9142-M10 was the only one displaying a great variability of adhesion extent with heparin pre-contact times assayed.

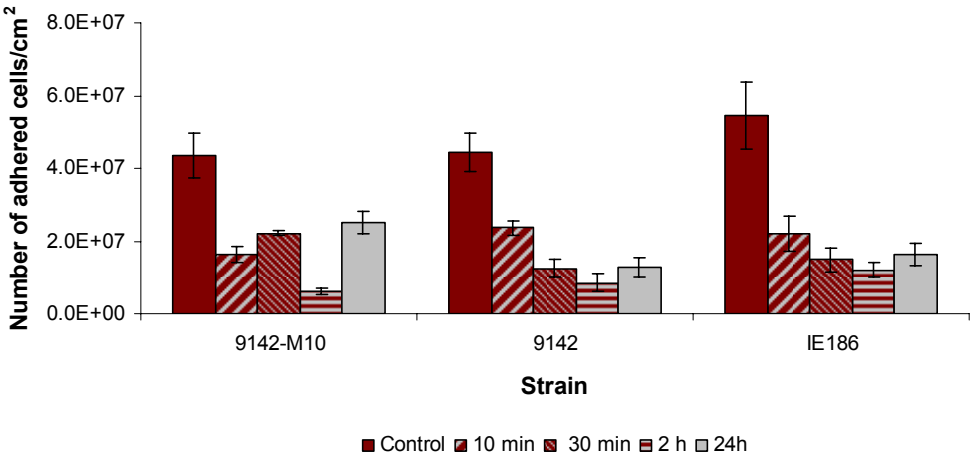


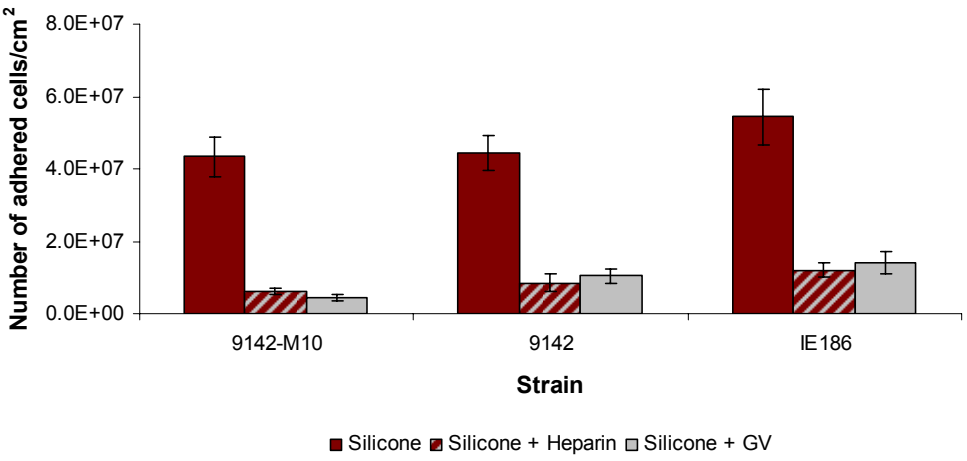
Figure 3.3 Number of *S. epidermidis* cells adhered to control silicone coupons and to silicone coupons subjected to different heparin pre-contact times (10 min, 30 min, 2 h and 24 h), for the three strains assayed (9142-M10, 9142 and IE186). Results represent means plus standard deviations (error bars) from three independent experiments.

3.3.2 Adhesion of *Staphylococcus epidermidis* to silicone and acrylic: effect of heparin and gentian violet pre-contact

Considering the above results, the subsequent assays were performed with 2 h pre-contact, for both conditioning substances.

In the case of silicone, a significant decrease ($p<0.005$) in the extent of bacterial adhesion to coupons pre-contacted with heparin or GV was observed in comparison to control coupons (Figure 3.4), for all strains under study ($p<0.001$). Besides, no statistical differences ($p>0.05$) were found between the extent of adhesion on silicone coupons pre-contacted either with heparin or GV, for each of the strains tested. However, on those pre-contacted with gentian violet, strain 9142-M10 adhered in significantly lower extent ($4.48\text{x}10^{6} \pm 9.44\text{x}10^{5}$ adhered cells/cm^2), comparing to strains 9142 ($1.04\text{x}10^{7} \pm 1.84\text{x}10^{6}$ adhered cells/cm^2, $p<0.001$) and IE186 ($1.39\text{x}10^{7} \pm 3.07\text{x}10^{6}$ adhered cells/cm^2, $p<0.001$).

(a)



(b)

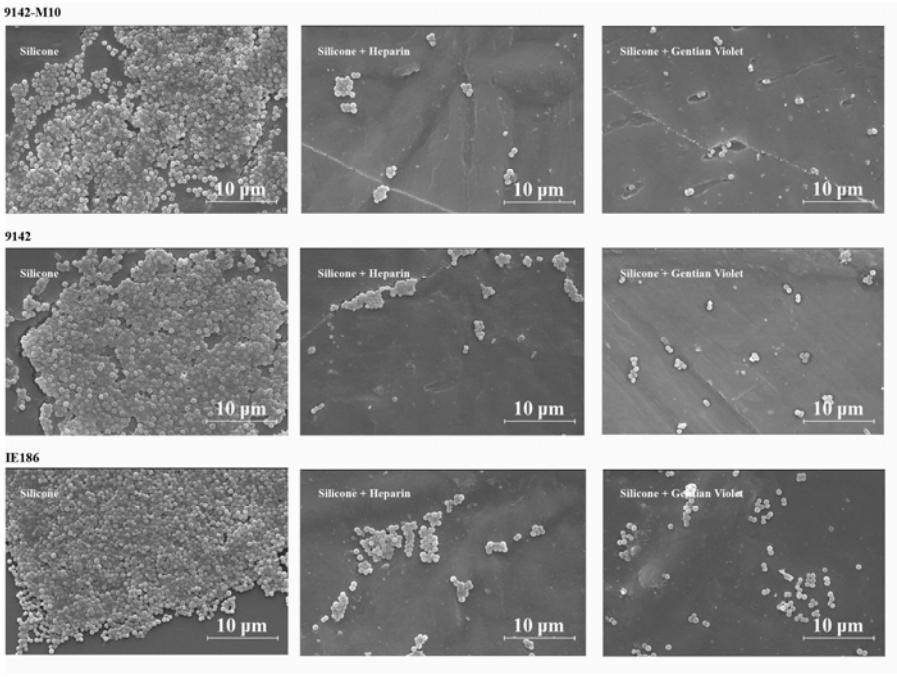


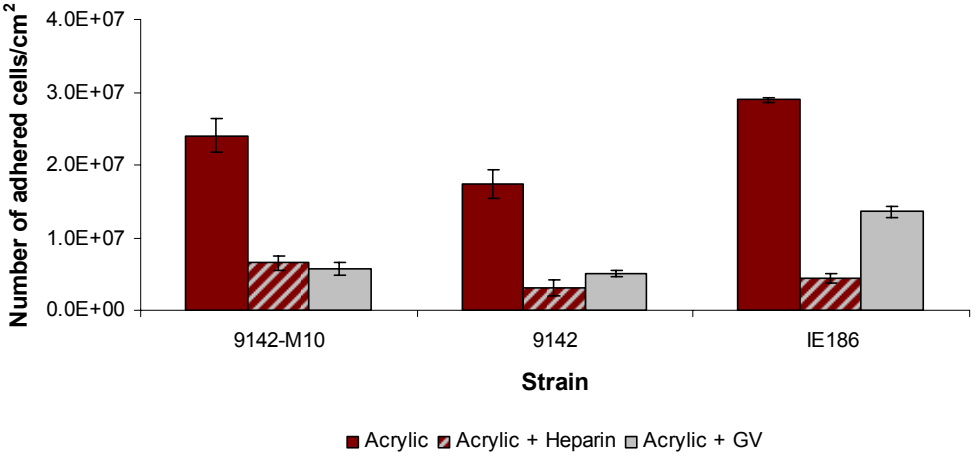
Figure 3.4 Effect of heparin and gentian violet pre-contact on *S. epidermidis* extent of adhesion to silicone. **(a)** Number of *S. epidermidis* adhered cells to non-conditioned silicone coupons (control) and to silicone coupons pre-contacted with heparin or gentian violet (GV), for the three strains assayed (9142-M10, 9142 and IE186). Results represent means plus standard deviations (error bars). **(b)** Representative scanning electronic microscopy photographs of the extent of adhesion, of the three *S. epidermidis* strains studied, to silicone pre-contacted with heparin or gentian violet (original magnification x3000).

In the case of silicone pre-contacted with heparin, a statistical difference ($p<0.001$) was observed between strain 9142-M10 ($6.16x10^6 \pm 7.98x10^5$ cells/cm^2) and strain IE186 ($1.21x10^7 \pm 2.17x10^6$ cells/cm^2), with strain 9142 presenting an intermediary level of reduction in the number of adhered cells ($8.51x10^6 \pm 2.50x10^6$ cells/cm^2, $p=0.047$).

Regarding acrylic conditioning (Figure 3.5), both pre-contacted surfaces also showed significantly lower levels of adhered cells, in comparison with controls ($p<0.001$), for all strains. In the case of strain 9142-M10, the number of adhered cells to acrylic was almost independent of the conditioning substance, either heparin or GV ($p=0.984$). Concerning strain 9142, the number of attached cells was less pronounced on acrylic pre-contacted with heparin ($3.07x10^6 \pm 1.07x10^6$ cells/cm^2), than with GV ($5.04x10^6 \pm 5.26x10^5$ cells/cm^2). In the case of IE186, heparin showed a significant ($p<0.001$) highest efficacy in acrylic conditioning, with only $4.36x10^6$ cells/cm^2 adhered, against $1.35x10^7$ cells/cm^2 when conditioned with GV. Comparing the behaviour of acrylic pre-conditioned with heparin and GV, it can be considered that heparin is slightly more efficient in reducing *S. epidermidis* adhesion than GV.

In the acrylic surface control, and in the pre-incubated GV samples the extent of adhesion of strain IE186 was significantly higher than in the case of strains 9142-M10 and 9142 ($p<0.001$). On the other hand, in the pre-incubated heparin coupons, *S. epidermidis* 9142-M10 was the strain that adhered at a higher extent ($p<0.001$) comparing to strains IE186 and 9142. Therefore, *S. epidermidis* 9142 was the most affected strain by the pre-conditioning of acrylic with heparin and GV.

(a)



(b)

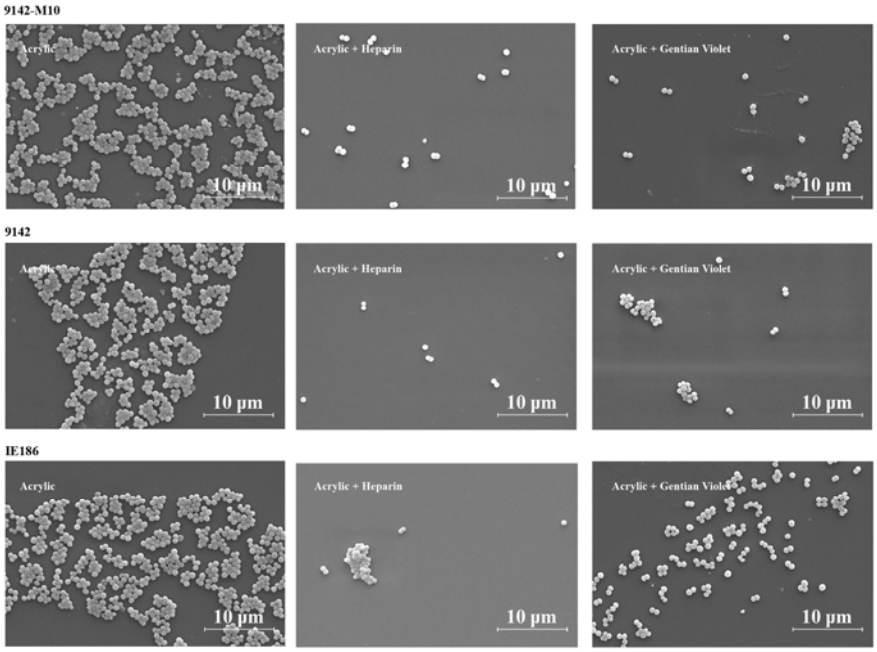


Figure 3.5 Effect of heparin and gentian violet (GV) pre-contact on *S. epidermidis* extent of adhesion to acrylic. **(a)** Number of *S. epidermidis* adhered cells to non-conditioned acrylic coupons (control) and to acrylic coupons pre-contacted with heparin or gentian violet (GV), for the three strains assayed (9142-M10, 9142 and IE186). Results represent means plus standard deviations (error bars). **(b)** Representative scanning electronic microscopy photographs of the extent of adhesion of the three *S. epidermidis* strains studied to acrylic pre-contacted with heparin or gentian violet (original magnification x3000).

The number of adhered cells of all three *S. epidermidis* strains was markedly lower ($p<0.001$) on acrylic than on silicone when the surfaces were not pre-conditioned. Moreover, when surfaces were pre-contacted with heparin, cells adhered also in a higher extent to silicone than to acrylic, especially strains 9142 ($p<0.001$) and IE186 ($p<0.001$). The same difference was observed for strain 9142-M10 ($p=0.009$) and strain 9142 ($p<0.001$) adhered to material coated with GV.

3.4 Discussion

One of the main sources of contamination of indwelling medical devices, such as catheters, is the skin insertion site. From this point microorganisms drift towards the intracutaneous tract on the exterior surface of the catheter, subsequently colonizing it and causing sepsis (Worthington *et al.*, 2000). Therefore, the heparin or GV pre-conditioning of both inner and outer surfaces of such devices can act as a preventive, effective, measure in infection development.

Actually, the results of the present study are in agreement with previous works where the use of such substances to prevent bacterial proliferation was assayed (Arciola *et al.*, 1994; Nomura *et al.*, 1997; Saji *et al.*, 1995; Tenke *et al.*, 2004). The results of Rose *et al.* (2005) also confirmed the ability of heparin pre-conditioning to reduce bacterial adhesion to cationic MPC copolymers. Another study (Abu El-Asrar *et al.*, 1997) regarding *S. epidermidis* adhesion to intraocular lenses (IOLs), demonstrated that a heparin treatment significantly reduced the extent of adhesion of these bacteria to regular polymethyl methacrylate (PMMA) IOLs, opposed to the inefficacy of antibiotic treatments. Similar results were obtained by Portolés *et al.* (1993) when performing adhesion studies of *S. epidermidis* to PMMA IOLs, in the presence of heparin in solution. These studies, along with the results of the present work, where the reduction of the extent of adhesion to acrylic and silicone pre-contacted with heparin reached the maximum

values of 85% (*S. epidermidis* IE186) and 86% (*S. epidermidis* 9142-M10), respectively, indicate that the pre-conditioning of indwelling medical devices with heparin might constitute a valid alternative to antibiotics, which have been revealing non efficient to reduce adhesion and biofilm formation on such surfaces.

Nevertheless, the mechanism by which heparin inhibits bacterial adhesion is not yet fully understood, although being known that it is not related with its action as an anticoagulant (Hanno *et al.*, 1981). The long hydrophilic chains and negative charge of heparin are likely to play a role in inhibiting adherence (Pringle and Fletcher, 1986). *S. epidermidis* cells are negatively charged which, when in contact with the also negatively charged heparin, may lead to a bacteria repelling action and consequently decreasing the extent of adhesion.

Concerning GV, Saji *et al.* (1995) demonstrated its *in vitro* significant bactericidal effect on methicillin resistant *S. aureus* (MRSA), isolated from clinical specimens, as well as in the treatment of decubitus ulcers infected with MRSA. The present results, that showed adhesion reduction levels in the order of 76% for acrylic (strain 9142-M10) and 90% (strain 9142-M10) for silicone pre-contacted, point out to a considerably GV efficacy also against CNS, such as *S. epidermidis*. Therefore, GV seems to possess a good antimicrobial activity that can be used against an important group of pathogens, normally associated with nosocomial infections.

In aqueous solutions, GV dissociates into positive and negative ions that break through the cell wall of gram-positive bacteria, such as *S. epidermidis*. The positive ions then interact with the negatively charged cell components such as peptidoglycan and DNA (Wishart *et al.*, 2006). Since GV is a mutagen and mitotic poison, cell growth is therefore consequently inhibited. This type of GV mode of action, as an antimicrobial agent, possibly explains its efficacy against bacteria and yeasts with mild to moderate effects on mammalian cells. In fact, Chaiban *et al.* (2005) evaluated the citotoxicity of an antiseptic combination of gentian violet and chlorhexidine, designated Gendine, having not found any citotoxicity against L929 mouse fibroblast cells.

Analysing the adhesion behaviour of the different strains studied, some differences were observed, particularly between strain 9142-M10 and strains 9142 and IE186. It should be

pointed out that strains 9142 and IE186, which presented similar adhesion ability (except on GV pre-contacted acrylic), have been defined as biofilm producers (Cerca *et al.*, 2005b; Mack *et al.*, 1994), while 9142-M10, with significantly different results, is an isogenic mutant of the biofilm negative strain 9142. Cerca *et al.* (2005b) also explained that adhesion and biofilm formation are two different features of the pathogenicity of medical devices infections and high levels of initial adherence do not necessarily lead to strong biofilm formation. This can be due to the fact that biofilm formation depends mostly of cell-to-cell adhesion phenomenon which, in case of CNS, is mediated by specific molecules such as PIA. On the other hand, initial adhesion also depends on several other bacterial cell surface factors. Thus, it is important to note that biofilm formation is not solely dependent on the extent of initial bacteria adherence to the substrate, but also that adhesion is a strain and not a species-dependent factor, as corroborated by the present results.

3.5 Conclusions

Nosocomial infections caused by CNS, such as *S. epidermidis*, may occur due to several factors, such as the use of indwelling medical devices for long periods of time; previous antibiotics administration (increased antibiotic use generally leads to increased resistance); patient state-of–health; and infection control practices by medical personnel (Koksal *et al.*, 2007).

The results presented in this work have a potential clinical significance, showing how both heparin and GV are effective in reducing bacterial adhesion. Moreover, the inoculum concentration used in the present experiments is very high comparing with a real situation of early state of infection. Thus, higher values of reduction in adhered cells can be expected. Pre-treatment of indwelling medical devices, suitable habitats for bacterial colonization, with such substances can therefore act as an infection control measure and may constitute a successful

and expedite method in reducing the incidence of nosocomial infections, especially during the insertion of this sort of devices into the patients.

CHAPTER 4 - *Staphylococcus epidermidis* adhesion to modified polycarbonate surfaces: gold and SAMs coated

This chapter describes specific modifications on polycarbonate outer layer utilized as model surfaces for the study of the adhesion of *Staphylococcus epidermidis*. The effect of gold coating on staphylococcal adhesion was assessed, as well as of subsequent coverage with different self-assembled monolayers (SAMs): two SAMs with a methyl terminal group and hydrophobic character and two hydrophilic SAMs with a carboxylic acid terminal group. Variations in the aliphatic chain length were also tested. A SAM with a calix-crown molecule, where a specific protein and its antibody were immobilized, was as well investigated in terms of bacterial adhesion.

4.1 Introduction

The risk of infection of indwelling medical devices due to initial adhesion of bacteria and subsequent colonization of the biomaterial surface, as a biofilm, constitutes nowadays a challenge to the development of less adherent surfaces.

In the last decades intensive research has been carried out in order to determine the parameters which control the interaction between bacterial cells and surfaces. Many surface properties have been shown to play an important role, such as hydrophobicity (Teixeira and Oliveira, 1999; van Loosdrecht *et al.*, 1987), electrical charge (Oliveira *et al.*, 2003), the presence of proteins (Baumgartner and Cooper, 1998; Wassall *et al.*, 1997), and surface chemistry (Cooper *et al.*, 2000; Margel *et al.*, 1993). A rigorous study of the effect of these properties on the adhesion process requires a model system that allows precise control of the type and the configuration of functional groups of the substrate surface (Tegoulia and Cooper, 2002). The advent of self-assembled monolayers (SAMs) has provided a way to fabricate well-defined model surfaces of known structures and properties that can be carefully regulated and manipulated.

SAMs are formed when surfactant molecules spontaneously adsorb in a monomolecular layer on surfaces (Pradeep, 1999). The order in these two-dimensional systems is produced by a spontaneous chemical synthesis at the interface, as the system approaches equilibrium (Ulman, 1996). The first gold-alkylthiolate SAM was produced by Nuzzo and Allara (1983), which realized the utility of combining a relatively inert gold surface with a bifunctional organic molecule in well-ordered, regularly oriented array. SAMs are highly ordered and densely packed assemblies of linear alkane molecules that are thermodynamically driven to form high-coverage films of molecular dimensions (Nealey *et al.*, 1997).

They comprise a number of attractive properties that make them suitable to be used as model surfaces: their preparation and biophysically relevant properties are well developed (Liang *et al.*, 2000); the density of ligands and non-specific adsorption can be controlled using

available synthetic techniques (Lahiri *et al.*, 1999; Mrksich and Whitesides, 1996); and SAMs are mechanically rigid and, thus, their deformation contributes less to the force required to detach bacteria (Liang *et al.*, 2000). Therefore, SAMs have been widely used to study interactions between biological environments and synthetic biomaterials, and eventually to improve the biocompatibility of materials (Deng *et al.*, 1996; López *et al.*, 1993; Patel *et al.*, 1997; Prime and Whitesides, 1993; Prime and Whitesides, 1991).

SAMs formed by the adsorption of alkyl trichlorosilanes on titanium (Sukenik *et al.*, 1990), silicon (Margel *et al.*, 1993), polystyrene (Vogler *et al.*, 1995), and poly(dimethyl siloxane) (Silver *et al.*, 1995); and the adsorption of alkanethiols onto the gold substrate (Deng *et al.*, 1996; Lestelius *et al.*, 1997; Lin and Chuang, 2000; López *et al.*, 1993; Prime and Whitesides, 1991; Tidwell *et al.*, 1997), are the most common and most highly ordered surfaces currently available for studying the interaction of cells and proteins with substrata of different surface chemistry. However, the monolayer of alkanethiol on gold is the most studied SAM mainly due to the moisture sensitivity of the alkyl trichlorosilane, as well as the ease of working with the crystalline gold surface (Hulman, 1996).

In general, the preparation of SAMs consists in the deposition of a solution of the molecule of interest onto the substrate surface and a final wash to remove the excess material. For instance, for the preparation of the broadly used gold-thiol SAM, a 1-5 nm film of titanium is evaporated onto a glass coverslip or silicon wafer to promote adhesion of gold to the surface. A 10-200 nm film of gold is then evaporated onto the surface. The resulting gold surface is then immersed into a solution of terminally substituted alkanethiols (Bain and Whitesides, 1989; Bain *et al.*, 1989) (Figure 4.1).

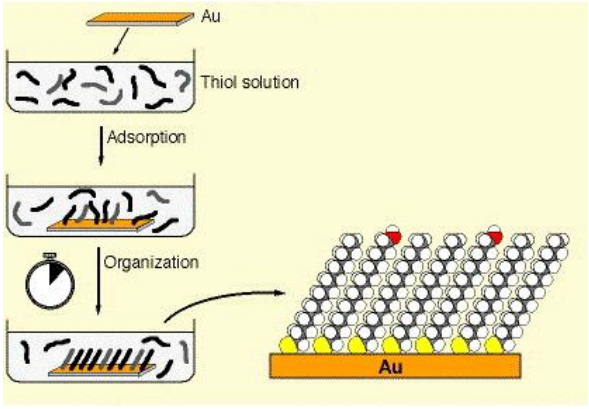


Figure 4.1 Preparation of SAMs. The substrate, gold (Au), is immersed into an ethanol solution of the desired thiol(s). Initial adsorption is fast (seconds); then an organization phase follows which should be allowed to continue for >15 h for best results. An illustrative scheme of a fully assembled SAM is shown to the right.
Adapted from: http://www.ifm.liu.se/applphys/ftir/sams.html.

The sulphur moiety of the alkanethiol has a very good affinity for gold, and the alkanes with a thiol head group bind to gold due to the strong sulphur-gold interaction. Also, molecular interactions such as van der Waals forces start to develop, as the molecules become closer to which other. These interactions orientate the molecules in a nearly perpendicularly way to the surface of the gold film, exposing their terminal functional group (Bain and Whitesides, 1989; Bain *et al.*, 1989; Bain *et al.*, 1998; Tegoulia and Cooper, 2002). The alkylchains can thus be imaged by a tightly packed hydrocarbon monolayer, and each alkylchain is oriented at an angle of about 28–40° to the surface normal (Nuzzo *et al.*, 1990). Gold-thiol SAMs are stable when exposed to air and aqueous or ethanolic solutions for several months (Bain *et al.*, 1989).

Intramonolayer hydrogen bonding is crucial in the SAMs of thiols or silanes bearing a terminal functional group, which can form hydrogen bondings between themselves. For example, terminal carboxyl groups in the SAMs of ω-carboxyalkanethiol can easily form hydrogen bonding within the monolayers (Aoki and Kakiuchi, 1999; Crooks *et al.*, 1993; Crooks *et al.*, 1997; Zhao *et al.*, 1999).

The terminal group of the precursor molecules from which SAMs are formed determines the surface chemistry (Franco *et al.*, 2000). With the versatility of functional groups

introduced by organic synthesis, the surface of SAMs can be tailored with a wide variety of chemical and physical properties while the well-ordered monolayer structure that is absent in other types of surface modifications is maintained. For instance, the functional group can be chosen so that certain properties are obtained (e.g., properties that can change the wettability of the surface so that it is hydrophilic (hydroxyl groups) or hydrophobic (methyl groups) (Hou *et al.*, 2007).

In addition to the terminal group, it has been reported that the alkyl chain length of a SAM also exerts a significant influence on its behaviour (Cooper *et al.*, 2000) such as, for example, the variation of its wettability according to the chain length (Bain and Whitesides, 1989; Laibinis *et al.*, 1991). The wettability of a SAM can also be influenced by the van der Waals forces of the metallic substrates, such as gold, that support the SAM (Miller and Abbott, 1997).

In recent years many studies have been focused on the use of SAMs to evaluate the effect of surface charge, wettability and topography on protein adsorption and cell behaviour using *in vitro* assay systems (Ito, 1999; Jenney *et al.*, 1998; Kapur and Rudolph, 1998; Webb *et al.*, 2000). According to Prime and Whitesides (1991), SAMs of alkanethiols on gold are useful model systems for investigating mechanisms of protein adsorption. Silin *et al.* (1997), e.g., showed that much less human immunoglobulin G and bovine serum albumin was adsorbed onto a SAM with an oligoethylene oxide terminal group than to other SAMs. More recently, Lee *et al.* (2006) used SAMs with different functional groups (X=OH, COOH, NH_2 and CH_3) to study the effect of the physicochemical characteristics of a material surface on the $\alpha5\beta1$ integrin-mediated adhesion of a cell line of erythroleukemia cells to fibronectin, one of the physiological fluids proteins that adsorb onto the biomaterial surface immediately after exposure to it. Wang and co-workers (2006) also used a cell line of bovine aortic endothelial cells and SAMs of alkanethiolates, to study how surface parameters, such as surface charge, affects the structure and activity of adsorbed proteins and, consequently, cell adhesion.

A special focus has also been given to the strength of cell adhesion and spreading on SAMs (Healy *et al.*, 1994; Sukenik *et al.*, 1990). For example, McClary *et al.* (2000) showed that

hydrophobic methyl-terminated alkanethiol SAMs on gold induce minimal cell attachment and cannot support spreading and the formation of focal contacts by mouse fibroblasts. Finlay *et al.* (2002) employed alkanethiolate SAMs on a gold surface to study the adhesion strength of diatom cells to SAMs, with their results clearly demonstrating that the adhesion of diatom cells (*Amphora*) was affected by the wettability of SAMs.

The advent of molecules called calixarenes, revealed to be extremely useful for the study of interactions between biological systems and synthetic biomaterials (Gutsche, 1989; Shinkai, 1991). Calixarenes are a class of cyclooligomers whose chemical versatility and conformational properties allow the design of flexible and preorganized molecular receptors such as calix-crowns (used in this work) and calix-spherands (Ungaro and Pochini, 1991). The name "calixarenes" was given due to the similarity between the shapes of these cyclic tetramers and a calix or a vase (Gutsche, 1989). Each calixarene contains a repeating phenolic unit formed into a macrocycle via methylene bridges (Gutsche, 1998; Mandolini and Ungaro, 2000).They are formed after phenol-formaldehyde condensation, with defined upper and lower rims and a central annulus (Jose and Menon, 2007) (Figure 4.2).

The polar and nonpolar features of cavities enable calixarenes to interact with a wide range of host species, depending on the binding groups substituted at each rim and the number of repeating units in the macrocycle (Shinkai, 1991). They can, thus, be used as sensors for metal ions, organic/neutral molecules, and drugs recognition (Jose and Menon, 2007).

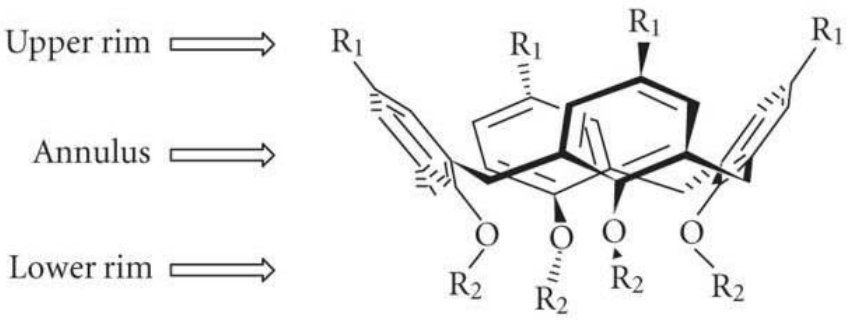


Figure 4.2 Typical structure and division of calixarenes. Adapted from: Jose and Menon, 2007.

Compared with other applications, the study on the complex formation between proteins and calixarene derivatives is less common. However, the derivative calix-crown molecule comprises a well–characterized linker system able to immobilize proteins efficiently (Lee *et al.*, 2003; Vitalii *et al.*, 1997). The organization of these artificial linker molecules on the solid substrates surface can be carried out with SAM technique, which guarantees each molecule to exhibit its original function on the surface (Esplandiú *et al.*, 2001), besides allowing to control the density and orientation of captured proteins and to obtain better reproducibility on a solid surface (Heyse *et al.*, 1995; Tsai and Lin, 2001).

While SAMs have great potential for controlling cell-surface interactions, their applications in biofilm control have not been well investigated. To control biofilm formation using SAMs, it is necessary to compare the effects of different functional groups, to analyze the biofilm structures on SAMs, and to explore the mechanism by which biofilm formation is reduced (Hou *et al.*, 2007). Wiencek and Fletcher (1995) used methyl and hydroxyl terminated SAMs for the study of the effect of surface free energy on *Pseudomomas* species adhesion. Also, Ista *et al.* (1996) reported that SAMs presenting hexa(ethyleneglycol) resist the attachment of *Staphylococcus epidermidis* and *Deleya marina* based on cell counting. Thus, despite the few studies available, SAMs presenting different functional groups on gold-coated surfaces constitute promising model surfaces for mechanistic studies of cell-surface interactions and the development of novel biofilm control strategies (Hou *et al.*, 2007).

4.1.1 Aims

The aim of the present work was to use modified polycarbonate surfaces to assess *S. epidermidis* adhesion ability. The polycarbonate surface alterations included coating of polycarbonate surface with gold and its subsequent coverage with hydrophobic and hydrophilic SAMs, both with variations in the aliphatic chain length. A calix-crown SAM was also created to

immobilize a specific protein and its antibody, for evaluation in terms of bacterial adhesion as well.

4.2 Materials and Methods

4.2.1 Bacteria and growth conditions

The strain used in this work was *S. epidermidis* 9142 (sub-chapter 2.2.1) and growth conditions were followed according to the procedure described in sub-chapter 2.2.2.

4.2.2 Substrata preparation

The polycarbonate substrate (PC) was cut into 2.0 cm x 2.5 cm and the gold film (G) was deposited by ion sputtering (Sputter Coater SC502, Fisons Instruments, UK) to a thickness of approximately 75 nm. At the same time, solutions of octanethiol [$HS(CH_2)7CH_3$] (OT), hexadecanethiol [$HS(CH_2)15CH_3$] (HDT), mercaptoacetic acid [$HSCH_2COH$] (MAA), mercapto-propionic acid [$HS(CH_2)2CO_2H$] (MPA) and calix-crown (CC) were prepared in Petri dishes, at a ratio (wt/wt) of 99.9% ethanol to 0.1% of the respective compound. The polycarbonate gold covered samples were washed in ultra-pure water, dried and placed in the respective solutions. The Petri dishes were sealed with parafilm, left overnight at 25 °C and protected from light. Next, only the coupons with the calix-crown monolayer were immersed for 1 h in a phosphate buffered saline solution (PBS) (pH 7.4), with a monoclonal antibody, anti-C-Reactive Protein (CRP) (100 μg/ml). Following this step, coupons were washed again with ultra pure water (Millipore Direct-Q, 18 MΩ/cm resistivity) and immersed, for 1h, in a PBS solution (pH 7.4) with the respective antigen, CRP (100 μg/ml).

4.2.3 Bacteria and substrata hydrophobicity

Hydrophobicity of bacteria and substrata was evaluated through water contact angle measurements. For contact angle measurements on substrata, a micropipette was adapted to a module enabling the movement along the vertical and horizontal axis, connected to an optical microscope in order to deposit a liquid drop (2 µl) onto the coupon, which was in a saturated atmosphere chamber. The drop image was captured by an optic system consisting of a CCD (Charge-Coupled Device) video camera (640 × 480 pixels), with amplification lenses.

In the case of bacteria, contact angle measurements were performed on bacterial layers deposited on membrane filters, according to the method described by Busscher *et al.* (1984) and using the sessile drop technique, as already described in sub-chapter 2.2.7. All measurements were performed at room temperature, with ultrapure water with an electrical resistivity of 17.8 MΩ/cm (Nanopure, Barnstead).

4.2.4 Initial bacterial adhesion

Initial adhesion of bacteria to substrata was allowed to occur as described in sub-chapter 2.2.4.

4.2.5 Image analysis

Image analysis and enumeration of adhered cells was performed as previously described (sub-chapter 2.2.5).

4.2.6 Statistical analysis

The resulting data were analysed using the Statistical Package for the Social Sciences Software (SPSS Inc., Chicago). The comparison was performed through one-way analysis of variance (ANOVA) by applying the Bonferroni analysis as a post hoc test. All tests were performed with a confidence level of 95%.

4.3 Results

4.3.1 Surface characterization

Considering that SAMs were prepared on gold films supported on polycarbonate surfaces, the polycarbonate (PC) and gold coated (G) surfaces were also tested as substrata and used as control surfaces.

The values of water contact angles (in degrees) on the different substrata assayed are presented in Table 4.1.

Table 4.1 Water contact angles (in degrees) of the materials and the *S. epidermidis* strain. Results are presented as mean ± standard deviation (SD).

	Water contact angle ± SD
***S. epidermidis* 9142**	25.6 ± 0.9
PC	87.2 ± 3.4
G	87.5 ± 2.0
OT	96.7 ± 4.6
HDT	101.9 ± 0.5
MAA	59.6 ± 3.8
MPA	53.8 ± 1.7
CC	85.0 ± 1.2
CRP	45.8 ± 1.1

According to Vogler (1998), hydrophobic surfaces exhibit water contact angle values higher than 65°, whereas hydrophilic ones exhibit water contact angles lower than 65°. Thus, PC, G, OT, HDT and CC surfaces are hydrophobic, while MAA, MPA and CRP surfaces are only slightly hydrophilic, with an almost hydrophobic character. They can be considered moderately wettable surfaces.

4.3.2 Cell adhesion to substrata

A crucial factor for the preparation of high quality SAMs is the surface condition of the gold coated substrate, because this metal is easily contaminated by the carbonaceous contaminants present in the atmosphere (Tsai and Lin, 2001). *S. epidermidis* adhered to the gold control surface and to the OT SAM in a lower extent compared to all the other surfaces (Figure 4.3 and Figure 4.5). On the other hand, the number of bacterial cells adhered to PC was slightly higher when compared with that adhered to HDT and MAA SAMs and statistically higher ($p<0.05$) in comparison to G and OT SAMs.

Analysing the adhesion values of the two methyl (CH_3) (OT and HDT) and carboxylic acid (COOH) terminated SAMs (MAA and MPA) it can be seen that, in the latter, cells adhered to a higher extent than to OT or HDT (Figure 4.3 and Figure 4.5). However, this difference was only statistically significant ($p<0.05$) in the case of MPA.

The results of the present study are in agreement with other literature reports (Cooper *et al.*, 2000; Haddow *et al.*, 1999; Tidwell *et al.*, 1997), where high levels of cellular attachment were observed to carboxylic acid terminated SAMs as opposed to low levels of attached cells to methyl terminated SAMs.

Regarding the effect of the aliphatic chain length on bacterial adhesion to hydrophobic SAMs (OT and HDT), the present results might suggest that an increase in chain length promotes a slight increase in the number of adhered cells (Figure 4.3). However, no statistical significance ($p>0.05$) was found. Conversely, in the case of hydrophilic layers (MAA and MPA), a slight

increase in the alkyl chain length promotes a substantial increment in the number of adhered cells.

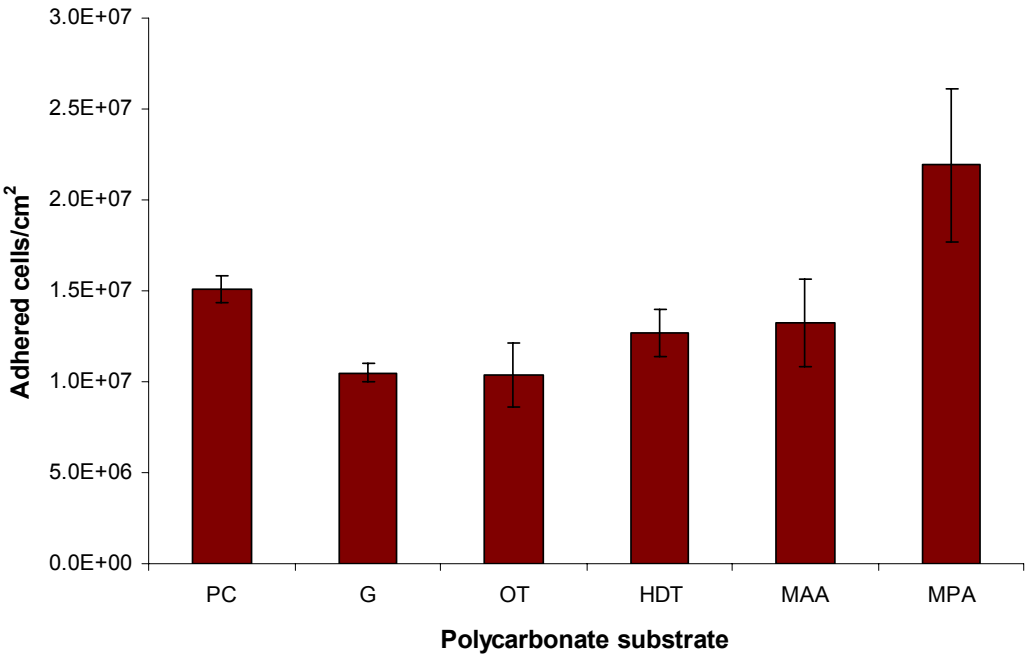


Figure 4.3 Number of *S. epidermidis* cells adhered per cm^2 on PC (polycarbonate) coupons and on polycarbonate modified surfaces: G (gold), OT (octanethiol), HDT (hexadecanethiol), MAA (mercaptoacetic acid) and MPA (mercaptopropionic acid).

Regarding the calix-crown SAM, it enables a non-specific immobilization of protein/antibodies and, as a result, it should favour bacterial adhesion due to its non-specific protein binding nature. Thus, the extremely high levels of *S. epidermidis* adhered to the CC SAM were expected. The number of cells attached to the complex anti-CRP - CRP immobilized by the calix-crown was approximately double of that to the gold surface. However, *S. epidermidis* cells adhered to a much lower extent to the anti-CRP - CRP substrate when compared to the number of cells adhered to the CC SAM (Figure 4.4 and Figure 4.5).

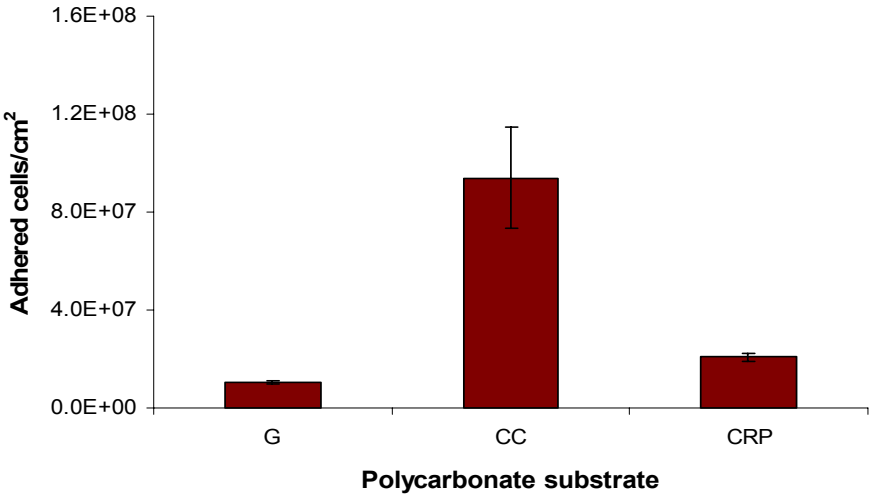


Figure 4.4 Number of *S. epidermidis* cells adhered per cm^2 on polycarbonate modified surfaces: G (gold), CC (calix-crown) and CRP (calix-crown with the antibody anti-CRP and the respective antigen, CRP).

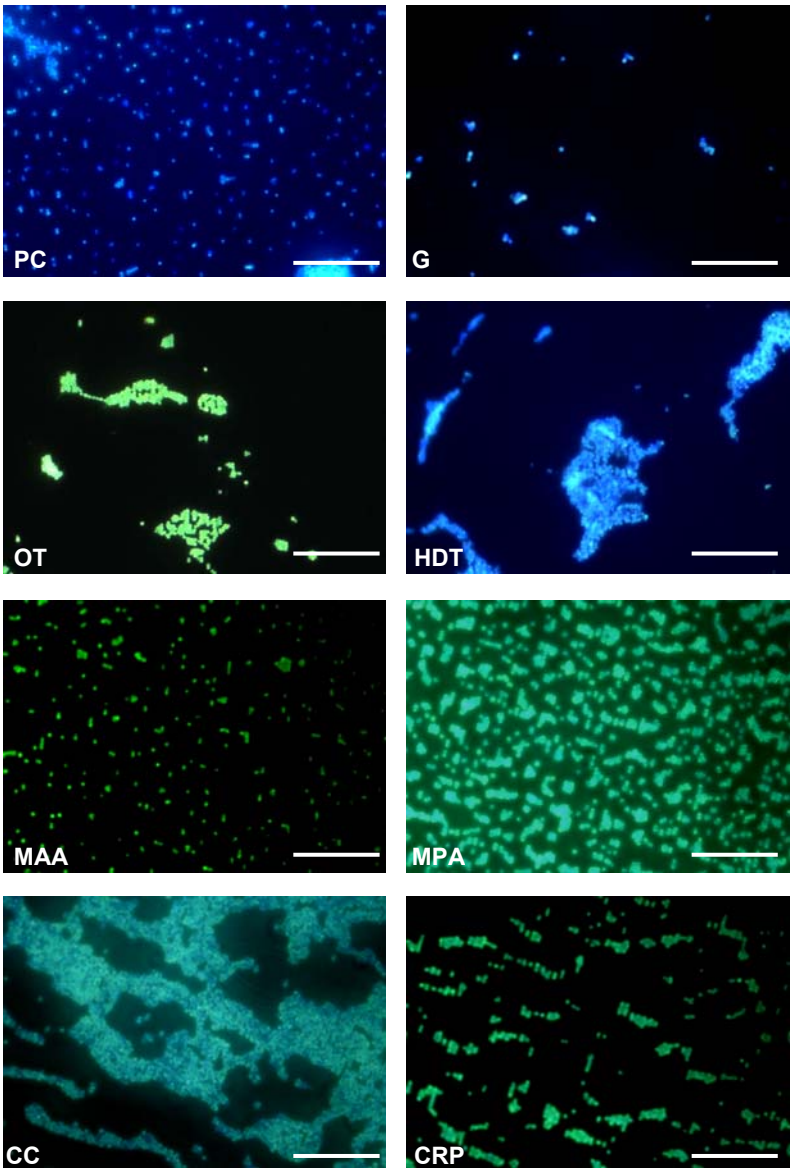


Figure 4.5 Representative epifluorescence microscopy photographs of the extent of adhesion of *S. epidermidis* cells to the PC (polycarbonate) coupons and to the polycarbonate modified surfaces: G (gold), OT (octanethiol), HDT (hexadecanethiol), MAA (mercaptoacetic acid) MPA (mercaptopropionic acid), CC (calix-crown) and CRP (calix-crown with the antibody anti-CRP and the antigen, CRP). Magnification ×1000, bar = 5 μm.

4.4 Discussion

Adhesion of bacteria to a given surface can be influenced by several factors such as hydrophobicity, the nature of the material and the immobilization of proteins on the surface. However, very subtle changes in the surface layer structure are also important, with the terminal group and the length of the alkyl chain playing a significant role in this process.

According to Arima and Iwata (2007), one of the factors that mostly affects cell adhesion is the wettability of the substrate surface and it has been considered that wettability of SAMs varies with chain length (Bain and Whitesides, 1989; Laibinis et al, 1991). Faucheux *et al.* (2004) found that SAMs terminated with CH_3 produced hydrophobic surfaces (water contact angle > 80°), while those with -COOH formed moderately wettable surfaces (water contact angle between 48-62°), which is in agreement with the results obtained in the present work (Table 4.1). According to the study of Lee *et al.* (1998), the maximum cell adhesion to polyethylene surfaces with a wettability gradient was found for a water contact angle of 55°, which is very close to the MPA water contact angle value determined in this study (53.8 ± 1.7°). It must be noted that on MPA SAM (with the exception of CC SAM) *S. epidermidis* adhered to the highest extent. In fact, some authors report that cell adhesion to polymer surfaces is favoured with moderate wettability and water contact angle between 40-70° (Lee *et al.*, 1997; Tamada and Ikada, 1993).

The water contact angle determined here for PC surface is very similar to the value determined by Rios *et al.* (2007) (81.3 ± 0.7°). Also, the values of water contact angles determined on the gold and methyl-terminated surfaces are close to the ones found in the literature where the values varied between 71-83° and 108-116° for gold and methyl terminated surfaces, respectively (Barbosa *et al.*, 2005; Cooper *et al.*, 2000; Lin and Chuang, 2000; Scotchford *et al.*, 2001; Tsai and Lin, 2001). However, in the case of the carboxylic acid terminated SAMs, the water contact angle was higher than most of the literature reported values: Scotchford and co-workers (2001) as well as Cooper *et al.* (2000) obtained water contact angle on MPA less than 15°, while Tsai and Lin (2001) and Lin and Chuang (2000) obtained for the

COOH SAMs the values of 4.3° and 33.5°, respectively. These differences were probably due to the highly polar nature of the carboxylic group as well as to its predisposition to rapidly adsorb laboratory contaminants (Tegoulia and Cooper, 2002). Another important point to stress is that, in this study, the gold coating was made on polycarbonate substrate while the majority of the studies available in the literature are about SAMs deposited on gold films on coverglass or silicon wafers, with different surface roughness/topography. Such differences might have led to variations in the surface characteristics of the SAMs.

The modification of the polycarbonate surface with gold and with SAMs lowered the levels of *S. epidermidis* adhesion in all four of the studied situations: simply by coating with gold and by layering with OT, HDT and MAA SAMs, although the last two ones had a less significant effect. The gold layer constitutes a high-quality support for the adsorption of the different SAMs, forming an extremely ordered and stable monolayer with a rigorous control on the terminal group functionality and surface chemistry (Cooper *et al.*, 2000). However, the present results show that the gold layer *per se* is very effective in lowering microbial adhesion. In fact, the antimicrobial effects of gold and its use in medicine are already well known (Saygun *et al.*, 2006) and once again are corroborated by the present work.

Regarding the behaviour of the terminal group, the results of the present study are in agreement with the values of the extent of adhesion described in the literature, despite the difference in the nature of the adherent specimens. Lin and Chuang (2000) observed that platelets adhered in much higher extent to a COOH SAM than to CH_3 terminated SAM. Also Tsai and Lin (2001) showed that the platelet adhesion intensity increased in the following order: gold<CH_3<COOH terminated SAMs. According to the work of Scotchford *et al.* (2001), osteoblasts adhered preferentially to COOH and then to CH_3. Faucheux *et al.* (2004) demonstrated that after a 2-h period, fibroblasts adhered more to SAMs with COOH terminal group than to SAMs terminated with CH_3. Nevertheless, it is important to note that a large amount of the available adhesion studies to SAMs concern different cell types which display distinctive responses to a particular surface. The behaviour and the mechanism by which an animal tissue cell adheres to a

specific surface may be very different from the one of a *S. epidermidis* cell. Regarding the effect of the alkyl chain length on the adhesion of *S. epidermidis* cells, no effect was detectable in methyl terminated SAMs, while on carboxylic SAMs, a subtle increase in chain length from MAA to MPA seems to exert some influence, with MPA exhibiting high levels of adhesion in comparison to MAA. Hence, it becomes difficult to understand which one of these factors has the strongest influence in *S. epidermidis* adhesion. In this particular case, it seems that the nature of the terminal group in combination with a moderate wettability exert a critical effect in *S. epidermidis* adhesion.

Calix-crown SAM is a particular case, due to its conformation and its non-specific protein binding nature. Cell wall proteins of the bacteria, such as adhesins, have a perfect niche for binding, so the adhesion is extremely enhanced. In this case, cell proteins bind in a non-specific way to the calix-crown molecule, and without competition with other proteins. This molecule can also be used as an artificial linker system for protein immobilization (Chen *et al.*, 2007) by pre-binding a specific antibody to promote the selective linkage of a given protein. This is very important because protein adsorption onto the surface of a foreign material is the early event for a set of varied applications such as biosensors, biochips, bioreactors and diagnostic techniques (Vitalii *et al.*, 1997).

In this work, the C-reactive protein (CRP), a biomarker for acute levels of inflammation, was immobilized on the calix-crown molecule by means of protein-protein interaction with anti-CRP. In this case, the extent of *S. epidermidis* adhesion was approximately double when compared to the gold coated surface. In a certain way, this is in agreement with the work of Arima and Iwata (2007) where the cellular adhesion was studied both on hydrophobic and hydrophilic SAMs with pre-adsorbed albumin. These authors observed that on hydrophilic SAMs, such as the CRP here used, albumin was replaced by cell adhesive proteins and, as a result, SAMs with moderate wettability became more suitable surfaces for cell adhesion. However, in comparison to the levels of bacterial adhesion to the calix-crown molecule, the values were significantly lower. The CRP and its antibody blocked the sites of bonding of the calix-crown

molecule, preventing the linkage of bacterial proteins. This type of SAM may constitute a good alternative to avoid bacterial adhesion to biomaterials. In addition, it is important to stress that under physiological conditions, most of the substrate properties are masked by the presence of an adsorbed protein layer and the effect of substrate properties on bacteria adhesion is minimal (Tegoulia and Cooper, 2002).

4.5 Conclusions

The use of SAMs constitutes an excellent method to determine the effect of surface modifications on bacterial adhesion, mainly due to the fact that they are structurally the best-organized and controllable surfaces available. The adhesion of *S. epidermidis* to modified polycarbonate surfaces is strongly determined by parameters such as the nature of the functional terminal group along with surface wettability.

The lowest levels of *S. epidermidis* attachment were observed, in addition to gold covered surfaces, on the methyl terminated SAMs, OT and HDT, demonstrating that methyl terminated SAMs constitute more suitable surfaces in preventing bacterial adhesion. The calix-crown molecule allows high levels of adhesion due to its non-specific binding nature. However, the pre-linkage of a particular protein blocks the sites of adhesion for the cell proteins, resulting in a decrease in the number of attached cells. This can constitute an alternative to control the levels of bacterial adhesion to biomaterials.

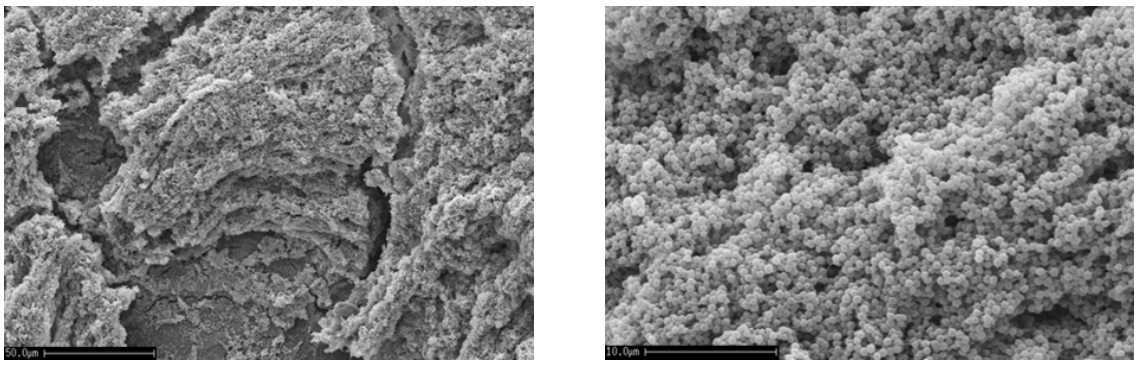
CHAPTER 5 - *Staphylococcus epidermidis* biofilms

Staphylococcus epidermidis is now well established as being a major nosocomial pathogen, associated with indwelling medical devices. Its major virulence factor is related with the ability to adhere to indwelling medical devices and there form biofilms. Thus, in this chapter, an attempt to better understand *S. epidermidis* biofilms is made. Essential differences between sessile and planktonic cells are also addressed, particularly those concerning metabolic activity.

5.1 Introduction

After the initial attachment of *Staphylococcus epidermidis* cells to the polymer surface, bacteria multiply and accumulate as multilayered clusters, embedded in self-excreted polymeric substances (EPS) and separated by water channels, then constituting a mature biofilm (Figure 5.1). The ability of *S. epidermidis* to form biofilms represents the most important virulence factor (Vuong and Otto, 2002). A biofilm can thus be defined as an interdependent community of microorganisms, and their associated extracellular products, at an interface and typically attached to an abiotic or biotic surface (Dunne, 2002; Wilson, 2001).

The biofilm architecture facilitates the exchange of nutrients, enabling biofilm communities to develop considerable thickness and complexity. It allows for individual cells, some of which physiologically specialized, to be kept in optimal nutrient conditions throughout the biofilm (Stoodley *et al.*, 2002). According to Otto (2008), biofilm formation involves surface proteins that mediate initial attachment to host matrix proteins. Following that, in the case of staphylococci, the expression of a cationic glucosamine-based exopolysaccharide enables bacterial cells aggregation.



(a) **(b)**

Figure 5.1 SEM observation of a general view of a mature biofilm.
(a) Magnification ×500, bar = 50 µm; **(b)** Magnification ×3000, bar = 10 µm.

Once firmly adhered to the abiotic surface, cells adapt to growth in these hydrated surface-associated communities, expressing phenotypic characteristics that differ completely from those expressed during planktonic growth (Stewart and Franklin, 2008). Such occurrence on the surface of surgical implants or other foreign materials ultimately leads to its colonization, translated in patient's infection and associated with considerable morbidity due to frequent hospitalizations, surgery and antimicrobial treatment (Presterl *et al.*, 2005). As the biofilm thickness increases, there is a progressive sloughing of single cells and/or bacterial clusters of different sizes which can be considered to be the main promoters of the infectious process (Donelli *et al.*, 2007). These detached cells may become septic emboli that, dispersing throughout the bloodstream, can cause metastatic infections and sepsis (Donelli, 2006).

Another issue is that bacteria in biofilms are more resistant (1000 up fold) to antibiotics and to the host immune defence system than their planktonic counterparts (Ceri *et al.*, 1999), indicating an altered metabolic activity (Resch *et al.*, 2005). This strong resistance requires regular replacement of the infected biomaterial and leads to substantial morbidity and mortality (Mack *et al.*, 2004).

Thus, it is extremely important to know the essential differences, especially concerning physiology and metabolic activity, between a planktonic cell and its sessile counterpart adhered to a surface, living within a biofilm.

5.1.1 Extracellular polymeric substances (EPS)

The presence of exopolymeric substances (Donlan, 2001) and particularly, the altered polysaccharides production, has been described as one of the physiological adaptations in biofilm-associated bacteria (Davies and Geesey, 1995). Most biofilm volume is actually composed of this extracellular polymeric substance rather than cells (Figure 5.2), a fact that was confirmed by ruthenium red staining and transmission electron microscopy (Jones *et al.*, 1969).

Generally, the biofilm matrix comprises several extracellular polymeric substances (EPS), connected through ionic interactions, such as polysaccharides, proteins, glycoproteins, phospholipids, nucleic acids, and humic acids (McSwain *et al.*, 2005). In some cases, significant amounts of extracellular DNA (e-DNA) have also been detected in the EPS matrix (Palmgren and Nielsen, 1996; Allesen-Holm *et al.*, 2006). Böckelmann *et al.* (2006) reported the formation of e-DNA as a spatial structure forming a filamentous network in biofilms acting, according to Yang *et al.* (2007), as an intercellular connector, stabilizing the biofilm matrix. Another recent study (Rice *et al.*, 2007) stated the importance of the genomic DNA released as a structural component of *S. aureus* biofilms. Also, the biofilm matrix can comprise cellular debris and products of the extracellular hydrolytic activity, as well as adsorbed chemicals and particles (Wingender *et al.*, 1999).

In the specific case of some gram-positive bacteria such as *S. epidermidis*, the chemical composition of EPS seems to be primarily cationic (Hussain *et al.*, 1993) and to comprise polysaccharides (Donlan, 2001; Sadovskaya *et al.*, 2005), proteins (Cucarella *et al.*, 2001; Lasa and Penades, 2006; Rohde *et al.*, 2005), considerable amounts of extracellular teichoic acids (Sadovskaya *et al.*, 2005; Sadovskaya *et al.*, 2004) and also extracellular DNA (Qin *et al.*, 2007).

EPS are moreover highly hydrated due to their ability to incorporate large amounts of water into their structure through hydrogen bonds (Donlan, 2002). Besides this, the matrix-enclosed microcolonies are separated by water channels that provide a flow system of nutrients and oxygen within the biofilm (Donlan and Costerton, 2002). The EPS determine the immediate life conditions of biofilm cells living in this microenvironment by affecting porosity, density, water content, charge, sorption properties, hydrophobicity, and mechanical stability (Flemming and Wingender, 2002).

From a general point of view, different organisms can produce different amounts of EPS, which increases with the age of the biofilm (Prakash *et al.*, 2003). Moreover, depending on the environment in which the biofilm has developed, the biofilm matrix composition can vary

greatly (Donlan, 2002). For instance, EPS production is sensitive to the nutrient status of the growth medium, e.g., excess available carbon and the limitation of nitrogen, potassium or phosphate promote EPS synthesis (Sutherland, 2001). Deighton and Borland (1993) showed that *S. epidermidis* increased slime production in iron-limited medium and late in growth phase when nutrients were limited.

EPS are responsible for providing mechanical stability and cohesive strength to the biofilm structure, forming a three-dimensional, gel-like, highly hydrated environment in which the cells are immobilized (Flemming *et al.*, 2000; Körstgens *et al.*, 2001; Sternberg, 1999).

The polymeric matrix interacts with the environment by attaching biofilms to surfaces and by its sorption properties, which allows them to remove dissolved substances from the environment and thus provide nutrients for biofilm bacteria (Flemming *et al.*, 2007). Furthermore, the extracellular matrix plays a major role in the intercellular connection process during surface colonization (Hussain *et al.*, 1991), in the protection against the host immune system and resistance to antibiotics (An and Friedman, 1998). EPS acts as a physical/chemical barrier thus contributing to the antimicrobial resistance properties of biofilms, by impeding the antibodies penetration or the antibiotics transport throughout them, probably by binding directly to these agents (Donlan, 2000).

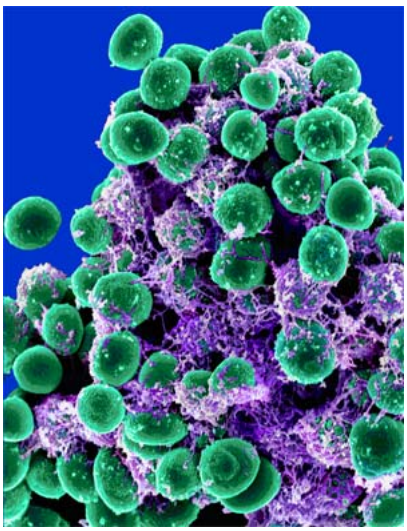


Figure 5.2 Scanning electron microscopy of *S. epidermidis* cluster embedded in exopolysaccharide matrix. Adapted from: http://www3.niaid.nih.gov/labs/aboutlabs/lhbp/pathogenMolecularGeneticsSection/.

5.1.1.1 Polysaccharide intercellular adhesin (PIA)

In *S. epidermidis*, the polysaccharide intercellular adhesin (PIA) is the major exopolysaccharide building up the biofilm matrix. It is a homopolymer of ß-1,6-linked *N*-acetyl glucosamine residues, located in fibrous strands on the *S. epidermidis* cell surface, that has been described as crucial for the process of cell-to-cell adhesion and biofilm accumulation (Mack *et al.*, 1996b).

PIA mediates the contact between bacterial cells, resulting in the accumulation of a multilayered biofilm, constituting an important virulence factor of *S. epidermidis* (Heilmann *et al.* 1996). Besides functioning as an intercellular adhesin, PIA is also responsible for the haemagglutination of erythrocytes, a common property of *S. epidermidis* (Fey *et al.*, 1999). The molecule also protects the pathogen from innate host defence (Vuong *et al.*, 2004b).

PIA is encoded by the *icaADBC* gene locus, which consists of the *icaA*, *icaD*, *icaB*, and *icaC* genes (Heilmann *et al.*, 1996). IcaA and IcaD form a UDP- *N*-acetyl glucosamine-transferase

located in the cellular membrane. Then, another putative membrane protein, IcaC, is required for the formation of longer polymers and might be involved in the export of the growing PIA chain (Gerke *et al.*, 1998).

It has been shown that the expression of the *ica* locus is phase-variable (Ziebuhr *et al.*, 1997; Ziebuhr *et al.*, 1999). The molecular basis for this variation appears to be the insertion and excision of the transposon IS256 in the structural operon, although it is not clear whether these events are in any way regulated by changes in cell physiology (Ziebuhr *et al.*, 1999). Insertion of the IS256 in genes encoding proteins that regulate ica expression such as rsbU and sarA have also been reported (Conlon *et al.*, 2004). Thus, by contributing to the release of planktonic cells from mature biofilms, this phase variation property may have important implications in the pathogenicity of persistent and recurrent *S. epidermidis* infections (O'Gara and Humphreys, 2001). However, these mechanisms are reversible, i.e., after repeated sub-culturing, the biofilm-positive phenotype can appear from biofilm-negative inocula. In a recent report (Nuryastuti *et al.*, 2008), it was suggested that *S. epidermidis* clinical isolates can irreversibly switch from biofilm-positive to biofilm-negative phenotype by spontaneous mutations of the *lexA* gene, which results in deregulation of *recA* expression leading to deletion of *icaADBC*.

The variation in the expression of the intercellular adhesion genes (*icaADBC*) can be experimentally illustrated by a change of colony morphology on Congo red agar (CRA) and altered biofilm formation: the presence of the intercellular adhesion genes is correlated with the exhibition of black colonies on Congo red agar, whereas the adhesin-negative strains form red colonies (Ziebuhr *et al.*, 1997).

PIA represents a very unusual EPS molecule because some *N*-acetyl glucosamine residues become deacetylated, producing a positive net charge of the polymer. In fact, according to Vuong *et al.* (2005), the presence of deacetylated PIA is essential for biofilm formation, immune evasion, adhesion to epithelial cells and virulence in an animal model of implant infection. In order to prove the dependence of PIA for biofilm formation in *S. epidermidis*, Li *et al.* (2005), transferred a plasmid containing the *ica* locus to three ica-negative strains and through *in*

vitro biofilm assays and a rat central venous catheter infection model, confirmed the importance of the *ica* locus for biofilm production and pathogenesis of *S. epidermidis*. Other studies have also reported the importance of PIA production for virulence in animal biofilm infection models (Rupp *et al.*, 1999a; Rupp *et al.*, 1999b).

5.1.2 EPS production as a virulence factor

The production of EPS by *S. epidermidis* has been related to *in vivo* colonization of indwelling medical devices such as prosthetic joints, prosthetic cardiac valves or intravascular devices, among others (Diaz-Mitoma *et al.*, 1987; Quie and Belani, 1997) and thus it is considered an important virulence factor in staphylococcal infections (Nayak and Satpathy, 2000). In fact, CNS slime has been associated with sepsis, including intravenous-catheter-related bacteremia (Etienne *et al.*, 1988; Ishak *et al.*, 1985; Rupp and Archer, 1994).

The results reported in the work of Arslan and Özkardeş (2007), in which staphylococci clinical isolates were evaluated in terms of slime production and susceptibility to antibiotics, corroborate the fact that EPS production has an important role as a virulence marker for clinically significant *S. epidermidis* isolates. Bacterial strains that do not produce extracellular matrix are less adherent and less pathogenic (Katsikogianni and Missirlis, 2004). This matrix is especially important for events after the initial phase of adhesion, which include protection against phagocytosis, interference with the cellular immune response and reduction of antibiotic effects (Costerton *et al.*, 1999; Costerton, 1999).

In addition to slime production, multi drug resistance has also been observed as one of the virulence determinants in *S. epidermidis* (Younger *et al.*, 1997). Such antibiotic resistance may be partly due to the slow growth rate of bacteria within the biofilm or to the limited transport of nutrients, metabolites, and oxygen to and from the biofilm surface (Donlan and Costerton, 2002; Duguid *et al.*, 1992; Mah and O'Toole, 2001; Monzón *et al.*, 2002; Stewart and Costerton, 2001). In the study of Nayak *et al.* (2007), among 78.9 % of the adherent organisms isolated,

detected as slime positive, 47.4 % were identified as multi drug resistant. This more frequent multiple resistance to antibiotics among exopolysaccharide-forming strains was also verified in the work of Arciola *et al.* (2005b), where the resistance of 342 clinical strains of *S. epidermidis* from orthopaedic implant infections to a panel of 16 different antibiotics was investigated.

These studies emphasize the importance of extracellular matrix in the colonization of bacteria to host tissue by preventing access of host defence mechanisms, but also in the protection of antibiotics effect. The difficulty in eradicating a chronic infection associated with slime formation has thus been reported, and slime-producing bacteria have been shown to resist higher antibiotic concentrations than non-slime-producing bacteria (Gristina *et al.*, 1987).

5.1.3 Biofilms *versus* planktonic cells

Bacteria within biofilms are intrinsically more resistant to antimicrobial agents than planktonic cells due to lower rates of mass transport of antimicrobial molecules to the biofilm associated cells (Suci *et al.*, 1994) or because biofilm cells differ physiologically from planktonic cells (Evans *et al.*, 1991). Antimicrobial concentrations sufficient to inactivate planktonic organisms are generally inadequate to inactivate biofilm organisms, especially those deep within the biofilm, potentially selecting for resistant subpopulations (Donlan, 2001).

While it is generally assumed that the physiological activities of the bacterial cells in a well-mixed planktonic culture are uniform, in biofilms, chemical (De Beer *et al.*, 2004; Ramsing *et al.*, 1993; Rani *et al.*, 2007; Xu *et al.*, 1998; Zhang *et al.*, 1995) and physical (Huang *et al.*, 1998; Huang *et al.*, 1995; Wentland *et al.*, 1996) microscale heterogeneities have been reported. Chemical gradients of nutrients, waste products and signalling compounds develop within the biofilm, resulting in unique environmental niches (Stewart and Franklin, 2008). Bacteria within the biofilms can respond to these local environmental conditions in various ways, such as altering gene-expression patterns (Boles *et al.*, 2004; Cooper *et al.*, 2005; Drenkard and

Ausubel, 2002) by turning on or off certain genes in order to adapt to a particular biofilm location (Sauer *et al.*, 2002).

Some studies have been focused on the identification of differences between planktonic and sessile cells (Donlan 2001; Møller *et al.* 1995). A number of works have been done in the area of proteomics and genomics by comparing the protein and gene patterns of sessile and planktonic organisms (Lazazzera, 2005; Jouenne *et al.*, 2004). According with Jouenne *et al.* (2004), cells in highly structured matrix-enclosed communities express different protein patterns from their planktonic counterparts. Moreover, DNA microarray data suggest that biofilms may have a unique pattern of gene expression (Lazazzera, 2005). In a recent study of Wang *et al.* (2008), the transcriptional regulator *sarZ* was identified as a novel important determinant of biofilm formation and biofilm-associated infection, on the basis of its significant impact on the transcription of the biosynthetic operon for biofilm exopolysaccharide. The influence of *sarZ* in the expression of several virulence genes, such as genes responsible for lipases and proteases expression, resistance to an important human antimicrobial peptide, and hemolysis was also proved.

All these recent advances constitute an important basis for the development of anti-staphylococcal drugs and vaccines. Nevertheless, there is still much to comprehend in this area.

5.1.4 Aims

The main goal of the work described in this chapter was the study of biofilm composition and metabolic activity of eight *S. epidermidis* strains. For this purpose, total biofilm biomass was determined through crystal violet assay, cell concentration by colony forming units (CFUs) and biofilm matrix composition was assessed for polysaccharides and proteins content. Biofilm metabolic activity was evaluated by two distinct methods: glucose uptake and XTT reduction assays. Metabolic activity of cells in suspension was also determined in order to try to understand the differences between sessile and planktonic cells.

5.2 Materials and Methods

5.2.1 Bacterial strains

Eight *S. epidermidis* strains were studied in this work: 9142, 9142-M10, 1457, 1457-M10, IE186, IE214, IE75 and LE7, which were already described in section 2.2.1.

5.2.2 Media and growth conditions

For all the assays, growth conditions were followed according to the procedure described in sub-chapter 2.2.2.

5.2.3 Substrate preparation

Poly(methylmethacrylate) (PMMA) (Repsol, Brønderslen, Denmark), also known as acrylic, was used as substratum in the subsequent assays and prepared as described in section 2.2.3.

5.2.4 Biofilm formation

Each clean acrylic coupon was placed into an individual well of a 6-well tissue culture plate containing 4 ml of TSB enriched with 0.25% of glucose (Merck). For every strain, a 50 µl $1x10^9$ cells/ml inoculum was added per well. The plates were incubated for 8 days at 37 °C in an orbital shaker (120 rpm) and the spent medium replaced by fresh TSB + 0.25% glucose every 12 h (fed-batch mode).

All experiments were performed in triplicate, in three independent occasions.

5.2.5 Total biomass quantification

The total attached biomass to the coupons was measured by crystal violet staining. After biofilm formation, the coupons containing the biofilm were removed from each well and non-adherent cells removed by washing the coupons twice with sterile ultra-pure water. Coupons were then transferred to new well plates and fixated with 4 ml of methanol, which was removed after 15 min of contact. After withdrawing the methanol, the coupons were allowed to dry at room temperature before adding 4 ml of crystal violet (1%, v/v). After 5 min coupons were gently washed with sterile, ultra-pure water and transferred to a new 6-well tissue plate. Acetic acid (33%, v/v) was added to each well to release and dissolve the stain. The eluted dye was removed from each well and placed in a 96-well microtiter plate and its absorbance was read in triplicate in an ELISA reader (Bio-Tek® Synergy HT, Izasa, Portugal) at 570 nm.

5.2.6 Biofilm cell concentration

The biofilm cell concentration was determined by CFUs enumeration. After biofilm formation as described in section 5.2.4, the acrylic coupons with biofilm were washed twice with NaCl 0.9%, and then the biofilm from each coupon was scrapped and resuspended into 25 ml of NaCl + 5 ml 0.05% Tween 20 (Merck) and vortexed vigorously for 5 min for disruption of matrix. Next, the suspension was sonicated (20 s with 22% of amplitude), centrifuged (5 min, 10 500 g, 4 °C), resuspended in 30 ml of NaCl 0.9% and sonicated again (20 s, 22% amplitude) to promote biofilm disaggregation. The absorbance was read at 640 nm. Then 100 µl of the appropriate dilution of each suspension were plated onto TSA plates, in triplicate. Prior to colony enumeration the plates were incubated for 24 h at 37° C.

5.2.7 Scanning Electron Microscopy (SEM)

The coupons with the biofilms were treated for SEM observation as described in sub-chapter 2.2.6. All photographs were taken using a magnification of x500.

5.2.8 Biofilm matrix extraction

The extraction of the biofilm extracellular matrix was performed using the cation exchange Dowex resin (50x8, Na^+ form, 20-50 mesh Aldrich-Fluka 44445), according to the procedure described by Frølund *et al.* (1996). Prior to extraction, the Dowex resin was washed with the extraction buffer [2mM Na_3PO_4 (Merck); 4 mM NaH_2PO_4 (Merck); 9 mM NaCl and 1 mM KCl (Merck); pH 7.0]. Then, the biofilms previously scrapped off the acrylic coupons were washed with phosphate buffer (0.01M; pH 7.0) and centrifuged for 5 min, at 9 000 g. The extraction was performed using 2 g of washed Dowex resin and 10 ml of extraction buffer per g of biofilm and stirring for 2 h at 400 rpm and -4 °C. The extracellular polymers (supernatant) were obtained by centrifugation at 9000 g for 20 min.

5.2.8.1 Proteins quantification

The concentration of the proteins extracted from the matrix was determined using the Bicinchoninic Acid Protein Assay Kit (BCA) (Sigma) (Smith *et al.*, 1985), with bovine serum albumin (BSA) as standard. Briefly, 25 µl of each sample (in triplicate) were placed into wells of a 96-well microtiter plate. Then, 200 µl of a work solution composed of 50 parts of BCA A solution and 1 part of BCA B solution were added to the wells and the plate was incubated for 30 minutes, at 37 °C. After cooling down to room temperature, the absorbance was read in an ELISA reader at 562 nm.

5.2.8.2 Polysaccharides quantification

The biofilm matrix polysaccharides were quantified by the method of Dubois *et al.* (1956). Briefly, 0.5 ml of aqueous phenol (5 % w/v) (Panreac Quimica SA) were added to 0.5 ml of the sample and agitated. Then, 2.5 ml of sulphuric acid (Aldrich-Fluka, Germany) were added to the mixture, agitated, and left to air-dry at room temperature for 15 min. The absorbance was read in a spectrophotometer (Spectronic 20 Genesys, Spectronic Instruments, USA) at 490 nm. The polysaccharides concentration present in the samples was determined through a calibration curve using glucose as standard.

5.2.9 Glucose uptake

5.2.9.1 Biofilm

In order to remove the cells loosely attached and residual medium, each coupon was gently transferred to a 100 ml glass beaker containing distilled water, and was allowed to rest there for approximately 10 s. Afterwards, a new transfer was made to a different glass beaker containing distilled water, followed by a third transfer 10 s later (Cerca *et al.*, 2004). Then the coupons were transferred to a new 6-well tissue plate containing 5 ml of 0.10% glucose (1g/l) solution. The glucose concentration in the medium was measured immediately after transferring the coupons and also 60 min later, maintaining the plates under slow agitation. Glucose was quantified with the enzymatic kit Glucose - TR (Spinreact, SA, Spain) using the ELISA reader at 505 nm.

5.2.9.2 Planktonic cells

All strains were incubated in 15 ml of TSB (inoculated with bacteria grown on TSA plates not older than 2 days) and grown for 24 (±2) h at 37 °C in an orbital shaker (130 rpm). Then, 100 µl of each cell suspension were transferred to 60 ml of fresh TSB and incubated for 18 h (to reach late exponential phase) at 37 °C and 130 rpm. Cells were harvested by centrifugation (for 5 min at 10 500 g and 4 °C) and resuspended in TSB to the same concentration as previously determined by CFU plating of biofilm cells. For all strains, 1 ml of the suspension obtained was centrifuged at 9 500 g and the pellet resuspended in 1 ml of 0.10% glucose solution. These samples were then transferred to the wells of a 6-well tissue culture plate containing 4 ml of 0.10% glucose solution. Glucose present in the medium was measured at time zero and after 60 min with the enzymatic kit Glucose - TR, as described for biofilm assays.

5.2.10 XTT reduction assay

5.2.10.1 Biofilm

The quantification of biofilm cellular activity was assessed through the XTT reduction assay as previously described (Logu *et al.*, 2003), with some modifications. Accordingly, the coupons containing the biofilm were washed twice with 4 ml of 0.9% NaCl and transferred to a new microtiter plate with each well containing 1 ml of XTT {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2Htetrazolium hydroxide}(200 mg/l) solution (Sigma, USA) plus PMS (phenazine methosulfate) (20 mg/l) (Sigma). The microtiter plates were incubated under agitation (120 rpm) for 3 h at 37 °C, in the dark. Following that, each solution was centrifuged for 5 min at 9 500 g and the absorbance read at 490 nm.

5.2.10.2 Planktonic cells

Cells were inoculated in 15 ml of TSB and grown for 24 (±2) h at 37 °C in an orbital shaker (130 rpm). Then, 100 µl of each cell suspension were transferred to 60 ml of fresh TSB and incubated for 18 h (to reach late exponential phase) at 37 °C and 130 rpm. After incubation, cells were harvested by centrifugation at 10 500 g for 5 min at 4°C, washed twice with NaCl 0.9% and resuspended in NaCl 0.9% to the same cellular concentration as previously determined by CFU plating of biofilm cells. For all strains, 1 ml of the suspension obtained was centrifuged at 9 500 g and the pellet resuspended in 900 µl ml of sterile ultra-pure water. To each sample, a 100 µl aliquot of the solution constituted by XTT (200 mg/l) (Sigma) and PMS (20 mg/l) (Sigma) was added. The suspensions were then incubated in the dark for 3 h at 37° C under agitation of 120 rpm. Following that, each solution was centrifuged for 5 min at 9 500 g and colorimetric changes were measured in the microtiter plate reader at 490 nm.

5.2.11 Statistical analysis

The results from all the assays were compared by the one-way analysis of variance by applying the Levene's test of homogeneity of variances and the Tukey multiple comparisons test, using SPSS (Statistical Package for the Social Sciences Inc., Chicago). All tests were performed with a confidence level of 95%.

5.3 Results

5.3.1 Biofilm formation

Strain variability in terms of biofilm formation ability was assessed through the crystal violet assay (Figure 5.3). It can be observed that the PIA-negative strains 9142-M10 and 1457-M10 produced very low biofilm biomass amounts in comparison with their wild-type strains, 9142 and 1457, respectively ($p<0.001$, for both pairs of strains). In fact, strains 9142 and 1457 were the highest biofilm producers being significantly different from almost all others ($p<0.05$) (except IE214). Strain IE186 also produced moderately high amounts of biomass while strains IE75 and LE7 formed thinner biofilms, with lower amounts of biomass on the acrylic coupons ($p<0.05$). Thus, strains 1457, 9142, IE214 and IE186 can all be considered as being good biofilm producers.

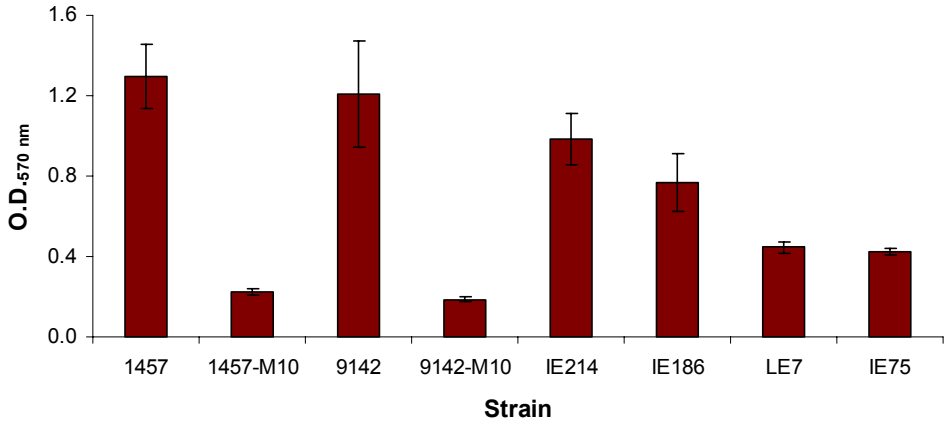


Figure 5.3 Biofilm biomass of the eight *S. epidermidis* strains, accumulated over 8 days, expressed as crystal violet optical density ($O.D._{570\ nm}$). Results represent means plus standard deviations (errors bars) of three independent experiments.

Figure 5.4 presents SEM images of 8-days biofilms of all the strains grown in TSB enriched with 0.25% glucose.

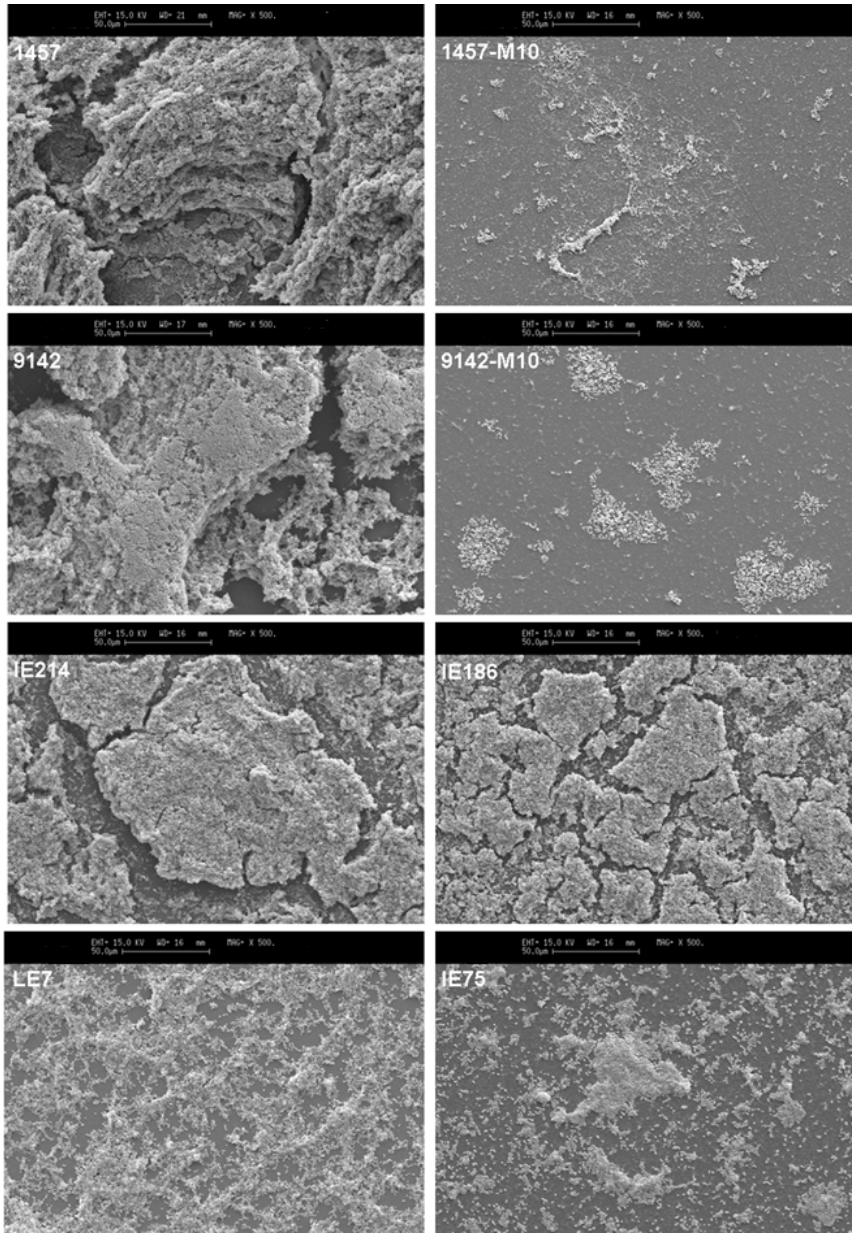


Figure 5.4 SEM photomicrographs of a general view of mature biofilms (8 days-old) of *S. epidermidis* 1457; 1457-M10; 9142; 9142-M10; IE214 ; IE186; LE7 and IE75 formed on acrylic coupons. Magnification ×500, bar = 50 µm.

The images reveal the thickness of biofilms, particularly high for strains 9142 and 1457. Biofilms of strains IE186 and IE214 also produced considerable biofilms, although with fewer bacterial layers and a more smooth appearance. These images also corroborate the crystal

violet results, since it is visible the extremely low amount of cells attached to the abiotic surface for the non-producing biofilm strains 9142-M10 and 1457-M10. The low ability of strains LE7 and IE75 to produce biofilm is, as well, visible in the photomicrographs.

5.3.2 Biofilm cell concentration

As was stated before, the glucose uptake and XTT reduction assays to determine the metabolic activity of cells in suspension were performed with the same cellular concentration of the biofilm assays. This cellular concentration was determined by CFU plating of biofilm cells and is presented in Figure 5.5, in terms of Log CFU/ml, for the eight *S. epidermidis* strains.

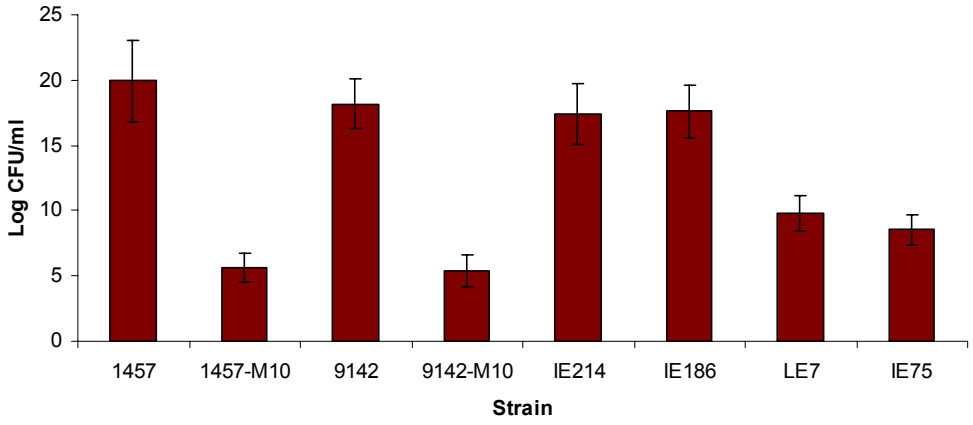


Figure 5.5 Biofilm cellular concentrations of the eight *S. epidermidis* strains, expressed as Log CFU/ml, used for the glucose uptake assays and XTT reduction assays in the sessile and planktonic form. Results represent means plus standard deviations (errors bars) from three independent experiments.

As it can be seen, strains 1457, 9142, IE214 and IE186 presented the highest cellular concentrations with no statistical differences between the respective values, but statistically different from the four remaining strains. It must be noted that these are also the strongest biofilm producer strains. The number of cells on LE7 and IE75 biofilms is significantly higher

($p<0.05$) than the number of cells of strains 1457-M10 and 9142-M10 on the acrylic surface, which were the strains with the lowest number of cells attached to the inert surface (Figure 5.5).

5.3.3 Characterization of biofilm matrix

Afterwards, in order to evaluate the contribution of matrix composition to the total biofilm biomass, the exopolymeric matrix of the four higher biofilm producer strains (IE214, IE186, 9142 and 1457) and of a lower biofilm producer strain (LE7) was extracted and quantified in terms of polysaccharides and proteins content. The amount of biofilm formed by the remaining strains was so low that did not allowed exopolymeric matrix extraction. According to the results presented in Figure 5.6, the matrix composition of biofilms varies significantly with the strain. Concerning polysaccharides content, strain 1457 was the one that produced larger amounts of these molecules, immediately followed by strain IE214, and differing significantly from strains 9142 and IE186 ($p<0.05$). *S. epidermidis* LE7 produced the lowest amount of polysaccharides comparing with the other four strains ($p<0.05$).

Regarding the presence of proteins in the extracellular matrix, *S. epidermidis* 9142 was the strain with the significant highest content ($p<0.05$), followed by strain IE214, then by strains 1457 and IE186, these two with very similar values, and finally by strain LE7, with the lowest protein concentration ($p<0.05$).

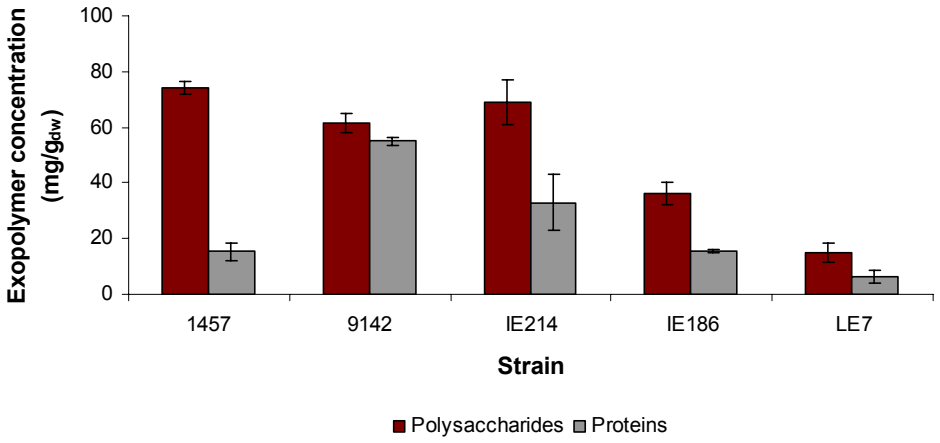


Figure 5.6 Concentration [mg *per* g of biofilm dry weight (dw)] of polysaccharides (glucose as standard) and proteins (BSA as standard) extracted by Dowex resin method from biofilms of *S. epidermidis* strains 9142, IE214, IE186, 1457 and LE7.

It should be noted that polysaccharides and proteins were determined against different standards. Consequently, the concentrations determined are not absolute values. Thus, comparing in relative terms, the ratio of polysaccharides/proteins (Table 5.1) determined for the biofilm matrix of strain 1457 was 4.3 times greater compared to the same ratio in strain 9142, 2.3 times higher compared to polysaccharides/proteins ratio in IE214 matrix, and approximately 2 times than in strains IE186 and LE7.

Table 5.1 Ratio of polysaccharides/proteins (mg/mg) obtained in the biofilm matrix of the eight *S. epidermidis* strains studied.

Strain	Polysaccharides/Proteins (mg/mg)
1457	4.9
9142	1.1
IE214	2.1
IE186	2.4
LE7	2.4

5.3.4 Glucose uptake in biofilms and in planktonic cells

The present results show that biofilm glucose uptake can differ inside the species (Figure 5.7). In absolute terms, *S. epidermidis* 9142 biofilms removed from the liquid phase the highest amount of glucose (23.98%) ($p<0.05$), followed by the biofilms of strains IE186 (12.70%) IE214 (8.29%) and 1457 (6.87%), while strains 1457-M10 and 9142-M10 displayed significantly ($p<0.05$) less glucose uptakes (3.53 and 4.09% respectively). On the other hand, strains IE75 and LE7 displayed even lower glucose uptake values (2.14 and 1.11%, respectively).

The % of glucose uptake by planktonic cells (Figure 5.7) shows the same tendency but is significantly ($p>0.05$) higher than their biofilm counterparts, for most of the strains under study (9142, 1457, IE186, IE214, LE7 and IE75). Strains 1457-M10 and 9142-M10 demonstrated higher values of glucose uptake in suspension, but in a non-significant way ($p>0.05$), comparing to biofilm results. Also, contrary to what was observed in sessile cells, in the planktonic form strains IE75 and LE7 displayed a higher glucose uptake compared to strains 1457-M10 and 9142-M10.

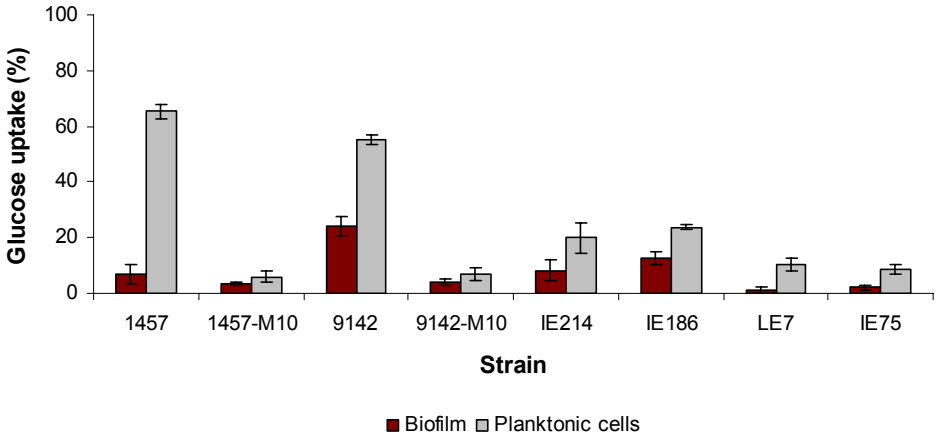


Figure 5.7 Glucose uptake expressed in % for the eight *S. epidermidis* strains in biofilm and in the planktonic form. Results represent means plus standard deviations (errors bars) from three independent experiments.

5.3.5 XTT reduction in biofilms and in planktonic cells

The results of the XTT reduction assay, indicative of the metabolic activity of cells within the biofilm and in the planktonic form are presented in Figure 5.8. Concerning biofilms, strain IE186 showed the highest cellular metabolic activity, followed by strains 9142, IE214 and 1457 ($p<0.05$). Cells within biofilms of strains 9142-m10, 1457-M10, IE75 and LE7 revealed significant lower levels of metabolic activity ($p<0.05$) comparing to others strains.

In respect to the planktonic state, cells behaviour was fairly distinct from their sessile counterparts (Figure 5.8). As it can be seen, in the planktonic form, cells are metabolically more active ($p<0.05$) than within biofilms for most of the strains under study (1457, 9142, IE214, IE186, LE7 and IE75). Thus, the two strains with the highest values of metabolic activity were *S. epidermis* 1457 and 9142 (2.53 and 2.32 optical density values, respectively), immediately followed by strains IE186 (2.01) and IE214 (1.83). As observed in the glucose uptake assays, *S. epidermidis* LE7 and IE75 planktonic cells are slightly more active than the mutant strains 9142-M10 and 1457-M10 cells, which are in a similar metabolic state either in biofilm or in the planktonic form ($p>0.05$).

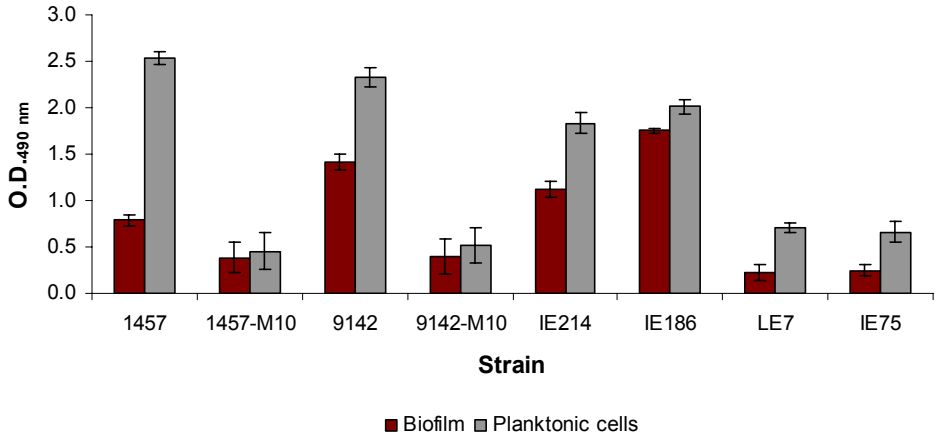


Figure 5.8 Cellular metabolic activity, measured by XTT reduction assay ($O.D._{490\ nm}$) from the biofilms of the eight *S. epidermidis* strains studied: 1457, 1457-M10, 9142, 9142-M10, IE214, IE186, LE7 and IE75. Results represent means plus standard deviations (errors bars) from three independent experiments.

5.4 Discussion

In this chapter, *S. epidermis* biofilms formed on acrylic coupons were characterized in terms of total attached biomass, cellular concentration and extracellular proteins and polysaccharides content. In a second part of the work, metabolic activity of biofilms was evaluated through two different methods: quantification of glucose uptake and XTT reduction assays. Metabolic activity of cells in suspension was also determined in order to try to understand the differences between sessile and planktonic cells.

Thus, in the first part of this work the biofilm formation capability of the eight strains was evaluated through crystal violet staining, which allows the quantification of the total biomass attached to the acrylic coupons. According to the results (Figure 5.3), there is strain variability in terms of biofilm formation ability among *S. epidermidis* species. Strains 1457 and 9142 formed very thick, patchy biofilms (Figure 5.4), while strains IE214 and IE186 formed smoother biofilms however, with significant amounts of biomass attached to the abiotic surface. The formation of thick, mature biofilms is a very important feature of *S. epidermidis* pathogenicity, indicating the strong virulence of these strains (Arciola *et al.*, 2005c; Mack *et al.*, 2000; Mack, 1999; Rupp *et al.*, 1999b). The low ability of biofilm formation of strains LE7 and IE75, demonstrated by the crystal violet assays, was as well perceptible in Figure 5.3, where much less cellular aggregates were visible on the surface, comparing to the good biofilm producer strains. The non-biofilm producing character of the isogenic mutant strains 1457-M10 and 9142-M10 was also demonstrated either by the low levels of total biomass quantified by the crystal violet assay and by the photomicrographs, where only few bacterial cells attached to the inert surface were visible. The residual biofilm production by these PIA-negative mutant strains corroborates the importance of this molecule in the second phase of *S. epidermidis* biofilm accumulation, by mediating cell-to-cell adhesion (Mack *et al.*, 1994). In fact, PIA is expressed by the majority of biofilm-producing clinical *S. epidermidis* isolates (Mack *et al.*, 1996b; Mack *et al.*, 1994; Mack *et al.*, 1992), which confirms its importance as a virulence factor in *S. epidermidis* infections.

Therefore, one of the main conclusions of this first part of the work is that there is heterogeneity between biofilms of strains of the same staphylococci species. This is in agreement with previous studies that showed that the amount of biofilm produced by individual *S. epidermidis* strains is highly variable phenotypically and regulated by several factors (Cramton *et al.*, 2001; Mack *et al.*, 2004). Also, these results showed that crystal violet staining is a reliable biofilm quantifying method like the biofilm dry-weight determination or CFU plating having the advantage of being used directly in the biofilm, with no need to remove it from the surface. This feature becomes the method more easily and expedite, i.e. providing the results almost immediately, with a great objectivity and accuracy.

The next step was the determination of biofilm cell concentration by CFU plating (Figure 5.5). The objective was to use the same number of cells in the glucose uptake and XTT reduction further assays in the sessile and planktonic form in a way that an accurate comparison between these two cellular states could be made. Analysing Figure 5.3 and Figure 5.5, a good correlation could be seen between total attached biomass and number of cells, i.e., both methods obtained almost the same order of results according to the strain. Hence, the strongest biofilm producers were, in decreasing order: 1457> 9142> IE214> IE186> LE7> IE75> 9142-M10> 1457-M10. Concerning the number of cells in each biofilm, the same order was obtained, excepted for strains IE186 and IE214, whose order was switched, despite the values were not significantly different. Therefore, the number of cells within a biofilm can also be used as a measure of biofilm formation capability.

The method chosen for EPS extraction can strongly influence the yield and chemical composition of extracellular polymers, and also can lead to different degrees of cellular lysis and consequent contamination of exopolymers with intracellular components (Bura *et al.*, 1998; Frølund *et al.*, 1996). Several methods have been reported for extracting exopolymers from activated sludge flocs, some of them also assayed in biofilms (Jahn and Nielsen, 1995). These include high-speed centrifugation, steaming, ultrasonication and the use of chemical agents such as: NaOH or EDTA (Brown and Lester, 1980), Tris/HCl buffer (Goodwin and Foster, 1985),

phosphate buffer and heat (Schmidt and Ahring, 1994), formaldehyde (Fang and Jia, 1996), cation exchange Dowex resin (Frølund *et al.*, 1996) and glutaraldehyde (Azeredo *et al.*, 1998). In the present work, Dowex extraction was the method chosen due to the fact that is a very smooth method, with no significant cell lysis for up 2 h of extraction (Jahn and Nielsen, 1995; Jahn and Nielsen, 1998). In fact, in the work of Azeredo *et al.* (2003) very small amounts of ATP, used as an indicator of cell lysis, were measured even after 4 h of extraction.

Thus, concerning biofilm matrix composition of the five more representative biofilm forming strains, in terms of polysaccharides and proteins content and, according to the results obtained that revealed different polysaccharides/proteins ratios among the different extracellular matrices studied (Figure 5.6; Table 5.1), it seems clear that the matrix composition of biofilms varies significantly according to the strain (Chaignon *et al.*, 2007). It must be noted that, between strains whose matrix was extracted, *S. epidermidis* 1457 was the strain that produced the larger amount of biofilm and strain LE7 was the lowest biofilm producer (Figure 5.3), and these are also the highest and the lowest polysaccharides producers, respectively. This suggests a certain degree of correlation between exopolysaccharides production and total amount of biomass formed. These results are in accordance with the work of Arslan and Özkardeş (2007), which suggests that the production of an extracellular matrix among a majority of clinical CNS isolates and its association with the strains ability to produce thicker biofilms may suggest a role of slime in pathogenesis.

In what concerns to proteins produced in the biofilm extracellular matrix, it seems that, depending on the strain, proteins might constitute an important part of the biofilm matrix, which is the case of *S. epidermidis* 9142. However, no direct relation was found between the proteins concentration determined and the amount of biofilm formed (Figure 5.3). These proteins detected in the biofilm matrix are probably some cell wall associated proteins released to the extracellular matrix but also secreted enzymes with proteolytic activity whose function is to help in the invasiveness into host tissues (Oleksy *et al.*, 2004). A more detailed study of biofilm matrix proteins is enclosed in chapter six.

The importance of the extracellular matrix to the biofilm recalcitrance is well known (Al-Fattani and Douglas, 2006; Donlan, 2000; Mah and O'Toole, 2001). According to some studies (De Beer *et al.*, 1994; Hoyle *et al.*, 1992; Suci *et al.*, 1994) the biofilm matrix can act as a barrier to the penetration of antibiotics. In the work of Cerca *et al.* (2005a), the inhibition of biofilm formation of three *S. epidermidis* strains (including the strains of the present study, 9142 and IE186) on acrylic, in the presence of sub-MICs of three antibiotics and other three combinations of antibiotics, was tested. In 4 of the 6 situations studied, strain 9142 was more resistant to the antibiotics effect than strain IE186. Thus, the results of the present work, that show the richer composition of the *S. epidermidis* 9142 extracellular matrix, in terms of polysaccharides and proteins content, compared to strain IE186, might be related to its resistance to antibiotics. These biofilm cells are immersed in a thick matrix which provides the bacterial population protection from the host defence mechanisms and antimicrobial agents. Matrix polymers seem, thus, to largely contribute towards biofilm defence, a fact that, according to the present results, appears to be partially related with the amount of exopolymers that each strain has the ability to produce.

However, the failure of antimicrobial agents to penetrate the biofilm matrix can not be seen as the sole reason of biofilms recalcitrance (Al-Fattani and Douglas, 2006). A number of studies have demonstrated that reductions in the diffusion coefficients of antibiotics within biofilms are insufficient to account solely for the observed changes in susceptibility (Al-Fattani and Douglas, 2006; Gilbert *et al.*, 2002) given that antimicrobial agent access is supported, such as in the case of nutrients and oxygen, by the presence of water channels in the biofilm structure. Thus, the different susceptibilities of *S. epidermidis* 9142 and IE186 to antibiotics can probably be due to factors that are intrinsic to each individual strain. Depending on the chemical nature of both the antimicrobial agent and the matrix material, phenomena of drug adsorption or neutralization (Al-Fattani and Douglas, 2006) can be favoured in 9142 biofilms. The expression of surface antigens (Cramton *et al.*, 1999) as well as the possible existence of certain percentage of persister cells, which are extremely tolerant to antibiotics (Keren *et al.*, 2004), can also help to

explain the lower susceptibility to antibiotics by this strain. A better capacity of horizontal transfer of resistance genes and recombination potential between the biofilm embedded bacteria has, as well, been referred as one of the reasons of the success of some *S. epidermidis* strains as pathogens in hospitals (Ziebuhr *et al.*, 2006). Besides, *S. epidermidis* 9142 showed lower levels of metabolic activity, a fact that has also been pointed as related to biofilm antibiotics resistance (Williams *et al.*, 1997).

In a second part of the work, metabolic activity of biofilms was evaluated through the measurement of glucose uptake as well as by XTT reduction assay. The results were also compared to those obtained for planktonic cells. From the analysis of the biofilm glucose uptake results (Figure 5.7) it can be said that biofilm glucose uptake is dependant on the strain. It should be pointed out that the glucose uptake determined for strain 1457 was the lowest compared to the glucose uptake of strains 9142, IE186 and IE214 biofilms. Therefore, the higher amount of polysaccharides detected in the extracellular matrix of strain 1457 may be responsible by the low concentrations of nutrients, such as glucose, that reach bacteria within the biofilm, reducing their uptake and, as a consequence, decreasing the levels of metabolic activity. Another important aspect to retain from these results is that the amount of glucose uptake of biofilm cells is always lower compared to the glucose uptake from cells in suspension. In fact, the reduction of the percentage of glucose uptake from planktonic cells to biofilm cells is on average threefold for the highest biofilm producing strain (*S. epidermidis* 1457). The only strains in which this difference is non-significant ($p>0.05$) are the mutant strains 1457-M10 and 9142-M10, whose total biofilm biomass determined was very low, as well as the number of cells. It has already been shown that cells in sessile form display a lower metabolic activity (Walters *et al.*, 2003). The surface attached populations, involved by the extracellular matrix, are exposed to a different nutrient environment from the planktonic cells (Møller *et al.*, 1995). Thus, lower levels of glucose uptake in biofilms might be indicative of less nutrients availability, which consequently leads to a lower metabolic state and slow growth, which has been considered one of the main reasons for biofilm resistance and success against antimicrobial agents (Fux *et al.*, 2005).

Comparing the results obtained, in terms of exopolysaccharides production (Figure 5.4), and biofilm activity determined by XTT reduction assay (Figure 5.8), it seems clear that a strong production of exopolysaccharides can lead to a decrease in the metabolic activity of cells. It has been suggested that the biofilm matrix itself could constitute a barrier to the penetration and diffusion of the nutrients, oxygen and even antibiotics (De Beer *et al.*, 1994; Henriques *et al.*, 2006; Hoyle *et al.*, 1992; Suci *et al.*, 1994). However, other studies suggest that diffusion of nutrients and oxygen through the biofilm matrix is almost equivalent to water (Stewart *et al.*, 1998). This makes sense taking into account that water is considered to be the major component of the biofilm matrix - up to 97% (Zhang *et al.*, 1998).

Thus, the present results obtained are most probably due to the fact that in biofilms with larger quantities of EPS the amount of metabolically active cells is less concentrated, leading to lower levels of total biofilm activity, measured by the XTT reduction assay. Besides, biofilms exhibit considerable structural, chemical and biological heterogeneity, with concentration gradients of nutrients, oxygen and signalling compounds. As a result, cells within a biofilm are in a wide range of physiological states (Stewart and Franklin, 2008). This can be explained by the fact that nutrients and oxygen, e.g., are consumed by cells in the upper layers of the biofilm, leading to a decrease in their concentration with increasing depth into the biofilm and distance from the nutrient source (Stewart and Franklin, 2008). Thus, on thicker biofilms, the concentration of nutrients that will reach the basal cell layers will be minimal, due to their previous consumption by cells in the outer layers, while in thinner biofilms, higher amounts of nutrients will reach these deeper cell layers. Consequently, the metabolic activity of cells in different locations within the biofilm will be necessary different, which will ultimately result in different total metabolic rates between biofilms with different thickness. Nonetheless, diffusional limitations due to the mesh generated by the EPS must also contribute, at least in some extent, to the metabolic activity final results. This is corroborated by the present results that showed that *S. epidermidis* 1457 and IE214, both with substantial exopolymeric matrices, were the strains that revealed the lowest levels of metabolic activity.

In the case of strain LE7, this effect was not so pronounced due to the lower amounts of polysaccharides production as well as to the low cellular concentration within the biofilm, compared to the strains to which exopolymers extraction was made. These facts leaded to the formation of a thin biofilm matrix, which was not sufficient to affect the metabolic levels of the few cells within the biofilm. However, it should be pointed that both PIA negative strains showed slightly higher levels of biofilm metabolic activity than strain LE7, either in glucose uptake or in XTT reduction assays. This can be explained by the fact that these strains do not produced extracellular matrix and therefore, the cells attached to the surface are more available to receive nutrients, and their metabolic rates are almost not altered, in comparison to their planktonic state.

Therefore, comparing the results obtained in terms of total biofilm mass and biofilm activity (glucose uptake or XTT reduction) it can be concluded that biofilm metabolic activity is not solely dependent on the cell number but also, and probably most important, on the thickness of the extracellular matrix formed. According to the work of Kuhn *et al.* (2003), in the case of a mature biofilm, the number of total cells might be high but their activity can be low, and it cannot be assumed that there is necessarily a linear relationship between the number of cells and the colorimetric signal emitted by XTT reduction. This is probably due to the fact that most of the total biofilm biomass of the strongest biofilm producing strains is composed of higher proportions of EPS (Figure 5.4). As a result, the metabolically active cells within the biofilm become more diluted, their concentration diminishes, and the resultant colorimetric signal is weaker, thus justifying the lower levels of metabolic activity. Thus, XTT reduction assay can not be seen as a method to quantify biofilm formation, as previously suggested (Jin *et al.*, 2003) since it measures metabolic activity, which relies on the localization of the cell inside the biofilm. This assay should be used as a complementary method to the crystal violet assay in order to allow the most accurate possible characterization of biofilms. Also, the results of the glucose uptake assay, herein developed, correlated well with the XTT reduction assay results and therefore can as well be considered a suitable method to study metabolic activity of planktonic and sessile cells.

Concerning glucose uptake and XTT reduction results from planktonic cells it is important to note that a strong correlation is observed between the number of cells and the absorbance of both metabolic activity evaluation methods, for all the strains studied. These results reinforce the fact that cells within a biofilm, enclosed in an exopolymeric matrix is determinant for biofilm physiology and metabolic activity.

5.5 Conclusions

This work provided a reliable approach to lead to a better understanding of biofilms composition and metabolic activity. One of the main conclusions of this work is the fact that *S. epidermidis* strains revealed different abilities of biofilm formation, i.e., biofilm formation is strain dependent. In fact, some strains were able to form thick, mature biofilms, which is very important once it is one of the pathogenic virulence factors of *S. epidermidis* species.

Biofilm extracellular matrix composition, in terms of polysaccharides and proteins content, is also strain dependent and a virulence factor and, overall, the results herein presented suggest that the production of extracellular polymers largely affects the biofilm total biomass as well as cellular metabolic activity. XTT reduction assay and determination of glucose uptake by cells in suspension and in biofilm are both expedite methods to evaluate the different behaviour of *S. epidermidis* strains in both forms. Strains producing larger amounts of polysaccharides formed thicker biofilms, with higher total biomass and lower metabolic activity. Extracellular protein production seems not to be so significant for biofilm total biomass. According to both methods, planktonic cells are in a higher metabolic state comparing to sessile cells, which seems dependable on strain specific properties of the biofilm such as the cell concentration and the thickness of the extracellular matrix produced.

CHAPTER 6 - Cell wall and extracellular matrix proteins related to *Staphylococcus epidermidis* adhesion and biofilm formation

Cells within biofilms present protein profiles different from those of their planktonic counterparts. Their depiction is therefore of extreme importance for the definition of new targets for bacterial infections control, and role in cell adhesion and biofilm formation processes. In this chapter, the protein profile of the cell wall extracts of eight *Staphylococcus epidermidis* strains is analysed. The protein pattern of the extracellular matrix of *S. epidermidis* good biofilm producer strains is also drawn. The main goal was to relate these protein patterns with their bacterial adhesion and biofilm forming ability.

6.1 Introduction

Some of the most important features concerning adhesion and biofilm formation of *Staphylococcus epidermidis* have been discussed in previous chapters. In the present chapter, an insight in to the role of cell wall (CW) proteins and proteins excreted to the biofilm extracellular matrix (EM) in *S. epidermidis* virulence is given. As has already been mentioned along this thesis, *S. epidermidis* infection of indwelling devices comprises two main steps: *S. epidermidis* cells adhesion to the biomaterial surface and cellular accumulation, both leading to biofilm formation, the most relevant pathogenic mechanism of staphylococcal infection (Batzilla *et al.*, 2006; Handke *et al.*, 2004).

Various cell surface-associated macromolecules, including proteins, have been recognized as being closely involved in these phases (Sun *et al.*, 2005). The CW anchored proteins of Gram-positive pathogens constitute a family of surface exposed proteins that often interact with targets in the host (Schneewind *et al.*, 1995). These interactions are frequently important for bacterial adherence and evasion from the host immune system. According to their involvement in the two main steps of biofilm formation these molecules have been divided in two main groups: proteins involved in primary attachment to the surface and proteins related to bacterial cell and biofilm accumulation.

6.1.1 Primary attachment

When bacteria first attach to the biomaterial, the first adhesion events involve non-specific physicochemical forces, such as hydrophobic interactions and van der Waals forces (Hogt *et al.*, 1983), but are also mediated by several proteins on the bacterial surface that establish the initial contact with the new environment within the host (Josefsson *et al.*, 2001; Navarre and Schneewind, 1999; Swiatlo *et al.*, 2003). In fact, most likely, bacterial cell surface hydrophobicity is determined for the most part by surface-associated proteins (Vadyvaloo and

Otto, 2005).Those involved in the first process include specific adhesins such as staphylococcal surface proteins (SSP-1 and SSP-2) (Veenstra *et al.*, 1996) and the major autolysin AtlE (Heilmann *et al.*, 1997).

SSP-1 and SSP-2 have been described as specific adhesins that contribute to the mediation of *S. epidermidis* 354 to biomaterials, namely polystyrene, and that are localized on the cell surface and on fimbria-like structures (Timmerman *et al.*, 1991; Veenstra *et al.*, 1996). According to Veenstra *et al.* (1996), SSP-1 and SSP-2 seem to play a structural role in providing an interaction interface for polymerization of fiber-like structures, in addition to a role in adhesion. SSP-1 exhibits a molecular mass of approximately 280 kDa, whereas SSP-2 has an estimated molecular mass of 250 kDa.

The major autolysin AtlE, which helps cells binding to bare polymer surfaces via hydrophobic interactions, is a 148 kDa protein, composed by two bacteriolytically active domains, a 60 kDa amidase and a 52 kDa glucosaminidase domain, generated by proteolytic processing (Heilmann *et al.*, 1997). The amino acid sequence exhibits a high similarity (61% identical composition) to the *S. aureus* autolysin Atl. The 60 kDa amidase is located at the cell surface, which constitutes the prerequisite for its function as an adhesin. In addition, this protein is also capable of binding to vitronectin, a host matrix protein, which indicates an involvement of AtlE during later stages of adherence, and not only in the primary attachment phase. The importance of AtlE in *S. epidermidis* pathogenicity was demonstrated by the work of Rupp *et al.* (2001), with an intravascular catheter-associated infection rat model, which showed that an *atlE* mutant strain was significantly less virulent than the wild-type. Another important function of AtlE was recently reported by Qin *et al.* (2007). According to this study, most of the extracellular DNA detected in *S. epidermidis* cultures and biofilms is generated through activity of the autolysin AtlE, by lysis of a subpopulation of the bacteria, and the extracellular DNA promotes biofilm formation of the remaining population.

The Bhp protein is a CW anchored protein, homologue to the biofilm-associated protein (Bap) of *S. aureus* that also seems to promote primary attachment to a biomaterial surface

(Cucarella *et al.*, 2001). All members of the Bap family seem to share the following characteristics: (i) a high molecular mass; (ii) a signal sequence for extracellular secretion; and (iii) a core domain of repeats, the number of which varies among different isolates (Shankar *et al.*, 1999) and throughout the course of an infection (Cucarella *et al.*, 2004). Disruption of the *bap* gene in *S. epidermidis* eliminated its ability to form a biofilm, whereas heterologous complementation of a biofilm-negative strain of *S. aureus* with the Bhp protein from *S. epidermidis* conferred the capacity to form a biofilm on a polystyrene surface (Tormo *et al.*, 2005a). This study clearly shows the importance of Bhp in *S. epidermidis* adhesion to polystyrene surfaces.

However, once inserted into the body, in later stages of adherence *in vivo*, implanted material rapidly becomes coated with host extracellular matrix and plasma proteins, such as fibronectin (Fn), fibrinogen (Fg), vitronectin (Vn), thrombospondin, collagen, von Willebrand factor, laminin and elastin (Cottonaro *et al.*, 1981; Dickinson and Bisno, 1989; Kochwa *et al.*, 1977). These host factors deposited on the implanted material can act as specific receptors for specific protein adhesins of the colonizing bacteria (Banner *et al.*, 2007; Hartleib *et al.*, 2000; Herrmann *et al.*, 1997; Lopes *et al.*, 1985).

The autolysin/adhesin Aae is a 35kDa surface-associated protein that has bacteriolytic activity and has the ability to bind vitronectin, fibrinogen and fibronectin (Heilmann *et al.*, 2003). It exhibits lytic activity against *Staphylococcus carnosus* and *S. epidermidis* cells. AtlE and Aae are bacterial autolysins, i.e., peptidoglycan hydrolases that are important in cell-wall turnover, cell division, cell separation and antibiotic-induced lysis of bacterial cells (Buist *et al.*, 1995; Doyle *et al.*, 1988; Höltje, 1996).

The fibrinogen-binding protein Fbe, also called serine-aspartate repeat protein G (SdrG) is a 93.7 kDa protein that can bind Fg by specifically recognizing the Bß chain of this molecule (Bowden *et al.*, 2005; Davis *et al.*, 2001, McCrea *et al.*, 2000; Nilsson *et al.*, 1998). Although they are not identical in primary sequence, the basic structure and organization of Fbe protein is almost identical to that of fibrinogen binding proteins from *S. aureus*, the clumping factors A and

B (ClfA and ClfB, respectively) (Bowden *et al.*, 2005; Davis *et al.*, 2001; Navarre and Schneewind, 1999). The role of Fbe in adhesion was demonstrated in a study (Pei and Flock, 2001b) where a *fbe*-deficient mutant showed reduced adherence to fibrinogen immobilised on a polyethylene surface and to peripheral venous catheters that were removed from patients. Moreover, according to Bowden *et al.* (2008), the *S. epidermidis* Fg-binding adhesin SdrG is necessary and sufficient for the attachment of this pathogen to Fg-coated materials. Studies with Fbe antibodies also showed their efficacy in hindering the adherence process, by increasing macrophage phagocytosis and preventing rigorous systemic infection *in vivo* when *S. epidermidis* was pre-opsonized with anti-Fbe antibodies prior to inoculation (Rennermalm *et al.*, 2004), suggesting the possibility of immunoprophylactic therapy against device associated infections (Pei and Flock, 2001a; Pei and Flock, 2001b; Pei *et al.*, 1999). According to Ponnuraj *et al.* (2003), it is possible that staphylococci express proteins that bind to Fg in order to prevent the release of chemotactic elements, thus reducing the influx of phagocytic neutrophils to the infection site and enhancing the bacterial survival into the host.

SdrG belongs to the serine-aspartate repeat family (Sdr) which also includes SdrF and SdrH and that are all characterized by the distinctive serine-aspartate dipeptide (SD) repeats (McCrea *et al.*, 2000). Like SdrG, SdrF and SdrH are expressed during *S. epidermidis* infection (McRea *et al.*, 2000). According to the recent study of Arrecubieta *et al.* (2007), SdrF is responsible for mediating binding to type I collagen while SdrH specific ligand has not been identified yet (Bowden *et al.*, 2005). GehD is a *S. epidermidis* general extracellular lipase also capable of binding to collagen type I, as well as to collagen type II and IV, thus mediating the adherence of *S. epidermidis* cells to immobilized collagens (Bowden *et al.*, 2002). Staphylococcal lipases have been implicated as possible virulence factors in localized infections such as abscesses (Hedström and Nilsson-Ehle, 1983; Hedström, 1975; Rollof *et al.*, 1987) and there is evidence that they are highly expressed during infection in a murine model (Lowe *et al.*, 1998).

A fibronectin-binding protein from *S. epidermidis*, called Embp, was also identified (Williams *et al.*, 2002). Williams *et al.* (2002) also showed that a recombinant protein containing

this fibronectin-binding domain prevented binding of *S. epidermidis* to fibronectin. In a recent study of gene profiling of *S. epidermidis* isolates from patients with ocular infections and normal individuals (Duggirala *et al.*, 2007), it was demonstrated the presence of the gene encoding Embp in isolates from endophthalmitis, indicating their potential to serve as virulence marker. Binding of *S. epidermidis* to fibronectin occurs at a single site in the C- terminus of fibronectin and can be inhibited by heparin (Arciola *et al.*, 2003).

All these surface proteins capable of binding to components of the EM or to other elements of the host tissues were defined as MSCRAMMs, i.e., microbial surface components recognizing adhesive matrix molecules (Patti *et al.*, 1994). MSCRAMMs have been found in almost all pathogenic gram-positive species, and their modular design and common binding domains suggests that they have arisen from a series of recombinational events and horizontal gene transfer (Navarre and Schneewind, 1999). In most cases, MSCRAMMs are covalently anchored to the cell wall by a mechanism requiring a COOH-terminal sorting signal with a conserved LPXTG motif (Patti *et al.*, 1994). The cleavage between the threonine and the glycine of the LPXTG motif liberates the carboxyl of the threonine to form an amide bond with the pentaglycyl cross-bridge in the staphylococcal peptidoglycan (Ton-That *et al.*, 1998).

A typical feature among MSCRAMMs is the cell sorting motif, a hydrophobic region that spans the bacterial membrane and a C-terminal cluster of positively charged residues (Bowden *et al.*, 2005). Many MSCRAMMs are capable of binding to more than one host matrix components, and a single strain often possesses several different proteins that bind the same host component (Navarre and Schneewind, 1999). Investigating the mechanism by which these proteins are able to attach to the bacterial surface and to host matrix proteins will advance our understanding of the initial attachment process. Moreover, antisera against binding proteins may establish as useful in the treatment or prevention of *S. epidermidis* infection.

In the recent study of Bowden *et al.* (2005) seven other CW anchored proteins were identified: the *S. epidermidis* surface proteins (Ses) SesA, SesB, SesC, SesE SesG, SesH and SesI. The analysis of the primary sequences of these proteins indicates that they have a similar

structural organization to CW anchored proteins from *S. aureus* and other Gram-positive cocci, despite the fact that not all Ses proteins are direct homologues of the *S. aureus* proteins. According to the same study, PCR analysis indicates that certain *Ses* genes may be more frequently found in disease isolates compared to strains isolated from healthy skin and that patients recovering from *S. epidermidis* infections had higher antibody titres against some Ses proteins, implying that these proteins are expressed during human infection.

6.1.2 Biofilm accumulation

After initial adherence, certain strains of *S. epidermidis* multiply and build up as multilayered cell clusters, a process that requires intercellular adhesion (von Eiff *et al.*, 2002). The polysaccharide intercellular adhesin (PIA), a polymer of *N*-acetyl glucosamine (Mack *et al.*, 1996b) synthesized by enzymes encoded by the *ica* operon (Mack, 1999), is crucial for this cell-to-cell adhesion process and biofilm accumulation (McKenney *et al.*, 1998; Ziebuhr *et al.*, 1997). However, some proteins are also important for biofilm formation.

Hussain *et al.* (1997) showed that the 140 kDa accumulation-associated protein (AAP) is essential for the accumulative growth on polymer surfaces, which results in visible biofilm formation of some *S. epidermidis* strains. This study showed that a biofilm-negative mutant, *S. epidermidis* M7, generated from *S. epidermidis* RP62A by mitomycin mutagenesis, lacked the 140-kDa protein and was unable to accumulate as a biofilm. Moreover, it was proposed that AAP plays a role in anchoring PIA to the *S. epidermidis* cell surface since a mutant carrying an inactivated *aap* produced PIA that is loosely attached to the bacterial surface (Hussain *et al.*, 1997). In a more recent study (Rohde *et al.*, 2005), it was proposed that the 140-kDa AAP found in *S. epidermidis* 5179 is the truncated isoform of a larger 220-kDa AAP and that the smaller protein is functional in biofilm accumulation independently of PIA. This truncation was shown to be necessary for biofilm formation, as the AAP-negative, biofilm-negative strain *S. epidermidis* 1585 was able to form a biofilm when it expressed the truncated form of AAP but remained

biofilm negative when full-length AAP was expressed (Rohde *et al.*, 2005). AAP is prevalent in clinical isolates of *S. epidermidis* (Rohde *et al.*, 2004). Also, antiserum raised against the purified protein inhibited biofilm accumulation of several biofilm-producing *S. epidermidis* strains in a concentration dependant manner, whereas pre-immune serum had no effect (Hussain *et al.*, 1997). However, the means by which AAP mediates biofilm formation is still not known. Sun *et al.* (2005) discovered that AAP is both secreted into extracellular fluid and expressed on the *S. epidermidis* cell wall. AAP is a cell wall-anchored protein with an N-terminal signal sequence and a C-terminal sorting signal including an LPXTG motif followed by a hydrophobic transmembrane region and a positively charged cytoplasmic tail (Bowden *et al.*, 2005; Roche *et al.*, 2003).

The protein Bhp has also been proposed to promote the intercellular adhesion during biofilm formation (Cucarella *et al.*, 2001). Sequence similarity may suggest that the *bhp* gene is involved in biofilm formation in human isolates of *S. epidermidis* in a way similar to *bap* in animal isolates of *S. aureus*. However, the mechanism by which Bap and Bhp contribute to biofilm formation is not known (Vadyvaloo and Otto, 2005).

In Table 6.1 are summarized the main *S. epidermidis* surface proteins and their putative functions.

Table 6.1 *S. epidermidis* surface proteins and their putative functions. Adapted from: von Eiff *et al.*, 2002.

Surface protein	Putative function
SSP-1, SSP-2	Adherence to unmodified polystyrene
AtlE	Adherence to unmodified and/or Vn-coated polymer surface
Bhp	Biofilm formation
Fbe	Binding to the ß chain of fibrinogen
SdrG, SdrF, SdrH	SdrG: binding to the ß chain of fibrinogen and inhibition of thrombin-induced fibrinogen-clotting; SdrF binding to type I collagen; SdrH: unknown
AAP	Biofilm accumulation

The establishment of an infection and the survival of the bacteria in the host depends on the ability to invade host tissues and to evade host defence systems, respectively. Thus, during the process of proliferation and accumulation as multilayered cell clusters, staphylococci, especially *S. aureus*, have developed multiple mechanisms for this purpose, such as: production of several extracellular proteins and enzymes, like protein A, lipases, proteases, esterases, phospholipases, fatty-acid modifying enzymes (FAME), as well as production of haemolysins and toxins, and toxic shock syndrome toxin-1 (TSST-1) (von Eiff *et al.*, 2002).

Contrary to *S. aureus*, *S. epidermidis* is much less toxigenic and there is only one toxin, the haemolytic N-formylated alpha-helical peptide δ-toxin, described for *S. epidermidis* (McKevitt *et al.*, 1990). It is encoded by the *hld* gene located in the regulatory *agr* locus (Otto *et al.*, 1998). The δ-toxin causes the lysis of erythrocytes and other mammalian cells by forming pores in the cytoplasmic membrane (Gemmell and Thelestam, 1981).

Additionally, proteases seem to play a crucial role in proteolytic inactivation of host defence mechanisms such as antibodies and platelet microbicidal proteins (PMPs), as well as in destruction of tissue proteins which leads to increased invasiveness (von Eiff *et al.*, 2002). An extracellular metalloprotease of 32 kDa and a cysteine protease, both with elastase activity, have been described in *S. epidermidis* (Sloot *et al.*, 1992; Teufel and Götz, 1993).

The *S. epidermidis* cysteine protease has been demonstrated to degrade several host matrix proteins, such as fibrinogen and fibronectin, and components of the immune system *in vitro*, and is thus considered to be a virulence factor (Sloot *et al.*, 1992). An extracellular serine protease is involved in the processing of epidermin (Geissler *et al.*, 1996), a lantibiotic that plays an important role in bacterial interference on skin and mucous membranes by excluding competing organisms that are sensitive to their bactericidal activities (Kupke and Götz, 1996).

The genes of two very similar lipases, GehC and GehSE1, as well as of the already mentioned GehD (Longshaw *et al.*, 2000), have been cloned and sequenced, and it seem to be important for skin colonization (Rosenstein and Götz, 2000). The characterization and expression

of FAME in *S. epidermidis* has also been described (Chamberlain and Brueggemann, 1997), acting by esterifying fatty acids to cholesterol, thereby destroying their bactericidal properties.

Some of these proteins are potential targets for immunotherapy, which provides a novel strategy for control of *S. epidermidis* infection, and could potentially reduce the rates of infection and have a significant impact on human health.

6.1.3 Aims

The purpose of the present work was to relate the protein profile of the cell wall (CW) extracts of eight *S. epidermidis* strains and the protein pattern of the extracellular matrix (EM) of four *S. epidermidis* strains (good biofilm producers) to their adhesion and biofilm formation capability.

6.2 Materials and Methods

6.2.1 Bacterial strains

Eight *S. epidermidis* strains were studied in this work: 9142, 9142-M10, 1457, 1457-M10, IE186, IE214, IE75 and LE7, which were already described in section 2.2.1.

6.2.2 Cell wall (CW) fraction preparation

The preparation of the CW fractions of the eight *S. epidermidis* strains was performed as previously described (Bowden *et al.*, 2005). Cells were grown for 24 h in 15 ml of tryptic soy broth (TSB; Merck, Darmstadt, Germany) at 37 °C under agitation (120 rpm). After this period, 50 µl of each suspension were transferred into 30 ml of fresh TSB and incubated for 18 h (late

exponential phase), at 37 °C and 120 rpm. The cells were then centrifuged for 10 min, at 7 000 g and 4 °C and washed twice with PBS, pH 7.4 (140 mM NaCl, 270 μM KCl, 430 μM Na_2HPO_4 and 147 μM KH_2PO_4). After, cells were resuspended in the digestion buffer solution containing Tris/HCl (50 mM; pH 7.5) (BioRad Lab, USA), NaCl (145 mM)(Merck), 30% (w/v) raffinose (Sigma), lysostaphin (100 μg/ml) (Sigma), lysozyme (100 μg/ml) (Sigma), DNase (10 μg/ml) (Sigma), iodoacetamide (1 μg/ml) (BioRad Lab) and phenylmethylsulfonyl fluoride (PMSF) (1 mM) (BioRad Lab) and adjusted to a concentration of approximately $1x10^9$ cells/ml, determined by optical density at 640 nm.

In order to release the proteins from the bacterial CW, cell suspensions were incubated for 30 min, at 37 °C, under soft agitation (60 rpm). Finally, the protoplasts were removed by centrifugation at 3 000 g for 20 min at 21 °C, and the supernatant recovered for SDS-PAGE.

6.2.3 Biofilm extracellular matrix (EM) fraction preparation

In order to obtain the EM proteins of the four strongest biofilm producers, biofilms of *S. epidermidis* strains 9142, 1457, IE214 and IE186 were formed as previously described in chapter 5 (section 5.2.4) as well as the biofilm matrix extraction (section 5.2.8).

6.2.4 Protein quantification

The concentration of the CW and EM proteins was determined according to the procedure described in sub-chapter 5.2.8.1.

6.2.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

CW proteins and EM proteins were separated in a 12% (w/v) acrylamide resolving gel and a 4% (w/v) acrylamide stacking gel of 20 cm x 20 cm, according to the method of Laemmli

and Favre (1973). Each sample was dissolved in an equal amount of 2 x SDS loading buffer [125 mM Tris-HCl (BioRad Lab), pH 6.8; 20% (w/v) glycerol (Sigma); 4% (w/v) SDS (Riedel-deHaën); 0.01% (w/v) bromophenol blue (BioRad Lab) and 1% (v/v) β-mercaptoethanol (Sigma)] and the solution was heated at 100 °C for 5 min prior to gels loading. A molecular size standard with the range 10–250 kDa (Precision Plus Protein Standards – Kaleidoscope, Bio-Rad Lab) was ran along with the samples. After electrophoresis, gels were stained with silver nitrate (Sigma) and analysed with the software Quantity One (BioRad Lab, USA).

6.2.6 Statistical analysis

The results from all assays were compared using one-way analysis of variance (ANOVA) by applying Levene's test of homogeneity of variances and the Tukey multiple comparisons test, using the SPSS software (Statistical Package for the Social Sciences Inc., Chicago). All tests were performed considering a confidence level of 95%.

6.3 Results

6.3.1 Analysis of *Staphylococcus epidermidis* cell wall (CW) proteins

SDS-PAGE profiles of the cell wall (CW) associated proteins from the eight *S. epidermidis* strains studied are presented in Figure 6.1. From a total of 103 bands obtained, 59%, ranging between 7 and 86 kDa, were found to be common to all isolates. As lysostaphin and DNAse are present in the lyses buffer used, a sample of the lyses buffer was included in all the gel runs being the enzymes visualized at 25 and 30 kDa, respectively (Figure 6.1(a) - lane J). It must also be noted that the differences among the eight *S. epidermidis* strains were most pronounced in proteins higher than 80 kDa [Figure 6.1(b)]. As it can be seen, the 280 kDa

protein was only detected in the CW extract of IE186, the 258 kDa in strains 1457, 1457-M10, IE186 and IE214 while the 148 kDa protein was solely detected in strain IE214.

Moreover, the strains presenting the highest % of the total number of proteins found were: 1457 (89.3%), IE186 (86.4%), IE214 (83.5%), IE75 (82.5%), 9142 (79.6%), 1457-M10 (69.9%), LE7 (68.0%) and 9142-M10 (67.0%).

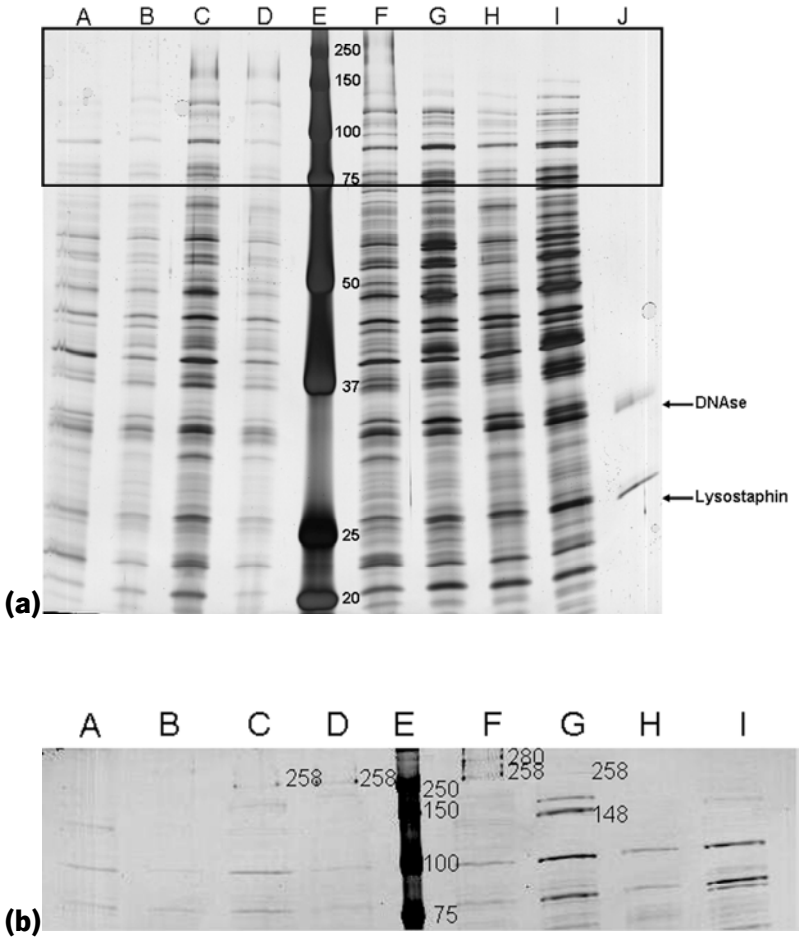


Figure 6.1 SDS-PAGE profiles of cell wall proteins, stained with silver nitrate, of the eight *S. epidermidis* strains assayed. Molecular mass markers are shown in the centre (Lane E), in kilodaltons. A – 9142; B – 9142-M10; C – 1457; D – 1457-M10; E - molecular standard; F – IE186; G – IE214; H – LE7; I – IE75; J – Lysis buffer sample. **(a)** Molecular weight ranging from 20 – 250 kDa; **(b)** Detail of a complete gel with a molecular weight ranging from 75 – 250 kDa.

6.3.2 Analysis of *Staphylococcus epidermidis* extracellular matrix (EM) proteins

The extracellular matrix (EM) of the four highest biofilm producer strains (IE214, IE186, 9142 and 1457) was extracted and studied in terms of proteins content. The EM protein profile of these strains can be seen in Figure 6.2.

Analysing the SDS-PAGE profiles of EM proteins of the four good biofilm producer strains studied, a total of 76 bands was detected among all strains. Around 84.2% of the proteins were also found in the CW extracts, while the remaining 15.8% were only observed in the EM extract of one or more strains studied. This value mostly includes low molecular weight proteins, with the sole exception for the 103 kDa protein observed for strain IE214. About 72.4% of the 76 bands detected in the protein profiles of the four EM extracts were found in strain IE214; 65.8% in strain 1457; 35.5% in strain 9142 and 31.6% in IE186, showing a higher array of bands in strains IE214 and 1457. For instance, a 115 and a 56 kDa bands were only observed in strain IE214 profile and a 51.8 kDa protein solely detected in strains IE214 and 1457. Strain 9142, as well as 1457, revealed the presence of a 31 kDa protein, which was not detected in the other two strains.

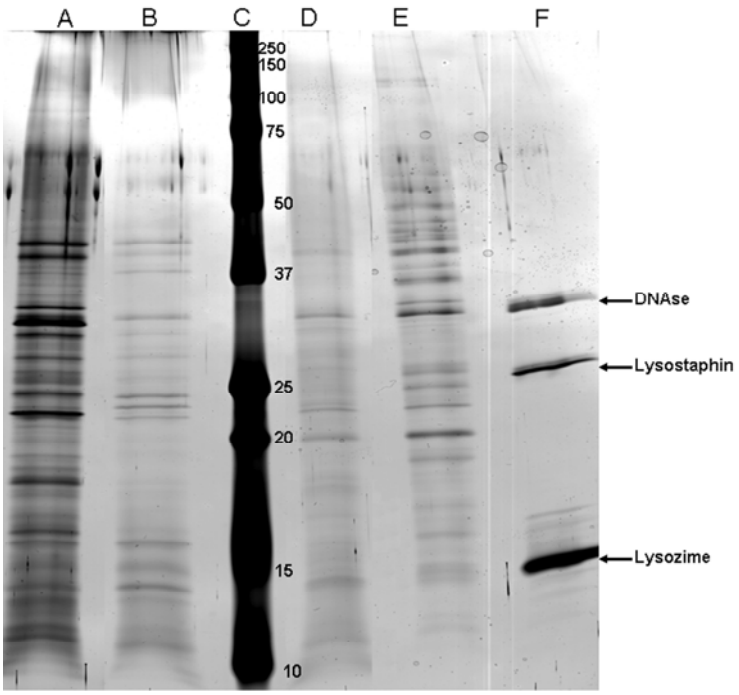


Figure 6.2 SDS-PAGE profiles of extracellular matrix proteins of the biofilm of four *S. epidermidis* strains, stained with silver nitrate. Molecular mass markers are shown in the centre (Lane C), in kilodaltons. A – 9142; B – 1457; C – molecular standard; D – IE186; E – IE214; F – Lysis buffer sample (DNAse, lysostaphin and lysozime, are visualized at 30, 25 and 15 kDa, respectively).

6.4 Discussion

Biofilm formation has been shown to be a major feature determining *S. epidermidis* virulence in device-associated infections, normally of complex treatment. It is estimated that sessile bacteria within biofilms are up to 1,000-fold more resistant to antibiotics than their planktonic counterparts (Ceri *et al.*, 1999). Cell Wall (CW) proteins of *S. epidermidis* are of major importance due to their potential role in virulence and host defence. They are responsible for functions such as environment sensing within the host; nutrients acquirement for bacterial survival; defence against the host immune system and proteins binding, or other components of the host tissues (Josefsson *et al.*, 2001; Swiatlo *et al.*, 2003).

In this work, the first goal was to study the possible relation between the bacterial adhesion capacity, the initial biofilm formation step, and CW proteins profile of eight *S. epidermidis* strains. It is expected that *S. epidermidis* surface proteins play a significant role in the primary steps of infection as well as in biofilm formation. For instance, *S. aureus* has the ability to attach to host ligands by virtue of different surface proteins, namely adhesins, being well-established as pathogenic factor in its invasiveness (Hussain *et al.*, 2001).

In the present work, a 60 kDa protein was found to be common to all CW protein profiles of *S. epidermidis* strains assayed (Figure 6.1), which is known to be an amidase and a fragment of the AtlE protein (Heilmann *et al.*, 1997; Mack, 1999). This protein has been indicated as sufficient for initial attachment (Heilmann *et al.*, 1997), and thus might be related with the adhesion of all strains to the acrylic coupons (Chapter 2; Figure 2.3). Several other proteins can also be highlighted as relevant in cell adhesion to surfaces, namely the cell surface protein ebpS with ≈ 51 kDa, which has been recognized as being an elastin binding protein important in adherence to host tissues (Gill *et al.*, 2005; Park *et al.*, 1996) and a corresponding band was present in the profile of all strains, as well as a 45 kDa protein, likely the degradation product of the 60 kDa amidase (Heilmann *et al.*, 1997).

It was also identified in all strains a CW associated protein of 42 kDa, potentially the transferring binding protein (Tpn), which is necessary for the iron acquisition within hosts, as an essential metal for bacterial growth (von Eiff *et al.*, 2002). Furthermore, this protein has the ability to bind to human transferrin as well to plasmin, thus contributing to the *S. epidermidis* entry into the host during the infection process (Modun and Williams, 1999). Likewise, a 32 kDa protein was found in all CW extracts, which is expected to be the *S. epidermidis* surface (Ses) protein SesH, recently described by Bowden *et al.* (2005) and detected among several types of clinical isolates including those colonizing skin flora and bacteraemic patients.

A 20 kDa CW associated protein was also common to all strains assayed and it can correspond to the cysteine protease (Ecp), known as staphopain, normally expressed in the CW

and in a soluble form. It is able to cleave elastin, insulin, myoglobin, fibronectin and fibrinogen, thus contributing to the colonization and infection of human tissues (Oleksy *et al.*, 2004).

Finally, a small protein of the size of SesI (14.8 kDa), previously identified in clinical isolates of healthy individuals but also recognized by the sera of infected patients (Bowden *et al.*, 2005) was present in the protein expression profile of all strains [Figure 6.1(a)] as well. Albeit the function of Ses proteins is still unclear, they seem to contribute to the pathogenicity of *S. epidermidis*.

Therefore, the presence of all these proteins on the surface of *S. epidermidis* strains herein studied corroborates their virulence connection and shows that besides adhesion to bare acrylic, here demonstrated, they also have potential for adhesion to host tissues.

According to the results of the adhesion assays, IE214 and IE186 were the strains that adhered at highest extent to the acrylic substrate (Chapter 2; Figure 2.3). Remarkably, these strains revealed the presence of the highest number of CW proteins that might be related with the adhesion process. Furthermore, strain IE214 exhibited a 258 kDa band that possibly corresponds to the Bhp protein, homologous to the biofilm-associated protein (Bap) of *S. aureus*, which helps promoting primary attachment to the biomaterial surface (Cucarella *et al.*, 2001). The band with 148 kDa, only detected in IE214, is expected to correspond to the major autolysin AtlE, indicated as being involved in the initial attachment of the cells to unmodified polymer surfaces (Heilmann *et al.*, 1997). In addition to the 60 kDa amidase fragment from AtlE, already mentioned as being present in all strains protein profile, a 52 kDa glucosaminidase fragment has also been described (Heilmann *et al.*, 1997) and was detected in this strain. In this clinical isolate protein profile the bands visualized most likely indicate the presence of the Fbe protein precursor (118 kDa) as well as the low molecular weight form of Fbe, also termed SdrG (≈94 kDa). SdrG has revealed to be sufficient for *S. epidermidis* attachment to biomaterials coated with human fibrinogen (Ponnuraj *et al.*, 2003). One of the *S. epidermidis* surface proteins recently described by Bowden *et al.* (2005) as being important for the biomaterial colonization,

the SesC protein, was also, presumably distinguished in the IE214 CW protein profile, corresponding to the 68 kDa band.

In a similar way, the protein profile of strain IE186 also showed the presence of Bhp, Fbe precursor and SesC as well as the presence of the 280 kDa surface protein SSP-1, a fimbria-like polymer that aids in the adhesion of some *S. epidermidis* strains to polystyrene (Veenstra *et al.*, 1996), and the 209 kDa SesG protein, apparently associated with disease isolates (Bowden *et al.*, 2005), which is the case of strain IE186, obtained from a patient with infective endocarditis.

Regarding the infected central venous catheter isolate, strain 1457, which also adhered in a high extent, the SDS-PAGE analysis revealed the presence of the proteins Bhp, SesG, SesC, SdrG and the SdrG precursor.

The PIA-negative strains, 9142-M10 and 1457-M10, adhered at a similar extent to the acrylic surface, but their protein profiles showed some differences: strain 1457-M10 produced Bhp, while strain 9142-M10 profile did not reveal any specific protein that might be related with adhesion, except the ones common to all strains.

Strain 9142, that adhered less to the substrate, produced a 68 kDa protein (SesC) and the 52 kDa glucosaminidase (AtlE fragment), besides the proteins produced by all strains. In opposition, strain IE75, which adhered less to the substrate, exhibited a protein profile containing only SdrG and its precursor, SesC and the 52 kDa AtlE fragment, from the described above.

The strain with the lowest number of adherent cells, LE7, showed in its profile the 52 kDa AtlE fragment. From these results, it can be said that the adhesion ability can, in fact, be ruled by the presence of some specific CW associated proteins.

Concerning cell accumulation and biofilm formation, the behaviour of the eight *S. epidermidis* strains (Chapter 5; Figure 5.3) was different compared to the adhesion process. This was already observed in the work of Cerca *et al.* (2005b) and, according to these authors, adhesion and biofilm formation are two different features of the pathogenicity of medical devices

infections because high levels of initial adherence do not necessarily lead to strong biofilm formation.

Regarding CW proteins involved in the biofilm formation process, besides Bhp (detected in IE214, IE186, 1457 and 1457-M10 profiles) and AtlE (only observed in IE214) whose importance in initial attachment but also in bacterial accumulation was already mentioned (Cucarella *et al.*, 2001; Heilmann *et al.*, 1997), also the extracellular 140 kDa protein (AAP) was found in 1457, 9142 and IE186 CW protein profiles. AAP protein has been described as crucial for accumulative growth of some *S. epidermidis* strains on polymer surfaces (Hussain *et al.*, 1997), even independently of the PIA presence (Rohde *et al.*, 2005). In fact, this 140 kDa AAP is a truncated isoform resulting of the proteolytic cleavage of a full length 220 kDa AAP (Rohde *et al.*, 2005), which was only detected in 1457-M10. However, this full-length AAP does not lead to a biofilm-positive phenotype unless it is proteolytically processed, as here corroborated by the low extent of biofilm formation by 1457-M10. From this cleavage also resulted a partially truncated form of the 220 kDa AAP, a 180 kDa AAP, observed on strain IE214, but whose function has not yet been determined (Banner *et al.*, 2007).

A 35 kDa bacteriolytic autolysin/adhesin (Aae), capable of binding to vitronectin and being associated to the colonization of host factor-coated material or host tissue by *S. epidermidis* (Heilmann *et al.*, 2003), was found in all CW extracts analysed. It must be noted that the presence of autolysins is considered to be a virulence factor.

Given the fact that for the PIA-negative mutant strains 9142-M10 and 1457-M10 the amount of biofilm formed was low, it is once again demonstrated that, besides proteins, PIA also plays a major role in the process of cell-to-cell adhesion and biofilm accumulation.

Nevertheless, the strains that produced the largest amounts of biofilm (1457, 9142, IE214 and IE186) showed the presence of the proteins Bhp, AtlE or AAP, combined or alone, which have been described as essential for the cell accumulation process (Cucarella *et al.*, 2001; Hussain *et al.*, 1997). Strains IE75 and LE7, low biofilm producers, did not show any of these proteins, therefore suggesting their importance in the biofilm accumulation process.

The extracellular matrix (EM) of the most important biofilm producers (strains 1457, 9142, IE214 and IE186) was extracted and their protein content was also analysed by SDS-PAGE electrophoresis (Figure 6.2). From the analysis of matrix protein profiles, only approximately 16% of the proteins detected were exclusive of the EM, compared to the CW. The majority of this % corresponded to low molecular weight bands, most likely degradation fragments of larger proteins.

Several tissue-damaging secretory proteases and other exoenzymes were detected in the EM of some strains. These proteases are important in the proteolytic inactivation of host defence mechanisms, such as antibodies, as well as in the damage of tissue proteins leading to amplified invasiveness (von Eiff *et al.*, 2002). Therefore, the band with ≈ 115 kDa detected in the IE214 profile seems to correspond to the Serine-Aspartase rich fibrinogen-binding, bone sialoprotein-binding protein. Its main function is protein binding, i.e., selective interaction with any protein or protein complex (Zhang *et al.*, 2003). The 56 kDa band, also present in strain IE214, might correspond to an extracellular metalloprotease with elastase activity (Teufel and Götz, 1993). Thus, this protease is able to hydrolyze elastin, a protein present in connective tissues, contributing to the disruption and damage of the tissue, and it also cleaves immunoglobulin A and G and CR1, a neutrophils receptor involved in phagacytosis. This leads to a decrease of the ability of neutrophils to kill bacteria by phagocytosis and therefore reduces the defence mechanisms of the host (Horwitz *et al.*, 1999).

The 51.8 kDa protein detected in IE214 and 1457 seems to match the serine protease EpiP, involved in the processing of epidermin, a lantibiotic that helps to exclude competing organisms that are sensitive to their bactericidal activities (Geissler *et al.*, 1996). On the other hand, the 31 kDa protein detected on 9142 and 1457 must be the glutamyl endopeptidase that shows activity towards human fibronectin and type 1 collagen and is considered a virulence factor (Dubin *et al.*, 2001; Ohara-Nemoto *et al.*, 2002). An important factor to be taken into consideration is that these two *S. epidermidis* strains are the highest biofilm producers. Therefore, despite the crucial production of proteins for the adhesion and biofilm formation

processes, the ability to secrete proteins with protease and bacteriolytic activity is fundamental for inactivation of host defence mechanisms and for the successful prevalence of *S. epidermidis* biofilms and infections.

Moreover, not all of the CW proteins associated with the initial attachment and cell accumulation were detected in the matrix extracts, presumably indicating a degradation of proteins during the 8 days process of biofilm maturation.

According to the present results and concerning the proteins found in both CW and EM extracts of each strain studied, the SDS-PAGE profiles of strain IE214 demonstrated ca. 67.1% similarity, followed by strain 1457, with 56.6% of analogy. Regarding strains IE186 and 9142, the EM and CW extract protein profiles matched by ca. 43.7%. Thus, between the four strains selected, *S. epidermidis* IE214 was the strain with more proteins expressed simultaneously in the EM and CW. Among these, were visible: 148 kDa AtlE; 94 kDa SdrG; 54 kDa SdrH, whose function is still not clear yet (Arrecubieta *et al.*, 2007); 45 kDa degradation product of the 60 kDa amidase; 42 kDa Tpn; 38 kDa degradation product of the 52 kDa glucosaminidase; 35 kDa Aae and the 32 kDa SesH. The only proteins found to be common to IE186 CW and EM extracts were the 45 kDa degradation products of the AtlE fragment of 60 kDa and the SesH protein. Moreover, the 60 kDa AtlE fragment is present in both extracts of all strains. In strain 1457, the most distinguishable bands that are also common to CW extract are essentially the degradation products of the AtlE, fragments of 60 and 52 kDa, as well as staphopain.

Therefore, a better knowledge of the roles of all these proteins which might be considered *S. epidermidis* virulence factors constitutes them appropriate targets for the development of novel therapies against staphylococcal infections.

6.5 Conclusions

The presence of proteins such as Bhp (strains IE214, IE186, 1457 and 1457-M10) and AtlE (strain IE214) seems to be essential in promoting initial adhesion to the substrate, since they were present in the most adhering strains. For the remaining strains, the presence of other specific proteins, such as the 60 kDa amidase seems to be sufficient to trigger their lower levels of adhesion. Biofilm formation seems to be mainly dependent of the presence of AAP, AtlE or Bhp on the CW of bacterial cells. However, it was evident the importance of the PIA molecule in biofilm formation, as revealed by PIA-negative mutants biofilm formation results. The EM extracts of the best biofilm producer strains revealed the presence of some exoenzymes produced and secreted by *S. epidermidis* cells, to aid the host defence systems invasiveness. Moreover, a large percentage of the CW proteins were secreted to the EM, despite the fact that only a few, related with the adhesion and biofilm processes, were detected.

CHAPTER 7 - Concluding remarks and work perspectives

In this last chapter, the most important conclusions withdrawn from the present thesis are addressed. Also, considering the conclusions of the work developed, some suggestions for further research in this field are given.

7.1 Concluding remarks

The aim of the present thesis was to clarify some of the phenomena that are involved in *Staphylococcus epidermidis* adhesion and biofilm formation upon biomaterial surfaces, as well as the implementation of new strategies for the prevention of bacterial colonization to inert surfaces. In order to achieve these goals, several aspects were studied throughout this thesis, namely: the influence of cell and material surface properties on the adhesion phenomenon **(a)**; the reduction of *S. epidermidis* adhesion either by pre-conditioning of the biomaterial surface with anti-adhesive substances **(b)** and by surface modification with self-assembled monolayers with specific properties **(c)**.

A detailed look was given to biofilm properties **(d)** in terms of total biomass, number of cells, extracellular matrix composition and also metabolic activity. At last, a descriptive study of proteins pattern of the cell wall and of the extracellular matrix of *S. epidermidis* biofilms was carried out in order to relate them with bacterial adhesion and biofilm forming ability **(e)**. The main conclusions that can be extracted from the work presented are the following:

a) All *S. epidermidis* strains studied were determined as being hydrophilic and adhered (at different levels) at a higher extent to silicone, which is more hydrophobic and rougher, than acrylic. This highlights the importance of biomaterial surface characteristics on bacterial adhesion. Conversely, cell surface physicochemical properties have no significant effect in the adhesion process, suggesting the importance of other cell surface factors in the initial adhesion process. *S. epidermidis* IE214 showed very unique adhesion behaviour, with cells highly aggregated between them, as a result of its higher values of surface tension components and its specific surface features. This corroborates that initial adhesion to an inert surface is strain dependent.

b) The number of adhered *S. epidermidis* cells to silicone and acrylic after pre-contact with heparin and gentian violet was statistically lower compared to the number of adhered cells to bare materials. Therefore, immersion of acrylic and silicone in heparin or gentian violet represents a promising, simple and effective strategy to reduce *S. epidermidis* adhesion, particularly during the insertion procedure of medical devices into patients.

c) The extent of staphylococcal adhesion to polycarbonate surface coated with methyl terminated SAMs was lower compared to the number of cells adhered to the carboxyl acid terminated SAMs, demonstrating that methyl terminated SAMs constitute more suitable surfaces in preventing bacterial adhesion. When the surface is coated with calix-crown molecules, bacterial adhesion is enhanced, due to the non-specific binding nature and geometrical configuration of these molecules. However, when a specific protein is linked to calix-crown, it blocks the sites of adhesion for bacterial cell wall proteins and adhesion occurs to a much lower extent. Thus, the use of certain SAMs as surface modifiers may, in the future, constitute a successful method in the reduction of bacterial adhesion to biomedical surfaces.

d) *S. epidermidis* strains revealed different abilities of biofilm formation corroborating that biofilm formation is strain dependent. The results herein presented prove that the production of extracellular polymers, especially polysaccharides, largely affects the biofilm total biomass as well as cellular metabolic activity. EPS production is also a virulence factor and is strain dependent. This work clearly demonstrated the phenotypic variability of *S. epidermidis* strains and the different behaviour patterns between sessile and planktonic cells, particularly as the metabolic state is concerned (free cells are metabolically more active than biofilm cells).

e) A significant percentage of the cell wall proteins detected was common to all the *S. epidermidis* strains studied. However, the most adhering strains (strains IE214, IE186, 1457) expressed a higher number of proteins associated with the initial adhesion process (Bhp and AtlE, e.g.), and the strongest biofilm producers expressed proteins, such as AAP, that were absent in the protein profile of the strains that produced lower amounts of biofilm. This study provided important highlights into the strain variability in terms of proteins expressed in the cell wall and how these proteins might be associated to the virulence of the strain. However, the work was also important to prove the importance of PIA: PIA-negative strains (1457-M10), although having some cell wall proteins related to biofilm formation, were unable to form biofilms.

Some of the cell wall proteins were also present in the extracellular matrix protein pattern, though in a smaller amount, as well as several exoenzymes important for the host immune system invasiveness.

7.2 Work perspectives

The work described in this thesis provided an insight into several aspects of *Staphylococcus epidermidis* pathogenicity, leading to interesting new questions for further research. Some of the suggestions that should be taken into consideration for future investigation are given below:

- Being *S. epidermidis* a strong nosocomial pathogen, it is of all interest to carry out studies using the greatest possible number of clinical strains. The clinical strains should be obtained from different sources, such as from patients with infective endocarditis but also from several types of indwelling medical devices (catheters, prosthetic heart valves, joint replacements, contact lenses) that were removed from the patient after infection with *S. epidermidis*. A well characterized collection of clinical isolates could thus provide a better understanding about the strain variation observed in this work.

- Infections of indwelling medical devices, such as catheters, have been associated with mixed bacterial-fungal biofilms. With the knowledge herein acquired about *S. epidermidis* biofilms, it would be very interesting to study the interspecies interactions between *S. epidermidis* and *Candida* species, e.g., *Candida albicans*.

- Another suggestion would be the study of the process of staphylococcal biofilm formation in the medical device itself, such as a catheter, to mimic the conditions under which it is normally used.

- Other biomedical polymers that are also used in the manufacture of implants or devices should be assayed, such as, polyethylene; poly(dimethylsiloxane), also known as silastic; poly(ethyleneterephthalate); polypropylene or polyurethane.

- The use of phage therapy to diminish or to prevent biofilm formation constitutes a research challenge as well as the study of the effect of other new antimicrobial agents, such as antimicrobial peptides.

- After implantation of a medical device, the polymer material rapidly becomes coated with plasma and extracellular matrix proteins such as fibronectin, fibrinogen and vitronectin. The use of these proteins in the adhesion assays would be a good way to approximate the laboratory tests with *in vivo* conditions. The same concept is applied to the use of cell lines related to the endocardium or to other tissues that are close to implants usually infected by *S. epidermidis*.

- Construction of negative mutants of *atlE*, *bhp*, *aap* or other genes responsible for the synthesis of proteins necessary for adhesion or biofilm formation and the evaluation of the impact of the lack of one or more of these genes in the *S. epidermidis* biofilm formation ability will also be an interesting challenge.

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