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Polymer designs to control biofilm growth on medical devices

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Abstract

Indwelling and temporary medical delivery devices (i.e. catheters) are increasingly used in hospital settings, providing clinicians with useful tools to administer nutrients, draw blood samples and deliver drugs. However, they can often put patients at risk for local or systemic infections, including bloodstream infections and endocarditis. Microorganisms readily adhere to the surfaces and colonize them by forming a slimy layer of biofilm. Bacteria growing in biofilms exhibit an increased antibiotic resistance in comparison with planktonic cells. Consequently the antibiotic treatment of these medical device-associated infections frequently fails. Detechment resulting in the formation of microemboli is a further biofilm related complication. Since infections often involve increased morbidity and morality, prolonged hospitalization and additional medical costs, various strategies to prevent biofilm formation on implanted medical devices have been developed over the last two decades. In this paper we review and discuss the most significant experimental approaches to inhibit bacterial adhesion and growth on these devices.

1. Introduction

It is well known that microorganisms can colonize a wide variety of medical devices, which can often put patients at risk for local and systemic infectious complications, including local site infection, catheter-related bloodstream infection (BSI) and endocarditis (Bouza et al. 2002; O' Grady et al. 2002). The potential for central venous catheters (CVCs) to cause morbidity and mortality resulting from infectious complications has been estimated in several studies. For example, in the United States, 15 million CVC days (i.e., the total number of days of exposure to CVCs by all patients) occur in intensive care units (ICUs) each year. In addition, the American Center for Disease Control has extimated an average rate of CVC-associated BSIs of 5.3 per 1,000 catheter days in ICU (O' Grady et al. 2002), with approximately 80,000 CVC-associated BSIs occuring in ICUs each year in the United States.

In modern medicine, artificial surfaces are used for a wide range of applications. Several medical devices, including heart valves, artificial joints and pace makers, are permanently implanted to repair or to replace damaged parts of the body. Other devices, including intravascular catheters, urinary catheters and ventilator tubes, allow clinicians to deliver drugs and nutrition fluids into the body, to expel fluids out of the body and to monitor the status of critically ill patients. In particular, intravascular devices can be divided into two broad categories: (i) those used for short-time (temporary) vascular access, such as peripheral venous or arterial catheters, and non tunnelled CVCs; (ii) those used for long-term (indwelling) vascular access, such as tunnelled CVCs and totally implanted intravascular devices. Indwelling catheters usually require surgical insertion, while temporary devices can be inserted percutaneously.

Although there is a broad spectrum of available biomaterials performing different functions, all of

them have the potential to act as a point of microbial infection. Recent studies have shown that a wide range of persistent medical device-related infections may be related to the ability of infectious organisms to form biofilms. Biofilms consist of surface adhering microorganisms encased in a hydrated matrix of polysaccharides and proteins, and nucleic acids.

Different factors play a role in microbial contamination and colonization of devices. First, the implanted device causes a local attenuation of antimicrobial immune responses, thereby facilitating microbial colonization. Second, most patients, expecially those admitted to ICUs, are immunocompromised and, therefore, more susceptible to microbial infections.

Treatment of medical device-related infections with conventional antimicrobials frequently fails because microrganisms growing in biofilms are much more resistant to antimicrobial agents than planktonic cells (Mah & O'Toole 2001; Stewart & Costerton 2001). Potential mechanisms responsible for microbial resistance include: (i) the polymer matrix encasing the bacteria presenting a diffusional barrier that can consequently inhibit the penetration of antimicrobial agents into the biofilm (Suci et al. 1994); (ii) biofilms have been shown to grow more slowly than planktonic cells and, as a result, take up antimicrobial agents more slowly (Evans et al. 1990); (iii) the presence of a subpopulation of persisters, i.e. a subpopulation of cells highly protected, perhaps in a spore-like state (Kaeberlein et al. 2002).

Several strategies to prevent biofilm growth on medical devices have been suggested. Among these, chemically modified polymers which prevent initial microbial adhesion and antimicrobial-releasing biomaterials which inhibit the growth of surface attached bacteria have been shown to be the most promising.

2. Mechanism of biofilm formation

Understanding the mechanisms of biofilm formation has been of fundamental importance in the design of new biomaterials able to prevent biofilm growth on their surfaces.

The use of confocal laser scanning microscopy (CLSM) has clarified the complex morphology that surface-adherent bacteria assume during growth (Stoodley et al. 2002). Biofilms have come to be defined as microbial sessile communities irreversibly attached to a substratum and embedded in a matrix of extracellular polymer substances (EPS) produced by the bacteria themselves (Costerton et al. 1995). Originally, biofilms were thought to be homogeneous monolayers of adherent microbial cells. More recently, however, it has been shown that they are characterized by a heterogeneous structure in which individual microcolonies are located in matrix-enclosed mushroom-like structures (Lewandowski 2000). Open water channels are also present between the microcolonies which function to allow nutrients and oxygen to reach the bacteria present in the center of the biofilm.

The basic formation of a biofilm can be described as a three-stage process (Figure 1):

Stage 1: initial and irreversible attachment of cells to the surface.

Stage 2: early development and maturation of biofilm architecture.

Stage 3: detachment of single cells or cell aggregates from the biofilm.

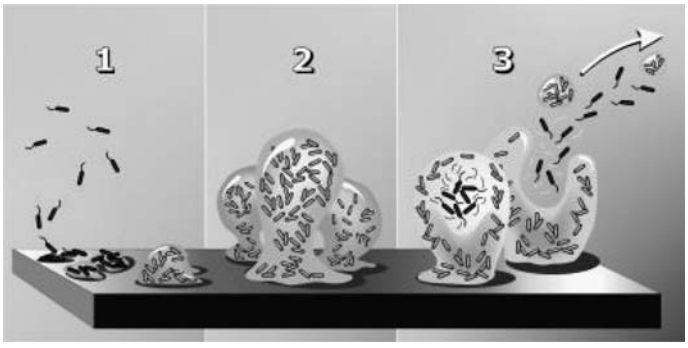

Figure 1. Schematic representation of the three stages involving biofilm formation. Schematic supplied by Peg Dirckx, Center for Biofilm Engineering, MSU.

2.1. *Initial microbial adhesion*

A common approach to interpret initial microbial adhesion to non-conducting surfaces, such as polymers and glass, has been to apply concepts of the Derjaguin, Landau, Verway & Ocerbeek (DLVO) theory (DerJaguin & Landau 1941). In fact, because of their small size, low density, net negative surface charge, and variable degrees of cell surface hydrophobicity, bacteria can be considered as living colloids. Then, the force to which bacteria are subjected to any separation distance is the sum of the van der Waals forces, electrostatic forces, acid–base interactions and Brownian motion forces. In particular, van der Waals forces are generally attractive and result from induced dipole interactions between molecules in the colloidal particle and molecules in the surface. Upon the approach of a surface, microorganisms will be attracted or repelled by the surface, depending on the resultant of these different non-specific interaction forces.

As the magnitude of these contributions depends on the properties of both the interacting partners, the rate of microbial adhesion to surfaces is highly influenced by surface hydrophobicity and the charge of the bacteria and, of most interest, by the physico-chemical properties of the materials.

2.2. *Irreversible microbial attachment and biofilm maturation*

The change from reversible to irreversible attachment involves specific and selective binding between bacterial adhesions and substratum receptors (Characklis 1990). Profound physiological changes may accompany the transition to a permanent attachment at a surface. As an example, *Pseudomonas putida* irreversible attachment has been shown to occur by switching from flagella-based motility to type IV pili-based twitching motility (Sauer & Camper 2001). In addition, Gerke et al. (1998) have shown that *Staphylococcus epidermidis* adherent cells produce a polysaccharide intercellular adhesion (PIA) that bonds the cells together and facilitates the formation of microcolonies.

Moreover, the generation of the complex architecture characterizing mature biofilms has been shown to be under genetic control. For example, *P. aeruginosa* showed a different protein profile when growing in a biofilm than when growing in a planktonic state (Brozel et al. 1995). DNA microarray results (Whiteley et al. 2001) demonstrated that over 70 genes unexpressed in mature biofilms were genes encoding proteins involved in translation, metabolism, membrane transport and gene regulation.

The regulation of gene transcription during biofilm growth is possible by the accumulation of signal molecules that allow bacteria to communicate with each other. In this regard, quorum sensing (QS) is defined as a cell-density dependent bacterial intercellular signaling mechanism that enables bacteria to coordinate the expression of certain genes able to coordinate the group behavior.

Understanding the role of QS systems in clinically relevant strains and consequently the possibility to interrupt cell-cell communication by the use of specific quorum sensing inhibitors, such as furanone compounds (Hentzer et al. 2002; Ren et al. 2001), could represent a new and interesting strategy to inhibit biofilm formation. In *P. aeruginosa*, it has been demonstrated that the ability to form the characteristic mushroom-structured and resistant biofilms is affected by QS (Davies et al. 1998). *P. aeruginosa* possesses two QS systems: the LasR-LasI and the Rh1R-Rh1I, with the cognate signal molecules *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL) and *N*-buturyl-L-homoserine lactone (BHL), respectively. The two QS circuits have been found to be related to the expression of virulence factors by this microrganism, including exoproteases, siderophores, exotoxins and several secondary metabolites (Passador et al. 1993; Winson et al. 1995). Similar AHL based QS systems have been shown to be important in biofilm formation of Gram negative Proteobacteria such as *Escherichia coli* and *Vibrio cholerae* (Schauder et al. 2001)

As a consequence, it may be possible to attenuate bacterial pathogenicity by interfering with bacterial QS systems. Several findings showed that halogenated furanone natural compounds isolated from the algae *Delisea pulchra* inhibit quorum sensing in *P. aeruginosa* bacteria (Hentzer et al. 2002; Ren et al. 2001). Moreover, synthesized furan-2-one analogues of natural compound products have been shown to inhibit quorum sensing-dependent swarming motility in *Serratia liquefaciens* (Hjelmgaard et al. 2003). In addition,

Hentzer et al. (2003) demonstrated that the use of quorum sensing inhibitors, including halogenated and synthetic furanones, produced an attenuation of *P. aeruginosa* virulence. In particular, *P. aeruginosa* biofilms treated with furanones have been found to be more susceptible to tobramycin.

2.3. *Cell aggregate detachment from mature biofilms*

Detachment has only recently been considered as an active process in biofilm development. This finding is based on the demonstration that active detachment is a physiologically regulated event (Stoodley et al. 2001). Sauer et al. (2002) showed, by comparing two-dimensional-gel protein patterns, that dispersed cells of *P. aeruginosa* are more similar to planktonic than to mature biofilm cells. Their data indicates that detachment represents a distinct process in the biofilm developmental life cycle. Not only does detachment allow biofilm cells to disperse to colonize new sites but can also preserve the network of nutrient exchange channels that run through many biofilms. Time-lapse microscopy has shown that biofilms can "shed" a wide size distribution of material ranging from single cells to visible clumps containing thousands of cells (Stoodley et al. 2001). In these studies a biofilm colonized flow system was able to maintain an effluent concentration of as much as 10^8 CFU/ml through detachment alone. While detachment may have some benefits for biofilm development the detachment of clumps from medical devices may have serious implications for dissemination of infection. Therefore, prevention of biofilm formation on the surfaces of medical devices not only reduces the likelihood of local infections but may also reduce detachment related complications such as recurrent bacteraemia and metastasis of infection.

3. Experimental approaches to control biofilm growth

Eradication of unwanted biofilms with disinfectants or antibiotics is hampered by the inherent resistance of biofilm organisms to these agents (Costerton et al. 1999). Removal of biofilms is facilitated when they are directly accessible, as in the case of biofilms growing on the exterior regions of the human body. For example, dental biofilms can be removed by mechanical cleansing together with the use of surface-active substances, including toothpastes and mouthwashes. Consequently, the most efficient strategy to control biofilms is to prevent their formation in the first place.

So far, the efforts to inhibit biofilm growth on medical devices have been mainly focused on preventing biofilm formation, including prevention of initial microbial adhesion or inhibition of growth of surface adherent bacteria.

3.1. *Engineering biomaterials inhibiting microbial adhesion*

As biofilm development requires the initial step of bacterial attachment to surfaces, efforts have been made to understand the chemico-physical properties of materials influencing bacterial adhesion. As a result, materials have been engineered which possess different properties, including surface hydrophilicity and charge, and tested for their ability to inhibit bacterial adhesion.

3.1.1. *Chemical modification of polymers*

Chemical modifications of polymers have improved either the anti-adhesive properties or biocompatibility of existing biomaterials (Table 1). Modifications mainly involved the incorporation of ionic functional groups in the polymer repeating unit, including sulphonate, carboxilate and quaternary amino groups.

Experiments performed by Baumgartner et al. (1997) showed that the introduction of phosphonate groups in polyurethanes, by the use of glycerophosphorylcholine as polymer chain extender, produced a strong effect on cell adhesion. Results indicated that hydrophilic surfaces were less colonized by bacteria then hydrophobic ones, due to

Table 1. Polymers mainly employed in medical devices

Polymers	Patent names
Poly (dimethyl siloxane)	Silastic (SR)
Polyethylene	Polyethylene, alkathene (PE)
Poly (ethylene terephtalate)	Mylar, melinex (PET)
Polypropylene	Propathene, prolene (PP)
Poly(tetrafluoro ethylene)	Teflon, fluon (PTFE)
Polyurethane	Texin, avcothane (PUR)

the increased ability of the phosphonate polyurethane to absorb water.

Similar findings were shown by Park et al. (2002) in the study of *S. epidermidis* adhesion on poly(ethylene oxide)-based multiblock copolymer/segmented polyurethane blends. The number of adherent bacteria was directly correlated with the amount of copolymer additive, indicating that the ability of the polymers to form hydration shells affected bacterial adhesion.

Another important finding concerned the understanding that physico-chemical properties of materials can also influence the growth rate of surface-adherent bacteria. In this regard, Gottenbos et al. (2000), showed that generation times of *Staphylococcus aureus* and *S. epidermidis* adhering to surfaces were shorter than that of planktonic bacteria and depended on the kind of interacting biomaterial. The suggestion was that surface growth of bacteria adhering to a biomaterial surfaces was affected by physico-chemical interactions between nutrients, bacteria and the biomaterial itself.

The influence on bacterial adhesion of specific functional groups has also been studied by Flemming et al. (1999). Their data indicated that the sulphonate and phosphonate groups were the most active while the quaternary amino groups were the less efficient in inhibiting bacterial adhesion. In contrast, a following study (Grapski & Cooper 2001) showed that the introduction in the polymer chain of *N*,*N*-bis(2-hydroxyethyl)isonicotinamide, a quaternary amino group, allowed the realization of a non-leaching biocidal polyurethane. In particular, experiments performed on *S. aureus* showed that the quaternized polymers were able to kill up to 95% of the surface adherent cells. Consequently, the results of the two studies suggest that the presence of quaternary amino groups in polymers did not inhibit bacterial adhesion but influenced the growth of surface-adherent bacteria. In fact, quaternary ammonium compounds have been shown to have a biocidal action mainly due to their ability to disrupt the bacterial cytoplasmic membrane.

3.1.2. *Hydrophilic polymer coatings*

Experimental findings concerning the hydrophobic characteristics of bacteria (Van der Mei et al. 1988) suggested hydrophilic polymer coatings as a possible strategy for reducing bacterial adhesion. Studies assessed either on Hyaluronan (Bridgett et al. 1993) or poly-*N*-vinylpirrolidone (Morra & Cassineli 1999) coated polyurethane catheters showed a reduction in *S. epidermidis* adhesion. However, limits of this strategy arise from the fact that physico-chemical properties of microorganisms can vary widely among species and generalizations at the species or even strain level are often impossible.

3.1.3. *Polymer coatings with albumin and heparin*

When a device is implanted, the human body reacts by coating the device with a protein layer mainly composed of fibronectin, laminin, fibrin and albumin. Some of these proteins can promote or inhibit microbial adhesion by binding to substrata or bacterial surfaces. Among them, fibronectin was recognized for its ability to mediate surface adhesion of *S. aureus*, as a consequence of the presence on the *S. aureus* cell-wall of specific fibronectin-binding proteins (Jeng et al. 2003) Moreover, as many bacteria interact with activated platelets (Wang et al. 1993), catheters were coated with anticoagulant compounds, including heparin (Paulsson et al. 1994) or albumin (Pratt-Terpstra et al. 1987). However, heparin failed to inhibit microbial adhesion, some microorganisms, including *Staphylococcus* spp. and *Candida* spp., possess receptors for this protein. However, when heparin coatings were performed by the use of benzalkonium chloride, a reduction of catheter-related bloodstream infections has been detected (Mermel et al. 1993). In this case, the antimicrobial activity was provided by benzalkonium chloride.

3.1.4. *Polymer embedded with electroconductive materials*

A novel approach to inhibit microbial adhesion on polymers has been developed by Liu et al. (1993) and consisted of the development of electroconducting polymers by incorporating carbon particles into the polymer matrix. In particular, two modified catheters were placed vertically in a nutrient agar plate and connected to an electrical device which generated a 10 μA electric current, with one catheter acting as a cathode and the other as a anode. Following seeding with either *S. epidermidis* or *S. aureus* and incubation overnight at 37 °C, a zone of inhibition of bacterial growth was

detected around the cathode. The bactericidal activity possessed by negative charged electroconducting polymers was explained by the establishment of electrostatic repulsions between the negatively charged bacterial cell wall and the polymer.

3.2. *Antiseptic and antimicrobial coated catheters*

Coating catheter surfaces with an antiseptic or antimicrobial drug, or incorporating such a substance into the catheter material itself has been demonstrated to be an important technological innovation for the prevention of medical device-related infections. However, surface modification with antimicrobials has had variable clinical success, with differences in the degree of protection attributed to: (a) the location of the device, (b) the type of antimicrobial agents used for the coatings and (c) the concentration of antimicrobial agents on the surface of the coated device.

3.2.1. *Silver coating*

Silver ions have been demonstrated to possess antimicrobial activity, particularly against Gram-negative bacteria. Its action is due both to the inhibition of bacterial replication, by its binding to microbial DNA, and to the disactivation of metabolic enzymes by its binding to sulphydryl groups (Petering, 1976).

Initially, Ag^+ ions were employed to chelate attachable cuffs made of biodegradable collagen. The cuff was attached to catheters immediately prior the insertion and created a mechanical barrier against invasion by resident bacteria of the skin. Silver ions provided an additional antimicrobial barrier. However, *in vitro* and clinical studies performed to assess the efficacy of silver-coated cuffs gave controversial results. Cook et al. (2000) conducted an *in vitro* study to assess the antimicrobial activity of a silver-coated sewing cuff versus uncoated cuff using confocal scanning laser microscopy. *S. epidermidis* adhered more to the surfaces of the silver-coated cuff compared with the uncoated cuff. These *in vitro* results cast a doubt on the anti-infective efficacy of silver-coated cuffs and suggest further assessment should be carried out using animal studies. Two prospective randomized multicenter clinical trials (Maki et al. 1988; Flowers et al. 1989) reported that the silver-impregnated cuff is effective in preventing CVC-related infections. In the first study, a threefold reduction of catheter colonization (28.9% versus 9.1%; $p = 0.02$) and a fourfold lower risk of catheter-related bloodstream infection (3.7% versus 1%; $p = 0.12$) was observed with the use of the silver cuffed catheters compared with traditional noncuffed CVCs. In the second study, a 78% reduction in catheter colonization and a 100% reduction in catheter-related bloodstream infections was reported. On the contrary, the silver-impregnated collagen cuff did not show benefits in a prospective randomized clinical trial of 200 tunnelled catheters implanted for about 143 days in cancer patients (Groeger et al. 1993). Therefore, the cuff appears efficacious only when catheters are implanted for a short time; this is probably due to the biodegradable nature of the collagen cuff to which the silver ions are chelated.

Catheters with a uniform silver layer on their surfaces have been developed by using ion-beam-assisted deposition (Groeger et al. 1993). Clinical studies (Bambauer et al. 1998; Trerotola et al. 1998; Bach et al. 1999; Lai & Fontecchio, 2002) have shown that silver coatings are effective in the reduction of catheter-related infections only when urinary catheters are concerned.

The absence of protection of silver ions on intravenous catheters may be due to the absence of silver ion antimicrobial activity against Gram-positive bacteria, the most involved microorganisms in intravenous catheter-related infections. Moreover, albumin present in the blood can bind silver ions, resulting in a lost of silver ion antimicrobial activity.

Lately, an interesting silver coated catheter system was developed by Raad et al. (1996a). The system consisted of a standard catheter with two silver wires wrapped in an helical fashion around the external circumference of the catheter. When the silver wires were connected to a power source and a current flow (ranging from 20 to 70 μA) was applied, silver ions were continously generated. This silver-iontophoretic catheter (SIC) showed *in vitro* activity against bacteria and yeasts and reduced catheter colonization in an *in vivo* rabbit model (Raad et al. 1996b). Moreover, a more recent (Hachem et al. 2003) *ex vivo* study showed that they are as safe as the clinically used chlorhexidine/silver sulfadiazine coated catheters. How-

ever, clinical trials are necessary to determine any associated side effect before use in humans.

3.2.2. *Catheter coated with chlorhexidine and silver sulfadiazine*

Chlorhexidine (CH) and silver sulfadiazine (SS) are antiseptic agents commonly used in topical applications, due in part to their synergistic activity. CH induces alterations in the bacterial membrane allowing lethal amounts of silver ions to enter the bacterial cell. Catheters having the external surface coated with CHSS have been produced (Arrowguard Blue®, Arrow International Inc, Reading, Pa., USA) and extensively studied both *in vitro* (Bach et al. 1993), *ex vivo* (Greenfeld et al. 1995) and clinical trials (Bach et al. 1994; Trazzera et al. 1995; Bach et al. 1996; Ciresi et al. 1996; Pemberton et al. 1996; Logghe et al. 1997; Maki et al. 1997; Tennenberg et al. 1997; Heard et al. 1998; Collin, 1999; Hannan et al. 1999; Sheng et al. 2000) to assess their ability in reducing catheter-related infections. Results of these clinical studies showed that CHSS coated catheters were effective in reducing catheter-related infections only when catheters were implanted for a short-term (less than 7 days). The main reasons included the low release rate of chlorhexidine after 48 h of elution (Schierholz et al. 1999) and the absence of an intra-luminal antimicrobial coating.

3.2.3. *Catheter coated with antibiotics*

In the last two decades, a number of antimicrobial-loaded catheters have been developed and studied for their ability to decrease bacterial adhesion and biofilm formation (Kamal et al. 1991; Gahtan et al. 1995; Thornton et al. 1996; Raad et al. 1996c; Schierholz et al. 1997; Kamal et al. 1998; Darouiche et al. 1999; Marconi et al. 2001; Donelli et al. 2002). Toward this end, Sherertz et al. (1989) demonstrated the efficacy of catheters coated with a variety of antimicrobials. These included dicloxacillin, clindamycin, fusidic acid, ciprofloxacin, cefuroxime, cefotaxime, and chlorhexidine, *in vitro* and in animal models.

So far, most antimicrobial coated catheters suffer from numerous limiting conditions (Donelli & Francolini, 2001) including, rapid release of the adsorbed antibiotic in the very first hours following implantation and, as a result, a relatively short persistence of antibacterial action. Development of a polymer-antibiotic system with long-term antibacterial activity depends on the catheter's ability to adsorb antibiotic molecules and to release them under clinically controlled conditions.

In this regard, a milestone in development of antimicrobial catheters with long-term protection was associated with the introduction of a bonding cationic surfactant, tri-iododecylmethyl ammonium chloride (TDMAC). Catheters precoated with this cationic surfactant are then coated with an antibiotic or antiseptic agent that is anionic in nature. Raad et al. (1996c) utilized TDMAC for the development of minocycline and rifampin-coated (M/R) catheters which were subsequently shown to prevent catheter colonization in an *ex vivo* study when implanted for 7 days in rabbits. Subsequent clinicals trials also showed their ability to reduce catheter colonization and catheter-related infections over a period of 7 days of implantation (Darouiche et al. 1999). Moreover, comparisons between minocycline and rifampin-coated and chlorhexidine and silver sulfadiazine-coated catheters showed that M/R catheters were less likely to be colonized than those impregnated with CH/SS either when the catheters remained in place for seven days or less (6% versus 21.4%) or for more than 7 days (10.8% versus 24.4%).

Polymers able to absorb high amount of antibiotics on their surfaces have been also obtained by Donelli et al. (2002) by introducing to the side chain of urethane polymers, specific functional groups able to interact with the drugs. This approach is similar to the one concerning the use of TDMAC. However, in the latter case, the antimicrobial chelant groups are directly inserted in the polymer chain.

A natural compound producted by lichens and possessing antimicrobial activity, (+)-usnic acid, was used to embed polyurethane matrices (Francolini et al. in preparation) resulting in polymers with biocidal activity against *S. aureus* and possibly other Gram-positive bacteria.

Figure 2 shows confocal images in which *S. aureus* growth on the untreated polymer is compared to its growth on the usnic acid loaded polymer, 3 days post bacterial inoculation. In the treated polymer (Figure 2b) only a few groups of cells was observed, demostrating the ability of (+)-usnic acid to inhibit *S. aureus* biofilm formation.

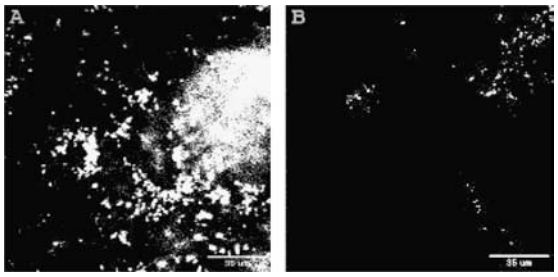

Figure 2. CSLM images showing *S. aureus* biofilm on the surface of the untreated polyurethane (A) compared with only single cells and small groups of cells attached to the surface of the (+)-usnic acid-loaded polyurethane (B) after 3 days from the inoculation. Viability staining showed that these cells were quickly killed. Scale bar = 35 μm.

To address the issue of uncontrolled antimicrobial leaching that most catheters suffer, Kwok et al. (2001), developed a controlled release system consisting of a drug-containing polymeric monolith coated with an ultrasound-responsive coating. The novelty of this system is associated with the presence of a coating based on ordered methylene chains. This coating formed an ultrasound-activated an "on–off switch" able to control drug release on demand, while keeping the drug inside the polymer carrier in the absence of ultrasound. The development of such a system could allow the release of antibiotics only under clinically controlled conditions.

In conclusion, antimicrobial loaded catheters still represent the most promising tools to reduce microbial catheter colonization and medical device-related infections. However, the risk of emerging multidrug-resistant pathogens is continuously growing. Consequently, to avoid the development of multi-resistant microorganisms, some factors should be taken into consideration, including: (i) the development of a catheter coated with antibiotics able to exhibit high local activity together with low systemic levels; (ii) coating with antibiotics not used in systemic therapies of bacterial or fungal infections; (iii) the use of synergistic antibiotic combinations possessing a broad-spectrum inhibitory activity.

4. Methods for screening anti-infective biomaterials

As the number of new biomaterials is steadily growing, highly efficient *in vitro* methods are required for quality control, screening and product improvement. Testing of the antimicrobial efficacy of anti-infective biomaterials should consider three independent aspects: (i) adhesion; (ii) proliferation; (iii) detection of bactericidal and bacteriostatic activity. One of the most common laboratory systems for the study of biofilm formation on surfaces is the flow cell reactor (Gottenbos et al. 1999; Stoodley & Warwood 2003). The substratum material of interest can be fixed in the flowcell and both initial adhesion and surface growth of bacteria can then be studied.

Biofilm formation can be followed *in situ* by using different microscopy techniques, including optical microscopy, confocal scanning laser microscopy and epifluorescent microscopy. The light microscope is one of the most traditional techniques for studying microbial adhesion on surfaces. As an example, Figure 3 shows clusters of a one day old biofilm of *P. aeruginosa* growing on the surface of a 2-hydroxyethyl methacrylate polymer.

The more recent application of confocal scanning laser microscopy (CSLM) has clarified the complex morphology that surface-adherent bacteria assume during growth and allows the examination of living fully hydrated biofilms in real time.

CSLM allows to visualize bacterial cells directly either on transparent or opaque surfaces and it also allows the possibility to study the three dimensional structure of biofilms. The use of laser illumination, confocal imaging together with computer based image processing allows the

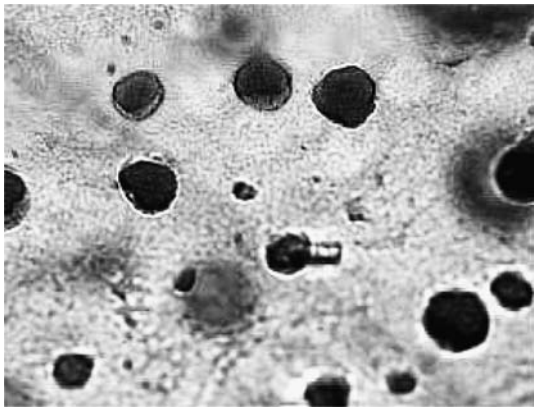

Figure 3. Biofilm clusters of *P. aeruginosa* (pMF230 gfp strain) growing on the surface of 2-hydroxyethyl methacrylate polymers and observed by ligth microscopy.

generation of three dimensional images of the specimen.

Figure 4 shows a confocal image of *P. aeruginosa* biofilm growing on the surface of a polyurethane matrix. The *P. aeruginosa* strain (pMF230) was characterized by the presence of a constitutive green fluorescent protein (GFP)-producing plasmid that allowed the direct observation of the bacteria without the use of fluorescent stains. The plasmid was constructed to carry a carbenicillin resistant cassette. The biofilm thickness and roughness can then be estimated by analysis of the Z-cross section of the images using software packages such as COMSTAT (Heydorn et al. 2000).

The CSLM and the simultaneous use of specific molecular probes also allows the determination of identity and physiological state of the adherent bacterial cells. As an example, if bacteria are stained by Live/Dead BacLight Viability kit (Molecular Probes), it is possible to estimate the number of dead and live attached bacteria. The kit differentially stains live cells vs. dead cells by using two nucleic acid stains, SYTO 9 and Propidium Iodide. The SYTO 9 green fluorescent nucleic acid stain (excitation maximum 508 nm, emission maximum 527 nm) labels all cells whether living or dead, while the red fluorescent nucleic acid stain, propidium iodide (excitation maximum 536 nm, emission maximum 620 nm), only enters cells with damaged membranes.

When both dyes are used, living cells will stain green and damaged/dead cells will stain red. Subsequent optical configuration of the confocal microscope by using appropriate excitation lasers and filters allows the qualitative observation and the quantitative determination of live and dead attached bacteria (Figure 5).

The number of bacteria attached to biomaterials can also be assessed by indirect methods, by biofilm detachment and by counting the number of colony forming units recovered from the detachment procedure. However, these methods may underestimate the actual number of living bacterial cells because of incomplete dispersal of aggregates and the presence of injured viable but non-culturable cells within the biofilm population.

As far the antimicrobial activity of anti-infective biomaterials is concerned, it is usually detected *in vitro* by the Kirby Bauer test. The test consists

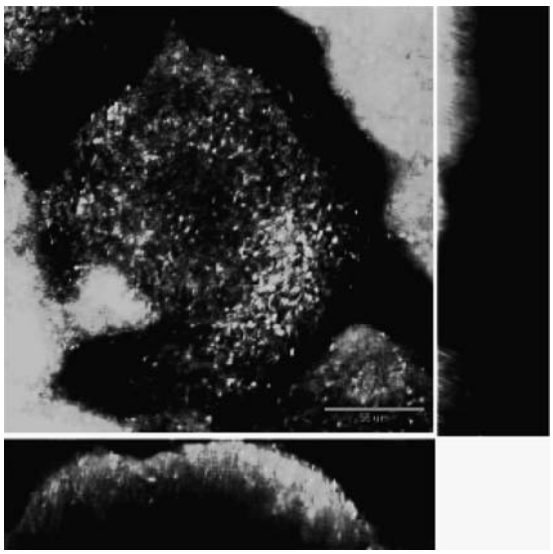

Figure 4. Biofilm of *P. aeruginosa* GFP expressing strain growing on the surface of a polyurethane disc. The image was taken by a Leica TCS-NT confocal microscope by using the 488 nm excitation laser. The square panel is the XY plan view and the saggital XZ and YZ cross sections are shown below and to the right respectively. Scale bar = 55 μm.

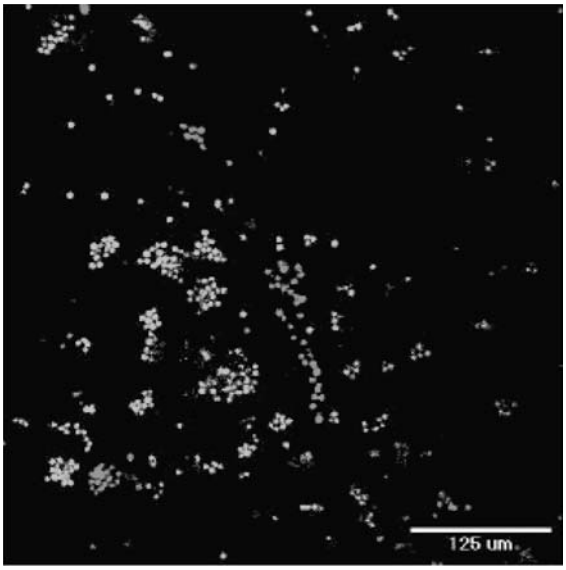

Figure 5. *Staphylococcus aureus* cells attached on the surface of a urethane polymer and stained with Live/Dead Viability Kit. The image was taken by a Leica TCS-NT confocal microscope by using the 488 nm excitation laser with a 488/568 nm/633 nm dichroic mirror and relative short pass filter of 580 nm in the first beam splitter position. A band filter allowing wavelengths of 525–550 nm to pass to the first detector was used for the SYTO 9 stain. A long pass filter of 645 nm was used for imaging the propidium iodide stain. Scale bar = 125 μm.

of placing antimicrobial loaded-polymers on solid nutrient media plates seeded with a specific concentration of bacteria, usually corresponding to 0.5 McFarland. Following incubation at 37 °C for 18 h, the antimicrobial activity of biomaterials is evaluated by the measure of the inhibition zone of bacterial growth formed around the materials and caused by the diffusion of the drug in the agar.

The correlation between the *in vitro* efficacy of biomaterials, evaluated by the Kirby Bauer test, and its activity *in vivo* was found by Sherertz et al. (1989). Results showed that only materials able to give *in vitro* inhibition zones ranging from 10 to 15 mm were efficacious in preventing *S. aureus* infection of catheters percutaneously inserted in rabbits.

However, the Kirby Bauer test was found not to be adequate for testing the antimicrobial activity of biomaterials loaded with poorly water soluble antimicrobials, since the inhibition zone was influenced by the diffusion of the drug in the agar. In this regard, Bechert et al. (2000) developed a new technique for testing antimicrobial properties of biomaterials, including anti-adhesive and antiproliferation activity. The system consisted of a microplate lid equipped with test probes forming a comb. The teeth of the comb were constructed of the biomaterials to be tested and fit precisely into the wells of commercial 96-well microplates. The teeth are colonized by turning the lid upside down and filling it with a microbial suspension. With this system up to 96 different materials can be investigated in a single experiment and under identical conditions. Adherent bacteria are detected by a standard enzyme-linked immunosorbent assay (ELISA). The antibiofilm activity of the material is measured by counting the number of daugther cells released by the attached bacteria over time. The larger the number of the released cells the less the antibiofilm activity of the material. Materials without antibiofilm activity will not only accumulate more biofilm biomass, which in itself will make the cells more difficult to kill, but may also result in greater detachment rates with consequences for dissemination of pathogens into and around the body.

5. Conclusions

Implantable medical devices are indispensable in the management of critically ill patients. Implants, including artificial heart valves, prosthetics and intravascular catheters, save human lifes and enhance quality of life. However, they are a leading cause for millions of primary nosocomial bloodstream infections. The persistence of device-related infections is mainly related to the ability of infectious organisms to form biofilms. Thus, understanding the characteristics of biofilm formation has allowed the development of biomaterials which can reduce the adhesion or inhibit proliferation of surface-attached bacteria. An increasing knowledge of the processes involved in the various stages of biofilm development promises the design of sophisticated new materials able to interfere or modify biofilms at each developmental stage.

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