Prevention of *Candida albicans* Biofilm Formation

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Abstract: Biofilm formation is often considered to be the underlying reason why treatment with an antimicrobial agent fails and as an estimated 65-80% of all infections is thought to be biofilm-related, this presents a serious challenge. Considerable attention has been devoted to the development of modified materials that prevent (or at least drastically reduce) microbial biofilm formation. In this review we present an overview of the approaches that have been used to prevent biofilm formation by the fungal pathogen *Candida albicans*.

Keywords: Candida albicans, biofilm, prevention, treatment, impregnation, functionalisation.

MICROBIAL BIOFILMS

Biofilms are highly structured, hydrated microbial communities containing sessile cells embedded in a self-produced extracelllular polymeric matrix (containing polysaccharides, DNA and other components) [1,2]. In comparison to their planktonic counterparts (i.e. free floating cells in suspension), sessile cells are often much more resistant to antimicrobial agents and this increased resistance has a considerable impact on the treatment of biofilm-related infections [2-5]. Several mechanisms are thought to be involved in biofilm antimicrobial resistance including (i) slow penetration of the antimicrobial agent into the biofilm, (ii) changes in the chemical microenvironment within the biofilm, leading to zones of slow or no growth, (iii) adaptive stress responses, and (iv) presence of a small population of extremely resistant "persister" cells [2,4,5]. The development of a biofilm occurs in several distinct phases [1,2,6]. Adhesion between cell-surface components and another surface is mediated by reversible hydrophobic and electrostatic forces and microbial attachment is the result of a balance between attraction and repulsion. Adhesion to abiotic surfaces is primarily mediated by hydrophobic interactions, whereas microbial adherence to biological surfaces is controlled by adhesins, *e.g.* lectins. Adherence is not limited to one single species as most biofilms in nature are polymicrobial [7]. For more information on the pathogenesis of polymicrobial biofilms, the readers are directed to the review of Jabra-Rizk in this special issue. Shortly after colonization, the adhered cells already show an altered phenotype (*e.g.* reduced motility, increased resistance). Secondly, the cells start to multiply and aggregate into communities. Microcolonies are formed on the surface and these will further develop and form complex, three-dimensional structures. This premature biofilm is anchored to the surface by more irreversible forces such as Van der Waals forces. The subsequent phase is characterized by the production and secretion of polymers, which form the extracellular polymeric substance (EPS). In later stages, micro-organisms can detach from the surface (either as single cells or in aggregates), become planktonic and colonize new surfaces (dispersal of the biofilm). The mature biofilm provides a three-dimensional network of cells entrapped in a polymer matrix on a colonized surface. The organization and structure of a biofilm highly depend on the species involved and the surface on which the biofilm is formed. Several factors affect the formation of a biofilm and its final architecture, including the substrate, the micro-organism, oxygen supply, availability of nutrients, the presence of saliva or a conditioning film and the EPS [6,8,9].

RESISTANCE OF BIOFILM CELLS

Life in a microbial biofilm offers considerable advantages over the planktonic mode of growth. Biofilm cells are significantly more tolerant to antibiotics and biocides. The biofilm provides protection for embedded cells against external stress such as antibacterial and/or antifungal agents and to human defense mechanisms in cases of biofilms formed in the human body. Considerable differences in susceptibility to antimicrobial agents between planktonic and biofilm cells have been reported and various underlying mechanisms for the increased resistance of biofilm cells have been described/proposed. First, environmental gradients within the biofilm structure may result in different antibiotic concentrations reaching the individual cells. Moreover, chemical and pH gradients may affect the antimicrobial activity [5,10]. The increased resistance of biofilm cells has been explained by a delayed penetration of the antimicrobial agent through the biofilm matrix. Antimicrobial agents have to diffuse through the extracellular matrix *via* water channels in order to reach the cells. The matrix may acts as a barrier to antimicrobial compounds as the target cells are located in the biofilm biomass [11]. In addition, matrix components may also bind antimicrobial agents directly [12-14]. Another hypothesis is that metabolically quiescent sessile cells grow more slowly than their planktonic counterparts, making them refractory to antimicrobial treatment. Furthermore, nutrient limitation and the production of toxic metabolites are thought to favor the formation of a biofilm [15]. Finally, the presence of persisters may underlie the resistance of biofilms to antimicrobials. Persister cells are phenotypic variants of

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wild-type strains and can survive concentrations of antibiotics or antifungal agents far above the MIC [16]. They occur in both planktonic cultures and bacterial biofilms produced by *Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus* [17] and may account for more than 1% of the population [18,19]. Highly antifungal tolerant cells have been identified in *Candida albicans* biofilms but not in planktonic cell suspensions [20].

BIOFILM FORMATION BY *CANDIDA ALBICANS*

Candida spp. occur as asporogenous yeast cells but can also form hyphae or pseudohyphae under certain conditions [21]. In the human body, *Candida* species occur as commensals on the skin as well as in the oral cavity, the gastrointestinal tract, the urogenital tract and the vagina [21]. Under certain circumstances these micro-organisms become pathogens and cause infections ranging from superficial mucous membrane infection (candidiasis) to life-threatening systemic diseases [22], particularly in immunocompromised patients with AIDS, cancer and diabetes mellitus [6,23]. Virulence factors of *C. albicans* include proteases, adhesins and the morphological conversion from a budding yeast to a filamentous form. The increasing use of indwelling medical devices in conjunction with an ageing/increasingly immuno-compromised population has resulted in a surge of hospital acquired *Candida* spp. infections, *C. albicans* ranking high among nosocomial pathogens. *Candida* infections are frequently associated with the formation of biofilms on implantable medical devices [15]. These devices readily support biofilm formation and are responsible for a considerable percentage of clinical candidiasis cases. Several experimental parameters such as the nature of the surface material [24,25], the growth medium [26,27] and conditions of incubation [28,29] influence *C. albicans* biofilm formation and structure [15]. Chandra *et al*. studied *C. albicans* biofilm formation on PDMS and polymethyl-methacrylate (PMMA) and found that for both polymers biofilm formation typically occurs in three distinct phases, i.e. an early (0 to 11 h), intermediate (12 to 30 h) and late (12 to 30 h) phase [30]. The development of a biofilm starts with the adhesion of primarily blastospores (yeast cells) to a surface. This adherence is mediated by non-specific hydrophobic and electrostatic interactions and specific adhesins on the fungal cell surface, *e.g.* glycoproteins belonging to the agglutinin-like sequence family (*ALS* family) [31-34]. The blastospore layer is in close contact with the surface and will anchor the final three-dimensional structure to the colonized substrate. The initial adherence is followed after appr. 3 to 4 h by the formation of microcolonies on the colonized surface. After 11 h, a thick fungal *C. albicans* growth can be observed. The intermediate phase (12-24 h) is mainly characterized by the synthesis of the extracellular matrix, covering the *C. albicans* cells of the premature biofilm and increasing with longer incubation times [30]. After 24 to 48 h of incubation, a complex network of yeast cells, pseudo-hyphae and true hyphae is established, with the upper layers of the biofilm mainly consisting of hyphae. Mature *Candida* biofilms show an extensive spatial heterogeneity, with a typical microcolony/water channel architecture and cells embedded in an extracellular polymer matrix. The heterogeneous build-up of mature biofilms allows an influx of water and nutrients and efflux of metabolites/waste products [35,36]. Based on non-destructive inspection of the biofilm structure using CLSM, the thickness of mature biofilms has been estimated to range from 25 up to more than 250 μm [30,35].

IN VITRO BIOFILM MODEL SYSTEMS

Several model systems have been described in the literature to grow biofilms *in vitro* on a variety of surfaces. Basically, these systems can be divided into so-called static (*e.g.* microtiter plate [MTP]) and dynamic (*e.g.* Modified Robbins Device [MRD] and Centers for Disease Control [CDC] bioreactor) systems.

Microtiterplate (MTP)

In these systems, biofilms are either grown on the bottom and the walls of the microtiter plate (most commonly a 96-

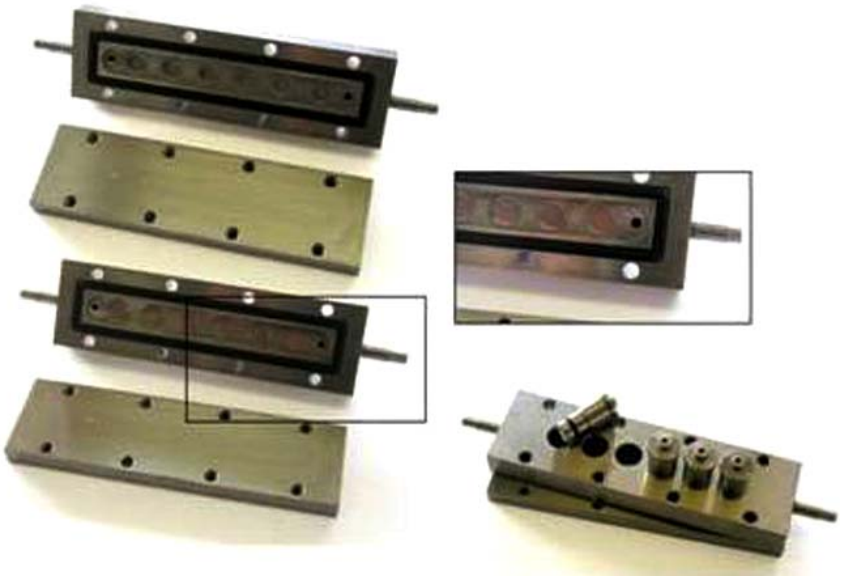

Fig. (1). Close-up of the Modified Robbins Device (MRD) loaded with PMMA disks.

well plate) or they are grown on the surface of a coupon placed in the wells of the microtiter plate (most commonly a 6, 12 or 24-well plate) [see for example references 37-40]. Microbial cell suspensions are added to the wells and the plate is incubated to allow adherence of the cells. After the adhesion phase, the cell suspensions are removed and the wells are rinsed to remove non-adherent cells. Finally, growth medium is added and biofilms are allowed to develop [38]. In this high throughput system, biofilms are formed with increasing incubation times but during their formation the growth medium is typically not replaced.

Modified Robbins Device (MRD)

Unlike static systems, colonized surfaces in a dynamic model system are exposed to a flow of a liquid medium, continuously removed by means of a peristaltic pump. The MRD (Fig. **1**) and CDC bioreactor (Fig. **2**), are two examples of dynamic biofilm model systems.

The set-up used in our research group consists of six homemade, separate stainless steel devices [41] to study the biofilm formation on disks. Prior to use, the disks are decontaminated (with ethanol) or sterilized (by autoclaving). One MRD contains six individual ports in a linear array along a channel of rectangular cross-section. Each port accepts a press-fit plug holding a disk. The six MRDs, containing the disks (36 in total) are placed in an aluminium heating block. Feedback from a Pt electrode placed in the heating block ensures a constant temperature in the MRDs. The tubing, valves and MRDs are washed, assembled and autoclaved prior to each run. Assembly is done in a Laminar Air Flow cabinet to prevent contamination and the tubing is connected to a peristaltic pump. Bottles either with growth media or inocula are connected to the MRDs and the setup includes a bypass to allow rinsing of the tubing at the inlet side of the devices and the removal of air bubbles. At the outlet side, a clamp placed on the tubing prevents the drainage of the liquid when the pump is switched off (*e.g.* during adhesion). After introduction of the inoculated solutions into the MRDs, the latter are flipped over to improve the adhesion of the planktonic cells to the disks. Once the devices are filled with the suspensions, the tubing at the inlet and outlet side is clamped off and the remaining cell suspension in the tubing at the inlet side is flushed out through the bypass. After the adhesion, the devices are flipped back, the clamps are loosened and the pump is started to allow a continuous flow of the growth medium and biofilm development on the disks. The set-up of the MRD allows a comparison of biofilms simultaneously grown under various conditions or on different surfaces. Antibiofilm strategies, *e.g.* strategies to prevent *Pseudomonas aeruginosa* biofilm formation have been studied in MRDs [42,43]. The potential of antibiotic lock therapy to remove biofilms from colonized surfaces has been evaluated in the MRD [44-46]. In the MRD, surfaces releasing active compounds can be tested in parallel without affecting biofilm development on control (unmodified) surfaces. To create a closed circuit, MRDs can be connected to glass vessels, such as a chemostat, in which the micro-organisms are grown continuously before they are introduced in the device [47-51].

Centers for Disease Control (CDC) Biofilm Reactor

The CDC biofilm reactor (Fig. **2**) consists of a glass vessel, in which three coupons (or polymer surfaces containing the developing biofilms) are held by each of eight rods. Each polypropylene rod is adjusted in such a way that the coupon is perpendicular to the rotating baffle [52]. In this reactor, the magnetic stirrer in the center of the vessel provides a continuous flow of nutrients (introduced in the reactor by means of a peristaltic pump) over the colonized surfaces [53]. As all surfaces are exposed to the same growth medium in one and the same bioreactor, this model system

Fig. (2). Set-up of the CDC reactor.

lends itself very well to monitor biofilm formation as a function of time [54-56].

Calgary Biofilm Device (CBD)

The Calgary Biofilm device (CBD) is an *in vitro* biofilm model system which has been extensively used to test the susceptibility of biofilm cells to antibiotics. The CBD has been described by Ceri *et al*. (2001) and is a 96-well plate based two-part reaction vessel : the top component consists of a lid with 96 pegs, while the bottom part of the vessel serves to channel the flow of growth medium, hence creating a consistent shear force at each biofilm-developing peg site. After biofilm formation, the biofilms on the pegs can be removed by sonication for cell counting or the lid with the biofilm-containing pegs can be introduced in a standard 96-well MTP for susceptibility testing. This *in vitro* biofilm model system has been widely used for studying biofilm formation by one [57] or multiple bacterial species [58], and by *Candida* spp. [59], as well as for the efficacy testing of disinfectants [60], antibiotics [61] and antimicrobial peptides [62]. The CBD is commercially available as the MBEC assay system to be used for testing the susceptibility of biofilm cells to antibiotics and biocides [63].

IN VIVO BIOFILM MODEL SYSTEMS

Although *in vitro* systems are frequently used, they also have their limitations, the major one being that they do not include the interaction between biofilms and the host immune system. In addition, the milieu surrounding the implanted medical device is extremely complex, which makes it difficult to mimic *in vivo* conditions using *in vitro* models. Therefore, animal model systems are necessary to study the pathogenesis of *C. albicans* biofilm-related diseases. Several animal models have been developed so far, utilizing rabbits [64], rats [65,66] or mice [67]. The model systems developed to mimick central venous catheter (CVC) infections [64,65,67] involve the placement of a CVC, followed by direct inoculation of *C. albicans* into the lumen of the catheter. In the subcutaneous rat (SCR) model, catheter segments are incubated with *C. albicans* prior to implantation [66]. Nett *et al*. recently described an *in vivo C. albicans* biofilm denture model in rats [68].

In vivo grown biofilms are structurally similar to biofilms described *in vitro*, except for the possible presence of host cells in the biofilm. In the CVC rat model it was shown that biofilm formation results in seeding of the kidneys with *C. albicans*, demonstrating that biofilms provide a niche for disseminated disease [65]. Despite the strengths of *in vivo* model systems, they are expensive, labour intensive and not easy to implement in the laboratory.

SUBSTRATES PRONE TO BIOFILM FORMATION

Several medical devices have been introduced in the human body. They include indwelling vascular catheters, cardiac pacemakers, artificial heart valves, peritoneal dialysis catheters, prosthetic hips, joints and tracheo-oesophageal voice prostheses (VPs). However, their increased use in healthcare settings has been accompanied by the formation of microbial biofilms at their surface, ultimately resulting in device-related infections. In medical settings, biofilms readily form on polymer substrates such as polydimethylsiloxane (PDMS) (also called "silicone"). PDMS is frequently used in the production of tracheo-oesophageal VPs [69]. Other polymers used in medical devices and susceptible to biofilm development are polyurethane (central venous catheters), natural rubber used for the production of urinary catheters [70] and polymethylmethacrylate (PMMA) in denture acrylics [71]. In addition, bacteria can easily form biofilms on stainless steel surfaces used in the food industry [72]. Infections associated with the use of medical devices can be localized at the site of insertion or be disseminated to the blood (septicaemia) and the organs (*e.g*. endocarditis). Both diagnosis and treatment of biofilm-associated infections are difficult. Moreover, antimicrobial therapy is usually ineffective [73].

C. ALBICANS BIOFILMS AND DEVICE-RELATED INFECTIONS

Introduction

With an ageing population, the demand for medical devices such as catheters and prostheses has increased considerably over the last years. Commonly used medical devices prone to *C. albicans* colonization and biofilm formation include central venous catheters, joint prostheses, dialysis materials, prosthetic heart valves, pacemakers, central nervous system shunts, urinary catheters, intra-uterine devices, tracheo-oesophageal VPs and dental prostheses (dentures) [15,36,74-76]. Half of all nosocomial infections are associated with the use of medial devices, i.e. the insertion of foreign, indwelling devices into the human body. Medical device related infections are extremely difficult to treat as microbial cells are protected from external influences inside the biofilm structure. Consequently, these infections are often life threatening and can also lead to failure of the device. Often, once a *Candida* biofilm has been formed *in vivo*, removal of the device is almost always required to fully eliminate the infection [36].

In Table **1** an overview of the relative infection risk associated with implantable devices in or on which *Candida* spp. biofilms develop frequently is shown [36]. *C. albicans* and *C. parapsilosis* are the most common cause of fungal biofilms on medical devices.

Tracheo-oesophageal voice prosthesis

Laryngopharyngectomy or total laryngectomy is the common treatment of laryngeal or hypolaryngeal carcinoma [77]. Malignant tumors of the vocal cords in humans are mostly removed by a total laryngectomy. Voice restoration can be achieved in alaryngeal speakers with oesophageal speech, the use of an electrolarynx and tracheo-oesophageal speech, using an artificial device. In the past few decades, the latter has been the preferred method for voice rehabilitation [78]. In order to restore speech of laryngectomized patients, Blom and Singer have created a tracheo-oesophageal puncture between the posterior wall of the tracheostome and the upper oesophagus, for placement of a VP [79]. This tracheo-oesophageal puncture is also called shunt or fistula. Tracheo-oesophageal speech is produced during expiration, i.e. air from the trachea is shunted into the

Table 1. Implantable Devices Prone to Formation of *Candida* spp. Biofilms (*Taken from Reference [36]*)

Device	Usage per year in the US	Infection risk (%)	Species involved
Central and peripheral venous catheters	5 million	3-8	*C. albicans* *C. glabrata* *C. parapsilosis*
Hemodialysis and peritoneal dialysis catheters	240,000	1-20	*C. albicans* *C. parapsilosis*
Urinary catheters	Tens of millions	10-30	*C. albicans* *C. glabrata*
Endotracheal tubes	Millions	10-25	*C. albicans*
Intracardiac prosthetic devices	400,000	1-3	*C. albicans* *C. glabrata* *C. parapsilosis* *C. tropicalis*
Prosthetic joints	600,000	1-3	*C. albicans* *C. glabrata* *C. parapsilosis*
Neurosurgical shunts	40,000	6-15	*C. albicans*
Voice prostheses	thousands	50-100	*C. albicans* *C. tropicalis*
Dentures	> 1 million	5-10	*C. albicans* *C. glabrata*

pharynx through the VP containing a one-way silicone valve. Vibration of the oesophageal wall and articulation of the produced sound using the tongue, teeth and lips create artificial tracheo-oesophageal speech. The first VPs were designed as non-indwelling devices, so that they could be removed by the patient for cleaning. The second generation involves the indwelling VPs, including Blom-Singer, Panje, Staffieri, Groningen buttons, Bordeaux and Provox 1 and 2 VPs. They are surgically inserted between the trachea and the oesophagus by an otolaryngologist, under local anaesthesia [78,80]. Nowadays Provox VPs are widely used in laryngectomees. Provox 1 VPs are placed in a retrograde manner, which is generally uncomfortable for the patient and which can be complicated in case of stenosis of the pharyngo-oesophageal segment. A Provox 2 VP is inserted in an anterograde manner. Blom-Singer VPs are similarly inserted in the tracheo-oesophageal puncture using a dissolvable gel cap. The Blom-Singer VP has been described in 1979 by Blom and Singer and the Provox VP by Hilgers and Schouwenburg in 1990 [81]. The Provox VP is commonly used in Europe, while the Blom-Singer device is more popular in the US [81]. Leakage around indwelling VPs is found in 13% up to 27% of all replacements. Custom fit Blom-Singer VPs with enlarged flanges have been designed to prevent periprosthetic leakage after tracheo-oesophageal voice rehabilitation [82]. In addition, fluid leakage through the valve is one of the most common problems related to the maintenance of the tracheo-oesophageal puncture and the functioning of the VP. Deformities on the VP surface and/or a negative pressure in the oesophagus (due to swallowing) may open the one way valve, so that liquid enters the respiratory tract. All VPs are highly susceptible to colonization by microorganisms, particularly by *Candida* spp., growing in biofilms on the surface [83,84]. Microbial examination of early explanted devices showed a predominance of *C. albicans,* as opposed to *C. tropicalis* isolates for late explanted VPs [85]. These biofilms cause malfunctioning of the valve, an increase in air flow resistance and possibly fluid leakage [86,87]. Consequently, frequent replacement of the VP is necessary [88]. The life span of VPs is different for each patient but generally not more than 3 months.

Dentures

Candida spp. including *C. glabrata*, *C. tropicalis* and particularly *C. albicans* are frequently recovered from the oral cavity. *C. albicans* easily colonizes inserted surfaces such as acrylic dentures. There is strong evidence that stomatitis, an infection of the oral mucosa, is associated with the use of dentures in the presence of *Candida* spp. in the mouth (*Candida*-associated denture stomatitis) [89]. A proper maintenance of the device and a correct hygiene are prerequisites for the prevention of stomatitis. Mouth rinses containing topical antifungal agents (nystatin or amphotericin B) to treat oral candidosis have limited efficacy. Moreover, they can lead to side effects and recurrent stomatitis if antifungal therapy is discontinued [90-92].

PREVENTION OF MICROBIAL BIOFILM BUILD-UP ON POLYMERIC SURFACES

The resistance of biofilm cells to conventional antibiotics or antifungals has prompted researchers to focus on the nature of the medical device rather than on attempts to remove or kill the micro-organisms. These approaches aim at altering the polymer surface using passive or active strategies for the prevention of biofilm formation.

Passive Antifouling Coatings to Inhibit Surface-Associated Microbial Life

An early step in the development of a biofilm is the adhesion of bacteria and/or fungi to surfaces, initiated by the interaction between compounds in the cell wall and the surface. Coatings have been developed to alter the physico-chemical properties of the surface so that micro-organism/ substrate interactions are reduced or even prevented and that subsequent biofilm development is inhibited [93]. Various polymers have been coated by applying a passive coating, *e.g.* alkanethiols on gold [94] and fish protein coatings on glass or vinyl plastic coverslips [95]. These coatings are "passive" because their effect is not due to antimicrobially active functional groups [96]. Polymers have also been modified using passive coatings of other polymers, *e.g.* polyethylene glycol, polyethylene oxide brushes and hydrophilic polyurethanes. However, the efficacy of these approaches is limited because the coatings are rapidly masked by adsorbed conditioning films [96].

Prevention of Biofilm Development by Antimicrobial Compounds Released from Modified Surfaces

Various antimicrobial compounds have been incorporated in polymers. They include antibiotics [97-101], antifungals [37,102], polyethyleneimines, quaternary ammonium compounds, silver ions or silver nanoparticles [103-105], antibodies [106-108] and nitric oxide [96,109-112]. In several studies the *in vivo* effect of catheters impregnated with antibiotics (often rifampicin) was evaluated (for an extensive review see reference 113). Negatively charged antibiotics can be electrostatically bound to a surface containing an adsorbed cationic surfactant, *e.g.* tridodecylmethylammonium chloride. The latter is immobilized by hydrophobic interaction between the long alkyl chain of the surfactant and alkyl groups on the surface. In a second procedure for preparing drug impregnated catheters, the antimicrobial compound is incorporated in the polymer by adding it prior to injection molding or extrusion (admixture) [37,114-116]. Finally, polymers can be soaked or dipped in solutions to load the antimicrobial in the polymer network (impregnation). Modified material should release high concentrations of the antimicrobial in early phases, to combat early colonization and preferably sustain this release over a sufficiently long period of time.

The effect of this approach on the ability of *C. albicans* to form biofilms on silicone was investigated by De Prijck *et al.* [37]. Biofilm formation on unmodified silicone was compared to biofilm formation on silicone loaded with nystatin (prepared both by admixture and impregnation) and silicone impregnated with miconazole, tea tree oil and zinc pyrithione. Nystatin-medicated silicone disks exhibited a concentration-dependent inhibitory effect on biofilm formation in a MTP but not in a MRD, indicating that a small fraction of free nystatin was released, which killed *C. albicans* cells in the limited volume of a MTP well. In contrast, biofilm inhibition amounted to more than 90% in the MRD on disks impregnated with miconazole, tea tree oil, and zinc pyrithione. In another study, polyethylene (PE) and polypropylene (PP) were functionalized at their surfaces with cyclodextrins (CDs) [102]. Functionalization with CDs provided PE and PP with the capability to incorporate the anti-fungal drug miconazole leading to reduced *C. albicans* biofilm formation in a MRD (up to 97% reduction).

Quaternary Ammonium Compound Coatings

Quaternary ammonium compound (QAC) coatings have been immobilized onto titanium surfaces by immersing the substrates in QAC containing solutions. Results showed an overall reduction of 98.8% of adhered cells for 4 *C. albicans* and 2 *Streptococcus mutans* isolates. Moreover, the results suggested that QAC-titanium exhibited its antimicrobial action through at least two mechanisms. The octadecyl chain inhibited initial adherence and the quaternary ammonium salt resulted in the killing of the cells that did manage to adhere, resulting in delayed or reduced microbial growth [117].

Silver Coatings

The antimicrobial properties of silver are well documented and its mechanism of action has been explained in multiple ways. Nucleophilic sulfhydryl, hydroxyl and amino groups present in proteins, enzymes and membranes easily form coordination bonds with silver cations (Ag^{+}). This results in the disruption of cell membranes and the loss of essential protein and enzyme functions, finally leading to cell death. Furthermore, silver cations are able to displace other positively charged ions, *e.g.* zinc (Zn^{2+}) and calcium (Ca^{2+}) cations, which are crucial for regulating cellular functions. The broad antibacterial spectrum of silver and the rare development of resistance together with its nontoxic effect on mammalian tissues, make it useful for antimicrobial coating of medical devices. Silver has found application in urinary catheters and in the management of wounds and burn wounds (dressings) [118]. Silver sulfadiazine is currently the most widely used topical antimicrobial agent for treatment of burn wounds [119]. Central venous catheters and orthopaedic fixation pins have also been coated with silver and have been shown to reduce adhesion of bacteria *in vitro*, but not *in vivo*. These coatings are probably unable to release the oxidized form of silver, which is believed to be responsible for the antibacterial effect. Surfaces coated with metallic silver do not release Ag^{+}. Silver loaded polymers, designed to act as a reservoir of Ag^{+}, are able to release this cation for extended periods of more than three months. Silver ions have been incorporated into polyamide *via* a melt-mix process [104] and medical grade PDMS has been loaded with organic silver complexes (nanoparticles) [120]. Silver has also been immobilized in various polymers using carriers such as zeolite [121,122] or zirconium phosphate [123]. Zirconium phosphate carrying multivalent silver, having the molecular formula $AgNaZr_6(PO_4)_9.H_2O$ (with a +2 valence for silver) showed

both *in vitro* (susceptibility testing) and *in vivo* (in a rat burn wound model) a higher antimicrobial effect against various bacteria (*S. aureus*, *P. aeruginosa* and *E. coli*) than silver sulfadiazine [124]. Roe *et al*. [120] have modified the surface of central venous catheters with a combination of silver nitrate, a surfactant and tetramethylethylenediamine. These silver nanoparticle coated catheters released silver ions over a period of more than 10 days and prevented biofilm formation by both Gram-positive and Gram-negative bacteria as well as yeasts, including coagulase-negative staphylococci, *Enterococcus* spp., *E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans* [120]. Statically grown 72 h old biofilms were inhibited for 95% (*E. coli*), 95% (*S. aureus*), 86% (coagulase-negative staphylococci), 91% (*P. aeruginosa*) and 98% (*C. albicans*), respectively. To increase the life span of the Blom-Singer tracheo-oesophageal VP, 7% silver oxide has been incorporated into the silicone matrix of the flap valve. *In vivo* results indicated a markedly longer mean lifetime of the silver coated VP, increasing from 36 days (uncoated VP) to 110 days [125]. The use of VPs with a valve containing silver oxide would be advantageous for laryngectomized patients requiring a frequent VP replacement due to fungal growth on the one way valve [126].

PREVENTION OF BIOFILM FORMATION ON POLYMERIC SURFACES FUNCTIONALIZED WITH ANTIMICROBIAL GROUPS

The purpose of covalent binding or grafting is to functionalize a surface, hence providing the material with specific properties such as enhanced hydrophilic, antimicrobial or biocompatible characteristics. Polymeric materials with antimicrobial activities, so called polymeric biocides can be prepared in different ways. A first method consists of synthesizing polymers with biocidal functional groups at their surface, *e.g.* by covalent binding of antimicrobial moieties on a preformed polymer network (grafting). The methods used can be plasma-induced or photo-induced polymerization or covalent binding using amino moieties. The second method is copolymerization. The antimicrobials are covalently bound to a surface by adding them as a block monomer during the polymerization process (see Table **2**).

Covalent Binding of Antimicrobial Moieties on a Preformed Polymer

Photo-induced grafting or photo-induced polymerization has gained a prominent place in surface modification, i.e. for the functionalization of polymeric materials. This technique

Table 2. Examples of Functionalized Surfaces with Activity Against *C. albicans*

Compound	Substrate	Procedure	Organism	Evaluation of the effect		Refs.
				Non-biofilm[a] (% reduction)	Biofilm[b] (% reduction)	
3-(trimethoxysilyl)propyl dimethyloctadecyl ammonium	Silicone	Argon plasma polymerization	*C. tropicalis* *C. albicans* Bacteria	-	Parallel plate flow chamber (64 % [total bacteria]) (88 % [total yeast])	[144]
Fluoroalkyltrichlorosilanes	Silicone	Argon plasma polymerization	*C. tropicalis* *C. albicans* Bacteria	-	Parallel plate flow chamber (< 90%)	[145]
N-alkylated PEI	Cotton, wool, nylon, PET, glass slides with amino groups	Acylation	*C. albicans* *S. cerevisiae* Bacteria	Agar overlay method (90-99 %)	-	[146]
Peptides (homopolymers)	Silicone	Coupling with AFB	*C. albicans*	-	MRD (up to 93%)	[141]
Peptides (salivary peptide histatin 5 and synthetic variants Dhvar 4 and Dhvar 5)	Silicone	Coupling with AFB	*C. albicans*	-	MRD (up to 96%)	[141]
QAP	Linear copolymer CEVE + VBC	Copolymerization	*C. albicans* Bacteria	Cut plug method Survival ratio *C. albicans* (71-100 %)	-	[143]
QAP	Crosslinked copolymer MMA + VBC + DVB CEVE + VBC +DVB	Copolymerization	*C. albicans* Bacteria	Cut plug method Survival ratio *C. albicans* (100 %)	-	[142]
Quaternised dimethylaminoethylmethacrylate	Silicone	Grafting	*C. albicans*	-	MRD (up to 92%)	[140]
Quaternised PEI	Silicone	Grafting	*C. albicans*	-	MRD (up to 74%)	[140]
Quaternised PEI	PMMA	Grafting	*C. albicans*	-	MRD (up to 74%)	[140]

[a] : procedures not involving growth in a biofilm model system

[b] : procedures involving growth in a biofilm model system effects are expressed as percent or log reduction.

Abbreviations: PET : polyester [poly(ethylene terephtalate)] ; ATRP : atom transfer radical polymerization, QAP : quaternary ammonium and phosphonium compounds, CEVE: chloroethylvinylether; VBC : vinylbenzylchloride ; MMA : methylmethacrylate ; DVB : divinylbenzene ; MPC : 2-methacryloyloxyethyl phosphorylcholine ; AFB : 4-azido-2,3,5,6-tetrafluoro-benzoic acid

allows the introduction of graft chains to a polymer without changing its bulk properties [127]. For surface modification, various polar, hydrophilic monomers have been grafted onto hydrophobic surfaces, such as polyolefins using photo-initiated polymerization. These hydrophilic monomers include acrylic acid, hydroxypropyl acrylate, methacrylic acid and methylmethacrylate [128]. Another method for grafting polymer chains, containing the biocide onto polymeric materials is plasma-induced graft polymerization. Poly(2-methacryloyloxyethyl phosphorylcholine) has been grafted onto the surface of PDMS using argon plasma as an activator of the surface [129] and onto the surface of Co-Cr-Mo alloys using oxygen plasma [130]. Oxygen and atmospheric plasma were used to graft acrylic acid or vinyl sulfonic acid onto solvent cast chitosan membranes [131] and polyethylene terephthalate [132]. Copper metallization (using poly[4-vinylpyridine] as metal ion trapping polymer) of polytetrafluoroethylene surfaces was carried out using helium plasma to design the interface of electronic devices [133]. Other examples of plasma-induced polymerization include graftings onto PDMS [134], polytetrafluoroethylene membranes [135], polyvinylidene surfaces [136] or polyurethanes [137]. An oxalate degrading enzyme, i.e. oxalate oxidase has also been covalently bound to PDMS. It was hypothesized that the enzymatic activity of oxalate oxidase could reduce the amount of oxalate crystals near the PDMS surface, hence preventing calcium oxalate crystal formation and subsequent encrustation of urinary catheters [138]. Various microbicidal surfaces have been synthesized by introducing positively charged groups on glass slides or nanoparticles. To this end, alkylated polyethyleneimines (PEIs) have been coupled by acylation to glass slides containing amino groups [139]. N-alkylated PEI containing glass showed a high bactericidal efficacy (93%) against airborne *S. aureus* with the highest reduction observed for octadecyl derivatized PEI moieties [139]. De Prijck *et al*. [140] evaluated whether quaternised PEI covalently bound to silicone or PMMA could reduce *C. albicans* biofilm formation. Although the use of C1 or C4-quaternised PEI reduced biofilm formation to some extent (reductions ranging from 35 to 74%), reductions were lower than expected based on sensitivity of planktonic cells. Several cationic peptides were covalently bound to silicone by De Prijck *et al*. [141]. The salivary peptide histatin 5 and two synthetic variants (Dhvar 4 and Dhvar 5), as well as polylysine, polyarginine, and polyhistidine were used to prepare peptide functionalized silicone surfaces. Dhvar 4 functionalized silicone yielded the highest reduction of the number of sessile *C. albicans* cells in the MRD. Poly-D-lysine PDMS, in particular the homopeptides with low molecular weight (2500 and 9600) showed the highest activity against *C. albicans* biofilms, with reductions of 93% and 91%, respectively. De Prijck *et al*. [140] covalently bound dimethylaminoethylmethacrylate (DMAEMA) and PEI moieties to silicone and PMMA surfaces and subsequently and subsequently quaternized these moieties. Covalently bound quaternized polyDMAEMA and PEI inhibited *C. albicnas* biofilm growth, with reductions up to 92%.

Copolymerization

In copolymerization, a polymerizable monomer, which contains the biocide is mixed with another monomer. Most attention has been paid to the polymeric quaternary "onium" salts. Both phosphonium and ammonium containing copolymers have been prepared [142,143]. A series of compolymers (Fig. **3**) derived from the monomer dimethylaminoethylmethacrylate with four different hydrophobic monomers (ethyl, butyl, cyclohexyl, octyl methacrylates) has been synthesized using free radical copolymerization with azobis-isobutyronitrile (or benzophenone) at a temperature of 60°C. The resulting copolymer was further modified with 1,3 propanesulfone to yield polysulfopropylbetaine derivatives. Broth dilution methods showed antibacterial activities against *S. aureus* and *E. coli,* with MIC values ranging from 1125 to 2000 μg per ml.

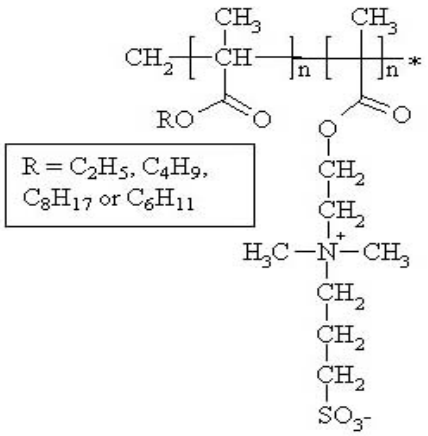

Fig. (3). Copolymer consisting of 2-dimethylaminoethylmethacrylate, with different hydrophobic methacrylate monomers (inset).

CONCLUDING REMARKS

Prevention or reduction of biofilm formation by antimicrobial compounds covalently bound to a surface requires that the antimicrobial moieties are effectively immobilized, densily grafted, remain stably bound to the surface long enough and are available to cells that freshly adhere to the surface (i.e. are not "masked" by the build-up of a layer of dead cells). The number of new candidate molecules for covalent binding to polymers is restricted, as several anionic, cationic, hydrophilic and hydrophobic compounds immobilized on polymers have previously been studied. Grafting of antimicrobially active compounds is preferred over their incorporation in the polymer as biofilm development is a process which occurs at the surface and not in the bulk. The overall reductions of the number of sessile cells in a biofilm are usually not higher than 90%. However, further research is needed to increase the density of antimicrobial compounds on the surface. In addition, antibiofilm effects will have to be determined not only against monospecies biofilms but also against biofilms consisting of more than one microbial species. Cytotoxicity tests are necessary to demonstrate that the modified substrates are not toxic when introduced in patients. Besides using grafting strategies, polymers can be modified by incorporation of compounds in the bulk polymer. For this purpose, solvent-based approaches are mostly used which may result in residues inside the polymer. Innovative impregnation approaches should focus on solvent-free approaches (*e.g.* using supercritical carbon dioxide).

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