

Biofilms: Recent Developments on an Old Battle

Carla C. C. R. de Carvalho*

IBB–Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

Received: September 4, 2006; Accepted: November 7, 2006; Revised: November 10, 2006

Abstract: Microbial cells are able to adhere to surfaces and through an exo-polymeric matrix they establish microbial communities known as biofilms. This form of immobilised biomass can be responsible for heat and mass transfer limitations in industrial processes and be a source of contamination and proliferation of infections in water supply systems and medical devices.

Several processes to prevent and destroy biofilms in surfaces and tissues have been patented and the new developments are reviewed. Most of the patents propose the use of UV radiation, high temperatures and addition of oxidant compounds to clean surfaces, which may be protected by antimicrobial coatings containing metal ions, non-pathogenic bacteria, time-release agents and biocides. Several biocidal compositions, comprising mixtures of disinfectants and biocides, are also presented. Mechanical, chemical and enzymatic procedures are discussed and particular emphasis is given to the cleaning and protection of medical devices and water supply systems.

Keywords: Biofilm, micro-organism, exopolymeric matrix, decontamination, disinfection, deterrence, biocide, detergent, water supply, ultra-pure water, medical devices, haemodialysis, catheter, antimicrobial surface, cell adhesion.

INTRODUCTION

Nearly 99% of the micro-organisms living on the Earth live in microbial communities known as biofilms [1]. They are formed by adhesion of cells to surfaces through an exopolymeric matrix. This matrix is important both in the formation and structure of the biofilm, and also on the protection of the cells since it may prevent the access of antimicrobials and xenobiotics to the cells inside the biofilm and confer protection against environmental stresses such as UV radiation, pH shifts, osmotic shock and desiccation [2-4]. Fossilised biofilms with 3.5 billion years are among the oldest records of life on Earth [5]. Biofilms are the world's most successful form of colonialism as they live on soils, sediments, mineral, plant and animal surfaces, even under extreme environments, from glaciers to hot vents. Biofilms are even able to thrive in highly irradiated areas of nuclear power plants [6].

Cell aggregation is advantageous and required in several processes, namely in biological wastewater treatment and bioremediation systems. However, they constitute a serious problem in many industrial processes (*e.g.* paper, food, cosmetic and pharmaceutical industries) because they can cause corrosion and limit mass and heat transfer in pipes and tubes and mainly in water distribution systems and healthcare environments as they are a source for microbial infections. Furthermore, biofilms can also bioaccumulate metals and toxic compounds [7]. Biofilms can contaminate contact lenses, catheters, endotracheal tubes, mechanical cardiac valves, prosthetic joints, surgical sutures, etc. It is

estimated that 750,000 surgical site infections occur annually in the US, resulting in 3.7 million extra days of stay and more than US\$1.6 billion in hospital expenses [8]. Furthermore, cells in biofilms have higher resistance to antibiotics and biocides than planktonic cells and gene transfer is possible horizontally, which improves the exchange of genes between resistant and non-resistant strains [9].

Bacterial strains that do not produce exopolymeric substances (EPS) present lower adherent abilities than slime-producing strains. EPS is particularly valuable after the initial phase of adhesion in organisms, conferring protection against phagocytosis, interference with the cellular immune response and reduction of antibiotic potency [10,11]. In fact, the host immune system is, in general, capable of rapidly kill non-adherent bacteria. The slow growth rate observed in biofilms and/or transport limitations of nutrients, metabolites and oxygen between the surface and the interior of the biofilm could be responsible for an increased antibiotic resistance over planktonic cells [12,13]. Furthermore, the EPS matrix acts as an anchor, securing the cells and preventing their detachment under flow conditions [13], although detachment of cells may also be an important process for propagation and formation of new colonies.

In this review, the later processes to control biofilm proliferation in medical devices and water supply systems, and the new developments on anti-microbial surfaces, detergents and biocides will be addressed.

1. FIGHTING BIOFILM FORMATION IN SURFACES

Metal and metal alloys are particularly susceptible to microbial action, the result being corrosion and fouling and ultimately material failure. Although plastic and rubber are less prone to biocorrosion, environmental factors such as temperature and solar radiation can trigger the microbial

*Address correspondence to this author at the IBB-Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal; Tel: +351-21-8417681; Fax: +351-21-8419062; E-mail: ccarvalho@ist.utl.pt

attack. Virtually any type of surface on Earth can be colonised by bacteria, yeasts, viruses or fungi.

Biocorrosion, which is the term used to name deterioration of metal as a result of microbial activity (microbiologically influenced corrosion, MIC, has also been used), can affect kinetics of cathodic and/or anodic reactions [14] and disrupt chemically protective layers [15]. Nevertheless, biofilms have also been used as a protective layer against corrosion [16] and this point will be discussed further on the paper. Among the groups of bacteria reported to cause metal corrosion in terrestrial and aquatic environments are sulphate-reducing bacteria, sulphur-oxidising, iron-reducing and iron-oxidising bacteria, manganese-oxidising bacteria and bacteria able to excrete organic acids [17].

Bacterial adhesion to surfaces include (i) an initial attraction of the cells towards a surface due to van der Waals attractions forces, Brownian motion, gravitational forces, electrostatic charges and hydrophobic interactions [18], (ii) molecular and cellular interactions by use of microbial surface polymeric structures such as capsules, fimbriae or pili and EPS [19,20]. A recent review [21] discusses the three theories describing bacterial-material interactions: the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, the thermodynamic approach and the extended DLVO theory.

Several attempts have been made throughout time to protect materials, instruments and equipment by plating, painting and application of enamel. Protection of surfaces include: addition of a non-adhesive or antimicrobial coating, release of a toxic agent at the surface, introduction of a "shape-shifting" surface or surface modifying additives and addition of biocidal substances.

1.1. Antimicrobial Surfaces

To prevent outbreaks of diseases by transfer of micro-organisms between materials such as food and textiles, investment has been made on the development of antimicrobial surfaces. The addition of organic or inorganic compounds to superficial coatings may directly inactivate or kill the micro-organisms attached to the surface and/or prevent their adhesion. A suitable biocide should be selected to provide a broad activity against the micro-organisms commonly found in the target application area and may be added to polymers during its manufacture.

Incorporation of anti-microbial agents into plastics although effective to prevent biofilm formation hampers their application in food and medical packaging unless only very small amounts, usually ineffective, are released. To overcome this, Seabrook and Heymann [22] added chemical controllers, in particular, vitamin E to polymeric materials to control the rate of release of 10,10'-oxybisphenoxarsine and 2-(4'-thiazolyl) benzimidazole to keep their concentration low enough to use the polymers in agricultural and food products. Phytochemicals and phytonutrients, which may be even extracted from plants and herbs, may be used as safe antimicrobial compounds in food contacting surfaces, combined with safe chemical releasers such as citric acid [23]. Daeschel and McGuire [24] suggested the application of a polypeptide bacteriocin, such as nisin. To protect a surface intended at protecting food, an aqueous bacteriocin solution is placed in contact with the surface so that

bacteriocin is adsorbed to the surface. These treated surfaces can kill susceptible bacteria in food packaging, even for extended periods of time.

It should be noticed that antimicrobial surfaces may prevent cross contamination between *e.g.* food, beverages and cosmetic products but should not replace good hygienic practices in product handling.

Fitzpatrick and co-workers developed ionene polymers, which are cationic polymers with a large number of positively charged nitrogen atoms in the main polymeric chain [25]. The antimicrobial polymer may be used in the treatment of microbial infections of the skin, oral mucosa and gastrointestinal track in mammals, as well as in the prevention or eradication of micro-organisms on a susceptible surface.

Besides slow-releasing systems, release-on-command systems and non-leaching systems have also been developed. These systems present the advantage of decreasing the risk of favouring microbial tolerance and resistance to biocides, as the biocide is released only when required. Monomeric oxazolidinones or hydantoin after homopolymerisation or copolymerisation with other monomers produce materials or coatings, which acquire biocidal properties whenever exposed to solutions of chlorine or bromide [26]. These coatings may be applied to glass, plastic, metals, fibres, food wrappers, pipes, tiles, etc. Surfaces to control the release of biocides can also be applied, *e.g.*, by converting liquid gelatine capsules into multi-coated controlled released systems [27].

Halogen-releasing compounds have been proposed by McCarthy *et al.* [28] by application of sodium hypobromite stabilised with a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one. 1,3-dibromo-5,5-dialkylhydantoin is capable of releasing a higher amount of chlorine in aqueous medium than N,N'-bromo-chloro-5,5-dimethylhydantoin [29]. The use of two biocides, such as isothiazolones and sodium hypobromite, allows an environmental and economic solution to control biofilms as small amounts of each biocide may be used. Furthermore, a better result may be achieved as isothiazolones are effective against bacteria and sodium hypobromite is able to penetrate and disrupt adherent biofilms.

Some coatings may not be able to inhibit cell attachment, acting only on attached cells: (-)-usnic acid-loaded polymers did not prevent attachment of *Staphylococcus aureus* cells but the adherent cells were killed, preventing the formation of a biofilm [30]. Reactive groups may also be linked to the polymers such as polypropylene, rubber, silicone, polyester and polyurethane to produce surface functionalised polymers to be used in the production of fabrics [31].

1.2. Addition of Metals to Surfaces

Corrosion may also be prevented by chemicals that include some salts present in hard water, such as those observed in mineral deposits inside Roman water systems. Before the understanding of the processes involved, copper was used in the Roman Empire to build pipe systems to deliver drinking water. Romans had observed that water delivered through copper was safe to drink and copper

utensils and cookware prevented the spread of diseases. Phoenicians preferred silver, using it to make containers to preserve water, wine and vinegar during their long journeys in the Mediterranean Sea.

As cleaning procedures with liquid and gaseous disinfectants were shown ineffective to prevent and destroy biofilms in surfaces, especially at long term, the idea of introducing biocidal agents, such as metals, within the surface material became an appealing solution. Silver coatings may be used in *e.g.* implanted devices to prevent infections in tissue pockets [32], dressings [33], textiles [34] and films [35]. In the fabrication of artificial gel matrixes (based on polyethyleneglycol or lactose) to heal wounds, silver was used both as the catalyst for gelation and anti-microbial agent [36].

Silver is also a very good disinfectant of drinking water, being used in tablets for rapid water purification that have been developed mainly due to the presence of militaries from developed countries in conflicts in the third world and the outspread of tourism in remote areas of the World. Tablets using silver are a good alternative to those using decolourised iodine tincture as some people are allergic to iodine and thyroid abnormalities related to the use of iodine to purify water have been reported [37]. Nevertheless, the application of metals in pipes for water distribution must take into consideration present water regulations concerning the concentration of aluminum, silver, copper, etc. Although the European directive 98/83/EC concerning drinking water does not state the limit for metal's concentration, many European countries limit the silver concentration to 0.05-0.1 mg/L. The US Environmental Protection Agency limits, in the National Drinking Water Regulations, the concentrations of copper to 1.0 mg/L and of silver to 0.1 mg/L.

1.3. Addition of Cells and/or Enzymes to Surfaces

Bacteria can have, surprisingly, a beneficial role in preventing corrosion. The bacterial protection of stainless steel can be the result of (i) production of inhibitors that are retained in the biofilm matrix [38], (ii) reduction of oxygen concentration at the metal surface [39], (iii) secretion of antimicrobial proteins [40], (iv) production of enzymes able to degrade the biofilm matrix [41]. Micro-organisms can interfere with others while competing for an ecological space. The relations between bacteria can be synergistic or antagonistic and bacterial interference can have a major role in proliferation of infections [42].

Polsenski and co-workers proposed a process of layering a coating material with micro-organisms and enzymes [43]. The micro-organism (*e.g.* *Saccharomyces*, *Aspergillus*, *Bacillus*), chosen so it can survive and grow under the pretended environment, should produce at least one amylolytic or proteolytic enzyme and the enzyme in the second layer may be produced by the micro-organism added or not. A layer containing nutrients for the micro-organism in the coated surface should be placed in an under layer, away from a competing or disruptive fouling community.

Homoserine lactone blockers, such as *N*-(3-oxo-decanoyl)-L-homoserine lactone and *N*-butyryl-L-homoserine lactone or analogs, have been proposed by Davies and co-workers to control, prevent and remove biofilms produced

by bacteria, alga, fungi and protozoa in surfaces [44]. Homoserine lactone autoinducers are known to influence "quorum sensing", which is a signalling system that allows bacteria to regulate gene expression depending on the number of individuals in the population. Previous patents had involved lactone autoinducers for diagnosis [45] and therapeutic purposes [46] but not to regulate biofilms.

2. BIOFILM DETERRENCE WITH BIOCIDAL AGENTS

Biocides are non-antibiotic chemical compounds with disinfecting and antiseptic properties since they are able to inhibit microbial growth or even kill micro-organisms. Biocides are being added to washing powders and liquid detergents, plastics for food packaging, fabrics, mouth washes, etc (Table 1). For that reason, they should be effective at low concentrations, be non-toxic and biodegradable. The biocidal action depend on (i) chemical properties (*e.g.* optimum pH and temperature of activity, reactivity), (ii) micro-organism (*e.g.* tolerance/resistance, metabolic status, number of organisms in the population), (ii) environment (*e.g.* surface type, water activity, presence of other reactive compounds). The biocide should therefore have a wide range of activity, both in terms of type of micro-organisms susceptible and conditions of action and should not be easily inactivated.

Table 1. Examples of Biocides Used in Patents Concerning Biofilm Deterrence

Biocide group	Examples of compounds	Examples of Patents
Peroxides	Hydrogen peroxide, Potassium permanganate	[90, 97], [52,53]
Halogen releasing compounds	Sodium hypochlorite, Sodium hypobromite	[97,27], [52]
Quaternary ammonium compounds	Didecyl dimethyl ammonium chloride, Dioctyl dimethyl ammonium chloride	[55]
Phenolic compounds	Phenol, Triclosan, Thymol	[52,54]
Terpenes	Carvone, Saponin	[48,52]

Manyak and co-workers developed a method to prepare multiple-specific hydrolytic enzyme mixtures by growing bacterial strains (*Microbulbifer* or a *Marinobacterium*) using more than one polysaccharide present in the targeted industrial or disease-related biofilm as carbon source and consequent isolation of the enzymes required [47]. The enzymes can be applied in the removal and prevention of biofilms in *e.g.* pipes, heat exchangers, cooling towers, swimming pools, teeth and even in the treatment of cystic fibrosis' patients and those with infections in implanted medical devices. Enzymes can also be used to remove and prevent the formation of dental plaque, without damaging tissues. Tsuchya patented compositions for oral care products, containing a dextranase derived from *Paecilomyces lilacinum* and a mutanase isolated from *Trichoderma*

harzianum. The dextranase is aimed at degrading the α -1,6-glycosidic linkage in dextran whilst the mutanase is target at destroying the α -1,3-glycosidic linkages in “mutan”, which is a major component of water-insoluble extracellular polysaccharides in dental plaque.

By studying the adhesion processes, Hultgren and co-workers developed compounds to mimic a chaperone G1 α -strand or an amino terminal motif of a pilus subunit. Pili structures anchor many Gram-negative bacteria to surfaces, beginning the process of adhesion. The presence of mimic compounds prevent or inhibit the pilus assembly to its hydrophobic groove and thus cell adhesion.

The tripeptide saponin, which reduces the surface tension of the biofilm promoting its detachment, can be used in combination with sodium lactate so that its acidic form kill the bacteria as the cells are released from the matrix [48]. The procedure is gentle enough to be used to wash and decontaminate eggs [49]. Terpenes are natural compounds which may be considered as GRAS (Generally Recognised As Safe) substances. For example, carvone, a component of caraway, dill and spearmint, has both antibacterial and antifungal activity and has been used for centuries in seed oil extracts, long before the mechanism of action was understood [50].

Mixtures of biocidal agents and disinfectants (either of natural or synthetic sources) seem to be the best and most efficient solution to destroy biofilms, according to the patents published. The mixtures usually contain effective amounts of a detergent to decrease the surface tension of the biofilm, a denaturing agent to attack the extracellular matrix and a biocide to kill the micro-organisms. Prevost *et al.* [51] proposed dodecyl sulphate (SDS) at a concentration of 2% both as detergent and denaturing agent and lactic and mandelic acids or a potent oxidant as hydrogen peroxide as bactericidal agent. A mixture containing a terpene or sodium hypochlorite, a detergent cetylpyridinium chloride (at a concentration of at least 0.1%) and an effective dislodging amount of salt (or corresponding acid that at the required pH will form the salt) was proposed by Barbeau and co-workers [52]. A further bactericide, for instance, hydrogen peroxide (about 5%), phenol derivatives (at least 0.1%) or sodium hypochlorite (at least 0.5%) and biofilm dislodging enhancer agents such as chaotropic agents or calcium chelators, can also be added. A similar biocide composition to clean surfaces was proposed by Day [53], comprising a peroxide and a hypochlorite compound at a ratio of at least 10:1(w/w) peroxide:hypochlorite.

A proposed “biocidal complex” aimed at killing bacteria and destroying at least part of the biofilm contains: a free-radical generating compound (*e.g.* hydrogen peroxide), a GRAS disinfectant (*e.g.* thymol) and an acid sulphate (*e.g.* sodium bisulphate) to catalyse the free radical formation [54]. The compounds should be maintained separately until use. This formulation may be used to clean and disinfect water lines in dental equipment. Mixtures of furanones and other disinfectants, such as at least one organosilane with quaternary ammonium functionality and/or at least one quaternary ammonium compound can make a synergistic antimicrobial composition effective against bacteria, yeasts and fungi [55].

3. BIOFILM DETERRENCE IN MEDICAL DEVICES

Biofilms can colonise almost all surfaces, from glass to steel, from cellulose to silicone, which are the main materials used to produce medical devices (Fig. 1). For fifty years, medical instruments have been sterilised by the medical industry with gaseous agents such as ethylene oxide and chlorine dioxide, which are commercialised in nonflammable blends with inert carrier gases to overcome their explosive character [56]. However, many of the corrosion or fouling processes which take place after adhesion and growth of micro-organisms occur inside the human body. Harsh treatments of the surfaces of the devices to prevent and/or destroy cell adhesion are, therefore, hampered.

Protective coatings have been the most wide spread method to prevent corrosion in metallic surfaces. However, cathodic protection appears as a good protective technique, especially in the medical field. Cathodic protection acts by providing an electrical current from external sources to counteract the normal electrochemical corrosion reactions.

Anti-corrosion and anti-fouling, combined with anti-rejection and antibiotic properties of medical devices for intra and extra body application were achieved by placing a semiconductive coating in metallic devices [57]. The technique uses semiconductor technology with no external anode, electrolyte or current flow. An electronic filter, connected to the conductive coating, monitors and minimises the corrosive noise generated by the coated conductive structure.

Other invention uses “oligodynamic iontophoresis” to prevent microbial adhesion to intraocular lens [58]. This technique involves the movement of ions from a metal such as silver to a conductive medium (saline, blood or urine) by application of an electrical current. Silver is effective against

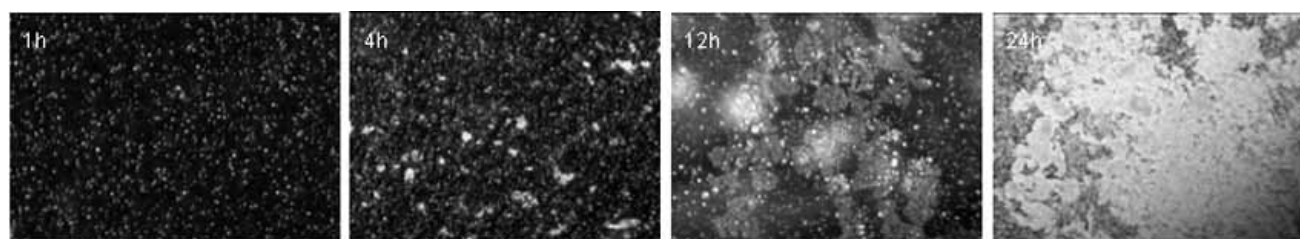


Fig. (1). Adhesion of *Rhodococcus erythropolis* cells to glass. Images taken with brightfield transmitted light microscopy (10x lens; numerical aperture = 0.25; horizontal field width = 0.8 mm; vertical field width = 0.6 mm).

a broad range of bacterial, yeast and fungal cells since the positively charged silver ions can interact with thiol groups of membrane-bound enzymes and proteins, uncouple the respiratory chain from oxidative phosphorylation or collapse the proton-driving force across the cytoplasmic membrane [59,60]. The current required to remove a bactericidal amount of silver ions from electrodes into solution is in the range of 1-400 μ amperes. Since an external electric power supply is required, oligodynamic iontophoresis has had limited use in medical devices. However, a composite material made of a conductive organic polymer matrix, in which two metals with a chemical half-cell potential difference are suspended, can act as an iontophoretic material [61]. The voltage potential generated between the two dissimilar metals generates a current of electrons in the conductive matrix after exposure to an electrolyte solution such as body fluids.

An improvement to an implantable port and other devices such as pacemakers and artificial joints, also encompass the presence of metallic silver, an inorganic silver compound, a silver salt of an organic acid or other antimicrobial compounds as taurolidine on the surface of the port or device [32]. The improved implantable port, including a housing, contains a silver coated surface and is implanted within a subcutaneous tissue pocket, or uses a separate container, in the form of a pouch, that is placed over the device before implantation in the subcutaneous pocket or used as a reservoir to hold the antimicrobial solution (*e.g.* taurolidine).

Antimicrobial substances required to destroy biofilms are not only toxic to micro-organisms but may also be toxic to the patient, causing allergic reactions, whilst some micro-organisms may produce specific compounds able to destroy the biocide molecule. To overcome these disadvantages, biocompatible acid precursors may be used to inhibit microbial attachment and/or growth on the surface of medical devices [62]. This is achieved once the device is placed inside the patient's body, as acid moieties are produced from the acid precursor (examples include glycolide, lactide, *p*-dioxanone, glycol glycolate and lactyl lactate), lowering the pH of the coating and adjacent device. The acid precursor may (i) diffuse through the coating and hydrolyse at the surface or (ii) first hydrolyse and the resulting acid diffuse through the coating to the surface.

Biodegradable microshapes, such as microspheres, containing time-release agents effective against bacterial biofilms can be placed into the gingival crevice or periodontal region to combat bacteria adhesion to teeth [63]. Bacterial plaque is the main cause of several periodontal diseases, including gingivitis and periodontitis. This technique may overcome the difficulties of periodontal prevention and therapy based on the individual motivation and skill to use toothbrushes, dental floss and other oral hygiene instruments.

Bacterial interactions, which may be synergistic or antagonistic, have a major role in maintaining the flora of skin, intestines, uroepithelial cells and mucous membranes, and thus in preventing the establishment of pathogenic bacteria. Several mechanisms of bacteria interference have been described, *e.g.*, production of antagonistic substances, competition of nutrients, changes in the microenvironment

and lack of available adhesion area for the pathogenic bacteria due to the presence of the non-pathogenic strains [42]. A recent patent describes how an antimicrobial and a non-pathogenic bacterial coating layer may be effective to demote the infection of surfaces by pathogenic micro-organisms [64]. The non-pathogenic bacteria, resistant to the antimicrobial used, should interfere with pathogenic strains trying to colonise the surface and dominate the ecological space. The antimicrobial agent, to be used with a kit applied to the medical device before implantation, can be an antibiotic, an antiseptic, a disinfectant or a combination of the three. Non-pathogenic gram-negative bacterium should be selected from *Enterobacteriaceae* (*e.g.* *Escherichia*, *Salmonella* and *Yersinia*), *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, *Gardnerella vaginalis* and *Acinetobacter* species. In the patent's context, "non-pathogenic bacteria" refers to known non-pathogenic bacteria and to pathogenic bacteria that have been mutated or converted to non-pathogenic strains.

3.1. Biofilm Prevention in Catheters

Catheters for vascular access and haemodynamic monitoring (*e.g.* infusion of electrolytes, drugs or chemotherapy agents; draw of blood for analysis and haemodialysis) are one of the most used type of medical devices and responsible for a high number of nosocomial infections [65]. There are four potential sources of catheter infection: (i) the presence of microbial cells at the site where the catheter is inserted through the skin; (ii) the catheter hub; (iii) pathogenic cells travelling through the blood stream from a distant infection site; (iv) contamination of the infusion fluid [66]. The degree of pathogenicity will depend upon microbial adherence, which is also related to the catheter material, and the host defence system.

Peritoneal dialysis catheters for acute use (application for less than 4 days) are usually made of relatively rigid nylon or polyethylene, whilst those for chronic afflictions are fabricated with soft materials, such as silicone rubber or polyurethane. The chronic catheters have extraperitoneal cuffs that cause local inflammation and tissue growth, that helps positioning the catheter while prevents fluid leaks and bacterial colonisation. Nevertheless, since silicone is a hydrophobic polymer, it is susceptible to biofilm formation. However, the hydrophilic polyurethane has also been reported to be attacked by micro-organisms. An antimicrobial agent such as triclosan or butyl paraben can be dispersed in medical grade silicone elastomer used to fabricate prosthetics and parts of voice prosthesis [67].

During peritoneal dialysis, the catheter is introduced directly in the peritoneal cavity of the patient and the catabolites migrate from the blood, across the peritoneal membrane, to the dialysis solution. An expected complication of peritoneal dialysis is peritonitis, being the catheter the major access for infection as it makes the bridge between the sterile inside the peritoneal cavity and the exterior of the body. Addition of 0.5-4% taurolidine into peritoneal dialysis solutions, and to lock and flushing solutions, may reduce or prevent microbial colonisation [68].

Besides risk of infection, clotting of catheters may also occur, since these devices may rest in the patient's body for a significant time, being used on a weekly or daily basis. To prevent the formation of thrombus, catheters are usually filled with a lock solution. The anticoagulant normally applied is heparin, which is injected into each catheter lumen immediately after each use. The heparin solution should be maintained in the lumen but must be withdrawn before the next application because heparin may cause haemorrhages.

Several innovations have been presented related to the lock solution. In one method, a syringe containing a lock solution of citrate salt (1.5-50%, w/w) is used to infuse the lumen of an indwelling catheter [69]. As alternatives, polyethylene glycol, glycerol, polyglycerol, polygeline or mixtures of them, may be added to the lock solution to increase its viscosity and density to expand the time of permanency, or the lock solution may be prepared to have a pH lower than 6.5.

A method to treat infections related to indwelling catheters was developed based on the application of an electric field through two electrodes applied to the internal and external surfaces of the catheter [70]. An antimicrobial drug solution is inserted in the catheter and in the receptacle of the electrode placed on the skin and around the exit area of the catheter. The permeability of the catheter to antimicrobial drugs increases both in the internal and external surfaces, helping to kill micro-organisms in difficult to access places.

Crossley proposed a technique that uses photodynamic therapy: light of a selected wavelength or wavelength band is coupled to the medical device and activates the release of a toxic substance from at least one photosensitizer compound embedded in the surface [71]. The photosensitizer may be a natural compound (such as porphyrins, polyynes or anthraquinones), a dye (rhodamines, methylene blue, etc) or other substance that reacts to light (such as cyanine compounds). Antimicrobial activity of these compounds may be inherent or acquired upon exposure to light.

4. BIOFILM DETERRENCE IN WATER SUPPLY SYSTEMS

Several methods have been proposed to prevent and destroy biofilms in water supply systems, including mechanical (*e.g.* rasping, sonication, freezing and thawing), chemical (*e.g.* biocides, detergents, surfactants) and enzymatic means.

4.1. Mechanical and Physical Processes

Filtration has been the most common way to remove solid particles from water, carbon filters being even able to remove both organic and inorganic compounds. However, to remove micro-organisms such as bacteria, yeasts and fungi the porous of the filter must be smaller than 0.2 μm , which contributes to a rapid fouling of the filters. To remove viruses and microbial particles, the pores must be even narrower. A study carried out in Karachi, Pakistan, showed that the combination of both boiling and filtering systems was only efficient to disinfect 38% of the samples collected from distinct houses [72]. Recent patents have described systems associating filtration techniques to ultraviolet (UV)

radiation. In the range of 200-300 nm, UV radiation is very effective in killing micro-organisms, and UV lamps have been extensively used, since almost no by-products are produced, contrarily to what happens during chlorination and ozonation (for a review on inactivation of viruses, bacteria and protozoan oocysts see [73]).

Filtered water may be cleaned of micro-organisms such as *Legionella* by radiating the water as it leaves storage tanks through a shower head, sprinkler, irrigation nozzle or similar openings [74]. To destroy and prevent biofilms inside conduits, Korin [75] presented an integrated filtration and sterilization unit using a wavelength (120-242 nm) of the UV light source that enabled both the sterilisation of the previously filtrated liquid and the generation of ozone from an oxygen containing gas. Albelda and co-workers [76] had suggested a method that included filtration and/or exposure of the liquid (to be used inside tubes) to UV radiation prior addition of an oxygen solution containing at least 0.2% (w/w) oxygen.

Costerton [77] proposed to freeze the biofilm in fouled pipes sufficiently slow to allow the formation of large (0.5-20 μm) sharp-edged ice crystals within the polysaccharide matrix. To achieve this, the pipes may be cooled with dry ice or liquid nitrogen. The frozen biofilm is subsequently thawed and removed, for instance, by a liquid flow through the pipes.

The opposite situation, the use of high temperatures, may also be used to disinfect surfaces and prevent the formation of biofilms. Micro-organisms such as *Legionella* can be eradicated from domestic hot water supply systems and similar devices, connected to water distribution circuits, using a heating cell that may also be used to heat the primary circuit [78]. In sea vessels and exterior places, the temperature of the pipes may be considerably lower than inside heated water tanks, decreasing the success of the process. In such cases, a heating ribbon, wire, rod or an elongated heating spiral may be applied inside the tubes to maintain the water at an efficient temperature (around 60°C), while decreasing energy and water consumption [79].

By circulating heated water at around 80°C through the fluid circuit of a dialysis machine comprising a water treatment module, a dialysate preparation module and circuit and an extracorporeal circuit (including the dialyser, arterial and venous blood lines to connect to the patient) during a certain period (around one hour) disinfection of all circuits may be achieved [80]. Suddath and co-workers developed a self-cleaning system with a boiler to provide sanitized water or steam to a dental workstation [81]. The steam is used to sterilise the delivery line and workstation and destroy adherent cells.

Small diameter tubes are difficult to clean, especially if the tubes are long, because fouling decreases flow velocities. Haemodialysis hollow fibres have length/diameter (L/D) ratios of about 1000-1500 and tubular membranes of 500-1500, dental chair tubes have L/D of 2000-3000, industrial pipes have usually L/D ratios of 1000-3000 and in endoscopes the ratio is about 500-2000 [82]. Tabani and co-workers patented a process for removing adherent contaminants from hollow porous fibres which consists in

back-flushing a liquid to fill the pores and application of a gas flow. The mixture of gas and bubbles provokes enough turbulence to remove the adherent particles into the liquid phase. The process may be applied in tubes with diameters from *c.a.* 0.2 mm to 10 cm or more, depending on a sufficient gas supply. Bubbles are able to remove mature biofilms at the point collision due to the combined effect of fluid dynamic shear forces and thermodynamic forces that pull bacteria from a surface when the bubble contacts the biofilm [83]. The fraction of biofilm removed per bubble is about 0.4 and this technique may be applied by powered toothbrushes to remove bacterial biofilms from teeth.

4.2. Chemical Processes

The application of oxidation processes, *e.g.* through the usage of oxidants such as ozone, hydrogen peroxide, chlorine or chlorine dioxide, is a well known process of water treatment, being able to remove organic and inorganic compounds in water while improving taste and colour. However, ozone low water solubility and stability, high cost and inefficiency to oxidise some organic compounds hamper its application, in particular economically [84].

Electrochemical generation of ozone for "point-of-use" applications (osmosis systems, refrigerators, drinking fountains, etc) to provide disinfected water, ozone-containing water and/or ozone gas was presented by Andrews and co-workers [85]. Disinfected water, produced by introduction of ozone into purified water, finds its application in anti-microbial and cleansing applications at consumer-level, *e.g.*, to wash food, cloths, toys, bathrooms, etc, as well as to wash and disinfect medical devices. Water enriched in ozone is also effective in eliminating micro-organisms and prevent biofilm formation in water circuits delivering water to a patient's mouth during dental procedures [86]. In ozone treatment of dialysis feedwater, the ozone should be applied to the water storage tank and removed prior the use of the water in dialysis treatment using UV light [87]. Water for dialysis and other processes requiring ultra-pure water can also be cleaned and disinfected by maintaining an acidic pH with a high carbon dioxide concentration in solution [88].

Chlorine dioxide is a gas with effective disinfectant, bleaching and oxidizing properties, although explosive in contact with air at concentrations above 10%. Kross and co-workers suggested the application of 25-2500 ppm chlorine dioxide solutions to decontaminate small diameter water pipes (such as those in dental units, ranging 6-19 mm) and concentrations 1-10 ppm to maintain the circuit clean [89].

To control biofilms and micro-organisms in general in systems supplying water to large medical or dental devices, a filtration system containing filters to remove particles, organic matter and bacteria can be combined with a pressurised storage tank to which antimicrobial agents are applied [90]. The biocidal agent being a mixture of hydroperoxide ions, a phase transfer catalyst and a trace colour or an antiseptic agent from citrus fruits, such as grapefruit seed extract.

4.3. Enzymatic Action

The polymeric matrix that anchors the cells constitutes a penetration barrier to biocides, decreasing their potency in comparison to that observed with planktonic cells while promoting microbial resistance [91]. The cells inside the biofilm have a lower access to nutrients and thus a slower growth rate, becoming more protected to the majority of antibiotics and biocidal agents since they act primarily upon dividing cells. The use of substances capable of destroying the physical integrity of the matrix, interfere with bacterial adhesion or initiate cell detachment from surfaces are good alternatives to biocides and/or disinfectants. The latter contribute to the propagation and spread of resistant strains [92] and its use may be restricted by environmental regulations [93].

Especial attention has been given to enzymes able to destroy polysaccharides, which are the primary building blocks of slime. Among these are proteases, such as alkaline proteases, and α -amylases from various *Bacillus* strains [94], acidic proteases and glucoamylases from *Aspergillus niger* [41], acidic and alkaline proteases from pineapple stem and cellulases [95]. In the late 1980s and early 1990s, combinations of biocides and enzymes were used: the enzymes were responsible for destroying the polysaccharide matrix to enhance the biocide action. Pedersen and Hatcher [96] used methylene-*bis*-thiocyanate, dimethyl dithiocarbamate or disodium ethylene-*bis*-dithiocarbamate as biocide and amylase, a dextran degrading enzyme or a levan hydrolase as the polysaccharide degrading enzyme. Robertson *et al.* [97] proposed the application of chlorine, hypochlorite, bromine, hydrogen peroxide, etc., in a concentration of 0.5-500 ppm and trypsin and/or endo-protease and/or chymotrypsin in about 0.01-1000 units to inhibit the growth of filamentous organisms. More environment friendly solutions have been proposed since then, including: (i) mixtures of enzymes and a surface active agent, preferentially anionic [98]; (ii) at least one enzyme belonging to carbohydrases, proteases, glycol proteases or lipases and a short-chained glycol component [99]; (iii) enzyme blending in 2-100 ppm of cellulase, α -amylase and protease [100].

5. CURRENT & FUTURE DEVELOPMENTS

The increasing understanding of how a biofilm is formed and the role of each mechanism involved in cell adhesion is providing precious information to the development of sound strategies to combat cell colonisation. Interferences (i) in the initially cell-to-surface and cell-to-cell contact, responsible for the formation of the first microcolonies at the surface, (ii) with the molecules responsible for cell-to-cell communication or quorum sensing and (iii) with the formation of EPS, responsible for the structure of the biofilm, can disrupt the process of biofilm formation and proliferation.

The approach and adhesion of cells to surfaces is facilitated by the cell surface hydrophobicity [101-103]. Cells growing in alcohols, hydrocarbons and terpenes, as sole carbon and energy sources, are able to change their surface hydrophobicity [104]. The routinely use of biocides in industrial and household products can act as a selective

pressure upon exposed bacteria. The more tolerant individuals will, in that case, have an increased contribution to the reproduction of the population under stress [105]. This could be responsible to the observed increased number of hospital infections [106]. Development of biocides and their extensive application should be conscientious and aimed at health and environment friendly, effective and economically feasible compositions.

ACKNOWLEDGEMENTS

This work was supported by a post-doctoral grant (SFRH/BPD/14426/2003) awarded to Carla da C. C. R. de Carvalho by Fundação para a Ciência e a Tecnologia, Portugal.

REFERENCES

- [1] Costerton JW, Stewart PS, Greenberg EP. Bacterial bio-films: a common cause of persistent infections. *Science* 1999; 284: 1318-22.
- [2] Davey ME, O'Toole GA. Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev* 2000; 64: 847-67.
- [3] Gilbert P, Das J, Foley I. Biofilms susceptibility to antimicrobials. *Adv Dent Res* 1997; 11: 160-67.
- [4] Flemming H-C. Biofilms and environmental protection. *Water Sci Technol* 1993; 27: 1-10.
- [5] Schopf JW, Hayes JM, Walter MR. Evolution on earth's earliest ecosystems: recent progress and unsolved problems. In: Schopf JW Ed, *Earth's earliest biosphere*. New Jersey, Princeton University Press. 1983; 361-84.
- [6] Satpathy KK. Effects of biofouling on the cooling water quality of a nuclear power plant. *Bull Electrochem* 1999; 15: 143-47.
- [7] Hope CK, Bott TR. Laboratory modelling of manganese biofiltration using biofilms of *Leptothrix discophora*. *Water Res* 2004; 38: 1853-61.
- [8] Edmiston CE, Seabrook GR, Goheen MP, *et al.* Bacterial Adherence to Surgical Sutures: Can Antibacterial-Coated Sutures Reduce the Risk of Microbial Contamination?. *J Am Coll Surg* (in press).
- [9] Hoyle BD, Costerton JW. Bacterial resistance to antibiotics: the role of biofilms. *Prog Drug Res* 1991; 37: 91-105.
- [10] Costerton JW. Introduction to biofilm. *Intern J Antimicrob Agents* 1999; 11: 217-21.
- [11] Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999; 284: 1318-22.
- [12] Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet* 2001; 358: 135-38.
- [13] Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microb Rev* 2002; 15: 167-93.
- [14] Jones DA, Amy PS. A thermodynamic interpretation of microbiologically influenced corrosion. *Corros* 2002; 58: 638-45.
- [15] Little B, Ray R. A perspective on corrosion inhibition by biofilms. *Corros* 2002; 58: 424-28.
- [16] Ornek D, Wood TK, Hsu CH, Sun Z, Mansfeld F. Pitting corrosion control of aluminum 2024 using protective biofilms that secrete corrosion inhibitors. *Corros* 2002; 58: 761-67.
- [17] Beech IB, Coutinho CLM. Biofilms on corroding materials. In: Lens P, Moran AP, Mahony T, Stoodly P, O'Flaherty V Eds, *Biofilms in Medicine, Industry and Environmental Biotechnology - Characteristics, Analysis and Control*. IWA Publishing of Alliance House. 2003; 115-31.
- [18] Gottenbos B, Busscher HJ, Van Der Mei HC, Nieuwenhuis P. Pathogenesis and prevention of biomaterial centered infections. *J Mat Sci: Mat In Med* 2002; 13: 717-22.
- [19] Pratt LA, Kolter R. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* 1998; 30: 285-93.
- [20] Wingender J, Neu T, Flemming H-C. What are bacterial extracellular polymer substances? In: Wingender J, Neu T, Flemming H-C Eds, *Bacterial extracellular polymer substances*. Berlin, Springer. 1999; 1-19.
- [21] Katsikogianni M, Missirlis YF. Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. *Eur Cells Mater* 2004; 8: 37-57.
- [22] Seabrook, S.G., Heymann, R.C.: US5554373 (1996).
- [23] Seabrook Jr, S.G., Craver III, W.E.: US5906825 (1999).
- [24] Daeschel, M.A., McGuire, J.: US5451369 (1995).
- *[25] Fitzpatrick, R.J., Shackett, K.K., Klinger, J.D.: US20056955806 (2005).
- [26] Worley, S.D., Eknoian, M.W., Li, Y.: US20026469177 (2002).
- [27] Wong, P.S.L., Dong, L.C., Wan, J.: US20056929803 (2005).
- [28] McCarthy, R.E., Dallmier, A.W., McCoy, W.F.: US20016322749 (2001).
- [29] Howarth, J.N., Nalepa, C.J., Sanders, M.J.: US20036641828 (2003).
- [30] Francolini I, Norris P, Piozzi A, Donelli G, Stoodley P. Uronic acid, a natural antimicrobial agent able to inhibit bacterial biofilm formation on polymer surfaces. *Antimicrob Ag Chemother* 2004; 48: 4360-65.
- [31] Jansson, R.E., O'Brien, R.N., Visaisouk, S.: US5104649 (1992).
- [32] Prosl, F.R., Polaschegg, H.-D., Estabrook, B.K., Sodemann, K.: US20036575945 (2003).
- [33] Olson ME, Wright JB, Lam K, Burrell RE. Healing of porcine donor sites covered with silver-coated dressings. *Eur J Surg* 2000; 166: 486-89.
- [34] Yuranova T, Rincon AG, Bozzi A, *et al.* Antibacterial textiles prepared by RF-plasma and vacuum-UV mediated deposition of silver. *J Photochem Photobiol, A* 2003; 161: 27-34.
- [35] Fan F-RF, Bard AJ. Chemical, electrochemical, gravimetric, and microscopic studies on antimicrobial silver films. *J Phys Chem B* 2003; 106: 279-87.
- [36] Babu R, Zhang J, Beckman EJ, Virji M, Pasculle WA, Wells A. Antimicrobial activities of silver used as a polymerization catalyst for a wound-healing matrix. *Biomater* 2006; 27: 4304-14.
- [37] Khan LK, Li R, Gootnick D, Peace Corps Thyroid Investigation Group. Thyroid abnormalities related to iodine excess from water purification units. *Lancet* 1998; 352: 1519.
- [38] Ornek D, Jayaraman A, Wood TK, Sun Z, Hsu CH, Mansfeld F. Pitting corrosion control using regenerative biofilms on aluminium 2024 in artificial seawater. *Corros Sci* 2001; 43: 2121-33.
- [39] Jayaraman A, Earthman JC, Wood TK. Corrosion inhibition by aerobic biofilms on SAE 1018 steel. *Appl Microbiol Biotechnol* 1997; 47: 62-68.
- [40] Jayaraman A, Mansfeld FB, Wood TK. Inhibiting sulphate-reducing bacteria in biofilms by expressing the antimicrobial peptides indolicidin and bactenecin. *J Ind Microbiol Biotechnol* 1999; 22: 167-75.
- [41] Orgaz B, Kives J, Pedregosa AM, Monistrol IF, Laborda F, SanJosé C. Bacterial biofilm removal using fungal enzymes. *Enz Microb Technol* (in press)
- [42] Itzhak B. The Role of Bacterial Interference in Otitis, Sinusitis and Tonsillitis, *Otolaryngology - Head and Neck Surgery* 2005; 133: 139-46.
- [43] Polsenski, M.J., Leavitt, R.I.: US20067041285 (2006).
- [44] Davies, D.G., Costerton, J.W.: US20067094394 (2006).
- [45] Bycroft, B.W., Williams, P., Stewart, G.S.A.B., Chhabra, S.R., Stead, P., Winson, M.K., Hill, P.J., Rees, C.E.D., Bainton, N.J.: US5593827 (1997).
- [46] Pearson, J.P., Gray, K.M., Passador, L., Tucker, K.D., Eberhard, A., Iglewski, B.H., Greenberg, E.P.: US5591872 (1997).
- [47] Manyak, D.M., Weiner, R.M., Carlson, P.S., Quintero, E.J.: US20046759040 (2004).
- *[48] Wiersma, J.G.: US5882916 (1999).
- [49] Wiersma, J.G.: US5753493 (1998).
- [50] de Carvalho CCCR, da Fonseca MMR. Carvone: Why and how should one bother to produce this terpene. *Food Chem* 2006; 95: 413-22.
- [51] Prevost, A., Barbeau, J., Cote, L., Charland, R., Faucher, E.: US5759970 (1998).
- [52] Barbeau, J., Gravel, D., Habi, A.: US20046762160 (2004).
- [53] Day, D.F.: US20056866870 (2005).
- [54] Day, D.F., Ott, C.M., Mayo, J.A., Kim, D.: US20046692757 (2004).
- [55] Charaf, U.K., Avery, R.W.: US20036528472 (2003).

- [56] Aguilera, A.M., Bitney, R.G., Conviser, S.A., Decaire, B.R.: US20036605254 (2003).
- [57] Bonaventura, J., Ignarro, L., Dowling, D.B., Spivack, A.J.: US20036524466 (2003).
- *[58] Christ, F.R.: US5843186 (1998).
- [59] Holt KB, Bard AJ. Interaction of silver(I) ions with the respiratory chain of *Escherichia coli*: an electrochemical and scanning electrochemical microscopy study of the antimicrobial mechanism of micromolar Ag⁺. *Biochem* 2005; 44: 13214-23.
- [60] Darouiche RO. Anti-infective efficacy of silver-coated medical prostheses. *Clin Infect Dis* 1999; 29: 1371-77.
- [61] Milder, F.L.: US5725817 (1998).
- [62] Jamiolkowski, D.D., Rothenburger, S.J., Spangler, Daniel J.: US20036514517 (2003).
- [63] Jernberg, G.R.: US20046726898 (2004).
- *[64] Darouiche, R.O., Hull, R.: US20046719991 (2004).
- [65] Saint S. Clinical and economic consequences of nosocomial catheter-related bacteriuria. *Am J Infection Control* 2000; 28: 68-75.
- [66] Öncü S, Sakarya S. Central venous catheter - related infections: an overview with special emphasis on diagnosis, prevention and management. *Internet J. Anesthesiol* 2003; 7 (N. 1).
- [67] Seder, E.V., Nelson, J.N.: WO02083031A3 (2002).
- [68] Polaschegg, H.-D.: US20046803363 (2004).
- [69] Ash, S.R.: US20056958049 (2005).
- [70] Stephen, R.L., Rossi, C., Eruzzi, S.: US5401239 (1995).
- [71] Crossley, K.: US20036551346 (2003).
- [72] Luby SP, Syed AH, Attullah N, Faizan MK, Fisher-Hoch S. Limited effectiveness of home drinking water purification efforts in Karachi, Pakistan. *Int J Infect Dis* 2000; 4: 3-7.
- [73] Hijnen WAM, Beerendonk EF, Medema GJ. Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: A review. *Water Res* 2006; 40: 3-22.
- [74] Ter Stege, S.: WO05124236A2 (2005).
- *[75] Korin, A.: US20046773610 (2004).
- [76] Albelda, D., Moshe, K.: US5925257 (1999).
- [77] Costerton, J.W.F.: US4419248 (1983).
- [78] Aussudre, C., Berthou, M., Chopard, F.: WO06037868A1 (2006).
- [79] Korstanje, J.C.: WO06059898A1 (2006).
- [80] Kenley, R.S., Treu, D.M., Peter, Jr., F.H., Feldsein, T. M., Pawlak, K. E., Adolf, W. F., Roettger, L.: US5591344 (1997).
- [81] Suddath, J.N., Piskorowski, W., Kasbrick, J.J.: US6821480 (2004).
- [82] Tabani, Y., Labib, M.E.: US20056945257 (2005).
- [83] Parini MR, Pitt WG. Dynamic removal of oral biofilms by bubbles. *Colloids Surf B* 2006; 52: 39-46.
- [84] Kasprzyk-Hordern B, Ziólek M, Nawrocki J. Catalytic ozonation and methods of enhancing molecular ozone reactions in water treatment. *Appl Catal B Environ* 2003; 46: 639-669.
- [85] Andrews, C.C., Murphy, O.J., Hitchens, G.D.: US20026458257 (2002).
- [86] Engelhard, R., Kasten, S.P.: US5942125 (1999).
- [87] Van Newenhizen, J.: US5585003 (1996).
- [88] Smith, S.D.: US20056908546 (2005).
- [89] Kross, R.D., Wade, W.: US20036599432 (2003).
- [90] Chandler, J.W.: US20026423219 (2002).
- [91] Marion-Ferey K, Pasmorey M, Stoodley P, Wilson S, Husson GP, Costerton JW. Biofilm removal from silicone tubing: an assessment of the efficacy of dialysis machine decontamination procedures using an *in vitro* model. *J Hosp Infect* 2003; 53: 64-71.
- [92] Ofek I, Hasty DL, Sharon N. *FEMS Immunol Med Microbiol* 2003; 38: 181-91.
- [93] Chen X, Stewart PS. Biofilm removal caused by chemical treatments. *Water Res* 2000; 34: 4229-33.
- [94] Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microbiol Biotechnol* 2002; 59: 13-32.
- [95] Napper AD, Bennett SP, Borowski M, Holdridge MB, Leonard MJC, Rogers EE, Duan Y, Laursen RA, Reinhold B, Shames SL. Purification and characterization of multiple forms of the pineapple-stem-derived cysteine proteinases ananain and comosain. *Biochem J* 1994; 301: 727-35.
- [96] Pedersen, D.E., Hatcher, H.J.: US4684469 (1987).
- [97] Robertson, L.R., LaZonby, J.G., Krolczyk, J.J., Melo, H.R.: US5324432 (1994).
- [98] Hollis, C.G., Terry, J.P., Jaquess, P.A.: US5411666 (1995).
- *[99] Eyers, M.E., Van Pee, K.L.I., Van Poele, J., Schuetz, J.F., Schenker, A.P.: US5789239 (1998).
- [100] Wiatr, C.L.: US4936994 (1990).
- [101] van Loosdrecht MCM, Lyklema J, Norde W, Schraa G, Zehnder AJB. The role of bacterial cell wall hydrophobicity in adhesion. *Appl Environ Microbiol* 1987; 53: 1893-97.
- [102] Yaskovich GA. The role of cell surface hydrophobicity in adsorption immobilization of bacterial strains *Appl Biochem Microbiol* 1998; 34: 373-76.
- [103] Kos B, Suskovic J, Vukovic S, Simpraga M, Frece J, Matosic S. *J Appl Microbiol* 2003; 94: 981-87.
- [104] de Carvalho CCCR, Parreño-Marchante B, Neumann G, da Fonseca MMR, Heipieper HJ. Adaptation of *Rhodococcus erythropolis* DCL14 to growth on n-alkanes, alcohols and terpenes. *Appl Microbiol Biotechnol* 2005; 67: 383-88.
- [105] Mulvey M, Diamond SA. In: Newman MC, McIntosh AW Eds, *Metal Ecotoxicology*. Chelsea, Lewis. 1991; 301-21.
- [106] White DG, McDermott PF. Biocides, drug resistance and microbial evolution. *Curr Opin Microbiol* 2001; 4: 313-17.