Zheng Zhang · Victoria E. Wagner
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Preface

Modifying medical devices with antimicrobial coatings and biomaterials is one of the most effective approaches to prevent device-associated infections. This book explores current and emerging antimicrobial technologies, biofilm-related evaluation techniques, and regulatory challenges, with particular emphasis on the coatings and modifications on medical devices. An overview of antimicrobial technologies on critical care implants followed by the public health and the regulatory science challenges associated with these devices are discussed. Progress in characterization and evaluation of infection on medical devices, such as diagnostic assays evaluating cell adhesion and biofilm formation including molecular-based approaches, is introduced. This book covers coatings and modifications incorporating various antimicrobial agents, including traditional antibiotics and antiseptic agents, as well as alternative agents through "anti-antibiotic" approaches. Delivering antimicrobial agents with porous materials is introduced afterwards as an example of many efforts to improve loading and delivering efficacy. Antifouling surfaces with characteristics to reduce bacteria colonization and physical approaches such as light-induced antimicrobial modifications are reviewed.

The surface of medical implants is considered as a critical battleground of bacterial colonization, biofilm formation, and immune reactions. It is also an intensive target for applying antimicrobial coatings and modifications. Since catheter-related bloodstream infection (CRBSI), ventilator-associated pneumonia (VAP), and catheter-associated urinary tract infections (CAUTIs) comprise the most frequent and expensive device-associated infections, vascular catheters, endotracheal tubes, or urinary catheters usually are the ultimate test fields for many promising antimicrobial techniques to achieve in vivo and clinical results. The regulatory landscape provides clear pathways to market acceptance of several types of antimicrobial devices, with examples of devices on the market described in this book. These include medical devices with eluting antimicrobial compounds such as chlorhexidine and triclosan, traditional antibiotics including rifampin and minocycline, or combinations (e.g., chlorhexidine/silver sulfadiazine). Standardized test methods and regulatory designations for appropriate claims are well understood for these types of products. What is less clear and still under investigation is how the

understanding of biofilms and their role in infection will impact claims and testing for more novel devices still under development. For example, claims language such as "reduction in biofilm" or "prevent microbial colonization" are less well defined and indeed, there exist few standardized, reproducible test methods to demonstrate such claims. This book provides a concise review of those regulatory challenges facing device manufacturers today.

Key to designing these next-generation devices are a clear understanding of the mechanisms of attachment, proliferation, and dissemination of microorganisms at the device interface. Several authors review the currently known dogma of biofilm development as well as many traditional and novel techniques to characterize the interaction of microbes with device surfaces. Methods to quantify cell adhesion and proliferation at interfaces, including confocal scanning microscopy, atomic force microscopy, Raman spectroscopy, surface plasmon resonance imaging, and quartz crystal microbalance, are discussed. Several commercially available biofilm reactor systems are presented and their utility for specific applications described. Molecular techniques, such as ribosomal 16S RNA sequencing and 16S rRNA fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis, fluorescent activated flow cytometry (FACS), and amplification of DNA and RNA using polymerase chain reaction (PCR), are important tools used to identify microbial species, understand genetic regulation, and characterize microbial behaviors and colonization of device surfaces in vitro and in vivo. These techniques are not only critical in understanding how the device design affects performance, but such data may provide evidence for mechanisms of action, efficacy, and safety claims as these devices are brought to market.

Several approaches to generating antimicrobial devices are presented. Immersing medical implants in an antimicrobial solution or coating the device with antimicrobial agents is commonly used to control over infections. The achievement of local delivery of significant quantities of active agents, and release of the drug throughout the period of implantation, represents a strong point in favor of this approach. These include the use of antibacteriostatic agents, such as chlorhexidine, or antimicrobials such as rifampin. A concise review of currently marketed antimicrobial medical devices, such as vascular access or urological devices, are discussed in terms of clinical efficacy, if known, as well as advantages or disadvantages of their use. However, not all of the antimicrobial agents are compatible with the substrate with enough loads, or can be released in a desirable manner. One key limitation of antimicrobial technologies marketed today is the duration of effect, as many devices are eluting by design and therefore have limited life once the agent is exhausted. Delivering agents as a response to infection, and covalently binding antimicrobial agents have been applied on medical device surfaces as one strategy to mitigate this issue. Various porous additives or biodegradable polymers have been developed to achieve controlled and prolonged release. Porous materials are discussed in a chapter as an example of achieving effective antimicrobial delivering. Additionally, novel approaches that do not rely on an eluting agent or an antimicrobial killing effect, including stimulating a targeted host immune response, either by modification of device surfaces to promote phagocytosis or the use of artificial opsonins, and

interference of microbial metabolism such as iron utilization, are discussed. An understanding of the molecular components and regulation of microbial biofilms has generated a number of antiadhesive molecules and disruptors, such as quorum sensing signal interference or amyloid inhibitors, to prevent microbial proliferation and biofilms on surfaces. Together, these topics cover a wide array of traditional and nontraditional pathways with the same goal in mind: prevention or mitigation of device-associated infections.

This book also addresses the growing threat of antibiotic resistance by presenting antifouling and physical approaches. Antifouling surfaces reduce bacterial attachment through unique surface characteristics. With no antimicrobial agents leaching from surfaces, antifouling surfaces achieve antimicrobial performance without introducing toxicity and drug resistance. In addition, some antifouling polymers also significantly reduce protein adsorption to a level that can inhibit thrombus formation or other device-related complications. Physical approaches such as light, acoustic energies, and mechanical stress are thought to be effective without concerns of side effects from active antimicrobial agents. Advances in light technology highlight the potential for light inhibition of biofilm formation in medical devices, which may be combined with photosensitizers, photocatalysts, or photocleavables. However, for these technologies, concerns such as reduced in vivo efficacy and safety make obstacles to get antimicrobial claims these devices. Clinical relevance between bacterial resistance and infection reduction still needs to be established.

Currently, more than a million infections are acquired in US hospitals each year including more than half associated with medical implants. The situation is being challenged by expanding application of invasive devices, increasing aging population, and diminishing effectiveness of antibiotics. Solutions may largely count on development of better and safer medical devices, together with improvement of diagnostic methods, regulatory science, and hospital operations. Hopefully, this book can provide the readers an overview of the exciting area from a few important perspectives, and inspire further research and stewardship that battle the device-associated infections.

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Chapter 1
Antimicrobial Modifications on Critical Care Implants

Zheng Zhang, Victoria E. Wagner, and John C. Victor

1.1 Introduction

Medical devices are defined by the FDA as an "instrument, apparatus, implement, machine, contrivance, implant, *in vitro* reagent, or other similar or related article, including a component part, or accessory which is intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or other animals, or intended to affect the structure or any function of the body of man or other animals, and which does not achieve any of its primary intended purposes through chemical action within or on the body of man or other animals and which is not dependent upon being metabolized for the achievement of any of its primary intended purposes" [1]. Medical devices can be divided into indwelling and implantable device dependent upon intended functionality and duration of use. Examples of medical devices include prosthetic hip and knee joints, pacemakers, chronic hemodialysis catheters, intraocular lenses, and vascular catheters. Globally the medical device industry is more than $180 billion and expanding at a rapid pace. In the United States alone, more than five million medical devices are used per year [2].

While most medical devices function as designed, there can be serious and often severe complications associated with their use. These include wear, thrombosis, and occlusion that can impair the device performance and impact the health and mortality of the patient. Healthcare acquired infections (HAIs) are a leading cause of mortality and morbidity globally. In 2007, HAIs were the *fourth leading cause of death in the United States*, with an estimated 1 in 25 patients in the United States per year alone suffering from HAIs [2]. In Europe there were more than 4.5 million cases of HAIs, with approximately 37,000 attributable deaths per year. HAIs result in significant healthcare costs, estimated at more than $5 billion alone in the United States

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and €7 billion in Europe. About 60–70% of all HAIs are associated with a medical device [2]. These infections are classified by device, including catheter-related bloodstream infection (CRBSI), ventilator-associated pneumonia (VAP), and catheter-associated urinary tract infection (CAUTI). Etiology is usually device dependent and rates of infection tend to differ between device and location [3, 4].

1.1.1 Bacterial Colonization and Biofilm Formation on Medical Implants

Biofilms are communities of microorganisms encased in extracellular polymeric substances (EPS) attached to a surface. Biofilm development is ubiquitous and the pathway is fairly well understood and described in discrete stages [2, 4, 5]. When planktonic microbes encounter a surface, reversible attachment may take place, followed by irreversible attachment. Cellular division and aggregation lead to microcolony formation which is followed by maturation to a robust biofilm. Finally, microbes may leave the biofilm through dispersion or dispersal and revert to a planktonic lifestyle. Biofilm formation is a complex orchestrated process. Research has elucidated several mechanisms that govern this process, including physical and chemical characteristics of the surface; exogenous physical, chemical, and biological stimuli from the microenvironment; cellular communication (quorum sensing); and nutrient limitation [2, 4–7]. Chapter 6 describes these phenomena in more detail.

Indwelling devices are especially prone to biofilm formation. Surfaces on indwelling devices usually are quickly coated with host proteins and glycoproteins, such as fibronectin, fibrinogen, vitronectin, albumin, and immunoglobulin [4]. Microbes can attach by non-specific or specific receptor–ligand interactions to these surfaces [4]. Once initial attachment occurs, biofilm development proceeds at a rapid pace, with most biofilms reaching a mature state within days dependent on species [5]. The type of indwelling device and its environment dictate which microbes are more likely to colonize the surface. For example, coagulase-negative staphylococci and *Staphylococcus aureus* tend to be recovered from vascular infections, such as prosthetic heart valves and central venous catheters, while *Escherichia coli* and *Enterococcus* sp. are dominant pathogens in urinary catheter infections [3, 4].

Microbes living in biofilms are more recalcitrant to antibiotic and antiseptic treatment versus free-floating planktonic cells [2]. This is theorized to be due to a number of factors, including shift in metabolism, exchange and upregulation of genes that encode proteins involved in antimicrobial resistance such as efflux pumps or enzymes like beta-lactamases, and the protective encasement of EPS that forms a physical barrier to exogenous antimicrobials. Therefore, most strategies to prevent biofilms usually seek to inhibit the initial adhesion events when microbes tend to still be susceptible to antimicrobial treatment.

1.1.2 Device-Associated Infections, Clinical Importance, and Regulatory Issue

In the early 1980s, clinical evidence implicated microbial biofilms as causative agents in device infections. Microscopic imaging demonstrated that microbial biofilms were found on medical devices, such as pacemaker leads and prosthetic joints, recovered from infected patients [8–11]. Today there is a greater understanding of the role of biofilms and their relation to disease [2–4, 12, 13]. It is estimated that more than 90% of all diseases are caused by biofilms. Biofilms are difficult to eradicate as microbes grown in biofilms are more resistant to traditional antimicrobial treatments [2, 4, 12].

Typically, the medical device serves as a nidus for the formation of microbial biofilms which are correlated with a majority of device infections [2, 4]. Microbes are introduced to the device during insertion or implantation or though repeated exposure to the environment, such as blood draws from a central catheter line. Current state-of-the art medical devices seek to prevent infection by inhibiting microbial attachment and colonization of the device. Strategies include the incorporation of antibiotics or antiseptics into the device to kill or inhibit microbial growth on the surface and surrounding environment and non-fouling device surfaces which inhibit microbial attachment [12]. Many of these approaches are described in more detail throughout this book and not discussed here. Despite best efforts, clinical treatment to resolve biofilms usually involves replacement of the device.

New therapies (as well as the limited usefulness of current ones) to address biofilm-centered device infection have been hampered in development from the reliance of test results on free-floating, planktonic-grown cells. This includes measures such as minimum inhibitory concentration (MIC), which describes the effects of antimicrobials on planktonic cells. The understanding that most microbes adopt a biofilm lifestyle in nature has resulted in a paradigm shift in both industry and government regulatory agencies. These include the development of more relevant in vitro and in vivo test methodology that utilizes biofilm-grown cells to characterize new treatments as well as an entire new language of treatment efficacy and standards. Chapter 2 outlines several of the more recent advances in guidance from federal agencies which govern device approval and the implications for the medical device industry.

1.2 Device-Associated Infections and Complications in ICUs

HAIs currently are the most common complications affecting hospitalized patients [14]. Intensive care unit (ICU)-acquired infections represent the majority of these infections [15]. Bloodstream infections (BSIs, usually associated with the use of an intravascular device such as vascular catheters, CRBSI), pneumonias (usually ventilator-associated pneumonias or VAPs), and urinary tract infections (UTIs, usually

Table 1.1 Typical device-associated infections, related devices, etiologic agents, and other device-relevant complications that related to infections

Device-associated infections	Related devices	Main etiologic agents	Other device-relevant complications
Catheter-related bloodstream infection (CRBSI)	Central venous catheters (CVCs) such as peripherally inserted central catheters (PICCs) and jugular axillo-subclavian central catheters (JACCs) hemodialysis catheters, etc.	*S. epidermidis*, *S. aureus*, *Candida albicans*, *P. aeruginosa*, *K. pneumoniae*, and *E. faecalis*	Catheter-related thrombosis (CRT), catheter occlusion
Ventilator-associated pneumonia (VAP)	Endotracheal tubes (ETTs), tracheostomy tubes (TTs)	*P. aeruginosa*, *S. aureus*, Enterobacteriaceae, *Haemophilus* spp., *Streptococcus* spp., *Acinetobacter* spp., *S. pneumoniae*, *Neisseria* spp., *Stenotrophomonas maltophilia*, coagulase-negative staphylococci	Device occlusion
Catheter-associated urinary tract infections (CAUTIs)	Urinary catheters such as Foley catheters and intermittent catheters ureteral stents	*S. epidermidis*, *E. faecalis*, *E. coli*, *P. mirabilis*, *P. aeruginosa*, *K. pneumoniae*, and other Gram-negative organisms	Encrustation, catheter blockage

catheter-associated UTIs or CAUTIs) are healthcare-associated infections most often associated with the use of invasive devices. Applying the invasive devices can also risk the patients with other complications that closely related to infection. Table 1.1 lists the three device-associated infections, devices, main etiologic agents, and other device-relevant complications.

1.2.1 Catheter-Related Bloodstream Infection (CRBSI)

CRBSI is defined as the presence of bacteremia originating from an intravenous catheter [16, 17]. It is estimated that more than 80,000 CRBSIs occur each year in ICUs, making CRBSIs a leading cause of nosocomial ICU infections [18]. CRBSIs lead to an increase in patient care cost and have an associated mortality of 12–25% annually [19, 20]. Application of different types of CVCs, peripheral venous catheters, and arterial catheters is the main cause of CRBSI. There are four potential sources of catheter colonization and catheter-related infections: the skin insertion

site, the catheter hub, hematogenous seeding of the catheter tip from a distant site of infection, and infusate contamination [21]. Microorganisms from both skin at the insertion site (extraluminal source) and the catheter hub/connector (intraluminal source) can colonize the catheters and infect the bloodstream. For short-term catheters (in place less than 10 days), microorganisms from the extraluminal source are the most common source of infection [22]. These organisms can migrate along the surface of the catheter into the cutaneous catheter tract surrounding the catheter, resulting in colonization at the catheter tip. With more prolonged dwell time (>30 days), CRBSIs arising from the intraluminal source dominate the catheters which usually happens when the IV system contacts with IV solution connection sites, access hubs, needleless connectors, or tubing junctions, or contamination with the patient's own body fluids or the skin [23, 24]. Contamination from other infection sites of infusate can be the intraluminal source but less commonly.

The most common organisms causing CRBSIs are staphylococci (both coagulase-negative staphylococci and *S. aureus*), enterococci, aerobic Gram-negative bacilli, and yeast. Certain pathogens are found to be associated with specific host, treatment, catheter site, and catheter characteristics. *S. aureus* infections are a significant cause of morbidity and mortality in patients using hemodialysis catheters [25]. Gram-negative bacilli have been associated with infections of patients with solid tumor [26]. Hub colonization of coagulase-negative staphylococci has been related to CRBSI during parenteral nutrition [27, 28]. Gram-negative bacilli and yeast have been affiliated with catheters placed in femoral veins [29]. Catheter materials can be especially vulnerable to certain microbial colonization. For example, *C. albicans* occurs more readily on silicone elastomer catheter surfaces than polyurethane catheters.

1.2.1.1 Catheter-Related Thrombus (CRT)

Thrombosis and infection complications are common and associated with substantial morbidity and cost. In a study of cancer patients with CVCs, about 41% required device removal before the end of treatment owing to such complication infection, thrombosis, blockage, and leakage [30]. Venous thrombosis may manifest in pain and swelling of the arm and is found in 1–7% of all patients with peripherally inserted central catheters (PICCs), with higher frequencies occurring in cancer patients [31, 32]. While the cumulative incidence of symptomatic thrombosis is 5.8% [33], the incidence of asymptomatic venous thrombosis is substantially higher, with more than 39% of PICC patients having detectable thrombosis [34]. Patients with PICC-associated thrombosis are reported to have hospital stays doubled, resulting in substantial additional costs per patient [35].

It has been demonstrated that CRT and CRBSI are related and the relationship seems to be bidirectional [36]. The catheter-related thrombi, usually form a fibrin sheath or occlude the inner lumen, are composed of fibrin, laminin, collagen, fibronectin, and immunoglobulins. The adsorbed proteins can provide acceptors for both Gram-positive and Gram-negative bacteria, especially *S. aureus* and *S. epidermidis*,

to enhance bacteria attachment on the surface [37]. Moreover, the proteinaceous surface also facilitates biofilm formation that contributes to the CRBSI. In vitro study showed that fibrin sheath formation around central venous catheters can significantly enhance catheter-related infection and persistent bacteremia [38]. It has been confirmed by clinical analysis that thrombotic complications are often associated with catheter sepsis [39]. A clinical analysis showed the risk of catheter-related sepsis was 2.62-fold higher when thrombosis occurred [40]. On the other hand, the attached microorganisms and biofilms are able to produce a coagulase enzyme that enhances the thrombogenic process. In patients with CVC-related infection, the risk of thrombosis increased markedly in comparison with those without infection [41].

1.2.2 Ventilator-Associated Pneumonia (VAP)

VAP is defined as pneumonia occurring more than 48 h after patients have been intubated and received mechanical ventilation. The device for orotracheal or nasotracheal intubation such as endotracheal tube (ETT) is considered as the most important factor related to the development of VAP [42, 43]. The insertion of an ETT disrupts the cough reflex, compromises mucocilliary clearance, injures the tracheal epithelial surface, provides a direct conduit for rapid access of bacteria from upper into the lower respiratory tract, and allows the formation of biofilm on the ETT surface [43]. It is believed that bacteria from the mouth and upper respiratory tract gain entrance into the lower respiratory tract via the artificial airway. As a result, pneumonia develops due to microbial invasion of the normally sterile lower respiratory tract and lung parenchyma [44].

During implantation, ETT has a high frequency of biofilm buildup. This biofilm comes from microorganisms from both exogenous and endogenous bacterial inoculum in the tracheal mucosa. Among the microorganisms, aerobic, Gram-negative bacilli (GNB) are associated with high mortality rate and account for more than 60% of VAP from previous reports [42]. The high-risk Gram-negative pathogens include *Pseudomonas aeruginosa*, *Acinetobacter* spp., and *Stenotrophomonas maltophilia* [42]. Gram-positive pathogens, especially methicillin-resistant *S. aureus* (MRSA), also were reported to be involved in infection in VAPs [44].

1.2.2.1 ETT Occlusion

Patency of the endotracheal tube during mechanical ventilation (MV) is often compromised by the accumulation of luminal mucus and debris [45]. While life-threatening occlusion is not very common, partial occlusion due to secretion accumulation is ubiquitous, with a decrease of ETT diameter and loss of intraluminal ETT volume [46, 47]. Biofilm is always present in intubated patients whatever the duration of intubation and appears quickly after intubation. Even after soft rinse, a small but measurable part of biofilm remains always present and seems strongly

adherent to the ETT lumen. It contains potentially pathogenic bacteria for the lung [48]. Pathogens-laden secretions stationed within the tube may migrate and colonize the lower respiratory tract, causing pneumonia. It has been reported the increase in ETT occlusion was associated with an increased incidence of pneumonia and atelectasis [49].

1.2.3 Catheter-Associated Urinary Tract Infection (CAUTI)

A UTI is an infection involving any part of the urinary system, including the urethra, bladder, ureters, and kidney [50]. Most nosocomial UTIs are associated with urinary catheters, known as catheter-associated UTIs (CAUTIs) [51, 52]. The insertion of a urinary catheter into the bladder through the urethra increases the susceptibility of a patient to UTIs, as these devices serve as the initiation site of infection by introducing opportunistic organisms into the urinary tract [51, 52]. The preferred mechanism of bladder entry during CAUTIs is extraluminal (66%), where organisms ascend from the urethral meatus along the catheter–urethral interface [51]. The longer the urinary catheter remains in place, the greater the tendency of these organisms to develop biofilms and result in urinary tract infections. For example, 10–50% of patients undergoing short-term urinary become infected catheterization (7 days). However, it is believed that patients undergoing long-term urinary catheterization will inevitably develop UTI (>28 days) [51, 52].

The majority of these uropathogens are fecal contaminants or skin residents from the patient's own native or transitory microflora that colonize the periurethral area [52]. The organisms commonly contaminating devices and developing biofilms are *S. epidermidis*, *Enterococcus faecalis*, *E. coli*, *Proteus mirabilis*, *P. aeruginosa*, *K. pneumoniae*, and other Gram-negative organisms [53]. Bacterial entry into the bladder can occur at the time of catheter insertion, through the catheter lumen, or along the catheter–urethral interface [51]. Through the inner lumen, microorganisms may ascend into the patient's bladder in 1–3 days. This rate may be influenced by the presence of swarming organisms such as *Proteus* spp. [50]. For long-term catheter indwelling, a large proportion of the bacteriuria species are *Proteeae*, including *Proteus mirabilis*, *Morganella morganii*, and *Providencia stuartii* [54].

1.2.3.1 Encrustation and Catheter Blockage

The long-term catheterization is complicated by encrustation that may lead to urothelial damage and/or blockage of urinary catheters or stents [51, 55]. Another complication of encrustation is weakening of the substrate material, which could make it difficult to remove. Encrustation usually starts with adherence of crystalloids and colloids to the surface of the implants. The microcrystals may include struvite (magnesium ammonium phosphate) and apatite (a hydroxylated form of calcium phosphate in which some of the phosphate ions are replaced by carbonate) [56].

Many researchers believe infection by urease-producing bacteria, particularly *Proteus mirabilis*, is most commonly associated with catheter encrustation and blockage [57, 58]. The urease of *P. mirabilis* is a particularly active enzyme, being able to hydrolyze urea several times faster than those produced by the other species [59]. Urease hydrolyzes urea in the residual bladder urine to produce ammonia causing a rise in pH. At higher pH, crystallization of the magnesium and calcium phosphates is induced. Crystals from the urine are deposited on the catheter or trapped in the organic matrix which can eventually block the catheter [60]. In addition to bacteria attachment and biofilm formation, some adsorbed protein layer can also enhance encrustation on urinary stents [61]. By analyzing stents removed from patients, it was found encrusted devices exhibited higher number of inflammatory and adhesion/motility proteins, compared with non-encrusted devices [62].

1.3 Typical Critical Care Implants: Materials and Functional Coatings

1.3.1 Vascular Catheters

Vascular catheters are made of synthetic polymers that are chemically inert, biocompatible, and resistant to chemical and thermal degradation. The most widely used polymers are polyurethanes and silicones. Currently more polyurethanes are used for vascular catheters because they allow for high catheter strength, while still can maintain a delicate catheter design. In terms of infection tendency, some study have suggested silicone catheters may have a greater risk of grossly apparent infection compared with polyurethane catheters using in vivo infection models, such as a rabbit model of subcutaneous *S. aureus* infection [63]. However, these comparisons have not been clinically proved in tunneled infusion catheters [64]. For long-term venous access devices usually implanted over 6 weeks, the material of choice is still silicone elastomer, and the alternative material of choice could be polyurethane coated with hydrophilic coatings [65]. In a clinical study with implantable venous access ports of the two materials, PU catheters exhibit a higher rate of infections and thrombogenicity, while silicone catheters exhibit a trend toward decreased mechanical stability [66].

A larger variety of thermoplastic polyurethanes have been used in the vascular catheter market and the diversity is significant. Polyurethanes for vascular catheters usually include polyester-, polyether-, and polycarbonate-based varieties, as well as aromatic and aliphatic grades. In general, the aliphatic polyetherurethanes are selected for their softening characteristics, processability, colorability, and versatility in filler selection. The aromatic polyetherurethanes are selected for strength, chemical resistance, and kink resistance [67]. Both aliphatic and aromatic polyether-based polyurethanes soften at body temperature, which promotes patient comfort and reduces the risk of vascular trauma. These PUs are commonly used for

indwelling catheters, such as CVCs and PICCs. Polycarbonate-based polyurethanes exhibit excellent long-term biostability and are commonly used in applications that are in the body for long periods. Additionally, polycarbonate polyurethane is resistant to chemicals (i.e., iodine, peroxide, or alcohols), thereby increasing the longevity of the catheter. It is an ideal material for hemodialysis catheters [68, 69].

1.3.1.1 Antimicrobial Coatings

Both polyurethane and silicone cannot resist complications related to biofilm formation and thrombus formation. To date, two types of catheter coatings have been developed—those with antimicrobial coatings and those with antithrombotic coatings [70]. Selected commercial vascular/ventricular catheters with antimicrobial claims are listed in Table 1.2, most of which have shown clinical antimicrobial outcomes. All of these catheters are based on releasing antimicrobial agents including antiseptics, antibiotics, or anticancer agents. It should be stated that while impregnated catheters are effective in reducing CRBSI and catheter colonization, they may not be effective across all blood infections [71].

Chlorhexidine is a cationic bisbiguanide and used as an antiseptic agent. It can bind to negatively charged bacterial cell walls and kill a broad spectrum of microorganisms at a certain concentration. Composition of chlorhexidine and silver salt exhibits synergistic, enhanced antibacterial activity [72]. Antiseptic catheters with chlorhexidine/silver sulfadiazine (CH-SS) have been used to prevent catheter-related bloodstream infection with a strong clinical track record [73]. Compared with antibiotic-loaded catheter, catheters impregnated with the combination antiseptic agents be less susceptible to antibiotic resistance [74]. Three generations of chlorhexidine/silver modification on vascular catheters have been developed. The first generation of the chlorhexidine-based antiseptic catheter protects only outside of the catheter (ARROWg+ard® Blue). The second-generation CH-SS catheter protects both outside and inside of the catheter, as well as the entire fluid pathway including the inside of the extension lines and hubs (ARROWg+ard Blue PLUS®). The concentration of chlorhexidine on the outside surface is three times higher than on first-generation catheters, and the inside surface is coated with chlorhexidine only [75]. From a clinical study with 158 adults, CVC with CH-SS reduces bacterial colonization by 44% and catheter-related bacteremia by an even greater 79% [76]. The third-generation technology (Chlorag+ard®) is a chlorhexidine solution bonded to the catheter surface with a controlled release. In addition to the antimicrobial protection, the technology also provides antithrombogenic protection, protection against thrombotic occlusion, and reduction in phlebitis and intimal hyperplasia. Not all of these claimed performances have been clinically reported.

Silver compounds have a long history of use as septic agents and people believe only the ionized form have antimicrobial properties [77]. It has been observed that bacterial DNA lost its replication ability and the protein became inactivated after Ag+ treatment [77]. Silver sulfadiazine provides a steady supply of silver ions over a long period of time, so it has been used to modify vascular catheters (BioBloc®,

Table 1.2 Antimicrobial modifications on vascular catheters

Antimicrobial agents	Modification description and example devices	Ref.
Modification with antiseptics		
Chlorhexidine/silver sulfadiazine (ARROW+gard Blue®, Teleflex)	Modifications are along the entire dwelling surfaces Pressure injectable CVCs, acute hemodialysis catheters	[73, 107–110]
Chlorhexidine/silver sulfadiazine (ARROWg+ard Blue PLUS®, Teleflex)	Modifications are on both outside and inside including extension lines and hubs Pressure injectable CVCs	[111–113]
Chlorhexidine (Chlorag+ard®, Teleflex)	Both the internal and external catheter surfaces are treated with chlorhexidine protection JACCs (ARROW®JACC), peripheral venous catheter (Arrow® Midline), PICCs (ArrowEVOLUTION™ PICC)	Teleflex internal report
Silver sulfadiazine coating (BioBloc®, CR Bard)	Tunneled long-term hemodialysis catheters (HemoGlide®, HemoSplit, and HemoStar), tunneled cuffed catheters (TCCs)	[78]
Silver ion sleeve with or without heparin coating (Covidien-Medtronic)	The catheter contains a silver-impregnated sleeve permanently bonded to the outer surface of the device from the hub to the cuff. Chronic hemodialysis catheters (Palindrome™ HSI, Palindrome™ SI)	[78, 79]
Inorganic silver powder and inert ceramic zeolite (AgION®, Sciessent)	CVCs, temporary dialysis catheters (Vygon)	[82]
Silver/platinum/carbon black (Oligon, Edwards Lifesciences)	Polyurethane extrusions combined with natural silver and platinum metals and carbon black with or without heparin coating Oligon Vantex® silver CVCs	[81]
Nanocrystalline silver film (Spi-Argent, Spire)	A silver film is deposited by ion beam-assisted deposition with an active thickness of 1 μm or less Split-tip chronic dialysis catheters (XpressO Silver™ and RetrO Silver™)	[84, 85]
Benzalkonium chloride (BZK)	BZK with and without hydrophilic coating, BZK-impregnated CVCs (Multi-Med®, Baxter-Edwards)	[86–88]
Modification with antibiotics or antibiotic–antiseptic combinations		
Minocycline/rifampin (MR, Spectrum® Cook)	Impregnated MR with hydrophilic coating consisting of polyacrylamide and PVP to enhance insertion (EZ-Pass® Cook) CVCs (both polyurethanes and silicones), silicone PICCs (Cook Spectrum Glide®)	[92–94, 96]

(continued)

Table 1.2 (continued)

Antimicrobial agents	Modification description and example devices	Ref.
Rifampicin/miconazole (Multistar+, Vygon)	Acute CVCs (Multistar+, Vygon)	[99, 100]
Cefazolin/benzalkonium chloride	CVCs were pretreated with the cationic tridodecylmethylammonium chloride, then anionic antibiotic cefazolin.	[102]
Modification with anticancer drugs		
5-Fluorouracil (5-FU, Angiotech)	CVC externally coated with 5-FU 5-FU CVCs (Angiotech)	[105, 106]

CR Bard) [78]. Silver ion-impregnated sleeves have been applied on the chronic hemodialysis catheters (Palindrome™ SI, Medtronic) [78, 79]. While silver in its nonionized form is inert, distributed metallic silver particles in polyurethane can release silver ions as contact with moisture [80]. In a clinical trial, CVCs from polyurethane combined with natural silver have reduced incidence of catheter colonization and may decrease the risk of CRBSI (Oligon Vantex®, Edwards Lifesciences) [81]. The silver-doped polyurethane can protect both inner and outer surfaces of the catheter. Another antimicrobial technology is based on a polyurethane compound with silver ion bonded with an inert ceramic zeolite. The zeolite cage holds silver ions, thereby enabling a controlled release (AgION® technology). CVCs have been prepared with this technology (Multicath Expert, Vygon). However, from some clinical analysis, the use of silver-impregnated catheters in adult intensive care patients is not associated with a lower rate of colonization than the use of standard multi-lumen catheters [82, 83]. A nanocrystalline silver film is deposited by ion beam-assisted deposition (IBAD, Spi-Argent, Spire), a vacuum-based thin film deposition process at low temperature under high vacuum. The affected layer in the typical films deposited by the IBAD process is in the order of 1 μm or less. The coating exhibits very low silver elution rates and is consequently long-lasting. In an early clinical test, bacterial colonization was observed in 8% of the treated catheter compared with 46.4% of untreated catheters. The SEM investigations also showed all treated catheters developed low thrombogenicity [84]. However, in a recent study based on the results of the bacteriological examination and patient diagnosis or outcome, the anti-infection efficacy could not be confirmed [85].

Benzalkonium chloride (BZK) is an antiseptic, which is believed to act by disrupting the cell membrane like many other quaternary ammonium compounds. An antiseptic central venous catheter (Becton Dickinson) had a hydrophilic coating which consists of polyvinylpyrrolidone (PVP) molecules bound to the polyurethane surface and cross-linked to form a network throughout the layer. The BZK molecules are entrapped within this network in an anhydrous state but diffuse into the surroundings when the PVP comes into contact with an aqueous solution. This mechanism ensured that BZK is continuously present on both the internal and external surfaces of the catheters. In vitro studies demonstrated that the antiseptic central

venous catheter inhibited the adherence of a range of bacterial species [86]. A clinical trial analysis demonstrates that the BZK-impregnated catheter significantly reduced the incidence of microbial colonization on both the internal and external catheter surfaces [87]. For another BZK-impregnated CVC (Multi-Med®, Baxter-Edwards), in vitro study shows reduced microbial colonization by a range of organisms [86]. However, statistical testing from a clinical trial showed no significant differences between the study and control group [88].

Minocycline is a broad-spectrum tetracycline antibiotic, which inhibits bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome [89]. Rifampin is an antibiotic acting its bactericidal effect by inhibiting DNA-dependent RNA polymerase [90]. The minocycline/rifampin (M/R) combination showed synergy by in vitro checkerboard testing, and the combination has been frequently combined for the prevention of catheter-related infections [91]. Both polyurethane [92] and silicone [93] catheters have been impregnated with M/R combination and shown to reduce the rates of catheter colonization and catheter-related bloodstream infections in clinical trials (Spectrum®, Cook) [94]. The anti-infection efficacy has been approved in an ICU environment with low rates of CLABSIs. The same study found the catheter did not increase the incidence of resistant organisms [95]. The technology can be combined with a hydrophilic coating consisting of polyacrylamide and PVP (EZ-Pass®, Cook) to facilitate insertion (Cook Spectrum Glide®). The hydrophilic coatings are originally based on a light-activated formulation that enables a covalent bond to form between lubricious coatings and device substrates (PhotoLink®, SurModics). An embolization of the hydrophilic coating to the lung that resulted in cavitary lung nodules was reported as a very rare case [96].

A second-generation M/R catheter was developed by adding chlorhexidine (CHX-M/R). CVCs and PICCs were impregnated with CHX-M/R and compared with first-generation M/R catheters. The second-generation catheter presents extended biofilm resistance compared with M/R catheters [97]. However, no clinical studies have been found.

Rifampicin has also been combined with miconazole and applied on the surface of CVCs (Multistar+, Vygon). Miconazole is an imidazole antifungal agent that inhibits ergosterol biosynthesis. Ergosterol is a critical component of fungal cell membrane [98]. The combination of rifampicin and miconazole leads to protection against a broad spectrum of microorganisms such as *Staphylococci*, Enterobacteriaceae, and *Candida*. A CVC that has been supersaturated with rifampicin and miconazole was associated with significantly lower risk for catheter colonization and catheter-related infections compared to standard catheters [99–101].

Cefazolin is a semisynthetic cephalosporin analogue with broad-spectrum antibiotic action due to inhibition of bacterial cell wall synthesis. Central venous catheters were fist pretreated with the cationic tridodecylmethylammonium chloride. Then anionic antibiotic cefazolin was then immobilized. A clinical trial has shown the safety and efficacy of this antibiotic formulation in reducing infection rate [102]. However, no commercial product was found.

Due to the similarities between cancer cells and pathogenic bacteria, it is expected that some anticancer drugs would be also effective against bacteria. The pyrimidine analogue 5-fluorouracil (5-FU) is an effective anticancer drug and widely used in the clinic. In concentrations well below those used in cancer therapy, 5-FU has been shown to inhibit growth of both Gram-positive and Gram-negative bacteria as well as *Candida* species [103, 104]. 5-FU has been coated on the outside of CVC and approximately releases 1 mg cumulative dose eluted over 28 days from a 20 cm CVC based on in vitro and in vivo data [105]. From a clinical trial with a total of 960 CVC patients for up to 28 days, results suggest CVCs with 5-FU are a safe and effective alternative to catheters externally coated with chlorhexidine and silver sulfadiazine [106].

1.3.1.2 Antithrombogenic Coatings

Heparin is a naturally occurring anticoagulant and one of the most intensively studied glycosaminoglycans. As a mixture of linear anionic polysaccharides, heparin can be immobilized to a substrate by physical adsorption, by covalent chemical methods, or by photochemical attachment [114]. Heparin can be partially depolymerized and coupled to the surface by endpoint attachment (Carmeda® Bioactive Surface or CBAS) [115]. It is believed that the heparin is covalently bonded since insignificant amounts of heparin were released from the surface. In vitro study revealed that the surface is able to highly reduce platelet adhesion, inhibit thrombin, and prevent complement activation [115, 116]. Endpoint attachment of heparin on materials in contact with blood has been shown to result in a high degree resistance to thrombosis in vivo which lasts more than 16 weeks [117]. A small animal test indicates that the covalently bound heparin molecules can significantly prolong patency and cause less pathologic damage to the catheterized vessel [118]. This has led to the application of antithrombogenic coatings on long-term hemodialysis catheters (e.g., Decathlon Gold and Alta Gold from Spire Biomedical).

Such heparinization of CVCs appears to have a great impact on both in vitro and in vivo bacterial colonization and can be a practical and economical approach to prevent catheter-associated bacteremia or fungemia [119]. An early clinical trial has shown that heparin-coated catheters decrease protein and platelet deposition on catheter surfaces and inhibit the early stages of catheter-related infections [120]. A clinical analysis on tunneled dialysis catheters confirmed heparin coating decreases the frequency of catheter-related bacteremia. However, the coatings have not significantly reduced the frequency of catheter malfunction from this analysis [121].

A coating comprises hydrophilic polyethylene oxide (PEO) layer with heparin, and sulfate/sulfonate groups covalently bonded which was developed to reduce thrombus formation on medical devices (Trillium®, BioInteractions). An ex vivo animal test at low systemic heparinization has shown the amount of clots significantly reduced with the coating of cardiopulmonary bypass circuits [122]. Related heparin-coated chronic hemodialysis catheter includes the Palindrome™ series from Covidien-Medtronic. For example, the Palindrome™ Emerald™ chronic

hemodialysis catheter is coated from the tip of the catheter to the cuff on the external surface and throughout the entire length on the internal surface (tip to luer adapters).

By adding fluorine-containing polyurethane surface-modifying macromolecules (SMMs) into polyurethane substrate, a heparin-free technology was developed to change surface characteristics of polyurethane [123, 124]. Fluorinated polymers have attracted interest as a passive strategy to reduce thrombus because of their low surface energy, relative blood compatibility, and oxidative stability. The technology is based on blending low molecular weight fluorinated polyurethane additives into the polyurethane from which the catheter is made. The polymer is present throughout the catheter, including the extraluminal and intraluminal surfaces, and remains present for the life of the catheter (Endexo™, Interface Biologics Inc.). The technology has been applied on vascular catheters such as PICCs, dialysis catheters, and midline catheters (AngioDynamics). In vitro blood flow loop test has demonstrated an average of 87% less thrombus accumulation on its surface, compared to commonly used PICCs based on platelet count. Results of an in vivo sheep study during 31-day indwelling time demonstrated that the BioFlo DuraMax dialysis catheter has thromboresistant characteristics comparable to the Palindrome H hemodialysis catheter (AngioDynamics website).

1.3.2 Endotracheal Tubes

Most endotracheal tubes are made of polyvinyl chloride (PVC), and others are made of silicone, latex rubber, and metal [125]. Concerns of latex allergy have let latex ETTs coated with a thin layer of silicone for the tube and cuff (e.g., Silkolatex-coated technology). To keep flexibility and softness, PVC for ETTs is highly plasticized with plasticizers. Di(2-ethylhexyl) phthalate (DEHP) is one of the most commonly utilized plasticizers. While DEHP has been used in medical devices for many years without reports of adverse effects, released DEHP from ETTs and other airway systems have caused some concerns, especially for vulnerable patient populations [126, 127]. Alternative plasticizers for ETT application are considered such as citrate-based plasticizer, trioctyltrimellitate (TOTM), and epoxidized soybean oil [128, 129].

Currently the antimicrobial-coated ETT on the market is based on dispersing silver ions in a hydrophilic polymer and applying the coating on both the inner and outer lumens of an ETT (Agento™, CR Bard). The silver-hydrogel coating formulation has been challenged using a dog model with buccal administration of *P. aeruginosa*. During a 96 h mechanical ventilation (MV), it was found that silver coating of ETTs may delay the onset of and decrease the severity of lung colonization by aerobic bacteria [130]. The coating has shown the feasibility and safety of the respiratory infection control [131], as well as a statistically significant reduction in the

incidence of VAP and delayed time to VAP occurrence compared with those receiving a similar, uncoated tube [132].

Using a formulation similar to that on CVCs, a polyurethane coating with silver sulfadiazine and chlorhexidine was applied on ETTs. The ETTs were tested on a sheep model with MV for 24 h. The coated ETTs induced a nonsignificant reduction of the tracheal colonization, eliminated or reduced bacterial colonization of the ETT and ventilator circuits, and prevented lung bacteria colonization. The study also shows the coated ETTs potentially be highly beneficial in the prevention of VAP during surgical procedures during general anesthesia requiring intubation and MV and particularly during expected prolonged postoperative MV [133]. In another in vivo study, the ETT coated with silver sulfadiazine in polyurethane was specifically selected for animal study and showed lower respiratory tract colonization in sheep mechanically ventilated for 24 h [134].

Ceragenins are cationic steroid compounds consisting of a sterol backbone with amino acids and other chemical groups attached to them, mimicking function of antimicrobial peptides [135]. Ceragenin CSA-13, one of the cationic steroids, is reported to show a concentration-dependent bactericidal/bacteriolytic activity against pathogenic streptococci, including multidrug-resistant *S. pneumoniae* [136]. The silicone–CSA-13 coating has prevented infection in a sheep model of a simulated open fracture wherein well-established biofilms were used as initial inocula [137]. A ceragenin-coated endotracheal tube was able to prevent bacterial colonization and biofilm development for 21 days, while C. R. Bard's Agento™ endotracheal tubes lost their ability to prevent bacterial colonization and biofilm development after 3 days when challenged with daily high inocula (10E6) of *P. aeruginosa* (CeraShield™, Ceragenix Pharmaceuticals).

1.3.3 Urinary Catheters

Urinary catheters are originally manufactured from natural rubber latex to be inserted into the bladder through the urethra to drain urine. Latex is still commonly in use due to its flexibility and low cost [138]. However, latex catchers exhibit tendency to form urethral stricture under long-term application [139, 140]. Concerns also include lack of biocompatibility and latex allergies. Moreover, the high water content of latex could reduce the size of inner lumen during implantation. Due to these reasons, latex catheters are usually applied for an indwelling time less than 14 days [141]. With the mechanical benefit of latex, silicone has been widely applied in urinary catheters, providing chemical inertness, biocompatibility, and water resistance. In addition to full silicone catheters, silicone-coated latex catheters were developed to mitigate rigidity of all-silicone catheters and reduce cost [142]. PVC and polyurethane are also used in different types of urinary catheters and stents.

1.3.3.1 Antimicrobial Coatings

A few silver-coated urinary catheters have been developed to resist UTI and other device-associated complications. Urinary catheters coated with silver alloy, silver oxide, or silver salt such as phosphate ion silver have been developed. Some of these silver compounds can be incorporated within a hydrogel coating to facilitate silver ion release. In vitro study reported silver citrate was compounded with lecithin in silicone coatings to make hydrophilic surfaces and achieve a more potent antibacterial efficacy [143]. Urinary catheters impregnated with chlorhexidine and silver sulfadiazine exhibited broad-spectrum, long-term microbial resistance in an in vitro urinary tract model [144]. A systematic review of randomized controlled trials revealed that silver alloy- but not silver oxide-coated catheters were associated with a significant reduction in bacteriuria in comparison with standard catheters [4]. A study showed silver oxide-coated catheters have failed to demonstrate the efficacy in prevention of catheter-associated bacteriuria and have even shown a significantly increased incidence of bacteriuria [145]. Another systematic review that aimed to determine which type of indwelling urinary catheter is best to use for long-term bladder drainage in adults found that all trials were small and with methodological weaknesses. The evidence from this systematic review was not sufficient as a reliable basis for practical conclusions [5].

Catheters impregnated with antimicrobial agents including nitrofurazone, and a minocycline/rifampicin combination has been evaluated for their anti-infection performance. From short-term in vitro test with 11 UTI-associated microorganisms, nitrofurazone-coated catheters significantly outperformed silver alloy-coated catheters for inhibitory activity, according to both inoculum broth and catheter sonicate counts, whether compared directly or against the corresponding control catheters [146]. During clinical trials, nitrofurazone-impregnated catheters reduced the risk of symptomatic CAUTI in patients who have indwelling urinary catheter for 5–7 days [147]. Nitrofurazone-impregnated urinary catheters reduced the incidence of catheter-associated bacteriuria and funguria (CABF) in adult trauma patients, reducing the need to change or prescribe new antimicrobial therapy [148]. However, from these studies, the magnitude of reduction was low and hence may not be clinically important [147–149]. In another clinical study, bladder catheters impregnated with minocycline and rifampin significantly reduced the rate of Gram-positive catheter-associated bacteriuria up to 2 weeks after catheter insertion [150]. The minocycline and rifampicin catheter is no longer manufactured [149].

1.3.3.2 Lubricious Coatings

There are two different types of lubricious coatings on silicone or latex catheters: hydrophobic polytetrafluoroethylene (PTFE) coatings and hydrophilic hydrogel coatings. Both coatings are successful to improve lubricity that silicone or latex catheters lack of, which contributes to discomfort as inserting or removing the devices. PTFE-coated catheters have been long investigated with their surface

characteristic of hydrophobicity and low friction [151]. The strong cohesive forces of PTFE molecules make their surfaces resistant to van der Waals forces, which are main causes of friction. Hydrophilic hydrogel coatings entrap water molecules on the surfaces, forming a water layer that reduces the friction. The non-covalently bond hydrophilic polymers can also serve as lubricants during insertion or removal, while the unbound polymers are not very desirable. Poly(vinylpyrrolidinone) (PVP) is a typical hydrophilic polymer for hydrogel coatings. The PVP-coated devices are significantly more lubricious than uncoated catheters [152]. A clinical trial also suggested that the use of a hydrogel-coated latex catheter rather than a silicone catheter may be better tolerated for the need for early removal [153].

In vitro models of bacterial adherence and encrustation have been tested to compare urinary catheters with above materials and coatings. An early study on bacterial adherence of CAUTI strains showed none of the strains adhered to the catheter with the hydrophilic coatings, while all strains adhered to the uncoated silicone catheter. The study also showed bacterial adherence was variable to the Teflon and elastomer surfaces [154]. A broad spectrum of bacteria resistance of hydrophilic coatings was further proved by incorporating a more anti-fouling segment, PEO, within the coating [155]. However, other researchers believed the more hydrophobic the bacteria, the more they are able to colonize hydrophobic materials, whereas hydrophilic cells are able to colonize hydrophilic materials more easily [156]. Increased adherence of hydrophilic bacterial strains on PVP-coated catheters was also reported [152]. In a bacterial adherence study involved two uropathogenic strains, the hydrogel-coated catheters did not reduce any bacterial adhesion compared with uncoated catheters [151].

In terms of encrustation, in vitro study results showed little evidence of PTFE coatings can resist encrustation due to the attachment of *Proteus* spp. [151, 154]. The encrustation resistance of hydrophilic coating is inconsistently reported, depending on the encrustation assays and bacterial strains. Some research showed PVP coating could be useful in preventing encrustation deposition [152], while other studies showed both hydrogel-coated catheters and unmodified catheters blocked rapidly in urine inoculated with a clinical strain of *Proteus mirabilis* [157]. It should be stated that the mechanism of encrustation on urinary devices is not completely understood. In addition to hydrophobicity, other factors such as morphology, chemical composition, defects, additives, and contamination may also contribute to encrustation [158, 159]. While clinical trials with enough patients would be desired to make a solid conclusion, it looks like current lubricious coatings are not effective enough to present comparative benefit to resist encrustation.

From a clinical trial evaluating the tendency to develop bacteriuria, there is no significant difference between the hydrogel-coated catheters and the uncoated catheters [160]. Recent systematic reviews comparing different types of standard catheters indicated confidence intervals were too wide to rule out clinically important differences in reducing UTI on hydrogel-coated catheters [149, 161]. Other antimicrobial strategies, such as incorporating antimicrobial agents, need to be combined with hydrogel coatings, to achieve antimicrobial claims.

1.4 Advances in Antimicrobial Technologies on Medical Implants

1.4.1 Delivery and Immobilization of Antimicrobial Agents

As above mentioned, antimicrobial-loaded medical implants are currently part of standard medical procedures for both local treatment and prevention of implant infections. Almost all the implant devices have been approved with antimicrobial claims are devices with antimicrobial agents. The antimicrobial agents and their application for medical devices, such as metals, antiseptics, and antimicrobials, are reviewed in Chap. 5. Progress on biofilm-specific antimicrobial agents, which include metabolite disruption such as iron, stimulation of the immune system, and prevention of extracellular matrix components of the biofilms, is reviewed in Chap. 6. These antimicrobial agents need to be incorporated within the medical device and maintain enough concentrations in the vicinity of the device during the implantation. In this part, advances in antimicrobial delivery, including prolonged and controlled release, responsive release, and surface immobilization of the antimicrobial agents, are covered.

1.4.1.1 Controlled and Prolonged Release

Imbibing medical implants in antimicrobial solutions or coating the device with antimicrobial agents is commonly used to control over infection. The achievement of local delivery of significant quantities of active agents, and release of the drug throughout the period of implantation, represents a strong point in favor of this approach. The imbibed or coated agents, usually small molecules compatible with substrates or coatings, can keep their antimicrobial efficacy for a long period of time [162]. However, not all of the antimicrobial agents are compatible with the substrate with enough loads or can be released in a desirable manner during implantation. Various porous additives or biodegradable polymers have been developed to achieve the controlled and prolonged release.

Macroporous (pore diameter > 50 nm) structure is designed to accommodate a large amount of drugs and achieve a controlled release from burst release to prolonged release [163, 164]. Active ingredients such as peptides, proteins, and genetic materials such as plasmid DNA and cells can be loaded within the structure. For example, an antimicrobial peptide was loaded within a TiO_2 structure with about 80 nm in diameter processed on titanium surface, with a slow release profile from 4 h up to 7 days [165]. Microporous structure with pore diameters smaller than 2 nm and mesoporous structure with diameters between 2 and 50 nm have also been developed for antimicrobial agent delivery. These structures can be ordered structure with well-defined pore size, or amorphous materials with a range of pore-size distribution. They were used to deliver agents such as nitric oxide, metal nanoparticles, and metal ions in a controlled rate and prolonged period. Chapter 7 provides a detailed review on their antibacterial application.

The dispersal of antimicrobial agents into biodegradable polymer coatings has proven effective for a prolonged and controlled release on medical implants, especially on orthopedic implants. A rat model has shown poly(D,L-lactide) (PDLLA) coating loaded with 10% gentamicin on an orthopedic device (titanium K-wire) prevents implant-related osteomyelitis after 6-week implantation [166]. Poly(L-lactide) (PLLA) coating with a thickness of about 30 μm was applied on titanium plates. Either antibiotics (combination of rifampicin and fusidic acid) or antiseptics (combination of Octenidin and Irgasan) were loaded in the coating. Implanted within rabbits after 28 days, devices with both coatings exhibit significant infection reduction [167]. Eight patients with open tibia fractures have been treated with an unreamed tibial nail (UTN) coated with PDLLA and gentamicin (PROtect, Synthes). In the 1-year follow-up, none of the patients developed an infection [168]. Another clinical study with 21 patients also showed gentamicin-coated UTNs were associated with an absence of deep wound infections after 6 months [169].

Polyhydroxyalkanoates (PHAs) are natural biodegradable polymers derived from bacteria. Thus the need for PHAs has arisen due to their tailorable mechanical properties, biocompatibility, and biodegradability [170]. It was reported bulk PHAs with antibiotics (Sulperazone® or Duocid®) reduce implant-related osteomyelitis (IRO) in rabbit tibia [171]. A PHA coating on the top of the antimicrobial-doped rods can be used to extend the release of the antibiotic agents [172]. Another in vitro test shows PHA/gentamicin formulations effective to reduce implant-related *Staphylococcus* infections in blood [173].

For the coating on Foley urinary catheters, polycaprolactone (PCL)-degrading enzyme lipase B was embedded within a PCL-based coating, and antibiotic gentamicin sulfate (GS) was co-impregnated within the coating. The coated Foley urinary catheters exhibited sustained in vitro release of GS over a 60h period. The results suggest that the antibiotic-plus-enzyme-loaded polymer can be used as tunable self-degrading antimicrobial biomaterial coating on catheters [174].

1.4.1.2 Infection-Responsive Release

Even with the prolonged release, the prophylactic inhibitory amounts of antimicrobial agents will be inevitably reduced and depleted. Moreover, delivery of sublethal dosage of antibiotics can lead to enhanced biofilm formation and induced factor expression [2]. An appealing device is made of a surface that releases antibiotics only during microbial infection. However, due to the complicity of infection, most infection-responsive release study is still at an early stage of development.

It was found that *S. aureus*-infected wound fluid showed high thrombin-like activity. Gentamicin was bound to poly(vinyl alcohol) hydrogel through thrombin-sensitive peptide linker. The conjugate released gentamicin and reduced the bacterial number in a rat model of *S. aureus* infection [175].

Within the urinary tract, usually the colonization of implanted devices is associated with production of urease. The urease-catalyzed hydrolysis of urea into ammonia can elevate urine pH to pH 9.1. An antimicrobial quinolone, nalidixic acid,

exhibits significantly increased solubility in alkaline media. Loaded within a catheter coating, the surface-localized nalidixic acid was released 50- and tenfold faster at pH 9, compared to release at pH 5 and pH 7, respectively [176]. Another pH-responsive hydrogel coating covalently bond nalidixic acid through a hydrolysable ester bond. The formulation demonstrated up to 20-fold faster rates of drug release at pH 10, representing infected urine pH, than at pH 7 and achieving reductions of up to 96.5% in in vitro bacterial adherence [177].

Another example of infection-responsive release takes advantage of the extracellular bacterial lipases abundant at sites of infection. An antibiotic, ciprofloxacin, was covalently bonded on the PEG-based materials through anhydrides, a lipase-sensitive linkage. The complex can completely kill wild-type strain of *P. aeruginosa*, but insignificantly affect the population of lipase-defective mutant. The research confirms the chances of developing a self-regulating system which only releases antibiotics as bacteria surrounding the medical devices [178].

1.4.1.3 Covalent Immobilization

Cationic agents such as quaternary ammonium-containing siloxanes [86], alkylated poly(4-vinylpyridine) [179], quaternary ammonium-containing (meth)acrylates [180], alkylated poly(ethylene imines) [181], phosphonium [182], amines [183], chitosans [184], chlorhexidine [185, 186], guanidine polymers (e.g., polyhexamethylene biguanide (PHMB) and polyhexamethylene guanide (PHMG)) [187], and antimicrobial peptides [188] have been covalently immobilized on substrates and exhibited different level of antimicrobial performance, mostly from in vitro tests. While the full mechanisms are still not fully understood, it is believed that the cationic groups penetrate through the negatively charged peptidoglycan layer of the cell and destruct the bacteria. The cationic polymers may have less toxicity to mammalian cells which was attributed to the different structure of cell membrane [189]. Among the limited reports of in vivo performance, a 3-(trimethoxysilyl)-propyl dimethyl octadecyl ammonium chloride (QAS)-modified silicone rubber was evaluated for their performance in a rat model for 3 or 7 days. The study showed antimicrobial properties against adhering *S. aureus* from subcutaneously implanted samples [190]. From a relevant in vitro study, the QAS modification reduced the viability of adherent staphylococci from 90% to 0% and of Gram-negative bacteria from 90% to 25%, while the presence of adsorbed plasma proteins had little influence.

Covalently binding antibiotic agents on the implant surfaces to reduce infection have also been investigated. The reported agents include penicillin [191], ampicillin [192], gentamicin [193], vancomycin [194], ciprofloxacin [195], and cefotaxime [196]. In these studies, the antimicrobial agents were usually immobilized on various substrates through a PEG spacer on substrates. Interestingly, some formulations have shown antimicrobial performance both on the surfaces and in solutions. The performance under in vivo condition is needed to show their potential on implanted medical devices.

Halogenated furanones isolated from the red marine alga *Delisea pulchra* can interfere with several bacterial communication systems particularly the quorum sensing (QS) systems of Gram-negative bacteria [197]. Furanones have potential to be used as a coating for biomaterials to control infection caused by *S. epidermidis*. In a study, furanone-coated catheters were prepared through a plasma-1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC) reaction. Biofilm formation by *S. epidermidis* was inhibited by 78% with furanone-coated catheters. In an in vivo sheep model, it is found furanones were effective at controlling infection for up to 65 days [198]. Synthetic furanone analogues based on dihydropyrrolones (DHPs) exhibit low cytotoxicity toward mammalian cells while retaining a broad-spectrum antimicrobial efficacy. DHP-coated polyacrylamide substrates were prepared using a reaction from azlactone groups on the polyacrylamide beads with ethylenediamine, followed by reaction with DHP. The substrates are effective in reducing the number of clinical isolates of *S. aureus* in vitro in a dose-dependent manner and are able to reduce the pathogenic potential of staphylococcal infection in a subcutaneous infection model [199].

Superoxide radicals can inhibit bacterial attachment to the solid surface. It was found selenium compound can be covalently bound to a solid matrix and retain its ability to catalyze the formation of superoxide radicals. In one study, a selenium compound was covalently attached on silicone contact lenses. The modified lenses decreased bacterial colonization in vitro while not adversely affecting the corneal health of rabbits in vivo [200]. In another example, selenocyanatodiacetic acid (SCAA) was immobilized to the surface of a hemodialysis catheter. *S. aureus* failed to develop biofilms on SCAA-modified catheters in either static or flow models. The SCAA coating also inhibited the development of *S. aureus* biofilms on HDCs in vivo for 3 days [201].

Immobilization of antimicrobial agents on medical devices is very attractive since there is no leakage into surrounding tissue, which reduces concerns of cytotoxicity and drug resistance. Moreover, a long-term efficacy is expected due to no depletion of the agents during the implantation. However, fouling from the biological media, such as blood proteins, mucins, and cellular materials, may deactivate the antimicrobial performance. Especially for the surfaces with cationic immobilization, considering many antimicrobial efficacies are related to a positive charge density threshold [180] and a large portion of above mentioned foulants are negatively charged, the fouling-induced deactivation could be especially true. Another concern is that immobilization chemistry is likely to reduce the activity of antimicrobial agents, such as antimicrobial peptides [202]. Loss of antimicrobial efficacy from immobilized furanones was also reported [203]. Optimization of immobilization such as conjugation chemistry, grafting density, and spacer length is crucial to improve the antimicrobial efficacy. While many microbial agent-immobilized surfaces have shown in vitro antimicrobial activity, the performances need to be evaluated against blood, human cells, and ultimately in vivo biocompatibility and antimicrobial efficacy [204].

1.4.2 Anti-fouling Surfaces

Anti-fouling surfaces reduce bacterial attachment through unique surface characteristics. With no antimicrobial agents leaching from surfaces, anti-fouling surfaces achieve antimicrobial performance without introducing toxicity and drug resistance. In addition, some anti-fouling polymers such as polybetaines also significantly reduce protein adsorption to a level that can inhibit thrombus formation or other device-related complications. The surfaces are especially intriguing for medical device application. Coatings with fouling resistance include both hydrophilic polymers (either nonionic or charged) and hydrophobic polymers. Structured surfaces that create unique surface characteristics such as superhydrophobicity and superhydrophilicity also exhibit microbial resistance. Various anti-fouling surfaces and their application in reducing microbial attachment on medical devices are reviewed in Chap. 8.

However, anti-fouling coatings cannot kill any microorganisms or inhibit their growth in the surrounding environment. For coatings with defects or coatings with less stability, bacteria may overcome the anti-fouling layer and finally find a way to grow onto the surface. For example, a PEG brush coating with a thickness of 7–17 nm has shown strong in vitro protein and bacteria resistance. However, after introducing defects (~10 nm), microorganisms can grow on the surfaces by adhering to the defects and compressing the PEG brushes [205]. Moreover, with a complex cascade of cellular events governing foreign body reaction and inflammatory activation, it is not uncommon that many anti-fouling surfaces notably reduce their in vitro bacterial resistance during in vivo tests. For many medical devices, clinical reliance between bacteria resistance and infection reduction still needs to be established. All these concerns make obstacles to get antimicrobial claims for applying anti-fouling surfaces to medical devices.

To enhance the antimicrobial efficacy as well as reduce other complications on medical devices, anti-fouling modifications are combined with other antimicrobial techniques. Delivering antimicrobial agents through an anti-fouling layer has been investigated. As mentioned previously, coatings with hydrophilic hydrogels in combination with antimicrobial agents such as minocycline/rifampin and silver have been applied on vascular catheters, endotracheal tubes, and Foley catheters. Antimicrobial efficacy was also reported for silver nanoparticles combined with PEG hydrogels [206], polybetaine coatings [207], and superhydrophobic coatings [208]. A combination of anti-fouling coating and antimicrobial agents is promising to achieve a long-term and multiple foulant-resistant surfaces in a complex environment.

Antimicrobial activity of anti-fouling surfaces can also be enhanced by covalently binding antimicrobial agents on anti-fouling polymers. The anti-fouling linkers help the antimicrobial agent to kill bacteria and also reduce non-specific biofouling. Quaternary ammonium-containing polymers [209] and various antibiotics [191–196] have been immobilized on the surfaces using an anti-fouling PEG linker. Antimicrobial peptides have been tethered on polybetaine brushes and demonstrated broad-spectrum antimicrobial activity [210]. Among all the anti-fouling polymers, carboxybetaine polymers are unique by providing functionalizable

carboxylic groups which could bond antimicrobial agents covalently [211]. In addition, anti-fouling polymers can be designed to achieve a regenerative killing-releasing strategy. For example, polycarboxybetaine esters can kill bacteria on the surface with the quaternary ammonium moieties. The killed bacteria can be further released from the surface after the polymer was hydrolyzed into a non-fouling polybetaine, releasing killed bacteria from surfaces [212].

1.4.3 Physical Antimicrobial Control

Physical approaches have been applied to control microbial growth with a long history. Heat, radiation (both ionic such as gamma and e-beam and nonionic such as UV), low temperature, high pressure, desiccation, osmotic pressure, and filtration have been widely used in many areas. Physical approaches are thought to be effective without concerns of side effects from active antimicrobial agents. However, they have to be delivered safely and not all of these physical methods are applicable for medical implants during implantation.

1.4.3.1 UV/Visible Light

Both ultraviolet (UV) and certain range of visible lights have shown bacterial resistance or bactericidal effect. The ultraviolet C (UVC) spectrum, especially those among the wavelength of 250–270 nm, is strongly absorbed by the nucleic acids of a microorganism and exhibits most lethal effect to microorganisms. UVC irradiation has been investigated to treat localized infections in vivo and found germicidal activities, including resistance to infections caused by multidrug-resistant microorganisms [213]. It is possible to disinfect throughout the entire lumen of a catheter in 2 min using a UVC LED diode source [214]. While UVC may selectively inactivate microorganisms and preserve mammalian cells with appropriate doses, light radiation under a safer wavelength range is more preferable for many implantable devices. In Chap. 9 of the book, the effects of light on microorganisms and potential application of light to prevent bacterial biofilm are reviewed.

A novel antimicrobial design of medical implants integrated with helical side emission optical fibers is developed (Fig. 1.1). The devices with an ultraviolet irradiation system comprise a medical device having a central lumen, an optical fiber having a longitudinal length, and an ultraviolet wave generator, wherein ultraviolet waves generated by the wave generator are dispersed along the longitudinal length of the optical fiber to disinfect the central lumen of the medical device. In this application, a relatively narrow band source of light is used in the irradiation devices. For example, the frequency bandwidth of ca.10 nm with a spectral peak at 366 nm is used due to a combination of poly(methylmethacrylate) (PMMA) optical fiber transmissivity, cost, availability, and antimicrobial efficacy. This peak frequency corresponds to an energy value range of 3.5–3.6 eV which is also the disruption energy

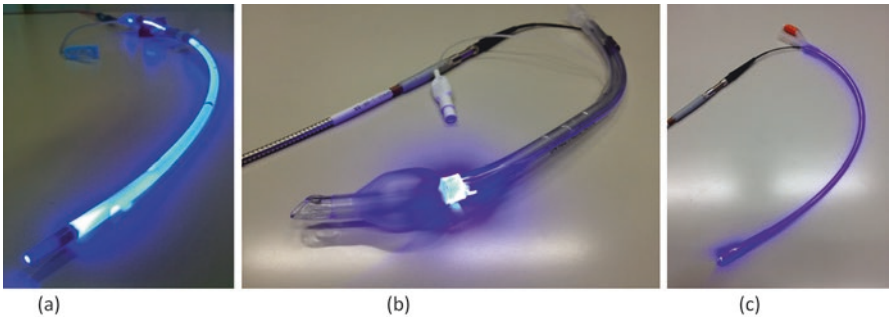

Fig. 1.1 A helical side emission fiber, with a controllable light source, is incorporated within (**a**) a cardiovascular catheter, (**b**) an endotracheal tube, and (**c**) a Foley catheter

associated with the S-H and C-C molecular covalent bonds within the organism genetic material. The theoretical eradication dose level for *S. aureus* is ca. 2.3 J/cm^2. This value corresponds to an optical energy fluence value of ca. 80 μW/cm^2 for an 8 h therapeutic exposure over the surface of the fiber. Experimentally, significantly lower optical fluence values have shown complete inhibition in 7 log inoculums of several species, including *S. aureus*. This may be attributable to the integration of energies associated with the full spectral band of the irradiation source [215].

The above light is under the range of UVA, which is absorbed weakly by DNA and considered much safer to mammalian cells compared with UVB and UVC. The primary mechanism of microbial inactivation by UVA is via the formation of reactive oxygen species (ROS), which cause the oxidation damage of the microorganisms. To further enhance the antimicrobial efficacy on the implant surfaces, photosensitizers or photocatalysts have been applied on the medical surfaces on which ROS can be triggered by UV or visible light. Antibiotic-resistant polymicrobial biofilms of *P. aeruginosa* and MRSA were grown in ETTs and treated with a methylene blue (MB) photosensitizer and 664 nm nonthermal activating light. Cultures of the lumen of the ET tube were obtained before and after light treatment to determine efficacy of biofilm reduction [216]. Another example includes a rose bengal-coated ETT, on which rose bengal is activated with UV light which causes singlet oxygen production and photosensitization [217]. During mechanical ventilation, a probe was connected to a UV–visible light source and introduced inside the ETT in vitro, and animal studies indicated some bacteriostatic and bactericidal effects of this technology [134]. In addition to ROS, other active antimicrobial agents can be released through photocleavable linkages. More details about light-triggered anti-infective materials are reviewed in Chap. 10, which include the surfaces containing photosensitizers, photocatalysts, and photocleavables.

1.4.3.2 Acoustic Energies/Ultrasound

Acoustic energies such as ultrasound have been explored to reduce biofilm formation on medical implants [218]. Low-power acoustic waves generated from piezo actuators were tested for their antimicrobial efficacy. It was found certain power intensities (<1.1 mW/cm^2 and frequencies of 100–300 kHz) appear to constitute the optimal acoustic energy levels for preventing biofilm formation on urinary catheters. In an in vivo test, Foley urinary catheters attached with elastic wave-generating actuators were inserted into the urinary tracts of male rabbits. The treatment with the elastic acoustic waves maintained urine sterility for up to 9 days compared to 2 days in control catheterized animals [219].

Ultrasonication has been found to increase transport of antibiotics across biofilms, enhancing the killing of bacteria within the biofilm encasing. For example, ultrasonication significantly increases transport of gentamicin across biofilms that normally blocked or slowed gentamicin transport when not exposed to ultrasound. This enhanced transport may be partially responsible for the increased killing of biofilm bacteria exposed to combinations of antibiotic and ultrasound [218, 220].

Sonoantimicrobial chemotherapy (SACT) was recently explored as a novel antimicrobial strategy [221]. Like photosensitizers, sonosensitizers produce ROS while being activated at low-intensity ultrasound instead of light. *S. epidermidis* membrane integrity was notably damaged, and the level of intracellular ROS level was remarkably increased after sonodynamic treatment [222]. As the low-intensity ultrasound is used in clinical diagnoses and penetrates deeper into the body than light, it is potential to incorporate sonosensitizers to medical devices and activated using a low-intensity ultrasound.

1.4.3.3 Mechanical Stress and Deformation

Deformation of elastomer surfaces under electrical or pneumatic actuation can debond various biofilms. A prototype of a model urinary catheter demonstrated release of mature *P. mirabilis* biofilms by ca. 90% from strained surfaces [223]. Another in vitro test using designed catheters to generate greater than 30% strain in the majority of the luminal surface when subjected to pressure is achieved. The catheter prototypes are able to remove greater than 80% of a mixed community biofilm of *P. mirabilis* and *E. coli* on-demand and furthermore are able to remove the biofilm repeatedly [224]. No in vivo results have been reported.

1.5 Summary

HAIs currently are the most common complications affecting hospitalized patients. Medical devices are responsible for a large portion of HAIs, particularly in ICU patients with critical implants. Generally, the infections have strong relationship

with bacterial colonization and biofilm formation on medical implants. Bacterial colonization can be a prelude to infections and normally recognized as the first step in biofilm formation. Microbes living in biofilms are more recalcitrant to antibiotic and antiseptic treatment. Clinical treatment to resolve biofilms usually involves replacement of the device.

Classified by implants, CRBSI, VAP, and CAUTI are the most common HAI infections from ICU. The pathogenesis of the infections is attributed to different etiologic agents that colonize the device. Other device-associated complications, such as catheter occlusion, thrombus formation, and encrustation, may correlate with infection and malfunction of the devices. Polymer materials used for manufacturing critical care implants, such as polyurethane, silicone, and PVC, could not resist bacterial colonization and biofilm formation. Antimicrobial modifications are the most controllable avenues to prevent the infection. Eluting antiseptics or antibiotics has been dominantly applied especially on vascular catheters and shown clinical advantages in reducing infections. These agent-eluting technologies can be combined with heparin coatings or hydrophilic coatings to reduce other complications or facilitate device insertion. However, not all modifications with eluting antimicrobials are effective. Some modified devices have not shown expected anti-infection efficacy with inconclusive or controversial clinical results. Moreover, antimicrobial-resistant pathogens become increasing concerns to the agent-eluting coatings.

Researchers are exploring new technologies to improve or replace current agent-eluting coatings. Controlled release system, responsive release, agent immobilization, anti-fouling surfaces, and physical infection control approaches have been developed, but for most of them, the anti-infection performance needs to be further evaluated. Most of these technologies have shown in vitro antimicrobial activity, or resistance to bacterial colonization and biofilm formation. Nevertheless, the effectiveness of many of these coatings still needs to be evaluated under clinically relevant environment and against a broad spectrum of bacteria, considering the diverse environments into which the devices are placed and the multiplicity of ways in which different organisms can colonize surfaces. In addition, both in vitro and in vivo biocompatibility are needed to confirm the safety of the developed system. Ultimately, in vivo antimicrobial evaluations with a relevant infection model are crucial to put these technologies into application in medial implants.

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Chapter 2
Antimicrobial and Anti-Biofilm Medical Devices: Public Health and Regulatory Science Challenges

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2.1 Public Health Challenge

Healthcare associated infections (HAIs) are one of the top 10 causes of death in the United States (~100,000 deaths per year) [1, 2] and impose a significant financial burden ($28–45 billion in 2007) [3]. HAIs can happen anywhere in the continuum of settings where patients receive health care (e.g., long-term care, home care, ambulatory care) [4]. A subset of HAIs acquired in healthcare—with some estimates over 60% [5, 6]—are related to medical device use (MD-HAIs). In fact, three of the four HAIs that are "areas of focus" in the 2013 HHS *National Action Plan to Prevent Health Care-Associated Infections: Road Map to Elimination* are MD-HAIs [7]. These are catheter-associated urinary tract infections (CAUTI), central line-associated bloodstream infections (CLABSI), and ventilator-associated pneumonia (VAP). The fourth HAI focus area, surgical site infections (SSI), also includes MD-HAIs such as prosthetic joint infection (PJI). These infections are called out because of their significant human and financial burden. CAUTIs cost the US healthcare system more than $2900 per episode, a total of $2.9 billion per year [8].

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CLABSIs costing US healthcare $1.2 billion and are associated with 56,000 deaths/year [9, 10]. PJIs costs an average $60,000 per case, resulting in estimated costs of $1 billion/year in 2014 [11]. PJI results in a 5-year survival rate (~75%) [12] similar to that of myocardial infarction (72%) [13] or colon cancer (~ 65%) [14]. Finally, VAPs total more than 250,000 cases per year at a cost of more than $5,000 per case [15]. Although there are many more MD-HAIs than just the ones that were called out in the *National Action Plan*, the collective financial burden of just these MD-HAIs alone (>$6.3 billion/year) more than suffices to show that MD-HAIs are a public health challenge that needs to be addressed.

There are many more MD-HAIs, some with much higher infection rates than those discussed above. Due to the large range of device types, the relatively low infection rate associated with many devices, and the lack of diagnostics to study MD-HAI pathogenesis clinically, it is extremely challenging to accurately assess the overall public health impact of MD-HAIs. A few authors have undertaken the arduous task of trying to get a better estimate of the overall impact of MD-HAIs. A 2012 paper by Busscher et al. focused on the infection incidence for 20 types of medical devices [16] (Table 2.1). The devices were grouped by implant site, with most devices having about 1–10% infection incidence with the exception of urinary catheters (33%) and abdominal wall patches (up to 16%). Nearly all voice prosthesis will eventually fail due to leakage caused by biofilm buildup. Incidence alone doesn't tell the full story of the impact of MD-HAIs. Another review paper in 2010 by Wolcott et al. provided economic costs and mortality for a subset of MD-HAIs

Table 2.1 Incidence of biomaterial-associated infection for different implants and devices

Tissue implant site	Implant or device	Infection incidence over lifetime (%)
Urinary tract	Catheter	33 (per week)
Percutaneous	Central venous catheter	2–10
	Temporary pacemaker	4
	Short indwelling catheter	0–3
	Peritoneal dialysis catheter	3–5
	Fixation pin or screw	5–10
	Sutures	1–5
	Voice prosthesis	25 (per month)
	Dental implant	5–10
Subcutaneous	Cardiac pacemaker	1–7
	Penile prosthesis	2–5
Soft tissue	Mammary prosthesis	1–7
	Abdominal wall patch	1–16
	Intraocular lens	0.1
Eye	Contact lens	0.1–0.5
Circulatory system	Prosthetic heart valve	1–3
	Vascular graft	1.5
Bone	Prosthetic hip	2–4
	Prosthetic knee	3–4
	Tibial nail	1–7

Adapted from Ref. [16]

including urinary catheter infections, infected cardiovascular devices, contact lens associated keratitis, orthopedic device infections, and ventricular shunt infection [17]. The review summarized information from journal articles, US government agency reports, and professional societies. The total estimated annual deaths were 82,000, with direct costs of $18 billion. This impact is commensurate with the scale of many major diseases such as breast cancer ($16.5 billion in 2010) [18, 19]or colorectal cancer ($14 billion) [19, 20].

The incidence and impact of MD-HAIs depend on numerous factors (e.g. the type of device material, the anatomic location, the use of prophylactic antibiotics) and are extremely diverse in terms of the microbes involved, the morbidity, mortality, chronicity and treatment modalities. A major area of research in MD-HAI pathogenesis is the increasingly well understood role of biofilm [21] [23]. Biofilm is defined as self-assembling multicellular communities that behave differently from their free floating (planktonic) counterparts [22]. The number of microbes required to initiate biofilm formation on a medical device surface can be as low as 100 [23]. Normally the microbes must adhere to the exposed surfaces of a device long enough to become irreversibly attached to form a conditioning film. This largely depends on the device surface properties and the aqueous environment [24]. In recent years, there is an increasing understanding of how colonization and biofilm may play a role in the pathogenesis of medical device associated infections [25, 26] as well as development of drug resistant microbes [27, 28].

Device colonization and biofilm have unique clinical features such as persistence that make them challenging to address. This challenge requires a coordinated response from medical device manufacturers, clinicians and public health/regulatory authorities. Many efforts have been made to prevent HAIs at the first line of defense: hygiene and sterility. These include, for example, handwashing, facility cleaning and decontamination, and efforts to ensure a sterile surgical field. Despite the tremendous impact that these efforts have had in reducing the incidence of HAIs, there are still significant human and financial costs associated with MD-HAIs. Thus, it is not surprising that researchers and medical device companies are interested in developing antimicrobial technologies to prevent MD-HAIs [5, 29]. Because of the potential for colonization and biofilm to lead to MD-HAIs, many antimicrobial technologies employed on medical devices are specifically targeted to this aspect of microbial life.

In this chapter, we introduce the *regulatory science* of antimicrobial and anti-biofilm technologies designed to prevent MD-HAIs. In the United States, *regulatory science* is the science of developing new tools, standards, and approaches to assess the safety, efficacy, quality, and performance of all FDA-regulated products [30]. Although the focus of regulatory science is not the invention of new medical products or technologies, in the total product life cycle (TPLC) of a medical device, the role of regulatory science can be very important. Good regulatory science can facilitate consumer access to innovative medical products that are safe and effective. In Section II, we first take a broad view beyond antimicrobial coatings to consider the range of possible medical therapies (e.g., device coatings, antimicrobials, vaccines) to prevent MD-HAIs, their use, limitations and safety. In Section III, we discuss regulatory definitions of the different types of technology and discuss mechanisms

of action and the importance of understanding combination products. Then in Section IV, we focus specifically on the regulatory science of antimicrobial technologies for medical devices. We show how the paradigm shift from a planktonic model of microbial life to a biofilm model introduces significant challenges to the scientific assessment process.

2.2 Antimicrobial/Anti-Biofilm Technologies to Prevent MD-HAIs

As discussed in Sect. 2.1, strategies to prevent MD-HAIs include antimicrobial and anti-biofilm technologies. Anti-biofilm technologies are differentiated from conventional antimicrobial strategies in that they are designed to target biofilm aspects of microbial life. They can be "enhanced" versions of antimicrobials that better penetrate biofilm or kill organisms in biofilm; whereas others may not even be lethal to organisms but may prevent colonization through a number of physical or chemical approaches. Conventional antibiotics or antimicrobials may also become anti-biofilm when they are released from a device surface to prevent colonization. A promising—but more challenging approach—is the development of technologies that are both biocompatible and encouraging to host integration while simultaneously resisting harmful bacterial colonization [31]. While these goals have been the source of much research progress over the past few decades, device coatings are not the only medical intervention to address MD-HAIs. Other types of technologies include vaccines, physical strategies for preventing or removing biofilm, and combinations of various modalities. Table 2.2 summarizes examples, effects and limitations of these technologies. It is separated into four major categories: (1) coating technologies/antimicrobials, (2) vaccines, (3) biofilm removal and (4) combined modalities. Systemic antimicrobials are beyond the scope of this discussion. Finally, although we don't address them in this chapter, it is important to mention the increasing interest in the use of probiotic, "beneficial colonization" strategies which might compete with harmful bacteria [32, 33]. Although this work is still nascent, it may be recognized in the near future as a type of strategy to prevent MD-HAIs in some cases.

2.2.1 Antimicrobial/Anti-Biofilm Coatings

Coatings are one of the most common types of antimicrobial medical device technologies seen in the research literature. There are a number of types of coating strategies (as described below) and the diversity of strategies continues to increase as creative new technologies are developed.

Anti-adhesive Anti-adhesive coatings are designed to prevent the first stage in biofilm formation, colonization, eliminating the threat at the outset. Bacteria can adhere

Table 2.2 Types of anti-biofilm technologies in medicine and their characteristics

Anti-biofilm technologies		Examples	Mechanisms	Limitations	Ref.
Coatings	Anti-adhesive	PEO, zwitterionic polymer, topographical structure, superhydrophobic coating	Low surface energy chemistry and nano-/micro-textured morphology reduce fouling, passive repelling	Stability, oxidation damage, in vivo efficacy may vary	[35–42]
	Antimicrobial loaded	Materials loaded with small molecule biocides, heavy metal, antibiotics, etc.	Active inhibition at the surface	Issues with optimizing release for effectiveness in preventing resistance, in vivo efficacy	[46, 47]
	Controlled/active release	Temperature-responsive copolymer, hydrolytically degradable film, PH-sensitive releasing	Active inhibition, release in response to stimuli	Release profile, stability, in vivo efficacy	[48–52]
	Dual/multifunctional	Differentially adhesive surfaces, low fouling, and antibacterial coating	Prevent colonization while promote tissue integration, combines modalities to reinforce efficacy	Complex to optimize	[53–55]
Antimicrobial/anti-biofilm agents	Antibiotics	Gentamicin, rifampicin, minocycline, doxycycline, etc.	Bactericidal through multiple genetic and biochemical pathways	Resistance	[56–59]
	Metal ions, oxides, nanoparticles	Silver zeolite, copper oxide, zinc oxide, ferric ammonium citrate	Release metal ions that target bacterial cells, some actions not well understood	Allergy	[61–72]

(continued)

Table 2.2 (continued)

Anti-biofilm technologies		Examples	Mechanisms	Limitations	Ref.
	Cationic compounds	Cationic surfactant, polymers, peptides, lipids, etc.	Disrupting bacterial membranes, allowing the free exchange of intra- and extracellular ions	Complex process in extraction, isolation and purification, expensive	[73–86]
	Quorum sensing inhibitors (QSI)	Triazolyldihydrofuranone, cinnamaldehyde, hamamelitannin	Inhibit virulence factors and biofilm formation	New entities chemical, limited information	[87–93]
	Dispersing enzymes	DNase I, DspB, a-amylase, restriction endonucleases	Cause biofilm detaching	expensive	[94, 95]
	Bacteriophage	Caudovirales, ligamenvirales, some unassigned viruses	Viruses that infect bacterial cells	Host specified, purification	[96–99]
	Natural compounds	Phenol, phenolic compounds, etc.	Diverse, not well studied	Complex composition add difficulties to optimize efficacy	[100] [101]
Biofilm removal techniques/ physical strategies		Manual debridement, pulsed electrical fields, ultrasound therapy, and other topical and combination therapies	Remove multispecies bioburden or devitalized host tissue	Promising yet the efficacy has yet to be proven in the clinic	[102–106]
Vaccines		Live, attenuated; toxoid; killed, whole cell; polysaccharide; polysaccharide-–protein conjugate	Leveraging immune system	Critical phenotypes and factors are not adequately addressed	[107, 108]
Combined Modalities		Polyphenolic compounds and antimicrobial agent, enzyme-based compounds combined with metal ions, antimicrobials with non-contact ultrasound therapy	Synergistic antibacterial mechanism	Complexity	[104, 109–114]

and grow on natural and synthetic surfaces in an aqueous environment. Both specific and non-specific interactions play important roles in the bacterial adhesion and biofouling [34]. Biofouling in the context of medical devices includes non-specific adsorption of biological molecules that happens at the moment that a medical device comes into contact with biological fluids. Many anti-adhesive coatings, such as polyethylene oxide (PEO) and zwitterionic polymers, draw on the substantial body of literature on coatings designed to prevent biofouling [35–37]. More recent developments in superhydrophobic coatings have also been employed to reduce microbial adhesion[38]. In addition to these chemical strategies, a number of physical strategies also exist [39–41]. Fabrication of coatings with nano and micro-textured morphologies has been optimized at length scales that can discourage microbial adhesion [40, 42]. For all types of anti-adhesive coatings, an important limitation is the impact of biofouling on coating effectiveness. Nearly all devices are subject to biofouling by body fluids as well as elements of the foreign body response. In situations with large numbers of microorganisms and relatively static fluid dynamics, biofilm can form on surrounding surfaces first and then cover the coating. For anti-adhesive coatings that work through chemical means, stability is another key challenge. For these reasons, anti-adhesive coatings are often not regarded to be as effective as active coatings in vivo. In general, covalent coatings with a history of biocompatibility are seen as less of a challenge for safety testing than novel materials or eluting coatings [43]. Nanostructured materials, fundamentally different in their biological interactions than nanoparticles, can significantly change cell morphologies that direct their differentiation and survival [44]. Recent work by Kumar et al. has elucidated how a nanofibrous structured surface can change cell behavior through the control of cell shape [44]. Properties such as this may result in grouping of nanostructured surfaces with nanoparticles as an area of concern [45].

Antimicrobial Loaded and Active/Controlled Release The most common type of coating is antimicrobial loaded. Numerous types of antimicrobials have been loaded into polymer and hydrogel coatings [46–49]. The coating material is tuned to release these antimicrobials at varying rates, depending on the application. Controlling and ensuring reproducible release rates is one of the limitations of these types of coatings, and can result in significant differences in outcomes of performance testing. As a result, more sophisticated controlled/active release coatings have been developed to respond to signals such as temperature, pH or other changes caused by the presence of microorganisms[50–52]. The ultimate goal of this technology is to release antimicrobials only when microorganisms are present. Two key benefits to active release are (1) preserving the antimicrobial until needed, and (2) reducing the potential for development of resistant organisms.

Multifunctional Multifunctional coatings, still in the early stages of development, are designed to combine any of the above concepts and may also combine host cell integration with antimicrobial function [53–55]. These coatings are especially promising for applications where an implant requires successful integration with host tissue to achieve the best long-term functionality. The added complexity of these products can make their safety profile more challenging to predict.

2.2.2 Antimicrobial Agents Included in Anti-Biofilm Strategies

Most of the antimicrobial agents that have been used in legally marketed medical device–drug combination products—such as antibiotics and metal ions—already have a long history of use against planktonic organisms.

Antibiotics Some of the antibiotics used on devices include gentamicin, tobramycin, rifampicin, and clindamycin [56–58]. Antibiotic resistance is an important concern with many of these agents. For example, bacteria on gentamicin-loaded bone cement in vivo have been found to have gentamicin resistance [59]. Further research needs to be conducted with the goal of learning how these coatings should be used in view of good stewardship principles. The Infectious Disease Society of America (IDSA) states that antimicrobial stewardship is the selection of the optimal antimicrobial drug regimen, dose, duration of therapy, and route of administration. "Antimicrobial stewards seek to achieve optimal clinical outcomes related to antimicrobial use, minimize toxicity and other adverse events, reduce the costs of health care for infections, and limit the selection for antimicrobial resistant strains" [60].

Metals Silver is the most commonly used metal seen in antimicrobial coatings. Minimum inhibition concentration (MIC) testing has shown a high level of silver antimicrobial activity in vitro [61, 62]. Some silver-coated dressing products have also shown rapid bactericidal action and can achieve a five-log reduction in a comparatively short time [63]. Other metals have been used but suffer from toxicity limitations at higher concentrations [64]. A combination of metals has sometimes been employed [65]. There are numerous reviews on silver-containing medical devices [67–71]. Current in vitro tests for performance of silver-containing coating technologies are not always good predictors of how they will perform in vivo [67, 68]. The outcome of these studies depends on the type of device, the specific use of the device, the type of silver coating, and the test conditions. The "apparent" performance of silver-containing products depends on the test methods employed [69]. Silver is one of the few antimicrobial technologies on medical devices that have been the subject of multiple clinical trials. The outcomes of these trials depend on factors such as duration of use, patients, bacterial species present, materials, and catheter care mistakes [70, 75–77]. An important consideration is the form of silver, i.e., as a salt or as a nanoparticle. Although there was initially very little information on the safety of nano-silver vs. ionic silver, significant research in the area of toxicology/biocompatibility has helped clarify how risk assessment might be performed [72].

Cationics Another group of conventional antimicrobial agents that have been researched and/or employed in device coatings is cationics, including quaternary ammonium compounds (QAC) [73], antimicrobial peptides (AMPs) [74], chlorhexidine [75], poly(hexamethylene biguanide) (PHMB) [76, 77], and chitosan [78, 79]. Antimicrobial peptides (AMPs) are found in a variety of organisms as a native defense against bacteria and are the source of inspiration for synthetic versions with higher efficacy [79, 80]. A common strategy for implementing AMPs involves sur-

face functionalization with a heterobifunctional cross-linker [81]. Compared with other cationic compounds such as PHMB, comparatively higher concentrations of these biocides are needed for efficacy and lasting biofilm inhibitory effect, which may lead to potential bacterial resistance [82, 83]. A number of studies show that cationics, especially PHMB, have a favorable toxicity profile with regard to skin irritancy and hypersensitivity at typical topical use levels [84, 85].

Many antimicrobials still in the research and development phase are specifically targeted to medical biofilms:

Quorum Sensing Inhibitors One of the earliest concepts to be tested was based on inhibition of "quorum sensing." Bacteria colonizing materials communicate when they reach a critical mass using small molecules called quorum sensing molecules, which can regulate biofilm formation and virulence factor secretion [92–94]. Both in vitro and in vivo tests showed that quorum sensing inhibitors could shut down this communication process and thereby prevent biofilm formation and infections associated with antibiotic-resistant strains [87, 88]. It has been proposed in the literature to use these agents on medical devices in combination with conventional antimicrobials, to reduce biofilm formation and thereby enable antimicrobials to work more effectively [28]. Since most of these inhibitors are small molecules, cytotoxicity is often a safety concern [89]. Because inhibitors are not lethal to microorganisms, it is sometimes proposed that "resistance" can't develop over time [90, 91]. However, this theory is controversial as it is not known if there are other factors that would give resistant clones a selective population advantage over longer periods of time. Quorum sensing inhibitors have been proposed for use in direct application to a colonized surface or as part of device coatings [92, 93].

Dispersing Enzymes Another approach is to remove biofilm after it is formed using dispersing enzymes. A number of enzymes are specifically targeted toward breaking down the molecular cross-linking found in biofilm matrices. Because these enzymes are naturally derived, they are relatively expensive to produce [94]. Although the enzymes are very effective at detaching many biofilms, an important safety concern is that the detached bacteria may travel to distant sites and reinitiate colonization, causing satellite infections [95].

Bacteriophage Bacteriophage is a naturally sourced antimicrobial that can be specifically strain targeted [96]. Minimal impact on nontarget bacteria or tissues was reported [97]. The immune system may inactivate phage in vivo [98]. While bacterial resistance to phage is possible, a growing number of engineered phages are emerging to provide alternatives [99].

Natural Compounds A number of natural small molecules have been isolated from plants that might be used in, on, or in conjunction with medical devices. These may be compounds under investigation for other properties, which are discovered to have antimicrobial function. Some of them may have beneficial "probiotic" effects by favoring commensal colonizers while being harmful to more virulent organisms [100, 101].

2.2.3 Biofilm Removal

Manual debridement is the centuries old technique for biofilm in accessible superficial wounds. It is considered a very effective way of managing the biofilm in chronic wounds by transforming "non-healable" wounds to healable wounds [102]. Because many medical device surfaces are inaccessible and can't easily be removed/cleaned without additional surgery, one focus of removal has been to use long-range physical methods such as ultrasound therapy [103]. Another potential approach for partially accessible devices is application of pulsed electric field with or without combined antibiotic therapy [112–114]. Electric fields have been applied (or generated through redox reactions) to interfere with important electrostatic factors in adhesion. A potential safety concern with physical removal methods is that biofilm clusters containing bacteria and endotoxins from physical removal procedures can migrate to other locations in a wound or become systemic, potentially resulting in life-threatening sepsis [105]. Another concern is to ensure safe use of electric fields, given the low threshold for tissue damage. More development is needed to translate this technology for the clinic [103].

2.2.4 Vaccines

More recently, vaccination and immunization have been explored with specific focus on effectiveness for preventing medical device-associated infection [106]. Specifically, prevention of prosthetic joint infection (PJI) associated with *S. aureus* infection has been targeted through the use of preoperative passive immunization with neutralizing antibodies. Vaccines have also been developed specifically to target upregulated antigens found in biofilm [107, 108]. A multivalent vaccine combining antigens upregulated in the biofilm and planktonic forms of *S. aureus* has shown efficacy in an animal model of infection without the use of antibiotic therapy. In the future, vaccines may play an important role in helping prevent device-associated infections without inducing drug resistance. However, it would be premature to assume that these technologies will be successful before their safety and effectiveness are successfully demonstrated in human clinical trials.

2.2.5 Combined Modalities

Combination therapy has been successful in other areas of medicine (HIV, cancer) because of the ability to target multiple biological mechanisms, thereby preventing a resistant population from emerging [109, 110]. In a similar manner, it may play an important role in fighting device-associated infections successfully due to the benefits of diverse modes of action and improvements in preventing development of microbial resistance. Many antimicrobial agents have been combined with other

antimicrobials to increase efficacy. For example, polyphenolic compounds have been combined with antibiotic and calcium modulators for acute pneumonia infection in vitro [111], enzymatic removal has been combined with metal ions [112], electrical fields have been combined with antimicrobials, and ultrasound has been combined with antibiotic therapy [113, 114].

2.3 Types of Antimicrobial/Anti-Biofilm Technologies: Device, Drug, Biologic, and Combination Products

Anti-biofilm technologies for medical applications such as those described above may be drugs, devices, biological products, or any combination of two or more of the above (combination product). Therefore it is helpful to review the definitions of these terms:

Drug (FD&C Act, 21 U.S.C. § 321(g)):

The term "drug" means (A) articles recognized in the official United States Pharmacopoeia, official Homoeopathic Pharmacopoeia of the United States, or official National Formulary, or any supplement to any of them; and (B) articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals; and (C) articles (other than food) intended to affect the structure or any function of the body of man or other animals; and (D) articles intended for use as a component of any article specified in clause (A), (B), or (C) [115].

Device (FD&C Act, 21 U.S.C. § 321(h)):

The term "device" (except when used in paragraph (n) of this section and in sections 301(i), 403(f), 502(c), and 602(c)) means an instrument, apparatus, implement, machine, contrivance, implant, in vitro *reagent, or other similar or related article, including any component, part, or accessory, which is—*

1. *recognized in the official National Formulary, or the United States Pharmacopeia, or any supplement to them,*
2. *intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or other animals, or*
3. *intended to affect the structure or any function of the body of man or other animals, and which does not achieve its primary intended purposes through chemical action within or on the body of man or other animals and which is not dependent upon being metabolized for the achievement of its primary intended purposes* [115].

Biological Product (PHS Act, 42 U.S.C. 262):

The term "biological product" means a virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, protein (except any chemically synthesized polypeptide), or analogous product, or arsphenamine or derivative of arsphenamine (or any other trivalent organic arsenic compound), applicable to the prevention, treatment, or cure of a disease or condition of human beings (Public Health Service Act Sec. 351(i)).

Combination Product (21 CFR 3.2(e)):
The term combination product includes:

1. *A product comprised of two or more regulated components,* i.e.*, drug/device, biologic/device, drug/biologic, or drug/device/biologic, that are physically, chemically, or otherwise combined or mixed and produced as a single entity;*
2. *Two or more separate products packaged together in a single package or as a unit and comprised of drug and device products, device and biological products, or biological and drug products;*
3. *A drug, device, or biological product packaged separately that according to its investigational plan or proposed labeling is intended for use only with an approved individually specified drug, device, or biological product where both are required to achieve the intended use, indication, or effect and where upon approval of the proposed product the labeling of the approved product would need to be changed,* e.g.*, to reflect a change in intended use, dosage form, strength, route of administration, or significant change in dose; or*
4. *Any investigational drug, device, or biological product packaged separately that according to its proposed labeling is for use only with another individually specified investigational drug, device, or biological product where both are required to achieve the intended use, indication, or effect.*

Many devices which incorporate antimicrobial technologies have been determined to be combination products. A combination product is assigned to an agency center or alternative organizational component that will have primary jurisdiction for its premarket review and regulation. Under section 503(g)(1) of the Act, assignment to a center with primary jurisdiction, or a lead center, is based on a determination of the "primary mode of action" (PMOA) of the combination product. For example, if the PMOA of a device–biological combination product is attributable to the biological product, the agency component responsible for premarket review of that biological product would have primary jurisdiction for the combination product. A final rule defining the primary mode of action of a combination product was published in the August 25, 2005, Federal Register. The final rule defines primary mode of action as "the single mode of action of a combination product that provides the most important therapeutic action of the combination product." In some cases, neither the FDA nor the sponsor can determine the most important therapeutic action at the time a request is submitted. A combination product may also have two independent modes of action, neither of which is subordinate to the other. To resolve these types of questions, the final rule describes an algorithm FDA will follow to determine the center assignment. The algorithm directs a center assignment based on consistency with other combination products raising similar types of safety and effectiveness questions, or to the center with the most expertise to evaluate the most significant safety and effectiveness questions raised by the combination product. The final rule is effective November 23, 2005 [116] (Fig. 2.1).

The classification of the anti-biofilm technology, as a device, drug, biological product, or a combination product, and the lead center assignment govern the regulatory requirements for the product. Information on the resources for medical devices can be found at: http://www.fda.gov/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CDRH/CDRHOffices/ucm115879.htm [118].

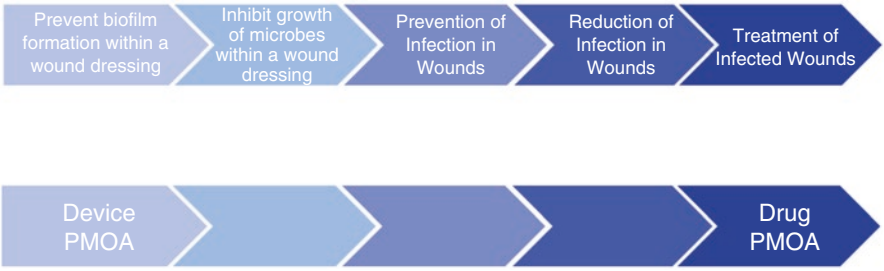

Fig. 2.1 Hypothetical example: subtle differences can make a difference in the classification of an anti-biofilm wound dressing [117]

Information on the resources for drugs can be found at: http://www.fda.gov/ForIndustry/FDABasicsforIndustry/ucm238040.htm [119]. Resources for biological products can be found at: http://www.fda.gov/BiologicsBloodVaccines/DevelopmentApprovalProcess/ucm2005991.htm

FDA maintains a public database of products cleared through the 510(k) premarket notification pathway (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMN/pmn.cfm) [120]. This database can be used to search for examples of antimicrobial/anti-biofilm technologies. It may be helpful to limit the scope of products searched by searching using a three-letter product code (procode) which is specific to a device type. If you don't know the procode for a device type, you can search for it here: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPCD/classification.cfm. It is prudent to understand the regulatory process which would apply to a specific product type and to obtain early feedback, when necessary, through appropriate pre-submission programs. For medical devices, the Center for Devices and Radiological Health (CDRH) Division of Industry and Consumer Education (DICE) has an email response form online for assistance, available at: https://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/ContactDivisionofIndustryandConsumerEducation/default.htm.

2.4 Regulatory Science Challenges of Antimicrobial/Anti-Biofilm Technologies in Medical Devices and Combination Products

2.4.1 Paradigm Shift

To better understand the challenges associated with the regulatory science of biofilms and anti-biofilm technology, it is important to consider the paradigm shift in microbiology from a planktonic to biofilm understanding of microorganisms, in particular bacterial and fungal organisms that are among the key causative agents of MD-HAIs. Until the late twentieth century, wound care (wound debridement) and dental applications (plaque) were the primary medical areas where biofilm was

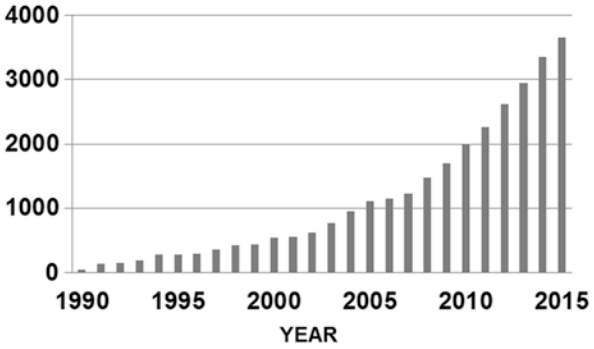

Fig. 2.2 The number of scientific publications per year with topic of "biofilm" (Web of Science, accessed 04/30/2015)

studied, due to it being visibly present as a persistent source of infection and inflammation. The increase in biofilm publications (Fig. 2.2) shows how in the 1990's work began to show a paradigm shift. Between 2006 and 2015, publications on biofilms nearly quadrupled, reflecting increasingly widespread interest.

Many classic microbiological studies relied heavily upon the study of planktonic cells because of the capability and convenience to perform experiments with suspended bacterial culture. As microbiologists realized that the majority of microbes on earth are found in structured biofilm ecosystems and not in the planktonic form [121], they began to realize the importance of biofilm in the etiology of device-associated infections. In colonized medical devices, bacterial life is a dynamic process in which cells grow in biofilm and may be shed into the environment to colonize other surfaces downstream of the first. Biofilm formation on medical devices comprises a number of physical, biological, and chemical processes—protein fouling, macromolecule adsorption and transportation, cell–material interaction, quorum sensing, and interaction with mammalian cells and immune system. It is important to understand the total process as well as the relationships between each stage of the cycle. Each stage in this process has a unique potential to play a role in medical device failure or patient harm [5].

The composition of biofilm is significantly different from planktonic organisms and may exacerbate the ability of a microbe to cause device failure and patient harm. The extracellular matrix (ECM) is composed of polysaccharides, proteins, nucleic acids, and other biomolecules. Water (97%), ions, soluble low- and high-molecular mass products, and cells are trapped in this matrix [122]. In addition to the physical and chemical protection afforded to cells living in biofilm, the potential for emergence of drug-resistant organisms may also be increased. The close association of bacteria in biofilms increases the potential for sharing genetic information that encodes for antimicrobial resistance. Even the structure of multi-species biofilms seems to be optimized for synergistic metabolism [123]. Bacteria living in a thick biofilm have a reduced metabolic rate (long stationary phase), which reduces the effectiveness of common antibiotic mechanisms and enables persister cells.

As our understanding of the importance of biofilm has increased, there is now a shift to studying how biofilm affects the clinical path of infections [28]. The role of biofilm in medical device-associated infections is difficult to study in vivo because it is microscopic and internalized. Non-specific inflammatory markers cannot distinguish between infections caused by planktonic cells and biofilm infections [124, 125]. Explants [130] of samples are difficult to study and may have false negative or false positive results [126]. In the past, swabs and samples that yielded negative results were considered clean. But now we are learning that these techniques do not always detect biofilm colonization which can cause long-term low-level sequelae [127]. Colonized devices do not always meet the definition of "infection" because they don't have the clinical signs and symptoms of an infection [128]. Yet studies are increasingly showing examples of how the presence of biofilm may impact aspects of device function directly (such as by blockage) or indirectly (by secondary interactions such as inflammatory processes). Chronic, subclinical infection due to biofilm has also been suggested as playing a possible role in changes to the immune system [136–139].

A lot of the information that we have on biofilm comes from the areas of wound care, where biofilm is externalized, as well as from studies of explanted devices and anecdotal reports of biofilm observed in surgery. We also see the inevitable development of biofilm in indwelling devices such as urinary catheters and endotracheal tubes. Despite the difficulty of detecting and measuring biofilm on many implanted devices, what we do know from clinical treatment of external biofilms (e.g., diabetic foot ulcers) is that they are extremely resistant to drugs and even physical removal and present an expensive and protracted battle that is often life-threatening for those with comorbidities. This paradigm shift in the understanding of biofilm's clinical role portends a need to think differently about the role of antimicrobial/anti-biofilm technologies in medical devices.

2.4.2 Impact of Paradigm Shift on Regulatory Science

The biofilm paradigm shift may have implications for medical device safety and performance. However, this shift is only now being integrated into microbiological studies in general. Some of the areas in biofilms and anti-biofilm technologies such as antimicrobial coatings needing further scientific research and development are discussed below.

2.4.2.1 Standardized Terminology

The definitions and claims involved in biofilms and anti-biofilm technology need to be generally agreed upon by all of the parties involved—industry, regulators, trade groups, and standards groups. Biofilm, from the very beginning, is more challenging to define than planktonic life because of the dynamic process that the term represents. The late Dr. Bill Costerton, one of the pioneers of biofilm research, defines

biofilms in *The Biofilm Primer* as "self-assembling multicellular communities that behave differently from their free floating (planktonic) counterparts" [22]. While this captures one stage of the biofilm life cycle, other stages such as adhesion, colonization, and dispersion are also an important part of the process. Many questions need to be clarified in the change from a planktonic to biofilm paradigm. For the purpose of quantifying antimicrobial effectiveness, planktonic microbiology was mainly concerned with counting bacteria and log reductions. The dynamic biofilm life cycle introduces nuanced and complex interrelated phenomena that challenge conventional scientific understanding. For example, when does adhesion become colonization? When does colonization become biofilm? What does it mean to "eradicate" biofilm—is it removing just the bacteria, part of the ECM or all traces of organic material? Metrics (e.g., how long, how many, how much) need to be developed for many of the terms surrounding medical device biofilms.

2.4.2.2 Performance Goals

It is important to understand what performance goals are associated with anti-biofilm technologies. Performance testing of planktonic-targeted antimicrobials typically involves minimal inhibitory concentration (MIC) or log reduction of colony forming units. But there are additional goals that have been targeted for anti-biofilm medical device technologies. Some examples of goals noted in the literature are:

- "inhibit bacterial adhesion/colonization" [36, 138]
- "prevent biofilm formation" [129]
- "control of bacterial biofilm growth" [130]
- "penetrate and kill bacteria in the biofilm" [131]
- "reduce biofilm viability" [114]
- "degradation of biofilm matrix components" [132]
- "removing medically important biofilms" [133]

We have underlined the main action verb in each of these statements because they are ordered from early stage (inhibition) to post-biofilm formation (removal). This shows how the dynamic life cycle of bacteria becomes much more important in performance goals associated with biofilms vs. goals associated with planktonic bacteria. There are still many questions associated with understanding these goals. The tremendous diversity of anatomic locations and uses of medical products means that the claims may not be "one size fits all." For example, what if an anti-biofilm technology on a long-term catheter can reduce biofilm over very short time periods (hours) but makes little difference in the infection incidence over the total period of device use (weeks)? Or what if an anti-biofilm technology works well to prevent biofilm in a wound dressing, but not on a urinary catheter? In some cases, there are questions about whether achieving the performance indicated by the claim may undermine the overall performance or safety of the product. For example, what if an antimicrobial coating on a material placed in the oral environment kills harmful bacteria but also kills commensal, nonpathogenic organisms that would normally

colonize surfaces and protect the host? Or what if an antimicrobial coating on an orthopedic implant leads to development of drug-resistant organisms?

2.4.2.3 Antimicrobial Mechanisms

With planktonic cells, there is usually significant literature evidence for how antimicrobials exert their effects (such as through receptor–ligand interactions, cell membrane disruption, etc.). However, for anti-biofilm technologies, the mechanism of how a technology works at the molecular scale may span scientific disciplines, making it challenging to study and understand. This is complicated by the fact that many anti-biofilm technologies may also be multimodal, meaning that they exert chemical and/or mechanical action in more than one modality. An example might be an anti-adhesion strategy that is also toxic to bacteria. Experiments examining only adhesion may not take into account the significant effect of bacterial toxicity. As a result, experimental studies of anti-biofilm technologies need to be carefully designed to ensure that they consider multimodal antimicrobial mechanisms. If there is more than one possible modality, they should be carefully controlled to account for confounding variables from the alternate modality.

Because of the potential for anti-biofilm technologies to be multimodal, it is also important to understand how a particular technology leads to a specific performance goal. An example given at a recent workshop illustrated this principle. The specific example involved wound dressing with a silver coating: If the dressing serves as a wound covering and the silver helps prevent bacterial colonization within the wound dressing, the wound dressing is a combination product with a medical device PMOA. If the same product serves to treat an infected wound, it is a combination product with a drug PMOA (Fig. 2.1) [134].

2.4.2.4 Antimicrobial/Anti-Biofilm Test Methods

In Vitro

Test methods found in voluntary consensus standards (not necessarily recognized by FDA) related to antimicrobial technologies fall into the two major categories of either conventional antimicrobial performance (planktonic) or anti-biofilm performance (Table 2.3).

Test methods for planktonic performance are mature and highly differentiated. Often an antimicrobial preservative may be included as part of a medical device material or formulation. The USP 51 preservative test measures the amount of bacterial growth in planktonic solution. Thus, it can be used to measure inhibition of microbial growth in a product. The test is most relevant for testing of unused solutions to show that they remain sterile before use. The Kirby–Bauer test (zone of inhibition, ZOI) is a rapid and simple way to evaluate susceptibility of a specific organism to an antimicrobial [135]. This format is more often used to help guide

Table 2.3 Standard test methods for antimicrobial performance

	Test ID	Test method/ Reactor platform	Measurement process	Limitations
Planktonic	CLSI M02-A11	Anti microbial disk susceptibility	Measure zone of inhibition	Not appropriate for coating performance, doesn't test biofilm
	USP 51	Preservative	Plating directly	Only measure bacteriostatic effectiveness in solution
	CLSI M07-A9	Antimicrobial susceptibility	Plating directly	Doesn't test biofilm
Biofilm	ASTM E2196	Rotating disk	Plating from coupons	High variability
	ASTM E2647	Drip flow reactor	Confocal microscopy or plating from coupons or direct coatings	Low throughput
	ASTM E2562	CDC flow reactor	Plating from coupons	Large volumes
	ASTM E2799	MBEC assay	Plating from plastic pegs	Mostly for liquids
	ASTM VVk32449	Single tube assay	Plating directly	For liquid disinfectants only

The tests in this table are not necessarily recognized by FDA

decision making on therapeutic treatment or in research for rapid screening of potential antimicrobial chemistries. The agar diffusion assay is not necessarily indicative of the effectiveness of an antimicrobial strategy against colonization or biofilm. Another test often used to show antimicrobial susceptibility is MIC testing. Because of the widespread use of this test, it is good for comparing potential antimicrobials in terms of understanding the minimum concentration needed to have an effect. Both ZOI and MIC tests may not be as relevant when an antimicrobial is used in the context of biofilm. These assays were not designed to be used for testing anti-biofilm strategies, but are often used in the literature to assess novel anti-biofilm technology.

There are a number of ASTM standards specifically dealing with biofilm that are sometimes used in the literature to evaluate anti-biofilm technologies for medical devices [136–138]. These assays were mostly developed for the assessment of environmental cleaners and other nonmedical technologies. While these standards may be a good starting point for development of appropriate test methods, in most cases they lack correlation with in vivo outcomes. The endpoint measured in biofilm assays is also an important consideration. The endpoints used in many biofilm assays often have limited correspondence with in vivo performance. Biofilm test standards are usually in the biofilm reactor format or biofilm assay format. Reactor standards tend to focus more on the reproducible creation of a biofilm. These tests

were developed as biofilm reactors and are not meant to simulate real-world in vivo use. Reactor standards often rely on plating and colony counting to evaluate results. While this is the gold standard for quantifying bioburden in planktonic solutions, it is more challenging and burdensome with biofilm. The process of recovering bacteria from biofilm on a surface may introduce error due to poor recovery or failure to grow after extraction. Biofilm assays are more suitable for measuring anti-biofilm performance but are often inappropriate for the context of medical device use. An ideal test method should reflect, first and foremost, how and where a product will be used. The type of microorganism(s), inoculum, composition of artificial soil, temperature, time of exposure, and endpoint measurement should be carefully designed and validated with matching in vivo or clinical data. It is important not to underestimate the impact that biofouling during actual device use might have on performance [139]. Anti-biofilm technologies that are covalently attached to a surface (non-eluting) may be rapidly passivated by proteins which then become a base for colonization. More challenging questions surround the effect of growth media on the test outcome. Although growth media have often been used to achieve reproducible and measurable results, in the real-world scenario, biofilm may have different persistence traits if it develops in different in vivo microenvironment with different types of nutrient sources. Many biofilm ecosystems also involve synergistic polymicrobial metabolism [140]. Testing a monoculture system may not adequately replicate these advantages. Questions have also been raised about differences in the biology (gene regulation and expression) in in vitro test environments versus in vivo [132]. Little has been done to understand how the phenotypic state of biofilm bacteria on a plastic surface in a microplate compares with a biofilm in vivo or even in an infected tissue (ex situ). Finally, the lack of immune response in test environments is a crucial missing link to understanding why some anti-biofilm technologies might be more effective than others in real-world use.

More realistic in vitro models are being developed and have great promise to improve on current tests. A porcine ex vivo model has been used to grow biofilms on the actual tissue that is similar to a wound, creating biofilm that is more robust than what is seen in current testing formats [141]. The model is used to test potential anti-biofilm strategies for infected wounds. When compared with biofilm on plastic plates, the model found that many effective anti-biofilm compounds in plastic plates were not effective to prevent biofilm in the ex vivo model. The results are currently being validated with an animal model. Another important step toward increasing clinical relevance is the use of co-culture models with human immune cells [141, 142]. While it is challenging to keep microbes and cells healthy in the same test environment, doing so can yield unique insights into how materials or solution components affect the ability of the immune system to clear microbial threats. A very different approach to improve reproducibility and throughput of anti-biofilm testing is the use of microfluidic devices [143, 144], including medical-device-on-a-chip (MDoC) [145, 146]. Ultimately, lab-on-a-chip technology may be able to provide a next-generation platform for a simulated test environment with numerous advantages over current strategies.

2.4.3 Animal Studies

Although we do not extensively review animal testing in this chapter, there is a substantial body of literature on animal biofilm models. Although many animal models are complicated by the same lack of biofilm specific diagnostics as clinical assessment, there are a number of promising test methods that take advantage of differences in scale for small animals to achieve real-time monitoring. One of these is OCT imaging that can quantify biofilm on an implanted device [147, 148]. The technique is promising for potential clinical use as well as on devices near the skin's surface, such as breast implants and dermal fillers. Another promising strategy is the use of luminescence to monitor both the bacterial bioburden and the immune response to implants simultaneously in mice [149]. This technique has been used to study how anti-biofilm technology can potentially prevent colonization of implanted materials while retaining biocompatibility. The ability to use this model for extended periods of time (as long as 1 year) allows for potential studies of late-term infections and chronic biofilm that are not achievable with most models. There are also a number of large animal models which are thought to be more comparable with human outcomes due to anatomic and immune system similarities [150, 151].

2.4.4 Clinical Testing

There are an increasing number of studies that correlate in vivo and clinical outcomes with in vitro anti-biofilm testing. Entire books have been written about this burgeoning field, which is too large to discuss in detail in this chapter [6, 152, 153]. It is worth noting that most of these studies are relatively small, and the diversity of animals, tests, and anti-biofilm technologies tested makes it difficult to assemble sufficient metadata. These studies show that in some but not all cases, there is a clear correlation between in vitro testing and clinical outcomes. Typically the bioburden from an in vivo sample is used to compare with in vitro reductions in biofilm or organisms. A few studies have found correlations between in vitro reduction of bioburden and mortality or morbidity. Fully powered clinical trials with anti-biofilm technology are rarely achieved due to the small number of participants that can be enrolled. One strategy to get around this challenge might be to use data from the few clinical trials that have been done to back correlate with test methods being developed for anti-biofilm technology. Another strategy that has been suggested is to initially develop anti-biofilm technologies for revision surgery, where the percentage of device-associated infections is typically much higher. Once a strategy has shown benefit for revisions, it may be possible to collect clinical data over time to support more general use.

2.4.5 *Questions About Safety: Antimicrobial Resistance and Biocompatibility/Toxicology*

An important item often missing in current anti-biofilm technology testing relates to assessing new issues of safety related to these technologies. Drug resistance is one of the chief public health concerns of the twenty-first century [154]. It is not clear if the use of antimicrobials on medical devices, particularly where low concentrations may elute over time, has an impact on the emergence of antibiotic resistant bacteria. To our knowledge, no standard formats have been developed to study if concentration gradients found in these technologies can lead to resistance or cross-resistance. Another concern mentioned above is the preservation of native colonizing microbiota [155]. There is little to no information on how to assess anti-biofilm technology's effect on commensal microbial communities. We know from the case with drug therapy's effect on the healthy colonizers in the gut that altering the microbial ecosystem can have harmful effects on human health. Some researchers have even tried to turn this concern on its head by developing ways to colonize medical devices with healthy, non-virulent microbial strains –"probiotics" that help protect from colonization by pathogenic organisms [156]. Finally, as mentioned above, many anti-biofilm technologies involve new nanotechnology such as nanoparticles or nanotopology that has not been previously been used in the clinic.

2.5 Conclusions

The science of antimicrobial and anti-biofilm technologies on medical devices is a diverse, interdisciplinary field associated with increasing understanding of the role of biofilms in MD-HAI pathogenesis. The public health challenge presented by MD-HAIs is a key driving force for the need to continually improve the regulatory science associated with these technologies. Antimicrobial coatings and anti-biofilm technologies on medical devices in general are not the only weapon in the arsenal of modern medicine. Other technologies should be considered when more appropriate or when combinations can be employed with synergistic potential. Device design and instructions for use should be thoughtfully considered to minimize potential for contamination and biofilm. While there are many novel technologies with demonstrated potential in vitro, it is important to understand the use of these technologies in the context of achieving overall best clinical outcomes with minimal cost. Many of the current challenges in the regulatory science of antimicrobial technologies might be addressed through research on the pathogenesis of device associated infections. Two keys to this research are (1) improved diagnostic methods to identify and quantify biofilm and its role in infections and (2) further development of in vitro methods that are correlated with specific in vivo outcomes. It is important to think about antimicrobial technologies not only from the perspective of microbicidal properties but also their interaction with the host immune system, effect of medical device integration and function, and antimicrobial stewardship (i.e., contribution to or prevention of drug resistance) [157].

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Chapter 3
Characterization of Bacterial Adhesion and Biofilm Formation

Nil Tandogan, Pegah N. Abadian, Bowen Huo, and Edgar D. Goluch

3.1 Introduction

For well over 100 years, researchers have been growing bacteria in test tubes as liquid cultures and on petri dishes as colonies. These two approaches have provided us with a wealth of information; however, they are of limited value for studying bacterial adhesion and biofilm formation. We are now aware of the significant cellular and molecular-level differences between planktonic and adherent cells that necessitate new strategies for generating and characterizing biofilms [1]. Biofilms are a crucial survival mechanism for bacteria. As it is now well known, bacterial cells become more virulent and more resistant to antibiotics when they are inside of a biofilm. Hence, patients with chronic infections are often suspected of having a biofilm that prolongs their recovery.

Further complicating the situation is the fact that the properties of biofilms and the cells inside of them change with time and environmental conditions. For example, cells exposed to certain flow geometries will generate biofilms, known as streamers that extend far away from the attachment point and cause severe problems in pipelines [2, 3]. In other flow profiles, such as ship exteriors, the same species of bacteria can form biofilms that are extremely adherent, increasing the drag force and corroding the surface. Biofilms are not always virulent and destructive. Some bacteria involved in nutrient cycling and biodegradation form biofilms at the air/water interface [4–7]. As you can imagine, many different techniques are required to study all of the various types of biofilms.

We will first discuss each stage of biofilm formation in some detail, and then we will focus on characterization methods and how they are used to analyze various stages of the biofilm life cycle.

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3.2 Biofilm Life Cycle

Biofilms were first analyzed in the 1930s. One of the first biofilm studies was reported by Henrici et al. who described the process of biofilm formation as "The deposit of bacteria becomes apparent in a few days and increases progressively, eventually becoming so thick that individual cells may be distinguished with difficulty. That the cells are actually growing upon the glass is indicated by their occurrence in microcolonies of steadily increasing size. They are fairly firmly adherent to the glass, not removed by washing under a tap." This description highlights the three main components required to identify a biofilm: bacterial cells, an extracellular matrix, and a surface or interface [8]. Other factors, such as environmental conditions and cell-to-cell signaling, affect the properties of the biofilm.

Biofilm formation begins with initial weak interactions between individual bacterial cells and the surface, followed by a strong adhesion step. The cells then begin to excrete various biomolecules, which are collectively referred to as extracellular polymeric substance (EPS), as they grow and divide. The EPS matrix significantly increases the robustness of the biofilm. The biofilm reaches a maximum size and enters a stasis stage during which it is referred to as "mature." In the final stage, cells detach from the biofilm and move to new locations [9, 10]. A schematic of the process is shown in Fig. 3.1.

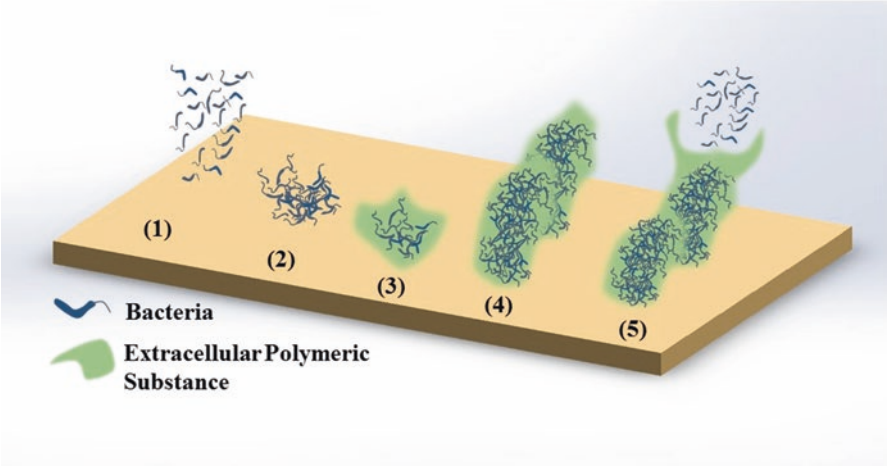

Fig. 3.1 The stages of a biofilm: (*1*) reversible attachment of bacterial cells, (*2*) irreversible attachment of the cells, (*3*) production of extracellular polymeric substance, (*4*) maturation of the biofilm, and (*5*) dispersal of bacterial cells from the biofilm

3.2.1 Adhesion

Biofilm formation starts with the adhesion of cells to a surface. As bacterial cells swim, or move in their environment by Brownian motion, they continuously sense and assess chemical cues through receptors embedded in their membranes. Adhesion to surfaces is advantageous for bacteria, as it provides access to nutrients precipitated on surfaces and protection from predators and environmental hazards [11, 12]. Since they have such a significant impact on survival, the mechanisms bacteria use to initiate adhesion to surfaces have been an important subject of many studies.

Bacterial adhesion to surfaces starts with initial weak attractions, which are reversible and can be broken fairly easily, using, for example, an increase in fluid shear. There are three theories that incorporate chemical interactions and thermodynamic principles to predict the possibility of reversible adhesion to surfaces. Once the weak adhesion is achieved, stronger chemical bonds form and bacteria secrete polymeric substances to strengthen the adhesion. We will now cover the steps in detail.

3.2.1.1 Reversible, Weak Adhesion

The theories that explain the reversible bonding mechanism, which initiates the adhesion process, are the DLVO (Derjaguin, Landau, Verwey, Overbeek) theory of colloid stability, the thermodynamic approach, and the extended DLVO [13]. In this section, we provide an introduction to each of the theories, which are frequently used for analyzing cell adhesion and biofilm formation. More in-depth explanations are provided in the referenced papers [13–16].

3.2.1.2 DLVO Theory

The DLVO theory of colloid stability employs the change in Gibbs free energy between colloid particles and surfaces as a function of distance and has been used to explain the reversible interactions between bacterial cells and surfaces, as bacteria can be approximated as large colloidal particles. The theory quantitatively describes the initial reversible interactions between bacteria and surfaces by summing the attractive hydrophobic Van der Waals bonds and repellent Coulomb double layer interactions, which occur due to charges on bacterial membrane and the surface [17]. DLVO does not take into account steric hindrance or hydrogen bond formation; however, these phenomena occur when the separation distance is less than 1 nm [18]. Adhesive forces between bacteria and surfaces have been measured when the two are as far as 20 nm apart, demonstrating the value of DLVO theory [19]. When the separation distance between bacteria and surface becomes less than 1 nm, steric hindrance and hydrogen bonds start to form, and DLVO theory is no longer applicable [20].

As planktonic bacterial cells swim or randomly move around via Brownian motion, they sense and approach within a few nanometers of the surface. Depending on their distance from the surface, their interaction with it varies. Van der Waals bonds are very weak hydrophobic interactions formed between the cell and the surface. The weakness of the bonds gives flexibility to bacterial cells as they can still exhibit Brownian motion and be detached from the surface when exposed to mild shear stress [18]. Coulomb interactions depend on the amount of charge on the bacterial cell membrane and the surface. Both Gram-positive and Gram-negative bacteria carry negative charges on their membrane [21, 22]. Teichoic acids embedded on the peptidoglycan wall of Gram-positive bacteria give the cells a net negative charge; while the lipopolysaccharides (LPS) on the outer membrane of Gram-negative bacteria are responsible for their net negative surface charge. In nature, surfaces also have negative charges. Hence, the Coulomb interactions are repellent, but the intensity varies with the ionic strength of the electrolyte solution. As a cell approaches a charged surface, an electrical double layer forms between the cell and the surface, which consists of two parallel layers. The counter ions in the aqueous solution are attracted to the charges on the surface, creating the first layer. The second layer is comprised of the free ions in the solution that are attracted to the bacterial membrane. Repulsion occurs when the electrical double layers overlap [23]. As the concentration of counter ions increases and interacts with the negatively charged surface, the electrostatic double layer thickness (the inverse Debye length) on the surface decreases, changing the net attraction to positive and thus promotes bacterial adhesion [20].

Beyond this ionic energy barrier, there is a second energy minimum, and its distance from the surface varies with the ionic strength of the sample solution, as shown in Fig. 3.2 [24]. Bacteria can reach this second energy minimum by using their appendages or by secreting extracellular polymeric substance (EPS) to adhere to the surface reversibly. As the contact radius decreases, the secondary energy minimum is lowered and adhesion can be induced [23]. At low ionic strength, however, the thickness of the electrical double layer increases; thus bacterial EPS or appendages cannot pass through the secondary energy minimum and reach the sur-

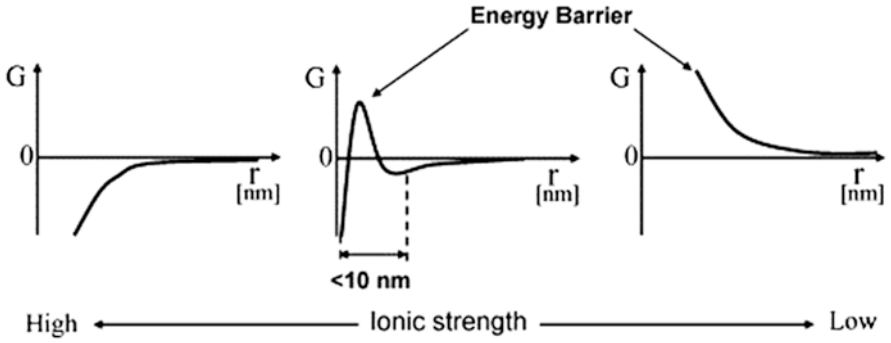

Fig. 3.2 Effect of ionic strength on total interaction energy between a bacterial cell and a surface (Adapted with permission from Ref. [24])

face. The secondary energy minimum is therefore an important part of the early reversible interactions exhibited by bacteria. Redman et al. experimentally demonstrated this phenomenon by flowing *E. coli* through a packed bed column with quartz grains as porous media [25].

Concerning the properties of bacterial cell membranes, DLVO theory does not account for several factors that play a role in the adhesion process. Hydrophobicity is one such factor. Rijnaarts et al. demonstrated that bacteria adhered more to hydrophobic Teflon surfaces than to glass [26]. This parameter is particularly important in wastewater treatment plants. Zita et al. showed that surface hydrophobicity promotes adhesion to sludge flocs in wastewater treatment processes [27]. In another work, the authors used fluorescent microspheres that could attach to the membrane surface of bacteria to measure the hydrophobicity of the bacterial cell surface, and their results indicated that the majority of bacteria showed hydrophilic membrane surface properties [28]. Van Loosdrecht et al. employed a more common approach, contact angle measurement, which we will discuss later in this chapter, to determine cell surface hydrophobicity and reported that the cellular growth phase affects the hydrophobicity of bacterial cells and biofilms [29]. It is important to note that depending on the species, bacterial cells can vary significantly between being hydrophobic or hydrophilic. DLVO theory also does not consider steric interactions between bacterial cells and the surface.

3.2.1.3 Thermodynamic Theory

Thermodynamic theory is another approach to explain the possibility of reversible interactions between bacteria and surfaces. This theory assumes that the interactions are always reversible, so it cannot be used to explain irreversible interactions [24]. The theory uses the Dupré equation, which evaluates the changes in free energy of adhesion using the interfacial free energy in the substrate microorganisms, aqueous phase microorganism, and substrate aqueous phases [23]. The basic thermodynamic principle utilized is that the system will always favor the minimum free energy conditions. This is also the case when determining the initial adhesion behavior of bacteria. The theory employs a very simple premise: bacterial adhesion should only be observed when the change in free energy is negative [13, 30].

Experimentally, surface energy or surface tension can be estimated via contact angle measurement, which is a common technique to predict the wettability of a surface. In one such example, Qu et al. analyzed the adhesion of bacterial species *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococci*, and *Serratia* to different contact lenses using thermodynamic approach. They calculated interfacial free energies from the contact angles [31]. As thermodynamically expected, they noted that bacterial adhesion is greater as the change in interfacial free energies is more negative. However, there are several studies where the thermodynamic approach led to contradictory results, when compared to the experimental results [23, 32]. One limitation of the theory is that bacterial cells may contact the surface only through surface appendages with a very small contact region, which are not accounted for in thermodynamic theory.

3.2.1.4 Extended DLVO

Both classic DLVO theory and the thermodynamic approach fail to explain interactions that could play a bigger role in bacterial adhesion, thus potentially resulting in misleading estimations of bacterial adhesion [33]. The classic DLVO theory only considers Van der Waals and electrostatic forces, and the thermodynamic approach only takes into account electrostatic interactions and interfacial free energies. Interactions, including Lewis acid-base, electron accepting/donating, and osmotic interactions, in some cases, can be the most important factors for determining bacterial adhesion characteristics [25, 34, 35]. Though these interactions require closer proximity to the surface than Van der Waals bonds or electrostatic interactions, they are stronger. For instance, Lewis acid-base interactions are one to two orders of magnitude stronger than electrostatic forces [36]. Van Oss proposed an extended version of DLVO, which estimates the changes in Gibbs energy of adhesion by including these interactions [36].

Sharma et al. evaluated all three approaches and compared them by experimentally testing the adhesion of *Paenibacillus polymyxa* bacteria onto minerals [33]. The results of this study revealed that the adhesion was governed primarily by Lewis acid-base interactions, which are accounted for in extended DLVO theory. Classic DLVO theory partially explained the observed behavior. The thermodynamic approach, however, predicted that no bacterial adhesion would occur.

Although extended DLVO approximations are relatively accurate, each case should be evaluated carefully, as there are cases, such as complex nanoscale structures on bacterial cell surfaces, that can make it difficult to explain the results even with extended DLVO [14]. Ong et al. illustrated the difficulties associated with modeling the bacterial adhesion process using extended DLVO theory when the cell surface contains complex structures [37]. A theory that takes into account all of the variables involved in bacteria adhesion would be quite complicated to derive and use, particularly as many of the factors are difficult to measure, but such a comprehensive theory would be incredibly beneficial to researchers working with bacteria in nearly every basic and applied field.

3.2.2 Surface Characteristics Affect Bacterial Adhesion

Surface characteristics such as roughness, free energy, and hydrophobicity manipulate bacterial adhesion on surfaces. This has led many researchers to focus on altering the surface chemistry of substrates to deter bacterial attachment [38]. The addition of nanoparticles to surfaces and changes in the surface chemistry have been shown to deter bacterial attachment to surfaces [39–41]. Self-assembled monolayers (SAMs) also effectively alter bacterial adhesion properties, either enhancing or preventing them as needed. Liu et al. examined the interaction of *Staphylococcus epidermidis* (*S. epidermidis*) with different surface protein layers: fibronectin and fetal bovine serum (FBS) [15]. Calculation of Gibbs free energy values revealed that while the thermodynamic approach estimated bacterial adhesion to surfaces with

non-protein layers and FBS layers well, its estimation of adhesion strength of bacteria to fibronectin-covered surfaces was not as accurate, which could be due to strong interaction between *S. epidermidis* and fibronectin. Ista et al. tried SAMs terminated with different chemical groups, including hexa(ethylene glycol), methyl, carboxylic acid, and fluorocarbon on solid substrates and tested the attachment behavior of *S. epidermidis* and a marine species *Deleya marina*. While the two species showed different preferences for the hydrophilicity of the surface, the SAM with oligo(ethylene glycol) end group on the surface significantly prevented the attachment of both species [42]. In addition to ethylene glycol functional groups, Ostuni et al. focused on determining different SAMs that hinder the attachment of proteins, bacterial cells, and mammalian cells [43]. Among the SAMs they tested, they concluded that SAMs terminating with –tri(sarcosine), N-acetylpiperazine, and an intramolecular zwitterion prevented the adhesion of *S. aureus* and *S. epidermidis* as comparable as to SAMs ending with ethylene glycol.

3.2.3 Irreversible Adhesion and EPS Production

Once bacterial cells have their initial contact with the surface, they continue to strengthen their attachment with irreversible bonds. In order to do so, they create a matrix of extracellular polymeric substances (EPS), which contains several complex polysaccharides, proteins, nucleic acids, and phospholipids [10]. EPS is the major component of a biofilm and provides numerous advantageous to cells. The structure immobilizes cells onto the surface, provides a robust shield against antibacterial agents, and creates a close network between the cells so that the cells can communicate with each other and exchange nutrients and other important molecules [44]. The structure of the matrix is very dynamic and complex, and its composition and morphology varies significantly between species. The structure can range from flat and smooth to rough and filamentous. Among the most common biofilm shapes are the mushroom-like structure of *P. aeruginosa* and the fruiting shape of *Myxococcus xanthus* [45].

In order to initiate the synthesis of numerous polymer blocks, significant modifications in gene expression occur. Several genes are turned on once the bacterial cells achieve their initial contact with the surface. The density of cells near the surface also affects gene regulation. As we mentioned earlier, bacterial cells continuously communicate with their environment and neighboring cells through self-signaling molecules. When the self-signaling molecules reach a certain threshold concentration, they activate genes that will express quorum sensing molecules (QSMs). QSMs regulate genetic expressions, modulate the synthesis of the EPS matrix, or induce virulence. The mechanism of quorum sensing has been widely studied. It was initially believed that quorum sensing starts only when a critical number of cells are present. However, advances in technology provided the opportunity to examine this phenomenon more closely at the single cell level [46]. Connell et al. created picoliter-sized microcavities and observed the quorum sensing behavior starting from a single cell [47]. Their results suggested that bacterial cells could

start processes for developing antibiotic resistance, which are also a quorum sensing response, with only 150 cells. Cell density therefore becomes the critical factor when the cell number is low.

QSMs are believed to begin playing a role in the regulation of cell function only after cell adhesion takes place [48], thus marking a distinct stage in the biofilm life cycle. To test this theory, wild-type *P. aeruginosa* cells were compared to the ones that had a mutation in a gene that controls cell-to-cell signaling. The results showed that while both cell types attached to the surface, the wild-type cells formed thick biofilms, whereas the mutated ones formed only a thin sheet of growth. Davies et al. investigated changes in the genetic regulation of alginate biosynthesis pathway between planktonic and biofilm *P. aeruginosa* cells. Alginate is one of the well-studied constituents of the EPS matrix, and their results indicated that the genes associated with alginate synthesis were upregulated after planktonic cells attached to a surface [49, 50]. Hence, there is a distinction between cell adhesion and EPS production.

Other factors, including the adhesive appendages of bacterial species and gene expression, also contribute to the adhesion process [51]. The appendages, fimbriae, found in many species in *Enterobacteriaceae* family are specific to mannose groups which are present on human epithelial cells [52]. One example is that *E. coli* has pili with FimH adhesin at the tips that adheres to the mannose groups of oligosaccharides located on the surface of epithelial cells [53–55].

3.2.4 Biofilm Maturation, Disassembly, and Dispersal

Bacterial cells producing EPS eventually create a biofilm that has a set size and shape for a given set of environmental conditions. A biofilm at this stage is referred to as being "mature." A mature biofilm is thought to be at steady state, where a balance is achieved between nutrient transport and cellular activity in the biofilm. The amount of time needed for a mature biofilm to form ranges from several hours to several weeks. Bacterial cells in a biofilm are known to differentiate their functions, with a fraction going dormant [56, 57]. The regulatory mechanism involved in the process of differentiation is not yet well understood, neither are the mechanisms by which the dormant cells are reactivated. When the protein expression of planktonic *P. aeruginosa* cells and the ones at the maturation stage in a biofilm was compared, expression of 50% of the entire proteome was increased sixfold, highlighting the complexity of cell activity at this stage and the potential for heterogeneity in cellular function [58].

The final stage of the biofilm life cycle is called disassembly or dispersal. In this stage, cells in the biofilm produce enzymes that dissolve the EPS, releasing them from the biofilm. Relatively little is known about the mechanisms that regulate biofilm disassembly and dispersal [59]. This knowledge gap for the final two stages of the biofilm life cycle is the result of two limitations. First, the experimental setup for growing large quantities of biofilms that have the reproducible physical and chemical properties is complicated relative to liquid and plate cultures. Second, it

is challenging to analyze what is happening to the biofilms and the cells inside of them as the processes of interest are dynamic, varying in both time and location within the biofilm. The new techniques that are being developed to address these analytical challenges are described in the next section of this chapter.

3.3 Techniques for Making Biofilms

Before we can analyze a biofilm, we must first create it. The specialized techniques, which are required for creating biofilms, are described in the following sub-sections.

3.3.1 Biofilm Reactors

While biofilms grow in a variety of environmental conditions, one technique has become the standard for creating biofilms. The general approach is to seed bacteria on the surface of interest and then flow fluid past it. The cells grow on the surface and form a biofilm. Alternatively, the fluid can contain bacteria and the bacterial attachment and subsequent biofilm formation occur simultaneously while the fluid is flowing.

The Center for Biofilm Engineering (CBE) at Montana State University has created many of the instruments and protocols associated with bulk biofilm production and analysis during the last 25 years. One of the most used instruments is the CDC Biofilm Reactor, which allows various species of biofilms to grow on sample surfaces [60, 61]. The reactor is a vessel with rods that hold the biofilms extended outward into the fluid in the container (Fig. 3.3). The fluid is rotated, resulting in the application of shear to the biofilm, using a stir bar. The reactor is able to grow multiple biofilm samples under high shear stress simultaneously, and the biofilms can be harvested individually for testing. The CDC Biofilm Reactor is most frequently used to analyze biofilm removal. The biofilm is grown on a surface of interest. Then, the surface coated with biofilm is removed from the reactor and exposed to a cleaning solution. The biofilm is then removed from the surface using sonication and the eluent is tested for microbial growth using culture plates. The American Society for Testing and Materials has approved protocol E2562, which is a method for the quantification of *P. aeruginosa* biofilm growth using the CDC Biofilm Reactor [62]. The reactor has also been used to grow biofilms for in vivo implantation in animal studies [63]. The limitation of the reactor design is that it does not allow for in-line analysis of the biofilm.

Like the CDC Biofilm Reactor, the Drip Flow Reactor (DFR) was also developed by the CBE. The DFR grows biofilms under low shear stress conditions by dripping bacteria onto a slide, while the device is held at an angle to cause gravimetric flow [64]. The methods used for the DFR were accepted by the ATSM with the designation E2647. A schematic of the DFR is shown in Fig. 3.4. The DFR can be combined

Fig. 3.3 Photograph of a 1 liter glass CDC Biofilm Reactor (Adapted with permission from Ref. [63])

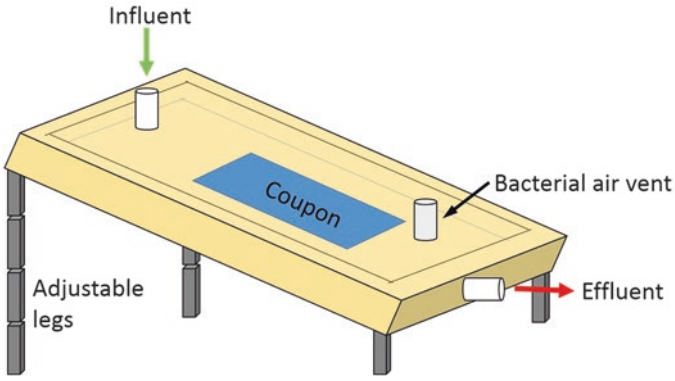

Fig. 3.4 Schematic of a Drip Flow Reactor. The coupon is the material of interest on which the biofilm forms

with some microscopy setups to perform in-line analysis if the surface material (coupon) is transparent.

Liu et al. (2001) used their own annular reactor made of PVC to grow biofilms and observed that the biofilms were able to respond metabolically and physically change under shear stress [65]. In the reactor, the changes in shear stress affected the anabolism and catabolism rate as well as the density and size of the biofilm. However, this setup only showed a macroscopic effect on biofilms because the reactor volume was 4 L, which was much larger than the scale of bacteria. They were able to show that biofilm characteristics changed depending on the shear stress applied. As shear stress increased, the biofilms became smoother and denser [65]. The denser biofilm correlates to the finding of the DFR biofilm reactor, where biofilms grown under

lower shear stress were less tolerant to treatment methods [64]. These results highlight how shear stress not only affects the rate of formation but also the properties of the formed biofilm.

3.3.2 *Modified Microwell Plates*

Microwell, or microtiter, plates are a staple of microbiology research. Microwell plates allow from 6 to 1536 experiments to be performed simultaneously. One of the main benefits of using microwell plates for biofilm experiments is that the plates allow for in situ sample analysis. Foncesa et al. used a microwell plate assay to evaluate biofilm adhesion during antibiotic treatment [66]. Orbital shaking applied a shear force to create dynamic conditions for the assessment of biofilm adhesion. Their experiments began with planktonic bacteria and observed the adhesion and formation of the biofilm. They showed that under dynamic conditions, the antibiotic was more effective at preventing biofilm formation. This study successfully demonstrated that the combination of shear stress and a chemical treatment affected biofilm formation more than each one does individually.

The Calgary Biofilm Device (CBD), shown in Fig. 3.5, is the most important advancement to microwell plates that has been made for biofilm analysis. This device grows biofilms in a 96-well plate format, which can then be tested using standardized molecular and quantitative analysis techniques. The CBD has been used to demonstrate the differences between the removal of bacteria in biofilms and

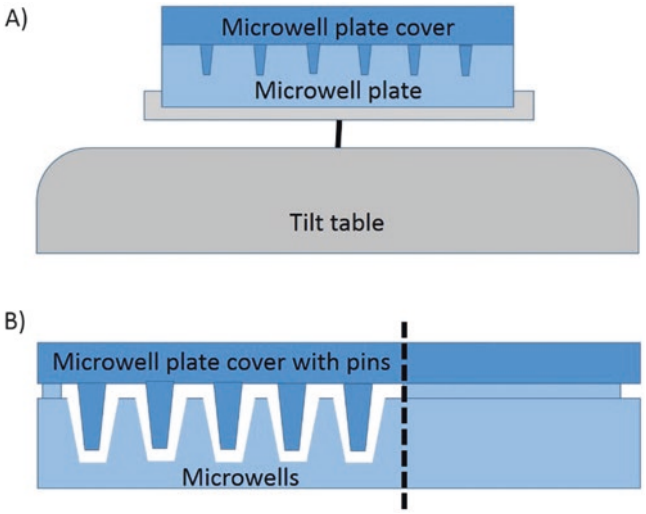

Fig. 3.5 (**a**) A tilt table that creates shear during biofilm formation by rotating the microwell plate, which causes the fluid inside each microwell to move. (**b**) Cutaway view of a Calgary Biofilm Device (CBD) showing the pins sitting inside of the wells of the microwell plate

planktonic bacteria. Ceri et al. (1999) used the Calgary Biofilm Device (CBD) to test the susceptibility of biofilms to antibiotics [67]. They observed that antibiotic concentrations necessary to remove biofilms were 100 to 1000 times higher than concentrations to remove their planktonic counterparts. The CBD can further be combined with a phenotypic microarray to assess metabolic activity through the use of a dye [68]. However, the CBD only grows biofilms in a monolayer and cannot evaluate biofilms grown in multiple layers. Furthermore, the CBD does not combine chemical treatment with application of a shear.

Annular reactors are another method of evaluating biofilm removal, which can be monitored by using laser-based focused beam reflectance measurements. Choi et al. (2003) attempted to establish which detachment process dominates the removal of a biofilm subjected to fluid flow [69]. The use of annular reactors successfully determined that most of the biofilm removal due to shear stress was done through erosion, which is the transfer of small particles from the biofilm into the bulk fluid [69]. This result indicates that at steady state, gradual removal of the biofilm should be expected. While the annular reactors evaluated biofilm removal under shear stress, they were not used to evaluate the efficacy of chemical treatment.

The biofilms grown with both CBDs and DRF devices have been used to evaluate biofilm removal [62, 64]. Once the biofilms were grown in both devices, the sample surfaces were removed from the device and tested. The biofilms that were formed under higher shear stress were more resilient to treatment methods [64]. These results support the work done by Liu et al. (2001), where biofilms formed under higher shear stress were denser than biofilms formed under low shear stress [65]. The studies conducted by Ceri et al. and Choi et al. addressed chemical treatment and shear stress, respectively. By first growing a biofilm and then testing the properties of the biofilm, they ensured that they were testing the biofilm and not planktonic bacteria.

3.4 Techniques for Analyzing Adhesion and Biofilm Properties

We will now describe the techniques used for studying cell adhesion and biofilms. We group them unofficially into two categories: traditional and emerging. Traditional techniques primarily analyze the bulk properties of biofilms and assess the effectiveness of chemicals to kill cells in biofilms. These techniques generally focus on biofilms found in the outdoor environment and industrial settings. As the medical community became aware of biofilms and their role in human disease, a need has emerged for high-throughput analysis and techniques that measure the mechanisms involved in the biofilm life cycle. New high-tech approaches, such as microfluidics, label-free, and real-time sensors, are required to meet these requirements. Methods for high-throughput and mechanistic analysis are still primarily in the development stages and have not yet been adopted by industry or regulatory agencies, hence the “emerging” nomenclature.

3.4.1 Contact Angle Measurements

One of the first and simplest techniques for evaluating biofilm and surface properties is contact angle measurement. The contact angle is the angle between liquid-vapor and liquid-solid interfaces of the liquid-solid-vapor system, which can then be inserted into Young's equation to thermodynamically determine interfacial energies [70]. The wettability of the surface reveals information about its hydrophobicity [71]. Contact angles are measured by placing a drop of water on the surface of interest, which can be either the surface on which the bacterial cells will attach or on top of the biofilm after it has formed on the surface. The angle that the drop makes with the surface on the inside of the drop is measured optically as shown in Fig. 3.6. The angles above 90° indicate a non-wetting or a hydrophobic surface, whereas angles below 90° show wetting or hydrophilic surfaces [72]. In one such example, Wang et al. evaluated the hydrophobicity of *P. aeruginosa* PAO1, *Pseudomonas putida*, and *E. coli* by measuring the contact angles of DI water, ethylene glycol, and methylene iodide on bacterial lawn with a Rame-Hart Goniometer, and results elucidated that PAO1 is more hydrophobic than the other two strains [14]. It is worth noting that there are concerns over the accuracy of contact angle measurements for bacterial hydrophobicity, due to the experimental challenges, including the dehydration of the bacterial lawn and the number of bacterial layers needed for the measurements [73].

Two types of contact angles can be measured: static contact angle and dynamic contact angle. Static contact angle is the angle obtained when the liquid droplet is still on the surface, and the boundary of liquid–solid–vapor phases is stagnant, whereas the dynamic contact angle is measured when the three-phase boundary is changing. This could be either due to the change in volume of the droplet or the tilting of the liquid droplet. As a result of this change, the formed contact angles are called the receding and the advancing angles, and the contact angle lies between these two angles.

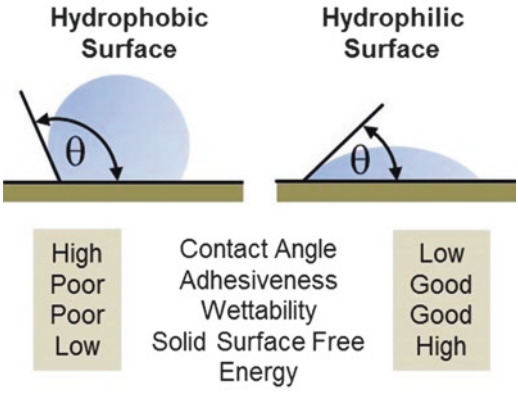

Fig. 3.6 Contact angle measurements provide information about the hydrophobicity of clean surfaces and biofilms

3.4.2 Microscopy Techniques

Different microscopy techniques have been implemented to visualize and detect biofilms and bacterial cells. Selection of the right microscopy technique varies with the experimental setting and the level of information needed from the sample, since each technique provides different details depending on the magnification and resolution limitations of the microscopes. The following sections highlight the most widely applied microscopy techniques on biofilm studies.

3.4.3 Confocal Scanning Laser Microscopy (CLSM)

Several studies incorporate confocal scanning laser microscopy (CLSM) to visualize biofilms and to understand more about the EPS structure as it offers high-resolution images by optically sectioning the specimen into planes and scanning one plane at a time with laser illumination [74]. The specimen can be imaged without chemical fixation or dehydration, which makes this technique stand out from other methods with more destructive sample preparation steps. The sections are combined to construct a 3D model to obtain additional information from the sample, which is not possible with conventional fluorescence microscopy [75]. An example of a biofilm formed inside of a microfluidic channel that is imaged with CLSM is shown in Fig. 3.7.

Several groups have used confocal microscopy to construct three-dimensional images of a biofilm [76, 77]. Using CLSM, Lawrence et al. identified and compared the bacterial and EPS regions of biofilms among different species [78]. The biofilms they measured were hydrated and cells only account for 2–23% of the biofilm volume.

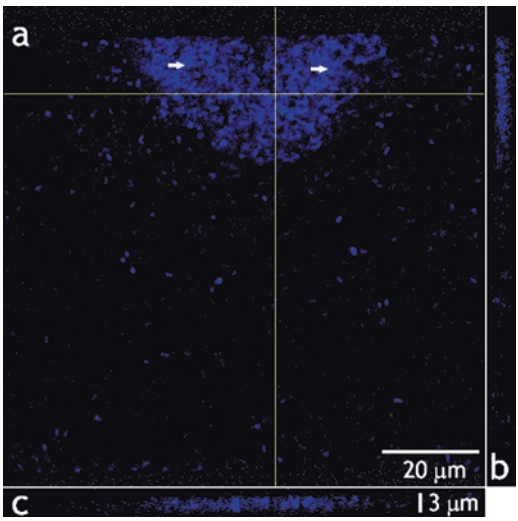

Fig. 3.7 Confocal images of a biofilm inside of a microfluidic channel: (**a**) 3D projection, (**b**) yz cross section and (**c**) xy cross section. The cross sections were taken at the respective *yellow lines*. The *white arrows* indicate areas where bacterial cells are on top of each other

Caldwell et al. pioneered the use of CLSM for imaging bacterial cells and biofilms in the 1990s [79, 80]. The use of CLSM was followed by several other researchers, including Wood et al. illustrating of the structure of intact biofilm from human dental plaque [81], Kim et al. studying the effectiveness of antimicrobials on biofilm [82], and many more [83, 84]. One of the drawbacks of this technique, however, is the ability to damage cells with the high intensity of the laser beam. The complexity and cost of confocal systems have also limited their use thus far.

3.4.4 Molecular Methods

There are numerous molecular techniques that can be coupled with microscopy to learn about how individual cells function in biofilms. Commonly used techniques include gene chips, fluorescent tagging, and fusion proteins. We will not go into the details of these techniques here, as they are applicable to all cellular analysis and generally do not require modification for bacterial research.

3.4.5 Atomic Force Microscopy (AFM)

AFM is a scanning probe technique that provides information about the surface properties of a sample, including topography, roughness, and height at sub-nanometer scale resolution. For bacterial cells and biofilms, it is a great method for determining membrane structure, stiffness, adhesion characteristics, and even observing cellular growth and division in 3D (Fig. 3.8) [85–88]. A major advantage of AFM is that it requires minimal sample preparation such as no chemical treatments to fix or dehydrate cells as it runs at atmospheric conditions. Thus it elucidates a more realistic profile regarding biofilms and allows real-time imaging of live cells at very high resolution. Nevertheless, it is often necessary to use linkers such

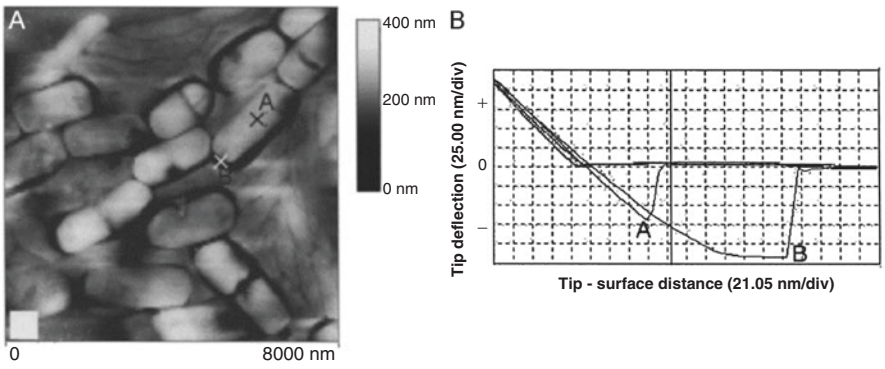

Fig. 3.8 (**a**) AFM image of a developing biofilm of sulfate-reducing bacteria, (**b**) force–distance curves at locations *A* and *B* (Adapted from Ref. [86])

as poly L-lysine to immobilize cells, particularly the motile cells, to surfaces, or porous membranes to trap cells. Information is gathered by detecting the oscillations of a cantilever as the surface topography changes. On the tip of a cantilever, there is a sharp probe and as it scans the surface of a sample, the forces between the probe and the sample lead to oscillations on the cantilever. These oscillations are measured with photodiodes by detecting the movement of a laser beam that is reflected off of the cantilever [89].

Information about the sample can be collected using different AFM modes: contact mode and tapping mode. Contact mode is the most extensively used mode to analyze bacterial cells and biofilms. As the name implies, the probe contacts the specimen while it scans the surface. The interaction between the specimen and the probe, however, causes drag forces, which can alter or damage the specimen [90]. Tapping mode is an alternative mode that scans the surface by briefly contacting the specimen at a very high frequency. The brief contact avoids the drag forces between the probe and the specimen and minimizes damage to the sample [87].

One example application of AFM is a series of studies of composite materials used in dental procedures to investigate their interaction with bacteria [91–94]. The effect of surface roughness on the adhesion of *Streptococci* onto dental composite resins was investigated in a study by changing the roughness of the composite resins [95]. Not only is the roughness of the surface determined using AFM, but the strength of the adhesion of bacterial cells onto the surface can also be measuring with AFM. Analysis of the adhesion forces showed that the roughness of the surface increases the adhesion, and the adhesion forces vary with bacterial species.

Another study focused on the adhesion of *P. aeruginosa* on fungi *Candida albicans* (*C. albicans*) [96]. Authors analyzed the surface thermodynamics, tested surface interactions with AFM, and concluded that the mannoprotein layer expressed on *C. albicans* allows initial acid–base and Van der Waals interactions with *P. aeruginosa*, but that quorum sensing molecules also play a crucial role in the attachment of *P. aeruginosa*. Differences in surface properties of bacteria vary the strength of adhesion on surfaces. Simoni et al. stated that the heterogeneity of LPS distribution in bacterial populations changes the adhesion characteristics [19].

When the information obtained from CLSM and AFM images is compared, CLSM gives lower resolution due to the limitations of light diffraction in optical microscopes whereas AFM can detect sub-nanometer size differences by the oscillations on the probe. It is important to note that AFM can produce artifacts on images. Depending on the shape of the tip and the height of the cells, it has been observed that the side of the probe can contact the cell and lead to misleading images. Thus the selection of the right tip shape to image bacteria is very crucial for accurate results [97].

3.4.6 Scanning Electron Microscopy (SEM)

Scanning electron microscopy is another effective imaging tool that is used to elucidate biofilm structure and morphology [98]. Figure 3.9 shows a scanning electron micrograph of a *P. aeruginosa* biofilm formed on a polymer surface. An

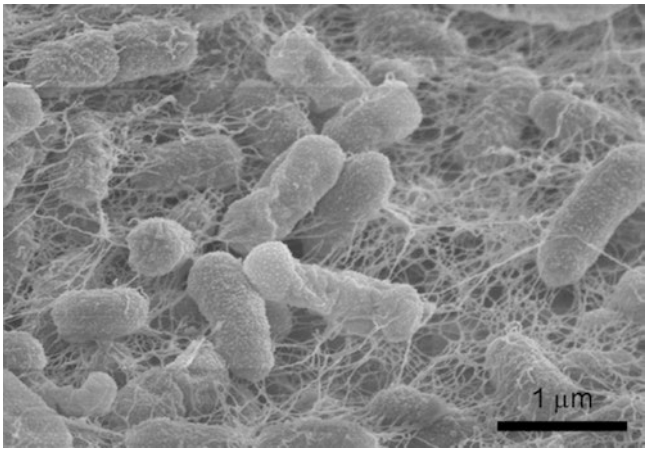

Fig. 3.9 SEM image of a robust *P. aeruginosa* biofilm formed on a polydimethylsiloxane surface

interesting study was conducted by Wang and Libera (2013), which showed that biofilm formation can be inhibited by patterning the surface of a device. They took scanning electron micrographs (SEM) of patterned surfaces, which showed that when the size of the patches was smaller than 1 μm, the bacteria had trouble adhering to the surface [99]. They argued that when the *Staphylococcus aureus* tried to adhere to the surface, they were bent and curved by the pattern, which created a morphology that was energetically unfavorable for adhesion. The SEM images from the study showed that the patterned surfaces prevented adhesion and proliferation by showing groups of bacteria in biofilms on unpatterned surfaces, while there was a noticeable decrease in bacteria on the patterned surfaces. Their result suggests that biofilm formation can be reduced simply by changing the geometry of a surface. One major disadvantage of SEM imaging is the destructive sample preparation steps, which include the use of chemicals to fix and dehydrate the biofilm. The surface must also be conductive, which requires the deposition of a metal or other conductive coating. The preparation of the sample for SEM imaging can potentially alter the biofilm structure and bacterial morphology, so care must be taken during the process.

An alternative scanning electron microscopy has been introduced to improve the analysis of environmental species. Unlike SEM, environmental scanning electron microscopy (ESEM) runs at lower vacuum conditions, and the humidity can be controlled inside the chamber, which enables the maintenance of the sample properties in their original state [100, 101]. Though the resolution of ESEM is lower than SEM and the samples still must be coated with a conductive material, this technique is a very promising advancement for observing biofilm and the EPS structure [102].

3.4.7 Optical Tweezers

Optical tweezers are a scientific instrument that is used extensively to trap and manipulate single cells by focusing optical forces generated by photons in a laser beam to a certain location in a fluidic solution [103]. The cells can be inside of a channel as long as it is transparent. The refractive index mismatch between the cell and surrounding solution allows users to hold and move the cells. The light beam is focused by directing it through a microscope objective. The narrowest point of the focused beam contains a very strong electric field gradient. Dielectric particles, such as cells and proteins, are attracted along the gradient to the region of strongest electric field, which is located at the center of the beam.

Ashkin and Dziedzic demonstrated the ability to trap and move *E. coli* cells using optical tweezers for the first time in 1987 [104]. Other studies have utilized this method to analyze the adhesion forces between bacterial cells and surfaces [105]. Liang et al. implemented optical tweezers to orient *E. coli* cells and experimentally measure the forces required to detach the cells' pili from mannose groups on surfaces [54].

3.4.8 Microfluidics for Fluid Shear Stress Studies

Microfluidic technology has become a powerful tool for studying biofilms and bacterial cells. These miniaturized systems allow the observation of cells at single cell level by confining them to micrometer to sub-micrometer dimensions. There is an extensive body of literature describing the use of microfluidic devices to study biofilm formation along with excellent summaries of the topic [106]. Of particular relevance to biofilms is the use of microfluidics for shear stress analysis. Shear stress is known to remove biofilms, but recent research indicated that the relationship is likely much more complex. Lecuyer et al. showed that at early stages of adhesion, shear stress can increase the residence time of bacteria on the surface, inducing their attachment [107]. Thomas et al. elucidated that the attachment strength of *E. coli* cells to guinea pig erythrocytes increased tenfold upon exposure to shear stress [108]. Depending on cellular motility and the shear rate, shear stress has a trapping effect on cells, which can promote cell adhesion. Rusconi et al. highlighted that lower shear stress promotes cell accumulation around surface regions and induces biofilm formation [109]. Their experiments with *P. aeruginosa* PA14 demonstrated that the increase in shear stress led more surface attachment. This phenomenon becomes very critical in chronic infections seen with patients using medical devices such as catheters that incorporate fluid flow, which can induce this type of bacterial behavior. Another example of the shear stress effect on biofilm formation is seen with streamer bacteria. It is now known that bacteria form filamentous biofilms under certain flow conditions. To study this, a group of researchers designed a microfluidic device that contains zigzag-shaped channels to create vortices. At these

hydrodynamic regions, they observed the initial accumulation of polymeric substances and then the formation streamer biofilms around these regions [2, 3].

Park et al. (2011) used a polydimethylsiloxane (PDMS)-based microfluidic device to grow biofilms and assess the biofilm formation under a fluid shear. In the device, biofilms were formed by flowing bacteria into the device at varying shear stresses. Biofilm growth in the microfluidic devices was affected by the shear stress applied by the fluid containing the bacteria being deposited on the surface [110]. The study concluded that the time required to form a biofilm decreased as the velocity increased up to a threshold value, above which there was a sufficient shear force exerted such that the dissociation of the bacteria from the surface outweighed the increase in number of bacteria that reach the surface. They found that any shear stress applied in the device above 0.17 dyn/cm^2 (0.00017 mbar) would remove more bacteria than the flow added. This study demonstrated the mechanics of biofilm adhesion in fluid flow.

3.4.9 Raman Microscopy

Raman microscopy is increasingly being utilized to map the spatial distribution of chemicals in biofilms. Raman microscopy provides chemical information about the material, via fingerprint spectra, in combination with the high spatial resolution by combining a Raman spectrometer with an optical microscope. Raman spectroscopy is a spectroscopic technique based on inelastic scattering of monochromatic light, usually from a laser source. Inelastic scattering means that the frequency of photons in monochromatic light changes upon interaction with a sample. The light beam from the spectrometer is focused onto the biofilm using the optics in the microscope. The photons are absorbed by the sample and then reemitted. The frequency of the reemitted photons is shifted up or down relative to the original monochromatic frequency, which is known as the Raman effect. This shift provides information about vibrational, rotational, and other low-frequency transitions in molecules. The light emitted back from the sample is collected and measured a charge-coupled device (CCD) or photomultiplier tube (PMT). Near-infrared (NIR) lasers are typically used for analyzing biological specimens as they emit lower energy light that is not as destructive to the sample. The main feature of this technique is that it requires little or no sample preparation as water has a very weak Raman signal [111].

Each molecule generates a unique Raman spectrum. Since biofilms contain thousands of different molecules, the complexity of sample does not allow for detailed chemical analysis, but concentration gradients can be mapped by looking at changes in the fingerprints obtained from different locations on the sample surface. This is done quantitatively with principal component analysis. One way to improve selectivity or sensitivity for this technique is to incorporate functionalized nanoparticles into the biofilm matrix that bind to targets of interest [112]. The best results are achieved by adding the nanoparticles during the biofilm formation process so that they can be incorporated uniformly.

3.4.10 Fourier Transform Infrared (FTIR) Spectroscopy

This technique is occasionally utilized to study biofilms. Infrared spectroscopy is performed by shining infrared light on the sample and measuring the absorbance of light in a range of wavelengths from 0.8 to 1000 micrometers. In FTIR, the absorbance information from all of the wavelengths is collected simultaneously to allow the information to be processed faster. Similar to Raman spectroscopy, FTIR instruments can be coupled with a microscope to image a sample surface. However, because of the intense absorption of infrared light by water, the biofilms are generally dried prior to imaging. To improve sensitivity, a special type of FTIR, known as attenuated total reflection (ATR), is generally utilized in these situations. As the name implies, ATR uses total reflection to guide the incident light along the sample surface, so that it can have more interaction with the sample than with the traditional setup, where the light travels through the sample. Using ATR, chemical information can be obtained from the sample even if it remains hydrated. One example of using FTIR spectroscopy was to measure diffusion of drug molecules through a fungal biofilm [113]. The chemical complexity of biofilm samples requires that fingerprinting or principle component analysis techniques be utilized to process the data similarly to Raman spectroscopy [114].

3.4.11 Surface Plasmon Resonance (SPR)

SPR sensors use a relatively simple instrument for measuring changes in biomass on a surface, but the phenomena employed in the measurement are quite complex. The most common instrument configuration has incident light shining on a prism made of high refractive index glass coated with 50 nm of gold [115, 116]. A microfluidic channel is attached over the gold surface to deliver solution to the sensor surface. At a certain angle, referred to as the resonance angle, the photons from the incident light entering the prism are transferred to the free electrons in the metal creating surface plasmon polaritons (SPPs) that extend approximately 200 nm above the surface. In this state, no light is reflected back out from the prism. When biomass, such as bacterial cells or extracellular matrix, attaches to the gold, the water on the surface is displaced, the refractive index is changed, the SPPs cannot form, and the incident light is reflected back out of the prism. The amount of reflected light is measured with a CCD. The intensity of the light is proportional to the amount of biomass on the sensor surface.

SPR has been used to study the adhesion of *P. aeruginosa* on bare and modified gold surfaces [117]. The results of these experiments showed that differences in binding kinetics could be distinguished for different surfaces and strains of cells. SPR is being used to determine the binding mechanisms of bacterial species by testing their adhesion kinetics to various natural and synthetic materials [118, 119]. SPR is being tested in pilot plants to detect biofilm formation on reactor surfaces [120].

3.4.12 Surface Plasmon Resonance Imaging (SPRi)

In a traditional SPR system, the average intensity of the reflected light from the entire surface is measured, and the results show the average refractive index variation of the sample on the entire surface. In SPR imaging, the intensity of the reflected light is analyzed at each position on the sensing surface. The output of this sensor is a grayscale image, which is called difference image and represents the refractive index changes of the dielectric media above the metal film pixel by pixel (Fig. 3.10). The pixel size determines the resolution of the device.

SPRi offers the unique advantages of measuring attachment of molecules onto a surface accompanied by large area imaging (~1 cm^2) [122, 123]. No other technique can provide these two attributes simultaneously, which are vitally important for investigating biofilm formation and removal [124]. The large area is necessary because the simultaneous movement of hundreds or thousands of cells, as well as the insoluble polysaccharides and proteins excreted by the cells, in a biofilm must be tracked during each stage of the biofilm life cycle [125]. Its simplicity, rapid analysis, low cost compared to confocal microscopy, and potential miniaturization [126, 127] make it an ideal technique for studying biofilm formation and decomposition in clinical and industrial environments.

The use of both SPR and SPRi for bacterial analysis is becoming increasingly prevalent [128]. The rapid imaging capabilities of a SPRi system are particularly important for multicellular and bacterial movement investigations. By using chambers and channels that confine cells near the sensor surface, it is possible to observe physical activity inside the chambers, such as cell movement and growth [123]. Even though the cells are much larger than the approximately 200 nm electromagnetic field that extends from the sensor surface, a significant portion of the cell is located within the field and is detected. Abadian et al. [129, 121] exploited this fact

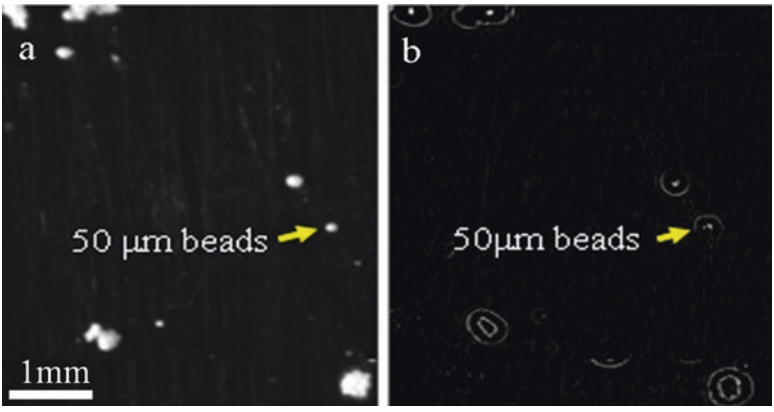

Fig. 3.10 (**a**) A microscope image of 50 µm beads on the prism surface. (**b**) The SPRi difference image from the same surface. The bright spots are where beads attached the surface and changed the refractive index at those locations (Adapted from Ref. [121])

to image the movement, adhesion, and removal of cells in biofilms in real time at the sensor surface. At the end of each experiment, the PDMS made chamber was removed and sensor surfaces were analyzed using fluorescence microscopy and scanning electron microscopy.

3.4.13 Quartz Crystal Microbalance (QCM)

Another approach for analysis of biofilm formation and removal is to use a quartz crystal microbalance. Very simply, quartz crystal microbalance (QCM) sensors measure the mass attached to a surface using changes in vibrational frequency. Applying an alternating current to quartz crystal sandwiched between two electrodes induces oscillations through the crystal in the direction parallel to the electrodes (known as a shear wave). The alternating current can be selected such that it matches the natural frequency of the crystal, thus generating a resonant frequency in the megahertz range. The frequency at which the sensor oscillates changes as mass is added or removed.

Frequency measurements are easily made with high precision; hence, it is easy to measure mass densities on the sensor surface that are as low as 1 μg/cm^2. When working with liquids, it is important to also measure the dissipation factor to help in the analysis and the viscoelastic properties of the liquid affect the sensor performance and the mass density it measures. Figure 3.11 shows a schematic of the sensor.

There is a substantial amount of work demonstrating the ability to use QCM as a biosensor to detect the presence of bacteria [130]; however, we are focused on characterization of biofilms. Nivens et al. measured the rate of biofilm formation for *Pseudomonas cepacia* using a QSM [131]. Castro et al. investigated the growth of *S. epidermidis* biofilms using a QCM sensor [132]. They observed that the dissipation factor, which is equivalent to the resonance bandwidth, increased dramatically for a mutant strain that did not produce extracellular matrix, while wild-type strains forming robust biofilms caused smaller changes. The mutant strain also caused the greatest frequency shift. Reipa et al. coupled QCM with optical detection to simultaneously measure changes in the viscoelastic properties and thickness of the biofilm in real time over the course of several days [133].

More recently, Ollson et al. used a QCM with dissipation (QCM-D) to determine the effect that bacterial appendages have on these types of measurements [134].

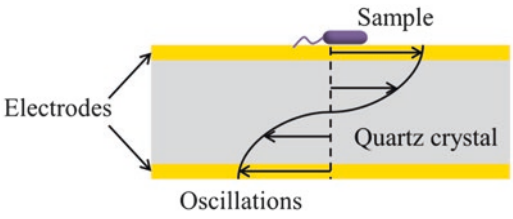

Fig. 3.11 Schematic demonstrating the sensing principle of a QCM

They found that bacteria devoid of surface appendages registered a frequency decrease. Adhesion of bacteria possessing surface appendages yielded either a smaller decrease or even an increase in frequency, despite the fact that more cells adhered. Vanoyan et al. measured changes in bacterial deposition and attachment when the cells were exposed to molecules inhibiting quorum sensing [135].

3.5 Final Remarks

There are many analytical methods available for characterizing bacterial cell adhesion and biofilms, but care has to be taken to select the appropriate approach for the particular property that is being investigated. The dynamic and highly variable properties of biofilms also require that experimental conditions mimic, as precisely as possible, the natural environment where the biofilm is found. Existing techniques are also frequently combined to provide a more accurate representation of the biofilm properties. It is expected that additional techniques will be developed in the future as the importance of biofilms is realized throughout the scientific community.

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Chapter 4
Molecular Approaches for Studying Medical Device-Associated Biofilms: Techniques, Challenges, and Future Prospects

Hongyan Ma and Kristy N. Katzenmeyer-Pleuss

4.1 Introduction

Bacteria dwelling within biofilms, such as medical device-associated biofilms, are remarkably difficult to treat with antibiotics or antimicrobials. The reasons for this are not entirely clear, but recent work has provided some insights [1–3]. Antimicrobials may be readily degraded by specific enzymes secreted by the adherent bacteria, or the chemical agent may fail to penetrate completely into the biofilm due to mass transfer rate limitations [2, 3]. In addition, bacteria within biofilms may be less susceptible to antimicrobial compounds through a variety of defense mechanisms that are not manifested by their planktonic counterparts. For example, biofilm-bound bacteria may enhance the segregational stability and rate of conjugative transfer of plasmid-DNA (often containing antibiotic resistance genes) between bacteria [4–6]. This is important since antibiotic-resistant bacteria, in a biofilm, may transfer these resistance genes more readily to neighboring bacteria [7, 8]. Treatment of biofilms on indwelling medical devices is further complicated by the fact that they are frequently multi-species communities, composed of Gram-positive and/or Gram-negative bacteria. For example, the organisms commonly found on urinary catheters as developing biofilms are *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* [9]. To better engineer and develop strategies to control these medical device-based biofilms, there is considerable interest in developing new types of diagnostic methods to study the architecture of, and metabolism within, such biofilms.

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Conventional studies of biofilm formation on medical devices have been carried out using selective plating techniques with destructive samples [10]. However, this technique is based on cultivation methods that may underestimate the overall extent of the population, since it has been estimated that only 1–5% of the total number of bacteria determined by direct counts are readily culturable [11–14]. The method also only provides cell population measures over the spatially averaged population instead of providing an insight on the local scale of the population. Over the past few decades, molecular techniques, especially coupled with other tools such as microscopy, flow cytometry, etc., have revolutionized the ability to rapidly detect, identify, and evaluate microorganisms in medical device-associated biofilms. These culture-independent approaches provide more powerful analyses to understand microbial diversity and their function within biofilms.

Herein, the advances of molecular tools that are currently available for analysis of the complex structure and development of medical device-associated biofilms are discussed as well as the advantages, limitations, and impact of such techniques on the future study of biofilm adhesion and formation on medical devices.

4.2 Background

Microbial cells (predominantly bacteria) and their extracellular polymers adherent on a substratum are called biofilms [1, 15, 16]. Biofilms can create serious problems by causing significant increases in both frictional and heat transfer resistances between the device and the body [17], by promoting biomaterial deterioration [18], by contaminating medical devices [19–21], and by causing serious, perhaps fatal infections (e.g., cystic fibrosis) [22]. Biofilms are medically important since few diseases are caused by microbes that are planktonic, that is, non-adherent and free-floating. Most microbial infections in the body are caused by bacteria growing within biofilms [23]. Examples include *P. aeruginosa* biofilms in the trachea and lungs of cystic fibrosis patients [24]; *P. aeruginosa* biofilms that form on contact lenses can lead to eye infections known as keratitis [25]; *E. coli* and *P. aeruginosa* form biofilms in the urinary tract, intestine, and within urinary catheters [9]; *Staphylococcus* spp. biofilms colonize various cardiovascular implants and devices [26]; and biofilms are involved in the pathogenesis of dental caries [27], periodontitis [28], dental implant failures [29], denture stomatitis, and oral yeast infections such as candidiasis [30].

Over 65% of hospital-acquired infections are associated with implants or indwelling medical devices, with a case-to-fatality ratio between 5% and 50% [31]. It is estimated that over 5 million medical devices are implanted per annum in the USA alone [32]. Microbial infections have been observed on a variety of medical devices, including prosthetic heart valves, orthopedic implants, intravascular catheters, artificial hearts, left ventricular assist devices, cardiac pacemakers, vascular prostheses, cerebrospinal fluid shunts, urinary catheters, ocular prostheses, contact

lenses, and intrauterine contraceptive devices [32]. An implant is susceptible to surface colonization, with the adherent bacteria being capable of forming biofilm at the implant-tissue interface [33]. Bacterial colonization of medical devices can lead to sepsis or septic/bacterial embolism, potential failure or removal of the device, or death of the patient [34].

Traditional strategies to control medical device-associated biofilm infections are based on the use of compounds that kill or inhibit the growth of freely suspended bacteria. However, "biofilm-bound" bacteria tend to be significantly less responsive to antibiotics and antimicrobial stressors than planktonic organisms of the same species [35–37]. In fact, studies have shown that sublethal doses of antibiotics can even exacerbate biofilm formation [38]. Bacteria embedded within biofilms are protected from many natural host defenses. The structure of a biofilm is such that immune responses may be directed only at those antigens found on the outer surface of the biofilm while antibodies and other serum or salivary proteins may fail to penetrate the biofilm matrix [39]. While antibody- or complement-activated phagocytosis is highly effective in killing planktonic bacteria, studies have shown antibodies are unable to opsonize bacteria in a biofilm. Also, phagocytes are unable to effectively engulf bacteria growing within a complex polysaccharide matrix attached to a solid surface. This causes the phagocyte to release large amounts of pro-inflammatory enzymes and cytokines, leading to inflammation and destruction of nearby tissues [40]. Consequently, systemic antibiotic treatment may fail to clear a biofilm infection and may require removal of the device. Moreover, the risk of antibiotic resistance development is drastically increased under the current standard use of systemic antibiotic treatment of medical device infections [41]. Therefore, developing a means to prevent bacterial colonization and biofilm formation is imperative. Antimicrobial-eluting coatings on medical devices are a promising strategy that has been attempted to prevent implant-associated infection without being toxic to host cells [42, 43].

4.3 Process of Biofilm Formation on Medical Devices

Numerous medical devices cannot tolerate biofilm formation past a certain stage, in which the adherent bacterial cells secrete cell signaling molecules and upregulate the production of virulence factors and extracellular polymers [17]. With this constraint, antimicrobial device coatings have focused on the control strategies of preventing bacteria adhesion and biofilm formation.

A number of physical, biological, and chemical processes are involved in the process of biofilm formation on medical devices in combination with the changing environmental and hydrodynamic conditions within the biofilm [1]. Specifically, for widely used medical devices made from non-fouling biomaterials, the body typically reacts to the device through the following several processes (Fig. 4.1): ① coating of the device surface with a film consisting of proteins and glycoproteins,

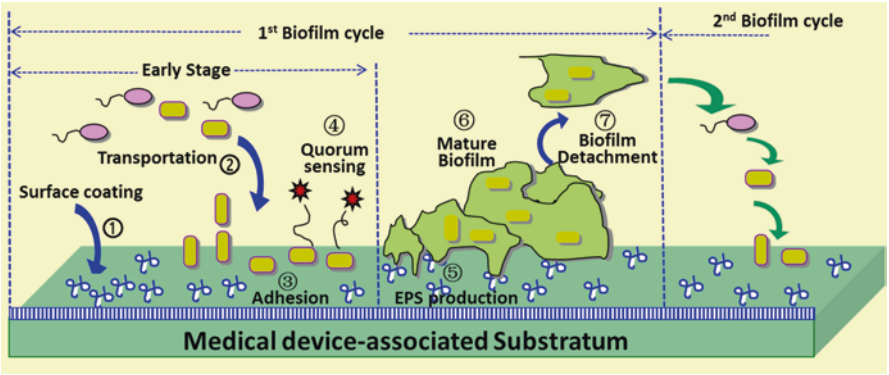

Fig. 4.1 Processes of biofilm formation on non-fouling biomaterials widely used for medical devices

such as fibronectin, fibrinogen, albumin, and immunoglobulins, many of which serve as binding ligands to receptors on colonizing bacteria or incoming mammalian cells. Regardless of the original device material (e.g., metal, polymer), the surface chemistry displayed to incoming cells is altered by this substratum preconditioning layer [1, 44]; ② cells are then transported to the substratum: liquid interface, and then biofilm formation continues with the following steps: ③ cell adhesion on the surface; ④ secretion of cell signaling molecules and subsequent quorum sensing initiation of community-wide phenotypic changes; ⑤ upregulation of virulence factors and secretion of extracellular polymers; and finally, once established, the biofilm continues to mature ⑥ by consuming soluble nutrients and recruiting other bacterial species or mammalian cells. Depending on other cell signals and the culture environment, ⑦ fragments of the biofilm may detach, and bacterial cells can be carried downstream and initiate a new cycle of biofilm formation (potentially leading to metastatic infection).

Before stage ④, accumulation of bacteria on medical devices is often at a low grade, producing indolent and clinically silent infections. However, when the biofilm reaches maturity, past stage ⑦, patients are facing high-grade infections involving the production of toxins and virulence factors [45]. Furthermore, the planktonic cells may start to detach from the surface of the device and spread throughout the body and blood, which may result in inflammatory reactions and further patient symptoms (e.g., fever, persistent local pain, erythema and edema, etc.) [46]. Due to these different stages of biofilm development on medical devices, multiple diagnostic methods have been used to study device-associated infections. However, as stated previously, difficulties are still encountered during the process of diagnosing and testing infections associated with bacterial colonization on medical devices, including low specificity and sensitivity, long processing time, and interpretation errors related to the enormous amount of data generated.

4.4 Biofilm Characterization Techniques

4.4.1 Conventional Approaches

Investigation of bacterial adhesion and biofilm formation on medical devices is currently accomplished by multiple test and diagnostic methods, including conventional culturing, microscopy, and optical studies on destructive or noninvasive samples. The first use of appropriate selective culture media led to the improved success of identifying and characterizing the causative organism at the species level. These studies revealed an initial picture of the predominant culturable species in such medical devices [47–49]. However, these conventional microbiological methods are only a fundamental approach for invasive samples collected from infected medical devices, for example, endotracheal tubes and urinary catheters, which are possible to be removed for culture compared to permanent implants. Frequently, incubation procedures are required for the organisms to grow and form visible colonies, such that at least 24 h are needed for the results to be available. Moreover, much more sophisticated facilities and longer times are required to culture anaerobic organisms collected from devices such as periodontal materials for the diagnosis of oral infection [50]. These bacterial species can take up to 14 days for culture, followed by procedures to test for antibiotic sensitivity test to guide the selection of antibiotics for patient treatment.

In some cases, such as more complex devices (e.g., artificial heart valves, joint replacements), conventional culturing methods have been hampered by the fact that noninvasive removal and collection of samples from the infected medical devices is difficult and even impossible. Alternative noninvasive microscopic methods have been applied to investigate medical device-associated infections on these devices. Imaging of biofilms was initially used by van Leeuwenhoek's to observe dental plaque in the seventeenth century [51]. The development of scanning electron microscopy (SEM) in the last 40 years has allowed imaging of the detailed structures of a wide range of biofilms; generally, such studies have shown that biofilms contain an apparently densely packed three-dimensional structure [52]. The preparation of samples for SEM studies, however, involves extensive dehydration of the samples, whereas natural biofilms usually exist in a fully hydrated state. More recent studies have used other microscopic techniques, in particular confocal laser scanning microscopy (CLSM), which allows the study of biofilms without drying procedures. Such studies have shown biofilms to have a more open architecture [53, 54], in which aggregates of biofilm biomass were shown to be interspersed with water channels of lower density. CLSM has more recently been used to examine biofilms of dental plaque bacteria developed in vitro [55, 56]. To study device-related biofilm infections in a clinical setting, a variety of imaging approaches have been used, including radiography, computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound [57]. Plain-film radiology and ultrasonography are currently considered as the only two imaging approaches that are safe and cheap with the potential to use routinely for screening device-related infections. However,

its usage has been limited for detecting biofilm infections in the late phase since they can only recognize morphological changes in the infected area, when the tissues have been damaged with the formation of erosion at the sites. CT, MRI, and other radiology methods share the same disadvantages as above, but with even more limitations on expense. Further, MRI has limited usage to the scanning of patients with pacemakers, implants, and other devices. Scintigraphy, a diagnostic test in nuclear medicine, has been successfully applied for imaging implant-associated bone infection [58]. As a highly sensitive approach, scintigraphy uses the radioactive tracers to detect small lesions of neutrophils at the infection site. However, this approach has shown low specificity for recognizing septic inflammatory infection. Moreover, the time-consuming labeling procedures and exposure to radiation also limits its usage in specific patients, such as children [59]. In summary, due to limitations in cost, specificity, and availability, none of these imaging methods are used routinely to diagnose medical device-associated infections. These imaging techniques also cannot identify the species of the microorganism causing the development of biofilm infections on devices.

In summary, conventional culture techniques and imaging methods to identify and characterize microorganisms on medical devices have many limitations. A large number of the bacterial organisms associated with device infections are not culturable or may require special laboratory facilities for their cultivation [11, 13, 50]. In addition, diagnostic imaging methods cannot identify bacterial species within biofilms. However, molecular-based diagnostic techniques have overcome the main limitation for detection of no- and low-growth organisms, with the ability to identify microorganisms related with device infections rapidly and specifically.

4.4.2 Advances in Diagnostic Techniques: Molecular Analysis

Medical device-associated biofilms are often complex, multi-species bacterial communities that are colonized on the device surface in a three-dimensional structure with communication pathways (e.g., water channels). As stated earlier, due to the dynamic lifestyle, conventional microbiological techniques are inadequate for the analysis and characterization of biofilms. This has led to the development of alternative techniques to assess device-related biofilms. With the advent of new molecular methods over the past few decades, the ability to detect and identify microbes embedded within biofilms has been revolutionized, even for those organisms that are not culturable or are slow-growing species.

Examples of such molecular analysis methods are the polymerase chain reaction (PCR)-based techniques. These methods include 16S rRNA gene sequencing, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), denaturing high-performance liquid chromatography (DHPLC), and pyrosequencing. Much research is currently underway to further investigate and understand microbial diversity and function within biofilms using these culture-independent molecular approaches. Examples of the molecular

techniques used to study biofilms include application of DNA probes (checkerboard DNA-DNA hybridization) or 16S rRNA probe hybridization (FISH) coupled with microscopy, which initially allowed the detection and enumeration of bacterial species with the developed function for multi-parametric analysis. Other innovative methods, such as flow cytometry (FCM) and its derivative fluorescence-activated cell sorting (FACS) and imaging flow cytometry (IFCM), have also paved the way for new possibilities in biofilm research through gaining a wide range of data on specific proteins related to biofilm function and biochemical measurement.

Herein, an overview of the major molecular techniques currently used in device-related biofilm analysis was provided. By learning more about the genetic and biochemistry of microbial biofilms, better strategies for infection prevention and treatment can be developed that would, in return, provide better healthcare for patients. Table 4.1 summarizes selected molecular-related methods for biofilm monitoring and examination and provides information about the performance and limitations of each method.

4.4.3 PCR-Based Methods

PCR is a powerful diagnostic method to identify the presence of specific genetic sequences related to individual bacterial species. 16S rRNA is highly conserved with unique sequence differences, which allows the recognition and discrimination of different bacterial species [60]. Figure 4.2 shows the original process of 16S rRNA gene sequencing. Fragments of 16S rRNA gene or a housekeeping gene are amplified with PCR for identification of the species present by comparing the sequence derived from the unknown sample to databases.

A culture-independent bacterial survey of Foley urinary catheters obtained from patients was investigated by this PCR-based method. Sequencing was performed using a MegaBACE 1000 (Amersham Biosciences, Piscataway, NJ) automated DNA sequencer. Microorganisms present in catheter biofilms were identified by a BLASTN search of rDNA sequence databases and molecular phylogenetic analysis [104]. The study was not able to provide quantitative results since indifferent amplification of DNA from both live and dead cells cannot be used for enumeration of living cells. Thus, Real Time Quantitative-Reverse Transcription-PCR (qRT-PCR) has been adopted to overcome this problem. QRT-PCR has been applied not only to detect, but also to quantify, a specific microorganism in a biofilm [105, 106]. To detect and quantify viable, but non-culturable (VBNC) staphylococci, in biofilms from central venous catheters (CVC), the qRT-PCR assay targeting bacterial 16S rRNA was utilized. The sequencing results demonstrated that VBNC species were found on the CVC, while negative test results were obtained from routine microbiological assays. The potential role of VBNC bacterial cells on the indwelling medical devices is associated with their metabolic activity, as well as their clinical performance [107]. Numerous studies have used 16S rRNA gene sequencing methods to investigate dental microbiota profiles in a culture-independent manner [61, 62]. One

Table 4.1 Summary of molecular-based techniques for device-related biofilm analysis

Molecular method	Useful purpose	Advantages	Limitations	References
16S rRNA sequencing	Identifies unculturable species Determines gene regulation level	High sensitivity Direct gene measurement	Requires specific DNA fragments Large database needed Low specification at species level	[60–64]
DGGE	Identifies bacterial populations with denaturing gel bands	Lower cost Less intensive labor	Low reproducibility of results Less quantitative results	[65–70]
T-RFLP	Identifies bacterial populations with peaks for fluorescence intensity Determines populations with peak areas	Rapid detection of genetic diversity	Expensive instruments High computation Large database needed	[71–76]
DHPLC	Detects point mutations in DNA	Fast exclusion of DNA sequence variation Reliable results	Not well-optimized High computation	[77–79]
Pyrosequencing	Identifies bacterial species with nucleotide peaks	Rapid detection High-throughput Low cost	Limited sequence length Does not sequence full length of 16S rRNA gene; thus, not suitable for identification	[80–83]
Checkerboard DNA-DNA hybridization	Identifies bacterial species with database comparison	Simultaneous process multiple samples	Labor intensive Limited to database from culturable species	[84–86]
FISH combined with imaging	Identifies bacterial phylogeny In situ monitoring of biofilms	Rapid detection Low cost High reliability Semiquantitative	Hard to detect slow-growing species False results from probes' permeability	[87–92]
FCM, IFCM, FACS coupled with imaging	Enumeration of cell populations Multi-parametric analysis	Rapid detection Quantification of results	High cost High level of expertise to operate No in situ results	[93–96]

(continued)

Table 4.1 (continued)

Molecular method	Useful purpose	Advantages	Limitations	References
ELISA	Identifies biofilm-related surface proteins Identifies released antigens	Early detection Low cost	Specificity lowered by detached planktonic cells from biofilms Lower sensitivity	[97–103]

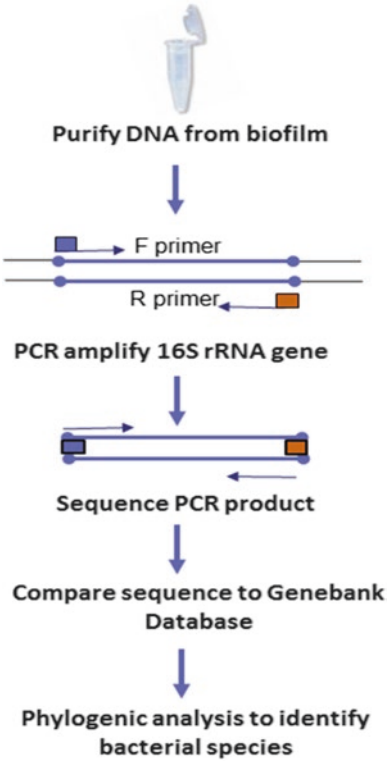

Fig. 4.2 Schematic of 16S rRNA gene sequencing procedure

of the major works in this area is the collection of 16S rRNA gene sequences to develop the Human Oral Microbiome Database, the goal of which is to catalogue all dental-associated bacterial species [108]. CORE, another phylogenetically curated 16S rDNA database of the oral microbiome, was also set up to provide comprehensive and redundant collection of oral bacteria at the genetic level [109]. These databases provide strong support for the discovery of microbiota profiles in the diagnosis of periodontal disease, as well as biofilm profiles from dental implants.

The culture-independent 16S rRNA gene sequencing method has the ability to process a large number of samples and can reveal a wealth of new information about device-associated biofilms with high specificity. For example, compared to

traditional culturing methods, more than 300 new bacterial species specific to particular oral surfaces have been identified [63]. However, a drawback of this method is low resolution in distinguishing those genetically closed and recombinant bacteria at the species level, such as certain streptococci [64]. These related species may share identical 16 s rRNA sequences or have very small differences of less than 0.5% [60]. Other weaknesses of the 16S rRNA gene sequencing technique include the difficulty of extracting DNA from all species, as well as biased results from the nonunique copies of the 16S rRNA genes within one bacterial species. Moreover, due to the higher cost and the tedious work of gene cloning, only low sample numbers can be carried out to reveal the predominant organisms in a sample.

Denaturing gradient gel electrophoresis (DGGE) is a combined method of PCR and electrophoresis-based techniques for biofilm analysis. Various marker genes (typically 16S rRNA) from mixed bacterial communities have been amplified using PCR and then subsequently analyzed and separated on a denaturing electrophoretic gel. The resulting band profiles can be analyzed to reveal differences in the predominant bacterial composition of biofilm samples. Fisher et al. initially reported the use of this technique, in which each band on a DGGE gel theoretically represents a different bacterial population [65]. 16S rRNA PCR-DGGE and species-specific PCR have been used extensively in the analysis of many different microbial communities, both for the study of environmental conditions [110, 111], as well as medically in association with wounds, neonatal endotracheal tubes, corneal ulcers, the gastrointestinal tract, and the oral cavity [66–70].

There are strengths and limitations associated with this non-culture, PCR-based DGGE approach. Some of its strong points include that it is less technically demanding, less labor intensive, and of lower cost than other methods. These advantages facilitate the use of the DGGE technique to analyze bacterial communities simultaneously for multiple samples. Previous studies have shown that DGGE can be used to generate a community profile of the microorganisms associated with medical device biofilms and further reveal the significant microbial diversity in a sample [66–70]. A limitation of DGGE is that most often only descriptive data are obtained rather than quantitative results. In addition, the sensitivity of DGGE is approximately 10^3 cells [112]; thus, only predominant organisms can be identified in a mixed community. More detailed results on bacterial profiles within biofilm communities may be gained if additional sensitive approaches are applied to the same samples.

Terminal restriction fragment length polymorphism (T-RFLP) is another alternative PCR-based molecular approach that can be applied to the study of complex bacterial communities and provide rapid comparison of community structures within biofilms. The method was first reported for use in a study of environmental microbial communities and was later used for the assessment of medical-related systems, such as microbial communities from oral spaces and urinary catheters [71–73]. T-RFLP analysis was performed as described previously [74, 75]. Gene markers, including 16S rRNA, were amplified by PCR using broad-range gene-specific primers labeled with fluorescent probes. Subsequently, the amplified products were digested using restriction endonucleases. The generated individual

terminal fragments of varying lengths were detected by measurements of the fluorescence intensity and analyzed using fragment analysis software. The resulting total number of peaks indicated the number of unique species present in the community, and the area of each peak represented the amount of each species [76].

T-RFLP provides a rapid tool for the identification of diverse microorganisms within a biofilm community. As a promising non-culture-dependent molecular-based approach, T-RFLP analysis is useful for evaluation of the effects of microbial profiles in many medically relevant biofilms. However, despite claims of shorter operating times, this high-throughput technique requires expensive equipment with a high level of operator expertise. Furthermore, large databases and computational power are needed to compare and count the microbial genetic differences in the communities [71].

Recent advances in technology of molecular methods have allowed the development of high-throughput sequencing or next-generation techniques for a rapid expansion of our knowledge on microbial profiles within medical device-associated biofilms. *Denaturing high-performance liquid chromatography (DHPLC)* is a new technology designed to reduce the number of clones necessary for DNA sequencing. It has been used for the identification of microbial pathogens from mixed samples of genitourinary infections [77]. Other applications of this approach involved the direct detection and identification of yeast species both in blood cultures and colonization in the gastrointestinal tract in transplant patients, with potential to apply the technique to microbial detection on the skin and dental surfaces for epidemiological purposes [78, 79]. It is a basic knowledge that the positive correlation was observed between percent GC and retention time in general. Thus, the strains were distinguished from each other based on the retention time of their corresponding amplicons. DHPLC was used to recognize clones that exhibited identical retention times from the same sample, by deleting unknown DNA sequence variations, so that the number of clones necessary for DNA sequencing could be reduced.

It is a fast and reliable method to detect and identify yeast or other bacterial species in biofilm-associated diseases or on medical devices. However, as a fairly new technology, database expansion and optimization are still major hurdles that will need improvement for broad application of the method.

The *pyrosequencing* method, a next-generation sequencing (NGS) technique, is a powerful culture-independent molecular approach based on the detection of pyrophosphate release during DNA synthesis that can be used for massive sequencing of microbial populations in a cost-effective and rapid manner [80, 81]. Figure 4.3 illustrates the pyrosequencing approach of the Roche 454 system (Roche Applied Science, Basel, Switzerland), the first and one of the most widely used NGS system. In general, DNA libraries are constructed from the sample, and then a mixture of the single-stranded DNA template are amplified with emulsion PCR. Sequencing by synthesis occurs in a massively parallel manner, in which two substrates, adenosine 5′ phosphosulfate (APS) and luciferin, are converted to visible light that is detected as a peak by a charge coupled device camera. The height of each peak is proportional to the number of nucleotides incorporated in the sample, such that a gigabyte of nucleotide sequence data can be generated [82]. The massive output data is then

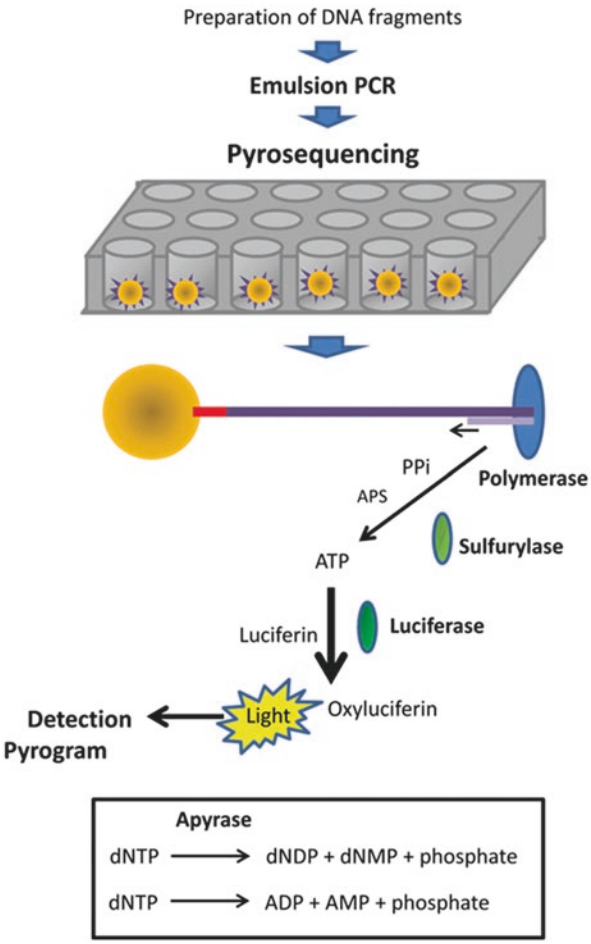

Fig. 4.3 The pyrosequencing approach used by the Roche 454 system [82] (*Abbreviations*: pyrophosphate (*PPi*), adenosine 5′ phosphosulfate (*APS*), adenosine triphosphate (*ATP*), dideoxynucleotides (*dNTP*), ribonucleotide triphosphate (*dNDP*), nucleotide monophosphate (*DNMP*), adenosine pyrophosphate (*ADP*), adenosine monophosphate (AMP))

processed to remove low-quality reads by using the advanced bioinformatics system. After comparing the sequences with available databases, the remaining useful data are classified and separated into operational taxonomic units [83]. The online tools and databases used for the assignment of taxonomy to sequences and functional analysis include BLAST programs from GenBank at the National Center for Biotechnology Information (NCBI), the Ribosomal Database Project (RDP), and the Human Oral Microbiome CORE [109, 113].

With the development of massive computing power and data analysis techniques, many efforts have been made to discover highly efficient NGS platforms. Other NGS platforms include the Illumina platform (Illumina, San Diego, CA, USA) and

SOLiD (Applied Biosystems, Foster City, CA, USA) [114, 115]. These platforms are all based on physical separation of RNA in a flow cell to perform massively parallel sequencing of clonally PCR-amplified products [116–119]. Advanced pyrosequencing systems have been successfully applied to determine biofilm-associated microbial profiles within the human body, such as in chronic wounds [120–122], periodontal implants [123–125], cochlear implants [126], diabetic foot ulcers [121], urinary catheters [127], endotracheal tubes [128], and venous leg ulcers [129]. Many other environments have also been assessed with pyrosequencing, including items such as bedding, surrounds, and furnishings from intensive care units (ICUs) [130], soil [131], and various environmental ecosystems such as hydrothermal vents of a deep marine biosphere [132].

The key advantage of the parallel pyrosequencing method is the ability to obtain massive amounts of sequencing information data with a much lower unit cost; for example, the technique generates data of a magnitude larger than the Sanger sequencing method [133]. As a pyrosequencing approach, the barcoding multiplex technique can be used to identify sequences from different samples in the same run; thus, efficiency of the data processing is increased and the costs are reduced [134–136]. Another strength of the pyrosequencing technique is the avoidance of biases inherent to the cloning procedure. Accurate results can be obtained rapidly to identify microbes and further determine their antibiotic resistance genotype [82]. However, a major weakness in the pyrosequencing method is the sequencing of long length genes. The most used platform for pyrosequencing currently produces an average read length of 400 bp, which results from the ability to sequence the entire 1500 bp 16S rRNA genes. It has been well accepted that more phylogenetic information from near full length sequence reads of 16 s rRNA gene can be acquired than from short length. Indeed, some bacterial species still require sequencing of the whole 16S rRNA to obtain reliable data for identification [137]. However, the increasing efficiency of data generation is likely to render this technology a promising future in the study of microbial biofilms. A more recent pyrosequencing system offers read lengths from 700 bp up to 1 kb [82], which could meet the general requirement for distinguishing a large number of human-associated microorganisms with the initial 500 bp region of the 16S rRNA genes [137].

4.4.4 Checkerboard DNA-DNA Hybridization

The checkerboard DNA-DNA hybridization technique, developed in 1994, has been used extensively as the gold standard to identify bacterial species [84]. The original method relies on the binding of isolated DNA from bacterial samples to a membrane, followed by hybridization with DNA probes specific to numerous species known from available databases. At least 40 bacterial species within oral biofilm samples were able to be detected and enumerated using this method [84]. This culture-independent approach has the advantage of processing multiple samples simultaneously. However, the weaknesses in this method include labor-intensive

procedures, low specificity, and limited recognition of bacterial species. The whole genomic DNA probes for genetically diverse bacterial species are mainly responsible for the low specificity in hybridization. In addition, available databases that relied on for this method were obtained from studies using cultural-dependent conventional methods.

The increasing development of computational power and genome sequencing techniques has rendered a replacement of DNA hybridization with NGS methods. PCR-based sequencing methods, such as pyrosequencing, are now generally applied with computational comparisons to distinguish genetic differences and identify bacterial phylogeny and taxonomy [85]. Before the discovery of 16S rRNA PCR-based sequencing methods, the checkerboard DNA-DNA hybridization approach had been widely applied to periodontal health, such as chronic periodontitis, periodontal disease-associated biofilms [86], oral biofilms on dental materials such as titanium [138], and peri-implants [139].

4.4.5 Molecular-Based Methods Coupled with Microscopy

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has emerged as a versatile reporter gene and in situ cell marker. Advantages, such as species independence and the lack of a requirement for substrates and cofactors, make GFP unique as a reporter gene [140]. GFP has become an especially valuable marker for nondestructively visualizing cells, particularly in microbial biofilms. Use of GFP in combination with imaging techniques, such as CLSM, has led to new insights into biofilm processes [141]. Several GFP variants with excitation and emission properties different from those of the wild-type protein have been developed [142]. One such protein, GFPuv [143] (Clontech, Palo Alto, CA), emits bright green light (maximum at 509 nm) when exposed to UV or blue light (395 or 470 nm). Mutant proteins GFPmut2 and GFPmut3 [144] have emission maxima of 507 and 511 nm, respectively, when excited by blue light (481 and 501 nm, respectively). A multiple-labeling technique based on two GFPs has been used for epi-fluorescent microscopy [145] and for one-photon confocal microscopy [146] for (a) mixed cultures of bacterial cells, where one species contained GFPuv and another species contained GFPmut2 or GFPmut3, and (b) a single species containing both GFPuv and GFPmut2 in the same cell. In all cases, these studies are qualitative, in that location and existence of the various GFP-expressing species (strains) are reported, but exact local concentrations of each microorganism are not provided.

The use of GFP and its variants in combination with CLSM provide powerful tools for investigating biofilm architecture and colonization on devices [147, 148]. The use of CLSM and fluorescent stain combinations [5] has dramatically improved the ability to noninvasively dissect a bacterial biofilm. With normal fluorescence microscopy, one cannot resolve structures within a thick sample because of light emitted and scattered outside the focal plane. By placing small apertures in the light path at points confocal to the focal point within the specimen, almost all of the out-of-focus fluorescence is blocked, allowing detection of just the plane of interest.

By making a series of such optical "slices" through the thickness (*Z*-direction) of the sample, a three-dimensional representation can be generated and manipulated with image-processing software. Coupled with the development of various fluorescent stains, CLSM can provide observations of various cellular and biofilm community processes (e.g., multiple species enumeration, plasmid presence and transfer, bacterial cell viability, and specific gene expression via in situ 16 s rRNA hybridization). However, the penetration depth of CLSM into these samples is limited to around 20–40 μm, depending upon the density of the samples analyzed.

In imaging studies of biofilms, the limitation on penetration depth may be the most critical. Scattering and adsorption of both the excitation and emission light result in a loss of signal as depth increases. CLSM problems can be obviated with two-photon (2PE) excitation microscopy [149]. Instead of using a light source emitting a continuous intensity of a few mW, 2PE uses a laser emitting ultrashort pulse of light with peak intensities per pulse in kW. Gerritsen and De Grauw [150] compared depth of penetration between CLSM versus 2PE microscopy as applied to mixed culture oral bacterial biofilms. Average overall biofilm thickness was approximately 110 μm. In addition, three-dimensional imaging for the 2PE was evaluated by determining the two-photon excitation point spread functions at various depths (0–90 μm) using 220 nm diameter fluorescent latex beads. In a second report, Vroom et al. quantitatively compared CLSM to 2PE microscopy for their respective abilities to noninvasively dissect deep, in vitro bacterial biofilms [51]. Furthermore, pH gradients were determined by fluorescence lifetime imaging with the fluorescent pH-sensitive stain carboxyfluorescein. A defined mixed culture bacterial biofilm was cultivated in a constant depth biofilm system at a fixed depth of approximately 180 μm. 2PE was able to collect clear images with greater resolution at depths four times deeper (140 μm versus 23 μm) than CLSM.

The use of CLSM and 2PE microscopy to study device-associated biofilms is further facilitated by a comprehensive range of molecular-based techniques. *FISH (fluorescence in situ hybridization),* a technique based on rRNA sequences to visualize and quantify different microbial species in situ, has also been widely used for biofilm imaging. In situ hybridization was first introduced into bacteriology in 1988 by using radioactive reporters [87], which was then replaced with fluorescent markers [151]. The development of the FISH technique has revolutionized the study of biofilms with more quantitative and sensitive studies [88, 89]. It also has been coupled with other techniques to discover information on microbial metabolic activity and genetic potential within biofilms [90–92]. Importantly, the FISH technique can provide structural insight of microbial biofilms in detail. For example, FISH has been used to assess the three-dimensional organization of natural biofilms and plaque accumulation in oral medical materials [152, 153]. By using fluorescently labeled 16S rRNA probes, the cells were stained in their native biofilm environment without disturbing their natural structure. The FISH technique provides an inexpensive and straightforward labeling tool to discover different bacterial species in a mixed culture biofilm. In comparison, alternative techniques, such as enzyme-linked immunosorbent assay (ELISA), although presenting the advantages of similar or even higher specificity, are more complex, expensive, and time-consuming [154, 155].

CLSM has been used subsequently to analyze samples and collect in situ images; the FISH technique here provided well-known configurations of typical biofilm structures, including mushroom-like and bacterial cell clusters of coccoid embedded in channels [156]. Furthermore, the combined technique provided an analyzed function to reveal bacterial composition and generate straightforward 2D and 3D images [152]. To directly identify and visualize biofilm bacteria in a species-specific manner, Nistico et al. developed a CLSM-based 16S rRNA FISH protocol, which located biofilm bacteria in the middle ear and upper respiratory tract mucosa [157]. The group found that as a powerful molecular technique, FISH could identify the three-dimensional structure of the biofilm as well as the involved bacterial species, without the involvement of traditional culture techniques. Nistico et al. specifically identified both Gram-positive and Gram-negative bacterial pathogens directly associated with host mucosal epithelia cells by using the species-specific 16S rRNA probes.

In summary, FISH is a cultivation-independent tool based on molecular techniques to identify and characterize microorganisms within biofilms in situ; the technique has the ability to identify bacterial cells' phylogeny and the 3D morphology of biofilms. FISH has been widely applied to the study of bacteria in biofilm communities found in natural habitats as well as those on medical device surfaces and is specifically suitable to identify the presence of non-culturable microorganisms [12, 158, 159]. Despite claims of lower costs, shorter operating times, and reliability, the difficulties of FISH techniques are in the detection of slow-growing bacteria since the low number of ribosomes produces a low fluorescent signal and limits the detection efficiency. In addition, a major hurdle lies in the differences in FISH probes' permeability to the bacterial cells embedded within condensed biofilms. A less aggressive permeable technique may leave some bacterial species unstained and result in false results that do not account for the undetectable species. The development of cryosectional techniques has overcome this problem by immobilizing biofilm sections on glass slides, such that enough signal can be localized by remaining ribosomes even if cells are lysed during the preparation procedure. To avoid errors from the detachment during the fluorescent staining and hybridization procedure, advanced fixation processes have been developed by embedding biofilms in agarose or polyacrylamide gel [160]. In addition to advances in cryosectioning and fixation, new imaging analysis techniques for confocal microscopy of multi-species labeled biofilms [161], the development of brighter fluorochromes, and the new generation of CARD-FISH [89] highlight the importance of the FISH technique for the detection, visualization, and semi-quantification of biofilms associated with medical devices.

4.4.6 Molecular-Based Methods Coupled with FCM and FACS

Developed in the late 1960s, FCM is a powerful analytical tool that utilizes light to count and profile cells and many other parameters from a heterogenous fluid rapidly, accurately, and simply. As a new approach to study medical device-related biofilms, FCM combined with reporter gene technology or fluorescent dyes also

provides culture-independent enumeration and analysis of differences in bacterial species or strains within biofilms. Further, as a derivative of FCM, FACS analysis simultaneously provides data on enumeration and sorts different fluorescently stained bacterial populations within a heterogeneous mixture of bacteria cells.

As a multi-parametric technique, FCM brings together various abilities, such as biologically analyzing the internal and external structures of cells, as well as chemically measuring the amount of specific proteins and biochemical in the cells. It is considered as one of the most useful tool for sophisticated cell analysis, not for the sole purpose of cell counting. Traditional studies of biofilms associated with medical devices have largely relied on various selective plating techniques followed by more specific analysis using PCR or immunological serotyping. These techniques are labor intensive with quite considerable processing time to generate a complete data profile. Several recent studies suggest that FCM could offer some advantages over conventional approaches, including shorter processing times [93, 94, 162].

When combined with phylogenetic and immunological analysis, FCM and its derivative FACS are enabled with new and exciting features to gain a wide range of data based on multi-parametric analysis. These features are especially of interest to investigate biofilms associated with medical devices due to the specific bacterial population heterogeneity within biofilms. To study the heterogeneity of multicellular fungal communities, protein-GFP fusions or promoter-lacZ fusions have been developed to recognize genes that are expressed in different parts of the colony (e.g., the outside versus the inside cell layers) [163, 164]. By using FACS-based sorting, the outside colony layers were fractionated from the cells in the center to gain insight into the contributions of cell heterogeneity to colony and biofilm phenotypes [162]. Plasmid transfer within biofilms, mainly responsible for antibiotic resistance in clinical environments, was analyzed by flow cytometry and FACS [7]. *Pseudomonas putida* KT2442 recipients received a GFP-marked conjugative TOL plasmid from *P. putida* TUM-PP12 donors. Transconjugants were able to be isolated rapidly from mixed populations of biofilms by using FCM coupled with FACS and GFP-based reporter gene technology. The transconjugant cells, sorted from the other populations, subsequently were characterized by cloning and multiplex PCR of conjugatively transferred genes (Fig. 4.4). A previous study also used this approach to evaluate the host range of a natural barley rhizosphere plasmid (GFP-labeled) in indigenous rhizosphere bacteria [13]. It was found that one-third of all transconjugants (bacteria containing plasmid transferred from a *P. putida* donor to indigenous recipient Gram-negative cells of *Proteobacteria*) were Gram-positive bacteria of *Arthrobacter spp.* Such surprising reports indicated the necessity of detecting non-culturable bacteria in this type of study. It also indicated that conjugal gene transfer might be far more efficient than was previously considered [165]. Recently, a combination of FCM cell sorting coupled with pyrosequencing has been used to identify bacteria that are coated with IgA, IgG, or IgM antibodies, with the intention to avoid the sample bias imposed by bacterial culture [166]. In this study, clinical samples (saliva, feces, urine, mucosa, milk, etc.) were analyzed with flow cytometry (to recognize active and Ig-opsonized cells and obtain cell counts) and pyrosequencing (to detect microbial composition). In this way, features of the

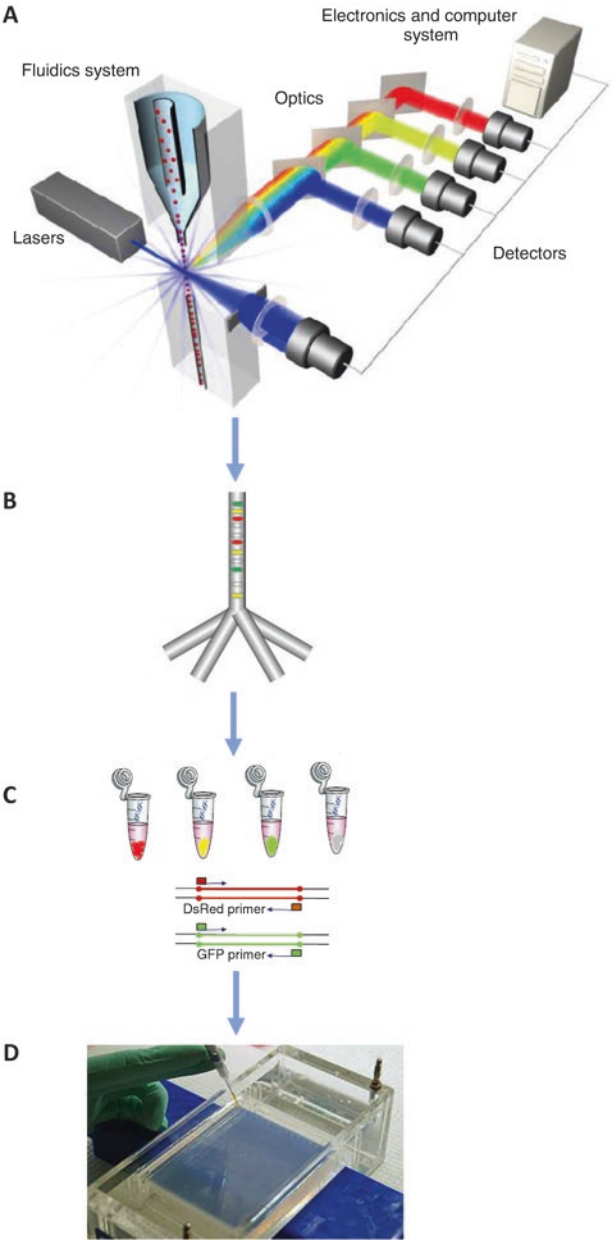

Fig. 4.4 FACS combined with molecular-based PCR to analyze plasmid transfer within biofilms. (**a**) Primary components of FACS systems. (**b**) Populations of the transconjugant (*green*), donor (*yellow*), recipient (*non-color*), and segregational lost (*red*) cells can be detected by flow cytometry and isolated by FACS. Plating assorted cells to different plates and forming single colonies. (**c**) PCR can produce and amplify GFP- and DsRed-containing sequences from assorted transconjugant cells. (**d**) Agarose gel electrophoresis can detect GFP- and DsRed-containing sequences

immune response represented by Ig-opsonized cells can be correlated to microbial composition and density, providing insights of the immune response to microbial infections and suggesting the relationship between potential biomarkers of health with disease conditions. For example, it was revealed that aggregation of microorganisms in biofilms may be less susceptible to antibiotics and partially avoid opsonization by Ig antibodies [166].

The beginning of the era of FCM techniques truly started with the human clinical application for immunological analysis involving mammalian cells. Microbial FCM brings the technique into a new and challenging area since these technologies are highly useful to obtain multi-parametric microbial community profiles within biofilms. However, these microbial FCM and FACS methods for investigating biofilms are not perfect and come with many drawbacks, including cost, the high level of expertise required to process samples and carry out data analysis, biased results from invasive sample collecting procedures, and the potential effects of staining on cell viability or metabolic activities. Moreover, these methods provide FCM profiling and other physiological data over spatially averaged populations instead of providing insights on a local scale. Finally, no morphology information exists for cell subpopulations.

Fortunately, a new and exciting technology, IFCM, has become a commercial reality. The Image Stream X Mark II from Amnis Corp, (Seattle, WA, USA) is one commercially available system.

As an impressive new cutting-edge procedure, IFCM combines FCM with advanced cell imaging techniques; thus, individual cells within a flow stream can be differentiated rapidly based on their shape, integrity, and morphology [95]. Furthermore, cellular responses can be monitored at the genetic level by imaging analysis of cell morphology and the degree of uptake of fluorescent metabolic dyes or expression of GFP reporter genes. These IFCM systems can rapidly provide information for an individual cell in a flow system using various modes, for example, image generating speeds of 2000 images/second are possible [96]. It has been reported that the application of IFCM mainly involves mammalian cells, with the ability to study cell-cell interactions, phagocytosis, apoptosis and autophagy, characterization of tumor cells, and many others [167]. However, a number of papers also show the progression of IFCM to the study of bacterial infection of mammalian cells. Therefore, IFCM technologies have current applications in the characterization and diagnosis of medical device-associated biofilms.

The application of FCM, FACS, and IFCM techniques to the study of medical device-associated biofilms has many advantages over conventional culturing methods. These methods can provide instant output of data related to the enumeration of various cell subpopulations as well as gain a range of data based on multi-parametric analysis, such as recombinant proteins and biochemical production. In this way, potential biomarkers of health and disease can be associated to microbial compositions, thus, revealing the contributions of each biological feature, such as the immune response to the microbial infections and related diseases. With the recent advances in combination FCM and cell imaging techniques, IFCM is leading the way to the development of rapid molecular-based biofilm quantification approaches at the genetic level.

4.4.7 Molecular-Based Methods Combined with Immunological Methods

The use of a combined molecular-based technique with immunological approaches have paved the way for new possibilities in medical device-associated biofilm research through understanding the function and activities of taxa present in the biofilms, rather than just recognizing microbes. Enzyme-linked immunosorbent assays (ELISA) have been performed to obtain early detection and diagnosis of biofilm colonization and infections associated with medical devices [155]. As an additional approach, molecular-based qRT-PCR could provide a quantitative method to detect pathogens in these infections. These combined methods can provide both serological and genetic information on biofilm infections, revealing a new tool that could allow the early detection and treatment of biofilms associated with many medical devices.

The use of ELISA truly started with the discovery of the specific nature of antibodies. In the early 1970s, the production of monoclonal antibodies (mAb), producing the same specific antibody for one cell line, revolutionized the use of the ELISA method in microbiology and biofilm research. "Hybridoma" cell lines generated from the fusion of vaccinated animal spleen cells with myeloma cells can produce antibodies specifically conjugated to different moieties, such as enzymatic or fluorescent molecules. ELISA is performed by coating a solid surface with antigen and then adding specific antibody conjugated with an enzymatic or fluorescent molecule. Later, a substrate may be added that is catalyzed by the enzyme to produce a colored product that can be detected by a spectrophotometer. Besides the basic steps mentioned above, many variations for the ELISA assay have been developed, including both direct ELISA and indirect ELISA. The direct ELISA provides a simple method to detect antigen specifically since only one mAb is used, eliminating the potential crosslink of a secondary antibody. However, the main limitation of this method is the assay sensitivity. The process of labeling may reduce the immunoreactivity of the primary antibody, the only involved antibody without any potential of signal amplification from other antibodies. The Indirect and sandwich ELISA provides a solution to this problem. A wide variety of commercially available labeled secondary antibodies may be used to avoid affecting the immunoreactivity of the primary antibody by enzyme or fluorescent labeling. The increased signal results in higher sensitivity since the primary antibody contains more free epitopes that can be bound by the labeled secondary antibody. While the sensitivity of the method is increased, other drawbacks are the nonspecific binding due to the cross-reactivity from the secondary antibody and a longer processing time that is required for the additional incubation with secondary antibody [168].

ELISA can also be used in the detection of infection-related products. Instead of targeting whole bacterial cells, the method is mainly focused on the evaluation of released proteins, such as inflammatory cytokines due to biofilm infection on site. To evaluate the relation of a biofilm with chronic infection, ELISA and molecular-based PCR are frequently used to quantify the production of cytokines in response

to different stages/levels of implant-associated biofilm infections. For example, the role of osteoblasts participating in the defense mechanisms of implant-associated *Staphylococcus* biofilm infections has been determined by evaluating the expression of pro-inflammatory cytokines, such as IL-8, IL-6, and CCL2, by quantitative RT-PCR and ELISA [97]. Another example is evaluation of the role played by *Staphylococcus aureus* biofilm in sinus inflammation. Expression of the inflammatory cytokine IL-6 has been measured at both the messenger RNA (mRNA) level using quantitative RT-PCR and the protein level using ELISA approaches [98]. The results presented in these studies provide strong evidence for the utility of a combined molecular and immunological method to better understand biofilm infections.

Another common ELISA study has relied on the detection of biofilm-related proteins or cell markers on whole bacterial cells. For example, binding of medically relevant soluble heparin to *Candida albicans* bacterial cells has been evaluated by ELISA [99]. The interaction between heparin, widely used for anticoagulation in central venous catheters, and microbes indicated catheter-associated infection [100]. In another study, multiple methods were used to characterize the *ebp*^{fm} pilus-encoding operon of *Enterococcus faecium*, an important microbe that causes catheter-associated urinary tract infections. Cell surface expression of $EbpC_{fm}$, the putative major pilus subunit protein of the *ebpABC*^{fm} operon, in different growth stages were evaluated by using whole cell ELISA. Flow cytometry was further used as a complimentary method to quantify cell surface expression of $EbpC_{tm}$ [101]. The expression of streptococcal fibronectin adhesin CshA was investigated by whole cell ELISA to determine the biofilm-related dental plaque developed by *Streptococcus sanguis* [102]. These examples are all based on an ELISA approach in which cell surface proteins and cell markers are screened and quantified to better understand their biofilm-related functions. Compared with other methodologies, such as PCR method analysis at the genetic level, the strengths of the ELISA method include the ability of direct protein level determination rather than estimation based on mRNA levels. Moreover, identification of biofilm-related surface protein expression may lead to discovery of their direct relevance in disease intervention. For example, detection of periodontal pathogens in dental plaque has been possible when both ELISA and RT-PCR techniques are applied with a quantification sensitivity of 10^4 and 10^2, respectively [103].

4.5 Implications for Enhanced Molecular Technology (Conclusion)

Molecular-based techniques for investigating biofilms provide innovative insight that is necessary to provide more accurate and detailed results for medical device-related biofilm diagnosis and treatment. Compared with conventional culture methods, molecular techniques have gained considerable interest during the past two

decades. The intriguing potentials involve the development of more rapid and personalized biofilm treatment methods through a better understanding of microbial genetics, pathogenicity, and population structures. Milestones have been achieved in our understanding of both the composition and structure of complex biofilms. Not only can they provide species information present in biofilms, molecular techniques can also help to understand the function and activities of the medical device-associated biofilms. Although culture-based methods still play an essential role in research labs and in the clinical setting for device-associated biofilm evaluation and diagnosis, molecular approaches have been successfully applied to biofilm bacteria to create diagnostic tests that are more sensitive to biofilms.

Herein, the common molecular methods that are currently used in biofilm studies have been summarized. As described previously, these approaches are not perfect and come with some weaknesses, including high cost, time-consuming nature, lower specificity, potential for artificial results, and high requirements for user expertise in instrument operation and sample processing. To obtain more accurate results with non-culturable bacteria growing in multi-species biofilms, it is necessary to simultaneously apply a combination of experimental approaches. To gain insightful information about the bacterial and fungal populations in biofilms on silicone rubber voice prostheses, PCR-DGGE and sequence analysis was used to identify microorganisms. Additionally, the architecture of the mixed biofilms on the implants was obtained by applying FISH with rRNA-targeted oligonucleotide probes and CLSM microscopy [169]. In another study, multiple 16S rRNA analysis methods, including DGGE, T-RFLP, and pyrosequencing were used to examine biofilms isolated from patients' urinary catheters. It was found that better results were obtained when multiple approaches were applied to avoid the high risk of sample contamination during biofilm processing and errors from a limited number of catheter samples [71]. Moreover, it has been shown that FISH can be combined with immunological techniques for rapid detection of various bacterial pathogens in clinical specimens. In a study of the specific detection and differentiation of *Chlamydiae*, the sensitivity of FISH was confirmed by combination with the direct immunofluorescence antibody technique [154].

Despite many big challenges ahead, molecular-based techniques for the analysis of medical device-associated biofilms are likely to become more important in determining taxa present in biofilms as well as help to understand the function and pathogenicity of microbial populations within biofilms. The combined usage of molecular-based experimental approaches with other methods, such as immunological analysis and microscopy, will further broaden its applications in clinically relevant diagnosis and treatment of biofilm infections associated with medical devices. Further advancements in molecular-based techniques will likely focus on more complex analysis of microbial genetics, pathogenicity, structures, and functions of medical device-related biofilms.

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Chapter 5
Implantable Medical Devices Treated with Antimicrobial Agents

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5.1 Introduction

With the rapid progress made in the medical sciences and increasing aging population, thousands of diagnostic and therapeutic implantable medical devices have been developed. According to the Medical Device Guidance document, MEDDEV 2.4/1 Rev.9 for the classification of medical devices [1], an implantable medical device is any device which is intended to be totally introduced into the human body or to replace an epithelial surface or the surface of the eye, by surgical intervention which is intended to remain after the procedure, and any device intended to be partially introduced into the human body through surgical intervention and intended to remain in place after the procedure for at least 30 days is also considered an implantable device. At present, implantable medical devices are being used in many different parts of the body for various applications such as orthopedics, pacemakers, cardiovascular stents, defibrillators, neural prosthetics, drug delivery stents, and long-term central venous catheters such as tunneled hemodialysis catheters. These devices improve the quality of life of the recipients and in certain cases even save lives. However, since implantable devices involve surgical procedures and direct contact with body tissues, there are several complications that can occur. The most common complications include immune system response leading to rejection, device failure, device breakdown or overuse, device migration, material sensitivity, bleeding, blood clots, nerve damage, and infection [2]. Information presented in this

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chapter is mainly focused on the device-associated infections (DAIs) and the strategies taken to address DAIs including the devices treated with antimicrobial agent(s).

5.2 Device-Associated Infections

Presently, one of the most vexing and morbid complications of device therapy is the development of infection. According to World Health Organization (WHO), prolonged and inappropriate use of invasive medical devices is the most common factor responsible for hospital-acquired infections (HAIs) [3]. According to the Multidisciplinary Alliance Against Device-Related Infections (MADRI), at least one-half of all HAI cases are medical device associated. This is primarily due to the fact that medical devices are placed on damaged skin or they are inserted into the body causing injury, which provides an ideal environment for disease-causing organisms. Unlike the human body, device polymers do not have an immune system to fight off microorganisms. As a result, microorganisms colonize the surfaces of the devices, multiply, and develop a biofilm, which is an organized community of bacteria protected by a slime layer. In the biofilm form, microorganisms undergo cellular changes that can make them resistant to antibiotic treatment [4].

There are four categories of infections that account for three-quarters of the HAIs that occur in acute care hospitals. These four categories of infection are the surgical site infections (SSIs), central line-associated bloodstream infections (CLABSIs), ventilator-associated pneumonia (VAP), and catheter-associated urinary tract infections (CAUTIs).

National Healthcare Safety Network (NHSN) at the Centers for Disease Control and Prevention (CDC) summarized the hospital-acquired infections (HAIs) data related to medical devices from the participating hospitals during 2009–2010 [5]. The summary was based on the data collected on CLABSIs, VAPs, CAUTIs, or SSIs that occur in patients staying in various types of patient care location. Overall, 2039 hospitals reported one or more HAIs; 1749 (86%) were general acute care hospitals, and 1143 (56%) had fewer than 200 beds. There were 69,475 HAIs and 81,139 pathogens reported. Eight pathogen groups accounted for about 80% of reported pathogens were as follows: *Staphylococcus aureus* (16%), *Enterococcus* spp. (14%), *Escherichia coli* (12%), coagulase-negative staphylococci (11%), *Candida* spp. (9%), *Klebsiella pneumoniae* (and *Klebsiella oxytoca*; 8%), *Pseudomonas aeruginosa* (8%), and *Enterobacter* spp. (5%). Nearly 20% of pathogens reported from all HAIs were multidrug-resistant phenotypes. These resistant organisms can be extremely difficult to treat and increase the treatment cost significantly. A study showed that the cost of each CLABSI case was $45,814, which increased by 27% to $58,614, when drug-resistant bacteria were involved [6].

The incidence of DAIs is increasing at steady state. Until 2004, the rate of DAIs was constant at approximately 1.5% per year, but then it steadily increased to a rate of 2.5% per year as reported in 2008 [7]. DAI typically occurs during procedures that breach the skin or introduce a foreign object into the body (i.e., catheterization,

intubation, intravenous access, and the use of surgical implants and devices). Many of these devices provide a breeding ground for bacteria and slime-like aggregations of bacteria known as biofilms, which are nearly impossible to kill with conventional antibiotics. Infections do not only occur upon the implantation of a medical device but also during regular surgical interventions. However, implantation of a device followed by a surgical procedure is associated with higher infection rates. For instance, the SSI rate for mastectomy procedure for the removal of one or two breasts due to breast cancer was reported to be 2% overall by the CDC. In a small study, SSI rate increased to 12.4% with immediate placement of a breast implant post mastectomy when compared to infection rates at 4.4–6.2% with more conventional mastectomy interventions [8]. It shows that implantation of a synthetic device is associated with a higher rate of infection.

5.2.1 Infection Prevention Strategies

The cost of these infections is substantial, both in terms of morbidity and financial resources expended. Additionally, there is a significant mortality risk with DAIs, especially with cardiovascular implantable electronic device infections (CIEDs [9], CAUTIs, and CLABSIs) [10]. With 250,000 CLABSIs occurring annually, mortality rate is estimated at 12–25% [11]. A meta-analysis of patients in the intensive care unit found that mortality rates were significantly higher when catheter-related blood stream infection occurred [12]. Each infectious episode significantly increases hospital length of stay, with additional healthcare costs ranging from $4000 to $56,000 per episode [13, 14]. The overall annual direct medical cost of all HAIs to US hospitals ranges from $28.4 to $45 billion [15].

To reduce this huge economic burden of HAIs and to improve the quality of care and patient safety, healthcare institutions are mandated to implement infection prevention programs. Implementation of such programs in several studies has shown dramatic decrease in the rates of nosocomial infections, particularly catheter-related infections in the medical intensive care unit. One such study showed a significant decrease in Foley-related UTIs from 6.23/1000 device days to 0.63/1000 device days. This decrease was still significant when adjusted for device utilization [16]. The CDC has shown the economic benefit of infection control interventions ranging from $5.7 (considering 20% infections preventable) to 31.5 billion (considering 70% infections preventable) [15]. An infection prevention program involves development of a care bundle which is a group of evidence-based practices that improve the quality of care when consistently applied to all patients. Bundles are developed for a range of conditions and disease processes [17–20]. These programs include education and training the staff, maintaining hand hygiene and aseptic technique, environmental hygiene, consistent screening of preoperative patient health evaluation, selection of appropriate device and placement site, and regular assessment of institutional protocols for infection prevention and surveillance [5, 21, 22]. Implementation of care bundles allows multidisciplinary teams and individual

wards/units to measure, target improvements, and demonstrate their compliance against key practices, thereby improving care for all patients.

The CDC, as well as the Center for Medical Services, has called for zero tolerance for hospital-associated infections such as CLABSI because of the seriousness of catheter-related bloodstream infections and as they are considered to be completely preventable [23]. In 2008, Medicare began to encourage US hospitals to adopt infection prevention strategies by instituting a policy of nonpayment for reasonably preventable HAIs, and more recently due to the Healthcare reform law, from 2015 hospitals in the United States will face an additional 1% reduction in Medicare reimbursement payments if they fall into the top 25% of national risk-adjusted hospital-acquired condition (such as SSI, CAUTI, and CLABSI) rates for all hospitals in the previous year. With these legislative, biologic, and economic factors hospitals are under great pressure to control the HAI epidemic. Compounding this problem is the rise in antibiotic-resistant organisms or superbugs, including Methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA kills an estimated 20,000 people in the United States each year, and since the mid-1990s, there has been an explosion in the number of MRSA infections [24].

5.2.2 Role of Antimicrobial Devices in Infection Prevention

One approach to achieve zero HAI rates is using devices that have antimicrobial properties. Several studies have shown benefits of adding antimicrobial devices in the bundle especially for patients requiring long-term devices. Based on the studies that have used the infection prevention bundle alone, the level of DAIs could not be reduced to zero. For instance, despite the use of bundle, CLABSI could not be reduced below 1.4 cases/1000 catheter days in critically ill patients [25, 26]. However, if the aseptic bundle is used with a combination of antimicrobial CVCs, the rate of CLABSI could be lowered to 0.25–0 case/1000 catheter days, [27, 28, 29]. Antimicrobial central venous catheters (CVCs) treated with chlorhexidine/silver sulfadiazine or minocycline/rifampin have now become the standard of care [27, 30–33]. Utilization of these antimicrobial catheters, in patients whose device is expected to remain in place for greater than 5 days, is among the CDC recommendations (Category 1A) for prevention of CLABSI [11]. Similarly, the Society for Healthcare Epidemiology of America recommends the use of silver zeolite-impregnated umbilical catheters in preterm infants [34] based on the 2012 study by Bertini [35]. More recently, the use of antibacterial envelop for cardiac implantable electrical devices (CIED) [36, 37] and chlorhexidine-treated peripherally inserted central catheters (PICCs) [38, 39] have also resulted in significant reduction in infection rates and economic benefits.

5.3 Antimicrobial Technologies for Implantable Medical Devices

5.3.1 Passive Technologies

There are several technologies developed to impart antimicrobial features to implantable medical devices. Selection of a technology for antimicrobial treatment on a device is dependent on factors like nature of the device material (metal, polymeric, ceramic, composite etc.), its intended use (blood contacting versus non blood contacting), technological factors (design complexity, functionality, reproducibility, manufacturability), characteristics of the antimicrobial substance if used, and financial factors (cost versus added value, regulatory path, certification) [40–42]. Broadly, current antimicrobial technologies on implantable devices can be divided into two categories—passive technologies which involve device surface modifications without the use of an antimicrobial substance and active technologies which involve an antimicrobial substance.

The passive technologies are based on modifications of the surface properties such as surface-free energy, polarity, and topography, which increase the biocompatibility and decrease the susceptibility to bacterial adhesion. Since these technologies do not rely on leaching of an antimicrobial substance, they are considered safe. As the passive surface modification approaches are described in great depth in Chapter 8, this chapter is primarily focused on the antimicrobial substances and the processes that constitute the active technologies for implantable medical devices.

5.3.2 Active Technologies

In contrast to passive technologies, antimicrobial medical device manufactured with an active technology involves incorporation of an antimicrobial substance either on the device surface or in the bulk of the device. Antimicrobials can either be applied on the surface by impregnation, soaking, imbibing, painting, dip coating, brush coating, die coating, rolling, wiping, spraying, and painting or can be incorporated in the bulk of the device by compounding and extrusion methods.

They can incorporate a large variety of antimicrobial compounds on the surface and modify the existing devices easily and inexpensively without changing the device bulk properties. Coatings with antimicrobial compounds have shown promising results and appear to increase biocompatibility and resist the adhesion of the bacteria on surfaces of indwelling medical devices (12). The current surface-treatment technologies incorporating antimicrobial compounds can be classified into three categories: depositing a thin film of antimicrobial compounds on the surface, ionic bonding anti-infective agents, and entrapping antimicrobial compounds in a polymer matrix.

Studies have revealed that changing the surface properties of a medical device significantly influences the ability of certain bacteria to adhere to the device; however, changing bacterial strains and the physiological media that the device contacts can also alter the adhesion process. For instance, modifying device surfaces to become more hydrophilic may reduce the adherence of some bacterial strains but may also increase the adherence of some other strains [43]. Incorporating antimicrobial substances, such as antiseptics, antibiotics, silver, and other pharmaceutical agents that are highly effective in killing bacteria on the device surface, provide an alternate approach to assure a surface-free of bacteria. The presence of such substances provides devices with an active chemical barrier against the intruding organisms. Such technologies provide high concentration of antimicrobial compounds in the local environment, killing bacteria that enter with the medical device during placement or that encounter the device later through any other route before they have a chance to generate a more resistant biofilm on the device surface. Antimicrobials can either be applied on the surface by impregnation, entrapment, dip coating, brush coating, die coating, rolling, wiping, spraying, and painting or can be incorporated in the bulk of the device by compounding and extrusion methods. Some antimicrobial substances which are utilized on commercially available antimicrobial medical devices are listed below.

5.3.3 Antimicrobial Substances

(a) *Metals*

Silver, copper, zinc, and some other metals have been used as antimicrobial agents since antiquity. Karl Wilhelm von Nägeli discovered the oligodynamic effect as the toxic effect of metal ions on viruses, algae, molds, spores, and prokaryotic and eukaryotic microorganisms, even in relatively low concentrations. Recent studies indicate that different metals cause discrete and distinct types of injuries to microbial cells as a result of oxidative stress, protein dysfunction, or membrane damage [44]. Metal, oxide, or salt compounds based on silver are among the most widely applied antimicrobials in healthcare industry because of their broad-spectrum antimicrobial activity and low toxicity to the human body. Silver compounds are applied on device surfaces through direct deposition method [45]. Silver has been proven efficacious in topical applications such as antibacterial creams and wound dressings. However, clinical effectiveness of silver containing implantable medical devices has been debated. A meta-analysis of 11 randomized controlled trials and 1 cohort study on silver-impregnated CVCs concluded that silver-impregnated CVCs did not reduce bacterial colonization or CRBSI rates [46]. Limited evidence suggests that endotracheal breathing tubes coated with silver reduce the incidence of ventilator-associated pneumonia and delay its onset, although no benefit is seen in the duration of intubation, the duration of stay in intensive care, or the mortality rate [47–50]. It is unknown if they are cost-effective [50] and more high-quality scientific

trials are needed [49]. Similarly, evidence does not support an important reduction in the risk of urinary tract infections when silver-alloy catheters are used, and these catheters are associated with greater cost than other catheters [51]. Pin tract infection is another complication associated with the use of external fixation and has been reported to occur in up to 63% of pins [52, 53]. In two randomized clinical studies, the effect of silver coating of external fixator pins was assessed on pin infection by comparing against non-coated pins. No significant differences were observed between the two types of pins in the rate of pin tract infection, clinical appearances of the pin sites, bacteriology of the pin tracts, torque to remove the pins, or radiographic lucency around the pin [54, 55].

(b) *Biguanides*

Chlorhexidine (CHX) is a broad-spectrum bactericidal agent against both on Gram-positive and -negative bacteria and fungi [56]. Given that CHX has a strong antimicrobial activity but relatively low levels of toxicity to mammalian cells [57, 58], it is regarded as the most useful and safe disinfectant. Its propensity to bind to the surface of tissues offers a long-lasting antimicrobial effect [59]. This property of CHX also makes it suitable for use as a preservative in some pharmaceutical or medical products, such as ophthalmic solutions, and as a disinfectant of medical instruments and hard surfaces. The effect of CHX on bacteria has been studied extensively in recent years. Though the in vivo and in vitro antimicrobial activities of CHX have been reported, the exact mechanism of action exerted by CHX on bacteria and the differences in activity on Gram-positive and -negative bacteria are still not very clear. It is generally thought that the cationic CHX interacts with the anionic phosphate residue of the lipid molecules in the cell membrane by adsorption. It has been postulated that CHX bypasses the cell wall exclusion mechanism, perfuses to cytoplasmic membrane to cause leakage of low molecular weight components through cell membrane, and precipitates cytoplasm content through the formation of complexes with phosphate moieties [60, 61].

The first antimicrobial implantable medical device treated with a combination of CHX and silver sulfadiazine (SSD) was a central venous catheter, which became commercially available in 1992. CHX-SSD CVC has been shown to be effective against wide array of gram-positive, gram-negative, and fungal pathogens responsible for CLABSI. In a randomized controlled trial, the first generation of the CHX-SSD CVC (treated only externally) was shown to reduce bacterial colonization of the catheter by 44% and CRBSI by 79% [62]. The second-generation CHX-SSD CVC (treated both externally and internally) was also proven effective in another randomized controlled trial, to prevent catheter colonization as compared to the untreated control catheter [27]. There are more than 30 human studies in support of CHX-SSD CVCs [27, 29–31, 62, 63] showing significant reduction in the CRBSI incidence and cost savings. Because of the effectiveness, Chlorhexidine-silver sulfadiazine CVC is a CDC 1A recommendation for CRBSI prevention [11]. Almost a decade later in 2010, another CHX-treated vascular catheter product, a peripherally inserted central catheter (PICC) received approval from the FDA with the antimicrobial efficacy claims for up to 30 days. The same catheter was approved with the

antithrombogenic efficacy claims in 2012. The extended duration efficacy from this device is achieved by coating the device surfaces with CHX entrapped in a polymer matrix resulting in slow release of this agent. The antithrombogenic efficacy of the product is attributed to thrombin inhibition by CHX; inhibition of thrombin does not allow the conversion of soluble fibrinogen to fibrin clot, the final step of the common pathway of blood coagulation. In a single hospital study, the use of antimicrobial/antithrombogenic CHX PICC resulted in a statistically significant decrease in the rate of CLABSI from 4.18/1000 catheter days to 0.47/1000 catheter days. Treatment cost savings were found to be an additional benefit of using these antimicrobial catheters [38]. In another recent study conducted at a long-term acute care hospital, 100 CHX PICCs were inserted over a 2-year period with a total of 1705 line days without any reported CRBSI [39] demonstrating the clinical effectiveness of this relatively new product.

Polyhexamethylene biguanide (PHMB) is another biguanide which is successfully applied on implantable long-term vascular catheters. The active antimicrobial component, PHMB, is copolymerized with non-thrombogenic poly(ethylene glycol) (PEG) and a stabilizing monomer to provide a stable, non-leaching coating assembly, which displays a contact-kill mechanism for antimicrobial activity and has demonstrated long-term protection against infection. The mechanism of antimicrobial action for PHMB is by phase separation and domain formation of membrane lipids [56]. Broxton et al. [64, 65] demonstrated that maximal activity of the PHMB occurs at pH 5–6 and that initially the biocide interacts with the surface of the bacteria and then is transferred to the cytoplasm and cytoplasmic membrane. The cationic PHMB is shown to have its effect mainly on the acidic negatively charged species where it induced aggregation leading to increased fluidity and permeability. This results in the release of lipopolysaccharides from the outer membrane, potassium ion efflux, and eventual organism death [66]. The clinical efficacy of the PHMB CVC in terms of reducing blood stream infection rate was demonstrated in a prospective randomized controlled, double-blind clinical trial [67]; however, the study showed no reduction of the catheter colonization rate.

(c) *Antibiotics (minocycline, rifampin, nitrofurazone)*

Single or combination of antibiotics with device polymeric coating has been in the market for some time. Examples include nitrofurazone-coated catheters and the combinational minocycline- and rifampin-coated catheters (e.g., Bard Magic$^{3®}$ intermittent urinary catheter; Cook Spectrum® minocycline + rifampin PICC), both of which have demonstrated antimicrobial efficacy. Nitrofurazone is a broad-spectrum antimicrobial agent that is typically used as a topical treatment for preventing or curing burn and wound infections but has found application in the device field. Nitrofurazone-impregnated urinary catheters have been reported to decrease the incidence of UTIs during patient use [68]. In a randomized clinical trial, nitrofurazone catheters performed slightly better than silver-coated or standard urinary catheters in preventing CAUTI and importantly resulted in significant estimated cost reduction with their use [68]. Similarly, the combination of minocycline/rifampin vascular access devices has demonstrated an advantage in their use in

terms of clinical outcomes and cost. The exchange of standard CVCs with a minocycline/rifampin combinatorial CVC (M/R-CVC) within 7 days in patients with *Staphylococcus aureus* (SA)–CLABSI resulted in lower mortality, as well as complete eradication of SA biofilms in a comparable in vitro infection model [69].

Although antibiotic-containing products have demonstrated clinical efficacy, the increase of antimicrobial resistance in recent years has led to a growing concern with their use in devices. The Center for Disease Control (CDC) estimates that over 2 million people in the United States become infected with microbes harboring resistance to one or more traditional antibiotics, and over 23,000 of these patients die [70]. Microorganisms have been found to have one or more resistance mechanisms to all traditionally prescribed antimicrobials in use today. The classic example is methicillin-resistant *Staphylococcus aureus*-mediated infections (MRSA), which are difficult to treat [71], but in recent years the headlines have been rife with stories of vancomycin-resistant *Enterococci* sp. (VRE), vancomycin-resistant *Staphylococcus aureus* (VRSA), and multidrug-resistant infections. Gram-negative microorganisms, such as *Pseudomonas aeruginosa*, Enterobacteriaceae, and Acinetobacter, are becoming increasing problems in HAIs due to their inherent antimicrobial resistance and their capacity to rapidly gain resistance to many classes of antibiotics. These include β-lactams, cephalosporins, carbapenems, fluoroquinolones, and even polymyxins, a class of compounds that had fallen out of disfavor with physicians, but is now being used as the "last resort" treatment option for multidrug resistance gram-negative infections [70]. For these reasons, emphasis on alternate strategies to prevent or resolve device-mediated infections besides use of traditional antibiotics is becoming more prevalent.

(d) *Triclosan*

Triclosan is a bisphenol with wide-spectrum antimicrobial activity that has been widely used for the past 30 years. Triclosan can be found in many consumer and healthcare products, including hand soap, toothpaste, and household items such as cutting boards (Microban®) to surgical drapes [72]. Triclosan has been used in numerous medical devices, including ureteral stents and surgical sutures [73, 74]. Its efficacy has been debated as typically infection rates are similar between devices that contain triclosan to those that do not contain the active agent [73]. However, a randomized clinical trial using the Triumph® ureteral stent demonstrated superior performance in decreasing incidence of ureteral stent infection symptoms, such as pain upon urination and lower abdominal discomfort, versus the Percuflex® control device [75].

Triclosan has been facing head winds from regulatory bodies and environmental agencies from both the United States and Canada. There is clinical and in vitro evidence that by itself, triclosan is not effective in preventing microbial contamination of device surfaces or mitigate infection [72, 74]. It has been shown in animal models that it impairs development in young animals [72]. More importantly there has been a growing concern that its widespread use may promote antimicrobial resistance [72, 76, 77]. Triclosan is thought to interfere with fatty acid biosynthesis in microbes [72]. Some microorganisms, such as *Pseudomonas aeruginosa*, have an inherent

resistance to low levels of triclosan [72]. However, exposure to sublethal concentrations of triclosan has demonstrated selection of populations of microorganisms with mutations in pathways traditionally found in impact sensitivity to antibiotics such as altered cell wall synthesis and upregulation of efflux pumps [72]. Evidence exists that triclosan exposure confers resistance to commonly used antibiotics through these mechanisms [72, 76, 77]. The EU banned use of triclosan in 2015, and in 2016 its use was finally banned by the FDA in soaps, although it is still approved for use in the healthcare industry and other products [73].

(e) *Zinc pyrithione*

Zinc pyrithione is a broad-spectrum antimicrobial and antimycotic and has wide use in various products. Compounding this agent into medical polymer materials for device applications seems to be a potentially viable antimicrobial path. Its problem is that it has very low solubility in water or saline, rendering itself noneffective antimicrobial agent using it alone; however, with combination with another agent, it may provide some synergy in antimicrobial efficacy. There is some evidence that zinc pyrithione may promote outer membrane changes in *Pseudomonas aeruginosa* that render them less susceptible to planktonic killing [78]. However, research incorporating zinc pyrithione into polydimethylsiloxane (PDMS) along with other antifungals demonstrated modest efficacy in preventing *Candida albicans* biofilm development in an in vitro biofilm model system [79]. This agent may become relevant as increasingly fungal pathogens are found in medical device-associated infections.

(f) *Nitric oxide*

Nitric oxide (NO) is a gas with anti-inflammatory and antimicrobial activity [80]. NO is a key modulator of the immune response, and low levels of nitric oxide are important in maintaining homeostasis, while high levels of NO promote inflammatory responses and can be toxic to tissues [81]. NO is important in regulating vasodilatation, vasoconstriction, vascular permeability, chemotaxis and leukocyte migration and adhesion, and tissue and endothelial cell damage [81]. Endogenous NO produced by macrophages is an important host defense mechanism and demonstrates broad-spectrum activity in killing microorganisms [81].

Recent genomics analysis has identified homologues between eukaryotic and prokaryotic NO-sensing protein domains, suggesting that prokaryotes can respond to the presence of NO [82]. Low-level nitric oxide has been shown to sensitize established biofilms of *Pseudomonas aeruginosa* to traditional antibiotic therapy such as tobramycin and agents such as hydrogen peroxide, as well as promote biofilm dispersal [82]. This is thought to be due to induction of general stress response upon exposure of the microorganisms to sublethal concentrations of NO, which may stimulate a phenotype more similar to planktonic growing cells that are more sensitive to antimicrobial action.

Due to its pleiotropic action, academic research on nitric oxide as a viable agent in medical devices has led to increased publications in recent years. Nitric oxide, though promising in mitigating biofilms in vitro and controlling adverse

pro-inflammatory immune responses, presents a practical challenge as release of the agent is difficult to control [83]. Current research efforts are aimed at overcoming this and other short comings of the technology [83]. Some methods to store and control NO release rates are discussed in Chapter 7 (Morris).

(g) *Bismuth thiols*

Bismuth thiols possess broad-spectrum antimicrobial activity. Bismuth appears to have great promise as an anti-biofilm agent and has been shown to be particularly effective against gram-positive biofilms [84, 85]. Its mode of action is thought to be by inhibiting extrapolysaccharide (EPS) production, an essential component of the biofilm matrix, through interference with iron metabolism [84–86]. Microbion, a Montana company, has engaged in research on using various bismuth thiols as antimicrobial agents on devices and as antimicrobial agents in other applications. Research has demonstrated both in vitro and in vivo efficacy in animal models in reducing attachment, biofilm formation, and infection in the presence of bismuth thiols [86]. Microbion has entered phase two trial using the compound bismuth-ethanedithiol (BisEDT). An issue with the compounds is that they are not very soluble in saline or blood, making them potentially not a viable path for vascular applications, but for dermal applications, it has some potential. However, the thiol moiety appears to influence the solubility of bismuth and impact its activity, suggesting a pathway to feasibility [86].

5.4 Summary and Limitations

Medical devices treated with active antimicrobial treatments face multiple technical challenges. First, to be efficacious, antimicrobial agents need to have sustained amount of release over time, preferably covering the entire device implant period to provide antimicrobial protection. Technology-wise, it is hard to achieve this as the antimicrobial agent tends to stop eluting out of the medical device typically in a week or two. Second, loading of antimicrobial agents on the device can be challenging. The amount of the antimicrobial agent could be too high, and the toxicity level may pose potential harm to the human body. This normally is the case for compounding the antimicrobial agent to the polymer material of the medical device. A lot antimicrobial agents such as biocides are very toxic to human body and have not been approved for use as antimicrobial agents on implantable devices by regulatory agencies. Third, application of antimicrobial agents has technical challenges to overcome. Most antimicrobial agents are applied to medical devices as a polymeric coating. Long-term stability and delamination of the coating with the device may be barriers for practical applications, and adverse reactions of coating degradation due to enzyme lying of coating as foreign matter in the blood stream is a probable possibility. To have a successful antimicrobial agent-medical device combinational product, a team of dedicated coating engineers, microbiologists, chemists, and material specialists is needed to overcome these difficulties.

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Chapter 6
Anti-antimicrobial Approaches to Device-Based Infections

James D. Bryers

6.1 Introduction

Biofilms are surface-associated communities of microbial cells that are embedded in a microbe-generated extracellular matrix (**EM**) of polysaccharides, proteins, and DNA. The overall financial impact of biofilm-based infections is estimated to be in the tens of billions of dollars per year [69]. Medical-device-based infections are further complicated in that most, if not all, causative bacterial species are now resistant to multiple antibiotics [43].

Unfortunately, *biofilm infections* are typically treated using the systemic application of antibiotics; compounds are chosen based on their ability to kill or inhibit the growth of *freely suspended microorganisms*. A major concern with this approach is the frequent development of resistance to antibiotics [49]. In fact studies have shown that sublethal doses of antibiotics can actually *exacerbate* biofilm formation [12]. As stated above, biofilm communities tend to be significantly less responsive to antibiotics and antimicrobial stressors than planktonic organisms of the same species [76, 103]. Our work has documented a further complication that the spread of antibiotic resistance genes borne on plasmid DNA, within and between species, is greatly exacerbated within biofilm communities [18, 19, 79]. As a consequence to this increase in resistance, researchers have turned to a number of alternatives to antibiotics, including bacteriophage [104] and bacteriophage lytic enzymes [45], probiotics [59, 87], and human antimicrobial peptides (defensins, cathelicidins, and histatins) [38]. The success of these alternatives awaits much development and optimization.

Unfortunately, most of these alternatives are still based upon some mechanism of killing or terminating the target bacteria, an approach some feel preordains the

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development of resistance in bacteria. It has been recently proposed to develop substances that specifically inhibit bacterial virulence [2]. Such "anti-pathogenic" drugs, in contrast to antibacterial drugs, do not kill bacteria or stop their growth and are assumed not to lead to the development of resistant strains. A very elegant approach comprises the inhibition of regulatory systems that govern the expression of a series of bacterial virulence factors, such as biofilm formation.

Consequently, we review here some recent alternative anti-biofilm approaches that do not necessarily kill bacteria but rather negate biofilm formation. There are numerous prospects of *anti-antibiotic* therapies that are emerging from research; not all of these will be addressed in this chapter. Therapies that will not be discussed here and the reader is urged to explore on their own are: (*a*) biofilm matrix disruption via DNA extraction; (*b*) quorum sensing interference; (*c*) immunotherapies, i.e., vaccines that target bacterial adhesins; and (*d*) biomaterials that heal with such fidelity that they prevent infection similar to the natural healing process.

Here we focus on three novel anti-biofilm strategies: (*1*) disruption of bacterial iron metabolism, (*2*) enhancing phagocytosis, and (*3*) preventing amyloid fibril production within the biofilm extracellular matrix.

6.2 Iron Metabolism Disruption

6.2.1 The Competition for Iron

Iron is critical for bacterial growth and the function of key metabolic enzymes [7, 15, 16, 114], and sequestration of iron is an early evolutionary strategy of host defense [44, 64]. Recent work has also shown that even when sufficient levels are available for bacterial growth, iron limitation blocks development, perhaps by a signaling mechanism [98, 97]. Iron limitation has also been shown to act at several stages of biofilm formation [20]; low iron inhibits bacterial attachment, microcolony formation, and mature biofilm development. Thus, strategies that disrupt iron metabolism present a therapeutic potential against infections caused by biofilm and planktonic bacteria.

One approach to combating infection is to exploit those stresses already imposed on organisms by the in vivo environment or host defenses. Fe metabolism is a major vulnerability for infecting bacteria for two reasons. First, in almost all pathogens, Fe is essential for growth and the functioning of key enzymes, such as those involved in DNA synthesis, electron transport, and oxidative stress defense [23]. Second, free Fe levels are extremely low in vivo (approximately 10^{-18} M) due to multiple host mechanisms that sequester Fe [23]. The importance of Fe limitation in blocking acute infection has been established for numerous bacterial species. In these studies, increasing the amount of available Fe markedly increased acute infections [23, 53]. For example, a single injection of Fe decreased the lethal dose of a *P. aeruginosa* strain (in a murine acute infection model) from $\geq 10^4$ organisms to $\leq 10^1$ [46]. Work

by a number of laboratories has linked Fe metabolism to the pathogenesis of chronic infections, in that high Fe levels (i.e., more Fe than is required for growth) promote biofilm development. High levels of Fe are required for the formation of cell clusters early in biofilm development and for the maturation of biofilms into three-dimensional structures [13, 92, 97]. Fe sequestration may also be protective during infection, as bacteria sampled from infection sites show gene expression profiles indicative of Fe starvation [21, 99, 117]. That host defenses severely limit available Fe and the critical role of Fe in infection suggest that invading organisms may be susceptible to interventions that further disrupt Fe acquisition or metabolism. Exploiting this Fe vulnerability has proven difficult. Fe chelation therapy has been tried, but most microorganisms can also use Fe when it is bound to chelators [115]. Targeting bacterial Fe uptake mechanisms is also problematic because most pathogens have many redundant uptake systems, e.g., *P. aeruginosa* has more than 30 genes encoding different Fe receptors [33]. This redundancy reduces the likelihood that any single therapy could block all Fe uptake systems.

6.2.2 Iron Replacement

Gallium and zinc (group IIIA transition metals) have many atomic features similar to Fe3+, including a nearly identical ionic radius, such that biological systems are unable to distinguish Ga from Fe3+. Unlike Fe3+, Ga does not undergo reduction/oxidation cycling that is critical for Fe to function in many enzymes. Thus, replacing Fe with Ga in such enzymes renders them nonfunctional [29, 84]. Ga can bind to the many siderophores of *Pseudomonas* sp. [11] and can be taken up by other bacteria including *S. aureus*, *S. epidermidis*, *E. coli*, *E. faecalis*, and *S. typhimurium* [15, 41, 40, 61]. Zinc and manganese may also interfere with iron metabolism in bacteria because of similar physicochemical properties and shared uptake pathways. Ga enters cells via the same mechanism used to acquire Fe [30, 31, 85]. Kaneko et al. [65] show that concentrations >1 μM $Ga(NO_3)_3$ inhibited *P. aeruginosa* suspended growth in 1/100 strength TSB medium (Fig. 6.1a). Since the authors were only interested in the specific anti-biofilm effects of Ga, the effects of Ga on biofilm formation were investigated at a low concentration of $Ga(NO_3)_3$ (1 μM), a level that did not impair the growth of suspended *P. aeruginosa* (Fig. 6.1a). In a clinical application, both suspended growth inhibition and anti-biofilm efficacy would be desirable. At concentrations of $Ga(NO_3)_3$ that were sub-inhibitory to suspended cultures, *P. aeruginosa* weakly attached to a glass surface, but biofilm formation was completely negated (Fig. 6.1b) at 0.5 μM.

To determine if Ga would actually kill and eliminate existing *P. aeruginosa* biofilms, Kaneko et al. [65] report growing biofilms for 3 days (with no Ga present) and then switching to medium containing Ga at various concentrations for 48 h. Bacterial viability was assayed using a live–dead stain. Most antimicrobial agents show markedly less activity against biofilms than against planktonic organisms (~1000–10,000-fold less activity, depending on conditions) [103]. In the Kaneko et al. [65] study,

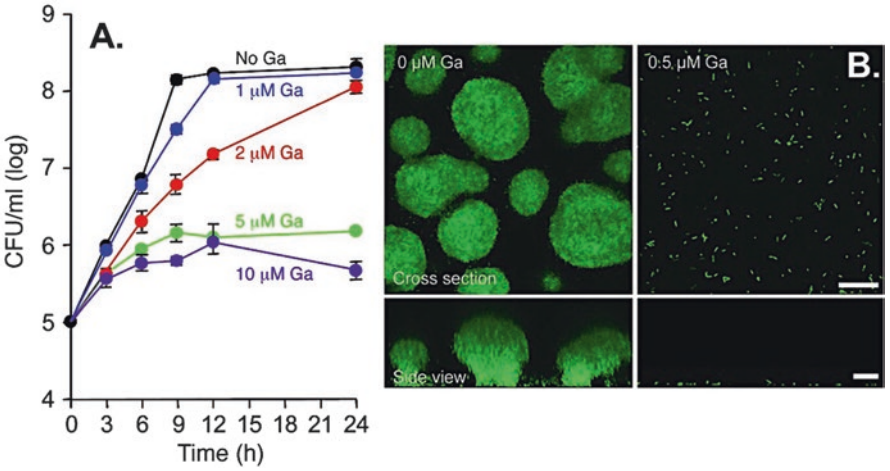

Fig. 6.1 (**a**) Effect of gallium on *P. aeruginosa* growth. $Ga(NO_3)_3$ inhibits *P. aeruginosa* growth in a concentration-dependent manner. Experiments were performed in biofilm medium at 37 °C, and data are the mean of four experiments; error bars indicate SEM. (**b**) Ga prevents *P. aeruginosa* biofilm formation. Confocal microscopic images of GFP-expressing *P. aeruginosa* in flow cells perfused with medium without (*left*) and with (*right*) Ga, 5 days after inoculation. Experiments were performed at 25 °C using 0.5 μg/ml $Ga(NO_3)_3$; this concentration did not inhibited suspended growth (see frame A). Top images, top-down views (*x*-*y* plane); bottom images, side views (*x*-*z* plane); scale bars, 50 μm (Adapted from Kaneko et al. [65])

bacteria within mature biofilms were killed to varying degrees by concentrations of Ga similar to those that killed planktonic cells: 10, 100, and 1000 μM.

Subsequently, there have been a few reports of incorporating gallium into the formulation of certain biomedical implants. In a series of four papers from the Valappil group [95, 108–107], gallium in the form of Ga_2O_3 was incorporated into phosphate-based glasses by a conventional melt quenching method. Intended for the treatment of periodontal disease, the efficacy of the various gallium-loaded glasses was assessed using the bacterial species *Porphyromonas gingivalis*, *Streptococcus gordonii*, *Streptococcus mutans*, and *Pseudomonas aeruginosa* cultivated both in suspension and in biofilms. While certain formulations reduced the planktonic concentration of cells significantly, gallium-loaded glass formulations had only minor-to-no effects on biofilm populations.

6.2.3 Enhanced Cellular Uptake Using Siderophore–Gallium Complexes

In response to sequestered iron in mammalian hosts, successful pathogens can acquire iron within the host via four strategies that target specific iron sources: (1) iron acquisition by degrading heme and heme-containing proteins; (2) iron acquisition by degrading transferrin, lactoferrin, and ferritin; (3) ferric iron acquisition by

siderophores; and (4) uptake of ferrous iron. Many bacteria and fungi (and perhaps mammals) produce *siderophores* (low molecular weight, high-affinity iron chelators) to acquire and transport iron, as detailed elsewhere [6, 55, 82, 116]. Further, many microorganisms have evolved the transport mechanisms to use heterologous siderophores produced by other microbes (xenosiderophores) [116], which is true for the opportunistic pathogen *P. aeruginosa* that produces two different siderophores, pyoverdine and pyochelin [34], but can utilize a variety of heterologous siderophores from other bacteria and fungi, including ferrioxamine B, ferrichrome, and enterobactin [35, 88]. Many pathogenic microorganisms produce siderophores that are directly implicated in their virulence [48, 82]. In these cases, siderophores of bacterial and fungal pathogens can directly remove iron from host proteins such as transferrin to support proliferation in invertebrates [71]. A schematic of Gram-negative and Gram-positive siderophore uptake mechanisms of ferric ion is shown in Fig. 6.2.

While Kaneko et al. [65] document efficacy of $Ga(NO_3)_3$ as both an anti-biofilm and antimicrobial, dosages were still relatively high. Banin et al. [14] report a novel approach for the delivery of gallium, in their case to *P. aeruginosa*, where they used a strong siderophore, desferrioxamine (**DFO**), to bind gallium ion. DFO is a bacte-

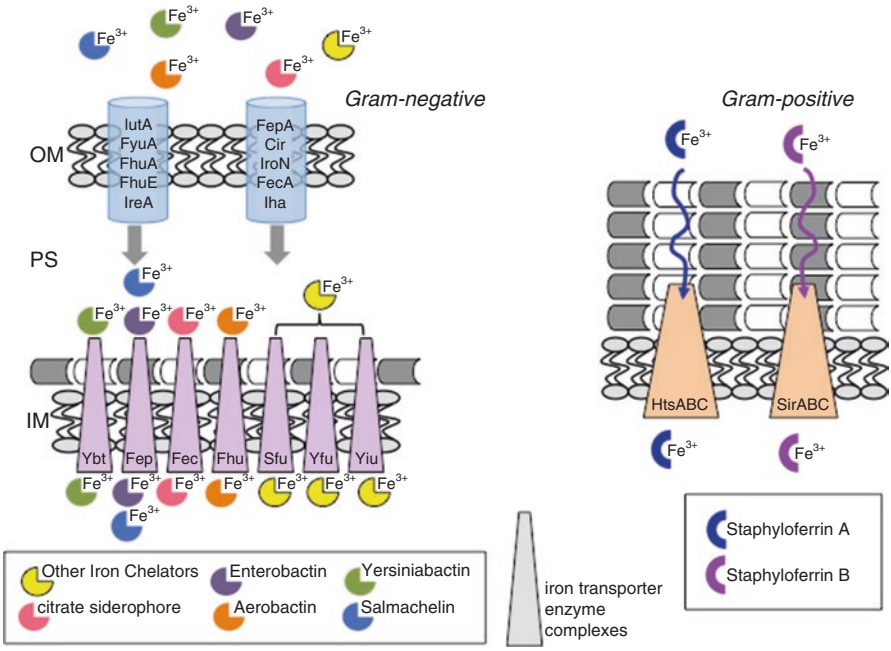

Fig. 6.2 Schemes for ferric iron uptake via siderophores in Gram-negative and Gram-positive bacteria. For Gram-negative (*left panel*), outer membrane receptors (*in blue*) import chelated iron to the periplasmic space where several inner membrane enzymatic transporter complexes (*in pink*) bring chelated iron into the cytoplasm to be uncoupled. In Gram-positive bacteria (*right panel*) the ABC transporters HstABC and SirABC import iron chelated by the siderophores staphyloferrin A and staphyloferrin B, respectively

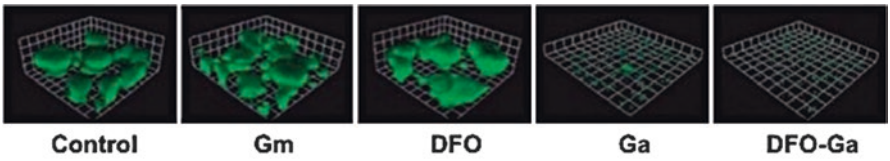

Fig. 6.3 Biofilm formation in flow cells with sub-inhibitory concentrations of DFO (0.001 mM), Ga (0.001 mM), DFO-Ga (0.001 mM), or gentamicin (Gm) (0.1 μg/ml). Shown are 3D reconstructed confocal microscope images of 6-day biofilms grown in the presence of the indicated agent (a side of each square on the grids is 23 μm). *P. aeruginosa* cells are expressing GFP (Reproduced from Banin et al. [14])

rial siderophore produced by the Actinobacteria *Streptomyces pilosus* and is used clinically to treat iron poisoning. DFO was selected as a siderophore carrier of Ga because *P. aeruginosa* possesses two uptake systems for DFO-Fe. Thus, DFO-Ga could deliver gallium to *P. aeruginosa* cells in preference to uncomplexed Fe via either of the two DFO uptake systems. Banin et al. [14] quantify the efficacy of both Zn-DFO and Ga-DFO against *P. aeruginosa* PAO1 growing under normal planktonic conditions and growing as biofilms. A flow cell system was used to examine the influence of sub-inhibitory concentrations of the DFO complexes (5 μM) on biofilm formation. As controls, DFO plus $GaCl_3$ or $ZnCl_3$ was applied separately. *P. aeruginosa* forms mature, thick, structured biofilms when cultivated in flow cells. Although addition of $ZnCl_3$ or DFO (5 μM) does not influence biofilm architecture, Zn-DFO (5 μM) complex does impair biofilm formation, while the use of free gallium (5 μM) or Ga-DFO (5 μM) complex completely blocks biofilm formation (Fig. 6.3). The ability of the DFO complexes to eradicate existing, mature PA (PAO1) biofilms was also examined. Survival of biofilms was measured as viable cell counts. Results show the antibiotic gentamicin (10 μM), about 2x the MIC for planktonic cultures, facilitated a 2 $\log_{10}$ decrease in cell counts, whereas Ga-DFO (1 μM) and Zn-DFO (1 μM) caused a 3–4 $\log_{10}$ decrease in cell counts. When the complexes were combined with antibiotic treatment, the DFO-Zn and gentamicin together were very effective in killing biofilm cells, reducing the viable count by almost 6 $\log_{10}$.

Ma et al. [80] describe the development of two novel anti-biofilm agents, gallium(Ga) and zinc (Zn), complexed with protoporphyrin IX (PP) or mesoprotoporphyrin IX (MP) that are both highly effective in negating suspended bacterial growth and biofilm formation. These chelated gallium or zinc complexes act as iron siderophore analogues, supplanting the natural iron uptake of most bacteria. Ma et al. describe development of a poly(ether urethane) (PEU) film that released either Ga or Zn complexes for a sustained time period; such loaded polymer systems could be developed into entirely new implants (catheters, shunts, tissue engineering scaffolds) or used as outer coatings applied to existing devices, prior to implantation. A segmented biomedical-grade poly(ether urethane) PEU (FDA accepted as BioSpan®) was used as the base polymer because of its excellent mechanical properties. PEU is an FDA-approved blood-contacting material and is

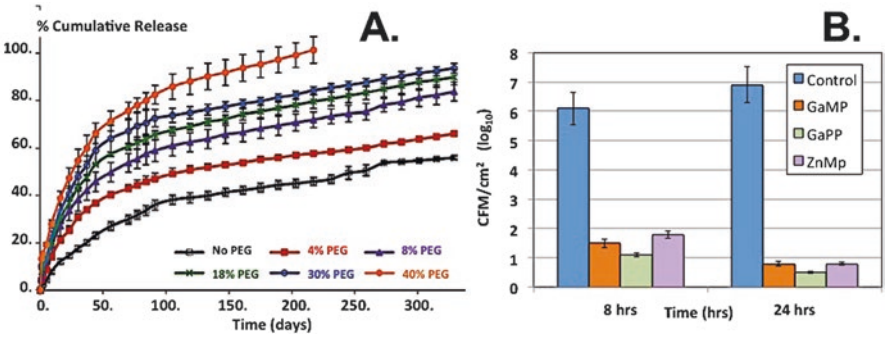

Fig. 6.4 (**a**) Cumulative percent release profiles of gallium/mesoporphyrin (MP) from porous pHEMA scaffolds as function of pore former, PEG, weight percentage. Each specimen contains 0.55% of Ga-MP and PEG (*MW* = 1450) in amounts varying from 2% to 40% (w/w). Temperature = 37 °C *pH* = 7.2. Data is taken from two separate experiments, each with $n = 3$. Error bars are standard deviations (SDs). (**b**) Adhesion of *Staphylococcus epidermidisSE* RP62A on porous pHEMA scaffolds releasing gallium MP complexes. = control pHEMA, no release; = Ga-MP; = Ga-PP; and = ZnMP (Adapted from Ma et al. [80])

commonly used in devices such as heart valves and spinal implants. Poly(ethylene glycol), PEG, was chosen as a pore-forming agent because it dissolves upon hydration, creating pores in the PEU through which drugs can escape. PEG was determined to be a superior pore-forming agent after extensive comparison with bovine serum albumin (BSA) (work previously shown by Kwok et al. [74]). An optimum formulation containing 8% PEG (*MW* = 1450) in the PEU polymer effectively sustained the release of Zn or Ga complexes for at least 3 months (Fig. 6.4a). All drug-loaded PEU films exhibited in vitro ≥ 90% reduction of Gram-positive (*Staphylococcus epidermidis*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria in both suspended and in biofilm culture versus the negative control PEU films that released nothing (Fig. 6.4b). Cytotoxicity and endotoxin evaluation demonstrated no adverse responses to the Ga or Zn complex releasing PEU films. Finally in vivo studies were carried out where PEU films releasing Ga-PP or PEU films releasing nothing were implanted subcutaneously in the dorsal area of mice; 24 h after implantation, mice were injected with 10^6 cell/mL bacterial suspension of either *S. aureus* or *P. aeruginosa* at the site of implantation. Mice implanted with empty PEU films required euthanasia 16 h after bacterial challenge due to obvious signs of local infection; mice that received Ga-PP releasing PEU films survived 3-weekly applied bacterial challenges with no apparent signs of infection.

Since this initial development of Ga siderophore complexes, there have been publications further developing their utility. Abdalla et al. [1] report the results of a study to quantify the growth-inhibitory activity of different Ga compounds against an ATCC strain and clinical isolates of *Mycobacterium abscessus*. Ga-protoporphyrin completely and significantly inhibited both an ATCC strain and clinical isolates of *M. abscessus* at much lower concentrations than $Ga(NO_3)_3$. When *M. abscessus* growth inside the human macrophage THP-1 cell line was assessed, Ga-protoporphyrin

was >20 times more active than $Ga(NO_3)_3$. Arivett et al. [9] similarly examined the antibiotic activity of gallium protoporphyrin IX (Ga-PP) against a collection of multidrug-resistant *A. baumannii* strains. Susceptibility testing demonstrated that Ga-PP inhibited the growth of all tested strains when cultured in cation-adjusted Mueller–Hinton broth, with a MIC of 20 μg/ml. This concentration significantly reduced bacterial viability, while 40 μg/ml killed all cells of the *A. baumannii* ATCC 19606(T) strain and a multidrug-resistant clinical isolate after 24 h incubation. Recovery of ATCC 19606(T) and ACICU strains from infected A549 human alveolar epithelial monolayers was also decreased when the medium was supplemented with Ga-PP, particularly at a 40 μg/ml concentration. Similarly, the co-injection of bacteria with Ga-PP increased the survival of *Galleria mellonella* larvae infected with ATCC 19606(T) or the clinical isolate. Ga-PP was cytotoxic only when monolayers of larvae were exposed to concentrations 16-fold and 1250-fold higher than those showing antibacterial activity, respectively. Chang et al. [28] report on the activity of gallium meso- and protoporphyrin IX against biofilms of multidrug-resistant *Acinetobacter baumannii* isolates. $Ga(NO_3)_3$ was moderately effective at reducing planktonic bacteria (64–128 μM) with little activity against biofilms (≥512 μM). In contrast, Ga-MPIX and Ga-PPIX were highly active against planktonic bacteria (0.25–8 μM). Cytotoxic effects in human fibroblasts were observed following exposure to concentrations exceeding 128 μM of Ga-MP and Ga-PP. Finally, Richter et al. [91] recently report similar results of gallium protoporphyrin in combination with deferiprone (an iron chelate). Deferiprone (20 μM) and Ga-PP (200 μg/mL) monotherapy for 2 h showed 35% and 74% biofilm removal, respectively, whereas simultaneous Def/Ga-PP administration showed 55% biofilm removal. In contrast, the consecutive treatment (2 h deferiprone followed by 2 h Ga-PP) achieved 95% biofilm removal. Cytotoxicity studies indicated no cell hazard in all treatments.

6.3 Enhancing Phagocytosis

6.3.1 Avoiding the Innate Immune Response

The immune system has evolved to protect the host from infection in two ways: *innate* and *adaptive* immunity. *Innate immunity* is the ability to produce a response within minutes or hours after infection through the recognition of molecules expressed by pathogens (e.g., microbial cell wall components, bacterial nucleic acids, formylated peptides, and viral double-stranded RNA). Such molecules are recognized by specialized receptors on cells of the innate immune system, which include dendritic cells, macrophages, neutrophils, natural killer cells, and gamma–delta T cells. Binding of microbial components to innate immune cell receptors (TOL-like receptors, NOD-like receptors, RIG-like receptors, C-type lectin receptors, and N-formyl met-leu-phe receptors) triggers signaling cascades within the cells that induce phagocytosis and the production of antimicrobial products.

Phagocytes also produce growth factors that can regulate adaptive immune responses. While innate immune responses are immediate (and may induce cross-protective immunity), they are typically short-lived.

The primary defense against infection is the innate immunity provided by neutrophils, macrophages, and dendritic cells particularly by activating the complement system. The role of complement activation is to control infections by eliminating microorganisms by opsonization and then clearance from the bloodstream [112]. Components of the complement system also interact with B and T cells to coordinate the adaptive immune response by regulating antigen presentation, promoting the formation of specific antibodies, and maintaining immunological memory [24, 68]. Complement evasion by many Gram-positive bacteria involves incorrect binding of complement recognition factors by bacterial cell wall proteins (proteins A, G, M) [94]. Gram-negative bacteria have also evolved secretory proteins that can degrade complement factors or their binding components or that prove anti-chemotactic or toxic to immune cells. For example, *S. aureus* has the ability to thwart neutrophils and macrophages by (*a*) inhibiting chemotaxis (blocking formylated peptide recognition, blocking C5a binding, secreting leukotoxins, and blocking LFA1–ICAM1-mediated extravasation), (*b*) negating opsonization (via protein G binding IgG antibody molecules by way of their Fc segments, plasmin degradation of bound IgG and C3b, and blocking C3b binding), and (*c*) thwarting phagocytosis (cell wall modifications to resist low endosomal pH, enzymatic degradation of endosomes) [47]. *Staphylococcus epidermidis*, another Gram-positive bacteria and the main species isolated in the majority of nosocomial infections, avoids the immune system through adhesion and biofilm formation [105, 109]. Otto and co-workers [77] have documented that Gram-positive bacteria actually sense antimicrobial peptides released by neutrophils and macrophage and can coordinate a directed defensive response. They discovered an antimicrobial peptide sensor system that controls major specific resistance mechanisms; the sensor contains a classical two-component signal transducer and an unusual third protein, all of which are indispensable for signal transduction and antimicrobial peptide resistance.

Some pathogens obviate the immune response by specifically targeting C-type lectin receptors, particularly dendritic cell-specific ICAM-3-grabbing non-integrins and mannose receptors, which benefits the pathogen by downregulating intracellular signaling and inhibiting maturation and cytokine secretion [72]. For example, entry of the Gram-negative oral pathogen, *Porphyromonas gingivalis*, into monocyte-derived dendritic cells in vitro leads to suboptimal DC maturation. This process requires *P. gingivalis* to express the major fimbriae, FimA [42, 62]. *P. gingivalis* also expresses unique immunosuppressive lipopolysaccharides (LPS) [32, 63, 90] and proteolytic gingipains [89]. The lipopolysaccharide of *P. gingivalis*, relative to those of *E. coli*, stimulates dendritic cells to secrete IL-10, but not IL-12 in vitro [63] and in vivo [90]. These two factors (suboptimal DC maturation and truncated cytokine expression) lead to the induction of a Th2 effector response, which suggests that *P. gingivalis* may target dendritic cell C-type lectin receptors (e.g., dendritic cell-specific ICAM-3-grabbing non-integrins) for entry and for blunting dendritic cell maturation.

6.3.2 *Artificial Opsonins*

Opsonization is the process where microorganisms and inanimate colloids (e.g., liposomes, particulates) are coated with host-produced proteins and lipids (immunoglobulins, complement factors), thus facilitating the binding of the opsonized bacteria or particle to specific receptor molecules present on phagocytes (i.e., neutrophils, macrophage, dendritic cells). IgG antibodies bind to their antigens on the surface of bacteria through coupling of the variable binding sites in the Fab region of the antibody, leaving the Fc region exposed. Phagocytes possess Fc gamma receptors and therefore can bind to the Fc-coated bacteria or particles and then internalize them. Complement fragment, C3b, also specifically binds to surface proteins or polysaccharides on microorganisms thus mediating binding to C3b receptors on the phagocytes. As described above, bacteria have evolved numerous ways to avoid opsonization by IgG and complement and thus avoid phagocytic elimination.

One possible alternative anti-biofilm biomaterial defense is one where the biomaterial would release factors that enhance neutrophil or macrophage phagocytosis of bacteria. There are a number of reports of synthetically derived "opsonins" enhancing bacterial phagocytic clearance.

The Taylor group, in a series of elegant papers, reports the use of several different bispecific fusion proteins that enhanced phagocytosis by macrophage of various pathogens, including *E. coli* [73], *P. aeruginosa* [78], and *S. aureus* [54]. In all cases, their artificial opsonins consisted of (*1*) a molecule that recognizes a surface marker on the pathogen that was chemically coupled with (*2*) a Mab that is specific to the complement receptor 1 (CR1) present on primate erythrocytes. In in vitro and in vivo studies, this series of works from the Taylor group demonstrated that their opsonins promoted binding of the target pathogen first to circulating erythrocytes, which then enhances macrophage phagocytosis of the bacteria. This phagocytosis did not apparently harm the erythrocyte, as verified in both in vitro and in vivo experiments [73].

Kobayashi et al. [70] report improved in vivo and in vitro phagocytosis of a periodontal pathogen, *Porphyromonas gingivalis*, using an artificial opsonin composed of two monoclonal antibody fragments: one against (*a*) the hemagglutinin domain of *P. gingivalis* (anti-r130k-HMGD antibody) and (*b*) the polymorphonuclear leukocyte (PMN) FcαRI (CD89) receptor (FcR). The Kobayashi work selectively targeted Fc receptors that were dominant on PMNs collected from gingival crevicular fluid of chronic periodontitis patients versus Fc receptors dominant on peripheral blood PMNs. Data shows that PMNs exhibited a higher capacity to phagocytose and kill *P. gingivalis* when treated with an opsonin that targeted *P. gingivalis* r130k-HMGD to leukocyte Fc RI as compared to opsonizing the bacteria with only the anti-r130k-HMGD antibody.

Encapsulated bacteria such as virulent strains of *Bacillus anthracis* impair phagocytosis with their capsules unless opsonized by antibodies. Poly-gamma-D-glutamic acid (gamma-PDGA) is the major component of the *B. anthracis* capsule. Bruno et al. [22] used poly-alpha-D-glutamic acid (alpha-PDGA)-coated magnetic beads as surrogates to simulate vegetative *B. anthracis* cells and avoid the hazards

of working with virulent bacteria. They report developing DNA aptamers against the alpha-linked PDGA-MBs. Four of the most frequent candidate aptamer sequences in the pool were coupled at their 5′ ends to Fc fragments of murine IgG to act as artificial opsonins. The effects of candidate aptamer-Fc conjugate addition on macrophage attachment and internalization of alpha-PDGA-coated beads were quantified using P388D1 and RAW 264.7 murine macrophage lines. P388D1 cells were not able to internalize the alpha-PDGA-coated beads, but attachment of the alpha-PDGA-coated beads was enhanced by the conjugates to varying degrees. Ingestion of alpha-PDGA-coated beads by RAW 264.7 cells in the presence of several different candidate aptamer-Fc conjugates demonstrated a statistically significant ($p < 0.01$) increase in phagocytic index, up to threefold in the first 30 min of exposure.

The major disadvantage of these original artificial opsonins described above was their use of bacterial species- or strain-specific Mabs or DNA aptamers; hence they lacked broad bacterial species recognition. The other limitation to these early constructs was that they used a phagocyte recognition moiety (e.g., Mab) that simply bound to the phagocytes, but did not necessarily activate phagocytosis.

Katzenmeyer and Bryers [67] describe a first-generation artificial opsonin that exhibits broad recognition of most Gram-positive bacteria and a phagocytic cell-targeting molecule that stimulates phagocytosis. Here, the antibiotic vancomycin, which binds to peptides terminated in D–Ala–D–Ala that are present in all Gram-positive bacterial peptidoglycan cell wall, was used as a bacterial recognition ligand. To negate the antibiotic membrane penetrating action of vancomycin, multiple copies of vancomycin were coupled to a poly-L-lysine (pLL) branched polymer (MW_N = 47,900) along with copies of the Fc portion of IgG. Vancomycin, when coupled to the large pLL, only acted as a Gram-positive bacteria recognition molecule and not an antibiotic. The vancomycin-based opsonins also exhibited higher affinity for MRSA and VRSA strains versus wild-type *S. aureus*. Pretreatment of Gram-positive strains with this multivalent opsonin *triples* (3X) in vitro phagocytosis compared to untreated controls (Fig. 6.5). These artificial opsonins were lyophilized, crushed into a fine powder, and then incorporated into a hydroxyethyl methacrylate (HEMA) monomer used to form polyHEMA films. In vivo data shows that upon hydration, films releasing the artificial opsonins were able to provide their intended protection to an initial bacterial challenge (10^7 cells/mL *SE*), *but as expected* (opsonin release was designed to terminate after 3 days), polyHEMA films depleted of opsonins failed against a second bacterial challenge at day 3.

6.3.3 *Biomaterial Surface Decorations that Influence Phagocytosis*

Macrophages (**MØ**) are the predominant cell type present at the tissue/biomaterial interface [66], and they are known to play a pivotal role in steering the outcome of implanted biomaterials. Following device implantation, macrophages infiltrate the

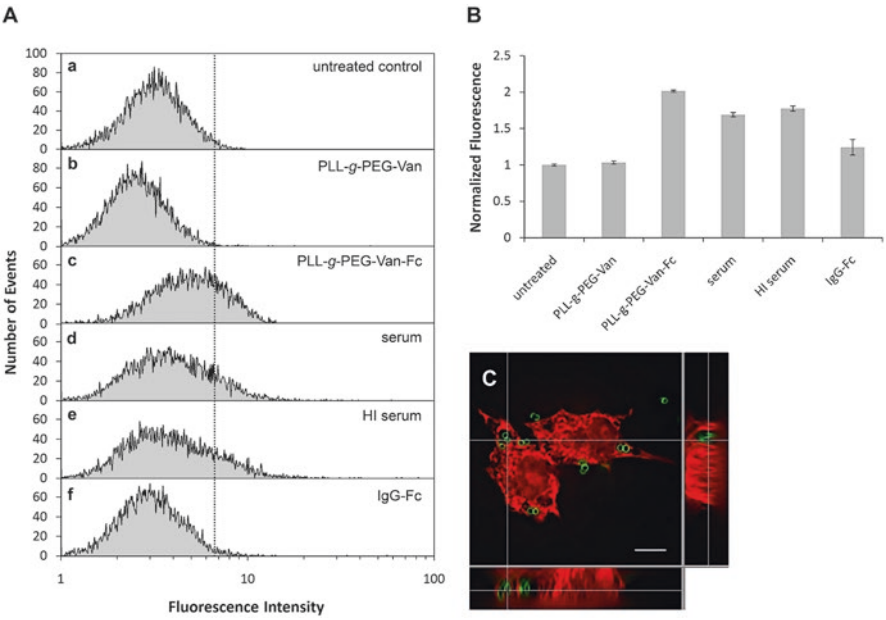

Fig. 6.5 Phagocytosis of *S. epidermidis* by HL-60 human neutrophil cell line. (**a**) Flow cytometry histograms showing the cell-associated fluorescence of the HL-60 population (~10,000 events). *Dotted line* indicates the threshold for identification of fluorescence-positive cells for an untreated negative control. Fluorescence of extracellular FITC-labeled *S. epidermidis* was quenched with trypan blue. (**b**) *S. epidermidis* opsonization with PLL-*g*-PEG-Van-Fc shifts HL-60 population to higher fluorescence intensity indicating a 3x greater extent of phagocytosis compared to opsonization with PLL-*g*-PEG-Van. Opsonization with human IgG-Fc alone does not significantly enhance phagocytosis. (**c**) CLSM image of live FITC-labeled *S. epidermidis* (*green*) ingested by a neutrophil (*red*). *Upper left* image is in the horizontal *x*-*y* plane. *Upper right* (*y*-*z* plane) and lower (*x*-*z* plane) images confirm that the selected bacterium is contained within the phagocyte. Scale bar = 5 μm (Adapted from Katzenmeyer and Bryers [67])

site of injury and mount an inflammatory response to clear wound debris and any pathogens introduced during the implantation procedure; later, they mediate the foreign body reaction, dictating the integration of the implant into the surrounding tissue [5]. The early response of MØ to a bacterial challenge is critical not only for the prevention of catastrophic infections but also to the proper healing and integration of the implant: persistent bacterial presence, even if asymptomatic, can cause prolonged inflammation at the implant site, leading to biomaterial degradation and/or damage to host tissue [110]. Unfortunately, the presence of implanted foreign bodies has been shown to increase host susceptibility to infections [8, 58]. Consequently, macrophage clearance of bacteria at the implant surface during the early post-implantation period is of key importance to the prevention of infections.

Activated MØ are most commonly categorized into two broad subtypes: classically activated (M1) and alternatively activated (M2) MØ. While M2 MØ are considered to play an important role in angiogenesis, neovascularization, and tissue

repair, M1 MØ are primarily responsible for microbial killing [83]. M1 MØ are characterized by markedly enhanced intracellular pathogen killing as well as the production of reactive oxygen and nitrogen intermediates and pro-inflammatory cytokines such as tissue necrosis factor (TNF)-α, interleukin (IL)-12, IL-1, and IL-6 [83]. In the murine system, M1 MØ are easily distinguished by their production of nitric oxide (NO) [56, 81]. M1 activation requires two distinct signals, interferon (IFN)-γ and a microbial stimulus such as bacterial lipopolysaccharide (LPS), both of which trigger the endogenous production of tissue necrosis factor (TNF)-α [36, 51, 83]. Interestingly, recent work has revealed that implant sites are characterized by a predominantly immunosuppressive microenvironment [57]. Areas of implants were shown to express high levels of anti-inflammatory cytokines IL-4 and IL-10, while lacking expression of TNF-α. Notably, IFN-γ, the primary signal necessary for M1 activation, was not detected near the implant site at any time point [57]. Considering the predominant role of M1 MØ in pathogen clearance, it has been suggested that such an immunosuppressive microenvironment may result in the susceptibility of the implant surface to bacterial colonization.

However, the importance of M1 activation on macrophage microbicidal function has never been fully elucidated in the context of implant-associated infections. In fact, controversies exist in the literature regarding the effect of M1 activation on macrophage phagocytic and microbicidal capacity. For example, Speert and Thorson [100] reported that IFN-c treatment diminished both the phagocytosis and killing of *Pseudomonas aeruginosa* by human monocytes, whereas Gratchev et al. [52] found that IFN-c/LPS treatment decreased the overall phagocytic capacity of human monocytes, but increased their killing of *Escherichia coli*.

There are a number of excellent papers that address modulating M1–M2 polarization using biomaterials (e.g., [4] review; [101, 102]), but almost all of these focus on manipulating MØ phenotype for the purpose of promoting tissue regeneration and healing; very few consider the goal of eliminating bacteria at the implant interface by enhancing MØ innate response.

The first such study was an excellent series of three papers that quantified the interaction between MØ and bacteria at the surfaces of materials that were designed to attract and modulate MØ behavior. Well before the concept of MØ polarization, Wagner et al. [113] describe the development of a series of biomaterials designed to promote MØ adhesion and subsequent activation. This paper presents the details behind the design and synthesis of the base PEG-g-PA copolymer, describes the surface modifications by peptides and Mab fragments using various PEO tethers, and provides results of surface analysis for the various materials. The efficacy of these various materials to control (*a*) random protein fouling and (*b*) bacterial cell adhesion and biofilm formation was also determined. A companion paper [110] evaluated the response of monocyte/MØ only (adhesion, cytokine expression, and oxidative burst) to PEG-g-PA copolymers that were modified with either (*a*) adhesion-promoting peptides (YRGDS, YRGES, and YEILDV) or (*b*) fragments of monoclonal antibodies specific to macrophage integrin receptors (anti-VLA4, anti-β1, anti-β2, and anti-CD64). Peptides bound to PEG-g-PA may facilitate macrophage adhesion, but they may also activate the cells, thus leading to inflammation.

Consequently, fragments of monoclonal antibodies (Mabs), known not to be involved in inflammatory stimulus, were also tethered to the based substratum. A third article [111] quantified MØ response to these decorated material interfaces, now in the presence of bacteria (*Staphylococcus epidermidis* and *Pseudomonas aeruginosa*). Materials modified with adhesion peptides marginally enhanced (2x) MØ attachment versus controls, but these materials tended to activate MØ to rapidly overexpress pro-inflammatory cytokines such that upon bacterial challenge they were less effective at phagocytosis. Conversely, PEG-g-PA materials modified by fragments of monoclonal antibodies significantly enhanced (7x) MØ adhesion, but initial "per cell" activation levels were markedly reduced compared to peptide-modified materials; thus these materials promoted significant phagocytosis upon bacterial challenge. MØ adhering to antibody fragment modified surfaces also exhibited sustained enhanced phagocytic response and higher bacterial killing efficiencies when compared with peptide-modified materials.

Park and Bryers [86] report a study on the effect of M1 activation on surface-adherent MØ interactions with bacteria relevant in implant-associated infections. To model the interaction of implant-adherent, M1-activated MØ with bacteria, an in vitro biomaterial platform was developed to evoke surface-adherent macrophage M1 activation, i.e., M1-activating ligands IFN-c and LPS were co-immobilized onto a model surface. Such a system was designed to evoke the specific activation of adherent macrophages—and macrophages only—by the surface-immobilized ligands, throughout the duration of the macrophage–bacteria interaction. Glass was silanized with a silane-PEG-biotin base layer, followed by an intermediate layer of streptavidin, to which biotin-conjugated ligands IFN-γ and LPS were coupled. These M1-activating ligands IFN-γ and LPS were immobilized to the substratum, each individually or in combination. Adherent macrophage response to the ligand-functionalized surfaces was evaluated using primary mouse bone marrow-derived MØ (BMDM). Finally, the phagocytic and microbicidal capacity of surface-adherent BMDM was assessed using the bacterium, *S. epidermidis*.

PEG–IFN-c/LPS-coated substrata enhanced the production of both IL-12(p40) and NO, indicating M1 activation of the adherent MØ. PEG:LPS, on the other hand, elicited an intermediate increase in the production of IL-12(p40) but did not markedly affect NO production, indicating a MØ phenotype distinct from M1 activation. Overall, ligand-presenting surfaces elicited lower levels of MØ activation compared to treatment with soluble ligands, which was attributed to differences in the total amounts of IFN-γ and LPS presented to the cells. The phagocytic capacity of IFN-γ-primed, innately activated, and M1-activated adherent macrophages was evaluated using live *S. epidermidis* cells. Although the differences were not statistically significant, a consistent trend toward lower phagocytosis (relative to untreated macrophages) was observed in M1-activated MØ at all time points (Fig. 6.6a). M1 activation has been shown to downregulate the expression of non-opsonic receptors such as mannose and scavenger receptors; Park and Bryers [86] suggest that non-opsonic pathways are important in the phagocytosis of *S. epidermidis*, since M1 activation appears to decrease their internalization. Bacterial killing assays were

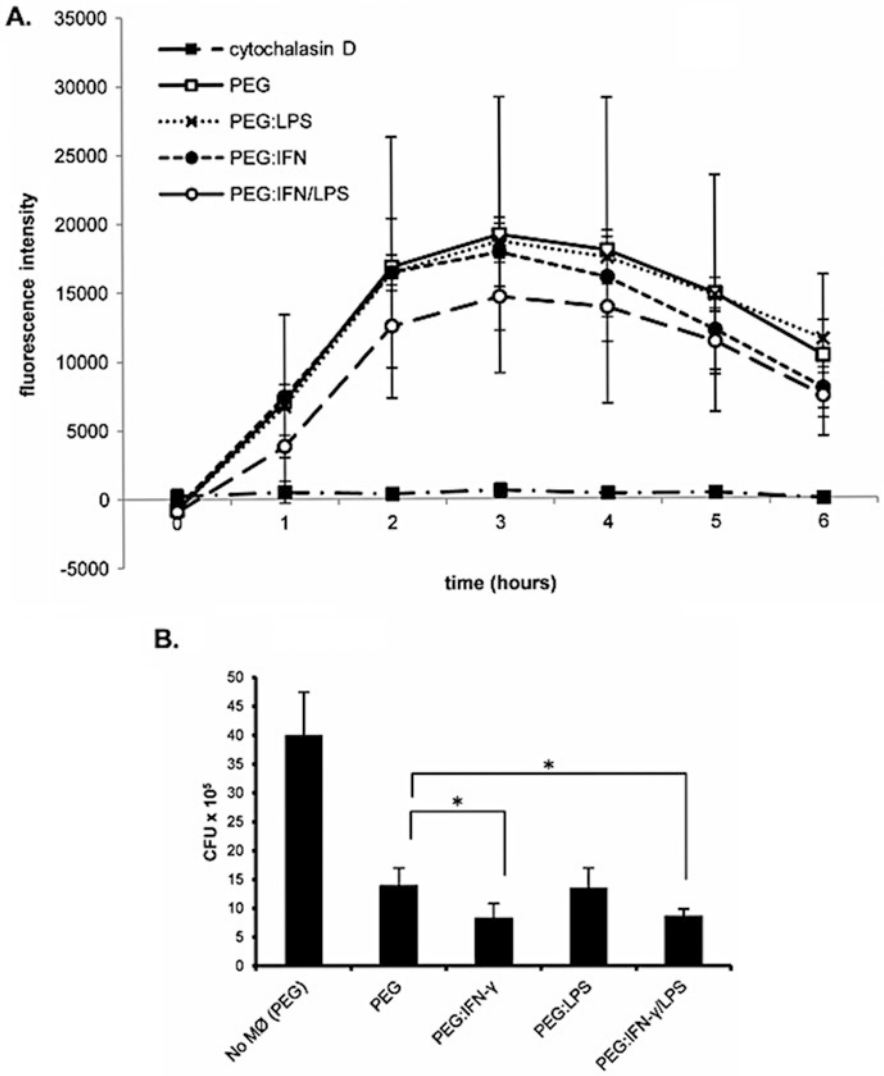

Fig. 6.6 (**a**) BMDM phagocytosis of *S. epidermidis* RP62A. BMDM were pre-cultured on the ligand-presenting surface for 24 h or with cytochalasin D (10 lM) for 1 h. pHrodoTM-labeled *S. epidermidis* RP62A cells were added to the macrophages at bacteria/macrophage ratio of 20:1, in 5% mouse serum, and incubated at 37 °C, 5% CO_2. The fluorescence of the wells (e.g., 550 nm/em 595 nm) was measured every hour for 6 h. All values are expressed as the mean value 6 SD ($N = 3$ for all treatments). One-way ANOVA was performed to test significance of differences among mean values; no significant differences were found (at $p < 0.05$). (**b**) BMDM killing of *S. epidermidis* RP62A. BMDM were cultured on the ligand-presenting surface for 24 h and then challenged with *S. epidermidis* RP62A at a bacteria/macrophage ratio of 1:1 in the presence of 5% mouse serum. After 4 h co-incubation at 37 °C, 5% CO2 BMDM were lysed, and surviving bacteria were quantitated via standard plate count method. Data is expressed as the mean colony-forming unit count (CFU × 10^5) per well ±SD. One-way ANOVA with post hoc testing was performed to test significance of differences among mean values. All macrophage-containing wells had significantly fewer surviving bacteria than bacteria-only wells ($p < 0.05$); *asterisks* (*) denote values significantly different relative to PEG ($p < 0.05$) (Reproduced from Park and Bryers [86])

also performed using a low bacteria: MØ ratio (1:1) (Fig. 6.6b). Compared to untreated macrophages, IFN-γ-primed and M1-activated MØ were shown to have increased ability to kill *S. epidermidis* cells.

6.4 Disrupting Biofilm Extracellular Matrix Amyloid Formation

Biofilms are surface-associated communities of microbial cells that are embedded in a microbe-generated extracellular matrix (**EM**) consisting of polysaccharides, proteins, and nucleic acids. Proteins in the EM can take on many structures, but the most commonly found is *amyloid*. While β-sheet amyloid fibers are the hallmark of human neurodegenerative diseases, increasing research suggests numerous Gram-positive and Gram-negative bacteria, including many multidrug-resistant (MDR) strains, e.g., *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*, resort to amyloid fibril formation to instigate bacterial aggregation, biofilm formation, adherence, and colonization of mucosal surfaces as an underlying mechanism of virulence [39]. As such, these functional amyloids, actually the soluble oligomeric intermediate precursors en route to fibril production, represent a novel target to prevent or disrupt biofilm formation.

6.4.1 Amyloid Fibril Formation Within Biofilm EM

Amyloids are remarkably stable protein polymers that form β-sheet-rich fibrils with a diameter of 5–10 nm. The *amyloid fold* is unique in that a range of proteins with totally different primary sequences can change structure, aggregate, and ultimately form the same fibrillar structures. The first step in amyloid fibril formation is aggregation of monomers into oligomeric intermediates, or "seeds" that share a common intermediate structure known as an α-sheet. Once seeds form, they nucleate fibril elongation, with the final amyloid structure being essentially a stack of β-sheet-rich monomers, aligned so that each β-strand is perpendicular to the fibril axis.

Because amyloid fibrils were identified in connection with various diseases, it was assumed that the fibrils themselves were toxic [25]. However, accumulating data suggest that mature amyloid fibrils are relatively inert, non-cytotoxic, and even protective. Instead, amyloid-related toxicity is caused by small soluble oligomers formed as an intermediate step in fibril polymerization. These structurally dynamic oligomeric intermediates can perforate lipid membranes [75]; hence disrupting existing fibrils is not advised. Thus, one novel approach to prevent biofilm formation would be by sequestering the α-sheet intermediates prior to their shift to the inert β-sheet fibril.

Microbes have harnessed the inherent toxicity of amyloid oligomers to kill surrounding cells or negate phagocytic cell chemotaxis. For example, the small hydrophobic microcin E492 (MccE492) is a soluble protein produced by *Klebsiella pneumonia*, and it exerts toxicity by forming pores in lipid membranes [39]. Fibril formation of MccE492 completely ablates toxicity to susceptible target cells. Changes in environmental pH also affect the aggregation of the listeriolysin O (LLO) protein of *Listeria monocytogenes*. LLO forms pores in the phagolysosome, allowing *L. monocytogenes* to escape into the cytoplasm during infection. Under alkaline pH, LLO can readily aggregate into fibril structures that bind the amyloid dyes thioflavin T (*ThT*) and Congo red. As with MccE492, LLO does not demonstrate pore-forming capabilities when in the fibril form. Indeed, since the small oligomers are considered the toxic species in amyloid formation, one obvious mechanism bacteria have for avoiding self-toxicity has been the rapid passage through the oligomeric stage to fibril formation.

Microbial amyloids are important in mediating mechanical invasion of abiotic and biotic substrata. In animal hosts, evidence indicates that these amyloid structures also contribute to tissue colonization by activating host proteases that are involved in hemostasis, inflammation, and remodeling of the host extracellular matrix (*ECM*) [39]. Activation of proteases by amyloids is also implicated in modulating blood coagulation. *Enterobacteriaceae* assemble adhesive amyloid fibrils termed *curli* at the bacterial cell surface to mediate cell–cell and cell surface interactions that promote bacterial adhesion to mammalian and plant cells as well as inert surfaces such as glass, stainless steel, and polymers. Curli also serve as an adhesive and structural component of the biofilm ECM [17, 27]. Amyloid adhesins and amyloid-integrated biofilms, in particular, are prevalent among diverse phyla (e.g., *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria*) that thrive in drinking water reservoirs and other environmental habitats. In addition to curli, bacteria can assemble hundreds of extracellular adhesive fibrils known as *pili*, which contribute to bacterial pathogenesis in the human host. Type I pili, crucial to uropathogenic *E. coli* infections, contain the *FimH* adhesin at their tip, which mediates binding to mannosylated receptors present on the luminal surfaces of mammalian bladder epithelial cells—an event that is critical in the pathogenesis of urinary tract infections.

Recently amyloidogenic extracellular fibrils composed of small peptides called *phenol soluble modulins* (*PSMs*) were identified as components in Gram-positive *Staphylococcus aureus* biofilms [96]. While *Staphylococcus aureus* is a commensal organism in the nasal pharynx, the species can also cause a variety of illnesses (minor skin infections, bacteremia, and sepsis), many of which involve biofilm formation within host cells. That PSMs form amyloid fibrils is particularly novel because soluble PSMs have a variety of reported functions. PSMs, isolated either from *Staphylococcus aureus* or *S. epidermidis*, reportedly recruit, activate, and lyse human neutrophils and can kill competing bacteria. Soluble PSMs also effectively act as a biofilm dissociation factor, but upon amyloid fibril formation, PSMs lose that ability. However, PSM fibrils are required for *Staphylococcus aureus* biofilms to tolerate (*a*) various dispersion agents (e.g., dispersin B, DNAse I, protease K) and (*b*) elevated mechanical

stress, again demonstrating functional roles in both the monomeric and fibrous states. The recently described *B. subtilis* amyloid TasA protein may also perform roles as a toxin and as a biofilm stability factor, as prior to its described amyloid properties, TasA was reported to display antimicrobial activity [93].

It was previously assumed that mycobacteria did not produce pili. However, Alteri et al. [3] recently reported that *Mycobacterium tuberculosis* produces fine (2–3 nm wide), aggregative, flexible *pili* that are recognized by IgG antibodies contained in sera obtained from patients with active TB, indicating that *Mycobacterium tuberculosis* produce pili or pili-associated antigen during human infection. Purified *Mycobacterium tuberculosis* pili are composed of low molecular weight protein subunits encoded by the *Mycobacterium tuberculosis* H37Rv ORF, designated *Rv3312A*. *Mycobacterium tuberculosis* pili can bind to the extracellular matrix protein laminin in vitro, suggesting that *Mycobacterium tuberculosis* pili possess adhesive properties. Isogenic pili mutants lose the ability to produce pili in vitro and demonstrate decreased laminin binding. *Mycobacterium tuberculosis* pili share morphological, biochemical (bind ThT and Congo red), and functional properties attributed to other bacterial pili, especially with curli.

6.4.2 Biofilm Prevention/Disruption with Amyloid Inhibitors

Romero et al. [93] screened a subset of bioactive molecules archived at the BIOMOL–ICCB Known Bioactives collection at the ICCB Longwood Screening Facility (Harvard Medical School, Boston, MA), and they found that two molecules, AA-861 (a benzoquinone derivative) and parthenolide (a sesquiterpene lactone), inhibited the formation of *B. subtilis* biofilms by preventing the assembly of TasA oligomers into functional amyloid-like fibrils. Both small molecules were also able to disrupt, to varying degrees of effectiveness, preformed 12-h-old *B. subtilis* biofilms, albeit at relatively high concentrations (100–200 μM), although this disruption may inadvertently disperse the toxic oligomers if carried out in vivo. Both small molecules were also able to inhibit biofilm formation of *B. cereus* and *E. coli* at high concentrations, but they had no effect on *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms. Cegelski et al. [27] recently reported on two different ring-fused 2-pyridones with pilicide activity that inhibited *EC* curli polymerization. This inhibition of both curli and type I pili was correlated with reduced virulence and a decreased ability of *E. coli* to colonize host tissues and form biofilms. They did not test these two compounds on preformed biofilms. The downside of these four amyloid inhibitors is that their inhibitory effects are not broad spectrum. While they inhibit the biofilm formation of the species to which they were screened, the molecules have no, or the opposite, effect on other species. AA-861 and parthenolide inhibited *B. subtilis* biofilm formation but stimulated amyloid production in other species; a similar reversal of effects was reported for the two ring-fused 2-pyridones when applied to species other than *E. coli* [27]. Finally, all four compounds alone (no bacteria present) exhibited significant mammalian cell cytotoxicity at the concentrations used.

6.4.2.1 Designer Amyloid Inhibitors

What is really required is an amyloid fibril inhibitor that is designed based upon the ability to bind to the soluble oligomer of any protein via recognition of a common nonstandard secondary structure shared by all oligomers. Structural similarities between soluble oligomers from a range of unrelated proteins have been demonstrated by generation of an antibody that recognizes a common backbone conformation [50]. However, the amorphous nature of the toxic oligomer prevents its high-resolution structural characterization. Atomistic molecular dynamics (MD) simulations, however, provide a means to obtain information about these states. Based on previous simulations, Daggett and co-workers [10, 37] proposed that toxic intermediates from different amyloid proteins adopt a common, nonstandard secondary structure called the *α-sheet* and proposed that the oligomer-specific antibody above [50] binds to this structure. The Daggett group has now designed, synthesized, and experimentally characterized a series of small peptides that adopt stable, monomeric α-sheet structure complementary to the α-sheet structure observed in amyloid proteins [60]. These α-sheet peptides are recognized by the A11 oligomer-specific antibody introduced above [50], and they inhibit aggregation in three different human amyloid systems: transthyretin, which is implicated in systemic amyloid disease and a major contributor to heart disease; the amyloid β-peptide, which is linked to Alzheimer's disease; and amylin (or IAPP), which is linked to type 2 diabetes. In effect, these anti-α-sheet peptide inhibitors are themselves α-sheets.

Our research group has hypothesized that the α-sheet structure in the toxic soluble oligomers associated with human diseases also forms during bacterial amyloid fibril production during biofilm formation. Thus, these designed α-sheet inhibitors should recognize and bind the bacterial soluble oligomers, regardless of the species and original protein structure since they assume a common structural intermediate form prior to fibrillization.

The effects of various anti-α-sheet peptide inhibitors on amyloid fibril formation and overall biofilm formation for a series of amyloid-forming bacterial species were quantified as a function of inhibitor concentration. After a 1.5 h inoculation of wells in a 24-well tissue culture plate with suspended cells, cell suspensions were aspirated and any remaining planktonic bacteria removed by gentle PBS rinses. Then, appropriate sterile fresh medium is added to each well, along with varying concentrations of the various amyloid inhibitors to be screened. Control wells did not receive an inhibitor. The shift in ThT (10 μM per well) fluorescence intensity upon binding to any amyloid fibrils (a classic fibril formation assay) within the biofilm matrix was measured directly from the microwell plates. Transmission EM and AFM images were collected on intact biofilm.

We have some preliminary data supporting our hypothesis that anti-α-sheet compounds inhibit aggregation and amyloid formation in bacterial systems and that our common α-sheet oligomer intermediate hypothesis is valid. We have tested a number of α-sheet inhibitors against a series of amyloid-forming bacterial species. We used the ThT binding assay described above to determine the ability of α-sheet designs to inhibit amyloid formation in a series of different bacterial species biofilms;

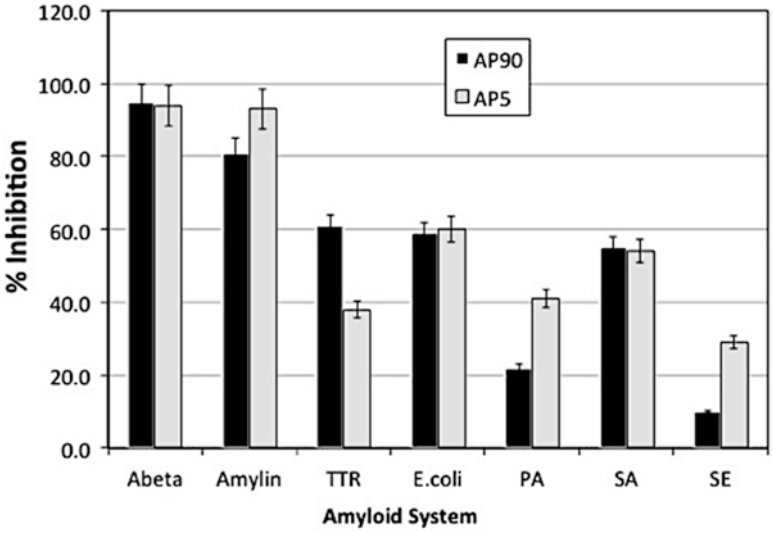

Fig. 6.7 Inhibition of amyloid formation in seven unrelated systems by two different α-sheet designs. Aβ and amylin aggregation were probed with ThT and TTR with Congo red, and the bacteria were assessed by crystal violet staining. Details, including SDs, have been presented for Aβ, amylin, and TTR; the bacterial results are very preliminary and merely suggest there is some effect upon adding α-sheet designs. Bacterial systems: *E. coli*, *P. aeruginosa* (*PA*), *S. aureus* (*SA*), and *S. epidermidis* (*SE*)

several designs did significantly reduce the amount of amyloid formed (Fig. 6.7), which also resulted in less biofilm formed in the case of *S. aureus* secreting PSMs (Fig. 6.8). It is important to emphasize that our best performing compound, A90, shows activity against both *S. aureus* and *P. aeruginosa*, bacterial species that express two totally different amyloid precursor proteins. This result supports our contention that the α-sheet structure is a critical intermediate in amyloid formation independent of the targeted proteins, sequences, structures, or even organisms.

6.5 Concluding Remarks

Existing anti-infective biomaterials can "deliver" anti-infective agents in one of two basic ways: agents are either directly tethered to the surface of the biomaterial or agents entrapped within the base biomaterial are released upon hydration into the adjacent surroundings. Unfortunately, no matter how lethal the anti-infective agent or how novel the "release" technology, anti-infective biomaterials based on toxic drug release *can never* provide active protection over the lifetime of indwelling implants. For example, heart valves can become infected by *Enterococcus faecalis* 20 years after implantation.

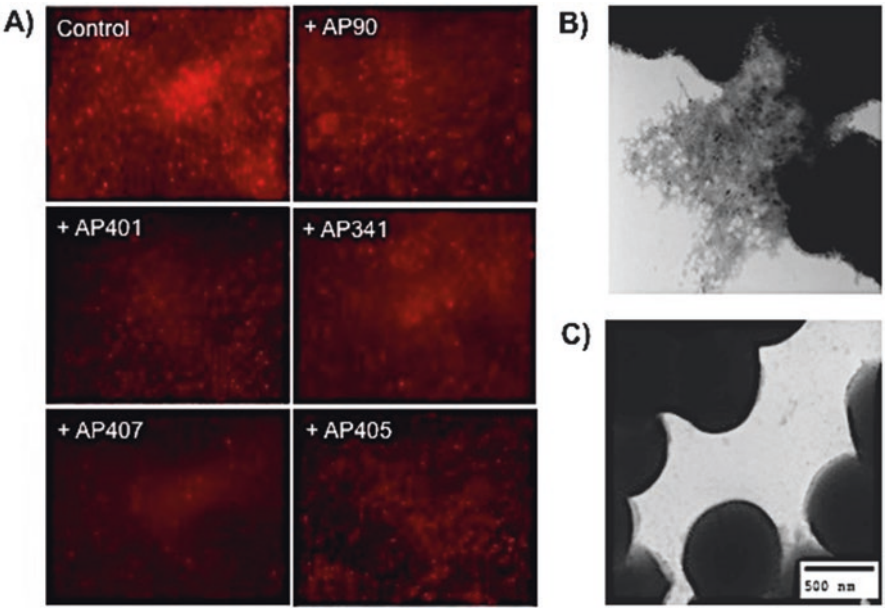

Fig. 6.8 *S. aureus* biofilm structures become less robust when grown in the presence of designed anti-α-sheet peptide inhibitors. (**a**) *S. aureus MN8* + mCherry biofilms were grown on glass substrates for 24 h and then cells were washed and fixed. Addition of peptide inhibitors (80 μM) reduced adhesion to the slide in some cases (e.g., design AP407), causing cells to detach during the wash step. Images are representative of duplicate wells. (**b**) In *S. aureus* SH1000 WT strain biofilms grown in regular LB medium, PSM amyloid fibrils are visible (TEM images) as deposits in spaces between cells. (**c**) Upon addition of the peptide design AP90 (80 μM), no extracellular fibril deposits were observed (Adapted from Bleem et al. in press)

What is required is anti-antibiotic or a non-killing anti-biofilm approach that seeks to negate bacteria colonization and reduce inflammation at an implantation site. Such approaches include (*a*) biofilm matrix disruption via DNA extraction; (*b*) quorum sensing interference; (*c*) immunotherapies, i.e., vaccines that target bacterial adhesins; (*d*) biomaterials that heal with such fidelity that they prevent infection similar to the natural healing process; (*e*) disruption of bacterial iron metabolism; (*f*) enhancing phagocytosis; and (*g*) preventing amyloid fibril production within the biofilm extracellular matrix; the latter three are detailed here.

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Chapter 7
Microporous Materials in Antibacterial Applications

Russell E. Morris

7.1 Introduction

Porous materials with pore sizes of less than about 2 nm are generally referred to as microporous solids. These materials form one of the most interesting and industrially important classes of compound, with large-scale industrial applications that cover a wide range of application [1, 2]. Porous materials comprise a wide ranging family of materials. They can be structurally well ordered with very well-defined pore sizes, or they can be structurally disordered with a wide variety of different pore sizes. Whatever the form of the pore distribution, it is generally the presence of pores that makes the materials so interesting, offering the opportunity to store active species within the pores and then release them at the appropriate time and at the correct rate.

One particular class of crystalline microporous material, the zeolite family, which is based on silicate chemistry, is used in different industries for a wide variety of purposes. However, there are several other types of microporous material that are of interest in the area of antibacterial coatings, and several of these are shown in Fig. 7.1.

Zeolites [3, 4] (porous aluminosilicates [5, 6], aluminophosphates [7, 8]) are perhaps the archetype for crystalline solids in this class. They are used in many different applications, from ion exchange materials in washing powders all the way through to catalysts in oil refining. Naturally occurring and synthetic zeolites have been studied extensively for many different types of gas manipulations and are particularly well known for separations (e.g., of O_2 from N_2 in air). As in many areas zeolites are the most advanced of the porous materials in terms of their utility in medical applications, including antibacterial coatings. However, several other types of material are also of great interest for antibacterial application.

Porous coordination polymers, generally built from metal ions connected by organic linkers (giving rise to the name metal-organic frameworks or MOFs), are a

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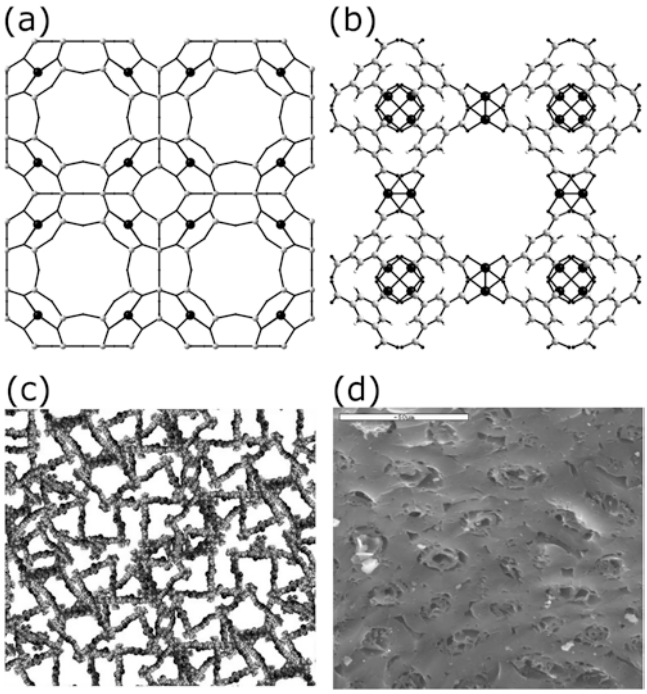

Fig. 7.1 Schematic representations of a zeolite with the dehydrated LTA structure (**a**), a metal organic framework with the dehydrated HKUST-1 structure (**b**), and a polymer of intrinsic microporosity (**c**). A scanning electron micrograph of a porous carbon (**d**) (Figure is reproduced from Ref. [31])

more recent addition to the ranks of highly crystalline porous materials [9, 10]. The large number of possible organic linkers combined with the quasi-infinite ways in which they can be used to connect metal ions and metal ion clusters leads to a huge range of potential materials. The most interesting feature of these materials is that they can be prepared as highly porous materials with internal surface areas exceeding 5000 m^2 per g in the most porous materials [11, 12]. This is significantly higher than zeolites, which typically have surface areas of several hundred m^2 per g.

The great advantage of highly crystalline materials is that they can be characterized extremely well using diffraction techniques to yield crystal structures—accurate three-dimensional representations of the time and space averaged structure, from which the maximum possible porosity can be calculated. This means that, in principle, the capacity of the porous solid is precisely known and that the payload of any active agent stored within the porosity is also precisely controllable.

However, the maximum porosity is not always accessible in practice perhaps because of problems removing guest molecules (which are present from the synthesis) from inside the materials, defects in the crystalline structure, or even the presence of impurities. For MOFs in particular, the rather lower thermal stability of the frameworks versus inorganic materials such as zeolites means that many potentially very interesting solids cannot be made at all porous because the structures collapse on thermal treatment before the guest molecules are removed. Early literature on MOFs

is littered with examples of reported "maximum adsorption capacity" that was lower than expected from the structure, caused most likely by incomplete removal of guest molecules [13]. Great care must be taken over this "activation" step to ensure as many of the guest molecules are removed as possible. However, there are now many examples where MOFs can be rendered highly porous, and these materials show great possibilities of storage and delivery applications [14–16].

Noncrystalline materials also have a great part to play in applications of porous materials. The most important of these are probably activated carbons, but nanoporous polymers are of increasing interest. They are often not so easy to characterize and perhaps do not have the visual impact of crystalline nanoporous solids, but each of these types of materials has its own particular advantages and disadvantages. Activated carbon [17, 18] is probably the original useful adsorbent material and has been known as a storage material for many years. The pyrolysis of any number of carbonaceous starting materials (coal, wood, coconut husks, etc.) can lead to polymeric materials with large surface areas, often well in excess of 1000 m^2 per g and even up to >3000 m^2 per g [19]. Unfortunately, the internal surfaces of activated carbons are often quite poorly defined in chemical terms, and the pore sizes can vary widely. However, this has not stopped them from being used extensively for filtering, adsorption, and other applications. In recent time, other carbon structures, in particular single-walled carbon nanotubes (SWCNT), have been prepared and their gas adsorption properties studied [20, 21].

Various types of cross-linked, network polymers can be prepared that possess intrinsic nanoporosity (sometimes called polymers of intrinsic microporosity, PIMs) [22, 23]. The great advantage of organic polymers is their wide range of chemical functionality (stemming from the great choice of monomers available) and their processability, which could lead to both tunable and easily manufactured and formed solids.

Some of the most interesting porous materials show other properties that greatly affect their gas adsorption and storage properties. Flexibility is one such property that promises to be extremely important in this context. Most inorganic frameworks are generally regarded to be fairly rigid, although even zeolites show some flexibility that gives rise to unusual effects such as negative thermal expansion [24, 25]. Most MOFs and carbons are also regarded as quite rigid. However, some notable MOFs, such as MIL-53 [26] and MIL-88 [27, 28], and many polymers exhibit considerable flexibility. Such properties clearly affect how much of any guest materials can be stored and can also affect how much, and under what conditions, they can be released [29, 30].

In this chapter, will look to explore how porous materials are used in antibacterial applications, explaining how the particular properties of the porous solid can be used to great effect.

7.2 Zeolites as Antibacterial Agents

The major property that marks zeolites as useful as antibacterial materials is their ion exchange capacity. The basic chemical composition of an aluminosilicate (the most important type of zeolite) is based on a silica framework (whose chemical formula would be SiO_2) where a proportion of the silicon atoms are replaced by aluminum. Each aluminum leads to a negative charge on the framework, which

needs to be compensated by a positive charge from a cation. These cations do not form part of the framework itself but sit in the pores and channels of the structure and are therefore mobile and can be exchanged out of the material as long as they are replaced by other cations [32]. Zeolites can therefore be viewed as excellent materials for the uptake, storage, and delivery of cationic species. This process is important in water softening applications (such as in detergent powders) where calcium ions in hard water are replaced by softer sodium cations.

In antibacterial applications, the dominant metal cations used are Ag^{+}, although there are several examples of Cu^{2+} and Zn^{2+} also being used [33–38]. The relatively low cost of preparing zeolites and the ease with which the cations can be introduced into the zeolites make them commercially feasible materials for antibacterial agents. Zeolites can accommodate both the oxidized silver cations (ie Ag^{+}) and nanoparticulate metallic silver (Ag^{0}) on reduction (Fig. 7.2) [39–41].

However, zeolites are also able to store other types of antibacterial agent. The gas nitric oxide, one of the body's own defenses against bacterial infection, has been stored very successfully in zeolites and used for several different applications [42, 43]. Interestingly there is little in the way of using zeolites to deliver antibiotic drugs, but much more on their use to adsorb antibiotics from water in a bid to reduce their presence in the environment [44, 45]. This maybe because the pores in zeolites are actually quite small and therefore more suited to the storage and delivery of small species (like cations or small gases) rather than drugs, which tend to be larger organic molecules. More promising materials for real drug delivery are the metal-organic frameworks discussed below.

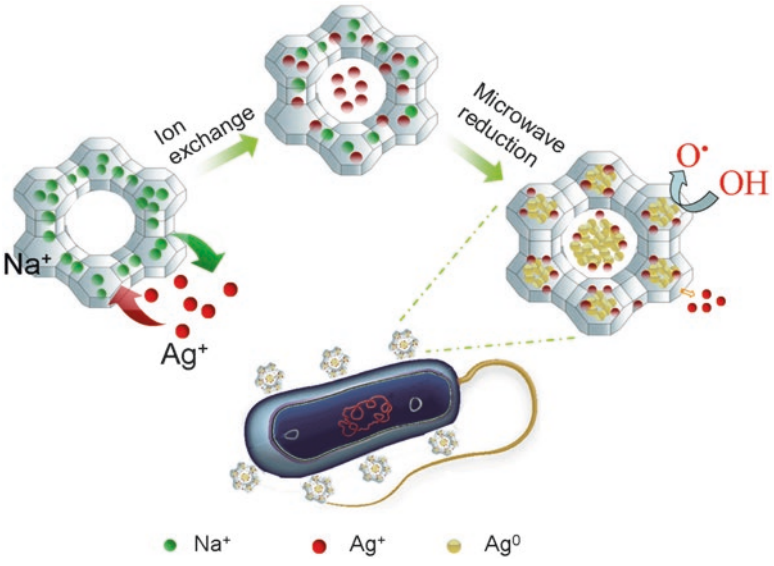

Fig. 7.2 An illustration of how, starting from the synthesized Na-zeolite, ion exchange can lead to Ag + encapsulated within the zeolite, which on further reduction can lead to nanoparticle silver metal (Ag^{0}) that can be used in antibacterial applications (Figure reproduced from Ref. [39])

7.2.1 Zeolite/Polymer Composites in Antibacterial Coatings and Medical Devices

Zeolites are generally prepared as powdered materials. Given their inorganic silicate-based composition, they suffer from an inherent lack of "processability." Therefore in order to form useful materials for application, the most common method is to incorporate the zeolites in composite materials with polymers. Zeolites have therefore been incorporated into many different polymeric materials [46, 47]. The literature in this area is extremely extensive, and the following discussion picks out only a small fraction of studies that have been undertaken.

Using inorganic compounds like clays and zeolites is extremely well known in polymer formulation chemistry, where they are often termed fillers. Composite zeolite-containing materials can be prepared by many of the standard polymer formulation techniques—spin casting, electrospinning, etc.—and the variety of polymers that have been "filled" with inorganics in this way is very wide indeed [46, 47] (Fig. 7.3).

Antibacterial materials such as zeolite/polymer composites have been utilized in three main ways: in wound healing dressings, as coatings for medical devices such as catheters, and as coatings for "consumer" goods.

7.2.1.1 Wound Healing Antibacterial Applications

The fact that silver cations or other antibacterial agents can be incorporated into zeolite/polymer composites offers the opportunity to develop dressings to prevent wound infection [49]. The advantage of encapsulating the antibacterial agents inside the zeolites is that this allows for more controllable delivery than if the compounds were directly incorporated into the polymers (which is of course also possible). Zeolites are already well-known additives in hemostatic interventions as contact of blood with zeolites can instigate clotting, which has been applied successfully in life-threatening trauma situations to staunch severe bleeding. QuikClot® brand zeolite products are the most famous in this area [50, 51].

The most prevalent use of zeolites in the antibacterial area comprises silver zeolites, but copper-exchanged zeolites have also been used [52]. Ninan and co-workers have been particularly prevalent in developing wound healing models using metal zeolites, with several studies of different zeolites. These have included studies using the zeolites clinoptilolite and faujasite, two readily available materials. These solids showed good antibacterial activity against a wide range of organisms (both gram positive and negative) and showed that composites with polymers such as gelatin are equally effective [53].Yu and co-workers showed that the antibacterial efficacy of silver zeolite-A/chitosan composites were effective against *E. coli*, reducing 9×10^6 CFU/mL to undetectable levels after 4 h [54].

In recent times, other antibacterial agents that can be stored in zeolites for wound healing applications have been of interest. Nitric oxide (NO) is one of the body's own defenses against pathogens, and there is evidence that lack of NO in the system

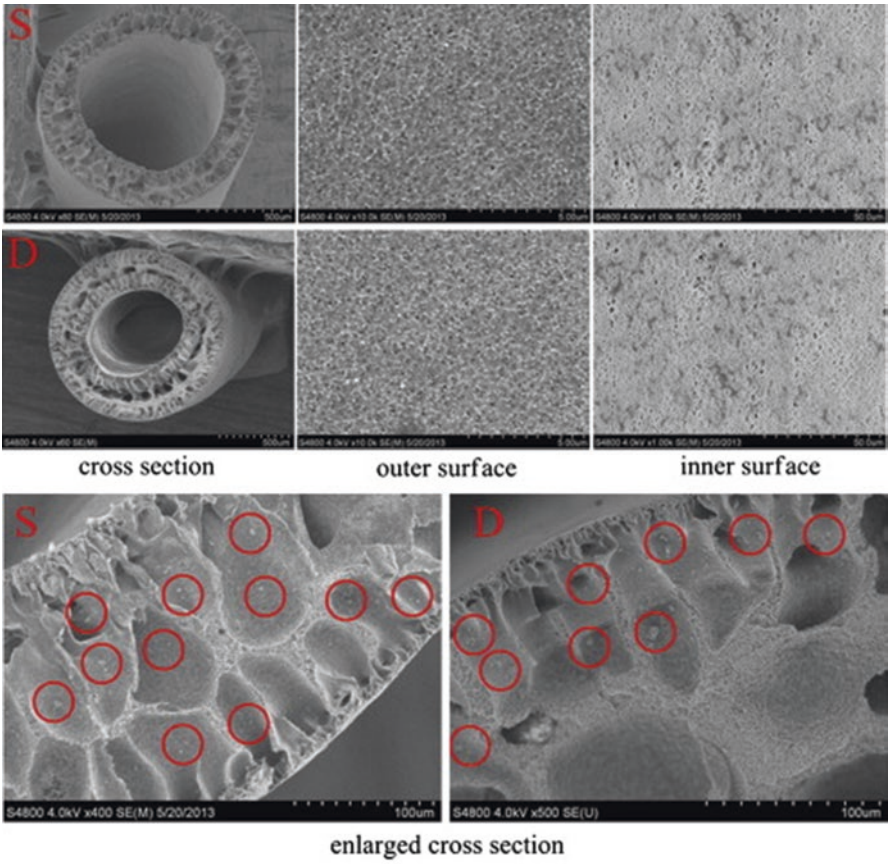

Fig. 7.3 Electron microscopy of hollow fibers of poly(vinylidene fluoride) containing silver zeolites. The different views show materials with zeolite particles throughout the polymer (*S*) and only on the outer surface (*D*). Individual zeolite crystallites can be seen in the enlarged cross sections (marked with the *red circles*) (Reproduced with permission from Ref. [48])

is correlated with poor wound healing, especially of infected wounds [55]. Wheatley and co-workers showed how zeolites could be used to store and deliver biologically significant amounts of NO [42, 56]. Others have since shown that delivery of NO to human skin using zeolites causes less inflammatory response than other methods [57] and that electrospun polylactic acid/zeolite composites could be used to release NO over significant time periods [58, 59].

Niedrauer and co-workers [60, 61] established that NO-releasing zeolites were strongly antibacterial against several different organisms (Fig. 7.4), reducing the colonies of bacteria to below detectable levels in most cases in a few hours, with log reductions of between 5 and 8.6 versus untreated colonies for *E. coli*, *A. baumannii*, *S. epidermis*, and MRSA. They also then showed that the NO-releasing zeolite had significant wound healing promotion effects in an obese rat study.

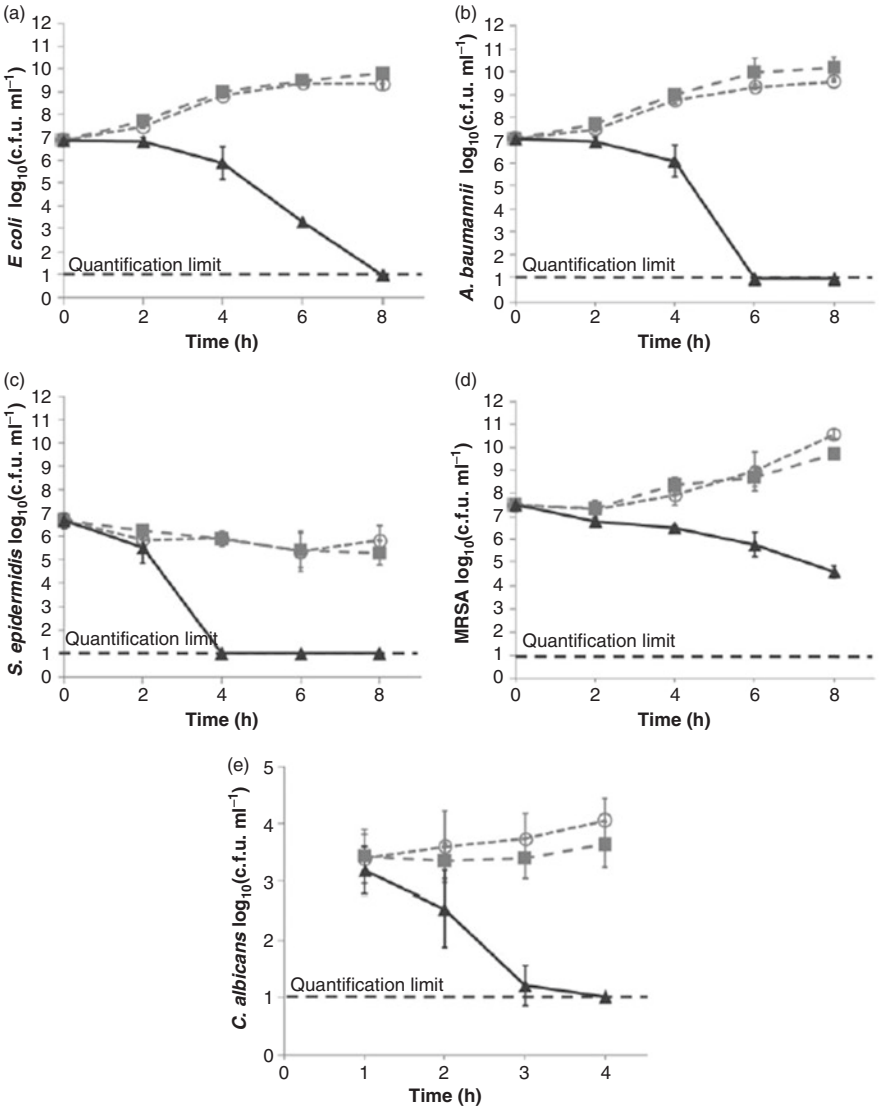

Fig. 7.4 The time dependency of antibacterial activity of NO-releasing zeolites against (**a**) *E. coli*, (**b**) *A. baumannii*, (**c**) *S. epidermidis*, (**d**) MRSA, and (**e**) *C. albicans*. The *solid triangles* are the results treated with the NO-zeolite other data are controls (Figure reproduced with permission from Ref. [60])

7.2.1.2 Coatings for Catheters and Other Medical Devices

It is a commonly held view that nosocomial, hospital-acquired infection is a severe challenge to patient well-being. The prevention of such infection is therefore an obvious target, and there are many examples of academic studies of the efficacy of silver zeolite-based coatings, as well as successful commercial trials.

There have been many academic studies looking at the antibacterial properties of silver zeolite/polymer composites. Many of these are really looking at the properties of the composites, rather than looking at the overall device effectiveness. However, from any of the laboratory-based studies, it is clear that the inclusion of the zeolites inside polymer films and coatings can be done without adversely affecting the antibacterial efficacy of the agents. This offers a very effective method of producing antibacterial materials.

In recent times, commercialized silver zeolite coatings (using the AgION™ technology) have been tested in trials to reduce catheter-related infections in adult and infant populations. These trials have shown some success. For example, in a trial of 86 infants where approximately half received the silver zeolite-coated catheter and the other half an uncoated polyurethane catheter, only 2% of the former developed infection, while 22% of the latter. This is a significant improvement that demonstrates nicely the potential of the commercial zeolite-coated catheters [62]. A similar study in adults also showed a significant reduction in bacterial colonization [63]. One study on the use of zeolite-coated catheters used in kidney transplants indicated a reduction from more than 50% of all uncoated catheters colonized to one where the incidence of bacterial colonization was about 6% [64].

7.2.1.3 Coatings on Nonmedical and Consumer Devices

Of course, antibacterial applications extend well beyond the direct healthcare sectors, and there are many reports of the use of zeolites as antibacterial paints on stainless steel, for instance. One interesting recent study looked at the effectiveness of silver zeolite as a coating for door handles, which showed a significant, but not complete, reduction in bacterial load over a significant time period [65].

7.2.2 Multifunctional Zeolite-Based Antibacterial Materials

A potential advantage of porous solids as delivery agents is the scope for multifunctional properties. That is, there is scope for the zeolite to do more than one job in any application. The two (or more) properties that can be utilized may both be antibacterial in nature, or one may be antibacterial and the other something completely different. For example, one of the most important applications of zeolites is as adsorbents. They are extensively used to remove odorous and toxic compounds from the environment. Sciessent, the company that produces the AgION technology described above, has used this application in combination with the delivery of silver ions as antibacterial agents to produce materials that both kill bacteria and adsorb malodourous chemicals (the so-called Agion Active XL™ technology).

In a similar vein, Fox and co-workers combined two antibacterial functions for zeolites, delivering both a metallic cation (in this case Zn^{2+}) and nitric oxide, and demonstrated that this could be an excellent bifunctional approach to antibacterial applications [66]. This type of approach may be particularly important where

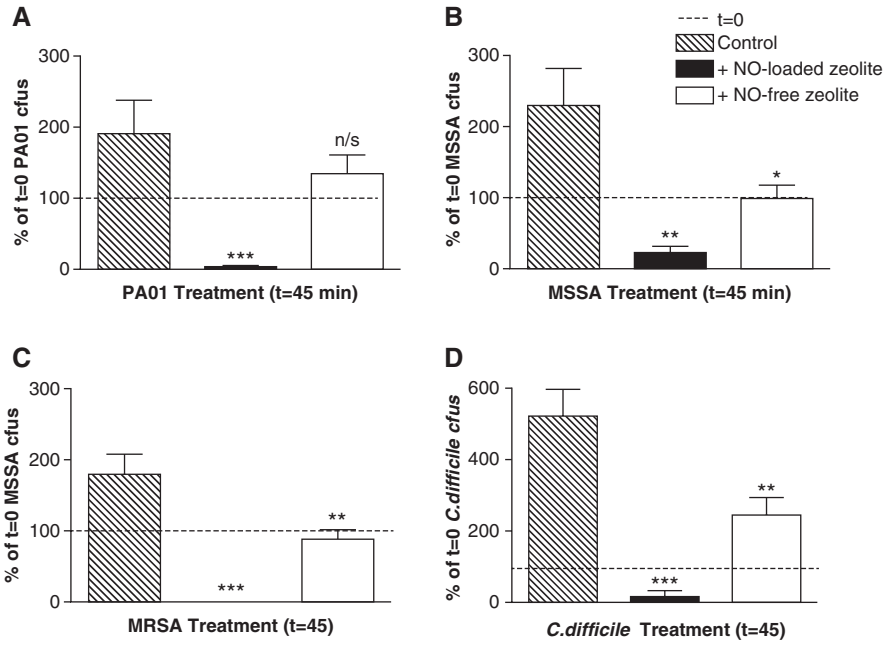

Fig. 7.5 The bactericidal effects of NO-loaded Zn^{2+}-exchanged zeolites against *P. aeruginosa* (PA01), MSSA, MRSA, and *C. difficile*. Bacterial growth following NO-loaded Zn-zeolite (*black bars*) and NO-free Zn-zeolite disk (*white bar*) treatments following incubation times of 45 min in suspensions (5% LB:PBS; 150 ll) of PA01 (**a**), MSSA (**b**), MRSA (**c**), and *C. difficile* (**d**; grown in 5% BHI broth to PBS). Data are expressed as % of cfus at $t = 0$ (*broken line*). *Bars* indicate the mean ± SEM of an average of 3–6 plates per treatment for $n = 3$–6 independent experiments. *Asterisks* denote statistical significance from control (*striped bar*) growth ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) (Figure reproduced from Ref. [66])

resistance is an issue, as having multiple modes of action may be beneficial in such situations. Figure 7.5 shows that the antibacterial activity of Zn-zeolite can be significantly improved by adding NO.

7.3 Metal-Organic Frameworks

Metal-organic frameworks (MOFs) are some of the most exciting chemical entities to have emerged in science over the last decade or so. They are solids comprised of metal or metal-cluster nodes linked by organic ligands into three-dimensional networks, which often have very high porosity in the nanopore (micropore to mesopore) regime. These materials have aroused much excitement for their ability to adsorb very large quantities of fuel vector gases such as hydrogen and methane, environmental gases such as carbon dioxide, as well as for their fundamental properties. Unlike the zeolites, which are essentially all of the same silicate-based composition, there is a huge variety of chemical composition in MOFs—it seems most

metals can be combined with suitable organic linking groups to form these unusual materials. This means that there is a very large set of solids to choose from, and many of these will have suitable chemical compositions that will make them applicable in biological situations. The field of "BioMOFs" [67, 68] is therefore beginning to become an important development in the field. Three MOF structures that have been of interest for BioMOF applications are shown in Fig. 7.6.

The great advantage of MOFs as delivery agents lies in their structural architecture and in their chemical (and structural) flexibility. In recent times, their very high adsorption capacities, coupled with their chemical flexibility, which allows the preparation of many toxicologically acceptable variants, has led to a burgeoning interest in using MOFs to adsorb and deliver large payloads of therapeutic agents, notably anticancer drug molecules [69, 70] and biologically active gases such as nitric oxide [13, 71–74].

The antibacterial properties of MOFs themselves are very little studied [75], but as they are generally known to be slightly unstable in aqueous solutions (although this varies from one MOF structure to the next), we would expect the MOFs to deliver metal ions into the environment and so act as antibacterial agents in their own right. However, the high porosity in MOF structures can also be used to store and deliver biologically active guests. One particular MOF, known by the acronym M-CPO-27, shows exceptional performance for the delivery of therapeutic gases such as nitric oxide (NO) and hydrogen sulfide (H_2S) [71, 76]. CPO-27 MOF, whose structure is shown in Fig. 7.6, is formed from linking metals (M = Mg, Fe, Mn, Co, Ni, or Zn) with a dihydroxy terephthalate linker. The biological properties of the metals range from very toxic (Co) to relatively benign (Fe and Mg). Metal ions such as Ni^{2+} and Zn^{2+} however have antibacterial behavior, and as MOFs are also well known to be somewhat unstable in aqueous solutions, we would expect the MOFs to deliver metal ions into the environment and act as antibacterial materials in their own right.

However, the very large pore sizes in MOFs make them even more useful than zeolites for the storage of larger organic molecules such as pharmaceuticals. In addition, the large porosity, coupled with the fact that the MOF itself can be made of antibacterial metal ions, offers even greater scope for multifunctionality [77].

A recent study by McKinlay and co-workers [77] showed how a CPO-27 MOF could be used to adsorb, store, and deliver an antibiotic drug (metronidazole) and a

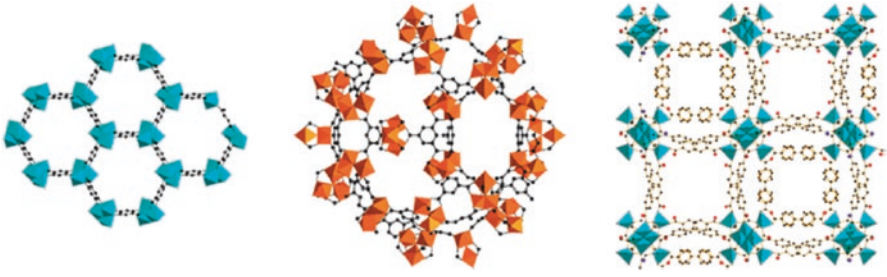

Fig. 7.6 View of the structures of a few topical MOFs, here CPO-27(Mg, Zn) (*left*), MIL-100(Fe) (*center*) and Bio-MOF-1 (*right*), based on exogenous linkers for bioapplications. Metal polyhedra and carbon atoms are in *blue* (Zn, Mg) or *orange* (Fe) and *black*, respectively

different antibacterial guest, nitric oxide (Fig. 7.7). Importantly, the various agents are released at different rates (Fig. 7.8) giving the antibacterial activity shown in Fig. 7.9, so that the time scales of action are complementary. The different therapeutic agents also have different modes of action, which can mitigate against resistance or inactivity toward any particular agent. Such approaches offer great potential for the development of new types of antibacterial solid. The results from antibacterial testing showed excellent potential for these materials, comparing very well with standard antibiotic compounds and in several cases showing considerably enhanced activity.

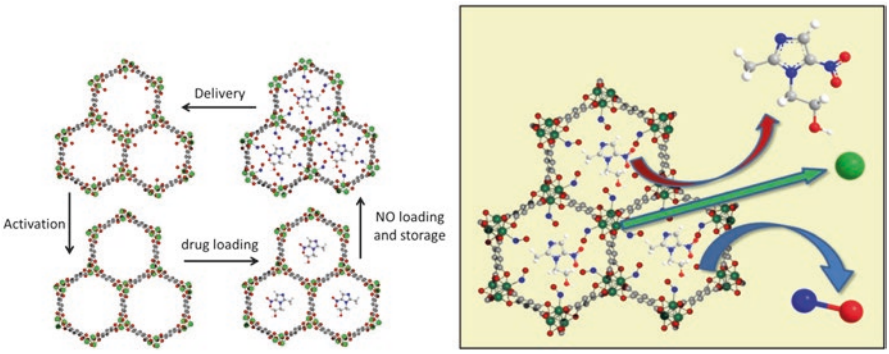

Fig. 7.7 The assembly strategy for loading of multiple therapeutic agents in MOFs. The loading of the guests is a stepwise process; activation (the thermal dehydration) followed by loading of a drug (in this case the antibiotic metronidazole). A further activation step (not explicitly shown) is then followed by loading of the gas (in this case nitric oxide). Delivery of the therapeutic agents is then triggered by exposure to water (Figure reproduced from Ref. [77])

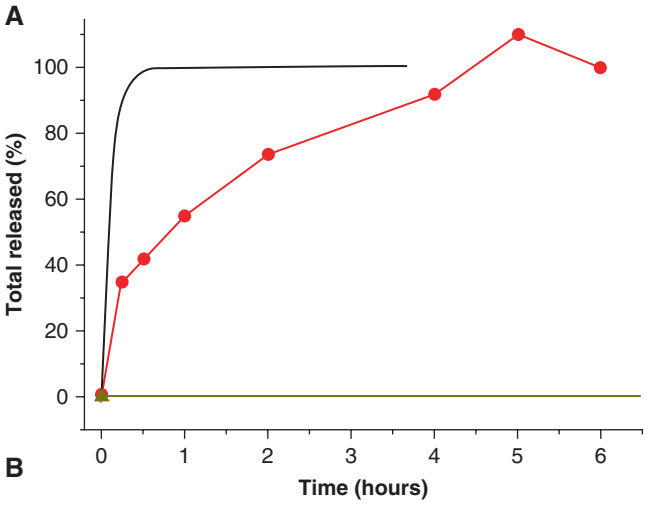

Fig. 7.8 Relative rates of release of NO, metronidazole, and metal ions from Ni-CPO-27 key. *Black line* NO release, *Red line* metronidazole release, *green line* Ni release, *blue line* Cu release. <4% of the Ni is lost after 6 h. Note that the error on the drug measurement is about ± 5%. Errors on the NO and metal release are much smaller (~1%) (Figure reproduced from Ref. [77])

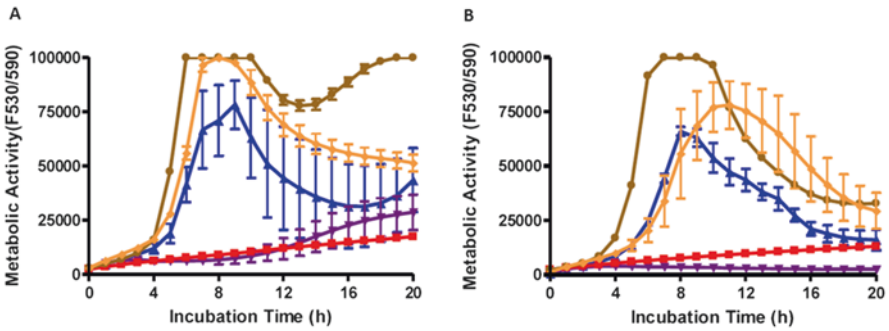

Fig. 7.9 The antibacterial activity of Ni-CPO-27 against planktonic *P. aeruginosa* and *S. aureus,* respectively. Key for (**a**) and (**b**): *Brown line* growth control, *Blue* MOF only, *Orange* metronidazole-loaded MOF, *Purple* NO- and metronidazole-loaded MOF, *red line* antibiotic control (Figure reproduced from Ref. [77])

7.4 Noncrystalline Porous Materials: Carbons and Mesoporous Silicas

The zeolites and MOFs described in the previous sections are both classes of crystalline materials. This means the pores in the materials are all nicely ordered, and the solids display very narrow pore size distributions. This is not necessary for many applications, and there are many examples of other types of porous materials being used in antibacterial applications. The most common of these are the carbon materials, often referred to as activated carbons [78]. Unlike zeolites activated carbons tend to be uncharged, and therefore incorporation of silver into the materials tends to be as nanoparticles. The lack of electrostatic interaction between the carbon and the silver particles can cause some issues with fast, uncontrolled release of silver. However, there is such a large set of materials prepared from widely different precursors and with different porosity characteristics that carbons are still a very widely studied class of solid for antibacterial applications, especially as delivery agents for silver. Figure 7.10 shows electron microscope images of silver nanoparticles encapsulated in a carbon aerogel [79]. Such materials are effective antibacterial agents.

Mesoporous silicas, a class of solid originally prepared for use in the oil industry, are zeolite-like in the chemical composition, but rather than having crystalline arrangements of atoms, they are atomically disordered walls [80]. However, the pore sizes tend to be significantly larger, allowing larger molecules to be delivered from the porous materials. There are, of course, many examples of silver nanoparticles incorporated into mesoporous materials, but there are also examples of mesoporous silicas being used to store and deliver other metallic and metallic oxide nanoparticles, antibacterial gases such as nitric oxide, and larger organic antibacterial agents such as antibiotics [81, 82]. Figure 7.11 shows a complex synthesis technique which uses carbon nanotubes as templates for mesoporous silicas that are used to host nanoparticle titania [83]. The carbon is removed at the end of the process to leave a hollow mesoporous silica tube that shows photocatalytic antibacterial activity against *E. coli*.

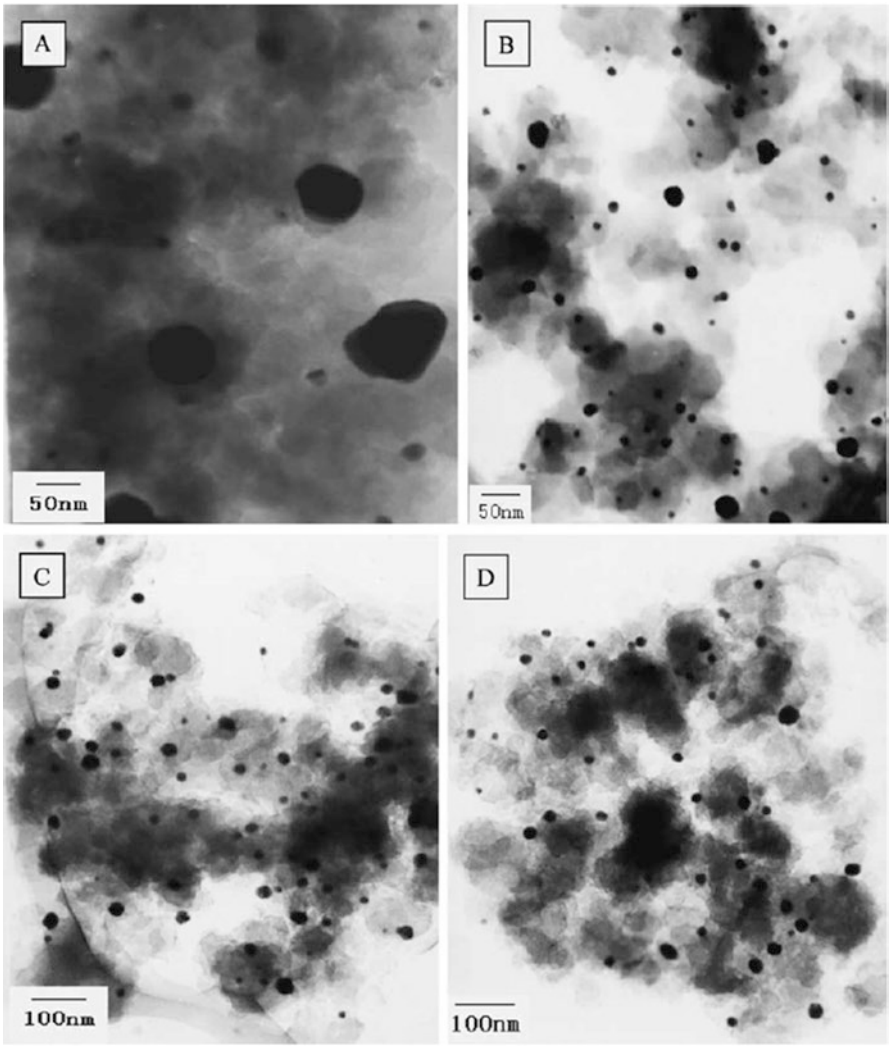

Fig. 7.10 TEM micrographs of silver nanoparticles dispersed in carbon aerogels (Figure reproduced by kind permission from Ref. [79]). The four samples show the different sizes of nanoparticle available using different conditions. (**a**) shows large particles prepared using high temperature carbonization; (**b**) shows smaller particles prepared at lower temperatures; (**c**) and (**d**) show intermediate size particles prepared at intermediate temperatures

7.5 Conclusions and Future Directions

Zeolite-based porous materials have already made a significant mark on the landscape of antibacterial products. Their growth in utility has been remarkable over recent years, and there seems considerable potential to increase the situations in which they are applicable. The rise of metal-organic frameworks as materials of

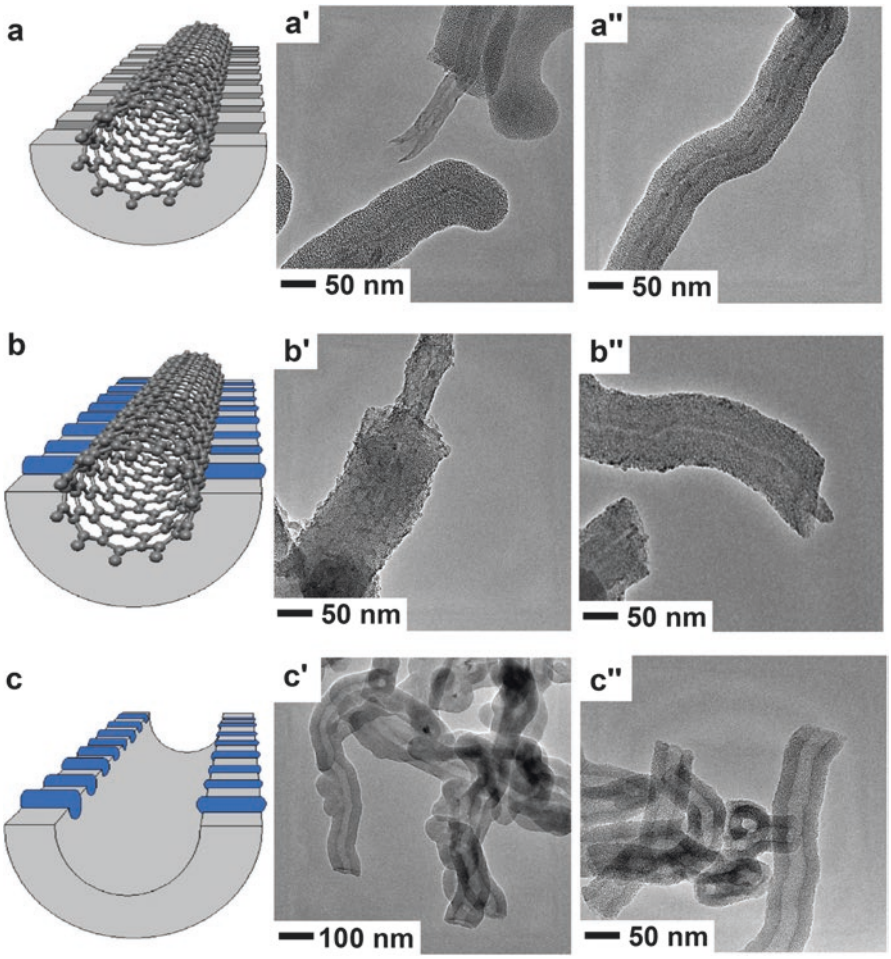

Fig. 7.11 Schematic and high-resolution transmission electron micrographs of the formation of titanium-incorporated mesoporous silica nanotubes. In *step a*, the mesoporous silica is formed around a carbon nanotube template. In *step b*, the titanium nanoparticles (shown in *blue*) are incorporated into the silica, and in *step c*, the carbon nanotubes are removed to leave hollow silica nanotubes (Figure reproduced by kind permission from Ref. [83])

great interest for many different applications is a much more recent phenomenon. However, the sheer range of different structures available, including many that will be toxicologically very suitable for medical and environmental applications, offers a real chance to develop a great many new uses and concepts in biologically active solids including antibacterial materials. I believe that this area will be one of the significant growths in the next few years.

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Chapter 8
Anti-fouling Medical Coatings

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8.1 Introduction

Biofouling is the gradual deposition and growth of undesired biomolecules, cells, and living organisms including bacteria, fungi, protozoa, algae, and invertebrates on a surface. The attachment of such biologicals to a surface facilitates subsequent microbial colonization resulting in the formation of self-organized multicellular communities called biofilms that typically develop at the solid–liquid interface of a large variety of living and inanimate substrata [1]. The formation of biofilms poses a serious problem in many industrial, environmental, and medical applications. For example, biofilm on heat exchangers, filters, pipelines, or separation membranes opposes heat and mass transfer and increases frictional resistance [2], resulting in decreased production rates and increased operation costs. In marine environments, biofouling has been a problem in particular for the shipping industry since ancient times because it increases drag on a moving vessel, thus resulting in longer voyages and higher fuel consumption by up to 40%. Biofouling by bacteria in particular raises an even greater concern in biomedical applications, such as biosensors, biomedical implants, and institutional equipment. Bacterial biofilm formation on medical devices like artificial organs, knee and hip implants, voice and vascular prostheses, catheters, and contact lenses poses a significant risk of infection and can not only reduce functionality or cause outright device failure—which might lead to the removal and replacement of the contaminated devices—but can also increase additional healthcare costs due to prolonged antibiotic therapy [3]. More seriously, medical device biofouling leads to increased mortality [4].

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A variety of concepts and approaches have been developed in modifying surfaces to be anti-fouling to bacteria; each strategy is broadly being categorized as either biopassive or bioactive [5–8]. Biopassive, also known as "nonadhesive" or "repellent," surface modifications aim to prevent the initial adhesion of bacteria. The alternative approach, bioactive surface modifications, actively kills bacteria in the vicinity or immediate contact with the surface. This approach typically involves the use of biocides such as quaternary ammonium compounds, *N*-halamines [9], antimicrobial peptides and antibiotics [8, 10, 11], or broad-spectrum antimicrobials like silver ions [12] and nitric oxide [13]. These biocides can be covalently bound to the surface or physically entrapped or adsorbed onto the surface and released into the environment. The use of biocides, however, can pose a substantial environmental risk, one example being the use of tributyltin [14] in past marine coatings—the toxicity of which leads the collapse of vast populations of marine organisms. In the medical field, antibiotic therapy resulting in incomplete eradication of biofilm has been linked with the emergence of antibiotic-resistant bacteria, which ultimately compromises the effectiveness of these agents—even for non-biofilm-mediated infections [15–17]. In fact, the use of the antibiotics has been shown to support the formation of methicillin-resistant strains of *Staphylococcus aureus* (MRSA), which cause the majority of nosocomial infections and notably more deaths in the United States than HIV [18].

In addition to the above two methods, there is another approach called fouling release, but it is only used in some areas. One example in marine applications applied silicone-based paint to release accumulated biofouling, but required vehicle speeds of 10–15 knots to impart the mechanical shearing action required for foulant release [19]. This approach will not be discussed in this chapter. Instead, this chapter will focus on the application of "nonadhesive" polymers in making anti-fouling (non-fouling) surfaces for medical devices.

It is believed that most of biofouling begins with adsorption of proteins [20–23]; therefore the intervention of this initial adsorption is critical in making a surface anti-fouling. Both hydrophilic and hydrophobic polymers have been studied and used for anti-fouling applications in biomedical areas, either as bulk materials or more widely used only as surface materials to generate anti-fouling surface layer through surface modification while retaining favorable bulk properties.

8.2 Surface Modification with Polymers

Surface modification with polymers is the simplest and most universal method to change the surface characteristics of a bulk substrate and achieve a "nonadhesive" performance. In general, surface modification of medical devices with polymers can be divided into two general categories based on the interaction between the bulk materials and surface polymers: physical adsorption and chemical attachment.

8.2.1 Physical Adsorption

Physical adsorption involves depositing a layer of anti-fouling polymer onto a substrate surface without the formation of covalent bonds between the substrate and polymeric film. The polymer film can be simply prepared by immersing a substrate surface into polymer liquid to form one or more monolayers of Langmuir–Blodgett film [24] or made by various traditional coating techniques such as spin coating [25], dip coating [26], spray coating [27], and roller coating [28]. The polymers can be homopolymers, random copolymers, or block copolymers, generally dissolved in appropriate solvents before applying on the substrates. Another method, known as layer-by-layer, involves depositing alternating layers of oppositely charged materials on solid surfaces [29]. Here, positively charged and negatively charged polyelectrolytes are used to form multilayer thin films with wash steps between each deposition. Depending on the technique used, the obtained polymer film can be as thin as a few nanometers (Langmuir–Blodgett film or layer-by-layer) or as thick as several millimeters (dip and spray coating). As there is no chemical bonding, the films are maintained by the weak forces between the substrate and polymer layer, mainly van der Waals forces, hydrogen bonding, and/or charge interaction. Therefore these coatings generally suffer from poor stability. These surface modification techniques are, however, simple to fabricate in large scale with low cost to commercialization.

8.2.2 Chemical Attachment

With regard to chemical attachment, the modification polymer layer is covalently linked to the substrate via a chemical reaction, and the obtained polymer film therefore generally exhibits improved robustness and stability. A model modification of this type is a self-assembled monolayer (SAM) [30], where polymer/oligomer molecules are organized with their head groups chemically attached to the substrate surface and their tail groups assembled from the attached head groups to form an "ordered" structure. SAMs can be formed in liquid as well as in vapor. They are easy to make, offer a good control of surface density and length, and contain fewer defects—making them an excellent candidate for mechanism study. SAMs are compatible only with specific substrates, however, such as silicon/glass or metals. Instead, graft polymerization, where polymer chains are covalently attached at one end to a surface, is the most conventional and versatile method to get a stable polymer layer. It can be divided into two types, "graft-from" and "graft-to" [31] strategies. In "graft-from" method, an initiator is first covalently bonded to the surface, from which the polymer chains grow [32]. It is therefore also known as surface-initiated polymerization. With this approach, the generation of surface-bonded initiator is usually the key step as it may involve multistep traditional chemistry reactions or high-energy irradiation such as UV [33], plasma [34], corona [35],

gamma radiation [36], electron beam [37], and ion beam [38]. Alternatively, in the "graft-to" method, polymer is synthesized first and then reacted with substrate to attach to the surface [39]. Due to the relatively big size of polymer chains, the grafted density and chain length are generally limited in "graft-to" method. To obtain the surface with well-defined graft polymer architectures, controlled polymerization techniques including atom transfer radical polymerization (ATRP) [40, 41] and reversible addition fragmentation chain transfer (RAFT) [42], nitroxide mediated radical polymerization (NMRP) [43], or ionic polymerization [44, 45] have recently been widely adopted.

8.2.3 Chemical Vapor Deposition

Differing from the above approaches, chemical vapor deposition (CVD) polymerization converts vapor phase monomers into polymer coatings in a single step [46, 47]. Compared with conventional graft polymerization, which is not practical on certain substrates due to the use of solvents and/or elevated temperature, CVD can be applied to nearly any substrates as it is solvent free and occurs at relatively low temperatures. In addition, CVD has other advantages including conformal coverage regardless of complex geometry of the surface and simple scale-up and is substrate independent. It is the chemical reaction that makes it different from physical vapor deposition such as evaporation and sputtering for small organic molecule coatings (polymers are not used due to their decomposition in vaporization). The reactions in CVD processes can occur in the vapor phase and/or at the surface. In CVD process, the polymer films range from as thin as a few nanometers to as thick as hundreds of micrometers, covalently bonded to the surface and/or in cross-linked organic networks. To obtain polymers by chain growth reactions, generally a volatile initiator is introduced in vapor phase together with the monomer, which can then be activated by heat (initiated CVD or iCVD) [48], UV light (photo-initiated CVD), or plasma (initiated plasma-enhanced CVD or iPECVD) [49, 50]. Oxidative CVD (oCVD) [51], vapor-phase polymerization [52], molecular layer deposition [53], and oxidative molecular layer deposition [54] are used to make polymers from step-growth polymerization through functional groups.

8.2.4 Plasma-Enhanced Chemical Vapor Deposition

Instead of using initiator, plasma-enhanced chemical vapor deposition (PECVD) was developed to utilize the volatile monomer fragmentation created by plasma to self-initiate deposition polymerization [55]. Therefore, a wider range of monomers including those for iCVD and other gases that react upon fragmentation are used in this method. At the same time, the fragmentation of monomers may lead to branching, a higher degree of cross-linking, and some defects such as reactive unpaired

electrons. Fragmentation can be limited by reducing plasma power and/or the use of pulsed, atmospheric, or downstream PECVD; although these processes may also reduce the film growth rate. It should be pointed out that PECVD is different from plasma treatment to make grafted polymer brushes for chemical linkage/attachment. In PECVD the monomer [56] or polymer [56–58] is either coated on the substrate followed by plasma treatment to bond it to the surface, while in plasma treatment active species are created by plasma to initiate a subsequent graft polymerization with the addition of monomers [59–62].

8.3 Anti-fouling Polymers

8.3.1 Hydrophilic Polymers

Hydrophilic polymers, so named due to their chemical affinity to water, have a long history in biomedical applications. With their ionic or other functional groups, hydrophilic polymers are able to participate in dynamic bonding with surrounding water. As a result, a hydration layer of water molecules from the environment is formed on the polymer surface [63]. This hydration layer works as a physical and energetic barrier to resist protein adsorption, leading to reduced bacterial adhesion.

It is proposed that water molecules residing on and/or penetrating into hydrophilic anti-fouling materials maintain "surface-bound" water via either hydrogen bonding or ionic solvation. Ionic solvation is thought to be a stronger interaction than simple hydrogen bonding. Expulsion of water molecules from both the polymer surface and protein is the first and obligatory step in permitting protein adsorption (in so doing, reducing the free energy barrier from dehydration entropic effects [64]). The strength of surface hydration is primarily determined by the physiochemical properties of a material (i.e., molecular weight and surface chemistry) and its surface packing (i.e., film thickness, packing density, and chain conformation [65]). Aside from surface hydration, chain flexibility also plays an important role in protein resistance, especially for long-chain polymers. When proteins approach the material surface, the physical compression of the polymer chains also causes steric repulsion—further resisting adsorption due to an unfavorable decrease in entropy [66]. Although most of water-soluble polymers can reduce protein adsorption to some extent, the best anti-fouling ability of polymers can only be achieved when surface hydration and steric repulsion work together.

Many medical devices are in general hydrophobic and have relatively high coefficients of friction that sometimes compound the risk of bacterial colonization. Hydrophilic polymers, particularly hydrogels made of cross-linked hydrophilic polymers to absorb water up to several times of their own weight without dissolving, are used to modify the surface of medical devices with improved wettability and lubricity. Some devices modified with hydrophilic coatings also exhibit reduced bacterial adhesion. For example, medical devices such as catheters and guidewires

benefit from this type of lubricious surface treatment because it reduces the insertion force and allows them to transverse the vasculature more easily, avoiding possible puncture damage and severe abrasion between the device surface and vessel walls and reducing the potential for thrombus formation. Common guidewires such as the Terumo Glidewire are known to employ hydrophilic coatings for this purpose. Polyvinylpyrrolidone (PVP) hydrogels have exhibited reduced bacterial adhesion on indwelling urinary catheters [67]. PVP hydrogel coatings on Pellethane central venous catheters were shown to reduce protein adsorption and bacterial attachment as well as decreased surface roughness [68]. Recently, Telford et al. reported anti-fouling behavior comparable to poly(ethylene glycol) (PEG) from cross-linked PVP thin films made via spin coating followed by simple thermal annealing [69]. Thin layers of PVP polymer brushes made by surface-initiated ATRP on silicon or gold also showed promising protein resistance for use in biosensors [70, 71]. Similarly, gold and silicon modified with hydrophilic polyacrylamide brushes by ATRP were shown to exhibit substantial reduction to the adhesion of Gram-negative and Gram-positive bacteria, as well as ultralow protein adsorption [72, 73].

Among all the hydrophilic polymers, poly(ethylene glycol) (PEG) systems are the most extensively studied for their anti-fouling properties in resisting protein adsorption and cell adhesion. Some initially attributed its non-fouling properties to the steric exclusion effect [64, 65], although more recently it has been generally agreed that its hydration ability or hydrophilic nature plays a key role in its non-fouling properties [74, 75]. Molecular simulation of oligo(ethylene glycol) (OEG) self-assembled monolayers (SAMs) has shown a tightly bound water layer around OEG, generating large repulsion forces toward proteins approaching its surface [76, 77]. Different attachment strategies have been developed for optimal PEG coverage and surface stability to prevent adhesion of bacteria. Kingshott et al. demonstrated that a PEG layer covalently bonded to a substrate can reduce bacterial adhesion at least two orders of magnitude better than previously reported surfaces coated with physio-adsorbed PEG—probably due to inherently improved stability and high coverage [78]. A multicomponent, cross-linked PEG-based coating which combined covalent surface attachment and internal matrix cross-linking chemistries developed by Harbers et al. inhibited the non-specific adsorption of proteins, bacteria, and mammalian cells [79]. Prime and Whitesides made a breakthrough in developing non-fouling surfaces based on SAMs with OEG groups [66, 80]. Using mixed SAMs from thiols such as $HS(CH_3)_{11}(OCH_2CH_2)_nOH$ and $HS(CH_2)_{10}CH_3$, highly protein-resistant surfaces were generated. While previous studies had suggested that only surfaces grafted with very long PEG chains can resist protein adsorption [81–83], Prime and Whitesides reported remarkable protein resistance with SAMs of alkanethiolates with as few as two ethylene oxide groups. The benefit of using long chains is that they can cover the surface more effectively. In the case of SAMs, it is possible to incorporate a large number of chains per unit surface area than with most other grafting technologies, thus making it possible to obtain a more effective surface coverage even with shorter chain lengths. In addition, Prime and Whitesides found that replacing the terminal-OH of PEG by more hydrophobic-OCH_3 did not decrease the ability of the surface to resist protein adsorption [66]. Using single-chain

mean field theory to explain the protein resistance of SAMs from shorter OEG chains, Szleifer et al. proposed that the density of polymer molecules in the region close to the substrate is the key parameter to determine protein resistance of the PEG layer [84]. The kinetics of protein adsorption, however, depends on the thickness of polymer layer. In graft polymerization, only a limited number of polymer chains can be attached per unit area. Therefore, the increased polymer layer thickness by the increase of chain length creates a kinetic barrier to prevent protein adsorption to the surface. Even though SAMs are by far the best known systems in terms of ease of fabrication and excellent surface coverage to generally result in lowest protein adsorption than other surface techniques, they are prone to defects and lack of robustness [85, 86]. On the contrary, PEG or other polymers covalently attached to the substrates are robust, but their protein resistance is not as effective as SAMs [85]. Therefore, an approach combining of the advantages of high surface density and easy formation from SAMs with thicker and more robust attachment from grafted polymers was proposed by Chilkoti et al. [86]: ATRP initiator with mercaptoundecyl group was used to form SAMs, followed by grafting PEG chains by surface-initiated ATRP polymerization. Recently, biomimetic strategies were used to attach PEG to various substrates [87–90]. 3,4-Dihydroxyohenylalanine (DOPA), an unusual catecholic amino acid abundantly found in marine mussels, is believed to be largely responsible for the strong adhesive characteristics of mussel adhesive proteins. The ability of mussels to adhere to a wide range of surfaces, including rocks, wooden piers, and metal ship hulls, suggests that DOPA may be an excellent anchor for modification of a variety of surface chemistries. Messersmith et al. developed PEG–DOPA polymers by conjugating terminated PEG with peptides containing up to three residues of DOPA [88]. The resultant PEG–DOPA was attached on TiO_2 substrates and exhibited excellent protein resistance.

Ether-containing reagents were also used to prepare PEG-like coatings to make anti-fouling surfaces, including plasma-induced deposition (PECVD) of triethylene glycol monoallyl ether [91], diethylene glycol methyl vinyl ether [92, 93], low molecular weight cyclic ethers [94–96], and commonly used oligoglymes [95] such as diglyme [97–99], triglyme [94, 100], and tetraglyme [101]. The choice of the glyme-based monomers and the plasma power density can affect the chemistry of the coatings. It was found that plasma polymers made from linear oligoglyme exhibited a better anti-fouling behavior than those from crown ethers with reduced protein adsorption [95], bacterial attachment, and biofilm formation [94].

Although the anti-fouling property of PEG is better than most of other hydrophilic polymers, its lack of stability against oxidation due to its intrinsic ether linkage limits its utility in long-term applications. PEG decomposes in the presence of oxygen and transition metal ions found in most biochemically relevant solutions [85], resulting in the loss of surface functionality in terms of hydrophilicity and the ability to resist protein adsorption.

Recently, great efforts have been made for the development of alternative polymers as a PEG substitute with good anti-fouling combined with long-term stability, including polyglycerol [102, 103], polysaccharides [104, 105], and more promisingly poly(2-oxazolines) [106, 107]. Poly(2-oxazolines) or poly(*N*-acyl ethyleneamines) were firstly reported in 1966 and 1967 [108–111]. Due to their

excellent biocompatibility, possibility of responsiveness, low dispersity, and high modulation of solubility, architecture, and functionality, they have emerged as novel polymers for biomedical applications [112–116]. Compared with PEG, poly(2-oxazolines) have the advantages of low toxicity and tunable hydrophilicity. The hydrophilicity can be tuned via the alkyl chain length in the 2-position, and some of poly(2-oxazolines) also exhibit thermosensitivity with different lower critical solution temperatures (LCST) [117].Most importantly, poly(2-oxazolines) show greatly enhanced stability [118–120] while still retaining their anti-fouling effect [106, 121]. Konradi et al. have directly compared the poly(2-oxazolines) with "gold standard" PEG for their anti-fouling and stable properties [122]. In their study, poly(2-methyl-2-oxazoline) (PMOXA) was selected as the example of poly(2-oxazolines). PMOXA and PEG were prepared as polymer brushes with the similar architecture through the same chemistry. Both the modified surfaces have demonstrated their equally excellent non-fouling properties in resisting adsorption of proteins from human serum and preventing adhesion of *E. coli*; however, a significant difference among these two polymers was found in their stability against oxidation. When exposed to 10 mM of hydrogen peroxide for 7 days, the thickness for PEG was reduced by over 50%, compared to less than 20% for PMOXA, due to the peptidomimetic structure from the latter [123] (which is believed to be stable against biological degradation [124]). It was reported recently that poly(2-ethyl-2-oxazoline) (PEtOXA) exhibited negligible hydrolysis at 37 °C even in the presence of digestive enzymes [125].

8.3.2 Zwitterionic Polymers

Zwitterionic polymers have been regarded as better candidates to replace PEG for anti-fouling applications. The use of zwitterions in anti-fouling surfaces was inspired by the external surface of the mammalian cell membrane, rich in phospholipids bearing zwitterion head groups—notably phosphatidylcholine (PC)—and was found to exhibit non-thrombogenic as well as biocompatible properties [126]. The anti-fouling properties of a single monolayer of a lipid zwitterion are even more impressive considering it rests on an extremely hydrophobic blanket of hydrocarbon chains [127]. The zwitterionic methacrylate monomer, 2-methacryloyloxylethyl phosphorylcholine (MPC), was first invented by Nakabayashi's group in 1977 [128], which facilitated a simple method to synthesize zwitterionic-based materials through polymerization chemistry. In 1990, Ishihara et al. [129] further improved the synthesis of MPC in high purity with higher yield to accelerate its commercial availability in the market. Since then, MPC polymers have been extensively synthesized, studied, and explored for various anti-fouling applications. A detailed review on PC polymers was written by Lewis and Lloyd in 2012 with emphasis on their biomedical applications [130].

An early study by Ueda et al. [131] proposed that the non-thrombogenic properties of PC could be attributed to the favored organization of plasma lipids into bilayered structures on PC surfaces, therefore preventing them from interacting with blood proteins (Mechanism 1, Fig. 8.1). Later studies, however, focused on the

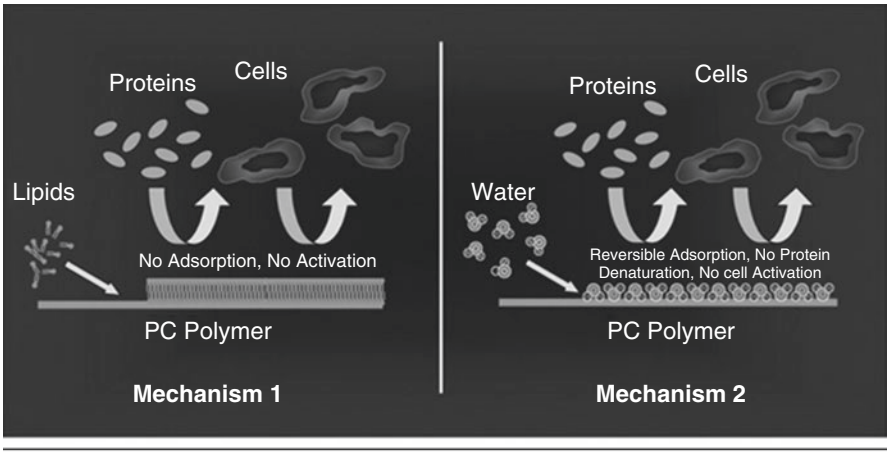

Fig. 8.1 Anti-fouling mechanisms of action for MPC polymers [130]

water-structuring abilities of PC polymers by different techniques, including differential scanning calorimetry and circular dichroism spectroscopy [132], Raman [133, 134], infrared (IR) [135, 136], and NMR [137]. They suggested that the high free water fraction from PC polymers allowed proteins to interact reversibly with the materials without inducing any conformation change in their ability to resist protein adsorption and cell adhesion (Mechanism 2, Fig. 8.1).

Due to the poor structural integrity of pure zwitterionic polymers, MPC generally is copolymerized with other monomers and/or coated/grafted on other substrates to improve their surface properties without altering their desired bulk properties. The copolymers of MPC with n-butyl methacrylate (BMA) were studied and suggested for use as membranes for implantable glucose sensor [138]. Indeed, MPC copolymer-based coatings have been extensively studied as anti-fouling materials, especially in medical device applications. In one such study, the linear copolymer of MPC, lauryl methacrylate (LMA), 3-trimethoxysilylpropyl methacrylate, and 2-hydroxylpropyl methacrylate were synthesized, dissolved in a suitable solvent, applied to substrates by various coating methods, and cured into cross-linked networks by heating or gamma irradiation for application as exceedingly robust coatings on expandable devices such as stents [139–141]. The studies showed that the coating was still present at the same thickness after 6 months of implantation.

In ophthalmic applications, for example, contact lenses of omafilcon A developed by Biocompatibles Ltd. (UK) were based on the copolymers of MPC and HEMA and used as extended-wear anti-fouling lenses [142]. However, its poor oxygen permeability made this kind of contact lens less than ideal for continual wear periods over 1 month. For this reason, silicone hydrogels with excellent oxygen transmissibility are exclusively used in extended-wear contact lenses. Biocompatibles Ltd. reported the grafting of MPC polymer on silicone hydrogels by means of an in-mold coating technique [143]. The uniform and stable PC coating provided improved lens wettability and lower protein adsorption. For other ophthalmic applications, MPC

polymers are also being studied to use as polymer coatings in glaucoma drainage devices [144, 145] and intraocular lenses (IOL). An IOL is a lens implanted in the eye used to treat cataracts or myopia. The studies showed that the use of MPC coating on acrylic IOL inhibited fibroblast and bacterial adhesion, therefore potentially reducing the risk of endophthalmitis [146, 147].

Due to the inherent non-thrombogenicity of PC polymers, they have been extensively studied and used in cardiovascular applications. A MPC and LMA copolymer-coated coronary guide wire was one of the first commercial products using PC polymers in cardiovascular applications [130]. Biocompatibles Ltd. also applied the cross-linked MPC in thin coatings on Bio*divYsio*™ coronary stents, which was approved by Conformité Européenne (CE) and the US Food and Drug Administration (FDA). Several subsequent clinical studies suggested in vivo safety, effectiveness, and long-term stability of the PC coatings. Later, Biocompatibles Ltd. launched two new types of Bio*divYsio*™ stents, known as Matrix LO™ and Matrix HI™, to the market. In these drug-eluting coronary stents, thicker PC-based polymer coatings were used, allowing for drug loading and delivery. In mid-2001, Abbott Laboratories introduced the PC-coated Bio*divYsio*™ coronary stents in the United States. In 2002, Biocompatibles Ltd. sold its cardiovascular stent business to Abbott Laboratories. Following the CE mark approval in December 2002, the Dexamet™ stent, developed by Abbott Laboratories, was launched in Europe. The PC coating coupled with anti-inflammatory drug dexamethasone was used in this drug-eluting coronary stent. Later, a ZoMaxx drug-eluting stent consisting of non-drug-eluting stent TriMaxx™ (also by Abbott Laboratories), a unique PC-based polymer, and a patent-protected immunosuppressant drug ABT-578 (synthesized by Abbott Laboratories) came to market. Abizaid et al. summarized the clinical study involving 4 months of implantation for 40 patients and demonstrated its safety and inhibition of neointima formation [148]. The Endeavor stent, a drug-eluting stent with PC polymer coating developed by Medtronic, was approved by the FDA in 2007.

A ventricular assist device (VAD) is a mechanical pump that is used to support heart function and blood flow in heart failure patients. It generally faces complications including infection, bleeding, and thromboembolism. In collaboration with several universities, Sun Medical Technology Research Corporation, Japan, developed EVAHEART® VAD, in which a titanium alloy is used as the main base material. A anti-fouling copolymer of MPC-co-BMA was applied on the titanium alloy, and the obtained EVAHEART VADs were evaluated for their blood compatibility in animal preclinical studies [149, 150]. The results showed the MPC copolymer-coated devices exhibited anticoagulative properties with significantly fewer activated platelets. After this, the first implantation of EVAHEART® VAD in humans was successfully performed in Tokyo Women's Medical University on May 7, 2005. The company completed a clinical trial consisting of 18 patients in 2008, and final regulatory approval was granted by the Japanese Pharmaceuticals and Medical Devices Agency (PMDA). By the end of 2012, more than 100 patients have been supplied with EVAHEART®. In December 2014, the first surgery outside Japan for implantation of EVAHEART® left ventricular assist devices was successfully completed at the University of Alabama at Birmingham Heart & Vascular Clinic.

In other vascular applications, PC-based polymers have been studied for use as vascular grafts and coatings on expanded poly(tetrafluoroethylene)(ePTFE) to reduce neointimal hyperplasia [151, 152] and grafted on ePEFE prostheses [153]. In addition, PC-based polymer coatings have been used on extracorporeal circuits [154, 155] and Synthesis® oxygenators (Sorin Biomedica, Italy)—which have been in clinical use in Europe since the spring of 2002 and released by the FDA and Health Canada for use in North America in 2003. The clinical study of the latter suggested promising performance with PC-coated oxygenators [156].

Another application of PC-based materials in medical devices utilizes the superhydrophilicity of zwitterionic polymers—making them a promising candidate for surfaces where lubricity is a key requirement. In hip joint replacement, for instance, the lubrication of artificial joints is critical for maintaining the long-term durability of hip replacements. Moro and Ishihara et al. developed a method to modify the surface of cross-linked ultrahigh molecular weight polyethylene (CLPE) in hip acetabular liners in artificial joints by grafting polymerization of MPC using photochemistry [157–159]. The MPC-grafted surface was exceptionally lubricous, and wear of the artificial hip joint was significantly reduced, both on the production of wear debris and bone resorption caused by wear debris. Based on this technology, Aquala®(Kyocera Medical Co) was approved in Japan to use this novel hip joint with MPC-grafted CLPE cup as long-term hip replacement. It is stated that "in gait loading tests equivalent to 15 years or more, the production of wear debris was reduced by about 99% compared to KYOCERA Medical Corporation's conventional products". From in vitro simulations, there was no significant change on this non-wearable MPC-modified PE even over 70 million cycles of loading. From 2011 to 2013, there were over 6500 hip joints implanted in Japan with this technology.

PC-based copolymers have also been investigated for biosensor applications. For example, MPC and BMA copolymers have been used as membranes for intravascular oxygen sensors [160] and glucose sensors [138], respectively. Ishihara and his colleagues also used different MPC-based copolymers for the preparation of miniaturized glucose sensors [161–163]. In an antimicrobial application, PC-coated polyurethane ureteral stents showed reduced biofilm formation [164]. PC-based coatings on inert polymers of silicon tympanostomy tubes and fluoroplastic ventilation tubes (known as PacifiC) were commercialized by Grace Medical and Gyrus ENT, respectively.

MPC tends to be quite costly, however, due to its multistep synthetic route and overall low yield—therefore limiting their potential applications. New alternative zwitterionic polymers have been explored based on betaine monomers carrying both positively and negatively charged atoms on the same repeat unit, such as sulfobetaine methacrylate (SBMA) and carboxybetaine methacrylate (CBMA). All of these polybetaine materials have proved to be both biocompatible and hemocompatible—reducing non-specific protein adsorption and consequent platelet adsorption in various biological applications. Zhang et al. demonstrated that both polysulfobetaine and polycarboxybetaine brush-modified gold substrates yielded performance close to ideal ultralow fibrinogen adsorption of less than 0.3 ng/cm^2, below the detection limit of surface plasmon resonance (SPR), and protein adsorption levels from plasma, below 10 ng/cm^2 [165, 166]. Further studies showed polysulfobetaines and polycarboxybetaine do not exhibit cytotoxicity in both in vitro tests and in vivo implantation [167, 168].

Recently, polySBMA has been widely studied due to the lower cost of SBMA monomer and its easy synthesis in different forms such as homopolymers, hydrogels, and copolymers, through conventional and controlled free radical polymerizations. In the 1990s, SBMA was mentioned in patents for making blood-compatible coatings on polymers, or copolymerized with other monomers for contact lenses exhibiting reduced protein adsorption. In the early 2000s, SBMA was investigated as a grafted layer to modify polyurethane and cellulose surfaces to exhibit antithrombogenicity [169–171]. During the past decade, academic labs have done extensive studies creating various polySBMA coatings to demonstrate the ultra antifouling properties of polySBMA [165, 172–177]. Despite this, however, commercialized products in medical devices using sulfobetaine are still rarely seen. Semprus Biosciences, acquired by Teleflex Inc., has developed technologies to generate zwitterionic polymer layers on polymer substrates to make anti-fouling medical devices [178]. In their work, polySBMA layers were covalently grafted on polyurethane peripherally inserted central catheters (PICCs), modifying both the external and internal lumen surfaces. Compared with control catheters, the polySBMA-modified catheters reduced protein adsorption by 99%, quantified via radio-labeled fibrinogen and enzyme-linked immunosorbent assay (ELISA). Using four cell types including human platelets, lymphocytes, monocytes, and polymorphonuclear leukocytes for testing, all of their respective attachments were reduced by over 98%, and the activation of these cells by modified PICCs was also significantly reduced. In vitro studies using bovine blood-based loop assays found thrombotic accumulation to be reduced by 99% with polySBMA-modified PICCs, even when they were preexposed to serum in vitro for 60 days. A subsequent in vivo study in highly thrombogenic canine model did not reduce the performance of modified PICCs in preventing device- and vessel-associated thrombus. The polySBMA-modified PICCs also resisted a broad spectrum of microorganism adsorption, with *A. baumannii*, *C. albicans*, MRSA, *S. aureus*, and *S. epidermidis* attachment being reduced by 97%–99.9% (Fig. 8.2a). For the bacteria commonly associated with catheter infections such as Gram-negative *E. coli* and Gram-positive *S. aureus*, the modified catheters exhibited 96% and 97% reduction in biofilm accumulation, respectively (Fig. 8.2b). Two polysulfobetaine-modified PICCs were approved by the FDA in 2012 and 2015, respectively. In addition to vascular catheters, zwitterionic polybetaine coatings on other medical devices including endotracheal tubes (ETTs) and orthopedic devices are being developed by Teleflex to reduce bacterial infections and other device-associated complications. In the context of contact lens applications, chemistries generating a durable polybetaine surface on various silicone hydrogels have been developed by Semprus Biosciences. Starting with various commercial silicone hydrogel contact lenses, they successfully modified contact lenses with polybetaine using a one-step polymerization process to improve the surface wettability for user comfort without affecting bulk properties.

When the anionic sulfonate group from sulfobetaine is replaced with carboxylic group, it changes to carboxybetaine. Structurally, carboxybetaine is similar to glycine betaine, one of the compatible solutes, and is essential to the osmotic regulation of living organisms. The use of carboxybetaine for non-thrombogenic coatings on

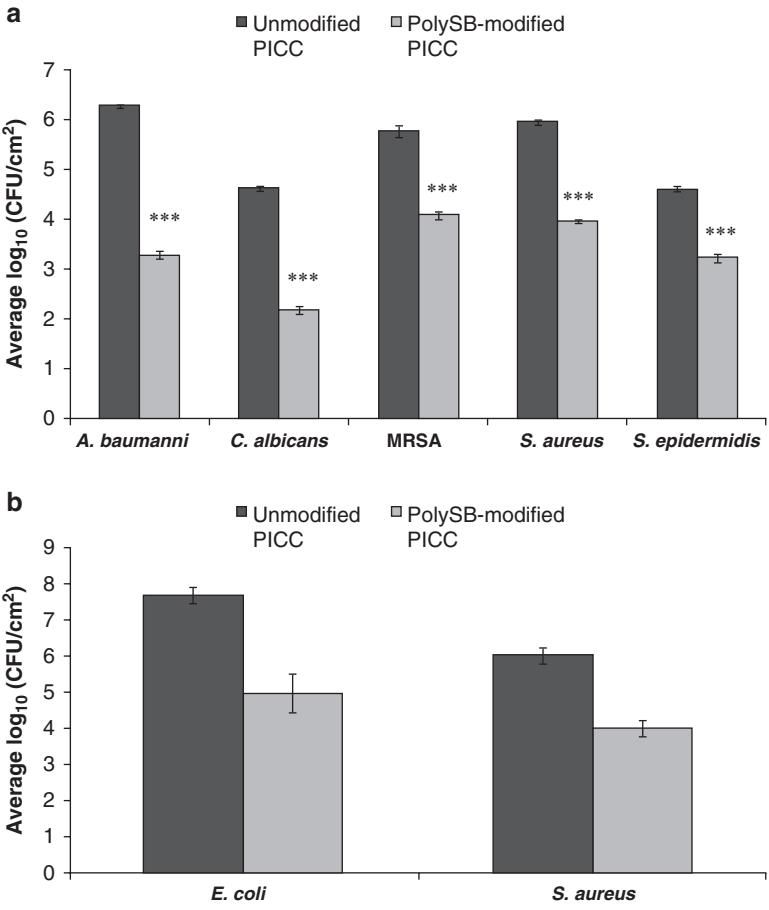

Fig. 8.2 Reduced adhesion of microorganisms to polySB-modified PICC samples. (**a**) Adhesion onto external PICC surfaces (n = 30 separate PICC samples). MRSA, methicillin-resistant *S. aureus*. (**b**) Intraluminal biofilm formation in PICCs (n = 3 separate PICC samples). Data are average log10 CFU/cm^2 ± SEM. ***$P < 0.001$, student's t test [178]

polyurethane was reported in 2003 [179]. Later, more carboxybetaine-based polymers including CBMA and carboxybetaine acrylamide (CBAA), mainly as hydrogels and surface coatings for non-fouling applications, were also investigated [167, 172]. These works demonstrated that the polycarboxybetaine polymers had a superior resistance to non-specific protein adsorption from undiluted blood plasma and serum. In 2013, a carboxybetaine hydrogel subcutaneous implant in mice was claimed to prevent capsule formation for over 3 months due to the ultralow fouling properties of the zwitterionic hydrogels [168]. Here a carboxybetaine-based crosslinker was used to avoid introducing other materials. On top of the anti-fouling properties, Zhang also found the functionalizable carboxylic group in carboxybetaine provides unique advantage over other betaines [180]. For example, carboxylic groups

are easily converted into ester groups for immobilization of biomolecules or drugs onto a surface. Under physiological conditions, the obtained ester groups are reversibly changed back to carboxylic groups upon hydrolysis to release biomolecules or drugs—resulting in dual functional non-fouling zwitterionic materials [181].

Zwitterionic materials also include polyampholytes and some polypeptides. Polyampholytes from 1:1 ratio of positive and negative charges on two different monomer subunits, such as mixed charge complexes of $-N^+(CH_3)_3$ and $-SO_3^-$/$-COO^-$, are structurally very similar to polybetaines. Thus similar anti-fouling effects of betaine-based zwitterionic polymers could also be obtained from polyampholyte-based materials. Practically there is the possibility of minor defects with two or more same charge units connected together in ion-pair comonomer polymer, but recent work suggests that such defects have negligible impact on protein adsorption [182, 183]. As there are many different charged monomers to be chosen, it is possible to design a wide range of new anti-fouling polyampholytes, even recreating the dual functionality of drug-carrying polycarboxybetaines. When alternating negatively charged amino acids (such as Glu or Asp) and positively charged amino acids (Lys or Arg), another type of zwitterionic polymer of natural peptides is obtained with excellent anti-fouling properties. For example, Chen et al. proved the surface from the peptides of Glu/Lys or Asp/Lys also had ultralow adsorption of fibrinogen, lysozyme, and albumin (<0.3 ng/cm^2) [184]. Plus, since they are constituted of naturally occurring peptides, they are nontoxic and biodegradable.

8.3.3 Hydrophobic Polymers

Contrary to hydrophilic materials' relying on the formation of highly hydrated layer, hydrophobic materials, which resist hydrogen bonding, have also been investigated for fouling-resistant performance. With low surface energy, hydrophobic materials tend to repel the attachment of water and biomolecules alike. However, adsorbed particles can increase surface energy to promote subsequent adhesion and fouling on hydrophobic surfaces. Due to the general inclusion of toxic components in material coatings needed to render the surface hydrophobic, hydrophobic surfaces used to be considered toxic to the host environment when used on medical devices. Recently, some biocompatible hydrophobic molecules have been developed for anti-fouling applications. For example, in the early 2000s, a fluoroligomer was developed in Dr. Paul Santerre's lab at the University of Toronto to be used as an additive mixed into polyurethane to reduce thrombus formation on catheters. Differing from most surface modification techniques mentioned, this hydrophobic fluoroligomer surface-modifying molecule is added directly to base polyurethane at 1–5% during manufacturing process. The fluoroligomer molecules then migrate to the catheter surface to significantly alter surface properties of segmented polyurethane by formation of new hydrophobic domains. The non-eluting fluoro-compound therein provides a passive surface to reduce protein adsorption, platelet adhesion and activation, and eventually thrombus formation. This technology, known as

Endexo™ technology, owned by Interface Biologics Inc. (IBI), is currently licensed to AngioDynamics for vascular access devices and Fresenius Medical Care for chronic dialysis systems. With this technology, both the AngioDynamics BioFlo PICC and the BioFlo Port (an implantable port catheter) were approved by FDA. In vitro blood loop results show that on average the BioFlo Port catheter had 96% less thrombus accumulation on its surface compared to non-coated conventional port.

8.3.4 Featured Surface

Inspired from the discovery that the arrangement of shark dermal denticles in a distinct diamond pattern with millions of tiny ribs is able to protect shark skin from algae, barnacle, and bacterial growth, Dr. Anthony B. Brennan, materials science and engineering professor at the University of Florida, invented the "Sharklet Technologies" texture pattern of "ridges" and "ravines" at nano-/microscale on plastic materials. Instead of using chemical methods to change the surface's adsorption affinity, it utilizes topography to induce mechanical stress on settling bacteria, known as mechanotransduction. Nanoforce gradients caused by surface variations induce stress gradients within the lateral plane of the surface membrane of a settling microorganism during initial contact. This stress gradient disrupts normal cell functions, forcing the microorganism to provide energy to adjust its contact area on each topographical feature to equalize the stresses. This expenditure of energy is thermodynamically unfavorable to the settler, inducing it to search for a different surface to attach to [185]. Sharklet is claimed to be "the world's first technology to inhibit bacteria growth through pattern alone" and is reported to inhibit the growth of bacteria such as *staphylococci* (including MRSA), *Pseudomonas aeruginosa*, vancomycin-resistant *enterococci*, *E. coli*, and other organisms commonly responsible for device-associated infection. Based on this licensed anti-fouling technology, Sharklet Technologies is developing medical devices in several areas including adhesively backed film and wound dressings. In a recent publication [186], they compared micropatterned Sharklet™ ETTs with commercial standard ETTs on secretion accumulation as well as bacterial attachment. Using their in vitro biofilm model, the polyurethane Sharklet™ ETTs exhibited a 71% reduction of biofilm from *P. aeruginosa* compared with commercial PVC ETTs. By using the Sharklet™ ETTs, the calculated lumen occlusion was reduced by 81–85% (depending on the analyzed locations) from an in vitro airway patency model, and the mucus accumulation was reduced by 61% from an in vivo model using Dorset sheep. These one-day test results were obtained from a study with a limited duration. It will be important to observe the long-term performance of such micropatterned ETTs in the future. Now partner with Cook Medical, they are developing a Sharklet Foley Catheter (silicone) to reduce the risk of catheter-associated urinary tract infections (CAUTIs). The company also has a Sharklet-patterned central venous catheter (CVC) being developed to prevent platelet adhesion and activation as well as catheter-related bloodstream infections.

8.3.5 Superhydrophobic Surfaces

The leaves of the *Nelumbo nucifera* (lotus) plant possess natural self-cleaning properties that are a result of their very high water repellence and have inspired the development of anti-fouling surfaces with similar superhydrophobic (also referred to as *lotus effect*) characteristics. This repulsion of water droplets from the superhydrophobic surface first greatly reduces the risk of being encumbered by water, especially in or near aquatic environments [187, 188], and second, the low adhesion from the superhydrophobic surface allows these droplets to easily roll off the surface while picking up contaminants and carrying them away, so-called self-cleaning [189, 190]. Superhydrophobic surfaces are in essence extremely difficult to wet, defined as having a water contact angle above 150° and a roll-off angle/contact angle hysteresis below 10°, and are believed to be created by the combination of hydrophobic (low surface energy) materials and hierarchical surface structure roughness (i.e., a microscale surface with typically nanoscale features). The air trapped in the spaces of the micro-/nanostructures of superhydrophobic surface significantly reduces the contact area between water droplets and material surfaces and likewise decreases the adhesion of fouling organisms. Therefore, superhydrophobicity is a very attractive characteristic to mimic for an anti-fouling surface in order to reduce the adhesive force between bacteria and the surface—facilitating the easy removal of bacteria before a biofilm is formed. Although some superhydrophobic coatings have been available on the market, such as Rust-Oleum® NeverWet®, none are currently being used for medical devices commercially. Current superhydrophobic surfaces generally suffer from poor long-term durability. When the micro-/nano-surface roughness is altered or lost, its superhydrophobicity is significantly reduced. Typically the micro-/nanostructure is mechanically fragile, being damaged under forces of abrasion and twisting or under low temperature or external pressure. Surface contamination [191] or the reaction of the surface layer with chemicals in the environment could also lead to the degradation of low surface energy [192, 193].

Following a similar rationale, Dr. Aizenberg's lab at MIT developed a technology of slippery liquid-infused porous surfaces(SLIPS) [194] inspired by the method with which the pitcher plant utilizes an entrapped liquid instead of air on the plant's surface to make it "slippery" to insects crawling along it [195, 196]. The lubricating liquid immobilized into the micro-/nanoporous structure of their substrates (such as glass, metals, and polymers) creates a very slick film with repellent, anti-fouling properties. This lubricating liquid needs to be stable, preferentially wets the substrates, stably adheres within the substrate, and is immiscible to the expelled liquids. For biomedical applications, this infused liquid also needs to be nontoxic, such as fluorinated oil. This SLIPS exhibited excellent reduction of biofilm attachment against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* over a 7-day period. Also, they claimed the surface coating by this technology on medical devices using FDA-approved materials showed good blood repellency as well as biofilm reduction, suggesting potential applications for biomedical applications.

8.4 Summary

It is believed that the hydration layer plays critical role for anti-fouling of the hydrophilic polymers. Many hydrophilic polymers have shown promising anti-fouling properties with optimized chemistries, such as PEG, polyamides, and polysaccharides. That being said though, their surface hydration layer is formed by hydrogen bonds, and these hydrogen bonds are relatively easily to break and reform. These hydrophilic polymers therefore often lose their anti-fouling properties upon a change in surface hydration. Zwitterionic polymers, which can bind water molecules more strongly and stably by electrostatically induced hydration, have become an attractive candidate for developing the next generation of anti-fouling and antimicrobial materials. Although under certain harsh conditions such as very low ionic strength and extremely high or low packing density the hydration of zwitterionic polymers could be compromised and affect the surface's anti-fouling properties to some degree, their favorable behavior in the majority of conditions is quite robust compared to the long-term durability issues faced for current hydrophobic anti-fouling surfaces.

The hydrophilic coatings, together with other strategies mentioned in the review, have been designed to utilize the anti-fouling characteristics of certain materials in modifying biomedical devices to resist biofouling. While promising performance can be demonstrated even in long-term in vitro studies, the ultimate metrics is in vivo relevancy, and there remains ample area for improvement.

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Chapter 9
Exploring the Potential of Light to Prevent and Treat Microbial Biofilms in Medical and Food Applications

Tara L. Vollmerhausen, Alan J. Conneely, and Conor P. O'Byrne

9.1 Introduction

Bacterial biofilms have great significance for public health and have a major impact in medical settings. It is estimated that more than 60% of human infections involve biofilm formation [11, 64]. Biofilm infections affect millions of people in the developed world. Bacteria are able to form biofilms on medical devices, such as in implant and catheter-associated infections, complicating healthcare delivery, prolonging the suffering of patients, and increasing healthcare costs. The most common healthcare-associated infections (HAIs) include ventilator-associated pneumonia, lower respiratory tract infections, catheter-associated urinary tract infections (CAUTI), and surgical site infections [35]. These infections are associated with Gram-positive bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis, Enterococcus faecalis,* and Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* [15]. These infections are often recalcitrant to antibiotic treatment both because of the physiological properties of the biofilm and because of the increasing prevalence of antibiotic-resistant strains. Thus, there is an urgent need for new interventions to help solve this problem. In this review, we discuss the potential for using UVA and visible light as a means of eradicating bacterial biofilms. We evaluate some of the emerging technologies that can be used to deliver the light and consider some of the limitations of the technology. We discuss the antimicrobial mode of action and outline the extrinsic and intrinsic factors that influence the sensitivity of bacteria to

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light. Finally, we review some recent examples of successful light-based antimicrobial treatments that demonstrate the real promise of this technology.

9.2 Pathogenesis of Device-Associated Infections

In the healthcare setting biofilms are able to contaminate ventilators, catheters, and medical implants. Device-associated infections cause significant morbidity and increase the duration of hospital stays. Catheterization, for example, may be applied to acutely ill patients after surgery, to monitor urine output or to treat patients with urinary retention [38]. A urinary catheter, often referred to as a Foley catheter, is a tube usually made of latex or silicone that is inserted into the bladder via the urethra to drain urine. The incidence of infection is proportional to the duration of catheterization, with between 10% and 50% of patients getting an infection after 7 days of catheterization and almost all developing an infection after 30 days [38].

Bacteria responsible for the infection may originate from healthcare personnel during device insertion or can be derived from the patient's own microflora. Initial attachment of bacteria is mediated by surface adhesins binding to a host-produced matrix consisting of proteins, polysaccharides, and other components that are present at the device-epithelial interface; see Fig. 9.1 [31]. Once attached to the catheter surface, bacteria are able to replicate to form complex and often multi-species communities known as biofilm [16, 77]. Upon adhesion, the production of exopolysaccharides leads to irreversible attachment and the formation of a matrix consisting of polysaccharides, proteins, and nucleic acids [11, 95]. The bacteria in the biofilm then replicate and develop into a fully mature biofilm, after which single motile bacteria are able to disperse to infect new sites (Fig. 9.1). Unlike planktonic cells, bacteria existing as part of a biofilm experience high cell density and oxygen and nutrient limitations. These sessile bacteria are able to communicate by a process called quorum sensing, allowing bacteria in a biofilm to exhibit coordinated multicellular behavior to respond and adapt to environmental changes [63]. This makes biofilm formation beneficial to bacteria, providing protection from stress and making it harder for the immune system to detect and inactivate them.

9.3 Antibiotic Resistance and Chronic Infections

Biofilm-mediated infections are often untreatable and can develop into chronic infections which are associated with increased morbidity and mortality. Bacteria existing as part of a biofilm show enhanced resistance to antibiotics compared to planktonic bacteria and are able to evade the immune system of the host [66]. Intrinsic mechanisms of antimicrobial resistance of biofilms may be due to limited diffusion of antimicrobial agents through layers of the biofilm, deactivation of antimicrobials in the outer layers of the biofilm, and the quiescent nature of cells in the

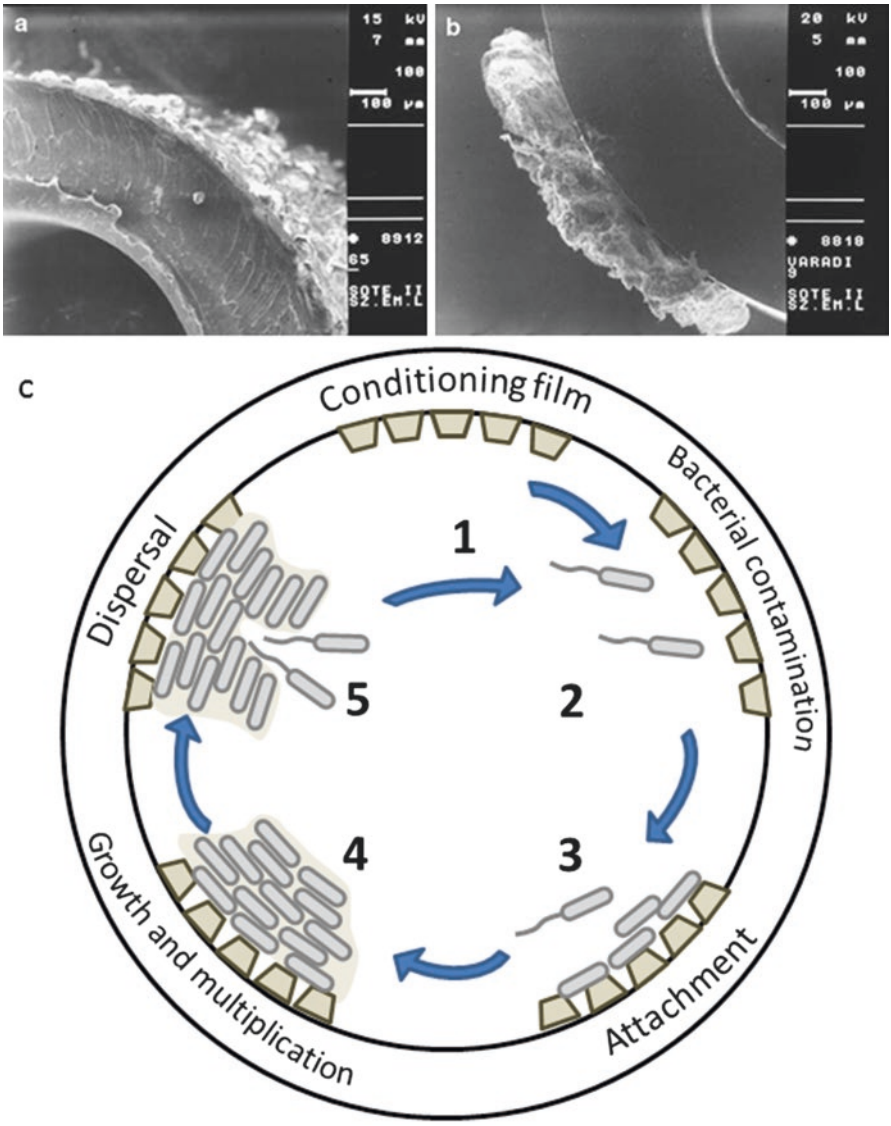

Fig. 9.1 Biofilm formation on the surface of a catheter. (**a**, **b**) Scanning electron microscopic image of a developing biofilm (Republished from Ref. [82]). (**c**) The process of biofilm formation on a catheter surface. After catheter insertion, host proteins form a conditioning film on the substratum in *stage 1*. In *stage 2*, bacteria contamination occurs and bacteria begin to reversibly attach to the surface in *stage 3*. In *stage 4*, the production of exopolymeric substances helps bacteria to irreversibly attach and the bacteria in the biofilm begin to multiply to form a mature biofilm. In *stage 5*, single motile bacteria disperse from the biofilm to infect distant sites

biofilms. Resistance may also be acquired through transfer of extrachromosomal DNA in the biofilm [14, 66]. These factors make clinical biofilm formation difficult to eradicate and can lead to the development of chronic infection despite antibiotic therapy. In view of the high level of antimicrobial resistance of biofilms, light-based antimicrobial therapy is gaining attention [4, 18, 21, 22, 24, 32, 48, 58] as a promising alternative approach to help prevent and treat these infections.

9.4 Photodynamic Therapy and Photoinactivation

Photodynamic therapy (PDT) is a treatment that has been used successfully for cancer and other diseases including bacterial infections. Inactivation of pathogens using light is a promising technology for microbial disinfection of medical devices. Both ultraviolet (UV) [3, 5, 48] and visible light [53, 56, 58] have been demonstrated to reduce microbial counts in planktonic cultures and on bacteria adhered to surfaces as part of a biofilm. Photodynamic therapy uses a combination of exogenously applied light-sensitive photosensitizers, light, and oxygen. The photosensitizers absorb light and react with molecular oxygen to produce cytotoxic species which can cause cell damage. Common photosensitizers used to inhibit microbial growth include toluidine blue O, methylene blue, and azure dyes [32]. Bacteria are also known to possess naturally produced light-sensitive endogenous photosensitizers including porphyrins, cytochromes, flavins, and NADH [65]. Photoinactivation is the inactivation of microorganisms by means of light, whereby the photoexcitation of endogenous photosensitizers in the presence of oxygen indirectly causes cell damage and death [32]. Photoinactivation is an attractive approach due to its intrinsic antimicrobial activity without the addition of externally applied photosensitizers. To be an effective antibacterial agent, light therapy needs to possess the ability to not only inhibit bacterial growth but also the ability to kill bacteria. Bactericidal agents are those which kill bacteria, whereas bacteriostatic agents are those which merely inhibit their growth. Viability of bacteria is traditionally defined as the ability to grow and multiply, such as the formation of colonies on solid agar; however, some viable cells are not able to proliferate but maintain cell membrane integrity and metabolic activity [43]. In contrast, cells which are dying or are dead have irreversibly lost the ability to grow and reproduce.

9.5 Light-Based Technologies

9.5.1 Terminology of Light Properties

Electromagnetic radiation, which includes visible light, is a form of energy that propagates as an oscillating wave of electric and magnetic fields. The electromagnetic spectrum describes the continuous wavelength range of electromagnetic radiation from radio waves through infrared light, visible light, UV light, and on to

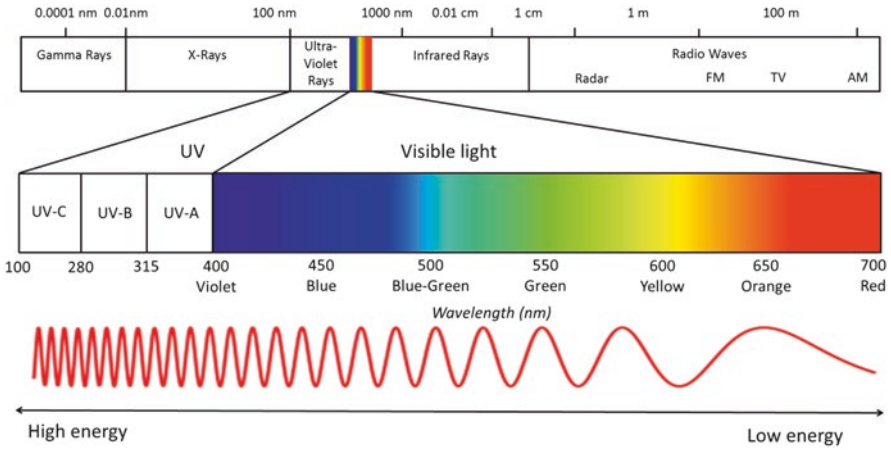

Fig. 9.2 The characteristics of the electromagnetic spectrum, highlighting the visible and UV light spectrum. Diagram shows the range of wavelengths and type of radiation for the electromagnetic spectrum

gamma rays; see Fig. 9.2. The various wavelength regions of the spectrum are somewhat arbitrarily assigned based on the properties of the radiation energy in each particular region. As an example, visible light is typically defined as the wavelength range from 400 to 700 nm as the human eye is effectively sensitive to light energy in this range. The energy in a light wave, as described by quantum mechanics, is carried by discrete massless particles called photons with the energy of each individual photon being inversely proportional to its wavelength.

The interaction of light with matter or organisms occurs by many complex photophysical mechanisms but can be generally described by basic principles of photochemistry:

The *Grotthuss–Draper law* states that only radiation that is *absorbed* can impact a chemical change, and this is important to consider in relation to experimental methodology and the measurement of light. An experimental light source can deliver energy at a certain rate (irradiance) to the sample; however, only a certain fraction of this energy will be absorbed by the organism of interest and produce a chemical event [1].

The *Stark–Einstein law* states that *each photon* that is absorbed reacts with *one molecule*. This allows an understanding of the relationship between the power of the light source, photon energy, and the reaction of the object.

Light can interact with biological systems (media, organisms, biofilms, etc.) through the absorption of photons with energies that are matched to the discrete energy levels of the atoms, ions, or molecules in the system. Only photons that provide the quanta of energy required to raise the molecule, to a specific higher energy level are absorbed. An energy level diagram, known as a Jablonski diagram, can be used to illustrate the energy states in a molecule and the possible absorption and emission mechanisms; see Fig. 9.3.

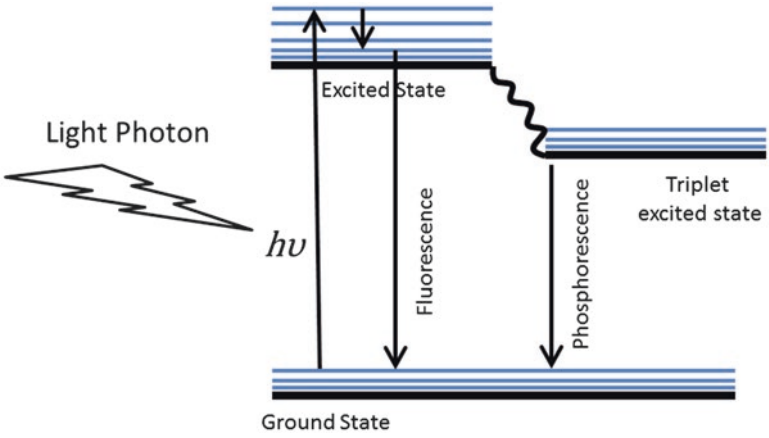

Fig. 9.3 Jablonski energy level diagram representing photon absorption and emission

The equation for photon energy is

$$E = h\frac{c}{\lambda}$$

where E is the photon energy in Joules (J), Planck constant $h = 6.626 \times 10^{-34}$ Js, speed of light in a vacuum $c = 2.998 \times 10^{8}$ m s^{-1}, and λ is the photon wavelength (m).

As $\frac{c}{\lambda}$ represents the frequency of the photon, the equation can also be simplified as follows:

$$E = h\nu$$

where E is the photon energy in Joules (J) and ν is the frequency of light in Hertz (s^{-1}).

Assuming all photons are absorbed, the total energy required to react with a mole of a substance can be calculated by

$$\Delta E_{\text{mol}} = N_{\text{A}} h\nu$$

where Avogadro's number $N^{A} = 6.022 \times 10^{23}$ mol^{-1} is the number of molecules in one mole.

As outlined above, when light interacts with matter, including biological systems, the discrete electronic energy levels of the atoms or molecules are important. Therefore, it is common to use the electron volt (eV) as the unit of energy in place of the Joule; the electron volt more readily describes the energy level band gap in molecules. The electron volt is defined as the energy required to raise an electron through 1 V; hence, 1 eV = 1.602×10^{-19} J. The energy of a photon in eV can be calculated using the formula:

$$E(eV) = \frac{1.2398}{\lambda(\mu m)}$$

where E is the photon energy in eV and λ is the photon wavelength in microns (μm).

Table 9.1 Summary of light measurement terminology commonly used in the literature related to bacterial inhibition

Terms	Units	Description
Fluence	$J\,m^{-2}$	Radiant energy traversing a small transparent imaginary spherical target containing the point under consideration, divided by the cross sectional area of this target
Energy density	$J\,m^{-2}$	Alternate term sometimes used for fluence or energy delivered per unit area
Dose	$J\,m^{-2}$	Generally the total energy received by a sample per unit area. May also be strictly referred to as total energy absorbed by a sample. In the literature, it is typically calculated as the irradiance multiplied by the exposure time. Sometimes used interchangeably with fluence
Fluence rate	$W\,m^{-2}$	Fluence per unit time
Irradiance	$W\,m^{-2}$	Radiant power received by a surface per unit area. Irradiance is the value that is typically calculated using standard light sensing equipment (Power divided by area)
Intensity	W/m^2	Alternate traditional term sometimes used to describe fluence rate or irradiance. Recommended for qualitative descriptions only due to ambiguity of definition
Power density	$W\,m^{-2}$	Alternate traditional term used to describe fluence rate or irradiance. More commonly used in optics or physics

For light-based experiments, the amount of light energy applied to a sample must be expressed and measured. In the literature, however, there are a number of inconsistencies related to the definitions of light exposure particularly across scientific disciplines, see Table 9.1.

Fluence is defined as the total radiant energy traversing a small transparent imaginary spherical target containing the point under consideration, divided by the cross section of this target (units: $J\,m^{-2}$) [91]. The *fluence rate* is the fluence per unit time (units: $W\,m^{-2}$). An extension of fluence is *irradiance* which is the energy per unit time applied over a planar surface area (units: $W\,m^{-2}$). It is generally more straightforward to measure light on a plane rather than a sphere; therefore, irradiance is often the quantity that is measured and may sometimes be used interchangeably with fluence rate. The term *intensity* is often used to quantify light in the literature but it can be considered an ambiguous term due to a variation in definition across scientific disciplines.

In relation to biological systems, the fluence may be termed the *dose* although in some disciplines dose is defined as the total amount of radiant energy absorbed rather than simply received by the exposed sample. In most cases, it is more accurate and straightforward to measure the applied light exposure rather than the absorbed dose. For example, planktonic bacterial cells in a liquid medium will only absorb a small fraction of the total incident energy with the remainder being transmitted, reflected, or scattered by the media.

9.5.2 Ultraviolet Light

A variety of light sources have been reported in the literature, many of which vary considerably in their emission spectra. From the electromagnetic spectrum, both visible and UV light have been investigated for their ability to inhibit microbial growth. The UV spectrum can be further divided into UVA (400–315 nm), UVB (315–280 nm), and UVC (280–100 nm) light. UVC light has the most germicidal action of the spectrum and is often used to disinfect wastewater [20]. UVC has also been used to effectively disinfect airborne pathogens [6] and to treat wounds infected with methicillin-resistant *S. aureus* [83]. However UVC light is limited in its clinical applications due to damage caused to mammalian cells [40]. Light in the UVC and UVB range can cause direct DNA damage in an oxygen-independent manner. Inside bacterial cells, UVC and UVB radiation is absorbed by DNA, causing the formation of thymine dimers leading to irreversible damage of the genetic material [10]. It has been demonstrated that high-density biofilms are able to attenuate UVC light by absorption of light by DNA [5]. In contrast to UVC and UVB, UVA light is absorbed weakly by DNA. The primary mechanism of microbial inactivation by UVA light is via the formation of reactive oxygen species (ROS) which cause oxidative damage of macromolecules such as proteins, DNA, and lipids, ultimately leading to loss of function and cell death [90].

9.5.3 Violet and Blue Light

Violet or blue light, in the visible spectrum of light with a wavelength range between 400 and 500 nm, can also be used to inactivate pathogens. It is an attractive alternative to UV light since it doesn't produce a significant DNA-damaging effect and it induces an antimicrobial effect without the addition of exogenous photosensitizers. Light of this wavelength is able to induce an antimicrobial effect by the excitation of endogenous intracellular porphyrins and other endogenous photosensitive compounds [2, 32, 55]. Irradiation with blue light has less detrimental effects to mammalian cells than UV light [44], with a greater dose required for blue light to produce similar damage caused by UV irradiation [40].

9.5.4 Mechanism of Inhibition by Light

Visible light produces a bactericidal effect by the absorption of light by naturally occurring intracellular molecules, or endogenous photosensitizers, that are excited by light and lead to the generation of ROS [65]. In the visible light spectrum, the violet and blue regions (400–500 nm) have been reported to be effective at killing various pathogens [19, 54]. Absorption of a photon excites electrons in the

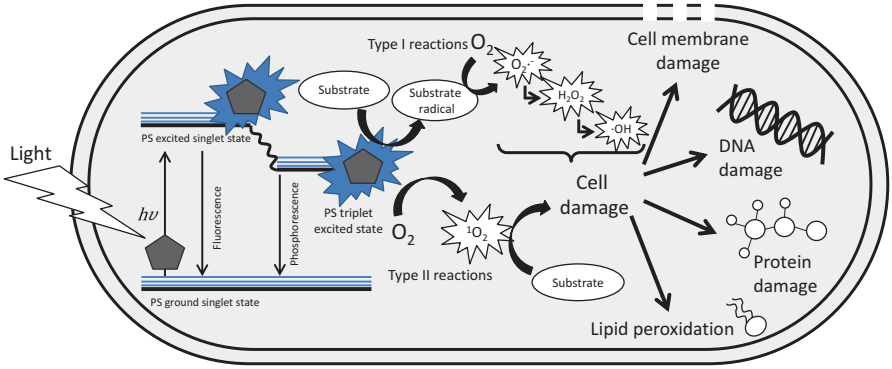

Fig. 9.4 The inhibitory mode of action of visible light. A light photon excites the ground singlet state photosensitizer (PS) to an excited singlet state which can further undergo intersystem crossing to the triplet state. PS in the triplet state can then react with organic substrates (type I reaction), to produce organic radicals that subsequently interact with molecular oxygen to generate superoxide anion radicals ($O_2\cdot^-$), which in turn leads to the production of other ROS such as hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH). Alternatively the triplet state PS can transfer energy directly to molecular oxygen ($^3O_2$) to form singlet oxygen ($^1O_2$; type II reaction). In both cases, the species produced are highly reactive and lead to damage of macromolecular components of the cell and ultimately death

photosensitizer molecule such as a porphyrin to the excited singlet state. This excited state may then undergo intersystem crossing to the triplet state, which is longer lived but with slightly less energy. From the triplet state, the photosensitizer molecule can then react via type I or type II photoprocesses, both of which are dependent on oxygen [32]. In type I reactions, the excited-state photosensitizer reacts directly with biomolecules to produce free radicals that can react with oxygen to produce cytotoxic species including superoxide and hydroxyl radicals. In type II reactions, the excited-state photosensitizer reacts with the ground state molecular oxygen (triplet) to produce the highly reactive singlet oxygen which is able to oxidize biomolecules including proteins, nucleic acids, and lipids; see Fig. 9.4 [32, 71]. Singlet oxygen is the main ROS produced in response to light [71] and is the major species responsible for phototoxic effects. High levels of ROS production are lethal to the cell [52]. The phototoxic effect of visible light is dependent on the presence of oxygen, with no phototoxicity observed when bacteria were exposed to visible light under anaerobic conditions [19]. Due to the unspecific mode of action and the multiple unspecific biochemical targets for the generated ROS, pathogens are unlikely to acquire resistance mechanisms [41, 53]. To investigate the potential for bacteria to acquire resistance, a multidrug-resistant *A. baumannii* strain was exposed to 10 repeated cycles of sublethal inactivation by blue light (415 nm, 70.2 Jcm^{-2}). Resistance did not develop and the strain was actually found to be more susceptible to light upon repeat exposure [98]. Similar results were found for *Vibrio fischeri* and *E. coli* after 10 cycles of sublethal white light [80]. Thus light-based therapies are an attractive alternative method to inactivate pathogens including multidrug-resistant pathogens that are not treatable by traditional antibiotics.

9.5.5 Endogenous Porphyrins

Bacteria possessing endogenous photosensitizers, such as porphyrins, cytochromes, flavins, and NADH, are more susceptible to killing by visible light [65]. Intracellular photosensitizers are able to absorb light over the entire visible range; however photoinactivation by endogenous porphyrins is most effectively achieved by blue light. Porphyrins are heterocyclic macrocycle organic structures composed of four modified pyrrole subunits connected by methine bridges [73]. Common naturally occurring endogenous porphyrins among bacteria include 5-coproporphyrin, carboxyporphyrin, uroporphyrin, 7-carboxyporphyrin, coproporphyrin, and protoporphyrin [65]. The most well-known porphyrin is heme, the pigment present in red blood cells, consists of four porphyrin molecules identified as protoporphyrin IX and is involved in oxygen transport in the blood. Similarly, among microorganisms protoporphyrin IX is involved in the respiratory pathway, being a precursor to heme production [94]. The production of this endogenous porphyrin can be enhanced using the precursor 5-aminolevulinic acid (ALA) [65], which is a naturally occurring metabolite in heme production leading to the production of protoporphyrin IX. This increased porphyrin accumulation has been shown to enhance microbial sensitivity to killing by blue light [65]. In fact, microbes which naturally possess high amounts of endogenous porphyrins, such as *Bacillus cereus* and *S. aureus*, are more susceptible to treatment by blue light [65]. Targeting endogenous photosensitizers is an attractive approach for light therapy, especially for Gram-negative bacteria which are less susceptible to light inactivation by exogenous applied photosensitizers due to the physical and functional barrier of their outer membrane [32].

9.5.6 Irradiation Parameters

Light can be produced from many different sources including halogen light, light-emitting diode (LED), laser diode, and helium-neon (HeNe) laser. Light-emitting diodes (LED) are a narrow band light source that generate light over a small range of wavelengths (~30 nm wide band), while lasers are a monochromatic light source which generate light at a specific wavelength (~0.002 nm wide band for a HeNe laser) [72]. A comparison into the use of LED or laser diode as a light source for photodynamic therapy found both sources were able to cause significant microbial inactivation [72]. This is likely due to the approximately equal photon energy level generated by both light sources, suggesting that it is the properties of the light rather than the light source itself that plays the major role in microbial inactivation.

Light energy as a function of time can be delivered in a number of forms: Continuous Wave (CW) is where the light is delivered at a continuous level over time. Modulated light is CW light that is delivered intermittently based on the duty cycle of the light source. Pulsed light refers to short bursts of high-intensity light. Although modulated and pulsed light are considered to be different in relation to the

duration and intensity of light delivery, in some cases in the literature, the modulated and pulsed terms may be used interchangeably. In certain methods, pulsed light is generated by an electrical charge stored in a capacitor [47, 48]. This light is then released in intermittent short pulses which increase the instantaneous energy intensity. Therefore, pulsed light is able to provide more instantaneous energy than continuous light for the same amount of total energy, making pulsed light an attractive method for microbial inactivation [47]. Pulsed irradiation has been demonstrated to have greater microbial inactivation in comparison to continuous irradiation [7, 48]. Modulated/pulsed UVA-LED irradiation has been shown to have a strong bactericidal effect with a 1 Jcm^{-2} dose (100 Hz, 365 nm) leading to 0.1% of survival of *E. coli* in biofilms treated with pulsed light compared to 1% survival for biofilms continuously exposed to light [48]. These results suggest that low-frequency pulsed light is a promising method for disinfection of microbial biofilms.

9.6 Microbial Response to Light

Pathogens are able to sense and respond to environmental cues, allowing them to modulate functions such as biofilm formation and motility which contribute to persistence in clinical settings. Light at higher intensities can kill microorgansims; therefore, the ability to sense this potentially threatening environmental condition can allow bacteria to respond and modulate their behavior. Microorganisms possess photoreceptors which sense light and enable them to regulate a variety of cell functions including motility, adherence, and virulence in response to light [29]. Therefore the ability to sense light may effectively help bacteria to move away and protect themselves from the light.

9.6.1 Light Responsiveness in Bacteria

Bacteria are able to sense light via light-responsive proteins that bind to an organic cofactor or chromophore. Light absorption via the chromophore leads to conformation changes, photoreceptor activation, and signal transduction. Some bacteria possess blue light sensors which are able to detect light in the UVA and blue wavelength spectrums. Blue light sensors include flavoproteins known as light, oxygen, or voltage sensing (LOV), blue light-utilizing flavin adenine dinucleotide (FAD) (BLUF) domains, and the cryptochrome photoactive yellow protein (PYP) [28, 36]. Flavin-based blue light receptors are among the most common bacterial photoreceptors, with LOV being present in 13.4% and BLUF in 11.4% of sequenced bacteria [51]. The frequency of the BLUF domain photoreceptor among *E. coli* species suggests an important role for light sensing in their survival [29]. It has been hypothesized that bacteria have evolved with the ability to sense light to avoid damage to the cell

caused by solar UV radiation [29], but protection against increased temperature and increased salinity may also be important aspects.

Light has been shown to modulate bacterial behavioral responses including motility, adhesion, and biofilm formation. The ubiquitous second messenger cyclic di-GMP (c-di-GMP) is central to regulating the transition between planktonic and sessile lifestyles and plays an important role in biofilm formation [74, 79]. The GGDEF and EAL domains (designated according to their conserved central sequence pattern) are involved in controlling the intracellular levels of c-di-GMP. The photoreceptors LOV, BLUF, and PYP are associated with or lead to the activation of GGDEF, involved in the c-di-GMP synthesis, and EAL, involved in c-di-GMP degradation [85]. Increased levels of c-di-GMP lead to reduced motility and increase the production of components involved in adherence and biofilm formation. The coupling of photoreceptors with c-di-GMP metabolic domains allows bacteria to sense light and subsequently modulate cell behavior in response. In *E. coli*, blue light sensing by the BLUF domain has been demonstrated to indirectly decrease intracellular c-di-GMP and modulate biofilm formation [85]. A subset of LOV domains have also been predicted to regulate c-di-GMP [36], and the location of a putative PYP gene next to a gene-encoding diguanylate cyclase in the deep-sea bacterium *Idiomarina loihiensis* suggests that PYP may also be part of a pathway capable of modulating intracellular c-di-GMP [69, 88].

Among environmental microorganisms, the freshwater-dwelling bacterium *Caulobacter crescentus* has shown enhanced attachment upon growth in blue light [70]. In contrast, the deep-sea-dwelling *Idiomarina loihiensis* has shown decreased biofilm formation in response to light [88]. A well-studied example of bacterial responses to light is in the opportunistic human pathogen *Acinetobacter baumannii*, which is able to sense and respond to blue light via the BLUF protein BlsA [62]. Blue light has been shown to inhibit motility of this pathogen in semi-solid agar and prevent early biofilm formation on glass surfaces [62]. Temperature dependence of this photoregulation suggests an important role for blue light sensing in the environmental locations outside of the human host, with light regulation lost at 37 °C. In fact, blue light has been found to modulate motility and biofilm formation among many species of the *Acinetobacter* genus [27]. The wide distribution of light sensing among the *Acinetobacter* genus suggests that light provides an important cue to this bacterium to facilitate acclimation to new environments.

The food-borne pathogen*Listeria monocytogenes* has also been shown to sense light via the blue light receptor Lmo0799, which is a potent inducer of the stress-sigma factor σ^B [67, 84]. This bacterium has been observed to form opaque and translucent rings on agar plates in response to light and dark growth conditions, respectively [84]. Bacteria isolated from the light-exposed opaque rings produced higher amounts of extracellular polymeric substances (EPS) and showed more resistance to stresses such as increased ROS [84]. These interesting results suggest that the blue light receptor Lmo0799 is able to sense light, alerting the bacterium to help protect against potentially destructive environmental conditions caused by the formation of ROS by light [84].

9.6.2 Differences in Susceptibility Among Microorganisms

Differences in the susceptibility of Gram-positive and Gram-negative bacteria toward light have been observed. Among a collection of clinically important pathogens, Gram-positive species were generally found to be more susceptible to 405 nm light and required a lower dose of light for inactivation than the Gram-negative species [56, 60]. Differences in sensitivity to light between pathogens have also been observed. Violet light at a wavelength of 405 nm has been demonstrated to inhibit *S. aureus* growth [56]. The bactericidal effect was attributed to the production of reactive species via photostimulation of endogenous intracellular porphyrins. It has been proposed that differences in photoinactivation rates by blue light are attributable to the types of porphyrins produced by different bacteria, rather than the overall quantity of porphyrin production [65]. Coproporphyrin is the predominant porphyrin in Staphylococcal strains, with the amount produced being 2–3 times higher than Gram-negative strains [65]. Therefore the enhanced susceptibility of Gram-positive strains to killing by light may be due to the carriage of more photosensitive endogenous porphyrins.

9.6.3 Factors Influencing Sensitivity

The human host offers a growth environment that is known to affect disease, for which bacteria are able to adapt and respond to changing environmental conditions [8]. Bacterial responses to host environmental conditions can impact their metabolic pathways, growth mode, population size, and age, factors which also influence microbial sensitivity toward antimicrobial treatments [14, 66]. It has been observed that bacteria in the stationary phase of growth are less susceptible to antibacterial agents than log-phase cells. Bacterial growth mode is a factor that has been shown to affect the susceptibility of *S. epidermidis* and *S. aureus* toward light [23]. In this study log-phase cultures were more sensitive to killing by light than stationary-phase cultures. Physiological changes during stationary phase, such as increased production of extracellular polysaccharides, have been shown to contribute to increased resistance to killing by photodynamic inactivation [23]. However the effect of growth mode on sensitivity to light has not been consistently supported, with others finding that growth phase has no significant effect on susceptibility to photodynamic inactivation [30, 45, 93].

Properties of the suspension medium have also been implicated in the susceptibility of microorganisms to light. The pH of the medium has been shown to have a marked effect on survival in light. Photoinactivation by red light and the photosensitizer toluidine blue O was shown to be greatest in an alkaline environment rather than an acidic environment [45]. In this study, it was postulated that pH may influence the penetration of photosensitizers into cells. Furthermore, the increased lifetime in the triplet state at high pH values may influence the oxidative damage caused

by light [86]. Differences in the sensitivity of Gram-negative and Gram-positive bacteria to light under acidic and alkaline pH conditions have led to the speculation that cell wall composition may play an important role in sensitivity to light. A recent study found Gram-negative *E. coli* O157:H7 was more sensitive to blue light illumination at an alkaline pH, while the Gram-positive *Listeria monocytogenes* was more sensitive at an acidic pH [25]. It has been postulated that under alkaline pH conditions, hydroxyl ions may lead to a weakened cell membrane which can be further damaged by ROS generation by light [25].

The biofilm mode of growth is known to provide bacteria with a survival advantage, helping the bacterial community to adapt to environmental changes. It has been shown that bacteria existing as part of a biofilm are better able to survive photodynamic inactivation using the photosensitizer methylene blue than their planktonic counterparts [87]. Given that chronic infections are more often associated with biofilm formation, these findings have clinical implications for the use of light to treat device-associated infections.

Oxygen is an essential component of photodynamic inactivation. In fact, the addition of oxygen during blue light exposure has been shown to increase the rate of inactivation of *S. aureus*, whereas decreased levels of ROS were generated in oxygen-depleted environments [55]. Similar results have been found for *P. gingivalis* and *F. nucleatum* during exposure to blue light under anaerobic conditions [19]. Despite the fundamental role of oxygen in photoinactivation, the addition of oxygen scavengers to media during light only partially protects bacteria from photoinactivation, which is perhaps due to the fast acting nature of excited singlet states [19]. Interestingly, less oxygen-tolerant microbes have been speculated to lack many of the key regulators that are used to counteract oxidative damage in aerobes, a factor which may make them more susceptible to reactive species produced during photoinactivation [61]. Biofilms are known to possess a heterogeneous oxygen gradient due to poor penetration of oxygen into the deepest layers of a biofilm [13, 76]. It would be expected that outer layers of the biofilm exposed to atmospheric oxygen would be more susceptible to light-induced formation of ROS than interior bacteria with limited oxygen. Limited oxygen concentrations in the deeper layers of the biofilm may limit ROS formation by light. The bactericidal effect of photodynamic inactivation has been demonstrated to occur predominantly in the outermost layers of the biofilm, with bacteria deeper in the biofilm surviving treatment [42]. This may also be due to limited diffusion of exogenous photosensitizers into deeper layers of the biofilm and stratification of light exposure by the biofilm [42].

9.7 Biofilm-Based Infections and Clinical Applications

The addition of light-activated photosensitizer molecules is used clinically to treat certain malignancies, some skin conditions, and gastric, dental, and viral conditions [32]. However the use of light without the addition of exogenous photosensitizers is gaining interest as an alternative antimicrobial therapy. While much work has

focused on the effectiveness of visible light to treat planktonic cultures [6, 23, 54, 56, 87], there is limited research into the effectiveness of visible light to treat biofilms [5, 58]. Unpublished data generated by the authors has shown that blue light can disrupt biofilm formation of *E. coli* on materials used to make catheters. Several studies have investigated the application of UVA and visible light to control biofilm formation on artificial surfaces including medical devices and food packaging material.

9.7.1 Visible Light Inactivation of Clinical Pathogens

Inactivation of pathogens by visible light is a new methodology that is a potential alternative to conventional antibiotics. A range of clinically important Gram-positive and Gram-negative bacteria species have been found to be inactivated using a visible 405 nm light LED array without the addition of exogenous photosensitizers [56]. Visible light has shown reduced germicidal efficiency in comparison to UV light, with one study examining inactivation of *E. coli* using UV irradiation at 270 nm showing 430 $\log_{10}$ per J cm^{-2} reduction [92], compared with a germicidal efficiency of visible light at 405 nm of 0.14 $\log_{10}$ per J cm^{-2} [56]. Despite the reduced germicidal efficiency of visible light in comparison to UV light, photo inactivation by visible light is considerably safer in terms of patient safety without the photodegradation of materials associated with light of the UV region, making it attractive for clinical applications [56].

Research into the effectiveness of 405 nm light to inactivate both Gram-negative and Gram-positive bacterial pathogens demonstrated inactivation not only in liquid suspension but also on agar plates and exposed inert surfaces [60]. In liquid cultures, *L. monocytogenes* was the most susceptible tested pathogen to light and was also readily inactivated when seeded onto agar surfaces. This study found that 405 nm light was able to inactivate aerosolized bacteria that had been deposited onto the surface of acrylic and polyvinyl chloride surfaces. In contrast to findings in liquid culture, *S. enterica* was more readily inactivated than *L. monocytogenes*, with both pathogens being more susceptible to 405 nm light inactivation on PVC surfaces, with an average dose of 45 Jcm^{-2} leading to more than 90% inactivation. The rate of inactivation was dependent on the dose of light, with inactivation of bacteria using higher intensity light achieved over a shorter duration. These findings suggest the potential for 405 nm light to be used in clinical settings or in the food industry to treat bacterial surface contamination.

Further study by this group investigated the bactericidal potential of 405 nm light for the inactivation of biofilms generated on glass and acrylic surfaces [58]. In this study, it was observed that 405 nm light was able to inactivate bacteria in both monolayer and mature biofilm populations. For *E. coli* monolayer biofilms that had been established for 4 h, a dose of 252 Jcm^{-2} was sufficient for complete inactivation on both glass and acrylic surfaces. The more densely populated biofilms required a greater dose. For mature biofilms that had been established for 48 h, a

higher dose of 504 Jcm^{-2} was required for near-complete (<1 CFU/mL) inactivation. These results demonstrate the potential for visible light to treat and prevent biofilms caused by pathogens and suggest the possibility of applying this approach in a clinical setting.

9.7.2 Medical Devices

Bacterial colonization of catheters is a significant problem that leads to severe complications in patients. As a result, new methods are needed to disinfect catheters and reduce the incidence of catheter-associated infections. One such method investigated by a group of Danish researchers is the use of UVC light to disinfect soft polymer tubes of a catheter [3]. In this study, UVC light from an LED diode was inserted into a polymer of ethylene vinyl acetate (EVA) tubes for intraluminal disinfection of the catheter. To simulate an aseptic breach, the device was contaminated for 3 h with a range of pathogenic microorganisms including *Candida albicans*, *S. aureus*, *E. coli*, and *P. aeruginosa*. UVC disinfection (275 nm) effectively killed bacteria after 2 min irradiance with 12.1 mJ cm^{-2} at the distal end of the catheter. However a longer irradiation treatment of more than 20 min was required to reach complete kill for *C. albicans*. The efficiency of UVC light to kill the multi-species microorganisms in the early stages of the biofilm was dependent on both the dose of light and duration of exposure.

These researchers also found that the dose required to achieve killing was dependent on the bacterial growth mode, whether growing as planktonic cells or as part of a biofilm. Due to the high doses required to effectively treat mature and opaque biofilms, it was suggested that frequent UVC light treatments starting from the time of catheter insertion would be more effective at preventing bacterial colonization and treat early catheter contamination [4]. This disinfection method has also been applied to silicone urinary catheters [5], which frequently cause the most common type of nosocomial infection CAUTIs. In this study, contaminated urinary catheters collected from patients were treated with UVC light. It was found that high doses (15 kJ m^{-2}) and longer treatment times (~60 min) of UVC light were required to effectively kill 99% of a mature biofilm. The dose required for a 99% reduction in biofilm was 100–1000 times greater than the lethal dose for planktonic cells. This may be due to the thick layer of bacterial cells and extracellular polymeric substances that composed the biofilm. This biofilm layer was found to attenuate light delivery with deceased disinfection efficiency as the bacterial content of the biofilm increased. It was later found that media can affect the light transmittance through the intraluminal space [3]. In this later study, the media that the bacteria were suspended in absorbed UVC light, which had an adverse effect on disinfection efficiency. Thus the local environment can influence the efficacy of light treatment, a factor which may have implications when trying to implement light as an antimicrobial treatment in a clinical setting, such as in catheters that are in close contact with bodily fluids [3].

9.7.3 Control of Food Pathogens on Surfaces and Packaging Materials

Food-borne disease outbreaks are very common worldwide. *B. cereus* is a spore-producing bacterium which is naturally found in soil environments. It is also a common food-borne pathogen frequently associated with contaminated foods including fresh fruit and vegetables and ready-to-eat foods. The use of light in the food industry is a promising method to inactivate microorganisms without adverse effects on food quality. Various studies have demonstrated that light can be used to inactivate food-borne pathogens on packaging materials [9, 53]. One such study investigated photoinactivation of *B. cereus* adhered to the surface of packaging material [53]. In this study, the production of endogenous porphyrins was stimulated by the addition of ALA. It was found that up to 20 min of illumination with 400 nm light with an intensity of 20 mW cm^{-2} (24 Jcm^{-2} dose) was sufficient to inactivate *B. cereus* that had been incubated with ALA. This method achieved more than a 4 log reduction in adherence of *B. cereus* to packaging material. The inhibitory effect of light without the addition of ALA was negligible. Light was also effective at killing spores of *B. cereus*, which are particularly troublesome due their tolerance of extreme environmental conditions. Incubation in ALA was also shown to kill *L. monocytogenes* upon illumination with the same dose of 400 nm visible light, with a up to 3.7 log decrease in recoverable of cells adhered to packaging material and 3.1 log decrease in biofilms [9]. These studies highlight the importance of both the amount of endogenous porphyrins and the illumination dose for inactivation of these food-borne pathogens adhered to packaging materials.

9.7.4 Photodynamic Inactivation of Biofilm

Photodynamic therapy enables the inactivation of pathogens using longer wavelengths which provides an advantage for clinical applications due to the increased potential for light to penetrate biological tissue and treat deep infections. Photodynamic inactivation of microbial biofilms has been demonstrated for prosthetic joint infections and dental infections involving implants. Biofilms on medical implants can lead to chronic infections, some of which require long-term suppressive antibiotic treatment and in some cases the removal of the implant [39].

Prosthetic joint infections are a growing concern as more people undergo procedures such as joint replacement. Prosthetic implants consist of a variety of materials including metal, plastic, and ceramic. A diverse range of pathogens are able to form biofilms on prosthetic implants. Photodynamic therapy using tetracationic ZN(II) phthalocyanine chloride as a photosensitizer, illuminated with a 689 nm laser diode, led to a reduction in biofilm mass and microbial counts for *S. aureus* and *P. aeruginosa* in vitro [89]. This suggests that photodynamic therapy may be a useful approach for prosthetic joint infections.

Peri-implantits is associated with inflammatory changes in the tissue surrounding dental implants and is mainly caused by pathogens that are also associated with various periodontal diseases. Immediately following a dental implant, a layer of host proteins cover the surface for which oral microorganisms adhere and form a biofilm. The biofilm formed on the surface of teeth is known as dental plaque and is associated with a range of periodontal diseases. A group of researchers have investigated the effect of photosensitizer toluidine blue ortho (TBO) in combination with either laser or LED light (620–660 nm) on the viability of biofilms of the oral pathogen *Streptococcus mutans* [81, 96, 97]. The combination of TBO and light was effective to reduce viability of biofilms grown on hydroxylapatite disks. Mature biofilms were more difficult to eradicate with photoinactivation occurring predominately in the outermost layers of the biofilm [96]. Similarly, photoactivation of TBO produced a 95% reduction in the viability of 5 day old *S. mutans* biofilms grown on enamel slabs [97]. The effectiveness of TBO in the treatment of dental biofilm has also been tested in situ. Multi-species biofilms were grown on human enamel slabs worn in the mouth of human volunteers for 7 days [81]. Illumination of TBO showed a slight reduction in the total streptococcal microbiological counts; however, this was not a statistically significant decrease. Other studies have also found that photodynamic therapy was unable to completely destroy complex multi-species biofilms [18]. An in vivo study investigating peri-implantitis among dogs found that photodynamic therapy using a paste-based azulene photosensitizer and laser light (660 nm) leads to a significant reduction in the amount of contamination on the surface of implants [34]. A clinical study of 15 volunteers with peri-implantitis around dental implants found toluidine blue O irradiated with a diode laser at a wavelength of 690 nm for 60 seconds led to a 2 log reduction in bacterial counts on implant surfaces [17]. Photosensitizers methylene blue [22, 50] and porfimer sodium [57], which is a complex mixture of porphyrin-modified oligomers commercially sold as Photofrin to treat cancer, have also been demonstrated to reduce biofilms by oral pathogens. Cumulative blue light treatment (455 nm LED) without the addition of photosensitizers has also been found to suppress biofilm growth in vitro by targeting endogenous porphyrins of black-pigmented oral pathogens [21]. These species accumulate photosensitive cell surface black pigments that consist of μ-oxobisheme of iron protoporphyrin IX or monomeric iron protoporphyrin IX [75]. These studies suggest that photodynamic therapy is a promising non-invasive method to reduce microbial adherence to dental implants; however, more evidence is needed into the effectiveness against multi-species biofilms.

9.8 Effect of Light on Mammalian Cells

Microbial inactivation by light holds tremendous opportunity to treat difficult infections caused by antibiotic-resistant or biofilm-producing microorganisms. However the safety of this treatment needs to be evaluated to ensure there is no collateral damage to host cells and tissue. Studies in human cell lines and animal models have shown that UV radiation can lead to mutations and cell death [40, 68, 78]. At shorter

wavelengths, DNA absorbs UV radiation [40]. The first step in host cell mutation by UV radiation is DNA damage which leads to a cascade of cell responses for DNA repair, mutation, and transformation [68]. Investigation into the illumination dose required to give 37% survival of human epithelial P3 cells found that for UVC light (254 nm) a fluence of 11 Jm^{-2} was required; however, for UVA light (365 nm), a higher fluence of 17×10^5 Jm^{-2} and 3×10^6 Jm^{-2} for blue light (434 nm) was needed to achieve a similar amount of killing [40]. This study found no increased mutation frequency at 334 nm; however there was clear mutagenic effect at 365 nm [40]. In the visible region, 405 nm light showed a weak mutagenic response with no mutagenesis observed using 434 nm light. These findings suggest that prolonged exposure to UV radiation may lead to malignant changes within host cells, with visible light inactivation having a clear advantage in terms of its safety.

More recent in vitro studies have found blue light to induce damage in mammalian cells [26, 37]. Cytotoxic effects of blue light on mammalian cells is thought to occur via photo-excitation of intracellular chromophores that are sensitive to blue light, leading to the generation of ROS [12, 37]. In an in vitro study [26], 6 h illumination (390–500 nm, 60.5 Jcm^{-2}) of human primary retinal epithelial cells was found to induce ROS-mediated damage to mitochondrial DNA and subsequently cell death. Further studies found high irradiance blue light (412–426 nm, 3×66 Jcm^{-2}) had a detrimental effect on human keratinocytes and skin-derived endothelial cells, while higher wavelengths (632–940 nm, 3×100 Jcm^{-2}) caused no damage [49]. In contrast to these in vitro studies, a clinical investigation into to effect of blue light (390–460 nm) on the skin of human volunteers found that a daily dose of 20 Jcm^{-2} over five consecutive days showed no photodamage to the skin or changes in inflammatory cells [44]. Further in vivo studies are needed to determine the safety of blue light for clinical applications.

The safety of photodynamic therapy with the addition of exogenous photosensitizers for microbial inactivation has been investigated in human cell and animal models [46, 59, 98]. For long-term use in chronic infections, it is important that photodynamic therapy is effective at inhibiting microbial growth and biofilms without causing mutagenic or carcinogenic effects on host cells. Histological evaluation of mice treated for oral candidiasis by photodynamic therapy found illumination with 455 nm or 630 nm (305 J cm^{-2}) LED light and showed no adverse effect on adjacent tissue the day after treatment [59]. Additionally, photodynamic therapy of infected wounds of the skin were shown to heal as well as control wounds, suggesting there was no damage to host cells [33]. Despite these promising safety results, many studies investigating host cell damage have been performed on short-term studies with no evidence on the long-term effects of photodynamic therapy on host cells.

9.9 Future Prospects

Recent studies in this exciting field have already demonstrated the potential of light-based technologies to be used in antimicrobial applications, both in the healthcare and food sectors. Photodynamic therapy is already available commercially for the

treatment of acne, with Levulan using the active ingredient ALA. However, there are challenges that still need to be overcome, not least of which is ensuring the safety of the patient in the case of medical applications. Regulatory authorities will have to approve any new light-based clinical interventions that are developed for use in or on patients, and presumably inpatient clinical trials will be a necessary part of this process. The optics to deliver defined doses of fixed wavelength light will have to be engineered to suit individual clinical or food applications. But the widespread availability of low cost LED and laser diode light sources should mean that this is not an insurmountable obstacle. The question of whether or not to use exogenous photosensitizing compounds as part of the treatment will have to be carefully evaluated for each application. Overall the enormous promise of light-based antimicrobial technologies should provide a sufficient motivating force to ensure that solutions are found to overcome these obstacles. An interdisciplinary approach involving biologists, physicists, engineers, and surface chemists will be vital to ensuring that this innovative antimicrobial treatment reaches its full potential.

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Chapter 10
Light-Triggered Anti-Infective Surfaces

Rebecca A. Craig and Colin P. McCoy

10.1 Introduction

Light is an attractive stimulus for conferring antimicrobial activity to surfaces due to its abundance in the environment and the ability to tailor its wavelength and dose, thus allowing tailored antimicrobial properties according to requirements. Light has been used in conjunction with light-sensitive compounds in medical treatment from as early as 2000 BC, when the psoralen-containing plants *Ammi majus* or *Psoralea corylifolia* were used to treat hyperpigmentation and leukoderma of the skin by Egyptians and in the Indian Ayurvedic system [1]. Although unknown at the time, psoralens in conjunction with sun-derived UVA light are now understood to cause photocyclizations of nucleic acid bases and generation of highly reactive singlet oxygen ($^1O_2$), thus representing a quasi-photodynamic therapy [2]. The medical applications of light in combination with photoactive compounds were not further exploited until the nineteenth century when Downes and Blunt discovered the bactericidal actions of sunlight [3], followed by light-induced curing of lupus vulgaris by Niels Finsen in 1896. Since that time, substantial progress has been made, with a growing understanding of light-sensitive compounds and their synthesis, thus providing a range of compounds which can respond to light of different wavelengths. As a result, a number of compounds are being investigated for development and have found utility in the modification of medical device surfaces for benefit to the patient. These compounds can be divided into three main groups: photosensitizers, photocatalysts, and photocleavables.

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10.2 Photosensitizers

Photosensitizers were first discovered by Oscar Raab in 1900, observing the lethal effects of acridine orange and light on *Paramecium* protozoan cells [4]. Further studies by von Tappeiner demonstrated the effects of topically applied eosin in conjunction with light on skin cancer and showed that the photodynamic effect arose due to dye-photosensitized photo-oxygenation of cellular components and peroxide accumulation [5, 6]. The term "photodynamic effect" was coined by von Tappeiner in 1904 to describe the reaction of light with photosensitizer in the presence of oxygen [6].

10.2.1 Mechanism of Action

As described by von Tappeiner, three main components are necessary for photosensitization: photosensitizer, molecular oxygen, and light. Delivery of light of the appropriate wavelength to the photosensitizer will, following its absorption, cause excitation of the photosensitizer from the ground state ($^1PS_0$) to the first excited state ($^1PS_1^*$). This can then undergo intersystem crossing to the excited triplet excited state ($^3PS_1^*$). The excited photosensitizer can then transfer energy or electrons to ground state molecular oxygen ($^3O_2$) to produce reactive oxygen species (ROS). Two main photochemical pathways are possible: type I pathways, involving electron or hydrogen atom transfer, and type II reactions involving energy transfer to molecular oxygen [7–9]. Type I reactions will lead to the formation of superoxide ($O_2^{\bullet-}$), which can further react to form H_2O_2 and hydroxyl radicals ($^\bullet OH$). The type II pathway is believed to be the predominant mechanism of photosensitizer-mediated cell death in photodynamic therapy (PDT) and photodynamic antimicrobial chemotherapy (PACT) [10–12], involving transfer of energy from the excited triplet state photosensitizer to ground state (triplet) molecular oxygen ($^3O_2$), generating excited singlet oxygen ($^1O_2$), which is highly reactive and strongly electrophilic, with a longer lifetime than other ROS [13]. The reaction is catalytic, with the photosensitizer remaining unconsumed in the process [10]. Type I reactions, however, increase in importance where oxygen concentrations are lower [14] or in more polar environments (Fig 10.1).

Although both pathways can occur simultaneously, they may occur in different proportions depending on the structure of the photosensitizer and on the microenvironment [16, 17]. ROS such as $^1O_2$ are highly reactive and can indiscriminately initiate oxidative reactions with a number of cellular components, most notably polyunsaturated fatty acids, but including lysosomes, mitochondria, plasma membranes, and Golgi apparatuses [18]. As $^1O_2$ is widely accepted as the predominant ROS, this will form the basis of ensuing discussions in this chapter.

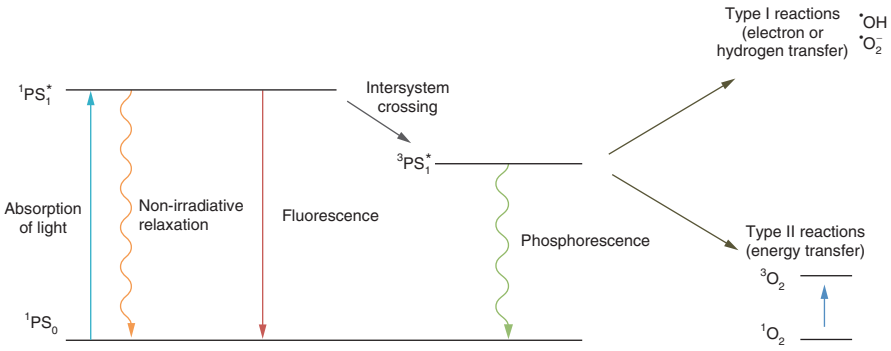

Fig. 10.1 Jablonski diagram illustrating the main photophysical processes occurring on irradiation of a photosensitizer with the appropriate wavelength of light (From [15])

10.2.1.1 Photosensitizer Classes

Photosensitizers are planar, unsaturated organic molecules with a delocalized electron system, causing the photosensitizer to be deeply colored [19]. One of the earliest used photosensitizers was hematoporphyrin [20], with its derivative, hematoporphyrin derivative (HpD), becoming widespread in the clinic. Although HpD demonstrated success, it was associated with a number of disadvantages including its retention by human cutaneous tissue for many weeks, causing significant skin photosensitivity [21]. Additionally, the targeted absorption peak at 630 nm is weak, thus resulting in the photosensitization reaction being less efficient than desired. Further research has led to the development of a number of newer-generation compounds.

The photosensitizers most frequently used in either clinical practice or research are from the porphyrin, texaphyrin, phenothiazinium, chlorin, and phthalocyanine classes. An additional compound is 5-aminolevulinic acid (ALA), a prodrug for endogenous production of protoporphyrin IX. Some examples of compounds from these classes are shown in Fig. 10.2.

10.2.1.2 Photodynamic Therapy

In photodynamic therapy (PDT), the photosensitizer is administered to the patient systemically or topically, allowing time for its selective localization in the target tissue, before irradiation of the tumor site with the appropriate wavelength of light. Selective localization occurs due to the physiological differences between healthy tissue and tumor tissue. The photosensitizer is thought to interact with low-density lipoprotein receptors, which are elevated in cancer cells. This allows selective accumulation of the photosensitizer in the tumor tissue, further facilitated by the leaky microvasculature and poor lymphatic drainage of tumors [22, 23]. Damage to normal tissue is therefore minimal in comparison with traditional chemotherapy, although skin photosensitivity is a commonly encountered side effect, requiring sun protection for a period of time following treatment [24].

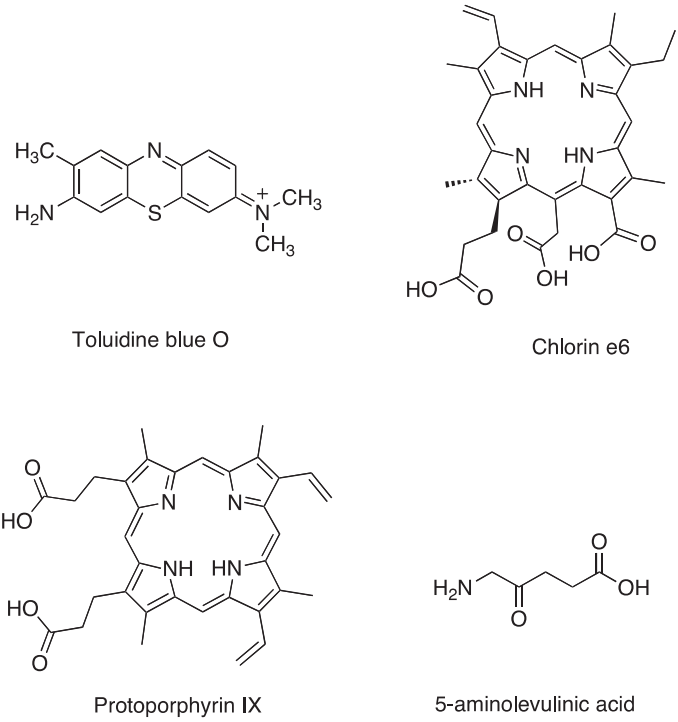

Fig. 10.2 Some examples of photosensitizers from the phenothiazinium class (toluidine blue O), the chlorin class (chlorin e6), the porphyrin class (protoporphyrin IX), and the prodrug 5-aminolevulinic acid

Wavelengths of light corresponding to the absorption maxima of the photosensitizers are chosen for use, but as PDT involves delivery of light across the barrier of the skin to reach the treated tumor, wavelengths in the red region are of greatest interest. For example, although blue light will more efficiently activate porphyrin compounds due to their strongly absorbing Soret band, the associated photobleaching and poor penetration through tissue have led to light in the red region being more frequently used, thus allowing activation of the Q bands. Although the Q bands are more weakly absorbing, the use of a higher fluence circumvents any decrease in efficacy [25].

Photosensitizers have been approved for the clinical treatment of a number of conditions including cancerous conditions and cosmetic indications such as actinic keratosis [26–28]. A table of a number of those approved is shown in Table 10.1. Many other photosensitizers are currently undergoing clinical trials, and it is expected that the number approved for a range of conditions will continue to increase over the coming years.

For a thorough review of photosensitizers currently marketed and used clinically in PDT, reference can be made to Allison et al. [29]. An area of interest for PDT is that of phototheranostics, involving the use of single nanoentities with the capability for targeted delivery, optical imaging, and photodynamic treatment. This is reviewed in [30].

Table 10.1 Photosensitizers and approved clinical uses

Photosensitizer	Condition
Hematoporphyrin derivative	Esophageal, lung, bladder, gastric, skin, breast, oropharyngeal, and cervical cancer; Barrett's esophagus with dysplasia, cervical dysplasia, psoriasis, prophylaxis in corneal transplant opacity
Methylene blue	Sterilization of frozen plasma
Methyl aminolevulinate	Actinic keratosis, nodular or superficial basal cell carcinoma, Bowen's disease
5-Aminolevulinic acid	Actinic keratosis
Hexaminolevulinate	Diagnosis of bladder cancer
8-Methoxypsoralen	Psoriasis

10.2.1.3 Photodynamic Antimicrobial Chemotherapy

The development of antibiotics in the 1940s stalled research into the antimicrobial uses of photosensitizers until the late twentieth century, when they were applied to the treatment of the herpes simplex virus [31–33]. Photodynamic antimicrobial chemotherapy (PACT) is based on PDT but is specifically applied to target microorganisms. The underlying principle of PACT is that if selective demonstration of live microbial cells is possible with a particular dye, which is also photosensitive, then illumination of the stained microbial cell should result in death of that cell when within a human subject or other environment [34]. Traditionally, PACT involves administration of photosensitizer in solution to microorganisms, allowing time for non-specific uptake by the microorganism, followed by administration of light of an appropriate wavelength. Photosensitizers have been found to be effective against bacteria [35] including hypervirulent *Clostridium difficile* [36], viruses [37], fungi [38], protozoa [39, 40], and bacterial spores [41–46]. The reasons for such high efficacy are twofold. Firstly, due to the nonselective nature of attack by $^1O_2$, any part of the microbial cell is a potential target, thus making resistance almost impossible. Development of resistance usually requires targeting of a specific site [47]. Secondly, microbial species have no effective defense mechanisms against such a degree of attack from ROS. The redox imbalance inflicted by ROS is greater than that which can be dealt with by cellular antioxidant species, and antioxidant enzymes present in cells are inactivated by $^1O_2$, thus compromising the cell ability to deal with the attack [39]. Despite attempts to induce photosensitizer resistance in microorganisms, to date none has been found [48]. This makes photosensitizer-mediated antimicrobial strategies particularly appealing in a time when drug-resistant microorganisms are widespread and posing an increasing challenge to conventional antibiotic-mediated treatments, to the extent that in 2014 the World Health Organization declared it to be a serious worldwide threat to public health [49]. Indeed, photosensitizers have similar efficacy against drug-resistant organisms as nonresistant organisms [50–53].

The range of photosensitizers used clinically for PACT is narrow and restricted mostly to those of the porphyrin and phenothiazinium class, in addition to neutral red, and a conjugate between chlorin (e6) and polyethylenimine. A comprehensive review of the uses of PACT can be found in [54] and [37]. Current uses of PACT include blood product disinfection, acne and rosacea treatment [55–57], peptic ulcer

disease [58, 59] and verruca [60, 61] treatment, nasal decolonization [62], dental treatment, and blood decontamination [63–65], in addition to nonmedical uses such as water disinfection [66, 67].

Bacteria often exist in a biofilm—a community of bacterial cells growing on a surface and encased in an exopolysaccharide matrix. Biofilms are found in approximately 80% of infections and are known to be around 1000-fold more resistant to antibiotics, antiseptics, and detergents [68]. PACT has shown high efficacy against both bacterial and fungal biofilms [69–74]. It is for this reason that PACT has been employed in the treatment of periodontitis [75–79]. Other areas that are highly susceptible to biofilm formation include catheters, contact lenses, and permanent indwelling devices such as joint prostheses, and therefore employing photosensitizer-mediated inactivation in such situations would be of great benefit. Surface immobilization of photosensitizers on such materials is a growing area of interest, with potential for preventing or eradicating biofilms, and is discussed in depth in the next section.

10.2.2 Surface Immobilization

Traditional PACT, while beneficial in a number of areas, is difficult to apply to the decontamination of medical devices when in situ in the patient due to the requirement for photosensitizer uptake by the microorganism. Immobilization of photosensitizers to polymer surfaces, or within the polymer structure, enables photosensitizer-mediated medical device surface decontamination. A large number of medical devices are amenable to such treatment, with devices such as contact lenses and intraocular lenses being naturally exposed to light through the eye. For other devices, a light source can potentially be inserted through the lumen, without discomfort to the patient. Photosensitizer-mediated antimicrobial strategies therefore provide significant advantages in an area with which significant cost and mortality are associated, and the overuse of antimicrobials can encourage the development of resistant strains.

Penetration of the photosensitizer into or direct contact with the microbial cell is not required for efficacy, with $^1O_2$ alone being sufficient for cidal action [80, 81]. The mechanism of action differs only marginally to traditional PACT in that, rather than requiring uptake by the bacteria, the photosensitizer remains immobilized on the surface of the material. Application of light leads to production of ROS such as $^1O_2$ at the material surface that can act prophylactically to cause death of approaching microbial cells, thus preventing adherence and device infection. Generated $^1O_2$ can also exert cidal action toward adhered microbial cells and indeed biofilms, from underneath. The lifetime of $^1O_2$ is short, in the region of 10^{-5}–10^{-6} s, limiting its effective distance to a few micrometers [13]. This is sufficient to prevent bacterial adhesion to the surface, the reinforcement of which is only thought to occur within 1 nm of the surface [82], but sufficiently short to avoid damage to surrounding tissue. This provides an attractive approach as it circumvents the necessity to achieve sufficiently high antibiotic concentrations in body fluids to eradicate biofilms and the risk of exposing bacteria to subtherapeutic levels of antibiotic, which would other-

wise increase the risk of development of drug-resistant strains. Their use would not only be limited to implanted medical devices but could be expanded to any surface within a clinical environment where light can easily be applied. For the purpose of this chapter, only materials relevant to medical devices will be discussed. A review of photosensitizer-mediated antimicrobial surfaces for other uses, such as antimicrobial packaging and textiles, can be found in Alves et al. [83] and Brovko [84].

A growing number of groups have been investigating photosensitizer modification of medical device materials. Some of the most prolific are the McCoy and the Wilson and Parkin groups.

Exploiting the natural light entering the eye, McCoy et al. have developed anti-infective intraocular lens materials [13, 85, 86]. These materials are based on acrylate copolymer hydrogels with ionic pendant groups at the surface. This facilitated electrostatic interaction with the tetracationic porphyrin tetrakis-(4-*N*-methylpyridyl) porphyrin (TMPyP) or the anionic porphyrin tetrakis-(4-sulfonatophenyl)porphyrin (TPPS) following a simple dip modification. TPPS photochemical properties appeared to be adversely affected by binding to the polymer, but TMPyP was still found to generate $^1O_2$ when illuminated with white light. A highly localized layer of TMPyP was achieved at the material surface, with promising antibacterial results with Gram-positive and Gram-negative organisms.

Also using a dip incorporation method, but employing a swell–shrink mechanism rather than electrostatic interactions, is the Wilson and Parkin group. A number of photosensitizers have been successfully incorporated into silicone and polyurethane by this method, materials that are commonly used for urinary and central venous catheters. Toluidine blue O, a phenothiazinium photosensitizer, was incorporated into silicone and polyurethane with and without nanogold using acetone–water mixtures [87]. Highly colored samples were attained which, on irradiation with a 634 nm laser, were capable of significant antibacterial activity. A greater uptake of TBO into polyurethane than silicone was achieved, with correspondingly higher $^1O_2$ generation. Polyurethane samples were therefore more strongly antimicrobial, with >5 log reductions of methicillin-resistant *S. aureus* (MRSA) and *E. coli* adherence after 1 and 2 min irradiation, respectively. Reductions on silicone were lower but still very efficient, with only 3 min irradiation required for a >5 log MRSA kill and 1.5 log reduction of *E. coli* achieved in the same time. No dark toxicity was observed, and the nanogold did not significantly enhance the cidal effect of the TBO. The same groups have also incorporated methylene blue, another phenothiazinium photosensitizer, and nanogold into polyurethane, irradiating with white light for 24 h to achieve antibacterial effects [88], and methylene blue into polyvinyl chloride catheters, irradiating with 660 nm laser light for up to 8 min [89], thus demonstrating the versatility of polymer substrates and light sources. One disadvantage of the method is the long incorporation times (usually 24 h) required to achieve sufficient photosensitizer levels. Nanogold was found in some studies to synergistically enhance the efficacy of the photosensitizer-mediated bactericidal action of the polymers [88, 90], but other experiments found no significant difference on its addition [87].

Phenothiazinium and porphyrin photosensitizers appear to be among the most effective photosensitizers when immobilized in medical device polymers; however other photosensitizers have shown promise. Using a similar preparation method, the

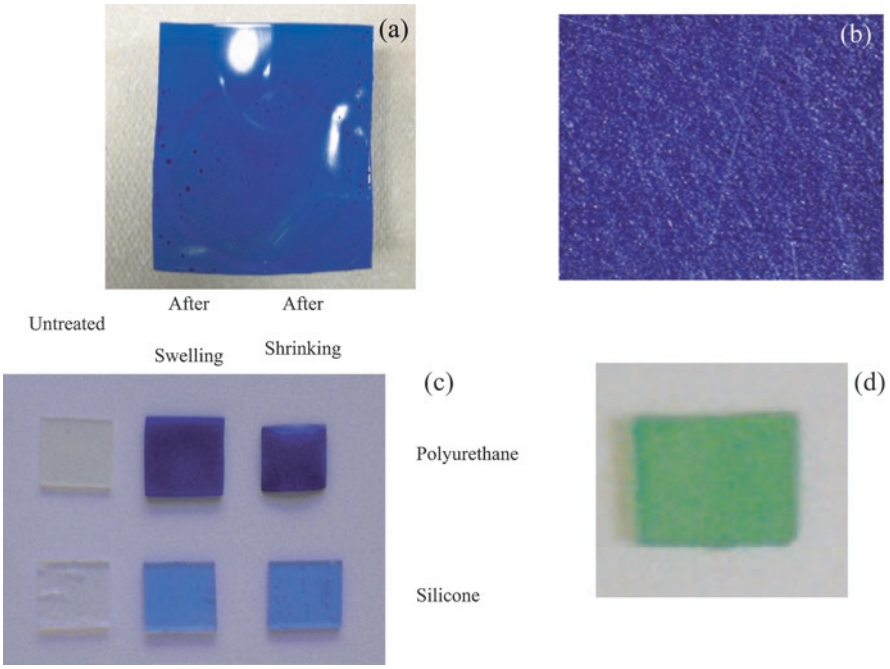

Fig. 10.3 Examples of photosensitizer-incorporated medical device materials. (**a**) Methylene blue-incorporated polyurethane, following 24 h swell in acetone–water [88]; (**b**) co-extruded TBO (0.4%) in polyethylene [93]; (**c**) TBO-incorporated polyurethane and silicone, incorporated by the swell–shrink method [87]; (**d**) indocyanine green-incorporated polyurethane, incorporated by the swell–shrink method [91]

groups have also incorporated indocyanine green into polyurethane [91] and crystal violet with nanogold into silicone [92]. The reductions in bacterial adherence with indocyanine green were lower than observed with phenothiazinium sensitizers, 2 log reduction of MRSA and *Staphylococcus epidermidis* and a 0.5 log reduction of *E. coli* following 15 min exposure to near infrared light (808 nm), but this demonstrates promise for further studies (Fig. 10.3).

Efficacy against *S. epidermidis* biofilm with a methylene blue-containing silicone has also been demonstrated by the same group. A 50% reduction of biofilm coverage was achieved when irradiated with 660 nm laser light, delivering 117 J cm^{-2} in doses of 10 min, with 60 min intervals over a 6 h period [94]. This confirms the efficacy of photosensitizer-incorporated surfaces in both the prophylaxis and also the arresting and reduction of biofilm formation.

Using a different technique, Funes et al. fabricated electrochemically generated polymeric films composed of porphyrin units [95]. The porphyrin, 5,10,15,20-tetra(4-N,N-diphenylaminophenyl)porphyrin, was either used alone or complexed with palladium chloride. A 3 log reduction of *E. coli* viable count and a 2 log reduction of *C. albicans* viable count were achieved following 30 min irradiation.

Co-extrusion of photosensitizers has also been employed as an incorporation method, with success. McCoy et al. extruded polyethylene with TMPyP, TPP

(5,10,15,20-tetraphenyl-21H,23H-porphine), TBO, and methylene blue in the development of multipurpose materials, whose potential uses include tubing, collection bags, and other inanimate hospital surfaces [93]. Negligible leaching of photosensitizer from the material was noted, and good antimicrobial performance was seen, with a 3.62 log reduction of MRSA adherence and a lower reduction of *E. coli*, following 2 h exposure to white light. The potential to extrude polymers containing photosensitizers provides a method whereby solvent damage to sensitive materials can be avoided. A similar method involving hot-pressed poly(vinylidene fluoride) with a variety of photosensitizers has also been used to achieve a white light-activated antibacterial layer on polyethylene [96]. The efficacy was related to the bacterial inoculum, with >4 log reductions in *S. aureus* observed following 6 h irradiation of materials containing rose bengal, when challenged with 10^3 cfu/ml, but increasing the inoculum to 10^5 cfu/ml or 10^7 cfu/ml significantly reduced the efficacy. This is likely related to the agglomeration of bacterial cells at higher concentrations, reducing the available O_2 for photosensitized reactions due to bacterial metabolism and hindering the diffusion of $^1O_2$ to cells at the center of the agglomerates [11].

A further incorporation method is the copolymerization of photosensitizers with medical device polymers. Felgentrager et al. functionalized and polymerized TPP with polyurethane, before spraying an approximately 30 μm layer onto a polymethylmethacrylate polymer plate [97]. A 3 log kill of *S. aureus* was achieved relative to dark control following 30 min irradiation with a broadband light source (> 400 nm). Similarly, Piccirillo et al. covalently bound methylene blue or TBO to silicone polymers via an amide condensation reaction, achieving a 5 log reduction of *S. epidermidis* and a 1–2 log reduction of *E. coli* following 4 min irradiation of low-power red light [98].

To date, there are no photosensitizer-incorporated polymers currently marketed for antimicrobial purposes (or any other purpose), but the experimental results are promising, and it is expected that in the coming years, a number of such materials will be available for use in the clinical setting. To this end, a number of patents have been filed including those detailing antimicrobial conjugates that can be applied to materials as a coating or incorporated into polymers to make antibacterial materials [99], porphyrin derivatives in coatings for implantable devices including stents and catheters [100], catheters coated with porphyrin or phthalocyanine [101], photosensitizer-incorporated medical device materials [102], and design of a catheter with an imbedded light source for photosensitizer-mediated antimicrobial activity [103].

10.3 Photocatalysts

10.3.1 Background

The photoactivity of titanium dioxide (TiO_2) has been known for approximately 100 years. As a commonly used white pigment in paints, it had been noted that exposure to sunlight caused flaking of TiO_2-containing paints. On the discovery in 1938 of the production of reactive oxygen species at surfaces containing TiO_2, and

the resultant photobleaching of dyes [104], TiO_2 was initially described as a photosensitizer until the 1950s, when further knowledge about its mechanism of action led to it being correctly described as a photocatalyst. One of the first studies reporting the photocatalytic effect of TiO_2 with UV irradiation was published in 1964 (as cited in [105]). Following this, in 1972, the photocatalysis of the decomposition of water by TiO_2 was described by Fujishima, working under the supervision of Honda [106]. The reaction is now known as the Honda–Fujishima effect. Following from the pioneering work by these, and other researchers, substantial efforts have been made in the understanding and design of photocatalytic systems.

A number of photocatalysts are known, including TiO_2, ZnO, SnO_2, and CeO_2. Of these, TiO_2 is the most frequently used due to its efficacy, low cost, high photoactivity, and availability [107, 108]. Three main polymorphic forms of TiO_2 are known: anatase, rutile, and brookite. Anatase is widely known to be the most effective photocatalyst for anti-infective applications, although mixtures of anatase and rutile have been shown to be more effective photocatalysts than 100% anatase [109]. Due to the frequency of the use of TiO_2 in comparison with other photocatalysts, TiO_2 will form the main focus of this section.

10.3.2 Mechanism of Action

TiO_2 is a semiconductor. Semiconductors are characterized by a band energetic structure, with a bandgap between the electron-filled valence band and the unoccupied conduction band [108]. Adsorption of a photon of light, whose energy at least equals that of the band gap, causes promotion of electrons from the valence band to the conduction band, leaving behind a positively charged "hole" in the valence band. The electrons can then migrate freely within the conduction band. The holes may be filled by electron migration from an adjacent molecule, allowing the process to be repeated. Holes and electrons can recombine in a nonproductive reaction (bulk recombination) or can react with dissolved water and oxygen to produce reactive oxygen species. Hydroxyl radicals (•OH) are produced by the oxidation of water, and superoxide radicals (O_2^-) are produced by reduction of oxygen [110]. These can react further in solution to generate hydroxyl radicals, hydroperoxyl radicals, and H_2O_2. A schematic representation of the mechanism of photocatalytic reaction is shown in Fig. 10.4, and the reactions are detailed in Scheme 10.1.

The energy required to promote an electron in anatase (the bandgap) is approximately 3.2 eV, and therefore photocatalysis can be activated with UVA light (< 385 nm). The requirement for UVA light can limit the utility of photocatalysis in disinfection, but doping with metals such as copper, lead, tin, Fe^{3+} [112], Cr^{6+}, Mo^{5+} [113], silver ions [114], and rare earth ions [115] can cause a bathochromic shift in the energy required for electronic excitation, reducing the bandgap and thus allowing visible light activation, as can dye sensitization with dyes such as 8-hydroxyquinone [116] and doping with nitrogen [117]. A review of the detailed mechanism of photocatalytic reaction and methods of optimization can be found in Carp et al. [118].

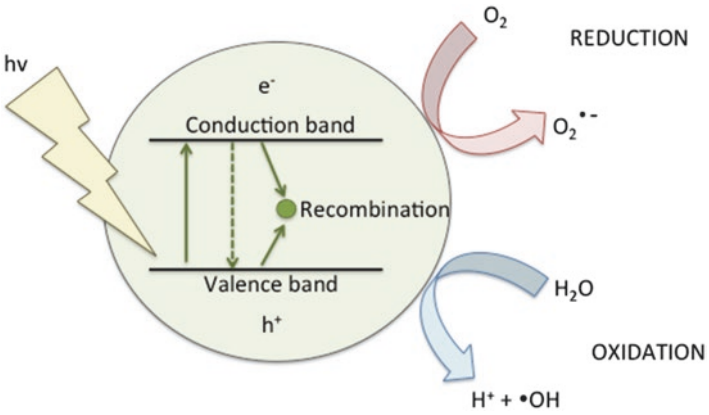

Fig. 10.4 Schematic representation of the basic photocatalytic reaction. Irradiation of light (hv) causes promotion of an electron (e^-) from the valence band to the conduction band, leaving behind a positively charged hole (h^+) in the valence band. The holes and electrons can take part in oxidation and reduction reactions, respectively, leading to the generation of reactive oxygen species, or can recombine in a nonproductive reaction (Adapted from [111])

Scheme 10.1 Photochemical, oxidative, and reductive reactions occurring in photocatalysis

$$TiO_2 + hv \rightarrow e^- + h^+$$

$$e^- + O_2 \rightarrow O_2^{\cdot -}$$

$$h^+ + H_2O \rightarrow \bullet OH + H^+$$

$$h^+ + OH^- \rightarrow \bullet OH$$

$$\bullet OH + \bullet OH \rightarrow H_2O_2$$

$$2O_2^{\cdot -} + 2H_2O \rightarrow 2\bullet OH + 2OH^- + O_2$$

10.3.3 Antimicrobial Properties

Antimicrobial activity of TiO_2 was first demonstrated in 1985, where complete elimination of *E. coli*, *Lactobacillus acidophilus*, and *Saccharomyces cerevisiae* was achieved following irradiation of platinum–TiO_2 [119]. Following from this, a vast and ever-increasing amount of research has been conducted into the use of photocatalysis for eradication of a number of microorganisms in a variety of situations. TiO_2 has found use as a photocatalytic antimicrobial agent in self-cleaning surfaces, water disinfection, air purification, pharmaceutical and hospital applications, and food production. A comprehensive review of the varying antimicrobial applications of TiO_2 can be found in [120].

$$\text{Organic compound} \xrightarrow{\text{TiO2, hv}} CO_2 + H_2O + \text{mineral acid}$$

Scheme 10.2 Mineralization of organic compounds in photocatalysis

The only components required for antimicrobial activity are photocatalyst, light, oxygen, and water [108]. Similar to photosensitizer-mediated microbicidal action, the main mechanism of cell death effected by photoactivated TiO_2 is thought to be via oxidative damage to the cell membrane and cell wall. This can be mediated by direct contact with the photocatalyst, contact with hydroxyl radicals in close proximity to the catalyst (within approx. 1 μm of the catalyst), or contact with H_2O_2 close to or at a distance from the catalyst. It is generally accepted that •OH is the main cause of cidal actions [108, 121].

Photocatalysis has been shown to exert cidal actions toward bacteria [122, 123], endospores [124, 125], fungi [126, 127], viruses [128, 129], protozoa [130, 131], algae [132], and prions [133, 134]. Resistance has been shown by *Acanthamoeba cysts* and *Trichoderma asperellum* conidiospores [135, 136], but it is possible that higher TiO_2 concentrations or longer time periods may enable effective eradication. Close contact between the bacteria and TiO_2 has been demonstrated to increase the extent of oxidative damage. An advantage of photocatalysts over conventional anti-infective measures is their ability to cause complete bacterial cell mineralization to CO_2 and H_2O following cell damage and death [121], preventing buildup of cell debris on the surface (Scheme 10.2).

10.3.3.1 Antimicrobial Surfaces

Photocatalytic TiO_2 surfaces have long been used as anti-fouling and self-cleaning surfaces in industry, and their uses in medical applications are being increasingly investigated. The total oxidation of organic substances by TiO_2, as already described, acts to prevent the adhesion of bacteria and biofilms to material surfaces. Due to the already described resistance known by a small number of organisms toward TiO_2, it is only appropriate to refer to TiO_2 surface properties as self-disinfecting rather than self-sterilizing. One disadvantage of TiO_2 is that it will degrade all organic material over time, which may result in production of harmful by-products and intermediates [118, 137]; however its antimicrobial benefits may outweigh this.

TiO_2-containing silicone catheters have been developed by Sekiguchi et al. [138]. A decrease in survival rate of *E. coli*, *P. aeruginosa*, and MRSA locked inside the catheters was observed following UVA irradiation, although only a 40% difference in rates of positive culture of bacteria on catheter tips was observed between treated and untreated catheters. Of the 18 patients who took part in the clinical arm of the study, 3 found a worsening of clinical symptoms, while 15 did not perceive a difference between TiO_2 catheters and conventional catheters. A more recent study by Yao et al. investigated the efficacy of Ag–TiO_2 silicone catheters [139]. Dark activity was observed, with UV a 2 log reduction in *E. coli*, *P. aeruginosa*, and *S. aureus* adherence following 20, 60, and 90 min, respectively. The dark activity of Ag–TiO_2

catheters against *E. coli* was greater than the activity of TiO_2 catheters when irradiated. With UV illumination, only 3–5 min was required for a similar reduction in *E. coli* adherence, although results were not detailed. Self-cleaning properties of Ag–TiO_2 catheters were also noted with UV irradiation. This system therefore shows promise as an antimicrobial catheter material, particularly in view of the dark activity displayed. Haghighi et al. have also developed TiO_2 catheter materials, looking both at silicone and PVC [140], showing a small decrease in *C. albicans* biofilms, presumably in dark conditions.

Surgical implant coatings have been developed by cathodic arc deposition of nanostructured anatase thin films, with potential uses in orthopedics and dentistry [141]. A 1 log reduction of *S. epidermidis* adherence was achieved following 2 min UV irradiation (365 nm); however following the full 13 min irradiation period, the bacterial reduction on TiO_2-coated surfaces did not differ substantially to that seen on the untreated Ti surfaces. This is attributable to the intrinsic antimicrobial effect of UV light. Materials suitable for orthopedic and dental implants have also been developed by Hu et al., with good ability to inhibit the growth of *S. aureus* and *E. coli* [142].

TiO_2 has also found use in orthodontic applications. Chun et al. have modified the surface of orthodontic wires with TiO_2, using a solgel method, demonstrating bactericidal activity toward *Streptococcus mutans* and *Porphyromonas gingivalis*, the causative organisms of dental caries and periodontitis, respectively, following 60 min UVA irradiation [143]. TiO_2 orthodontic ceramic brackets have also shown good antimicrobial activity against *S. mutans* following 1 h UVA irradiation [144], while N-doped TiO_2 ceramic brackets prepared by magnetron sputtering displayed the greatest activity against *C. albicans*, from all organisms tested [145]. Thin films of N-doped TiO_2 have also been deposited on stainless steel brackets using radio-frequency sputtering, conferring antimicrobial activity toward a number of organisms, including *S. mutans* [146]. As *S. mutans* adherence is one of the main causative factors for dental plaque, and therefore orthodontic failure [143], materials that can successfully prevent adherence of this organism may be of great benefit.

Additional medical uses include anti-infective metal pins for skeletal traction [123, 147], antibacterial cellulose nanocomposites for wound healing and tissue regeneration [148], dental implants [149], biofilm-inactivating dental adhesive [150], self-sterilizing lancets for blood glucose monitoring [151], and surgical implants [141].

In general, while displaying promise as effective antimicrobial strategies for medical devices, the bacterial reductions seen with TiO_2 materials are lower than those achieved with photosensitizer-mediated strategies. This could be due to insufficient light dosing or TiO_2 concentrations of the material surface, or could be a function of the greater resistance of organisms to TiO_2-mediated strategies. Another difficulty is the high inoculums used, often in the region of 10^6–10^9 cfu/ml, which are much higher than would be expected clinically. The reductions seen with TiO_2 surfaces, while not as large in number as those achieved with photosensitizer-incorporated surfaces, are still clinically relevant due to the low numbers of microorganisms expected to be present at the time of implantation. If the photocatalytic surface is able to sustain the prevention of adherence of low numbers of microorganisms, this will prevent the formation of a biofilm and clinical infection.

10.4 Photocleavables

The ability to tailor the properties of a polymeric system in response to an externally applied light trigger is particularly appealing for medical device applications such as drug release as, unlike many other stimulus-responsive systems, a high level of control can be exerted on the light stimulus, allowing localization in time and space, remote activation, and control in terms of wavelength and intensity [152, 153]. Light-induced transformations in organic compounds were first described in 1885 by Ciamician and Silber [154]. Since then, a number of photoremovable groups have been explored for use as protecting groups in chemical synthesis. The majority of these groups are sensitive in the UV range, which could impose some limitations for in vivo uses due to the restricted tissue penetration, but this may be circumvented by the use of two-photon irradiation. Two-photon irradiation allows delivery of light in the near infrared (NIR) region of the spectrum. NIR light, unlike UV, is innocuous and penetrates tissue readily, thus allowing application of light externally to the body.

The use of photocleavable compounds in the development of anti-infective materials and surfaces is poorly studied, but is a field in which there is much potential. While not antimicrobial in themselves, their use within materials can enable the timely delivery of effective anti-infective agents. A number of photolabile compounds are well known in the field of organic synthetic chemistry, and these are being exploited for drug delivery purposes. Many of these can easily be translated to the development of surface-eluting materials, allowing delivery of conventional antimicrobials, or of photosensitizers, which have proven safety profiles. Some such compounds are *o*-nitrobenzyl compounds, coumaryl groups, cinnamate esters, benzyl groups, α-substituted acetophenones, and benzoins [155]. A number of these compounds are shown in Fig. 10.5. Two main strategies have been employed to use photocleavable linkers in antimicrobial or drug delivery applications. The predominant strategy is to attach the reagent to the polymer via a photocleavable linker, allowing its release on application of light. The second is to functionalize the photocleavable compound with two polymerizable groups to employ it as a cleavable crosslinker of the polymer with two where, on irradiation of light, one of the bonds between the linker and the polymer chain is broken, allowing elution of trapped drug from the polymer (Fig. 10.6).

Among the most widely studied photocleavable compounds are the *o*-nitrobenzyl esters, which have found application in polymer science research including the development of photoresists. Kloxin et al. have applied *o*-nitrobenzyl photochemistry to the synthesis of polymerized photodegradable macromers. The o-nitrobenzyl group was dual functionalized, allowing it to act as a photodegradable cross-linker. Upon UV or two-photon irradiation, channels rapidly develop within the gel, allowing migration of encapsulated cells [156]. Such a system could be applied as a medical device coating, allowing diffusion of drugs or other treatments on demand. Aside from this, the majority of published phototriggered *o*-nitrobenzyl-based drug delivery strategies have focused on nano- or micro-particulate systems [157–164].

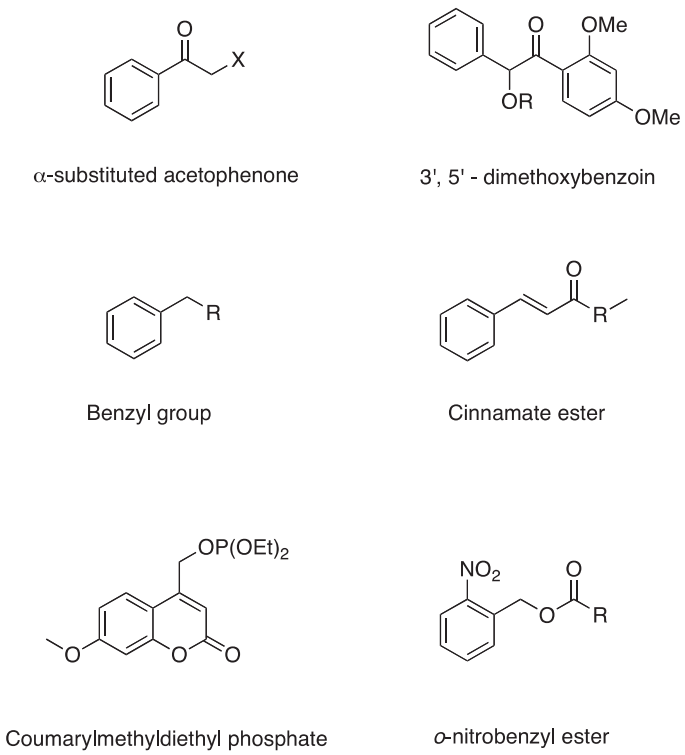

Fig. 10.5 The structures of some well-known photocleavable groups

With the advances in polymer coating technology, it may be possible to coat such systems onto the surface of medical devices to confer antimicrobial properties.

Of particular interest to antimicrobial systems, Velema et al. have developed a system that allows orthogonal control of antibacterial activity, thus allowing selection of the class of antibiotic to be released [165]. The photocleavable groups, based on derivatives of 7-dialkylaminocoumarin and 7-alkoxycoumarin, were alkylated with two complementary carboxylic acid-containing antibiotics: a fluoroquinolone and benzylpenicillin. Irradiation with light of 381 nm and 322 nm, respectively, caused selective phototriggered release of the antibiotics and resultant bactericidal activity. Such a system could be useful if incorporated into a polymeric system to allow wavelength-dependent targeting of specific bacterial species.

Hampp et al. have extensively researched the field of photolabile polymers for drug delivery, employing a number of different photocleavable compounds. Their strategies mainly involve attachment of the reagent to be released to the polymer, via the photocleavable linker. A number of polymers they have studied are particularly relevant to the area of medical devices and include intraocular lens materials. A patented

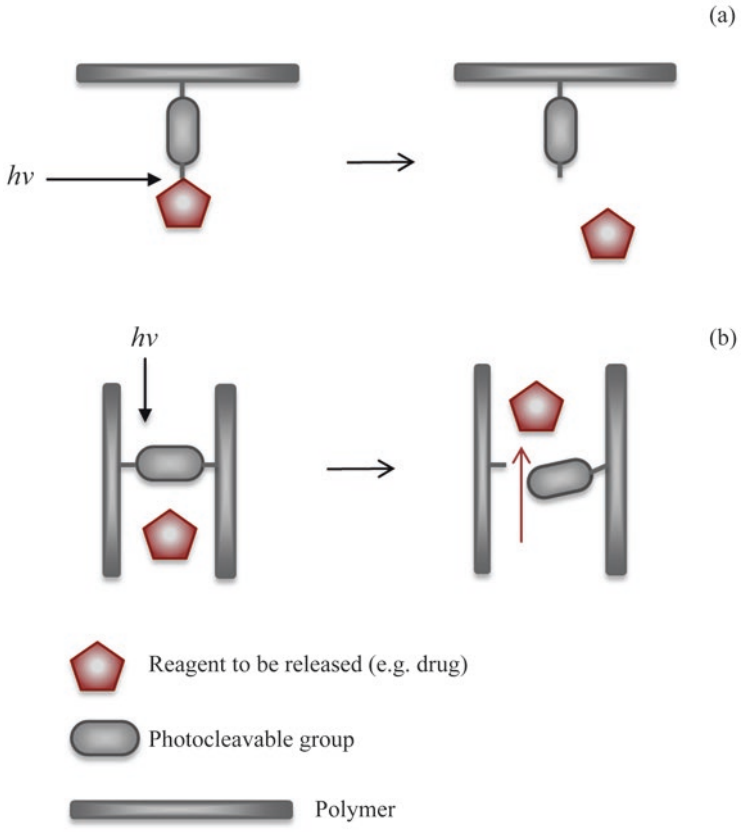

Fig. 10.6 The two main strategies for drug release from a polymer using photocleavable compounds. (**a**) Attachment of the drug to the polymer backbone via a photocleavable linker. Application of light causes cleavage of the bond between the drug and the photocleavable group, releasing the drug. (**b**) Polymerization of the photocleavable group as a polymer cross-linker. Application of light causes cleavage of one of the bonds, leading to an increased porosity and drug elution from the polymer network

system involving photocleavable polymers such as poly(MMA) intraocular lenses based on cinnamic acid derivatives allows drug release on two-photon irradiation [166]. Although drug treatment for posterior capsular opacification was the goal of the work, such a system could be of use for the release of antibacterial agents to prevent the development of postsurgical endophthalmitis. Two-photon-triggered drug delivery from an intraocular lens acrylic polymer with *o*-nitrobenzyl linkers was also described [167]. Steady release of the model drug, 5-fluorouracil, was achieved without attached auxiliary groups, and the presence of a UV absorber only decreased the release rate by 6%. This group has also developed photocleavable polymers based on coumarin linkers, allowing the release of the chemotherapeutic drug chlorambucil via one- or two-

photon irradiation [168]. One-photon irradiation was noted to cause degradation of the drug, something that could be expected upon irradiation of a number of aromatic compounds, but this was largely circumvented by the use of two-photon irradiation. Additionally, the Hampp group has synthesized a novel photocleavable coumarin-analogue cross-linker, to which they conjugated 5-fluoro-heptanoyluracil, before polymerizing into a HEMA/methyl methacrylate copolymer [169]. One- and two-photon release of the drug was achieved, allowing multidose drug release, with a view for cataract treatment.

A recent patent by Victor et al. [170] details one of the first designs for a medical device containing a photolabile coating to allow delivery of reagents to intravascular or intraluminal locations. The coating contains photocleavable linkers possessing dual functionality. One functional group covalently binds the agent to be released; the other remains fixed to the coating. On irradiation of the appropriate wavelength of light (preferably >300 nm) from an optical fiber inserted into the lumen of the device, the linkage to the agent is cleaved. This allows the release of the reagent attached, but the linker remains on the coating. Suggested reagents to be held by such linkers include cytotoxics, antibacterials, antivirals, and therapeutic antibodies.

The successful use of photocleavable compounds in the delivery of medicinal compounds, including antimicrobial agents, indicates the vast potential of this group of compounds possesses in the area of anti-infective medical devices.

10.5 Conclusions

The three main strategies for conferring light-triggered anti-infective properties to surfaces (photosensitizers, photocatalysts, and photocleavables) have shown benefit in both the research environment and, in the case of photosensitizers and photcatalysts, the clinic. The antimicrobial reductions conferred on activation of photosensitizer-incorporated systems appear to be greater than those obtained with photocatalytic systems. Due to the small numbers of microbial cells expected to be present on a medical device surface, however, such high microbial reductions may not be necessary, and the level of antimicrobial activity conferred by TiO_2 appears to be sufficient to effectively decontaminate devices. In addition, the ability of TiO_2 to cause cell mineralization confers an advantage, allowing the development of anti-fouling medical device materials. Research in the area of photocleavable compounds for application to medical device polymers is not as well developed as with the other two approaches, but the potential for widespread use is large, as indicated by the ease of release of bound or entrapped drugs in laboratory research. Due to an already large bank of well-studied photocleavable compounds, it is anticipated that the development of phototriggered anti-infective medical devices incorporating photocleavables can progress with ease.

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