Review

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Journal of
Biomedical Nanotechnology
Vol. 10, 1–22, 2014
www.aspbs.com/jbn

Medical Biofilms—Nanotechnology Approaches

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Biofilms are colonies of bacteria or fungi that adhere to a surface, protected by an extracellular polymer matrix composed of polysaccharides and extracellular DNA. They are highly complex and dynamic multicellular structures that resist traditional means of killing planktonic bacteria. Recent developments in nanotechnology provide novel approaches to preventing and dispersing biofilm infections, which are a leading cause of morbidity and mortality. Medical device infections are responsible for approximately 60% of hospital acquired infections. In the United States, the estimated cost of caring for healthcare-associated infections is approximately between $28 billion and $45 billion per year. In this review, we will discuss our current understanding of biofilm formation and degradation, its relevance to challenges in clinical practice, and new technological developments in nanotechnology that are designed to address these challenges.

KEYWORDS: *Biofilms, Nanotechnology, Nanoparticles, Nanomaterials, Medical Devices, Infection.*

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INTRODUCTION

Biofilms are colonies of bacteria or fungi that adhere to a surface by means of flagellar proteins, type IV pili, and secretion of an extracellular polymeric substance (EPS) composed of polysaccharides and extracellular DNA (eDNA).[1] Prokaryotes' ability to form adherent colonies was first described by Antonie van Leeuwenhoek in 1650, but the term biofilm was not coined until 1977 when William Costerton observed that the vast majority of bacteria in an Alpine lake were adherent to the rocks on the bottom, rather than free-floating, and described these colonies as biofilms.[3] Until the emergence of this cooperative model, bacterial cells were thought to operate with relative independence from one another aside from occasional conjugation and competition for nutrients.

By this time, the field of microbiology had evolved based on planktonic bacterial models. A great deal of what was understood about the behavior, structure, function, and antimicrobial susceptibility of bacteria was based on an exception, and the biofilm state was really the rule.

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Received: 14 January 2014
Accepted: 27 January 2014

Biofilms are distinct in many aspects of their biology, including stress tolerance, metabolism, use of adhesion proteins, and gene sharing. Hence, the need arose for novel approaches, such as nanoscale modulation of the surfaces where biofilms grow, as a means of reducing clinical infections.

The biofilm paradigm has extensive implications in the medical field. We have only just begun to understand our relationship with the beneficial biofilms that grow on our skin and in our gastrointestinal tract. Alterations in these protective barriers can lead to infections with pathological bacteria, and alterations in our own innate defenses can allow our nosocomial bacteria to spread and cause pathological infections. A classic example of this is intravenous catheterization, which provides a direct route for skin bacteria to infect the blood. Increased use of a variety of medical devices also increases the number of opportunities for infection. Five years after he described lake biofilms, Costerton reported an analysis of an infected pacemaker, determined to be covered with a thick biofilm.[4] The pacemaker infection did not respond to treatment with antibiotics and ultimately required explanation.

Pacemakers are only one example of devices prone to biofilm related complications. Prosthetic valves and ventricular assist devices can also harbor biofilms, as can orthopedic and other surgical implants. In hospitalized patients, there is significant infection risk from devices such as vascular access catheters, endotracheal tubes, and urinary catheters. The field of dentistry has long been aware of biofilms that cause dental caries and sometimes more extensive orthodontic infections with maxillofacial reconstructions. Biofilms colonize wounds and lead to prolonged healing and scar formation. In gastric ulcers, *Helicobacter pylori* form biofilms that erode the stomach lining. For patients with cystic fibrosis, difficulty clearing the mucus in the lungs results in increased risk of lung biofilm infection and death due to pneumonia.

Nanotechnology such as nanoparticles and nanotextured surfaces provide unique advantages for preventing

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attachment of biofilms and delivering antimicrobial agents. Treated implant surfaces can kill microbial species and improve healing and tissue integration. Antimicrobial metals, polymers, peptides, and combinations thereof show promising efficacy at eradicating biofilms while causing minimal damage to the host tissue. Importantly, nanotechnology has also afforded developments in biofilm detection. Miniaturization of devices such as chip calorimeters has implications in early detection of clinical infections as well as improvement and standardization of sensitive real-time monitoring of biofilms in the laboratory setting.

SCOPE OF THE REVIEW

This review addresses current nanotechnology approaches to medical biofilms. The first section provides an overview of biofilm biology, including the genetic and phenotypic qualities of formation, quorum sensing, and dispersion. The second section focuses on individual biofilm influenced clinical applications in cardiology, orthopedics, dentistry, critical care, wound healing, ulceration, and cystic fibrosis. Third, we discuss current developments in nanotechnology for clinical applications, including antimicrobial surfaces, treatments, and biofilm detection.

BIOFILM BIOLOGY

Biofilms are ubiquitous in nature, and nearly all species of bacteria are able to form biofilms[5] with few exceptions.[6] This is also true of fungal biofilms such as *Candida* and *Aspergillus*. An analysis of 84 strains of *Candida* determined that all of them were able to form biofilms.[7] In response to environmental stimuli, including stimuli from neighboring biofilm forming cells, planktonic cells undergo a series of genetic and phenotypic changes. These changes have been qualitatively described in five general stages of biofilm development (Fig. 1):

(i) reversible attachment,
(ii) irreversible attachment,
(iii) maturation-1,
(iv) maturation-2, and
(v) dispersion.[8]

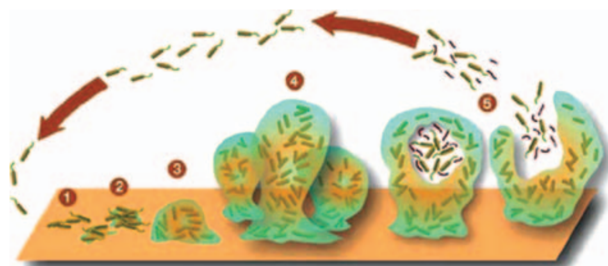

Figure 1. The stages of biofilm formation. Stage 1 is reversible attachment; stage 2 involves the secretion of the EPS. Stage 3 is characterized by 3-D architecture, with complex architecture in Stage 4. Stage 5 is the dispersion stage, with release of planktonic bacteria. Reprinted with permission from [214], D. J. Musk and P. J. Hergenrother, Chemical counter measures for the control of bacterial biofilms: Effective compounds and promising targets. *Curr. Med. Chem.* 13, 2163 (2006). © 2006, Bentham Science Publishers.

Maturation-1 and maturation-2 are distinguished by architectural complexity. In maturation-1, there is the emergence of 3-D architecture, while maturation-2 stage biofilms achieve their full complexity. There is ongoing disagreement in the field about whether biofilm formation is a developmental process[9] or a natural part of the life cycle as described by O'Toole et al.[10] Conversely, Knudsen et al. point out that the proteins produced are highly conserved, and none are specific to biofilm formation, suggesting that biofilm formation is simply an adaptive process of survival.[11, 12]

In contrast to planktonic bacteria, sessile bacteria in biofilms demonstrate a unique degree of resistance to environmental stressors like ultraviolet light,[13] antibiotics,[14] desiccation,[15] pH extremes,[16] and immune system attack.[17, 18] The secreted extracellular polymer matrix (EPS), is composed of polysaccharides and extracellular DNA (eDNA),[1] and may help to encapsulate bacteria and protect them from the environment. On the other hand, it also may induce adaptive changes for cells that are separated from nutrients, including slower metabolism and adaptations for low oxygen and pH.

The stimuli that induce planktonic bacteria to form a biofilm can be a favorable growth environment and detection of an environmental stressor. *Pseudomonas aeruginosa* requires adequate iron concentrations to form their mushroom shaped biofilms, and formation is prevented by iron chelation or mutations to the iron uptake pathway.[19] *Pseudomonas fluorescens* requires phosphate in order to express adhesion molecule LapA.[20, 21]

In contrast, biofilms may also form in order to allow a colony to achieve a persistent state in the presence of nutrient deprivation. *Mycoplasma* forms biofilms in the presence of limited nitrogen conditions.[22] *Francisella* is a fastidious species that requires supplementation with iron, cysteine, and 12 other nutrients in order to grow, and biofilm formation may help it to persist until nutrients become available.[23] Antibiotics are another class of biofilm inducing stressor. Subinhibitory doses of various antibiotics with diverse mechanisms of action cause transition from planktonic form to sessile form in a number of models. Low doses of tobramycin induce biofilms in *P. aeruginosa* and *E. coli*,[24] and low doses of beta lactam antibiotics induce biofilm formation in 15 different *S. aureus* strains, both methicillin resistant and sensitive.[25]

Extracellular Polymer Matrix and Extracellular DNA

The extracellular polymer matrix (EPS), or glycocalyx, is composed of polysaccharides, proteins, lipids and extracellular DNA (Fig. 2). The matrix constitutes up to 50–90% of the biomass of a biofilm[26] and provides a number of

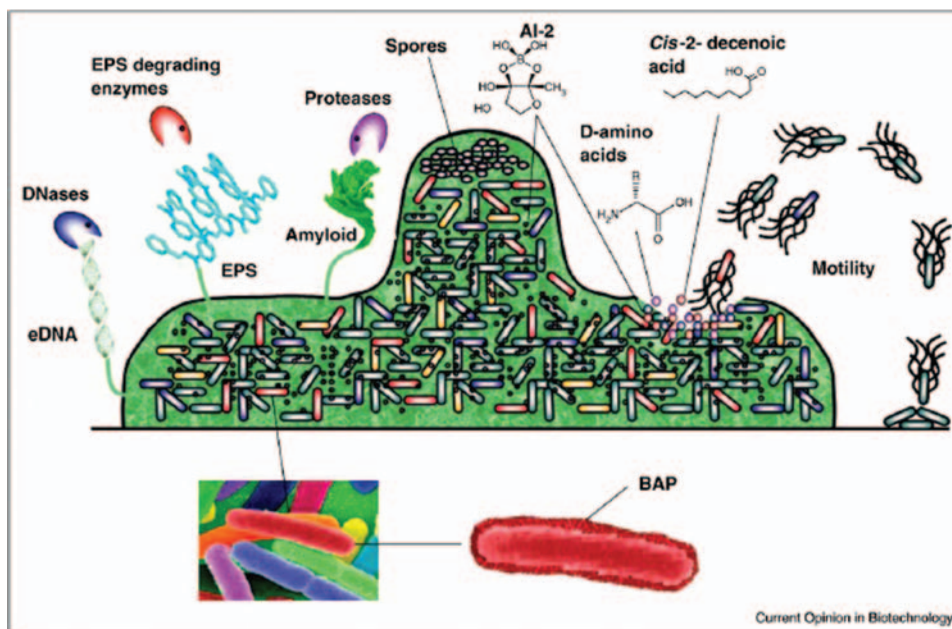

Figure 2. Schematic of extracellular polymer matrix mediated virulence mechanisms, including eDNA, polysaccharides, amyloid fibers, quorum sensing molecule autoinducer 2 (AI-2), dispersal inducers including amino acids and cis-2-decanoic acid. Molecules that degrade the EPS components include DNAse, protease, and other enzymes. Reprinted with permission from [213], T. Abee, et al., Biofilm formation and dispersal in gram-positive bacteria. *Curr. Opin. Biotechnol.* 22, 172 (2011). © 2011, Elsevier Inc.

functions. The EPS also enhances adhesion to a static surface or a free floating organic microgel.[27] In addition to adhesion, the EPS enhances hydration and retention of extracellular digestive enzymes to aid metabolism. This includes metabolism of pronutrients as well as degradation of harmful substances. William Costerton, who coined the term biofilm, was one of the first to propose that the biofilms resistance to antibiotic treatment may be due to the protection provided by the EPS.[28]

In addition to protection and structure, the EPS acts a signaling stimulus to induce additional cells to join the biofilm and aid in its construction. Cyclic di-guanosine monophosphate (c-di-GMP) is a bacterial second messenger that regulates cell surface adhesiveness.[29] The EPS polysaccharide Psl produced by *P. aeruginosa* acts as a positive feedback signal by stimulating the production of additional EPS proteins in a c-di-GMP dependent mechanism. Psl acts on diguanylate cyclases SiaD and SadC to produce c-di-GMP, stimulating increased production of additional Psl.[30] A polysaccharide stimulus may also be derived from the surface of attachment. Plant polysaccharides can induce biofilm formation through kinase activation of master regulator gene Spo0A.[31]

The EPS environment facilitates communication and cooperation between organisms in the biofilm. Genes are shared more efficiently due to increased rates of plasmid dispersal via conjugation.[32] Within the EPS, bacteria form a complex network of nanotubes for sharing plasmids, even between cells of different species. Nanotubes can even form between organisms of different gram staining classes; *B. subitlis* forms nanotubes with *S. aureus* as well as *E. coli*.[33] The plasmids transferred may confer a survival advantage such as antibiotic resistance genes. Mixed culture biofilms are common in nature, and they show enhanced formation efficiency when grown as a mixed species culture rather than a single species.[34] In addition to enhancing biofilm related gene expression and protein production, the presence of mixed species enhances *S. epidermidis* autolysis and eDNA release.[35]

The eDNA in the EPS was recently found to have a role in directing the shape of growth of the biofilm. In 2013, using time-lapse phase-contrast microscopy, Gloag et al. observed that eDNA can be used as a tract to direct cell migration and biofilm expansion. In expanding *Pseudomonas* biofilms, clusters of "bulldozer" cells were observed to create a radial tract to the outside edge of the film. The tract was lined with eDNA, which allowed following cells to orient and migrate out in an organized manor.[36]

The protection provided by the EPS also makes it a prime target for biofilm dispersal. Agents that disrupt the EPS such as polysaccharide depolymerases, DNAse, protease, cellulase, and alginate lyase may come to play a more prominent role in combating biofilm infection.[37] For example, in *Aspergillus fumingatus*, treatment with DNAse increased susceptibility to antifungal treatment.[38] Targeting the EPS also has the potential advantage of specificity. Dispersin B detaches *S. epidermidis* biofilms but not *S. aureus*, while DNAse I detaches *S. aureus* but not *S. epidermidis*.[39] Some populations of planktonic bacilli

propelled by flagella tunnel into biofilms create pores which are thought to increase nutrient flow in the matrix. When these bacilli were altered to express a bactericide, they eradicated a *S. aureus* biofilm and occupied the space created.[40]

Gene Expression Changes

Using DNA microarrays to compare planktonic to biofilm *P. aeruginosa*, Whitely et al. found that only 1% of genes showed differential expression.[41] However, later papers indicated a much larger number of genes were differentially regulated. Sauer et al. reported as many as 800 proteins with differential expression when planktonic *P. aeruginosa* was compared to maturation-2 stage biofilm.[8] Microarray data in 2012 showed that there were 89 genes differentially expressed between planktonic and biofilm cells.[42] The explanation for the difference in findings may be population heterogeneity within the biofilm, the difference in species studied, the use of a greater number of probes, or improved knowledge about genes of unknown function.

In response to environmental signals, sigma factor proteins and small non-coding RNAs (sRNAs) transmit regulatory signals to various genes to help the bacteria adapt.[43] The first step for many biofilms appears to be induction of a master regulatory gene that triggers the subsequent genetic changes necessary for biofilm formation. In *Listeria monocytogenes*, the master regulator switch is called PrfA, which is a transcriptional activator found to directly activate 175 genes for the biofilm's transition out of the 627 genes that were differentially regulated according to microarray analysis.[44] In *Bacillus subtilis*, the master regulator switch is called SinR, and it is known to down-regulate flagella expression.[45]

Those arguing against biofilm formation as a developmental process argue that gene expression changes observed during the biofilm formation process are not specific or unique to biofilm formation. Instead, the bacteria make adaptive use of innate mechanisms from multiple pathways in order to alter their phenotype for biofilm formation.[11,12] For example, *Acinetobacter baumannii* 17978 makes a pentasaccharide for its capsule that is also used in the biofilm matrix glycoproteins. Disrupting the gene prevents capsule formation, and may be a target for disrupting biofilm formation.[46]

Many of the gene expression changes are indeed highly conserved. Most relate to adhesion, and reflect an ability to adapt to the surface in question. For example, biofilms of *X. fastidosa* express adhesion protein XadA1 when adhering to both silicone and ethyl cellulose, but not cellulose acetate. They noted that the more homogenous the surface, the better the bacteria were able to adhere and proliferate.[47] In *Vibrio cholera*, adhesion protein RbmA is used for cell to cell adhesion, Bap1 is used for adhering to the surface. In combination with a mixture of polysaccharides, RmbC and Bap1 also made up a flexible envelope encasing clusters of cells.[48] Similarly, *Saccharomyces cerevisiae* gene FLO11 encodes a cell-surface adhesion specifically implicated in biofilm colony formation and morphology.[49]

Additional biofilm related gene changes are observed in genes of metabolism. Within the different regions of the biofilm, bacteria make specific adaptations for nutrient availability. The combined treatment of *S. mutans* with sucrose and starch induces formation of a biofilm that expresses genes for sugar metabolism, but this does not occur with sucrose alone.[50] *S. mutans* increases expression of genes associated with glucan synthesis, binding, and remodeling as well as fatty acid biosynthesis and branched chain amino acid metabolism.[34] Biofilms upregulate genes involved in the mevalonate pathway, cell wall biosynthesis, and purine and pyrimidine nucleotide metabolism pathways.[42] *P. aeruginosa* biofilms express genes for complex type IV pili and glucose metabolism as well as genes consistent with limited oxygen and iron.[51]

Flagella have a surprising role in biofilms as a non-motile structural component utilized by cells on the edge of the colony. Flagella as well as type IV pili can act as adhesins that attach bacteria to a surface or to other bacterial cells.[52] Mutation of flagellar proteins reduces biofilm formation capacity.[8] Loss of flagella was initially thought to be a mechanism of immune system evasion, but it was recently found that nonmotile flagella are also tolerated by the immune response. This suggests that it is not the flagellar protein, but rather the motility of the bacteria that activates phagocytotic cells.[53]

Colony Structure

The composition of the matrix and the shape of the biofilm is determined by the types of microorganisms present, the shear forces on the colony, the temperature and the availability of nutrients.[54] When biofilms form in tubular devices such as catheters or stents, the colonies take the form of streamers, which can grow to clog the device.[55] In microfluidic environments, the formation of biofilms can be predicted based on the Reynolds number, a measure of flow turbulence. High Reynolds values are associated with more turbulent flow, and have been found to suppress the growth of biofilms, while laminar flow was more favorable for biofilm formation.[56] Genome-wide assays have identified several signaling cascades that play a role in colony morphology, including MAPK, TORC, SNF1, and RIM101.[49] Alterations in environmental stimuli can alter colony structure, such as low doses of clarithromycin which inhibit twitching motility in *P. aeruginosa*.[57]

Biofilm colonies can take several shapes besides streamers and may be flat, wrinkled, or mushroom shaped. *B. subtilis* strain NCIB 3610 contains a plasmid that lends to its characteristically wrinkled biofilm architecture (Fig. 3).[58] The formation of the wrinkles is secondary to patterns of cell death that focus mechanical forces within the biofilm. Control of cell death influences where wrinkles form.[59]

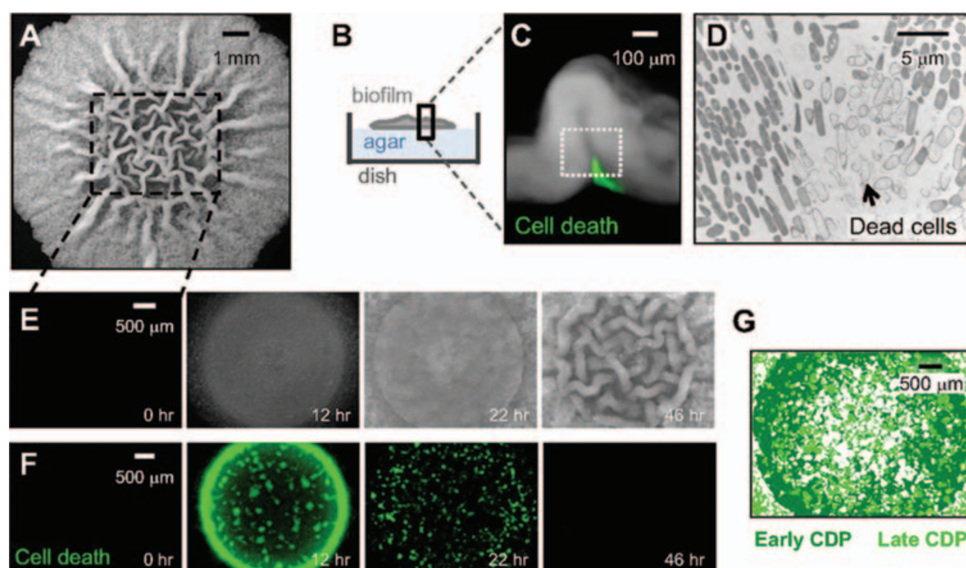

Figure 3. ***B. subtilis*** **biofilm colony morphology (A)** ***B. subtilis*** **biofilm at 3 days. (B) Schematic of cross section. (C) Cross section florescence image of a biofilm wrinkle. (D) Transmission electron micrograph (TEM) of the biofilm wrinkle showing dead cells (black arrow). (E) Biofilm morphology over time. (F) Marker of cell death (CDP) over time (G) Early (dark green) and late (light green) CDP detection. (H) Control of cell death alters the wrinkle pattern in the colony. Reprinted with permission from [59], M. Asally, et al., Localized cell death focuses mechanical forces during 3D patterning in a biofilm.** ***PNAS*** **109, 18891 (2012). © 2012, Proceedings of the National Academy of Sciences.**

The shape of the biofilm colony yields heterogeneous populations within the structure. The colony edges are more susceptible to selective pressures, promoting mutations that can resist environmental stressors such as antibiotics.[60] Microbes on the inside of the colony adapt to have slower metabolism and tolerance of nutrient shortage and hypoxia. The edges of the biofilm structure have the most access to resources, but are also the most unprotected. In *C. albicans* biofilms, the outer colony layers were found to be actively growing by fermentative metabolism, while the inner layers had changes in mitochondrial activity consistent with a resting state. Genes associated with translation, glycolysis, and ergosterol biosynthesis were present on the outside layers, while the inside layers upregulated genes for sulfur assimilation. Outer cells expressed cell wall synthesis proteins, while inner cells expressed cell wall degrading enzymes.[61]

In addition to variations in metabolism within the biofilm, the location of a cell within the colony structure determines the type of attachment proteins expressed. For *E. coli* biofilms, amyloid curli fibers are expressed most significantly on the outer layer, while the bottom layer expresses a mesh of flagella. On the rim of the colony, cells express flagella that wrap around the colony.[62] Immune detection of curli, which is also produced by the typhoid serotype of *Salmonella enterica*, requires corecognition by Toll-like receptors (TLR) 1 and 2.[63]

Quorum Sensing

Quorum sensing mechanisms allow collections of bacteria to behave in a coordinated way. For example, in gram negative bacteria, quorum sensing peptide acylated homoserine lactones (AHLs) and *Pseudomonas* quinolone signal (PQS) induce gene changes that lead to biofilm formation.[64] In gram positive bacteria, competence-stimulating peptide (CSP) induces a subset of cells to lyse, thereby providing the extracellular DNA of the EPS.[65] Disabling quorum sensing molecules or their receptors is an emerging strategy for preventing biofilm formation.[66,67] Rhamnolipid is a quorum sensing, controlled virulence factor in *P. aeruginosa* that destroys polymorphonuclear (PMN) cells by lytic necrosis and inhibits PMN chemotaxis.[68] This explains why newly established biofilm colonies are more susceptible to PMN attack than established biofilms.[69] Rhamnolipid is inhibited by Ajoene, a sulfur containing compound in garlic.[68] Quorum sensing homoserine lactones (HSL) bind to HSL quorum sensing receptors such as LuxR-type proteins, including LasR and RhlR. The receptors can be blocked by meta-bromo-thiolactone in order to inhibit quorum sensing.[70]

Biofilm Dispersal

There are multiple methods of inducing biofilm dispersal, including nutrient depletion, interfering with attachment, degrading the EPS, interfering with quorum sensing, and imitating naturally produced dispersal signal proteins. Deprivation of glucose is a simple means of causing rapid biofilm dispersal.[71] Interfering with attachment can be effective, but there is significant strain variation and redundancy in attachment proteins. For example, polysaccharide intercellular adhesin (PIA) is an important cell to cell

adhesion molecule in gram positives *S. aureus* and *S. epidermidis*. However, 27% of *S. epidermidis* strains isolated from orthopedic implants form biofilms without PIA.[72]

Some species secrete peptides that induce self-dispersal, dispersal of self and other species, or act specifically on the species they compete with for nutrients and space. LapA is a surface adhesion and a biofilm matrix component that is elevated in the presence of the second messenger c-di-GMP. In *Pseudomonas putida*, when c-di-GMP falls, the cysteine proteinase produced by the gene LapG degrades the membrane associated protein LapA, promoting dispersal.[73] *B. subtilis* releases D-amino acids to trigger the dispersal stage, including D-leucine, D-methionine, D-tyrosine, and D-tryptophan. These amino acids also induce dispersal of *S. aureus* and *P. aeruginosa*.[74] Rhamnolipid produced by *P. aeruginosa* induces dispersal of Bordetella bronchiseptica.[75] Similarly, the fatty acid cis-2-decenoid acid from *P. aeruginosa* causes dispersal of *Escherichia coli*, *Klebsiella pneumoniae, Proteus mirabilis, Streptococcus pyogenes, Bacillus subtilis, Staphylococcus aureus,* and the yeast *Candida albicans*.[76]

Bacteriophages are an effective vector for inhibiting bacteria that work more effectively in the biofilm setting than antibiotics. The efficacy of bacterophages has been known for a long time, but antibiotics were simpler to produce. Although phages are much larger than antibiotic molecules, phages are able to diffuse through the EPS matrix. Therefore biofilm formation does not confer phage resistance.[77] Animal and human trials indicate that phages are often well tolerated in their host.[78] Bacteriophages often produce depolymerases that hydrolyze biofilms; and they are often extremely host specific. Drawbacks to phage treatment include the potential for the development of resistant bacterial strains and inactivation of the phage by the host immune system.

Targeting the EPS is also an extremely effective means of biofilm dispersal, often rendering the bacteria susceptible to traditional planktonic treatments. DNAse inhibits the formation of biofilms in *P. aeruginosa*,[1] and low frequency ultrasound increases the efficacy of antibiotic treatment in *E. coli* infections, but not for *P. aeruginosa*.[79] Activating the nonspecific ClpP protease via acyldepsinpeptide antibiotic (ADEP4) in combination with rifampicin was effective for killing biofilm persisters.[80]

Bacteria express DNABII family proteins as a structural component of their DNA, and thus it is present in the eDNA of the EPS. A vaccine against the DNABII family proteins was effective at initiating a targeted immune response and debulking biofilms in an animal model of established biofilm infection.[18] However, due to the prevalence of natural and beneficial biofilms, vaccination against biofilm matrix proteins may not be a clinically useful practice unless highly specific targets in pathological strains are identified.

BIOFILMS IN CLINICAL PRACTICE

Medical devices are associated with approximately 60% of hospital acquired infections,[2] and up to 80% of infection related deaths.[81] In the United States, the estimated cost of caring for healthcare-associated infections is between $28 billion and $45 billion per year.[2] Biofilms resist traditional antibiotic treatment and adhere readily to a wide variety of clinical apparatuses such as catheter tubing and surgical implants. The biofilms on these devices constantly shed planktonic bacteria, acting as a source for recurrent infections. Often, removal of the device is the only effective treatment.

Complicating the problem of biofilm eradication is the fact that some biofilms are beneficial to our physiology, and so anti-biofilm treatments need to be highly specific to the pathogenic bacteria. Biofilms are an intrinsic aspect of the normal commensal bacteria that populates the skin and the gut. In these roles, they perform many beneficial functions, such as catalyzing the breakdown of long chain fatty acids for our absorption and production of essential metabolic cofactors such as folate and vitamin B_{12}. Additional evidence shows that our commensal bacteria may serve as an immunological barrier, crowding out otherwise pathological bacterial species by competing for nutrients and secreting antimicrobial peptides that target competitors.[82] Gut biofilms are critical for protecting against pathological infections such as *Clostrium difficile*. A single dose of clarithromycin profoundly reduces the heterogeneity of gut flora and increases susceptibility to infection with *C. difficile*.[83]

There are a number of clinically relevant biofilm forming species to consider (Table I). Wounds and implants are typically infected with *Streptococcus*.[34,84] or *Staphylococcus*.[17,35,69,85,86] *Staphylococcus* is also a leading cause of hospital acquired pneumonia. In the lungs, biofilms of *P. aeruginosa*[8,51,87] form, as well as *Burkholderia cenocepacia*.[88] In the gastrointestinal tract, *Helicobacter pylori*[89–91] and C*lostridium difficile*[83] compete with the natural commensal biofilm species found in the gut. Fungal infections are distinct from bacterial infections. They must be treated differently than bacterial infections due to their differences in biochemical structure and metabolism. Fungal infections, particularly *Candida albicans*, are also widespread and capable of causing co-infection in multi species biofilms.

The immune system response to biofilms is characterized by a period of initial inflammation followed by long term tolerance. Mouse model studies of orthopedic biofilm infections reveal that this allows biofilms to become chronic infections, even when treated with vancomycin antimicrobial therapy. Early in the infection, a proinflammatory Th_1 response is mounted, evident by cytokine release of IL-2, IL-12, p70, TNF-α, and IL-1β. The authors propose that tissue damage in the acute inflammatory phase actually enhances biofilm attachment. There is

Table I. Summary of causative organisms of biofilm based infections in various clinical applications.

Applications	Typical organisms	Outcome
Pacemakers	*S. aureus, S. epidermidis, Streptococcus, Candida*	Fibrosis and inhibition of lead conduction, destruction of leads
Prosthetic valves	*S. aureus, S. epidermidis, Streptococcus, Enterococcus*	Valve calcification, regurgitation
Ventricular assist devices	*S. aureus, S. epidermidis, Candida, P. aeruginosa*	Sepsis
Orthopedic implants	*S. aureus, Streptococcus, Corynebacterium*	Inhibited tissue integration, loosening of implant
Contact lenses	*P. aeruginosa, Serratia marcescens, S. aureus*	Redness, irritation, corneal damage
Dental implants	*Streptococcus, Actinomyces, Porphyromonas, Prevotella*	Loss of fixation
Vascular access catheters	*S. aureus, Staphylococcus, Enterococcus, Candida, Klebsiella*	Sepsis
Urinary catheters	*E. coli, Candida, Enterococcus faecalis, Proteus mirabilis*	Urinary tract infection, kidney stones
Cystic fibrosis	*P. aeruginosa*	Pneumonia
Wounds and ulcers	*P. aeruginosa, H. pylori, S. aureus*	Delayed healing, scar formation, necrosis

a significant Th_{17} response throughout the infection, characterized by IL-6 and IL-17 release. On day 28, mice had significantly increased surface marker expression of CD44, indicating a tolerant T_{reg} and Th_2 response.[17]

Antibiotic and Biocide Resistance

Eradication of biofilms often requires an antibiotic dose up to 1000 times higher than the lethal dose for planktonic bacteria.[92] Several authors have suggested that clinical antibiotic susceptibility testing should be performed on bacteria cultured as biofilms rather than planktonic species. Rather than a minimal inhibitory concentration, such testing would yield a "biofilm eradicating concentration" (BEC).[93,94] Not only is it clear that the indicated lethal doses for biofilms are normally much higher than indicated by planktonic susceptibility studies,[95] but even the drug of choice can change based on the use of the biofilm assay.[96] However, the only randomized, double-blind placebo-controlled clinical trial to compare dosing by biofilm susceptibility versus planktonic assay did not find a difference in *P. aeruginosa* colony forming units.[94,97] This may be a result of the heterogeneity of the clinical cultures.

There are four widely cited predominant theories regarding why biofilms resist antibacterial treatment. The first is that the EPS provides mechanical shielding from the environment, reducing the exposure of the bacteria to antimicrobial agents in the environment. For example, the ECM appears to protect *P. aeruginosa* against the antibiotic tobramycin.[98] The second theory is that the EPS maintains a concentrated assortment of antimicrobial peptides within the EPS matrix, such as beta lactamases, thus providing active digestion of antimicrobials. An experiment supporting this theory showed that extracts of the EPS of *S. epidermidis* interfered with the antimicrobial activity of vancomycin and teichoplanin. The antibiotics had to be given at a five times the normal dose to be effective against either planktonic or biofilm bacteria.[99] The third theory is that due to the reduction in access to nutrients and tolerance of anaerobic conditions, many of the bacteria in biofilms are dormant and thus insensitive to drugs blocking the cell cycle.[100–103] In the literature, this dormant subpopulation of bacteria are known as "persisters."[104] The fact that more established biofilm colonies are more effective at resisting antimicrobials despite an identical EPS is consistent with the development of persisters in stationary growth phase.[87] Another theory is that the biofilm structure supports gene sharing, particularly genes of antibiotic resistance. In this context, the specifics of the structure appear to be less important than the activation of antibiotic resistance gene.[14] However, other authors maintain that the selection of antibiotic resistant strains is a laboratory phenomenon and is not the key mechanism of antibacterial resistance in multispecies biofilms.[92] The four mechanisms are not mutually exclusive, and may all play a role in antibiotic resistance.

Disinfectants, or biocides, such as alcohol, iodine, and hydrogen peroxide, typically damage the cell membrane of bacteria. Chemical treatment with 2-Bromoalkanoic acid is effective at inhibiting biofilm growth of *P. aeruginosa*.[105] Biofilms also show varying resistance to treatment with disinfectants. *S. epidermidis* biofilms were found to be sensitive to alcohols of concentrations greater than 60%, but resistant to 30 minutes of exposure to povidone-iodine, or 5% hydrogen peroxide.[106] These types of sterilization techniques are common for routine care of contact lenses. Traditional contact lens care solutions are not as effective as hydrogen peroxide based solutions against *P. aeruginosa*, *S. marcescens*, and *S. aureus* biofilms.[107]

Dental Plaque

Dental plaques are biofilms that can cause dental caries (cavities), inflammation of the mouth and gums, and infection of dental implants. In *S. mutans*, the EPS helps to

maintain a pH gradient, which helps *S. mutans* compete against other bacteria. However, the pH gradient is also the mechanism responsible for enamel erosion and cavitation.[16] Effective biofilm dispersal is thus an important part of routine dental care as well as more advanced procedures such as root canals and fillings. In the latter case, if the area sealed is not sterile, secondary caries underneath the sealant can form.

Chlorhexidine is a cationic bisbiguaninidine found in many commercial mouthwash formulas. It is highly effective against planktonic oral bacteria, but biofilms are protected, possibly due to interactions with the negatively charged EPS.[108] Mouth rinse solutions containing silver nanoparticles were found to be ineffective against *E. faecalis* biofilms, but a 0.02% gel solution treatment was effective.[109] Silver nanoparticles are also effective as anti-biofilm tooth surface treatments.[110] The nanoparticles had similar efficacy to silver nitrate, but did not cause discoloration.

Quaternary ammonium polyethylenamine nanoparticles in dental resin demonstrated antimicrobial effects against oral biofilms. The resin composite with nanoparticles was found to be coated almost exclusively with dead bacterial cells, while the control resin accumulated a thinner biofilm of live cells.[111]

Vascular Access Catheters

Blood stream infections due to central intravenous catheters (CVCs), or central lines, occur in the US at a rate of 2 infections per 1000 central-line-days and globally at a rate of 6.8 per 1000 central-line-days.[112] A rat model of biofilms on an indwelling venous access port showed 100% mortality among immunosuppressed rats, and 30% mortality in immune competent rats.[113] In clinical practice, catheters are replaced at frequent intervals to reduce infection risk, but this carries an increases cost to healthcare systems and an increased procedure risk to patients.

Venous catheters have to exhibit biocompatibility with the blood, while resisting colonization by external bacteria such as the commensal bacteria on the skin. Activation of the complement cascade and coating of the catheter with platelets and serum proteins inadvertently provides an ideal surface for bacterial adhesion.[84] Vascular catheters are at risk for colonization by several gram positive and gram negative species as shown in Figure 4.

Pacemakers and Heart Valves

In the US, 85,000 heart valves and 300,000 pacemaker-defibrillators are implanted annually.[114] The mortality for heart valve infections exceeds 30%.[115] The commensal skin organism *Staphylococcus epidermis* is the most common cause of cardiac implant infections.[116] *S. epidermis* biofilms isolated from infected pacemakers, defibrillators, prosthetic valves, and coronary artery bypass grafts were noted to grow thicker biofilms than isolates cultured from skin.[106] Besides *S. epidermidis*, a variety of bacterial strains can be isolated from cardiac devices. Primarily gram negative species are seen such as *P. aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumonia*, *E. coli*, and *Proteus*. Less often, gram positive bacteria were seen including *Staphylococcus*, *Streptococcus*, and *Enterococcus*, followed by yeast.[117] One of the earliest descriptions of an implant related biofilm infection was of an *S. aureus* infection on a cardiac pacemaker.[4]

Calcification is an important cause of bioprosthetic valve failure.[118] Infections can cause the host cells integrating onto the device to deposit calcium, which may cause valve regurgitation and stenosis. Heart valve biofilms disrupt flow, create a source for continuous infection of the blood stream, and cause a risk of embolus to other organs. Heart valves become infected when injury leads to clot formation, which provides an ideal surface for bacterial adhesion. Biofilms on heart valves caused by *Mycobacterium*

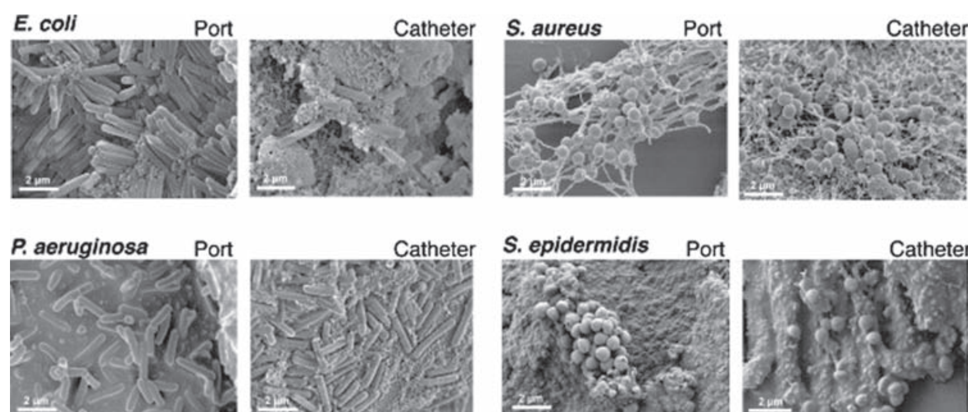

Figure 4. **SEM images of IV port and catheter surfaces infected with single clinical strains. Reprinted with permission from [113], A. Chauhan, et al., A rat model of central venous catheter to study establishment of long-term bacterial biofilm and related acute and chronic infections. *PLoS ONE* 7, e37281 (2012). © 2012, Public Library of Science.**

fortuitum have been noted to cause systemic infection without causing vegetations on the valve.[119]

Orthopedic Implants

Infection is the cause of removal in 14.8% of hip implant failures.[120] The inflammation and tissue destruction caused by the growth of bacteria around an implant have been compared to gingivitis due to bacteria on teeth. The bacteria lead to inflammation and destruction of the supporting tissues, however in peri-implant disease this occurs at a much faster rate with more extensive damage than it does in gingivitis.[121] Altering the microscale surface texture of biomaterials by sand blasting, sintering, or plasma spray has improved tissue integration in orthopedic implants.[122–124] Nanoscale TiO_2 and ZnO both have twice as much osteoblast adhesion than microscale surfaces.[125]

Urinary Catheters

Urinary catheterization is a routine procedure for surgical and intensive care unit patients. The complication of urinary tract infection, particularly with biofilm forming bacteria, can result in the formation of kidney stones. Urease positive bacteria such as *Proteus*, *Klebsiella*, and *Psuedomonas* create an alkaline environment in the presence of urea, which cause the precipitation of minerals into struvite ($Mg(NH_4)PO_4 \cdot 6(H_2O)$) and carbonate-apatite ($Ca_{10}(PO_4)6(CO_3)$). Examination of struvite stones shows biofilm on the surface and the inside. Infected stones can cause relapsing urinary tract infections until removed surgically.

One of the strategies for preventing urinary catheter biofilms is silver coating, which is designed to elute out over time. However, in patients scheduled for short term hospital stays, a large randomized controlled trial comparing PTFE catheters to silver alloy catheters or nitrofural catheters did not demonstrate a significant difference between the groups.[126] In light of insignificant benefit in preventing UTIs, silver catheters were deemed not cost effective.[127] One proposed alternative strategy is to precoat catheters with non-pathological bacterial strains.[128]

Contact Lenses

Biofilm colonization of polymeric contact lens materials is treated with regular cleaning using contact lens cleaning solutions. Although there are several kinds of lens materials, biofilms grow on at least six of the most common types.[129] Biofilms of *Fusarium* and *Candida* resist killing by typical solutions including MultiPlus and MoistureLoc.[129] Similarly, biofilms of *P. aeruginosa, S. marcescens*, and *S. aureus* isolated from contact lenses are resistant to traditional contact lens care solutions but susceptible to hydrogen peroxide based solutions.[107]

Neutrophils dramatically enhance biofilm formation of *P. aeruginosa* on contact lenses. *P. aeruginosa* is capable of utilizing the DNA and F-actin as a starting scaffold for the EPS, and treatment with DNAse reduced biofilm formation.[130] Contact lens hydrogels designed to elute ceragenin were capable of resisting bacterial colonization with *P. aeruginosa* for 15 days and *S. aureus* for 30 days.[131]

Wounds

In wounds, biofilm infection results in delayed healing. Wounds often harbor anaerobic species, which are difficult to culture using standard clinical methods, and thus detection can be delayed. Topical treatment with antibiotic ointments including mupirocin, triple antibiotic ointment, and gentamicin are effective at reducing biofilms.[132] although *S. aureus* has some degree of resistance.[133] Silver based wound dressings are effective against *P. aeruginosa*, *Enterobacter cloacae*, and *S. aureus*.[134]

Endotracheal Tubes

Endotracheal tubes are a source of pneumonia infection and a major cause of mortality in intubated patients. In a study of 10 endotracheal tubes, all had biofilms growing in the lumen, and neutrophils were found layered in the EPS.[135] There are several commercial silver coated endotracheal tubes.[135, 136] Silver coated endotracheal tubes placed in ventilated patients were found to prevent colonization with bacteria, whereas three out of seven untreated endotracheal tubes were colonized.[136]

Cystic Fibrosis Airway Infections

Cystic fibrosis is a common genetic disorder in which chloride channel malfunction leads to difficulty maintaining an osmotic gradient, resulting in thicker secretions. In the lungs, the thick secretions are difficult to clear, and there is an increased risk of infection. Cystic fibrosis is associated with an annual healthcare cost of $15,500 per patient.[137] The microbiota present in the upper airways of normal and cystic fibrosis patients is more diverse than previously thought, containing several previously undetected obligate anaerobes.[138] According to PCR analysis of the infecting strains, colonization with a given strain can last for years.[139] The opportunistic species *P. aeruginosa* is the leading cause of death among individuals with cystic fibrosis. *P. aeruginosa* is resistant to beta-lactam and aminoglycoside antibiotics.[140] Targeting the extracellular DNA in the EPS of the biofilm directly through DNase or indirectly by targeting associated factors such as integration host factor (IHF) has been shown to be highly effective at debulking biofilms in CF patients.[141]

Ulcer Causing Biofilms

Certain biofilms create an acidic environment in the low oxygen regions in the center of the colony, causing tissue damage and ulceration.[16] This is the mechanism for pressure ulcers, diabetic ulcers, and gastric ulcers. Medicare data from 2007 estimates that the cost of care of a single pressure ulcer is $43,000, adding up to 11 billion dollars in annual healthcare costs in the US.[142]

Helicobacter pylori is a leading cause of gastric ulcers, which are a risk factor for gastric cancer induced by the bacterial cagA gene.[143] Approximately half of the human population harbors *H. pylori*, but it only rarely causes symptoms. This may be related to differences in strains, as biofilms composed of mixed strains were found to form much more complex biofilms than single strains.[89] Alternatively, serum starvation has been noted to induce biofilm formation in *H. pylori* and mimics the environment in the gut where *H. pylori* biofilms form.[90]

Antibiotic treatment is a major risk factor for ulcer causing biofilms. This may be due to the loss of diversity of competing species, or as a direct result of antibiotic stress induced biofilm formation. In diabetic foot ulcers, infections with biofilms constitute 68% of the infections, and factors that were most strongly associated with biofilm infection included previous treatment with antibiotics, polymicrobial infection, and ulcers that were necrotic.[144] In the pathogenic species, there is evidence of antibiotic resistance mechanisms. In *H. pylori* biofilms, antibiotic efflux pump expression is significantly increased, resulting in a higher minimum bactericidal concentration for clarithromycin.[91] However, there is also evidence that the complete eradication of *H. pylori* can be associated with an increased risk of esophageal adenocarcinoma.[143]

NANOTECHNOLOGY SOLUTIONS FOR BIOFILM RESISTANCE, TREATMENT, AND DETECTION

Nanotechnology will provide some of the most important advancements in medical devices and biomaterials in the coming years. Reduction of device related adverse events will depend on enhancing antimicrobial activity and improving biocompatibility through nanoscale modifications.[145] Nanomaterials are defined as having at least one dimension less than 100 nm. They provide an advantage over traditional materials because their scale is more similar to that of biological reactions occurring on the cellular level. Increased surface area to volume ratio enhances the efficacy of chemical reactions by providing a greater reaction surface. Nanoparticles are also capable of puncturing micrometer sized bacterial cell membranes without doing harm to larger host cells.[146]

Biocompatibility plays an integral role in biofilm resistance. Although several surfaces have antimicrobial activity, they may also be damaging to human cells. A summary of strategies for biocompatibility and infection resistance is provided in Table II. For instance, cationic bactericidal polymers are believed to exert their effect via membrane lysis. Unfortunately however, cationic materials are also harmful to human cells.[147] Additional modifications are necessary to make the material safe for human cell interaction, such as embedding the cationic compound into a 20 peptide MAXI hydrogel. This material was antibacterial against gram negative (*E. coli, K. pneumonia*) and gram positive bacteria (*S. aureus, S. epidermidis, S. pyogenes*) without causing harm to NIH 3T3 fibroblasts or red blood cells. The fibroblasts were able to adhere and proliferate on the hydrogel surface, and the red blood cells did not demonstrate hemolysis.[148] Designing materials that do not harm the host tissue is crucial to the design of antibiofilm coatings.[149]

Table II. Biomaterial science in combating biofilm-related infections.

Strategy	Approaches
First strategy: Modification of the biomaterial surface to confer anti-adhesive properties	• Coatings based on hydrophilic polymeric brushes based on poly(ethylene glycol) (PEG) and/or poly(-ethylene oxide) (PEO) • Polyamidoamine dendrimers • Biosurfactants
Second strategy: Doping the material with antimicrobial substances	• Antibiotic loaded biomaterials • Loading with disinfectants and bactericidal substances (e.g., NO, Ag, Zn, Cu, chlorexidine, lysozyme, metal nanoparticles) • Biofilm-disgregating agents (e.g., Dispersin B, DNase I, N-acetylcysteine) • Grafted chitosan and its quaternised derivatives • Hydrophobic polycationic coatings (e.g., *N*,*N*-hexyl, methyl-polyethylenimine and other tetralkylammonium functionalized polymers) • Immobilized antimicrobial peptides • Titanium oxide (TiO_2) and silver oxide (Ag_2O) nanoparticles with photocatalytic activity enhanced by irradiation with visible light
Third strategy: Combining anti-adhesive and antimicrobial coatings	• Multilayer film constructed by assembling layer-by-layer heparin and chitosan • Covalent conjugation of antimicrobial peptides immobilized onto a hydrophilic polymer
Fourth strategy: Material able to oppose biofilm formation and, at the same time, to support tissue integration	• Silver containing hydroxyapatite coatings • Poly(L-lysine)-grafted-poly(ethylene glycol) functionalized with adhesive peptides such as RGD • Bioglasses doped with gold nanoparticles

Source: Reprinted with permission from [86], C. R. Arciola, et al., Biofilm formation in Staphylococcus implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. *Biomaterials* 33, 5967 **(2012)**. © 2012, Elsevier Inc.

The wide variety of medical device applications implies a variety of technical specifications. Some devices, such as orthopedic implants and heart valves, require tissue integration and mechanical resilience. Others, such as vascular access catheters, require flexibility and resistance to clot adhesion. Thus, modern nanomaterials consist of a large array of metal, polymer, and composite designs.

Antimicrobial Metals

Several metals have been recognized for intrinsic antibacterial properties, including silver, zinc oxide, titanium oxide, iron, iron oxide, copper, and aluminum oxide. The antimicrobial properties of metals provide an alternative to antibiotics, without significant risk of resistance mutations. This is important considering that the development of new antimicrobials has been relatively unsuccessful.[150] It is presently well established that nanoparticles are better than microparticles at resisting biofilm formation.[125, 151, 152] Nanoparticles may be delivered free floating in nanoparticle gels or suspensions, as particles designed to elute from a surface or bound to a nanotextured surface.

For metal and metal coated biomaterials, the surface can be textured by sand blasting, sintering, plasma spray, anodization, electron beam evaporation, or pulsed microplasma cluster source. Texturing provides an antibiofilm surface while improving tissue integration.[153, 154] When comparing rough titanium surfaces produced by electron beam evaporation versus non-textured titanium or nanotube anodization or nanotexturing, the nanorough surface decreased adhesion *of S. aureus, S. epidermidis, and P. aeruginosa*. Meanwhile, the anodized surfaces actually had higher bacterial accumulation than the control non-nanotreated surface.[153] Pulsed microplasma cluster source (PMCS) allows surface deposition of varying surface roughness from 16–32 nm. Increased roughness resulted in increased protein adsorbtion, but reduced biofilm formation by *E. coli* and *S. aureus*.[155]

Silver Nanoparticles

Silver is a classic antimicrobial metal and is routinely incorporated into burn treatments and wound dressings. Silver nanoparticles have a broad range of applications, resisting biofilm formation by *E. coli*, *Enterococcus* sp., *S. aureus*, coagulase-negative *staphylococci*, and *Candida albicans*.[156] A recently developed chip calorimetry technique showed that silver nanoparticles are able to completely eradicate *P. aeruginosa* biofilms when treated with a concentration of 0.5 ug/mL.[157] It is even possible to achieve extended release of silver nanoparticles from a surface for a period of several days. A nanocomposite coating using AgCl particles embedded in a TiO_2 matrix showed extended release of silver particles, and prevention of biofilm formation by *E. coli, S. epidermidis*, and *P. aeruginosa*.[158]

The mechanism of silver toxicity is that it is able to bind with DNA, RNA, and amino acids. When silver interacts with thiol groups, there is inactivation of respiratory enzymes.[159] Silver also causes the production of reactive oxygen species (ROS).[160] These destructive mechanisms may be responsible for the relative toxicity of silver to host tissues. Nanoparticles of 20 nm have higher toxicity in fibroblasts than those of larger sizes,[161] and osteoblast proliferation is inhibited by high silver concentrations.[162]

Zinc Nanoparticles

Zinc nanoparticles have the advantage of maintaining antimicrobial activity while exhibiting a low toxicity for mammalian cells. Scanning electron microscopy shows that the ZnO nanoparticles damage the cell wall of bacteria. Compared with other metal oxides (MgO, TiO_2, Al_2O_3, CuO, CeO_2), zinc oxide nanoparticles were found to have the most effective growth inhibition against *S. aureus*[151] and are also effective against *S. epidermidis, S. pyogenes, B. subtilis, E. faecalis,* and *E. coli*.[151] Fluids containing ZnO nanoparticles are bacteriostatic against *E. coli*.[163] Zinc oxide nanotextured surfaces are also more resistant to bacteria than titanium oxide.[125] Zinc oxide nanoparticles embedded in PVC from commercial endotracheal tubes were found to grow 87% less *S. aureus* than control PVC.[164] Not all bacteria are sensitive to zinc. In particular, *P. aeruginosa* and *Proteus* are resistant to treatment with zinc.

As with other nanoparticles, the size influences the efficacy. Nanometer sized particles (200 nm) were much more effective at preventing bacterial growth than micrometer sized particles (2 um).[165] Comparing ZnO particles of 1 um, 50–70 nm and 8 nm, the smallest particles were the most effective at inhibiting *S. aureus*. The 8 nm particles inhibited 99% of growth while the larger particles inhibited 50%.[151]

Titanium Nanoparticles

Titanium oxide is the most common material for orthopedic and dental implants due to its mechanical strength, chemical stability, and excellent biocompatibility.[166] Surfaces treated with titanium oxide nanotube structures are favorable to osteocyte adhesion, while resisting *S. epidermidis* colonization.[167] Titanium is more resistant to bacterial colonization than stainless steel. Osteoblasts show greater calcium deposition and alkaline phosphatase activity on titanium oxide than ZnO, leading to better tissue integration *in vivo*.[125] One of the unique properties of TiO_2 is that it has enhanced bactericidal activity ($< 60\%$) upon photoactivation with UV light.[151]

Iron Nanoparticles

Iron oxide nanoparticles are routinely used as MRI contrast agents.[168] Superparamagnetic iron oxide nanoparticles (8 nm) were found to suppress *S. epidermidis* growth.[169]

The antibiotics penicillin, streptomycin, and vancomycin, when bound to magnetic nanoparticles of Fe_3O_4, showed enhanced antibacterial activity against both planktonic and biofilm forms of *E. faecalis*.[170] Magnetic FePt nanoparticles conjugated to vancomycin have been shown to be useful for quantifying bacteria *in vitro*. The nanoparticles bind to the bacteria, which can then be separated from the culture by magnetic force.[171]

Metal Polymer Composites

Some polymers offer favorable properties for resisting bacterial attachment but do not have the mechanical properties desired for the application. Polymer coatings can be applied by dip coating, spin coating, layer-by-layer plasma polymerization or Langmuir–Blodgett extrusion.[147] Silicone covalently bonded to titanium nanoparticles reduced *S. aureus* adhesion by 93% compared to untreated silicone.[172] Composites of metals with polymers such as polyvinyl alcohol (PVA) and polyethylene glycol (PGA) are relatively simple to produce via reactions involving the reduction of a metal salt using PVA or PGA as reducing agents.[173]

Polyether urethane (PEU) with 8% polyethylene glycol (PEG) was used as a scaffold for sustained release of zinc or gallium particles. The metals act as iron analogs, interfering with bacterial iron uptake. They were bound to chelating agents protoporphyrin IX or mesoprotoporphyrin IX. The gallium particles were much more effective than the zinc particles. PEU discs releasing gallium mesoprotoporphyrin IX (GaMP) were implanted into mice. *P. aeruginosa* infections were not detected in the blood of mice with GaMP discs compared to controls, which became septic.[174]

Silver bromide nanoparticles can be formed from reactions within a polymer matrix. Added bromohexane was dehalogenated by the polymer base, resulting in an even distribution of bromine ions within the matrix. Next, silver was added to the material to induce formation of silver bromide particles. Particles formed were 20–40 nm in size. In this case, the polymer was the amphiphilic graft copolymer of poly(vinylidene fluoride-co-chlorotrifluoroethylene)-g-poly(4-vinyl pyridine).[175]

Titanium oxide and siloxane polymer doped with silver was applied to intramedullary nails. Two goats underwent tibial bone fracture, followed by fixation with treated or control nails. The animals with coated nails were able to ambulate on the broken limb after 5 weeks, while controls were not. Mechanical analysis of the tibia showed much higher mechanical loading capacity in the treated fracture.[162]

Sterilization of materials can be achieved using a nanoenergetic coating which can be sprayed on top of a biofilm infected surface and ignited for sterilization. This represents an innovation in point of care and ease of use where access to other sterilization methods is unavailable, environmentally undesirable, or time or labor intensive. The coating consists of aluminum nanoparticles in a THV-220A fluoropolymer oxidizer matrix, which were tested on metal and glass.[176]

Polymeric Nanomaterials

Polymers are highly versatile biomaterials and can be engineered to have a wide variety of properties. Polymeric devices, including those with antimicrobial surfaces, can be printed using 3D printing. A proof of concept demonstration used the antimicrobial nitrofuratonin mixed with the thermoplastic polymer poly-L-lactic acid before being extruded in preparation for 3D printing.[177] Materials can be modified to tether antimicrobials or to form biodegradable drug eluding scaffolds. For example, surface modification of low density polyethylene (LDPE) and silicone rubber (SR), using Poly-2-dimethylaminoethyl methacrylate (pDMAEMA) followed by treatment with methyl iodide, resulted in a 99% reduction in *C. albicans* and *S. aureus* colonization. The surfaces could be loaded with the antimicrobial drug nalidixic acid for sustained release over several hours.[178] One of the potential drawbacks of unmodified polymeric material is that it can be degraded extensively by a biofilm. For example, the AKS2 strain of *P. aeruginosa* was shown to be capable of degrading polyethylene succinate (PES).[179] Increasing the hydrophobicity of the *P. aeruginosa* cell surface by varying the concentrations of glucose and ammonium sulfate in the media demonstrated that more hydrophobic bacteria were more prone to biofilm formation and increased polymer surface degradation.[179]

Experiments combining porous PTFE membranes (pore size $\geq$ 200 nm) with perfluorinated lubricating fluids have shown improved biofilm resistance compared to the PTFE surfaces alone. The surface resists biofilm formation by *P. aeruginosa* (99.6%), *S. aureus* (97.2%), and *E. coli* (96%). In mild flow conditions, the attachment of bacteria was completely prevented. In contrast to materials that release nanoparticles, this surface does not break down over time.[180] Some metals have increased reactivity after photoactivation, and this is also true of some polymeric biomaterials. Chitosan is a naturally occurring cationic biopolymer. When chitosan nanoparticles were bound to a rose-bengal dye as a photosensitizer, the treatment had improved antimicrobial properties and reduced toxicity to fibroblasts.[181] Hydrogels are another excellent biomaterial. Hydrogels can be engineered to release vitamin E-functionalized cationic polycarbonate from a block copolymer of MTC-VE and PEG. The hydrogel killed 99.9% of *S. aureus*, and *E. coli*. Fluconazol could be added for activity against *C. albicans*. No toxicity was detected against human fibroblasts.[182]

Silicone can be functionalized by tethering compounds to the polydimethylsiloxane (PDMS) surface. This construct, called a brush design, is particularly effective against biofilms.[183] For example, an arginine-tryptophan-rich peptide was covalently bound to the silicone substrate (PDMS) using a polyethylene glycol (PEG) spacer

Table III. Summary of antibiotics, their target species, class and mode of action.

Antimicrobial	Target species	Class	Mode of action	Refs.
Rifampicin	*Mycobacterium, S. aureus*	Rifamycin	Inhibits RNA synthesis	[80]
Vancomycin	Gram positives: *S. aureus, S. epidermidis*	Glycopeptide	Inhibits cell wall synthesis	[85, 193, 194]
Tobramycin	*Pseudomonas*	Aminoglycocide	Inhibits mRNA translation	[24, 98, 150, 192]
Clindamycin	*S. aureus, S. epidermidis, Bacteroides, Fusobacterium*	Lincosamide	Inhibits mRNA translation	[83]
Ciprofloxacin	Broad spectrum, gram positive and negative	Quinolone	Inhibits bacterial replication	[190]

bound to an allyl glycidyl ether (AGE) polymer brush. The surface was effective against *E. coli*, *S. aureus*, and *P. aeruginosa*. arginine- and lysine-rich peptide mixtures were immobilized on a polymethylsiloxane (PDMS) surface using an AGE polymer brush linker. The surface showed antimicrobial activity for *E. coli*, *S. aureus*, and *C. albicans* without mammalian cytotoxicity.[185]

Antifungal biomaterial design presents a challenge because fungi are also eukaryotes, so most bactericidal strategies are ineffective while effective treatments may also be toxic to the host cells.[186] Silicone based brush designs have also proven to be effective against fungal infections. An antifungal material composed of a synthetic variant of a salivary peptide called Dhvar 4, with a 4-azido-2,3,5,6-tetrafluoro-benzoic acid (AFB) linker to a polydimethylsiloxane (PDMS) inhibited *C. albicans* colonization at a concentration of 7 μM.[187] The same group showed 92% reduction in *C. albicans* colonization of PDMS surfaces with dimethylaminoethylmethacrylate (DMA-EMA), or alternatively polyethylenimine (PEI) moieties.[188]

Impregnated Antibiotics

Materials can be impregnated with antibiotic molecules that are either tethered to the surface or designed to elute out. A summary of common antibiotics is provided in Table III. The number of synthetic and natural antimicrobial peptides is enormous, leading some to use bioinformatics approaches to finding suitable peptides.[189] Localized antibiotic delivery decreases the risks of systemic toxicity while allowing for high doses of antibiotic to be delivered to the site of the implant.

Polydioxanone (PDS) polymer scaffolds containing metronidazole or ciprofloxacin demonstrated sustained drug release for 48 hours. Ciprofloxacin impregnated materials resisted biofilm formation by *Porphyromonas gingivalis* and *Enterococcus faecalis*, while the metronidazole material resisted only *P. gingivalis*. Importantly, cytotoxicity only occurred with high dose ciprofloxacin.[190] Carbon nanotubes are not antimicrobial, but can be coated with PEG bound to the antimicrobial peptide nisin. This surface showed enhanced resistance to *E. coli, P. aeruginosa, S. aureus*, and *B. subtilis*.[191]

Antibiotics can also be delivered using liposomes. Negatively charged liposome nanoparticles carrying tobramycin and polystyrene nanoparticles were more effective than free tobramycin. Negatively charged particles accumulated in the biofilm, while positively charged particles did not.[192]

Vancomycin

Vancomycin is a versatile antibiotic against some of the most common gram positive infections including *S. aureus*. One of the concerns for vancomycin surfaces is that they can decrease osteointegration, but this has not be observed in all cases.[195] By tethering nanoparticles to vancomycin, antibacterial efficacy is increased. Gold nanoparticles are 4–5 nm in diameter, making them much smaller than bacterium, and a single nanoparticle can anchor approximately 30 vancomycin molecules to its surface. Therefore, the enhanced toxicity of vanomycin nanoparticles may be due to enhanced drug presentation to the bacterial surface.[193]

Although *E. coli* is not normally inhibited by vancomycin, it is sensitive to vancomycin with gold

Table IV. Methods of detecting biofilms.

Technique	Comments	Refs.
Cresyl violet	Gold standard for quantifying colonies, but requires termination of the sample. Does not provide real time information.	[197]
PCR	Gold standard for identifying the presence of bacterial strains.	
AFM	High resolution, provides information about the mechanical properties of the colony.	[200]
FRET	Provides a mechanism of detecting quorum sensing.	[201, 202]
FISH	Provides spatial information about several species, capable of detecting culture negative species.	[203, 204]
Chip calorimetry	Very sensitive means of monitoring metabolic activity.	[157, 205, 206]
Sensors	Capable of detecting biofilm specific properties such as autoflorescence, oxygen, H_2O_2 or nitrate.	[208–212]
Magnetic nanoparticles	Can be used for fractionating bacteria bound to vancomycin nanoparticles.	[171]

nanoparticles.[194] Vancomycin resistant strains of bacteria such as vancomycin resistant *Staph aureus* (VRSA), vancomycin resistant *enterococci* (VRE), and *E. coli*, become sensitive to vancomycin when it is incorporated with nanoparticles.[85, 193, 194] Titanium alloy with vancomycin covalently attached to the surface reduced colonization by *S. epidermidis*, without the development of vancomycin resistant strains.[85]

Methods for Detecting Biofilm in the Laboratory and the Clinic

Clinically, biofilm infections can be very difficult to detect, as there is no serum marker to distinguish planktonic bacterial infections from biofilm infections.[196] Furthermore, laboratory methods of detecting and quantifying biofilms are key to the development of established, standardized experimental methods as well as the practical application of detecting infections and monitoring response to treatment. Typical traditional methods for detecting biofilms are staining with cresyl violet, quantitative polymerized chain reaction (qPCR), flow cytometry, scanning electron microscopy, and confocal scanning laser microscopy. Table IV provides a summary of traditional and emerging biofilm detection technologies.

Cresyl violet staining and quantification with a spectrophotometer is considered the most reliable routine laboratory method of biofilm measurement.[197] There is also a less common qualitative culture method for biofilms in which biofilm forming colonies grow black on a congo red agar.[198] Biofilms in fluids can be detected with high sensitivity using a coulter counter, which exhibits greater sensitivity than cresyl violet. The coulter counter distinguishes between bacterial cells, aggregates, and extracellular polymer matrix (EPM). Measurements of the ratio between cells and EPM may provide a quantitative indication of biofilm formation.[163]

Atomic force microscopy is capable of nanometric resolution of the topography of a surface. AFM operated in digital pulsed force mode provides additional information regarding the elastic, electrostatic, and adhesive properties of the sample.[199] The response of an *Aspergillus fumigatus* biofilm to treatment with alginate lyase was monitored using AFM in digital pulsed force mode.[200]

Förster resonance energy transfer (FRET) takes advantage of conformational change to bring fluorescent molecules into close enough proximity to transfer energy and emit light. In the bacteria *Vibrio fischeri*, the quorum sensing autoinducer N-(3-oxo-hexanoyl)-L-homoserine lactone (3OC6HSL) causes a conformational change in LuxR. LuxR was therefore inserted between a YFP/CFP FRET pair and attached to a chip. The sensor was able to detect concentrations of 3OC6HSL as low as 100 μM.[201] A similar FRET system using LuxP to detect binding of quorum sensing autoinducer-2 (AI-2).[202]

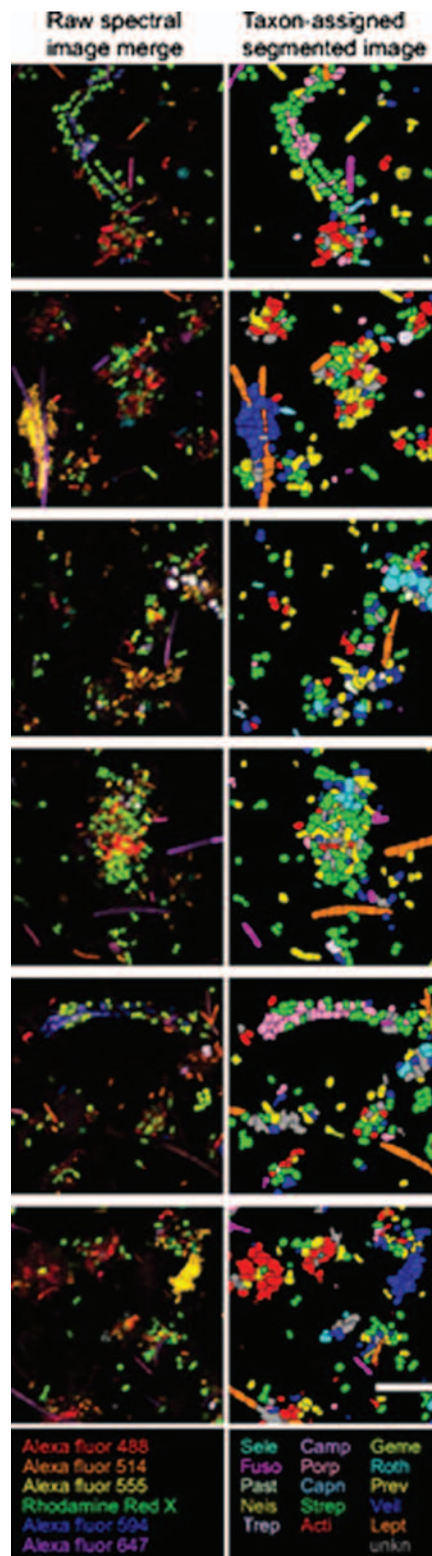

Figure 5. FISH analysis of dental plaque biofilms shows interspecies interactions and colony morphology. Reprinted with permission from [203], A. M. Valm, et al., Systems-level analysis of microbial community organization through combinatorial labeling and spectral imaging. *PNAS* 201101134 (2011). doi:10.1073/pnas.1101134108. © 2011, Proceedings of the National Academy of Sciences.

In a variation on FISH (CLASI-FISH), combinatory labeling of up to 15 bacterial taxa can be used to investigate interaction within and between species in multi species biofilms (Fig. 5).[203] In a study of heart valve infection, fluorescence *in situ* hybridization (FISH) was conducted using probes against common bacteria. Out of 13 cases that were blood culture negative for infection, FISH was able to identify 5 infections. Out of the 37 cases that were culture negative, FISH identified 11 false negatives.[204] However, it should be noted that FISH does not replace PCR as the gold standard.

Chip calorimeter monitors heat production as a surrogate for metabolic activity in biofilms.[157] Chip calorimeters provide a real-time, non-invasive measurement of a given biofilm, while other methods are more terminal and involve sonication or similar disruption. Chip calorimetry has been used to monitor response to antibiotics. Results were compared to counts of colony forming units (CFU), ATP measurement, and quantitative confocal laser scanning microscopy. Chip calorimeters were more sensitive for detecting small numbers of bacteria than counting CFUs.[205] Chip calorimetry was used to detect elimination of *Pseudomonas* by the bacterial predator *Bdellovibrio bacteriovorus*.[206] Validated by confocal laser scanning microscopy.

Biofilms can be grown on chips for high throughput drug screening. One platform prints yeast cells of *C. albicans* encapsulated in a collagen matrix onto a surface treated microscope slide. When incubated, the cells form biofilms that exhibit typical resistance to antifungal drugs. The slide contains 768 separate individual biofilm samples.[207]

Sensors have been developed to detect various specific signals that indicate biofilm infection. For example, an optical sensor is able to distinguish the autoflorescence signal of reduced nicotinamide adenine dinucleotide (NADH).[208] Similarly, the unique fluorescence signal of tryptophan can be detected by optical sensors following UV excitation.[209] The use of an oxygen detection sensor using photobacterium *kishitanii* was demonstrated with a *P. aeruginosa* biofilm.[210] Another sensor detects H_2O_2 using a glassy carbon electrode embedded in a silver titanium oxide composite.[211] Finally, sensors exist that can detect nitrate by means of embedded nitrate reductase in a Polypyrrole/Carbon nanotube film.[212]

ABBREVIATIONS

AGE, allyl glycidyl ether
AFM, atomic force microscopy
AI-2, autoinducer-2
BEC, biofilm eradicating concentration
CVC, central intravenous catheters
CFU, colony forming units
DMA-EMA, dimethylaminoethylmethacrylate
EPS, extracellular polymer matrix
FISH, fluorescence *in situ* hybridization
FRET, Förster resonance energy transfer
IHF, integration host factor
LDPE, low density polyethylene
NADH, nicotinamide adenine dinucleotide
pDMAEMA, poly-2-dimethylaminoethyl methacrylate
PDMS, polydimethylsiloxane
PDS, polydioxanone
PES, polyethylene succinate
PEG, polyethylene glycol
PEI, polyethylenimine
PIA, polysaccharide intercellular adhesion
PMN, polymorphonuclear
PTFE, polytetrafluoroethylene
PVA, polyvinyl alcohol
PMCS, pulsed microplasma cluster source
TLR, toll-like receptors
UV, ultraviolet

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