ISSN: 2577 - 8005

Review Article

Medical & Clinical Research

Factors Associated with Biofilm Persistence on Different Surfaces, Spread and Pathogenicity

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Submitted: 17 Dec 2020; **Accepted**: 23 Dec 2020; **Published**: 04 Jan 2021

Abstract

The conglomeration of microbial life on a self-produced extracellular polysaccharide (EPS) matrix for mutual co-existence and protection against external aggression and adverse environmental conditions best describe biofilms. This community of microorganisms confers a number of survival and nutritional benefits to members while at the same time portend great ecological and health concern. Biofilms can form on virtually any surface; terrestrial, aquatic, plants, animals and on medical devices and implants. The ability of biofilms to disperse from the parental stalk ensures continuous survival and spread within their ecological niche. Biofilm organisms therefore possess unique survival mechanisms over their plancktonic form and have contributed to our understanding of the mechanisms of pathogenicity of infectious microorganisms. This review highlights trends in the understanding of biofilms and emphasized their health significance.

Keywords: Biofilm; Antibiofilm Compound; Antimicrobial Resistance; Implantable Devices, Microbial Pathogenesis; Microbiologically Influenced Corrosion

Introduction

The ecological significance of biofilms especially in microbial pathogenesis and water quality has resulted in greater interest in their formation, dispersion, clinical and environmental implications and control. These aggregates of microorganisms formed on self-generated polymeric substances possess unique survival propensity over their planktonic counterparts; stressful conditions of fluctuating temperature and presence of antimicrobials and immune factors [1-4]. Biofilm organisms express differing property in respect to growth and gene composition from their freeliving counterparts and as such, any detachment from the consortium present in medical instrument or water distribution system may results in clinical infection and decline in water quality respectively leading to possible disease outbreak [2, 5]. It has been variously reported that biofilms account for majority of the chronic antibiotic resistant nosocomial and device –attributed infections [6, 7]. Therefore, understanding of the property of biofilms and conditions favoring their formation and persistence is crucial in harnessing their benefits and preventing potential hazards.

The magnitude in terms of burden of infection and cost implication of biofilms associated infections can be appreciated from the following data. Biofilm associated bacteria have been shown to be responsible for most outbreaks of waterborne diseases as the biofilms in water distribution systems ensure availability of nutrients to the microbes in addition to protection against water disinfectants [8]. Jayaraman et al. highlighted the economic significance of biofilm corroding sulfate reducing bacteria in water pipelines as costing the industry 4-6 billion dollars annually [9]. Furthermore, the growth of pathogenic microorganisms on medical instruments as biofilms has been strongly linked to healthcare associated infections which account for some 1.7 million infection cases and approximately 100,000 deaths resulting to an estimated expenditure of $30 billion in the US annually [10]. Biofilms are critical in the pathogenesis of infections associated with the use of medical devices; including intravascular catheters, urinary catheters and orthopedic implants. In the United States, 1.7 million healthcare associated infections was estimated for the year 2002, The number of associated deaths reported was 98,987; of these, 30,665 were from bloodstream infections, 13,088 from urinary tract infections and 8205 from surgical site infections including those associated with orthopedic implants [10]. Central venous catheters (CVCs) are responsible for the highest proportion of bloodstream infections, their wide use being associated with a substantial risk of infectious complications that prolong hospital stay and increase healthcare costs [11]. More than five million CVCs are implanted each year in the United States, and there are approximately 200,000 cases of bloodstream infections related to their use with catheter-related infections

being the most common cause of nosocomial endocarditis [12-14]. According to the Transparency Market Research (2014) the global medical device reprocessing market is growing at an estimated rate of 19.3% annually and may hit the $2.6 billion mark by 2020. This astronomic growth is however confronted with the potential infection arising from biofilm contamination in reprocessed devices [15].

A biofilm is a composite of surface-associated microbial cells embedded in an extracellular polymeric substance matrix. Jones et al. described biofilm bacteria on trickling filters in a waste water treatment plant with the aid of the Scanning and Transmission Electron Microscopy [16]. Characklis was among early investigators that describe biofilms resistance to chlorine [17]. Much of the work in the last two decades has relied on tools such as Scanning Electron Microscopy (SEM) or standard microbiologic culture techniques for biofilm characterization. Non-cellular materials such as mineral crystals, corrosion particles, clay or silt particles, or blood components, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix. Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems [18-20].

Mechanisms of Formation of Biofilms

The attachment of microorganisms to surfaces is a very complex process, with many variables affecting the outcome. In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface "conditioning" films in addition to increase in flow velocity, water temperature, or nutrient concentration [19, 21, 22]. The physico-chemical nature of the aqueous medium which includes pH, nutrient levels, ionic strength, and temperature, are essential determinants in biofilm formation [21]. Fletcher found that an increase in the concentration of several cations (sodium, calcium, lanthanum, ferric iron) affected the attachment of *Pseudomonas fluorescens* to glass surfaces, presumably by reducing the repulsive forces between the negatively charged bacterial cells and the glass surfaces [22]. Properties of the cell surface such as presence of fimbriae, flagella, and surface-associated polysaccharides or proteins, are important and may possibly provide a competitive advantage for one organism where a mixed community is involved.

The solid surface may have several characteristics that are important in the attachment process. Characklis et al. observed that the extent of microbial colonization appears to increase as the surface roughness increases. This is because shear forces are diminished, and surface area is higher on rougher surfaces. The physicochemical properties of the surface may also exert a strong influence on the rate and extent of attachment. Earlier reports have indicated that microorganisms attach more rapidly to hydrophobic, non-polar surfaces such as Teflon and other plastics than to hydrophilic materials such as glass or metals [23-25].

A prerequisite for surface colonization by microorganisms is priming also known as conditioning provided by organic substances within the environment [26]. Loeb and Neihof were the first to report the formation of these conditioning films on surfaces exposed in seawater [27]. They found that the films were organic in nature, formed within minutes of exposure, and continued to grow for several hours. In the oral cavity of man, the priming material is a proteinaceous substance called acquired pellicle which develops on tooth enamel. Pellicle comprises albumin, lysozyme, glycoproteins, phosphoproteins, lipids, and gingival crevice fluid and provides the substrata on tooth enamel for biofilm formation. Other conditioning materials described include blood, tears, urine, saliva, intervascular fluid, and respiratory secretions [28, 29].

Ofek and Doyle stated that the surface energy of the suspending medium may affect hydrodynamic interactions of microbial cells with surfaces by altering the substratum characteristics [30]. The velocity of the suspending medium may determine the rate of settling of microbial cells. Under very low linear velocities, the cells must traverse the sizeable hydrodynamic boundary layer, and association with the surface will depend in large part on cell size and cell motility. As the velocity increases, the boundary layer decreases, and cells will be subjected to increasingly greater turbulence and mixing. Higher linear velocities become necessary for rapid cell association with the surface, at least until velocities become high enough to exert substantial shear forces on the attaching cells, resulting in detachment from the composite [23, 31].

Four cell inherent factors influence biofilm formation and these include surface hydrophobicity, presence of fimbriae and flagella, and production of extracellular polymeric substances (EPS) [19, 32-37]. The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with an increasing nonpolar nature of one or both surfaces involved [19, 32, 33]. Fimbriae play a role in cell surface hydrophobicity and attachment, probably by overcoming the initial electrostatic repulsion barrier that exists between the cell and substratum [34, 35, 37]. Most fimbriae that have been examined contain a high proportion of hydrophobic amino acid residues [36].

Bendinger et al. found that mycolic acid-containing organisms (*Corynebacterium, Nocardia, and Mycobacterium*) were more hydrophobic than were non-mycolic acid-containing bacteria, and increase in mycolic acid chain length generally coincided with increase in hydrophobicity [38]. The O antigen component of lipopolysaccharide (LPS) has been shown to confer hydrophilic properties to gram-negative bacteria [39]. Williams and Fletcher showed that mutants of *P. fluorescens* lacking the O antigen adhered in greater numbers to hydrophobic materials. Korber et al. used motile and non-motile strains of *P. fluorescens* to show that motile cells attach in greater numbers and attach against the flow more rapidly than do non-motile strains [40]. Nonmotile strains also do not colonize or seed vacant areas on a substratum as evenly as do motile strains, resulting in slower biofilm formation by the former.

Substantial evidences show the presence of genes regulating bacterial interaction with substratum. These genes can be up or down regulated [41]. Davies and Geesey demonstrated *algC* upregulation in individual bacterial cells within minutes of attachment to surfaces in a flow cell system. Prigent-Combaret et al. found that 22% of these genes were up-regulated in the biofilm state, and 16% were down-regulated. Becker et al. showed that biofilms of Staphylococcus aureus were up-regulated for genes encoding enzymes involved in glycolysis or fermentation

(phosphoglycerate mutase, triosephosphate isomerase, and alcohol dehydrogenase) and conclude that the up-regulation of these genes could be due to oxygen limitation in the developed biofilm, favoring fermentation [42, 43]. Pulcini also showed that *algD, algU, rpoS*, and genes controlling polyphosphokinase (PPK) synthesis were upregulated in biofilm formation of *P. aeruginosa*. Prigent-Combaret et al. suggested that the expression of genes in biofilms is evidently modulated by the dynamic physicochemical factors external to the cell and may involve complex regulatory pathways [42, 44].

Biofilm Structure

The principal components of biofilms are microbial cells and extracellular polymeric substances (EPS) [3], the latter accounting for between 50% and 90% of the total organic carbon of biofilms [45]. EPS vary in chemical and physical properties, from Gram negative to Gram positive bacteria but predominantly is composed of polysaccharides. Some of these polysaccharides are neutral or polyanionic, as is the case for the EPS of gram-negative bacteria [3]. The presence of uronic acids (D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pryruvates confers anionic property which allows association of divalent cations such as calcium and magnesium, which subsequently cross-link with the polymer strands and provide greater binding force in a developed biofilm [45, 46]. In contrast, the EPS of gram-positive bacteria such as the staphylococci and streptococci is cationic [5, 47]. Hussain et al. has earlier reported that the slime of coagulasenegative staphylococci consists of a mixture of teichoic acid and proteins [48].

EPS is highly hydrated a property that prevents desiccation in some natural biofilms just as its complexity contributes to antimicrobial resistance by impeding the mass transport of antibiotics through the biofilm, probably by binding directly to these agents [49-51]. Majority of EPS share both hydrophilic and hydrophobic properties a few are hydrophobic [46]. EPS may also vary in solubility; for instance, many bacterial EPS possess backbone structures that contain 1,3- or 1,4β-linked hexose residues and tend to be more rigid, less deformable, and in certain cases poorly soluble or insoluble while some may be readily soluble in water. Furthermore, the EPS of biofilms is not generally uniform but may vary spatially and temporally [46]. Leriche et al. used the binding specificity of lectins to simple sugars to evaluate bacterial biofilm development by different organisms. Their results showed that different organisms produce differing amounts of EPS and that the amount of EPS increases with age of the biofilm. EPS may associate with metal ions, divalent cations, other macromolecules (such as proteins, DNA, lipids, and even humic substances) [45, 52, 53]. EPS production is known to be affected by nutrient status of the growth medium; excess available carbon and limitation of nitrogen, potassium, or phosphate promote EPS synthesis in as much as slow bacterial growth do enhance EPS production [46].

Ecological Significance of Biofilms

The micro-colony represents the structural unit of a biofilm. Proximity of cells within the microcolony or between micro-colonies creates a suitable environment for nutrient assimilation, exchange of genetic materials, and quorum sensing [54-56]. Since micro-colonies may be composed of multiple species, the cycling of various nutrients such as nitrogen, sulfur, and carbon) through redox reactions can readily occur in aquatic and soil biofilms [20].

Conjugation, the mechanism of plasmid transfer, occurs at a greater rate between cells in biofilms than between planktonic cells [57]. Ghigo showed that the F conjugative pilus (encoded by the *tra* operon of the F plasmid) acts as an adhesion factor for both cell-surface and cell-cell interactions, resulting in a three-dimensional biofilm of *Escherichia coli* [58]. Plasmid-carrying strains develop more adherent biofilms as opposed to non-plasmid borne strains as a result of increase tolerance to shear and a closer cell-cell contact. Since plasmids may encode for resistance to multiple antimicrobial agents, biofilm association also provides a mechanism for selecting for, and promoting the spread of, bacterial resistance to antimicrobial agents.

Cell-to-cell signaling has been demonstrated to play a role in cell attachment and detachment from biofilms. Xie et al. showed that certain dental plaque bacteria can modulate expression of the genes encoding fimbrial expression (*fimA*) in *Porphyromonas gingivalis. P. gingivalis* would not attach to *Streptococcus cristatis* biofilms grown on glass slides. *P. gingivalis*, on the other hand, readily attached to *S. gordonii. Streptococcus cristatus* cell-free extract substantially affected expression of *fimA* in *P. gingivalis*, as determined by using a reporter system. *S. cristatus* is able to modulate *P. gingivalis fimA* expression and prevent its attachment to the biofilm [59, 60]. Davies et al. showed that two different cell-to-cell signaling systems in *P. aeruginosa, lasR-lasI* and *rhlRrhlI*, were involved in biofilm formation. At sufficient population densities, these signals reach concentrations required for activation of genes involved in biofilm differentiation. Stickler et al. described acylated homoserine lactone signals in biofilms of gram-negative bacteria on urethral catheters [61, 62]. Yung-Hua et al. demonstrated that induction of genetic competence is mediated by quorum sensing in *S. mutans* and reported transformational frequencies to be 10–600 times higher in biofilms than planktonic cells.

Predation by protozoa, bacteriophage and polymorphonuclear leukocytes (PMNs) biofilm bacteria has to contend with. Murga et al. demonstrated the colonization and subsequent predation of heterotrophic biofilms by *Hartmannella vermiformis*, a free-living protozoon and also by *Acanthamoeba* spp. in contact lens storage case biofilms [63, 64]. That biofilms may play a role in bacterial pathogenicity has been demonstrated with true pathogens such as *Legionella pneumophila, S. aureus*, *Listeria monocytogenes, Campylobacter* spp, *E. coli* O157:H7, *Salmonella typhimurium*, *Vibrio cholerae*, and *Helicobacter pylori* [63, 65-71]. The ease with which these pathogens dislodge from the biofilm may facilitate their spread within ecological niche and aid in their transmission. The mechanism of interaction and growth apparently varies with the pathogen, and at least for *L. pneumophila*, appears to require the presence of free-living protozoa to grow in the biofilm [63]. Survival and growth of pathogenic organisms within biofilms might also be enhanced by the association and metabolic interactions with indigenous organisms. Camper et al. showed that *Salmonella typhimurium* persisted in a model distribution system containing undefined heterotrophic bacteria from an unfiltered reverse osmosis water system for >50 days, which suggests that the normal biofilm flora of this water system provided niche conditions capable of supporting *Salmonella typhimurium* [68].

Biofilms in Water System

Biofilms are common features in water distribution systems irrespective of the nature of the plumbing material. Natural organic matter and residual chlorine may serve as precursors for biofilm formation in water distribution system when microorganisms are present. Studies have shown that biofilms in drinking water systems can serve as reservoirs for *Helicobacter pylori, Legionellae* species and *Mycobacterium avium* [72, 73]. Free-living protozoa can be part of biofilm ecosystems and are increasingly recognized for harbouring pathogens. Biofilms are also possible contributors to coliform regrowth. While biofilms can be a source of concern for water utilities, in drinking water distribution systems they have been associated with the removal of some haloacetic acids (HAAs) including mono-halogenated compounds and di-halogenated species (but not trihalogenated species) [74]. The dominant HAA degraders in drinking water system enrichment cultures are *Afipia* spp. [75].

Biofilm growth can increase localized cast iron pipe corrosion by changing oxygen concentration and electrical potential of the pipe wall [76]. While biofilm growth in a water pipe can be beneficial as a barrier to corrosion, it is generally considered to be detrimental in most aspects of iron corrosion. Additionally, anaerobic sulfate-reducing bacteria can contribute to microbiologically induced corrosion by generating hydrogen sulfide gas that accelerates corrosion processes [77]. Other group of microorganisms of importance in water distribution system are the ammoniaoxidizing bacteria, actinomycetes, iron and sulphur bacteria. Biofilms formed by these organisms are usually resistant to chlorine and chloramines and are usually associated with offensive odor taste and color in pipe distributed water. Current practice by water utility companies to control biofilm formation includes flushing, swabbing/pigging, chemical treatments, and ice pigging. Flushing is the most popular method, but it is not always effective. It is suited to water mains less than 12" in diameter and may not work well on some deposits, such as manganese coatings or adherent corrosion scale.

Health Significance of Microbial Biofilms

There is growing interest of the role of biofilm formation in the pathogenesis of a number of chronic clinical conditions notably cystic fibrosis, native valve endocarditis, otitis media, periodontitis, and chronic prostatitis [6, 78-80]. A spectrum of indwelling medical devices or other devices used in the health-care environment have been shown to harbor biofilms, resulting in measurable rates of device-associated infections [6, 81]. Biofilms of potable water distribution systems have the potential to harbor enteric pathogens, *L. pneumophila*, nontuberculous mycobacteria, and possibly *Helicobacter pylori* [72, 73]. Characteristics of biofilms that can be important in infectious disease processes include: detachment of cells or biofilm aggregates may result in bloodstream or urinary tract infections or in the production of emboli; cells may exchange resistance plasmids within biofilms; cells in biofilms have dramatically reduced susceptibility to antimicrobial agents; biofilm-associated gram-negative bacteria may produce endotoxins; biofilms are resistant to host immune system clearance [81]. Biofilm may represent a critical reservoir for the shedding and dissemination of bacteria hematogenously. For instance, Raad et al. showed a positive correlation between biofilms on central venous catheters and septicemia [65].

Resistance to antimicrobial agents

Biofilms are highly resistant to most antimicrobial agents and disinfectants [81]. In addition, organisms within biofilms can readily acquire resistance through the transfer of resistance plasmids. Such resistance could be especially acute in the health-care environment for patients with colonized urinary catheters and collection bags. Many of the enteric organisms shown to colonize urinary catheters carry plasmids encoding resistance to multiple antimicrobial agents [82]. Transfer of plasmids within biofilms has been well established. Resistant organisms such as methicillin-resistant *Staphylococcus aureus* have also been shown to form biofilms [63].

Treatment of biofilm related infections is more complex and may require both tissue debridement and antibiotics and over a protracted period. This treatment regimen encourages emergence of antibiotic resistant bacterial strains [7, 83, 84]. Another phenomenon is the occurrence of persisters (dormant cells) within biofilms that are tolerant to antibiotics during antibiotic therapy and become active proliferative cells when therapy is discontinued [49, 85, 86]. Bacterial resistance exemplifies a state of active growth and multiplication during antibiotic therapy while tolerance refers to a state of survival or persistence in the presence of antibiotics but do not proliferate [49]. Several studies have shown that sessile bacteria are 500-5000 times more tolerant towards antibiotics in comparison to their planktonic state [79, 87].

Examination of the degree of bacterial resistance to antibiotics showed that the processes are facilitated in a biofilm consortium and less favored in planktonic cells [6]. A summary of these mechanisms was given by Khatoon et al. and represented here.

1. As biofilms mature, oxygen and nutrient concentration decreases which affect negatively the penetration of antibiotics within biofilms, resulting in persister cells and consequently tolerance to antibiotics [7, 49].
2. Biofilm bacteria respond more to stress factors which they are habitually exposed to by expressing stress response genes such as the s-factors that protect them from antibiotics, host immune factors and environmental toxins [88].
3. Differences in the physiological properties of established biofilms and actively growing planktonic cells can also explain the decreased sensitivity of biofilms towards antibiotics, which are known to target active cell processes [55].
4. Aminoglycosides that require oxidative process for this class of antibiotics to cross the cell membrane may be inactive in a biofilm environment depleted of oxygen [79].
5. Extracellular DNA (eDNA) released by autolysis in the EPS has the capacity to neutralize the activity of antimicrobial drugs such as tobramycin via its cation chelating properties, as seen in P. aeruginosa biofilms [89]. This mechanism also accounts for biofilm bacteria tolerance to metals such as zinc, copper, and lead [7].
6. Extracellular β-lactamases, are known to degrade antimicrobials, preventing them from reaching the biofilm [49].
7. Bacteriophages including filamentous phage particles help in releasing eDNA and the development of antibiotic tolerant colonies within biofilms [49].
8. Several biofilms are also able to inhibit or block leukocytic predation through various mechanisms. One such mechanism

is the quorum sensing (QS) induced production of rhamnolipids by P. aeruginosa in response to phagocytic leukocytes [7].

Biofilm Dispersion and Resistance to Host Immune Mechanism

Like the dehiscent tree biofilms survive and proliferate by spreading into favorable environment. This process of releasing some components of the biofilm to commence a new existence is referred to as dispersion. Thus, dispersion is a survival mechanism that enables bacteria in the consortium to discover and establish new ecological niche. By so doing infection spread through the body. A biofilm has two layers, the base film layer where the microbial cells exist, and the surface film where they get dispersed into their surroundings for expansion and continued existence [90]. This stage causes chronic infection and other severities like embolic complications, which require immediate treatment [90]. As such, this process is often referred to as metastatic seeding [5, 7, 79, 91]. The dispersion of cells occurs either as single cells or as clumps of cells which are sloughed off the biofilm [79]. This is said to be a programmed process that is initiated by oxygen level (in case of aerobic biofilms) or nutrient starvation. This starvation stimulates small molecules like fatty acid DSF (cis-11-methyl-2-dodecenoic acid), which triggers auto phosphorylation and leads to activation of c-di-GMP phosphodiesterase that degrades c-di-GMP. Degradation of c-di-GMP leads to the tearing of clusters by shear forces or the release of planktonic cells that dissolve a portion of the EPS [7, 90, 92]. While this is one mechanism, there are others apart from gene regulation pathways involved in the dissolution of EPS [6, 90, 92].

Evidence has been provided to support the hypothesis that microorganisms detaching from biofilms on indwelling medical devices could overcome the host immune system and cause an infection [93]. Shiau and Wu showed that the extracellular polymeric substance matrix produced by *S. epidermidis* interfered with macrophage phagocytic activity [94]. Meluleni et al. found that opsonic antibodies made by patients with chronic cystic fibrosis were unable to mediate phagocytosis and eliminate bacterial cells growing in biofilm microcolonies [95]. Yasuda et al. demonstrated that re-suspended biofilm cells of *Escherichia coli* were less sensitive to the killing activity of human polymorphonuclear leukocytes (PMNL) *in vitro* and were of the opinion that this was due to resistance of the biofilm organisms to the active oxygen species produced by the PMNL. This indicates that cells detaching from biofilms in indwelling medical devices may have the ability to survive the PMNL phagocytic activity in the bloodstream to initiate a bloodstream infection.

Biofilms on Medical Devices

Strong biofilms forming bacteria on medical devices are *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis,* the viridans streptococci, *E. coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa* [96]. *S. aureus* and *S. epidermidis* are associated with about 40-50% of prosthetic heart valve infections, 50-70% of catheter biofilm infections and 87% of bloodstream infections [96]. About 75% of implantable device associated infections are caused by Staphylococcus species while. *P. aeruginosa* has become a model candidate for studying biofilm formation in gram negative bacteria [97, 98, 53, 54]. In humans, biofilms account for up to 80% of the total number of microbial infections according to National Institute of Health [78, 99], including endocarditis, cystic fibrosis, periodontitis, rhinosinusitis, osteomyelitis, non-healing chronic wounds, meningitis, kidney infections, and prosthesis and implantable device related infections [3, 7, 26, 79, 80].

Gram-negative bacteria within biofilms of indwelling medical devices will produce endotoxins. Production of such toxins could be potentially harmful for patients undergoing hemodialysis as espoused by Vincent et al. who measured endotoxin levels on hemodialysis tubing and showed a correlation with bacterial biofilm counts [100]. Other studies have also measured endotoxin levels of biofilms and these further corroborate the possibility of endotoxins from dialysate diffusing across the dialysis membrane of the dialyzer [101, 102].

Resistance associated with biofilm has become nightmare in healthcare delivery especially in treating biofilm causing infections including implant associated infections. Prosthetic and implantable devices can get contaminated during surgery or post-surgery and the colonization rate for implantable devices is higher and faster than that of native human tissue primarily because of adequate vascularization of the human tissues [26, 80, 87, 103, 104]. Factors such as differences in implant surface hydrophilicity, surface charge, surface energy, and biomaterial composition also play a role in increasing the rate of infection in implants [26, 103, 105].

Methods that detect and quantify biofilms on the inner (luminal) as well as outer surfaces of catheters will provide true picture of biofilm colonization. A widely practiced procedure to detect bacterial colonization on catheter tips is the roll plate method developed by Maki et al [106]. This technique is based on the premise that biofilm-associated bacteria on the outside of the catheter tip can be reproducibly recovered by rolling the tip over the surface of an agar plate. However, organisms that are not removed by contact with the agar or organisms on the inner lumen of the catheter are not detected, and this makes clinical interpretation difficult. A much more reliable, more quantitative method is to use mechanical forces (e.g., sonication or vortexing) to remove the biofilm-associated organisms, which can then be quantified by means of plate count or fluorescent staining techniques [81, 107, 108]. Ward et al. also proposed an endoluminal brush technique for the quantification of biofilm-associated organisms on catheters [109]. Any of these procedures should be useful for the recovery and quantification of biofilms on other medical devices, such as prosthetic joints and mechanical heart valves. However, once the catheter tip is removed, some symptoms may resolve. For all patient populations, the roll-plate method may provide clinically relevant data, especially if culture of a venous blood specimen is not readily available.

Treating infections that involve biofilms

The pathology of polymicrobial infections involving an anaerobe and facultative anaerobic bacteria is aggravated by biofilms formed at pathologic sites and is a synergy of various interacting mechanisms [110]. In one study *Prevotella bivia* failed to co-aggregates or co-aggregated weakly with other anaerobic Gram-negative bacilli such as *Prevotella disiens or Bacteroides fragilis* but co-aggregated strongly with facultative bacteria such as

Escherichia coli and *Staphylococcus aureus.* The study further demonstrated enhanced subcutaneous abscess formation in mice injected with a mixed inoculum of *P. bivia* and *E. coli* while abscess formation was absent in mice injected with mono-culture of *P. bivia.* From the above study it is evident that the inducing factor for *P. bivia* and *E. coli* interaction is oxygen gradient created by the facultative bacterium that enabled the anaerobe to establish in the infection site with reduced redox potential. In the study being described *P. bivia* was able to aggregate at infection site and this was facilitated by calcium ions but not sodium or ferrous ions. The ability of *P. bivia* to form aggregates in the presence of calcium ions may explain why certain bacteria, especially oral microflora, can result in dental plaque formation. Failure of *P. bivia* to form aggregates in the presence of sodium and ferrous ions which are important component of body fluids, especially blood, may explain in part why it has not been implicated in septicemia and disseminated diseases. Earlier studies in this area have described bacterial aggregation as a possible mechanism by which bacteria evade killing by host phagocytes and antimicrobial agents in vivo [111, 112]. The ability of *P. bivia* to form aggregates and also coaggregate with facultative organisms may explain partly while it is predominant in infections of the female genital tract [113]. While aggregate formation may help retain *P. bivia* in pathologic sites, co-aggregation with facultative organisms serves as a link through which organisms in mixed infections interact with possible exchange of genetic materials.

The importance of bacterial slime in biofilm formation and antibiotic resistance cannot be overemphasized [114]. Ayepola et al found a strong correlation between antibiotic resistance and biofilm formation in coagulase negative staphylococci. This confirmed an earlier report that methicillin resistance occurred more in *S. epidermidis* strains that produced slime [115].

Biofilm age may influence susceptibility. If an indwelling medical device is colonized by a biofilm, the problem will inevitably get worse, and the aging biofilm will become increasingly difficult to treat. Old biofilms have been shown to be even less susceptible to antimicrobial agents than are younger biofilms [81]. In addition, if organisms with acquired resistance are present in the biofilm, the probability of resistance plasmid transfer might increase over time.

The concentrations of antimicrobial agents required to inactivate biofilm associated organisms are much higher than the concentrations sufficient to inactivate systemic organisms in the standard *in vitro* micro-dilution test [116]. In addition to the issue of concentration differences, it is possible that certain categories of antibiotics may be more effective against biofilm-associated organisms than are others. For example, Ceri et al. showed that ciprofloxacin and trobramycin were more effective as treatment against biofilms of *P. aeruginosa* than a number of other antibiotics, such as piperacillin, imipenem, and ceftazidime [117]. Gentamicin was more effective against *S. aureus* biofilms than were any of the other agents tested, including oxacillin and vancomycin. If antimicrobial therapy is considered a viable option against biofilm colonization, then susceptibility testing should be performed with biofilm-associated organisms. It may, in fact, be difficult or impossible to achieve inhibitory concentrations of the antimicrobial within the biofilm at the site of infection (i.e., tissue or blood).

Prevention of Biofilm Formation

Gram-positive and gram-negative bacteria communicate with each other using small diffusible signal molecules called autoinducers. The most common classes of signal molecules are oligopeptides in gram-positive bacteria, N-acyl homoserine lactones in gram-negative bacteria and a family of autoinducers known as AI-2 in both gram-negative and gram-positive bacteria. This communication process among cells, known as quorum sensing (QS), plays a significant role in modulating not only the expression of genes associated with the production of specific enzymes, virulence factors and metabolites but also the development of microbial communities as biofilms. Thus, QS is described as a regulatory mechanism allowing sessile microorganisms to respond to needs that are related to the increasing population density through the expression of specific sets of genes [11].

In many bacterial species, QS play a significant role in biofilm survival [116, 118]. In *P. aeruginosa* the Las QS system is involved in the development of antibiotic tolerance; the Lassystem-induced tolerance is regulated by the *rpoS* gene and Rasmussen and Givskov identified three of these signal points in gram-negative bacteria to include; the signal generator, the signal molecule and the signal receptor [119, 120]. The signal receptor being the most investigated for application purposes and sufficient data show that, QS can be prevented by inhibiting the signal molecule from binding to the receptor via analogues of the signal molecules [121, 122]. Thus, the use of molecules interfering with QS is a promising strategy to inhibit microbial adaptation to the host environment and initiate infectious processes [123, 124]. QS inhibitors and antagonists is postulated to be the most promising therapeutic tools for the treatment of biofilm-based infections [11].

Potent inhibitors of gram-negative QS are the halogenated furanone purified from *Delisea pulchra* and a series of related synthetic derivatives, reported to be efficacious as anti-infective drugs in animal models [125-127]. Usnic acid, a naturally occurring dibenzofuran derivative, was demonstrated to be able to affect the morphology (thickness and roughness) of *P. aeruginosa* biofilm without inhibiting bacterial growth, this phenomenon presumably indicating its interference with bacterial signaling pathways [128]. In gram-positive bacteria, the QS inhibitor RNAIII inhibiting peptide (RIP) has been demonstrated to be very efficacious in preventing and treating staphylococcal infections associated with CVCs (central venous cathethers), orthopedic implants and ureteral stents. Using a rat model, Cirioni et al. reported that RIP coated CVCs significantly reduced bacterial load and enhanced the effect of tested antibiotics in the treatment of CVC-associated S. *aureus* infections [129]. When exposed to RIP, biofilm *S. aureus* cells become as susceptible to antibiotics as planktonic cells [129].

Regarding orthopedic implants, RIP-loaded polymethylmethacrylate beads were implanted in rats and were demonstrated to be able to prevent *in vivo* methicillin-resistant *S. aureus* (MRSA) biofilm formation either alone or combined with vancomycin, highlighting this QS inhibitor as an alternative or an additional agent to be used for the prevention of orthopedic infections [130]. Ureteral stents coated with the QS inhibitor RIP were implanted in rat bladders and shown to inhibit *S. aureus* biofilm formation on the stent surfaces. In addition, stent coating with RIP and teicoplan in increases the antibiotic efficacy in

preventing ureteral stent-associated staphylococcal infections [129].

Biofilms, Corrosion and Mitigation

Corrosion is a major engineering dilemma and had accounted for huge financial investment in all industries that utilize metal, concrete or plastics in their operations. Many recorded engineering disasters and failures were association with corrosion. These include; the collapse of the Mianus bridge in 1983, when the bearings rusted internally and pushed one corner of the road slab off its support; another was the Silver Bridge disaster of 1967 in West Virginia when a steel suspension bridge collapsed within a minute resulting in the loss of lives. Furthermore, the corrosion of metal prosthetic devices (pins, plates, hip joints, pacemakers and other implants) which may be microbiologically influenced has dire health consequences [131].

An overview on corrosion will expand our understanding of the role of microorganisms especially as biofilms in modulating or enhancing corrosion. From the engineering perspective, corrosion is the deterioration, degradation or destruction of a material or its properties by chemical or electrochemical reactions due to the interaction between the environment and material's surface. All metals when exposed to a corrosive environment corrode resulting in reduced mechanical strength. Metals are refined from their ores. The ores are of lower energy level than the corresponding metal and are equally more stable than metals which are highly reactive. Therefore, corrosion is a process of metals trying to revert to their original forms. Corrosion is an electrochemical reaction and may occur as chemical or atmospheric corrosion. When acidic substances including water come in contact with metals, such as iron and/or steel, rust begins to form. Rust is the result of corroding steel after the iron (Fe^{2+}) particles have been exposed to oxygen and moisture. This environment around the metal provides the nutrient, chemical and physical ambience for organisms such as sulfate reducing bacteria, Sulfur oxidizing bacteria and iron bacteria to grow and form biofilms on metal surface [132, 133].

Metals such as structural steel and copper alloys tend to corrode generally over the entire surface in the absence of crevices or galvanic effects. In such cases, corrosion is determined by the rate at which dissolved oxygen can be delivered to the metal surface. Microorganisms present in the aqueous medium often have the potential to increase or decrease oxygen transport to the surface; consequently, the organism has a role in increasing or decreasing general corrosion [134]. Most MIC, however, manifest as localized corrosion because the organism in question do not form in a continuous film on the metal surface. These organisms settle on the metal surface as discrete or spotty colonies [133]. Corrosion may be aerobic caused most by *Desulfovibrio* (Sulfur reducing bacteria- SRB) or aerobic caused by *Thiobacillus* (sulfur oxidizing bacteria).

Microbiologically influenced corrosion occurs on metals exposed to different environments including sea water, fresh water, soils, foodstuffs, demineralized water, sewage, aircraft petrol, human plasma, and process chemicals [134]. Stainless steel is covered with a highly protective film of chromium oxyhydroxide and is resistant to corrosion in many aggressive environments; however, acidic solutions are aggressive to this film layer and results in severe pitting formation [135]. Mineral acid solutions used in industry for pickling, descaling, acid cleaning and oil-well acidizing create a favorable environment for acidic corrosion, hence it is necessary to add corrosion inhibitors to the aqueous medium to protect the surface of metals from corrosion. Many organic compounds are being evaluated as inhibitors of corrosion and these are used as biocides. These compounds adsorb to the metal surface forming protective films against corrosive agents. Thiourea and thiadiazole have been studied in great details with great promise as anticorrosion compounds [135]. Thiourea contains one sulfur and two nitrogen atoms. The availability of lone pair electrons in the inhibitor molecules facilitates electron transfer from the inhibitor to the metal, forming a coordinated covalent bond. The corrosive inhibitor acts as a protective film of which the strength of the adsorption bond depends on the electron density, the donor atom of the functional group and also on the polarizability of the group. The organic substances belonging to this group contain mainly oxygen, sulfur, nitrogen atoms, and multiple bonds in the molecules that facilitate the adsorption on the metal. Thiadiazole and its derivatives are non-toxic and therefore may emerge as suitable corrosion inhibitor candidates. The planarity and pairs of free electrons in heteroatoms are important in the adsorption of thiadiazole to metal surface.

Selection of appropriate metallic materials for use in different environments against MIC is very important. Microbiologically influenced corrosion does not involve new corrosion mechanisms. Hence, the resistance of stainless steel to this corrosion type increases with increasing content of the alloying elements, which are beneficial for resistance pitting corrosion and crevice corrosion.

The standard austenitic steels of the ASTM 304L and 316L types are therefore susceptible to both types of MIC. Thus, the use of steel that are regarded as immune to MIC in seawater, such as hyper-duplex, super-duplex and high-alloy austenitic stainless steel grades are important [136]. The use of very highly alloyed super austenitic stainless steels such as 904L (08904), 254SMO(S31254), and 1925HMO(N908925) for such corrosion resistance in seawater has been reported but the AL6XN (NO83677) alloy tube was susceptible [137].

Bacterial have the propensity to form biofilm on any material from stainless steel to glass, plastic or rubber [138, 139]. Irrespective of material in question, the physico-chemiccal environment will dictate the degree and sustainability of biofilm on the material [139]. In their study, Egwari et al observed that rubber and plastic coupons attracted bacterial aggregation more compared to glass coupons, though no reason was adduced for this occurrence. However, it was evident from their study that nutrient and the presence of inhibiting compounds in the aqueous medium were the overriding factors in bacterial adherence to surfaces. It therefore follows that an environment hostile to microbial growth and proliferation will mitigate biofilm formation.

Many studies have explored this fact in considering natural organic compounds as possible candidates to prevent biofilm formation on different surfaces. Considerable attention is focused on discovering antibiofilm compounds from plants since these compounds are less toxic and thus environmentally friendly. A large pool of organic compounds has been identified and characterized and evaluated

for antimicrobial, antibiofilm and antifouling activities. In a very recent study, the mitigating activity of *Salvia officinalis* extract against *Pseudomonas aeruginosa* MIC of 304L stainless steel (SS) was reported [138]. *P. aeruginosa* was found to speed up MIC of 304L SS while S. officinalis extract produced 97.5 ±1.5 % inhibition of MIC by P. aeruginosa. The authors were able to demonstrate from electrochemical data obtained and the HPLC-Q-TOF-MS characterization of the extract active antimicrobial and antibiofilm compounds which resulted in the inhibition of further development of biofilms already formed and prevention of the formation of new biofilms on the metal surface. The authors attributed the prevention of MIC on 304L SS to the adsorption on the metal surface some of the active compounds in the *S. officinalis* extract. This protective coating prevented *P. aeruginosa* from forming biofilms on the 304L SS surface. In another study, Ru Jia et al described the mechanisms of biofilm-induced corrosion and mitigation pathway as is briefly stated below in collaborating other earlier reports in this field: first, biofilms cause MIC and biofouling and that MIC may occur via extracellular electron transfer in order to sequester energy from the environment (cathodic depolarization); that some microorganisms secrete corrosive metabolites that lead to MIC (examples of these metabolites include sulfuric acid from *Thiobacillus* spp., sulfide ions by anaerobic bacteria mostly sulfurreducing bacteria, volatile phosphorus compounds by SRB that produce black precipitate in the medium, Fe(OH)2, FeS, oxygen depletion and hydrogen ion removal; use of biocides and biocide enhancers for MIC mitigation and finally embracing emerging technologies for prevention of MIC and these include bacteriophage technology, quorum sensing inhibitors, and assessing biocide efficacy through application of electrochemical methods [139-143].

Plant Extracts and Organic Compounds with Antibiofilm Properties. A comprehensive review of natural products with antibiofilm activity was done by Song *et al* and here highlights are given. Song grouped natural products into seven categories and these are alkaloids, polyphenols, terpenes, essential oils, sugar alcohols, other chemicals and plant extracts [144].

Alkaloids

Alkaloids are present in high concentration in many parts of higher plants and have been demonstrated to possess anti-cancer, anti-microbial or anti-virus activities. Berberine inhibited the growth of oral pathogens such as *F. nucleatum* at 31.25 µg/mL, *P. intermedia* at 3.8 mg/mL and *E. faecalis* at 0.5 mg/mL, but demonstrated poor antibiofilm activity against multispecies cultures, but had demonstrable biofilm inhibiting property against *E. faecalis* at 2 mg/mL when combined with 1% chlorhexidine [145]. Another study has shown that berberine could significantly prevent the formation of *S. epidermidis* biofilm at the concentration 30 µg/mL. The possible mechanism is that berberine may bind to amyloid proteins associated with EPS within *S. epidermidis* biofilms [146]. Reserpine predominant in the plant genus *Rauwolfia*, was found in one study to inhibit at 0.0156 mg/mL *Klebsiella pneumoniae* biofilm, which was 64-fold lower than its minimum inhibitory concentration [147]. Zhao *et al*. reported that tetrandrine inhibited *Candida albicans* biofilm formation by breaking down hyphal structure through the Ras1p-cAMP-PKA pathway which plays an important role in promoting hyphal growth [148]. The antibiofilm activity of cinchona alkaloids against *Staphylococcus aureus* was reported by Skogman [149].

Polyphenols

Polyphenols are secondary metabolites in plants with good antimicrobial activities especially against oral pathogens and have potential for inhibiting plaque formation on tooth surface. Sato et al. isolated from *Artocarpus heterophyllus*, artocarpin and artocarpesin with inhibitory activities against *S. mutans* and other plaque-forming streptococci at concentrations ranging from 3.13-12.5 µg/mL [150]. Other polyphenols with antimicrobial activity and potential as antibiofilm agents include embelin (from *E. ribes*), Isopanduratin A, (from the rhizome of *Kaempferia pandurata*) [151, 152]. Prabu *et al*. found that guaijaverin, a flavonoid isolated from the leaves of Psidium guajava Linn., had the ability to prevent the adherence of *S. mutans* to smooth surfaces with 83.7% inhibition against CLSM 001 at 500 µg/mL, and naringin, a flavonoid widely found in grapefruit, had inhibitory effect on viable *S. mutans* at 20 µg/mL within 1 min [153]. A recent study has shown that cyanidin can inhibit the quorum signaling pathway of *K. pneumoniae*, a species capable of causing severe infections. The possible mechanism is that cyanidin is capable of competing with the signaling molecule for LasR receptor protein binding thereby interrupting QS regulation. LasR is a key QS signal receptor which is involved in the transcriptional activation of several pathogenic factors. Thus, cyanidin is potentially a lead compound for therapy of infections caused by *K. pneumoniae* [154].

Terpenes

Terpenes are hydrocarbons found in microorganisms, plants, and animals with proven antimicrobial and antibiofilm activities mostly against oral pathogens of the *Streptococcus mutans* group, *Actinomycetes actinomycetocomitans* and *Porpyromonas gingivalis*. Among the well characterized and studied terpenes are bakuchiol obtained from the seeds of *P. corylifolia* Linn, *ent*-rosane diterpenoids and labdane diterpene obtained from the whole plant *Sagittaria sagittifolia* [155]. The bactericidal activities of Sagittine A, B, C and D against *S. mutans* and *Actinomyces naeslundii* was established at 62.5 to 125 µg/mL while Sagittine E only had an inhibitory effect on the growth of *A. naeslundii* at 62.5 µg/mL [156]. Xanthorrhizol, isolated from the rhizome of C*urcuma xanthorrhiza* Roxb., in addition to its broad-spectrum antimicrobial activity was found to destroy up to 76% of *S. mutans* biofilm in the presence of chlorhexidine gluconate [157].

Essential oils

Takarada *et al.* found that essential oils from manuka, tea tree, eucalyptus, lavandula, and romarinus could significantly inhibit the adherence of *S. mutans* (>50%). Tea tree oil and manuka oil displayed obvious inhibitory effects on the adherence of *P. gingivalis,* and most of these oils were capable of interfering with adhesion and primary biofilm formation of *S. mutans* and *P. gingivalis* [158]. The essential oils from *Artemisia lavandulaefolia*, containing 10 main oil compounds, have considerable inhibitory effects on 15 strains of oral anaerobic bacteria [159, 160].

Other natural chemicals

Srivastava *et al*. demonstrated that colostrum hexasaccharide (CHS) from mare colostrum could interfere with bacterial quorum sensing as it relates to staphylococcal pathogenicity [161]. The mechanism is that CHS can degrade acylhomoserine lactones (AHLs), which can bind to the receptor LasR and trigger the production of violacein. The activities of several QS-regulated

virulence factors such as toxins, proteases and lipase have been demonstrated to be inhibited by CHS at a concentration of 5 mg/mL [160]. A compendium of plants extracts with antimicrobial and antibiofilm properties are detailed in the work of Song *et al* for further reference [144]. The body of knowledge available and emerging line of research on organic compounds especially from higher plants species is of great promise in considering them as emerging control strategies for biofilm control and also drug discovery research and development.

Methods for Evaluation of Anti-Biofilm Compounds

The importance of biofilms in infectious disease development and persistence, corrosion of metals and other materials have necessitated continuous research into methods for monitoring biofilm formation, quantification of biomass on surfaces and assessing the effects of antibiofilm compounds. Though there exist presently a number of methods; each method is limited in scope as they individually target different components of biofilm structure and component, for example, total biofilm biomass, on surface, viability biomass, toxicity to biofilm, cost implication and EPS quantification [144]. According to Song *et al*, none of the available methods could simultaneously measure the effect of compounds on viability, biomass and the EPS layer and recommended that the new direction should be the development of new quantitative assessment methods that incorporate total biomass detection, viability of biomass and effect on EPS layer [162]. Traditional methods can be classified as static or flow; where the flow cell systems afford the biofilm continuous supply of nutrients but has the disadvantage of high cost that makes application at large scale impracticable [144]. Both static and flow system use turbidity as measurement index and cannot quantify biofilm nor determine biofilm dispersion rate.

The microtitre plate methods that use dyes have become widely acceptable as they are more amenable to routine investigations and research studies. One of the common stains that has been extensively used is crystal violet (CV) as an indicator of total attached biofilm biomass [163-165]. CV staining measures total biomass, but not the viable biomass. Thus, CV is considered to be useful for monitoring removal of biofilm [166]. To overcome the limitation of dyes that can only measure biomass, the use of viability markers such as resazurin has been employed, which allows drug screening assays to be efficiently performed on different strains. The resazurin-based assay offers a simple, rapid, non-laborious and sensitive measurement for the viability of microbes. Living bacteria metabolize resazurin into a fluorescent product named resorufin. The application of the resazurin metabolism assay has been widely used for the evaluation of compounds on biofilms [167].

Other viability probes have also been used to determine general biofilm viability: 2, 3-bis-(2methoxy-4-nitro-5-sulfophenyl)-2htetrazolium-5-carboxanilide (XTT), fluorescein diacetate (FDA) and fluorogenic dye SYTO 9 (LIVE/DEAD). After a comprehensive evaluation of multiple microtiter plate assays for quantification of microbial biofilms, it was concluded that the resazurin and FDA assays were most favorable [168]. Both XTT and SYTO 9 assays are widely exploited approaches for measuring biofilm viability but they are expensive [168]. Resazurin is the most commonly used and cost-effective reagent because it has no toxicity towards the biofilms. Because biofilms are surrounded by self-produced EPS, Pitts et al. introduced methods of using fluorescent dyes to measure the quantity of EPS [166]. However, currently there are no existing methods that can simultaneously measure the effects of a compound on viability, biomass and the EPS layer. Therefore, there is a need to develop new quantitative assessment methods with those features. Since resazurin has the advantage of being non cytotoxic, its use allows the bacteria to also be used for CV staining for measurement of biomass [168].

Conclusion

Biofilm will remain a subject of interest for a very long time as it is shedding new light on the pathogenesis of infectious diseases. Biofilms have defined the strength inherent in communal existence especially where it is made up of heterogeneous species. An increasing understanding of the interplay of all the components that make up a biofilm will be a major breakthrough in the development of drugs and preventive measures against diseases associated with biofilms. It is evident that many drugs and therapeutic regimens are targeted to counter the complex nature of cells that constitute a biofilm in addition to the use of protective coating devices that inhibit biofilm formation. What however should be borne in mind is the propensity of microbial life to easily modify their lifestyle thereby evading whatever measures are in place against their existence. It is therefore of utmost importance that the various therapeutic introductions to fight against biofilm formation and diseases be monitored and evaluated continually for efficacy and possible emergence of resistance mechanisms.

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Citation: *Effiok Warrie William, Egwari Louis Osayenum, Olasehinde Grace Iyabo, Akinnola Olayemi Oluseun, and Kilani Adetunji Musbau (2021). Factors Associated with Biofilm Persistence on Different Surfaces, Spread and Pathogenicity. Journal of Medical & Clinical Research 6(1):330-343.*

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