Review Article

Microbiology Section

# Biofilms
## A Challenge To Medical Science

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## ABSTRACT
Biofilms are a group of microbes which are encased in an exopolysaccharide matrix on both biotic and abiotic surfaces. Various changes occur during their transition from a planktonic to a surface attached community. This causes a number of persistent infections which respond poorly to conventional antibiotic therapy. This review summarizes the nature, prevalence, detection and the treatment of biofilm associated infections.


**Key Words :** Biofilms, Indwelling medical devices, Tissue culture plates, Multi drug resistant

## INTRODUCTION
The first recorded observation concerning biofilms was probably given by Henrici in 1933, who observed that water bacteria were not free floating, but that they grew on submerged surfaces.[1]
A biofilm is a sessile community of microorganisms which are attached to an interface or to each other and are embedded in an exopolysaccharide matrix. It manifests an altered growth rate and transcribes genes that free floating organisms do not transcribe. [2] After adherence to a surface, these microorganisms adapt to the environment of the biofilm by increasing the secretion of exopolysaccharide. This helps the microorganisms to escape their killing by antibiotics.[1]

## THE MECHANISM OF BIOFILM FORMATION
There are five steps of biofilm formation on medical devices.[3]
In steps 1 and 2, the identification and association with a surface is followed by strong adhesion. The time taken by this is 1 to 2 hours post – implantation. These reversible, non specific cellular associations occur through long and short range forces, e.g., van der Waal's forces, gravitational forces, hydrogen bonds, hydrophobic interactions, etc.

In steps 3 and 4, microbial cells aggregate to form micro colonies. Thereafter, further growth and maturation of the biofilm takes place in the next 2 – 3 hours. Specific chemical reactions between the compound of the microbial cells and the substrate surfaces result in strong adhesion and irreversible molecular bridging. The biofilm which is formed can be of a flat or mushroom shape, which depends upon the nutrient source. Microbial polysaccharide and adhesin proteins promote the attachment of organisms to the substrate surfaces.

In step 5, sloughing of the biofilm into small pieces occurs and these pieces move transiently to form daughter cells. The daughter cells which are thus formed, travel down through the blood stream to various new attachment sites.

Transitions to a sessile state of bacteria occur in response to the limitation of essential nutrients. Biofilm formation is commonly regulated by inter and intraspecies quorumsensing mechanisms. Availability of nutrients, chemotaxis towards the surface, the motility of bacteria, surface adhesion and the presence of surfactants, influence biofilm formation in microorganisms.[4]

By using bacillus subtilis, Dr Stanley Wall has shown that a protein called Deg U regulates biofilm formation.[4]

Certain surface proteins, extracellular proteins, capsular polysaccharides and adhesins PS/A and autolysin (encoded by the atl E gene) regulate biofilm production. The ica genes also code for PS/A and intracellular adhesion.[5]

Biofilms may be formed by one or several types of microorganisms. Studies on polymicrobial biofilms which are formed by Candida albicans and Staphylococcus epidermidis indicate that biofilms which are produced, may protect the fungus from antifungal action.[6]

Many researchers have shown that the regulation of ica operon and the formation of biofilms depend upon various environmental factors like anaerobicity, carbon dioxide level and glucose and osmotic levels. Sodium chloride is a known activator of ica operon transcription. Some workers have found that sodium chloride mostly induced biofilm formation among methicillin sensitive staphylococcus aureus. They also found that biofilm production among methicillin resistant staphylococcus aureus was mainly glucose induced.[7]

There was a variable adherence of the microorganisms to the polystyrene surfaces in vitro. This may be due to the variation in different strains and due to the expression of the genes which are responsible for biofilm production. Another hypothesis is that, in the inserted catheter under in vivo conditions, several host proteins coat the catheter surface. The microorganisms lodge to the coat by using multiple receptors.[7],[8]. The various factors such as surface area, the type of surface (rough/smooth), porosity, charge on the surface and surface hydrophobicity play a role in the formation of the biofilm. A rough surface is more favourable for the colonization of bacteria. The hydrophobicity in polymeric materials increases biofilm formation. Microorganisms get attached easily on porous surfaces. Electrostatic interactions cause biofilm cohesion. Cations contribute to the cross linking of the biofilm matrix.[3]

The extent of initial adhesion depends upon the adherence property of the receptacle, the duration and the number of bacteria coming in contact with the test surfaces and the fluid turbulence of the test media.[9]

Exopolysaccharides are formed under selective pressure and are controlled by diffusible chemical signals (quorum sensing) of the cells within the biofilm. So, the biofilm is not homogeneous. [3],[11].

A study showed that the addition of pheromones to biofilm forming Enterococcus faecalis yielded a high amount of biofilm formation.[12]
Another group of workers demonstrated a strong association between the biofilms which were produced by the clinical isolates of Acinetobacter baumanni and multiple drug resistance. The presence of extended spectrum beta lactamases (bla PER1) is likely to facilitate cell adherence.[13]

The prevalence and the expression of F-like conjugative pili, adherence fimbri and curly, which are known to promote biofilm formation in Escherichia coli K12, cannot totally account for the increased biofilm formation of non domesticated Escherichia coli in vitro.[14]

## BIOFILM ASSOCIATED INFECTIONS AND THEIR IMPLICATIONS IN HEALTH CARE

According to a recent public announcement from the National Institutes of Health, more than 60% of all the infections are caused by biofilms.[15] As described by Prasanna et al, about 40-50% of adults had biofilm related gingival infections. Among 4000 infants with cerebrospinal- fluid shunts, 15-20% had biofilm related infections. 95% of the urinary tract infections were associated with urinary catheters. 86% pneumonias were associated with mechanical ventilation and 85% of the blood stream infections were closely related to intravascular devices.[3]

## THE SUGGESTED ROLES OF THE BIOFILMS IN PRODUCING INFECTIONS ARE –

a. Detachment of the cells – the cells may get detached from the biofilm. This may cause blood stream and urinary tract infections.[16]
b. Resistance to the host immune system– Biofilm coated bacteria escape the damaging effect of the antibodies produced by the infected host cells.[17]
c. Production of endotoxins – Gram negative bacteria which are encased in biofilms, produce endotoxin.[18]
d. The generation of resistant organisms – Bacteria can transfer plasmids by conjugation within the biofilm. So, resistance factors may be exchanged through a plasmid.[19]

Two types of biofilm associated infections can occur –
1. Foreign body infections
2. Native tissue infections

Foreign body infections – These are more commonly associated with the colonization of microbes on indwelling medical devices (IMD). IMDs may cause the haematogenous spread of infections throughout life if the devices are in place.

For surgical IMDs, tissue damage and clot formation are associated with surgical implantation, thus causing increased microbial biofilm formation.

For non surgical IMDs, e.g. Urinary catheters, colonization may occur from skin or through or around catheters, once they are implanted.
Native tissue infections – Some biofilm related infections involve no foreign bodies eg. Urinary tract infections by uropathogenic Escherichia coli, cystic fibrosis by Pseudomonas aeruginosa, native valve endocarditis by streptococcus viridians, etc.[1],[2]

## MECHANISMS OF ANTIMICROBIAL RESISTANCE OF BIOFILMS

Microbial biofilms have been associated with a variety of persistent infections which respond poorly to conventional antibiotic therapy. This also helps in the spread of antibiotic resistant traits in nosocomial pathogens by increasing mutation rates and by the exchange of genes which are responsible for antibiotic resistance. Antibiotic therapy against device associated biofilm organisms often fails without the removal of the infected implant. An elevated expression of the efflux pump is another mechanism for the development of antibiotic resistance in biofilm bacteria. The specific up regulation of genes which encode antibiotic transporters, has been seen in biofilms which are formed by Pseudomonas aeruginosa, Escherichia coli and Candida albicans. Physiological heterogeneity is another important characteristic which is observed in biofilm bacteria. This phenomenon affects the rate of growth and metabolism of the bacteria and is reflected by interbacterial quorum signals, the accumulation of toxic products and the change in the local micro environment. These so called persister cells are not resistant to antibiotics per se, but become resistant when associated with the biofilm.[9]

The overall healthcare mechanisms of the underlying antimicrobial resistance of biofilms are:

1. Trapping of antibiotics in the exopolysaccharide matrix – The exo polysaccharide slime causes a diffusion barrier by restricting the rate of molecule transport to the interior of the biofilm, or by chemically reacting with the molecules themselves. The exopolysaccharide which is negatively charged, restricts the penetration of the positively charged molecules of antibiotics by chemical interactions or by molecular binding. This also dilutes the concentration of the antibiotics before they reach to the individual bacterial cells in the biofilm, thus making the antibiotics less effective against microorganisms.[1], [2]
2. Bacteria which are coated with biofilms escape the host immune system – Biofilm bacteria escape the damaging effect of the antibodies which are produced by the host immune system in response to infections.[16]
3. Quorum sensing and genotyping adaptations alter the metabolism and decrease the growth rate of bacteria- A cell to cell communication in bacterial biofilms is established through chemical signaling. Small, diffusible molecules of class of N – acylated homoserine lactones (AHLs) are liberated by biofilm bacteria into their surrounding environment. These AHLs are associated with DNA binding proteins. As the amount of AHLsreaches a threshold level, it induces the transcription of specific genes throughout the population. The regulation of this type is known as quorum sensing (Requirement of a specific population of bacteria that is nessesary for the activation of the AHL – responsive genes). The cells lying deep within the biofilm have less metabolic activity and growth rates. This makes the biofilm organisms inherently less susceptible to antibiotics. Due to the consumption of oxygen and glucose, relative anaerobiasis is created at the deeper layers of the biofilm, where in order to survive, the microorganisms transform into slow growers and non growers. Older biofilms are relatively more resistant than newer biofilms.[3]

   After the attachment to a biotic or an abiotic surface, the bacteria undergo further adaptation, i.e, increased synthesis of exopolysaccharide and increased antibiotic resistance. They also develop an increased resistance to UV light, increased genetic exchange, altered metabolism and increased secondary metabolic production.[1], [2]

## THE DETECTION OF BIOFILM PRODUCING MICROORGANISMS

Early biofilm formation detection might result in a greater success in the treatment, because in long standing cases, they may be very damaging and may produce immune complex sequelae.[2]

There are two methods for the detection of biofilms –

1. The Phenotypic method

a. The tissue culture plate (TCP) method – The wells of the tissue culture plates are inoculated with a bacterial suspension along with positive and negative controls and these are incubated for 24 to 48 hours. Planktonic cells are removed by washing with phosphate buffered saline. Biofilms are fixed with 2% sodium acetate and are stained with 0.1% crystal violet. The excess dye is washed away with deionised water. The plates are dried properly and the optical densities of the stained biofilms are obtained spectrophotometrically.

b. The tube method(TM) – 10 ml of Tripticase soy broth with 1% glucose is inoculated with a loopful of test organisms, along with positive and negative controls. The broths are incubated at for 24 – 48 hours. The culture supernatants are decanted and the tubes are washed with phosphate buffered saline. The tubes are dried and are stained with 0.1% crystal violet. The excess stain is washed away with deionised water. The tubes are dried in an inverted position.

c. The Congo red agar (CRA) method – The Congo red stain is prepared as a concentrated aqueous solution and is autoclaved at 1210c for 15 minutes. This is added to autoclaved Brain heart infusion agar with sucrose at 550c. The plates are inoculated with the test organisms along with positive and negative controls and are incubated at 370C for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicate biofilm production.

Various studies have established that TCP is a better screening test for biofilm production than the TM and the CRA methods. The test is easy to perform and to assess biofilms, both qualitatively and quantitatively. [20],[21].

2. The Genotypic method

Sonications and PCR amplification methods have been shown to improve the detection of biofilms. Biofilm non producers are negative for ica A and ica D and lack the entire ica ADBC operon. But this requires specialized equipments and techniques.[22],[23]

## PROPHYLAXIS AGAINST BIOFILM FORMATION

This includes systemic perioperative and local antibiotic prophylaxis. The aim of the local antibiotic prophylaxis is to inhibit the colonization of microorganisms on devices and the contamination of the surrounding tissues. Antimicrobials can be applied locally in various forms, such as: -

1. Device coating – Devices are coated with antibiotics or quorum sensing inhibitors, which are either covalently bound to the device, or are locally eluted from it. Device coatings are of two types – active and passive. Passive coating such as ethylene glycol, poly ethylene oxide and hydrophilic poly urethane can be used. The effectiveness of passive coating is limited. In active coating, the release of anti microbial agents in high fluxes occur to inhibit the initial adhesion of bacteria.[3], [9], [24], [25]
2. Device immersion – The dipping of the device in antimicrobial solution, e.g., rifampicin dipped vascular graft.[25]
3. Surgical site irrigation – Skin antisepsis and the antimicrobial irrigation of the surgical field.[25]
4. Antibiotic loaded cements – The use of antibiotic loaded bone cements (usually in joint arthroplasties) provides the local delivery of antibiotics, the stabilization of soft tissues, scope for an easier re implantation and better clinical outcome.[26]
5. Antibiotic lock therapy – The catheter lumen is filled with the concentrated antibiotic solution and is then “locked” into place for an extended period, when not in use. This is done to prevent the colonization by bacteria.[9]
6. Antimicrobial carrier – Antimicrobials can be added onto a carrier either preoperatively or during surgery. Biodegradable (polylactide or polygalactide) or non biodegradable (poly methyl methacrylate) polymers which are impregnated with antimicrobials are used in orthopaedic prostheses. The resulting effect of the antimicrobials persist for weeks to months.[25]

Antibiotic prophylaxis is controversial, but is increasingly common in the high risk patient group.[9]

## TREATMENT

The common treatment against persistent infections which are produced by biofilm producers is the removal of an infected IMD, combined with antibiotic/antifungal therapy.

In case of IMD in non surgical patients, long term antibiotic therapy is required.[24], [27], [28]

## EXPERIMENTAL THERAPY

1. The in vitro use of ultrasound electric fields to enhance the penetration of antibiotics through microbial biofilms – Devices, which emit low energy surface acoustic waves, electric currents, or pulsed ultrasound reduce the colonization of the devices and enhance the release of locally applied antibiotics.[3], [9]
2. Proteolytic enzyme treatment, e.g, alginate lysate in case of the polysaccharide biofilm of Pseudomonas aeruginosa.[1]
3. The evaluation of newer antibiotics and microbicide immersion practices. [3]
4. The disruption of signaling molecules (acylhomoserine lactones) acting as quorum sensing systems. These are involved in the biofilm architecture and detachment, e.g. palulin and penicillin acid, which are secondary metabolic products of the Penicillium species, act as quorum sensing inhibitors.[29]
5. Inhibition of biofilms by small molecules – Designing small molecules which can prevent biofilm formation at some specific point. Amino imidazole, triazole and tether units together form a conjugate which can disperse bacterial biofilms without causing bacterial death. Short carbon chain molecules (decanol, decanoic acid and dodecanol) can inhibit and disrupt biofilms in a concentration dependent manner.[3], [30]
6. In the future, the treatment that inhibits the transcription of the biofilm regulatory genes might be able to completely inhibit biofilms. Identifying the virulent factor and genes which cause biofilm formation, can help in preventing the colonization of the microorganisms.[1], [9]
7. Use of sensors – The inhibition of biofilms after their complete formation is difficult because of the presence of ‘persister’cells. Sensors which can detect biofilm formation as early as possible, are a great help for the treating clinicians. Research is going on to make several types of sensors for biofilm monitoring, such as bacterial touch sensors and electro chemical sensors (non bacterial sensors). Overexpression of VPsS, a hybrid sensor kinase, enhances biofilm formation in Vibrio cholerae (bacterial sensor).[31]
8. Chitosan, a polymer which is isolated from the crustacean exoskeleton, inhibits candidal biofilm formation in vivo. It damages the fungal cells. Therefore, it can be considered for the

prevention or the treatment of the fungal biofilms of the central venous catheters and other medical devices.[32]

## CONCLUSION

Many biofilm infections develop slowly, producing very few symptoms initially, but in the long run, they may produce immune complex sequelae and may act as reservoirs of infection.[2]

Standard, in vitro antibiotic susceptibility tests are not predictive of the therapeutic outcome of biofilm associated infections. [28] The overall healthcare costs which are attributed to the treatment of biofilm associated infections are much higher due to their persistence. Besides, a longer hospital stay is another factor for higher costs. Early detection of biofilm associated infections and newer treatment options for the management of the same are needed.

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