

BIOFILM IN DIALYSIS WATER SYSTEMS

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1. What is a biofilm?

Basically, a biofilm is formed when a structure or a surface has a growth on it of a community of active microorganisms. The growth means that there is cell metabolism going on. At start, the number of organisms in the biofilm is low and the surface area covered by the biofilm is small. This will change over time, depending on the nutritional situation.

In this paper the phenomena of surface growth (biofilm) will be reviewed and commented from different aspects.

2. Where do you find biofilm?

Surface growth will be present in many situations. In nature we find microbial biofilm in the interface between water and solid surfaces. In living organisms we find microbial biofilm for example on epithelial cells and on teeth. In human beings microbial biofilm may be found on implants. In many industrial applications a microbial biofilm is found in various water systems.

This paper takes the water systems of dialysis clinics as examples.

3. Why do microorganisms prefer surfaces?

When water systems show a very low content of nutrients available in the free-floating fluid phase, the amount of nutrients on the surfaces can be hundred times higher. Many nutrients attach to surfaces, forming physical-chemical bonds. For example many larger molecules show electrical charges, positive or negative, in different areas of the molecule. Surfaces also have electrical charges, differing from one microenvironment to another, attracting nutrients.

Microorganisms want to be where the food is found. Consequently, microorganisms will try with all means to come to a surface and settle down. Not only that, they want to remain on the surface to fulfill the basic role that nature has given to any specie, that is to multiply.

4. How can microorganisms attach to a surface?

There are numerous mechanisms and strategies in the microbial world how to solve the problem to settle down on a surface and remain there.

On a sub molecular level there are different chemical binding types, like hydrogen bonds, van der Waals forces, ionic, and covalent binding. All these types of binding mechanisms may be involved when a sub molecular structure bind to a surface.

On a molecular level the polysaccharides and proteins of the outer cell envelop, especially the slime capsule, are of importance. Hydrophobic and hydrophilic properties are important for interactions.

On a structure level the proteins and polysaccharides can be organized in thin threads and other forms to facilitate the attachment to a surface.

On cellular level special structures like stalks, prosthecas and the cell shape enable the cells to attach more effectively to surfaces. Also flagella can be of assistance.

On an organism level the organism can use principles like chemotaxis and motility in order to find a suitable environment.

On a community level there might be signal substances for inter-cell communication that facilitate the finding of a suitable environment.

All of these strategies will be used in different combinations depending upon the individual specie. Some combinations are more effective in a certain type of environment and will consequently be

dominating in that environment while other combinations are more favorable in other environments. This is an area where the multiplicity of nature shows up.

5. Does the surface material matter?

The properties of a surface are much in discussion, and from time to time materials appears that are said to be more or less free from biofilm formation. So far one must say that this is not true. Biofilm will occur on all materials. It is just a question of time and type of organism. Material properties like surface roughness and surface properties (hydrophobic or hydrophilic) may facilitate colonization.

In an experiment to investigate attachment to different materials with a *Flavobacterium* sp as test organism, isolated from a water system, the following was found. The material with the lowest microbial attachment was Teflon, followed by cross-linked polyethylene (PEX), in turn followed by glass and stainless steel that showed the highest degree of attachment. It can be noted that most polymer materials, like PEX and PVDF, have smooth surfaces as they are extruded, while metal surfaces mostly show traces of the manufacturing tools.

6. What nutrients do the organisms in the biofilm use?

All organisms require energy for whatever activity. Nutrients of all kinds can offer energy and building material for microorganism, if only they have the necessary tools, that is enzymes, to use the existing possibilities.

When this is the case the nutrients can be used, resulting in metabolism and growth. This will go on until the available nutrients are consumed or until some essential growth factor is lacking. The growth will in most cases be rather slow, with long generation times compared to what we are used to find in pure test tubes cultures and optimal conditions. A natural community always balances on the borderline to starvation as available types of nutrients may run out.

When the primary nutrient is consumed, the microorganisms investigate if they can form the enzymes necessary to metabolize what other nutrients that may be in the environment. Such a procedure we can note as a nutritional shift when we study a culture with more than one nutrient present. For example, when glucose is present it is used first as almost all organisms have systems to metabolize glucose. When glucose is used up the culture stops to increase in number. The culture may now prepare itself to use another nutrient, for example galactose. When the enzymatic systems are prepared and operating, growth starts again and we see a new raise in the growth curve. The same procedure may occur for a third, a fourth, and etcetera source of nutrient.

In a surface growth this procedure is going on continuously in very small communities.

7. What characterizes surface growth?

The behavior of microorganisms in a community of a biofilm is different to that of planktonic (free floating) organisms. The growth is different as they are more close to each other. Resistance to disinfectants differs as the community can organize more protection than the single cell.

Proof that inter-cell communication occurs is such a simple thing as the surface of a colony on an agar plate. Some species do not only form a round colony with a smooth slimy surface. Instead they show colony morphology with a pattern of rings or radial lines. The pattern occurs as a result of the colony growth. The colony is the result of multiplication and co-operation of about one billion individual cells. Moreover, other colonies of the same strain under the same cultivation conditions gives exactly the same colony morphology. How does the single cell know how the colony morphology is to be and that it can be repeated?

8. What characterizes a biofilm?

The properties of the individual biofilm are totally dependent upon the species participating. This means that it is controlled by the hygiene, i.e. what microorganisms enter the system.

The strength of attachment, the mode of attachment, the water solubility of the slime capsule will be of importance for the occurrence of organisms coming off the surface and found in the environment.

When the biofilm lives the cells metabolize and produce different kinds of molecules. The metabolites of a culture in an exponential growth phase are normally simple compounds like low-molecular weight fatty acids. These are primary metabolites.

When a culture is in a stationary growth phase more complex metabolites are formed. These are secondary metabolites. Examples are pigments, antibiotics and extra-cellular toxins. The amounts of metabolites formed are totally determined by the nutritional situation. In principle, the cells make only the most essential processes. Only when an excess of nutrients is available, larger amounts of metabolites are formed.

On the other hand microorganisms are small. The dry weight of 10^9 cells of a typical Gram-negative bacteria cultivated in a laboratory is around 0.32 mg. A cell in the natural environment is much smaller. A cell grown in the laboratory may have a length of 3 μm and a diameter of 1 μm , which gives a volume of 2.4 μm^3 . A cell from a natural nutrition-poor environment may be 0.5 μm in length and 0.2 μm in diameter. This gives a volume of 0.016 μm^3 , which is 150 times smaller. The weight is of course smaller too. This means that in principle there will always be enough nutrients present for slow growth in all environments, as no environment has a cleanliness of this degree.

9. How many microorganisms can be in the biofilm?

What are the potential of a biofilm in a water system? The following example can give an idea. In a water system it is determined, by microscopy, that the cell density on the inner surface is 1 cell per 10 μm^2 , i.e. 0.1 cells per μm^2 . Assume that the system has a piping length of 100 meter, which gives a total inner surface area of 50 000 cm^2 . As 1 cm^2 equals $10^8 \mu\text{m}^2$ there will then be $0.1 \times 10^8 \times 50\,000 = 500\,000\,000\,000$ cells in the system. These will not be detected by a water analysis. For this system the water analysis gave 100 CFU/ml. As the water system had a total volume of 32 liters, the total number of CFUs can be calculated to 3 200 000. When the two figures are compared it is seen that a water analysis may underestimate the microbiological quality of the complete system with a factor of 100 000.

Assume that all cells of 1 cm^2 surface come off the surface simultaneously, are distributed evenly and measured. The result would be $0.1 \times 10^8 / 32\,000 = 312$ cells/ml, which is above most alert and action limits.

10. How can you test for biofilm?

In most cases when a water system for purified water is installed, a validation work starts in order to find out how the system is to be maintained and disinfected. Typically, water samples in large numbers are taken and analyzed for their content of cultivable microorganisms. From these findings decisions are made.

There is one problem with this approach. The inner surfaces of the system are not sent to the laboratory. So even if just a few organisms are seen in the fluid samples there may be growth on the inner surfaces of the system, with low amounts of material coming off the surface. Moreover, what comes off the surface is normally 'pieces of growth' that can contain everything from a couple of cells to many thousands. This will normally just form one colony-forming unit. So with traditional water sampling there is a low likelihood to reveal the real situation.

Instead some kind of surface sampling should be made. The simplest way is to use a cotton swab or have special test pieces in the system that can be taken out for analysis.

In all analysis one must also consider using the appropriate cultivation technique. For instance, if a system contains sodium bicarbonate or sodium chloride, the cultivation media should be adapted to this, otherwise nothing may be found. This is valid for fluid as well as surface investigations.

But, actually, acting on the results of analyses is not the appropriate principle to maintain a water system. Instead one should initiate a proactive disinfection program from the very beginning where method and frequency is based on microbiological knowledge.

11. What organisms are found in biofilm?

Not only bacteria can be found in biofilm but also fungi, yeast and even protozoas. When fungi growth occurs, the hyphae of the fungi act as steel reinforcement bars in reinforced concrete. Many water

born fungi are very difficult to become sporulating in the laboratory and hence our knowledge about them is limited.

An observation made is that when yellow colored colonies, resembling the genus *Flavobacterium* and *Caulobacter*, are present in biofilm they are almost not to be detected in the water unless the sample volume is large, 100 ml or more.

12. Can you remove biofilm by disinfection?

When a biofilm already exists, it is hard to eradicate. Chemical disinfectants have difficulties to diffuse into the biofilm and only the top layers are damaged. For chlorine disinfectants a loss of more than 80% of the active substance was recorded under the surface in a biofilm. Long exposure times must be used in order to be successful with chemical disinfection. Heat is the best method (if the materials can stand it) as all organisms are too small to chill down their environment but must adopt the temperature applied. Decisive for the success is the frequency of disinfection - and to be proactive!

13. Can you prevent the formation of biofilm?

In some dialysis water systems it is suggested that no biofilm will develop due to high flow rates and a turbulent flow. This is likely not true. What is forgotten is that in the zone closest to the surface, where the nutrients are, there is another physical phenomenon - simple friction. In this zone the fluid velocity is low even if the flow rate in the center of the piping is high. (Compare with the flow of a river.) The issue of materials is further discussed in item 5.

The fact is that biofilm cannot be prevented by any other mean than *proactive disinfection*.

Conclusive remarks

Surface growth is something that normally develops quite slowly and is hard to detect. Due to this fact many technical water systems are judged to be in far better condition than they really are.

Once a biofilm is formed it is difficult to remove. Often polysaccharides are part of the film and these are difficult to destroy. Even if a disinfection procedure will be quite effective, the growth is soon restored, as the inactivated microflora will serve as nutrients for the successors. That means that there might be two microflora present, one old that is inactivated and one new that is actively growing.

The biofilm serves as a reservoir not only of microorganisms but also for endotoxin. After disinfection the endotoxin content may increase. This increase is not caused, as many believe, from the fact that there are many dead bacteria. The endotoxin content is about the same regardless if the cells are alive or dead as endotoxin is more to be considered a structural part of the cell.

The first organisms to settle down on a surface must be very tolerant to harsh conditions in order to survive. Successors have it more easily and then there is a higher risk of having more demanding organisms present.

Microbiological analyses of water samples will never be able to give a correct picture of the reality in a water system for purified water of the reasons given above.

The only way to handle the problem of surface growth in water systems is to apply the principle of proactive disinfection. This means disinfection by suitable method and with such a high frequency that microbial growth will not occur.

Sampling and analyses do not improve the quality, nor give the truth, but is only costly and cumbersome. The quality is assured by maintenance actions i.e. disinfection. Use the resources in a way that they give the best benefit.

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