Biofilm evaluation methods outside body to inside - Problem presentations for the future -

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Abstract:

Biofilms are formed at interfaces between solid materials/environments or organisms' tissues/ environments by bacterial activities. They are produced not only inside bodies, but also outside them. Inside bodies, the biofilm formation would lead to infection and chronic diseases, while it would lead to stickiness on various industrial materials followed by daily problems. However, the phenomena in both cases have essentially the same and common root. Therefore, it would be very informative for us to compare both cases to each other, when one would like to understand the mechanism, characteristics and to establish countermeasures. We authors have pursued the biofilm formation and growth in the case of problems outside body so far. Generally, the in-vitro biofilm research and evaluation should be composed of biofilm formations and the following quantitative measurements. And recently, we have gradually applied the concepts, methodology and principles to the research for biofilms formed inside the body, modifying them little by little. This paper will explain the modification process with many real successful and unsuccessful examples and propose the unsolved problems together with the history. Then we would like to give the reference guideline to design experimental processes for biofilm problems inside the body.

Keywords: Biofilms, Laboratory Biofilms Reactor, micro fouling, crystal violet

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1. Introduction

Biofilms [1-8] can be defined as a microorganism group in film-like polymeric substances. They are not only the aggregate of bacteria, but are also embedded into exopolymeric substances composed of polysaccharides, proteins, lipids and nucleic acids. Water is the dominant component and occupies more than 80% of all constituents. The cross sectional schematic figure is shown in Fig. 1.

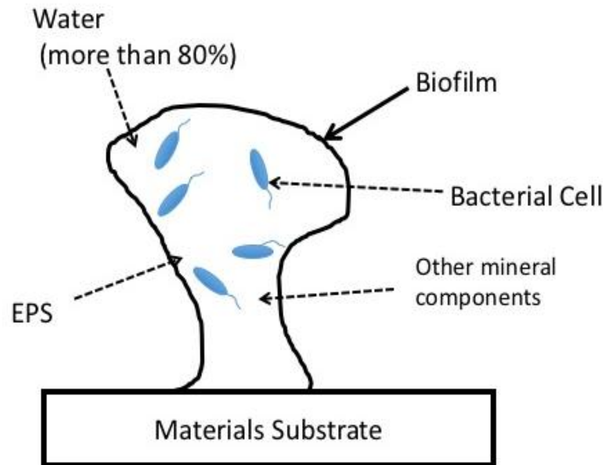

Fig.1 Schematic illustration of biofilms formed on materials.

Biofilms can be formed at the interface between solid-liquid, liquid-liquid and gas-solid interfaces. When we focus on substrates for biofilms' formation, two possibilities can be mentioned mainly. It is shown in Fig. 2 schematically – biofilms on animated solids (materials) and organisms' tissues.

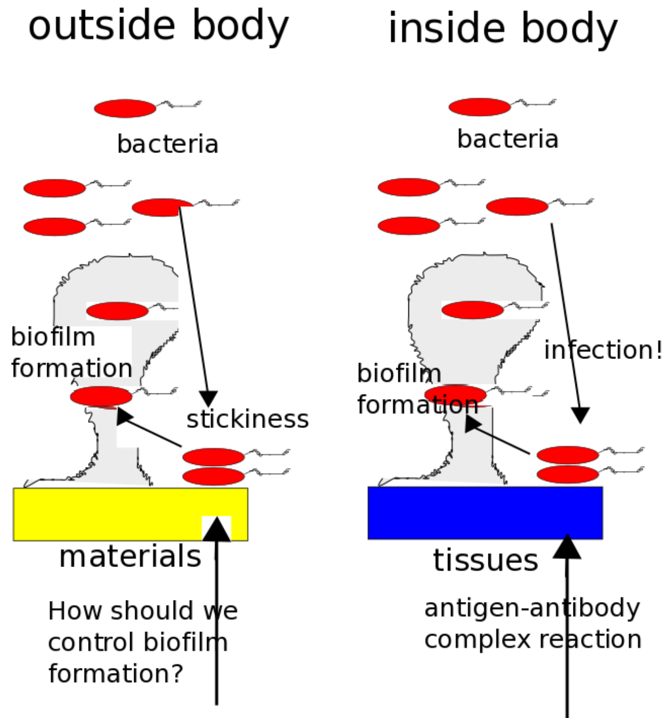

Figure 2. Biofilms' formation outside and inside body.

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Both are compatible to each other. Planktonic bacteria in oligotrophic environments always seek for nutrients and attach to interfaces, since carbon compounds as nutrient exist on them. Then bacteria attach to interfaces and begin to grow proliferously. When the number of attached bacteria at the interface reaches the threshold value, quorum sensing would occur and bacteria begin to excrete polysaccharides outside their bacterial cells.

When the phenomenon occurs inside bodies, it is called infection. On the other hand, the same phenomenon occurs outside the body, leading to the formation of stickiness. Both cases bring about many troublesome problems (and sometimes beneficial events). We started our biofilm research to solve various problems outside bodies and have devised various evaluation methods for biofilms' formation. And we are gradually going to move into medical science and to develop proper evaluation methods for biomaterials and medical instruments such as implants, catheters, stents etc. which will be used in the medical scene. Since both phenomena outside and inside the body are very similar and sometimes quite the same from the viewpoint of biofilms. Therefore, the knowledge, information and "tricks" could be applied to a large extent. When both would be compared to each other, the results for both must be very informative. Therefore, we introduced our successful evaluation methods for research and development of antifouling materials and tried to show how they could be applied to a case inside the body in this paper.

2. Evaluation methods for biofilms

Biofilm evaluation should mainly be composed of two steps. One of them is the process to produce biofilms on materials' surfaces. And it should be followed by the identification/quantification processes as shown in Fig. 3 schematically.

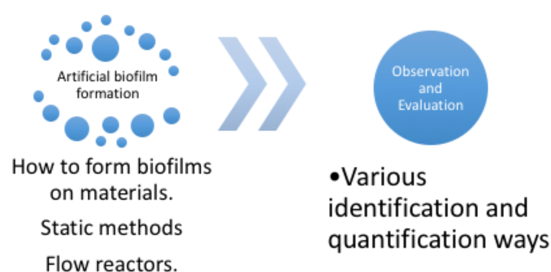

Figure 3. The concept for evaluation of biofilms in laboratories.

As for the first step, some ASTM standards have already been fixed. E2562[1] fixes biofilm formation methods and the quantification using a continuous biofilm reactor (CDC biofilm reactor). The quantification is based on the colony counting. The target bacteria are mainly *Pseudomonas aeruginosa*. E2871 [2] is the standard for biofilm evaluation for antiseptic substances. It uses a CDC biofilm reactor too. E2196 [3] is the standard for biofilm formation methods and quantification ways using rotation-type reactors. E2647 [4] is also a standard for biofilm formation and

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quantification ways. It utilizes drip-flow biofilm reactors. E2799 [5] is an evaluation standard for antiseptic substances using MBEC assay. For all of these ASTM standards, the target bacteria are *Pseudomonas aeruginosa.* The extent of biofilm formation is evaluated by the count of viable bacteria. Therefore, the series of standards focus on the biofilm formation method rather than on the type of evaluation. However, the biofilm evaluation for biofilms and biofouling should be composed of two steps – how to produce biofilms (naturally or artificially) and how to identify them quantitatively, as shown in Fig. 3. Both steps should carry the same weight and correlate with each other. For these standards, the quantification of biofilms is done only by the counting of bacteria.

There are two weak points for the standards. One of them is their model bacteria used for their evaluation methods. Actually, *Psuedomonas aeruginosa* is generally easy to form biofilms. And in addition, the diseases such as cystic fibrosis in lungs have been issued in European countries very often. However, they are not always primary causative organisms in other organs and other areas/countries. Since *P.aeruginosa* is a Gram-negative bacterium, should we check the biofilm formation behavior by one of Gram-positive bacteria? The second problem is the quantification process. The number of bacteria is very important. However, we have experienced that the number did not always correspond to the extent of biofilm formation, since the concept of aggregation and firm attachment were missing. Fig. 4 shows the situation schematically. Lots of Bacteria may exist on a material surface. However, they may be homogeneously distributed on the surface. In such a case, EPS are not detected sometimes. On the other hand, inhomogeneous distribution of bacteria would be related to EPS on materials very often (Fig. 4-(2)). For the former, bacteria might be just put on materials without any firm attachment. Such a phenomenon may occur, when bacteria might exist not on materials, but in the surrounding environment. In such a case, the number of bacteria would be doubtful.

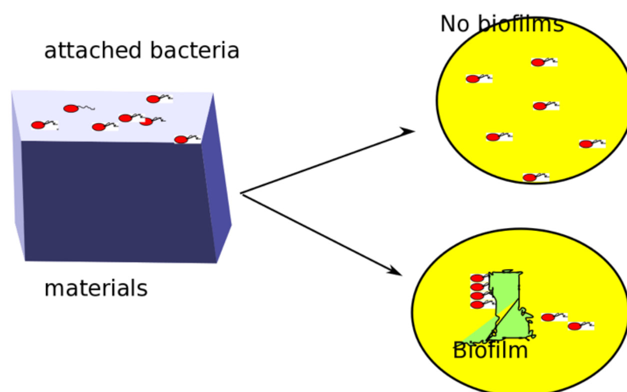

Figure 4. The distribution and the number of bacteria for biofilm formation.

Various types of biofilm reactors on laboratory scales have been devised so far [14-19]. They could be classified into two main categories, static methods and flow ones. For the former, preculture bacterial solutions fill containers with specimens. Or the wall of the container might be the specimen itself. In a certain time, biofilms are produced on specimens or walls. For such a purpose, various well plates could be

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used. In the case of specimens, they are put at the bottom or stood against walls of wells. However, the flow and the following shear force, the important factor, is missing for the method, even though the thick biofilms in tens of micrometers could be obtained in those static methods.

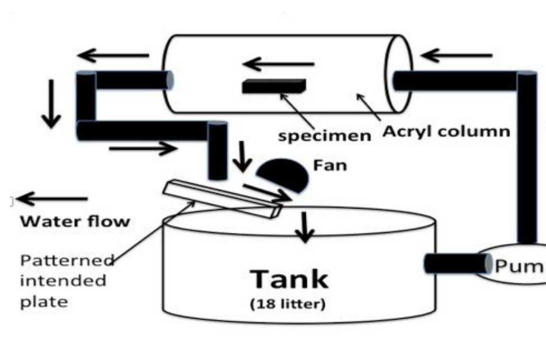
The methods in the second category introduce flow in various ways. In those cases, biofilms produced on materials are generally thinner than those in static methods (several micrometers in many cases). After the formation process, even bacteria could not be observed by microscopes. They might be flown away with the streaming of liquids. The cause for the difference of biofilm qualities might be attributed to the difference of constitution of biofilms (e.g. the ratio of polysaccharides to proteins in EPS etc.).

3. Our evaluation processes proposed from the viewpoint of materials science

3.1. Laboratory biofilm reactors to produce biofilms artificially

As described before, the in-vitro evaluation process is composed of two main parts – artificial production of biofilms by laboratory biofilm reactors (LBRs) and the quantitative evaluation for biofilm formation on materials. We started our biofilm research with the laboratory biofilm reactor with residential biota. Fig. 5 shows the laboratory biofilm reactor using residential biota [6] [7] [8] [9] [10] [11] which we have used to evaluate biofilms on various materials.

(1) the appearance



(2) schematic illustration

Figure 5. The laboratory biofilm reactor with environmental biota

The LBR is composed of two main parts – the tank at the bottom and the acrylic cylindrical column (length: 440mm, inner diameter: 40mm) where specimens are put inside in parallel with the flowing direction of the liquid. The tank is filled with liquid (20L – 40L) to mimic the environments. For example, you could use sea water or discharged water if you wish. Usually, we use tap water as the liquid. The circulated liquid flows inside of the system and flows out and down onto the intermediate plate where the environmental air is mixed with residential biota. In this way, germs in the environment enter the LBR system constantly. The liquid is circulated by the pump (an electrodynamic pump) at 6L/min for a certain time period such as one week, one month, etc. After the immersion for a certain period, specimens are taken out of the system and the biofilm formation behavior is evaluated in various ways.

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Generally, it is easier to form biofilms with residential biota as compared with certain bacteria. Therefore, residential biota must contain some kinds of bacteria that make it easier for biofilms to survive. In this case, biofilm could form on materials' surfaces easily, so that one could differentiate the biofilm formation tendency among materials. However, it is almost impossible to determine which kind of bacteria would form biofilm primarily and mainly, since residential biota are composed of diversified bacteria and the bacteria in biofilms are generally viable but non-culturable ones (VNBC) [12]. On the other hand, a certain kind of bacteria (cultivated continuously in laboratories) often lose their characteristics to form biofilms. Therefore, this method can be used as a screening test to tell the relative difference among specimens.

As mentioned above already, the apparatus was the starting point for us. One may feel from the viewpoint of biology and medical science that the apparatus might be a little bit insufficient or "rough-mannered". However, it was in fact a reasonable and "lucky" one for us, since pretty many new antifouling materials or coating were produced in industrial fields, using this apparatus. The part of intermittent plate in this apparatus actually corresponds to the incubator for the biological system. The incorporated bacteria flora must be composed of many kinds of bacteria. Bacteria in real environments (residential biota) are generally the best to form biofilms, since they have developed and kept the capability to form biofilms in oligotrophic environments. Therefore, this apparatus has brought us a good chance to analyze biofilm constantly. The demerit is that we cannot determine which bacteria could form biofilms primarily. A certain (unknown) bacterium could surely form biofilms first of all and other secondary bacteria would enter the biofilm to build and grow more biofilms.

The other weak point for this apparatus is that the environmental bacteria flora (residential flora) might change from the site to the site where the evaluation process would be carried out. Therefore, this apparatus should be used to compare specimens with positive and negative controls. And the results should be also compared with those in real environments by in-vivo experiments.

When we came to think about the application to the cases inside the body, the apparatus had to be modified, even though the apparatus could solve the problems and questions for the development of biomaterials first of all. The biofilm problem inside the body is usually the simpler flora rather than that outside body. And from the pathogenic viewpoint, one or two bacteria would be often responsible for the research purpose in many cases, even though the patient might suffer from other pathogens at the final stage of his/her life. For example, E-coli is the most responsible at the beginning stage for urinary systems, and the flora generally changes with time to be dominated by *P. aeruginosa*. Therefore, the LBR should be modified for biofilm research from the viewpoint of medical science, so that a single kind of bacteria would exist in the LBR system. The situation often needs the whole LBR system to be sterilized. The problem might lead to difficulties and often put some limitations on the design of the system.

For example, Fig. 6 shows another laboratory biofilm reactor we used with a certain kind of exiting bacteria [13]. The LBR in Fig. 6 is similar to that in Fig. 5 from the structural viewpoint. It is composed of two parts – a reservoir and a column. The reservoir is filled with diluted culture fluid containing a certain kind of bacteria. Specimens are placed inside a column made of polycarbonate. The pump is a peristaltic one in this case. And the components are

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connected to each other by silicone tubes. Before the test, all of these components except the pump itself are autoclaved at 121°C for 20 minutes, so that the contamination would be avoided carefully. The reservoir (250ml) has three mouths. Two of them are used as inlet and outlet for the liquid. The third one is used for moving air. The ambient air without environmental bacteria can enter and exit through a filter (the pore size is below 0.2 micrometer.), so that the system would not burst as a result of inner pressure by bacterial respiration. Since the size of the column (length: 140mm, inner diameter: 16mm) is much smaller than that in Fig. 5, the size of the system is pretty compact. Also due to the necessity for autoclave treatment, the compactness is especially needed. However, the autoclave treatment was still hard for us to carry out sometimes.

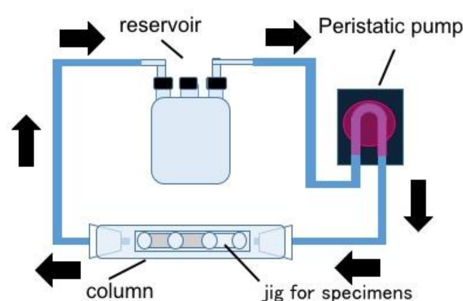

Figure 6. Circulation type LBR for a monospecific bacterium.

From the viewpoint of compactness, we developed a smaller flow tip instead of the column as shown in Fig. 7. Also in the flow tip, liquid enters through the inlet and exits from the outlet to produce flow inside like the apparatuses in Fig. 5 and 6. The specimen is put in the rectangular hollow at the bottom of the tip. Since this apparatus is small, one can carry out experiments on the table. It can also be autoclaved very easily, as long the material constituting the flow tip is available for autoclaves (acrylic and polycarbonate one). On the other hand, each tip treats only one specimen as a coupon, which might lead to ineffectiveness. However, this might be just a simple technological problem. For example, a couple of tips could be connected to a reservoir in parallel.

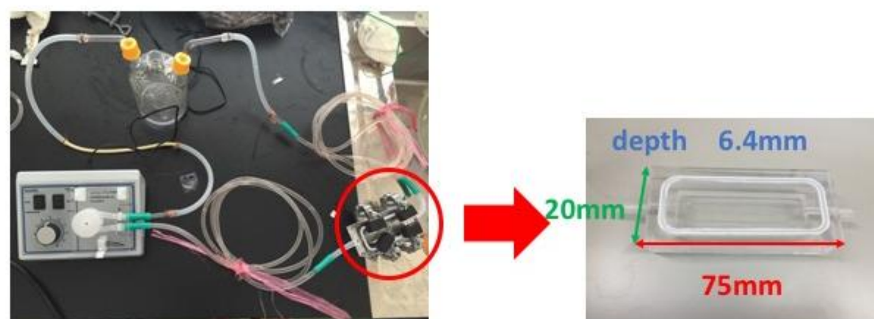

Figure 7. Flow tip type LBR

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The LBRs shown in Fig. 8 is a rotation type LBR. In the circulation type LBR as shown in Fig. 5, 6 and 7, the reservoir for incubation stays aloof from the column for biofilm formation geographically. Even though a pump could send the liquid with bacteria, it is sometimes hard for bacteria to constantly reach the specimens in the column. And the possibility for and risk of contamination is generally high. Originally, the reason why we devised the circulation type LBR was to provide shear stresses for bacteria by flow. From the viewpoint, the flow is the essence for biofilm formation. To utilize the advantage and also to overcome the disadvantage, we devised a new rotation type LBR shown in Fig. 8. In this apparatus, specimens are attached to the rotator hooked in the center of the LBR made of a separable flask. And the rotator with specimens is immersed in a culture solution. It means that the incubation reservoir and column in the circulation type LBR were integrated into one, so that the disadvantage of the circulation type LBR would be overcome. The rotator is driven by an external electric motor. The rotation brings specimens shear stresses depending on the rotation number. The entire separable flask could be autoclaved at 121°C for 20 minutes.

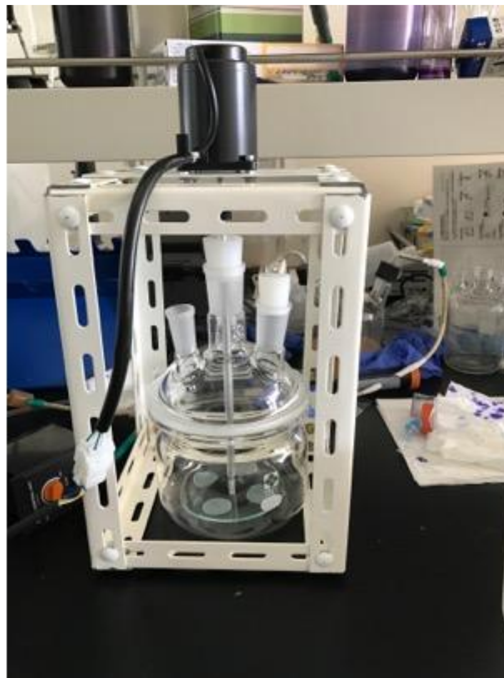

Figure 8. Rotation type LBR.

Fig. 9 is another rotation LBR where the rotator is placed vertically. Specimens are attached to its perimeter and the rotator is driven by an external electric motor moving at a certain rate. In the half cycle, specimens are exposed to the ambient air and in the other half cycle, they are immersed in the liquid where specimens could have shear stresses. This process makes it possible for specimens to form biofilms on them also by ambient germs. However, the sterilization and the experiments by a monospecific germ might be impossible [14]. The type is very different from the circulation type LBR. This might be used for the biofilm problem-solving outside the body. However, it would be the future task how to apply it to those inside the body.

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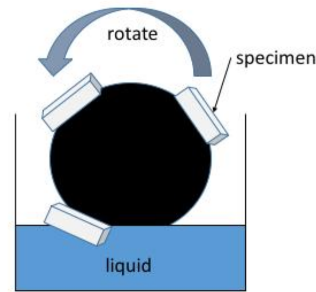
Waterwheel type Laboratory Biofilm Reactor

Specimens touch with water and air consecutively

(1) the appearance



(2) the schematic principle

Figure 9. Waterwheel-type LBR

The LBR where one can investigate biofilm formation by a certain monospecific bacterium is very important particularly for biomaterials. This is because the target is always infection and the prevention/ treatment against it is required. For such a purpose, it is very important to avoid contamination. As already described, sterilization is very important and it has been the main point for the modifications, when we applied the LBR to the biofilm problem inside the body. The sterilization process by autoclaves has been solved so far, and we have devised many kinds of LBRs. However, the final unsolved problem will be how to sanitize/sterilize specimens themselves. For advanced materials, high temperatures and pressure might change qualities and characteristics of materials before the evaluation tests. The problem will be unsolved also in the future.

3.2. Qualitative and quantitative evaluation for biofilm formation

The quantitative measurement for biofilm formation is the key component for biofilm research. As described in section 2 and shown in Fig. 4, it has been very difficult to pursue generally due to some unavoidable reasons. Bacteriologists and biologists usually pick up the method to measure the count of bacteria, since quorum sensing needed for biofilm formation regulates the process. Even though the number of bacteria is important to estimate the extent of biofilm formation, the number of bacteria remaining on materials' surfaces after the LBR test is completed, may have no direct relation to the extent of biofilm formation in some cases. In addition to reasons mentioned in section 2, in the flow type LBR such as a loop type, etc. is sometimes hard to find the bacteria after the test, while the number of bacteria often corresponds pretty well to the extent of biofilm formation in immersion type (static) tests. From the viewpoint of materials science, the counting measure should be examined and investigated further.

As for visualization of biofilm, the confocal laser microscope has been heavily used so far. This method makes it possible for us to visualize biofilms in three dimensions. It provides us with lots of useful information about biofilms. In addition, various instrumental analyses using SEM-EDX [15] [16], FIB-SEM [17], AFM [18, 19], etc. have been used for biofilm investigations. Recently, the complexity system analysis such as metabolomics based on mass spectrometry appeared and has attracted attention.

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We have also investigated some instrumental analyses. For example, low vacuum SEM-EDX indirectly confirmed the existence of biofilms on a steel specimen. This is because the elements incorporated into biofilms produced inhomogeneous element distributions on materials' surfaces. Fig. 7 [20] is an example showing biofilm traits by environmental biota formed on a glass slide sample in tap water.

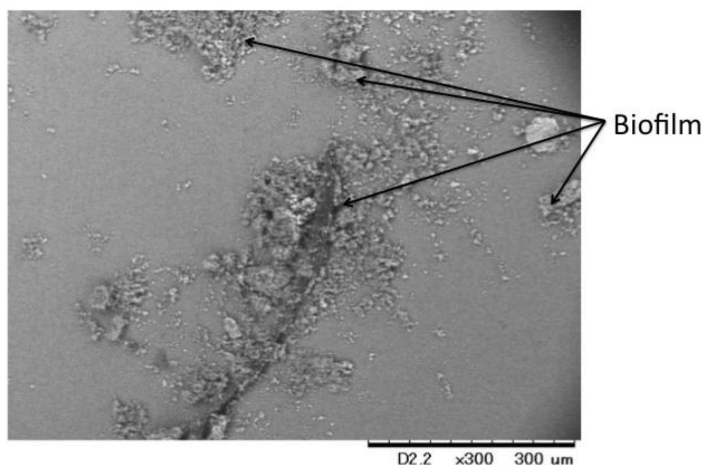

Figure 10. Biofilms formed on a glass specimen and the concentration of elements in them, observed and measured by a low vacuum SEM-EDX.

On a glass surface site without biofilm, sodium (13.6%) – magnesium (5.6%) – aluminum (1.8%) – silicon (73.2%) – calcium (5.9%) were observed. However, the composition changed with biofilm present. Several metal components were detected, including zinc, iron, magnesium, tin, etc. They were incorporated into biofilm from the water. On a site with biofilm, the composition was 7.2%Na – 6.9%Mg – 1.3%Al – 64.4%Si – 9.5%Ca – 5.8%Fe – 1.3%Zn – 3.8%Sn [20]. The observation was possible for the research using the apparatus with environmental biota in Fig. 5.

However, one would have a serious problem to "observe" biofilms, using SEM-EDX and other high vacuum analyses. Under the vacuum chamber of those analytic apparatuses, biofilms and bacteria themselves lose their original shapes and one would observe only the traits, as mentioned above. At this point, we utilize the phenomena that biofilms generally incorporate many organic and inorganic matters from environments. Therefore, the concentration difference would be produced on materials' surfaces. Then, we could observe the trait for biofilms. However, it is more desirable for us to get the morphological information for biofilms.

To solve the serious problem, we now have two alternatives. One of them is the freeze dehydration process. The process is shown in Fig. 11 schematically. Since biofilms contain lots of water (more than 80% constituents are water!), the water has to be substituted with ethanol, first of all. To achieve the purpose, specimens are filled in the mixed solutions of water and ethyl alcohol for a certain time (for example, 15 minutes each), so that the mixture ratio is changed gradually. Finally, the specimen is immersed into almost complete ethanol solution and then they are immersed in the mixed one of ethyl alcohol and t-butyl alcohol, so that the ethyl alcohol in biofilms is exchanged with t-butyl alcohol finally. When the biofilms are filled with t-butyl

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alcohol completely, they are frozen at -20 degrees Celsius, and the frozen biofilms are put into vacuum. Finally, we get the freeze dehydrated biofilms. They can be observed in the vacuum chamber morphologically. They can keep a certain fixed shape, when they are put in the vacuum chamber. And probably, the freeze dehydrated biofilms would contain lots of pores on their surfaces. Therefore, they are easily stained much more than non-freeze hydrated ones. However, the original shape might be lost, as shown in Fig. 11. That is still another problem remained for us.

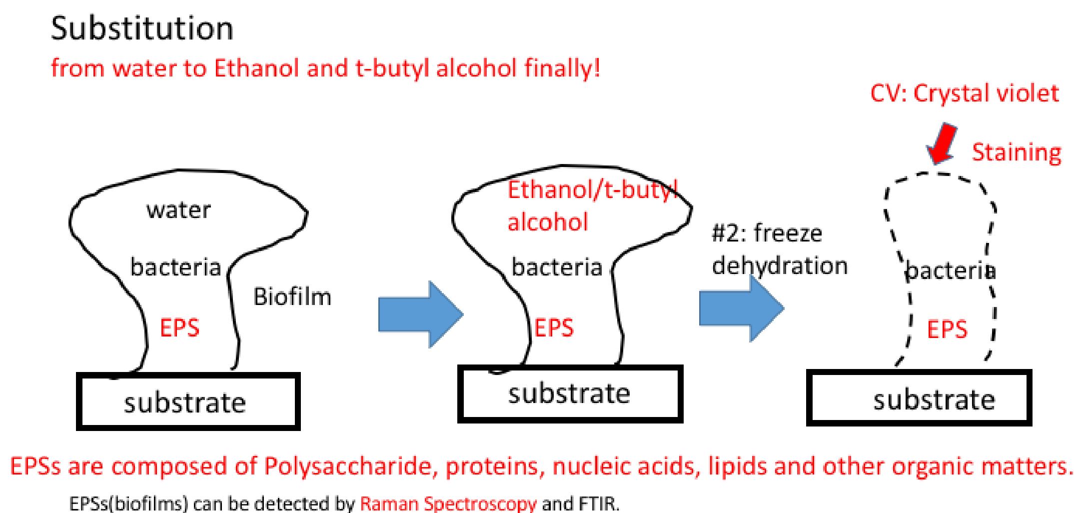

Figure 11. Freeze dehydration process of biofilms

Another alternative is the utilization of ionic liquids. This is still under investigation. However, the most serious weak point for usual freeze dehydration process may be avoided, using ionic liquids. In such a case, the original shape might be kept after the substation of water with ionic liquids, since they are stable in vacuum.

The methods we have paid attention to for direct observation using usual reflection-type optical microscopes are different from conventional ones and aim to establish the new evaluation systems, so that materials scientists and engineers could use them for their speedy R&D activities. Therefore, the simple method is favorable and from the viewpoint, optical microscopes are the best, if they would be available. Even though biologists usually use fluorescence microscopes, they are not so easy or simple for materials scientists and engineers to utilize. They are pretty complicated and too expensive sometimes, which may beat down the motivations for R&D. Therefore, we chose optical microscopes which materials scientists and engineers have very often used. Even though biofilms could be originally observed by optical microscopes as obscure contamination, they were too touchy-feely and non-scientific. Then, we paid attention to the asperity of biofilms formed on materials' surfaces. Using some optical microscopes, one can collect plural images around the focal point for each specimen put on the observation stage of the microscopes. These images shifting from the focal point could be overlapped and integrated into one and show the stereoscopic image (Depth from Defocus method [21]). Fig. 12 shows some of those images for biofilms formed on a steel specimen. Fig.12-(1) is the apparatus which we use for the purpose. When the specimen does not form biofilm so much, the

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image shows just a continuous color change (Fig.12-(2)). In this case, the image corresponds to the specimen's gradient on the observation stage. When biofilms form on a specimen on the other hand (Fig.12-(3)), each part has its own color assigned by the computer depending on the height. As shown in the figure, biofilm corresponds to the convex parts (reddish parts) and the surface with biofilms generally shows the sea and island colored structures like this one. This method makes it possible for us to screen the specimens from the viewpoint if they could form biofilms or not. This may be a pretty rough screening method. In several micrometer orders (<10 micrometers, close to the limitation of resolution), the PC installed on the microscope has the difficulty to fix the focal point and often gives us artifacts. Therefore, we would say that this method might be hard to be quantified.

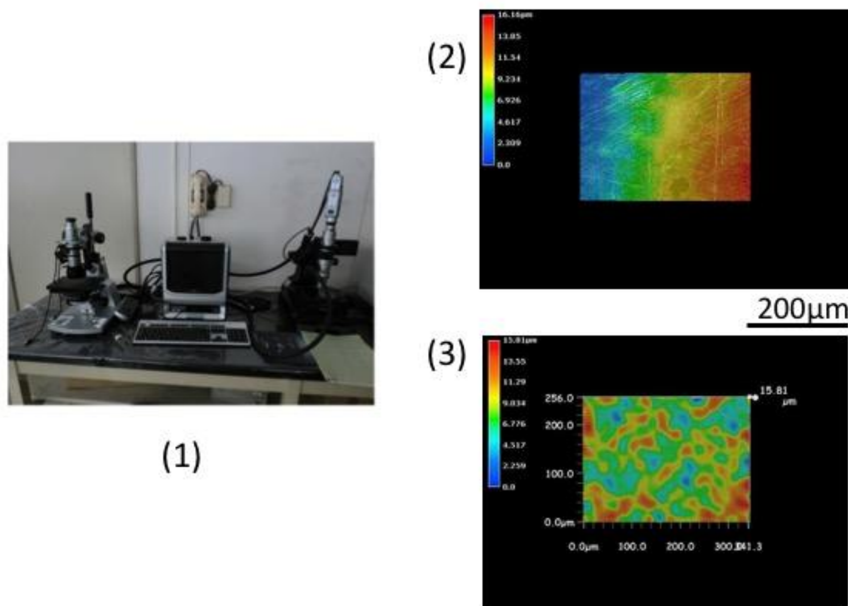

Figure 12. 3D images of biofilms by Depth from Defocus method of optical microscope
(1) Optical microscopy (Keyence VW-9000), (2) No biofilms, (3) Biofilm pattern

On the other hand, staining is a favorite method, since it is simple and ready to specimens with relatively large areas. Crystal violet is one of the prospective staining agents for biofilms. It is well-known for gram stain. However, this agent itself could stain polysaccharide and other organic polymers, even though the mechanism of staining is not clearly understood. Probably, it stains the negatively charged part of polymers. Fortunately, biofilms have complicated components generally – polysaccharide, protein, nucleic acids, lipids, other incorporated organisms from the environments - and most of them including bacteria seem to be stained by crystal violet. Therefore, this agent could be favorable for biofilm staining, since biofilms are composed of complicated and plural components.

Fig. 13 shows the schematic illustration for the staining by crystal violet [22] [23]. Commercial crystal violet powder was dissolved into distilled water and 0.1% solution was prepared. The specimens were immersed for 30 minutes and then rinsed using clean water. Commercial semi-transparent sticky tapes were attached to the

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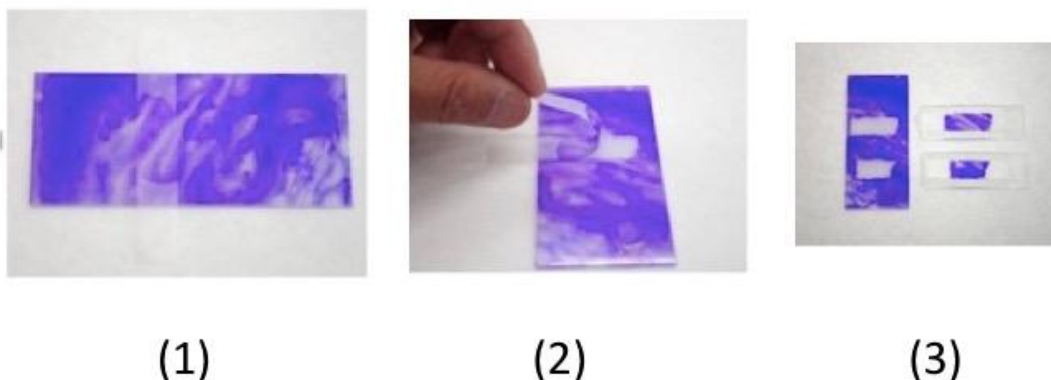
specimens for about 30 minutes. Then they were peeled off and attached to transparent glass slides. The stained parts on the specimens were copied through the process almost completely and the extent of staining on the glass was observed and evaluated using the analytical equipment. The copied crystal violet could be evaluated by absorbance/transmittance, color difference, the amount of agents attached to the polymer tape, etc. [24]



(1) (2) (3)

Figure 13. Staining of crystal violet and the copy

However, the copy of the stained crystal violet may inevitably contain errors to some extent, since the tape could not make crystal violet attach to itself, when crystal violet might penetrate into holes and pores at surfaces. In such a case, some appropriate solvents might play an important role. The dissolved crystal violet from the materials' surfaces could change the color of solutions and their absorbance or permeability could be measured to evaluate the extent of biofilm formation.

Even though the staining by crystal violet is very useful and effective for biofilm formation, it is very important to fix the stained matters if they would be components of biofilms or not. For the purpose, Raman spectroscopy and FTIR-ATR are very effective for the confirmation and identification, since EPS is composed of organic compounds. Fig. 15 shows an example of Raman shift peaks corresponding to EPS in biofilms formed on a pure iron sheet.

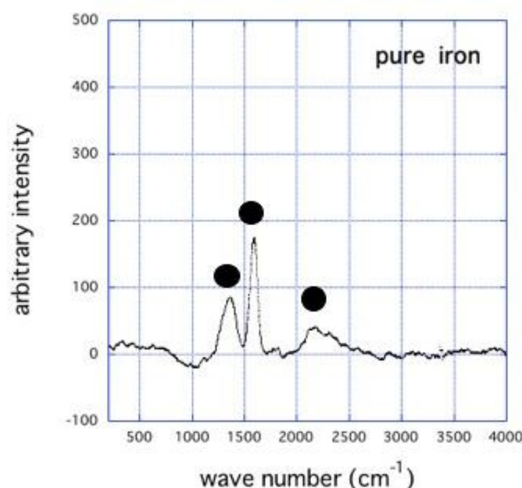


Figure 15. Raman spectroscopy for pure iron specimen after exposure in LBR.

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Three peaks at 1400cm^{-1}, 1600cm^{-1} and 2300cm^{-1}, were observed. Comparing this to the non-coated reference sample, we assume each peak corresponds to biofilm components. The peak at 1400cm^{-1} is assigned to lipids [25-27] , that at 1600cm^{-1} to Amid I [26-30] and the broad peak at 2200 cm^{-1} to DNA, RNA or Phospholipid [31]. These peaks could confirm the stained specimens formed biofilms.

Even though the Raman spectroscopy is a very powerful tool to detect EPS in biofilms, the quantification of biofilm formation on large materials' surfaces, since it is also one of the local analyses. The quantification is still now remained as unsolved problem for this analysis. The combination of crystal violet staining and the spectrographic method might be more desirable, since it would bring us qualitative and quantitative, localized and holistic information simultaneously.

4. Conclusions

We described our short research history, showing concrete examples outside the body and modification ways for problems inside the body. Both are similar and have the same principles and concepts applicable to each other. The evaluation process is basically composed of two steps, the biofilm formation using laboratory biofilm reactors (LBRs) and the following quantitative measurements. As for the former, the type of LBRs can be classified into two types, the static immersion test and flow type ones. And as for the latter, biofilms have been measured by the count of viable bacteria quantitatively so far, since it might have a reasonable meaning from the viewpoint of quorum sensing. However, more convenient and novel industrial methods oriented more to materials science and engineering are needed for the biofilm research. In this paper, we proposed some methods composed of biofilm formation and quantitative measurements, showing our short history of modification. We hope that this mini review will be helpful for readers to devise and to design experimental processes for problem solving of biofilms inside the body in the near future.

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