EVALUATING BIOFILMS ON MATERIAL SURFACES: A NEW INTERNATIONAL STANDARD

A new ISO standard could help spur development of new surface treatments and innovative products that can help defend against harmful bacteria and biofilms.

What is sometimes called slime is a soft and slippery surface coating that often forms in areas experiencing heavy water use, such as kitchens and bathrooms. This can lead to poor hygiene as well as material deterioration including corrosion. Slime is similar to biofilm, which is mainly produced by bacteria adhering to a material's surface and developing into a community of microorganisms that produce a slimy matrix in which to thrive (Fig. 1). Bacteria are everywhere. One type known as planktonic bacteria float individually in various environments as they seek a nutrient source for survival. For most bacteria, the nutritional requirement for continued growth is a carbon compound. Because material surfaces are energetically unstable, they often adsorb trace amounts of carbon compounds. Therefore, microorganisms such as bacteria try to attach themselves to material surfaces to ingest the carbon source as nutrition.

Material surfaces must cross energy barriers to connect, but so called nanowires-such as the cilia of bacteria-make this relatively easy. As the number of adherent bacteria gradually increase, a phenomenon called quorum sensing, or interbacterial communication, occurs. Next, the concentration of a signal molecule called an autoinducer (a relatively low molecular weight chemical secreted by the bacteria) increases with the higher concentration of adhesive bacteria. This signal molecule reenters the bacterial cell and through several reactions stimulates certain parts of the DNA, resulting in the discharge of polysaccharides. Thus, areas with high concentrations of adherent bacteria are covered with a thin, wet, heterogeneous film of water. This is the formation process of a biofilm.

Although early biofilms are composed of bacteria, environmental moisture, and polymers derived from bacteria, many biological studies have shown that biofilms provide a favorable environment for bacterial growth. Bacteria can share nutrients and drugs are ineffective in preventing this process. Biofilms continue to grow, but when nutrients are depleted, they collapse. Bacteria that had been slowly growing inside the biofilm now swim back into the environment as planktonic bacteria and reattach themselves to another material surface.

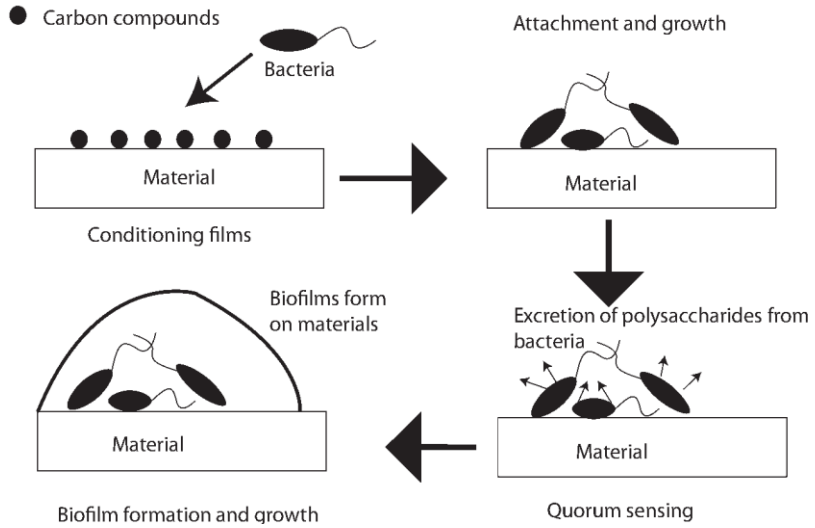

Fig. 1 — Schematic illustration of biofilm formation and growth.

This repeated process gradually spreads the biofilm over the material's surface. When a biofilm forms on the surface of a metal, for example, the metal of the substrate dissolves into the biofilm as ions. These ions react with polymer components in the biofilm, leading to corrosion and scale formation. Likewise, polymers and ceramics experience other problems, such as material degradation, deterioration of hygiene, and serving as a breeding ground for infectious diseases (Table 1).

BIOFILM EVALUATION

Destroying the biofilms that lead to material degradation is essential for materials science and engineering progress. However, effective countermeasures can only be achieved by developing new materials. To this end, creating a technology to quantitatively evaluate biofilms from an engineering perspective is necessary. Until now, evaluation methods for biofilms have relied on basic qualitative techniques.

Biofilms may be observed by the naked eye as well as touching a surface where they exist. However, their exact identity cannot be determined until various components such as water, bacteria, and extracellular polymeric substances (EPS) are known. Of these, moisture is insufficient to determine whether a suspected biofilm is indeed a true biofilm, and the presence of bacteria needs to be verified. Even if bacteria are present, they may not form biofilms. Biofilm formation cannot be confirmed until the local concentration of bacteria increases to some extent, and it becomes clear that the water is fully hydrated due to the discharge of polysaccharides. Therefore, confirmation of EPS is the most important indicator of a true biofilm.

Confocal laser microscopy, Raman spectroscopy, and Fourier transform infrared spectroscopy (FTIR) are the most commonly used instrumental analysis methods to confirm the presence of EPS. From a materials science viewpoint, microorganisms are systems in which organic substances are formed on the surface of the substrate material. Over the course of numerous research studies, biofilms have been examined using various material analyzers and expensive biological instruments. Genetic analysis and mass spectrometry are examples of biological characterization techniques. From a materials science perspective, confocal laser microscopy is a typical analysis method, as are optical microscopy, SEM-EDX, AFM, FIB-SEM, Raman spectroscopy, FTIRATR, and other instruments. These methods are very effective as they can confirm the biofilm components and may provide new insights. For this reason, these techniques are still valid and necessary. However, from a practical standpoint there is still a need for more intuitive, inexpensive, and quantitative evaluation methods.

MOVING TOWARD STANDARDIZATION

The lead author of this article, Hideyuki Kanematsu, FASM, and his colleagues at the National Institute of Technology (KOSEN) in Japan began studying biofilms to address microbial corrosion around 2007. Kanematsu was part of a small research group working on microbial corrosion issues and antimicrobial research. Around 2012, executives from The Society of International Sustaining Growth for Antimicrobial Articles (SIAA) visited Kanematsu's laboratory at Suzuka National College of Technology to discuss biofilms. During the meeting, it was agreed that a unified standard was needed with regard to this research.

Several standards related to biofilm evaluation already exist in the United States (ASTM E2196, E2562, E2647, E2871, E2799, E3151, E3161) and also in the EU (BS EN 1276, EN 1040, EN 1275, EN 13717, EN 13697, EN 1500). However, these generally address an evaluation standard for biocides and are not standards for

TABLE 1 – PROBLEMS CAUSED BY BIOFILMS

Phenomena/ Environment	Examples/Results	Materials
Corrosion	Atmospheric corrosion Marine corrosion Corrosion in oil environments Corrosion of building materials	Metallic materials (iron, steel, copper), concrete
Scale and slime	Buildup of scale and slime on cooling towers and pipes	Copper and copper alloys, hot dip galvanized steels, iron and steel, cast iron, PVC
Ships and marine structures	Biofouling (such as attachment of oysters and barnacles)	Carbon steels, polymers, stainless steels
Food processing	Decline in public health/hygiene	Stainless steels
Medical field	Infection, chronic diseases, nosocomial infection	Titanium alloys, stainless steels, hydroxyapatite
Material degradation in soil	Deterioration of soil pipes	Concrete, cast iron
Fishing	Seagrass beds and fish reefs	Concrete, metallic materials, slags
Heat exchangers	Air conditioners, washing machines, humidifiers	Various polymers
Kitchen and bathroom	Dirt around water edge, clogged sinks	Metallic materials, ceramics, polymers

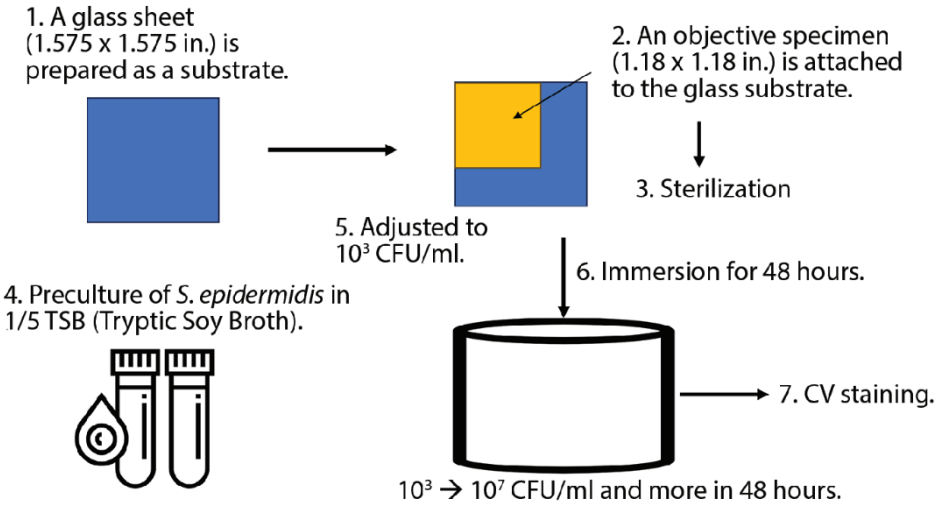

Fig. 2 — Biofilm formation according to new ISO standard, ISO 4768:2023.

materials or products. In contrast, SIAA has focused on materials used in products-and their surfaces-and has investigated biofilm formation and growth as one of the material's characteristics. SIAA organized a committee and several subcommittees to carefully study this issue, and after several preliminary votes and discussions, a new ISO standard (ISO 4768:2023) was successfully adopted by a final vote on July 18, 2023.

BIOFILM EVALUATION PROCESS

The authors determined that the most effective method for biofilm evaluation is staining, which is practical, intuitive, easy to perform, and inexpensive. Various staining procedures are possible. Biofilms are mainly composed of bacteria, EPS, and water, and many reagents have been developed to stain bacteria in biofilms. However, as discussed previously, the essence of a biofilm is EPS. Therefore, creating a staining agent that can stain EPS, bacteria, and other components of biofilms in the broadest time range is important. From this perspective, crystal violet is the most favorable. Crystal violet is a stain with a triphenyl-methane backbone, which is also used as a pH indicator and in Gram stains for dyeing bacteria. The cations with triphenyl-methane groups are ionized to chloride ions in aqueous solution. Therefore, the crystal violet is electrically attracted to polarizable polymers, where it adsorbs and develops color. Crystal violet is ideal because it can stain the entire biofilm.

Figure 2 shows the biofilm formation process according to the new standard (ISO 4768:2023) as follows: Prepare a 4-cm glass plate; place a 3-cm square specimen of the material to be tested onto the glass plate and attach it with double-sided tape; then place specimen in a polyethylene container with a dilution of 103 CFU/ml of Staphylococcus epidermidis precultured in 1/5 TSB medium.

After 48 hours of immersion, specimens are removed, rinsed with sterile water, and stained with 0.1% crystal violet for 30 minutes. The specimen is then rinsed with sterile water again, wiped with a nonwoven cloth moistened with alcohol to remove the crystal violet, and immersed in 1% sodium dodecyl sulfate to extract the purple-stained biofilm. The biofilm is then irradiated with light at 590 nm and the absorbance is measured.

The absorbance is quantitatively related to the biofilm on the material surface. However, the problem is that a higher absorbance value indicates a higher amount of biofilm. Although biofilms can be beneficial in some cases, they typically tend to be harmful to human health and negatively impact the products they attach to. Therefore, the index should be set so that the higher the value, the less likely a biofilm will form. In addition, the absolute value of absorbance varies depending on the device, production lot, and environment, and using correlation values is more practical than absolute values. For this reason, an index of anti-biofilm activity, R, was proposed in the new ISO standard, and is shown in equation (1).

$$R=\{(A_0- A_1)/A_0)x\ 100(\%)\ (1)$$

where A_0, = absorbance of control specimen; and A_1= absorbance of target specimen.
This formula allows for relative evaluation and eliminates many of the drawbacks of using absorbance values.

FUTURE CONSIDERATIONS

SIAA is actively considering the possibility of certifying products based on this new international standard, using a system that should be complete by mid-2024. In the meantime, the authors plan to introduce their ideas on using the standard from the perspectives of materials science, materials engineering, and especially materials surface engineering.

Fig. 3 — Application of ISO standard to development of new anti-biofilm materials. If the difference between samples in the anti-biofilm index is greater than 20%, this would indicate that the anti-biofilm properties are approaching the target value for the new material.

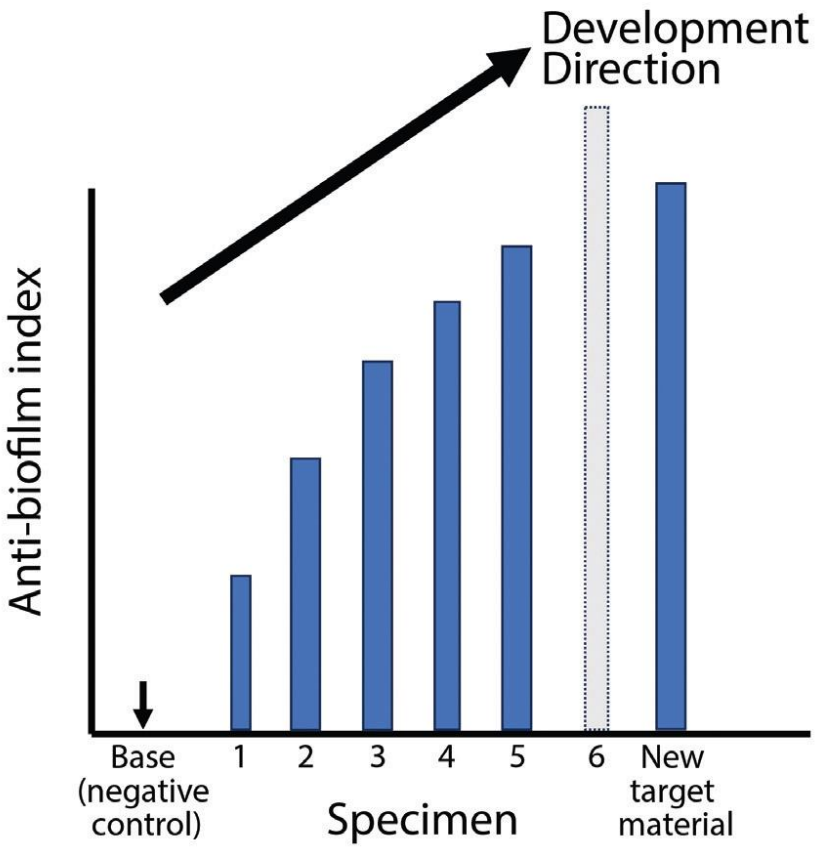

Figure 3 shows a schematic of potential antibacterial materials development. In this example, the base material represents a material that is currently in production and serves as the negative control. The R-value is zero. Suppose that the goal is to increase the anti-biofilm properties of this material through alloy addition or surface modification. By experimenting with N = 3 according to the standard, the R-value can be obtained. One guideline for development is to bring the R-value close to the value of the new target material.

The authors believe that this new ISO standard is important from a materials perspective in terms of both product development and quality assurance, and that it can serve as a guideline for related industries in the future.

For more information: Hideyuki Kanematsu, professor of materials science and engineering, National Institute of Technology (KOSEN), Suzuka College, Japan, kanemats@ suzu ka.kosen-ac.jp.

Authors: Hideyuki Kanematsu, FASM,* National Institute of Technology, Suzuka College, and Osaka University, Japan
Tomokatsu Ota, Japan Food Research Laboratories, Osaka
Naoki Nakatsugawa and Susumu Hiranuma, Society of International Sustaining Growth for Antimicrobial Articles, Tokyo
Source: ADVANCED MATERIALS & PROCESSES | JANUARY / FEBRUARY 2024