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BIO-FOULING: PREVENTION BY THE USE OF FUNCTIONALIZED MATERIALS

Key words

Biofilm, bio-fouling, quorum sensing, material functionalization, surface modification.

Abstract

Microbial biofilm formation called *bio-fouling* causes both epidemiological and technological problems by increasing material and energy demand. Due to growing microbial resistance, currently used methods of prevention have become less effective. The use of novel functionalized materials is considered a stable, non-specific, and widely effective approach. Material modification can be achieved by changing its chemical composition and surface properties such as roughness and charge. This paper presents the current state of the art focusing on the potential application in the industry and methodology for testing such novel materials. The antimicrobial activity was examined against representatives of Gram–positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria in static (plate assay) and dynamic conditions (batch culture).

Introduction

Contrary to common belief, microorganisms do not tend to exist as separate cells, suspended in a liquid environment [1]. More often, they form multicellular aggregates on interfacial layers of liquid-liquid, liquid-gas, and liquid-solid state structures called biofilms or biolayers [2]. The tendency to concentrate is a survival strategy by building a stable and secure microenvironment for the growing microbial population. For this purpose, microorganisms use many of their adaptation skills regulated by the alteration of genome activity. This is clearly visible observing differences between freely suspended (planktonic) and the 'resident' form of the same organism. This phenomenon is strongly associated with concentration of the cells in a given environment [3]. Such 'a control system of alteration' is called *quorum sensing* (QS) and relates mainly to bacteria. A microbial cell constantly produces extracellular signalling molecules, so-called “autoinductors.” Specific concentration of these molecules in the surrounding environment (depending on the species) stimulates membrane or cytoplasmic receptors that trigger the activation or suppression of specific parts of the genome [4]. The microbial cell changes its phenotype by, e.g., the loss of the ability to move and the increased production of EPS (*extracellular polymeric substances*). These substances are mainly in the form of long-chained carbohydrates, combined with proteins, nucleic acids, surfactants and lipids that form the matrix, which stabilizes the structure of biofilm, fortifies the connection with the surface, retains water, and protects cells from toxins [5]. The process of multispecies biofilm maturation involves the attachment of ‘pioneering’ cells followed by successive colonization by other, more demanding microorganisms (Fig. 1). Microbial cells growing in biofilm form microcolonies that are separated by the network of water channels that provide nutrients and collect products of metabolism. In each microcolony, a gradient of size and metabolic activity of forming cells is observed. Outer cells are large, active and dividing, whereas inner ones (due to the lack of oxygen and nutrients) are small, and show little activity or even antibiosis [6]. This makes them resistant to many antibiotics and disinfectants. Biolayers can form on glass, metals, plastics, and building materials, but also on other organisms or the interfacial phase of liquids. The roles of properties such as roughness, porosity, microdamages, surface charge, etc. are essential.

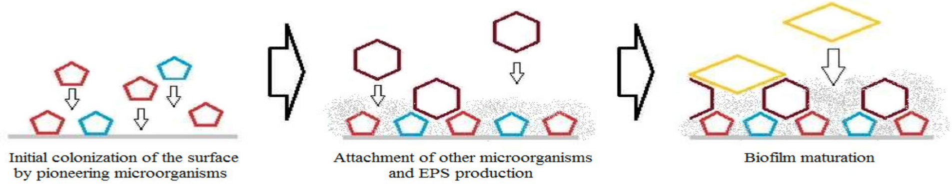

Fig. 1. Crucial stages of microbial biofilm maturation

1. Biofilm formation in industrial processes

The presence of biofilms can be considered a positive phenomenon, especially where microbial cells are intended to be immobilized biocatalysts, e.g. in the production of lactic acid in membrane bioreactors (MBR's), in the biological stage of wastewater treatment plants, biological gas purifiers, and in the bioremediation of contaminated soil [7–10]. More often, biofilms impede industrial processes, deteriorate both substrate and product properties, hinders maintaining proper hygiene of public facilities, disrupt the stability of ecosystems, damage tools and installations, and make processes more material and energy consuming. This is particularly visible in the case of drinking water distribution systems, where biofilms cause jamming and lead to biocorrosion and the perforation of components such as tubing and heat exchangers. Moreover, the presence of biolayers entails hydraulic material and hydraulic loss and epidemic risk, for example, by the presence of *Legionella pneumophila*, which is etiological factor of Legionnaires' disease [11]. Biocorrosion is not only the problem of water systems, because biolayers are also present in seaports and marine units causing frequent damage and malfunctions [12]. Material degradation by microbial activity also concerns architecture, where microorganisms contaminate, impair aesthetic qualities, and destroy buildings, monuments, etc. [13]. In fact, many of these microbes are dangerous for human health producing metabolites of allergic or even carcinogenic properties [14]. A common industrial problem is the deterioration of operational liquids such as fuels, oils, lubricants, coolants, etc. Microorganisms living in such environments deteriorate operational liquids' properties. Organic acids produced by microorganisms additionally increase the corrosiveness of lubricants, whereas the biofilm structure obstructs components of, e.g., fuel systems [15]. Bio-fouling is also a bane wherever membrane techniques are used. The specificity of membrane filtration is the concentration of some components (including potentially existing microorganisms) on one side of the system. This situation creates a favourable environment for biofilm formation due to the QS-phenomenon and the presence of porous material as a support. In the especially threatened food industry, bio-fouling concerns process disrupting (growth on surfaces and installations made of stainless steel, glass, or PTFE) and the possibility of product contamination [16]. Serious problem of implantology is pathogenic biofilm formation on implants causing opportunistic infections from residual microorganisms. What should be underlined is the fact of a growing amount of multi-drug-resistant bacteria, on which commonly used disinfectants are insufficient [17].

2. Material functionalization in order to biofilm prevention

The dense and structured form makes biofilm extremely hard to remove; therefore, complex efforts should be undertaken towards the inhibition of microbial aggregation and maintaining the number of bacterial cells at a low level [3]. In order to avoid microbial adaptation, a potentially efficient approach is the application of combined techniques, including the use of novel functionalized materials. Sophisticated features of these materials include unique surface properties and/or the presence of immobilized or released biocide.

Substances of active antimicrobial properties can be utilised in the mass of a modified material or constitute its functional coating. As an example, some metal ions or nanoparticles can are used. Silver nanoparticles, with their ability to disrupt microbial growth and metabolism are commonly used as a compound of catheters coating [18]. Other metals with such properties are gold and copper [19–20]. Inorganic agents of antimicrobial activity are also metal oxides: zinc oxide, copper oxide, and iron (III) oxide. Azam et al [21] reported the effectiveness these substances against Gram-positive and Gram-negative representatives with considerable domination of zinc oxide. Fujishima et al [22] also indicate titanium dioxide (TiO_2), which due to its photocatalytic properties also acts as an anti-corrosion agent for stainless steel type 304. In another report, McBride et al [23] used a 1% addition of triclosan in a simple releasing system. Triclosan showed nearly 100% antimicrobial effectiveness against tested microorganisms and retained its quality over a long period of time. Chitosan – polysaccharide, having remarkable properties such as non-toxicity, biocompatibility, and miscibility with many substances (including polymers), also attracts researchers' attention due to its antimicrobial potential. It is already used in food packaging systems, but new applications are tested in the textile industry and as an antimicrobial coating of medical devices. Another strategy is to immobilize specific peptides onto surface, such as Magainin I – peptide of 23 amino acids, showing a wide spectrum of non-specific properties against Gram-positive and Gram-negative bacteria but also against viruses and fungi [24]. Many experiments involve surface grafting by substances of enzymatic activity. Immobilized DNase I acting on nucleic acids present in EPS efficiently inhibits growth of *S. aureus* and *P. aeruginosa* of 95 and 99%, respectively, and remains stable over 14 hours [25]. However, this period, in some cases, is sufficient for protection of implants and medical devices. The solutions described above are only few examples of bioactive compounds present in the wealth of nature. Development in this matter – although prolonged – is essential in the consideration of constantly growing resistance to biocides and disinfectants currently used. Functionalization of materials towards the improvement of their anti-biofouling properties can also be achieved by physical treatment. Since a great number of microorganisms are characterized by negative surface charge, limitation of biofilm formation by the alteration of the electrical properties of surface is possible [26]. However, bacteria, fungi, and other microbes may produce extracellular organelles and substances to overcome this line of defence [27]. Microorganisms prefer hydrophobic surfaces. Davidson et al [28] proved fluorinated substances (including polymers) subjected to oxidation characterizes better wettability and thereby less susceptibility to biofilm formation. Moreover, the composition of a given material influences the properties of EPS and the stability of created biofilm, and this knowledge can be applied at the stage of material composition [6]. An important factor of biofilm formation is also micro-roughness of the surface and the modification of surface nanostructure should be adjusted to the actual application [29]. These considerations may potentially reduce biofilm formation and enrich the surfaces by new functionalities. Commonly used techniques for material modification are exposition to flame, a wide range of radiation, plasma treatment, chemical treatment, and the use of ion, electron or photon beams [18]. These efforts are intended to change hydrophobicity, increase crosslinking rate, boundary layer removal, and for the implantation of additional chemical groups of applied usefulness [30].

Because of the generality of the discussed subject, authors of this paper focused on a reliable and adequate method for testing bacteriostatic and bactericidal qualities of novel functionalized materials as a crucial step in their selection and deployment. The methodology of such experiments is developed in the Institute for Sustainable Technologies – National Research Institute by Team of Industrial Biotechnologies.

3. Materials and methods

Samples for testing in the form of surface-modified, capillary membranes were obtained in cooperation with the Faculty of Chemical and Process Engineering of Warsaw University of Technology[1]. These membranes, characterized by different kinds of surface modification were conventionally named P1-P4 with the control of unmodified polypropylene membranes (K). Two representative microorganisms of both Gram-positive (*Bacillus subtilis* PCM2021) and Gram-negative (*Escherichia coli* PCM2560) were used for testing. Antimicrobial properties of these samples were examined in static conditions (plate assay, LB medium, 1% agar, inoculated with 100 µl of night culture) as well as dynamic conditions (batch cultures of 8 ml of LB culture medium in 50 ml Erlenmeyer flasks, inoculated and incubated with sample materials over 24 hours). The evaluation of results obtained in batch cultures was conducted by the comparison of optical density values (OD_{550}, wavelength of 550 nm) measured at zero- and 24-hours of incubation and related to the control (K). Susceptibility to biofilm formation was estimated both by the measurement of optical density and by the number of colony forming units of liquids used for biofilm removal.

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[1] Samples used in these experiments were prepared and provided by M. Eng. Marta Bojarska (Faculty of Chemical and Process Engineering at Warsaw University of Technology) according to methodology developed during realization of her PhD thesis.

4. Results and discussion

In the plate assay, examined samples showed differential effectiveness observed as the change of medium turbidity near samples, caused by living cells (Fig. 2). Sample P1 demonstrates no considerable differences in comparison to the control sample, whereas P2 caused (especially visible in the case of *B. subtilis*) an appreciable increase of medium turbidity. The desirable properties (from anti-biofouling point of view) were demonstrated by samples P3 and P4. Almost total absence of microorganisms near this membranes on a relatively large area and the fact of the presence of little UV-radiation (strip light) suggested the possibility of creating a diffusing antimicrobial agent *in situ* by membrane coating, e.g. free radicals.

Experiments using batch cultures of microorganisms provided results comparable to those obtained in the test. Evaluation of antimicrobial properties of membranes present in culture medium was conducted by the comparison of optical density values (OD_{550}, wavelength of 550 nm) at zero- and 24-hours of incubation (Fig. 3). An Initial increase of OD_{550} in each flask confirms the hypothesis of biocide generated by a particular sample during the test and its potentially photocatalytic background. In addition, in this case, samples P3 and P4 showed the best effectiveness. These results are likely associated with partial inactivation of the inoculum, considerable inhibition of growth, and the division rate of survived cells and their constant lysis. Moreover, in the ideal case, antimicrobial properties of functionalized materials should be associated with a decrease of susceptibility to biofilm formation. For this purpose, the amount of the microorganisms attached to a given material was examined. This test involved the measurement of the optical density of the liquid used for biofilm detachment (Fig. 4). In comparison to the control sample, all of the examined samples incubated with *E. coli* indicated a decrease of cells adhered to the membrane. This fact was confirmed by the plate assays for the determination of number of colony forming units (Table 1).

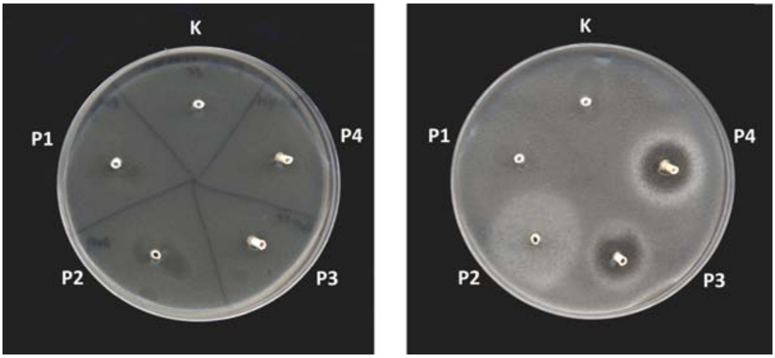

Fig. 2. Results of plate assay with use of *Escherichia coli* (left) and *Bacillus subtilis* (right)

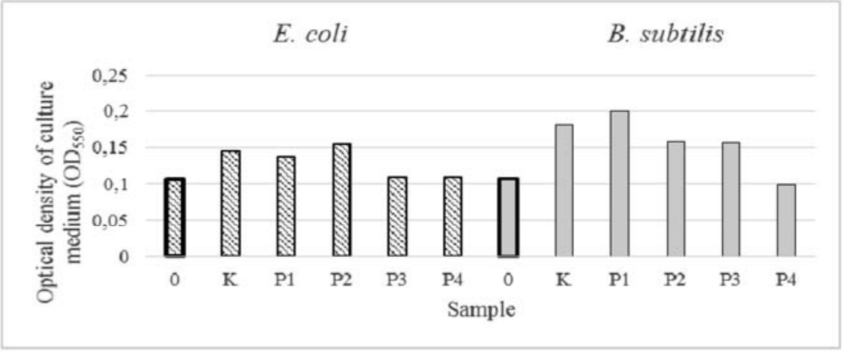

Fig. 3. Changes of optical density of cultures incubated with samples in comparison to initial value (0) and control material (K)

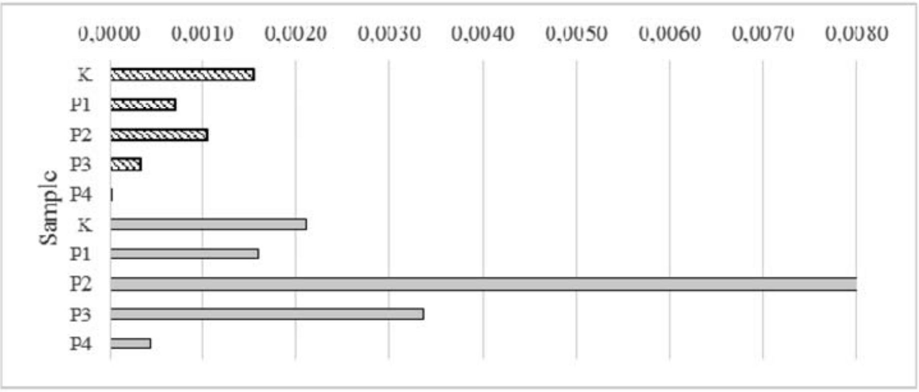

Fig. 4. Optical density of the liquids used for biofilm removal. (K) is a control sample

Table 1. Number of colony forming units of liquids used for biofilm removal

Colony forming units (n* 10^8 in 1 cm^3 of suspension)									
Escherichia coli					*Bacillus subtilis*				
K	P1	P2	P3	P4	K	P1	P2	P3	P4
12.0	–	27.6	0.12	0.004	3.4	0.81	34.8	0.13	0.03

Differences in bactericidal effect observed in comparison to *B. subtilis* cultures are likely to be caused by unequal toxicity against used microorganisms or by differences in EPS composition (species-dependent feature), determining the stability of biofilm on a given substrate. This observation emphasizes the necessity of searching for material modification of wide-ranging, universal, and effective antimicrobial qualities.

Conclusion

Material functionalization may be a powerful tool in the battle with the problem of biofilm formation and has a great applicatory potential in many industrial technologies. Current achievements in this matter indicate the need of a complex approach that is suitable for a concrete purpose. Development, production, and reliable testing methods become crucial for biofilm control in the face of the constantly growing adaptation of the microorganisms to the currently used methods.

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Problem bio-foulingu oraz możliwości przeciwdziałania temu zjawisku materiałami powierzchniowo funkcjonalizowanymi

Słowa kluczowe

Biofilm, bio-fouling, quorum sensing, funkcjonalizacja materiałów, modyfikacje powierzchniowe.

Streszczenie

Powstawanie biofilmu mikrobiologicznego na powierzchni materiału, zwane bio-foulingiem, stwarza potencjalne zagrożenie epidemiologiczne i/lub utrudnia przebieg procesów przemysłowych przez zwiększenie ich materiało- i energochłonności. Wobec rosnącej odporności drobnoustrojów na stosowane obecnie metody zapobiegawcze konieczne staje się poszukiwanie materiałów funkcjonalizowanych, zdolnych do stabilnego, niespecyficznego i efektywnego hamowania rozwoju bakterii na ich powierzchni. W niniejszym opracowaniu zaproponowano metodykę oceny właściwości przeciwdrobnoustrojowych prototypowych membran funkcjonalizowanych. Ich skuteczność określano względem reprezentatywnych szczepów *Escherichia coli* i *Bacillus subtilis.*