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Physical Approach How *P. Aeruginosa* Build Biofilms

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ABSTRACT
Treatment of bacterial infections that are accompanied by bacterial biofilm formation represents a challenge due to the increase in bacterial resistance to antibiotics. Therefore, it is essential to determine the mechanisms of the biofilm formation at initial stages, and to investigate the biophysical and chemical factors involved in this process in order to effectively fight and prevent the biofilm-associated infections. To the study of the bacterial biofilms rotational viscometric method was used and to observe bacterial growth turbidimetric method was used. The results, were analyzed according to Origin 6.1. Escherichia coli K12 and Pseudomonas Aeruginosa PAO1 strains were used in experiments. A new approach was proposed while studying the bacterial biofilms formation by using the rotational viscometric method. It is shown that the relationship between the number of P. aeruginosa bacteria, which was added to the LB broth and the time of the biofilm formation is not linear. Observing the process of bacteria multiplication in uninterrupted time regime gives opportunity to determine the mechanism of creation the biofilm. Mathematical modeling of the results reveals that the initial process of the biofilm formation consists at least two stages. From our point of view the first stage of the bacterium P. aeruginosa reproduction process (in the logarithmic stage) the bacterium synthesizes and releases into the environment a certain amount of special hydrophobic molecules that accumulate on the surface of the broth. In the second stage, the hydrophobic sites of bacterial cells interact with the hydrophobic molecules on the surface through hydrophobic bonds, forming the bacterial biofilm.
Keywords: *biofilm; biofilm surface; process of biofilm formation; turbidimeter; viscometer.*

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INTRODUCTION

The formation of biofilms by bacteria causes diseases such as cystic fibrosis [1], pulmonary infections [2], and valve endocarditis [3], as well as infections caused by the formation of biofilms on medical devices [4] and represent an important challenge in the treatment and management of the infection. The main problem in the treatment of such infections is that antibiotics cannot penetrate through the biofilms, and consequently, bacterial resistance to antibiotics is increased [5]. There are various approaches explaining the mechanisms of the biofilm formation by bacteria which begin with the adhesion of bacteria to the surface [6,7]. The formation of the biofilm consists of several stages: reversible adhesion and irreversible adhesion, the formation of the 3D structure of the biofilm, which contains extracellular polymeric substance (EPS), and finally the detachment process [8]. In addition, there are many factors, which influence the adhesion process of bacteria to the surface. In a test tube, bacteria can form biofilm only between liquid growth medium and air. It is obvious that the surface properties of bacterial membranes should play an important role in biofilm formation [9]. It is shown that bacterial flagella and pili play an important role in activating bacterial motility which stimulates the formation of the biofilms that are defined by special properties of bacterial flagella and pili [10]. For example, one of the most common types of bacteria, *Pseudomonas aeruginosa* has a special type IV pili on the surface of the outer membrane. Hydrophobic pili, due to its hydrophobic properties, determines the special orientation of the cell on the interface of broth [11,12]. Therefore, increasing the hydrophobicity of the bacterial cell surface increases

the adhesiveness of the bacteria. In the case of *P. aeruginosa,* the main role in this process was attributed to rhamnolipid. However, the hydrophobicity may be non-uniformly distributed across the membrane surface [13]. Bacterial fibrils affect biofilm formation. The presence of amyloidal fibrils, especially for *P. aeruginosa,* determines the formation of extensive biofilms on the air-medium surface [14]. It should be mentioned that amyloids may contain hydrophobic residues. Thus, hydrophobic features of the cell surface may have an important role in the initial stages of biofilm formation [15]. Over the years many different methods have been developed for the in vitro study of biofilm formation [16]. Most of these methods allow the results to be observed only after a certain period. High-resolution technological microscopes can detect the creation of the first layers of biofilms more quickly [17]. However, in order to determine the mechanisms of the initial stages of biofilm formation it is necessary to observe this process in an uninterrupted time regime. For this purpose, biophysical devices such as a rotational viscometer and turbidimeter were used. The modified viscometer is based on the rotation of a fluid-floating rotor, which floats in the center of the fluid due to surface tension forces, and changes in the surface properties affect the nature of its rotation [18], while the turbidimeter can measure the turbidity and observe any kind of cell growth in uninterrupted time regime [19].

MATERIAL AND METHODS

Escherichia coliK12 [20] and *Pseudomonas Aeruginosa PAO1* strains were used in the experiment. *E. coli K12* is nonpathogenic and *PAO1* is a human pathogen [21,22].

Viscometric method

A Zim-Croisser type viscometer, modified at the Department of Biophysics, was used. The mechanism of the modified viscometer is based on measuring the time of one rotation of the rotor, which is immersed in liquid due to surface tension forces, the rotor is placed in the center of the stator. It should be mentioned that the rotor has no mechanical contact with the stator, which allows to detection small changes in the viscosity of the solution as well as the surface tension characteristics with high sensitivity. In addition, the viscometer can operate in automatic mode, which allows observation of mechanisms of the biofilm formation and records experimental data in an uninterrupted time regime [18]. The reason for choosing this method for the biofilm study was that in the process of the biofilm formation on the broth surface, the surface tension characteristics change. As a result, rotor rotation speed also changes (decreases) (Fig. 1. b). Figure 1. a shows the working principle of the viscometer, where the rotor rotates inside the stator with the help of a rotating magnetic field that acts on a bronze plate that is placed inside the rotor. The workspace of the viscometer, between the stator and the rotor, is filled with the standard bacterial LB broth. By using a thermostat (Fig. 1. b) the operating temperature was set to 37 °C [18].

Turbidimetric method

Experiments on bacterial culture growth were carried out on a turbidimeter device, which was created at the Department of Biophysics at I. Javakhishvili Tbilisi State University. The device can observe the process of bacteria multiplication in an uninterrupted time regime. This allows us to determine the interval of time between the moment when the bacterial culture was added to the broth and the beginning of the multiplication process. The working volume of the turbidimeter is 15 cm3[19]. The bacterial overnight culture was diluted in the broth until the optical density of the culture became 0.5 OD (at 600 nm). The results, which are associated with the beginning of the biofilm formation process were analyzed according to Origin 6.1.

RESULTS

The viscometric method used in the study allows us to observe the process of biofilm formation in an uninterrupted time regime as well as to characterize it. As it was mentioned above, biofilm formation depends on the type of bacteria. That is why *E. coli,* due to the ability to produce a poor biofilm [20], and *P. aeruginosa* bacteria, due to the ability to produce a strong biofilm, were chosen for the research [21,22]. In the viscometer, when the bacterial culture was added to the LB broth, the bacterial multiplication process started, but the viscosity of the solution remained the same as it had been before adding the bacteria, suggesting that the addition of a high titer of bacteria to the broth does not change the viscosity. The rotation speed of the viscometer rotor in LB broth with inoculated bacteria was high (viscosity was small), since the rotor speed depends not only on the viscosity of the solution but also on the state and properties of the liquid surface where the rotor is moving. The aim was to show that the formation of the bacterial biofilm in the viscometer should have led to changes in the surface properties. And, any physical change in the properties of the biofilm surface will affect the rotor speed. Figure 2. a shows the viscometric curves which show that the ability of bacteria to form the biofilms is reflected in the rotor speed. In particular, it was shown that *E. coli* could not form the biofilms (the rotor rotation speed was not changed), while in the case of *P. aeruginosa* at a certain stage of bacteria multiplication, the time of one turnover of the rotor

increased sharply, which is likely to be associated with the formation of the biofilm between rotor and stator. It should also be mentioned that the time when bacteria begin to form the biofilm depends on the number of bacteria which is added to the LB broth. If this number is large, the biofilm formation will start in a short time. To address this issue, the same number of bacteria was used for both *E. coli* and *P. aeruginosa*. Figure 2. a shows the dependence of the rotor rotation period on time for the bacteria *P. aeruginosa* growing in the broth. The data reveals that at the begging of the experiment, the rotation period of the rotor is short which indicates that increasing the concentration of bacteria in the broth (the bacterium multiplies) does not increase the viscosity of the LB broth. An increase in the rotor rotation period occurs when the *P. aeruginosa* bacteria start to form the biofilm. As it was mentioned above, the rotor of the viscometer is immersed in the stator due to the surface forces.During the bacterial biofilm formation, the rotor rotation speed slows down. For this reason, it becomes unambiguously clear that a decrease in the rotation speed is not associated with an increase in the viscosity of the broth. This process is associated with the biofilm formation on the surface and not with the multiplication of bacteria in the broth.Experiments that were carried out to prove this hypothesis showed that if the biofilm integrity is destroyed mechanically the rotor starts to rotate in the stator at high speed (with a short period, $\sim$ 25 seconds). Moreover, the rotor rotation speed becomes the same as it was prior to the biofilm formation (Fig. 2.b). After destroying the biofilm integrity, the rotor speed again starts to decrease in a much shorter time and finally, the rotor stops. The rapid decrease in the speed can be explained by the fact that there were already all the necessary conditions for the bacteria to form the biofilm again which had previously been mechanically destroyed. To find out at what stage of reproduction *P. aeruginosa* bacterium biofilm starts the formation, the data of the viscometric experiment is compared with the data of the turbidimetric one. The same bacterium/LB broth ratio was used in both experiments. Figure 3 shows the results of these experiments, where the bacteria /LB broth ratio in both methods was 0.17. In particular, 0.2 ml of *P. aeruginosa* bacterium (1 OD of optical density) in the viscometer was added to 1.2 ml of LB broth. For the turbidimeter, 2 ml of *P. aeruginosa* bacterium (also the same bacterium) was added to 12 ml of LB broth. According to the viscometer data, the biofilm formation starts no earlier than 4 hours after the addition of the bacterial culture (Fig. 3. b), while according to the turbidimeter data, the stationary phase of *P. aeruginosa* multiplication starts after 2 hours (Fig. 3. a). According to the obtained experimental data, the process of biofilm formation is not directly connected to the process of bacterial multiplication. From our point of view, the bacterial biofilm formation process starts before a stationary phase. While the time needed to achieve the stationary phase depends on the number of bacteria that were added to the broth, increasing the number of bacteria added to the broth allows the stationary phase to be reached faster. Therefore, the time of the biofilm formation should depend on the number of bacteria that are added to the broth. The relationship between the biofilm formation time and the number of bacteria was found by using the viscometric experiments (Fig. 4). In particular, it is shown that the relationship between the number of *P. aeruginosa* bacteria which was added to the LB broth and the time of the biofilm formation is not linear, suggesting that when the bacterium starts the biofilm formation other conditions besides the bacterial concentration should also be present. According to the results obtained in Figures 2, 3, and 4, in the process of biofilm formation, the surface tension properties should play an important role, which manifests itself in changes in the surface properties during bacterial reproduction due to the release of amphiphilic substances from bacteria and bacteria bounding itself to these molecules.

DISCUSSION

A new approach was proposed while studying bacterial biofilm formation by using the rotational viscometric method. It allows monitoring the kinetics of the biofilm formation in an uninterrupted time regime, in order to enable observation of the mechanisms of the biofilm formation. The surface properties of the fluid are changed during the biofilm formation, which is fully reflected in the rotation period of the rotor of the viscometer. According to the experiments carried out on *P. aeruginosa*, the results indicate that the biofilm formation starts when the bacterium synthesizes and releases into the environment a certain amount of special hydrophobic molecules that accumulate on the surface of the broth. This was indicated by comparing experiments using two different methods. The turbidimetric experiment shows that *P. aeruginosa* bacteria reach the initial stage of the stationary phase after approximately 2.5 hours (Fig. 3. a), while the viscometric data have revealed that the process of the bacterial biofilm formation starts after 4 hours (Fig. 3. b). It should be emphasized that the difference between the achievement of the maximum titer by bacteria and the biofilm formation is approximately two hours. It should be emphatically mentioned that in the present experiments, the *P. aeruginosa*/LB broth ratio in the turbidimeter and viscometer was the same (1 OD bacterium in LB with a ratio of 0.14 bacterium /LB in both devices). The experimental results shown in Figure 4 indicate that the time of the bacteria biofilm formation is not linearly proportional to the number of bacteria added to the broth. (Fig. 4. b). Mathematically analyzed

results of the initial process of the biofilm formation as shown in Figure 5, reveal that the mechanism of the beginning of the biofilm formation can be described by the sum of two exponents: It can be assumed that the initial stage of biofilm formation is at least a two-stage process.

$$T = 0.13e^{-\frac{t}{443.6}} + 86.6e^{\frac{t}{1.8}}$$

There are many factors of biofilm formation described in the existing literature [23]. One of them is the presence of hydrophobic sites on the surface of a bacterial membrane. In particular, the presence of the hydrophobic sites on the membrane surface for *P. aeruginosa* bacteria which form the biofilms are confirmed [6,11]. In agreement with this idea, it is assumed that due to the hydrophobic regions of bacteria can form hydrophobic bonds with the specific amphiphilic molecules, which are synthesized by bacteria and are already described in the literature [14]. Due to its amphiphilicity, hydrophilic groups of molecules are immersed in the broth (aqueous environment) and its hydrophobic groups are located above the surface of the broth. In such a way, they promote the formation of bacterial associates with the surface.

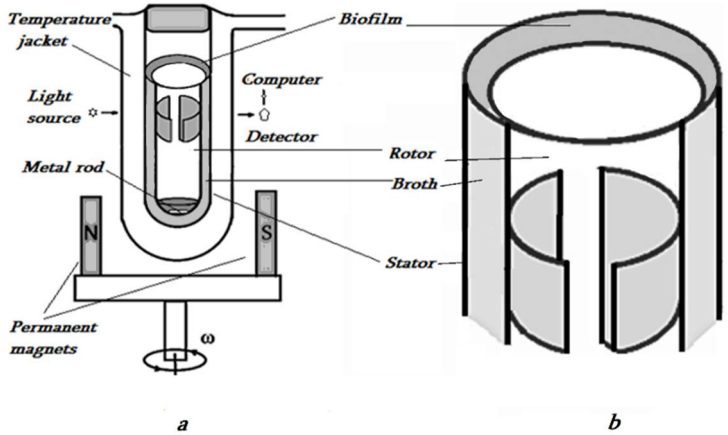


Figure 1. Description of the modified viscometer, which can follow the process of biofilm formation. a. The principal diagram of the viscometer shows the operation of the device. b. Area of biofilm formation on the surface of the rotor

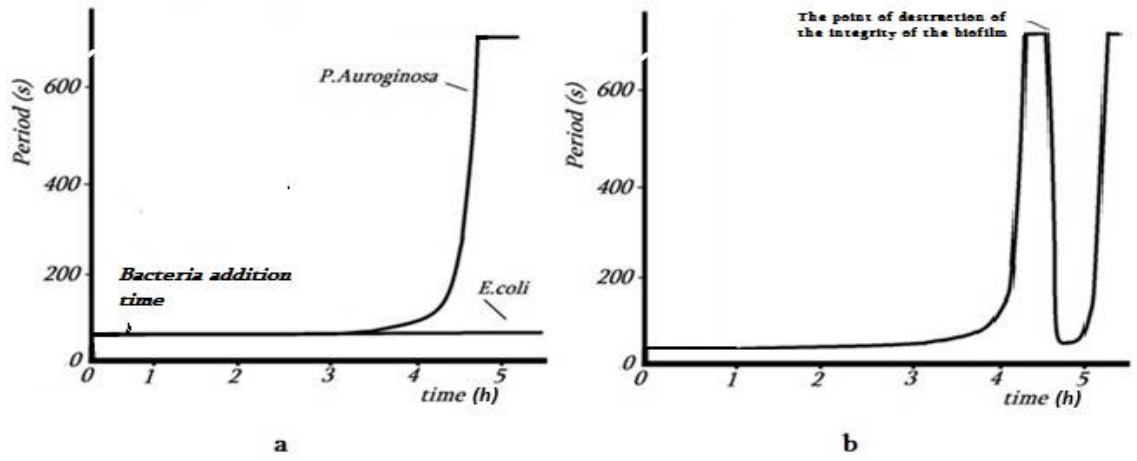
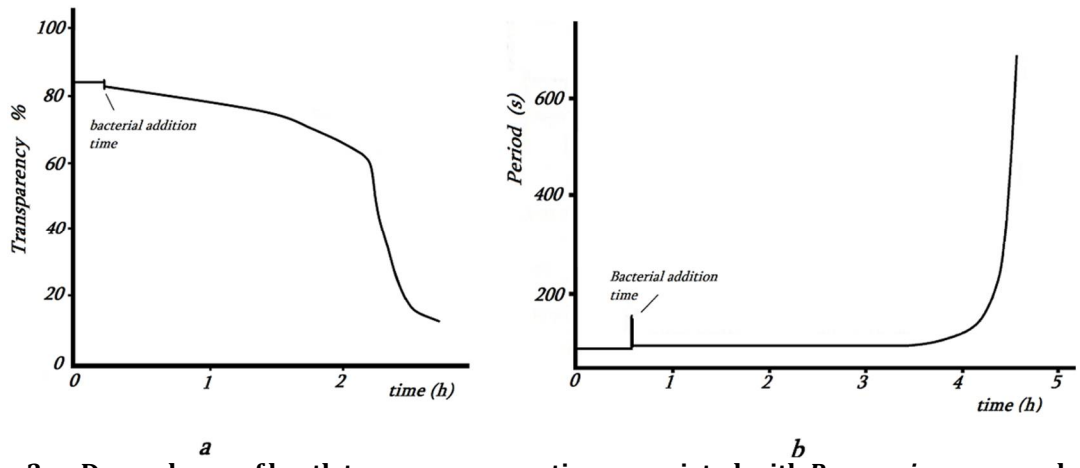
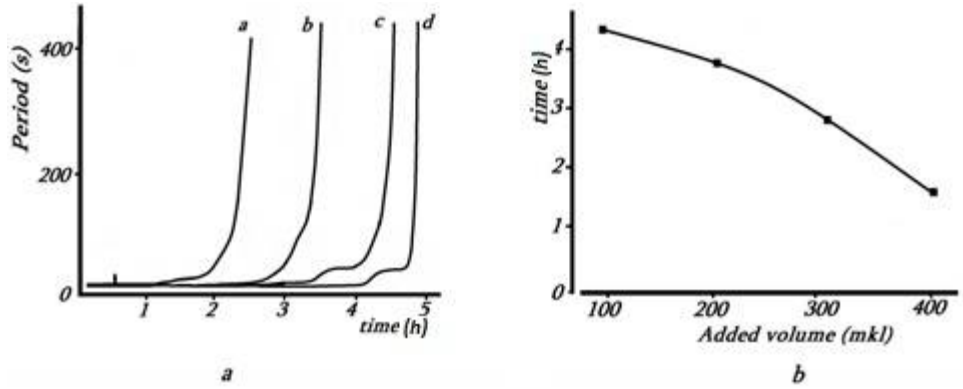


Figure 2. The viscometric curve of biofilm formation. a. Dependence of the period of rotation of the rotor on time for the bacteria *P. aeruginosa* and *E. coli*. b. Is shown the moment of mechanical destruction of biofilm and its re-formation.



a b

Figure 3. a. Dependence of broth transparency on time associated with *P. aeruginosa* reproduction in the turbidimeter; b. Dependence of viscometer rotor rotation speed on time for the same bacteria/broth ratio.



a b

Figure 4. a. Viscometer data of biofilm formation for different concentrations of bacteria; b. Dependence of the biofilm formation time on the initial amount of bacteria

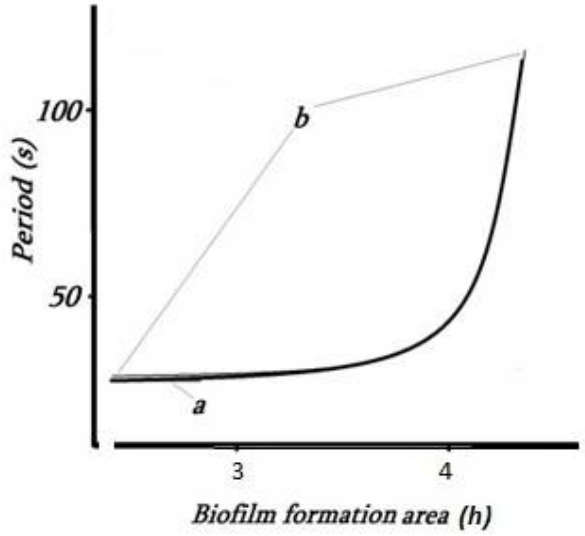


Figure 5. a. Experimental curve of the beginning process of biofilm formation; b. Theoretical curve.

CONCLUSION

Mathematical modeling of the results reveals that the initial process of biofilm formation consists of at least two stages. From our point of view the first stage of the bacterium P. aeruginosa reproduction process (in the logarithmic stage) the bacterium synthesizes and releases into the environment a certain amount of special hydrophobic molecules that accumulate on the surface of the broth. In the second stage, the

hydrophobic sites of bacterial cells interact with the hydrophobic molecules on the surface through hydrophobic bonds, forming the bacterial biofilm. It is believed that if bacteria use the above-discussed mechanisms for biofilm formation this will make it possible to more purposefully fight against biofilm-related bacterial infection and solve this problem more easily. This new experimental approach can be extended to other types of bacteria that can form biofilms.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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