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Review

Methods to study microbial adhesion on abiotic surfaces

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Abstract: Microbial biofilms are a matrix of cells and exopolymeric substances attached to a wet and solid surface and are commonly associated to several problems, such as biofouling and corrosion in industries and infectious diseases in urinary catheters and prosthesis. However, these cells may have several benefits in distinct applications, such as wastewater treatment processes, microbial fuel cells for energy production and biosensors. As microbial adhesion is a key step on biofilm formation, it is very important to understand and characterize microbial adhesion to a surface. This study presents an overview of predictive and experimental methods used for the study of bacterial adhesion. Evaluation of surface physicochemical properties have a limited capacity in describing the complex adhesion process. Regarding the experimental methods, there is no standard method or platform available for the study of microbial adhesion and a wide variety of methods, such as colony forming units counting and microscopy techniques, can be applied for quantification and characterization of the adhesion process.

Keywords: biofilms; experimental methods; microbial adhesion; predictive methods

1. Introduction

Biofilms are known as a bacterial population growing attached to a surface and confined in a polymeric matrix, normally of microbial nature. Important characteristics of biofilms include: (i) their resistance to antibiotics, disinfectants and dynamic environments; and (ii) their ability to grow even in nutrient-deficient conditions [1–4]. These bacterial communities can occur in both biotic and abiotic environments, on a wide variety of surfaces, such as living tissues, medical devices, industrial pipes and natural aquatic systems [5,6]. Several problems reported in industry, such as product spoilage, infection, reduced production efficiency, corrosion, unpleasant odours, pipe blockages and equipment failure are associated to the presence of biofilms [7,8]. However, not all biofilms are undesirable [9]. The use of biofilm reactors in industry can be advantageous due to the possibility to immobilize cells, keeping higher biomass concentrations inside the reactors and consequently improving the reaction rates [10]. Nowadays, there are several industrial processes that are employing biofilms. These processes include the production of vinegar [10], ethanol [12,13] and butanol [14]. Additionally, biofilms can be effectively applied in wastewater treatment processes [11], microbial fuel cells [15] and in the production of biosensors. The above referred biofilm properties difficult the removal process. However, they are responsible for the wide variety of applications described for biofilms [16]. In this sense, there is an increased interest in developing new strategies able to control and prevent formation of undesirable biofilms, as well as to improve the formation and stability of beneficial biofilms. Therefore, it is important to understand the physicochemical aspects involved in microbial adhesion, which corresponds to the initial step of biofilm formation. This manuscript provides an overview about the microbial adhesion process to abiotic surfaces with emphasis on the methodologies used to study this step of biofilm formation.

2. Adhesion Process

Biofilm formation comprises the following steps [17]: (i) transport of planktonic cells from the bulk liquid to the surface; (ii) adsorption of the cells at the surface; (iii) extracellular polymeric substances (EPS) and cell-cell signalling molecules production; (iv) irreversible adsorption of cells; (v) biofilm maturation; (vi) detachment of some biofilm cells; and (vii) biofilm recolonization (Figure 1) [3,18]. Microbial adhesion, in turn, corresponds to the first stages of biofilm formation and can be divided into reversible and irreversible adhesion. In the first one, planktonic cells are transported from the bulk liquid to the surface, reversibly attaching to the surface. At this stage, cells are transported as a result of physical forces, such as attractive van der Waals forces, repulsive electrostatic forces, gravitational forces, Brownian motion and hydrophobic interactions, or as a result of bacterial movements determined by the presence of appendages, such as flagella, fimbriae and pilli. In irreversible adhesion, chemical reactions between the cells that remain immobilized and the surface may occur, determining firmer adhesion of bacteria to the surface by the bridging function of microbial surface polymeric structures [19].

2.1. Factors influencing microbial adhesion

Microbial adhesion can be influenced by several factors, which can be grouped in three categories: (i) properties of the bulk medium; (ii) properties of the surface; and (iii) properties of the

microorganism [21,22]. The properties of the culture medium that mostly influence the adhesion process include the presence of conditioning substances or antimicrobial compounds, pH, temperature, flow velocity, exposure time, microorganisms concentration, surface tension and ionic strength. The physicochemical and morphological properties of the surface also contribute to the effectiveness of microbial adhesion. For example, the prevalence of attractive van der Waals forces or repulsive electrostatic forces strongly depends on surface charge and hydrophobicity [23]. Additionally, chemical composition, porosity and roughness of the surface determine the higher or lower affinity of microorganisms to it. As well as the properties of the surface, physicochemical properties of microorganisms' surface are also responsible for the adhesion process. Other characteristics of microorganisms that are also known for their important role in the adhesion process include their ability to produce EPS and the presence of extracellular appendages [24].

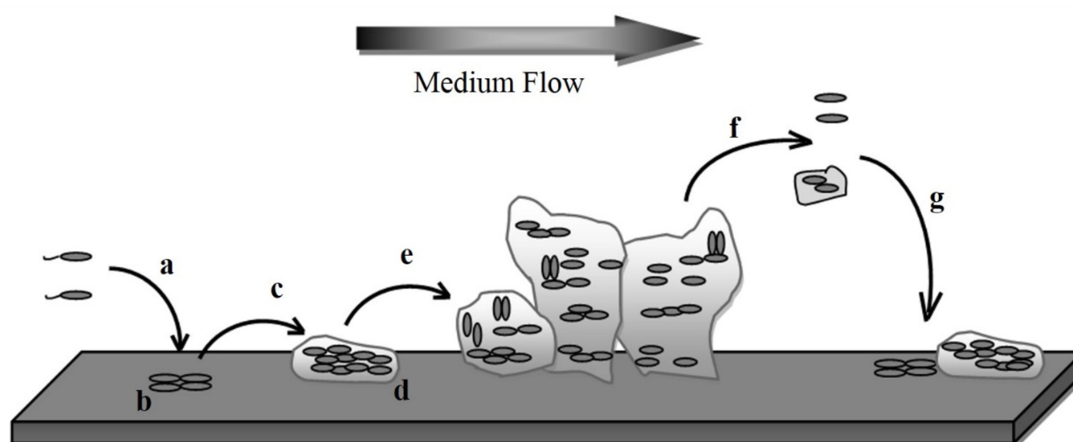

Figure 1. Biofilm formation steps: (a) transport of planktonic cells from the bulk liquid to the surface; (b) adsorption of cells at the surface; (c) starting of EPS formation and production of cell-cell signalling molecules; (d) irreversible adsorption of cells; (e) biofilm maturation; (f) biofilm removal by detachment or sloughing; and (g) biofilm recolonization. Steps (a) to (d) correspond to the different steps involved in bacterial adhesion. Based on Simões and Simões [20] and Qureshi *et al.* [10].

2.2. Predictive models to explain microbial adhesion

Although microbial adhesion cannot be explained exclusively by surface physicochemical properties, different theories have been developed to explain microbial adhesion based on these properties and on colloidal stability [25]. One of the most studied is the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of colloid stability, which states that the net interaction between two surfaces is the result of the combination of two forces: the van der Waals forces, generally attractive, and the electrical double layer interactions, usually repulsive [26]. Another theory used to explain the adhesion process is the thermodynamic theory of adhesion, which explains the interaction of a microbial cell and a cell substratum from a thermodynamic point of view, assuming that this interaction is only possible when a decrease in the surface Gibbs free energy is observed.

The previously described theories have been combined, resulting in the Extended Derjaguin-Landau-Verwey-Overbeek (XDLVO) theory. The XDLVO theory results from an extension of DLVO theory by the inclusion of acid-base interactions, which account for the hydrophobicity of the surfaces involved [27]. Although these theories can explain microbial adhesion assuming the interaction between colloidal particles, they ignore the microbiological aspects involved in the adhesion process. An example is the excretion of EPS, which can be crucial in the irreversible adhesion of microorganisms [28].

Surface hydrophobicity can be determined using the approach of van Oss [29], which allows the assessment of the absolute degree of hydrophobicity of any surface in comparison with their interaction with water. In this approach, the degree of hydrophobicity of a given surface (s) is expressed as the free energy of hydrophobic interaction between two entities of that surface when immersed in water (w): ΔG_{sws}^{TOT}, in mJ m^{-2}. When $\Delta G_{sws}^{TOT} < 0$, the interaction between the two entities is stronger than the interaction of each entity with water and the material is considered hydrophobic. Alternatively, if $\Delta G_{sws}^{TOT} > 0$, the material is hydrophilic. ΔG_{sws}^{TOT} can be calculated through the surface tension components of the interacting entities, according to Equation 1 [30–32]:

$$\Delta G_{sws}^{TOT} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+} - \sqrt{\gamma_s^+\gamma_s^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad (1)$$

where γ^{LW} accounts for the Lifshitz-van der Waals (LW) component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base (AB) component (γ^{AB}), being $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$. LW forces, usually attractive, result from instantaneous asymmetrical distribution of electrons in molecules (the higher the value of LW component, the more apolar is the surface and, therefore, the lower would be its affinity for polar liquids) [33]. Electron donor and acceptor parameters give information about the molecules present in the studied surface: higher γ^+ indicates the presence of positively charged molecules and higher γ^- indicates the presence of negatively charged molecules [29]. AB forces result from electron transfer interactions between polar components of the involved surfaces. These interactions can be attractive (hydrophobic attraction) or repulsive (hydrophilic repulsion), depending on the free energy of hydrophobic interaction [33,34].

The surface tension components of a surface (s) can be obtained by measuring the contact angles of different pure liquids (l), of known values of surface tension, followed by the simultaneous resolution of three equations of the form of Equation 2 [35].

$$(1 + \cos\theta)\gamma_l^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^+\gamma_l^-} + \sqrt{\gamma_s^-\gamma_l^+}\right) \quad (2)$$

where θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$.

The free energy of adhesion can be calculated through the surface tension components of the entities involved in the adhesion process by the thermodynamic theory, expressed by the Dupré equation. This equation (Equation 3) states that the total energy of interaction between a bacterium (b) and a substratum (s) that are immersed in water (w) can be expressed by the interfacial tension components.

$$\Delta G_{bws}^{TOT} = \gamma_{bs} - \gamma_{bw} - \gamma_{sw} \quad (3)$$

where γ_{bs} is the interfacial tension component of bacterium/substratum, γ_{bw} is the interfacial tension component of bacterium/water and γ_{sw} is the interfacial tension component of substratum/water.

The interfacial tension for a diphasic system of interaction bacterium/substratum, γ_{bs}, can be defined according to the following equations:

$$\Delta G_{bws}^{TOT} = \gamma_{bs} - \gamma_{bw} - \gamma_{sw} \quad (4)$$

$$\gamma_{bs} = \gamma_{bs}^{LW} + \gamma_{bs}^{AB} \quad (5)$$

$$\gamma_{bs}^{LW} = \gamma_{b}^{LW} + \gamma_{s}^{LW} - 2 \times \sqrt{\gamma_{b}^{LW} \times \gamma_{s}^{LW}} \quad (6)$$

$$\gamma_{bs}^{AB} = 2 \times \left(\sqrt{\gamma_{b}^{+} \times \gamma_{b}^{-}} + \sqrt{\gamma_{s}^{+} \times \gamma_{s}^{-}} - \sqrt{\gamma_{b}^{+} \times \gamma_{s}^{-}} - \sqrt{\gamma_{b}^{-} \times \gamma_{s}^{+}}\right) \quad (7)$$

3. Laboratorial Systems to Study Microbial Adhesion

The most commonly used laboratorial systems to study microbial adhesion include flow chambers, also known as small-scale flow cells [36], and microtiter plates [37,38]. Flow chambers are commonly used with image acquisition systems, enabling real-time observation of microbial adhesion, the replication of biofilm cells and the production of EPS. Typical geometries known for small-scale flow cells include flat plate and glass capillary flow cells [39]. A microtiter plate is a flat plate containing multiple wells arranged in a rectangular array. The wells work as independent test tubes, allowing the simultaneous study of different conditions. The use of microtiter plates in laboratory studies is very common, since these devices present some advantages, such as the requirement of small amounts of reagents and the simple control of temperature and hydrodynamic conditions [40]. Table 1 includes the main advantages and disadvantages of the described laboratorial systems.

4. Methods to Assess Microbial Adhesion

Microbial adhesion can be assessed experimentally, however, there are still few techniques to measure it [41,42]. The conventional methods are based on microscopic or plate counting and are named direct methods. The indirect methods are essentially spectrophotometric. The direct methods are more laborious and bacteria have to remain culturable, while indirect methods are less sensitive and less accurate [41]. The main advantages and disadvantages of the methods applied to observe microbial adhesion are summarized in Table 2.

There is not a standardization of the methods used to assess the adhesion process. Furthermore, the strains commonly used to study and predict microbial adhesion are reference strains, which can be considered inadequate and not representative of real case strains [43].

Table 1. Advantages and disadvantages of the most commonly used laboratorial systems to study microbial adhesion.

	Advantages	Disadvantages
Flow chamber	• Allows direct microscopic investigation of microbial adhesion; • The hydrodynamic conditions and the environment can be carefully controlled and easily changed; • Can operate in batch or continuous mode.	• The testing surface must be transparent, limiting its application to specific surfaces.
Microtiter plates	• Allows direct microscopic investigation of microbial adhesion; • The environment can be carefully controlled and easily changed; • A relevant number of surfaces can be tested simultaneously.	• Operates under low hydrodynamic stress conditions; • Can operate only in batch or in fed-batch mode.

4.1. Direct methods

Microbial adhesion can be observed through microscopic techniques, such as light, epifluorescence (Live/Dead *Bac*Light stain, 4′,6-diamidino-2-phenylindole—DAPI, the tetrazolium salt 5-cyano-2,3-ditolyltetrazolium chloride—CTC, acridine orange, fluorescence *in situ* hybridization with oligonucleotide probes—FISH) [44], transmission electron (TEM), scanning electron (SEM) [45], confocal laser scanning (CLSM) [46] and atomic force (AFM) [47] microscopy. It can also be evaluated with spectrophotometry [47], plate counting (colony forming unit (CFU) counting), coulter counter [47], radiolabelling [45] and flow cytometry [51].

One of the most used techniques is light microscopy, since it is simple, fast and efficient. This method is applied to observe and enumerate bacteria in translucent surfaces and normally it can be achieved by staining the bacteria with dyes, such as Gram stain and crystal violet. However, this method does not give any information about cells viability [47]. Light microscopy can also be used to visualize bacteria when using a flow chamber system [62]. On the other hand, epifluorescent microscopy is a fastest method that can be used with opaque surfaces (metal, ceramic, etc.), having the disadvantage of being more expensive. This method is able to distinguish live cells from dead ones, allowing the determination of cell viability [44]. TEM allows the study of the internal and external structures involved in cell adhesion, while SEM allows the observation of microbial attachment to the surfaces, as well as the morphology of both bacteria and surface material. As disadvantages: in TEM the samples cannot be reused and the assessment of cells viability is not possible; in SEM the resolving power is inferior to TEM and it is a less sensitive method [45,46,47]. To overcome some of the light microscopy limitations, a laser-scanning confocal microscope can be used. Confocal microscopy encompasses confocal imaging and laser illumination, as well as an advanced image processing technique that provides high resolution images [46]. With this technology, it is possible to observe the cells *in situ*, as well as their structure and distribution [47].

Table 2. Advantages and disadvantages of the direct and indirect methods used to study microbial adhesion.

	Methods	Advantages	Disadvantages	References
Direct	Light Microscopy	• Fast; • Simple; • Inexpensive; • Efficient.	• The surfaces have to be translucent; • The sample cannot be reused; • Does not give information about cells viability.	[44]
	Epifluorescent microscopy	• Opaque surfaces can be used; • Fast (faster than light microscopy).	• Expensive; • Adjustments are necessary to the clusters of bacteria.	[44]
	TEM	• Allows the visualization of internal and external adhesion structures.	• Restricted to a soft substratum; • The sample cannot be reused; • Does not give information about cells viability.	[45-47]
	SEM	• Opaque materials can be used.	• Time consuming; • Less sensitive; • Lower resolving power than TEM.	[45-47]
	CLSM	• Examination can be performed *in situ*; • Samples can be sectioned optically exposing the 3D structure.	• Expensive; • The light wavelength limits the resolving power of the microscope.	[46]
	AFM	• Nanometer resolution; • Cell adhesion forces can be quantified.	• Technical difficulties associated with live-cell experiments; • Requires extensive expertise; • The interior of living cells cannot be assessed.	[47,48]
	CFU counting	• Quantitative results; • Rapid results; • Does not involve specialised technology.	• The detachment from a surface may not be complete and can be harmful to the cells; • The true number of cells attached may not be reflected (due to cell aggregation and loss of ability to replicate on a solid medium).	[44,49]
	Coulter counter	• More accurate than microscopic techniques; • Simple.	• Counts the total number of bacteria and not the viable ones.	[47]
	Radiolabelling	• More versatile and sensitive; • Rapid processing.	• Unstable; • Limited to longer experiences; • Hazardous materials; • Expensive;	[45,46,50]

			• Requires specialised equipment.	
	Flow citometry	• Multiparameter data acquisition; • Rapid.	• Expensive; • Requires specialised operators; • Only quantitative.	[51]
Indirect	Spectrophotometric	• Rapid; • High throughput.	• Incompleteness; • Bacteria can be non-homogeneously stained; • The dye can be adsorbed to the abiotic materials.	[44,47,52,53]
	BTA	• Does not require any manipulation of the samples; • Easy; • Low cost.	• Cannot be compared with other methods.	[44,49,54]
	ATP	• Accurate; • Sensitive; • Rapid.	• Not specific; • The quenching of emitted light can adversely affect the measurements.	[55-57]
	QCM	• High resolution.	• Low throughput; • Susceptible to contaminations.	[58]
	MSS	• Allows the use of different surface materials; • Non-invasive, avoiding contaminations.	• Detects deposits that are really attached to the surface.	[59,60]
	SPR	• Requires cheap reagents and an UV-vis spectrophotometer.	• Low throughput; • Inability to handle many samples simultaneously.	[61]

AFM enables the study of the surfaces to a submicron level and evaluates the forces between biological molecules and surfaces on a molecular level [47]. The tip can be functionalised with the microorganism in study (or other molecule) and the force between the tip and the surface is measured. Additionally, the interior of the living cells cannot be assessed [48]. CFU counting is a simple and fast standard method applied to evaluate the viability and cultivability of the cells, however, it cannot be reliable when studying adhered cells and biofilms, or even microbial aggregates. Therefore, it is only used to determine the viable cells [44,49]. Other method that can be used is Coulter counting. This method cannot distinguish viable bacteria from the non-viable ones, but it can be used to measure the bacteria size and to count the number of total cells. The mode of operation is based on the resistance of a conducting solution when a cell (or particle) passes in an opening [47]. In addition, radiolabelling involves labelling of cells with radiotracers, which can be expensive to purchase and to disposal and involves a risk to the operator [45,46,50]. As a quantitative approach there is available the flow cytometry. In this quantitative technique a fluid passes through a detector that counts the particles present at a rate of 1000 cells per second. This method is rapid and allows the acquisition of multiparameter data [51].

4.2. Indirect methods

The indirect methods include the spectrophotometric methods, biotimer assay (BTA), adenosine triphosphate (ATP) assay, quartz crystal microbalance (QCM), mechatronic surface sensor (MSS) and surface plasmon resonance (SPR).

Spectrophotometric methods are based on the staining of the adhered cells and the consequent spectrophotometric measure of the dye that stained the microorganisms [44]. They are based on a relationship between the number of cells and the absorbance obtained from the spectrophotometric measure. There are several dyes that can be applied, such as crystal violet, safranin, congo red [47], tetrazolium salts [52] and alamar blue. Despite the high throughput, this method is not accurate, since it measures biofilm formation at the bottom of the wells [53]. Another colorimetric assay is BTA. This method allows indirect counting of viable bacteria in the attached form. It involves a specific reagent that contains phenol red and measures the microbial metabolism. The colour of the specific reagent switches from red-to-yellow due to the microbial products of primary fermentative metabolism [44,49,54]. ATP is a constituent of all living cells responsible for the regulation of metabolic pathways. In dead cells this molecule is lysated in few minutes. Since it occurs proportionally to the cellular mass, it can be used to calculate the number of viable cells [55,56,57]. QCM is an acoustic biosensor which senses mass and energy changes of the studied material that is coupled to the sensor crystal, in the nanogram range. It can be used to study a variety of surface processes including bacterial adhesion [58]. MSS is another sensor, which is similar to the QCM, however, it is not in direct contact with the fluid (allows the use of different surface materials and avoids the presence of an additional source of contamination). It detects and evaluates the cells that are attached to the surfaces [59,60]. Other biosensor used is the SPR. This is an optical biosensor, with low throughput, that detects mass concentration at a metal sensor chip surface by measuring changes in the refractive index [61].

5. Conclusions

The study of microbial adhesion is an important issue to consider both in the prevention of biofilm formation and in the development of biofilms for beneficial applications. Studying the adhesion process involves a theoretical prediction using mathematical models based on surface

physicochemical properties and experimental approaches for formation, characterization and quantification of biofilms. This study has shown that conventional physicochemical approaches based on Lifshitz-van der Waals, electrostatic and acid-base interactions provide important models for the study of bacterial adhesion, but have a limited capacity in the complete understanding of the adhesion process. There is no standard method and/or platform available for the study of microbial adhesion. Additionally, a wide variety of methods can be applied for direct and indirect quantification and characterization of the adhesion process. Of all described methods, CFU counting and microscopy are the most commonly used.

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Conflict of Interest

All authors declare no conflicts of interest in this paper.

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