9

The importance of physicochemical properties in biofilm formation and activity

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9.1 INTRODUCTION

Microbial adhesion to solid surfaces is the *sine qua non* condition for the formation of biofilms. This process is mainly governed by the physicochemical properties of both microbial cells and solid surfaces. However, other factors can be also involved and might ultimately have a strong influence on the overall process. For instance, high levels of shear stress have been shown to reduce bacterial adhesion (Gjaltema *et al.* 1997). Thus, parameters like the porosity or surface roughness can have a determinant role in shielding the cells from the effects of shear.

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The importance of the studies on initial microbial adhesion on biofilm formation has been questioned, because the number of cells in a mature biofilm after growth can be several times higher than the number involved in initial adhesion. Those studies are only justified as a contribution to improve the understanding of the adhesion phenomenon (Marshall 1985; Absolom *et al.* 1983). From another point of view, this approach overlooks the importance of the microorganisms that initially adhere as being the link between the colonised surface and the biofilm (Busscher *et al.* 1995a).

Those working with biofilm reactors are aware that the reactor performance is dependent on the stability and activity of the biofilm. This chapter reflects on the role of surface properties on biofilm formation and on the possibility of using knowledge of them to predict the stability and eventually the activity of a biofilm.

9.2 HOW ADHESION HAS BEEN PREDICTED

The process of bacterial colonization on surfaces immersed in aqueous media has been extensively studied because of the importance of attached bacteria in fields like medicine, biotechnology, biofouling and geochemistry. Different approaches have been used to describe and to simultaneously predict bacterial adhesion to solid surfaces. It can be argued that bacterial colonization of immersed surfaces is always to be expected, because it is considered to be ubiquitous. However, in practical terms it is important to determine the density of cell attachment and the ease of removal of the attached cells. A detailed outline of the most common methodologies used to describe bacterial adhesion is presented below.

9.2.1 Thermodynamic approach

The interaction between a microbial cell and a solid substratum is only possible from a thermodynamic point of view if it leads to a decrease in the surface Gibbs free energy (Absolom *et al.* 1983; Busscher *et al.* 1984). This means that adhesion is favorable if the variation of the total Gibbs energy is negative ($\Delta G < 0$).

The interfacial free energy of interaction of two surfaces 1 (microbial cell) and 2 (substratum) immersed in a medium 3 is given by the Dupré equation (van Oss 1991):

$$\Delta G_{132} = \gamma_{12} - \gamma_{13} - \gamma_{23} \tag{9.1}$$

γ_{12} is the interfacial tension between surfaces 1 and 2 and the other parameter γ have a similar meaning, according to the interacting surfaces indicated in subscript.

The calculation of the surface tension of a solid has been a controversial subject, mainly owing to it being considered a whole entity (Spelt and Neumann 1987) or being formed by components, and due to questions about the nature of those components (van Pelt *et al.* 1983; van Oss *et al.* 1988). Most authors now accept the approach of van Oss *et al.* (1988), in which the surface tension is the sum of the apolar electrodynamic Lifshitz – van der Waals (LW) interactions and the polar interactions, owing to electron-acceptor/electron-donor interactions, also designated as (Lewis) acid–base (AB). Thus for a given substance, i, the total surface tension is given by:

$$\gamma_i^{Tot} = \gamma_i^{LW} + \gamma_i^{AB} \tag{9.2}$$

The polar (AB) component comprises two non-additive constituents: the electron acceptor (γ^+) and the electron donor (γ^-) parameters.

As the surface tension components, LW, and, AB, are additive, the Dupré equation can be written in a different form to obtain the interfacial free energy of adhesion among the interacting entities (ΔG_{132}^{adh}):

$$\Delta G_{132}^{adh} = \Delta G_{132}^{LW} + \Delta G_{132}^{AB} \tag{9.3}$$

The relation of both ΔG_{132}^{LW} and ΔG_{132}^{AB} with the individual surface tension components (γ_i) is fully described in the literature (van Oss 1991).

Calculation of the surface tension of a solid can be obtained via contact angle determination, employing at least one apolar and two polar liquids of well known surface tensions and solving Young's equation three times (Adamson 1982; van Oss 1991). When the solids are in granular form (e.g. sand), the contact angle can be indirectly determined by the thin layer wicking technique, using the Washburn equation (Constanzo *et al.* 1990; Teixeira *et al.* 1998).

From all the literature where the thermodynamic approach has been considered, we can conclude that this criterion cannot be used in a straightforward fashion. It happens that in many situations where $\Delta G_{132}^{adh} > 0$, adhesion of bacterial cells does occur. This can be shown by the results obtained using different approaches for the calculation of the surface free energy.

Example 1. Studies on the adhesion of three different strains of oral streptococci to glass, polymethylmethacrylate and polytetrafluorethylene, considering that the surface tension comprises a dispersion and a polar component, showed that adhesion occurs even when $\Delta G_{132}^{adh} > 0$ (Busscher and Weerkamp 1987). However, the evaluation of the number of adhering bacteria yielded the conclusion that, for each bacterial strain, adhesion increased with decreasing ΔG_{132}^{adh}.

Example 2. Studies done to select the most suitable carrier for nitrification in an air-lift reactor gave similar results (Teixeira and Oliveira 1998). In this case, the surface tension calculations followed the approach of van Oss (1991), considering the LW and AB components. The carriers under evaluation were particles of sand, pumice stone, poraver (foam glass), basalt and limestone. The nitrifying consortium was composed of *Nitrosomonas* and *Nitrobacter*. All of the obtained interfacial free energies of adhesion were positive. However, the two materials giving the lower values of ΔG_{132}^{adh} (limestone and basalt) were the ones showing higher nitrifying activity. This can be attributed to a higher number of adhered bacteria, giving rise to a more efficient biofilm. It must be stressed that the evaluation of the nitrifying efficiency was followed during 20 days in an airlift reactor. The biofilm had previously formed around the particles of each type of carrier during one month in flasks that had been inoculated with the consortium and with the medium having been replenished every seven days.

Summarizing, the thermodynamic criterion cannot be generalized, but can point to a trend for adhesion of a given bacterial strain to different types of support.

9.2.2 DLVO theory

Since the advent of DLVO theory (named after Derjaguin, Landau, Verweu and Overbeek (Oliveira 1992)), formulated to explain the stability of lyophobic colloids 50 years ago, several attempts have been made to use this approach to describe bacterial adhesion, considering the cells as 'living colloids' (Marshall *et al.* 1971; van Loosdrecht *et al.* 1988; Azeredo *et al.* 1999). According to this theory the total energy of interaction arises from the balance between the LW forces and electrostatic forces, effective between a cell and the substratum, changing with the distance from the surface. Most of the substances, with the exception of some metallic hydroxides, display a net negative charge when immersed in a liquid medium with pH near neutrality. So, the inter-penetration of the electrical double layers of like charge of two approaching surfaces gives rise to a repulsive force (EL), whereas the LW forces are generally attractive. The total free energy of interaction is given by:

$$\Delta G^{TOT} = \Delta G^{LW} + \Delta G^{EL} \tag{9.4}$$

The energy profile of such an interaction can have two minima separated by an energy barrier (Oliveira 1992; van Loosdrecht *et al.* 1988). The minimum more distant from the surface (secondary minimum) is usually not very deep (the decrease in the total energy of interaction is small), and the cells accumulating in this minimum (stabilizing in this energy state) are only loosely

associated with the surface. In this case adhesion is considered reversible; this means that the cells can be easily removed. Those cells that are able to overcome the energy barrier and progress to the primary minimum (close to the surface and theoretically of infinite depth) become more firmly attached; they attain a very low energy state and adhesion is irreversible.

It must be noted that the mentioned energy profile is strongly dependent on the ionic strength of the medium. A low ionic strength can promote a very high energy barrier, while a high ionic strength levels the profile; the secondary minimum and the energy barrier disappear and a firm adhesion is facilitated.

Both DLVO forces depend on the geometry of the interacting surfaces. The interaction between a sphere and a smooth flat surface is made solely by one point, whereas two smooth flat surfaces enter into entire contact. Apart from geometrical assumptions, others have to be made, namely for the calculation of *Hamaker* constants (Visser 1972; van Oss 1994) and for the determination of the electrical surface potential of the interacting entities (Hunter 1981). But, probably, the most difficult to establish is the electrical behavior of both surfaces while approaching. This is to say that they can keep both their surface potentials constant, the surface charge constant, or they can interact in a mixed mode with one at constant surface potential and the other at constant charge (Oliveira 1992). The last situation is considered the most probable in biological systems (Rajagopalan and Kim 1981).

So far, it has been difficult to find in the literature a situation where bacterial adhesion can be fully explained by DLVO theory, because other types of interaction, rather than DLVO forces, can play an important role in the overall process. This happens even if the different modes of double-layer interactions are considered.

Example 3. The experimental tests done to select a suitable carrier for *Alcaligenes denitrificans* in an inverse fluidized bed reactor studied several polymeric materials: high density polyethylene (HDPE), polypropylene (PP), polyvinyl chloride (PVC) and polymethylmethacrylate (PMMA) showed that adhesion occurs to the greatest extent to PP followed by PVC, HDPE and lastly to PMMA (Teixeira and Oliveira 2000). The corresponding DLVO energy profiles for the two extreme situations, adhesion to PP and to PMMA, are presented in Figure 9.1. As can be seen, considering both interacting surfaces either at constant charge (Figure 9.1a) or at constant potential (Figure 9.1b), the interaction is always repulsive ($\Delta G^{TOT} > 0$) and a high-energy barrier is formed at close contact, which would prevent adhesion. Furthermore the energy barrier is more pronounced for PP than for PMMA, which is not in accordance with the experimental results. If a mixed mode of interaction is assumed (Figure 9.1c), the formation of a secondary minimum is notorious. A plausible explanation

relies on reversible adhesion taking place at distances of more than 1 nm. However, the energy barrier for PMMA is smaller than for PP, and the reversibility of adhesion is also questionable. All the supports were vigorously rinsed in distilled water after the adhesion experiments and before cell enumeration. Hence it can be assumed that only the cells strongly attached to the surface remained.

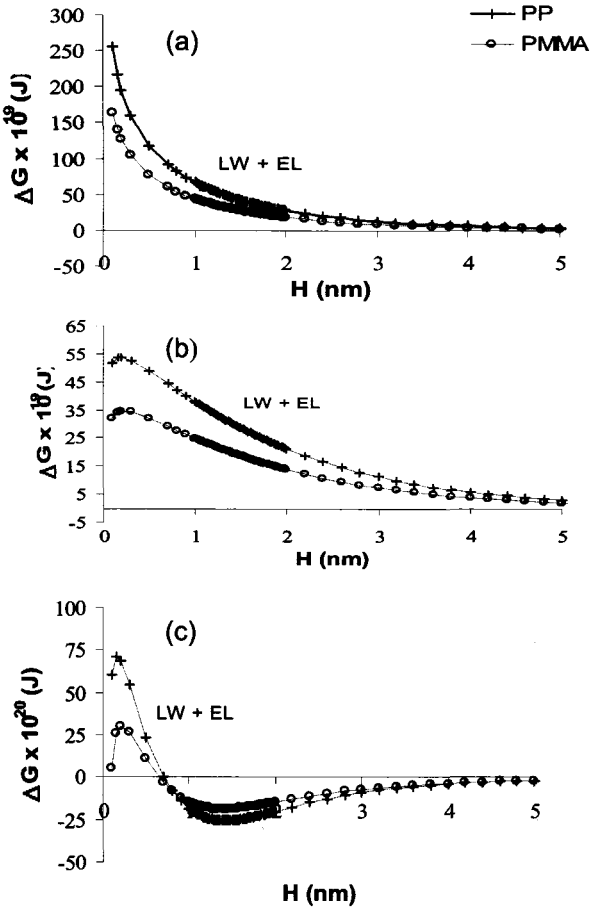

Figure 9.1. Variation of the DLVO total free energy of interaction between *Alcaligenes denitrificans* and the polymeric supports (PP and PMMA) as a function of the separation distance (H) under the condition of constant charge (a), constant potential (b), and mixed case (c).

9.2.3 XDLVO theory

Considering that in many non-metallic condensed materials, liquids and solids, polar interactions of the hydrogen bond type (AB) often occur, van Oss (1994) proposed an extension of the DLVO theory; it also accounts for the interactions due to Brownian movement forces (BR), and is generally known as the XDLVO theory. Accordingly, the total free energy of interaction is expressed by:

$$\Delta G^{TOT} = \Delta G^{LW} + \Delta G^{EL} + \Delta G^{AB} + \Delta G^{BR} \quad (9.5)$$

When LW, EL and AB interactions are measured individually, the total free energy of interaction (ΔG^{TOT}) can be obtained by summing the values of those entities plus + 1 kT for ΔG^{BR} (for systems with two degrees of freedom). If the ΔG^{TOT} is measured as a whole (by interfacial tension determination), then ΔG^{BR} is already included (van Oss 1994).

The omission of the AB forces, which are generally one or two orders of magnitude greater than the EL and LW forces, is the origin of most of the anomalies that were observed if the DLVO theory was used to interpret interfacial interactions in polar media (van Oss *et al.* 1990).

Example 4. Three mutants (TR, CV, and F72) of the gellan (polysaccharide) producer *Sphingomonas paucimobilis* were isolated and used in adhesion experiments. TR is the highest producer of exopolymer (EPS), followed by CV, whereas in F72 this ability is almost repressed. The adhesion assays were performed in two types of medium: in phosphate buffer saline (PBS) and in solutions of the excreted and isolated exopolymers of each mutant (Azeredo *et al.* 1999). This means that in some of the experiments the glass slides were preconditioned with the exopolymer of the mutant being tested. The number of cells per square millimeter adhering to bare glass slides (in PBS) and coated glass slides (in the EPS solutions) is shown in Table 9.1.

Table 9.1. Number of cells per square millimeter (± standard deviation) of each mutant of *Sphingomonas paucimobilis* adhered to glass in phosphate buffer saline (PBS) and in the corresponding solution of extracellular polymeric substances (EPS).

Mutant	Adhesion medium	
	PBS	EPS
TR	323±36	2513±215
CV	539±72	1508±144
F72	646±72	826±72

Figure 9.2 presents the total free XDLVO energy of interaction as a function of the distance of separation for both types of experiment. The energy profiles for the interactions in PBS show a secondary minimum at 12–15 nm from the surface. The stabilization of the cells at this minimum is due to weak forces and an irreversible adhesion would not be expected, on account of the energy barrier at the minimum distance of separation. This may be an explanation for the small number of adhered cells (Table 9.1), which is in accordance with the depths of the secondary minima. An increase in the number of adhered cells corresponds to an increase in the secondary minimum depth.

The energy profiles obtained for adhesion in the EPS solutions clearly show a discrepancy with the practical results. This anomaly can be explained if adhesion is considered to be preferentially mediated by polymeric interactions. The polymers adsorbed to the glass surface can bind to the polymers of the EPS layer surrounding the cells. A fluorescence microscopic observation of the three mutants after binding calcofluor white and a lectin (conA) showed that mutant TR had a very thick EPS layer, which was not so large around mutant CV and was almost non-existent in mutant F72.

Although the inclusion of the AB forces is considered a drastic correction of the DLVO theory (van Oss *et al.* 1990), failures are to be expected when dealing with 'living colloids'. Apart from other phenomena, they may possess attached portions of exopolymers or surface appendages that may be able to overcome the energy barrier establishing a stable interaction with the substratum, which are difficult to account for in the calculations. This is why both DLVO and XDLVO theories are most suited to explain adhesion *a posteriori.*

9.3 SURFACE PROPERTIES RELEVANT FOR ADHESION

9.3.1 Hydrophobicity

The most well-known work on the effect of substratum wettability on bacterial adhesion is attributed to Dexter *et al.* (1975), who studied bacterial attachment in marine systems. The following studies, either in marine or medical systems, also indicate that the wettability of solid surfaces influences adhesion of bacteria, eukaryotic cells and proteins (Busscher and Weerkamp 1987; Margel *et al.* 1993; Prime and Whitesides 1993; Wiencek and Fletcher 1997; Taylor *et al.* 1997).

The wettability of a surface is now more generally referred to as hydrophobicity. In aqueous medium, adhesion is favored between hydrophobic surfaces, which can enter into closer contact by squeezing the water layer between them.

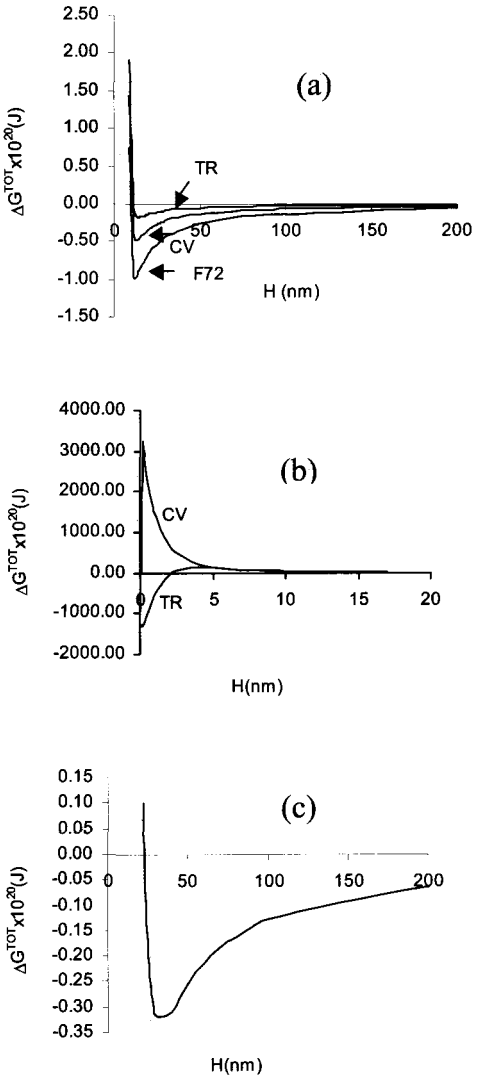

Figure 9.2. Variation of the XDLVO total free energy of interaction as a function of the distance of separation (H) for the interaction: (a) between the mutants TR, CV and F72 and glass in PBS medium; (b) between the mutants TR, CV and glass in EPS solutions; (c) *ibid.* for the mutant F72.

In biological systems, hydrophobic interactions are usually the strongest of all long-range non-covalent interactions, and can be defined as the attraction between apolar or slightly polar molecules, particles or cells, when immersed in water. Their sole driving force is the hydrogen bonding (AB forces) energy of cohesion between the surrounding water molecules (van Oss 1997). This means that the AB forces, if strongly asymmetrical or monopolar, are responsible for the orientation of water molecules adsorbed on the surfaces, and, as expected, water molecules oriented on the surface of one particle will repel water molecules oriented in the same manner on the surface of an adjacent particle (Parsegian *et al.* 1985; van Oss 1994). If the orientation of the water molecules is sufficiently strong, the two particles will not approach each other. On the other hand, if the surface is more weakly apolar, its capacity for orienting the most closely adsorbed water molecules is less pronounced and the particles will approach each other under the influence of their net LW attraction. Accordingly, 'hydrophobic' compounds or surfaces do not repel water: they attract water with rather substantial binding energies, albeit not quite as strongly as very hydrophilic ones (van Oss 1995). It should be stressed that hydrophobic attractions can prevail between one hydrophobic and one hydrophilic site immersed in water, as well as between two hydrophobic entities.

In the words of Busscher (1995), hydrophobicity is ubiquitously accepted to be a major determinant in biointerfacial reactions, but, on closer inspection, we all give different meanings to the word 'hydrophobicity' and we all use different techniques to measure 'hydrophobicity'.

Actually, several techniques have been used to determine the degree of hydrophobicity of bacterial cells or particulate materials. For materials that can be obtained in a flat plate shape, hydrophobicity has very often been expressed in terms of the contact angle formed by a sessile drop of water. In the case of bacterial cells, one of the most frequently used techniques to assess hydrophobicity is the so-called BATH (bacterial adherence to hydrocarbons) method, proposed by Rosenberg (1984), which is now more generally known as MATH (microbial adherence to hydrocarbons). In a study to characterize the hydrophobic properties of streptococcal cell surfaces (van der Mei *et al.* 1987), the following methods were compared: MATH, hydrophobic interaction chromatography, salting-out aggregation and contact angle measurements. Although these methods are commonly used in hydrophobicity determinations, the results obtained led the authors to the conclusion that it was not possible to define the surface 'hydrophobicity' of a bacterium, other than on a comparative level with closely related strains. Other authors (van Loosdrecht *et al.* 1987), studying the role of bacterial cell-wall hydrophobicity in adhesion, have also used different methods – contact angle measurements and partitioning of cells in two-phase systems (water-hexadecane and PEG-DEX) – to determine the degree

of hydrophobicity of 23 bacterial strains. As a conclusion they proposed the water contact angle as the best method to quantify cell hydrophobicity, because they found some drawbacks in the utilization of two-phase systems. Later, it was observed that the zeta potentials of the hydrocarbons could be highly negative in the various solutions commonly used in MATH (Busscher *et al.* 1995b). So, MATH may measure a complicated interplay of long-range van der Waals and electrostatic forces and of various short-range interactions (van der Mei *et al.* 1995), rather than pure hydrophobic interactions.

As was already mentioned, with the techniques described above it is only possible to assess hydrophobicity in qualitative terms. However, according to van Oss (1997), it is possible to determine the absolute degree of hydrophobicity of any given substance (i) compared with water (w), which can be precisely expressed in applicable S.I. units. When the free energy of interaction, ΔG_{iwi}, between two entities (i) immersed in water (w) has a positive value, i is hydrophilic, and when ΔG_{iwi} has a negative value, i is hydrophobic. More precisely (in cases of a negligible LW interaction), ΔG_{iwi} expresses the degree to which the polar attraction of entities i to water is greater (hydrophilicity) or smaller (hydrophobicity) than the polar attraction that water molecules have for each other. When the net free energy of interaction between two entities i immersed in water is sufficiently attractive (i.e., $\Delta G_{iwi}<0$), the surfaces of i are genuinely hydrophobic. The more negative ΔG_{iwi}, the more hydrophobic that entity is; the more positive ΔG_{iwi}, the more hydrophilic.

ΔG_{iwi} is simply related to the interfacial tension between i and water, γ_{iw}, as:

$$\Delta G_{iwi} = -2\gamma_{iw} \tag{9.6}$$

where γ_{iw} can be determined by contact angle measurements or thin layer wicking.

Using this last criterion, it has been easier to relate hydrophobicity with the capacity of bacteria to colonize different types of surface.

Example 5. Table 9.2 summarizes the results obtained in the study referred to in Example 3, which are relevant to discuss the effect of surface hydrophobicity of the polymeric materials on the attachment of *Alcaligenes denitrificans*. This bacterial strain has a ΔG_{iwi} = 18.2 mJ/mm^2, and this means that the interaction occurred between hydrophilic bacterial cells and hydrophobic polymeric materials.

Table 9.2. Surface tension components (γ^{LW} and γ^{AB}) and surface free energy of interaction between two surfaces of material i immersed in water (ΔG_{iwi}), in mJ/m^2, and the average number of adhered bacterial cells per square millimeter (± standard deviation) (adhesion in citrate minimal medium).

Material	γ^{LW} (mJ/m^2)	γ^{AB} (mJ/m^2)	ΔG_{iwi} (mJ/m^2)	Average cell number ($mm^2 \times 10^{-3}$)
PP	40.3	0	−67.2	32.1±1.6
HDPE	39.5	3.8	−59.2	20.0±1.1
PVC	37.5	0	−22.0	13.7±1.0
PMMA	43.5	0	−16.8	3.1±0.1

Table 9.2 also shows that an increase in the hydrophobicity of the polymeric supports promotes an increase in the number of adhered cells. In a closer inspection, if only the supports with $\gamma^{AB} = 0$ are considered it is possible to draw a linear correlation between the degree of hydrophobicity (ΔG_{iwi}) and surface colonization. HDPE is out of this correlation because of the finite value of γ^{AB}, which is a measure of the degree of residual hydration (van Oss 1997). Thus, in spite of the intermediate hydrophobicity of HDPE, bacterial adhesion is not favored, because the bound water layer has to be removed before complete contact can occur.

Example 6. Four porous microcarriers, clay, foam glass, pozzolana and sepiolite, used in an anaerobic fluidized bed reactor, were compared for their ability for biomass colonization (Alves *et al.* 1999). The results showed that sepiolite had the greatest microbial retention capacity, followed by clay, pozzolana and finally foam glass, when expressed as mass of volatile solids per internal porous volume ($gVS/L_{\text{internal porous volume}}$). In a further development from this study, the surface tension of the carriers was determined by the thin-layer wicking technique and the ΔG_{iwi} value for each type of material was calculated. The relation between ΔG_{iwi} and the amount of attached biomass is represented in Figure 9.3. In this case all the ΔG_{iwi} values are positive, meaning that all the assayed carriers have a hydrophilic nature. However, a decrease in ΔG_{iwi} corresponds to an increase in hydrophobicity. Thus, it can be said that Figure 9.3 expresses the linear correlation between support hydrophobicity and biomass retention capacity.

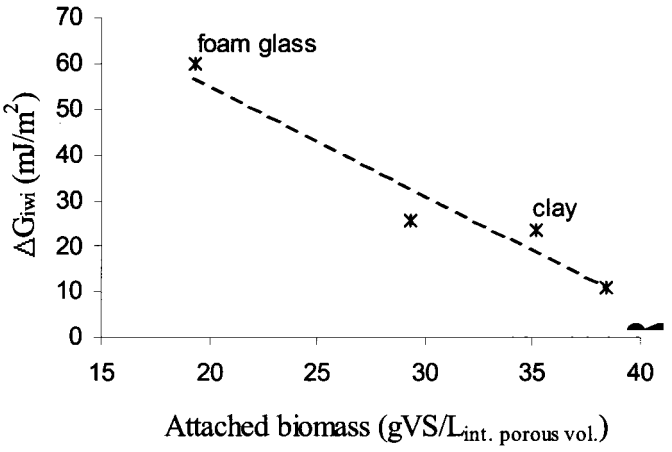

Figure 9.3. Relation between the attached biomass and the degree of hydrophobicity, expressed as ΔG_{iwi}.

9.3.2 Surface charge and cation bridging

The electrical charge of the interacting surfaces is very often mentioned in adhesion studies. However, rather than surface charge, what is usually experimentally determined is the zeta potential, but both quantities can be directly related. When a particle moves through an electrolyte solution, under the influence of an electric field, part of the diffuse electrical double-layer of ions moves with the particle, while the outer region remains with the bulk phase of the environment. The interface between these two ionic regions is known as the hydrodynamic slip-plane, and the potential at this plane, with respect to an electrode at an infinite distance away in the environment, is the zeta potential (ζ). The surface potential (ψ_o), assuming a linear Poisson–Boltzmann distribution of ions, is related to the zeta potential through

$$\psi_o = \frac{1}{4\pi\varepsilon}\,\zeta\,[1 + (d/R)]\,\exp(\kappa d) \tag{9.7}$$

where d is the distance between the particle surface and the slip-plane, R is the particle radius and κ is the reciprocal double-layer thickness or Debye–Hückel parameter (Oliveira 1992). The total surface charge (Q) can be related to the surface potential by

$$Q = 4\pi\varepsilon R\,(1 + \kappa R)\,\psi_o \tag{9.8}$$

where ε is the dielectric constant of the medium. This demonstrates that a given material has a very negative surface charge when its measured zeta potential has a high negative value.

As was briefly alluded to before, in the usual operational pH range in biofilm reactors, most of the materials (bacteria + supports) display a negative zeta potential (negative surface charge). This means that, in most the cases, bacterial adhesion is not mediated by electrostatic interactions, because these assume a repulsive effect. Nevertheless, it is possible to modify the surface of certain types of support to render them positively charged, which can be important in laboratory studies but is almost economically prohibitive for large-scale applications.

Example 7. The adhesion of *Pseudomonas putida* to different types of glass bead was assayed, namely: normal beads (Tamson ballotini 31/10), beads etched with 5% HF to roughen the surface, silanized beads with increased hydrophobicity, and beads positively charged by treatment with diethylaminoethyl-dextran (Gjaltema *et al.* 1997). The tests, performed under batch conditions with occasional gentle rocking, showed that the cells adhered best to the hydrophobic or positively charged beads.

The bridging effect of divalent cations, especially Ca^{2+}, in biological systems has been frequently referred to in the literature. These cations have a hydrophobizing effect on negatively charged particles (van Oss 1994), and according to some authors are related to increased reversible adhesion (McEldowney 1994; Takeuchi *et al.* 1997).

Referring to Example 2, the higher efficiency of limestone particles, followed by basalt, which was attributed to a higher number of adhered cells, can be a consequence of the hydrophobizing effect of Ca^{2+}, strongly present in limestone and in smaller quantities in the plagioclases of basalt. In Example 6, the support displaying a higher amount of adhered biomass was sepiolite, which is the material richest in surface divalent cations, in this case Mg^{2+}, as determined by electron dispersion spectroscopy (EDS).

Another interesting effect is the paradoxically facilitated adhesion between very negatively charged surfaces when divalent cations are present in the liquid medium.

Example 8. Referring once more to the study mentioned in Examples 3 and 5, the adhesion assays were done in two different liquid media: sodium phosphate buffer saline (NaPBS), containing only monovalent cations and citrate minimal medium (containing Ca^{2+}, Fe^{2+} and Mg^{2+}). The ionic strength was the same in both cases. The values of the zeta potential for bacteria and the polymeric materials used as supports are presented in Table 9.3. They did not show significant deviations from one liquid medium to another.

Table 9.3. Zeta potential (± standard deviation) of *Alcaligenes denitrificans* and of the polymeric materials used as carriers at pH 7.3.

Material	Zeta potential (mV)
Bacteria	−34.8±2.2
Polypropylene (PP)	−45.0±1.9
High-density polyethylene (HDPE)	−38.3±1.6
Polyvinylchloride (PVC)	−37.7±2.1
Polymethyl-methacrylate (PMMA)	−29.8±1.5

The average number of bacterial cells per square millimeter adhering to each type of polymeric material, in NaPBS and in citrate minimal medium, is shown in Figure 9.4.

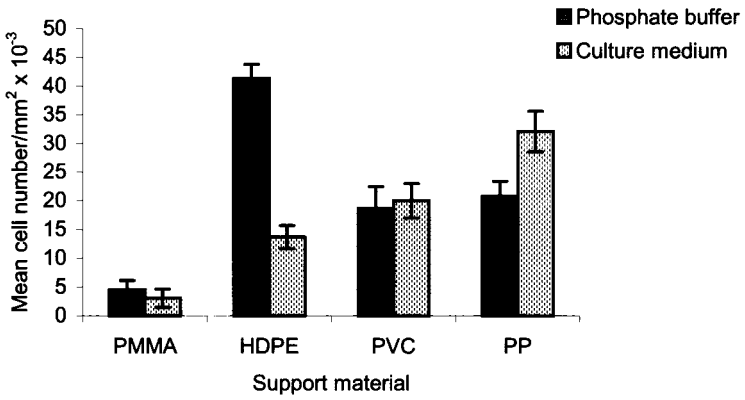

Figure 9.4. Average number of cells of *Alcaligenes denitrificans* per square millimeter adhering to the polymeric supports, in PBS and culture medium.

In the presence of divalent cations (citrate medium), adhesion to the more negatively charged materials is more favorable. In this situation, the number of adhered cells is linearly dependent on the increasingly negative values of zeta potential (Figure 9.5).

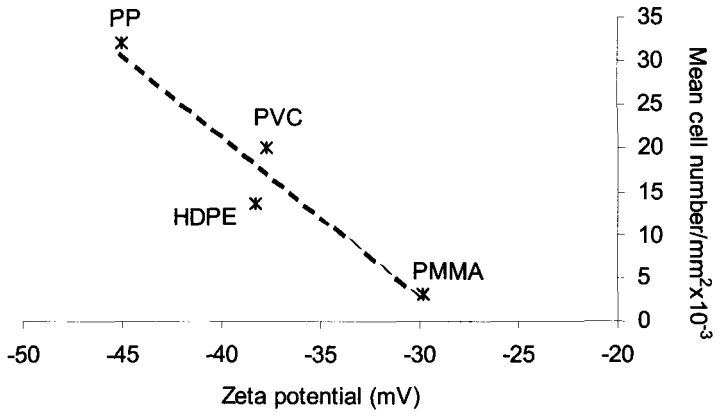

Figure 9.5. Relation between the number of adhering cells of *Alcaligenes denitrificans* per square millimeter and the zeta potential of the polymeric supports.

In the absence of divalent cations (NaPBS), this tendency disappears and it is only possible to directly relate bacterial adhesion with the decrease of the total XDLVO energy of interaction.

Although the properties mentioned above are determinant for adhesion, the amount of biomass present in a carrier also depends on the available surface area for microbial attachment, which is related to other physical properties.

9.3.3 Surface roughness and porosity

There are many reports in the literature about the advantages of using porous and rough supports for biofilm development. Apart from displaying a high surface area, a rough surface and/or internal pore space may provide a more hydrodynamically quiescent environment, thereby reducing the detachment of immobilized cells by hydraulic shearing forces (Bryers 1987; Characklis 1990; Quirynen and Bollen 1995).

The accumulation of microorganisms in porous structures is dependent upon the cell dimensions, the mode of reproduction and the pore diameter of the material (Messing and Oppermann 1979a). Accordingly, to achieve high accumulation of microbes that reproduce by fission, at least 70% of the pores of an inorganic carrier should have pore diameters in the range of one times the smallest major dimension through five times the largest major dimension of the cell. If the microbes reproduce by budding, the highest accumulation is achieved if at least 70% of the pores have diameters in the range of one times the smallest

dimension of the cell to less than four times the largest cell dimension. The microorganisms that form spores and exhibit mycelial growth are considered in another report (Messing and Oppermann 1979b). For those, the highest accumulation is considered to be attained when 70% of the pores have diameters in the range of one times the smallest dimension of the fungal spore to less than about 16 times the largest dimension of that spore. Shimp and Pfaender (1982) also reported that microbe size crevices favor surface colonization. Later, Wang and Wang (1989) mathematically calculated the theoretical maximum cell retention capacities of microcarriers with different pore sizes and concluded that a mean pore diameter within a range of 2–5 times the mean cell diameter would yield the maximum immobilized cell densities. In methanogenic fluidized bed reactors, under similar start-up conditions, porous microcarriers were capable of reducing the start-up times by more than 50% compared with sand (Yee *et al.* 1992).

Some authors consider that surface roughness is even more important for colonization than internal surface area (Petrozzi *et al* 1991). Fox *et al.* (1990) concluded that surface roughness is critical in biofilm development during the start-up of an expanded bed reactor. The biofilm began in crevices that were protected from shear forces.

Example 9. The four porous microcarriers of Example 6 were characterized in terms of roughness, pore size, attached biomass and specific methanogenic activity (SMA). The latter was expressed as mass of volatile fatty acids (as COD) removed per mass of adhered volatile solids per day. The corresponding values are presented in Table 9.4. As was already mentioned, sepiolite had the highest hydrophobicity, a surface with the higher concentration of divalent cations and good cell crevice size. It is the most favorable carrier for biomass attachment and consequently for biofilm development. Despite that, it shows a paradoxical behavior, displaying the smallest methanogenic activity.

Table 9.4. Surface characteristics, attached biomass (g VS/$L_{\text{internal porous volume}}$) and specific methanogenic activity (SMA) of the attached biomass (g VFA-COD/g VS_{attached}·day) (± standard deviation).

Material	Roughness	Pore size	Attached biomass	SMA
Sepiolite	++++	cell size crevices	38.4±2.4	0.173±0.007
Clay	+++	10–100 μm	35.1±1.0	0.329±0.003
Pozzolana	++	10–300 μm	29.3±1.3	0.340±0.038
Foam glass	+	20–1000 μm	19.3±1.4	0.289±0.010

+, Lowest degree of roughness; ++++, highest degree of roughness.

This example suggests that a great accumulation of biomass, or the accumulation inside pores of very small size, can give rise to strong limitations in mass transfer through and from the inner biomass. This will lead to a decrease in the expected activity. It is important to be aware of this fact, especially when working with expanded bed reactors, where by an adequate manipulation of the operating conditions the unwanted excessive growth of biofilms can be controlled.

9.4 CONCLUDING REMARKS

In biofilm reactors, the selection of the supports for biomass immobilization is of great importance to obtain a stable biofilm leading to high overall reactor efficiency. The support must favor microbial adhesion, must be hard if subjected to high hydrodynamic shear stress, must have a low cost and must be easily available. It should also be noted that a quick, strong and uniform attachment of microorganisms to the support surface is essential to lower the start-up time of the reactor. The physicochemical properties of the support generally required to promote a stable adhesion are: a high degree of hydrophobicity; the existence of divalent cations (Ca^{2+} and Mg^{2+}) at the surface; and a certain degree of roughness. To account for biomass detachment due to hydrodynamic shear or abrasion between carriers, roughness and porosity are of great importance. In faster-growing and higher-yielding biofilm cultures, the biofilm efficiency is less dependent on these two surface characteristics. On the contrary, slow-growing and low-yielding biofilm cultures should benefit from immobilization on rough and porous supports. However, bacteria retained in the internal surface area or inside a niche may experience diffusional resistance to the flux of substrates and products. The use of supports with large pores can overcome this problem, because in this situation the transport of metabolites can also be mediated by internal convective flow.

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