THE ROLE OF HYDROPHOBICITY IN BACTERIAL ADHESION

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*In biological systems, hydrophobic interactions are usually considered to be the strongest of all long-range non-covalent interactions. Considering hydrophobicity as the energy of interaction, ΔG_{iwi}, between two entities (i) immersed in water (w): then a positive value means that i is hydrophilic, and when ΔG_{iwi} has a negative value, i is hydrophobic. In other words, an increase in ΔG_{iwi} means a decrease in hydrophobicity. The above concept was used in the interpretation of various adhesion experiments: (I) adhesion of a denitrifying strain (**Alcaligenes denitrificans**) to polymeric surfaces; (II) adhesion of an anaerobic consortium to porous microcarriers; (IV) adhesion of **Staphylococcus epidermidis** to polymeric materials, used in medical indwelling devices. In all the mentioned studies a linear correlation was obtained between the degree of hydrophobicity of the supporting surfaces and the number of adhered cells.*

Introduction

The effect of substratum wettability uppon bacterial adhesion has been known for a long time, especially after the studies of Dexter *et al.* (1975) on bacterial attachment in marine systems. The wettability of a surface is now more generally expressed in a reverse sense and is referred to as hydrophobicity. More recent studies have shown that the hydrophobicity of solid surfaces influences adhesion of bacteria, eukaryotic cells and proteins (Busscher & Weerkamp 1987; Margel *et al.* 1993; Prime & Whitsides 1993; Wiencek & Fletcher 1997; Taylor *et al.* 1997). On the other hand, bacteria and other microorganisms, including viral particles, have evolved many different ways to use the hydrophobic effect in order to adhere to substrata (Doyle 2000). In fact, there are compelling reasons to believe that the hydrophobic effect may be the primary driving force for the adhesion of most pathogens (Duncan-Hewitt 1990).

Despite the recognized importance of the hydrophobic effect, it has been difficult to give it a satisfying definition (Doyle 2000). Definitions of hydrophobicity have been given in terms of thermodynamic principles or from a hypothetical point of view. The latter arose mainly from the attempts to explain biological recognition on the basis of hydrophobic interactions occurring between enzyme-substrate, antigen-antibody, lectin-carbohydrate or adhesin-receptor. From a chemical point of view, Blokzijl and Engberts (1993) gave the following detailed definition of hydrofobicity. "*At moderate temperatures and pressures, apolar compounds are poorly soluble in water. Traditionally, the reluctance of apolar compounds to dissolve in water has been attributed to the hydrophobicity of these compounds, in other words, their fear of water. In fact, the term hydrophobicity is misleading. The London dispersion interactions between water and apolar compounds are favourable and quite substantial. It is more appropriate to point out that the apolar compound must intrude into a liquid that is characterised by an extremely*

high cohesive energy density. Each water molecule is strongly inclined not to sacrifice any of its hydrogen bonds, leading inevitably to significant reorientation of water molecules at the surface of the nonpolar solute molecule".

According to van Oss (1997), 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. Its sole driving force is the hydrogen bonding (also designated AB forces or Lewis Acid-Base) energy of cohesion between the surrounding water molecules. This means that the AB forces, if strongly asymmetrical or monopolar, are responsible for the orientation of water molecules adsorbed on the surfaces. As a result of this 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. If on the other hand 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 Lifshitz-van der Waals (LW) attraction. "Hydrophobic" compounds or surfaces do not repel water rather they attract water with a substantial binding energy, albeit not quite 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*".

Techniques to determine hydrophobicity

Several techniques have been used to determine the degree of hydrophobicity of bacterial cells or paticulate materials. For materials that can be obtained in a plate flat shape, hydrophobicity has been very often expressed in terms of the contact angle formed by a sessile drop of water. In the case of bacterial cells, one of the most 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 characterise 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 determination, 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*. 1988), 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.

Since some drawbacks were found in the utilisation of two-phase systems, they concluded that water contact angle measurements were the best method for the quantification of cell hydrophobicity. Subsequently, it was observed that the zeta potentials of those hydrocarbons that were commonly used in MATH could be highly negative (Busscher *et al*. 1995). MATH may therefore measure a complicated interplay of long-range van der Waals and electrostatic forces and various short-range interactions (van der Mei *et al.* 1995), rather than reflect solely hydrophobic interactions. In a recent survey (Doyle 2000) of the methods employed to assess microbial cell surface hydrophobicity, which included methods other than those already mentioned, it was also concluded that ~contact angle methods are the most definitive descriptors of cell surface hydrophobicity. These other methods were based on the adhesion of cells to either liquids or solid materials were dependent on factors such as temperature, time, pH, ionic strength and relative concentration of interacting species. All of these factors conspire to influence the adhesive event (Ofek & Doyle 1994).

Quantification of hydrophobicity

As expressed earlier, using the techniques described above it is only possible to assess hydrophobicity in qualitatively. According to van Oss (1997), however, it is possible to determine the absolute degree of hydrophobicity of any given substance (i) vis-à-vis 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 the case 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 which 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., $\Delta G_{iwi}<0$) then the surfaces of i are genuinely hydrophobic. The more negative ΔG_{iwi} becomes then the more hydrophobic that entity is; the more positive ΔG_{iwi} becomes then the more hydrophilic that entity becomes.

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

$$\Delta G_{iwi} = - 2\gamma_{iw} \qquad \text{(I)}$$

Whilst direct surface tension measurements are possible for liquid-gas interface, the determination of surface free energy (i.e. surface tension) of solids can only be obtained by indirect measurements. Therefore, γ_{iw} can be determined by contact angle measurements or thin layer wicking, the latter being appropriate when the solid material is in particulate form (e.g. sand).
Considering the approach of van Oss et al. (1988, 1991), the surface free energy of a solid or a liquid, γ_i^{TOT}, is the sum of apolar Lifshitz-van der Waals γ_i^{LW}, and polar acid-base interactions, γ_i^{AB}:

$$\gamma_i^{TOT} = \gamma_i^{LW} + \gamma_i^{AB} = \gamma_i^{LW} + 2\left(\gamma_i^- \gamma_i^+\right)^{1/2} \qquad \text{(II)}$$

The polar interactions are mainly due to London dispersion interactions, but induction (Debye) and orientation (Keesom) interactions may also be involved (van Oss et al., 1988). In many situations the polar acid-base interactions consist entirely in hydrogen bonding and in the most general sense they are electron donor, γ_i^-, and electron acceptor, γ_i^+, interactions. Thus, the interfacial free energy between entity i and water (w) can be expressed as:

$$\gamma_{iw} = \gamma_i^{LW} + \gamma_w^{LW} - 2\left(\gamma_i^{LW}\gamma_w^{LW}\right)^{1/2} + 2\left[\left(\gamma_i^+\gamma_i^-\right)^{1/2} + \left(\gamma_w^+\gamma_w^-\right)^{1/2} - \left(\gamma_i^+\gamma_w^-\right)^{1/2} - \left(\gamma_i^-\gamma_w^+\right)^{1/2}\right] \quad \text{(III)}$$

The surface free energy components of water are known (Table 1), but the corresponding values for the entity i have to be determined.

Contact angle measurements

If entity i is a solid (e.g. substratum or microbial cells), then the surface tension components can be determined by measuring the contact angles (θ) formed by three different liquids (for which apolar and polar components are known) on its surface. Thereafter, three forms of the following equation, resulting from Young's equation, are obtained. These can be solved simultaneously to calculate, γ_i^{LW}, γ_i^+ and γ_i^-:

$$W_a = \gamma_l(1+\cos\theta) = 2\left(\gamma_i^{LW}\gamma_l^{LW}\right)^{1/2} + 2\left(\gamma_i^-\gamma_l^+\right)^{1/2} + 2\left(\gamma_i^+\gamma_l^-\right) \quad \text{(IV)}$$

Where W_a is the work of adhesion and the subscript *l* means liquid.

Table 1 Surface tension parameters (mJ/m^2) of the liquids commonly used in contact angle measurements and thin-layer wicking.

Liquid	γ^{TOT}	γ^{LW}	γ^+	γ^-
Water	72.8	21.8	25.5	25.5
Glycerol	64.0	34.0	3.9	57.4
Formamide	58.0	39.0	2.3	39.6
Diiodomethane	50.8	50.8	0	0
n-Decane	23.8	23.8	0	0
α-Bromonaphthalene	44.4	44.4	0	0

In order to measure contact angles on microbial cells it is necessary to provide these cells as a homogeneous cell lawn. This is usually achieved by collecting the cells onto a cellulose filter membrane (Busscher *et al*. 1984).

Thin-Layer Wicking

When the substratum is particulate, a situation that is very common in biofilm reactors using suspended carriers, then it is not possible to use contact angle measurement techniques. In such instances the solid material must be ground to a powder (average particle size <0.38 μm), before using the thin-layer wicking

technique to determine the surface free energy components of the solid (Van Oss 1991; Chibowski & Holysz 1992; Teixeira *et al*. 1998). This technique is based on the penetration of a liquid (wicking) into a porous solid. The velocity of liquid penetration into the solid depends upon the dispersion forces of the liquid and capillarity forces of the solid,and is expressed by Washburn's equation:

$$x^2 = \frac{rt}{2\eta}\Delta G \qquad \text{(V)}$$

Where, x is the penetrated distance, r is the capillary radius, t is the penetration time of the distance x, η is the liquid viscosity and γ_{lv} is the liquid surface tension. Experimentally, a suspension of the powdered material is deposited on a glass plate (e.g. microscope slide), to form a porous layer. In this case, an "effective radius", R, must replace the capillary radius. It has to be noted that the Washburn's equation is valid only when the solid surface possesses a duplex film of the penetrating liquid ahead of the liquid front. According to the experimental conditions and the characteristics of the liquid the following four situations have to be considered (Teixeira *et al*. 1998).

(i) The liquid used is apolar with low surface tension (e.g. n-decane, Table 1) and completely wets the solid surface that was formerly equilibrated with the liquid vapour (pre-contacted).

In this situation, when the liquid penetrates the thin porous layer a liquid film forms ahead of the liquid front. Thus, no contact angle is formed and the free energy variation accompanying the process is equivalent to γ_l and the relationship $x^2 = f(t)$ is described by:

$$x^2 = \frac{Rt}{2\eta}\gamma_l \qquad \text{(VI)}$$

and the effective radius, R, can be determined.

(ii) The liquid has the same characteristics as above, but the thin layer was not exposed to the saturated vapour (bare plate).

In this case, there is no liquid film on the surface ahead of the liquid front and a single liquid layer is formed and a modification of the Washburn's equation is needed to describe the function $x^2 = f(t)$.

$$x^2 = \frac{Rt}{2\eta}\Delta G_b \qquad \text{(VII)}$$

$\Delta G_b = W_a - W_c$, is the specific free energy change that takes place during the liquid penetration. Where $W_a = 2\left(\gamma_s^{LW}\gamma_l^{LW}\right)^{1/2}$ is the work of adhesion of the apolar liquid to the surface, and $W_c = 2\gamma_l$ is the work of liquid cohesion.

Combining these relationships it is possible to calculate the apolar component of the surface tension of the solid:

$$\gamma_s^{LW} = \left(\frac{\Delta G_b + 2\gamma_{yl}}{2\sqrt{\gamma_l^{LW}}} \right) \quad \text{(VIII)}$$

(iii) The liquid is polar with high surface tension and does not completely spread onto the pre-contacted solid surface.

A duplex film of the liquid is formed on the surface (for instance by adsorption of the vapour) before the liquid penetrates the porous layer and a contact angle (θ) is formed. The relationship $x^2 = f(t)$ is then expressed by:

$$x^2 = \frac{Rt}{2\eta}(\gamma_l \cos\theta) = \frac{Rt}{2\eta}\Delta G_p \quad \text{(IX)}$$

ΔG_p means the free energy change that accompanies penetration along the pre-contacted plate.

(iv) The same liquid as in (iii), but the thin layer of the solid material was not equilibrated with the saturated vapour of the liquid.

The liquid does not totally wet the surface and a contact angle is formed between the liquid and the surface. The maximum pressure exerted by the liquid film is equivalent to the work of dispersion. The function $x^2 = f(t)$ is:

$$x^2 = \frac{Rt}{2\eta}(W_a - W_c + \Delta G_p) \quad \text{(X)}$$

In the case of polar liquids

$$W_a = 2\left(\gamma_s^{LW}\gamma_l^{LW}\right)^{1/2} + 2\left(\gamma_s^{-}\gamma_l^{+}\right)^{1/2} + 2\left(\gamma_s^{+}\gamma_l^{-}\right)^{1/2} \quad \text{(XI)}$$

Using two polar liquids a system of two equations can be obtained in order to calculate γ_s^+ and γ_s^-.

All these forms of the Washburn's equation should give a linear dependence of $x^2 = f(t)$, with the slope depending on the free energy changes accompanying the liquid penetration into the porous medium.

Usually three probe liquids are used for each liquid. Six plates (covered with the porous layer) are assayed: three bare plates and three pre-contacted with the liquid vapour. The pre-contact is performed by placing the plates in a closed vessel and by allowing them to equilibrate with the saturated vapour of the liquid for 20-24 hours (Teixeira *et al*. 1998).

The Hydrophobic Effect on Bacterial Adhesion

*Adhesion of **Alcaligenes denitrificans** to Polymeric Supports*

Experimental tests were performed in order to select a suitable carrier for *Alcaligenes denitrificans* in an inverse fluidised bed reactor. The polymeric materials included high density polyethylene (HDPE), polypropylene (PP), poly(vinyl chloride) (PVC) and polymethylmethacrylate (PMMA). These tests showed that adhesion occured to the greatest extent onto PP followed by PVC, HDPE and least to PMMA (Teixeira & Oliveira 1999). The hydrophobicity of the polymeric materials was determined by contact angle measurements, and the numbers of adhered bacterial cells enumerated automatically by image analysis. Table 2 summarises the relevant results obtained that are required to discuss the effect of substrate surface hydrophobicity on the attachment of *Alcaligenes denitrificans* (Gram negative).

A. denitrificans has a $\Delta G_{iwi} = 18.2$ mJ/mm^2. This implies that the interaction occurred between hydrophilic bacterial cells and hydrophobic polymeric materials. Table 2 also shows that an increase in the hydrophobicity of the polymeric supports promotes increased numbers of adhered cells. If only those supports with $\gamma^{AB} = 0$ are considered, then it is possible to draw a linear correlation between the degree of hydrophobicity (ΔG_{iwi}) and surface colonisation. HDPE falls out of this correlation due to 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 favoured, since the bound water layer has to be removed before complete contact can occur.

Table 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 cells of *Alcaligenes denitrificans* per mm^2 (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}x10^{-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

Those materials were also characterized in terms of their surface charge (Teixeira & Oliveira 1999). It was found that between pH 6 and 9 they are all negatively charged, including the bacterial cells, as would be expected (Oliveira 1992). The polymer that displayed the greatest negative charge was PP, although it was also the material that showed more attached cells, but was also the most hydrophobic. These observations further enhance the effect of hydrophobicity in the process of bacterial adhesion.

Adhesion of an Anaerobic Consortium to Inorganic Carriers

Experiments were conducted in order to selecting an appropriate carrier to be used in an anaerobic fluidised bed reactor. The following materials were tested: clay, foam-glass, pozzolana, and sepiolite, were compared in terms of their ability for

biomass accumulation (Alves *et al*. 1999). Biomass accumulation, expressed as mass of volatile solids per internal porous volume ($gVS/L_{internal\ porous.volume}$), showed that sepiolite had the greatest microbial retention capacity followed by clay, pozzolana and foam-glass. In a further development of 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 1. In this case, all the ΔG_{iwi} values were positive, meaning that all of the carriers were hydrophilic. Decreases in ΔG_{iwi}, however, correspond to increases in hydrophobicity. It can therefore be said that Figure 1 expresses the linear correlation between the degree of support hydrophobicity and biomass retention capacity.

*Adhesion of **Staphylococcus epidermidis** to Polymeric Supports*

S. epidermidis (Gram positive) is a commensal organism associated with skin and a common etiological agent associated with infections of indwelling medical devices, such as catheters and intracardiac prostheses (Dickinson & Bisno 1989; Cramton *et al*. 1999). Coagulase-negative staphylococci commonly express a polysaccharide/adhesin, that is responsible for the increased adherence of such strains (McKenney *et al.* 1998). We therefore investigated the ability of different strains of *S. epidermidis* to colonise four polymeric materials, commonly used in indwelling devices: polyethylene (PE), silicone (SI), expanded polytetrafluorethylene (ePTFE) and cellulose diacetate (CDA). The bacterial strains used were: *S. epidermidis* ATCC 35984 (RP62A) and strains M187 and M187-Sn3 kindly donated by Gerald B. Pier (Channing Laboratory, Harvard Medical School, Boston, MA, USA). Strains RP62A and M187 both have capsule and are polysaccharide-adhesin positive (PS/A+), whilst M187-Sn3 is an isogenic mutant of M187 and is polysaccharide-adhesin negative (PS/A-). The polymeric materials and the bacterial cells were characterised in terms of their hydrophobicity by contact angle measurements.

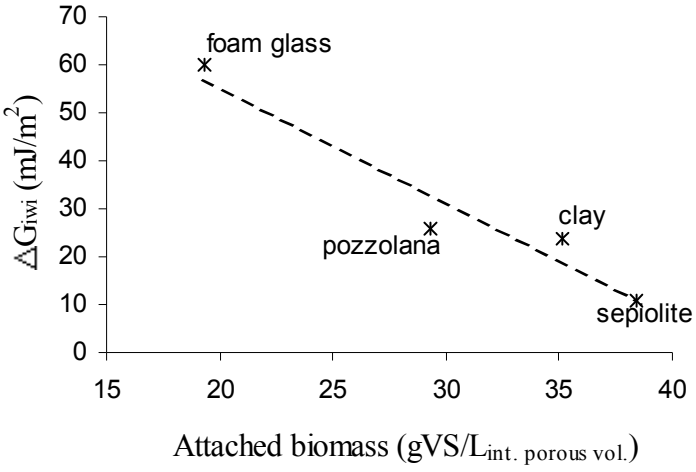

Figure 1 Relation between the attached anaerobic biomass and the degree of hydrophobicity of various inorganic carriers, expressed as ΔG_{iwi}.

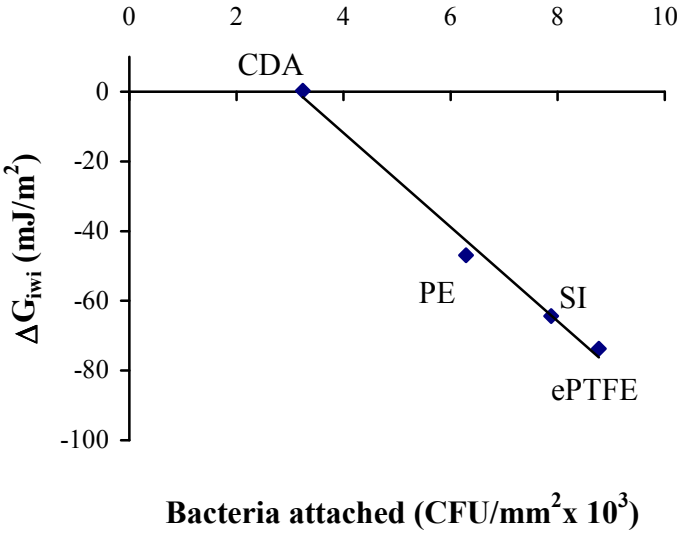

Figure 2 Relation between the number of attached cells of *Staphylococcus epidermidis* ATCC 35984 (RP62A) and the degree of hydrophobicity (ΔG_{iwi}) of various types of substrata.

Adhesion of these staphylococcal strains to cellulose diacetate (Fonseca *et al*. in press) showed higher numbers of adherent cells for PS/A+ strains (Table 3). These strains were also more hydrophobic than the PS/A- phenotype.
When the adhesion of the RP62A strain was assessed against the four polymeric materials a linear relationship was obtained between the numbers of attached bacterial cells and the hydrophobicity of the surface (Figure 2). In other words, the most hydrophilic material (CDA) was also the least adherent. It has to be pointed out that the polymeric materials were also characterised in terms of their surface charge, using zeta potential measurements (Zeta Meter 3.0+, USA), and roughness determined by a laser rugosimeter (Perthometer S3P, Perthen, Germany). No direct correlation could, however, be found between these properties and cellular attachment.

Table 3 Number of *S. epidermidis* cells expressed as colony forming units (CFU) adhered to diacetate cellulose after 1 hour of incubation in phosphate buffer saline (PBS), for each phenotype assayed and the respective ΔG_{bwb} (mJ/m^2)[a]

Strain	N. of cells adhered (CFU/mm^{2}x10^3	ΔG_{bwb} (mJ/m^2)[a]
RP62A	3.31±0.17	17.5
M187	3.33±0.39	17.4
M187-Sn3	2.08±0.40	31.9

[a] ΔG_{bwb} is a measure of bacterial cells hydrophilicity, because all the values are positive, but a higher degree of hydrophilicity means a lower degree of hydrophobicity.

The hydrophilicity of CDA was enhanced by chemical treatments that involved deacetylation and phosphorylation (Fonseca et. al., in press) Deacetylated CDA

(CDA-D) and phosphorylated CDA (CDA-P) were submitted to similar physical characterization as the earlier materials. The adhesion assays, performed with strain RP62A, showed that the number of adhered cells decreased significantly in the case of CDA-D and was even more pronounced for CDA-P (Figure 3).

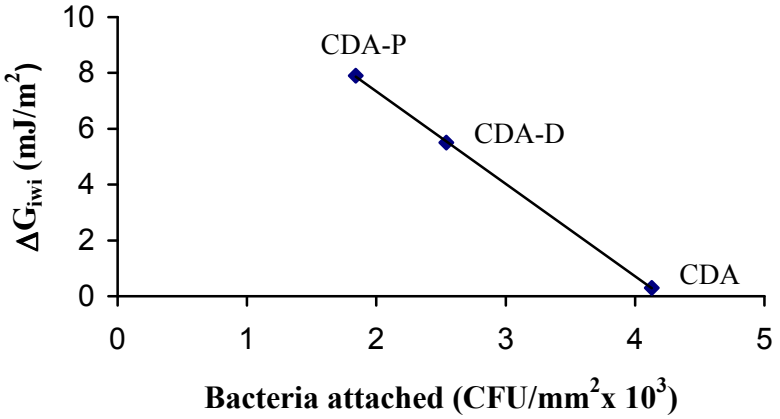

Figure 3 Relation between the number of attached cells of *Staphylococcus epidermidis* ATCC 35984 (RP62A) and the degree of hydrophobicity (ΔG_{iwi}) of cellulose diacetate (CDA) surfaces.

Once again, a linear correlation was obtained between the number of attached cells and surface hydrophobicity. More precisely, because CDA and its derivatives are hydrophilic (all with $\Delta G_{iwi} > 0$), it can be said that an increase in the degree of hydrophilicity lowers the number of attached cells.

Conclusion

The linear correlations obtained between the numbers of attached cells and the degree of hydrophobicity of the substrata (above) were only possible through quantification of "hydrophobicity". Even, if these observations were found to the co-incidental, then is still no doubt that hydrophobic interactions plays an important role in the adhesion process. In very simple words, it can be said that the interaction between two hydrophobic entities is favoured because they can enter into closer contact through the facilitated "squeezing of water" in between.

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