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Thermodynamic analysis of marine bacterial attachment to oligo(ethylene glycol)-terminated self-assembled monolayers

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Abstract

Colloidal models are frequently used to model the thermodynamics of bacterial attachment to surfaces. The most commonly used of such models is that proposed by van Oss, Chaudhury and Good, which includes both non-polar and polar (including hydrogen bonding) interactions between the attaching bacterium, the attachment substratum and the aqueous environment. We use this model to calculate the free energy of adhesion, ΔG_{adh}, for attachment of the marine bacterium *Cobetia marina* to well defined attachment substrata that systematically vary in their chemistry and their ability to attach bacteria, namely a series of oligo(ethylene glycol) (OEG) terminated self-assembled monolayers that vary in the number of OEG moieties. For this system, the values of ΔG_{adh} calculated using VCG do not correlate with observed attachment profiles. We examine the validity of a number of assumptions inherent in VCG and other colloidal models of adhesion, with special attention paid to those regarding bacterial surfaces.

Keywords: Fouling resistance; Thermodynamics; Oligo(ethylene glycol); *Cobetia marina*; Surface tension; Interfacial tension; Bacterial attachment

Background

Attachment of microorganisms to a submerged solid support is the first step in development of a biofilm [1-3]. Interactions of attaching bacteria with the substratum may strongly influence the final properties of the subsequent biofilm including structure [4], adhesion strength [5] and global developmental processes, such as quorum sensing [6] and exopolysaccharide production [7]. As such, attachment is the most logical place to prevent, as in the case of biofouling, or engineer, as in the case of microbial biofuel cells, biofilm formation. Accurately modeling initial attachment events is, therefore, critical not only to understanding a fundamental biological process, but also to optimizing the formation of biofilms for a variety of applications.

Colloidal models remain a popular approach to predicting attachment for bacteria that do not have specific attachment peptide sor sugar binding proteins commonly found on pathogenic or commensal organisms [8-11], or for which such specific attachment mechanisms are unknown [12,13]. Bacteria exhibit some colloid-like properties: their sizes fall within the upper limits of colloid particles as generally define (~1 μm) and they, like colloidal particles, tend to collect at interfaces. If and until selection pressure requires specific attachment mechanisms, bacteria may be best served by exploiting colloidal interactions at surfaces rather than expending metabolic energy to encode and express specific attachment molecules, thus allowing them a greater range of likely supports for biofilm formation.

A number of colloidal models have been proposed to explain biomolecular attachment in general, and bacterial attachment in particular [12,14-17]. Of these the van der Waals-Lewis-Acid–base model proposed by van Oss, Chaudhury and Good (VCG) in the late 1980s [18] 0020 seems to most accurately reflect the interfacial processes most likely to be important in biological attachment: nominally apolar Lifshitz-van der Waals interactions and polar Lewis acid–base interactions, including the special

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cases of hydrogen-bonding [13,19,20] and electrostatic interactions [13]. In addition to the VCG model being used itself to study microbial interactions at the interface, it further informs the extended Derjauin-Landau-Verwey-Overbeek model, currently in widespread use [21-23] and also the recently developed Chen/Qi ratio [24].

We recently used VCG to examine the role of the substratum-water interfacial tension (γ_{SL}) in elucidating differences in fouling resistance between oligo(ethylene glycol) (OEG)-terminated self-assembled monolayers (SAMs), correlating $\gamma_{SL,}$ and its components, with increased degrees of hydrogen bonding between the SAM and water [25]. In that study, the increase in hydrogen bonding between OEG-SAMs and water correlated with increasing units of EG within the SAM and with decreased bacterial attachment. In this manuscript, we expand these studies to calculations of free energy of adhesion (ΔG_{adh}), based on the VCG model, for attachment of the marine bacterium, *C. marina*, to OEG-SAMs.

SAMs of alkanethiolates on gold terminated with varying lengths of OEG [26] are a particularly attractive model system for studying the relationship between bacterial attachment and an estimated ΔG_{adh}. The number of ethylene glycol (EG) units (n = 1-6) in a SAM determines its resistance to protein adsorption [27], as well as attachment of mammalian cells [28], algal zoospores [29] and marine bacteria [30,31]. The difference between OEG-SAMs that resist and permit cellular adsorption is one EG moiety [32]; for example, for the marine organism, *Cobetia marina,* an OEG-SAM with three OEG moieties (EG n = 3) permits bacterial attachment, whereas one in which EG n = 4 resists bacterial attachment [25]. The OEG system is also unique in that the relatively low attachment observed on these surfaces means that over the course of our experiments (2 hr) it is extremely unlikely that an attachment maximum will be encountered, resulting in consistent attachment kinetics throughout the course of the study. Previous studies [33] suggest ΔG_{adh} for non-specific attachment to SAMs that attach bacteria will be negative.. Because the difference between OEG-SAMs that attach and those that do not attach microbes is one residue, the VCG model predicts that ΔG_{adh} be negative for those SAMs attaching *C. marina* and positive for those not attaching the organism, with the transition from negative to positive being associated with an increase of one EG residue. We observed no correlation between number of attaching bacteria and ΔG_{adh} of OEG-SAMs calculated using the VCG model of microbial attachment. In the process, we examined several parameters that might lead to this non-correlation and conclude that both errors endemic to the laboratory use of VCG and assumptions, common to all colloidal models of attachment, about the bacterial surface are the main source of the observed nonconcordance.

Methods

Preparation of self-assembled monolayers

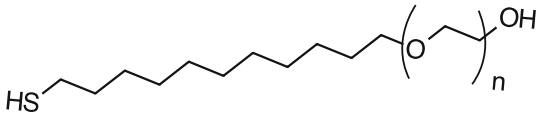
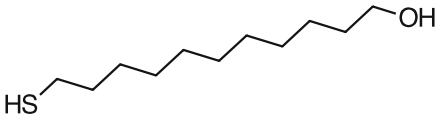
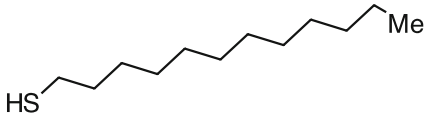
SAMS were prepared as described previously [31]. Briefly, glass coverslips (Fisher, Fairlawn, NJ) were treated with 70:30 H_2SO_4/H_2O_2 for at least 1 hour, rinsed in copious amounts of deionized water, and dried under dry nitrogen. The samples were then loaded into a thermal evaporator. After evacuating the chamber to 1 millitorr, 15 Å Cr was deposited followed by 300 Å Au. For Wilhelmy plate contact angle evaluation, metal was deposited on both sides of the sample.

After metal deposition was complete, samples were immersed in 1 mM ethanolic solutions of OEG-terminated alkanethiols (EG n = 1-6; all from Prochimia, Poland; a kind gift from the lab of M. Grunze), 1-mercaptoundecanol (OH; Aldrich, St. Louis MO), undecanethiol (CH_3; Aldrich, St. Louis, MO), 1-mercaptoundecyl trimethylamine (NMe_3^+; Prochimia, Poland) or poly(ethylene glycol) substituted undecanethiol ($EG_{500;}$ MW 2000, Rapp Polymere, Tübingen) and incubated for at least 2 hours. Prior to use, samples were sonicated in fresh ethanol for 5 minutes and dried under a stream of dry nitrogen immediately before analysis. Structures of thiols used for this study are shown in Table 1.

Bacterial strains and culture conditions

All media and buffers were prepared with de-ionized water generated by a system using tap water processed sequentially through water softening, reverse osmosis and

Table 1 Structures of thiols used in this study

Thiol	Structure
OEGn = 1,2,3…	
OH	
CH_3	
NMe_3^+	

ion exchange (Barnstead-Thermolyne RoPure/Nanopure system). The final resistivity of the processed water was greater than 18MΩ cm^{-1}. Marine Broth 2216 (MB, Difco, Franklin Lakes, NJ) was prepared according to manufacturer's instructions. Marine Agar (MA) was prepared by the addition of 1.5% Bacto agar (Difco) to MB. Artificial sea water (ASW) contained 400 mM NaCl, 100 mM $MgSO_4$, 20 mM KCl, 10 mM $CaCl_2$ [34]. Modified basic marine medium plus glycerol (MBMMG) contained 0.5× ASW plus 19 mM NH_4Cl, 0.33 mM K_2HPO_4, 0.1 mM $FeSO_4 \cdot 7H_2O$, 5 mM Trishydroxyaminomethane hydrochloride pH 7, and 2 mM glycerol [34,35]. *Cobetia marina* (basonym, *Halomonas marina*) ATCC 25374, is stored in frozen (−70°C) stock aliquots, made from first generation cultures of the original ATCC lyophilate, in MB containing 20% glycerol. Experimental stock preparations were maintained on MA slants and were stored at 4°C for up to 2 weeks. Prior to inoculation into a chemostat, a single colony from a MA slant was inoculated into 50 mL of MB and grown overnight with shaking at 25°C. A chemostat culture was established by inoculating 3 mL of the overnight culture into MBMMG. The chemostat was maintained at a flow rate of 1 mL min^{-1} (dilution rate, 0.16 h^{-1}) with constant stirring. The concentration of the subsequent culture was ~10^7 cells mL^{-1}.

Bacterial attachment to surfaces

SAMs prepared on gold films coated on 60 × 24 mm coverslips were placed into a flow-cell apparatus [31] which was then mounted onto the stage of an optical microscope (Axioskop, Zeiss, Jena) and connected to the outflow of the chemostat. The *C. marina* culture was allowed to flow through the cell at a rate of 1 mL min^{-1} for two hours. Bacterial attachment was monitored through a CCD camera attached to the microscope. The images were fed to a computer using Axiovision software (Zeiss). At the end of the attachment time, images of 10 fields of view within 10 mm of the horizontal midline of the slide were captured, the number of attached bacteria counted and the average cell density for each slide determined.

Contact angle measurements

Contact angles of SAMs were measured using the Wilhelmy Plate method on a Krüss K100 tensiometer with Lab Desk 303 (Krüss, Jena) software. Contact angle liquids were water (18 MΩ cm^{-1}; Millipore Academic System; Millipore, Billerica, MA), diiodomethane (99% ReagentPlus; Sigma-Aldrich, St. Louis,MO), formamide (Omipure; EMD; Gibbstown, NJ), glycerol (anhydrous; J.T. Baker, Phillipsburg, NJ) and hexadecane (Sigma Aldrich). Samples were double-sided SAMs made on 22×40 mm, thickness 1 coverslips (Fisher). For measurement, SAMs were immersed to a depth of 1 cm, with contact angle measured on the sample six times per mm (a total of 60 data points per sample). For each SAM formulation, a minimum of three samples was measured per probe liquid (deionized water, diiodomethane, formamide, glycerol or hexadecane).

Contact angles of bacteria were taken on mats of bacteria supported on cellulose acetate filters (0.2 μm pores; Millipore, Billerica) [33,36]. Approximately 120 mL of chemostat culture were filtered, followed by an equal volume of deionized water to remove residual salt. The filtered bacteria were then allowed to air dry before contact angle analysis. To ensure that the surface of the mat was dry without being totally dehydrated, water contact angles were initially taken every 10 minutes during the drying cycle until they became stable; contact angles for analysis were taken during the time period in which it was determined that the water contact angle did not change, in our case, 40–70 minutes after drying commenced [33]. Contact angles were determined, using the angle analysis tool on ImageJ image processing software (NIH; [37]), from photographs taken with DROPImage software(Ramé-Hart, Succasunna, NJ) linked to a Ramé-Hart contact angle goniometer. Bacterial contact angles were measured with the same liquids as used for SAMs (deionized water, diiodomethane, formamide, glycerol and hexadecane).

Calculation of surface and interfacial tensions using VCG

Surface tension and components were calculated using the van Oss-Chaudhury-Good (VCG) equation [18]:

$$1 + \cos\theta = \frac{2(\sqrt{\gamma_{SV}^{LW}\gamma_{LV}^{LW}} + \sqrt{\gamma_{SV}^{+}\gamma_{LV}^{-}} + \sqrt{\gamma_{SV}^{-}\gamma_{LV}^{+}})}{\gamma_{LV}} \quad (1)$$

where: $\Upsilon_{SV}{}^{LW}$ and $\Upsilon_{LV}{}^{LW}$ are the Lifshitz-van-der-Waals components of the surface tensions of the substratum and the probe liquid, respectively, $\Upsilon_{SV}{}^{-}$ and $\Upsilon_{LV}{}^{-}$ are the Lewis basic (electron donating, hydrogen bond accepting) components, and $\gamma_{SV}{}^{+}$ and $\Upsilon_{LV}{}^{+}$ are the Lewis acidic (electron accepting, hydrogen bond donating) components. Υ_{LV} is the total surface tension of the probe liquid. Because there are 3 unknowns, contact angles were taken with three different probe liquids and the unknowns $\gamma_{SV}{}^{LW}$, $\Upsilon_{SV}{}^{-}$ and $\gamma_{SV}{}^{+}$ calculated by simultaneously solving the three equations using MATLAB software (Mathworks, Natick). Values for the surface tension of bacteria (γ_{BV}) were obtained by substituting the contact angle of the probe liquids on bacterial mats into Equation (1).

Interfacial tensions between the bacterium and the substratum (γ_{BS}), the bacterium and water (γ_{BL}) or the substratum and water (γ_{SL}) were calculated by inserting

the relevant components of γ_{SV}, γ_{BV} and γ_{LV} into the following equation (shown here for γ_{SL}) [13]:

$$\gamma_{SL} = (\sqrt{\gamma_{SV}^{LW}} - \sqrt{\gamma_{LV}^{LW}})^2 - 2[(\sqrt{\gamma_{SV}^{+}} - \sqrt{\gamma_{LV}^{+}})(\sqrt{\gamma_{SV}^{-}} - \sqrt{\gamma_{LV}^{-}})] \quad (2)$$

ΔG_{adh} calculations. ΔG_{adh} was calculated by two different methods in order to insure fidelity of sometimes-complicated equations and for further analysis of components of ΔG_{adh}. The basic equation for calculating ΔG_{adh} using colloidal models is a special case of the Dupré equation [14,18,38]:

$$\Delta G_{adh} = \gamma_{BS} - \gamma_{BL} - \gamma_{SL} \quad (3)$$

and is quickly calculated using the values for γ_{BS}, γ_{BL}, and γ_{SL} obtained from Equation (2).

The VCG model, however, specifies that ΔG_{adh}, like surface and interfacial tension, can be divided into two components, one apolar (ΔG_{adh}^{LW}) and one polar (ΔG_{adh}^{AB}) [13,18]:

$$\Delta G_{adh} = \Delta G_{adh}^{LW} + \Delta G_{adh}^{AB} \quad (4)$$

Both components of ΔG_{adh} can be derived according to Equation (3), with the interfacial tensions being calculated using iterations of Equation (2). The resulting components of ΔG_{adh} are [13,18]:

$$\Delta G_{adh}^{LW} = \left(\sqrt{\gamma_{BW}^{LW}} - \sqrt{\gamma_{SV}^{LW}}\right)^2 - \left(\sqrt{\gamma_{BV}^{LW}} - \sqrt{\gamma_{LV}^{LW}}\right)^2 - \left(\sqrt{\gamma_{SV}^{LW}} - \sqrt{\gamma_{LV}^{LW}}\right)^2 \quad (5)$$

And

$$\Delta G_{adh}^{AB} = 2[\sqrt{\gamma_{LV}^{+}}\left(\sqrt{\gamma_{BV}^{-}} + \sqrt{\gamma_{SV}^{-}} - \sqrt{\gamma_{SV}^{-}}\right) + \sqrt{\gamma_{LV}^{-}}\left(\sqrt{\gamma_{BV}^{+}} + \sqrt{\gamma_{SV}^{+}} - \sqrt{\gamma_{LV}^{+}}\right) - \sqrt{\gamma_{BV}^{+}\gamma_{SV}^{-}} - \sqrt{\gamma_{BV}^{-}\gamma_{SV}^{+}} \quad (6)$$

Results and discussion

Attachment of C. marina to SAMs

The number of cells attached to OEG-SAMs after 2 hrs exposure to *C. marina* (7.5×10^7 cells/mL) is shown in Figure 1. Included for comparison is the number of cells attached to a methyl terminated SAM (CH_3-SAM), which we have previously shown to attach *C. marina* in relatively large numbers [31,39]. As previously reported [25], the number of *C. marina* attached to OEG-SAMs over a 2 hour period decreases with increasing length of EG, with no attachment occurring when $EG_n \geq 4$ These data are consistent with those reported for zoospores of the macroalga. *Ulva linza,* on a similar series of OEG-SAMs [32].

Contact angles

Advancing contact angles (θ_{AX} where "X" is the liquid) of SAMs are summarized in Table 2. θ_{AW} for OEG-SAMs with EG n = 2-6 were statistically identical (~34°; $p = 0.33$), but were different from EG n = 1 and OH (EGn = 0) ($p \leq 0.01$); the latter two SAMs had statistically similar ($p = 0.14$) contact angles (average = 26°). There was no significant difference for advancing contact angles of formamide (θ_{AF}) or dioodomethane (θ_{AD}). Although differences were observed between advancing

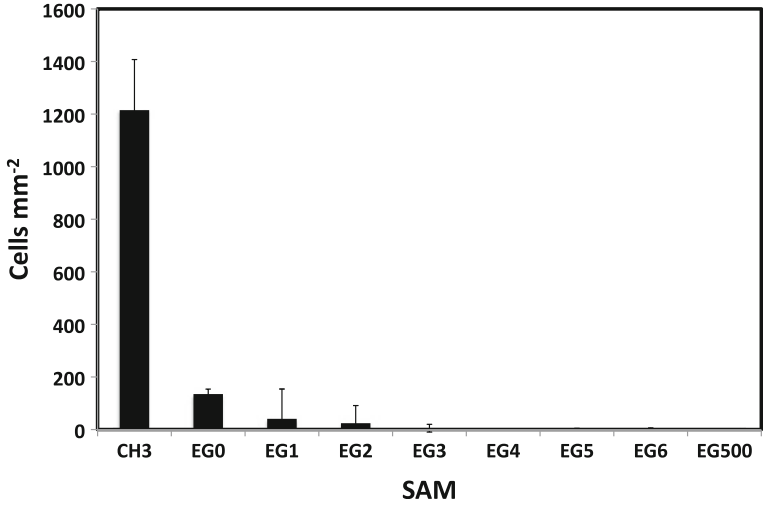


Figure 1 Two hour attachment of *Cobetia marina* to EG-SAMs and a methyl-terminated positive control. Attachment was considered to be 0 when the number of cells mm^{-2} was less than one ($EGn \geq 1$). Error bars represent 95% confidence levels.

Table 2 Contact angles of SAMs with different contact angle liquids

SAM	Formamide	Glycerol	Hexadecane	Water	Diiodomethane
OH	35 ± 1°	24 ± 1°	39 ± 2°	5 ± 3°	24 ± 2°
OEG_1	25 ±1°	29 ± 2°	35 ± 2°	7 ± 2°	28 ± 2°
OEG_2	25 ±2°	29 ± 1°	39 ± 1°	15 ± 1°	32 ± 1°
OEG_3	27 ± 2°	23 ± 2°	38 ± 3°	18 ± 1°	33 ± 1°
OEG_4	24 ± 3°	27 ± 2°	42 ± 1°	1 ± 1°	33 ± 1°
OEG_5	32 ±2°	26 ± 2°	44 ± 3°	11 ± 1°	34 ± 1°
OEG_6	27 ± 2°	24 ± 3°	47 ± 2°	11 ± 1°	33 ± 2°
PEG	29 ±1°	21 ± 1°	60 ± 1°	3 ± 2°	37 ± 1°
CH_3	66 ± 1°	90 ± 1°	96 ± 0°	36 ± 1°	108 ± 2°
NMe_3^+	26 ± 2°	25 ± 1°	39 ± 2°	9 ± 3°	18 ± 2°

contact angles of hexadecane (θ_{AH}), all were less than 20°, and thus, few differences were observed when $\cos\theta_{AH}$, the input into all subsequent calculations, was compared. Glycerol contact angles (θ_{AG}) were statistically different between all EG-SAMs. As previously observed [25], θ_{AG} was able to differentiate between fouling (OH-, EG- (n = 1-3)) and nonfouling (EG- n = 4-6, PEG) SAMs.

Contact angles of *C. marina* bacterial mats supported on cellulose acetate filters using the five probe liquids from this study are presented in Table 3. Uncoated cellulose acetate filters were permeable to all liquids, rendering them effectively completely wettable ($\theta_{AX} = 0°$).

ΔG_{adh} calculations

ΔG_{adh} was calculated using both the Dupré Equation (3) and by the method proposed by VCG, as outlined above and in Equations (4), (5) and (6). To calculate ΔG_{adh} according to the Dupré equation, the interfacial tensions γ_{BS}, γ_{BL} and γ_{SL} were first calculated according to Equation (2). γ_{BS} on all surfaces was statistically zero, γ_{BL} was −6.2 ±3 mJ m^{-2}. γ_{SL} for this series of OEG-SAMs has been reported previously [25] and is large and negative, driving ΔG_{adh} in a positive direction. Both methods of calculation yielded identical results (p = 1.0) for all SAMs; results are summarized in Figure 2.

As demonstrated in Figure 2, ΔG_{adh} as calculated from contact angles of water, diiodomethane and glycerol on bacteria and OEG-SAMs did not reflect the resistance to attachment of bacteria to OEG-SAMs with OEG > 3, nor was attachment correlated in a systematic way to ΔG_{adh}. Based on previous applications of the VCG model, [35,40] one would predict that ΔG_{adh} for SAMs with EG ≤ 3 would be negative; they are not. Clearly ΔG_{adh} as calculated from contact angles and the VCG model is either not physically meaningful or is not capturing all the relevant information in the system. We now consider how the inputs into the equations for ΔG_{adh} ((3) and (4)) influence this value and from where the discrepancy between attachment of *C. marina* and estimates of ΔG_{adh} may stem.

Table 3 Contact angles (θ) of mats of logarithmic phase *C. marina* supported on cellulose acetate filters

Liquid	θ
Diiodomethane	34 ± 2°
Formamide	53 ± 2°
Glycerol	64 ± 2°
Hexadecane	2 ± 1°
Diiodomethane	34 ± 2°

We have previously demonstrated [31,39] that *C. marina* attaches in far greater numbers to SAMs terminated with a methyl group (CH_3-SAM) when compared to OH-SAMs. When we calculated ΔG_{adh} for *C. marina* attaching to a CH_3-SAM using liquid combination water, diiodomethane and glycerol (WDG), a value of −32.7 ± 5 mJ m^{-2} was obtained; the value is negative, as would be expected for a SAM attaching large numbers of bacteria (average coverage 1,121 ± 192 cells mm^{-2} under the same experimental conditions as for OEG-SAMs). When we calculated ΔG_{adh} for attachment of *C. marina* to a trimethylamine-terminated SAM (NMe_3^+-SAM; average coverage 662 ± 44 cells cm^{-2} under the same experimental conditions as for OEG-SAMs) using inputs from WDG, however, ΔG_{adh} = 24 ± 4 mJ m^{-2} is positive and statistically similar to OH-SAMs (ΔG_{adh} = 21 ± 4 mJ m^{-2}) that attached one fifth of the cells attached to NMe_3^+-SAMs (Figure 1). Because the ΔG_{adh} calculated for CH_3-SAMs is due only to Lifshitz-van-der-Waals interactions, with no polar components, and it had at least the expected negative sign for ΔG_{adh}, we explored the possibility that the VCG method of calculating ΔG_{adh} misses information about the polar components.

Our first examination was whether the polar liquids used to measure contact angles on SAMs and bacteria affected the resulting values of ΔG_{adh}. The three contact liquids used for VCG type analysis usually include one

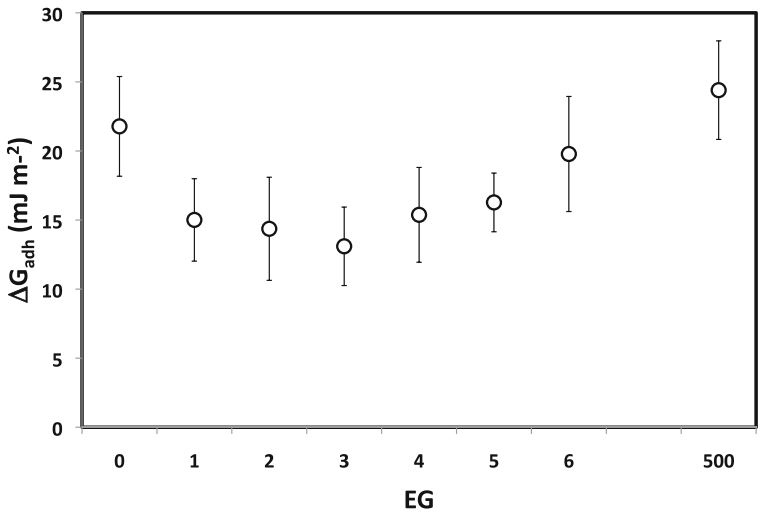


Figure 2 ΔG_{adh} of OEG-SAMs as a function of number of EG groups. Data from contact angles taken with water, diiodomethane and glycerol were modeled using the VCG model of bacterial attachment. Error bars represent 95% confidence levels.

apolar and two polar liquids, although, if the model is robust, any combination of contact angle liquids should result in the same value for ΔG_{adh}. As can been seen in Figure 3, this was clearly not the case; ΔG_{adh} values calculated from θ_{AX} taken three different sets of liquid: (i) water, diiodomethane and formamide (WDF), (ii) diiodomethane and glycerol (WDG) and (iii) water, glycerol and formamide (WGF) revealed different trends for ΔG_{adh}.

One criticism of the VCG method is that it tends to underestimate γ_{SV}^{+} on surfaces [12], which could significantly alter values calculated relying on this component of surface tension. For the OEG series, there was no statistical difference between different OEG surfaces using either WDG or WDF inputs, although the values obtained were less than one [25] which is much lower than the literature values for ethylene glycol (γ_{LV}^{+} = 1.6 mJ m^{-2}) [41]. To see how well this method captures γ_{SV}^{+} on a surface for which γ_{SV} is expected to be dominated by Lewis acidic interactions, we tested all liquids on a SAM terminated with trimethylamine (NMe_3^{+}), which, as an onium, should be Lewis acidic. Figure 4 shows γ_{SV} and its components for NME_3^{+} SAMs for all liquid groups. That NMe_3^{+} -terminated SAMs appear to be hydrogen bond accepting is highly unexpected since the lone pair of electrons that normally mediate hydrogen bond formation with nitrogen are fully occupied. Surface analysis including X-ray photoelectron spectroscopy (data not shown) confirms that

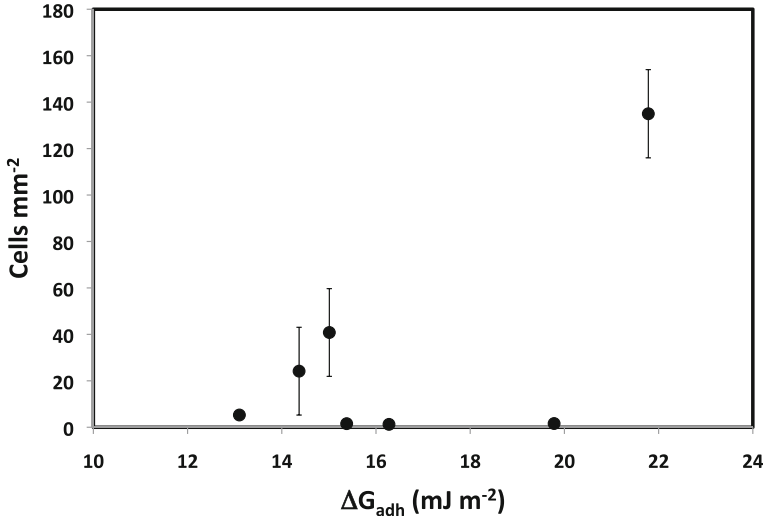


Figure 3 Attachment of *C. marina* as a function of ΔG_{adh} calculated from contact angles taken with water, diiodomethane and glycerol and calculated using the Van VCG model of bacterial attachment. Error bars represent standard error.

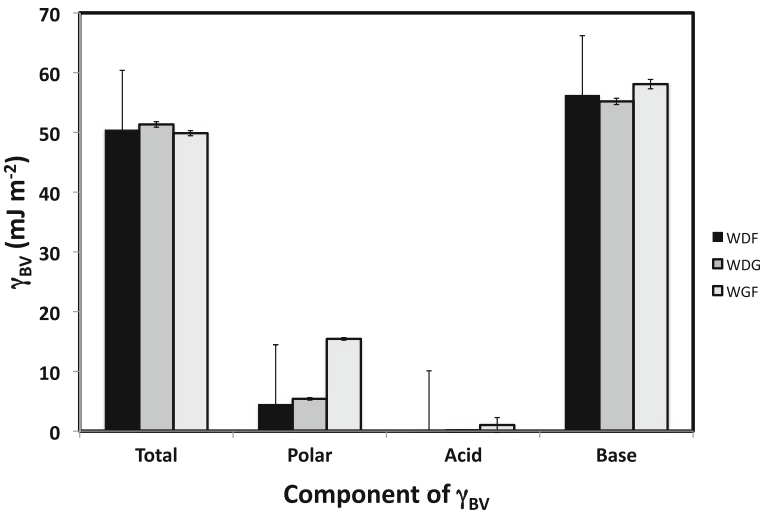


Figure 4 Total, polar, Lewis acidic and Lewis basic components NMe_3^+ (γ_{SV}) as calculated using the VCG model of bacterial attachment from contact angles measured using water, diiodomethane and formamide (WDF), water diiodomethane and glycerol (WDG) or water, glycerol and formamide (WGF). Error bars represent 95% confidence levels.

no adducts are found on this surface, so this observation is not a result of surface contamination. We suspect that this underestimation of γ_{SV}^+ is due to the fact that, although water and glycerol have significant Lewis acidic components, they are not sufficiently large or dominant for their contact angles to result in accurate estimation of γ_{SV}^+. The lack of polar organic liquids with a significant Lewis acidic monopole was noted by van Oss, Chaudhury and Good in their original proposal of their model [18,20] as a possible shortcoming and the situation has not noticeably improved in the last two decades. Only bromoform ($\gamma_{LV}^+ = 1.72$ mJ m^{-2}) has a γ_{LV}^+ value >1 mJ m^{-2}, the minimum value considered by VCG to be useful in calculations, but it cannot be used in a general laboratory setting as its safe handling requires a respirator.

Most applications of the VCG model, and indeed the original model itself, assume that for water $\gamma_{LV}^+ = \gamma_{LV}^- = 25.5$ mJ m^{-2} [13,20,33,40], whereas others, most notably Lee [42], have shown that above 0°C, water is, in fact, much more likely to donate hydrogen bonds (be more Lewis *acidic*) than accept them and that, at room temperature, $\gamma_{SV}^+ = 1.8\gamma_{SV}^-$. Because the acid and base components for all other liquids were calculated based on the VCG assumption, surface tensions for other liquids, and the resulting surface tensions for measured solid surfaces, overestimated the Lewis-basic component [12,42]. When we used WDF contact angle values to calculate ΔG_{adh}, the use of Lee values resulted in lower values for ΔG_{adh}, whereas there was no statistical difference ($p \geq 0.05$) for ΔG_{adh} when calculated using contact angles from the WDG liquid set. Use of the Lee values in calculations did not, however, shift ΔG_{adh} such that it was negative for SAMs attaching cells, nor did it sufficiently raise the values of γ_{SV}^+ for NMe_3^+-SAMs such that they now were predominantly Lewis acidic. We must, therefore, conclude, in agreement with others [35], that using the modified values of the components of γ_{LV} does not have a substantial effect on VCG calculations.

An assumption that, as far as we know, remains unchallenged with regard to the VCG model and, more accurately, its application to analysis of surface tension has to do with the way the apolar components of substratum and bacterial surface tension, γ_{SV}^{LW} and γ_{BV}^{LW}, are calculated. VCG includes in these values not only attractive London dispersion interactions resulting from fluctuating dipoles and resulting induced dipoles, but also possible repulsive interactions between fixed dipoles (Keesom interactions) and fixed dipole (Deybe) interactions [13]. Although the latter are considered to be insignificant [13], we maintain that they are neglected, particularly given that the apolar liquid of choice for most VCG analysis [13,19,35,40,43], diiodomethane, although considered strictly apolar [13,19,33,40,42,43], has a small, but possibly significant, acid monopole (0.72 mJ m^{-2}) [44]. To test the significance of this monopole, we examined the effect of including this monopole on calculations of surface tension components, ΔG_{adh}^{LW} and ΔG_{adh}^{AB} As an added test, we compared values obtained with diiodomethane, both including and ignoring the acid monopole, with those obtained with hexadecane, which is known to be completely apolar.

The differences in γ_{SV}^{LW} of polar SAMs as measured with hexadecane and diiodomethane is substantial, whether or not the acidic monopole of the latter is considered; γ_{SV}^{LW} calculated from diiodomethane is ~45 mJ m^{-2}, if the acid monopole is included that value drops to ~35 mJ m^{-2},

whereas that using hexadecane is ~27 mJ m^{-2}, similar to that for the CH_3-SAM. The inclusion of the acid monopole calculation of γ_{SV}^{LW}, thus, has a profound effect on γ_{SV}^{LW}. These results indicate that either the supposition that either Keesom or Debye interactions are insignificant may be false (as they seem to account for nearly 10 mJ m^{-2} when the acid monopole included in the calculation) or the assumption that acid or base components <1.0 mJ m^{-2} are insignificant [13,18] is unwarranted; in either case, further re-examination of these assumptions is suggested by these results.

The value of γ_{SV}^{LW} for organic polymers and biopolymers is noted by van Oss to be universally ~45 mJ m^{-2} [13,18], but we propose that this may be an artifact based on the use of diiodomethane as an apolar liquid that seems to always result in this value (see also the value of γ_{BV}^{LW} in Figure 4). Examination of the γ_{SV}^{LW} of SAMs as calculated with diiodomethane and hexadecane seems to indicate that this value may be an artifact. γ_{SV}^{LW} of an OH-SAM, is, for example, about 20 mJ m^{-2} higher than γ_{SV}^{LW} of an CH_3-SAM using contact angles of diiodomethane and ignoring the acid component. If we compare γ_{LV}^{LW} for n-decane (23.8 mJ m^{-2}) and 1-decanol (22 mJ m^{-2}) we see no such increase [41]. More to the point, the total surface tension for dodecane (similar to CH_3-SAM) is 25.6 mJ m^{-2}, whereas that for dodecanol is 28.6 mJ m^{-2}. Taking into account that γ_{LV}^{AB} for most alcohols [41] is 3–6 mJ m^{-2}, it seems very unlikely to us that a similar change on a SAM surface would nearly double the value of γ_{SV}^{LW}. On the other hand, a SAM surface is well ordered, and the main surface exposed would be OH (or NMe_3^+) so a slightly higher value for OEG-SAMs might be expected. If we take into account, however, the published values of γ_{LV}^{LW} for (21.8 mJ m^{-2}), glycerol (34 mJ m^{-2}) and ethylene glycol (29 mJ m^{-2}) [41], are still much lower than those proposed for most polymer surfaces calculated using diiodomethane. We propose, therefore, that hexadecane or some other completely apolar liquid is the most relevant when analyzing SAMs. We should note, however, that using hexadecane alone is insufficient to bring about a correlation between ΔG_{adh} and attachment.

The second part of the system that must be considered in reconciling attachment to ΔG_{adh} is the surface energetic components of the attaching bacteria, presented in Figure 5. Based on preliminary data from microbial adhesion to solvents (MATS; data not shown), a qualitative method of assessing general surface tension of bacteria [19] and also its affinity for CH_3-terminated SAMs, we expected *C. marina* to be quite hydrophobic, rather than only moderately hydrophobic (θ_{AW} =52˚) as results from contact angles on bacterial mats suggest. In this analysis, *C. marina* was also Lewis basic, which would explain its low attachment to Lewis basic surfaces (OH, COO^-, short chain OEG-SAMs [25,39]) and its propensity to attach to amine-containing surfaces. The value of γ_{BS} with regard to the polar SAMs was statistically zero, regardless of which polar liquids were used for contact angle analysis. For comparison, when γ_{BS} is calculated for the interaction between *C. marina* and CH_3-SAMs, which attach *C. marina* in relatively large numbers (in this study 1,215 bacteria mm^{-2} compared to 135 ± 19 bacteria mm^{-2}

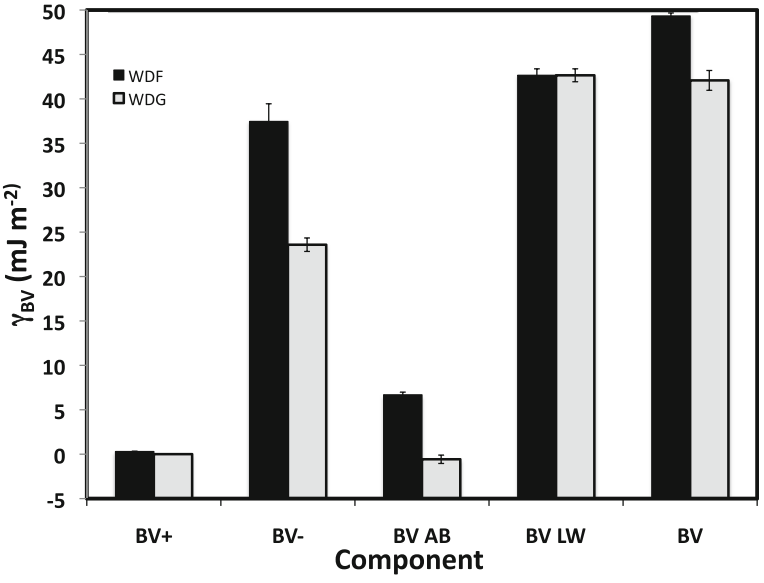


Figure 5 The surface tension and components of logarithmic phase *Cobetia marina* calculated from contact angles of water, diiodomethane and formamide and water, diiodomethane, and glycerol (circles) using the VCG model. Error bars represent 95% confidence levels.

for OH-SAMs), it is ~6 mJ m^{-2}; unfortunately, this value is also positive, which, according to the Dupré equation [3] would tend to disfavor attachment. γ_{BL} between *C. marina* and water was ~6.7 mJ m^{-2}, which would tend to favor attachment (γ_{BL} is subtracted from γ_{BS} in equation [3]). Clearly, then ΔG_{adh} is dominated by γ_{SL}, which we have shown previously to be negative, and increasing in magnitude with longer length OEG-SAMs, while highly positive for CH_3-SAMs.

The role of the organization of water around the OEG-SAMs as it relates to thermodynamics has been extensively discussed both in our previous work on the relationship between γ_{SL} and attachment of *C. marina* [25] and its included references, with the conclusion that γ_{SL} calculated using VCG supports current mathematical theories that suggest hydrogen bonding between OEG moieties and water renders bacterial attachment to these surfaces entropically disfavored. The observation that bacterial attachment to a CH_3-SAM is increased in this system is energetically favored might lead one to make similar arguments that attachment of bacteria is entropically favored near a hydrophobic surface; in other bacterial systems [31,39,43], however, attachment to CH_3-SAMs is lower than to other SAMs, suggesting that the influence of the increased entropy upon bacterial attachment is not significant.

We suspect, however, that calculations of γ_{BV} and the resultant interfacial tensions γ_{BS} and γ_{BL} do not capture information most relevant to attachment. An assumption common to even the most carefully considered models of non-specific bacterial attachment is that surface tension is uniform across the bacterial cell (and, indeed, across a monoculture population). This assumption is simply invalid. The role of extracellular appendages such as flagella and pili in attachment are well known [45,46], and even though we deliberately chose *C. marina* as a model organism partly due to the lack of observable extracellular structures [47], years of observation have led us to conclude that the surface is very likely not uniform as we have observed the cells orient themselves differently on different SAMs during attachment. We and others have also proposed that bacteria have different attachment mechanisms on different surfaces [4,35,48]. Furthermore an emerging field of study, based on observations of attachment of *Caulobacter crescentus*, has made a strong case that during cell division the biochemistry of the cell envelope of the two daughter cells may be very different, and that genetically programmed biochemical heterogeneity on individual cells is present [49]. The relevant γ_{BV} and components to include in a free-energy calculation are, therefore, less likely to be those of the whole bacterium, but rather that of the part of the cell which is interacting with the SAM. We are currently developing a method by which the areas of individual cells involved in attachment to each SAM may be accurately assayed; preliminary results indicate that different regions of the *C. marina* cell surface do, indeed, interact differently with when SAM surface chemistry is altered.

We also initially assumed that attachment of *C. marina* to SAMs is nonspecific, *i.e.,* unmediated by receptor-ligand interactions. The lack of correlation ΔG_{adh} and attachment called this assumption into question. It has been demonstrated previously that ΔG_{adh} calculated using VCG does not correlate with attachment when the interaction between the bacteria and the surface is ligand mediated, *i.e.,* specific [33,40]. It is possible that *C. marina* possesses receptors that interact specifically with components of SAMs. We also considered the possibility that exopolymeric substances (EPS) secreted by planktonic *C. marina* might form conditioning films that would present specific ligands for attachment. Marine bacteria are known to produce exopolymeric substances while growing planktonically [50] and to attach to conditioning films of EPS formed on surfaces [51]. It was not hard, therefore to envision a scenario in which *C. marina* could produce EPS, even while in carbon-limited chemostat conditions, that could form conditioning films on SAMs, to which *C. marina* could bind specifically.

We tested for the presence of EPS deposited onto SAMs from filtered (0.45 μm nylon) chemostat and also tested the filtered effluent directly for carbohydrate, DNA and protein. The lectin concavalin A (ConA) binds specifically to α-D-mannosyl and α-D-glucosyl groups in carbohydrates and glycoproteins. These residues are frequently found in the EPS of *Pseudomonas aeruginosa* [52] and that of marine pseudomonads [50]. Alexa-dye-conjugated ConA staining of CH_3-SAMs exposed for two hours under flow to filtered chemostat effluent showed no discernable deposits, whereas the same SAMs exposed to 1 μg mL^{-1} dextran stained easily. The amount of dissolved carbohydrate in filtered chemostat effluent was estimated using the phenol-sulfuric acid method as modified by Jain for use in salt water [53] and no detectable (i.e. < 1 μg/mL) carbohydrate was found when compared with a glucose standard. Bradford assays for protein of the filtered effluent were similarly negative and no absorption peak was detected at 260 nm indicating the absence of nucleic acid. We thus conclude that, at least under our experimental conditions, EPS-derived conditioning films do not play a role in attachment of *C. marina* to SAMs.

A final criticism of VCG may lie in the ability of calculations based on so many contact angles and their attendant errors may make error propagation an issue, particularly for low contact angles (as in OH-SAMs in this study) where accuracy and precision during contact angle measurement is difficult. A model developed for much simpler systems may not be applicable to more

complex systems involving heterogeneous cells and populations. We believe that the measurements required to accurately model this system are becoming increasing possible, however, and that more comprehensive explanations will be rapidly forthcoming.

Conclusions

We have undertaken a systematic study of VCG model as it pertains to bacterial attachment and ΔG_{adh}. The key to this investigation is a series of SAMs differing only in the length of ethylene glycol chains presented at the surface, resulting in profoundly different attachment profiles. ΔG_{adh} as calculated using the VCG model, however, did not reveal even a qualitative correlation between this value and attachment. We conclude that the VCG model as currently utilized is insufficient to describe the relevant interaction occurring between the bacteria, attachment substratum and water. These results call into question the generalized use of contact angles and colloidal models based on them, such as VCG, as a substitute for surface energy in thermodynamic analyses of bacterial attachment. Because VCG is used not only in its own right, but also informs other models of attachment, most notably, the highly popular extended DLVO model, caution should be taken when drawing conclusions regarding the effect of substratum surface energy on attachment of microbes, at least until such time as contact angle probe liquids are identified which can accurately account for all components of surface tension.

In addition to the limitations VCG as applied to calculating γ_{SV}, we suggest a second important factor preventing accurate modeling of microbial attachment is inherent assumptions made about the surface energy of bacteria themselves. To date, all surface energy analyses of microbial cells assume that the population is uniform and that the surfaces of cells themselves are homogenous [12]; the average surface energy of both the population and individual cells is, thus the relevant information needed for input into VCG or any colloidal model. We and others, however, have demonstrated that different part of the cell are relevant for attachment to different substrata [34,48,54]. We are currently developing a method to probe which parts of the bacterial surface are relevant to attachment to different substrata is currently underway that might eventually lead to a more nuanced view of the bacterial cell surface and its relevance in attachment.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LKI designed and carried out all experimental protocols and drafted the manuscript. GPL conceived the idea of using OEG SAMs as a systematic tool for these studies and participated in the drafting of the manuscript. Both authors read and approved the final manuscript.

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