Zeta potential of motile spores of the green alga *Ulva linza* and the influence of electrostatic interactions on spore settlement and adhesion strength

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The zeta potential of the motile spores of the green alga (seaweed) *Ulva linza* was quantified by video microscopy in combination with optical tweezers and determined to be -19.3 ± 1.1 mV. The electrostatic component involved in the settlement and adhesion of spores was studied using electret surfaces consisting of PTFE and bearing different net charges. As the surface chemistry remains the same for differently charged surfaces, the experimental results isolate the influence of surface charge and thus electrostatic interactions. *Ulva* spores were demonstrated to have a reduced tendency to settle on negatively charged surfaces and when they did settle the adhesion strength of settled spores was lower than with neutral or positively charged surfaces. These observations can be ascribed to electrostatic interactions. © *2009 American Vacuum Society.* [DOI: 10.1116/1.3110182]

I. INTRODUCTION

When an aquatic micro-organism approaches a surface in order to settle and adhere, the forces experienced change with respect to those present during motion in the bulk water. These additional forces are determined by various surface parameters including chemistry, charge, mechanical properties, and morphology.[1] One of the forces presented by a surface is electrostatic interaction, which should influence settlement of spores, larvae, bacteria, and their interfacial adhesion strength.

Ulva linza is a marine alga (seaweed) of cosmopolitan distribution and a common member of biofouling communities. It disperses itself through the production of large numbers of spores ("zoospores"). These are small (5–7 μm diameter), motile, single cells possessing four flagella (the organelles responsible for motility) and bounded by a lipoprotein cell membrane: there is no polysaccharide-rich cell wall at this stage. The spores "settle" (i.e., attach) into a solid substrate from the water column, at which point they lose their flagella, secrete a glycoprotein adhesive which permanently attaches the spore to the substrate, and form a cell wall.[2] At a later stage the attached spore germinates, undergoes cell division, and eventually grows into a new plant. Zoospores of *Ulva* have been used extensively as a model system to study how fouling organisms of this type respond to surface properties or "cues" for settlement.[3,4] Such studies provide a basic understanding of fouling processes that may prove valuable in the development of novel antifouling coatings. In one particular study it was shown that protein-resistant hydrophilic oligothylene glycol surfaces[5] also resist the settlement and adhesion of zoospores of *Ulva*.[6] If such surfaces are rendered hydrophobic by aliphatic end groups, resistance to protein adsorption is reduced and the surfaces become more attractive for the settlement of the spores.[6] The resistance of hydroxyl terminated oligoethylene glycol surfaces toward protein adsorption is partly explained by electrostatic interactions.[5,7] Another indication that electrostatic interactions induced by charged end groups may affect adhesion of zoospores of *Ulva* is given by experiments of Ista *et al.*[8] Mixed self assembled monolayers (SAMs) consisting of methyl and hydroxyl terminated alkylthiols were compared to mixed SAMs composed of methyl and carboxylic acid terminated alkylthiols. In both cases the wettability of

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the surfaces was systematically varied by the mixture ratio of the two components. Comparison of the settlement of spores of *Ulva* on the two types of mixed SAMs showed that for equivalent wettabilities, settlement on the carboxyl group-containing SAMs tended to be lower than for the hydroxyl terminated ones.[8] The observed difference might be either due to selective response to the carboxylic acid groups or due to electrostatic repulsion.

Besides self-assembled monolayers, discharge treatment can be used to create carboxylic acid groups on polymer surfaces. Jansen and Kohnen[9] showed that this surface modification led to a significant reduction of biofilm growth of *Staphylococcus epidermidis* KH6 compared to the pristine polymer. This effect was discussed in terms of the repulsive electrostatic interactions of the mainly negatively charged bacteria[10,11] with the discharge-treated surfaces.[9,12] Mutants of *Staphylococcus aureus* with increased negative charge in their cell wall were also unable to form biofilms on slightly negatively charged polystyrene or glass surfaces.[12] The general affinity of biomacromolecules for charged surfaces has been demonstrated by selective immobilization of proteins to locally charged surfaces.[13] Electrostatic fields in the form of electrets based on positively charged fluorinated ethylenepropylene have been observed to influence differentiation of cultivated neuron cells.[14]

The goal of the present study was to consider the influence of electrostatic interactions between spores of *Ulva* and quasi-permanently positively or negatively charged polytetrafluoroethylene (PTFE), so-called electrets. Electrets are materials that can retain electrical charge or polarization.[15] As the chemistry is identical for all surfaces and only the net charge is changed, any observed differences in the adhesion of the spores is caused by electrostatic forces.

In parallel with these studies on charged films, we also measured the zeta potential of swimming spores. The zeta potential is a combination of the surface charge on the body of a cell or particle plus any adsorbed layer at the interface and is specific to the medium in which the surface is immersed.[16] Along with other surface properties, such as hydrophobicity, the zeta potential is important in determining the likelihood that single celled algae and bacteria will adsorb to surfaces.[17–21] Many studies on bacteria and cyanobacteria have shown that in most natural situations they have a negative zeta potential.[10,22–24] Unicellular algae also tend to be negatively charged[25] and this is usually attributed to the presence at the surface of polysaccharide materials with associated negatively charged carboxyl groups. This was demonstrated for diatoms by Gelabert *et al.*[26] who showed differences between the electrophoretic mobility of whole cells and isolated siliceous frustules. Examples of the zeta potential of unicellular green algae are -20 mV for *Selenastrum capriconutum* in buffered 0.1*M* sodium perchlorate at *p*H 8.0 (Ref. 27) and -27 and -30 mV, respectively, for *Chlorella* in raw and tap water at *p*H 7.0.[28] The surface physiology of these planktonic algae is dominated by the presence of cell walls which are lacking in the unsettled spores of *Ulva*. Instead the spore is surrounded only by a cell membrane. Cell walls and membranes are chemically quite different and this could cause the zeta potential of spores to differ from that of other unicellular algae.

II. MATERIALS AND METHODS

A. Preparation of volume-charged polytetrafluoroethylene films

Samples were cut from 25 μm thick PTFE films bought from Goodfellow. The samples were charged by means of a positive or negative corona discharge in air with a control grid for limiting and controlling the surface potential of the samples. The high voltage at the corona tip was plus or minus 15 kV and the grid voltage was plus or minus 3 kV.[15,29,30] The PTFE films had an area of 72 cm^2. In order to obtain high volume-charge densities, the samples were charged for 30 min at 200 °C and then cooled down to room temperature under the electric field. After charging, the surface potential was measured with a bipolar Trek 314 electrostatic voltmeter in air. The surface potential measured was approximately plus or minus 2800 V. The volume-charge distribution was measured in a piezoelectrically generated pressure step (PPS) experiment.[29,31] A piezoelectric x-cut quartz plate was driven by a 100 ns long square voltage pulse with an amplitude of 300 V. The resulting pressure step was coupled into the sample. The upper surface of the sample was contacted with a conducting-rubber electrode of 5 mm diameter. The PPS response was measured between the grounded quartz metallization and the rubber electrode and was fed into a Telemeter TVV-679-1-SMA three-stage amplifier with 1 GHz bandwidth and 38.6 dB gain. The output of the preamplifier was connected to a digital storage oscilloscope from Tektronix with 1 GHz bandwidth. The oscilloscope signal was averaged over 500 runs in order to enhance the signal-to-noise ratio. The positively charged samples showed a charge density of about 40 C/m^3 and the negatively charged samples around 30 C/m^3. After the charging experiments and the first measurements, the samples were attached to a microscope slide with double-sided adhesive tape from Conrad Electronics. The side that had faced the corona discharge during charging was glued directly to one side of the adhesive tape. Photoelectron spectroscopy was used to reveal possible chemical changes during the charging process. The chemical composition and the absence of carboxylic acid groups showed that all surfaces (charged and uncharged) do have equal surface chemistry. Chemically identical surfaces are very important for our study as the discharge treatment often leads to chemical alteration of the surface which in itself could influence adhesion, as demonstrated for the adhesion of mammalian cells to fluorinated polymer films.[32] Thus, we used the reverse bonding of the PTFE films, which was sufficient to avoid any influence of chemical surface modification caused by the corona discharge and thus reduces the experiment to electrostatic interactions. The charge still remaining in the films after preparation, bioassay, and return by mail, approximately 6 weeks, was measured by the above described technique and shown to be on average 85% of the initial value.

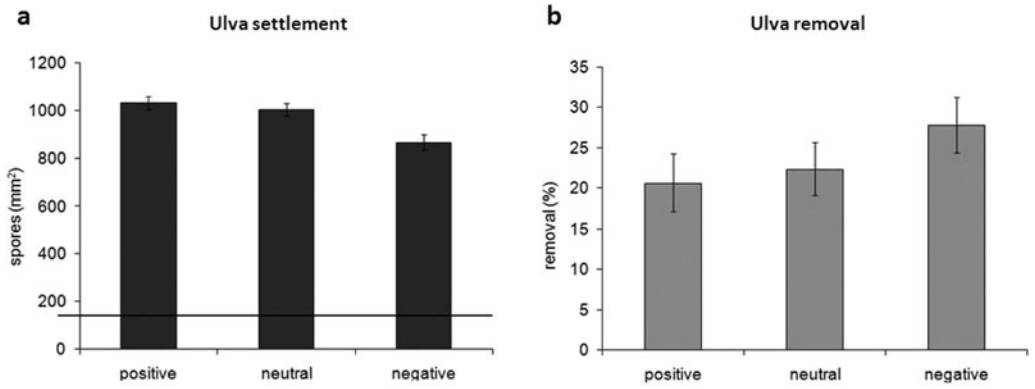

FIG. 1. Electret surfaces: (a) The density of spores of *Ulva* settled on the electret surfaces. Means are from 90 spore counts, 30 from each of three replicates; bars show 2× standard error of the mean (SEM). The level of settlement on Nexterion glass slides is shown by the horizontal line. (b) Percentage removal of spores after exposure to a wall shear stress of 52 Pa. Bars show 2× SEM, calculated from arcsine transformed data.

B. Assays for settlement and adhesion strength of zoospores of *Ulva linza*

Fertile plants of *Ulva linza* were collected from Llantwit Major beach, Glamorgan, Wales (51°40′ N; 3°48′ W). *Ulva* zoospores were released and prepared for settlement and adhesion experiments as described previously.[33] 10 ml of freshly released spores in Tropic Marin® artificial seawater (ASW) (1.5×10^6 spores per ml) were added to individual compartments of a sterile Quadriperm dish each containing a test surface. Six replicates of each test sample were immersed simultaneously. The slides were incubated in darkness for 45 min and then washed gently in ASW to remove unsettled, i.e., motile, spores. Three replicates were used to determine the number of settled (attached) spores. Spores were fixed in 2.5% glutaraldehyde in ASW, washed in deionized water, and dried. Spore counts were taken using a Kontron 3000 image analysis system attached to a Zeiss epifluorescence microscope. Spores were visualized by autofluorescence of chlorophyll and counts were recorded for 30 fields of view on each slide as described by Callow *et al.*[34] To determine the adhesion strength of attached spores the remaining three replicates were exposed to a wall shear stress of 52 Pa in a calibrated water channel using methods previously described.[35] The number of spores remaining after flow was compared to the unexposed samples.

C. Zeta potential measurement

Zoospores were released as described above. Measurements of zeta potential were carried out using a small flat walled electrophoretic cell, 150 μm deep and 5 cm long. The cell was filled with the seawater spore suspension and a potential difference of 100 V applied across it, providing a field strength of 24.46 V cm^{-1}. Spores of *Ulva* are motile (150–200 μm/s) and frequently change the direction of swimming.[33,36] Thus, tracking individual spores is difficult by conventional microscopy. The method of measuring zeta potential requires observations on electrophoretic mobility of individual spores and therefore a method was required to position individual spores into the desired locations within the electrophoretic cell. For this purpose a laser trap (optical tweezers) was used to capture spores and to move them with precision to different points within the cell. Fontes *et al.*[37] used optical tweezers to perform similar measurements on red blood cells.

The laser beam was produced by a continuous wave Coherent Innova 90 laser operating at 514.5 nm and 100 mW power, which was directed into an inverted Leica DM-IRB microscope as described by Ward *et al.*[38] The position of the stationary layer (level) of the electrophoretic cell was established by trial and error using the laser trap to release spores at fixed vertical increments within the cell. Measurements of true electrophoretic mobility, unaffected by electro-osmotic errors, were carried out at the stationary level. The velocities of spores released at this point were determined by recording their passage across the electrophoretic cell as digitized video clips. The time to travel a known distance was measured and spore velocity calculated. The time taken to trap each spore and to position it within the electrophoretic cell was <30 s and the measurement of mobility after the trap was switched off was less than 1 s. The released spores did not begin swimming immediately; hence the parameter being measured was electrophoretic mobility rather than motility due to normal swimming behavior. Prior observations of spores trapped under these laser operating conditions showed that spores were not obviously damaged by the power of the laser.

III. RESULTS

A. Settlement and adhesion of spores to charged surfaces

Figure 1 shows the results for the spore settlement and removal bioassay following the protocol described above. Settlement of spores was high on all PTFE substrates compared to Nexterion glass. Comparing the three surfaces with different net charges, the settlement of spores was less on the negatively charged surfaces. The effect was relatively small (approximately a 15% reduction compared to the neutral surface) but one-way ANOVA with Tukey's pairwise compari-

son showed that the effect was statistically significant ($P=0.001$), with spore density on the negatively charged surface being significantly lower than on the neutral and positively charged surfaces. Spore density on the neutral and positively charged surfaces was not significantly different. The effect was reproducible, similar results being obtained in a repeat experiment performed at a different time with a separate batch of electrets.

The adhesion strength of settled spores was determined hydrodynamically in a flow channel, being expressed as a percentage of the initial spore attachment density after exposure to 52 Pa wall shear stress. Attachment strength was significantly lower ($P=0.01$) on the negatively charged PTFE compared to the positively and the uncharged surfaces [Fig. 1(b)]. Again, Tukey's pairwise analysis suggests that spore attachment strength to the positively charged and neutral surfaces did not vary significantly.

B. Zeta potential determination

For zeta potential determination, analysis of the video footage showed that the mean electrophoretic spore velocity was 27.99 μm s^{-1} ($n=10$). From this, the spore mobility in the electrophoretic cell after the optical trap was switched off and hence the zeta potential was calculated. Field strength (E) [defined as the product of the applied voltage (V)/effective interelectrode distance (l)] was calculated to be 24.46 V cm^{-1}. Spore mobility (υ) was calculated from spore velocity (ν) (microns per second)/applied field strength (E) (V/cm) and determined as -1.144×10^{-8} m^2 s^{-1} V^{-1}. Zeta potential was derived using the Smoluchowski equation[16] [i.e., zeta potential (ζ)=spore mobility (υ)$\times$viscosity (η)/permittivity of the suspending medium (seawater) (ε)]. The value of η for seawater was taken as 1.085×10^{-3} Pa s at 20 °C.[39] The value used for permittivity (ε) of seawater was the relative permittivity [72.5 F m^{-1} (Ref. 40)] multiplied by the permittivity of vacuum (8.854×10^{-12} F m^{-1}). The zeta potential for spores in sea water at pH 8.2 was thus calculated to be -19.3 ± 1.1 mV (mean $\pm$ standard error, $n=10$).

IV. DISCUSSION

The zeta potential of -19.3 mV for swimming spores of *Ulva* is a relatively low value but not dissimilar to that for other unicellular algae and bacteria in high ionic strength media at midrange pH values. For example, the green alga *Dunaliella parva* at pH 7.6 in a glycerol buffer has been recorded as having a zeta potential of -30 mV.[41] A *Pseudomonas* sp. and a marine sulfate-reducing bacteria sp. in seawater at pH 7.0 had zeta potentials of -2.6 and -5.6 mV respectively,[42] and various *Staphlococcus* sp. and *Actinomyces* sp. in buffered 0.05M KCl at pH 6.2 had zeta potentials between -50.8 and -95.44 mV.[22]

At the time of settlement, the spore of *Ulva* is a naked protoplast, the outermost layer of which is a cell membrane. Little information is available regarding the precise composition of the membrane for these spores, but like most eukaryoric cell membranes they will be composed mainly of lipids and proteins organized into a bilayer structure. Proteins make up a small part of most membranes and their contribution to the overall charge is likely to be small. The bulk of the membrane is almost certainly composed of phospholipids. The head group on these molecules, which forms the outer layer, is a phosphorylated alcohol. The phosphate group will be negatively charged in seawater. Other groups, some with positive charges (e.g., choline) may be present, but these will be heavily outnumbered by the negative charges, giving the spore an overall negative charge.

Settlement of the spores was investigated with respect to PTFE surfaces with different net charges. As surface chemistry is the same for all three differently charged surfaces, the experimental results isolate the influence of surface charge and thus electrostatic interactions. The results indicate a relatively small ($\approx$15%) but reproducible and statistically significant reduction in settlement on the negatively charged surfaces, which suggests that some component of settlement is regulated by electrostatic repulsion of these negatively charged spores. Although the effect is small it must be remembered that spores respond to multiple surface cues and the observed level of settlement will reflect the balance of settlement-stimulating and settlement-inhibiting cues. In this context, the base PTFE of the electrets is in itself attractive to spores since it presents a hydrophobic surface, which previous studies (e.g., Schilp *et al.*[6]), including those on fluorinated substrates,[43] have been shown to stimulate settlement.

Many aquatic organisms have stages in their lifecycles that involve the attachment of propagules to surfaces. The surface charge on such a propagule will greatly affect the interactions which take place at the interface between themselves and the substrate. Temporary adhesion is governed by long range forces (>2 nm) which depend on the free energy of the surfaces involved (providing the force) and their distance. The free energy is typically described in terms of the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory combining the free energy associated with van der Waals forces and that associated with the electrical double layer from the charges on the surface. The high salinity of sea water leads to a very small Debye screening length of 0.4 nm.[44] On this basis it is interesting that zoospores are still able to sense a difference in surface potential and settlement is different for the three charged surface.

Surface charge will also be important in determining the interactions between the substrate and the adhesive polymers secreted by the spores once they have committed to settlement and again it was noted that adhesion strength of attached spores was less on the negatively charged PTFE surfaces. Unfortunately the adhesive polymers themselves are not sufficiently well characterized to know whether this effect is reflected in the charge properties of these molecules.

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