Influence of Substrate Wettability on the Attachment of Marine Bacteria to Various Surfaces

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The effect of the initial substrate surface condition, as indicated by the critical surface tension for wetting, on the rate of attachment of marine bacteria to a variety of solid surfaces has been measured. The techniques used to determine the number of bacteria attached per unit surface area were a lipopolysaccharide test utilizing Limulus lysate and direct examination of the surface by scanning electron microscopy. The results obtained by the two techniques are compared and their significance to the control of microbiological slime film formation (biofouling) is discussed.

Marine biofouling and its control have been the subjects of a large number of scientific and technical publications. The majority of these have dealt with the life histories of individual fouling species or with the influence of leachable toxicants from the substrate on the rate at which large sedentary fouling organisms attach to it. As a result of this effort, a large number of toxic agents have been developed, and are available commercially, which successfully inhibit the attachment to, and growth of these organisms on, the surfaces of structural materials for periods ranging up to 3 years.

The use of toxic agents has, however, been less successful in preventing growth of the primary or microbiological slime film. It is known that microorganisms, such as marine bacteria and diatoms, will attach to and reproduce on all but the most highly toxic surfaces (11, 13, 17). A number of biocidal chemicals are available that will kill bacteria in a closed system, but the volume of chemical needed to control primary film formation in an open system typical of most sea water fouling situations is prohibitive. Moreover, many of these treatments are coming under increasing attack for use in open systems due to environmental concerns.

The effect of several parameters other than toxicity on the rate of attachment of microorganisms to solid surfaces has been discussed in the literature. These parameters include: Van der Waals forces (8), the shape of the electrical double layer (8, 9), chemotaxis (18), the ability of the organism to produce polymeric fibrils (5, 9, 10), the surface texture of the substrate (14), surface charge (5, 12), and the wettability of the substrate (2, 17). The latter of these parameters, the substrate wettability, is the subject of the experiments reported in this paper. It was hypothesized (17) as early as the late 1940s that the wettability of the substrate should have an influence on the attachment rate of marine bacteria to structural materials. Only within the past few years has there been experimental evidence to support this hypothesis. Baier and coworkers (1, 2), in their studies of the attachment of cells from human blood to the surfaces of implant materials, have shown that the attachment rate is a function of the initial surface properties of the substrate. Using liquid droplet contact angle techniques developed earlier by Zisman (20, 21), Baier and coworkers measured the wettability of a variety of substrates and correlated this with the rate of attachment. The wettability was represented by the empirical parameter, \( \gamma_{\text{crit}} \), which is the critical surface tension for wetting of the substrate. \( \gamma_{\text{crit}} \) is determined by measuring the contact angle, \( \theta \), between each of a series of liquid droplets of known surface tension and the substrate in question, and plotting the cosine of these angles against the droplet surface tension. The critical surface tension for wetting of the substrate is then defined as the intercept of the best straight line through the data with the cos \( \theta = 1 \) axis. Physically, \( \gamma_{\text{crit}} \) separates the liquids which form contact angles with the substrate of less than about one degree (i.e., spread spontaneously) from those forming higher contact angles and not spreading.

Baier (2) correlated \( \gamma_{\text{crit}} \) with the rate of attachment of cells from human blood to candidate metallic and polymeric implant materials.

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and found that there appeared to be a range in \( \gamma_{\text{crit}} \) from 25 to 30 dynes/cm corresponding to a minimum rate of attachment. This was called the biocompatible range and corresponded to a substrate surface composed mostly of close-packed methyl and hydroxyl groups. Substrates with values of \( \gamma_{\text{crit}} \) both above and below the biocompatible range were said to fall in the bioadhesive range. This grouping encompassed nearly all structural materials (metallic oxides and polymers as well as the common coating materials), and it was shown that such substrates acquired glycoprotein type conditioning films during the first few minutes of exposure to either human blood (2) or sea water (6). Deposition of these conditioning films shifted the value of \( \gamma_{\text{crit}} \) toward the biocompatible range but always left the surface well within the bioadhesive range. It was reasoned that, since the primary fouling organisms such as bacteria and algae attach by means of excreting a bioadhesive cement which is primarily glycoprotein and polysaccharide in character (5), the glycoprotein conditioning films provided a favorable surface for their attachment. The critical surface tension would then, presumably, influence the rate of attachment through its influence on the formation of the conditioning films.

It has been suggested (6) that, since glycoprotein conditioning films have been shown to form on substrates immersed in both sea water and human blood, an influence of critical surface tension on the rate of attachment of microorganisms to substrates immersed in natural sea water should be observed similar to that demonstrated in blood. This suggests further that it may be possible to control primary film formation by manipulation of the initial substrate surface properties rather than by using coatings with leachable toxicants. Additional work is needed, however, before a realistic evaluation of the practical utility of this concept can be made. The mechanism of the formation of the biocompatible range in human blood is still poorly understood, and, for sea water, it has not yet been verified experimentally that there is a range of \( \gamma_{\text{crit}} \) corresponding to a minimum rate of attachment.

The work reported in this paper was undertaken to provide some of the data needed to establish whether or not there is a biocompatible range in sea water. The number of bacteria per unit area attached to substrates of various \( \gamma_{\text{crit}} \) values were measured at prescribed time intervals by a recently developed lipopolysaccharide (LPS) test utilizing Limulus lysozyme and by direct examination of substrates in the scanning electron microscope (SEM). Limulus lysozyme is an aqueous extract derived from the blood cells (amoebocytes) of the horseshoe crab, Limulus polyphemus. This extract contains an enzyme which is activated by lipopolysaccharides (19) derived from the outer cell wall of gram-negative bacteria. Once activated, this enzyme causes a second protein in the lysate to polymerize and clot (19). This test can be used quantitatively (15) and can detect less than 10\(^{-11}\) g of LPS per ml. Since most bacteria in sea water are gram-negative, and since LPS comprises about 10% of the dry weight of a bacterium, the LPS test can be used to determine bacterial biomass (S. W. Watson, unpublished data).

MATERIALS AND METHODS
Sample panels, 1 by 2 inches (ca. 2.54 by 5.08 cm), were cut from the materials listed in Table 1 and were exposed at 3 m depth to natural sea water in Woods Hole harbor. All exposures were made between 24 July and 20 August 1974, during the height of the fouling season at this location. The mean water temperature, measured daily during this period, was 22.2°C with the standard deviation of all measurements being 0.5°C. The glass slides were exposed for periods of up to 504 h, and all other samples for periods of up to 366 h.

The plastic substrates used in this study were all molded from the pure polymer with no plasticizers, coloring agents, or other additives (Table 1). Pure polystyrene, polypropylene, and polytetrafluoroethylene are characterized in the literature (3, 4) as being nontoxic and safe for use in culturing marine microorganisms. There are no data available for polyvinyl fluoride (PVF) but it should be nontoxic in the pure form without plasticizers or fillers. The adhesable grade of PVF was used rather than the release grade which has additives in the surface layers to make the film strippable.

The metal and glass samples were prepared by washing in warm soapy water, degreasing ultrasonically in benzene, and drying in air. The glass slides were then wrapped in aluminum foil and baked for 3 h at 100°C. The polymer samples were prepared in a similar manner except that they were not baked. All specimens were attached in duplicate to metallic holders by nylon machine screws, and the metal samples were isolated from the holders by PVC spacers. The glass slides were enclosed in sterile 300-μm mesh nylon pouches and the pouches were attached to the holder. Details of the holder configurations are shown in Fig. 1.

Upon recovery after each exposure period, one of the duplicate samples was prepared for scanning electron microscopy by (i) fixing for 10 s in a 5% solution of glutaraldehyde in sea water filtered through a 47-mm, 0.22-μm membrane filter (Millipore) and (ii) rinsing through a series of ethyl alcohol-sea water solutions to 100% absolute ethanol followed by rinsing in a series of ethanol-freon 113 solutions to 100% freon 113. They were then critical
TABLE 1. Configurations and Suppliers of Materials Useda

| Material                   | γ<sub>c</sub> (dyne/cm) | Condition                                      | Supplier                                  |
|----------------------------|-------------------------|------------------------------------------------|-------------------------------------------|
| Glass                      | 46 to 70                | Microscope slide                               | Fisher Scientific Co.                    |
| OFHC copper (99.9% copper) | 45                      | 0.005-inch (ca. 0.013 cm)-thick cold            | Sommers Thin Strip, Waterbury, Conn.     |
| 270 Nickel (99.97% Ni)     | 45                      | 0.005-inch (ca. 0.013 cm)-thick cold            | Sommers Thin Strip, Waterbury, Conn.     |
| Polystyrene                | 33                      | Clear crystalline, pure polymer less than       | Monsanto Corp., Springfield, Mass.      |
| Polypropylene              | 29                      | 0.1% monomer 1- or 2.5-mm-thick sheet           | Hercules Corp., Wilmington, Del.         |
| Polyvinyl fluoride         | 28                      | 0.15-mm-thick film adherable grade, no plasticizers or fillers | E. I. duPont DeNemours & Co., Wilmington, Del. |
| Polytetrafluoroethylene (Teflon) | 18                  | 1-mm-thick sheet skived from molded billet (used molded side) white | E. I. duPont DeNemours & Co., Wilmington, Del. |

*a Values of critical surface tension shown are those published by Lee (7) and Zisman (20, 21).

Watson (16) and was found to clot with 2 × 10⁻¹⁰ g of a Klebsiella pneumoniae standard LPS furnished by the Food and Drug Administration. All glassware was baked out at 180 C for 3 h, and pyrogen-free water (Travenol) was used for dilutions. Substrates were first boiled for 1 min in 10 ml of pyrogen-free water to extract the LPS. Ten-fold serial dilutions were then made from this solution in disposable test tubes (10 by 75 mm), and the contents of each tube reacted with an equal volume (0.1 ml) of the Limulus lysate. Before running each test, the lysate sensitivity was determined by reacting with the LPS standard. Each test tube was then inverted slowly to test for clot formation. If a firm clot formed which would not run when the tube was inverted, the tube was scored as positive, indicating the presence of at least 2 × 10⁻¹⁰ g of LPS per ml in the sample.

The highest dilution of each sample that formed a firm clot was recorded. The number of grams of LPS in the original sample was determined by multiplying this dilution factor by 2 × 10⁻¹⁰. To convert the amount of LPS in the samples to the number of bacteria attached to the substrate surface from which the sample was derived, the amount of LPS measured was divided by the estimated amount of LPS contained in an average marine bacterium. For this investigation, it was estimated that a typical bacterium the size of Escherichia coli would contain about 10⁻¹⁴ g of LPS, and this number was used in calculating all the data reported in this paper.

RESULTS

The number of bacteria attached per square centimeter of sample surface is shown as determined by the LPS test in Fig. 2. Due to the 10-fold dilutions used in this experiment, each data point represents a one order of magnitude range, the extremities of which are upper and lower bounds to the actual number of bacteria attached at the particular exposure time. These ranges are shown as vertical lines. The data point actually determined in the test is plotted...
At any given time, the number of organisms attached to the nontoxic, high energy glass surface was one and a half to three orders of magnitude higher than for any other material tested. For the first 20 h of immersion, the nontoxic polymer and nickel surfaces and the highly toxic copper surface were indistinguishable in this test. Beyond 20 h, the curves for these materials gradually diverged with the copper curve remaining the lowest.

The rate of attachment was calculated from the curves in Fig. 2 as follows. Assume that a given curve on the log-log plot may be represented by a series of straight line segments. Each segment may be described by an equation of the form: \( y = Kt^n \) (i) where \( y \) is the number of attached bacteria per unit area, \( t \) the elapsed exposure time, \( B \) the slope, and \( K \) the intercept with the \( y \) axis when \( t = 1 \). The instantaneous rate of attachment at a given time, \( t \), (expressed as the number of bacteria attached per unit area per unit time) is then the time derivative, \( dy/dt \), of equation (i) evaluated at \( t \) with the appropriate constants \( K \) and \( B \).

The curve for glass was represented by two segments, the first from 1 to 5 h \( (K = 10, B = 4.8) \) and the second from 6 to 300 h \( (K = 2 \times 10^4, B = 1.7) \). The remaining curves were all represented by the same segment from 1 to 15 h \( (K = 20, B = 2.4) \). Beyond 15 h, the curve for nickel, polystyrene, and polypropylene remained the same; that for PVF and Teflon approached a straight line with \( K = 100 \) and \( B = 1.7 \); and that for copper approached a straight line with \( K = 700 \) and \( B = 1.0 \). The results of the calculations are summarized from 3 to 300 h in Fig. 3. The rate of attachment was always highest on glass and lowest on OFHC copper. The rate continually increased for all materials except copper, for which it decreased to a constant value of about 600 cells/cm² per h after 15 to 20 h.

Bacterial counts per square centimeter of exposed substrate surface were also determined by direct observation in the SEM. Figures 4 through 7 are typical micrographs from which such counts were taken. Each of these micrographs represents about 1,200 square micrometers of specimen surface. Figure 4 shows a nearly continuous film of rod-shaped bacteria on commercial purity nickel after 14 days. The density of organisms on this surface was representative of about 40% of the total specimen surface observable in the SEM. Fifty percent of the specimen surface was more densely populated than that shown in Fig. 4, and 10% of the surface had no organisms attached. Figure 5 shows scattered individual cells on polystyrene after

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**Fig. 2.** Number of attached bacteria per square centimeter (measured by LPS test) as a function of duration of exposure in natural sea water. The vertical lines represent one order of magnitude error bands through which the curve must pass. These are due to the 10-fold serial dilutions used in the LPS test.
Figure 3. Rate of attachment of bacteria to various substrates as a function of duration of exposure to natural sea water.

For each substrate, the final count of organisms per square centimeter for a given surface was determined by first scanning the entire specimen to determine what types of distributions of organisms were to be found (e.g., scatter of individual cells, discrete colonies) and what percentage of the total area each type of distribution represented. Individual counts for each type of distribution were then added together and weighted by the proper area percentage.

The results of the SEM counts are tabulated for the four substrates examined by this technique in Table 2. The critical surface tension of each substrate, as tabulated by Zisman (20, 21) and Lee (7), and the LPS test data (including the upper and lower bounds due to the 10-fold dilutions) are shown for comparison.

The agreement between the two sets of data was satisfactory (it was excellent for polystyrene), and the same trends with critical surface tension and with time of exposure were revealed by both methods. Where the two sets of data did not agree, the LPS test usually gave the lower reading.

Figure 8 shows the number of bacteria attached per square centimeter as determined by SEM plotted against the critical surface tension of the substrate. In agreement with previous work in human blood, the data show a decrease in attachment with decreasing critical surface tension, a minimum in attachment between 20 and 30 dynes/cm, followed by an increase in attachment for lower critical surface tensions.

**DISCUSSION**

The data presented clearly show an influence of the critical surface tension of the substrate on the attachment of marine microorganisms to solid surfaces. Compared to the high energy, nontoxic glass surface, the effect is a decrease of 1.5 to 3 orders of magnitude in the number of attached organisms per square centimeter for exposure times between 1 and 500 h.

The most dramatic effect occurs during the first 10 h of exposure where the rate of attachment for the glass surface, as shown in Fig. 3, is 10 to 100 times higher than that of the low energy and toxic surfaces tested. This suggests, in agreement with earlier published work, that the events occurring at the surface within the first few hours are critical and are the most likely to be influenced by the surface properties of the substrate.

The shape of the curve of attachment versus critical surface tension shown in Fig. 8 is consistent with the earlier work (6) in human blood. The minimum in the curve, centered at about 25 dynes/cm, indicates the probable existence of a biocompatible range corresponding to a minimum rate of attachment of microorganisms from sea water. This range appears to be slightly lower than the biocompatible range (25 to 30 dynes/cm) found in blood, but the resolution of the present data is not sufficient to show whether there is a statistical significance to this difference. The term biocompatible range should, perhaps, be used with caution, as the number of attached bacteria at the 7-day exposure interval was less than 10^4/cm^2 only on the highly toxic copper surface. The minimum in the curve may extend lower than that shown in Fig. 8, but proof of this must await further experimental data.

The mechanism for the existence of a min-
The minimum in the curve of Fig. 8 is not clearly understood. It has been shown that the bonding forces responsible for the interfacial tension of the substrate are so localized that only the first monolayer of material is important (2, 20). Thus, the first monolayer of adsorbed organic conditioning film that forms from the sea water masks the original surface and its critical surface tension. Since this first monolayer is usually well formed before any bacteria are observed to attach (6), it is unlikely that the critical surface tension, or wettability, of the pure clean substrate has any direct influence on the attachment process. The influence must, rather, be secondary. The initial surface composition (i.e., $\gamma_{crit}$) probably determines the character of the conditioning film that forms, and this, in turn, influences the attachment of fouling organisms. If these ideas are correct, one would expect that, compared to substrates in the bioadhesive range, a substrate whose initial value of $\gamma_{crit}$ was within
the biocompatible range should either take a very long time to acquire a conditioning film, acquire no film at all, or acquire a film of substantially different character. This has yet to be tested experimentally.

The resolution along the abscissa of Fig. 8 can probably be improved by doing an experiment with an increased number of substrates whose critical surface tensions fall in the range from 20 to 35 dynes/cm. These materials should include at least polytetrafluoroethylene ($\gamma_{\text{crit}}$, 18), polytrifluoroethylene ($\gamma_{\text{crit}}$, 22), polyvinylidene fluoride ($\gamma_{\text{crit}}$, 25), PVF ($\gamma_{\text{crit}}$, 28), and polyethylene ($\gamma_{\text{crit}}$, 31). The resolution along the ordinate of Fig. 8 can perhaps be improved by as much as a factor of two by increasing the number of photographs from which each data point is determined and performing a statistical analysis. It is not clear, however, that this small gain in resolution

Fig. 5. Scattered bacteria on a polystyrene substrate after 14 days in Woods Hole harbor. Spherical coci are seen at "A", rod-shaped bacteria at "B", and stalked rods at "C".
would be worth the effort. Electronic image analyzing and particle counting techniques would be difficult to apply reliably in this case because of the number of inorganic particles appearing on the surface that are roughly the same size as a bacterium. The resolution of the LPS test can be improved by using twofold dilutions. Doing this, the LPS test should be able to distinguish between nickel and polystyrene or between polyvinylfluoride and polytetrafluoroethylene (Teflon), as it could not do in the present experiment.

The only anomaly in the data is that determined by the LPS test for nickel. The SEM data shown in Table 2 place nickel in approximately the position relative to glass and polystyrene that was originally expected, whereas the LPS test placed nickel nearly an order of magnitude lower, and close enough to polystyrene that it was unable to distinguish between them. Although nickel has been known to show toxic effects under some cir-
FIG. 7. Bacteria embedded in a slime layer on a polytetrafluoroethylene (Teflon) substrate after 14 days of exposure in Woods Hole harbor.
INFLUENCE OF SUBSTRATE WETTABILITY

Table 2. Number of bacteria per square centimeter as determined by the SEM and the LPS test at 7 and 14 days

| Substrate                        | \( \gamma_{eq} \) (dynes/cm) | 7 days (168 h) No. of bacteria/cm\(^2\) | 14 days (336 h) No. of bacteria/cm\(^2\) |
|----------------------------------|-------------------------------|----------------------------------------|----------------------------------------|
| Glass                            | 46                            | LPS test: Max \( 1.2 \times 10^9 \), Curve \( 1.2 \times 10^9 \), Min \( 1.2 \times 10^9 \) | SEM count: No data | LPS test: Max \( 6.0 \times 10^9 \), Curve \( 3.0 \times 10^9 \), Min \( 6.0 \times 10^9 \) | SEM count: No data |
| Nickel                           | 45                            | LPS test: Max \( 2.2 \times 10^9 \), Curve \( 2.2 \times 10^9 \), Min \( 2.2 \times 10^9 \) | SEM count: \( 2.5 \times 10^7 \) | LPS test: Max \( 1.5 \times 10^9 \), Curve \( 9.0 \times 10^8 \), Min \( 1.5 \times 10^9 \) | SEM count: \( 9.1 \times 10^7 \) |
| Copper                           | 45                            | LPS test: Max \( 7.5 \times 10^9 \), Curve \( 7.5 \times 10^9 \), Min \( 7.5 \times 10^9 \) | SEM count: No data | LPS test: Max \( 7.5 \times 10^9 \), Curve \( 1.6 \times 10^9 \), Min \( 7.5 \times 10^9 \) | SEM count: No data |
| Polystyrene                      | 33                            | LPS test: Max \( 2.2 \times 10^9 \), Curve \( 2.2 \times 10^9 \), Min \( 2.2 \times 10^9 \) | SEM count: \( 2 \times 10^9 \) | LPS test: Max \( 1.5 \times 10^9 \), Curve \( 9.0 \times 10^8 \), Min \( 1.5 \times 10^9 \) | SEM count: \( 7 \times 10^9 \) |
| Polypropylene                    | 29                            | LPS test: Max \( 2.2 \times 10^9 \), Curve \( 2.2 \times 10^9 \), Min \( 2.2 \times 10^9 \) | SEM count: No data | LPS test: Max \( 1.5 \times 10^9 \), Curve \( 9.0 \times 10^8 \), Min \( 1.5 \times 10^9 \) | SEM count: No data |
| Polyvinyl-fluoride               | 28                            | LPS test: Max \( 5.5 \times 10^9 \), Curve \( 3.5 \times 10^9 \), Min \( 5.5 \times 10^9 \) | SEM count: \( 1.0 \times 10^9 \) | LPS test: Max \( 8 \times 10^8 \), Curve \( 1.0 \times 10^9 \), Min \( 8 \times 10^9 \) | SEM count: \( 3 \times 10^8 \) |
| Polytetrafluoroethylene (Teflon) | 18                            | LPS test: Max \( 5.5 \times 10^9 \), Curve \( 3.5 \times 10^9 \), Min \( 5.5 \times 10^9 \) | SEM count: \( 1.6 \times 10^9 \) | LPS test: Max \( 8 \times 10^8 \), Curve \( 1.0 \times 10^9 \), Min \( 8 \times 10^9 \) | SEM count: \( 5.6 \times 10^8 \) |

*Values of critical surface tension shown are those published by Lee (7) and Zisman (20, 21). The maximum and minimum values shown for the LPS test are due to the 10-fold dilutions used. Max, maximum; Min, minimum.

In some circumstances, it is unlikely that the anomaly can be explained in this manner because nickel has been shown to be safe for use in culturing marine phytoplankton (4). Since only the samples used in the LPS test were effected, it is considered more probable that either these samples became unintentionally contaminated with a low surface tension organic film before exposure, or a slight dissolution of nickel ions into the test solution during the 1-min boil in pyrogen-free water interfered with the LPS test itself.

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Fig. 8. Number of attached bacteria per square centimeter from the SEM data as a function of the critical surface tension for wetting.
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