The influence of substratum topography on bacterial adhesion to polymethyl methacrylate

R. L. TAYLOR, J. VERRAN, G. C. LEES*, A. J. P. WARD‡
*Department of Biological Sciences, *Department of Materials Technology, and ‡Department of Mechanical Engineering, Manchester Metropolitan University, John Dalton Building, Oxford Road, Manchester, M1 5GD, UK*

The effect of substratum roughness on the adhesion of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* was investigated using PMMA. A small increase in R_a values (0.04–1.24 μm) resulted in a significant increase ($P < 0.05$) in bacterial attachment. Subsequent increases in surface roughness ($R_a = 1.86$–7.89 μm) resulted in a decrease in adhesion, although adhesion was still higher than to the smooth surface. When the PMMA surfaces were coated with protein (bovine serum albumin), no difference ($P < 0.05$) could be determined in the amount of protein adsorbed, irrespective of surface topography. However, the influence of the underlying topography on adhesion was still evident. Substratum topography is an important parameter affecting bacterial adhesion to surfaces.

1. Introduction

The surface roughness of a substratum, indicated by the parameter R_a, can be defined as the relatively fine-spaced deviations or irregularities that establish the predominant surface pattern. Manufacturing processes involved in the production of thermoplastics [1] and contact lenses [2] and the subsequent use of such materials [3, 4] often result in the surface becoming flawed and therefore roughened. In addition, surface texture can markedly affect the performance of contact lenses, in particular the wetting, debris accumulation, comfort and quality of a lens [5].

Surface texture or roughness has been shown to influence bacterial adhesion to catheters [4, 6] dentures [7, 8] and industrial systems [9]. Materials used in industry must comply with British standards of acceptable roughness which operate a grading system [10].

Light and electron microscopy studies have shown that surface irregularities serve as preferential starting points for attachment [11, 12] providing niches in which micro-organisms are protected from shear forces [13, 14]. This is believed to allow the microbial cell time in which to attach irreversibly to a surface [15]. The contribution of surface topography to attachment is believed to be greater than physico-chemical interactions such as surface free energy and hydrophobicity [15].

This study describes the effect of type and degree of roughness on the adhesion of rods and coccal bacteria to PMMA.

2. Materials and methods

2.1. The production of roughened PMMA

PMMA (ICI, Acrylics Division, Darwen, UK) was selected for the series of experiments, as it is a standardized thermoplastic with a smooth finish. It is also a pure polymer and therefore the polymer composition would remain unaltered. Silicone carbide abrasion of PMMA was achieved by contra rotation of a Buehler Motopol 12 (Buehler UK Ltd) polisher. Silicone carbide paper grades of P1200 (particle size 15.3 μm), P400 (particle size 35 μm) and P120 (particle size 125 μm) were applied with a constant pressure of 178 N at a speed of 120 r.p.m.

Bead and shot-blasting of PMMA was performed using a Honermaster blaster (Slough, Bucks.) set at 36 $1.66\ m^3\ s^{-1}$ for 10 s. Glass beads (Potters, Ballotini, Barnsley, Yorkshire, UK) had a smooth surface and uniform shape with a diameter range of 90–190 μm. The grains of metal used were uneven in shape and surface with a diameter of 200–400 μm.

Following roughening, the PMMA samples were rinsed twice in distilled water, ultrasonicated in distilled water for 15 min, rinsed once more and then dipped in methanol. These processes attempt to remove embedded grinding material from pits and grooves. All surfaces were stored dry at room temperature in sterile air-tight containers.

2.2. Laser profilometry

A laser perthometer S8P (Perthen, Mahr, Germany) was used to provide information on the surface topography of both smooth and rough PMMA. The principle of the system involves the dynamic focusing of a laser light reflected from a surface.

Seven sample lengths of 0.8 mm were measured, analysis being performed on the central five sections. The average roughness values, R_a, R_z, R_{max} and R_{lo} were calculated. R_a is the arithmetic mean of the departures of the roughness profile from the profile

centre-line. R_z is the average distance between the five highest peaks and the five lowest valleys, and R_{max} is the largest single peak-to-valley height (Romney, 1990). R_{lo} is the distance of the peaks and valleys that make up the tracing length.

2.3. Microbial adhesion to roughened PMMA

Cultures used were *Pseudomonas aeruginosa* (medical isolate) and *Staphylococcus epidermidis* (NCTC 11047) [16]. Each culture was inoculated into 10 ml BHI broth and incubated for 18 h at 37 °C without agitation. Cells were harvested and washed three times in phosphate buffered saline (PBS). Bacteria were resuspended in the same buffer to an optical density of 1.0 (540 nm) which represents approximately $1–4 \times 10^8$ colony-forming units (cfu) per ml as determined by culturing and plate counting. Methods were described in detail in a previous publication [16].

Following the assay, PMMA pieces with adherent bacteria were rinsed by dipping three times in phosphate buffered saline (PBS). Adherent bacteria were fixed with methanol for 30 s, stained with crystal violet and water (1 : 1) for 10 s, rinsed with water and air-dried, lying horizontally. Three replicates of each type of roughened PMMA were used in each assay and the experiment was repeated twice.

To determine the effect of a BSA conditioning film on adhesion, PMMA pieces were placed into 10 and 50 mg/ml BSA solutions for a 48 h period. Quantitative analysis of BSA was achieved using the Bio-Rad protein assay which is based on the method of Bradford [17]. Comparison to a standard curve provides a relative measure of protein concentration. Four standards of BSA, from 1–10 $\mu g\,ml^{-1}$, were used. For each sample, 40 µl of the extraction pool were added to 160 µl of the diluted dye reagent (1 : 4).

Image analysis was performed to give percentage area figures [18]. The microscope was focused for an upper and lower plane and the sum of the percentage area gained for both images was recorded. Thresholding of the image highlighted only those cells that were in focus and ignored those cells that were out of focus.

2.4. Scanning electron microscopy

Roughened samples of PMMA with and without BSA and adherent bacteria were rinsed in PBS and fixed with methanol and air dried. The test pieces were mounted on aluminium stubs and sputter-coated with gold/palladium alloy. The specimens were examined on a Cambridge Stereoscan 250 scanning electron microscope.

2.5. Dynamic contact angle (DCA) analysis

The DCA analysis system (Cahn Instruments, Cerritos, CA, USA) was used to record the solid/liquid/air interface via an electrobalance as a function of time and immersion depth. Two wetting liquids were used for each piece of PMMA; distilled water with a surface tension of 72.6 Nm^{-1} and bromonaphthalene with a surface tension of 44.6 Nm^{-1}.

3. Results

The roughness values of smooth and roughened PMMA were measured using laser profilometry (Table I). An increase in the grit size used for roughening resulted in an increase in R_a, R_z and R_{max} values. The R_{lo} values for the roughened PMMA were higher than for smooth PMMA, although there was no relationship between the type of roughness (abraded and blasted) and the R_{lo} value.

The contact angle and surface free energy of roughened surfaces was different from that of the smooth PMMA surface (Table II). The largest increase in the advancing contact angle was observed in the P1200 surface. However, with the other roughened surfaces the higher the R_a value, the lower the contact angle became. No relationship could be determined between the advancing contact angle and the arithmetic mean deviation, R_a, of smooth and roughened PMMA.

TABLE I The surface topography of smooth and roughened PMMA was measured using laser profilometry ($n = 10$). In addition to the R_a (arithmetic mean of the departures of the roughness profile from the profile centre-line) and R_z (average distance between the five highest peaks and the five lowest valleys), R_{max} (largest peak-to-valley height) and R_{lo} (distance of the peaks and valleys that make up the tracing length) values were also obtained

Type of roughness	R_a (µm)	R_z (µm)	R_{max} (µm)	R_{lo} (cm)
Smooth	0.04	0.94	2.61	4.01
P1200	1.24	10.1	13.8	5.34
P400	1.86	14.5	20.9	5.38
P120	3.66	25.3	28.8	5.34
Bead-blasted	2.16	17.0	23.4	5.15
Shot-blasted	7.89	44.4	53.9	5.21

TABLE II The contact angle and surface free energy of smooth and roughened (silicone carbide paper grades P1200, P400 and P120 with particle sizes of 15.3, 35 and 125 µm, respectively, bead and shot-blasted) PMMA were measured by dynamic contact-angle analysis. Advancing and receding contact-angle measurements were obtained in distilled water and bromonaphthalene, from which the surface free energy was calculated

PMMA	Advancing contact angle (deg)	Receding contact angle (deg)	Total surface energy (Nm^{-1})
Smooth	63	41	54
P1200	87	46	46
P400	77	49	48
P120	77	60	48
Bead-blasted	70	43	50
Shot-blasted	68	30	52

3.1. Bacterial adhesion to smooth and roughened PMMA with and without the presence of BSA

The adhesion of *P. aeruginosa* and *S. epidermidis* to PMMA was significantly greater when PMMA was abraded with P1200 silicone carbide particles ($P < 0.05$) (Fig. 1). There was no significant difference between the number of adherent bacteria on the remaining roughened surfaces and the smooth surface for both organisms, although the numbers on the roughened surfaces were slightly higher than on the smooth PMMA.

Using light microscopy, bacteria were observed in the pits and gullies produced on the abraded and blasted surfaces when viewed at $\times 1000$ magnification (Figs 2 and 3). However, at higher magnification, cells were observed to have adhered evenly over the surface (SEM) (Figs 4 and 5).

There was no significant difference in the amount of BSA (10 and 50 mg ml^{-1}) adsorbed on to smooth and roughened PMMA after 2 d (Fig. 6). However, there was a slight but not significant increase in the amount of protein deposited on the surface with the highest R_a value (shot blasted) and the silicone carbide-abraded PMMA, respectively, compared with the other surfaces. No relationship could be determined between surface roughness, R_a, of PMMA and the amount of protein adsorption for either concentration of BSA used.

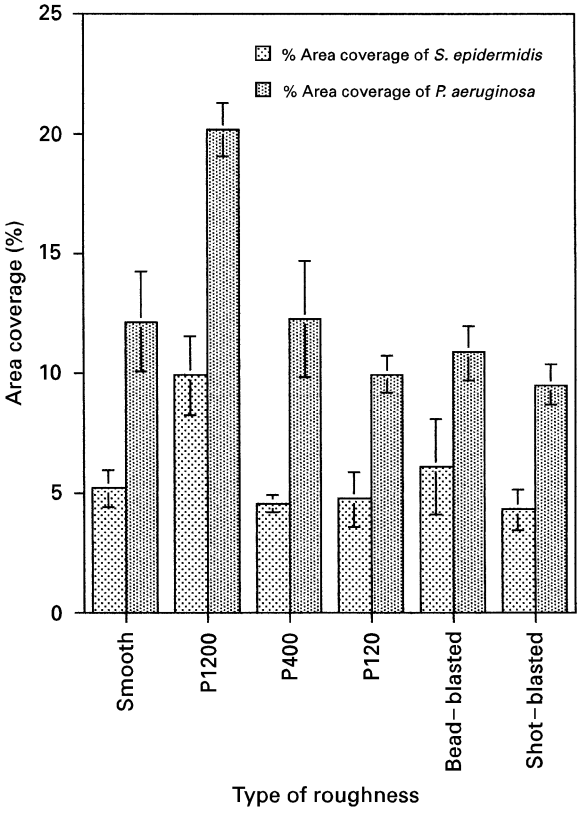

Figure 1 The adhesion of *P. aeruginosa* and *S. epidermidis*, following 1h incubation, to smooth, abraded and blasted PMMA ($n = 6$). Image analysis of the samples provides a percentage area figure which refers to the coverage of cells in a microscopic field. Significantly greater bacterial adhesion was recorded on P1200-abraded PMMA ($P < 0.05$). There was no significant difference between the number of adherent bacteria on the remaining roughened surfaces and the smooth surface for both organisms.

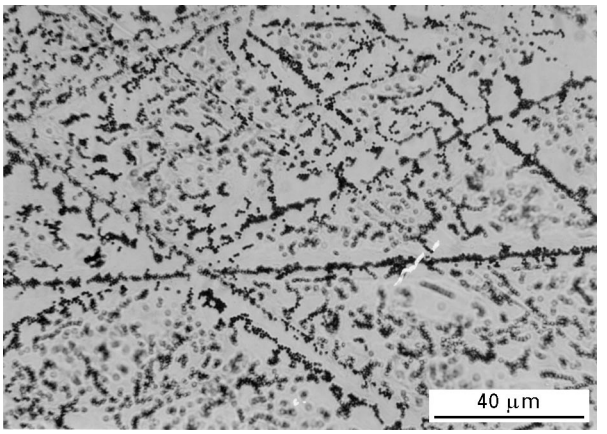

Figure 2 Light microscopy of *S. epidermidis* cells that adhered to P400-abraded PMMA after 1 h incubation. Cells appear to be attached predominantly to gross surface defects.

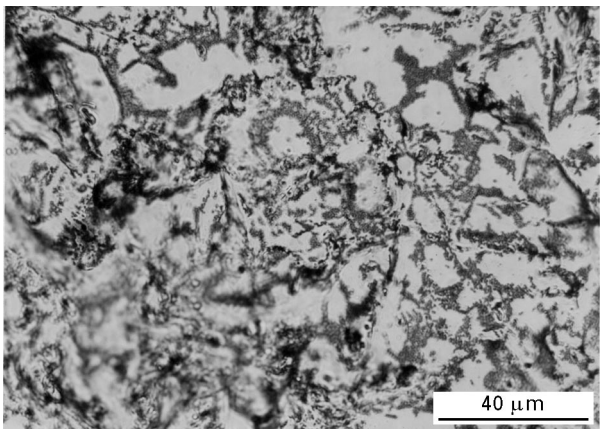

Figure 3 Light microscopy of *P. aeruginosa* cells that adhered to shot-blasted PMMA after 1h incubation. Cells appear to be attached predominantly to gross surface defects.

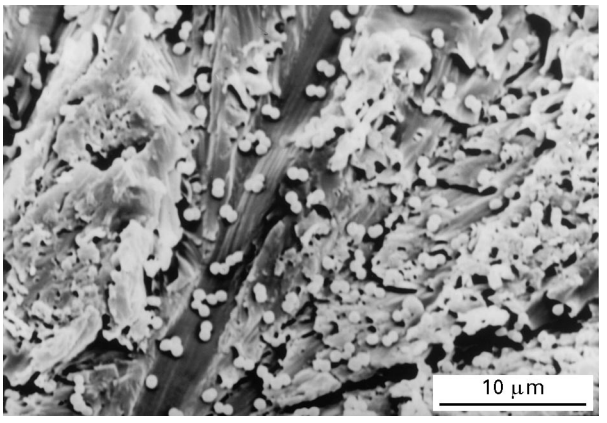

Figure 4 Scanning electron micrograph of *S. epidermidis* cells that adhered to P400-abraded PMMA after 1h incubation. A higher magnification reveals cells to be attached to both gross and minor defects on the PMMA surface.

The presence of a BSA coating on PMMA caused a significant decrease in *S. epidermidis* adhesion (Fig. 7) but did not significantly affect *P. aeruginosa* adhesion (Fig. 8). The overall pattern of adhesion was the same as for the naked PMMA, i.e. highest on the P1200-abraded surface.

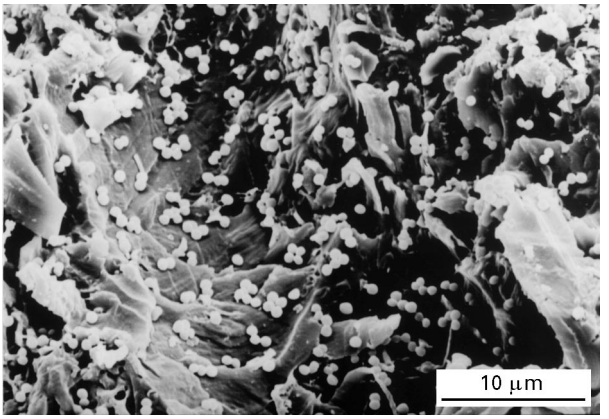

Figure 5 Scanning electron micrograph of *S. epidermidis* cells that adhered to shot-blasted PMMA after 1h incubation. A higher magnification reveal cells to be attached to both gross and minor defects on the PMMA surface.

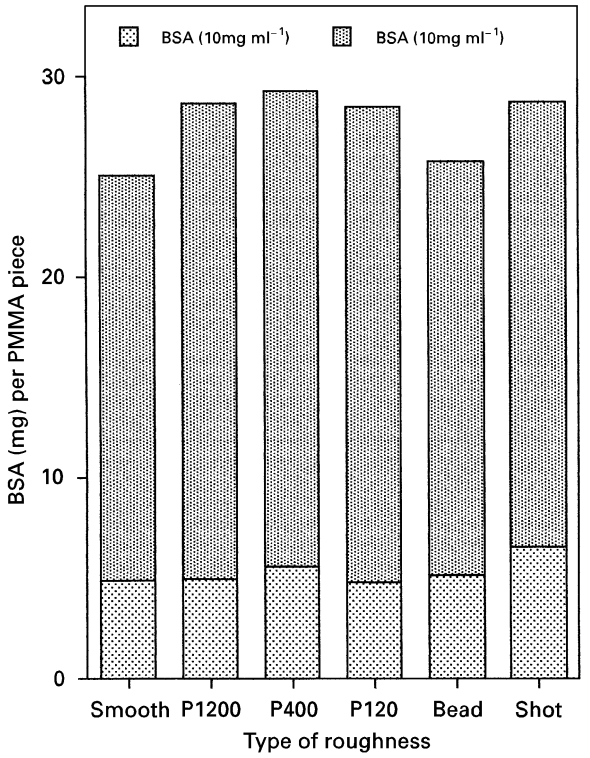

Figure 6 BSA adsorption (10 and 50 mg ml^{-1}) was quantified using a Bio-Rad protein assay to PMMA following a 2d incubation period ($n = 6$). There was no significant difference in the amount of BSA (10 and 50 mg ml^{-1}) adsorbed to smooth and roughened PMMA.

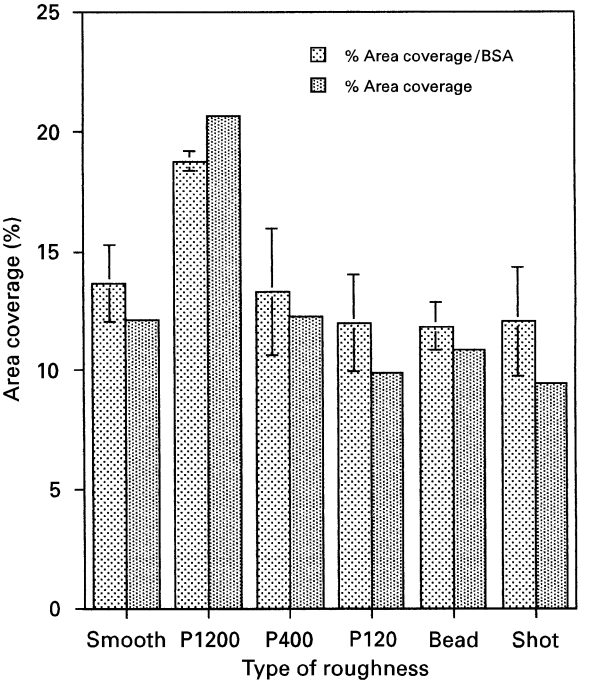

Figure 7 The adhesion of *P. aeruginosa* (medical strain) to smooth and roughened BSA-coated (10 mg ml^{-1}) PMMA following a 1h incubation period was quantified using image analysis ($n = 6$). Previous results of adhesion to uncoated PMMA (Fig. 1) are included for comparison. The presence of a BSA coating did not significantly alter the adhesion pattern as for naked PMMA.

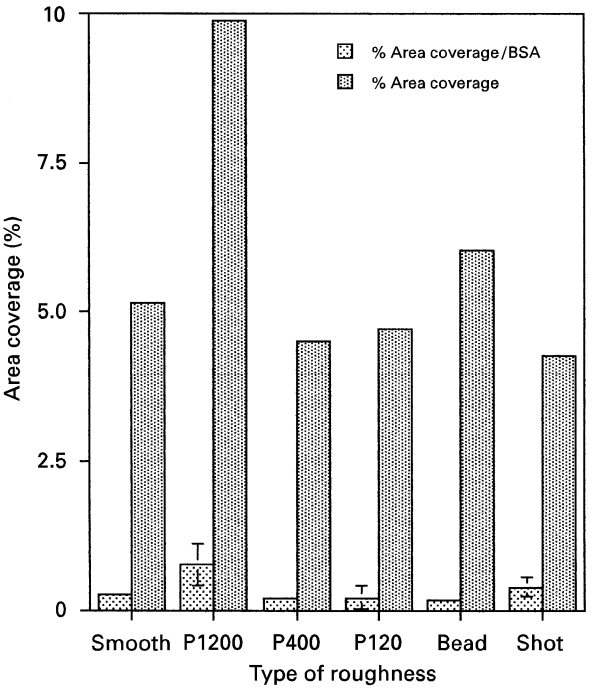

Figure 8 The percentage area of *S. epidermidis* (NCTC11047) to smooth and roughened BSA-coated (10 mg ml^{-1}) PMMA following a 1 h incubation period was quantified using image analysis ($n = 6$). Previous results of adhesion to uncoated PMMA (Fig. 1) are included for comparison. The presence of a BSA coating on PMMA, significantly reduced adhesion ($P < 0.05$), although most attachment was still evident on the P1200-abraded PMMA.

4. Discussion

Even at a molecular level, the surface topography of appliances used in medicine and industry has been demonstrated to affect the rate of microbial attachment. The results of this study have shown that a small increase in surface roughness from smooth has a significant effect on microbial adhesion to that surface. Previous publications using bacteria and yeast [6, 8, 19] have also shown greatest adhesion to surfaces with an R_a value ranging from 1.12–1.29 μm. This increase in adhesion may be due to the protection afforded to the cells from shear forces by microscopic niches. Surface roughness can induce immobilization of the cell [20] and irreversible attachment may be established more easily [21].

Bacterial adhesion to rougher surfaces, pitted (blasted) and abraded, scratched surfaces decreased as the R_a values of these surfaces increased. In addition, the enumeration of bacteria became progressively more difficult, in particular on the blasted surfaces. The thresholding function of an image analyser can be modified to ignore cells which are out of focus. Thus a method was developed which successfully outlined cells in one of two distinct focal planes.

Light microscopy revealed the location of the bacteria to be mainly in the pits and gullies of the roughened surfaces. Surface defects increased in size on samples with higher R_a values thus offering the micro-organisms less protection from shear forces. SEM showed cells attaching to micro-defects on heavily abraded surfaces, indicating again that it is small changes in surface properties which are important in altering attachment sites for pioneer species, rather than gross defects.

The effect on adhesion of an increase in surface area caused by roughening needs to be considered. Laser profilometry has shown that a similar increase in the trace length, R_{lo}, occurred for all of the roughened surfaces when compared with the smooth surface, despite differences in their R_a values. Although it must be taken into consideration that R_{lo} is a line measurement of the peaks and troughs rather than an area measurement, one might extrapolate that the overall surface-area increase is similar for all roughened surfaces. Scanning electron microscopy revealed that peaks and troughs are larger for rougher surfaces though less frequent in number. Thus, again the type and degree of roughness are affecting the results, not merely an increase in surface area and resultant potential attachment area.

The R_a values for roughened surfaces have been shown to differ due to the instrumentation used to measure this parameter. This may have important implications in industry where regulations on the quality of surface finish are imposed. Lower values were obtained using laser profilometry (0.04, 1.24, 1.86, 3.66, 2.16 and 7.89 μm) as the beam of laser light is roughly ten times smaller than the stylus used in talysurf instrumentation and therefore has an improved resolving power. The radius of the laser is 2 μm which is small enough to probe into crevices that could accommodate bacteria.

The influence of polymer surface topography on contact angle hysteresis was first researched by Wenzel [22]. Dynamic contact analysis was originally developed for smooth surfaces, although useful information can be ascertained by using the technique on roughened surfaces. Busscher *et al.* [23] showed that differences in the contact angle hysteresis of surfaces with an R_a greater than 0.1 μm was related to roughness of a surface, whereas for surfaces with an R_a less than 0.1 μm, any difference in hysteresis could not be related to surface roughness but might be due to chemical heterogeneity of the surface. The roughened surfaces in this study had an R_a value higher than 0.1 μm and therefore any change in contact angle may be related to surface texture. This potentially provides another parameter with which to describe roughness.

There was a large increase in the contact angle of PMMA abraded with silicone carbide grade P1200 compared with smooth PMMA. Subsequent increases in the R_a values of the remaining roughened samples produced a decrease in the contact-angle measurement, although the measurement was still higher than that for smooth PMMA. In the case of PMMA, the change in contact angle reflects a physical effect, rather than an alteration in the hydrophobicity, as the chemical composition of the polymer will not have altered. The dynamic contact angle reflects the ease with which a liquid of known surface tension can spread over the surface of a material. The effect of roughening will impede the flow of a liquid over the surface and thus produce a different measurement. It appears therefore that fine roughening produces a greater deterrent to the liquid phase than the surfaces with higher R_a values. Little work has been performed to date to clarify a relationship between the type and orientation of roughness and the contact angle.

The influence that surface roughness has on polymer characteristics would also depend on the composition of the polymer. For example, the roughening of thermoplastics with additives, e.g. PVC, may result in alterations to the surface chemistry and the release into the immediate environment of chemicals which may affect bacterial adhesion.

All surfaces immersed in a fluid environment become coated with a conditioning film that alters not only the surface properties of the polymer but also the adhesion of bacteria to the surface. It was assumed that surface irregularities would cause the conditioning film to be more easily retained [24] and therefore increase in quantity. However, there was no significant difference in adsorption of BSA (10 and 50 mg ml^{-1}) to smooth and roughened PMMA to any surface.

This failure to detect a difference in protein deposition, despite the increase in surface area of the roughened surfaces, may be due to the small surface area of the test pieces. All of the test pieces used were 10 mm × 10 mm × 3 mm, and only one side was roughened, hence only a small amount of BSA was adsorbed to all surfaces. Thus no difference in BSA deposition between smooth and rough samples was detected.

The presence of BSA did not influence the effect of topography on adhesion, although the overall amount of adhesion of *S. epidermidis* was considerably reduced, whereas that of *P. aeruginosa* was unchanged. PMMA coated with BSA is more hydrophilic than uncoated PMMA (results not shown). This change is probably due to differences between the two bacterial strains, as *S. epidermidis* was shown to be more hydrophobic than *P. aeruginosa* [16]. This suggests that, despite the different surface interactions that occur due to the presence of a protein layer, the influence of surface topography on adhesion was still apparent. Examination of uncoated and protein-coated roughened PMMA using SEM revealed little difference in the appearance of the two types of surface. The use of a replica model visualized using transmission

electron microscopy [25] may enable the degree of coverage of the conditioning film to be determined.

The degree of surface roughness has a considerable influence on the amount of microbial adhesion, with small increases in roughness producing maximal adhesion. However, surfaces with larger pits and gullies corresponding to an increased R_a value did not have the ability to retain bacteria as effectively. These effects were still apparent despite the presence of a conditioning film. Information relating the cell size, degree of attachment and surface topography of materials may be beneficial to industry and medicine where a smooth surface cannot always be guaranteed and where the limits of tolerance of surface roughness need to be defined.

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