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An atomic force microscopy investigation of bioadhesive polymer adsorption onto human buccal cells

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Abstract

Atomic force microscopy (AFM) was used to examine the buccal cell surface in order to image the presence of adsorbed bioadhesive polymers identified from previous work. Isotonic saline solution (5 ml) containing either polycarbophil (pH 7.6), chitosan (pH 4.5) or hydroxypropyl methylcellulose (pH 7.6) (0.5% w/v) was exposed to freshly collected buccal cells (ca. 48×10^4 cells/test) for 15 min at 30°C. The cells were then rinsed with a small volume of double distilled water, allowed to air-dry on a freshy cleaved mica surface and imaged using contact mode AFM. Untreated cells showed relatively smooth surface characteristics, with many small 'crater-like' pits and indentations spread over cell surfaces. Cells that had been treated with all the investigated polymers appeared to have lost the crater and indentation characteristic and gained a higher surface roughness. These results suggest that polymer chains had adsorbed onto the cell surfaces. Quantitative image analysis of cell topography showed significant increases ($P < 0.05$) in arithmetic roughness average (R_a) for all the investigated polymer treated cells surfaces with respect to untreated control specimens. The changes in surface topography indicate the presence of adsorbed polymer, confirming previous work. This study demonstrates the suitability of AFM as a powerful and sensitive technique for detecting and imaging bioadhesive polymers present on mucosal cell surfaces.

Keywords: Atomic force microscopy; Bioadhesion; Mucoadhesion; Oral cavity; Polymers

1. Introduction

Over 400 different types of oral cavity disorder exist, such as oral candidiasis, gingivitis, xerosto-

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mia, dental caries, and oral lesions, which are usually treated by local therapy (Zegarelli, 1987). Conventional drug therapy is limited by the relatively short retention times of delivery systems, such as dental gels and mouthwashes, as a result of the flushing action of saliva and the consumption of foodstuffs (Washington and Wilson, 1989; Harris and Robinson, 1992; Smart, 1993; Rathbone et al., 1994). Solid and semisolid bioadhesive dosage forms can be unacceptable to the patient due to difficulty in eating, drinking and/or communication during treatment (Washington and Wilson, 1989; Smart, 1993). Some areas of the oral cavity may not receive therapeutic levels of drug due to their non-uniform distribution in saliva (Weatherell et al., 1992). The overall aim of these investigations is to develop a liquid formulation containing therapeutically active components (i.e. macromolecular carriers) that could be distributed throughout the oral cavity, or targeted to specific regions, and then retained for extended periods. The adsorption and binding of macromolecules from solution onto mucosal cells has been the subject of comparatively few studies (e.g. Park and Robinson, 1984) relative to the study of solid and semisolid mucoadhesion. Therefore in our previous work, a lectin-binding inhibition technique was developed to investigate polymer adsorption to human buccal mucosal cells (Patel et al., 1999). Certain polymers were identified in this study as being highly 'retentive', i.e. produced the greatest inhibition of lectin binding, and their presence was confirmed using staining techniques.

The atomic force microscope shown in Fig. 1, is one of several types of scanning probe microscope that can be used to obtain topographic information of surfaces with very little sample preparation and in an almost completely non-invasive manner (Binning et al., 1986). The atomic force microscope does not require the specimen to be electron- or ion-conductive and hence, has been widely used in biological structural studies. The microscope has the additional advantage in being able to image under near-physiological conditions

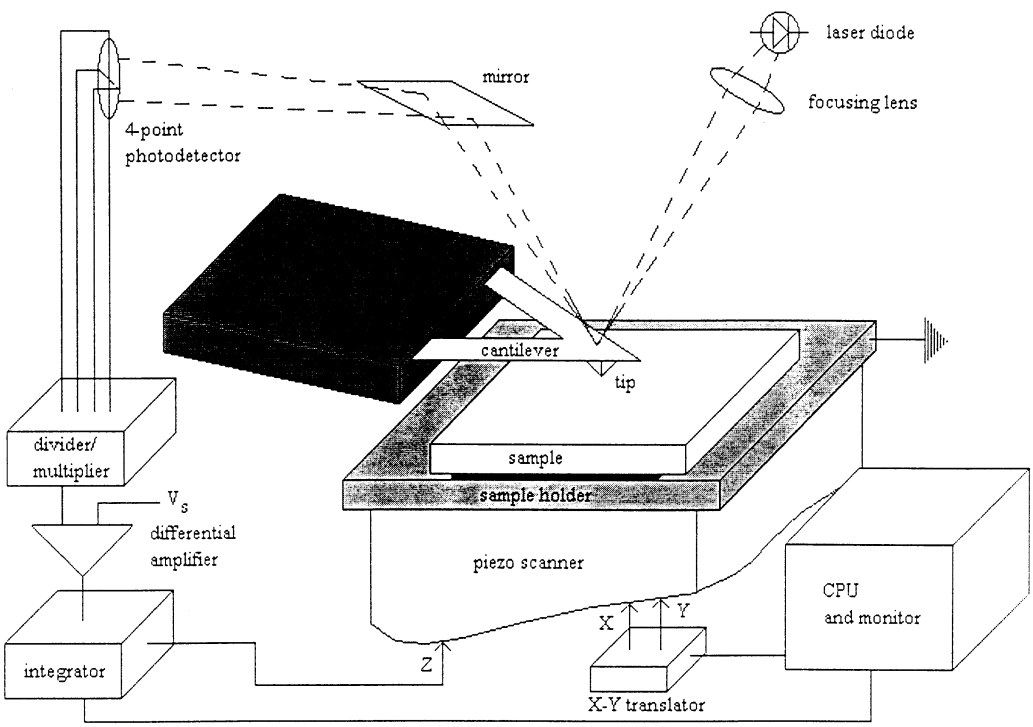

Fig. 1. A schematic diagram showing the essential features of an atomic force microscope.

and in environments in which no other technique can achieve comparable resolution of such non-crystalline specimens (Binning et al., 1986). The atomic force microscope uses a cantilever-mounted tip to sense the surface topography of a specimen. In contact mode, the small silicon nitride, square pyramidal tip is brought into contact with the specimen surface, where short-range, van der Waals repulsive interactions, of the order of a few nano-Newtons, cause the flexible cantilever to deflect. The probe is then raster-scanned across the surface of the specimen and the tip-sample forces are kept constant by means of a feedback circuit to the piezoelectric scanner to which the sample is mounted. Thus, movement of the probe in three-dimensions can be monitored and converted to a digital, topographic image of the surface under investigation.

This paper describes the use of atomic force microscopy (AFM) to image the binding to oral mucosal cell surfaces of candidate macromolecular carriers identified in our previous work using a lectin binding inhibition techniques (Patel et al., 1999), namely, polycarbophil, hydroxypropyl methylcellulose and chitosan. The topography of the human buccal cell surfaces will be investigated and compared to those that have been exposed to solutions of these bioadhesive polymers.

2. Experimental

2.1. Materials

Human buccal cells were obtained fresh from the oral cavity of healthy volunteers of both sexes, aged between 18 and 40, from the University of Portsmouth. Hydroxypropyl methylcellulose (Methocel 65HG) was obtained from Fluka Chemicals, Gillingham, UK. Polycarbophil was obtained from BF Goodrich Co., Chemical Group, Cleveland, USA. Chitosan (Sea Cure 240) was obtained from Pronova Biopolymer, Oslo, Norway. Nalgene graduated conical centrifugation tubes were obtained from Nalge Company, New York. All other agents were of analytical grade, purchased from Sigma Chemical Co., Poole, UK.

2.2. Methods

The methods for cell and solution preparation, described below, were the same as those used in our previous study (Patel et al., 1999).

2.3. Standardisation of buccal cell numbers

Donors were required not to eat or drink for 30–60 min prior to harvesting the buccal cells. Cells from 8–10 male and female volunteers were removed by gently scraping the inner cheeks of the oral cavity with a wooden spatula. The cells were mixed together by immersing into 10 ml isotonic 0.05M Tris buffer saline (TBS), pH 7.6. An aliquot of the cell suspension (0.9 ml) was added to 0.5% w/v trypan blue solution (0.1 ml). The concentration of cells was determined using a haemocytometer so that appropriate volumes could be taken to give 48×10^4 cells per test. The cells were stored at 4°C and used within 4 h of harvesting.

2.4. Preparation of polymer solutions

The polymers used in this investigation were dissolved in 0.9% saline to give a 0.5% w/v solution. The polymers were initially dissolved using gentle heating and stirring, or if necessary, a high intensity ultrasonic bath was used. Once dissolved

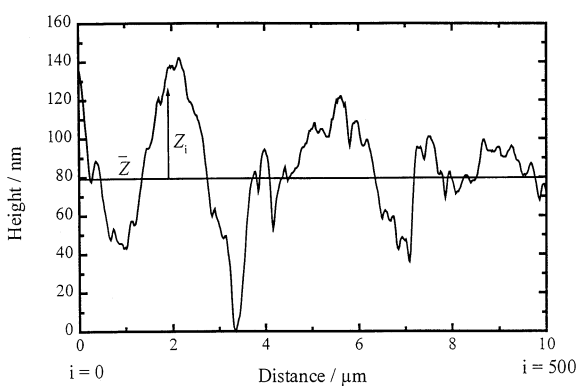

Fig. 2. A typical single-line profile transect obtained from an atomic force micrograph. Quantitative surface roughness data can be obtained from the measured heights at each pixel position across the profile (Z_i) and the average height (Z). Length of transect, 10 μm.

the pH of the polymer solutions was adjusted to 7.6 (pH 4.5 for chitosan) using 0.1 M sodium hydroxide or 0.1 M hydrochloric acid. The polymer solutions were stored between 2 and 4°C for at least 48 h to allow the polymer chains to fully hydrate prior to use.

2.5. Treatment buccal cells

The buccal cell suspension was centrifuged in a graduated conical centrifugation tube at 2000 rpm for 5 min, after which all but 2 ml of the supernatant was removed. An aliquot of the solution (5 ml) containing 0.5% w/v polymer solution in 0.9% w/v saline at pH 7.6 (pH 4.5 for chitosan) was added to the cell suspension and incubated for 15 min at 30°C. The cells were separated from solution by centrifuging at 2000 rpm for 5 min, after which all but 2 ml of the supernatant was removed. The cells were then vigorously stirred using a vortex mixer and washed using 0.05 M TBS (12 ml, pH 7.6) to remove unbound polymer, followed by centrifugation at 2000 rpm for 5 min. The washing step was repeated twice using distilled water. A single drop sample of the cell suspension in water was applied to a freshly cleaved mica surface (1×1 cm^2) and allowed to air-dry (ca. 10 min) prior to examination using AFM.

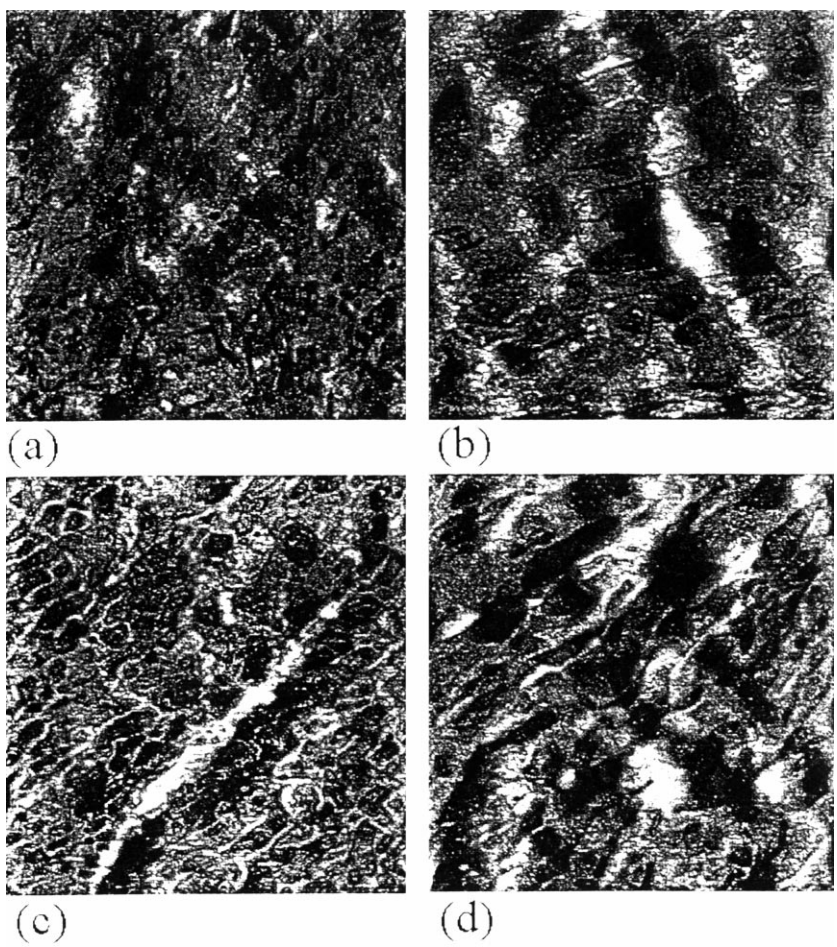

Fig. 3. Some typical, top-view atomic force micrographs of human buccal cells that have been subjected to various treatments: (a) untreated cells (control); (b) 0.5% w/v polycarbophil (pH 7.6); (c) 0.5% w/v methocel 65HG (pH 7.6); and (d) 0.5% w/v chitosan (pH 4.5). Scan range, $x = y = 10$ μm.

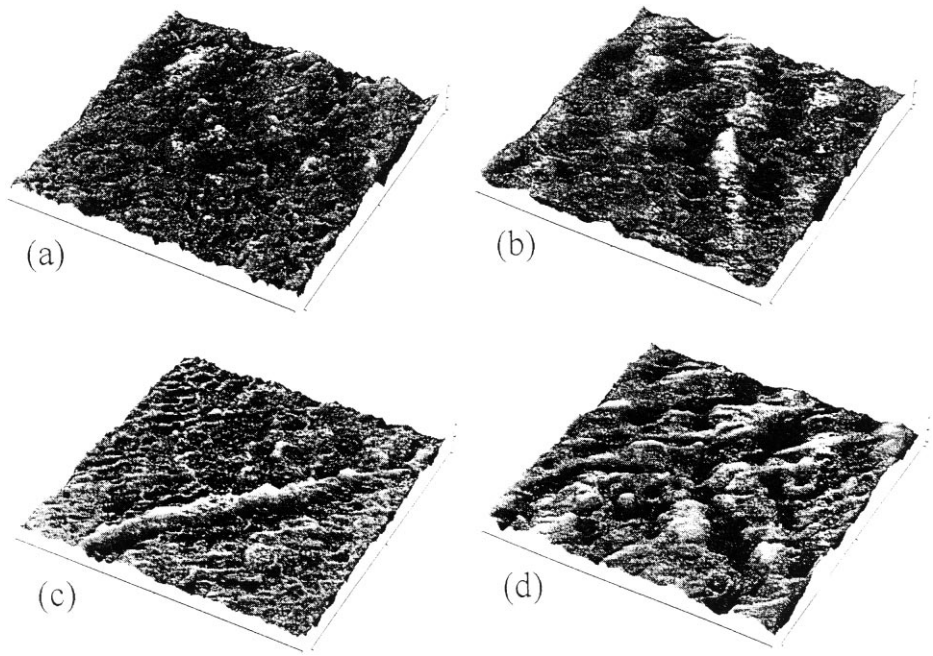

Fig. 4. Pseudo-three dimensional renditions of the top view images shown in Fig. 3 provides a greater appreciation of the relative topographic changes resulting from the adsorbed bioadhesive coatings. Legends (a–d) are indentical to those for top-view images.

The control was prepared in a similar manner, omitting the polymer exposure step.

2.6. AFM studies

AFM studies were performed in air under normal atmospheric conditions, at a temperature of 20°C and a relative humidity of 50%, using a Discoverer TopoMetrix TMX2000 Scanning Probe Microscope (SPM) instrument (TopoMetrix Corporation, Saffron Waldon, UK). A scanner capable of a maximum x, y, z-translation of $70 \times 70 \times 12$ μm was used. Imaging was performed in contact mode using forces in the range of 1–10 nN, using standard-profile, pyramidal, silicon nitride tips mounted on cantilevers of spring constant 0.036 Nm^{-1}. Quantitative data, such as height measurements and surface roughness, were obtained using TopoMetrix image analysis software (TopoMetrix SPM Lab, Version 3.06.06, 1996). Images were levelled using a sixth-order horizontal algorithm to remove the effects of 'image bow' prior to surface roughness measurements. Surface roughness measurements were recorded using the arithmetic roughness average term, R_a, which is the arithmetic average of the absolute values of the measured profile height deviations, given by:

$$R_a = \frac{1}{n} \sum_{i=1}^{n} |Z_i - \bar{Z}|$$

Where: n, number of height positions along line profile; Z_i = height at position i (nm); and $\bar{Z}$, average height (nm). For this study, ca. 100 line profiles of length 10 μm were analysed for the control and each of the treated cell images. The arithmetic roughness average, R_a, was extracted from the surface roughness profile, an example of which is shown in Fig. 2.

3. Results and discussion

Isolated human buccal cells have been used as model mucosal surfaces in several previous studies (e.g. Gibbons and Dankers, 1983; Nantwi et al., 1997; Patel et al., 1999). Typical, top-view atomic force micrographs of untreated and exposed hu-

man buccal cells are shown in Fig. 3. Untreated cells appear to have a level surface (Fig. 3a), although many small, crater-like pits, such as those shown in the bottom-right quadrant of the figure, and valleys were observed. Buccal cells treated with polycarbophil (Fig. 3b), hydroxypropyl methylcellulose (Fig. 3c) and chitosan (Fig. 3d) appear rather different, in that they have lost these crater and valley topographic features. In addition, a rippled overlayer was observed on polymer exposed cells, the topography of which seemed to be characteristic of each specific polymers. For example, the surface cells treated with hydroxypropyl methylcellulose shows a fine, rippling effect whereas those exposed to chitosan display a more coarse, undulating surface feature. The differences in topography can be appreciated further from pseudo three-dimensional renditions of the micrographs (Fig. 4). Here, it can be seen that chitosan exposed cells have a greater peak-to-valley roughness compared to cells subjected to other treatments.

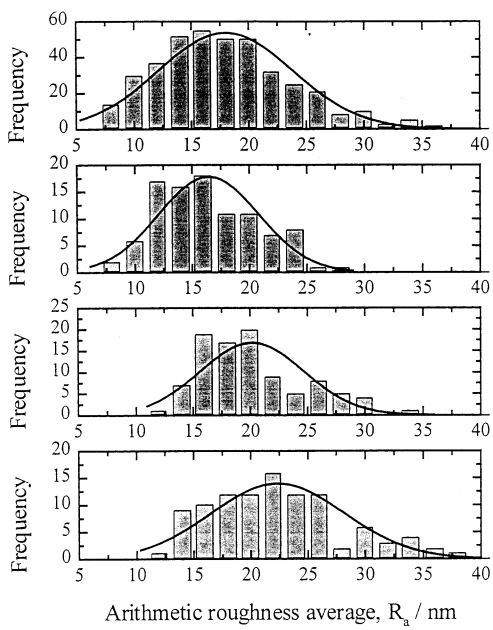

Fig. 5. Histograms showing arithmetic roughness average (R_a) frequency distributions obtained from many images transects directly show the increase in surface roughness of human buccal cells resulting from exposure to bioadhesive polymers. Plots from top to bottom show untreated cells (control) and those treated with 0.5% w/v polycarbophil (pH 7.6), 0.5% w/v hydroxypropyl methylcellulose (pH 7.6) and 0.5% w/v chitosan (pH 4.5), respectively.

Quantitative image analysis, incorporating one-way analysis of variance (ANOVA), showed significant differences in arithmetic roughness average (R_a) of cell surfaces that had been subject to each of the polymer-exposure treatments ($P < 0.05$, $n = 100$). For example, the combined arithmetic roughness averages for chitosan treated cells (22.3 ± 5.8 nm) was significantly greater than that of untreated cells (12.9 ± 3.5 nm). Frequency distribution histograms of the R_a roughness parameters on untreated and treated cells are also shown in Fig. 5.

4. Conclusions

This investigation has shown AFM to be a sensitive technique for imaging the presence of adsorbed bioadhesive polymers on mucosal cell surfaces under the conditions of minimal sample preparation. The imaging methods described are relatively simple, in that they make use of contact mode, topographic operation, and as such, they are only able to provide qualitative and semi-quantitative information with regard to cell coverage. Changes in surface topography were indicative of the presence of bound polymer, whose ability to adsorb onto buccal cell surfaces has already been identified using direct staining and lectin-binding inhibition techniques (Patel et al., 1999). Although there is a potential danger of operator bias in all imaging techniques, when used in conjunction with other experimental procedures this provides strong additional supporting evidence. We believe that this is the first time AFM has been used in this way.

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