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SUBSTRATE HYDROXYLATION AND CELL ADHESION

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SUMMARY

Adhesion of BHK cells to a variety of polymer surfaces carrying measured densities of hydroxyl and carboxyl groups was studied. The effects on cell adhesion of blocking hydroxyl groups by acetylation and carboxyl groups with diazomethane were measured. Hydroxyl groups were required for cell adhesion, though the very high surface densities of these groups diminished cell adhesion. The optimal surface density of OH groups for BHK adhesion was 2000 per 1×10^{-11} cm^2. Carboxyl groups slightly inhibit cell adhesion, since blocking of these groups by methylation increased adhesion. The role of oxidizing systems of cellular origin in conditioning of the substrate, in serum-free conditions, was demonstrated for leucocytes and BHK cells, in particular by the result that oxidizable substrates such as phytane and poly(1,2-butadiene) could be made suitable for cell adhesion by contact with cells.

INTRODUCTION

In a recent paper, Curtis *et al.* (1983) suggested that hydroxylated polystyrene surfaces had very great adhesiveness for leucocytes and BHK cells. The evidence was based on the use of a novel oxidation system for rendering the polystyrene very adhesive. This oxidation system generated high densities of hydroxyl groups on the plastic surface, which could be demonstrated by X-ray photoelectron spectroscopy (XPS) and by attenuated total internal reflection spectroscopy (ATR) as well as by radioactive binding methods. Blocking of the hydroxyl groups by acylating reagents such as acetic anhydride diminished the adhesiveness of the surfaces but blocking carboxyl groups by esterification had little effect on adhesion. However, it is desirable to provide further tests of the correctness of these deductions, because several reports have ascribed the adhesiveness of 'tissue culture' grade plastic to the presence of carboxyl groups. We are faced with the possibility that hydroxyl groups make a major contribution to cell adhesion to such surfaces and that carboxyl groups have no such role, or with the reverse position, or with the possibility of important contributions from both types of group. This paper sets out to provide further tests on these points.

In addition we have explored the question of whether a reasonable density of hydroxyl groups is a sufficient requirement for cell adhesion or whether other features of the surface on which the hydroxyl groups are borne could be of

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importance. To test this we have used hydroxylated polypropylene and polyisopropenyl alcohol, which are both polymers with hydroxyl groups at the tertiary carbon site, partially hydrolysed polyvinyl acetate (which bears secondary hydroxyl groups), poly(hydroxyamino acids) and partially hydrolysed cellulose triacetate, which carries secondary and primary hydroxyl groups.

A further problem raised by the previous paper (Curtis *et al.* 1983) is that high adhesiveness is ascribed to a high density of hydroxyl groups on a substrate while it is well known that surfaces such as agarose, which are heavily hydroxylated, are non-adhesive (Maroudas, 1977). Lydon *et al.* (1985), who examined cell adhesion to surfaces formed of mixtures of poly(hydroxymethyl) and poly(ethylmethacrylate) found that cells would not adhere to surfaces formed from mixtures of more than 90 % of one or the other polymer; in other words, weakly and very highly hydroxylated surfaces were non-adhesive but those of intermediate hydroxylation were adhesive. Thus in this paper we report on the relationship between density of hydroxylation and adhesion.

Curtis *et al.* (1983) pointed out that appreciable cell attachment took place on untreated polystyrene in the absence of serum. Since it would be expected that such polystyrene would have few or no surface hydroxyl groups a problem for investigation exists, which we approach in this paper.

We have used four main approaches in this work. In the first, very specific and reactive reagents have been used to react with hydroxyl groups and carboxyl groups. These have been used both to determine their effects on cell adhesion and to measure the surface density of hydroxyl or carboxyl groups. In the second approach we have prepared surfaces of known chemistry having variable levels of hydroxyl groups and investigated cell adhesion on such surfaces. In the third approach we have studied cell adhesion onto surfaces that should be susceptible to hydroxylation by oxidation systems possessed by the cells themselves, while in the fourth we have looked for direct evidence of substrate hydroxylation by cells.

MATERIALS AND METHODS

Cells

BHK21 C13 cells were obtained and grown, and human leucocytes were obtained by the methods described previously (Curtis *et al.* 1983). BHK cells were trypsinized to provide cell suspensions using trypsin at a level of 200 BAEE units per ml for 5 min at 37 °C. Trypsin action was stopped by addition of the serum-containing culture medium in those experiments in which cells were tested for their adhesion in the presence of serum, but when cell adhesion in the absence of serum was being tested tryptic activity was stopped by adding 25 μg of leupeptin (Sigma Chemical Co., Poole, UK) to each ml of cell suspension, followed by washing of the cells in a serum-free Ham's F10 medium. The BHK cells were suspended in either Ham's F10 saline or Ham's F10 medium plus 3 % foetal calf serum plus the insulin/transferrin/selenite supplement (ITS) (Collaborative Research, Waltham, MD, USA) with insulin and transferrin, each at 5 $\mu g\,ml^{-1}$ at a density of 2×10^5 cells ml^{-1}. Leucocytes were suspended at a density of $1{\cdot}5\times10^6$ cells ml^{-1} in Hepes-buffered Hanks' balanced salts solution with or without 10 % foetal calf serum (Gibco-Biocult, Paisley, UK).

Culture dishes

Polystyrene culture dishes, bacteriological and tissue culture grade were purchased from the following makers: Sterilin, Teddington, Middlesex, UK and Nunc through Gibco-Biocult, Paisley, UK.

The bacteriological dishes were given the following treatments: chloric acid treatment by adding 3 ml of 70% perchloric acid and 1 ml saturated aqueous potassium chlorate to the dishes and allowing these to react for 10 min at 37°C. Control dishes (bacteriological) were stripped of residual mould-release agent by treatment for 30 min with ethanolic NaOH (5 M in 70% ethanol) followed by extensive washing with tap water. This treatment was shown (Curtis *et al.* 1983) to produce surfaces identical in behaviour to pure polystyrene.

Other polymers

Polyvinyl acetate surfaces and partially hydrolysed polyvinyl acetate surfaces were prepared by making 5% (w/v) solutions of polyvinyl acetate (medium molecular weight grade, Aldrich Chemical Co. Ltd, Gillingham, Dorset, UK) in acetone; 3 ml of this solution was placed in a 60 mm diameter glass Petri dish and the acetone was allowed to evaporate. The films were then pumped under high vacuum to remove residual acetone. In order to obtain partial hydrolysis of the surface layer to produce surfaces rich in polyvinyl alcohol the films were exposed to 2 M-NaOH solutions for varying periods. The films were then washed free of residual NaOH and used for cell adhesion measurements.

Surfaces of poly(1-2,butadiene), poly(1-4,butadiene) and poly(isobutylene) (obtained from Polysciences, Ltd, Northampton, UK) were cast from solutions of these polymers in petroleum ether. Polypropylene surfaces were prepared by pressing films at 170°C from homopolymer (high M_r grade), a gift from Shell, UK, Manchester, England, in a press (Specac Ltd, Orpington, Kent, UK) using poly(tetrafluorethylene)-coated platens at a pressure of 0·5 tonne cm^{-2} for 15 min. The films were then trimmed to fit Petri dishes, and treated with the chloric acid reagent at 70°C for 10 min. Poly(methylmethacrylate) sheet containing about 3% poly(ethylacrylate) was purchased from ICI Ltd (Welwyn Garden City, UK) and oxidized by exposure to an oxygen plasma at 150 millitorr (1 torr ≈ 133·3 Pa) for 3 min at 60 W in a barrel etching machine (Electrotech plc, Warrington, Avon, UK). Poly(methylacrylate) solution in toluene was purchased from Aldrich Chemical Co. (Gillingham, Dorset, UK) and films were cast directly from the solution on glass formers.

Cellulose triacetate, poly(1-butene) and poly(methylpentene) were purchased from Aldrich Chemical Co., Ltd (Gillingham, Dorset, UK). Films of the cellulose acetate were cast from solution and films of poly(1-butene) (PB) and poly(methylpentene) (PMP) were prepared in the same manner as polypropylene films but at 90°C and 220°C, respectively, above the glass transition temperature of the polymer. Poly(isopropenyl alcohol) was a gift from Professor G. Kirby, Department of Chemistry, University of Glasgow. This polymer (PIP) was formed into films by solution casting from acetone.

The polymer films were trimmed to fit within polystyrene Petri dishes or cast onto the surfaces of glass Petri dishes.

The poly(amino acids) used in this work were purchased from Sigma Chemical Co. (Poole, UK) and dissolved in trifluoracetic acid at 100 $\mu g\,ml^{-1}$. Samples (1 ml) were placed in polystyrene bacteriological Petri dishes (18·9 cm^2 area) and allowed to adsorb for 20 min. Excess polymer and the trifluoracetic acid were washed off with sterile Ham's F10 saline.

Treatment of dishes with diazomethane and with acetic anhydride

Diazomethane was used to methylate carboxyl groups and acetic anhydride to esterify hydroxyl groups in order to discover whether blocking these groups changed cell adhesion. [^{14}C]diazomethane and H^{36}Cl gas (both in solution in dry petroleum ether) were used to measure the extent of binding. Diazomethane was prepared by dissolving 0·1 g of 'Diazald' (Aldrich Chemical Co., Ltd, Gillingham, Dorset, UK) in either petroleum ether (when the reagent was to be used on polystyrene surfaces) or diethylether (when the reagent was to be used on polypropylene surfaces) and adding ethanolic NaOH slowly to the solution. The diazomethane was distilled over with the petroleum or diethylether and stored at −20°C. Radioactive diazomethane was prepared

on a micro-scale using [^{14}C]Diazald (Amersham International plc, Amersham, UK) by the same general method. Diazomethane treatment was carried out by exposing the surface to be treated to diazomethane vapour for 1 h.

Acetylation was carried out in ultra-dry conditions using acetic anhydride at a level of 1 μmol ml^{-1} in ultra-dry petroleum ether with the addition of 1 μmol of 4-dimethyl aminopyridine as a catalyst for the acetylation of tertiary alcohols (Scriven, 1983) for a duration of 20 min. The treated surfaces were then washed three times in petroleum ether to remove unreacted reagents.

Detection of tertiary hydroxyl groups

Tertiary hydroxyl groups, unlike primary and secondary alcohols, react with dry HCl gas to form tertiary chlorides (Fessenden & Fessenden, 1979). This reaction was used with H^{36}Cl to detect tertiary hydroxyls on polymers that had not been exposed to cells. Samples of this reagent were titrated to measure the concentration of HCl. Solid samples of H^{36}Cl (Amersham International plc, Amersham, UK) were treated with known quantities of the unlabelled acid to give a product with a specific activity of around 0·02 μCi μmol^{-1}. Each batch made had a specific activity close to this value. Samples were treated with portions of this reagent for 10 min, washed with dry petroleum ether, and then counted.

When hydroxyl group density was being measured on surfaces that had been in contact with protein or possible sources of protein, such as cells, a modification of this procedure was used. Tertiary chlorides were formed with cold HCl gas in petroleum ether solution at 1×10^{-4} M. Protein hydrochlorides would also be formed if protein was present. Excess HCl gas was removed under high vacuum. The samples were then immersed in dry nitrobenzene and reacted by the Friedel-Crafts procedure (Olah, 1963) with [^{14}C]benzene (Amersham International plc, Amersham, UK) using anhydrous aluminium chloride as a catalyst for 1 h. Subsidiary experiments showed that this reaction has an efficiency of about 10 % under these conditions for surface labelling.

In some experiments surface hydroxyl group density on polymers that had not been exposed to sources of protein was measured with [^{14}C]phenylisothiocyanate (Amersham International plc, Amersham, UK) using the method described by Matsunaga & Ikada (1980). This reagent detects primary, secondary and tertiary hydroxyls.

Superoxide treatment of surfaces

Potassium superoxide was purchased from Pfaltz and Bauer, Inc. (Stamford, Conn. 06902, USA). Materials to be treated were placed in small samples of 0·2 M-phosphate buffer, pH 7·0, and sufficient potassium superoxide was added to bring the concentration to 0·005 M. The samples were left in the reactant for 30 min at 25 °C.

Measurement of cell adhesion

In order to measure cell adhesion $0\cdot6\times10^6$ BHK21 C13 cells (in a final volume of 3 ml) were placed in each culture dish, which was then incubated at 37 °C for 15 min. Cell spreading during these incubation periods is inappreciable on control surfaces. The cells that had not adhered during these culture periods were removed from the dishes by washing with Hepes-buffered Hanks' balanced salts solution three times at an average shear rate of 30 s^{-1} and a peak rate of 100 s^{-1} for 10 s on each wash. The number of adherent cells in each of 10 standard counting areas was then counted using phase-contrast microscopy to detect the cells and a Quantimet 720 image-analysing computer to count the cells. The counting areas were 0·0026 cm^2. The results are expressed in terms of the number of cells adhering per cm^2.

Zymosan activation of polymorphonuclear leucocytes

Zymosan particles (Sigma Chemical Co., Poole, UK) were preopsonized by incubation for 1 h with 25 % autologous human serum at 37 °C in a rotating flask. The opsonized particles were washed free of serum by centrifugation with three changes of Hanks' saline. Polymorphonuclear leucocytes (3×10^6 per ml) in Hanks' saline were plated out onto polystyrene Petri dishes and incubated at 37 °C with the opsonized zymosan particles (4 mg per ml; final volume 3 ml per dish) with constant shaking. Control dishes were plated similarly but without zymosan particles.

Further dishes were treated with zymosan-activated leucocytes that had been pre-incubated with superoxide dismutase (1·5 mg per 1×10^7 cells) for 5 min at 22°C. Dishes were washed after incubation with the leucocytes for 2 h, using Hanks' saline, and the adherent leucocytes were removed by treatment with a 0·01 % Versene solution in calcium- and magnesium-free Hanks' saline.

Scanning electron microscopy

Suspensions of BHK cells in Ham's F10 medium plus 3 % foetal calf serum and the ITS supplement were plated out on either 'tissue culture' grade or on chloric-acid-treated polystyrene under the same conditions as those used for cell adhesion measurements. After 15 min incubation at 37°C the cultures were fixed in 2·5 % (w/v) glutaraldehyde in 0·11 M-phosphate-buffered saline (0·145 M-NaCl, 0·05 M-$CaCl_2$, 0·05 M-$MgCl_2$, pH 7·3). After an initial fixation of 60 min at 37°C the fixation was continued at 4°C for 12 h. Material was then washed in phosphate-buffered saline and fixed for 1 h in 1 % (w/v) osmium tetroxide, followed by a rinse in distilled water. Each sample was then rapidly cooled in liquid propane at −196°C, mounted in a precooled sample holder and placed in a liquid-nitrogen-cooled stage of the vacuum chamber of a modified high-vacuum coating unit (Edwards High Vacuum, Crawley, UK). The samples were kept at −80°C and 1×10^{-6} torr pressure for 16 h. After slow rewarming (10 deg.C h^{-1}) to 25°C the samples were sputter-coated with gold to a thickness of ≈ 20 nm in a Polaron E5000 sputter coater (Polaron plc, Watford, UK) and mounted for examination in a Philips SEM 500 at 6 or 12 kV.

RESULTS

In earlier work (Curtis *et al.* 1983; Curtis & Forrester, 1984) we suggested that hydroxylation of the surface of polystyrene resulted in a considerable increase in cell attachment. Does this effect of hydroxyl groups extend to other surfaces that might bear them? Do carboxyl groups play an appreciable role in adhesion?

The surface chemistry of a range of polymer surfaces before and after various types of surface treatment is listed in Table 1. These results are confirmed by XPS data described by Curtis & Kirby (1983).

Cell adhesion to a variety of hydroxyl-group-rich surfaces

The polymer poly(isopropenyl alcohol) was prepared especially for this work, as a test surface of high hydroxylation, for comparison with poly(propylene), poly(1-butene) and poly(methylpentene) (PMP = TPX) treated by a modification of the chloric acid process described by Curtis *et al.* (1983), partially hydrolysed polyvinyl acetate and acid-hydrolysed cellulose triacetate, as well as polystyrene and polypropylene. Structures for monomer units of these polymers are shown in Fig. 1.

The results of measurements of the adhesion of BHK cells to these substrates and appropriate controls are shown in Tables 2, 3 and 4. It is clear that poly(isopropenyl alcohol), poly(1-butene), hydroxylated poly(propylene) and poly(methylpentene) provide after surface hydroxylation, adhesive surfaces for cells even in the presence of serum, unlike their control surfaces. Blocking of hydroxyl group function by acetylation diminishes adhesion.

It should be noted that the untreated polymer surfaces that are relatively free of hydroxyl groups (Table 1) permit appreciable cell attachment in serum-free conditions (Tables 3, 4). This result is apparently discrepant with the interpretation

Table 1. *Chemistry of surfaces treated by various oxidation processes*

Polymer	Treatment	Hydroxyl group density (nM cm^{-2})	Carboxyl group density (nM cm^{-2})
Polystyrene	Untreated	0·028	0·145
	Chloric acid	0·46	0·518
	Corona discharge	ND*,†	1·44
PMMA	Untreated	0·06	0·027
	Oxygen plasma	1·74	0·76
Polypropylene	Untreated	0·024	ND
	Chloric acid	0·26	ND
PIP	Untreated	0·90	ND

Measurements are the means of counts of at least three samples. Hydroxyl group density was measured with dry $H^{36}Cl$ gas; sp. act. 0·32 μCi μmol^{-1}. Carboxyl group density was measured with [^{14}C]diazomethane; sp. act. 3·6 μCi μmol^{-1}. Hydroxyl group densities were also measured with labelled acetic anhydride and with labelled phenyl isothiocyanate reagents, which gave similar results. PMMA, poly(methylmethacrylate); PIP, poly(isopropenyl alcohol).

* A value of 0·04 nm cm^{-2} was obtained in earlier work (Curtis *et al.* 1983).

† ND, not determined.

of the role of hydroxyl groups advanced here but is the subject of further experiment described below.

Poly(serine) and poly(hydroxyproline) provide surfaces of greater adhesion than poly(glycine) when adsorbed to polystyrene (Table 4). Partially hydrolysed cellulose acetate can provide an adhesive surface, though the extent of adhesion depends on the duration of hydrolysis, there being an optimum after a short period of time (see Fig. 2). However, partially hydrolysed polyvinyl acetate is a non-adhesive surface in both the absence or the presence of serum, even though a variety of hydrolysis conditions were tried. The clear evidence is that the precise substrate chemistry has little effect on cell adhesion thereto, provided that a reasonable density of hydroxyl groups is present. However, very highly hydroxylated surfaces such as poly(isopropenyl) alcohol, partially hydrolysed polyvinyl acetate and hydrolysed cellulose triacetate (Fig. 2) are of low or zero adhesiveness. This suggests that the precise surface density of hydroxyl groups may be of importance in adhesion.

Hydroxyl group density on surfaces and cell adhesion

In Fig. 3 are plotted the adhesiveness of the surface for BHK cells and the hydroxyl group density for treated polystyrene. The results show that adhesion increases to a maximum asymptotically and then falls as further hydroxylation is effected.

The role of carboxyl groups in cell adhesion

Carboxyl groups are to be found on the surface of many polymers as a result of chain scission by photo and other oxidation processes (Schnabel, 1981). Furthermore, the oxidative processes by which surfaces are made suitable for cell culture

might well be expected to result in chain scission. So we measured the surface density of carboxyl groups on a variety of polymers before and after exposure to oxidative environments (Table 1) and also investigated cell attachment on these surfaces before and after blocking of the carboxyl groups with diazomethane (Table 5). It is clear from the results that though oxidative processes such as oxygen plasma treatment produce additional carboxyl groupings on the surfaces of these polymers they do not increase cell adhesion.

Cell adhesion to unoxidized polymer surfaces in serum-free conditions

In this and earlier work (Curtis *et al.* 1983) it was noted that BHK cells and leucocytes will attach to untreated polystyrene in the absence of serum proteins,

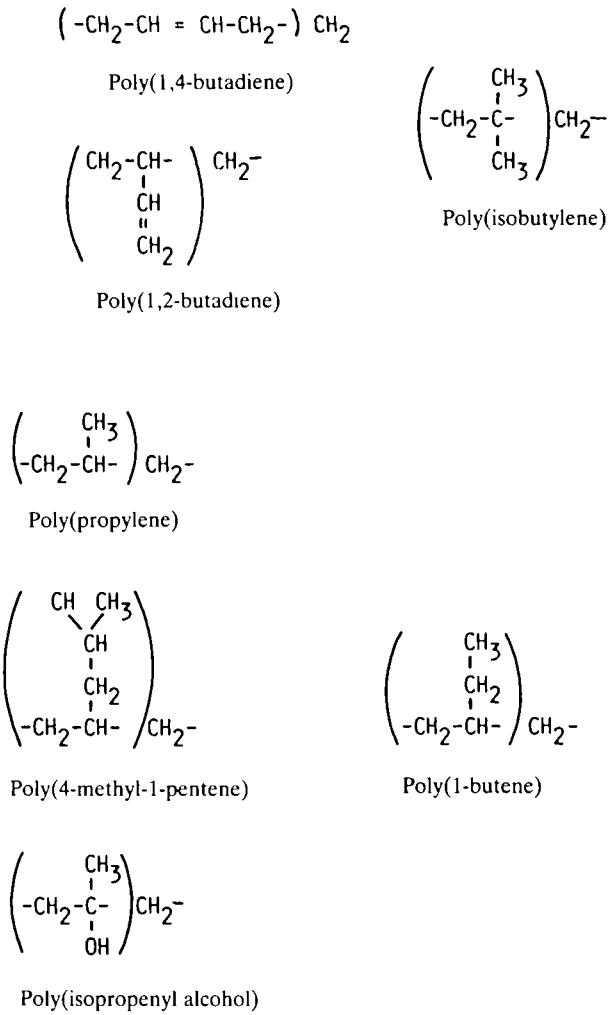

Fig. 1. Structures for polymers used. The monomer units together with link bonds to next unit outside brackets are shown.

although the attachment is less rapid and extensive than is found for hydroxylated polystyrene. However, the density of hydroxyl groups on untreated polystyrene (Table 1) or on untreated PB, PMP or polypropylene (see Table 1) is very low. This

Table 2. *The role of hydroxyl groups in cell adhesion*

Polymer	Cell adhesion (cells cm^{-2}) Free hydroxyl groups	Acetylated hydroxyl groups
Polystyrene	971 (34)	471 (140)
Polystyrene, hydroxylated	30261 (3·6)	731 (148)
Polypropylene	3458 (12)	636 (56)
Polypropylene, hydroxylated	24916 (19)	1191 (5)
PIP	6517 (27)	756 (55)

Ham's F10 + 3 % serum medium. Measurements of cells attached per cm^2; means of at least 30 measurements; standard deviations in parentheses as a percentage of the mean. Maximal possible cell attachment 31750 cells cm^{-2}. PIP, see Table 1.

Table 3. *Adhesion of BHK cells to various polyolefin polymer surfaces*

Polymer	Cell adhesion (cells cm^{-2}) Serum medium	Serum-free medium
Polystyrene	234 (91)	7880 (24)
Polystyrene, hydroxylated	12905 (19)	25399 (17)
Polystyrene, TC grade	12996 (23)	22159 (19)
Polypropylene	3234 (8)	10506 (4)
Polypropylene, hydroxylated	19667 (6)	45286 (4)
PMP	0 (0)	6680 (33)
PMP, hydroxylated	21942 (10)	35016 (19)
PB	3930 (28)	16781 (28)
PB, hydroxylated	18471 (23)	32226 (22)
PIP	13440 (23)	ND

Adhesion measurements are described in the text and legend to Table 2.

Table 4. *Adhesion of BHK cells to other polymer surfaces*

Polymer	Cell adhesion (cells cm^{-2}) Serum medium	Serum-free medium
Control	589 (90)	6091 (17)
Poly(L-serine)	3851 (48)	19021 (11)
Poly(L-leucine)	ND	4144 (29)
Poly(L-glycine)	ND	9101 (27)
Poly(L-hydroxyproline)	4120 (11)	11790 (28)
Cellulose triacetate	393 (85)	2534 (96)
Polyvinyl acetate		
Unhydrolysed	0	0
Hydrolysed, 0·5 min	0	0

Media: Ham's F10 with or without 3 % foetal calf serum. Adhesion measurements were as described in the legend to Table 2.

apparent paradox could be explained either by assuming that some other mechanism of adhesion operates in such conditions or that the cells are able to hydroxylate the surface to some extent. The latter suggestion is testable. In order to do so the following series of experiments was carried out.

Since stimulated leucocytes produce superoxide anions in the surrounding medium (Claster *et al.* 1984) we tested whether superoxide treatment of polystyrene rendered it suitable for cell attachment and produced hydroxyl groups on the plastic (see Table 6). As an alternative demonstration, leucocytes were stimulated with

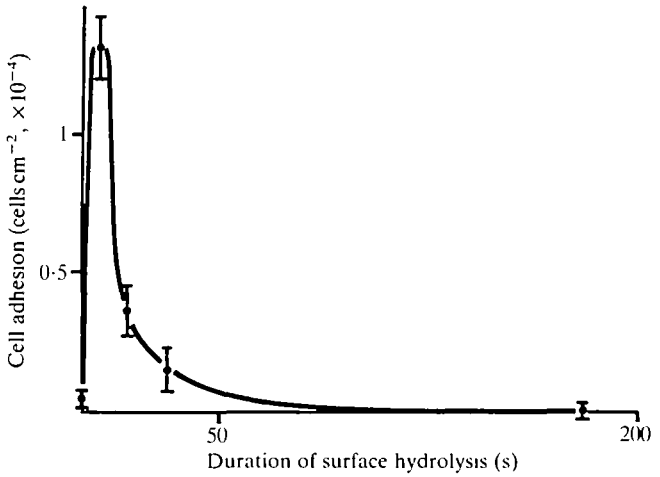

Fig. 2. Duration of acid hydrolysis of cellulose triacetate surfaces and extent of cell adhesion thereto. Vertical bars show standard deviation.

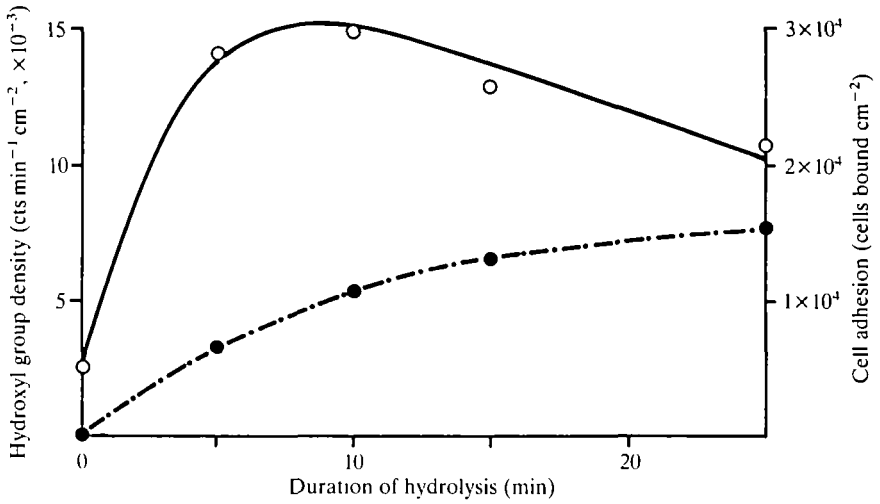

Fig. 3. Hydroxyl group density and BHK cell binding. Hydroxylation of polystyrene by increasing duration of chloric acid treatment. Hydroxyl group density measured with $H^{36}Cl$ reagent (for sp. act., see the text). (O——O) Cell adhesion, cells cm^{-2} adhering; (●-·-·-●) hydroxyl group density.

zymosan and incubated in a polystyrene dish; the leucocytes were then removed and the dish used for attachment of BHK cells. Further experiments were done in which the zymosan–leucocyte treatment was carried out in the presence of the antioxidant, butylated hydroxytoluene, or of the enzyme superoxide dismutase. Results are shown in Table 6 and are consistent with the oxidation of the polystyrene by an oxidizing product of activated leucocytes.

Table 5. *Effects of surface oxidation of polymers and of blocking carboxyl groups on cell adhesion*

Polymer	Oxidation system	Cell adhesion (cells cm^{-2}) Carboxyls free	Carboxyls blocked
A. *Serum medium*			
Polystyrene	None	355 (150)	196 (134)
	Chloric acid	28 571 (8)	27 916 (14)
	Corona discharge	25 545 (7)	23 973 (9)
PMMA	None	14 462 (5)	17 423 (21)
	Oxygen plasma	12 261 (9)	17 292 (16)
B. *Serum-free medium*			
Polystyrene	None	6 170 (20)	8 441 (31)
	Chloric acid	28 492 (14)	29 475 (20)
	Corona discharge	17 292 (15)	19 473 (20)
PMMA	None	3 576 (52)	26 391 (11)
	Oxygen plasma	22 165 (23)	28 571 (13)

Cell adhesion in Ham's F10 + 3 % foetal calf serum, or for serum-free results in Ham's F10 medium. Carboxyl groups blocked by pretreatment with diazomethane. Cell adhesion measurements were as described in Table 2. PMMA, see Table 1.

Table 6. *Superoxide treatment of polystyrene: effects on cell adhesion*

Treatment	Cell adhesion (cells cm^{-2})	Hydroxylation (nm cm^{-2})
A. *Exogenous superoxide*		
None	1 732 (22)	0·02
5 mM-superoxide, 15 min	10 100 (13)	0·06
B. *Leucocyte-derived superoxide*		
Control	1 397 (35)	0·025
Zymosan-stimulated leucocytes	18 205 (31)	0·08
+BHT at 1×10^{-5} M	1 257 (65)	ND
+BHT at 5×10^{-5} M	550 (80)	ND
+superoxide dismutase	987 (22)	ND

Cell adhesion measured in Ham's F10 + 3 % foetal calf serum medium. Hydroxyl group density measured with [^{14}C]phenylisothiocyanate reagent; sp. act. 3·1 μCi μmol^{-1}. BHT, butylated hydroxytoluene.

Direct demonstration of substrate oxidation by BHK cells

This was carried out by allowing BHK cells to settle onto plain poly(methylpentene) substrates for varying periods in the absence of serum. PMP substrates were used, because polystyrene is incompatible with the Friedel-Crafts reaction system, to detect hydroxyl groups formed on the polymer rather than proteins released by the cells. The cell suspensions and attached cells were then removed and after intensive drying the dishes were treated with dry hydrochloric acid gas in solution followed by conversion of any tertiary chlorides through the Friedel-Crafts reaction to bind labelled benzene. Though the cells may be depositing proteins and glycosidic products on the substrate, none of these molecules will contain tertiary alcohols. The results are shown in Table 7.

Attachment of cells to other potentially oxidizable surfaces

Substrates containing tertiary carbon atoms should be especially open to oxidation by many systems (Schnabel, 1981). Thus it is interesting to discover whether cells can render such surfaces suitable for their attachment while being unable to attach to other surfaces that are less oxidizable. Poly(1-2,butadiene), unlike its near analogues poly(1-4,butadiene) and poly(isobutylene), has tertiary carbon atoms (see Fig. 1). Similarly phytane, a C_{26} hydrocarbon, contains six tertiary carbon atoms whilst *n*-hydrocarbons do not contain any. Substrates of these materials were exposed to suspensions of BHK cells in serum-free media at 37 °C for 30 min. Cell attachment by the end of this period was measured. The results (Table 8) show that the oxidizable substrates now support cell attachment whilst the less reactive surfaces do not.

Scanning electron microscopy (SEM) of adhering cells

Fig. 4 shows cells attaching to tissue culture grade and to chloric-acid-treated polystyrene only 15 min after plating out. It is clear that the cells spread more rapidly on the chloric-acid-treated polystyrene and that they do so by the projection of microspikes and microfilopodia. Note that the polystyrene surface appears similar in both types of surface.

DISCUSSION

The results we describe confirm and extend those reported earlier (Curtis *et al.* 1983; Curtis & Forrester, 1984). It is clear that carboxyl groups play a small role in

Table 7. *Hydroxylation of poly(methylpentene) by BHK cells*

Duration of exposure of polymer surfaces to cells (min)	[^{14}C]benzene binding (disints min^{-1} cm^{-2})	(pmol cm^{-2})
0	80	1·2
90	607	9·1

Means of measurements on seven dishes; 160 000 cells cm^{-2}; serum-free Ham's F10 medium at 37 °C. Hydroxyl groups were detected by formation of tertiary chlorides followed by Friedel-Crafts reaction with labelled benzene; sp. act. 30·5 μCi μmol^{-1}.

Table 8. *Adhesion of cells to tertiary carbon polymers and to their unoxidizable analogues*

Polymer	Presence of tert. C	Cell adhesion (cells cm^{-2})
Poly(1-2,butadiene)	Y	18404 (27)
Poly(1-4,butadiene)	N	5901 (43)
Poly(isobutylene)	N	0 (0)
Phytane	Y	4996 (37)
Triacontane (an *n*-alkane)	N	1164 (38)
Poly(methylacrylate)	Y	22143 (14)

Cell adhesion in serum-free Ham's F10. For further details on mode of expression of cell adhesion data see legend to Table 2.

cell adhesion for BHK cells in either serum or serum-free media. This conclusion is at variance with studies in which non-specific methods of oxidation have been used and in which carboxyl groups have been estimated by techniques such as X-ray photoelectron spectroscopy (Ramsey *et al.* 1984). These authors found that adhesion increased as polymer surfaces were oxidized and as carboxyl groups, detected by photoelectron spectroscopy, increased; interestingly, the authors note the possibility that alcohols are formed. It should be pointed out that the non-specific oxidation methods would produce a certain density of hydroxyl groups on polymers such as polystyrene, and that the deconvolution methods used in interpretation of such spectra are not wholly free from the possibility of misinterpretation. The results of the dye-binding methods used in such work to estimate surface charge are almost certainly erroneous, if only because the dye binding level is several hundred dye molecules per $Å^2$ (Rappaport *et al.* 1969; Arnstein & Hartman, 1975). Direct evidence that blocking carboxyl groups by highly reactive reagents such as diazomethane has no effect on adhesion appears to be more conclusive (Tables 1, 5).

Hydroxyl groups are of considerable importance in providing an adhesive surface, the chemistry of the substrate to which they are attached being of less consequence. This finding confirms, in an entirely different manner, the results of Lydon *et al.* (1985), who showed that mixtures of various proportions of poly(2-hydroxyethylmethacrylate) and poly(ethylmethacrylate) were adhesive substrates for cells but that the pure polymers or mixtures containing more than 90% of one or the other polymer did not support cell adhesion. Similar results were reported by another group (Hattori *et al.* 1985).

Oxidation of polymers with relatively non-specific agents such as radio-frequency plasma, superoxide anions or ultraviolet radiation in the presence of oxygen is likely to proceed through the formation of hydroperoxide groups (Kamiya & Noki, 1983). These may decompose by a variety of mechanisms: if tertiary carbons are present macroion transfer will produce tertiary alcohols as one of the products. Tertiary carbons will be considerably more reactive than secondary or primary carbons to these oxidizing reagents (Wiberg & Foster, 1961). It should be noted that storage of the polymers in light in the presence of oxygen would probably account for the

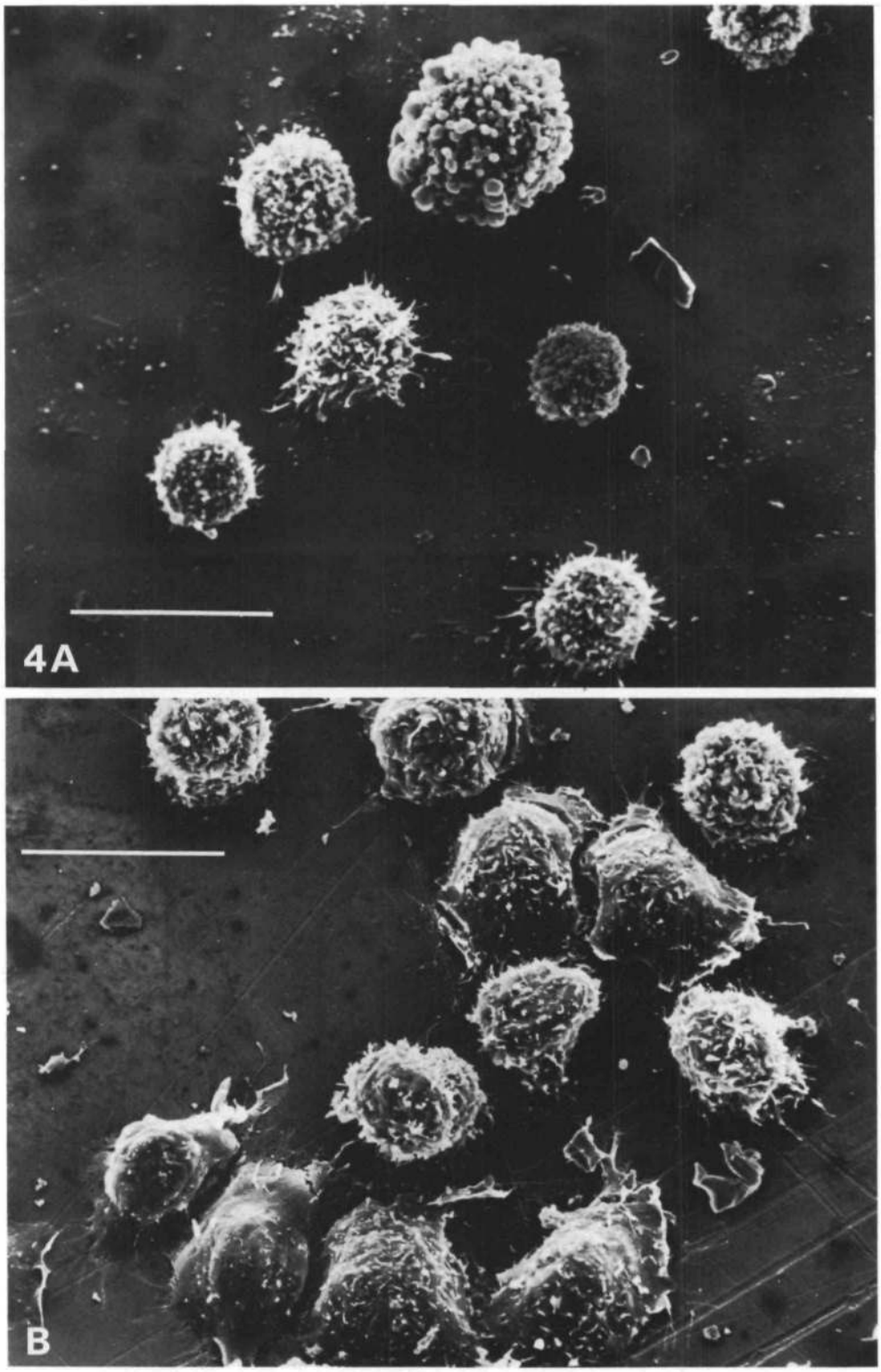

Fig. 4. The adhesion and spreading of BHK cells on tissue culture grade polystyrene (A) and on chloric-acid-treated polystyrene (B) after 15 min at 37 °C in Ham's F10 + 3 % serum medium. Note the greater degree of spreading on the chloric-acid-treated material. Bars, 20 μm.

low levels of hydroxyl and carboxyl groups found in this work for the untreated polymers. The reaction mechanism for the chloric acid reagent is not known, but it is likely to be a more specific and direct hydroxylating reagent. At first sight it may seem surprising that both the untreated and the radio-frequency oxidized poly(methylmethacrylate) bear hydroxyl groups on their surfaces (Table 1), but it should be borne in mind that the commercial polymer used contains about 3% poly(ethylacrylate), which is a 'tertiary carbon' polymer.

The presence of a high surface density of hydroxyl groups on these surfaces (Matsunaga & Ikada, 1981) could be expected to result in strong adsorption of proteins by hydrogen bonding, and the binding of many serum proteins to such surfaces has already been demonstrated (Curtis & Forrester, 1984). Our finding that adhesion is maximal at a level at which the surface is not fully hydroxylated parallels that described by Lydon *et al.* (1985). Maroudas (1977) reported that dried agarose surfaces were adhesive for cells and suggested that this effect arose because the surfaces then had a high dry weight concentration of material. A related explanation is that during drying an appreciable proportion of the hydroxyl groups has become directed to face inwards into the gel so that the surface density of hydroxyl groups is low enough for adhesion to take place.

Our results for adhesion to hydroxylated polystyrene suggest that adhesion is maximal at a hydroxyl surface density of about $0{\cdot}5\,\mathrm{nmol\,cm^{-2}}$, while adhesion is lower on poly(isopropenyl alcohol) at a surface density of nearly $1\,\mathrm{nmol\,cm^{-2}}$. These values should be compared with data published by Chmielowiec & Morrow (1983) and by Hartig & Huttinger (1983). The optimal hydroxyl group density is $0{\cdot}5\,\mathrm{nmol\,cm^{-2}}$ (equivalent to 3000 groups per $1000\,\mathrm{nm^2}$). It may be relevant to note that the surface density of serine and threonine hydroxyls on plasma fibronectin, treating it as a $2\,\mathrm{nm} \times 120\,\mathrm{nm}$ rod (Holly *et al.* 1984) and using data on amino acid composition from Sekiguchi *et al.* (1981), is, assuming that hydroxyl distribution is equal on either surface, 710 per $1000\,\mathrm{nm^2}$. Thus, though it is customary to think of the fibronectin molecule as having a specific binding site (Yamada *et al.* 1985), it is clear that it might contribute considerably to cell adhesion simply by reason of its high hydroxyl group density, as well as its low surface charge. In this connection it should be noted that Curtis & Kirby (1983) found that 'bare' hydroxylated polystyrene was appreciably more adhesive than adsorbed plasma fibronectin surfaces. It should be appreciated that though polystyrene and polypropylene surfaces were 'flat' under SEM and although these materials as well as cellulose triacetate and poly(1-butene) were shown by subsidiary measurements to have an immeasurably small porosity, some uncertainty about the exact values of surface densities of groupings must arise because of 'roughness' and the presence of cavities in the surfaces.

One interpretation of the surface density optimum of hydroxyl groups is that at higher density the surface becomes too mobile for effective stabilization to take place. We find that partially unhydrolysed polyvinyl acetate or cellulose acetate are non-adhesive; Forrester & Wilkinson (1981) have found that the highly hydroxylated molecule hyaluronic acid is non-adhesive.

Tentatively we suggest that water structuring and the resultant forces of attraction may explain our results. We should note that one major problem is posed by the results. While adhesion to these surfaces in the presence of serum is probably due to the interposition of a layer of one or more serum proteins to which the cells adhere, adhesion in the absence of serum may either be direct bonding between cell and substrate or adhesion between cell and proteins that it has secreted or shed (Culp, 1974). Our earlier work (Curtis *et al.* 1983) and work described in the accompanying paper (Curtis & McMurray, 1986) shows that cell attachment to highly hydroxylated surfaces can take place in the absence of serum even for cells whose synthetic abilities for producing fibronectin and other export proteins, and whose pools of such proteins, are small.

Thus the question arises as to whether the cells attach directly to the surface hydroxyl groups or to released surface components that adsorb to the hydroxyl groups to which the cells later attach. Even if components are adsorbed that are of cellular origin, one wonders if the chemistry of the substrate relatively remote from a cell surface 'protrudes' through to influence interaction.

The work has also shown that BHK cells possess an ability to modify appropriate surfaces by oxidation. This was not suspected. Such oxidations may explain some of the conditioning effects that cells have upon substrates and raise questions about the extent to which cells can modify their lipids and those of their neighbours by producing oxidation products such as leukotrienes. This phenomenon is, of course, already well known for leucocytes (Hoover *et al.* 1984), where these agents have substantial effects on cell adhesion. A short report (Gallagher & Curtis, 1984) indicates that similar phenomena can be found for lymphocytes.

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