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SURFACE TREATMENTS AND CELL ATTACHMENT

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SUMMARY

Photoelectron spectroscopic examination of treated plastic surfaces showed that surface oxidation, primarily as carboxyl groups, was responsible for formation of good growth surfaces. Gas-plasma studies indicated that only very short exposures were required and that the effect was confined to a thin surface layer that produced adhesive surfaces. Highly adhesive surfaces were produced using oxidizing chemicals. Studies with a polymeric ester demonstrated the importance of unesterified carboxyl groups for high adhesiveness.

Key words: adhesion; surfaces; plastic; plasma; Vero; fibroblast.

INTRODUCTION

The vast majority of cell cultures are classed as anchorage dependent and grow only when attached to a suitable surface. Glasses are suitable for many anchorage-dependent cells whereas air plasma- or corona discharge-treated polystyrene seems to be nearest to a universally suitable surface. Most commercial polystyrene tissue culture vessels are plasma- or corona-discharge treated. Rubin reported in 1966 that polystyrene petri plates could be made suitable as substrate for growth of attachment-dependent vertebrate cells through treatment with concentrated sulfuric acid (11). This effect was attributed to an increase in negative surface charge due to sulfonation. Electrical glow discharge was reported by Amstein and Hartman to make polystyrene plates suitable for tissue culture use (1). Kim and Goring have described the effect of a corona discharge on the surface morphology (9). They suggested three possible mechanisms for this effect, viz. increase in negative charges on the polystyrene molecule itself, accumulation of negative charges in impurities on the plastic surface (such as mold-release agents), or removal of surface impurities, perhaps by imparting a similar charge to both surface and impurity. Two widely used microcarriers, on the other hand, are composed of DEAE-dextran or dimethylaminopropyl-arylamide. At physiological pH these microcarriers would be expected to have a net positive surface charge. In earlier studies both surface negative charge (1) and surface positive charge (10) have been proposed as being desirable for cell growth.

The object of this study is to clarify the role of the surface on adhesiveness by characterizing the changes in the surface chemical composition of plastics due to electrical discharge and chemical treatments. The surfaces were chemically characterized by electron spectroscopy analysis (ESCA). It was found that physical or chemical treatments, which oxidized the plastic surfaces, also increased cell adhesiveness. Most of the effect is attributed to the presence of carboxyl on the treated plastic surfaces.

MATERIALS AND METHODS

Cells. Green Monkey kidney cells, Vero (American Type Culture Collection, Rockville, MD) (ATCC no. CCL 81, Lot F 1323, refrozen in house at Passage 131 and used at Passages 138 and 139), were used to determine effects of surface treatment on cell growth. No mycoplasmal contamination tests were done. The medium used was 95% Medium 199 with Earle's salts, 5% fetal bovine serum, and Gentamycin (Schering Corp., Kenilworth, NJ) at 100 μg/ml. All cultures were incubated at 36° C in an atmosphere of 95% air:5% CO_2 and 95% relative humidity.

Sterile slides were placed in sterile rectangular culture vessels with removable lids. Slides less dense than the medium were anchored to the

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vessel bottom with silicone grease. The slides were inoculated with Vero Green Monkey kidney cells at a final concentration of approximately 10^5 cells/cm^2 surface area. Cultures were incubated 24 h and then tested for adhesion by the centrifugal method.

Surfaces were tested for suitability for growth by inoculation at 10^3 cells/cm^2 surface area (9.6×10^3 cells/4 ml medium). Plates were examined at 3 and 6 d; representative fields were photographed using a Nikon inverted microscope and Polaroid film. In all experiments, control plates of untreated and O_2-plasma-treated plates were included.

Plasma treatments. Untreated polystyrene plates were Falcon 1008 petri dishes (Falcon Plastics, Los Angeles, CA) or untreated tops of Corning tissue culture petri dishes (Corning Glass Works, Corning, NY). Plasma treatments were carried out in house rather than using commercially treated plates, to eliminate any batch-to-batch variability. A Plasmod (Tegal Corp., Richmond, CA) plasma chamber was used. Flowing oxygen at a pressure of less than 1 torr was introduced into the chamber. Unless noted otherwise, the plasma generator was used at 50 W rf at 13.56 MHz. These conditions provided a stable oxygen plasma (12). The operating procedure involved a 10 min equilibration with gas flow followed by 5 min with the plasma generator on.

Chemical treatments. Slides approximately $2.54 \times 7.62 \times 0.2$ cm and ESCA coupons of $1.27 \times 1.09 \times 0.2$ cm were prepared, and identification marks were added. The samples were left untreated, treated with a gas plasma, or chemically treated as listed in the Results section. Samples were rinsed with sterile distilled water after treatment. Each treatment caused the initially hydrophobic surfaces to become hydrophilic.

The nitric acid treatment imparted a yellow tint to the treated plastics. It was necessary to use a short treatment at room temperature to avoid excessive reaction giving a "foamed" surface that was not transparent.

Adhesion measurements. The centrifugal method described in an accompanying paper was used (6). In brief, cells were grown for 24 h on slides, which were then centrifuged at various speeds and stained. The gravity force necessary to remove cells was determined and is used as a measure of adhesiveness. Cultures of each trial in Table 3 were done in triplicate. Those in Table 4 were done in duplicate.

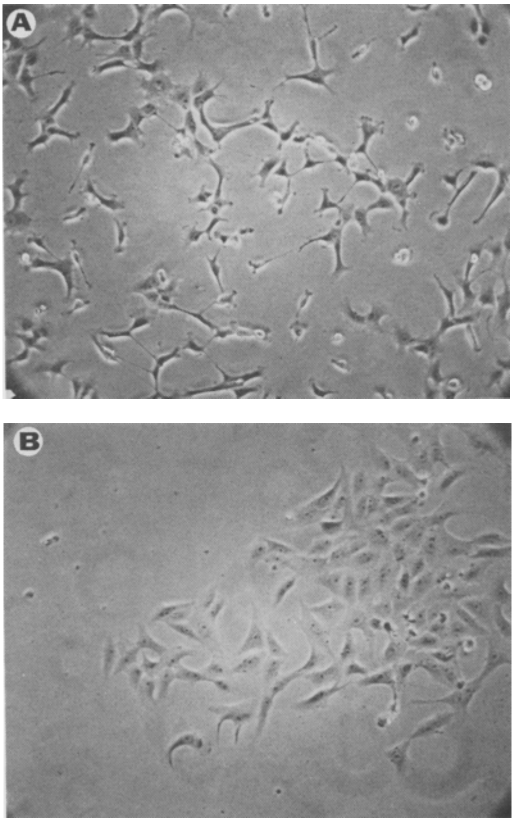

FIG. 1. Appearance of representative 7-day Vero cultures on (*A*) untreated and (*B*) O_2-plasma-treated polystyrene plates.

Electron spectroscopy for chemical analysis. Spectra were recorded using a Hewlett-Packard model 5950A electron spectrometer (5) (Hewlett Packard, Palo Alto, CA). The technique involves placing a sample in a high vacuum (10^{-9} torr) and irradiating the surface with a monoenergetic beam of $AlK_{\alpha 1,2}$ x-rays (1486.6 eV), which results in the ejection of electrons from a surface layer less than about 50 Å thick (3). The energy of the ejected electron is characteristic of the element and the specific functional group and thus may be used for chemical analysis of surfaces. The surface is flooded with low energy electrons (5 to 10 eV) to eliminate the charging problem encountered with insulating samples. For the soft radiation used with this technique, x-radiation damage for the ESCA samples is generally not considered a problem and none was observed. Samples for ESCA showed no differences in their surface chemical composition with short or long irradiation times in the spec-

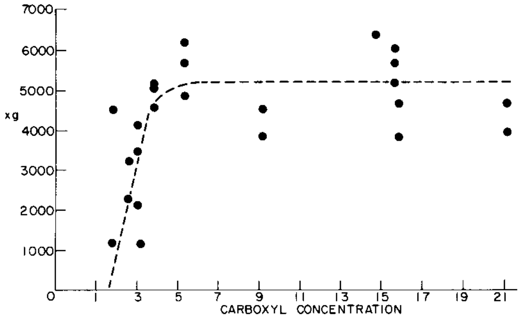

FIG. 2. Cell adhesiveness measured by gravity force necessary to remove cells from surface versus measured normalized surface carboxyl concentration. Units are carboxyl carbon-to-total carbon ratio in parts per thousand as measured by ESCA.

trometer. All measured band areas were corrected for scattering cross section.

The powered reference compounds (*see* Table 1) were pressed into solid discs at 15 tons/in.^{2} The plastic samples of polyethylene, polystyrene (PS), polymethyl methacrylate, and polymethylpentene were cut from commercially available sheets.

RESULTS

ESCA of the surfaces. Wide ESCA scans give information about the elemental composition of the surface for all elements present at concentrations of >1 atomic percentage (except H and He), as each element gives peaks at characteristic energies. The intensities of the bands, when corrected for electron cross sections, were used to obtain the ratios of the elements present on the surface. A typical wide scan of a polystyrene sample that had been treated with an oxygen plasma is shown in Fig. 4. The bands, due to the carbon and oxygen, are labeled.

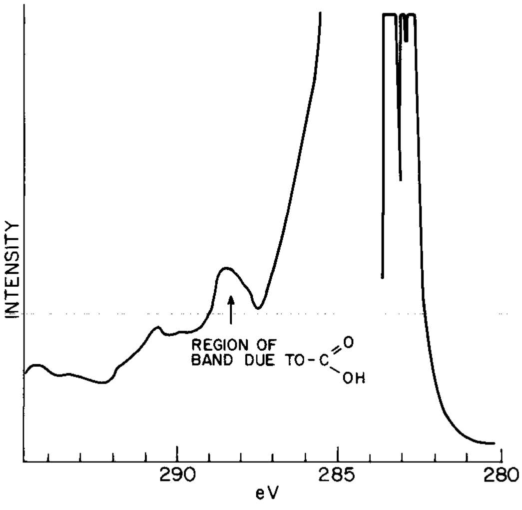

FIG. 3. Photoelectron spectroscopic narrow scan spectrum of O_2-plasma-treated polystyrene showing bands due to alkyl carbon near 284 eV and carboxyl carbon near 288 eV.

TABLE 1

NARROW ESCA SCANS OF REFERENCE COMPOUNDS, TREATED AND UNTREATED POLYSTYRENE AND POLYETHYLENE

Compound[a]	Number of Oxygen Bands	Energy Shift[b] From Alkyl Carbon
Polyethylene (alkyl)	0	0
Polyvinylalcohol (alcohol)	1	1.3
Polyox (ether)	1	1.6
5,5 Dimethyl 1,3 Cyclohexadione (carbonyl)	1 (Possibly 2)	2.2
Malonic acid (carboxyl)	2	1.0 4.75
Polymethylmethacrylate (ester)	2	0.7 1.3 3.6
Polyethylene (untreated)	0	0
Polyethylene (plasma)	2	2.0 4.5
Polystyrene (untreated)	0	0
Polystyrene (plasma)	2	1.6 4.5
Polystyrene (untreated)	0	0
Polystyrene (plasma)	2	1.6 4.5

[a] The six reference compounds were used to determine the position of the band due to a given functional group.

[b] The position of the functional group band is expressed in electron volts as the shift from the position of the alkyl band at 284 eV (cf Fig. 3).

In most of the surfaces studied here, only a few elements are present (e.g., carbon, hydrogen, oxygen). However, the carbon can be present in the form of quite different functional groups. These can be determined by narrow ESCA scans of the C_{1s} bands near 284 eV, the difference in energy at which a band appears being characteristic of a given functional group (3). When oxygen functional groups are present, the number

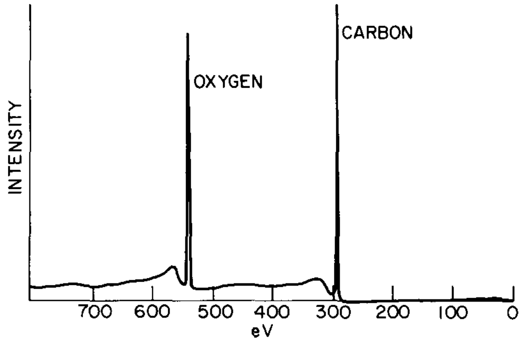

FIG. 4. Photoelectron spectroscopic wide scan spectrum of O_2-plasma-treated polystyrene showing bands due to carbon in polystyrene and oxygen bonded on surface.

of oxygen bands near 530 eV observed in narrow scans aids in identifying the groups. A correlation was developed between the specific functional groups and the observed energy shifts from the alkyl group by taking a series of well-defined reference compounds each of which had only one type of functional group present. A typical narrow ESCA scan for the treated sample shown in Fig. 1 is given in Fig. 3 and the band due to the surface carboxyl group is labeled. The correlation obtained between the functional group and the shifts in energy of the observed bands is given in Table 1.

Increased wettability accompanies plasma treatment (7). The effects of plasma treatments on wettability were determined by measuring the contact angles of sessile water drops. A drop of water was placed on the surface and the angle between the surface and the drop was measured at the point of contact using a telescopic goniometer. Water drops on untreated polystyrene had a contact angle of approximately 90° whereas plasma treatment reduced the angles to 18 to 38°. This is consistent with the report that bubble contact angles of untreated polystyrene were 90° but those of tissue culture polystyrene were 29° or less (4).

Gas-plasma treatments. Electron spectroscopy for chemical analysis was used to quantitate the effect of O_2-plasma treatment on the ratio of oxygen to carbon in treated polystyrene. The effect of duration of O_2-plasma treatment is shown in Table 2. It is seen that for times >30 s no additional oxygen is incorporated on the surface.

To determine the depth of effect of O_2-plasma irradiation, a treated plate was ablated at the rate of 10 Å/min by argon ion bombardment in the ESCA machine. After 90 s, corresponding to a depth of 30 Å, greater than 90% of the oxygen was removed.

Untreated plastics typically have an O-to-C ratio of 0.1 or less. After an O_2-plasma treatment the O-to-C ratio is typically about 0.3. When treated in a He or N_2 plasma, the O-to-C ratio was also about 0.3. To determine whether the O_2 in the plasma reacts directly with the surface or whether the plasma reacts with the plastic surface to produce surface radicals, which then react preferentially with any oxygen molecules (7), the following experiment was carried out in the ESCA spectrometer. Before and after Ar^+ bombardment of the surfaces at 10^{-4} torr (Ar plasma) the spectrometer vacuum was at least 10^{-9} torr. A scan was taken that showed only a trace of oxygen. The same sample was then exposed to pure dry nitrogen and another scan was taken, which resulted in some oxygen incorporation, presumably due to traces of oxygen in the nitrogen gas. The sample was then exposed to air and a large amount of oxygen was picked up.

This experiment unambiguously demonstrated that it was sufficient to use the gaseous ions to create a surface radical, resulting in oxygen incorporation on subsequent exposure to atmospheric oxygen. Because no nitrogen was detected on the surfaces, this experiment also demonstrated that molecular oxygen is considerably more reactive with respect to surface radicals than is molecular nitrogen.

Appearance of cells on plasma-treated polystyrene. Figure 1 *A* and *B* shows the appearance of 7-d Vero cultures on untreated and O_2-plasma-treated polystyrene plates. Growth on the un-

TABLE 2

EFFECT OF DURATION OF EXPOSURE TO O_2-PLASMA ON THE LEVEL OF OXYGEN ON POLYSTYRENE SURFACES

Duration of Treatment	O-to-C Ratio
None	0.00
On/off (0.1 s)	0.02
30 s	0.32
2 Min	0.31
5 Min[a]	0.57
30 Min	0.33

[a] Five minute sample also contained Si, apparently due to contamination.

TABLE 3

CHEMICAL TREATMENT OF POLYSTYRENE AND EFFECTS ON SURFACE CHEMICAL COMPOSITION AS MEASURED BY ESCA AND ADHESIVENESS AS MEASURED BY CENTRIFUGATION

	Conditions				Adhesiveness $\times g$		
Treatment	° C	hr	O-to-C	COOH-to-C (Parts Per Thousand)	Trial 1	Trial 2	Trial 3
None	—	—	0.08	2.56	2275	—	3223
O_2-plasma, 50 W	23	0.08	0.29	15.6	6028	5185	5686
Concentrated HNO_3	25	1	0.06	3.0	4140	2129	3494
70% $HClO_4$	32	20	0.34	5.3	4170	6188	5686
30% H_2O_2	57	16	0.30	3.0	4570	5185	5034

treated surface is characterized by sparse coverage; by cells not closely adherent to the surface, as indicated by areas of high phase-refractility (white areas) near the cells, and by cells distorted lengthwise with a spindle appearance. Growth on plasma-treated surfaces is characterized by more nearly complete coverage of the surface and cells close to the surface. The appearance of Vero cells on treated and untreated plates is consistent with that reported by Amstein and Hartman (1).

Functional group analysis of the treated surfaces. Table 1 lists the observed shifts in the carbon core-electron binding energies and the number of oxygen bands observed for the reference compounds. The intensities and number of oxygen bands observed were those expected from the structures of the reference compounds. Also included in Table 1 are the observed shifts for the O_2-plasma-treated polystyrene and polyethylene.

Comparison of the shifts in the binding energies and number of oxygen bands observed in the treated polymers leads to the conclusion that the plasma-treated polymers have surface carboxyl groups. Also, the band intensities for plasma-treated polyethylene and polystyrene were essentially identical, indicating that the surface functional groups were the same on both polymers. Examination of the aromatic satellite band in polystyrene that occurs near 290 eV showed only minor changes when normalized, suggesting that most of the oxidation takes place on the side chains rather than on the aromatic ring.

It was concluded that the oxygen incorporated on plasma-treated polystyrene and polyethylene under our experimental conditions consisted of carboxyl groups, with the possibility of some incorporation as alcohol or ether groups.

Cell adhesiveness of chemically treated surfaces. The data given above suggested that oxidizing chemical treatments might duplicate the chemical effects of gas plasma on adhesiveness. Polystyrene was examined because it is used in most tissue culture ware. Polymethylpentene was tested because, like PS, it contains only C and H atoms and yet had a high melting point and was expected to withstand vigorous chemical treatments at elevated temperatures. Polymethylmethacrylate was tested because its ester composition allowed testing the role of esterified versus free carboxyl groups. The chemical treatments used along with the O-to-C ratios obtained from wide ESCA scans, level of carboxyl groups obtained from narrow ESCA scans, and the cell adhesiveness as measured by the centrifugal force method are listed in Tables 3 and 4. Adhesiveness to

TABLE 4

CHEMICAL TREATMENT OF POLYMETHYLPENTENE AND EFFECTS ON SURFACE CHEMICAL COMPOSITION AS MEASURED BY ESCA AND ADHESIVENESS AS MEASURED BY CENTRIFUGATION

	Conditions				Adhesiveness $\times g$	
Treatment	° C	hr	O-to-C	COOH-to-C (Parts Per Thousand)	Trial 1	Trial 2
None	—	—	0.13	3.19	0	1159
O_2-plasma, 50 W	23	0.08	0.34	15.8	3845	4683
Concentrated HNO_3	65	1	0.02	1.8	1170	4507
0.2 M $KMnO_4$ in 2 M H_2SO_4	65	2	0.11	14.7	6400	—
70% $HClO_4$	65	20	0.20	21.1	3955	4687
30% H_2O_2	65	16	0.12	9.17	3845	4533

TABLE 5

REGRESSION ANALYSIS OF DATA FROM TABLES 3 AND 4

Polystyrene Data	Correlation Coefficient	T-Ratio	Correlation Significance
Carboxyl versus adhesiveness	0.824	2.52	>0.90
O-to-C versus adhesiveness	0.696	1.68	<0.90
Polymethylpentene data			
Carboxyl versus adhesiveness	0.846	3.18	>0.95
O-to-C versus adhesiveness	0.305	0.64	<0.90

HNO_3-treated samples had a very high variability. This may reflect the apparent high sensitivity of these surfaces to reaction with HNO_3.

The data in Tables 3 and 4 were analyzed statistically and included the variabilities in both the ESCA and the adhesiveness measurements. The analysis is summarized in Table 5. It is clear that the surface carboxyl group concentration is a better predictor or indicator of cell adhesiveness than the O-to-C ratio. A plot of the surface carboxyl group concentration against the cell-removal force is given in Fig. 2. The general trend shows that the cell-removal force increases with increasing surface carboxyl group concentration.

The methylmethacrylate polymer is completely esterified. By treating with strong acid or alkali the ester is hydrolyzed to give methacrylic acid or ionized methacrylate groups. The results of various treatments designed to hydrolyze surface esters of polymethylmethacrylate on surface chemical properties and on adhesiveness are given in Table 6.

Examination of the polymer structural formula gives an O-to-C ratio of 0.4 and a carboxyl carbon-to-alkyl carbon ratio of 0.25. For complete hydrolysis within the volume of polymer sampled by the ESCA (down to about 50 Å) these ratios would increase to 0.50 and 0.33, respectively. For the untreated and hydrolyzed polymer the values found fell generally in these ranges. The O_2-plasma treatment resulted in a substantial increase in both the O-to-C and COOH-to-C ratios, which probably arises from oxidation of the methyl side groups.

TABLE 6

EFFECT OF VARIOUS TREATMENTS OF POLYMETHYLMETHACRYLATE ON SURFACE CHEMICAL COMPOSITION AS MEASURED BY ESCA AND ADHESIVENESS AS MEASURED BY CENTRIFUGATION

Treatment	O-to-C	COOH-to-C	Adhesiveness (×g)
None	0.46		2300
NaOH	0.48	0.26–0.33	5600
H_2SO_4	0.54[a]		4740
O_2-plasma	0.60	0.39	>6400

[a] Corrected for $-SO_2$ groups from measured S-to-C = 0.12.

Hydrolysis of the methyl ester on polymethylmethacrylate to give carboxyl groups resulted in increasing the cell adhesion strength by greater than two times. These results are consistent with the idea that incorporation of oxygen per se in the plastic surface (e.g., as an ester or hydroxyl) is inadequate for good adhesiveness. They suggest the oxygen is most effective in enhancing adhesiveness when in the carboxyl form.

DISCUSSION

Plasma or oxidant treatment of the hydrocarbon polymers resulted in oxygen incorporation and cell growth compatability. These treatments gave O-to-C ratios up to about 0.3 and COOH-to-C ratios up to about 0.021. Inasmuch as each carboxyl group has two oxygens, this indicates that of the total oxygen incorporated about 14% is in the carboxyl form. The ablation experiments showed that after treatment the incorporated oxygen is present to a depth of 30 Å or slightly greater. This depth is commensurate with the penetration depth of the x-ray beam used for analysis (about 50 Å). These data suggest that the oxygen incorporated below the surface is not in the carboxyl form and that the carboxyls are present mainly on the surface. As the adhesiveness data showed, the best correlation is obtained by comparing adhesion with carboxyl content, rather than with total oxygen content.

Oxygen constituents in the polymer itself are not necessarily sufficient to give high adhesiveness because adhesiveness of polymethylmethacrylate (an ester) was enhanced by treatment with strong acid or base, each of which would hydrolyze the methyl ester with formation of a carboxylic acid or salt. Immersion of these surfaces with carboxyl groups at pH 7.2 would result in the ionized salt as the predominant species. The observation that untreated polymethylmethacrylate and untreated polycarbonate, both of which have relatively large amounts of oxygen on the surface but no free ionized groups and also have relatively low adhesiveness, further supports the idea that carboxyl groups contribute to adhesiveness.

To put the adhesiveness data in context the adhesiveness of various materials was measured (6). Untreated polycarbonate, which has a 0-to-C ratio of 0.19 in the polymer structure, had relatively poor adhesiveness, as did the polymethylmethacrylate with an O-to-C ratio of about 0.4 where the oxygen is present as ester groups.

All common glasses have a backbone of polymerized SiO_2 chains with the percent composition of SiO_2 increasing in the following order: soda lime (72%), borosilicate (81%), and fused quartz (100%). Inasmuch as adhesiveness also increases in this order, it seems reasonable to suggest that the silica content of glass is important for adhesiveness to these materials.

The surface charge of silica at $pH > 2$ is negative (8). Measured zeta-potentials for glasses are usually negative in aqueous solutions, so that these materials have a net negative charge under physiological conditions (Mizzoni, M.S.; personal communication).

Additional data on the relation between surface charge and growth is provided by the composition of microcarriers. Diethylaminoethyl modified dextran and dimethylaminopropyl modified acrylamide, constituents of two commonly used microcarriers, would be expected to carry a net positive charge at physiological pH. It seems clear that if charged groups are necessary for good cell attachment, the nature of the charge is not of prime importance. These results are consistent with the theoretical conclusions that the adhesion of cells to polystyrene increased with the polarity of the polystyrene surface (2).

Because it was not possible to obtain quantitative ESCA data on the relative amounts of ether and hydroxyl groups on the surface, one cannot exclude a role for these groups in cellular adhesiveness. The level of carboxyl groups, which could be quantitatively measured, correlated better to adhesiveness than did the level of total surface oxygen (which included ether and hydroxyl as well as carboxyl groups). In addition, the observation that polymethylmethacrylate, an ester, had poor adhesiveness but hydrolysis of the surface, exposing carboxyl (but not ether or hydroxyl) groups increased adhesiveness, suggested the efficiency of carboxyl groups for good adhesion. The observation of similar effects of surface treatments on both polystyrene and polymethylpentene indicates the benzene ring of polystyrene is not necessary for good adhesiveness to plastic surfaces.

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