Cell adhesion to plasma-treated polymer surfaces

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Polymer surfaces were modified by glow discharge to study the effect of surface treatment on cell adhesion using polyethylene, polytetrafluoroethylene, poly(ethylene terephthalate), polystyrene, and polypropylene films. The surface wettability of all the films, evaluated by the water contact angle, decreased with respect to the length of plasma treatment. For each of the polymers, a different dependence of cell adhesion on the length of plasma treatment was observed, but, in each case, the optimal water contact angle for cell adhesion was approximately 70°. The X-ray photoelectron spectroscopy (XPS) of the plasma-treated surfaces using a derivatization method suggested that hydroxyl groups were primarily introduced onto the surfaces of the polymer by plasma treatment. Formation of carboxyl groups by plasma treatment was also observed from XPS, although streaming potential measurements could not identify the newly generated groups.

(Keywords: plasma discharge; cell adhesion; contact angle; polymer surface)

INTRODUCTION

It has been reported that cell adhesion is reduced with the increasing wettability of the substrate surface[1–4]. On the other hand, there have been a few published papers which give almost opposite results[5–8]. This discrepancy may be partly due to differences in the chemical structure among the substrate polymers used for the cell adhesion and the plasma treatment. Our previous studies[9,10] indicated that cell adhesion became maximal on the surface having a certain range of contact angle against water and decreased with the higher or lower wettability than that angle. However, these studies used various polymers of different chemical compositions. To study the effect of water wettability on cell adhesion, it would be better to use a single polymer having different surface wettabilities. Such polymer surfaces can be obtained by exposing a polymer to plasma discharge for different periods of time. Some researchers[11,12] have reported that glow discharge treatment alters a polymeric material to a substrate with a higher propensity for cell adhesion.

In this paper we employ five kinds of polymer with different surface wettabilities and investigate cell adhesion, focusing on the relationship between the number of cells attached and the surface wettability. The low-temperature plasma used for surface modifications in this study has recently received much attention not only in industrial but also in biomedical fields[12–16]. One of the advantages over other modification methods is that only the surface region of a polymer can be modified without affecting the bulk properties of that polymer[17].

EXPERIMENTAL

Materials

Polyethylene, polytetrafluoroethylene, poly(ethylene terephthalate), polystyrene, and polypropylene films are all commercial products. The thickness of the films used in this study ranged from 10 to 50 μm. For purification, all the films were subjected to Soxhlet extraction with methanol for 12 h, followed by drying in vacuum at room temperature. All the films had smooth surfaces when observed by SEM.

Plasma discharge

The plasma discharge treatment of films was carried out using a glow discharge reactor equipped with a bell-jar-type reaction cell (a model LCVD12, manufactured by Shimazu Seisakusyo Co. Ltd, Japan)[18]. The frequency applied was 5 kHz, so that this reactor did not require any impedance matching unit. Two internal electrodes each with a surface area of 14×14 cm^2 were placed 6.5 cm apart in the glass bell-jar and two magnets were placed between the two electrodes to confine the plasma. The films to be treated with plasma were fixed onto a stainless steel sample holder placed between the two electrodes. The holder was rotated at 68 rev min^{-1} by a motor to ensure homogeneous plasma treatment over the whole film surface. The pressure in the bell-jar was reduced to 10^{-3} torr, followed by introduction of Ar gas into the bell-jar at a flow rate of 20 ml min^{-1}. When the pressure in the bell-jar had been adjusted to about 0.04 torr after introduction of gas, plasma was generated at 24 W and the films were exposed to plasma for predetermined periods of time. After plasma treatment, the films were reserved in a desiccator. Contact angle and zeta potential measurements, as well as cell attachment tests, were carried out within 1 h after plasma treatment.

Contact angle and streaming potential measurement

The water contact angles of the original and plasma-treated films were measured at 25°C and 65% relative humidity (r.h.) with the sessile drop method using

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redistilled water. At least five readings were made on different parts of films 1 min after placing 10 μl of water droplets on them and averaged. Streaming potentials were measured on films of $3 \times 5\,cm^2$ at 25°C using the cell unit described by Andrade and coworkers[19]. The electrode was made of platinum and the pH of the electrolyte solution used for the potential measurement was adjusted to 7.4 by mixing HCl and KOH aqueous solutions, the ionic strength always being kept at 1×10^{-3}. All the aqueous solutions were prepared from redistilled water and the zeta potential was calculated from the streaming potential according to the Helmholtz–Smoluchowski equation.

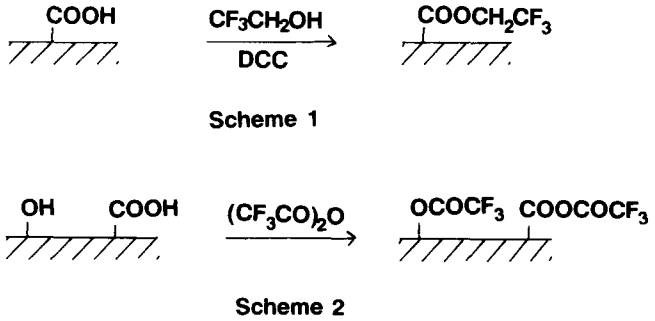
X-ray photoelectron spectroscopy and chemical derivatization of glow discharged surfaces

An ESCA 750 spectrometer manufactured by Shimazu Seisakusyo Co. Ltd was employed to carry out the X-ray photoelectron spectroscopy (XPS) measurement of plasma-treated films at a pass energy of 1253.6 eV with a MgK_α X-ray source. Chemical derivatizations for XPS analysis of surface functional groups on the films were carried out according to the method of Everhart and Reilly[20]. For the determination of carboxyl groups, 2,2,2-trifluoroethanol (TFE) and dicyclohexylcarbodiimide (DCC) were used (*Scheme 1*). In a solution containing 500 μl of TFE, 1 ml of pyridine, and 200 mg of DCC in 15 ml of methylene chloride, a plasma-treated film was immersed and allowed to react for 15 h at 25°C. The film was then washed with anhydrous diethyl ether and immersed in diethyl ether for 12 h to extract the unreacted reagents. This derivatization reaction resulted in the introduction of fluorine atoms to the carboxyl groups generated by the plasma treatment. Another derivatization was carried out using trifluoroacetic anhydride (TFAA) to determine both the hydroxyl and carboxyl groups (*Scheme 2*). In a solution containing 1 ml of TFAA and 1 ml of pyridine in 15 ml of benzene, a plasma-treated film was placed for 1.5 h at 25°C, washed with benzene, and then immersed in diethyl ether for 12 h. The F_{1s} peak area of the functional groups was determined by a computer curve fitting method for the chemically derivatized films.

Cell culture and cell adhesion test

L cells, which are established cell lines of mice fibroblast, were used for the cell adhesion test. Cultures were maintained in a 37°C water-jacketed incubator equilibrated with 5% CO_2 and kept at approximately 99% r.h. The cells were routinely grown in Eagle's MEM supplemented with 10 v/v% foetal calf serum (FCS, M. A. Bioproducts, Maryland, USA) and 60 ml l^{-1} Kanamycin on a 250 ml plastic culture flask (Nunc, Denmark). The cultured L cells were trypsinized from the culture flask, washed once in a medium with 10% FCS and in a medium without serum, and then collected by centrifugation at 1000 rev min^{-1} for 5 min. The pellet of cells was suspended in a medium without serum and the cell density was adjusted to 1.76×10^5/ml by a medium without serum.

Each plasma-treated film was placed in separate wells of a Nunc multi-dish (24 wells, 15 mm diameter). Prior to the cell adhesion test, PBS solution was added to each well and pre-incubated for 2–3 h at room temperature. After pre-incubation, the PBS solution was aspirated and then 1 ml of cell suspension was immediately added to each well and the solution incubated further for 60 min at 37°C. After incubation, the films were taken from the well with a forceps and dip-rinsed twice in PBS solution in order to remove non-adhering cells and then placed in a test tube to count the number of adherent cells by the LDH activity method as described in a previous paper[10].



RESULTS

Contact angle and zeta potential

The change of water contact angle induced upon plasma discharge treatment is shown in *Figure 1* for the different polymer films. As is seen, the water wettability of polymer surface is enhanced in every case by the plasma treatment since the contact angles decrease with the treatment time, but their dependence on the plasma treatment time is slightly different among the polymer films[21]. For instance, the contact angle of polystyrene film decreases from 80° to 40° upon exposure to plasma discharge for 10 s, whereas that of poly(ethylene terephthalate) changes from 80° to 60°. The decrease in contact angle of polyethylene is larger than that of polypropylene. These differences may be explained in terms of the extent of oxidation of the polymers. In this study, the plasma treatment time was kept to shorter than 30 s, as the prolonged plasma treatment produced a rough surface when observed by SEM.

The zeta potential change of polymer surfaces by the plasma treatment is shown in *Figure 2*. No significant

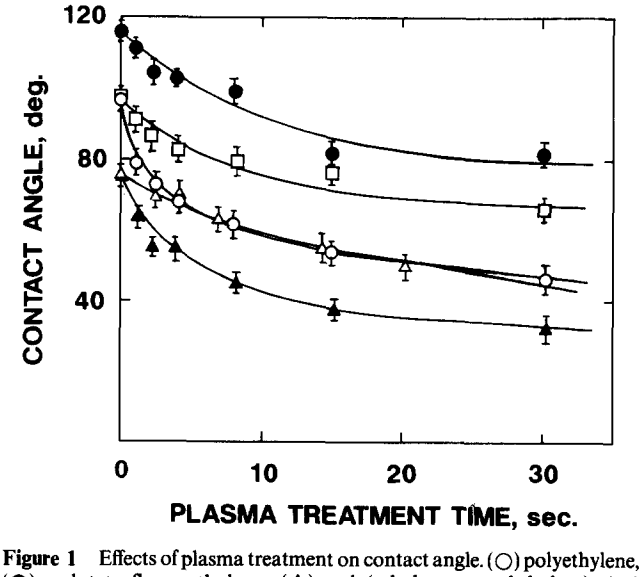


Figure 1 Effects of plasma treatment on contact angle. (○) polyethylene, (●) polytetrafluoroethylene, (△) poly(ethylene terephthalate), (▲) polystyrene, (□) polypropylene

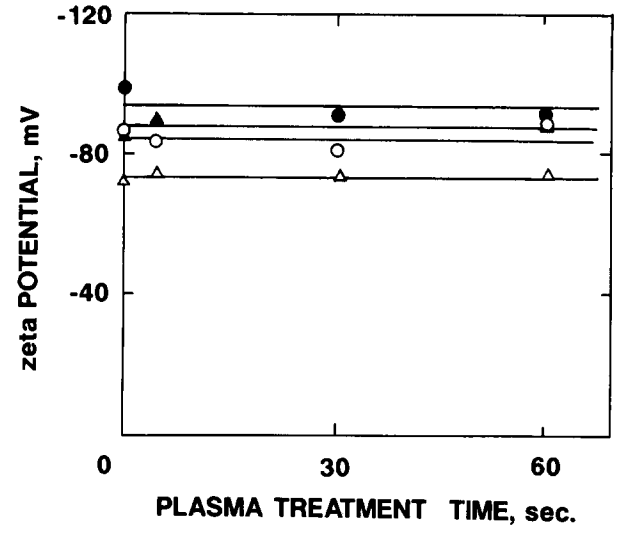


Figure 2 Effects of plasma treatment on zeta potential. (○) polyethylene, (●) polypropylene, (△) poly(ethylene terephthalate), (▲) polystyrene

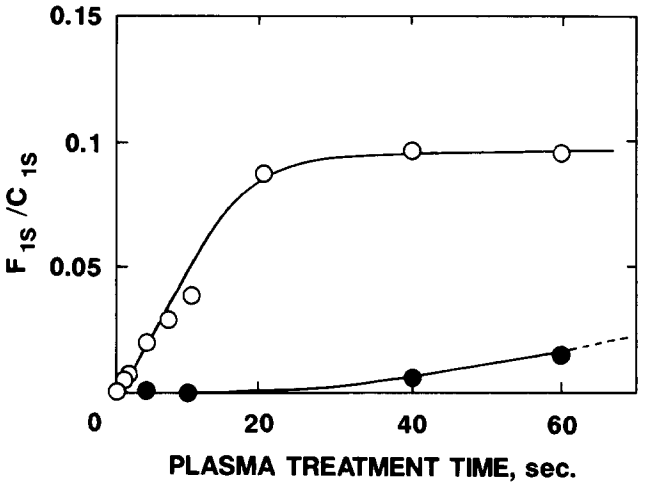


Figure 3 F_{1s}/C_{1s} intensity ratios after derivatization for the polyethylene film exposed to Ar plasma (24 W). (○) derivatization of both –OH and –COOH, (●) derivatization of –COOH

change of zeta potential is observed for any treated polymer surface under conditions of pH 7.4 and an ionic strength of 1×10^{-3}. It is reported that various functional groups (including ionic groups) are generated on polymer surfaces by plasma discharge treatment[20,21]. If carboxyl groups are abundantly generated by exposure to Ar plasma discharge, the zeta potential of polymer surface may become larger in absolute value. However, it is also likely that any appreciable change of zeta potential is not observable, even if anionic groups are formed, because the adsorbed ions which give highly negative zeta potentials will overshadow the newly generated carboxyl groups or will be simply replaced by them.

Functional groups introduced by plasma discharge

XPS spectra of the treated polyethylene film did not show any N_{1s} peak but an increase in height of O_{1s} peak and in the area of the C_{1s} peak (data not shown). As this observation implied that oxygen was introduced on the plasma-treated surface, a derivatization technique was employed for assessment of hydroxyl and carboxyl groups. The result on the plasma-treated polyethylene film is shown in *Figure 3*. It is seen that the sum of hydroxyl and carboxyl groups linearly increases with the discharge time up to 20 s, whereas the concentration of carboxyl group gradually increases with the plasma treatment time.

Cell adhesion

Figures 4 and *5* show the effect of plasma discharge treatment on cell attachment to the surfaces of polyethylene, polystyrene, polypropylene, and polytetrafluoroethylene. As is seen, the plasma treatment caused a noticeable change in cell adhesion for all the films. The change is roughly classified into two types. In one type, the number of cells attached initially increases with the plasma treatment time, but, after passing through a maximum, decreases or remains constant, as seen in *Figure 4*. In another type, as in *Figure 5*, a monotonous increase in cell attachment is observed with the plasma discharge time.

To examine whether there is any distinct correlation between the contact angle and the cell adhesion, the

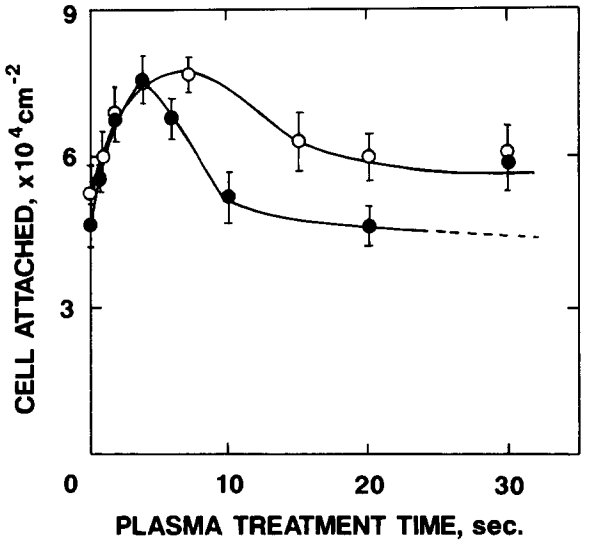


Figure 4 Effect of plasma treatment on L cell adhesion to polyethylene and polystyrene films. (○) polyethylene, (●) polystyrene

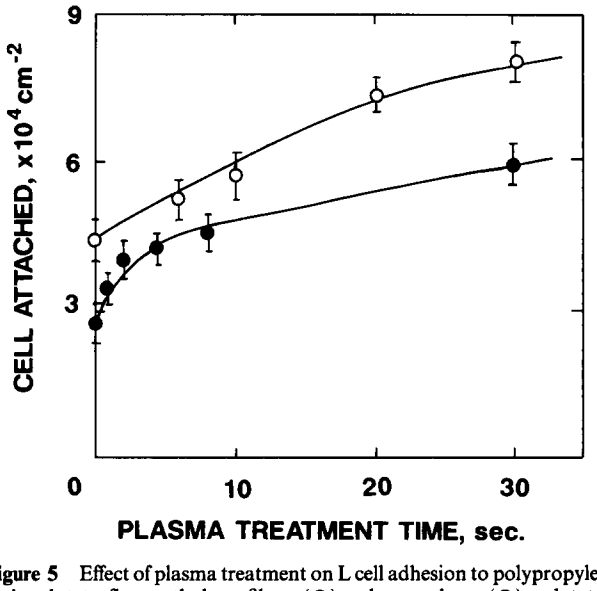


Figure 5 Effect of plasma treatment on L cell adhesion to polypropylene and polytetrafluoroethylene films. (○) polypropylene, (●) polytetrafluoroethylene

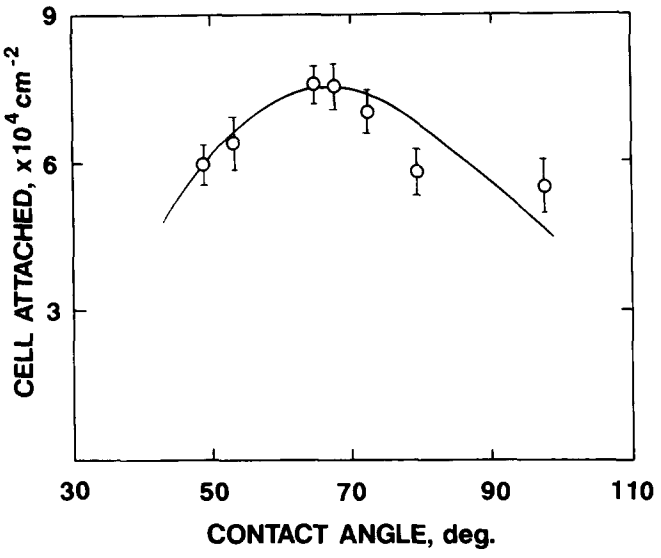


Figure 6 Relationship between the contact angle of plasma treated surfaces and L cell adhesion for polyethylene film

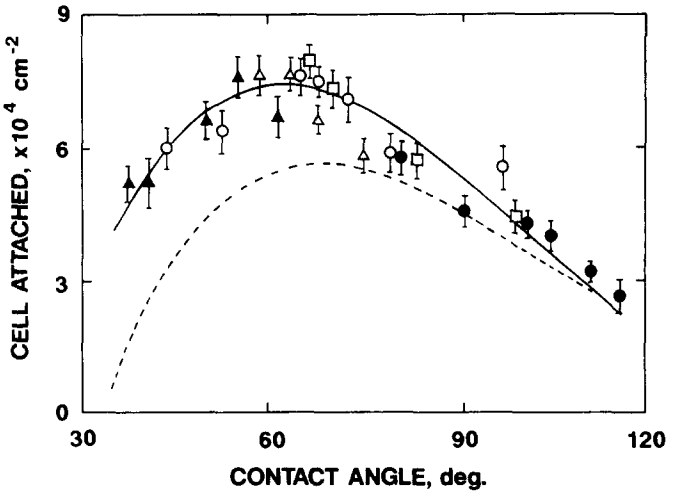


Figure 7 Relationship between the contact angle and L cell adhesion. —— with plasma treatment, --- without plasma treatment. (○) polyethylene, (●) polytetrafluoroethylene, (△) poly(ethylene terephthalate), (▲) polystyrene, (□) polypropylene

number of cells attached was plotted against the contact angle of the plasma-treated polymers. The result for polyethylene is shown in *Figure 6*. The cell adhesion exhibits a maximum at the contact angle around 70°. When the water contact angle of polymer surfaces becomes lower or higher than 70°, L cell adhesion decreases, similar to the results obtained in a previous paper[9]. All the cell adhesion data are plotted as a function of the contact angle of the plasma-treated films in *Figure 7*. Interestingly, almost all the data fit on a single master curve. It is obvious that the surface with the water contact angle around 70° is the most suitable for cell adhesion, whereas the more hydrophilic or the more hydrophobic surfaces become less adhesive to cells. This tendency agrees well with our previous result[10], which is again represented as a dotted line in *Figure 7*. Although the dependence of cell adhesion on the contact angle of the surface is similar for both the cases, the number of cells attached is larger for the plasma-treated surfaces by 30% than for the non-treated when compared at the same contact angle around 70°. In other words, plasma discharge treatment improves cell adhesion to all the polymer surfaces as reported by other researchers.

DISCUSSION

It is widely accepted that surface wettability greatly affects cell attachment. We have also found that there is an optimal wettability for cell adhesion, that is, approximately 70° water contact angle[9,10], when cell adhesion is studied using a wide variety of substrate polymers. The present study showed a similar dependence of the cell adhesion on the surface wettability even for single-substrate polymers such as polyethylene when the contact angle was changed by plasma discharge treatment. As is well known, plasma discharge treatment alters the water wettability of polymer surfaces through production of oxidized groups. In the present case it is likely that hydroxyl groups were introduced mainly on the surfaces by plasma treatment, resulting in enhancement of water wettability of the polymer films.

It is often pointed out that the improvement of cell attachment by plasma discharge is due to the increased water wettability. In fact, we observed an increase in cell adhesion with an increase in water wettability by the plasma treatment as seen in *Figure 5*. However, in the case of polystyrene and polyethylene, the plot of number of attached cells against the plasma treatment time showed a maximum, as is apparent in *Figure 4*, although the wettability continuously increased with the plasma treatment time (*Figure 1*). These results clearly denote that hydrophilization of polymer surfaces by discharge treatment causes a significant change in cell adhesion but does not always improve cell adhesion.

The most interesting finding in this work is that a master curve satisfying all the experimental data was obtained, regardless of the kind of polymer, when the number of cells attached was plotted against the water contact angle of the plasma-treated films as clearly shown in *Figure 7*. It is also worth noting that cell attachment takes place more dominantly on the plasma-treated surfaces than on the non-treated ones, when compared at the same contact angle, i.e. the same water wettability. The reason for this is not clear, but it seems that exposure to plasma made the film surfaces microscopically more rough to increase the total surface area of films. It is also highly possible that functional groups such as carboxyl are generated by plasma treatment to enhance cell attachment. However, a further study is needed to make this clear.

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