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Effect of Cold Plasma-Surface Modification on Surface Wettability and Initial Cell Attachment

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***Abstract*—**A thin coating of hexamethyldisiloxane and subsequent O_2-plasma treatment was performed on mirror-polished titanium in order to regulate the wide range of wettability including 106 and almost 0 degrees of contact angles. The adsorption behavior of fibronectin and albumin in both individual and competitive mode, and initial attachment of fibroblasts and osteoblasts were investigated. Individually, fibronectin adsorption showed a biphasic inclination, whereas albumin showed greater adsorption to hydrophobic surfaces. In competitive mode, in solution containing both fibronectin and albumin, fibronectin showed greater adsorption on hydrophilic surfaces, whereas Alb predominantly adsorbed on hydrophobic surfaces. Initial attachment of both cells increased with increase in surface wettability, in particular, on super-hydrophilic surface, which correlated well with fibronectin adsorption in competitive mode. These results suggest that a cold plasma-surface modification enabled to regulate the surface wettability, and fibronectin adsorption may be responsible for increasing cell adhesion on hydrophilic surfaces in a body fluid.

***Keywords*—**cold plasma-surface modification, wettability, protein adsorption, initial cell attachment.

I. INTRODUCTION

THE vital reaction of biomaterials is greatly affected by the surface chemistry, which involves surface wettability and electrokinetic potential. Especially, surface wettability could regulate the protein adsorption and subsequent cell behavior. When a material is exposed to a body fluid or culture media, protein adsorption plays an important role in initial cell attachment. However, it is difficult to obtain consistent results of correlation between surface wettability and protein adsorption as well as subsequent initial cell attachment. The reason for inconsistency were considered to be based on the different materials used, different surface topographies employed, and the narrow ranges of surface wettability employed.

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Thus, it should be more helpful for understanding this property, if a wide range of wettability were employed with a single material and without change in surface topography.

A cold plasma-surface modification including a plasma polymerization and plasma surface treatment is suitable for controlling the surface chemistry. This approach is superior in that it is environmentally pollution-free, it yields safe products, and good quality control can be maintained, thus ensuring defect-free films. In particular, plasma polymerization using organosilicon monomers, hexamethyldisiloxane (HMDSO), has come into wide use [1]-[3]. These monomers are of interest because of their high deposition rates and the ability of varying the deposition conditions to control their structure and properties. At the same time, plasma surface treatments using various gases such as Ar, O_2, N_2, SO_2 have been utilized to modify blood compatibility, to influence cell adhesion and growth, and to control protein adsorption [4]-[6]. Notably, O_2-plasma treatment was reported to be able to control hydrophilicity/hydrophobicity and to introduce various functional groups, leading to applications such as humidity sensors, enzyme immobilization, and polymer bonding without the use of adhesives [7], [8]. Accordingly, a cold plasma-surface modification may be useful to evaluate the influence of surface wettability on protein adsorption and cell behavior.

It is believed that the efficiency of cell adhesion and growth is dependent on the balance between adhesion-promoting versus adhesion-inhibiting proteins which competitively adsorb to a surface. In general, a cell adhesion protein such as fibronectin and cell adhesion inhibiting protein such as albumin are both included in the serum used for cell culture. Therefore, the competitive adsorption behavior of these proteins on material surfaces should be quantitatively analyzed using a mixed solution containing both proteins [9], [10]. The influence of surface wettability on the adsorption behavior of proteins and subsequent cellular adhesion remains to be clarified.

This study aimed to investigate the influence of cold plasma-surface modification on the surface wettability and the initial cell attachment as well as protein adsorption using a single material without changing surface topography.

II. MATERIALS AND METHODS

A. HMDSO coating with plasma polymerization.

Hexamethyldisiloxane [HMDSO, $(CH_3)_3SiOSi(CH_3)_3$] monomers were plasma-polymerized onto titanium plates using a commercially available plasma-surface modification apparatus (VEP-1000, ULVAC, Kanagawa, Japan). Mirror-finished titanium disks were positioned in a chamber at room temperature. The reservoir of HMDSO was heated to 70°C and the pipe for introducing the HMDSO was heated to

80°C. The HMDSO gas was then introduced into the equipment chamber through the heated pipe at a gas flow rate of 45 sccm (mL/min). The plasma was generated using a radio-frequency generator operating at 13.56 MHz at a power level of 200 W. The chamber pressure was maintained at about 10 Pa throughout the plasma polymerization process. Plasma polymerization was performed for periods of 20 min. After the plasma was turned off, nitrogen gas was used to purge the chamber, which was then allowed to return to ordinary atmospheric pressure. The process resulted in the deposition of plasma-polymerized film on the titanium with a film approximately 100 nm thick as determined by a Dektak Stylus Profiler System (Veeco Instruments Inc., Woodbury, NY, USA).

B. O_2-plasma treatment.

Using the same apparatus as the plasma polymerization, HMDSO-coated specimens were used to create a wide range of wettability with the following procedure. The polymerized samples were re-introduced into the chamber of the plasma-surface modification apparatus and subjected to low-energy O_2-plasma treatment (10W, 1.8 Pa, gas flow rate of 50 mL/min) at room temperature for different time durations. Five specimens for the each experiment were prepared with different contact angles of 106, 80, 40, and 0 degrees by controlling the O_2-plasma time durations of 0, 10, 28 and 80 seconds, respectively, according to the preparatory experiment. It was reached almost zero degree, i.e. super-hydrophilicity, when the plasma duration exceeded 80 seconds. The contact angle of this condition was represented by 0 degrees. These specimens had mirror-like topographies with a mean surface roughness (Ra) of less than 0.1 μm. Surface characterization, protein adsorption assay and cell culture were started within one hour after the O_2-plasma treatment.

C. Surface characterization.

XPS analysis (ESCA-750, Shimadzu, Kyoto, Japan) were conducted for surface characterization of as-coated specimen (HMDSO), O_2-plasma treated specimens (HMDSO+O2), as-polished titanium (Ti) and O_2-plasma treated titanium (Ti+O2). The O_2-plasma treatment was carried out the time duration of 80 seconds. The XPS analysis was carried out with Mg-Kα as the X-ray source at 8 kV and 30 mA to determine the intensity of Si2p, O1s, and C1s. The binding energy of each spectrum was calibrated with C1s of 284.8 eV. FT-IR-RAS analysis (FT-IR-430, Jasco Corp., Tokyo, Japan) analysis was also conducted at a resolution of 4-cm^{-1} for HMDSO and HMDSO+O2 specimens. The incident angle of the infrared ray to the specimen was 75 degrees. A bare Ti surface was used as the background while calculating the spectrum of the specimen in order to obtain a spectrum that was limited to the substances formed on the titanium by plasma polymerization. Each surf ace type was measured three times.

D. Protein adsorption assay

Both individual and competitive adsorption of fibronectin (Fn, Wako Pure Chemical Industries, Japan) and albumin (Alb, Wako Pure Chemical Industries, Japan) to the surfaces was determined by immunofluorescence. Three kinds of solution were employed in this study: 100mg/L Fn, 400 mg/L Alb for individual adsorption, and a mixture of the two proteins (100 mg/L Fn and 400 mg/L Alb) for competitive adsorption. The concentrations of proteins were determined according to the preparatory experiment. All the protein was dissolved in a PBS solution with a pH of 7.2. Firstly, 1.0 mL each solution was dropped onto the surfaces with different wettability, and incubated for 20 minutes at 37°C. After washing twice with PBS for 5 minutes, the specimens were incubated with primary antibody of rabbit anti bovine Fn (LSL Co. Ltd., Japan) at a dilution of 1:200 for 1 hour. The specimens were rinsed twice with PBS for 5 minutes. Then, the mixed solution of sheep anti-bovine Alb (FITC labeled Rhodamine, 1:500, Bethyl Laboratories, USA), and goat anti-rabbit IgG (RITC labeled, 1:200, ICN Pharmaceuticals, USA) was incubated in a dark place for 1 hour. The samples were then observed under confocal laser scanning microscopy (LSM-MRC1024, Bio-Rad, Japan). The two channel fluorescence images were obtained simultaneously (red channel for Rhodamine and green channel for FITC). Four parallel samples were employed for each observation. Red (RITC, Rhodamine) and green (FITC) channel images obtained at each observation were stored. For quantitative evaluation of protein adsorption, the red (RITC, Rhodamine) and green (FITC) channel images obtained at each observation were opened with an imaging software (Adobe Photoshop) and the L*a*b* value (ISO 105-J03) was calculated in 4 randomly selected areas (5 x 5 dots each) with a color sampling tool. The red channel of the Alb-only sample and the green channel of the Fn-only sample served as a negative control (L_0, a_0, b_0) in calculating fluorescence intensity (ΔE*ab) according to the following formula: $\Delta E^*ab = [(L_x-L_0) +(a_x-a_0)+ (b_X-b_0)]^{1/2}$. Here, L_x, a_x, b_x represents the L*a*b* value of the red or green channel of the sample.

E. Initial cell attachment assay

Mouse fibroblast cell lines L929 and mouse osteoblast-like cell line MC3T3-E1 were used for initial cell attachment assay. Cells were cultured in culture flasks at 37°C in a 5% CO_2 supplied incubator. The culture media was MEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). One percent penicillin/streptomycin was added to the media. The specimens were put into a 24-well culture dish. Freshly confluent flasks of cells were incubated in 1% trypsin (Gibco) for 5 minutes, and then suspended in full culture medium. The cells were seeded into the wells at a density of 2.5 x 10^4/well (1 mL). After 60, 120, or 360 minutes of incubation for L929 cells and 30, 60, or 180 minutes of incubation for MC3T3-E1 cells, respectively, cell number was measured with the following steps. First, the specimens were washed gently three times with the culture media in the wells to remove any unattached cells; the media was pipetted out; and the specimens were washed once with 2 mL PBS. Second, the washed discs were put into a new 24-well culture dish, 0.5 mL trypsin was added, and another 0.5 mL MEM + 10% FBS was added after 5 min. Finally, the cells in each well were counted three times by a Coulter Counter Z1 (Beckman Coulter, Inc. USA) with the mean representing the attached cells. Before cell counting, cell morphology was observed by an inverted

optical microscope.

The cells were rinsed with PBS and fixed with 2% paraformaldehyde at 4°C for at least 1 hour, followed by dehydration through an ethanol series. The samples were then freeze-dried and sputter coated with a thin layer of Au-Pd alloy. Specimens were observed by a scanning electron microscope (JSM-6340F; JEOL, Japan).

III. RESULTS

The XPS spectra of HMDSO, HMDSO + O2, Ti and Ti+O2 specimens are shown in Fig. 1. The C1s peak of the outermost layer of the HMDSO surface appeared at 284.4 eV, and shifted to 285.9 eV on the HMDSO + O2. These peak shifts indicate a change from the dominant CH_3-Si-O- group of the HMDSO

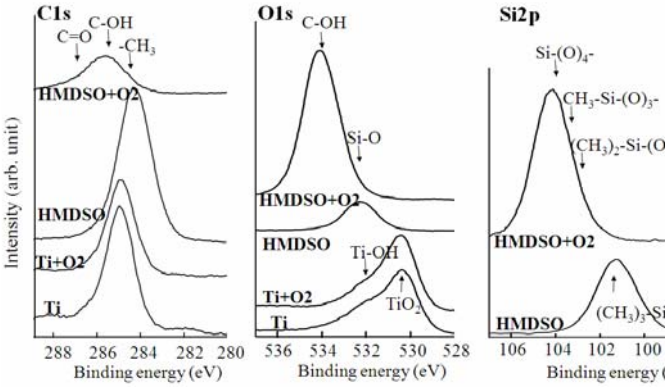

Fig. 1 XPS spectra of HMDSO, HMDSO+O2, Ti and Ti+O2 specimens.

surface to the Si-$(O)_4^-$ with OH group of HMDSO + O2 surface. The O1s peak of the outermost layer of the HMDSO surface was observed at 532.4 eV, and shifted to 534.3 eV on the HMDSO + O2. On the Ti and Ti+O2 specimens, O1s peaks appeared at around 533.0 eV and 530.5 eV, which corresponded to the Ti-OH and TiO_2, respectively. The Si2p peak of the outermost layer of HMDSO was observed at 101.3 eV, and shifted to 104.3 eV on the HMDSO + O2.

The FT-IR-RAS spectra of the HMDSO and HMDSO + O2 specimens are shown in Fig. 2. A peak attributed to Si-O-Si bonds appeared at around 1080 and 1100 cm^{-1} in the HMDSO and HMDSO + O2 specimens, respectively, showing a higher shift in comparison with the reported wavelength of 1050 cm^{-1} of the Si-O-Si bonds of the monomer. The O_2-plasma treatment caused this peak to broaden. The peaks at around 800 cm^{-1}, 850 cm^{-1}, 1250 cm^{-1}, and 1400 cm^{-1} are attributed to the Si-$(CH_3)_x$ bonds. The O_2-plasma treatment decreased the intensity attributed to the Si-O-Si bonds and Si-(CH_3)x. No appreciable peaks were observed on the Ti+O2 specimens.

Fluorescence intensities (ΔE*ab) for individual protein adsorption for Fn and Alb according to contact angle are shown in Fig. 3. Surface wettability (contact angle) affected Fn and Alb adsorption pattern, with the former showing biphasic features, with higher adsorption levels at both hydrophilic and hydrophobic surfaces. With Alb, adsorption increased with hydrophobicity from 0 degrees to 80 degrees, but not at 106 degrees.

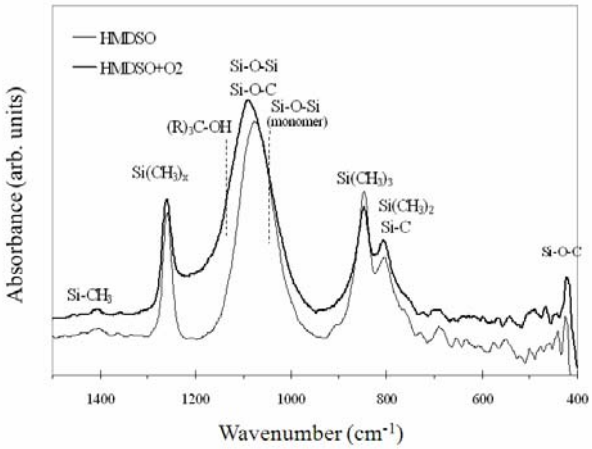

Fig. 2 FT-IR spectra of HMDSO and HMDSO + O2 specimens.

Fluorescence intensities for competitive adsorption for Fn and Alb, where a mixture of both kinds of protein was used, according to contact angle are shown in Fig. 4. The former showed higher adsorption levels on hydrophilic surfaces, whereas Alb showed adsorption predominantly on hydrophobic surfaces, except at a surface angle of 106 degrees.

The results of the initial cell attachment of L929 cells with different contact angle are demonstrated in Fig. 5. The typical images of the SEM results are also shown in Fig. 6. L929 cells showed different speeds of attachment with different surface wettability. The hydrophilic surfaces showed more cell attachment during the initial stages (60, 120 and 360 minutes. Even after 360 minutes of incubation, cells attached very poorly on the 106-degrees surface and most cells remained round. The hydrophilic surface attracted more cells and the cells seemed to be spreading.

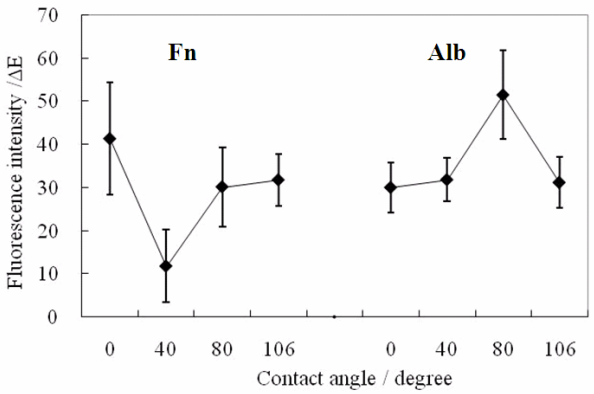

Fig. 3 Fluorescence intensities (ΔE*ab) for individual protein adsorption for Fn and Alb.

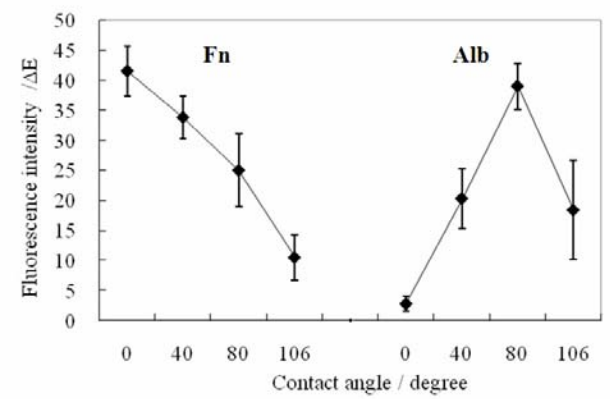

Fig. 4 Fluorescence intensities (ΔE*ab) for competitive protein adsorption for Fn and Alb , where a mixture of both kinds of protein was used.

Initial cell attachment MC3T3-E1 cells increased with increase in surface wettability at 30 and 60 minutes incubation (Fig. 7). Cell attachment at 180 minutes incubation was no significant differences among contact angles except 106 degrees. Typical SEM images of cell morphology at 30 minutes incubation are shown in Fig. 8. On hydrophilic surfaces, cells showed good spreading. Especially, cell spreading in super-hydrophilic condition looks more notable than other conditions. On the other hand, most of cells showed a poor spreading morphology in the 106 degrees of contact angle due to very poor adherence.

IV. DISCUSSION AND CONCLUSIONS

A cold plasma-surface modification allowed precise control of surface wettability without change in surface morphology in the present study. Surfaces with a wide range of contact angle (wettability) of between 106 degrees (hydrophobicity) and almost 0 degrees (super-hydrophilicity) were obtained by HMDSO coating and subsequent O_2-plasma treatment with altering the time duration of O_2-plasma treatment [7], [11].

XPS and FTIR-RAS analyses revealed that the increase of surface wettability by O_2 plasma treatment was due to the introduction of O_2-functional groups [12],[13]. The change of the HMDSO monomer structure caused by plasma polymerization and the O_2-plasma treatment that followed can be presumed to be as follows. That is, the fragmentation of the Si-C and C-H bonds of the HMDSO monomers were caused by plasma polymerization and changes in the hydrophilic surfaces were concomitant with introduction of O_2 functional groups during O_2-plasma treatment.

In the present study, we evaluated the adsorption behavior of Fn (cell adhesion protein) and Alb (cell adhesion inhibiting protein) in both individual and competitive mode, and initial attachment of fibroblast and osteoblast on surfaces with a wide range of wettability. In individual mode of protein adsorption, Fn and Alb showed different adsorption tendencies, depending on surface wettability; that is, Fn adsorption showed biphasic features, with higher levels of adsorption on both hydrophilic and hydrophobic surfaces.

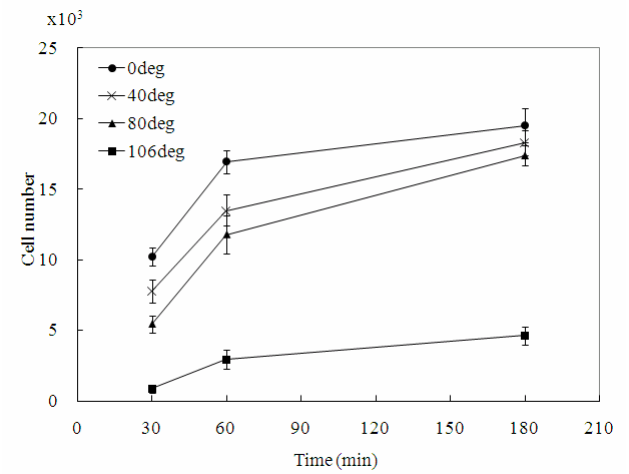

Fig. 5 Initial cell attachment of L929 cells with different contact angle.

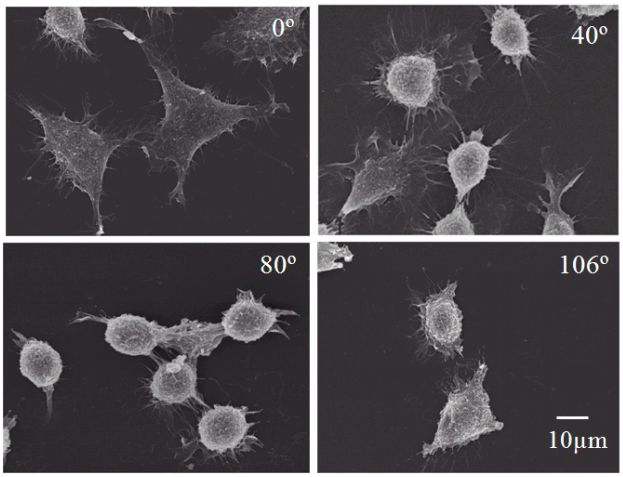

Fig. 6 Typical SEM images of L929 cells morphology with different contact angle at 360 minutes incubation.

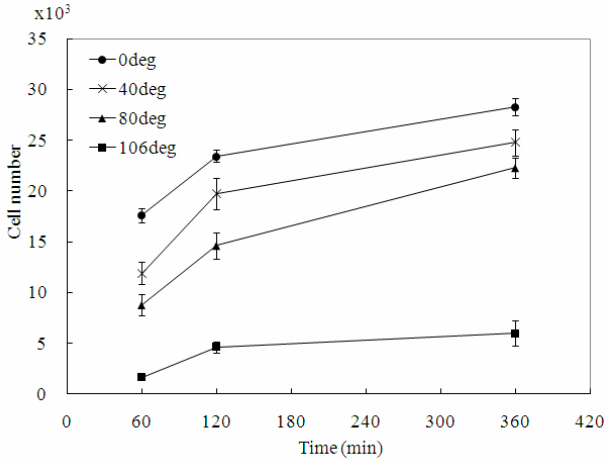

Fig. 7 Initial cell attachment of MC3T3-E1 cells with different contact angle.

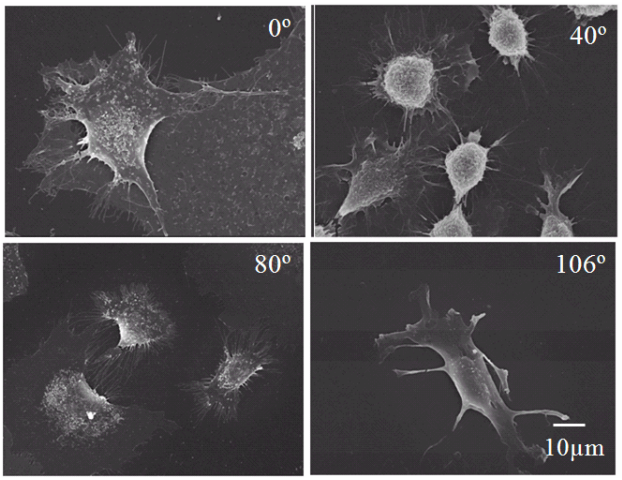

Fig. 8 Typical SEM images of MC3T3-E1 morphology with different contact angle at 30 minutes incubation.

With Alb, protein adsorption increased with hydrophobicity from 0 degrees to 80 degrees, but not at 106 degrees due to hydrophobic interaction. These results are in accordance with the results of Grinnell *et al.*[14] and Hao *et al.* [15]. On the other hand, Fn showed high levels of biphasic adsorption on hydrophobic and hydrophilic surfaces, but lower levels on moderate hydrophilic surfaces. That was explained by hydrophobic interaction on hydrophobic surfaces and ionic bonds to hydrophilic surfaces [16]. In addition to amount, protein conformation also affects the function of attached adhesive molecules. Adsorbed Fn showed two different conformations according to surface wettability. Antibodies tend to bind to a much greater degree to proteins with a more active conformation on a hydrophilic surface than they do to those on a hydrophobic surface. It has been suggested that an adhesive sequence such as RGD is in an active state on hydrophilic surfaces [14]. In contrast to individual mode, in competitive mode, Fn showed much higher adsorption on hydrophilic surfaces, whereas Alb predominantly adsorbed on hydrophobic surfaces, except at 106 degrees.

Initial attachment of both fibroblast and osteoblast increased with increase in surface wettability, which correlated well with Fn adsorption in competitive mode. Therefore, in culture medium including FBS, Fn is prone to adsorb preferentially on hydrophilic surfaces, resulting in high cell attachment, whereas Alb preferentially adsorbs on hydrophobic surfaces, interfering with cell attachment. Consequently, Fn adsorption may be responsible for increasing cell adhesion on surfaces with hydrophilicity in a body fluid or culture media under physiological conditions. In addition, the results in the present study revealed that super-hydrophilic surfaces were a promising method for enhancing the rapid attachment and spreading of both fibroblastic and osteoblastic cells.

In summary, a cold plasma-surface modification including HMDSO coating with plasma polymerization and oxygen plasma treatment enabled to regulate the protein adsorption and initial cell attachment by controlling the surface wettability of the materials. The findings in this study may contribute to the assessment and design of new surfaces for biological applications, suggesting that surface wettability be taken into account in the design of new biomaterial surfaces, especially in orthopedic and dental implants.

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