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Abstract
In this work, nitrogen, oxygen and air glow discharges powered by 50 Hz AC power supply are used for the treatment of type-A gelatin film cross-linked by a dehydrothermal (DHT) process. The properties of cross-linked gelatin were characterized by contact angle measurement, atomic force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS) analysis. The results showed that the water contact angle of gelatin films decrease with increasing plasma treatment time. The treatment of nitrogen, oxygen and air plasma up to 30 s had no effects on the surface roughness of the gelatin film as revealed by AFM results. The XPS analysis showed that the N-containing functional groups generated by nitrogen and air plasma, and O-containing functional groups generated by oxygen and air plasmas were incorporated onto the film surface, the functional groups were found to increase with increasing treatment time. An in vitro test using rat bone-marrow-mesenchym-derived stem cells (MSCs) revealed that the number of cells attached on plasma-treated gelatin films was significantly increased compared to untreated samples. The best enhancement of cell attachment was noticed when the film was treated with nitrogen plasma for 15–30 s, oxygen plasma for 3 s, and air plasma for 9 s. In addition, among the three types of plasmas used, nitrogen plasma treatment gave the best MSCs attachment on the gelatin surface. The results suggest that a type-A gelatin film with water contact angle of 27–28° and an O/N ratio of 1.4 is most suitable for MSCs attachment.

Keywords
50 Hz AC discharge, cross-linked gelatin, plasma treatment, surface modification, stem cell

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1. Introduction

Tissue engineering using cell transplantation appears to be the most promising alternative to existing therapies for restoring tissue and organ function. Tissue substitutes should restore biological functions of damaged tissues using cells with proliferation and differentiation potential. Cell affinity is the most important factor to be concerned with when biodegradable polymeric materials are utilized as scaffolds in tissue engineering [1]. Tissue integration is conditioned by the adhesion and spreading ability of cells on implant surfaces. Cell behavior on biomaterial surfaces depends on implant–cell interactions in correlation with surface properties. Surface hydrophilicity, roughness, texture, chemical composition, charge and morphology strongly affect cellular responses in contact with the implants [2, 3].

Biomaterial is one of the key considerations for tissue engineering and medical applications. Usually, materials used to form a scaffold should be biocompatible and biodegradable. Furthermore, it should correctly regulate tissue functions. Among various types of biomaterials, gelatin is one of the most dominant materials in tissue-engineering applications. Gelatin derived from hydrolysis of collagen has been of interest because gelatin solution is much cheaper and easier to obtain than collagen. Gelatin is biocompatible, biodegradable, non-immunogenic and non-antigenic [4–6]. Also, the gelatin molecule contains the Arg–Gly–Asp (RGD)-like sequence that promotes cell adhesion, migration and proliferation [7]. Gelatin can also be used as a base material substituting a large portion of collagen to produce scaffolds without affecting the biological properties [8]. As the main drawbacks of gelatin are its water solubility and weak mechanical properties. These result in high instability of scaffold in vivo. Cross-linking is often employed prior to its use in cell culture and biomedical applications. However, cross-linked gelatin films have been reported to have high hydrophobicity and low surface energy which do not favor cell adhesion [9, 10]. Therefore, surface modification was introduced. Many surface modification techniques have since been developed. Plasma treatment is one of the surface modification methods that is widely employed for surface modification of materials since it is a versatile, fast and environmentally benign technology [11]. Surface properties modified by plasma are wettability and chemistry, which can greatly affect cell response. For example, cold plasma, such as radio frequency (RF) generated plasma, has been reported to modify the surface of synthetic polymers such as poly(lactide-co-glycolide) (PLGA) and poly(l-lactic acid) (PLLA) by introducing reactive groups onto the surface, resulting in higher hydrophilicity of the surface [12–14]. In our previous study, the affinity of L929 mouse fibroblasts on the cross-linked gelatin films treated with pulse inductively coupled plasma (PICP) was greatly enhanced because of hydrophilicity improvement of the surface [10]. Lee et al. revealed that plasma treatment of polycaprolactone films using different gases, including argon, nitrogen and oxygen, exhibited a different human prostate epithelial cell attachment and growth [15].

In this work, alternating current (AC) glow discharge, one of the non-thermal plasma systems, is used. It can be used to modify the surface of polymers [18].
The design of 50 Hz AC glow discharge is much simpler than a typical inductively-coupled RF plasma reactor as a high-frequency power supply is not required. Operating at a power line frequency (50–60 Hz) requires only an inexpensive power supply. Recently, Wong et al. have reported the adhesion strength of polyimide film that could be enhanced by polymerization process using a 50 Hz AC glow discharge system [16]. Carrino et al. also found that a 50 Hz AC plasma system could be used to improve polypropylene wettability and adhesion properties [17]. Based on our best knowledge, glow discharge using a 50 Hz AC system has not been applied to treat natural polymers.

This work aimed to employ three different types of gas, nitrogen, oxygen and air, in 50 Hz AC glow discharge to treat cross-linked gelatin film. Various surface properties of gelatin film, including wettability and surface chemistry, were investigated. These effects on the in vitro attachment of rat bone-marrow-derived stem cells (MSCs) were explored. The specific information of gelatin surface properties suitable for the attachment of MSCs was also investigated.

2. Materials and Methods

2.1. Materials

Type-A gelatin with an isoelectric point (IEP) of 9 was obtained from Nitta Gelatin. Alpha-modified Eagle minimal essential medium (α-MEM) and trypsin/EDTA from Sigma-Aldrich were used as received. Other chemicals used were all of analytical and cell-culture tested grade.

2.2. Plasma Experimental Setup and Characterization

The setup of glow discharge plasma using an alternating-current (AC) system, modified from our previous study [18], is shown in Fig. 1. The plasma chamber was...
made of cylindrical glass. The plasma deposition process occurred between two
circular parallel plate electrodes of 8 cm in diameter with an inter-electrode dis-
tance of 6 cm. The 50 Hz sinusoidal high voltage was applied to one electrode
while the other was connected to the ground. The applied voltage was generated
from a power supply consisting of a Variac and a transformer. The voltage across
the electrodes ranged from 3 to 15 kV. Before operation, the chamber was evacuated
to less than 0.3 mbar before the operating gas was filled to the required pressure.
After the pressure was stabilized at 1 mbar, glow discharge plasma was produced
by applying the AC power. The applied voltage was measured using a high-voltage
probe. The discharge current was determined using an AC ammeter. The average
power \( W \) was calculated according to equation (1):

\[
W = \frac{1}{T} \int_{t}^{t+T} I(t)V(t) \, dt,
\]

where \( W \) is the average power (W), \( I \) the discharge current (mA), \( V \) the applied
voltage (kV) and \( T \) is the cycle time.

In order to characterize the N and O species of the plasma produced, an Ocean
Optics USB4000 charge couple device (CCD) spectrometer was used to monitor
the light emitted from the plasma in the wavelength range of 200–1000 nm. The
plasma emissions were collected using an optical fiber placed about 1 cm in front
of the glass chamber. The fiber was connected to the spectrometer through a 10 µm
entrance slit. The data were acquired with the Ocean Optics’ Spectra Suite software.

2.3. Preparation of Plasma-Treated Cross-Linked Gelatin Films

Gelatin was dissolved in deionized water to achieve a 0.05 wt% solution. Gelatin
film was prepared by solution casting technique. Gelatin solution (100 µl) was
dropped onto a glass cover slip 15 mm in diameter and air-dried in clean condi-
tions. After solvent evaporation, the film was cross-linked using dehydrothermal
treatment in a vacuum oven at 140°C for 48 h [10]. The cross-linked gelatin film
was placed inside the cylindrical tube of the plasma apparatus and treated with the
glow discharge using three different types of gas: nitrogen, oxygen and dry air. The
pressure and the discharge power were fixed at 1 mbar and 4 W, respectively, while
the treatment times were varied from 3 to 30 s. All the different sets of plasma
treatment conditions used are summarized in Table 1.

2.4. Characterization of Untreated and Plasma-Treated Gelatin Films

2.4.1. Contact Angle and Surface Energy of Gelatin Films

Contact angles of cross-linked gelatin films were measured by sessile dropping
technique at room temperature using a contact angle meter (Cam-Plus Miccro, Tan-
tec) equipped with an optical microscope. Deionized water and ethylene glycol
were used as liquid media. The contact angles of the sample were averaged from 5
measurements at 30 s dropped. The surface free energy was calculated according to
Table 1.
Atomic compositions and relative ratios of C, N and O calculated from the XPS spectrum of plasma-
treated and untreated cross-linked gelatin surfaces

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type of gas used, treatment period</th>
<th>Atomic composition (%)</th>
<th>Atomic ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>O</td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td>70.0</td>
<td>20.4</td>
</tr>
<tr>
<td>N-3</td>
<td>N₂, 3 s</td>
<td>66.4</td>
<td>23.3</td>
</tr>
<tr>
<td>N-9</td>
<td>N₂, 9 s</td>
<td>64.2</td>
<td>22.8</td>
</tr>
<tr>
<td>N-15</td>
<td>N₂, 15 s</td>
<td>57.5</td>
<td>21.1</td>
</tr>
<tr>
<td>N-30</td>
<td>N₂, 30 s</td>
<td>62.7</td>
<td>21.4</td>
</tr>
<tr>
<td>O-3</td>
<td>O₂, 3 s</td>
<td>67.7</td>
<td>23.3</td>
</tr>
<tr>
<td>O-9</td>
<td>O₂, 9 s</td>
<td>64.6</td>
<td>25.9</td>
</tr>
<tr>
<td>O-15</td>
<td>O₂, 15 s</td>
<td>61.0</td>
<td>28.3</td>
</tr>
<tr>
<td>O-30</td>
<td>O₂, 30 s</td>
<td>59.2</td>
<td>30.6</td>
</tr>
<tr>
<td>A-3</td>
<td>Air, 3 s</td>
<td>65.0</td>
<td>24.8</td>
</tr>
<tr>
<td>A-9</td>
<td>Air, 9 s</td>
<td>63.7</td>
<td>25.9</td>
</tr>
<tr>
<td>A-15</td>
<td>Air, 15 s</td>
<td>62.5</td>
<td>26.6</td>
</tr>
<tr>
<td>A-30</td>
<td>Air, 30 s</td>
<td>63.0</td>
<td>25.2</td>
</tr>
</tbody>
</table>

Young’s equations as follows [14]:

\[(1 + \cos \theta_1)\gamma_{L1} = 2\left((\gamma_S^d \gamma_{L1}^d)^{1/2} + (\gamma_S^p \gamma_{L1}^p)^{1/2}\right)\]

\[(1 + \cos \theta_2)\gamma_{L2} = 2\left((\gamma_S^d \gamma_{L2}^d)^{1/2} + (\gamma_S^p \gamma_{L2}^p)^{1/2}\right)\]

where \(\gamma_S^d\) is the surface energy of dispersive component, \(\gamma_S^p\) the surface energy of polar component, \(\theta_1\) the contact angle of water and \(\theta_2\) the contact angle of ethylene glycol. For water, \(\gamma_{L1} = 72.8\) mJ/m², \(\gamma_{L1}^d = 22.1\) mJ/m² and \(\gamma_{L1}^p = 50.7\) mJ/m². For ethylene glycol, \(\gamma_{L2} = 48.0\) mJ/m², \(\gamma_{L2}^d = 29.0\) mJ/m² and \(\gamma_{L2}^p = 19.0\) mJ/m².

2.4.2. Surface Topology of Gelatin Films
Surface topography of the plasma-treated and untreated samples was examined using atomic force microscopy (AFM, Nanoscope IV, Veeco) in a tapping mode. In order to ensure that the cross-linked gelatin films were not altered by scanning tip, the specimens were kept at 80°C for 2 h prior to AFM measurement. Three-dimensional images and surface topography parameter data were acquired using Nanoscope image-processing software. The surface parameters were averaged from 4 areas of each sample and expressed as the mean ± SD (n = 4).

2.4.3. Surface Chemistry of Gelatin Films
The chemical bonding states and atomic ratio of untreated and plasma-treated cross-linked gelatin surface were examined by X-ray photoelectron spectroscopy (XPS: ESCALAB250, VG Scientific) using an Al-Kα (1486.6 eV) X-ray source. The pressure in the sample chamber was controlled at 10⁻⁸–10⁻⁹ Torr. The photo-emitted electrons were collected at a take-off angle of 90°. In order to calculate the atomic
composition of plasma-treated and untreated gelatin films, the deconvolution process was performed by curve fitting of the C\textsubscript{1s}, O\textsubscript{1s} and N\textsubscript{1s} peaks derived from XPS spectra.

2.4.4. Surface Isoelectric Point (IEP) of Gelatin Films
The IEP of gelatin surface was determined using contact angle titration, modified from the goniometry technique [19]. Contact angles of plasma-treated and untreated gelatin films were measured by sessile dropping technique using a contact angle meter equipped with an optical microscope. Phosphate buffers with different pH values, ranging from 4 to 10, were used as liquid media. The contact angle was measured at the 30 s drop and averaged from 3 measurements.

2.5. Isolation of Rat Bone-Marrow-Derived Stem Cells (MSCs)
In accordance to Chulalongkorn University Animal Care and Use Committee (CU-ACUC) regulations and with ethics approval from the research ethical committee, Faculty of Medicine, Chulalongkorn University, bone-marrow-derived stem cells (MSCs) employed for biocompatibility test of untreated and plasma-treated films were isolated from the bone shaft of femurs of 3-week-old female Wistar rats according to the technique reported by Takahashi \textit{et al.} [6]. After killing and sterilization, both ends of rat femurs are cut away from the epiphysis and the bone marrow is flushed out with a syringe (16-gauge needle) with 1 ml medium. The cell suspension is then cultured in tissue-culture plates containing α-MEM supplemented with 15 vol% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin at 37°C and 5% CO\textsubscript{2}. The medium is changed on the 4th day after isolation and every 3 days thereafter. When the proliferated cells become subconfluent, usually for 7–10 days, the cells are trypsinized using 0.25 wt% trypsin-EDTA. The cells of the second- and third-passage at sub-confluence are used.

2.6. Cell-Culture Test
Plasma-treated and untreated gelatin films were placed into 24-well cell-culture plates and sterilized in 70 vol% ethanol for 30 min. To remove ethanol, the samples were extensively rinsed with phosphate-buffered saline (PBS). MSCs in α-MEM containing 15 vol% FBS and 100 U/ml penicillin/streptomycin were seeded onto the films at 2 × 10\textsuperscript{4} cells/film and incubated at 37°C in 5% CO\textsubscript{2}. After cell culture for 3, 6, 12 and 18 h, the culture medium was removed and the films were then rinsed twice with PBS. Cell viability was determined using DNA assay [9]. Briefly, the cell samples were lysed at 37°C overnight in 30 mM sodium citrate-buffer saline solution (SSC, pH 7.4) containing 0.2 mg/ml sodium dodecylsulfate (SDS). Cell lysates were then mixed with a fluorescent dye solution (Hoechst 33258 dye) in a black 96-well plate. The fluorescent intensities of mixed solution were immediately measured at the excitation and emission wavelength of 355 and 460 nm, respectively. The results were reported as the number of cells using a standard curve prepared from MSCs. The same treatment of the films without cells was used as
control. All data were expressed as mean ± SD (n = 3). In addition, the percentage of cell attachment was calculated according to the following equation:

$$\text{Attachment (\%)} = \left( \frac{N_1}{N_0} \right) \times 100,$$

where $N_1$ and $N_0$ are the number of attached cells at a specific culture time and seeded cells, respectively.

2.7. Cell Morphology and Spreading

After 6 h of culture, attached MSC on the films were fixed in 2.5 vol% glutaraldehyde solution at 4°C for 1 h. Gelatin films were then serially dehydrated by series of ethanol (30, 50, 70, 80, 90, 95 and 100 vol%) for 5 min at each concentration. Hexamethyldisilazane (HMDS) was added to dry the dehydrated gelatin films at room temperature. The morphology of cell attached was observed using scanning electron microscopy (SEM, JEOL JSM-6400). The dry gelatin films were carefully fixed on stubs and gold-coated using a JEOL JFC-1100 sputtering device prior to SEM observation. In addition, the cell spreading area of attached MSC was evaluated from the photographs taken using an optical microscope (10× magnification). Cells spreading area was determined using Image J-Analysis software [9]. A minimum of 40 cells was examined for each sample.

2.8. Statistical Analysis

Significant levels were determined by the paired $t$-test. All statistical calculations were performed on SPSS system for Windows (SPSS version 13.0). $P < 0.05$ was considered significant.

3. Results

3.1. Optical Emission Analysis of 50 Hz AC Plasma

The emission spectra of 50 Hz AC plasmas generated using nitrogen, oxygen and dry air are presented in Fig. 2. The results show that nitrogen plasma mainly consists of nitrogen radicals ($N_2^*$) and nitrogen ions ($N_2^+$), as indicated by the strong peaks at 315.9, 337.13 and 357.8, 391.44 nm, respectively [20]. For oxygen plasma, strong peaks are observed at 777.42 and 844.56 nm, corresponding to the oxygen radical ($O^*$) [21]. For air plasma, both nitrogen and oxygen species were observed. In addition, the hydrogen radical ($H_\alpha^*$) was noticed for all gases used as indicated at 656.30 nm.

3.2. Effects of Plasma Treatment on the Water Contact Angle and Surface Energy of Cross-Linked Gelatin Film

The water contact angle and surface free energy of untreated and plasma-treated cross-linked gelatin films as a function of treatment time and type of gas are shown in Figs 3 and 4, respectively. It can be noticed that the water contact angle of untreated cross-linked gelatin films is 87°. With 3 s plasma treatment, the water
Figure 2. Optical emission spectrum of (a) nitrogen plasma, (b) oxygen plasma and (c) air plasma generated by 50 Hz AC glow discharge at 4 W and 1 mbar.
contact angle sharply decreased to 55°. Longer treatment time resulted in a further decrease until the water contact angle of the cross-linked gelatin films reached 23° at the treatment time of 15 s for all gases used. The decrease of water contact angle as a function of plasma treatment time did not significantly alter when various gases were used.

The total surface free energy of untreated cross-linked gelatin was 38.6 mJ/m². After plasma treatment for 3 s, the total surface free energy significantly increased.
to 47.5 mJ/m². However, after plasma treatment for 15 min, the surface free energy of gelatin film seemed to be consistent at 70.0 mJ/m² for all gases used. The results of the variation in the polar and dispersive component of surface free energy (Fig. 4) showed that after treatment with plasma the polar component of gelatin film was increased, while the dispersive component of the surface was decreased. A similar trend was observed for all gases used.

3.3. Effects of Plasma Treatment on the Surface Topology of Cross-Linked Gelatin Film

The surface topology and the quantitative parameters of surface roughness of cross-linked gelatin films before and after oxygen plasma treatment investigated by AFM...
Figure 5. Surface topology of (a) untreated cross-linked gelatin films and oxygen plasma-treated cross-linked gelatin films at 4 W and 1 mbar for (b) 3, (c) 9, (d) 15 and (e) 30 s. This figure is published in colour in the online edition of this journal, which can be accessed via http://www.brill.nl/jbs

are shown in Fig. 5. The surface of the untreated cross-linked gelatin was smooth (Fig. 5a) with the calculated mean surface roughness (Rms) of 0.65 nm. After treatment with oxygen plasma for 3–30 s, the surface topology remained unchanged
No significant difference between the Rms of untreated and plasma-treated cross-linked gelatin films was noticed. The results on surface topology of plasma-treated gelatin films using nitrogen and dry air were similar to those using oxygen (data not shown).

3.4. Effects of Plasma Treatment on the Surface Chemistry of Cross-Linked Gelatin Film

The N₁s, O₁s and C₁s spectra of plasma-treated and untreated cross-linked gelatin films, obtained from the XPS results, are shown in Fig. 6. It can be seen that after treatment with nitrogen plasma, oxygen and air plasma, the N₁s, O₁s and C₁s spectra were presented at the same binding energies as observed in the case of untreated film. In order to quantify the composition of gelatin surface, the deconvolution of the N₁s, O₁s and C₁s spectra of plasma-treated and untreated cross-linked gelatin films was performed. Four peaks, corresponding to N₁s (400 eV), O₁s (532 eV) and C₁s (285 and 288 eV), were observed. Based on the peak-fitting the N₁s spectrum represents C–NH, O₁s –COOH or –OH, C₁s at 285 eV C–CH and C₁s at 288 eV C–O–O [15]. The same N₁s, O₁s and C₁s spectra of gelatin films were also obtained after treatment with oxygen and air plasma. To quantitatively compare the composition change of the film before and after plasma treatment, the relative N/C and O/C ratios were calculated and summarized in Table 1. The N/C, O/C and O/N ratios of untreated film were 0.14, 0.29 and 2.1, respectively. After treatment with nitrogen plasma for 30 s, N/C and O/C ratios were increased to 0.25 and 0.34, respectively, while the O/N ratio decreased to 1.4. Similar phenomena were also observed with air plasma treatment. After treated with air plasma for 30 s, the N/C and O/C ratios of gelatin films were increased to 0.19 and 0.40, respectively, while the O/N ratio increased slightly to 2.2. When gelatin films were treated with oxygen plasma, the

![Figure 6. N₁s, O₁s and C₁s spectra of untreated and plasma-treated cross-linked gelatin films at the treatment time of 30 s. This figure is published in colour in the online edition of this journal, which can be accessed via http://www.brill.nl/jbs](http://www.brill.nl/jbs)
O/C and O/N ratios continuously increased with increasing treatment time, resulting in the highest O/C and O/N ratios at 0.52 and 2.9, respectively, after 30 s of treatment.

3.5. Effects of Plasma Treatment on the IEP of Cross-Linked Gelatin Film

The contact angle of untreated and plasma-treated gelatin film as a function of the pH of the PBS used is shown in Fig. 7. The IEP of the films can be determined from the pH at which the maximum contact angle occurs. It was obvious that the IEP of untreated film was 8. After treatment with nitrogen plasma for 30 s, the IEP of gelatin films was shifted to 9. In contrast, the IEP of gelatin films treated with both oxygen and air plasma for 30 s tended to decrease to approx. 6–7.

3.6. Attachment of MSCs Cultured on Gelatin Film

In this section the biocompatibility of untreated and plasma-treated cross-linked gelatin films using MSCs is presented, especially the attachment behavior of cells. The number of MSCs attached on untreated and plasma-treated gelatin films cultured for 3, 6, 12 and 18 h is shown in Fig. 8. In the early stages of attachment (after 3 h culture), the number of cells attached on nitrogen-, oxygen- and air-plasma-treated gelatin films was observed to be higher than on the untreated sample. Especially in the samples treated with nitrogen plasma for 9–30 s, oxygen plasma for 3 s and air plasma for 9 s, a significant difference of the number of cells attached as compared to the untreated sample was observed. After longer culture periods, it was evident that treatment with nitrogen plasma for 9–30 s, oxygen plasma for 3 s and air plasma for 9 s could greatly enhance the attachment of cells on the gelatin films. However, longer treatment by O-containing plasma, such as oxygen and air, could not further enhance cell attachment on the gelatin surface. The number of cells attached on plasma-treated gelatin film, when oxygen and air plasma was ap-
Figure 8. Number of MSCs attached on (a) nitrogen plasma-treated gelatin films, (b) oxygen plasma-treated gelatin films and (c) air plasma-treated gelatin films, determined by DNA assay. *P < 0.005, significant difference relative to untreated films. **P < 0.05, significant difference relative to N-15 and N-30. P < 0.05, significant difference relative to O-3. †P < 0.05, significant difference relative to A-9.

plied for 9–30 and 15–30 s, respectively, tended to decrease compared to that on untreated sample.

3.7. Morphology and Spreading Area of MSCs Attached on Gelatin Film

After 6 h of cell seeding, the spreading areas of MSCs attached on untreated and plasma-treated cross-linked gelatin were observed as presented in Table 2. A significantly larger spread area was noticed on the surface of plasma-treated gelatin films compared to that on the surface of untreated sample. The maximum spread-
Table 2.
The spreading area of MSCs, and percentage of cell attachment after 6 h cultured on plasma-treated and untreated gelatin films

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell spreading area (µm²)</th>
<th>Cell attachment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>151 ± 26</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>N-3</td>
<td>243 ± 24*</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>N-9</td>
<td>301 ± 27*</td>
<td>57 ± 10*</td>
</tr>
<tr>
<td>N-15</td>
<td>336 ± 32*</td>
<td>69 ± 11*</td>
</tr>
<tr>
<td>N-30</td>
<td>320 ± 30*</td>
<td>76 ± 1*</td>
</tr>
<tr>
<td>O-3</td>
<td>269 ± 55*</td>
<td>64 ± 13*</td>
</tr>
<tr>
<td>O-9</td>
<td>229 ± 40*</td>
<td>48 ± 10</td>
</tr>
<tr>
<td>O-15</td>
<td>227 ± 48</td>
<td>27 ± 3*</td>
</tr>
<tr>
<td>O-30</td>
<td>190 ± 40</td>
<td>27 ± 5*</td>
</tr>
<tr>
<td>A-3</td>
<td>250 ± 38*</td>
<td>43 ± 10</td>
</tr>
<tr>
<td>A-9</td>
<td>210 ± 28*</td>
<td>66 ± 4*</td>
</tr>
<tr>
<td>A-15</td>
<td>249 ± 29*</td>
<td>30 ± 13*</td>
</tr>
<tr>
<td>A-30</td>
<td>200 ± 25</td>
<td>33 ± 5</td>
</tr>
</tbody>
</table>

* P < 0.05, significant difference relative to untreated films.

The spreading area of MSCs on the gelatin surface was obtained when the nitrogen, oxygen and air plasmas were applied for 15–30, 3 and 9 s, respectively. The corresponding morphology of maximum spread MSCs attached on the gelatin film treated with each plasma type is shown in Fig. 9. The cells attached to plasma-treated surface exhibited a more elongated and flattened structure compared to the rounded shape of the untreated sample (Fig. 9a).
Figure 9. Morphology of MSCs cell attached after 6 h culture. (a) Untreated, (b) nitrogen plasma treatment for 15 s, (c) oxygen plasma treatment for 3 s and (d) air plasma treatment for 9 s.

4. Discussion

In this work, 50 Hz AC plasma was introduced to treat the surface of cross-linked gelatin film in order to enhance biocompatibility. The results from in vitro cell culture using MSCs showed that the number of adhered cells on plasma-treated gelatin film was higher than that on untreated sample. The plasma-treated gelatin film induced cell attachment with a large spreading area (Table 2, Fig. 9). This revealed that the plasma-treated gelatin films were more favorable to cell attachment than untreated gelatin films. From the result of contact angle and surface energy measurements (Figs 3 and 4), the plasma-treated gelatin film was shown to be more hydrophilic than untreated gelatin. This might result from the polar groups incorporated into the surface of gelatin during plasma treatment, resulting in a highly hydrophilic gelatin surface [14]. The hydrophilic surface was generally known to support cell attachment [22]. The wettability of the polymer surface was reported to be possibly caused by material cross-linking, surface roughness and surface chemistry [23, 24]. However, our previous study found that short time treatment of plasma had no effect on the degree of cross-linking of gelatin film [10]. In case of surface roughness, the observation of surface topology (Fig. 5) revealed that the surface of gelatin films remained unchange after nitrogen, oxygen and air plasma treatment. Generally, the enhancement of surface roughness caused by plasma treat-
ment depended on the operating conditions such as discharge power, pressure and treatment time [25]. In this study, the applied 50 Hz AC plasma at the operating discharge power of 4 W could not alter the surface roughness of cross-linked gelatin films since the low density of high-energy active species at low operating discharge power could not physically etch the surface of gelatin film [25].

In order to observe whether the alteration of surface chemistry of plasma-treated gelatin surface was the reason for the more hydrophilic surface, we have considered the formation of N and O species obtained from nitrogen, oxygen and air plasma. The observation on optical emission spectroscopy in Fig. 2 revealed that the functionalization process of N and O containing functional groups was possibly performed by N and O reactive species on the surface of cross-linked gelatin films. The relative ratios of C, N and O of plasma-treated and untreated gelatin films are summarized in Table 1. It was evident that the N/C ratio generated by nitrogen plasma and O/C ratio generated by oxygen plasma increased with increasing treatment time. This suggested that hydrophilic functional groups, such as NH2 and COOH, might be incorporated onto the surface of gelatin films. The increase of N/C and O/C ratios of plasma-treated gelatin films could result in the decrease of the water contact angle. It could be concluded that plasma treatment with 50 Hz AC using all three gases was able to quantitatively alter the surface chemistry of the films, resulting in the change of wettability of gelatin surface films.

Many studies have reported the interaction of different types of cells with various substrates having different wettability. Tamada et al. stated that a polymer surface with a water contact angle of 70° provided the most suitable surface for cell adhesion. However, the surface used in those reports was not well controlled in terms of roughness, ionic charge, etc. [26]. Lee et al. also reported fibroblast and endothelial cells adhesion to a polyethylene surface with gradient of wettability performed by corona discharge and found the maximum cell adhesion at a water contact angle of 55° [27]. According to our results, it was shown that the water contact angle of the gelatin surface suitable for MSCs cell attachment depended on the type of gas used to generate plasma. In other words, differences in surface chemistry which were introduced by various types of gas plasma provided a specific water contact angle suitable for MSCs attachment. In the case of nitrogen plasma treatment, it was obvious that MSCs could greatly adhere on the highly hydrophilic gelatin surface. The maximum attachment of MSCs on gelatin surface was found for the film plasma-treated for 15 and 30 s (water contact angle 27–28°). It was also reported in our previous study that treatment with nitrogen pulse inductively coupled plasma (PICP) having a contact angle of 27–28° provided the highest attachment of L929 mouse fibroblasts [10]. The improved attachment could be attributed to the increase of N-containing functional groups, such as NH2, on the surface of gelatin during nitrogen plasma treatment. Since adhesive glycoproteins, such as fibronectin and vitronectin, play important roles in the initial cell attachment, the N-containing groups were capable of efficient interaction with protein by hydrogen bonding, which could affect the adsorption of serum adhesive glycoproteins [28].
addition, the increase of N-containing functional groups could affect the change of surface charge of gelatin film. As observed in Fig. 7, after treatment with nitrogen plasma for 30 s the IEP of gelatin film tended to increase compared to that of untreated film. This revealed that the surface charge of gelatin film became positive at physiological pH and could interact with negatively charged cell surface, promoting better cell attachment [9, 29].

Considering the plasma treatment using oxygen-containing gas, such as oxygen and air, it was clearly observed that, as the surface wettability of gelatin films increased with increasing treatment time, the number of adhered cells increased and then decreased at longer treatment time. This suggested that the moderately hydrophilic surface of type-A gelatin film was most favorable to MSCs attachment. The maximum attachment was observed at the treatment time of 3 s (water contact angle 55°) for oxygen plasma treatment and 9 s (water contact angle 40°) for air plasma treatment, as summarized in Table 3. The increase of cells attachment on oxygen and air plasma-treated gelatin surfaces with moderate hydrophilicity could be attributed to the hydrophilic part of protein existing in the outer region of cell membrane due to the repulsion of hydrophobic part of protein and phospholipids in the inner region of cell membrane [29]. Therefore, the hydrophilic property of the plasma-treated gelatin surface could promote cell attachment by increasing the affinity between the protein and gelatin surface. This corresponded to the report by Shin et al. [30]. They examined human bone-marrow-derived stem cells (hBM-SCs) adhesion to a polyethylene surface with a gradient of wettability prepared by oxygen-containing gas corona discharge and found the maximum cell adhesion on substrate with moderate hydrophilicity at a water contact angle of 57°.

Interestingly, the high hydrophilicity of oxygen and air plasma-treated gelatin surface could not further enhance cell attachment (Fig. 8b and c). This could be attributed to the high O/C ratio introduced onto gelatin surface. Daw et al. reported that an increase of O/C ratio and carboxylic acid reduced the number of osteoblast-like cells attached [31]. This might result from the increase of O/C ratio leading to a highly negative charge of the gelatin surface, as seen in Fig. 7. The IEP of gelatin film treated with oxygen and air plasma for 30 s was decreased to 6–7, compared to that of untreated film. This suggested that the gelatin surface would exhibit a

### Table 3.
Summary of the conditions of plasma treatment at which % attachment of MSCs on gelatin films was maximal and their corresponding surface properties

<table>
<thead>
<tr>
<th>Type of gas</th>
<th>Attachment (%)</th>
<th>Treatment period (s)</th>
<th>Water contact angle (°)</th>
<th>O/C ratio</th>
<th>N/C ratio</th>
<th>O/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>38</td>
<td>–</td>
<td>84</td>
<td>0.29</td>
<td>0.14</td>
<td>2.1</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>69–76</td>
<td>15, 30</td>
<td>27–28</td>
<td>0.34–0.36</td>
<td>0.25–0.27</td>
<td>1.4</td>
</tr>
<tr>
<td>Oxygen</td>
<td>64</td>
<td>3</td>
<td>55</td>
<td>0.34</td>
<td>0.13</td>
<td>2.6</td>
</tr>
<tr>
<td>Air</td>
<td>66</td>
<td>9</td>
<td>40</td>
<td>0.41</td>
<td>0.16</td>
<td>2.5</td>
</tr>
</tbody>
</table>
slightly negative charge at physiological pH and prohibit the adhesion of negatively charged cell through electrostatic repulsion [32].

The conditions of plasma treatment which provided suitable surface properties of gelatin films for best attachment of MSCs after 6 h of seeding are also summarized in Table 3. There were more MSCs attached to the plasma-treated surface (64–76%) compared to untreated films (38%). It was obvious that the type of gas used to generate plasma greatly affects the attachment behavior of MSCs. Among the surfaces plasma-treated using various types of gas, the gelatin surface treated with nitrogen plasma for 15–30 s could best enhance the attachment of MSCs (69–76%). The most suitable contact angle and O/N ratio of nitrogen plasma-treated gelatin film for optimal attachment of MSCs were 27–28° and 1.4, respectively.

5. Conclusion

50 Hz AC plasma is being introduced to treat gelatin, one of the most widely used biomaterials, in this study. The hydrophilicity and surface energy of plasma-treated gelatin was promoted in comparison with untreated samples. 50 Hz AC glow discharge could alter the surface chemistry but not surface roughness of gelatin film. From in vitro MSCs cell culture, the results revealed that cells could adhere to plasma-treated samples better than untreated samples. In this work, we have shown that the specific water contact angle and oxygen to nitrogen (O/N) ratio of the plasma-treated gelatin surface suitable for MSCs attachment depended on both the type of gas used and the treatment time. Moreover, among the three types of plasmas used, nitrogen plasma treatment could best enhance the biocompatibility of cross-linked gelatin films.

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