

CHAPTER 2

IMPROVING THE ATTACHMENT AND PROLIFERATION OF UMBILICAL CORD MESENCHYMAL STEM CELLS ON MODIFIED POLYSTYRENE BY NITROGEN-CONTAINING PLASMA

2.1 Introduction

Recently, cell therapy using mesenchymal stem cells (MSCs), can be used to treat many human diseases. This can be achieved by the transplantation of cells in *In vitro* system. Thus, the cell culture substrates must optimize for cells have survive long enough to restore normal function. Polystyrene is the popular culture vessels used due to its excellent durability, good optical and non-toxicity. However, PS itself is unsuitable for eukaryotic cell culture because of its hydrophobic nature. Therefore, surface treatments are required to optimize cell adhesion, proliferation and differentiation. Most of the present commercialize PS culture vessels are normally pretreated, using variety of techniques including corona discharge, ultraviolet radiation, gamma irradiation and gas plasma modification [1], are well developed for common types of cells in nature, but more specific cell types require more advanced modifications. MSCs from different sources, such as bone marrow cavity, so called BM-MSCs, umbilical cord blood, CBMSCs, or umbilical cord Wharton's jelly, WJMSCs has been identified as a highly potential of similar differentiation.

This section investigates the nitrogen-containing gas plasmas process by using a 13.56 MHz inductively coupled discharge plasma reactor modified onto polystyrene (PS) culture dish surface. A mixture of N-containing gas (N_2 and NH_3) and noble gas (He and Ar) has been used. This was expected to introduce the N-containing functional groups and the more hydrophilic groups on PS surface and favored the Wharton's Jelly mesenchymal stem cells (WJMSCs); BCP-K1.

These techniques provide hydrophilicity to PS surfaces either by oxidizing and chemical functional group alteration. This is a consequential factor defining the surface energy, polarity, wettability and zeta potential, and accordingly the character of the cell-material interaction. There is optimal cell adhesion to temperately hydrophilic and positively charged substrates, because of the adsorption of cell adhesion-mediating molecules accessible to cell adhesion receptors (e.g. integrins). Cell adhesion to positively charged surfaces is better than to negatively charged surfaces. The reasoning is that the cell surfaces adhesive-molecules are negatively charged and consequently adhere preferentially to positively charged surfaces such as amine-containing surface [2].

However, previous studies showed that the concentration of functional groups, introduced on a polymer surface by plasma treatments, may change over time,

depending on the environment and temperature, especially amine functional groups [3].

In this study we focus on the long-term effect of physico-chemical properties of the noble gas mixture on enhancement of reactive species in nitrogen-containing plasma and on hydrophobic recovery of treated PS surfaces over 30 days. Hydrophobic recovery means surface restructuring of the polymer, a reorientation of nonpolar groups (hydrophobic property) from the bulk to the surface and condensation of surface hydroxyl groups. Addition of inert gases such as argon (Ar), neon (Ne) and helium (He) in nitrogen plasma, enhances the concentration of active species through Penning excitation and ionization processes [4, 5]. He has higher metastable energies than any other inert gas. This making it a powerful Penning reagent for exciting plasma species through inelastic collisions, and thus can be added in nitrogen-containing plasma to achieve a considerable increase in the reactive level. He or Ar gas are commonly used in the laboratory because of their low cost, whereas Ne, xenon (Xe), and other noble gases are of limited application.

The process was performed in a mixed gas of N_2 or NH_3 and He or Ar, in order to understand which kinds of mixed gas and the optimum power play an important role in the control of reactions between surface reactive species and plasma reactive species.

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2.2 Experimental Setup and Methods

2.2.1 PS Sample Preparation

Commercial PS culture dishes (Nunclon®, Denmark; Cat#153066), 35 mm in diameter, were used as the material model in this study. The bottom part of each dish was carefully cut free from its surrounding high edge to obtain a circular PS-membrane sample, 14 mm in diameter, using a stainless-blade, mounted on a motor-operated instrument. The sample membranes were carefully handled before, during and after the cutting process to prevent fingerprints and any other surface disrupting dirt. After being cut, these sample membranes were cleaned in ethanol with ultrasonic for 5 min and sterilized in a plasma chamber. They were used in this experiment as re-treated PS (NH_3 -treated PS or N_2 -treated PS) with the assigned plasma gases. In our experiments, the PS-membrane samples were randomly separated into 2 groups;

the first group for the long-term 30-day course of physico-chemical evaluation and the second for the short-term 7-days course, with the WJMSCs interaction studies.

The PS-control material for the cell bioassay was the surface of 24-well-bottom plates, pretreated commercially with oxygen (Nunclon®, Denmark; Cat#142475). The plates used were the originally sterilized products from the producer.

2.2.2 Physico-chemical Properties Study

PS-membrane samples were treated and evaluated by the following techniques;

1) Plasma Treatment

The plasma reactor system used in this section was shown in Figure 2.1 and Figure 2.2. A self-made inductively-coupled discharge plasma system operated at 13.56 MHz (as described by Chaiwong et al. (2010) [6]) was employed to treat the samples. The original pretreated PS-membrane samples were introduced into a chamber, which was evacuated by a rotary pump to a base pressure of 1.6 mTorr (2.1 Pa). The samples were cleaned by Ar plasma sputtering with 100 mTorr and 100 W for 10 min before treatment. Nitrogen-functional groups were deposited onto the PS-membrane surface, using a mixture of noble gas (He and Ar) and nitrogen-containing gas (N₂ and NH₃). A suitable treated condition was found with change 3 parameters, the total pressure of the gas mixture was kept constant at 100 mTorr (13.33 Pa), the RF power was adjusted at 50 and 100 W for 15 min. The ratio of N₂ and NH₃ to He or Ar was fixed at 10% by volume.

2) Evaluation

2.1) Surface Analysis

Deposition surfaces were characterized by Fourier transform infrared (FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS) and water contact angle measurement (as shown in Figure 2.3). We also investigated the short-term effect, over 7 days, on cellular behavior including attachment (adhesion), proliferation rate and stemness of WJMSCs on plasma-treated PS surfaces compared with commercial oxygen-containing pretreated PS culture dishes.

Contact angle was obtained using the sessile drop technique with DI water droplet of 10 μl. A micropipette was used to drop the water on the surface of PS-membrane samples. The image of the water droplet was then captured and exported to an image analyzing software to determine the contact angle. Water contact angle measurement demonstrates the relationship between the properties and chemistry of a surface by wettability. The contact angle (in degrees) is the angle at which a liquid interface meets a solid surface. The greater the angle, the higher is the surface hydrophobicity.

An ATR-unit of a Nicolet 6700 FTIR spectrophotometer (Bruker, Germany) was used to investigate chemical bonding on the sample surfaces. The spectra were collected by averaging 64 scans at a resolution of 4 cm^{-1} from $400\text{-}4,000\text{ cm}^{-1}$.

X-ray photoelectron spectroscopy (XPS) was carried out to determine the quantitative and qualitative elemental surface composition, using an Ultra DLD spectrometer (Kratos Analytical Ltd, UK) with a monochromatized $Al\text{-}K_{\alpha}$ x-ray source ($h\nu = 1,486.6\text{ eV}$). The anode voltage and current were 15 kV and 10 mA .

Survey spectra were collected using a pass energy of 160 eV with 1 eV/step , while region scans were collected with a pass energy of 40 eV , at a rate of 0.1 eV/step . The pressure in the analysis chamber was maintained at $7 \times 10^{-7}\text{ Pa}$. Binding energy was referenced to the $C1s$ neutral carbon peak at 284.6 eV .

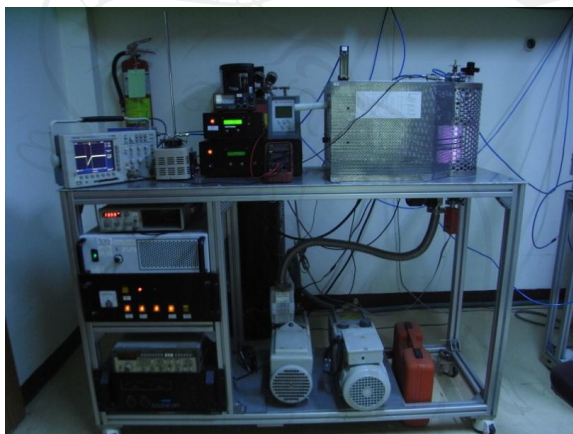


Figure 2.1 A 13.56 MHz ICP plasma reactor.

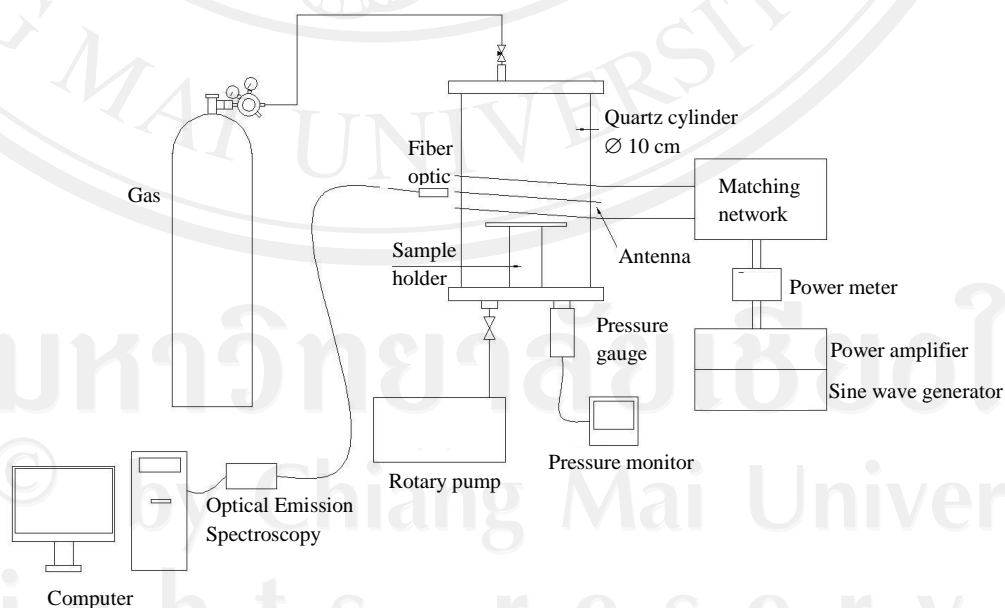


Figure 2.2 Schematic of a 13.56 MHz ICP plasma reactor.



Figure 2.3 Contact angle measurement at PBP laboratory, Physics and materials science Department, CMU.

Table 2.1 N-containing gas plasmas conditions.

The mixed gases of N-containing gas and noble gases plasmas	Total Pressure (<i>mTorr</i>)	RF power (<i>W</i>)	Time (<i>min</i>)
1. 10%N ₂ +Ar	100	50	15
2. 10%N ₂ +Ar	100	100	15
3. 10%N ₂ +He	100	50	15
4. 10%N ₂ +He	100	100	15
5. 10%NH ₃ +Ar	100	50	15
6. 10%NH ₃ +Ar	100	100	15
7. 10%NH ₃ +He	100	50	15
8. 10%NH ₃ +He	100	100	15

2.2) Cell Behavior Study

Two treated PS-membrane types were compared with the PS-control group. The chosen criterion was mainly considered on the highest attachment efficiency of the cells at day 1.

2.2.1) Cell Culture and Seeding

The BCP-K1, a line of WJMSCs, was provided from The Human and Animal Cell Technology Research Laboratory, Department of Biology, Faculty of Science, Chiang Mai University. The cells were cultured on 25 cm² pretreated PS cultured

flasks (Nunc®), Denmark; Cat#156340) in DMEM (Dulbecco's modified Eagle's medium) (Gibco, USA) containing 20% (*v/v*) FBS (fetal bovine serum) (Gibco, USA), 10 *ng/ml* bFGF (basic fibroblast growth factor) (Sigma, USA) and 5 *mg/ml* bovine insulin (Sigma, USA). Cultures were maintained at 37°C, 5% CO₂ and 95% relative humidity. The culture medium was refreshed every 2 days before achieving 80% cell-population confluence. On detaching from the cultured flasks, they were exposed to 0.05% (*v/v*) trypsin-EDTA (Gibco, USA) for 10 *min*. The cells were collected and washed with phosphate buffer saline (PBS) then centrifuged and resuspended in the medium prior to cell seeding. The cells were counted and the cell density adjusted to 30,000 *cells/ml*. Then the cells were seeded onto each PS-membrane sample, which was previously settled on the bottom of each well of a 24-well PS culture plate. The diameter size of a PS-membrane sample was actually made to match and set onto the diameter of a well on this 24-well culture plate. Cell attachment and proliferation were evaluated 1, 3, 5 and 7 days after seeding. The experiment was performed in 5 replicates of the same type of PS-membrane sample. Similar cell density, culture conditions, replications and evaluated periods were carried out on the PS-control 24-well-bottom plates.

2.2.2) Cell Attachment Efficiency and Proliferation Assay

Attachment efficiency and proliferation assay of BCP-K1 were quantitatively evaluated by using the Vybrant® MTT Cell Proliferation Assay Kit (Molecular Probe, Netherlands). The PS-membrane samples containing attached cells, were transferred to a new 24-well plate, filled with 500 μ l fresh medium in each well. This was to avoid false evaluation on the cells over flown from PS-membrane samples. The assay for cells on PS-control groups was performed in the initial 24-wells culture plate (no transfer). According to the manufacture's instruction, 50 μ l MTT-solution was added to each well (final concentration 0.5 *mg/ml*) and incubated at 37°C for 4 *h*. Thereafter, 500 μ l of the solubilization mixture (1 *g* SDS: 0.1 M HCl) was added into each well to dissolve formazan crystals, produced by active cells, and left overnight at 37°C. The colorimetric optical density (OD) of liquid in each well was obtained by spectrophotometry at 570 *nm* using Spectra™ MR Microplate Spectrophotometer (Dynex Technologies, USA). The proliferation assay was analyzed using the OD570 value from each PS membrane sample at various culture periods in comparison to the control at 1 day, which was presented as percentage of attached and proliferated cells. A higher percentage of cell attachment and proliferation corresponds to a higher number of viable cells.

2.2.3) FAK-ELISA

FAK (focal adhesion kinase) was assessed using the enzyme-linked immunosorbent assay (ELISA) method to measure an anchorage-dependent protein in cells, which adhered on each PS-membrane sample. After seeding cells onto PS-

membrane samples, the FAK-ELISA was performed at every time period, at 1, 3, 5 and 7 days, as mentioned above. Each sample was washed with PBS for 2-3 times prior to fixation by 4% (*v/v*) formaldehyde for 20 *min* at room temperature. The sample was washed twice thereafter with washing buffer (0.1% (*v/v*) Triton X-100 in PBS) then incubated with the quenching buffer (wash buffer containing 1% (*v/v*) H₂O₂ and 0.1% (*v/v*) Azide) for 30 *min* and washed with washing buffer. Blocking buffer (5% (*v/v*) BSA (bovine serum albumin, Invitrogen, USA) was added for 1 *h* before adding anti-FAK primary antibody (1:2,000) (Sigma, USA) and incubated at 4 °C overnight. The HRP (horseradish peroxidase)-labeled secondary antibody (1:20,000) was added in each sample, incubated for 2 *h*. The SIGMAFAST™ OPD (o-phenylenediaminedihydrochloride) (Sigma, USA), a substrate for HRP, was added to the samples then incubated for 30 *min* in the dark to allow color development. The samples were then measured at OD_{492 nm}, using a microplate spectrophotometer as described previously.

2.2.4) Immunocytochemical Analysis

Immunocytochemistry was carried out on the 3 groups of BCP-K1 growing on PS surfaces as mention in the short-term cell behavior study. Cells were examined at the initial stage (day 1) and when the experiment finished (day 7). The method was adapted from Brohlin et al. (2009) [7]. In brief, cells on a PS-membrane surface were rinsed with 1 M PBS (*pH* 7.4), followed by fixation with 4% formaldehyde in PBS for 15 *min*. The cells were then washed 3 times with PBS and permeabilized with 0.1% Triton X-100 in PBS in 37°C for 30 *min* followed by 10 *min* at room temperature. The cells were gently washed 3 times with PBS at room temperature and blocked in 10% Bovine Serum Albumin (BSA; Invitrogen, USA) for 1 *h* at room temperature in humidified container. Cells were then incubated overnight at 4°C with mouse monoclonal antibody for human CD105 (1:2,000), human CD34 (1:2,000), SSEA-4 (1:2,000) (all from Molecular Probes, USA) and human CD9 (1:500) (US biological, USA). After rinsing in PBS for 3 times, secondary goat anti-mouse antibodies Alexa Fluor® 488 goat anti-mouse IgG1 (1:200), Alexa Fluor® 594 goat-anti mouse IgG3(γ3) (1:200) and Alexa Fluor® 546 goat anti-mouse IgG2b(γ2b) (1:200) (all from Molecular Probes, USA), were added and incubated for 1 *h* at room temperature in the dark. Cells were washed a further three times in PBS and incubated in 300 nM 4',6-diamidino-2-phenylindole (DAPI) and mounted with ProLong® Gold anti-fade reagent (both from Molecular Probes, USA). The mounted and stained cells were allowed to cure for 24 *h* at room temperature in the dark before photomicrography under an epifluorescent microscope (Olympus DP-50, Japan).

2.2.5) Cell Morphology

Cell morphology was observed under a light microscope equipped with digital photomicrography devices. A scanning electron microscope (SEM) (JSM 633S Jeol,

Japan) was also used to observe the fine details of the attachment mechanism of cells on the surfaces.

Data Analysis

All data were presented as mean \pm SD. Analysis of differences in the means between different PS membrane samples and controls were performed by the One-Way ANOVA procedure, followed by multiple comparisons using Duncan adjustment at $p \leq 0.01$.

2.3 Results and Discussion

2.3.1 Surface Wettability and Aging Effect of Plasma Treated-PS

Table 2.2 Water contact angle of PS-control and plasma treated PS-membrane.

Nitrogen-containing plasma	Contact angle ($^{\circ}$) \pm SD
Control PS	82.8 \pm 1.4
10% N ₂ +Ar, 50 W	20.1 \pm 5.8
10% N ₂ +Ar, 100 W	17.5 \pm 3.6
10% N ₂ +He, 50 W	14.3 \pm 2.6
10% N ₂ +He, 100 W	13.4 \pm 5.0
10% NH ₃ +Ar, 50 W	17.3 \pm 2.0
10% NH ₃ +Ar, 100 W	11.1 \pm 1.5
10% NH ₃ +He, 50 W	13.4 \pm 3.5
10% NH ₃ +He, 100 W	14.6 \pm 4.1

The surface hydrophilicity of treated PS-membrane was significantly improved in both N₂ and NH₃ plasma treatments (Table 2.2). Treated samples had a considerably lower contacted angle of 20 $^{\circ}$ (compared with 82.8 $^{\circ}$ for the PS-control), indicating good spreading of water on the material surface and low hydrophobicity of the material surface. The nitrogen-containing plasma treated PS-membranes were more hydrophilic, resulting in more polar groups ($-NH_2$ and oxidized structures e.g. carbonyl, carboxyl and ester groups) being grafted onto the surface during the plasma treatment [1, 8]. Nonpolar molecules are exhibited by London forces because of the correlated movements of the electrons in interacting molecules. These forces reveal weak intermolecular forces. If the material is too hydrophobic, molecules of extracellular matrix (ECM) are absorbed in a denatured and rigid state. This geometrical appearance is unsuitable for binding to cells, since specific sites on these molecules are less accessible to cell adhesion receptors, e.g. integrin. The polar component of surface energy consists of all other interactions due to non-London forces. Polar molecules interact through dipole-dipole intermolecular forces and hydrogen bonds. On hydrophilic surfaces, cells adhered in higher numbers to more

hydrophilic materials and were spread over a large area. At the same time, ECM proteins were adsorbed in a more flexible form, which allows them to be rearranged by the cells and thus provides access for cell adhesion receptors to the adhesion motifs on these molecules. The incorporated amine groups are capable of efficient interaction with ECM proteins by hydrogen bonding, resulting in covalent attachment between the positively charged groups ($-NH_2$) and the cells that carry a negative charge [8, 9].

However, in many cases the amine surfaces had a rather limited shelf life after plasma oxidation reactions. When the plasma-treated surface was preserved at elevated temperature or storage in air, the contact angle increased greatly. The general conclusion is that migration of treated-chains surface into deeper layers or migration of hydrophobic non-treated chains to upper surface layers, hydrophobic regions can be generated at the surface during the aging process [10].

The stability of a plasma-treated polymer surface is an important issue for biomedical use. Structural elements (chains and chain segments) can move between polymer surfaces and deeper layers (bulk) by rotational and translational motions. The surface composition can change to minimize the interfacial energy between the polymer and its environment. Figure 2.4 shows decay of wettability over 30 days after N_2 and NH_3 plasma treatments. The contact angle of treated-PS surfaces with higher RF power (100 W) were lower than that of 50 W RF power treated-surfaces. Moreover, the decay of 100 W RF power treated surfaces was less than of 50 W RF power treated-surfaces. It can conclude that the optimum RF power for mixed gases of He and N_2 or NH_3 was 100 W.

In Figure 2.4 a), the decay of N_2 +He plasma treated surface was lower than that of N_2 +Ar plasma treated surface. Similarly, in Figure 2.4 b) the decay of NH_3 +He plasma treated surface was also lower than that of NH_3 +Ar plasma treated surface. Therefore it is suggested that He gas plasma can be used to create stable covalent binding sites for the attachment of amine functional groups over a long time. Inert gas plasma treatments introduce no new detectable chemical species onto the polymer surface, but they can induce degradation and rearrangement of the polymer surface. During inert gas plasma exposure, the created functional sites produces dangling bonds (by removal of H atoms and breaking chemical bonds in polymer chains), which may recombine to cross-link neighboring polymer chains [3, 11]. He plasma is well known to efficiently produce cross-linking reactions [5, 12]. We expected that the formation of a cross linked polymer surfaces, preventing reptation of the modified polymer chains into the bulk, could be the reason for the improved stability observed for the mixed He gas-treated plasma. Ar pre-treatment, before N-containing functionalized, breaks bonds in the polymer chains to create functional sites by extracting hydrogen from $C-H$ bonds in polymers. The functional sites subsequently couple with free radical sites, with active species from the N-containing plasma phase, to form N-containing functional groups [3, 11, 13]. Reactive gas plasma (e.g., NH_3 ,

N_2 , O_2 , CF_4 and SF_6) treatments create new chemical species, which bring about chemical reactivity of the polymer surface [14]. However, the most important surface population of chemical species, formed after plasma treatment, is dependent on both the chemical structure of the polymer and the plasma gas.

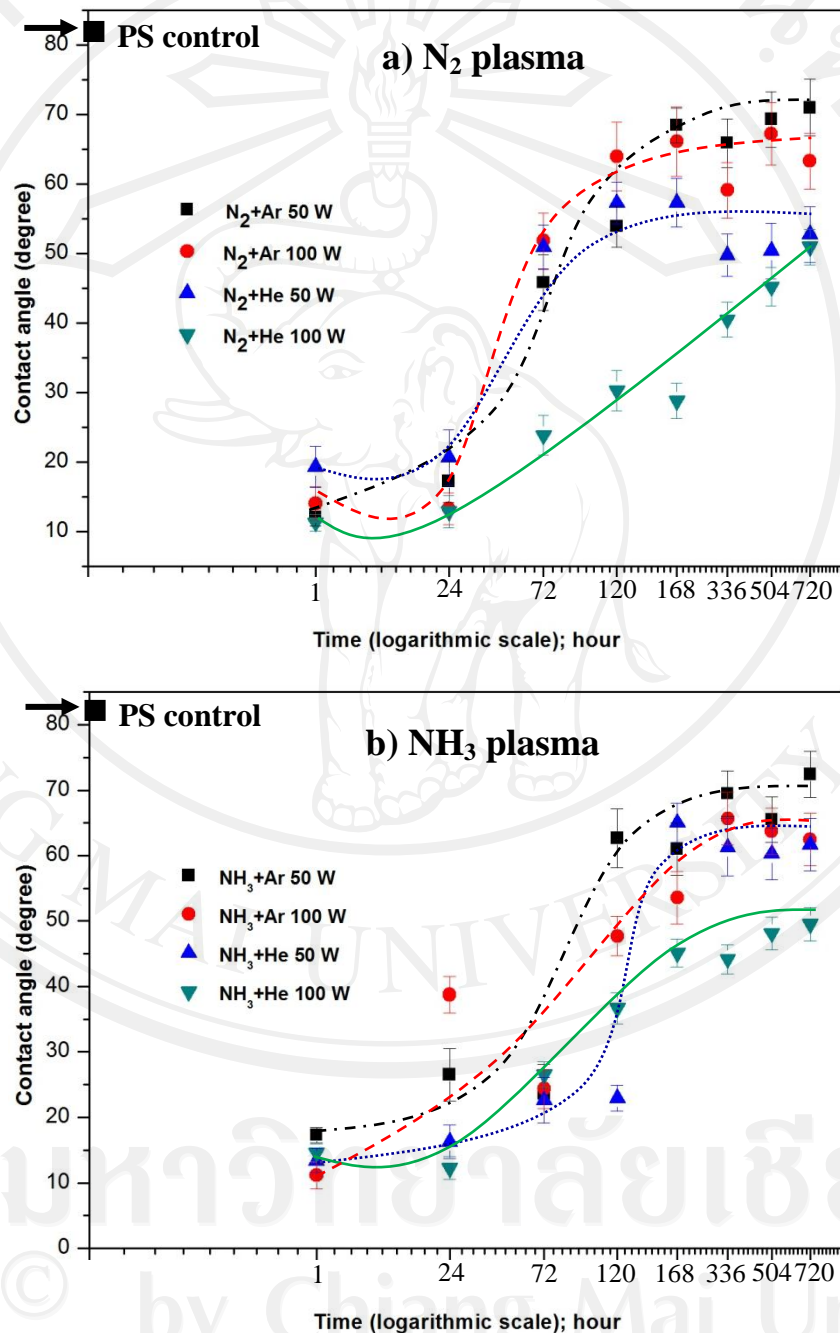


Figure 2.4 Decay of wettability of PS surfaces along 30 days after N_2 - (a) and NH_3 -plasma treatment (b)

2.3.2 Surface Characterization

For FTIR analysis, the plasma-treated PS membranes have been characterized by ATR-FTIR. The absorption spectra of the plasma polymerization amine-containing functional layers in nitrogen-containing plasma are shown in Figure 2.5. The presence of amine functional groups on NH_3+He and N_2+He plasma treated PS was indicated by the peak of the primary amine ($-\text{NH}_2$) stretching absorption at the $3,440\text{ cm}^{-1}$ region and the CN stretching absorption at the $1,267\text{ cm}^{-1}$ region. The aromatic ring of PS structure is indicated by the peak of the absorption at the $1,598\text{ cm}^{-1}$ region [8]. The following FTIR spectrographic facts were used to explain the weak peak of N-H and C-N in Figure 2.5. These peaks are only a guide to the chemical composition of the plasma-modified surface. The detected signals were always lower than the actual amount, because the layers of N-H and C-N formed were very thin [10]. However, in combination with the XPS result, it can be clearly seen that up to 9% of functional groups were replaced on the PS surface. On the other hand, amine signals on the treated PS surface were from the gas phase treatment. No residual gas without chemical reaction was left on the surface after that. Anyway, if there should be some noncovalently- bound amine on the surface, they would have been taken away by the rigorously ethanol and ultrasonic cleaning, which was carried out after the plasma treatment.

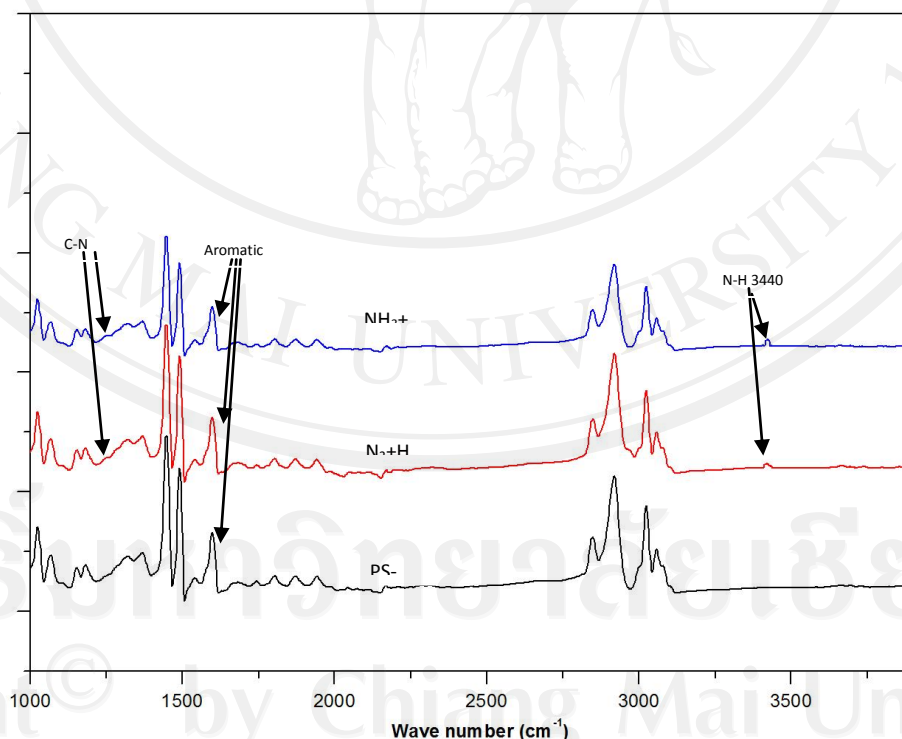


Figure 2.5 FTIR spectrum of the plasma treatment amine-containing functional groups with PS-control, N_2+He and NH_3+He treated PS-membrane.

On the XPS analysis, we considered and discussed our results here in relation to several previous studies [15, 16]. XPS characterized the treated surfaces to obtain the information on chemical structure. Figure 2.6 shows the survey scan spectra of PS-control, N_2+He treated PS-membrane and NH_3+He treated PS-membrane. The survey spectra confirmed the presence of $C1s$ and $O1s$ on PS-control, demonstrating the oxygen-containing pretreatment onto the PS surface and the presence of $C1s$, $O1s$ and $N1s$ of treated PS-membranes. Figure 2.7 shows high-resolution $C1s$ (Figure 2.7a) spectra of PS-control, $C1s$ (Figure 2.7b) of N_2 -treated PS-membrane, $C1s$ (Figure 2.7c) of NH_3 -treated PS-membrane, $N1s$ (Figure 2.7d) of N_2 -treated PS-membrane and $N1s$ (Figure 2.7e) of NH_3 -treated PS-membrane. The $C1s$ peak of PS-control was deconvoluted into five peaks with binding energies of 284.6, 285.2, 285.9, 286.7 and 291.3 eV, which were attributed to carbons in $C-C$ or $C-H$, $C=C$, $C-H_2$, $C-O$ or $C-OH$ and $\pi-\pi^*$, respectively (Figure 2.7a) (Yasushi et al. 2008 [17]). The $\pi-\pi^*$ $C1s$ peak is due to “shake-up” excitations taking place in the π orbitals on the benzene rings. NH_3 -treated PS membrane produced two new peaks with binding energies of 287.7 and 288.6 eV, which were attributed to $C=O$ and $N-C=O$ (amide) groups, respectively (Fig. 2.7b). In addition, two peaks were identified of $N1s$ spectra shown in Fig. 2.7e. The peak at 398.5 eV was assigned to $N=C$ or NH_2 bonds and peak at 400.5 eV was assigned to $NHC=O$, NH_2 or $N-H$ of NH_3^+ [3]. N_2 -treated PS-membrane produced two peaks with binding energies of 287.7 and 288.6 eV same as NH_3 -treated PS-membrane but $N1s$ spectra was deconvoluted into one peak with binding energies of 400.3 eV.

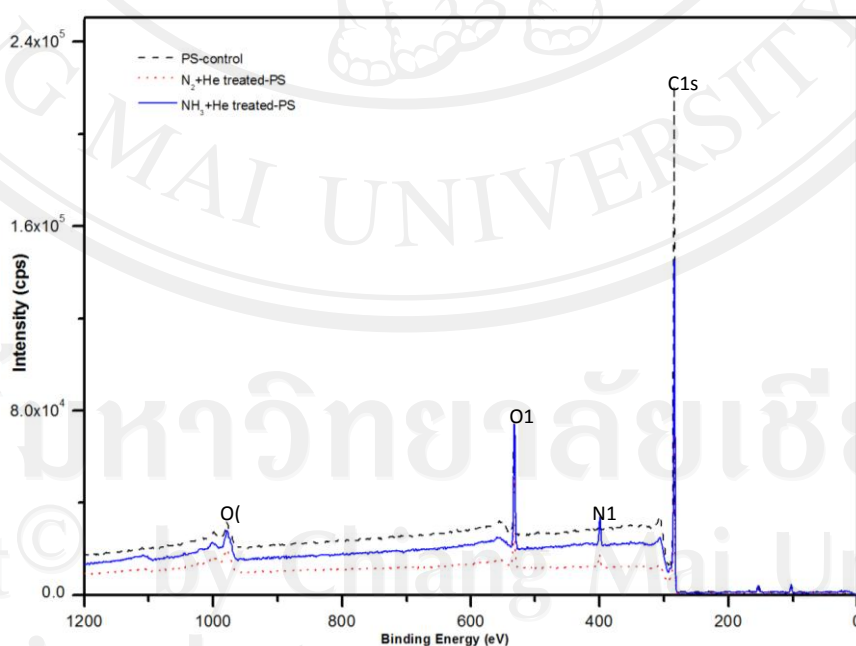


Figure 2.6 XPS scan spectra of PS-control, N_2+He treated-PS and NH_3+He treated-PS.

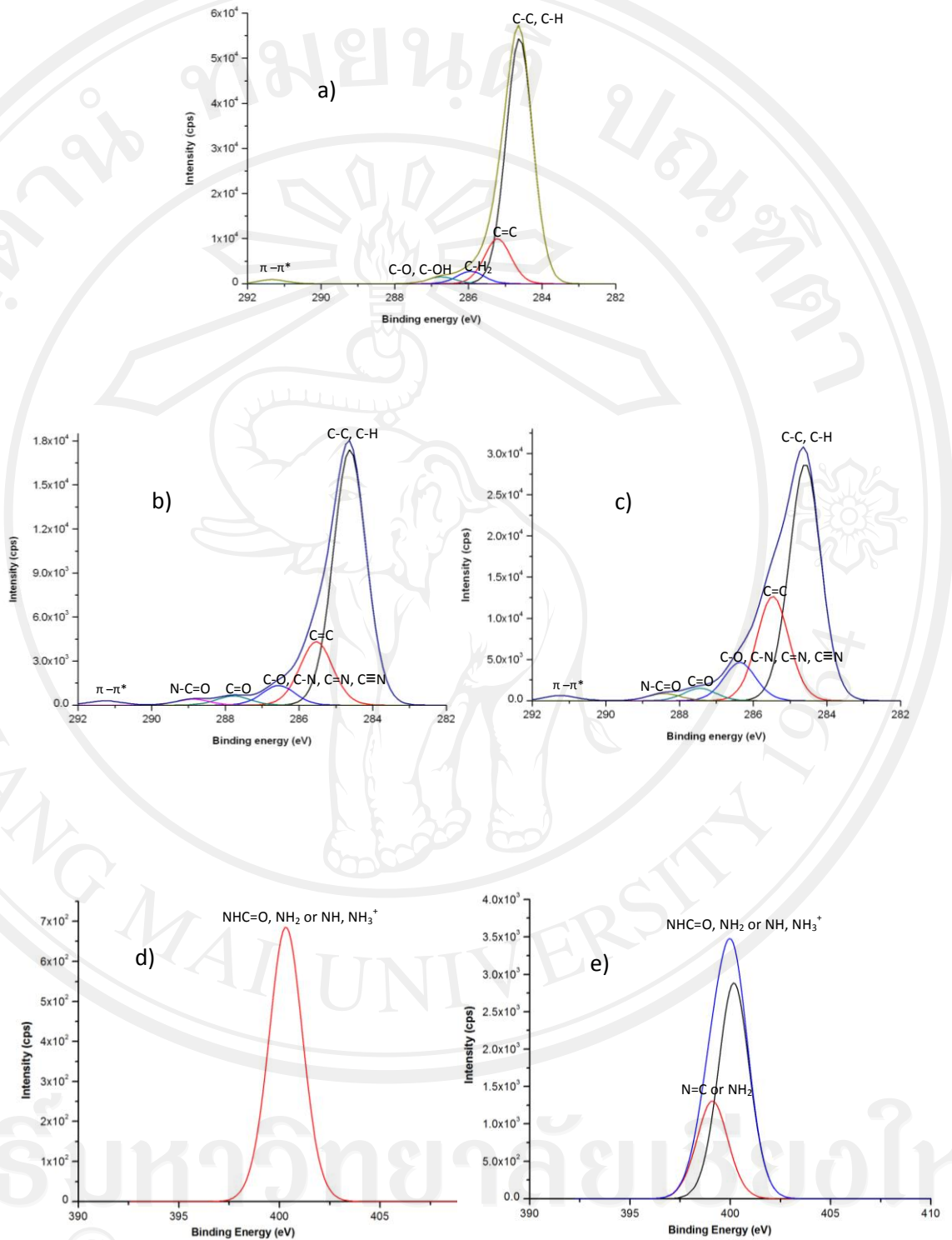


Figure 2.7 High resolution peaks of $C1s$ for PS control (a), N_2 +He treated PS-membrane (b), NH_3 +He treated PS-membrane (c) and $N1s$ for N_2 +He treated PS-membrane (d) and NH_3 +He treated PS-membrane (20x of d) (e).

Table 2.3 summarizes the results of a deconvolution analysis of *C1s* peaks, shown in Figure 2.7. The NH₃ plasma and N₂ plasma were successfully N-functionalized on PS surface and a larger amount of oxygen functional groups and nitrogen functional groups were introduced by NH₃ plasma than by N₂ plasma, because the *C1s* peak at 285.0-286.9 eV assigned to C-O, C-N, C=N and C≡N bonds were significantly increased when observed in the NH₃ plasma-treated PS-membrane, indicating the formation of carbonyl, amide and amine functional group on membrane surface, especially with NH₃ plasma.

Table 2.3 Estimated percentage of carbon functional groups from *C1s* XPS spectra.

PS sample	Binding energy (eV)	assignments	Peak area (%)
PS control	284.6	C-C, C-H	78.2
	285.2	C=C	14.4
	285.9	-CH ₂ -	4.0
	286.7	C-O, C-OH	2.2
	291.3	π-π*	1.2
NH ₃ -treated PS	284.6	C-C, C-H	78.3
	285.6	C=C	11.4
	286.5	C-O, C-N, C=N, C≡N	4.5
	287.7	C=O	2.7
	288.6	N-C=O amide	1.6
	291.3	π-π*	1.2
N ₂ -treated PS	284.6	C-C, C-H	79.6
	285.6	C=C	10.3
	286.5	C-O, C-N, C=N, C≡N	4.4
	287.6	C=O	2.4
	288.7	N-C=O amide	2.2
	291.1	π-π*	1.1

2.3.3 Cell Behavior

Cell behavior experiments were performed over 7 days using BCP-K1. Figure 2.8 shows the percentage of attached cells on the PS-membrane samples when cultured for 1 day. Compared with cells from the PS-control group, almost all groups of cells on the treated PS-membrane expressed significantly more adhesion, especially the mixed-He groups. Less adhesion was observed on the two groups of mixed-Ar at 50 W ($p \leq 0.01$). In addition, BCP-K1 tended to proliferate and spread over surfaces of PS-membranes at the 50 W N₂+He, 100 W NH₃+Ar and 100 W NH₃+He when cultured for 3, 5 and 7 days as shown in Figure 2.9. The relative percentage of cell proliferation on the PS-control exhibited only 80 on day 7 compared with day 1. In

contrast, cell proliferation on the treated PS-membrane samples increased about 150 on day 7 compared with those on day 1. The plasma treatments of 50 W N₂+He and 100 W NH₃+He were therefore chosen for further studies, since they were the best two treatments on PS surfaces, in which provoked cell adhesion and proliferation efficiency. Note that measurement of water contact angle (Table 2.2) was carried out immediately after surface preparation, under the dry surface conditions. Submerging the surface into the culture medium over the 7 day experiment (Figure 2.9) caused the “hydrophobic recovery” and also a “decay effect” (Figure 2.4) and eventually resulted in the cell proliferation. Furthermore, BCP-K1 showed very good attachment on NH₃+He 50 or 100 W surface at day 1 (Figure 2.8), but showed less ability to proliferate by day 7 (Figure 2.9). It was possible that the culture medium and culture conditions over the 7 days of cell culture caused leakage of functional groups, due to “hydrophobic recovery” and also the “decay effect”. Ar and He are precursor gases to assist and retain plasma condition [19]. Neither of them alone generated amines on the treated surfaces [19] and they were therefore not evaluated for cell behavior in this experiment.

Cell adhesion and proliferation was observed by light microscope and photomicrograph taken in the serial time courses as shown in Figure 2.10. The photomicrographs clearly displayed more cells on the treated PS membrane than on the PS-control, indicating more effective cell adhesion and enhanced proliferation on the treated PS-membrane than the commercialize PS-controls. Furthermore, cells attached to all treated PS membrane surfaces exhibited similar normal fibroblast-like morphology to those on the PS-controls.

Cellular behavior on any biomaterial is an important indication to determine its biocompatibility. The whole process of adhesion and spreading of cells after contact with biomaterials consists of cell attachment, filopodia growth, cytoplasmic webbing and flattening cell mass, and the ruffling peripheral cytoplasm, which progress in a sequential fashion [20]. As seen in Figure 2.11, the typical SEM images along the time courses showed different attachment of cells on different PS surfaces. Cells on the treated PS-membrane surfaces adhered and spread more than on the PS-control surfaces. Incomplete attachment of BCP-K1, with ragged cytoplasmic boundary, was often observed on the PS-control surfaces at day 1.

FAK or focal adhesion kinase involved multi-protein complex formation, which links ECM to the actin cytoskeleton and related signaling proteins. FAK is also involved in the regulation of cellular migration, proliferation, survival and invasion in some cell types [21]. Examination of FAK indicates cellular adhesion, proliferation and spreading on the PS surfaces. Figure 2.12 shows the amount of FAK protein as the OD492 value, i.e. the higher the OD value, the greater is the amount of FAK protein secreted from the cells. Considering the two patterns of FAK amount exhibited among the PS surfaces. Firstly, at day 1 and 7, the significantly highest FAK was observed on the cells adhered to the 50 W N₂+He-treated PS-membrane,

while the cells on the 100 W NH₃+He-treated PS-membrane secreted similar amounts of FAK as the PS-control. This display might imply that the N₂+He-treated PS-membrane enhanced more cellular activities related to FAK secretion than the other surfaces. Apparently, such cellular activities on day 1 and 7 were not activated during day 3 and 5, when significant similarities appeared among the three types of surfaces. It is possible that the FAK might be activated via different pathways and play different roles [22, 23]. Secondly, on day 3 and 5 was significantly less than on days 1 and 7, indicating the similar pattern of cellular activities on the PS surfaces. This FAK pattern (Figure 2.12) supported the explanation of related proliferation behaviors of the cell along the time courses (Figure 2.9). Considering on day 1, the cells needed FAK for initial attachment and that raised the total amount of FAK. On day 3 to 5, the cells virtually expressed a lag period of intracellular growth adjustment, almost without proliferation; hence the lower amount of FAK. After that, on day 7, the cells regained their activity entering the exponential growth phase. Actually, this is the explanation of the FAK amount related to the pattern of MSC growth, in which similar among other cell types in the in vitro condition [24, 25].

From immunocytochemical analysis, the stemness markers were performed to monitor the stability of BCP-K1 on the three types of PS surfaces used. Cells on all PS surfaces were positive for mesenchymal stem cell marker; CD105, undifferentiated WJMSC markers, CD9, and SSEA-4 and negative for hematopoietic stem cell marker; CD34. This result agrees with [26], who report expression of the major characteristic criterion of human mesenchymal stem cell (hMSC). The samples of fluorescent photomicrography from this experiment are shown in Figure 2.13.

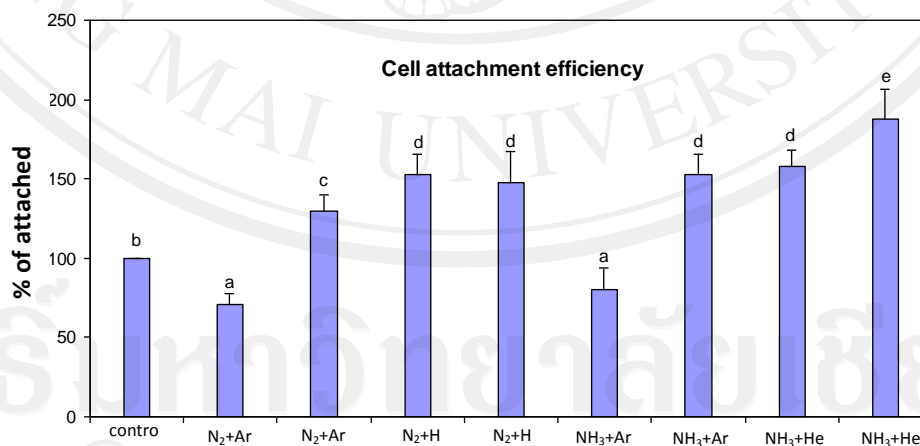


Figure 2.8 Attachment efficiency of BCP-K1 on PS surfaces when cultured for 1 day.

The data was calculated and converted to percentage (mean±SD).

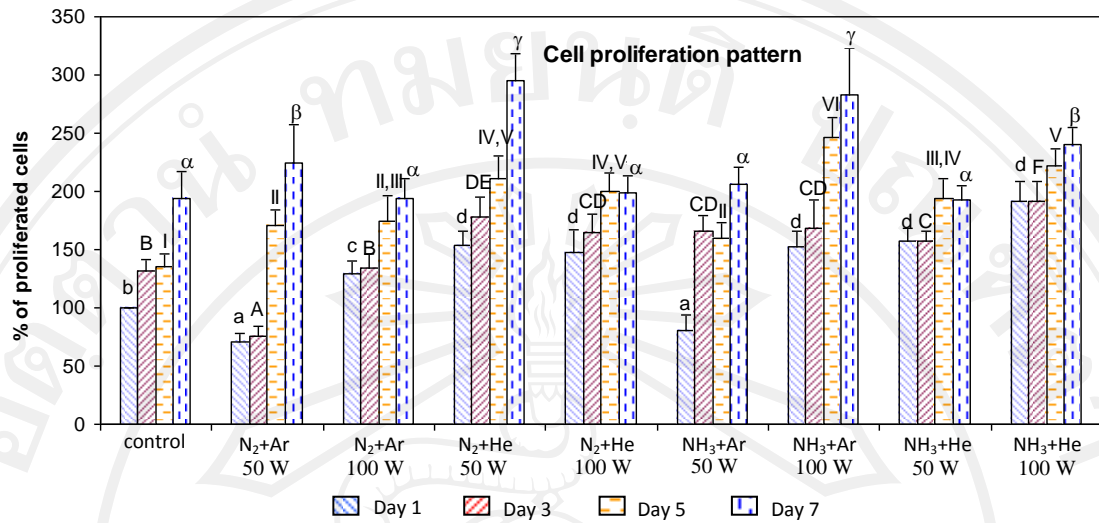
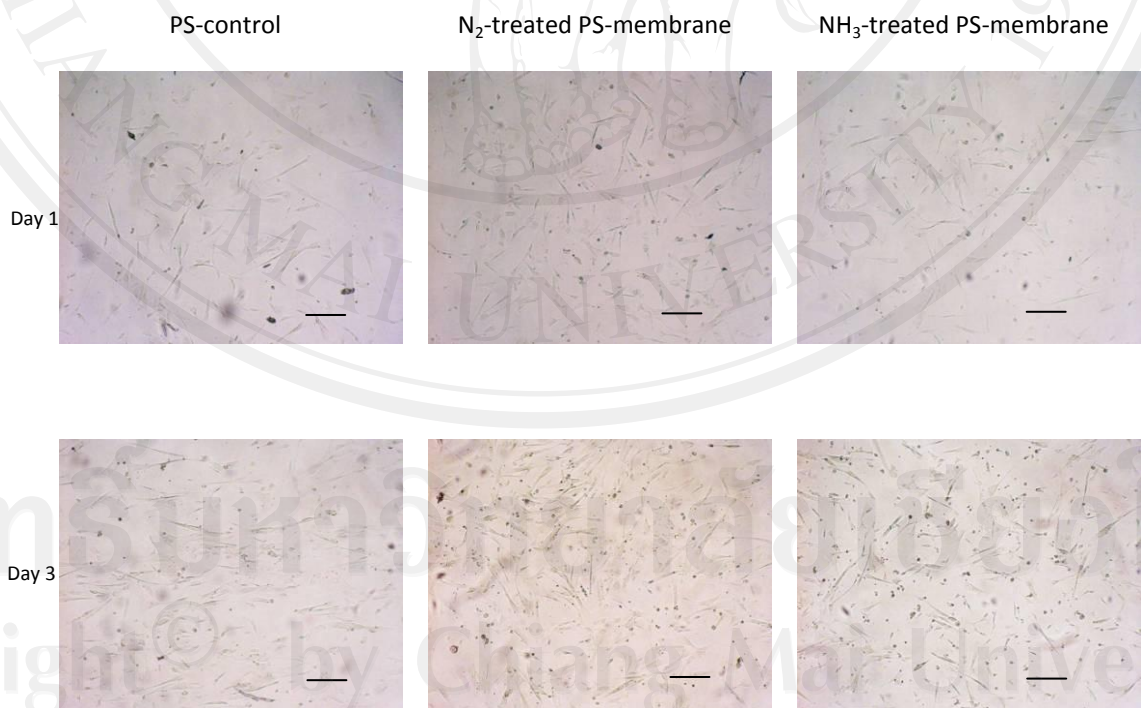


Figure 2.9 Cell proliferation pattern of BCP-K1 on PS surfaces when cultured for 1, 3, 5 and 7 days. The letters on the bar top assume the same result within 5% ($p \leq 0.05$).

(a, b, c, and d are in 1 day, A, B, C, D, E, and F are in 3 days, I, II, III, IV, and V are in 5 days, and α , β , and γ are in 7 days of culturing).



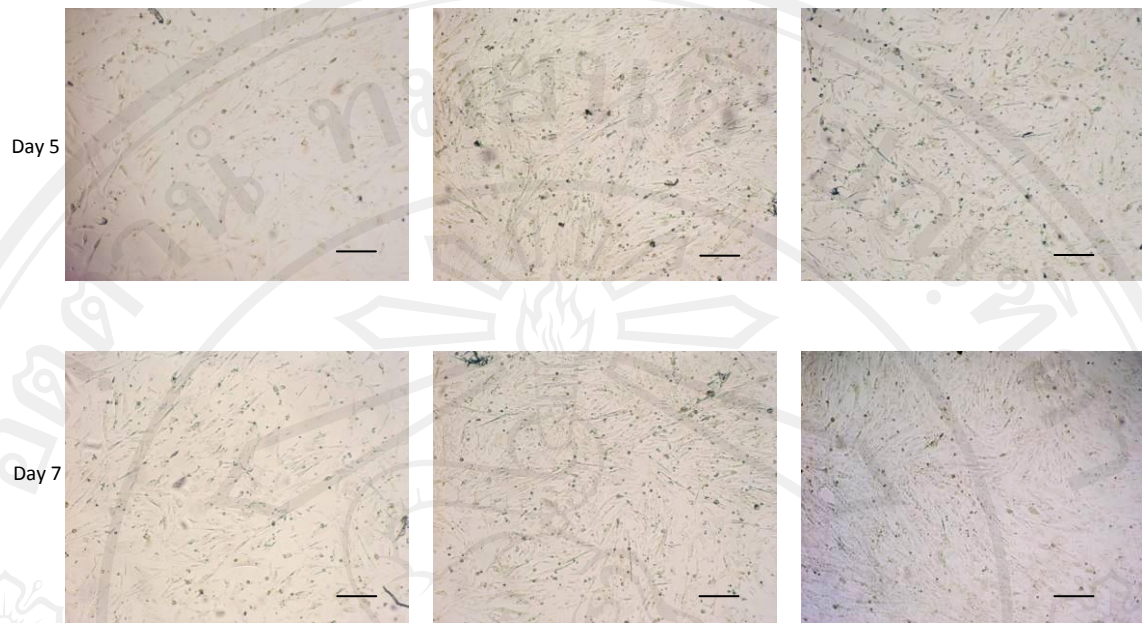
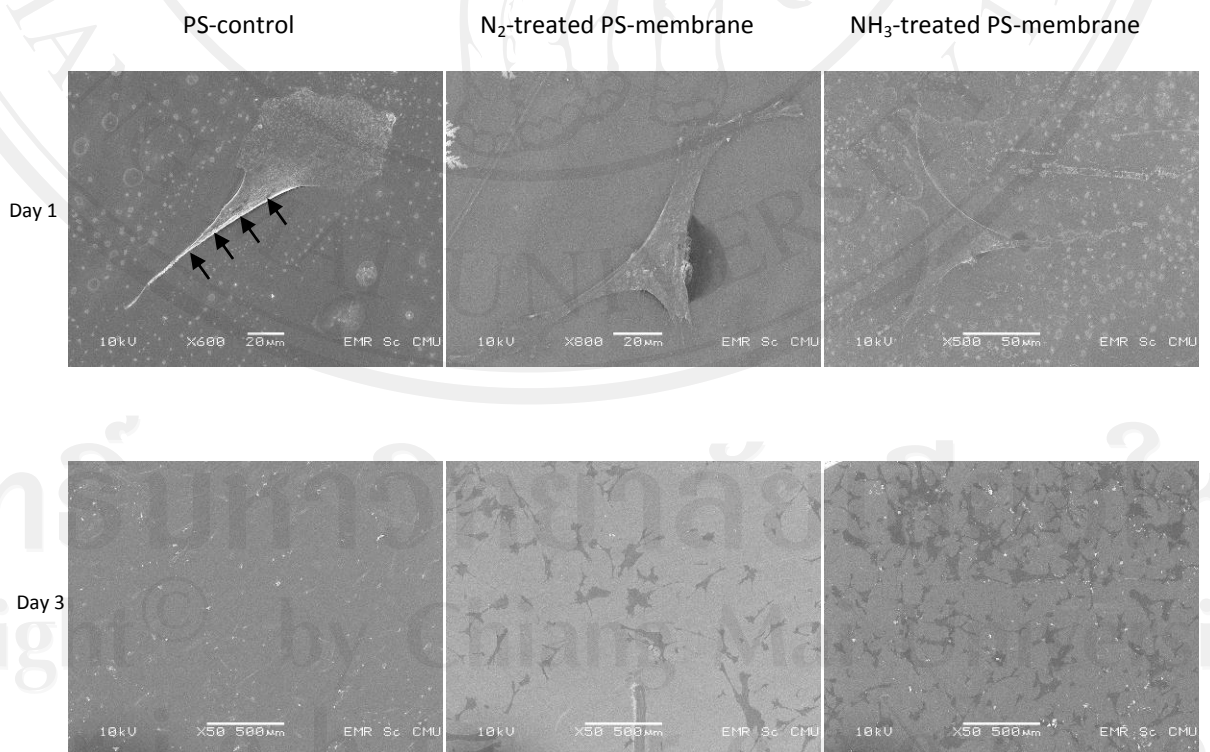


Figure 2.10 Optical micrographs of attachment and proliferation of BCP-K1 when cultured on PS-control, N₂+He treated PS-membrane and NH₃+He treated PS-membrane for 1, 3, 5 and 7 days. (Scale bar = 20 μ m).



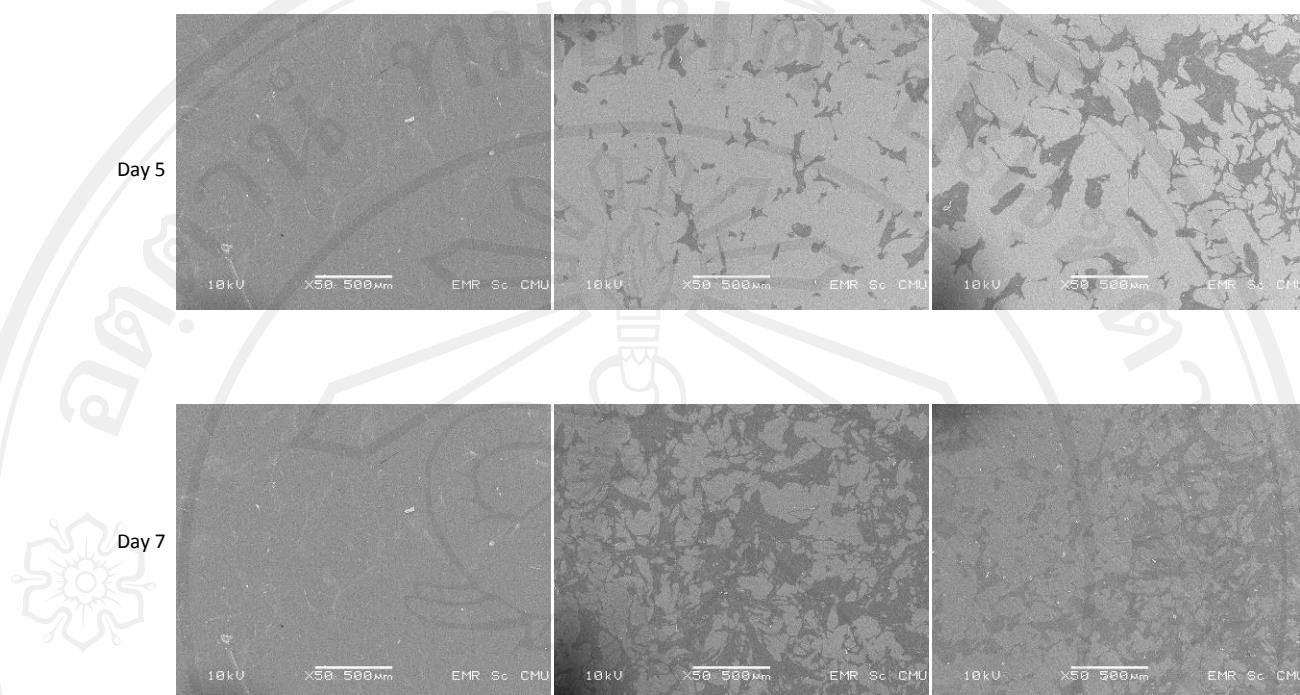


Figure 2.11 SEM micrographs of attachment of BCP-K1 when cultured on PS-control, N_2+He treated PS-membrane and NH_3+He treated PS-membrane for 24 h. PS-control, ragged boundary (arrows) was often seen among cells, indicating the incomplete attachment.

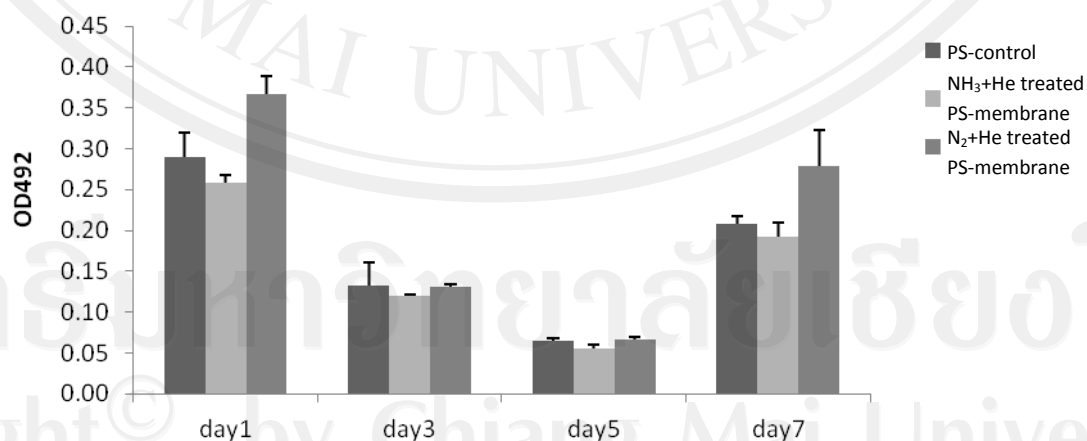


Figure 2.12 FAK-ELISA of BCP-K1 culturing on different PS samples for 1, 3, 5 and 7 days (mean \pm SD).

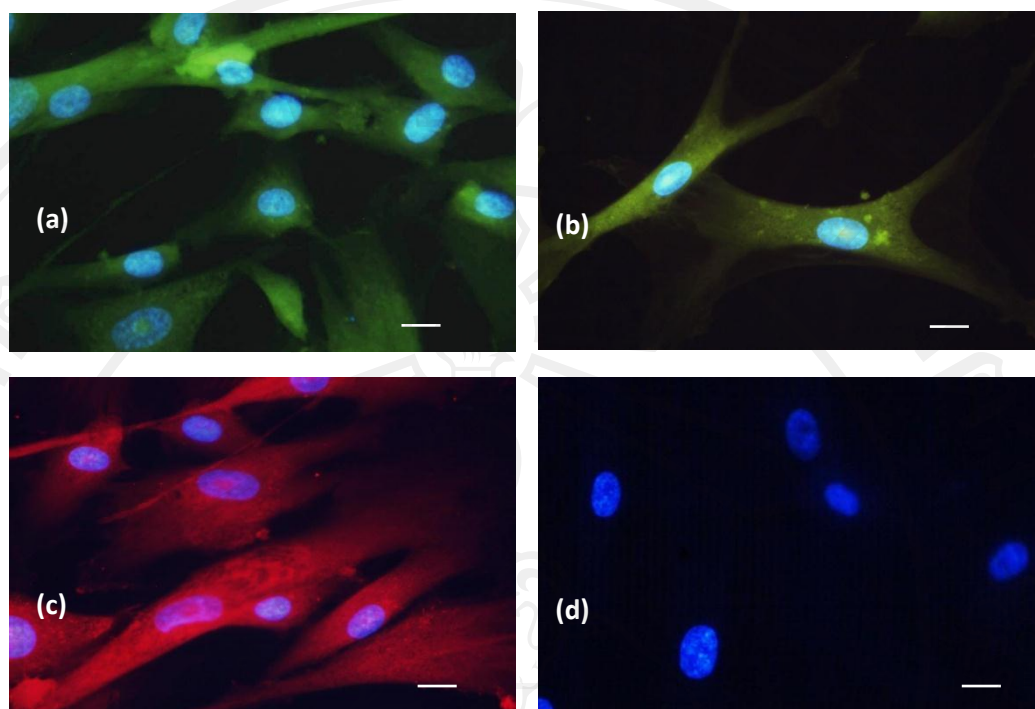


Figure 2.13 Immunocytochemical analysis of BCP-K1 for (a) the mesenchymal stem cell markers, CD105, (b) undifferentiated Wharton's jelly stem cell marker, CD9, and (c) SSEA-4. Negative result (d) for hematopoietic stem cell marker, CD34, showed blue nuclei from DAPI. (Alexa Fluor®488: green, Alexa Fluor®546: yellow, Alexa Fluor®594: red, DAPI: blue) (scale bar = 100 μ m).

2.4 Conclusions

In this study, the surface properties of PS-membrane samples were successfully modified with nitrogen- and ammonia-containing plasma, in which the amine functional groups deposited onto the PS surface. Stability of amine functional groups on the PS surface was achieved by using a N_2+He or NH_3+He gas mixture. The plasma created stable covalent binding sites for attachment of amine functional groups. Cellular adhesion and proliferation were significantly improved and also stemness stability. Results could be explained by the changes in wettability and surface functionalization and the creation of carbonyl, amide and amine functional groups on the PS surface. The stability of a plasma-treated surface was found via crosslink polymer using N-containing gas mixed with He gas. The present findings should be used as a guideline for surface modification of PS culture vessels for biomedical applications.

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