การปรับปรุงเจลาตินที่เชื่อมขวางแล้วโดยการปรับพื้นผิวด้วยพลาสมาเพื่อการทดสอบกับเซลล์ในระดับห้องปฏิบัติการ

นาย อิศราวุธ ประเสริฐสังข์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรดุษฎีบัณฑิต
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IMPROVEMENT OF CROSSLINKED GELATIN FOR IN VITRO CELL CULTURE BY PLASMA SURFACE TREATMENT

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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อิศราวุธ ประเสริฐสังข์ : การปรับปรุงเจลาตินที่เชื่อมขวางแล้วโดยการปรับพื้นผิวด้วยพลาสมาเพื่อการทดสอบกับเซลล์ในระดับห้องปฏิบัติการ (IMPROVEMENT OF CROSSLINKED GELATIN FOR IN VITRO CELL CULTURE BY PLASMA SURFACE TREATMENT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. ศิริพร ดำรงค์ศักดิ์กุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.ดร. รัฐชาติ มงคลนาวิน, Professor Chiow San Wong, Ph.D., 101 หน้า.

งานวิจัยนี้มุ่งเน้นการศึกษาผลกระทบของพลาสมาที่มีต่อสมบัติทางกายภาพและชีวภาพของเจลาตินที่ผ่านการเชื่อมขวางแล้ว ในส่วนแรกของการศึกษา ระบบพลาสมาไนโตรเจน ออกซิเจน และอากาศกระแสสลับความถี่ 50เฮิร์ตได้ถูกนำมาใช้ในการดัดแปรพื้นผิวของเจลาตินชนิด เอ ที่ผ่านการเชื่อมขวางด้วยกระบวนการทางความร้อน จากผลการทดลองพบว่าค่ามุมสัมผัสกับน้ำของฟิล์มเจลาตินมีค่าลดลงเมื่อเพิ่มเวลาในการดัดแปรพื้นผิวด้วยพลาสมา การดัดแปรพื้นผิวด้วยพลาสมาไนโตรเจน ออกซิเจน และอากาศเป็นเวลา 30 วินาทีไม่ส่งผลกระทบต่อความขรุขระของฟิล์มเจลาติน หมู่ฟังก์ชันที่มีไนโตรเจนเป็นองค์ประกอบที่ถูกสร้างขึ้นโดยพลาสมาไนโตรเจนและอากาศ และหมู่ฟังก์ชั่นที่มีออกซิเจนเป็นองค์ประกอบที่ถูกสร้างขึ้นโดยพลาสมาออกซิเจนและอากาศได้ถูกชักนำให้ยึดติดบนพื้นผิวของฟิล์มเจลาติน ทั้งนี้ยังพบว่าปริมาณของหมู่ฟังก์ชันเหล่านี้เพิ่มขึ้นตามเวลาที่ใช้ในการดัดแปร อุณหภูมิที่ใช้ในการเก็บตัวอย่างฟิล์มที่ผ่านการดัดแปรพื้นผิวด้วยพลาสมาส่งผลต่อการเปลี่ยนแปลงสมบัติความชอบน้ำของฟิล์มเจลาติน จากการทดสอบความเข้ากันได้ทางชีวภาพในระดับห้องปฏิบัติการด้วยเซลล์ต้นกำเนิดไขกระดูกพบว่า จำนวนเซลล์ที่ยึดเกาะบนพื้นผิวของฟิล์มเจลาตินที่ผ่านการดัดแปรพื้นผิวด้วยพลาสมาเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับพื้นผิวที่ไม่ได้ผ่านการดัดแปร นอกจากนี้ยังพบว่าการดัดแปรพื้นผิวเจลาตินด้วยพลาสมาไนโตรเจนสามารถส่งเสริมให้เกิดการยึดเกาะของเซลล์บนพื้นผิวได้ดีที่สุดเมื่อเทียบกับการใช้พลาสมาออกซิเจนและอากาศ ค่ามุมสัมผัสกับน้ำและอัตราส่วนของ ออกซิเจนต่อไนโตรเจนที่เหมาะสมต่อการยึดเกาะของเซลล์ต้นกำเนิดบนพื้นผิวฟิล์มเจลาตินคือ 27-28 องศาและ 1.4 ตามลำดับ สำหรับส่วนที่สองของการศึกษา พลาสมาพลังงานสูงชนิดการเหนี่ยวนำแบบพัลซ์ได้ถูกนำมาใช้ในการดัดแปรพื้นผิวของฟิล์มเจลาติน แก๊สไนโตรเจนได้ถูกเลือกใช้ในการผลิตพลาสมาเนื่องจากสามารถชักนำให้เกิดการยึดเกาะของเซลล์บนพื้นผิวฟิล์มเจลาตินได้ดีที่สุดดังเห็นได้จากผลการทดลองของส่วนแรก จากการทดลองพบว่าการดัดแปรพื้นผิวด้วยพลาสมาชนิดการเหนี่ยวนำแบบพัลซ์ไม่ส่งผลต่อสมบัติทางความร้อนและระดับการเชื่อมขวางของฟิล์มเจลาติน ทั้งนี้ความชอบน้ำ ค่าพลังงานพื้นผิว และความขรุขระของฟิล์มเจลาติน ที่ผ่านการดัดแปรพื้นผิวมีค่าสูงขึ้นตามจำนวนซ้ำของการยิงพลาสมา จากการทดสอบความเข้ากันได้ทางชีวภาพในระดับห้องปฏิบัติการแสดงให้เห็นว่า พลาสมาชนิดการเหนี่ยวนำแบบพัลซ์สามารถส่งเสริมให้เกิดการยึดเกาะของเซลล์บนพื้นผิวฟิล์มเจลาติน ผลการศึกษาโดยสรุปชี้ให้เห็นว่าพลาสมาระบบกระแสสลับความถี่ 50 เฮิร์ตและชนิดการเหนี่ยวนำแบบพัลซ์สามารถส่งเสริมสมบัติทางกายภาพและชีวภาพของฟิล์มเจลาติน

ภาควิชา วิศวกรรมเคมี	ลายมือชื่อนิสิต อิศราวุธ ประเสริฐสังข์
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ISARAWUT PRASERTSUNG: IMPROVEMENT OF CROSSLINKED GELATIN FOR IN VITRO CELL CULTURE BY PLASMA SURFACE TREATMENT. THESIS ADVISOR: ASSOC. PROF. SIRIPORN DAMRONGSAKKUL, Ph.D., THESIS CO-ADVISOR: ASST. PROF. RATTACHAT MONGKOLNAVIN, Ph.D., PROF. CHIOW SAN WONG, Ph.D.,101 pp.

This research aimed to study the effects of plasma treatment on the physical and biological properties of crosslinked gelatin. In the first part, nitrogen, oxygen, and air glow discharges powered by AC 50Hz were used to treat type A gelatin film crosslinked by dehydrothermal process. The water contact angle of gelatin films was decreased with increasing plasma treatment time. The treatment of nitrogen, oxygen, and air plasma up to 30 seconds had no effects on the surface roughness of gelatin film. 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. These functional groups were found to increase with increasing treatment time. The storage temperature of plasma-treated samples had an influence on the recovery of wettability. *In vitro* test using rat bone marrow mesenchymal derived stem cells (MSCs) revealed that the number of cells attached on plasma-treated gelatin films was significantly increased compared to untreated samples. In addition, among three types of plasmas used, nitrogen plasma treatment provided best MSCs attachment on gelatin surface. The results suggested that a type A gelatin film with water contact angle of 27-28° and the O/N ratio of 1.4.is most suitable for MSCs attachment. In the second part, PICP plasma system, one of high energy plasma system, was introduced to treat gelatin films. Nitrogen was selected to generate PICP plasma since it induced best cell attachment as found in the first part. PICP could alter the surface properties of gelatin film but does not influence the thermal property and the degree of crosslinking of the film. The hydrophilicity, surface energy, and surface roughness of gelatin films were promoted by increasing the number of applied pulses of PICP. Biocompatibility results showed that PICP treatment could enhance cell attachment on the surface of gelatin film. The study implied that both AC 50Hz and PICP can be used to enhance the physical and biological properties of gelatin film.

Department : Chemical Engineering	Student's Signature Isarawut Prasertsung
Field of Study : Chemical Engineering	Advisor's Signature
Academic Year : 2010	Co-Advisor's Signature
	Co-Advisor's Signature

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CHAPTER I

INTRODUCTION

1.1 Background

Tissue engineering using cell transplantation appears to be the most promising alternative to exist therapies for restoring tissue and organ function. Tissue substitutes should restore biological functions of damaged tissues using cells with proliferative and differentiative potentials. Cell affinity is the most important factor to be concerned with when biodegradable polymeric materials are utilized as cell scaffold in tissue engineering [1]. Tissue integration is conditioned by the adhesion and spreading ability of cells on implant surfaces. The cell attachment belongs to the first phase of cell/materials interactions and the quality of this phase will influence the cell‟s capacity to proliferate and differentiate itself on contact with the material. The interaction of cells with the material is a result of specific recognition among cell surface adhesion receptors, which are integrins and extracellular matrix (ECM) that have a cell-binding domain containing the Arg-Gly-Asp (RGD) sequence. Cell behavior on biomaterial surfaces depends upon implant–cell interactions, correlated with surface properties [2-3].

For biomedical application, surface properties including surface charge, chemical composition, topography and hydrophilicity/hydrophoblicity are very important because most biological reactions occur on surfaces or interfaces [4]. Many research groups have extensively studied the effects of surface properties of substrate on the interaction of biological species with surfaces. Schakenraad J.M. *et al.* reported that human fibroblast cells spread poorly on hydrophobic surface but more extensively on hydrophilic surfaces [5]. Tamada Y. *et al.* stated that a polymer surface with a water contact angle of 70^{o} provided the most suitable surface for cell adhesion [6]. Van Wachem P.B. *et al.* found that human endothelial cell could adhere greatly on the moderate hydrophilicity polymer substrate. Fibroblasts were found to orient themselves along the length of grooves in a surface, a phenomenon called„„contact guidance““ [7,8]. In a comparative study concerning different parallel micro groove sizes on a poly (L-lactic acid) surface, it was revealed that PLA surface with a groove

depth of 1.0 mm and groove widths of 1 and 2 mm induced formation of the most mineralized extracellular matrix (ECM) of osteoblast-like [9]. These examples suggested the surface properties, including wettability, surface chemistry, surface roughness and surface energy can greatly affect cell response to the nature of substrate. Therefore, in order to design the scaffold used in tissue engineering, the surface properties of the substrate must be considered as the important factor.

Biomaterial is one of key consideration for tissue engineering and medical applications. Usually, materials used to form the scaffolds should be biocompatible and biodegradable. Furthermore, it should regulate tissue functions correctly. Among various types of biomaterials, gelatin is one of the most dominant materials in tissue engineering applications. Gelatin, derived from hydrolysis reaction of collagen, has been of interest because it is much cheaper and easier to obtain solution than collagen. Gelatin is biocompatible, biodegradable, non-immunogenic and non-antigenic [10-12]. Also, gelatin molecule contains Arg–Gly–Asp (RGD)-like sequence that promotes cell adhesion, migration and proliferation [13]. It was reported in previous study that gelatin can be used as a base material substituting a large portion of collagen to produce scaffolds without affecting the biological properties [14]. As the main drawbacks of gelatin are its water solubility and weak mechanical properties, these result in high instability of scaffold *in vivo*. Crosslinking is therefore employed prior to its use in cell culture and biomedical applications. However, crosslinked gelatin films had been reported to have high hydrophobicity and low surface energy which do not favor to cell adhesion [15-16]. Therefore, to overcome these problems, the surface modification was introduced.

Many approaches have been developed in order to improve cell affinity of hydrophobic polymers, including surface and bulk modification. Surface modification can be done by incorporating or coating some biologically active molecules onto the surface of the materials [17]. Plasma treatment is one of the surface modification methods that are widely employed for surface modification of materials since it is versatile, fast and friendly to environment. Surface properties that can be modified by plasma are wettability, roughness and chemistry. For example, cold plasma such as radio frequency (RF) generated plasma has been reported to modify the surface of poly (lactide-co-glycolide) (PLGA) by introducing reactive groups onto the surface as well as improve cell adhesion [18-20]. Yang J. *et al*. also reported that the surface characteristics of poly(l-lactic acid) (PLLA) films is changed dramatically after a

plasma treatment in the presence of various gases. In addition, the adhesion force and affinity of mouse 3T3 fibroblasts on the PLLA films treated with ammonia plasma were greatly enhanced by hydrophilicity improvement of the surface [21]. However, the disadvantage of plasma treatment is storage effect [22]. If the plasma-treated polymer surface is exposed to atmospheric air, the plasma-activated surface quickly absorbs the moisture. The radicals which exist on the surface after plasma treatment would react with oxygen species, storage effect then occurs [23-24]. Therefore, in order to use the plasma-treated samples in biomedical applications, the storage effect must be explored.

In this work, alternating current (AC) glow discharge and pulsed inductively coupled plasma (PICP), are introduced to treat gelatin surface. The AC glow discharge is one of non-thermal plasma systems which could be used to modify the surface of polymers [25]. The design of glow discharge using AC 50Hz is much simpler than typical inductively coupled RF reactor plasma since 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 C.S. *et al*, have reported the adhesion strength of polyimide film that could be enhanced by polymerization process using a 50Hz alternating current glow discharge system [26]. Luigui C. *el al*, also found that, AC 50Hz plasma system could be used to improve polypropylene wettability and adhesion properties [27]. Pulse inductively coupled plasma (PICP) is one of high energy plasma systems which could be used in surface modification due to the fact that the surface treatment by PICP does not require lengthy treatment period to achieve desired effects [28]. The pulse treatment is more practical where the design of PICP is much simpler than typical inductively coupled RF reactor plasma. In addition, inductively coupled plasma produced in a closed environment provides clean environment for treatment of biomaterials. Single pulse plasma minimizes the thermal load of the sample. Moreover, the PICP system has flexibility in reducing and rising electron temperature of the plasma through variation of electrical energy stored and operating gas pressure. Based on our best knowledge, glow discharge using AC 50Hz system and PICP have not been applied to treat natural biopolymers.

The purpose of this research is to apply glow discharge using AC 50Hz and PICP system to improve the crosslinked gelatin films for *in vitro* cell culture. The research is divided into 2 parts. Part I is the study of AC 50Hz plasma effects on the physical and biological properties of crosslinked gelatin film. Three different types of

gas; nitrogen, oxygen and air, used in AC 50Hz glow discharge were employed to treat crosslinked gelatin film. Various surface properties of gelatin film, including surface roughness, wettability and surface chemistry, were investigated. The effects of storage time and temperature on the surface properties of plasma-treated gelatin were also investigated. The effects of plasma treatment on *in vitro* attachment of rat bone marrow-derived stem cells (MSCs) and L929 mouse fibroblast were explored. The specific information of gelatin surface properties suitable for the attachment of bone marrow-derived stem cells (MSCs) and L929 mouse fibroblast was also investigated. Part II emphasized on the effects of pulsed inductively coupled plasma (PICP) on the physical and biological properties of gelatin films. In this part, the specific information, i.e. type of gas for best cell attachment obtained from Part I was chosen to treat crosslinked gelatin. The properties of PICP-treated gelatin film including water contact angle, degree of crosslinking, surface roughness, and thermal properties were investigated. In addition, the cell affinity of PICP-treated gelatin films was evaluated using L929 mouse fibroblast as a model cell *in vitro*.

1.2 Objectives

1. To set up the glow discharge using AC 50Hz and pulsed inductively coupled plasma (PICP) system and characterize the plasma produced
2. To investigate the effects of plasma condition and storage on the properties of plasma-treated crosslinked gelatin films
3. To investigate the improvement of crosslinked gelatin for *in vitro* cell culture using plasma surface treatment
4. To correlate specific information of gelatin surface properties suitable for the attachment of bone marrow-derived stem cells (MSCs) and L929 mouse fibroblast

1.3 Scopes of research

Part I: Plasma treatment of crosslinked gelatin films using AC 50Hz plasma system

The scopes of research was sub-divided into 2 sub-parts as follows.

A. Effects of plasma condition and storage on the properties of gelatin

1. Set up of glow discharge using AC 50Hz system and its characterization
   1.1 Measurement of plasma species by optical emission spectroscopy
   1.2 Observation of the uniformity of plasma

2. The gelatin films were prepared by solution casting and dehydrothermal crosslinking technique. The surface of gelatin film was modified by oxygen glow discharge using AC 50 Hz. Parameters of plasma treatment to be investigated were
   2.1 treatment time,
   2.2 gas flow rate,
   2.3 plasma pressure,
   2.4 discharge power.

3. Characteristics of gelatin films modified by glow discharge using AC 50 Hz were investigated including:
   4.1 hydrophilicity/hydrophobicity by water contact angle,
   4.2 surface topology by atomic force microscopy (AFM),
   4.3 surface chemistry by Fourier transform infrared spectroscopy (FT-IR),
   4.4 Storage effects on plasma-treated crosslinked gelatin.

B. Effects of plasma treatment on *in vitro* biological properties of gelatin

1. The gelatin films were prepared by solution casting and dehydrothermal crosslinking technique. The surface of gelatin film was modified by glow discharge using AC 50 Hz. Parameters of plasma treatment to be investigated were
   - 1.1 treatment time,
   - 1.2 type of gas; nitrogen, oxygen, and dry air.

2. Isolation of rat bone marrow-derived stem cells (MSCs) from 3-week-old Wistar rat was performed using medium selection technique.

3. Characteristics of gelatin films modified by glow discharge using AC 50 Hz were investigated including:
   - 3.1 hydrophilicity/hydrophobicity by water contact angle,
   - 3.2 surface energy calculation,
   - 3.3 surface chemistry by X-ray photoelectron spectroscopy (XPS),
   - 3.4 surface topology by AFM,
   - 3.5 *in vitro* biological characterization.

     Two types of cell including L292 mouse fibroblast and rat bone marrow-derived stem cells (MSCs) were used. The behaviors of cells on the surface of gelatin films to be investigated were
     - 3.5.1 attachment and proliferation test by DNA assay,
     - 3.5.2 cell morphology by scanning electron microscopy (SEM),
     - 3.5.3 cell spreading area calculation.

Part II: Plasma treatment of crosslinked gelatin films using PICP system

1. Set up of glow discharge pulsed inductively coupled plasma system (PICP) and its characterization.

2. The gelatin films were prepared by casting and dehydrothermal crosslinking technique. The surface of gelatin film was modified by nitrogen glow discharge using PICP. Parameter to be investigated was number of applied pulses.

3. Characteristics of gelatin films modified by glow discharge using PICP were investigated including:
   - 3.1 hydrophilicity/hydrophobicity by water contact angle,
   - 3.2 surface topology by AFM,
   - 3.3 Thermal property by differential scanning calorimeter (DSC)
   - 3.4 Degree of crosslinking by TNBS assay
   - 3.5 *in vitro* biological characterization.

     L929 mouse fibroblast cell was used. The behaviors of cells on the surface of gelatin films to be investigated were
     - 3.5.1 attachment and proliferation test by MTT assay,
     - 3.5.2 cell morphology and spreading area calculation.

CHAPTER II

RELEVANT THEORY

2.1 Plasma

2.1.1 Plasma state [29]

Plasma is the forth state of the matter and constitutes more than 99% of the universe. It is ionized gas and consists of electron, ion and neutrons which are fundamental and excited states. From a macroscopic point of view, plasma is electrically neutron. However, it contains free charge carriers and electrically conductive.

Plasma state can be divided in two main categories: *hot plasma and cold plasma*. Hot plasma has extremely high energy content, which induces fragmentation of all organic molecules to atomic level. Consequently, this plasma can only be used to generate extremely high caloric energy or to modify thermally stable inorganic materials (metal, metal oxides, etc.). High gas temperature in hot plasma is obviously not suitable for moderate surface treatments like deposition of films, cleaning, dry etching, etc. For this purpose, cold plasma is required. Cold plasma has been widely used for surface treatment such as surface activation, fictionalization, cleaning and grafting.

2.1.2 Method of plasma generation [30]

Different plasma methods and plasma source have been reported to modify the surface and characteristic of materials such as biocompatibility and mechanical/chemical properties. To produce plasma, electron separation from atoms or molecules in the gas state, or ionization, is required. When an atom or a molecule gains enough energy from an outside excitation source or via interaction (collisions) with one another, ionization occurs. The production processes of plasma usually depend on the type of plasma generating devices. In this section, DC electrical

discharge, AC electrical discharge, microwave plasma, and pulsed inductively coupled plasma (PICP) are described in details.

2.1.2.1 DC electrical discharge

2.1.2.1.1 Electrical breakdown

Usually, the natural form of gas is an insulator and will not conduct electrical current. When an electric field is applied across it, stray charges are always present in the neutral gas. The stray charge is produced by the ionization of the gas particles by cosmic ray or any other background radiation from the environment.

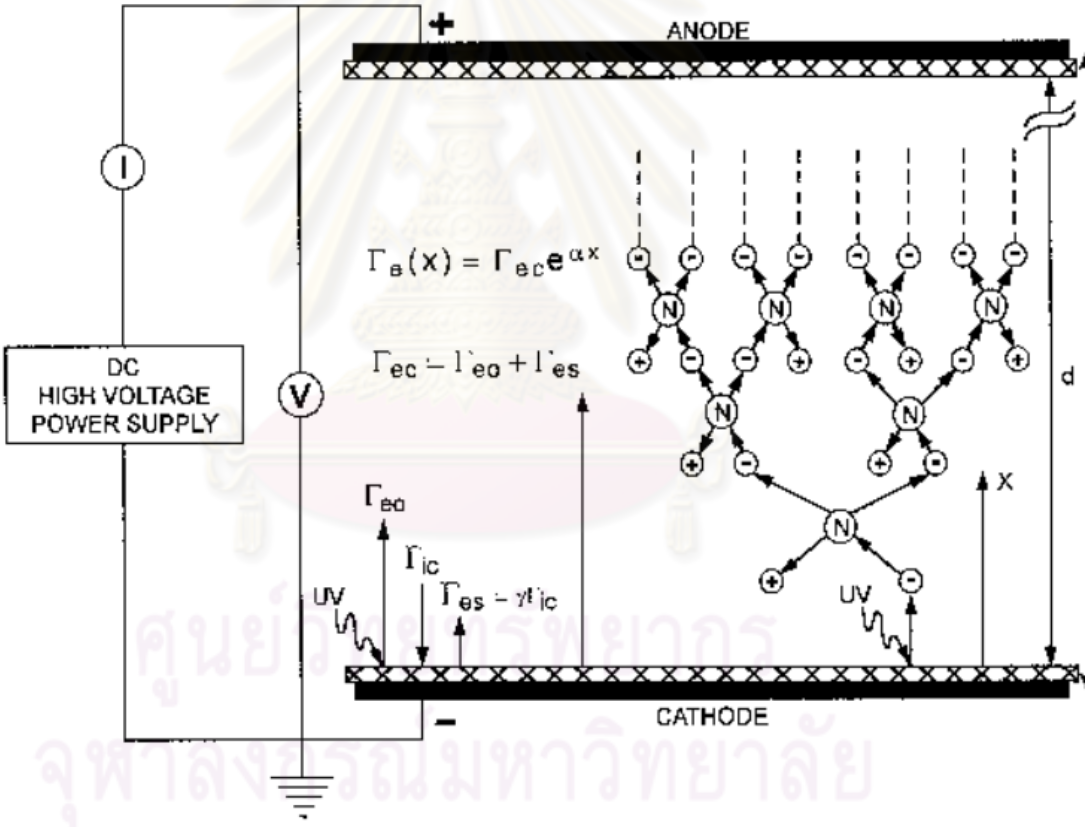


Figure 2.1 The simplest configuration of DC electrical discharge [30]

If the electric field is applied to the gas using electrodes, the stray charges (electrons) can be produced by the photoelectric effect at the cathode surface due to the absorption of UV photons. The presence of stray electron plays an important role in electrical discharge. They can be accelerated to high energy to produce ionizing

collision resulting in new charge particles. Without the presence of stray electrons, electrical discharge may not occur. The simplest configuration to produce an electrical discharge through a gas is to apply a potential difference across a pair of parallel electrodes placed inside a chamber filled with the gas at suitable pressure as shown in Figure 2.1.

Consider an electron originated from the cathode due to the absorption of UV photon with the presence of electric field, the collision process between electron and atom can be generated. The electron will be accelerated to high enough energy for excitation or ionization. When an ionizing collision occurs, the colliding electron together with the new electron will be further accelerated by the electric field. They may produce further ionizing collisions. The number of electrons will be multiplied as progress from the cathode to the anode.

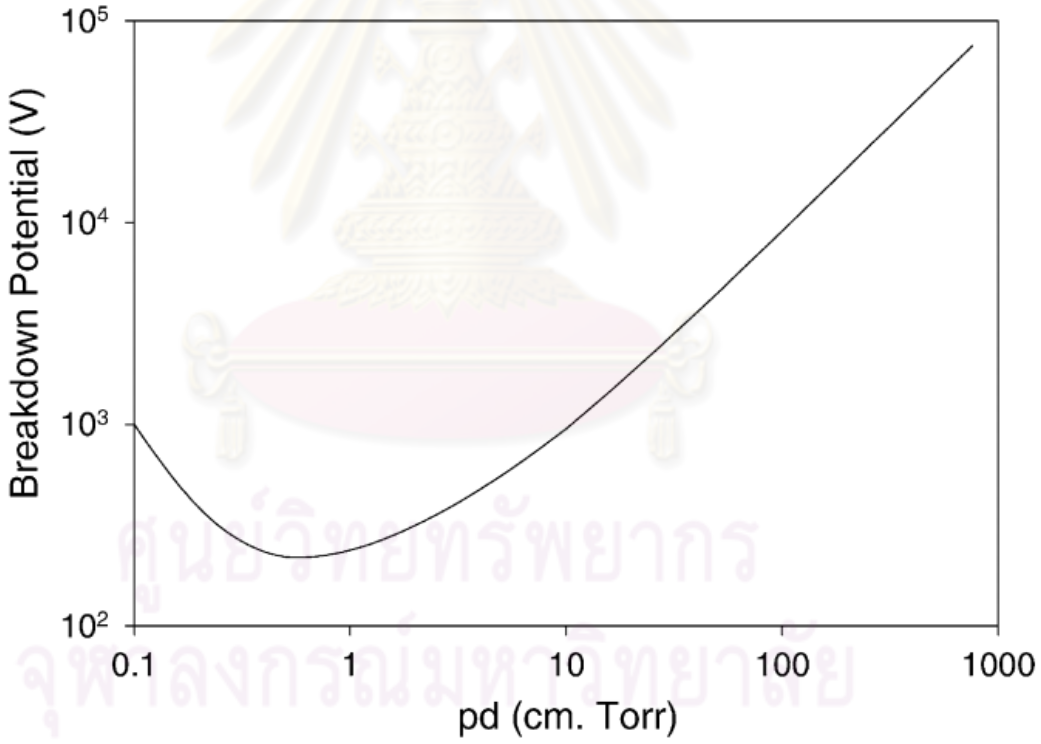


Figure 2.2 The relationship between the breakdown electric field of air and pressure [31]

In general, gaseous plasma is ignited by applying a potential through the gas. The breakdown potential depends on the pressure and discharge gap width. Figure 2.2 depicts the relationship between the breakdown potential of air and pressure. The breakdown potential has a minimum value when the pressure is 0.7 Torr, and

deviation from this value leads to an increase of the critical breakdown electric field [31].

2.1.2.1.2 The *I-V* characteristic of electrical discharge

The variation of the current flowing in the electrical discharge circuit with applied voltage Vs can be summarized by the *I-V* characteristic curve as shown in Figure 2.3. The vertical axis in the figure is the voltage drop across the discharge tube.

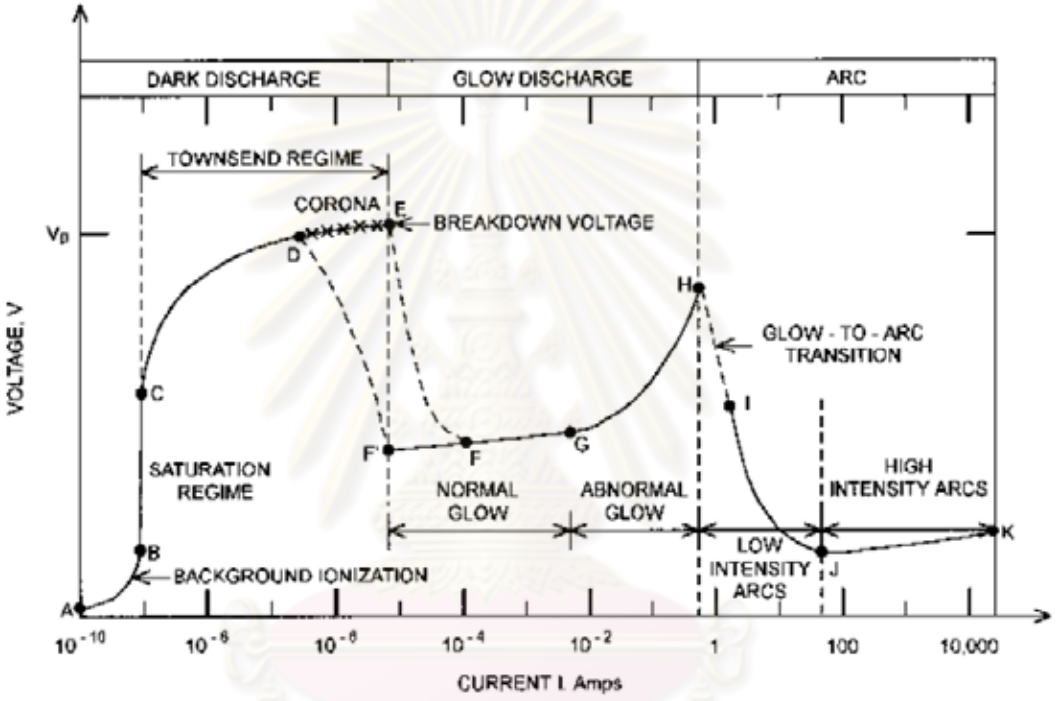


Figure 2.3 *I-V* characteristic curve [30]

The first part of the characteristic, AB, is caused by charges produced by ionization of the gas by environmental stray radiation or photoelectric effect at the cathode surface due to UV radiation. At low voltage, electrons available may be accelerated toward the anode to constitute to the current. If no ionizing collision by electron occurs due to the low potential (and hence low electric field), the maximum current is determined by the total number of initial electron available. This current is in the region below nanoampere and increases with applied potential. It reaches a saturation value corresponding to the maximum number of electron available, section BC in the *I-V* characteristic.

With increasing applied potential, the electrons may be accelerated to have enough energy to produce the excitation and ionization process. New charge particles, both ions and electrons, will be produced by ionization. This gives rise to an increase in the discharge current. As the potential is further increased, the discharge current will increase and then breakdown will occur. After electrical breakdown is occurred, electrical discharge is formed at potential V_B.

The region before breakdown, region ABCDE of the *I-V* curve is often referred to the dark discharge region. It is subdivided into the background current (ABC), the Townsend region (CDE), and the corona region (DE). A corona discharge is maintained by controlling the current at the micro ampere region. The voltage drop across the discharge tube when the discharge is in the dark discharge region, is roughly equal to the applied potential.

After breakdown, the discharge will try to draw infinite current from the power supply. It is essential to have a current limiting resistor (R_L) in series between the source and the discharge. The type of discharge will depend on magnitude of the discharge current, which is controlled by the combined effect of limiting resistor (R_L) and the plasma impedance. After breakdown, the plasma resistance is negligible compared to R_L. This means that the voltage drop across the discharge tube will be zero and the full voltage will be developed across R_L. When R_L is adjusted to limit the current to be in the region of mA, a voltage drop across the discharge tube will occur. It is roughly constant when the discharge current is varied. This is the normal glow discharge region (region FG of the *I-V* characteristic) and the voltage across the electrodes is called glow voltage, Vg. The normal glow region may be extended down to 10^{-5} A when the current is reduced gradually from mA (from F-F‘‘).

When the current is included from point G, the voltage across the electrodes will increase. The glow discharge is changed and become abnormal. When the current is increased to greater than 1 A (point H), the voltage across the electrodes suddenly drops to lower than the glow voltage. The discharge has changed into the arc discharge. As a summary, three types of discharge obtained by controlling the current are:

10^{-7} - 10^{-5} A	=	Corona discharge
10^{-5} - 1 A	=	Glow discharge
> 1 A	=	Arc discharge

2.1.2.2 AC (radiofrequency) discharge [30]

When DC power supply is varied from zero up to the breakdown voltage of the gas discharge and turn down to zero, the discharge will develop following the *I-V* characteristic curve (Figure 2.3) from point A to E. Beyond point E the obtained discharge is a glow discharge with glow voltage and current given by point F. Further increase in the supply voltage, the discharge current will increase while the glow voltage remains almost constant. This can be assumed that the voltage will begin to decrease when it has reached a value where the discharge is operating at point G. On reducing of the voltage, the path of discharge is followed by G to F to F‟ then to D when the discharge will disappear and return to the dark discharge condition.

At low frequency, gas discharge powered by an AC source can be considered not difference from that maintained by a DC source as described above. The criterion of “low” frequency is that the characteristic time of the voltage variation (usually taken to be the periodic time) should be larger than the transit time of the ions from anode to cathode. This is normally in the region of below 1 kHz. For high frequency, the discharge behavior becomes different from the DC source. First, the breakdown voltage will be lower. In a self-sustained glow discharge, new charged particles are produced by the ionization of the gas and the secary emission. Secary emission can generate at cathode by ion bombardment. This is balanced by the loss of electrons at the anode. For sufficiently high frequency (probably above 1 MHz) source, the loss of electrons at the anode will be reduced (or even becomes zero loss) since the alternating field may reverse some or all of the electrons before they reach the electrodes. Although there will be a reduction of the electron production by ion bombardment at the cathode surface, this will be compensated by the reduction of loss of electrons at the anode.

In order to ensure that the oscillation time of the electrons caused by the alternating electric field is shorter than their transit time between the electrodes, sufficiently high frequency AC power supply must be used. This also depends on the pressure of the gas. For pressure of a few torr and discharge distance of several cm, the use of frequency in the radiofrequency range is appropriated. A commonly used frequency is 13.56 MHz as agreed by international communication authorities. Under this condition, electron collides many times within one oscillation of the electric field so it may be able to transfer the energy it absorbs from the field to other particles. For

lower pressure, there may not be sufficient collision for the electrons to achieve equilibrium with other particles. This requires frequency in the range of microwave region (>GHz).

In RF discharge, electrons are expected to travel back between the electrodes as the field changes direction. As a result, steady plasma is formed between the electrodes. The condition of plasma is expected to be similar to that the DC discharges except that the potential distribution between the electrodes may vary during each cycle. RF discharge can be produced by using two types of configuration: capacitively coupled or inductively coupled. The capacitively coupling can be implemented with a set of parallel plates such as those used for the conventional DC glow discharge. For RF power source, an electrodeless discharge can be obtained by placing the electrodes outside the plasma chamber, as can be seen in Figure 2.4. This can eliminate the contamination of the plasma by the electrode materials.

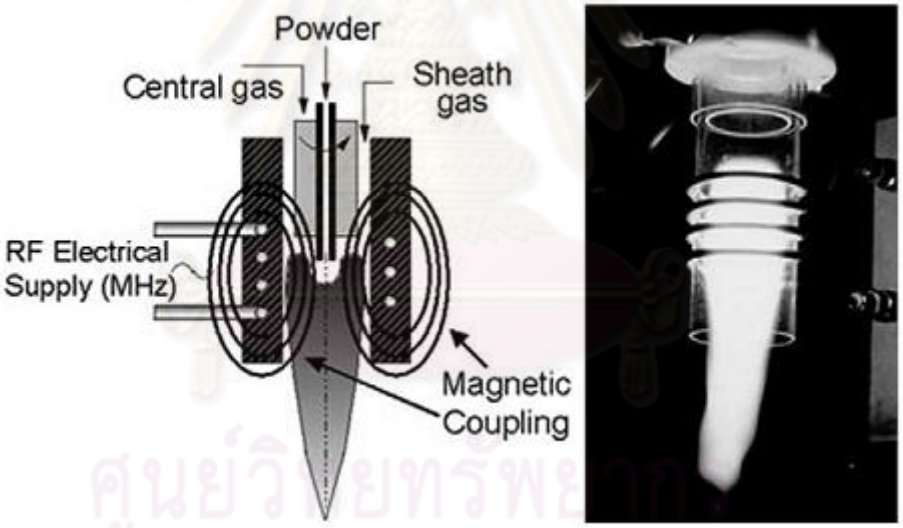


Figure 2.4 RF plasma system [29]

2.1.2.3 Microwave plasma [29]

The microwave is the region of electromagnetic spectrum with frequency above 1 GHz up to 300 GHz corresponding to the wavelength of 30 cm to 1 mm, respectively. Microwaves are guided along the system and transmitted energy to the plasma gas electrons. Elastic collisions between electrons and heavy particles occur. Due to the large mass of heavy particles, the collided electrons rebound whereas the

heavy particles remain static. The electrons are thus accelerated (they get kinetic energy) and the heavy particles are slightly heated. After several elastic collisions (which follow probabilistic laws), the electrons get enough energy to produce inelastic exciting or even ionizing collisions. The gas is partially ionized and becomes plasma which supports microwave propagation. The electron density obtained from microwave plasma is in the range of 1 x 10^{10} cm^{-3} to 1 x 10^{15} cm^{-3}.

2.1.2.4 Pulsed inductively coupled plasma (PICP)

Pulse inductively couple plasma device originated from the research in the field of controlled thermonuclear fusion. The PICP discharge can produce the pulsed high-density plasma. The "high-density plasma" here means a fully ionized gas with density in excess of about 10^{16} particles/cm^3 and with temperature in excess of 10^6 K (1 eV=11,600K). PICP device is one of the most useful and effective plasma processes. For example, this device has been utilized in the deposition of thin-films, including amorphous carbon film, diamond-like carbon film, etc. It has also been used to process the super-conducting films for lithography, which reveals its potential advantages over other methods. Such plasma could be used as light sources giving copious emission of electromagnetic radiation at short wavelengths. In addition, the technique associated with the generation of pulsed high-density plasma has applications in a broad scope [32].

The dynamic of pulse inductively coupled plasma

The dynamic process of PICP discharge is shown in Figure 2.5. After switch is closed, discharges through a single-turn coil are generated by power supply. The discharge current produces an axial magnetic field in the coil-encircled area (Figure 2.6). The rapidly increasing magnetic field induces an electric field opposite to the direction of the discharge current in the coil. The electric field in tube produces a plasma current sheath near the tube wall. The force exerts the plasma current sheath and rapidly compresses it toward the tube axis. Meanwhile, the plasma particles pass the surface of fibers and escape from the tube end (end loss). The plasma experiences shocks wave heating, adiabatic compression heating, and others. [32]

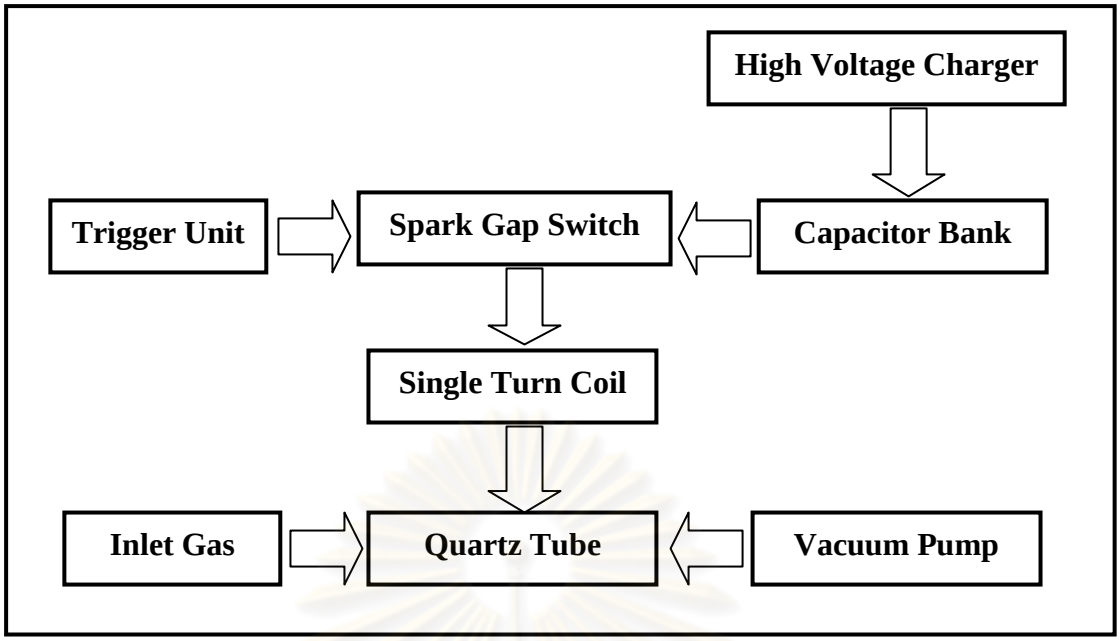


Figure 2.5The dynamic process of PICP system [32]

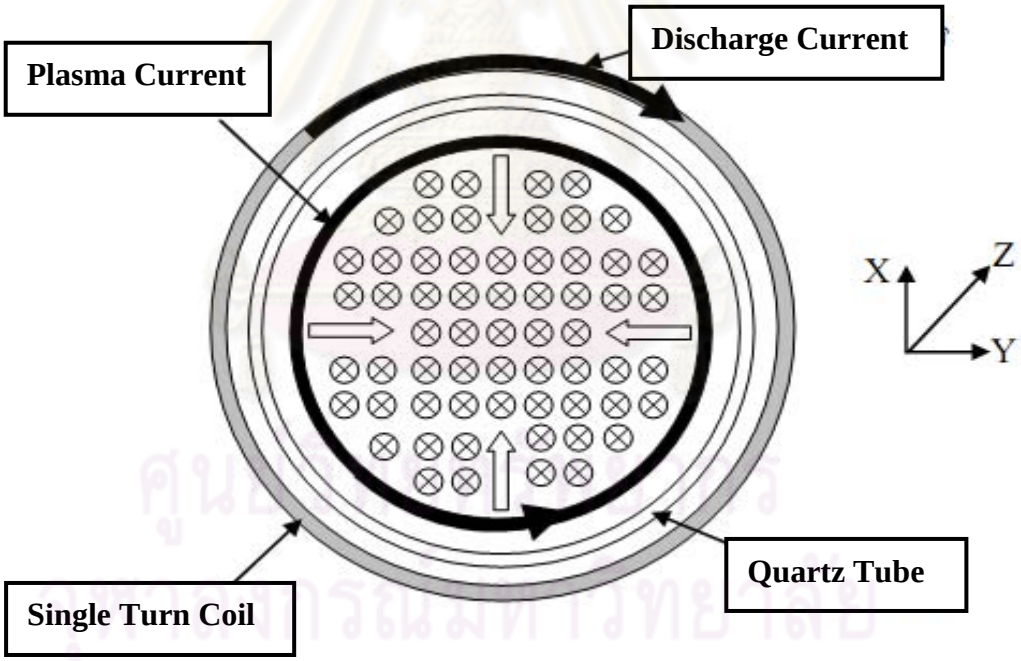


Figure 2.6 PICP discharge [32]

2.2 Surface modification using plasma technique [29]

The plasma technique is a convenient method for modifying surface properties of a material. There is an advantage to using the plasma technique when treating surfaces of complex shape because the plasma treatment is conducted in vacuum and it tends to be pervasive [31]. In this section, the principle and application of plasma sputtering, etching, implantation, polymerization, and grafting copolymerization are described.

2.2.1 Plasma sputtering and etching

In plasma sputtering and cleaning processes, materials are removed from the surface by chemical reactions to form volatile products and physical sputtering. Inert gases such as neon and argon are used for cleaning the surface of materials. Argon is one of the most common inert gases used because of its relatively low cost and high sputtering yield. Plasma sputtering is a simple plasma surface treatment method. During the sputtering process, a negative voltage (about 1 to several kV) is applied to the substrate and argon plasma is generated by radio frequency glow discharge (RFGD). The ions are accelerated by the applied electric field. Since the energy is not very high, the argon ions cannot go very deeply into the substrate. A big portion of their energy is transferred to the surface atoms via elastic and inelastic collisions with the materials. Some surface atoms will acquire enough energy and escape from the substrate into the vacuum chamber. After the first layer of atoms has been sputtered off, the underlying layers will be exposed and gradually etched. With sufficient sputtering time, surface contamination will be cleaned off. This process can be used as a pretreatment for subsequent implantation and deposition. The weight loss in the etching process is mainly bond scission of polymers and reactions of the radicals generated in the polymer chains upon plasma exposure. In addition to the chemical etching process, physical sputtering occurs frequently as well when polymers are exposed to plasma.

2.2.2 Plasma implantation

When a polymer is exposed to plasma with sufficient plasma density and treatment time, many functionalities will be created near the surface. Crosslinked polymer chains can be formed. In a typical plasma implantation process, hydrogen is first abstracted from the polymer chains to create radicals at the midpoint of the polymer chains. The polymer radicals then recombined with simple radicals created by the plasma gas to form oxygen or nitrogen functionalities. Radical species are created in the plasma zone play an important role in the implantation process. Generally, polymers are hydrophobic, and the conversion of these polymers from being hydrophobic to hydrophilic usually improves adhesion strength, biocompatibility, and other pertinent properties. Formation of oxygen functionalities by ion implantation is one of the most useful and effective processes of surface modification. In general, oxygen plasma is used, but plasma of other compounds consisting of carbon dioxide, carbon monoxide, nitrogen dioxide, and nitric oxide can make the polymer surface hydrophilic as well. Besides oxygen functionalities, chlorine functionalities that can contribute to an increase in the hydrophilicity are formed using CF_2C and CCl_4 plasmas. On the other hand, if one wants to improve the hydrophobic properties of the polymer, higher degree fluorinated compounds such as SF_6, CF_4, and C_2F_6 are used as plasma gases.

2.2.3 Plasma polymerization

In plasma polymerization, the transformation of low molecular weight molecules (monomers) into high molecular weight molecules (polymers) occurs with the assistance of energetic plasma species such as electrons, ions, and radicals. Plasma polymerization is chemically different from conventional polymerization involving radicals and ions. In many cases, polymers formed by plasma polymerization have different chemical compositions and physical properties compared to conventional polymerization. This uniqueness results from the reaction mechanism of the polymer-forming process. Polymer formation in plasma polymerization includes plasma activation of monomers to radicals, recombination of the formed radicals, and reactivation of the recombined molecules. Plasma polymers do not comprise repeating monomer units, but instead complicated units containing crosslinked, fragmented, and

rearranged units from the monomers. In most cases, plasma polymers have a higher elastic modulus and do not exhibit a distinct glass transition temperature. Hydrocarbons such as methane, ethane, ethylene, acetylene, and benzene are widely used in the synthesis of plasma polymerized hydrogenated carbon films. The enhanced microhardness, optical refractive index, and impermeability result in good abrasion resistance. Plasmas of fluorine-containing inorganic gases, such as fluorine, hydrogen fluoride, nitrogen trifluoride, bromine trifluoride, sulfur tetrafluoride, and sulfur hexafluoride monomers are used to produce hydrophobic polymers. Plasma polymers fabricated using organo-silicon monomers have excellent thermal and chemical resistance and outstanding electrical, optical, and biomedical properties. The common organo-silicon precursors include silane, disilane (SiSi), disiloxane (SiOSi), disilazane (SiNHSi), and disilthiane (SiSSi).

2.2.4 Plasma grafting copolymerization

When polymeric materials are exposed to plasma, radicals are created in the polymer chains. These radicals can initiate polymerization reactions when put in contact with monomers in the liquid or gas phase. As a result, grafted copolymers are formed on the surface. In plasma grafting copolymerization, polymers are first exposed to the plasma to create radicals on the surface. Inelastic collisions between the electrons in the plasma and polymer surface produce radicals in the polymer chains. Afterwards, the polymers are exposed to a vapor of the monomer or an aqueous or organic solution of the monomer. Since the plasma produces radicals only close to the surface of the polymers, plasma grafting copolymerization is restricted to the near surface. Plasma grafting copolymerization is often employed to alter the surface hydrophilicity of polymers [26]. It is usually conducted by first exposing a polymer to plasma such as argon, helium, or nitrogen for a short time (a few second). The process introduces many radicals to the surface of the polymer and experimental results reveal that these radicals can survive for several days. Afterwards, the polymer is brought into contact with the vapor of a monomer at an elevated temperature for a period of time. Oxygen in the monomer vapor or dissolved in the monomer solution inhibit the reactions and should be avoided.

2.3 Gelatin

The selection of biomaterials plays important role in the design and development of tissue engineering product. A biomaterial must interact with tissue to repair, rather than act simply as a static replacement. Furthermore, biomaterials used directly in tissue repair or replacement applications must elicit a desirable cellular response. The two main material types which have been successfully used in developing scaffolds for tissue engineering include:

a) Natural polymers, such as collagen, gelatin, glycosaminoglycan, starch, chitin and chitosan.

b) Synthetic polymers, such as polylactic acid (PLA), polyglycolic acid (PGA) and their co-polymers (PLGA).

In this study, the natural polymer such as gelatin is focused for film fabrication.

2.3.1 The nature of gelatin [34-35]

Gelatin is a protein which does not occur in nature. It is derived from collagen, a main component found in connective tissue, skin and bone of human and animals such as fish, bovine and porcine. Gelatin can be obtained by partial hydrolysis of collagen with subsequent purification, concentration, and drying operations.

Gelatin is a polypeptide, series of amino acids joined together by peptide bonds as shown in Figure 2.7.

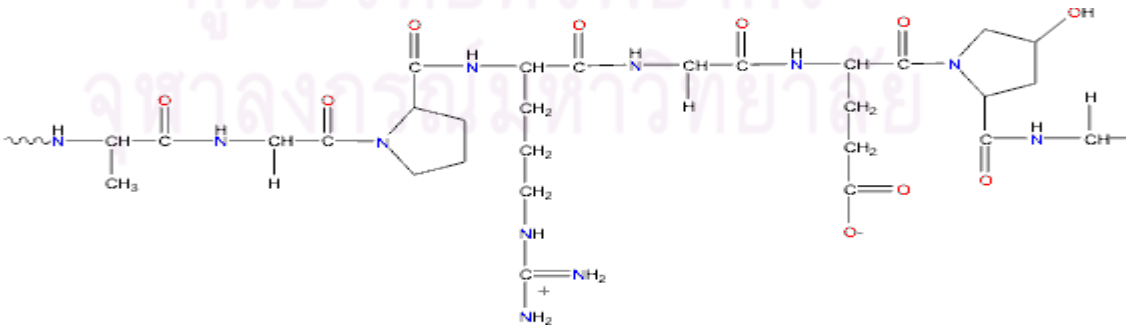


Figure 2.7 Molecular structure of gelatin [34]

2.3.2 Types of gelatin

Gelatin can be divided into 2 types depending on the production process.

a) Type A gelatin is produced from an acid process. This process is mainly applied to porcine skin, in which collagen molecule is young. The isoelectric points (pI) of type A gelatin are in the range of 7-9. High gel strength (bloom strength) gelatins normally have the higher pI and the low bloom strength gelatins have a pI closer to 7.

b) Type B gelatin is obtained form an alkaline process. This process is mainly applied to cattle skin and bone, in which the triple helix collagen molecule is more densely crosslinked and complex. Type B gelatin has a pI value around 5. The differences in the properties of both gelatin types are shown in Table 2.1.

Table 2.1 Properties of type A and type B gelatin [35]

	Type A	**Type A**
pH	3.8-5.5	5.0-7.5
Isoelectric point (PI)	7.0-9.0	4.7-5.4
Gel strength (Bloom)	50-300	50-300
Viscosity (cp)	15-75	25-75
Ash (%)	0.3-2.0	0.5-2.0

2.3.3 Properties of gelatin

Gelatin is nearly tasteless and odorless. It is colorless or slightly yellow, transparent and brittle. It is soluble in hot water, glycerol, and acetic acid, and insoluble in organic solvents. Gelatin swells and absorbs 5-10 times its weight of water to form a gel in aqueous solutions at low temperature. The viscosity of the gel increases under stress and the gelation are thermally reversible. Gelatin has a unique protein structure that provides a wide range of functional properties. These proteins can form a triple helix in aqueous solution.

Gelatin is amphoteric, meaning that it is neither acidic nor alkali, depending on the nature of the solution. The pH at which charge of gelatin in solution is neutral is known as isoelectric point (pI). The isoelectric point (pI) of a protein is the pH at

which the protein will not migrate in an electric field. This is due to the fact that at that pH the molecule carries an equality of positive and negative charges.

Gelatin, is rather unique as it can have an isoelectric point anywhere between pH 9 and pH 5, depending upon the source and method of production.

The properties of gelatin from various sources can be different, for example, fish gelatin is distinguished from bovine or porcine gelatin by its low melting point, low gelation temperature, and high solution viscosity.

2.3.4 Amino acid composition

Gelatin is a heterogeneous mixture of water-soluble proteins of high molecular weight. On a dry weight basis, gelatin consists of 98 to 99% of protein. The molecular weight of these large protein structures typically ranges between 20,000 and 250,000. Coils of amino acids are joined together by peptide bonds. As a result, gelatin contains relatively high levels of amino acids, as presented in Table 2.2. The predominant amino acid sequence is Glycine-Proline-Hydroxyproline (Gly-Pro-Hyp).

2.3.5 Characteristics of gelatin for tissue engineering applications

1. Suitable for cell attachment, proliferation and differentiation.
2. Biodegradable or bioresorbable with a controllable degradation and resorption rate to match cell/tissue growth *in vitro* and/or *in vivo*.
3. Not induce an inflammatory response or an immune rejection of the tissue.
4. Since gelatin derived from hydrolysis reaction of collagen, it is much cheaper and easier to obtain solution than collagen.

Table 2.2 Amino acid composition in gelatin [35]

Amino acid	%
Alanine	8.9
Arginine	7.8
Asperic acid	6.0
Glutamic acid	10.0
Glycine	21.4
Histidine	0.8
Hydroxylysine	1.0
Hydroxyproline	11.9
Isoleucine	1.5
Leucine	3.3
Methionine	0.7
Phenylanine	2.4
Proline	12.4
Serine	3.6
Theronine	2.1
Tyrosine	0.5
Valine	2.2
Total	100

2.4 Crosslinking methods [36]

To increase the biostability and decrease biodegradation rate, gelatin films have to be crosslinked. Crosslinking can be done by various methods such as chemical treatment, dehydrothermal treatment, ultraviolet irradiation, or electron beam irradiation. Two widely used methods including chemical and physical crosslinking are described as follow.

2.4.1 Chemical crosslinking

Among the chemical crosslinking agents, glutaraldehyde is the most widely used due to its high efficiency in the stabilization of collagenous materials.

Crosslinking of collagenous samples with glutaraldehyde involves the reaction of free amino groups of lysine or hydroxylysine residues of polypeptide chains with the aldehyde groups of glutaraldehyde. It is likely that glutaraldehyde can crosslink between the two amino residues of gelatin chains.

2.4.2 Physical crosslinking

2.4.2.1 Dehydrothermal crosslinking

Dehydrothermal treatment (DHT) is a physical method of crosslinking gelatin molecules that avoid potentially cytotoxic reaction products. This treatment can be done in a vacuum oven at the temperature above 100^{o}C. Formation of crosslinks during DHT treatment is dependent on the exhaustive removal of bound water from gelatin molecule. The removal of water results in condensation reaction between the amino and carboxyl groups of gelatin molecules. Dehydrothermal crosslinking can occur only if the amino and carboxyl groups are close to each other. Thus, it is possible that dehydrothermal treatment allows gelatin molecules to crosslink to a less extent than glutaraldehyde treatment.

2.4.2.2 UV irradiation

UV irradiation generates radicals at the aromatic residues of gelatin amino acids, such as tyrosin and phenylalanine. The binding of these radicals will react to each other, resulting in crosslinking formation. The primary limitation to the rate and exent of crosslinking may be the number of aromatic amino acid residues. The degree of crosslinking of gelatin films can be controlled by the time of treatment and dose of UV irradiation. When the irradiation time is short, UV irradiation will enable gelatin to crosslink intermolecularly. However, it is possible that irradiation for longer time preferably acts on the chain scission of gelatin molecules. A balance of the crosslinking and chain scission will result in unchanged density of crosslinking.

Comparing the above crosslinking methods, chemical crosslinking provides the greatest degree of crosslinking for protein. However, the disadvantage of chemical crosslinking is that some chemical agents, which are toxic to cells, can be left over and can cause irritation to patient when using the scaffolds in human bodies. Thus, dehydrothermal treatment is chosen in this study, since there is no toxicity problem to cells.

2.5 Mesenchymal stem cell [37]

Mesenchymal stem cells are defined as self-renewable, multipotent progenitor cells with the capacity to differentiate into tissues of mesodermal origin such as adipocytes, osteoblasts, chondrocytes, tenocytes, skeletal myocytes and visceral stromal cells, tissues of ectodermal origin such as neurons, and tissues of endodermal origin such as hepatocytes, depending on factors such as cell density, basal nutrients, spatial organization, growth factors, and cytokines.

2.5.1 Sources of mesenchymal stem cells

Mesenchymal stem cells have been initially identified in bone marrow with the amount of 1 mesenchymal stem cell per 100,000 nucleated cells. Source of mesenchymal stem cells is not only bone marrow, but also adult tissues such as fat, hair follicles and scalp subcutaneous tissue, periodontal ligament, thymus and spleen, as well as pre-natal tissues, such as placenta, umbilical cord blood, fetal bone marrow, blood, lung, liver and spleen.

2.5.2 Phenotypic characteristics of mesenchymal stem cells

When stem cells are isolated and cultured *in vitro*, the adherent population indicating mesenchymal stem cells tends to form colonies of spindle-shaped cells (fibroblast-like) termed as colony-forming unit-fibroblast (CFU-F). The CFC-F property is frequently used when studying the proliferative capacity of mesenchymal stem cells in culture. Initially, the cultured fibroblast-like cells are observed to be ALP-positive, collagen IV-positive and fibronectin-positive. In addition, type I collagen and laminin of the basement membrane are found in the extracellular matrix surrounding the cultured mesenchymal stem cells. A wide range of cell-surface antigens and peptides such as CD105, CD73 as well as other adhesion molecules and growth factor/cytokine receptors including CD29, CD90, CD117, CD166, CD54, CD102, CD121a,b, CD123, CD124, and CD49 have been targeted in these initial mesenchymal stem cells populations using a wide panel of antibodies.

2.5.3 Growth and differentiation of mesenchymal stem cells

Mesenchymal stem cells and colony forming units-fibroblastic (CFU-F) can be induced *in vitro* to expand and differentiate into the osteoblastic lineage using dexamethasone, ascorbate, and β-glycerolphosphate. Under these conditions the cells

undergo sequential: 1) growth, 2) differentiation, and 3) maturation. Growth or proliferation is marked by a many-fold increase in cell number. Synthesis of collagen I and alkaline phosphatise (ALP) indicate differentiation. Maturation is noticed by formation of multilayer nodules, secretion of osteocalcin and mineralization of the extracellular matrix (ECM). It has been reported that increase in ALP activity indicating early osteoblastic differentiation stage can be seen after 4 days, with activity peaking at 7-10 days and then slowly decreasing. Deposition of calcium and secretion of osteocalcin defined as late osteoblastic differentiation stage are usually quantified after 15 days of the culture in osteogenic medium [38].

CHAPTER III

LITERATURE REVIEWS

In 1991, the cell attachment behavior on plasma-treated polymer surfaces was examined by Ikada Y. *et al.* [6]. Polymer surfaces including polyethylene, polytetrafluoroethylene, polyethylene terephthalate, polystylene, and polypropylene were modified by glow discharge in order to study the effect of surface treatment on cell adhesion. The result showed that the surface wettability of all the films, evaluated by water contact angle, decreased with respect to the time period of plasma treatment. The X-ray photoelectron spectroscopy (XPS) of the plasma-treated surfaces using a derivatization method suggested that hydroxyl and carboxyl groups were introduced onto the surface of the polymer by plasma treatment. The results on mouse fibroblast cell culture showed that a different dependence of cell adhesion on the time period of plasma treatment was observed. They also suggested that the optimal water contact angle for cell adhesion was approximately 70^{o}.

In 1997, Lee J. H. *et al.* [39] have studied the interaction of cells on chargeable functional group gradient surface. The functional group gradient surface of polypropylene was prepared by corona discharge treatment with gradually increasing power and graft copolymerization of acrylic acid (AA), sodium *p*-styrene-sulphonate (NaSS), and *N,N*-dimethyl aminopropyl acrylamide (DMAPAA). AA and NaSS are negatively chargeable and DMAPAA is positively chargeable in phosphate buffer saline or cell culture medium at pH 7.3-7.4. The functional group gradient surfaces were characterized by water contact angle, fourier transform infrared spectroscopy in the attenuated total reflectance mode, and electron spectroscopy for chemical analysis. The interaction of Chinese hamster ovary cell with the functional group gradient surface was also investigated. The results showed that the functional groups were grafted on the surface with gradually increase of their density. The results on *in vitro* cell culture indicated that a grater quantity of cells had adhered and grown on the surface with moderate density of functional groups. This may be related to the hydrophilicity of the surface. The DMAPAA-grafted surface showed a large amount of cell attachment probably owing to the positive charge character, while AA and

NaSS-grafted surface, which are negatively charged, showed poor cell attachment. These results implied that surface functional group and their charge character as well as wettability play important roles for cell adhesion, spreading, and growth.

In 1997, Latkany R. *et al*. [40] have studied plasma surface modification of artificial cornea for optimal epithelialization. They have demonstrated that the optimal surface treatment of a polyvinylalcoholcopolymer hydrogel for epithelial cell migration and proliferation is an argon radio frequency (RF) plasma treatment. The surface chemistry of the material was determined prior to each cellular evaluation in order to compare the biological response with a known surface chemistry. The results showed that cells became confluent on argon-plasma-treated surfaces under several different reaction pressures, and after 2 weeks they became multilayered. Moreover, argon plasma-treated sample could enhance proliferated cells, extracellularmatrix and adhesion proteins compared to that acetone- and ammonia-treated surfaces.

In 2001, the enhancement of cell affinity of poly (D,L-lactide) by combining plasma treatment with collagen anchorage was reported by Yang J. *et al*. [21]. The changes of surface properties were characterized by contact angles, surface energy, X-ray photoelectron spectra and scanning electron microscopy. The mouse 3T3 fibroblasts were used as model cells to evaluate the cell affinity of poly (D,L-lactide) before and after modification. Effects of different modification methods including plasma treatment, collagen coating and combining plasma treatment with collagen anchorage were investigated and compared. The results showed that the hydrophilicity was improved while surface-free energy was reduced, after each modification. They revealed that plasma pre-treatment could improve the roughness as it incorporated the polar groups and positively charged groups onto the sample surface. The plasma pre-treated surface would then benefit in anchoring collagen tightly. As a result, cell affinity of PDLLA modified by combining plasma treatment with collagen anchorage was greatly improved, which would facilitate further application when the modified materials were used as cell scaffolds in tissue engineering.

In 2002, Yang J. *et al.* [41] fabricated poly(D, L-lactide) films and investigated the effects of anhydrous ammonia gas plasma treatment on surface

properties of films. They demonstrated that, O-containing and N-containing groups such as hydroxyl and amine were incorporated onto the surface of films. The effect of preserving method for maintaining the hydrophilicity was also investigated. The results indicated that preserving at low temperature (0-4^{o}C) was sufficient to maintain the plasma-modified surface properties. Mouse 3T3 fibroblast cells were used as model to compare cell affinity of modified and neat films. The results revealed that ammonia plasma treatment could promote the cells attachment and growth. After 4 days culture, the plasma treated films yielded almost two magnitudes cell compared to the control.

In 2002, the effects of plasma treatment of poly(L-lactic acid) and poly(L-lactic-co-glycolic acid) (70/30) scaffolds on human skin fibroblast cells were examined by Yang J. *et al*. [23]. Anhydrous ammonia plasma treatment was used to modify surface properties. The results showed that hydrophilicity and surface energy were improved. The polar N-containing groups and positive charged groups also were incorporated into the sample surface. A low-temperature treatment was used to maintain the plasma-modified surface properties effectively. The plasma-treatment method also helped to resolve the problem of cell loss during cell seeding. The negative effects of the ethanol trace on cell culture were avoided. They also suggested that anhydrous ammonia plasma treatment enhanced the cell affinity of porous scaffolds.

In 2002, Yang J. *et al*. [42] studied the effects of collagen-anchored polylactone on cell affinity. Poly(l-lactic acid)(PLLA)/poly(l-lactic-co-glycolic acid) (PLGA) 85/15 scaffolds were modified by plasma treatment. Then they were collagen anchored (PT/CA), and the cell affinity was evaluated by cell culture under shear or shear-free conditions. They also proposed the dyeing method for measuring the modified depth when plasma treatment is applied for the treatment of porous scaffolds. The results showed that a porous scaffold could be modified by plasma treatment and that a depth of about 4.0 mm for this modification can be reached with ammonia plasma treatment. PT/CA modification is an effective surface modification method for facilitating cell transplantation and improving the cell affinity of three-dimensional porous cell scaffolds in tissue engineering. It could solve the problem of non-uniform cell distribution in most synthetic porous scaffolds. The results on mouse

3T3 fibroblast cell culture indicated that the quality of cell attachment on PT/CA-modified PLLA scaffolds is better than that on unmodified PLLA.

In 2004, the surface properties of poly (lactide-co-glycolide) (70/30) modified by oxygen plasma treatment was reported by Yuqing W. *et al*. [43]. The surface structure, topography and surface chemistry of treated PLGA (70/30) films were characterized by contact angle measurement, scanning electron microscopy observation, atomic force microscopy, and X-ray photoelectron spectrum analysis. The cell affinity of the oxygen plasma treated films was evaluated under dynamic conditions by Parallel Plate Flow Chamber (PPFC). The results showed that the hydrophilicity increased greatly after oxygen plasma treatment. High quantities of –C–O groups, such as hydroxyl andperoxyl groups could be incorporated into the surface of PLGA (70/30) by controlling appropriate plasma treatment conditions. Moreover, the oxygen plasma treatment resulted in formation of peaks and valleys on the sample surfaces. The roughness increased with treatment time. Cells stretched very well and the ability to endure the shear stress was improved greatly after the PLGA was modified by appropriate plasma treatment, i.e. under 50 W for 2 or 10 min. However, when the treatment time was increased to 20 min, the percentage of adherent cells on the roughest surface decreased because the content of polar groups incorporated onto the surface decreased. The results showed that improved cell adhesion was attributed to the combination of surface chemistry and surface topology of PLGA during plasma etching.

In 2006, the influence of ammonia plasma treatment on the modifying depth and degradation of scaffolds were investigated by Wan Q. *et al*. [18]. The results showed that the modifying depth of the scaffolds increased with treatment time. The plasma power ranging from 20 to 80W slightly influenced the modifying depth. However, the degradation of the scaffolds increased with increasing treatment time and plasma power. The results also showed that the plasma intruded the scaffolds gradually from top to bottom. For a 4mm thick scaffold, the optimized treatment condition was 20W of power in a 30 Pa ammonia atmosphere for 30 min of treatment time. Under this condition, the integrity of scaffold could be relatively well kept. The results on M3T3 fibroblast cell culture showed that ammonia plasma treatment enabled the penetration of cells into scaffolds and facilitated the proliferation of cells.

In 2007, Hong S. *et al.* [20] investigated the effects of combining oxygen plasma treatment with anchorage of cationized gelatin on poly (L-lactide-co-glycolide) (PLGA) surface. The effects of modification method were compared with other methods including oxygen plasma treatment, cationized gelatin or gelatin coating and combining oxygen plasma treatment with anchorage of gelatin. The change of surface property was compared by contact angles, surface energy, X-ray photoelectron spectra (XPS), attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) and scanning electron microscopy (SEM) measurement. They found that the optimum oxygen pretreatment time determined by surface energy was 10 min when the power was 50 W and the oxygen pressure was 20 Pa. Analysis of the stability of gelatin and cationized gelatin anchored on PLGA by XPS, ATR-FTIR, contact angles and surface energy measurement indicated the cationized gelatin was more stable than gelatin. The result using mouse NIH 3T3 fibroblasts as model cells to evaluate cell affinity *in vitro* showed the cationized gelatin-anchored PLGA (OCG-PLGA) was more favorable for cell attachment and growth than oxygen plasma-treated PLGA (O-PLGA) and gelatin-anchored PLGA (OG-PLGA). Moreover, cell affinity of OCG-PLGA could match that of collagen-anchored PLGA (AC-PLGA). So the surface modification method combining oxygen plasma treatment with anchorage of cationized gelatin provides a universally effective way to enhance cell affinity of polylactone-type biodegradable polymers.

In 2007, Qu X. *et al.* [44] investigated the effects of oxygen plasma treatment on the formation of bone-like apatite on poly(lactide-co-glycolide) (PLGA)(70/30). Biodegradable PLGA(70/30) films and scaffolds were first treated with oxygen plasma and then incubated in a modified simulated body fluid 1.5SBF0 to prepare a bone-like apatite layer. The formation of the apatite and its influence on osteoblast-like cells growth were investigated. They found that the bone-like apatite formability of PLGA(70/30) was enhanced by plasma pretreatment. The changes of surface chemistry and surface topography induced by oxygen plasma treatment were both effective for apatite formation. The apatite formability increased with increasing plasma-treating time. Under a treating condition of 20W for 30min, oxygen plasma treatment could penetrate into the inner scaffold. After 6 days of incubation, the apatite formed in plasma-treated scaffold was better distributed than in untreated scaffold, and the weight and mechanical strength of the plasma-treated scaffold were

both enhanced. Compared with PLGA(70/30), the apatite layer formed on oxygen plasma-treated PLGA(70/30) surface enhanced adhesion and proliferation of MC3-T3 osteoblast-like cell, but had no significant effect on the ALP activity of cells at day 7.

In 2008, Hyun-Uk L. *et al.* [17] reported the surface modification of a biocompatible poly e-caprolactone (PCL) film treated by atmospheric cold plasma (ACP) with reactive gases. The change in wettability and surface topology of the PCL film after the plasma treatment with the reactive gases (Ar, H_2, N_2 and O_2) were determined using contact angle and surface roughness measurements. The chemical bonding states and molecular vibration modes of the activated organic groups on the polymer surface were examined by X-ray photoelectron spectroscopy and Fourier-transformation infrared techniques. The surface of the ACP-treated PCL films was also examined for their in vitro cell attachment and proliferation using human prostate epithelial cells (HPECs). The increase in the hydrophobicity of the Ar+H_2 plasma-treated PCL film resulted in a lower cell loading in the initial step of cell culture as well as a decrease in the level of cell attachment and proliferation compared with the pristine film. However, the hydrophilic properties of the Ar+N_2, Ar and Ar+O_2 plasma-treated PCL film improved the adhesion properties. Therefore, the Ar+N_2, Ar and Ar+O_2 plasma-treated PCL films showed a better cell distribution and growth than that of the pristine PCL film. They suggested that ACP-treated PCL film is potentially useful as a suitable scaffold in biophysics and biomedical engineering applications.

In 2008, Corona discharge treatment was applied to modify the surface of polyethylene (PE) by Shin Y. N. *et al.* [45]. The results showed that the wettability of PE surface was gradually increased by power increase of a corona treatment along the PE length, indicating that the hydrophilicity of PE surface increased gradually. The adhesion and proliferation behavior of human bone marrow stem cells (hBMSCs) on the gradient PE surface was also evaluated. They found that hBMSCs were adhered and proliferated on better highly hydrophilic than hydrophobic surfaces. The plot of proliferation rate vs. the water contact angles was parabolic. These results indicated that surface wettability played an important role in the cell attachment and proliferation.

In 2009, Lim J. *et al.* [15] examined the effects of plasma treatment of silk fibroin nanofiber on the cellular activities of normal human keratinocytes and fibroblasts. Silk fibroin nanofibers were prepared by electrospinning and treated with plasma in the presence of oxygen or methane gas to modify their surface characteristics. The surface characteristics of silk fibroin nanofibers after plasma treatment were examined using contact angle measurements and XPS analysis. They found that the hydrophilicity of the electrospun silk fibroin nanofibers decreased slightly by the methane plasma treatment. On the other hand, the hydrophilicity of the silk fibroin nanofibers increased greatly by oxygen plasma treatment. The oxygen plasma-treated silk fibroin nanofibers showed higher cellular activities for both normal human epidermal keratinocytes and fibroblasts than the untreated ones.

In 2010 Wu Y. C. *et al.* [46] treated chitosan film with RF plasma using argon gas and characterized both its physical and chemical properties. They found that argon plasma increased nano-scale roughness and introduced a carbonyl group to the chitosan surface. Meanwhile, the contact angles also decreased as the duration of the plasma treatment on chitosan was prolonged, indicating that the increased roughness and carbonyl group promote the hydrophilicity of the chitosan surface. Moreover, the number of osteoblasts adhering to plasma-treated chitosan films increased and their morphology became flatter with longer plasma treatments. They also demonstrated that plasma treatment on chitosan was capable of triggering the initial attachment of osteoblasts to chitosan surfaces.

CHAPTER IV

EXPERIMENTAL WORKS

4.1 Materials

1. Type A gelatin (pI 9, Nitta Gelatin Co., Osaka, Japan)
2. Glutaraldehyde solution (25% v/v, MW 100.12 g/mol)
3. β-Alanine ($C3H_7NO_2$, MW 89.09 g/mol)
4. 2,4,6-trinitrobenzene sulfonic acid (TNBS, $C_6H_3N_3O_9S$, MW 293.17 g/mol)
5. Sodium hydrogen carbonate ($NaHCO_3$, MW 84.01 g/mol)
6. Sodium chloride (NaCl, MW 58.44 g/mol)
7. Sodium hydroxide (NaOH, MW 40.00 g/mol)
8. Ethanol (C_2H_5OH, MW 46.04 g/mol)
9. Sodium citrate (HOC(COONa)(CH_2COONa)2.2H2O, MW 294.10 g/mol)
10. Sodium dodecylsulfate (SDS, $C_{12}H_{25}OSO_3Na$, MW 288.38 g/mol)
11. Phosphate buffer saline (PBS, pH 7.4)
12. Dulbecco‟s modified eagle medium (DMEM, 10%medium + L-glutamine + AB, Hyclone, USA)
13. Alpha-modified Eagle minimal essential medium (α-MEM, 10%medium + L-glutamine + AB, Hyclone, USA)
14. Fetal bovine serum (FBS, Hyclone, USA)
15. Penicillin/streptomycin antibiotic (100 U/ml, Hyclone, USA)
16. Trypsin-EDTA (0.25% trypsin with EDTA · Na, Gibco BRL, Canada)
17. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, USB corporation, USA)
18. Dimethylsulfoxide (DMSO, (CH3)2SO, MW 78.13 g/mol)
19. bisBenzimide fluorescent dye (Hoechst 33258, C25H24N6O.3HCl, MW 533.88 g/mol)
20. Tissue culture plates (Corning, USA)
21. Centrifugal tubes (Corning, USA)
22. Glass slip (15 mm in diameter, Matsunami, Japan)

23. Lint-freed paper (Kimwipe)

4.2 Equipments

1. Pulsed inductively coupled plasma apparatus
2. AC 50Hz plasma apparatus
3. An OceanOptics USB4000 charge couple device (CCD) spectrometer
4. Freezer (-40°C, Heto, PowerDry LL3000, USA)
5. Vacuum drying oven and pump (VD23, Binder, Germany)
6. Scanning electron microscopy (SEM, Jeol JSM 5400)
7. UV Spectrophotometer (Versa Max, Molecular Device Japan Co., Japan)
8. Microplate reader (1420, Perkin Elmer, UK)
9. Fourier transform infrared spectroscopy (FT-IR, Spectrum GX, Perkin Elmer, UK)
10. Water contact angle and video contact analyzer (Camplus Micro, Tantec Inc, USA)
11. X-ray photoelectron spectroscopy (XPS) (ESCALAB 250, VG Scientific, UK)
12. Atomic force microscopy (AFM, Veeco, Nanoscope IV, USA)
13. Centrifuge (AG 22331, Eppendorf, Germany)
14. Digital balance
15. Water bath
16. Auto-pipettes (10, 100, 1000, 5000 μl)
17. Multi-channel pipette

4.3 Preparation of crosslinked gelatin films

4.3.1 Preparation of gelatin film on teflon mold

Type A Gelatin was dissolved in deionized water to achieve 10 wt.-% solution. 2.5 ml of the solution was cast on a teflon mold. After solvent evaporation at room temperature, the film with the thickness of 0.12 mm was removed and crosslinked using dehydrothermal treatment in a vacuum oven at 140^{o}C for 48 hr [16].

4.3.2 Preparation of gelatin film on glass slip using casting technique

100 µl of 0.05 wt.-% gelatin solution was used to prepare film on a glass cover slip (diameter = 15 mm.). After solvent evaporation for overnight, the film was crosslinked using dehydrothermal treatment in a vacuum oven at 140^{o}C for 48 hr [47].

4.4 Set up and characterization of plasma system

In this work, two types of plasma systems including AC 50Hz plasma and pulsed inductively coupled plasma (PICP) were used. The setup of both plasma systems and their characteristics were described as follows.

4.4.1 Set up of glow discharge using AC 50Hz system

The setup of AC 50Hz plasma or glow discharge using alternating-current (AC) system was shown in Figure 4.1.

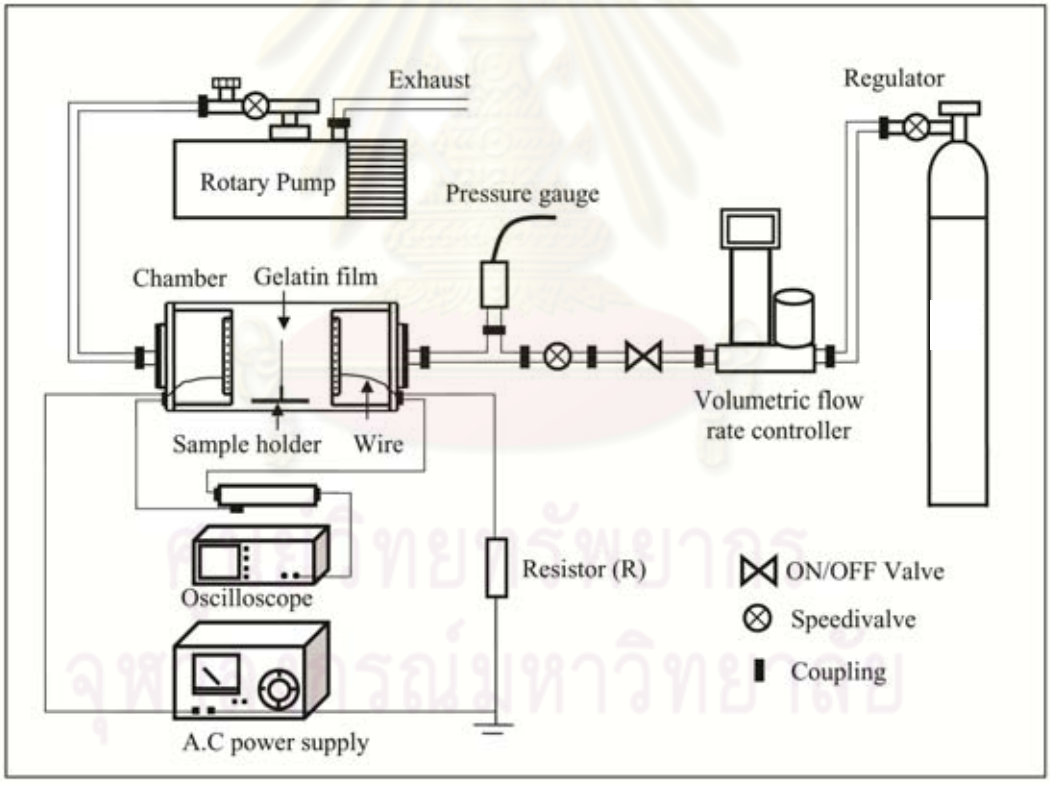


Figure 4.1 Schematic diagram of glow discharge using AC 50Hz system

The system consisted of a chamber connected with an AC 50Hz power supply, a cylinder of operating gas and a rotary pump. 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 distance 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 a 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).

$$W = \frac{1}{T} \int_{t}^{t+T} I(t)V(t)d(t) \qquad (1)$$

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

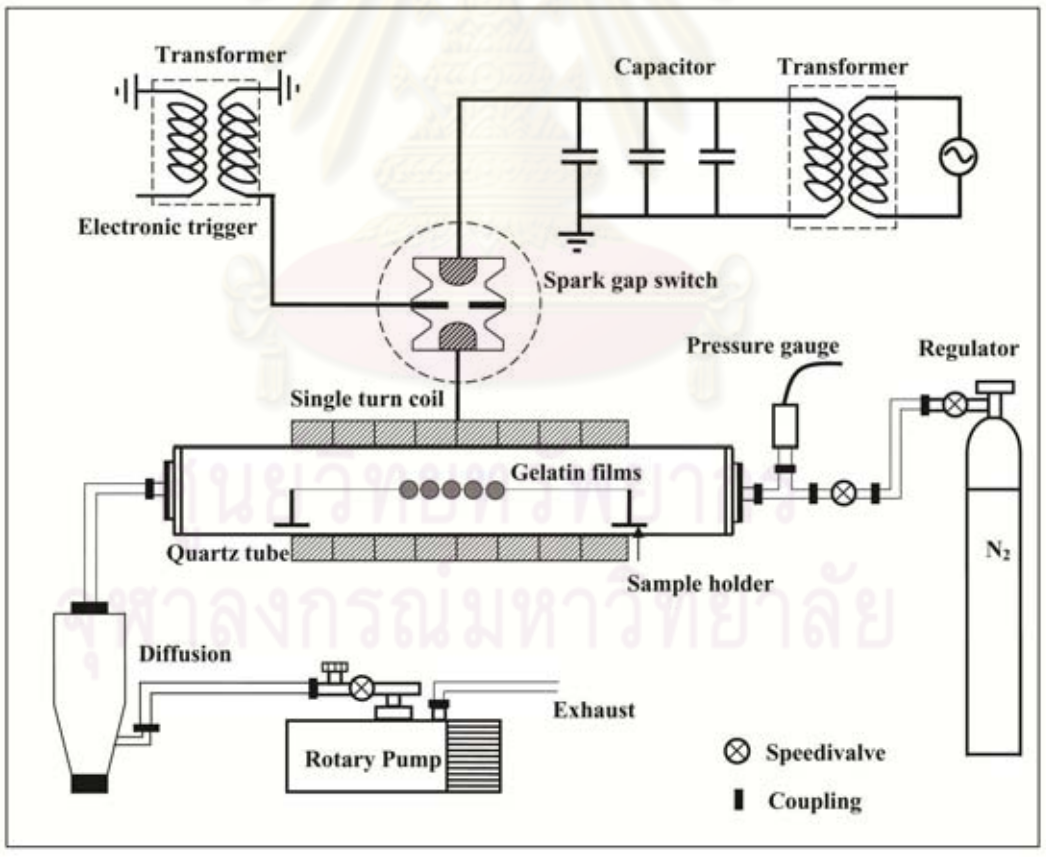


Figure 4.2 Schematic diagram of pulsed inductively coupled plasma (PICP) system.

4.4.2 Set up of pulsed inductively coupled plasma (PICP) system

A pulse inductively couple plasma (PICP) was setup as shown in Figure 4.2, where a 12 cm long cylindrical quartz tube was used to enclose gelatin films in a vacuum. PICP was driven by discharging 130 kA of current from three 4 μF capacitor bank. The initial store energy of the system was 2.4 kJ when the charging potential is 20 kV. For each operation, operating gas was filled at low pressure of 5 Pa after a pre-evacuation of the quartz tube below 2 Pa by a system of diffusion and rotary pump. When operating, the current was discharged through a single turn steel coil around the quartz tube producing plasma inside through electromagnetic induction process. The plasma produced was in single short pulse where the duration was 12 μs.

4.4.3 Characterization of plasma species

In order to characterize N and O species of plasma produced, an OceanOptics USB4000 charge couple device (CCD) spectrometer was used to monitor the light emitted from the plasma in the wavelength of 200 nm – 1,000 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 OceanOptics' Spectra Suite software.

4.5 Plasma treatment of crosslinked gelatin films

Plasma treatment of crosslinked gelatin films was divided into two parts. Part I was the study of AC 50Hz plasma effects on the physical and biological properties of crosslinked gelatin film. This part consists of the effects of plasma condition and storage on the properties of gelatin film and the effects of plasma on the improvement of *in vitro* biocompatibility of gelatin film. Part II was the investigation on the effects of pulsed inductively coupled plasma (PICP) on the properties of gelatin films. The schematic diagrams of plasma treatment in part I and II were summarized in Figure 4.3 and 4.4, respectively.

Part I: Plasma treatment of crosslinked gelatin films using AC 50Hz plasma system

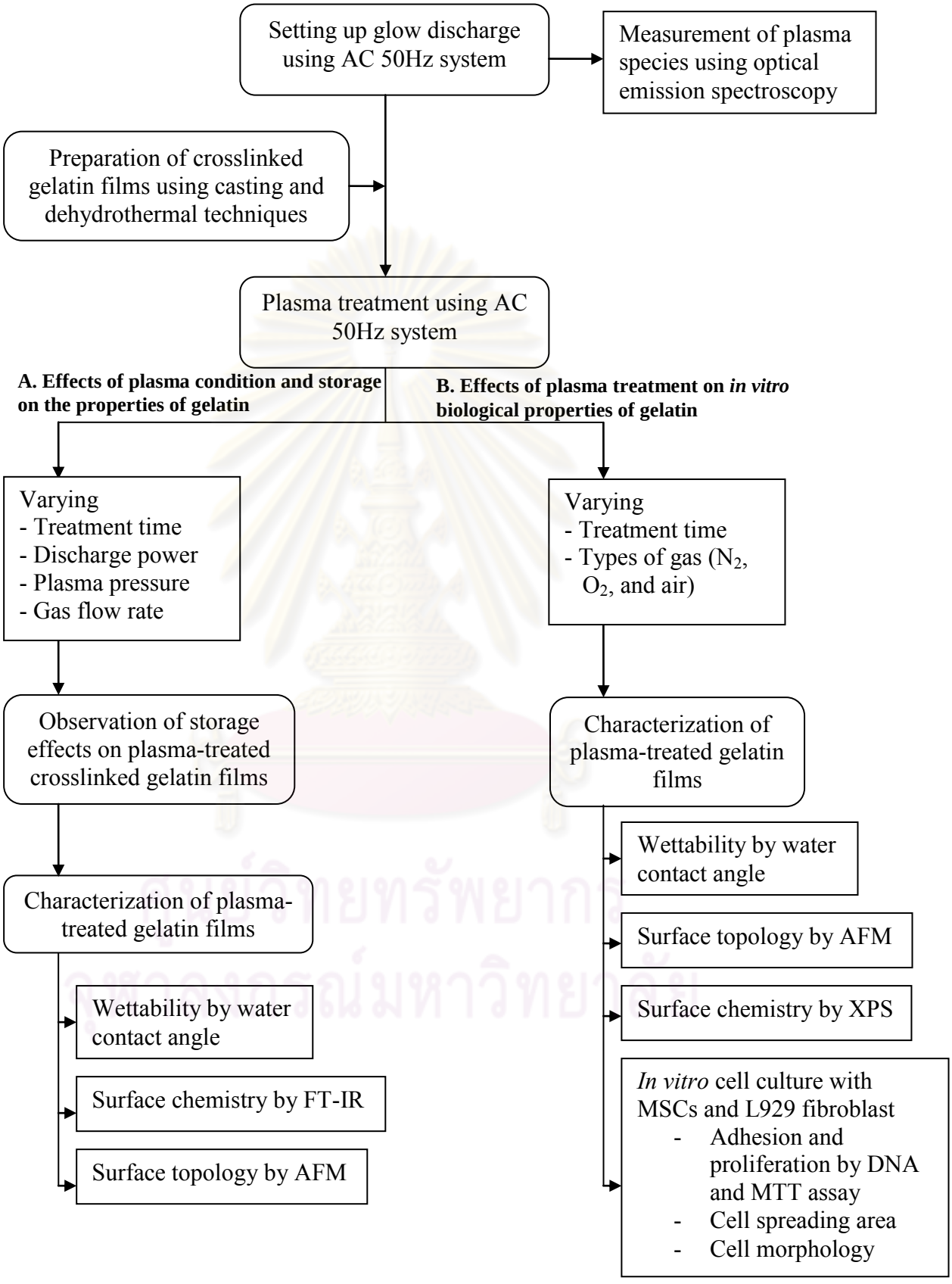


Figure 4.3 Diagram of plasma treatment using AC 50Hz system

Part II: Plasma treatment of crosslinked gelatin films using PICP system

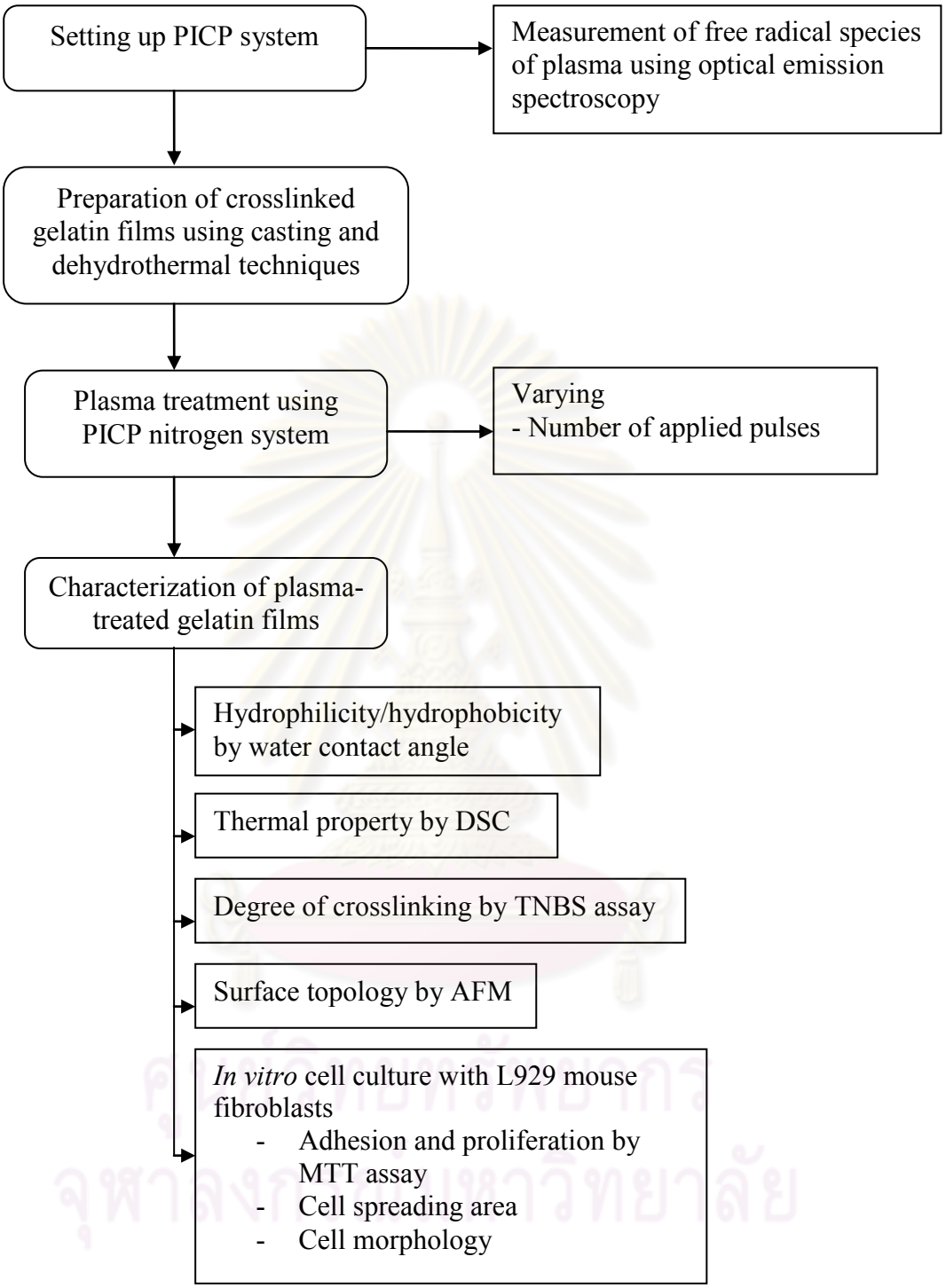


Figure 4.4 Diagram of plasma treatment using PICP system

Part I: Plasma treatment of crosslinked gelatin films using AC 50Hz plasma system

The experimental in this part was divided into two sub-parts. The first part focused on the effects of plasma condition and storage behavior on the properties of crosslinked gelatin films. In the second part, the effects of plasma treatment on the *in vitro* biocompatibility of crosslinked gelatin were investigated.

A. Effects of plasma condition and storage on the properties of gelatin

Crosslinked gelatin film prepared in section 4.3.1 was cut into pieces with a dimension of 2.5x2.5 cm^2 and placed inside a cylindrical tube of plasma apparatus. The film was treated with oxygen glow discharge using AC 50Hz system. The electrical discharge, pressure, and oxygen flow rate were varied in the range of 3-12 watt, 0.4-2 mbar, and 3-10 sccm, respectively.

After plasma treatment, the oxygen plasma-treated crosslinked gelatin samples (at 6 watt, 5 sccm of oxygen flow rate, and 1 mbar for 13 sec) were stored at different conditions in order to explore the storage effect on the properties of gelatin films. The plasma-treated crosslinked gelatin samples were kept at various storage temperatures of 50^{o}C, 25^{o}C, and 5^{o}C with the controlled relative humidity of 30±1%. The storage effect was studied over the period of 168 hr (7 days) via water contact angle measurement and FTIR-ATR analysis.

B. Effects of plasma treatment on *in vitro* biological properties of gelatin

In this sub-part, the crosslinked gelatin film prepared in section 4.3.2 were plasma treated with three different types of gasses including nitrogen, oxygen and dry air, prior to *in vitro* cell culture. The film was placed inside the cylindrical tube of the plasma apparatus and treated with the glow discharge. The pressure and the discharge power were fixed at 1 mbar and 4 watt, respectively, while the treatment times were varied from 3 to 30 sec. The set of the investigated samples were summarized in Table 4.1. The details of *in vitro* test were described in section 4.7.2.

Table 4.1 The set of AC 50Hz plasma-treated and untreated gelatin samples

Samples	Type of gas plasma	Treatment time (sec)
Untreated	-	-
N-3	Nitrogen	3
N-9	Nitrogen	9
N-15	Nitrogen	15
N-30	Nitrogen	30
O-3	Oxygen	3
O-9	Oxygen	9
O-15	Oxygen	15
O-30	Oxygen	30
A-3	Air	3
A-9	Air	9
A-15	Air	15
A-30	Air	30

Part II: Plasma treatment of crosslinked gelatin films using PICP system

Crosslinked gelatin film was cut into pieces with a diameter of 14 mm and placed inside a cylindrical quartz tube of PICP apparatus. The film was PICP-treated by a single pulse discharge generated by electrical energy storage. The repeated pulse discharge for 10 and 20 times was applied to the samples, in order to investigate the accumulating effect on the biological and physical properties of the gelatin films. The set of tested samples were summarized in Table 4.2

Table 4.2 The set of PICP-treated and untreated gelatin samples

Samples	Number of repeated pulsed
G-0	0
G-1	1
G-10	10
G-20	20

4.6 Characterization of plasma-treated gelatin films

4.6.1 Wettability

Contact angles of crosslinked gelatin films were measured by sessile dropping technique at room temperature. Deionized water and ethylene glycol were used as liquid media. After 30 sec of dropping, the contact angle of the samples was measured and averaged from 5 measurements (±standard deviation). The surface free energy was calculated according to Young"s equations as follows [21]:

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

$$(1+\cos\theta_2)\gamma_{L2} = 2\left(\left(\gamma_S^d\gamma_{L2}^d\right)^{1/2} + \left(\gamma_S^p\gamma_{L2}^p\right)^{1/2}\right) \quad (3)$$

where γ^d_S is the surface energy of dispersive component; γ^p_S is the surface energy of polar component; θ_1 is the contact angle of water; and θ_2 is contact angle of ethylene glycol. For water, γ_{L1} = 72.8 mJ/m^2, γ^d_{L1} = 22.1 mJ/m^2, and γ^p_{L1} = 50.7 mJ/m^2. For ethylene glycol, γ_{L2} = 48.0 mJ/m^2, γ^d_{L2} = 29.0 mJ/m^2, and γ^p_{L2} = 19.0 mJ/m^2.

4.6.2 Surface topology

Surface topography of the plasma-treated and untreated samples was examined using atomic force microscopy (AFM, Veeco, Nanoscope IV, USA) in a tapping mode. In order to ensure that the crosslinked gelatin films were not altered by scanning tip, the specimens were kept at 80^{o}C for 2 hr 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 area points of each sample and reported as the mean ± standard deviation (n = 4).

4.6.3 Surface chemistry

Fourier transform infrared attenuated total reflection spectroscopy (FTIR-ATR)

FTIR-ATR spectra were collected on a Bruker Tensor-27 FTIR single beam spectrometer. A KRS-5 crystal with a dimension of 50 x 20 x 5 mm^3 was used. Each spectrum represents 32 scans ratio against a reference spectrum obtained by recording scans of an empty ATR cell.

X-ray photoelectron spectroscopy

The chemical bonding states and atomic ratio of untreated and plasma-treated crosslinked gelatin surfaces were examined by X-ray photoelectron spectroscopy (XPS: VG Scientific, ESCALAB250, UK) using Al-K_α (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^o. The deconvolution process was performed by the curve fitting of C1s, O1s and N1s peaks.

4.6.4 Thermal property

The thermal properties of gelatin films were examined using a differential scanning calorimeter (NETZSCH DSC 204 F1, Germany). 5 mg of gelatin film was sealed in an aluminum pan and then heated at the rate of 10^oC/min from room temperature up to 250^oC.

4.6.5 Degree of crosslinking

The determination of degree of crosslinking was carried out by modifying the method of Bubins *et al*.[48]. The concept of this method was to react free amino groups of gelatin, which indicated uncrosslinking groups, with 2,4,6-trinitrobenzene sulphonic acid (TNBS). Briefly, about 5 mg of gelatin films was weighed into a test tube where 1 ml of 0.5 wt./vol.-% TNBS solution and 1 ml of 4 wt./vol.-% sodium hydrogen carbonate ($NaHCO_3$, pH8.5) were added. It was then heated at 40^oC for 2 hr. The uncrosslinked primary amino groups of gelatin would react with TNBS and form a yellow soluble complex. This solution was further treated with 2 ml of 6 N HCl at 60^oC for 1.5 hr. The absorbance of the solutions was determined at 415 nm after suitable dilution spectrophotometrically. The degree of crosslinking was then calculated using the following equation and the values were expressed as the mean±standard deviation (n=4).

$$\text{Degree of crosslinking (\%)} = 1 - \left(\frac{\text{Absorbance of crosslinked films}}{\text{Absorbance of uncrosslinked films}} \right) \times 100 \quad (4)$$

4.7 *In vitro* cell culture

In order to investigate the effects of plasma treatment on the biological properties of gelatin films, both L929 mouse fibroblast and rat bone marrow-derived stem cell were used. The details of in vitro cell culture are described as follow.

4.7.1 In vitro cell culture using L929 mouse fibroblast

Plasma-treated and untreated gelatin films were placed into 24-well plates and sterilized in 70 vol.-% ethanol for 30 min. To remove ethanol, the samples were extensively rinsed with phosphate-bufferd saline (PBS). L929 mouse fibroblast cells were seeded at a density of $2x10^4$ cells per film in DMEM containing 10 vol.-% fetal bovine serum and incubated at 37^oC in a 5 vol.-% CO_2 incubator. After cultured for 3, 6, 24 and 72 hr, the culture media was then removed and the films were rinsed with PBS. Cell morphology was observed under an optical microscope. The spreading area of cells after cultured for 24 hr was determined using Image J-Analysis software. A minimum of 80 cells were examined for each sample. Cell viability was determined using MTT assay [49]. Briefly, 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetra zolium bromide (MTT) was added and incubated for 30 min. Dimethylsulfoxide (DMSO) was used to elute complex crystals and the absorbance of solution was measured at 570 nm using a spectrophotometer. The results were reported as the number of cells using a standard curve prepared from L929 mouse fibroblast. The same treatment of the films without cells was used as control. All data were expressed as mean ± standard deviation (n=3). The population doubling time (PDT) was calculated from logarithmic of cell proliferation from 24 to 72 hr according to the following equation [50].

$$\text{Multiplication rate } (r) = 3.32 \times (\log N_2 - \log N_1) / (t_2 - t_1) \quad (5)$$

$$\text{PDT} = 1/r \quad (6)$$

where N_1 and N_2 are the number of cells at the culture time of t_1 and t_2, respectively.

4.7.2 In vitro cell culture using rat bone marrow-derived stem cell

4.7.2.1 Isolation of rat bone marrow derived stem cells (MSCs)

Rat 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 Y.

et al. [12]. After sacrify and sterilization, both ends of rat femurs were cut away from the epiphysis and the bone marrow was flushed out by a syringe (16-gauge needle) with 1 ml of medium. The cell suspension was then cultured in tissue culture plates containing Alpha-modified Eagle minimal essential medium (α-MEM) supplemented with 15 vol.-% fetal bovine serums (FBS) and 100 U/ml penicillin/streptomycin at 37°C, 5% CO_2. The medium was changed on the 4^{th} day after isolation and every 3 days thereafter. When the proliferated cells become subconfluent, usually for 7 to 10 days, the cells were trypsinized using 0.25 wt.-% trypsin-EDTA. The cells of the sec- and third-passage at sub-confluence were used.

4.7.2.2 Cell culture

Plasma-treated and untreated gelatin films were placed into 24-well cell culture plates and sterilized in 70 vol.-% ethanol for 30 minutes. To remove ethanol, the samples were extensively rinsed with phosphate-buffer saline (PBS). MSCs in α-MEM containing 15 vol.-% fetal bovine serum and 100 U/ml penicillin/streptomycin were seeded onto the films at 2 x 10^4 cells/film and incubated at 37°C in a 5 % CO_2 incubator. After cell culture for 3, 6, 12 and 18 hr, the culture media were removed and the films were then rinsed twice with PBS. Cell viability was determined using DNA assay [12]. Briefly, the cell samples were lysed in 30 mM sodium citrate-buffer saline solution (SSC, pH 7.4) containing 0.2 mg/ml sodium dodecylsulfate (SDS) at 37°C for overnight. Cell lysates were then mixed with a fluorescent dye solution (Hoechst 33258 dye) in 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 cell was used as control. All data were expressed as mean ± standard deviation (n=3). In addition, the percentage of cell attachment was calculated according to the following equation:

$$\%attachment = \frac{N_1}{N_0} x100 \qquad (7)$$

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

4.7.2.3 Cell morphology and spreading observation

After 6 hr of culture, attached MSC on the films were fixed in 2.5 vol.-% glutaraldehyde solution at 4^{o}C for 1 hr. Gelatin films were then serially dehydrated by series of ethanol 30, 50, 70, 80, 90, 95 and 100 vol.-% for 5 minutes 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 JEOL JFC-1100 sputtering device prior to SEM observation. In addition, the photographs of cell attached on each film were taken at 10X magnification using an optical microscope. Cells spreading area was determined using Image J-Analysis software. A minimum of 40 cells were examined for each sample.

CHAPTER V

RESULTS AND DISCUSSION

5.1 AC 50Hz plasma characterization

Optical emission spectroscopy was used to monitor the excited plasma reactive species generated by AC 50Hz plasma. The optical emission analysis is expected to explicate the reaction of reactive species that may contribute to plasma surface modification. The emission spectra of AC 50Hz plasmas generated using nitrogen, oxygen, and dry air, were presented in Figure 5.1. The results showed that nitrogen plasma mainly consisted of nitrogen molecule radical ($N_2^{\bullet}$) and nitrogen molecule ion (N_2^{+}) as indicated by the strong peaks at the wavelengths of 315.9, 337.13 and 357.8, 391.44 nm, respectively [51]. For oxygen plasma, the strong peaks were observed at the wavelengths of 777.42 and 844.56 nm corresponding to oxygen radical ($O^{\bullet}$) [52]. For air plasma, both nitrogen and oxygen species were observed. In addition, the hydrogen radical (H_{α}) was noticed at the wavelength of 656.30 nm when all types of plasma were generated. The results on optical emission spectroscopy implied that the N and O containing functional groups were performed by N and O reactive species on the surface of crosslinked gelatin films.

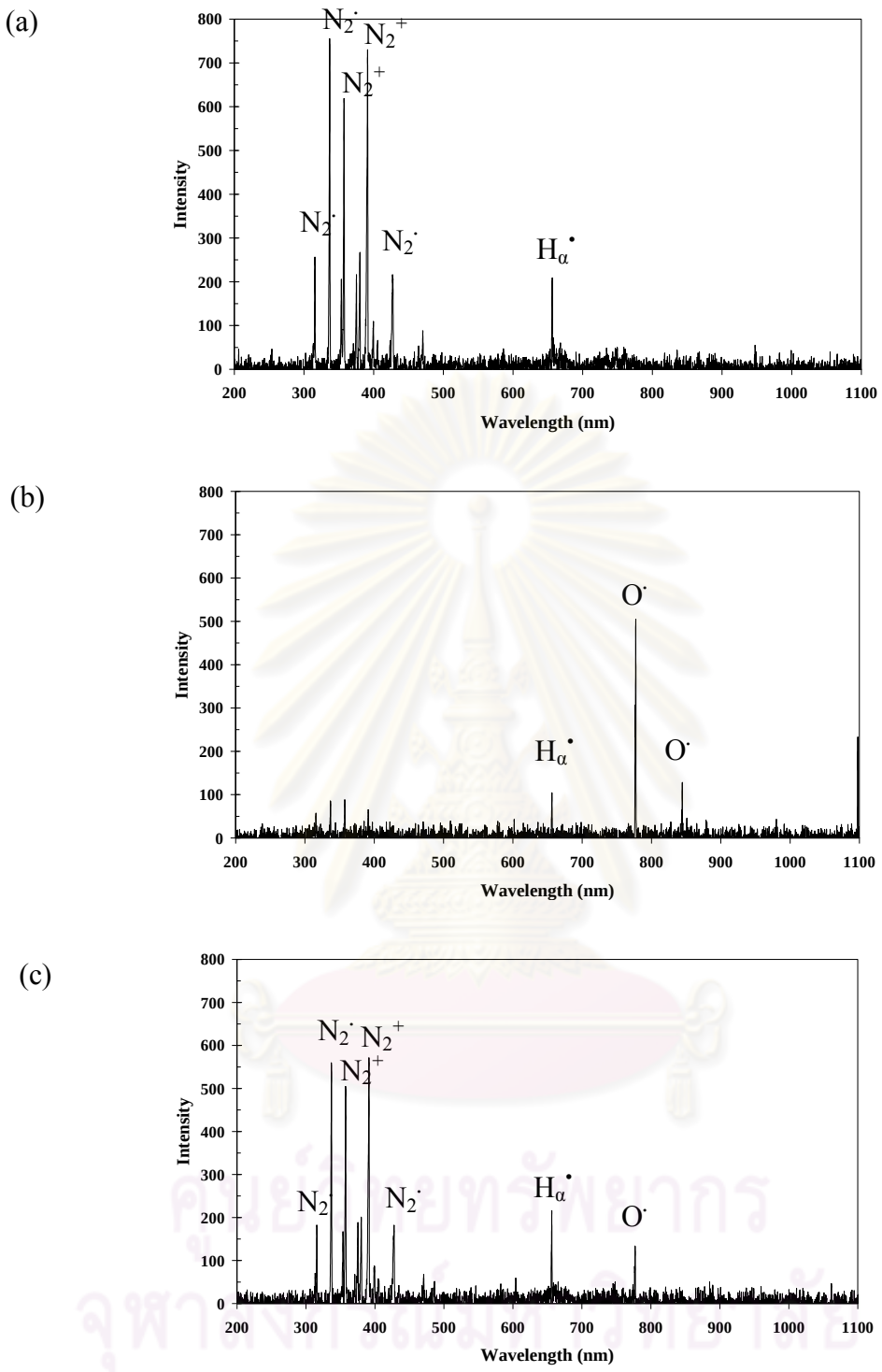


Figure 5.1 Optical emission spectrum of (a) nitrogen plasma, (b) oxygen plasma, and (c) air plasma generated by AC 50Hz glow discharge.

Part I: Plasma treatment of crosslinked gelatin films using AC 50Hz plasma system

In this part, the results were presented and discussed into two sub-parts; A: the effects of plasma condition and storage on the properties of crosslinked gelatin films, and B: the effects of plasma treatment on the enhancement of in *vitro* biocompatibility of crosslinked gelatin films, as described in section 4.5.

A. Effects of plasma condition and storage on the properties of gelatin

AC 50Hz oxygen plasma was introduced to treat crosslinked gelatin films. The effects of plasma conditions including treatment time, discharge power, plasma pressure, and gas flow rate on the properties of crosslinked gelatin were reported. Also, storage effects of plasma-treated crosslinked gelatin films were discussed.

5.2 Wettability of crosslinked gelatin films

The wettability of the plasma-treated substrate is known to be affected by plasma operating conditions such as treatment time, discharge power, plasma pressure, and gas flow rate [53]. Figure 5.2 depicted the water contact angle measured from the plasma-treated and untreated crosslinked gelatin films as a function of discharge power and treatment time. The oxygen flow rate and pressure were fixed at 5 sccm and 1 mbar, respectively. It could be noticed that the water contact angle of untreated crosslinked gelatin films was 86.9^{o}. After plasma treatment for 1 sec, the water contact angle of crosslinked gelatin was significantly decreased compared to untreated film. Increasing treatment time resulted in continuous decreasing of water contact angle. When the crosslinked gelatin films were treated longer than 13 sec, the contact angle seemed to be consistent. This implied that the hydrophilicity of plasma-crosslinked gelatin films was saturated by plasma treatment. The decreasing of water contact angle after plasma treatment revealed the strongly increased hydrophilicity of crosslinked gelatin surface induced by oxygen glow discharge. This could be attributed to the oxygen-containing functional groups that were incorporated onto the surface during plasma treatment [54]. As reported by Morent R. *et al*, treatment of oxygen-containing plasma could introduce C=O and O-C=O polar groups on the surface of polyethylene terephthalate (PET), resulting in enhanced surface hydrophlicity [53].

When discharge power was increased from 3 to 9 watt, the water contact angle of crosslinked gelatin films was faster decreased than that of discharge power 3 watt. However, when discharge power was increased from 9 to 12 watt, the water contact angle of crosslinked gelatin films was unchanged, as seen in Figure 5.2. This could be explained that the increasing of discharge power could promote the amount of reactive oxygen species. This led to more incorporation of oxygen-containing polar groups, resulting in faster decrease in contact angle [53].

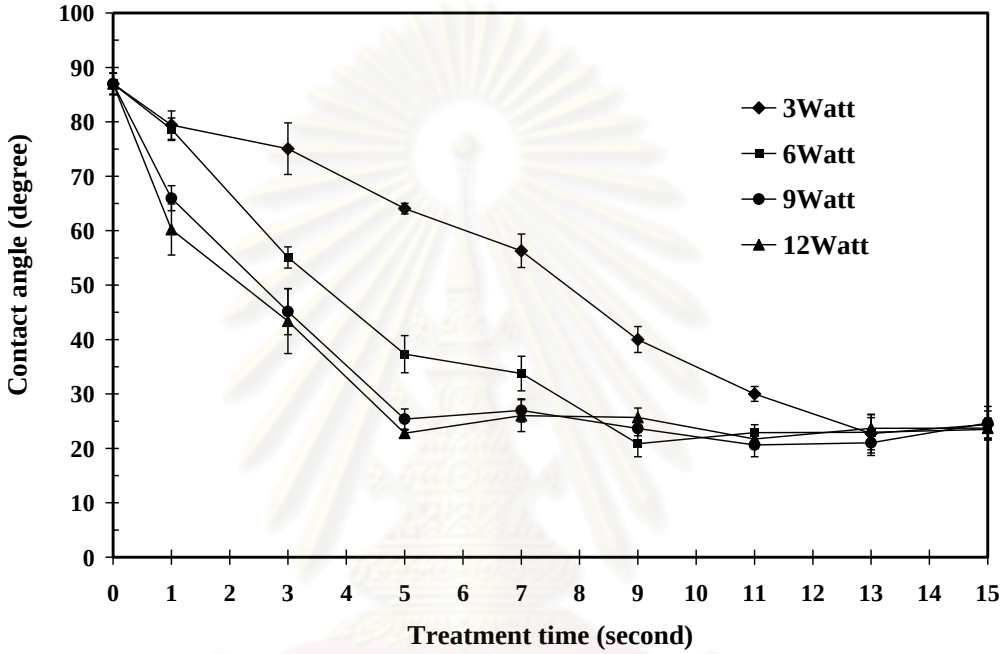


Figure 5.2. The water contact angle of oxygen plasma-treated crosslinked gelatin films as a function of discharge power and treatment time.

The effect of operating pressure on water contact angle of plasma-treated crosslinked gelatin films was shown in Figure 5.3. The oxygen flow rate and discharge power were fixed at 5 sccm and 6 watt, respectively while the pressure was varied from 0.4 to 2 mbar. The result showed that when the plasma treatment was performed at high operating pressure, the water contact angle of plasma-treated crosslinked gelatin films were sharply decreased compared to that at low operating pressure. The water contact angle of crosslinked geatin films at the operating pressure of 0.8, 1, and 2 mbar reached the saturation value after treated with plasma for 9 sec, while at the operating pressure of 0.4 mbar, the water contact angle was unchanged after treated with plasma for 11 sec. This behavior could be attributed to the great number of active oxygen species at high operating pressure [53]. High concentration

of active species could easily incorporate onto the crosslinked gelatin surface, leading to a much lower contact angle.

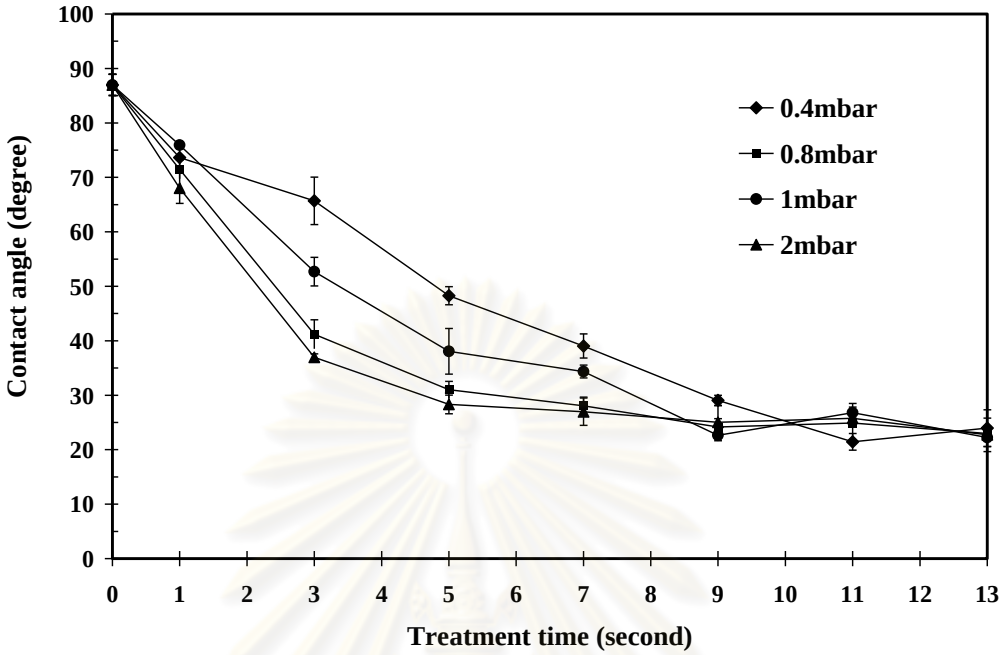


Figure 5.3. The water contact angle of oxygen plasma-treated crosslinked gelatin films as a function of operating pressure and treatment time.

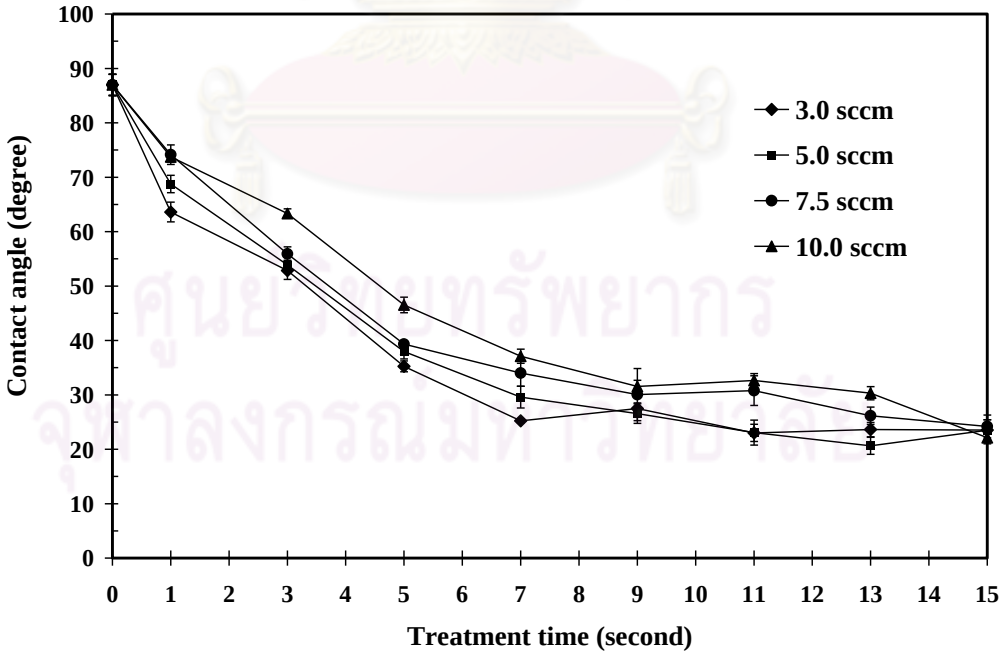


Figure 5.4. The water contact angle of oxygen plasma-treated crosslinked gelatin films as a function of oxygen flow rate and treatment time.

The effect of oxygen flow rate on water contact angle of plasma-treated crosslinked gelatin was presented in Figure 5.4. The pressure and discharge power were fixed at 1 mbar and 6 watt, respectively. It could be noticed that the water contact angle of plasma-treated crosslinked gelatin films at high oxygen flow rate was slowly decreased compared to that at low oxygen flow rate. For the treatment period of 1-13 sec, the water contact angle of film treated by plasma at the oxygen flow rate of 3 sccm was noticed to be significantly lower than those at 10 sccm. At higher oxygen flow rate, the number of reactive oxygen-containing species in the plasma region decreases, leading to slower decrease in water contact angle [53]. The result suggested that, to enhance hydrophilicity of crosslinked gelatin surface, the used gas flow rate should be as low as possible.

5.3 Surface topography of crossliked gelatin film

The surface topography of crosslinked gelatin films before and after oxygen glow discharge treatment investigated by AFM was shown in Figure 5.5 while the calculated mean surface roughness (Rms) of surface roughness was presented in Figure 5.6. The surface of untreated crosslinked gelatin was smooth (Figure 5.5a) and the mean surface roughness was 0.53 nm. After treated with oxygen glow discharge, the gelatin surface became obviously rough and irregular (Figure 5.5b-5.5f). The mean surface roughness was significantly increased with increasing plasma treatment period from 3 to 15 sec. The maximum mean surface roughness, obtained from plasma-treated gelatin film for 15 sec, was 4.63 nm. In general, oxygen plasma treatment is known to have etching effect on polymer surface and produce nanostructure surface topography [44]. Enhanced roughness of crosslinked gelatin surface could be obtained when increasing the plasma treatment period. From the result on surface roughness and water contact angle, it could be noticed that when the contact angle was decreased, the surface roughness was increased. This trend corresponded to the previous study reported by Litte U. *et al.* [55]. This suggested that plasma treatment could affect both water contact angle and surface roughness of crosslinked gelatin films. The alteration of water contact angle of plasma-treated gelatin film could be affected by the change of surface roughness.

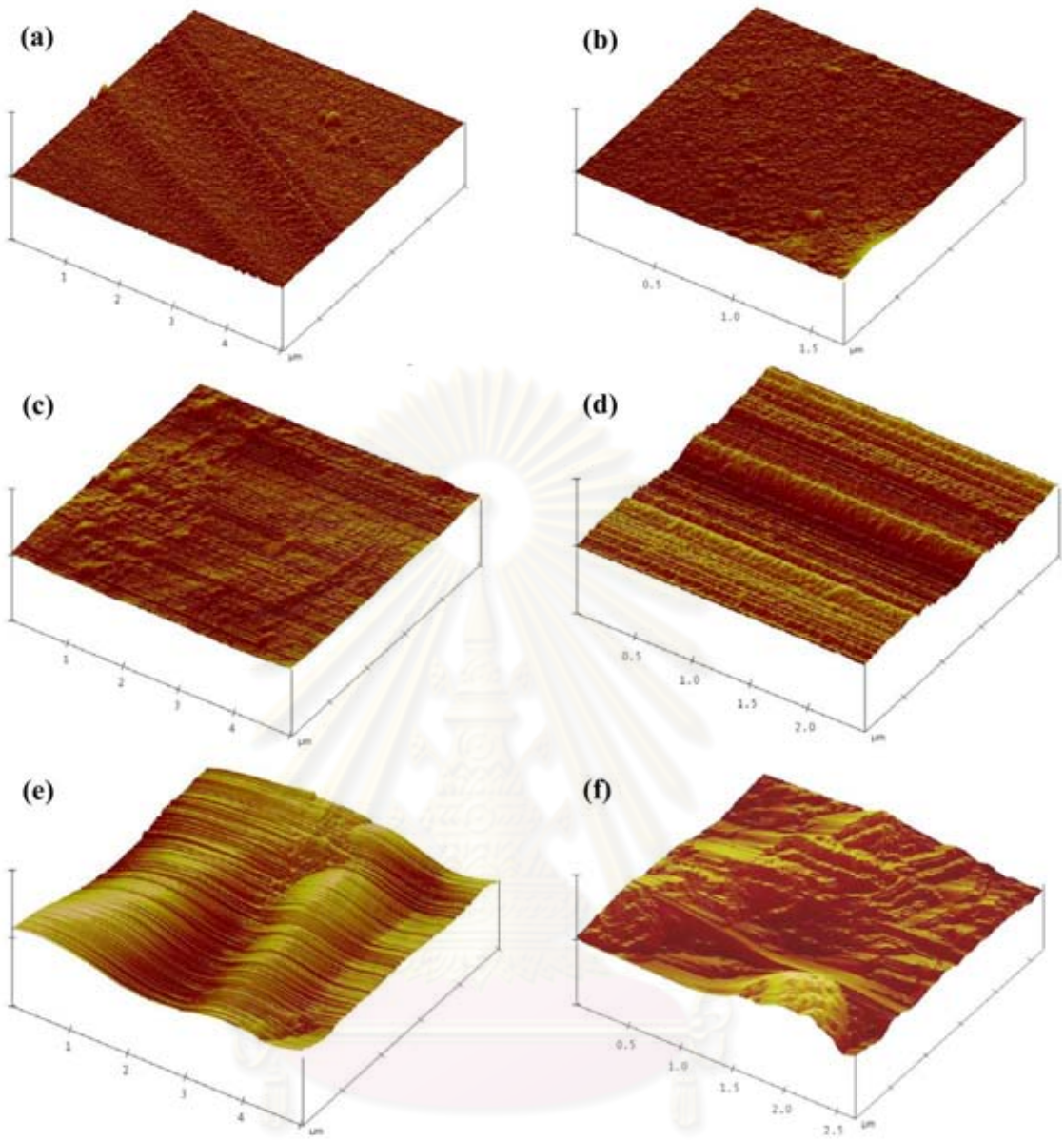


Figure 5.5. Surface topography of (a) untreated crosslinked gelatin film and oxygen plasma-treated crosslinked gelatin films at the treatment time of (b) 3 sec, (c) 5 sec, (d) 9 sec, (e) 13 sec and (f) 15 sec (plasma operating condition: 6 watt, 5 sccm and 1 mbar) (data scale 10 nm.).

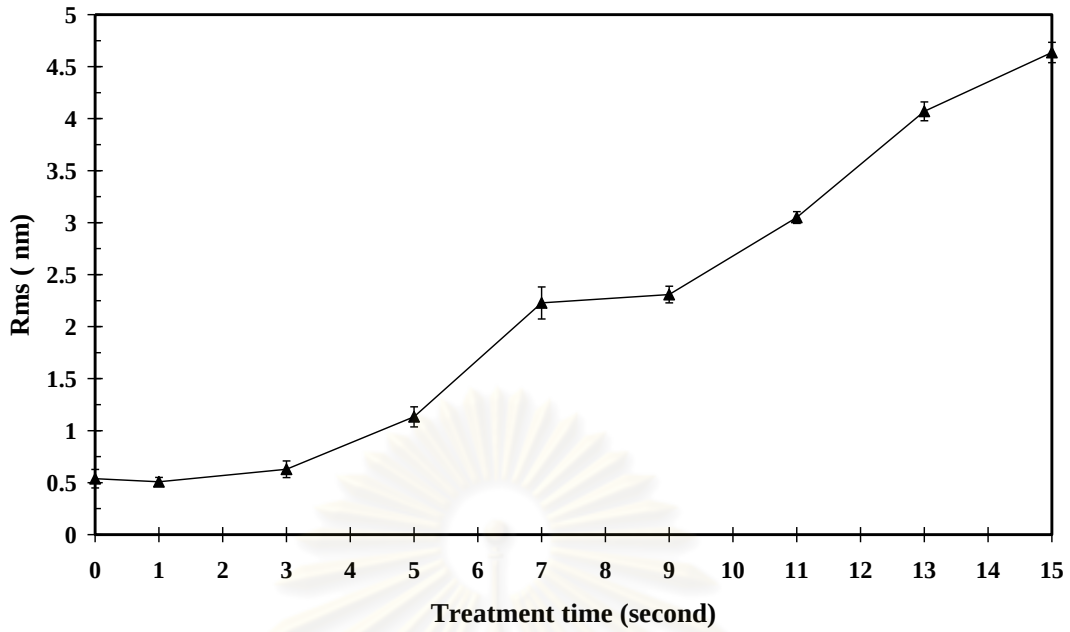


Figure 5.6. The calculated mean surface roughness (Rms) of oxygen plasma-treated crosslinked gelatin films as a function of treatment time.

5.4 Storage effect on plasma-treated crosslinked gelatin films

To investigate the effect of storage, the oxygen plasma-treated gelatin film was stored at different time periods and temperatures. Figure 5.7 showed the water contact angle of oxygen plasma-treated crosslinked gelatin films as a function of storage time and temperature. From the result, it was evident that the contact angle of plasma-treated crosslinked gelatin films was increased after storage at all storage temperatures. After storage for 72 hr, the contact angle of the plasma-treated gelatin films reached the saturation value. The films aged at high temperature (20^{o}C, 50^{o}C) exhibited a faster recovery of contact angle compared to that at low temperature (5^{o}C). Especially at 50^{o}C, the contact angle of the plasma-treated crosslinked gelatin film approached the value of the untreated crosslinked gelatin surface. It has been reported that increasing the storage temperature could enhance the polymer chain mobility and surface rearrangement [53]. This led to a decrease in the polar groups on the surface of gelatin films [26]. At the storage temperature of 5^{o}C which was far below the glass transition temperature (T_g) of gelatin, the low mobility of polymer chain occurred resulting in less hydrophobic recovery of plasma-treated crosslinked gelatin films.

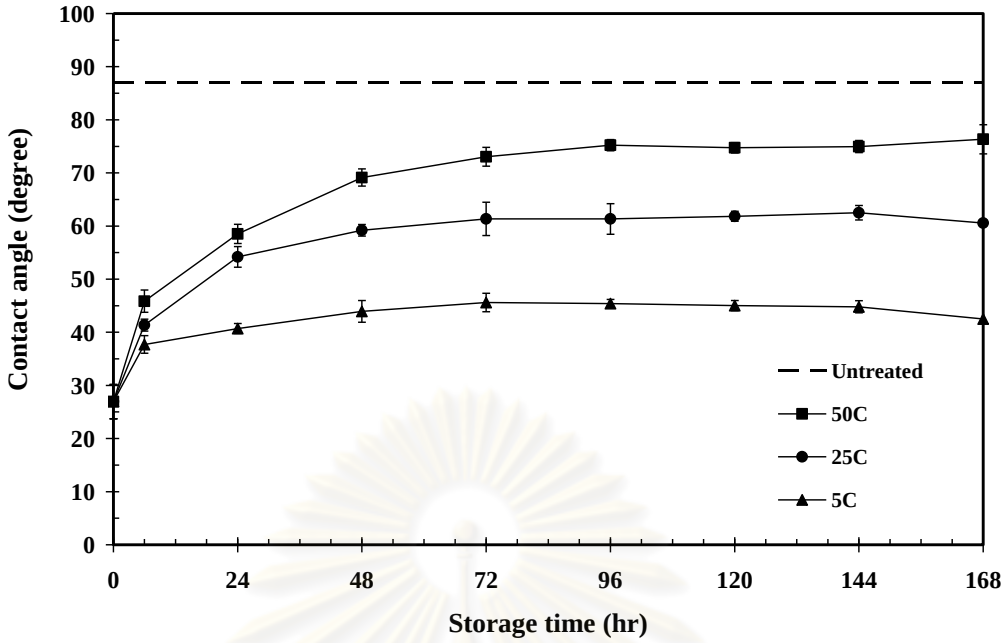


Figure 5.7. The water contact angle of oxygen plasma-treated crosslinked gelatin films after storage at different storage time and temperature.

After storage at 25°C and 5°C for 168 hr (7 days), the alteration of surface chemistry was examined by FTIR-ATR. The results in Figure 5.8 showed that the characteristic peaks of crosslinked gelatin films were at 1655 and 1537 cm^{-1}, corresponded to COO-asymmetric stretching and amide II of gelatin, respectively [56]. After treated with plasma, an obvious transmittance band at 3293 cm^{-1}, corresponded to OH group vibration [57], was observed. This suggested that the oxygen-containing groups such as OH could be incorporated into the crosslinked gelatin surface during plasma process. After storage at the temperature of 25°C and 5°C for 168 hr, the transmittance peak of plasma-treated crosslinked gelatin films at 3293 cm^{-1} tended to slightly decrease. The characteristic peak of the samples stored at 25°C seemed to be similar to that of untreated film. The results from FTIR-ATR revealed that, storage at high temperature could recover the surface chemistry of plasma-treated crosslinked gelatin.

In Figure 5.9, the calculated mean surface roughness (Rms) of plasma-treated crosslinked gelatin films after storage at 25°C for 3 months was compared to that without storage. This depicted that the surface roughness of films caused by plasma treatment was not recovered. The results suggested that the hydrophobic recovery during storage process could be likely due to the recovery of surface chemistry, not

the surface roughness [55,58]. In the other words, the surface roughness of gelatin films obtained from plasma treatment was a non-recovery effect.

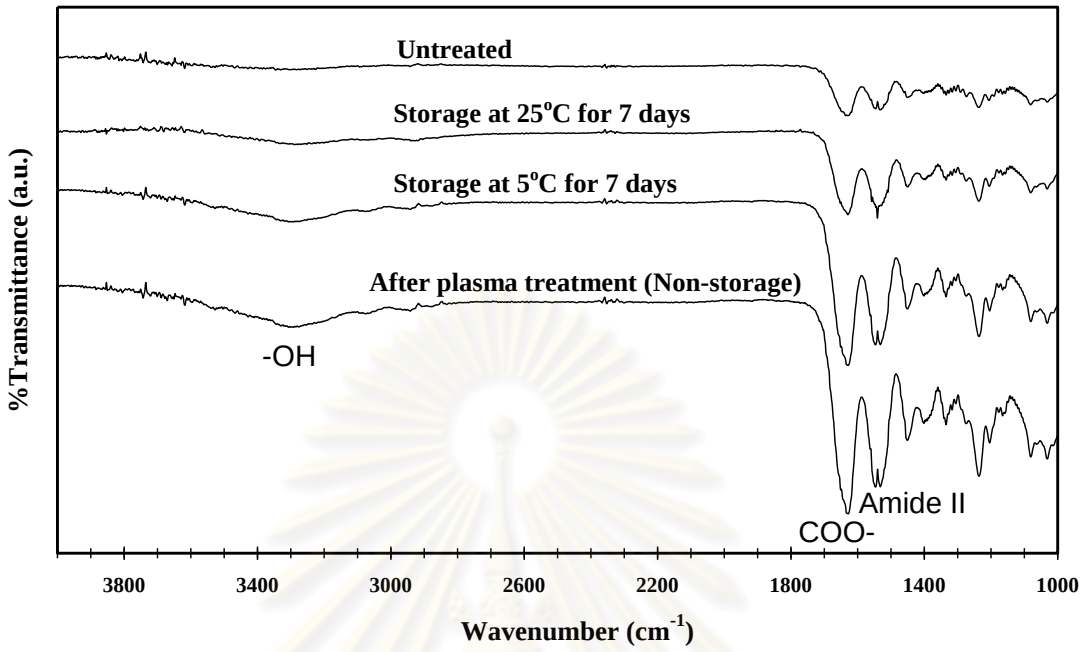


Figure 5.8. The FTIR-ATR spectra of untreated and oxygen plasma-treated crosslinked gelatin films after storage process.

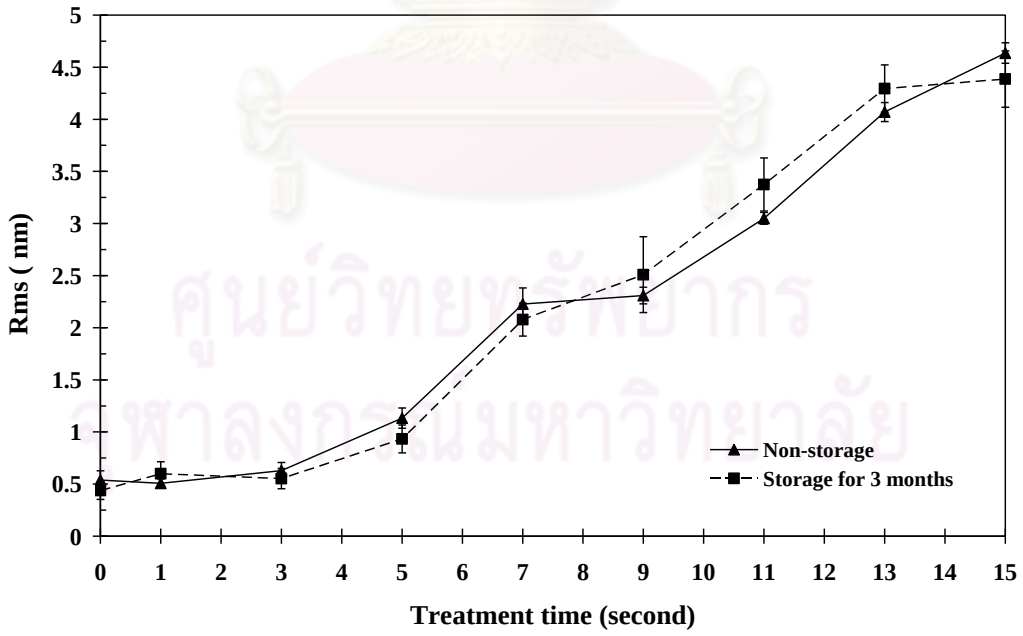


Figure 5.9. The calculated mean surface roughness (Rms) of storage and non-storage oxygen plasma-treated crosslinked gelatin films.

5.5 Summary

Oxygen glow discharge using AC 50Hz was being introduced to treat gelatin, one of the widely used biomaterials. The hydrophilicity of plasma-treated crosslinked gelatin was promoted in comparison to untreated samples. The surface roughness of gelatin film was noticed after plasma treatment. The roughness was further increased with increasing plasma treatment period. We have demonstrated that the reduction in contact angle of plasma-treated crosslinked gelatin films, which indicated the improvement of hydrophilicity, could be due to the cumulative effects of changes in the surface chemistry and surface roughness. The storage process at various temperatures was found to recover the hydrophilicity of the film at different rates, due to the recovery of surface functionality of plasma-treated crosslinked gelatin film. It was suggested that oxygen glow discharge using AC 50Hz could be a potential method to manipulate the surface properties of crosslinked gelatin.

B. Effects of plasma treatment on *in vitro* biological properties of gelatin

In this part, three different types of gas plasmas including nitrogen, oxygen, and air plasma were introduced to treat the crosslinked gelatin films. The effects of plasma treatment on the wettability, surface roughness, and surface chemistry of crosslinked gelatin were elucidated. Lastly, the behavior of rat bone marrow-derived stem cell and L929 mouse fibroblast on plasma-treated gelatin surface were reported, compared to that on untreated surface.

5.6 Wettability and surface energy of crosslinked gelatin films

The water contact angle and surface free energy of untreated and plasma-treated crosslinked gelatin films as a function of treatment time and type of gas were shown in Figure 5.10 and Figure 5.11, respectively. It could be noticed that the water contact angle of untreated crosslinked gelatin films was 84.3±5.2^{o}. After plasma treatment for 3 sec, the water contact angle was sharply decreased to 55.6±4.4^{o}. Longer treatment time resulted in a further decrease till the water contact angle of the crosslinked gelatin films reached 23.4±1.6^{o} at the treatment time of 15 sec 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 crosslinked gelatin was 38.6±2.1 mJ/m^2. After treated with plasma for 3 sec, the total surface free energy was significantly increased to 47.5±1.7 mJ/m^2. However, after treated with plasma for 15 sec, the surface free energy of gelatin film seemed to be consistent at 70.0±1.1 mJ/m^2 for all gases used. Comparing the variation in polar and dispersive components of surface free energy (Figure 5.11), it was shown that after treated with plasma, the polar component of gelatin film was increased, while the dispersive component of the surface was decreased. Similar trend was observed for all gases used.

From the results, it was obvious that the polar groups could be incorporated onto the surface of gelatin during plasma treatment, resulting in highly hydrophilic gelatin surface. In addition to the introduction of polar groups, the wettability of polymer surface was reported to be possibly caused by material crosslinking, surface roughness, and surface chemistry [55,59]. However, our previous study found that short time treatment of plasma had no effect on the degree of crosslinking of gelatin film [16]. Therefore, the decreasing in water contact angle might be resulted from the change of surface roughness and surface chemistry. In order to observe whether the

alteration of surface roughness and chemistry of plasma-treated gelatin surface were the reasons of increased hydrophilicy, the observation of surface topology and chemistry of the gelatin film treated by nitrogen, oxygen, and air plasma must be considered.

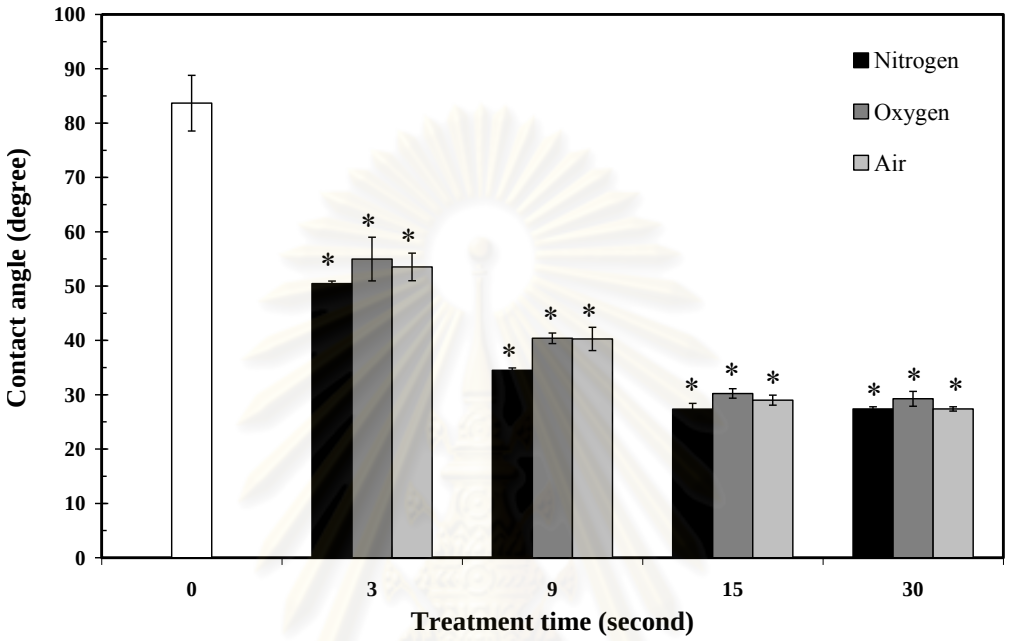


Figure 5.10 Water contact angle of untreated and plasma-treated crosslinked gelatin films using three gases; nitrogen, oxygen, and air. *represented a significant difference at $p<0.05$ relative to untreated films.

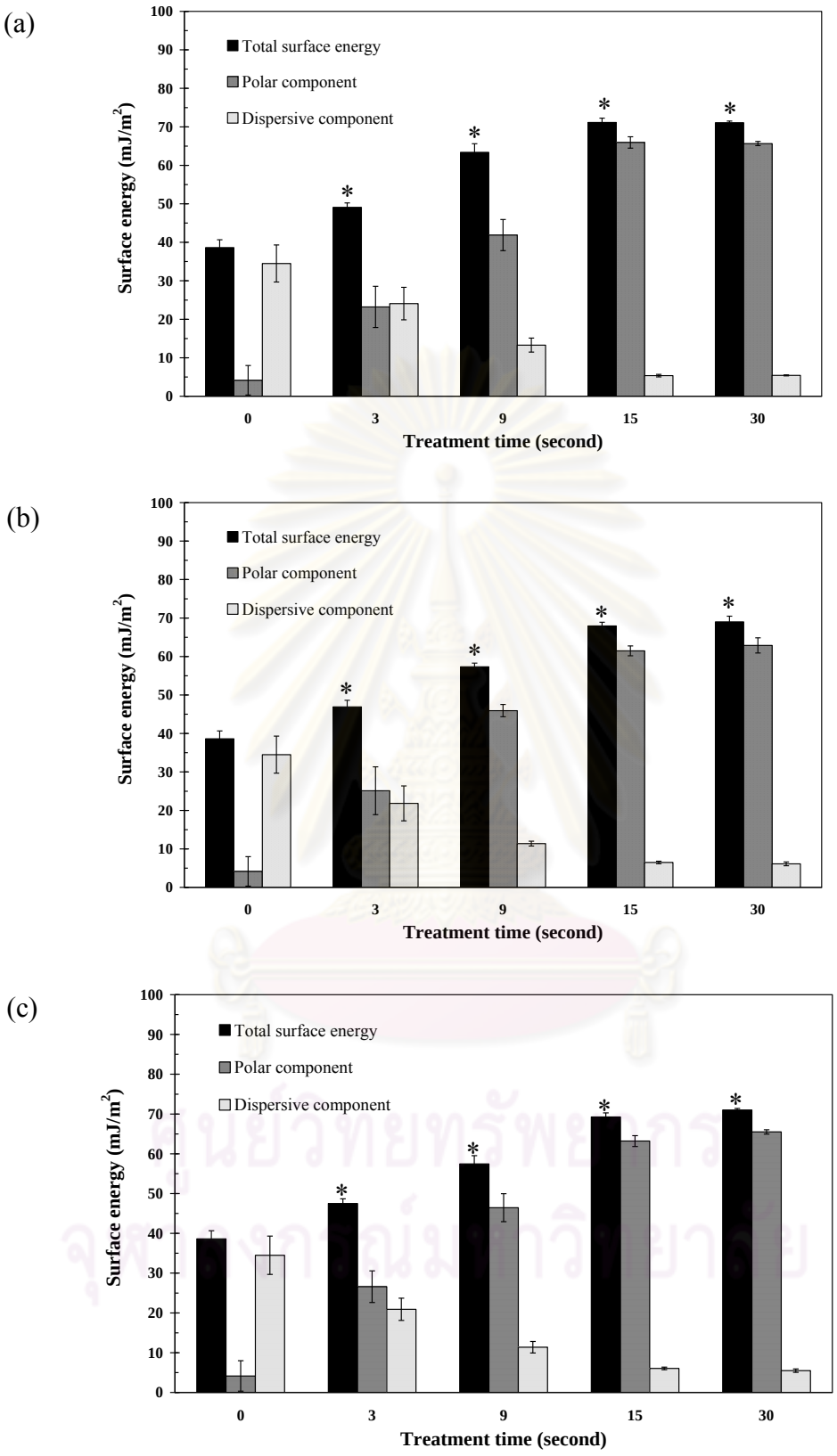


Figure 5.11 The surface energy, polar component and dispersive component of (a) nitrogen plasma-treated gelatin film, (b) oxygen plasma-treated gelatin film and (c) air plasma-treated gelatin film. *represented a significant difference at $p<0.05$ relative to untreated films.

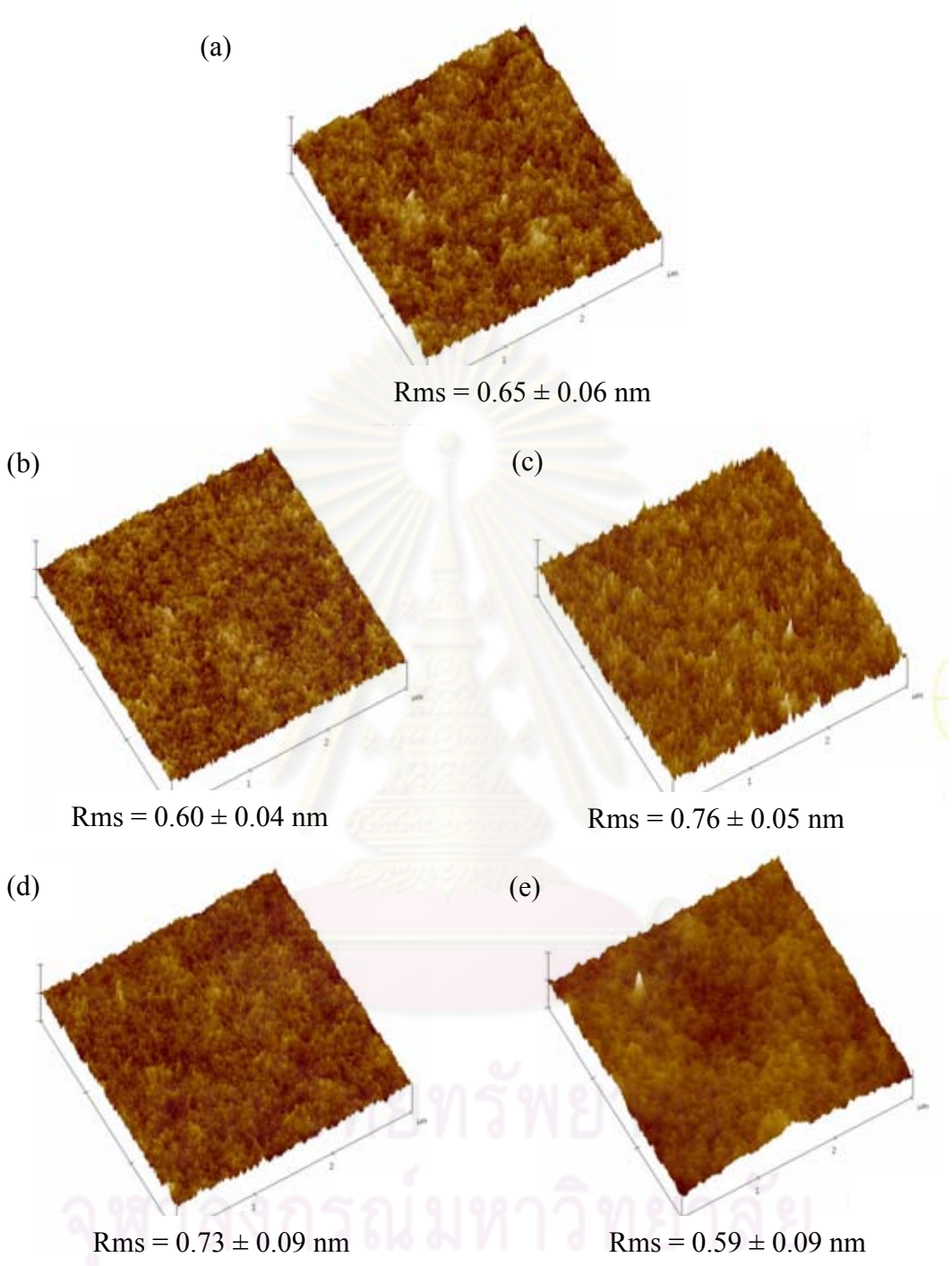


Figure 5.12 Surface topology of (a) untreated crosslinked gelatin films, and oxygen plasma-treated crosslinked gelatin films at 4 watt and 1 mbar for (b) 3 sec, (c) 9 sec, (d) 15 sec and (e) 30 sec (data scale 10 nm.).

5.7 Surface topology of crosslinked gelatin films

As mentioned earlier that surface topology could affect the wettability of the polymer surface. So the effect of plasma treatment on the surface topology of crosslinked gelatin films was investigated. The surface topology and the quantitative parameters of surface roughness of crosslinked gelatin films before and after oxygen plasma treatment investigated by AFM were shown in Figure 5.12. The surface of the untreated crosslinked gelatin was smooth (Figure 5.12a) with the calculated mean surface roughness (Rms) of 0.65±0.60 nm. After treated with oxygen plasma for 3 to 30 sec, the surface topology was unchanged (Figure 5.12b-5.12d). No significant difference between the Rms of untreated and plasma-treated crosslinked 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).

The observation of surface topology in Figure 5.12 revealed that the surface of gelatin films remained unchange after nitrogen, oxygen, and air plasma treatments were applied. Generally, the enhancement of surface roughness caused by plasma treatment depended on the operating conditions such as discharge power, pressure, and treatment time [60]. In this study, the applied AC 50Hz plasma at the operating discharge power of 4 watt could not alter the surface roughness of crosslinked gelatin films since the low density of high energy active species could not physically etch the surface of gelatin film [60]. These results ensured that the alteration of the wettability of plasma-treated crosslinked gelatin films was not affected by surface topology of films.

5.8 Surface chemistry of crosslinked gelatin films

In order to observe whether the alteration of surface chemistry of plasma-treated gelatin surface caused higher hydrophilicity of surface, we have investigated the surface chemistry by XPS. The survey spectra of plasma-treated and untreated were showed in Figure 5.13. It could be seen that after treated with nitrogen, oxygen, and air plasmas, 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 nitrogen plasma-treated and untreated crosslinked gelatin films were performed as showed in Figure 5.14. Four peaks corresponding to N1s (400 eV), O1s (532 eV), C1s (285 eV) and C1s (288 eV) were observed. Based on the peak-fitting, N1s spectra

represents C-NH, O1s represents –COOH or –OH, C1s at 285 eV represents C-CH and C1s at 288 eV represents C-O-O [17]. The same N1s, O1s and C1s spectra of gelatin films were also obtained after treated with oxygen and air plasma.

To quantitatively compare the change of the film before and after plasma treatment, the relative ratios of O/C, N/C and O/N were calculated and summarized in Table 5.1. The result showed that O/C, N/C, and O/N ratios of untreated film were 0.29, 0.14 and 2.1, respectively. After treated with nitrogen plasma for 30 sec, O/C and N/C ratios were increased up to 0.34 and 0.25, respectively, while O/N ratio was decreased to 1.4. Similar phenomena were also observed in air plasma treatment. After treated with air plasma for 30 sec, O/C and N/C ratios of gelatin films were increased up to 0.40 and 0.19, respectively, while O/N ratio was slightly increased to 2.2. When gelatin films were treated with oxygen plasma, the O/C and O/N ratios were 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 sec of treatment.

It is well known that the functional groups such as N-containing and O-containing groups could be generated onto the surface of substrate during plasma treatment [17]. These functional groups might be performed by the formation of N and O species obtained from nitrogen, oxygen, and air plasma. As seen in Table 5.1 that the N/C ratio generated by nitrogen plasma and O/C ratio generated by oxygen plasma was increased with increasing treatment time. This suggested that hydrophilic functional groups such as NH_2 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 water contact angle. It could be concluded that plasma treatment by AC 50Hz 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.

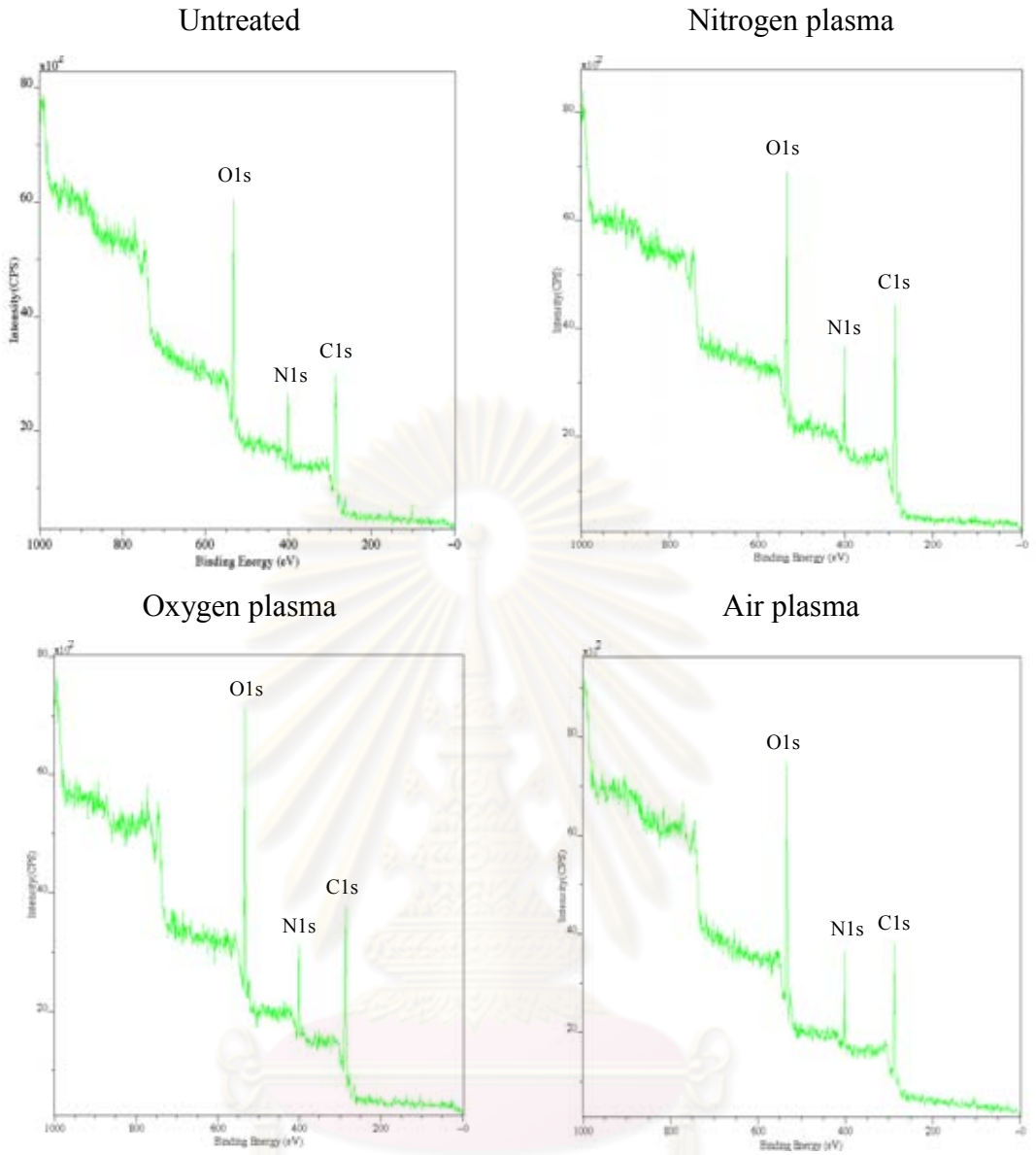


Figure 5.13 XPS survey spectra of untreated and plasma-treated crosslinked gelatin at the treatment time of 15 sec.

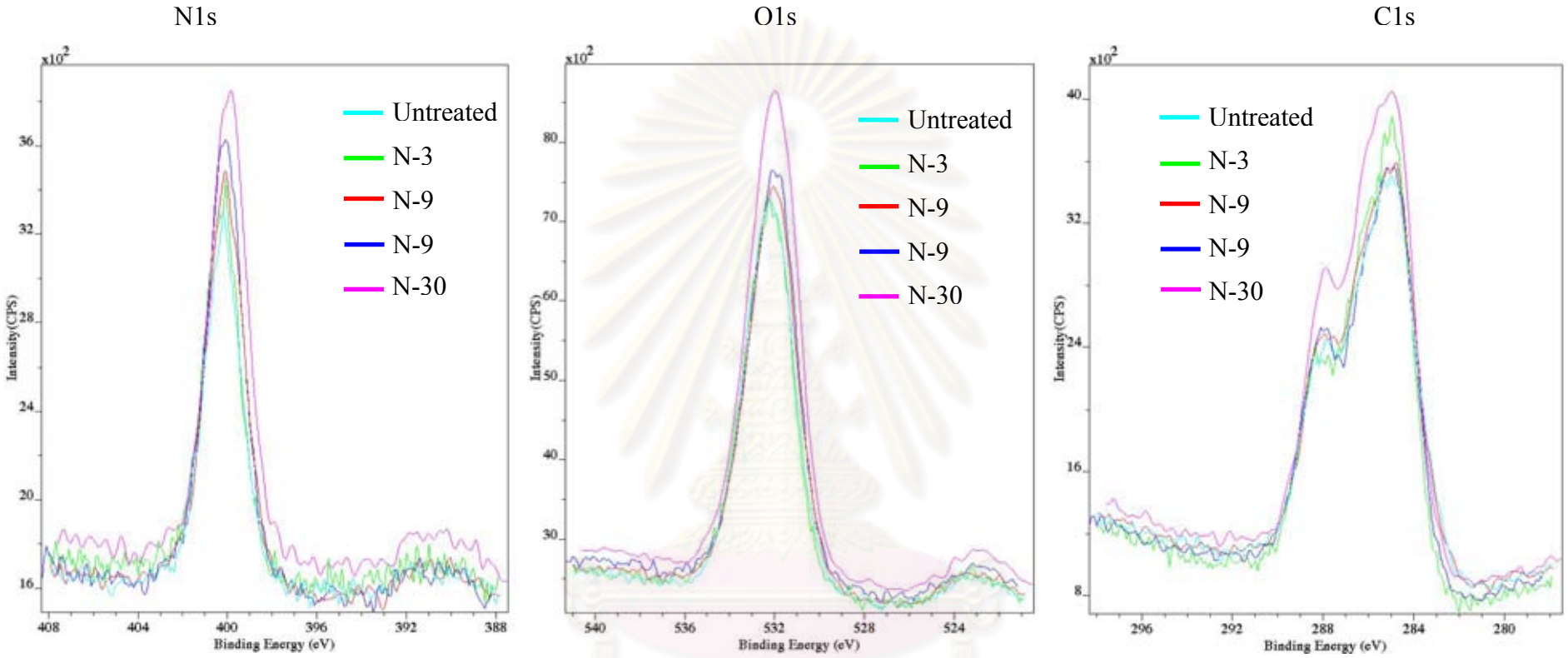


Figure 5.14 N1s, O1s, and C1s spectra of untreated and nitrogen plasma-treated crosslinked gelatin films.

Table 5.1 Atomic compositions and relative ratios of C, N, and O calculated from XPS spectrum of plasma-treated and untreated crosslinked gelatin surface.

Samples	Atomic compositions (%)			Atomic ratio		
	C	O	N	O/C	N/C	O/N
Untreated	70.0	20.4	9.5	0.29	0.14	2.1
N-3	66.4	23.3	10.6	0.35	0.16	2.2
N-9	64.2	22.8	12.9	0.36	0.20	1.7
N-15	57.5	21.1	15.4	0.37	0.27	1.4
N-30	62.7	21.4	15.8	0.34	0.25	1.4
O-3	67.7	23.3	8.8	0.34	0.13	2.6
O-9	64.6	25.9	9.4	0.40	0.15	2.7
O-15	61.0	28.3	10.0	0.46	0.17	2.7
O-30	59.2	30.6	9.7	0.52	0.18	2.9
A-3	65.0	24.8	10.1	0.38	0.16	2.4
A-9	63.7	25.9	10.3	0.41	0.16	2.5
A-15	62.5	26.6	10.8	0.43	0.17	2.4
A-30	63.0	25.2	11.67	0.40	0.19	2.2

5.9 Biocompatibility test

The biocompatibility of untreated and plasma-treated crosslinked gelatin films using MSCs and L929 fibroblast was presented in this section. Due to a limit in cell number, MSCs was used to emphasize on the effects of plasma treatment on the attachment, while L929 was used to investigate the cell proliferation.

5.9.1 MSC cell culture

Attachment of MSC on crosslinked gelatin films

The number of MSCs attached on untreated and plasma-treated gelatin films after cultured for 3, 6, 12, and 18 hr were shown in Figure 5.15. At early stage of attachment (after cultured for 3 hr), the numbers of cells attached on nitrogen, oxygen, and air plasma-treated gelatin films were observed to be more than that on untreated sample. Especially the samples treated with nitrogen plasma for 9 to 30 sec, with oxygen plasma for 3 sec, and with air plasma for 9 sec, significant differences on the number of cells attached were observed, as compared to that on untreated sample. After longer period of culture, it was evident that treatment by nitrogen plasma for 9 to 30 sec, oxygen plasma for 3 sec, and air plasma for 9 sec 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 gelatin surface. The number of cells attached on oxygen and air plasma-treated gelatin films tended to decrease when longer treatment was applied.

Morphology and spreading area of MSCs attached on gelatin film

After 6 hr of cell seeding, the spreading areas of MSCs attached on untreated and plasma-treated crosslinked gelatin were observed as presented in Table 5.2. A significantly larger spreading area of MSCs was noticed on the surface of plasma-treated gelatin films compared to that on untreated surface. The maximum spreading area of MSCs on gelatin surface was obtained when the nitrogen, oxygen, and air plasmas were applied for 15 to 30 sec, 3 sec, and 3 sec, respectively. The corresponding morphology of maximum spread MSCs attached on gelatin film treated with each type of plasma was shown in Figure 5.16. The cells attached on plasma-treated surface were more elongated and flattened compared to rounded shape of cell on the untreated sample (Figure 5.16a).

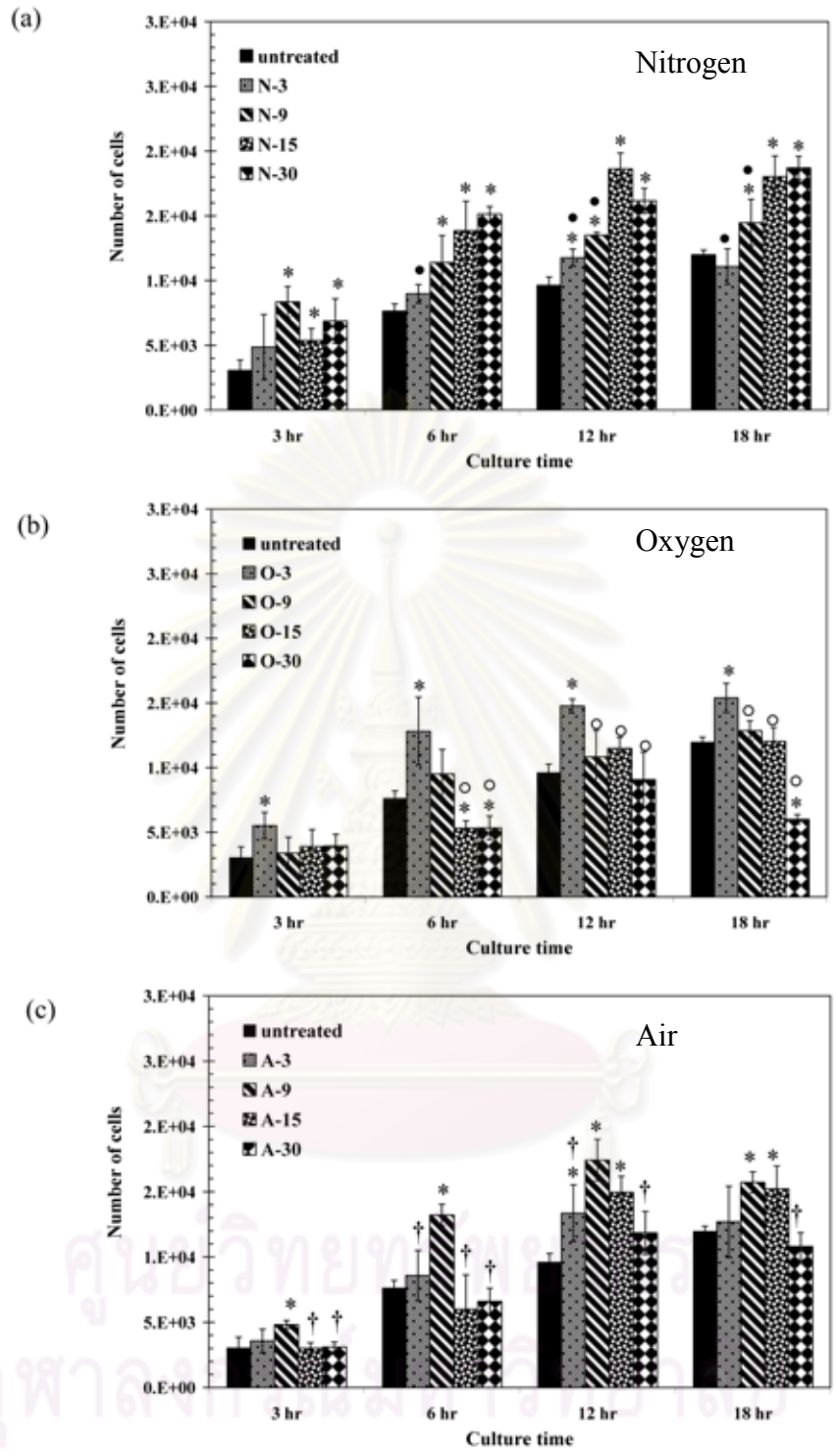


Figure 5.15 Number of MSCs attached on (a) nitrogen plasma-treated gelatin films, (b) oxygen plasma-treated gelatin films, and (c) air plasma-treated gelatin films, determined by DNA assay. * represented a significant difference at $p<0.05$ relative to untreated samples. • represented a significant difference at $p<0.05$ relative to N-15 and N-30. ° represented a significant difference at $p<0.05$ relative to O-3. †represented a significant difference at $p<0.05$ relative to A-9.

Table 5.2 The spreading area of MSCs, and percentage of cell attachment after 6 hr cultured on plasma-treated and untreated gelatin films. * represented a significant difference at $p<0.05$ relative to untreated films.

Samples	Cell spreading area (μm^2)	Percentage of cell attachment (%)
untreated	151±26	38±3
N-3	243±24*	45±4
N-9	301±27*	57±10*
N-15	336±32*	69±11*
N-30	320±30*	76±1*
O-3	269±55*	64±13*
O-9	229±40*	48±10
O-15	227±48	27±3*
O-30	190±40	27±5*
A-3	250±38*	43±10
A-9	210±28*	66±4*
A-15	249±29*	30±13
A-30	200±25	33±5

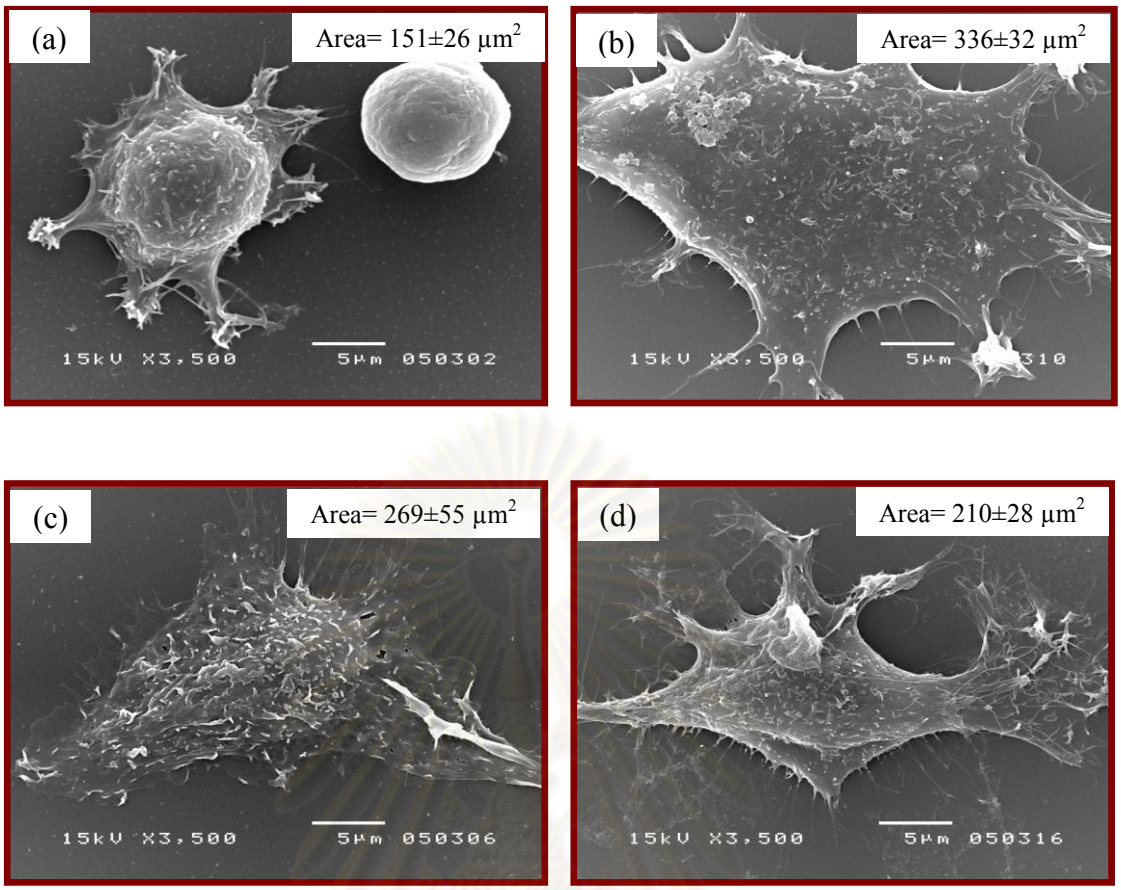


Figure 5.16 Morphology of MSCs cell attached after 6 hr of culture on (a) untreated gelatin film, and plasma-treated gelatin films using (b) nitrogen for 15 sec, (c) oxygen for 3 sec, and (d) air for 9 sec.

The results from *in vitro* cell culture using MSCs ensured that the plasma-treated gelatin film induced cell to attach with large spreading area as elucidated in Table 5.2 and Figure 5.16. These revealed that the plasma-treated gelatin films were more favorable to cell attachment than untreated gelatin films. This could be explained by appropriate surface property such as wettability that was suitable for cell attachment. Many studies have reported the interaction of different types of cells with various substrates having different wettability. Tamada Y. *et al.* stated that a polymer surface with a water contact angle of 70^{o} provided the most suitable surface for cell adhesion [6]. However, the surface used in their report was not well controlled in term of roughness, ionic charge, etc. Lee J. H. *et al.* also reported the adhesion of fibroblast and endothelial cells on polyethylene surface with wettability gradient produced by corona discharge. They found the maximum cell adhesion on the surface with the

water contact angle of 55^{o} [39]. According to our results, it was shown that the water contact angle of gelatin surface suitable for MSCs cell attachment depended on the type of gas used to generate plasma. In other words, differences in surface chemistry introduced by various types of gas plasmas provided different 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 MSC on gelatin surface appeared at the film plasma-treated for 15 and 30 sec (water contact angle = 27^{o} to 28^{o}). It was also reported in our previous study that gelatin surface treated with nitrogen pulse inductively coupled plasma (PICP), having the contact angle of 27^{o} to 28^{o} provided the greatest attachment of L929 mouse fibroblast [16]. The greater attachment behavior could be attributed to the increase of N-containing functional groups such as NH_2 on the surface of gelatin during nitrogen plasma treatment. Since the adhesive glycoproteins such as fibronectin and vitronectin played 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 [61]. In addition, N-containing functional groups incorporated into the gelatin surface could provide positively charged surface at physiological pH, inducing better interaction with negatively charged cell surface [47,62].

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 was continuously increased with increasing treatment time, the number of adhered cells was increased and then decreased at longer treatment time. This suggested that 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 sec (water contact angle=55^{o}) for oxygen plasma treatment, and at the treatment time of 9 sec (water contact angle=40^{o}) for air plasma treatment, as summarized in Table 5.3. 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 [62]. 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 previous report by Shin Y. N. *et al.* [45]. They examined human

bone marrow derived stem cells (hBMSCs) adhesion to polyethylene surface with a gradient of wettability prepared by oxygen-containing gas corona discharge. It was reported the maximum cell adhesion on substrate with moderate hydrophilicity at water contact angle of 57^{o}.

Interestingly, as observed in Figures 5.15b-5.15c, high hydrophilicity of oxygen and air plasma-treated gelatin surface could not further enhance cell attachment. The highly hydrophilicity could be resulted from the high O/C ratio introduced onto gelatin surface. Daw R. *et al.* reported that an increase of O/C ratio and carboxylic acid reduced the number of attached osteoblast-like cell. In addition, increase of O/C ratio led to highly negative charge of the gelatin surface [63], which prohibited the adhesion of negatively charged cell through electrostatic repulsion [64].

From each gas plasma treatment, suitable surface properties of gelatin films for maximum attachment of MSCs after 6 hr of seeding were summarized in Table 5.3. There were more MSCs attached on plasma treated surface (64-76%) compared to untreated films (38%). It was obvious that the types of gas used to generate plasma greatly affect the attachment behavior of MSCs. Among the plasma treated surface using various types of gas, gelatin surface treated with nitrogen plasma for 15 to 30 sec could best enhance the attachment of MSCs (69-76%). Corresponding water contact angle and O/N ratio of nitrogen plasma-treated gelatin film for best attachment of MSCs were 27-28^{o} and 1.4, respectively.

Table 5.3 Summary of the conditions of plasma treatment at which maximum %attachment of MSCs on gelatin films was noticed and their corresponding surface properties.

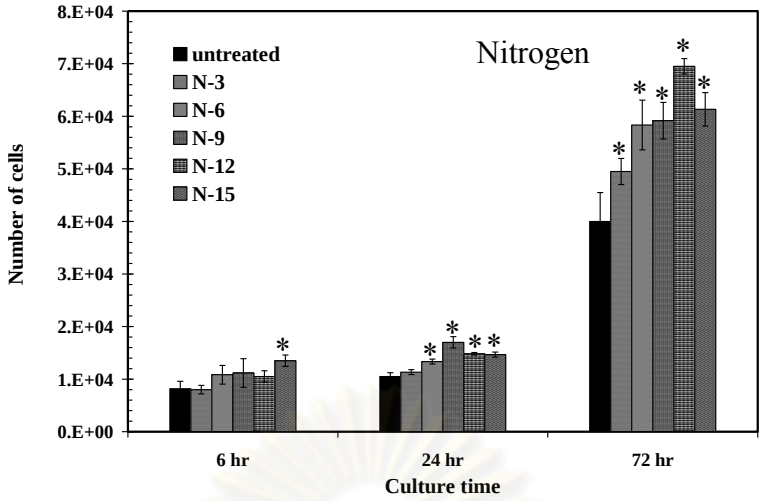
Type of gas	%attachment	Treatment period (sec)	Water contact angle (^{o})	O/C ratio	N/C ratio	O/N ratio
Untreated	38	-	84	0.29	0.14	2.1
Nitrogen	69-76	15, 30	27-28	0.34-0.36	0.25-0.27	1.4
Oxygen	64	3	55	0.34	0.13	2.6
Air	66	9	40	0.41	0.16	2.5

5.9.2 L929 fibroblast cell culture

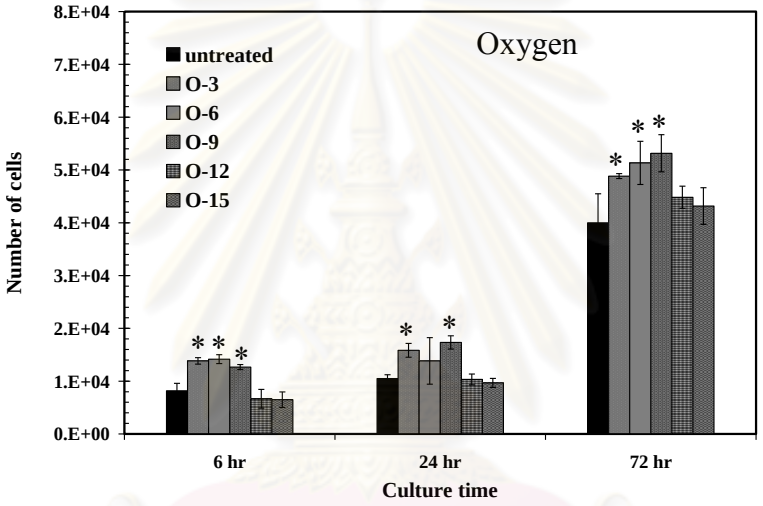
The number of L929 attached and proliferated on untreated and plasma-treated gelatin films after cultured for 6, 24, and 72 hr was shown in Figure 5.17. After cultured for 6 and 24 hr, the numbers of cells attached on nitrogen, oxygen and air plasma-treated gelatin films were observed to be more than that on untreated sample. Especially the samples treated with nitrogen plasma for 6 to 15 sec, with oxygen plasma for 3 to 9 sec and with air plasma for 6 sec, significant differences in the number of cells attached were observed, as compared to that on untreated sample. The %attachment of cell on nitrogen, oxygen and air plasma-treated films were significantly increased compared to that on untreated samples, as showed in Table 5.4. However, longer treatment by oxygen and air plasma could not further enhance cell attachment on gelatin surface. The number of cells attached on plasma-treated gelatin film, when oxygen and air plasma was applied for 12 to 15 sec, seemed to decrease compared to that on untreated sample. This corresponded to the result obtained from the attachment of MSC on plasma-treated gelatin film as described in section 5.9.1. After 72 hr of culture, it was evident that treatment by nitrogen plasma for 6 to 15 sec, with oxygen plasma for 3 to 9 sec and with air plasma for 3 to 6 sec could greatly enhance the growth of cells on the gelatin films. The population doubling time of cells on nitrogen, oxygen and air plasma-treated gelatin samples were decreased compared to that untreated sample, as seen in Table 5.4.

Considering the proliferation of L929 after 72 hr of culture, it was evident that more proliferated cells on plasma-treated films were remarkably observed. The shorter population doubling time of cells cultured on plasma-treated film indicated the higher growth of cells, compared to ones on untreated films. These revealed that the plasma-treated gelatin films were more favorable for cell proliferation than untreated gelatin films. In addition, the maximum in the number of proliferated cell were observed at the treatment time of 6 to 15 sec for nitrogen gas, 3 to 9 sec for oxygen gas, and 3 to 6 sec for air. This could be the results from more adhered L929 on plasma-treated surface after culture for 6 hr.

(a)



(b)



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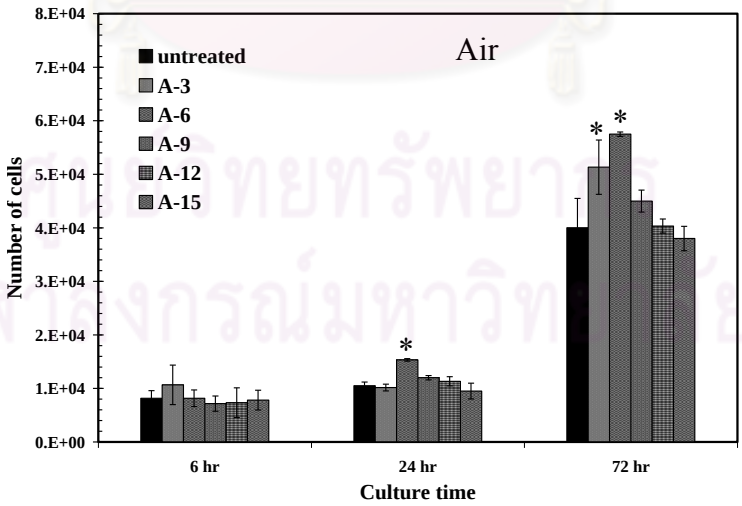

Figure 5.17 Number of L929 attached on (a) nitrogen plasma-treated gelatin films, (b) oxygen plasma-treated gelatin films, and (c) air plasma-treated gelatin films, determined by MTT assay. * represented a significant difference at $p<0.05$ relative to untreated samples.

Table 5.4 The spreading area, percentage of cell attachment after 6 hr of culture and population doubling time of L929 fibroblast on plasma-treated and untreated gelatin films. * represented a significant difference at $p<0.05$ relative to untreated films.

Samples	Cell spreading area (μm^2)	Percentage of cell attachment (%)	Population doubling time (hr)
untreated	126±20	40±7	29.5
N-3	149±26	40±4	24.5
N-6	195±28*	54±9	25.5
N-9	221±34*	56±4*	26.6
N-12	235±21*	53±5*	21.5
N-15	258±31*	68±5*	23.2
O-3	246±25*	69±3*	25.5
O-6	251±37*	71±4*	25.3
O-9	236±26*	63±2*	26.7
O-12	139±30	33±9	26.6
O-15	147±31	33±7	24.2
A-3	206±20*	53±3*	23.6
A-6	241±27*	41±4	24.2
A-9	226±26*	36±2	24.2
A-12	149±30	37±9	27.2
A-15	147±31	39±7	28.0

5.10 Summary

AC 50Hz plasma is being introduced to treat gelatin, one of the widely used biomaterials, in this study. The hydrophilicity and surface energy of plasma-treated gelatin was promoted in comparison with untreated sample. AC 50Hz glow discharge could alter the surface chemistry, but not surface roughness, of gelatin film. From *in vitro* MSCs and L929 fibroblast cell culture, the results revealed that cells could adhere and proliferate on plasma-treated samples better than untreated samples. In this work, we first reported the specific water contact angle and oxygen to nitrogen (O/N) ratio of plasma-treated gelatin surface suitable for MSCs and L929 fibroblast attachment which depended on both the type of gases used and the treatment time. Moreover, among the three types of plasmas used, nitrogen plasma treatment could best enhance the biocompatibility of crosslinked gelatin films.

Part II: Plasma treatment of crosslinked gelatin films using PICP system

PICP plasma was introduced to treat crosslinked gelatin films. Nitrogen was selected to generate PICP plasma since it induced best cell attachment as found in Part I. The properties of PICP-treated gelatin film including water contact angle, degree of crosslinking, surface roughness, and thermal properties were reported. In addition, the cell affinity of PICP-treated gelatin films was evaluated using L929 mouse fibroblast as a model cell *in vitro*.

5.11 PICP plasma characterization

The emission spectrum of PICP plasma generated using nitrogen was presented in Figure 5.18. The results showed that the spectrum mainly consisted of nitrogen molecule radical ($N_2^{\bullet}$), nitrogen atom radical ($N^{\bullet}$), nitrogen molecule ion (N_2^{+}), nitrogen atom ion (N^{+}), as indicated by the strong peaks at the wavelengths of 567.75 nm, 746.83 nm, 391.44 nm, and 491.03 nm respectively [51]. Moreover, the hydrogen radicals at the wavelength of 657.08 nm and 485.96 nm were also observed. This result was similar to the spectrum peak obtained from the use of nitrogen AC 50Hz plasma system. However, the intensity of the spectrum obtained when PICP was applied, was much higher than that AC 50Hz system. The results on optical emission spectroscopy implied that the N-containing functional groups were performed by N reactive species on the surface of crosslinked gelatin films during PICP treatment.

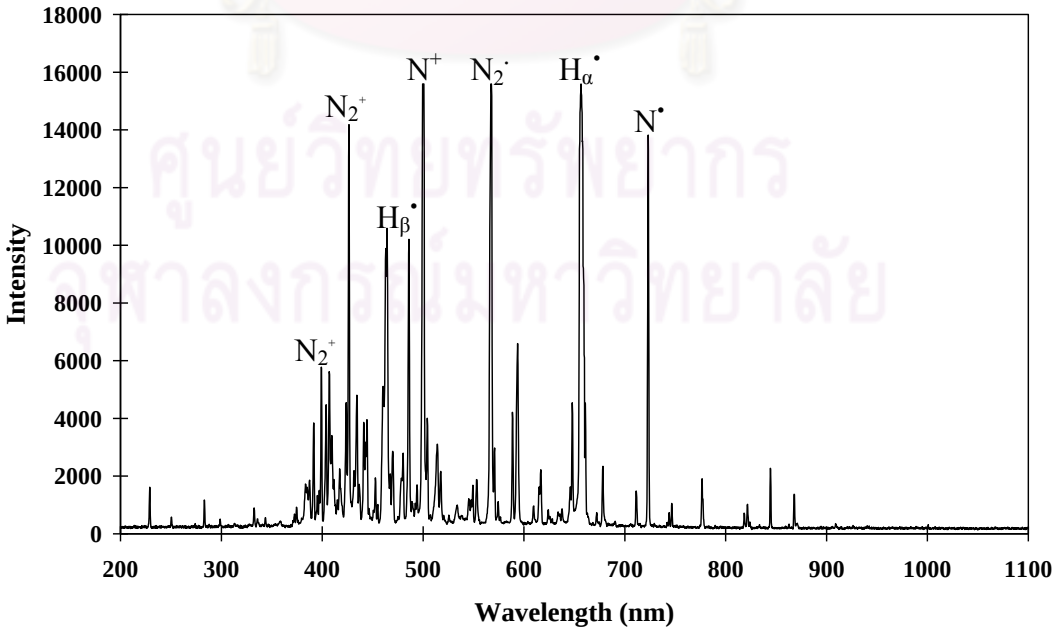

Figure 5.18 Optical emission spectrum of nitrogen plasma generated by PICP system.

5.12 Wettability of crosslinked gelatin films

A comparison of contact angles of untreated and PICP-treated gelatin films was shown in Figure 5.19. It could be noticed that the contact angles to water and ethylene glycol of PICP-treated gelatin films were lower than those of untreated samples. The contact angle to water and ethylene glycol of PICP-treated gelatin films was decreased with increasing the number of repeated discharges.

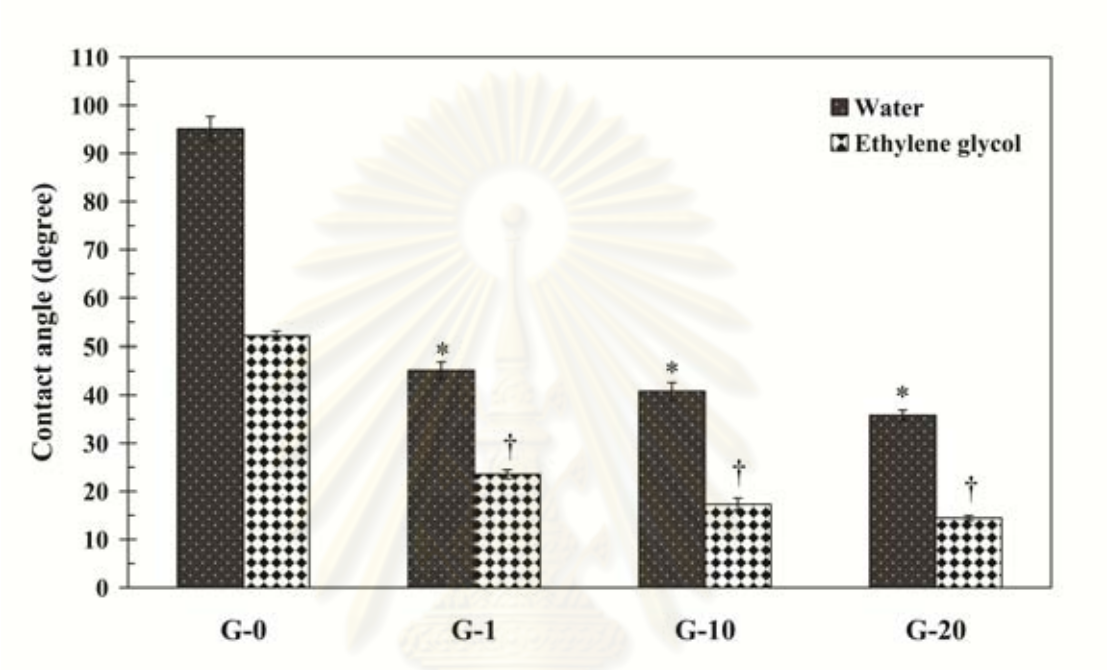

Figure 5.19 Water and ethylene glycol contact angle on PICP-treated and untreated gelatin films. * and † represented a significant difference at $p<0.05$ relative to G-0.

The calculated surface free energy of untreated and PICP-treated gelatin films was compared in Figure 5.20. The surface free energy of crosslinked gelatin was significantly increased from 36.5±1.5 to 53.8±2.1 mJ/m^2 after a single pulse discharge of PICP. When the pulse discharge was repeated to 10 and 20 times, the surface free energy of crosslinked gelatin film was slightly increased to 60.0±1.5 and 60.2±1.8 mJ/m^2, respectively.

The results revealed that the crosslinked gelatin film was more hydrophilic after PICP treatment. Hydrophilic functional groups were possibly introduced. Suggestions were made by Yang J., et al. where surface of hydrophobic materials, such as poly (D,L-lactide), treated by nitrogen gas plasma yielded hydrophilic functional groups [21]. This might be attributed to the increase in polar components after the nitrogen plasma treatment was introduced [23].

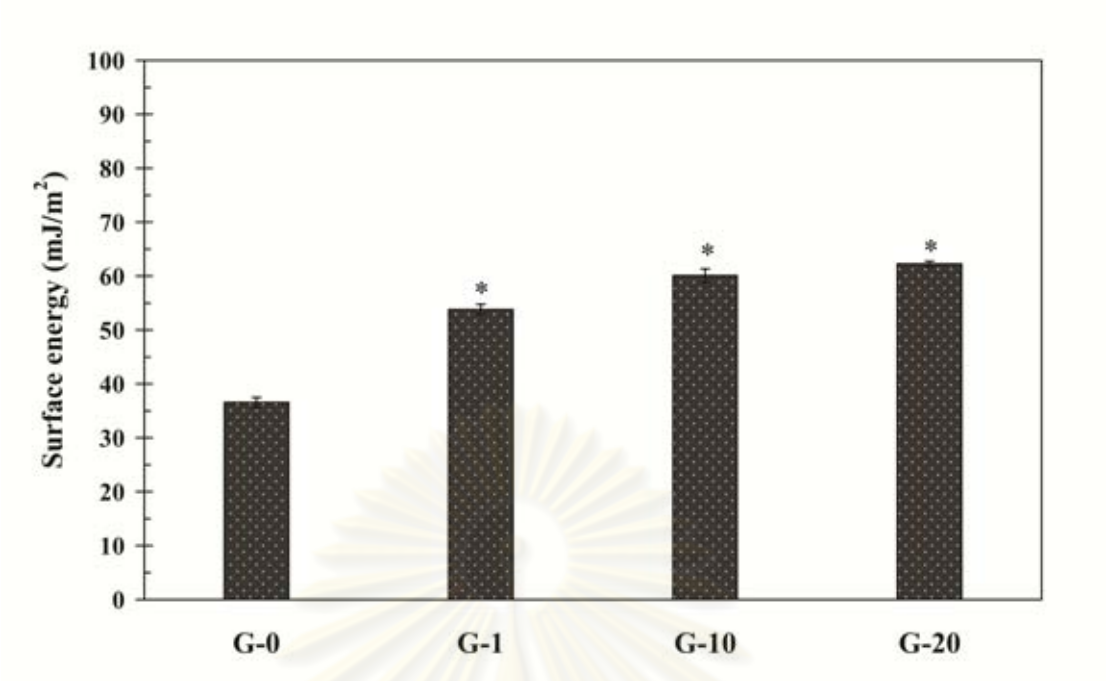

Figure 5.20 Surface energy of PICP-treated and untreated gelatin films. * represented a significant difference at $p<0.05$ relative to G-0.

5.13 Thermal property of crosslinked gelatin films

The thermal behavior of untreated and plasma-treated crosslinked gelatin films examined using differential scanning calorimetry technique was presented in Figure 5.21. It could be seen that all untreated and PICP-treated gelatin showed an endothermic melting peak at around 221^{o}C. No change in the thermal property of untreated and PICP-treated gelatin was noticed. The result implied that the plasma treatment did not induce any significant thermal behavior changes.

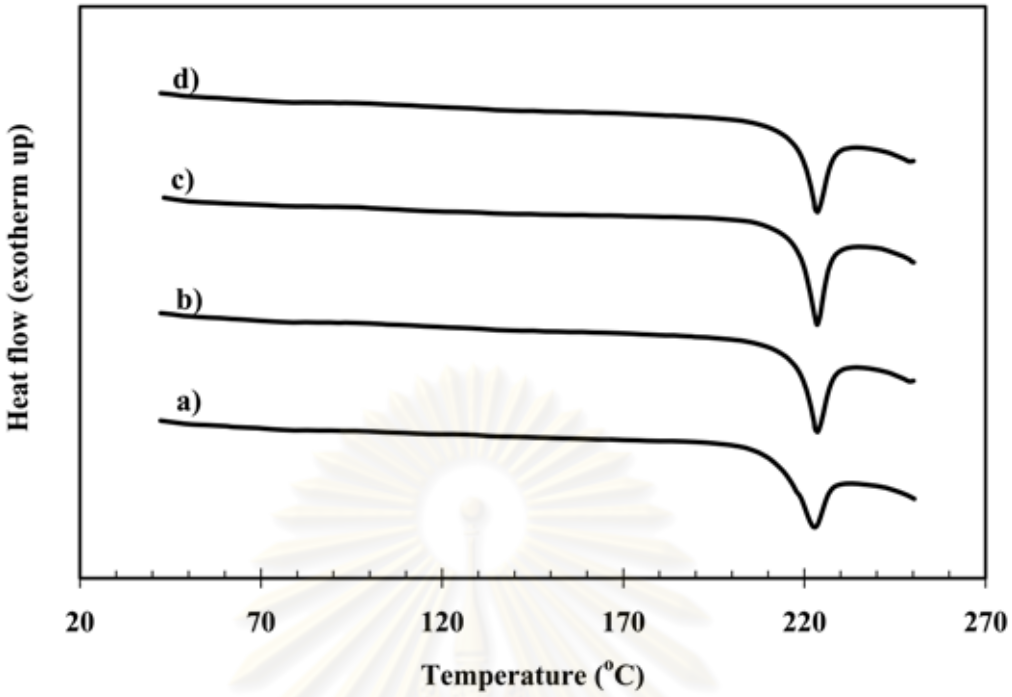

Figure 5.21 DSC thermograms of PICP-treated and untreated gelatin films: a) G-0, b) G-1, c) G-10, and d) G-20.

5.14 Degree of crosslinking of crosslinked gelatin films

The degrees of crosslinking of untreated and PICP-treated gelatin films presented in Figure 5.22 showed that the percentage of croslinking of dehydrothermal crosslinked gelatin before PICP treatment was 40.0±5.2%. This corresponded to the previous work reported on the degree of crosslinking of Type A gelatin by dehydrothermal technique [13]. After a single and repeated pulse PICP treatment, the degree of crosslinking of gelatin films remained unchange, implying that the application of PICP treatment on the surface of crosslinked gelatin did not induce any further crosslinking in gelatin films. This revealed that PICP could not create further crosslinking of gelatin films. This was possibly due to the short exposure time in PICP treatment.

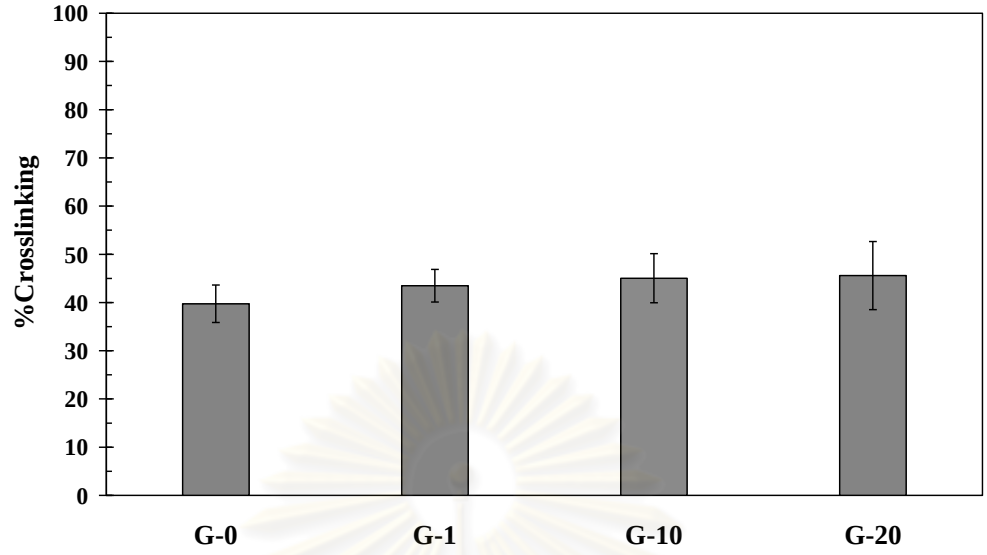

Figure 5.22 Degree of crosslinking of PICP-treated and untreated gelatin films. *represented a significant difference at $p<0.05$.

5.15 Surface topology of crosslinked gelatin films

The surface topography of crosslinked gelatin films before and after nitrogen PICP treatment investigated by AFM was shown in Figure 5.23 while the quantitative parameters of surface roughness were presented in Table 5.5. The surface of the untreated gelatin was smooth (Figure 5.23a) and the calculated mean surface roughness Ra and Rms were 0.390±0.026 and 0.488±0.033 nm, respectively. However, after a single PICP discharge, the sample surface became obviously rough and irregular (Figure 5.23b). The mean surface roughness was significantly increased to Ra = 0.838±0.031 nm and Rms = 1.075±0.087 nm. Many large valleys and peaks with nano-scale were produced. When the PICP discharge was repeated in succession for 10 and 20 times, the roughness of the gelatin film decreased (Figure 5.23c and 5.23d). The mean surface roughness was slightly decreased to Ra = 0.683±0.071 nm and Rms = 0.868±0.101 nm after 10 discharges, and Ra = 0.577±0.044 nm, and Rms = 0.710±0.087 nm for 20 discharges**.**

The AFM results on surface topography shown in Figure 5.23 indicated that the surface roughness of gelatin films occurred after treated with a single PICP discharge. In general, plasma treatment is known to have etching effect on polymer surface and produce nanostructure surface topography [18,44]. However, in this case,

repeating number of discharges did not further enhance the surface roughness of gelatin film. Large cavities on the polymer surface were observed, resulting in the low surface roughness when increasing number of repeated PICP discharges was applied.

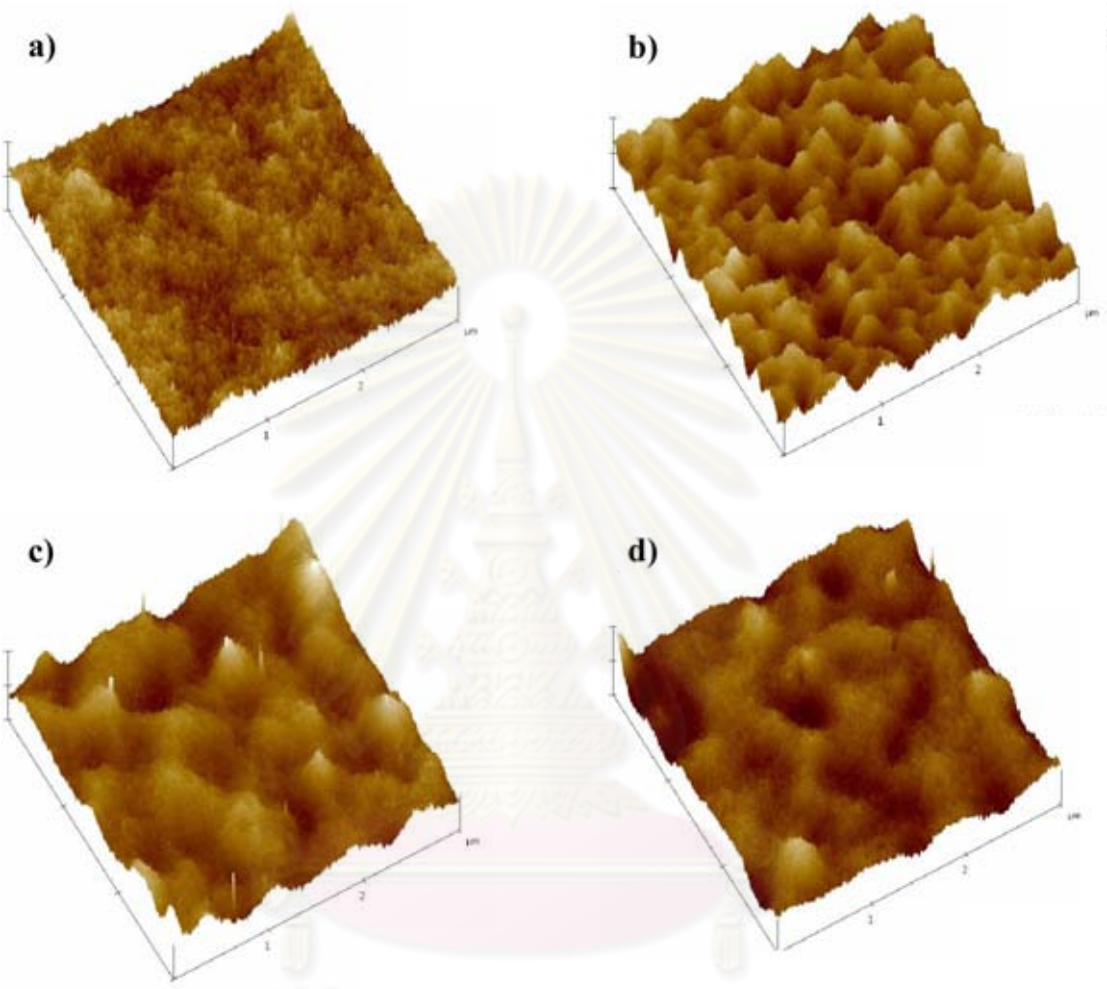

Figure 5.23 Surface topography of PICP-treated and untreated gelatin films: a) G-0, b) G-1, c) G-10, and d) G-20 (data scale 5 nm.).

Table 5.5 Surface parameters and the spreading area of cells after 24 hr cultured on PICP-treated and untreated gelatin films. (* represented a significant difference at $p<0.05$ relative to G-0).

Samples	G-0	G-1	G-10	G-20
Surface parameters				
*R*a (nm)	0.390±0.026	0.838±0.031*	0.683±0.071*	0.557±0.044*
*R*ms (nm)	0.488±0.033	1.075±0.087*	0. 868±0.101*	0.710±0.087*
Cell spreading area (μm^2)	143.83±28.42	280.88±53.42*	259.71±62.73*	274.00±71.27*
Population doubling time (hr)	27.02	22.22	24.39	24.93

5.16 In vitro cell culture

Figure 5.24 presented the number of L929 mouse fibroblasts attached and proliferated on untreated and PICP-treated gelatin films after cultured for 6, 24, and 72 hr. After 6 hr of culture, the number of cells attached on PICP-treated gelatin films tended to be higher than that on untreated sample. No significant difference in the number of cells on various films was observed. However, the more elongated shape of cells was remarkably observed on PICP-treated surface, compared to those on the untreated gelatin films, as observed in Figure 5.25. After 24 hr of culture, it was obvious that introducing of PICP treatment could enhance the number of cells on the film surface. The morphology of cells on both PICP-treated and untreated gelatin films exhibited spindle shape and elongated form, but a significantly larger spread cells were observed on the surfaces of PICP-treated gelatin films as presented in Table 5.5. After 72 hr of culture, the number of proliferated cells on PICP-treated gelatins films was significantly higher than that on untreated samples. No significant difference in the number of cells on gelatin films treated with a single or repeated discharges were observed. As seen in Table 5.5, the population doubling time of cells on untreated and PICP-treated gelatin films for 1, 10 and 20 repeated discharges was found to be 27.02, 22.22, 24.39 and 24.93, respectively,.

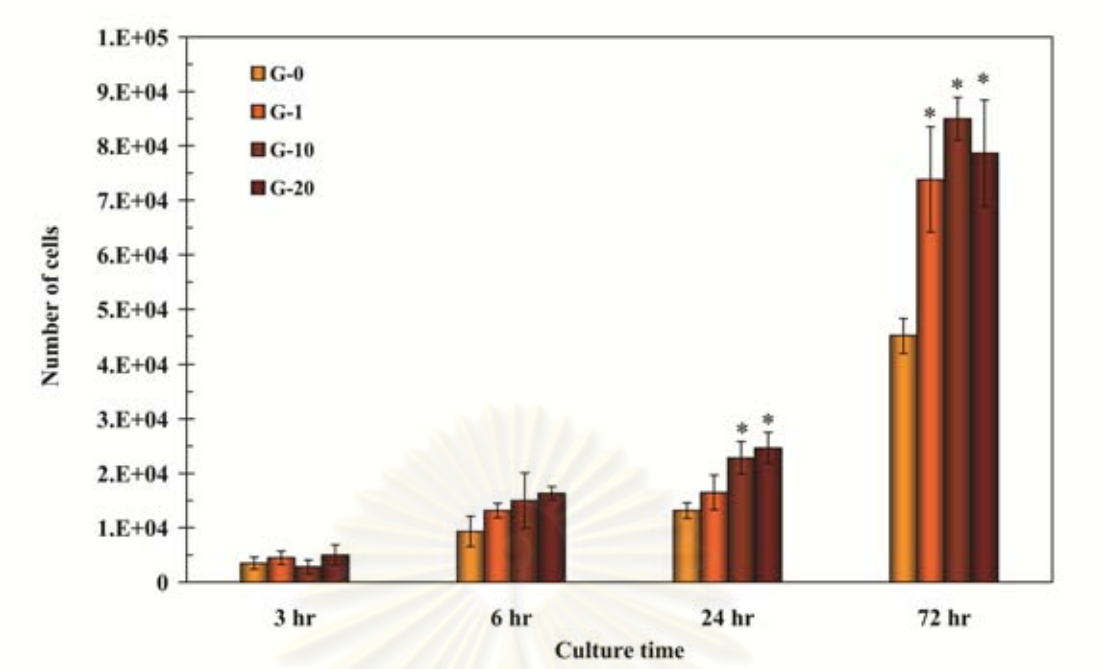

Figure 5.24 The number of L929 mouse fibroblast cells attached and proliferated on plasma-treated and untreated crosslinked gelatin film. * represented a significant difference at $p<0.05$ relative to G-0 of each group.

The results from *in vitro* cell culture using L929 mouse fibroblasts implied that the cells well adhered on PICP-treated surface, resulting in great proliferation observed after 24 and 72 hr of cell culture. The shorter population doubling time of cells cultured on PICP-treated film indicated the higher growth of cells, compared to ones on untreated films. These revealed that the PICP-treated gelatin films were more favorable for cell proliferation than untreated gelatin films. This could be attributed to the changes in surface properties caused by PICP treatment as previously discussed, i.e. more hydrophilicity, higher surface energy and surface roughness. As discussed in Part I that hydrophilicity of material surface could facilitate greater MSC cell attachment, it might be attributed to the protein of cell membranes, which contain hydrophilic amino acids, existing in the outer region due to the repulsion caused by the protein and hydrophobic components at the inner region. Therefore, the hydrophilic property of the PICP-treated gelatin surface could promote cell attachment by increasing the affinity between the protein and polymer surface. In addition N-containing groups could be incorporated into PICP-treated crosslinked gelatin surface by nitrogen plasma treatment. Since the 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 glycoprotiens [61]. Finally, roughness of gelatin film could provide more surface area which was known to support cell attachment.

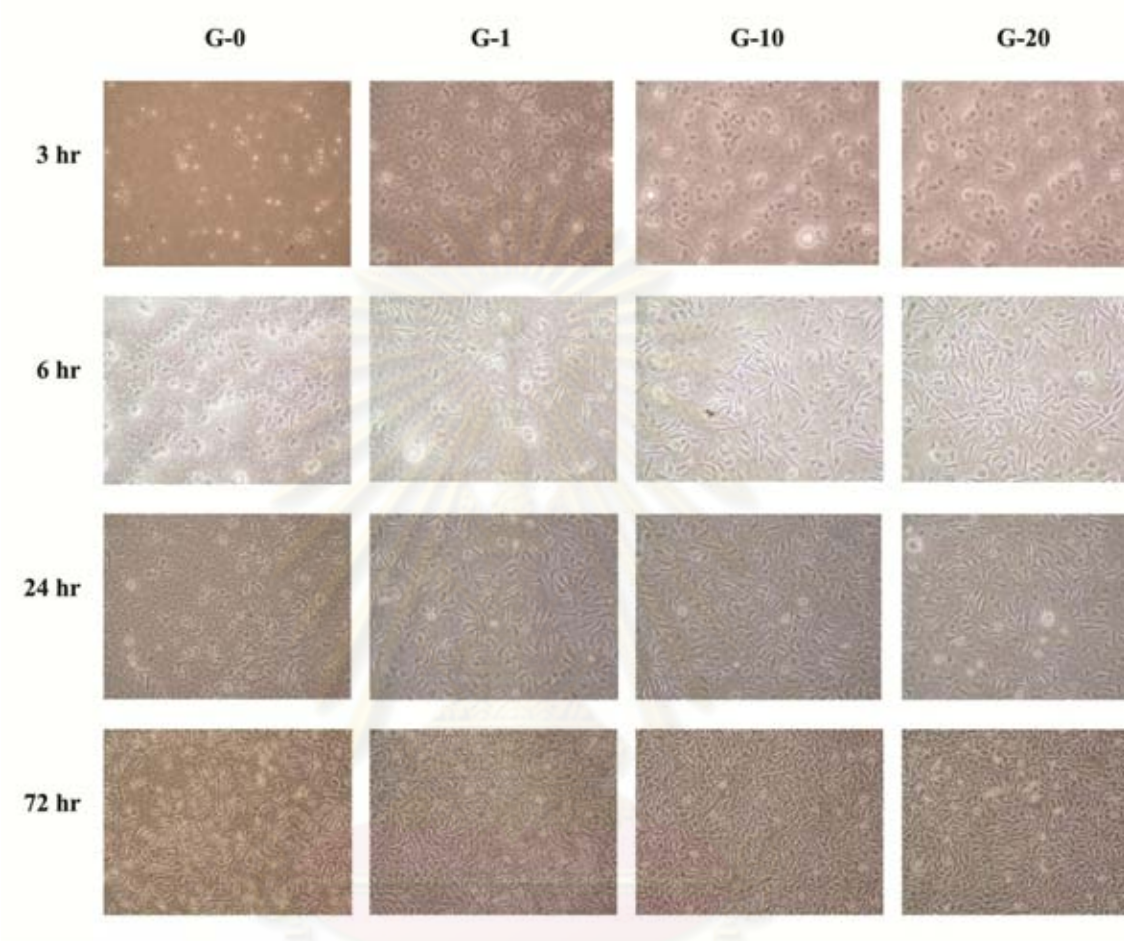

Figure 5.25 Morphology of L929 cells after 3, 6, 24 and 72 hr of seeding on the surface of PICP-treated and untreated gelatin films.

5.17 Summary

In this study, the gelatin film was treated by PICP plasma system. PICP could alter the surface properties of gelatin film but does not influence the thermal property and the degree of crosslinking of the film. The hydrophilicity and surface energy of PICP-treated gelatin was promoted in comparison to untreated sample. This was similar to the result obtained when AC 50Hz plasma was applied to treat gelatin films. The surface roughness of gelatin film occurred after a single PICP discharge. However, increasing in the number of repeated discharge could not further enhance surface roughness of gelatin film. From *in vitro* L929 cell culture, the results revealed that cells could adhere and proliferate on PICP-treated samples better than that on untreated samples. This corresponded to the MSC attachment on the AC 50Hz plasma-treated gelatin film, as found in Part I. This study has indicated that PICP treatment could be a potential surface modification method of crosslinked gelatin for future tissue engineering application.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

This research successfully investigated the effects of plasma treatment using AC 50Hz and PICP on the physical and biological properties of gelatin films. It was discovered from the first part of study that physical and biological properties of crosslinked gelatin films were affected by AC 50Hz plasma parameters such as treatment time and type of gases. We have demonstrated that the reduction in the contact angle of plasma-treated crosslinked gelatin film, which indicated the improvement of hydrophilicity, was due to the combined effect of changes in the surface chemistry and surface roughness. The degree of surface roughness was found to increase with increasing plasma treatment time. In order to maintain the properties of plasma-treated gelatin surface for longer period, the gelatin samples must be preserved at low temperature. We also found that the change in hydrophilicity during storage process was mainly due to the recovery of surface chemistry, not the surface roughness. Biocompatibility test revealed that the percentage of MSC and L929 cell attachment on plasma-treated gelatin films depended on type of gas and treatment time. Among the three types of plasmas used including nitrogen, oxygen, and air, nitrogen plasma treatment could best enhance the biocompatibility of crosslinked gelatin films. The treatment time, water contact angle and O/N ratio of nitrogen plasma-treated gelatin film for best attachment of MSCs were 15-30 sec, 27-28^{o}, and 1.4, respectively. The results ensured that the surface properties such as wettability and surface chemistry of materials strongly affected and regulated cell response.

In the second part of this work, PICP plasma system, one of high energy plasma system, was introduced to treat gelatin films. Nitrogen was selected to generate PICP plasma since it induced best cell attachment as found in the first part. We have demonstrated that PICP could alter the surface properties of gelatin film but does not influence the thermal property and the degree of crosslinking of the film. The hydrophilicity, surface energy, and surface roughness of gelatin films were promoted by increasing the number of applied pulses of PICP. Biocompatibility test showed that

PICP treatment could enhance L929 attachment on the surface of gelatin film, similar to the use of AC 50Hz plasma system.

In conclusion, the use of AC 50Hz and PICP system could change the surface properties of crosslinked gelatin film including wettability, surface roughness and surface chemistry which strongly affected and regulated the behaviour of cell. The attachment and proliferation of cells were enhanced by AC 50Hz plasma treatment as well as PICP. Considering the plasma system generation, AC 50Hz plasma provided the advantage in term of cost reduction, time saving and ease of handling, compared to PICP system. Thus, it could be concluded that both AC 50Hz and PICP plasma systems can be employed to improve *in vitro* biocompatibility of crosslinked gelatin film.

5.2 Recommendations

In this work, the surface roughness of material did not change after plasma treatment under the desired variable parameters of plasma generation. Only wettability and surface chemistry were shown to alter after plasma treatment, causing differences in cell growth. However, the surface roughness of the material has been reported as an important factor that influences cell behavior. Therefore, the effects of surface roughness of film on the behavior of cells should be further investigated in order to obtain the comparable results. In addition, the effects of plasma treatment on the differentiation of cells should be explored in further study.

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APPENDICES

APPENDIX A

Standard curve for TNBS assay

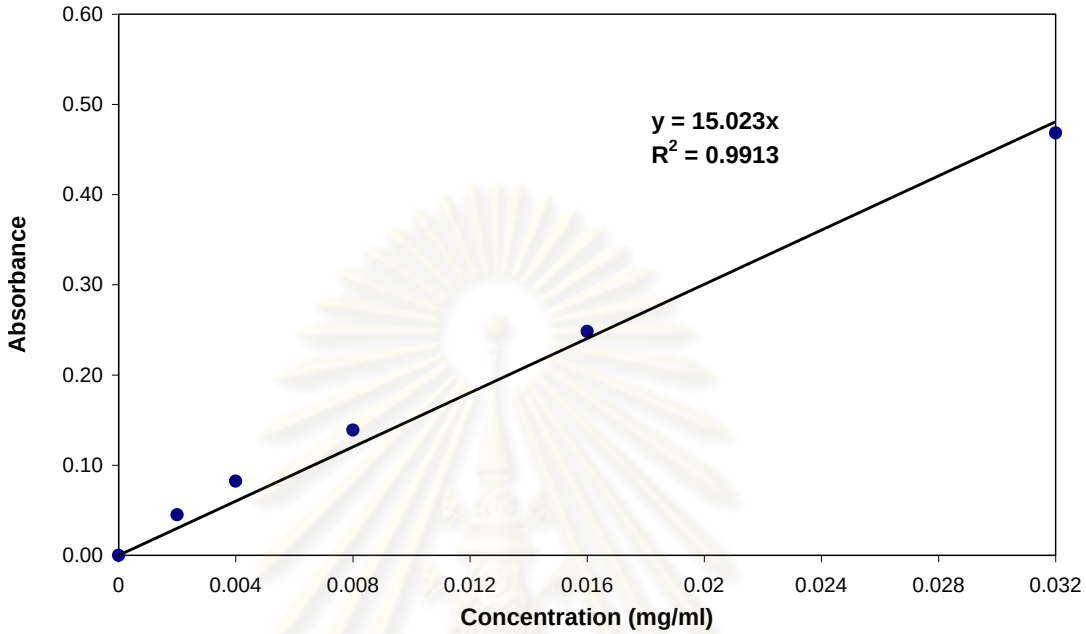

Figure A-1: β-alanine standard curve for TNBS assay

APPENDIX B

Standard curve for MTT assay

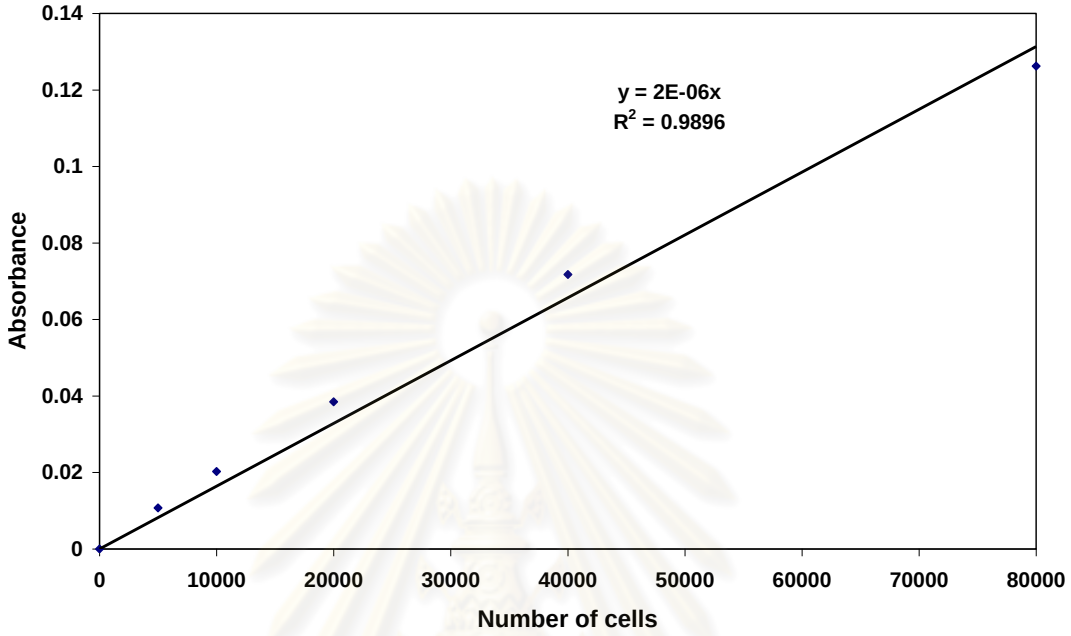

Figure B-1: Standard curve of L929 fibroblast cell number for MTT assay

APPENDIX C

Standard curve for DNA assay

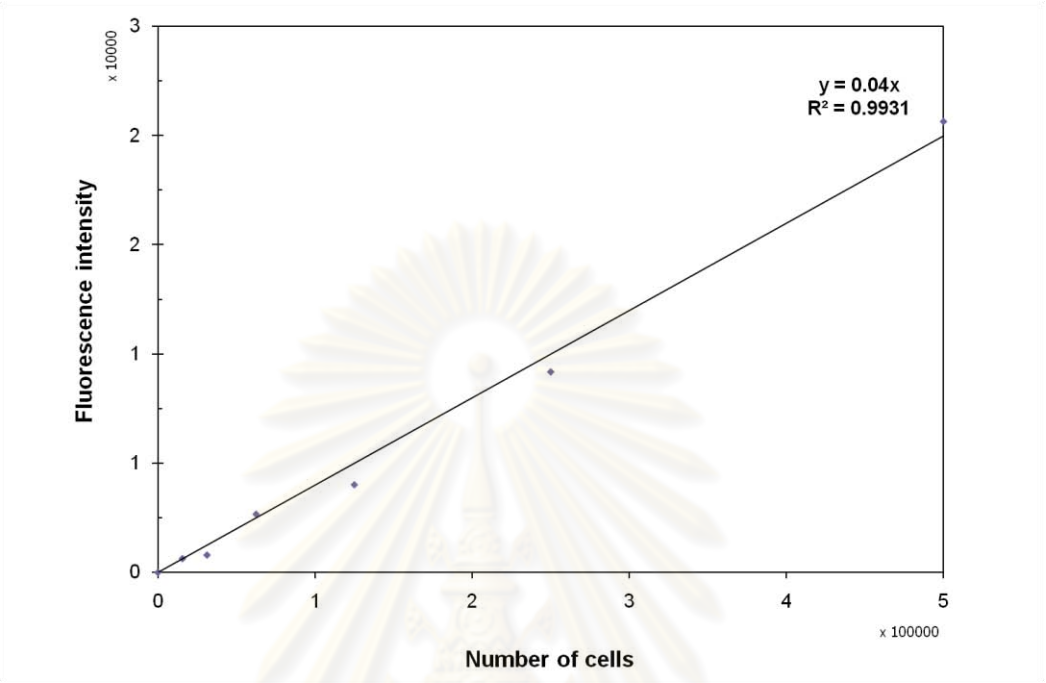

Figure C-1: Standard curve of cell number for DNA assay

BIOGRAPHY

Mr. Isarawut Prasertsung was born in Roi-Et, Thailand on January 31, 1980. He graduated at the high school level in 1996 from Roi-Et Wittayalai School. In 2003, he received his Bachelor Degrees of Engineering with a major of Plastic Engineering from Faculty of Engineering, Rajamangkala University of Technology Thanyaburi. In 2006, he received his Master Degrees of Engineering with a major of Chemical Engineering from Faculty of Engineering, Chulalongkorn University. Field of his master"s thesis was skin tissue engineering. After graduation, he pursued his graduate study to doctoral degree in the same field at Chulalongkorn University and continued his dissertation on plasma surface treatment of biomaterial. During his Ph.D. study, he has been conducting some parts of his Ph.D. research at the Plasma Research Laboratory, Department of Physics, Faculty of Science, University of Malaya, Malaysia.

Two papers from this dissertation were published in two international journals as follows:

- *International Biological Macromolecules* **46**: 72-78 (2010).

- *Surface Coating and Technology*, **205**: S133-S138 (2010).

Also, some parts of this work were accepted for oral and poster presentations as follows:

- The 13th International Conferrence on Biomedical Engineering (ICBME2008), Suntec Singapore International Convention and Exhibition Centre, Singapore, 3-6 December 2008 (Poster presentation).

- Seventh Asian-European International Conference on Plasma Surface Engineering (AEPSE2009), BEXCO convention center, Busan, Korea, 20-25 September 2009 (Poster presentation).

- Tissue Engineering & Regenerative Medicine International Society Asia Pacific Chapter Meeting (Termis AP 2010), Sheraton on the Park, Sydney, Australia, 15-17 September 2010 (Oral presentation).