CHAPTER 3

Experimental Procedures

Plasma process is an effective and economical technique to modify surface with various plasma sources, such as glow discharge plasma, inductive coupled plasma or atmospheric pressure plasma for etching, deposition or cleaning on various surfaces. The main burden was carried by capacitive and inductive coupled RF plasma. In this study, we used inductive coupled RF plasma (ICP) to modify PLA surface using ammonia plasma for protein adsorption. The research facilities available for this study at Plasma-Bio and Clean Energy Laboratory of Plasma and Beam Physics Research Facility (PBP), Department of Physics and Materials Science, Chiang Mai University (CMU), Thailand. The using of plasma enhanced chemical vapor deposition (PECVD) to enhance the surface wettability of silicon oxide (SiO_x) films for protein adsorption and cell attachment and proliferation were contributed by Institute for Plasma-Nano Materials, Center of Advanced Plasma Surface Technology, Sungkyunkwan University, Korea. The biological part was obtained from Department and Research Institute of Dental Biomaterials and Bioengineering, College of Dentistry, Yonsei University, Korea. This section describes processing methodology used for overall experiments.

3.1. Inductively Coupled Plasma System

The home-made 13.56-MHz inductively coupled plasma reactor was designed using a cylindrical 0.6 liter vacuum chamber made from quartz (Fig. 3.1). This system normally is called low pressure plasma due to it working on the rough vacuum. There are three main subsystems for this plasma source: the vacuum system, the gas holding system and the discharge power, as shown in Fig. 3.2. Optical emission spectroscopy (OES) was used to observe the plasma parameter mainly in the active plasma species, plasma temperature and plasma density. In OES the plasma radiation was collected by a lens, focused onto an optical fiber and input to a S2000 Miniature Fiber Optic

Spectrometer (Ocean Optic, Inc., USA). Spectra were recorded from 200 nm to 800 nm in 0.6 nm steps.

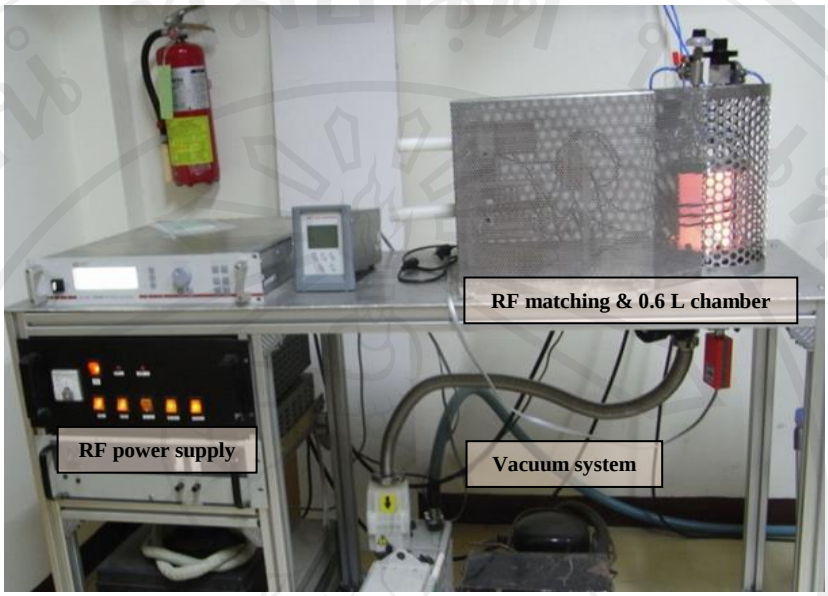

Figure 3.1 The home-made 13.56-MHz inductively coupled plasma reactor at CMU.

Vacuum System

The chamber was evacuated to the base pressure down to at least 1.4×10^{-2} torr by using a rotary pump. The sample holder was held on the cover. The sample was hung on the sample holder as thoroughly all over the surface.

Gas Holding System

The system can be used with any gases or liquids. In this process, we used argon and ammonia gases. The gas flow rate was controlled by an MKS-Type 247D Mass Flow controller. The gas pressure was read out by pirani gauges. The pirani gauge can work under the range of 5×10^{-1} – 1×10^{-4} torr.

RF Discharge Plasma Source

The RF power supply from the Dressler 13.56 MHz RF generator model of HPG 1365B was connected with a matching box via a RG 393/U coaxial cable of 50 Ω

impedance. In practice, most RF glow discharge systems operate at 13.56 MHz due to this frequency allowed by international communications authorities as it can radiate certain energy without interfering with communications. An RF antenna is made from a braided copper wire, which was in 6 mm in diameter with three loops around the quartz chamber. The matching box was composed of an inductor and variable capacitor. The role of the matching network was to add a correct amount of reactance to the network to made the combination of the matching network and the plasma look like 50 Ω.

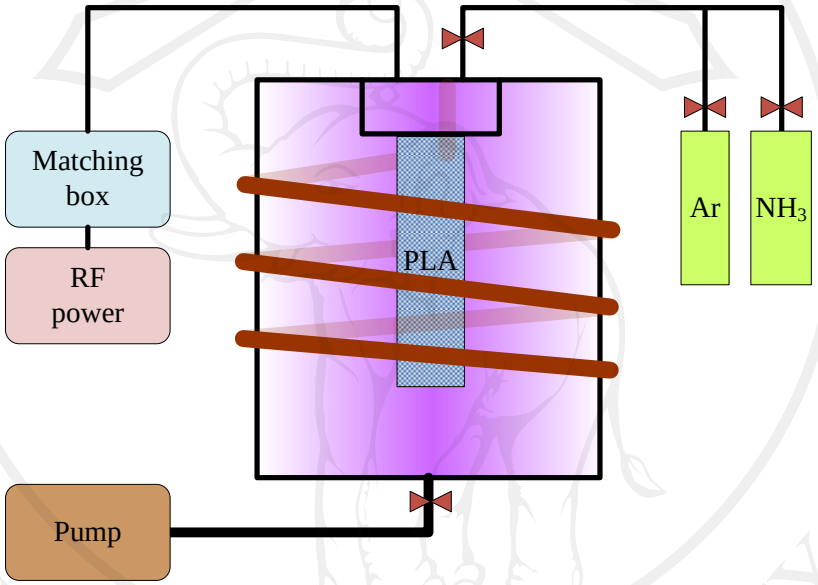

Figure 3.2 Schematic drawing of the inductively coupled RF plasma system.

3.2. Plasma Enhanced Chemical Vapor Deposition System

The PECVD technique is widely used to modify polymer surface at low temperature. It is advantageous to modify the surface properties of polymer or biomaterial without altering the bulk characteristic of materials. The plasma was generated with RF electromagnetic field. This technique is famous for producing plasma polymerization and so has been used to synthesize thin films of polymeric, organic and inorganic coating. The physical and chemical structure and film characteristics can be controlled by the plasma process parameter. PECVD was used extensively to deposit amorphous silicon oxide thin films. Due to the films properties

can varies with turning the deposition parameters. The process parameters depend on the type of precursor, gas ratio, substrate and deposition temperature, pressure, flow rate, reactor geometry and deposition time. Moreover, the thickness of the films can be controled with nanometers to micrometers.

The plasma polymerization films were formed by the monomers. It was carried out by carrier gas, such as argon or hydrogen gas, then the electrons break up the monomers to promote chemical reaction. The inelastic collisions raised the more excited electrons, ions and free radicals. However, the atoms and radicals were found more ions or electrons due to the energy required for excitation and dissociation was lower than the ionization. Then the atoms and the radicals adsorbed onto the surface and formed an amorphous film.

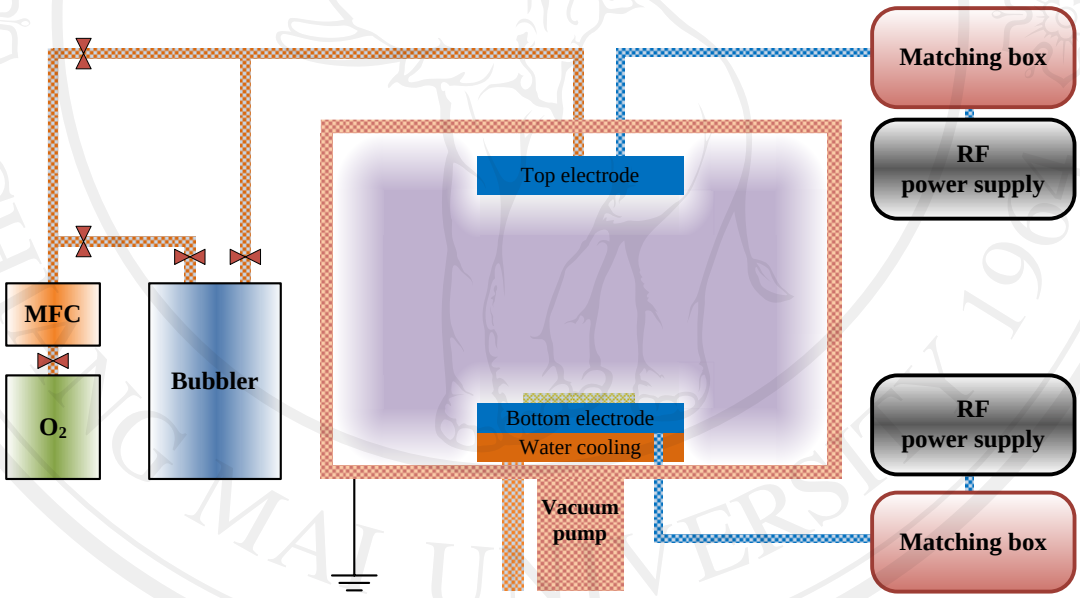

Figure 3.3 The schematic diagram of the plasma enhanced chemical vapor deposition system.

The schematic diagram of the PECVD configuration was shown in Fig. 3.3. The cylindrical vacuum chamber was made from stainless steel of 40 cm in diameter and 40 cm in length. Two circular electrodes of 20 cm in diameter were covered with ceramic plate and placed in the cylindrical chamber in vertical and parallel. The distance

between top and bottom electrode was 7 cm. The bottom electrode was used like a sample holder.

Vacuum System

The vacuum system was started by using a rotary pump. Then a few seconds later, the pressure evacuated down to around 10^{-2} torr. Next, the booster pump continued working. The rotary pump was used as a backing pump to prevent the booster-pump motor overload. However, the booster pump was fast enough to make the system go down to base pressure with the couple motors pumping mechanisms. The pirani gauge was used to read the pressure.

Gas Holding System

The system can be used with any gases or liquids. In this process we used oxygen gas for pre-treatment plasma process and used as a carrier gas. The gas flow rate was controlled by Mass Flow controller. The gas pressure was read out with pirani gauges. The precursor was in the bubbler which has the temperature control for controlling the flow rate of the monomers which evaporate into the system. The oxygen gas was used as a carrier gas by flowing into the bubbler. Then the vapors of the monomers flowed out with oxygen gas to the shower at the top electrode.

Cooling System

While the plasma processing was started, a lot of heat was generated. Water cooling was used to remove the heat from various components. A wire was flowing through the water to them all. Many electronic devices require cooling by water, such as booster pump, matching box, and the sample holder.

RF Discharge Plasma Source

The 13.56 MHz RF powers were applied to both electrodes with maximum powers 600 watt. The RF power supply was connecting with a matching box before applied to the electrodes. The matching box was composed of an inductor and variable

capacitor. The role of the matching network is to add a correct amount of reactance to the network to make the combination of the matching network and the plasma look like 50 Ω.

The figures of PECVD chamber are shown as below, Fig. 3.4-b shows the plasma treatment used by both electrodes and Fig. 3.4-c shows the plasma treatment by using only the bottom electrode.

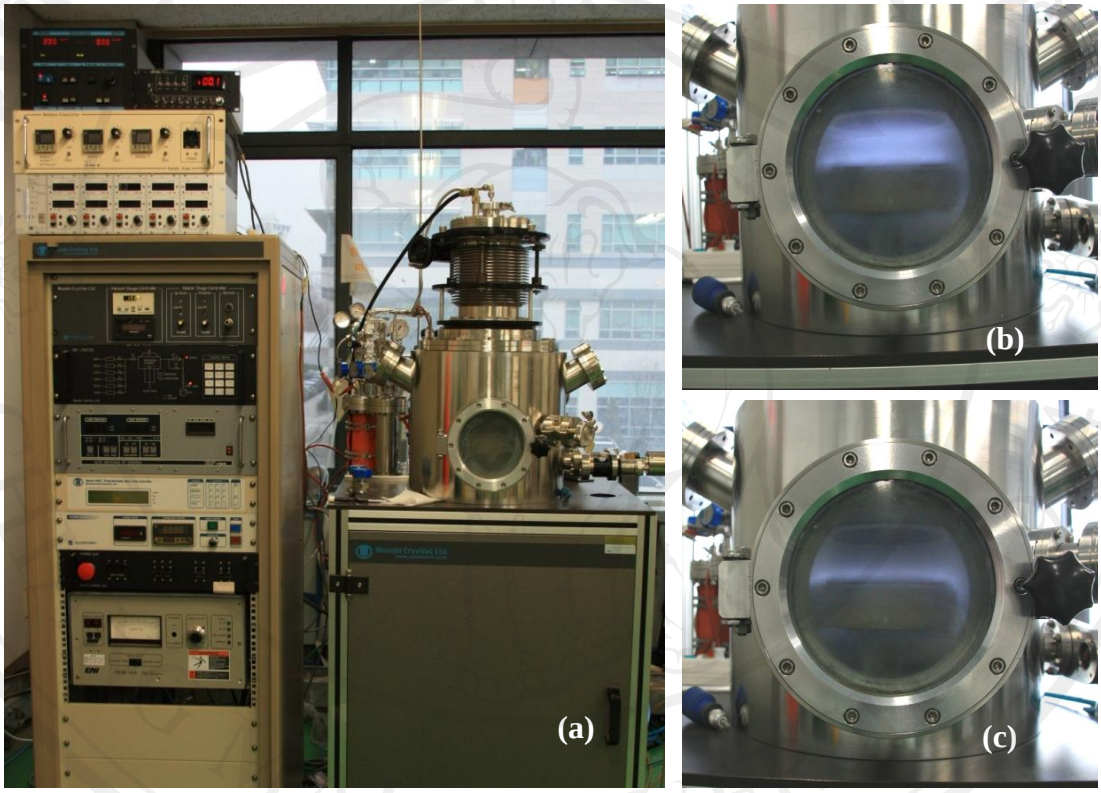

Figure 3.4 The schematic diagram of the plasma enhanced chemical vapor deposition system at Institute for Plasma-Nano Materials, Center of Advanced Plasma Surface Technology, Sungkyunkwan University, Korea.

3.3. Material and Methods

3.3.1. Material

In this study, the PLA films were obtained from Adcharaporn Boonma from the School of Mechanical Engineering, Institute of Engineering, Suranaree University

of Technology, Thailand. There were prepared from PLA resin of the NatureWork® with L/D ratios from 24:1 to 30:1 by the standard calendering process. The film is clear with 50µm thickness.

3.3.2. Experiments

The PLA film was investigated in two major parts; surface function and plasma polymerization. The flow chart as in Fig. 3.5 gives you an overview about this work.

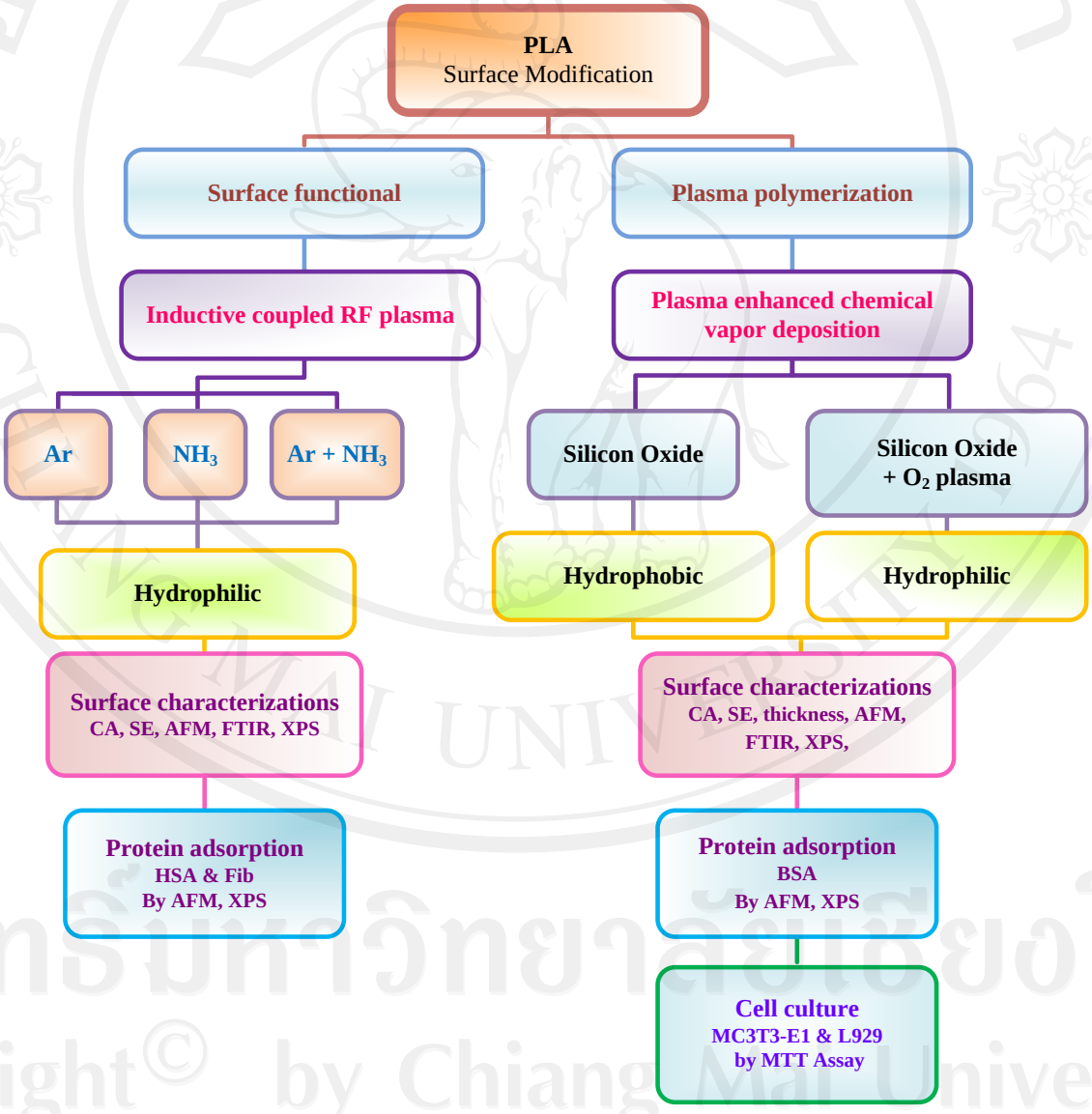

Figure 3.5 The overview of experiments.

The surface function of amine or amine groups was functioned by using the ammonium plasma treated with ICP technique, in which more detail will be explained in Chapter 4. The film was hung on the sample holder. Then the film samples were first sputtered by Ar plasma to remove residues. After that, the films were treated with NH_3 and mixture Ar and NH_3 plasma by variations of RF power. The Ar gas was added to mix with the NH_3 plasma to increase the NH_3 associated. Optical emission spectroscopy was used to observe the active plasma species. The surface was characterized by contact angle, surface energy, ATR-FTIR, AFM and XPS before the protein adsorption test. The protein adsorption of human serum albumin (HSA) and fibrinogen (Fib) on plasma treated PLA was characterized by AFM and XPS.

The effect of surface wettability to protein adsorption was studied by plasma polymerization of silicon oxide which these films gives hydrophobic surface and silicon oxide with oxygen plasma post-treatment films gives hydrophilic surface. The surface was characterized the same as previous study and its more detail will be explained in Chapter 5. Protein adsorption was detected by using AFM and XPS. Moreover, cell attachment and cell proliferation of MC3T3-E1 mouse pre-osteoblasts cells and L929 mouse fibroblasts cells were studied on the film surfaces via MTT assay.

3.3.3. Protein Adsorption and Cell Culture

Protein Adsorption

The protein powders were prepared into the water solution with the concentration of 0.5% W/V. Then mix gently by inverting the tube 10 times. Do not vortex, avoid shearing genomic protein. The films were cut into 1 cm × 1 cm and placed into wells of a 4-well cell culture plate. Then protein was immobilized onto the films by loading 1 ml of protein solution onto each well, beware the bobble and we checked to see that the films were not flooding over the solution. Then it was left incubated for 20 min on a rocking shaker at room temperature and sucked slowly by micropipette. After incubation, the films were washed in distilled water, first soaking by adding distilled water 1 ml onto each well as slowly as possible to prevent damage the protein bonding layer. Then it was left for 20 min on a rocking shaker at room temperature. Next, it was

washed with distilled water 3 times with soft pipetting. The films were dried in a new plate and left at room temperature for 12 h before characterized by XPS and AFM.

There are several techniques to analyze the protein adsorption, such as surface plasmon resonance fluorescence spectroscopy (SPFS), surface plasmon resonance spectroscopy (SPS) [93-97], FTIR [93, 98-100], quartz crystal microbalance (QCM) [101-103], ellipsometry [87, 104-107], total internal reflection spectroscopy [108-112], XPS [99, 113-116] and AFM [117-120] which this study has a monolayer of protein adsorption that the thickness in the range of a few nm as this reason XPS was selected. XPS is the surface-sensitive quantitative technique that measures element composition. The XPS measures the kinetic energy and counts the electron from the core level of the sample from the top surface to 10 nm. Moreover, XPS was used to follow the fingerprint of the protein adsorption via the characteristics of the nitrogen N_{1S} and relative concentration by signal intensity. AFM is important to characterize technique for biomaterial. AFM determins the surface morphology and parameter of such a molecular shape, surface coverage, protein cluster and cluster size. For the monolayer of the protein adsorption [121], the height of the valley can tell the size of protein. The roughness of the surface can tell us the conformations and concentration of the protein adsorption.

Cell Culture

Mouse cells were used as established *in vitro* model in tissue engineering part. In this investigation, mouse fibroblast cells (L929) [122-124] and mouse pre-osteoblast cells (MC3T3-E1) [125-127] were used to study cell cultivation on the surfaces of the as-deposited SiO_x and O_2-plasma treated SiO_x films. Use of the fibroblast cells was aimed at artificial tissue test and use of the osteoblast cells was aimed at scaffold test. Fibroblast cells are normally used as a model of cell attachment and proliferation since they are the most common cells of connective tissues in animals and able to grow at a high rate. These cells can differentiate into different kinds of connective tissue. Fibroblast was important in the tissue repair. When the body had a wound, fibroblasts were produced quickly and as much as needed to repair while the other prevents

infection. Various cell culture models have been employed to study human osteoblast cells. MC3T3-E1 is a mouse pre-osteoblast cell which represents a popular osteoblast cell line. MC3T3-E1 or primary mouse cell represents a reliable alternative to the primary human osteoblast cell model for the *in vitro* research. Additionally, both cell lines growth occurs at the same rate due to the fact that they have the similar in molecular regulation of gene expression.

The sample was sterilized under ultraviolet light for 1 h before cell culture. The cell attachment and proliferation were evaluated using MTT assay. Both cell lines were diluted to 5×10^3 cells/ml. The culture medium was supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic solution (WelGENE, Korea). The cells were cultured at 37°C in a 5%-humidified CO_2-atmosphere incubator. Then cell seeding films were measured with a fluorescence spectrometer at wavelengths of 544 and 590 nm. In these experiments, three replicas were used for calculating the percentage of each experiment. The cell attachment was observed in 1 day and cell proliferations were observed in 3 and 7 days.