# CHAPTER III

# EXPERIMENTAL

## 3.1 Material

1. High ammonium natural rubber : Thai rubber latex Public Co., Ltd., Chonburi
2. Poly (ethylene glycol) methacrylate, $M_w$=360 (PEGMA) : Aldrich
3. *N*-Vinyl pyrrolidone (VPy) : Aldrich
4. 2-Methacryloyloxyethyl phosphorylcholine (MPC) : NOE cooperation, Japan
5. Bovin serum albumin : Aldrich
6. Bicinchonic assay kit (QuantiPro™ BCA assay) : Sigma
7. Phosphate buffer saline (PBS) : Aldrich
8. Sodium dodecyl sulfate : Fluka
9. Benzophenone : Fluka
10. Acetone : Merck
11. Methanol : Merck
12. Ethanol : Merck

13. 50 % Dispersion Sulfur : Chemical grade, Kijphaiboon, Co., Ltd.
14. 50 % Dispersion ZDEC : Chemical grade, Kijphaiboon, Co., Ltd.
15. 50 % Dispersion Zinc Oxide : Chemical grade, Kijphaiboon, Co., Ltd.
16. Potassium oxide : Chemical grade; Kijphaiboon, Co., Ltd.
17. Sodium laurate : Chemical grade; Kijphaiboon, Co., Ltd.
18. Platelet- poor plasma (PPP) : Thai Red Cross Society
19. Platelet-rich plasma (PRP) : Thai Red Cross Society
20. Ultrapure distilled water : Mill-Q Lab system



## 3.2 Equipment

### 3.2.1 Attenuated Total Reflectance Infrared Spectroscopy (ATR-IR)

All spectra were collected at resolution of 4 $cm^{-1}$ and 64 scan using Nicolet-Magna 750 series II FT-IR spectrometer equipped with a DTGS detector. A hotizental plate ATR (Spectra Tech Inc.) accessory with a Germanium (Ge) prism was employed for all ATR spectral acquisitions.

### 3.2.2 UV-Spectroscopy

UV on Microtiter plate reader, Model Sunrise, Tecan Austria GmbH, was used for determining the amounts of absorbed protein using bicinconic assay by reading UV absorbance at $\lambda = 562$ nm.

UV spectroscopy Model Techna, specgene was used to observe the benzophenone dissolved and residual after graft copolymerization by reading UV absorbance at $\lambda = 251$ nm.

### 3.2.3 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) Model JSM-5800L, was used to observe the morphology of surface-adherent platelets.

## 3.3 Experimental

### 3.3.1 Preparation of Vulcanized and Unvulcanized Latex Film

Two types of natural rubber latex films were prepared from high-ammonia natural rubber latex (HANR). The unvulcanized NR latex films were cast directly from HANR and dried in the dark for 3-4 days at ambient temperature. The vulcanized NR latex films were prepared by accelerated-sulfur vulcanization process at 60°C. The cast films were dried in the dark for 7-10 days at ambient temperature. The curing formulation is shown in Table 3.1.

**Table 3.1** Formula of vulcanization process for vulcanized rubber

| Reagent | Quantity of mixing (phr) |
|---|---|
| HANR | 100.00 |
| 50% dispersion sulfur | 0.90 |
| 50% dispersion zinc oxide | 1.20 |
| 50% dispersion zinc | 1.20 |
| 20% potassium laurate | 0.75 |
| 10% potassium oxide | 3.00 |

### 3.3.2 Surface Grafting of Hydrophilic Monomers onto NR Latex Films

NR latex films were washed with hot DI water, followed by methanol and acetone, respectively for 30 min each in an ultrasonic bath. The NR latex films were immersed in 1% (w/v) benzophenone in acetone for a desired period of time then dried in the dark under vacuum for 2 h. The NR latex films having absorbed benzophenone were immersed in a glass tube containing 25 mL monomer solution in degassed water. Degassing was done by boiling the deionized water for a few minutes prior to bubbling with nitrogen gas until the water was cooled down to ambient temperature. The glass tube was then capped with a septum before exposed to UV light having $\lambda$ = 350±50 nm, 500 Watts at 60°C. The nitrogen gas was bubbled into the monomer solution throughout the course of reaction. After a desired period of reaction time, the NR latex films were stirred in water overnight then rinsed thoroughly with hot ethanol (50°C) to remove the ungrafted polymer before dried under vacuum overnight.

### 3.3.3 Determination of Benzophenone Residue After Graft Copolymerization

NR latex films, having the dimension of 1.0 x 1.0 $cm^2$, were put in ethanol in an ultrasonic bath for 30 min and further soaked overnight in ethanol. The absorbance of the ethanol solution was measured at 251 nm by UV spectroscopy. The amount of benzophenone dissolved was calculated from the benzophenone concentration in the ethanol solution. The data are expressed as mean ± standard deviation (S.D).

### 3.3.4 Contact Angle Measurements

Milli-Q water used as a probe fluid was dropped onto the film surface by a Gilmont Syringe with a 24-gauge flat-tipped needle. Images of water droplets on the film surface were taken with a digital camera (Sony, Model F707). The contact angles were measured with Adobe Photoshop 6.0 Software.

## 3.4 Protocols for Blood Compatibility Test



### 3.4.1 Determination of Total Amount of Adsorbed Human Plasma Protein

The controlled and modified NR latex films, having the dimension of 1.0 x 1.0 $cm^2$ were placed into a 24-well tissue culture plate containing deionized water in each well. The samples were allowed to stand in the wells overnight to reach an equilibrium hydration. Each sample was removed from deionized water and suspended in the wells containing 2.0 mL PPP before incubated at 37°C for 3 h. Three pieces of samples were analyzed for each condition. The samples were removed from PPP and rinsed thoroughly with phosphate buffer saline solution

(PBS) (2x) to remove any loosely attached protein. The adsorbed protein on the sample surface was detached by soaking each film in 2.0 mL of 1 % aqueous solution of sodium dodecyl sulfate (SDS) for 30 min. A protein analysis kit based on the bicinchonic acid (BCA) method was used to determine the concentration of the protein dissolved in the SDS solution. 100 μL (0.1 mL) of SDS solution that soak each samples was added into 96-designated wells. 100 μL of BCA working solution was then added in each well, before the well-plate was incubated at 37°C for 2 h. The absorbance of the solution was measured at 562 nm by UV plate reader. The amount of protein adsorbed on the samples was calculated from the protein concentration in the SDS solution. The data are expressed as mean ± standard deviation (S.D).

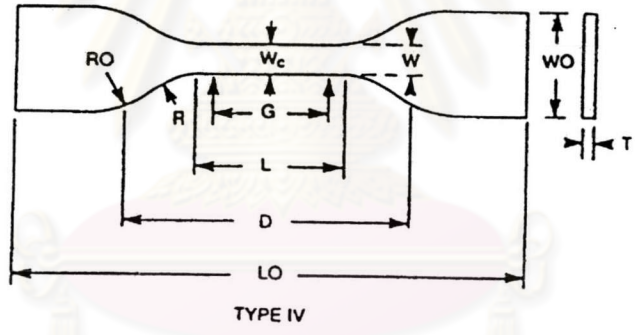
### 3.4.2 Evaluation of Platelet Adhesion

The controlled and modified NR latex films, having dimension of 1.0 x 1.0 $cm^2$ were placed into 24-well tissue culture plate containing PBS in each well. The samples were allowed to stand in the wells overnight to reach an equilibrium hydration. The PRP (2.0 mL) was added into each well via a micropipet. The well plate was incubated for 1 h at 37°C. After the PRP was removed using a micropipet, the substrates were rinsed with PBS (3x). The saline solution containing 2.5% (v/v) glutaraldehyde was added to each well in order to fix the platelets adhered on the sample surfaces. The samples were rinsed with PBS (3x) followed by deionized water (3x) prior to dehydration by sequentially soaking in 30, 50, 70, 90, 99 and 100 % (v/v) ethanol in water for a period of 10 min interval. The samples were dried under vacuum for 24 h then sputtered with gold before analyzed by scanning electron microscopy (SEM).

### 3.5 Tensile Stress- Strain Properties (ASTM D412)

The properties of vulcanized rubber, which were determined, are the tensile strength, the elongation at break and the stress at a given elongation (modulus). The vulcanized rubber was stamped with a compress air sample cutter (Model SDAP-100-N) using a dumbbell (Type IV). The stress-strain curve of samples was measured using the following condition. Tensile machine model LLOYD 468 K, was used to determine the mechanical properties.

| | |
|---|---|
| Temperature | 25 °C |
| Humidity | 60 % |
| Sample Rate | 10.00 pts/sec |
| Crosshead speed | 500 mm/min |




W : 6 mm WO : 19 mm G : 25 mm R : 14 mm T : 4 mm or under
L : 33 mm LO : 115 mm D : 65 mm RO : 25 mm

**Figure 3.1** Schematic diagram of tensile test specimen (Type IV).