CHAPTER III

MATERIALS AND METHODS

3.1 Biological materials

3.1.1. Fresh field natural latex from RRIM 600 clone was obtained from Pan Asia Biotechnology Co., Ltd., Rayong, Thailand. It was preserved with .03% (v/v) ammonia

3.1.2. Human gingival fibroblast

3.2 Chemicals for natural rubber grafting and testing

Acetone

Ethyl methacrylate monomer (EMA)

Lauric Acid

Light petroleum ether

Methyl methacrylate monomer and polymer

Normal buthylacrylate (*n*-BA)

Potassium hydroxide

Sodium sulphite (anhydrous)

Sodium azide

Coffee solution

Tea solution

Capsaicin solution

3.3 Chemicals for cell culture and Scanning electron microscopy

Dulbecco's Modified Eagle Medium (DMEM) (GIBCO BRL,USA)

DMEM without phenol red (GIBCO BRL, USA)

Antibiotic- antimycotic solution (GIBCO BRL, USA)

L-glutamine (GIBCO BRL,USA)

Fetal bovine serum (GIBCO BRL, USA)

Normal saline solution

Glutaraldehyde

Ethyl alcohol

Osmium tetroxide

3.4 Apparatus

Fourier-transform infrared spectroscopy (Perkin Elmer, Model Spectrum One)

Universal testing machine (Lloyd Universal Testing Machine, Model LR 10 K, UK)

Durometer (Shore A) (Pacific Transducer, USA)

CO_2 incubator (Shel Lab, USA)

Biohazard Hood (MDH, UK)

Co-60 radiation source (Office of Atom for Peace, Thailand)

Spectrophotometer (Hunter Lab, USA)

Spectrophotometer (Ultrospec 3000, UK)

Inverted phase contrast microscope (Olympus ck2, Japan)

Scanning electron microscope (JEOL, JSM-5410 LV, Japan)

Transmission Electron Microscopy (TEM Model JEM-200CX)

Contact Angle Measurement (Model CAM-Micro, USA)

Differential Scanning Calorimeter (Model DSC 200, Germany)

Digital balance

Bench-top centrifuge

Hemocytometer

Orbital shaker

3.5 The methods were divided into three parts:

Part I:Preparation of high-ammonium concentrated natural rubber latex from fresh field latex, preparation of gamma irradiated prevulcanized natural rubber latex (IVNRL), grafting of EMA monomer onto IVNRL by gamma irradiation, and determination of its particle morphology and grafting efficiency

Part II: Study of physical and mechanical properties testing of graft copolymer. These properties include consisting of, hardness, tensile strength, tear strength, water absorption, contact angle, and staining test.

Part III: In vitro study of the cellular response to the graft copolymer.

In this study Coe Supersoft ®, soft acrylic resin, was prepared according to manufacturer recommended procedures and used for the control material.

3.5.1 Part I

3.5.1.1 Preparation of High-ammonium concentrated natural rubber latex

Fresh field latex was purchased from Pan Asia Biotechnology Co., Ltd., Rayong, Thailand in the form of milky like emulsion with ammonia as a preservative (Figure 3.1). Fresh field latex was determined for dried rubber content (DRC) (Figure 3.2 a, b) and total solid content (TSC). Then the water soluble chitosan and ammonium hydroxide were added to the latex for the final concentrations of 0.1% and 0.3% w/w, respectively to preserve and anti-coagulate of the latex. The ammoniated latex was again tested for DRC and TSC as well as volatile fatty acid (VFA) (Figure 3.3), NH_3, and Mg^{++} content. Reduction of Mg^{++} content was perform by adding 50 ppm (maximum) of diammonium hydrogen phosphate (DAP) into the latex. To increase mechanical stabilization time (MST) ammonium laulate was added into DAP-ammoniated latex for the final concentration 0.05% w/w. The whole mixture of latex was kept standing over night. The precipitate was discarded and the

supernatant latex was determined for DRC, TSC, VFA, NH_3, and Mg^{++} content again. Then the soluble alginate and KOH were added into the supernatant latex for the final concentration of 0.01 phr and 0.1% w/w, respectively. The latex was adjusted to 25% DRC by 0.3% NH_4OH. After being kept standing for 3 hours the 25% DRC latex was centrifuged at 7,000 x g (Figure 3.4). This concentrated latex was finally adjusted to 60% DRC and 0.6% NH_3. The final preparation of latex was called high ammonium concentrated latex and was adjusted for TSC, VFA, KOH number, and Mg^{++} content to comply with the ISO specification for high ammonium concentrated latex.

Figure 3.1. The fresh field latex contained in plastic container was preserved with ammonium hydroxide as a preservative.

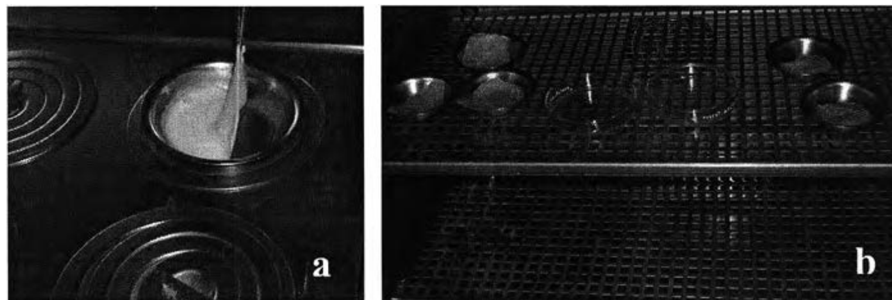


Figure 3.2. The determination of DRC a) The natural latex was coagulated and turned into soft rubber by acetic acid and heat on water bath at 100 °C, b) The rubber was creped and dried in an oven at 60 °C for 10-12 hours then it was weighed for DRC calculation.

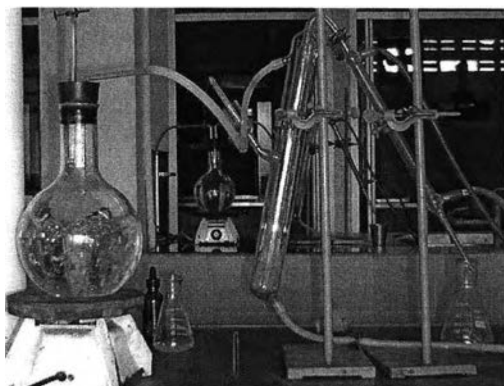


Figure 3.3. The process of determination of volatile fatty acid by modified equipments

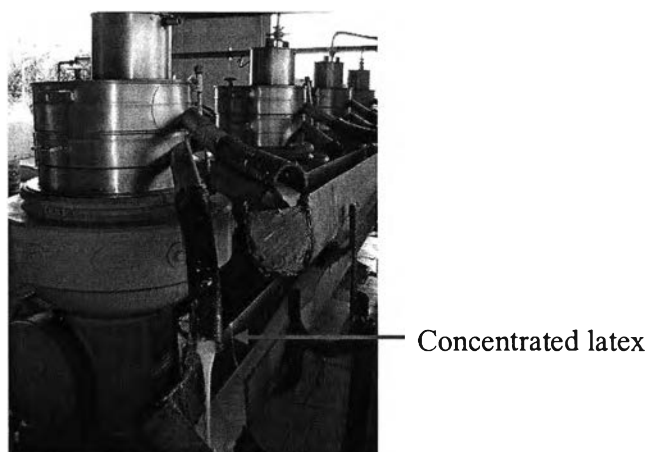


Figure 3.4. The latex was centrifuged using a factory scale centrifuge machine at 7,000 x g to produce the concentrated latex

3.5.1.2 Preparation of gamma irradiated prevulcanized natural rubber latex

Before vulcanization process high-ammonium concentrated natural rubber latex containing 60% DRC was diluted with 1 % ammoniumhydroxide to make up the 50% DRC and stabilized by 0.2 phr KOH .

For vulcanization process 5 phr of normal buthylacrylate (*n*-BA) was added into 50% DRC latex with slowly continuous stirring (Figure 3.5) and then irradiated at room temperature by gamma ray from a Co-60 source (Office of Atom for Peace, Bangkok, Thailand) at the irradiation dose of 10, 11, 13, 14, 15, 16,17, and 18 kGy. After irradiation the prevulcanized latex (IVNRL) was casted in glass mold, air dried in room temperature for 24 hours and heat dried in hot air oven at 80 °C for 48 hours. The dry rubber sheet was cut into a dumbbell shape according to American Society for Testing and Materials (ASTM) D638. Evaluation of the tensile strength is performed using a Universal testing machine with a crosshead speed of 50 mm/min at room temperature. The data were evaluated and the appropriated dose of gamma ray that produced the tensile strength about 6 MPa was chosen. In this study, the total dose of gamma ray using for vulcanization and grafting process was chosen at 15 kGy.

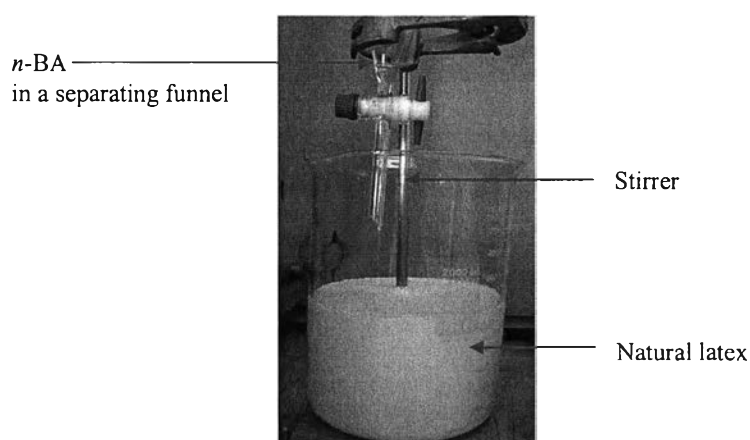


Figure 3.5 The *n*-BA was added into 50%DRC latex with slowly continuous stirring.

3.5.1.3 Grafting of ethyl methacrylate (EMA) monomer onto prevulcanized natural rubber latex by gamma irradiation

Different concentrations of EMA monomer in the form of emulsion were mixed thoroughly into the IVNRL by mole ratio of IVNRL to EMA equal to 90:10, 80:20,

70:30, 60:40, and 50:50 (Figure 3.6) and then keep the mixture standing for 24 hours. They were then irradiated by gamma ray using a Co-60 source at room temperature and at a total dose of 5 kGy. The irradiated IVNRL/EMA product was call the graft copolymer in this study.

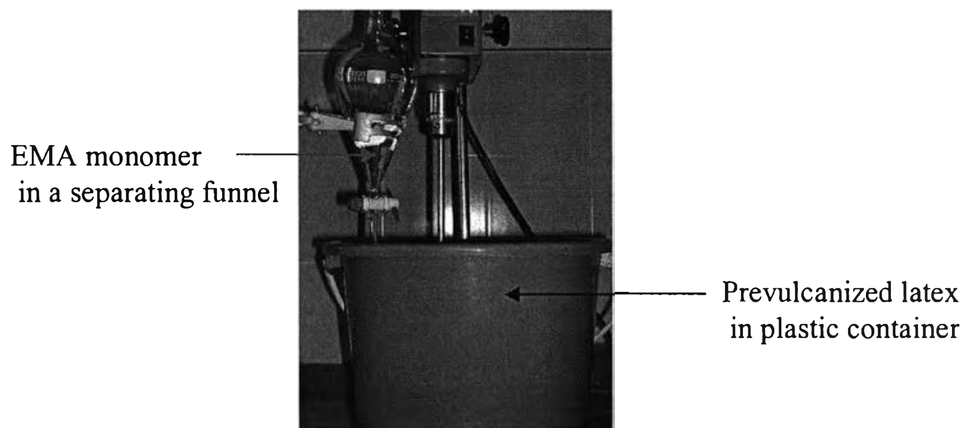


Figure 3.6 The EMA monomer was added into prevulcanized latex with slowly continuous stirring.

3.5.1.4 Determination of the morphology of the graft copolymer particle by Transmission Electron Microscopy (TEM)

The graft copolymer latex was diluted 400 times with distilled water. Then, 2 drops of 2 % aqueous of osmium tetroxide was incubated with 200 cm^3 of diluted latex for 1 day to stain the rubber particle. A drop of the stained latex was applied on a carbon-coated Formvar film depositioned on a grid and dried in a dessicator. The morphology of the graft copolymer was examined by Transmission Electron Microscopy at 120 kV.

3.5.1.5. Determination of grafting efficiency

To determination the grafting efficiency the graft copolymer had to be casted in an open glass tray (170 x 170 x 3 mm^3) and dried at room temperature for 48 hours (Figure 3.7 a). Once the graft copolymer sheet turned clear (Figure 3.7 b), it was removed from the tray and continuously dried in an oven at 80 ^{o}C for 24 hours. After the graft copolymer sheet was dried out, the water-soluble impurities were leached out by distilled water. Finally, the sheet is thoroughly dried again in the oven at 70 ^{0}C for 24 hours.

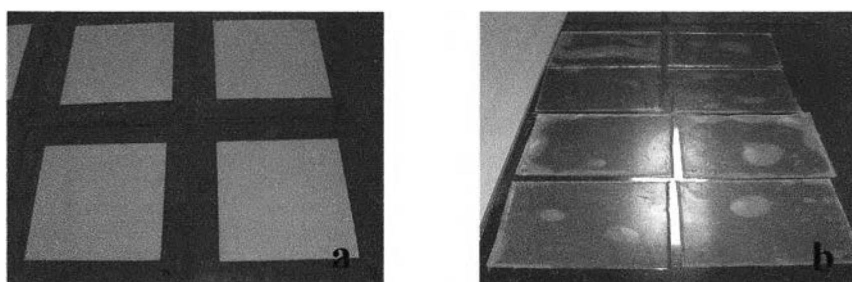


Figure 3.7 a) The graft copolymer latex was casted in open glass trays and dried at room temperature for 48 hours. b) The graft copolymer sheets turned clear before moving to hot air oven.

Soxhlet extraction procedures were carried out to assess the amounts of the residual of ungrafted natural rubber and residual of EMA in the final product (Figure 3.8). The residual of ungrafted natural rubber was extracted in a Soxhlet extractor by light petroleum ether for 24 hours and residual of EMA was extracted with acetone for 24 hours. After each extraction, the graft copolymer is determined by the residual weight. The grafting efficiency was defined by the formula below.

I22777489

$$\text{\% of grafting efficiency} = \frac{\text{Weight of extracted graft copolymer x 100}}{\text{Total weight of graft polymer}}$$

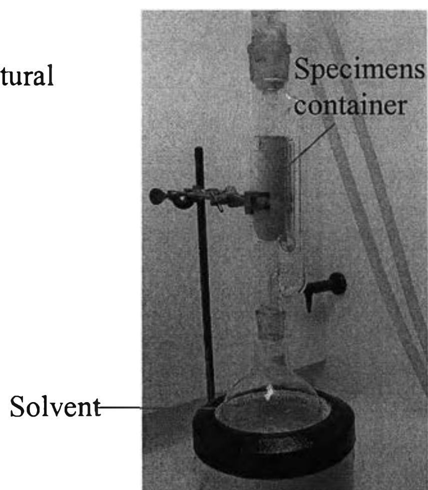


Figure 3.8 The residual of ungrafted natural rubber and residual EMA were extracted by Soxhlet extraction procedures

3.5.1.6 Characteristics of graft copolymer

This study was performed to investigate the functional groups of EMA that presented in the graft copolymer. After the removal of residual polyethyl-methacrylate and residual natural rubber by Soxhlet extraction. The graft copolymer was dissolved with methyl ethyl ketone (MEK) and toluene. The graft copolymer solution was then prepared into a thin film on potassium bromide (KBr) (Figure 3.9) and characterized using infrared spectroscopy.

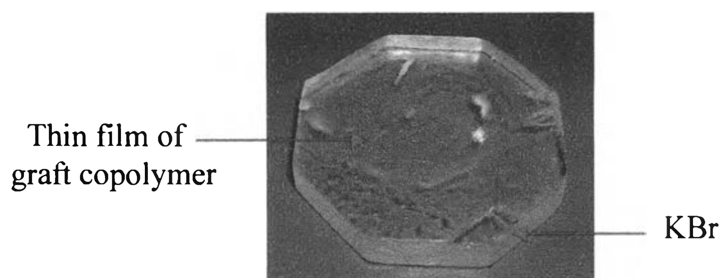


Figure 3.9. Thin film of graft copolymer was smeared KBr before characterization by Infrared spectroscopy.

3.5.1.7 Glass transition temperature (T_g) examination

This study was performed to investigate the transition temperature that the rubber would change from soft to hard phase. The graft copolymer and Coe Supersoft® specimen were prepared in the circular disk of 5 mm in diameter and 3 mm thick (Figure 3.10). They were determined for the transitional glass temperature (T_g) by Differential Scanning Calorimeter.

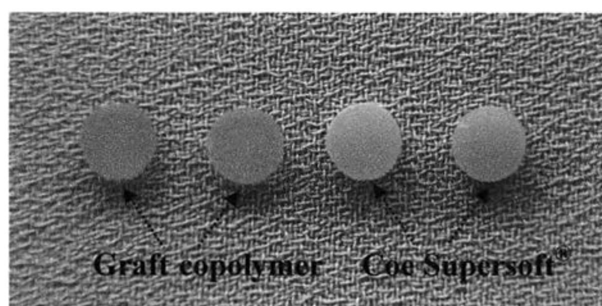


Figure 3.10. The graft copolymer and Coe Supersoft® specimens were prepared in the disk shape for determination of T_g value.

3.5.2 Part II: Test of physical and Mechanical properties

The specimens used in these tests were graft copolymer prepared in different form. The specimens of graft copolymer and the Coe Supersoft® were used for each testing.

3.5.2.1 Hardness test

The hardness test was performed according to ASTM D 2240-91 (Standard Test Method for Rubber Property Durometer Hardness) base on the measurement of the penetration of a rigid plunger into the rubber specimen under specified condition. Ten flat and smooth rectangular sheets ($70x70x3mm^3$) of tested materials were prepared. Then the specimens were immersed in distilled water at 37 ^{o}C at the different immersion times (30minute, 1hour-6hours, 1week-3weeks, 1month-10 months). The specimen from each immersion time was placed under the plunger of Durometer (Figure 3.11) then the plunger was pressed with the minor force on the specimen, the scale showed the value of the hardness in Shore A value at room temperature. Made five measurements of hardness at different positions on the specimen at least 6 mm apart and determined the arithmetic mean.

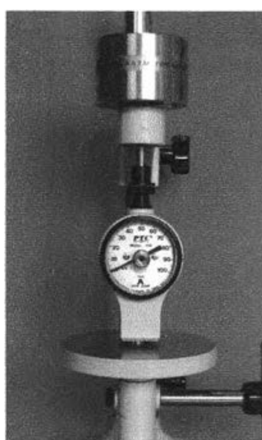


Figure 3.11. The Durometer apparatus was used for measuring the surface hardness.

3.5.2.2 Tensile strength test

Tensile strength was measured according to ASTM D638-01. The flat and smooth rectangular sheet test specimen of the graft copolymer and control group material with 3 mm thick were prepared (Figure 3.12 a). The specimens were cut with one of the steel dies conforming to the dimensions shown in Figure 3.12 b. For the tensile strength test, twenty specimens of each material were performed by the universal testing machine (UTM) with a crosshead speed of 50 mm/min at room temperature (Figure 3.13).

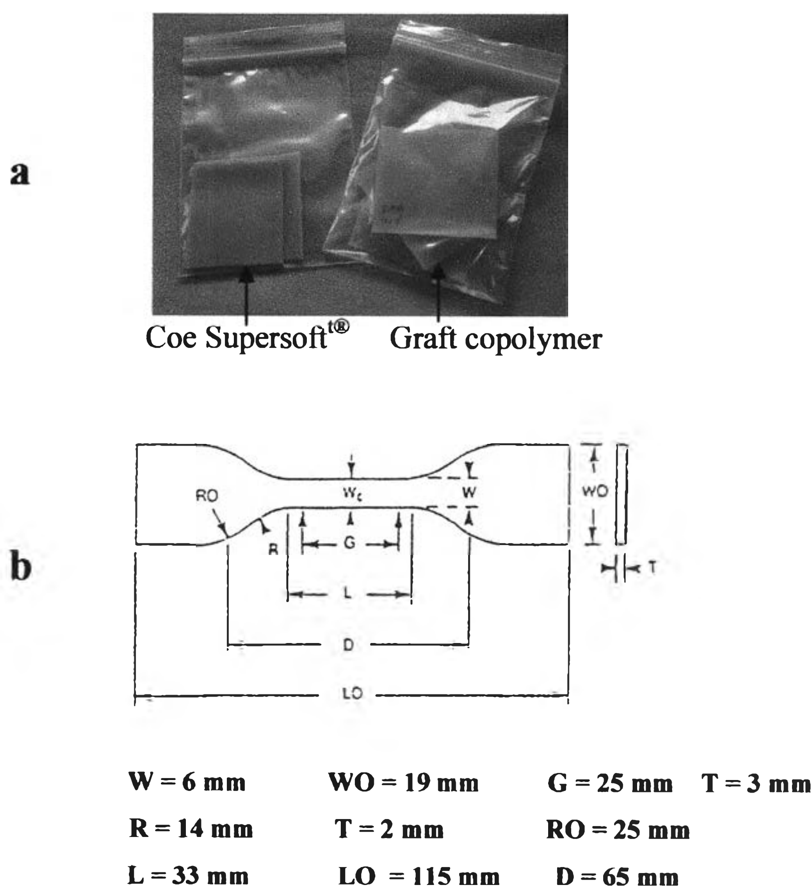
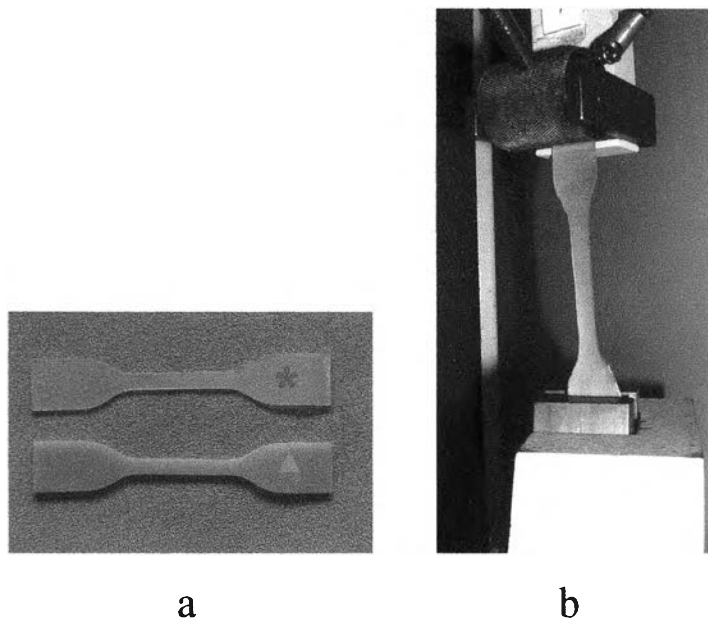


Figure 3.12 a) The flat and smooth rectangular sheet of tested materials

b) Dumbbell-shaped test piece (type IV) with its dimensions



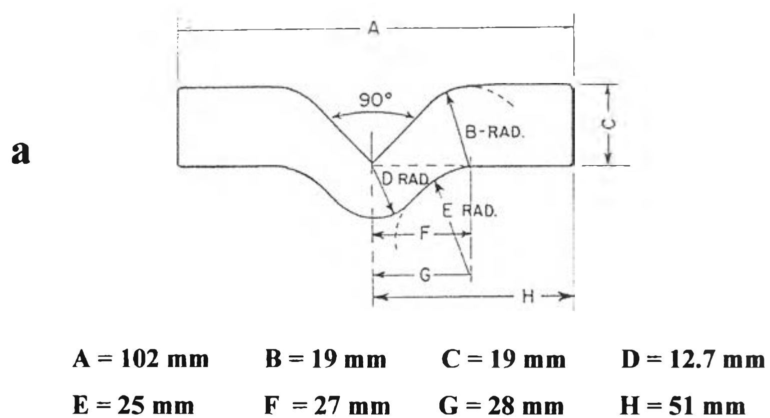
a b

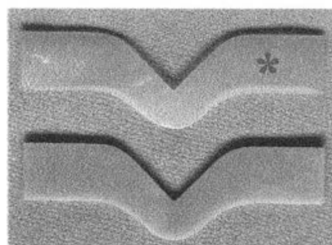
Figure 3.13 a) The dumbbell-shaped test piece of graft copolymer ✻ and Coe Supersoft® ▲ b) The specimen was fixed to the holder of the UTM for determination tensile strength.

3.5.2.3 Tear strength test

Tear strength was measured according to ASTM D624. The flat and smooth rectangular sheet of the graft copolymer and control material with 3 mm thick were prepared. The specimens were cut with one of the steel dies conforming in shape according to Die C (Figure 3.14 a, b). Twenty specimens of each material were measured the tear strength by the UTM with a crosshead speed of 500 mm/min at room temperature (Figure 3.14 c)

a





b c

Figure 3.14 a) The shape and dimensions of test piece for tear strength testing
b) The graft copolymer * and Coe Supersoft® ▲
c) The test piece was fixed to the holder of the UTM for tear strength measurement.

3.5.2.4 Tensile bond strength testing

The rectangular silicone dies (10x10x73 mm^3) were invested in gypsum mould to perform the rectangular spaces after removal of silicone dies, the small pieces of silicone (3x10x10 mm^3) were placed in the middle of the space (Figure 3.15 a). Separating media was applied on the gypsum mould then the heat polymerized PMMA powder and methyl methacrylate monomer (MMA) were mixed according to the manufacturer's instruction and pressed into the rectangular space. The small piece of silicone was removed to provid 3 mm of the space distance between the PMMA bars. The latex of graft copolymer or dough state of Coe Supersoft ® was packed into the remaining space (Figure 3.15 b.) and compressed at 211 kg/cm^2 for 10 minutes. The mould and specimens were then heated in the hot air oven at 74 ^{0}c for 10 hours. After removing the cured specimen from the mould, it was stored in distilled water at 37 ^{0}C and tested at 24 hours. For the tensile bond strength, fifteen specimens of each material were measured by the UTM at crosshead speed of 5 mm/min (Figure 3.16).

In order to study the nature of the interface between the PMMA and either the graft copolymer or Coe Supersoft®, the specimens were prepared for scanning electron microscopic (SEM) examination by cutting the specimens into small pieces. Each pieces composed of 2 mm of soft liner bonded to 2 mm of PMMA. Investigation by SEM was performed and the interfaces between the PMMA and the soft lining materials were recorded.

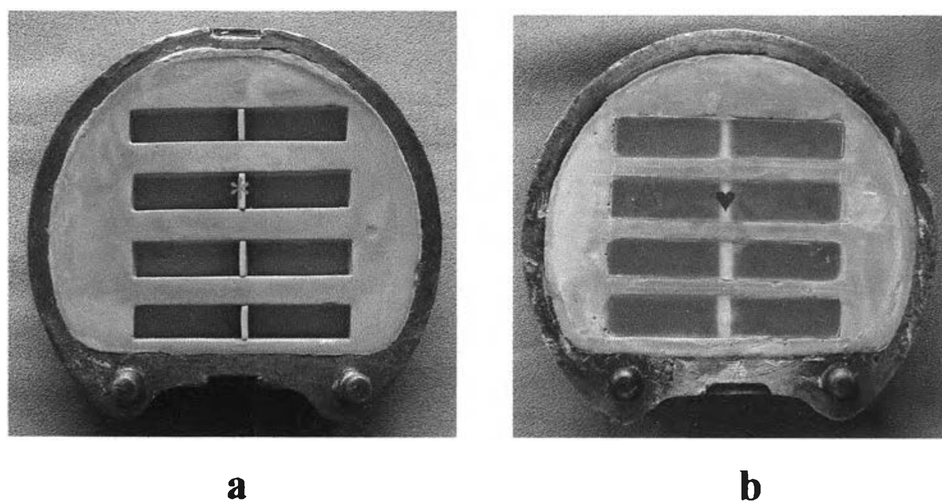


Figure 3.15 a) The rectangular space moulds with the small pieces of silicone ⁎ placed in the middle of the space.

b) The tested material ♥ was packed between the denture base materials. ▲

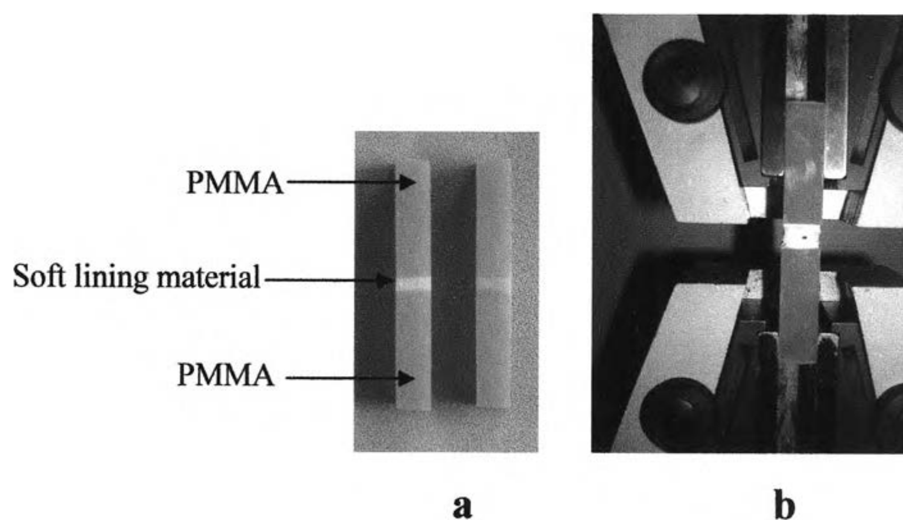


Figure 3.16 a) The tested specimens after immersion in water for 24 hours.

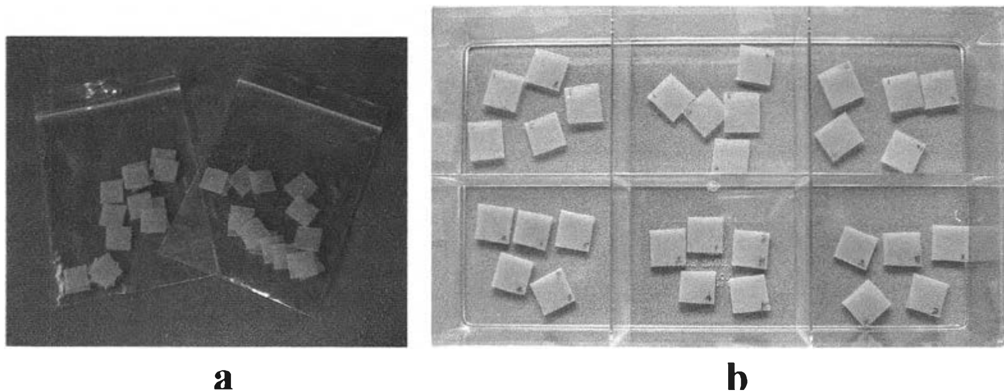
b) The specimen was fixed to the holder and measured the tensile bond strength by a Lloyd UTM.

3.5.2.5 Water absorption testing

The flat and smooth rectangular sheet of the graft copolymer with 2 mm thick was prepared. The specimens were cut with one of the steel dies conforming in square-shaped 20x20x3 mm^3 (Figure 3.17 a.). Fifteen specimens of each material were placed in a desiccator at 37 ^0C with silica gel. Daily weights of the desiccated specimens were obtained until a stable weight reading (m_o) was obtained. The specimens were placed in distilled water and stored at 37 ^0C in the dark (Figure 3.17 b.). At each storage interval time: 30 minutes, 1 day-14days, 3 weeks- 4 weeks, 2 months-10 months, the specimens were removed. Excess moisture on the specimen was wiped off quickly and the specimens were weighed (m_t).

The percentage of water absorption (M_T) was determined by the following formula:

$$M_T = \frac{(m_t - m_o) \times 100}{m_o}$$



a b

Figure 3.17 a) The specimens were cut into a square shape.

b) The specimens were immersed in distilled water at 37 ^oC.

3.5.2.6 Contact angle test

The flat and smooth rectangular sheet test specimen of the graft copolymer and control group material with 3 mm thick were prepared and cut into 20x40x3 mm^3. After preparation, fifteen specimens of each material were stored in distilled water at 37 °C for 24 hours before testing. The specimen was placed horizontally on the platform of a contact angle meter (Figure 3.18). One drop of deionized distilled water was placed carefully from a hypodermic syringe onto the surface of each specimen. The drop was placed near the edge of the specimen to aid the viewing. The contact angle from each drop of water presented on the scale board was measured and calculated. Three measurements were made for each specimen.

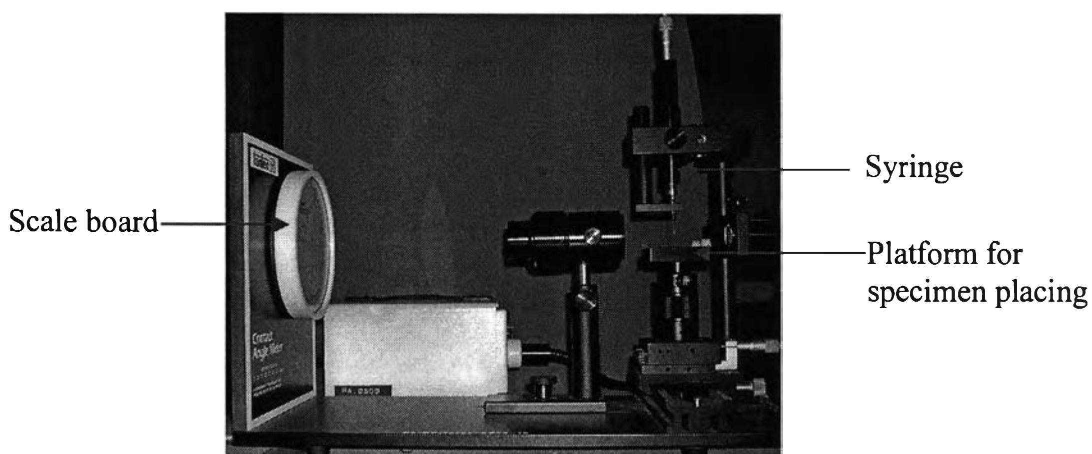


Figure 3.18. The contact angle meter was used for measuring the wettability of the specimen surface.

3.5.2.7 Staining test

The square-shaped specimens of 20x20x3 mm^3 were fabricated (Figure 3.19). The test was conducted by measuring the color change of the specimens after immersion in three staining solutions (instant coffee solution, tea solution and capsaicin dissolved in vegetable oil). To prepare a standard solution of coffee, 15 g of

coffee was poured into 500 ml of boiling distilled water. After 10 minutes of stirring, the solution was passed through filter paper. The tea solution was prepared by immersing 5 tea bags into 500 ml of boiling distilled water for 10 minutes. For the capsaicin in vegetable oil was prepared by adding 30 g of capsaicin (Neo Pacific, Thailand) with 130 g of vegetable oil the stirred until the color was homogeneous. To prevent fungal growth, 0.3 g of sodium azide was added to each 500 ml of each solution. Color measurement of ten specimens of each material before immersion were performed using a spectrophotometer according to CIE L*a*b* uniform color scale [2]. After immersion in the staining solutions at 37 ^{0}C for different intervals of times (30 minutes,1 hour, 1day-7days, 2 weeks-8 weeks), excess solution on each specimen surface was removed with paper towel and color measurements were performed again. The mean value of three randomly selected sites on each specimen represented its changed color value. Color changes (ΔE) were determined by calculating in this formula. $\Delta E = \{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2\}^{½}$

when **L** is lightness, **a** is redness-greenness and **b** is yellowness-blueness

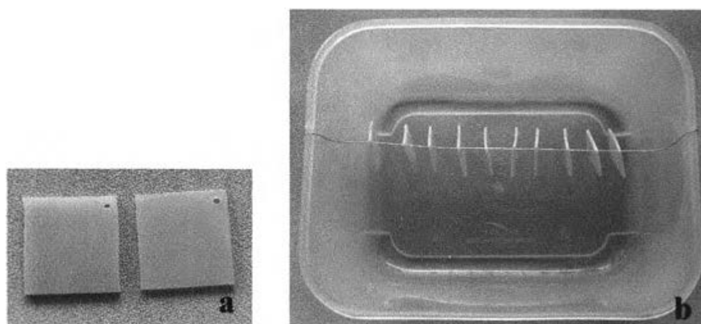


Figure 3.19 a) Each square-shaped specimen was drilled at one corner to perform a hold for hanging with stainless steel wire

b) The specimens were hanging in a plastic container containing the staining solution.

3.5.2.8 Statistical analyses

The data from physical and mechanical properties of graft copolymer and Coe Supersoft® used as a control group were statistically analyzed by independent T-test and One-Sample T-test at 95% confidential level.

3.5.3 Part III: In vitro study of the cellular response to the graft copolymer

3.5.3.1 Cell culture

Clones of normal human gingival fibroblasts were prepared from healthy gingival tissues. Briefly, the tissue sample from gingivectomy were cut into small pieces and transferred to 35 mm culture plates (Falcon, Germany). The tissue samples were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 2mM L-glutamine, 100 units/ml penicillin,100 μg/ml streptomycin and 5 μg /ml amphotericin B and maintained at 37 °C in humidified atmosphere of 95% air and 5% CO_2. All these ingredients for culture medium were purchased from Gibco BRL (Gibco Laboratories, USA). The medium was replaced every day. The explant was observed under the inverted microscope daily. On day 3 to 10 after tissue explant, a lot of cells migrated out of the tissue. Subculture of the cell clusters was performed in order to reduce the density of cell population and evenly distribute the cells in the new culture plates. This process allowed the cells to receive enough nutrients from the growth medium. In addition, this process could help eliminate other cell types except fibroblasts. Since the fibroblasts adhere on the substrate faster than other cell types do.

The method of subculture was described below:

1. Remove the culture medium and wash twice with simple DMEA to remove the traces of serum that would interfere the action of trypsin.

2. Detach the cell with trypsin-EDTA. This reagent will destroy the protein promoting the adhesion between cell to cell and cell to the substrate. As the result, the cells were freed from the attached surface.

3. Stop the trypsin-EDTA reaction with trypsin inhibitor.

4. The cell suspension was then filtered with lens paper. The filtered cell suspension was centrifuged at 2,000 g to pellet the cells and the supernatant was discarded. The pellet was resuspended in the fresh medium.

5. The cells were counted by hemocytometer and replated at the cell density of 2 $\times 10^4$ per ml in culture medium. The culture plate was kept at 37 °C in humidified atmosphere of 95% air, 5% CO_2.

6. After leaving the cells to attach the plate for 1 hour, the old culture medium with unattached cells was replaced with a fresh one. The medium was replaced very 2 days. When the population of cells was high in density, the subculture was repeated as described above. With this method of selective attachment of the cell, clone of fibroblasts can be established approximately at the 5 th passage. For this study, cells from the fifth passages were used in the study the reaction of fibroblasts with graft copolymer and Coe Supersoft®.

3.5.3.2 Cytotoxicity test

For cytotoxicity test, 4 x 10^4 cells/well were seeded in 24-well plates and incubated at 37 °C in humidified atmosphere of 95% air, 5% CO_2 for 24 hours. Cell cultures were immediately exposed to vulcanized graft copolymer sheets (3 mm in diameter, 3 mm thick) stabilized in the well. The control groups were the well with out the graft copolymer and the wells with Coe Supersoft®. After another 24 hours of incubation, the viability of cells was immediately evaluated using the MTT assay. In each test, different formulas of graft copolymer were tested and each formula was test in 3 wells. The experiment was repeated 3 times with cells from different patients.

Concentrations corresponding to 50% death of cell were evaluated from the standard curves.

3.5.3.3 Study the cellular response of fibroblasts to the graft copolymer by scanning electron microscopy.

The specimens (20x20x2 mm^3) were sterilized by autoclave before being fixed onto the 35 mm culture plates with sticky wax. Then, the cells from the 5th passage were plated into the prepared culture plates at the concentration of 10,000 cells /ml. The culture plates were maintained at 37 ^{o}C in humidified atmosphere of 95% air, 5% CO_2 for 72 hours. The specimens were investigated with inverted phase contrast microscope everyday. After 72 hours of co-culture, they will be prepared for SEM examination. Briefly, the specimens were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.2 at 4 ^{o}C for 1 hour and 1% osmium tetroxide for 1 hour. After fixation, the specimens were dehydrated with graded ethanol. The specimens were then dried at critical point drying, fixed on the stubs and coated with gold particles before investigation under the SEM.