CHAPTER III

METHODS AND MATERIALS

3.1 Experimental Design

This work was divided into three parts. The first part was to synthesize the two-chitosan derivatives, TMC and HTACC, by varying equivalent of reacting agent and reaction times. In addition, the cytotoxicity of chitosan and its derivatives were examined. IC_{50} or 50% inhibitory concentrations values were determined with human keratinocyte cell line (HaCaT) by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The second part was to prepare five "leave-on" formulations i.e. leave-on formulation without cationic polymers (LO), leave-on formulation with polyquaternium-10 (LO+quat), leave-on with chitosan (LO+chitosan), leave-on with TMC (LO+TMC), and leave-on with HTACC (LO+HTACC). Moreover, four types of damaged hairs i.e. waved, straightened, dyed, and UV damaged were used as test substrates. The last part, the roles of leave-on were studied in terms of physical and mechanical properties of leave-on coated hairs. The presence of the compounds coated on hair was determined by scanning electron microscopy (SEM) and microscope-attached attenuated total reflection-Fourier transform infrared (ATR FT-IR) microspectroscopy with a slide-on germanium internal reflection element (IRE).

3.2 Materials

3.2.1 Sample and reagents

Black hairs from an Asian thirty two-year old female used in this study were supplied by Life Science Cosmetics Research Center Co., Ltd. All reagents and materials are analytical grade. Chitosan with a weight-average molecular weight of 45,000 Da was purchased from Seafresh Chitosan (Lab) Co., Ltd., Thailand. The degree of deacetylation (DD) was 85% as determined by ^1H NMR. A dialysis tubing cellulose membrane (Sigma) with molecular weight cut-off of 12,400 g/mol (avg. diameter. 49 mm; avg. flat width 76 mm) was used to purify all modified chitosans. Iodomethane,

CH_3I (Riedal-deHaen); glycidyltrimethylammonium chloride, GTMAC (Fluka); *N*-Methyl-2-pyroridone, NMP (Merck); acetic acid, glacial (Merck); acetone, analytical grade (Merck); silver nitrate, $AgNO_3$ (Merck); sodium chloride, NaCl (Merck); sodium hydroxide, NaOH (Merck); sodium iodide, NaI (Aldrich); sodium sulfide, Na_2S (Merck); sulfuric acid, conc. (Merck) were used as received.

3.2.2 Instruments

1. Labconco Corporation freeze dryer model 7753501
2. Mercury Varian 400 MHz nuclear magnetic resonance (NMR) spectrometer
3. Perkin Elmer Fourier transform infrared (FT-IR) spectrometer model system 2000
4. Rayonet Photochemical Reactors model RPR-100 and UV lamps with 313 nm (UVB)
5. Nicolet 6700 FT-IR spectrometer equipped with a mercury-cadmium-telluride (MCT) detector (Thermo Electron Corporation, Madison, WI, USA)
6. Contiuμm™ infrared microscope with 15X Cassegrain infrared objective and 10X glass objective
7. Homemade slide-on germanium (Ge) μIRE
8. Scanning electron microscope (SEM) model JEOL, JSM-6480LV
9. Industrial presses (Hand Toggle) (HMC-Brauer Ltd, Milton Keynes, England)
10. Miniature tensile tester (MTT)
11. Laser scanning micrometer (LSM-series 500)
12. TA.XT.Plus Stable Microsystems Texture Analyzer
13. Instron 5564 tensile test tester equipped with Bluehill software version 2.0
14. Brookfield digital viscometer model DV-I+ (Brookfield Engineering Laboratories, Inc., Middleboro, USA)

3.3 Experimental Methods

3.3.1 Synthesis of *N,N,N*-trimethylammonium chitosan chloride (TMC)

TMCs were synthesized by methylation of chitosan with iodomethane in the presence of NaOH as described by Sieval *et al.*[2] Chitosan (~5 g) was dispersed in NMP at 50°C for 18 h. NaI (3.8 g, 1 equiv) and 15%w/v aqueous NaOH were added and stirred at 50°C for 15 min. Subsequently, iodomethane (9.52 mL, 6 equiv) was added in two equal portions (4.76 mL each) at 6 h intervals at 50°C. Finally, the mixture was stirred at 50°C for 18 h. After methylation, the product was precipitated in acetone. The precipitate was then isolated by centrifuge. the solid product was dispersed in 15%w/v NaCl solution in order to exchange the I^- counter-ions of the TMC for Cl^-. The final suspension was dialyzed with deionized water for three days, followed by freeze-drying to obtain a cotton-like material. The amounts of iodomethane were varied to 4.76 mL (4 equiv) or 7.93 mL (5 equiv) or 14.28 mL (12 equiv) in order to synthesize TMC having different *DQs*.

Moreover, NMP solvent was replaced by DMF:H_2O (1:1) or H_2O in order to increase methylating efficiency of iodomethane on chitosan.

3.3.2 Synthesis of *N*-[(2-hydroxyl-3-trimethylammonium)propyl] chitosan chloride (HTACC)

HTACCs were synthesized by reacting chitosan with GTMAC as described by Seong *et al.*[3] Chitosan (~5 g) was dissolved in 1%v/v acetic acid at room temperature. Then GTMAC (7.7 g, 2 equiv) was added into the chitosan solution. The reaction was performed at 70°C for 24 h. After the reaction, products were dialyzed with deionized water for three days, followed by freeze-drying to obtain a cotton-like material. HTACCs with different *DQs* were synthesized by starting with 5 g of chitosan but using 15.3 g (4 equiv) or 23.0 g (6 equiv) of GTMAC and varying the reaction times (2, 4, 8, 12, and 24).

3.3.3 Characterization of chitosan and its charged derivatives

Chemical structures of chitosan and its charged derivatives were determined by ^1H NMR spectroscopy. Chemical shifts (δ) were reported in part per million (ppm) relative to tetramethylsilane (TMS) or using the residual protonated solvent signal as a reference. All measurements were performed at 300K, using pulse accumulations of 128 scans. D_2O/CF_3COOD was the solvents for 10 mg chitosan and its derivatives. According to the literature, the hydrogen atom bonded to carbon 2-6 of the glucopyranose ring is responsible for the set of signals ranging from 3.25 to 4.25 ppm (for TMC) and from 3.40 to 3.80 ppm (for HTACC) while those signals observed at 3.10 ppm are attributed to the hydrogen atoms of the three methyl groups (9 H′s). From the ratio between the area under a reference signal and that under the signals of the quaternary ammonium group, it is possible to determine the degree of quaternization ($\%DQ$) of the TMCs and HTACCs by using expression:

$$\textit{Degree of quaternization} = \%DQ = \left[\frac{\int N^{+}(CH_3)_3/9}{\left(\int H-2',3,4,5,6,6'/6\right)\times DD}\right]\times 100 \qquad (3.1)$$

where, $\%DQ$ is the degree of quaternization; $\int N^{+}(CH_3)_3$ is the integral of the 9 H's on the three methyl groups (9 H′s) attached to the quaternary ammonium atom. $\int H-2',3,4,5,6,6'$ is the integral corresponding to the H-2′,3,4,5,6 and 6′ protons from 3.25 and 4.25 ppm (for TMC) and from 3.40 to 3.80 ppm (for HTACC). The DD of chitosan determined by ^{1}H NMR was 0.85.

In addition, the signals observed at 2.85 and 2.65 ppm are attributed to the hydrogen atoms of the methyl groups pertaining to di- and monomethylated amino groups, respectively in TMC. The average degree of dimethylation ($\%DS_{N(CH_3)_2}$) and degree of monomethylation ($\%DS_{NHCH_3}$) of the methylated product were analyzed by using expression:

$$\textit{Degree of monomethylation} \quad \%DS_{NHCH_3} = \left[\frac{\int NHCH_3/3}{\left(\int H-2',3,4,5,6,6'/6\right)\times DD}\right]\times 100 \qquad (3.2)$$

$$\textit{Degree of dimethylation} = \%DS_{N(CH_3)_2} = \left[\frac{\int N(CH_3)_2/6}{\left(\int H-2',3,4,5,6,6'/6\right)\times DD}\right]\times 100 \qquad (3.3)$$

where, $\int N(CH_3)_2$ is the integral of the dimethyl peak (6 H's). $\int NHCH_3$ is the integral of the monomethyl peak (3 H's). $\int H-2',3,4,5,6,6'$ is the integral corresponding to the H-2′,3,4,5,6 and 6′ protons from 3.25 and 4.25 ppm.

In addition, undesired methylation at the hydroxy groups on chitosan (3.20 and 3.15 ppm) was also found in TMC. The degrees of methylation at 3-O and 6-O (*DOM-3* and *DOM-6*) of the TMCs were calculated by using the following equation:

$$\%DOM-3=\left[\frac{\int 3-OCH_3/3}{(\int H-2',3,4,5,6,6'/6)\times DD}\right]\times 100 \quad (3.4)$$

$$\%DOM-6=\left[\frac{\int 6-OCH_3/3}{(\int H-2',3,4,5,6,6'/6)\times DD}\right]\times 100 \quad (3.5)$$

In addition, the $\%DQ$ of TMCs and HTACCs were determined by titration method. The Cl^- counter ions of TMC and HTACC were titrated with aqueous silver nitrate ($AgNO_3$) as described by Lim *et al.*[56] Briefly, 0.1 g of TMC or HTACC was dissolved in 100 mL deionized water. The conductivity of the TMC or HTACC solution was measured as a function of the volume of 0.017 M $AgNO_3$ added using a conductivity meter. The volume of 0.017 M $AgNO_3$, V_{AgNO_3}, that resulted in the lowest conductivity for TMC or HTACC solution was employed to calculate the $\%DQ$ using the following equation:

$$\%DQ_{(TMC)}=\frac{1.7\times 10^{-5}\times V_{AgNO_3}}{\left(\frac{W_w-(1.7\times 10^{-5}\times V_{AgNO_3}\times m_{CH_3Cl})}{(m_G\times DD)+m_{AG}(1-DD)}\right)\times DD}\times 100 \quad (3.6)$$

$$\%DQ_{(HTACC)}=\frac{1.7\times 10^{-5}\times V_{AgNO_3}}{\left(\frac{W_w-(1.7\times 10^{-5}\times V_{AgNO_3}\times m_{GTMAC})}{(m_G\times DD)+m_{AG}(1-DD)}\right)\times DD}\times 100 \quad (3.7)$$

Specifically, 1.7×10^{-5} corresponds to the number of moles of $AgNO_3$ in 1 mL of solution. W_w is the weight of TMC or HTACC in 100 mL (0.1 g). m_{CH_3Cl} is the molecular weight of CH_3Cl (50 g/mol). m_{GTMAC} is the molecular weight of GTMAC (151 g/mol). m_G is the molecular weight of glucosamine (161 g/mol). m_{AG} is the molecular weight of *N*-acetyl-glucosamine (203 g/mol). The DD of chitosan is 0.85.

The FT-IR spectra of chitosan and its derivatives were recorded with a Perkin Elmer model system 2000 Fourier Transform Infrared (FT-IR) spectrometer, with 32 scans at resolution 4 cm^{-1}. A frequency of 4000-400 cm^{-1} was collected by using TGS detector. All samples were prepared as potassium bromide pellets.

3.3.4 Solubility tests of the charged derivatives of chitosan

Solubility was monitored visually. Solid samples of TMCs or HTACCs (100 mg) were dispersed in H_2O (20 mL) according to a method of Sashiwa *et al.*[58] The pH of the solution was adjusted with 0.5%w/v aqueous HCl and NaOH.

3.3.5 Cytotoxicity of chitosan and its charged derivatives

Cytotoxicity in terms of IC_{50} of chitosan, TMCs, and HTACCs were examined on human keratinocyte cells line or HaCaT using MTT assay at pH 6.0. Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, pH=7.4) solution containing *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanosulphonic acid) (HEPES), $NaHCO_3$, 1%v/v penicillin/streptomycin, 10%v/v fetal bovine serum in sterilized H_2O, was used as culture medium. HaCaT cell cultures of passage numbers 5-13 were used for all of the experiments. The cells were seeded in 96-well plates (Costar) at a seeding density of 1×10^5 cells/mL. The test sample was dissolved in the above-mentioned solution at concentrations ranging from 0.5-5000 μg/mL. HaCaT cells were treated with varying concentrations of the test sample for 24 h in humidified atmosphere with 5% CO_2 at 37°C.

Statistical analysis of IC_{50} comparison between concentrations of the test sample was performed using Statistical Package for the Social Science (SPSS) version 14.0 software. Statistical comparisons made by the One-Way Analysis of Variance (ANOVA) with the Fisher's Least Square Difference (Fisher's LSD) tests. Each experiment was performed at least twelve times. All data were presented as a mean value with its standard deviation indicated. Differences were considered to be statistically significant when the *p* values were less than 0.05.

3.3.6 Preparation of leave-on conditioner

Five formula of leave-on conditioners were prepared; leave-on without cationic polymers (LO), leave-on with polyquaternium-10 (LO+1%quat), with chitosan (LO+1%chitosan), with TMC (LO+1%TMC), and with HTACC (LO+1%HTACC).

Table 3-1 Chemical composition of leave-on formulation without cationic polymer and leave-on formulation with 1%w/w polyquaternium-10, chitosan, TMC or HTACC

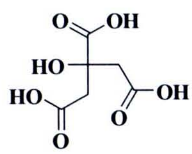
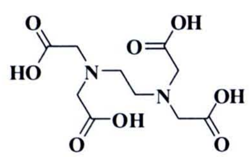
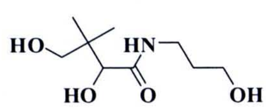
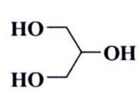
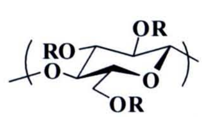
Composition of leave-on formula	**Amount (%w/w)**	
	LO	**LO+cationic polymers**
PART A: Water phase		
Citric acid  Citric acid	q.s.	q.s.
Disodium EDTA  EDTA	0.100	0.100
DI water	84.630	83.630
DL-panthenol  DL-panthenol	0.150	0.150
Glycerine  Glycerine	5.000	5.000
Hydroxyethyl-cellulose  R=H or CH_2CH_2OH Hydroxyethylcellulose	0.020	0.020

Table 3-1 *continued*

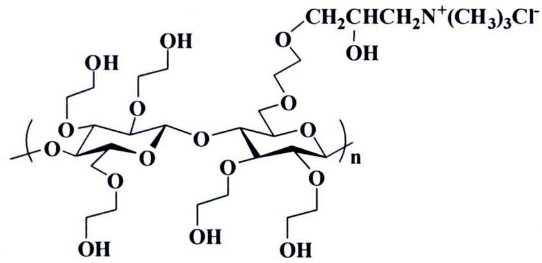
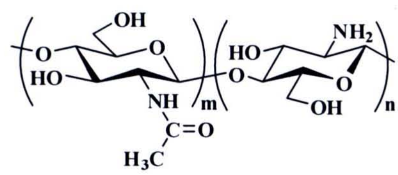
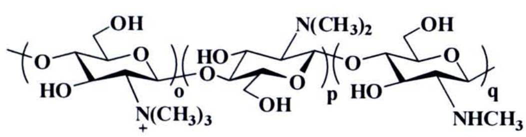
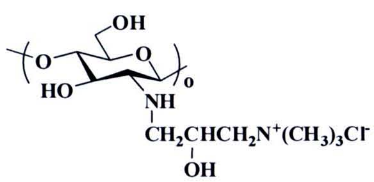
Composition of leave-on formula		**Amount (%w/w)**	
		LO	**LO+cationic polymers**
Polyquarternium-10, Chitosan, TMC, or HTACC	 **Quaternized hydroxyethyl cellulose (polyquaternium-10)**  **Chitosan**  ***N,N,N*-trimethylammonium chitosan chloride (TMC)**  ***N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride (HTACC)**	-	1.000
PART B: Oil phase			
Ceteareth-6	a polyoxyethylene ethers of a mixture of cetyl alcohol and stearyl alcohol	2.000	2.000
Cetyl alcohol	 **Cetyl alcohol**	2.000	2.000

Table 3-1 *continued*

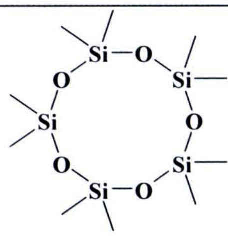
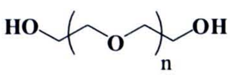
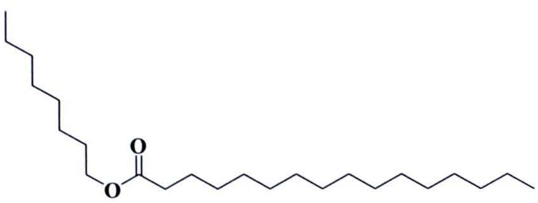
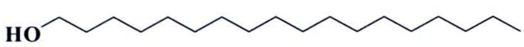
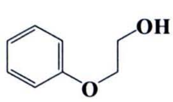
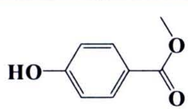
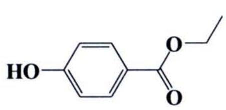
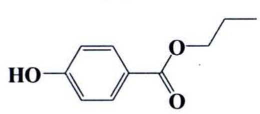
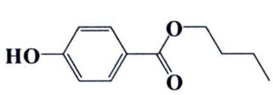
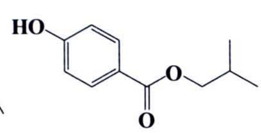
Composition of leave-on formula		Amount (%w/w)	
		LO	LO+cationic polymers
Cyclomethicone	 Cyclomethicone	2.000	2.000
Glyceryl stearate and PEG-100	 Glyceryl stearate  Polyethylene glycol	1.000	1.000
Octyl palmitate	 Octyl palmitate	1.000	1.000
Stearyl alcohol	 Stearyl alcohol	1.000	1.000
PART C: Additive			
Euxyl K300	 Phenoxyethanol  Methyl paraben  Ethyl paraben  Propyl paraben  Butyl paraben  Isobutyl paraben	0.500	0.500

Table 3-1 *continued*

Composition of leave-on formula		Amount (%w/w)	
		LO	LO+cationic polymers
Hydrolyzed wheat protein		0.100	0.100
Hydrolyzed silk protein		0.100	0.100
Quatermium-79 hydrolyzed collagen	a cationic conditioning agent from virgin palm oil combine with collagen	0.100	0.100
Sunshine		0.300	0.300
Total		**100.00**	**100.00**

3.3.7 Preparation of cosmetically-treated hairs

Waved, straightened, and dyed hairs were prepared by treating hair strands with Angela™ Cold wave lotion (Just Modern), Caring® Hair straightener cream, and Caring® Beauty hair colour cream, respectively. A UV lamp with 313 nm (UVB, RPR-3000A° lamp) was used as an irradiation source to prepare UV-damaged hairs. Hair strands were clamped vertically at a distance of 1.5 inches from the UV lamp (as shown in Figure 3-1).

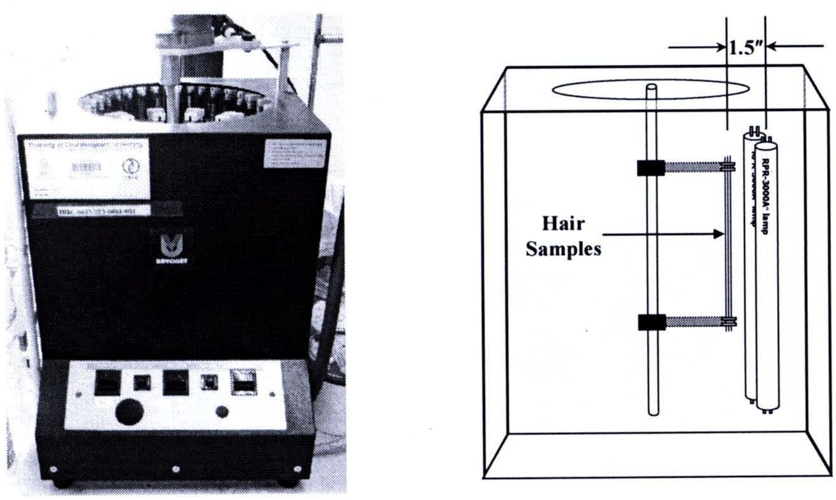


Figure 3-1 Rayonet Photochemical Reactors model RPR-100 and the position of hairs in reactors.

3.3.8 Analysis of hair samples by attenuated total reflectance-Fourier transform infrared (ATR FT-IR)

All human hair samples were air-dried prior to analyses. All ATR spectra of hair surface were collected with a Continuμm™ infrared microscope equipped with a liquid N_2 cooled mercury-cadmium-telluride (MCT) detector. The microscope was connected to a Nicolet 6700 FT-IR spectrometer (Thermo Electron Corporation, Madison, WI, USA). Spectra in the mid-infrared region (4000-650 cm^{-1}) at a spectral resolution of 4 cm^{-1} were collected with 128 co-addition scans. A homemade ATR accessory with a slide-on miniature germanium (Ge) IRE was lightly pressed on the hair sample during spectral acquisitions.

Cysteic acid content of hair surface

Normalization of IR spectra of virgin and treated hairs was carried out based on amide I band at 1657 cm^{-1}, in which the peak area was large and not influenced by the chemical and photochemical treatment on the hair. The cysteic acid content of the hair surface was determined from the area ratio of the S=O band (calculated from the peak to a baseline, which was drawn between 1020 and 1070 cm^{-1}), and the amide I peak. (calculated from the peak to a baseline, which was drawn between 1580 and 1730 cm^{-1}).

Secondary structure of chemical constituents on hair surface

The information of secondary structure of keratin proteins were acquired by curve fitting analysis. The amide I region between 1580 and 1730 cm^{-1} was deconvoluted by fitting with Gaussian and Lorentzian functions. The peak position and peak area of the fitted peak were identified and used to elucidate the secondary structure information.

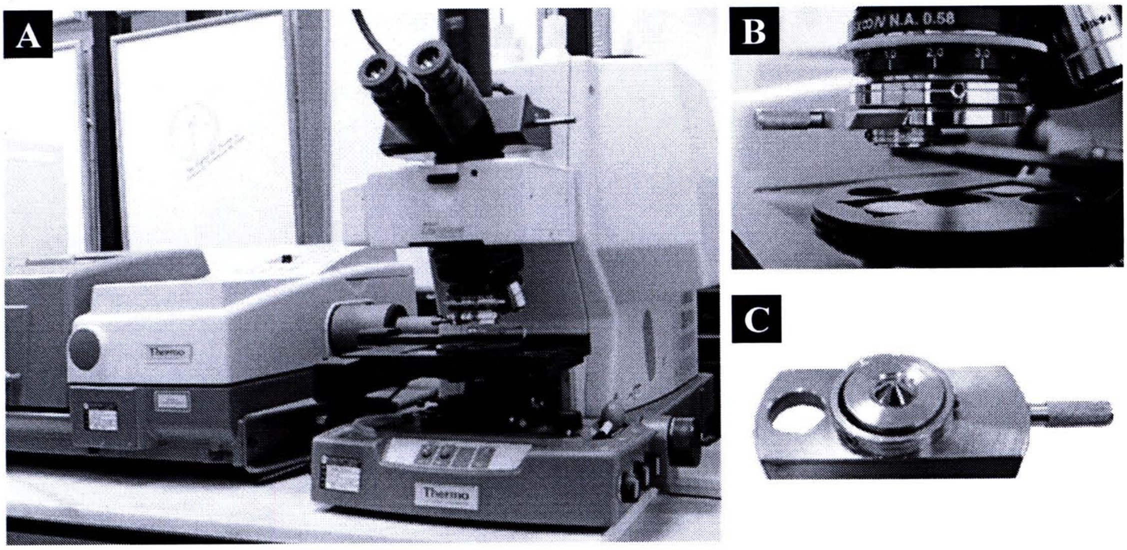


Figure 3-2 ATR FT-IR microspectroscope: (A) Continuμm™ infrared microscope attached to the Nicolet 6700 FT-IR spectrometer, (B) the slide-on Ge μIRE is fixed on the position of slide-on housing on the infrared objective, and (C) homemade slide-on Ge μIRE.

<u>Default Spectral Acquisition Parameter</u>

Nicolet 6700 FT-IR Spectrometer

<u>Instrumental Setup</u>

Source	Standard Globar™ Infrared Light Source
Detector	MCT
Beam splitter	Ge-coated KBr

<u>Acquisition Parameters</u>

Spectral resolution	4 cm^{-1}
Number of scans	128 scans
Spectral format	Absorbance
Mid-infrared range	4000-650 cm^{-1}

<u>Advanced Parameters</u>

Zero filing	none
Apodization	Happ-Genzel

Phase correction Mertz

Continuμm™ Infrared Microscope

<u>Instrumental Setup</u>

Detector MCT

Objective 15X Schwarazschild-Cassegrain

Aperture size 150 μm × 150 μm

3.3.9 Morphology of hair samples by scanning electron microscopy (SEM)

All human hair samples were air-dried on a glass slide. The samples were sputter-coated by gold films, and moved to specimen chamber. The morphology of hairs was then analyzed. The operating accelerating voltage was 15 kV for capturing the image of samples.

3.3.10 Tensile testing of hair samples by miniature tensile tester (MTT)

Hair samples were randomly selected. The single hair was threaded onto ferrules. Then, the hair was laid on the sample-mounting block as shown in Figure 3-3. One side of hair was clung and the remaining of hair was also clung while gently pulling the hair straight. After the hair block was placed under press, the hair was released from the sample-mounting block and uses a sharp tool to remove the crushed ferrules from the block.

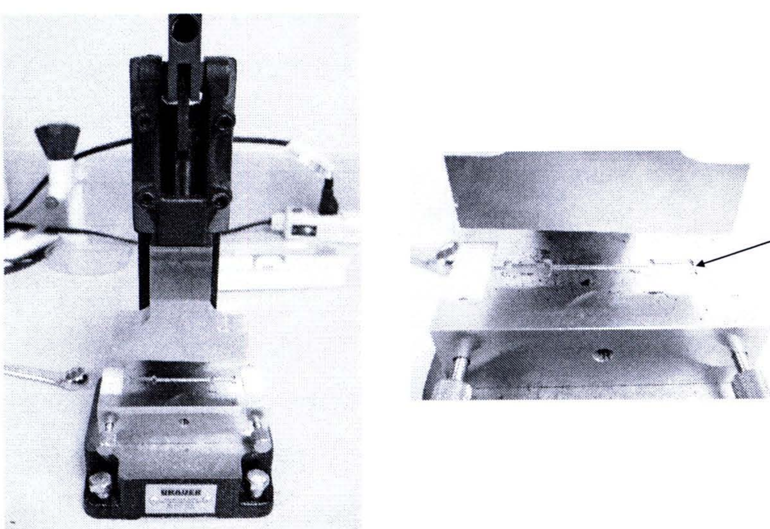


Figure 3-3 Industrial presses (Hand Toggle).

The hair sample was extended to break on miniature tensile tester (MTT) as shown in Figure 3-4. The rate of extension was 20 mm/min. All the single hair was initially conditioned at temperature and humidity control room (22°C, 50%RH). Ten specimens were tested for each sample type. The cross-sectional area (sq.µm) of the hair sample was determined by laser scanning micrometer (LSM).

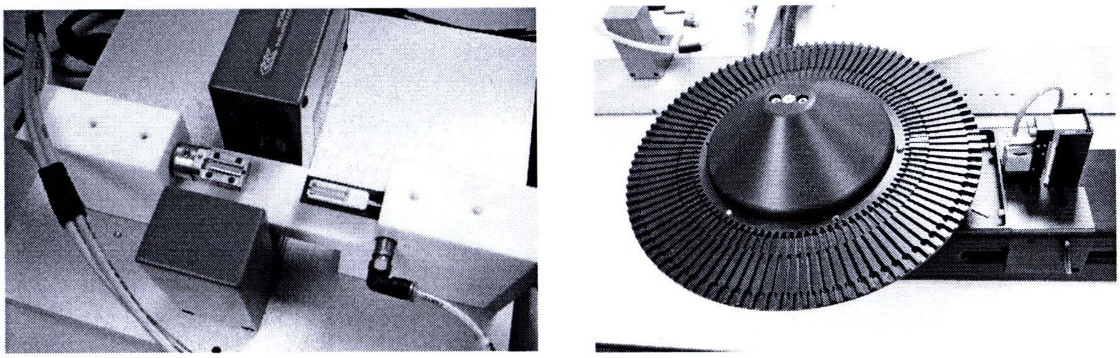


Figure 3-4 Laser scanning micrometer, LSM-series 500 (left); Miniature tensile tester, MTT (right).

Elastic modulus (N/m^2; Pa), plateau load (gmf/sq.µm), break extension (%strain), and break load (gmf/sq.µm) that are associated with hair strength were determined. The mean and standard deviation values of each parameter were recorded for each sample. Data is analyzed by Fisher's Least Square Difference (Fisher's LSD) at 95% confidence level.

3.3.11 Hair texture analysis

3.0 g of virgin hairs with 7 inches and 1.5 inches wide dimension were aligned in parallel. Then hairs were secured at the root end by aluminium clip and glue. The remaining hair tip was trimmed out, to obtain 2.5 g of hair tress with 6 inches long for the test. Five tresses were wetted together under tap water at a flow rate 4 L/min, at 37°C for 5 sec. The tresses were washed with 1.25 g of 14% sodium lauryl ether sulfate (SLES).2eo, twice before treatment. Then the tresses were combed through until the tresses were aligned. An amount of 2.15 g of leave-on conditioner was applied for 1 min. Each sample was tested wet by rolling neoprene probe. Five specimens were tested for each sample type by Texture Analyzer as shown in Figure 3-5. All tested tresses were left overnight in a temperature and humidity control room (22°C, 50%RH) before measuring by the same procedure but at dry state.

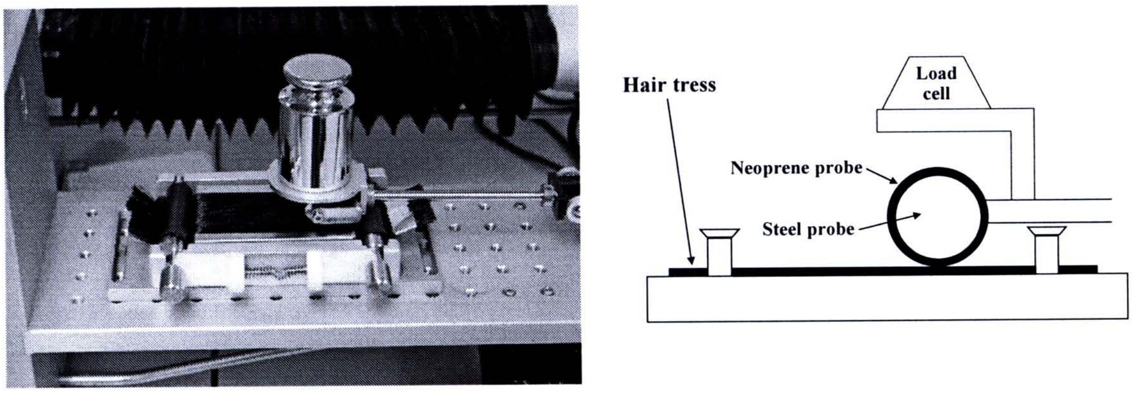


Figure 3-5 Texture Analyzer (TA.XT.Plus Stable Microsystems)

<u>Texture Analyzer Settings</u>

Test mode	: Compression
Pre-test speed	: 1.0 mm/sec
Test speed	: 10.0 mm/sec
Post-test speed	: 10.0 mm/sec
Target mode	: Distance
Distance	: 40.0 mm
Trigger type	: Auto (force)
Trigger force	: 0.1 g
Break mode	: Off
Stop point at	: Start position
Tare mode	: Auto
Advanced option	: On

The area of each friction loops was calculated as the measure of the hair friction and was typically reported in the unit of g.mm. Five replications of hair tresses were collected for each treatment. Mean and standard deviation values of each parameter were recorded for each sample. Data is analyzed by Fisher's Least Square Difference (Fisher's LSD) at 95% confidence level.

3.3.12 Wet combing test

3.0 g of virgin hairs with 7 inches and 1.5 inches wide dimension were aligned in parallel. Then hairs were secured at the root end by aluminium clip and glue. The remaining hair tip was trimmed out, to obtain 2.5 g of hair tress with 6 inches long for the test. Five tresses were wetted together under tap water at a flow rate 4 L/min, at 37°C for 5 sec. The tresses were washed with 1.25 g of 14% sodium lauryl ether sulfate (SLES).2eo, twice before treatment. An amount of 2.15 g of leave-on conditioner was applied for 1 min. The treated tress was suspended on the load cell and inserted into the middle of the fine toothed comb. The treated tress was passed through a comb at a speed of 40 inch/min and the friction force was measured as a function of distance. All the tresses were tested by Instron 5564 tensile tester equipped with Bluehill software version 2.0. Five combing were measured per tress and five tresses were measured in each treatment.

After analysis, the graph showed the force measured by the load cell as a function of the distance traveled by the crosshead. The calculated parameters such as the maximum combing force (gmf), the average combing force (gmf), and the combing energy (mJ) were obtained automatically. The maximum combing force was the highest load recorded during the experimental. The average energy was an averaged value between two pre-determined points, while the combing energy was the energy under the curve. The mean and standard deviation values of each parameter were recorded for each sample. Data was analyzed by Fisher's Least Square Difference (Fisher's LSD) at 95% confidence level.