CHAPTER III

RESEARCH METHODOLOGY

This chapter presents the methodology of this research including the materials, apparatus and the experiments. The details of each part are given below.

Materials

1. Primary human skin keratinocyte (HaCaT, Cell Lines Service, Eppelheim, Germany)
2. Isolated type I collagen from bovine tendon
3. Bovine tendon type I collagen (Analytical Grade, Sigma-Aldrich Co., St.Louis, Missouri, USA)
4. Chitosan
   - 4.1 Shrimp MW 30,000, DD 85-90% (Seafresh Chitosan (Lab) Co., Bangkok, Thailand.)
   - 4.2 Shrimp MW 100,000, DD ~95% (Seafresh Chitosan (Lab) Co., Bangkok, Thailand.)
   - 4.3 Shrimp MW 100,000-1,000,000, DD > 90% (Bannawach Bio-Line Co., Ltd., Chonburi, Thailand)
   - 4.4 Crab MW 100,000-1,000,000, DD> 90% (Bannawach Bio-Line Co., Ltd., Chonburi, Thailand)
   - 4.5 Squid MW 100,000-1,000,000, DD > 90% (Bannawach Bio-Line Co., Ltd., Chonburi, Thailand)
5. Polyvinyl alcohol (PVA) (Analytical Grade, Fluka Chemie GmbH, Industriestr, Buchs SG, Switzerland)
   - 5.1 MW 72,000
   - 5.2 MW 145,000

6. Dulbecco's Modified Eagle's Medium (high glucose) (Cell Lines Service, Eppelheim, Germany)

7. Fetal bovine serum; FBS (Analytical Grade, California, USA)

8. Glutaraldehyde 25% in water (Analytical Grade, Fluka Chemie GmbH, Industriestr, Buchs SG, Switzerland)

9. Sodium dihyfirogen orthophosphate (Analytical Grade, Merck, Germany)

10. Trypsin EDTA (Analytical Grade, GIBCO, California, USA)

11. Glacial acetic acid (Analytical Grade, VWR International Ltd., Poole, England)

12. Sodium chloride (Analytical Grade, VWR International Ltd., Poole, England)

13. DC Protein assay (BIO-RAD, Philadelphia, USA)

14. Tris (hydroxymethyl)-aminomethane (Analytical grade, MERCK, Darmstadt, Germany)

15. Concentrated hydrochloric acid (Analytical grade, MERCK, Darmstadt, Germany)

16. *N,N,N',N'*-Tetramethylethylenediamine (TEMED) (BIO-RAD, Philadelphia, USA)

17. Sodium dodecyl sulfate (BIO-RAD, Philadelphia, USA)

18. Glycerol (BIO-RAD, Philadelphia, USA)

19. Bromophenol blue (BIO-RAD, Philadelphia, USA)

20. 2-Mercaptoethanol (BIO-RAD, Philadelphia, USA)

21. Acrylamide (BIO-RAD, Philadelphia, USA)

22. *N,N'*-Methylene-bis-acrylamide (BIO-RAD, Philadelphia, USA)

23. Ammonium persulphate (APS, Pro Pure, Amresco, Ohio, USA)

24. Glycine (BIO-RAD, Philadelphia, USA)

25. Coomassie brilliant blue (BIO-RAD, Philadelphia, USA)

26. Ethanol (Analytical grade, MERCK, Darmstadt, Germany)

27. Methanol (Analytical grade, Labscan, Bangkok, Thailand)

28. MilliQ water (the water produced exceeds the standard requirements for type II analytical grade water; resistivity > 5M.cm, TOC levels < 30ppb)

29. Sodium dodecyl sulfate (SDS, BIO-RAD, Philadelphia, USA)

30. Hydroxyproline (Analytical Grade, Fluka Chemie GmbH, Industriestr, Buchs SG, Swizerland)

31. Hydrochloric acid (Analytical Grade, J.T. Baker, USA)

32. Collagenase from *Clostidium histolyticum* (Gibco, California, USA)

33. Trypsin (Analytical Grade, Sigma-Aldrich Co., St.Louis, Missouri, USA)

34. Bovine serum albumin (BSA, Sigma-Aldrich Co., St.Louis, Missouri, USA)

35. One step RT-PCR Taq (Superscript™ with platinum® Taq, Invitrogen, USA)

36. Primers for KGFR, Keratin 4-14 and GAPDH (Invitrogen, California, USA)

Instruments

1. Fourier transform infrared (FTIR) spectroscopy (Model GX series, Perkin Elmer, Connecticut, USA)
2. Freeze drying apparatus (FTS system Dura dry type FD 95C12, FTS Systems Inc., New York, USA)
3. Microplate spectrophotometer (Spectra Count, Perkin Elmer, Massachusetts, USA)
4. Scanning electron microscopy (Leo Electron Microscopy, Inc, Cambridge, England)
5. Tensometer (Instron 3342, Instron Ltd., Buckinghamshire, England)
6. UV-visible spectrophotometer (Cary 1E, Varian, Inc., Palo Alto, California, USA)
7. Vertical gel electrophoresis (BIO-RAD, Philadelphia, USA)
8. Incubator (model 311, ThermoForma, Marietta, USA)
9. Inverted microscope (model TS100, Nikon Corporation, Tokyo, Japan)
10. Vertical laminar air flow cabinet (model BHG2004S, Faster s.r.l., Ferrara, Italy)
11. Water bath (model LWB-211A, Daihom LabTech Co. Ltd., Korea)
12. PCR system (GeneAmp® PCR system 9700, Applied Biosystems, California, USA)
13. UV-spectrophotometer (Biochrom, Cambridge, England)

14. UV transilluminator (UVP Inc., California, USA)

Methodology

1. Preparation of bovine type I collagen

1.1 Isolation of type I collagen from bovine tendon

Type I collagen is the most common of mammal collagen and is also widespread all over the body as well as skin. From this reason, type I collagen is used to fabricate the matrix which would mimic the natural human skin. The bovine tendon is one of the interesting sources of type I collagen because most of the tendon composed of type I collagen in this study. Additionally, the bovine tendon is largely available. Therefore, it was used as the source of type I collagen. After removing the fat and muscle impurity substances, the bovine tendon was cut into small pieces and digested with 0.25% trypsin solution at 37°C for 24 hr. The pieces of tendon were removed and incubated with 0.5M acetic acid and incubated at 4°C for 48 hr. The swollen tendon was agitated, and the collagen solution was centrifuged to separate the insoluble impurity. The supernatant was collected and precipitated by 5% NaCl solution. The precipitated collagen was dialyzed with deionized water for 72 hours and then lyophilized.

1.2 Characterization of isolated type I collagen

Composition and purity of type I collagen were characterized and confirmed by fourier transform infrared (FTIR) spectroscopy and sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). Total proteins of the isolated collagen were analyzed in comparison with standard type I collagen.

1.2.1 Chemical characterization of isolated collagen

The isolated collagen was investigated for chemical composition and purification using fourier transform infrared (FTIR) spectroscopy.

1.2.2 Determinaton of total proteins of isolated collagen

DC protein assay was used to determine the amount of total protein. Bovine serum albumin (BSA) was used as the standard protein. BSA was dissolved in distilled water in various concentrations: 1.5, 1.2, 1, 0.6, and 0.2 mg/ml. The isolated collagen was dissolved in 0.5 M acetic acid in different concentrations. After individually pipetting 5 μl of standard solution and collagen solution into 96-well plate, reagent A, an alkaline copper tartrate solution, (25 μl) was added into each well and then followed by 200 μl of reagent B (dilute Folin reagent). This plate was incubated at room temperature for 15 min and then evaluated for absorbance at 750 nm by a microplate spectrophotometer.

1.2.3 Determination of purity of isolated collagen

The determination of purity of isolated collagen was performed by using SDS-PAGE technique. The isolated collagen was dissolved in 0.5 M acetic acid. The collagen solution (25μl) was mixed with 2x sample buffer and heated at 95°C for 5 min. This solution was mixed by vortex for 20 min and centrifuged at 13,000 g for 8 min. An aliquot (20 μl) of the obtained solution was loaded into an acrylamide gel (8% lower gel), and run with 200V for 1 hr. The gel was rinsed 3 times with milliQ water and stained with the Coomassie brilliant blue (SDS-PAGE gel stain) for 1-3 hr. The gel was removed to gel destain solution for 1-2 hr. The determination of the molecular weigth of the sample was performed by comparing with that of the standard's band (type I collagen from Sigma-Aldrich).

2. Matrix preparation

2.1 Preparation of chitosan and PVA matrix

This study was performed to optimize the amount of polymer and type of chitosan or PVA for matrix preparation. Casting technique on a glass Petri dish was used to fabricate the matrix. Chitosan from several sources including the shell wastes of shrimp, crab and exoskeleton of squid were dissolved in 0.5 M acetic acid solution in

various concentrations. The PVA with different molecular weights were dissolved in hot water in various concentrations. The formulations of the matrix preparation are shown in Table 3 and 4. Chitosan or PVA solution was cast into a Petri dish and left to dry at room temperature. The matrix film was formed over time. The thickness of the prepared matrix was controlled to be in rage of 100±20 μm.

To select the type and amount of polymer for the blended matrix preparation, the physical properties; surface morphology according to scanning electron microscopy (SEM) and mechanical property of the prepared matrix were determined. The type and amount of polymer, which had provided strong and high porosity matrix, were taken for further studies.

Table 3 Formulations for chitosan matrix preparation.

Formulation	Chitosan (g)	Acetic acid (ml)	Distilled water (ml)
Chitosan 0.5% w/v - Shrimp MW 30,000 - Shrimp MW 100,000 - Shrimp MW100,000 - 1,000,000 - Crab MW 100,000 - 1,000,000 - Squid MW 100,000 - 1,000,000	0.5	2.87	92.13
Chitosan 1% w/v - Shrimp MW 30,000 - Shrimp MW 100,000 - Shrimp MW 100,000 - 1,000,000 - Crab MW 100,000 - 1,000,000 - Squid MW 100,000 - 1,000,000	1	2.87	91.63
Chitosan 2% w/v - Shrimp MW 30,000 - Shrimp MW 100,000 - Shrimp MW 100,000 -1,000,000 - Crab MW 100,000 - 1,000,000 - Squid MW 100,000 - 1,000,000	2	2.87	90.63
Chitosan 3% w/v - Shrimp MW 30,000 - Shrimp MW 100,000 - Shrimp MW 100,000 -1,000,000 - Crab MW 100,000 - 1,000,000 - Squid MW 100,000 - 1,000,000	3	2.87	89.63

Table 3 (cont.).

Formulation	Chitosan (g)	Acetic acid (ml)	Distilled water (ml)
Chitosan 4% w/v - Shrimp MW 30,000 - Shrimp MW 100,000 - Shrimp MW 100,000 -1,000,000 - Crab MW 100,000 - 1,000,000 - Squid MW 100,000 - 1,000,000	4	2.87	88.63

Table 4 Formulations for PVA matrix preparation.

Formulation	PVA (g)	Distilled water (ml)
PVA 0.5% w/v - MW 72,000 - MW 145,000	0.5	99.5
PVA 1% w/v - MW 72,000 - MW 145,000	1	99.0
PVA 2% w/v - MW 72,000 - MW 145,000	2	98.0
PVA 3% w/v - MW 72,000 - MW 145,000	3	97.0
PVA 4% w/v - MW 72,000 - MW 145,000	4	96.0

2.2 Preparation of the blended collagen/chitosan and collagen/PVA matrix

This study was aimed to optimize the blending ratio between collagen and chitosan or collagen and PVA by observing the surface morphology.

Type I collagen and chitosan were individually dissolved in 0.5 M acetic acid solution. The PVA was partly dissolved in hot water. Collagen solution was blended with chitosan or PVA in various ratios. The total amount of polymer used was in the range of 0.5-4% w/v, according to the previous study. The formulations of the casting solution are shown in Table 5-8. The obtained solution was subsequently cast into a Petri dish for film formation. All matrices were controlled their thickness to be in rage of 60±20 μm.

To select the ratio of polymer blended (collagen/chitosan or PVA), physical properties and surface morphology obtained from SEM of the blended matrix were determined. The ratio which provided the flexibility and high porosity of the matrix was selected for further studies.

Table 5 Formulations for preparation of the matrix from collagen/shrimp chitosan MW 30,000 at 1% w/v concentration.

Composition	Ratios of Collagen/Chitosan			
	8:2	7:3	6:4	5:5
Collagen (g)	0.80	0.70	0.60	0.50
Chitosan (g)	0.20	0.30	0.40	0.50
Acetic acid (ml)	2.87	2.87	2.87	2.87
Distilled water (ml)	96.13	96.13	96.13	96.13
Total (ml)	100	100	100	100

Table 6 Formulations for preparation of the matrix from collagen/shrimp chitosan MW 100,000 at 0.5% w/v concentration.

Composition	Ratios of Collagen/Chitosan			
	8:2	7:3	6:4	5:5
Collagen (g)	0.40	035	0.30	0.25
Chitosan (g)	0.10	0.15	0.20	0.25
Acetic acid (ml)	2.87	2.87	2.87	2.87
Distilled water (ml)	96.63	96.63	96.63	96.63
Total	100	100	100	100

Table 7 Formulations for preparation of the matrix from collagen/crab chitosan MW 100,000 - 1,000,000 at 3% w/v concentration.

Composition	Ratios of Collagen/Chitosan			
	8:2	7:3	6:4	5:5
Collagen (g)	2.40	2.10	1.80	1.50
Chitosan (g)	0.60	0.90	1.20	1.50
Acetic acid (ml)	2.87	2.87	2.87	2.87
Distilled water (ml)	94.13	94.13	94.13	94.13
Total	100	100	100	100

Table 8 Formulations for preparation of the matrix from collagen/ PVA at 0.5% w/v concentration.

Composition	Ratios of Collagen/PVA	
	9:1	8:2
Collagen (g)	0.45	0.40
PVA (g)	0.05	0.10
Acetic acid (ml)	2.87	2.87
Distilled water (ml)	96.63	96.63
Total (ml)	100	100

2.3 Preparation of collagen/chitosan crosslinked with different crosslinking agent

This study was performed to observe the effect of crosslinking agent on the mechanical properties of the matrix.

Glutaraldehyde (GA) or β-glycerol phosphate (GP) was used as a crosslinking agent in this study. The blended collagen/chitosan solutions with various ratios were prepared by the similar method, according to the previous evaluation. This blended polymer solution was crosslinked with GA or GP by variation of concentration of the crosslinking agents (Table 9-12). The optimum matrices were determined from their physicochemical properties.The desired matrix was selected for testing with primary human skin keratinocytes to evaluate cytotoxicity including cytocompatibility, cell adhesion and cell proliferation. The concentration of GA used was varied in rage of 0.05-0.15% w/w of total polymer while that of GP used was varied in rage of 0.5-1.5% w/w of total polymer. The selected concentration was determined from the previous studies [46] and the viscosity of the polymer solution. Using high concentration of crosslinking agent caused viscous or gel-liked polymer solution.

Table 9 Formulations of collagen/chitosan matrix at 0.5% w/v of total polymer in the percent of and using GA as a crosslinking agent.

Content	Collagen/Chitosan (8:2)			Collagen/Chitosan (7:3)		
GA concentration (w/w of total polymer)	0.05%	0.10%	0.15%	0.05%	0.10%	0.15%
Collagen (g)	0.40	0.40	0.40	0.35	0.35	0.35
Chitosan (g)	0.10	0.10	0.10	0.15	0.15	0.15
Acetic acid (ml)	2.87	2.87	2.87	2.87	2.87	2.87
25% GA (ml)	0.001	0.002	0.003	0.001	0.002	0.003
Distilled water (ml)	96.629	96.628	96.627	96.629	96.628	96.627
Total (ml)	100	100	100	100	100	100

Table 10 Formulations of collagen/chitosan matrix at 1% w/v of total polymer in the percent of and using GA as a crosslinking agent.

Content	Collagen/Chitosan (8:2)			Collagen/Chitosan (7:3)		
GA concentration (w/w of total polymer)	0.05%	0.10%	0.15%	0.05%	0.10%	0.15%
Collagen (g)	0.80	0.80	0.80	0.70	0.70	0.70
Chitosan (g)	0.20	0.20	0.20	0.30	0.30	0.30
Acetic acid (ml)	2.87	2.87	2.87	2.87	2.87	2.87
25% GA (ml)	0.002	0.004	0.006	0.002	0.004	0.006
Distilled water (ml)	96.128	96.126	96.124	96.128	96.126	96.124
Total (ml)	100	100	100	100	100	100

Table 11 Formulations of collagen/chitosan matrix at 0.5% w/v of total polymer in the percent of and using GP as a crosslinking agent.

Content	Collagen/Chitosan (8:2)			Collagen/Chitosan (7:3)		
GP concentration (w/w of total polymer)	0.5%	1.0%	1.5%	0.5%	1.0%	1.5%
Collagen (g)	0.40	0.40	0.40	0.35	0.35	0.35
Chitosan (g)	0.10	0.10	0.10	0.15	0.15	0.15
Acetic acid (ml)	2.87	2.87	2.87	2.87	2.87	2.87
25% GP (mg)	2.50	5.00	7.50	2.50	5.00	7.50
Distilled water (ml)	96.628	96.628	96.628	96.628	96.628	96.628
Total (ml)	100	100	100	100	100	100

Table 12 Formulations of collagen/chitosan matrix at 1% w/v of total polymer in the percent of and using GP as a crosslinking agent.

Content	Collagen/Chitosan (8:2)			Collagen/Chitosan (7:3)		
GP concentration (w/w of total polymer)	0.5%	1.0%	1.5%	0.5%	1.0%	1.5%
Collagen (g)	0.80	0.80	0.80	0.70	0.70	0.70
Chitosan (g)	0.20	0.20	0.20	0.30	0.30	0.30
Acetic acid (ml)	2.87	2.87	2.87	2.87	2.87	2.87
25% GP (mg)	5.00	10.00	15.00	5.00	10.00	15.00
Distilled water (ml)	96.125	96.120	96.115	96.125	96.120	96.115
Total (ml)	100	100	100	100	100	100

2.4 Sterilization of the matrix

The selected matrix was soaked in 10% NH_4OH to neutralize the exceed acidity, and rinsed 3 times with sterilized distilled water. Then it was immersed into 75% ethanol for 24 hr for sterilization followed by rinsing with PBS for 3-4 times [46] and immersed in the culture medium for 4-10 hr before using as a matrix for cultivation.

3. Evaluation of physicochemical properties of the matrix

3.1 Surface morphology

To determine the surface morphology, scanning electron microscope (SEM) was used to evaluate porosity of the matrices. The matrices were cut into small piece, coated with ultrathin gold layer and observed at 3.50 KX. The porosity of the matrices was observerd by visualization.

3.2 Mechanical properties

Tensile strength and % elongation at break of the prepared matrix were measured to determine their mechanical properties using a tensometer. The tensile

strength was used to evaluate the matrix strength and % elongation at break was used to evaluate the flexibility. The tensometer investigation conditions are listed below:

Temperature	25°C
Humudity	50%
Tensile force	100 N
Rated of separation	12.5 mm/min

The tensile strength and % elongation at break were calculated by using the following equations:

$$\text{Tensile strength} = \frac{\text{The force at break (kgf)}}{\text{Area of enforcement (mm}^2\text{)}}$$

Whereas the area of enforcement was derived by multiply the matrix's thickness and the matrix's width.

% elongation at break = maximum of tensile strain x 100

The optimum tensile strength of the matrix for skin tissue engineering should not be more than 7.7 MPa (785 kgf/mm^2) [47].

3.3 Swelling property and enzymatic degradation [39]

The matrix samples were measured to obtain the initial weight (W_0), thickness (T_0) and diameter (D_0). The samples were incubated in the enzyme solution of 10 ml phosphate buffer saline (PBS), pH 7.4, and containing collagenase at the concentration of 200U/5 g of collagen at 37°C. The matrix size samples was hourly measured untill the constant size was observed. The matrix samples were rinsed thoroughly with distilled water to remove buffer and collagenase remaining on the surface and blotted to remove the surface water. Individual samples were measured to obtain thickness (T_t) and diameter (D_t), and dried at room temperature to obtain weight after degradation (W_t). The swelling of matrix should not be more than 80 times [46].

The swelling degree was calculated from:

$$\% \text{ swelling} = 100 \times (D_t/D_0)^2 \times (T_t/T_0)^2$$

The remaining weight of the matrix was calculated after enzymatic degradation using the following equation to evaluate the degradation time.

$$\% \text{ weight remaining} = 100 \times (W_t/W_0)$$

After the matrix was immersed in the enzyme solution, all supernatant was removed at the fix time interval (2, 4, 6, 16, 24, 48, and 168 hr). The enzyme solution (10 ml) was subsequently replaced. After hydrolyzed with 6 M hydrochloric acid at 120°C for 12 hr, 6 ml PBS (pH 7.4) was added in the dried sample to re-dissolve. The released hydroxyproline was then measured with the UV-visible spectrophotometer at 202 nm. The degradation time of the matrix in the skin tissue engineering should be about 1-2 months. The matrix was degraded within less than 3-4 weeks would hider the wound healing process [46].

4. Cytotoxicity test

4.1 Cytocompatibility test [35]

This study was aimed to evaluate the compatibility of the derived matrix to primary human skin keratinocyte. HaCaT cells at a density of $1x10^5$cells/ml were cultured on 24-well plastic plate and incubated at 37°C in humidified atmosphere containing 5% CO_2 for 24 hr. The sterilized matrix was then deposited on the confluence HaCaT. HaCaT cells morphology were observed by the light microscopy for 5 days after deposition without any change in culture medium. The obtained results would be compared with HaCaT cultured on plastic well plate without the matrix.

4.2 Cell adhesion test [35]

After the matrix was placed in 96-well plate, HaCaT cells were seeded on the matrix at a density of $1x10^4$ cells/ cm^2 and incubated at 37°C in humidified atmosphere containing 5% CO_2 for 3 hr. The matrix area was 0.25 cm^2. Three hours later, the matrix was rinsed with steriled PBS to remove free cells. Then, it was placed into a new well and 200 μl of culture medium was added. Cell viability was evaluated by adding 50 μl of sodium 3'-[1-(phenylaminocarbonyl)-3, 4-tetrazolium]-bis (4-methoxy-6nitro) benzene sulfonicacid hydrate (XTT) assay (appendix E) and measuring the absorbance at 490 nm. Cell adhesion on the plastic plate was used as the control.

XTT is useful assay for the quantification of viable cells. The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formation dye by metabolic active cells. Therefore, this conversion only occurs in viable cells. The formazan dye formed is soluble in aqueous solutions and directly quantified using a microplate spectrophotometer. The wavelength used to measure the absorbance of the formazan product is between 450-500 nm. An increase in the number of living cells resulting in an increase in the overall activity correlates with the amount of the orange formazan formed.

4.3 Cell proliferation test [35]

As previously described, HaCaT cells were seeded on the matrix at a density of $1x10^4$ cells/ cm^2 (96-well plate), and incubated at 37°C in humidified atmosphere containing 5% CO_2. The culture medium was changed on day 3. On day 5, the matrix was rinsed with steriled PBS to remove dead cells. Then, it was placed in a new well and 200 μl of culture medium was added. Cell viability was evaluated by adding 50 μl of XTT and measuring the absorbance at 490 nm (appendix F). Cell proliferation on the plastic plate was used as the control.

4.4 Morphology of cell adhesion on the matrix [48]

After the cultivation, HaCaT cells adhering to the matrix were washed with PBS (pH 7.4) and then fixed in the buffer containing 2.5% GA for 12 hr at 4°C. This

matrix was washed with PBS to remove the residual GA. The HaCaT cells adhering to the matrix were post fixation with OsO_4 for 2 hr and then washed with PBS. This matrix was dehydrated through a series of ethanol (30, 50, 70, 90, 100%) for 15 min and followed by soaking in acetone for 2 times with 15 min each. After the matrix was dried by the critically point dried system and coated with an ultrathin gold layer, it was observed by SEM.

4.5 Chemical characteristic of the matrix

This study was aimed to investigate the GA residues, which is the toxic component. After the matrix was neutralized, sterilized and washed, it was analysed for the chemical structure using FTIR spectra.

4.6 Rate of cell proliferation on the matrix

The obtained matrix was observed the rate of HaCaT cells proliferation by using XTT assay. The cultivation was observed at 1-5 days for calculation of the percent proliferation of HaCaT cells on the matrix.

4.7 Characterization of keratinocyte growth on the developed matrix [49-51]

To determine whether keratinocytes function is normal, the keratinocyte growth factor receptor (KGFR) and keratins expression were used as the marker of natural keratinocyte. Keratinocyte growth factor (KGF) stimulates proliferation of the keratinocyte, therefore, KGFR plays an important role in binding with KGF. For keratin, it is the keratinocyte's skeleton. In this study, the gene expression of KGFR and keratin was determined by reverse transcriptase polymerase chain reaction (RT-PCR) method. The keratinocytes were collected during the proliferation phase. Total RNA was extracted from the keratinocytes, and the expression pattern of KGFR and keratins in cell cutured in the matrix was compared to that on the plastic plate. The characterization was performed by the following method.

4.7.1 RNA isolation

HaCaT cells at a density of $1x10^5$cells/cm^2 were seeded on the developed matrix adhered on the 24-well plate and incubated at 37°C in humidified atmosphere containing 5% CO_2 for 1, 3, 5, 7, 14, 21, 28, and 56 days. The matrix area was 2.25 cm^2. For RNA isolation, cells on the matrix was trypsinized by 0.25% trypsin – EDTA and washed with PBS (pH 7.4). Cells were pelleted by centrifugation at 1,200 rpm for 5 min. The cells were lysed with 250 µl TRIzol reagent by pipetting and incubated at room temperature for 5 min. Then, 67 µl of chloroform was added, mixed by vortex, and incubated at room temperature for 5 min. The sample was centrifuged at 12,000 rpm for 5 min at 4°C. After the centrifugation, the mixture was separated into three phases. RNA remained exclusively in a colorless upper aqueous phase. This phase was removed and put into a new tube and 167 µl of isopropanol was added and gently mixed. After being incubated at room temperature for 10 min and centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was then removed. The RNA pellet was washed once with 500 µl of 75% cold ethanol and mixed by vortex. The mixture was centrifuged at 8,000 rpm at room temperature for 5 min at 4°C. The supernatant was removed and the pellet was left to dry at room temperature. The pellet was redissolved by adding 50 µl of DEPC water and kept at -80°C. RNA quantity was determined by UV spectrophotometer at 260 and 280 nm.

4.7.2 RT-PCR

RT-PCR method was used to determine the gene expression of KGFR and keratin 4-14. The details of the primers are shown bellow. HaCaT cells growth on plastic plate were used as the positive control.

Gene	Primer	Primer sequence
KGFR	Sense	5' CAATGCAGAAGTGCTGGCTCTGTTC 3'
	Antisense	5' AAGCTGACACTGGGCAAGCCCCTG 3'
Keratin 4-14	Sense	5' ATGGTAAACTCCTGTTGTGGCTCCG 3'
	Antisense	5' TCAGCAGCAAGAGGAGGCACAGCAC 3'
GAPDH	Sense	5' ACCACAGTCCATGCCATCAC 3'
	Antisense	5' TCCACCACCCTGTTGCTGTA 3'

The cDNA synthesis was performed on 200 ng total RNA with SuperScript™ one-step RT-PCR system. KGFR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were amplified by PCR in a first tube, and keratin 4-14 and GAPDH were amplified in a second tube. In the first tube, PCR was performed using the GeneAmp(R) PCR system for 35 cycles under the following cycle condition: 1 min at 94°C for denaturation, 1 min at 54°C for annealing, 2 min at 72°C for extension, and 5 min at 4°C for holding. The second one was performed in the same condition excepted the annealing step was performed 1 min at 55°C. The PCR products were seperated by electrophoresis on a 1% agarose gel.

5. Statistic analysis

The statistical analysis was performed by one-way analysis of variance (ANOVA) test. Independent samples t - test was used to compare two groups of samples. The *p* - value of less than 0.05 were considered as statistically significant. The data are expressed as mean ± standard deveation (SD).