CHAPTER 2

Literature Reviews and Relative Theories

This chapter provides two main sections which are the literature reviews and relative theories. In the literature reviews section, many publications had been reviewed in terms of applications of the scaffold, materials for fabricate scaffolds, fabrication methods, and material parameters identification, and etc. The relative theories are describing the biology of skin including its anatomy, and bioengineered skin, the theory of contact model for test scaffold.

2.1. Literature Reviews

The acute injury, genetic skin disorders, and chronic wounds are the main problems that contribute to the normal skin loss integrated functions and structures. Numerous researchers have found that the burn wound and scalds are the dominated problems that lead to the fast, extensive, deep wounds. Meanwhile, those severe wounds involve with the damage of substantial layer of skin then evolutionally lead to the difficult of skin regeneration. As so far, these problems have not been successfully solved by just using the traditional therapeutic methods, such as the skin grafting techniques, or even worse that will be life-threatening (Shevchenko, Eeman et al. 2014).

The applications of engineering within the field of biological science have been increased quickly in terms of the capacity to solve the trauma or wound healing problems. For the main purpose of skin regeneration is that to look for the proper skin repair processes to decrease the long-term consequences of scarring, which is meaningful to the patients. Compare to the normal skin, the scar is the abnormal phenomenon since the skin architectures, chemical components, and cells' phenotype are abnormal. As so far, the tissue engineering (the application of the scaffolds and cells) plays as the key potential role for tissue or skin regeneration. And the tissue engineering

has been used to skin and tissue regeneration for a few decades while this technique's achievement of the clinical applications still need to be improved. To obtain a better understanding of this developing technology, the researchers require the alignment of the biology science, tissue engineering, and the commercial upscaling of production to build a safe environment for the clinical applications. In addition, the training for the introduction of new technologies is necessary for management of this health problem (Wood 2014).

Previous research had shown that the excise on time could regulate the complex fluid status of the patients' burned wounds so the preparation of the wound affected tissues was not postponed, which could avoid the consequences of a poor healing result. This allows the application of a topical method in terms of the controlling of both fluid and heat loss and the occurring of re-epithelialization. Furthermore, the treatments subsequently should be able to facilitate the return of the environment that near the skin's original appearance and function of those patients. For those reasons, the materials are applied in this field that should follow these four main principles as below and that is associated with individual patient's specific requirements: be able to mimic the original skin's conditions to provide a normal wound healing environment; be able to replicate the original skin's chemical environment for the efficient wound healing processes; be able to replace the damaged parts of skin and tissues; be able to continue benefits to the promptly skin regeneration. Normally, the topical antimicrobial therapy followed by either temporary or permanent coverage of the wound. And historically, the autografts that are sourced from the healthy and unaffected donors have acted as the mainstream of treatment (Auger, Lacroix et al. 2009).

The first step for preparing the chitosan solution is that weigh the chitosan powder and then dissolve the chitosan powder (between 2 and 10% w/w) with the water: acetic acid mixed solution (the ratios of 99:1, 97:3 and 95:5). After that, the solutions will be stirred at 100 rpm and be heated at 50ºC until the solution became clear and homogenous. For the next step, the mixed solutions will be poured into steel containers (the internal diameter of 2cm and height of 1cm). After that, those specimens will be phase-separated at the temperature of −20 ºC for 24 hours. Subsequently, those

specimens will be transferred into a bath of acetone at −20 ºC for another 24 h in order to replace the water. Then the resulted specimens will be dried by using the supercritical CO_2 (SC-CO_2). Here the processes of SC-CO_2 drying will be performed as the following procedures: first, the steel containers will be loaded on a metallic support; and then put in the high-pressure vessel. The vessel will be closed and be filled totally that begins from the bottom by using the SC-CO_2. When the requirement of both pressure and temperature are obtained (200 bar and 35 ºC respectively), the drying stage will be started, and with the flow of SC-CO_2 at a flow rate of nearly 1kg/h; and the correspondence of a residence time inside the vessel is about 4 min; the drying stage will be lasted five hours. The depressurization time is about 20 min in order to bring back the system to atmospheric pressure (Baldino, Cardea et al. 2014).

The polyvinyl alcohol (PVA) has the average molecular weight (Mn) of 74,800 g/mol. To obtain the mixed PVA, sodium alginate and gelatin solutions (the total dosage: 10g), all of the materials were dissolved in distilled water (100mL) under the specific temperature separately before dropping into the mixed aqueous solution of saturated boric acid and $CaCl_2$. Then followed with the mixture was kept until form spherical beads for two hours. After the last washing of the formed beads by using the distilled water, then the mixed solution can be stored for a further using (Hui, Zhang et al. 2015).

PVA is a man-made material, which has very excellent characteristics, such as convenient preparation, good biodegradability, excellent chemical resistance, and good mechanical properties. Nowadays, PVA has been used in a lot of biomaterial applications. For example, PVA membranes have been used in the artificial pancreas, hemodialysis, and implantable biomaterials. Meanwhile, because of the chitosan contains hydroxyl and amine groups so it is potentially miscible with PVA by the formation of hydrogen bonds. Therefore, polyvinyl alcohol (PVA) was blended with chitosan frequently to enhance the physical properties of chitosan films by adopting the solution blend method in the previous research (Chen, Wang et al. 2008).

The procedures of synthesizing porous gelatin scaffolds were as the following steps: the gelatin powder was dissolved in de-ionized water at 50 ºC to obtain the

20%w/v gelatin aqueous solution. Then the gelatin solution was transferred to the stainless-steel molds. The sodium hydrogen carbonate was added into the steel molds as the foaming agent and blended with the gelatin solution. After that, acetic acid was added into the molds with blending to promote the formation of gelatin foam. After obtaining the purpose-solution, all the specimens were transferred into the freezer (−25 ºC) for one hour. After freezing of those specimens, those solidified specimens were removed out from the metallic molds and instantly be plunged into a 4 ºC water bath to extract traces of the remained unreacted compounds. Herein, the cross-linked gelatin scaffolds were prepared by immersion in 0.25, 0.50, 0.75, and 1.00% v/v GTA solutions separately, and kept that for three hours. This step skipped the control specimens because the non-crosslinked condition was used as the comparison group. After that, samples were washed again and kept with de-ionized water overnight in order to remove the excess cross-linker agent. The resulting samples were transferred to the fridge and then to the lyophilizer for lyophilization for 24 hours (Poursamar, Hatami et al. 2015).

The method of blending two or more different polymers together, which has gradually become an important technique to improve the performance and reduce the cost so that people would be able to find the better ways for the commercial products. A few researchers had studied on the CS/PVA binary blended film before while the study associated with the CS/PVA/GA ternary blended film that was not able to give enough useful information. Therefore, the CS/PVA/GA ternary blended system is becoming the major purpose of this research (Chen, Wang et al. 2008).

The CS/PVA/GA ternary blended films were obtained from a variety of blending ratios and blending methods. The previous study had illustrated that the weight ratio of CS/PVA was equal to 1, the mechanical properties of CS/PVA binary blended films were better than those of other weight ratios. For this reason, the weight ratio of CS/PVA will be kept to 1 and followed the binary mixture that was blended with a variety of amounts of gelatin (GA) solution within this research. Then the characters of those samples were tested, such as thermal properties, chemical structures, and morphology of surface of the CS/PVA/GA ternary blended films were examined with

differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), X-ray diffractometer (XRD), scanning electron microscopy (SEM), and the contact angle instrument, separately (Chen, Wang et al. 2008).

The promising approach to achieve a better function for the processes of wound healing, which utilize a sustained release carrier to deliver the angiogenic factors, such as the VEGF. As the previous study reported, the PLGA microsphere is a pretty good choice to deliver the VEGF. The antimicrobial performance plays a vital role within the long-term process of angiogenesis, and the PLGA microsphere is a promising way that could be applied in the delivering of the antimicrobials, such as the gentamicin that could avoid the wound getting the infection from bacterial or other reasons (Wang, Wang et al. 2015).

The previous research had shown that the scaffold freezing cast technique was applied to synthesize the different hydroxyapatite (HA) scaffolds with porous holes before. For the preparation of the PVA solution: the PVA powder was dissolved in de-ionized water (10wt.% PVA) at 80 ºC and with the constantly stirring to make sure the solution becomes homogeneous. After the PVA solution became clear and homogeneous, the solution was cooled down to 30 ºC. Then the gelatin solution (15wt.%) was mixed with the PVA solution with continuous stirring for two hours. Following by the HA nanoparticles was slowly added into the resulting PVA–gelatin solution with continuous stirring until the solution was homogenous. The final HA slurry was taken in the glass molds and was pre-freezer for 12 hours at −5 ºC with a refrigerator then followed by freeze-drying for another 24 hours under the conditions with −53 ºC and 77 torrs. Those freezing cast HA-gelatin-PVA scaffolds were designed as HGPS, HGPR, and HGPF with different morphologies, such as the spherical, rod and fibroid, respectively. The different grades of scaffolds were prepared with a variety of ratios that include 30, 40 and 50 wt.%. Within those scaffolds, the nanorod HA and the 40wt.% solid loading scaffolds exhibited the highest compressive strength and 70 vol.% porosity. Therefore, this type of ratio was considered as the optimized composition method, which will be able to enhance the mechanical strength through the cryo-

treatment. Normally, the recommended time for cryo-treatment is five hours (Swain and Sarkar 2014).

The scanning electron microscopy (SEM) was applied for observation of the surface structure and morphology of the scaffolds. The degree of cross-linking, porosity, the elastic property of those scaffolds was characterized as mentioned previously. For measuring the degradation rate: all of the samples were weighed (m0) then degassed and immersed into 10 mL of PBS (pH 7.4). After that, all samples were stored in a shaker at 37 ºC and 60 rpm for a variety of times. During the different time point, those scaffolds were removed from the shaker and were rinsed with deionized water a few times to clean the extra PBS. Then the freeze-drying was operated, and then the weight (mt) of samples was weighed again. The maintenance of mass was calculated according to the ratio of mt and m0 (n = 5) (Kakkar, Verma et al. 2014).

The ethanol infiltration method was applied to measure the porosity of the keratin composite and collagen scaffolds. First of all, the weight of the dry samples (W_o) was measured. Then these samples were soaked with the ethanol until all of the air bubbles were cleared away. Then these scaffolds were taken out from the ethanol and the extra ethanol on the surface was removed then their weight was measured again immediately (W_e). The porosity of the scaffolds was calculated with this following equation:

$$\%\,porosity = \left[\left(W_e - W_o\right)/\rho V_s\right] \times 100 \qquad (1)$$

Within this function, the ρ represents the density of ethanol at the room temperature (0.789 mg/ml) and the V_s represents the volume of the scaffold that will be calculated according to the geometry of the scaffold (Han, Dong et al. 2014).

To test the compression capacity and the tensile strength, scaffolds were analyzed with the Texture Analyzer Pro CT V1.4 Build 17 (Brook field Engineering

Labs,Inc.). Within this process, these specimens were analyzed in triplicates. The compression strength was in the dried state. The circular punches were cut with the thickness of 7-10 mm and the diameter of 15 mm then compressed until the thickness of these samples were reduced to 50 % of the original thickness. For analyzing the compression strength in the wet state, scaffolds were immersed into the PBS (PBS with pH-7.4) one hour and then analyzed the data with the same method as previously. For analyzing the tensile strength of the specimens, the rectangular sections were cut with a cross-sectional area of approximately 7×4 mm^2. When the samples were in the dry state, the scaffolds needed to be clamped vertically using the gauge length of 10 mm then loading with the trigger (7 g) at the test speed was 0.5 mm/s. All of the scaffolds were stretched until they could not maintain the original shapes. In order to analyze the tensile strength when the samples were wet, all of the scaffolds were immersed into the PBS (pH-7.4) for about 1 hour then followed by the measurement of tensile strength (Han, Dong et al. 2014).

According to the ASTM method (F-1635-95) through the measurement of the changing of the specimens' weight within a variety of time intervals and those scaffolds were simulated with specific physiological conditions to test the biodegradation rate. The samples used in the study of Han et al. had sizes and weight which were between 40 to 45 mg. The original dry weight of the scaffolds was weighed (W0). Then those scaffolds were immersed in the 0.1 M PBS (pH 7.4) at 32 ºC and shaken by using a horizontal shaker with 50 rpm. Those scaffolds were removed out from the degradation medium with the interval of three days and rinsed thoroughly with the deionized water before transferring into the freezer. After the above steps, the resulting scaffolds were freeze-dried for 24 h. And then all of the samples were weighed again at each time interval and determined as Wt. Therefore, the percentage weight remaining can be calculated by using the equation below:

$$D(\%) = (W_O - W_t)/W_t \times 100 \quad (2)$$

The W_0 means the original dry weight of scaffold before the degradation, and the Wt means the dry weight of scaffold after degradation. This study had been lasted for 28 days (Han, Dong et al. 2014).

The previous study by Wang et al. demonstrated that in vitro culture of mouse fibroblasts with the collagen/chitosan-based scaffold that can facilitate cell adhesion and proliferation. And this study used the mouse fibroblast with a density of 1×104 cells/mL in a HyClone RPMI-1640 medium and supplemented with 10% fetal bovine serum in an incubator with the conditions of 5% CO2 and 37 ºC (Wang, Wang et al. 2015).

In order to measure the cell viability, adhesion, and proliferation by culturing with these scaffolds: the methyl thiazolyl tetrazolium (MTT) assay was applied to evaluate the relative viability of cells (Wang, Wang et al. 2015). The principle of the MTT assay is the measurement of the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide to a colored formazan through the viable cells.

In the previous study by Baniasadi et al, all of the prepared scaffolds were immersed in the 75% ethanol liquid for two hours and then rinsed them with the PBS solution three times. After that, those samples were transferred to the 24-well tissue culture dishes and followed with immersed to the DMEM for 24 hours prior to the cells seeding onto the specimens with the density of 2×104 cells/cm². Then those cell-seeded scaffolds were placed in the incubator at 37 ºC and 5% CO2. The MTT assay was applied to test the viability of those cells on the first, the third, the seventh, and the fourteenth day of culturing separately (Baniasadi, Ramazani et al. 2015).

2.2. Relative Theory and Hypotheses of the study

2.2.1. Structure and functions of the skin

Skin is the largest organ or tissue of the mammalian. It is the soft outer coverage of vertebrates that shows significant regional variations in terms of both structures and functions. For the human being, the skin accounts approximately 2 m^2 of the total surface area and the thickness of skin is around 2.5 mm. Meanwhile, the skin represents almost 6% of the total body weight of the human, which is higher than the other organs (not including muscle, bones, fat tissue and circulation systems).

The skin is consisted of four different layers, such as the epidermis (the outermost layer of skin), basement membrane (the thin fiber sheet to separate the epidermis and dermis apart), dermis (the beneath skin layer that consists of the connective tissue and provides elastic ability to release stress and strain), and hypodermis (the key part to guarantee that the skin can connect with bone and muscles). The skin contains various specialized tissues and organs, which includes the follicle cells, eccrine, sebaceous, apocrine glands, nails and glands of mammary. The skin of human plays a significant role because of its differences histoarchitectures and functions. The Fig 2.1 shows the significant differences in terms of the histology and physiology of skin (the upper one is human skin and two lower are the skins of the laboratory mice) (Slominski, Manna et al. 2015).

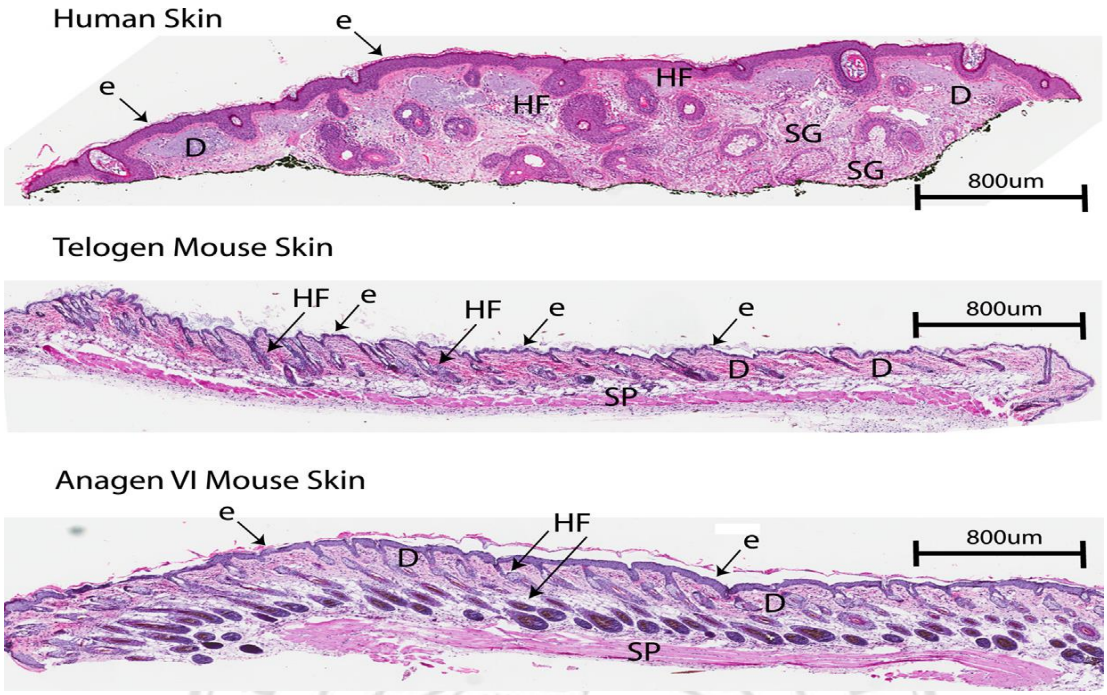

Figure 2.1 The differences between the histostructure of human and mouse skin at telogen and anagent VI phases of the hair cycle. The meaning of those characters inside the figure are defined as follow: e means epidermis; D means dermis; HF means hair follicles; SG means sebaceous glands; SP means panniculus carnosus.

2.2.2. Bioengineered skin

As the result of the development in the past a few years, the bioengineering had constructed a variety of skin substitutes that were applied in the clinical treatment of the deep burns and the others skin-associated problems. The effort of those researchers of the bioengineered skin substitutes also enlightens the understanding of the skin's functions and structures. Furthermore, through these engineered materials, people are able to understand the physiological processes of skin regeneration, and then the three-dimensional models of skin will be fabricated soon. This Figure 2.2 simply illustrates the theory of the bioengineered skin substitute (Huang and Fu 2011).

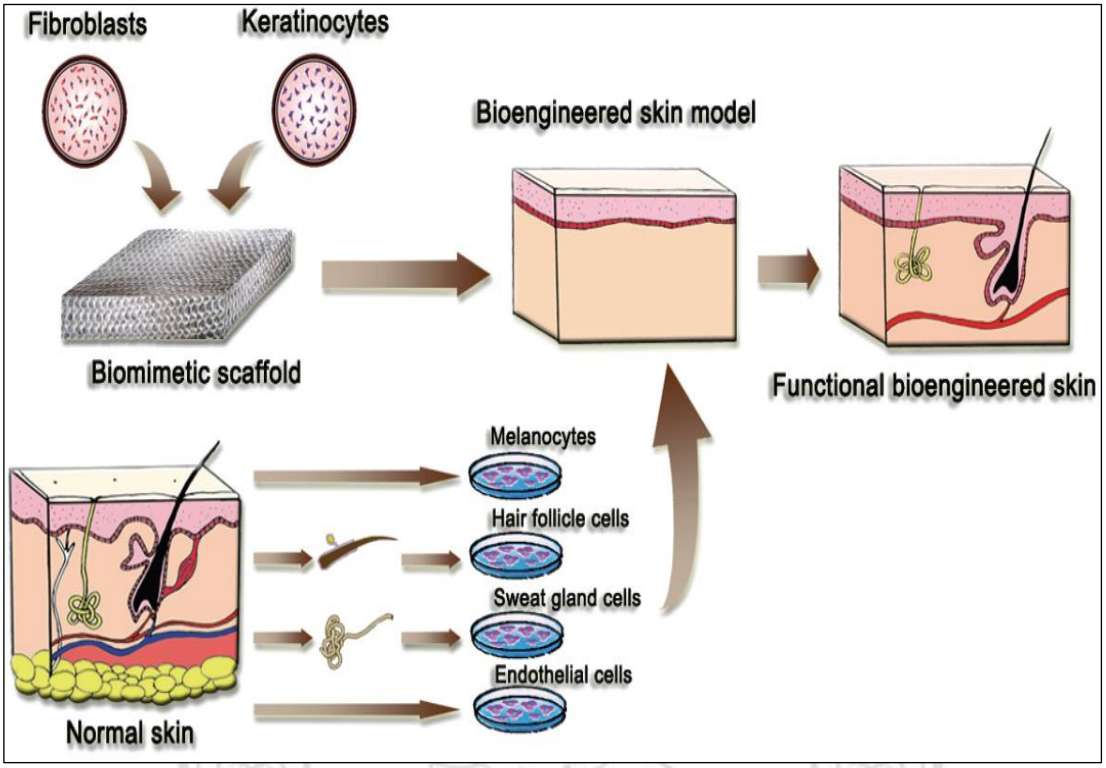

Figure 2.2 The strategy for bioengineered skin constructs which is a promising strategy for a complete restoration of functional skin.

Generally, the scaffolds of medical applications should have enough amount of pores with the appropriate pore size. At the same time, those pores of the scaffolds should have a good connectivity to each other. Besides, the scaffolds should be able to present a high surface area-to-volume ratio. Additionally, the three-dimensional (3D) shape of the scaffolds is also very important since that will promote the tissue regeneration.

The previous research by Lawrence and Madihally had illustrated that the two-dimensional scaffold was restricted the spreading ability of cells and those cells were only able to attach to the surfaces of scaffolds. This problem led to most of the biophysical properties problems of the substrate due to the cells could not have the same action while they were inside the body. On the contrary, the cells were cultured with a three-dimensional scaffold that had shown very good biophysical and biological properties that cells contacted with the substrate completely. This phenomenon had proven the significant influence of a 3D environment because of this environment

promoted the cells to have a better adhesion ability and more activated status. Besides, the three dimensions substrates provided both the physical and chemical signals that guided the cells colonization and supported cells attachment and proliferation, respectively (Association 2002, Baniasadi, Ramazani et al. 2015).

2.2.3. According to the JKR model to express the contact radius as follows:

$$a^3 = \frac{3R}{4E^*}\left(P + 3\gamma\pi R + \sqrt{6\lambda\pi R + (3\lambda\pi R)^2} \right) \quad (3)$$

where g is the combined surface energy, *P* is the appliedload, *R* is the effective be obtainedfrom the following equation:

$$\frac{1}{E^*} = \frac{1-\upsilon_1^2}{E_1} + \frac{1-\upsilon_2^2}{E_2} \quad (4)$$

E represents the Young's modulus and y represents the Poisson's ratio. The subscripts 1 and 2 represent the two contacting bodies.

When surface adhesion is incorporated into the applied load, the total interfacial force (P_1) is given by the following equation:

$$P_1 = P + 3\gamma\pi R + \sqrt{6\gamma\pi R + (3\gamma\pi R)^2} \quad (5)$$

When the applied load, *P* is equal to zero, and the contact radius a_0 can be obtained from:

$$a_0^3 = \frac{9\pi R^2\gamma}{2E^*} \quad (6)$$

The pull-off force ($P_{pull\text{-}off}$) is determined as the force needed to separate the two contacting surfaces and can be expressed as

$$P_{pull-off} = -\frac{3}{2}\gamma\pi R \quad (7)$$

Since the pull-off force can be directly measured from the force-displacement curve that obtained from the AFM, the combined surface energy will be determined from the above equation.

Furthermore, the specimen deformation (δ) due to contact can be obtained from the following equation:

$$\delta = \frac{a^2}{R}\left[1 - \frac{2}{3}\left(\frac{a_0}{a}\right)^{3/2}\right] \tag{8}$$

By using the above equations, the relationship between E^* and δ can be derived as follows:

$$E^* = \frac{3P_1}{4R^{1/2}\delta^{3/2}}\left[1 - \frac{2}{3}\left(\frac{6\pi R\gamma}{P_1}\right)^{1/2}\right]^{3/2} \tag{9}$$

The specimen deformation can be obtained by subtracting the deflection of the cantilever from the displacement of the specimen stage as follows:

$$\delta = D_{piezo} - D_{def} \tag{10}$$

Within above function, the D_{pieco} represents the vertical displacement of the specimen stage and the D_{def} is the deflection of the cantilever. In order to adjust the displacement of the stage, indentation tests are conducted on a relatively rigid Si wafer. In this case, it is thought that the stage displacement is equal to the cantilever deformation since the deformation of the rigid Simaterial is assumed to be negligible under very low indentation forces (KG 2008).

Finally, by using the specimen deformation, E^* will be calculated by using the Hertz contact model as the reference value:

$$E^* = \frac{3P}{4R^{1/2}\delta^{3/2}} \tag{11}$$