CHAPTER V

DISCUSSION AND CONCLUSION

1. Discussion

1.1 Physicochemical properties of the matrix from chitosan and PVA

Chitosan is the polycationic biopolymer derived from several sources of chitin, such as shell of shrimp, crab, and exoskeleton of squid, by alkaline deacetylation of chitin. Chitosan is easily soluble in inorganic and organic acid (pH < 6) since it has both free amino groups and hydroxyl groups on its backbone. The main parameters influencing the characteristics of chitosan are its degree of deacetylation (DD), molecular weight (MW) and the source of chitosan [52, 53]. In this study, % DD of chitosan did not different (85-90%), thus, the chitosan characteristics were not depend on the % DD of chitosan.

In general, the larger MW of chitosan provides the chitosan matrix with more tensile strength and % elongation at break; these probably involved the chain length of polymer chain. The high MW is longer than low MW; therefore, chitosan can extend to the stress and exhibited more elongation [52]. As this study, the chitosan matrix at the same concentraion, the tensile strength of high MW chitosan matrix tended to be higher than that of low MW chitosan matrix. However, focussing in the matrix obtained from the same source of chitosan, the different concentrations of chitosan did not affect on mechanical strength of the matrix.

The arrangement of molecules of chitin from various sources is different due to their physicochemical properties. α-chitin is the chitin from shell waste of shrimp and crab shell. The polymeric chains in this chitin are inversely arranged. This structure has strong intermolecular forces because of the hydrogen bond between the polymer chains. In contrast, β-chitin from squid is characterized by week intermolecular forces arising from loose arrangement of the molecules because the polymeric chains in β-chitin is parallelly arranged. Moreover, β-chitin exhibited high chemical reactivity compared with

α-chitin [32]. Similarly, the matrix prepared from squid chitosan MW 100,000 - 1,000,000 was more flexible than the matrix prepared from shrimp and crab at the same MW because free molecules can move within the chitosan network.

PVA is a highly hydrophilic polymer due to the abundant hydroxyl groups on its molecule. The available PVA shows good physicochemical properties [8-10]. The stability and degradation rate of PVA are dependent on their MW [43]. That is, increase in MW of PVA results in increasing the mechanical properties of the PVA matrix. Moreover, the mechanical properties are related to the amount of PVA. Therefore, the PVA matrix with high concentration and/or high MW was brittle. Although, the different of MW did not exhibit obvious difference in the tensile strength, % elongation at break significantly decreased when the MW increased.

Indeed, the polymeric network is corresponded with the flexibility and porosity of the matrix. The matrix from loose network probably provides the matrix with higher porosity and flexibility than the matrix from strong network [54]. The porosity of chitosan and PVA matrix were evaluated from their surface morphology. The flexibility was evaluated by the stability of matrix after peeled off from the Petri dish and % elongation at break. In this study, the porosity of the matrix is the first consideration to obtained and optimized type and amount of polymer, chitosan and PVA for blending with collagen. It was found that the matrix prepared from high MW (100,000 - 1,000,000) shrimp chitosan did not show the porosity. Moreover, it was found that the amount of polymer did not affect the porosity and mechanical properties of the matrix. The squid and shrimp chitosan at the same MW (100,000 - 1,000,000) did not show the surface porosity, while their cross section revealed the sheet characteristic. On the contrary, the crab chitosan provided the matrix with interconnected porosity. Moreover, their stability and flexibility were greater than that of squid and shrimp matrix. It is interesting to further investigate the structure of polymeric chain after a matrix formation.

Although, the matrix from PVA MW 72,000 and 145,000 did not show the porosities, their flexibility was high (% elongation at break as 50-100%). This may be

because the hydroxyl groups of the PVA network allow the free diffusion of water and enhance the mechanical properties of the matrix [55].

1.2 Physicochemical properties of the collagen/chitosan or collagen/PVA matrix

The collagen matrix prepared by casting technique was very brittle. Incorporation of collagen with chitosan or PVA significantly improved the mechanical properties of the collagen matrix. Moreover, the results of the tensile strength and % elongation at break indicated that introducing PVA into the collagen matrix significantly increased the tensile strength and % elongation at break of the matrix which was higher than that introduced with chitosan. This result correlated with the tensile strength and % elongation at break of the PVA or chitosan matrix.

In collagen, the hydroxyl groups of hydroxyproline are involved in hydrogen bonds between chains. These side chains are capable of forming hydrogen bonds with –OH and $-NH_2$ groups of chitosan. Moreover, the –COOH and $-NH_2$ groups in the collagen may also form hydrogen bonds with –OH and $-NH_2$ groups of the chitosan. The long chain of chitosan can wind around the collagen triple helix. Two different molecules (collagen-chitosan) may form a complex, which have much higher viscosity than the single component [56]. In addition, collagen and chitosan may be bonded ionically. In an acidic solution, polycationic groups ($-NH_2$) of chitosan forms complexes with the polyanionic groups (-COOH) of the collagen [57-59]. Therefore, the ratio of collagen and chitosan are critical for the stability of the matrix that is determined by the crosslinking density inside the matrix [55].

Addition of the GA crosslinkers (Figure 33A) allows the matrix more stable because the aldehyde groups of GA form covalent imine bonds with the amine groups of the collagen and chitosan [46, 55, 60]. These crosslinking models are shown in Figure 34.

In GP crosslinking, GP (Figure 33B) combines with chitosan and collagen via hydrogen bonding, hydrophobic and ionic interaction. In acidic solution, chitosan becomes positively charge due to the protonation of free amine ($-NH_3^+$) groups. When protonated, the collagen exhibits positive ($-NH_3^+$) and negative charges ($-COO^-$). The

positive charge has a potential ionic interaction with $-OPO(O^-)^2$ in the GP. This interaction model is shown in Figure 35. The collagen and chitosan contain hydrogen bonding favoring groups (-OH, $-NH_2$, and –C=O). The interaction among the matrix of collagen, chitosan and GP are probably the hydrogen bonding [61, 62]. Therefore, the tensile strength and % elongation at break of the collagen/chitosan matrix crosslinked with GA or GP were higher than those of the uncrosslinked matrix.

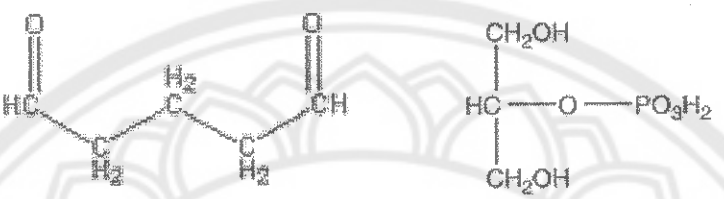

Figure 33 Chemical structures of glutaraldehyde (A) and β-glycerolphosphate (B).

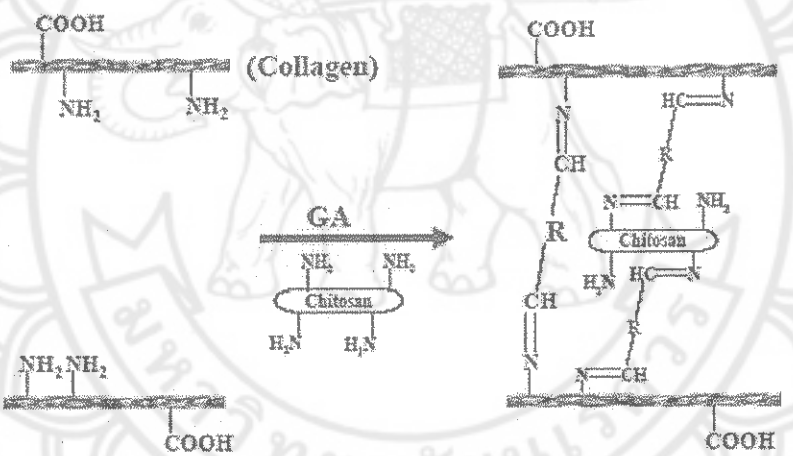

Figure 34 Schematic of collagen and chitosan crosslinked with glutaraldehyde [46].

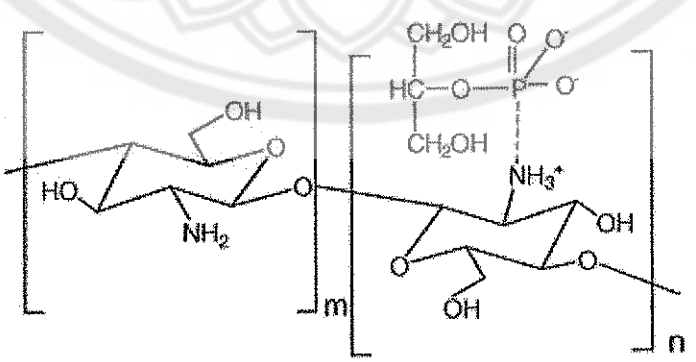

Figure 35 Ionic interactions of chitosan and β-glycerolphosphate [61].

Crosslinking with GA might decrease the porosity of the matrix due to the increased crosslinking density. However, changing the amount of GA slightly changed the porosity. The porosity of the matrix crosslinked with GP was very low; this may probably involve the interaction inside the polymer chains. Most of the interactions within these polymers are reversible link such as hydrogen bonding and ionic interaction. Such interactions occur when the molecules are in the close vicinity therefore the porosity of the matrix is low. The reversible link is a weak interaction between polymer chains and provides non stable structure. Therefore, the mechanical properties (tensile strength and % elongation at break) of the matrix crosslinked with GP tended to be low.

The main interactions inside the collagen/PVA networks are hydrogen bonds. These interactions occur between the hydroxyl groups of PVA and the hydroxyl or amino groups of collagen [57]. Additionally, the hydroxyl group can form hydrogen bond with free water molecules therefore the free PVA molecules and free water can move within network of the matrix. This interaction correlates with the high flexibility of the collagen/PVA matrix.

1.3 Swelling property and degradation

Because the obtained matrices are expected to be used in the skin tissue engineering, its water absorbing property becomes a critical factor. This is because that the matrix may involve in the absorption of body fluid and the transfer of cell nutrients and metabolites through the matrix. The networks containing covalent crosslinked chitosan are considered as porous. This term is used to describe networks containing free water that can diffuse through the matrix. Indeed, the percent swelling relate with the mesh size of the network [55]. The swelling change of matrix translates into a change in the mesh size of the matrix.

GA not only enhanced the strength of the matrix, but also decreased the swelling of the matrix because the addition of GA increased the crosslinking density of the matrix. Generally, water diffusion depends mainly on the crosslinking density. The polymeric chains form a network containing a number of pores filled with small molecules such as water, which can be free or bound to the hydrophilic groups of the network [55].

As the crosslinking density is increased, water content, swelling capacity and mesh size of the network decrease. Indeed, increasing the amount of crosslinker decreases the ability of collagen and chitosan to form hydrogen bonds with water molecules [55, 63]. This study showed that the matrix crosslinked with GA exhibited decreased the swelling property. Moreover, increasing the chitosan tended to decrease the matrix swelling property, which correlated with the influence of chitosan on the porosity of the matrix.

The hydrogen bonding and ionic interaction among collagen, chitosan and GP lead to reduction of hydrophilic interaction and hydrophibic increases [62]. Therefore, the water absorption of GP-crosslinked matrix decreased resulting in low swelling property.

The swelling of collagen/PVA matrix as shown in this study was more than 100% due to high hydrophilic property of the PVA chains and low crosslinking density of this matrix. However, high molecular weight PVA was more stable in shape and had lower swelling property compared to that of the low molecular weight PVA, which corresponding to the previous study [64].

In addition, the swelling results of the matrix correlated with the result of the matrix degradation. The result suggested that increasing swelling property refered to the increase in the degradation of the matrix.

1.4 Cytotoxicilty test

After a prolonged direct contact of the matrices with primary human keratinocyte (HaCaT), neither cell death nor growth disorder was observed. Therefore, the matrices were cytocompatible and none of cytotoxic substances was released into the culture medium, even if it contained GA. Although the free GA molecule is toxic, in this study, GA was used in a small amount. Such GA molecules were completely polymerized with collagen and chitosan chains. Moreover, the matrices neutralization completely excluded the GA residues from the matrix before being cultured with HaCaT.

The initial event in seeding cell to the matrix is cell adhesion on the surfaces of the matrix, which is a direct contact between cell membrane and the surface of the matrix. The cell adhesion occurs via the interaction with cell surface receptor such as

integrins. The integrins act as a physical link between the cell surface and the extracellular matrix, and also connect the extracellular matrix to the intracellular cytoskeleton. The integrin receptors bind the extracellular molecules via the short amino sequences, which is recognize region (such as Arginine-Glycine-Asparagine) and many cell adhesion sequences [2, 65]. Collagen also contains the recognize region. The matrices in this study were composed of collagen. Therefore, these matrices were able to stimulate cell adhesion on the surface of the matrices. In addition, the matrix containing chitosan may enhance the ability of cell adhesion due to the interaction between cells and chitosan. This interaction may be due to the electrostatic interaction between the cells and the amino group of the chitosan molecule, since the biospecific interaction between a cell receptor and an *N*-acetylglucosamine of the chitosan molecule has been reported [8]. The percent cell adhesion correlated with the matrix swelling property. This may involve the hydrophilic interaction between cell membrane and water molecules inside the matrix. The matrix with excess hydrophilic or excess hydrophobic affected the cell adhesion and proliferation because this involves the water absorption and transfer of cell nutrients, which are important for cell growth.

The HaCaT cells attached on the matrix survived and proliferated on the surface of the matrix although the pore size of the matrix was small. Jianbio M. et al [66] revealed that the pore sizes in an area were less than 5 μm; cell would stand at the edges of the small pores instead of getting into the pores. Because chitosan contained positive charges, the negative surface of the cells would bind tightly with chitosan and grow *in situ* in the presence of a medium. It was surprised that the matrix prepared from collagen/crab chitosan MW 100,000 - 1,000,000 in ratio of 7 to 3 and crosslinked with 0.1% GA could promote HaCaT proliferation on such matrix. This result related with its high mechanical (tensile strength and % elongation at break) and swelling properties. The enhancing mechanism on HaCaT proliferation on the matrix was still unclear. It may be results of the interaction between chitosan and growth factors in the serum metal ion, such as calcium [39]. It has been reported that chitosan may open the tight junction of cell membrane, so the growth factor and other important protein can pass into the cell

[66-68]. However, there is no report that the sources of chitosan influence cell adhesion and growth. Therefore, this study is the first to report this event. The adhesion of HaCaT on the GP crosslinked matrix was low. This may be because of the increased hydrophobic interaction of this matrix. Furthermore, the low proliferation on this matrix may correlate with the low swelling of the GP crosslinked matrix. Because of water absorption is important for a transfer of cell nutrients, oxygen, and bioactive substances. Therefore cells attached on the GP crosslinked matrix were slowly grown.

For collagen/PVA matrix, although it could induce cells adhesion, cell proliferation was slow. HaCaT cultured on the collagen/PVA matrix tended to be narrow spread on the surface of the matrix [44, 70]. The obtained results corresponded with the previous report. Such report indicated that the adsorption of proteins and adhesion of HepG2 cells are reduced on highly hydrophilic surface as well as the PVA matrix [44, 70].

It is known that the expression of KGFR and keratins present as the normal skin keratinocyte. Keratin filaments are a marker of the keratinocyte and acts as a cytoskeleton of the keratinocyte [12, 16]. For example, keratin 5, 10, and 14 are secreted by keratinocyte, and they are not express in other cell types [40]. KGF is a potent mitogen for keratinocytes and stimulates proliferation of the keratinocyte therefore KGFR plays an important role in binding with KGF [12]. After 2 months of culture on the developed matrix, keratinocyte revealed the tendency to produce the keratin 4-14 and KGFR as reflected by keratin 4-14 and KGFR mRNA expression. It was assumed that after 2 months, the keratinocyte culture on the developed matrix could express their normal phenotype. However, the mRNA expression is quantitative characterization, which can not be directly implied to their functional protein. Therefore the further investigation to confirm this result, such as enzyme-linked immuno sorbent assay (ELISA) or immunohistochemistry, should be performed.

2. Conclusion

The results from this study indicated that not only amount and molecular weight of polymer and amount of crosslinker, but also the type of polymer (e.g. chitosan) were parameters that influenced on the physicochemical of the matrix prepared by casting technique. On the other hand, blending collagen with high molecular weight polymer and crosslinker tended to decrease the swelling and degradation time of the matrix. However, their porosity and flexibility tended to decrease. It is interesting to note that using the different sources of chitosan provided the matrix with different porosity and mechanical properties. Therefore, preparation of the matrix with appropriate ratio of collagen to the blended polymer, amount of crosslinker and size of molecular weight of blended polymer provide the matrix with the desirable properties.

It can be concluded that the matrix prepared from collagen/crab chitosan MW 100,000 - 1,000,000 in the ratio of 7 to 3 and crosslinked with 0.1% GA would be applicable to acute wound healing due to this matrix could promote adhesion and proliferation of HaCaT more than the other formulations as well as the plastic plate. This matrix exhibited the highest strength, flexibility, and percent of swelling. Moreover, it showed the degradation time of about 43 days. Therefore, this matrix has a potential for application in skin tissue engineering. A good skin matrix should have a biodegradation time constant required for acute wound healing which is about 25-42 days for burns and skin [18, 61]. In addition, the matrix prepared from collagen/shrimp chitosan MW 30,000 in the ratio of 8 to 2 crosslinked with 0.15% GA and collagen/shrimp chitosan MW 100,000 in the ratio 8 to 2 crosslinked with 0.15% GA exhibited good strength with the degradation time of about 53 and 64, respectively. However the matrix prepared from collagen/shrimp chitosan MW 30,000 in the ratio of 8 to 2 crosslinked with 0.15% GA would be more appropriate for acute wound healing, whereas another one would be more appropriate for chronic wounds where 8 weeks degradation time is recommended [18]. The matrix prepared from collagen/chitosan crosslinked with GP and collagen/PVA from this study was inappropriate in skin tissue engineering application.