CHAPTER V

DISCUSSION

Lyophilized extract

In this study, light brown powder of tamarind fruit pulp's extract was obtained from lyophilization process. This extract could be easily solubilized in water. Even though this crude extract has many kinds of AHAs constituent such as lactic acid, citric acid and malic acid but the main constituent is tartaric acid. So we determined only the amount of tartaric acid in the extract and also in further systems. The results shown that our crude extract had tartaric acid about 20- 30% (w/w) that closes to the previous study (8-23%) (Greave M.W.,1990). However, the different amount of tartaric acid from tamarind fruit pulp's extract is generally varying from different source of the tamarind. In addition, the sugar form of this crude extract is sensitive with the humidity in air. It can absorb water very quickly, leading to the changing in appearance that will turn to darker color and higher weight. Therefore, the lyophilized crude extract is recommended to be kept in a tight container at low relative humidity condition (such as at freeze temperature).

Liposomes preparation : uncoated and coated liposome

The preparation of liposome loaded tamarind fruit pulp's AHAs could be done successfully by method modified from the previous study (Weiner,1989). The extract solution had pH of about 4 at the concentration of 2% tartaric acid. This pH range is weak acid. Beneficially, several literatures confirm that liposomes are more stable in acidic condition than basic condition.

Although we did not investigate the physical characteristics of the system occurred during process of liposomes preparation by reverse phase evaporation method, the process that might be occurred during emulsification was the formation of the "inverted micelle". Such inverted micelle or droplets were collapsed into a viscous

gel like state when the organic phase had been removed by evaporation. The critical point in this procedure was probably when the gel state collapsed. At this point some of the inverted micelles disintegrated, releasing their encapsulated material; the excess lipid contributed to a complete bilayer around the remaining micelles, resulting in the formation of vesicle; this step was done by shaking to accelerate the formation of vesicles. The optimum duration of each procedure including sonication, evaporation and shaking time were 7, 60 and 1 min, respectively. However, changing the equipment model may result in different results. Mostly, the vesicles obtained from the reverse phase evaporation are large unilamella vesicle (LUVs) (Weiner, 1989).

The experiment results showed that the ratio of lipid dispersed in organic solvent and extract solution (oil:water ratio) had profound effects on inverted micelles formation. The optimum O:W ratio was 5:1 since the higher ratio of water phase led to the aggregation of an emulsion and/or the low entrapment efficiency. That might be a result of uncomplete formation of emulsion.

Use of phospholipids and incorporation of cholesterol into the bilayer of liposomes generally improves stability (Sulkoski, 2005) therefore the appropriate amount of lipoid®: cholesterol molar ratio was investigated. The difference of lipoid® and cholesterol molar ratio resulted in the difference of entrapment efficiency. The highest entrapment efficiency achieved by incorporation of cholesterol in lipoid® with the ratio of lipoid® to cholesterol 2 to 1. The incorporation of lower ratio of lipoid® might not be enough to entrap all extract solution. The use of higher ratio of lipoid® might cause the aggregation, resulting from the excess phospholipid content.

Characterized liposomes by optical micrograph at 1000 fold indicated the well-form vesicles with lipid bilayer. These vesicles had various sizes with the range of 300-500 nm. To achieve narrow size distribution, some batches of liposome loaded tamarind pulp's extract was extruded through an Extruder device LipoFast™-100 equipped couple with a 100 nm pore size polycarbonate membrane. The obtained resulted showed the

homogeneous and smaller size of liposomes (100-200 nm). These indicated that, the rearrangement of liposome vesicles occur via this device.

To improve the stability of liposomes even in the storage or usage condition, liposomes was coated with chitosan. The coating process was modified from the previous study (Perugini., 2000). Such study reported that degree of acetylation of chitosan did not affect coating properties of chitosan on liposomes. In this reason, we had been interested whether the usage of the different sources (crab, squid and shrimp), concentration (0.1, 0.5 and 1%w/v) and amount (1, 2 and 3 ml) of chitosan did affect the coating properties on liposomes. One of major effect that we investigated was the encapsulation efficiency of the coated and uncoated liposome. The results showed that the encapsulation efficiency of each batch was not significantly different when various sources and/or amount of chitosan solution were used. On the contrary, the encapsulation efficiency of chitosan coated liposomes tended to increase as the concentration of chitosan was increased (from 0.1 to 1 % w/v). That may be a result of the entrapment of tartaric acid between the filaments on the high thickness of chitosan coated liposomes.

Transmission electron micrograph (TEM) indicated clear chitosan film coated on liposomes surface. Since chitosan molecule possess positive charge, they can interact with negative stain which was uranyl acetate resulting in black surface over the liposomes vesicle. Unfortunately, the high tension of TEM (120 kV) caused the force on liposomes vesicle resulting in non-spherical shape and repture finally. However, the result obtained from the TEM micrograph indicated that the coating liposomes with chitosan tended to increase vesicle size of about 2-3 times (200-300 nm). That may be a result of liposome vesicle surrounding by the thin film of this polymer.

The zeta potential of the uncoated liposomes was negative, whereas that of the chitosan coated liposome was positive. Therefore, the main interaction between liposomes and chitosan was electrostatic attraction. The mechanism of coating liposomes by chitosan probably involved hydrogen bonding between the polysaccharide

and the phospholipids head group. Since chitosan carried high positive charge, the absorption of chitosan on the liposome surface increased the density of positive charge, resulting in the appearance of the net positive charge. Additionally, the zeta potential increased as the chitosan concentration was increased.

The zeta potential of particles is thought to play an important role in the resistance to flocculation and coagulation (. Since all such chitosan coated liposomes possessed high positive zeta potential (6-29 mV), it's still in the recommended range of the stabilized particle (-30–30 mV) (Woodle, 1992). The structure of adsorbed chitosan film stabilized the particles against particle-particle interaction presumably by the mechanism of steric stabilization (Kellaway, 1981). Additionally, the thickness of the adsorptive layer of chitosan on the liposomes would keep the particles apart, leading to more stabilization of the coated liposome.

The determination of chitosan reacted with liposomes indicated the coating efficiency of chitosan on liposome's shell. Figure 29 showed the coating efficiency of chitosan tended to increase as the concentration of chitosan was increased (from 0.1 to 0.5%w/v). After that, the saturated state appeared, which corresponded to the encapsulation efficiency and zeta potential results. This may be assumed that the thickness of chitosan film coated on liposome is determined by the concentration of chitosan.

It is interesting to note that, at high concentration of chitosan (0.5, 1% w/v), varied amount of chitosan solution did not affect on the amount of chitosan reacted with liposome's shell. This may because the amount of chitosan contained in those solutions exceeded to react with the liposome's shell. However, at the lower concentration of chitosan (0.1% w/v), the total amount of chitosan contained in 1 ml solution was lower than that contained in 2 or 3 ml solution. Such lower amount may not completely cover the shell, resulting in the lower coating efficiency.

In vitro release of tartaric acid

It has been recommended as a rule of thumb that the drug concentration in the sink phase in release experiments should be kept below 10% of saturation (Nounou M.M. et al., 2006). Therefore, 1 ml of vesicles suspension was placed in 10 ml of PBS medium (pH 5.5) to provide sink conditions. The pH range was selected to mimic the skin condition. The strength, which corresponds to the sustained release ability of the coated liposome and the stability of the coated liposome during storage in preparation of the carrier system was investigated. The reproducibility and efficacy of the release study were confirmed by using a control sample of tartaric acid solution. This can ensure that the dialysis membrane is not a barrier of the release process. The result indicated that the release pattern of tartaric acid from liposomes was prolonged. The increasing in the percentage of chitosan (from 0.1 to 1 %w/v) decreased the release rate. Approximately 20–30% of the tartaric acid was released at a relatively rapid rate during the first 2 hours, followed by slower release rates over the next ten hours. The initial rapid phase of tartaric acid released was less evidence in the case of chitosan coated liposomes. The initial fast rate of release is commonly described to substance detachment on liposomal surface while the later slow release results from sustained substance release from the inner lamellae (Henriksen et al., 1995). Zero-, first-order and Higuchi's equations were applied to *in vitro* release results. Correlation coefficient values of Higuchi's model was found to be the best fitting model suggesting that tartaric acid transported out of the liposomes was driven mainly by a diffusion-controlled mechanism. In general, the release profiles of liposomal dispersions were biphasic showing a relatively large burst effect over the first two hours followed by a slower release phase. The burst effect varies with the strength of the liposome's shell (Vemuri S., 1995). Generally, tartaric acid is a low molecular weight molecule and easily leached out from the bilayer lipid structure of the liposomes to the external aqueous buffer. Therefore, the exhibition of the low released rates of the chitosan coted liposome probably causes by the increase in rigidity of that vesicle's shell.

There are very few published reports on long-term stability of liposomes and nopublished reports on the establishment of detailed protocols for stability testing. The release profile of the coated and uncoated liposomes may be used to indicate the stability of the modified vesicles in holding the active ingredient during the storage in the preparation. Since we found the lower rate and amount of tartaric acid released from the coated liposomes, this may indicate higher stability of the coated liposome than the uncoated liposome.

In vitro efficacy of tamarind's AHAs on keratinocyte proliferations

HaCaT human keratinocyte cells were selected as an *in vitro* model to investigate the influence of the tamarind fruit pulp's AHAs, liposomes and chitosan coated liposomes loaded tamarind fruit pulp's AHAs on the keratinocyte proliferation.

AHAs have been extensively used in cosmetic and dermatologic formulations, Recently, AHAs have been recognized as important adjunctive therapeutic elements in a variety of skin disorders including photodamage (Funasaka et al., 2001), hyperpigmentation (Tung et al., 2000) and acne (Atzori et al., 1999). Repeated use of AHAs formulation has been demonstrated to alter the structure of the stratum corneum. Therefore, the using of human keratinocyte cells are the best condition for this study.

The experimental results showed that the tamarind fruit pulp's extract solution at the concentration of 50-500 ug/ml tartaric acid did not significantly promote the proliferation of HaCaT. However, the concentrations of tartaric acid did not damaged cells compared to control. On the contrary, the highest concentration of tartaric acid 1000 ug/ml in tamarind fruit pulp's extract reduced cell viability up to 50% of cells. That may be a result of the acidic condition (pH 4) of the medium.

Surprisingly, the highest increase in HaCaT cell proliferation occurred when the cells were treated with the liposomes or chitosan coated liposome encaped tamarind fruit pulp's extract containing tartaric acid at various concentrations. Especially, at the concentration of 1000 mg/ml, the proliferation of cell increased up to 2 folds, as

compared with the control. A previous study also reported that the keratinocyte proliferation increases by treating with lactic acid; one kind of AHAs(Yamamoto et al., 2005 and Reeder L. et al., 2001). The coating liposomes with chitosan did not affect the ability of the delivery system to promote the cell growth.

From the clearly different result of treatment with extract solution and delivery system, we assumed that the hydrophilic molecule and acidic of tamarind's AHAs caused the difficulty in cell uptake. In the other hand, the amphiphatic molecule and the major compound in liposomes, phospholipids, can promote the uptake ability of the cells probably via phagocytosis pathway.

Even though the result clearly indicated the ability in increasing keratinocyte proliferation of tamarind's AHAs, the mechanism of action of AHAs has not been fully determined. It is hypothesized that AHAs act as a chelating agent and thereby decrease local calcium ion concentrations from cation dependent cell adhesion molecules. This calcium loss from cadherins of desmosomes, adherens junctions, and tight junctions causes a decrease in desmosomal attachments. This makes the usually protected endogenous stratum corneum chymotryptic enzymes on cadherins vulnerable to proteolysis. When calcium is decreased then cellular adhesions are disrupted and exfoliation takes place. Another proposed mechanism for AHAs induced exfoliation is an increase in apoptosis. In one study, lactic acid was shown to cause a concentration dependent increase in apoptotic cells. In this same study, vascular endothelial growth factor (VEGF) was increased at least 2.5 fold over vehicle control with either a 1.5 or 3% concentration of lactic acid (LA). Angiogenin secretion was decreased by Lactic acid in a concentration dependent manner. It was concluded that topical AHAs modulate secretion of cytokines by keratinocytes and that this regulation may account in part for their effects in skin disorders as well as photoaging. Another study in 2003 confirms that glycolic acid directly accelerates collagen synthesis by fibroblasts, and modulates matrix degradation and collagen synthesis through keratinocyte released cytokines. The primary mediator for this matrix degradation is interleukin 1α (IL-1 α). It was assumed then that AHAs may cause upregulation of epidermal and dermal markers by stimulating

transforming growth factor beta (TGF-β) which in turn causes activation of dermal dendrocytes and mast cell release (Ditre CM. et al., 1996).

However, the successfully results from *In vitro* model could not accurately predict the advantage of the developed liposomes on human skin. Actually, liposomes vesicle will be broken when applied on skin and intact vesicle can not possible to transport across the skin. From many studies reported clearly that, the possibility that vesicle components; phospholipids may themselves permeate through the membrane and even disrupt its barrier function (they may act as penetration enhancers) (Betz, 2001 and Olga, 2002). Furthermore, it is conceivable that vesicles may break down and reform in situ incorporating thereby, for example, lipids originating from the skin surface (e.g. sebaceous lipids) or lipids from the intercellular domains of the stratum corneum (Alvarez-Roman R. et al., 2004). Theses findings may use to explain the possible occurrence of the inner skin cell uptaking the developed liposome after application on the skin.

In vitro efficacy of tamarind's AHAs on melanogenesis inhibition

Interestingly, the *in vitro* study of previous experiment (Usuki et al., 2003) showed that glycolic acid and lactic acid in dose of 300 or 500 ug/ml suppressed melanin formation by directly inhibiting tyrosinase activity. Adjusting the pH up to 5.6 did not affect tyrosinase activity and this effect was then deemed independent of these AHAs acidic nature. AHAs may work on pigment lesions by accelerating epidermal turnover and by directly induceing skin turnover which improves the appearance of hyperpigmentation due to hyperkeratinization. Moreover, AHAs may also directly inhibiting melanin formation in melanocyte.

MML-1 human melanocyte cells were selected as an *in vitro* model to investigate the influence of the tamarind fruit pulp's AHAs, liposomes and chitosan coated liposomes loaded tamarind fruit pulp's AHAs on the melanogenesis inhibitory. Although no work was done to investigate the effect of AHAs on melanogenesis of human melanocytes *in vitro*, we are interested to use human melanocyte cell line because it

mimics to the human skin cell. In this study, we determined the melanin content of MML-1 after treated with our samples comparing kojic acid, well known whitening agent.

With one exception, our results showed none of the formulation even kojic acid affected the melanogenesis of human melanocytes. Unfortunately that MML-1 produces light color and less amount of melanin than mouse melanoma. In this reason, the absorbance determination of melanin by spectrophotometer did not provide clarified results.

Mouse melanoma cells of the B16 cell line are widely used in studies of the regulatory mechanisms of melanogenesis (Ando, 1999) because these cells produce, unlike the vast majority of commercially available human melanocyte, ample melanin during serial passage of the cells. It remains unclear why kojic acid affects the pigmentation of murine melanoma cells but not that of human melanocytes. The main conclusion from our work showed the fact that melanin modulating agents differ in their actions on melanogenesis when tested in different *in vitro* cell culture model. Thus, the hypothesis if tamarind's AHAs do affect human pigmentation should be proven by an *in vivo* model.