CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problems

The natural hair color is under the complex genetic control and the result from the amounts and types of melanins produced by the follicular melanocytes. Follicular melanocytes derived from the neural crest cells locate in the hair follicles and produce the melanins through the melanogenesis pathway. Melanogenesis, a complicated pigment biosynthesis, involves the oxidative reaction of tyrosine to be either brown/black eumelanin or yellow/red pheomelanin depending on the cysteine or glutathione existing. These produced melanins are packed into granules known as melanosomes and then transferred to the cortical keratinocytes. The individual hair generally contains both eumelanin and pheomelanin. The perception of different hair colors is due to the mixture of the two types of melanin with physical phenomena that give the reflection and refraction of the incident light. Follicular melanocytes are active only during the hair shaft production or anagen phase of the hair growth cycle. During the resting period of hair or telogen phase of the hair growth cycle, the pigment producing cells are in the shrunken adendritic forms at the surface of the dermal papilla[1-3]. Canities, the lack of hair pigment condition on the scalp, is the physical characteristic that is significantly associated with the chronological aging and genetics. The incidence of canities is found in Caucasians earlier than in Asians and Africans. The onset of white hair in Caucasians is in the mid of 30 years old (34.2$\pm$9.6), whereas in Asians and Africans are at the late 30 years old and in the mid

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of 40 years old (43.9±10.3), respectively[3]. Currently, there is no treatment regimen for this condition and the masking of white hairs with hair dye is popularly used. Nevertheless, the hair masking with hair dye has been reported to have the numerous harmful effects, such as dermatitis, hair loss and cancer[4]. Many researchers have investigated the low toxic compounds which can induce the melanogenesis pathway. Among these compounds, saturated fatty acids, such as palmitic acid and stearic acid, as well as methyl ester derivatives have been reported to stimulate the melanogenesis, while the unsaturated fatty acids, such as linoleic acid and oleic acid, decrease the melanin production[5-6]. The lotus flower essential oil composing of palmitic acid methyl ester as the major component has been reported to have melanogenesis stimulation with the increased tyrosinase activity as well as the expression of the tyrosinase, microphthalmia-associated transcription factor (MITF) and tyrosinase-related protein-2 (TRP-2). Also, the methyl ester of saturated fatty acid has been demonstrated to have lower cytotoxicity than its corresponding saturated fatty acid[7].

Follicular penetration, one of routes of percutaneous delivery, is the penetration of topically applied substance through the pilosebaceous unit consisting of hair shaft, hair follicle, arrector pili muscle and sebaceous glands. This penetrating route is accounted for only approximately 0.1% of the total skin surface area. Although the limited openings and small surface area of the transport of this route have been reported, the applied substance can be rapidly absorbed. The fast absorption is due to the location of the bottom part of pilosebaceous unit that is in the dermis and the nerve innervated region without any absorption barrier[8]. However, the physicochemical characteristics of the penetrating substances, including the compatibility with the secreted sebum and the proper particle size, should be taken

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into account. Recently, liposomes and micro-/nanoparticles have been used as transfollicular delivery systems due to their follicular penetration efficiency[9-10]. The polymeric microparticles with the diameter in the range of 3-10 μm have been reported on their selective penetration into the follicular ducts, whereas particles larger than 10 μm remain on the skin surface. Also, the 5 μm diameter microspheres are specifically targeted to the follicular ducts and can diffuse down to the depth of 400 μm below the skin surface[11-13]. The confocal laser scanning microscopy has been used for the visualization of the penetrating particles through the tissue. The accumulation of the fluorescent polystyrene nanoparticles in the follicular openings of pig skin has been found[14]. The non-ionic liposomes are successfully employed to facilitate follicular delivery of both minoxidil and plasmid DNA in the *in vivo* model[15]. In this study, the saturated fatty acid methyl esters were synthesized and evaluated their cytotoxicity and melanogenesis induction activity as well as entrapped that methyl ester in the proper niosomal formulations in order to enhance transdermal delivery via transfollicular route. The development of hair lotion containing the synthesized methyl ester loaded in the developed niosomal formulation for canities treatment was performed and the *in vivo* rabbit skin irritation and melanogenesis induction assays in aged mice of the developed hair lotion were also evaluated.

1.2 Objective

To entrap the saturated fatty acid methy ester in niosomes for canities treatment.

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1.3 Scope of the study

This study was divided into 8 steps as follows:

1. Synthesis of four saturated fatty acid methyl esters from their parent saturated fatty acids, which contained 12-18 carbons by Fischer esterification, as well as characterization of the synthesized saturated fatty acid methyl esters by Fourier transform infrared spectroscopy (FTIR) and gas chromatography-mass spectrometry (GC/MS).

2. Evaluation of the *in vitro* cytotoxicity and melanogenesis induction activity of the synthesized saturated fatty acid methyl esters in B16F10 melanoma cells for the selection of the saturated fatty acid methyl ester with low cytotoxicity and high melanogenesis stimulation activity.

3. Development of three charges of blank niosomal formulations, including neutral, cationic and anionic charges, as well as physical characterization and stability evaluation of the developed blank niosomes for the selection of each charged blank niosomes with high stability.

4. Loading of the selected methyl ester in the selected three charged niosomes as well as physical and chemical stability investigation of the selected methyl ester loaded in three charged niosomes.

5. Evaluation of the *in vitro* cytotoxicity, melanogenesis induction activity and transfollicular penetration of the selected methyl ester loaded in three charged niosomes for the selection of the most appropriate charged niosomes loaded with the selected methyl ester to prepare the hair lotion product.

6. Development of the hair lotion bases and evaluation of the physical characteristics and thermodynamic stability of these bases for the selection of the base forrmulation with high stability to incorporate the selected methyl ester loaded in the

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selected charged niosomes.

7. Development of the hair lotion containing the selected methyl ester loaded in the selected charged niosomes as well as investigation of physical and chemical stability of the hair lotion containing the selected methyl ester loaded in the selected charged niosomes.

8. Evaluation of hair lotion containing the selected methyl ester loaded in the selected charged niosomes in the *in vitro* transfollicular penetration as well as *in vivo* rabbit skin irritation and melanogenesis induction assay in aged mice.

1.4 Literature reviews

1.4.1 Hair

Hair, a part of personal adornment, is the characteristic feature of all mammals and covers the entire body. Although hair appears to be a simple structure, it is actually complicated anatomy. Hair is formed from small organs (follicles) located in the complex environment of skin composing of multiple layers and continuously grows in the hair growth cycle.

1.4.1.1 Hair structure[1,16-18]

Hair structure develops between 9 weeks and 4 months after conception. All hair structure of human embryo is formed in sequence from the head down to the caudal part of the body. New hair structure is formed continuously after viviparity and although it develops uniformly in the first period, the difference in the density depends on the growth of the skin as the body develops and the body part. Three types of hairs based on the length, pigmentation and composition of hair shaft, are lanugo, vellus and terminal hairs (Fig. 1). Lanugo hair, the early hair, is fine, non-

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medullated and short. The lanugo is lost in the eighth month after viviparity to be replaced by relatively thick vellus hair up to about 2 cm long. The entire human body at birth is covered with vellus hair, which is short (less than 2 cm), thinner than 30 μm, silky and non-pigment. This hair is finally replaced during growth after birth by long thick terminal hair, which is longer than 2 cm, thicker than 30 μm, pigment and medullated. The appearance of the terminal hair depends on the age that puberty is reached, location and sex. With the exception of the palms of hands, soles of feet, lips, nipples and mucous membranes of sex and other organs, hair covers the entire body and the length and thickness depend on the location.

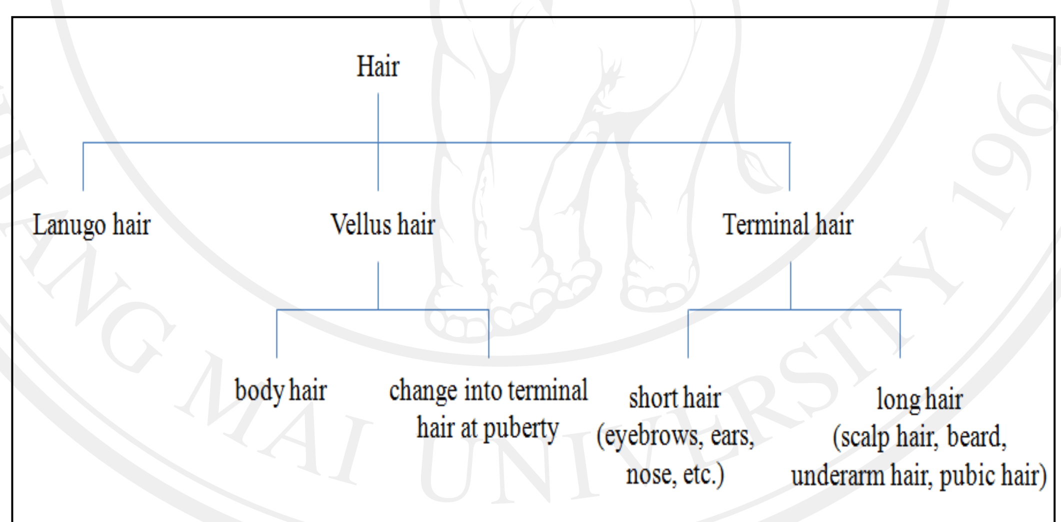

Figure 1 Hair classification based on the length, pigmentation and composition of hair shaft[16]

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Hair is an epidermally derived structure comprising of the hair follicle, the hair shaft and the other components. The hair follicular structure is divided into two parts based on the functional compartment, which are the dermal and the epithelial compartments (Fig. 2). The dermal compartment locates in the lower

portion of hair follicle and composes of the dermal sheath and the dermal papilla. The epithelial compartment, a dynamic portion of hair follicle, includes the hair matrix and three concentric structures named the outer root sheath, the companion layer and the inner root sheath. For the hair shaft, three layers of hair shaft are cuticle, cortex and medulla, while the other components of hair follicle are melanocytes, Langerhans cells and Merkel cells.

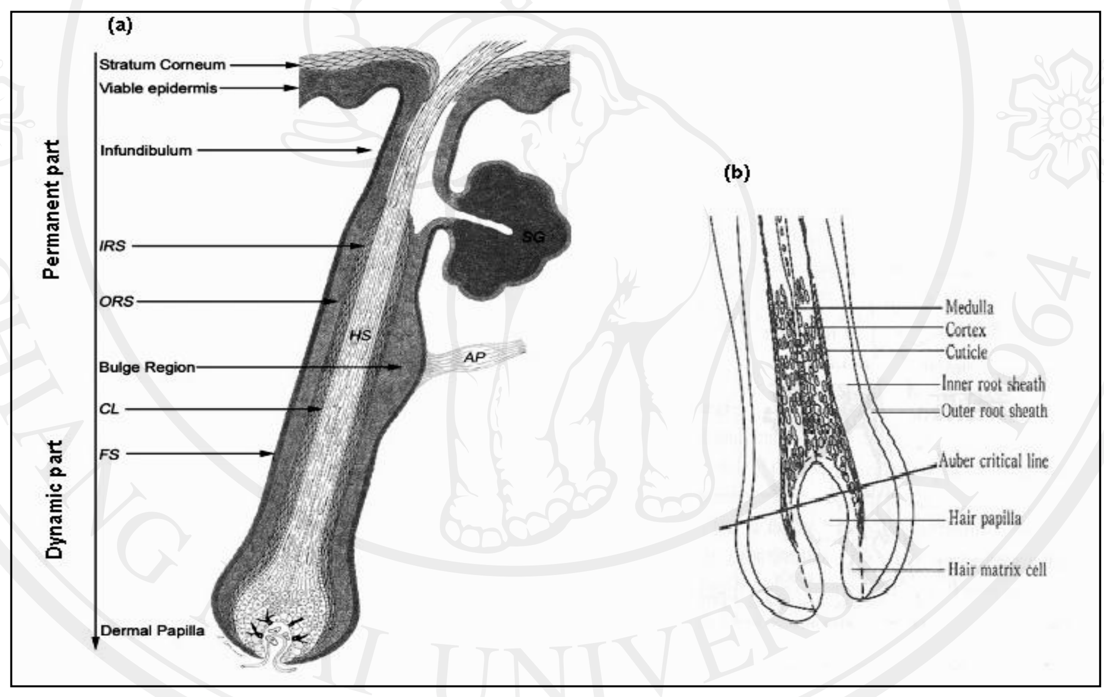

Figure 2 Hair follicle structure (a) and the enlarged hair bulb (b). HS: hair follicle, ORS: outer root sheath, IRS: inner root sheath, CL: companion layer, FS: fibrous sheath, SG: sebaceous gland and AP: arrector pili muscle[16-17]

A. Hair follicle

Dermal compartment of hair follicle: Figure 2a demonstrated the lower part of hair follicle located in the dermis layer with the network of blood

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microvessels. The entire length of hair follicle is surrounded by the dermal sheath, which is made of a connective tissue sheath produced by fibroblasts and a basement membrane. The dermal papilla, almost totally embedded into the matrix epithelium of the lower part of hair follicle, is an oval mass of extracellular matrix harboring spindle-shaped fibroblasts and has the small blood vessels cross over. This oval mass is a reservoir for hair growth and the real heart of follicle due to its size apparently controls the size of follicle and its capability for the induction of follicular development.

Epithelial compartment of hair follicle: At the hair bulb or the lower part of the follicle, the hair matrix consists of epithelial cells closely surrounded by the dermal sheath at outside and the dermal papilla at inside (Fig. 2b). At a critical level corresponding to the widest part of papilla named Auber line, the matrix cells enter the various differentiation programs that give rise to the outer root sheath, inner root sheath and the hair shaft. Under the Auber line of hair bulb, the matrix cells are poorly differentiated and actively divided. The outer root sheath lies in continuity with epidermis and is further divided into 3 parts; lower, central and upper. Inner root sheath, the next major epithelial compartment, separates the outer root sheath from the hair shaft. The multistep differentiation programs characterize the rigid structure in the inner root sheath which may serve as a mold for the growing shaft and as a diffusion barrier involved in the maintenance of morphogenic gradients inside the hair follicle.

B. Hair shaft

Hair shaft is in the center of hair follicle and is consisted of three different epithelial cell types, which are cuticle, cortical and medulla cells (Fig. 3).

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Cuticle, the outer surface of hair shaft, covers the entire hair from the root to the tip. It has a scale-like structure and encloses the inner cortex. It is consisted of translucent and non-pigmented cells. The cuticle comprises about 10-15% of hair and has a rough surface composed of cystine-rich keratin protein. This layer prevents the loss of cortical water and protein as well as forms the path for the diffusion of water and chemical agents into the cortex. Cortex, the inner layer of hair shaft, contains the keratinized cortical cells aligned along the long axis of hair. The cortex comprises about 85-90% of hair and includes the pigment granules. The cortical cells are important in the smoothness and softness of hair. Medulla, the center of hair shaft, is consisted of honeycomb-like cells with empty spaces aligned along the longitudinal axis of hair and contains melanin. Thick hair may have thick medulla, the cells may appear like continuous pencil or they may be broken in places. In vellus and infant hairs, there is no medulla layer.

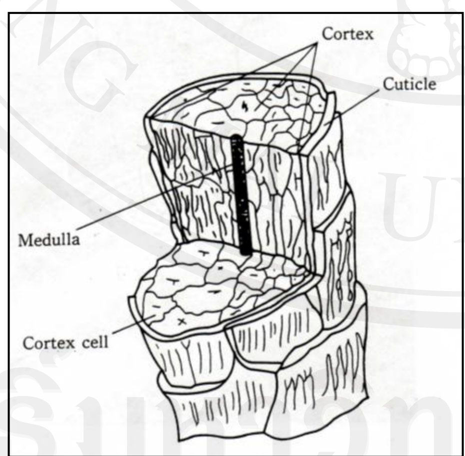

Figure 3 Hair shaft composition[16]

C. **Other components of hair follicle**

Melanocytes: Figure 4 showed the numerous dendritic melanocytes

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on top of dermal papilla (Mel-BMc). These melanocytes lie in the basal layer and closely interact with the basement membrane by extending their dendrites into the intercellular spaces of bulb and to the cortical cells. Melanosomes, the pigment granules, are transferred from melanocytes into the hair cortex. Another set of melanocytes located in the basal layer of the upper third of outer root sheath are amelanotic (AMel-BMc) and represent as the reservoir for epidermis and cyclically renewed follicles (Fig. 4).

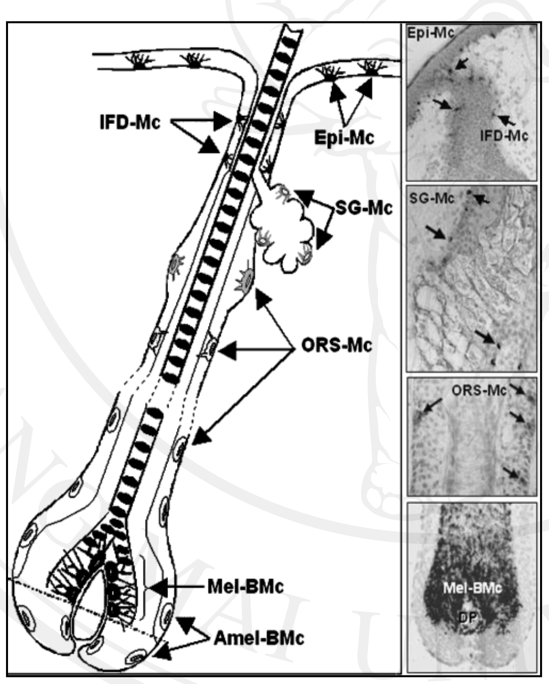

Figure 4 Melanocyte distribution in the human anagen scalp hair follicle. Epi-Mc: epidermal melanocytes, IFD-Mc: infundibular melanocytes, SG-Mc: sebaceous melanocytes, ORS-Mc: outer root sheath melanocytes, Mel-BMc: melanogenic bulbar melanocytes, Amel-BMc: amelanotic bulbar melanocytes and DP: dermal papilla[19]

Langerhans cells: Langerhans cells locate in the upper part of outer root sheath at the level of isthmus, an area between the duct opening of sebaceous

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gland and the attached area of arrector pili muscle, and in the sebaceous gland. These cells play the important role in the immune system.

Merkel cells: Merkel cells can be detected at the level of isthmus and involve the network of sensory nerve convergence at this level.

1.4.1.2 Hair functions

Hair functions include the hair growth cycle and the hair pigmentation. Three phases of hair growth cycle, which are anagen, catagen and telogen phases, are significantly associated with the hair pigmentation occurring in the bulbar melanocytes.

A. Hair growth cycle[1,16, 18]

Hair separately grows following the continuous cyclic pattern and the life of each hair is independent of other hair. Three phases of hair growth cycle are anagen or the growth period, catagen or the growth cessation period and telogen or the resting period (Fig. 5).

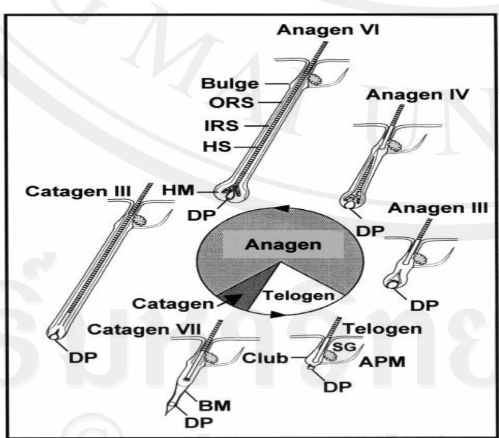

Figure 5 Hair growth cycle. DP: dermal papilla, ORS: outer root sheath, IRS: inner root sheath, HS: hair shaft, HM: hair matrix, mel: melanocyte, BM: basement membrane, APM: arrector pili muscle[1]

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Anagen phase: During the period of hair growth or anagen, the dermal papilla is large and the hair matrix actively divides. The hair elongation is observed and the hair bulb reaches into the sub-dermal tissues. After a cycle of hair growth, a new anagen is turned on and the lowermost part of follicle has to reform for production of a new hair shaft. The anagen phase is driven by deep remodeling events involving both follicle and the immediate dermal environment. Depending on the body region, the anagen phase can last from 1 to 6 months (arms, fingers, eyelashes), 5 to 7 months (legs) or up to 1 to 10 years (adult scalp). The latency period, which is about 80% of hair cycles, lasts from 2 to 5 months on average before a new hair cycle is engaged.

Catagen phase: The most obvious and early feature of this catagen phase is the cessation of pigment production and mitosis of bulb matrix cells as well as the arrest of inner root sheath differentiation. The lower part of follicle undergoes the apoptotic degeneration and becomes the club-shaped, moving upwards to reach the level of arrector pili muscle insertion. The bulbar region of follicle collapses and a minimal program of differentiation in the epithelial column can be observed under the club hair. Concomitantly, the membrane becomes thickened and dermal papilla physically separates from the club hair. However, dermal papilla ultimately follows the upward movement of the lower follicle in the follicular sheath. At the end of this phase, macrophages clean up the follicular remnants and only the dermal papilla with a residue of the matrix remain. One-third to one-half of the follicular length is lost during the process in this phase.

Telogen phase: The resting phase of the hair cycle is characterized by the lack of cell division and the presence of inactive melanocytes. Dermal papilla

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becomes atrophic and lacks of extracellular matrix and microvessels. After a few months, the spontaneous onset of a new anagen appears. The stimulus for new hair cycle initiation is that an arising of induction signal in the follicular epithelium on the resting papilla, thereby re-establishing the communication between epithelial and dermal compartments of follicle and initiating a new morphogenetic process. The onset of anagen phase is marked by proliferation of matrix cells and down-growth of follicle through the dermis and along a pathway of fibrous streamers. Also, the proliferation of a melanocyte subset is observed after the onset of anagen and those melanocytes, which will co-migrate with the newly formed bulb in close position with dermal papilla, will actively function in melanogenesis process. The different compartments are progressively backed in place, while the neurocutaneous and vascular networks are remodeled.

B. Hair pigmentation[19-21]

Hair pigmentation or hair color is from the presence or absence of melanin in the cortex of hair fiber. Melanin obtained from a complex biochemical pathway called melanogenesis is produced by hair bulb melanocytes, the neural crest-derived cells and then packed into granules known as melanosomes. The melanosomes are then transferred to cortical keratinocytes by the dendritic projections of melanocytes during the hair growth period or anagen phase. In the hair bulb, one melanocyte interacts and fills melanosomes into five keratinocytes, while in the epidermal pigmentation one melanocyte interacts with 35 viable keratinocytes.

Melanogenesis process starts from hydroxylation and oxidation reactions of tyrosine to dopa and dopaquinone, respectively, by tyrosinase enzyme. Dopaquinone is then oxidized to produce either eumelanin or pheomelanin depending

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on the existing of cysteine or glutathione (Fig. 6). The pigment biosynthesis involves at least three melanogenic enzymes, which are tyrosinase, tyrosinase-related protein-1 (TRP-1) or 5,6-dihydroxy carboxylic acid (DHICA) oxidase and tyrosinase-related protein-2 (TRP-2) or dopachrome tautomerase. These melanogenic enzymes are the products of tyrosinase gene family expression which specifically takes place in melanocytes. Tyrosinase, the rate limiting enzyme of pigment biosynthesis, is synthesized by ribosomes of rough endoplasmic reticulum and transported through the smooth endoplasmic reticulum to the golgi apparatus. Tyrosinase is then released within membrane-bound vesicles, while the structural melanosomal proteins are also synthesized and then incorporated into vesicles at the smooth endoplasmic reticulum. These fused two vesicles, including tyrosinase and structural melanosomal proteins, result in the formation of a melanosome. As a melanosome composing of the deposited melanin matures, it will pass into the dendrite of melanocyte. Two types of melanin, which are eumelanin or brown/black melanin and pheomelanin or yellow/red melanin, are packed into melanosomes called eumelanosomes and pheomelanosomes.

C. Association of hair growth cycle and hair pigmentation[18, 20]

Since the hair follicles display a cyclical activity, the hair follicle melanocytes follow the similar process through hair cycle phases. However, the origin of melanocytes present in the hair bulb after hair regeneration or telogen phase is not fully understood. In bulbar region, the upper outer root sheath of hair follicle contains the inactive melanocytes. Both bulbar and outer root sheath melanocytes can be identified by the expression of premelanosomal protein pMel-17. In pigment hair follicles, the expression of tyrosinase and TRP-1 can be detected only in melanocytes located at the bulb in anagen phases III/IV to VI of hair growth cycle. During catagen

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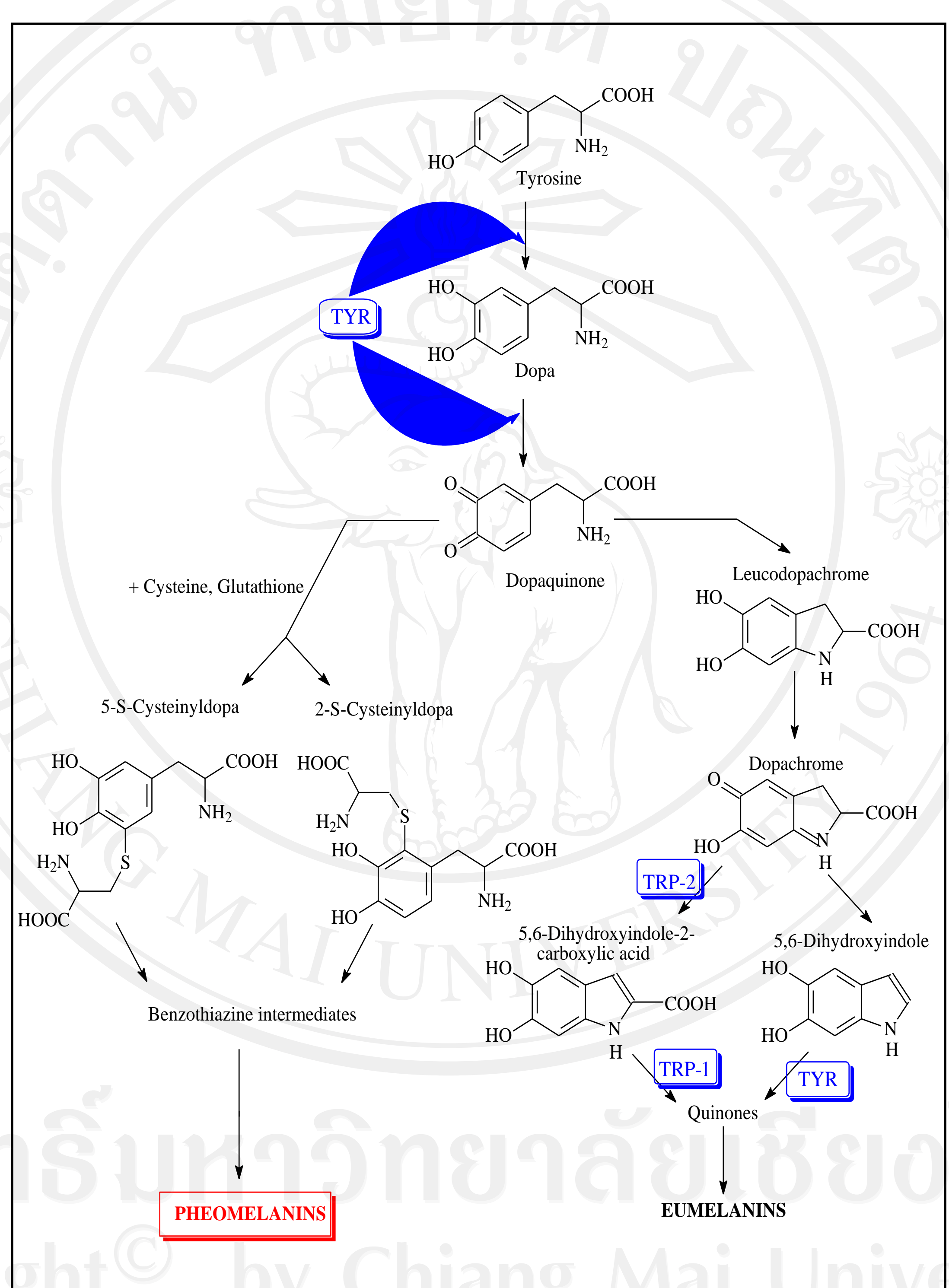

Figure 6 Melanogenesis process[21]

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phase, two melanogenic enzymes are undetectable although melanocytes are in the bulbar area. The epithelial column of catagen follicle and capsule of telogen follicle also contain the inactive melanocytes. At the induction stage of a new anagen hair follicle, some melanocytes located in the nascent bulb, which is close to the dermal papilla, are committed to cell division. Thus, melanogenic proteins are differentially expressed in bulbar melanocytes in relation to their activation state in anagen hair follicle. The bulbar melanocytes are positive for pMel-17, tyrosinase and TRP-1, while the outer root sheath melanocytes are pMel-17 positive but both tyrosinase and TRP-1 negative. In human, the melanogenesis is restricted to anagen hair follicle because of the expression of melanogenic enzymes. In addition, the cell division commitment and both tyrosinase and TRP-1 expression are observed only in melanocytes located in the nascent bulb in the new developing anagen hair follicles.

1.4.2 Canities[1-2]

Canities, a condition of having scalp gray/white hairs, is a physical appearance occurred after the decreased function of hair bulb melanocytes together with the defective transfer of melanin from melanocytes to cortical keratinocytes. The onset of canities is hereditary and usually develops in the age of 40. The average age for Caucasians is mid-30s, while for Orientals and Africans are late-30s and mid-40s, respectively. The completely loss of melanin is observed in white hairs, while the admixture of pigment and white hairs is found in gray hairs.

1.4.2.1 Causes and factors affecting hair depigmentation

A. Regulation of hair pigmentation[19-20]

A complex regulatory control of pigment biosynthesis involves hormones, neurotransmitters, cytokines, growth factors, eicosanoids, cyclic

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nucleotides, nutrients and the physicochemical environment. Positive regulators or melanogenesis stimulators act sequentially or in parallel through pathways involving activation of G-protein coupled receptors, receptors coupled to kinase activities or nuclear receptors. In addition, the nutritional factors tyrosine and dopa function as substrates for melanin and also stimulate the melanogenic apparatus in a proper genetic and environmental background. The signal transduction pathways, including stem cell factor and its receptor (c-Kit) and endothelins and their G-protein coupled receptors, play the important role in normal follicular melanogenesis. The pro-pigmentary activity is initiated by the binding of locally produced proopiomelanocortin (POMC) gene products, such as POMC-derived adrenocorticotropic hormone (ACTH), α-MSH and β-MSH peptides, and MC1 receptor, the receptor of α-MSH. The activation of adenyl cyclase produces cAMP that subsequently stimulates the proliferation, melanogenesis and dendritic formation of melanocytes. The accumulation of POMC products is predominantly observed in the outer root sheath of scalp follicular keratinocytes rather than the overlying epidermis. Also, mRNAs coding for POMC and MC1 receptor predominantly express during the hair growth period which is related to the activity of tyrosinase. The activity of the local POMC/MC1 receptor axis plays a role in the physiological regulation of anagen-associated hair pigmentation.

B. Causes of hair depigmentation[1-2,19-20,22-29]

Canities, the loss of melanin content in the hair shaft, has affected the individual hair follicle with either gradual loss of pigment over several cycles, gradual loss of pigment along the same hair shaft or the fully depigment hair shaft. The incidence of hair depigmentation occurs irrespective of sex, hair color and initial

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content of melanin. To date, the molecular and cellular origin of hair graying remains unclear. The origin involves the various factors, both endogenous and exogenous factors. Endogenous factors include the inherited diseases, autoimmune diseases and melanogenic enzyme malfunction, whereas exogenous factors include the environmental effects, medicine consuming and nutritional state. Environmental effects, such as pollution, ultraviolet (UV) irradiation and psychoemotional stress, consider as the accelerating agents or stressors. These agents generate the numerous free radicals. The free radical theory is associated with the aging process. The age-related loss of hair color has involved the melanogenesis clock in hair bulb melanocytes. Since the melanogenesis process involves the various steps in oxidation reactions, thereby producing the large amounts of reactive oxygen species. The very high levels of free radicals are needed to diminish with the efficient electron transfer, such as the antioxidant system. If there is not adequate free radical removal system, the accumulation of free radicals will cause the significant oxidative stress in the melanocyte and in the highly proliferative anagen hair bulb epithelium. In this circumstance, melanogenic bulbar melanocytes are perhaps assumed the post-mitotic, terminally differentiated (pre)senescence status to prevent cell transformation.

The age-related loss of melanin is also related to the morphology study of gray and white hairs. The gray hairs demonstrate the much reduced active melanocytes, but detectable dopa oxidation reaction, while white hairs do not contain those active pigment producing cell. The gray and white hairs also exhibit the decreased amounts of amelanotic melanocytes in comparing to the pigmented hairs. The reduced amelanotic melanocytes may be associated with the cell signaling impairment in anagen phase of hair growth cycle. Furthermore, the decreased cell

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signaling in the bulge region containing the numerous melanocyte stem cells may cause the immature melanocytes that eventually cannot function. Sarin et al. have reported that the white hairs in aged people are associated with the lack of melanocytes and melanocyte stem cells[28]. In addition, tyrosinase, a key melanogenic enzyme in melanogenesis process, also plays a crucial role in the melanogenesis process. The activity of tyrosinase in the pigment hair of aged people is less than in the pigment hair of middle aged people. Besides, the transfer of pigment granules is taken into account. The decreased transfer of melanin granules into cortical keratinocytes have been reported in the involvement of canities.

1.4.2.2 Treatment of hair depigmentation

Treatment of hair depigmentation with the approved medicine in nowadays is not indicated. Many studies have investigated both *in vitro* and *in vivo* models for the causes as well as the reversal of canities, including the compounds possessing the melanogenesis induction activity and the radiation activation of melanocyte reservoir.

A. Radiation[29-31]

The loss of hair pigmentation is not due to the total loss of all follicular menalnocytes. Melanocytes located in the outer root sheath of senile white hair follicles have the unclear function with the negative for both dopa and the melanocyte specific markers. These melanocytes are available for repigmentation/ repopulation of the epidermis if necessary. Their lack of contribution to hair pigmentation may indicate the defect in the inductive micro-environment necessary for bulbar pigment production. There is the evidence of induced scalp hair repigmentation after radiation therapy of cancer or scalp inflammatory events. This

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reversal of canities may result from radiation/cytokine-induced activation of outer root sheath melanocytes. This demonstrates the possibility that these melanocytes may be induced to migrate and differentiate to naturally repigment graying hair follicles.

B. Vitamin and mineral supplement[32-34]

Some studies have reported that the canities is related to the vitamin and mineral deficiency. The taking of the high doses of *p*-aminobenzoic acid, pantothenic acid or zinc shows the reversal of pigment to gray hair. The mechanism of *p*-aminobenzoic acid may be from the directly enhanced hair bulb melanocyte activity, while the latter two substances involve the function of melanogenic enzymes.

C. Topical treatment with minoxidil[35]

In the treatment of androgenetic alopecia with topical minoxidil, the darkening of hairs has been observed. The mode of action may be due to the prolonged anagen and follicular enlargement, thereby increasing the melanogenesis.

D. Melanogenesis induction compounds from natural and synthesis[5-7,36-45]

The various substances from both natural and synthesis sources have been reported on the melanogenesis stimulation with low toxicity. The hydroalcoholic extract from fresh human placenta[38-39], coumarin constituents from Umberiferae plant extracts[36], piperine and cubebin from *Piper nigrum*[37,40-41], palmitic acid methyl ester from lotus flower essential oils[7] are some compounds obtained from natural source with melanogenesis stimulation activity, whereas retinoic acid[46], saturated fatty acids with 16-18 carbons[5-6,42-45], tyrosine sulfate are substances obtained from synthesis reaction.

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1.4.3 Fatty acids and esters[16,47-48]

Fatty acids or carboxylic acids, the compounds containing of a linear carbon chain skeleton of variable length with a carboxyl (-COOH) group at one end, are essential for energetic, metabolic and structural activities. Esters are the carboxylic acid derivative in which the hydroxyl group (-OH) is replaced by an alkoxy group (-OR). Esters are commonly found both in nature and in the chemical industry. Animal fats and vegetable oils are the mixture of esters as the waxy materials. Plants often synthesize esters that give the characteristic tastes and odors to their fruits and flowers.

1.4.3.1 Classification of fatty acids and esters

A. Classification of fatty acids[47-48]

Fatty acids can be classified into groups on the basis of the presence of double or triple bonds called saturated and unsaturated. Saturated fatty acids, the single bonded carbon chain fatty acids, have the general formula R-COOH, in which R represents a straight-chain hydrocarbon of the form $CH_3(CH_2)_x$ or C_nH_{2n+1}. Most of saturated fatty acids occurred in nature have unbranched structures with an even number of carbon atoms. These saturated fatty acids range from short-chain-length volatile liquid to waxy solids having chain lengths of ten or more carbons. Fatty acids from 2 to 30 carbons (or longer) do occur, but the most common and important acids contain carbons in range of 12-22 and are found in many different plant and animal fats. The branched chain saturated fatty acids have also found in the natural. They are divided into the iso and anteiso acids. Iso acids contain the methyl group in the ω-1 position from carboxyl group, while anteiso acids have the methyl group in the ω-2 position. The most common type of branch is a single methyl group, but sometimes

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there are longer branches or more than one branching methyl. Iso and anteiso acids normally occur in small amounts in animal fats, waxes and marine oils.

For the unsaturated fatty acids, they may contain one or more double or triple bonds. They can be divided into monounsaturated, polyunsaturated and acetylenic fatty acids. The unsaturated fatty acid with a double bond has two possible configurations, either *cis* or *trans*, according to the relative positions of the alkyl groups (Fig. 7). Many naturally occurring unsaturated fatty acids have the *cis* orientation. More than 100 naturally occurring monounsaturated fatty acids have been identified, but most of them are very rare compounds. In general, these fatty acids have an even number of carbons and the double bond has the *cis* conformation.

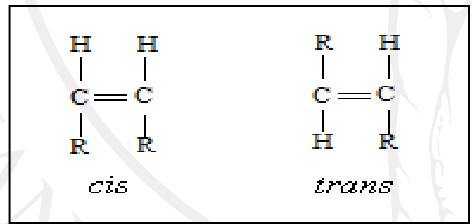

Figure 7 Configurations of unsaturated fatty acid[47]

Polyunsaturated fatty acids, which are sometimes referred to PUFAs, can be classified into a number of categories according to the relative positions of the double bonds. One of the most familiar and widespread unsaturated fatty acids is linoleic acid (18:2), which is also nutritionally essential. Natural linoleic acid is predominately the *cis-cis* isomer and presents in almost every vegetable fat, animal fats and fish oil. Another important unsaturated fatty acid is α-linolenic acid. This acid is a main fatty acid of plant leaves, stems and roots and is also a significant constituent of many photosynthetic organisms.

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B. Classification of esters[47-50]

Since esters are derived from the reaction of alcohols with acids, a diversity of esters with very different characteristics can be obtained and make them to be the biggest group of lipids. Three categories of esters, which are classified according to the acid part and the substituted group, are esters of aliphatic acids, esters of aromatic acids and esters containing nitrogen. With variation of the chain length and grade of branching on either side of the molecule, the physical properties of esters can be modulated and also obtained both solid and liquid ester products. Table 1 showed the examples of esters and their physical properties. These esters have widely been used in various applications, such as medicines and cosmetics.

Table 1 Examples of esters and their physical properties[50]

Alcohic part	Acidic part	Melting point (°C)	Spreading	Polarity (mM/m) interfacial tension against water
Butyl	Stearate	22	-	-
Cetearyl	Stearate	55	-	-
Myristyl	Myristate	41	-	-
Isocetyl	Stearate	-2	Medium	17
Ethylhexyl	Stearate	10	Good	32
Isopropyl	Palmitate	14	Good	29
Isopropyl	Stearate	19	Good	22
Diisopropyl	Adipate	-6	Very good	14
Lauryl	Lactate	-3	N.D.	N.D.
Myristyl	Lactate	13	N.D.	N.D.
Ethylhexyl	Hydroxystearate	15	Poor	N.D.
Pentaerythrityl	Tetraisostearate	< -10	Poor	10
Isodecyl	Neopentanoate	< -30	Medium-good	30
Butylene glycol	Caprylate/Caprate	-10	Good	21.5

N.D.:not determined

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1.4.3.2 Application of fatty acids and esters in medicines, pharmaceutics and cosmetics

Since the principal lipids in the body include the free fatty acids, the esters of glycerol and fatty acids (tri-, di- and mono-acylglycerol), the esters of glycerol that incorporate with fatty acid containing a phosphate group (glycerophospholipids), the esters that contain a long chain hydroxylic base esterified to a long-chain fatty acid and a phosphate group (sphingolipids), the lipids that contain a sugar group (glycolipids) and cholesterol and its esters, the application of fatty acids and esters are closely related in medicines, pharmaceutics and cosmetics.

A. Application of fatty acids [44,47-54]

Fatty acids are essential for normal growth and maintenance as well as have the various important functions in living cells, such as cell signaling, composition of cell membrane and epidermal lipids. Thus, fatty acids, both saturated and unsaturated, have been widely used in medicines as the main or combined therapy for various conditions, such as the malabsorption syndrome, dermal symptoms, migraine and borderline personality disorder. In pharmaceutics, fatty acids have also been employed as the excipients in the pharmaceutical production, such as tablets, capsules, injections and topically applied formulations. Aungst et al. have prepared the topical naloxone formulation using various vehicles and penetration enhancers[53]. Fatty acids and fatty alcohols were very effective promoters of naloxone flux. In both the acid and alcohol series, maximum flux was with C_{12} adjuvants and for C_{18} acids and alcohols unsaturated adjuvants were more effective than saturated ones. For cosmetics, fatty acids are generally used to form soaps with either alkaline earth metal salts, ammonium hydroxide or alkylolamines, for *in situ* acting as emulsifiers. Also,

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they have been incorporated in the cleansing formulations, color cosmetics, body creams and lotions.

B. Application of esters[49,54-61]

Esters are the most widespread of all naturally occurring compounds. Esters, the constituents in volatile oils, are responsible for the therapeutic and psychological effects of oils. Examples of esters used in medicinal application are methyl salicylate found in the oil of Wintergreen with local irritation, antirheumatic and antiseptic properties, linalyl acetate found in lavender essential oil with sedative effect and geranyl acetate found in various plant volatile oils with antimicrocial activity. In pharmaceutics, esters have been widely applied for improvement of solubility and stability, increasing of drug penetration, decreasing of adverse effects as well as extracting of active metabolites from plants. *L*-alkylazacycloalkan-*2*-one esters of indomethacin are prepared to obtain the stable derivative in aqueous media and improve the delivery through human skin[59]. Ethyl acetate is widely used solvent in various plant extractions, while tocopheryl phosphate, an esterified vitamin E, is a water soluble form of vitamin E with various cellular functions, such as oxidant-protected intracellular transport and cell signaling[60]. For cosmetics, esters have been generally employed in perfume industry and cosmetic formulations as fragrances, emollients, dye solvents and clouding agents. Paraben or *p*-hydroxybenzoate is a class of chemicals widely used as preservative in cosmetic and pharmaceutical industries. As known, various esters have been synthesized, thus their physicochemical properties are taken into account in cosmetic preparation. Gorcea et al. have evaluated four cosmetic emollient esters, including diisopropyl adipate, isodecyl neopentanoate, isocetyl stearate and

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octyldodecyl stearoyl stearate, in the *in vitro* and *in vivo* models for prediction of their sensorial benefits and reported the results as the guidance for selection of specific emollient ester to incorporate in cosmetic formulation[61].

1.4.3.3 Sources of fatty acids and esters

A. Natural sources[47-48,55]

Approximately 99% of the consumed fatty acids are as acyl esters, usually triacylglycerols and to a lesser extent, phospholipids and other complex lipids found in plant and animal tissues. Animal fats are the rich source of saturated medium-chain fatty acids and oleic acid (18:1). The short chain saturated fatty acids (4:0-10:0) are occurred in milk fats and in a few seed fats. Bovine milk contains butanoic acid as well as small amounts of 6:0, 8:0, 10:0 and 12:0 acids. Milks from sheep and goat also contain these acids, but decanoic acid presents in the larger amounts. Lauric acid (12:0) and myristic acid (14:0) are major components of seed fats of the Lauraceae and Myristiceae families. Palmitic acid (16:0) is the most prominent saturated fatty acid occurring in fish oil, in the milk and storage fat of many mammals as well as in vegetable fats. Stearic acid (18:0) is a minor component in most vegetable fats. The long chain saturated fatty acids (19:0 and greater) are major components in only a few uncommon seed oils.

For the unsaturated fatty acids, linoleic acid (18:2) is found in various plant seed oils, while arachinoic acid (20:4) is found in all animal tissues and animal-based food products. Brain and several other organ meats contain hydroxyl fatty acids in small amounts and long-chain saturated fatty acids up to 26 carbons in length can also be found in small amounts in most animal tissues. The conjugated linoleic acid (18:2) and γ-linolenic acid (18:3) are important fatty acids that are found

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in the small amounts in diets. Gamma linolenic acid is also found in primrose, borage and black currant seed oils. Alpha-linolenic acid (18:3) is found in flax and perilla seed oils, while the deep ocean fish and fish oil are the good source of eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6).

For esters, they are widely found in nature. The fruits and flowers with fragrant odors are composing of the pleasant-smelling esters. Also, esters are found in animal source. Cetyl palmitate is the main ingredient of spermaceti, a wax obtained from the sperm whale. Oleyl oleate is mainly ester in whale oil, while myricyl palmitate is found in beeswax. Examples of esters in plants are myricyl cerotate found in carnauba wax, ethyl cinnamate found in cinnamon oil, ethyl butyrate containing in pineapple oil and isoamyl acetate containing in banana oil.

B. Synthesis

Both fatty acids and esters can be synthesized from biotechnology and chemical reactions as shown in the following methods.

Biotechnology[47-48,62]: The lipid biotechnology is used for improvement of agronomic properties and alteration of fatty acid and lipid compositions (Fig. 8). The microorganisms used in the lipid biotechnology are the eukaryotic cells (yeasts, molds and algaes) and the prokaryotic organisms (bacteria) for production of triacylglycerols and more specific lipids, e.g., wax, esters, polyesters, poly-β-hydroxybutyrate.

Esterification reactions can also be produced by several microorganisms. Many reports demonstrate the production of wax esters by *Acinetobacter* sp. H01-N grown on a wide variety of *n*-alkanes of chain lengths ranging from 16-20 carbons, while a non-specific lipase B from *Candida antarctica* is

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commercially used in the production of various fatty acid esters (Fig. 9).

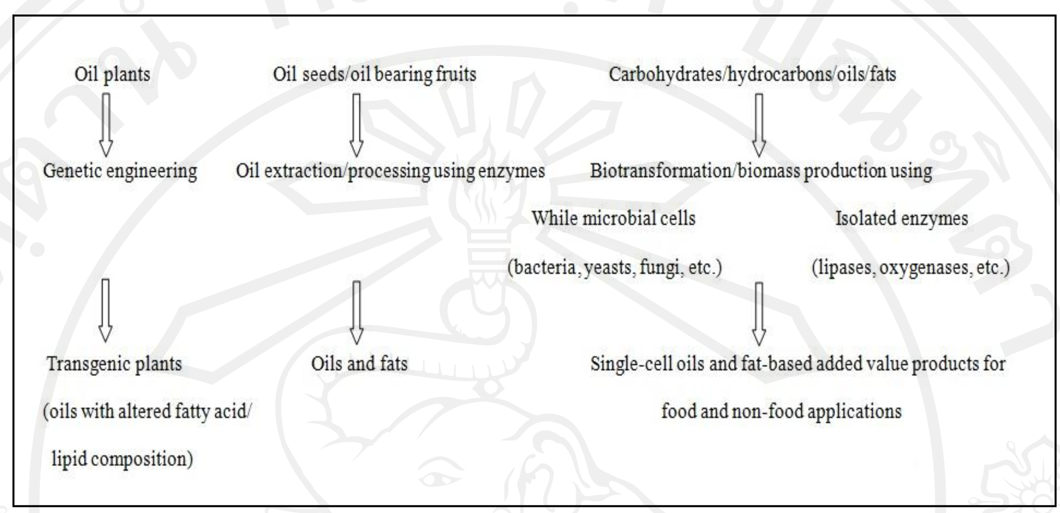

Figure 8 Major areas of lipid biotechnology[48]

Acids (8:0-14:0) + Isopropanol $\xrightarrow{\text{Lipase}}$ (8:0-14:0)-Isopropyl esters + Water

Bioester

Figure 9 Lipase-catalyzed synthesis of bioesters[47]

Chemical reactions[55-56]: The long chain aliphatic acids can be obtained from the hydrolysis of fats and oils. The fatty acids found in fats and oils are generally straight-chain acids with even numbers of carbons ranging between 6-18. The hydrolysis of animal fat gives mostly saturated fatty acids, while plant oils give large amounts of unsaturated fatty acids with one or more olefinic double bonds. The examples of acetic acid, an important commercial fatty acid used as vinegar, can be prepared by the fermentation of sugars and starches acetic as well as the chemical reaction of ethylene with a catalytic oxidation to form acetaldehyde, followed by another catalytic oxidation to acetic acid. Methanol can also serve for an industrial

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synthesis of acetic acid. The rhodium-catalyzed reaction of methanol with carbon monoxide requires the high pressure, which is not proper for a laboratory synthesis (Fig. 10).

(a) sugar and starches $\xrightarrow{\text{fermentation}}$ $CH_3{-}CH_2{-}OH$ (ethyl alcohol) $\xrightarrow[O_2]{\text{fermentation}}$ $CH_3{-}C({=}O){-}OH$ (acetic acid)

(b) $H_2C{=}CH_2$ (ethylene) $\xrightarrow[PdCl_2/CuCl_2 \text{ (catalyst)}]{O_2}$ $CH_3{-}C({=}O){-}H$ (acetaldehyde) $\xrightarrow[\text{cobalt acetate (catalyst)}]{O_2}$ $CH_3{-}C({=}O){-}O{-}H$ (acetic acid)

(c) CH_3OH (methanol) + CO $\xrightarrow[\text{heat, pressure}]{\text{Rh catalyst}}$ CH_3COOH (acetic acid)

Figure 10 Chemical synthesis of acetic acid by fermentation (a), catalysis of ethylene (b) and rhodium-catalyzed reaction of methanol (c)[56]

For esters, the nucleophilic acyl substitution of carboxylic acid under acidic catalyst called the Fischer esterification is widely employed (Fig. 11). In the acidic condition, the carbonyl group of acid is protonated and activates toward nucleophilic attack. It is then lost of proton and gives the hydrate of ester. Due to the Fischer esterification is an equilibrium reaction, thus, the addition of excess one reactant or removal one of the products may drive the reaction to yield the high ester product. Also, esters can be synthesized by other chemical reactions, such as the reaction of acid chloride with an alcohol, the reaction of an acid with an ether solution of diazomethane and the reaction of acid anhydride with alcohol.

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$$R-O-H + H-O-\overset{O}{\overset{\|}{C}}-R' \underset{}{\overset{H^+}{\rightleftharpoons}} R-O-\overset{O}{\overset{\|}{C}}-R' + H-O-H$$

alcohol acid ester

Figure 11 Synthesis of ester by the Fischer esterification[56]

1.4.3.4 The relationship between fatty acids, esters and hair melanogenesis[5-7,42-45,63-65]

Various studies have indicated the involvement of fatty acids and ester derivatives in melanogenesis. Saturated fatty acids, such as palmitic and stearic acids, have been reported on their melanogenesis induction, while unsaturated fatty acids, such as linoleic and linolenic acids, reduce the pigment formation. These fatty acids have demonstrated their melanogenesis regulation through the ubiquitin-proteasome pathway. Saturated fatty acid has exhibited the decreased ubiquitination of tyrosinase protein and decelerated the proteasomal enzyme degradation, resulting in the increased amount of tyrosinase protein. Meanwhile, unsaturated fatty acid has shown the increased ubiquitination of tyrosinase and accelerated the spontaneous tyrosinase proteolysis, thereby decreasing a key role enzyme in pigment biosynthesis process. Also, the proportion of saturated to unsaturated fatty acids, especially the markedly increased unsaturated fatty acids, has affected the process of tyrosinase to the golgi apparatus, resulting in the increased tyrosinase degradation after maturation in the golgi apparatus[5-6,42-45].

For the role of ester on melanogenesis, palmitic acid methyl ester, a major constituent containing in lotus flower essential oil, has been demonstrated on the melanogenesis induction by the increased expression of tyrosinase, MITF-M and

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TRP-2 proteins, but not tyrosinase mRNA. Also, this melanogenesis regulation includes the increased phosphorylation of cAMP responsive element binding protein (CREB)[7]. Methyl/ethyl linoleate and linolenate, the unsaturated fatty acid alkyl esters isolated from *Oxalis triangularis*, have shown the inhibition of melanogenesis and tyrosinase activity, while methyl/ethyl palmitate and stearate increase the melanogenesis in B16 mouse melanoma cells[63]. Saccharide fatty acid esters including nonioside P, nonioside Q, nonioside R, nonioside S and nonioside T obtained from the methanol extract of fruits of *Morinda citrifolia* (noni), demonstrate the melanogenesis inhibitory activity in B16 melanoma cells induced with α-melanocyte-stimulating hormone[64-65].

1.4.3.5 Problems of fatty acids and esters for the application in medicines, pharmaceutics and cosmetics[56,66-69]

Since the fatty acids and ester derivatives are used in various applications, including medicines, pharmaceutics and cosmetics, some complications of these compounds have been identified and classified into 2 types, in-process product preparation and the administration of products.

A. In-process product preparation[56,66]

As known, both fatty acids and ester derivatives are lipophilic substances with high partition coefficient values (log Ko/w). Most fatty acids and esters are miscible with oily materials and organic solvents, but not water soluble compounds. When they have been incorporated in the product preparations, including medicines, pharmaceutics and cosmetics, the good emulsifying system is needed for preparation of semisolid and liquid dosage forms to give the high compatibility and stability products. In addition, these acids and esters may react with other compounds

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and give their degradable products. In the pharmaceutical preparation of tablet and capsule dosage forms, the addition of fatty acids and salts of ester derivatives may affect the hardness and the release drug profile. In the preparation of microemulsion system as the novel transdermal drug delivery system, the microemulsion system containing sucrose esters, cetearyl octanoate, alcohols and water, with various different surfactant/cosurfactant at different surfactant/cosurfactant mass ratios and different hydrophilic-lipophilic balance (HLB) values, were investigated. The long and unsaturated oleic chain of surfactant improved the extent of microemulsion zone and the combination of laurates at HLB value of 7 increased the water and oil solubilizing capacity[66].

B. The administration of products[67-69]

The parenteral administration of compounds, including intravenous and intramuscular injection, may cause the phlebitis, sepsis, pneumothorax and infection[67]. Meanwhile the topical administration of product containing high contents of fatty acids and ester derivatives may cause greasy stain and the problem on percutaneous absorption. As known, high lipophilic compounds can penetrate through lipid components of skin more readily than water soluble compounds, the strong lipophilic substances will be hampered by the hydrophilic regions in the lipid bilayer[68]. Biesakski et al. has developed an inhalable vitamin A-ester and administered through a tracheostoma in rats for treatment of vitamin A deficiency[69]. The retinyl ester was appeared in blood and detected in various tissues after inhalation. Thus, the study has demonstrated that the inhalative application circumvents problems of oral administration and gives the sufficient supply to sensitive target tissues.

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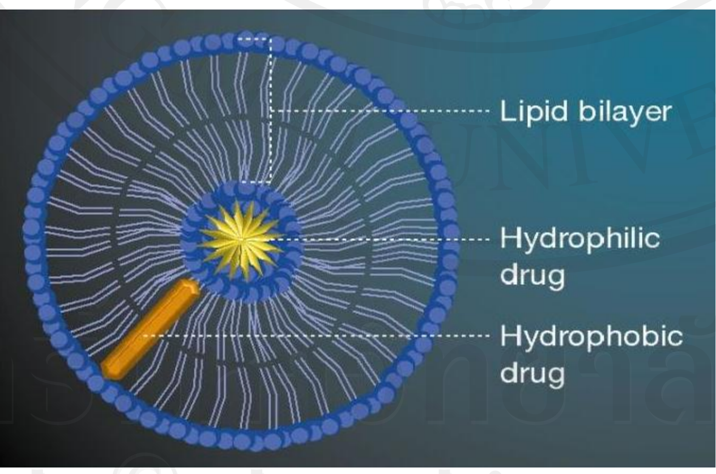
1.4.4 Niosomes[70-72]

Non-ionic surfactant based vesicles (niosomes) are formed from the self-assembly of non-ionic amphiphiles in aqueous media, resulting in the closed bilayer structures (Fig. 12). The assembly into closed bilayers usually involves some input of energy, such as physical agitation and heat. The result is an assembly in which the hydrophobic parts of the molecule are shielded from the aqueous solvent and the hydrophilic head groups prefer the maximum contact with same. These structures are similar to phospholipid vesicles or liposomes and are able to encapsulate both hydrophobic and hydrophilic molecules and function as drug carriers. The advantages of niosomes including low cost, great stability and ease of storage have led them to be used as the alternative for phospholipids. Niosomes are first reported as a feature of the cosmetic industry in the seventies, but they have been studied and employed as drug targeting agents since then.



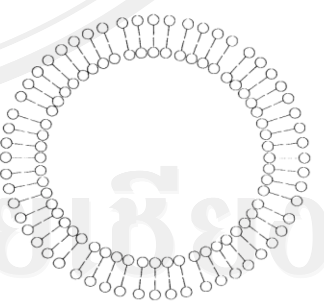


Figure 12 Schematic representation of a non-ionic surfactant vesicle and the loaded hydrophilic and hydrophobic drugs. O represents the hydrophilic head group and -- represents hydrophobic side chain[70,72]

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1.4.4.1 Compositions and characteristics

A. Niosomal compositions[70-71,73-75]

Niosome formation requires the presence of non-ionic surfactant and aqueous solvent. The vesicle forming non-ionic surfactants compose of the hydrophilic head group and the hydrophobic tail. The hydrophobic moiety, which generally has the length of alkyl group ranging from 12-18 carbons, may consist of one or two alkyl or perfluoroalkyl groups or a single steroidal group and a wide variety groups, such as glycerol, ethylene oxide, crown ether, polyhydroxy, sugar and sugar with amino acid, may present as the hydrophilic part. These two portions of molecule may be linked through ether, amide or ester bonds. Cholesterol, a natural steroid that is an important component of cell membrane, is incorporated in vesicle formulation to change the stability, fluidity and permeability of bilayers. Also, the charged molecules, such as dicetyl phosphate and stearylamine, often include in the bilayers to stabilize the system against the formation of aggregates by repulsive steric or electrostatic effects. The level of surfactant and lipid used to make niosomal dispersions is usually 10-30 mM. The alteration of surfactant and water ratio during the hydration step may affect the system's microstructure and properties. The increased level of surfactant and lipid increases the total amount of compound encapsulation.

Manosroi et al. have prepared the niosomes with hydrated mixture of various non-ionic surfactants, including polyoxyethylene-4-lauryl ether (Brij30), polyoxyethylene-2-stearyl ether (Brij72), polyoxyethylene sorbitan monostearate (Tween61), sorbitan monostearate (Span60), glyceryl monostearate, diglyceryl monolaurate and tetraglyceryl monolaurate, and cholesterol as well as studied the

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bilayer formation under polarization microscope and the entrapment efficiency of vesicles[73]. The entrapment efficiency of vesicles and microviscosities of the vesicular membrane depended on alkyl chain length of non-ionic surfactants and amount of cholesterol used to prepare vesicles. The stearyl chain (C_{18}) non-ionic surfactant vesicles showed higher entrapment efficiency than the lauryl chain (C_{12}) non-ionic surfactant vesicles. Cholesterol was used to complete the hydrophobic moiety of single alkyl chain non-ionic surfactants for vesicle formation. Cable et al. has incorporated either the anionic dicetyl phosphate or the cationic stearylamine in the bilayer to achieve greater protection against flocculation in vesicle dispersion[74]. Minoxidil, a poor water soluble drug used for treatment of androgenetic alopecia, has been formulated for topical application by loading in niosomes prepared from polyoxyethylene alkyl ethers (Brij) and sorbitan monoesters (Span) with cholesterol and dicetyl phosphate. The results showed that the higher entrapment efficiency was obtained with niosomes prepared from Span60 and cholesterol at 1:1 molar ratio using 25 mg minoxidil. Also, drug loaded in niosomes increased the percentage of minoxidil absorbed in the skin layers more than the commercial and control formulations[75].

B. Physical characteristics of niosomes[76-82]

The frequent physical characterization of niosomes, including morphological study, vesicular size, zeta potential value and entrapment efficiency, are investigated as following:

Morphological study: The structure of nanovesicular system can be visualized with the high-magnification power of the electron microscope. Transmission electron microscopy (TEM) is a powerful tool for the investigation of

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the microstructure of materials, providing crystallographic information and composition at the nanometer scale. TEM specimen preparation involves the thinning of the specimens to the electron transparent thickness. The morphology of nanovesicles is performed on small copper disc called grids casted with a fine mesh. After placing of a small drop of the sample on the hydrophilic grid, the staining process is needed to easily differentiate the sample from the background. However, aqueous samples do not survive the high vacuum of an electron microscope and water loss occurs leading to microstructure changes. Therefore, special techniques of sample preparation are necessary prior to electron microscopy. Freeze fracture has been established to be successful at overcoming these problems[76].

Vesicular size: The particle size is an important parameter in in-process control and particularly in quality assurance, because the physical stability of vesicle dispersions depends on particle size and particle size distribution. Dynamic light scattering (DLS) is a relatively fast method of vesicular size characterization in solution. DLS detects the fluctuations of the scattering intensity due to the Brownian movement of molecules in solution. DLS measurements are made on the fixed scattering angle of Zetasizer Nano system (Malvern Instruments Ltd., Malvern, UK). Samples are measured in cuvette and the resulting data are analyzed by the DLS software[77]. In fact, the small size of the niosomes can increase the physical stability of the formation according to the Strokes' law, indicating the velocity of a droplet is proportional to the square of its radius[78]. DLS software can analyze the size distribution in the term of polydispersity index (PDI). The maximum value of PDI is limited to 1.0. The PDI value of 1 indicates that the sample has a very broad size distribution and may contain large particles or aggregates.

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Zeta potential value: The zeta potential of the nanovesicular formulation depends on the movement of the vesicles (electrophoretic velocities). Zeta potential is measured by application of an electric field across the dispersion. The charge particles within the dispersion migrate toward the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential. Samples are measured in the disposable capillary cell which is the only cell with entirely disposable cuvette and electrodes. If the zeta potential is reduced below a certain value (in the range of $\pm$30 mV), the attractive forces between the vesicles, owing to the Van der Waal's force, overcome the forces of the repulsion and then, the particles come to form aggregation[79]. The suitable range for the stable nanovesicular formulation should be out of the range of $\pm$30 mV[80].

Entrapment efficiency: This characteristic is the amount of the drug loaded inside the nanovesicles and can be obtained from the total amount of loaded drug divided by the total initial input of drug. To calculation the entrapment efficiency of nanovesicular formulation, it needs to separate the free drug from the entrapped drug using various separation techniques, such as centrifugation, size exclusion chromatography and dialysis. The ultracentrifugation method is the most widely used technique for determination of the entrapment efficiency, owing to its fastest and simple method. The nanovesicular dispersion is centrifuged and then the clear supernatants are carefully removed to separate free drug. Size exclusion chromatography or gel filtration is a separation technique used to separate molecules on the basis of size and shape. The gel filtration matrices, such as Sephadex®, Sephacryl® and Sepharose®, are composing of pores with tunnels in which the size can be controlled depending on the size of molecules to be separated. Small

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molecules can penetrate the pores and enter the beads. However, larger molecules do not enter the beads and are excluded first from the column. Therefore, the large molecules of drug loaded in nanovesicles are eluted before the small molecules of free drug[81]. Currently, the microdialysis technique has also been used to determine the entrapment efficiency of nanovesicles. The free drug would diffuse into the probe because there is a concentration gradient of the free drug from the outside to the inside of the microdialysis fiber. The molecular weight cutoff of the microdialysis membrane, such as nanostructures, and the incorporated drug cannot cross the membrane[82]. After separation, the vesicular structure is ruptured by addition of surfactant or solvent before the analysis of drug content. The entrapment efficiency can be calculated as follows:

$$\%\ \text{Entrapment efficiency (EF)} = \frac{(\text{Amount of entrapped compound})}{(\text{Total amount of loading compound})} \times 100$$

1.4.4.2 Classification of niosomes[83-84]

Niosomes can be classified into 3 groups based on the size of vesicles, multilamellar vesicles (MLV), large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV).

A. Multilamellar vesicles

The size of vesicles is larger than 0.05 μm diameter. These vesicles are the most widely prepared due to the simple preparation and mechanically stable for long-time storage. These vesicles are appropriate used as drug carrier for lipophilic compounds.

B. Large unilamellar vesicles

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The size of these vesicles is larger than 0.10 μm diameter. This niosomal type contains the high aqueous and lipid compartment ratio, thus, the large volumes of compound can be encapsulated.

C. Small unilamellar vesicles

The size of these small vesicles is 0.025-0.05 μm diameter. These vesicles are mostly prepared from multilamellar vesicles by sonication, extrusion or homogenization methods. The vesicles are thermodynamic unstable and tend to aggregation and fusion. Also, the entrapment volume is small and the entrapment efficiency is quite low.

1.4.4.3 Niosome preparation methods[83-86]

Niosomes can be prepared by different methods based on the sizes of the vesicles, number of bilayers, entrapment efficiency of aqueous phase and permeability of vesicle membrane. Table 2 summarized the niosome preparation methods and type of the obtained vesicles. The different techniques have been used to prepare the different type of nanovesicles. The order of high to low entrapment efficiency is MLV, LUV and SUV, whereas the order of loaded compound permeation rate is SUV, MLV and LUV[85].

1.4.4.4 Application of niosome in medicines, pharmaceutics and cosmetics

Niosomes have been studied and demonstrated as the potential compound delivery system in various applications.

A. Application in medicines

Immunization: The topical immunization with the niosomes loaded with hepatitis B surface protein using as an antigen and cholera toxin B as an adjuvant

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Table 2 Niosome preparation methods[83-86]

Method	Principle	Type of nanovesicles
Hand-shaking method (Thin-film hydration method)	The thin lipid mixture deposited on the wall of round bottom flask is hydrated with the excess aqueous phase and gently shaken. MLV are formed spontaneously when an excess volume of aqueous phase is added to the dry lipid film.	MLV
Supercritical carbondioxide fluid technique ($scCO_2$)	Supercritical carbon dioxide has been used for the substitution of organic solvent to prepare bilayer vesicular formulations with the advantages of not only being environmental friendly, non-toxic and nonflammable.	MLV
Ether injection method	The ether solution of surfactant and lipids is slowly injected into the aqueous phase using a gauze needle at 60°C. The formation of LUV is from the slow vaporization of solvent, resulting in the ether gradient extending towards the interfaces of aqueous-non-aqueous interface.	LUV
Solubilization and detergent removal method	The detergent (surfactant) is used to solubilize the lipid mixture. The detergent is then removed by dialysis or column chromatography.	LUV
Reverse phase evaporation technique	The vesicles can be prepared by forming water in oil emulsion. An aqueous phase is added into the lipid mixture in the excess organic solvent. The solvent is removed under reduced pressure and the two phases are emulsified by sonication. The removal of organic solvent causes the phospholipid or surfactant coated water droplets to cool and eventually form a viscous gel. The next step is to bring about the collapse of certain proportion of water droplets.	LUV
Dehydration/rehydration of SUV	The sonicated vesicles are mixed in an aqueous solutions with the solute desired to be loaded and the mixture is dried under a stream of nitrogen. As the sample is dehydrated, the small vesicles fuse to form a multilamellar film that effectively sandwich's the solute molecules between successive layers. Upon rehydration, large vesicles are produced loading a significant proportion of the solute. The optimal mass ratio of lipid to solute is approximately 1:2 to 1:3. The method has the potential application to large scale production, since it depends only on controlled drying and rehydration processes and does not require extensive use of organic solvents, detergents or dialysis system.	LUV
Sonication method	The MLV and LUV are sonicated either with a bath type sonicator or a probe sonicator under an inert atmosphere to get the SUV. During sonication, large vesicles are broken down and SUV with high radius of curvatures are formed.	SUV
Microfluidization method	It is based on submerged jet principle in which two fluidized streams interact at ultra-high velocities within the interaction chamber. Here, the energy supplied to the system remains within the area of vesicle formation, resulting in the greater uniformity, smaller size and better reproducibility method.	SUV
French press method	The size reduction of nanovesicles is performed by passage through a small orifice under high pressure. MLV dispersion is placed in the French press and extruded at about 20,000 psi at 4°C. On passing through the French pressure cell, the heterogenous populations of vesicles are formed ranging from several micrometers in diameter to SUV size. Multiple extrusions results in a progressive decrease in the mean particle diameter (30-80 nm) depending on the pressure used. The obtained vesicles are more stable than sonicated ones and can be used advantageously as drug delivery carriers.	SUV

Note MLV: multilamellar vesicles, LUV: large unilamellar vesicles and SUV: small unilamellar vesicles

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have been demonstrated as the topical delivery of vaccine[87]. The mannosylated niosomes have also exhibited as both topical and oral vaccine delivery carrier and adjuvant for the induction of humoral and cellular immunity[88-89].

Gene delivery: Antisense oligonucleotides were effectively delivered using cationic niosomes of sorbitan monoesters that showed positive cellular of the antisense oligonucleotides from the prepared niosomes[90]. Polysorbate cationic niosomes exhibited the binding capacity and the gene transfer study showed high efficiency in mediating cellular uptake and transferred gene expression[91]. Elastic cationic niosomes were used to enhance transdermal absorption of luciferase plasmid (pLuc) by application of iontophoresis or stratum corneum stripping method. The prepared nanovesicles showed high degree of flux, thus, presenting niosomes as suitable carriers for luciferase plasmid transdermal delivery[92].

Diagnosis: Niosomes are considered as carriers of iobitridol, a diagnostic agent used for X-ray imaging. The vesicles composing of D-α-tocopheryl polyethylene glycol 1000 succinate, polyoxyethylene glycol 4000 stearate, sorbitan monostearate, cholesterol and dicetyl phosphate were prepared by the film-hydration and sonication method. The rate of encapsulation and the stability of vesicles were found to be satisfactory[93].

B. Application in pharmaceutics

Antineoplastic agents: The problems of chemotherapy are side effects and lesser therapeutic efficiency. Various strategies have been developed to overcome these problems including application of niosomes as a novel drug delivery system. Paclitaxel loaded in negatively charged niosomes have shown the slow release profile which is beneficial in storage, drug administration, the decreased toxic

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side effects and efficient oral delivery[94]. 5-fluorouracil indicated for treatment of actinic keratosis and non melanoma skin cancer has exhibited the enhanced percutaneous absorption after topical application of drug loaded in niosomes[95]. Doxorubicin loaded niosomes demonstrated the prolonged release with double tumoricidal activity, 10 times decreased clearance and increased levels of metabolites in liver. Preparation of doxorubicin loaded niosomes has also shown the slow release profile with peak plasma concentration same as the free drug without pulmonary side effects, reduced IC_{50} in doxorubicin resistant cells and increased activity in ovarian cancer cells[96-97].

Anti-inflammatory agents: Diclofenac loaded niosomes prepared from Tween61, cholesterol and ethanol have been developed and incorporated in the gel formulation. The gel containing diclofenac loaded niosomes exhibited the skin permeation in the stratum corneum, deeper skin layer and the receiver chamber in the vertical Franz diffusion cell as well as inhibition of the ethyl phenylpropiolate-induced rat ear edema higher than the commercial gel[98]. Flurbiprofen, an another non-steroidal anti-inflammatory agent, loaded niosomes have shown the increased bioavailability and the reduced carrageenan induced-rat paw edema after oral administration or topical application of drug loaded niosomes in a hydroxypropyl methyl cellulose semisolid base[99]. Indomethacin encapsulated niosomes composed of Tween60, cholesterol and stearylamine have been developed and evaluated the sustained anti-platelet aggregation effect. The niosomes loaded with indomethacin exhibited the more efficient effect in inhibition of platelet aggregation than the free drug, probably due to greater quantity of the drug reaching the specific site of inhibition in the interior of the platelets and acting directly on the cyclooxygenase

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system to prevent thromboxane formation[100].

Anti-infective agents: One of the earliest diseases for which niosomal formulations proved particularly beneficial is to treat parasitic infection. The intraveneous administration of sodium stibogluconate loaded niosomes has shown higher level of antimony when compared with administration of drug in solution. In the study, drug loaded niosomes also significantly exhibited more active in the reduction of parasite burden from liver than free drug[101]. Niosomes loaded with isoniazid, anti-tuberculosis drug, prepared by reverse phase evaporation method has demonstrated a potential to remain at the treated site for the long periods and maintain the drug concentration for 30 h. The cellular uptake of drug loaded niosomes was 61.80% by macrophage cells, indicating the effective treatment of tuberculosis. In addition, the drug loaded niosomes demonstrated the benefits in dose reduction, toxicity reduction, frequency of drug administration reduction and increased patient compliance[102]. Niosomes loaded with rifampicin consisting of Span85 and cholesterol have been prepared and exhibited the drug delivery to lung, the target site, with the reduced toxicity and effective drug uptake. The niosomal formulation showed the site specific targeting by controlling of niosome size[103]. Griseofulvin, a low oral bioavailability anti-fungal drug, has been loaded in niosomes in order to improve poor and variable oral bioavailability. The drug loaded niosomes composing of Span 60, cholesterol and dicetyl phosphate indicated the high entrapment efficiency as well as the significant retarded *in vitro* drug release as compared with free drug. *In vivo* study of drug loaded niosomes, the significantly improved oral bioavailability with decreased fluctuation of plasma drug was found after a single oral dose. The peak plasma concentration of drug loaded in niosomes was approximately two times

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of free drug[104]. Preparation of cefuroxime, a low oral bioavailability cephalosporin antibiotic, in niosomal formulation has been exhibited high entrapment efficiency as well as the good stability in bile salts and release of drug profile *in vitro* study[105].

Vitamin A derivative: Tretinoin, an effective treatment of different skin diseases with several systemic side effects, has been prepared in niosomes for topical application in order to decrease the systemic toxicity as well as increase the thermodynamic stability of drug. The negatively charged niosomes loaded with tretinoin were prepared to obtain MLV, LUV and SUV. *In vitro* drug release using Franz diffusion cells, the rate of drug release from drug loaded niosomes was faster than from a drug solution and the tretinoin delivery increased from MLV to LUV to SUV[106]. Also, the drug loaded niosomes retarded the drug photodegradation and gave higher cutaneous drug retention than commercial formulation[107-108].

C. Application in cosmetics

Niosomes of N-acetyl glucosamine were prepared to enhance percutaneous penetration in order to treat hyperpigmentation disorders. Drug loaded niosomes significantly improved the extent of drug to be localized in the skin in compared to free drug solution[109]. Gallic acid loaded in both elastic and non-elastic niosomes have been developed and exhibited that compound loaded elastic niosomes demonstrate the increased permeation through the skin that is beneficial for topical antiaging application[110]. Minoxidil, the approved topical treatment of androgenetic alopecia, has been loaded in niosomes and evaluated for their skin permeation in compared to commercial formulation. Minoxidil loaded niosomes enhanced drug accumulation in the skin as compared to commercial, indicating a promising approach for topical delivery of hair loss treatment[75]. Finasteride loaded niosomes have been

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formulated for topical treatment of androgenetic alopecia. *In vitro* permeation and *in vivo* deposition studies of finasteride loaded niosomes, the potentials of niosomal dispersion loaded with drug for successful delivery were indicated through the drug delivery to pilosebaceous units containing androgen receptors at sebaceous glands and dermal papilla[111].

1.4.5 Transfollicular delivery[9-10,112-114]

Percutaneous absorption, a complex biological process, is the passage of topically applied compound into and through the skin. Stratum corneum, the outermost layer of skin, has been regarded as the major barrier for compound penetration as well as a main pathway for penetrants. This skin layer is consisted of a heterogeneous two compartment system of protein-enriched cells embedded in lipid domains. The lipid regions demonstrate as the possible pathways for hydrophobic substances, whereas the corneocytes may show a pathway for hydrophilic compounds. Although the proposed transport pathways in stratum corneum have been well defined, the existence of other pathway still remains and is increasing its role in percutaneous transport. Transfollicular penetration is the passage of compound through pilosebaceous unit composing of hair follicle, hair shaft, sebaceous gland and arrector pili muscle. Hair follicles and sebaceous glands are occupied only about 0.1% of total human skin surface area. However, hair follicle is an invagination of the epidermis, which may allow for a much greater potential area for absorption. Recently, hair follicle is gaining recognition as a complex and dynamic structure which may contribute to passive transport of compounds into the skin.

1.4.5.1 Target sites of transfollicular delivery[10,112-113]

Figure 13 demonstrated the specific target sites of follicular delivery.

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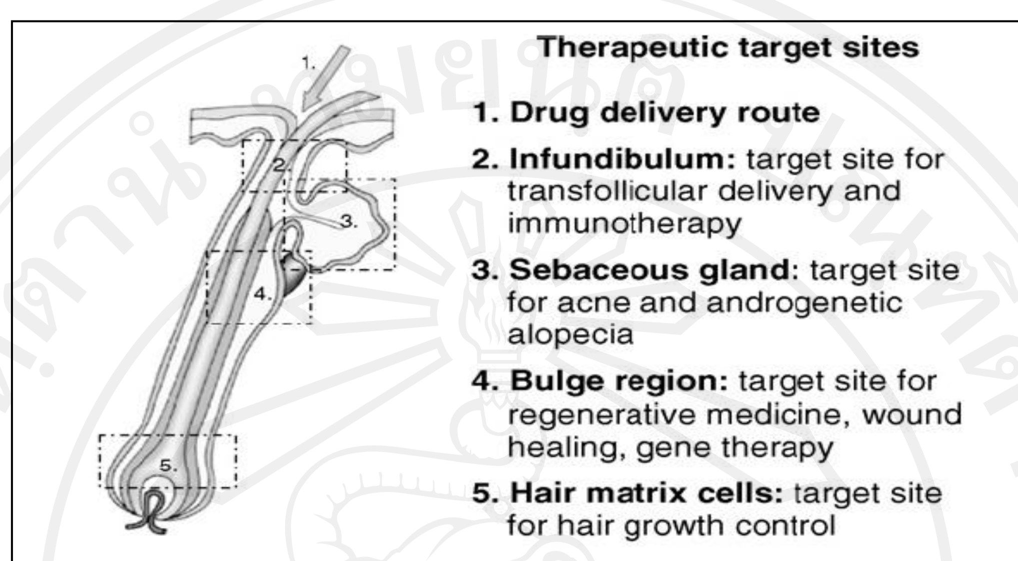

Figure 13 Target sites of transfollicular delivery[113]

Follicular targeting offers opportunities to treat hair disorders and hair follicle-associated diseases as well as gene therapy and immunotherapy.

A. Follicular infundibulum[9,114-116]

The infundibulum of follicle especially the lower part, which is an interrupted barrier, offers the increased permeability. At the disruption of the skin barrier, the antigen presenting cells, mast cells and other immune cells are found in the high density at the upper part of hair follicle, thus, this area can potentially be used as a target for topical vaccination. Also, an extensive capillary network associated with upper dermal vasculature has supplied the upper part of follicle. This region of follicle is appropriate used as the rapid transport shunts that permit topically applied substances to pass the continuous stratum corneum and readily reach either the viable skin layers or the systemic circulation[9].

B. Sebaceous gland[10,113,117-119]

Sebaceous gland, which is connected with the hair follicle through

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the follicular duct in the lower infundibulum, is an important formation site of active androgens. Androgens exert their influence on hair growth and sebum secretion by interacting with receptors at several locations, including the follicular papilla and sebaceous glands. Due to the association with etiology of acne, androgenetic alopecia and other sebaceous gland dysfunctions, this gland represents an obvious target site. For the treatment of these pathologies, it may be important to increase the distribution of certain drugs in the hair follicles. RU 58841-myristate, a new anti-androgen prodrug, has been synthesized and converted to RU 58841, a potent anti-androgen metabolite, for topical treatment of acne and androgenetic alopecia. The compound was loaded on solid lipid nanoparticles in order to enhance the dermal targeting effect. The permeation study using Franz diffusion cells showed only negligible permeation of epidermis and excised porcine skin within 6 h, indicating the more topical action of drug to hair follicle[118-119].

C. Bulge region[113,120-121]

The bulge region, the closest part of outer root sheath, provides the insertion point for the arrector pili muscle, marks the bottom of the permanent portion of hair follicle and is the host of epithelial stem cells. These stem cells play the important role in the reconstitution of hair follicles during hair regeneration[120]. In addition, the bulge cells involve the wound repair in which they do not contribute to normal epidermis homeostasis in non-wounded skin[122-123]. Being highly prolific and multipotent, the bulge cells offer opportunities as a stem cell source for cutaneous regenerative medicine. Promising goals may be the treatment of alopecia with new hair follicles bioengineered from the patient’s bulge stem cells or gene delivery to specific bulge stem cells to facilitate long-term gene correction of congenital hair

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diseases or genetic skin disorders, such as epidermolysis bullosa, which is associated with extensive wounding[121,124]. Recently, melanocyte stem cells have also found at the bulge region from where they migrate to the bulbar area to generate pigment hair shafts. The exhaustion or incomplete maintenance of melanocyte stem cell supply results in a loss of hair shaft pigmentation[120].

D. Hair matrix cells[9-10,113]

The follicular papilla and hair matrix cells located at the base of hair follicle are the regions of key cellular interactions that are important in the regulations of hair growth and undergo continuous remodeling during hair cycle.

1.4.5.2 Factors affecting transfollicular delivery[9-10]

Although the pilosebaceous unit may be desirable as therapeutic target sites and shunts for drug delivery, the access to these structures can be problematic due to architectural and physicochemical constraints. Table 3 showed the potential barriers and the corresponding resolving strategies.

Table 3 The potential barriers and resolving strategies associated with transfollicular delivery[9]

Barrier	Resolving strategy
Hair cycle	Apply penetrant during anagen
Sebum	1. Use a lipophilic pernetrant
	2. Use a sebum-miscible vehicle
Size selectively	Optimise microparticle diameter

A. Hair cycle[9-10]

Various studies have emphasized that the penetration properties vary not only with hair follicle morphology, but also with the functional status of hair

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follicle. Each hair follicle undergoes continuous cycling, which includes the complete remodeling of its non-permanent portion, extending from the lower end of the bulge region down to the bulb. As hair growth cycle is composed of 3 phases, including anagen, catagen and telogen, the duration of each phase depends on the type and localization of hair follicle. Under physiological conditions, 85% of scalp hair is in anagen phase and approximately 15% is in telogen phase. During each cycle, the hair follicle undergoes substantial changes in the immune and gene expression status as well as in its vascular supply[125]. In addition, the active and inactive follicles have affected the penetration of topically applied substances into the infundibulum[126]. The active follicles demonstrate the sebum flow and/or hair growth, whereas inactive follicles show neither. Also, the orifices of inactive follicles have been covered with a mixture of dry sebum, desquamated corneocytes and other cell debris. Thus, only the active hair follicles are open for compound penetration, owing to the removal of hair follicle covering by hair growth and/or sebum production[127-128].

B. **Sebum**[9-10]

Sebaceous gland is connected to upper part of hair follicle via the inserted duct and releases the sebum to follicular canal. The secreted sebum that creates the enriched lipid environment in the canal has affected the follicular drug penetration. Meidan et al. have applied mild heating to 42°C or low intensity ultrasound to skin of Wistar rats as well as guinea pigs and observed the sebum from the sebaceous gland[129]. The discharged sebum filled the hair follicle shafts has blocked the penetrating hydrophilic compound through follicular route. However, the significant variations in sebum chemistry between species should be considered before extrapolating data from animal to human studies. The synthesis of artificial

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human sebum composing of oils, wax esters, triglycerides and fatty acids, has been prepared and evaluated by differential scanning calorimetry[130-131]. Also, the *in vitro* sebum fluxes of three model compounds, including ethyl 4-hydroxybenzoate, butyl 4-hydroxybenzoate and hexyl 4-hydroxybenzoate, were measured and demonstrated that the fluxes of those model compounds were closer to that of human sebum. The results of this study have exhibited that the artificial sebum can be used as an alternative to human sebum for study of penetrating compounds through follicular delivery[131]. With the secreted sebum in general, the formulation with a suitable wetting agent has also been evaluated and demonstrated that the vehicle gives the good contact with the sebum across the vent of duct[132].

C. **Size selectivity**[9-10,112-113]

The size selectivity of follicular openings represents the potential barrier for compound penetration through hair follicle. The previous studies have been reported that the particles less than 1 μm can be found in upper layers of stratum corneum and in follicular orifices, whereas particles of 3–10 μm can be observed only in follicular orifices. Larger particles than 10 μm completely remain on the skin surface. Currently, transfollicular penetration has been widely studied and used various visualization techniques, including magnetic resonance imaging, video microscopy, ultrasound backscatter microscopy, confocal laser scanning microscopy and confocal Raman spectroscopy, to detect the penetrating compound in or through the skin and hair follicle. Among those techniques, the confocal laser scanning microscopy has been widely used as a well-established, non-invasive method for deriving high resolution images from skin as well as other biological tissues. Principal advantages of this technology include its capacity for *in vivo* application,

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good time-resolution and the ability to visualize at multiple depths parallel to the sample surface without the need for mechanical sectioning[133].

Two types of human hairs, terminal and vellus hairs, have been studied as the influenced factors for follicular penetration. Terminal hairs are macroscopically long (> 2 cm), thick (>30 μm), pigment and mostly contain a medullary cavity, whereas the non-pigment vellus hairs are thinner than 30 µm, silky and do not grow longer than 2 cm[134-135]. On the scalp, hair follicles are arranged in follicular unit, which contains pilosebaceous unit as well as 1–4 terminal hairs, 1–2 vellus hairs and encircled by branches from the same arrector pili muscle[9]. Patzelt et al. have determined the penetration depths of two different types of particles sized 122 to 1000 nm *in vitro* on porcine skin and found that the particles of medium size (643 and 646 nm, respectively) penetrate deeper into the porcine hair follicles than smaller or larger particles[136]. Also, the varying size of penetrants can selectively deliver to the desire target, owing to the different target sites found in specific depth of each hair type as shown in Fig. 14.

1.4.5.3 Transfollicular delivery system

A. Penetration enhancement formulations[9-10,112-113,137]

The active substances and vehicles have the influences on follicular penetration effectiveness. Concerning on vehicles, many investigations have reported that ethanol, dimethylsulfoxide, propylene glycol as well as lipophilic rather than hydrophilic vehicles are able to improve follicular penetration[138-139]. In recent years, micro-/nanoparticles and liposomes/niosomes have been attracted attention as a result of their competency to improve follicular penetration. Liposomes and niosomes are the closed vesicular structures comprising a bilayer membrane as a hydrophobic

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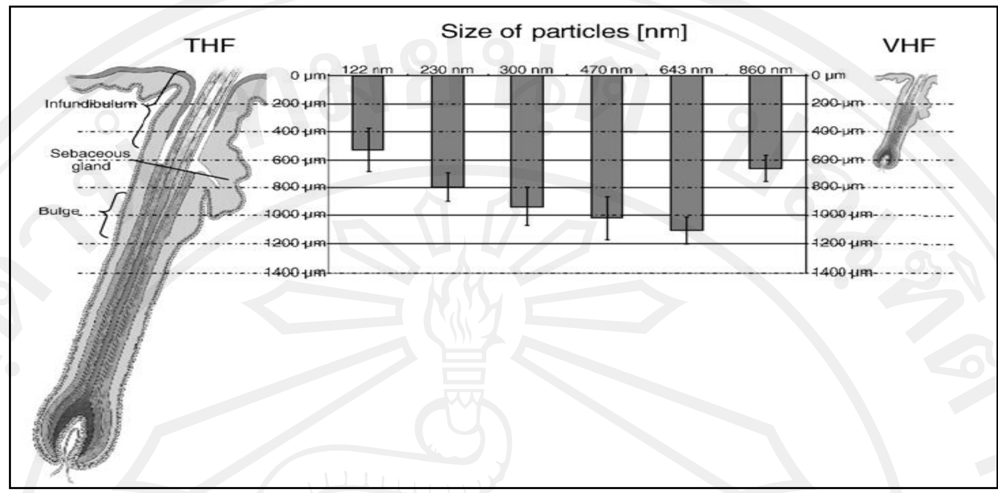

Figure 14 Penetration depths in micrometer of different sizes of particles in relation to the target sites within terminal hair follicle (THF) and vellus hair follicle (VHF)[136]

compartment and an aqueous core. As liposomes and niosomes have hydrophilic and lipophilic parts, hydrophilic substances can be enveloped in their inner part and lipophilic substances can also insert in the membrane. Liposomal and niosomal formulations have been found to enhance the penetration of several agents into the skin and to be useful systems for follicular drug targeting. Nanoparticles have also been proposed as topical systems for transfollicular delivery, owing to the good stability and the controlled release.

Liposomes entrapped with DNA have been demonstrated the successful delivery into hair follicles in animal models and human skin[140-141]. Also, melanins, proteins and small molecules entrapped liposomes have been selectively targeted to the hair follicle and hair shafts, while free molecules are trapped in the stratum corneum as well as cannot enter the follicle in the *in vivo* models[140,142]. Finasteride, an oral 5α-reductase inhibitor drug indicated for treatment of

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androgenetic alopecia, loaded in liposomes and niosomes for topical application have been exhibited the delivery of finasteride to pilosebaceous unit[111].

For microspheres, various active compounds have been loaded and exhibited the follicular delivery. Adapalene loaded microspheres with 5-μm diameter have been specifically targeted to the follicular ducts and demonstrated the controlled release of drug into hair follicles both *in vitro* and *in vivo* on hairless rats and human skin[12]. Similarly, methylene blue and rhodamine-6G loaded in 5-μm microspheres have been demonstrated the penetration into follicular duct and sebaceous gland without permeation within stratum corneum[13,143].

B. Penetration enhancement instrumentations[9,144-146]

Iontophoresis, one of methods used for enhancement of transdermal drug delivery, is an application of electric potential gradient to the skin surface, resulting in the increased flux of ionic compounds. Three enhanced drug transport mechanisms are as follows: (1) the driving electrode repels oppositely charged species, (2) the electric current increases skin permeability and (3) electro-osmosis moves uncharged and large polar molecules[147]. Although the exact routes for ion transport have not yet been fully understood, hair follicles are implicated as possible ion channels that may ultimately be useful in localized or systemic drug delivery into the skin[146, 148]. During electrophoresis of excised human skin, the charge flow across pores localized by fluorescein has been detected[145]. Also, the identification of hair follicles as ionic channels has been reported during iontophoresis across excised hairless mouse skin. The high ionic flow has been detected at sebaceous glands and hair follicles, especially those with small hairs. These observed data have demonstrated that iontophoresis may be particularly important for delivery of ionic,

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hydrophilic and high molecular weight compounds, which have been problematic in conventional topical and transdermal delivery strategies[149-150]. In additionally, follicular penetration can be enhanced by cyanoacrylate skin surface biopsy that is used to remove the superficial part of the stratum corneum as well as open the follicular orifices. After this pre-treatment, 40 nm particles have been observed the penetration through hair follicle epithelium *in vitro*[151].

1.4.5.4 Transfollicular delivery evaluation methods

A. Skin sandwich system[10,113]

Skin sandwich system is an *in vitro* technique used for quantification of follicular transport. The role of follicular transport or shunts in percutaneous absorption is determined by comparison of drug flux across hydrated epidermal membrane to that through a hydrated "sandwich" of epidermal membrane plus adhering extra stratum corneum on top[152]. Figure 15 showed the skin sandwich system that the additional stratum corneum was placed on top of skin for follicular blockage.

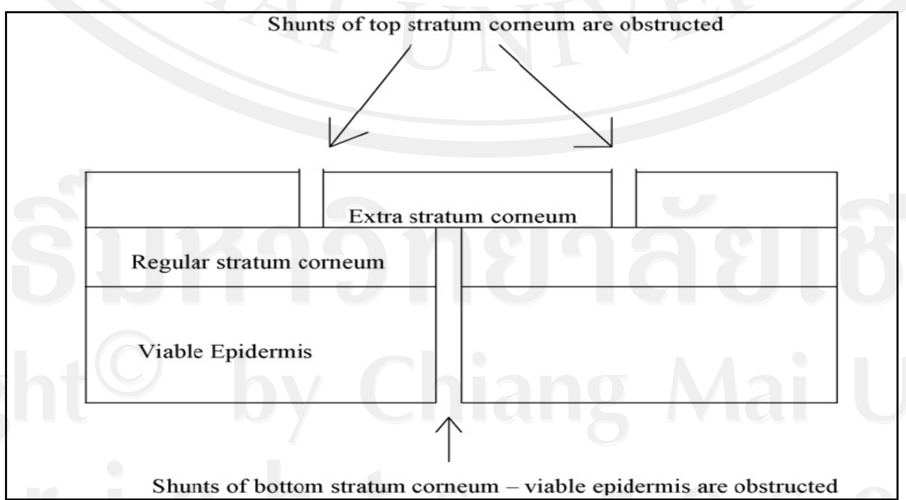

Figure 15 Skin sandwich system[153]

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The flux through single membrane and skin sandwich system is calculated and compared. If the shunts play no part in the permeation process, steady state flux through the sandwich is then half that of the single membrane. If shunts are responsible for all drug transport, flux through the 'sandwich' is then zero. Hence, the decreased magnitude of flux allows the quantifiable identification of shunt contribution to total absorption. The shunts are believed to correspond to the hair follicles since the much smaller sweat duct openings close over when the tissue is fully hydrated[149,152]. Figure 16 demonstrated the scheme of transfollicular evaluation through epidermal membrane and skin sandwich system. This sandwich system has been used to evaluate the penetration of estradiol loaded liposomes and the flux ratio of compound is close to 0.5. This flux value has demonstrated the very minor contribution of estradiol delivery through follicular route[149]. Also, the skin permeation of mannnitol, a hydrophilic compound, and estradiol, a lipophilic drug, has been investigated using the skin sandwich model and found that mannitol transport is entirely mediated by follicles, whereas estradiol penetration is almost entirely non-follicular in nature[154].

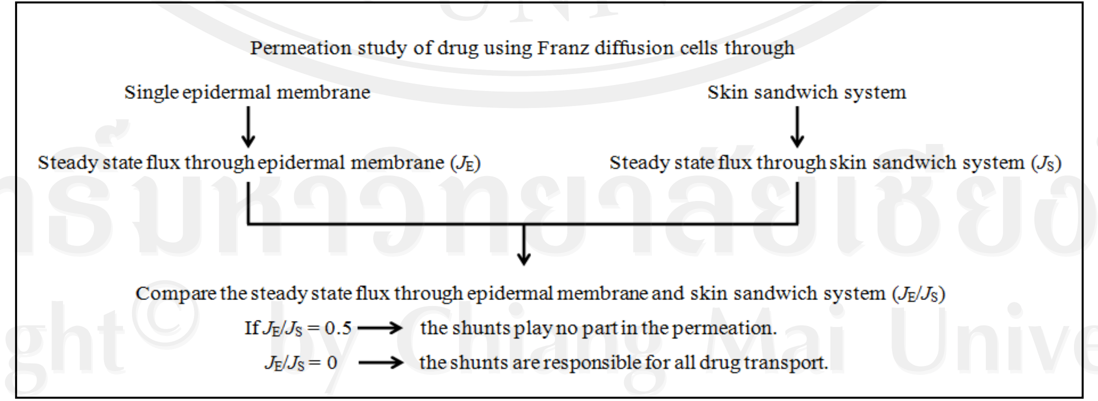

Figure 16 Scheme of transfollicular evaluation through epidermal membrane and skin sandwich system[152]

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B. Selective follicular blocking[9-10]

This developed method allows the selective measurement of the follicular rate on the penetration process in order to clarify the role of follicles. The method is applicable both *in vitro* and *in vivo* conditions. The nail varnish or wax mixture is selectively applied into every single follicular orifice on human or animal skin as shown in Fig. 17[155]. The follicular block after application of nail varnish or wax mixture represents a selective method to close the hair follicles for the penetration study of topically applied substances that the tape stripping or other mechanical interferences are avoided[156]. Figure 18 showed the scheme of transfollicular evaluation using selective follicular blocking method. Caffeine, a naturally occurring purine based alkaloid, has been used as a hydrophilic model drug for investigation of this follicular blocking method and exhibited that the caffeine penetration after topical application is permeated through hair follicles faster and higher than that via interfollicular route[155,157].

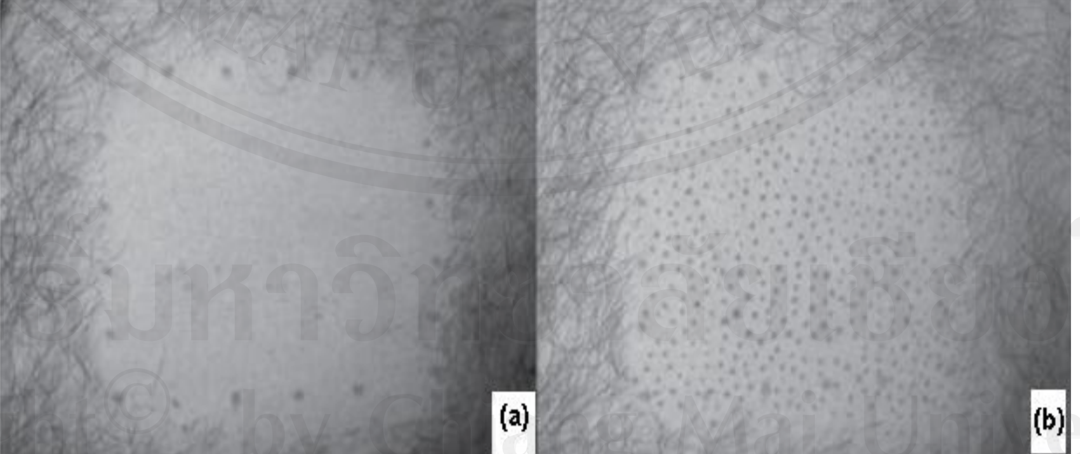

Figure 17 Selective follicular blocking before (a) and after (b) nail varnish application[155]

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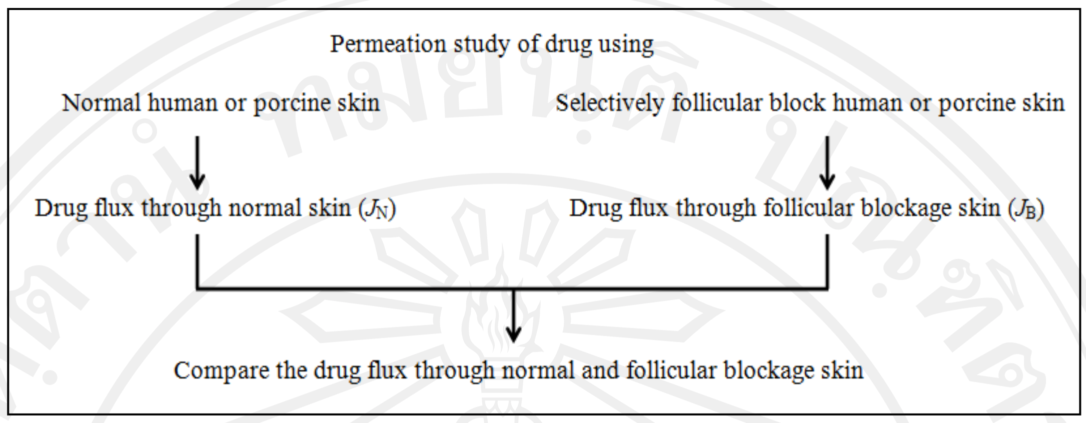

Figure 18 Scheme of transfollicular evaluation using selective follicular blocking method[155-156]

C. Differential stripping[10,113,144]

The differential stripping, a non-invasive and direct determination of penetrating substance into the hair follicle infundibulum, combines the tape stripping and cyanoacrylate skin surface biopsy. Sequential tape stripping is allowed the removal and quantification of the contents of the stratum corneum. Cyanoacrylate skin surface stripping (CSSS) is subsequently performed for selective removal of hair follicular located compound and debris[158]. The cyanoacrylate skin surface stripping using the liquid cyanoacrylate glue was shown in Fig. 19. A drop of cyanoacrylate glue is dropped on the surface of a glass slide and pressed on to skin. After glue polymerization, the glass slide is removed from the skin with corneocytes and follicular casts[144]. Figure 20 illustrated the scheme of transfollicular evaluation by differential stripping[126,158]. Tiechmann et al. have used the combined stripping for evaluation of topically applied fluorescent dye into the hair follicles *in vitro* and *in vivo* models and found that the fluorescein dye can be detected in the follicular infundibula after 30 min and 48 h[158]. The differential stripping has been used for

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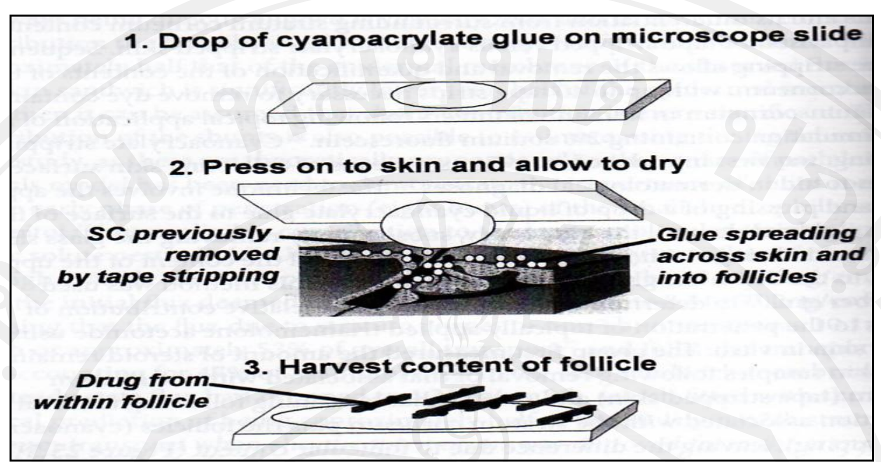

Figure 19 Cyanoacrylate skin surface stripping technique after tape stripping[144]

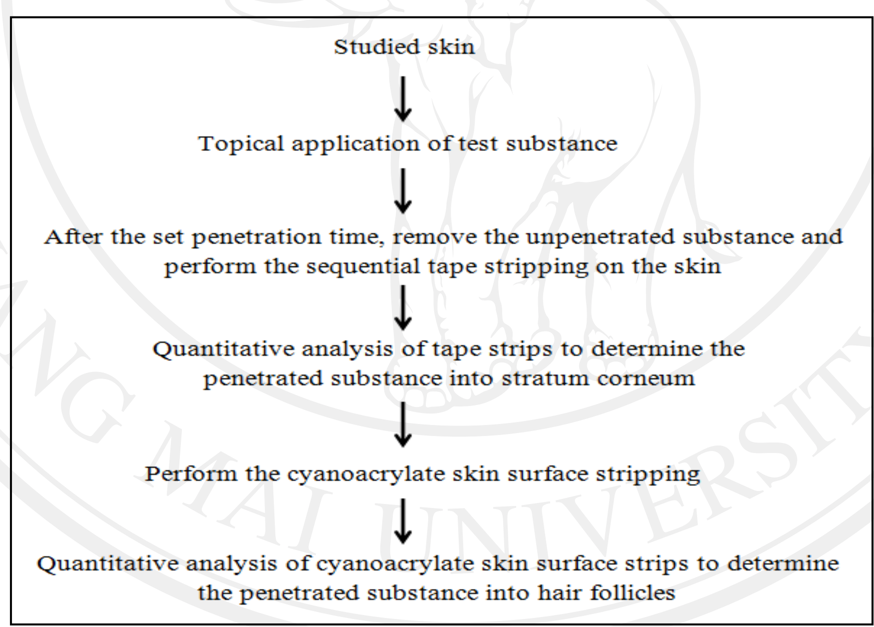

Figure 20 Scheme of transfollicular evaluation by differential stripping technique consisted of tape stripping and cyanoacrylate skin surface stripping[126,158]

investigation of human follicular reservoir *in vivo* and *in vitro*. The results revealed a significantly reduced follicular reservoir *in vitro*, which was only 10% of the *in vivo* reservoir. Thus, the authors have suggested that *in vitro* excised skin are suitable for

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penetration studies only to a limited extent as follicular penetration is greatly diminished, due to the contraction of elastic fibers of skin[159].

1.4.6 *In vitro* melanogenesis induction assays

1.4.6.1 Cell cytotoxicity by sulforhodamine B[160-161]

The sulforhodamine B (SRB) assay developed in 1990 is one of the most widely used methods for *in vitro* cytotoxicity screening. The assay relies on the binding of SRB to cellular protein and that binding is consequently quantitative analyzed. SRB is a bright pink aminoxanthene dye with two sulfonic groups that bind to basic amino acid residues under mild acidic condition and dissociate under basic condition. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell mass. Figure 21 showed the scheme of cell cytotoxicity by SRB assay[160]. This SRB assay has been widely used for drug-toxicity testing against different types of cancerous and non-cancerous cell lines. Generally, the cancerous cell lines, such as melanoma cells, HT-29 colon adrenocarcinoma cells and human mouth epidermal carcinoma cells, are used to screen the anti-cancer/anti-tumor potential of substances, whereas the non-cancerous cell lines, such as fibroblasts, keratinocytes and melanocytes, are employed to evaluate the substance toxicity[7,162-163].

The effectiveness of SRB assay is frequently compared to that of another method using tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The MTT assay is required cellular metabolic activity to convert the colorless tetrazolium to the purple-colored formazan dye, therefore, only viable cells can be detected, whereas the SRB method does not distinguish between viable and dead cells. This difference does not compromise the ability of SRB assay to detect

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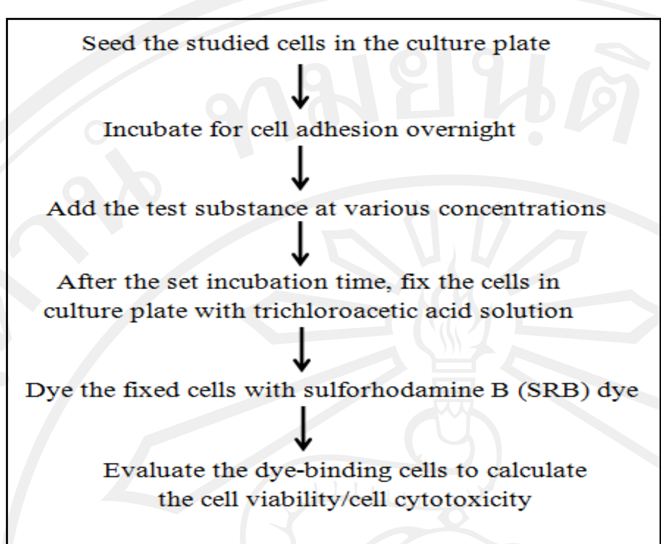

Figure 21 Scheme of cell cytotoxicity by SRB assay[160]

cytotoxic effects of a compound. The studies undertaken by several groups have been reported that results from SRB assay correlate well with those of the MTT assay, although the half maximal inhibitory concentration (IC_{50}) values of compounds tested using the SRB method are slightly higher. However, the SRB assay has several advantages over the MTT assay. For example, some compounds can directly interfere with MTT reduction without effect on cell viability, while SRB staining is rarely affected by this type of interference. In addition, SRB staining is independent of cell metabolic activity, therefore, fewer steps are required to optimize assay conditions for specific cell lines than in the MTT assay.

1.4.6.2 Melanin content[6,41,164]

Melanin plays the important role in the physiology, pathology and toxicology of the skin, eye and brain. In addition, melanin is an antioxidant and has an affinity for metals and other toxic chemical substances. The quantity and quality of melanin are the most important factors that determine the color of the iris, hair and

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skin. The studies on the modulation of quantity and quality of melanin are important in theoretical and practical aspects. Dermal and ocular melanocytes and ocular pigment epithelial cells can be successfully cultured *in vitro*. Melanin content and melanin production in pigment cells correlate with those of corresponding cells *in vivo*. Thus, pigment cells have been used as an *in vitro* experimental model system for study of the modulation of melanogenesis[164].

Figure 22 showed the scheme of melanin content evaluation *in vitro*. The melanin content measurement from pigment cells is easily compared with melanin analysis in pigment whole tissues because there is not interference from other tissue components present in cells and melanin obtained from culture cells is easily dissolved. The spectrophotometric method based on the absorption of light at a special wavelength by melanin is the most popular and simple method for measurement of melanin content in culture cells. Two different parameters, melanin content per cell and melanin content per culture, have been widely used to evaluate melanin content. Melanin content per cell, a determination of the melanin quantity in each pigment cell, can be calculated by dividing the amount of melanin by the number of melanin containing cells. Melanin content per culture, a determination of melanin content per cell and the cell density in a given area, is calculated by multiplying melanin per cell by the total number of cells in the flask. These two different parameters have been used for the description and evaluation of melanin content in a given pigment cell line at a certain growth period or for the evaluation of the changes in melanin content in a pigment cell line after treatment with a special chemical, physical or biological stimulator or inhibitor[164].

1.4.6.3 Tyrosinase activity[6-7, 41]

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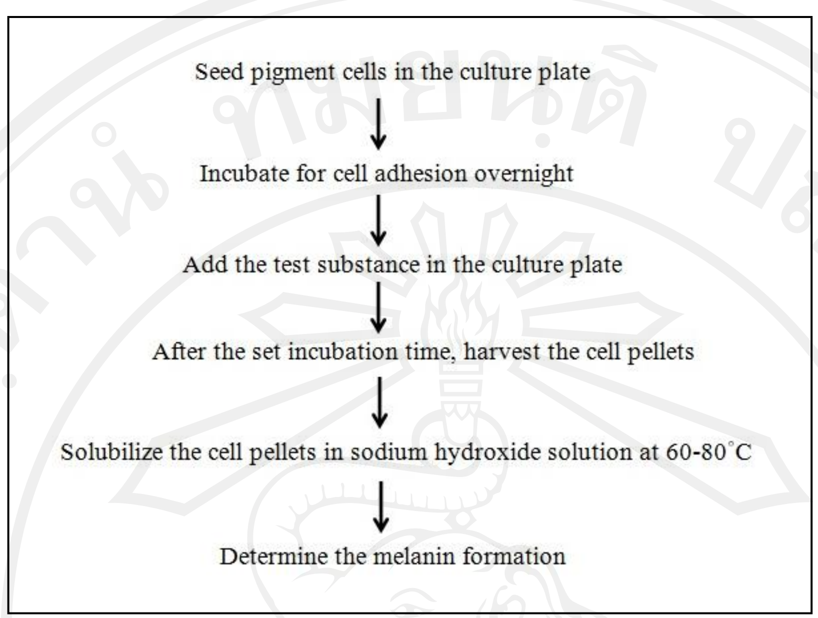

Figure 22 Scheme of melanin content evaluation *in vitro*[6-7,39,41,164-165]

Since tyrosinase is the rate-limiting enzyme of melanogenesis, the most logical regulatory point to control pigment formation is by inhibiting or stimulating that activity. Tyrosinase activity can be assayed in both cell-free and cell culture systems with various detection methods as shown in Fig. 23. In cell-free system, the test compound is tested to evaluate the direct effects on enzyme activity[166]. The test compound is mixed with substrate, tyrosine or *l*-dopa, and the standard tyrosinase enzyme is then added and incubated for a set time. The absorption of reaction mixture is measured and the dopachrome formation is calculated to determine the tyrosinase activity. For cell culture system, the pigment cells are treated with the test substance and incubated at 37°C for a set time[7,41,167]. The cells are then lysed to obtain tyrosinase enzyme from pigment cells. The enzyme substrate is added and the absorbance of reaction mixture is measured the determine enzyme activity.

1.4.6.4 Tyrosinase-related protein-2 activity[168-170]

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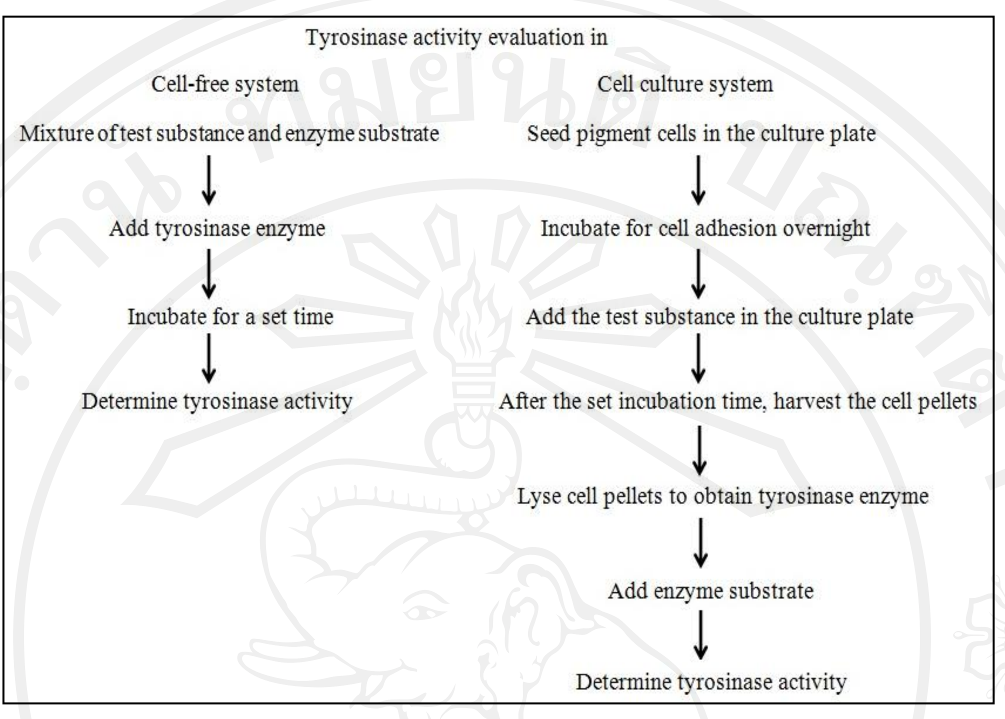

Figure 23 Scheme of tyrosinase activity evaluation[7,41,166-167]

Dopachrome tautomerase or TRP-2 catalyzes the rearrangement of dopachrome to the carboxylated derivative DHICA (5,6-dihydroxyindole-2-carboxylic acid). DHICA can also be generated from dopachrome by certain divalent metal cations. In the absence of these factors, dopachrome spontaneously forms the structurally distinct product, DHI (5,6-dihydroxyindole). However, activity of TRP-2 can be analyzed in both cell-free and cell culture system as shown in Fig. 24. In cell-free system, the test compound is mixed with dopachrome produced from the mixture of tyrosinase and *l*-dopa and incubated for a set time. Hydrochloric acid is added to stop the reaction and the mixture is then centrifuged. The obtained precipitate is washed and dissolved. The mixture is measured the absorbance to evaluate for the amount of melanin or the remaining dopachrome substrate[166]. For cell culture system, pigment cells are treated with the test substance and incubated at 37°C. After a set

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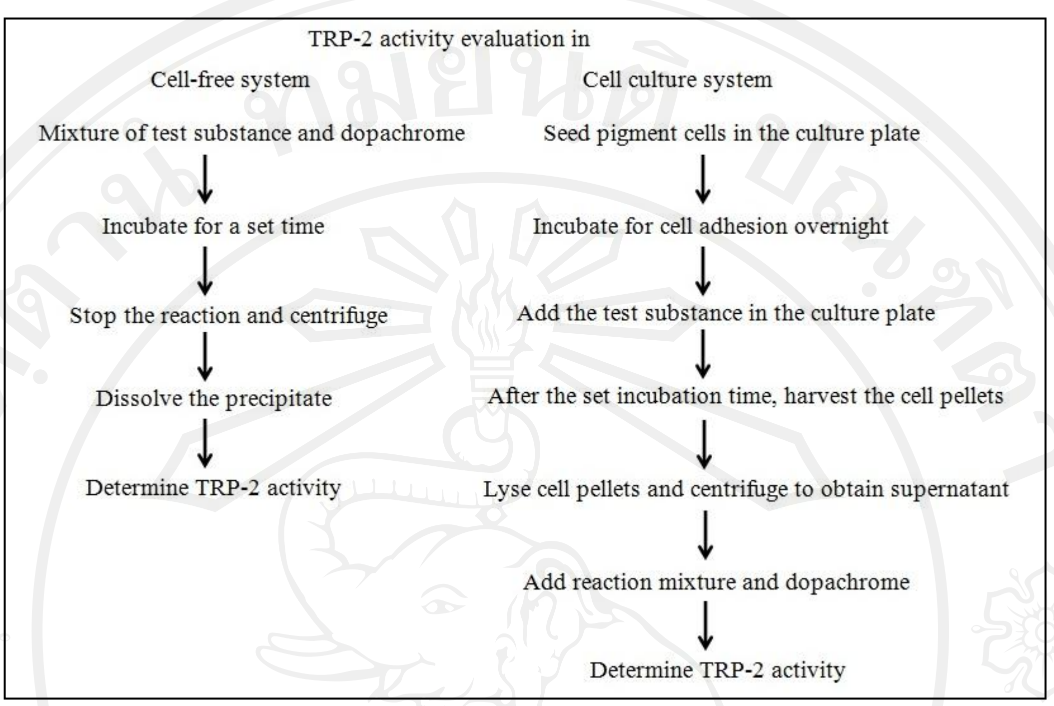

Figure 24 Scheme of TRP-2 activity evaluation[166,169-171]

time, the cells are then lysed and the obtained supernatant is mixed with the mixture of phenylthiourea, ethylenediamine tetraacetic acid (EDTA) and sodium phosphate buffer. Dopachrome prepared from *l*-dopa and sodium periodate is added into the mixture and incubated. The decreased absorbance based on the consumption of dopachrome is measured and calculated in compared to bovine serum albumin, a negative control[169-170]. HPLC is also used for the detection of TRP-2 activity. The evaluation system has been employed a hydrophobic C18 column and isocratic elution with sodium borate buffer, pH 2.5, containing of 25% methanol at a flow rate of 0.5 ml. Elution of peaks is monitored at 280 nm and the peaks of dopachrome, DHI and DHICA can be detected at 8.5, 10.4 and 12.7 min, respectively[170].

1.4.6.5 Total protein content[7,41,172-173]

Protein quantification is an integral part of laboratory involved protein

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analysis. Cell lysates are determined to verify the success of lysis step and to normalize multiple samples for side-by-side comparison. The simple direct protein analysis can be obtained by the absorbance measurement at 280 nm. Nevertheless, amino acids containing aromatic side chains, such as tyrosine, tryptophan and phenylalanine, exhibit strong UV-light absorption and may give the false determination of proteins and peptides consisting of aromatic amino acids. Thus, several colorimetric, reagent-based protein assay techniques have been developed and commonly used in protein research[173].

Figure 25 demonstrated the scheme of total protein content evaluation[173]. The sample is added to the total protein reagent and produced the color change in proportion to the added amount. The protein concentration is determined by reference to a standard curve consisting of known concentration of a purified reference protein. The Bradford protein assay is very fast, accurate and sensitive to detect the small amount of protein. The principle of Bradford protein assay is based on the observation that the maximum absorbance for an acidic solution of Coomassie brilliant blue G-250 shifts from 465 to 595 nm when the binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing the visible color change[173]. This assay is recommended for protein determination in the presence of melanin. Although melanin can adsorb a small amount of dye from the reaction system in the procedure, their sensitivity to protein makes the melanin interference negligible[172].

1.4.7 *In vivo* irritation and melanogenesis induction evaluation

1.4.7.1 *In vivo* irritation determination[174-175]

The evaluation of skin irritation potential is essential to ensure the safety

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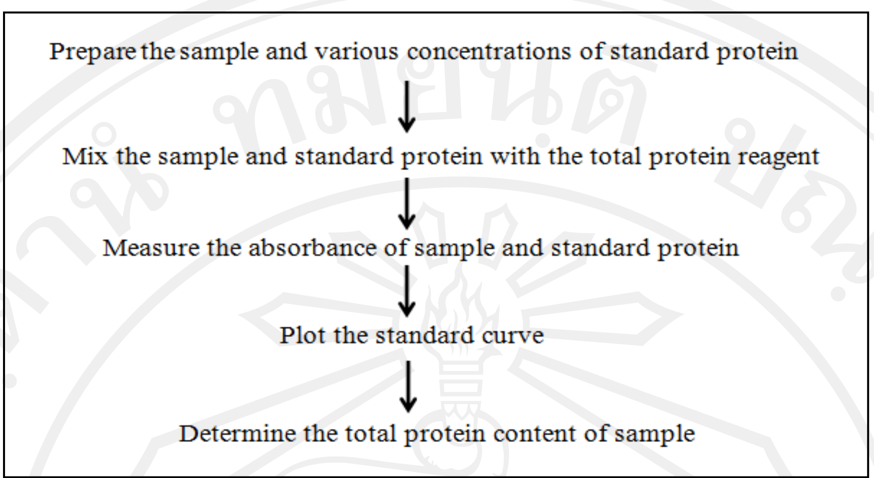

Figure 25 Scheme of total protein content evaluation[41,166,173]

of individual in contact with a wide variety of substances designed for industrial, pharmaceutical and cosmetic uses. Before application on human skin, the irritation evaluation of chemicals or formulations is necessarily tested. The most common test is the rabbit skin irritation assay described in the Organization for Economic Cooperation and Development (OECD) test guideline 404 that is initially introduced by Draize[174, 176]. Acute skin irritation can be evaluated *in vivo* on the shaved rabbit skin as shown in Fig. 26. The test product is applied in a single dose on the rabbit skin and covered with a gauze patch for 4 h. After patch removal, the residual test compound is removed and washed with distilled water. The irritation symptoms, including erythema and edema, are then evaluated and graded at 1, 24, 48 and 72 h using Draize scoring system as shown in Table 4. The primary irritation index (PII) value of test compound is calculated from the erythema and edema grades as the following formula:

$$\text{PII} = \frac{[(\Sigma \text{ erythema grades at 1,24,48,72 h} + \Sigma \text{ edema grades at 1,24,48,72 h})]}{(\text{number of animals} \times 4 \text{ scoring intervals})}$$

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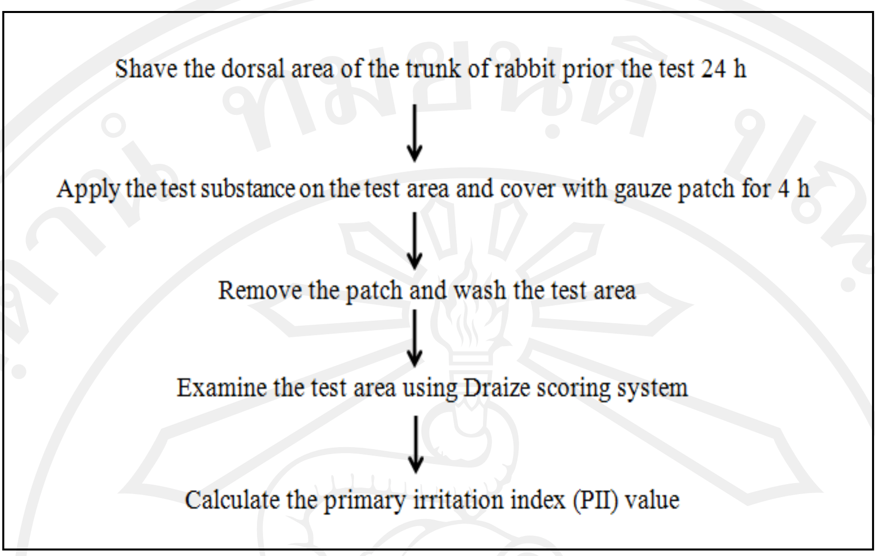

Figure 26 Scheme of *in vivo* irritation determination[174, 176]

Table 4 Draize scoring system[175]

Skin reactions	Score
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edge of area well defined by definite raising)	2
Moderate edema (raised > 1 mm)	3
Severe edema (raised > 1 mm and extending beyond the area of exposure)	4

The irritation degree is categorized based on the PII value as non-irritation (PII = 0), slight (PII = 0.01-1.99), moderate (PII = 2.00-5.00), or severe (PII = 5.01-8.00) irritation[177].

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Currently, an alternative strategy and the associated protocol for the use of human volunteers to identify skin irritation hazard have been described. *In vitro* skin irritation, the reconstructed human skin equivalents have been developed as the three-dimensional skin models composing of growing keratinocyte cultures at the air-liquid interface on de-epidermized dermis, acellular or fibroblast-populated dermal substrates, such as inert filters or collagen matrix. These cultures exhibit the well-stratified and cornified epidermis with basal, spinous and granular layers along with a functional stratum corneum, mimicking the normal human skin. The test compound is directly applied on the skin cultures and measured the cytotoxic effect by using MTT reduction assay, extracellular release of the pro-inflammatory mediator (interlukin-1α) and the cytosolic enzyme (lactate dehydrogenase) in the assay medium[175,178].

For human volunteers, if corrosive and other toxicologically unacceptable hazards do not exist for the substance or preparation, it is then possible to conduct an irritation test in human volunteers with the highest ethical standard. The human 4-h patch test provides the opportunity to identify substances with significant skin irritation potential without recourse to the use of animals. The protocol should be designed to avoid the production of more than mild irritant reactions and the value of experimental data in providing gold standard data for identification of test substance should be discussed to obtain acceptance data[175,179].

1.4.7.2 *In vivo* melanogenesis induction evaluation[38,40]

The investigation of *in vivo* melanogenesis induction can be performed in the pigment mice as shown in Fig. 27[38,40]. Pal et al. have evaluated effect of human placental extract on the pigment recovery in the skin[38]. The C57BL/6J black mice

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with age-induced early gray body coat hair, pinkish skin and histologic melanocyte disappearance, indicating the initiation of a prolonged telogenic phase of hair growth, were used to assess the pigment-inducing activity of extract. The resurgence of blue skin, followed by shiny black hair at the extract application area demonstrated the reversal of the age-induced prolonged telogenic phase of hair growth to the anagenic phase, whereas the unchanged skin color was observed at the vehicle treated site. The histological examination also showed the significant enhancement in the number of melanizing centers together with invagination at the top of upper epidermis, resembling the initiation of hair follicle growth in the extract treated group.

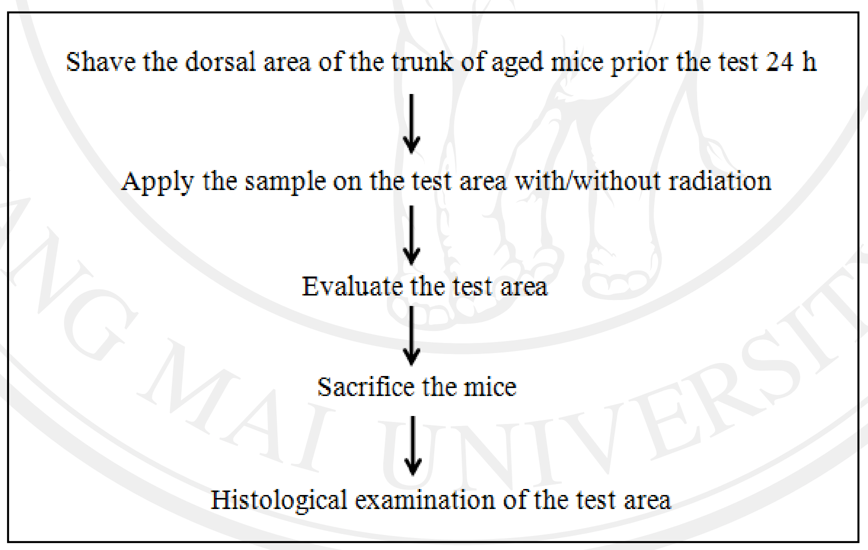

Figure 27 Scheme of *in vivo* melanogenesis induction evaluation[38,40]

Another *in vivo* model of pigmentation induction has been performed in male inbred HRA.HRII-c/+/Skh hairless pigment mice[40,180]. The mice were treated with piperine in combined with UV radiation, only piperine, only UV radiation and solvent. The pigmentation was assessed visually by an investigator blinded to the

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treatment that the animals had received with the scoring system. The results showed that the pigmentation of piperine in combined with UV radiation treated group was better than mice treated with either agent alone, indicating the usefulness of their concomitant use.

1.4.8 Commercial products for canities treatment

The commercial products claimed for treatment of canities are available both orally administration and topically application. Table 5 demonstrated the examples of canities treatment products and their active compositions[181-186]. Most products are composed of various plant extracts, vitamins and minerals, and claimed for nourishing and blackening of hairs. Some plant extracts, such as *Radix polygoni* and *Angelica sinensis* (Chinese angelica), have been reported on their melanogenesis induction activity[187-189]. However, those products have also been composed of the plant extracts reported on the decreased tyrosinase activity, such as extracts of *Panax ginseng*, *Ganaderma lucidum*, mulberry fruit, noni and aloe vera[64,190-192]. Thus, the selection of product is needed to take the product compositions into account.

1.4.9 Research on canities treatment

1.4.9.1 Natural approaches

The hair graying is the most obvious sign of aging in humans, its mechanism is largely unknown. Nishimura et al. and Steingrimsson et al. have reported that the hair graying is caused by the defective self-maintenance of melanocyte stem cells[31,171]. The process is accelerated dramatically with B-cell lymphoma 2 (Bcl 2) deficiency, which causes selective apoptosis of melanocyte stem cells, but not of differentiated melanocytes, within the niche at their entry into the dormant state. This finding may benefit for treatment of hair graying with stem cell

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Table 5 The examples of the commercial products available in the market for canities treatment[181-186]

Product and manufacturing company	Dosage form and appearance of product	Active ingredients of product	Mechanisms of action	Price per package (US Dollar)
Get Away Grey® Get Away Grey company	oral capsule	catalase enzyme, plant extracts, chlorophyll, vitamins and minerals	cell protection from antioxidant substances and plant extracts as well as supplement of vitamins and minerals to nourish hair	60 cap/bottle $ 29.99
Bawang® hair blackening & strengthening shampoo with Chinese herbal extracts Bawang (Guangzhou) Co., Ltd.	shampoo and conditioner	*Radix polygoni*, Chinese angelica, ginseng extract, mulberry fruit extract	enrichment of hair follicles with Chinese herbal extracts to retard the premature graying	200 ml shampoo and 55 ml hair blackening conditioner $ 22.73
BSY® Noni black hair magic Guangzhou Maykay Cosmetics Co., Ltd.	shampoo	*Ganoderma lucidum* extract, wild ginseng extract, noni extract, black olive extract, aloe vera extract, Ayurvedic herbs	nourishment of hair root and prevention of hair loss with herbal extracts	20 sachets/pack (20 ml/sachet) $ 60
O'LIYE® herbal blackening shampoo Guangzhou Sanzi Cosmetics Co., Ltd.	shampoo	extracts from various plants, such as fleece flower root, glossy privet fruit extract, medlar and others, *m*-aminophenol, *p*-phenylenediamine	coloring of gray hair and supplement of nutrients to hair follicle as well as production of melanin	125 ml/bottle $ 22.30
Natural-in-Black® hair cream Naturo® Health International	hair cream	*Quercus infectoria* extract, *Ligustrum lucidum* extract, sesame oil, clove oil, coconut oil	supplement, repairment and production of melanin to the hairs	150 g/bottle $ 78
Desyham® Hair darkening oil Desyham Marketing	hair oil	coconut oil, various herbs	nourishment of gray hair to turn into black hair	125 ml/bottle $ 6.57

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therapy.

In addition, many plants and active compounds extracted from plants have been widely investigated for the melanogenesis stimulating potential to treat the pigment formation disorder. The Chinese medicinal plants, *Herba menthae, Lignum dalbergiae*, *Radix polygoni*, and *Angelica sinensis*, exhibited their effects on the up-regulation of melanin biosynthesis[187,189,193]. The results found that the water extracts of all test compounds increased the rate of melanin biosynthesis *in vitro*. The extracts of *Herba menthae* and *Lignum dalbergiae* demonstrated strong absorption of both UVA and UVB, whereas the recipe showed strong absorption of UVB. Cubebin, an active lignan isolated from *Piper nigrum*, has stimulated the melanogenesis production substance without any significant effects on cell proliferation[41]. The mechanism of cubebin is mediated through the activation of p38 mitogen-activated protein kinase (MAPK), resulting in the increased melanin amount and tyrosinase expression. The furocoumarins including psoralen, xanthotoxin, bergapten, isopimpinellin and sphondin, isolated from the seven Umbelliferae crude drugs have shown their potent melanogenesis stimulation activity *in vitro* study[36]. These compounds may be useful ingredients in cosmetic preparation for the prevention of gray hair. Also, the lotus flower essential oil extracted from *Nelumbo nuficera* flowers has exhibited the increased melanin content as well as tyrosinase activity[7]. The lotus essential oil consisting of palmitic acid methyl ester, a major constituent, is regulated the pigment stimulation via the expression of tyrosinase, MITF and TRP-2 proteins.

Fatty acids, the major components of biological cell membranes, have been reported on their effects on melanogenesis. Ando et al. have investigated the

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effects of saturated and unsaturated fatty acids on melanogenesis[6,42,44-45]. The saturated fatty acids have induced the pigment production through the retardation of tyrosinase degradation and the decreased ubiquitination of many cellular proteins, while the unsaturated fatty acids exhibit the opposite effects on pigmentation. The human placental alcoholic extract have been prepared and tested the effect on melanogenesis both *in vitro* and *in vivo* models[38-39]. *In vitro* study, the placental extract increased the mitogenesis and melanogenesis, while *in vivo* study the recovery of pigmentation on skin and hair was observed after topical application on the aged mice.

1.4.9.2 Synthetic approaches

The synthetic substances have been synthesized and investigated for their effects on the melanogenesis both *in vitro* and *in vivo* studies. Bafilomycin A1 and concanamycin A, two vacuolar type H^+-ATPase inhibitors, have been shown the melanogenesis stimulation effect through the activation of tyrosinase and melanin content[194]. The retinoic acid, a vitamin A derivative, has been exhibited its melanogenesis induction effects in both murine B16F10 and human Hs 939 melanoma cells[46,195]. This substance interfered at the subcellular level by alteration of melanosomes density as well as the relative volume of the nucleus and nucleolus. Also, the cells displayed the altered morphology and the increased relative volume of melanosomes, leading to melanin formation. Piperine analogues are the derivatives of piperine, a pepper alkaloid isolated from *Piper nigrum.* Those analogues have investigated their effects on melanogenesis and shown the increased melanin content as well as the alteration of cell morphology by increasing of dendritic formation[40,196].

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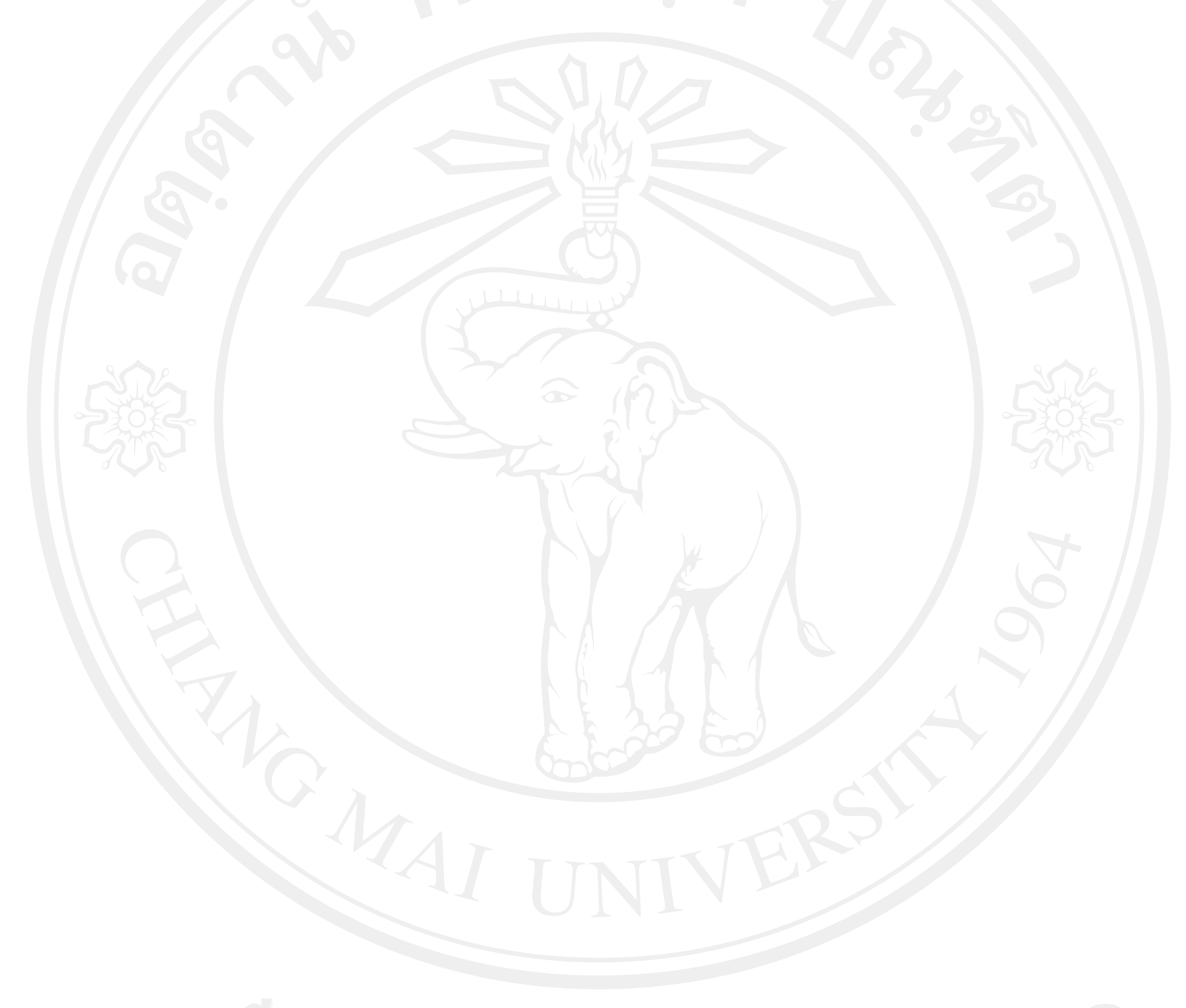
In vivo study, the combination of piperine and UV radiation showed the better pigmentation than only piperine treatment.



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