CHAPTER II

LITERATURE REVIEWS

2.1 Skin structure and function

As the outermost layer of protection for the body, the skin is constantly exposed to chemicals and environmental influences that affect its health and appearance. The top layer of skin is the epidermis, which performs an important barrier function between the external and internal environments. The next layer is the inner thicker layer called the dermis that is prominent for collagen and elastic tissue. Figure 2.1

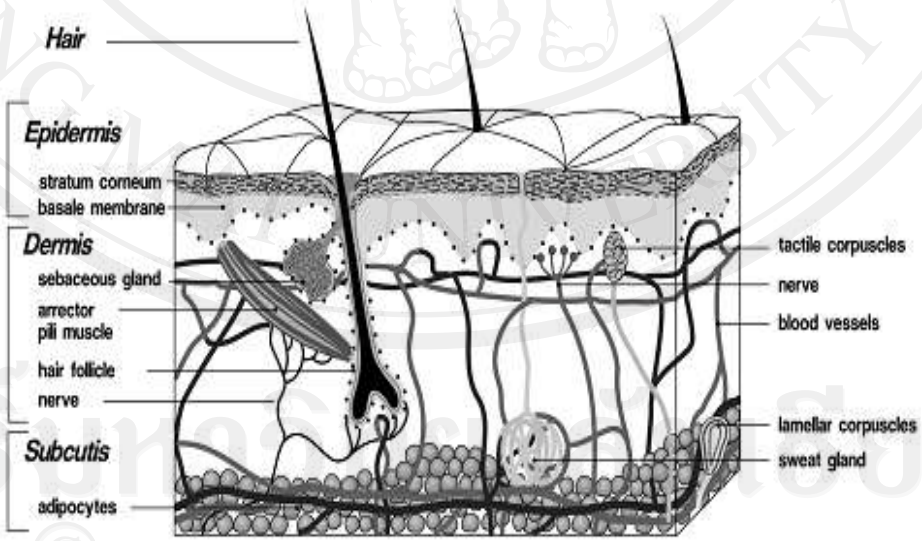

Figure 2.1 Diagrams of Skin Layers. (Copy from http://www.cognis.com/veris)

2.1.1 Structure of the skin [8, 11]

The skin has several layers and sits on muscle which is covered by subcutaneous fat. In this fat layer there are blood vessels, and the roots of the hair follicles and sweat glands.

Epidermis

The epidermis is the outer layer of skin. The thickness of the epidermis varies in different types of skin. The epidermis contains 5 layers; from bottom to top the layers are named:

- stratum basale or stratum germinativum
- stratum spinosum
- stratum granulosum
- stratum lucidum
- stratum corneum

The bottom layer, the stratum basale, has cells that are shaped like columns. In this layer the cells divide and push already formed cells into higher layers. As the cells move into the higher layers, they flatten and eventually die. The top layer of the epidermis, the stratum corneum, is made of dead, flat skin cells that shed about every 2-3 weeks. There are three types of specialized cells in the epidermis.

- The melanocyte produces pigment (melanin)
- The Langerhans' cell is the frontline defense of the immune system in the skin
- The Merkel's cell's function is not clearly known

Figure 2.2 Layers of the epidermis (copy from http://dermatology.about.com/od/anatomy/ss/epidermis_2.html)

Dermis

The dermis also varies in thickness depending on the location of the skin. The dermis is composed of three types of tissue that are present throughout - not in layers. The types of tissue are collagen, elastic tissue and reticular fibers. The dermis is composed of two layers, the papillary and reticular layers.

- The upper, papillary layer contains a thin arrangement of collagen fibers.
- The lower, reticular layer is thicker and made of thick collagen fibers that are arranged parallel to the surface of the skin.

The dermis contains many specialized cells and structures such as hair follicles, sebaceous (oil) glands and apocrine (scent) glands, sweat glands, blood vessels and nerves.

Subcutaneous Tissue

The subcutaneous tissue is a layer of fat and connective tissue that houses larger blood vessels and nerves. This layer is important in the regulation of temperature of the skin itself and the body. The size of this layer varies throughout the body and from person to person.

The skin is a complicated structure with many functions. If any of the structures in the skin are not working properly, a rash or abnormal sensation is the result. The whole specialty of dermatology is devoted to understanding the skin, what can go wrong, and what to do if something does go wrong.

2.1.2 Functions of the skin

The skin is the largest organ of the human body, and performs many functions:

- it cushions the body against external forces
- it provides waterproof protection through the lubricating activity of the sebaceous glands that produce sebum
- it help retain vital fluids within the body
- it regulates temperature-cooling the body when it is hot and conserving heat when it is cold: when hot, the sweat glands produce perspiration, and the blood vessels widen(dilate) to dissipate heat; when cold, the blood vessels narrow(constrict) to conserve body heat
- it shields internal organs from harmful rays from the sun

- it provides a barrier against bacteria, viruses, other microorganisms and chemical pollutants
- it contains many cells that identify-touch, pain, pressure, itching
- it excretes waste and toxins

2.2 Skin aging [4-5, 12, 14-16]

The researches show that there are, in fact, two distinct types of aging. Aging caused by the genes we inherit is called *intrinsic* (internal) *aging*. The other type of aging is known as *extrinsic* (external) *aging* and is mostly caused by environmental factors. [4]

Intrinsic skin aging

Intrinsic aging, also known as the natural aging process, is a continuous process that normally begins in our mid-20s. Within the skin, collagen production slows, and elastin, the substance that enables skin to snap back into place, has a bit less spring. Dead skin cells do not shed as quickly and turnover of new skin cells may decrease slightly [4]. The intrinsic skin aging process is characterized by increasing of cellular catabolic activities, deficiency of antioxidant defense mechanisms (genetic polymorphism), deficiency of melanin synthesis (genetic polymorphism), deficiency of detox capacity (genetic polymorphism), decreasing in sexual hormones supply, (age related) and lowering of blood perfusion (arteriosclerosis, lack of exercise). [4, 13]

Biomarkers of the intrinsic skin aging include the hyaluronic acid depolymerization, a reduced melanogenesis and estrogen dependent collagen synthesis, lowered ATP generation and wound repair capabilities, impaired antioxidant defense and increased lipofuscin generation, (age spots).

Extrinsic skin aging [4, 13]

The extrinsic aging is related to:

- The photoaging process induced by sunlight or artificial UV-exposure, which has the major impact on skin appearance through an obvious free radical generation in the skin.
- Toxic environmental exposure via smoking, industrial exhausts, heavy metals, detergents, all of which are known to be potent free radical inducers.
- Chronic infection/ inflammatory states associated with an increased free radical attack, (superoxide, peroxinitrite and hypochlorite).
- Inappropriate nutrition, (excess of refined carbohydrates, fats, food additives, alcohol, low water intake) and last, but not least;
- Sleep deficiency and stress.

Biomarkers of the extrinsic skin aging process includes products of lipid peroxidation, collagenase activation, glycation/ oxidation of proteins (AGE products), low DNA repair capacity and cumulative DNA damage/ mutations leading to skin cancer and others. According to skin is direct contact with environment, it undergoes changes as a consequences of external factors. Exposure to UV light (photoaging) is considered to be the most significant factor of the extrinsic aging of the skin. [17]

As the outermost layer of protection for the body, the skin is constantly exposed to chemicals and environmental influences that affect its health and appearance. Skin can be exposed to a greater extent than other body tissues to excess cold or heat, ultraviolet (UV) and ionizing radiation and prooxidant air pollutants such as ozone. The skin is rich in lipids, protein and DNA, which are especially vulnerable to damaging free radicals generated by a number of physiological and biological processes. Free radicals are believed to have an important role in skin aging and in development of many inflammatory skin disorders and skin cancers. Skin aging processes are overlapped and strongly related to an increased generation of free radicals in the skin. [18]

2.3 Free radical

In chemistry, radicals; often referred to as free radicals**,** are atomic or molecular species with unpaired electrons on an otherwise open shell configuration. These unpaired electrons are usually highly reactive, so radicals are likely to take part in chemical reactions. Thus a free radical can be defined as any species capable of independent existence that contain one or more unpaired electrons, and unpaired electron being one that is alone in an orbit. Most biological molecules are non-radicals, containing only paired electrons. [19] A radical can react with both radicals and non-radical molecules in several ways and may donate its unpaired electron to a non-radical molecule or it might take an electron from another molecule in order to form a paired electron. Free radicals are generally very reactive molecules possessing an unpaired electron. They are produced continuously in cells either as by-products of

metabolism, or for example, by leakage from mitochondrial respiration. [20] The term "Reactive oxygen species (ROS)" is often used to include both the radical and non-radical species as shown in Table 1. [21]

Table 2.1 Reactive oxygen species found in vivo.

Name	Singlet/formula	Radical(R) or non-radical (NR)
Molecular oxygen	O_2	NR
Nitric oxide	$NO^{\bullet}$	R
Nitrogen dioxide	$NO_2^{\bullet}$	R
Superoxide	$O_2^{\bullet-}$	R
Peroxyl	$ROO^{\bullet}$	R
Singlet oxygen	$1\Delta gO_2$	NR
	$1\Sigma g^{+}O_2$	R
Hydrogen peroxide	H_2O_2	NR
Hydroperoxyl	$HOO^{\bullet}$	R
Alkoxyl	$RO^{\bullet}$	R
Hypochlorous acid	HOCl	NR
Name	**Singlet/formula**	**Radical(R) or non-radical (NR)**
Peroxynitrite	HNOO	NR
Hydroxyl	$OH^{\bullet}$	R

Reactive oxygen species are potentially very toxic to cells. Due to their highly reactive nature, they can readily combine with other molecules, such as enzymes, receptors, and ion pumps, causing oxidation directly, and inactivating or inhibiting their normal functions. Some of the products of reactive oxygen species attack of other molecules can interfere with nucleic acid function, generating alterations in the base sequence with the potential for mutations. Changes in normal proteins and other structures by free radical species can also generate novel immunogenic structure.

Production of free radicals in human body

Free radical formation occurs continuously in the cell as a consequence of both enzymatic and non-enzymatic reactions. Enzymatic reactions which serve as soures of free radicals include those involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis and in the cytochrome P450 system. Free radicals also arise in non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing radiation. Some internally generated sources of free radicals are mitochondria, phagocytes, xanthine oxidase, reactions involving iron and other transition metals, arachidonate pathways, peroxisomes, exercise, inflammation and ischeaemia/reperfusion. Some externally generated sources of free radicals are cigarette smoke, environmental pollutants, radiation, ultraviolet light, Ozone and certain drugs; pesticides, anaesthetics and industrial solvents. Figure 2.3

With electrons unhinged, free radicals roam the body, wreaking havoc. The free radical, in an effort to achieve stability, attacks nearby molecules to obtain another electron and, in doing so, damages those molecules.

If free radicals are not inactivated, their chemical reactivity can damage all cellular macromolecules including proteins, carbohydrates, lipids and nucleic acids. Their destructive effects on proteins may play a role in the causation of cataracts. Free radical damage to DNA is also implicated in the causation of cancer and its effect on LDL cholesterol is very likely responsible for heart disease. In fact, the theory associating free radicals with the aging process has also gained widespread acceptance.

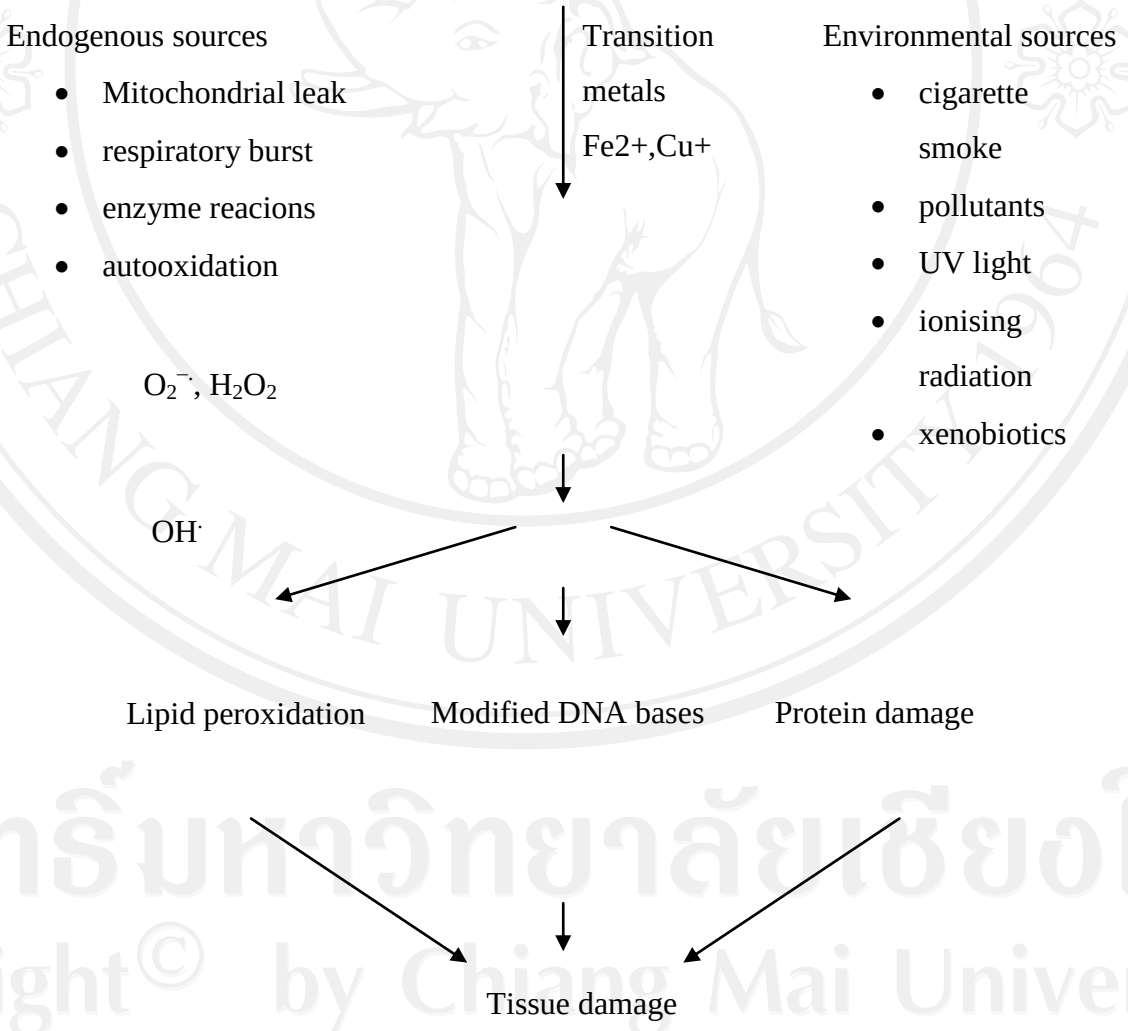

Figure 2.3 Production of free radicals in human [6]

In chemistry, free radicals take part in radical addition and radical substitution as reactive intermediates. Chain reactions involving free radicals can usually be divided into three distinct processes; initiation, propagation and termination. [22]

- Initiation reactions are those that result in a net increase in the number of free radicals. They may involve the formation of free radicals from stable species or they may involve reactions of free radicals with stable species to form more free radicals.

- Propagation reactions are those reactions involving free radicals in which the total number of free radicals remains the same. It occurs when the number of radicals is conserved. There are four major mechanistic types of propagations have been identified that may be relevant to biology; atom or group transfer, electron transfer, addition and β scission

- Termination reactions are those reactions resulting in a net decrease in the number of free radicals. Typically two free radicals combine to form a more stable species. There are three molecular processes which can terminate a chain of radical reaction; homolinking and cross-linking of radical, radical scavenging and electron transfer.

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process whereby free radicals steal electrons from the lipids in cell membranes, resulting in tissues damage Figure 2.3.This process proceeds by a free radical chain reaction mechanism. It often affects polyunsaturated fatty acid because they contain multiple double bonds between methylene-CH_2- groups that posses especially reactive hydrogen. As with any radical reaction, the reactions consists of three major steps as

mentioned above the lipid peroxidation is also the important reaction that can cause aging of skin. Active oxygen species can initiate and propagate lipid peroxidation reactions. Free radical mediated lipid peroxidation generally requires radicals and weakly bonded polyunsaturated fatty acids for the initial steps. Oxygen and a lipid radical are necessary for the propagation step which produces lipid peroxidation byproducts.

2.4 Antioxidant [6, 19-21, 23-24]

An antioxidant molecule is a molecule capable slowing or preventing of the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from substance to an oxidizing agent. Oxidation reaction can produce free radicals which starts chain reaction that damage cell or tissues. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. Antioxidant defenses, both endogenous and exogenous, are present to protect cellular components from free radical induced damage. These can be divided into three main groups: antioxidant enzymes, chain breaking antioxidants, and transition metal binding proteins. Antioxidants are often reducing agent such as thiols, ascorbic acid or polyphenol. Antioxidants can be either endogenous compounds, produced by the organism as part of its ROS defense, or can be exogenous compounds acquired from diets. The endogenous system includes both enzymatic and non enzymatic antioxidants. These can be divided into three main groups: antioxidant enzymes, chain breaking antioxidants, and transition metal binding proteins

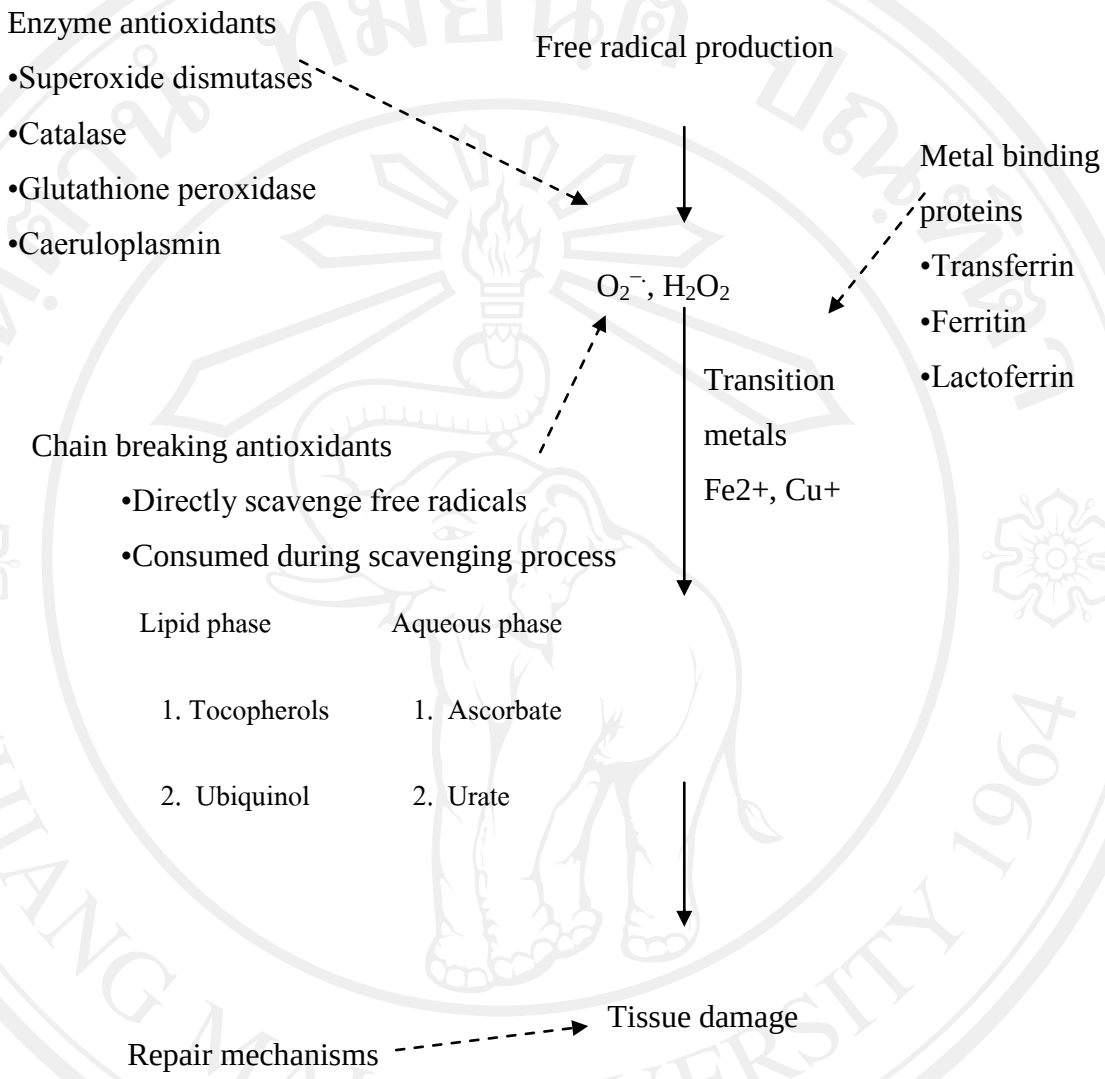

Figure 2.4 Antioxidants defenses against free radicals attack. [6]

- The enzyme antioxidants

One important line of defence is a system of enzymes, including glutathione peroxidases, superoxide dismutases and catalase, which decrease concentrations of the most harmful oxidants in the tissues.

- The transition metal binding

Transition metal binding proteins (ferritin, transferrin, lactoferrin, and caeruloplasmin) act as a crucial component of the antioxidant defence system by sequestering iron and copper so that they are not available to drive the formation of the hydroxyl radical. The main copper binding protein, caeruloplasmin, might also function as an antioxidant enzyme that can catalyse the oxidation of divalent iron.

$$4Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O$$

Fe^{2+} is the form of iron that drives the Fenton reaction and the rapid oxidation of Fe^{2+} to the less reactive Fe^{3+} form is therefore an antioxidant effect.

- The chain breaking antioxidants

An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. Some such antioxidants, including glutathione, ubiquinol and uric acid, are produced during normal metabolism in the body. Other lighter antioxidants are found in the diet. Although about 4000 antioxidants have been identified, the best known are vitamin E, vitamin C and the carotenoids. Many other non-nutrient food substances, generally phenolic or polyphenolic compounds, display antioxidant properties and, thus, may be important for health.

The most used synthetic antioxidants are butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA) and tertiary butylhydroquinone (TBHQ). These compounds have been reported as health risks and their use is restricted in several countries. For example, BHA may induce hepatic activity, liver enlargement, growth reduction and carcinoma formation. BHT was toxic to the liver, kidney and lungs, and

reduced weight gain, among other effects observed in animals. Meanwhile, there is a growing interest in natural antioxidants found in plants because of the world-wide trend toward the use of natural additives in food and cosmetics. A medicinal plant is one of the most important targets to search for natural antioxidants from the point of view of safety. [25] Natural antioxidants of plant origin are generally classified as vitamins, phenolic compounds including flavonoids and phenolic acids.

Flavonoids and other polyphenol belong to the recently popular phytochemicals, i.c., chemicals derived from plant material with potentially beneficial effects in human health. [26] The compounds are known as secondary plant metabolites, which indicate that most of these substances have been regarded as nonessential and therefore secondary in function. Polyphenolic compounds are the important and large group of naturally-occurring antioxidants. They are widely distributed in higher plants, but also occur in mosses, fungi, algae and lichens. Many polyphenolic compounds show antioxidant activities against ROS.

2.5 Nanostructured Lipid Carriers

Lipid nanoparticles with solid particle matrix are derived from o/w emulsions by simply replacing the liquid lipid (oil) by a solid lipid, i.e. being solid at body temperature. The solid lipid nanoparticles (SLN), first generation of lipid nanoparticles, was developed at the beginning of the nineties. [27] They were produced from solid lipids only. By preparing the particles from a solid lipid, especially highly purified solid lipids, the particle matrix tends to form a relatively perfect crystal lattice leaving limited space to accommodate the actives. This limits the loading capacity and

can lead to expulsion of active from the lipid matrix during storage. [29] In the second generation, the nanostructured lipid carriers (NLC) was developed to minimizes or avoids those potential problems associated with SLN. NLC particles consist of a mixture of solid lipid and liquid lipid and are in particular appropriate for drugs/active compounds with higher solubility in oils than in solid lipids. A blend of liquid and solid lipid creates a less perfect crystalline structure with many imperfections providing more space for drug accommodation. That means to say "the perfectness" of the NLC system is its "imperfectness" in its crystalline structure. [2, 27-29]

2.5.1. Nanostructured lipid carriers preparation techniques [30]

The production process is identical for both particles SLN and NLC. Many different techniques for the production of lipid nanoparticles have been developed. These methods are

- High pressure homogenization (hot or cold homogenization)
- Microemulsion technique
- Emulsification-solvent evaporation
- Emulsification-solvent diffusion method
- Solvent injection or solvent displacement method
- Phase inversion, multiple emulsion technique

However, high pressure homogenization (HPH) is widely use to prepare NLC and has many advantages compared to the other methods, e.g. easy scale up, avoidance of organic solvent and short production time. High pressure homogenizer; are widely used in many industries including the pharmaceutical industry. Therefore,

no regulatory problems exist for the production. [2, 27] NLC can be produced by either hot or cold high pressure homogenization technique demonstrated in Figure 2.5. The active compound is dissolved or dispersed in a mixture of liquid lipids and melted solid lipids. In the hot homogenization method the lipid melt containing active compound is dispersed in a hot surfactant solution by high speed stirring. The obtained pre-emulsion is then passed through a high pressure homogenizer adjust to the same temperature. The obtained nanoemulsion is cooled; the lipid phase solidifies and forms an aqueous suspension/dispersion of lipid nanoparticles. In cold homogenization method, the active containing lipid melt is cooled down. After solidification the mass is crushed and grounded to obtain lipid microparticles. The lipid microparticles are then dispersed in a cold surfactant solution yielding a cold pre-suspension of micronized lipid particles. This suspension is passed through a high pressure homogenizer at room temperature to obtain an aqueous suspension/dispersion of lipid nanoparticles same as hot homogenization. [3]

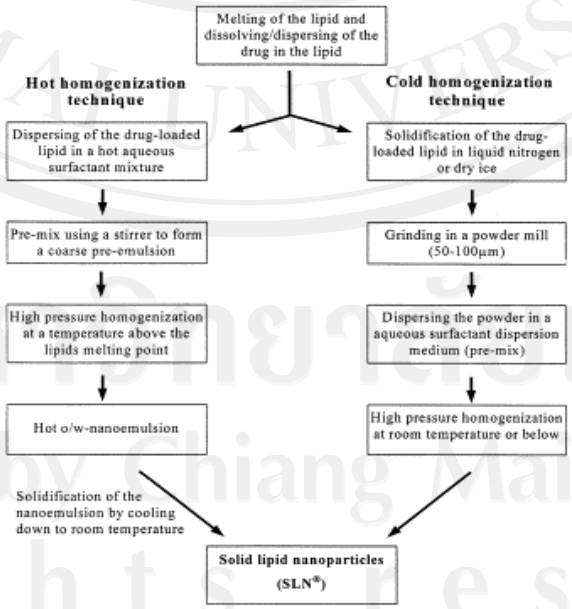

Figure 2.5 Schematic procedure of high pressure homogenization techniques [1]

2.5.2 Types of nanostructured lipid carriers [28]

The type of NLC depends on the chemical nature of the active ingredient and lipid, the solubility of actives in the melt lipid, nature and concentration of surfactants, type of production, and the production temperature. The three types of NLC can be summarized. Figure 2.6

2.5.2.1 Type I: The imperfect type

This model is obtained when mixing solid lipids with small amounts of liquid lipids (oils). The matrix of NLC is not able to form a highly ordered structure due to the using of different molecules distorts the formation of a perfect crystal. The particle matrix contains many imperfections providing space to accommodate the active in molecular form or as amorphous clusters. That means to say "the perfectness" of the NLC system is its "imperfectness" in its crystalline structure.

2.5.2.2 Type II: The amorphous type

It is created when mixing special lipids that do not recrystallize anymore after homogenization and cooling down such as isopropylmyristate and hydroxyoctacosanylhydroxystearate. It's just solid in an amorphous state which can avoid the occurrence of crystallization and minimizing drug expulsion.

2.5.2.3 Type III: The multiple type

It is an oil-in-solid lipid-in-water dispersion. The solid lipid matrix contains tiny liquid oil nanocompartments. This NLC type uses the fact that for a number of drugs, the solubility in oils is higher than their solubility in solid lipids.

This model obtained when mixing solid lipid with liquid lipid in such a ration that the solubility of the oil molecules in the solid lipid is exceeded

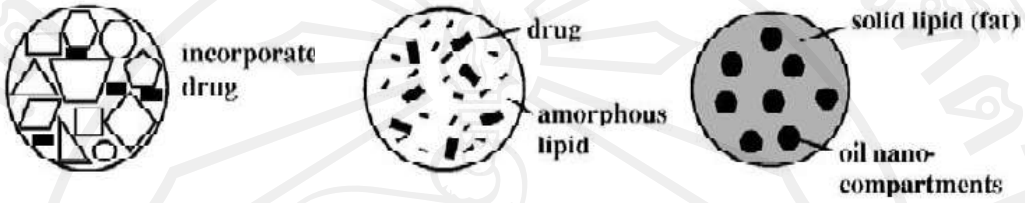

Figure 2.6 The three types of NLC: imperfect type (left), amorphous type (middle), multiple type (right) [28]

2.6 Marigold (*Tegetes erecta Linn.*) [31-34]

Figure 2.7 *Tagetes erecta* Linn.

Scientific name *Tagetes erecta* Linn.

Family name Compositae/Asterceae

Common name Marigold**,** American marigold, African marigold, คำปู้จู้หลวง Khampuu chuu luang, ดาวเรืองใหญ่, พอทู

Physical Characteristics

Marigold (*Tagetes erecta* Linn.) is one of the common well known plants in Family Compositae. The lower leaves are broad and spatula shaped. Upper leaves may be oblong, are smooth at the edges, and are arranged alternately along the stem. Seeds are crescent to horseshoe shaped with the rough exterior. Its branching stem grows to the height of 30- 60 cm. Marigold plant is propagated by the seeds. Marigold does not need cultivation but the soil should be free from the weeds. The seeds are germinated in two to three weeks. It needs full sunlight for its growth.

Medicinal uses

Marigold is used for stomach upset, ulcers, menstrual period problems, eye infections, inflammations, and for wound healing. It is antiseptic. If the Marigold flower is rubbed on the affected part, it brings relief in pain and swelling caused by a wasp or bee. A lotion made from the flowers is most useful for sprains and wounds and a water distilled from them is good the sore eyes. The infusion of the freshly gathered flowers is beneficial in fever. Externally it is used in the treatment of alopecia. Internally it is used to treat bladder and kidney problems, blood in the urine, uterine bleeding and many more. Yellow dye has also been extracted from the flower, by boiling. The burning herb repels insects and flies. Pigments in the Marigold are sometimes extracted and used as the food coloring for humans and livestock. In traditional medicine it has been used for conjunctivitis, dizziness, edema, stomach and intestinal diseases etc. For the antioxidant property, there are some studies that

mentioned about the antioxidant activity of marigold and other plants in Family Compositae

Wang et.al, 2006 had studied on lutein which was extracted and purified from marigold flower (*Tagetes erecta* L.). The antioxidant activity of lutein was examined by using the photochemiluminescence (PCL) assay and the β-carotene-linoleic acid model system (β-CLAMS). The mutagenicity and anti-mutagenicity of lutein were also examined. Lutein showed a greater antioxidant acvtivitty than the other two common carotenoids, β-carotene and lycopene. [35]

The antioxidant activity of the essential oil from flowers of *Tagetes erecta* was evaluated using diphenyl-1-picrylhydrazyl (DPPH), thiocyanate, β-carotene bleaching, free radical scavenging activity and oxidation of deoxyribose assay. The results demonstrated that essential oil from *Tagetes erecta* at the dose level proved possesses significant antioxidant activity less than α-tocopherol. The antioxidant activity of the essential oil may be due to the presence of the camphor and methyl eugenol. Both are a chemical compound occurring naturally in a variety of spices and herbs. [36]

Wei et.al determined major phytochemical contents and antixodant activities of 11 Chinese cultivars of marigold. Dried marigold flowers were extracted with ethanol, ethyl acetate, and *n*-hexane and the extracts were analyzed by high-performance liquid chromatography–mass spectrometry and chemical methods to determine their lutein esters, phenolic and flavonoid contents, and antioxidant activity. The different cultivars of marigold showed considerable variations in their

lutein ester contents, ranging from 161.0 to 611.0 mg/100 g of flower (dry basis). The different cultivars of marigold also showed marked variations in total phenols and flavonoids, as well as antioxidant and radical-scavenging activities. Ethanol was confirmed to be the best solvent for extracting both phenols and flavonoids from marigold flowers, while *n*-hexane was the worst. The ethanolic extracts also exhibited the highest antioxidant and radical-scavenging activities. The cultivar *Xinhong* had the highest phenolic and flavonoid contents and radical-scavenging activity, as well as one of the highest lutein contents and antioxidant activities. [37]

Calendula arvensis L. and *Calendula officinalis* L. are also well known plants in Family Compositae as marigold. Cetkovic et.al had investigated the antioxidant activity of the methanolic and water extracts of growing wild marigold, *Calendula arvensis* L. (GWM) and cultivated marigold, *Calendula officinalis* L. (CM), in a concentration range of 0.10-0.90 mg/ml, The GWM and CM extracts were evaluated on three different free-radical species: 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH), hydroxyl radical and lipid peroxyl radical using electron spin resonance (ESR) spectroscopy. These extracts of CM and GWM, scavenged all types of investigated radicals in dependence on their applied concentrations. Generally, CM extracts possessed better scavenging and antioxidant activity than GMW extracts, while methanolic extracts exhibited lower activities than water extracts. Water extracts of CM had the best antioxidant properties; 0.75 mg/ml extracts completely eliminated hydroxyl radical. The same concentration of this extract scavenged 92% DPPH and 95% peroxyl radical during lipid peroxidation. Antioxidant properties were in correlation with the contents of total phenolic compounds and flavonoids in

extracts. The formation of o-semiquinone radicals from rutin and caffeic acid in lipid peroxidation system proved the mechanism (hydrogen donating and/or one-electron reduction) of free-radical scavenging activity. The ESR data demonstrate that methanolic and water extracts of CM possess similar free radicals scavenging and antioxidative activity as synthetic antioxidants BHA. [38]