# CHAPTER 1

# Introduction

## 1.1 Historical Background

Teleost fish are of high interest for humans in three areas including, as resource of diet, as ornamental pet, and as animal model for research purposes. For being human food resource, wild stocks of fish were dramatically decreased due to overconsumption and other environmental factors. Therefore, aquaculture has been grown continually to respond to fish declining and to respond to increasing consumer demand (Gormaz, 2014). To reach those purposes, all-male fish high production is desirable in many fish species due to higher growth rates and giving better coloration with controlling unwanted breeding. Many researchers emphasize on fish reproductive mechanism therefore to develop new strategy for inhibiting estrogen synthesis.

The reproductive system of female fish is influenced by estrogens in the early phase, such as growth of oocytes, and by the maturation inducing steroid (MIS) in the late phase, such as promoting oocyte maturation and then ovulation. In the early phase, the follicle-stimulating hormone (FSH) from pituitary stimulates the follicular cells to secrete estrogen into the circulation, and stimulates the synthesis of vitellogenin in the liver (Kime, 1993). Vitellogenin is glycolipophosphoprotein which function as nutritional provision for the developing embryo (Kohli, 2005) and down-regulation by function of ovarian aromatase (Lubzens, 2010). While the concentrations of female sex hormone and vitellogenin are dropped, the 20β-hydroxysteroid dehydrogenase (Hsd20b) is up-regulation in the granulosa cells and stimulated by luteinizing hormone (LH) from pituitary resulted initiation the processes of oocyte maturation by MIS (Nagahama, 2008) which is a prerequisite for fertilization (Tokarz, 2015).

These mechanism knowledge leading to develop blocker of each critical point for fish masculinization especially aromatase (Cyp19) which responsible for generating of

C18 female sex steroid hormones and is thus the most important key in regard of control sexual development in teleost fish (Böhne, 2013; Mills, 2014).

## 1.2 Objectives

1.2.1 To screen the aromatase inhibitors (AIs) from Thai plant.

1.2.2 To investigate the cytotoxic activity of the plant AIs

1.2.3 To investigate masculinize activity of the plant AIs

1.2.4 To develop the plant AIs nanoparticles

1.2.5 To investigate the effect of plant AIs nanoparticles on masculinization

## 1.3 Literature Review

In aquaculture, sex reversal can occur by social conditions or by ecological factors, such as water temperature, photoperiod, nutrition, endocrine disrupting chemicals or by man-made chemicals. Several sex reversal techniques have been employed for fish production of monosex or sterile population, especially male fish, due to faster growth, better coloration, or enhanced marketability (Lee, 2006). Therefore, the utilization of synthetic and natural compound to promote sex differentiation by inhibiting estrogen synthesis was issued for fish masculinization.

### 1.3.1 Fish masculinization

Fish masculinization can be manipulated by 3 large techniques included using genetic modification technology for super male production, implementation of androgen or androgen-like compound to increase male fish hormone, and administration of aromatase inhibitor.



A super male fish (YY) could be obtained from feminizing males (XY) by using estrogens prior their sexually identifying stage through progeny test and after that breeding with normal males (XY), which resulted three genetically possibilities: super males (YY), males (XY), and females (XX). However, identification stage of sex-reversed female is complicate and taking long time according to karyotype and morphology are not different from genetically female sibling (Mair, 1997; Alcántar-Vázquez, 2014). Furthermore, this strategy is not effective absolutely with taking high costs (Green, 1997).

Hormonal supplementation is the most effective techniques for fish masculinization, Fish is fed with exogenous sex steroids e.g., androgens or androgen-like compound during sex differentiation to genetically male and then transforms the sexual phenotype to the male gender. The chemicals treatment must be performed throughout the critical period to induce sex reversal in an effective manner (Nakamura and Takahashi, 1973). The 16 natural or synthetic androgens have been used to masculinization in over 35 species within the Anabantidae, Cichlidae, Cyprinidiae, Cyprinodontidae, Poecilidae, and Salmonidae. The common administration is dietary supplementation. The chemicals first dissolved in alcohol and then mix with the diet. This recipe is easy manipulation but has many disadvantages such as the chemicals can be degraded while passage through the digestive tract, the lack of uniformity of the chemicals in feeding and resulted variability in dose among treated individuals. Furthermore, excessive doses result sterility or paradoxical feminization (Goudie, 1983). Immersion techniques also have been employed successfully and revealed more efficient than oral administration in small fish and in some species (Baker, 1988). The injection or implantation methods also have been not successful and are not easy to manipulate in small fish. Although the treatments are widely manipulated in masculinization, it also takes costs with not friendly to environments (Andersson, 1999; Golan, 2014).

Interestingly, aromatase inhibitors (AIs) administration seem to be the most practical for both small and large all male production with environment-friendly due to their specificity on enzyme. This technology is challenged in aquaculture since 1987 (Steele, 1987) and recognized as one of the most effective means to verify endogenous estrogen in ovarian differentiation. The AIs, include exemestane (Ruksana, 2010; López, 2014), fadrozole (Ankley, 2002; Komatsu, 2006), letrozole (Gao, 2010; Shen, 2013) have been used on masculinization in non-mammalian animals including teleost fish due to reducing estrogen biosynthesis (Afonso, 1997). Furthermore, the AIs from natural resources are also interesting according to their biodegradable properties.

### 1.3.2 Aromatase

Aromatase is the rate limiting NADPH-cytochrome P450 (CYP450) enzyme which response to conversion of androgens (testosterone and androgen) to estrogens (estradiol and estrone), and in fish, is most active in microsomal membrane of endoplasmic reticulum of gonad and brain tissue (Ahima, 2000). However, it is also found in other tissues, such as liver, kidney, adrenals, brain, muscle and fat. Unlike mammals, some teleost fish species, including tilapia and zebrafish, contain multiple copies of the aromatase gene. These genes have differential expression in different tissues and have high expression in the brain, and the ovary. The enzyme activity in fish is also varying during development in brain tissue preceding in the gonads (Kishida, 2001) and play important role in sex differentiation. The structure and function of aromatase were influenced from two distinct gene loci, cyp19a, and cyp19b in ovary and brain, respectively (Tong, 2001). However, the amino acid sequences of aromatase encoded from these genes have high similarity of different teleost fish (Lee, 2014). Interestingly, the active site residue (in β4 sheet) glutamine remains highly conserved among all fish species but exhibits divergence: histidine in amphibians, reptiles, birds, and mammals (Hong, 2009).

Aromatase catalyzes the conversion androstenedione (ASD) to estrone (E1) in the presence of oxygen and NADPH, through the reaction in the synthesis of estradiol (E2) (Meunier, 2004). The enzyme also has the important role in biotransformation of xenobiotics, drugs, steroids (Guengerich, 2004) and has attractive target for a selective inhibition of estrogen biosynthesis while its blockade is not interfere the other steroids production. Several classes of aromatase inhibitors have been developed as potential therapeutic agents (Brodie, 1997). The pathway of steroidogenesis in teleost fish is indicated as Figure 1.1.

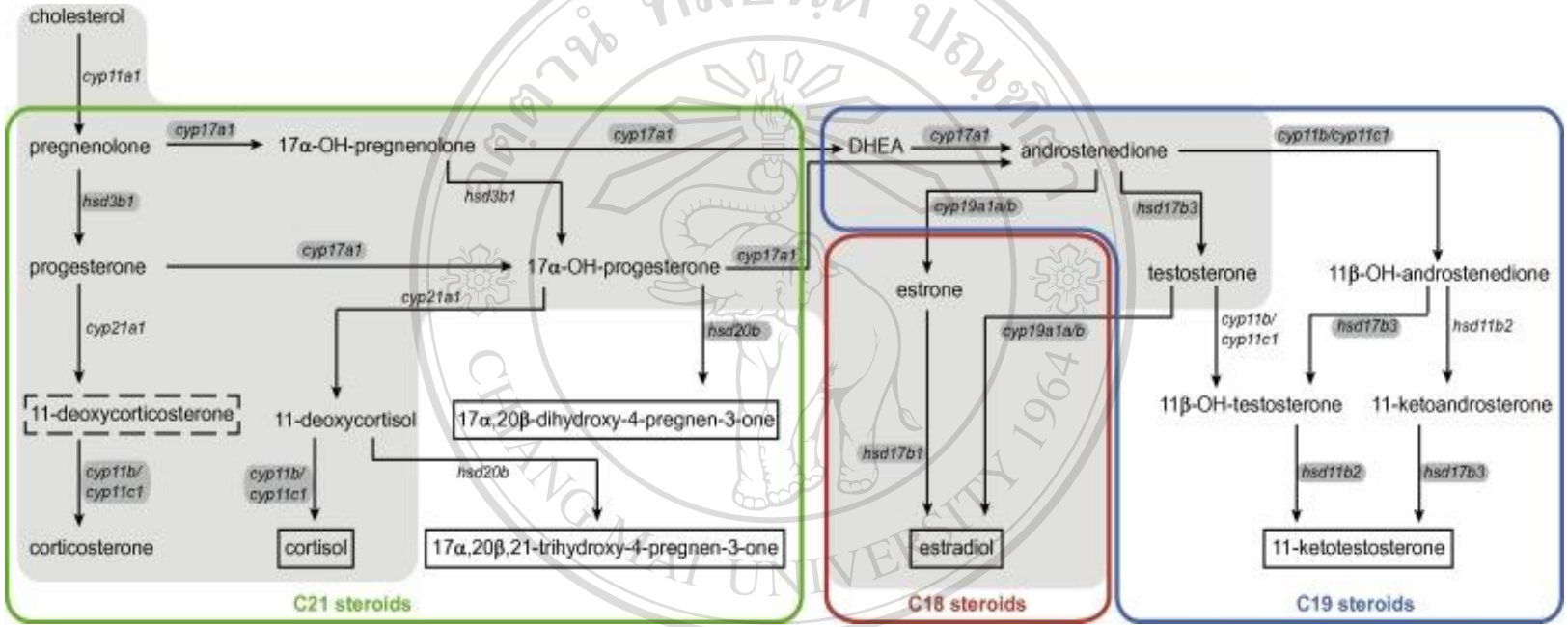



Figure 1.1 The pathway of steroidogenesis in teleost fish. The highlight in gray is main pathway of steroidogenesis, which is similar to humans. Cyp11b gene is commonly found in the most teleost fish, and cyp11c1 is found only in zebrafish (Tokarz, 2015).

### 1.3.3 Aromatase inhibitors

Aromatase inhibitors (AIs) developed for human therapeutic, used in treatment of estrogen-dependent cancer, have been shown effectiveness in studies on many classes of non-mammalian vertebrates (Forlano, 2006). There are three-generation AIs nowadays including type I steroidal AIs and type II non-steroidal AIs, which have been approved by United States FDA for treatment of hormone-dependent breast cancer in ER-positive woman. Aminoglutetimide is a first-generation AIs for the treatment of advanced breast cancer but was terminated using due to high toxicity and low selectivity towards aromatase. The second-generation (formestane and fadozole) and third-generation (exemestane, anatrozole, letrozole) revealed lower toxicity and higher selectivity with greater oral bio-availability. The structure of these AIs was revealed in figure 1.2.

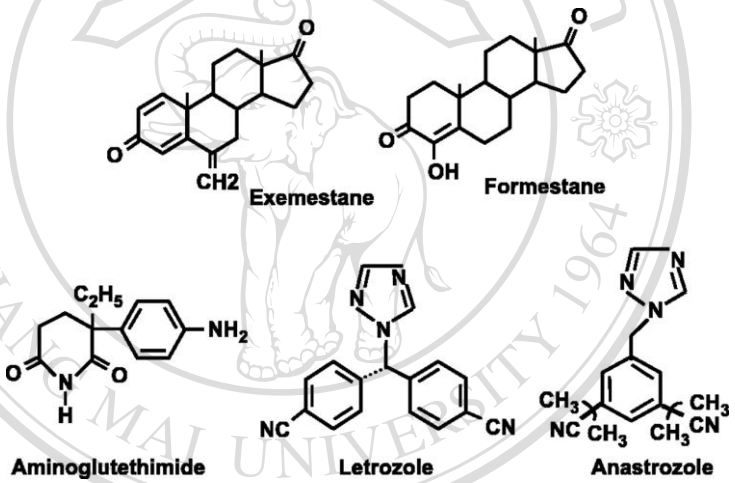



Figure 1.2 The molecular structure of the first-generation AIs; aminoglutethimide, the second-generation AIs; formestane and the third-generation aromatase inhibitors; anastrozole, letrozole, and exemestane (Lønning, 2005).



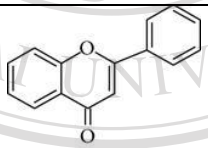
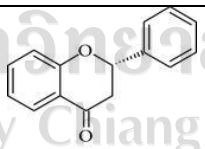
The development of non-steroidal AIs for fish masculinization is considered to be of strategic value to the aquaculture industry. Although potent steroid and non-steroid AIs are present available, flavonoid as natural compound with less side effects has been described as the candidate for biosafety aspect and cost effectiveness.

Flavonoids represent as a group of phytochemicals exhibiting many functions of biological activities arising mainly from their antioxidant properties and ability to modulate several enzymes or cell receptors. Among the enzymes that interact with

flavonoids, cytochromes P450 (CYPs) monooxygenases, metabolizing endogenous substrates (e.g. steroids), play a prominent role (Hodek, 2002). Certain classes of flavonoid compounds are assigned as phytoestrogens since their structure similar skeleton of estrogens. These flavonoids can bind to estrogen receptor and modulate its activity. Some flavonoids are having an anti-estrogenic effect, exhibit an anti-cancer activity, especially in tissues (breast, ovary) which exposed to sex hormones (Chen, 1998). Thus, flavonoids as the steroidogenic enzyme inhibitors are extensively studied for utilization in fish masculinization.

Naturally occurring flavonoids proved to be potent aromatase inhibitors (Table 1.1) shows the compound structures of flavones and flavanones that have greater aromatase inhibitory activity than isoflavones and isoflavanones, which are known to exhibit affinities to bind to estrogen receptor (kumar, 2013). Unfortunately, the low solubility of the flavonoids in water often presents for its applications. Hence, the development of water-soluble and slow-releasing entrapped flavonoid by using nanotechnology has been implicated for fish masculinization.

Table 1.1 The structure of potent aromatase inhibitory activity flavonoids. (Kumar, 2013)

| **Group of flavonoid** | **Structure backbone** | **Examples** |
|---|---|---|
| Flavone |  | Apigenin, Luteolin |
| Flavanone |  | Hesperetin, Naringenin |

### 1.3.4 Nanocarriers

Nanocarrier is the materials in nano-sized range which being used as a transport module for another substance, such as hydrophobic drug. The commonly used nanocarriers include carbon-based materials, noble metals, polymeric micelles, and lipid-based materials. Interestingly, the most common types of nanoparticles used for drug delivery are polymeric nanoparticles, solid lipid nanoparticles (SLNs), crystal nanoparticles, liposomes, micelles, and dendrimers. Interestingly, micelles are able to contain either hydrophilic or hydrophobic drugs depending on the orientation of the phospholipid molecules (Rezaei, 2012).

Nanotechnology (e.g. polymeric micelles) has benefits of achieving site specific delivery especially for enzyme inhibition. Polymeric nanoparticles provided flexibility in design because different polymers from synthetic or natural sources are available include cyclodextrin, poloxamer, and polylactide-co-glycolide (PLGA) and bilosome.

#### 1.3.4.1 Cyclodextrins

Cyclodextrins (CDs) are a family of cyclic oligosaccharides, consisting of a number of (1→4)-linked d-glucopyranose subunits. The common CDs, named α-, β- and γ-CDs, are composed of six, seven and eight glucoses, respectively. The shape of CDs are look like a truncated cone with central cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges, which give it a lipophilic character as shown in figure 1.3.

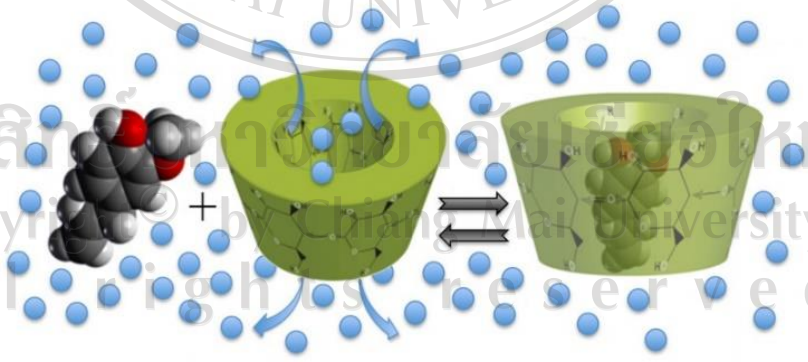




Figure 1.3 The formation of a typical cyclodextrin inclusion complex (Mura, 2014).

The three-dimensional structure of the CDs molecules, characterized by a hydrophilic outer surface, and an internal relatively hydrophobic cavity, is responsible for both water solubility and ability to entrap within their cavities hydrophobic

molecules of suitable size. Polymeric particles or nanoparticles, taking cyclodextrin as the polymer, can improve the solubility of drug, and modulate drug releasing rate without cytotoxicity against fibroblasts (Memisoglu-Bilensoy, 2006). However, αCD was prohibited by United State Pharmacopoeia 28th Edition and βCD also has been reported in some nephotoxicity (Brewster, 2007). Thus, hydroxypropylbetacyclodextrin (HPβCD) was employed in this studies due to its safety and hydroxypropyl groups enhanced the interactions stability of hydrophobic structure with flavonoids (Nguyen, 2013; Yoa, 2014) without toxicity (Gould, 2005; Rasheed, 2008) and HPβCD can solubilized in water more than the others as indicated in Table 1.2.

Table 1.2 Specifications and properties of some cyclodextrins (modified from Loftsson, 2007).

| **Cyclodextrin [a]** | **MW (Da)** | **Solubility in water (mg/ml)[b]** | **Ph.Eur [c]** | **USP/NF [d]** |
|---|---|---|---|---|
| αCD | 972 | 145 | yes | no |
| βCD | 1135 | 18.5 | yes | yes |
| HPβCD | 1400 | >600 | yes | yes |
| γCD | 1297 | 232 | yes | yes |
| HPγCD | 1576 | >500 | no | no |

[a] αCD: α-cyclodextrin, βCD:β-cyclodextrin, HPβCD: 2-hydroxypropyl-β-cyclodextrin, γCD: γ-cyclodextrin, HPγCD: 2-hydroxypropyl-γ-cyclodextrin.

[b] Solubility in pure water at 25 °C.

[c] Ph.Eur.: European Pharmacopoeia 5th Edition (2005)

[d] USP/NF: United States Pharmacopoeia 28th Edition/National Formulary 23rd Edition (2005)



**1.3.4.2 Pluronics**

Pluronics (Poloxamer, Synperonics, or Kolliphor) are the common pharmaceutical triblock polymers used to fabricate polymeric micelles (Oh, 2004), composed of hydrophobic parts of polyoxypropylene (polypropylene oxide; PPO) fringed by two hydrophilic parts of polyoxyethylene (polyethylene oxide; PEO). The PPO core can incorporate of water-insoluble drugs (Kozlov, 2000) while PEO cover the

micelles in a dispersed state and decreases undesirable hydrophobic interactions (Batrakova, 2004). The amphiphilic block copolymer of Pluronics can entrap non-covalent incorporation of hydrophobic drugs into the hydrophobic core of Pluronic micelles resulted in an increase of solubility, stability, and bioavailability. Hence, this polymer has been used for the encapsulation of various water insoluble drugs into the nanoparticles in the form of polymeric micelles (Chen, 2013, Sahu, 2011). Moreover, Pluronics are reported to be bio-compatibility, low toxicity, and low degradation (Mayol, 2008; Morlan, 2008). In addition, Pluronics can minimize adsorption to surfaces due to hydrophilicity (Jindal, 2015). Thus, Pluronics micelles were development by using pluronics F-68 (poloxamer 188) and pluronics F-127 (poloxamer 407) incorporate with non-ionic surfactant to improve solubility of selected flavonoid. The chemical structure and micelles of pluronics were indicated in Figure1.4.

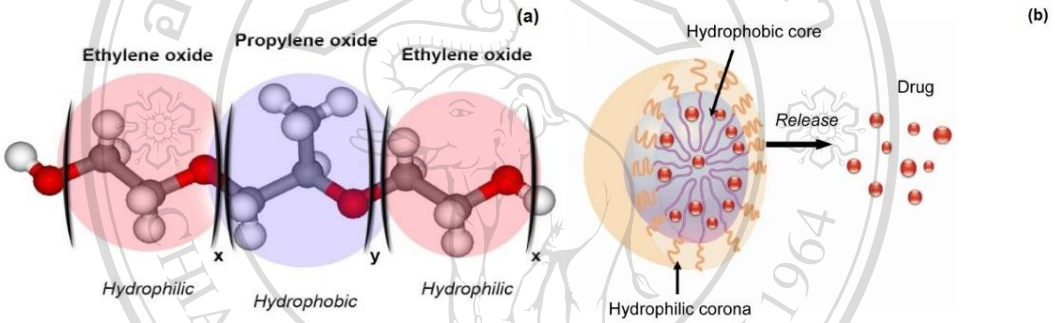



Figure 1.4 Pluronic block copolymer molecules (a) and micelle with drug (b) (Batrakova, 2008).

The coding of Pluronic starts with a capital letter to indicate its physical form at room temperature (L = liquid, P = paste, F = flake) followed by two or three digits. The first digit multiplied by 300 define the rough molecular weight of the hydrophobic parts; and the last digit multiplied by 10 gives the percentage PEO content. Their specifications of Pluronic F-68 and F-127 were provided in Tables 1.3 (Pitto-Barry, 2014).

Table 1.3 Specifications and properties of Pluronic F-68 and F-127

| **Properties** | **Pluronic F-68** | **Pluronic F-127** |
|---|---|---|
| Total MW (Da) | 7,680-9,510 | 9,840-14,600 |
| PEO content (%) | 79.9-83.7 | 71.5-74.9 |
| No. of PEO repeats | 152.73 | 200.45 |
| No. of PPO repeats | 28.97 | 65.17 |
| Physical form | Flake | Flake |
| pH (dissolved in water) | 5.0-7.5 | 5.0-7.5 |
| CMC (Molar) | $4.8 \times 10^{-4}$ | $2.8 \times 10^{-6}$ |
| HLB | 29 | 22 |

CMC = Critical Micelle Concentration; HLB = Hydrophilic-Lipophilic Balance

#### 1.3.4.3 Poly(dl-lactide-co-glycolide)

PLGA is a copolymer that can be prepared at different ratios between its inherent monomers, lactic (LA) and glycolic acid (GA). Depending on the ratio of LA to GA used for the polymerization, different forms of PLGA can be obtained regarding to the monomer ratio used. PLGA can be dissolved by a wide range of general solvents, including acetone, chlorinated solvents, and tetrahydrofuran. PLGA is available as D-, L-, and D, L-enantiomers whereas GA deficits the methyl side group resulted in lacking isomer. PLGA copolymers are degraded by heterogeneous erosion, hydrolysis of its ester linkages in liquid environment. After the degradation process, LA and GA are formed as by-products. The degradation rates can be affected by divergent parameters such as molecular weight, the ratio of GA to LA, stereochemistry (mixtures of D and L lactic acid monomers) and end-group functionalization (Wu, 2001; Lu, 2000). Moreover, the shape of the device and acidic environment are strongly affects PLGA degradation because of initial autocatalysis (Holy, 1999).

Poly (dl-lactide-co-glycolide), PLGA, is a candidate polymer for drug entrapment into nanosize which is biocompatible, biodegradable and approved by EMA (European Medicines Agency) and US FDA (Food and Drug Administration) for human use (Toro, 2004). Low molecular weights of PLGA with higher glycolide content are more hydrophilic and amorphous, and have a shorter deterioration time. Glycolic acid is hydrophilic and absorbs a large amount of water. In the other hands, higher lactic acid content PLGA is more hydrophobic, absorb less amount of water and degraded in a more gradual manner (Schliecker, 2003). Therefore, PLGA has been used for the encapsulation of water insoluble drugs incorporate with surfactant into the nanoparticles in the form of polymeric micelles. The general structure is indicated as figure 1.5.

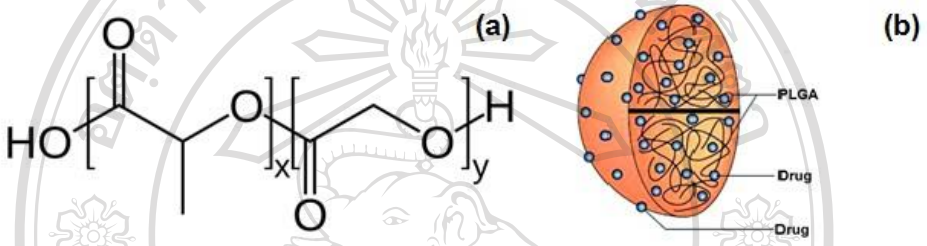



Figure 1.5 PLGA block copolymer molecule (a) and micelle with drug (b) (modified from Vashist, 2014).

By changing the lactide:glycolide ratio and lactide stereoisomeric composition, polymers having *in vivo* degradation times from a few weeks to more than 6 weeks as indicated in Table 1.4.

Table 1.4 Specification and properties of PLGAs (Gentile, 2014)

| polymer | Modulus (GPa) | Solvent | Crystallinity (%) | Degradation time (week) |
|---|---|---|---|---|
| PLGA 85/15 | 2.0 | acetone, chloroform, ethyl acetate, THF | amorphous | 5-6 |
| PLGA 75/25 | 2.0 | acetone, chloroform, ethyl acetate, DMF, THF | amorphous | 4-5 |
| PLGA 50/50 | 2.0 | acetone, chloroform, ethyl acetate, DMF, THF | amorphous | 1-2 |

In this thesis, PLGA 50:50, identified as copolymer consisted of 50% lactic acid and 50% glycolic acid, was used by purpose of enhancing solubility of compound.

**1.3.4.4 Bilosome**

Bilosome, the lipid-based vesicle system for oral route drug delivery (Aburahma, 2016) and transdermal delivery (Al-mahallawi, 2015), is one of recent tools in drug delivery that consist of various lipids, non-ionic amphiphiles (niosomes) forming a closed bilayer structure and incorporating bile salts resulted making them biocompatible (Conacher, 2001). Among the different nanocarriers, Bile salts in bilosomes provides to stabilize the outer surface against the detrimental effects in gastrointestinal tract and enzymatic degradation in nanometric size range with negative surface charge and hydrophobic properties (Jain, 2014). The lipid bilayer encloses an aqueous space where drug candidates may become entrapped (Mann, 2006). In addition, the system is feasibility to scale up resulted can successfully apply the system into a clinical trial. This advance system are also reported in which vesicles have been decorated by anchoring ligand for the variety of receptors such as galactosyl, antigen presenting cells (Ahsan, 2002), mannosyl (Jain, 2014), and vaccines as indicate in Table 1.5.

Table 1.5 The bilosomal formulations used in oral immunization

| Bilosome composition | Vaccine/antigen | reference |
|---|---|---|
| 1-monopalmitoyl glycerol, cholesterol, dicetyl phosphate, deoxycholic acid | influenza subunit | Conacher, 2001 |
| 1-monopalmitoyl glycerol, cholesterol, dicetyl phosphate, deoxycholic acid | influenza vaccine | Mann, 2004 |
| sorbitan tristearate, cholesterol, dipalmitoyl phosphatidyl ethanolamine, succinimidyl (4-N-maleimidomethyl) cyclohexane-1-carboxylate, bovine serum albumin, nadeoxycholate | cholera toxin B subunit | Singh, 2004 |
| 1-monopalmitoyl glycerol, cholesterol, dicetyl phosphate, sodium deoxycholate | tetanus toxoid vaccine | Mann, 2006 |
| sorbitan tristearate, cholesterol, dicetylphosphate, sodium deoxycholate | hepatitis B vaccine | Shukla, 2008 |
| 1-monopalmitoyl glycerol, cholesterol, diacetyl phosphate, deoxycholic acid | influenza vaccine | Bennett, 2009 |
| 1-monopalmitoyl glycerol, cholesterol, dicetyl phosphate, sodium deoxycholate | influenza A antigen | Mann, 2009 |
| sorbitan tristearate, cholesterol, dipalmitoyl phosphatidyl ethanolamine, succinimidyl (4-N-maleimidomethyl) cyclohexane-1-carboxylate, sodium deoxycholate | recombinant hepatitis B surface antigen | Shukla, 2010 |
| Sorbitan monooleate, cholesterol, sodium deoxycholate | Recombinant hepatitis B surface antigen | Arora, 2011 |
| sorbitan tristearate, cholesterol, dicetyl phosphate, sodium deoxycholate | diphtheria toxoid | Shukla, 2011 |
| 1-monopalmitoyl glycerol, cholesterol, dicetyl phosphate, sodium deoxycholate | H3N2 subunit protein | Wilkhu, 2013 |
| sorbitan tristearate, cholesterol, dicetyl phosphate, sodium deoxycholate | recombinant human enterovirus 71 vaccine | Premanand, 2013 |
| glucomannan, non-ionic surfactant vesicles, stearyl amine, sodium deoxycholate | tetanus toxoid vaccine | Jain, 2014 |

The ratios of selected lipids and surfactants make a difference to the vesicle sizing, entrapment efficiency percentage (Shukla, 2016), surface charge and their stability. Generally, bilosome are prepared by adopting either thin film hydration method (Shukla, 2011) or hot homogenization method (Wilkhu, 2013).

Thus, AIs could be protected from the effects of fish digestion in gastrointestinal tract and effectively released AIs at the target cells of zebrafish. The structure of bilosome was revealed in Figure 1.6.

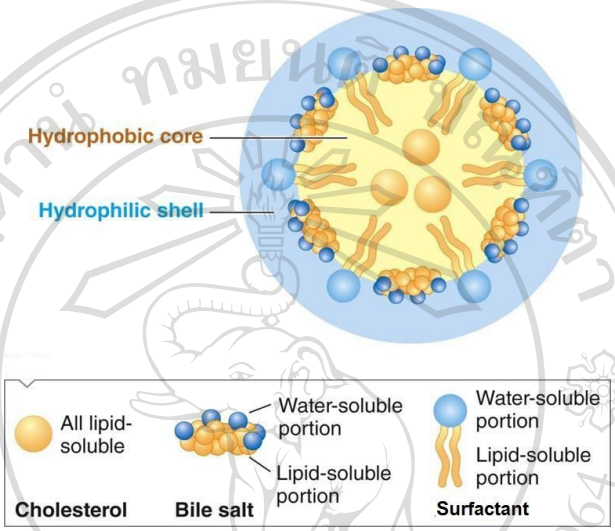



Figure 1.6 The structure of bilosome (modified from www.studyblue.com)

### 1.3.5 Surfactants

Surfactants can be are amphiphilic molecules which have hydrophilic part and lipophilic part that give them two behaviors—adsorption and self-assembly in solution. The applications of surfactants such as wetting, spreading and stabilization of particles resulted in formation of nanoscale structures from polymer to micelles. The structure of surfactants composes the polar, water soluble part (term the "head group") and the non-polar, oil soluble part (termed the "tail group"). The head group should be charged or be polar because of inclusion of oxygen or similar atoms. Depending on the nature of the polar group, surfactants for bilosome development can be classified into four groups: anionic, cationic, zwitterionic and non-ionic as indicated in Table 1.6 (modified from Vieira, 2008).

Table 1.6 The classification and properties of surfactants

| Surfactants | Properties | examples |
|---|---|---|
| Anionic | Negative charge in head group. | (a) Carboxylates: Alkyl carboxylates-fatty acid salts; Carboxylate fluorosurfactants,<br>(b) Sulfates: Alkyl sulfates (e.g., Sodium lauryl sulfate), Alkyl ether sulfates (e.g., Sodium laureth sulfate),<br>(c) Sulfonates: Docusates (e.g., Dioctyl sodium sulfosuccinate), Alkyl benzene sulfonates,<br>(d) Phosphate esters: Alkyl aryl ether phosphates, Alkyl ether phosphates, (e.g., Sodium lauryl sulphate) |
| Cationic | Positive charge in head group. | $RN^+H_3Cl^-$ , $RN^+(CH_3)_3Cl^-$ (e.g., Cetyl trimethyl ammonium bromide) |
| Zwitterionic | Positive or negative depended on pH | $RN^+H_2CH_2COO^-$, $RN^+(CH_3)_2CH_2CH_2SO_3^-$ (e.g., Amino acids, Betaines, Sulfobetaines, Phospholipids, Phosphatidylcholine) |
| Non-ionic | Hydrophilic head group with hydrophobic tail(s) | Polyol esters (e.g., glycol and glycol esters), Polyoxyethylene esters (e.g., Spans, Tweens), Poloxamers. |



For special purpose in pharmaceutical, various exotic surfactant structures have been developed include the bolaform surfactants, which have the head group at either end of non-polar tail (Glaser, 2006), and the gemini surfactants, which have two hydrophilic surfactant units joined by a spacer to make a “twinned” molecule possess a number of superior properties when compared to conventional single-headed, single-tailed surfactants (Chevalier, 2002). Interestingly, biosurfactants, which are surface-active substances synthesized by living organisms, are interesting in recent years due to

their diversity, environmentally friendly nature, possibility of large-scale production (Banat, 2000), and potential applications in pharmaceutical purpose especially bile acids and bile salts (Shukla, 2015).

However, to assist in polymeric nanoparticle synthesis, the most frequently used non-ionic surfactants (e.g. tweens, spans, poloxamer) and bile acid/salt surfactants have been used to develop micelles (Yunomiya, 1998), reversed micelle microemulsions, and other surfactant self-assembly systems. These surfactants provided flexibility in design according to their different length and polarity are available include

#### 1.3.5.1 Tween 20

Tween 20 is registered trademarks of Croda Americasa non-ionic surfactant formed by the ethoxylation of sorbitan prior adding lauric acid with 20 repeat units of polyethylene glycol (Ayorinde, 2000). Its stability and relative nontoxicity allows it was used as detergent, stabilizer and emulsifier in pharmacological applications. Tween 20 is heat sensitive and will be darken by higher temperatures. Interestingly, It was commonly used in development of polymeric micelles (Yamagata, 2009), niosome and bilosome nano-size ranges (Bayindir, 2010; Al-mahallawi, 2015).The chemical structure was shown in Figure 1.7 (Gelain, 2000).

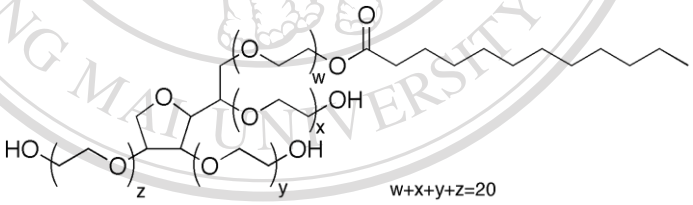



Figure 1.7 The chemical structure of Tween 20
(source https://en.wikipedia.org/)



#### 1.3.5.2 Tween 80

Tween 80 or Polysorbate 80, viscous water-soluble yellowish liquid, is a nonionic surfactant derived from polyethoxylated sorbitan and oleic acid. The hydrophilic parts are polyethers (polyoxyethylene groups) and the lipophilic part is oleic acid. It is often used in foods and cosmetics to form polymeric micelles (Choi, 2015). Interestingly, It was found that polysorbate 80 coated-polymeric nanoparticles pass through the brain faster and the uptake is 35% higher than regular nanoparticles

(Lobenberg, 1998; Xu, 2011). The chemical structure was shown in Figure 1.8 (Chou, 2005).

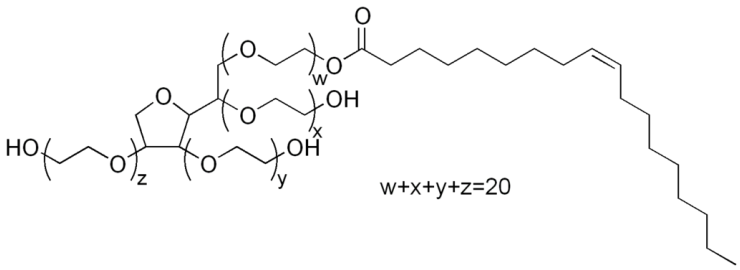



Figure 1.8 The chemical structure of Tween 80
(Source: https://en.wikipedia.org/)

**1.3.5.3 Span 60**

Span 60 or Sorbitan monostearate, brownish yellow wax or beige flakes, is an ester of sorbitan and stearic acid. It is solubilized in hot ethanol, benzene and hot oil; slightly soluble in ether and petroleum ether. Span 60 is water/oil type emulsifier. There was no evidence of carcinogenic activity and had no adverse effects on the death rate or body-weight gain in mice which receiving 2% of this surfactant (Hendy, 1978). Span 60 is a commonly used non-ionic surfactant in bilosome formation (Al-mahallawi, 2015). The structure of Span 60 was revealed in Figure 1.9.


Figure 1.9 The chemical structure of Span 60
(source: https://commons.wikimedia.org/)



**1.3.5.4 Span 80**

Span 80 or Sorbitan monooleate, is a mixture of the partial esters of sorbitol and oleic acid. It is an amber-coloured oily viscous liquid, light cream to tan beads or flakes which solubilized in aniline, dioxane, ethanol, ether, ethylacetate, petroleum ether, toluene and slightly soluble in warm water. Span 80 is water/oil type emulsifier. There was some evidence of adverse effects on body-weight gain in mice which receiving 10% of this surfactant (Ingram, 1978). Span 80 is also the generally used non-ionic surfactant

in niosome formation (Jain, 2014) and bilosome formation due to enable encapsulated hydrophobic drugs (Bayindir, 2010; Al-mahallawi, 2015). The structure of Span 80 was revealed in Figure 1.10.

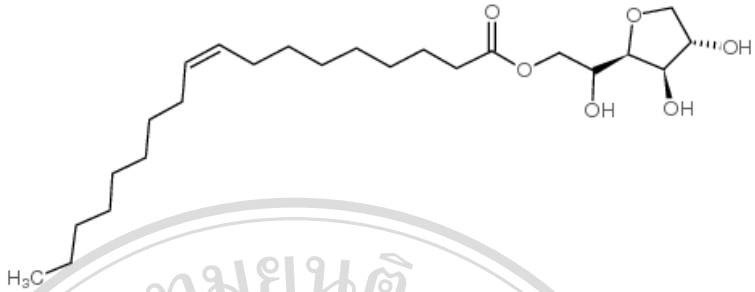



Figure 1.10 The chemical structure of Span 80
(source: http://www.chemicalbook.com/)

**1.3.5.5 Bile salts and Bile acids**

Bile acids are steroid acids found predominantly in different molecular forms in mammals and other vertebrates (Hofmann, 2010). It can be synthesized in the liver and conjugated with taurine or glycine in the liver, forming bile salts. The conjugated salts of their 7-alpha-dehydroxylated derivatives, deoxycholic acid and lithocholic acid are also found acting as a surfactant that emulsifies them into micelles. Bile acids are weak acids which apparent various pKa values due to the main structure of steroid nucleus. The various bile acids used in drug delivery system are revealed in Figure 1.9.

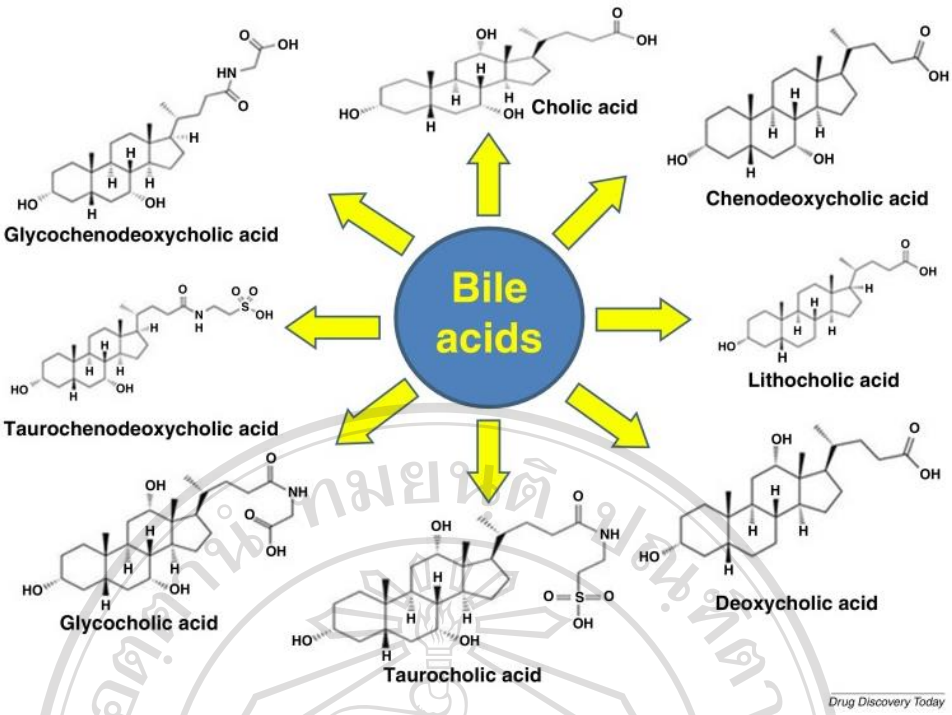



Figure 1.11 The structure of bile acid derivative (Shukla, 2016)

Bile salts are amphipathic steroidal biological surfactants derived from cholesterol (Moghimipour, 2015) that contain a hydrophilic steroid nucleus encompassing hydroxyl groups and methyl groups. Bile salts play important role in emulsifying and solubilizing fats by forming of mixed micelles resulted in improved the passage of hydrophobic molecules through biological membranes, thus enhancing the oral bioavailability (Holm, 2013).

Base on their structure and properties, various mixed micelle systems were successfully employed bile acids or bile salts for improving solubility of hydrophobic compounds such as silybin (Yu, 2009), rifaximin (Darkoh, 2010), Itraconazole (Le Dévédec, 2013). In another study, the bile acid coated nanoparticles revealed improved delivery of docetaxel in cervical cancer (Zeng, 2013). The deoxycholic acid is also used to enhance the oral bioavailability of biodegradable nanoparticles (Samstein, 2008).

### 1.3.6 Zebrafish

The National Institute of Environmental Health Sciences (NIEHS) in the United States and The Institute for Environment and Sustainability (IES) in Europe approve the zebrafish as animal model due to the success of using zebrafish in environmental

research compared to other established model systems. On the other hand, zebrafish is a well-known established model species which has numerous benefits for studying due to a high degree of human genome homology and cost-effective to work (Bar-Ilan, 2009), short generation time, easily manipulate in a relative small space (Oliveira, 2011). It also provides conceptual insights into many aspects of vertebrate biology and genetics (Segner, 2009). A further advantage of zebrafish is the availability of a variety of different strains with different properties which enables selection of strains specifically for the experimental purpose (Loucks, 2004). Zebrafish can be housed in small facilities for large numbers production resulted reduces husbandry costs. The short generation time is also an advantage for chronic or genetic studies.

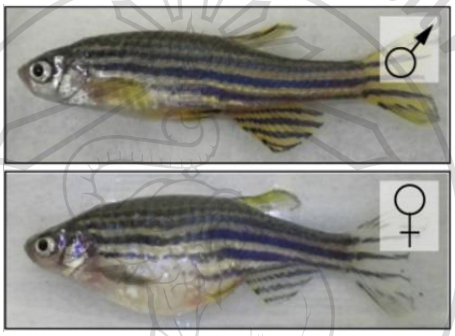


Figure 1.12 Male and female zebrafish with secondary sex characteristics: body shape and fin color (Baker, 2013).

Identifying male and female zebrafish is distinguished from their body, coloring and behavior (Figure 1.12). The body shape of a male zebrafish is slender compared to the female. Female tend to move slower than male and also be notable from males by a silvery-blue line on the lateral side of the body (Spence, 2008). The zebrafish embryos develop outside the mother so that they can be easily manipulated, for instance, by water exposure. The crucial advantages of the zebrafish model for phenotypic screening promoted its use as pharmacological tool in drug discovery (Goldsmith, 2004; Hill, 2005) and as a screening model to study the effects of EDCs on vertebrate organogenesis (Li, 2016).

## 1.4 Rationale and Hypotheses

Masculinization of zebrafish can be produced by direct synthetic hormonal treatment that is efficient and straightforward (Santos, 2006) however synthetic hormones are more expensive than plant extracts and their fate in water, sediment and there is many providing information on the potential risks of using synthetic hormones (Contreras-Sanchez et al., 2001). An alternative technique for all-male production is perhaps to use plant extracts as aromatase inhibitor for masculinization. Many kinds of flavonoids were found potentially on inhibiting aromatase enzyme *in vitro*. However, the limitation of flavonoids solubility is the big problem for utilization in masculinization due to lack of bioavailability in fish. To overcome this point, nanotechnology is immersed therefore increasing solubility and targeting the tentative natural compound to aromatase in female zebrafish. Hence, there is high possibility to achieve the expected positive results from using the nanocarriers for zebrafish masculinization.