MECHANISTIC STUDY FOR ANTI-MELANOGENIC PROPERTY
OF TETRAHYROCURCUMIN AND ENHANCEMENT OF
BIOACCESSIBILITY THROUGH BOTH TOPICAL DELIVERY AND
ORAL DELIVERY BY INNOVATIVE FORMULATIONS

by

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ABSTRACT OF THE DISSERTATION

MECHANISTIC STUDY OF THE ANTI-MELANOGENIC PROPERTY OF TETRAHYROCURCUMIN AND EVALUATION OF ITS BIOACCESSIBILITY THROUGH TOPICAL ROUTE AND ORAL ROUTE

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Tetrahydrocurcumin (THC) is one of the major metabolites of curcumin, and it possess a variety of health-related benefits, such as anti-cancer, antioxidant, anti-inflammation, etc., Skin hyperpigmentation could be induced and severly by the agressive accumulation of reactive oxygen species (ROS) generated by the external stresses such as constant exposure to UV, air pollution etc., Based on the anti-oxidant properties of THC, it might help to protect skin from any deleterious effects associated with ROS. However, because of its hydrophobic chemical structure and low aqueous solubility, its application has been quite limited. In order to solve this, two innovative formulation strategies were used including nanoemulsion and
polymer conjugate. The objective is to solve the aqueous solubility challenge for THC and enhance its bioaccessibility through both the topical and oral route respectively.

In the first part of this work, efficacy of THC as an anti-melanogenic agent was explored. *In vitro* tyrosinase activity method was utilized in order to study the efficacy of THC on the inhibition of tyrosinase, the key enzyme involved in the melanogenesis process. Later *in vitro* B16F10 melanoma cell model was studied, the data confirmed that THC is capable of reducing the α-MSH (melanocyte-stimulating hormone) induced production of melanin. Additionally, the protective role of THC on the keratinocytes was also investigated using the *in vitro* H$_2$O$_2$ induced oxidative stress model. Through this study, the data showed that THC could significantly reduce the ROS level after the oxidative stress. In addition, THC was able to reduce the production of α-MSH in the H$_2$O$_2$ induced oxidative stress keratinocytes model. As this hormone is the messenger between keratinocytes and melanocytes controlling the melanogenic process. Therefore, THC’s anti-melanogenic property and its mechanism was confirmed.

Secondly, phospholipid based nanoemulsion was designed to enhance topical delivery efficacy of THC. The solubility of THC in three different carrier oils were determined. Medium Chain Triglyceride (MCT) showed the maximum solubility for THC, which is around 9.4 mg/mL compared to 1.2 mg/mL in sunflower oil and 0.6 mg/mL in argan oil. Later, a penetration enhancer, Diethylene Glycol Monomethyl Ether (DEGEE, commercial name: Transcutol®), was introduced and combined with MCT in order to find the optimized solubility for THC as well as reasonable solvent-solute effect. Last, the final formula was composed of MCT, Diethylene Glycol Monomethyl Ether, water and phospholipids. The stability of the formula was monitored at 50°C and room temperature.
The permeation performance of this nanoemulsion vs. THC suspension was conducted using \textit{in vitro} Franz diffusion cell model. This study confirmed that delivery efficacy for THC in the optimized phospholipid based nanoemulsion vesicle could be significantly increased compared to the free THC suspension.

Thirdly, tetrahydrocurcumin-hyaluronic acid (THC-HA) conjugate was synthesized for the purpose of increasing the bioaccessibility of THC through oral route. Characterizations including $^1$H-Nuclear Magnetic Resonance ($^1$H-NMR), differential scanning calorimetry (DSC) and x-ray diffraction (XRD) were used to confirm the formation of the conjugate. \textit{In vitro} dissolution drug release in both simulated gastric fluids (SGF) and simulated intestinal fluids (SIF) was studied to evaluate the stability and integrity of the conjugate in the gastrointestinal tract. The result concluded that the conjugate remained stable with less than 10\% w/w THC released from the conjugated form in both of the conditions up to 4 h. Later, the bioaccessibility was evaluated using \textit{ex vivo} Franz cell model using small intestine from porcine and \textit{in vitro} TNO dynamic gastrointestinal model-1 (TIM-1). Both models confirmed that THC-HA conjugate was able to enhance the overall bioaccessibility for THC. This could be explained by both the solubility enhancement and mucoadhesive property from hyaluronic acid that contribute directly to the bioaccessibility enhancement.
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CHAPTER 1. Introduction

Skin is the largest organ in the human body, and it interacts directly with the external environment. Its main functions include the regulation of the moisture content and the temperature within the body, protection from external microorganism, UV radiation, chemicals etc. [1,2]. The wellness of the skin not only represents our identity, but also it has a dramatic effect on mental health. Skin related disease is ranked as the fourth most frequent cause of human disease globally and regardless of the age, skin disease could make an impact on a newborn or older generation [154].

Skin is composed of three main layers, including epidermis, dermis, and hypodermis. Epidermis, as Fig. 1 shows, is the outermost layer and functions as a physical barrier to protect the body. Within the epidermis layer, there are three types of cells, i.e., keratinocytes, melanocytes and langerhans cells. More than 90% cells in the epidermis are keratinocytes, they are responsible to produce keratin and synthesize epidermal lipids that form the skin barrier. Epidermal lipids are composed of ceramides, cholesterols and free fatty acids protecting the skin from water loss, intrusion from pathogen etc., [65]. They are located in the stratum corneum surrounding the corneocytes, which is always described as the “brick-mortar” structure. When the barrier is disrupted, it might lead to the skin disease such as atopic dermatitis. Additionally, it was studied before and concluded that barrier disruption might also activate T-cells, which casus inflammation [124]. Keratinocytes also provide pro-inflammatory cytokines and chemokines to defend against infection [102]. Additionally, Keratinocytes cross-talk with other cells such as melanocytes, fibroblasts, langerhans etc., to play a vital role in maintaining the skin homeostasis. For example, it was pointed out that keratinocytes-fibroblasts work together in the wound healing process [168]. Keratinocytes
interacts with melanocytes to control the production of melanin, the detailed process was presented in Fig. 2. Alpha-MSH secreted from keratinocytes works as agonists for the melanocortin 1 receptor on melanocytes, then expressions for the enzymes involved in the melanogenic process would be activated leading to the melanin production [15]. Another type of the cells in the epidermis layer is langerhans cells. They work as defenders and protect skin from the infections. When foreign matter is introduced, langerhans cells would evaluate and decide the level of the immune response. Both langerhans and keratinocytes are critical to the immune property of the epidermis [14, 125]. Melanocyte is located at the boundary between epidermis and dermis layer. the key function of melanocyte is to produce melanin that is responsible for skin pigmentation.

Dermis is the layer connecting the epidermis and the subcutaneous tissue. The main functions of the dermis layer are to provide the skin strength and elasticity, regulate the body temperature and sensation. There are four main cells in this layer including fibroblasts (primary), adipocytes, mast cells and histocytes [105]. Among those, fibroblasts are particularly interesting as they are in charge of the biosynthetic process for collagen, which is critical as it is in charge of building the structure for many tissues including skin. The biosynthetic rate of collagen decreases significantly during the ageing process. Besides the intrinsic ageing process, excessive UV is also known to jeopardize the structure in the dermis layer as well. For instance, it was demonstrated that after the UV induced damage, collagen-degrading enzymes would be up regulated leading to the damage to the skin structure. Additionally, the generation of ROS after the absorption of UVA (320-400 nm) would degrade the existing collagen as well, causing the damages to the skin structure and bringing the threats to other cells [112, 113]. Today, collagen supplements are very popular
among the consumers. In general, there are three main types, including injectable collagen products, topical collagen products and oral collagen supplements. Many studies demonstrated that oral intake of collagen peptide could increase skin elasticity as well as some moisturization effect, those two effects were more pronounced compared to collagen applied through topical route \([114, 115]\). The large molecular size of collagen and hydrophilic nature make them almost impossible to permeate through the stratum corneum, which might be the main reason about low efficacy from topical application.

Hypodermis is the deepest layer in the skin, and it is the home for adipose tissue. Unlike dermis layer, where it is nearly made up of 60% water. The majority of the hypodermis layer is made of fat. This layer also interlaced with blood vessel and nerves. Adipocytes are the main cells in this layer \([129]\). Unlike epidermis and dermis, hypodermis have been given much less attention and were treated simply as reservoirs of energy, as thermal., or as structural support \([130]\). Instead, it was found that dermis, specifically adipocytes in this layer is involved with the hair follicle growth and skin wound healing.
1.1 Oxidative stress and melanogenesis

Oxidative stress is defined as the imbalance of accumulation of reactive oxygen radicals (ROS) and insufficiency of the antioxidants. Oxidative stress is one of the main challenges that threatens human’s health. At varying degrees, it could lead to the inflammation or even carcinogenesis [155]. Melanogenesis is the process taking place in melanocytes to produce melanin, and this process could be impacted by the oxidative stress. Today, the role of oxidative stress on melanogenesis has been well studied and confirmed that oxidative stress could stimulate the melanogenesis causing the skin hyperpigmentation [15].

Specifically, when excessive ROS is generated and started to accumulate in the epidermis layer, it permeates through nuclear membrane in keratinocytes, reacts with DNA-associated transition metals to generate hydroxyl radical and cause DNA base damages for the cells [6, 7, 8, 9] Additionally, many biochemical factors are synthesized excessively as
well, such as alpha-Melanocyte-stimulating hormone (α-MSH), endothelin-1 (ET-1), stem cell factor (SCG), etc., they are then transferred from keratinocytes to melanocytes [10, 11]. When melanocytes receive the excessive biochemical factors from keratinocytes, as shown in the below Fig. 2 and Fig. 3, it leads to the activations of expressions for the tyrosinase-related proteins including tyrosinase-related protein-1 (TRP-1) & tyrosinase-related protein-2 (TRP2). This then leads to the overproduction of melanin as a result that causes the skin hyperpigmentation [15,60].

Melanocyte itself is also vulnerable to oxidative stress [60]. After the ROS attack, more melanin are generated as a result. The main is the expressions for tyrosinase, trp-1 and trp-2 involved in this process, are usually up-regulated after the accumulation of ROS. Among those, tyrosinase is particularly important. The reason is that during the melanin synthetic process, the first two steps are the rate-limit steps, which include the conversions from tyrosine to L-dihydroxyphenylalanine (L-DOPA) and/or to dopaquinone. Tyrosinase is the enzyme in charge of the rate of those two reactions [60, 109]. When melanin starts to be accumulated in the epidermis, it functions as a shield to protect skin from UV induced damages by absorbing ultraviolet radiation. However, during the biosynthetic process for melanin, as Fig. 4 shows, ROS including hydrogen peroxide and superoxide anions are generated, which makes the melanocytes more susceptible to the oxidative stress.

When melanin is over produced, it could also be dangerous to skin. Additionally, melanin might work as a UVA and UVB sensitizer that is responsible for the cell death [110]. Also, it has been speculated that pheomelanin, as one type of melanin, might have a carcinogenic effect leading to melanoma, a serious type of skin cancer [111]. Therefore, it is critical to maintain a tolerable rate for the generation of melanin. There are a variety of
different sources that cause the hyperpigmentation, as previously discussed, oxidative stress could be viewed as one of the main sources that impacts the homeostasis of melanocytes and triggers over production of melanin. Based on this, it is critical to maintain the ROS level within the epidermis layer to prevent the potential damages to the cells, inhibit the excessive melanogenesis triggered by the oxidative stress [15].

**Figure 2.** Pathways of UV or VL triggered ROS that induced pigmentation, reprinted from [15].
Figure 3. Graphical presentation of keratinocyte-melanocyte communication pathway reprinted from [59].

Figure 4. Biosynthetic pathway of melanin and the generation of reactive oxygen species reprinted from [60].
1.2 Polyphenol and its application in the relief of oxidative stress induced skin threats

Polyphenol is defined as the secondary metabolites of plants and characterized by multiple phenol units [99]. Based on the structure, it could be further categorized into the following groups including flavonoids, stilbenes, lignans, phenolic acids. As natural or naturally-derived ingredients are becoming more and more popular in the cosmetic industry, polyphenol is getting a lot of attentions not only because of its natural characters but also their countless health-related benefits. Below summarized some potential mechanisms for the beneficial functions of polyphenol.

1.2.1. Antioxidant

Our skin directly interfaces with the external environment, which frequently brings the stress to the skin. A clinical study demonstrated that the sunlight and the particle matter pollution would dramatically deplete the vitamin C and vitamin E from the skin surface, which left our skin fragile to the oxidative stress [100]. Additionally, constant exposure to the UV light leads to the generation of ROS. Aggressive generation and then the accumulation of ROS in the skin would cause damages to the skin cells, or even lead to skin cancer. Additionally, ROS could also react with the skin surface lipid and initiate the lipid peroxidation, which damages the skin barrier [169].

Most polyphenols are known to possess excellent antioxidant properties. For example, topical supplement of the green tea constituent(−)-epigallocatechin-3-gallate (EGCG) was able to inhibit the UV-induced ROS production, which demonstrated the protective function of EGCG against oxidative stress for the skin [101]. Curcumin is another example that is well-known for its antioxidant property. It was concluded before that curcumin was able to
mitigate the $\gamma$-radiation induced ROS threats in the mice by the molecule’s own free radical scavenging and upregulating Nrf2 expression properties [150]. In general, polyphenols are known to participate in the hydrogen atom transfer (hydrogen-donation), single electron transfer, sequential proton loss electron transfer, and transition metal chelation. By which, they could quench the free radicals to exhibit the antioxidant properties. Previously the relationship between the structure of the polyphenol to the antioxidant property was studied and found out that the unsaturation in the C ring, the 3-OH group, orthodiphenolic structure in B ring were essential for the superior antioxidant property of flavonoids and phenolic acids [103,104]. For instance, the unsaturation in the C ring found in quercetin has better antioxidant properties than catechin though they share the same arrangement of the hydroxyl groups. The role of the 3-OH group has also been examined and found out that it is essential for the antioxidant property as well.

1.2.2. Anti-ageing

Ageing is no doubt a very complicated process, both intrinsic and extrinsic factors contribute to this process. Based on the definition from World Health Organization (WHO), ageing is a result from the impact of accumulation of varied molecular and cellular damages over time. Many researchers pointed out that oxidative stress is the main cause of ageing during normal metabolism [130]. For intrinsic factors, such as normal metabolism, their impacts on skin ageing could not be changed. However, for some other extrinsic factors that induce the oxidative stress to human skin, their impacts could be minimized or avoided to certain extent with proper treatment [153].

UV damage is one of major external threats that leads to the ROS generation, which then causes the skin ageing effect, such as the formation of wrinkles. UV, UVA (315-400
nm) specifically reaches to the dermis of the skin and generates ROS that causes damages to cells such as fibroblast, which is responsible for the generation of collagen. When collagen was produced less, wrinkles and other unfavorable skin conditions will take place. Additionally, ROS is also responsible for increasing the synthesis of hyaluronidase and elastase that then degrade the structure builders including hyaluronic acid, elastin, collagen etc., [133]. Diet, a high-fat diet for instance, might also contribute to the increasing levels of oxidative stress [3].

Regardless the source of the generation for ROS, there is strong evidence that over generation and accumulation of the ROS would lead to skin ageing. Below Fig. 5 is a summary of different pathways for skin ageing from the literature [133].
Figure 5. Summary of the mechanisms of both intrinsic and extrinsic factors induced oxidative stress on skin ageing, reprinted from reference [133].

Based on the antioxidant property of polyphenol, it might help to relieve the extrinsic factors induced oxidative stress and mitigate the skin ageing phenomena. A recent review summarized about the potential mechanisms for polyphenol’s anti-ageing properties [131]. One is the antioxidant properties of polyphenols that have been discussed above contributing to the anti-ageing effect. What is also interesting, is that polyphenol could also upregulate the endogenous antioxidant, oxidase enzyme production and activity, besides its own antioxidant properties. For instance, resveratrol could protect skin from UV-induced wrinkle formation through the activation of Nrf2/HO-1 signaling pathway to relieve the oxidative stress. Nrf2 is a transcriptional factor regulating the expressions of enzymes such
as superoxide dismutase (SOD), glutathione (GSH), heme oxygenase-1 (HO-1) etc., that could be used to defend against oxidative stress \[131\]. In conclusion, skin ageing is a result of oxidative stress and polyphenol could be used to mitigate this effect primarily based on its own antioxidant properties. Also, an interesting \textit{in vitro} study on fibroblast proved that treatment with EGCG could prevent UV-B irradiation inducted collagenase activation, which helped to prevent the collagen degradation \[151\].

1.2.3. Anti-melanogenic (skin whitening)

There are many different mechanisms to explain why polyphenols could help mitigate the over production of melanin. One explanation is that their potential capacities to impact on the activity of tyrosinase, including chelating copper at the active site of tyrosinase and/or alternating the structure of the enzyme. Ellagic acid is a good example that function as anti-melanogenic agent based on that mechanism to chelate the cooper at the active site that further changes the enzyme activity. Oxyresveratrol could not chelate the copper ion of tyrosinase, instead it forms the van der Waals forces and hydrogen bonds between itself and the enzyme, as a result it limited the activity of tyrosinase by altering the enzyme’s structure \[119, 120, 121, 122\]. Other mechanism includes the role of polyphenol to inhibit the melanocytes proliferation \[123\]. Last, as previously discussed, the excessive ROS generation and accumulation in the epidermis will lead to the increasing levels of tyrosinase, TRP-1 and TRP-2, which directly would cause more melanin to generate as a result. As shown in \textbf{Fig.4}, melanogenic biosynthetic process is a summary of oxidative process. Therefore, the antioxidant properties of polyphenols would be another mechanism to explain why polyphenol could work as an anti-melanogenic agent.
1.2.4 Anti-inflammatory

When ROS was produced dramatically and accumulated within the skin, this would lead to the secretion of inflammatory cytokine causing skin inflammation [164, 165]. There are two possible ways to explain the mechanisms of polyphenol’s anti-inflammatory properties. One of many popular theories is that polyphenol is able to inhibit the activation of transcription factors that are responsible for the secretion of proinflammatory cytokines that promote the inflammation [134]. Avenanthramide, a unique polyphenol from oat, has proved to be effective in terms of inhibiting the release of inflammatory cytokines associated with pruritic skin disease [117]. The polyphenols fractions in Cymbopogon citratus leaves, including luteolin 7-o-neohesperidoside, cassiaoccidentalin B, carlinoside, cymaroside and tannins, have been evaluated using in vivo carrageenan-induced rat paw edema model and found that there was a significant reduction of edema [118]. Quercus mongolica Fisch. Ex Ledeb. (QM) showed great potential as anti-inflammatory agent, specifically the fraction has demonstrated to be efficient in terms of inhibiting the activities against MCP-1 (Monocyte chemoattractant protein-1), IL-6 (Interleukin-6), IL-10 (Interleukin-10) etc., in the keratinocytes model after UVR irradiation treatment. Another mechanism is based on the antioxidant properties as both might have an effect on the similar biomarkers. From a preliminary clinical study, resveratrol showed a significant decrease in terms of inflammation caused by acne vulgaris [135]. Therefore, polyphenols’ strong antioxidant property as well as the capacities to inhibit the release of proinflammatory cytokines enable them as anti-inflammatory agents.
1.3 Tetrahydrocurcumin

Tetrahydrocurcumin (THC) is a metabolite of curcumin, it was first identified back to 1978 by Dr. Holder et al. Compared to curcumin, the major functional groups such as phenolic hydroxy groups, methoxy groups and keto-enol moiety still remain intact in THC as shown in the below Fig. 6. This might be the main reason why THC has many health related benefits similar to curcumin.

THC is well known for the superior antioxidant property. Some researchers pointed out that the hydrogenation promotes the hydrogen-donating ability, thus led to a relatively higher antioxidant capacity compared to the parent molecule, curcumin [16,106]. Previously based on the results from DPPH radical scavenging assay as well as AAPH induced linoleic acid oxidation, it was concluded that THC was more effective compared to curcumin and Trolox (water soluble derivative of vitamin E) in terms of antioxidant property [107]. Clinically, THC was also found to effective to relieve the oxidative stress on the male diabetic wistar rats [157,158]. Many studies have demonstrated that THC has better efficacy, compared to curcumin, in terms of quenching the ROS to relieve the oxidative stress. Besides the quenching capacities from the molecule, THC could also upregulate the enzymes such as SOD, GST etc., which could be used to enhance the defense against oxidative stress [136].

In addition to the antioxidant property, THC is also known to possess many other biological properties such as anti-inflammatory, anti-cancer, etc., As previously discussed, the anti-inflammatory property might derive from antioxidant property of the polyphenol. Additionally, a previous study showed that THC could bind to the enzyme PLA2 (phospholipase A2), which releases the arachidonic acid as the substrate for the
proinflammatory mediators \[137, 138\]. In a lipopolysaccharide (LPS) induced RAW 264.7 macrophage cell study, THC and curcumin demonstrated their anti-inflammatory efficacies to inhibit the production of nitric oxide, inhibit the release of prominent cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 \[139\]. In an \textit{in vivo} study on mice, THC could also facilitate the wound healing process by the combination of its impacts on increasing the levels of SOD, decreasing the levels for glutathione and glutathione. Also, THC’s superior antioxidant property contributes to the healing process as well. Based on above findings, THC could be an effective anti-inflammatory agent.

Besides the above properties, THC also has anti-cancer property. Cancer is a very complicated process from the initial growth to the further progression. A recent review summarized the proposed mechanisms of THC as an anticancer agent and shown in \textbf{Fig 7}. Briefly, during the development of cancer, THC could participate to inhibit the cancer growth by its own properties of reduction of oxidative damage, inhibition for cell proliferation, migration etc.,

Based on the superior antioxidant, along with anti-inflammatory and many other countless health-related properties of THC, it is speculated that THC could be an effective agent to relieve the oxidative stress that generated and accumulated within the skin. Additionally, as a result of its lipophilic nature (log P =2.8) and relatively small molecular weight (372.4 g/mol), THC could be absorbed from the skin directly or through the oral delivery to reach systemic circulation. However, THC still has the challenge of low aqueous solubility, which needs to be solved in order to reach a higher bioaccessibility regardless of the delivery methods.
1.4 Skin delivery vs. Oral delivery

Delivery of the compounds through skin route including topical and transdermal delivery, has the advantages of improving the patient’s compliance, decreasing the dosing frequency, bypassing the hepatic first-pass metabolism etc., compared to the traditional oral
delivery. In order to maximize the efficacy of targeted compounds that intend to be absorbed in the skin, an optimized delivery system is always needed. Today there are variety of different formulas that are designed in order to enhance the skin delivery efficacy. More details were discussed in the Chapter 4 introduction section.

Besides the above topical and transdermal delivery methods, delivery of the targeted compounds for skin health via oral route has also gained a lot of attentions recently not only among the researchers but also from consumers as well. Compared to topical and transdermal delivery, oral delivery could eliminate the challenge of low skin penetration for certain molecules of interest. It could also allow a better distribution within all the skin layers, including epidermis, dermis, hypodermis, etc., [142]. Ingestible food and nutrition for a better skin health is now on a popular trend and is defined as “nutricosmetics” [115]. It is assumed that ingestible food and nutrition must be able to be absorbed by the intestinal barrier, reach the systemic circulation and distribute to the skin in order to bring the biological benefits. For example, a research demonstrated the protective role of drinking tea on reducing the UVB-induced skin inflammation and acute oxide stress, it could also reduce the wrinkle formation by showing the green tea’s anti-ageing effect using the in vivo mouse model [152]. One way to explain is based on the systemic delivery to reach the skin. Another way is related to the connection between gut health and skin health. Some studies demonstrated that when there is an intestinal structure change or intestinal flora imbalance, this could lead to skin hemostatic disorder as well. Therefore, gut health is linked to the skin health. While it might still require further evidence to confirm the dominant role between these two possible mechanisms or even propose more mechanism. However regardless of
the mechanism, efficient delivery of beneficial nutrient through oral route does prove its role to enhance skin health status.

There are many evidence demonstrating this conclusion, an earlier study showed that both the oral and topical application of vitamin E were effective to reduce UV-induced pigmentation [126]. Hydrolyzed collagen is a popular ingredient in the nutricosmetic category. Many studies demonstrated that collagen-derived peptide could be absorbed and recirculated in the blood stream, clinical studies confirmed that skin elasticity as well as skin hydration level were increased over the test period [115, 127]. However, collagen and other hydrolyzed collagen with relatively large molecular weight could not be absorbed through skin due to the high molecular weight and hydrophilic nature, therefore oral delivery would be considered as a better delivery pathway. Another example is L-ascorbic acid, a form of Vitamin C, its hydrophilic nature limits its efficient skin absorption [141]. Based on the analysis of the current market, there are 5 key markets segments in the nutricosmetics category, including Omega-3 fatty acids, Collagen peptides, Hyaluronic acid, Minerals, Vitamins [128]. Their benefits in the skin care products have been well studied, which is also the main reason contributing to their success in the nutricosmetic category. While it has always been a popular topic regarding the efficacy between the two methods (topical delivery vs. oral delivery). The molecules’ own properties itself play a critical role in determining the ultimate efficacy between topical delivery and oral delivery.
CHAPTER 2. HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Skin, the largest organ in the human body, and it works as a barrier to protect us. The integrity and well-being of the skin not only impacts skin health, but also plays a vital role for our overall health status. Polyphenol is a group of molecules that are extracted from the plant and it is reported to possess many properties such as antioxidant, anti-inflammatory, anti-ageing etc., that could be beneficial to the skin. Both academia and industry are conducting intensive research to explore the benefits of using polyphenol enriched products for the skin. In addition, as there are growing interests from consumers today to pursue more natural or naturally-derived ingredients instead of from the synthetic source, polyphenols could be considered great candidates with high potential commercial value.

Curcumin is a type of polyphenol with many biological properties. However, its application has been limited based on its extremely low water solubility and low stability. Tetrahydrocurcumin (THC) is a major colorless metabolite of curcumin and it is well known to possess many bioactive functions based on the similar structure compared to curcumin. Additionally, it was reported that the hydrogenation promotes the hydrogen-donating ability leading to a higher antioxidant property of THC as a result. The superior antioxidant property of THC would help relieve the oxidative stress generated and accumulated in the skin, which makes it a great candidate as an effective agent to protect skin from oxidative stress-induced skin disorder such as hyperpigmentation, skin inflammation etc. Besides, THC has a relatively better aqueous solubility, higher stability as well as bioavailability.
compared to the previous unsaturated curcumin. Therefore, it would be less challenging for
the scientists to formulate.

Based on the above analysis, I hypothesized that THC could relieve the oxidative stress
in the skin due to its own superior antioxidant property. Secondly since the level of
melanogenesis could be significantly impacted by the reactive oxygen species (ROS), THC
might work as anti-melanogenic agent to relieve the over production of melanin. Thirdly,
innovative delivery system is needed to further enhance the bioaccessibility for THC. This
is mainly due to the fact that THC still has relatively low water solubility, it is speculated
that a delivery system would be needed to be proposed to resolve this challenge in order to
increase the overall bioaccessibility.

2.2 Objectives

In order to test and validate above hypothesis, the whole project has been divided into
several specific goals. The overall objective for this project is to first evaluate and
demonstrate the benefits of THC in the skin under oxidative stress. Secondly, it is to utilize
different formulation strategies to develop the optimum platform for THC to be efficiently
absorbed either via topical or oral route.

Aim 1. Evaluation of the potential efficacy of THC as anti-melanogenic agent

We first used mushroom tyrosinase as the targeted enzyme and studied the inhibitory
effect of THC on the enzyme’s activity. Then the protective role of THC in the H$_2$O$_2$
induced-oxidative stress in keratinocyte was studied. Additionally, as one of the key
functions for keratinocyte is to synthesis epidermal lipids during differentiation, THC’s
impact on the lipid synthesis was also evaluated. Last murine B16F10 melanoma cell was used to evaluate the anti-melanogenic mechanism of THC.

**Aim 2. Design of lecithin-based nanoemulsion delivery system for THC to be delivered via skin**

The formulation was developed based on the previously published work from our group with modifications, which is a lecithin-based nanoemulsion and fabricated using the combination of high shear and high pressure homogenization methods. The formulation was originally designed to enhance the bioaccessibility of polyphenol via oral route, its capacity to interact with skin in order to deliver the compounds via topical route has not been studied yet. After the fabrication of the nanoemulsion, the prototypes were then characterized using particle size analyzer, polarized light microscope, LumiSizer etc., Their permeation enhancement performance was evaluated using *in vitro* Franz diffusion cell model.

**Aim 3. Design of hyaluronic acid-based conjugate for THC to be delivered via oral route**

Hyaluronic acid (HA) is a natural polymer that could be found in the human body and it is known to possess many health promoting benefits. As oral supplement is becoming more and more popular method for skin health, THC-HA conjugate was designed and fabricated with the purpose to enhance the bioaccessibility for THC in the solid dosage aimed for oral delivery. After fabrication, the conjugate was characterized using advanced analytical tools including $^1$H-Nuclear Magnetic Resonance ($^1$H-NMR), X-ray diffraction (XRD), Differential scanning calorimetry (DSC) to confirm the formation of the conjugate.
Later, the conjugate’s bioaccessibility for THC was studied using both the *in vitro* TNO dynamic gastrointestinal model-1 (TIM-1) and *ex vivo* Franz diffusion cell using the small intestine from porcine.
CHAPTER 3. EXPLORATION OF
TETRAHYDROCURCUMIN AS ANTI-MELANOGENIC AGENT.

Part of this work has been accepted for publication as “Tang, X., Dong, Q., Li, J., Li, F., Michniak-Kohn, B., Ho, C.T. and Huang, Q., 2021. Anti-Melanogenic Mechanism of Tetrahydrocurcumin and Enhancing its Topical Delivery Efficacy Using a Lecithin-Based Nanoemulsion. Pharmaceutics”.

3.1 Introduction

Melanogenesis is the process in which, melanin is synthesized from the melanocyte and transported to the skin surface. This process could be significantly stimulated when the skin is attacked by external factors such as UV radiation, visible lights, or even air pollution [10, 11, 143]. Currently there are many molecules with anti-melanogenic properties based on different mechanisms [144]. One of the common mechanisms is related to the molecule’s antioxidant properties. When the skin is challenged by the external factors as described above, ROS would be generated aggressively, and it will activate the enzymes involved in the biosynthetic process of melanogenesis, which leads to the over production of melanin. As a result, antioxidants would be efficient to relieve the oxidative stress and control the melanogenic process. Another mechanism for the anti-melanogenic property is based on the inhibitory effect on the enzyme activity, such as tyrosinase, involved in the melanogenic process. Since tyrosinase is the key enzyme involved in the rate-limiting step for generation
of the melanin. When its activity is controlled, the overall biosynthetic rate of melanin would be reduced as well. In addition, other mechanisms including the inhibition of melanosome transfer from the melanocytes to keratinocytes and acceleration of epidermal turnover are also commonly used to explain how a compound could function as anti-melanogenic agent. In this chapter, THC’s potential anti-melanogenic effect and mechanism was explored based on the combination of in vitro mushroom tyrosinase activity assay, in vitro B16F10 melanoma cell and in vitro keratinocyte cell studies.

3.2 Materials and Methods

3.2.1 Materials

Tetrahydrocurcumin was a gift from Sabinsa (East Windsor, NJ, USA). Methanol and acetonitrile were purchased from Sigma-Aldrich Company (St. Louis, Mo, USA). Milli-Q water (18.3 MΩ) was used in all experiments. HaCaT human keratinocytes (KCB200442YJ) were purchased from Kunming cell bank, the Chinese Academy of Sciences (Kunming, China). B16F10 melanoma cell (CRL-6475™) was purchased from ATCC (Manassas, VA, USA). α-melanocyte stimulating hormone (α-MSH), L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Yuanye Biotechnology (Shanghai, China). Dulbecco’s modified Eagle’s medium (DMEM), penicillin and streptomycin were obtained from Gibco Laboratories (Life Technologies Inc., NY, USA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) was purchased from sigma (St. Louis, MO, USA).
3.2.2 *In vitro* mushroom tyrosinase activity assay and kinetics study

In order to evaluate THC’s potential impact on the activity of tyrosinase, *in vitro* mushroom tyrosinase activity spectrophotometric assay is used. Here, 0.01 M L-DOPA, 100 Units/mL tyrosinase (TYR) solution was prepared in 0.2 M PBS (pH 7.4). Tetrahydrocurcumin was first dissolved in Dimethyl Sulfoxide (DMSO) and then diluted with PBS to the following concentrations, including 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL. Then, 160 µL L-DOPA solution was added in a 96-well plate, 20 µL test sample was added. Last 20 µL tyrosinase solution was added to the above mixture. The plate was protected from light and incubated at 30 °C for 30 minutes. As below Fig. 8 showed, L-DOPA is oxidized o-dopaquinone. This reaction rate, indicated the tyrosinase activity, was monitored using a 96-well microplate reader with UV absorbance ($\lambda$=475 nm) and calculated with below formula (eq. 1).

![Figure 8. Conversion from L-DOPA to Dopaquinone](image)

$$TYR \text{ activity (\%)} = \frac{A-B}{C-D} \times 100 \quad (1)$$

Where A is the absorbance of reaction mixture, B is the absorbance for the reaction mixture without TYR, C is the absorbance of reaction mixture without test sample and D is the sample with only L-DOPA.

Following the similar method from above *in vitro* mushroom tyrosinase activity assay, THC’s inhibitory kinetic study against mushroom tyrosinase was studied. Briefly, selected
THC concentrations (0, 100, 200 μg/mL) and selected L-DOPA concentrations (0.25, 0.5, 1, 2, 4, 5, 10 mM) were incubated respectively. Then the activity was measured using the same conditions as in vitro mushroom tyrosinase activity assay method. The inhibition type was analyzed using Lineweaver-Burk plots, and the inhibition constant was determined by second plots of apparent \( K_m/V_m \) or \( 1/V_m \) versus the concentrations of THC [66], where \( K_m \) represents the Michaelis constant and \( V_m \) is the rate when enzyme is fully saturated with substrate. The formula was described in eq.2.

\[
\frac{1}{V} = \frac{K_m + [S]}{V_{max}[S]} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}
\]  

3.2.3 In vitro B16F10 cell study.

3.2.3.1 Cell culture.

B16F10 were cultured in Dulbecco’s modified Eagle medium, supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin and 1% (v/v) streptomycin at 37°C in a humidified incubator at 5% CO₂.

3.2.3.2 B16F10 cell viability studies

Cells were seeded in a 96-well plate at a density of \( 1 \times 10^4 \) cells per well and cultured for 48 h without or with selected concentrations of THC (0.05, 0.5, 1, 10, 20, 40 μg/mL). Then 20 μL 5mg/mL MTT solution was added and culture for another 4 h. Later, the medium was discarded and 150 μL DMSO was added in order to dissolve the formazan crystals. Finally, the absorbance of each well was measured at a wavelength of 490 nm using a microplate reader. Cell viability was calculated using the follow formula (eq. 3),
Cell viability \% = \frac{OD_{\text{sample}} - OD_{\text{blank}}}{OD_{\text{control}} - OD_{\text{blank}}} \times 100\% \quad (3) .

Where OD is the optical density.

3.2.3.3 Measurement of melanin content

Measurement of melanin content in B16F10 was determined based on published method with slight modifications [17]. Briefly, B16F10 cells were seeded at a density of \( 1 \times 10^4 \) cells per well in a 96-well plate. Then cells were treated with the following groups, including two selected concentrations of THC (1, 10 \( \mu \)g/mL) with \( \alpha \)-MSH (0.5 \( \mu \)M), cells with \( \alpha \)-MSH (0.5 \( \mu \)M) and untreated cells, which were free of THC and \( \alpha \)-MSH as the control. They were all cultured for a total of 48 h. Finally, all the cells were washed with PBS, harvested, lysed, and melanin was solubilized with 1N NaOH (including 10\% DMSO v/v) at 80°C for 1 h. Melanin contents were determined by measuring the absorbance using a microplate reader with UV absorbance (\( \lambda = 475 \) nm). The quantity of cells in each well were measured using cell counter, and the melanin contents were determined based on the absorbance value/cell number.

3.3.3.4 In vitro cellular tyrosinase activity assay

B16F10 cells were seeded at a density of \( 10^6 \) cells per well in a 6-well plate. Cells were treated with 2 mL of the following groups including, 10 \( \mu \)g/mL THC with 0.5 \( \mu \)M \( \alpha \)-MSH, 0.5 \( \mu \)M \( \alpha \)-MSH free of THC, free of THC/\( \alpha \)-MSH for 48 h as control, respectively. After incubation, the medium was removed, cells were washed twice with PBS. Then cells were detached with 0.25\% v/v trypsin in PBS, counted, and centrifuged at 1,000 rpm for 5 mins. Later, cell pellets were re-suspended in PBS containing 1\% v/v Triton X-100 (with 1 \( \mu \)g/mL Leupeptin, 100 \( \mu \)g/mL phenylmethylsulfonyl fluoride) at 4 °C. After that, the suspension
was centrifuged at 12,000 rpm for 10 mins. Total protein contents from the supernatant were
determined using the enhanced BCA protein assay kit (Beyotime, Shanghai, China). The
mixture containing equal 90 μL of the protein in the supernatant was collected and mixed
with 10 μL L-DOPA solution, incubated for 20 mins at 37°C. Absorbance was measured at
475 nm using a microplate reader [18]. Tyrosinase activity could be calculated using eq. (4).

\[
TYR \text{ activity } \% = \frac{OD_{475 \text{ of test group}}}{OD_{475 \text{ of control group}}} \times 100 \text{ equation (4)}
\]

3.2.3.5 Real-time Polymerase Chain Reaction (RT-PCR)

The mRNA expressions of tyrosinase, trp-1, trp-2 in B16F10 cells were determined by
RT-PCR using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal
standard. Cells were cultured at a density of 10^6 per well in a 6-well plate. Below groups
were pretreated without α-MSH as the control, with 0.5μM α-MSH, and with both 0.5μM
α-MSH + 10 μg/mL THC as the test samples. After 48 h, cells were washed with PBS,
detached with trypsin and then centrifuged at 1,000 rpm for 5 mins. Supernatant was
removed and cell pellets were collected. Total RNA was extracted from cell pellets using
the TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa Bio, Japan) following
manufacturer’s instruction. cDNA was then synthesized using PrimeScript RT reagent kit
with gDNA eraser (Takara, Japan) following the manufacturer’s instruction. RT-PCR was
performed using an CFX Connect Real-Time PCR detection system (BIO-RAD, USA).
Lightcycler® 480 SYBR Green I Master (Roche, Penzberg, Germany) was used for
amplification and detection of DNA. The qRT-PCR primer sets for tyrosinase, TRP1 and
TRP2 were purchased from Applied Biosystems (Foster city, CA, USA) and summarized
in the below Table 1. Target gene expression was normalized to the internal standard, GAPDH. Relative quantization was performed using the comparative $\Delta\Delta^{Ct}$ method according to the instructions provided with the manufacturer’s instructions. Each experiment was performed in triplicate.

Table 1. The primer sequences used in the real-time polymerase chain reaction measurement for GAPDH (internal standard), tyrosinase, tyrosinase related protein-1 (trp-1) and tyrosinase related protein-2 (trp-2)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'-TGTTTCCTCGTCCCCGTAAG-3'</td>
<td>5'-GATGGCAACAAATCTCCACTTTG-3'</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>5'-TCCAAGAGTCAGATCCAGGC-3'</td>
<td>5'-TCCTGGGGTGCTTCTTCTT-3'</td>
</tr>
<tr>
<td>TRP1</td>
<td>5'-AGTGGCTCGTTGTACTTG-3'</td>
<td>5'-TGGAGTTGGATTCGGG-3'</td>
</tr>
<tr>
<td>TRP2</td>
<td>5'-CTTTTGACCATGTTGCAGGA-3'</td>
<td>5'-AGGAGTTGATCATGGCA-3'</td>
</tr>
</tbody>
</table>

3.2.3.6 Western blot

B16F10 cells were seeded at a density of $10^6$ per well in a 6-well plate and pre-treated without $\alpha$-MSH as the control, with 0.5µM $\alpha$-MSH, and with both 0.5µM $\alpha$-MSH + 10
μg/mL THC as the test samples. After 48 h, cells were washed with PBS, detached with trypsin and then lysed, harvested on cold ice. Then cell lysates were centrifuged at 12,000 rpm for 5 mins at 4 °C and the supernatant was collected. The total protein content was determined by the BCA protein assay kit (P0009, Beyotime, China). The controlled equal amounts of proteins were separated by 12% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with 5% non-fat skim milk in Tris Buffered Saline with Tween (TBST) with shaking for 1 h. Then it was rinsed with TBST for 2 min each time. The membrane was later incubated with primary antibodies including anti-tyrosinase antibody (AF5491, Affinity Biosciences, Cincinnati, OH, USA), anti-TRP-1 antibody (ab178676, ABCAM, Cambridge, MA, USA) and anti-TRP-2 antibody (Proteintech group, Wuhan, China), GAPDH polyclonal antibody (ATPA00013Rb, Atagenix, Wuhan, China) at 4°C for overnight. Later the cells were washed three times with PBST for 10 mins each time. Finally, the cells were incubated with secondary antibodies, goat anti-mouse IgG (H+L), HRP conjugate (SA00001-1, Proteintech, Wuhan, China), goat anti-rabbit IgG (H+L), HRP conjugate (SA00001-2, Proteintech, Wuhan, China) for 1 h at room temperature. The membrane was further washed three times with PBST for 10 mins each time. Protein bands were visualized using an Enhanced Chemiluminescence (ECL) kit (Yitong biotech, Wuhan, China) and analyzed using ChemiDoc™XRS+ imaging system (Bio-Rad, California, USA). Quantification of relative protein expressions, i.e., grey values were carried out using Image Lab 3.0 software and GAPDH as internal standard (Bio-Rad, California, USA).
3.2.4  In vitro cellular protective effect of THC on H₂O₂ induced cell damage - HaCaT study.

3.2.4.1 Cell culture

HaCaT Cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% (v/v) fetal bovine serum, 4 mM glutamine, 1% (v/v) penicillin and 1% (v/v) streptomycin (Hyclone Laboratories, Logan, UT, USA) in a 37°C humidified incubator under 5% CO₂ air.

3.2.4.2 HaCaT cell viability studies

Viability of HaCaT cells treated with different concentrations (0.25, 0.5, 1, 2, 4 µg/mL) of THC were evaluated using MTT colorimetric assay. Briefly, HaCaT cells were seeded at the density of 1× 10⁴ per well in a 96-well microtiter plates, they were cultured in the medium containing selected concentrations of THC for 24 h. Later, the medium was removed, the cells were washed with PBS and MTT solution (0.5 mg/mL) was added, incubated for 4-6 h. Last, the medium was discarded and 150 µL DMSO was added in order to dissolve the formazan crystals. The absorbance of each well was recorded at 490 nm using a microplate reader, and the cell viability was determined using the eq.(3).

3.2.4.3 Protection against hydrogen peroxide induces cell death

We first cultured HaCaT cells (1× 10⁴ cells per well) with different concentrations of hydrogen peroxide solution (150, 300, 450, 600 µM) for 24 h in order to evaluate the effects of hydrogen peroxide induced oxidative stress on the cell viability. Then, the medium was removed, and the cells were washed with PBS, MTT solution was added and incubated for another 4-6 h. Last, the medium was discarded and 150 µL DMSO was added. The cell viability was determined using eq.(3).
For protection against hydrogen peroxide induced cell death, HaCaT cells (1× 10^4 cells per well) were seeded and cultured with the selected concentrations of THC (0.25, 0.5, 1, 2, 4 µg/mL) for 2-3 h first. Then the medium was removed, different concentrations of hydrogen peroxide (150, 300, 450, 600 µM) was added respectively and cells were cultured for another 24 h [20]. Later, the medium was removed, cells were washed with PBS and incubated with MTT solution (500 µg/mL) for another 4-6 h. Last, the medium was discarded and 150 µL DMSO was added in order to dissolve the formazan crystals. The cell viability was determined using eq.(3).

### 3.2.4.4 Measurement of ROS

In a 6-well plate, HaCaT cells (2×10^5/well) were seeded and cultured using the method described above. Varied concentrations of (0.5, 1, 2, 4 µg/mL) THC were added to the cells and cultured for another 12 h. Subsequently, 600 µM hydrogen peroxide solution was added to the cells and incubated for one hour to induce the oxidative stress. Then, 10 µM 2′-7′dichlorofluorescin diacetate (DCFH-DA) was added to the cells and incubated for 1 h. The fluorescence strength of the cells was measured using a flow cytometry (CytoFLEX Flow Cytometer, Beckman Coulter, Indianapolis, Indiana, USA) with an excitation wavelength of 490 nm and an emission wavelength of 530 nm, respectively. The peroxide levels in the cells were plotted as one-parameter histograms with cell count on the y-axis and fluorescence on the x-axis.

### 3.2.4.5 Lipid synthesis in HaCaT

In order to study if THC has impacts on the lipid synthetic process in the HaCaT cells, which is a critical function in the epidermis, the following published method was used with
slight modifications [63]. In a 96-well plate, HaCaT cells (5000 cells per well) were seeded and cultured with selected concentrations of THC (0.025, 0.05, 0.1 µg/mL) for 5 days. Then the culture medium was removed and nile red (9-diethylamino-5H-benzo[a]phenoxazine-5-one, Sigma-Aldrich GmbH) solution (1 µg/mL) was added to each well. The cells were protected from light and gently mixed for 10 mins. Finally, the fluorescence intensity was monitored using microplate reader with an excitation wavelength of 485 nm and an emission of 590 nm, respectively.

3.3 Results and discussion

Figure 9. Inhibitory effect of THC on mushroom tyrosinase activity.
As shown in the above Fig.9, the inhibitory effect of THC on the mushroom tyrosinase activity was demonstrated and the effect was concentration dependent. The activity of the mushroom tyrosinase decreased as the concentration of THC increased. Lineweaver-Burk plot in Fig.10 revealed that THC functioned as mixed-competitive inhibitor by showing different values of the slope and y-intercept between the reaction with and without inhibitor (THC). This indicated THC could freely bind to tyrosinase and a tyrosinase-L-DOPA complex. Though THC is not targeting at the active site of tyrosinase, THC could change the conformation of the free enzyme or tyrosinase-L-DOPA complex to inhibit the continuous, efficient binding between the tyrosinase and L-DOPA, thus reduce the overall activity.

Murine B16F10 melanoma cell is a very commonly used in vitro model today in both academic and industrial research to evaluate the skin-whitening potential of a compound. Cell viability was first conducted using MTT assay method in order to ensure that the proposed concentrations for the tested compound would not inhibit cell growth (> 90%
viability). As shown in the below Fig.11, THC inhibited the growth for the B16F10 cells when the concentration of THC was at or higher than 20 µg/mL and the inhibition was concentration dependent. Therefore, THC below 10 µg/mL was deemed as safe for the B16F10 cells. In addition, α-MSH was frequently used to stimulate the production of melanin in B16F10 cells. It was proposed that α-MSH could bind to 6-BH₄, a molecule known to regulate the availability of L-tyrosine and the activity of tyrosinase [163]. Fig.12 indicated that the 0.5 μM α-MSH could significantly increase melanin synthesis compared to the control group. Specifically, α-MSH increased almost 5 times higher melanin production. Two different concentrations of THC (1, 10 µg/mL) were added to the cells together with α-MSH in order to evaluate the potential inhibitory effect of THC on the melanin synthetic process. The result confirmed the inhibitory effect, and the effect is dose dependent (Figure 12).

![Figure 11](image)

**Figure 11.** B16F10 cell viability results of THC, data was calculated from three wells for each concentration, expressed as mean ± SD (n=3).
Next, the effects of THC on the tyrosinase activity in the α-MSH stimulated B16F10 cells were investigated. As expected, α-MSH significantly promoted a higher tyrosinase activity compared to the control group (absence of α-MSH), which might explain the increased production of melanin as observed in the Fig. 12. When THC was added to the culture medium together with α-MSH, tyrosinase activity was significantly reduced compared to the group with α-MSH as presented in Fig.13. Specifically compared to the control group, α-MSH increased the tyrosinase activity to 185.2%. When 10 μg/mL THC was added together with α-MSH, TYR activity reduced to 121.1%. This study demonstrated that THC was effective to inhibit the tyrosinase activity in the α-MSH stimulated B16F10 cell model. As shown in Fig. 14, gene expression levels of enzymes, including tyrosinase,
trp-1 and trp-2 were studied by the RT-PCR. This result indicated THC reduced all the mRNA levels.

**Figure 13.** Effect of α-MSH, THC on the activity of tyrosinase (TYR) in B16F10 cells. Data was calculated from three wells for each concentration, expressed as mean ± SD (n=3). ***p<0.001 compared to the group treated with 0.5µM α-MSH.
**Figure 14.** Effects of THC on mRNA expressions of TYR (A), TRP-1 (B), TRP-2 (C) in B16F10 cells. Levels of mRNA were determined by PCR and GAPDH was used as the internal reference. Results are expressed as mean SD (n = 3). ***p < 0.001 compared to group treated with α-MSH.

Finally, western blot was also used to investigate the effect of THC on the levels of the key enzymes that are involved with the biosynthetic process for melanin, including tyrosinase, tyrosinase related protein-1 (trp-1), tyrosinase related protein-2 (trp-2) [60]. GAPDH was used as the internal standard. The result was presented in **Fig.15.** ImageJ was used to quantify the grey value from the western blot results and summarized in the below **Table 2.** The result concluded that THC could reduce the protein levels of tyrosinase, trp-1 and trp-2 in the α-MSH stimulated B16F10 cells model.

![Western blot analysis](image)

**Figure 15.** Western blotting analysis of protein expressions for TYR, TRP1, TRP2, and GAPDH as the internal reference in B16F10.
Table 2. Quantitative results of the grey values from the western blot analysis. Grey values for tyrosinase (A); tyrosinase related protein-1 (B); tyrosinase related protein-2 (C). GAPDH acted as the internal standard.

<table>
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</tr>
<tr>
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<td>1097892</td>
<td>0.963</td>
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<tr>
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<td>0.554</td>
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<td>0.594</td>
</tr>
</tbody>
</table>
HaCaT cell viability was first studied. As shown in the Fig. 16, the selected concentrations of THC (0.25, 0.5, 1, 2, 4 μg/mL) did not impact on the cell viability compared to the control group (free of THC).

**Figure 16.** HaCaT cell viability results of THC, data was in triplicate, expressed as mean ± SD (n=3).

**Figure 17.** Effects of H$_2$O$_2$ on the viability of HaCaT cells.
*In vitro* H$_2$O$_2$ induced cell damage model has been widely used in order to evaluate any protective role against oxidative stress of certain compounds [85]. In this work, a series of varied H$_2$O$_2$ concentrations (150, 300, 450, 600 µM) were used to induce oxidative stress for HaCaT. After HaCaT cells were exposed to H$_2$O$_2$, cell viability was measured using the same eq.(3) and concluded that H$_2$O$_2$ induced damage was concentration dependent. Specifically, compared to the cells absence of H$_2$O$_2$ treatment, cell viability for 150 µM H$_2$O$_2$ was reduced to 50.8%, for 300 µM H$_2$O$_2$ it was reduced to 47.7%, for 450 µM H$_2$O$_2$ it was reduced to 44.5%, and for 600 µM H$_2$O$_2$ it was reduced to 42.6%. Later, pretreatment of selected THC concentrations (0.25, 0.5, 1, 2, 4 µg/mL) were added to the cells first and then, similar as described above, different levels of H$_2$O$_2$ was added to induce the oxidative damage and cell viability was evaluated. As *Fig. 18-Fig. 21* indicated, THC could protect HaCaT from H$_2$O$_2$ induced cytotoxicity in different degrees.

**Figure 18.** 150 µM THC protective roles on H$_2$O$_2$ induced HaCaT cytotoxicity, expressed as mean ± SD (n=5). ***p<0.001 compared to group free of THC, treated with H$_2$O$_2$ only.
**Figure 19.** 300 µM THC protective roles on H$_2$O$_2$ induced HaCaT cytotoxicity, expressed as mean ± SD (n=5). ***p<0.001 compared to group free of THC, treated with H$_2$O$_2$ only.

**Figure 20.** 450 µM THC protective roles on H$_2$O$_2$ induced HaCaT cytotoxicity, expressed as mean ± SD (n=5). ***p<0.001 compared to group free of THC, treated with H$_2$O$_2$ only.
Figure 21. 600 µM THC protective roles on H₂O₂ induced HaCaT cytotoxicity, expressed as mean ± SD (n=5). ***p<0.001 compared to group free of THC, treated with H₂O₂ only.

Figure 22. Effect of THC on the H₂O₂ induced ROS levels in HaCaT.
Figure 23. Effect of pretreatments of THC of different concentrations on H$_2$O$_2$-induced intracellular ROS level in HaCaT assessed by flow cytometry.

This could be explained by the antioxidative property of THC that protects the keratinocytes from the H$_2$O$_2$ induced oxidative stress. As shown in Fig. 22 and Fig. 23, in the presence of 600 μM H$_2$O$_2$, ROS level significantly increased in the HaCaT cells, almost three times higher compared to the control group (absence of H$_2$O$_2$), which led to the cell death observed (Fig. 17). There are some serious consequences if ROS accumulates aggressively in the epidermis, including skin hyperpigmentation, disruption to the skin lipids responsible for the skin barrier function, induction of skin inflammation etc., [162]. When the selected concentrations of THC (0.5, 1, 2, 4 μg/mL) were prior to the H$_2$O$_2$ treatment, they all demonstrated the protective role by decreasing the ROS levels found in
the HaCaT cells and the effect was dose dependent. When THC was added at the level of 1 μg/mL or 2 μg/mL, the α-MSH level was reduced significantly as well (Figure 24). α-MSH is the messenger between the keratinocytes and the melanocytes, controlling the melanogenic process. Therefore, THC proved that it could reduce the oxidative stress induced melanin production.

In conclusion, the data collected that THC demonstrated to be an effective anti-oxidative agent and anti-melanogenic agent.

Figure 24. α-MSH levels found in the supernatant in HaCaT, after the cells were pretreated without or with the selected concentrations of THC (0.5, 1, 2, 4 μg/mL) for 12 h prior to the addition of H2O2. Results are expressed as the mean SD (n = 3). *P < 0.05.
As previously introduced, one of the key functions for keratinocyte is to secret epidermal lipids responsible for skin barrier. *In vitro* human keratinocyte skin cell model is frequently used to study the epidermal lipid metabolism, which is essential for the human skin barrier properties [64,65]. In this study, Nile red was used to detect the intracellular lipid droplets. Varied concentrations of THC (0.025, 0.5, 0.1 μg/mL) were cultured with HaCaT, as above Fig. 25 indicated, no noticeable difference was observed between the study group and blank (free of THC) from total lipid synthesis perspective. Therefore, it is concluded that THC did not influence the epidermal lipid metabolism.

3.4 Conclusion

The study in this chapter demonstrated that THC could act as anti-melanogenic agent by the inhibitory effects on the biosynthetic process for melanin. Mushroom tyrosinase was
first used as the model to examine the inhibitory effect of THC on its activity. In vitro mushroom tyrosinase activity assay and kinetics study confirmed the inhibitory role of THC on the activity of mushroom tyrosinase, and the inhibitor type is noncompetitive. In vitro B16F10 melanoma cell study was used later and concluded that THC could decrease the activity of tyrosinase, which is the key enzyme that is responsible for melanin synthesis. In addition, THC could also inhibit the gene expressions of the three major enzymes that are involved with melanin biosynthetic process, including tyrosinase, TRP-1 and TRP-2. Finally, the effect of THC on the HaCaT was studied in terms of lipid production, relief of H$_2$O$_2$ induced oxidative stress etc., As expected, based on the antioxidant properties of THC, it could help to relieve the H$_2$O$_2$ induced oxidative stress. Pretreatment of THC to the keratinocytes could efficiently reduce the ROS level after the H$_2$O$_2$ induced oxidative stress in the keratinocytes. This protective role of THC is critical to help maintain the wellness of keratinocyte, especially when it is under the oxidative stress. What is also interesting is that THC could reduce the levels of $\alpha$-MSH in the keratinocytes cells. As discussed above, this is critical as another potential mechanism explaining about how THC could act as an anti-melanogenic agent since $\alpha$-MSH is the messenger between the keratinocytes and the melanocytes controlling the melanogenic biosynthetic process.

In addition, as another important function of keratinocyte, THC’s impact on the lipid production was studied. As the result indicated, THC did not impact the total lipid synthesis overall compared to the control (in the absence of THC).

Based on all the above findings, THC’s anti-melanogenic role as well as anti-oxidant role have been confirmed. Therefore, the benefits to deliver THC to skin have been established. However, THC is crystalline in nature and has relatively low aqueous solubility,
which prevents it from being absorbed the skin. A proper solvent as well as a delivery system is needed to enhance the solubility of THC and enable THC to be permeate through the skin layers, which would be discussed in more details in Chapter 4.
CHAPTER 4. DEVELOPMENT OF A NANO EMULSION SYSTEM FOR TOPICAL DELIVERY


Part of this work has been accepted for publication as “Tang, X., Dong, Q., Li, J., Li, F., Michniak-Kohn, B., Ho, C.T. and Huang, Q., 2021. Anti-Melanogenic Mechanism of Tetrahydrocurcumin and Enhancing its Topical Delivery Efficacy Using a Lecithin-Based Nanoemulsion. Pharmaceutics”.

4.1 Introduction

Providing antioxidants to the skin would be a promising remedy to relieve the oxidative stress as previously discussed. Epidermis in the skin lack the blood vessels for the nutrients to be absorbed from the food, it mainly relies on the molecule’s own diffusion mechanism in order to be delivered to this layer through the traditional oral delivery route [5]. Skin delivery including both topical and transdermal delivery might work as alternative options based on the advantages of avoiding first pass metabolism, no or limited interaction with the complicated fluids that is composed of a variety of enzymes, different pH conditions
that might degrade the actives. In addition, it has better compliance, and it is easy to apply as well.

However, skin barrier, especially the outermost layer stratum corneum, is a major challenge that limits the molecule’s permeability [12]. Many properties of the compound such as molecular weight, partition coefficient, stability, solubility etc., all play important roles in terms of whether or not it could be absorbed and penetrated through the skin to become bioaccessible. Fick’s first law in eq. (5) describes the relationship between steady permeated flux J with molecule’s diffusivity D in the formulation and molecular permeation coefficient Kp, which, by certain assumptions, is a function of molecule own’s partition coefficient and molecular weight [145]. In addition to the molecule’s intrinsic properties, formulations (delivery system) could play a role in manipulating the diffusivity of the active, enhancing the permeation performance by the interaction with the stratum corneum within the epidermis, etc., which as a result, facilitates the delivery efficacy.

In terms of the skin delivery, there are currently two main types based on the targeted site. As Fig. 26 showed, topical delivery is targeted for the localized area within the skin. Transdermal delivery, on the other hand, is not just limited to the localized area within the skin layers, instead it aims to permeate through all the layers and reach systemic circulation [75].

\[
J = K \cdot \Delta C = \frac{D \cdot K_p \cdot \Delta C}{h}
\]  

(5)

Where Kp represents the permeability coefficient and D is the diffusion parameter. h is the thickness of the membrane and \(\Delta C\) is the concentration gradients.
Currently there are many different formulation technologies that can be used to facilitate the topical delivery efficacy, including but not limited to hydrogel, nanoemulsion, microneedle, liposome, etc. The impacts of different formulations are mainly used to increase the overall solubility of the drug and/or interact with the skin in order to enhance the efficacy for topical delivery efficacy based on Fick’s law [74].

![Figure 26](image)

**Figure 26.** Percutaneous absorption of drugs through topical and transdermal route, reprint from [75].

**Hydrogel**

Hydrogel is made from a group of polymeric materials, of which, hydrophilic groups retain water in the 3-D networks. Based on the type of the polymers, hydrogel could be derived either from a natural or synthetic source. There are several ways to produce hydrogel, including physical interaction of the polymers (e.g., entanglements, electrostatics, and crystallite formation), the linkage between polymer chains via chemical reaction, and the linkage between polymer chains via ionizing radiation which generates main-chain free
radicals to form cross-link junctions [23]. The hydrogel is usually limited to be applicable for hydrophilic molecules due to its hydrophilic nature [71]. In order to enable the platform suitable for hydrophobic drugs, the incorporation of other advanced formulations are needed, such as liposome, nanoemulsion, polymer conjugate etc., In that case, hydrogel could not be the only delivery system, it might have to combine with other formulation that is more compatible with hydrophobic molecules. Hydrogel in that case, is mainly used to manipulate the formulation’s rheological performance.

**Microneedle**

Microneedle (MN) is a device that consists of needles of micron size, arranged on a small patch. The skin is temporarily disrupted when MN is applied. It could bypass the barrier layer, which is SC, and will directly reach the epidermis or upper dermis layer [25]. Based on the nature of the device, it has the advantage of better permeation, faster onset of action etc., compared to the formulations that require to permeate through the epidermis layer first before reaching the targeted site based on the passive diffusion methods [68, 69]. The variables that determine the permeation performance usually include the materials of the needle, shape/length of the needle, fabrication techniques etc., this technology is widely used as a delivery system for transdermal delivery, however its delivery efficacy for topical site remains limited or it requires the combination of other formulation strategies in order to reach the desired efficacy.

**Liposome**
Liposomes are primarily made of phospholipids, which will form bilayer membranes when dispersed in the water phase. Liposomes can be prepared based on different methods, including reverse-phase evaporation, thin-film hydration, detergent-depletion, etc. Liposomes could be categorized based on size, including small, intermediate and large; Based on lamellarity, including uni-, oligo, and multilamellar vesicles [26]. Liposomes have the capacity to encapsulate and deliver both lipophilic and hydrophilic actives, where lipophilic actives remain in the lipid membrane and hydrophilic actives remain in the aqueous phase. The permeation performance depends on the size, composition, surface charge, elasticity, etc., [70]. The limitations are the relatively poor stability when in contact with the skin, and low % loading of the targeted compound [72].

**Nanoemulsion**

Nanoemulsion is a class of multiphase colloidal dispersions with particle sizes usually in the range of 20-200 nm. They are mainly composed of oil phases, emulsifying agents and aqueous phases. There are two major methods to prepare nanoemulsion, including high-energy and low-energy emulsification. The high-energy emulsification includes high shear and high pressure homogenization, ultrasonic emulsification, microfluidization, membrane emulsification, etc., The low-energy emulsification includes phase inversion temperature (PIT), emulsion inversion point, and spontaneous emulsification [24]. Nanoemulsion could enhance the permeation performance by incorporating a permeation enhancer and high drug loading for hydrophobic compounds leading to a high concentration gradient [71]. Regarding the mechanism for the nanoemulsion to be absorbed through
topical route, it was proposed that nanoemulsion first enters the intercellular area of the stratum corneum and then disrupts the corneocytes, absorbs into the skin [81].

Each formulation has its own unique advantages and disadvantages. Therefore, many researchers used the combination of two or several types of formulations to reach the optimized topical delivery efficacy, such as nanoemulgel, which is the combination of nanoemulsion and hydrogel. In this chapter, nanoemulsion was designed for their advantages of solubility enhancement, greater absorption, high active loading, and high compatibility with a variety of different product platforms such as creams, foams, liquids etc., [61].

Below reprinted Fig. 27. summarized the process flow diagram for developing a topical formulation [79]. In brief, our design of the proof of concept formulation followed the same procedure. First, ingredient candidates were screened to find the maximum solubility, later lecithin based nanoemulsion was then developed and optimized based on the previous published works from our group [44]. Finally, in vitro permeation performance and stability were conducted respectively.
As previously discussed, there are three main components for nanoemulsion including oil phase, emulsifier and aqueous phase. Though our original formulation from our group was designed and proven to show great stability [44], however that formulation was not intended to develop for skin application initially. In order to justify the rationale, literature research was first conducted to evaluate if the ingredients used in the previous work are acceptable in the skin application. Below summarized some of the findings of each ingredient’s application on the skin.

Lecithin, functions as the main emulsifier, is used in the nanoemulsion to stabilize the oil/water interphase, solubilize the hydrophobic compound, and facilitate the topical delivery. Lecithin, including various types of Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), Phosphatidylinositol (PI) and
Phosphatidic acid (PA), is able to interact with the lipid bilayer in the stratum corneum, thus facilitate the topical drug delivery [27]. Additionally, a recent study demonstrated that lecithin, specifically phosphatidylcholine used in their study (see Fig. 28), could only reach around 10% of the total stratum corneum depth, which made it a great candidate as an agent for topical delivery [80]. In our work, lecithin was used the main emulsifier based on the benefits of excellent emulsifying property as well as moisturizing effect, which is essential for the skin. Inactive Ingredient Database (IID) was also utilized to verify if the selected ingredient was approved by FDA. It was found that Lecithin is approved for topical drug and the maximum potency per unit dose for lecithin is between 1-1.4% w/w.

![Structure of saturated phosphatidylcholine](image)

**Figure 28.** Structure of saturated phosphatidylcholine

Oils or lipids, another main composition for the nanoemulsion, are usually used to solubilize the hydrophobic compounds and facilitate the topical delivery by interacting with lipid layers within the stratum corneum similar as a permeation enhancer. It was also reported that oil phase in the formulation could impact on the final viscosity of the formulation as well [78]. Additionally, different oil types, based on the amounts of triglycerides and free fatty acid, also have different levels of the skin health promoting benefits, such as restoring skin barrier, protecting skin barrier by the occlusive effect, and reliving the skin inflammation etc., [62]. MCT is a type of triglycerides that have an average of 6-12 carbon atoms. It is commonly used to increase the solubility, partition coefficient
and diffusivity for lipophilic compound [76,77]. It is known to remain on the upper layer of stratum corneum without deep penetration [62]. Per IID, medium-chain triglyceride is also approved by FDA as inactive ingredient and the maximum potency per unit dose for topical is around 10-15% w/w.

In order to further optimize the solubility for THC, Diethylene Glycol Monoethyl Ether (Transcutol®, DEGEE), a well-known skin permeation enhancer was considered. It is reported as an excellent solvent for water insoluble compounds. In addition, it is reported to be safe and biocompatible with the skin [32]. The mechanisms for DEGEE as permeation enhancer could be explained in the following steps, first it is a great solvent and could enhance the solubility of the targeted compounds within the vehicle. Secondly, it could interact with the skin lipid within the stratum corneum layer to facilitate the diffusion of targeted compound. What is also interesting is that DEGEE is a hydrophilic chemical permeation enhancer. It has the “pull” effect to facilitate higher absorption/retention of targeted compound within the skin. Specifically, due to the hydrophilic nature, DEGEE would stay near the polar head of the skin lipid in the stratum corneum and induce swelling. As a result, more accumulation would occur within the epidermis [83]. Per the IID, DEGEE is approved as inactive ingredient and the maximum dosage is up to 49.91 %w/w. In conclusion, all the ingredients are safe for the skin application.

![DEGEE structure](image)

**Figure 29.** Structure of DEGEE

After the formulation is developed, *in vitro* Franz cell diffusion cell (Shown in Fig.30) is a commonly used methodology today to evaluate the permeation rate for the targeted
compound through the skin. This test could help to provide valuable information about the impact of formulation variables such as concentration and physical state (viscosity, pH, crystallinity of the compound) of the targeted compound within the formulation.

Currently there are a variety of different membranes that are frequently used including the skin from human and animals (Porcine, rat, dog etc.,) or synthetic membrane such as Strat-M® membrane from Millipore. Among those, the skin from the animal or human always have the variability issue. On the other hand, synthetic polymer membrane might be a great alternative for the research if the correlation between it and human cadaver skin could be confirmed. Recently, several studies have confirmed that Strat-M® showed similar drug permeability behavior compared to the actual ex vivo human skin across a variety of formulation types, drug properties [29, 30]. Therefore the in vitro Franz permeation model using Strat-M® could be a reliable model to predict the future in vivo performance and study the formulation impacts.

In addition, in order to investigate the kinetics of the THC permeation in the lecithin-based nanoemulsion through Strat-M® membrane, the following models were applied [31].

**Figure 30.** Schematic representation of Franz diffusion cell (Left) and Cross sections images for the synthetic Strat-M® membrane (Right).
I. Zero-order model:

\[ Q_t = Q_0 + k_0 t \quad (6) \]

II. First-order model:

\[ \log Q_t = \log Q_0 - \frac{k_1}{2.303} t \quad (7) \]

III. Higuchi model:

\[ Q_t = k_H t^{1/2} \quad (8) \]

IV. Korsmeyer-Peppas model:

\[ \frac{M_t}{M_\infty} = k_p t^n \quad (9) \]

4.2 Materials and Methods

4.2.1 Materials

Tetrahydrocurcumin was a gift from Sabinsa (East Windsor, NJ, USA). Diethylene glycol ethyl ether (DEGEE, Transcutol®) was a kind gift from Gattefossè (Paramus, NJ, USA). Medium chain triglyceride was obtained from Stephan (Northfield, IL, USA). Lipoid S 75 was a kind gift from American lecithin (Oxford, CT, USA). Methanol and Acetonitrile were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Milli-Q water (18.3 MΩ) was used in all experiments.

4.2.2 Determination of THC’s solubility and diffusivity in selected solvents

In order to determine the solubility of THC in the solvents including argan oil, sunflower oil, MCT, DEGEE and the combination of MCT and DEGEE at varied weight ratios. An excess quantity of THC (approximately~700 mg) was added to 7 mL of each individual solvent or combination of both at certain ratios (6:1, 1:1, 1:6, v/v) and mixed for 24 h using
a magnetic stirrer at room temperature. Then the 1.5 mL samples were centrifuged at 9,000 rpm for 10 min. The supernatant was collected, filtered through a 0.45 µm filter and diluted with methanol. Finally, THC’s concentration in each solvent was determined by the following HPLC method in order to confirm the solubility in each vehicle. Once solubility was confirmed, their impacts on THC’s diffusivity, in other words, the solute-solvent effect was then evaluated using Franz diffusion cell.

Franz diffusion cell (FDC-6, Logan Instrument Corp., NJ, US) with a diffusion area of 1.32 cm² was used to study the diffusivity of THC from the solvent. 400 mg of THC was added to the 7 mL solvent. Later 1 mL of the previous samples were loaded in the donor, 8 mL pH 6.8 PBS (50% v/v ethanol) was filled in the receptor. The dialysis membrane (MWCO: 6,000-8,000 Da, Thermo Fisher Scientific, Inc.) was mounted between the donor and receptor medium. The temperature was maintained at around 37 °C. Then 1 mL sample was collected from the receptor at fixed time intervals (0.5, 1, 2, 4, 6, 8, 12, 24 h), the remaining medium was replaced with fresh medium. Concentration of the samples were analyzed using HPLC method.

4.2.3 High Performance Liquid Chromatography (HPLC) analytical conditions

THC was quantified by the Agilent 1100 series HPLC system (Santa Clara, CA, USA) with a UV detector (280 nm). A Phenomenex C18 column (5 µm, 4.6 × 150 mm) was used with the isocratic elution consisted of 50% acetonitrile and 50% water (including 0.1% v/v formic acid). The flow rate of 1 mL/min and the injection volume was 20 µL.

The calibration curve was prepared by dissolving THC in methanol and diluting using the same solvent to a series of varied concentrations (6.25, 12.5, 25, 50, 100 µg/mL).
4.2.4 Fabrication of nanoemulsion

THC was first solubilized in the oil phase containing diethylene glycol ethyl ether and MCT at 50-60°C. Once THC was fully solubilized, then the oil phase was added to the water phase containing lecithin PC 75. Two separated phases were kept mixing under magnetic stirring for 10 mins till homogeneity was reached. The coarse emulsion was formed using high-speed homogenizer (IKA Works Inc., Wilmington, NC) at 10,000 rpm for 1-3 mins. Finally, the nanoemulsion was formed through EmulsiFlex-C6 high-pressure homogenizer (AVESTIN Inc., Ottawa, Canada) at 120 MPa. The schematic fabrication process was also presented in the Figure 31.

![Figure 31. Schematic process for fabricating the nanoemulsion](image)

4.2.5 Particle size measurement

The particle size of the nanoemulsion was measured by dynamic light scattering mode-based Particle Size Analyzer (Model 90 Plus, Brookhaven Instrument Corp., Holtsville, NY,
USA) at a fixed scattering angle 90° and room temperature. Each time, 50 µL of the nanoemulsion was diluted to 25 mL distilled water to prepare the samples.

4.2.6 Polarized light microscopy

In order to monitor the presence of THC crystals in the formulations. Emulsions were analyzed using Leica DFC 550 (Wetzlar, Germany).

4.2.7 Accelerated and real time stability test

In order to evaluate the stability for the nanoemulsion, LumiSizer was first used for the purpose of screening the formulations summarized in table 3. The principle of this test is to give stress to the tested sample by centrifuge. The change in the transmission indicated the phase separation and the instability index was reported as a result of the change level of the transmission as a function of initial sample height.

The new prototypes were developed based on the results from LumiSizer data, their real time stabilities were monitored at room temperature and 50°C respectively. Phase separation and/or any presence of flowing particles were evaluated by the naked eye during storage, particle size and polarized microscopy data were collected as needed.

4.2.8 In vitro permeation test

Franz diffusion cell (FDC-6, Logan Instrument Corp., NJ, US) with a diffusion area of 1.32 cm² was used to study the permeation profile of THC in nanoemulsion. Briefly, 1.5 mL samples were loaded in the donor, 8 mL pH 6.8 PBS (50% v/v ethanol) was filled in the receptor. The synthetic membrane (Strat-M® membrane from Millipore) was mounted
between the donor and receptor medium. The temperature was maintained at around 37 °C. then 1 mL sample was collected from the receptor at fixed time intervals for the HPLC analysis. The cumulative permeation was calculated using below eq.(10).

\[ Q(\mu g/cm^2) = \frac{\sum M}{A} \]  

(10)

Where M is the cumulative mass and A represents the surface area, which in this case should be equal to 1.32 cm².

At the end of the experiment, the kinetic analysis was conducted based on fitting the data to the different kinetic models described above and the best correlated model was selected based on the highest regression coefficient (R²). In addition, all the membranes were rinsed with water twice front and back, the surface was gently wiped to remove any potential residues and then sonicated in 5 mL methanol for 30 mins. All the samples were filtered through 0.45 μm filter and analyzed using HPLC to quantify the retention of THC in the membrane.

Statistical analysis

All experimental results are shown as means ± standard deviations. The significance was recorded as P-value < 0.05.

4.3 Results and discussion

4.3.1 HPLC calibration curve for THC

THC was dissolved in methanol to a series of concentrations from 6.25 μg/mL to 100 μg/mL. The result for the HPLC profile and the calibration curve was obtained and summarized in the below Fig.32 and Fig. 33. Additionally, it was shown that two major peaks were found in the chromatogram, where one major (retention time at 4.9 min)
represented the keto form of the tetrahydrocurcumin, and another major peak (retention time at 8.4 min) represented the enol form of the tetrahydrocurcumin. This is because that the 3,5-dione structure of THC enables the keto-enol tautomerism [58].

![Figure 32. THC 0.1 mg/mL standard solution chromatograph](image)

4.3.2 Determination of THC’s solubility and diffusivity in the selected solvents

![Figure 33. HPLC calibration curve for THC](image)
Figure 34. THC solubilities in different solvents

THC has very limited water solubility, which is reported to be around 56 ng/mL [81]. In order to solve this challenge, different oils and surfactant (permeation enhancer) were selected and their solubility capacities were evaluated. Solubility test on each individual solvent was first studied and the result was presented in Fig. 34. Among the three candidates for the oil phase, it was found that THC has the highest solubility in MCT (9.4 mg/mL) compared to argan oil (0.6 mg/mL) and sunflower oil (1.2 mg/mL). DEGEE had approximately 126.4 mg/mL solubility for THC. As a result, MCT was selected as the oil phase, and DEGEE was selected as a co-surfactant in the formulation in order to maximize the solubility. Finally, the varied volumetric ratios between MCT and DEGEE were designed in order to explore if there are any synergistic effect on the solubility for THC. Interestingly to note, when MCT was mixed with DEGEE at 1:1 ratio (v/v), the mixture had a solubility of 104.5 mg/mL, which is almost similar to the neat DEGEE. The two ingredients at 1:1 v/v seem to produce a synergistic effect at this ratio for the solubility enhancement. In addition to the solubility capacity of the solvent, the solute-solvent
interaction is another factor that is critical to be considered. For example, if the solvent-
solute effect is too strong and the release of the targeted compound from the solvent would
be impacted. As a result, it becomes less available for the skin absorption [88,89]. As Fick’s
law indicated, the permeation performance depends on the diffusivity D and partition Kp.
For compounds that have a log P (partition coefficient) close to two or even higher, such
as tetrahydrocurcumin (log P =2.8), they usually have a high permeation due to their
lipophilic nature [89]. Therefore, diffusivity would be the rate-limiting step. In order to
select the optimum solvent, besides the solubility capacity, the vehicle-solute effect should
also be evaluated.

As indicated in the below Fig. 35, the samples tested below showed the similar solubility
for the THC originally, however their release profiles were significantly different.
Specifically, THC in the neat solvent DEGEE showed the lowest release indicating the
strong interaction between the solute and the solvent. When MCT was combined with
DEGEE, a much faster release behavior was observed. Additionally, the release data was
fitted, and the release pattern was found based on the highest correlation coefficient (R²).
It was concluded that the release pattern changed from zero order (in neat DEGEE) to first
order (in the combination of MCT and DEGEE). The zero-order indicated that the release
of THC from neat DEGEE is independent of the concentration, and the first-order release
concluded that the release of THC from the combination is concentration dependent. In
conclusion, when developing future nanoemulsion, the optimized ratio between MCT and
DEGEE should be considered in order to ensure a proper release performance as well as
solubility capacity.
4.3.3 Fabrication of the nanoemulsions

Nanoemulsion was prepared following the method as described in Fig.31. A series of formulations were produced, and their stability performance were first predicted using LUMiSizer. Samples were centrifuged, the particle movement or phase movement was detected and monitored during the process, the change in the % of the transmission as function of sample height was quantified and recorded as instability index. As below Table 3 indicated, when the concentration for lecithin dispersion decreased to 30% (w/w) or below, high instability index was observed. Phase separation phenomena for those samples at the end of the stressed test were observed as well. Comparing between S4 and S7, the concentration for lecithin dispersion were kept constant at 50% (w/w). different MCT oil loadings were used from 10% to 40% (w/w), it was concluded that within the test range, oil
loading did not make a major impact on the overall stability for the nanoemulsion. Therefore, S5 and S7 were selected as the prototypes based on the LUMiSizer stability results under stressed conditions and previous solubility study. As comparison and for the purpose to further study the impact of MCT on the formulation stability and permeation performance, free of MCT was selected as the control.

**Table 3.** Formula compositions for S1-S7

<table>
<thead>
<tr>
<th>Ingredients/ID</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
</tr>
</thead>
<tbody>
<tr>
<td>8% (w/v) Lecithin dispersion</td>
<td>20%</td>
<td>30%</td>
<td>40%</td>
<td>50%</td>
<td>50%</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>Water</td>
<td>49%</td>
<td>39%</td>
<td>29%</td>
<td>19%</td>
<td>9%</td>
<td>--</td>
<td>29%</td>
</tr>
<tr>
<td>DEGEE</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>THC</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>MCT</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>30%</td>
<td>40%</td>
<td>10%</td>
</tr>
<tr>
<td>Instability index</td>
<td>0.746</td>
<td>0.411</td>
<td>0.053</td>
<td>0.049</td>
<td>0.061</td>
<td>0.062</td>
<td>0.023</td>
</tr>
</tbody>
</table>

Below **table 4** summarized the final formulations of the nanoemulsion used in the stability study and following *in vitro* Franz cell permeation study.

**Table 4.** Formula compositions for F1, F2, F3, F4

<table>
<thead>
<tr>
<th>Ingredients/ID</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
</tr>
</thead>
<tbody>
<tr>
<td>8% (w/w) Lecithin dispersion</td>
<td>50%</td>
<td>50%</td>
<td>50%</td>
<td>--</td>
</tr>
<tr>
<td>Water</td>
<td>9%</td>
<td>29%</td>
<td>39%</td>
<td>99%</td>
</tr>
<tr>
<td>DEGEE</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>--</td>
</tr>
<tr>
<td>THC</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
</tr>
</tbody>
</table>
4.3.4 Stability study

After fresh nanoemulsions were prepared (see Figure 36), particle size of each formulation was measured except for F4 (THC water suspension). The time zero results were summarized in the below Fig 37. General distributions of the particle size for the samples showed that F2 & F3 had narrower distribution with smaller particle size, compared to F1 (with higher % w/w oil phase).

Then the samples were stored at both room temperature and 50 °C. Their stabilities were evaluated using the combination of visual observations, polarized microscopy and particle size measurements.

Figure 36. Pictures of formulation F1, F2, F3 and F4
As below results from the polarized microscopy indicated in Fig. 38, due to THC recrystallization, large crystals were observed for the sample F3 (free of MCT) and less amounts of the similar crystals were also observed in the formulation F2 after 7 days stored at 50°C. Comparing between F1, F2 and F3, MCT demonstrated the beneficial role to prevent re-crystallization of THC in the nanoemulsion. This could be explained because MCT provided the hydrophobic environment for THC to be dispersed evenly. DEGEE on the contrary, is a hydrophilic ingredient. It is miscible with water in nature, which is not favorable to THC. Therefore, though DEGEE provided highest solubility for THC, MCT demonstrated to be a critical component in the formulation to prevent recrystallization of THC during storage.
Similarly, this was also demonstrated by the following particle size data. As indicated in the Fig. 39, after 7 days at 50 °C, particle size distribution for F1 and F2 remained unchanged compared to the data collected at time zero presented in Fig. 36. F3, on the other hand, was observed with extremely large size of the particles mainly due to the formation of the THC crystals. After this, F1 was kept in the room temperature to monitor the stability continuously up to 1 month. At the end of 1 month, no physical separation or crystals was observed. Particle size results of F1 were summarized in Figure 40 and concluded that the formulation is stable within the test period. Impacted by the COVID-19, the lab for the polarized microscope was not available. TEM (Transmission electron microscopy) was used to capture the image of F1 at 1 month in room temperature (Figure 41). As shown, the formulation was free of any crystals and the oil droplets were uniformly distributed.

Figure 38. Polarized microscopy of F1(A), F2 (B) and F3 (C) after 7 days stored at 50 °C
Figure 39. Particle size results of F1(A), F2 (B) and F3 (C) after 7 days stored at 50 °C
**Figure 40.** Particle size results of nanoemulsion F1 fresh at time zero (A); 1 week in 50 °C (B); 1 week in room temperature (C); 4 weeks in room temperature (D).

**Figure 41.** TEM images of F1 at room temperature after 1 month.
4.3.5 *In vitro* permeation test

![Graph showing in vitro permeation test results](image)

**Figure 42.** Comparison of *in vitro* cumulative permeation results of THC in different carriers.
Figure 43. Comparison of *in vitro* cumulative permeation results of THC in different formulations (F1, F2, F3, F4)

Figure 44. Comparison of cumulative retention within the membrane results of THC in different formulations (F1, F2, F3, F4)
Before *in vitro* Franz diffusion cell permeation study, the solubility of THC in different mediums (1% v/v tween 80 in water, 3% tween 80 in water, 30% v/v ethanol solution, and 50% v/v ethanol solution) were conducted first in order to determine the optimized sink condition. It was found that THC has the highest solubility in 50% v/v ethanol solution which was around 5 mg/mL, compared to the 0.24 mg/mL solubility in the 1% v/v tween 80 solution, 0.27 mg/mL in the 3% v/v tween 80 solution and 0.45 mg/mL solubility in 30% v/v ethanol solution. In order to ensure that the medium was maintained at sink condition during the future test, 50% v/v ethanol solution was chosen as the receptor medium.

Then, the effect of different neat solvents on the permeation of THC was first studied (*Figure 42*). After 24 h, it was confirmed that THC in MCT permeated approximately 1.5-times slightly higher than in DEGEE. This might be explained by the fact that THC reached higher degree of the saturation in the MCT as it has lower solubility in MCT compared to DEGEE, and this led to higher thermodynamic activity of THC and thus permeated slightly more [82]. Or this might be due to the fact that MCT has better affinity to the skin lipids in the epidermis, which facilitated the better permeation. Also, when THC was solubilized in MCT or DEGEE, it showed around 87 times higher permeation compared to THC suspension in water as the control, which demonstrated that solubility is the first key factor for the efficient topical delivery for THC.

In addition, synergistic effect was also observed for the solvent of MCT: DEGEE =3:1 (v/v), showing the highest permeation performance among all the candidates. This is frequently observed for the binary solvents that have different mechanisms of penetration enhancement [84]. Previously, it was reported that the synergistic effect of the binary solvents for penetration was observed for the combination of hydrophilic propylene glycol
and lipophilic oils etc., however the exact mechanism remains to be answered. For the solvent of MCT: DEGEE = 1:1 (v/v), the overall permeation performance was similar compared to the neat DEGEE solvent except for the fact that THC in the combination of MCT and DEGEE (1:1, v/v) showed slightly higher cumulative permeation compared to neat DEGEE in the first 12 h. Previously, it was demonstrated that this specific combination has similar solubility compared to neat DEGEE, but they have relatively faster release, which might explain the phenomena about the higher cumulative permeation observed for this combination in the first 12 h. In conclusion, the solvent of MCT: DEGEE = 3:1 (v/v) has the highest potential to permeate through the skin barrier and becomes bioaccessible to skin.

Fresh nanoemulsions (F1, F2 & F3) were prepared again and immediately tested in vitro using Strat-M® membrane to compare the permeation performance. From Fig.43, nanoemulsion F1 fabricated with a volumetric ratio between MCT: DEGEE=3:1 (v/v) showed the highest permeation results. This is consistent with the result found and summarized in Fig.42, which could be explained by the unique synergistic effect of the combination of DEGEE and MCT at optimum ratio. In addition, the retentions of the formulations in the membrane were also evaluated (Figure 44), MCT also facilitated the retention within the membrane, which is ideal for the topical delivery. The kinetic analysis for the permeation rate was conducted, as it is critical to predict the permeation behavior in the long term. The kinetic analysis results showed that all the relevant lecithin-based nanoemulsion permeation rate followed the Korsmeyer-Peppas model as summarized in the Table 5. In addition, all of their respective n values were found to be greater than 1, indicating the permeation is based on case-II transport (zero order) mechanism [160, 161].
Figure 45. Schematic illustration about nanoemulsion topical delivery

Table 5 The regression coefficient of kinetic models for F1, F2, F3

<table>
<thead>
<tr>
<th>Kinetic model</th>
<th>Regression coefficient (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F1</td>
</tr>
<tr>
<td>Zero-order</td>
<td>0.9702</td>
</tr>
<tr>
<td>First-order</td>
<td>0.7697</td>
</tr>
<tr>
<td>Higuchi</td>
<td>0.9472</td>
</tr>
<tr>
<td>Korsmeyer-Peppas</td>
<td>0.9841</td>
</tr>
</tbody>
</table>

This is a promising result demonstrating the potential application of nanoemulsion used in a lotion or cream that is water based due to the high compatibility for the topical application. What is interesting is that, though nanoemulsion did not permeate as same as neat solvent, it certainly can mitigate the potential skin irritation when excipient such as DEGEE used in the high dosage [32].
4.4 Conclusion

Nanoemulsion is defined as biphasic dispersion of two immiscible liquids and is widely used in the area of drug delivery [71]. Four steps are usually involved in the process for formulations to be used in the topical delivery area, including (i) API/nutraceutical release from the vehicle, (ii) absorption into the outermost layer, stratum corneum, (iii) passive diffusion within the stratum corneum, (iv) penetration through the skin layers [86]. In this chapter, nanoemulsion was used as the main platform for topical delivery. First, a hydrophilic surfactant DEGEE and a variety of hydrophobic oils (MCT, argan oil, sunflower oil) were selected, their solubility capacities for THC were evaluated. It was found that THC showed relatively lower solubilities in the oils (for example, solubility for THC in MCT was around 9.4 mg/mL but significantly higher solubility in the DEGEE (~126 mg/mL) compared to its original solubility in water, which is reported around 56 ng/mL). Both oil and DEGEE were demonstrated to significantly increase the solubility of THC.

Secondly, the solvent-solute effect was evaluated using Franz diffusion cell on both dialysis membrane and Strat-M® membrane. Though DEGEE has the highest solubility for THC, its permeation behavior was slower and lower compared to neat MCT, which might be explained by the high thermodynamic activity of THC in MCT and the strong solute-solvent effect between THC and DEGEE that limits the release of THC from the solvent matrix. Based on the result from Franz diffusion cell on Strat-M® membrane, it was found that MCT : DEGEE = 3:1(v/v) showed the faster, higher cumulative permeation among all the candidates of interest. This demonstrated the observation of synergistic effect at this specific ratio as well as the advantage in terms of higher permeation profile from this.
Later, nanoemulsion was developed and fabricated using the optimum ratio between MCT and DEGEE (which is 3:1, v/v, F1) as stated before, and 1:1 (v/v, F2), free of MCT (F3) as the control. From the Franz diffusion cell study, it was shown that nanoemulsion fabricated using MCT and DEGEE (3:1,v/v) had the fastest, and highest cumulative permeation performance. The cumulative permeation of F1 is 1.5 times higher than the result from F2, and 2.3 times higher than F3. Besides, F1’s cumulative performance is about 9.4 times higher than unformulated THC suspended in water. Separately, the membrane retention was also analyzed at the end of Franz diffusion cell permeation study. Nanoemulsion fabricated using MCT and DEGEE (3:1,v/v) had the highest retention within the membrane. This confirmed that nanoemulsion was able to increase the cumulative permeation as well as retention. Therefore, this formulation is a promising approach to enhance the overall topical delivery efficacy. Future *in vivo* study might be needed to further confirm this point.
CHAPTER 5. DESIGN OF A HYALURONIC ACID BASED POLYMER CONJUGATE FOR ORAL DELIVERY

Part of this work has been published as “Tang, X., Zhang, M., Zhang, H., Pan, Y., Dong, Q., Xin, Y., Ho, C.T. and Huang, Q., 2021. Evaluation of the bioaccessibility of tetrahydrocurcumin-hyaluronic acid conjugate using in vitro and ex vivo models. International Journal of Biological Macromolecules”.

5.1 Introduction

Oral delivery still remains to be the most common way for administration. In addition to the traditional cosmetic products that can be applied directly on the skin, Consumers are now on the trend to look for ingestible supplements to boost their skin health, which leads to the booming growth for the beauty supplement industry. “Inside-out” is becoming the key claim in many beauty supplement products that are attractive to the consumers. From a scientific standpoint, many recent clinical studies also demonstrates that oral supplement of antioxidant could indeed help to improve skin radiance, skin whitening, and relieve skin inflammation etc., [33].

Compared to the topical/transdermal delivery, oral delivery relies on different mechanisms for the nutrients/drugs to be absorbed in order to produce the biological effects as expected. Bioaccessibility, as the key part of the bioavailability, is the fraction of an ingested compound that becomes accessible to be absorbed through the epithelia layer in the gastrointestinal (GI) tract. It is usually used to predict the bioavailability. Below eq.(10) explains the parameters that determine the bioavailability.
\[ F_{\text{Bioavailability}} = F_C \times F_B \times F_A \times F_T \quad (10) \]

Where, \( F_C \) is the fraction of targeted compound from food to ingestion, 
\( F_B \) is the bioaccessibility of targeted compound, 
\( F_A \) is the fraction of targeted compound that is absorbed in the intestinal wall, 
\( F_T \) is the fraction of target compound after chemical and or enzymatic transformation and enters the systemic circulation, also named as Bioactivity.

There are a variety of different \textit{in vitro} or \textit{in vivo} models that can be used to evaluate the above individual parameter in order to determine and predict bioavailability. \textbf{Fig. 46.} summarized the critical components, including bioaccessibility, absorption, metabolism and bioactivity, and current available models to evaluate the respective component.[35].

\textbf{Figure 46.} Current \textit{in vitro} and \textit{in vivo} models to evaluate bioavailability [35]

While it might be complicated to evaluate each component and their respective impact on the bioavailability, therefore many researchers and scientists are evaluating
bioaccessibility instead to predict the future bioavailability. Currently there are many different \textit{in vitro} methodologies that are frequently used to evaluate the bioaccessibility, such as Caco-2 cell permeation model, \textit{ex vivo} Franz diffusion cell using intestine tissue from animal, TNO dynamic gastrointestinal model-1 (TIM-1). Their mechanisms and applications are discussed in more details in the following sections.

\textbf{\textit{In vitro} Caco-2 transport}

Caco-2 cell, a human derived epithelial cell line, is primarily used to study the transport and uptake of targeted compounds through a variety of pathways, including paracellular, transcellular, efflux activities. \textbf{Figure 47} showed the schematic representation about this model, Caco-2 monolayer has the similar transporters and enzymes found in the intestine, which qualifies this model to be used for bioaccessibility evaluation. However, there are criticisms regarding to the accuracy of this model to predict \textit{in vivo} results, such as the acts that this model is static, lack of the movement of varied fluids in the GI tract [39].
**Figure 47.** Schematic representation of in vitro Caco-2 cell transport study [38]

**In vitro Franz diffusion cell**

Franz diffusion cell (shown in Figure 48) is commonly used in evaluating the permeability of topical products. Following the similar principal, many researchers are now using this model to evaluate the permeability of a compound or a specific formulation in the intestine in order to evaluate the bioaccessibility thus predict the bioavailability. There is evidence proving that this model could be an equivalent method compared to Caco-2 transport assay for the certain compounds [37]. This could be a very efficient model to provide valuable information to evaluate bioaccessibility. Additionally, besides the intestine from the animals that works as a membrane during the study, there are a variety of different synthetic membranes to be used as alternative options today and have the advantages of high reproducibility. For example, phospholipid vesicles-based permeation assay (PVPA) fabricated form liposome that simulates the intestinal cell membrane, Permeapad ® developed from University of Southern Denmark that is composed of phospholipids that
deposited between two sheets, and the artificial membrane system that is based on the cellulose with molecular weight cutoff weights of 12-14 kDa, they are intensively being evaluated by the industry and academia on the correlations between this cell free in vitro methods and in vivo results [39]. If an acceptable correlation could be found, this method based on synthetic membrane is no doubt a convenient, reproducible, and fast method for the initial screening of target compound to evaluate bioaccessibility.

Figure 48. Small intestine tissue from porcine (Left), schematic representation of Franz diffusion cell (Right).

In vitro TNO dynamic gastrointestinal model-1 (TIM-1)
TIM-1 model (shown in Fig. 49) consists of four compartments including stomach, duodenum, jejunum, and ileum. The system is controlled by a computer program to simulate the digestion through GI tract in human. During the run, samples were collected from jejunum and ileum after filtration by semipermeable fiber systems to mimic the bioaccessible portion of the targeted compound from the matrix. This in vitro model offers the advantages of high reproducibility and accuracy [36]. TIM-1 model is by far the closest system compared to the in vivo dynamics event within the GI tract [52,53]. The limitations of TIM-1 model are the lack of intestinal mucosa to mimic absorption in real conditions, the lack of metabolism and excretion, in addition to the unavailability of the food information such as energy density [53].

Previously the bioaccessibility in the oral delivery was defined and some common in vitro and in vivo methods were summarized in Fig. 46 as well. In the following works, the combination of ex vivo Franz diffusion cell and in vitro TIM-1 model were selected as the main methods to evaluate the bioaccessibility through oral route.
THC is crystalline in nature, therefore solubility is a major challenge in regard to the bioaccessibility. To overcome this, many innovative formulations have been proposed to first enhance the aqueous solubility of the targeted compound, and then increase the adsorption within the intestine in order to improve bioaccessibility as the target. Polymer-drug conjugate is intensively used especially in the pharmaceutical industry today for its advantages of water solubility enhancement, target delivery, controlled release [49]. Polyethylene glycol (PEG) is used frequently as the polymeric carrier. It has the advantages of the ability to enhance the drug solubility in water, reduce the clearance and their nanosize property when dispersed in the water exhibits a higher permeability and retention effect [170]. However, it is not biodegradable. Hyaluronic acid (HA), on the contrary, is a natural polymer with high biodegradability, biocompatibility and even bio-functionality. For example, HA is widely used in the cosmetic industry for its anti-wrinkle, moisturization benefits. It is also known that it could relieve the dry eye or promote the tissue regeneration. It is a linear polysaccharide with glucuronic acid and N-acetylglucosamine (see below Figure 50 for the molecular structure). It has many functional groups such as hydroxyl, carboxyl, N-acetyl that are available for chemical modification and reaction in order to form the polymer-API conjugate. For example, paclitaxel, a type of anticancer drug, was conjugated to HA between the hydroxyl groups in the drug and carboxylic groups in HA [50]. Lactoferrin-Hyaluronic acid (LF-HA) conjugate was formed between the carboxyl group in HA and amino group in LF by forming the amine linkage [51]. Molecular weight of HA is no doubt an important factor that determines the structure, viscosity, mucoadhesive property and penetration capacity of this macromolecule. Currently it still remains unclear about the mechanism of HA, especially HA with high molecular weight (HMW), to be
absorbed in the GI tract and there are many in vitro or in vivo results that are conflicting with each other [52]. One theory is that HMW HA could be absorbed by the TLR4 receptors located on the epithelial cells and subsequently, HMW HA is able to be absorbed. While other debated that HMW HA could not be absorbed at all based on the size of the molecule.

In this study, THC was first conjugated to HA with high molecular weight \((1.6 \times 10^6 \text{ Da})\) by forming the ester bond between hydroxyl groups in THC and carboxyl group in HA. The conjugate was then characterized using XRD, \(^1\text{H}-\text{NMR}, \text{DSC}\) to confirm the successful conjugation. Bioaccessibility of THC-HA conjugate was then evaluated using ex vivo porcine small intestine Franz diffusion cell model and TNO gastrointestinal model-1 (TIM-1). In addition, in vitro dissolution test in both simulated gastric conditions and simulated intestinal conditions were conducted to study the release of THC in order to monitor the integrity of the THC-HA conjugate. Finally, mucoadhesive assay and oscillatory rheological measurements for both HA and THC-HA conjugate were conducted to investigate their mucoadhesive properties.

![Structure of hyaluronic acid](image)

**Figure 50.** Structure of hyaluronic acid

---

**5.2 Materials and methods**

**5.2.1 Materials**
Hyaluronic acid (Mw∼1.6×10^6 Da) was a gift from DSM (Kaiseraugst, Switzerland). THC was a gift from Sabinsa (East Windsor, NJ, USA). 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were purchased from Sigma (St. Louis, MO, USA). Dime-thyl sulfoxide (DMSO) and hydrochloric acid solution were purchased from Pharmco-AAPER (Brookfield, CT, US). Potassium phosphate monobasic anhydrous, sodium chloride and sodium hydroxide were purchased from VWR (Radnor, PA, USA). Pepsin from porcine gastric mucosa, pancreatin from porcine pancreas were purchased from Sigma (St. Louis, MO, USA). Dialysis membrane (MWCO: 6,000-8,000 Da) was obtained from Spectra/Por, Thermo Fisher Scientific (Waltham, MA, USA).

5.2.2 Synthesis of Tetrahydrocurcumin-Hyaluronic acid (THC-HA) conjugate

THC-HA conjugate was synthesized following the published method with slight modifications [40,41]. Initially, 0.6 g HA were dissolved in 80 mL water under magnetic stirring at room temperature. In a separate beaker, 300 mg DCC and 150 mg DMAP were dissolved in 40mL DMSO. Then the two phases were combined and kept mixing for approximately 1 hour in order to activate the carboxylic group of the HA. Later 3.2 g THC was dissolved in 40mL DMSO, it was then added to the above phase and started heating to 60-65°C. When the desired temperature was reached, the beaker was kept mixing for approximately 6 hours under N₂. After 6 hours of mixing, the solution was then purified using dialysis membrane against DMSO for 1 day, and 3 days against water. Last, the conjugate was lyophilized and kept under refrigeration (-20°C) prior to other tests.

5.2.3 Characterizations of the THC-HA conjugate
$^1$H-Nuclear magnetic resonance ($^1$H-NMR)

THC-HA conjugate and hyaluronic acid were dissolved in Deuterium Oxide (Millipore, Burlington, MA, US), THC was dissolved in DMSO-d$_6$ (Millipore, Burlington, MA, US), they were all measured using Varian NMR (Varian Medical System, Inc., CA, USA).

Differential scanning calorimetry (DSC)

Thermal behaviors of THC, HA, physical mixture of THC & HA, THC-HA conjugate were analyzed by TA Instruments Discovery DSC (New Castle, DE, USA), data analysis was carried out by TRIOS software. The experiment was conducted by weighing around 5-10 mg of the sample in the aluminum pans and hermetically sealed. Then the system was heating from 25℃ to 150 ℃, at the speed of 10 ℃ per min under nitrogen.

X-ray diffraction (XRD)

XRD patterns including crystallinity for THC, HA, THC-HA conjugate, physical mixture of THC & HA were determined with a Cu-Ka radiation (k=1.54 Å) using Philips X’pert powder X-ray diffractometer at voltage of 40 kV and 40 mA. Scans were carried out at 5-55° (2θ) at the rate of 0.02 °/s.

5.2.4 Determination of tetrahydrocurcumin content in THC-HA conjugate

Excessive THC-HA conjugate was dispersed into water and mixed for 24 h under magnetic stirring to reach equilibrium. Then the sample was first diluted with water and
centrifuged at 9,000 rpm for 5 min. The supernatant was collected, diluted with methanol before HPLC assay. The result concluded that THC and HA was at the mass ratio of 16:100 in the THC-HA.

5.2.5 Mucoadhesive assay

Rheological measurements were performed using Advanced Rheometric Expansion System (Rheometric Inc., Piscataway, NJ, USA) at room temperature using parallel plate geometry with a diameter of 50 mm and gap height of 0.5 mm. A controlled shear rate $\gamma$ was deployed to determine the viscosity of the samples as a function of the shear rate ranging from $1 s^{-1}$ to $100 s^{-1}$. SIF without pancreatin was used (pH 6.8) to solubilize hyaluronic acid, THC-HA conjugate, and mucin respectively and kept mixing up to 4 h.

For measuring the bioadhesion component ($\eta_b$), polymer dispersion (12 mg/mL) was mixed with mucin suspension (0.1g/mL) at the ratio of 1:1 v/v, polymer dispersion (6 mg/mL), mucin (0.05 g/mL) were prepared respectively and kept at 37 °C before the test. Apparent viscosity vs. shear rate was studied. $\eta_b$ and mucoadhesion index were calculated using below eq. (11) and eq. (12) [45,46]. The apparent viscosity values were obtained at a fixed shear rate ($5 s^{-1}$).

$$\eta_{bio} = \eta_{Muc+P} - \eta_{Muc} - \eta_P; \quad (11)$$

$$\Delta(\%) = \frac{\eta_{Muc+P} - \eta_{Muc} - \eta_P}{\eta_{Muc} + \eta_P} \times 100 \quad (12)$$

In this equation, $\eta_{bio}$ is the Bioadhesion component, $\Delta(\%)$ is the mucoadhesion index.

$\eta_{Muc+P}, \eta_{Muc}, \eta_P$, are the apparent viscosity of mixture of Mucin and HA (or Mucin and THC-HA conjugate), Mucin and HA (or THC-HA conjugate) at defined shear rate ($5 s^{-1}$), respectively.
5.2.6 Dynamic oscillatory rheology measurements

In addition to the mucoadhesive assay, dynamic oscillatory rheology is another valuable and reliable tool to evaluate mucoadhesive properties [47]. Viscoelastic properties of mixture of HA or THC-HA conjugate with mucin solution were measured compared to the individual solution, respectively [48]. The angular frequency $\omega$ was performed between 0.5 rad/s to 100 rad/s, with a shear strain of 3% (linear-viscoelastic region, data not shown).

5.2.7 In vitro release

Release of free tetrahydrocurcumin from THC-HA conjugate was studied in both simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) based on the method from Wei et al [42]. For SGF, 3.2 g/L pepsin was dissolved in water containing 34 mM sodium chloride and 84 mM hydrochloride acid solution. The final pH of SGF was adjusted to 1.2. For SIF, 50 mM of monobasic potassium phosphate, 15 mM sodium hydroxide and 10 g/L pancreatin were mixed in water and the final pH of SIF was adjusted to 6.8. THC-HA conjugate (approximately 10 mg) was dispersed and mixed with 3 mL of SGF or SIF solutions and incubated at 37 °C. At each time intervals (0.5, 1, 1.5, 2, 3, 4 h), 0.5 mL was withdrawn from the solution and equal volume of fresh SGF, or SIF was added back. The aliquot was then filtered through Amicon® Ultra-0.5 mL centrifugal filter (MWCO ~3 kDa, Merck Millipore, Burlington, MA, USA) and centrifuged at 11,000 rpm for 16 mins. The concentration of the free THC in the filtrate was determined by the HPLC method. Cumulative THC release at subsequent time points is calculated using the eq.(13) and eq.(14) from below:
\[ m_i = C_i V_{total} + \sum_{1}^{i-1} C_n V_{re} \]  \tag{13}

\[
\% \text{THC release} = \frac{m_i}{m_t} \times 100 \quad (14)
\]

Where, \( m_i (\mu g) \) is the cumulative mass released at time point \( i \). \( m_t (\mu g) \) is the initial total weight of THC added to the medium. \( C_i (\mu g/mL) \) is the concentration of released THC at time point \( i \), \( C_n (\mu g/mL) \) is the concentration at previous time point \( n \), \( V_{re} \) is the volume removed at each time point (mL), \( V_{total} \) (mL) is the total volume of the release medium.

\[ 5.2.8 \text{ Ex vivo permeation study} \]

Porcine small intestine was acquired from the local slaughterhouse, jejunum was preprepared from it and used for the permeation study \[43\]. Before mounting the jejunum segment on the Franz diffusion cell, the adipose tissue was carefully removed, and the remaining jejunum was kept in pH 7.4 PBS for at least 30 min. A Franz diffusion cell (FDC-6, Logan Instrument Corp., NJ, USA) was used to study the permeation behavior of THC-HA conjugate compared to the free THC suspension (both samples contained equivalent 0.2 mg/mL THC) in order to predict intestinal absorption. 1.3 mL sample was loaded in the donor, 7 mL 50\% (v/v) ethanol in pH 7.4 PBS was filled in the receptor, which was maintained at 37℃. 1 mL from the receptor was collected at each different time intervals (0.5, 1, 1.5, 2, 3, 4 h) and the receptor was replaced with fresh medium. The cumulative intestinal permeation was calculated using eq. (15).

To assess the retention within the tissue, at the end of the 4 h experiment, jejunum was extracted with methanol. First all the membranes were rinsed with receptor medium twice front and back, the surface was gently wiped to remove any potential residues and then
sonicated in 5 mL methanol for 30 mins. All the samples were filtered through 0.45 μm filter and analyzed using HPLC, the % w/w of retention was calculated using eq. (16).

\[
\% \text{Cumulative intestinal permeation of THC} = \frac{\sum C_i \times V_r}{\text{mass of THC in the initial feed in the donor}} \times 100 \tag{15}
\]

\[
\% \text{Intestinal retention of THC} = \frac{\text{mass of THC in the membrane at the end}}{\text{mass of THC in the initial feed in the donor}} \times 100 \tag{16}
\]

Where \( C_i \) is the concentration of THC in the receptor at the time \( i \), and \( V_r \) is the receptor volume.

5.2.9 TNO gastro-intestinal model-1 (TIM-1)

The digestion behavior of THC-HA was further studied using the dynamic in vitro TNO gastrointestinal model-1 (TIM-1) (Zeist, Netherlands). This model is composed of four compartments including stomach, duodenum, jejunum, and ileum [44]. The temperature of the system was controlled at 37 ± 1 ℃, the detailed fluid compositions in each compartment was prepared according to the previously published paper [27]. Briefly in this study, physical mixture of crystalline THC and hyaluronic acid (w/w, 16:100) or THC-HA conjugate was mixed directly with gastric electrolyte solution and further diluted with water to 300 g containing equivalent 0.5 mg/g THC before being fed into the system. The dialysate fluids from the jejunum and the ileum were passed through semipermeable capillary membranes (Spectrum Minikros M80S-300-01P, Repligen, Waltham, MA, USA) and collected at different time interval (30, 60, 90, 120, 180, 240, 300, 360 min). The samples were then stored in the -20°C refrigerator before HPLC analysis. For
the extraction procedure of THC, 600 μL of sample was mixed with 600 μL of ethyl acetate and 20 μL 5 mg/mL resveratrol (as internal standard, from E.K.Herb, Changsha, China) following by vortex and centrifugation for 5 min at 9000 rpm, respectively. Later, 400 μL supernatants were collected, and ethyl acetate was removed using nitrogen. Last, the sample was reconstituted with 0.3 mL methanol for HPLC analysis. The experiment was conducted in duplicate and average cumulative bioaccessibility was calculated using the eq. (16) below.

\[
\text{%Bioaccessibility of THC} = \frac{\text{THC in Jejunum or Ileum or Jejunum+Ileum}}{\text{THC in the initial feed}} \times 100
\]  

(16)

Statistical analysis

All experimental results are shown as means ± standard deviations. The significance was recorded as P-value < 0.05.

5.3 Results and discussion

THC was conjugated to HA through carbodiimide reaction. After lyophilization, THC-HA conjugate showed a very fine, fluffy type of the structure and became very easily hydrated when dispersed in the water as shown in Fig. 51 and Fig. 52. Additionally, no visible particles were observed up to 3 mg/mL (approximately around 0.5 mg/mL THC). This might indicate the successful conjugation as THC has very limited water solubility.

In order to verify the formation of the conjugate, below characterization tests including XRD, DSC and \(^1\)H-NMR were conducted, and the results were summarized in the following section.
5.3.1 Characterizations results

XRD

As shown in Figure 53, the result confirmed that HA was amorphous indicated by the broad band absence of sharp peaks. The crystalline status of THC was found in the samples of physical mixture of THC and HA, THC itself by the observation of characteristic sharp peaks within the test range. This result is consistent with the reported data [57]. On the contrary, the sharp characteristic peaks of THC could not be found in the THC-HA
conjugate indicating the change of THC crystalline to amorphous structure when THC was conjugated to HA. As a result, this might lead to higher aqueous solubility, better absorption as well as potentially better bioavailability for the targeted compound [156].

![Figure 53. X-ray Diffraction results of I, THC. II, HA. III, physical mixture of THC and HA. IV, THC-HA conjugate.](image)

**DSC**

DSC results in Fig. 54 showed a sharp endothermic peak for THC at around 98-102 °C, which is the melting temperature required to break the crystal structure of THC. This characteristic peak could also be found in the result from the physical mixture of THC and HA, indicating that THC still exists in the crystalline structure in the physical mixture.
However, for THC-HA conjugate, the characteristic peak disappeared, therefore this study served as another evidence that THC was not crystalline in the THC-HA conjugate.

**Figure 54.** DSC results of THC, THC-HA conjugate, HA, Physical mixture of THC & HA

**\(^1\text{H-NMR}\)**

H-NMR or C-NMR is widely used to determine the structure of an unknown compound. In this case, we use proton NMR to evaluate the structure of THC-HA conjugate in order to find out if the conjugation is successful. As **Fig. 55** showed that the characteristic aromatic protons of THC were observed at 6.5-7.0 ppm, which could be found in both THC compound and THC-HA conjugate demonstrated that THC was in both of the samples [57,166]. For HA solubilized in D\(_2\)O, characteristic proton peaks (from acetyl groups) could be found at around 2.0 ppm, which was also seen in the THC-HA conjugate results. Based on this, along with the result from XRD and DSC, it could be concluded that THC-HA
conjugate was successfully formed, and the crystalline structure of THC was modified to amorphous status in the conjugate.

**Figure 55.** $^1$H-Nuclear Magnetic Resonance results of THC in DMSO-D$_6$, HA and THC-HA in D$_2$O.

5.3.2 Rheology

Mucoadhesive assay, the method to investigate the effect of the interaction between polymer and mucin, was first conducted. If the polymer is mucoadhesive, then the viscosity or rheology for the mixture should be higher than the sum of the individual value. The curves of viscosity vs. shear rate (1-100 s$^{-1}$) for HA, THC-HA conjugate, mucin, HA + mucin, THC-HA + mucin, were reported in the below **Figure 56.** As shown, all the samples exhibited pseudoplastic fluid behaviors, i.e., shear thinning behaviors. At a fixed shear rate
(5 s⁻¹), the mucoadhesive index, which is the measurement of the mucoadhesive strength [55,56], was calculated and summarized in the Table 6. By comparison, HA without modification shows a relatively higher mucoadhesive strength (~98%) than THC-HA conjugate (65%). However, the conjugate still inherited some levels of the mucoadhesive property of HA as shown in the results summarized in Table 6.

Later, linear oscillatory rheology was studied. As shown in Figure 57, the mixture of HA and mucin exhibited gel type behavior, with the evidence that G’>G” across all the test range. Mucin showed low degree of frequency dependence with G’ almost equal to G” and remained constant across the test range. For HA, transition (from liquid-like to solid-like) took place at around 70 rad/s. Similarly, the mixture of THC-HA conjugate and mucin gel type behavior, showing that G’>G” across all the test range. For THC-HA conjugate itself, G’>G” was observed across the test range demonstrating that the fluid remained liquid-like behavior. What is also interesting, is that similar to the result from previous viscosity test, the rheological value (both the elastic modulus G’ or viscous modulus G”’ of the mixture between the polymer (HA or THC-HA conjugate) dispersion and mucin is also significantly higher than the sum of the individual value. Based on the results from both the viscosity and rheology studies, it was concluded that THC-HA conjugate and HA all possess the mucoadhesive properties. For THC-HA conjugate specifically, this property might be beneficial in terms of enhancing the intestinal absorption, which makes positive impacts on increasing the bioaccessibility.
Figure 56. The plots of apparent viscosity versus shear rate curves for 6 mg/mL HA (solid triangles), 12 mg/mL HA+ 0.1g/mL mucin (1:1, v/v, solid circles), 6 mg/mL THC-HA conjugate (solid squares), 12 mg/mL THC-HA conjugate + 0.1g/mL mucin (1:1, v/v, solid crosses)

Table 6. Comparison of apparent viscosity, bioadhesion component and Mucoadhesion index for HA, HA+mucin, conjugate, conjugate+mucin, mucin.

<table>
<thead>
<tr>
<th></th>
<th>Apparent viscosity (Pa.s) at shear rate (s⁻¹)</th>
<th>Bioadhesion component (ηₐ, Pa.s)</th>
<th>Mucoadhesion index Δ(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>6.394</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>HA+mucin</td>
<td>13.158</td>
<td>6.515</td>
<td>98.073</td>
</tr>
<tr>
<td>conjugate</td>
<td>1.067</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>conjugate + mucin</td>
<td>2.171</td>
<td>0.855</td>
<td>64.970</td>
</tr>
<tr>
<td>mucin</td>
<td>0.249</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
Figure 57. The plots of storage modulus (G’, solid symbols) and loss modulus (G”, empty symbols) versus angular frequency for A: 0.05g/mL mucin (squares), 6 mg/mL HA (rhombuses), 12 mg/mL HA+ 0.1g/mL mucin (circles) and B: 0.05g/mL mucin(squares), 6 mg/mL THC-HA

5.3.3 In vitro release

In order to examine the integrity of the conjugate in the fluid, free THC release in both of the SGF and SIF was studied. Based on the literature, it is hypothesized that free THC might release from the THC-HA conjugate following two main steps, first it is the damage
from the fluids to the structure of the conjugate, and then the cleavage of the ester bond between HA and THC might be observed to release THC [54]. **Fig. 58** showed that the release in both SGF and SIF follows the sustained release behavior, no burst release is observed. Additionally, in both of the SGF and SIF conditions, less than 10% (w/w) of the THC has been released from the conjugate format. This provided the evidence that THC-HA is relatively stable during the general transit time (up to 4 h) in the GI tract.

**Figure 58.** The plot of in vitro release of THC from THC-HA conjugate in simulated gastric fluid (solid squares) and simulated intestinal fluid (solid circles). The solid lines are used to guide the eyes.
Figure 59. *ex vivo* Franz diffusion cell results of (A) Intestinal permeation between THC suspension and THC-HA conjugate; and (B) Intestinal retention result between THC suspension and THC-HA conjugate.

### 5.3.4 *Ex vivo* permeation study

As previously discussed, intestinal absorption is the key step in determining the bioaccessibility or even bioavailability. Franz diffusion cell could be used as an easy and efficient model to study the intestinal absorption of THC-HA conjugate compared to the free THC suspension [167]. In this experiment, sink condition was maintained using the 50% v/v ethanol in water solution as found in chapter 4, the result was analyzed based on the amount permeated through the porcine intestine over time and retention of the compound within the membrane at the end of the experiment. As the results indicated in Figure 59,
compared to the THC suspension, THC-HA conjugate significantly enhanced the intestinal permeation rate. This might be due to the combination of aqueous solubility enhancement and mucoadhesive property of the conjugate found in the previous studies. On the contrary, limited by the THC’s original crystalline structure, THC suspension showed much less permeation rate as well as tissue retention. Besides, the kinetic analysis for the permeation behavior between THC-HA conjugate and THC suspension were also conducted. As Fig 60 showed, that permeation rates of both THC-HA conjugate and THC suspension followed the zero order release kinetics. In conclusion, THC-HA conjugate exhibited approximately 6 times higher of THC absorbed in the intestine compared to the free THC suspension. This study demonstrated that THC-HA could enhance the bioaccessibility for THC observed by the higher permeation rate compared to the THC crystalline suspension.
5.3.5 *In vitro* TIM-1

TIM-1 model is by far the closest system compared to the *in vivo* dynamics events within the gastro-intestine tract, and it was widely used for the reliability, accuracy etc., The drawback of this model is the lack of intestinal mucosa to mimic the absorption in real conditions. In order to overcome this challenge, previous *ex vivo* permeation study was conducted on the porcine small intestine in order to study the absorption behavior close to the real condition. Here the *in vitro* TIM-1 model was mainly used to evaluate the overall bioaccessibility. In this experiment, resveratrol was used as the internal standard during the extraction process. One of the chromatographs was presented in Figure 61, the retention time for resveratrol is around 3.6 mins. Cumulative bioaccessibility between physical mixture of THC & HA and THC-HA conjugate solution found in Jejunum, Ileum and combination of both was summarized in the below Figure 62. As the results indicated that THC-HA conjugate could significantly enhance the bioaccessibility for THC, which was an approximately 10 times higher enhancement compared to the THC/HA physical mixture. This result, along with the previous intestinal absorption result, demonstrated that THC-HA is a successful delivery system that could significantly enhance the overall Bioaccessibility, which is one of the critical components for the future bioavailability.
Figure 61. Example peak from extract of TIM-1 sample. Resveratrol (internal standard) found at 3.6min THC peak found at 4.9min and 8.3min.
Figure 62. Cumulative bioaccessibility profiles of THC from (A) Jejunum; (B) Ileum; and (C) the combination of both Jejunum dialysate and Ileum dialysate of in vitro TNO dynamic gastrointestinal model -1 (TIM-1) system expressed as percent of input concentration.
5.4 Conclusion

![Image](image_url)

**Figure 63.** Proposed interaction mechanism between THC crystal, THC-HA conjugate, physical mixture of THC and HA, respectively with the intestinal epithelium.

Hyaluronic acid is a multi-functional carrier that could facilitate the uptake and sustain the release of the compound *via* the oral route. Additionally, it is also known to possess many biological functions that are beneficial to the consumers and patients. Phenolic compounds are well known for its many health beneficial properties, but their efficacy is usually limited by its poor aqueous solubility and low stability in the challenging environment throughout the GI tract when administered *via* the oral route.

In this work, high molecular weight HA was selected to synthesize the THC-HA conjugate. Characterization methods including DSC, XRD and $^1$H-NMR were used to confirm the formation of the conjugate. The bioaccessibility of THC in the THC-HA compared to free THC crystalline suspension was evaluated using the combination of the *ex vivo* Franz cell model and *in vitro* TIM-1 model. Franz cell study demonstrated that THC-HA conjugate increased the permeation rate up to 6 times higher than the free THC.
suspension. TIM-1 model showed about the 10 times higher of the bioaccessibility compared to the free THC crystallize suspension. This might be explained by the viscosity and rheology study that THC-HA inherited the mucoadhesive properties from HA to some extent and the conjugated could also increase the water solubility for the THC. Though some studies concluded that there were no interactions between high molecular weight HA and mucin, as there was no Significant change in both the elastic and viscous modules of the mixture compared to the sum of the individual polymer dispersion, our results showed the opposite conclusion. From both the mucoadhesive assay and rheology studies, we concluded that THC-HA still has the mucoadhesive properties.

As presented in Figure 63, the proposed interaction with the mucus layer within the small intestine was summarized. HA or THC-HA conjugate could restrain the mobility of the active, leading to a higher retention.

In conclusion, the conjugate works to increase the overall bioaccessibility based on the solubility enhancement for THC and mucoadhesive property it has.
CHAPTER 6. SUMMARY AND FUTURE DIRECTIONS

6.1 SUMMARY

Skin is the largest organ of human body, maintaining the wellness of skin is critical as it is the first defender to protect our skin or even the whole body from external stress. Recently the nutrition needed to the skin has gained many interests from the consumers, which has impacted today’s cosmetic and nutrition industry.

THC is a molecule that derived from a curcumin known for possessing many health related benefits. Additionally, it was demonstrated by many researchers that THC still has many similar biological activities, and it has superior antioxidant property compared to curcumin. Besides it has better stability, higher aqueous solubility, as well as higher enhanced bioavailability compared to the parent molecule. However, it still has relatively low aqueous solubility, which is around 56 ng/mL that limits its further applications.

In this research project, we used the α-MSH induced melanogenesis as the study model. THC’s inhibitory impact on the mushroom tyrosinase activity was first evaluated and confirmed. Later the protective role of THC in terms of relieving the ROS within the HaCaT cells were evaluated. Last in the murine B16F10 melanoma cells, THC’s impact on the melanogenesis was studied and confirmed that at the α-MSH stimulated condition, THC was efficient to control the overall melanogenesis.

In the previous works, two different formulations were designed including a lecithin-based nanoemulsion and hyaluronic acid-based conjugate. Nanoemulsion was used to apply topically and their stability, penetration enhancement performance compared to the unformulated THC crystals were studied. From the in vitro Franz diffusion cell study, THC in the nanoemulsion showed around 10 times higher permeation performance. In addition
to the topical nanoemulsion, THC-HA conjugate was designed and developed for the purpose for oral delivery. The delivery efficacy was evaluated using the combination of \textit{ex vivo} Franz diffusion cell using porcine small intestine and TIM-1. This combination is efficient to predict the overall bioavailability. Further \textit{in vivo} study is still needed to consolidate this result.

The work presented in this research confirmed about the health promoting role of THC in the skin. In addition, it used the innovative formulations to significantly enhance the delivery efficacy through both the topical delivery and oral delivery. This work laid a critical foundation about THC’s further applications in nutritional, cosmetic and pharmaceutical industry. Through the execution of this project, many new ideas were generated that could be helpful for future inspirations. Below summarized a few areas that might be worthwhile to investigate and some of them were evaluated about the potential feasibility through some preliminary study and the result was summarized below as well.

6.2 \textbf{IN VIVO PHARMACOKINETICS STUDY.}

\textit{In vivo} pharmacokinetics (PK) study is aimed to evaluate the overall bioavailability. Though there are many \textit{in vitro} methods available today such as TIM-1 system, \textit{in vivo} study remains important for the researchers to further evaluate the safety and efficacy. PK study is usually composed of absorption, distribution, metabolism and excretion. There are some common measurements used in the PK analysis, including $C_{\text{max}}$ (the maximum plasma concentration recorded), $t_{\text{max}}$ (the time needed to reach $C_{\text{max}}$), AUC (i.e., Area under the curve, it is a measurement of the exposure to certain actives or drugs), $t_{1/2}$ (elimination half-life, which stands for the time for the plasma concentration to fall by $\frac{1}{2}$ of its original value).
In the previous study, THC-HA conjugate was used for oral delivery and THC’s bioaccessibility has been evaluated using \textit{in vitro} TIM-1 model and \textit{ex vivo} Franz diffusion cell. In order to further validate the bioavailability enhancement by the conjugate formation, \textit{in vivo} study is needed to evaluate the bioavailability using animals.

\textbf{6.3 EXPLORATIONS OF OTHER APPLICATIONS OF THC-HA CONJUGATE.}

One interesting study is that high molecular weight HA (1.6 MDa) was previously conjugated to antibody and the conjugation, primarily derived from the HA property, limited cytokine diffusivity to control the intensity of the inflammatory responses [147]. Additionally, the conjugate also increased the topical delivery efficacy which could be demonstrated by the significant decrease of related inflammatory markers. Based on this, it is speculated that THC-HA conjugate might produce a synergistic effect of controlling the skin inflammation due to the THC’s own anti-inflammatory property as well as the high viscous property from the high molecular weight HA, which might be interesting for the future investigation.

\textbf{6.4 IMPACTS OF THC and THC-HA conjugate ON SKIN SURFACE LIPIDS.}

In addition to the skin whitening benefits, THC has been reported to possess many other benefits to skin such as anti-inflammatory and wound healing properties [81, 90]. Additionally, it was demonstrated that THC could enhance the production of extracellular matrix components, such as hyaluronic acid, collagen based on the result of \textit{in vitro} human foreskin fibroblast study [91]. When THC was delivered topically, it is critical to understand the safety and impact on other skin cells within the skin layer. In the epidermis layer, it is composed of keratinocytes, melanocytes and Langerhans cells. Keratinocyte is
of particular interest, not only because almost more than 90% cells in epidermis are keratinocytes, but also, they play essential roles to provide the skin structure, and works as defenders for skin inflammation. When keratinocytes differentiate (see Figure 64), lamellar bodies composed of phospholipids, glucosyleramides, cholesterol etc., were secreted into stratum corneum, which were later converted to ceramides and fatty acids [98]. Skin lipid integrity is critical for the maintaining of skin health, as it is responsible for skin barrier permeability. jeopardizing this layer would leave the skin or even the whole body fragile [93, 97]. In the chapter 3, keratinocytes with THC were cultured and lipid accumulation was not observed to increase with the existence of THC.

Figure 64. Pathway for the formation of lamellar bodies and secretion in keratinocytes [98]
For the explorational purpose, THC was applied on three separate volunteers. THC (in the final formula, 2% w/w) was dissolved in DEGEE first and then dispersed in (1% in the final formula, w/w) HA (Mw~1.6 MDa) water solution as test sample. Then free of THC hydrogel formula was used as the control group. To prevent THC from recrystallization, fresh solution was prepared every time prior to the experiment. Hyaluronic acid-based hydrogel formula was used to prevent the potential interference between any oil or lipid based formula with the final lipid analysis. After 30 days of continuous applications (once per day), the skin surface lipids were extracted using the published method [95]. In brief, the samples were first collected by tape stripping method and stored at -80 °C before use. Before analysis, lipids in the samples were extracted with different reagent mixtures. Last the samples were analyzed using UPLC-QTOF-MS.

As shown in the Fig.65, THC did not make any majors impacts on the average carbon chain lengths of ceramides and fatty acids. However, within the category of phospholipids, THC demonstrated that it could enhance the levels of all the different types of phospholipids including PC, PI, PE and PS. Based on the limited literature research and the results from this exploratory study[148,149], it might be interesting for the future to explore and confirm the role of THC in the cell growth and proliferation based on this phospholipidomic approach.
Figure 65. Skin surface lipids analysis results including (A) effect of THC on the average carbon chain length of ceramides and fatty acids; (B) effect of THC on the phospholipid production.

6.5 DEVELOPMENT OF THC-HA WITH DIFFERENT MOLECULAR WEIGHTS OF HA FOR TOPICAL DELIVERY

Hyaluronic acid is a well-known molecule for its superior moisturization benefits and ability to restore the skin barrier [92]. In cosmetic industry, hyaluronic acid has become one of the key ingredients that consumers find most attractive to. It has been proved that HA is able to improve the skin hydration, collagen production and face rejuvenation [94]. Besides, it has been used intensively as a delivery agent for topical and transdermal delivery based on its bioadhesive, hydration, ability to interact with stratum corneum and receptor affinity properties [22]. In our previous works, we discussed about the potential mechanisms of HA as an effective delivery agent and published this work titled “Applications and delivery mechanisms of hyaluronic acid used for topical/transdermal delivery- A review” in the journal “International Journal of Pharmaceutics” [22]. In this work, we pointed out the importance of molecular weight of HA when it was used to enhance the topical or transdermal delivery efficacy. In brief, HA with low molecular weight is able to permeate
through the stratum corneum and even reach the dermis layer. On the contrary, HA with high molecular weight cannot permeate through the stratum corneum and would remain on the surface of the skin. It would be extremely interesting to investigate if THC-HA could product any synergistic effect in terms of moisturization, anti-ageing, anti-wrinkle etc., compared to the physical mixture.
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