

FABRICATION OF NANOSPONGE FOR TOPICAL DELIVERY OF RETINOIC ACID FOR SKIN ACNE

BY

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ABSTRACT

Acne is a skin disease that causes physical and psychosocial impact for those that are affected. It is perceived as a common condition among adolescents, yet the prevalence in adulthood remains high. All-trans retinoic acid (ATRA) has high efficacy against acne but is insoluble in water, unstable in the presence of light, heat and air; and may cause side effect on the skin such as dryness, peeling and pruritus. Nanosponge with its porous structure can entrap ATRA and maybe able to enhance the solubility and stability of ATRA, improving its delivery while reducing side effects. Hence the objective of this study is to formulate a stable, safe and effective ATRA loaded nanosponge gel-cream formulation for the treatment of acne. Nanosponge was prepared with β -cyclodextrin (BCD) as the polymer and carbonyldiimidazole (CDI) as the cross-linker. ATRA was loaded into the nanosponge with varying ratios where the solubilization and entrapment efficiency was assessed. The final loaded nanosponge was then characterized through particle size and zeta potential analysis, surface morphology assessment, Attenuated Total Reflectance Fourier Transform Infra-Red (ATR-FTIR) spectroscopy, and Differential Scanning Calorimetric (DSC) analysis. The loaded nanosponge was then incorporated into a gel-cream formulation. A stability study was conducted for 12 weeks where the organoleptic properties, pH, zeta potential, particle size, polydispersity index (PDI), ATRA content and microbial limit were tested. Franz diffusion cell study with rat skin and an animal model weekly skin assessment, irritation and histology study were also conducted. Formulation was applied once daily for 21 days. Weekly skin tests to assess the trans-epidermal water loss (TEWL), hydration and erythema was done with Dermalab Combo® equipment. Human study involving 15 participants were done with nanosponge and commercial formulation with weekly skin assessment similar to animal study. Formulations were applied separately on different arms at the volar area of the forearm for 4 weeks. The ATRA nanosponge showed desirable characteristics with particle size of below 300 nm, polydispersity index below 0.5 and zeta potential value of -26.17 mV. Encapsulation efficiency obtained was 73.90% which is optimal as it is above 60%. Both ATR-FTIR and DSC study confirmed inclusion of ATRA in the cyclodextrin. The gel-cream formulation which ATRA loaded nanosponge was incorporated into remained stable in both real-time and accelerated chamber with small particle size and PDI, highly negative zeta potential, acidic pH and no phase separation nor physical changes. Animal study results show that the commercial formulation group caused significant increase in trans-epidermal water loss (TEWL) and erythema which is parallel to the physical observation whereby skin became dry, flaky, and developed wounds. Nanosponge group did show a slight increase in TEWL overtime, but it was not significant. The observation with Draize skin score and histology study further proved the irritation caused by commercial formulation and the ability of nanosponge to reduce such reactions. The results for human study also showed signs of skin barrier dysfunction similar to the findings in animal study. Reports from participants validated the findings as more participants experienced side effects with commercial formulation. As a conclusion the fabrication of ATRA loaded nanosponge gel cream that is safe, stable and effective was successful.

ملخص البحث

حب الشباب هو مرض جلدي يسبب تأثيراً جسدياً ونفسياً على الأفراد ويُعتبر شائعاً بين المراهقين خصوصاً في فترة البلوغ. أظهر حمض الريتينويك (ATRA) فاعلية عالية ضد حب الشباب ولكنه غير قابل للذوبان في الماء، وغير مستقر في وجود الضوء والحرارة والهواء، وقد يسبب آثاراً جانبية على البشرة مثل الجفاف والتقشر والحكة. يمكن لجسيمات النانو الإسفنجية أن تحتجز ATRA وربما تزيد من استقراره وقابليته للذوبان؛ مما يعزز فعاليته ويقلل من الآثار الجانبية. لذلك، هدفت هذه الدراسة إلى صياغة تركيبة مستقرة وآمنة وفعالة من جل كريم النانو الإسفنجي المحمل بـ ATRA لعلاج حب الشباب. تم إعداد جسيمات النانو الإسفنجية باستخدام بيتا-سيكلودكسترين (BCD) كبوليمر وكربونيلديميدازول (CDI) كعامل ربط. تم تحميل ATRA في النانو الإسفنجي بنسب متفاوتة وتم تقييم كفاءة التذويب والاحتجاز. ثم تم توصيف النانو الإسفنجي من خلال تحليل حجم الجسيمات وجهد زيتا وتقييم الشكل السطحي وتحليل الامتصاص بجهاز تحويل فورييه للطيف بالأشعة تحت الحمراء (ATR-FTIR) وتحليل الكالوريميتري التبايني (DSC)، وتم بعد ذلك دمج النانو الإسفنجي في تركيبة جل كريم. تم إجراء دراسة استقرار لمدة 12 أسبوعاً حيث تم اختبار الخصائص الحسية، ودرجة الحموضة، وجهد زيتا، وحجم الجسيمات، ومؤشر التشتت (PDI)، ومحتوى ATRA، والحد الأقصى للميكروبات. تم أيضاً إجراء دراسة الامتصاص الجلدي في جلد الجرذ المخبري ودراسة أسبوعية على حيوان لتقييم الجلد ودرجة تهيجه وأيضاً دراسته تشريحياً. وُضعت التركيبة مرة واحدة يومياً لمدة 21 يوماً، وأجريت اختبارات أسبوعية على الجلد لتقييم فقدان الماء عبر البشرة، والترطيب، والاحمرار باستخدام معدات Dermalab Combo. أجريت الدراسة كذلك على 15 فرداً مع النانو الإسفنجي والتركيب التجارية مع تقييم أسبوعي للجلد مماثل للدراسة على الحيوانات. تم تطبيق التركيبات على أذرع مختلفة في منطقة الساعدين لمدة 4 أسابيع. أظهر النانو الإسفنجي ATRA خصائص جيدة مع حجم جسيمات أقل من 300 نانومتر، ومؤشر تفرقة أقل من 0.5، وقيمة جهد زيتا تبلغ -26.17 مل فولت. تم الحصول على كفاءة التغليف 73.90% وهي مثلى حيث تتجاوز 60%. أكدت كل من دراسة ATR-FTIR و DSC إدراج ATRA في السيكلودكسترين. تركيبة جل الكريم بالنانو الإسفنجي ظلت مستقرة مع حجم جسيمات صغير ومؤشر DSC أقل، وجهد زيتا سالب بشكل كبير، ودرجة حموضة حمضية، ولم يكن هناك وجود لمرحلة لانفصال الجسيمات أو تغيرات في الخصائص الفيزيائية. أظهرت نتائج الدراسة الحيوانية أن مجموعة التركيب التجاري تسببت في زيادة كبيرة في فقدان الماء عبر البشرة والتهاب للجلد، حيث أصبحت البشرة جافة ومتقشرة وظهرت جروح. أظهرت مجموعة النانو الإسفنجية زيادة طفيفة في فقدان الماء عبر البشرة مع مرور الوقت،

ولكنها لم تكن كبيرة. أثبتت الملاحظة باستخدام Draize skin score ودراسة التشريح أن التركيب التجاري يسبب التهيج بشكل كبير وقدرة النانو الإسفنجي على تقليل مثل هذه الردود. أظهرت نتائج الدراسة البشرية أيضاً علامات على اضطراب حاجز الجلد مماثلة للنتائج التي تم العثور عليها في دراسة الحيوانات. أكدت المراجعات المقدمة من المشاركين صحة النتائج حيث تعرض عدد أكبر من المشاركين لآثار جانبية مع التركيبة التجارية. في الختام، نجح تصنيع جل الكريم النانو الإسفنجي المحمل بـ ATRA الذي سيكون آمناً ومستقراً وأكثر فعالية.



APPROVAL PAGE

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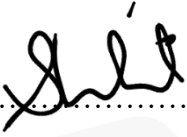
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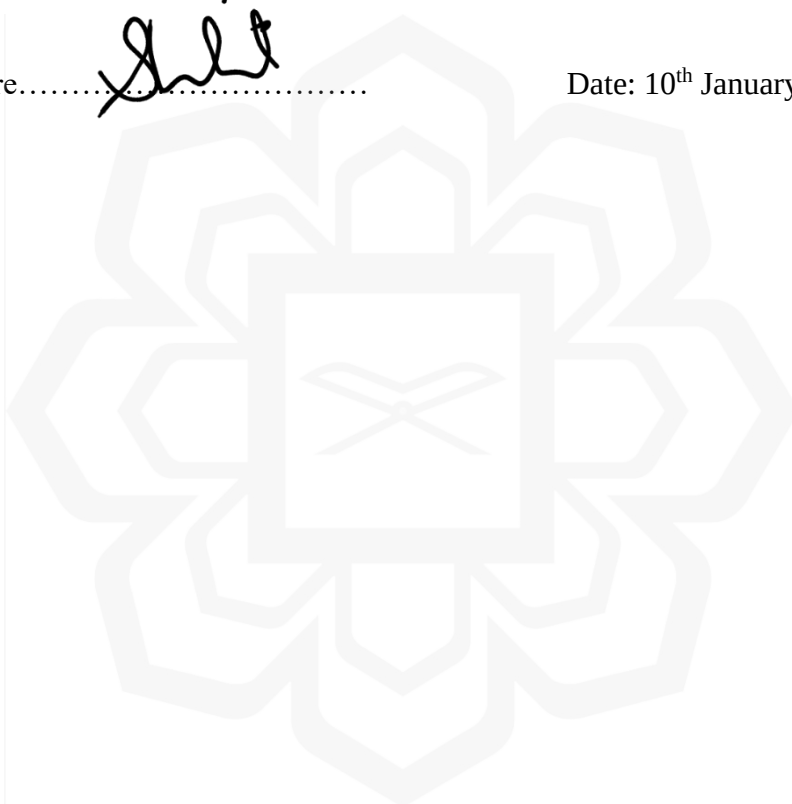
DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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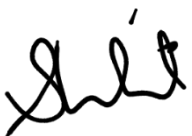
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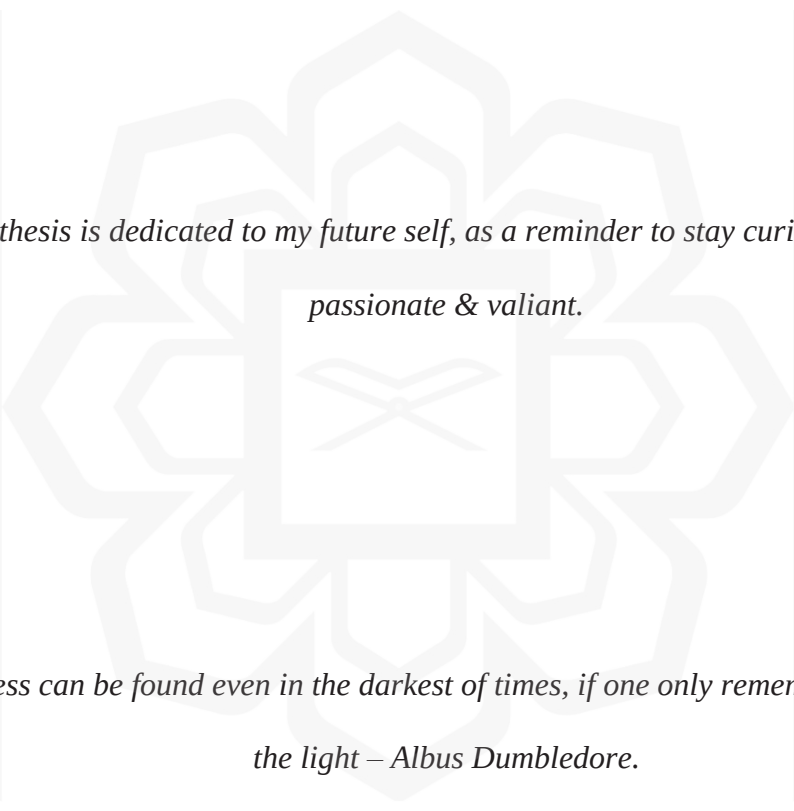


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*This thesis is dedicated to my future self, as a reminder to stay curious, humble,
passionate & valiant.*

*Happiness can be found even in the darkest of times, if one only remembers to turn on
the light – Albus Dumbledore.*

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LIST OF ABBREVIATIONS

ATRA	All-trans Retinoic Acid
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infra-red
BCD	β – cyclodextrin
CDI	Carbonyl diimidazole
DSC	Differential Scanning Calorimetry
TEWL	Trans-epidermal Water Loss



CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Acne is a common inflammatory skin disorder affecting 9.4% of the global population making it the eight most prevalent disease worldwide (Tan & Bhate, 2015). Narrowing the population to those aged between 12-25 years old, the prevalence of acne increases to approximately 85% (Stamu-O'Brien et al., 2021). Although it is perceived as a self-limited skin problem among adolescents, its prevalence remains high even in adulthood (Dawson & Dellavalle, 2013). The severity of acne effects not only manifests itself physically but also through psychosocial impacts. Physically acne appears as comedones, papules, pustules, nodules, or cyst depending on the severity and causes permanent scarring and disfigurement. A serious comorbid psychosocial effect of acne includes depression, social isolation, and even suicidal thoughts. Quality of life of acne patients can be improved with appropriate topical acne treatment and adherence (Gieler et al., 2015; Stamu-O'Brien et al., 2021).

Topical retinoids are currently the mainstay for all types of acne. All-trans retinoic acid (ATRA) or tretinoin is the most active form of vitamin A derivative that can be used as a monotherapy or in combination with benzoyl peroxide or a topical antimicrobial agent. Not only can it improve the appearance of active acne, but it can also prevent the formation of new microcomedones (Dawson & Dellavalle, 2013). ATRA can regulate epithelial cell growth, normalize desquamation, reduce sebum production, enhance collagen synthesis and impart comedolytic action. However, ATRA has some problems including insolubility in water, unstable in the presence of light, air and heat along with some distressing side effects (Charoenputtakhun et al., 2014). All these issues maybe alleviated and improved with the development of a formulation using novel drug delivery system (Leccia et al., 2015). Nanosponge with its porous structure maybe able to enhance the solubility of ATRA and improve its

delivery over all while reducing side effects due to its ability to provide sustained release (Bhowmik et al., 2018; Castro et al., 2011).

1.1.1 Layers of The Skin

As the largest organ in human, the skin plays multiple crucial roles to protect the body and maintain homeostasis. Being the outermost barrier, the skin protects from harmful physical or chemical agents and prevent fluid loss from the body. Vitamin D production and regulation of body temperature is also a vital role of skin. The skin is consist of three main layers namely the epidermis, dermis and hypodermis as shown in Figure 1.1 (Lai-Cheong & McGrath, 2017). Epidermis is the outermost layer which is composed of four different layers that is stratum basale, stratum spinosum, stratum granulosum and stratum corneum. Stratum basale is the innermost layer that is involved in cell division. This layer of the epidermis contains melanocytes, which transfer melanosomes to keratinocytes determining individual skin pigment; and Merkel cell which is related to sensation. Stratum spinosum has abundance of desmosomes and keratin filament which holds adjacent cells together. Langerhan cells are also present in this layer acting as an antigen presenting cell. The keratinocytes then lose their nucleus and become flattened at the stratum granulosum layer.

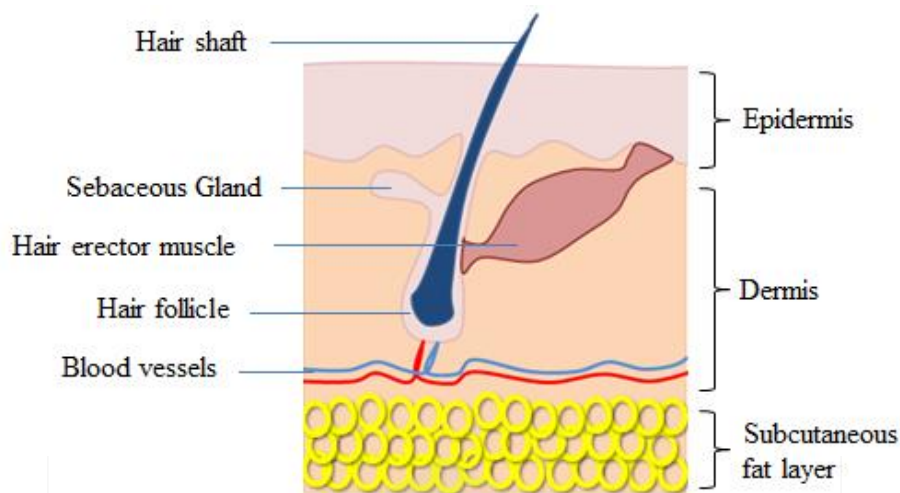


Figure 1.1 Layers of The Skin

Finally, in the stratum corneum is where the keratinocytes turn into corneocytes that are characterized by flattened and elongated cells without nucleus and organelles. This layer is the critical layer that protects the skin from external insults. Keratinocytes in the basal layer will undergo cell differentiation or also known keratinization where the cells change in each layer from a postgerminative state in stratum basale to a fully differentiated protein-filled hardened skin cells. Lamellar bodies produced by keratinocytes will secrete lipids, protease inhibitor, hydrolases and antimicrobial peptides into the upper epidermal layers. This secretion occurs at the stratum granulosum provides antimicrobial protection while also preventing fluid loss (Yousef & Sharma, 2018).

Dermis layer is the second layer located in between epidermis and hypodermis. This layer contains several appendages such as sweat glands, hair follicles and sebaceous glands. Blood vessels and nerves that provide nutrition and sensation are also located in this layer. Structural support is provided by the dermis layer due to its thicker appearance compared to epidermis and hypodermis. The dermis consists of two layers which are the papillary dermis and reticular dermis. The papillary dermis is the thinner layer which is located below the epidermis. Shear stress resistance in the dermal-

epidermal junction area is provided by this layer. The thicker reticular layer is located below the papillary dermis and above the hypodermis. Dermis is mainly composed of collagen fibres, elastic fibres and extrafibrillary matrix. Most of the dermis layer is taken up by collagen fibres that are loosely woven in the papillary layer and becomes thicker as it nears the hypodermis. Elastic fibres that give elasticity to the skin is mainly located in the reticular layer while the extrafibrillary matrix fills the space, ease movements of molecules, fluids and inflammatory cells; and resists compression. Hypodermis is the subcutaneous fat layer and the innermost layer of the skin made up of mainly fat cells. These fat cells acts as insulators, shock absorbers, energy source and contributes to buoyancy (Albanna & Holmes, 2016).

1.1.2 Acne and Acne Treatments

Acne can happen to anyone of all age groups, but it is especially common in teenagers. Acne can reduce patients' quality of life along with an increased risk of anxiety and depression due to its psychological consequences (Gieler et al., 2015; Stamu-O'Brien et al., 2021). Acne can be influenced by internal factors such as medications, diet and hormone; and external factors from the environment. Acne is a condition that happens as a result of hair follicles being clogged with sebum and dead skin cells. Increase in sebum production or also known as seborrhea can interfere with the natural skin desquamation process. The excess sebum binds the dead skin to the surface preventing it from shedding. Excess sebum that builds within the sebaceous glands also causes inflammation due to its composition. The blockage of hair follicles by dead skin cells on the other hand is caused by a process known as hyperkeratosis. Hyperkeratosis is described as a process where the external layer of the skin is abnormally thickened. This happens because of insufficient desquamation or shedding of dead skin cells as well as excessive production of new skin cells or corneocytes. These cells will then block the sebaceous gland by forming a plug. Due to the excessive sebum production, the skin becomes an ideal site for bacteria specifically *Cuticubacterium acnes* to breed and cause further inflammation. Androgen hormone that are present in both men and women can also exacerbate the condition by increasing the sebum production (Greydanus et al., 2021; Kunin, 2014). The causes of acne are shown in Figure 1.2.

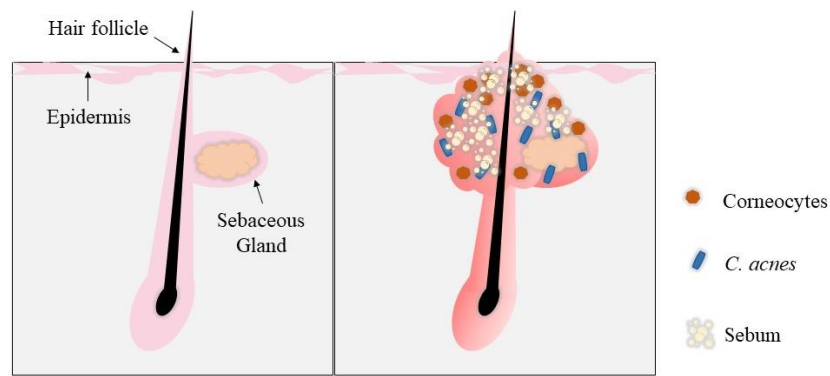


Figure 1.2 Pathogenesis Of Acne (Right) Illustrated in Comparison to a Normal Hair Follicle (Left). (Syed Omar & Hadi, 2022)

There are two types of acne lesion namely the noninflammatory lesion and inflammatory lesion. Blackhead or open comedone is a noninflammatory lesion that forms due to enlargement of follicular plug. Whitehead or closed comedone are similar except the pore remains closed. Inflammatory lesions include papules, pustules, and nodules. Papules are swollen and inflamed closed comedones while pustules are ones that are inflamed to the point of rupturing into skin. Nodules acne lesions that rupture into skin are severely inflamed with a large, tender and swollen appearance (Greydanus et al., 2021; Kunin, 2014). Acne can be divided into several types which include mild, moderate, and severe acne. Mild acne is usually characterized by the presence of open and close comedones that are limited to the face. Moderate acne includes more inflammatory lesions like papules and pustules on the face, maybe accompanied by some truncal disease. Lastly severe acne can be shown by the presence of cysts and nodules along with elaborate truncal disease. Severe acne is usually treated systemically while mild to moderate acne can be sufficiently treated with topical treatments (Dawson & Dellavalle, 2013; Ministry of Health Malaysia, 2022; National Institute of Health and Care Excellence, 2021)

The first line of therapy for mild acne is benzoyl peroxide, topical retinoids or topical combination therapy. For moderate acne, topical combination therapy; oral

antibiotic, topical retinoid and benzoyl peroxide; oral antibiotic and topical retinoid; or benzoyl peroxide and topical antibiotic. A combination of oral antibiotic with either topical combination therapy or oral isotretinoin is the recommended management for severe acne (Ministry of Health Malaysia, 2022; National Institute of Health and Care Excellence, 2021; Zaenglein, 2018). The topical combination therapy refers to the combination of two topical anti acne agents, for example topical retinoid with benzoyl peroxide or topical retinoid with a topical antibiotic. Benzoyl peroxide exerts its antibacterial activity by oxidizing bacterial proteins and it also has anti-inflammatory and comedolytic activity. Topical retinoids have comedolytic, anti-inflammatory activity as well as the ability to normalize desquamation. Topical antimicrobials such as clindamycin and erythromycin have bactericidal and bacteriostatic activity, respectively (Leccia et al., 2015; MOH, 2012; NICE, 2021). Other available acne treatments include azelaic acid, dapsone, salicylic acid, zinc, hormonal therapy and spironolactone (Leccia et al., 2015; Zaenglein, 2018). Shown in Figure 1.3 are some of the available products for the management of acne in Malaysia.



Figure 1.3 Marketed Products for the Management of Acne Available in Malaysia

1.1.3 Retinoids

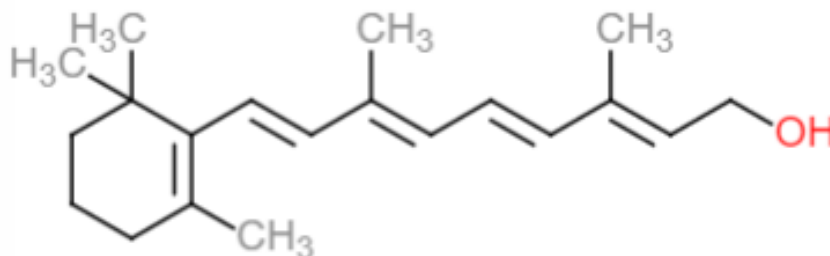


Figure 1.4 Chemical Structure of Retinol

Vitamin A or retinol as shown in Figure 1.4 is a fat-soluble vitamin crucial for a normal and healthy vision development which cannot be synthesized *in vivo* but must be acquired through diet. Main form of vitamin A that can be obtained through diet are beta carotene and retinyl esters. Retinol are cleared and absorbed by the intestines in chylomicrons and transported to the liver to be stored as retinyl esters. The absorption of vitamin A involves bile salts, dietary fat and pancreatic lipase (Riahi et al., 2016). If not stored, retinol is oxidized to tretinoin or ATRA through a two-step process with retinaldehyde as intermediate. The transformation of retinol into its active metabolites acts as an essential bioactive mediator of retinol's function (Hubbard et al., 2014).

The most important metabolites are ATRA which consists of two different isomers which are 11-cis retinal and ATRA. 11-cis retinal is a chromophore for visual function while ATRA is a regulator of gene transcription. Their metabolic pathway is as shown in Figure 1.5. In the body, retinyl esters are hydrolysed and results in vitamin A or also known as all-trans retinol (ATROL) which binds to retinol binding protein (RBP) forming ATROL/RBP complex. After the complex is taken up by target organs it is either re-esterified to retinyl esters or binds to cytosolic retinoid binding protein (CRBP). The ATROL/CRBP complex is also a storage form and a substrate that undergoes oxidation of ATROL into all-trans retinal (ATRAL) catalysed by retinol

dehydrogenase (RDH). The ATRAL is transported by CRBP in the cell and the oxidized to ATRA by various retinaldehyde dehydrogenases (RALDH). Degradation of ATRA is by CYP26 which is a member of the cytochrome hydroxylase P450 family which will turn ATRA into a less bio-active retinoid (Zasada & Budzisz, 2019).

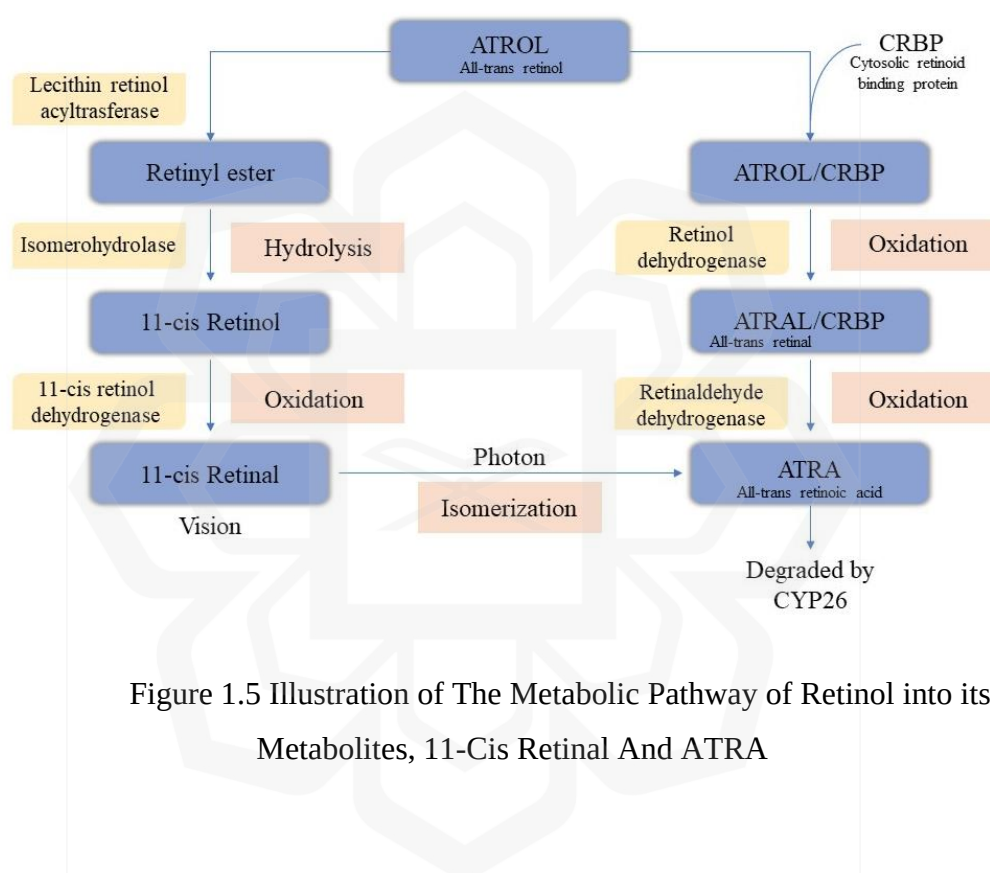


Figure 1.5 Illustration of The Metabolic Pathway of Retinol into its Metabolites, 11-Cis Retinal And ATRA

Retinol binding proteins that regulate the metabolism of retinols and retinoic acid by acting as carriers which activates receptors in the nucleus include CRBP and cytosolic retinoic acid binding protein (CRABP). Each protein has two subtypes CRBP I, CRBP II, CRABP I and CRABP II. CRBP I is the main regulator for retinoid homeostasis and immobilizes intracellular retinol for storage. It prevents ATROL from nonspecific oxidation and act as carrier protein for ATROL to be presented to RDH for oxidation or lecithin retinol acyltransferase (LRAT) for esterification (Napoli, 2017). While CRABP, although not the main regulator of homeostasis still plays a role in

preventing ATRA from non-specific degradation and ensure the solubility of retinoids in aqueous intracellular environment. CRABP I mainly regulates ATRA metabolism rate by presenting ATRA to CYP26A1 and CRABP II regulates ATRA signalling through negative feedback mechanism (Riahi et al., 2016).

Retinoids carried by carrier proteins interact with retinoid nuclear receptors (RNRs), which consist of two groups: the retinoic acid receptors (RARs) that recognize ATRA and 9-*cis* retinoic acid, and retinoid X receptors (RXRs) that recognize 9-*cis* retinoic acid only. Both groups have three isotypes— α , β and γ —and the RAR mediates retinoic acid signalling and RXR activates other signalling pathways with other receptors, such as liver X receptors, farnesoid X receptors and peroxisome proliferator-activated receptors. The binding of a ligand leads to dimerization of the RAR with RXR forming a heterodimer that binds to the retinoic acid response element (RARE) to initiate gene transcription in the promoter region of target genes as depicted in Figure 1.6 (Kam et al., 2012). Retinoids are involved in embryogenesis and play a role in the development of the nervous system, liver, heart, kidneys, intestines, eyes and limbs. Most RXR receptors are not critical for embryogenesis, but the RAR subtypes function redundantly and are critical in embryonic development (Zasada & Budzisz, 2019). ATRA, as the most active form of vitamin A, has been used and studied in the treatment of many diseases and multiple different formulations have been developed to enhance the efficacy and reduce the toxicity of ATRA.

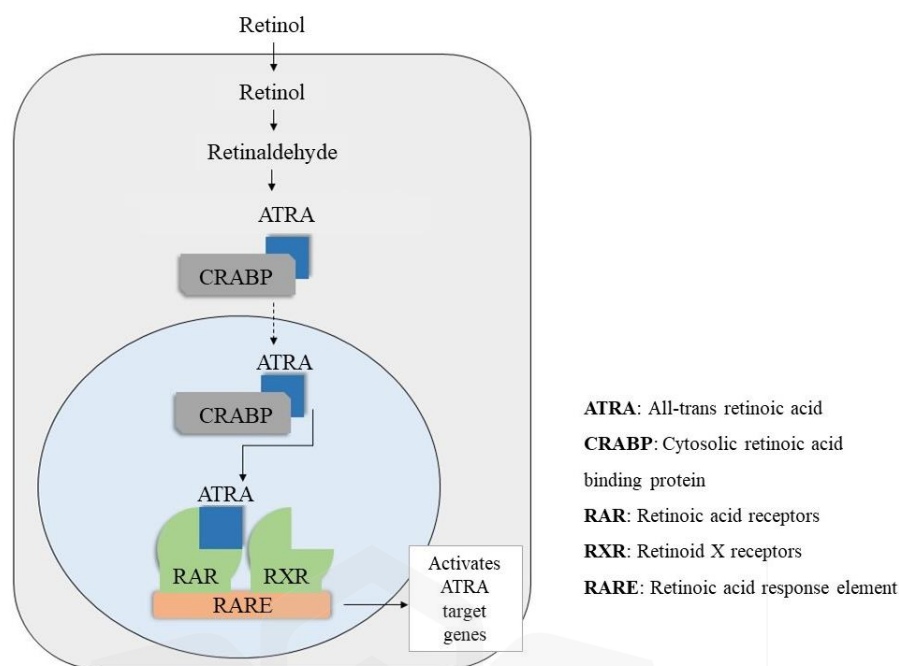
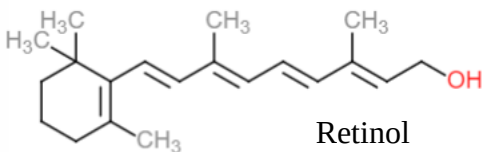
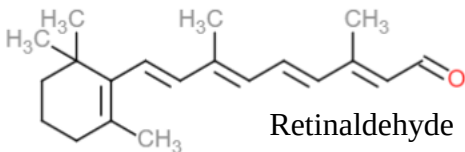
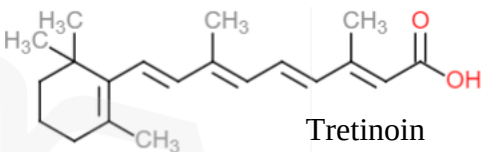
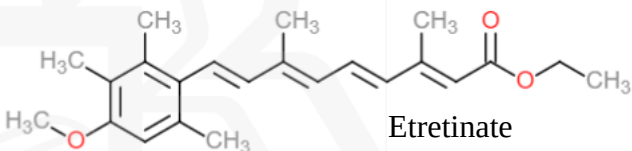
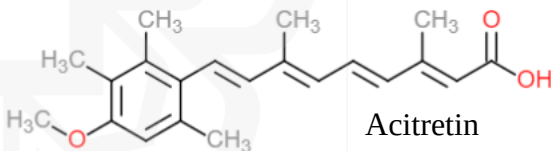
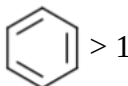
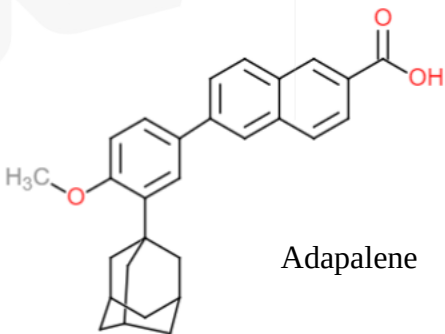
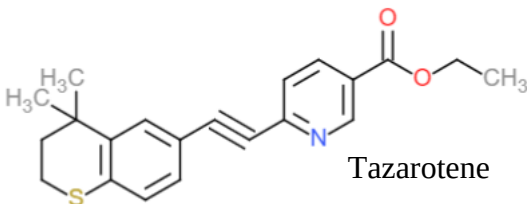


Figure 1.6 The Process of RNR (RAR, RXR) Activation in The Cell by ATRA
(Syed Omar & Hadi, 2022)

. Retinoids has been used widely to treat multiple diseases and has evolved into different generations. The first generation of retinoids include retinol, tretinoin, isotretinoin and alitretinoin. Etretinate and acitretin are part of the second generation and finally adapalene, bexarotene and tazarotene are the third generation of retinoids (Azulay & Vendramini, 2016; Syed Omar & Hadi, 2022). The generations of retinoids are as listed in Table 1.1. The first two generations contain compounds with a polyene tail, whereby the first-generation compounds contain a cyclohexane ring that is replaced by a benzene ring in the second generation. The polyene tail makes these compounds less selective towards receptors. Because there are multiple ATRA receptors with different activities, it is desirable for a retinoid to have selective affinity towards receptors that confer beneficial effects with less affinity towards receptors that act counter to these benefits or cause side effects. The third generation consists of a group of polyaromatic synthetic retinoids with a cyclized polyene side chain and includes adapalene and tazarotene. The cyclization of the polyene tail leads to selective activity (Hubbard et al., 2014; Zasada & Budzisz, 2019).

Table 1.1 Generation of Retinoids (Syed Omar & Hadi, 2022)

Generation	Retinoids	Structure
<i>First generation.</i> Natural non-aromatic retinoids.  Cyclohexane ring	Retinol	 Retinol
	Retinyl palmitate	
	Retinyl retinoate	 Retinaldehyde
	Retinyl N-formyl aspartamate	
	Retinyl acetate	 Tretinoin
	Retinaldehyde	
<i>Second generation.</i> Synthetic monoaromatic retinoids.  Benzene ring	Etretinate	 Etretinate
	Acitretin	 Acitretin
<i>Third Generation</i> Synthetic polyaromatic retinoids  Multiple benzene rings	Adapalene	 Adapalene
	Tazarotene	
	Bexarotene	 Tazarotene

More recently developed retinoids include seletinoid G and trifarotene. Seletinod G was designed and developed in 2005 using computer-aided molecular modelling to provide a compound with good efficacy and tolerability. In comparison with ATRA, seletinoid G showed barely any skin irritation or erythema, and similar effects towards inhibiting collagen degradation (Kim et al., 2005). Trifarotene is the most recently developed retinoid, claimed to be first in a fourth generation class, which has selective affinity towards the ATRA receptor-gamma (RAR γ), and superior comedolytic, anti-inflammatory and depigmenting activity compared with other retinoids (Balak, 2018).

Other than acne management, retinoids were also found to be beneficial in photoaging, psoriasis and certain cancers. Tretinoin and tazarotene have been FDA approved for the treatment of photoaging. From the term itself, photoaging is the exposure of skin to UV radiation leading to heightened production of reactive oxygen species (ROS) causing cell oxidative damage including collagen breakdown. Retinoids prevent dermal matrix degradation and increase collagen production and fibroblast proliferation hence improving appearance of wrinkles (W. R. Lee et al., 2022; Poon et al., 2015). Skin, oral, lung, prostate, breast, ovarian and bladder cancer can benefit from retinoids. Low dose of ATRA can cause cell cycle arrest while high dose ATRA triggers apoptosis of cancer cells. ATRA has been FDA approved for the treatment of lymphoma and leukemia (Chen et al., 2014; Szymański et al., 2020). In psoriasis, acitretin also a vitamin A derivative was found to be safe and effective especially in pustular psoriasis and erythroderma psoriasis either monotherapy or with combination therapy (Dogra & Yadav, 2014). Tretinoin is also widely used for the management of psoriasis (Chen et al., 2020; Raza, Singh, Lohan, et al., 2013).

1.1.3.1 ATRA

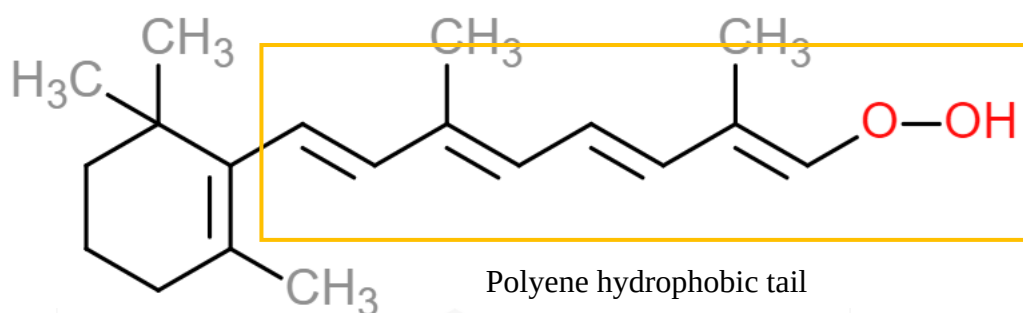


Figure 1.7 Chemical Structure of ATRA

The foundation used for the treatment of acne is topical retinoids. Retinoids are a group of compounds derived from vitamin A synthetically or from natural compounds. Naturally, ATRA or also known as retinoic acid can be derived from β -carotene (Butnariu, 2016; Yee et al., 2021). The structure of ATRA is as shown in Figure 1.7. ATRA consists of a polyene hydrophobic tail containing four isoprene units attached to a cyclic 6 carbon ring which makes a head-to-tail structure. The conjugated carbon-carbon double bonds in the polyene tail makes retinoids light sensitive (Barua & Furr, 1998; Kam et al., 2012). ATRA is also a highly lipophilic substance indicated by a high log P value of 6.3 and has low molecular weight of approximately 300 Da.

Tretinoin or ATRA was found to be one of the retinoids with highest efficacy against acne compared to more recent retinoid such as adapalene (Azulay & Vendramini, 2016; Castro et al., 2011). Retinoids act by normalizing desquamation through promoting differentiation and reducing keratinocyte proliferation. While the anti-inflammatory actions take place by blocking inflammatory pathways involving toll like receptors, leukocyte migration and AP-1 pathway which inhibits inflammation by reducing the release of cytokines and nitric oxide. Other than targeting acne, retinoids can also improve secondary acne lesions, in particular acne scarring and pigmentation.

Scarring is improved by upregulation of procollagen genes while pigmentation is reduced by accelerating epidermal turnover and inhibition of melanosome transfer (Leyden et al., 2017). However, it has greater skin irritation risk compared to other retinoids. The use of retinoids are limited by its side effects such as peeling, dryness, erythema, desquamation, burning and pruritis especially in those with sensitive skin (Leccia et al., 2015; Leyden et al., 2017). Chemical-wise, ATRA is insoluble in water, and unstable in the presence air, light and heat (Charoenputtakhun et al., 2014).

Due to its known side effects, poor solubility in water and sensitivity to external stimuli, scientists have researched a variety of carriers to improve the delivery and stability of ATRA whilst reducing the adverse effects. One of the many carriers extensively researched is liposomes due to their versatile nature. Liposomes are spherical carriers with an inner aqueous core enclosed by a lipid bilayer surrounding the entire vesicle (Bavarsad et al., 2016). They have the capacity to enhance drug efficacy and stability while simultaneously reducing toxicity with a crucial ability to facilitate targeted drug delivery (Alavi et al., 2017). ATRA in liposomes exhibited superior efficacy against non-inflammatory lesions and causes less irritation and redness in comparison to free ATRA and commercial ATRA formulation (Abdel Rahman et al., 2016). Nevertheless, challenges such as low solubility, leakage, drug fusion within the encapsulation and high production costs have spurred further research related to liposomes. As a result, liposomes have evolved into different types to suit different drugs, disease, and delivery. Study of ATRA encapsulation has extended into niosomes, ethosomes, transferosomes (deformable liposomes), hexosomes and glycerosomes. Promising results were seen with hexosomes, and deformable liposomes which showed superior efficacy in reducing side effects associated with ATRA (Bavarsad et al., 2016; Wang et al., 2014). More recent research has even developed liposomes as flexible liposomal gel dual-loaded with ATRA and betamethasone for the treatment of psoriasis. This formulation was able to provide sustained release, high transdermal efficacy and high skin retention whilst maintaining anti-psoriatic effect and reducing side effects (Wang et al., 2020).

Apart from liposomes which are vesicular carriers, lipid nanoparticles such as solid lipid nanoparticle (SLN) and nanostructured lipid carrier (NLC) have also been used to encapsulate ATRA. Lipid nanoparticles represent a modern alternative to conventional colloidal carrier systems used for delivering lipophilic drugs, such as emulsion. These lipid nanoparticles offer the advantages of colloidal carriers while minimizing their drawbacks. They are capable of effectively encapsulating both hydrophilic and lipophilic drugs (Mishra et al., 2018; R. Shah et al., 2015). SLNs for instance are composed of solid lipids with a melting point exceeding 40°C, ensuring that the lipid matrix remains solid under typical room and body temperatures (Charoenputtakun et al., 2014; Souto et al., 2020). SLNs offer benefits which include reduced toxicity, increased drug loading capacity, controlled drug release, enhanced stability, biodegradability, and ease of scaling up to an industrial level due to their cost-effectiveness and low organic solvent use (Arantes et al., 2020; Castro et al., 2011; Silva et al., 2016). Additionally, SLNs possess a substantial surface area, enabling improved skin penetration and controlled release of active ingredients (Charoenputtakun et al., 2014). Evaluation on the impact of ATRA on chronic wound healing utilizing a diabetic mouse model found that ATRA led to an enhancement in wound healing by reducing the presence of macrophages and neutrophils at the wound site. Furthermore, there was an increase in collagen deposition, particularly type iii collagen, which is linked to a healing process that results in reduced scar tissue formation. Interestingly, the application of ATRA-SLM directly to the wound bed was shown to improve wound healing without causing any harmful effects (Arantes et al., 2020). However, the encapsulation efficiency and loading capacity of ATRA in SLN is quite low (Arantes et al., 2020; Silva et al., 2016).

The emergence of NLC helped to overcome the limitations of SLNs. NLCs consist of a blend of solid and liquid lipid matrices, possessing lower melting point in comparison to SLNs, yet retaining a solid form under typical room and body temperatures. By incorporating a liquid lipid with varying density along its fatty acid chain, a less organized arrangement is achieved within the lipid matrix. This results in a significantly increased capacity for loading drugs. Additionally, this less structured state in the solid lipid matrix enhances the thermodynamic stability of the carrier system (Ghate et al., 2016; Silva et al., 2015; Souto et al., 2020). An ATRA formulation carried

within NLCs for topical use exhibited excellent entrapment efficiency. Skin irritation assessments involving ATRA in NLCs showed no signs of irritation or erythema after days of application, which stood in contrast to a commercially available formulation. Furthermore, the stability of ATRA was enhanced, as evidenced by the absence of alterations in particle size and the absence of substantial drug loss over time (Charoenputtakhun et al., 2014; Ghate et al., 2016). Enhancements in the characteristics of ATRA were achieved through the use of NLCs. Nonetheless, when NLCs were compared to SLNs, they exhibited lower levels of drug permeation and controlled release capabilities, but they could potentially outperform SLNs in terms of efficacy.

ATRA has also been encapsulated in hybrid nanoparticles to improve ATRA delivery. A study combined phospholipid and zein; which is a natural protein obtained from corn to encapsulate ATRA. The hybrid nanoparticle was able to protect ATRA from degradation in comparison to its free form. The pharmacological efficacy assessed through its antitumor activity was not compromised by the encapsulation (Gagliardi et al., 2021). Another example of a hybrid nanoparticle combined chitosan and tripolyphosphate lipid to encapsulate ATRA for the treatment of diabetic nephropathy. ATRA release was improved due to the better drug solubilization. Oral delivery of ATRA was also more promising due to the enhanced mucoadhesive property (Asfour et al., 2021). A lipid polymer hybrid encapsulating both ATRA and tripterine to treat arthritis also showed promising results through reduction of adverse effects associated with ATRA and improved overall drug efficacy (J. Li et al., 2021). Generally, ATRA has been encapsulated in different drug delivery systems to suit different needs of the specific diseases.

1.1.4 Nanoparticles and Nanosponges

Advancement of nanotechnology brought about the discovery of formulations based on nanoemulsion and nanoparticles. Nanoparticles which range from 10-1000 nm are either particle dispersion or solid particles (Mohanraj & Chen, 2007). Nanoparticles can

be classified into encapsulating nanoparticles, complexing nanoparticles and conjugating nanoparticles. Conjugating nanoparticles bind to molecules through strong covalent bonds. Complexing nanoparticles attach itself to molecules by electrostatic charges. While encapsulating nanoparticles such as nanosponge and nanocapsule has many pockets to carry molecules or able to entrap the molecules within itself (Shivani & Poladi, 2015; Subramaniam et al., 2012). There are also lipid nanoparticles used to entrap drug molecules such as liposomes, solid lipid nanoparticle (SLN) and nanostructured lipid carrier (NLC) as depicted in Figure 1.8.

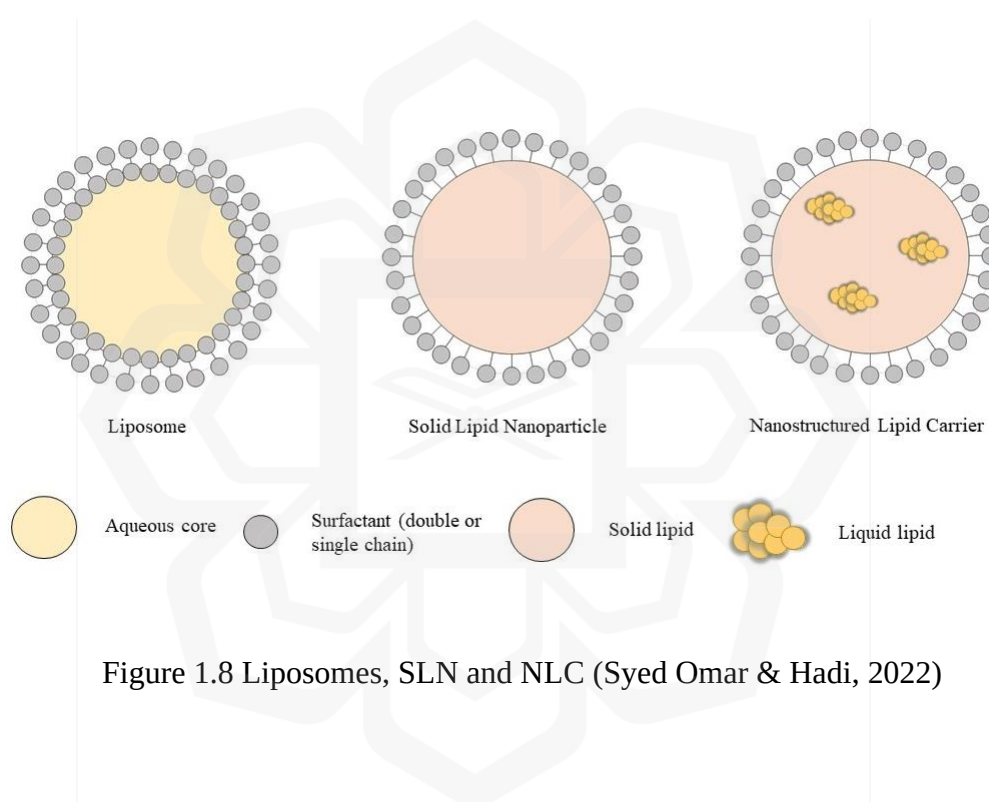


Figure 1.8 Liposomes, SLN and NLC (Syed Omar & Hadi, 2022)

Nanosponge is a type of nanoparticles made of polymers and cross-linkers to hold the polymer together. It is a novel three-dimensional network drug delivery system with sphere shaped nanoparticles and an abundance of nanosized cavities to store drugs (Panda et al., 2015). Nanosponges can be developed in crystalline or paracrystalline form which will affect and determine its loading capacity. The size and drug releasing property over time can be varied by adjusting the proportions of polymer and cross linkers (Shivani & Poladi, 2015; Tiwari & Bhattacharya, 2022). Nanoparticles specifically nanosponge has gained attention due to its many benefits that can improve drug delivery. Nanosponge can encapsulate both hydrophilic and lipophilic molecule

hence improve solubility of poorly soluble drugs. Easily degradable molecules can also use nanosponge to protect it from degradation improving its stability. Both advantages can help enhance delivery and efficacy of drug molecules (Patel & Oswal, 2012; Shivani & Poladi, 2015). Different formulations for different route of administration can be formulated using nanosponge as a delivery system. Size of the nanosponge as well as its drug release profile either slow or fast can be varied indicating the flexibility of nanosponges (Panda et al., 2015; Subramaniam et al., 2012). Moreover, nanosponge can also be used for specific target site delivery with predictable release. This technology is also desirable in oral route of administration to mask unpleasant taste of drug. Side effects and irritations can be reduced thus increasing tolerability which leads to better compliance. Nanosponges are also non-toxic, non-irritating, non-allergenic, non-mutagenic and biodegradable (Bhowmik et al., 2018; Kaur et al., 2015).

1.1.5 Nanosponge Preparation Methods

As there are various types of nanosponge, the preparation method for nanosponge also varies. As depicted in Figure 1.9 are all the different methods for nanosponge preparation (Tiwari & Bhattacharya, 2022).

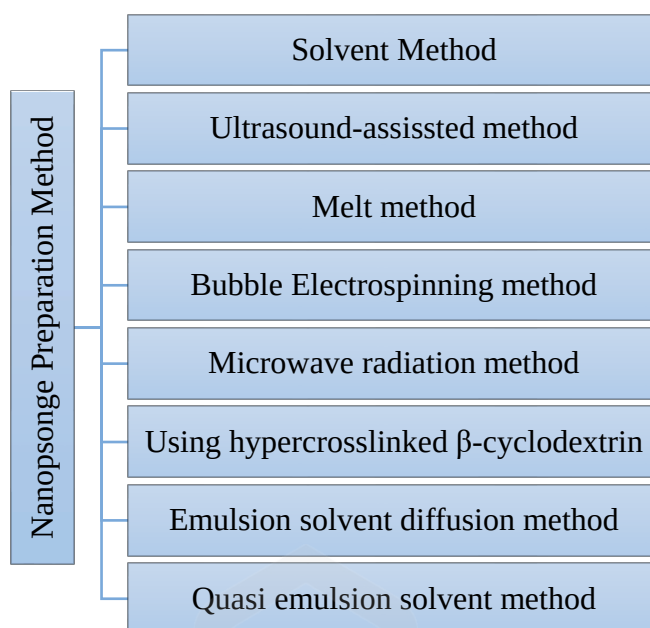


Figure 1.9 Different Methods of Nanosponge Preparation

1.1.5.1 Solvent Method

The chosen polymer is mixed in a suitable solvent. The mixture is then added to a surplus amount of cross linkers. The reaction is executed at a temperature ranging from 10°C to solvent's reflux temperature for a duration around 1- 48 hours. Following the reaction, the solution is then cooled to room temperature and an excess of distilled water is added to it. Product recovery is done through vacuum filtration and the purification is carried out by prolong Soxhlet extraction with ethanol. Lastly, product is dried using vacuum and ground to powder with a mechanical mill (Panda et al., 2015; Shringirishi et al., 2014; Tiwari & Bhattacharya, 2022).

1.1.5.2 Emulsion Solvent Diffusion Method

The dispersed phase containing ethylcellulose and drug is dissolved in dichloromethane. The continuous phase containing a definite amount of polyvinyl alcohol is dissolved in water through continuous stirring. The dispersed phase is then

added to the continuous phase or aqueous phase a little at a time and stirred at 1000 rpm for 2 hours. The nanosponge formed is collected and then filtered, washed and oven dried at 40°C for 24 hours. Finally, removal of residual solvents is done by storing nanosponges in a vacuum desiccator (Shivani & Poladi, 2015; Shoaib et al., 2018).

1.1.5.3 Hyper Cross-linked β -cyclodextrins

Nanosponge can be produced by reacting cyclodextrin with a cross linker. β -cyclodextrin is dissolved in dimethylformamide (DMF) before adding cross linkers. The reaction takes place at 100°C for 4 hours. The solid block formed from the reaction is then ground and later added with deionized water to aid the removal of DMF. Purification of the product is carried out through Soxhlet extraction with ethanol. Product obtained is dried and finally dispersed in water to separate the suspended colloids to be collected and lyophilized (Patel & Oswal, 2012; Pawar et al., 2019; Shivani & Poladi, 2015).

1.1.5.4 Ultrasound Assisted Synthesis

This method produces nanosponges without the use of solvent. A polymer and a cross linker are mixed in a flask and placed in an ultrasound bath at 90°C. The mixture is continuously sonicated for 5 hours and then left to cool. Product formed is broken roughly and then washed with water to remove unreacted polymers. Prolonged Soxhlet extraction method with ethanol is applied for the purification step. Lastly product is dried and stored at 25°C. (Shringirishi et al., 2014; Subramaniam et al., 2012; Trotta & Mele, 2019).

1.1.6 Applications of Nanosponges

Nanosponge with its porous structure is able to carry insoluble drugs hence improving its solubility, dissolution rate and stability (Tiwari & Bhattacharya, 2022). There are multiple drugs that have limited efficiency and bioavailability due to poor solubility. Itraconazole (Biopharmaceutical Classification System – Class 2) has limited dissolution causing poor availability of the drug. Application of nanosponge improved the solubility by more than 20-fold and with the addition of copolyvidonum the solubility exceeded 50-fold. (Patel & Oswal, 2012; Shivani & Poladi, 2015). Cyclodextrin based nanosponges are found to be suitable for carrying enzymes and proteins. In industrial processes, enzymes play a big role where it lowers energy consumption, reduce production of pollutant, and increase reaction speed at milder conditions in comparison to not using enzymes in the processes. The nanosponge allows enzyme to maintain its activity and efficiency at a longer operation time. It also helps expand the pH and temperature range for operations. Enzymes and protein are also beneficial therapeutically; however, these molecules have many limitations. Protein drugs are poorly absorbed due to large molecular size, degree of ionization, high surface charge, enzymatic instability, low permeability and hydrophilic nature. Cyclodextrin nanosponge is also able to adsorb protein drugs and protect it from breakdown, modify its pharmacokinetic and increase its stability (Panda et al., 2015; Pawar et al., 2019).

Nanosponges can also help improve effectiveness of anticancer agents in reducing tumour growth. The nanosponge loaded with drugs can target specific cells and release its content at the target site. This not only gives more effective treatment but also reduced side effects. Example of anticancer agent with nanosponge include paclitaxel and camptothecin (Panda et al., 2015; Shivani & Poladi, 2015). Nanosponge in cancer treatment is mainly used to improve the solubility of lipophilic drugs, protect drugs from degradation along with the benefits of being biocompatible, highly specific and having a prolonged release behaviour (Iravani & Varma, 2022b). Other than encapsulation of solids and liquids, nanosponge has the capability of encapsulating gases such as oxygen, and carbon dioxide. Nanosponge can form inclusion complexes with the gas and act as an effective carrier due to its known porosity. This application improves the delivery of oxygen and release it in a controlled manner and is suitable in

management of many disease that can cause hypoxia (Shringirishi et al., 2014). Nanosponge can also remove organic molecules from water by binding strongly onto them. The nanosponge can be embedded with porous ceramic filter creating a hybrid organic/inorganic filter to purify water from pollutants (Patel & Oswal, 2012). Pollutants are removed through absorption/adsorption or disinfection process making it a cost effective and also time and energy saving strategy compared to other water treatment methods (Iravani & Varma, 2022a).

In topical drug delivery nanosponge has been used to entrap varying drugs to improve treatment for different diseases. For example, in psoriasis, patients are usually treated with a super potent topical corticosteroid, clobetasol propionate. However, this steroid is associated with common side effects like skin atrophy, steroidal acne, hypopigmentation, and contact dermatitis which limits its usage for topical application. Hence the reason for developing clobetasol propionate loaded in cyclodextrin nanosponge based hydrogel. The results not only proved to alleviate side effects and provide controlled release, but anti-psoriatic activity was also increased along with improved solubility and stability (Kumar et al., 2021). Another lipophilic and poor solubility drug that has been entrapped in nanosponge is celecoxib. The entrapment of celecoxib in a β -cyclodextrin nanosponge hydrogel formulation for topical use was able to increase the solubility of celecoxib by 30-65 fold which resulted in an increase in drug bioavailability proving the novel formulation was more effective than plain gel for topical celecoxib application (Gangadharappa et al., 2017). Ethyl cellulose and polyvinyl alcohol based nanosponge has also been used to entrap the poorly water soluble butenafine for the treatment of fungal infection. Entrapment of butenafine in nanosponge led prolonged release of the drug which can helped butenafine to penetrate deeper within the skin layers and prevent recurrence of skin infection which is common with fungal infection. The nanosponge formulation showed higher antifungal activity compared to free butenafine and marketed formulation (Ahmed et al., 2021). The use of nanosponges significantly enhances the delivery of topical drugs, effectively reducing the risk of adverse effects, improving drug efficacy, and ultimately promoting better patient compliance.

1.2 PROBLEM STATEMENT

Acne is a major skin disease affecting people aged 11 to 30 years old which usually starts during adolescents and may continue into adulthood. This inflammatory skin disease manifests itself in different inflammatory and noninflammatory lesions that usually occurs on the face. Having acne especially on the face can be perceived as not fulfilling the beauty standards which may cause serious psychological distress. People affected by this disease are surrounded by negative emotions of feeling embarrassed, self-conscious, lack of confidence, sad, anger, frustration, anxiety, and despair which eventually may lead to reduced quality of life, major depression or even suicide. Retinoids is one of the most widely used and recommended topical treatment for all types of acne. ATRA or tretinoin specifically has the highest efficacy against acne but comes with a downside of unpleasant side effects which include dryness, irritation, and erythema. Novel drug delivery systems were found to reduce the side effects by localizing the drug release to the skin, improving its retention, and delivering drugs in controlled manner. However, formulations of ATRA using nanosponge as its delivery system for the management of acne is rather new and has not been explored. The development of ATRA loaded nanosponges should be explored despite the presence of other novel formulation due to its superiority in terms of stability, nontoxicity, and efficacy. Nanosponges can enhance ATRA stability by shielding it from degradation, ensuring prolonged drug efficacy. Entrapping ATRA within nanosponges may facilitate controlled skin penetration and gradual release, minimizing adverse effects. This approach can also improve solubility, allowing the formulation of ATRA in water-based preparations tailored to patient preferences. Hence, nanosponge offers a promising avenue for optimizing ATRA delivery.

1.3 RESEARCH OBJECTIVES

1.3.1 General Objective

The objective of this study is to develop a safe, stable, and effective ATRA loaded nanosponge formulation for the management of acne.

1.3.2 Specific Objectives

1. To develop and characterize a stable ATRA loaded nanosponge.
2. To develop and characterize a stable gel-cream formulation with incorporation of ATRA loaded nanosponges.
3. To assess the antimicrobial efficacy against *Cutibacterium acnes* of developed formulation.
4. To analyse penetration of ATRA using Franz cell diffusion studies to understand permeation and retention of ATRA.
5. To evaluate the effects of the formulation on the skin in animal model.
6. To investigate the topical effects on human skin and acceptability of the developed formulation.

1.3 RESEARCH HYPOTHESIS

A safe, stable and effective ATRA loaded nanosponge formulation for the management of acne can be developed.

1.4 SIGNIFICANCE OF STUDY

Nanosponge may enhance solubility of active ingredient, minimize side effects, increase stability and flexibility of the formulation. On top of that, nanosponges are self-sterilizing due to its small pore size, it is non-irritating and non-toxic (Bhowmik et al., 2018). Specifically, in topical drug delivery, irritation usually occurs due to penetration of active ingredient through the skin. With nanosponge application, an even and sustained rate of release with longer retention of active ingredient within the skin can be achieved. An already efficient acne treatment like ATRA, can be improved in terms of its characteristics, release, and side effects.



CHAPTER TWO

DEVELOPMENT, ENTRAPMENT AND CHARACTERIZATION OF ATRA LOADED NANOSPONGE

2.1 INTRODUCTION

Nanosponge is a novel drug delivery system developed with the aim to improve the delivery of existing drug. Studies have been done researching the use of nanosponge in delivering a diverse range of drugs with various delivery route gaining positive results (Kumar et al., 2018, 2021; Shende et al., 2013; Torne et al., 2013). Delivering drugs using nanosponge has been garnering attention due to the fact that it helps reduce dose, provide controlled and sustained release along with enhancement of stability duration (Bhowmik et al., 2018; Panda et al., 2015). These advantages in the formulation development phase will benefit the end-users of drugs in the long run. Cyclodextrin based nanosponges is one of the class of nanosponges that has been used immensely in current researches attributed to its nanocavities that has the ability to entrap lipophilic and hydrophilic chemical substances (Pawar et al., 2019).

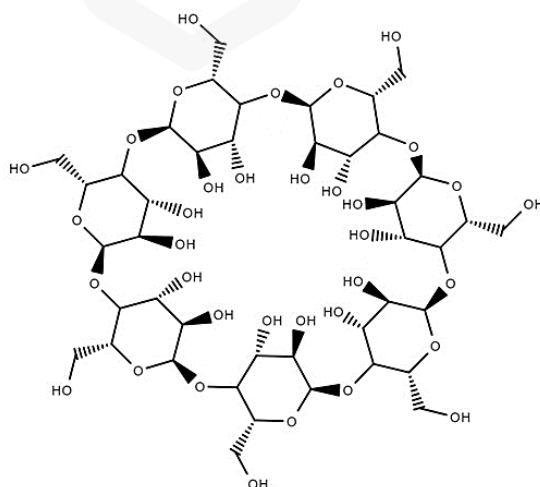


Figure 2.1 Chemical Structure of β -cyclodextrin

Cyclodextrins are part of cyclic glucopyranose oligomers that has a cage-like structure with lipophilic cavity. The chemical structure is as depicted in Figure 2.1. Cyclodextrin has an amphiphilic nature with a hydrophobic cavity and hydrophilic surface making them a polar compound and water soluble (Utzeri et al., 2022). The interaction of cyclodextrin with a cross-linker improves the formation of a solid material with high porosity and intertwined microchannels (Tannous et al., 2021). β -cyclodextrin is largely opt for development of nanosponges because of its nano-sized cavities, cost effectiveness, easy availability, and most importantly high complexing ability with cross-linking agents (Bhowmik et al., 2018; Pawar et al., 2019). β -cyclodextrin based nanosponge can be classified into carbamate, carbonate, ester, polyamidoamine, cyclodextrin-calixarene and modified nanosponge which differ based on the methods of preparation, solvents and crosslinkers used (Pawar et al., 2019).

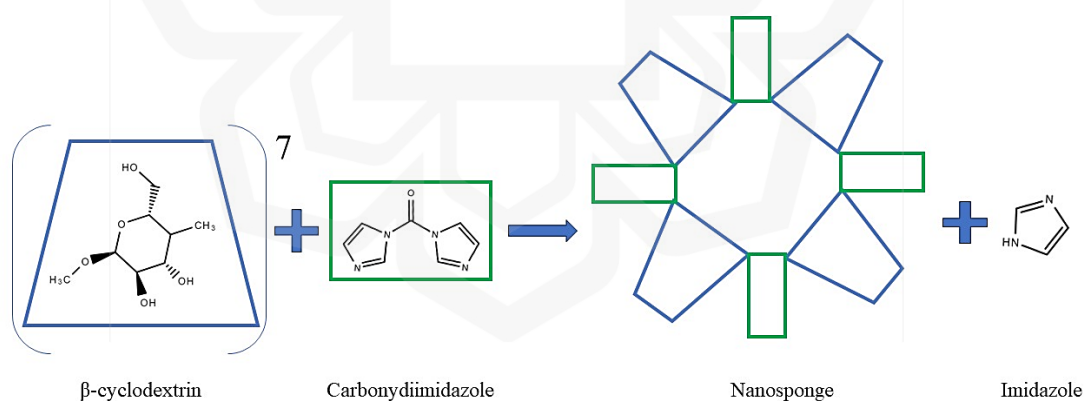


Figure 2.2 The Chemical Reaction of Nanosponge Preparation Involving β -cyclodextrin Polymer and Carbonyldiimidazole as the Crosslinker

In this study, β -cyclodextrin based carbonate nanosponge was prepared with solvent evaporation techniques where carbonyldiimidazole act as a crosslinker that links β -cyclodextrin together which is depicted in Figure 2.2. Nanosponge proves to be versatile and can be adjusted to suit the type of drug the requires improvement in its delivery. The use of nanosponge in topical delivery is also very promising due to its sustained release that can reduce skin irritation while maintaining constant efficacy (Aggarwal et al., 2016; Argenziano et al., 2019; Kumar et al., 2021).

2.2 METHODOLOGY

2.2.1 Nanosponge Preparation

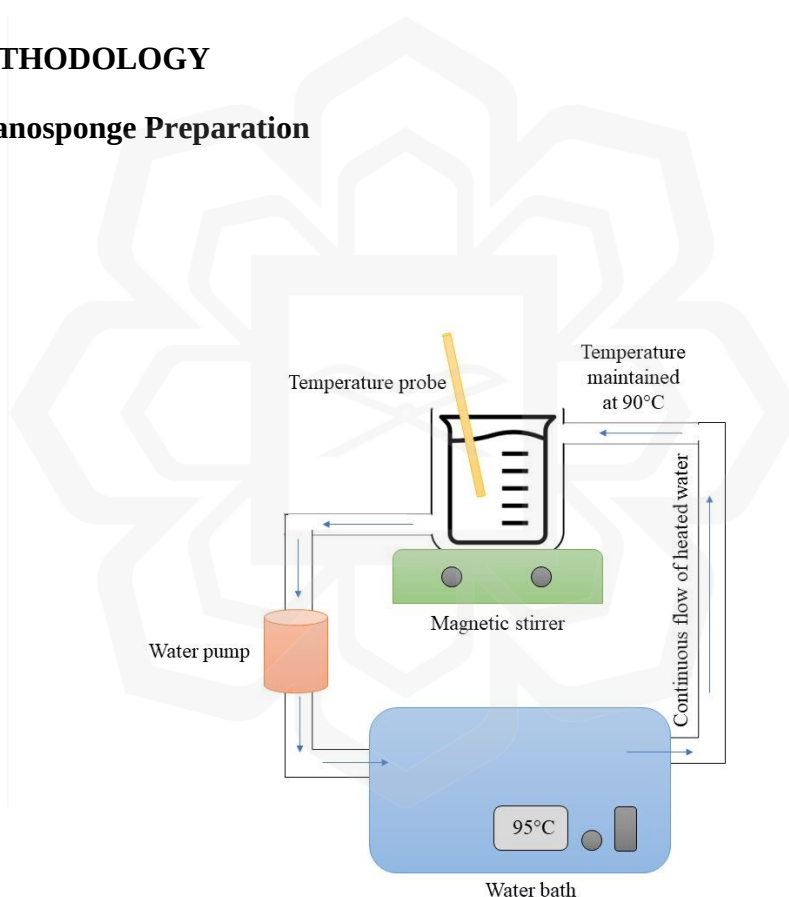


Figure 2.3 Schematic Representation of The Setup for Nanosponge Preparation

A jacketed beaker connected to a water bath maintained at 95°C was set up for the nanosponge development as shown in Figure 2.3. Dimethylformamide (DMF) (Fisher Scientific, UK) which acts as a solvent was added to the beaker and maintained at 90°C.

Polymer used was anhydrous β -cyclodextrin (BCD) (Acros Organics, UK) and carbonyldiimidazole (CDI) (Acros Organics, UK) as the crosslinker. BCD was dried overnight in an oven at 50°C. When apparatus set up reaches 90°C, BCD was dissolved in DMF forming a clear solution once completely dissolved. CDI was added to the clear solution where the colour changed slightly yellowish. The reaction was performed at 90°C for 3 hours producing a solid block. The solid produced was washed with water followed by acetone to remove unreacted polymers through a vacuum filtration system. The nanosponge was then purified through Soxhlet extraction with absolute ethanol (System, Malaysia) for 24 hours. The purified nanosponge was then crushed with a mortar and pestle. The ratio of polymer to crosslinker (polymer:crosslinker) was varied at 1:2, 1:4 and 1:8 as shown in Table 2.1.

Table 2.1 Variation of BCD and CDI at each ratio

Polymer:crosslinker	Amount of BCD		Amount of CDI	
1:2	4.4 mmol	5g	8.8 mmol	1.43 g
1:4			17.6 mmol	2.86 g
1:8			35.2 mmol	5.71 g

2.2.2 Particle Size Reduction of Nanosponge

Nanosponge from section 2.2.1 was then subjected to homogenization using WiseTis HG-15D digital homogenizer (Witeg Labortechnik, Wertheim, Germany) to reduce the particle size. 5g of nanosponge was suspended in 20 mL distilled water. The nanosponge was then homogenized at 2000 rpm with varying durations of 10 minutes, 15 minutes, and 20 minutes. The nanosponge was then centrifuged at 5000 rpm for 15 minutes with a benchtop centrifuge (Sartorius, Germany) and freeze dried for 24 hours. The factors investigated to optimize the nanosponge were the ratio of polymer:crosslinker and duration of homogenization as shown in Table 2.2. The particle size, polydispersity index and zeta potential were the dependant variable studied and

optimized with factorial design through minitab software. The best nanosponge was selected and used for entrapment of ATRA.

Table 2.2 Different Nanosponge Formulation Prepared with Varying Factors

Polymer:crosslinker	Duration of homogenization	Nanosponge preparation
1:2	10 minutes	NS1
	15 minutes	NS2
	20 minutes	NS3
1:4	10 minutes	NS4
	15 minutes	NS5
	20 minutes	NS6
1:8	10 minutes	NS7
	15 minutes	NS8
	20 minutes	NS9

2.2.3 ATRA Encapsulation in Nanosponge

The process is as depicted in Figure 2.4. A 10 mg/mL aqueous suspension of nanosponge was prepared where 20 mg of nanosponge was suspended in 2 mL distilled water. ATRA was then dispersed into the aqueous suspension at different ratios of 1:2, 1:4 and 1:6 (drug:nanosponge w/w). The suspension was then sonicated for 15 min and then proceeded with stirring for 24 hours in ambient temperature in a dark room. After 24 hours sample was centrifuged 3000 rpm for 10 minutes and the supernatant was collected. The supernatant is then freeze-dried using Alpha 1-2 LDplus Freeze Dryer (Martin Christ[®], Osterode am Harz, Germany) for 24 hours. Loaded nanospheres was stored at room temperature before further use (Dhakar et al., 2019; Osmani et al., 2016).

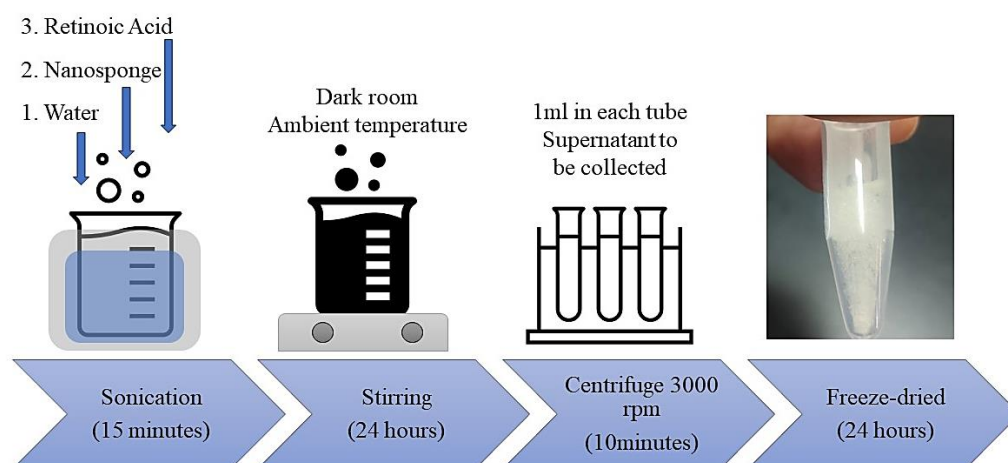


Figure 2.4 Illustration on the Entrapment Process of ATRA into Nanosponge

2.2.4 Characterization of ATRA-Loaded Nanosponges

2.2.4.1 Entrapment Efficiency

1 mg of loaded nanosponge was dispersed in 1 mL of methanol and then sonicated for 15 minutes at room temperature. The amount of ATRA loaded in the nanosponge was determined through quantitative estimate of ATRA by HPLC analysis after filtration of the sonicated sample. The percentage of ATRA loaded into the nanosponges was calculated as follows (Aldawsari et al., 2015; Kumar et al., 2018; Osmani et al., 2016)

$$\text{Drug loading (\%)} = \left(\frac{\text{Weight of drug in nanosponge}}{\text{Weight of loaded nanosponge}} \right) \times 100$$

Equation 2.1

$$\begin{aligned} &\text{Entrapment efficiency (\%)} \\ &= \left(\frac{\text{theoretical drug content} - \text{free drug}}{\text{theoretical drug content in nanosponge}} \right) \times 100 \end{aligned}$$

Equation 2.2

2.2.4.2 Solubilization Efficiency

The solubility of ATRA was assessed using the shake flask method. ATRA was added in excess (12.5 mg) to a 5 mL distilled water in a flask and in another flask, ATRA was added in excess (12.5 mg) to a flask containing fixed amount of nanosponge (5mg) dispersed in 5 mL distilled water. Both flasks were sealed and shaken for 24 hours at room temperature in the dark using a mechanical shaker at 100 rpm (Sherje, 2019). The sample was then centrifuged at 6000 rpm for 20 minutes and the supernatant collected. Methanol 2.5 mL was added to the supernatant to extract RA. Next, supernatant was sonicated for 15 minutes prior to filtration for analysis. The filtrate was analysed in triplicates with HPLC to determine the concentration of dissolved ATRA (Dhakar et al., 2019; Kumar et al., 2018; Osmani et al., 2016).

2.2.4.3 Particle Size and Zeta Potential

Particle size and zeta potential of the nanosponge was determined using Zetasizer (ZEN 1600, Malvern, UK). Nanosponges was diluted 10 times using filtered distilled water as a dispersant before being analysed in triplicate at 25°C (Aldawsari et al., 2015; Penjuri et al., 2016).

2.2.4.4 Surface morphology

The shape and surface topography of nanosponge and nanosponge loaded with ATRA was evaluated through Field emission scanning electron microscope (FESEM) JSM-7800F Prime (JEOL, Tokyo, Japan) using an acceleration voltage of 5-7kV. Samples were coated with thin gold palladium layer before being placed on a glass slide and kept under vacuum. Nanosponge was recorded under magnification ranging from 3000x to 30000x (Gupta et al., 2013; Srinivas & Sreeja, 2013).

2.2.4.5 Fourier-transform Infra-Red (FTIR) Spectroscopy Study

FTIR analysis was done to assess any interaction between polymers and ATRA. Analysis was done using Spectrum RX Two Instrument with a resolution of 0.5 cm^{-1} . (PerkinElmer Inc., USA) complete with Spectrum 3.5 software. Each sample was placed on the zinc selenide crystal and were run a total of 16 scans at a scanning range of 400 cm^{-1} - 4000 cm^{-1} with a temperature of 25°C .

2.2.4.6 DSC Study

Thermal analysis was carried out with DSC from Netzsch model DSC214 Polyma with the Netzsch Proteus® software (Selb, Germany). Samples of ATRA, blank nanosponge and ATRA loaded nanosponges were analysed using standard alumina with aluminium sample pans whereby an empty pan was used as the reference standard. DSC was used to analyse the compatibility of the active ingredient with the polymer and its thermal behavior. 5 mg samples in aluminium pans was heated at a range from 30°C to 300°C under nitrogen atmosphere with a heating rate of 10°C per minute and a nitrogen purging rate of 50 ml/min (Osmani et al., 2016; Swaminathan et al., 2010).

2.2.5 High-performance Liquid Chromatography (HPLC) Method Validation

Method was validated according to International Conference on Harmonization (ICH) on validation of analytical procedures. Parameters validated include specificity, precision, accuracy, linearity, limit of detection and limit of quantification (ICH, 1995). The HPLC analysis of ATRA was done using an isocratic system with HPLC system 1200 series from Agilent technologies, (California, USA) with an integrated controller, quaternary pumps, and autosampler, a solvent degasser unit, and a variable wavelength detector (VWD). The column used as the stationary phase was a C-18 silica-based

column (ZORBAX, Eclipse Plus, Agilent Technologies) which makes it a reverse phase chromatography. Methanol and 0.5% acetic acid in water with ratio of 90:10 was used as the mobile phase with a flow rate of 1.0 mL/min. Standard stock solution strength 0.2 mg/mL was prepared by dissolving 20 mg of ATRA standard in 100 mL methanol. Next, the standard solutions were prepared by diluting the stock solution in methanol to concentrations ranging from 5 - 90 µg/mL. All prepared samples were filtered through a 0.45 µm syringe filter. Samples were injected three times, and the chromatogram was detected at a wavelength of 350 nm.

Table 2.3 HPLC Specifications.

Parameters	Specifications
Column	C18 150mm x 4.6mm 5µm
Injection volume	20 µL
UV detection	350 nm
Flow rate	1 mL/min
Column temperature	25 °C
Run time	15 minutes
Retention time	7.5 minutes
Sample solvent	Methanol
Mobile Phase	Methanol and 0.5% Acetic acid (90:10)

2.2.5.1 Linearity

Linearity is when the results obtained are directly proportional to the concentration of analyte in sample. Several concentrations of the standard stock solution were prepared and injected in triplicate. A calibration curve was plotted where the linear regression obtained confirms linearity.

2.2.5.2 Accuracy

Accuracy is the ability to produce results that are in close agreement with the accepted reference value. A standard solution of concentration ranging from 50-100 times higher than unspiked sample was spiked into the sample and injected in triplicate. From the results, percentage recovery can be calculated, which should be in the range of 95-105%.

2.2.5.3 Specificity

Specificity is the ability to determine the presence of target analyte in the presence of other substances such as impurities or products of degradation. A blank, a placebo and a standard solution were injected to observe any peak interference at the retention time of standard solution.

2.2.5.4 Precision

Precision is the closeness of agreement between multiple measurements of the same sample. Inter-day and intra-day assay were conducted using three different concentrations. Intra-day assay was done by injecting all three concentrations in triplicates on the same day, while inter-day assay was done by injecting all three concentrations in triplicates on different days (Day 1, Day 2, Day 3). From the results, percentage of relative standard deviation (RSD) was calculated.

2.2.5.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD is the lowest amount of analyte that can be detected on a sample while LOQ is the lowest amount of analyte that can be quantitatively determined in a sample. The LOD and LOQ were determined through linear regression of the calibration curve.

2.2.6 Data Analysis

Data collected were recorded in triplicates and presented in the form of mean $\pm$ standard deviation. Data gathered on the development of nanosponge with various polymer:crosslinker ratio and homogenization duration was examined with the implementation of a 3^2 full factorial design by using the Minitab software. Data on characterization of nanosponge was analyzed with one-way ANOVA to determine any significant difference. Data is considered statistically significant when $p \leq 0.05$.

2.3 RESULTS AND DISCUSSION

2.3.1 Factorial Design Analysis of Nanosponge Development

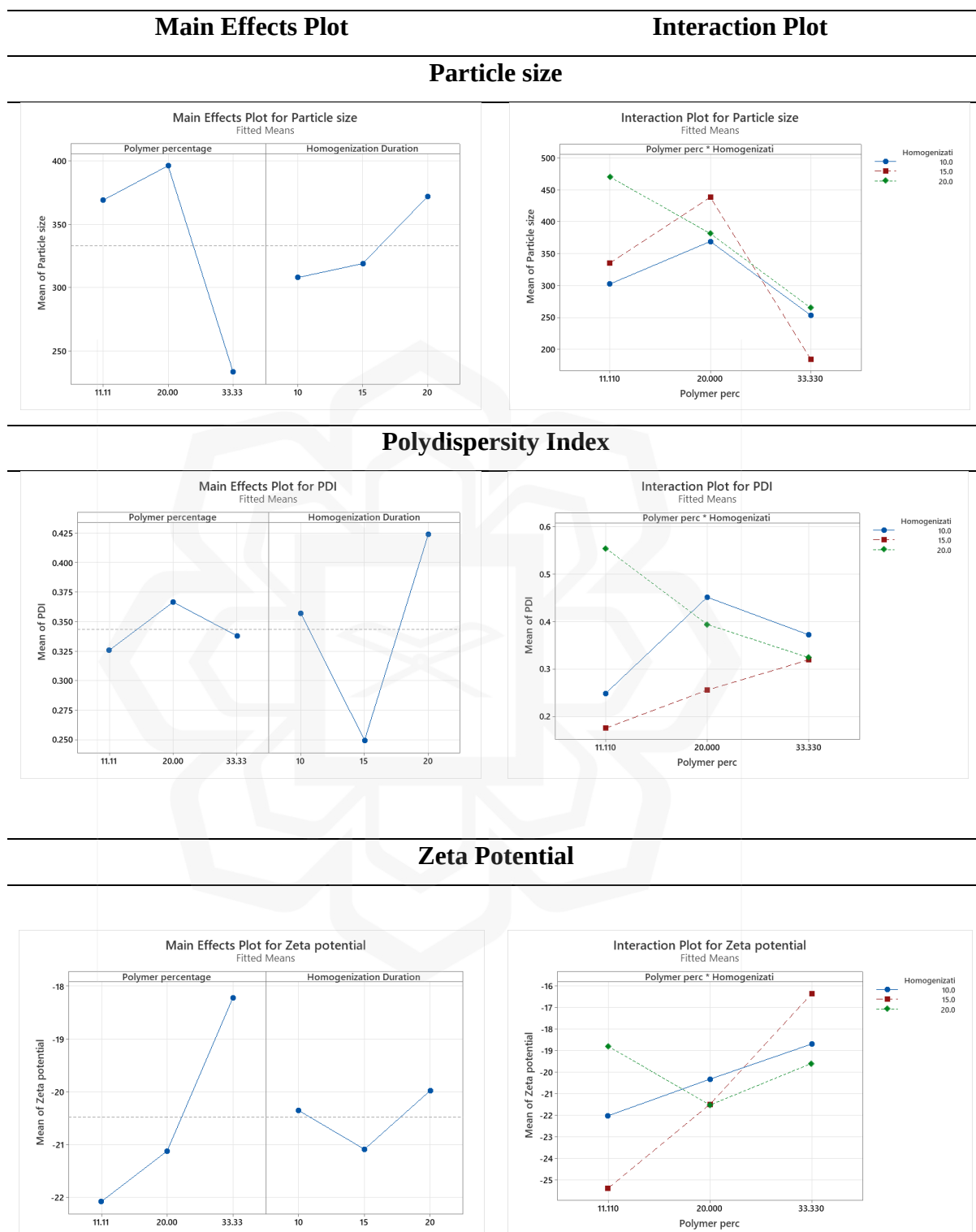
Table 2.4 shows the measured particle size, polydispersity index (PDI) and zeta potential for the varying polymer to crosslinker ratio and homogenization time which were analysed with 3^2 factorial design in design of experiment using Minitab software version 17. The independent variable or the cause in this analysis is the polymer:crosslinker ratio and homogenization time. Particle size, PDI and zeta potential are the dependent variables or effects.

Table 2.4 Particle size, PDI and Zeta Potential at Varying Parameters

Polymer ratio	Time		
	10	15	20
Particle size (nm)			
NS1 1:2	252.93 ± 19.12	183.93 ± 18.70	264.47 ± 18.75
NS4 1:4	368.67 ± 76.51	438 ± 23.96	381.07 ± 12.01
NS7 1:8	301.97 ± 5.82	334.7 ± 17.97	469.87 ± 62.44
Polydispersity Index			
NS2 1:2	0.371 ± 0.05	0.319 ± 0.06	0.323 ± 0.05
NS5 1:4	0.451 ± 0.13	0.255 ± 0.03	0.393 ± 0.01
NS8 1:8	0.248 ± 0.03	0.175 ± 0.01	0.554 ± 0.03
Zeta Potential (mV)			
NS3 1:2	-18.7 ± 0.17	-16.37 ± 0.25	-19.6 ± 0.17
NS6 1:4	-20.33 ± 0.25	-21.5 ± 0.3	-21.53 ± 0.25
NS9 1:8	-22.03 ± 0.66	-25.4 ± 0.26	-18.8 ± 0.4

The main effects plot and interaction plot are presented in Table 2.5. Main effect plot is the effect of an independent variable on the dependent variable while the interaction plot is the effect of independent variables interacting on the dependent variable.

Table 2.5 Main Effect and Interaction Plot for Parameters Varied in Nanosponge Development.



Note: polymer percentage used instead of ratio for analysis using minitab software whereby 11.11% is 1:8, 20.00% is 1:4 and 33.33% is 1:2

2.3.1.1 Particle Size

Particle size is significantly affected by polymer:crosslinker ratio and homogenization duration ($p < 0.05$). The interaction of these factors also has significant effect on particle size. The main effect plot shows smallest particle size at 1:2 and at 10 minutes, respectively. However, the homogenization duration does show an increasing trend in particle size with longer duration of homogenization. Increase in homogenization time causes an increase in applied total energy of the system which in turn increases particle coalition and leads to formation of larger particles.

A previous study found that with a mixing time more than 15 minutes, the increase in particles sizes were greater ($p < 0.05$) (Anarjan et al., 2015) The interaction plot depicts that the smallest particle size is obtained at ratio 1:2 with homogenization duration of 15 minutes while the largest size is at 1:8 with homogenization duration of 20 minutes. Generally, ratio 1:2 produced the smallest particle size due to the lower crosslinking density which is related to the polymer:crosslinker ratio. The crosslinking density affects the chemical composition, and physical properties which includes particle size. At ratio 1:2, the nanosponge has the lowest amount of crosslinker which means it has lower crosslinking density producing particles that are less hard and easier to be subjected to size reduction (Chaudhary & Sharma, 2019). While nanosponge at ratio 1:8 and 1:4 has more crosslinking density producing larger particle size as depicted in the graph.

2.3.1.2 Polydispersity Index

Polymer:crosslinker ratio was not significant to PDI, which can be seen from the main effect plot which shows that the PDI values fluctuates slightly from one another. On the other hand, homogenization duration shows great fluctuation from values to values in line with the data analysis which found homogenization duration having significant effect on PDI ($p < 0.05$). The interaction of the two factors were also found to have

significant effect on PDI ($p < 0.05$). From the interaction plot, the lowest PDI is obtained at ratio of 1:8 with 15 minutes homogenization duration and highest at ratio 1:8 with 20 minutes homogenization duration which is similar to results obtained for particle size at section 2.3.1.1. Based on these results, a large PDI measurement corresponds with a large particle size which was most likely produced through particle coalition. Coalition in a vessel can occur between particle and particle, particles and vessel or particles and repeller, that can lead to particle agglomeration hence the large particle size and size distribution (Dizaji et al., 2019; Nienow, 1992; D. Wang & Fan, 2013). At a higher crosslinker concentration, the particle size and polydispersity index increases compared to the lower concentration. This phenomenon maybe due to the action of crosslinker which facilitates crosslinking and leads to the formation of large size cross-linked polymeric agglomerates (Moin et al., 2020).

2.3.1.3 Zeta Potential

Both factors and their interaction significantly affected zeta potential ($p < 0.05$). At higher polymer:crosslinker ratio, zeta potential increases. From the main effects plot, higher polymer to crosslinker ratio (1:8) causes lower zeta potential, while homogenization duration shows lowest zeta potential at 15 minutes. The interaction plot shows that at 10 and 15 minutes there is an increasing trend of the zeta potential as the polymer:crosslinker ratio decreases. A previous study also showed similar trends on the effect of polymer:crosslinker ratio on zeta potential, whereby the zeta potential becomes more negative as the amount of crosslinker increases (Pedrazzo et al., 2020).

2.3.2 Characterization of ATRA Loaded Nanosponge

From the analysis, three solutions were selected as shown in Table 2.6. These three solutions were then entrapped with ATRA to determine the nanosponge with the best entrapment efficiency. From the main effects plot for homogenization time, the most

optimum homogenization time is 15 minutes which produced particles with small size, PDI and low zeta potential. The software then came up with solution A with the most optimum results for the lowest values of all parameters. While solution B is the optimum solution when the parameter allows larger particle size, and solution C is when the parameters allow higher zeta potential resulting in solutions with different BCD:CDI ratios. It is imperative to evaluate the impact of varying BCD: CDI ratios on both drug entrapment and solubility, given that the crosslinking density network varies between different ratios (Chaudhary & Sharma, 2019).

Table 2.6 Selected Nanosponge Formulations.

Solution	Ratio polymer:crosslinker	Homogenization time
A	1:2	15 minutes
B	1:4	
C	1:8	

2.3.2.1 Entrapment Efficiency and Solubilization Efficiency

Entrapment of ATRA was done by entrapping drug into three different nanosponge as mentioned in Table 2.6. Samples were analysed with HPLC whereby the methods are specified in section 2.2.5. The entrapment efficiency was then calculated using equation 2.2. The entrapment efficiency obtained for solution A, B and C shown in Table 2.7 and Figure 2.5 were an average of 75.32% (SD=0.0289), 73.69% (SD=0.0781) and 78.19% (SD=0.850), respectively. The different nanosponge used (1:2, 1:4, 1:8) has significant effect ($p < 0.05$) on the percentage of entrapment efficiency whereby there is no significant difference between solution A and B, but there is significant difference between solution A & B compared to solution C ($p < 0.05$). Hence solution C (1:8), which obtained the highest entrapment efficiency was selected for further determination of the best drug:nanosponge ratio for entrapment. Previous study which entrapped

paliperidone in NS 1:4 and NS 1:8 obtained similar results whereby NS 1:8 had higher EE% (Sherje et al., 2019). Another study that entrapped calcium carbonate in β -cyclodextrin nanosponge also showed that nanosponge with polymer:crosslinker ratio of 1:8 having the highest entrapment efficiency (Shende et al., 2013). The high entrapment efficiency and loading capacity obtained maybe the result of higher crosslinking between BCD and CDI which permits better entrapment of ATRA in the nanosponge (Shende et al., 2013; Sherje et al., 2019).

Table 2.7 Entrapment Efficiency of Different Solutions

Solution	Entrapment efficiency (%)	Loading Capacity (%)
A	75.32% (SD = 0.029)	1.80%
B	73.69% (SD = 0.078)	1.97%
C	78.19% (SD = 0.850)	14.83%

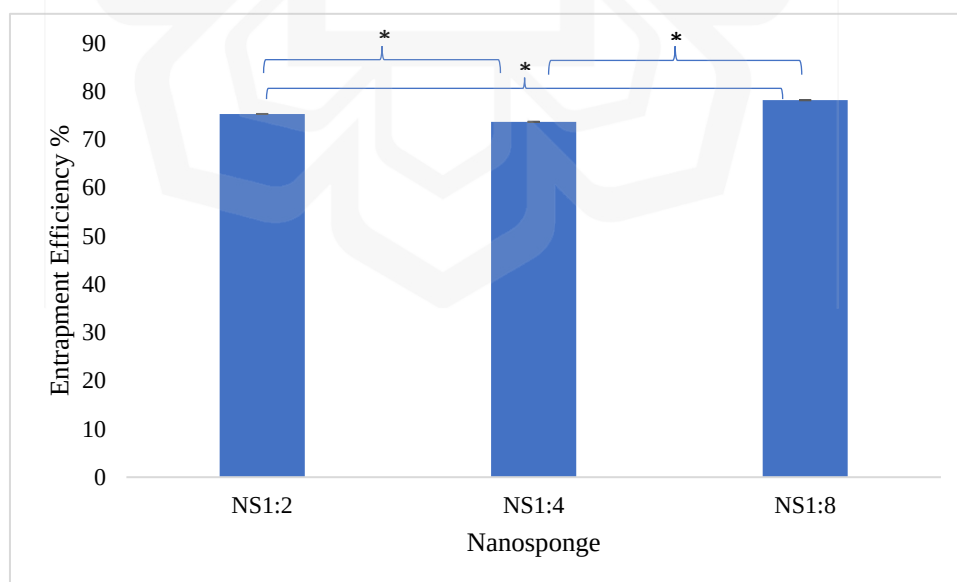


Figure 2.5 Entrapment Efficiency of Nanosponge with Varying Polymer:Crosslinker ratio (Mean $\pm$ SD, n=3, [*] = p<0.05)

The polymer to crosslinker ratio shows a significant effect on the solubility efficiency ($p < 0.05$) of the loaded nanosponge. Each ratio was significant to ATRA and was also significant to one another except for 1:2 to 1:4. All loaded nanosponge showed higher solubility than ATRA alone as presented in Figure 2.6 due to the structural characteristic of nanosponge. Nanosponge structurally has numerous pores that may help improve the wetting property of ATRA and improve its solubility (Sherje et al., 2019). Ratio of BCD:CDI may be a factor affecting the solubilization capacity of ATRA as can be seen from the graph which shows 1:8 having significant higher solubilization efficiency in comparison to other ratios. As the ratio increases, cross-linker concentration also increases which provides more sites for drug complexation hence improving solubilization efficiency (Zidan et al., 2018).

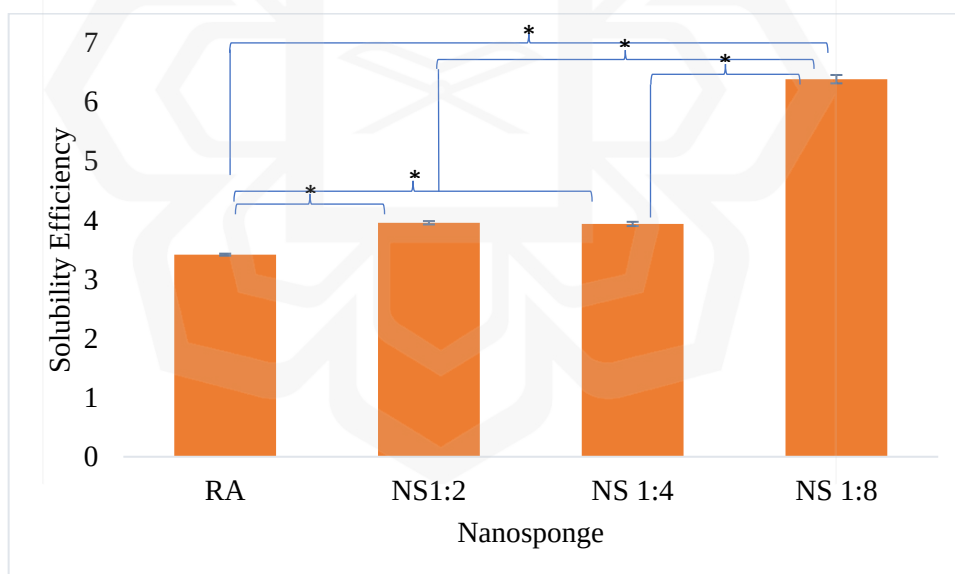


Figure 2.6 Solubilization Efficiency of Nanosponge with Varying Polymer:Crosslinker Ratio (Mean±SD, n=3, [*] = $p < 0.05$)

Based on the entrapment efficiency and solubilization efficiency NS 1:8 was selected for further investigations. Three different drug:nanosponge (RA:NS) ratios of

1:2, 1:4 and 1:6 was prepared. The entrapment efficiency values obtained for 1:2, 1:4 and 1:6 was 73.90% (SD=0.1457), 59.73% (SD=0.0351) and 51.86% (SD=0.401), respectively. One way ANOVA analysis done using minitab software showed that different RA:NS ratio has a significant effect ($p<0.05$) on the entrapment efficiency. Comparison between different ratio also was found to be significant to one another ($p<0.05$). This leads to nanosponge 1:8 with drug:nanosponge ratio of 1:2 has the highest entrapment efficiency. Graph in Figure 2.7 shows decreasing trend with RA:NS ratio 1:2 having the highest EE% well above the optimal EE% which is $>60\%$ while 1:6 has low EE% (El-Say, 2016; H. Shah et al., 2019). This could be due to the availability of nanosponge to entrap ATRA compared to higher drug concentrations which may lead to drug molecules displacing one another. A prior study recorded similar findings whereby nanosponge with the same crosslinking level did not show significant change in encapsulation efficiency with increasing drug ratio (Gaber et al., 2023). The final nanosponge selected for further formulation was nanosponge with polymer to crosslinker ratio of 1:8 which was prepared with ATRA to nanosponge ratio of 1:2 as it has high entrapment efficiency, solubility efficiency and loading capacity.

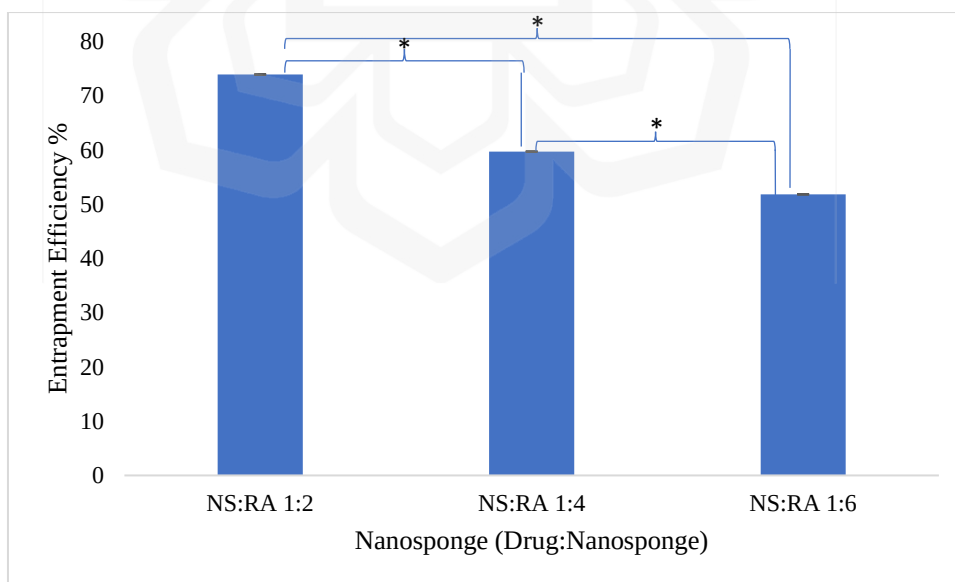


Figure 2.7 Entrapment Efficiency Of Nanosponge 1:8 With Varying Drug:Nanosponge Ratio (Mean $\pm$ SD, $n=3$, [*] = $p<0.05$)

2.3.2.2 Particle Size and Zeta Potential

Table 2.8 Parameters Assessed for Nanosponge Loaded with ATRA (n=3, mean $\pm$ SD)

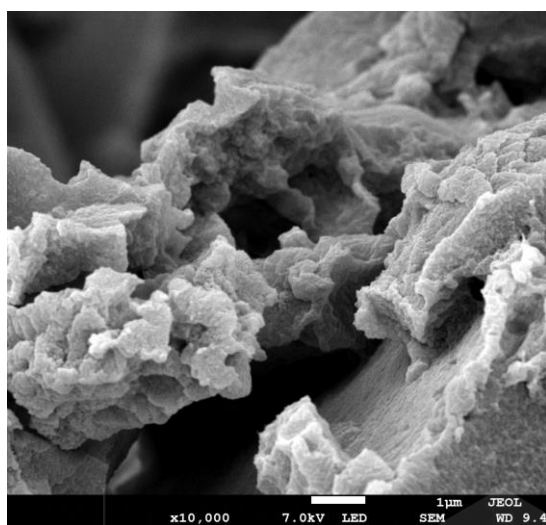
Parameter	Results	
Particle size	193.90 $\pm$ 2.74 nm	
Polydispersity index	0.322 $\pm$ 0.0346	
Zeta potential	BCD	-21.13 $\pm$ 2.57 mV
	CDI	-22.13 $\pm$ 0.55 mV
	ATRA	-37.37 $\pm$ 2.45 mV
	Plain nanosponge	-25.4 $\pm$ 0.26 mV
	ATRA-loaded nanosponge	-26.17 $\pm$ 0.513 mV

The characteristics of loaded nanosponge is as shown in Table 2.8. The ATRA-loaded nanosponge has a particle size of 193.90 $\pm$ 2.74 nm which can be used in topical treatment with ATRA as smaller particle size provides slow release and has better skin retention (Aggarwal et al., 2016; Kumar et al., 2021). The PDI of 0.322 $\pm$ 0.0346 is below 0.5 which indicates acceptable polydispersity (Torres et al., 2022). The closer the PDI value to 0 indicates a monodisperse particle distribution which is favourable for better stability and drug delivery (Rosyada et al., 2019). The zeta potential value was -26.17 $\pm$ 0.513 mV which is much lower than values obtained in previous studies which ranged from -8.74 to -20.21 mV (Ansari et al., 2011; Sherje et al., 2019). These prior researches employed similar nanosponge for entrapment of paliperidone and resveratrol (Dhakar et al., 2019; Sherje et al., 2019). The zeta potential of the loaded nanosponge is affected by various factors including the specific polymers, crosslinkers and drug incorporated into the nanosponge. For instance, a study that entrapped clobetasol propionate in the nanosponge observed a zeta potential of 6.19 mV for clobetasol alone, but this value turned negative ranging from -17.27 mV to -27.77 mV, when clobetasol was entrapped (Kumar et al., 2021). In our current investigation, the polymer, crosslinker and drug (retinoic acid) exhibited a zeta potential of -21.13 $\pm$ 2.57 mV, -

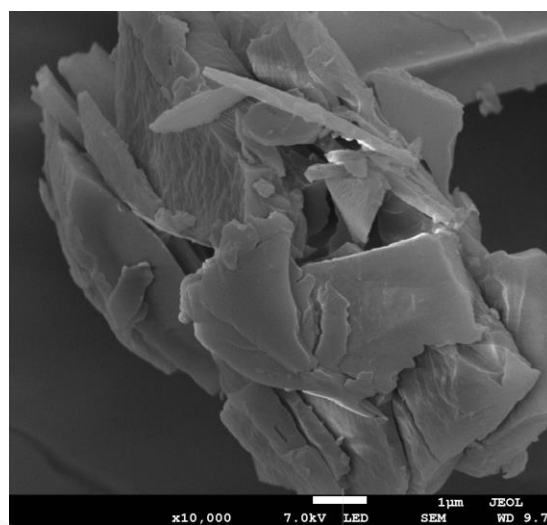
22.13±0.55 mV and -37.37±2.45 mV, all of which are negatively charged, leading to an overall increase negative charge of the loaded nanosponge.

2.3.2.3 Surface Morphology

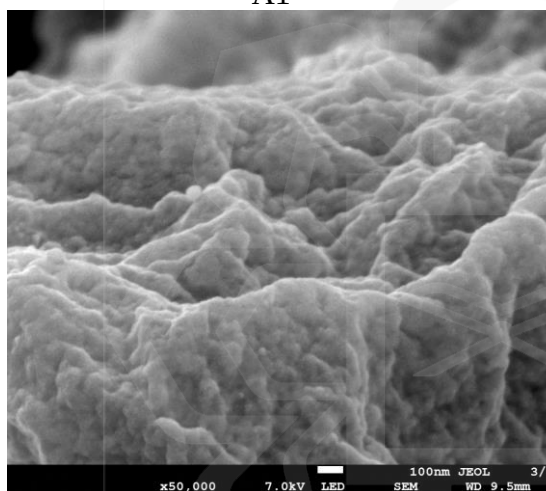
FE-SEM was done to gain more insights on the shape and surface morphology of the developed nanosponge. The surface morphology is as depicted in Figure 2.8 with spatial resolution of 1 µm and 100 nm. FE-SEM image of plain nanosponge as shown in image A1 and A2 shows irregularly shape crystals with a porous structure which is responsible to entrap ATRA. The ATRA loaded nanosponge on the other hand which is depicted in image B1 and B2 shows that the porous structure visible in plain nanosponge is no longer visible. From the figure, a layer can be seen on the surface demonstrating the entrapment of ATRA in nanosponge which occurs through the effects of freeze drying (Kumar et al., 2021).



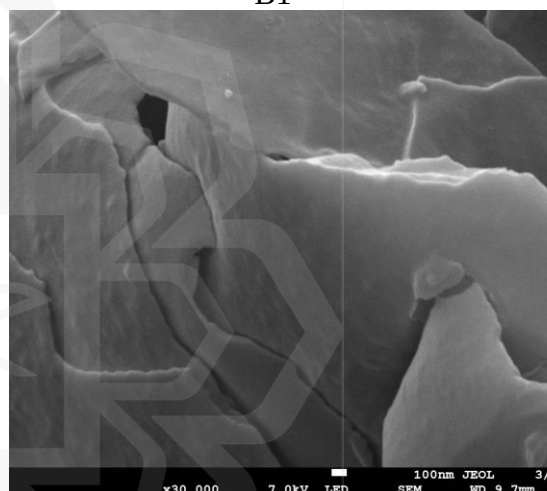
A1



B1



A2



B2

Figure 2.8 Field Emission Scanning Electron Microscopy of Plain Nanosponge at (A1) 1 μm; (A2) 100 nm And Loaded Nansoponge At (B1) 1 μm; (B2) 100 nm

2.3.2.4 Fourier-transform Infra-Red (FTIR) Spectroscopy Study

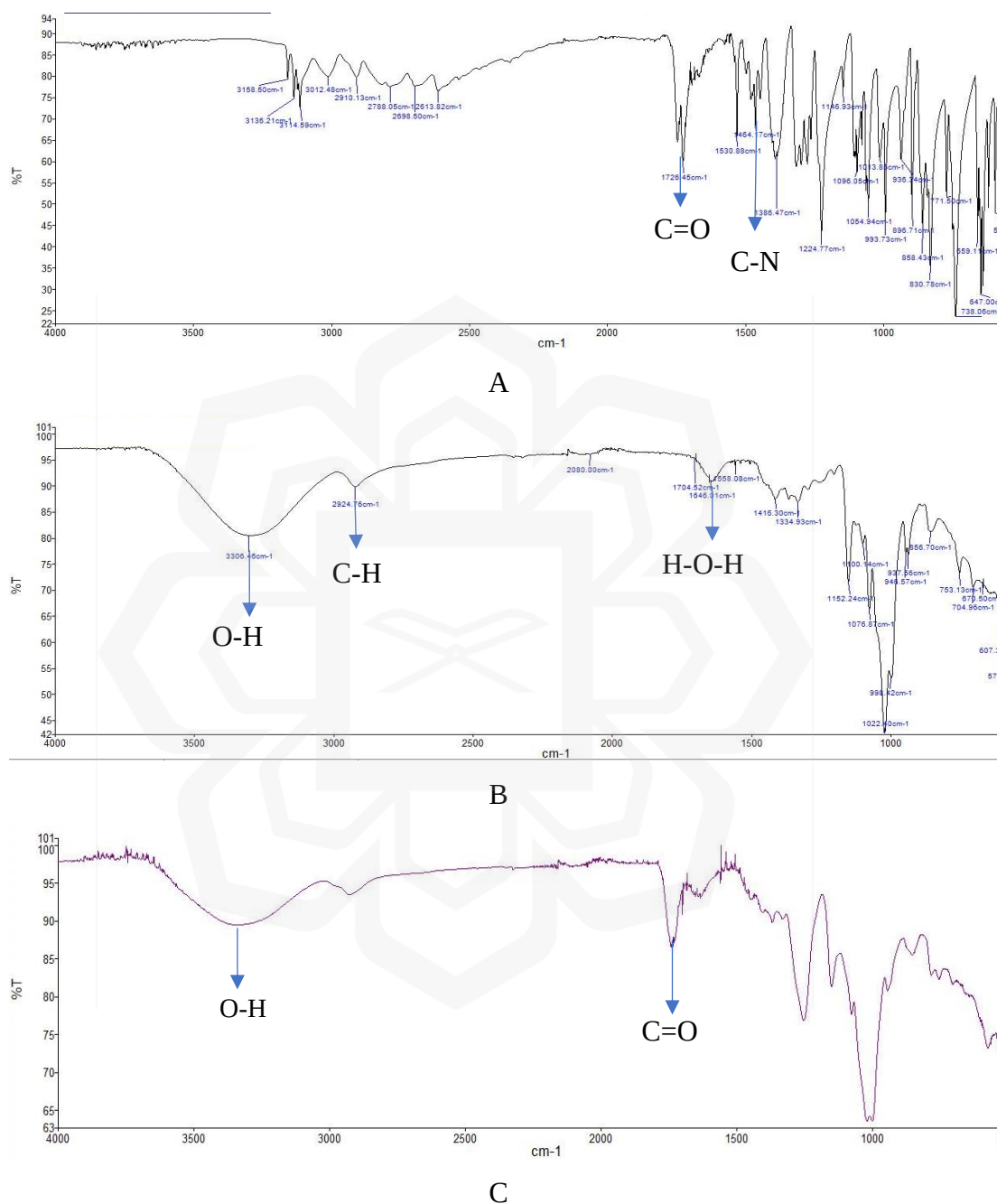


Figure 2.9 FTIR Fingerprint of (A) Carbonyldiimidazole, (B) β -cyclodextrin and (C) Plain Nanosponge*

*Plain nanosponge: refers to nanosponge that has not been loaded with any drug.

The FTIR spectrum of CDI in Figure 2.9 (A) shows peak at around 1745 cm^{-1} and 1726 cm^{-1} due to C=O stretching as well as peak at 1464 cm^{-1} representing C-N stretching (Xin et al., 2019). The FTIR spectrum of BCD in Figure 2.9 (B) shows characteristic peaks at $3300\text{--}3400\text{ cm}^{-1}$ due to the O-H group stretching, at 2924 cm^{-1} due to C-H stretching, at 1646 due to H-O-H deformation bands of water and at 1152 cm^{-1} and 1022 cm^{-1} indicated C-H overtone stretching (Rachmawati et al., 2013; Xin et al., 2019). Figure 2.9 (C) depicts plain nanosponge which retains O-H group stretch from cyclodextrin and C=O stretch from carbonyldiimidazole.

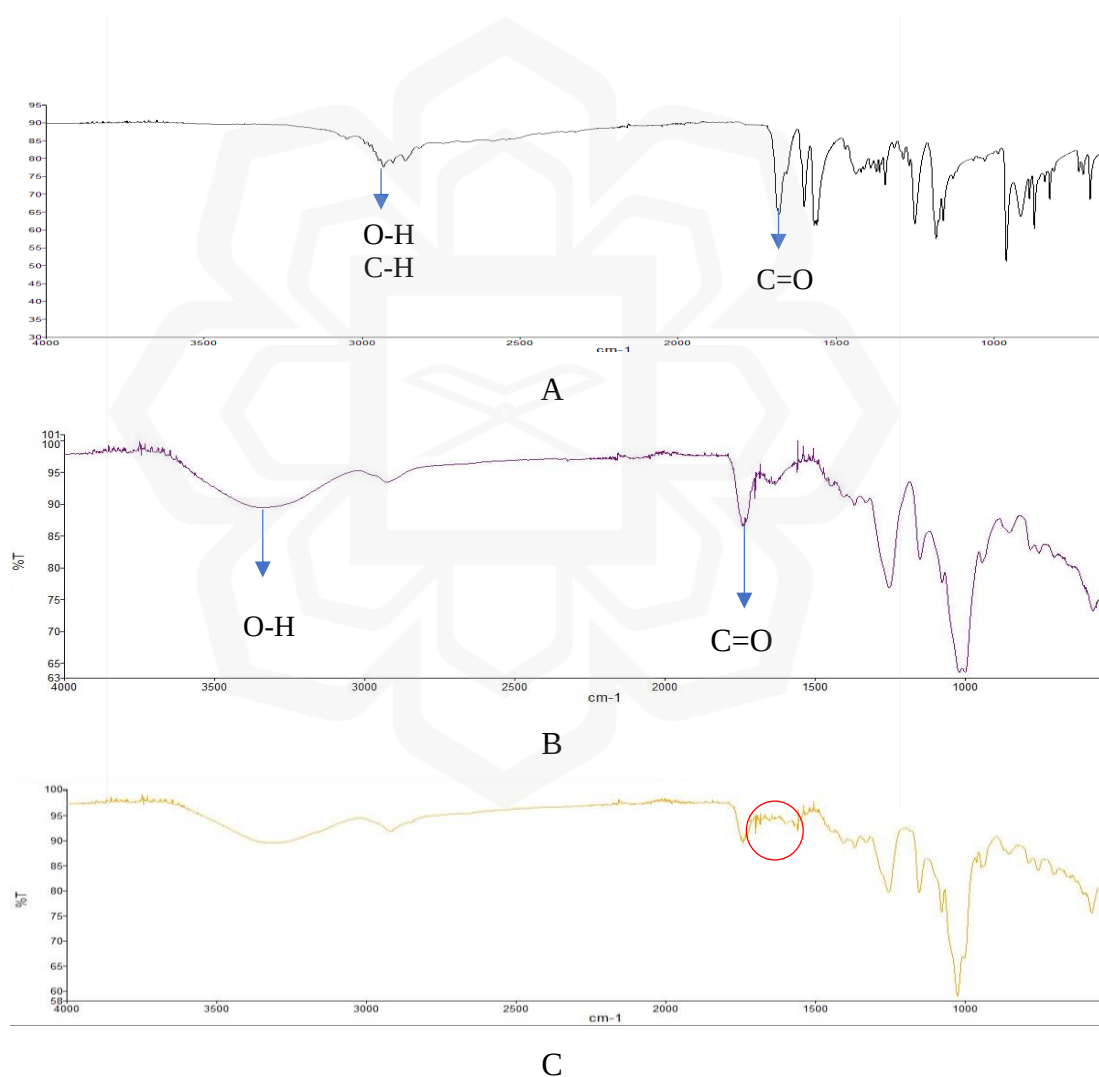


Figure 2.10 FTIR Fingerprint of (A) ATRA, (B) Plain Nanosponge* and (C) ATRA Loaded Nanosponge**

**Plain nanosponge: refers to nanosponge that has not been loaded with any drug, ** ATRA loaded nanosponge: refers to nanosponge that has been loaded with drug (ATRA)*

In Figure 2.10 (A) which is the IR spectra of ATRA peaks of C=O bonds of the carboxylic group are present at 1685-1666 cm^{-1} . The peaks attributed to OH functional group of carboxylic acid and C-H of the double bonds are seen at 2600-3429 cm^{-1} (Shakiba et al., 2017). The IR fingerprint of loaded nanosponge as depicted in Figure 2.10 (C) confirms molecular interaction as the spectra shows disappearance and shift of the ATRA peaks. In comparison to plain nanosponge in Figure 2.10 (B), loaded nanosponge shows a disappearance of peak circled in red as well as a reduce intensity of the peaks which may reflect the formation of new molecular bonds, hence reducing existing functional group. This interaction indicates inclusion of ATRA in the cyclodextrin cavity (Argenziano et al., 2019; Zidan et al., 2018).

2.3.2.5 Differential Scanning Calorimetric (DSC) Study

Figure 2.11 shows the thermogram of ATRA, plain nanosponge and loaded nanosponge. The DSC thermograms of ATRA, plain nanosponge and ATRA loaded nanosponge are also shown overlapping in Figure 2.12. DSC analysis of ATRA (Fig. 2.11A) depicted a sharp endothermic peak at 183.5°C which indicates its melting temperature (Abdel Rahman et al., 2016). In the case of plain nanosponge (Fig. 2.11B), a broad peak appeared around 77.2°C corresponding to the expulsion of water content from the β -cyclodextrin cavities (Zidan et al., 2018). The DSC thermogram of ATRA loaded nanosponge (Fig. 2.11C) showed the disappearance of the melting endotherm of ATRA. This suggests possible interaction of ATRA with nanosponge and the formation of inclusion and non-inclusion complex. After incorporation of ATRA in the nanosponge, the drug is entrapped and molecularly dispersed in the cyclodextrin cavity hence unable to crystallize reflecting the disappearance of its peak (Abdel Rahman et al., 2016; Argenziano et al., 2019; Osmani et al., 2016). The phenomenon can be seen clearly from the overlapping thermogram in Figure 2.12.

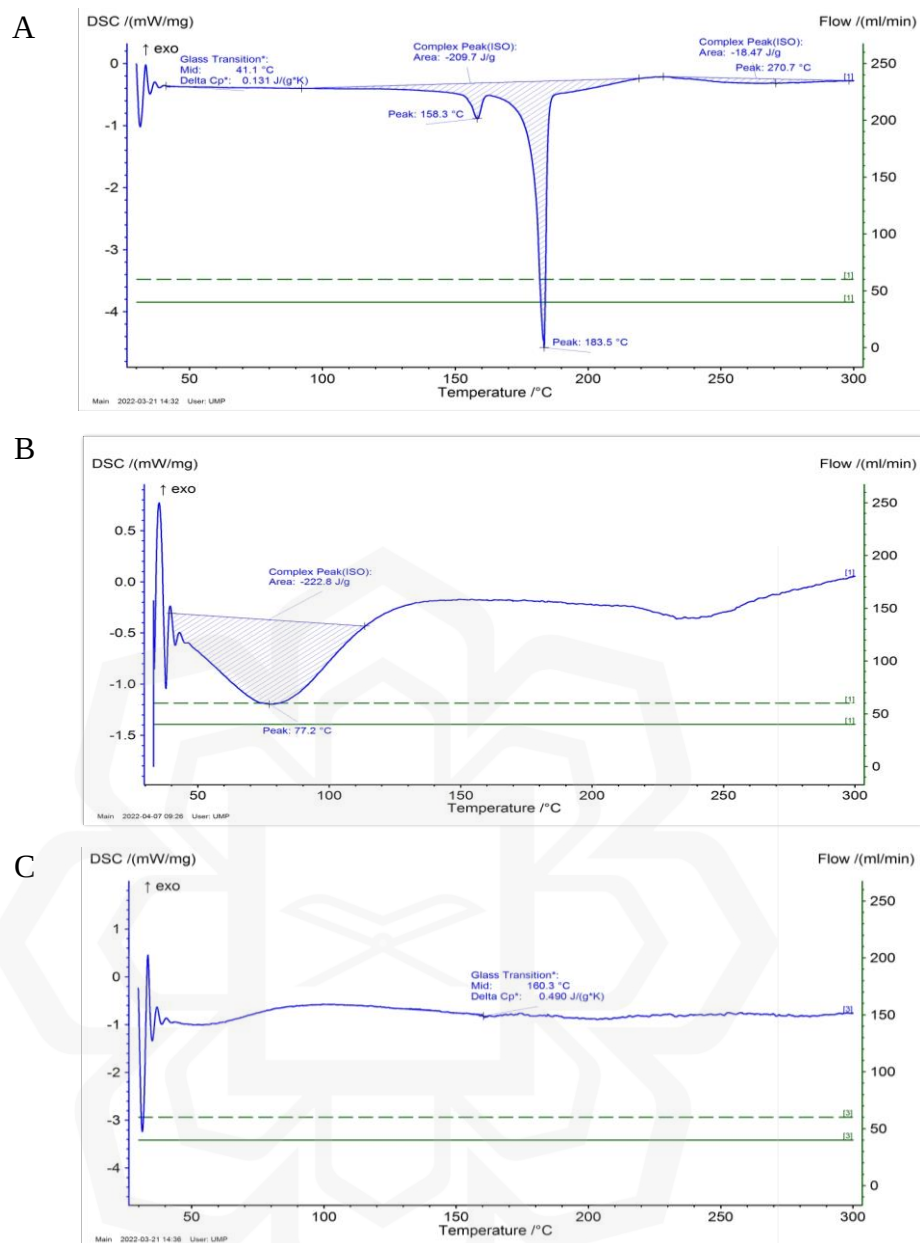


Figure 2.11 DSC Thermogram of (A) ATRA, (B) Plain Nanosponge and (C) ATRA Loaded Nanosponge.

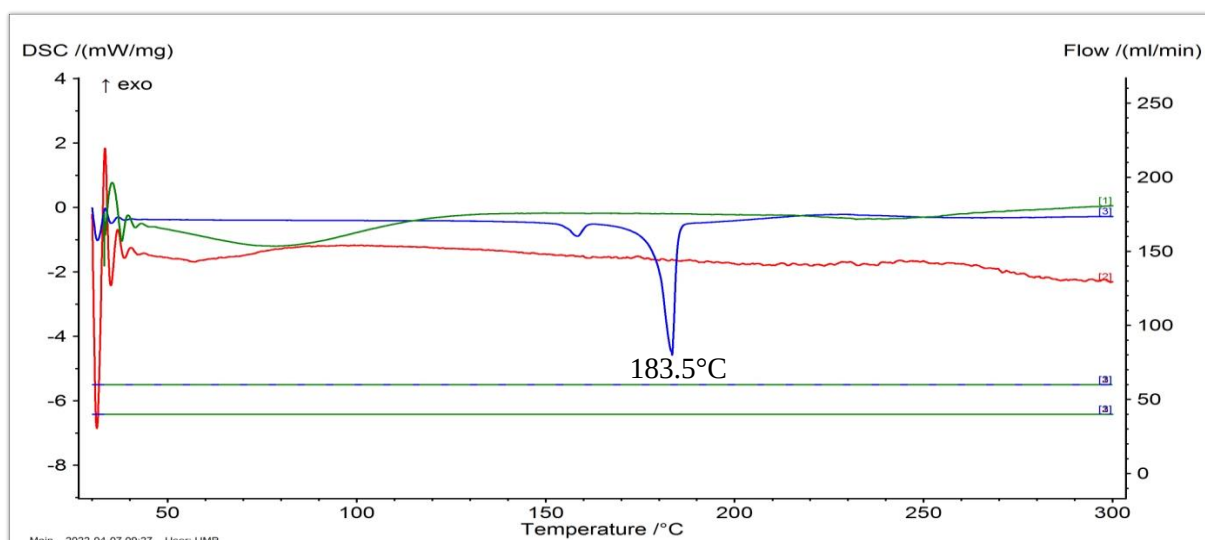


Figure 2.12 Overlapped DSC Thermogram of ATRA (blue), Plain Nanosponge (green), and ATRA Loaded Nanosponge (red)

2.3.3 High Performance Liquid Chromatography (HPLC) Method Validation

2.3.3.1 Linearity

A calibration curve as shown in Figure 2.13 with 6 concentration levels ranging from 5-90 µg/mL was plotted and demonstrated good linearity. The linear regression obtained based on the calibration curve is $r^2=0.9996$ which is in accordance with guideline that require $r^2>0.995$ (FDA, 2020).

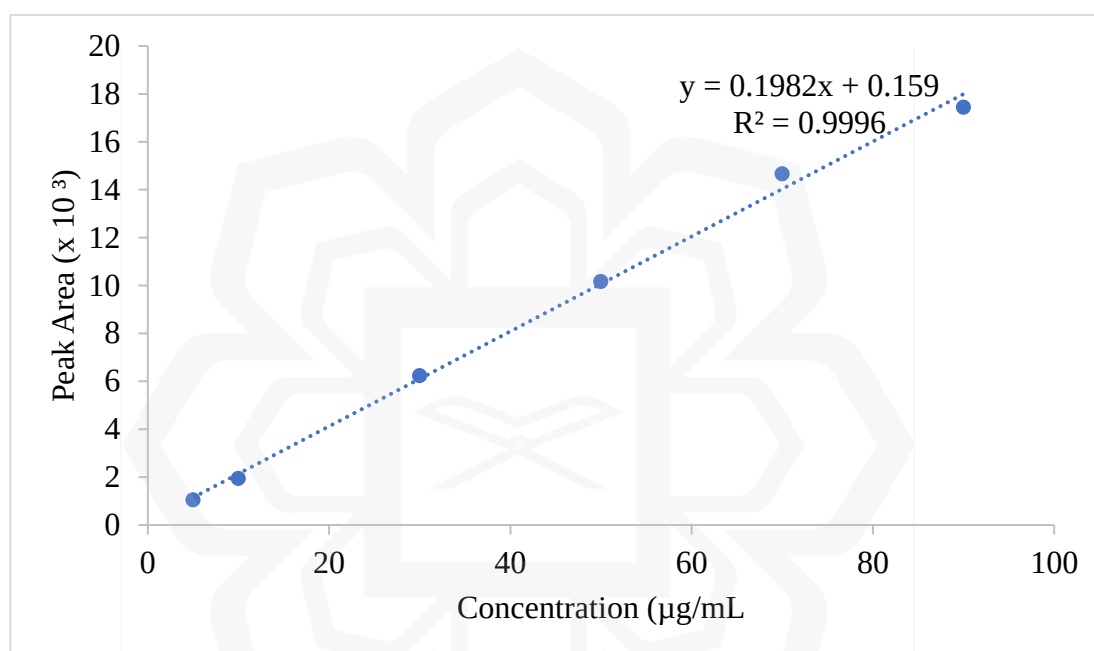


Figure 2.13 Standard Calibration Curve of Six Concentration Levels

2.3.3.2 Accuracy & Precision

Accuracy is the closeness of the values with accepted reference value represented by percentage recovery obtained which are well within the range of 97-103% (FDA, 2020). In Table 2.9, intra-day precision assay shows result of three ATRA concentration between 0.31-0.39 % while inter-day precision assay obtained values between 0.48 - 0.54%. Precision shown by intra-day and inter-day assay shows result of relative standard deviation percentage within acceptance criteria of $\pm 2\%$ (FDA, 2020).

Table 2.9 Accuracy and Precision Results

Concentration	Accuracy	Precision	
	Mean percent recovery (%)	Intra-day assay (%RSD) n=3	Inter-day assay (%RSD) n=9
30	99.88 $\pm$ 0.39	0.39	0.49
50	99.27 $\pm$ 0.32	0.32	0.54
70	98.14 $\pm$ 0.30	0.31	0.48

2.3.3.3 Specificity

Specificity of the HPLC method was validated though comparing peaks of blank methanol (Fig. 2.14), placebo (Fig. 2.15) and ATRA standard solution (Fig. 2.16) in the same retention time of ATRA. No peak interference observed for blank and placebo at the peak of ATRA which is at 7.5 min.

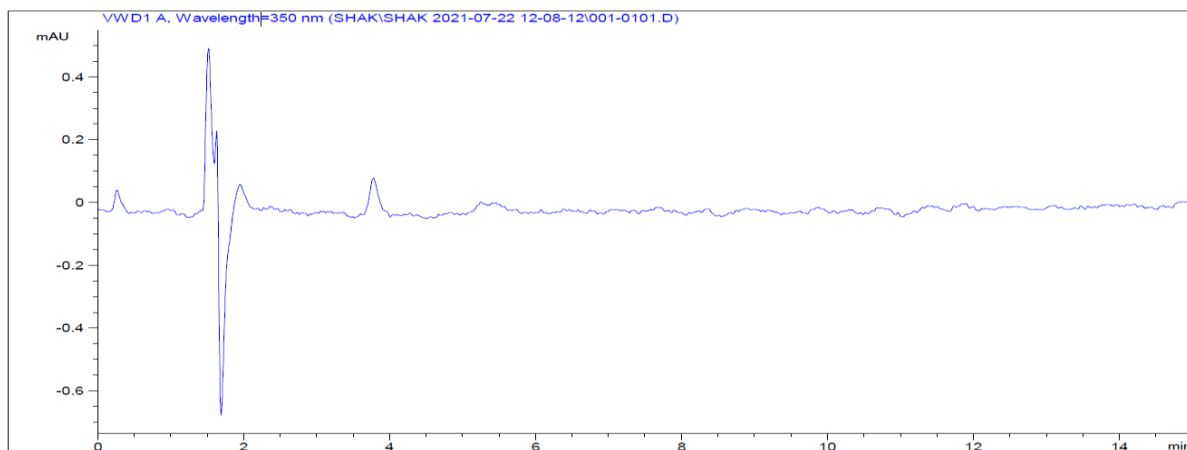


Figure 2.14 Chromatogram of Blank (Methanol)

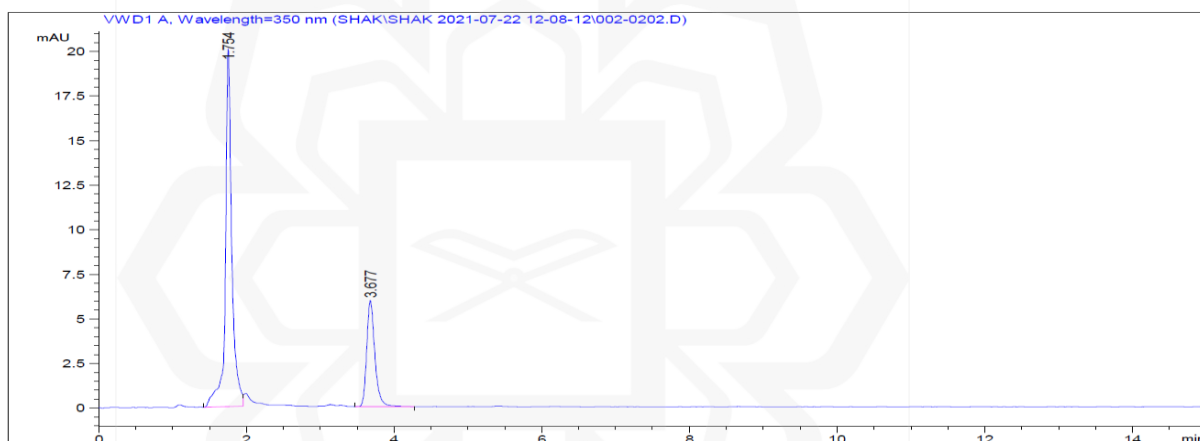


Figure 2.15 Chromatogram of Placebo

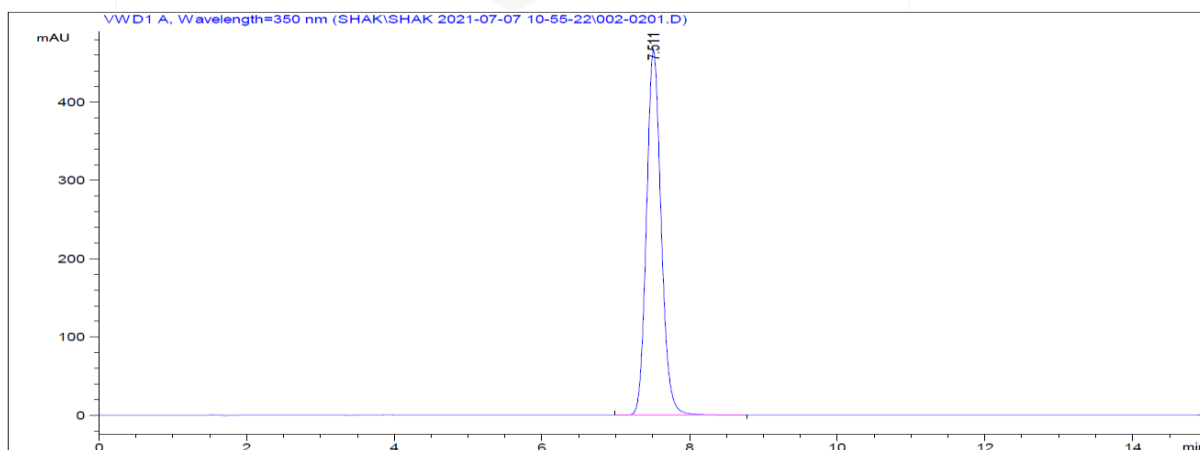


Figure 2.16 Chromatogram of ATRA Standard Diluted in Methanol

2.3.3.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Based on the standard deviation and the slope of curve from linear regression of the calibration curve, the LOD and LOQ was calculated from Equation 2.3 and 2.4. the lowest concentration of ATRA detected was 6.65 µg/mL while the lowest ATRA concentration quantified was 22.17 µg/mL

$$LOD = \frac{3\delta}{s}$$
$$= 6.65 \mu\text{g/mL}$$

Equation 2.3

$$LOQ = \frac{10\delta}{s}$$
$$= 22.17 \mu\text{g/mL}$$

Equation 2.4

2.4 CONCLUSION

As a conclusion, steps involved to produce a nanosponge requires tedious investigation in order to develop nanosponge with the most desirable properties for ATRA entrapment. Varying the polymer to crosslinker ratio as well as the duration of homogenization and the interaction of the two factors affected the properties of the nanosponge significantly. At the longest duration of homogenization of 20 minutes, an increase in particle size, PDI and zeta potential was observed. However, at the lowest duration of 10 minutes the results vary where the zeta potential and PDI is quite high compared to 15 minutes, yet it has lowest particle size, but not significantly different to 15 minutes. This made 15 minutes the most ideal duration for homogenization. The polymer to crosslinker ratio is a different story whereby high ratio resulted in small particle size but a zeta potential nearer to the agglomeration threshold. Analysis through design of experiment factorial analysis came up with three solutions which were entrapped with ATRA to further examine its EE% and solubility to determine the most preferred nanosponge for further formulation and investigation.

The solutions were nanosponge at ratio 1:2, 1:4 and 1:8 at 15 minutes. Out of all the three solutions, nanosponge 1:8 showed the highest EE% at 78.19% with 14.83% loading capacity. The solubilization was also the highest compared to the other solutions and free ATRA. Nanosponge 1:8 was further investigated by varying the drug to nanosponge ratio at 1:2, 1:4 and 1:6 where the final ratio that had the highest entrapment at 73.90% was ratio 1:2. Hence, the final nanosponge selected for further investigations were nanosponge 1:8 with drug to nanosponge ratio 1:2. The final entrapped nanosponge had a small particle size at 193.90 ± 2.74 nm and a PDI of 0.322 ± 0.0346 with zeta potential of -26.17 ± 0.513 mV. Entrapment of ATRA was confirmed through ATR-FTIR and DSC. In FTIR analysis, there were disappearance and shift of ATRA peaks which indicated inclusion of ATRA in cyclodextrin cavity. The same case with DSC where the melting endotherm of ATRA disappeared indicating interaction of nanosponge and ATRA. The step-by-step investigation of different factors affecting nanosponge development and ATRA entrapment helped determine the most ideal nanosponge to proceed for topical formulation.

CHAPTER THREE

DEVELOPMENT, OPTIMIZATION, CHARACTERIZATION AND STABILITY STUDIES OF GEL-CREAM FORMULATION WITH ATRA LOADED NANOSPONGE

3.1 INTRODUCTION

Topical dosage form is the main dosage form to manage cutaneous disorders which includes acne. New method and technologies are constantly being investigated and developed to improve this common route of administration in order to further enhance drug delivery and patient compliance (Sabalingam et al., 2022; Talat et al., 2021). Topical formulation is commonly categorized into solid, semi-solid and liquid formulation. Semi-solid is considered the most popular one as it is widely used in cosmetics and skincare as well. Examples include, ointments, creams, lotions, gels and the latest one is Gel-cream. Gel-cream which is also known as gelled emulsion or emulgel is defined as the combination of a classical gel with an emulsion. As it involves emulsions, it can be divided into oil in water or water in oil emulgel depending on the emulsion portion of the cream gel. Gel-cream formulation is becoming an emerging consumer favourite with high acceptability as it has properties of a cream and a gel where it is thixotropic, emollient, easy to remove, greaseless and pleasing (Talat et al., 2021).

A gel cream formulation was chosen as the base to carry the ATRA-loaded nanosponges due to its favourable properties. The benefits of a gel-cream formulation combine both the desirable qualities of a gel and a cream; where cream is moisturizing and emollient, and gel is cool and quick drying. The drawback of a gel is it is not suitable to carry hydrophobic drug while creams have reduced contact time on skin surface. The cream-gel formulation not only combines both benefits but removes both drawbacks (Sabalingam et al., 2022). ATRA is well known for its drying and irritation side effect, hence ensuring the formulation is sufficiently moisturizing as well as soothing will be

beneficial to further mitigate this side effects on top of loading it into nanosponge to enhance its delivery, stability, and patient acceptability.

Formulation prepared will usually be stored in certain condition and undergo a series of tests repeated over several time points to assess its stability. Guidelines have been established by different bodies to suggest appropriate conditions and test that should be done (ASEAN, 2013; WHO, 1996). Stability is crucial in ensuring that there is no physical, chemical or microbiological changes that can lead to product instability. Stability assurance is done through ensuring that the specifications set for the product is met and maintained throughout the stability study period. Some of the parameters monitored include appearance, odour, colour, texture, pH, size, zeta potential, drug content and microbial limit. Placing formulations in storage conditions based on guidelines and with frequent testing is important to ensure product is stable. Some of the product instability that can occur are flocculation, coalescence, creaming and phase separation (Omar et al., 2022). These instabilities can be caused by many reasons which can easily be known once product undergoes stability testing.

3.2 METHODOLOGY

3.2.1 Gel-cream Development

The gel cream formulation was adapted from previous study with slight modifications (Maia Campos et al., 2015). The formulation consists of four parts namely Part A, Part B, Part C and Part D. Part A consist of distilled water prepared in the lab with distiller Favorit W4L (PLT Scientific, Selangor, Malaysia) and hydroxyethylcellulose from Green herbology (Selangor, Malaysia). Lecithin (Acros Organics, Belgium) and glycerin in Part B is from Beauty360 (Selangor, Malaysia). Part C consists of squalane and propylene glycol from Beauty360. Tocopherol acetate is from Green herbology while preservative Euxyl-PE is from IMCD Malaysia Sdn. Bhd. (Malaysia) The composition of the gel-cream is as listed in Table 3.1.

Table 3.1 Composition of Gel-cream Formulation

Composition	Ingredients	Function	Amount %
A	Distilled water	Water phase	Qs 100
	Hydroxy ethylcellulose	Gelling agent	2.0
B	Lecithin	Emulsifier	1.0
	Glycerin	Humectant	3.0
C	Squalane	Oil phase	2.0
	Propylene glycol	Wetting agent	2.0
D	Tocopherol acetate	Anti- oxidant	0.5
	Euxyl-PE	Preservative	0.2

The step-by-step depiction of the process to make the gel-cream formulation is as shown in Figure 3.1. Hydroxyethylcellulose (HEC) was dispersed in water and heated up to 70°C in a water bath (Isotemp, Fisher Scientific, Ohio, USA). Once HEC completely dissolved and gel was formed, lecithin and glycerin were added to the mixture with continuous mixing. In a different beaker, propylene glycol and squalane was mixed and added to the first beaker and homogenized at 2000 rpm for 15 minutes. Once the mixture has cooled down to room temperature, tocopherol acetate, phenoxyethanol and ethylhexylglycerin (Euxyl-PE) were added to the mixture and homogenized for another 15 minutes at 2000 rpm.

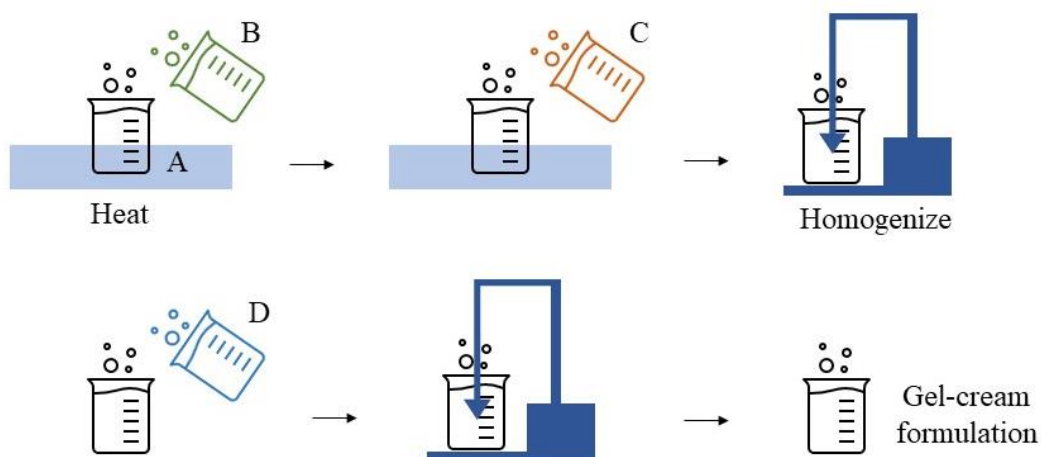


Figure 3.1 Step by Step Diagram of Formulating the Gel-Cream

3.2.2 Gel-cream Characterization & Preliminary Stability Study

The prepared gel-cream was then characterized with several parameters including physical appearance, particle size, zeta potential, pH, rheology profile and centrifugation. Preliminary stability study of the gel-cream was done by measuring parameters again after 6 months. The cream was left in ambient room temperature at about $\pm 25^{\circ}\text{C}$.

3.2.2.1 Physical Analysis

The physical and organoleptic properties of developed formulation were assessed to ensure it is suitable for users. It is essential to observe the appearance, colour, texture, and odour of the formulation (Apriani et al., 2019).

3.2.2.2 pH

pH analysis is an important part of characterization to ensure that it is a suitable pH to be applied on the skin. An acidic pH suits the skin best in order to protect the skin's acid mantle which is normally at pH 5.5 (Kuo et al., 2020). Hence through pH analysis the acidity or alkalinity of the formulation can be measured. The formulation was diluted with distilled water to 10% w/w before being analysed with a calibrated pH meter (Metler Toledo, Switzerland). Measurements were taken in triplicates (Apriani et al., 2019; Ijaz et al., 2022).

3.2.2.3 Particle Size and Zeta Potential

Stability of the formulation highly depends on the zeta potential and particle size. Zeta potential is a measure of attractive or repulsive charge between particles which reflect the potential of the formulation to destabilize into aggregation or flocculation. Particle size also has an important role where smaller particles are preferred as it gives higher viscosity and better stability. Samples were analysed using Zetasizer (ZEN 1600, Malvern, UK). Samples placed in capillary cells were diluted 1:100 using deionized water to prevent multiple scattering effect. Measurements were taken in triplicates at a temperature of 25°C (Apriani et al., 2019; Esteban et al., 2016).

3.2.2.4 Rheology Study

A stress-controlled rheometer (HAAKE MARS, Thermo Scientific, Germany) with a cone and plate having a diameter of 35 mm and a gap of 1 mm was used to analyse the rheological properties of the formulation. Samples loaded on to the lower plate of the rheometer and was allowed to equilibrate to a temperature of 25°C. The two main parameters that determines the flow properties of the formulation are shear stress and

apparent viscosity with application of shear rate of 0.01 to 1000 s⁻¹ (Athikomkulchai et al., 2020; Kirtil & Oztop, 2016).

3.2.2.5 Centrifugation Study

Forced centrifugation study was done to investigate the stability of the formulation against phase separation (Rodrigues et al., 2016). 10 g of the cream formulation was placed in a centrifugal tube and centrifuged using benchtop centrifuge (Megafuge 8, Thermo Scientific, Germany) at 5000 rpm (2739 xg) for 10 minutes at 25°C (Omar et al., 2021).

3.2.3 Inclusion of ATRA Loaded Nanosponge in Gel-cream Formulation

3.2.3.1 Optimization of ATRA Loaded Nanosponge in Gel-cream Formulation

From the plain gel-cream development the formulation was further optimized when ATRA loaded nanosponge was added to ensure the formulation has desirable characteristics with good stability. The method for gel-cream development was optimized by varying the homogenization speeds to 3 different speeds namely 2000 rpm, 3000 rpm and 4000 rpm for 15 minutes. The amount of ATRA loaded in the nanosponge was determined through previous findings. The maximum dose of ATRA is 0.1% applied daily. This study uses 0.05% as the difference in terms of reduction in inflammatory and non-inflammatory lesion is not significant, while the adverse events were higher in 0.1% in comparison to 0.05% (Lucky & Sugarman, 2011). For a 0.05% formulation, 0.34% of retinoic acid loaded nanosponge was added into the gel-cream formulation. The parameters analysed were zeta potential, particle size, polydispersity index and pH, centrifugation study and physical appearance.

3.2.4 Stability Study

Stability study was done over the span of 3 months with the final formulation. The study was carried out at temperatures of $30^{\circ}\text{C}\pm 2$ and $40^{\circ}\text{C}\pm 2$ at $75\%\pm 5\%$ RH for 3 months in an accelerated and real-time stability chamber, respectively (Binder KBF 240, Fisher Scientific, Germany). Properties of the formulation was assessed at each time points at 0 weeks, 2 weeks, 4 weeks and 12 weeks (ASEAN, 2013). The assessment includes physical appearance, pH, centrifugation, particle size, polydispersity index, zeta potential as mentioned previously and an addition of HPLC analysis for content analysis as well microbial limit count.

3.2.4.1 High-performance Liquid Chromatography (HPLC) Assay

Samples were analysed with HPLC using an isocratic system with HPLC 1200 series from Agilent technologies with an integrated controller, quaternary pumps, and autosampler, a solvent degasser unit, and a variable wavelength detector (VWD) (Agilent, Stevens Creek Blvd Santa Clara, USA). Samples were run at 355 nm using a reverse phase C-18 column ($3\mu\text{m}$, $150 \times 4.6\text{mm}$). The mobile phase consists methanol and 0.5% acetic acid in water with ratio of 90:10 v/v. A flow rate of 1.0 mL/min was employed (Raza, Singh, Lohan, et al., 2013; Raza, Singh, Singla, et al., 2013). Working standard solutions was prepared by diluting prepared standard solutions in methanol in concentrations ranging from 5 – 90 $\mu\text{g/mL}$. 0.5g of sample was added 90:10 methanol:water and then sonicated. All samples were filtered through $0.45\mu\text{m}$ syringe filter.

3.2.4.2 Microbial Limit Count

1 g of the sample was diluted using phosphate buffer solution pH 7.2 to a 1 in 10 dilution. Next, 10 mL of the sample was inoculated in the *Soybean-casein Digest broth* which was incubated at 30-35°C for 18-24 hours. Sample was then sub-cultured on a *Mannitol Salt Agar* (MSA) plate and incubated for 18-72 hours at 30-35°C. The presence of *S. aureus* can be confirmed if there are any growths of yellow or white colonies surrounded by a yellow zone. Same steps were repeated and sub-cultured on *Cetrimide Agar* (CA) incubated for 18-72 hours at 30-35°C. Presence of *Pseudomonas aeruginosa* can be confirmed if there is growth on this agar.

A dilution of 1:10 of the gel-cream in phosphate buffer solution pH 7.2 was done for the determination of Total Aerobic Microbial Count (TAMC) and Total Yeast and Mold Count (TYMC) using the pour-plate method. 1 mL of diluted sample was added to a plate that was poured with 15-20 mL of *Soybean-casein digest agar* for TAMC. Plate was then incubated at 30-35°C for 5 days or less. Similar steps were repeated on *Sabouraud dextrose* agar for TYMC. The plate for TYMC was then incubated at 20-25°C for 5 days or less (USP, 2021a, 2021b).

3.2.5 Antimicrobial activity

Freeze dried pellet of *Cuticubacterium acnes* (DSM 1897) was obtained from Deutsche Sammlung von Mikroorganism und Zellkulturen (DSMZ), Braunschweig Germany. The pellet was rehydrated in 0.5 mL brain heart infusion (BHI) broth and left for 30 minutes. The content of was gently mixed with an inoculation loop. Half of the amount was transferred to 5 mL of BHI broth. Each mL of the broth was mixed with 1 mL of glycerol and stored in -80°C freezer. The leftover rehydrated pellet was streaked on BHI agar. The plate was incubated at 37°C for 5 days. The whole process was done in an

anaerobic chamber filled with carbon dioxide 5%, hydrogen 10% and nitrogen 85% as *C. acnes* is an anaerobic microorganism.

The antibacterial activity was determined through a modified agar well diffusion method. An 8 mm borer was used to cut three wells in the plate. *C. acnes* bacterial population was standardized to 0.5 Mcfarland standard by measuring the turbidity in sodium chloride solution (0.85%) using densitometer. The bacteria were then streaked on to BHI agar. The nanosponge loaded with ATRA was prepared into a gel-cream formulation. Antimicrobial activity was done on the placebo gel-cream formulation, ATRA loaded nanosponge gel-cream formulation and a commercial formulation containing ATRA at the same dose. 1 g of each formulation was added to the wells. The plates were incubated for 72 hours at 37°C in anaerobic environment. They were then examined and the diameter of the zone of inhibition was measured.

3.2.6 Data Analysis

All the data obtained were recorded in triplicates and presented in the form of mean $\pm$ standard deviation. The data obtained from the development and optimization of gel-cream formulation underwent analysis using GraphPad Prism. To ascertain significance in the optimization process, a one-way ANOVA was applied. The stability study which spanned over a three-month period, was subjected to analysis using repeated measured ANOVA. This statistical approach ensures a comprehensive understanding of the formulation optimization and stability over time.

3.3 RESULTS AND DISCUSSION

3.3.1 Gel-cream Characterization & Preliminary Stability Study

The characterization of gel-cream and its assessment after 6 months are as shown in Table 3.2. The organoleptic properties of the gel-cream show that the formulation is off-white in colour, odourless, smooth, and shiny texture and shows no separation. These results remain the same even after 6 months as can be observed in Figure 3.2. The pH which was initially 6.00 ± 0.06 reduced to 5.03 ± 0.04 after 6 months is still considered suitable for skin application with potentially no irritation as it lies within the normal skin pH of 4-6 (Kuo et al., 2020). An acidic pH is preferred because human skin is covered with an acidic mantle that is lost with frequent washing (Khan, Akhtar, Khan, et al., 2013). Lower pH has been suggested as a therapeutic strategy in skin diseases (Proksch, 2018).

The zeta potential continuously had a value that is able to electrostatically maintain a stabilized suspension which is less than -30 and more than 30 mV (Gumustas et al., 2017). The particle size initially had a considerably larger particle size than desired. Due to its initial size, over time the size became larger most likely as a result of Ostwald ripening which is a frequent occurrence in emulsions (Simonazzi et al., 2018). Furthermore, the PDI values obtained at 0 month is larger than desired in order to maintain a stable formulation. As can be seen from the results at 6 month the PDI value increased to 0.8 which reflects high polydispersity as it is near to 1. An acceptable PDI for better stability of the formulation is below 0.5 (Torres et al., 2022).

Table 3.2 Gel-cream Characterization and Preliminary Stability Assessment Results
(Mean $\pm$ SD, n = 3)

Parameter		0-month	6-month
Organoleptic properties	Colour	Off-white	
	Odour	No scent	
	Texture	Smooth and shiny	
Centrifugation		No separation	
pH		6.00 $\pm$ 0.06	5.03 $\pm$ 0.04
Zeta Potential (mV)		-51.8 $\pm$ 7.05	-60.1 $\pm$ 7.19
Particle size (nm)		495.37 $\pm$ 1.62	869.3 $\pm$ 22.75
PDI		0.671 $\pm$ 0.01	0.800 $\pm$ 0.01

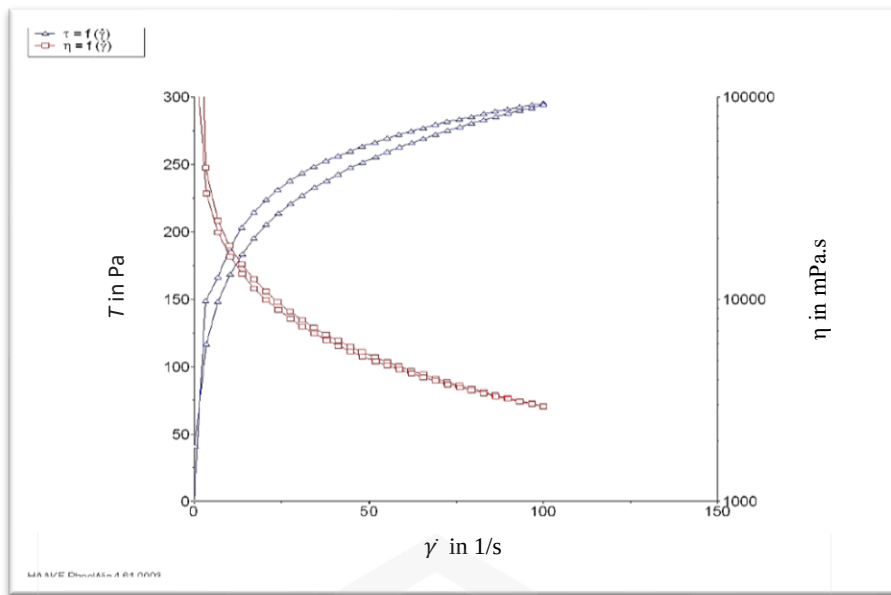


Figure 3.2 Centrifugation, Texture and Viscosity Observation of the Gel-Cream Formulation

The Rheology profile of the formulation is as shown in Figure 3.3. The formulation follows a non-Newtonian flow, where the relation between shear stress (mPa) and shear rate (1/s) is not linear. Non-Newtonian flow goes against the Newtonian law of viscosity where the relationship between shear stress and shear rate is linear with viscosity as the constant. Specifically, both formulations portray a pseudo plastic flow which is also known as the shear-thinning system. This system shows that increase in shear stress increases the shear rate hence reduce viscosity of the fluid (Rodrigues et al., 2016). The increase in shear rate surpasses the Brownian motion hence providing less resistance to flow which in turn reduces the viscosity of the formulation (Niu et al., 2016). This is shown by the red lines depicting a decreasing viscosity because of increasing shear rate.

From the graph the blue line forms a hysteresis loop which is a characteristic of thixotropic system. Thixotropy is a time-dependent shear thinning property where with stress, fluid flows as viscosity reduces. The longer the time the fluid undergoes stress, the lower the viscosity. The hysteresis loop is formed due to the breakdown of the formulation which does not return to its original state immediately after stress is removed. Instead, it takes time to return to its original viscous state. In the graph this feature is shown by the blue lines which are not superimposed upon each other depicting the thinning of the formulation and its return to the viscous state.

A



B

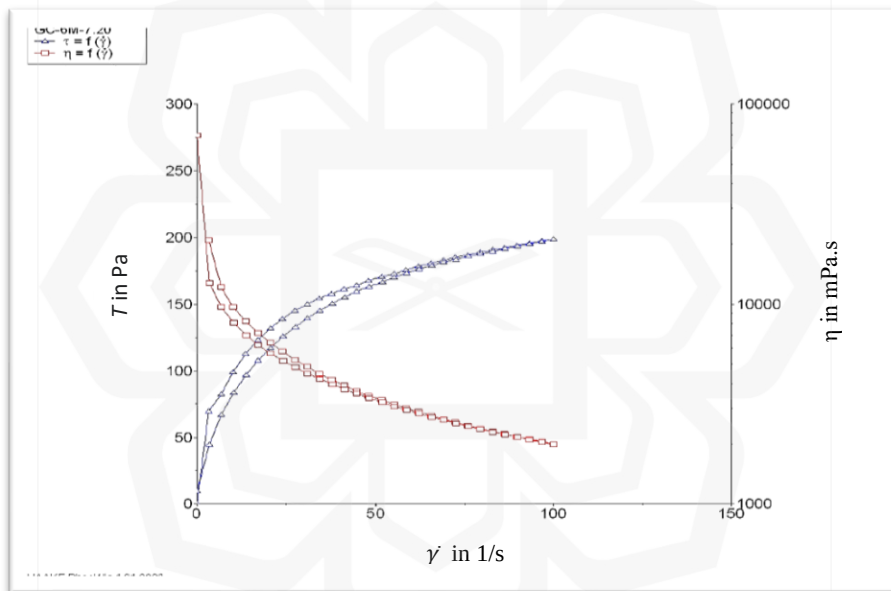


Figure 3.3 Rheology Profile of The Gel-Cream Formulation. (A; 0-Month Profile, B; 6-Month Profile. Red; Viscosity, Blue; Shear Stress)

3.3.2 Optimization of ATRA-loaded Nanosponge Gel-cream Formulation

Based on the gel-cream formulation in the previous section there were several parameters that required further improvement. Hence, to optimize the formulation, prior to stability study, gel-cream formulation containing ATRA loaded nanosponge was prepared with varying homogenisation speeds at (2000 rpm, 3000 rpm and 4000 rpm). From Table 3.3. the organoleptic properties and physical assessment of the formulations prepared with varying homogenisation did not show any differences from one another. However other parameters do show some varying results.

Table 3.3 Gel-cream with ATRA Loaded Nanosponge Physical Assessment at Varying Homogenization Speeds

Parameter	Homogenization speed	Results
Colour	2000	Off-white
	3000	
	4000	
Odour	2000	Odourless
	3000	
	4000	
Texture	2000	Smooth & shiny
	3000	
	4000	
Centrifugation study	2000	No separation
	3000	
	4000	

Based on Figure 3.4 at the pH increases slightly with increasing speed. However, the difference in values between the varying speeds had no significant difference ($p>0.05$) in the pH changes. The pH was 6.28 ± 0.02 , 6.34 ± 0.02 , 6.66 ± 0.05 , at 2000, 3000 and 4000 rpm, respectively. A previous study investigated the effect of homogenization pressure on pH of sesame vegetable cream and found no significant difference in pH. The results from this current study and prior study on cream formulations shows that pH is not a parameter that is easily influenced by technical parameters (Samani & Naji, 2019).

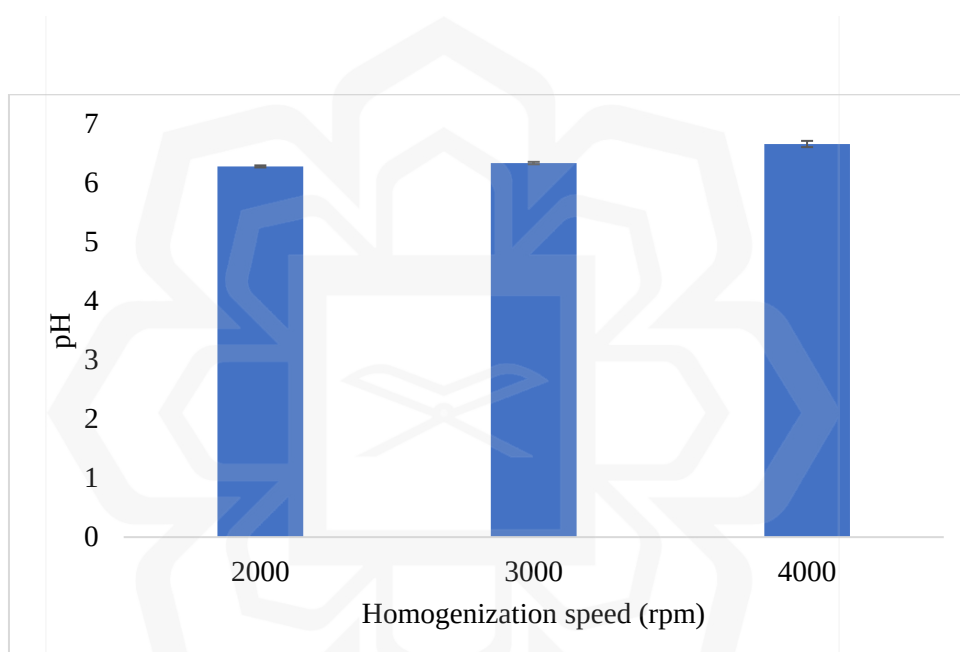


Figure 3.4 pH of Gel-Cream Formulation with ATRA Loaded Nanosponge at Varying Homogenization Speed (Mean $\pm$ SD, n = 3)

Figure 3.5 shows the different particle size when the homogenization speed was varied. The results were found to be significantly different from one another for particle size ($p<0.05$) but no significant difference of PDI ($p>0.05$). As can be observed from the graph, the most optimal speed to obtain a small particle size is at 3000 rpm. Smaller particle size is desired as it reduces the risk of Ostwald ripening hence improving the overall stability of the formulation (Simonazzi et al., 2018). Delivery-wise smaller

particle size leads to higher skin retention and contributes to sustained drug delivery (Kumar et al., 2021). From the graph the particle size reduced when homogenized at 3000 rpm (209.53 ± 1.56 nm) compared to 2000 rpm (263.5 ± 5.91 nm). Speed of homogenization affect the particle size and PDI of the formulation in which increasing the speed reduced the particle size formed. This is due to the higher energy density and shear stress exerted which are inversely proportional to the droplet size (Mulia et al., 2019; Peng et al., 2015).

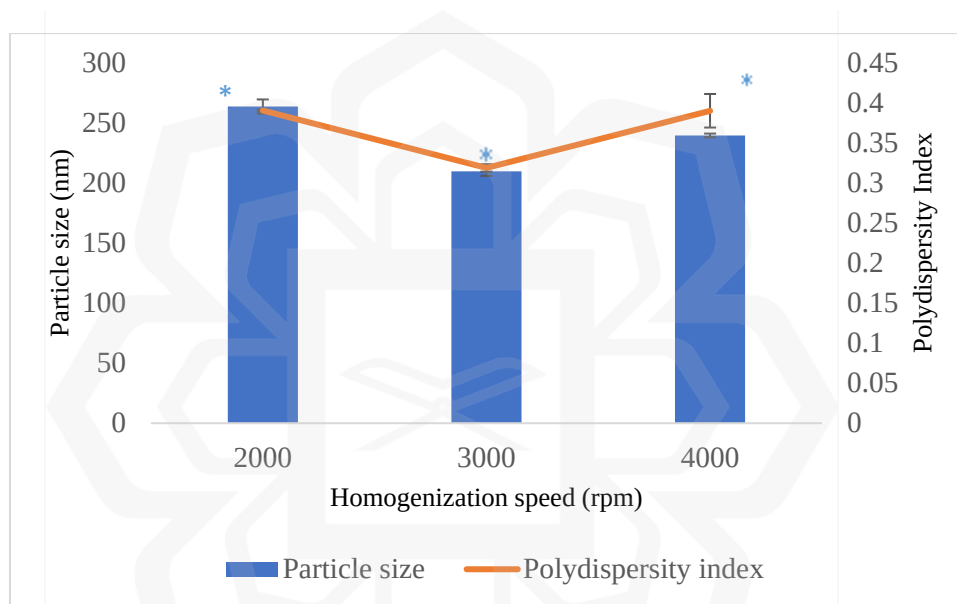


Figure 3.5 Particle Size and Polydispersity Index of Gel-Cream Formulation with ATRA Loaded Nanosponge at Varying Homogenization Speed (Mean $\pm$ SD, n =3, [*] = $p < 0.05$). Blue*: significance for particle size.

Results suggest that at 3000 rpm there were enough mechanical energy to break down the droplets and produce smaller droplets (disruptive force > cohesive force). However, high stirring speeds increase the chances of coalescence as the collision rate increase which is reflected by the results of the particle size at 4000 rpm which was 239.47 ± 1.56 nm (Bamba et al., 2018). The appropriate balance to achieve small particle size is at 3000 rpm. As the particle size reduces when homogenized at 3000 rpm, it can

be observed that the PDI also follows similar trends. As the particle size becomes smaller from 2000 rpm (0.3903 ± 0.0012) to 3000 rpm (0.3187 ± 0.0046), the presence of varying size also reduces. The PDI increases again at 4000 rpm (0.3900 ± 0.0209) is most likely due to the coalition of droplets forming more particles with varying size.

The zeta potential as depicted in Figure 3.6 were -46.2 ± 0.44 , -46.7 ± 1.04 , -45.97 ± 0.78 , at 2000, 3000 and 4000 rpm, respectively. There were no significant differences between the values ($p > 0.05$) yet 3000 rpm does measure the lowest value among all. The speed of homogenization does have an effect on zeta potential which is related to the particle size (Narayanan et al., 2014; Priyanka et al., 2018). As the particle size decreases, a higher zeta potential closer to the aggregation threshold is observed. Such effect is not very evident in this study most likely due to the range of speed used which is lower compared to previous studies. Generally, the formulation has negatively charged droplets which is related to the pH of the formulation and its composition. Non-stable behaviour occurs when the pH is very low, whereby the zeta potential is much closer to the isoelectric point which is the point where zeta potential is 0 (Romdhane et al., 2015; Večeř & Pospíšil, 2012). So, the pH and zeta potential obtained for this formulation is sufficiently low for skin application yet not too low as to compromise the formulation stability.

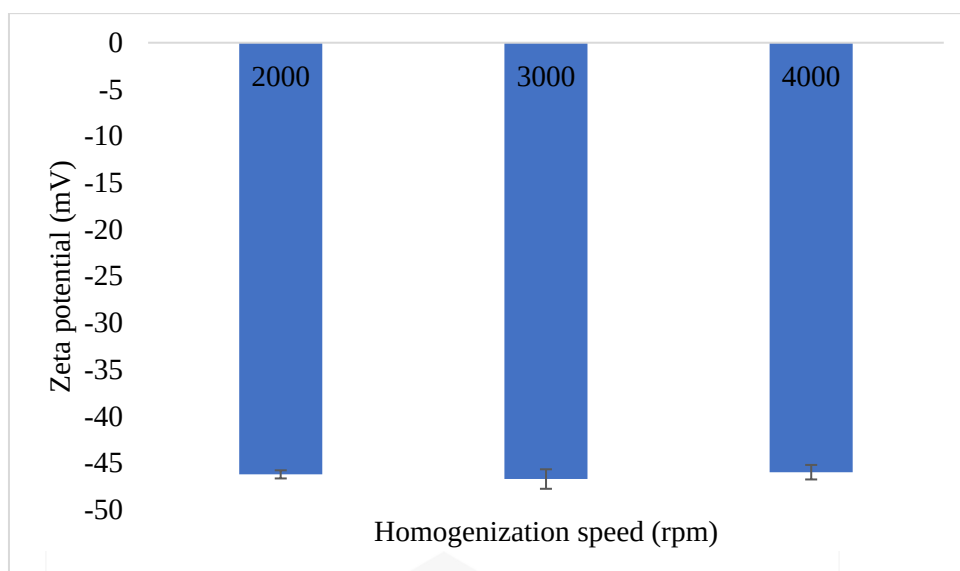


Figure 3.6 Zeta Potential of Gel-Cream Formulation with ATRA Loaded Nanosponge at Varying Homogenization Speed (Mean $\pm$ SD, n = 3)

3.3.3 Stability Study of ATRA loaded Nanosponge Gel-cream

3.3.3.1 Physical Analysis and Centrifugation Study

The gel-cream prepared was placed in stability chambers with two different environments namely the real-time stability chamber (30°C/75%RH) and the accelerated stability chamber (40°C/75%RH). Throughout the duration of the study of a total of 3 months, the cream was assessed 4 times a Week 0, Week 2, Week 4 and Week 12. The study was conducted for a duration of 3 months as a preliminary data for the stability of the formulation when subjected to accelerated and real-time stability study following parameters (temperature and humidity) suggested by guidelines for drug approval (ASEAN, 2013; ICH, 2003). Table 3.4 shows the observations and assessments made regarding the physical properties of the gel-cream throughout the period of the stability study. The colour of the formulation which is off- white maintains the same throughout the period of the stability study in both chambers. The colour is a result of the white base gel-cream colour mixed with the slightly yellowed nanosponge loaded with ATRA. Physically ATRA is a yellow coloured powder as a result of its structure which contains an alternating C=C double bonds in the polyene side chain

(Nofal et al., 2022). The texture and odour of the formulation also remained the same throughout the study period. The formulation maintained a smooth texture and had no odour since the beginning of the stability study.

Forced centrifugation study was applied on the formulation and showed that the formulation remained stable and did not display phase separation or any signs of instability such as creaming or foaming. The presence of emulsifiers like lecithin and propylene glycol helped in maintaining the stability of the formulation as it supports the mixing of oil based and water-based ingredients of the formulation. Lecithin is a zwitterionic surfactant that can effectively reduce interfacial tension of oil-water and propylene glycol has been used a co-surfactant in prior studies. A mixture of emulsifiers can help improve the formation of a stable emulsion with good properties (Cha et al., 2019; El Khayat et al., 2018). On top of that, presence of hydroxyethyl cellulose as a thickener helps suspends droplets and reduces the risk of destabilization (Costa et al., 2021).

Table 3.4 Physical Observation of ATRA loaded Nanosponge Gel-cream Stored in 30°C/75%RH and 40°C/75%RH at Week 0, Week 2, Week 4 and Week 12.

Characteristic	Storage	ATRA Loaded Nanospong Gel-Cream			
		Week 0	Week 2	Week 4	Week 12
Colour	30°C/75%RH	Off-white	Off-white	Off-white	Off-white
	40°C/75%RH	Off-white	Off-white	Off-white	Off-white
Texture	30°C/75%RH	Smooth	Smooth	Smooth	Smooth
	40°C/75%RH	Smooth	Smooth	Smooth	Smooth
Odour	30°C/75%RH	Odourless	Odourless	Odourless	Odourless
	40°C/75%RH	Odourless	Odourless	Odourless	Odourless
Phase	30°C/75%RH	X	X	X	X
separation	40°C/75%RH	X	X	X	X

Abbreviation: X is absent.

3.3.3.2 pH

From Figure 3.7 it can be observed that both formulations in both conditions maintained a pH well within the suitable range. The pH of formulation in real-time and accelerated stability chamber were between 5.13-6.34. The change in pH over time was found to be significant ($p < 0.005$) yet the gel-cream stored in both environments still remain acidic and suitable for skin application. The graph for both environments showed a decreasing trend compared to its baseline value at week 0. Tukey's multiple comparison found that there is significant difference $p < 0.005$ between all weeks for both real-time and accelerated stability study. An acidic pH is preferred in order to maintain the acidic mantle that covers the skin which can be affected with frequent washing, hence applying an acidic product will help return the skin to its normal condition (Khan et al., 2013; Omar et al., 2021).

It is highly agreed to acidify topical products to possess pH range between 4-6. This is because an adult skin typically exhibits a pH ranging between 4.1-5.8 (mean 4.9). The important function of an acidic pH of the stratum corneum is for skin barrier function, lipid synthesis and aggregation, epidermal differentiation and desquamation, maintenance of skin microbiome, reducing inflammation and as an antimicrobial barrier to prevent colonization (Lukić et al., 2021; Proksch, 2018). Inflammatory skin disease or disease that involves epidermis which includes acne has an increased pH and disturbed skin barrier. Hence why it is of great importance to ensure the formulation applied on the skin is acidic and remains acidic (Proksch, 2018).

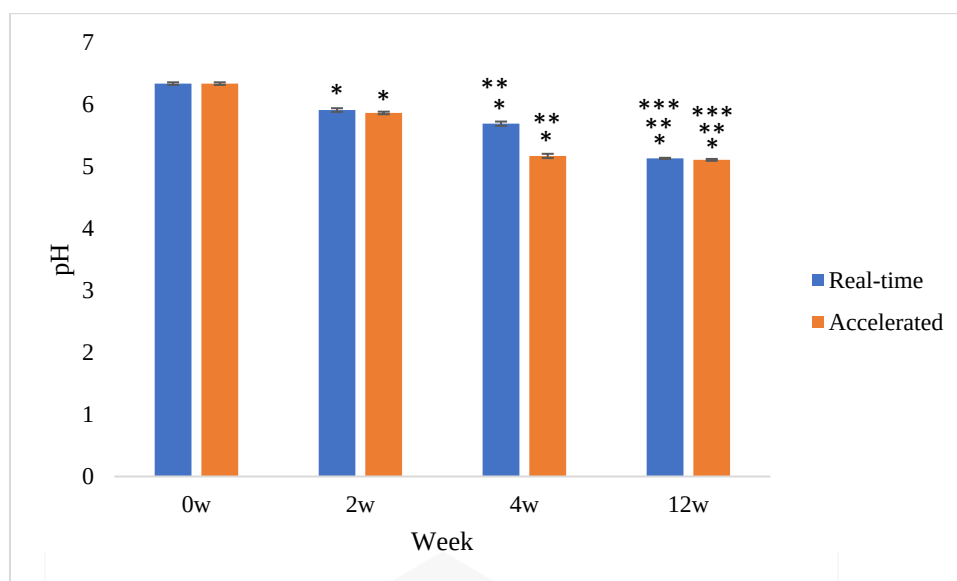


Figure 3.7 pH Values of ATRA Loaded Nanosponge Gel-Cream Stored in 30°C/75%RH and 40°C/75%RH for 12 weeks (Mean $\pm$ SD, n = 3, * = $p > 0.05$). (Abbreviations: 0w is Week 1; 2w is Week 2; 4w is Week 4; 12w is Week 12, * is significant to 0W, ** is significant to 2W and *** is significant to 4W)

3.3.3.4 Particle Size and Polydispersity Index

The particle size monitored throughout the stability study period are as depicted in Figure 3.8. The particle size for the formulation in both chambers ranged between 199.8-224.4 μm . The changes of the values measured over the period of 12 weeks did show significant change ($p < 0.05$). Pairwise comparison shows that for real-time chambers there were significant differences between week 2 to week 0 ($p < 0.05$) and week 12 ($p < 0.05$) as well as week 4 to week 12 ($p < 0.05$). While data from the accelerated chamber data found significant difference between all weeks except week 4 to week 12. Formulation in both chambers shows significant difference to week 12 indicating that over longer periods of time, the change in size is significant.

From the graph, particle size started to increase slightly which can be clearly seen from week 4. The increase is higher for samples stored in the accelerated stability

chamber compared to the real-time stability chamber. This increase in size is most likely due to process called Ostwald ripening which is where smaller particles present in a solution start to dissolve and deposit on to larger particles to minimize surface area ratio in order to reach a more thermodynamically stable state (Van Westen & Groot, 2018). The occurrence of Ostwald ripening is what eventually leads to creaming, flocculation, or phase separation. However, having a nano-sized formulation is extremely helpful in delaying and preventing this phenomenon due to its extremely small size that can remain stable for longer duration (Simonazzi et al., 2018). A small particle size provides higher stability and also increases viscosity (Khor, 2014).

Despite the slight increase in size, the formulation remained physically stable and did not show any signs of instability. Furthermore, the size did not change much over time, maintained well below 300 nm. Generally, smaller particle size is desired as it provides higher skin retention which helps deliver a more efficient amount of drug over a long period of time. Prior research have found that particle size approximately as high as 700 nm to as low as below 100 nm shows high skin retention (Argenziano et al., 2019; Liu et al., 2018; Moribe et al., 2010). Particle sizes below 100 nm has been shown to penetrate into the deep layers of the skin and at a faster rate (Adib et al., 2016; Xiang et al., 2022). The current size of the formulation is sufficiently small for skin retention, accumulation at the hair follicles and to deliver slow release ATRA to the target layer which is mainly the epidermis.

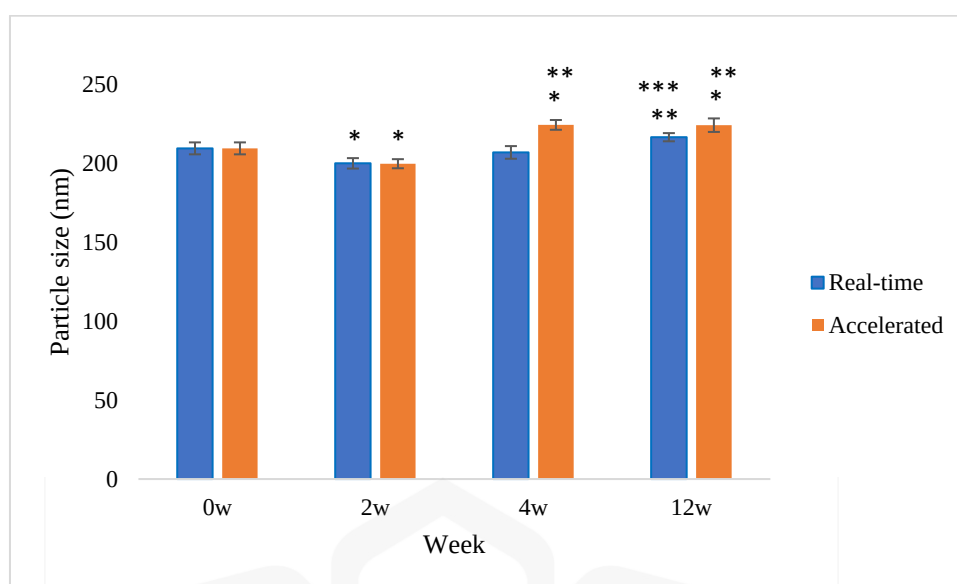


Figure 3.8 Particle Size Values of ATRA Loaded Nanosponge Gel-Cream Stored in 30°C/75%RH and 40°C/75%RH for 12 weeks (Mean $\pm$ SD, n = 3, $\ast$ = p <0.05). (Abbreviations: 0w is Week 1; 2w is Week 2; 4w is Week 4; 12w is Week 12, $\ast$ is significant to 0W, $\ast\ast$ is significant to 2W and $\ast\ast\ast$ is significant to 4W)

The PDI values obtained throughout the stability study ranged between 0.246 – 0.318 for real-time chamber and 0.255 – 0.335 for the accelerated chamber. Despite the fluctuations in values, the overall data was not significant (p >0.05). Pairwise comparison did show significant differences (p <0.005) between week 0 and week 2; week 2 to week 4 and week 2 to week 12 for both chambers. PDI below 0.5 indicates acceptable polydispersity (Torres,2021). The closer the value to 0 indicates a monodisperse particle size (Rosyada et al, 2019). The formulation showed stable PDI values during the stability study duration in both real-time and accelerated chamber. The slight increase in week 12 is parallel to the slight increase in particle size which may have contributed to the presence of variously sized particle in the size distribution data.

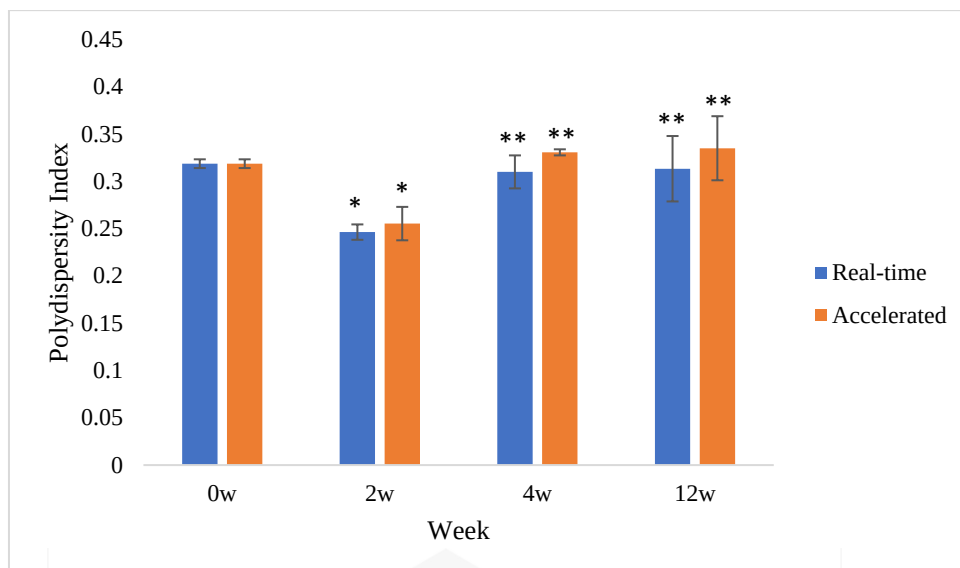


Figure 3.9 Polydispersity Index of ATRA Loaded Nanosponge Gel-Cream Stored in 30°C/75%RH and 40°C/75%RH for 12 weeks (Mean ± SD, n = 3, *=p<0.05). (Abbreviations: 0w is Week 1; 2w is Week 2; 4w is Week 4; 12w is Week 12, * is significant to 0W, ** is significant to 2W and *** is significant to 4W)

3.3.3.5 Zeta Potential

The zeta potential value measured ranged between -46.7 to -56.1 and -46.7 to -55.93 for real-time and accelerated chamber. The data collected showed significant difference (p<0.05). Pairwise comparison for both formulations in both chambers showed that the formulation has significant differences (p<0.05) between all weeks except for week 0 to week 2. Zeta potential values less than -30 mV and more than 30 mV is much preferred as it is further from the agglomeration threshold (0 mV) (Gumustas et al., 2017; Thakur et al., 2020). A large value either positive or negative indicates good stability as it reflects the highly charged particles. This is important as it prevents aggregation through electrostatic repulsive force that causes the droplets to repel one another (Joseph & Singhvi, 2019).

Maintaining zeta potential other than within -30 mV to +30 mV is very important in maintaining formulation stability as the closer the value is to the agglomeration threshold (0 mV), the higher the risk for the formulation to agglomerate or coalesce with one another due to the attractive van der Waals forces that act upon them (Samimi et al., 2019). This may finally lead to physical instability such as creaming or phase separation. The data in graph 3.10 shows that over time the zeta potential values goes further from the isoelectric point, or in other words becomes more stable. This phenomenon maybe contributed by the pH of the formulation that slightly reduces from week 0 to week 12. pH value is known to have influence on the zeta potential of a formulation.

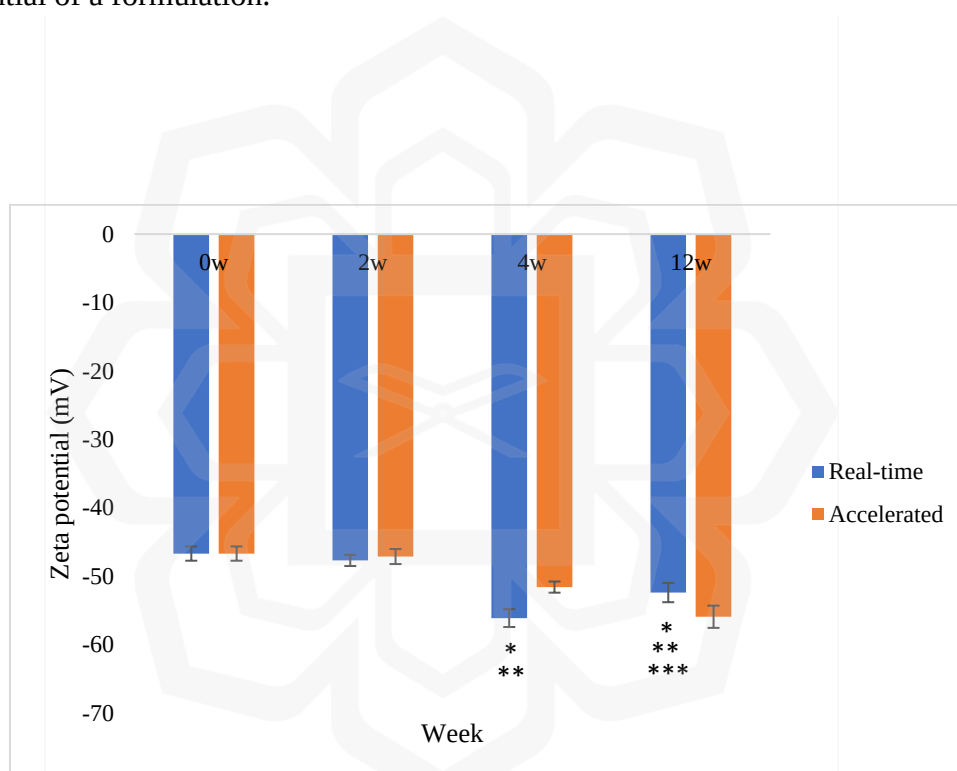


Figure 3.10 Zeta Potential Values of ATRA Loaded Nanosponge Gel-Cream Stored in 30°C/75%RH and 40°C/75%RH for 12 weeks (Mean $\pm$ SD, n = 3). (Abbreviations: 0w is Week 1; 2w is Week 2; 4w is Week 4; 12w is Week 12, * is significant to 0W, ** is significant to 2W and *** is significant to 4W)

3.3.3.6 ATRA Content

The ATRA content throughout the 12 weeks of stability study is as shown in Figure 3.11. Formulation stored in both real-time and accelerated chamber showed a declining trend from 100% to 95.8% and 95.4%, respectively. Previous studies encapsulating ATRA in different carriers also found that with increasing temperature, the degradation also increased which is in line with Arrhenius equation that describes the effect of temperature on the reaction rate. As temperature increases, the rate also increases and speeds up the process which in this case is speeds up degradation (Charoenputtakhun et al., 2014; J. Chen et al., 2020; Ghate et al., 2016, 2019). Past studies which encapsulated tretinoin in NLC found no significant different on the entrapped drug content after 4 weeks and 1 year (Ghate et al., 2016, 2019). Although the analysed data showed significant difference ($p < 0.05$) in the changes of the retinoic content, based on guidelines, degradation below 5% in comparison to initial assay is considered acceptable as it is not considered a significant change (ASEAN, 2013; WHO, 1996).

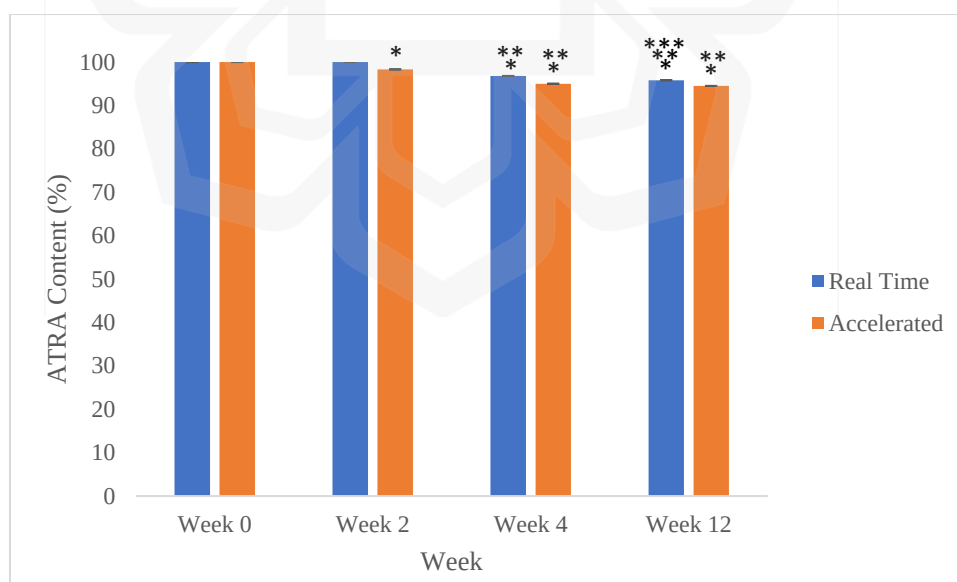


Figure 3.11 ATRA Content of ATRA Loaded Nanosponge Gel-Cream Stored in 30°C/75%RH and 40°C/75%RH for 12 weeks (Mean $\pm$ SD, $n = 3$, $* = p < 0.05$). (Abbreviations: 0w is Week 1; 2w is Week 2; 4w is Week 4; 12w is Week 12, * is significant to 0W, ** is significant to 2W and *** is significant to 4W)

Encapsulation of drug in a carrier has been proven to improve drug stability. ATRA has benefitted from encapsulation in different carriers whereby in a prior study the photostability of ATRA in NLC was assessed under UV lamp for 6 hours, which found that NLC prevented the degradation of ATRA in comparison to ATRA in methanolic solution (Asfour et al., 2019). Encapsulation of ATRA in a phospholipid/zein hybrid nanoparticle was also able to protect ATRA from UV induced degradation compared to its free form when subjected to UV for 360 minutes (Gagliardi et al., 2021). Different drugs that have been entrapped in nanosponge was also able to improve the drug stability. Clobetasol propionate entrapped in nanosponge had less degradation compared to pure clobetasol propionate when subjected to UV for 60 minutes (Kumar et al., 2021). The stability of piroxicam loaded nanosponge was assessed at 4°C, 25°C and 40°C for 3 months; and the results at 4°C and 25°C resulted in no significant degradation of piroxicam (Gaber et al., 2023). Butenafine has also been loaded into nanosponge whereby the loaded nanosponge was subjected to a stability study at 25°C and 50°C for 3 months. A drug release study was done at the beginning and the end of the stability study discovered that the changes in the release profile obtained a similarity factor ($f_2=75$). A value of f_2 between 50-100 indicates that the release profiles are considered similar (Ahmed et al., 2021). ATRA can benefit immensely from a drug carrier in order to improve its stability, whilst the use of nanosponge has been proven time and time again to improve stability of different drugs that has been loaded into nanosponge. From the results of this study where the degradation of ATRA is minimal over three months goes to show that the entrapment of ATRA in nanospoge was able to improve the stability of ATRA

3.3.3.7 Microbial Limit Study

Microbial limit study was done to ensure that there is no microbial growth or contamination of the formulation which is imperative part of stability study (Ilomuanya et al., 2018). The gel-cream formulated has an aqueous phase which makes the formulation more susceptible to microbial contamination (Simões et al., 2018). From

Table 3.5 it can be concluded that there was an absence of microorganism contamination in the gel-cream formulation. At four time points over 12 weeks the formulation was tested and proved to be free from microbial contamination. The antimicrobial agent or preservative of choice in this formulation is Euxyl-PE contributed to the microbial stability of this formulation. Euxyl-PE is a combination of phenoxyethanol and ethylhexylglycerin. Phenoxyethanol is a well-tolerated preservative with a wide spectrum of antimicrobial activity useful in preventing products from spoiling and losing its efficacy. It is effective against gram positive and; gram negative bacteria as well as yeast (Dréno, 2017). It's combination with ethylhexylglycerin makes it more effective as ethylhexylglycerin ruins the interfacial tension of microorganism cell membrane giving way for phenoxyethanol to act more efficiently (Langsrud et al., 2016).

Table 3.5 Microbiological Test Results of Formulation Containing Retinoic Acid Loaded Nanosponge Stored in 30°C/75%RH and 40°C/75%RH at Week 0, Week 2, Week 4, and Week 12.

Microbiological Tests	Storage Condition	Retinoic Acid Loaded Nanosponge Gel-cream			
		W0	W2	W4	W12
<i>S. aureus</i>	30°C/75%RH	X	X	X	X
	40°C/75%RH	X	X	X	X
<i>P. aeruginosa</i>	30°C/75%RH	X	X	X	X
	40°C/75%RH	X	X	X	X
TAMC	30°C/75%RH	X	X	X	X
	40°C/75%RH	X	X	X	X
TYMC	30°C/75%RH	X	X	X	X
	40°C/75%RH	X	X	X	X

Abbreviation: W0 is Week 0; W2 is Week 2; W4 is Week 4; W12 is Week 12; X is absent

3.3.4 Antimicrobial activity

Figure 3.12 shows the plate with the three different formulations tested against *C. acnes*. As seen from the image, placebo and nanosponge formulation showed a zone of inhibition of 12mm while the commercial formulation did not show inhibition towards *C. acnes*. Although both commercial and nanosponge formulation contain retinoic acid, the results were opposite of one another. Instead, the nanosponge formulation obtained a result similar to the placebo formulation whereby the similarity in these two formulations is the use of the same base gel-cream.

Prior studies done did not found ATRA to be effective against *C. acnes* on its own. However, acne formulations combining ATRA and other ingredients such as benzoyl peroxide, chitosan, clindamycin and erythromycin were found to have antimicrobial activity against *C. acnes* (Khoshbakht et al., 2020; Leyden, 2012; Ridolfi et al., 2012; Soni & Bharadwaj, 2020). A study that determined the ability for ATRA to reduce *C. acnes* discovered that the reduction of the bacteria is in concomitant with the reduction of sebum on the skin (Leyden & McGinley, 1982). This may indicate that the action of ATRA against *C. acnes* is indirect. The nanosponge and placebo formulation shows a slight action against *C. acnes* which may be a result of the preservative used in this study which is EUXYL-PE (phenoxyethanol & ethylhexylglycerin) which may have activity when combined with other ingredients in the formulation. A study tested formulations with a combination of preservatives which include phenoxyethanol and ethylhexylglycerin and it was found to have quite an extensive activity against several types of bacteria including *C. acnes* (Pinto et al., 2021; Wang et al., 2019).

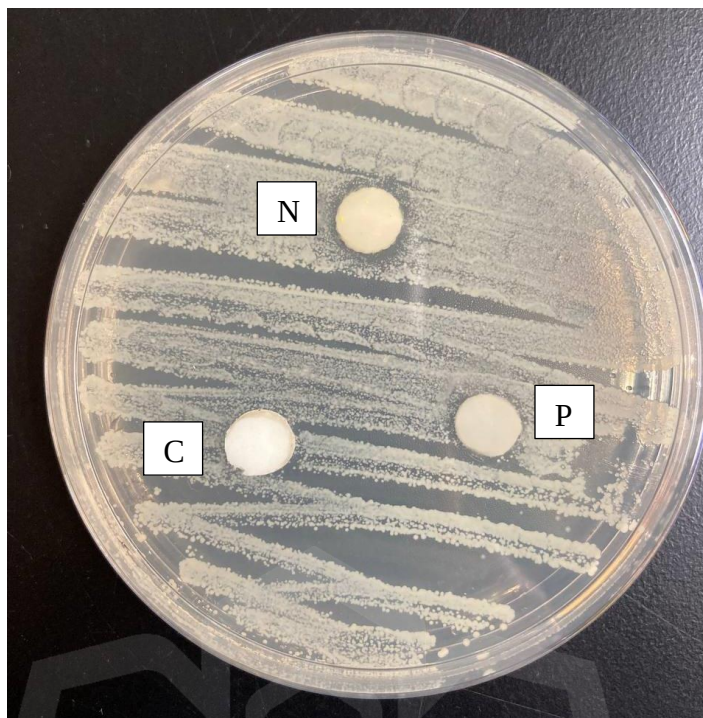


Figure 3.12 Zone of inhibition of ATRA against *C. acnes* (P; placebo, N; nanosponge, C; commercial).

3.4 CONCLUSION

The initial cream developed was proved to be stable and have acceptable organoleptic and physical properties, pH, zeta potential and rheology profile. Physically the formulation appearance has an off-white colour, smooth and shiny texture, with no odour. The pH is acidic suitable for skin application, a highly negative zeta potential far from the agglomeration threshold and has a non-Newtonian flow. However, the particle size was considerably large (495.37 nm) for an effective delivery to the skin layers. Moreover, its polydispersity index was initially near to 0.7 and continued to increase after some time. The large size and high polydispersity increased the risk of instability as the occurrence of Ostwald ripening is more common with large particle size. Due to these problems, optimization was done where the homogenization duration was increased and several speed of homogenization at 2000 rpm, 3000 rpm and 4000 rpm was tested on the formulation. Data obtained showed that homogenization at 3000 rpm for 15 minutes produced gel-cream formulation with the best characteristics. The

particle size reduced to 209.53 nm while PDI was at 0.318. The particle size and PDI obtained was ideal to maintain stability at longer periods of time and most importantly the size is suitable to effectively deliver drug to the skin layers.

Stability study was done for a duration of 12 weeks in 2 chambers, real-time (30°C/75%RH) and accelerated (40°C/75%RH). Throughout the stability study the pH slightly reduced to become more acidic, yet still suitable for skin application. The particle size increased slightly in the real-time chamber. Whereas the sample in the accelerated chamber increased at a rate slightly higher than the real-time chamber. The polydispersity index remained fairly the same for formulation in the real-time chamber and increased slightly in accelerated chamber. The zeta potential in both conditions became more negative. The ATRA content showed slight reduction over time below 5% which is in accordance with stability guidelines. Lastly TYMC, TAMC and specific microbial test for staphylococcus aureus and pseudomonas aeruginosa showed no growth. In a nutshell, the development and optimization of gel-cream with ATRA loaded nanosponge produced a stable formulation with acceptable characteristics after subjected to a 12-week stability study in two different conditions.

CHAPTER FOUR

EX VIVO, IN VIVO, AND HISTOLOGY STUDY OF ATRA LOADED NANOSPONGE GEL-CREAM ON RAT SKIN

4.1 INTRODUCTION

Assessment of percutaneous absorption is important in ensuring the quality and safety of drug whilst also obtaining a more in-depth behaviour of a drug or formulation. Franz cell diffusion study is a validated tool and one of the most well-known tools to determine penetration and retention of topical formulation. Franz cell not only assess skin permeability, but it also provides crucial insights into the relationship between skin, drug and formulation which is highly useful in the development of a novel formulation (Iliopoulos et al., 2020; Ng et al., 2010). The advantage of Franz diffusion cell method includes reduced tissue handling, no continuous sample collection and low amount of drug needed for analysis (Salamanca et al., 2018). Franz diffusion cell study is designed to mimic skin environment in terms of pH, temperature and solutions which are tested over a membrane either a dialysis membrane, synthetic skin substitute or real skin. As it models the skin environment, it can reproduce *in vivo* data in the laboratory using a robust model, hence the reason this study is favoured (Franz, 1975). The continuous flow cell and the static cell are two different kinds of diffusion cells. The continuous flow cell can determine the rate of clearance as the receptor medium is constantly pumped through the receptor chamber replicating *in vivo* blood flow. On the other hand, a static flow cell aids in assessing the flux, concentration, and uptake of a substance into a membrane. Static flow cells can be used for studies with infinite or finite doses (Benson & Watkinson, 2012). A large dose is applied to the membrane in an infinite dose study so that donor depletion is minimal, presuming no change in drug concentration throughout the experiment. While finite dose studies use small dose in the donor chamber to simulate *in vivo* or the amount that would be used in practical applications (Bartosova & Bajgar, 2012).

Determination of the irritation potential of an active ingredient or novel formulation on animal model is vital in order to gain insights on its safety while also understanding the behaviour and effects of the formulation on the skin. Skin irritation as defined by the Globally Harmonized System of Classification and Labelling of Chemicals is the occurrence of reversible damage to the skin occurring after exposure to a substance or mixture (REACH, 2006). Typically, skin irritation is characterized by the appearance of redness, itching or pain in the area that was in contact with the product. Draize skin irritation test is one of the tools known to assess skin irritation based on observation after application of product by scoring the appearance of erythema and oedema (Draize et al., 1944). Histopathology is also one of the important outputs of dermal irritation study which can support the findings of physical observations (OECD, 1981). In this chapter all the studies are done on rats or rat skin. The main reason on the usage of rat skin instead of others is because the appropriateness and ease of handling method as the rat is considered to have decent calmness. Plus, rat skin is the most structurally similar with the human skin compared to other rodents' skin (Todo, 2017). The thickness of stratum corneum and epidermis of the rat and human skin can be considered as approximately similar (SC rat=18 μ m, rat epidermis=32 μ m, SC human=17 μ m and human epidermis=36 μ m) (Marquet et al., 2019; Todo, 2017).

4.2 METHODOLOGY

4.2.1 Materials

The rat used in this study was white Sprague Dawley rats obtained from A Sapphire Enterprise, (Selangor, Malaysia). Ethical approval number was obtained from Institutional Animal Care and Use Committee (I-ACUC) of IIUM with approval number IACUC-2020-025 (Appendix II). Phosphate buffer saline (PBS) was procured from Sigma Life Science, Sigma Aldrich Co. (St Louis, USA) while the methanol was obtained from Fisher Scientific (Ohio, USA). The chemicals used for HPLC analysis were methanol and glacial acetic acid (Merck, Darmstadt, Germany). Deionized water for HPLC analysis and PBS preparation was obtained from Nex Power 4000 Ultra-Reversed Osmosis Water Purification System equipment (Human Corp., Seoul, Korea).

Dow corning high vacuum grease used in Franz cell was from Sigma Life Science, Sigma Aldrich Co. (St Louis, USA). The commercial cream used in this study was Tretinoin cream 0.05% from YSP Industries Sdn. Bhd. (Selangor, Malaysia). The ingredients of the commercial retinoic acid product consist of stearyl alcohol, polyethylene glycol monostearate, xanthan gum, stearic acid, isopropyl myristate, sorbic acid, butylated hydroxytoluene and titanium dioxide). The *in vivo* study with rats, sodium chloride 0.9% w/w irrigation solution from Ain Medicare Sdn Bhd (Kelantan, Malaysia), Ketamine (Ketamil) and Xylazine (Xylazil-100) brand Ilium from Troy Laboratories Pt Ltd (New South Wales, Australia). Chemicals for histology study include Formaldehyde, Toluene, Hydrochloric Acid, Reagent alcohol (Ethanol), Xylene, Hematoxylin, and Eosin are from Leica Biosystems (Illinois, USA). The DPX mountant for slide preparation was from R&M Chemicals (Selangor, Malaysia).

4.2.2 *Ex vivo*

4.2.2.1 *Receptor Medium & Skin Preparation*

PBS is a commonly used receptor medium due to its physiological pH of 7.4 and suitability for water soluble compounds (SCCP, 2006). PBS contains disodium hydrogen phosphate, sodium chloride, potassium chloride and potassium dihydrogen phosphate (Simon et al., 2016). Preparation of PBS was done accordingly based on the package label with instructions to dissolve a tablet in 200 mL of deionized water. Once the tablet completely dissolved PBS is mixed with methanol at a 2:1 v/v ratio to cater to retinoic acid as it is not water soluble. The prepared receptor medium was degassed using an ultrasonic sonicator for 30 minutes to remove air bubbles (Haque et al., 2017; SCCP, 2006).

The skin was obtained from male Sprague Dawley rats. Dorsal skin is the main area that was used as it is preferable compared to ventral skin if the study is design to correspond with *in vivo* usage (Marquet et al., 2019). The hair of the rats was removed by shaving and depilatory cream application at the required area a day prior to

euthanization. The rats were euthanized with an overdose of ketamine and xylazine which is 5x the normal dose (AVMA, 2020). After the rats were euthanized, the skin was obtained and stored in -20°C freezer immediately. The carcass of the rats was placed in a body bag and labelled for further disposal procedure. Before use for Franz skin study, the skin was thawed in PBS at room temperature for 30 minutes before use (Santos et al., 2020).

4.2.2.2 Franz Cell Apparatus Setup

Permeation of the ATRA was investigated by using static Franz diffusion cell depicted as in the setup in Figure 4.1. The rat skin was cut in a circular shape with a radius of approximately slightly larger than the opening of the receptor chamber. The opening of the receptor chamber has a radius of 0.5 cm with area of 0.79 cm². Dow corning high vacuum grease was applied around the mouth of both donor and receptor chamber to ensure the skin stays in place. The skin was mounted between the receptor and donor compartment using tweezers, then it is secured with a clamp with weight attached to it to stabilize the apparatus.

The receptor medium was filled up to 2.5 mL in a clean and dry receptor cell using an extended pipette tip. Great caution was taken while filling the receptor chamber in order to ensure no air bubbles were introduced. The assembled apparatus was then placed on a magnetic stirrer plate (Thermo Scientific, Massachusetts, USA) submerged in a water bath at 37°C (Fisher Scientific, Ohio, USA). The apparatus was left for 30 minutes to allow the receptor medium and the skin to equilibrate to the surrounding temperature (Santos et al., 2020). Temperature of the skin was maintained at 32°C by keeping the temperature of the apparatus at 37°C (SCCP, 2006). Temperature of the skin was inspected from time to time using a temperature probe. Magnetic stirrer (Fisher Scientific, Ohio, USA) placed in the receptor chamber was set at 300 rpm to maintain constant temperature and ensure homogenous distribution throughout the receptor medium.

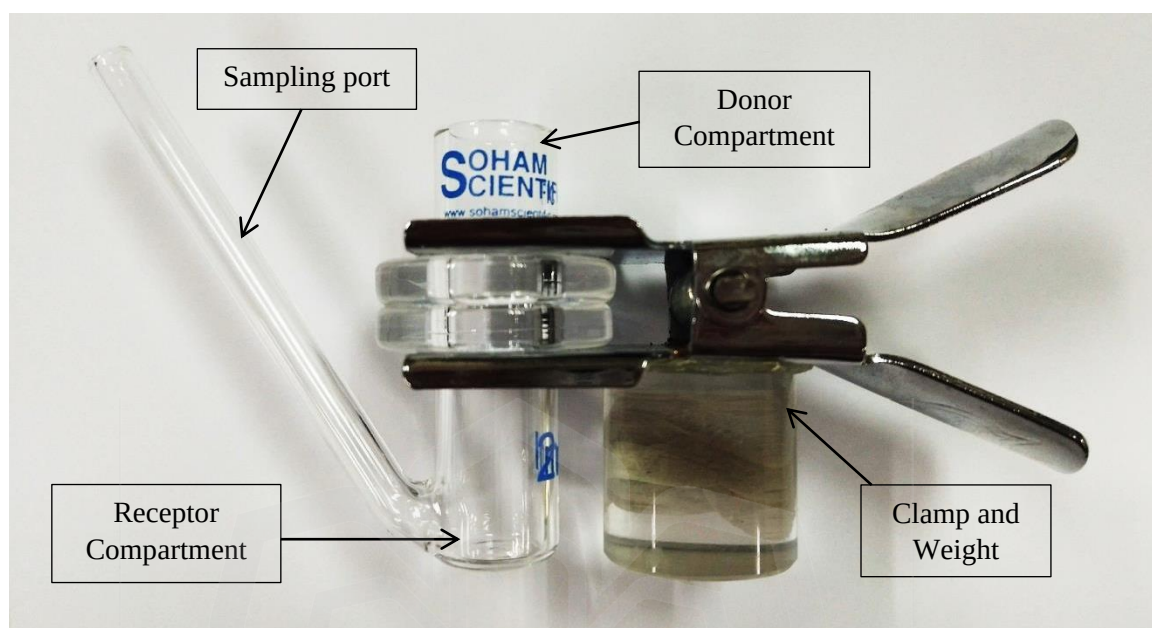


Figure 4.1 Franz Cell Apparatus Set Up

4.2.2.3 Franz Cell Study

Franz cell study was done using 8 membranes as the minimum number of replicates for acceptable data is 8 (OECD, 2019) and 6 (SCCP, 2006). The formulation was placed on the skin in the donor chamber and aliquots (300 μ L) of the sample were taken at time intervals 0.5, 1, 2, 3, 4, 5, 6, 8, 12 and 24 hours. The same amount of receptor medium was replaced after each sample is taken to keep the volume constant and maintain sink conditions (Simon et al., 2016). Samples were kept in labelled Eppendorf tubes and stored in -20 C freezer until HPLC analysis. A finite study was done to replicate *in vivo* application of the formulation where ATRA in the donor compartment was expected to deplete over time due to permeation or evaporation (Benson & Watkinson, 2012; Lau & Ng, 2017). 10 mg/cm² of the formulation was applied on to the membrane/skin and distributed homogenously using a small spatula. The donor top was not occluded to

expose the membrane to ambient condition. The receptor arm however, was occluded with parafilm to prevent evaporation of receptor fluid (Selzer et al., 2013).

4.2.2.4 Washing and Extraction

After completion of permeation study, the skin was washed and extracted to determine skin retention of ATRA. A washing solution of methanol: water (50:50) was prepared for washing and extraction. Washing was conducted a total of three times where the skin was rinsed with 1 mL of the washing solution. The rinsing step was repeated 5 times using the same solution before being transferred to a labelled Eppendorf tube (Haque et al., 2017). The skin was then cut into small pieces and soaked in methanol for 24 hours to allow extraction to take place. The samples were placed in an orbital shaker for 24 hours at 32°C with continuous shaking at 150 rpm. Both the washing and extraction samples were sonicated for 15 minutes and centrifuged another 15 minutes at 32°C with a speed of 12000 rpm. The supernatant was then collected, filtered, and analysed with HPLC.

4.2.2.5 Data Analysis

Data from HPLC was calculated to determine the cumulative amount of ATRA permeated (mg/cm^2) and flux of ATRA ($\text{mg}/\text{cm}^2/\text{h}$). A graph of cumulative amount of ATRA permeation was plotted against time. The equation to calculate cumulative amount is as written in equation 4.1. The washings and extractions from the skin were also analysed and the data obtained was calculated to plot a graph on drug recovery.

$$Q = \frac{\{C_n V + \sum_{i=1}^{n-1} (C_i S)\}}{A}$$

Equation 4.1

Whereby, Q is the cumulative amount of the compound released per surface area membrane ($\mu\text{g}/\text{cm}^2$), C_n is the concentration of compound determined at n^{th} sampling interval ($\mu\text{g}/\text{mL}$), V is the volume of individual Franz diffusion cell, $\sum_{i=1}^{n-1} (C_i)$ is the sum of concentration of compound determined at sampling intervals 1 through n-1 ($\mu\text{g}/\text{mL}$), S is the volume of sampling aliquot (mL) and A is the surface area of sample orifice.

Flux ($\mu\text{g}/\text{cm}^2/\text{hr}$) was calculated by dividing the amount of ATRA at a specified time with its duration. Recovery within the range of 85% - 115% (SCCP, 2006) 90% - 110% (OECD,2019) should be obtained from the dose applied. The recovery was calculated from the equation below where T is total recovery, P is permeation, W is washings and E is extraction.

$$T = P + W + E$$

Equation 4.2

4.2.3 *In vivo*

4.2.3.1 *Study Procedure*

A total of 12 rats was used and divided into four groups of 3 rats with different topical application. Group 1 was applied with ATRA loaded nanosponge gel cream formulation, group 2 with placebo formulation, group 3 with commercial ATRA product and group 4 with saline for control. Sample size was calculated using resource

equation which was based on the degree of freedom of analysis of variance (ANOVA) and also considering SCCS guidelines where a maximum of 3 rats per group is allowed (Arifin & Zahiruddin, 2017; European Comission, 2012).

For the weekly skin assessment, the rats were anaesthetized with ketamine (100mg/kg) and xylazine (10mg/kg) intraperitoneally. Before the study began, hair was removed by shaving and application of depilatory cream. Hair removal was also done weekly with the depilatory cream prior to skin testing days. Formulation was applied once daily for 21 days (Hamishehkar et al., 2015; OECD, 1981). Dorsal skin is the main area that was used as it is preferable compared to ventral skin if the study is designed to correspond with *in vivo* usage (Marquet et al., 2019). All the housekeeping of the rats was practiced in animal house, Kuliyah of pharmacy, IIUM Kuantan, Pahang. After 21 days, the rats were euthanized and skin from the test area was excised for further study. The carcass of the rats was placed in a body bag and labelled for further disposal procedure (AVMA, 2020).

4.2.3.2 Animal handling

The rats that were used in this study were male Sprague-Dawley rats, which were at the age of 57-70 days and which is in the phase of young adulthood and mature enough to be used for skin experiments (Mccutcheon & Marinelli, 2009). The weight of the Sprague-Dawley rats used were approximately 200-300g. The respective Sprague-Dawley rats was housed in cages separately and was fed with conventional lab diet and unlimited water supply. The rats were kept in the laboratory for one week before the start of research for acclimatization purposes. The surrounding temperature was kept at 22°C±3°C with controlled humidity of 50-60% and hygienic condition (OECD, 1981; OECD, 2004). The bedding of the rats was changed weekly. The management of the rats was monitored by experts to avoid any harm towards the animal.

4.2.3.3 Data Collection

Photos were taken weekly under the same light source to monitor signs of erythema and oedema. The Draize score for skin irritation was utilized to examine for any signs of oedema or erythema daily (S. H. Lee, 2013; Reisman et al., 2014). The Draize skin score is as shown in Table 4.1. The score was then calculated using primary dermal irritation index (Iris Betsabee et al., 2017). Further assessment with Dermalab combo was also used to assess the hydration, erythema, and skin barrier weekly. The description on the Dermalab Combo can be found in section 5.2.4.

Table 4.1 Draize irritation skin score

Formation of erythema and flaking	Grade	Formation of oedema	Grade
No erythema	0	No oedema	0
Slight erythema	1	Slight oedema	1
Defined erythema	2	Minor oedema	2
Moderate to severe erythema	3	Moderate oedema	3
Grave erythema and formation of pressure sores	4	Severe oedema	4

4.2.3.4 Data Analysis

All data gathered throughout the study obtained from all four groups were analysed with Graphpad prism 8. The data was analysed using repeated measures ANOVA to see if there was any significant difference in the parameters measured weekly over the span of the study. For a more detailed analysis, Tukey's and Sidak's multiple comparison test was also employed whereby the Tukey's test compared the means between weeks

within the same formulation, while Sidak's test compared the means of different formulation for each week.

4.2.4 Histology Study

4.2.4.1 Fixation, Dehydration, Clearing and Wax Infiltration

Specimen for this study was obtained from the previous irritation study on Sprague Dawley rats. After euthanasia the skin where formulation was applied was excised and soaked in 10% formalin solution. The formalin was prepared by mixing 1 part of formaldehyde with 9 parts distilled water. The excised skin was left to soak 24 hours to allow penetration into every part of the tissue. This step is very important as it preserves the tissue and provides protection for the following steps. Dehydration is an important step to ensure all water is removed from the specimen to allow paraffin wax to infiltrate the tissue. The process involves soaking the tissue in several increasing concentrations of ethanol to slowly replace the water within the specimen. The tissue soaked in formalin was taken out, cut to an appropriate size, and then placed in labelled cassettes to segregate them. The specimens were then soaked in 50%, 70%, 80%, 90% ethanol of 1 ½ hour at each concentration followed by three consecutive times in 100% ethanol for 1 hour each.

After removing as much water as possible, the specimen was prepared for wax infiltration. The ethanol in the tissue is not miscible with wax, hence the ethanol needs to be replaced with an intermediate solvent that can replace ethanol and then be replaced with wax. This is called the clearing step, and the clearing agent that was used in this step was toluene. Other than replacing ethanol, toluene is also important to ensure fat is removed from the tissue as much as possible for better wax infiltration. Specimens were soaked 30 minutes in a 1:1 mixture of ethanol and toluene and finally soaked overnight (12 hours) in 100% toluene (Feldman & Wolfe, 2014). The step-by-step process of dehydration and clearing is as shown in Figure 4.2

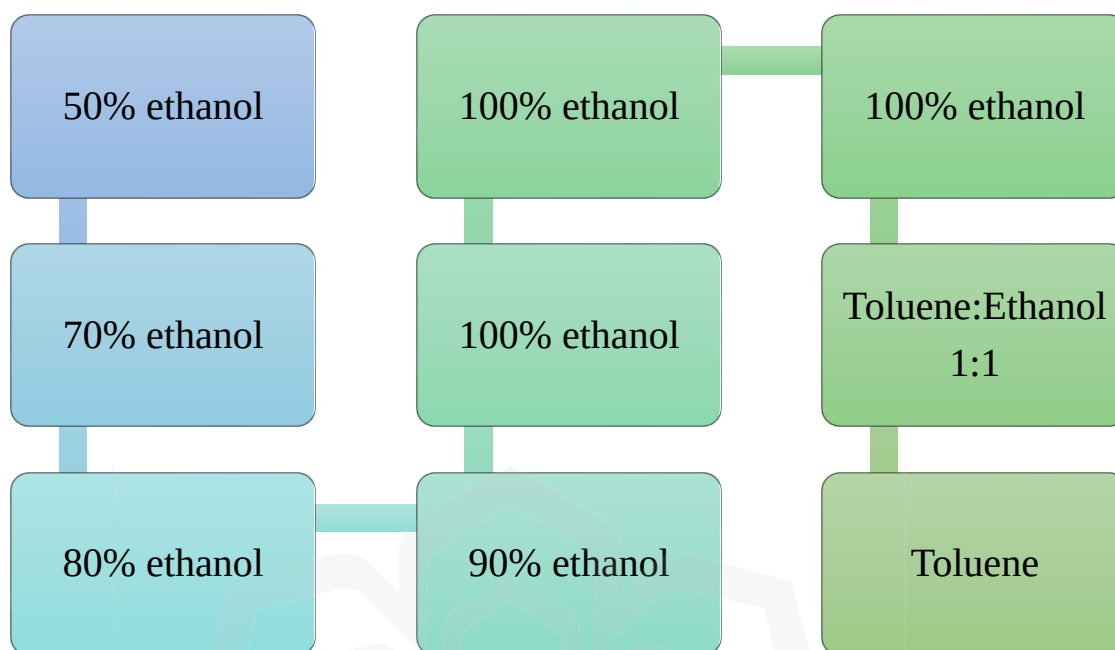


Figure 4.2 Step by Step Order of Skin Tissue Soaking for Dehydration and Clearing Process

Once the specimen has been completely dehydrated the specimen is ready for wax infiltration. Wax was melted in an oven at 60°C in three separate beakers. The specimens were soaked in each beaker. The specimens were soaked for 30 minutes, followed by another 30 minutes and a final 45 minutes. Changing beakers and solution instead of just soaking in one beaker for 1 hour and 45 minutes is important to ensure all the clearing agent is replaced with wax as much as possible.

4.2.4.2 Embedding, Microtomy and Slide Preparation

Once the specimen has thoroughly been infiltrated with wax, the skin tissue was embedded into a solid wax block to ease the cutting process later. The embedding process is done on a tissue embedding station (Leica Biosystems EG1160, Illinois,

USA) complete with a paraffin reservoir, paraffin dispenser, cassette bath for the specimens, mold warmer to prepare the blocks, a work surface as well a cold plate. The paraffin tank, cassette bath, mold tray and work surface were set at 60°C while the cold plate was set at -5°C and the paraffin flow rate was set at 80%. The skin tissue is placed into a mold at the desired orientation suitable with the cross section to be investigated later. The paraffin tank was filled with sufficient paraffin wax for the mold preparation. Melted wax was filled into the mold while ensuring the skin tissue maintains the desired orientation. Once fully filled, an embedding ring was placed on the mold, and more wax is filled to the brim. The molds are made out stainless steel to ease the block removal while the embedding rings are made of plastic which acts like a handle to hold the mold. The filled mold is placed on a cold plate to solidify.

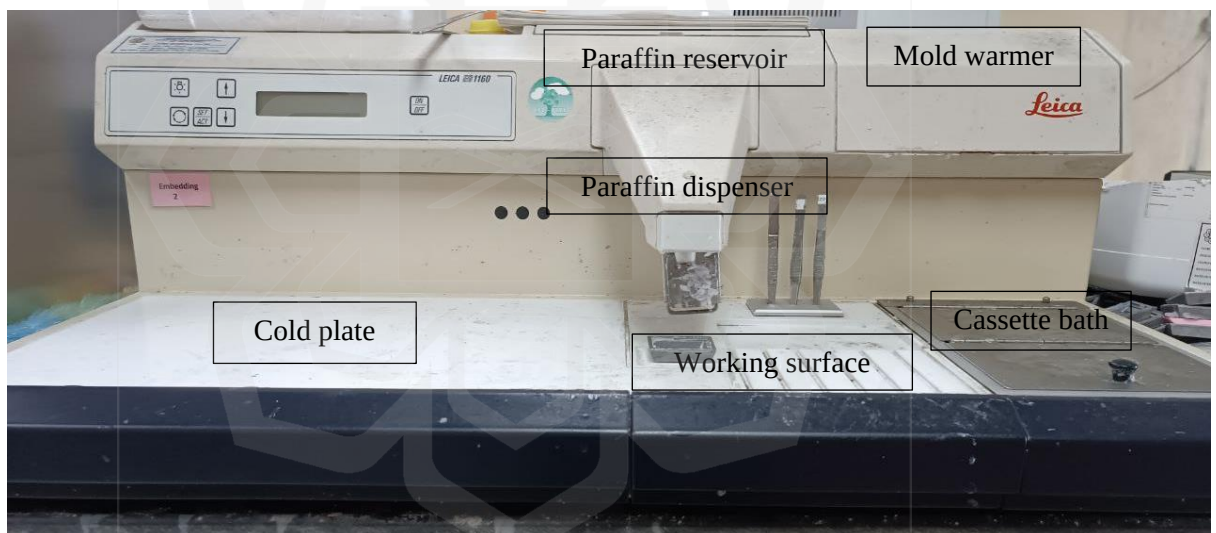


Figure 4.3 Tissue Embedding Station

The solid block removed from the mold was then clamped onto the manual rotary microtome from Leica Biosystems model RM2135 (Illinois, USA) to prepare ribbons. The microtome blade was replaced with a new one prior to the cutting process. The clamp was adjusted to meet the blade and produce thin cross-sections of the specimen. The ribbons are then cut into individual cross sections (paraffin sections) which was then floated in a warm water bath model Leica HI1210 (Illinois, USA). The

warm water bath was set at 40°C to help the tissue smooth out and flatten fully when mounted onto a slide. The paraffin section was mounted on the slide by dipping the slide in the water bath, slanting it at an angle that helps adhesion of the paraffin section onto the slide. The mounted slide was then dried on a flattening table from Leica biosystems model HI1220 (Illinois, USA) set at 30°C and left for 24 hours.

4.2.4.3 Staining

For the staining procedure, the slides were placed in a staining rack and transferred to a different working chamber filled with chemicals required for the staining procedure. First and foremost, the slides were dipped in xylene to remove excess paraffin, followed by ethanol and diluted ethanol to rehydrate the specimen on the slide and prepare it for the aqueous stain. Once rehydrated the slides were rinsed with water and then transferred to the hematoxylin stain. Excess hematoxylin was rinsed with water and then transferred to an 1% acid alcohol to remove nonspecific hematoxylin stain and finally rinsed with water once more before being soaked in eosin stain. The slides were once again dehydrated with diluted ethanol followed by pure ethanol and then cleared with xylene. Finally, a drop of DPX mountant was dropped on the slide and a coverslip was placed on top. Slides prepared were then observed under microscope (Cardiff et al., 2014; Feldman & Wolfe, 2014). The steps are as depicted in Figure 4.4

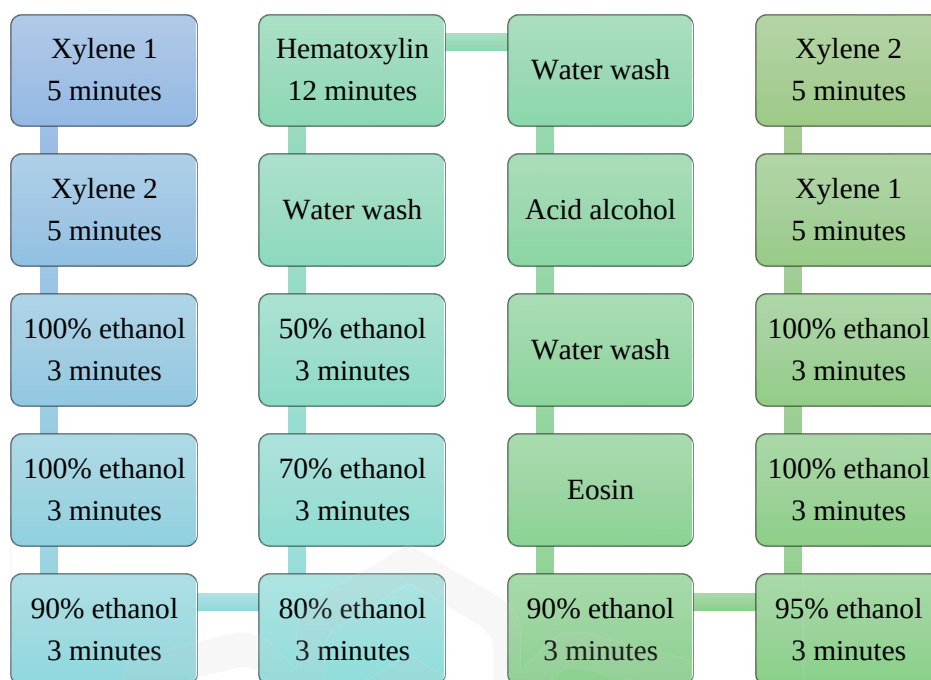


Figure 4.4 Hematoxylin and Eosin Staining Steps

4.3 RESULTS AND DISCUSSION

4.3.1 *Ex vivo* Skin Permeation and Retention Studies

4.3.1.1 *Cumulative Amount of ATRA*

Figure 4.5 shows the cumulative amount of ATRA from commercial formulation that permeated the rat skin in 24 hours where the amount does not increase proportionally over time. The total amount of ATRA permeated from the total dose applied was 68.35%. The graph depicts a plateauing effect whereby the permeation slowly reduces as a result of dose depletion which is a distinctive trait of a finite study. In a finite study the dose applied is typically determined at a normal skin application dose which will deplete over time. The ATRA encapsulated in nanosponge on the other hand may have permeated an infinitesimal amount after 24 hours and could not be plotted.

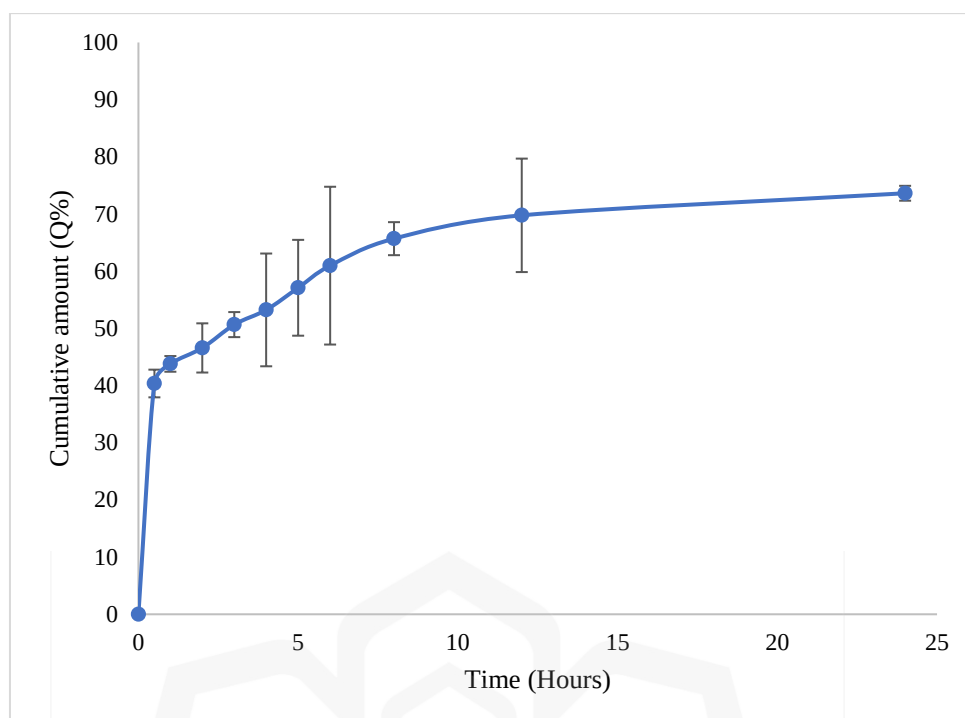


Figure 4.5 Estimated Cumulative Amount of ATRA ($\mu\text{g}/\text{cm}^2$) from Commercial Formulation Permeated Over Time (Mean $\pm$ SD, $n = 8$)

Prior studies that encapsulated ATRA in nanostructured lipid carrier and nanocapsule did not detect or found negligible values of ATRA in the receptor fluid compared to its commercial counterpart which is desirable for the treatment of acne as we want the action to remain within the skin layers (Ebrahimi et al., 2021; Ghate et al., 2019). Other studies that encapsulated ATRA in SLN and NLC which did lead to permeation into the receptor fluid discovered that the release of ATRA became slow and prolonged (Arantes et al., 2020; Ghate et al., 2016). The amount of drug release within the stipulated time was also much lower compared to the marketed formulation which released 100% of the drug by the end of the study (Ghate et al., 2016). These results align with the findings of this study which shows ATRA from commercial formulation permeating into the receptor chamber while the nanosponge formulation did not. Permeation into the receptor fluid is not ideal as physiologically this may indicate that ATRA can enter the blood stream.

4.3.1.2 Flux Profile of ATRA

The flux profile on Figure 4.6 depicts the flux of ATRA permeation from commercial formulation. Flux demonstrates the rate of a particular compound passing through a membrane/skin at a certain period. The flux peaked at 0.5 hour and started depleting gradually up to 24 hours. The reducing flux reflects dose depletion of ATRA which is as expected for a finite dose study.

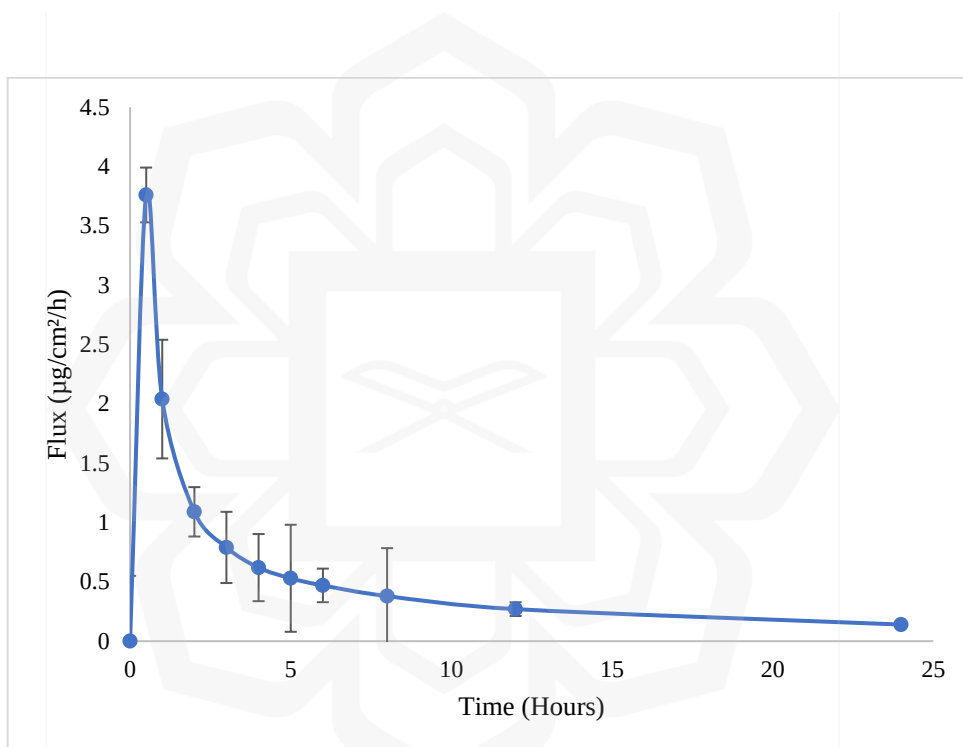


Figure 4.6 Mean Flux of ATRA (µg/cm²/h) from Formulation
(Mean ± SD, n = 8)

4.3.1.3 Total Recovery of ATRA

The total recovery recorded for commercial formulation is 93.03% while nanosponge formulation recorded a total of 94.17%. The recovery obtained for both formulations are in alignment with ranges suggested by both the Scientific Committee on Cosmetic

Product (SCCP, 2006) and Organization for Economic Cooperation and Development (OECD, 2004) which is 85-115% and 90-110%, respectively. Total recovery is an important tool in finite study to enhance permeation data's validity. The values are usually calculated from the amount of drug permeated, skin washings as well as skin extraction. However, for nanosponge formulation, the recovery was calculated from skin washings and skin extraction only. The results are as shown in Figure 4.7

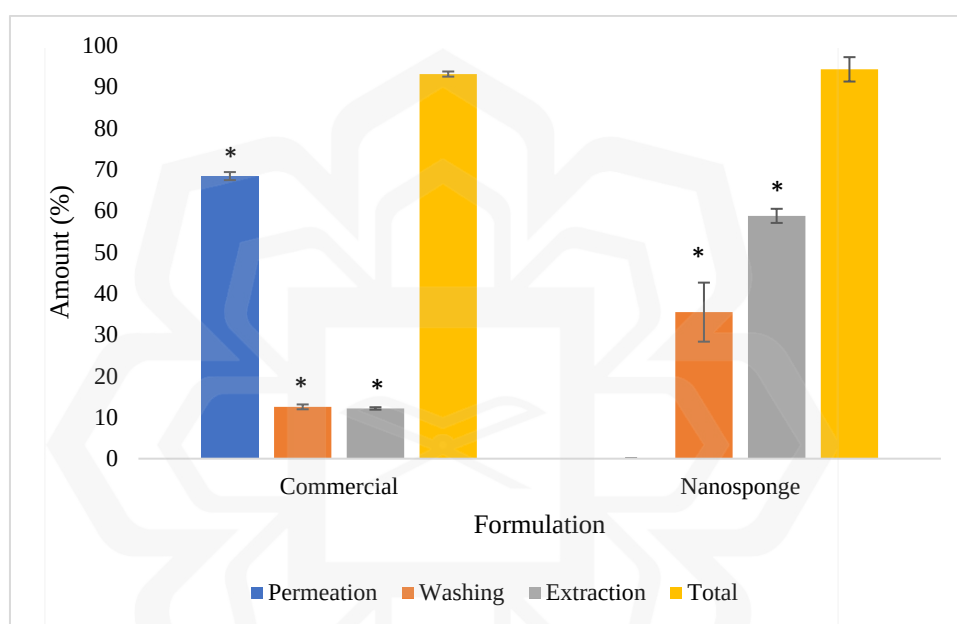


Figure 4.7 Total Amount of ATRA Recovered from Franz Cell Study (Mean $\pm$ SD, n = 8, * = $p < 0.05$)

As can be seen from the graph, permeation contributed a major percentage of drug recovery for commercial formulation compared to nanosponge formulation where the permeation was unable to be calculated. Contrary to permeation, nanosponge formulation had higher amounts of drug recovered from washings and extractions compared to commercial formulation. As for the total drug recovered, both obtained values within the stipulated guidelines. The differences between commercial and nanosponge group were found to be significant ($p < 0.05$) for permeation, washings, and extraction while the total recovery did not show any significant difference.

The major percentage of recovery in commercial formulation came from the permeation (68.35%) while for nanosponge formulation the major percentage came from the skin extraction (58.73%). This indicates that nanosponge formulation mostly remained within the skin layers while commercial formulation permeated into the donor compartment which reflects permeation into blood stream. The high skin retention of drugs in nanoparticles, as evidence in previous studies, is typically attributed to the nanosized particle that facilitate drug penetration through the stratum corneum and in to the deeper layers of the skin (Rapalli et al., 2023; Yokota & Kyotani, 2018). Furthermore, the extended duration of drug retention within the skin layers can be attributed to the gradual release of the drug from nansoponges, effectively slowing down the drug's movement across the skin as it is steadily released.

Previous research also discovered that the amount of drug extracted from the skin was much higher in the NLC formulation compared to the commercial formulation by almost double the value (Ghate et al., 2019). Entrapment of ATRA in novel drug delivery system was proven to retain drug within the dermal layers longer with minimal permeation ensuring the drug acts within the skin and lessen the occurrence of side effects. Skin retention combined with the slow and prolonged release show that entrapment causes a sustained release effect.

4.3.2 *In vivo*

4.3.2.1 *Erythema*

Erythema is a skin reaction that causes the skin to redden as a result of external stimuli or irritants such as chemical substance, allergens or UV (Abdlaty et al., 2021; Khosrowpour et al., 2019). The erythema baseline value for control group was at 1.733 and at W3 2.467 (MD=-0.733), for commercial group, the baseline was 1.667 and at W3 was 7.900 (MD=-6.233), for placebo group the baseline was 2.033 and ended at W3 with 3.33 (MD=-1.300) and finally nanosponge group had a baseline of 2.033 and

ended at W3 with 2.167 (MD=-0.133). The commercial group has the greatest difference between means while nanosponge group has the lowest. The erythema values were compared between groups whereby it was discovered that, in W1, the commercial group has a significant difference to control, placebo and nanosponge group ($p < 0.05$). The values of commercial group were once again significantly different to control, placebo and nanosponge group at W3 ($p < 0.05$). Compared to baseline (W0), there is a significant difference in control group between W0 and W2, in placebo group between W2 and W3, in commercial group between W0 to W1 and W3 and between W2 and W3 ($p < 0.05$).

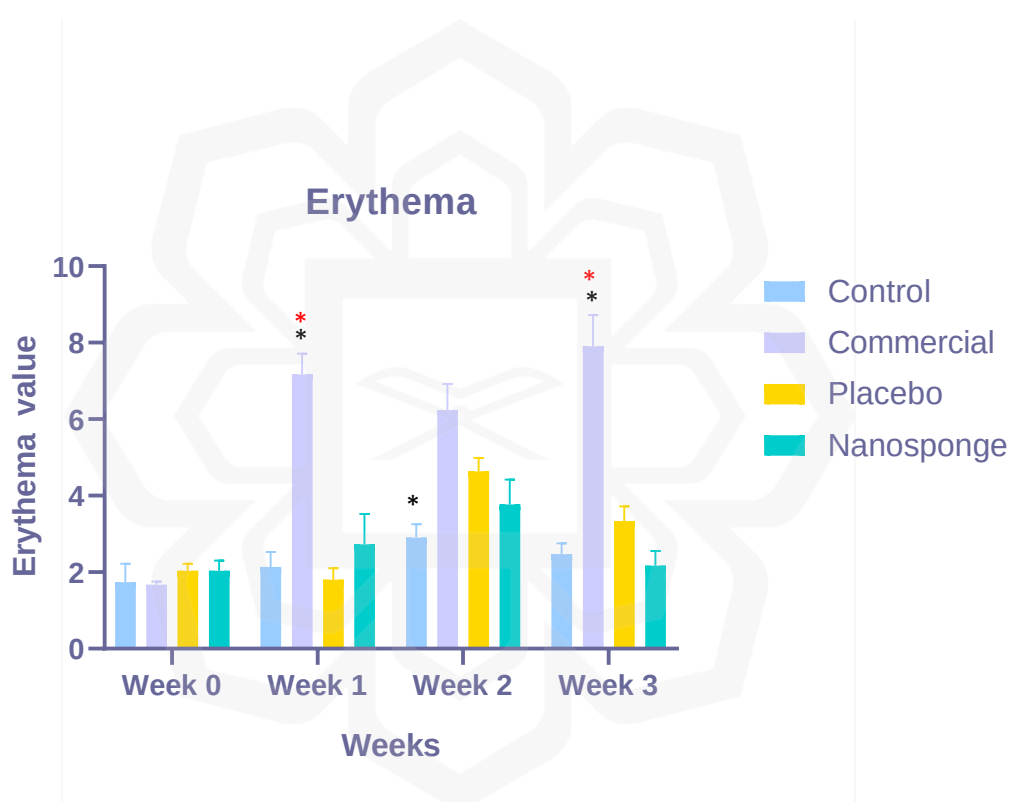


Figure 4.9 Erythema values measured from four groups (Control, Commercial, Placebo, Nanosponge) at different time intervals (Mean $\pm$ SEM, $n=3$, * = $p < 0.05$, * compared between formulations, * compared to baseline)

In general, it is evident in Figure 4.9 commercial formulation has higher values of erythema from W1-W3 compared to other groups and compared to its baseline. ATRA has been known to have several side effects or also known as retinoid dermatitis.

It is clinically characterized by dryness, peeling, erythema, scaling, oedema, stinging, burning, pruritus and photosensitivity. Erythema is one of the more common side effects that frequently occur (Harper et al., 2019; Ogé et al., 2019). Therefore, it can be postulated that significant increase in erythema value in the commercial group is due to retinoid dermatitis. Nanosponge group which also contains ATRA did not show a significant increase in erythema value which may indicate the effectiveness of nanosponge in reducing side effects associated with ATRA. Erythema also seems to increase from W0 to W2 in all groups. The increase of erythema value in other groups may be due to the repeated shaving which may have irritated the skin and cause redness (Sulzberger et al, 2016).

4.3.2.2 TEWL

TEWL represents the amount of water loss from the skin which also represents the condition of the skin barrier. A higher TEWL value indicates high water loss from the skin which reflects a skin barrier disruption. The skin barrier regulates water loss, helps retain moisture and as the name implies acts as a barrier between our body and external aggressors (Rosso et al., 2016). The results are illustrated in Figure 4.10. The baseline TEWL value for control group started at 4.90 g/m²/h and at week 3 the value was 5.37 g/m²/h (MD=-0.47), for the commercial group, started at 6.93 g/m²/h and ended at 31.13 g/m²/h (MD=-24.20), placebo group started at 5.70 g/m²/h and ended at 6.43 g/m²/h (MD=-0.87) and finally nanosponge group started at 6.46 g/m²/h and ended at 8.00 g/m²/h (MD=-1.60). The highest mean difference is commercial group followed by nanosponge group, placebo group and lastly control group. The Commercial and Nanosponge group has higher values compared to other groups, most likely due to its ATRA content. Placebo and control group both have no active ingredients nor any other ingredient that might irritate the skin hence the negligible difference between means.

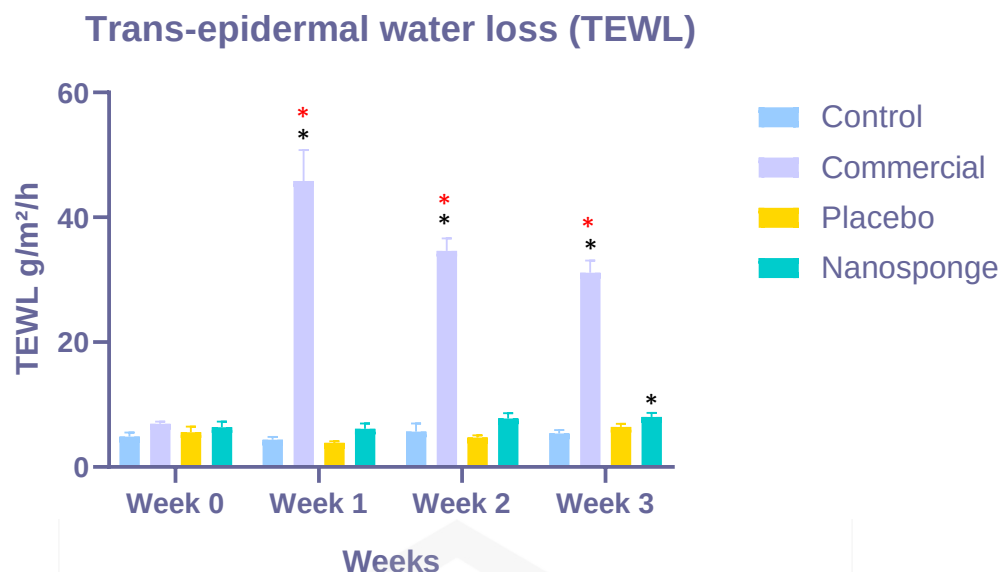


Figure 4.10 TEWL values measured from four groups (Control, Commercial, Placebo, Nanosponge) at different time intervals (Mean $\pm$ SEM, $n=3$, * = $p<0.05$, * compared between formulations, * compared to baseline)

In the Commercial group, W1, W2, W3 shows significant difference ($p<0.05$) to W0 of the same group. Nanosponge formulation also showed significant difference at W2 when compared to W0 ($p<0.05$). Commercial formulation shows significantly higher values compared to control group, placebo group & nanosponge group ($p<0.05$). From Figure 4.10 and data analysis, it is clear that the commercial formulation causes a significantly higher TEWL values which reflects the skin barrier dysfunction caused by ATRA. The side effects associated with ATRA such as dryness, erythema, rash, and scaling are all signs of skin barrier dysfunction (Hubbard et al., 2014; Szymański et al., 2020). In section 4.3.2.2, the elevated erythema values, a known side effect of ATRA, align with the observed increase in TEWL values, signifying a compromised skin barrier condition. Nanosponge group also showed an increase in TEWL value evident in W2, yet the increase is not as high as conferred by the commercial group which may be contributed by the entrapment of ATRA in nanosponge. These findings emphasize that the application of ATRA can have adverse effects on the skin. In contrast, the

entrapment of ATRA within nanosponges appears to mitigate such side effects offering a more skin-friendly approach.

4.3.2.3 Hydration

The changes of value over time and the effects different formulation were significant to the overall data ($p < 0.05$). The hydration value recorded over the span of 21 days for control group started with baseline 330.8 μS and ended with value 194.2 μS (MD=136.6), commercial group started with baseline 256.2 μS and ended at 215.6 μS (MD = 40.60), placebo group starts with a baseline 261.4 μS and ends at 222.9 μS (MD=38.53), and lastly nanosponge group started at baseline 285.1 μS and ended at 195.3 μS (MD=89.77). The highest mean difference is seen with control group which may be due to the application of normal saline on its own. A form of emulsion which is also an emollient was applied on other groups which may contain occlusives and humectants. These ingredients may facilitate skin hydration by binding water into stratum corneum, aids moisture retention by occluding water in the skin and concurrently combats skin dryness (Proksch et al., 2019).

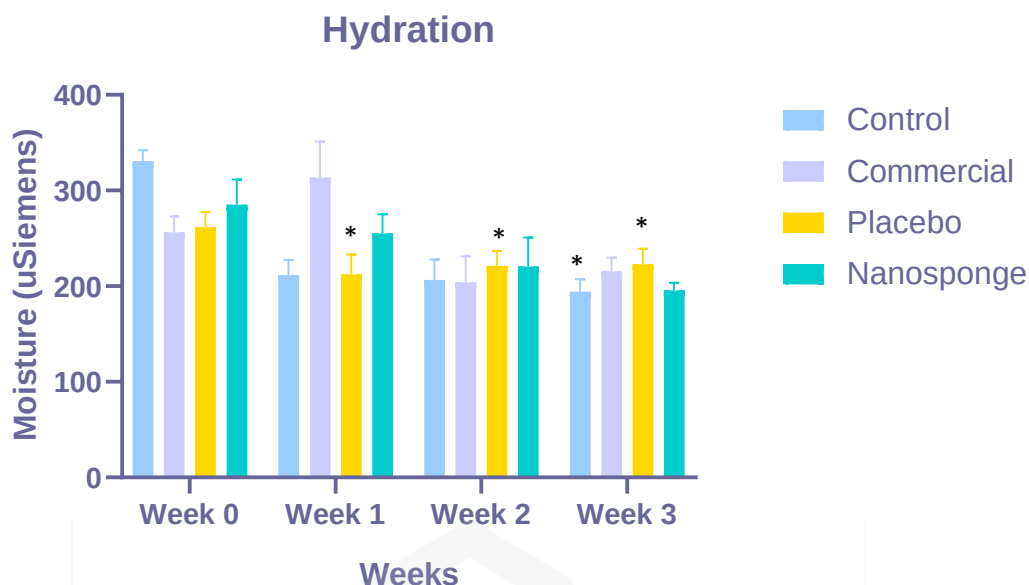
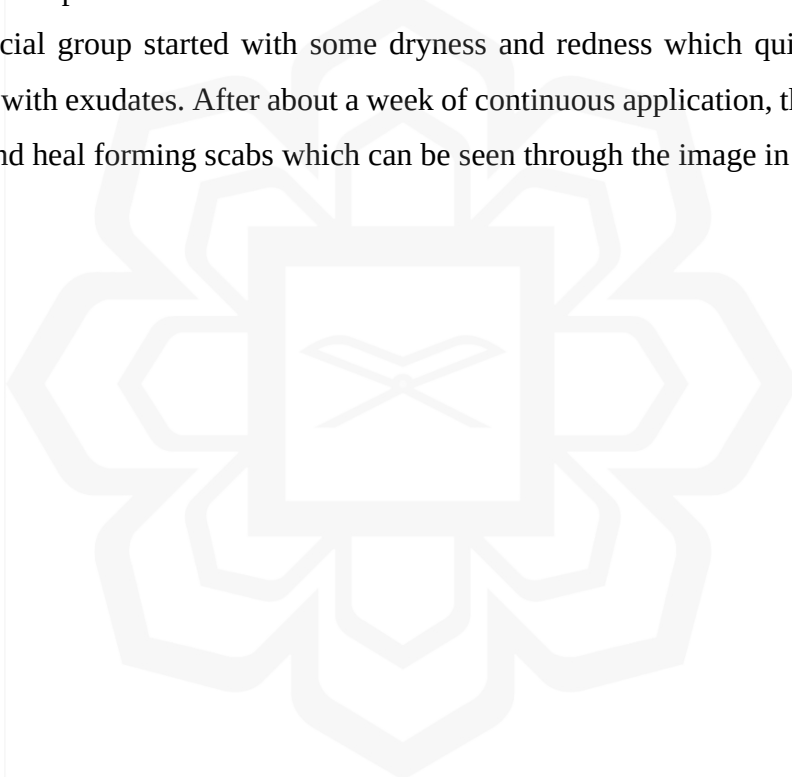


Figure 4.8 Hydration Values Measured from Four Groups (Control, Commercial, Placebo, Nanosponge) at Different Time Intervals (Mean $\pm$ SEM, $n=3$, * = $p<0.05$, * compared between formulations, * compared to baseline)

When compared to W0, control group shows significant difference in W3, while placebo group shows significant difference in W1, W2, W3 ($p<0.05$). The results are as shown in Figure 4.8. Decreased hydration in all groups maybe due to shaving induced irritation symptoms which include dryness, redness, stinging, tightness and itching (Cowley & Vanoosthuyze, 2012). Shaving induced irritation can be caused by either superficial skin abrasion caused by interaction of the skin with the shaver's cutter or; a neurogenic skin inflammation caused by mechanical stimulus applied on the skin that transmits signal from the perifollicular skin through sensory nerve ending (Maurer et al., 2016; Rietzler et al., 2016). An exception to W1 of commercial group, which shows increase in hydration maybe caused by wounds on the skin which has exudates (Spear, 2012). Hydration shows no significant difference when each formulation was compared to different groups each week ($p>0.05$). As hydration is influenced by individual variations which in this case is the amount of water intake, it can be difficult to see a particular trend or obvious significance between groups or within weeks (Akdeniz et al., 2018).

4.3.3 Irritation Potential and Histopathological Evaluation

The formulation was applied daily to the rats for a total of 21 days. The images in Figure 4.11 show the skin condition of the rats in each group after the duration of the study. The observations made reflect the results obtained from the weekly Dermalab combo skin analysis. The observation for the control and placebo grouped maintained similar throughout the study, while the nanopsonge group did show signs of dryness with the appearance of skin flakiness towards the end of the study yet after 2 days was not visible anymore. It is also clear that skin barrier of the rats on the commercial group were clearly disrupted as there are wounds visible even at the end of the study. The commercial group started with some dryness and redness which quickly turned into wounds with exudates. After about a week of continuous application, the wound started to dry and heal forming scabs which can be seen through the image in Table 4.2.



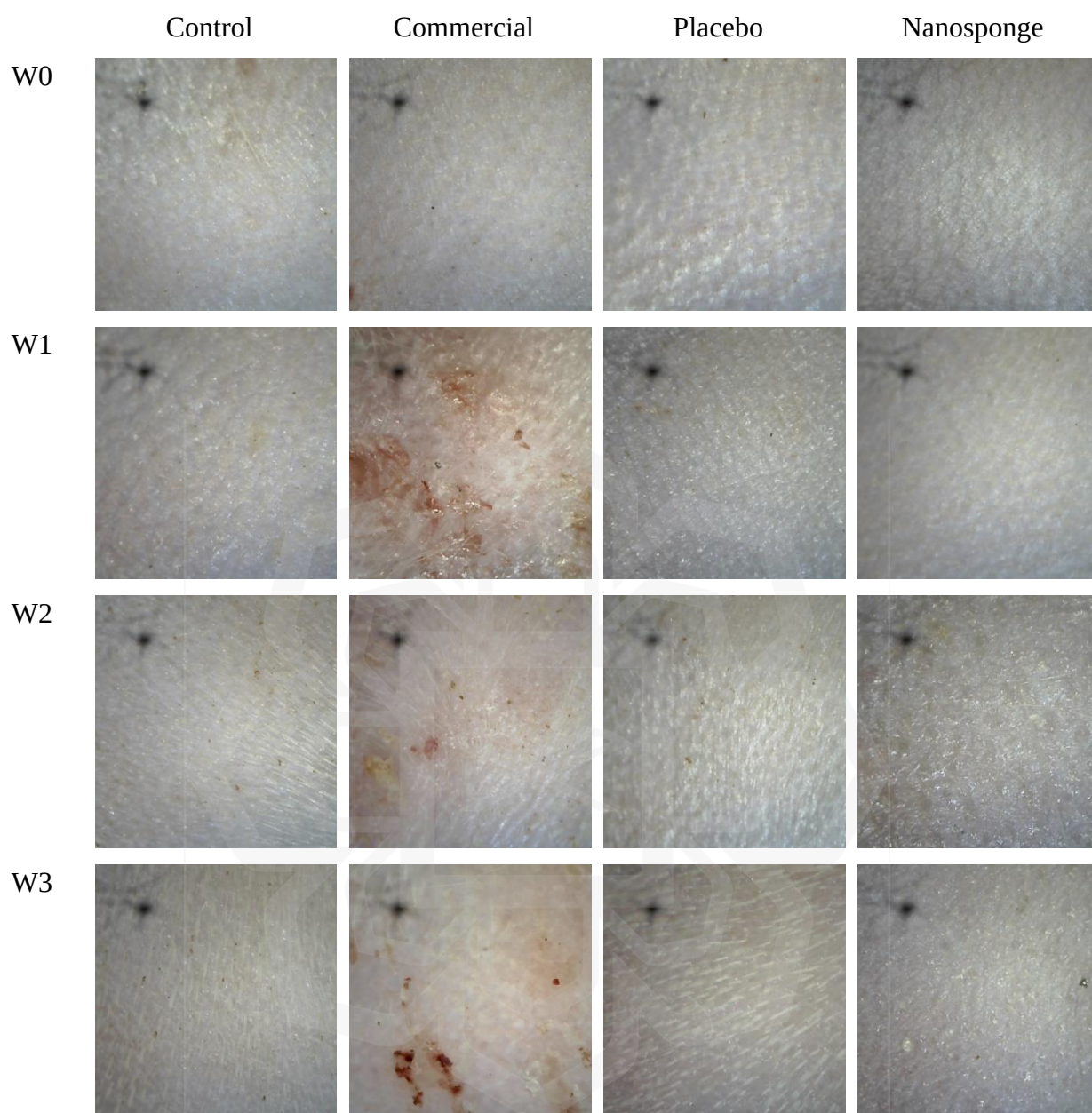
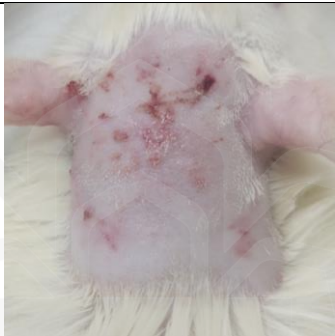
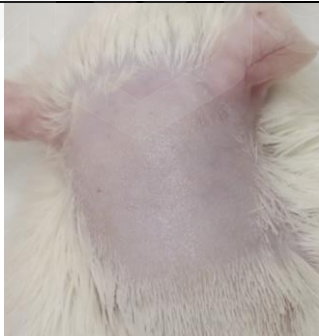


Figure 4.11 Weekly Image of Rat Skin According to Groups as Captured with Videoscope

Table 4.2 Rat Skin Condition After 21 Days of Formulation Application

<i>Group</i>		<i>Observation</i>
Control		No changes observed throughout the study period.
Commercial		Shown dryness on D1, peeling D2, wounds started to appear on D3.
Placebo		No changes observed throughout the study period.
Nanosponge		Shown visible dryness in the final week but disappeared after 2 days.

The Draize skin irritation score was utilized in this study to assess the irritancy of the formulation. Draize skin score was applied to the first 72 hours of the study, where the skin was observed at 24, 48 and 72 hours (Iris Betsabee et al., 2017; OECD, 1981). The results are presented in Table 4.3.

Table 4.3 Observation Recorded for 72 Hours Based on Draize Skin Irritation Score.

Commercial group		Observation period			Mean
		24h	48h	72h	
Rat 1	Erythema	1	2	3	2
	Oedema	0	0	0	0
Rat 2	Erythema	1	3	3	2.33
	Oedema	0	0	0	0
Rat 3	Erythema	1	3	3	2.33
	Oedema	0	0	0	0

The score was calculated with the primary dermal irritation index (PDII) which is the addition of the mean of erythema to the mean of oedema. The equation is shown below.

$$PDII = x^- erythema + x^- edema$$

Equation 4.3

The calculated scores for PDII was categorized as shown in the following Table 4.4 (Iris Betsabee et al., 2017).

Table 4.4 Scores for the Primary Dermal Irritation Index

Primary dermal irritation index	Category
0.0 – 1	Non-irritant
1.1 – 2	Slightly irritant
2.1 – 5	Moderately irritant
5.1 - 6	Severe moderated irritant
6.1 - 8	Severe irritant

The scores for Control, Placebo and Nanosponge group were 0.0 which is considered as non-irritant. The Commercial group, however, obtained a score of 2.22 which is scored as moderately irritant. This finding is similar to a previous study which also found the commercial product to be moderately irritant while their formulation entrapped in nanostructured lipid carrier shows similar results to its placebo counterpart which did not cause erythema to the skin (Ghate et al., 2019). The results using PDII are mainly based on the erythema scores as during the study period, observation done found that there was no visible oedema. Hence, a calculation with Simplified irritation index (SII) which is driven by only one of the observed effects which is erythema was calculated. The equation is as shown below.

$$SII_{SKIN} = \frac{\sum ERYTHEMA \text{ at } 24/48/72h \text{ of } n * \text{ animals}}{3 \times n *}$$

Equation 4.4

Whereby, 3 is the number of timepoints and n* is the number of animals calculated for the score which is 2 out 3 in this study. The results are then categorized and arranged to correspond with Classification, Labelling and Packaging (CLP) and Globally Harmonised System (GHS) skin irritant classification as shown in Table 4.5

(Charmeau-Genevois et al., 2021). These are systems under United Nations (UN) are used to identify hazardous chemicals. From the calculation the SII calculated for commercial group is 2.33 which is categorized as a skin irritant.

Table 4.5 SII Irritant Classification Corresponding to CLP and GHS Classification.

SII range	CLP Interpretation	GHS Interpretation
0 and < 1.5	Not irritant	Not irritant
1.5 and < 2.3	Not irritant	Potentially irritating
2.3 and < 3	Irritant	Irritant
3 and 4	Corrosive	Corrosive

After 21 days of the irritation study, the rat skins were excised for histology assessment. The histology images are as depicted in Figure 4.12. The control group appeared normal without any histopathological changes. Normal histological observation of skin layers such as thin epidermis and dermis layer, hypodermis with subcutaneous fatty tissues lined with subcutaneous muscles along with other normal cytological elements were seen through the image. The hair follicle, sebaceous gland, are also evident in the dermal layers. The placebo group also showed normal histological observations with well-defined layers and did not show any signs of inflammation.

For the commercial group, signs of inflamed skin were observed through the presence of epidermal thickening, vasodilation, oedema, infiltration of inflammatory cells as well as hyperkeratosis. Lastly the nanosponge group did show some signs of inflammation where epidermal thickening can be seen as well as infiltration of inflammatory cells (Asfour et al., 2019). Hyperkeratosis is the thickening of the stratum corneum while epidermal thickening is the increase in thickness of the epidermis or also known as hyperplasia which all occur as a response due to chronic inflammation.

Oedema can be seen through the increase in gaps between cells while vasodilation is the dilated appearance of the blood vessels. Increase in inflammatory cells infiltration can be seen especially in the commercial group from the appearance of small black spots indicating the presence of inflammatory cell such as neutrophils and macrophages (Sharma et al., 2019).

The results are similar to previous studies with ATRA in penetration enhanced vesicle whereby the entrapped formulation did not cause a severe inflammatory reaction when compared to commercial formulation or ATRA formulation with no carriers which induced more severe inflammatory reaction seen through the appearance of inflammatory signs. (Asfour et al., 2019; Bavarsad et al., 2016). A study entrapping ATRA in nano-lipoidal carrier also obtained similar results whereby ATRA encapsulated in phospholipid is well tolerated on mouse skin compared to the marketed product (Raza, Singh, Lohan, et al., 2013).

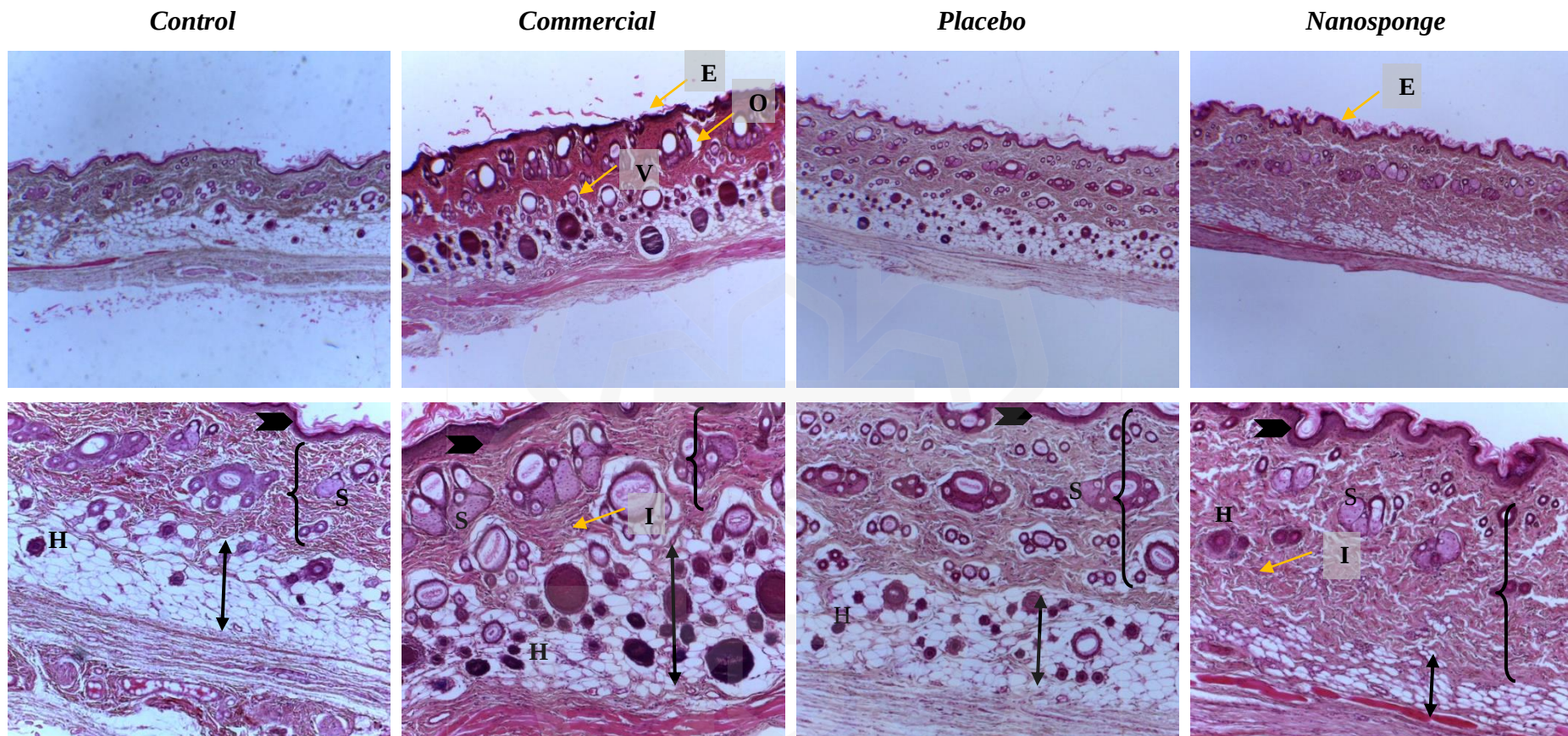


Figure 4.12 Rat Skin Histology After Control, Commercial, Placebo and Nanosponge Formulation Applied for 21 Days Whereby Signs of Inflammation Can Be Seen in Commercial and Nanosponge Group. Yellow arrows: signs of inflammation, arrowhead: epidermis, bracket: dermis, double ended arrow: hypodermis, E: epidermal thickening, O: oedema, V: vasodilation, I: infiltration of inflammatory cells, H: hair follicles, S: sebaceous glands

Previous studies also found results similar to our findings although encapsulating in different carriers. Entrapment of ATRA prevents direct exposure of ATRA to the skin by reducing the contact of the acidic group (COOH) of ATRA with the stratum corneum and alleviating the irritating side effects. The sustained release effect helps in decrease dose dumping which also reduces irritation and increase the dermal tolerability (Ebrahimi et al., 2021; Ghate et al., 2019). Additionally, franz cell permeation studies have indicated that ATRA exhibits a notable affinity for retention within the skin layers over a 24-hour period. This observation suggests a controlled and gradual release of ATRA, resulting in minimal systemic circulation. This slow-release mechanism has been associated with a reduction in the occurrence of potential side effects, as supported by observations, evaluation of irritation potential and histological assessment in rat models. TEWL and erythema both of which directly reflects the impact of ATRA on the skin barrier, exhibited fewer side effects when using the ATRA loaded nanosponge formulation in comparison to the commercial formulation. Thus, the incorporation of ATRA loaded nanosponge in gel-cream improves the skin's tolerance to ATRA, consequently promoting better patient compliance.

4.4 CONCLUSION

Ex vivo testing using Franz cell diffusion was done in order to investigate ATRA permeation behaviour and retention behaviour. A finite study using static flow cell was done using rat skin from male Sprague Dawley rats were used. Conditions were set to best mimic *in vivo* skin environment. The study was done both on ATRA loaded nanosponge gel-cream formulation and a commercial marketed formulation both at a strength of 0.05%. Results showed that commercial formulation permeated into the receptor fluid at a higher amount and rate compared to the nanosponge formulation. While the nanosponge formulation was found to retain more ATRA in the skin compared to the commercial formulation. ATRA loaded nanosponge gel-cream formulation obtained favourable results less permeation means that it most likely won't be absorbed systemically. Its high skin retention allows more ATRA to act at the target

site which is within the skin layers. These results also reflect the capability of nanosponge to provide slow release.

Irritation study on animal model using the same rat species was also done for 21 days. The rats were divided into four groups namely the control group, commercial group, placebo group and nanosponge group. The formulation was applied daily whereby daily observations and weekly skin test were done. For the first 72 hours the skin was observed for any sign of erythema and oedema and translated into scores using the Draize skin test, from there the PDII and SII were calculated. The results for nanosponge gel-cream were similar to control and placebo group indicating the formulation did not irritate the rats. However, the commercial formulation was categorized as moderately irritant based on the PDII and irritant based on SII. Observation throughout the study however did show some dryness in the nanosponge group towards the end of the study. The skin assessments showed significantly high TEWL and erythema value for the commercial formulation, opposite to the nanosponge formulation which shows result almost similar to control and placebo group. The histology was also done after 21 days. Observation under the microscope found that the control and placebo formulation appeared normal like a healthy skin cross section. The commercial formulation showed multiple signs of inflammation while the nanosponge formulation was also found to be slightly inflamed.

In a nutshell, these results point out that the nanosponge formulation was able to diminish the side effects that are well known to ATRA. The successful entrapment of ATRA in the nanosponge delivered through gel-cream formulation was able to give sustained delivery to the skin without systemic absorption. As a result, the formulation was able to improve skin tolerance towards ATRA while still delivering effective dose to the skin. Patient compliance toward the use of topical ATRA could be improved through this form of drug delivery system.

CHAPTER FIVE

ATRA LOADED NANOSPONGE GEL-CREAM FORMULATION STUDIES ON HEALTHY HUMAN SKIN

5.1 INTRODUCTION

Although Franz cell studies and irritation studies on animal give necessary comprehension on the mechanisms and behaviour of ATRA on the skin, *In vivo* studies on human are still as important. Application of *in vivo* studies whether on human can provide data and proof on the efficacy along with the safety of the formulation (Abd et al., 2016). Permeability of skin varies among species, where animal skin is more permeable compared to human skin. This along with other physiological differences in terms of metabolism, lymphatic and circulation system make human studies unable to be completely replaced with animal studies (Todo, 2017). The insights provided through human skin is valuable in predicting realistic conditions of formulation/drug in applications.

Skin also differs individually and can easily be affected by many internal and external factors hence recruitment of many participants will help gain more perceptions on how similarly and differently a drug acts within a group (Dąbrowska et al., 2018). This study assesses the skin using Dermalab combo which is a non-invasive skin assessment. It is advantageous and useful as it is not harmful and also cost effective. Being able to monitor the effects of formulation over time on the skin and assess it quantitatively can prove the benefits of a certain treatment or condition without bias. Observation with the naked eye can be perceived very differently from one person to another. Another plus point in human studies is the ability of gaining appraisal on personal inclination of each participant on the types of formulation they prefer. Patient adherence is very important in medicine as it reduces a lot of cost on healthcare burden. Therefore, providing treatment tailored to patient's predisposition is indispensable (Palmeira-de-Oliveira et al., 2022; Stegemann et al., 2016). That is why in this study the main aim is to prove that nanosponge formulation reduces side effects associated

with retinoic acid on human skin as well as discover the acceptance and inclination of participants towards a gel-cream formulation.

5.2 METHODOLOGY

5.2.1 Study Design and Setting

The study design that was an *in vivo* study which evaluated the effects of a gel-cream formulated with ATRA loaded nanosponge on healthy human skin compared to commercial ATRA formulation. This study combined both quantitative and qualitative methods to achieve its objective. For the quantitative approach, a skin condition assessment was done using DermaLab® Combo. The DermaLab® Combo consists of several probes that measured the erythema, melanin content, hydration, TEWL and skin elasticity of each participant. The qualitative approach on the other hand was done throughout the course of the study where the participants were given a questionnaire. The questionnaire included items which allowed the participants to report any adverse effect throughout the period of using the formulation and at the end of the study their opinion on the organoleptics properties of the formulation was also evaluated to determine consumer acceptability. The setting of the study was done in Dermatopharmaceutics Lab, Kulliyyah of Pharmacy, International Islamic University Malaysia, Kuantan, Pahang. Ethical approval was obtained from IIUM Research Ethics Committee (IREC) with approval number IREC 2021-059 (Appendix III). Research was also registered under National Medical Research Register (NMRR) with NMRR identification number NMRR ID-22-01827-IAS.

5.2.2 Study Participants

The participants of this study were recruited individually by approaching them personally one by one. The study was explained in detail to the potential participants. If they were willing to be a participant, an informed consent form was given to them.

They were given a few days to read through and reconsider before returning the informed consent form indicating their agreement. Participants were not forced into being a subject for this study. The number of participants required in the skin assessment study is 15 people tested on both hands making a total of 30 test sites. Based on past skin studies, the subject ranged from 10-30 people (Ryu et al., 2015; Teeranachaideekul et al., 2013; Watanabe et al., 2014).

In this study, we chose 30 subjects to obtain a significant result with minimum number of subjects. There are different ways to get an estimate of the sample size based on the type of analysis, power, effect size and alpha level. With a power of 0.8, an alpha value of 0.05, a beta of 0.2 and an estimate of results, the sample size calculated was a total of 12 subjects with 6 per group (Kane, 2019). Calculation including an effect size of 1 came up with a total of 28 subjects with 14 per group (AI-Therapy Statistics, 2023). A nomogram for sample size estimation using effect size and power had a value of 32 (Serdar et al., 2021), meaning 16 per group which is similar to normal approximation using the Z statistic calculation (Kohn & Senyak, 2021). As the study is non-invasive with application of commercialized topical product and nanosponge formulation that has been tested in animal study, the study proceeds with a total of 30 participants. The subjects were divided into two groups of 15. One of the groups received ATRA loaded nanosponge formulation while the other group received commercial formulation. There were inclusions and exclusions criteria to recruit the participants as tabulated in Table 5.1.

Table 5.1 Participants' Inclusion and Exclusion Criteria for the Study

Inclusion Criteria	Exclusion Criteria
▪ Normal skin condition ▪ Able to comply with daily use of the test product ▪ Has no active skin diseases ▪ Willing to stop using any product at the studied area. ▪ No damaged skin around test site	▪ Having any skin disease such as eczema, psoriasis or severe acne ▪ Had any skin disease in the last 3 months and was treated with medication. ▪ On any medication or medical product that may affect skin's response ▪ Exposed to sun excessively. ▪ Pregnant or planning to get pregnant. ▪ On any drugs that causes photosensitivity

5.2.3 Study Procedure

Participants were selected and asked to read and understand the informed consent form before signing the consent form indicating their agreement to participate. Once the informed consent forms were returned by the participants, they were briefed again regarding the study and how they contributed to the study. The briefing emphasized possible risks of the study and their right to withdraw at any point of the study. A week before the baseline skin condition assessment is done, participants were asked to stop using any products other than basic shower creams or soap at the volar area of the arm. This was to ensure that the baseline reading is not affected by the use of any topical skin products such as creams and lotions.

Baseline skin condition assessment was done with DermaLab® Combo probes as detailed out in section 5.2.4. Right after the baseline measurement, participants were given the gel-cream either containing the ATRA loaded nanosponge or a commercial formulation to be applied once daily during night-time at the volar area of the forearm.

A bottle of formulation was given weekly to the participant for 4 weeks study duration. The duration of four weeks is in line with the turnover time of stratum corneum layer which is around 2-4 weeks (Egelrud, 2000; Gordon, 2013). Participants applied 5 mg/cm² of formulation at an area of 30 cm². Participants were advised to withdraw 0.15 mL using a needle-less 1mL syringe for application on the designated study site. To ensure that the formulation was applied at the same area each time, as well as to make sure that the tested area has been applied with the formulation, a tool illustrated in Figure 5.1 was provided to the participants to aid the process. Using the tool, an exact area can be positioned to apply the formulation and to test different parameters with Dermalab combo. The participants were asked to come to the laboratory for a weekly skin condition assessment for 4 weeks on the same day each week after the baseline measurement to evaluate the effects of the formulation.

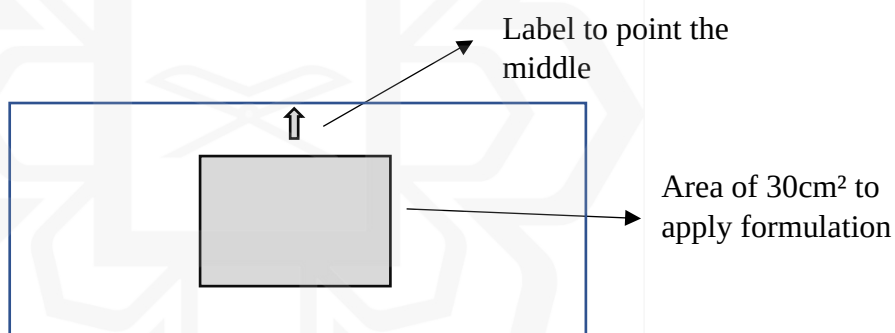


Figure 5.1 Tool for Participants to Apply Formulation

5.2.4 Weekly Skin Assessment Data Collection

Data collection was done through weekly skin evaluation. The skin assessment was done using DermaLab® Combo (Dermalab® Series Skinlab Combo), manufactured by Cortex Technology. Assessment of the skin was done on the volar aspect of the forearm between the wrist and the elbow whereby the probe was placed on the studied area.

Tests took an average of about 10 minutes for each participant. The parameters assessed were as follows:

5.2.4.1 Skin Colour Probe



Figure 5.2 Skin Colour Probe

The skin colour probe as shown in Figure 5.2 was used to measure the melanin content and erythema of the skin. The probe was calibrated using the white surface of the equipment calibrator. Next, assessment was done at three different areas within the study site to get an average value. Erythema values measured through this probe was compared to baseline values of each participant to detect any unusual redness. Based on the melanin values measured, results were classified according to melanin index as shown in Table 5.2.

Table 5.2 Classification Based on Melanin Content (Cortex Technology, 2012)

Melanin	Melanin index
Celtic type (I)	0 – 20.0
Caucasian white (II)	10.0 – 30.0
Mixed type (III)	20.0 – 50.0
Mediterranean type (IV)	30.0 – 60.0
Asian type (V)	40.0 – 70.0
Black type (VI)	60.0 – 99.9

5.2.4.2 Hydration



Figure 5.3 Hydration Probe

The hydration probe in Figure 5.3 was utilized to measure the hydration or moisture of the skin through the conducting properties of the upper layer of the skin when exposed to different voltage (Cortex Technology, 2012). The probe was calibrated to ensure accuracy of reading with the equipped calibrator. Then, the pin hydration probe was pressed onto the volar forearm at several times to get an average. Generally, moist skin would have a reading of 300 μ S (microSiemens) and above.

5.2.4.3 TEWL

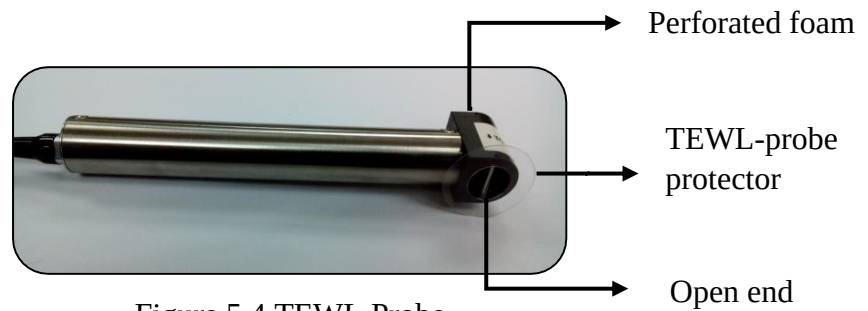


Figure 5.4 TEWL Probe

The TEWL probe in Figure 5.4 on the other hand was used to measure the water loss from the skin. This reflects the condition of the skin barrier through its ability to retain water. There are two sides of the probe, namely the perforated foam which faced up and the open end which is the side that was pressed against the skin. A TEWL- probe protector was attached at the open end to prevent cross contamination. The measurement of TEWL was presented in gram per square meter per hour ($\text{g/m}^2/\text{h}$). Normal skin values would be within the range of 2 – 10 $\text{g/m}^2/\text{h}$.

5.2.4.4 Elasticity

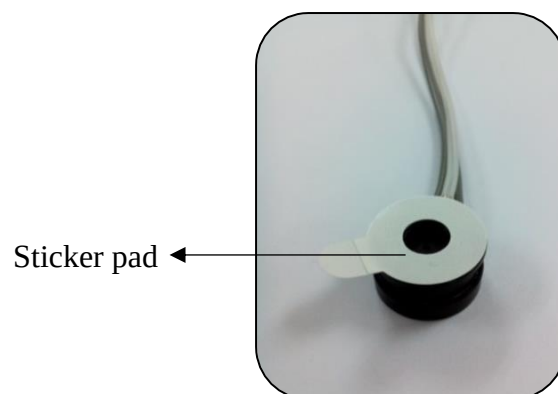


Figure 5.5 Elasticity Probe

The elasticity probe on Figure 5.5 was used to measure the skin retraction time by applying suction on the skin surface. A sticker pad was attached on the surface of the probe then placed on the skin. The normal elasticity score as stated in DermaLab Combo Manual is as follows in Table 5.3.

Table 5.3 Normal Score for Elasticity Parameter (Cortex Technology, 2012).

Age	Normal Score (ms)
20 – 30	300 – 550
30 – 50	600 – 800
60 and above	800 and above

5.2.5 Questionnaire Data Collection

Data collection through questionnaire were divided into three parts. The first part is the evaluation of skin condition where participants assess their skin condition throughout the study. Through this part also the occurrence of adverse events can be monitored. Common side effects of ATRA application are listed along with a arrange to assess each symptoms severity starting from absent, mild, moderate, severe, and very severe. Next part of the questionnaire is related to product stability. Participants are asked to evaluate the condition of the formulations received throughout the study to ensure they did not receive an unstable or contaminated formulation. Finally, the questionnaire evaluates the participants acceptability towards the formulations. Features of the formulation including colour, smell, texture, stickiness, application, cooling, and greasiness are assessed based on how pleasant it was to the participants. The form is attached in Appendix IV.

5.2.6 Data Analysis

Both baseline data and the data gathered after application of the formulation for 4 weeks was analysed using Graph Pad Prism. The data was analysed using repeated measures ANOVA based on data distribution to assess the significant difference in the parameters measured ($p < 0.05$) before and after application of the formulation. Sidak's and Tukey's test was also employed in this data analysis. The data obtained from the form on adverse events and participants acceptability was analysed with ordinal regression analysis where the parameter estimates was reported. All data was compared to the commercial formulation.

5.3 RESULTS AND DISCUSSION

5.3.1 Weekly Skin Assessment

The study started with a total of 15 participants. At week 2, 1 participant dropped out due to irritations and redness experienced on the skin applying commercial formulation. The study continued with 14 participants. At the end of the study several participants also started experiencing some side effects of dryness and irritations. The complaints were gathered through questionnaire and analysed in section 5.3.2

5.3.1.1 Erythema

Sidak's and Tukey's multiple comparisons test was done for all the data obtained from Dermalab weekly analysis. Tukey's test compared between weeks within each formulation while Sidak's test compared the different formulations from week to week. Comparing between the two formulations showed a significant difference on the overall data ($p < 0.05$). The changes of both formulation from baseline to week 4 within the group itself was not significant. However, when the comparison was done between the

two formulations week to week, there were significant difference at W3 and W4 ($p<0.05$). From the graph in Figure 5.6 it can be seen that the commercial formulation did show an increasing trend while the nanosponge formulation has a slight decreasing trend. The mean difference of W4 to baseline of nanosponge (MD=0.173) and commercial group (MD=-0.481) shows the difference in changes as well as the trends as reflected in the graph.

Use of ATRA to treat acne is well known and has been established through evidence-based guidelines. Despite its effectiveness the use of ATRA is still limited by its cutaneous side effects (erythema, peeling, itching, burning, dryness) (Harper et al., 2019). Erythema, one of ATRA's side effects is a reddening of the skin caused by an external stimuli (Abdlaty et al., 2021). In the case of this study the application of commercial formulation may have caused an internal reaction which although not quite visible to the naked eye, was detected through this probe as reflected by the erythema values. The erythema assessment results from the animal study also supports the finding that commercial formulation can cause erythema to the skin. Although nanosponge in the animal study did incur some reaction which is opposite to the finding in the human study the conclusion remains similar in which nanosponge formulation was able to prevent or reduce the occurrence of erythema to the skin compared to the commercial formulation.

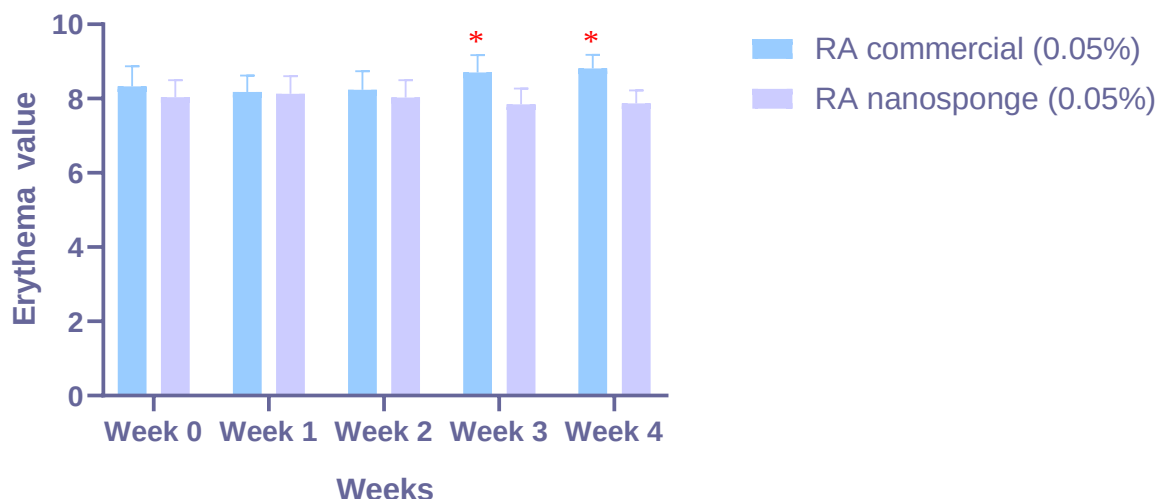


Figure 5.6 Erythema Values Measured from Two Groups (Commercial & Nanosponge) at Different Time Intervals (Mean $\pm$ SEM, $n=14$, * = $p<0.05$, * compared between formulations, * compared to baseline)

5.3.1.2 TEWL

Results for TEWL showed that there were significant differences of the data due to time, formulations and also the interaction of both variables ($p<0.05$). In the Commercial group, W2 shows significant difference ($p<0.05$) to W0 of the same group. Nanosponge formulation also showed significant difference at W4 when compared to W0 and W2 ($P<0.05$). Nanosponge formulation compared to the commercial group shows significant difference ($p<0.05$) at W4. From the graph in Figure 5.7, the values fluctuate yet the commercial group shows an increase from W0 to W4 (MD=-0.464) while the nanosponge group shows the opposite from W0 to W4 (MD=1.162) compared to baseline.

The values for both formulations generally fluctuated but at W2, W3 and W4 the difference between nanosponge formulation and commercial formulation was obvious. This increase of TEWL in commercial group are signs of skin barrier dysfunction which maybe associated ATRA side effects such as dryness, erythema, rash and scaling

(Szymański et al., 2020). As can be seen from section 5.3.1.1, there is an elevation in erythema values which corresponds with the observed rise in TEWL values with the use of commercial ATRA formulation. Similar trends were observed in animal study whereby the commercial formulation caused an increase in the TEWL values significantly. TEWL values for nanosponge fluctuated and did not show a clear trend although it did decrease compared to the baseline. The decrease in TEWL for nanosponge group demonstrates the advantages of ATRA nanosponge entrapment in action. Nanosponge is known to provide slow delivery preventing dose dumping of the drug. It also helps reduce direct contact of high dose ATRA on the skin which all works in favour of improving skin tolerability towards ATRA whilst reducing side effects (Ebrahimi et al., 2021). The decreases may also be supported by excipients of the gel-cream specifically glycerol. Glycerol can prevent damaging effect to the stratum corneum and cause rapid repair of the skin barrier by generating stimulus for repair through flux of water in the stratum corneum (Roussel et al., 2012).

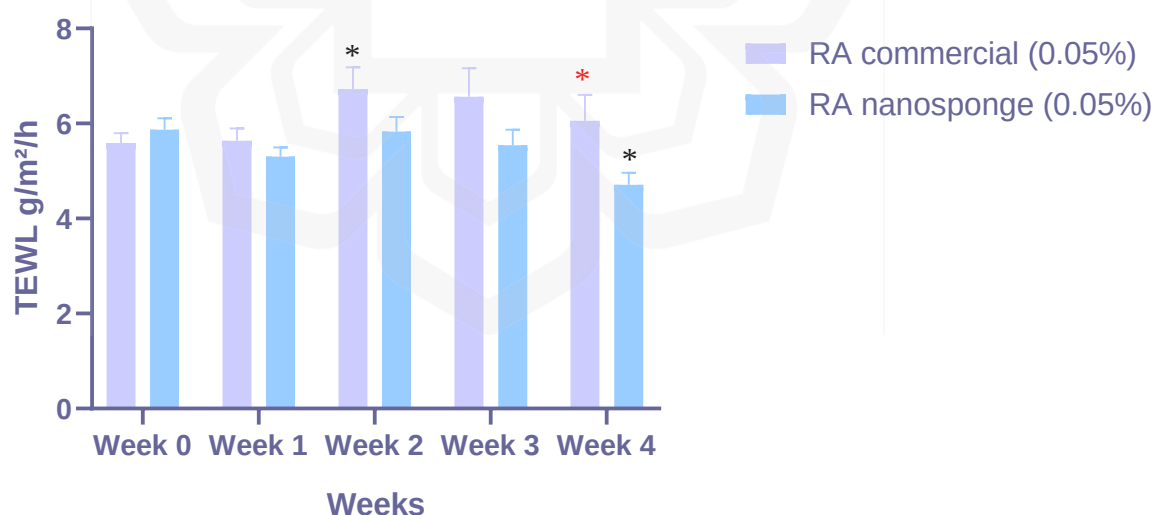


Figure 5.7 TEWL Values Measured from Two Groups (Commercial & Nanosponge) at Different Time Intervals (Mean $\pm$ SEM, n=14, * = $p < 0.05$, * compared between formulations, * compared to baseline)

5.3.1.3 Hydration

Hydration did not show any significant changes over time nor was there significant difference between the formulations in general. The results compared within weeks using Tukey's multiple comparison test did show some significant difference. When compared to W0, commercial and nanosponge group shows significant difference to W2 & W4 ($p < 0.05$). When compared to W1, commercial group shows significant difference in W2 & W4 while nanosponge group shows significant difference at W2 only ($p < 0.05$). Comparison between the groups from week to week showed no significant difference as well. Although there was no significance overall, Commercial and nanosponge showed an increase in hydration compared to the baseline (MD=-23.60, -24.26). The results are as depicted in Figure 5.8.

No significant difference in the overall data was most likely due to various individual factors that affect hydration such as age, gender, ethnicity, body mass index, lifestyle and skin type (Dąbrowska et al., 2018). In comparison to the study using rats, a decreasing trend can be seen which may be caused by repeated shaving. This hypothesis can be said with conviction as the rats were kept in a controlled environment and monitored daily minimizing the variables that may affect skin hydration. Whereas in the case of studies with humans, the variability and individual factors that affect skin hydration can only be controlled to a certain extent hence leading to difficulty to observe a clear trend. Nevertheless, a slight increasing trend with both formulations can be seen towards the end of the study. This result maybe contributed by the application of creams/emulsion which both contains emollients and humectants that can improve hydration in the commercial (stearyl alcohol, isopropyl myristate, stearic acid – emollient) and nanosponge formulation (glycerol, propylene glycol – humectant; lecithin, squalane – emollient) (Proksch et al., 2019).

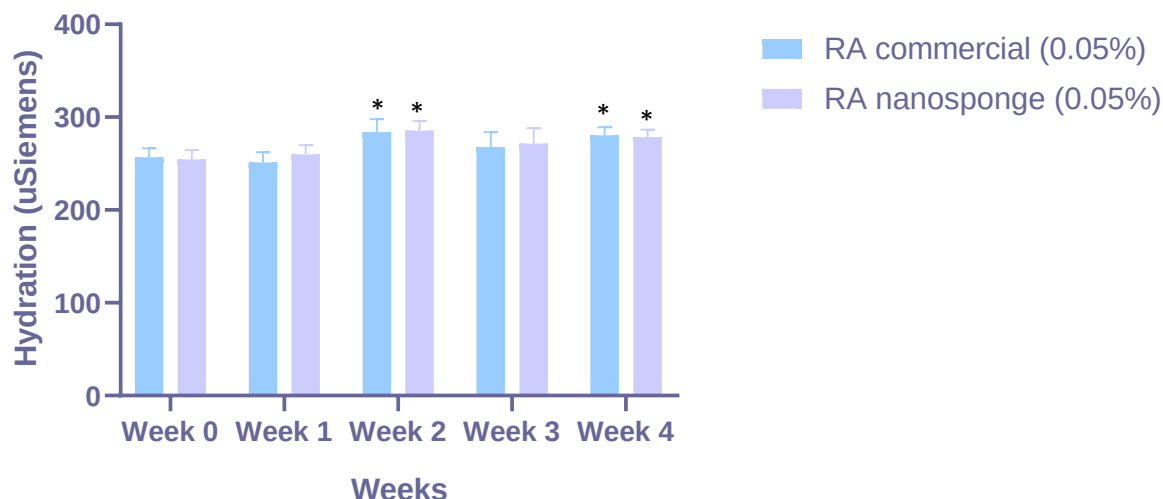


Figure 5.8 Hydration Values Measured from Two Groups (Commercial & Nanosponge) at Different Time Intervals (Mean $\pm$ SEM, $n=14$, * = $p<0.05$, * compared between formulations, * compared to baseline)

5.3.1.4 Elasticity

The overall data was significant contributed by the different formulation and also the interaction between time and formulations ($p<0.05$). Comparisons within week for each formulation was not found to be significant for commercial formulation. Nanosponge on the other hand, was found to be significant between W0 and W4 ($p<0.05$). The formulations compared to each other was significant at W3 and W4 ($p<0.05$). From the graph in Figure 5.9, a slight increasing trend from baseline can be seen for the commercial group (MD=-0.1429) while the nanosponge group (MD=47.50) has a decreasing trend. The increase in retraction time for commercial group suggest that the skin elasticity has reduced. A study comparing atopic dermatitis (AD) skin with normal healthy skin assessed elasticity and found that participants with higher SCORAD (scoring atopic dermatitis) which is associated with skin barrier dysfunction have lower skin elasticity compared to healthy skin. The TEWL and erythema values were also increased in the AD skin (Montero-Vilchez et al., 2022). The findings can be related to this current study whereby the side effects that causes skin barrier dysfunction has been

reflected through the increased erythema and TEWL values by commercial formulation. Consequently, a reduced elasticity presented through the higher retraction time for commercial formulation was recorded.

On the contrary nanosponge formulation showed a decrease in skin retraction time which imply the improvement in skin elasticity. ATRA can increase the production of collagen and elastic fibres thus improving skin elasticity. ATRA can inhibit matrix metalloproteinase (MMP) which is responsible for collagenolysis by acting on tissue inhibitor metalloproteinase (TIMP) and activator protein 1 (AP-1) (Riahi et al., 2016). ATRA action also leads to accumulation of extracellular matrix which enhances skin elasticity and prevents wrinkling (Z. Li et al., 2017; Syed Omar & Hadi, 2022). Although both formulations contain ATRA, the difference of the drug delivery direct exposure of skin to high dose of ATRA vs slow release from nanosponge is evident through the results. Instead of being able to reap the benefits of using ATRA, the use is masked by its side effects as seen through the results of commercial formulation. The entrapment of ATRA in nanosponge reduced the side effects and enhance the efficiency of the drug.

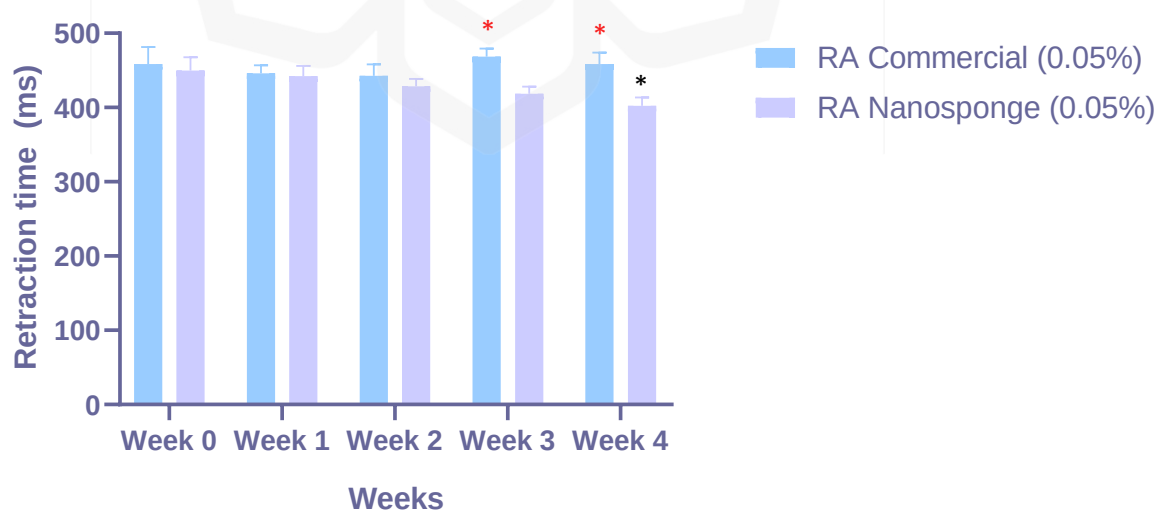


Figure 5.9 Elasticity Values Measured from Two Groups (Commercial &

Nanosponge) at Different Time (Mean $\pm$ SEM, n=14, * = $p < 0.05$, * compared between formulations, * compared to baseline)

5.3.1.5 Melanin

Melanin data analysis discovered a significance in the data over time ($p < 0.05$). Comparison within weeks found that the commercial formulation at W1, W2, W3 and W4 were all significant compared to W0. While nanosponge was found to be significant at W2, W3, W4 compared to W0 ($p < 0.05$). Comparison between the formulations did not show any significant differences. From the graph below in Figure 5.10, both formulations show a rise in melanin index at the end of the study. From the mean difference commercial formulation (MD=-3.519) did show a slightly bigger difference to the baseline which means higher increase compared to nanosponge formulation (MD=-2.517). Post inflammatory hyperpigmentation (PIH) also known as post inflammatory melannosis is skin hyperpigmentation triggered by an irritation or injury which causes an abnormal overproduction of melanin. Although ATRA is a known treatment for hyperpigmentation its irritating side effects may cause undesired post inflammatory response that may worsen skin hyperpigmentation (Callender et al., 2011, 2022). Despite the similar trend in melanin values, other skin assessment parameters show contrasting results between the two formulations. The increase in melanin values is slightly less in nanosponge group which may propose the ability of nanosponge to minimize side effect and maximize efficiency of ATRA.

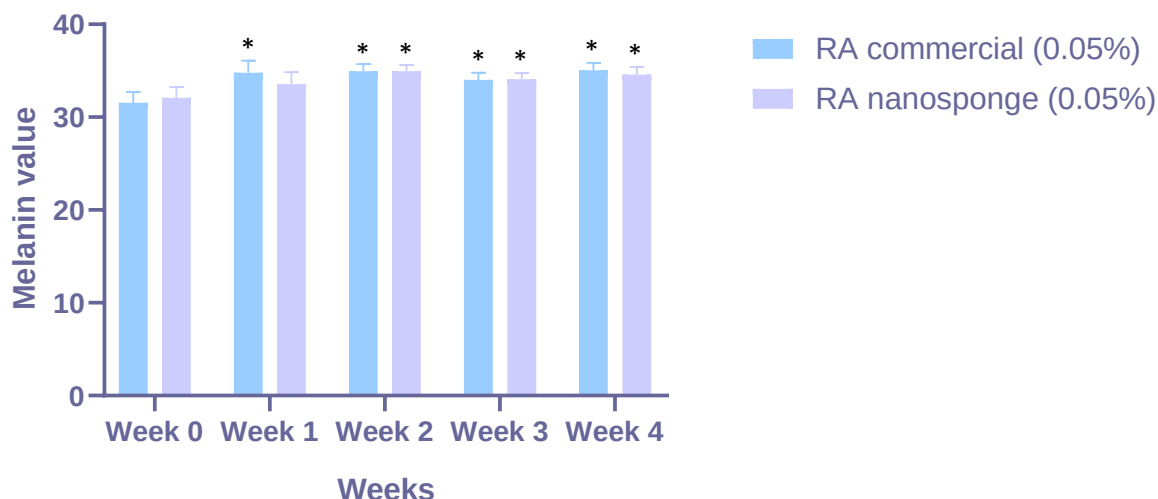


Figure 5.10 Melanin Values Measured from Two Groups (Commercial & Nanosponge) at Different Time Intervals (Mean $\pm$ SEM, n=14, * = $p < 0.05$, * compared between formulations, * compared to baseline)

5.3.2 Questionnaire Data Collection

5.3.2.1 Side effects

5.3.2.1.1 Redness

The commercial formulation (Formulation 1) caused more severe redness based on the positive estimate of formulation 1 as shown in Table 5.4. 0 out of 15 reported occurrences of redness in nanosponge group (Formulation 2) while 8 out of 15 reported mild-moderate redness (26.7%) caused by commercial formulation. 3 reported mild redness (10.0%) and 5 reported moderate redness (5%).

Table 5.4 Parameter Estimates for Redness.

		Estimate	Std. Error	Sig.
Threshold	[Redness = 0]	20.479	.518	.000
	[Redness = 1]	21.306	.548	.000
Location	[Formulation=1]	20.613	.000	.
	[Formulation=2]	0 ^a	.	.

5.3.2.1.2 Dryness

The commercial formulation (Formulation 1) caused more severe dryness which is based on the positive value of the estimates in Table 5.5. None of the participants in nanosponge group reported dryness while 5 out of 15 (16.6%) participants in commercial group reported mild-severe dryness. 3 person (10%) reported mild dryness, 1 person (3.3%) reported moderate dryness and 1 person (3.3%) reported severe dryness each.

Table 5.5 Parameter Estimates for Dryness

		Estimate	Std. Error	Sig.
Threshold	[Dryness = 0]	19.890	.548	.000
	[Dryness = 1]	21.068	.760	.000
	[Dryness = 2]	21.836	1.035	.000
Location	[Formulation=1]	19.197	.000	.
	[Formulation=2]	0 ^a	.	.

5.3.2.1.3 Irritation

Based on the estimates for irritation in Table 5.6, the commercial formulation (formulation 1) causes more severe irritation. 9 out of 15 participants (30%) in the commercial group reported mild to severe irritation of the skin. 4 people (13.3%) reported mild irritation, 3 people reported (10%) moderate irritation and 2 people (6.7%) reported severe irritation. Alternately, no participants reported irritation for nanosponge group.

Table 5.6 Parameter Estimates for Irritation

		Estimate	Std. Error	Sig.
Threshold	[Irritation = 0]	20.371	.527	.000
	[Irritation = 1]	21.470	.548	.000
	[Irritation = 2]	22.649	.760	.000
Location	[Formulation=1]	20.777	.000	.
	[Formulation=2]	0 ^a	.	.

5.3.2.1.4 Rash

The positive estimates for commercial formulation (formulation 1) as seen in Table 5.7, indicates that it causes more severe rashes than nanosponge formulation. 5 out of 15 (16.7%) participants in the commercial group experienced mild-moderate rash. Whereby 2 people (6.7%) reported mild rash and another 3 people (10.0%) reported moderate rash.

Table 5.7 Parameter Estimates for Rash

		Estimate	Std. Error	Sig.
Threshold	[Rash = 0]	19.890	.548	.000
	[Rash = 1]	20.583	.645	.000
Location	[Formulation=1]	19.197	.000	.
	[Formulation=2]	0 ^a	.	.

5.3.2.1.5 Discolouration

The estimate for commercial formulation (formulation 1) in Table 5.8, indicates that it causes more severe discolouration compared to nanosponge formulation. 4 out of 15 (13.3%) participants in the commercial group experienced mild to moderate skin discolouration with 3 participants (10.0%) reported mild discolouration and 1 participant (3.3%) reported moderate discolouration.

Table 5.8 Parameter Estimates for Discolouration

		Estimate	Std. Error	Sig.
Threshold	[Discolouration = 0]	20.095	.584	.000
	[Discolouration = 1]	21.723	1.035	.000
Location	[Formulation=1]	19.084	.000	.
	[Formulation=2]	0 ^a	.	.

Overall, more participants experienced some side effects when using the commercial cream formulation compared to the nanosponge formulation. Participants perception regarding the side effects is also in agreement with results from the weekly skin analysis. The combination of nanosponge delivery system and gel-cream dosage

form has helped reduce side effects and maintain good skin condition throughout the study.

5.3.2.2 Product acceptability

Based on the ordinal regression analysis, results for each parameter assessed showed a positive estimate value for commercial formulation (Formulation 1). This indicates that commercial formulation has a higher probability to get scores on the more unpleasant side. Table 5.9 shows that on the positive side of the table (Very pleasant, non-sticky, cooling and non-greasy) nanosponge formulation was selected frequently compared to the commercial formulation. While on the opposite end (very unpleasant, sticky, not cooling and greasy), in most parameters, commercial formulation gains more votes compared to nanosponge formulation. It can be concluded that most participants prefer the nanosponge gel-cream formulation over the commercial formulation. Prior studies developing a psoriasis formulation based on patient preference aimed to improve sensory attributes such as low residues, low stickiness, greaseless, good spreadability and good moisturizing properties. Psoriasis treatment is usually in the form of ointment, cream or solution. Based on the attributes mentioned, they formulated an emulgel and hydrogel which possess these attributes (Oliveira et al., 2022). A study comparing tretinoin branded lotion and generic cream was also done in the past where more participants agreed that lotion was more gentle, soothing, spreadable, less residue, not sticky and absorbent (Draelos et al., 2022). The outcome is similar to our current study in which the agreement scores on product acceptability were higher for nanosponge gel-cream formulation compared to the commercial cream.

The importance of assessing the patient's or participants perception on the formulation and side effects they might experience is to ensure that the development of this formulation includes patient centricity perspective. Patient centric pharmaceutical drug product design is a key factor in determining product efficacy as it is dictated by patient's adherence towards the therapeutic strategy (Stegemann et al., 2016). As each patient is individually different in terms of background, skills, capabilities, preferences,

skin conditions, comorbidities, disability; addressing patient's need or preferences in the real world may improve therapeutic outcome. In the case of ATRA. The unwanted side effects, unappealing formulations leads to low adherence. Despite it's known theoretical efficacy, the acceptance and preference of patients dictate the actual real-life efficacy. Considering patient's acceptance and preference in the early stages of a product development will make the product more likely to be chosen, used properly and in turn boost efficacy (Palmeira-de-Oliveira et al., 2022).

Table 5.9 Information on Participant's Acceptance Towards Formulation

Formulation	Range						
	Very pleasant	Pleasant	Fairly pleasant	Neutral	Fairly unpleasant	Unpleasant	Very unpleasant
	Colour						
C	7	4	0	2	1	0	1
N	10	5	0	0	0	0	0
	Smell						
C	6	5	0	2	2	0	0
N	7	3	1	3	1	0	0
	Texture						
C	6	3	2	1	1	1	1
N	10	4	1	0	0	0	0
	Application						
C	4	5	1	4	0	0	1
N	7	5	1	2	0	0	0
	Stickiness						
	Not sticky		→		Sticky		
C	4	3	0	5	2	0	1
N	8	5	1	0	1	0	0
	Cooling						
	Cooling		→		Not cooling		
C	3	2	1	0	0	2	7
N	5	1	1	4	0	0	4
	Greasiness						
	Non-greasy		→		Greasy		
C	6	5	0	2	0	1	1
N	9	5	0	1	0	0	0

5.4 CONCLUSION

In this chapter the formulated ATRA loaded nanosponge gel-cream formulation was applied on human skin to assess its effects on the skin and investigate its irritation potential in comparison to commercial ATRA cream. First part of the study, data was collected quantitatively where participants' skin was measured weekly using several probes on Dermalab combo. Participants applied the cream daily for a total of 4 weeks and came in weekly to check their skin's erythema, hydration, TEWL, elasticity and melanin values. The results indicate high erythema and TEWL values for commercial group, slight improvements in hydration and increase in melanin for both groups and an improved elasticity for nanosponge group. The increase in TEWL and erythema is an indication of the irritation potential caused by ATRA which is more prominent in the commercial group. The elasticity of the skin which can be improved by ATRA as shown by nanosponge group, can also be reduced due to ATRA side effects.

The qualitative assessment of this study done through questionnaire further strengthens the findings of the quantitative analysis. A higher number of participants reported side effects occurring with commercial formulation rather than nanosponge formulation. A number of participants reported redness, dryness, irritation, rash and discolouration in commercial group. The nanosponge group also had several reports yet it was less than the commercial group. The product acceptability assessment results demonstrated that more participants prefer nanosponge gel-cream formulation over the commercial cream formulation as it is less sticky, less greasy, pleasant application and texture. In conclusion, nanosponge may be able to increase patient's adherence towards ATRA therapy as it reduces side effects with the use of nanosponge and gains much more preference from patient through its gel-cream dosage form.

CHAPTER SIX

GENERAL CONCLUSION AND FUTURE WORKS

6.1 GENERAL CONCLUSION

Acne is an inflammatory skin condition that appears in the form of inflammatory and non-inflammatory lesion on the skin. It is usually physically visible due to its manifestations on the face which has a huge impact on sufferer's psychology. ATRA is a known treatment of acne with the highest efficacy yet limited due to its chemical properties and side effects on users. Chemically it is highly insoluble making it difficult to formulate; and it also is sensitive toward air light and heat. The side effects that occur due to ATRA usage include dryness, irritation, and erythema. In order to maximize the benefits of ATRA, its delivery system should be improved by employing advance drug delivery systems. Nanosponge can entrap ATRA within its nanocavities, easing the formulation process, providing sustained release which can help in reducing side effects while maintaining localized effect. Therefore, the aim of this study was to be able to produce a safe, stable, and effective ATRA loaded nanosponge formulation to be used in the management of acne.

In order to achieve the objective method to fabricate nanosponge was thoroughly investigated to ensure that the nanosponnge used to entrap ATRA has the best properties. The nanosponge was prepared by varying the ratios of polymer (β -cyclodextrin) and crosslinker (carbonyldiimidazole) at ratios of 1:2, 1:4 and 1:8. At the same time the duration of homogenization was also varied at 10, 15 and 20 minutes. All data obtained was analysed with design of experiment factorial analysis which came up with three solutions which are 1:2, 1:4 and 1:8 at 15 minutes. The entrapment efficiency and solubilization efficiency was examined and resulted in 1:8 being the selected nanosponge as it improved the solubility and has the highest entrapment efficiency. The entrapment process was further studied by varying the ratio of drug: nanosponge at 1:2, 1:4 and 1:6. The final nanosponge selected for further formulation into a dosage form was nanosponge ratio 1:8 (polymer:crosslinker) and 1:2 (drug:nanosponge) as it had the

highest entrapment efficiency at 73.90%. The entrapment was verified through ATR-FTIR analysis, DSC analysis and surface morphology analysis.

The dosage form selected to carry nanosponge s a gel-cream formulation. The initial development phase of the gel-cream produced formulation with a large particle size and polydispersity index which may affect stability in the long run. The formulation was then optimized by varying the speed of homogenization. the outcome of the optimization showed that 3000 rpm resulted in a gel-cream formulation with optimal characteristic preference. As ATRA loaded nanoosponge was incorporated into the gel-cream formulation, a stability study was carried out by storing the formulation in a real-time and accelerated stability chamber. The organoleptic properties, physical appearance, pH, zeta potential, particle size, polydispersity index and microbial limit count were assessed at baseline, week 2, week 4 and week 12. The formulation remained stable and maintained desirable characteristics throughout the stability study duration. Antimicrobial activity study against *C. acnes* was also done evaluated with placebo formulation, nanosponge formulation and commercial formulation. The results are comparable with prior studies which show that tretinoin does not have direct effect on *C acnes*.

To investigate the permeation and retention behaviour of ATRA, *ex vivo* tests were performed using Franz cell diffusion on rat skin from male Sprague-Dawley rats. Conditions were chosen to best mimic the skin environment *in vivo*. The study was conducted using both ATRA-loaded nanosponge gel-cream formulation and a commercial formulation at 0.05% strength. The results showed that the commercial formulation penetrated the receptor fluid to a greater extent and at a higher rate. Nanosponge formulation on the other hand retained more ATRA in the skin. The nansopong gel cream formulation loaded with ATRA produced favourable results because lower permeation means it's not absorbed systemically. The results reflect the ability of the nanosponge for slow release and localized effect at the target site, i.e., in the skin layers. An animal irritation study was also conducted for 21 days whereby rats were divided into four groups of three (control group, the commercial group, the placebo group and the nanosponge group). During the first 72 hours, the skin was

observed for signs of erythema and oedema using the Draize skin test. From this, the primary dermal irritation index (PDII) and simplified irritation index (SII) were calculated. The results for the nanosponge gel cream, placebo and control indicate no skin irritations. However, the commercial formulation was found to be moderately irritating based on PDII and irritating based on SII. Skin assessments showed significantly high TEWL and erythema scores for the commercial formulation, in contrast to the nanosponge formulation. Histology was also performed after 21 days. The commercial formulation showed several signs of inflammation, and the nanosponge group was also mildly inflamed. The control and placebo formulation looked normal and comparable with a healthy skin cross-section.

Finally *in vivo* studies on 15 human subject (30 arms) were conducted for 4 weeks to assess the effects of the nanosponge formulation in comparison to the commercial formulation. The participants were also asked regarding side effects they experienced and the acceptability toward the product. As a result, nanosponge cream incurred less side effects on the participants than the commercial formulation as the TEWL and erythema values did not increase as much. On top of that, nanosponge formulation increased skin elasticity through reduction of retraction time. Higher number of participants also reported having experienced side effects such as redness, irritation, rash, dryness, and discoloration with commercial formulation. The nanospnge gel-cream formulation was also highly favoured over commercial formulation in terms of its texture, application, less greasy and less sticky.

In general, a safe, stable and effective ATRA loaded nanosponge formulation was successfully fabricated. The first objective to develop and characterize ATRA loaded nanosponge was completed through thorough investigations on several factors that affected entrapment efficiency. The following objective of developing a stable gel-cream formulation and incorporation of the loaded nanosponge was also fulfilled by implementing a stability study with rigorous characterization of the formulation for 12 weeks. The antimicrobial activity was also assessed where the developed formulation started to be compared to commercial formulation completing the third objective. In the following chapter the fourth and fifth objective were achieved through animal studies.

The permeation of ATRA, irritation study, weekly skin assessment with Dermalab Combo for 21 days and histology study was done where an in-depth comparison was done between ATRA loaded nanosponge gel-cream formulation and commercial formulation on rat skin. Finally, skin assessment on human skin was done by once again utilizing Dermalab combo to assess the skin condition for 4 weeks. TEWL, erythema, hydration elasticity and melanin content were monitored throughout the study. At the end of the study, feedback from the participants on the desirability of the formulation as well as side effects were gathered and analysed which concluded the final objective.

Extensive research to obtain ATRA loaded nanosponge with good properties is important in order to ensure ATRA was able to be entrapped in a nanosponge that is suitable for further formulation and skin application. The final nanosponge had preferred features and confirmed its ability to entrap ATRA through FTIR, DSC and FESEM. Incorporating ATRA loaded nanosponge into gel-cream was a meticulous process as the stability of the gel-cream and ATRA was at stake. The gel-cream formulation had good zeta potential, particle size, PDI and pH to remain stable and deliver drugs to the skin layers effectively. The outcome of this study showed that ATRA loaded nanosponge gel-cream formulation can minimize side effects caused by ATRA. Sustained drug delivery to the skin led to high skin retention, low systemic absorption allowing ATRA to act at within the target site i.e., skin layers. An effective dose of ATRA was able to be delivered to the skin with improved skin tolerance. Patient adherence towards ATRA treatment can be improved tremendously as the occurrence of side effects reduced. In addition, a patient centric gel-cream formulation formulated to suit patients' preference adds more value in improving adherence to treatment while comprehensively improving acne therapy.

6.2 RECOMMENDATIONS AND FUTURE WORKS

The findings of this research were able to improve the action of ATRA on the skin by using nanosponge to deliver the drug slower to the skin and help reduce side effects. Future works could be more focused on grasping the mechanisms and action it has on human skin. Franz cell diffusion studies could be done with human skin to get a better idea how well it retains within the skin layers as the human skin is physiologically different from rat skin. Most importantly, exhaustive *in vivo* research can be done on subjects with acne of different severity through a clinical trial. Human study can give enlightenment on the effects of the formulation on acne such as acne progression and healing. Observation on how it improves the appearance of acne over time and the influence of the formulation on sebum will also be beneficial in understanding the improvements the formulation has compared to marketed formulation or provide ideas on how it can be further improved. Prior study on how ATRA affects C. acnes over time can also be documented to see how a slow delivery of ATRA differs from normal ATRA delivery. Furthermore, human study will allow the research to extend into post acne scarring and how the formulation acts in comparison to marketed formulations. Proving the benefits of this formulation on patients that require this treatment may trigger further research to scale up the formulation to market the product and maybe one day reach consumers.

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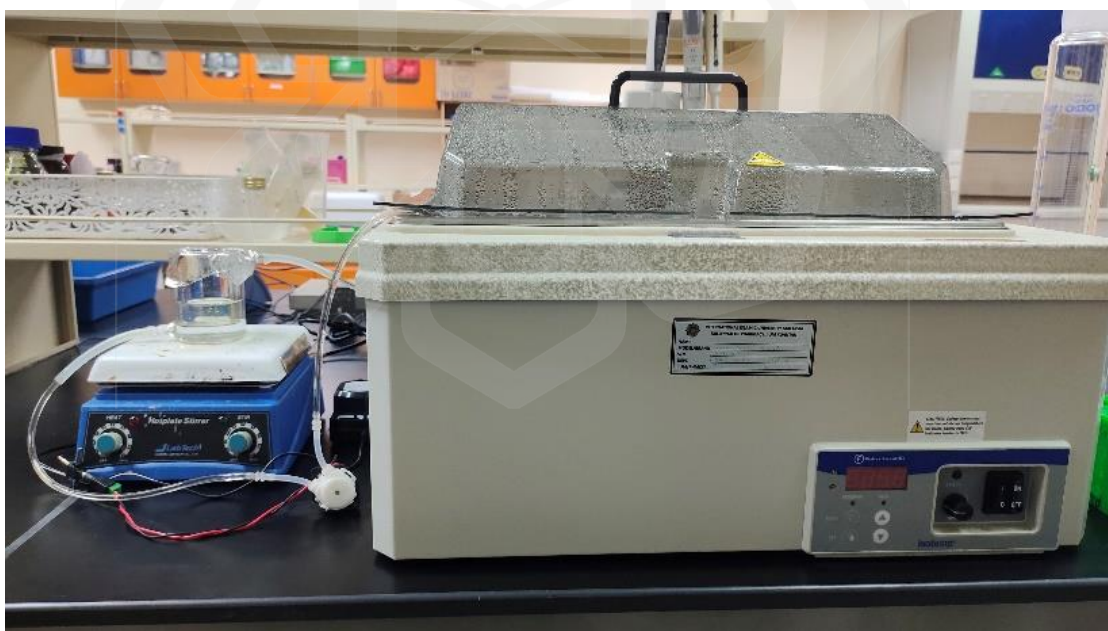
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APPENDIX I: NANOSPONGE PREPRARATION SETUP



APPENDIX II: ANIMAL ETHICS APPROVAL



الجامعة الإسلامية العالمية ماليزيا
INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA
يُؤْتِي السَّلَامَ إِذَا رَأَى الْيَتِيمَ فَلْيَسِّرْهُ
(Company No 101067-P)

RESEARCH MANAGEMENT CENTRE

Our Reference : IIUM/504/14/2/IACUC
Date : 01st December 2020

Assoc. Prof. Dr. Hazrina Ab Hadi
Department of Pharmaceutical Technology,
Kulliyyah of Pharmacy,
International Islamic University Malaysia.

Dear Dr,

السلام عليكم ورحمة الله وبركاته

IACUC- IIUM APPROVAL

The Institutional Animal Care and Use Committee (I-ACUC) held its 3rd 2020 meeting and has reviewed and commented of the above research project.

ID	: IACUC-2020-025
Study Title	: Evaluation Of The Permeation, Irritation Potential And Effects Of Retinoic Acid Loaded Nanosponge Gel- Cream Formulation On Rat Skin
Study Site	: Animal House, Kulliyyah of Pharmacy
Animal Details	: Rat (Sprague-dawley)
Animal Quantity	: 20
Principal Investigator	: Assoc. Prof. Dr. Hazrina Ab Hadi
Expiry	: 01 st December 2021

Thank you.

والسلام

ASSOC. PROF. DR. MUHAMMAD LOKMAN MD. ISA
Chairperson, I-ACUC

cc. Deputy Director RMC Kuantan

Appendix : Reviewing Committee list of IACUC Meeting 3/2020

DISCLAIMERS: This approval **ONLY** covers the Ethical aspect of your Study and **CANNOT** be use as permission/approval to use any facility (e.g. Animal Room) and authorization to store/handle any poison/chemical



SIRIM
CERTIFIED TO MS ISO 9001:2008
Registration No. AS 3492

Garden of Knowledge and Virtue

Office Address: Research Management Centre, 1st Floor, Block 2, Admin Building, International Islamic University Malaysia
Kuantan Campus, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang
Tel: +609 570 4220 / 4223 Fax: +609 571 6786 E-mail: rescentre@iiu.edu.my Website: <http://www.iiu.edu.my/research>

APPENDIX III: HUMAN ETHICS APPROVAL

 الجامعة الإسلامية العالمية ماليزيا INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA بني برسيق ايسلاما <i>Garden of Knowledge and Virtue</i>	LEADING THE WAY KHALIFAH · AMĀNAH · IQRA' · RAHMATAN UL-ĀLAMĪN SUSTAINABILITY INSTITUTION OF THE YEAR
RESEARCH MANAGEMENT CENTRE (RMC)	
Our Ref. Date	: IIUM/504/14/11/2/IREC 2021-059 : 17 May 2022
Assoc. Prof. Dr. Hazrina bt Ab Hadi (Principal Investigator) Kulliyah of Pharmacy, IIUM Kuantan Campus 25200 Kuantan Pahang	
Dear Assoc. Prof. Dr.,	
The IIUM Research Ethics Committee (IREC) has reviewed your study protocol as mentioned below:-	
ID NO. TITLE	: IREC 2021-059 : Evaluation of the Effect of Retinoic Acid Loaded Nanosponge Gel-cream Formulation on Healthy Human Skin
REGISTRATION DATE AMENDMENT NO. CO-INVESTIGATOR	: (Previous Approval Letter, dated 9 Mar 2021) : IREC 2021-059 (01) (Submission date: 09 May 2022) : Nil
NAME OF SITE	: Dermatopharmaceutics Lab, Kulliyah of Pharmacy, IIUM Kuantan Campus
ETHICAL EXPIRY DATE	: 9 Mar 2023
The IIUM Research Ethics Committee (IREC) operates in accordance to the Declaration of Helsinki, International Conference of Harmonization Good Clinical Practice Guidelines (ICH-GCP), Malaysia Good Clinical Practice Guidelines and Council for International Organizations of Medical Sciences (CIOMS) International Ethical Guidelines	
The following proposed amendment documents have been received and reviewed to the above study:-	
Amendment :- 1. Addition of Co-Investigator – Asst. Prof. Dr. Rasimah bt Ismail 2. Addition of advertisement poster for participant recruitment	



Decision by IIUM Research Ethics Committee (IREC):

(☒) Approved
(☐) Disapproved

Date of Approval: 14 May 2022

The investigator(s) are required to:

- a) submit the 'Continuing Review Form' 30 days before **EXPIRY DATE** to renew Ethical Approval.
- b) notify IREC of any change in protocol and obtaining further ethical approval as appropriate.
- c) report any adverse incident during the course of a study to IREC even if the incident is not directly related to the study.
- d) report to the IREC within 72 hours for all internal SAEs (occurring in IIUM PI site).
- e) report in a prompt manner if the information impacts the continued ethical acceptability of the trial for external SAEs (occurring in participants at other sites).
- f) provide information of minor protocol deviation in Progress Report or End Report whichever necessary.
- g) report any major protocol deviation occurs within 5 working days.
- h) submit Progress Report Form before the end of six (6) months given by IREC.
- i) complete and submit the End of Project Report Form to the IREC Secretariat's Office.
- j) All records and data subjects are **CONFIDENTIAL** and used only for the purposes of this study and all issues and procedures on data confidentiality must be observed.

Thank you.

Yours sincerely,



PROF. DR. NASSER MUHAMMAD AMJAD
Chairman,
IIUM Research Ethics Committee (IREC)

Copy : Protocol File – IREC 2021-059

DISCLAIMER: The approval letter only covers the ethical aspect of your study only. Any other permission/approval to use any facilities, data or human resource should fall under applicant's responsibility.

APPENDIX IV: FEEDBACK FORM

Version :1
Date: 19th February 2021

Participant Number:

Date:

Please tick boxes relevant to your answer.

EVALUATION OF SKIN CONDITION

Skin condition	Absent	Mild	Moderate	Severe	Very severe
Redness					
Dryness					
Irritation					
Rash					
Discolouration					

Others, please specify:

EVALUATION OF PRODUCT STABILITY

Change	Present	Absent
Odour		
Colour		
Texture		
Foreign material		

Others, please specify:

EVALUATION OF TEST PRODUCT

Colour	Pleasant								Unpleasant
Smell	Pleasant								Unpleasant
Texture	Pleasant								Unpleasant
Stickiness	Non-sticky								Sticky
Application	Pleasant								Unpleasant
Cooling	Cooling								Not cooling
Greasiness	Non-greasy								Greasy