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Proliposomes: Applications and Improvements

프로리포솜의 응용과 개선에 대한 연구

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Abstract

Proliposomes: Applications and Improvements

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The objective of this work was the development of novel applications and production methods of proliposomes. This drug delivery system offers a variety of advantages when compared to conventional liposomal formulations. Among them the ability to form liposomes upon resuspension, making dry formulation possible. This would be interesting for the formulation of unstable drugs. This approach was explored in PART I with the formulation of AAPE for topical delivery. Another advantage is the ability to promote the solubilization of otherwise low solubility drugs. Despite these advantages proliposomes also face challenges. Prime among these the low total drug content due to the need for a carrier material. These aspects, a low solubility drug (Itraconazole) and a method to increase the total drug content, were explored in PART II of this work.

Part I. AAPE Proliposomes for Topical Atopic Dermatitis Treatment

The objective of this study was the development of a proliposomal formulation of AAPE for the treatment of topical atopic dermatitis. The anti-inflammatory effect of advanced adipose stem cell derived protein extract (AAPE) could be improved by minimizing protein degradation. Therefore a dry proliposomal formulation was manufactured by evaporating a solution of soy phosphatidyl choline, AAPE and Poloxamer 407 in ethanol under vacuum on sorbitol powder. Characterization of proliposomes (zeta potential, diameter, stability and flowability) as well as *in vivo* efficacy in a dermatitis mouse model was investigated. Reconstitution of the proliposomal powder formed liposomes of 589 ± 3.6 nm diameter with a zeta potential of -51.33 ± 0.36 mV. Protein stability was maintained up to 90 days at 25°C in dry proliposomes. *In-vivo* studies on a atopic dermatitis mouse model showed a significant reduction in IgE levels after topical AAPE proliposome treatment. This suggested that AAPE proliposomes is a promising formulation for atopic dermatitis treatment.

Part II. A Layered Proliposomal Formulation With Increased

Itraconazole Content

Proliposomes can increase the solubility of the encapsulated drug but suffer from low total drug content. A practical oral formulation would have to reach 10% drug content to allow 100mg doses in 1g tablets. Therefore a proliposome production method capable of increasing the drug content was developed. Proliposomal powder based on a sorbitol matrix, coated with SoyPC and Poloxamer 407 as a surfactant was manufactured via a fluidized bed method. Multiple lipid / drug layers were separated by layers of Eudragit E100. Dissolution profiles were investigated according to USP. Physical characteristics including flowability, size, and zeta-potential of proliposomal powder as well as resuspended liposomes were measured. The surface morphology and flowability of multilayer proliposomes was similar to that of reference material. After resuspension liposomes of 859-1525nm with zeta-potentials of -16.8mV to -24.0mV were formed with encapsulation efficiency of >70%. Dissolution of 80% within 2 hours for multilayer proliposomes and one hour for single layer proliposomes was observed. This indicates that layering is a viable approach to increase the lipid and drug content of proliposomal formulations while maintaining good handling characteristics and rapid dissolution.

Keywords: ADSC, AAPE, Itraconazole, proliposomes, liposomes, atopic dermatitis, Layering, Fludized bed

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PART II. A Layered Proliposomal Formulation With Increased Itraconazole Content

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Part I. AAPE Proliposomes for Topical Atopic Dermatitis Treatment

1. Introduction

Topical atopic dermatitis is a condition of the skin involving an inflammatory response to external or internal stimuli. Common causes are sensitization with environmental toxins or induction by direct exposure to a wide range of allergens and inflammation-inducing agents as well as microbes (Leung et al., 2004). In recent years the prevalence of inflammatory skin diseases, in particular atopic dermatitis increased drastically (Boguniewicz and Leung, 2011). Among the suggested explanations are the increased concentration of allergens and environmental toxins as well as increased prevalence of obesity and changes to the human diet in westernized countries (Devereux, 2006). These trends were not only observed in countries with more advanced economies but also in developing countries (Liao et al., 2009). As there is currently no cure, the treatment of atopic dermatitis is often limited to managing the symptoms, especially the inflammatory response (Finch et al., 2010). A wide variety of drugs is used for this purpose, among which are topical applications of various cortical steroids. However, these steroidal anti-inflammatory drugs are associated with various side effects, which limits long term medication (Watson and Kapur, 2011; Aschoff et al., 2011). Several chemokines and cytokines might reduce the inflammatory response avoiding the risk of unwanted side effects from corticosteroids (Chi et al., 2006). A topical formulation containing these messenger molecules could thus be used for the treatment of atopic dermatitis.

Human adipose-derived stem cells (ADSC) are interesting subjects in regenerative medicine. They are considered an interesting source of therapeutic proteins due to the possibility of harvesting large quantities from adipose tissues as well as their low immunogenicity and the large number of soluble factors that can be derived (Tallone et al., 2011). While some properties, *e.g.* in the inhibition of t-cell

proliferation *via* t-cell contact-induced human leukocyte antigen excretion, require physical contact of ADSCs to target cells (Selmani et al., 2008), there is also a large variety of constantly excreted soluble factors such as HGF, TGF- β and IL-6, suggesting non-contact dependent activity (Di Nicola et al., 2002; Groh et al., 2005; Djouad et al., 2005). Recent publications have demonstrated that ADSCs excrete anti-inflammatory factors into the adipose derived stem cell culture media (ADSC-CM) (Newman et al., 2009), and furthermore anti-inflammatory and anti-oxidant properties of its lyophilisate “advanced adipose stem cell derived protein extract” (AAPE) have been observed (Kim et al., 2008).

Protein drugs in general are prone to degradation due to a variety of mechanisms including hydrolysis, aggregation as well as chemical and postranslational modification (Jiskoot et al., 2012). Moreover, AAPE as a complex mixture may also contain proteolytic factors expressed by ADSCs (Lai et al., 2012). The arising drug stability problem could be solved with a dry storage formulation. A systemic delivery *via* the oral route would expose the protein factors to a denaturing environment and thus a topical delivery would be necessary. However, this would lead to another challenge: low skin permeation and deposition of proteins due to the lipophilic character of the stratum corneum. Although the use of liposomal formulations to enhance skin deposition and penetration is a widely attempted concept (Jukanti et al., 2011; Gupta et al., 2008), they often face stability issues, leading to aggregation and/or drug leakage and reduced shelf life (Stark et al., 2010). This in combination with possible low stability of AAPE in solution makes this approach undesirable.

Proliposomes, a free flowing powder which upon hydration forms liposomes spontaneously, might be a solution for this dilemma (Song et al., 2002). This formulation would likely increase protein stability due to the absence of water, which would inhibit hydration-dependent degradation mechanisms. Furthermore, a dry formulation would prevent liposomal aggregation during storage, while at the

same time delivering a liposomal encapsulated drug, thus possibly resulting in increased skin permeation/deposition (Gupta et al., 2008). Additionally, compared to conventional preparation methods, proliposome manufacturing processes can be scaled up easily, which make this approach attractive to the industrial point of view. Herein, a proliposomal formulation of AAPE, which can enhance the protein stability, for the treatment of topical atopic dermatitis was investigated.

2. Materials and methods

2.1. Materials

Anhydrous ethanol of 99.9% purity was purchased from Daejung (Gyeonggi-do, Korea). Soy phosphatidyl choline (PC) was provided by Avanti (Alabaster, USA). Bovine serum albumin was purchased from Sigma-Aldrich (St. Louis, USA). AAPE was provided by Proteomics (Seoul, Korea). Poloxamer 407 was purchased from BASF (Ludwigshafen, Germany), Sorbitol was purchased from Kanto Chemical (Tokyo, Japan) while SDS was provided by TCI (Tokyo, Japan). Precision plus protein standard was purchased from BioRad (Hercules, USA). All other reagents were of analytical grade.

2.2. Preparation of AAPE proliposomes

Proliposomal powder was prepared by the “evaporation on matrix” method (Song et al., 2005): Briefly, soy PC (400 mg) was dissolved in 62.5 ml ethanol, while 25 mg AAPE and 75 mg poloxamer 407 were dissolved in 1 ml water. Both solutions were then added to a round bottom flask and sonicated for 10 minutes. Sorbitol (4.5 g) was added followed by a second sonication of 10 minutes. The solvent was then evaporated under vacuum in a rotary evaporator at 40°C until dryness. The resulting mixture was placed in a freeze dryer over night to remove residual solvent. The dry material was sieved, placed in polypropylene bottles and stored at 4°C.

2.3. Zeta potential and liposome size measurement

Proliposomal powder (10% w/v) was placed in PBS and hydrated for 1 minute followed by 30 seconds of vigorous agitation resulting in a liposomal solution. The average mean diameter, polydispertiy index and zeta potential values were analyzed with a dynamic light scattering (DSL) instrument (ELS-Z, Otsuka Electronics Co. Ltd., Osaka, Japan) according to the manufacturer's protocol.

2.4. SEM and TEM imaging

For scanning electron microscopy (SEM) imaging, dry proliposomal powder was fixated on aluminum stubs and sputter coated with gold for 250 seconds. Sample surface morphology was then observed in a JSM-5310LV scanning electron microscope (JEOL, Tokyo, Japan). For transition electron microscopy (TEM) imaging, samples were prepared by rehydrating proliposomal powder in double distilled water (10% w/v), subsequent loading of the resulting liposomal solution onto a TEM copper grids followed by uranyl acetate staining. The samples were then observed in a JEM 1010 TEM (JEOL, Tokyo, Japan).

2.5. Flowability

Powder flow properties were measured as the angle of repose, Carr index (C) and Hausner ratio (H). Powder was dropped through a funnel unto a metal disk, and the height as well as diameter of the settled cone was measured (Carr, 1965). The angle of repose (α) was calculated from the following formula:

$$\tan(\alpha) = \text{Height} / (0.5 \times \text{Base diameter})$$

For the Hausner ratio (H) and Carr index (C), a weighted quantity of proliposomal powder was filled into a volumetric cylinder and the bulk volume (V_{bulk}) as well as tapped volume (V_{tapped}) was determined.

$$H = 100 \times (V_{\text{bulk}} - V_{\text{tapped}}) / V_{\text{bulk}}$$

$$C = (\rho_{\text{bulk}} / \rho_{\text{tapped}})$$

2.6. Protein stability study by SDS PAGE

Stability of AAPE protein in aqueous solution and proliposomes was observed by using SDS PAGE. For the stability of unformulated AAPE in aqueous solution, 20 μg were dissolved in 2 ml PBS and stored at 4°C and 25°C. At each time point (1, 3, 7, 14 and 21 days), aliquot of samples (80 μl) were taken and SDS PAGE analysis was conducted. For the stability study of AAPE protein in dry proliposome, 50 mg samples which were being kept at 25°C were reconstituted in 500 μl PBS at each time point (1, 5, 30, 60 and 90 days). Stability of AAPE protein at 25°C after reconstitution was also determined for 90 days. The SDS PAGE analysis procedure was as followed: 80 μl samples was mixed with 20 μl sample buffer (containing 10% SDS), vortexed and incubated for 10 min at 80°C. ‘Precision Plus Protein’ internal standard (1 μl , 250 kDa, BioRad, Hercules USA) was then added. After vortexing for 10 seconds, 10 μl was loaded into each well of a 7.5% SDS PAGE. Gels were run at 200 V and silver stained with a ProteoSilver kit from Sigma Aldrich (St. Louis, USA). The gels were then photographed and images were densitometrically analyzed with GelEval (FrogDance Software, Dundee, UK). AAPE stability was measured as the intensity ratio between the internal standard band at 250 kDa and an AAPE protein band at 300 kDa. Percent of the marker protein intensity ratio at each time point was plotted, considering the initial value as 100%.

2.7. In vivo study

Atopic dermatitis was induced in NC/Nga mice by treating with 1% DNCB (2,4-dinitrochlorobenzene) dissolved in acetone/olive oil (3:1) (Traidl et al., 1999). The animal study was approved by the Institutional Animal Care and Use Committee of Inha University (Incheon, Republic of Korea). DNCB was applied 3 times per week for 2 weeks. Reconstituted liposomal solutions (200 μ l for dorsal skin and 100 μ l for ear) without (blank) or with AAPE (100 mg/ml) was then applied 5 times per week for 2 weeks, followed by serum IgE level determination using an IgE ELISA kit. At the end of the second week, all mice were sacrificed by ether anesthesia. The back skin and ear tissues were dissected and embedded in paraffin. For H&E staining, sections were stained with hematoxylin for 3 min, washed, and stained with 0.5% eosin for an additional 3 min. After an additional washing step with water, the slides were dehydrated in 70, 95, and 100% ethanol, and cleared in xylene. The sections were photographed using Nikon Eclipse TE2000-S inverted research microscope (Nikon Corp., Japan). To determine IL-5 mRNA expression in the dorsal skin biopsy of atopic dermatitis-like skin lesions, reverse-transcription PCR was performed. Briefly, total RNA was extracted from frozen dorsal skin with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Single-strand cDNA was synthesized from 1 μ g total RNA using a reverse transcription system (Promega, Madison, WI, USA) in a total volume of 20 μ l. Equal amounts of cDNA were subsequently amplified by PCR in a 25 μ l reaction volume containing 1X PCR reaction buffer, 200 μ M dNTPs, 0.5 pmol specific primer for IL-5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 1 units Taq DNA polymerase (TaKaRa Biotech, Shiga, Japan). GAPDH was used as an internal control of cDNA amplification. Amplification products were separated on 1.5% agarose gels and results were recorded by a molecular imaging system (Kodak, Rochester, NY, USA).

3. Results and discussion

3.1. Preparation of proliposomes

Proliposomes were prepared by evaporating a solution of AAPE, poloxamer and soy PC in ethanol on a sorbitol matrix under vacuum. This method was chosen based on the following preliminary studies with diverse matrix materials and methods. Mannitol and glucose gave similar results in regards to particle size, but exhibited less favorable handling characteristics due to its poor flowability (data not shown). Moreover, shock freezing of a matrix material soaked in a lipid, poloxamer and AAPE solution, followed by lyophilization under vacuum produced a fine and fiber like dust, but did not produce free flowing powder. On the other hand, the evaporation on matrix method based on the literature report (Song et al., 2005) could successfully produce the proliposome of AAPE with good flow property, and form liposomal solution upon reconstitution.

3.2. Characterization of proliposome particles

The proliposomal powder ranged from 0.5 mm to 1.5 mm in size. SEM imaging revealed that the crystal structure of the sorbitol particles, which was initially sharp and ragged, was successfully covered with a lipid film, resulting in a smoother surface (Figure 1A). Upon reconstitution of the proliposomal powder, a liposomal solution with bimodal polydisperse volume distribution of an average size of 589 ± 3.6 nm and 593 ± 21.2 nm for the AAPE containing liposomes and for the blank liposomes, respectively, were formed (Table 1). TEM imaging of the reconstituted liposomes revealed the formation of large spherical liposomes (Figure 1B). Zeta potentials of -51.33 ± 0.36 mV for AAPE proliposomes and -51.58 ± 2.29 mV for blank proliposomes were observed. Interestingly conventional film hydration liposome solutions with the identical composition exhibited a less negative zeta

potential of about -20 mV. The fact that there was no significant difference between the zeta potential of the blank and the AAPE containing proliposomes suggests that AAPE is not likely the cause of the low zeta potential. The correlation between anionic surfactant concentrations and liposome zeta potential is well known (Varshosaz et al., 2009) and could be the cause of the negative zeta potential observed (*e.g.*, by a higher association degree with the liposomal surface compared to conventional liposomes, resulting in higher surface charge density). Moreover, the observed zeta potential of -51.33 ± 0.36 mV in this study would suggest low aggregation of proliposomal powders during storage (Hanaor et al., 2012).

The ratio between the bulk and tapped density of a powder, or Hausner ratio (H), is a commonly used method of asserting the flow properties of a powder formulation. This in conjunction with the angle of repose and the Hausner ratio derived Carr index (C) gives a reliable estimate whether a formulation is free flowing or not. As shown in Table 1, the AAPE containing proliposomal formulation can be classified as free flowing or having fair flow properties based on its angle of repose ($35.1 \pm 0.3^\circ$), Carr index ($7.1 \pm 0.9\%$) and Hausner ratio (1.08 ± 0.01) (Carr, 1965).

3.3. Effect of proliposome on the stability of AAPE

Unformulated AAPE in aqueous solution exhibited poor stability at 4°C and 25°C when dissolved in PBS, resulting in less than 5% of marker protein remaining after 7 days at 25°C and below 25% remaining after 21 days at 4°C (Figure 2). However, increased stability was observed when AAPE was formulated as proliposomal powder, which resulted in $93.7 \pm 10.0\%$ of marker protein remaining after 90 days when stored at 25°C (Figure 3). Interestingly, when proliposomal powder was reconstituted in PBS and stored at 25°C , $7.3 \pm 4.5\%$ of marker protein remained after 90 days (Figure 3).

While a wide variety of denaturing factors might harm therapeutic proteins, most of them require an aqueous environment. Most prominent among them is common hydrolysis, followed by enzymatic degradation which also depends on the

hydration of the formulation. These results show that the elimination of water from the formulation significantly increased protein stability, possibly due to its non-hydrated state. It is also interesting to note that the stability of marker protein of AAPE in the reconstituted proliposomes is significantly higher than that of unformulated AASP in PBS at 25°C. About 40% of marker protein remained after 7 days at 25°C in the reconstituted AAPE solution (Fig. 3) while less than 5% of protein remained on the same day (Fig. 2). It appears that the proteins in the reconstituted liposomes are protected from degradation.

3.4. In vivo effect on atopic dermatitis mouse model

Previous reports have established that ADSCs secrete cytokines and exhibit diverse pharmacological actions (Raicevic et al., 2011; Lee et al., 2012; Ghajar et al., 2010). In this study, we investigated how far AAPE proliposomes may delay or inhibit the development of atopic dermatitis in DNCB treated Nc/Nga mice, which showed progressive diffuse erythematous changes and crusting on the back as well as histological findings of hyperkeratosis, parakeratosis, with spongiosis, exocytosis of mononuclear cells in the epidermis and infiltration of inflammatory cells into the upper dermis (Pokharel et al., 2008). When DNCB exposed mice were treated with AAPE proliposomes, the DNCB-induced dermatitis decreased in intensity (Figure 4). The extent of incrustation was particularly reduced by the administration of AAPE proliposomes. Moreover, histological studies revealed that thickening of the epidermis and infiltration of inflammatory cells was significantly lower in the AAPE proliposome-treated groups (Figure 5). The application of DNCB also elevated the serum IgE levels (Figure 6A), which is one of the indices for the development of dermatitis compared with normal mice (Vanoirbeek et al., 2006). It is interesting to note that the serum IgE levels were lower in the AAPE proliposome-treated group when compared to the DNCB control group (Figure 6A). T helper type 2 (Th2) cells release cytokines, such as IL-4, IL-5, and IL-13. These cytokines, in turn, induce class switching, making IgE producing plasma cell

(Fujita et al., 2012). To gain a better understanding of the molecular mechanism involved in the development of atopic dermatitis, we assessed the mRNA expression of IL-5 among Th2 cytokines, which are dominant in the acute phase (Leung and Bieber, 2003) in the dorsal skin lesions by RT-PCR. As shown in Figure 6B, repeated application of DNCB significantly increased mRNA expression of IL-5 in the DNCB-treated group versus the control group. This result indicates that AAPE proliposome treatment significantly inhibited the mRNA expression of cytokines.

4. Conclusions

These results showed that the proliposomes containing AAPE ameliorated the development of atopic dermatitis in Nc/Nga mice and thus may be applied as a treatment for atopic dermatitis. Additionally the formulation as a proliposomal powder facilitated long term stability of AAPE with rapid liposome formation upon reconstitution.

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Table 1: Characterization of AAPE and blank proliposomes after reconstitution in PBS and flow properties of dry proliposome powder (Mean \pm SD).

Parameter	unit	AAPE proliposome	Blank Proliposome
Diameter	nm	589 \pm 3.6	593 \pm 21.2
Polydispersity	-	0.307 \pm 0.005	0.323 \pm 0.013
Zeta potential	mV	-51.33 \pm 0.36	-51.58 \pm 2.29
Angle of repose (α)	degree	35.1 \pm 0.3	--
Carr index (C)	%	7.1 \pm 0.9	--
Hausner ratio (H)	--	1.08 \pm 0.01	--

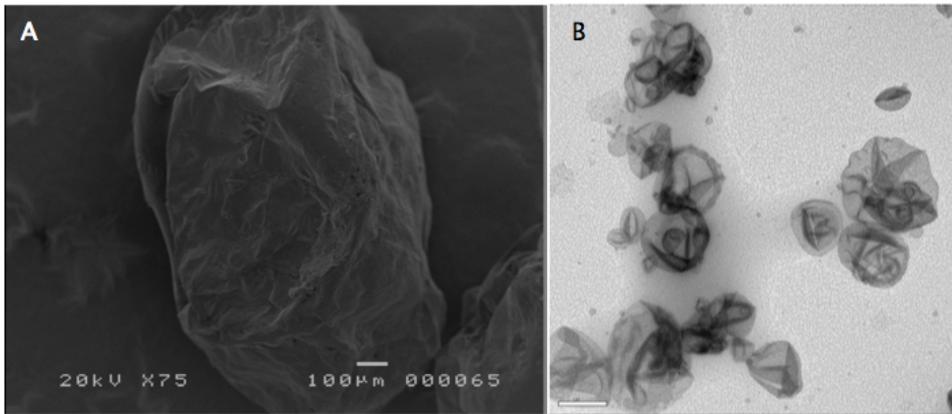


Figure 1. Morphology of AAPE proliposomes. (A) 75 x magnification SEM image of AAPE proliposome. (B) TEM image of uranyl acetate stained reconstituted AAPE proliposomes at 10k x magnification. The bar in the lower left corner represents 500 nm.

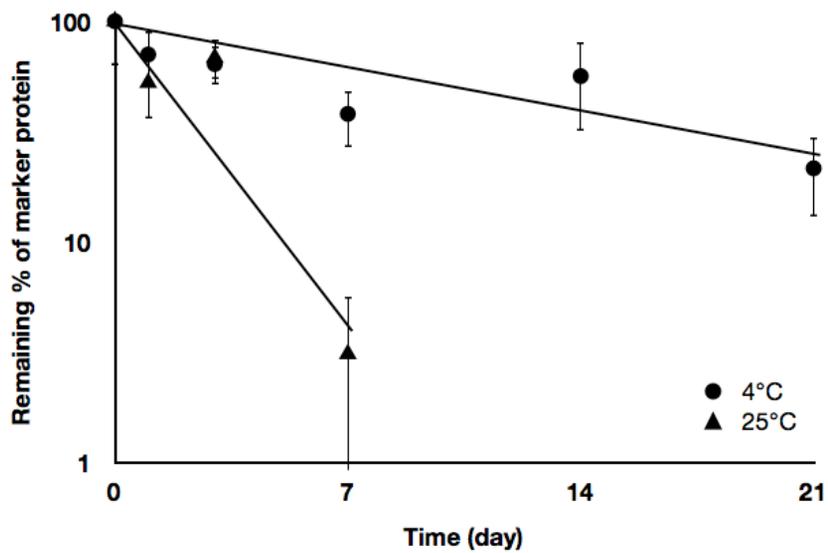


Figure 2. Stability of AAPE in PBS at 4°C and 25°C (mean±SD, n=3). Percent remaining of marker protein was determined by SDS PAGE.

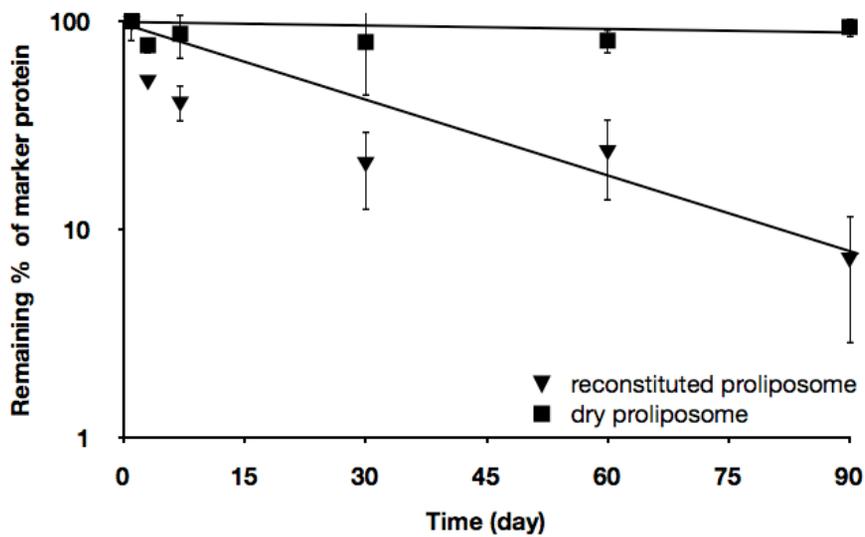


Figure 3. Stability of AAPE in dry proliposome at 25°C and after reconstitution in PBS. (mean±SD, n=3). Percent remaining of marker protein was determined by SDS PAGE.

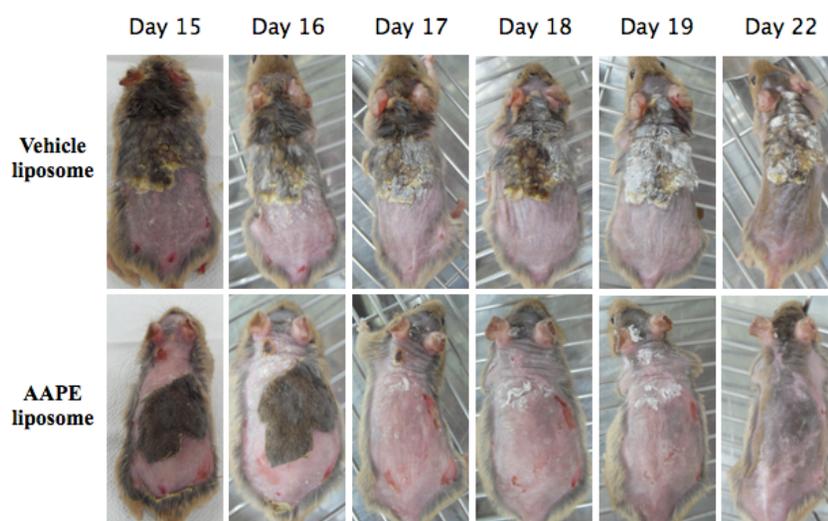


Figure 4. Skin condition of DNCB treated mice during blank and AAPE liposome treatment.

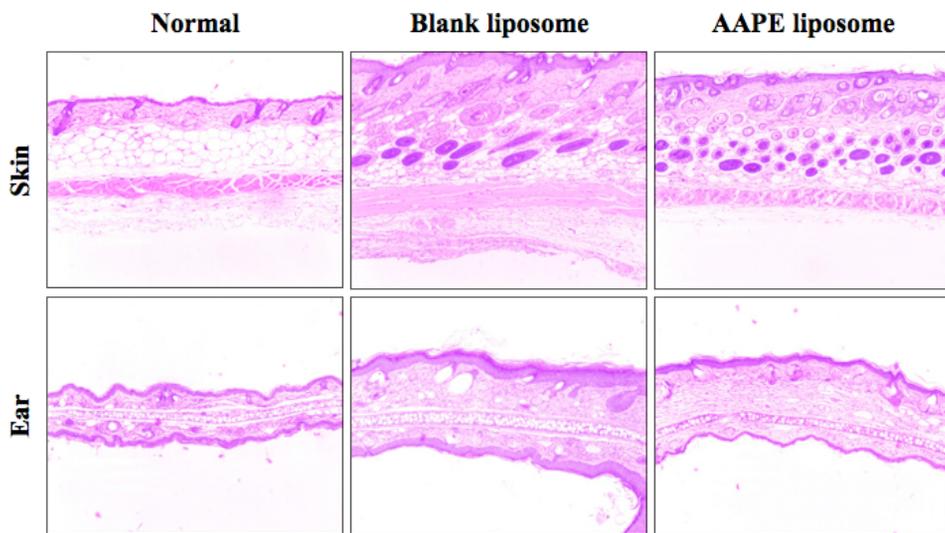


Figure 5. H&E stains of mice skin after treatment with AAPE proliposomes, following the induction of atopic dermatitis.

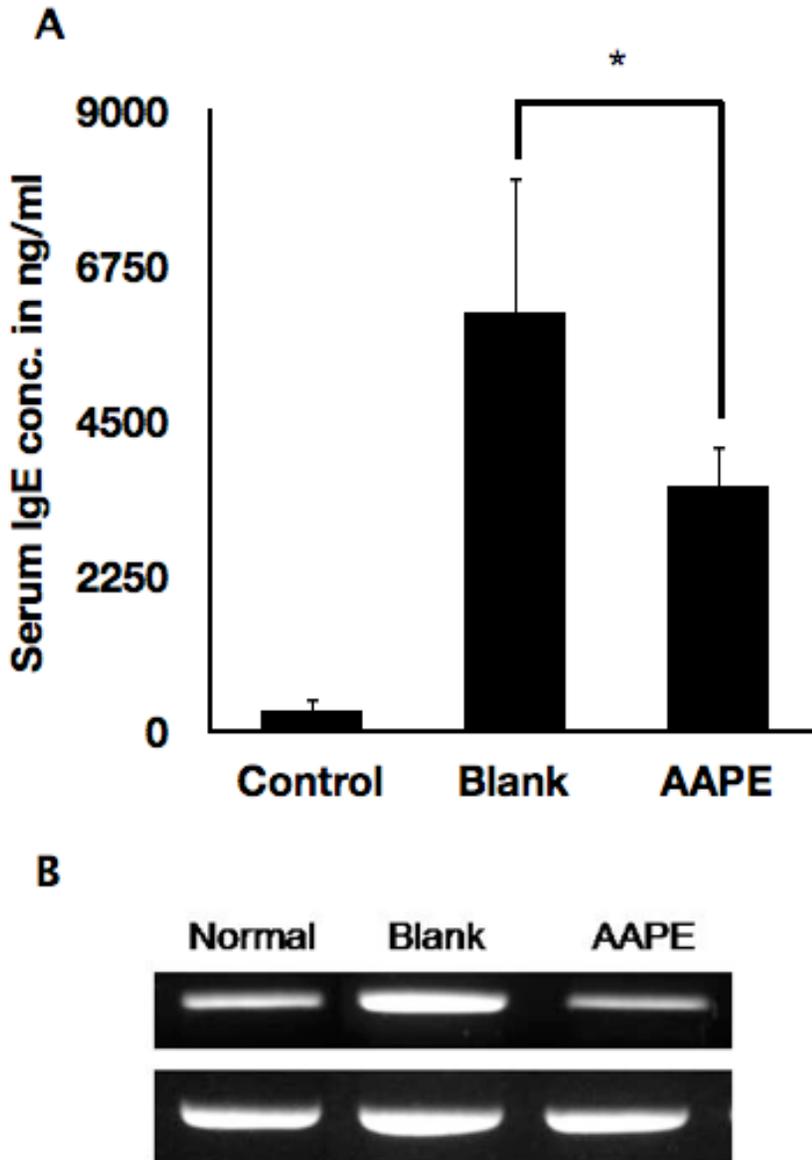


Figure 6. Serum IgE levels (A) and IL-5 mRNA expression (B) in mice after topical treatment with AAPE proliposomes and blank proliposomes following the induction of atopic dermatitis. *: IgE levels in the AAPE treated group are statistically significant, different from the blank treatment with a $p < 0.05$; $n=3$.

6. 국문초록

본 연구의 목적은 지방세포로부터 유리된 개선된 줄기세포 단백질 추출물 (advanced adipose stem cell derived protein extract (AAPE))를 프로리포솜 제형으로 개발한 후 이 제형이 국소적 아토피 피부염 치료에 어떠한 효과가 있는지에 대해 밝히는 것이다. AAPE의 소염효과는 단백질의 분해를 최소화함으로써 개선될 수 있다. 이러한 단백질 분해효과를 최소화하기 위하여 본 연구에서는 에탄올에 녹인 soy phosphadityl choline, AAPE와 폴록사머 407을 진공 상태에서 소비톨 파우더에서 기화시켜서 건조된 프로리포솜 제형을 만들었다. 그리고 이 제형의 물리적 특성(제타전위, 지름, 안정성, 유동성)과 더불어 피부염 마우스 모델에서의 효과를 평가하였다. 프로리포솜 파우더를 재구성하여 리포솜을 만들었을 때, 지름은 589 ± 3.6 nm, 제타전위는 -51.33 ± 0.36 mV였다. 프로리포솜에 봉입된 단백질의 안정성이 25°C에서 90일 동안 유지된 것을 확인하였다. 아토피 피부염 마우스 모델에서의 동물실험결과, AAPE 프로리포솜을 처리하였을 때 IgE 수치가 유의적으로 감소하는 것을 확인하였다. 이로서, AAPE 프로리포솜이 아토피 피부염 치료제로서 유망한 제형이 될 가능성이 있음을 알수있었다.

주요어: 자가지방유래 증배염성 줄기세포, 개선된 자가지방유래 증배염성 줄기세포 단백질 추출물, 프로리포솜, 리포솜, 아토피 피부염

학번 : 2009-31313

PART II. A Layered Proliposomal
Formulation With Increased Itraconazole
Content

1. Introduction

Liposomes, vesicles formed by lipid bilayers are a well researched drug delivery system mostly used for intravenous drug delivery (Chang and Yeh, 2012). Current approaches try to overcome key limiting factors like low circulatory times due to clearance by the reticuloendothelial system (RES) (Awasthi et al., 2003), short shelf life due to liposome aggregation and drug instability in aqueous environment (Lee et al., 2002; Quintilio et al., 2000). Furthermore targeted drug delivery is either attempted with passive methods like the utilization of the tumor related enhanced permeation and retention (EPR) effect or with surface modifications like lipid-targeting-moiety conjugates leading to increased drug accumulation on the target site (Gabizon et al., 2010).

However other aspects needed for the general popularization of liposomal drug delivery systems are often not considered: manufacturing aspects including up-scalability, poor patient compliance for non oral formulations, non solution formulations leading to tableted liposomes as well as the economic feasibility of production methods and materials (Storm and Crommelin, 1998).

Proliposomes are a dry, free flowing formulation which upon resuspension in water or related physiological liquid produces liposomes (Payne et al., 1986). These formulations can be grouped into two classes: lyophilized liposomes and lipid film proliposomes. Lyophilized liposomes are often freeze dried with added cryoprotectants and are then re hydrated upon application (Stark et al., 2010). These do offer benefits in stability and the possibility to produce dry formulations but do still require a multi step manufacturing process most commonly involving a

thin film deposition with solvent evaporation followed by film hydration and liposome formation as well as subsequent lyophilization (Misra et al., 2009; Stark et al., 2010). The major advantage is that the liposome production is a well established method and easily done in the lab scale with inexpensive equipment like a rotaevaporator and freeze driers for lyophilization. However this method does not allow the product to be pressed into tablets where the compression induced shear forces would disrupt lyophilized liposomes. On the other hand lipid film proliposomes are produced by depositing a film of lipid, drug and additional components like surfactants or stabilizers on a soluble matrix which when resuspended dissolves and liberates the lipid / drug film which then spontaneously forms liposomes (Song et al., 2002). This method offers the same benefits as lyophilized proliposomes but also allows tableting as well as economic interesting production methods like fluidized bed coating for a single step manufacturing process (Chen and Alli, 1987). Unfortunately this method is not without drawbacks. First among them is the necessity for a soluble matrix on which the lipid film is deposited. The carrier material is making up the bulk of the final formulation (often exceeding 90%)(Xu et al., 2009) and considering the maximum size for a oral tablet of about 1 to 1.5g in this leaves only about 100mg for active pharmaceutical ingredients. However the liposome forming lipid should also exceed the drug to ensure a high encapsulation efficiency (Agnihotri et al., 2010). This limitation in total drug amount per tablet makes this formulation unfeasible for most drugs except high efficacy drug needing very small doses. To improve the feasibility of proliposomal formulations an increased the drug and lipid amount would be desirable. Lipids like phosphatidyl cholin from egg or soya bean are sticky and lead to non free flowing powder when exceeding certain film thickness, prohibiting simple increased lipid percentage as a mean to increase the total drug amount. Adding layers of water soluble polymer between multiple, thin lipid layers might improve the flowability of proliposomal powder.

A practically water insoluble BCS 2 drug Itraconazole was chosen as a model drug in the investigation of the dissolution profiles as well as bioavailability and general feasibility of the multilayered proliposomal formulation. Itraconazole a member of the triazole group is an anti-fungal agent effective against a wide variety of fungi including *Cryptococcus*, *Candida*, *Aspergillus* and *Blastomyces* (Yamazaki et al., 2010). It is mostly utilized in the treatment of systemic, topical or intestinal infections. Itraconazole shows however poor transport across the blood brain barrier and is therefore not the first choice for the treatment of fungal meningitis (Imbert et al., 2003). While exhibiting good penetrating characteristics, Itraconazole's low solubility results in a maximum bioavailability of 55% (Prentice and Glasmacher, 2005). A formulation able to increase the solubility of itraconazole might be able to increase overall bioavailability and in turn reduce the necessary dose.

Herein a multilayer proliposomal tablet containing itraconazole for oral delivery and a practical production method for increased drug content in proliposomal formulations utilizing a fluidized bed coater was proposed.

2. Methods and Materials

2.1. Materials

Soya phosphatidyl choline was purchased from Lipoid (Ludwigshafen, Germany). Ethanol (99.99%; anhydrous), Dichloromethane (99.5%) and Sorbitol were obtained from Daejung (Shiheung, South Korea). Poloxamer 407 was kindly donated by BASF (Ludwigshafen, Germany) Water was double distilled and filtered with a MilliQ Plus system from Millipore(Billerica, US). Itraconazole was provided by DongKoo Pharm (Seoul, Korea). Tween 80 and monostearin were purchased from Tokyo Chemical Industry (Tokyo, Japan) Eudragrit E100 and L100 were donated by Evonik (Essen, Germany)and HPLC grade Acetonitril was purchased from Sigma Aldrich (St. Louis, US).

2.2. Preparation of proliposomes

Proliposomes were produced by depositing a coating solution on 600 μ m sorbitol particles in a Freund ‘Spiraflo’ fluidized bed coater. Inlet flow was kept at 37°C, stirrer speed at 60rpm with back pulsing on and spray air flow set to appropriate conditions to achieve nebulization. Coating solution was deposited at aprox. 3ml/min in continuous spray mode. The drug / liposome layer solution was prepared by placing the appropriate amount Itraconazole in a mixture of 100ml water free Ethanol and 100ml Dichloromethane, followed by 10g Soy PC and 1g Poloxamer 407 dissolved in 10ml water. The solution was kept agitated. Barrier layer coating solution was prepared by dissolving 3g E100 or L100 in 100ml Ethanol, followed by the addition of 0.3g Tween 80 as plasticiser and 0.15g Monostearin as an anti tacking agent.

2.3. Flow properties of proliposomal powder

Flow test measurements were performed with 8,10, 11.3 and 15mm nozzles. All experiments were performed in triplicates on a GTL flowtester (Erweka, Heusenstamm, Germany) .

2.4. Preparation and physical characterization of proliposomal tablets

Tablets were prepared from proliposomal powder without the addition of further excipients. An EP-1 tablet press (Erweka, Heusenstamm, Germany) with a 20.00 x 8.65 mm die / punch set was used. Hardness, height and width were measured with an Erweka TBH 300 tablet tester (Erweka, Heusenstamm, Germany).

2.5. Preparation of liposomes from proliposomal powder and tablets

Liposomal solution was prepared by resuspension of 0.1% (w/v) proliposomal powder or tablets in water or 0.1N hydrochloric acid under gentle agitation.

2.6. HPLC analysis of itraconazole

Samples were first diluted 1:10 with Methanol to disrupt liposomes and then separated on a Phenomenex Gemini-NX 5 μ C18 column with 70% Acetonitril, 30% water as mobile phase on a Waters E2695 HPLC instrument. Itraconazole concentration was measured with a Waters 2475 fluorescence detector set to 260nm excitation and 365nm detection.

2.7. SEM images of proliposomes

SEM was used to investigate the surface morphology of the proliposomes. Samples were mounted on brass stubs with conductive carbon tape and coated for 120 seconds with gold in a sputter coater. Thus prepared samples were observed in a JSM-5310LV Scanning electron microscope (JEOL, Tokyo, Japan) operating at an accelerator voltage of 15kV. Images were captured with the SemAfore 5.1 software (JEOL, Tokyo, Japan).

2.8. Liposome size, zeta potential and size distribution

Average size, size distribution and zeta potential of liposomes resuspended in water was measured via Electrophoretic Light Scattering, using a Photal ELS Z (Otsuka Electronics, Osaka, Japan). Solvent parameters for water were used.

2.9. TEM images of liposomes

Proliposomal powder was resuspended in water (1% w/v) and shaken gently. After dissolution liposomal solution was loaded onto copper grids, stained with uranyl acetate solution and washed twice with water. The copper grids were dried under ambient conditions and observed under a JEM 1010 TEM (JEOL, Tokyo, Japan).

2.10. Encapsulation efficiency

Encapsulation efficiency was measured as:

Encapsulation Efficiency in % = $\frac{(\text{Amount in solution before filtration} - \text{Amount in solution after filtration})}{\text{Amount in solution before filtration}} \times 100$

Drug was separated from the liposomes via centrifugation assisted ultrafiltration with Centrisart-1 ultrafiltration units (Sartorius Stedim Biotech GmbH, Goettingen, Germany) at 1500 RCF.

2.11. X-Ray diffraction

Powder XRD patterns of Itraconazole, Sorbitol and F-6 proliposomal formulation were recorded on a D8 FOCUS (Bruker AXS, Billerica, MA, USA). Scans were performed from 5 to 50° (2 θ) at 2° per minute. A Cu K α 1 radiation source with a wavelength of 0.154056nm was used.

2.12. Dissolution study

The USP apparatus 2 (Paddle) was used to investigate the dissolution behaviour of itraconazole proliposomal powder pressed into tablets. The dissolution media was 0.1 N hydrochloric acid kept at 37°C, stirring speed was set to 50 RPM and samples were drawn at 5, 10, 20, 30, 60, 120, 180, 300, 480 and 720 minutes.

3. Results and Discussion

The particle size of resuspended proliposomes was in the range of 800 to 1500nm as shown in table 1. The solvent parameter were not adjust to the amount of sorbitol, Poloxamer 407 or Eudragrit E100 in solution and therefore different formulations when resuspended have different optical densities as well as viscosities. The increasing average size with increasing drug / layer content could be thus be caused by increased viscosity when higher polymer concentrations are found in the solution leading to decreased particle mobility and therefore increased apparent particle size. This theory would be supported by the TEM images (figure 1) where no noticeable liposomes size difference is apparent. The apparent zeta potential of -16.8 to -24mV might be affected in a similar fashion. The size of the vesicles as well as the production method would suggest that large multi lamella vesicles were formed (Maestrelli et al., 2006) which may be advantageous for the inclusion of hydrophobic drugs in the inter lamella space due its relative lipophilic environment. However the ratio of drug to lipid did not change when the number of layers was increased (table 2) and therefore it is not likely that the increase in entrapment efficiency with increased number of layers is based on this mechanism. A recent paper by Alasino et al investigated Lipid - E100 interaction and reported that E100 can interact or penetrate lipid membranes or even form single layers them selves (Alasino et al., 2012). This could hint at interesting, until now not described interactions between E100 and liposome systems which might influence also the membrane - lipophilic drug interaction and is in our opinion worth further investigation.

The flow properties of powders are determined by a variety of factors and are difficult to predict accurately (Yu et al., 2011). Spherical particles of uniform size minimize contact surface and thus total friction, leading to improved flowability

(Kaerger et al., 2004). Furthermore the particle surface material determines the coefficient of friction and therefore also influences the flowability of a powder (Podczeczek and Mia, 1996). To investigate the effect of proliposomal coating on the flow properties of raw 650 μ m sorbitol we utilized a direct flow tester as recommended by the European pharmacopoeia (The European Pharmacopoeia). Sorbitol as well as proliposomal particles were roughly spherical with smoother surface in proliposomes compared to sorbitol (figure 2). Particle sizes appeared homogeneous but were not specifically measured. Sorbitol, a non cohesive powder, is generally considered a free flowing powder (Jivraj et al., 2000) and was used as reference. Proliposomal formulations with increasing number of layers and thus increasing amount of lipid as well as increasing mean particle diameter generally exhibited slightly worse flow properties but still were comparable to sorbitol (table 3). The possibly increased friction coefficient due to the increasing lipid content was apparently well compensated for by covering with the E100 layering material as well as the smoother surface produced by the coating process. We have made the experience that with 650 μ m sorbitol particles, a surface coat of above 5% w/w Soy PC becomes very sticky and does not flow at all. Formulation F4, the only formulation utilizing PVA as a barrier material showed poor flowability which correlates well with the observed rough surface morphology (data not shown). L100 coated proliposomes also exhibit fair flow properties which may be of use in future projects where enteric coated proliposomal particles are desired.

The E100 proliposomal powder was subjected to direct compression to form proliposomal tablets without further additives. Especially no additional lubricant was necessary due to the presence of soy pc and monostearin which facilitated good tablet release behavior. Tablets of 1g were manufactured and accurate weights as well as dimensions can be found in table 4. Hardness was not significantly different up to F6, which contains 3 layer proliposomal powder but significantly decreased when 5 layered proliposomal powder was used. This is likely caused by the increased Soy Pc, and E100 content, both materials with hardness significantly lower than that of sorbitol.

Itraconazole is practically water insoluble but slightly soluble under acidic conditions (Shin et al., 2004). Dissolution profiles of itraconazole tablets (drug content in figure 3) were analyzed in physiological relevant conditions (0.1N HCl). Under these conditions pure Itraconazole showed extremely low dissolution rates, resulting in 0.5% dissolution after 12 hours (figure 4), making dissolution unlikely before the formulation passes the gastric tract. Proliposomal formulations of itraconazole on the other hand show significantly improved dissolution rates and were able to bring large quantities of itraconazole into solution. After 60 minutes 80% of the single layer itraconazole (F2) was dissolved while increasing E100 content (F6 and F7) needed 120 and 360 minutes respectively to achieve the same relative dissolution. This increase in total dissolution as well as dissolution rate can be explained with the encapsulation of drug into liposomes as well as drug localization in to the lipid bilayer, which provides a hydrophobic environment likely to promote dissolution of the \log_p 5.66 drug itraconazole. Furthermore the change in physical state of itraconazole from crystalline to amorphous as indicated by the absence of the marked peak in the x-ray diffraction pattern of proliposomal formulation (figure 5) is likely to have increased the dissolution rate further. It could be also interesting to investigate whether the lipid - itraconazole film forms a solid dispersion or a lyotropic liquid crystal in which the itraconazole is dissolved. Contrary to our expectations did the inclusion of the disintegrant Polyvinylpyrrolidone (PVPP) not increase the dissolution rate. One possible explanations could be the coating of the PVPP particles with the hygroscopic barrier material Soy PC during tablet pressing. Making the lipids dissolution the rate limiting factor.

4. Conclusions

Layering via fluidized bed coating is a viable approach to increase drug content in proliposomal formulations while maintaining good flowability. Handling characteristics were excellent and the formulation could be tableted without additional excipients. Furthermore were dissolution rates depending on the number of barrier layers and subsequently the amount of barrier layer material in the final formulation. This also opens interesting new possibilities for the manufacturing of timed release proliposome granules. Overall the concept of layered proliposomes and tablets thereof was investigated and found to be a viable approach for oral drug delivery.

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Table 1. Size, polydispersity, zeta potential and encapsulation efficiency of prololiposomal formulations.

Name	Number of layers	Size \pm SD in nm	PI	Zeta potential in mV	Encapsulation Efficiency in %
F1	1	858.7 \pm 46.6	0.370 \pm 0.020	-24.0 \pm 7.3	-
F2	1	1022.0 \pm 60.6	0.354 \pm 0.015	-22.4 \pm 3.8	70.8 \pm 1.1
F6	3	1014.3 \pm 6.07	0.332 \pm 0.008	-16.8 \pm 0.5	87.7 \pm 0.7
F7	5	1524.7 \pm 63.3	0.389 \pm 0.02	-23.5 \pm 6.0	90.9 \pm 0.2

Table 2. Name, composition and drug content of proliposomal formulations.

Name	Layer	Soy PC	Poloxamer 407	Itra	PVA	L100	E100	Tween 80	Monoste arin	Sorbitol
F1	1	5	1	-	-	-	-	-	-	95
F2	1	5	1	2.5	-	-	-	-	-	92
F3	3	10	1	5	-	2	-	0	-	82
F4	3	10	1	5	2	-	-	0	0	82
F5	3	10	1	5	-	2	-	0	0	82
F6	3	10	1	5	-	-	2	0	0	82
F7	5	15	2	8	-	-	4	0	0	75

Table 3. Flow properties of various proliposomal powders and 650 μ m Sorbitol as reference. Values are given in g/s flow trough nozzles of different sizes.

Name	15mm	11.3mm	10mm	8mm
Sorbitol	25.6 \pm 1.5 g/s	13.3 \pm 0.0 g/s	9.6 \pm 0.5 g/s	*
F1	25.1 \pm 1.3 g/s	12.9 \pm 0.0 g/s	8.8 \pm 0.1	*
F2	25.0 \pm 0.0 g/s	12.5 \pm 0.0 g/s	8.5 \pm 0.0	4.3 \pm 0.1 g/s
F4	17.8 \pm 0.6 g/s	8.5 \pm 0.2 g/s	*	*
F5	22.6 \pm 0.6 g/s	11.0 \pm 0.1 g/s	7.6 \pm 0.1	4.0 \pm 0.1 g/s
F6	26.1 \pm 0.8 g/s	13.2 \pm 0.2 g/s	9.2 \pm 0.1	5.0 \pm 0.0 g/s
F7	21.8 \pm 0.5 g/s	10.2 \pm 0.3 g/s	6.9 \pm 0.5	*

*: Hangs up

Table 4. Weight, hardness, thickness and length of proliposomal tablets. n=5

	Weight \pm SD in mg	Hardness \pm SD in kp	Thickness \pm SD in mm	Length \pm SD in mm
F2	1021.20 \pm 19.76	18.12 \pm 1.86	6.41 \pm 0.05	20.03 \pm 0.33
F6	1016.20 \pm 7.36	21.86 \pm 1.20	6.03 \pm 0.01	20.01 \pm 0.42
F7	1009.00 \pm 15.56	9.99 \pm 1.46	6.32 \pm 0.12	20.25 \pm 0.51

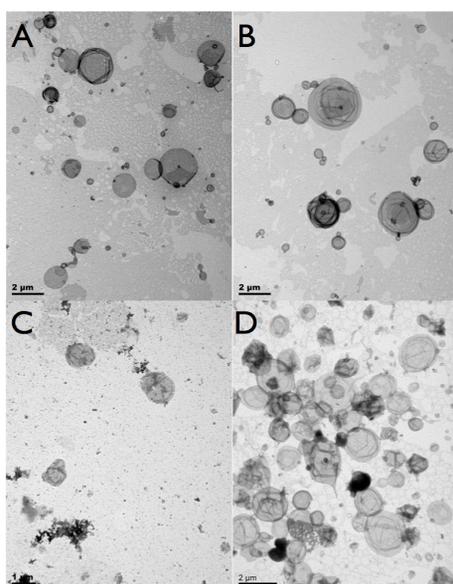


Figure 1: Transmission electron micrographs of blank, F2, F6 and F7 proliposomes. The bar in the lower left corner represents 2 μ m (panel A, B and D) respectively 1 μ m (panel C)

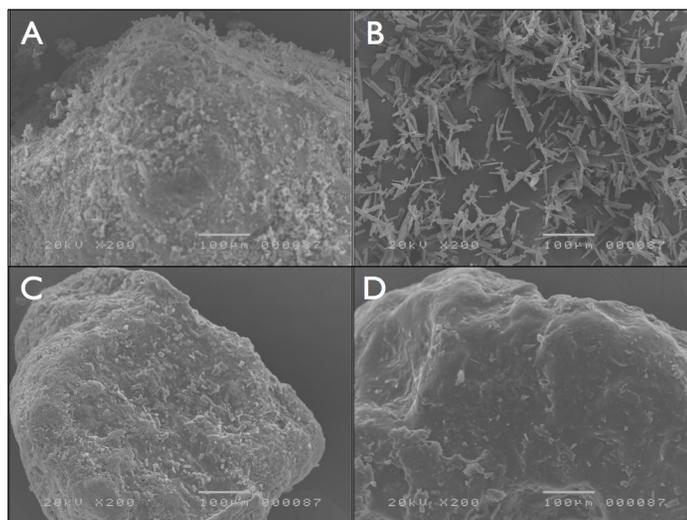


Figure 2: SEM images of sorbitol (A), itraconazole (B), F6 (C) and F7 (D). 200 x magnification, the bar equals 100µm.

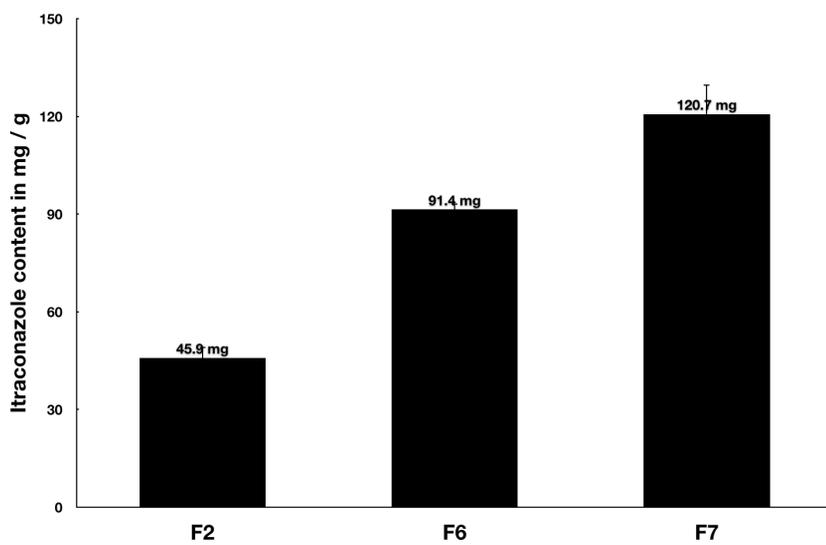


Figure 3: Itraconazole content in proliposomal tablets.

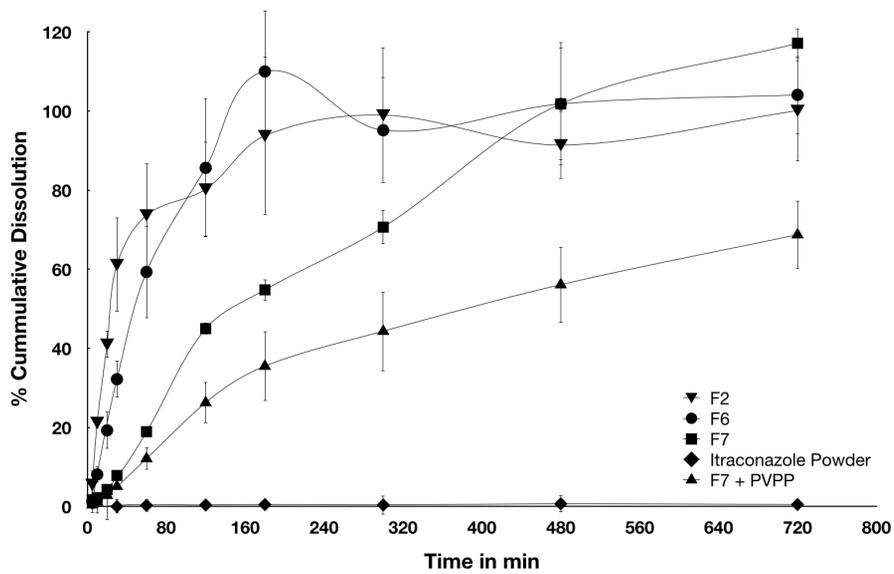


Figure 4: Figure 4: Dissolution profile of F2, F6, F7, and raw itraconazole in an USP type 2 (paddle) apparatus at 37°C in 0.1N HCl.

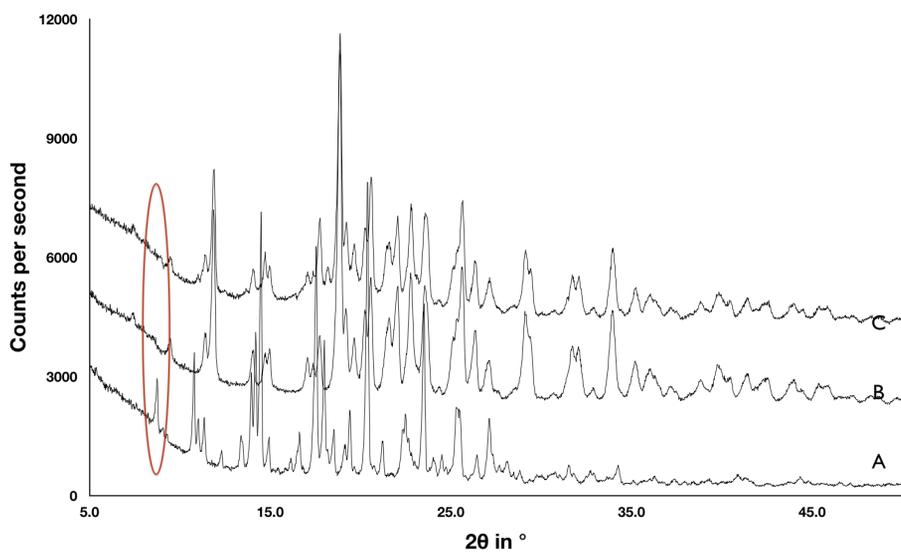


Figure 5: Powder X-ray diffraction pattern of Itraconazole (A), Sorbitol (B) and F-6 proliposomes (C)

6.국문초록

프로리포솜은 약물의 용해도를 증가시킬 수 있는 장점이 있지만, 봉입할 수 있는 약물의 총량이 적다는 단점이 있다. 그런데 실생활에서 사용되는 경구제제는 1g 정제에서 100mg의 약물함량(10%)를 포함 할 수 있어야 한다. 그러므로 높은 약물함량을 가질 수 있는 프로리포솜을 만드는 방법이 개발되어야 한다. 본 연구에서는 이러한 제형을 만들기 위하여, 소비톨을 기반물질로 하여 계면활성제로SoyPC와 폴록사머 407을 계면활성제로 사용하였고 유동층조립법으로 프로리포솜을 만들었다. 그리고 Eudragit E100을 이용하여 프로리포솜을 다층성 프로리포솜으로 만들었다. 용출실험은 USP기준에 따라서 진행하였다. 재분산된 리포솜을 포함하여, 프로리포솜의 유동성, 크기, 제타전위와 같은 물리적 특성을 확인하였다. 다층성 프로리포솜의 표면형태와 유동성은 기존 물질과 비슷한 값을 보였다. 이 프로리포솜을 재분산 시켰을 때 얻어진 리포솜의 크기는 859-1525nm, 제타전위는 -16.8mV to -24.0mV값을 가졌고 봉입 효율은 70%이상이 나왔음을 확인하였다. 단층성 프로리포솜은 한 시간에 80%의 용출율을 보였고, 다층성 프로리포솜은 2시간 안에 80%이상의 용출율을 보였다. 이러한 결과는 다층성 프로리포솜이 리피드와 약물의 함량을 증가시키고, 조작성이 쉬우며 빠른 용출을 보이는 제형이라는 것을 암시한다

주요어: 프로리포솜, 리포솜, 아토피 피부염, 다층화, 유동층조립법, 이트라코나졸

학번 : 2009-31313

Appendix

Curriculum vitae

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Education:

2009 - Present Ph.D in Pharmaceutics at Seoul National University,

relevant courses include: pharmacokinetics, physical pharmacy and soft nanomaterials

2005 - 2009 M.Sc. in Biotechnology at Dongseo University Busan

relevant courses include: genetics and chemical technical analysis

2002 - 2009 Dipl.Ing. in Biotechnology at Technical University of Berlin

relevant courses include: process engineering and mechanical engineering

Current Research Focus:

Liquid crystal microfluidic systems, design and manufacturing of pharmaceutical research instruments, particulate Drug Delivery Systems, self emulsifying Drug Delivery Systems, novel colloidal systems and their preparation, especially proliposomes.

Skills:

Chemical synthesis / analysis, chromatography (HPLC, GC, TLC etc.), computer assisted manufacturing (CAM, CAD, CNC and 3D printing), drug manufacture (FBG, pan coating, tableting, Spray drying), FACS, imaging (SEM, TEM etc.), in vitro / ex vivo studies (Franz and Ussing chamber), spectroscopy (IR, UV, NMR, MS) etc.

Software proficiency:

AutoCad, Inventor, Maya (dynamics / rendering), RhinoCAM, CAMBam, VisualMill, Mach3 etc.

Languages:

German:	Native
English:	Near Native (TOEIC 990 pts.)
French:	Basics
Korean:	Basics

Relevant experience:

- | | |
|-----------|---|
| 2013-2014 | Part time lecturer (CNC / CAM) at FABLAB / Tide Institute Seoul |
| 2006-2008 | Organized graduate level laboratory courses (chemical technical analysis) |
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Manuscripts in preparation:

Jahn A, Shim CK, Chung SJ, Kim DD. A Novel Multilayer Proliposomal Formulation with Increased Itraconazole Content. Manuscript in preparation

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Song CK, Lee JH, **Jahn A**, Choi MJ, Namgoong SK, Hong SS, Chong S, Shim CK, Chung SJ, Kim DD. In vitro and in vivo evaluation of N,N,N-trimethylphytyosphingosine-iodide (TMP) in liposomes for the treatment of angiogenesis and metastasis. International Journal of Pharmaceutics. 2012, 434(1-2), 191-198

Jahn A, Kim SY, Choi JH, Kim DD, Ahn YJ, Yong CS, Kim JS. A bioassay for mosquito repellency against *Aedes aegypti*: method validation and bioactivities of DEET analogue. *Journal of Pharmacy and Pharmacology*. 2010, 62(1), 91-97

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(Alexander JAHN, Jae Young LEE, Dae-Duk KIM)

2. Lyotropic liquid crystal systems in drug delivery: A review

(Dong-Hwan Kim, Alexander Jahn, Sung-Joon Cho, Jung Sun Kim, Min-Hyo Ki, Dae-Duk Kim)

RESEARCH ARTICLE

AAPE proliposomes for topical atopic dermatitis treatment

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Abstract

Context: Anti-inflammatory effect of advanced adipose stem cell derived protein extract (AAPE) could be improved by minimising protein degradation. **Objective:** To develop a proliposomal formulation of AAPE for the treatment of topical atopic dermatitis. **Materials and methods:** Proliposomal powder was manufactured by evaporating a solution of soy phosphatidyl choline, AAPE and Poloxamer 407 in ethanol under vacuum on sorbitol powder. Characterisation of proliposomes (zeta potential, diameter, stability and flowability) as well as *in vivo* efficacy in a dermatitis mouse model was investigated. **Results and discussion:** Reconstitution of the proliposomal powder formed liposomes of 589 ± 3.6 nm diameter with zeta potential of -51.33 ± 0.36 mV. Protein stability was maintained up to 90 days at 25 °C as proliposomes. *In vivo* studies on atopic dermatitis mouse model showed a significant reduction in IgE levels after topical AAPE proliposome treatment. **Conclusion:** AAPE proliposomes maintained protein stability and showed promising results for atopic dermatitis treatment.

Keywords

ADSC, AAPE, atopic dermatitis, liposomes, proliposomes

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Introduction

Topical atopic dermatitis is a condition of the skin involving an inflammatory response to external or internal stimuli. Common causes are sensitisation with environmental toxins or induction by direct exposure to a wide range of allergens and inflammation-inducing agents as well as microbes (Leung et al., 2004). In recent years, the prevalence of inflammatory skin diseases, in particular atopic dermatitis increased drastically (Boguniewicz and Leung, 2011). Among the suggested explanations are the increased concentration of allergens and environmental toxins as well as increased prevalence of obesity and changes to the human diet in westernised countries (Devereux, 2006). These trends were not only observed in countries with more advanced economies but also in developing countries (Liao et al., 2009). As there is currently no cure, the treatment of atopic dermatitis is often limited to managing the symptoms, especially the inflammatory response (Finch et al., 2010). A wide variety of drugs is used for this purpose, among which are topical applications of various cortical steroids. However, these steroidal anti-inflammatory drugs are associated with various side effects, which limit long-term medication (Aschoff et al., 2011; Watson and Kapur, 2011). Several chemokines and cytokines might reduce the inflammatory response avoiding the risk of unwanted side effects from corticosteroids (Chi et al., 2006). A topical formulation containing these messenger molecules could thus be used for the treatment of atopic dermatitis.

Human adipose-derived stem cells (ADSC) are interesting subjects in regenerative medicine. They are considered an interesting source of therapeutic proteins due to the possibility of harvesting large quantities from adipose tissues as well as their low immunogenicity and the large number of soluble factors that can be derived (Tallone et al., 2011). While some properties, e.g. in the inhibition of t-cell proliferation via t-cell contact-induced human leukocyte antigen excretion, require physical contact of ADSCs to target cells (Selmani et al., 2008), there is also a large variety of constantly excreted soluble factors such as HGF, TGF- β and IL-6, suggesting non-contact dependent activity (Di Nicola et al., 2002; Djouad et al., 2005; Groh et al., 2005). Recent publications have demonstrated that ADSCs excrete anti-inflammatory factors into the adipose derived stem cell culture media (ADSC-CM) (Newman et al., 2009), and furthermore anti-inflammatory and anti-oxidant properties of its lyophilisate ‘‘advanced adipose stem cell derived protein extract’’ (AAPE) have been observed (Kim et al., 2008). Additionally, a study of AAPE’s effect on human keratinocytes (HK) found that 64% of the protein present belongs to the collagen class and that HK proliferation and migration increased in the presence of AAPE (Moon et al., 2012).

Protein drugs in general are prone to degradation due to a variety of mechanisms including hydrolysis, aggregation as well as chemical and post-translational modification (Jiskoot et al., 2012). Moreover, AAPE as a complex mixture may also contain proteolytic factors expressed by ADSCs (Lai et al., 2012). The arising drug stability problem could be solved with a dry storage formulation. A systemic delivery via the oral route would expose the protein factors to a denaturing environment and thus a topical delivery would be necessary. However, this would lead to another challenge: low skin permeation and deposition of proteins due to the lipophilic character of the stratum corneum. Although the use

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Pharmaceutical Nanotechnology

In vitro and *in vivo* evaluation of *N,N,N*-trimethylphosphingosine-iodide (TMP) in liposomes for the treatment of angiogenesis and metastasisChung Kil Song^a, Ju-Hee Lee^b, Alexander Jahn^a, Myeong Jun Choi^c, Sung Keon Namgoong^d, Soon-Sun Hong^b, Saeho Chong^a, Chang-Koo Shim^a, Suk-Jae Chung^a, Dae-Duk Kim^{a,*}^a College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, South Korea^b School of Medicine, Inha University, Incheon 400-712, South Korea^c Phytos Co., Gyeonggi University, Suwon, Gyeonggi-do 443-760, South Korea^d Department of Chemistry, Seoul Women's University, Seoul 139-774, South Korea

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ABSTRACT

Phytosphingosine and methyl derivatives are important mediators on cellular processes, and are associated with cell growth and death. The antitumor activity of *N,N,N*-trimethylphosphingosine-iodide (TMP) as a novel potent inhibitor of angiogenesis and metastasis was evaluated in B16F10 murine melanoma cells. The results indicated that TMP itself effectively inhibited *in vitro* cell migration, tube formation, and the expression of angiogenic factors as well as *in vivo* lung metastasis. However, TMP slightly suppressed *in vivo* experimental tumor metastasis in its free form and induced side effects including hemolysis and local side effects. Therefore, in an attempt to reduce the toxicity and the undesirable side effects of TMP, a liposomal formulation was prepared and tested for its effectiveness. TMP liposomes retained the effectiveness of TMP *in vitro* while side effects were reduced, and both *in vivo* experimental and spontaneous tumor metastasis were significantly suppressed. These results support the conclusion that TMP effectively inhibits *in vitro* angiogenesis as well as *in vivo* metastasis, and a liposomal formulation is more efficient delivery system for TMP treatment than solution.

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1. Introduction

Angiogenesis is the process by which blood vessels are formed from pre-existing vessels for wound repair, the development of granulation tissue and tumors (Folkman, 1971; Ossowski and Reich, 1983). Various studies dealing with tumor angiogenesis have pointed out that tumor expansion with the formation of new blood vessels and tumor regression due to angiogenic inhibitors are correlated with metastasis (Holash et al., 1999; Hood et al., 2002; Weidner et al., 1991, 1993). During the process of tumor angiogenesis, highly permeable blood vessels are formed in and around solid tumors, thus providing an efficient translocation route for tumor cells to leave the primary tumor site, possibly inducing metastasis (Zetter, 1998). Therefore the use of angiogenic inhibitors for the suppression of metastasis has been an area of considerable interest (Ebos et al., 2009; Konno et al., 1995; O'Reilly et al., 1994; Pàez-Ribes et al., 2009). Sphingolipids and their metabolites,

such as ceramide, sphingosine, sphingosine-1-phosphate (S1P) and phytosphingosine, have been identified as important mediators of cellular processes, in particular, cell proliferation, differentiation, senescence and apoptosis (Merrill, 2002; Spiegel and Merrill, 1996; Spiegel and Milstien, 2002; Woodcock, 2006). For instance, it has been reported that sphingosine (SPN) is a negative modulator of transmembrane signaling through protein kinase C (PKC) as well as an inhibitor of sphingosine kinase-1 (SK-1), which is implicated in cell growth and inhibitory apoptosis (Cuvillier and Levade, 2001; Hannun and Bell, 1989). In addition, the inhibitory effect of SPN derivatives on metastatic potential has also been reported (Okoshi et al., 1991). Interestingly, *N,N,N*-trimethylsphingosine (TMS) showed a much stronger inhibitory effect on PKC activity than *N,N*-dimethylsphingosine (DMS) and unsubstituted SPN (Endo et al., 1991). Phytosphingosine, which is similar in structure to sphingosine, is a major component of membranes produced by plants, fungi, mammalian tissue and some types of cancer cells (Jo et al., 2003). The inhibitory effects of synthetic phytosphingosine derivatives (*N*-monomethylphytosphingosine and *N,N*-dimethylphytosphingosine) on SK-1 activity were recently reported to be stronger than that of DMS (Park et al., 2010). Moreover phytosphingosine and its methyl derivatives induce the apoptotic cell death of cancer cells through ROS generation, caspase activation and Bax translocation (Kim et al., 2009; Park

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A bioassay for mosquito repellency against *Aedes aegypti*: method validation and bioactivities of DEET analogues

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Abstract

Objectives Vector-borne diseases are still a major mortality factor in Africa and South-east Asia and effective mosquito repellents are therefore needed. An efficient and safe in-vitro assay system using artificial blood and skin substitute could facilitate the development of novel repellents, as most assays currently rely on human subjects or vertebrate whole blood. Moreover, examining the skin permeation profiles could provide safer mosquito repellents. The new assay system could serve as an initial system for testing new repellent candidates upon validation with DEET and its analogues.

Methods *N,N*-Diethyl-*meta*-toluamide (DEET) and five analogues were synthesised and used to validate a novel in-vitro bioassay using artificial blood and collagen membrane. Repellency against *Aedes aegypti* was correlated with lipophilicity and skin permeation.

Key findings The new in-vitro assay showed good reproducibility (interday relative standard deviation <10% at high concentrations). Four of the five DEET analogues showed repellency similar or superior to that of DEET. Repellency correlated linearly with lipophilicity but stronger repellents tended to permeate skin better.

Conclusions The new in-vitro assay using blood substitute and collagen membrane significantly simplifies screening of possible mosquito repellents. Lipophilicity as well as skin permeation profiles should be considered before testing of compounds that are candidates for mosquito repellents.

Keywords DEET; in-vitro assay; mosquito; repellent; skin permeation

Introduction

Female mosquitoes can transmit diseases through the transfer of pathogen-contaminated saliva when biting hosts to obtain blood, which is needed to develop eggs.^[1] One of the mosquito species commonly found in tropical and subtropical areas, *Aedes aegypti*, feeds during the day and multiple times while gravid, making it a potent disease vector.^[2] Common mosquito-borne and clinically important diseases include malaria (Africa, Central America, Asia), West Nile virus (Africa and north America) and dengue fever (Africa). It has been reported that mosquitoes feed on various vertebrates that attract mosquitoes via chemical attractants, which include carbon dioxide,^[3] lactic acid,^[4] ATP^[5] and colour plus heat.^[2]

Various means have been used to avoid being bitten by mosquitoes: the cultivation and use of mosquito predators,^[6] the pesticide dichloro-diphenyl-trichloroethane (DDT),^[7,8] use of insecticide-treated nets, which were able to reduce infections with vector-borne diseases in Africa,^[9] and the use of various repellents, including natural products like *Cyperus scariosus*, *Juniperus macropoda*, *Nigella sativa* and Neem oil.^[10–15]

The most common chemical repellent known today is *N,N'*-diethyl-*m*-toluamide (DEET), which entered civilian use in 1956.^[16] It offers complete protection time (CPT) ranging from 203 to 756 min, varying with climatic effects, mosquito species, physical activity, attractiveness of the host and design of the assay.^[17,18] However, DEET is associated with systemic toxicities and is suspected to be one cause of the Gulf War syndrome.^[19] A common alternative, popular in Europe and Australia, is hydroxyethyl isobutyl piperidine carboxylate, known under the trade name Icaridin,^[20] which though

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Patents

1. Multiple layer proliposomes and manufacturing method thereof. (under review)
2. 지방 줄기세포 유래 단백질 추출물을 안정화시킨 프로리포솜을 유효성분으로 포함하는 아토피 성 피부염 치료 또는 예방용 조성물

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발명의명칭 다중 프로리포솜 및 이의 제조방법

특 허 청 장

<< 안내 >>

1. 귀하의 출원은 위와 같이 정상적으로 접수되었으며, 이후의 심사 진행상황은 출원번호를 통해 확인하실 수 있습니다.
2. 출원에 따른 수수료는 접수일로부터 다음날까지 동봉된 납입영수증에 성명, 납부자번호 등을 기재하여 가까운 우체국 또는 은행에 납부하여야 합니다.
※ 납부자번호 : 0131(기관코드)+접수번호
3. 귀하의 주소, 연락처 등의 변경사항이 있을 경우, 즉시 [출원인코드 정보변경(경정), 정정신고서]를 제출하여야 출원 이후의 각종 통지서를 정상적으로 받을 수 있습니다.
※ 특허로(patent.go.kr) 접속 > 인원서비스다운로드 > 특허법 시행규칙 별지 제5호 서식
4. 특허(실용신안등록)출원은 명세서 또는 도면의 보정이 필요한 경우, 등록결정 이전 또는 의거서 제출기간 이내에 출원서에 최초로 첨부된 명세서 또는 도면에 기재된 사항의 범위 안에서 보정할 수 있습니다.
5. 외국으로 출원하고자 하는 경우 PCT 제도(특허-실용신안)나 마드리드 제도(상표)를 이용할 수 있습니다. 국내출원일을 외국에서 인정받고자 하는 경우에는 국내출원일로부터 일정한 기간 내에 외국에 출원하여야 우선권을 인정받을 수 있습니다.
※ 제도 안내 : <http://www.kipo.go.kr-특허/미양-PCT/마드리드>
※ 우선권 인정기간 : 특허-실용신안은 12개월, 상표-디자인은 6개월 이내
※ 미국특허상표청의 선출원을 기초로 우리나라에 우선권주장출원 시, 선출원이 미공개상태이면, 우선일로부터 16개월 이내에 미국특허상표청에 [전자직교환허가서(PTO/SB-39)]를 제출하거나 우리나라에 우선권 증명서류를 제출하여야 합니다.
6. 본 출원사실을 외부에 표시하고자 하는 경우에는 아래와 같이 하여야 하며, 이를 위반할 경우 관련법령에 따라 처벌을 받을 수 있습니다.
※ 특허출원 10-2010-0000000, 상표등록출원 40-2010-0000000
7. 기타 심사 절차에 관한 사항은 동봉된 안내서를 참조하시기 바랍니다.



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특허출원서

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【발명의 국문명칭】 다중 프로리포솜 및 이의 제조방법

【발명의 영문명칭】 Multiple layer proliposomes and manufacturing method thereof

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【성명의 국문표기】 김대덕

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(57) 요약

본 발명은 지방 글리세로 유래 단백질 추출물을 안정화시킨 프로리포솜을 유효성분으로 포함하는 아토피성 피부염 치료 또는 예방용 조성물에 관한 것으로, 상기 프로리포솜은 지방 글리세로 유래 단백질 추출물의 안정성을 증진시킨 뿐 아니라, 아토피성 피부염 치료 효과를 향상시키므로 아토피성 피부염 치료용 약제 또는 아토피성 피부염 개선을 화장품으로 유용하게 사용될 수 있다.

(74) 대리인
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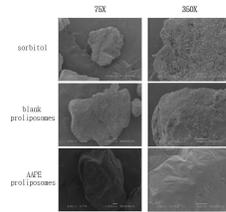
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(54) 발명의 명칭 지방 글리세로 유래 단백질 추출물을 안정화시킨 프로리포솜을 유효성분으로 포함하는 아토피성 피부염 치료 또는 예방용 조성물

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대표도 - 도1



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Defense presentation

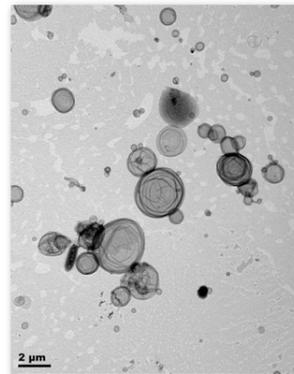
Proliposomes: Applications and Improvements

Thesis Defence
2013.11.26
Alexander Jahn

1

Background

- Liposomes
 - Lipid Bilayer Vesicle
 - Lyotropic liquid crystal
- Advantages
 - Encapsulation
 - Functionalization
 - Toxicity / delivery
- Challenges
 - Predominantly IV
 - Production cost

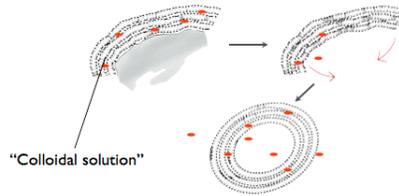


BANGHAM, A.D. & HORNE, R.W. (1964) NEGATIVE STAINING OF PHOSPHOLIPIDS AND THEIR STRUCTURAL MODIFICATION BY SURFACE-ACTIVE AGENTS AS OBSERVED IN THE ELECTRON MICROSCOPE. *J Mol Biol*, 8, 660-668.

2

Background

- Proliposomes
 - Analogue to self emulsifying
- Advantages
 - Dry formulation
 - Can help with solubility
- Potential for Optimization
 - Low total drug content
 - not functionalized



Payne, N.I., Timmins, P., Ambrose, C.V., Ward, M.D. & Ridgway, F. (1986) Proliposomes: a novel solution to an old problem. *J Pharm Sci*, 75, 325-329.

3

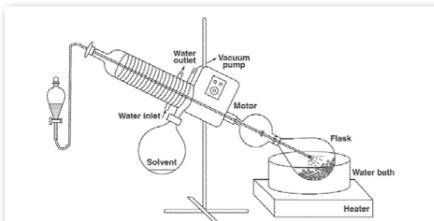
Part I. AAPE Proliposomes for the treatment of topical atopic dermatitis

- AAPE
 - “Lyophilized Adipose Tissue Derived Stemcell Culture Media”
 - ADSC excrete HGF,TGF- β , IL-6 etc.
- AAPE proliposomes
 - AAPE not stable
 - AAPE anti-inflammatory
 - Need practical topical application

Secreted
Acrap30, AgRP, Angiopoietin-2, bFGF, BTC, EGF-R, FAS, FGF-4, FGF-9, G-CSF, GTR, GTR-Ligand, GRO, HGF, ICAM-3, IGF-1SR, IGFBP3, IGFBP6, IL-2R α , IL-6R, IL-8, IL-11, IL-12p40, IL-17, Lymphotaktin, MCP-1, MIF, MIP-1 α , MIP-1 β , MIP-3 β , MSP α , NT-4, Oncostatin M, Osteoprotegerin, PIGF, spp130, sTNF RII, TIMP-1, TIMP-2, TRAIL R3, TRAIL R4, uPAR, VEGF, VEGF-D

Schinkothe, T., Bloch, W. & Schmidt, A. (2008) In vitro secreting profile of human mesenchymal stem cells. *Stem Cells Dev*, 17, 199-206.

4



Method: "evaporation on matrix"

- Soy PC (400mg) dissolved in 62.5ml EtOH
- AAPE (25mg) and 75mg poloxamer 407 in 1ml water.
- Unified solutions and sonicated for 10 minutes.
- Sorbitol (4.5g) added and solvent evaporated under vacuum at 40°C
- Product freeze dried over night to remove residual solvent and stored at 4°C.

Song, K.H., Chung, S.J. & Shim, C.K. (2002) Preparation and evaluation of proliposomes containing salmon calcitonin. *Journal of controlled release : official journal of the Controlled Release Society*, 84, 27-37.

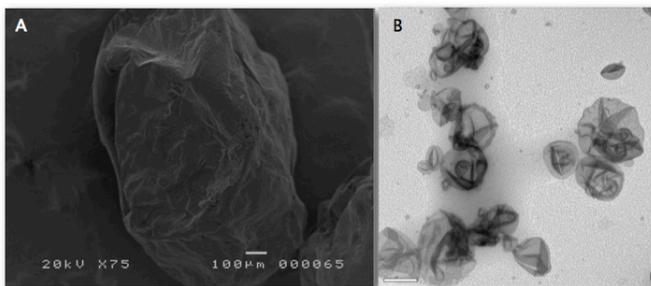


Figure 1. Morphology of AAPE proliposomes. (A) 75 x magnification SEM image of AAPE proliposome. (B) TEM image of uranyl acetate stained reconstituted AAPE proliposomes at 10k x magnification. The bar in the lower left corner represents 500 nm.

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Parameter	unit	AAPE proliposome	Blank Proliposome
Diameter	nm	589 ± 3.6	593 ± 21.2
Polydispersity	-	0.307 ± 0.005	0.323 ± 0.013
Zeta potential	mV	-51.33 ± 0.36	-51.58 ± 2.29
Angle of repose (α)	degree	35.1 ± 0.3	--
Carr index (C)	%	7.1 ± 0.9	--
Hausner ratio (H)	--	1.08 ± 0.01	--

Table 1: Characterization of AAPE and blank proliposomes after reconstitution in PBS and flow properties of dry proliposome powder (Mean ± SD)



Figure 2. Typical Intensity distribution for AAPE proliposomes

6

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6

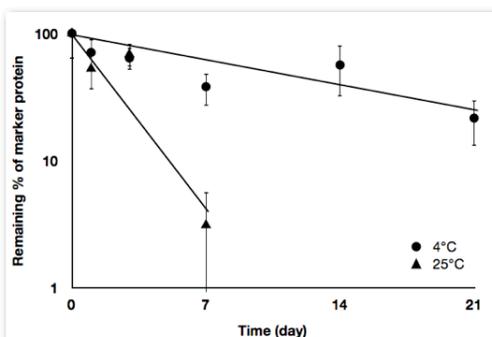


Figure 4. Stability of AAPE in PBS at 4°C and 25°C (mean±SD, n=3). Percent remaining of marker protein was determined by SDS PAGE.

Figure 5. Stability of AAPE in dry proliposome at 25°C and after reconstitution in PBS. (mean±SD, n=3). Percent remaining of marker protein was determined by SDS PAGE.

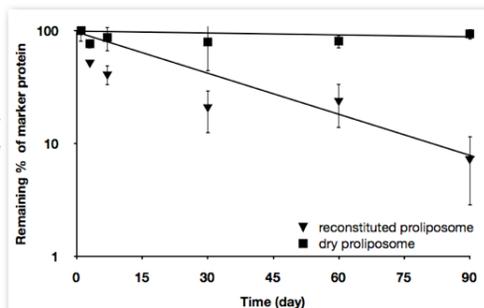


Figure 6. Skin condition of DNCB treated mice during blank and AAPE liposome treatment.

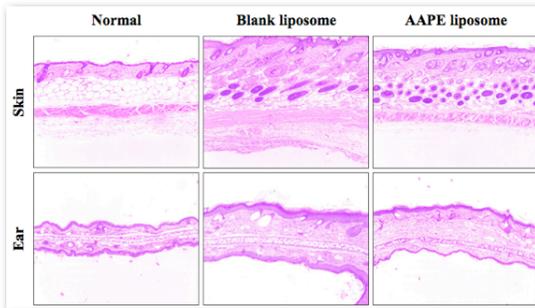


Figure 7. H&E stains of mice skin after treatment with AAPE proliposomes, following the induction of atopic dermatitis.

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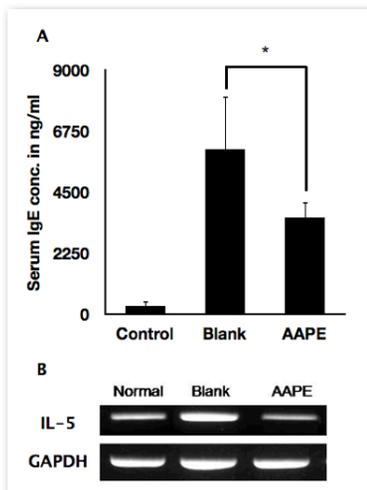


Figure 8. Serum IgE levels (A) and IL-5 mRNA expression (B) in mice after topical treatment with AAPE proliposomes and blank proliposomes following the induction of atopic dermatitis. *: IgE levels in the AAPE treated group are statistically significant, different from the blank treatment with a $p < 0.05$; $n=3$.

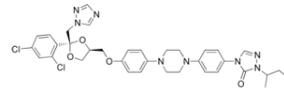
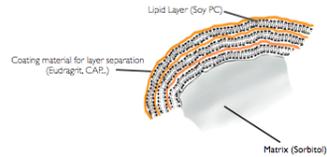
Conclusion

- Dry Formulation
- Increased stability of AAPE
- Convenient reconstitution
- In vivo* dermatitis treatment

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Part II. Layered Itraconazole proliposomes

- Itraconazole: BCS II (high permeation / Low solubility) drug
- Common dose 100mg / oral
- Solubility BA limiting factor
- Proliposome for increased solubility
- Layering for increased drug content

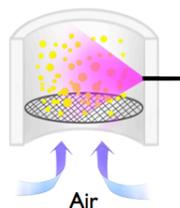


11

Fluidized Bed

Fluidized Sorbitol: Particle size < 650µm Geldard group B - easy fluidization High intermixing Tangential spray: Easier for medium sized particles Higher kinetic energy compared to top spray

- Even coating



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Preparation and Process parameters

Preparation: Proliposome Coating
 solution: Add Itraconazole to 200ml
 Ethanol 30 min Probe sonication Add Soy
 PC Add Poloxamer 407 from 10% aqueous
 stock solution Barrier solution: Dissolve
 Monostearin, Barrier material and Tween 80
 in Ethanol

Process parameter:
 Tangential spray
 37°C Inlet, 34°C Outlet temperature
 Spray: continuous (approx. 2ml/min)
 Back pulsing on
 Batch size 150g
 Rotor 60 rpm

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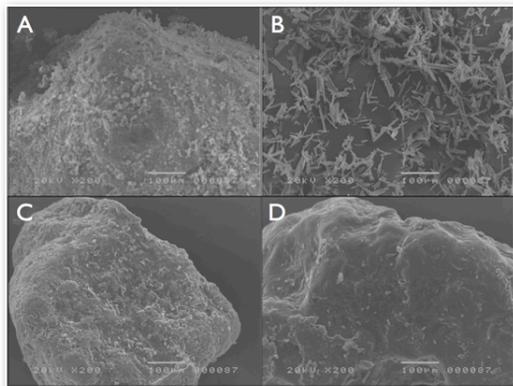
Table 2. Name, composition and drug content of proliposomal formulations.

Name	Number of Layers	Soy PC	Poloxamer 407	Itraconazole	PVA	L100	E100	Tween 80	Monostearin	Sorbitol
F1	1	5	0.5	-	-	-	-	-	-	94.5
F2	1	5	0.5	2.5	-	-	-	-	-	92
F3	3	10	1	5	-	2	-	0.2	-	81.8
F4	3	10	1	5	2	-	-	0.2	0.1	81.7
F5	3	10	1	5	-	2	-	0.2	0.1	81.7
F6	3	10	1	5	-	-	2	0.2	0.1	81.7
F7	5	15	1.5	7.5	-	-	4	0.4	0.2	75.4

Table 3. Size, polydispersity, zeta potential and encapsulation efficiency of proliposomal formulations, indicating increasing size, zeta potential and encapsulation efficiency of liposomes in correlation with increasing drug and lipid content.

Name	Number of layers	Size \pm SD in nm	PI	Zeta potential	Encapsulation Efficiency
F1	1	858.7 \pm 46.6	0.370 \pm 0.020	-24.0 \pm 7.3	-
F2	1	1022.0 \pm 60.6	0.354 \pm 0.015	-22.4 \pm 3.8	0.708
F6	3	1014.3 \pm 6.07	0.332 \pm 0.008	-16.8 \pm 0.5	0.877
F7	5	1524.7 \pm 63.3	0.389 \pm 0.02	-23.5 \pm 6.0	0.909

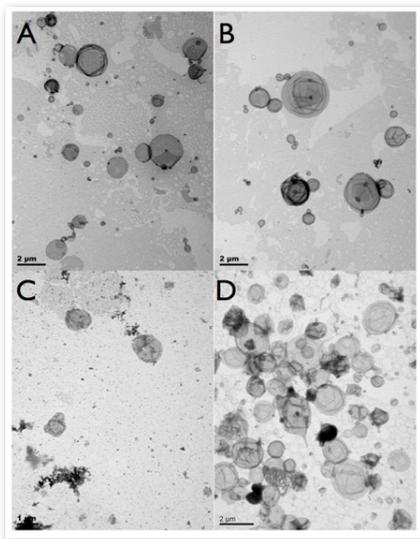
14



Sample preparation: Dry sample mounted with conductive tape on aluminum stubs. Sputtercoated for 250 sec.
SEM: JEOL JSM-5310LV at 15kV

Figure 9: SEM images of sorbitol (A), itraconazole (B), F6 (C) and F7 (D). 200 x magnification, the bar equals 100µm.

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Sample preparation: Resuspended in water (1% w/v), loaded onto copper grids, stained with uranyl acetate solution and washed twice with water. Dried under ambient conditions.

TEM: JEM 1010

Figure 10: Transmission electron micrographs of blank, F2, F6 and F7 proliposomes. The bar in the lower left corner represents 2µm (panel A, B and D) respectively 1µm (panel C)

16

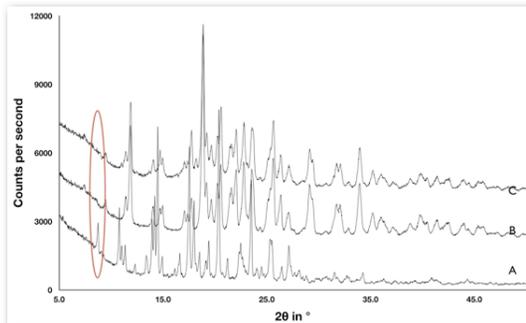


Figure 11: Powder X-ray diffraction pattern of Itraconazole (A), Sorbitol (B) and F-6 proliposomes (C)

pXRD: Scans were performed from 5 to 50° (2θ) at 2° per minute on a D8 FOCUS (Bruker AXS, Billerica, MA, USA). A Cu Kα1 radiation source with a wavelength of 0.154056nm was used.

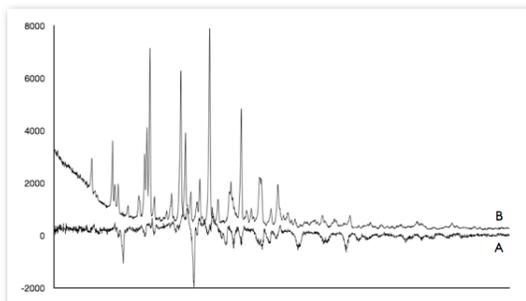


Figure 12: Powder X-ray diffraction pattern of F6 - Sorbitol (A) and Itraconazole (B)

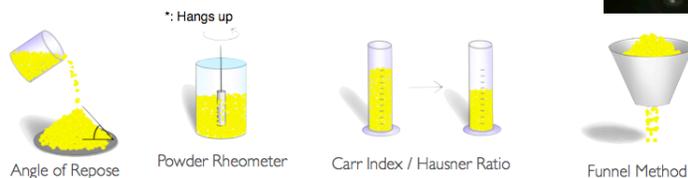
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How to measure flowability?

Table 4. Flow properties of various proliposomal powders and 650µm Sorbitol as reference.

Values are given in g/s flow trough nozzles of different sizes.

Name	15mm	11.3mm	10mm	8mm
Sorbitol	25.63 ± 1.51	13.30 ± 0	9.57 ± 0.48	*
F1	25.07 ± 1.31	12.90 ± 0	8.83 ± 0.09	*
F2	25.00 ± 0	12.50 ± 0	8.50 ± 0	4.27 ± 0.09
F4	17.83 ± 0.61	8.50 ± 0.16	*	*
F5	22.63 ± 0.61	11.00 ± 0.14	7.57 ± 0.09	4.03 ± 0.05
F6	26.13 ± 0.80	13.17 ± 0.19	9.23 ± 0.09	5.03 ± 0.09
F7	21.83 ± 0.52	10.20 ± 0.29	6.87 ± 0.45	*



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Table 5. Weight, hardness, thickness and length of proliposomal tablets. n=5

	Weight \pm SD in mg	Hardness \pm SD in kp	Thickness \pm SD in mm	Length \pm SD in mm
F2	1021.20 \pm 19.76	18.12 \pm 1.86	6.41 \pm 0.05	20.03 \pm 0.33
F6	1016.20 \pm 7.36	21.86 \pm 1.20	6.03 \pm 0.01	20.01 \pm 0.42
F7	1009.00 \pm 15.56	9.99 \pm 1.46	6.32 \pm 0.12	20.25 \pm 0.51



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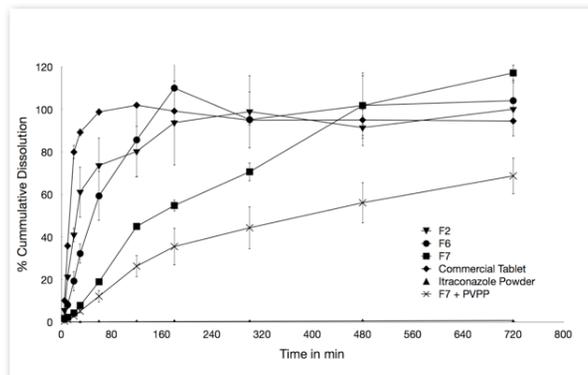


Figure 13: Dissolution profile of F2, F6, F7, and raw itraconazole in an USP type 2 (paddle) apparatus at 37°C in 0.1N HCl.

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Conclusion

- Increased drug content
- Layering kept good flowability
- Good dissolution times
- Increased Solubility
- Interesting for controlled release

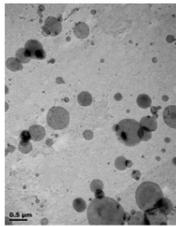


Figure 14: TEM image of liposomes formed by hydration of proliposomal cornflakes. The bar in then lower left corner represents 500nm

Onwards

- Functionalization
- Different carrier
- Frosted Cornflakes
- Vitamin Encapsulation
- 2 in 1 formulations

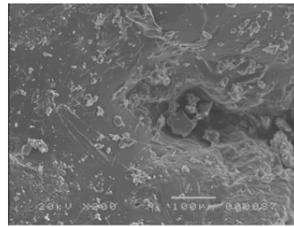


Figure 15: SEM image of proliposomal cornflakes surface. The bar represents 100µm

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Supplementary slides

References

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Fate of encapsulated Itraconazole in intestine

- Solubility BA limiting factor
- Very low gastric absorption
- Increased solubility at low pH
- Risk of precipitation in small intestine
- Liposome uptake not fully understood
- Endocytosis? Fusion? Peyer's Patch?
- Itra supposed to be BCS II
- Needs 90% absorbed in intestine after oral
- Most reports state 55% BA max - Not conclusive
- Liposomal or Free Itra better?

Unconclusive

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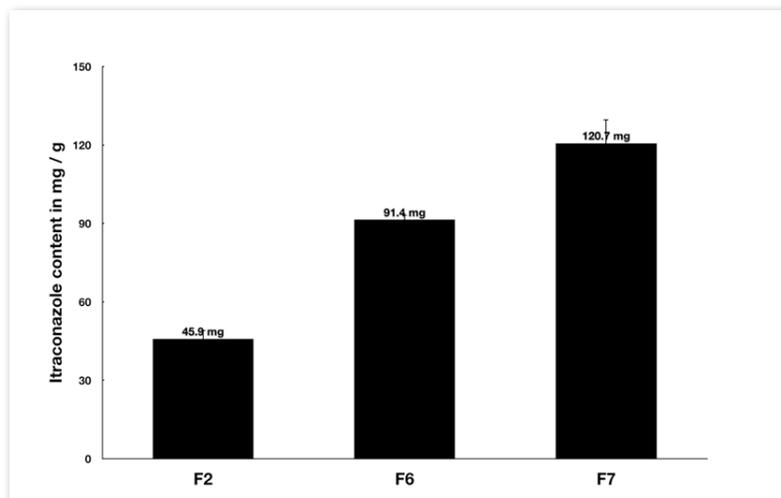


Figure 16: Itraconazole content in proliposomal tablets.

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Liposomes

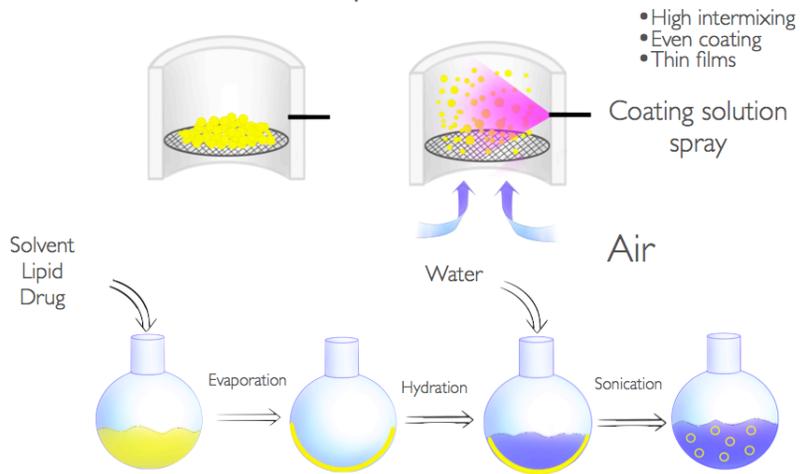


Figure 17: Illustrations.