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Transcutaneous drug delivery by liposomes using fractional laser technology

近赤外波長フラクショナル・レーザーアシストによる ナノリポソームの経皮デリバリー促進効果についての研究

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【緒言】近年、薬物の本来持ち得る薬理効果の発現や副作用の抑制など、薬物投与の最適化という考え方が強く認識されるようになり、ドラッグデリバリーシステム(Drug delivery system, DDS)の開発が注目され、新しい薬剤投与経路として経皮治療システムの研究が進められている。この利点 は、 1)高齢者や乳児などの経口投与の困難な患者においても投与可能である、2)肝臓での初回通過効果を回避できる、3)薬物濃度を長時間必要レベルに維持しやすい、4)容易に薬物の投与を中断できる、5)針を使用しない、6）組織内圧の高い部位に投与可能である、などがあげられる。しかし皮膚には強固なバリアとして最外層に角質層が存在しており、多くの薬剤の皮膚透過性は低い。経皮吸収の促進と制御方法には、 化学的手法(基剤や吸収促進剤等の利用：リポソーム、高分子ミセル、ナノエマルジョン等)と物理的手法(電場や超音波等の利用：イオントフォレーシス、エレクトロポレーション、超音波、テープストリッピング、マイクロニードル等)に大別され、それぞれの特徴を活かした吸収促進法が試みられてきた。それらを基に多角的なアプローチから開発が行われ、それぞれの特徴を活かした DDS について精力的に研究されている。なかでもレーザー（Light amplification by stimulated emission of radiation, LASER：輻射の誘導放出による光増幅）の機械的作用を利用した DDS が評価されつつある。レーザー光は指向性や収束性に優れており、また、発生する電磁波の波長を一定に保つ性質をもつ。レーザーの基礎的理論は、1917 年 Albert Einstein が論文 Zur Quantentheorie der Strahlung（放射の量子論について）にて、電磁放射の吸収、自然放出、誘導放出についての確率係数（Einstein 係数）に基づき、マックス・プランクの輻射公式から新たな公式を導き出したことに端を発する。一方で工学的な技術の完成には 1960 年 Theodore Maiman の最初のレーザー発生装置の開発まで 50 年を待たねばならなかった。レーザーの医学分野への応用は、大きく二つの分野に分けて考えることができる。一方はレーザー光の特徴である単位周期性の波長をセンサープローブとして用いる、がんの診断や細胞分析への情報応用、すなわち、光の「時間的」コヒーレンス(Longitudinal Coherence)の応用である。他方はレーザー光の集光性を利用し、微小な局所に集中できるエネルギー源としてメスのように用いる応用、つまり、光の「空間的」コヒーレンス（Transverse Coherence）の応用である。現在レ

ーザーは、医療現場において主に形成外科、眼科、歯科口腔外科、外科、泌尿器科の 5 分野で日常的に使用されており、特に皮膚形成外科領域での利用は群を抜いている。レーザーの皮膚形成外科的利用は、1970 年代に皮膚の蒸散への応用から始まった。CO_2 レーザーをはじめとした蒸散系レーザーを皮膚や臓器に焦点を合わせて照射すると、レーザー光は細胞や細胞外構成物質の水分に吸収され、熱エネルギーに変換される（Fig.1）。単位体積あたりのレーザーエネルギー密度が材料の蒸散閾値を越えると、蒸散飛散物による反力が発生する。さらにレーザー光強度が高くなり約 10^{12} W/cm^2 を越えると、光学的絶縁破壊が起きる。発生した高温・高圧のプラズマは、初期に極超音速(hypersonic)で膨張し、その膨張波が減速するときに衝撃波が発生する。またレーザーと媒質との 2 次的相互作用としてキャビテーションが発生し、その圧壊による音響波の発生も存在する。高出力のレーザーを生体に吸収させ生体自体に高温部を形成し切除を行なうため、吸収・切除に適切なレーザー光源を選択すると装置は大型となり、Nd:YAG（neodymium-doped yttrium aluminum garnet: 1064nm)、Er:YAG （erbium: 2940nm）などの波長をもつ巨大なレーザーが用いられてきた。近年の半導体レーザーなどの工学的進化により機器も小型化し、ドット状に照射したレーザー光を先端の微小領域で吸収させ、熱エネルギーに変換することで皮膚真皮まで入れ替えるフラクショナルレーザー照射技術が発達した。フラクショナルレーザーを「物理的」経皮吸収デバイスと考えれば、画期的な経皮 DDS の増強ツールとなりうる。これに加

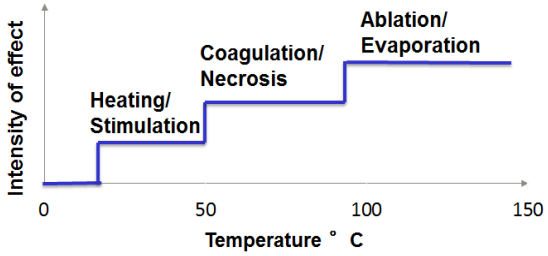

Fig. 1　組織の温度とレーザーパワーの相関

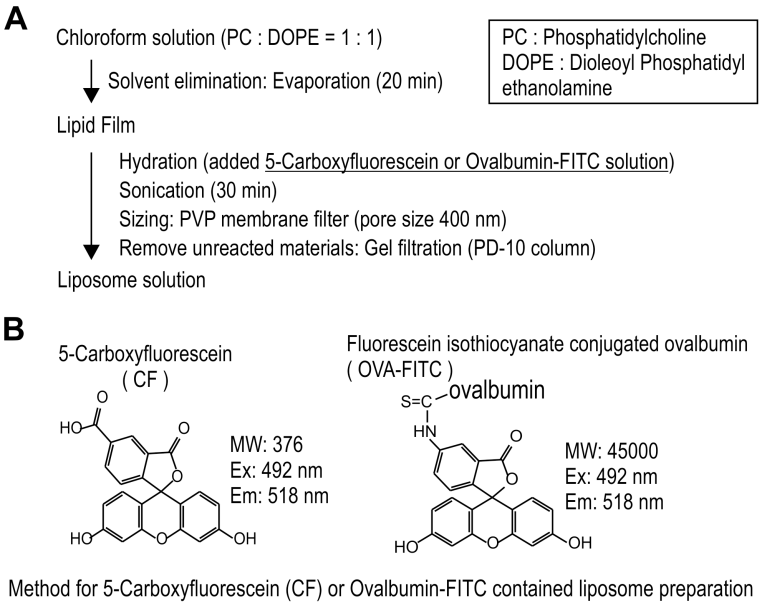

Fig.2　**A** リポソームの作成　**B** CF OVA-FITC 化学式

え「化学的」経皮吸収キャリアとして膜融合性リポソームを併用することでフラクショナルレーザーの経皮 DDS 増強効果を評価すべく、本研究を行った。

【実験方法】疎水性の増大による皮膚透過性の向上、細胞取り込みの向上を目的とし、ペプチド内封リポソームを作製した。リポソームの内封物として、CF（カルボキシフルオレセイン)、OVA-FITC を用いた。CF の分子量は 376.32 であり低分子のモデルとして、OVA-FITC は分子量が約 45,000 で、ワクチンのモデルタンパクとして使用した（Fig.2)。DDS には epidermal junction が大きくかかわっているために、まずユカタンピッグの皮膚を用い、角質の有無による CF 内封リポソームの皮膚透過率の比較検討を行いた。その後 OVA-FITC 内封リポソームを利用して、レーザー波長を 10,600 nm に固定したのち、パワー（W)、照射時間（μ秒)、照射密度（%）をそれぞれ変化させた場合の皮膚透過率及び透過速度の比較を、フランツセルを用いて行った。施術時の皮下の活性酸素の発生状況をヘアレスマウスの皮膚スライスサンプルにて ESR(electric spin echo)法を用いて調べ、生成されたフリーラジカルの量を測定した。3 次元人工培養皮膚（LabCyte EPI-MODEL：J-TEC）を用い、フラクショナルレーザーとリポソーム併用時の 48 時間毒性試験を行った。

【結果】レーザー波長を 10,600 nm、パルス幅を 500 μ秒に固定した場合は、0 W、10 W、20 W とそれぞれパワー依存性に透過率が上昇した。20 W 1,500 μ秒以上の長さで照射した場合は角質を除去した皮膚と、未処理の皮膚の透過率がほぼ同一になった。Fig.3 に示したように照射パワーが大きいほど穴の深さが深くなり、透過率、透過速度ともに上昇した。同様に照射時間が長いほど直径の大きな穴が生じるため、みかけの透過率、透過速度が増大した。照射密度が高いほど穴の個数が増加するため、透過率が増大した。ESR 法では皮下で発生した酸素ラジカル、ヒドロキシラジカル、アスコルビン酸ラジカルの発生比率が有意に変化することがわかり、近赤外線のフラクショナルレーザー波長で、皮下に反応を生じることがわかった。三次元培養皮膚による試験では、CO_2 フラクショナルレーザーとリポソーム併用による毒性は認められないと考えられた。

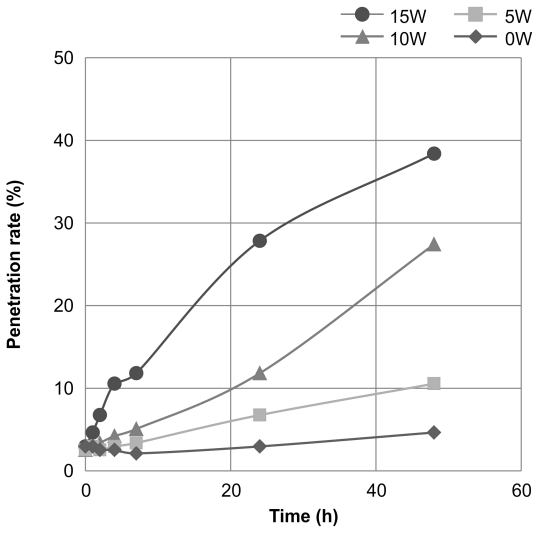

Fig.3 ユカタンピッグの皮膚に対するレーザーパワー依存性の OVA-FITC リポソーム通過率の違い

【考察】皮膚形成外科においてレーザー技術を大きく変えたのは、1983 年に Anderson および Parrish らが発表した、選択的光熱融解論（Selective Photothermolysis）である。選択的光熱融解論は、レーザー光の**波長（nm）**、**照射持続時間（Pulse duration：μ秒）**、そして単位面積当たりの**パワー（W）**（またはエネルギー量（Fluence：J/cm²）の 3 つのパラメータを調節して照射することにより特定の標的（メラニン色素、ヘモグロビン色素、水分子）、細胞内構築物を選択して融解するという理論である。

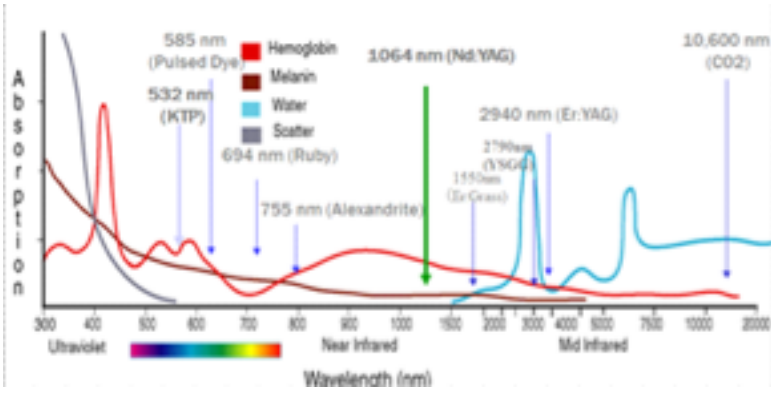

Fig.4　レーザー波長による組織吸収性　HW Lim, NA Soter, editors, Clinical Photomedicine, New York, 1993

Anderson らは、紫外線～赤外線領域の波長の光を皮膚に照射した際に、光をよく吸収する物質（Chromophore）と波長における吸光度を調べ詳細な吸収特性曲線を作成した（Fig.4）。この技術は、皮下の色素斑や刺青、レーザー脱毛などの治療技術に応用されており、工学的に多くの波長が作られてきたが、本研究では水の高吸収帯であり生体内の水に吸収され、皮膚のみを蒸散させる（穴をあける）性質をもつ CO2 レーザー（波長 10,600 nm）を利用した。通常、薬物の皮膚透過は受動拡散によって起こり、薬剤の濃度勾配が移動の駆動力となる。薬物の輸送経路は、角質層を通るルートと汗腺や毛穴のような皮膚付属器を経由するルートに大別される。付属器の有効表面積は皮膚全体の 0.1%程度であるので、高分子など特殊な薬物を除いて、角質層を通るルートの方が重要であると考えられている。角質層を経由するルートはさらに角質細胞の実質を経由するルートと細胞間隙の脂質を経由するルートに分けられるが、これら 2 つのルートの

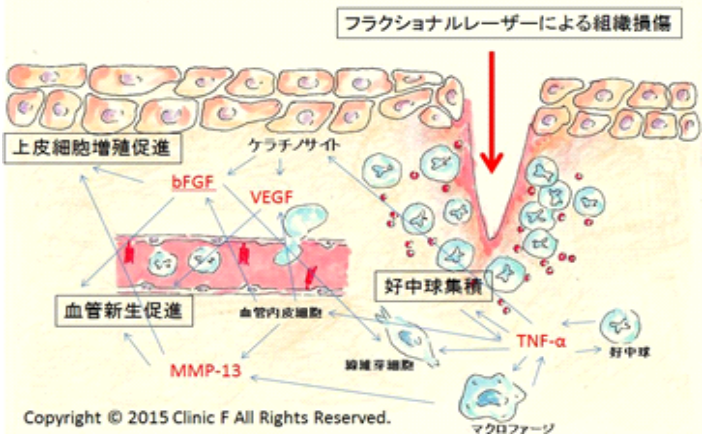

Fig.5　フラクショナルレーザー後の創傷治癒

寄与を組織学的に区別するのは困難である。レーザー照射方法で大きな革命を遂げたのは、2004 年に登場したフラクショナルレーザーである。この機器はレーザー光線を分離し、皮膚表面に 1 cm^2 あたり約 2000 個の、直径数 10 μm 単位のレーザー光をドット状に照射してすることで、表皮から真皮にかけて極小の凝固柱を作り、古い角質を除去（punch out）すると同時に、孔のすぐ周囲の正常な表皮基底細胞により創傷治癒効率を上げる効果を持つ。フラクショナルレーザー技術により、レーザー治療において新たな第四のパラメータとして照射密度（spots/cm^2）が登場した。創傷が非常に小さいため角質バリア機能は早期に復活し、炎症が少なく治癒が早い(Fig.5)。この手法を DDS に併用すると、従来の物理的手法と比較し、1）時間的・空間的制御性（照射密度依存性）に優れ、2）創面を湿潤もしくは乾燥にコントロール（波長依存性）ができ、3）穴の深さ（パワー依存性）および 4）大きさ（照射時間依存性）をコントロールできる点で、経皮ドラックデリバリーシステムの飛躍的な効率改善につながることが期待される。先行研究では、フラクショナルレーザーにより皮下への局所麻酔薬、NSAIDS、オピオイド、癌化学療法薬、コルチコステロイド、ワクチン、ビタミン C、ボツリヌストキシンを導入した報告があるが、本研究のようにフラクショナルレーザー機器を用いて自由な深さ及び大きさの穴を皮膚に開け、リポソームなどの化学的キャリアを併用すれば、多くのペプチドやメラノサイトなどの細胞を皮下にデリバリーすることが可能となる。本研究では、CO_2 レーザーの波長で 20 W 1500 μ 秒以上照射するとレーザーによる皮膚の蒸散が真皮まで到達し、角質を取り除いた皮膚と全く同じ経皮透過率を示した。さらに実際の皮膚の凍結切片を用いて、皮下への薬剤透過率の上昇を確認した。ESR 法を用いて、実際にこの近赤外線波長が生体に影響があることを突き止めたが、これは筆者の先行研究にて抗酸化剤フラーレンを使用して除去できることを報告している。3 次元培養皮膚の照射実験では毒性は認められず、本手法は人体に影響なく施術が可能であると考える。

【結語】本研究は、近赤外線フラクショナルレーザーがリポソーム併用による経皮ドラッグデリバリーの効率を上げることを確認した。物理的デバイスと化学的キャリアによる薬学ドラッグデリバリーの融合した研究分野においては、フラクショナル CO_2 レーザーとリポソームを用いた報告はなく、透過率上昇率も他の機器と比較して、効率が極めて高い。今後レーザーは美容目的だけではなく、インシュリンやワクチン投与の内科的疾患や、ケロイドなどの内圧の高い組織に対しての注入薬の改善などに利用が期待できる。フラクショナル・レーザーアシストによる経皮ドラッグデリバリーの改善は、生体情報の取得への応用、そして生体切除および改質への応用に次ぐ、レーザーの第三の医学的応用になりうると考える。

主論文

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Contents

Abbreviations	Name
AsA*	ascorbyl redical
CF	5-carboxyfluorescein
DOPE	dioleoylphosphatidylethanolamine
DPPMPO	5-dihydro-3H-pyrrole-N-oxide
EDTA	ethylenediaminetetraacetic acid
ESR	the electron spin resonance
FBS	fetal bovine serum
MnO	manganese oxide
mT	The g-value and distance
O_2*	the superoxideanion radical
OH*	hydroxyl radical
OVA-FITC	ovalbumin
PBS	phosphate-buffered saline
PLDD	Percutaneous Laser Disc Decompression
RFU	Relative Fluorescence Units
SC	the stratum corneum
TiO_2	the titanium oxide
TP	the tip-processed
YMP	Yucatan micro-pigs

Preface

The present investigation appeared in this Ph.D. thesis is the collection of studies which have been carried out under the direction of Professor Hideko Kanazawa.

Department of Pharmacy, Keio University. during 2014-2017.

These studies are concerned with the transcutaneous drug delivery by liposomes using fractional laser technology and high-temperature heat source generation with quasi-cw (continuous wave) semiconductor lasers at power levels of 6W for medical use.

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Chapter 1

Transcutaneous drug delivery by liposomes using fractional laser technology

1.1 Abstract

Objective: Transdermal delivery of hydrophilic peptides remains a challenge due to their poor cellular uptake and transdermal penetration. I hypothesize that combination of a CO_2 fractional laser to enhance percutaneous absorption and liposomes as transdermal carriers would improve skin penetration of hydrophilic drugs.

Methods: Liposomes were prepared using membrane fusion lipid dioleoylphosphatidylethanolamine, and used to deliver 5-carboxyfluorescein (CF) and fluorescein isothiocyanate-conjugated ovalbumin (OVA-FITC) as model hydrophilic peptide drugs. Liposome size was estimated by dynamic light scattering. Liposome uptake into murine macrophage cells and penetration or permeation into Yucatan micro-pig skin after irradiation by CO_2 fractional laser at varying energy levels (laser power and exposure duration) were investigated using Franz cell and fluorescence microscopy. Oxidative damage to the irradiated mouse skin was assessed by electron spin resonance.

Results: Size of CF and OVA-FITC encapsulated liposomes was 324±75 nm. Cellular uptake of OVA-FITC delivered by liposomes was 10-fold higher (1370 relative fluorescence units, RFU) than delivered in solution form (130

RFU). Fractional laser irradiation increased skin permeation rate of CF liposomes (0–10%) and OVA-FITC liposomes (4–40%) in a dose-dependent manner. While peeling off the stratum corneum facilitated CF liposome penetration at low energy levels (2.69–3.29 J/cm^2; 10-20 W for 500 μs), drug permeation was similar (7–8%) in peeled or untreated skin at higher laser energy levels (6.06 J/cm^2; 20 W for 1500 μs). FITC penetrated deeper in the skin after laser irradiation. However, OH, O_2–, and VC reactive oxygen species were generated upon irradiation of the skin with a fractional CO_2 laser. Conclusions: Increasing laser power and irradiation time increased liposome uptake by cells and penetration of peptide drugs across the skin in a dose-dependent manner. High-energy CO_2 fractional laser overcomes the rate-limiting barrier function of the stratum corneum. Further investigations are required to establish the safety and efficacy of fractional laser-irradiation assisted delivery of liposome-encapsulated drugs as a transcutaneous drug delivery system.

Key words: Fractional laser assisted drug delivery, liposome, microthermal zone, fractionated, fractional photo-thermolysis

1.2 INTRODUCTION

Transdermal drug delivery systems offer distinct advantages over the topical application and oral administration of drugs for dermatological therapy.[1-3] Besides being non-invasive, transdermal systems allow self-controlled application and dose adjustment by simply varying the patch area. The transdermal dose required for the therapeutic effect of a drug is lower than the oral dose because the transdermal administration avoids the first-pass metabolism in the liver.[4] The oral bioavailability of several drugs is low due to the first-pass metabolism in the liver and breakdown of active substances in the gastrointestinal tract.[5]

Interestingly, the cutaneous bioavailability of most topically applied drugs is relatively low, with only 1%–5% being absorbed into the skin.[6,7] Furthermore, most drugs and vaccines that are absorbed do not have sufficiently deep penetration to reach the desired target in tissue.[5,8,9] Many molecules are too large to penetrate the SC and require either injectable or systemic delivery. Particularly, large molecules with molecular weight > 500-Dalton[10] cannot traverse the stratum corneum (SC), the rate-limiting outermost keratinized skin layer.[7,11] The clinical application of transdermal patches has been limited to lipophilic drugs that can easily pass through the SC and reach therapeutic levels in the blood following topical delivery.[12] Different technologies, including drug carriers; such as liposomes, nanoparticles, and other complex systems; and physical and chemical enhancers; have been in-

vestigated to enhance the transdermal bioavailability of drugs.[5] Liposomes (Fig. 1) are spherical vesicles made of an endogenous phospholipid bilayer membrane that surrounds a hydrophilic core. Because liposomes are biologically inert, weakly immunogenic, and possess low intrinsic toxicity; they have been used as carriers to improve the transdermal delivery of hydrophilic peptides, nutrients, and pharmaceuticals that otherwise have poor cellular uptake and transdermal penetration. For example, carboxyfluorescein (CF) liposome formulations showed a nearly 2.8 fold higher delivery of CF through human skin compared to CF solutions.[13]

Besides liposome-mediated delivery, various chemical enhancers and physical methods have also been tested for their ability to increase the permeability across SC.[1,10,14] For example, iontophoresis and electroporation use electrical charge to force ionized drugs or vaccines across the SC layer.[10,15,16] Ultrasound and shock waves temporarily reduce the barrier property of skin by inducing cavitation or the production of pressure waves to increase the penetration of molecules.[10,14,17] Mechanical strategies such as microneedles and tape stripping have been developed to facilitate transcutaneous drug and vaccine delivery.[18,19] Recent studies have reported the use of fractional laser for transdermal drug delivery to improve delivery of peptide molecules by 8–15 fold.[20-22] A fractional laser irradiates cells with high precision by

controlling the area and degree of ablation through laser settings such as power, pulse duration, percentage of skin coverage, and ablation pattern. Lasers can also be used repeatedly without the risk of cross-contamination.

I hypothesized that a combination of CO_2 fractional laser to enhance percutaneous absorption and liposomes as transdermal carriers would improve the skin penetration of drugs. In the present study, liposomes were prepared using the membrane fusion lipid dioleoylphosphatidylethanolamine (DOPE) and loaded with CF or OVA-FITC as model hydrophilic drugs. Their cellular uptake and transdermal penetration was assessed.

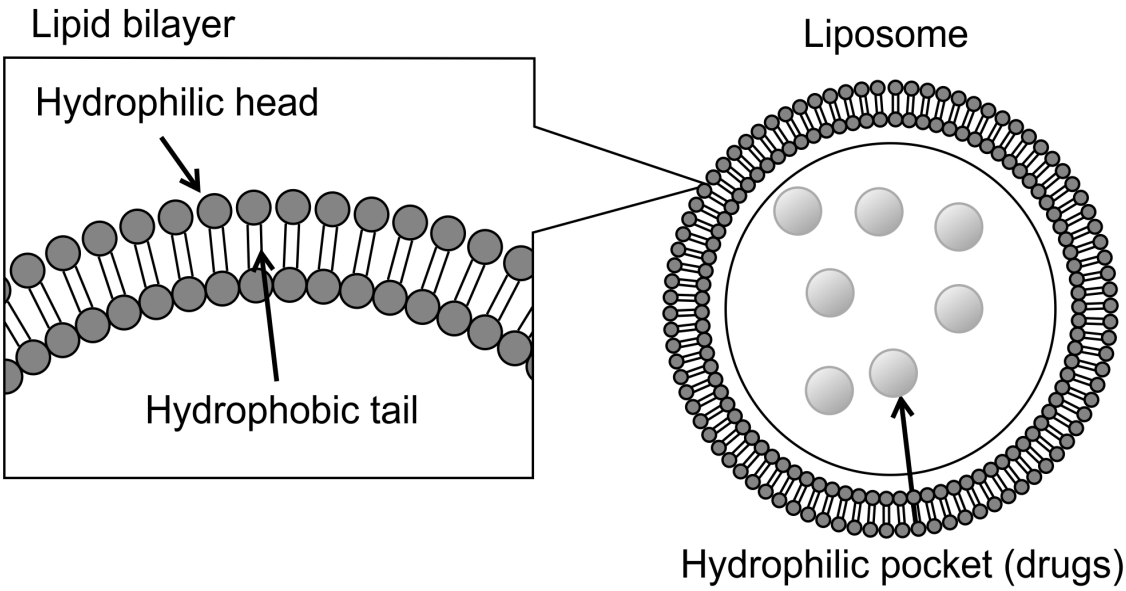

Figure 1. Liposomes as a carrier for drug delivery. A liposome is a spherical vesicle with at least one lipid bilayer and a hydrophilic pocket that can be used as a vehicle for administration of nutrients and pharmaceuticals. As drug carriers, liposomes have proved most effective for diseases affecting the reticuloendothelial system and blood cells in particular.

1.3 MATERIALS AND METHODS

1.3.1 FITC Labeling of OVA

OVA (10.06 mg, Sigma Aldrich, St. Louis, MO, USA) and FITC (11.78 mg, Sigma Aldrich) were dissolved in 4 mL $NaHCO_3$ (pH 9.0) solution and allowed to react at room temperature for 3 days at 4 °C. Unreacted FITC was removed and OVA-FITC was purified by dialysis using Slide-A-Lyzer dialysis cassettes (molecular weight cutoff, 20 kDa, Thermo Fisher Scientific, Waltham, MA, USA).

1.3.2 Liposome Preparation

CF-liposome and OVA-FITC liposomes were prepared using a previously described method.[23] Briefly, a solution of egg phosphatidylcholine and DOPE (1:1, w/w) in chloroform was evaporated in a flask to form a lipid membrane (Fig. 2). The membrane was dispersed with 1 mL OVA-FITC or 2 mM CF in phosphate-buffered saline (PBS) and sonicated for 30 min to prepare drug-loaded liposomes. The liposomes were extruded through a polycarbonate membrane (pore diameter, 400 nm). The free lipids, and CF or OVA-FITC were removed by gel permeation chromatography on a Sephadex G-25M column (Sigma-Aldrich Japan, Shinagawa, Tokyo) with PBS. The liposome suspension was stored at 4 °C until measurement.

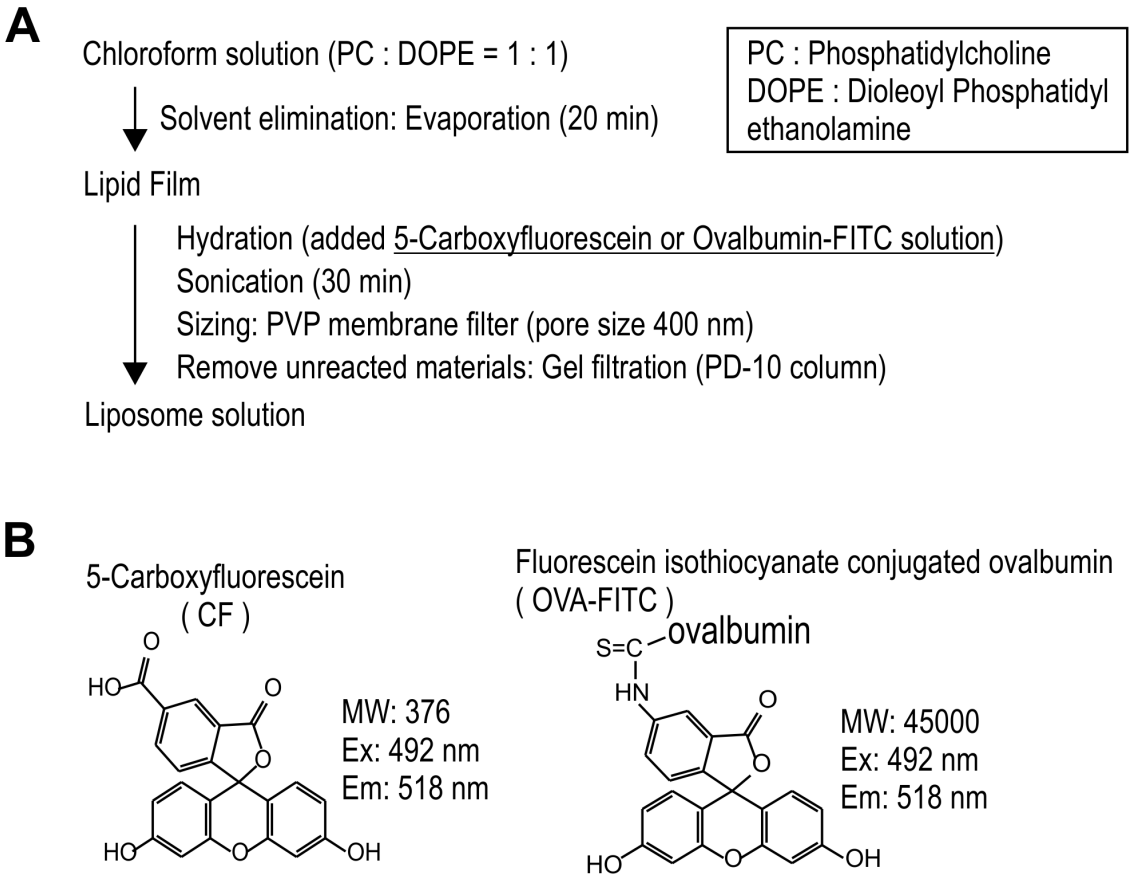

Figure 2. Protocol for liposome preparation. Liposomes were prepared by mixing the egg phosphatidylcholine and DOPE in chloroform, followed by evaporation, hydration, sonication, sizing, gel-filtration, and dialysis.

1.3.3 Determination of Liposome Size by Dynamic Light Scattering

The size of liposomes was measured by dynamic light scattering using previously described methods.[24] Liposomal suspensions (n=5) were analyzed using a Nano-ZS zetasizer (Malvern Instruments, Malvern, UK), and the data was averaged for analysis.

1.3.4 Cell Culture

RAW264.7 cells (murine macrophage cell line; ATCC, Rockville, MD) were cultured as sub-confluent monolayers in 75-cm^2 culture flasks with a vent cap in minimal essential medium supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator. Cells were dissociated using a cell scraper, (Techno Plastic Products, Trasadingen, Switzerland) and seeded in a flask for 2–3 days. Subsequently, cells were grown in 35-mm glass-bottomed dishes (for microscopic assessment of OVA-FITC cellular uptake) and in 60-mm dishes (for flow cytometric analysis).

1.3.5 Microscopic Analysis of OVA-FITC Cellular Uptake

RAW264.7 cells were grown overnight in 35-mm glass-bottomed dishes at a density of 2.0×10^5 cells/dish in 2 mL medium supplemented with 10% FBS. Cells were then incubated for 30 min with 100 μL OVA-FITC solution (control group) or OVA-liposome suspension at 37 °C, rinsed three times with PBS, and incubated with paraformaldehyde for 20 min at 4 °C. Subsequently, cells were rinsed three times with PBS, and visualized under an epifluorescence microscope (BZ-9000, Keyence, Osaka, Japan) to examine the cellular uptake of OVA-FITC from solution and liposome suspension.

1.3.6 Flow Cytometric Analysis of OVA-FITC Cellular Uptake

RAW264.7 cells were grown overnight in 60-mm dishes at a density of 5.0×10^5 cells per dish in 5 mL medium supplemented with 10% FBS. Cells were then incubated for 30 min with 100 μL OVA-FITC solution or liposome suspension at 37 °C, rinsed twice with PBS, and harvested with trypsin/ethylenediaminetetraacetic acid (EDTA). Subsequently, 1×10^4 cells from each group were re-suspended in PBS and their fluorescence intensity was measured by flow cytometry (excitation wavelength: 492nm, emission wavelength: 518nm) to quantify the cellular uptake of OVA-FITC solution and liposomal suspension. Untreated cells were used as a negative control.

1.3.7 Yucatan Micro-pig Skin Preparation

The skin from adult Yucatan micro-pigs (YMP) was used to study the transdermal delivery of liposomes because its density, thickness, and physicochemical properties are similar to human skin.[25,26] Frozen YMP skin samples (from 5-month-old female pigs, average weight, 25 kg) were obtained from Charles River Japan Inc. (Yokohama, Kanagawa, Japan). The subcutaneous fat was removed from the YMP skin sheets. Approximately 0.4 mm thick skin samples were used in the permeation experiments. YMP skin was used for studying the transdermal permeation profile using Franz cells and histological analysis after laser irradiation.

1.3.8 Transdermal Permeation of CF or OVA-FITC through YMP Skin

YMP skin samples maintained at 37 °C were mounted in vertical Franz cells (effective area and volume: 0.64 cm^2 and 5 mL, respectively; Perme Gear, Hellertown, PA, USA) (Fig. 3). The permeation of CF liposomes was assessed through a skin sample, in which the SC was peeled while keratin was left intact. The temperature of the solution was maintained at 37 °C in a circulating water bath. Liposome suspension (1 mL) was added to the donor chamber, and the transdermal permeation profile was obtained by measuring the fluorescence intensity as skin transmittance for 2 days using the

following formula (Fig. 3):

$$\text{Permeation rete (\%)} = \frac{F^r}{F^d} \times 100$$

where F^r is the fluorescence of sample from the receptor chamber and F^d is the fluorescence of sample from the donor chamber.

The steady-state flux and permeability coefficients of OVA-FITC liposomes were also calculated at 0, 6, 8, and 10 W and a 500-μs duration using the following formula:

$$P = \frac{J_{SS}}{(C_{DO} + C_{DE})/2}$$

where P is the permeability coefficient (mm/h), J_{SS} is the steady-state flux (μg/cm^2/h), C_{DO} is the initial donor concentration (μg/mL), and C_{DE} is the final donor concentration (μg/mL).

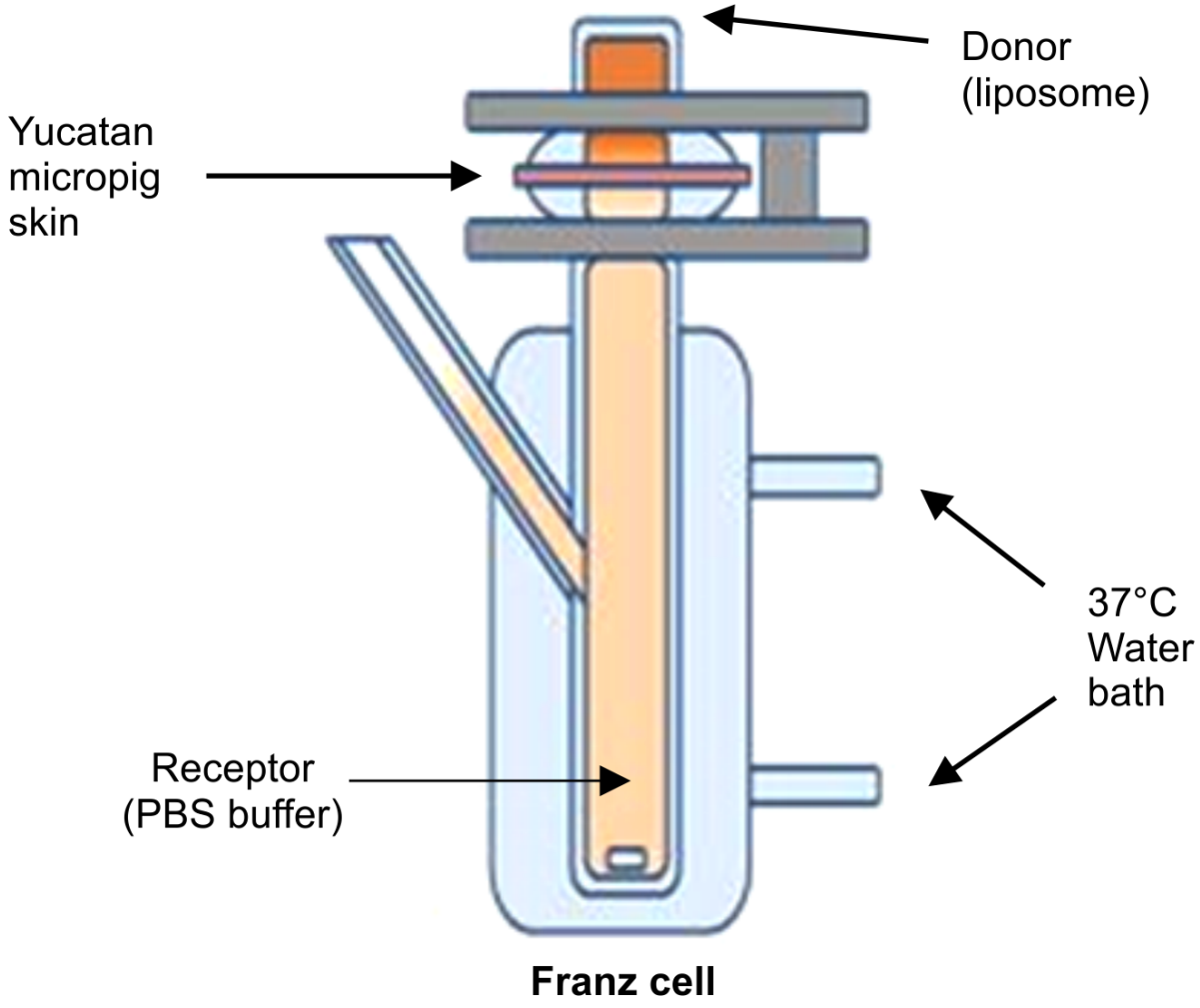

Figure 3. Mechanism of Franz cells. Franz cells are individually hand blown diffusion cells comprising two borosilicate glass components. The upper portion is referred to as the cell cap, cell top, donor chamber, or donor compartment. The lower portion is generally referred to as the body of the cell or sometimes the receptor chamber; however, in case of jacketed cells, this designation is misleading, since the receptor chamber is the innermost portion of the cell.

1.3.9 Laser Irradiation and Histological Analysis of YMP Skin

Intact YMP skin (SC not peeled) was irradiated with laser beams at a wavelength of 10.6 μm using a fractional CO_2 laser (SmartXide DOT (DEKA: Florence, Italy) for experiments involving CF liposomes; and eCO_2 (Lutronic: Seoul, Korea) for experiments involving OVA-FITC liposomes) with a moveable articulated arm.[27,28] Laser power was set at 0, 10, 20, or 30 W and pulse duration was 500 or 1500 μs. Irradiation was performed using a spot size of 10 μm and a spot density of 225 spots/cm^2, covering approximately 12% of the skin surface.

Full-thickness skin was sectioned (5 μm) at indicated times (0, 6, and 24 h) after laser irradiation (0, 15 W). The prepared sections were evaluated by fluorescence microscopy to visualize the penetration of FITC.

1.3.10 Electron Spin Resonance Analysis Using Mouse Skin

We have previously demonstrated that laser irradiation induces the production of reactive oxygen species in the skin.[29] Therefore, the present study aimed to investigate whether free radicals are generated by combined application of liposomes and fractional laser irradiation. Four-week-old male hairless mice (Nippon SLC, Shizuoka, Japan) were used for the electron spin resonance (ESR) experiments. The animals were housed individually in stainless steel cages at a temperature of 19.0 °C–25.0 °C and a mean relative

humidity of 35.0%–75.0% on a 12:12-h light:dark cycle (lights on at 7:00 AM), with free access to food and water. All procedures were carried out in accordance with the guidelines for the care and use of laboratory animals of the Japanese Pharmacological Society.

Using ESR, reactive oxygen species were detected 3 min after irradiation. The spin trapping agent 5-(diphenylphosphinoyl) 5-methyl-4,5-dihydro-3H-pyrrole-N-oxide (DPPMPO) was used at 50–500 mM in 10% w/w dimethyl sulfoxide. DPPMPO (100 mg/cm^2) was applied to the target area prior to irradiation. The irradiated skin sample was immediately removed and placed on an ice-cold plate after rinsing with ice-cold PBS (pH 7.2). Samples were cut into pieces measuring 3–5 mm × 10–20 mm, and weighed to normalize the ESR signal of each radical. Spin trap agent (10–50 μL) was added to the tissue samples (10–50 mg) immediately after weighing.

After precisely 5 min, ESR measurements were conducted as previously described.[30] ESR spectrometer was used in TE11 mode universal cavity (ES-UCX2; JEOL) with X-band microwave units (8.750–9.650 GHz); an ESR standard marker with MnO powder (DATUM MO7-FB-4; JEOL); and an aqueous sample cell (ES-LC12; JEOL) was used. Samples (20–100 μL) were analyzed in a tissue-type quartz cell (Labotec, Tokyo, Japan) with a home-made cover glass (40 × 5 × 0.5 mm). Multiple standard free radicals were generated and identified based on the g value and hyperfine splitting (hfcc) of each spin adduct. Peaks were identified by analyzing the signal used to

determine the g value and hfcc from the distance between peaks. The g value and distance between peaks for hfcc were measured using A-System v.l.40 ISAJ FA-manager v.l.20 software (JES, Tokyo, Japan) supplied with the ESR spectrometer (JES-FA200; JEOL, Tokyo, Japan). The signal for each sample was compared to that of manganese oxide (MnO), an internal standard, to obtain signal ratio, and the relative intensities of radicals were calculated by comparison with the third MnO signal intensity.

1.4 RESULTS

1.4.1 Cellular Uptake of OVA-FITC

The uptake of OVA-FITC liposomes by RAW264.7 cells was visualized by fluorescence microscopy. FITC fluorescence was not observed in cells of the control group (OVA-FITC solution); however, it was observed in cells treated with OVA-FITC liposomes (Fig. 4A). The efficiency of OVA-FITC uptake by RAW264.7 cells was confirmed by flow cytometry analysis. The FITC fluorescence intensity of the OVA-FITC liposomes-treated cells was 10-fold higher than the OVA-FITC solution-treated cells as shown by flow cytometric spectral analysis (Fluorescence intensity in Relative Fluorescence Units (RFU): 1370 RFU in the OVA-FITC liposome group compared to 130 RFU in the control group; Fig. 4B).

Dynamic light scattering analysis showed that the mean diameter of OVA-FITC liposomes was 324±75 nm (Fig. 4C).

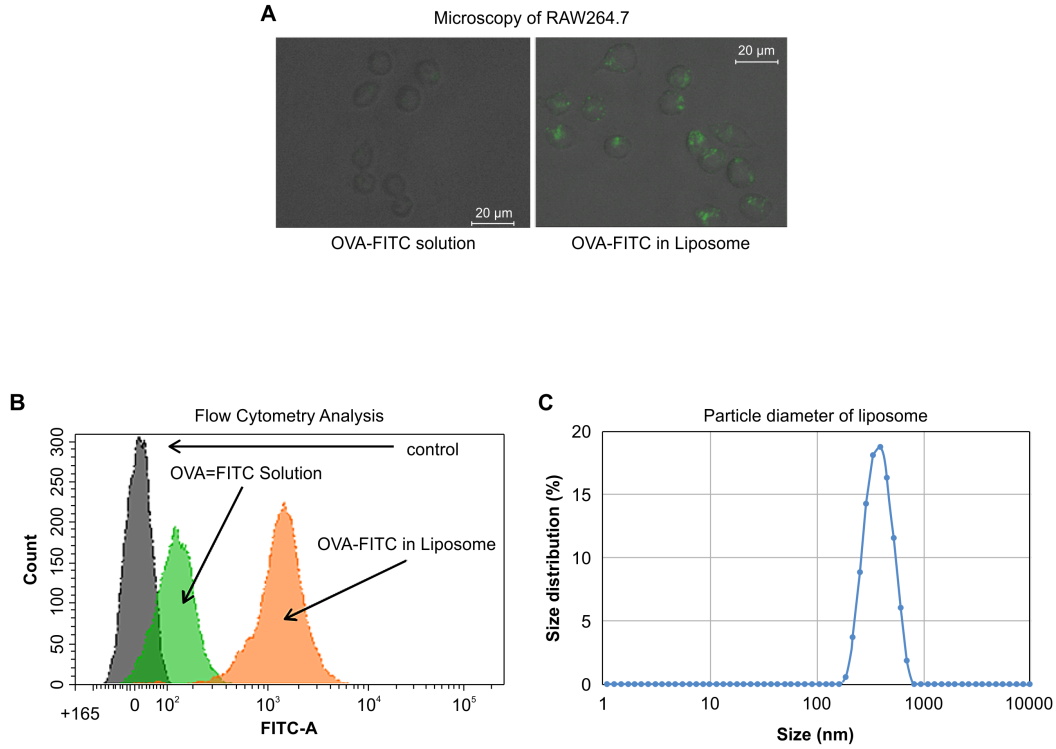

Figure 4. (A) The uptake of OVA-FITC with/without liposomes by RAW264.7 cells was visualized by fluorescence microscopy. FITC fluorescence was detected in the cells (A). The efficiency of OVA-FITC uptake by RAW264.7 cells was confirmed by flow cytometry. Encapsulation of OVA-FITC in the liposomes increased its uptake by 10 fold (1370 RFU in the OVA-FITC liposome group compared to 130 RFU in the OVA-FITC solution group); (B). Mean diameter of the OVA-FITC liposomes was 324 nm (C).

1.4.2 Transdermal Permeation

of CF Liposomes through YMP Skin

Without fractional laser irradiation, CF liposomes did not permeate through the skin in either the presence or absence of the SC (Fig. 5). It is similar to the inability of exogenous molecules to penetrate the basement membrane and tight junctions in the skin. However, laser irradiation increased the transdermal permeation of CF liposomes from 0–10% in an energy dependent manner; power (0, 10, and 20 W; DEKA SmartXide DOT fractional CO_2 laser), pulse duration (500 and 1500 μs), and time (1, 2, 4, 6, 20, 24, and 48 h). At high energy levels (6.06 J/cm^2; 20 W for 1500 μs), CF liposomes permeated through the skin to a similar extent (7–8%) in the presence or absence of keratin in epidermis/SC. This suggests that the high-energy fractional laser irradiation increases the permeability of the intact skin to CF liposomes, overcoming the inhibition by rate-limiting keratin/SC layer.

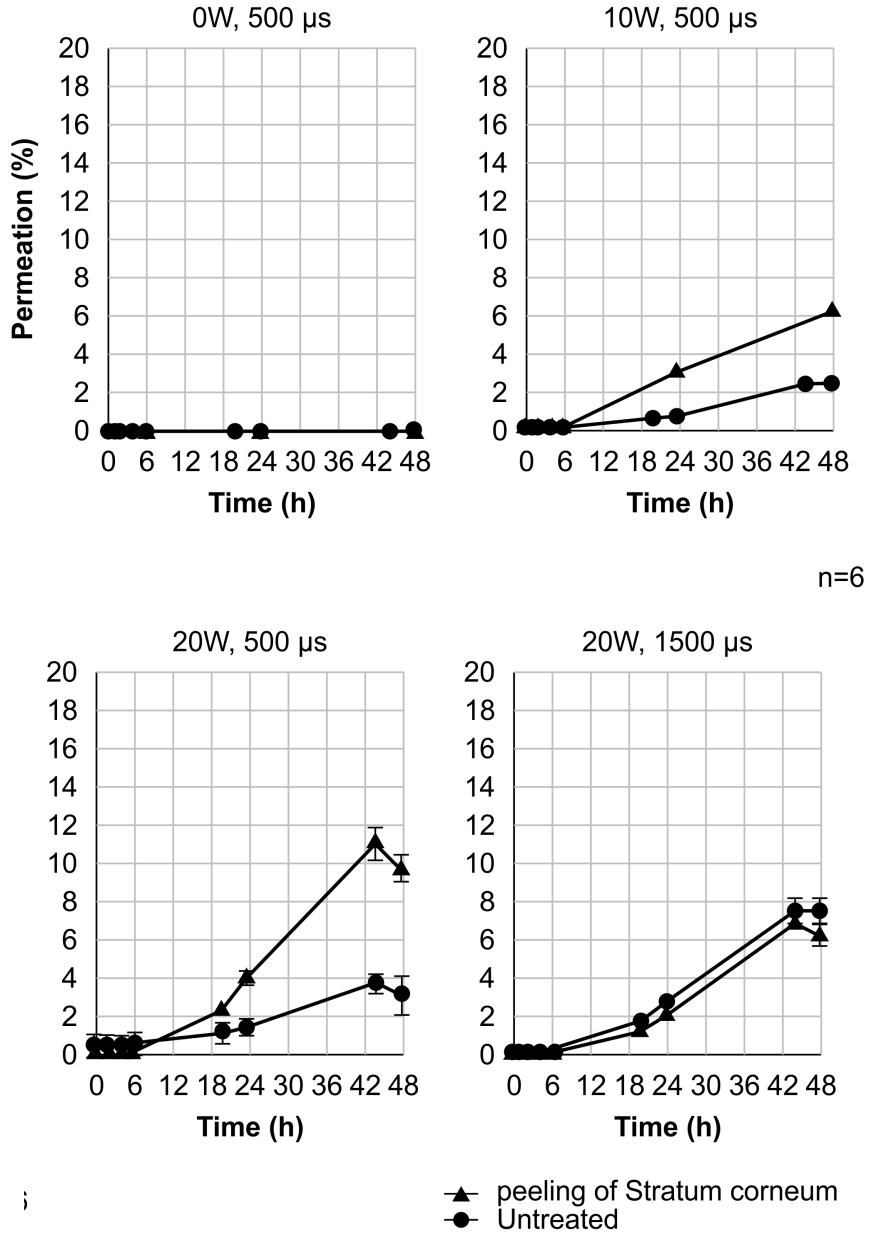

Figure 5. Transdermal permeation of CF liposomes through Yucatan micropig skin. Laser irradiation (DEKA SmartXide DOT fractional CO_2 laser) increased the permeation of CF liposomes through both untreated and SC-peeled skin in a manner that was dependent on the energy level; however, at high laser power and duration (20 W for 1500 μs), permeation rates were similar in the two groups indicating that peeling of the epidermis was not required at high energy levels. Values represent mean ± SD of 6 independent experiments.

1.4.3 Transdermal Permeation of OVA-FITC Liposomes through YMP Skin

The transdermal permeation of OVA-FITC-loaded liposomes through the *intact* YMP skin increased from 4–40% in a dose-dependent manner depending on laser power (0, 5, 10, and 15 W; Lutronic eCO_2 laser) and time (0-48 h) (Fig. 6). It indicates that a fractional laser can improve the delivery of small molecules such as CF as well as relatively large protein conjugates.

The steady-state flux and permeability coefficients of OVA-FITC liposomes increased in a dose-dependent manner at laser power of 0, 6, and 8 W. At laser power of 0, 6, 8, and 10 W, steady-state flux was 0.03, 2.00, 2.86, and 1.32, and the permeability coefficients were 0.003, 1.149, 0.192, and 0.080, respectively.

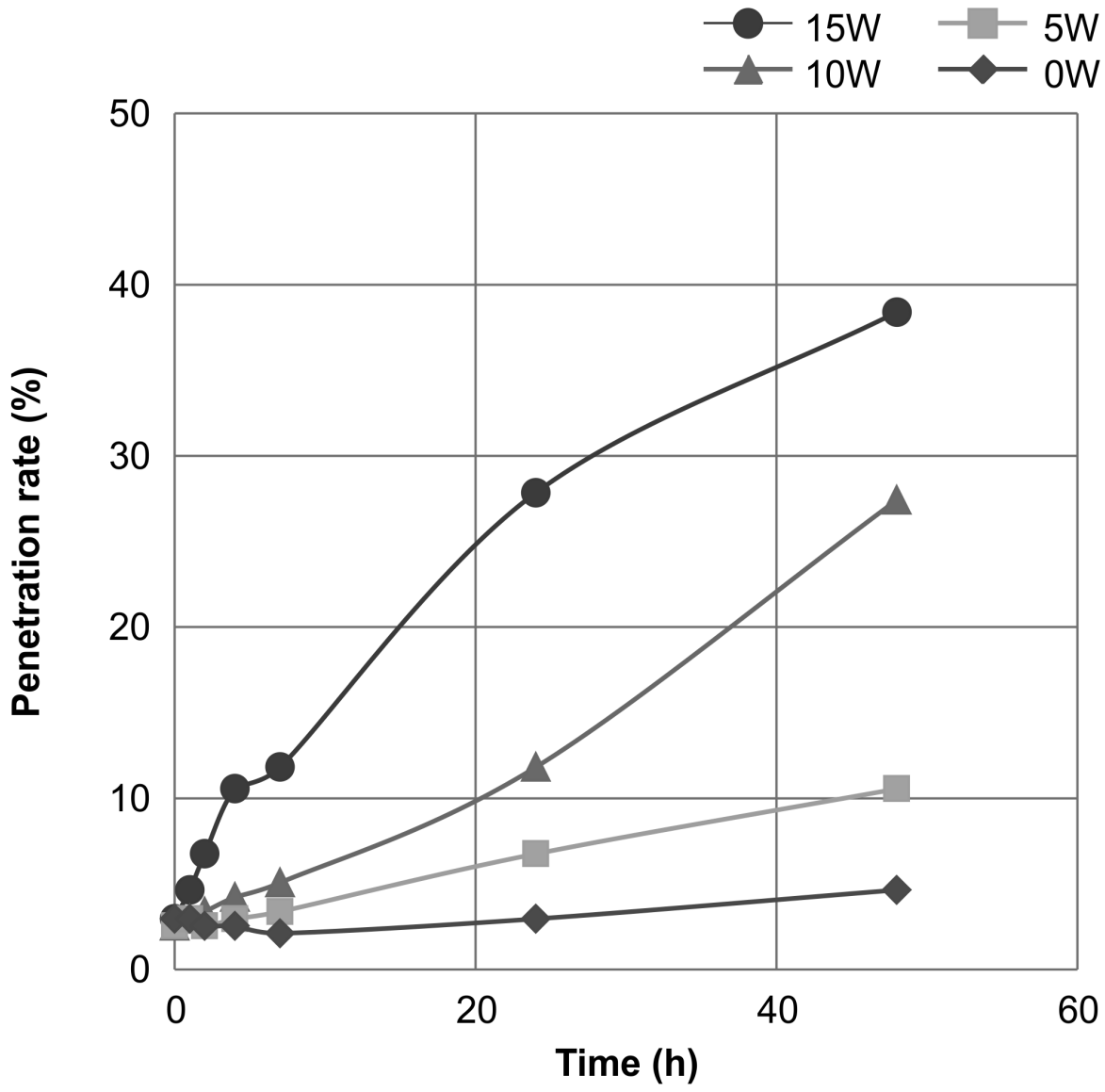

Figure 6. Transdermal permeation of OVA-FITC liposomes through intact Yucatan micropig skin increased in a laser power-dependent manner with 500 μs exposure at 0, 5, 10, and 15 W (Lutronic eCO_2 laser) between 0-48 h. Values represent mean ± SD of 6 independent experiments.

1.4.4 Laser Irradiation and Histological Analysis of Yucatan Micro-pig Skin

Fluorescence microscopic analysis of the frozen biopsy specimens revealed that FITC fluorescence was detected in the skin only upon laser irradiation (Fig. 7). Compared to the non-irradiated skin, FITC fluorescence penetrated deeper into the irradiated skin. Furthermore, the depth of penetration was higher in the skin irradiated for longer durations.

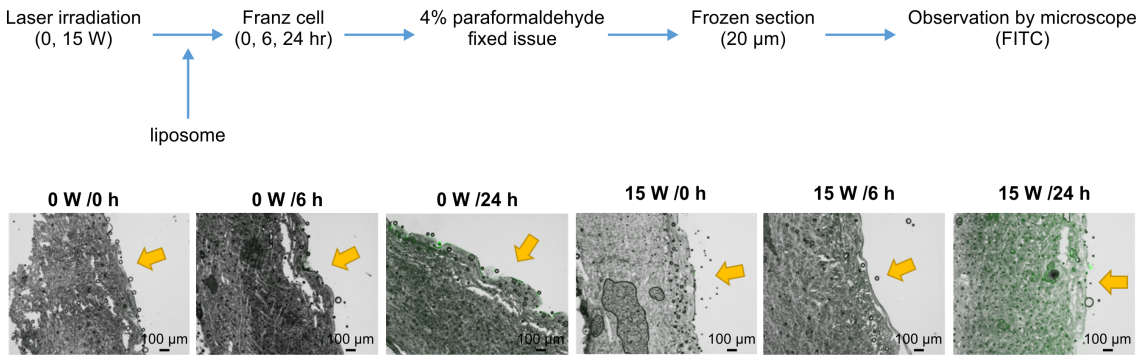

Figure 7. FITC fluorescence in Yucatan micropig skin with or without fractional laser irradiation. Arrows indicate the superficial surface of the skin. Compared to their penetration in the non-irradiated skin, fluorescent molecules penetrated deeper into the skin with laser irradiation.

1.4.5 Electron Spin Resonance Analysis Using Mouse Skin

ESR spectra of the living skin were obtained after CO_2 fractional laser irradiation using DPPMPO. They revealed waveforms corresponding to hydroxyl radical (OH), superoxide anion (O_2^-), OH+O_2^- (overlap between OH and O_2^-), and ascorbyl (VC) (Fig. 8) that were not observed in the spectra of non-irradiated samples. The relative amounts of OH−, O_2^-, and VC were significantly higher in the irradiated skin than in the non-irradiated skin ($P < 0.05$).

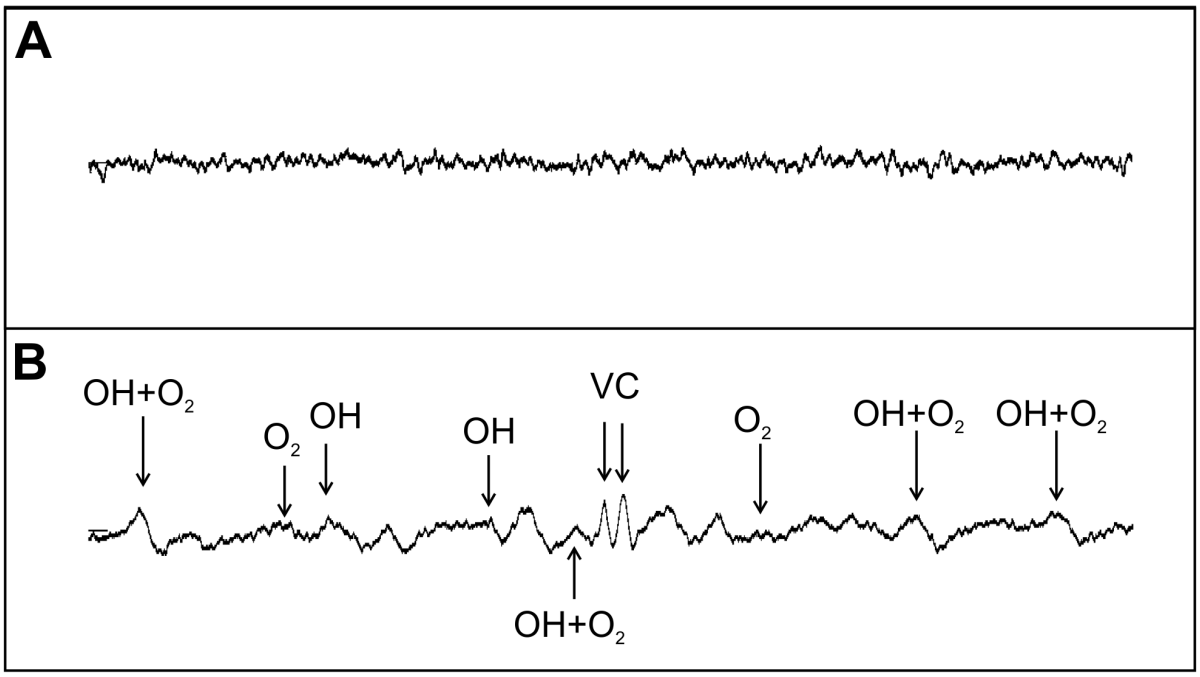

Figure 8 DPPMPO ESR spectra of fractional laser-irradiated skin. OH, O_2^-, $OH+O_2^-$, and VC were not observed in the non-irradiated skin (A) but were detected following irradiation with a CO_2 laser (B).

1.5 DISCUSSION

Human skin resists against the penetration by many molecules. Particularly, the 500-Dalton rule[10] stipulates that large molecular weight compounds do not traverse the corneal layer. Since peptides are hydrophilic, they cannot readily penetrate the hydrophobic skin surface.

In this study, I developed a method for improving the stability and cellular uptake of peptides by encapsulating them in liposomes, and using fractional laser to increase their dermal penetration. I demonstrated that CF and OVA-FITC could be encapsulated in nanoscale liposomes. Incorporation of the model-drug molecules in liposomes improved their cellular uptake as compared to delivery in the solution form. However, application of liposomal preparations alone did not result in the skin penetration of molecules. Interestingly, concurrent irradiation using fractional laser increased the skin permeation of liposome-encapsulated molecules in a dose-dependent manner. Peeling off the rate-limiting stratum corneum facilitated the skin permeation at low laser energy levels; however, drug penetration was similar in the peeled or untreated skin at higher laser energy levels. These results are particularly significant because they show that the high-energy CO_2 fractional laser overcomes the rate-limiting barrier function of the stratum corneum. Furthermore, they demonstrate the utility of combining CO_2 fractional laser (as a percutaneous absorption enhancer) and liposomes (as transdermal carriers) as an approach to improve the skin penetration of hy-

drophilic peptide drugs.

Such laser-assisted drug delivery could enable the topical medications to penetrate deeper, even for the systemic drug administration via a transcutaneous route. This method has advantage that unlike micro-needles, no biohazardous materials are produced.[31] Before the development of fractional laser treatment, laser beams were evenly delivered to a large area of the skin to disrupt the entire SC layer. However, this could potentially delay the skin recovery similar to full-surface laser skin rejuvenation.[22] In contrast, fractional photo-thermolysis, which was developed nearly a decade ago to replace the full-surface skin resurfacing, illuminates the skin with multiple distinct micro-laser beams that create micro-channels in the skin.[32] In this study, a spot size of 10 μm and a spot density of 225 spots/cm^2 was used, irradiating only approximately 12% of the skin surface. Ablative fractional lasers have been evaluated for their potential to enhance the transcutaneous delivery of hydrophilic drugs (e.g., methyl 5-aminolevulinate, 5-aminolevulinic acid, and imiquimod), polypeptides, and dextrans [27,33,34]; however, the previous studies used dissected skin as test samples. In this study, both cadaveric micropig skin and live mouse skin were used to study the transdermal permeation and oxidative effects of laser radiation on the skin.

In a previous study, I demonstrated that fractional CO_2 laser irradiation induces the production of reactive oxygen species in the skin.[29] The present study corroborated our previous results and ESR analysis showed that the

reactive oxygen species (OH, O_2−, and VC) were generated upon irradiation of the skin with a fractional CO_2 laser. A previous study reported that free radicals are also produced by irradiation with a high-energy, short-wavelength photon such as ultraviolet B radiation.[28] This indicates that the reactive oxygen species can be generated even by the exposure to low-energy waves if the conditions are identical. The generation of excessive reactive oxygen species has secondary effects on the skin pigmentation. However, they have certain beneficial effects. For example, OH and O_2^- are known to induce apoptosis and necrosis in the skin cells, thereby promoting the skin rejuvenation and contributing to the cosmetic effects of the laser. The concurrent use of liposomes, which have been verified to have no toxicity, offers a potential strategy to mitigate any laser-induced oxidative damage. For example, delivery of antioxidants loaded in the liposomes concurrently with fractional CO_2 laser irradiation may potentially mitigate the damage to the skin. I have previously reported that antioxidants such as fullerenes or ascorbyl derivatives inhibit the oxidative damage induced by laser irradiation.[29] Furthermore, albumin used in the liposomes in this study is an abundant and important circulating antioxidant,[35] and may also potentially mitigate the oxidative damage to the skin from the fractional laser treatment. The safety of fractional laser therapy and the possibility of using antioxidants to mitigate the oxidative damage to the skin warrant further investigation in preclinical and clinical studies using the human skin, with the eventual goal of achieving therapeutic gains without causing any toxicity.

In summary, an increase in the laser power and irradiation time increased the cellular uptake of liposomes and penetration of peptide drugs across the skin in a dose-dependent manner. High-energy CO_2 fractional laser overcame the rate-limiting barrier function of the stratum corneum. However, further investigations are required to establish the safety and efficacy of fractional laser irradiation-assisted delivery of liposome-encapsulated drugs as a transcutaneous drug delivery system.

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Chapter 2

Fractional laser-assisted percutaneous drug delivery via temperature-responsive liposomes

2.1 Abstract

Liposomes are used for transdermal delivery of drugs and vaccines. Our objective was to develop temperature-responsive (TR) liposomes to achieve temperature-dependent, controlled release of an encapsulated drug, and use fractional laser irradiation to enhance transdermal permeability of these liposomes. TR-liposomes prepared using a thermosensitive polymer derived from poly-N-isopropylacrylamide, N,N-dimethylacrylamide, egg phosphatidylcholine, and dioleoylphosphatidylethanolamine, delivered fluorescein isothiocyanate-conjugated ovalbumin (OVA-FITC) as a model drug. Effect of temperature on liposome size and drug release rate was estimated at two temperatures. Transdermal permeation through hairless mouse skin, with and without CO2 fractional laser irradiation, and penetration into Yucatan micro-pig skin were investigated using Franz cell and fluorescence microscopy. Dynamic light scattering showed that mean liposome diameter nearly doubled from 190 to 325 nm between 37 and 50 °C. The rate and amount of OVA-FITC released from TR-liposomes were higher at 45 °C that those at 37 °C. Transdermal permeation

of OVA-FITC across non-irradiated skin from both TR- and unmodified liposomes was minimal at 37 °C, but increased at 45 °C. Laser irradiation significantly increased transdermal permeation of both liposome groups at both temperatures. Fluorescence microscopy of frozen biopsy specimens showed deeper penetration of FITC from unmodified liposomes compared to that from polymer-modified liposomes. Rhodamine accumulation was not observed with polymer-modified liposomes at either temperature. Temperature-dependent controlled release of an encapsulated drug was achieved using the TR-liposomes. However, TR-liposomes showed lower skin permeability despite higher hydrophobicity. Fractional laser irradiation significantly increased the transdermal permeation. Additional studies are required to control liposome size and optimize transdermal permeation properties.

Keywords: Assisted drug delivery, controlled drug release, fractional laser, liposome, temperature-responsive polymer, thermosensitive polymer

2.2 INTRODUCTION

Transdermal delivery of drugs and vaccines offers significant advantages over traditional methods such as needle injection. Besides being painless, transdermal delivery systems eliminate biohazard risks associated with needles and can be self-administered. Several drugs such as nicotine, hormonal contraceptives, local anesthetics, and analgesics have been commonly used as transdermal patches. However, such use has largely been limited to lipophilic drugs that can penetrate and permeate through the stratum corneum of the skin. Several mechanical [1], chemical [2–4], and energy-based [5–7] strategies have been adopted to enhance transdermal penetration of hydrophilic drugs and peptides.

One of the chemical-based strategies commonly used to deliver hydrophilic drugs are closed bilayer phospholipid systems called liposomes. Liposomes comprise a lipid shell and a hydrophilic core that can be loaded with hydrophilic drugs including peptides and vaccines. The lipid shell facilitates cellular uptake and transdermal penetration, after which the drugs are released from the core into the cell or across the skin. Several natural and biocompatible materials such as egg phosphatidylcholine (egg PC) and dioleoylphosphatidylethanolamine (DOPE) have been used to create the lipid bilayer of liposomes.

To further improve the efficiency of transdermal drug delivery and expand the variety of drugs that can be delivered via liposomes, additional strategies such as the use of iontophoresis, electroporation, ultrasound, microneedles, and fractional laser have been employed to increase transdermal permeability [1,8–13]. In particular, fractional laser-based strategies appear promising as they precisely control the permeability by controlling the area and degree of irradiation through laser settings such as power and pulse duration [14–16]. Another innovative development in the field of liposomes is temperature-responsive (TR) liposomes. By incorporating a thermosensitive polymer into the liposome membrane, liposomes can be designed to trigger drug release only at a specified temperature [17–19]. For example, liposomes containing the thermosensitive polymer poly-N-isopropylacrylamide (PNIPAAm) that has a lower critical solution temperature (LCST) of 37.8 °C, would lose the hydration layer, become unstable, disintegrate, and release the encapsulated drug only at physiologic body temperature, but not during storage at lower temperatures. This is particularly attractive as the liposomes could be tailored to allow enhanced and targeted release of an encapsulated drug by controlling the environmental temperature.

The objectives of this study were to (1) develop a TR liposome by incorporating a thermosensitive polymer into the liposomal membrane as a strategy for temper-

ature-dependent controlled release of an encapsulated drug, and (2) investigate the enhancement in transdermal permeability of TR-liposomes by concurrent use of fractional laser irradiation.

2.3 MATERIALS AND METHODS

2.3.1 Fluorescein isothiocyanate labeling of ovalbumin

Ovalbumin (OVA; 10.06 mg, Sigma Aldrich, St. Louis, MO, USA) and fluorescein isothiocyanate (FITC; 11.78 mg, Sigma Aldrich) were dissolved in 4 mL NaHCO3 (pH 9.0) solution and allowed to react at for 3 days at 4 °C. Unreacted FITC was removed and fluorescein isothiocyanate-conjugated ovalbumin (OVA-FITC) was purified by dialysis using Slide-A-Lyzer dialysis cassettes (molecular weight cut-off 20 kDa; Thermo Fisher Scientific, Waltham, MA, USA).

2.3.2 Liposome preparation

OVA-FITC TR-liposomes were prepared using a previously described method with slight modifications (Figure 1) [20]. Briefly, 10% of a thermosensitive polymer (P[N-isopropylacrylamide-co-N,N-dimethylacrylamide 30%]-DOPE; MW 10000, Lower Critical Solution Temperature, LCST: 37.8 °C), where 30% N,N-dimethylacrylamide (DMAAm) was incorporated with PNIPAAm, was mixed

with a solution of egg PC and DOPE in chloroform at concentrations listed in Table 1. The solution was evaporated in a recovery flask to form a lipid membrane. The membrane was dispersed with 1 mL OVA-FITC solution and sonicated for 30 min to prepare liposomes. The liposomes were extruded through a 400 nm pore-sized membrane filter (Extruder®, 2 PVP membrane filters, 700 Industrial Park Dr, Alabaster, AL 35007). The free and decomposed lipids and OVA-FITC in the filtrate were removed by gel filtration using a PD-10 column (Sigma-Aldrich Japan, Shinagawa, Tokyo) with 1x phosphate-buffered saline (PBS). Liposomes containing a 1:1 ratio of egg PC and DOPE, but without the thermosensitive polymer, were also prepared to serve as controls. The liposome suspensions were stored at 4 °C until measurement.

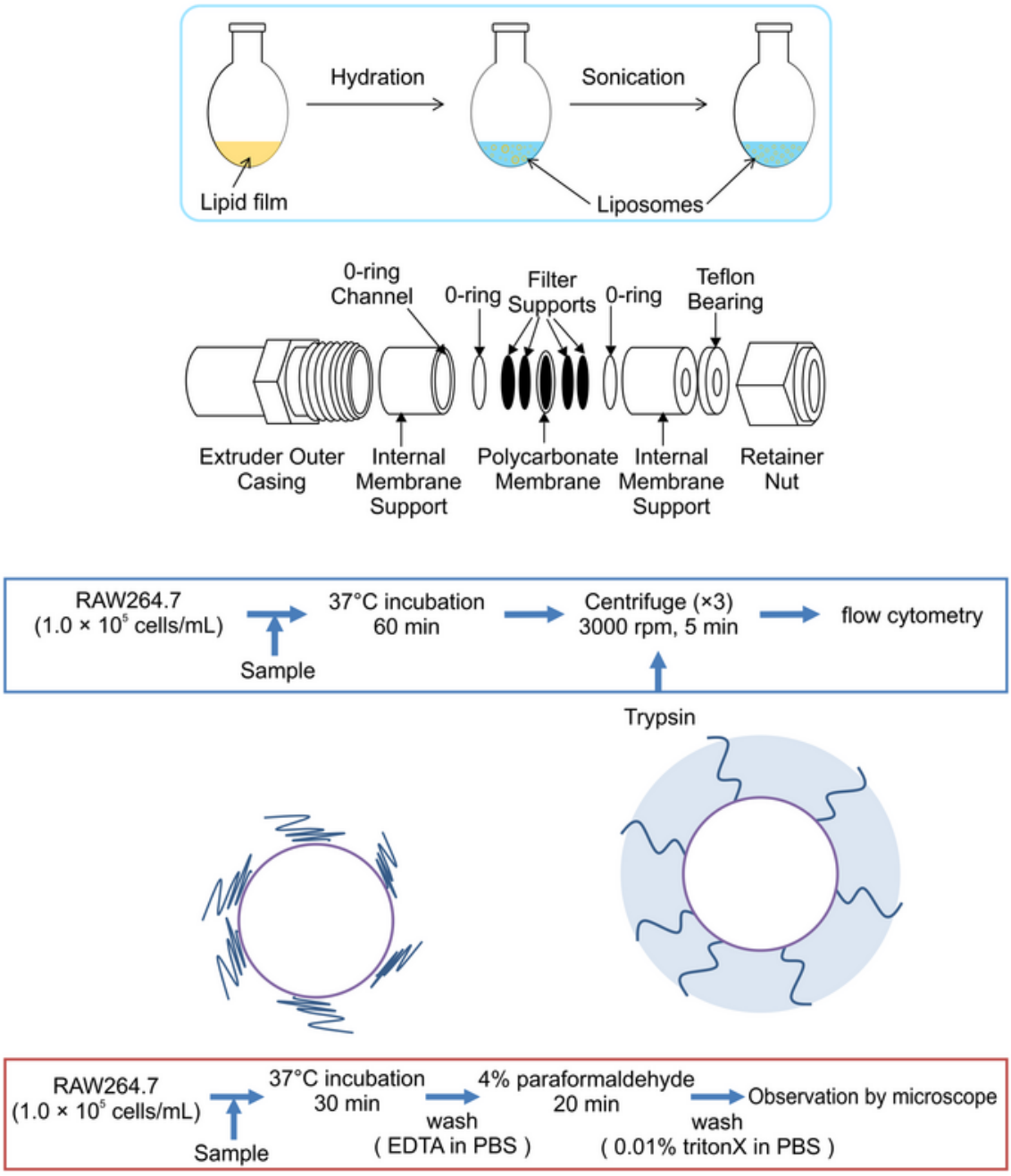

Figure 1. Steps involved in liposome preparation.

	Mole rate	Weight (mg)
P(N-isopropylacrylamide-co-DMAAm30%)-DOPE	10	28.9
Egg PC	50	10.0
DOPE	40	8.0

Table 1. Liposome composition.

2.3.3 Effect of temperature on liposome size

Liposome size was measured by dynamic light scattering using previously described methods [21]. The temperature of the liposome suspension (n = 6) was increased from 35 to 50 °C and the liposome diameter was measured using a Nano-ZS Zetasizer (Malvern Instruments, Malvern, UK).

2.3.4 Effect of temperature on temporal release rate of drug from liposome

Ten microliters of the liposome suspension obtained by gel filtration was diluted 10x in an extraction buffer (1x PBS with 10% TritonX) and incubated at 37 °C or 45 °C (n = 3/group). After specific times (1, 5, 10, 20, 30, and 60 min), 10 µl of the buffer was sampled to measure its fluorescence intensity (Infinite® M1000

TECAN; Tecan, Männedorf, Switzerland). The release rate was calculated using the formula:

$$\text{Drug release rate } (\%) = \frac{F^t - F^i}{F^f - F^i} \times 100$$

where Ft is the intermediary fluorescence at a given time point, Fi is the initial fluorescence, and Ff is the final fluorescence of the sample at 60 min.

2.3.5 Transdermal permeation of liposomes through hairless mouse skin

Hairless mouse skin (Laboskin®, Hos: HR-1 Male, 7 weeks old; Hoshino Laboratory Animals Inc., Ibaraki, Japan) was defrosted and cut into 15-mm-diameter specimens. Half of the skin specimens were irradiated with CO2 fractional laser to investigate the effect of laser irradiation on transdermal permeation of OVA-FITC from TR-liposomes. Irradiation was performed with laser beams at a wavelength of 10.6 μm using a fractional CO2 laser (Lutronic: Seoul, Korea) for experiments involving OVA-FITC liposomes) with a moveable articulated arm [14,22] at 15 W power, 12 mJ pulse energy, and a spot density of 200/cm2. The skin specimens were mounted in vertical Franz cells (effective area and volume: 0.64 cm2 and 5 mL, respectively; Perme Gear, Hellertown, PA, USA) (Figure 2). The

temperature of the PBS solution in the Franz cell was maintained at 37 °C or 45 °C using a circulating water bath. The transdermal permeation of OVA-FITC from TR- and control liposomes across non-irradiated and laser-irradiated skin was assessed at 35 and 45 °C (n = 2/group/condition). Liposome suspension (1 mL) was added to the donor chamber, and the transdermal permeation profile was obtained by measuring the fluorescence intensity (Infinite® M1000 TECAN; Tecan) as skin transmittance for 2 days using the following formula:

$$\text{Permeation rate } (\%) = \frac{F^r}{F^d} \times 100$$

where Fr is the fluorescence of sample from the receptor chamber and Fd is the fluorescence of sample from the donor chamber.

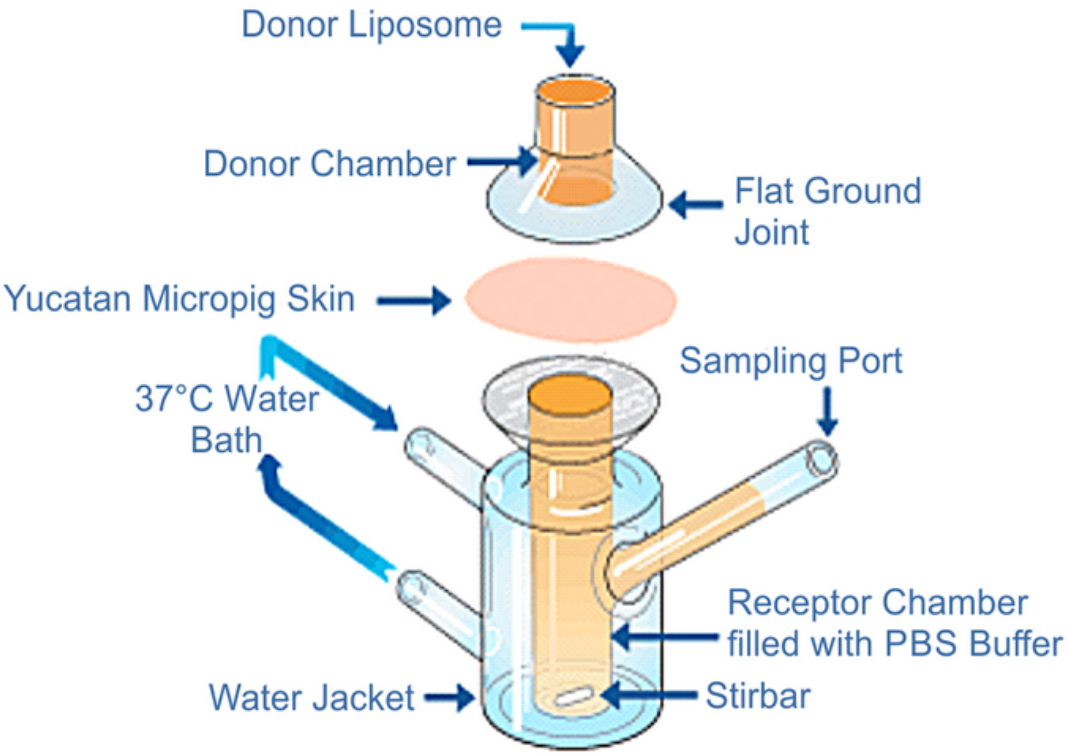

Figure 2. Franz cell setup used to measure transdermal permeation of liposomes through hairless mouse skin.

2.3.6 Transdermal penetration of liposomes through Yucatan micro-pig skin

The skin from adult Yucatan micro-pigs (YMP) was used to study the transdermal penetration of TR- and control liposomes at 35 and 45 °C. YMP skin was chosen because its density, thickness, and physicochemical properties are similar to those of human skin [23,24]. Frozen YMP skin samples (derived from 5-month-old female Yucatan micro-pigs, average weight, 25 kg) were obtained from Charles River Japan Inc. (Yokohama, Kanagawa, Japan). The subcutaneous fat was removed from the YMP skin sheets and approximately 0.4-mm-thick skin samples

were used in the permeation experiments using Franz cells at 35 and 45 °C as described above. After 48 h, the YMP skin sample was retrieved from the Franz cell, fixed with paraformaldehyde and processed for cryosectioning. Frozen sections (20-µm thick) were evaluated using bright-field and fluorescence microscopy to visualize the penetration of the liposomes and the distribution of FITC and rhodamine.

2.4 Results and discussion

2.4.1 Effect of temperature on liposome diameter

Dynamic light scattering analysis showed that the mean diameter of OVA-FITC liposomes increased steadily and nearly doubled as the temperature increased from 37 °C (close to LCST of the component thermosensitive polymer; liposome diameter, 190 ± 5 nm) to 50 °C (liposome diameter, 325 ± 7 nm; Figure 3). I posit that the increase in liposome size occurred because the thermosensitive polymer in the liposome membrane changes in shape and elongates inducing the liposome

surface to change from hydrophilic to hydrophobic at temperatures above LCST, causing the liposomes to aggregate together and making the apparent particle size larger [25–27]. These observations also confirmed the temperature-dependent properties of the tested TR-liposomes.

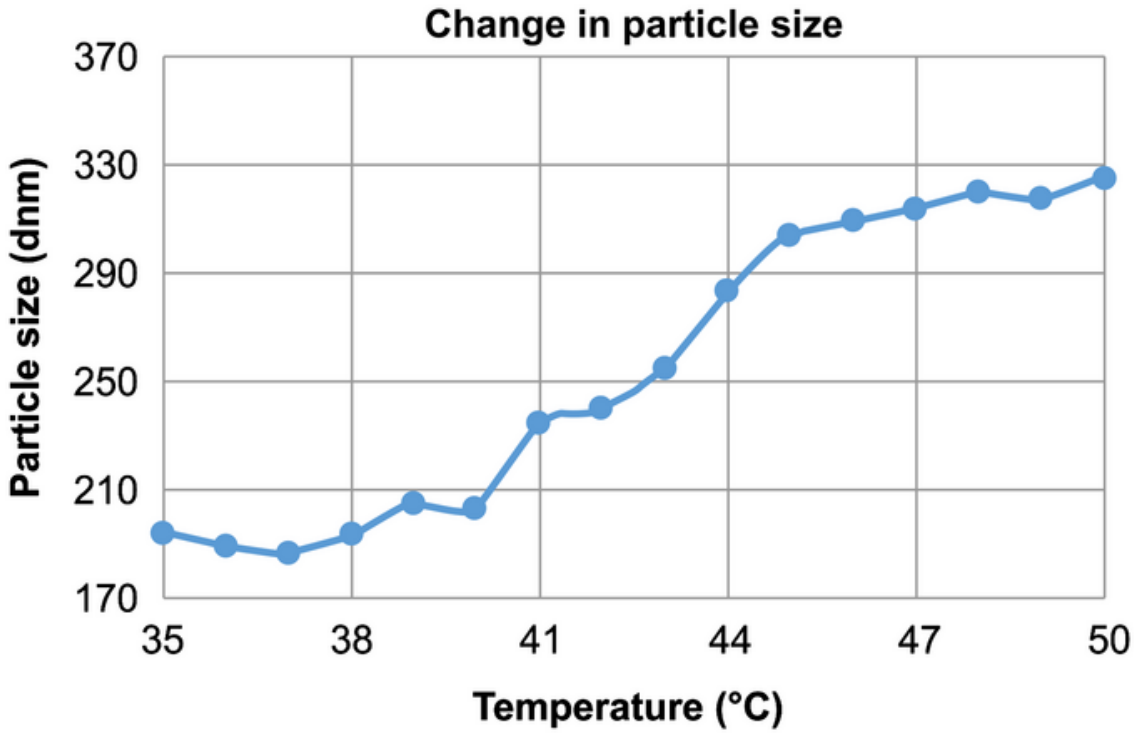

Figure 3. Changes in liposome particle size with temperature.

2.4.2 Effect of temperature on drug release from liposome

The rate and cumulative amount of OVA-FITC released from the TR-liposomes over 60 min of incubation were higher at 45 °C compared to those at 37 °C (Figure 4). The total amount of drug released over 60 min nearly doubled from 32% at

37 °C to 70% at 45 °C. These observations further confirmed the temperature-dependent properties of the TR-liposomes.

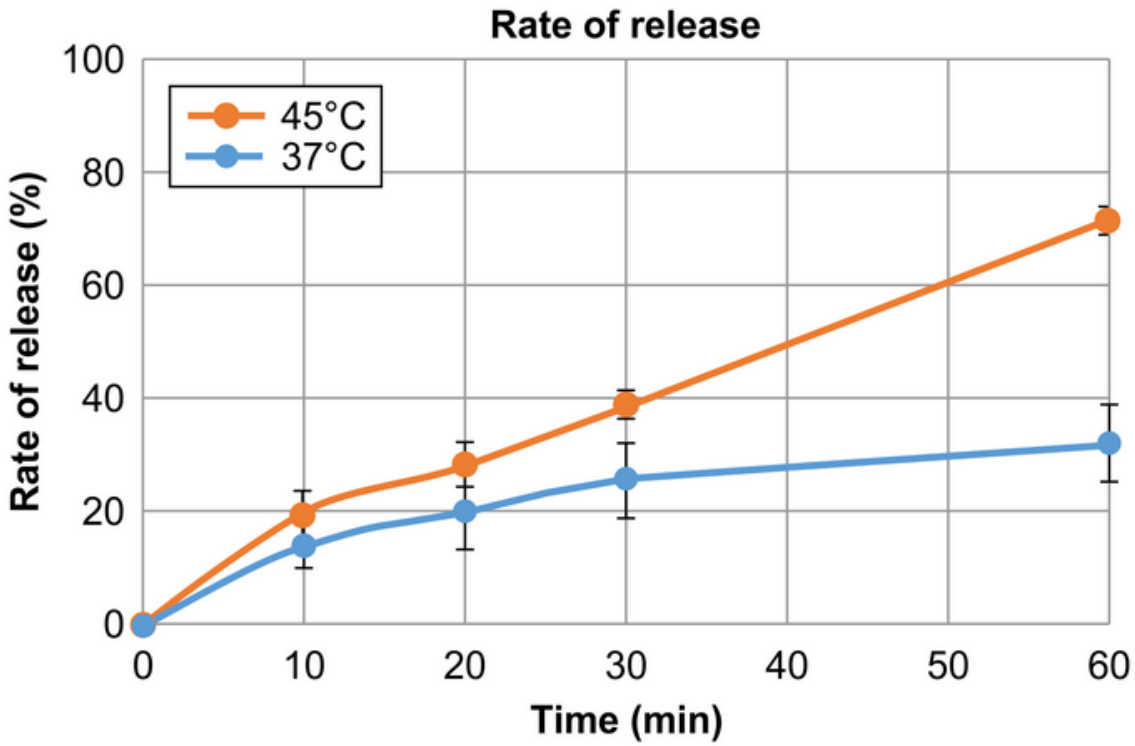

Figure 4. Effect of temperature on temporal release rate of drug from liposome.

2.4.3 Effect of fractional laser irradiation and temperature on transdermal permeation of liposome

Transdermal permeation of OVA-FITC across non-irradiated skin from both TR- and control liposomes was minimal at 37 °C, but increased at 45 °C (8% for TR-liposomes, and 30% for control liposomes after 48 h; Figure 5). The increase in transdermal permeation could be explained by (1) a softening of the liposomal

lipid membrane, and (2) an increase in hydrophobicity of the polymer (in the TR-liposomes) at a higher temperature, facilitating liposome absorption into the skin. The lower permeation of OVA-FITC from TR-liposomes could be explained by (1) their larger size, and (2) the development of a hydration layer by the polymer that stabilizes the liposome and impedes absorption of the liposome into the skin [28–30].

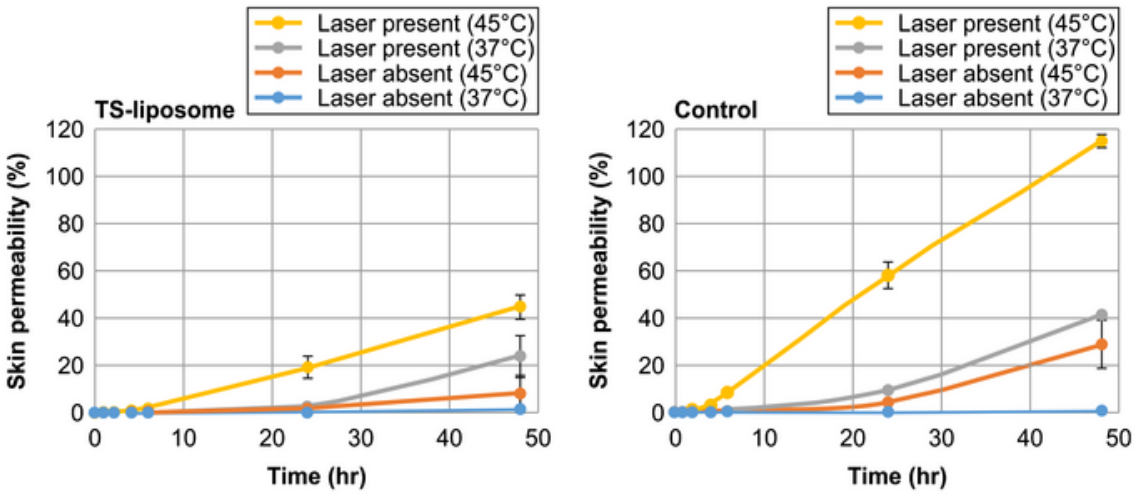

Figure 5. Transdermal permeation of (A) polymer-modified and (B) control liposomes at 37 and 45 °C.

Fractional laser irradiation of skin significantly increased the transdermal permeation for both liposome groups under both temperature conditions. The increase in transdermal permeation by laser irradiation could be explained by creation of microscopic holes in the skin. Furthermore, compared to control lipo-

somes, TR-liposomes continued to show lower permeation, suggesting that the larger size and the stabilizing effect of the polymer-derived hydration layer continued to impede absorption across irradiated skin.

2.4.4 Transdermal penetration of liposomes through Yucatan micro-pig skin

Fluorescence microscopy of the frozen biopsy specimens (Figure 6) showed deeper penetration of FITC fluorescence from unmodified liposomes compared to that from polymer-modified liposomes. Rhodamine fluorescence distribution and intensity showed interesting differences between the two groups. Rhodamine accumulation was not observed with TR-liposomes at either 37 °C or 45 °C. However, rhodamine was observed close to the epidermal surface with only an increase in intensity at 45 °C, whereas FITC penetrated deeper into the dermis at the higher temperature (Figure 7). These results suggest that permeation occurred after release of the encapsulated material from the TR-liposomes into the medium, whereas control liposomes adsorbed and fused with the skin surface prior to release of the encapsulated material into the skin.

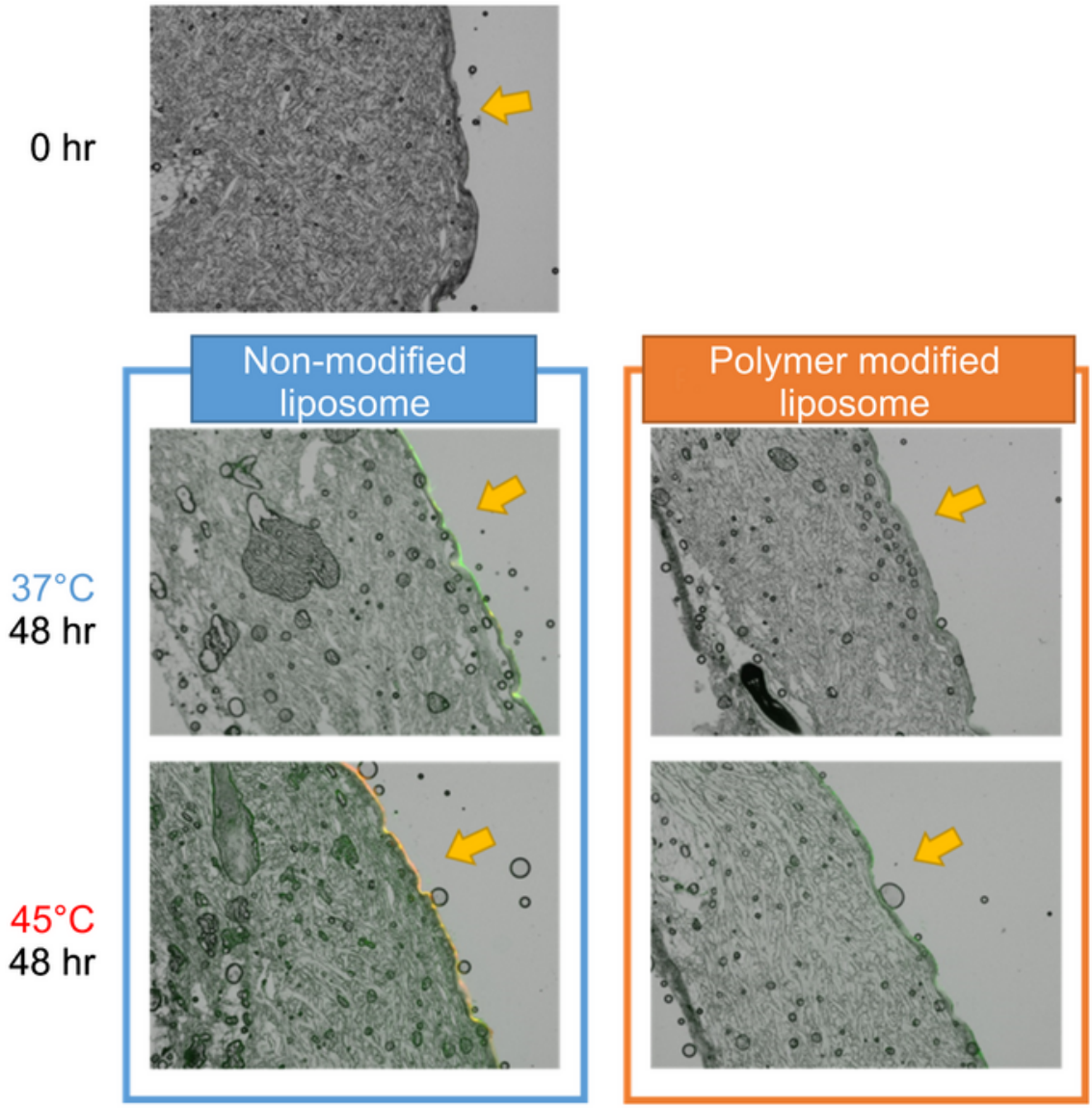

Figure 6. Fluorescence imaging of dermal cross-sections showing transdermal penetration of TR- and control liposomes. Light field images were superimposed with green (FITC) and red (rhodamine) fluorescence images for analysis.

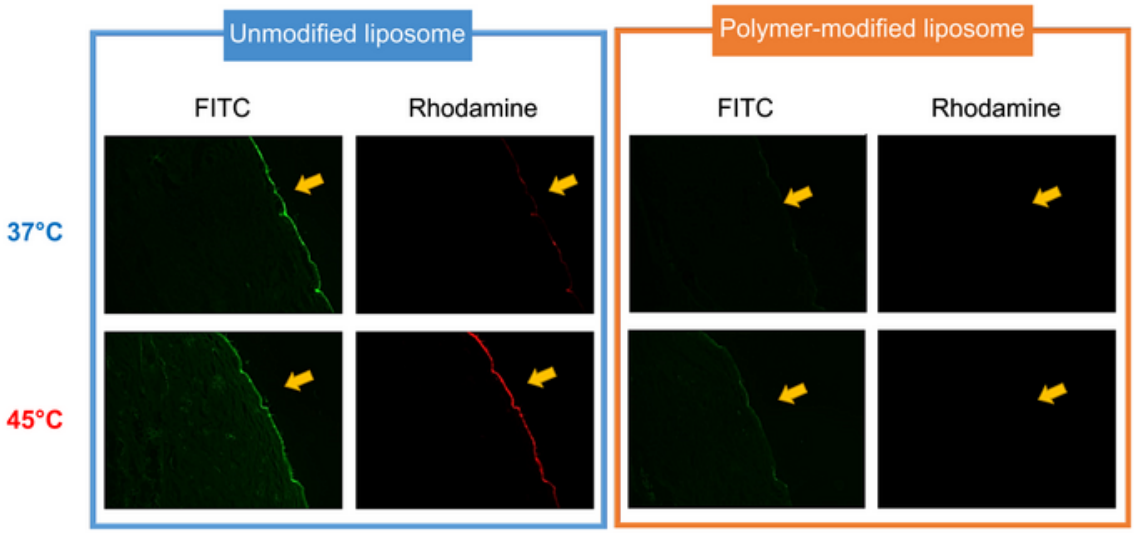

Figure 7. Fluorescence imaging of dermal cross-sections showing surface accumulation and transdermal penetration of TR- and control liposomes (green fluorescence: FITC and red fluorescence: rhodamine).

The accumulation of liposomes on the epidermal surface has been reported previously. It has been suggested that the liposome itself does not permeate into the skin, but rather forms a stacking structure on the surface of the stratum corneum to act as a local depot of the drug that is subsequently absorbed into the skin [31]. The phospholipids in the liposomal membrane interact with intercellular lipids in the stratum corneum and change its permeability to the drug, which then permeates the skin following the thermodynamic activity gradient between the stratum corneum and the skin [32,33]. Our results suggest that the presence of the

thermosensitive polymer hinders this step of liposome fusion with the stratum corneum, thereby reducing the permeability of the TR-liposomes.

In this study, I successfully developed a method for formulating TR-liposomes that increased drug delivery in response to an increase in temperature. The thermosensitive polymer used to prepare the liposomes was chosen such that their LCST was close to the physiologic body temperature (37 °C). This would ensure that drug release from the liposome would be triggered only after application on the skin surface – a feature that is important to improve the shelf life of the liposome preparation and allow controlled drug release. However, the drug release was somewhat lower in comparison to control liposomes without the thermosensitive polymer, likely due to the larger size of the TS-liposomes [29,30] and possible hindrance from the polymer-derived stabilizing hydration layer on the liposome surface. Interestingly, the mechanism of release also appeared to be different for the TS-liposomes. Unlike the control liposomes, which appeared to stack and fuse on the epidermal surface before releasing their encapsulated material into the skin, the TS-liposomes did not fuse but released their encapsulated material into the medium from where it was subsequently absorbed into the skin.

Drug permeation and penetration were therefore lower with TS-liposomes compared to non-modified liposomes.

Notably, CO2 fractional laser irradiation of the skin increased dermal penetration of drug from both types of liposomes with drug permeation from TS-liposomes reaching modest levels at 45 °C. These results show that the fractional laser largely overcomes the inhibitory effect of the large size and/or the stabilizing hydration layer on the TS-liposomes. Fractional lasers have been previously evaluated for their potential to enhance the transcutaneous delivery of hydrophilic drugs, polypeptides, and dextrans [14,15,34]. Our results demonstrate the utility of combining CO2 fractional laser and liposomes as an approach to enhance skin penetration of hydrophilic peptide drugs. The increased dermal permeability to drugs is likely achieved by creation of micro-channels within the area of skin illuminated with the multiple, distinct micro-laser beams of the fractional laser [35].

This study has several limitations. First, the size of liposomes prepared in this study was not uniform. Though I used a 400 nm filter for sizing, liposome diameter ranged from 190 to 325 nm. As smaller liposomes have higher dermal permeability [28–30], it is important that uniformly sized liposomes are synthesized and

evaluated to increase the consistency and reproducibility of results. Further studies are also required to optimize the liposome composition to improve drug permeation and penetration into skin, to permit effective application even in the absence of fractional laser treatment. Second, I did not investigate the safety and biocompatibility aspects of the liposome systems or their combination with dermal irradiation using fractional laser. However, PNIPAAm, egg PC, and DOPE used in the TR-liposomes have been extensively used in liposome and other therapeutic preparations, and their safety and biocompatibility are well documented. Fractional laser irradiation of skin is also commonly used clinically and is known to be mostly safe. There have been some concerns of oxidative damage by reactive oxygen species induced by laser irradiation of skin [36]. Concurrent delivery of antioxidants such as fullerenes and vitamin C derivatives via liposomes has been suggested as a strategy to mitigate any laser-induced oxidative damage. Further preclinical and clinical studies are required to optimize and establish therapeutic efficacy and address any safety concerns of these novel strategies.

In conclusion, I developed TR-liposomes by incorporating a thermosensitive polymer into the liposomal membrane, and achieved temperature-dependent controlled release of an encapsulated drug. I also demonstrated the possibility of concurrently using fractional laser irradiation as a strategy to increase the

transdermal delivery of drugs from the TR-liposomes. Additional studies are required to control liposome size and optimize their transdermal permeation properties.

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Chapter 3

3.1 Presentations

2016

The 36th ASLMS American Society for LASER Medicine and Surgery Annual Conference in Boston, USA

Fujimoto T, Baba K, Oki Y, Jian W, Kanazawa H : Facilitation of transcutaneous drug delivery using a fractional laser and Liposome technology

2015

The 35th ASLMS American Society for LASER Medicine and Surgery Annual Conference in Kissimmee, FL, USA

Fujitmoto T, Ito S, Tujino S, Kanazawa H: Comparison Study of Rapid Repetition Rate And Continuous Near Infrared (Nir) Light Devices: Detection Of The Different Reactive Oxygen Species Induced Oxidative Stress In Skin During Irradiation, Respectively

2014

The 34th ASLMS American Society For LASER Medicine and Surgery 2013 Annual Conference in Phoenix, Arizona, USA

Fujimoto T, Yamaguchi S, Kanazawa H, Ito S:Detection of the Different Reactive Oxygen Species Induced Oxidative Stress in Skin During CO2 Fractional Laser, RF, CO2 Fractional Laser and RF Combination Therapy Respectively

2013

The 33rd ASLMS American Society For LASER Medicine and Surgery Annual Conference in Boston, USA

Fujimoto T, Yamaguchi S, Kanazawa H, Ito S Detection of the different reactive oxygen species induced oxidative stress in skin during 1550nm & 1927nm Fractionated Laser therapy: Lasers in Surgery and Medicine

3.2 Papers

1) Fujimoto T, Baba K, Oki Y, Jian W, Ito S, Ito M, Hiruta Y, Kanazawa H: Transcutaneous drug delivery by liposomes using fractional laser technology. Lasers Surg Med. in print

2) Fujitmoto T, Ito S, Tujino S, Kanazawa H: Comparison Study Of Rapid Repetition Rate And Continuous Near Infrared (Nir) Light Devices: Detection Of The Different Reactive Oxygen Species Induced Oxidative Stress In Skin During Irradiation, Respectively: Lasers Surg Med.: 2015:47(S26),p9-10

3) Fujimoto T, Imai Y, Tei Y, Ito S, Kanazawa H, Yamaguchi S :High temperature heat source generation with 4-6W power level quasi-cw (continuous wave) semiconductor lasers for medical use: J. Biomed. Opt. 2014; 19 (10) :101502

4) Fujimoto T, Imai Y, Tei Y, Yamaguchi S Development of A Semiconductor Laser Based High Temperature Fine Thermal Energy Source in an Optical Fiber Tip for Clinical Applications Jpn. J. Appl. Phys. 2014; 52: 052501

5) Fujimoto T Metabolic syndrome treatment with the ACBODY low-frequency rotating stimulation device: Bio Clinica. 2014; 29 (4): p76-83

6) Fujimoto T, Nagata T, Yoshioka M, Ito S, Masaki H, Kanazawa H Effects of Novel Vitamin C Derivative GO-VC, After Treament of Fractional CO2 Laser in

Asian Patients: Lasers Surg Med. 2014; 46 (S25) : p9

7) Fujimoto T, Imai Y, Tei Y, Fujioka T, Yamaguchi S High temperature heat source generation with a very low power level quasi-cw (continuous wave) semi-conductor laser for medical use SPIE2013-Proceedings: 856569

8) Fujimoto T, Ito S, Ito M, Kanazawa H, Yamaguchi S :Induction of different reactive oxygen species in the skin during various laser therapies and their inhi-bition by fullerene: Lasers Surg Med. 2012; 44(8): pp685-94.