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Lipid cubic phases in topical drug delivery: Visualization of skin distribution using two-photon microscopy

Johanna Bender [a], Carl Simonsson [b], Maria Smedh [c], Sven Engström [a], Marica B. Ericson [d,e,*]

[a] Department of Chemical and Biological Engineering, Chalmers University of Technology, Göteborg, Sweden
[b] Department of Chemistry, Dermatochemistry and Skin Allergy, University of Gothenburg, Göteborg, Sweden
[c] Centre for Cellular Imaging, the Sahlgrenska Academy, University of Gothenburg, Göteborg, Sweden
[d] Department of Physics, University of Gothenburg, Göteborg, Sweden
[e] Department of Dermatology, Sahlgrenska University Hospital, University of Gothenburg, Göteborg, Sweden

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ABSTRACT

The distribution of sulphorhodamine B (SRB), a fluorescent hydrophilic model drug, was investigated in human skin after passive diffusion using four different topical delivery systems. The delivery vehicles applied were two bicontinuous lipid cubic systems, a commercial ointment and water. The lipid cubic systems consisted of either monoolein (MO) or phytantriol (PT) and water. The formulations were applied on full-thickness human skin during 24 h. Thereafter the samples were investigated using two-photon microscopy (TPM). The TPM system consisted of an inverted microscope with a 40× water-immersion objective, laser scan-box, and a pulsed femtosecond titanium:sapphire laser operating at 780 nm. The fluorescence was detected using a 560 nm long-pass filter. Sequential optical sectioning was performed, resulting in images obtained at different tissue depths. TPM revealed that SRB mainly penetrates the skin via the intercellular lipid matrix. Samples exposed to the cubic phases showed a higher accumulation of SRB in micro-fissures, from which a fluorescent network of threadlike structures spread laterally in the tissue. These structures were also detected in some of the ointment samples, but not as frequent. The penetration of SRB into the stratum granulosum was deduced from the fluorescence of SRB present inside polygonal keratinocytes with cell nuclei. Higher SRB fluorescence was obtained in the outermost layer of the epidermis using the bicontinuous cubic phases, compared to when using the reference formulations. Thus, our results suggest that the dominating delivery route using the cubic phases is via micro-fissures caused by microscopic clustering of the keratinocytes in the skin. From these micro–fissures hydrophilic compounds, here modeled by SRB, can diffuse into the surrounding intercellular lipid matrix acting like a source for sustained release.
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1. Introduction

Topical drug delivery is an attractive alternative to oral administration since, e.g., first-pass metabolism in the liver is circumvented and constant drug levels can be maintained in specific locations for prolonged periods of time. The main drawback is the limited uptake of drugs over the skin barrier, and studies of topical drug uptake are necessary in order to facilitate the design of efficient drug delivery systems. Traditionally, skin permeation is studied with diffusion cells, were the skin is considered as a biomembrane, and the permeation through the membrane is measured [1,2]. However, these studies lack information about how the substances are absorbed and distributed in the skin. Thus, in this study we use two-photon microscopy (TPM) to obtain information by visualizing the uptake in full-thickness human skin of the hydrophilic fluorescent model drug sulphorhodamine B (SRB) (Fig. 1).

Stratum corneum (SC) is considered to be the rate limiting barrier in transdermal drug delivery [3,4]. Various solutions to improve the drug uptake in skin from topical formulations have been proposed. One solution is to introduce a known penetration enhancer per se in the drug delivery vehicle [5], another is to use a complete drug delivery system. Examples of such systems are liposomes [6], lipid vesicles [7–9] and bicontinuous cubic phases [10,11].

The bicontinuous cubic phases are equilibrium phases consisting of a mixture of lipids and water and have proved effective for topical drug delivery [10,11]. We have earlier investigated the in vivo drug delivery potential of cubic phases compared to commercial ointment for topical delivery of aminolaevulinic acid and methyl aminolaevulinate, two hydrophilic compounds used for photodynamic therapy [12]. The investigated cubic phases contained either the lipid monoolein (MO) or phytantriol (PT), for which molecular structures are presented in Fig. 1. MO is a monoglyceride formed endogenously during oil digestion in the upper intestine [13]. Together with water, MO may form the liquid crystalline bicontinuous cubic phase [14–16]. The bicontinuous cubic phase is an isotropic, thermodynamically

* Corresponding author. Department of Physics, University of Gothenburg, SE-412 96 Gothenburg, Sweden. Tel.: +46 31 772 32 80.
E-mail address: marica.ericson@physics.gu.se (M.B. Ericson).

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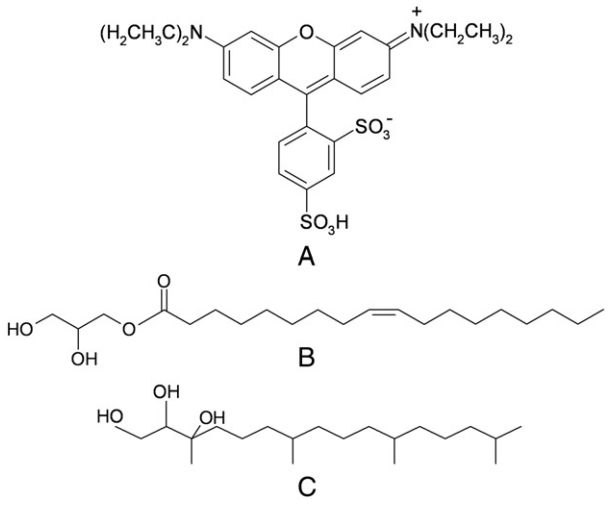

Fig. 1. Molecular structures of A) sulphorhodamine B (SRB), B) monoolein (MO) and C) phytantriol (PT).

stable and macroscopically stiff. It consists of one congruent lipid bilayer (3–-4 nm thick), extending in three dimensions, surrounded on both sides by 5–-6 nm wide water channels. Also the polar lipid PT, which has been used in the cosmetic industry for several applications [17,18], has been shown to have an aqueous phase behavior similar to that of MO [19]. Thus, PT may also form a bicontinuous cubic phase when mixed with water. Since the cubic phase is bicontinuous, it can dissolve both hydrophilic, amphiphilic and hydrophobic substances, which makes it interesting as a drug delivery vehicle. Bicontinuous cubic phases from both MO and PT and were shown to be superior for topical delivery of hydrophilic compounds in our former study [12]; however, the penetration pathways into skin using these formulations have not yet been clearly investigated. Therefore mechanistic studies are required to understand why the MO and PT cubic phases improve drug delivery.

The classical techniques for assessment of dermal drug delivery systems include investigation of systemic uptake in vivo by blood or urine, and in vitro permeability studies using diffusion cells [2,20,21]. However, these methods do not provide any information on how the substances are absorbed and distributed in the skin. By using confocal laser scanning microscopy (CLSM) fluorescent compounds can be visualized in skin [9,22,23], although scattering and absorption of the excitation and emission light, which lies in the UV and visible range, limit the imaging depths. Furthermore, photobleaching and photodamage are other major drawbacks using CLSM [22].

Two-photon excitation is an outcome of a nonlinear excitation process through simultaneous absorption of two photons [24]. This means that near infrared light (NIR) can be used for excitation of fluorophores with fluorescence in the visible range of light. Since the NIR wavelengths lie in the so-called "optical window" of biological tissue, TPM enables imaging of fluorophores much deeper into highly light scattering and light absorbing tissue compared to CLSM, with minimal photobleaching and phototoxic effects [25,26]. These features make TPM a powerful tool in skin research [7,27,28]. For example, Yu and coworkers used TPM for studying the effect of a penetration enhancer, using SRB and rhodamine hexyl ester as model drugs [27]. We have used TPM in a similar fashion, to visualize the skin distribution of SRB, topically applied using bicontinuous lipid cubic vehicles of MO and PT on full–thickness human skin. Water and a commercial ointment have been used as reference vehicles. The choice of the reference vehicles was made to be able to relate to our results from a former in vivo study of lipid cubic phases [12], and to be able to compare formulations with various internal structures, i.e. the ointment being an emulsion with discrete micrometer–sized aggregates and the water because it lacks the internal order. The hydrophilic model drug was chosen since it has been used in previous TPM studies [27].

2. Materials and methods

2.1. Chemicals

Glyceryl monooleate (RYLO™ MG 19 Pharma, Lot no. 2202/42) with a monoglyceride content of 95%, a diglyceride content of 3.8%, free glycerin content of 0.6%, a water content of 0.1%, and a fatty acid composition of oleic acid 89% / linoleic acid (C18-2) 5% / saturated C18 3% / saturated C16 1% / linolenic (C18-3) 1%, was kindly provided by Danisco Cultor (Brabrand, Denmark). Phytantriol (3,7,11,15-tetra methyl-1,2,3-hexadecanetriol, Lot no. PTL-02924), purity 99.03%, was a kind gift from Kuraray Co. Ltd (Tokyo, Japan). Sulphorhodamine B (Lot. no. 36086, Molecular probes Oregon, USA) was bought from Sigma Aldrich (Steinheim, Germany) and a commercial ointment (Unguentum M, Hermal, Reinbek, Germany) at the local pharmacy. Water was of Milli-Q quality. All other chemicals were of analytical grade and used as supplied.

2.2. Preparation of formulations

MO was melted in a block heater at 45 °C. When melted, the appropriate amount of lipid was weighed into a glass vial and left to cool. Meanwhile, SRB was weighed in together with water in another vial. When dissolved, the appropriate amount of the resulting SRB stock-solution was added to the lipid. The sample was sealed, vortexed and centrifuged back and forth several times before it was left to equilibrate in a dark environment for 2–3 days at 20–22 °C. The PT formulation was prepared in the same way as the MO sample, but the lipid was instead melted at 50 °C. The lipid/water composition in the MO cubic phase was 70/30 w/w, while the ratio for the PT cubic phase was 75/25 w/w. Both samples had an SRB concentration of 0.14% (w/w). Blank samples of both lipids were prepared in the same way but without addition of drug. Since the cubic phase is isotropic, the samples were examined between crossed polarizers after equilibration in order to detect if any anisotropy was present. The viscosity and visual appearance of the cubic samples were examined before, during and after each experiment in order to detect if any phase transitions had occurred. This would have been seen as anisotropy and/or a decreased viscosity of the samples. Since no such changes were observed, the samples were regarded as cubic.

For the reference vehicles, the appropriate amount of water or ointment was weighed into a vial and the proper amount of drug, i.e. 0.14%, was added. The liquid sample was vortexed and the ointment reference was carefully stirred with a glass-stick until the drug had dissolved. All reference samples were prepared at 20–22 °C and kept under dark conditions until used.

2.3. Application of the formulations on full-thickness skin

Full-thickness human skin was obtained from a breast reduction surgery of three Caucasian female patients at the Sahlgrenska University Hospital, Göteborg, Sweden. The skin was stored at −70 °C and used within 4 weeks after surgery. The skin was thawed at room temperature and excess fat was removed with a scalpel before application of formulations.

Approximately 60 mg of each of the cubic sample and the commercial ointment were applied with a small spatula in small amounts at a time onto the full-thickness skin, which was pre-washed with phosphate buffered saline (PBS) and thereafter dried with Kleenex. Application was performed so that a visually good contact between the formulation and the skin was obtained to allow for passive diffusion into the skin. The area was thereafter covered with transparent occlusive dressing (OpSite® Flexigrid, Smith & Nephew Medical Ltd, Hull, England) in order to simulate in vivo conditions, before being mounted in the diffusion cells (Franz-type). The exposed surface area was about 0.8 cm^2. For the water vehicle, the skin was directly mounted in the diffusion cells and 1 ml of PBS was added to

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