Liquid Crystal Nanodispersions Enable the Cutaneous Delivery of Photosensitizer for Topical PDT: Fluorescence Microscopy Study of Skin Penetration
Liquid Crystal Nanodispersions Enable the Cutaneous Delivery of Photosensitizer for Topical PDT: Fluorescence Microscopy Study of Skin Penetration

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Abstract: Topical photodynamic therapy (PDT) has been applied to almost all types of nonmelanoma skin cancer and numerous benign skin disorders [1-2]. PDT is based on the administration of a photosensitizing drug and its selective retention in malignant tissue and its subsequent activation by light at specific wavelengths, causing cell death by the production of free radicals and/or reactive oxygen species [3, 4]. Numerous strategies have been studied in recent years in attempts to improve the accumulation of photosensitizers and their precursors in the skin, including the use of microemulsions [5], micelles [6], liposomes [7], ceramic-based nanoparticles [8], gold nanoparticles [9], polymer nanoparticles [10], dendrimers [11], invasomes [12] and liquid crystalline phases [13-15]. Lipophilic phthalocyanines (e.g., zinc and chloroaluminum compounds) have been widely used as photosensitizers in preclinical studies of PDT for the treatment of skin cancer [16, 17]. In contrast, the water-soluble zinc phthalocyanine tetrasulfonate (ZnPcSO₄) has displayed high photodynamic efficiency and reduced phototoxic side effects in the treatment of brain tumors and ocular tumors [18]. However, with exception of a few studies [19-21], there is a lack of data on the application of ZnPcSO₄ in PDT for skin cancer.

Their ability to control the release of drugs and their excellent biocompatibility make liquid crystals based on polar lipids such as monoolein (MO) particularly attractive as delivery matrices for the topical and transdermal delivery of drugs [20, 22-25], including prodrugs and photosensitizers in PDT for skin cancer [13, 14]. Moreover, liquid crystalline lipid-water phases with an inverse structure (reverse hexagonal and the cubic phases) can co-exist in equilibrium with an excess of water, forming kinetically stable colloidal dispersions [26].

ZnPcSO₄ (MW 898.15 g/mol) presents four charged groups in its molecular structure, which makes the molecule hydrophilic and prevents its penetration into the lipophilic stratum corneum, the main skin barrier. The low passive penetration of the drug, therefore, motivates the use of nanoparticles to improve drug entry into deeper skin layers. Nanoparticles of cubic and hexagonal lipid-water phases have been used for the development of controlled-release formulations of biologically active agents in the field of drug delivery and have demonstrated their potential as a new topical delivery system [15]. In this context, we studied several bulk MO-based liquid crystalline phases and their nanodispersions in vitro and in vivo with respect to their ability to increase the skin uptake of ZnPcSO₄, a crucial condition for the effectiveness of topical PDT for skin cancer.

Keywords: Liquid crystalline phases, nanodispersion, skin cancer, skin penetration, photodynamic therapy, zinc phthalocyanine.

1. INTRODUCTION

Topical photodynamic therapy (PDT) has been applied to almost every type of superficial nonmelanoma skin cancer and numerous benign skin disorders [1-2]. PDT is based on the administration of a photosensitizing drug and its selective retention in malignant tissue and its subsequent activation by light at specific wavelengths, causing cell death by the production of free radicals and/or reactive oxygen species [3, 4]. Numerous strategies have been studied in recent years in attempts to improve the accumulation of photosensitizers and their precursors in the skin, including the use of microemulsions [5], micelles [6], liposomes [7], ceramic-based nanoparticles [8], gold nanoparticles [9], polymer nanoparticles [10], dendrimers [11], invasomes [12] and liquid crystalline phases [13-15]. Lipophilic phthalocyanines (e.g., zinc and chloroaluminum compounds) have been widely used as photosensitizers in preclinical studies of PDT for the treatment of skin cancer [16, 17]. In contrast, the water-soluble zinc phthalocyanine tetrasulfonate (ZnPcSO₄) has displayed high photodynamic efficiency and reduced phototoxic side effects in the treatment of brain tumors and ocular tumors [18]. However, with exception of a few studies [19-21], there is a lack of data on the application of ZnPcSO₄ in PDT for skin cancer.

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MO:OA:water of 77:5:18 (w/w/w). The preparations were loaded with 0.5 mg of ZnPcSO$_4$/3g formulation after solubilization in the aqueous phase. All preparations were kept at room temperature and protected from the light for 24 h. The formulations were examined through a polarized light microscope (Axioplan 2 Image Pol microscope, Carl Zeiss, Oberkochen, Germany) to characterize their liquid crystalline structure.

2.3. Nanodispersion Obtainment and Characterization

The cubic and hexagonal phase gels, obtained as described above, were dispersed by vortex-mixing in a pH 6.0 citrate buffer containing 1% or 1.5% of poloxamer in the proportion 10:90 (gel:buffer), respectively. The dispersions were loaded with ZnPcSO$_4$ at the same concentration used for the bulk gels. The resulting dispersions were sonicated in an ice bath for 2 min, centrifuged at 1,901 × g for 10 min and then filtered through a 0.8 μm membrane [21]. The mean diameter and particle size distribution of each dispersion were determined using a dynamic light scattering system (Zetasizer, NanoZS, Malvern, UK) at 90° with a He-Ne laser. For this procedure, samples were first diluted in particle-free purified water, and the measurements were performed at 25°C.

The degree of encapsulation was determined by size exclusion chromatography. Samples (500 μL) were loaded on a Sephadex LH-20 column (3.5 cm in diameter with a column packing length of 17 cm) and eluted using purified water as the mobile phase. The eluted fractions were monitored by turbidity measurements at 410 nm using a FEMTO 800XI spectrophotometer (Sao Paulo, Brazil). Aliquots of 1 mL of each fraction were lyophilized then solubilized in 3 mL of methanol and assayed for ZnPcSO$_4$ content by spectrophotometry ($\lambda_{ex}$ = 640 nm, $\lambda_{em}$ = 730 nm), as described in the spectrofluorimetric assay section. The encapsulation efficiency (EE) was calculated using the following equation:

$$EE = \frac{M_1}{M_t} \times 100$$

where, $M_1$ is the amount of ZnPcSO$_4$ encapsulated in the nanoparticles and $M_t$ is the amount of ZnPcSO$_4$ used in the formulation. The experiments were performed in triplicate.

The lamellar nanodispersions not was evaluated in this research because it has been studied by ours research group and in the future it may be demonstrated as promising skin delivery enhancer of a several lipophilic and hydrophilic photosensitizers.

2.4. In vitro Skin Penetration Study

ZnPcSO$_4$ topical delivery systems were assessed in an in vitro model of porcine ear skin, as previously described [24] in order to evaluate the skin retention of ZnPcSO$_4$ from nanodispersions of MO-based liquid crystalline hexagonal and cubic phases and the corresponding cubic, lamellar and hexagonal phase bulk gels.

The skin of a freshly excised porcine ear was carefully dissected (ensuring that the subcutaneous fat was completely removed), dermatomized at 500 μm (Dermaton, Nouvag, Switzerland) and stored at -20°C. On the day of the experiment, the skin was mounted in a Franz diffusion cell (with a diffusion area of 1.5 cm$^2$, delimiting by a template). After 6 h of application, the animals were euthanized with carbon dioxide vapor following the protocol previously authorized by the University of São Paulo Animal Care and Use Committee (Authorization number: 10.1.160.53.0). The treated skin regions were dissected.

2.5. Spectrofluorimetric Assay for ZnPcSO$_4$

ZnPcSO$_4$ present in the receptor solution and samples from size exclusion chromatography was assayed by spectrofluorometry ($\lambda_{ex}$ = 640 nm, $\lambda_{em}$ = 730 nm) using a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan). Analytical method was developed and evaluated with respect to linearity, precision, accuracy, limits of detection (LOD), and lower limit of quantification (LLOQ). The linearity of the assay was determined using methanolic standard solution with concentration ranging from 0.25–10.00 μg/mL, and the calibration curve was $y=0.1447x+0.0025$ ($r=0.999$). The error and accuracy of the method showed a variation coefficient not greater than 0.93% and 96%, respectively. The lower limits of quantification and detection of the method were 0.25 μg/mL and 0.08 μg/mL, respectively.

2.6. In vivo Skin Penetration Studies

These experiments were performed using six female hairless mice (strain HRS/J, Jackson Laboratories, Bar Harbor, ME, USA). One hundred microliters of MO-based hexagonal dispersion formulation was applied to the skin on the dorsal region of the hairless mice over an area of 1.5 cm$^2$, delimiting by a template. After 6 h of application, the animals were euthanized with carbon dioxide vapor following the protocol previously authorized by the University of São Paulo Animal Care and Use Committee (Authorization number: 10.1.160.53.0), and the treated skin regions were dissected.

2.7. Visualization of Cutaneous Penetration of Photosensitizer by Fluorescence Microscopy

The skin samples obtained from the in vitro and in vivo experiments were embedded in a matrix of Tissue-Tek® O.C.T.™ compound (Sakura, Zoeterwoude, The Netherlands), frozen at -17°C and sectioned to the skin surface into vertical slices 40 μm thick using a cryostat (CM 1900, LEICA, Nussloch, Germany). The presence of ZnPcSO$_4$ and its distribution in the skin layers was visualized by fluorescence microscopy (Axioskop 2 plus, Carl Zeiss, Göttingen, Germany) using 640 nm and 730 nm band-pass excitation and emission filters, respectively (filter Set 50, Carl Zeiss). Images were recorded with a light-sensitive charge-coupled device digital camera (AxioCam HR, Göttingen, Carl Zeiss) using identical sensitivity and exposure settings.

3. RESULTS

We investigated the enhanced skin penetration of a water-soluble zinc phthalocyanine provided by MO-based liquid crystalline formulations and their nanodispersions. Bulk gels of lamellar, cubic and hexagonal crystalline phases were initially identified by light microscopy with crossed polarizer, as well as the cubic and hexagonal phase dispersion systems. Polarized light microscopy observation showed patterned structures and birefringent textures of lamellar and hexagonal phases, such as the Maltese cross (Fig. 1A) and an angular texture (Fig. 1B), respectively. Photomicrographs of the nanodispersions of the cubic and hexagonal phases are shown in Fig. 1C and 1D, respectively and it is in agreement with Lopes et al., 2007 [25].

The particle size distribution, zeta potential and polydispersity of both the cubic and hexagonal phase nanodispersions loaded with ZnPcSO$_4$ are listed in Table 1. The light scattering analysis demonstrated the presence of nanosized particles with sizes of 224 ±10 nm and 188 ± 10 nm for the cubic and hexagonal nanodispersions, respectively. Both nanodispersion present negative zeta potential values, of which, hexagonal phase showed higher negative charge. The polydispersity index indicate monodispersed characteristic for both nanodispersions. The encapsulation degrees assessed by size exclusion chromatography were 50.1% and 38.5% ($\pm$ 2) ZnPcSO$_4$ loading in the hexagonal and cubic nanodispersion systems, respectively.
In vitro ZnPcSO₄ skin penetration is illustrated in Fig. (2). After 6 h of application, the MO-based hexagonal nanodispersion system promoted a greater penetration of photosensitizer in the deeper skin layers (Fig. 2F) compared with the cubic phase nanodispersion (Fig. 2E) and the bulk crystalline phases, i.e., the lamellar (Fig. 2C), cubic (Fig. 2C) and hexagonal phases (Fig. 2D).

Due to the improved penetration effect, the hexagonal nanodispersion was selected for a subsequent in vivo penetration study. (Fig. 3) shows sections of untreated skin with and without fluorescence excitation (Fig. 3A and B, respectively) and skin treated with the hexagonal nanodispersion system photographed immediately and 50 seconds after the application of the excitation light (Fig. 3C and 3D, respectively). The in vivo treatment with the hexagonal nanodispersion loaded with ZnPcSO₄ yielded an increased fluorescence in deeper skin layers. (Fig. 3A and 3B) are photomicrographs of untreated skin photographed without and with fluorescence excitation, respectively; a photomicrograph of skin treated with the hexagonal nanodispersion system was obtained in the same way (Fig. 3C). No fluorescence was observed in the untreated skin (Fig. 3B); however, the skin treated with the hexagonal nanodispersion system loaded with ZnPcSO₄ emitted a characteristic red light.

**DISCUSSION**

The water-soluble ZnPcSO₄ has shown high photodynamic efficiency and reduced phototoxic side effects in the treatment of brain and ocular tumors [18, 27]. However, there is a lack of data for its application in topical PDT for skin cancer. Aqueous dispersions of liquid crystalline phases of MO were previously obtained and characterized [23], but the use of a hexagonal phase dispersion as a colloidal carrier of ZnPcSO₄ for topical PDT is a new application in this field. Here, we describe the preparation, characterization and skin uptake of ZnPcSO₄ delivered by a liquid crystalline phase MO nanodispersion.

Fig. (1) shows characteristic patterns of birefringent liquid crystalline phases under polarized light. The hexagonal phase displayed an angular texture [28]. The lamellar phase showed distinct woven structures and/or a mosaic or Maltese cross pattern, whereas the stiff transparent cubic phase was nonbirefringent [28, 29]. The presence of ZnPcSO₄ did not change the liquid crystalline structure of these phases.

Liquid crystalline nanodispersions have many important characteristics for drug delivery due to their drug solubilization capability.
and release properties, which can improve skin penetration by the drug [30]. The sonication of cubic and hexagonal phase with an excess of the aqueous phase to form nanodispersion systems resulted in milky and low-viscosity dispersions, as reported previously [15]. In order to evaluate the influence of poloxamer in cubic and hexagonal phase structures, the formulations were observed by polarized light microscopy before and after addition of poloxamer. The presence of poloxamer did not disrupt the cubic or hexagonal phase structures and additionally yielded a stable nanodispersion in the presence of photosensitizer. Increasing the poloxamer concentration to 1.5% in the hexagonal nanodispersion system caused a discrete reduction in particle size as determined by light scattering, which is in agreement with previously published results [31, 32].

Demonstrating the penetration-enhancing effect of MO, a previous study reported an increase of 5-aminolevulinic acid (5-ALA) and release properties, which can improve skin penetration by the drug [30]. The sonication of cubic and hexagonal phase with an excess of the aqueous phase to form nanodispersion systems resulted in milky and low-viscosity dispersions, as reported previously [15]. In order to evaluate the influence of poloxamer in cubic and hexagonal phase structures, the formulations were observed by polarized light microscopy before and after addition of poloxamer. The presence of poloxamer did not disrupt the cubic or hexagonal phase structures and additionally yielded a stable nanodispersion in the presence of photosensitizer. Increasing the poloxamer concentration to 1.5% in the hexagonal nanodispersion system caused a discrete reduction in particle size as determined by light scattering, which is in agreement with previously published results [31, 32].

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