LIPID-BASED NANOCARRIERS IMPROVED ZnPcSO₄ CELLULAR UPTAKE IN HUMAN KERATINOCYTES FOR USE IN TOPICAL PHOTODYNAMIC THERAPY

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Abstract
Topical photodynamic therapy (PDT) involves both local administration of a photosensitizing drug followed by illumination or irradiation of visible light, resulting in light-drug combinations to kill cells and microorganisms. PDT using tetra-sulfonate phthalocyanine, a second generation of hydrophilic photosensitizers, has recently become a good treatment option for treatment of skin malignances. However, tetra-sulfonate phthalocyanine must be delivered into the target layers of the skin to achieve promising results. Thus, strategies to improve the phthalocyanine cell uptake based on lipid-based nanocarriers were evaluated in this work. The results showed that tetra-sulfonate phthalocyanine loaded lipid-based nanocarriers (microemulsion and liquid crystalline nanodispersion) have nanometric particle size, with low polydispersity, negative zeta potential and high ZnPcSO₄ encapsulation efficiency. Furthermore, both nanocarriers investigated presented little cytotoxicity against HaCaT cells, however the microemulsion presented higher toxicity upon higher concentrations. Finally, the formulations developed herein enabled high ZnPcSO₄ uptake, a promising result. Future studies will be conducted using tumor skin cells lines.

Introduction
Photodynamic therapy (PDT) is widely used for therapy of neoplastic and non-neoplastic diseases and has been investigated in the treatment of patients with either in situ or superficial cancers who are unable to tolerate or who refuse surgical resection¹. This noninvasive technique has specificity for the target tissue, and allows the treatment of multiple lesions simultaneously due to its low toxicity and good cosmetic results². PDT is based on the administration light-sensitive drugs or photosensitizers and their selective retention in the malignant tissue. Subsequent activation by exposure of a photosensitizing (PS) agent to light at a wavelength matching the absorption spectrum leads to a photophysical reaction resulting in cell death due to the production of free radicals and/or reactive oxygen species, especially oxygen singlets (O₂³). Commonly used PSs include porphyrin derivatives and their precursor 5-aminolevulinic acid (ALA), chlorins and phthalocyanines³⁹.

Phthalocyanines are second-generation PSs compounds that are highly colored, generally lipophilic, have shown high photodynamic efficiency in the treatment of tumors, and present reduced phototoxic side effects⁹. A high number of phthalocyanines are currently available with different central metal ligand (usually zinc, silicon or aluminum used to increase singlet oxygen production) and also vary in the type of side chains⁸.
Allen and cols (2001) discussed that the presence of zinc as the central metal ion in phthalocyanine-based photodynamic therapy induces an oxidative stress with short triplet lifetime, high triplet quantum yields and high singlet oxygen quantum yields, which increased photo activity, inducing apoptosis in several malignant and nonmalignant cell lines\textsuperscript{11}. In addition, high photosensitizing activity was also found for water-soluble sulfonated zinc phthalocyanines used against \textit{S. cerevisiae} suggesting that the presence of cationic charge is necessary for inactivation of this microorganisms\textsuperscript{12}. Furthermore, tetra-sulfonated zinc phthalocyanines had more affinity to cells than mono-substituted ones and, as a consequence, the tetra-substituted phthalocyanines exhibited a higher phototoxicity against three skin-derived cell lines such as HT-1080 transformed human fibroblasts, 3T3 mouse embryo fibroblasts and HaCaT human keratinocytes\textsuperscript{10}. This tetra-sulfonated zinc phthalocyanine (ZnPcSO\textsubscript{4}), with molecular weight of 898.15 g/mol, presents four charged groups in its molecular structure (Figure 1), which makes the molecule hydrophilic and prevents its penetration into the lipophilic stratum corneum, the main skin barrier, considering the topical administration.

![Figure 1. Molecular structure of zinc phthalocyanine tetrasulfonate.](image)

Topical delivery of ZnPcSO\textsubscript{4} through the skin is a promising strategy due to patient acceptance, restriction of side effects and potent efficacy, but the low passive penetration of the drug could hamper the effective treatment. Thus, nanoparticles could be used to improve drug entry into deeper skin layers, because they are able to overcome stratum corneum barrier, which is needed for successful topical photodynamic therapy using ZnPcSO\textsubscript{4}. In this context, ZnPcSO\textsubscript{4}-loaded lipid-based nanoparticles (microemulsion and liquid crystalline nanodispersion) were prepared and characterized in order to investigate their cytotoxicity and cellular uptake in HaCaT cell line, which has been reported for the first time.

**Material and methods**

**2.1 Materials**

The high purity ZnPcSO\textsubscript{4} was purchased from Frontier Scientific. Polysorbate 80 (HLB:15), sorbitan monooleate (HLB:4.3), propylene glycol (PG), Oleic acid (OA) and polyethylene glycol (PEG) were purchased from Sigma Aldrich Co. (St. Louis, MO, EUA). Canola oil of food grade (Cargill, São Paulo, SP, Brazil) was obtained at a local supermarket. A commercial grade of monoolein (MO) (Myverol 18:99) was purchased from Danisco Ingredients (Copenhagen, Denmark) and used as received. Poloxamer 407 was purchased from BASF (São Paulo, Brazil). Dimethyl sulfoxide (DMSO, analytical grade) was purchased from Merck (Darmstadt, Germany). Water was
purified using the Millipore Milli-Q® Water System (Millipore Corporation, Bedford, USA). Amicon® Ultra centrifugal filter devices were purchased from Millipore (Darmstadt, Germany).

2.2 Solubility and Partition coefficients

The aqueous solubility of ZnPcSO4 was confirmed at 25 °C through the addition of an excess amount of ZnPcSO4 in purified water at constant magnetic stirring (300 rpm) for 24 h, in order to reach the equilibrium. Samples were filtered through a 0.45 µm pore sized membrane (Millipore, Darmstadt, Germany) and diluted in methanol for quantification by an spectrophotometric assay (Praça et al, 2012). ZnPcSO4 was studied regarding its octanol-water partition coefficient. Three mL of a ZnPcSO4 solution (1 µg/mL) in purified water were added to the same octanol volume (previously saturated with water). This mixture was stirred at 300 rpm for 30 min, at 25 °C. Then the organic and aqueous phases were separated. ZnPcSO4 was assayed in the aqueous phases before and after the partitioning. The octanol-water partition coefficient was calculated according the following equation:

\[
\log P_{(o/w)} = \log (C_2)/(C_1 – C_2), \quad \text{where:}
\]

\[
P_{(o/w)} = \text{octanol-water partition coefficient}
\]

\[
C_1 = \text{Concentration of drug before partitioning}
\]

\[
C_2 = \text{Concentration of drug after partitioning}
\]

All the experiments were protected from light and done in triplicate.

2.3 Preparation of lipid-based nanocarriers

2.3.1 Microemulsion preparation

The microemulsion was prepared as previously reported by Aliberti et al., 2017. Mixtures of sorbitan monooleate and polysorbate80 (47.0%) at (3:1) were mixed using a magnetic bar at 1500 rpm during 1 hour and subsequently stored overnight at room temperature. Canola oil (38%) was then added and finally aqueous phase composed by propylene glycol/water (3:1, w/w) was also added to the vials, followed by vortexing at 2500rpm during 3 min at room temperature. The preparations were loaded with 0.5 mg of ZnPcSO4/3g formulation after solubilization in the aqueous phase. Formulations were kept at room temperature and protected from light for 24 h.

2.3.2 Nanodispersion preparation

The hexagonal liquid crystalline phase based on monoolein was prepared as previously reported by our research group (Praça et al., 2012). The lipid monoolein was first melted at 42°C followed by the addition of oleic acid and water at 77:5:18 (w/w/w). The preparations were loaded with 0.5 mg of ZnPcSO4/3g formulation after aqueous phase solubilization. Following 24 hours, the gel was visualized under a polarized light microscope for liquid crystalline phase identification (Axioplan 2 Image Pol microscope, Carl Zeiss, Oberkochen, Germany). Then, the formulations were dispersed by vortex-mixing in a pH 6.0 citrate buffer containing 1.5% of poloxamer in the proportion 10:90 (gel:buffer). The resulting dispersions were sonicated in an ice bath for 2 min. centrifuged at 1,901 xg for 10 min and then filtered through a 0.8µm membrane.

2.4 Physicochemical characterization

2.4.1 Size, polydispersity index and zeta potential by dynamic light scattering

The mean diameter and particle size distribution of unloaded and loaded lipid nanocarriers prepared were determined using a dynamic light scattering system (Zetasizer, NanoZS, Malvern, UK) containing a laser system of 4mW He-Ne, operating at a wavelength of 633 nm. Measurements were taken in a 173 ° detection angle and the measurement position within the cuvette was automatically determined by the software. The data represent the average values from three separate measurements. For this procedure, samples were first diluted in 1 mM Kcl (1:200, v/v) and the measurements were performed at 25°C. Measurements of the particle electrophoretic mobility were carried out using the same instrument. The equipment performs an average of 12 determinations for each analysis. The data represent the average values from three separate measurements.

2.4.2 Measurement of encapsulation efficiency

The encapsulation efficiency (EE) was determined after separation and removal of the unloaded drug by ultrafiltration process 13. Briefly, the formulation (0.25 mL) was individually added to the Amicon® Ultra centrifugal
filter devices (Millipore, Darmstadt, Germany) followed by centrifugation at 6000 x g (Centrifuge 5430R, Eppendorf, Hamburg-Eppendorf, Germany) for 15 min at 22 °C. After centrifugation, the unloaded drug was collected in the ultrafiltrate (M_{FD}) and then, it was quantified by fluorimetric assay as well the total drug amount from the formulation (M_{TD}) which was released by mixture with methanol. EE was calculated by the follow formula: 

$$EE(\%) = \frac{M_{TD} - M_{FD}}{M_{TD}} \times 100.$$ 

2.4.3 Quantification of ZnPcSO_{4} by Spectrofluorimetric Assay

In this work, ZnPcSO_{4} was assayed following a previously validated analytical method reported by Praça et al., 2012. Spectrofluorimetric assay (exc = 640 nm, em = 730 nm) using a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) was applied. The linearity of the assay was determinate using methanolic standard solution with concentration ranging from 0.25–10.00 g/mL, and the calibration curve was 
y=0.1447x+0.0025 (r=0.999).

2.5 Cell culture

HaCaT cells line were cultured in 75 cm² flasks with high glucose Dulbecco’s Modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum and antibiotics (100 IU/mL of penicillin, streptomycin and 250 ng/mL amphotericin-B) at 37 °C in which the CO_{2} level was kept constant at 5%.

2.6 Cell viability assay

Cell viability was assayed by resazurin reduction assay following the instructions of the manufacturer’s protocol. Cells were seeded in 96-well culture plate at 10^{4} well for the HaCaT cell line 24 hours before treatments. Serial dilutions of samples were freshly prepared in phosphate buffer solution (PBS), pH 7.4 in the range of 10 to 0.078 µg/mL (ZnPcSO_{4}). Cells were treated for 4 hours in the dark. After treatment, cells were washed twice with complete DMEM and incubated with 90 µL of DMEM and 10 µL of the resazurin reagent for 4 hours. Fluorescence intensities were measured at 540 nm in a SYNERGY-HT multiwell plate reader, Bio-Tek (USA) using KC4 software. Untreated cells were used as a control with 100% viability. The relative cell viability (%) compared to control cells was calculated by 

$$\frac{[abs]_{sample}}{[abs]_{control}} \times 100.$$ 

2.7 Cellular uptake

The in vitro quantitative cellular uptake of unloaded and loaded ZnPcSO_{4} delivery systems was evaluated in HaCaT cell line by flow cytometry using a Faccsanto BD FACSCalibur™ opening with excitation laser at 633 nm and emission line at 640 to 800nm. After seeding in 96-well culture plate at 10^{4} cells/well in DMEM with 10% heat-inactivated fetal bovine serum (FBS) and incubated for 24 h at 37 °C in 5% CO_{2}, cells were exposed to ZnPcSO_{4} at 1.25 µg/mL, following preliminary cell viability assay, and incubated again for 24 h. The cells were washed twice with PBS and detached by treatment with a trypsin solution. Then, cells were resuspended with PBS after centrifugation (129 g for 5 min). Propidium iodide (PI) was also used for analysis of cell viability according to the manufacturer’s instructions. Untreated cells were used as viability control and cells treated with free ZnPcSO_{4} were used as a positive control.

2.8 Statistical analysis

Results obtained in this work were presented as mean ± standard deviation (SD). Data were statistically analyzed by One-way analysis of variance (ANOVA) followed by Tukey multiple comparison test to compare all studied groups. Statistical significance was fixed at p < 0.05

Results and discussion

3.1. Obtainment and characterization of lipid-based nanocarriers

Knowing the physicochemical characteristics of nanostructured delivery system is a crucial first step for future in vitro assays once it helps to predict their performance. For this reason, we performed the measurements of solubility and partition coefficients for ZnPcSO_{4}, the characterization of the lipid-based nanocarries and finally, we evaluated their application on in vitro cellular uptake using human keratinocytes cells line. The obtained results were relevant findings to pave the way for future research in the field of topical photodynamic therapy to treat skin cancer.
The evaluations of solubility and partition coefficients are important preformulation parameters influencing the conduct of formulation development\(^{11}\). Octanol/water partition coefficients of ZnPcSO\(_4\) was Log \(K_{(o/w)}\) = 0.203 ± 0.028 (30 min) at 25°C. This coefficient shows molecule affinity between lipophilic/hydrophilic media and is important for cutaneous permeation studies and also for the development of topical formulations\(^{14}\). It is an indicative that the hydrophilic ZnPcSO\(_4\) could be incorporated into the lipid-based nanocarriers.

The use of ZnPcSO\(_4\) loaded in lipid-based nanocarriers is a promising strategy to solve problems based on self-aggregation of the hydrophilic phthalocyanine and also to improve its skin retention while preventing undesired generalized photosensitization\(^{15}\). The bulk gels of hexagonal crystalline phases formed by monoolein lipid as well as microemulsions formed mainly by vegetal oil and propylene glycol were initially identified by macroscopic and microscopic appearance (Figure 2). Macroscopically, the nanodispersions were heterogeneous and contained a gel phase and an excess of water that formed a milky, low-viscosity system after dispersion (Figure 2-A) while microemulsions were identified as isotropic and transparent dispersion even ZnPcSO\(_4\) incorporation (Figure 2-B). The microscopic observation by light polarized showed patterned birefringent textures of hexagonal phases (Figure 2-C). Our results are in agreement with previously reported studies\(^{5,13,16}\). The addition of ZnPcSO\(_4\) did not change the physical structure of both lipid-based carriers. In addition, both lipid nanostructured delivery systems developed in this work showed similar physicochemical characteristics considering small particle size (lower than 200nm), zeta potential, polydispersity and ZnPcSO\(_4\) entrapment degree (Table 1).

The light scattering analysis demonstrated the presence of nanocarriers with sizes of approximately 20 and 170 nm for the microemulsion and nanodispersion system, respectively, with low polydispersity index. However, the polydispersity of the microemulsion was much lower. In addition, both formulations presented negative zeta potential values, of which, the nanodispersion showed higher negative charge than the microemulsion system. This is a positive feature of the nanodispersion system, because the negative potential zeta values are indicative of colloidal stability\(^5\).

![Figure 2. Macroscopic visualization of lipid-based nanocarriers containing ZnPcSO\(_4\), nanodispersion (A) and microemulsion (B) and microscopic characterization of the liquid crystalline hexagonal phase containing ZnPcSO\(_4\) by polarized light microscopy (C).](image-url)
Table 1. Physicochemical properties of unloaded and ZnPcSO4 loaded in lipid-based nanocarriers.

<table>
<thead>
<tr>
<th></th>
<th>Unloaded Microemulsion</th>
<th>Microemulsion containing ZnPcSO4</th>
<th>Unloaded Nanodispersion</th>
<th>Nanodispersion containing ZnPcSO4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size (nm)</td>
<td>18.5 (± 1.5)</td>
<td>20.7 (±2.6)</td>
<td>187.2 (± 3.0)</td>
<td>168.2 (± 6.5)</td>
</tr>
<tr>
<td>Polydispersity</td>
<td>0.1 (±0.05)</td>
<td>0.05(±0.0)</td>
<td>0.2 (±0.03)</td>
<td>0.2(±0.0)</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>-15.7 (±1.5)</td>
<td>-13.8 (±1.3)</td>
<td>-22.0(±1.4)</td>
<td>-23.0(±0.5)</td>
</tr>
</tbody>
</table>

Results are represented as mean ± SD (n = 3).

The encapsulation degrees were in the range of 50% for ZnPcSO4 loaded in the both nanodispersion and microemulsion systems, respectively. Regarding the lipid source used for the nanocarries as well as the hydrophilic characteristics of ZnPcSO4, these results could be expected. Hydrophilic ZnPcSO4 is supposed to be retained in the polar lipid layers of the nanocarriers. Moreover, the nanocarriers studied, based on monoolein gel plus oleic acid with low surfactant concentration or canola oil with higher surfactant and co-surfactant concentrations, showed similar concentration of aqueous phase in the range of 18% and 15% for nanodispersion and microemulsion, respectively.

The potential of lipid nanocarriers for topical delivery of other hydrophobic photosensitizers, such as Chlorin and Protoporphyrin (PpIX) were recently reported. Petrilli and colleagues (2013) showed an encapsulation degree of 52.1% for Chlorin, which was similar to the encapsulation obtained herein, while higher encapsulation efficiency (88%) was reported for PpIX by Rossetti and colleagues (2016). However, this behavior can be explained by n-methyl-2 pyrrolidone presence which was used to dissolve the PpIX and may have favored the incorporation and arrangement of drug into lipid nanocarriers.

Therefore, small size of the nanocarriers obtained in this work in addition to the high encapsulation efficiency found may be favorable characteristics to facilitate ZnPcSO4 cell penetration. Noteworthy, in vitro cytotoxicity and cellular uptake evaluations ofZnPcSO4 lipid-based nanocarriers were also performed herein.

The effective absence or low cell cytotoxicity induced by ZnPcSO4 in aqueous solution (Free ZnPcSO4) or ZnPcSO4 loaded lipid-based nanocarriers were evaluated in vitro under dark light condition using a human skin cell line HaCaT. HaCaT cells are frequently used as non-target cells in photodynamic therapy and simulate the healthy skin cells.

In this work, none of the investigated formulations were able to induce cell cytotoxicity greater than 20% within the tested conditions of ZnPcSO4 during 4h cell exposure in the dark. When higher concentrations of ZnPcSO4 carried from microemulsion was used, from 2.5 to 10 µg/mL, the cell cytotoxicity values were more expressive (Figure 3). However, these results were also obtained for unloaded microemulsion, suggesting that the microemulsion composition was the cause of cell death. So, sorbitan monooleate and polysorbate 80 (3:1) present into microemulsion may had a negative effect on cell viability. However, when the microemulsion was diluted 10 times, this effect was less prominent. ZnPcSO4-loaded nanodispersions did not present cytotoxicity in higher concentrations, on the other hand.
Figure 3. HaCaT cell viability results determined by neutral red uptake assay (%) for the microemulsions and nanodispersions with or without ZnPcSO4 as well as free ZnPcSO4 aqueous solution after 24 h of incubation.

Ours results of absence or low ZnPcSO4 cell cytotoxicity are in agreement with other studies recently reported for different phthalocyanines using the same HaCaT cells line\textsuperscript{18}. Jancula and colleagues (2013) reported that the cytotoxic effects of several phthalocyanines on HaCaT cells were strongly dependent on illumination treatment as well as on the molecular structure of tested phthalocyanines derivates. In the dark, none of the investigated 31 phthalocyanines types were able to induce greater than 50\% cell inhibition of neutral red uptake within the tested concentration range 2- or 24-h cell exposure\textsuperscript{18}. Considering skin cancer cells line, the aluminum phthalocyanines tetrasulfonate chloride showed similar results using A431 cells (human epidermoid carcinoma) exposed in the dark to concentrations about 1 µg/L\textsuperscript{21}. Since several works demonstrated a good correlation of HaCaT cell line with damage to human skin cells evaluated \textit{in vivo}\textsuperscript{22}, our results suggest that applications of loaded ZnPcSO4 at concentrations about 1 µg/mL should not represent an acute risk of toxicity for human skin.
The cellular uptakes of unloaded (free ZnPcSO4) and loaded ZnPcSO4 lipid-based nanocarriers in HaCaT cell lines were also evaluated by flow cytometry. It was observed that the uptake of free ZnPcSO4 was low when compared with the uptake of ZnPcSO4-loaded lipid-based nanocarriers in HaCaT cell lines at 24 h (Figure 4). The highest cell uptakes were close to 100% for both microemulsion and nanodispersion. Due to this higher cell uptake of ZnPcSO4 in HaCaT cells line, these nanocarriers are promising candidates for PDT of the skin cancer. In general, our results indicated that both formulations studied are good carriers to improve cells uptake of ZnPcSO4 in normal human keratinocytes cells. On the other hand, the ZnPcSO4 microemulsion showed negative effect regarding cell viability when compared to the liquid crystalline nanodispersion containing ZnPcSO4 (Figure 4). These findings suggest an advantage in the use of the nanodispersion to deliver ZnPcSO4 compared to microemulsion.

Conclusion
ZnPcSO4-loaded microemulsion and liquid crystalline nanodispersion presented nanometric size, low polydispersity, negative zeta potential and high encapsulation efficiency. Furthermore, the delivery systems presented little cytotoxicity against HaCaT cell line, particularly the nanodispersion, and both formulations promoted high cell uptake, a promising result. The results obtained herein encourage future studies to investigate the efficacy of these nanocarriers for topical PDT of skin cancer. Further studies will be conducted by our research group to investigate the phototoxic effects on tumor skin cell lines.
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