

Iontophoretic transport of zinc phthalocyanine tetrasulfonic acid as a tool to improve drug topical delivery

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Phthalocyanines have been used as systemic photosensitizers because of their high affinity towards tumour tissue, and the high rates of reactive oxygen species produced when they are irradiated during photodynamic therapy. However, the topical administration of these compounds is limited by their large size, poor hydrosolubility and ionic character. This study aimed to investigate the iontophoretic delivery of charged zinc phthalocyanine tetrasulfonic acid (ZnPcS₄) from a hydrophilic gel to different skin layers by means of in-vitro and in-vivo studies. Six hours of passive administration was insufficient for ZnPcS₄ to cross the stratum corneum (SC) and to reach the epidermis and dermis. No positive effect was reached when anodal iontophoresis was performed, showing that the drug–electrode attraction effect was higher than the electro-osmosis contribution at a pH of 5.5. Cathodal iontophoresis, however, was able to transport significant amounts of the drug to the viable epidermis. In addition, the absence of NaCl in the formulation significantly increased (by five-fold) the amount of ZnPcS₄ that crossed the SC and accumulated in the epidermis and dermis. It was possible to visualize the

drug accumulation in the follicle openings and in the epidermis, even after SC removal. In-vivo experiments in rat skin showed that these results were maintained in an in-vivo model, even with only 15 min of iontophoresis. In addition, confocal analysis of the treated skin showed a homogeneous distribution of ZnPcS₄ in the viable epidermis after this short period of cathodal iontophoresis. *Anti-Cancer Drugs* 22:783–793 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Photodynamic therapy (PDT) has been clinically investigated for the treatment of skin pathologies such as actinic keratosis, Bowen's disease and basal cell carcinoma and squamous cell carcinoma. It is officially recognized in Canada, Japan, the United States and some European countries [1]. Topical PDT is a convenient and less invasive therapy for skin diseases, in which a photosensitizer (PS) agent is administered to the patient through the skin and accumulates in the tissue. The subsequent exposure of the tissue to light in the wavelength of the PS maximum absorption kills the abnormal cells. This event occurs because the PSs used in PDT absorb photons and start a photochemical reaction producing reactive oxygen species [2]. The most studied drug for topical PDT is 5-aminolevulinic acid, which is not a PS agent itself, but is a precursor of the photosensitive protoporphyrin IX [3]. The direct administration of a PS that is capable of crossing the skin and accumulating in a tumour, however, would be more adequate for topical PDT than a prodrug, considering that it would not be dependent on skin metabolism for conversion into an active molecule, thereby providing more regular skin absorption [4].

Porphyryns have been used as systemic PSs because of their high affinity to tumour tissue and because of their pronounced effect upon irradiation with light [5,6]. These compounds are also photo-stable, and have high quantum yield and a long triplet-state half-life. However, these molecules absorb light at relatively short wavelengths, which do not penetrate deep tissues efficiently. To overcome this disadvantage, most modern PSs being studied absorb light at higher wavelengths and are thus compatible with the therapeutic window of the skin. Among these PSs, the phthalocyanines can be highlighted [7].

Phthalocyanines are second-generation PS porphyrin derivatives. They have shown a high efficacy in the treatment of tumours in animals, and clinically with few side effects [8–10]. The presence of a metal ion, such as zinc or aluminium, in the molecule, increases its half-life in the form of a triplet state, therefore increasing its chances of reacting with the substrate. Phthalocyanines are structurally similar to porphyryns; however, they have a higher molar absorptivity ($\epsilon > 10^5$ /mol/l/cm, when fully monomerized) at wavelengths that allow greater

penetration of light in normal tissues (typically at 670–690 nm) compared with porphyrins (haematoporphyrin-purified derived-photofrin II, $\epsilon > 10^3 \text{ M/cm}$ at 630 nm) [11]. Among the nonsubstituted phthalocyanines, zinc phthalocyanine has been widely studied [12]. There are several studies evaluating zinc phthalocyanine photocytotoxicity against different cell lines [13–15]. This drug, however, is lipophilic and tends to aggregate under physiological conditions, which decreases its fluorescence and its production of singlet oxygen and reduces its photodynamic activity [16]. Moreover, it is well established that lipophilic compounds, when applied to the skin, tend to be retained in the lipophilic stratum corneum (SC), which decreases their permeation towards the deep layers of the skin where skin tumours are normally located.

Zinc II phthalocyanine tetrasulfonic acid (ZnPcS_4 , Fig. 1) is a hydrophilic substituted phthalocyanine [17] that presents four negative charges and, therefore, decreased aggregation. Its activity in PDT has been evaluated in a fibrosarcoma tumour model, with implantation of the tumours on the right flank of BDIX rats, and it was shown that this PS was able to induce tumour regression after its administration through the lateral caudal vein. It was also shown that there is a relationship between the regression rate and the excitation wavelength of drug molecules [18]. On the basis of these stimulating results and of all the photochemical advantages provided by this compound in PDT, such as its high efficiency for singlet oxygen cytotoxic photo degradation [19] and absorption in a phototherapeutic region, especially in the region of 650–800 nm [20], this study aims to investigate the

topical delivery of ZnPcS_4 focusing on its retention in different skin layers. Iontophoresis is used as a method to enhance the skin penetration of ZnPcS_4 , as it is known that hydrophilic compounds have difficulty in crossing the SC, and the treatment of cutaneous diseases using PDT depends on the accumulation of PS in the deep layers of the skin, where the tumours are usually located [15].

Iontophoresis is a technique widely used to enhance and control the penetration of polar substances through the skin by the application of a small direct electric current, usually less than or equal to 0.5 mA/cm^2 [21,22]. Charged molecules have their iontophoretic transport enhanced by an electrorepulsion mechanism, whereas electro-osmosis, that is, the convective anode-to-cathode solvent flux that occurs at physiological pH during the application of the current, is the main mechanism that controls the iontophoretic transport of polar uncharged molecules [23]. Iontophoresis is therefore a suitable alternative to transport the negatively charged ZnPcS_4 .

Iontophoresis has already been studied with PDT to increase the skin transport of the prodrug, 5-aminolevulinic acid, [24–29] and hydrophilic and charged porphyrins [4,30]. Studies with porphyrins showed that iontophoresis was able to increase both anionic and cationic porphyrin permeations through the skin without changing their stability. However, to the best of our knowledge, the quantification of charged PSs into the skin after iontophoresis has never been reported. Therefore, in this study, we focused on showing the main route of ZnPcS_4 skin penetration when administered topically along with its location, distribution and quantification in the different layers of the skin.

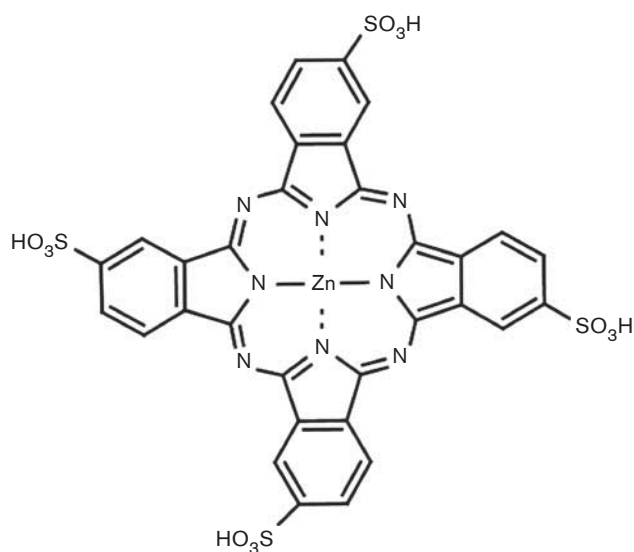
In this study, we evaluated (i) the in-vitro passive and iontophoretic transport of ZnPcS_4 using porcine skin to verify the influence of polarity (cathodal or anodal iontophoresis) and of competing ions on drug skin retention and (ii) the in-vivo ZnPcS_4 passive and iontophoretic skin retention using rat skin, by varying the time of application of the drug formulation. The presence of the drug in the rat skin was evaluated both by the quantification of the drug in the SC and in the viable epidermis and dermis and by confocal laser scanning microscopy. On the basis of our findings, we draw conclusions with regard to the potential of iontophoresis in enhancing the penetration and the distribution of ZnPcS_4 in the deep layers of the skin.

Materials and methods

Chemicals

ZnPcS_4 was obtained from Frontier Scientific (Logan, USA), Ag-wire (99.99%, $\varnothing = 1.5 \text{ mm}$) and AgCl (99.99%) were purchased from Sigma-Aldrich (Steinheim, Germany), dimethyl sulfoxide (DMSO) from Vetec (Rio de Janeiro, Brazil), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Fig. 1



Chemical structure of zinc phthalocyanine tetrasulfonic.

(HEPES) from J.T. Baker (Phillipsburg, USA), NaCl from Synth (Diadema, Brazil), Tissue Tek (O.C.T. Compound) from Sakura (Torrance, USA), Fluoromount from Sigma-Aldrich (St Louis, USA) and hydroxyethyl cellulose (HEC) was obtained from Galena (Campinas, Brazil). All other reagents used were BDH or high-pressure liquid chromatography reagents such as methanol (J.T. Baker) and ethanol (Synth). The water used in all preparations was of Milli-Q grade (Millipore, France).

Skin

Dermatomed skin (700 µm) from the ears of a pig was obtained less than 2 h after killing the animal (Olhos d'Água Indústria e Comércio de Carnes Ltda., Brazil), and was stored at -20°C for a maximum of 30 days before use.

Quantification

ZnPcS₄ retained in the skin in the in-vitro and in-vivo experiments was quantified using an ultraviolet/visual spectrophotometer (Femto-800XI, São Paulo, Brazil) operated at 679 nm. A linear calibration graph ($y = 0.1447x + 0.0025$; $r = 0.999$) was obtained in DMSO over a working concentration range of 0.25–10.00 µg/ml. Intra-day and inter-day precision and accuracy of the method showed a variation coefficient and a relative error not greater than 0.93 and 3.71%, respectively. It was also sensitive and selective during all of the analyses. The limits of quantification and detection of the method were 0.25 and 0.08 µg/ml, respectively.

ZnPcS₄ in the stability studies and in the receptor solution (that crossed the skin in the in-vitro experiments) was quantified using an ultraviolet/visual spectrophotometer (Femto-800XI) operated at 626 nm. A linear calibration graph ($y = 0.027x - 0.010$; $r = 0.998$) was obtained in physiological buffer (25 mmol/l HEPES, 133 mmol/l NaCl) over a working concentration range of 0.80–1.60 µg/ml. Intra-day and inter-day precision and accuracy of the method showed a variation coefficient and a relative error not greater than 2.80 and 0.40%, respectively. It was also sensitive and selective during all of the analyses. The limit of quantification of the method was 700 ng/ml.

Electrical stability of zinc II phthalocyanine tetrasulfonic acid

Before carrying out the iontophoresis experiments, we investigated whether there would be any degradation or reaction of the drug in the presence of electrical current. To do this, aqueous solutions containing 5.5 mmol/l of ZnPcS₄ were prepared and analysed in triplicate before and after being left in contact with an electric current of 0.4 mA for 6 h. The solutions were suitably diluted in a sufficient quantity of water for the analysis. Their pH values were also checked before and after the application of the electric current.

Formulation

ZnPcS₄ (1.1 mmol/l) was incorporated in an HEC gel. The HEC gel consisted of 1.5% (w/w) HEC dispersed in a propylene glycol/water [5:95 (w/w)] solution. Depending on the experiment to be performed, 89.5 mmol/l of NaCl was added to this formulation. The resulting pH of the formulation was always 5.5, so no pH correction was needed to be done.

In-vitro evaluation of ZnPcS₄ iontophoretic transport across and into the skin

Experiments were performed *in vitro* in vertical, flow-through diffusion cells (LG-1088-IC-Laboratory Glass Apparatus, Inc., Berkeley, California, USA). The area of skin exposed in each electrode chamber was 0.8 cm². Ag/AgCl electrodes were prepared in the usual manner described in the literature [31]. The cathodal compartment was filled with 1 g of a formulation containing or not containing NaCl. The anodal and receptor chambers of the diffusion cells simply contained a physiological buffer (25 mmol/l HEPES, 133 mmol/l NaCl) at pH 7.4. The receptor solution was stirred at 300 rpm and was kept at 37°C by a circulating water system (Ecoline 003, E100 from Lauda, Lauda-Königshofen, Germany). ZnPcS₄ transport from the cathode compartment was followed over a period of 6 h at a constant current of 0.5 mA/cm². The voltage of the complete circuit and of each cell was measured during the 6-h experiment with a voltmeter (Freak, MY-63, Zhangzhou, China) to guarantee that Ag/AgCl electrode reactions were occurring as expected, and that the skin was intact [4].

Anodal experiments were also performed by placing the formulation in contact with the positive electrode. A 'passive' experiment was performed with conditions identical to those described above, except that no current was applied.

Skin uptake in vitro

At the end of in-vitro experiments, the skin was removed from the diffusion cell and was pinned up on a piece of Parafilm (Pechiney, Chicago, USA) with the SC face up. The part of skin that had been in contact with the ZnPcS₄ donor formulation was then tape-stripped using 15 Scotch Book Tapes no. 845 (3M, St Paul, Minnesota, USA). The tape strips were subsequently immersed in 10 ml of DMSO and were shaken for 1 h to determine the amount of drug retained in the SC. The remaining skin epidermis and dermis was cut into small pieces, homogenized by a tissue homogenizer (Turratrec TE-102; Tecnal, Brazil) for 1 min with 10 ml of DMSO and sonicated in an ultrasound bath for 45 min. After this, the solution was filtered (solution 1) and the skin was submitted to another extraction for 30 min in an ultrasound bath with 2.5 ml of DMSO, later, this solution was also filtered (solution 2). Aliquots of the filtered homogenates (solutions 1 and 2) were then analysed for their ZnPcS₄ content. This drug extraction method from the skin showed recovery values above 85%.

In addition to the quantification of the drug retained in the skin, passive and iontophoretic experiments were performed using the same protocol to visually show the distribution of the drug in the skin. Pictures of the skin samples were taken using a conventional camera (Samsung NV15, 10.1 megapixels; Tianjin, China).

In-vivo evaluation of ZnPcS₄ iontophoretic skin penetration in an animal model

ZnPcS₄ skin penetration after passive and iontophoretic in-vivo administration of the formulation was investigated in a 4-week-old male Wistar rats ('Biotério Central', University of São Paulo, Brazil). The animals were housed at 24–28°C, exposed to daily 12:12 h light/dark cycles (lights on at 06:00), and had free access to standard rat chow and tap water. The animal protocol was approved by the University of São Paulo Animal Care and Use Committee (authorization number: 08.1.980.53.5).

The hair on the abdominal skin of the animals was trimmed off 48 h before the experiments were performed [30]. A few minutes before the experiments were performed, the rats were anaesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg), and were placed on their back. The formulation (1 g) containing 1.1 mmol/l ZnPcS₄ was applied to the skin surface by an open glass chamber (1.1 cm²), and was sealed to the skin with silicone grease. An AgCl electrode was then introduced and maintained at least 5 mm from the skin surface by means of a plastic lid that covered the chamber. An Ag counter electrode was applied to another part of the animal through a chamber that simply contained the buffer solution. Phoresor II (model PM 850; Iomed, Inc., Salt Lake City, Utah, USA) delivered a constant current density of 0.5 mA/cm² for 5 or 15 min. Passive experiments were also performed in the same way but the current was applied for a period of 5, 15 or 60 min. At the end of the experiments, the rats were killed using carbon dioxide vapour. The drug-exposed skin areas were cleaned with cotton, soaked in water, and removed from the animals for (i) quantification of the drug or (ii) confocal microscopy analysis.

For quantification of the drug, the diffusion area of the treated skin was dissected, pinned up on a piece of Parafilm with the SC face up and tape-stripped eight times. The SC removal was indicated by the glistening of the exposed (viable epidermal) surface.

The next steps for the determination of the amount of ZnPcS₄ retained in the SC and the epidermis and dermis were similar to those used in the in-vitro experiments.

Confocal scanning laser microscopy

Skin samples obtained after the in-vivo passive and iontophoretic treatments had their fluorescence preserved by an application of Tissue Tek solution (O.C.T. Compound) and were frozen at –20°C. Cryosections

(30 μm), perpendicular to the skin surface, were made by a cryostat, and subsequently, all slices received Fluoromount to support fluorescence stability, and to protect the slices against photobleaching effects. For confocal fluorescence microscopy, a Leica TCS SP2 confocal microscope (Mannheim, Germany) with a ×40 immersion objective was used. Samples were excited with a laser at 633 nm and the fluorescence was detected at 640–800 nm.

Data analysis

At least four to six replicates of each experiment were used. Results are presented in the text as the mean ± standard deviation. Data were analysed by analysis of variance followed by parametric Tukey's test. Statistical significance was fixed at a *P* value of 0.05.

Results and discussion

Study of the stability of ZnPcS₄ against electric current

The quantification and pH analyses of the ZnPcS₄ solutions assure that ZnPcS₄ will be stable during the iontophoretic experiments. The remaining percentage of ZnPcS₄ (100 ± 28%) and its pH value (6.03 ± 0.23) after 6 h of electric current application of 0.4 mA was not statistically different from the values analysed in the beginning of the experiment (pH: 5.86 ± 0.14), showing that there were no changes in the ZnPcS₄ chemical properties after the current application.

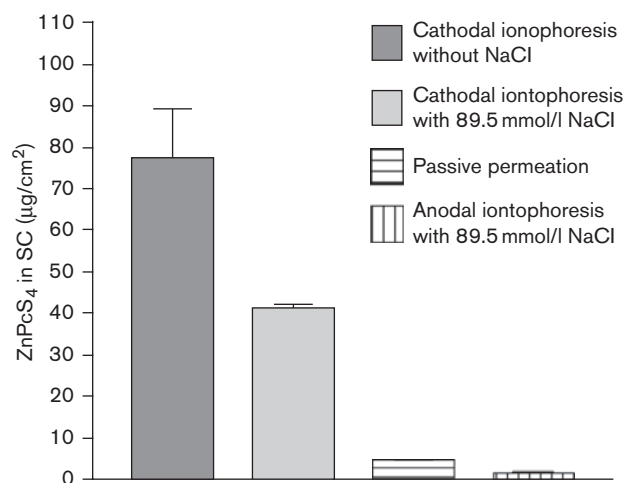
ZnPcS₄ skin retention after in-vitro iontophoresis

Figures 2 and 3 show that ZnPcS₄ retention in SC under passive administration is very low, restricting its transport to the deep layers of the skin epidermis and dermis in detectable amounts. Obviously, no ZnPcS₄ was detected in the receiving solution [physiological buffer containing 25 mmol/l HEPES, 133 mmol/l NaCl at a pH of 7.4] after 6 h of passive administration. In fact, this low topical penetration of the drug into the skin was expected in passive experiments because the ZnPcS₄ has a relatively high molecular weight (898.15 g/mol) inherent in tetraazaisoindole derivatives, which makes its diffusion through the SC difficult [32]. Indeed, ZnPcS₄ presents in its structure four charged groups, which makes the molecule hydrophilic and prevents its penetration into the SC that has lipophilic properties. The low passive penetration of the drug, therefore, justifies the use of iontophoresis to improve drug entry into the skin.

When placed in contact with the negative electrode (cathodal iontophoresis), the skin transport of ZnPcS₄ was significantly increased (*P* < 0.05). An electrorepulsive contribution is the only mechanism that explains the enhanced entry of the drug in the skin by iontophoresis because of the similarity of charges of the PS at pH 5.5 and the negative electrode.

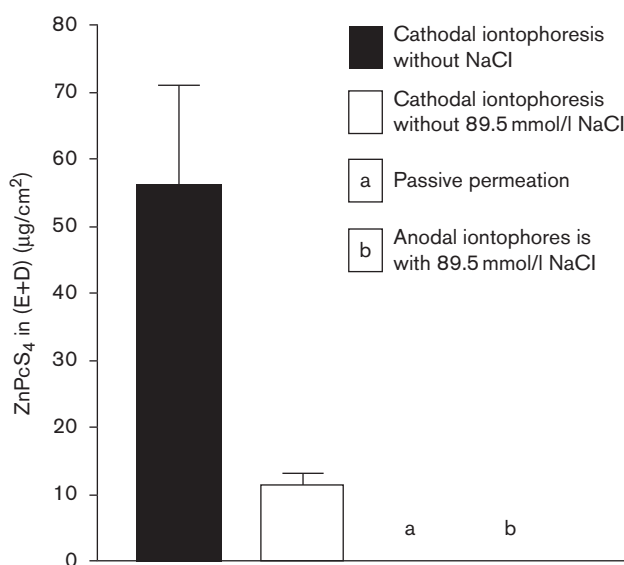
Cathodal delivery of ZnPcS₄ was also evaluated to consider the presence and the absence of competing ions

Fig. 2



Uptake of zinc phthalocyanine tetrasulfonic (ZnPcS₄) into the stratum corneum (SC) after 6 h of in-vitro passive permeation, anodal iontophoresis and cathodal iontophoresis experiments from the hydroxyethyl cellulose gel containing 1.1 mmol/l of the drug and 89.5 mmol/l or no NaCl at a pH of 5.5. The density of the current applied was 0.5 mA/cm². Data shown are the mean ± standard deviation of four to five replicates (Tukey's test, $P < 0.05$).

Fig. 3



Uptake of zinc phthalocyanine tetrasulfonic (ZnPcS₄) into the epidermis and dermis (E + D) after 6 h of in-vitro passive permeation, anodal iontophoresis and cathodal iontophoresis experiments from the hydroxyethyl cellulose gel containing 1.1 mmol/l of the drug and 89.5 mmol/l or no NaCl at a pH of 5.5. The density of the current applied was 0.5 mA/cm². Data shown are the mean ± standard deviation of four to five replicates. Values below limit of quantification (a and b; Tukey's test, $P < 0.05$).

(Cl⁻) in the donor formulation. As Cl⁻ ions can compete with negatively charged ZnPcS₄ molecules for current transport, the salt presence was studied to check the

influence of competing ions on the cathodal transport of the drug. Note that although the presence of Cl⁻ ions is necessary to guarantee the electrode reaction when anodal iontophoresis is applied [23], Cl⁻ ions are not required for this purpose when cathodal iontophoresis is performed because the negative AgCl electrode releases Cl⁻ during the passage of electric current. Nevertheless, as in anodal iontophoresis the presence of Cl⁻ ions in the formulation is necessary, cathodal iontophoresis in the presence of NaCl salt was performed in an attempt to compare ZnPcS₄ anodal and cathodal iontophoresis from solutions with the same ionic strength. In all of the situations, the voltage of each cell was monitored hourly during the 6 h of the experiment, and no voltage increase was observed, even when NaCl was absent.

Figure 2 shows that in the presence of NaCl, cathodal iontophoresis improved the drug retention in SC to eight-fold compared with the passive condition, and promoted its diffusion to the epidermis and dermis (Fig. 3). The absence of NaCl significantly improved ZnPcS₄ retention in SC (approximately two-fold) and in epidermis and dermis (approximately five-fold) compared with the cathodal iontophoretic experiments performed in the presence of salt. These results confirmed the competition between the high-mobility Cl⁻ ions and ZnPcS₄ by current transport, which decreased the skin transport of this PS. Other studies have also reported the influence of competing ions on cathodal iontophoresis [30,33] and showed similar results.

Anodal iontophoresis was performed with the expectation that ZnPcS₄ transport could take advantage of the anode-to-cathode electro-osmotic flow. Therefore, it is worth analysing its contribution toward the transport of the high-molecular weight ZnPcS₄. Figures 2 and 3, however, show that at pH 5.5, the electro-osmotic contribution to ZnPcS₄ skin penetration is not significant. The amount of drug present in the SC and in the epidermis and dermis was even lower than the amount quantified in those layers of the skin when the formulation was passively administered. It seems that the attraction forces between the highly negatively charged PS and the positive electrode avoided the drug transport by the anode, masking the electro-osmotic contribution and restricting its passive permeation. The same effect was reported for the porphyrin TPPS₄, a negatively charged PS [30].

Qualitatively, after removing the formulation from the skin (the skin was washed thoroughly with distilled water and dried), it was visualized to have a bluish colouration, characteristic of ZnPcS₄, in the areas of skin that had been in contact with the formulation. This colouration appeared to be homogeneously distributed on the surface of the skin in the passive and anodal iontophoresis experiments, but heterogeneously accumulated in the openings of hair follicles in the experiments involving cathodal iontophoresis (Fig. 4). From this observation, it

can be inferred that the main route for ZnPcS₄ penetration during cathodal iontophoresis is through the hair follicles (follicular transport), showing the importance of these structures as a route for drug penetration through the skin when iontophoresis is applied [34,35].

In addition, it can be observed in Fig. 4a that the drug accumulation in the skin is much more intense in the areas treated with cathodal iontophoresis in the absence of NaCl than in the presence of this salt, indicating the greatest ZnPcS₄ penetration and confirming what was

Fig. 4

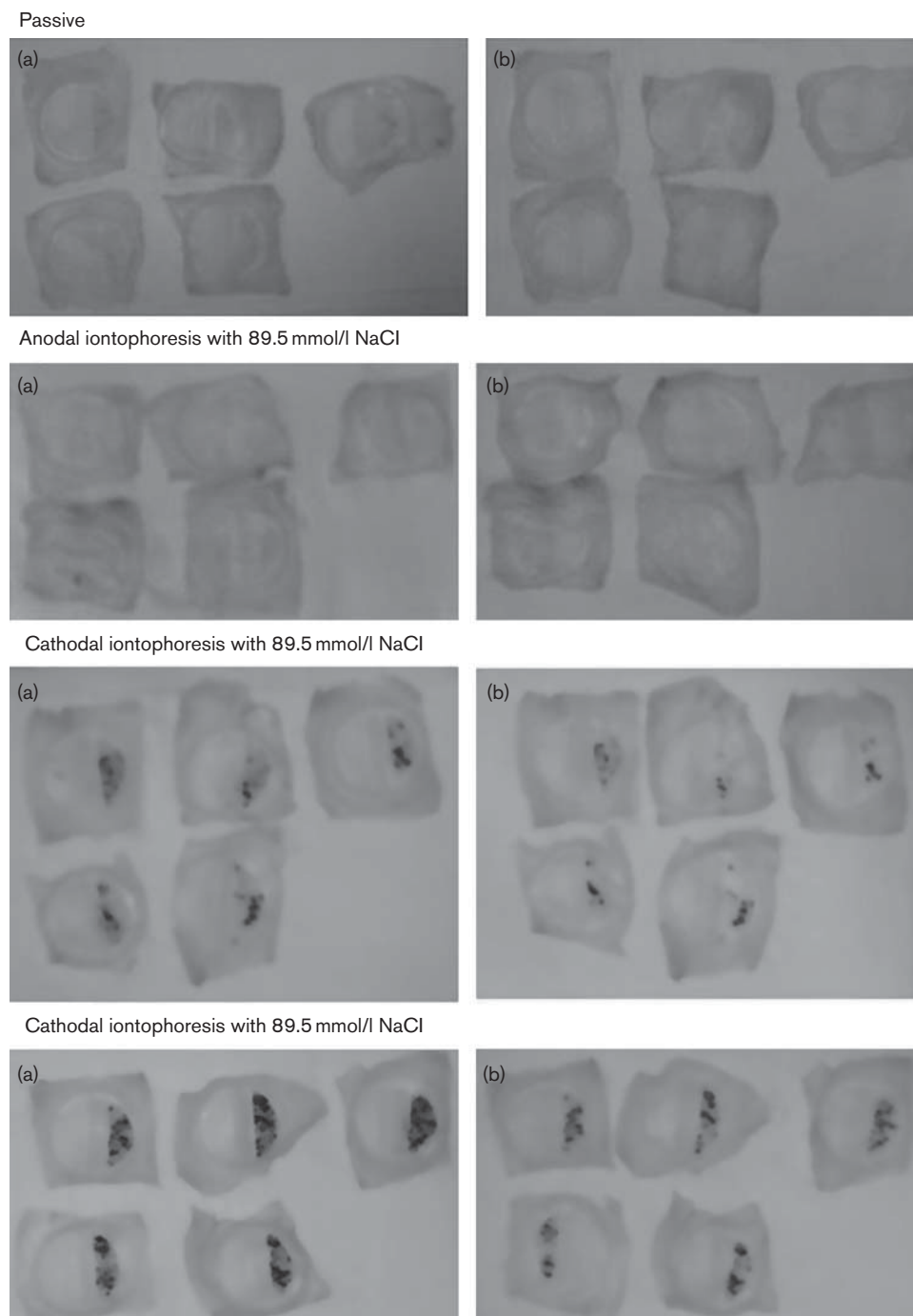


Image of the pig skin obtained after 6 h of passive permeation, anodal and cathodal iontophoresis from the hydroxyethyl cellulose gel containing 1.1 mmol/l of zinc phthalocyanine tetrasulfonic and 89.5 mmol/l or no NaCl. (a) Skin before removal of stratum corneum and (b) skin after removal of stratum corneum.

observed in the quantification studies. To verify whether the drug characteristic colour (blue) persisted in the deep layers of the skin or whether it was present only in the upper layers, the SC was removed and the epidermis and dermis was also visually analysed. Figure 4b shows an intense colouration of the drug in the epidermis and dermis of skins submitted to cathodal iontophoresis, but this colouration disappeared under the other conditions. These results indicate that cathodal iontophoresis increased the penetration of ZnPcS₄ not only in the SC but also in the deep layers of the skin, the target of PDT.

In-vivo skin ZnPcS₄ uptake after cathodal iontophoresis

On the basis of the promising results obtained *in vitro*, when cathodal iontophoresis in the absence of NaCl was performed, in-vivo ZnPcS₄ skin permeation experiments were realized in Wistar rats. This study aimed to observe whether the iontophoretic enhancement over passive permeation was kept even in a shorter period of drug administration in an in-vivo condition. In the analysis of these results, it is important to consider the differences between porcine and rat skins, such as SC thickness, which is higher in porcine skin, and the density of total hair follicles per area, which is approximately 123 times higher in the rat skin than in porcine skin [36]; these differences certainly affect the magnitude of drug skin permeation. This last feature of the rat skin hair follicles might influence the extension of drug transport by iontophoresis because the importance of the follicular route on ZnPcS₄ iontophoretic transport was observed in the in-vitro experiments.

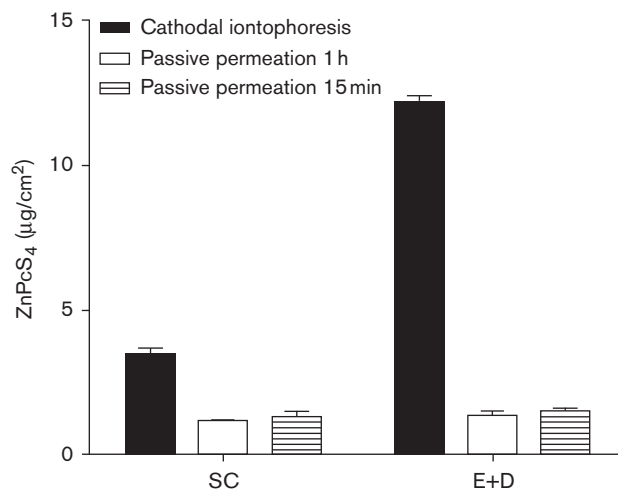
The data presented in Fig. 5 show that cathodal iontophoresis improved drug uptake in deep layers of the skin by approximately 11 times compared with the passive condition after only 15 min of treatment. It is important to point out that the treatment of skin diseases requires an efficient PS penetration in the deep layers of the skin, that is, it is necessary that the drug crosses the SC and reaches the epidermis and dermis [15]. Cathodal iontophoresis, therefore, seems to be a technique capable of transporting large amounts of ZnPcS₄ into the skin quickly, thus benefiting topical PDT for skin tumours.

Passive experiments for a longer period of application were performed to verify whether the drug was able to diffuse passively to the epidermis and dermis in the same extent as that observed after 15 min of iontophoresis. The results presented in Fig. 5 show that passive application for 1 h does not improve drug uptake in SC or in the deeper layers of skin, justifying the use of iontophoresis to enhance the delivery of ZnPcS₄.

Confocal scanning laser microscopy

Figures 6 and 7 show confocal scanning laser microscopy images of rat skin treated for different time periods with the ZnPcS₄ formulation passively and with cathodal iontophoresis, respectively. As ZnPcS₄ is a fluorescent

Fig. 5

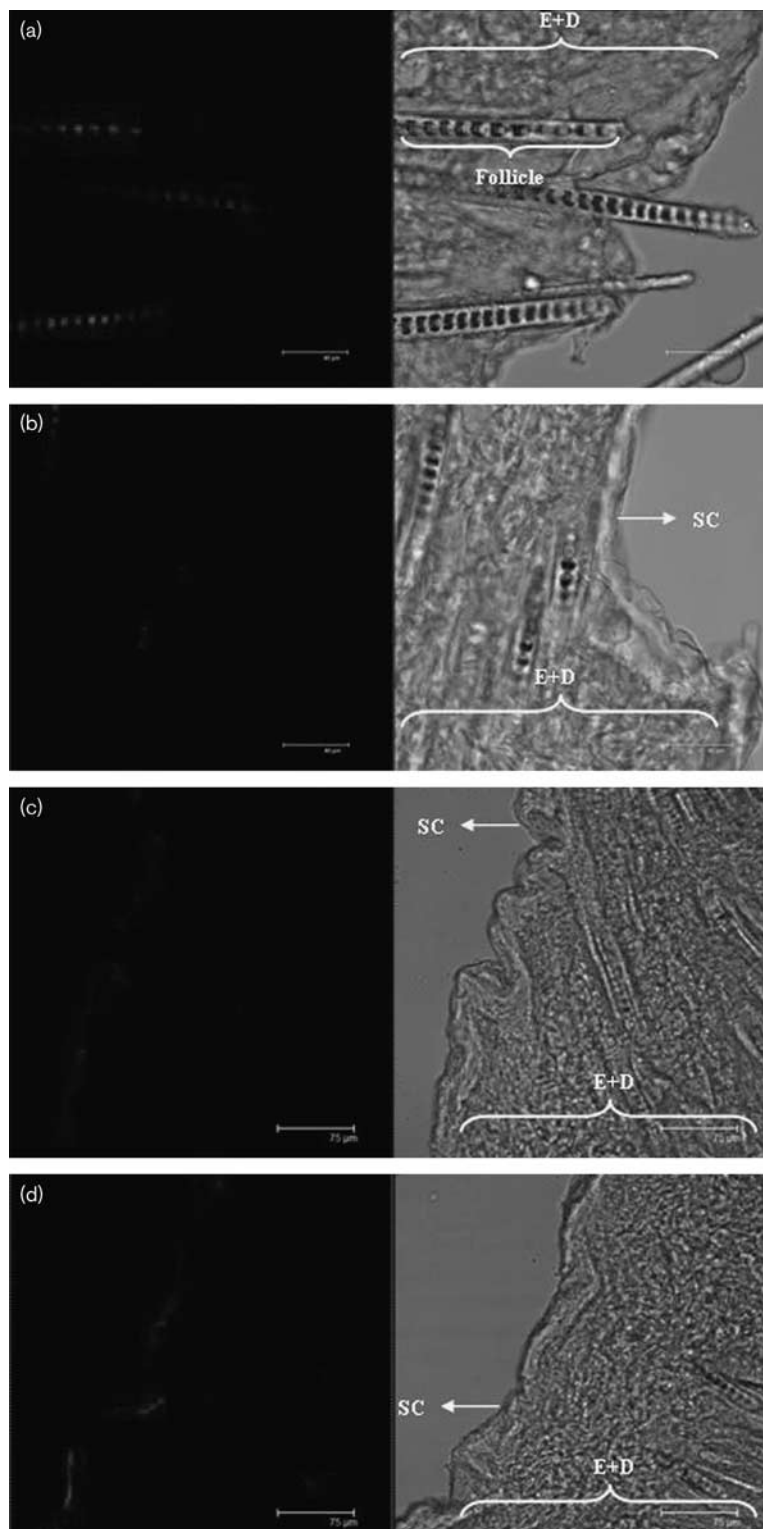


In-vivo uptake of zinc phthalocyanine tetrasulfonic (ZnPcS₄) into the stratum corneum (SC) and epidermis and dermis (E + D) after 15 min and 1 h of passive permeation and 15 min of cathodal iontophoresis permeation from the hydroxyethyl cellulose gel containing 1.1 mmol/l of the drug at a pH of 5.5. The density of the current applied was 0.5 mA/cm². Data shown are the mean \pm standard deviation of five replicates (Tukey's test, $P < 0.05$).

compound, it is visualized under excitation with a laser at 633 nm. It is shown in Fig. 6b that 5 min of passive application is not enough for the PS to penetrate the skin passively because the image from the passive treatment is similar to that of untreated skin (Fig. 6a). After 15 min of treatment, it is seen in Fig. 6c that ZnPcS₄ penetrated the SC passively, but the drug is concentrated mostly in the superficial layers of the skin. Increasing this passive treatment to 1 h (Fig. 6d) did not sufficiently increase ZnPcS₄ penetration, and the drug distribution continued to be observed only in the superficial layers of the skin. In contrast, only 5 min of cathodal iontophoresis was able to deliver ZnPcS₄ to the skin in depths that correspond to viable epidermis and dermis (Fig. 7b). After 15 min of iontophoresis (Fig. 7c), the PS was already homogeneously distributed in these deep layers of the skin.

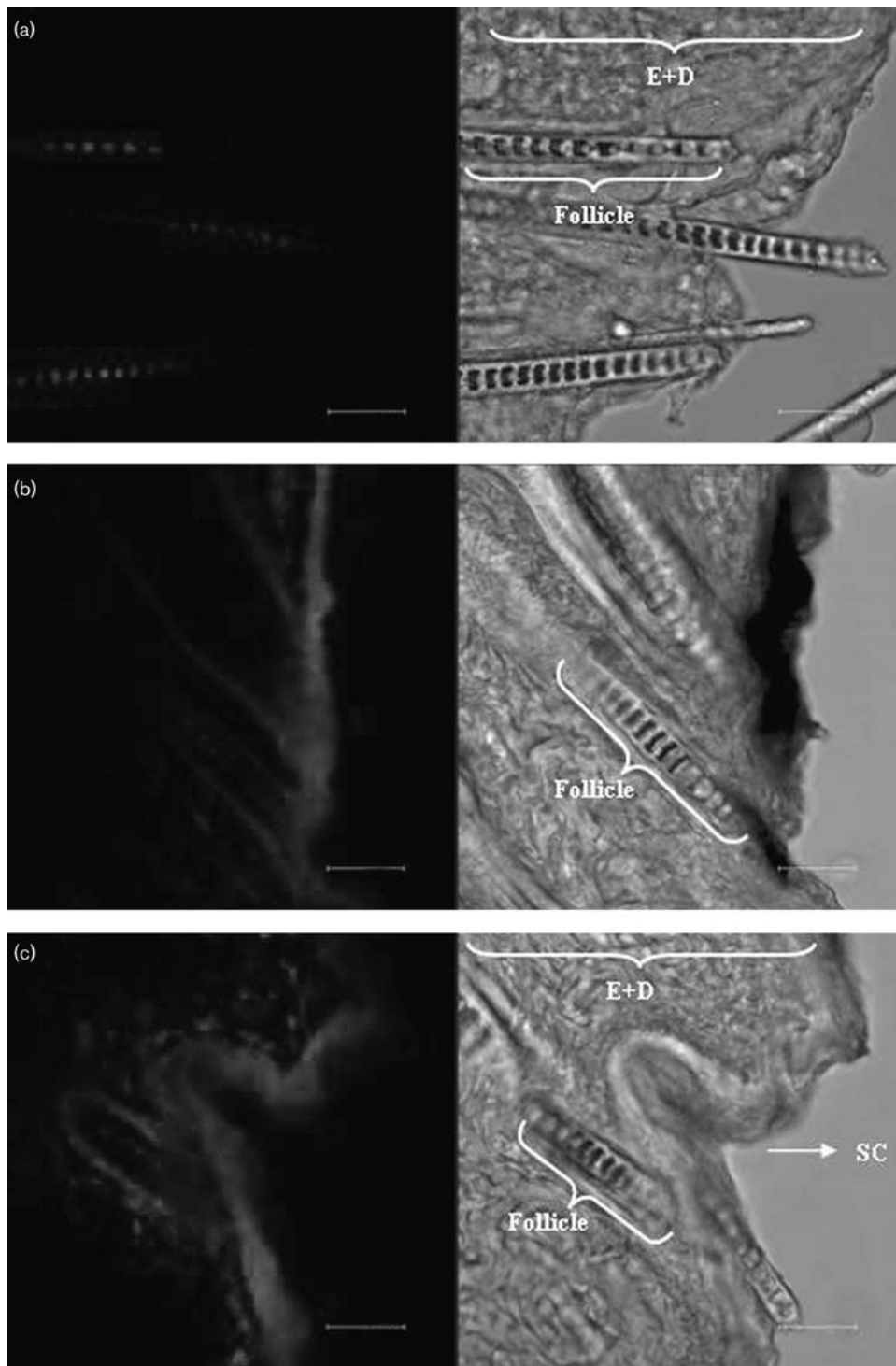
These experiments involving confocal scanning laser microscopy analysis of the skin also permitted us to visualize the skin permeation route of ZnPcS₄. It is well documented in the literature that drugs administered by iontophoresis tend to cross the skin through the follicular route, known as the appendageal pathway. This happens because the skin appendages offer reduced resistance to the passage of molecules when an electrical current is applied [34,35]. Indeed, in Fig. 7b, it is possible to see that ZnPcS₄ molecules are more concentrated around the follicular regions of the skin. This visualization became more difficult in Fig. 7c, when high amounts of the PS had already penetrated the skin and are homogeneously distributed in the entire tissue. It was already shown that iontophoresis has the ability to improve follicular

Fig. 6



Confocal scanning laser microscopy micrographs of Wistar rat skin (perpendicular series) treated passively for different times with a hydroxyethyl cellulose gel containing 1.1 mmol/l zinc phthalocyanine tetrasulfonic. Skin samples were analysed using a $\times 40$ objective and a laser source of 633 nm to excitation and an emission band at 640–800 nm. (a) Untreated skin, (b) 5 min of passive application, (c) 15 min of passive application and (d) 1 h of passive application. E + D, epidermis and dermis; SC, stratum corneum.

Fig. 7



Confocal scanning laser microscopy micrographs of Wistar rat skin (perpendicular series) treated with iontophoresis for different times with an hydroxyethyl cellulose gel containing 1.1 mmol/l zinc phthalocyanine tetrasulfonic. Skin samples were analysed using a $\times 40$ objective and a laser source of 633 nm to excitation and an emission band at 640–800 nm. (a) Untreated skin, (b) 5 min of cathodal iontophoresis and (c) 15 min of cathodal iontophoresis (0.5 mA/cm^2). E + D, epidermis and dermis; SC, stratum corneum.

penetration of other agents used as chemotherapeutics, such as 5-fluorouracil, for which iontophoretic delivery through follicular appendages seems to be more efficient than its passive transport [37].

From the results presented in this study, it was shown that iontophoresis not only improved ZnPcS₄ skin penetration in significant amounts but also increased the skin penetration depth, which seems to be related to the time of the electric current application. Porphyrin derivatives were the first PS to be used in PDT, but because of the lack of specificity when administered systemically, the result was a prolonged and generalized photosensitivity that could last for 6–10 weeks [38]. Thus, ZnPcS₄ topical administration by cathodal iontophoresis can be an alternative for the systemic PDT treatment of skin cancer, bringing benefits to this therapy with respect to the reduction of intrinsic systemic side effects of the PS when they are administered systemically.

Conclusion

In conclusion, it was shown that although 6 h of passive administration was insufficient for ZnPcS₄ to cross the SC and reach the epidermis and dermis, cathodal iontophoresis improved the PS topical delivery to the epidermis and dermis in in-vitro experiments. No positive effect was reached when anodal iontophoresis was performed, showing that the drug–electrode attraction effect was higher than electro-osmosis at a pH of 5.5. The absence of NaCl in the formulation significantly increased the amount of ZnPcS₄ by five-fold that crossed the SC and accumulated in the epidermis and dermis. Experiments in rat skin showed that these results were maintained in an in-vivo model, even over a short period of time. In addition, confocal analysis of the treated skin showed that iontophoresis enhanced the cutaneous transport of ZnPcS₄, which was transported through the skin by the transfollicular route, allowing a homogeneous distribution of the drug in the viable epidermis. The rapid accumulation of ZnPcS₄ in the deep layers of the skin at the studied conditions shows that this type of system is a very promising tool in topical PDT for skin tumour treatments.

Acknowledgement

Conflicts of interest

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References

- Gibson SL, Havens JJ, Foster TH, Hilf F. Time-dependent intracellular accumulation of d-aminolevulinic acid, induction of porphyrin synthesis and subsequent phototoxicity. *Photochem Photobiol* 1997; **65**:416–421.
- Robersts DJH, Cairnduff F. Photodynamic therapy of primary skin cancer: a review. *Br Plast Surg* 1995; **48**:360–370.
- Araújo LM, Thomazine JA, Lopez RF. Development of microemulsions to topically deliver 5-aminolevulinic acid in photodynamic therapy. *Eur J Pharm Biopharm* 2010; **75**:48–55.
- Gelfuso GM, Gratieri T, Souza JG, Thomazine JA, Lopez RFV. The influence of positive or negative charges in the passive and iontophoretic skin penetration of porphyrins used in photodynamic therapy. *Eur J Pharm Biopharm* 2011; **77**:249–256.
- Tita SPS, Perussi JR. The effect of porphyrins on normal and transformed mouse cell lines in the presence of visible light. *Br J Med Biol Res* 2001; **34**:1331–1336.
- Sekyia S, Kubota K, Kasai T, Iwasaki H, Yamauchi K, Takamisawa H, Tenjin Y. Cytocidal effects of hematoporphyrin derivative and argon dye laser on human gynecologic tumor cell in vitro. *Int J Gynecol Obstet* 1998; **26**:151–158.
- Kaestner L, Cesson M, Kassab K, Christensen T, Edminson PD, Cook MJ, et al. Zinc octa-n-alkyl phthalocyanines in photodynamic therapy: photophysical properties, accumulation and apoptosis in cell cultures, studies in erythrocytes and topical application to Balb/c mice skin. *Photochem Photobiol Sci* 2003; **2**:660–667.
- Van Leengoed HLLM, Cuomo V, Versteeg AAC, Van Der Veen N, Jori G, Star WM. In-vivo fluorescence and photodynamic activity of zinc phthalocyanine administered in liposomes. *Br J Cancer* 1994; **69**:840–845.
- Beeby A, Fitzgerald S, Stanley CF. Protonation of tetrasulfonated zinc phthalocyanine in aqueous acetonitrile solution. *Photochem Photobiol* 2001; **74**:566–569.
- Vittar NBR, Pucca CG, Strassert C, Awruch J, Rivarola VA. Cellular inactivation and antitumor efficacy of a new zinc phthalocyanine with potential use in photodynamic therapy. *Int J Biochem Cell Biol* 2008; **40**:2192–2205.
- Allémann E, Brasseur N, Kudrevich SV, Madeleine C, Van Lier JE. Photodynamic activities and biodistribution of fluorinated zinc phthalocyanine derivatives in the murine EMT-6 tumour model. *Int J Cancer* 1997; **72**:289–294.
- Cristóbal J, Stockert JC, Villanueva A, Rello-Varona S, Juarraz A, Cañete M. Caspase-2: a possible trigger of apoptosis induced in A-549 tumor cells by ZnPc photodynamic treatment. *Int J Oncol* 2006; **28**:1057–1063.
- Fadel M, Kassab K, Fadeel DA. Zinc phthalocyanine-loaded PLGA biodegradable nanoparticles for photodynamic therapy in tumor-bearing mice. *Lasers Med Sci* 2010; **25**:283–292.
- Ricci-Júnior E, Marchetti JM. Zinc(II) phthalocyanine loaded PLGA nanoparticles for photodynamic therapy use. *Int J Pharm* 2006; **310**:187–195.
- Fabris C, Soncin M, Miotto G, Fantetti L, Chiti G, Dei D, et al. Zn(II) – phthalocyanines as phototherapeutic agents for cutaneous diseases. Photosensitization of fibroblasts and keratinocytes. *J Photochem Photobiol B: Biol* 2006; **83**:48–53.
- Zhao B, Yin JJ, Bilski PJ, Chignell CF, Roberts JE, He YY. Enhanced photodynamic efficacy towards melanoma cells by encapsulation of Pc4 in silica nanoparticles. *Toxicol Appl Pharmacol* 2009; **241**:163–172.
- Reddi E, Zhou C, Biolo R, Menegaldo E, Jori G. Liposome- and LDL-administered Zn(II)-phthalocyanine as a photodynamic agent for tumours. I. Pharmacokinetic properties and phototherapeutic efficiency. *Br J Cancer* 1990; **61**:407–411.
- Griffiths J, Cruse-Sawyer J, Wood SR, Schofield J, Brown SB, Dixon B. On the photodynamic therapy action of zinc phthalocyanine tetrasulfonic acid in vivo. *J Photochem Photobiol B* 1994; **24**:195–199.
- Allen CM, Sharman WM, Van Lier JE. Current status of phthalocyanines in the photodynamic therapy of cancer. *J Porphyr Phthalocyanines* 2001; **5**:161–169.
- Ben-Hur E. Basic photobiology and mechanisms of action of phthalocyanines. In: Henderson BW, Dougherty TJ, editors. *Photodynamic therapy: basic principles and clinical applications*. New York: Marcel Dekker; 1992. pp. 63–78.
- Guy RH, Kalia YN, Delgado-Charro MB, Merino V, López A, Marro D. Iontophoresis: electrorepulsion and electroosmosis. *J Control Release* 2000; **64**:129–132.
- Jadoul A, Bouwstra JA, Pr at V. Effects of iontophoresis and electroporation on the stratum corneum: review on the biophysical studies. *Adv Drug Deliv Rev* 1999; **35**:89–105.
- Kalia YN, Naik A, Garrison J, Guy RH. Iontophoretic drug delivery. *Adv Drug Deliv Rev* 2004; **56**:619–658.
- Lopez RFV, Bentley MVLB, Delgado-Charro MB, Guy RH. Iontophoretic delivery of 5-aminolevulinic acid (ALA): effect of pH. *Pharm Res* 2001; **18**:311–315.
- Lopez RFV, Bentley MVLB, Delgado-Charro MB, Guy RH. Optimization of aminolevulinic acid delivery by iontophoresis. *J Control Rel* 2003; **88**:65–70.

- 26 Lopez RFV, Bentley MVLB, Delgado-charro MB, Salomon D, Van Den Bergh H, Lange N, Guy RH. Enhanced delivery of 5-aminolevulinic acid esters by iontophoresis in vitro. *Photochem Photobiol* 2003b; **77**:304–308.
- 27 Rhodes LE, Tsoukas MM, Anderson RR, Kollias N. Iontophoretic delivery of ALA provides a quantitative model for ALA pharmacokinetics and PpIX phototoxicity in human skin. *J Invest Dermatol* 1997; **108**:87–91.
- 28 Gerscher S, Connelly JP, Beijersbergen Van Henegouwen GMJ, Macrobert AJ, Watt P, Rhodes LE. A quantitative assessment of protoporphyrin IX metabolism and phototoxicity in human skin following dose-controlled delivery of the prodrugs 5-aminolaevulinic acid and 5-aminolaevulinic acid-n-pentylester. *Br J Dermatol* 2001; **144**:983–990.
- 29 Boddé HE, Roemelé PEH, Star WM. Quantification of topically delivered 5-aminolevulinic acid by iontophoresis across ex vivo human SC. *Photochem Photobiol* 2001; **75**:418–423.
- 30 Gelfuso GM, Figueiredo FV, Gratieri T, Lopez RFV. The effects of pH and ionic strength on topical delivery of a negatively charged porphyrin (TPPS4). *J Pharm Sci* 2008; **97**:4249–4257.
- 31 Green PG, Hinz RS, Kim A, Szoka FC, Guy RH. Iontophoretic delivery of a series of tripeptides across the skin in vitro. *Pharm Res* 1997; **8**:1121–1127.
- 32 Boehncke WH. Topical photodynamic therapy for psoriasis. In: Calzavara-Pinton P, Szeimies RM, Ortel B, editors. *Photodynamic therapy and fluorescence diagnosis in dermatology*. San Francisco: Elsevier Science; 2001. pp. 259–270.
- 33 Sylvestre JP, Guy R H, Delgado-Charro MB. In vitro optimization of dexamethasone phosphate delivery by iontophoresis. *Phys Ther* 2008; **88**:1177–1185.
- 34 Barry BW. Novel mechanisms and devices to enable successful transdermal drug delivery. *Eur J Pharm Sci* 2001; **14**:101–114.
- 35 Kassar DG, Lynch AM, Stiller MJ. Physical enhancement of dermatologic drug iontophoresis and phonophoresis. *J Am Acad Dermatol* 1996; **34**:657–666.
- 36 Stahl J, Niedorf F, Kietzmann M. Characterisation of epidermal lipid composition and skin morphology of animal skin ex vivo. *Eur J Pharm Biopharm* 2009; **72**:310–316.
- 37 Welch ML, Grabski WJ, Mccollough ML, Skelton HG, Smith KJ, Menon PA, Anderson LL. 5-Fluorouracil iontophoretic Bowen's disease. *J Am Acad Dermatol* 1997; **36**:956–958.
- 38 Dougherty TJ, Cooper MT, Mang TS. Cutaneous phototoxic occurrences in patients receiving photofrin. *Lasers Surg Med* 1990; **10**:485–488.