



Research paper

The influence of positive or negative charges in the passive and iontophoretic skin penetration of porphyrins used in photodynamic therapy

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ABSTRACT

Meso-tetra-(*N*-methylpyridinium-4-yl)-porphyrin (TMPyP) and *meso*-tetra-(4-sulfonatophenyl)-porphyrin (TPPS₄) are photosensitizing drugs (PS) used in photodynamic therapy (PDT). Based on the fact that these compounds present similar chemical structures but opposite charges at pH levels near physiological conditions, this work aims to evaluate the *in vitro* and *in vivo* influence of these electrical charges on the iontophoretic delivery of TMPyP and TPPS₄, attempting to achieve maximum accumulation of PS in skin tissue. The iontophoretic transport of these drugs from a hydrophilic gel was investigated *in vitro* using porcine ear skin and vertical, flow-through diffusion cells. *In vivo* experiments using rats were also carried out, and the penetration of the PSs was analyzed by fluorescence microscopy to visualize the manner of how these compounds were distributed in the skin after a short period of iontophoresis application. *In vitro*, both passive and iontophoretic delivery of the positively charged TMPyP were much greater (20-fold and 67-fold, respectively) than those of the negatively charged TPPS₄. TPPS₄ iontophoresis *in vivo* increased the fluorescence of the skin only in the very superficial layers. On the other hand, iontophoresis of the positively charged drug expressively increased the rat epidermis and dermis fluorescence, indicating high amounts of this drug throughout the skin layers. Moreover, TMPyP was homogeneously distributed around and into the nuclei of the skin cells, suggesting its potential use in topical PDT.

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1. Introduction

Photodynamic therapy (PDT) is a currently studied treatment for neoplastic diseases. It involves the administration of a photosensitizing drug (PS) that preferentially accumulates in target tissues [1]. After PS accumulation, the tumoral lesion is irradiated with secure doses of visible light that activates the drug and in the presence of oxygen generates cytotoxic and reactive oxygen species, such as singlet oxygen, hydroxyl radicals, superoxide anions and hydrogen peroxide. The presence of these toxic species will cause cell death in the neoplastic tissue [2,3]. A topical administration of PSs is well suited for the treatment of skin tumors by PDT because it would deliver the drug into the pathological site to increase the local bioavailability of the PS and reduce the occurrence of systemic side effects during treatment.

5-Aminolevulinic acid (ALA), a precursor of endogenous PS protoporphyrin IX (PpIX), is the most studied drug in topical PDT. Due to its relatively low molecular weight (168 Da), ALA can penetrate damaged skin rather easily, leading to PpIX accumulation,

especially in tumor cells [1]. However, there are many variables that may interfere with PpIX local bioavailability, such as irregular ALA skin penetration, dependence on the quantity of pro-drug that penetrates the skin and the local metabolism of ALA conversion in PpIX, and heterogeneous distribution of ALA-induced PpIX in the tumor [1]. Therefore, the direct administration of a PS instead of a pro-drug could be advantageous because the drug would already be photosensitive and would not be dependent on skin metabolism for conversion into an active molecule [4].

Porphyrins belong to a very important class of PS used in PDT by parenteral administration [5]. Although Photofrin[®], a non-charged porphyrin, is most often used in the PDT of tumors, charged porphyrins also have several interesting features that make them attractive for PDT. *Meso*-tetra-(*N*-methylpyridinium-4-yl)-porphyrin (TMPyP) (Fig. 1A), for instance, is a cationic water soluble porphyrin that is known to accumulate in tumors with a good degree of selectivity [6,7]. Since the positive charges of the TMPyP side chains are appropriate for electrostatic interactions with the negative charges of nucleic acids, TMPyP can promote high rates of tumor necrosis and has been shown to increase the survival of tumor-bearing animals [6–9]. Also, *in vitro* studies showed that cationic porphyrins are able to intercalate between DNA bases, thus inducing DNA lesions upon photoactivation [6]. *Meso*-tetra-(4-sulfonatophenyl)-porphyrin (TPPS₄) (Fig. 1B), first used

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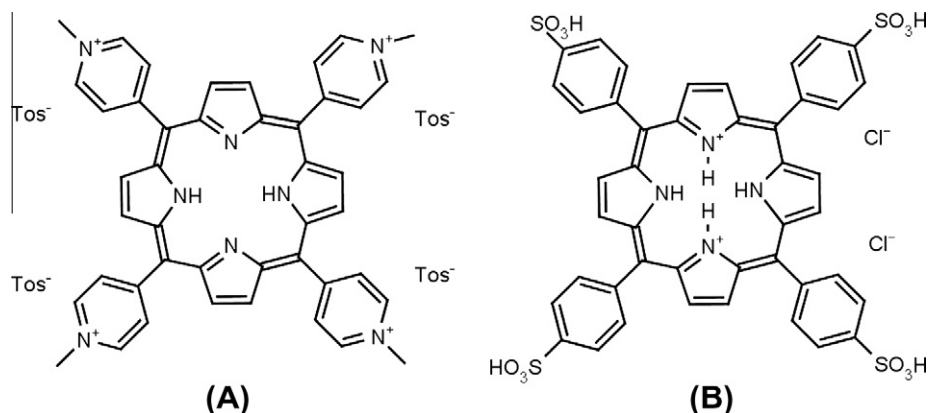


Fig. 1. Chemical structure of (A) TMPyP and (B) TPPS₄.

by Winkelman in 1962 [10], is a negatively charged stable drug that is water soluble at room temperature and provides a great yield of singlet oxygen when irradiated [11]. However, when TPPS₄ is placed in acidic media, it is protonated and suffers from aggregation, which drastically changes its spectral and energetic properties [12]. Furthermore, TPPS₄ permeation through the skin is widely affected by other environmental conditions like concentration and ionic strength [4]. Aside from the problem of aggregation, the highly lipophilic nature of the skin restricts the permeation of these hydrophilic high-molecular-weight charged compounds through the stratum corneum [13]. To overcome these obstacles, application of iontophoresis to promote their delivery was studied.

Iontophoresis is a technique that uses an electrical potential to maintain a constant low-level electric current (≤ 0.5 mA/cm²) to drive both charged and uncharged compounds into and across the skin at rates much greater than their passive deliveries [13–17]. The amount of compound delivered by iontophoresis is directly proportional to the quantity of charge passed, which depends on the intensity of the applied current, the duration of current application and the skin surface exposed to the active electrode compartment [17]. In this way, the topical application of TMPyP and TPPS₄ by iontophoresis could improve the skin permeation of these drugs.

Although iontophoresis of charged molecules has been thoroughly studied, the influence of electrorepulsion for delivering highly charged molecules (the porphyrins in study have four negatives or four positive charges when ionized) has never been investigated before aiming a dermal treatment. The most of the studies involving iontophoresis evaluate the contribution of the electroosmotic flow when molecules opposed in charge are studied, because these opposite charges are normally obtained with changes in the pH of the formulation. For instance, amino acids, peptides or proteins [18–20] that have an isoelectric point, or simply weak acids or bases [21–22], have the pH of their solutions altered to maintain the drug in the positive or negative form. Because changes in the solution's pH can modify electrorepulsive and electroosmotic contributions for the total drug's flux [17,23], studying the influence of these contributions for structurally similar drugs, as the porphyrins proposed to be studied in this work (Fig. 1), but opposed in charge, at a same specific pH, may assist in the understanding of topical iontophoretic delivery. It is also important to point that despite the obvious benefits that iontophoresis may offer for the treatment of a local cutaneous pathology, there have been limited applications of this technique in the treatment of dermal conditions [17,23]. Therefore, attempts to understand the influence of iontophoresis in drug's skin distribution, and not only in drug's

percutaneous absorption, can contribute for the potential use of iontophoresis to target the skin.

In this way, the aim of this work was to make use of iontophoresis to topically deliver TMPyP and TPPS₄, evaluating the influence of negative and positive charges presented by these two porphyrin-derivate compounds on their iontophoretic deliveries. This approach was also expected to achieve a high amount and homogeneous distribution of PS in the skin, which could translate to progress in topical PDT.

2. Materials and methods

2.1. Materials

TMPyP (TMPyP tetra tosylate salt) and TPPS₄ (TPPS₄ dihydrochloride salt) were obtained from Frontier Scientific Corporation (Logan, USA). Ag-wire (99.99%, $\phi = 1.5$ mm), AgCl (99.99%) and Pt-wire, all used for preparing the Ag/AgCl electrodes [24], were purchased from Sigma–Aldrich (Steinheim, Germany). Hydroxyethylcellulose (HEC) was purchased from Galena (Campinas, Brazil) and used for preparing gel formulations by weighting 1.5 g of the polymer and dispersing it in 98.5 g of water. HEPES was purchased from J.T. Baker (Phillipsburg, USA), NaCl from Synth (Diadema, Brazil) and Tissue-Tek® (O.C.T. Compound) from Sakura (Toorance, USA). All other reagents were of BDH or HPLC grades. The water used in all preparations was of Milli-Q grade (Millipore–France).

2.2. Skin

The skin used for the *in vitro* experiments was dermatomed (500 μ m) from porcine ear. It was obtained immediately after slaughter of the animal (Frigorífico Pontal Ltda., Pontal, Brazil) and stored at -20 °C for a maximum of 30 days before use.

2.3. Electrical stability

The electrical current influence on the stability of the porphyrin solutions was evaluated prior to the iontophoretic permeation studies. For this purpose, 10 mL of a TMPyP or TPPS₄ aqueous unbuffered solution at 5.0 mg/mL, containing 89.5 mM NaCl, was subjected to a current of 0.4 mA by placing them in contact with the positive (Ag) and negative (AgCl) electrodes connected to a power supply over 6 h (maximum period of iontophoresis application). Samples were collected before and after this experimental setup, and the TMPyP and TPPS₄ spectra in both situations were analyzed, as well as their pH.

2.4. *In vitro* iontophoresis

Experiments were performed *in vitro* using vertical, flow-through diffusion cells (LG-1088-IC-Laboratory Glass Apparatus, Berkeley, CA) and Ag/AgCl electrodes prepared as previously described [24]. The area of skin exposed in each electrode chamber was 0.8 cm². Dermatomed skin was mounted in the diffusion cell with the dermal side facing downward into the receiving medium of 6.0 mL of isotonic buffer (HEPES 25 mmol/L, NaCl 133 mmol/L) at pH 7.4. For anodal iontophoresis, the anode compartment was filled with 1.0 g of the gel formulation containing TMPyP or TPPS₄ and the counter-electrode compartment simply contained the isotonic buffer. For cathodal iontophoresis, the cathode was filled with 1.0 g of the gel formulation containing TPPS₄ and the anode was filled with the isotonic buffer. Iontophoretic transport of the drugs was followed over a period of 6 h at a constant current of 0.5 mA/cm² generated by a Kepco APH 500DM apparatus (Kepco Power Supply, USA). The voltage of the complete circuit and of each cell was measured hourly with a voltmeter (Freak, MY-63) to guarantee both the intactness of the skin and that the Ag/AgCl electrode reactions were occurring as expected.

During the experiments, the receiving solution was stirred at 300 rpm and kept at 37 °C by a circulating water system (Ecoline 003, E100 from Lauda, Germany). The receiving solution was also perfused continuously at 3 mL/h using a peristaltic pump (Pump Pro MPL580 – Watson-Marlow Bredel Pumps, United Kingdom). Samples were collected automatically every hour (Fraction collector PTFCL-Pharmatest, Germany), and the amount of drug that permeated the skin, i.e., the amount of drug in the receiving solution, was analyzed as following described.

“Passive” experiments were also performed. In that case, all conditions were identical to those described above except that no current was applied.

2.5. Sets of *in vitro* iontophoretic experiments performed

The cationic porphyrin TMPyP was incorporated into the non-ionic HEC gel at pH 5.5 and its anodal transport was measured as a function of drug concentration and ionic strength. Thus, two sets of experiments were performed for this drug. In the first series, the TMPyP anodal transport was measured from the HEC gel containing 89.5 mM of NaCl and different concentrations of the drug (5.0, 2.5 and 1.0 mg/g, corresponding to 7.5, 3.7 and 1.5 mM). In the second series, anodal transport of the drug at 5.0 mg/g was evaluated from the gel in the total absence of NaCl.

The TPPS₄ formulation comprised a HEC gel containing 5.0 mg/g (5.3 mM) of the drug (TPPS₄), without NaCl, at pH 5.5. The iontophoretic transport of this negative porphyrin was evaluated as a function of the polarity of the electrode compartment to evaluate electroosmosis and electrorepulsion contributions.

2.6. Quantitative analysis

Both of the drugs were quantified using a UV/Vis Spectrophotometer (Femto-800XI). A linear calibration graph ($y = 0.186x + 0.004$; $r = 0.999$) was obtained over the working concentration range of 0.1–1.0 µg/mL at 423 nm for TMPyP. The intra- and inter-day precision and accuracy of this method showed a coefficient of variation (CV%) and relative error (E%) not greater than 3.0% and 3.7%, respectively. It was also sensitive and selective during all of the analyses: the selectivity of the method was tested by the analysis of the receiving solution UV/Vis spectra when no drug was administered (blank experiment).

The method used to quantify the TPPS₄ had already been validated by our group [4]. A linear calibration graph for this drug

($y = 0.274x + 0.001$; $r = 0.999$) was also obtained over the working concentration range of 0.1–1.0 µg/mL at 412 nm.

2.7. Data analysis

At least 5 replicates of each experiment were used. Results are presented in text as means ± standard deviations (SD). Data were evaluated statistically using analysis of variance (ANOVA), and the Student's t-test was used to compare two data sets. Statistical significance was fixed at $P < 0.05$.

2.8. *In vivo* iontophoresis

TMPyP and TPPS₄ skin penetration after passive and iontophoretic *in vivo* experiments was investigated in male Wistar rats that were four weeks old (“Biotério Central”, University of São Paulo, Brazil). The animals were housed at 24–26 °C, exposed to daily 12:12 h light:dark cycles (lights on at 6 a.m.) 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: 06.1.492.53.9).

The hair on the abdominal skin of the animals was trimmed 48 h before the experiments. A few minutes before the gel administration, the rats were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg) and placed on their back. HEC gel formulation (1.0 g) containing 5.0 mg/g of TMPyP or TPPS₄, both at pH 5.5, was applied to the skin surface of the animals via an open glass chamber (1.03 cm²) and sealed to the skin with silicone grease. Ag or AgCl electrode (for TMPyP or TPPS₄, respectively) was then introduced and maintained at least 5 mm from the skin surface. Negative electrode patches (Return electrode patch, Iomed Inc., Salt Lake City, USA) (Fig. 2) or Ag electrode embedded in buffer (for TMPyP or TPPS₄, respectively) was used as counter-electrodes. A Phoresor II (model PM 850, Iomed Inc., Salt Lake City, USA) delivered a constant current of 0.5 mA for 10 min. At the end of the experiment, the rats were sacrificed with carbon dioxide vapor. The drug-exposed skin areas were cleaned with cotton soaked in water and removed from the animals for analysis by fluorescence microscopy (Zeiss Axioskop) with a suitable barrier filter (470–520 nm) (Microaktueal). For this operation, the sample fluorescence was preserved by applying Tissue-Tek (O.C.T. Compound) solution and freezing in liquid nitrogen. Cryosections (16 µm) were made with a cryomicrotome (Microm D-6900, Heidelberg, Germany) and, subsequently, all slices were mounted in a *p*-phenylenediamine mounting medium to support fluorescence stability and to protect slices against photobleaching effects. Skin slices were then fluorimetrically



Fig. 2. Picture illustrating the *in vivo* experiments involving anodal iontophoresis of TMPyP performed in animal models.

analyzed. The photographs were captured using a Zeiss MC 80-DX microscope camera system and a 4000x objective (Zeiss Plan-Neofluar).

3. Results and discussion

The porphyrins studied in the present work have shown many stimulating results as PS drugs for PDT. The TMPyP, for example, has excellent selectivity for neoplastic tissue [6,7,25], and TPPS₄ provides excellent yields of singlet oxygen [4,12,26]. Both compounds present tetrapyrrolic macrocyclic structures (Fig. 1), but different side chains, which confers opposite electrical charges to them. At pH 5.5, TPPS₄ molecules are non-protonated (H₂TPPS₄⁴⁻), presenting four net negative charges. TMPyP on its turn presents four positive charges (H₂TMPyP⁴⁺) at any pH between 1.5 and 12.5.

It is important to point out that porphyrins only permeate the skin when they are in their monomeric forms, as aggregation could prevent skin transport [4]. The aggregation of charged porphyrins depends on the pH of the formulation, on the presence of other ions and on their concentration. At the pH of the experiments (pH 5.5), the molecules are not aggregated [26–28]. It is also assumed that the hydrogel formulation employed to incorporate the drugs (HEC gel) for iontophoresis did not drain during its contact with the electrodes, yet permitted the passage of the electric current.

3.1. Electrical stability

The dosage and pH analysis of the samples guarantee that the charged porphyrins will be stable during all of the iontophoretic permeation experiments. The reminiscent percentage of TMPyP (102 ± 12%) and TPPS₄ (98 ± 10%) and their pH values (5.57 ± 0.07 and 5.62 ± 0.14, respectively) after 6 h of current application were not statistically different from those parameters analyzed previously in the beginning of the experiment (pH 5.58 ± 0.11 for TMPyP and pH 5.82 ± 0.10 for TPPS₄). No difference between the UV/Vis spectra of the porphyrins before and after the current application was observed (data not shown), proving that there were no changes in the chemical properties of the compounds after the current application.

3.2. *In vitro* iontophoresis

Positive molecules in contact with the anode at pH levels near physiological values are expected to be transported through the skin by iontophoresis due to two main mechanisms: (i) electromigration, in which the positively charged drug is repulsed through the skin by the positive electrode (anode), and (ii) electroosmosis, as at pH levels higher than 4.0, the skin is negatively charged and is cation permselective (1). Thus, current passage causes a net convective solvent flow in the anode-to-cathode direction, facilitating the transport of molecules despite their electrical charge [1,15,16,23,29]. It is thereby expected that TMPyP, with its positive electric charges, has its topical delivery favored by anodal iontophoresis by taking advantage of these two mechanisms, while the negatively charged TPPS₄ penetrates the skin only due to electroosmosis when in contact with the anode and only by electrorepulsion when in contact with the cathode.

3.2.1. TMPyP iontophoresis

TMPyP passive permeation, i.e., *in vitro* skin permeation of this drug without electric current application, from the HEC gel with 89.5 mM NaCl and 7.5 mM (5.0 mg/g) of the drug showed mild TMPyP transport across the skin. When the same formulation was placed in contact with the anode in the presence of a 0.5 mA/cm²

electric current, a significant TMPyP skin permeation improvement was reached, with an approximately 6-fold increase over its passive permeation after the 6 h of experiment (Fig. 3). These results are expected when a positive drug is iontophoretically delivered. Considering only experiments with the most currently used drugs in topical PDT (ALA and its derivatives, as examples), a series of positively charged ALA esters were submitted to the anodal iontophoresis, and a 50-fold higher flux over passive transport was observed for the methyl-ALA aqueous solution [16]. As the size and lipophilicity of the ester increased, the efficiency of electrotransport decreased. Iontophoresis of methyl-ALA from hydrophilic gels also showed around a 20-fold improvement over passive permeation when the anodal iontophoresis was applied [30].

Fig. 3 shows that passive and iontophoretic deliveries of TMPyP showed similar permeation profiles until the third hour of experiment, but from this time iontophoresis was able to dramatically increase the permeation of the positive porphyrin. It seems therefore that the lag time in passive conditions were not achieved in those 6 h of experiments, in contrast with iontophoresis in which it was achieved after 3 h. We decided then to choose the last hour of the experiment (6 h) to compare passive and iontophoretic delivery of the porphyrins.

To evaluate the drug concentration effect on TMPyP anodal iontophoresis, HEC gel formulations containing (i) 1.5, (ii) 3.7 and (iii) 7.5 mM of the drug with 89.5 mM NaCl at pH 5.5 were placed in the anode. The TMPyP iontophoretic flux, calculated from the third hour of experiment, was linearly increased with the drug concentration increase ($r = 0.99$), as can be seen in Fig. 4. Marro et al. [31] and Lopez et al. [15] also evaluated *in vitro* the transdermal iontophoresis of lidocaine and ALA, respectively, as a function of the concentration of drug in donor solution, and both of them achieved significant drug delivery improvement by increasing the concentration in the anodal formulation. The linear increase in TMPyP iontophoretic delivery with the concentration only occurs because there are other ions in the anode, such as Na⁺, which compete with the drug for transport through the skin. Increasing the drug concentration increases the drug ions in the donor compartment that compete proportionally with other ions in this compartment. This proportionality, however, does not always occur. Nair and Pachangnula [32] studied the effect of concentration on the iontophoretic flux of arginine-vasopressine during anodal iontophoresis and obtained a linear relationship, although

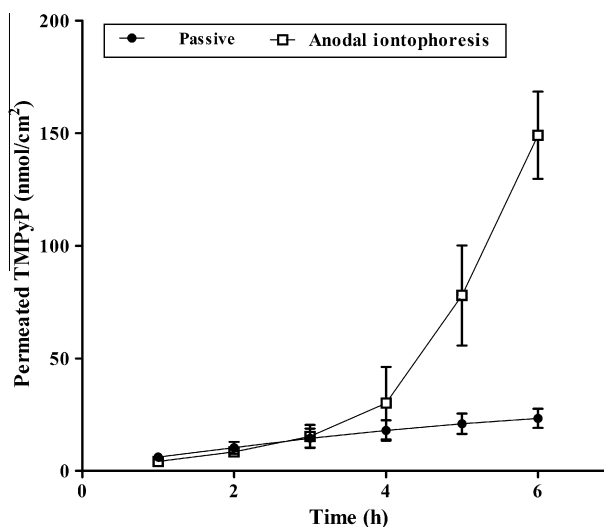


Fig. 3. Passive and anodal iontophoretic permeation profiles of TMPyP across porcine skin from a HEC non-ionic gel containing 5.0 mg/g TMPyP and 89.5 mM NaCl at pH 5.5 (mean ± SD; $n = 5$).

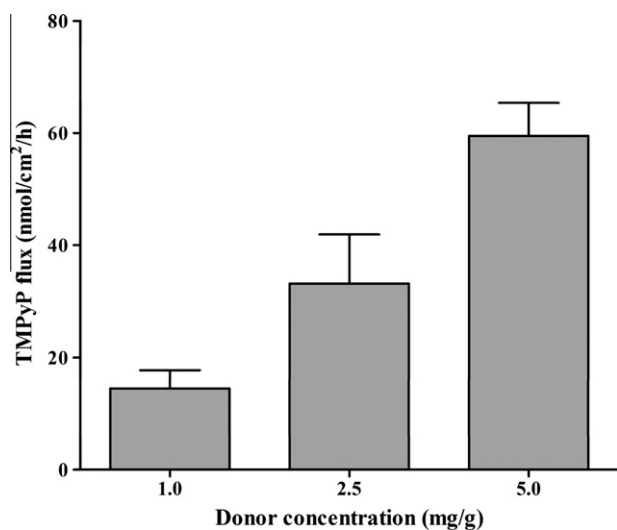


Fig. 4. Anodal iontophoretic fluxes of TMPyP across porcine skin as a function of its concentration in the donor chamber (1.0, 2.5 and 5.0 mg/g) (mean \pm SD; $n = 5$). Linear regression (calculated from a x - y scatter plot): $y = 74.65x + 2.74$, where y = iontophoretic flux of TMPyP and x = TMPyP percentage in donor chamber; (r) = 0.99. All of the formulations were comprised of an HEC gel with 89.5 mM NaCl at pH 5.5.

at higher concentrations, the flux of arginine-vasopressine was not yet proportional. In such cases, it was assumed that the pores of the skin may have been saturated when higher concentrations of the drug were transported by iontophoresis, decreasing the electroosmotic contribution. Therefore, it seems that TMPyP does not interact with the skin or saturate its pores, at least at the concentrations studied, as the iontophoretic flux was directly proportional to drug ion concentration. Electrorepulsion seems to be the dominant mechanism in this case, estimated to account for 80% of total iontophoretic transport [33].

The influence of ionic strength on TMPyP skin permeation was also studied because it is known that the presence of molecules or ions that could compete with the drug by current transport must reduce the electrorepulsive drug flux through the skin [15,17,23,34]. Therefore, an HEC gel formulation containing only 7.5 mM of TMPyP without NaCl, at pH 5.5, was placed in the anode compartment and its iontophoresis was evaluated. In Fig. 5, it can be observed the competitive ion absence significantly increased ($P < 0.05$) TMPyP iontophoretic transport through the skin. The flux increase was on the order of 29%. Marro et al. [31] also observed that in the absence of competitive ions, the iontophoretic transport of lidocaine, propranolol and quinine was independent of their concentration in the donor chamber. This occurs because the drug molecules are the only cationic species in the system able to transport current through the skin. It is important to point out that although 89.5 mM NaCl is the calculated amount that provides chloride ions for assuring the Ag electrode reactions, in the absence of the salt, the system voltage was constantly monitored and showed no significant changes, meaning the electrode reaction was occurring. The chloride ions that come from the skin towards the anode during the iontophoresis must be supporting this reaction during the experiment.

3.2.2. TPPS₄ iontophoresis

We have already studied the transdermal permeation of TPPS₄ as a function of its concentration, pH and ionic strength of the aqueous solutions [4]. The protonation of this molecule in acidic media drastically changes its spectral and energetic properties [12], as well as its skin penetration [4]. The presence of NaCl in aqueous solution at physiological pH also affects TPPS₄ skin pene-

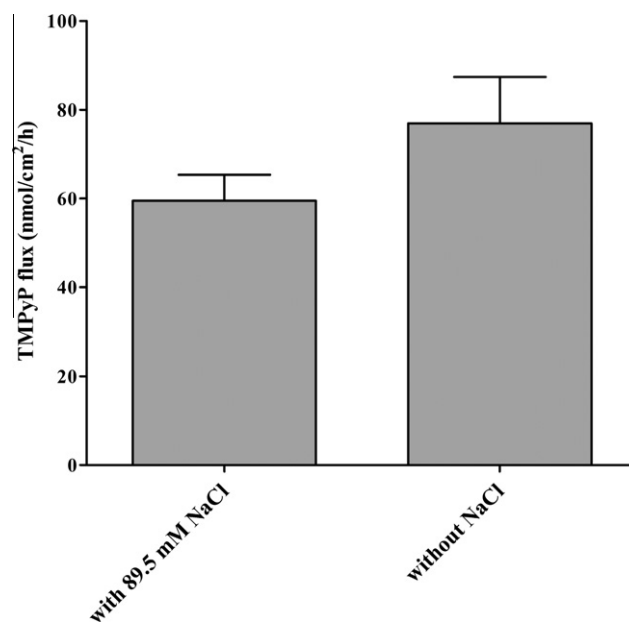


Fig. 5. Anodal iontophoretic fluxes of TMPyP across porcine skin from the HEC gel with 5.0 mg/g of TMPyP at pH 5.5 in the presence and absence of 89.5 mM NaCl (mean \pm SD; $n = 5$).

tration, significantly improving its passive transdermal permeation [4]. In the present work, to evaluate only the influence of the iontophoresis without the contribution of the passive flux and to compare this result to the iontophoresis of the cationic TMPyP porphyrin, the salt was not added to the drug donor gel formulation. In this way, the iontophoretic flux of the porphyrins in study can be compared despite the differences in their initial donor concentration because it is known that in the absence of competitive ions the iontophoretic transport is independent of drug concentration in the donor chamber [31]. Note that iontophoresis of TPPS₄ was investigated at 5.3 mM (5 mg/g), and not at 7.5 mM because TPPS₄ precipitate in the gel at this last molar concentration.

It is shown in Fig. 6 that the passive permeation of TPPS₄ from HEC gel was very low. When cathodal iontophoresis was applied, electrorepulsion between the negative charges of the molecule

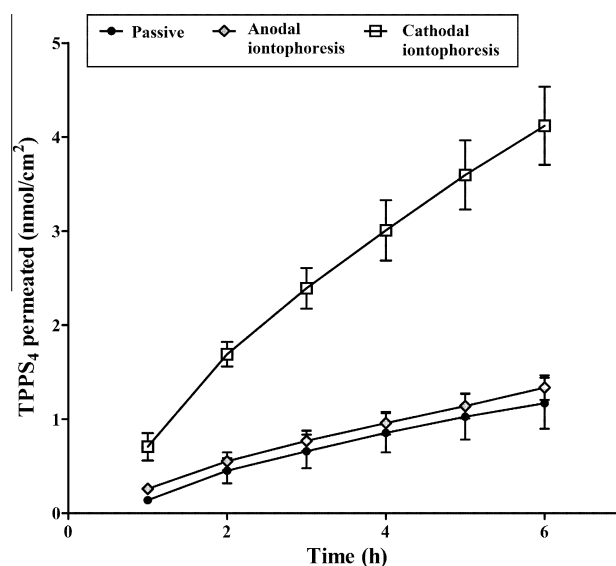


Fig. 6. Passive, cathodal and anodal iontophoretic fluxes of across porcine skin from a HEC gel containing 5.0 mg/g of drug at pH 5.5 (mean \pm SD; $n = 5$).

and the electrode was expected, and skin permeation of TPPS₄ after 6 h increased 3-fold over its passive permeation (Fig. 6). When compared to TMPyP iontophoresis, the iontophoretic amount of TPPS₄ permeated after 6 h was 67-fold lower (Table 1). However, over anodal iontophoresis of TMPyP, both electroosmotic and electrorepulsive fluxes contributed to its iontophoretic transport, but during cathodal iontophoresis of TPPS₄, only an electrorepulsive flux transports this drug through the skin since electroosmotic contributions are in the anode-to-cathode direction at pH 5.5 [4,23]. Because it is known that the electroosmotic flux of any substance is independent of its molecular weight and charge density [16,23], the anodal iontophoresis of TPPS₄ was studied to verify how much electroosmotic flux would improve the permeation of TPPS₄.

Fig. 6 shows that the anodal iontophoretic flux of TPPS₄ is not significantly different from its passive flux ($P > 0.05$). The inefficiency of anodal iontophoresis, i.e. of electroosmosis, in improving the permeation of TPPS₄ must be related to the electro-attraction that occurs between its charges and those of the positive electrode. Equally, Sylvestre et al. [22] studied the iontophoresis of the negatively charged dexamethasone phosphate and verified that its delivery from the anode was really inefficient for clinical purposes due to similar reasons as the observed in this work.

It is interesting to note that TPPS₄ iontophoretic permeation profile was very similar to its passive permeation one (Fig. 6), in contrast to the TMPyP iontophoretic permeation that clearly reduced drug's lag time when compared to the passive condition. From Fig. 6, it is possible to draw two opposing hypothesis about TPPS₄ lag time: (1) it is smaller than 1 h after passive and iontophoretic delivery and (2) the steady state was not achieved during the period of the experiment. It is hardly to believe that a charged molecule with a relatively high-molecular-weight ($MW_{\text{TPPS}_4} = 935.4 \text{ Da}$) would present a lag time smaller than 1 h. Therefore, we believe that the steady state for TPPS₄ was not attained during the 6 h of experiment, but longer period of diffusion would be necessary to prove that. In this work, however, we performed only 6 h of iontophoresis because one can increase drug permeation without compromising skin's barrier properties during this period of experiment [35].

In summary, under the studied conditions, passive permeation of TPPS₄ was about 20-fold lower than that of TMPyP after 6 h of experiment. The main reasons for this reduced passive permeation are the higher molecular weight of TPPS₄, as well as its four negative charges at pH 5.5, which make difficult its diffusion through the negatively charged skin at this pH [4,26]. Electrorepulsive flux contributed to a 3-fold increase over TPPS₄ passive permeation after 6 h, while TMPyP anodal iontophoresis (electrorepulsive plus electroosmosis) increased 12 times the permeation of this drug. Anodal iontophoresis had no effect on the transport of TPPS₄. Therefore, the contribution of the electroosmotic flux for a highly anionic drug must be counterbalanced by other electrostatic phenomenon that may occur during iontophoresis, as drug attraction by the electrode of the same polarity, and repulsion between the $\text{H}_2\text{TPPS}_4^{4-}$ and the known negatively charged stratum corneum of the skin [14] obstructing its skin penetration even when the ionto-

phoresis is applied. The calculated electrorepulsive contribution for TMPyP (calculated by subtracting the passive and the electroosmotic contributions, the last one estimated to be 20% of the total iontophoretic flux [33]) was around 70-fold higher than the calculated electrorepulsive contribution for the negative porphyrin (calculated by the difference between the cathodal iontophoretic amount and the passive amount). In this context, the iontophoresis of the positively charged tetrapyrrolic macrocycle TMPyP was much greater than that of the structurally similar, but negatively charged TPPS₄. Therefore, the study of the contribution of electrorepulsion for highly charged cationic (+4) and anionic (−4) drugs, in a specific pH, indicated that iontophoretic contribution, specifically the electrorepulsive one, is much more relevant for skin penetration of cationic than for similar, but anionic, drugs, at pH 5.5.

To verify the drugs distribution in the skin after passive and iontophoretic administration, *in vivo* experiments were performed, as discussed below.

3.3. *In vivo* iontophoresis

Iontophoretic *in vitro* experiments clearly showed that the studied porphyrins can overcome the stratum corneum barrier in greater amounts than through passive delivery. The presence and distribution of these drugs in the viable epidermis, the real site of interest in PDT, can be better evaluated by *in vivo* experiments since these layers of the skin are not viable in the *in vitro* experiments performed [36]. Also, there is no proportionality between the amount of drug accumulated inside the tissue and the amount permeated into the receptor compartment when iontophoresis is applied [4]. Therefore, to analyze how deep TMPyP and TPPS₄ penetrate into the skin after a short period of electrical current application and their tissue distribution, the porphyrin fluorescence was analyzed *in vivo* in Wistar rats after passive and anodal iontophoresis for TMPyP and passive and cathodal iontophoresis for TPPS₄ from gel formulations at 5.0 mg/g in the absence of NaCl at pH 5.5.

As can be seen in Fig. 7B, 10 min of passive permeation of the negatively charged TPPS₄ did not change the blue fluorescence of the skin. The application of cathodal iontophoresis (0.5 mA/cm²) on the TPPS₄ formulation for the same period of time made possible the visualization of fluorescence, typical of the PS studied, only in the superficial layers of the skin (Fig. 7C). In our previous work, the *in vivo* iontophoresis of TPPS₄ was also performed under the same experimental conditions, except for the presence of NaCl in the formulation. According to Aggarwal and Borissevitch [26], at neutral pH, NaCl addition can bind TPPS₄ molecules, forming $[\text{TPPS}_4 \times n\text{Na}^+]$ complexes that partially neutralize the negative charge of $\text{H}_2\text{TPPS}_4^{4-}$. The *in vivo* iontophoretic permeation of TPPS₄ in this case (in the presence of NaCl) was expressive, showing fluorescence throughout the whole skin tissue [4]. These results corroborate the theory that the negative charges of the TPPS₄ molecule are the major factor responsible for its passive and iontophoretically low skin penetration.

On the other hand, the presence of positive charges in the tetrapyrrolic macrocycle increased the passive and the iontophoretic delivery of TMPyP. In Fig. 7D, the TMPyP fluorescence can be observed in the superficial layers of the skin after 10 min of the passive experiment. Iontophoresis expressively increased the rat epidermis and dermis fluorescence, indicating high amounts of the drug was able to cross the SC barrier throughout the entire skin (Fig. 7E). In addition, it can be seen in the photomicrographs that TMPyP was homogeneously distributed around and into the nuclei of the skin cells. The accumulation of TMPyP at such cellular sites is advantageous for PDT because its photodynamic action is achieved as soon as it forms DNA complexes in the nuclei of the cutaneous cells [6,8,9].

Table 1
Positive and negative porphyrins delivered *in vitro* after 6 h of passive and iontophoretic experiments. Both formulation comprised a HEC gel contained only 5.0 mg/g of porphyrin at pH 5.5.

Porphyrin	Passive (nmol/cm ²)	Iontophoresis (nmol/cm ²)
TMPyP	23.4 (±4.2)	276.9 (±57.5) [Anodal]
TPPS ₄	1.2 (±0.3)	4.1 (±0.4) [Cathodal]

^aValues are presented as means ± SD of 5 replicates.

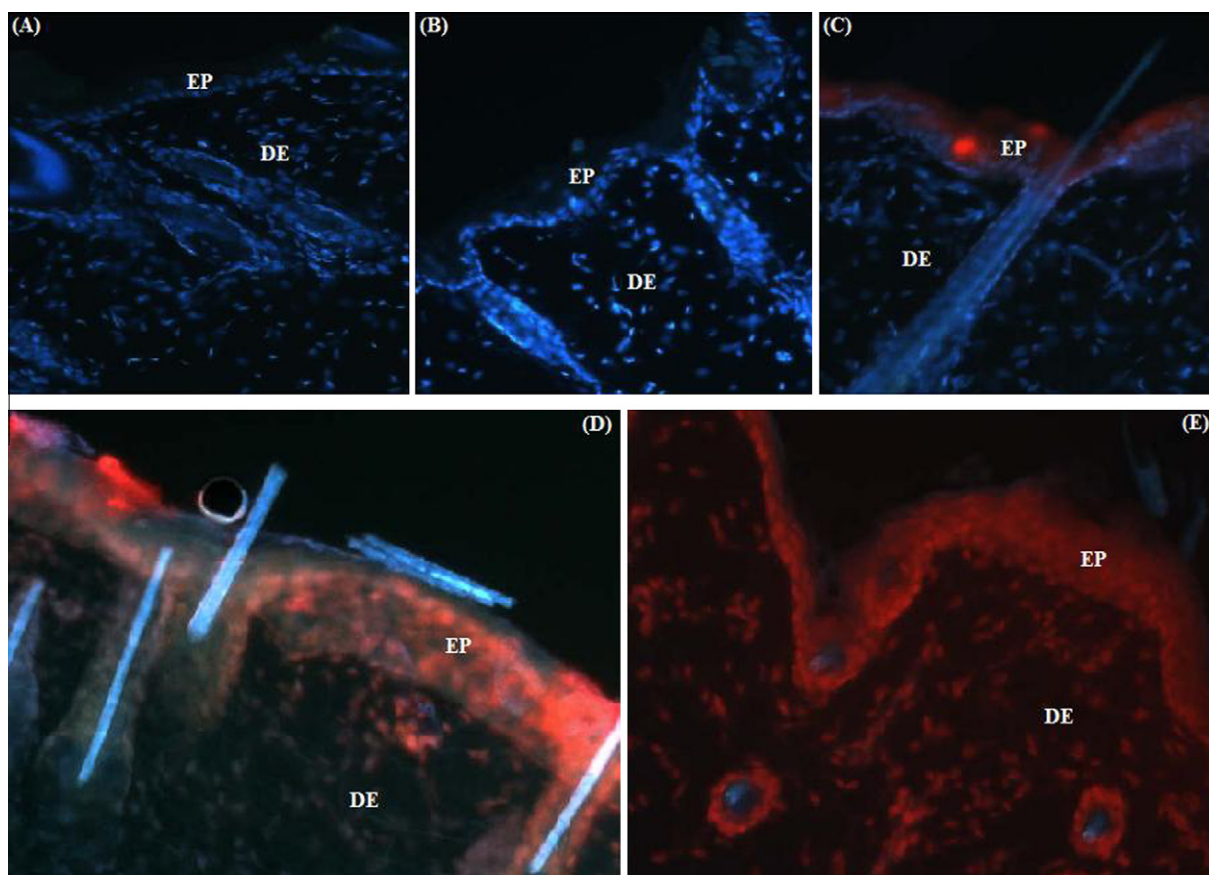


Fig. 7. Fluorescence microscopy photomicrographs of vertical slicing of Wistar rat skin before (A) and after 10 min of passive (B) and anodal iontophoresis (C) of a HEC gel containing 5.0 mg/g TPPS₄ at pH 5.5; and after 10 min of passive (D) and anodal iontophoresis (E) of a HEC gel containing 5.0 mg/g TMPyP. EP. Epidermis; DE. Dermis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

It is important to point out that the hair follicle is the main permeation route for drugs delivered by iontophoresis [37], and as Wistar rats are hairier than other skin models as porcine and human skin, the performance of this technique showed in the present work could be lower if a different animal model was used.

In conclusion, iontophoretic studies with the positively charged tetrapyrrole macrocycle TMPyP showed the expected results: a significant improvement in drug transdermal permeation when compared to passive permeation, significant TMPyP delivery by increasing the drug concentration in the anodal formulation and an enhanced drug amount delivered when the background electrolyte concentration was lowered. On the other hand, despite the fact that the cathodal iontophoretic delivery of the negatively charged tetrapyrrole macrocycle had improved TPPS₄ flux over passive permeation, this drug permeation was, both in passive and in iontophoretic experiments, much smaller than the positive drug permeation. *In vivo* experiments confirmed this difference by showing a deeper skin penetration and a homogeneous distribution of the positive TMPyP over the H₂TPPS₄⁴⁻ when anodal iontophoresis was employed. These results suggest that the iontophoresis of TMPyP is a promising technique for topical PDT.

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