The Effects of pH and Ionic Strength on Topical Delivery of a Negatively Charged Porphyrin ($TPPS_4$)

GUILHERME MARTINS GELFUSO, FÁBIO VASCONCELOS FIGUEIREDO, TAÍS GRATIERI, RENATA FONSECA VIANNA LOPEZ

Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café s/n, 14040-903, Ribeirão Preto, São Paulo, Brazil

Received 24 August 2007; accepted 27 November 2007

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.21295

ABSTRACT: *Meso*-tetra-[4-sulfonatophenyl]-porphyrin ($TPPS_4$) is a charged porphyrin derivate used in photodynamic therapy (PDT) by parenteral administration. This study means to investigate potential enhancement for its topical delivery by determining the $TPPS_4$ dependence on the environmental characteristics and applying iontophoresis. In order to accomplish this task, cathodal and anodal iontophoresis as well as passive delivery of the drug were studied *in vitro* and *in vivo* in function of its concentration, pH and ionic strength. A reduction in drug concentration as well as the NaCl elimination from donor formulation at pH 2.0 increased $TPPS_4$ passive permeation through the skin *in vitro*. Iontophoresis improved $TPPS_4$ delivery across the skin when applied in solutions containing NaCl at pH 2.0, regardless electrode polarity. However, at pH 7.4, the amount of $TPPS_4$ permeated by iontophoresis was not different from that one permeated after passive experiments from a solution containing NaCl. Despite the fact that iontophoresis did not improve $TPPS_4$ transdermal delivery at this specific condition, *in vivo* experiments showed that 10 min of iontophoresis quickly and homogeneously delivered $TPPS_4$ to deeper skin layers when compared to passive administration, which is an important condition for topical treatment of skin tumors with PDT.
 J Pharm Sci 97:4249–4257, 2008
Keywords: iontophoresis; topical administration; skin penetration; photodynamic therapy; $TPPS_4$

INTRODUCTION

Photodynamic therapy (PDT) is a new form of local cytotoxic treatment that may be effective for a wide variety of conditions: corneal neovascularization, sterilization of freshly frozen plasma, actinic keratosis, hair removal, psoriasis, Kaposi's sarcoma and several types of cancer which includes: lung, T-cell lymphoma, breast, esophageal, bladder, gastric, cervical, head and neck, brain, intestine and skin cancer among others.[1] Fundamentally, a photosensitizing agent (PS), which preferentially accumulates in target tissues, interacts with visible light and molecular oxygen. Then, two types of photodynamic reactions can occur: one involves the generation of free radicals (type I photochemical reactions), and the other involves the production of singlet molecular oxygen O_2 (type II), which is the main photoproduct responsible for cell inactivation. The type II reaction has an important effect on the cell death, although the photodynamic mechanism of the PS on neoplastic tissues is not fully understood.[2] PDT efficiency is affected by various factors, including PS photo physical properties, wavelength of the activation light, depth of the

Correspondence to: Renata Fonseca Vianna Lopez (Tel./Fax: +55-16-3602-4202; E-mail: rvianna@fcfrp.usp.br)

Journal of Pharmaceutical Sciences, Vol. 97, 4249–4257 (2008)

light penetration in the biological tissue and drug distribution in the specific tissue.[3]

The most widely applied PSs for PDT in human medicine are porphyrin derivatives. They are composed by tetrapyrrolic macro cycles, with distinct effects in biological systems.[4] *Meso*-tetra-[4-sulfonatophenyl]-porphyrin ($TPPS_4$; Fig. 1) was first used by Winkelman, in 1962.[5] It is a stable drug, water soluble at room temperature, which provides a greater yield of singlet oxygen.[6] However, depending on its synthesis and purification $TPPS_4$ can be neurotoxic.[6,7] Therefore, its topical administration, especially for the treatment of skin diseases, can avoid this problem. Nevertheless, $TPPS_4$ molecule can be protonated in acid media, which changes drastically its spectral and energetic properties[8] and can also affect its skin penetration. In order to administer this porphyrin topically with higher efficacy, it should be dispersed in an adequate formulation or drug delivery system and a complete understanding of its behavior in the formulation is required.

Iontophoresis is a method which transports hydrophilic and charged molecules into and through tissues, by application of a small direct current (no more than 0.5 $mA\,cm^{-2}$).[9] It has been widely used for topical anesthesia[10] and for topical and transdermal delivery of a great variety of drugs, including aminolevulinic acid (ALA), which is a porphyrin precursor widely used in PDT.[11–14] The two main mechanisms involved in iontophoretic delivery of drugs are electromigration and electroosmosis. Both positively or negatively charged molecules are typically delivered by electromigration, as the charged drug stands in the same charged compartment, while neutral molecules can be transported via electroosmosis.[15]

In the present study, the potential of iontophoresis as a method to enhance the $TPPS_4$ delivery for topical PDT was analyzed *in vitro* and *in vivo* in function of drug concentration, pH of the formulation and ionic strength in the donor solution by comparing the passive delivery with cathodal and anodal iontophoresis.

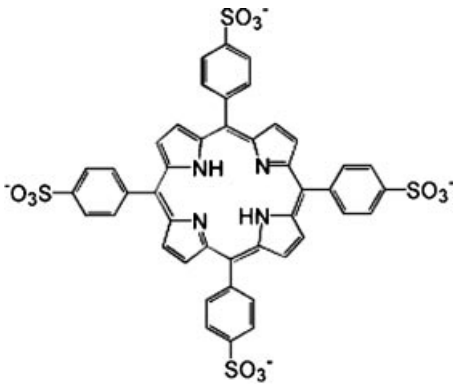

Figure 1. Chemical structure of $TPPS_4$.

MATERIALS AND METHODS

Materials

The $TPPS_4$ 2HCl ($TPPS_4$ acid dihydrochloride) was obtained from Frontier (Logan, USA), Ag-wire (99.99%, $\phi = 1.5$ mm), AgCl (99.99%) and Pt-wire were all purchased from Sigma-Aldrich (Steinheim, Germany), HEPES from J.T. Baker (Phillipsburg, USA), NaCl from Synth (Diadema, Brazil), Tissue Tek® (O.C.T. Compound) from Sakura (Torrance, CA) and *p*-phenyllenediamine from Sigma-Aldrich. All other reagents were BDH or HPLC reagents. The water used in all preparations was of Milli-Q grade (Millipore, France).

The membrane used for the *in vitro* experiments was fullthickness skin from porcine's ear. It was obtained less than 2 h after the slaughter of the animal (Frigorífico Pontal Ltda., Pontal, Brazil). The whole skin was removed from the outer region of the ear, it was separated from the underlying layer and stored frozen for a maximum of 7 days before use.

Analytical Chemistry

A method for $TPPS_4$ quantification was developed using an UV/Vis Spectrophotometer (Femto—800XI) operated at 412 nm. A linear calibration graph ($y = 0.274x + 0.001$; $r = 0.999$) was obtained over the working concentration range 0.1–1.0 μg/mL. Intra- and interdays precision and accuracy of the method showed a variation coefficient and a relative error not greater than 1.3% and 4.2%, respectively. The method was validated and showed precision and accuracy. It was also sensitive and selective during all the analyses.

In Vitro Iontophoretic Experiments

The skin was mounted between the upper and lower parts of vertical, flow-through iontophoretic cells (LG-1088-IC—Laboratory Glass Apparatus, Inc., Berkeley, CA). The area of skin exposed in

each electrode chamber was 0.8 cm^2. Ag/AgCl electrodes were prepared according to Green et al.,[16] and a constant current of 0.4 mA was passed between the electrodes from a Kepco APH 500 DM power supply (Kepco Power Supply, Flushing, NY). The voltage of the complete circuit and of each cell was measured hourly with a voltmeter (Freak, MY-63) to guarantee the both the intactness of the skin and that the Ag/AgCl electrodes reactions are occurring in according to the expected.

Three sets of experiments were performed. The first series aimed to determine the influence of drug concentration at pH 2.0. $TPPS_4$ transport from the cathode compartment was followed over a period of 6 h at a constant current of 0.5 mA/cm^2. The $TPPS_4$ formulation comprised a solution of the drug at 0.1% and 0.5% with 89.5 mM of NaCl. The anodal and receptor chambers of the diffusional cells simply contained a physiological buffer (133 mM NaCl, 25 mM HEPES) at pH 7.4. The receptor solution was stirred at 300 rpm and kept at 37°C by a circulating water system (Ecoline 003, E100 from Lauda, Lauda-Königshofen, Germany). The receptor was perfused continuously at 3 mL/h using a peristaltic pump (Pump Pro MPL580—Watson-Marlow Bredel Pumps, Falmouth, United Kingdom) and samples were collected automatically every hour (Fraction collector PTFCII—Pharmatest, Hainburg, Germany) for 6 h. At the end of the experiment, the amount of transported $TPPS_4$ was analyzed as described above.

In the second series of experiments the donor pH effect (2.0 and 7.4) in solutions containing 0.5% of the drug and 89.5 mM of NaCl was evaluated on both cathodal and anodal iontophoresis. The counterelectrode and receptor solution was maintained at pH 7.4. The same current conditions and background electrolyte (HEPES-buffered NaCl) were employed as before.

In the last series, the ionic strength was appraised by the presence or absence of 89.5 mM of NaCl in solutions containing also 0.5% of the drug at pH 2.0 and at pH 7.4. In the experiments realized in the absence of NaCl, the electrochemistry reactions within the Ag/AgCl electrodes were ensured by the presence of 10 mM of Cl^-, the $TPPS_4$ counterion, and by monitoring the voltage in each diffusion cell during all the period of the study. The donor solution pH was also measured after the experiment.

"Passive" experiments were also performed for all series of experiments: all conditions were identical to those described above except that no current was applied.

In Vivo Experiments

$TPPS_4$ skin penetration after passive and iontophoretic *in vivo* experiments was investigated in male Wistar rats, 4 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 abdominal skin of the animals was trimmed of 48 h before the experiments. Few minutes before the experiments the rats were anesthetized with an intraperitoneal injection of ketamine (50 mg/Kg) and xylazine (10 mg/Kg) and placed on its back. Formulations (1 mL) containing 0.5% of $TPPS_4$ and 89.5 mM of NaCl, at pH 7.4, were applied to the skin surface via open glass chamber (1.1 cm^2) and sealed to the skin with silicone grease. AgCl electrode was then introduced and maintained at least 5 mm from the skin surface by means of a plastic lid that covered the cell. Ag counterelectrode was introduced in another glass chamber, also sealed to the animal's skin surface, containing only buffer solution and 133 mM of NaCl. A Phoresor II (model PM 850, Iomed, Inc., Salt Lake City, UT) delivered a constant current of 0.4 mA for 10 min. At the end of the experiment the rats were sacrificed by carbon dioxide vapor. The drug-exposed skin areas were cleaned with cotton soaked in methanol and removed from the animals for fluorescence microscopy analysis.

Fluorescence Microscopy

Skin samples obtained following *in vivo* passive and iontophoretic treatment had their fluorescence preserved by an application of Tissue-Tek® (O.C.T. Compound) solution and frozen in liquid nitrogen. Cryosections (16 μm) were made by a cryomicrotome (Microm D-6900, Heidelberg, Germany) and, subsequently, all slices were mounted in a *p*-phenyllenediamine mounting medium to support fluorescence stability as well as to protect slices against photobleaching effects. Skin slices were then fluorimetrically analyzed by a Fluorescence Microscope (Zeiss Axioskop) with a

Microaktueal barrier filter (470–520 nm). The photographs were captured by using a Zeiss MC 80-DX microscope camera system and a 4000× objective (Zeiss Plan—Neofluar).

Data Analysis

At least 4–6 replicates of each experiment were used. Results are presented in text as means ± SDs. Data were analyzed by ANOVA followed by parametric Tukey's test. Statistical significance was fixed at $p < 0.05$.

RESULTS AND DISCUSSION

The 5-aminolevulinic acid (ALA), precursor of the endogenous photosensitive agent protoporphirin IX (PpIX), is the most used drug in topical PDT. Due to its relatively low molecular weight (168 Da) ALA can penetrate the damaged skin rather easily, leading to PpIX accumulation specially in tumor cells.[4] Nevertheless, there are many variables that may interfere in PpIX local bioavailability—the most noticeable ones are: (i) the irregular ALA skin penetration; (ii) the ALA conversion in PpIX, which depends on the quantity of pro-drug that penetrates the skin as well as on the local metabolism; and (iii) the heterogeneous distribution of ALA-induced PpIX in the tumor.[4] Therefore, the direct administration of a photosensitive agent, as $TPPS_4$, can be advantageous once the administrated drug is already photosensible and does not need to be converted into another molecule to be active. However, the efficacy of porphyrin in PDT depends on its excited state lifetime and quantum yields which, in turn, depend on formulation environment.[8,17]. It is probable that the known drug aggregation[8,17] that happens in some formulations conditions affects drug skin penetration. In this way, passive and iontophoretic permeation of $TPPS_4$ were evaluated as a function of its concentration, pH, electrode polarity, and ionic strength of the formulation.

Drug Concentration

To evaluate the drug concentration's effect on the $TPPS_4$ passive and cathodal iontophoresis, formulations containing (i) 0.1% and (ii) 0.5% of the drug with 89.5 mM NaCl at pH 2.0 were studied. This pH was chosen for this first study as it is the pH presented by the solution when the drug is dispersed without any adjustment. The NaCl was added in order to guarantee the electrical current (0.4 mA) transport for the 6 h of the iontophoretic experiments.

As it can be seen in Table 1, when only the passive delivery is observed, the increase in drug concentration seems to prevent its delivery. It is well documented[17–22] that in acid media $TPPS_4$ is being biprotonated ($H_4^{2+}TPPS_4^{4-}$), changing drastically its spectral and energetic properties. According to Aggawarl and Borissevitch,[17] $TPPS_4$ tends to form aggregates at high concentrations, in acid pH and also in the presence of NaCl. Our results have demonstrated that this aggregation also decreases porphyrins permeation across the skin, therefore affecting their efficacy in topical applications.

Table 1 also shows the cathodal iontophoretic delivery of the $TPPS_4$ at pH 2.0. As already described, at such pH $TPPS_4$ molecules are biprotonated ($H_4^{2+}TPPS_4^{4-}$), thus presenting two net negative charges which can suffer electrorepulsion in case they are put in contact with the negative electrode (cathode). Moreover, this pH was a recourse applied in attempt to reverse the skin negative charge in physiological conditions. Lopez et al.[11] showed that anode-to-cathode electroosmotic flow is significantly reduced at pH 4. Delgado-Charro and Guy[23] and Hoogstraate et al.[24] demonstrated that the skin negative charge can be reduced, neutralized, or even

Table 1. $TPPS_4$ Passive and Cathodal Iontophoretic Delivery, as a Function of Drug Concentration, From Solutions Containing 89.5 mM of NaCl, at pH 2.0

Drug Concentration in the Donor Compartment (%)	$TPPS_4$ in the Receptor Solution After 6 h of Experiment ($\mu g/cm^2$)	
	Passive	Cathodic Iontophoresis
0.1	2.64 (±0.34)	4.25 (±0.53)
0.5	1.44 (±0.19)	5.43 (±0.71)

Data shown are the mean ± SD of six replicates.

reversed by the iontophoresis of certain cationic species. In the present work, at pH 2.0, there are hydrogen ions in the donor solution which associate with the fixed negatively charged sites in the skin, leading to a net convective solvent flow in the cathode-to-anode direction, since skin is positively charged and anion permselective in this pH; and facilitating drug transport from the cathode besides electrorepulsion.

It is possible to notice in Table 1 that the amount of $TPPS_4$ delivered on cathodal iontophoresis was greater than those in passive experiments. When 0.5% of the drug was placed in the negative compartment there was an increase of about four times in the total amount delivered. Other studies have also reported that charged molecules, when placed in same charged compartment, tend to benefit from the electrorepulsion and this electrorepulsion is amplified with the increase of ions present in the formulation.[25] Nevertheless, a fivefold increase in drug concentration improves only 1.3 times drug iontophoretic delivery, probably due to the $TPPS_4$ aggregation phenomenon. This greater amount of the drug (0.5%) was chosen for further studies on the attempt to verify the real influence of the drug concentration in skin permeation when the drug is not in its aggregated form,[17] that is, at pHs above the $TPPS_4$ pK_a and in the absence of NaCl.

pH

It is well described by Aggawarl and Borissevitch[17] that $TPPS_4$ does not aggregate at any pH above the drug pK_a ($TPPS_4$ $pK_a = 4.8$), regardless its concentration and NaCl presence. In addition, at physiological pH drug molecules are nonprotonated ($H_2TPPS_4^{4-}$),[17] presenting four net negative charges. Therefore, passive and iontophoretic experiments were also realized at pH 7.4 to take advantage from the nonaggregate state of the molecule as well as from its four negative charges that may, theoretically, improve electrorepulsion when compared to the two negative charges presented by the molecule at pH 2.0.

Passive, cathodal and anodal iontophoretic fluxes of a solution containing 0.5% of $TPPS_4$ and 89.5 mM of NaCl at pH 7.4 are presented in Figure 2. It can be seen that the passive delivery at pH 7.4 was even greater than the iontophoretic delivery at pH 2.0. The most probable reason for this expressive passive permeation could be attributed to the nonaggregate state of the molecule at pH 7.4. The nonaggregated $TPPS_4$ permeated passively through the skin around 7.5 times more than the aggregated drug at pH 2.0 in the presence of NaCl.

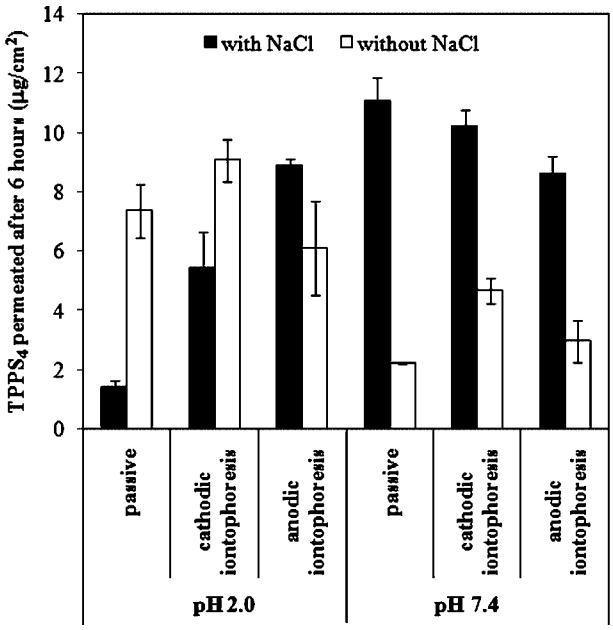

Figure 2. $TPPS_4$ permeated after 6 h of passive, cathodal and anodal iontophoretic experiments from solutions containing 0.5% of the drug in the presence or absence of 89.5 mM of NaCl, at different pHs. Data shown are the mean ± SD of six replicates.

Due to $TPPS_4$ great passive permeation at pH 7.4 solutions with NaCl, its cathodal iontophoresis at this same pH did not seem to be effective, once the amount passively delivered was numerically and not statistically different ($p > 0.05$) from the total amount delivered after the electrical current application. One suitable explanation is that in this case (at pH 7.4, in the cathode, in the presence of NaCl) the electroosmosis is in the counterdirection of the electrorepulsion, thus objecting the drug flow in cathode-to-anode direction. It must be emphasized that the skin at pH 7.4 is negatively charged and also that there is a solvent electroosmotic flow in anode-to-cathode direction.[26] Kochhar and Imanidis[14] obtained a similar result: besides the greater ionic valence of Leuprolid at pH 4.5 compared to pH 7.2, the permeation rate almost doubled at the latter, because at pH 4.5 the electroosmotic flow was in cathode-to-anode direction and, in that case, the drug was placed in the anode compartment.

The influence of the donor solution pH on the anodal iontophoretic flux of 0.5% of $TPPS_4$ was also studied and the results are shown in Figure 2

as well. It can be observed that the amount of drug delivered after 6 h of anodal iontophoresis in the presence of the salt from the pH 7.4 solution was even lower than the amount delivered passively. Despite the fact that $H_2TPPS_4^{4-}$ does not suffer electrorepulsion when in contact with the positive electrode, it was expected that at pH 7.4 the anodal iontophoresis could take advantage of the anode-to-cathode electroosmotic flow. Nevertheless, the positively charged electrode is probably attracting the negatively charged drug, objecting its delivery.

The anodal iontophoretic flux of $TPPS_4$ in the presence of NaCl was studied at pH 2.0 as well. It can be observed in Figure 2 that there was a considerable increase on the total amount delivered after anodal current application compared to cathodal iontophoresis also at pH 2.0. This fact is a difficult understanding matter, as in this case ($TPPS_4$ in the anode, pH 2.0) the electroosmotic flow is in the opposite direction (cathode-to-anode) and should hinder drug delivery. $TPPS_4$ is not expected to suffer electrorepulsion either, as it is placed in the opposite charged compartment. However, it can be speculated that the attractiveness by the electrode is somehow being compensated by the attractiveness performed by the skin, that at pH 2.0 has a positive charge residual.[14,26]

In conclusion, it has been found that at pH 7.4, in the presence of NaCl, when $TPPS_4$ is on its nonaggregated state, its passive permeation is favored. Otherwise, at this physiological pH, neither cathodal nor anodal iontophoresis has a significant role on $TPPS_4$ permeation. At pH 2.0 the aggregation reduces passive permeation of $TPPS_4$, and in this case, both cathodal and anodal iontophoresis have shown to increase significantly drug skin permeation.

Ionic Strength

It is known that NaCl presence can affect iontophoretic flux, normally decreasing drug permeation by competition.[27] Nevertheless, it also affects the $TPPS_4$ aggregation.[8,17] In this way, *in vitro* skin permeation experiments in the absence of this salt were also studied.

Passive Permeation

For passive permeation at pH 2.0, as it can be noticed in Figure 2, the absence of NaCl increases five times the $H_4^{2+}TPPS_4^{4-}$ skin permeation if compared to its permeation in the presence of the salt. According to Aggawarl and Borissevitch,[17] NaCl presence leads to the formation of "clouds" of Na^+ counterions around the $H_4^{2+}TPPS_4^{4-}$, which can reduce the electrostatic repulsion between the drug molecules, consequently increasing the drug aggregation at this pH. Therefore, it is clear that the aggregates of $H_4^{2+}TPPS_4^{4-}$, which are formed in acid pH in the presence of NaCl,[17] permeate the skin less than the free drug molecules.

At pH 7.4, however, Aggawarl and Borissevitch[17] observed an absorption band centered at 413 nm that was characteristic for deprotonated $TPPS_4$ form ($H_2TPPS_4^{4-}$). In their work they described that at pH 7.0 in the NaCl presence, this absorption band can be associated with a $[TPPS_4 \times nNa^+]$ complex, which NaCl addition could bind $TPPS_4$ molecules in that mentioned complex. Therefore, at pH 7.0 the presence of salt did not stimulate the aggregation of the nonprotonated form of the porphyrin in any concentration. Nevertheless, as it can be observed in Figure 2 the presence of the salt at pH 7.4 significantly increases the drug passive permeation if compared to the formulation that does not have NaCl at the same pH. It is likely that $[TPPS_4 \times nNa^+]$ complex formed in the presence of NaCl partially neutralize the negative charge of $H_2TPPS_4^{4-}$, increasing, in this way, its passive flux through the skin.

Finally, analyzing only the influence of pH in $TPPS_4$ skin passive permeation in the absence of the salt, Figure 2 shows that pH 2.0 environment leads to a greater drug permeation. The presence of four negative charges in the molecule at pH 7.4 instead the two negatives charges at pH 2.0 can explain this results once ionized molecules have more difficult in crossing physiological membranes.

Iontophoresis

Comparing the influence of the iontophoresis on the skin permeation of $TPPS_4$ from formulations at pH 7.4 in the absence of the NaCl to drug passive skin permeation at this same condition, it is possible to notice that cathodal and anodal iontophoresis expressively contributed to arise the $TPPS_4$ flux. In the cathodal iontophoresis, despite of the counterconvective flow at pH 7.4, the drug's 4 negative charges have contributed to its electrorepulsive transport. Furthermore, there was not any significant amount of competitive ions in this compartment that could have disturbed drug electrorepulsion. In the anodal

iontophoresis, electroosmosis is the only mechanism that may have contributed to the $H_2TPPS_4^{4-}$ iontophoretic flux, once the drug does not have any positive charge to suffer electrorepulsion at this pH. Nevertheless, all this improvement caused by iontophoresis over the passive drug permeation at pH 7.4 was still lower than the passive drug permeation in the NaCl presence.

NaCl absence influences on iontophoresis at pH 2.0 over passive nonaggregated drug permeation at this same pH can be seen in Figure 2 as well. At pH 2.0, cathodal iontophoresis was able to contribute to the drug permeation when it is compared to its passive delivery. The passive contribution after 6 h of experiment was 7.36 ± 0.89 $\mu g/cm^2$ while the cathodal iontophoresis delivered 9.08 ± 0.71 $\mu g/cm^2$. Therefore, the real increase caused by the cathodic iontophoresis was about 1.72 $\mu g/cm^2$. This increase was obtained by both electrorepulsive and electroosmotic fluxes, respectively due to the two negatives charges of $H_4^{2+}TPPS_4^{2-}$ electrorepulsion role and the cathode-to-anode convective solvent flux through the skin which must be present at pH 2.0. Also in the absence of NaCl and at pH 7.4, the cathodal iontophoresis increased about 2.44 $\mu g/cm^2$ the $TPPS_4$ skin permeation in comparison to passive delivery at same conditions. As at pH 7.4 the electrorepulsive contribution in the cathode should be dominant (the electroosmotic flux was in anode-to-cathode direction), it is concluded that the improved permeation conferred by cathodal iontophoresis in both studied pHs must be mainly related to the electrorepulsive contribution, since $TPPS_4$ is nonaggregated in the absence of NaCl. Therefore, there is a connection between the increase of iontophoresis contribution and the increase of the charge density of the molecule with the pH change (pH $2.0 = -2$, pH $7.4 = -4$).

Anodal iontophoresis at pH 2.0 in the absence of NaCl, however, was not significant different from passive drug permeation at this same condition. This result can be partially explained by both countereletroosmotic flow and drug-to-electrode interaction of the nonaggregated molecules. By comparing the anodal iontophoresis on either presence or absence of NaCl at pH 2.0, it can be noticed that the NaCl presence increased anodal iontophoretic drug permeation. It is possible that the presence of the Na^+, which in according to the literature[8,17] "neutralizes" in part the $H_4^{2+}TPPS_4^{2-}$, has lowered drug-to-electrode interaction, thus increasing the anodal iontophoretic flow.

It is relevant to point out that the well known influence of NaCl in both electrorepulsive and electroosmotic[9,12,27] iontophoretic transport, that is, NaCl competes with drug molecule for charge transport decreasing its flux, was rather different from $TPPS_4$ iontophoretic delivery, due to the dominant role of the electrostatic interaction at its aggregation. Therefore, $TPPS_4$ iontophoretic transport number seems to be more influenced by the great passive permeation than by the presence of NaCl. At pH 2.0, for instance, in the absence of NaCl (when the passive permeation is great), the cathodal iontophoresis was able to make $TPPS_4$ transports only 1.54% of the current applied against 3.57% transported in the presence of NaCl. On the other hand, at pH 7.4, the presence of NaCl, that expressively improved the passive permeation, leads to an insignificant contribution of the iontophoresis for drug permeation. The passive permeation, in its turn, seems to be strongly influenced by NaCl presence: at pH 2.0, the presence of NaCl leads the $TPPS_4$ to form aggregates, decreasing its passive permeation and increasing the iontophoretic transport number when compared to experiments realized in the absence of NaCl. At pH 7.4, the NaCl presence leads to the formation of $TPPS_4 \times n Na^+]$ complex that improved considerable drug passive permeation making iontophoretic transport insignificantly in 6 h of experiment.

In Vivo Studies

It is well known that when iontophoresis is applied, the main pathway for drug permeation is represented by hair follicles, sweat glands and other shunts which often give passage to epidermis. For that reason, there is no proportionality between the amount of drug accumulated inside the tissue and the amount permeated into the receptor compartment. Therefore, the presence of the fluorescent $TPPS_4$ into the skin was analyzed by fluorescence microscopy after passive and cathodic iontophoresis experiments from a solution containing 0.5% of the drug with 89.5 mM NaCl at pH 7.4. This pH was chosen based on high passive and iontophoretic *in vitro* permeation through the skin of $TPPS_4$ at this condition. In addition, Gonçalves et al.[8] showed that the nonprotonated $TPPS_4$ posses higher quantum yield of the triplet state and therefore, should be promising for PDT.

As it can be seen in Figure 3, only 10 min of iontophoresis increased expressively the rat epidermis and dermis red fluorescence compared to drug passive delivery. Furthermore, after iontophoresis, drug fluorescence showed to be homogenously distributed all over the skin surface and layers. After passive experiments, on the other hand, a heterogeneously red fluorescence can be observed only in superficial skin layers.

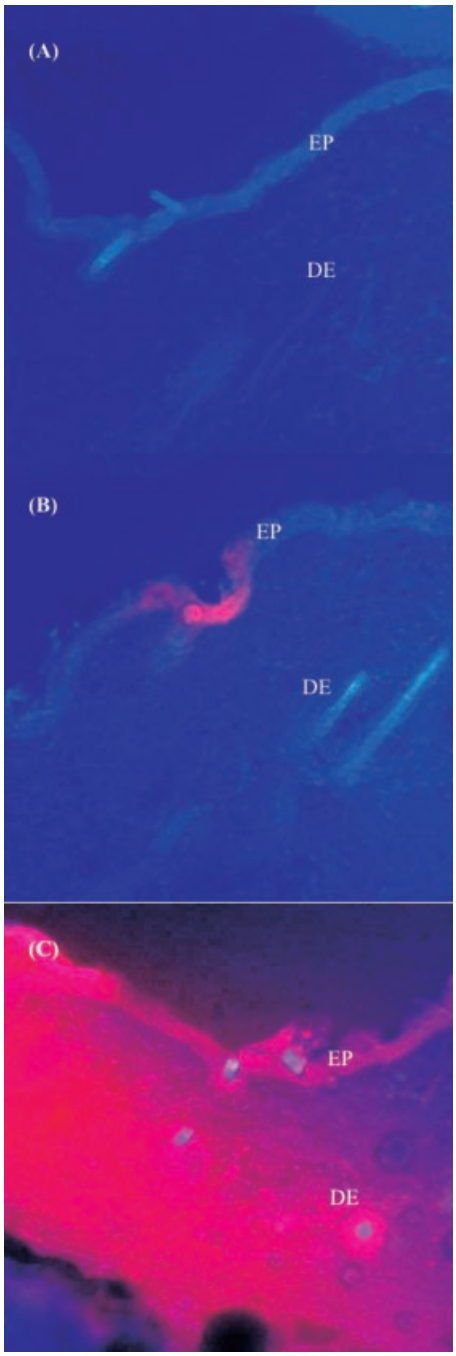

Figure 3. Fluorescence photomicrographs of vertical slicing of Wistar rat skin before (A) and after 10 min of passive (B) and cathodic iontophoresis (C) of a solution containing 0.5% of $TPPS_4$ and 89.5 mM of NaCl at pH 7.4; 4000×. (EP) Epidermis; (DE) Dermis.

Despite the fact that, on first demand, these *in vivo* results do not seem to be in accordance with the ones expected by the *in vitro* results, it must be taken into account that, in the *in vivo* experiments, the drug was monitored into the viable skin and not in the receptor solution as in the *in vitro* studies. Moreover, *in vivo* experimental time was plenty reduced to one that is considered suitable for an iontophoresis therapy. The cathodal iontophoresis, in this way, was able to increase the skin penetration of $TPPS_4$ in a short period of time.

CONCLUSIONS

In vitro experiments showed that the reduction of $TPPS_4$ aggregation by decreasing drug concentration, by increasing pH or by NaCl retreat from acidic pH formulations improved $TPPS_4$ passive skin permeation significantly. Reduction of drug negative residual charge by the addition of Na^+ to a physiological pH formulation also increased significantly its passive permeation. At pH 2.0, in the presence of NaCl, iontophoresis increased $TPPS_4$ skin permeation in comparison to passive permeation regardless the polarity of the electrode. At pH 7.4, however, $TPPS_4$ iontophoretic permeation through the skin in the presence of NaCl was not more efficient than its passive transdermal permeation that was already expressive. Finally, despite the fact that drug passive and iontophoretic delivery from the best formulation (pH 7.4 with NaCl) leads to almost the same amount of $TPPS_4$ in the receptor solution *in vitro*, *in vivo* experiments showed that the presence of $TPPS_4$ into the skin, characterized by tissue red fluorescence, was significantly improved and homogenously distributed by iontophoresis. It is still to be studied whether the formulation conditions for topical $TPPS_4$ administration revealed on this paper are finally transposed to drug skin tumor penetration.

ACKNOWLEDGMENTS

The authors would like to thank the Fundação para o Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil, for supporting this research.

REFERENCES

1. De Rosa FS, Lopez RF, Thomazine JA, Tedesco AC, Lange N, Bentley MX. 2004. In vitro metabolism of 5-ALA esters derivatives in hairless mice skin homogenate and in vivo PpIX accumulation studies. Pharm Res 21:2247–2252.
2. Allison RR, Downie GH, Cuenca R, Hu XH, Childs CJH, Sibata CH. 2004. Photosensitizers in clinical PDT. Photodiagn Photodyn Ther 1:27–42.
3. Szpringer E, Lutnicki K, Marciniak A. 2004. Photodynamic therapy—Mechanism and employment. Ann Univ Mariae Curie Sklodowska 59:498–502.
4. Lopez RF, Lange N, Guy R, Bentley MV. 2004. Photodynamic therapy of skin cancer: Controlled drug delivery of 5-ALA and its esters. Adv Drug Deliv Rev 56:77–94.
5. Winkelman J. 1962. The distribution of tetraphenylporphinesulfonate in the tumor-bearing rat. Cancer Res 22:589–596.
6. Zima T, Jirsa M, Jirsa M, Jr., Jiráscová M, Bradová V, Stádník B. 2004. Effect of chloroquine wash-out period of photosensitizers in the skin and selected organs in rats. Physiol Res 53:103–108.
7. Jiraskova M, Jirasek L, Stork J, Vosmik F, Jirsa M. 2003. Photodynamic diagnosis and therapy in dermatology: Experience with use of $TPPS_4$ in skin diseases. Cas Lek Cesk 142:439–493.
8. Gonçalves PJ, Boni LDE, Neto NMB, Rodrigues JJ, Zílio SC, Borissevitch IE. 2005. Effect of protonation on the photophysical properties of meso-tetra (sulfonatophenyl)porphyrin. Chem Phys Lett 407: 236–241.
9. Kalia YN, Naik A, Garrison J, Guy GH. 2004. Iontophoretic drug delivery. Adv Drug Deliv Rev 56:619–658.
10. Zempsky WT, Sullivan J, Paulson DM, Hoath SB. 2004. Evaluation of a low dose lidocaine iontophoresis system for topical anesthesia in adults and children: A randomized, controlled trial. Clin Ther 26:1110–1119.
11. Lopez RFV, Bentley MVLB, Delgado-Charro MB, Guy GH. 2001. Iontophoretic delivery of 5-aminolevulinic acid (ALA): Effect of pH. Pharm Res 18: 311–315.
12. Lopez RFV, Bentley MVLB, Delgado-Charro MB, Salomon D, Van Den Bergh H, Lange N, Guy GH. 2003. Enhanced delivery of 5-aminolevulinic acid esters by iontophoresis in vitro. Photochem Photobiol 77:304–308.
13. Lopez RFV, Bentley MVLB, Delgado-Charro MB, Guy GH. 2003. Optimization of aminolevulinic acid delivery by iontophoresis. J Control Release 88:65–70.
14. Kochhar C, Imanidis G. 2004. In vitro transdermal iontophoretic delivery of leuprolide under constant current application. J Control Release 98:25–35.
15. Merino V, Kalia YN, Guy RH. 2007. Transdermal therapy and diagnosis by iontophoresis. Trends biotechnol 15:288–290.
16. Green PG, Hinz RS, Kim A, Szoka FC, Jr., Guy RH. 1997. Iontophoretic delivery of a series of tripeptides across the skin in vitro. Pharm Res 8:1121–1127.
17. Aggawarl LPF, Borissevitch IE. 2006. On the dynamics of the $TPPS_4$ aggregation in aqueous solutions. Spectrochim Acta Part A 63:227–233.
18. Akins DL, Zhu HR, Guo C. 1994. Absorption and raman scattering by aggregated meso-tetrakis(p-sulfonatophenyl)porphine. J Phys Chem 98: 3612–3618.
19. Pasternack RF, Gibbs EJ, Collings PJ, De Paula J, Turzo LC, Terracina A. 1998. A nonconventional approach to supramolecular formation dynamics: The kinetics of assembly of DNA-bound porphyrins. J Am Chem Soc 120:5873–5878.
20. Borissevitch LE, Tominaga TT, Imasato H, Tabak M. 1996. Fluorescence and optical absorption study of interaction of two water soluble porphyrins with bovine serum albumin: The role of albumin and porphyrin aggregation. J Lumin 69:65–76.
21. Zhang Y-H, Chen T-M, He T, Liu FC. 2003. Raman and infrared spectral study of meso-sulfonatophenyl substituted porphyrins ($TPPS_n$, n = 1, 2A, 2O, 3, 4). Spectrochim Acta Part A 59:87–101.
22. Borissevitch IE, Gandini SC. 1998. Photophysical studies of excited-state characteristics of meso-tetrakis (4-N-methyl-pyridiniumyl) porphyrin bound to DNA. J Photochem Photobiol B 43:112–120.
23. Delgado-Charro MB, Guy RH. 1994. Characterization of convective flow during iontophoresis. Pharm Res 11:929–935.
24. Hoogstraate AJ, Cullander C, Nagelkerke JF, Senel S, Verhoef JC, Junginger HE, Boddé HE. 1994. Diffusion rates and transport pathways of fluorescein isothiocyanate (FITC)-labeled model compounds through buccal epithelium. Pharm Res 11: 83–89.
25. Pikal MJ. 2001. The role of electroosmotic flow in transdermal and iontophoresis. Adv Drug Deliv Rev 46:281–305.
26. Guy RH, Kalia YN, Delgado-Charro MB, Merino V, Lopez A, Marro D. 2000. Iontophoresis: Electrorepulsion and electroosmosis. J Control Release 64: 129–132.
27. Nugroho AK, Li GL, Danhof M, Bouwstra JA. 2004. Transdermal iontophoresis of rotigotine across human stratum corneum in vitro: Influence of pH and NaCl concentration. Pharm Res 21:844–850.