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Transdermal Drug Delivery of Adenosine by Microplasma with Iontophoresis and DMSO

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Abstract

Medication brings negative effects regarding the method of the drug administration, such as risk of infections and metabolism of drugs. Transdermal drug delivery can avoid these problems. Microplasma discharge treatment was used to overcome the skin barrier. TEM observation of the untreated and microplasma treated skin confirmed the effect of microplasma on lipid lamellae. Franz diffusion cell was used to investigate permeation of Adenosine through Yucatan micropig skin. The amount of the penetrated drug and the drug retained inside the skin was measured by HPLC. We compared Adenosine permeation through the untreated skin, iontophoreticaly pre-treated skin with $FeSO_4 \cdot 7H_2O$, iontophoreticaly pre-treated skin with $FeSO_4 \cdot 7H_2O$ followed by microplasma treatment, and DMSO treated skin. Iontophoretic pre-treatment with $FeSO_4 \cdot 7H_2O$ before microplasma treatment was used with idea to enhance a lipid oxidation inside the skin. Microplasma treatment was the most effective in permeation of Adenosine. On the other hand, DMSO treatment and iontophoreticaly pre-treated skin followed by microplasma treatment retained the most and the similar amount of the drug inside the skin.

Keywords: Microplasma • Transdermal Drug Delivery • Iontophoresis • Adenosine • DMSO

Introduction

Different types of medicaments have been used to support an immunity reaction of sick people. However, apart from the positive effects of these drugs, they also have a side effects related to the way of administration. For example, using injections, increase risk of an infection. A mathematical modeling based on the study in 2014 [1] showed that 1.67 million of Hepatitis B virus infections, 315 120 cases of Hepatitis C virus infections and 33877 of HIV infections were caused by unsafe injections use. However, this is not the only problem of the use of unsafe injections. Even in the case of the safe injection treatment, the risk is still present. A 6 months study in a Korean clinic in 2012 [2] showed that 61 people were infected after the repeating injection treatment. Another study in Japan in 2007 [3] showed that 0.5% of hospitalized people suffered from a catheter related blood stream infection (i.e. infusion). This represents thousands of infected people in Japan every year.

Oral drug delivery also causes serious problems. A size of tablets is limited by the required dose (for a high dose, large tablets or several tablets are required). A drug can also be metabolized in the gastrointestinal tract to an ineffective or a dangerous form. To ensure an effective amount of the drug and to compensate the metabolic effect, it is necessary to increase the dose and this can lead to liver cirrhosis. Moreover, drinks and foods can change the metabolism of the drug and expose the patient to an overdose or an under dose. The transdermal drug delivery represents an alternative which helps to avoid these problems. The number of transdermal patches is growing every year. FDA approved 32 patches in USA during last 20 years and currently, almost the triple amount of the patches is in a clinical or a pre-clinical testing.

The transdermal drug delivery is a very attractive possibility of drug administration. However, the skin has a very low permeability for foreign molecules because of a structured layer called stratum corneum, a lipid-rich matrix with embedded corneocyte cells. This is especially problematic for relatively large molecular drugs, which represent a large majority of active agents for therapeutic applications. An enhancement of the skin permeability has been studied using a chemical enhancer [4,5], an electroporation (using a high voltage) [6] or an iontophoresis (using an electric current). Plasma has the potential to use all these effects; moreover, it offers active species such as radicals and ions. The plasma discharge is still a relatively new technique in the drug delivery field.

An air DBD plasma discharge was used for the transdermal delivery of large molecules such as dextran with a molecular weight of 10 kDa that penetrated to a depth of 600 μm within one hour. Even larger molecules such as albumin (66 kDa), IgG human immunoglobulin (115 kDa) and SiO_2 nanoparticles with a diameter of 50 nm could penetrate to a depth of 200 μm within one hour [7]. The transdermal drug delivery of a various molecules such as fluorescent dye fluorescein (332 Da) [8] by an air plasma jet; phenol red (354 Da) [9,10]; Galantamine Hydrobromide (368 Da) [11], or Cyclosporine (1203 Da) [12] by an argon microplasma or a plasma jet; or DNA [13] by a helium plasma jet were successfully tested. The principle of this drug delivery method is not fully understood yet, and various effect of plasma have been observed and studied. Gelker et al. [14] delivered a set of drugs with different molecular weight through the human skin after the air atmospheric plasma treatment but this effect was caused by the plasma induced micro-holes in the skin. Gelker et al. [15] found out in another study that a μs-pulsed DBD was more efficient than a ns-pulsed DBD. Choi et al. [16] found out that plasma can influence a intercellular junction of the skin keranocyte cells and allow the penetration of a drug. Lee et al. [17] demonstrated that an argon discharge was more effective than a helium discharge. Chen et al. [18] used cold atmospheric plasma integrated with micro needles for improving the transdermal delivery of an immune checkpoint blockade.

The aim of our research is to study the improvement of the transdermal delivery of Adenosine as a model hydrophilic drug. As well as the investigation of the retention of Adenosine inside the skin using microplasma discharge combined with the iontophoresis, especially the influence of the supposed lipid oxidation as a one of the factors of the skin permeability improvement.

Adenosine has already been used as a hydrophilic model drug in several studies. Transdermal delivery of Adenosine was improved by using iontophoresis [19], micro needles [20], chemical enhancers [21] or micro-emulsions [22].

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Materials and methods

Adenosine

Adenosine is an organic compound consisting of an adenine attached to a ribose. Adenosine has a molecular weight of 267 Da (g·mol^{-1}) and a molecular formula of $C_{10}H_{13}N_5O_4$. It is used to identify and convert heart rhythm. Adenosine was ordered from Fujifilm Wako Pure Chemical Corporation.

Sample preparation

The Hairless Yucatan micropig's skin was ordered from Charles River Japan, Inc. (Yokohama, Japan). The skin samples were stored at 80°C. Before using them, the fat layer was removed by using a knife, and the skin was cut into pieces of dimensions 2 cm × 2 cm. The cut pieces were soaked at 4°C in phosphate buffered saline (PBS). After three hrs, the skin samples were placed in 60°C distilled water for 1 min. Finally, the epidermal layer was peeled.

Franz diffusion cell

The permeability of the skin was investigated using Franz diffusion cell. The procedure is illustrated in Figure 4. The skin sample was used as a membrane between the receptor and the donor compartment. The diffusion area was equal to 1.65 cm^2. The donor compartment was filled with 1 ml of Adenosine solution dissolved in water at a concentration of 3 mg/ml. The receptor compartment with a volume equal to 10 ml was filled with a solution of PBS and ethanol at a ratio of 70:30 (vol/vol). The Franz cell was kept in a water bath at 37°C with constant stirring. In the case of using DMSO in the experiment, DMSO was used as the solvent instead of water in the donor part.

HPLC analysis

The amount of Adenosine that penetrated through the skin was measured by using a high-performance liquid chromatograph (HPLC LC-2010AHT, Shimadzu). The mobile phase consisted of acetonitrile/water=10/90. The flow rate was set to 0.8 ml/min. A column Inertsil ODS-SP 5 µm with dimensions of 150 × 4.6 mm was used as a stationary phase. The column temperature was set to 25°C. Adenosine was detected at 260 nm with a retention time of 3.5 min.

Microplasma treatment

Dielectric barrier discharge was generated by a thin-film electrode Figure 1. Atmospheric argon microplasma was maintained by a voltage of 1.6 kV and a frequency of 4 kHz. A saw-shape function of the voltage was set by a function generator (Tektronix, AFG3102) and amplified by high voltage amplifier (Trek, model 5/80). The voltage and the current waveforms are depicted in Figure 2. The flow of argon was set to 5 L/min by a flow meter (Yamato). The skin

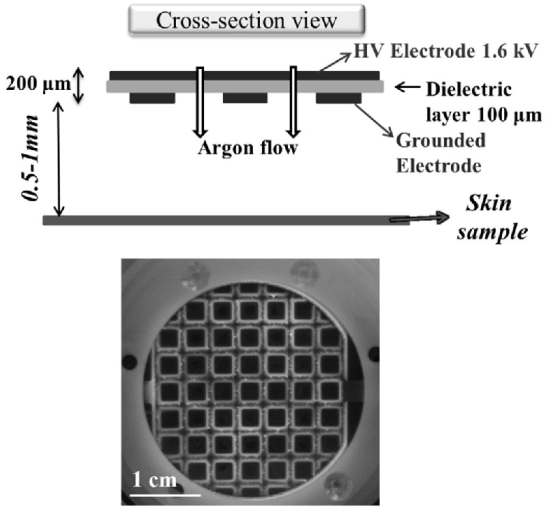

Figure 1. Up: Cross-section the view of the microplasma electrode – the white arrows indicate the flow of argon. Bottom: Surface discharge on the electrode. Argon gas is flowing through the openings (space inside of the dark squares).

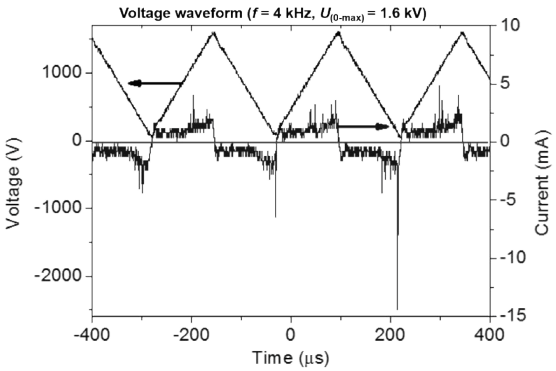

Figure 2. The voltage and the current waveforms during the microplasma treatment.

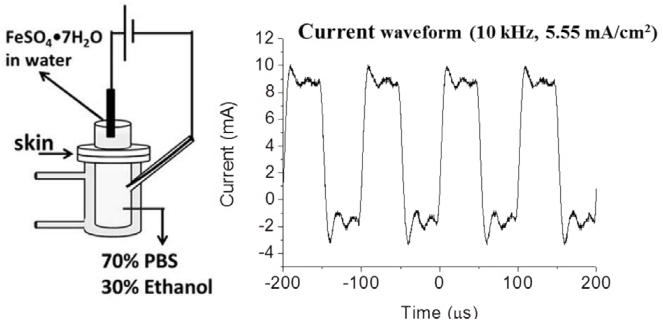

Figure 3. Left: Schematic description of iontophoretic pre-treatment of the skin. Right: The current waveform of the iontophoresis at 10 kHz and the current density 5.55 mA/cm^2.

treatment lasted for 5 minutes. The distance between the electrode and the skin was 0.5 – 1 mm (Figure 1).

Skin used for observation by transmission electron microscope was treated by atmospheric argon microplasma generated at a voltage of 800 V and a sine frequency of 25 kHz with a Neon transformer (ALPHA Neon M-5, LECIP). The gas flow of was set at 5 L/min by a flow meter (Yamato) see details [23].

Iontophoretic $FeSO_4 \cdot 7H_2O$ pre-treatment

The skin was pre-treated by $FeSO_4 \cdot 7H_2O$ in order to incorporate the iron inside the skin. The skin was inserted into Franz diffusion cell. The donor compartment was filled with 1 ml of a solution of $FeSO_4 \cdot 7H_2O$ dissolved in water at a concentration of 30 mg/ml. The receptor compartment with a volume equal to 10 ml was filled with a PBS and ethanol at a ratio of 70:30 (vol/vol). Iontophoretic DC current of 0.5 mA/cm^2 was applied for 15, 30, 60, 120 and 180 min. In the case of the pulsed current, 5.55 mA/cm^2 was applied for 40 min. Franz diffusion cell was kept in a room temperature during the treatment according to Figure 3 (left) and with the waveform in Figure 3 (right) in the case of the pulsed iontophoresis.

Treatment procedures of the skin

Our procedure of the skin treatment could be composed of four steps graphically described by Figure 4. The procedures of the treatments were as follows:

A: The prepared skin sample was pre-treated by the iontophoresis with $FeSO_4 \cdot 7H_2O$ to incorporate the iron into the skin (details in section Iontophoretic $FeSO_4 \cdot 7H_2O$ pre-treatment) and after the pre-treatment, the content of the receptor and the donor parts of Franz diffusion cell were disposed.

B: Excess water on the surface of the skin was removed with paper tissue to remove surface water. The prepared skin samples were treated by argon microplasma discharge for 5 minutes (Details in section Microplasma treatment).

C: A new receptor solution was prepared. The skin was inserted into Franz diffusion cell and 3 mg/ml of Adenosine in water was applied for 24 hrs into the

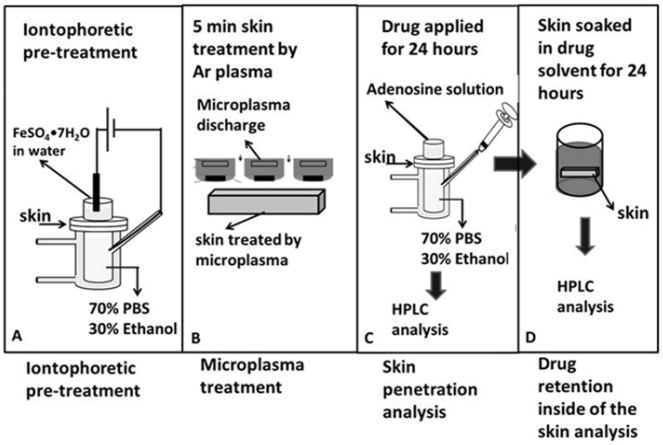

Figure 4. The treatment procedure and analysis for the penetration of Adenosine through the skin and the retention inside the skin with steps **A:** Iontophoretic pre-treatment by $FeSO_4 \cdot 7H_2O$, **B:** Microplasma treatment, **C:** The drug application and the penetration analysis, **D:** The elution of the drug from the skin and the retention analysis.

donor solution. After 24 hrs, the samples from the receptor part of the diffusion cell were analyzed by HPLC to determine the amount of the penetrated drug through the skin.

D: The skin samples from Franz diffusion cells were removed and put into distilled water for 10 minutes to remove the surface drug solution. After 10 minutes, excess water on the surface of the skin was removed using a tissue paper and one layer of stratum corneum was peeled off by one tape strip to remove residual drug on the surface of the skin. Afterwards, the skin was inserted in a solution of a PBS and ethanol for the elution of Adenosine from the skin. The elution was analyzed by HPLC to determine the retention of Adenosine by the skin.

The samples and methods of the skin treatment are denoted as follows: control samples (C), microplasma treated samples (MT), iontophoreticaly pre-reated samples (ITC), iontophoreticaly pre-treated samples followed by microplasma treatment (IMT), DMSO treated sample (DMSO).

Control samples (C): The procedure is characterized by the steps C and D in Figure 4.

Microplasma treated samples (MT): Procedure is characterized by the steps in B, C and D in Figure 4.

Iontophoreticaly pre-treated samples (ITC): The procedure is characterized by the steps in A, C and D in Figure 4. In the step A, the prepared skin sample was treated by the pulsed iontophoresis for 40 min.

Iontophoreticaly pre-treated samples followed by microplasma treatment (IMT): The procedure is characterized by the steps in A, B, C and D in Figure 4. In the step A, the prepared skin sample was treated by the pulsed iontophoresis for 40 min and from 15 min to 180 min by the DC iontophoresis.

DMSO treated samples (DMSO): The procedure is characterized by the steps in C and D in Figure 4. In step C, the prepared skin samples were inserted into Franz diffusion cell and 3 mg/ml of Adenosine in DMSO was applied for 24 hrs.

Transmission electron microscopy (TEM)

An investigation of the microplasma treated skin was observed by Transmission electron microscopy (Jeol Ltd, JEM1200EX). The preparation of the untreated and microplasma treated samples were prepared by the next procedure:

1. Pre-fixation: 2% glutaraldehyde (0.1 M phosphate buffer) at 4°C
2. Washing: 0.1 M phosphate buffer 4°C overnight
3. Post-fixation: 2% osmium solution at 4°C for 3 hrs
4. Washing: distilled water 4°C for 1.5 hrs
5. Pressing fixation: 2% ruthenium tetroxide aqueous solution, 3.6 kg Pressure 4°C for 3 hrs
6. Washing: distilled water 4°C overnight
7. Dehydration: Soaking in sequential high concentration of ethanol (50, 70, 90, 100, 100, 100%) at 4°C (50% only), rest in room temperature, for 15 minutes each
8. Substitution: Propylene oxide, room temperature for 45 min Mixture of propylene oxide + epoxy resin at room temperature for 1.5 hrs
9. Embedding: Epoxy resin (EPON 812) is placed in a capsule together with cured epoxy resin and hardened at 60 ° C for 48 hrs
10. Curing agent: Methyl nadic anhydride (MNA)
11. Curing agent: Dodecenyl Succinic anhydride (DDSA)
12. Accelerator: Dimethylaminomethyl phenol (DMP-30)
13. Slicing: Slicing with ultramicrotome
14. Dyeing: 2% uranyl acetate, stain section with lead staining solution
15. Reinforcement: Carbon deposition (Reinforcement of section relative to electron beam)

Results

The effect of microplasma treatment (MT) and iontophoretic $FeSO_4 \cdot 7H_2O$ pre-treatment (ITC)

Adenosine penetration

When the microplasma treatment (MT) was followed by the application of Adenosine solution, almost 38 µg of Adenosine permeated through the skin. 15 µg of Adenosine permeated after iontophoretic pre-treatment (ITC) and 4.5 µg in control sample(C), (Figure 5). Microplasma treatment (MT) appears to be more effective tool for the transdermal drug delivery than iontophoretic pre-treatment by $FeSO_4 \cdot 7H_2O$ (ITC).

Adenosine retention

Microplasma treatment (MT) did not improve the retention of the drug inside the skin (2.5 µg of Adenosine) and the amount was similar to the control sample (2 µg of Adenosine), (Figure 6). Iontophoresis (ITC) increased Adenosine inside the skin to 6.6 µg.

The effect iontophoretic $FeSO_4 \cdot 7H_2O$ pre-treatment followed by microplasma treatment (ITP) and DMSO treatment (DMSO)

Adenosine retention

Plasma is a medium with reactive species with the ability to oxidize skin lipids

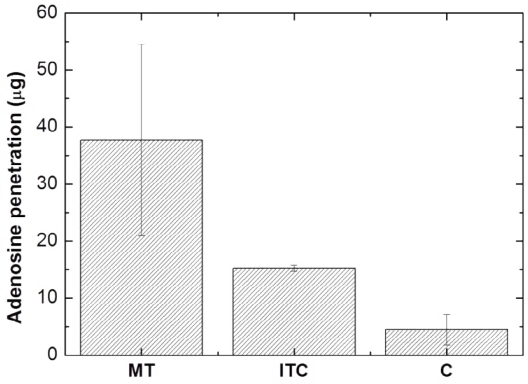

Figure 5. The amount of Adenosine permeated through the skin of control sample (C), sample pre-treated by the $FeSO_4 \cdot 7H_2O$ iontophoresis (ITC) and microplasma treated sample (MT).

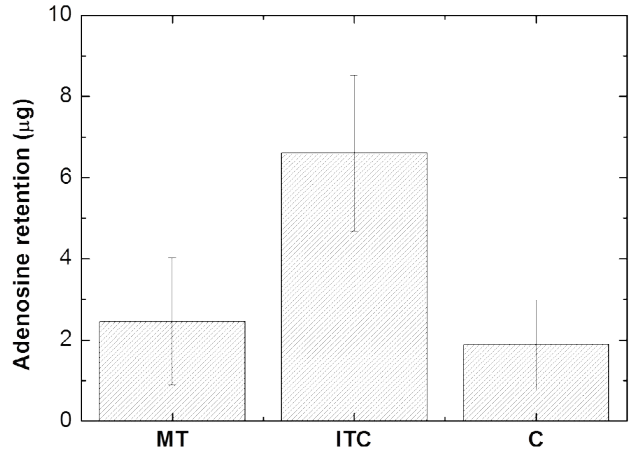

Figure 6. The amount of Adenosine retained inside the skin of control sample (C), the sample pre-treated by $FeSO_4 \cdot 7H_2O$ iontophoresis (ITC) and microplasma treated sample (MT).

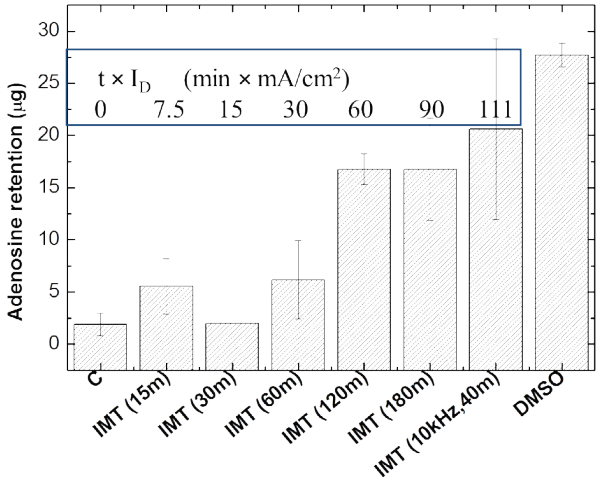

Figure 7. Amount of Adenosine retained inside the skin of control sample (**C**), sample pre-treated by iontophoresis (**IMT**) and DMSO (**DMSO**). Number in the brackets represents time of iontophoretic pre-treatment in minutes.

and weaken the barrier function of the skin. To study the effect of supposed microplasma induced oxidation, increase of the oxidation ability of the microplasma by incorporation of the iron atoms inside the skin was carried out. This iron will serve as a catalyst in the lipid peroxidation. The iron from $FeSO_4 \cdot 7H_2O$ was incorporated into the skin by the iontophoresis. Figure 7 demonstrates the retention of Adenosine inside the skin for different types of skin treatments. Every treatment is characterized by the product of $t \times I_D$, the pre-treatment time "t" of the iontophoresis in minutes and the current density "I_D" in mA/cm^2 to be possible to compare the continuous and the pulsed iontophoresis. In the case of the pulsed iontophoresis, the pre-treatment time was calculated as the sum of the delays of all the positive pulses during the treatment.

When the skin was pre-treated by iontophoresis of $FeSO_4 \cdot 7H_2O$ solution from 15 to 60 minutes ($t \times I_D$ values 7.5-30) followed by microplasma treatment and the drug application (IMT samples of Figure 7), there was no significant increase of the Adenosine observed inside the skin (close to 5 μg). The amount of the retained Adenosine significantly increased to a value 17 μg after 120, respective 180 minutes of iontophoresis of the $FeSO_4 \cdot 7H_2O$ solution ($t \times I_D$ values 60 – 90).

The limitation of the DC iontophoresis is the amplitude of the current which can be safely used for human therapy. The pulsed iontophoresis at frequency higher than 10 kHz allows using higher currents and thus reduces the treatment time. Pulsed iontophoresis was applied for 40 minutes (20 minutes of the active time -50:50 = ON: OFF ratio, $t \times I_D$ value 111) what was almost an equivalent to the direct current iontophoresis applied for 3 hrs. In the case of pulsed iontophoresis, close to 20 μg of Adenosine was retained inside the skin.

If we compare Adenosine retention in the case of microplasma treatment (MT), iontopohretic treatment with $FeSO_4 \cdot 7H_2O$ (ITC) and microplasma treatment with iontopohretic $FeSO_4 \cdot 7H_2O$ pre-treatment (IMT) in Figures 6 and 7, it is visible that the increase of the retained Adenosine could not be caused by microplasma (as the amount of the drug is low comparable to the control skin), neither by iontophoretic pre-treatment (which caused a very low increase to 6.6 μg) but by combination of microplasma treatment and iontophoretic pre-treatment. Iontophoretic pre-treatment with $FeSO_4 \cdot 7H_2O$ followed by microplasma treatment involves/amplifies another process that could initiate the lipid oxidation by microplasma.

When the chemical enhancer DMSO was used for dissolving Adenosine, the retained amount of the drug reached value of 26 μg.

Adenosine penetration

Figure 8 demonstrates the penetration of Adenosine through the skin for different types of the skin treatments. Comparison between control sample and the samples with iontophoretic pre-treatment followed by plasma treatment (IMT) shows that the amount of the penetrated drug increased from 5 μg (control sample) to 15 μg (3 hrs of DC iontophoretic or 40 min pulsed iontophoretic pre-treatment) with the time of iontophoretic pre-treatment. To analyze the effect of plasma, we compared pulsed iontophoresis of $FeSO_4 \cdot 7H_2O$ solution with and without plasma treatment prior to the application of the drug solution (Figures 6 and 8). The results demonstrated that the plasma treatment had no effect in this case. The increase of the penetrated Adenosine was due to the increase in treatment time or the current density of iontophoretic pre-treatment with $FeSO_4 \cdot 7H_2O$, as the values of with (ITC, Figure 6) and without (IMT (10 kHz, 40 min), Figure 8) followed by plasma treatment were comparable. The amount of the penetrated Adenosine after using DMSO enhancer was the same as that of control samples.

The skin cross section observation by Transmission emission microscopy (TEM)

Transmission emission microscopy allows observing a lamellar structure of the lipid matrix. This matrix can be observed as bright lines with a dark space in between. The bright lines represent a lipid tails and the dark spaces represent a hydrophilic head [24]. Figure 9 displays control sample (C) with an unaltered lipid lamella (Figure 9 A and 9B). Figure 9 C depicts the unaltered lipid lamellae between two cornedosmosomes denoted as "d". Corneodesmosomes appears as a dark space with bright lines between corneodesmosome and corneocytes.

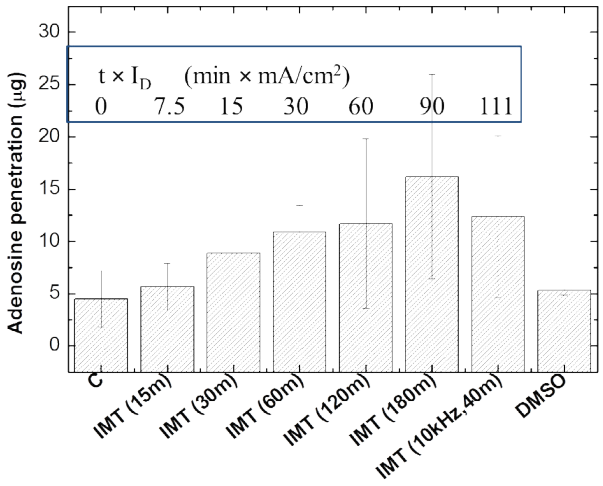

Figure 8. The amount of Adenosine penetrated through the skin of the control sample (**C**), the sample pre-treated by iontophoresis (**IMT**) and DMSO (**DMSO**). The number in the brackets represents time of the iontophoretic pre-treatment in minutes.

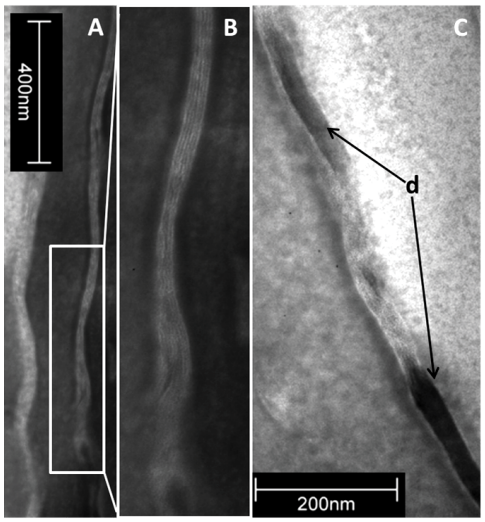

Figure 9. Display of control sample (**C**) **A:** the unaltered lipid lamellae, **B:** the detail of photo in A, **C:** the unaltered lipid lamellae between a two cornedosmosomes denoted as "**d**".

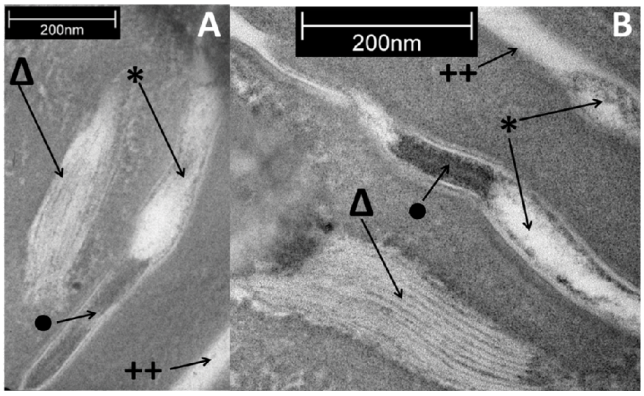

Figure 10. Display of microplasma treated sample (MT) in both A and B. "**Δ**" denotes the unaltered lipid lamellae, **"*"** denotes an unordered phase. "**++**" denotes the lipid lamellae with homogenous structure "**●**" can be corneodesmome with some bright structures.

Figure 10 A and 10B displays microplasma treated samples (MT). The symbol "Δ" denotes the unaltered lipid lamellae. The symbol "++" denotes the lipid lamellae with homogenous structure without alternating black and white space. The unordered phase is denoted as a **"*"**. The unordered phase was clearly separating from the cell walls on the sides, leaving an empty space there. The black area denoted as a "●" can be corneodesmome with bright structures.

Discussion

The skin cross section observation

Previous research has shown some variability of the lipid lamellae with an age, localization and a treatment method [25]. Usually, stratum corneum lipids of the upper part have lower ordering; higher number of the intercellular lipid lamellae or stratum corneum intercellular space is very often filled with an amorphous lipid material [25]. However, the skin of young individuals with no visible skin dryness contents good even lamellae structure regardless localization in stratum corneum. On the other hand, upper parts of the older skin do not contain even lipid bilayer structures in dry or moist condition but rather the non-lamellar amorphous/fibrous material [25]. Particles coming from plasma and impinging on the skin surface and the skin lipids can affect their lipid composition and structure [23]. Radicals and ions penetrating water layer of the lipid lamellae can induce creation of another radicals or ions [26]. These radicals can participate on lipid oxidation or interact with corneodesmosomes.

It was shown that if the lipid barrier contents the only one type of lipids, this barrier is very strong [27]. The effect of the lipid barrier (also ordering of the lipid lamellae) deceases with an increase of number of different types of lipids or with a number of unsaturated fatty acids [28]. The skin section of microplasma treated samples content several types of structures such as the homogeneous structure (denoted as a "++"). This structure can be naturally present in the skin as a result of ageing because of oxidation. However, it can be inducing by oxidizing agent such as plasma. Sankhagowit et al. [29] demonstrated that oxidation of a lipid molecule can change the ratio between head length and tail length of lipids of the lipid membrane and lipids loses their lamellar structure. The skin becomes more permeable for drugs.

Disorganized phase (denoted as a **"*"**) indicate very disturbed parts. These structures could be observed near cornedosmosomes or on corneodesmosomes, very often indicating their degradation [30,31]. Degradation of corneodesmosomes allows detachment of corneocytes. This effect also leads to the higher permeability of the skin. As it is seen from TEM observation (Figure 10), microplasma treatment can cause changes of the lipid bilayer. How exactly these changes influence drug delivery, it has to be object of the future systematic research. In the next sections, influence of different types of treatments on the drug permeability will be discussed.

Microplasma treatment (MT)

As the result shows in Figure 5, microplasma treatment (MT) is very effective in improving transdermal delivery, i.e. the fastest method and the highest amount of the drug was permeated during our study. As it was mentioned above, the reactive species of microplasma can affect lipids and corneodesmosomes. Corneodesmosomes are one type of intercellular junctions. The intercellular junctions between cells form the second barrier for the transdermal drug delivery. A cell adhesion molecule called E-cadherin is essential for the appropriate intercellular junction formation [32].

Choi et al. [16] observed that plasma treatment of the skin inhibits intercellular interactions and causes gaps between the epidermal cells by the dispersion of E-cadherin from the cellular membranes. E-cadherin localization was recovered in three hrs after the plasma treatment and the skin returned to its original state within 24 hrs.

Heartel et al. [33,34] also observed down-regulation of E-cadherin after air plasma treatment of HaCaT cells (cultured human keratinocyte). They observed no difference when they treated the cells in the culture medium or when the culture medium was treated separately, and then they added the medium to the cells. This suggests that the products between interaction of the medium and the plasma played a key role in this down-regulation of E-cadherin. In the case of the skin, water inside the skin can play role of this medium.

Lee et al. [17] compared the plasma effect on E-cadherin regulation with and without a grounded conductive mesh between the treated cells and the plasma electrode. The grounded mesh caught charged particles coming from the plasma and helped to separate their effect from radicals produced by the plasma. The plasma with the grounded conductive mesh (without charged particles) had no effect on regulation of E-cadherin, on the other hand; E-cadherin was down-regulated without the grounded mesh (with charged particles). It means that the charged particles play crucial role in the E-cadherin regulation and in the transdermal drug delivery application of the plasma, while the radicals do not. These researches suggest that the main interaction of the plasma with the skin occurred with intercellular junctions. However, more extensive research is necessary to clarify the mechanism of plasma ions function in the E-cadherin regulation.

Iontophoretic $FeSO_4 \cdot 7H_2O$ pre-treatment (ITC)

Iontophoresis is a non-invasive method of increasing penetration of ions through the skin by applying electric current (using low voltages). Several types of waveforms of the applied current have been investigated [35], and it has been shown that various waveforms have various efficiency on the skin permeability [36]. Iontophoresis is used for ionisable drugs and it is very effective for heavy molecules of weight 7-15 kDa [37, 38]. It can also be used for small molecules such as lidocaine (234 Da) [39]. The main changes in skin after the iontophoresis are an increase in the hydration of stratum corneum,

and a decrease in electrical resistance of the skin [40]. The time required for a recovery of the skin resistance can be influenced by the processes caused by the iontophoretic current inside the skin. The recovery time increases with the time of the current application and also with the current density. The decrease in the resistance depends on the current density but it is independent on the time of the current application [41].

Apart from affecting the drug, hydration and the resistance, Hama et al. [42] demonstrated that the skin barrier can be controlled by an electrical current with experiment with charged liposome. This treatment induced dissociation of the intercellular junctions; such as the tight junctions and the adherent junctions.

Dilation of the tight junctions increased the transportation of drug through a paracellular route of a rat intestinal tissue in the study by Leonard et al. [43]. This effect was reversible and confirmed by TEM and a confocal microscopy observation. Iontophoresis and microplasma treatment may have similar effect on the tight junctions based on studies mentioned above. Moreover, if iontophoresis is applied with the $FeSO_4 \cdot 7H_2O$, some oxidation with oxidants naturally present in the skin can also occur. Despite of the similar effects observed effectivity of iontophoresis was lower than during microplasma treatment (Figure 5). We hypothesize that the slight increase of penetrated Adenosine after iontophoresis could be explained by affecting the intercellular junctions. The slight increase in the retention of Adenosine inside the skin (Figure 6) could be explained by a natural oxidation, based on the description in the section Iontophoretic $FeSO_4 \cdot 7H_2O$ pre-treatment followed by microplasma treatment (IMT).

DMSO treatment (DMSO)

DMSO is a known solvent and a drug delivery enhancer and it needs only 15 min to penetrate stratum corneum [44]. DMSO is able to change a conformation of surrounding proteins from an α-helical to a more reactive β-sheet conformation [44]. Usually, it binds to free water but if there is not sufficient free water, protein-bound water is probably removed from the proteins to bind to DMSO and this effect force the proteins to change the conformation.

DMSO can change a gel phase structures of the skin ceramides to the more permeable liquid-crystalline phase. This behavior is induced by an accumulation of its molecules at the head group region of the ceramides. DMSO is competing with water molecules in creating hydrogen bonds with the ceramides [45]. Moreover, it causes a decrease of the bilayer thickness and an increase in the area per lipid molecule in the ceramide bilayer. The similar effect was observed in a molecular simulation of a phospholipid bilayer containing cholesterol [46]. At low concentration, it induces an expansion and thinning of the lipid bilayer. At higher concentrations, DMSO induces the formation of a transient “water pores” in the cell membrane [47]. The effect of DMSO is strongly concentration dependent and at high concentrations might not be reversible [48]. Using the high concentration of DMSO in our study improved the Adenosine retention inside the skin, not the permeation through the skin (Figures 7 and 8). As DMSO can penetrated through the skin relatively fast, the explanation can be that the diffusion speed of Adenosine in altered lipid matrix is a relatively slow, not allowing the penetration of a significant amount of the drug within 24 hrs. The solution for an increase the flow of the drug through the skin can be ti use a higher concentration of Adenosine to increase the drug gradient across the skin.

Iontophoretic $FeSO_4 \cdot 7H_2O$ pre-treatment followed by microplasma treatment (IMT)

Oxidation of the skin can cause changes that weaken its barrier effect. Molecular oxygen is not reactive enough to start the oxidation, therefore it must be changed to a more reactive state such as hydroxyl radical (OH), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroperoxyl radical ($HO_2\cdot$), lipid peroxyl radical (LOO·), alkoxyl radical (LO·), metastable singlet oxygen (O_2 (a)), or iron-oxygen complexes (ferryl-, perferryl radical) [49]. According to Pham et al. [50], the major source of a lipid peroxide in the skin is squalene and the lipid peroxidation can be enhanced by external factor.

In our research, we planned to investigate the effect of the supposed lipid oxidation of the microplasma treatment on transdermal drug delivery or the drug retention. The oxidation of the lipid membrane also increases water permeability [51]. It is possible that the lipid oxidation by the microplasma can have a minor effect, but it is still present and can affect the lipid barrier. As it is seen from Figure 10, despite of some altered distribution (homogenous structures) of the lipids in the bilayer barrier, there is still possible to see some unaltered areas. However, to maximize the effect of the oxidation, we incorporated the iron ions into the skin by iontophoresis and microplasma as an external source of the radicals. A hydroxyl radical (HO·) can be created by reaction of an iron complex with H_2O_2 or H_2O_2 with O_2^- [49]. HO· reactions are very fast, and they occur near the point of formation. A superoxide radical is very selective and cannot trigger the lipid peroxidation. On the other hand, it can react very fast with NO radical and create peroxinitrite ($ONOO_2$) or peroxynitrous acid (ONOOH), strong oxidative and nitrating agents [52].

A molecular simulation study of a cholesterol and phospholipid peroxidation in the lipid bilayer showed that the phospholipid hydro peroxides tend to bend and the cholesterol hydro peroxides tend to tilt. This leads to a partial degradation of its condensing and ordering properties [53].

Simulation of an oxidized lipids by Boonoy et al. [54] showed that depending on an aldehyde lipid or a peroxide lipids formation, different behavior of the lipid membrane can occur. A high formation of the aldehyde concentrations leads to unstable pores, evolving into micelles. No pores were observed when the peroxide lipids were present. The aldehyde lipids are polar groups and very mobile inside the bilayer allowing the pore formation. The oxidation of the lipids usually occurs near to the lipid heads group and they form hydrogen bonds with water. The area of the lipids depends on the position of the oxidation and the thickness of the lipid bilayer depends on the lipid tails.

Mason et al. [55] observed a reduction of the bilayer width after oxidation by XRD. Moreover, the oxidized skin lipids made the permeation of another reactive oxygen species easier to permeate along the lipid bilayer [56].

Yusupov et al. [57] using MD simulations showed that the reactive oxygen species can penetrate through the water layer and reach the head groups of a phospholipid bilayer, but only OH radicals can react with those head groups (OH, HO_2 and H_2O_2 were considered). They were able to detect two types of reactions:

1. Elimination of a fatty acid chain of Phosphatidylcholine.
2. Oxidation of the lipid side chain creating short fatty acid and residual Phosphatidylcholine bearing an aldehyde function.

The first reaction led to a decrease of the area per lipid, in an increase of the bilayer thickness and the higher ordering of the lipids, i.e. a stronger barrier effect. However, if both reactions occur, it will lead to an increase in the area per lipid, a decrease in the bilayer thickness and lower lipid ordering, i.e. a weaker barrier effect.

Yusupov et al. [58] in another study showed that the oxidized phospholipid layer helps to decrease the time of pore formation if a voltage is applied. This synergic effect was observed when the lipids were formed by aldehydes, not peroxides.

Thus microplasma treatment induced increase in the retained Adenosine inside the skin (Figure 7) similarly to DMSO. As it is seen from previous studies, DMSO and lipid oxidation works on different principles but finally cause very similar effects on the lipid barrier, i.e. the creation of pores and lipid bilayer disruption. However, we cannot increase the concentration of Adenosine as in the case of DMSO because its solubility in water or PBS is limited. The solution could be by using a better solvent that does not affect the lipids. Using DMSO would lead to a competing effect of DMSO and oxidation and it might not lead to an improvement. The comparable value of the retained Adenosine can support an idea of its slow diffusion through the disrupted lipid matrix caused by DMSO or the lipid oxidation.

The increase in drug penetration in the iontophoretic $FeSO_4 \cdot 7H_2O$ pre-treatment followed by the microplasma treatment (IMT Figure 8) could be attributed to the effect of the iontophoresis as it correlates with the treatment

time and the current density of the iontophoresis. The most probably the $FeSO_4 \cdot 7H_2O$ moved the interaction of the plasma particles with the intercellular junction towards the oxidation and the amount of the permeated drug remained the same as in the case of iontophoretic $FeSO_4 \cdot 7H_2O$ pre-treatment (ITC) (Figure 5). This leads to the speculation that the permeability of the skin after affecting the intercellular junctions can be higher than after affecting the lipids in the lipid matrix.

Conclusion

We investigated five procedures of the skin treatment, Control (C), Microplasma treated (MT), Iontophoreticaly pre-treated (ITC), Iontophoreticaly pre-treated followed by microplasma treatment (IMT), DMSO treatment (DMSO). We hypothesize that microplasma treatment (MT) and iontophoresis (ITC) can weaken the intercellular junctions and this could lead to a relatively fast permeation of Adenosine. On the other hand, the supposed oxidation of the lipids (IMT) or DMSO treatment can lead to the lipid disruption and creation of pores which led to a relatively slow permeation of Adenosine across the skin and the dominant amount of the drug remained inside the skin after 24 hrs. However, this theory has to be verified the other experiments. It has to be also highlighted that future experiments have to confirm if incorporation of $FeSO_4 \cdot 7H_2O$ by iontophoresis followed by microplasma treatment caused lipid oxidation, as the oxidation was only supposed in this paper. Microplasma treatment (MT) led to the highest permeation of Adenosine after 24 hrs equal to 38 µg (4.5 µg in control sample) and only 2.5 µg remained inside the skin, comparable to 2 µg in control sample. Iontophoretical pre-treatment (ITC) permeated 15 µg and 6.6 µg remained inside the skin. DMSO treatment allowed the permeation of 5 µg of Adenosine and 28 µg remained inside the skin. Iontophoreticaly pre-treatment followed by microplasma treatment (IMT) allowed 17 µg to penetrate the skin and 20 µg remained inside the skin at its maximum. The permeated amount in IMT procedure correlated with the time of iontophoretic pre-treatment and the current density. The observation of the skin by TEM showed microplasma treatment effects on the lipid portion and corneodesmosomes.

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