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3D-Printed, Pocket-Size Diffusion Cells for Skin Permeation Investigation †

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Abstract: Here we present a novel, compact 3D-printed diffusion cell as an *in vitro* tool for skin permeation investigation. As proof-of-concept, a diffusion cell for studying the permeation of a model molecule (FITC-dextran, 4 kDa) through explanted mice skin is fabricated and characterized. Good viability of the tissue up to 24 h incubation in the cell is demonstrated via MTT assays. The real-time diffusion of the molecule by means of fluorescence microscopy allowed the determination of its diffusivity through the skin (~$2 \cdot 10^{-10}$ m^2/s). Our results open the door for the real-time, high-throughput and cost-effective investigation of skin in any labs.

Keywords: transdermal drug-delivery; molecule diffusivity; bottom-up approach; lab-on-chip

1. Introduction

The high-throughput and cost-effective *in vitro* assay of skin permeation is crucial for several applications as important as pharmaceutics, toxicology, nanomedicine and cosmetics [1–3]. Traditional *in vitro* methods for cutaneous permeation analysis are based on the permeability of a test substance across human or animal skin via static diffusion cells (Franz cells) [4]. In this set-up, a skin membrane is placed between two glass compartments, namely the donor and the receptor, and the concentration profile of the compound under test is obtained by sampling the receptor solution at predetermined time-steps [5,6]. Though widely used, the large volume of the Franz cells (5–10 mL) and the time-consuming procedures necessary to obtain transport parameters (e.g., diffusivity) limit the chances for the high-throughput and cost-effective skin permeation assay.

To overcome these issues, we present a 3D-printed, pocket-size diffusion cell (3D-PDC) as an *in vitro* tool for skin permeation analysis. As depicted in Figure 1A, the skin is magnetically clamped between two elements: the top cover presents a donor chamber (350 μL), while the bottom receptor chamber (500 μL) integrates two microchannels and a glass cover for the coupling of the 3D-PDC with optical detection systems. Drugs and nanotherapeutics can be directly administrated from the top aperture, while the microchannels allow for nutrient delivery and retrieval of metabolites or diffused substances (Figure 1B). Remarkably, the pocket-size of the 3D-PDC, not only reduce reagents consumption and waste production, but also support the coupling with external instruments for the control of the environment (e.g.,

humidity, CO_2) and the real-time quantification of the cumulative amount vs. time of the compound of interest. Here we exploited this versatility by connecting our device with an inverted fluorescence microscope to study the permeation of a model molecule (FITC-dextran, 4 kDa) through two membrane models (healthy and microneedle-treated mice skins).

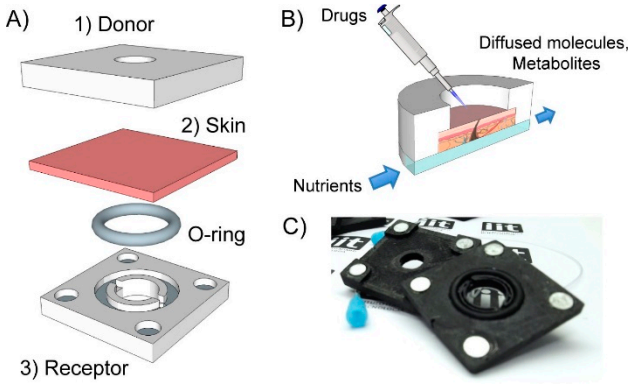

Figure 1. (**A**) Schematic of the 3D-PDC that consists of three parts: (1) the receptor chamber, integrating two channels and a glass window; (2) the skin tissue and (3) the donor chamber. An O-ring inserted under the skin seals the receptor and prevents leakage of liquids. Both components integrate 4 magnets to ease the assembling procedure. (**B**) 3D-PDC operational principle. Drug delivery systems (liquids, creams) can be directly applied to the epidermis through the donor. Two microchannels allow sampling of the receptor solution. (**C**) Photograph of the donor and receptor parts (3D-PDC size 1 cm × 3.5 cm × 3.5 cm).

2. Materials and Methods

2.1. Fabrication of the 3D-PDC

Diffusion cells were fabricated by fused deposition molding (FDM) of poly-lactic acid (PLA). A commercial 3D-printer (CubePro, 3D Systems with a resolution of 100 μm) was used to extrude PLA filament through a heated nozzle (T = 220 °C). The 3D-PDC was built layer-by-layer by moving the nozzle in the XYZ-plane according to a G-code. Finally, an optically transparent cover and 4 magnets completed the device.

2.2. Skin Tissue Preparation

All experiments with animals were carried out in accordance with the guidelines of the European Communities Council (Directive 2010/63/EU) and the Italian Ministry of Health. 8–12 weeks old male CD1 mice (Charles River, Italy) were sacrificed, the back of the animal was shaved, and the full thickness skin was isolated. For *in vitro* viability test we employed fresh skin, whereas for the diffusion test, the explanted skin was frozen at −20 °C. Before the experiment, the skin was thawed at 37 °C for 30 min and then rinsed in phosphate buffer saline (PBS, 1x) for 30 min. For the microneedle treatment, a commercial roller (250 μm stainless steel needle, Pearl Enterprises LLC) was rolled on the epidermis 6 times in an asterisk-like direction.

2.3. In Vitro Viability Test

Skin survival within the 3D-PDC was evaluated with Thiazolyl blue staining (MTT). Briefly, the skin was clamped between the donor and the receptor chamber, previously filled with culture media +20% Fetal Bovine Serum, and the 3D-PDC was transferred in a sterile incubator (37 °C, 5% CO_2). After 12 and 24 h of incubation, the skin was cut with a 6-mm skin biopsy punch and transferred in 24 well plates with 0.5 mL of serum free media and 5% MTT reagent. After 3.5 h of incubation at 37 °C, each skin piece was fragmented, and formazan was extracted from the tissue by immersion in absolute ethanol. After 2 h of incubation under gentle shaking (dark, room temperature) the absorbance was measured at 570 and 670 nm.

2.4. Diffusivity Analysis

Skin permeation of dextran solutions was studied though time-lapse fluorescence microscopy. A 2-mm-thick mice skin (2.5 cm × 2.5 cm) was inserted in the 3D-PDC with PBS filling the receptor. Afterwards, the 3D-PDC was positioned on a heated chamber (T = 35 °C) and fixed to the XY stage of an inverted microscope (Ti-E, Nikon, Tokyo, Japan). An external control unit (Okolab, Naples, Italy), connected to a CO_2 tank and a heated water bath, provided the correct gas mixture and humidity. Finally, the donor chamber was loaded with 350 µL of a FITC-dextran solution (4 kDa Sigma Aldrich, Milan, Italy, λ_{ex} = 490 nm, λ_{em} = 520 nm) at the concentration of 0.5 mg/mL. A glass cover was applied on top of the donor chamber to avoid evaporation. Time-lapse images, 1 frame every 5 min over 24 h, were collected with a microscope objective (Nikon Plan Apo 10×–0.45 NA) and a CMOS camera (Andor). A FITC filter (λ_{cutoff} = 505 nm) was used to cut off the excitation light and to capture only the fluorescence. The focal plane of the objective was 2.5 mm below the dermis.

3. Discussion

We designed, fabricated and tested a 3D-PDC for *in vitro* skin permeation assays. Specifically, we used FDM to fabricate the donor and receptor compartments of the device. The direct additive-manufacturing nature of this method allowed to rapidly prototype (printing time ~ 1 h) the entire device with minimal cost and, more importantly, to proper tailor the design to operate with skin membranes. As a matter of fact, once assembled, the 3D-PDC maintained the needed pressure to keep the skin stretched and avoid liquid spillage or uncontrolled perfusion during both the static and the flow-through operations. Furthermore, a simple visual inspection of the skin, up to 24 h incubation in the diffusion cell, reveled the absence of possible physical damages.

To gain a deep insight into the integrity and conditions of the skin during its incubation in the 3D-PDC, we performed viability assays. In this experiment, we studied the viability of various regions of the skin in contact with the 3D-PDC parts (Figure 2A). Importantly, we found that the skin in the central region of the receptor chamber remained viable, presenting up to 100% and 70% viability for incubation times of 12 and 24 h, respectively (Figure 2B,C). This result further demonstrates that the skin preserves its integrity and viability over moderately long incubation times in the 3D-PDC, thus ensuring that the data obtained during the diffusion experiments are valid and relevant. Notably, this encouraging result might extend the use of 3D-PDCs as tools for other skin-on-chip applications, such as wound healing, tissue engineering and organ-on-chip.

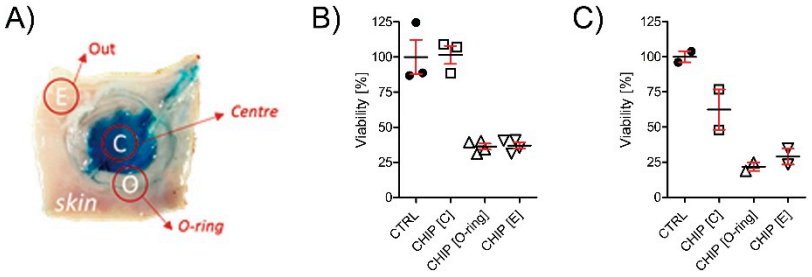

Figure 2. (**A**) Schematic representation of the 3 different skin regions taken into account for the viability evaluation by MTT assay. C, O and E denote the central part of the skin between the chambers, the part of the skin in contact with the O-ring and the outside region delimited by the O-ring, respectively. (**B,C**) Skin viability results after 12 and 24 h incubation in the 3D-PDC, with respect to the control samples.

The 3D-PDC is compatible with a standard fluorescence microscope, as schematically depicted in Figure 3A. This result, which is due to the pocket-size of our design, is key for the real-time detection of the concentration profile of the compound under test. Figure 3B shows

the results of the diffusion of the dextran molecules through the healthy skin membrane, as predicted by our theoretical model and investigated by time-lapse microscopy. The concentration profile is non-linear with an initial plateau up until 12 h. At this time-step the concentration starts to increase indicating that the dextran is accumulating in the receptor chamber. Remarkably, we found that the experimental profile is in really good agreement with the theoretical one that we calculated by solving the Fick's second law for the donor/skin/receptor system. Specifically, we found that the diffusivity of the dextran in the tested skin is about $2 \cdot 10^{-10}$ m^2/s, in agreement with the literature on this subject.

Finally, we extended our investigation to active transdermal drug-delivery (TDD) systems. As a case study, we assessed the functionality of a microneedles-roll as vehicle to enhance the skin diffusivity of FITC-dextran. Figure 3C shows the cumulative amount over time of dextran diffusing in the receptor for both passive and microneedle-assisted diffusion. A significant enhancement (~5 folds) in the dextran diffusion through the treated skin with respect to the untreated one is evident. This is not surprising, considering that microneedles help to cross the epidermis and accelerate the molecular diffusion. Importantly, this result clearly demonstrates that our method can be used to study and quantify the efficiency of a transdermal drug-delivery system.

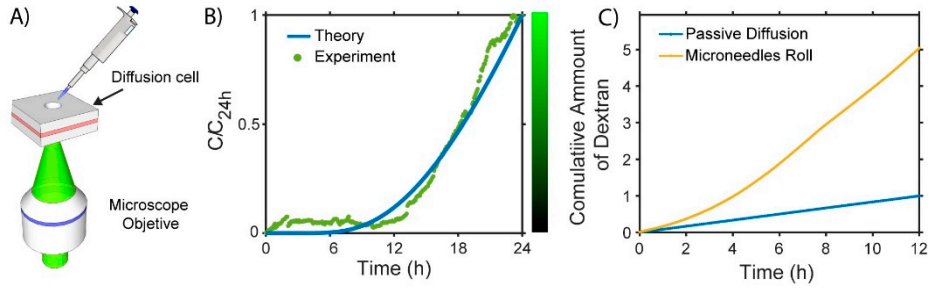

Figure 3. (**A**) Schematic (not in scale) of the experimental setup used for the real-time diffusion experiments. (**B**) Experimental and theoretical normalized FITC-dextran concentration vs. time. C_{24h} denotes the concentration detected at the focal plane of the objective after 24 h. (**C**) Assessment of a microneedle roller as TDD. Cumulative amount vs. time curve of FITC-dextran diffused through the untreated mice skin and the microneedle-treated mice skin. To better highlight the enhancement in diffusivity due to the microneedles' action, both curves were normalized with respect to the cumulative amount of FITC-dextran diffused through the untreated skin after 12 h.

4. Conclusions and Future Perspectives

3D-PDCs are suitable for *in vitro* skin permeation assays and quantitative assessment of TDD systems. Furthermore, the good viability of the incubated skin makes the device promising for applications where the viability of the tissue is an issue. In this regard, optimization of the current design and setup would also allow prolonged and advanced studies, such as tissue barrier diseases and wound healing.

Our compact design also offers the possibility of integrating the 3D-PDC with various detection instruments (e.g., photo-spectrometer), thus widening the potential applications of our method to other emerging fields, such as nanotoxicology, nanotherapeutics and regenerative medicine.

Conflicts of Interest: The authors declare no conflict of interest.

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