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Comparison Between Franz Diffusion Cell and a novel Micro-physiological System for In Vitro Penetration Assay Using Different Skin Models

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ABSTRACT

In vitro diffusive models are an important tool to screen the penetration ability of active ingredients in various formulations. A reliable assessment of skin penetration enhancing properties, mechanism of action of carrier systems, and an estimation of a bioavailability are essential for transdermal delivery. Given the importance of testing the penetration kinetics of different compounds across the skin barrier, several in vitro models have been developedThe aim of this study was to compare the Franz Diffusion Cell (FDC) with a novel fluid-dynamic platform (MIVO) by evaluating penetration ability of caffeine, a widely used reference substance, and LIP1, a testing molecule having the same molecular weight but a different lipophilicity in the two diffusion chamber systems. A 0.7% caffeine or LIP1 formulation in either water or propylene glycol (PG) containing oleic acid (OA) was topically applied on the Strat-M® membrane or pig ear skin, according to the infinite-dose experimental condition (780 ul/cm²). The profile of the penetration kinetics was determined by quantify the amount of molecule absorbed at different time-points (1, 2, 4, 6, 8 hours), by means of HPLC analysis.

Both diffusive systems show a similar trend for caffeine and LIP1 penetration kinetics. The Strat-M® skin model shows a lower barrier function than the pig skin biopsies, whereby the PGOA vehicle exhibits a higher penetration, enhancing the effect for both diffusive chambers and skin surrogates. Most interestingly, MIVO diffusive system better predicts the lipophilic molecules (i.e. LIP1) permeation through highly physiological fluid flows resembled below the skin models.

Introduction

The skin tissue is an effective barrier, representing a fundamental interface between the human body and the external environment. Based on its chemical-physical features, it forms a protective layer against harmful environmental influences such as ultra-violet light, microorganisms, pollutants and environmental toxins, pesticides, or other chemical drugs. Moreover, skin regulates temperature and homeostasis of the body, particularly by limiting the loss of water [1].

Dermal absorption assays are routinely adopted to predict risks from skin exposure to chemicals, but also to demonstrate benefits after topic application of cosmetics, medical device or therapeutic active ingredient. In this context, the Organization for Economic Co-operation and Development (OECD) and the United States Environmental Protection Agency have produced guidelines for the in vitro and in vivo assessment of percutaneous absorption [2], that establishes the passage of compounds across the skin. This process is basically divided in three steps [3]: i) penetration, which consists of the entry of the chemical compound into the stratum corneum, build up by an intracellular lipid matrix of mainly ceramides, cholesterol and free fatty acids [4,5]; ii), permeation , namely the gradual passage of the substance through the subsequent layers, which are both functionally and anatomically distinct from the stratum corneum; iii) the uptake t into lymphatic and blood vessels [5-8]. Interestingly, according to the skin absorption outcome, the classification of these testing compounds may range from cosmetics to "medical devices made of substances", that need to be absorbed in order to achieve their intended action [9-11].

Therefore, there is increasing demand for reliable and reproducible in vitro and ex vivo skin absorption methods that accelerate the chemicals testing and the measurement of their absorption percentage. In the last two decades, the European Union and national legislations have stipulated that animal experiments should be avoided whenever scien-

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tifically feasible, in line with the 3Rs (reduce, replace, refine) approach [2]. Furthermore, owing to the difference in skin structure, animal studies do not always reflect the human situation [2]. The estimation of percutaneous absorption of compounds using excised animal skin is widely accepted for the toxicological risk assessment [12]. Porcine skin has been re-cognized as an appropriate tissue for prediction of human skin permeability for some years, despite the lower barrier function of this tissue compared with human skin [13-17].

Recently, numerous skin surrogate systems and human skin equivalents (HSEs) have been developed to study skin penetration to overpass limits of animal sources. HSEs are typically constructed by culturing human keratinocytes on appropriate substrates [18], providing a good-quality control of the system and species relevance. Consequently, some reconstructed tissues (i.e. Episkin and EpiDerm) have already been validated under specific applications, such as for skin irritation [19,20]. In general, they have proved a lower barrier function than human skin [18,21,22], but for hydrophilic compounds (i.e. ethyl ascorbic acid [23] and caffeine [21]) some HSEs (i.e. LabSkin, EpiDerm, EpiSkin, SkinEthic) represent a valuable option to carry out transdermal delivery investigations, even though some regulation guidelines still have to be updated [10,24].

Artificial membranes have been also fabricated and employed as synthetic skin alternatives [25]. Non-lipid- and lipid-based membranes are cost-effective and reproducible tools to study the underlying physicochemical mechanisms of the passive drug diffusion, [24,26]. Moreover, being non-biological models, these skin models may support highthroughput screenings, without lot-to-lot variability, safety and storage limitations [4].

The parallel artificial membrane permeability assay (PAMPA) may be represent an alternative to simulate dermal absorption of some compounds [25,27,28], whose results displayed a good correlation with . full-thickness skin (R^2 >0.7).

The Strat-M[®] membrane is an animal free, multiple layer polyether sulfone membrane, which is coated with skin lipids (e.g., ceramides, cholesterol, and free fatty acids), especially designed to mimicking the skin structure for transdermal diffusion testing . The hydrophobic lipidic structure, which is coated on the membrane is composed of the main stratum corneum lipids, paired with the polyether sulfone membrane cut-off this membrane mimics a skin penetration more closely than other membranes, which are limited only on their cut-off definition [29]. These chemico-physical porperties make Strat-M[®] membrane a good skin model alternative to evaluate penetration flux and permeation of molecules, under infinite and finite dosing conditions [30]. However, due to their lower barrier function, artificial membranes typicaly lead to a higher penetration ability and penetration rate [12,13,31].

For ex vivo skin penetration studies the OECD guideline recommends pig ear skin as suitable skin surrogate to mimic human percutaneous penetration [24,32]. Pig ear skin shows similarities in morphology as well as penetration abilities and corresponds to that of human skin [33].Typically, the passive diffusion of active ingredients is tested by culturing surrogate skin in a Franz Diffusion Cell (FDC) setup [34]. The FDC system can be either a static or flow-through setup. Although both are compliant with the OECD Test Guidelines 428, static FDC setup remains simpler, lower-cost and more widely used diffusive system [12,24]. It consists of a receptor compartment filled with a physiological buffer solution, in which the compound is released after penetrating through the skin surrogate. Onto this surrogate a finite ($\leq 10\mu L/cm^2$) or infinite ($\geq 10\mu L/cm^2$) formulation dose can be topically applied into the donor compartment and allows the evaluation of penetration kinetics over time [35,36]. Finite dosing represents more closely application and usage condition, whereas an infinite dosing helps to understand and elaborate permeation abilities due to a steady state penetration and a constant high formulation concentration. The penetration kinetics interpretation underlines the predicted bioavailability of the active ingredient, which is important to ensure the efficacy and the exposure to the living cell entity and a targeted drug delivery. The hydrophilic alkaloid caffeine is recommended as a model compound by the OECD guideline for in vivo, ex vivo, and in vitro percutaneous penetration testing due to its well-known penetration behaviour [32]. To optimize and ensure targeted delivery of ingredients into the skin, the formulation needs to be designed individually [37]. Most ingredients are formulated into a standard formulation (vehicle) containing water, glycols and fatty acids for penetration testing based on their physico-chemical properties and solubility characteristics [38]. Thereby, propylene glycol (PG) is the most used glycol and is often combined with other penetration enhancers like oleic acid (OA) to test ingredient penetration [39].

In this work, we compared the FDC with a novel ready-to-use, compartmental technology , named MIVO® - Multi In Vitro Organ device, compliant with the OECD 428 guideline's definition of a diffusion cell and able to properly resemble the mono-directional physiological capillary-like flow below the tissue. MIVO has been already adopted to carry out diffusion studies in gut absorption [40], tumor cells intravasation [41] and cancer drug efficacy testing [42].

Here, molecules having different lipophilicity (expressed as log p values) although same molecular weight (i.e. caffeine and LIP1, acronym for 1,3-Benzodioxol-5-ylmethylurea lipophilic molecule) have been adopted as testing molecules for skin permeation assays through two human surrogate models, the Strat-M® membrane and the pig ear skin, carried out employing both FDC, as it is the standard in vitro method for this type of study, and MIVO®. Also, computational fluid-dynamic (CFD) simulations have been performed to inspect the flow field beneath the skin in the receptor of both diffusion cells considered.

Materials and method

Diffusive chambers

MIVO® system

The MIVO® device is a disposable cell culture chamber able to host living tissues (e.g. cellular monolayers, 3D reconstructed tissues, tissue biopsies) or artificial membranes under physiological conditions, providing a multiple fluidic circulation that mimics the human circulatory system with the vascularization of the tissue of interest. The diffusion cell designed and implemented in this work is schematically represented in Figure 1, panel A, showing features in compliance with the OECD 428 rule for in vitro skin absorption method. Specifically, the human skin surrogate separates the donor compartment from the receiver one, exposing a surface area of 0.43 cm² suitable for drug administration. The receiver compartment has been designed to be connected to a peristaltic pump inducing a monodirectional flow: then, a capillary velocity can be set up below the skin barrier, emulating the real physiological conditions. A three-way valve placed in the fluidic circuit allows the sampling of the media over time, without affecting the sterility environment and the tissue. The whole system is hosted within the cell culture incubator with controlled atmosphere (i.e., 32°C, 5% CO2).

Strat-M® membranes and pig skin biopsy discs were placed within MIVO®; an add-on equipped with a biocompatible o-ring avoiding any fluid leakage blocks the skin models inside the MIVO® chamber. The donor compartment was filled with a volume of caffeine formulations according to the infinite-dose experimental condition (780uL/cm²), and the receiver one was filled with 2.3 mL of calcium and magnesium enriched phosphate buffered saline (PBS), at a flow rate of 2 mL/min in order to have below the skin surrogate a mean flow velocity (of 0.1 cm/s), resembling the capillary flow [42,43]. The experiment with Strat-M® was performed in triplicate, while the one with pig ear skin was performed with 6 replicates. For both skin models, 400 μ L (or 20%) of the circulating solution in the receiver compartment were collected at different time-points (1, 2, 4, 6, 8 hours), in order to assess the quantity of caffeine/LIP1 accumulating into the receiver chamber over time. The samples were filtered through a cellulose acetate membrane filter (0.22 μ m pore size) and analyzed through High-Performance Liquid Chromatography (HPLC).



Figure 1. Diffusive chambers. Schematic representation of the diffusive chambers where the tissue (Strat-M® or pig skin) is placed for performing dynamic absorption studies. Panel A: the MIVO® fluidic chamber; Panel B: Franz Diffusion Cell.

l'able 1		
Technical featur	es of MIVO and FDC.	

Diffusive chamber	MIVO	FDC
Flow regimes	Laminar, monodirectional, spatially homogeneous	Rotational, slight turbulent, spatially non homogeneous
Reynolds number	~20 < 2000	~1700 < 2000
Driving force	Peristaltic pump	Stirring bar
Media Sampling system	Through a three-way valve	Through the sampling port
Receptor Media volume	Flexible, ranging from 2.5 to 5 ml	From 5 to 20 ml according to the model
	Cells monolayer on inserts, membranes (eg StratM, Permeapad), 3D	Membranes, biopsies
Skin models	reconstructed skin tissues (e.g. epiDerm, epiSkin SkinEthic,	
	Labskin), biopsies	
Skin clamping	Auto-centering and blocking of inserts, add-on with o-ring for	Metallic clamps
	biopsies	
Sterilization	Already sterile and ready to be used	Autoclavable, after a pre-clean/wash

FDC system

. .

The FDC system consists of a receiver compartment filled with 5 mL PBS, in which the compound is released after penetrating through the skin surrogate. Onto this surrogate the infinite-dose experimental condition (780μ L/cm²) is topical applied into the donor compartment and allows the evaluation of penetration kinetics over time [33-35,44]. Figure 1B shows a schematic illustration of the FDC with its magnetic stirrer and its thermostatically controlled water bath, to maintain a controlled temperature of 32°C.

The Strat-M[®] membranes and pig skin biopsy discs as human skin surrogates were placed onto the FDC (Ø 9 mm, diffusion area 0.64 cm²) from Logan Instruments Corp. (Somerset, USA). The Strat-M[®] membrane was placed shiny side up and the pig skin dermal side down onto the receiver compartment. The experiment with the Strat-M[®] membrane was performed with 6 replicates, while the one with pig ear skin was performed with 8 replicates. After an experimental equilibration for 30 min, different exposure times (1, 2, 4, 6, 8 hours) were adopted and 1000 μ L (or 20%) of the receiver compartment were collected and filtered through a cellulose acetate membrane filter (0.22 μ m pore size). The solution was HPLC analyzed and replaced with fresh PBS. A summary of the main features of MIVO and FDC was shown in Table 1.

Chemicals

Caffeine, 1,3-Benzodioxol-5-ylmethylurea (LIP1), Milli-Q® (water), acetonitrile, were purchased from Merck KGaA (Darmstadt, Germany). Propylene glycol (PG), Oleic acid (OA), and calcium and magnesium enriched PBS solution were purchased from Sigma Aldrich by Merck KGaA (St. Louis, USA). Formulations containing either water or PGOA (95:5) with either 0.7% caffeine (w/w) or LIP1 were prepared.

Caffeine and LIP1 were selected as reference molecules having same molecular weight and different lipophilicity (Table 2).

Skin models: Strat-M® and pig skin biopsies

The Strat-M® membrane purchased from Merck KGaA (Darmstadt, Germany) is a non-animal based synthetic membrane build up by multiple layers of polyether sulfones coated with skin lipids, which mimics the penetration ability of human skin (Figure 2). This lipid coating, which mimics the intercellular lipid matrix of the human stratum corneum, contains a combination of ceramides, cholesterol, free fatty acids, and other components with a similar specific lipid ratio to human skin surface [4].

Pig ear skin was used as a human skin surrogate for penetration testing. The pig ears (German domestic pigs, 6-month-old) were obtained from a local slaughterhouse (Brensbach, Germany). Freshly slaughtered, they were cleaned with water, dried using soft tissue and stored at $+4^{\circ}$ C. The skin from the back of the pig ear was dermatomed with an electrical dermatome from Humeca BV (Borne, Netherlands). 500 µm thick splitskin punches with a diameter of 26 mm were obtained from each ear and stored for the maximum of 6 month at -20°C.

HPLC

The quantitative concentration of caffeine and LIP1 was determined using a HPLC (VWR-Hitachi ELITE LaChrom system) system. A Chromolith® Performance RP-18e 100-4.6 mm (Merck KGaA, Darmstadt) column was used as the stationary phase at 30°C and at flow rate of 2.0

Table 2

Physico-chemical properties of caffeine and LIP1.

1 - 2 mm

2 - 25 mm

Dermis

Subcutis

~200 µm



Figure 2. Strat-M® skin model. Schematic illustration of the skin morphology and the multilayered structure of the Strat-M® membrane to compare the different skin layers with a scanning electron microscopic image of a Strat-M® cross-section.

mL/min, with an isocratic mobile phase of 90% water and 10% acetonitrile (HPLC gradient grade, Merck KGaA, Darmstadt) for caffeine and a mobile phase of 80:20 for LIP1. The caffeine amount was determined at a detection wavelength of 272 nm, and LIP1 at 285 nm, using a DAD l-2450 detection unit. Prior the analysis, the samples were mixed in auto sampler screw vials and analyzed with an injection volume of 60 μ L of each sample (n=4). The specificity of the HPLC run was controlled via a blank injection and an internal standard solution. The quantification linearity was confirmed by a six-point calibration series (0.5-250.0 μ g/mL) with linear regression confirmation of R²> 0.99 in all cases. Accuracy and precision of the HPLC run was determined within the internal acceptance criteria variation <2 %.

Statistical analysis

Calculations were made using Microsoft® Excel® Office 365 and statistical analysis were performed using GraphPad Prism 8.03. All data sets are shown as the mean \pm standard deviation (SD) with statistically significant differences determined by t-test (the Bonferroni method for multiple comparisons was also applied) with probability (p) values $<\!0.05.$

100 µm

Results

Caffeine penetration kinetics using Strat-M®

Layer of Polyolefin

"Subcutis'

The cumulative amount of caffeine penetrated through Strat-M[®] was derived for the two experimental conditions (PGOA and Milli-Q[®] as donor solutions), showing statistical difference between the two diffusive chambers, although similar trend were observed (Figure 4). The passage of caffeine was enhanced (~10X) when using PGOA as a vehicle (Figure 4A); moreover, the caffeine absorption through the Strat-M[®] membrane shows a linear trend during time in the Milli-Q[®] solution (Figure 4B), while in PGOA an initial plateau was observed after 6 hours.

Caffeine penetration kinetics using pig skin biopsies

The cumulative amount of caffeine penetrated through pig skin tissues was measured for both formulations (PGOA and Milli-Q®), compar-



Figure 3. Pig biopsy vs. Strat-M®. Image of a 500 µm thick pig split-skin biopsy (on the left) and the Strat-M® membrane (on the right).



Figure 4. Cumulative amount of Caffeine penetrated through the Strat-M[®] membrane. Comparison of the caffeine penetration kinetics through Strat-M[®] membrane in FDC and MIVO[®] systems with PGOA (A) and Milli-Q[®] water (B) as vehicle solution. Values are presented as mean ± SD.

ing the FDC and the MIVO® chambers (Figure 5) and showing statistical difference between the two diffusive systems only in MilliQ® conditions. The PGOA formulation (Figure 5A) led to an increasing amount of caffeine in the receiver chamber over time, despite the resulting penetration (ug/cm²) was overall lower than for Strat-M® (~12X for FDC and ~17X for MIVO®). Likewise, when caffeine was topically applied within the Milli-Q® vehicle (Figure 5B), its penetration over time through the pig skin was lower than the one resulted with Strat-M® membrane.

LIP1 penetration kinetics using Strat-M® and pig skin biopsies

Similarly to the caffeine in PGOA (Fig. 4A and Fig. 5A), the LIP1 in PGOA formulation displayed a gradual increase of its passage through Strat-M and pig skin, with an initial plateau observed at the ending points for Strat-M (Figure 6A) and a linear trend for pig skin (Figure 6B) for both diffusive systems. Like for caffeine, also LIP1 was more absorbed through the Strat-M than the pig skin (~6X for FDC and ~3X for MIVO®).

The cumulative amount of LIP1 was statistically higher in MIVO than in FDC already after 2 hours using Strat-M and 6 hours using pig skin.

Interestingly, values of caffeine in PGOA are much higher (~4X after 8hr) than those of LIP1 using Strat-M in FDC system (Figure 6C), while no significant differences are observed between caffeine and LIP1 using

pig skin in FDC system (Figure 6D). On the other side, as expected for lipophilic molecules applied onto skin tissues, MIVO displays a higher LIP1 permeation (~3X after 8hr) than caffeine using pig skin model (Figure 6D), while artificial Strat-M membrane displayed similar permeation behavior for caffeine and LIP1 (Figure 6C).

A comparison of the caffeine and LIP1 penetration for all implemented experimental conditions after 8 hours, with statistical analysis, was also shown (Figure 7).

Mathematical approach to determine the diffusion coefficient

Permeation of an active ingredient through the skin's stratum corneum is described as diffusion process in which active transport plays no role [45]. Mathematically, skin absorption can be described by Fick's laws of diffusion.

The first Fick's law is specific to an infinite dose condition [45], which is described for experiments with a topical amount higher than $100 \,\mu\text{L/cm}^2$ (or higher than $10 \,\text{mg/cm}^2$):

$$J = -DdC/dx \tag{1}$$

where J is the rate of transfer per unit area (flux) $(g/cm^2/h)$, dC is the concentration gradient (g/cm^3) , dx is the linear distance travelled (cm) and D is the diffusion coefficient (cm^2/h) .



Figure 5. Cumulative amount of Caffeine penetrated through pig skin. Comparison of the caffeine penetration kinetics through pig skin biopsies in FDC and MIVO® systems with PGOA (A) and Milli-Q® water (B) as vehicle solution. Values are presented as mean \pm SD.

Figure 6. Cumulative amount of LIP1 penetrated through the Strat-M[®] membrane and pig skin. Comparison of the LIP1 penetration kinetics through Strat-M[®] membranes (A) and pig skin biopsies (B) with FDC and MIVO[®] and in comparison with Caffeine penetration kinetics through Strat-M[®] membranes (C) and pig skin biopsies (D). Values are presented as mean \pm SD, the values obtained by using the two systems are compared using paired t-Test statistics (p < 0.05).

Thereby a steady-state flux, J_{ss} , is commonly assessed in vitro and ex vivo in diffusion cells (e.g., FDC and MIVO®), consisting of a donor compartment separated from the receiver compartment by a human skin surrogate. The ingredient is applied to the stratum corneum side of the skin, and an accumulation of active ingredient in the receiver compartment is monitored by repeated concentration measurements in the receiver medium over time.

to be constant. Therefore, a plot of the permeated mass per unit area versus unit time, yields a linear function with a slope which represents the steady-state flux.

The apparent permeation coefficient, P_{app} , which represents an independent measure of the skin resistance against permeation of the examined active ingredient, is frequently calculated as:

Under infinite dose conditions, dC can be replaced by the known donor concentration,
$$c_D$$
, and the permeated mass per time is assumed

$$P_{app} = J_{ss} / c_D \tag{2}$$

Figure 7. Caffeine and LIP1 permeation in all the experimental conditions. Quantity of caffeine penetrated after 8 hours through Strat-M[®] (A) and pig skin (B) with FDC and MIVO[®] and quantity of LIP1 through Strat-M[®] (C) and pig skin (D) with FDC and MIVO[®]. Values are presented as mean \pm SD, the values obtained by using the two systems are compared using paired t-Test statistics (p < 0.05).

Table 3

The table summarizes the values of the steady-state flux and the apparent permeation coefficient for all the experimental conditions.

		Skin Model			
		Strat-M®		Pig Skin	
		FDC	MIVO®	FDC	MIVO®
LIP1 in PGOA	J_{ss} (µg/cm ² /h)	83.59	123.13	14,53	31.97
	P_{app} (cm/s X 10 ⁻⁶)	3.32	4.88	0,63	1.27
Caffeine in PGOA	J_{ss} (µg/cm ² /h)	268.18	216.01	17.2	10.05
	P_{ann} (cm/s X 10 ⁻⁶)	10.64	8.57	0.68	0.40
Caffeine in Milli-Q®	$J_{ss}^{upp}(\mu g/cm^2/h)$	20.94	16.51	2.54	4.75
	P_{app} (cm/s X 10 ⁻⁶)	0.83	0.65	0.1	0.19

because this value only depends on the donor concentration used in the given experiment.

 J_{ss} and P_{app} were derived for the two skin models and the caffeine/LIP1 vehicle from the linear part of the respective absorption curves by linear regression, by using an automated approach [46], if at least four data points are within the linear part of a curve ($R^2 > 0.92$).

The time to achieve steady-state conditions, under infinite dose conditions is referred as lag time (t) and the preceding period is the lagphase. Lag time is a function of the active ingredient loading the stratum corneum and dermis, diffusivity, and thickness of the skin. Lag time is the time required for the diffusion flow to become stable. By using the Lag time calculation [47], the diffusion coefficient can be derived:

$$t = x^2/6D.$$
(3)

Table 3 and Table 4 report respectively the steady state flux and the percentages of caffeine and LIP1 absorbed after 8 hours under different conditions.

Table 4

Percentage values of penetrated caffeine and LIP1 through Strat-M® membranes and pig skin biopsies in all the experimental conditions.

% of penetrated through the skin barrier						
	Skin Mode	l				
	Strat-M®		Pig Skin			
	FDC	MIVO®	FDC	MIVO®		
LIP1 in PGOA Caffeine in PGOA Caffeine in Milli-Q®	11.6% 36.2% 3.2%	17.6% 28.6% 2.6%	2.5% 3.1% 0.4%	5.4% 1.7% 0.8%		

Computational fluid dynamic simulation of fluid flow within MIVO and FDC

Fluid dynamic simulations were performed both in MIVO and in FDC environments to simulate the fluid flow profiles, mean velocities

Figure 8. Computational Fluid-dynamic simulations of FDC and MIVO. Velocities distribution at the plane below the skin within FDC and MIVO (panel A). Velocity profiles at the cut line, compared to the physiological values, within FDC and MIVO (panel B).

and shear stresses below the skin model (Figure 8). The 3D domain was drawn based on the dimensions of the two diffusive chambers used for skin permeation assays. The physical outputs were modeled using Comsol Multiphysics 5.6 (Laminar Fluid Flow module). The fluid was supposed to be laminar, incompressible, and not turbulent. The velocity profiles were calculated according to Navier-Stokes and the continuity equation [48,49]. In the MIVO environment, the flow rate was set to Q=2 mL/min to generate velocity resembling the capillary blood flow, while in the FDC the flow was generated by a stirring bar (length of 7 mm, rotational frequency 10Hz). An iterative geometric multigrid (GM-RES) algorithm was used to solve the equations. A no-slip condition was fixed on the boundary of the geometry.

Discussion

In the fields of pharmaceutical industry, reliable skin penetration data of active ingredients are indispensable, since it affects the bioavailability, defined as the amount of molecules that reaches the systemic circulation [50]. Various studies have considered the impact of different physico-chemical formulations and skin models on permeation assays. Moreover, the use of in vitro diffusion cellss has evolved into a major research methodology, providing key insights towards more reliable, reproducible, and standardized in vitro and ex vivo methods.

In vitro models frequently involve the use of artificial membranes to model realistic human skin penetration features. Although these membranes do not model the cellular mediated phenomena affecting the molecule passage through a living tissue, diffusion studies can be carried out. In particular, artificial membranes may be preferred to skin biopsies as they are more easily resourced, less expensive, structurally simpler and lead to a faster outcome [51]. Another challenging approach is based on in vitro human epidermis and dermis cells models, building human skin equivalent (HSE). Some commercially available HSE like GraftskinTM, SkinEthicTM, LAbskinTM, EpiDerm and Episkin, have been already adopted for penetration assays [52,53] and to understand metabolic skin response [13,31] within exploratory assays, while regulatory bodies are currently involved in guidelines updates., Also artificial membranes, although to a higher penetration ability and penetration rate [12,13,31], are currently adopted to provide useful permeation measurement for multiple formulations with higher throughput [4]. Indeed, they exhibit superior data reproducibility, as in vivo variables such as donor age, sex and anatomical site are excluded [51]. In this context, the Strat-M® represents an interesting option among synthetic skin models, since it is a membrane-based model with diffusion characteristics well-correlated to human skin [4]. However, it does not fully resemble the heterogeneous multi-layer structure of human skin.

For these reasons, an effective alternative is represented by animal skin biopsies as indicated in the OECD TG 428 guidelines [32]. Among these, pig ear skin is currently the most widely used, given its histological similarities to human skin, with a comparable stratum corneum thickness [54,55].

In this work, a comparative study was performed by using both synthetic non-animal-based membrane (i.e., Strat-M®) and pig ear skin biopsies for assessing the penetration of two molecules with similar molecular weight but different lipophilicity: caffeine, a reference OECD 428 substance, and LIP1, a lipophilic test substance. By applying such testing molecules to the skin under infinite dose conditions, we expected that these penetrate into and diffuse through the stratum corneum, depending on their physico-chemical properties. [56,57] Indeed, a continuous increase of the caffeine and LIP1 penetration was observed up to 8 hours in all experimental conditions; in particular, Strat-M® turned out to be more permeable than pig biopsy, as already reported for synthetic membranes, especially for hydrophilic molecules [13]; this i can be due to a different morphological and histological structure of the skin tissue.

Moreover, despite artificial skin membranes do not fully resemble the proper passive route of molecule permeation, a lower variability, and therefore a higher reproducibility, was observed for the Strat-M® model than pig ear biopses, as expected [31]. Although the same anatomical site (i.e., pig ear) was selected for all donors, this high variability is intrinsic to the model because of the biopsy structure, age and hydration state of the skin that may differ from one donor to another [45].

Transdermal pharmacokinetic studies allow to recognize the fate of the new formulations/drugs applied to the skin, to evaluate what fraction of the applied doses have been effectively absorbed, and also to determine the bioequivalence of the generic products [58]. To better resemble the in vivo situation, where systemic circulation rapidly clears permeants , diffusion chambers hosting skin models have been widely adopted as more reliable alternative to the static condition [59]. Among these, FDC systems are used since many years, under the two available configurations: static and flow-through [34]. In both systems the receptor fluid is stirred in a non-physiological way, but the pivotal difference between the two configurations is the continuous fluid replacement in the flow-through type [10,24]. This further implementation makes the flow-through system more reliable than the static counterpart, mimicking the supply of fresh fluid media as it happens in vivo thanks to the continuous tissues vascularization. On the other side, flow-through cells are labor-intensive, introducing an additional lag time and augment experimental costs (for additional pumping equipment and larger volume of fresh media), making the use of the static device preferable and more widely diffused among testing laboratories [12,24]. Furthermore, it becomes fundamental that the fluid-dynamic environment implemented within the diffusion cell could be highly reliable and biomimetic in terms of flow velocities and mass transports profiles, that affect the diffusion kinetics of the testing ingredient [42].

For these reasons, MIVO® system has been recently employed as alternative diffusion cell, due to its ability in reproducing physiologically relevant flow culture conditions. MIVO®, as FDC systems, consist of inert non-adsorbing material with receptor compartment volumes of 2 - 10 mL and surface areas of exposed membranes of about 0.2 - 4 cm², according to the OECD 428 guidelines. The fluid flow is imposed within MIVO through the adoption of a peristaltic pump, which allows to finely regulate the flow rate, the flow direction and the velocity profile below the hosting tissue. In particular, based on the inner design of the receptor chamber, by setting an unidirectional flow rate of 2 mL/min it is possible to reproduce values within the capillary blood velocity range, providing a micro-physiological tool for pharmacokinetic and pharmacodynamic studies with high predictability and reproducibility outcome, as already reported for gut absorption assay [60] and drug efficacy assays [42].

As evidence of this, CFD simulations of velocity profiles below skin have been thus performed for both FDC and MIVO. A spatially homogeneous velocity profile consistent with the capillary blood flow was observed below the skin cultured within MIVO, whereas a rotational not physiological profile was detected within FDC, showing values ranging from the arterial to veins velocities in the outer and inner region, respectively, thus making MIVO a diffusion system more suitable to recapitulate the human blood flow dynamics (Figure 8). As a matter of fact, a laminar fluid flow was noticed within the MIVO apparatus without any vortex formation [42], whereas FDC exhibited a slight turbulence (Reynolds number approximately equal to 1700). In particular, this undesirable vortex, being far from reproducing capillary physiological settings, may lead to inadequate molecule distribution throughout the receptor compartment since it has a potential to disrupt the static fluid layer adjacent to the membrane; such an effect changes one of the assumptions of Fick's law, namely that the calculation of the diffusion coefficient includes a contribution from the boundary layer [61].

Importantly, MIVO® provides a similar permeation trend to FDC system when challenged with the Strat-M® for the caffeine in PGOA as well as the Milli-Q® formulation. According to the skin-related physical features of the Strat-M®, caffeine penetration across the membrane was dependent on the vehicle used : in particular, the PGOA vehicle enhanced the ingredient penetration through both the Strat-M® (~10X for both diffusive chambers) and the pig skin (~8X and ~3X for FDC and MIVO®, respectively).

Interestingly, the positive effect of PGOA was constant in the two diffusion cells where the artificial membrane was adopted, confirming the good reliability of MIVO® for permeationassays. Moreover, the percentage of caffeine permeated through pig skin in MIVO® was in line with the observed values in Schäfer-Korting [62]. However, statistically significant differences were observed between MIVO® and FDC, with higher permeation values measured in FDC. To better investigate these differences, a different molecule having the same molecular weight but different lipophilicity (i.e. LIP1) was used .

By using both Strat-M® and pig skin models, MIVO® showed higher cumulative amount of LIP1in PGOA permeation than that measured in FDC, although a similar slope was observed.

In particular, by using the skin tissue as a model, the lipophilic LIP1 was more absorbed than caffeine with MIVO® system, whereas FDC showed the same permeation of caffeine and LIP1 despite their different

lipophilic properties. This could be cross-correlated with a more physiologically relevant fluid flow conditions below the skin tissue hosted within MIVO rather than FDC, properly recapitulating the faster passage of lipophilic molecules than hydrophilic ones. Interestingly, a positive penetration enhancing effect of OA in increasing diffusion through skin was observed also for LIP1 molecule. This is mostly due to the hydrophobic lipidic structure of the Strat-M® top layer mimicking the tight epidermal stratum corneum [31]. Considering its barrier characteristics and water resistance, the stratum corneum is in fact the main layer that limits drug absorption through the skin [36,63,64]. The major route of skin permeation is through the intact epidermis, and two main pathways have been identified: the intercellular route through the lipids of the stratum corneum and the transcellular route through the corneocytes. In both cases, the molecules diffuses into the intercellular lipid matrix, which is recognized as the major determinant of absorption by the skin [65-69].

On the contrary, the Milli-Q® formulation led to a lower caffeine penetration by using the FDC, confirming the key chemical role of the PGOA: the effects of the OA penetration enhancer on skin barrier function have been widely studied. Recent studies suggested that OA may reduce reversible the stratum corneum lipid bilayer density and thickness [70] and disrupts the skin barrier facilitating water transport [71].

Besides comparing two human skin surrogate models and two vehicles, this paper aimed to compare a novel diffusion system (i.e., MIVO®) with the FDC under dynamic in vitro circumstances to provide reliable data on penetration of caffeine and LIP1, having same molecular weight but different lipophilicity, as suggested by the OECD recommendations.

The results of the comparative analysis highlighted that the MIVO® diffusion chamber shows comparable penetration trend with the standard FDC system (Table 3), and a possibly better prediction of the behavior of lipophilic molecules. Then, since the FDC has been adopted as a reference diffusive chamber by the OECD guidelines, the MIVO® device could be adopted as an efficient platform for predicting the penetration kinetics of different molecules, or to perform prescreening tests before OECD acceptance.

Conclusion

In this manuscript the FDC and a novel micro-physiological system (MIVO®) are compared as in vitro platform to determine percutaneous skin penetration. The penetration ability of caffeine and LIP1 through the artificial Strat-M® membrane and pig ear skin, as human skin surrogates, are tested. Both systems show a continuous increasing penetration up to 8 hours, with a higher overall penetration flux for the Strat-M® membrane than pig skin. The FDC and MIVO® system demonstrated similar penetration kinetic profiles for caffeine and LIP1 as penetrating ingredient, when topically applied. Pig skin tissue displayed a more permissive behavior for lipophilic molecules in the MIVO diffusive system, in line with in vivo data, highlighting the importance to properly resemble the capillary blood circulation within the diffusive systems. This study provided evidence on a reliable comparability for penetration testing, using the two diffusive chambers, both compliant with OECD 428 guidelines, to determine dermal delivery of active ingredients Fig. 3.

Declaration of Conflicting Interest

The authors declare that: Silvia Scaglione and Maurizio Aiello are scientists and shareholders of React4life, that commercializes MIVO device.

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