Video Article

A Method for Determination and Simulation of Permeability and Diffusion in a 3D Tissue Model in a Membrane Insert System for Multi-well Plates

Hao-Hsiang Hsu[1], John-Kevin Kracht[1], Laura Elisabeth Harder[1], Kerstin Rudnik[1], Gerd Lindner[2], Katharina Schimek[2], Uwe Marx[3], Ralf Pörtner[1]

[1]Institute of Bioprocess and Biosystems Engineering, Hamburg University of Technology

[2]Institute of Biotechnology, Department Medical Biotechnology, Technische Universität Berlin

[3]TissUse GmbH

Correspondence to: Ralf Pörtner at poertner@tuhh.de

URL: https://www.jove.com/video/56412
DOI: doi:10.3791/56412

Keywords: Bioengineering, Issue 132, Skin model, permeability, diffusion, membrane insert system, simulation, fluorescein isothiocyanate-dextran, fluorescein sodium salt

Date Published: 2/23/2018

Citation: Hsu, H.H., Kracht, J.K., Harder, L.E., Rudnik, K., Lindner, G., Schimek, K., Marx, U., Pörtner, R. A Method for Determination and Simulation of Permeability and Diffusion in a 3D Tissue Model in a Membrane Insert System for Multi-well Plates. *J. Vis. Exp.* (132), e56412, doi:10.3791/56412 (2018).

Abstract

In vitro cultivated skin models have become increasingly relevant for pharmaceutical and cosmetic applications, and are also used in drug development as well as substance testing. These models are mostly cultivated in membrane-insert systems, their permeability toward different substances being an essential factor. Typically, applied methods for determination of these parameters usually require large sample sizes (*e.g.*, Franz diffusion cell) or laborious equipment (*e.g.*, fluorescence recovery after photobleaching (FRAP)). This study presents a method for determining permeability coefficients directly in membrane-insert systems with diameter sizes of 4.26 mm and 12.2 mm (cultivation area). The method was validated with agarose and collagen gels as well as a collagen cell model representing skin models. The permeation processes of substances with different molecular sizes and permeation through different cell models (consisting of collagen gel, fibroblast, and HaCaT) were accurately described.

Moreover, to support the above experimental method, a simulation was established. The simulation fits the experimental data well for substances with small molecular size, up to 14 x 10^{-10} m Stokes radius (4,000 MW), and is therefore a promising tool to describe the system. Furthermore, the simulation can considerably reduce experimental efforts and is robust enough to be extended or adapted to more complex setups.

Video Link

The video component of this article can be found at https://www.jove.com/video/56412/

Introduction

Organo-typical 3D cultures have become powerful tools for drug development and substance testing[1]. In this respect, human skin models are of special interest due to regulatory requirements, such as those in the cosmetics industry. They have subsequently led to the development of numerous 3D skin models, for use either on their own as single-organ cultures in multi-well plates, or in multi-organ-chips in combination with additional organ models, *e.g.*, the liver[2].

With respect to cultivation of a skin equivalent, the air-liquid interface (ALI) is an essential element for proper epidermal differentiation[3]. Cell culture inserts composed of a vessel with a liquid-permeable membrane at the bottom are typically used to establish an ALI. ALIs are widely utilized in commercially available skin models such as EpiDerm[4], Phenion[5], and Episkin[6], for the culture of skin models with sizes from 96-well (4.26 mm in diameter) up to 12-well (12.2 mm in diameter) plates. The method described here determines the permeation of substances in a membrane insert system.

The permeability coefficient is a significant parameter for evaluating the quality of any cultured skin-model compared to native skin[5], and is used to assess how quickly active substances migrate through the skin. Especially if drugs or cosmetics products need to be applied to the skin, this parameter is essential to understand when precisely the active agents pass through it. A simulation can further help to predict the behavior of the system and to subsequently reduce the necessary time-consuming experimental effort, especially when a large set of substances is involved.

The Franz diffusion cell is state-of-the-art for permeation experiments with skin and skin models[5,6,7,8,9]. This device consists of two compartments with a fixed sample (diffusion barrier) in between. The substance to be tested is applied directly to the top of the sample (donor compartment) and the concentration of the permeating compound can be detected on the opposite (acceptor) compartment. On the acceptor side, constant temperature and homogeneous substance concentration are ensured through a temperature chamber and a magnetic stirrer. Samples can be taken from a sampling arm on the acceptor side of the Franz cell. With a height range between 19 cm and 179 cm, this system is relatively large[10,11]. Another method for determination of diffusion coefficients in gel-like substances and tissues is FRAP. This technique uses the principle

of bleaching fluorescently labeled particles in the gel and then determining the recovery time of the bleached area to calculate the diffusion coefficient[12,13,14].

Furthermore, Fourier-transform-infrared (FTIR) spectroscopy can be used to detect particle movement with infrared light absorbance in order to determine the permeation process of substances in skin[15,16]. However, these or other imaging methods (*e.g.*, two-photon fluorescence correlation spectroscopy[17]) need cost intensive instruments.

In this article, a method is presented to directly measure the permeability of a barrier within a membrane insert system, where a skin model can be cultivated. This method enables permeability experiments to be run with a large number of small samples (well size up to 4.26 mm) in a compact system. This is in contrast to the Franz diffusion cell, where a separate device is needed for each probe, which has to be mounted on the device and is difficult to realize for small samples (size of 4.26 mm). Furthermore, since the method does not require major instrumentation (*e.g.*, a confocal or multiphoton microscope), a reduction in both time and cost is achieved.

All the experiments were performed in microporous membrane insert systems with a sample (barrier) consisting of agarose gel or a collagen cell model established on the membrane. Fluorescent substances (donor) with varying molecular sizes were applied to the top of the sample, and the concentration of permeated substance was detected on the bottom (acceptor) using a fluorescence plate reader (see **Figure 1**). In order to validate the method and test the accuracy of this simulation, agarose gels were produced and used as a barrier. Hydrogels are generally used for the investigation of diffusion and permeation processes in porous medium in the biological sciences[13]. The method was then tested in a cell-seeded system consisting of a collagen matrix of primary fibroblasts and Human adult low Calcium high Temperature keratinocytes (HaCaT) cells (cell-matrix model), which is a simplified skin model[18,19].

Additionally, the permeation process was simulated by means of flow simulations with computational fluid dynamics. It was found that, by means of parameter optimization, the diffusion coefficient could be calculated from the experimental data. In general, this simulation offers different applications; for instance, it is possible to predict a permeation process based on short experiments and the simulation can significantly reduce the number of experiments.

Experimental method and simulation were designed for application to an organ-on-a-chip system[1,20,21], specifically the 2-organ-chip (2-OC) developed commercially[1,22,23,24,25]. In principle, the permeation process of any organ model based on membrane insert systems can be described in this way.

Protocol

1. Preparing the Sample for Permeability Studies

NOTE: In order to verify permeation measurements and simulations, a sample consisting of agarose gel or a cell matrix model based on cultivation of the skin model was used.

1. **Agarose gel**
    1. Dissolve 0.2 g of agarose high resolution powder in 10 mL of H_2O (double-distilled water).
    2. Mix the solution and heat it up to 80 °C. Maintain the temperature for 8 min.
    3. Apply 28.6 µL agarose gel on the membrane of the 96-well membrane insert system (4.26 mm in diameter) or use 226 µL for the 12 well membrane insert system (12 mm in diameter) (*e.g.*, Transwell system).
    4. Wait 10 min until the gel is solidified.

2. **Collagen gel**
    NOTE: All steps are performed under sterile conditions and the solutions are kept on ice to slow down the polymerization of the collagen gel.
    1. Mix 125 µL of Hanks' Balanced Salt Solution (HBSS) with 1 mL of 0.4% collagen R solution (rat tail collagen).
    2. Titrate the solution with 1 M NaOH (sodium hydroxide) (~6 µL) until the color of phenol red changes from yellow to red.
    3. Add 125 µL of Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal calf serum (FCS) to the collagen gel and mix carefully with a pipette tip.
    4. Apply 28.6 µL of collagen gel to the membrane of the 96-well membrane insert system or 226 µL for the 12-well membrane insert system.
    5. Leave the gel in an incubator (37 °C, 5% CO_2) for 30 min.

3. **Collagen cell model with fibroblast**
    NOTE: All steps are executed under sterile conditions.
    1. Prepare primary fibroblasts 5-7 days before the experiment. Cultivate the fibroblasts with DMEM + 10% FCS in cell culture flasks (75 cm^2) and change the medium every 2-3 days.
    NOTE: Depending on the experimental setup, a larger number of cells can be used.
    2. Remove the medium from the cell culture flask (80% confluent) and wash twice with 10 mL (culture flask 75 cm^2) of phosphate buffered saline (PBS). Add 3 mL of 0.05% Trypsin/ ethylenediaminetetraacetic acid (EDTA) and incubate for 3 min at 37 °C.
    3. Tap gently on the culture flask to detach the cells from the surface. Stop the reaction by adding 3 mL of DMEM + 10% FCS. Transfer the solution into a centrifuge tube.
    4. Centrifuge the cell suspension at 120 x g, remove the supernatant, and resuspend the cells with 0.5 mL of DMEM + 10% FCS.
    5. Count the cells and adjust to a concentration of 0.5 x 10^6 cells/mL.
    NOTE: The following steps are executed on ice to slow down the polymerization of the collagen gel.
    6. Mix 125 µL of HBSS with 1 mL of 0.4% collagen R solution.
    7. Titrate the solution with 1 M NaOH (~ 6 µL) until the color of phenol red changes from yellow to red.
    8. Add 125 µL of cell suspension (DMEM + 10% FCS + 0.5 x 10^6 cells/mL) into the collagen gel and mix carefully with a pipette.

9. Apply 28.6 µL of collagen gel on the membrane of the 96-well membrane insert system or 226 µL for the 12-well membrane insert system.
10. Leave the gel in an incubator (37 °C, 5% CO_2) for 30 min.
11. Apply 75 µL of DMEM + 10% FCS on the gel surface and 300 µL to the receiver plate of the 96-well membrane insert system. For the 12-well membrane insert system, use a volume of 590 µL for the surface and 1,846 µL for the receiver plate.
12. Remove the medium from the surface of the cell matrix model (air lift) and incubate the cell matrix model further for 7 days. Use 100 µl medium on the bottom and change the medium every day.

4. **Collagen cell model with HaCaT**
NOTE: All steps are executed under sterile conditions.
1. Prepare the HaCaT 5-7 days before the following steps. Cultivate the HaCaT with DMEM + 5% FCS in a cell culture flask (75 mm^2) and change the medium every 2-3 days.
NOTE: Depending on the experimental setup, a larger number of cells can be used.
2. Remove the medium from the flask and wash twice with 10 mL (culture flask 75 cm^2) of PBS. Add 3 mL of 0.05% Trypsin/EDTA and incubate for 10 min at 37 °C. Stop the reaction with 3 mL of DMEM + 10% FCS. Transfer the solution into a centrifuge tube.
3. Centrifuge the cell suspension at 120 x g, remove the supernatant and resuspend the cells with 0.5 mL of DMEM + 10% FCS.
4. Count the cells and adjust to a concentration of 0.5 x 10^6 cells/mL.
NOTE: The following steps are executed on ice to slow down the polymerization of the collagen gel.
5. Mix 125 µL of HBSS with 1 mL of 0.4% collagen R solution.
6. Titrate the solution with 1 M NaOH (~ 6 µL) until the color of phenol red changes from yellow to red.
7. Add 125 µL of DMEM + 10% FCS to the collagen gel and mix it carefully with a pipette.
8. Apply 28.6 µL of cell suspension on the membrane of the 96-well membrane insert system or 226 µL for the 12-well membrane insert system.
9. Leave the gel in an incubator (37 °C, 5% CO_2) for 30 min.
10. Apply 75 µL of cell suspension to the gel surface and add 300 µL of DMEM + 10% FCS to the receiver plate of the 96-well membrane insert system. For the 12-well membrane insert system, use a volume of 590 µL cell suspension for the surface and 1,846 µL of DMEM + 10% FCS for the receiver plate.
11. Incubate the cell matrix model for 3 days; exchange the medium after 2 days.
12. Remove the medium from the surface of the cell matrix model and incubate the cell matrix model for further 7 day. Use 100 µl medium on the bottom and change the medium every day.

5. **Collagen cell model with fibroblasts and HaCaT**
NOTE: All steps are executed under sterile conditions on ice to slow down the polymerization of the collagen gel. Prepare the fibroblasts as described in step 1.3 until step 1.3.5, and prepare a day later HaCaT as described in step 1.4 until step 1.4.4.
1. Mix 125 µL of HBSS in 1 mL of 0.4% collagen R solution.
2. Neutralize the solution with 1 M NaOH (~6 µL) until the color of phenol red changes from yellow to a red violet.
3. Add 125 µL of primary fibroblast cell suspension consisting of DMEM + 10% FCS + 0.5 x 10^6 cells/mL to the collagen gel and mix carefully.
4. Apply 28.6 µL of cell suspension to the membrane of the 96-well membrane insert system or 226 µL for the 12-well membrane insert system.
5. Leave the gel in an incubator (37 °C, 5% CO_2) for 30 min.
6. Next, apply 75 µL of DMEM + 10% FCS on the gel surface and 300 µL on to the receiver plate of the 96-well membrane insert system. For the 12-well membrane insert system, a volume of 590 µL is used for the surface and 1,846 µL for the receiver plate.
7. Incubate for 1 day at 37 °C and 5% CO_2.
8. Remove the medium from the surface and add a HaCaT cell suspension with 0.5 x 10^6 cells/mL. The volume is the same as described before under step 1.5.6.
9. Incubate the cell matrix model for 3 days; exchange the medium after 2 days.
10. Remove the medium from the surface of the cell matrix model and incubate the cell matrix model for further 7 day. Use 100 µl medium on the bottom and change the medium every day.
NOTE: For this investigation, 3 gel/cell-model samples were prepared for the 12-well membrane insert system. For the 96-well membrane insert system, we used 6 samples for the gel/cell model. For statistical means, 3 samples are common. But for the experiments in the 96-well membrane insert system with collagen matrix model we expected failures and deviations for the cell culture. Therefore, we chose a larger number of samples.

2. Permeability Studies in the Membrane Insert System

1. **Donor substance**
NOTE: Two fluorescein sodium salts (NaFl) are produced.
1. Dissolve NaFl in H_2O at a concentration of 0.1 mg/mL and 0.01 mg/mL. The different fluorescein isothiocyanate-dextranes (FD) with a molecular weight of 4,000, 10,000, 20,000, and 40,000 g/mol are dissolved in H_2O at a concentration of 2 mg/mL. Use these solutions as donor substance for the permeability experiments (see **Figure 1**) with agarose gel.
2. For the setup with the collagen cell model, prepare all solutions with DMEM + 10% FCS instead of water.
NOTE: Prepare stock solutions (10x higher concentration) of the donor substance. Small variations of the donor concentration can influence the results of the permeability experiment.

2. **Experimental method**
NOTE: The permeability experiment is executed at 37 °C and a humidity of > 90%. This parameter ensures the viability of the cells. Temperature influences the diffusion process so the same parameters are used for the experiments with the agarose gel, collagen gel, and collagen cell model. The volume information in the bracket refers to the 12-well membrane insert system.

1. Prepare a 96- (or 12-) well membrane insert system with a barrier consisting of agarose gel (see Protocol 1.1) or cell model (see Protocol 1.2-1.5) and the fluorescence donor substance.
2. Prepare dilutions of 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320 of the donor substance for establishing a standard curve. Pipette 300 µL (1,846 µL) of every dilution in three wells of the receiver plate. For the 12-well membrane insert system use a separate receiver plate. The serial dilutions are used to convert the measured fluorescence [RFU] into the equivalent concentration [mg/mL].
3. Add 75 µL (590 µL) of donor substance on top of the sample (agarose gel or cell model) and 300 µL (1,846 µL) of acceptor substance (H_2O or DMEM + 10% FCS) in the receiver plate (see **Figure 1**).
   NOTE: Ensure that the surfaces of the liquid in the membrane insert system and in the receiver plate have the same level to avoid hydrostatic pressure.
4. Transfer the entire system onto a shaker in the incubator. Adjust the shaking to achieve homogenous mixing (total stroke orbit is 1.5 mm, speed is adjusted at level 3.5, which is related to a rotation of ~ 480 1/min) to avoid a concentration gradient, which influences the diffusion process.
5. Determine fluorescence periodically every hour. To measure the fluorescence, transfer the membrane insert system into an empty plate and measure the fluorescence within the receiver using a plate reader. Use an excitation wavelength of 485 nm and emission of 535 nm for fluorescein.
6. Run the experiment for 5 h.
   NOTE: During the experiments, the liquid evaporates from the entire system. The evaporation changes the concentration in the donor and acceptor and influences the results. This effect is neglected in the case of a running time of 5 h, but for longer running times it should be considered.

3. **Calculating the permeability coefficient**
   1. To establish the standard curve, plot the fluorescence of the serial dilutions versus concentration and perform a linear regression over the data.
   2. Use the slope of the linear regression to convert the fluorescence data of the permeation experiment into concentration. For the purpose of the simulation, convert units into mol/m^3.
   3. Plot the concentration as a function over time and establish the linear segment of the data (see **Figure 2**).
   4. Determine the slope of this linear part and calculate the permeability coefficient according to the following equation (see the example in **Figure 2**):
      $$\frac{dc_A}{dt} = P \cdot A \frac{c_D}{V_A}$$
      where dc_A/dt is the change of the concentration of the substance within the acceptor side over time (the slope); C_D is the concentration on the donor side; P is the permeability coefficient; A is the permeation surface, and V_A is the volume of the acceptor. This equation is derived from Fick's First Law and can only be applied when $C_D \gg C_A$[6,22].
   5. NOTE: The concentrations in the donor have to be much higher than the concentration detected in the acceptor. This was verified in the experimental setup.

3. Simulation

NOTE: The simulation was done with COMSOL Multiphysics 5.1. A basic knowledge of this is assumed. For the diffusion simulation, the following assumptions are made: (a) the diffusion coefficient of the substances in H_2O is much higher in comparison to that in the gel. To compensate for this difference, the simulation uses a value of 1 x 10^{-9} m^2/s which is higher by a factor of 10 to 100 compared to the diffusion coefficient of NaFl through 2% agarose gel. (b) in the experiment, the substance diffuses through the barrier and then through the membrane of the membrane insert system. In contrast to the experimental setup, the virtual agarose gel or cell matrix and membrane are considered to be one homogenous phase. (c) boundary effects on walls are set to "no slip", all slipping effect on walls (not between liquid and gel or liquid and cell model) of the membrane insert system are neglected and are not significant for the diffusion process.

1. **Setup of the diffusion simulation**
   NOTE: These steps demonstrate the setup of the simulation of the permeability experiment. The simulations for the 96- and 12-well membrane insert systems were set up separately. The "Chemical Species Transport" module uses an equation based on Fick's second law of diffusion:
   $$\frac{\partial c}{\partial t} + u \cdot \nabla c = \nabla \cdot (D \nabla c) + R$$
   where c is the concentration of the substance, t is the time, u is the velocity, D is the diffusion coefficient, and R is the reaction rate. The reaction rate was neglected because no chemical reaction occurred in the diffusion process.
   1. Open the program and start a new model. Chose the "Model Wizard", select the 3D model, add "Transport of Diluted Species" to physics interface in the pull-down menu, click on "Study", select the "Time Dependent" study, and click on "Done".
   2. Go to the "Global Definitions" and add "Parameters" with right click. Enter the geometrical and physical parameters in the grid (see **Table 1**, **Table 2**, and **Figure 3e**).
      NOTE: The concave surface of the agarose gel in a 96-well membrane insert system was approximated with an immersion ball.
   3. Set up the geometry of the membrane insert system from the experiments. In Step 3.2, an example is shown of how to build the geometry of the 96-well membrane insert system. The unit of length is set to meter.
      NOTE: Do not build the whole geometry to save calculation time. Instead, the geometry can be reduced by using center lines of a quarter of the geometry (see **Figure 3a** and **Figure 3b**).
   4. Add two "Domain Probes" in "Definitions" (right click on "Definitions" and locate "Probe") and select one probe as the acceptor domain and the other as the donor domain. Choose for both type "Average" and the expression "c" with the unit "mol/m^3".
      NOTE: This step is optional and shows the concentration of the acceptor and donor during the simulation.
   5. Set the diffusion coefficient (Dc) in "Transport Properties 1" in "Transport of Diluted Species "as "Dif_w".

NOTE: "Transport Properties 1" is used for both acceptor and donor domain. In the next step, the barrier domain will be overwritten.

6. Add a second "Transport Properties 2" with right click on "Transport of Diluted Species" and select the barrier (2) in the "Domain Selection". Depending on the objective of the simulation the diffusion coefficient can be set as a value of the barrier or as a dummy variable "D". For the first test run, set a value of 2E-10 m^2/s.
   NOTE: "D" will be declared later in Step 3.3.
7. In "Transport of Diluted Species" for "Initial Values 1" define the concentration as zero.
   NOTE: "Initial Values 1" is used for barrier and acceptor domain. In the next step, the donor domain will be overwritten.
8. Add a second "Initial Values 2" with right click on "Transport of Diluted Species" and select the donor (3) as the domain. Set the concentration as the initial concentration of the donor substance (*e.g.*, C_fl from Table 2).
9. Add "Symmetry 1" with right click on "Transport of Diluted Species", add and choose all surfaces of the "Boundary Selection," which mirror the whole geometry (for the geometry example in step 3.2, it is the boundary number 1, 2, 4, 5, 7, 8).
   NOTE: This point can be neglected if the whole geometry was set up.
10. With a right click on "Mesh" add two "Free Tetrahedral". Select the barrier (2) as the domain (change the Geometric entity level into "Domain"). Add "Size" with right click on "Free Tetrahedral 1" and for the predefined mesh "Finer" for the 12-well membrane insert system or "extra Fine" for a 96-well membrane insert system.
11. In the second "Free Tetrahedral" select the acceptor and donor as domain and the predefined mesh "Normal" for a 12-well membrane insert system or "Finer" for a 96-well membrane insert system (see **Figure 3c** and **Figure 3d**).
    NOTE: It is also possible to choose a coarser mesh to save computation time. This might reduce the accuracy of the results. Click on "Build All" in "Mesh Setting" to mesh the geometry.
12. Start the simulation with "compute" in the "Study 1".

2. **Example setup for a geometry**
   NOTE: Here an example is given of how to set up the geometry of the 96-well membrane insert system (with concave barrier). All steps are executed in the geometry module of the program. The unit of length is set to meter.
   1. Generate a cylinder 1 (right click on "Geometry 1") with radius of d_w/2 and height of h_sp+h_b+h_a.
   2. Generate a cylinder 2 with radius of d_tran/2, height of h_b+h_a, and z position of h_sp.
   3. Use the "Difference" option (right click on "Geometry 1", locate "Booleans and Partition") to subtract cylinder 2 from cylinder 1. Chose "cyl1" in the "Objects to add", activate "Object to subtract" and chose "cyl2". The new volume is the geometry of the acceptor.
   4. Generate a cylinder 3 with radius of d_a/2, height of h_b, and z position of h_sp.
   5. Generate a sphere 1 with radius r and z position r_z.
   6. Use the "Difference" option to subtract sphere 1 (sph1) from cylinder 3 (cyl3). The new volume is called Difference 2.
   7. Generate a cylinder 4 with radius of d_a/2, height of h_b+h_a, and position z of h_sp.
   8. Use the "Difference" option to subtract cylinder 4 (cyl4) from Difference 2. The new volume is the geometry of the acceptor.
   9. Repeat steps 3.2.4-3.2.6 to build the barrier (the agarose gel or cell model in the experiment).
   10. Make a union 1 of all geometry elements (right click on "Geometry 1", locate "Booleans and Partition").
   11. Generate a block 1 with all edges set to a length of d_tran*2, position x of -d_tran and y of d_tran*2.
   12. Generate a block 2 with all edge length of d_tran*2, position x of -d_tran*2 and y of -d_tran.
   13. Use the "Difference" option to subtract union 1 from block 1 and block 2.

3. **Adding the Parameter Optimization to the Simulation**
   NOTE: With the help of parameter optimization the diffusion coefficient can be fitted to the previously generated experimental data. The following instructions show how to integrate the optimization part into the diffusion simulation. Make sure the diffusion simulation is working before starting these steps.
   1. Add the Physics-Module "Optimization" using "Add Physic" (Optimization can be found in "Mathematics" in the category "Optimization and Sensitivity") to the simulation. Click on "Add to Component".
   2. Add "Variables" with right click under "Definitions" (local in Component) and type in the variables from **Table 3**.
      NOTE: The parameter optimization uses real numbers, *i.e.*, the factor 1^{-10} of the diffusion coefficient must be defined separately.
   3. Add an "Average 1" with right click on "Definitions" in section "Component Coupling" and type in the operator name "Acceptor".
   4. Generate a separate text document containing the experimental data.
      NOTE: A semicolon separates columns; a line break separates rows. Time declaration is measured in seconds, concentration in mol/m^3. Remove the first and second data point in the lag phase (see **Figure 2**) of the experiments to avoid possible fitting errors. Here is an example how the text document might look like:
      3540; 0.00216
      7140; 0.00724
      12240; 0.01707
      15180; 0.02230
      18660; 0.02697
      21540; 0.02931
      This example can be used to test the simulation.
   5. Add "Global Least-Squares Objective" with right click on "Optimization", attach the text document from step 3.3.4 to the "experimental data" and define the first column as "Time Column 1" with right click on "Global Least-Squares Objective" and the second column as "Value Column 1" with right click on "Global Least-Squares Objective". In the "Expression" of "Value Column" type the variable "C".
   6. Add "Global Control Variables 1" with right click on "Optimization" and declare "D_search" as a variable with the initial value "1", lower bound "0", and upper bound "1000".
   7. Add "Optimization" with right click on "Study 1" and chose "SNOPT" as an optimization solver method. Set the optimality tolerance to 1E-9.
      NOTE: If the simulation did not converge, increase the optimality tolerance. Keep in mind that the simulation will be inaccurate if the optimality tolerance is too large.

8. Start the parameter optimization with "compute" in "study". Do not forget to set the diffusion coefficient on the barrier as "D".

Representative Results

Permeability experiments in a 96-well membrane insert system with 2% agarose gel as a barrier were conducted in order to evaluate the accuracy of a simulation. Fluorescein sodium salt (NaFl) and fluorescein isothiocyanate-dextranes (FD) were used to verify the impact of the molecular size of the diffusing substance from 5 x 10^{-10} m up to 45 x 10^{-10} m Stokes radius (376.27-40,000 mol wt). The simulation's native parameter optimization was used to fit the simulation to experimental data.

To that end, slopes of only the linear parts of the simulated permeability were compared to the experimental outcomes. For small molecular sizes, simulation and experimental data were in good agreement with 99.2% for NaFl and 80.2% for FD 4,000 (see **Figure 4a** and **Figure 4b**). Larger molecular size generated higher deviations showing correlations of 50.5% for FD 10,000, 79.7% for FD 20,000, and 53.6% for FD 40,000. Curve progression in the simulations showed a delay at the beginning and a stronger rise in the further course of the graphs (see **Figure 4c-4e**).

Permeability coefficients and simulated diffusion coefficients are shown in **Table 4.** The permeation coefficient decreases with increasing molecular size. Standard deviation was between 0.08 x 10^{-8} m/s and 0.47 x 10^{-8} m/s (N = 7), which corresponded to an absolute error of between 4.18% and 46.15%. Experiments with larger molecules showed a larger absolute error. The simulated diffusion coefficients behaved very similarly to experimental permeability coefficients. Substances with larger Stokes radii showed decreasing diffusion coefficients, and the absolute error ranged between 9.09% and 18.46% (N = 3).

In additional permeation experiments, four different collagen cell model types were used as barriers in a 12-well membrane insert system. These models comprise a cell-free model and a cell model with different combinations of primary fibroblasts in the collagen gel and HaCaT on the surface. The following combinations were used: Collagen (Col.) as a cell-free model, Collagen + Fibroblasts (Col.+F.), Collagen + HaCaT (Col. +H.), and Collagen + Fibroblasts + HaCaT (Col.+F.+H.). Fluorescein sodium salt with DMEM + 10% FCS was used as donor substance. For image analysis of the collagen cell model, staining with hematoxylin and eosin (HE) was used. This staining was done using the manufacturer's protocol. In **Figure 5**, such a stain with a representative Col.+F.+H. model is shown. The HE slightly stains the tissue structure of the collagen matrix. The fibroblasts are located in the matrix, and the nuclei of the fibroblast and HaCaT cells are stained in dark violet. On top of the collagen matrix, there is a layer containing many nuclei, which should be the nuclei of the HaCaTs, building an enclosing layer on the top of the model.

In **Table 5**, experimental permeation coefficients and simulated diffusion coefficients are listed. A trend can be seen for most of the models with HaCaT, which have lower permeation/diffusion coefficients in comparison to the models without HaCaT. The absolute error of the permeation coefficients is 10.9-24.4%, and for the diffusion coefficients 5.2%-12.9%.

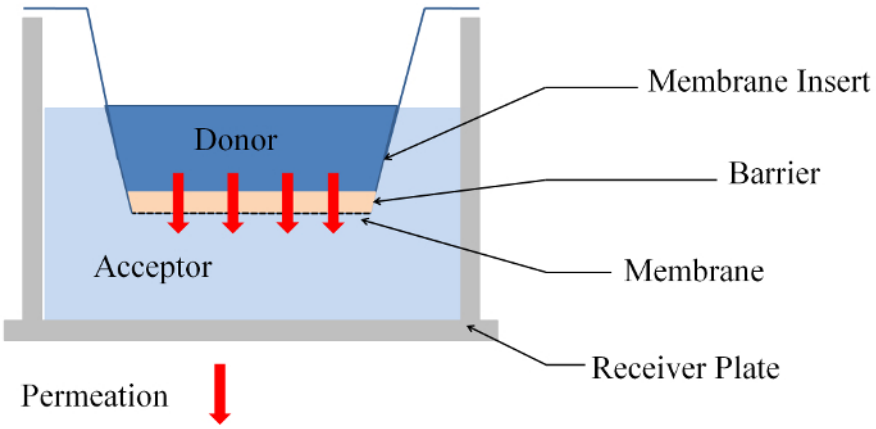

Figure 1: Side view of the permeability experiment in a membrane insert system. Please click here to view a larger version of this figure.

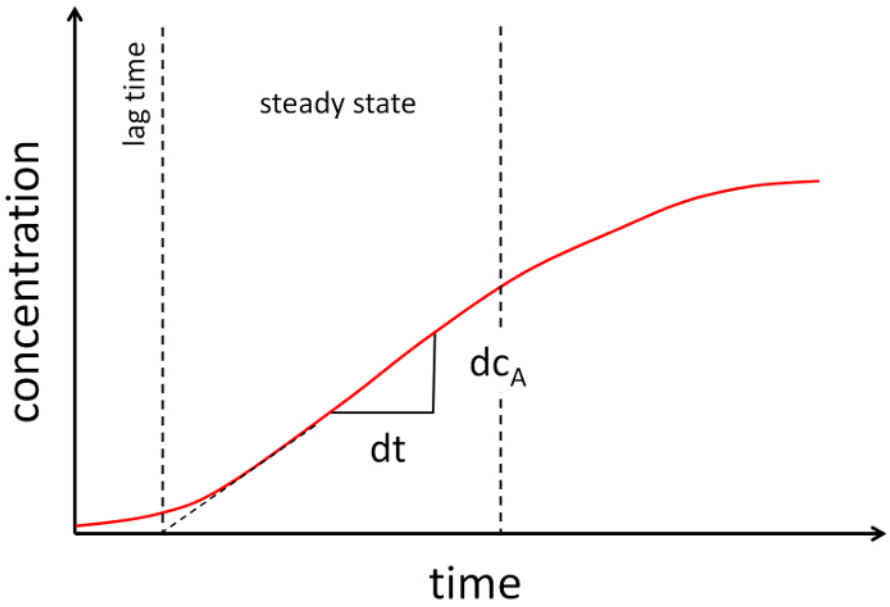

Figure 2: Exemplary graph of a permeability experiment. The concentration of the acceptor is plotted over time. Two dashed lines bracket the nearly linear part of the graph. The slope of the linear part is used to determine the permeability coefficient. Please click here to view a larger version of this figure.

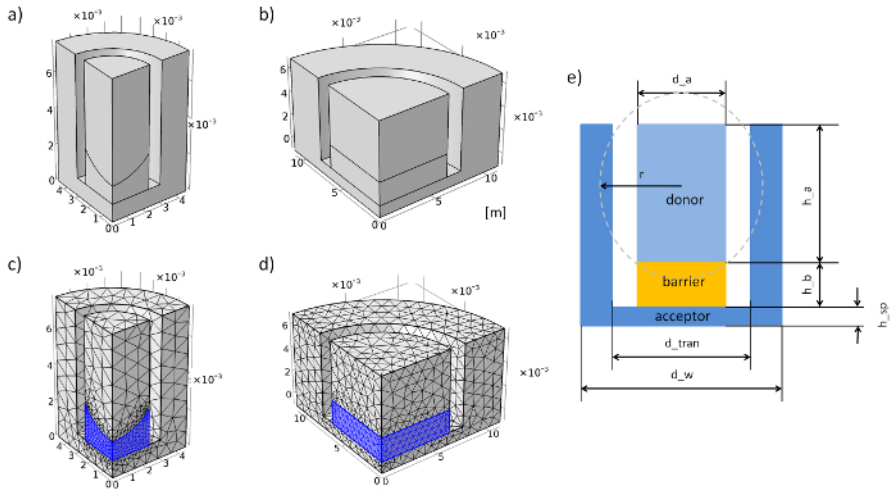

Figure 3: Geometry and mesh of the membrane insert system in the simulation. (**a**) Geometry of the 96-well membrane insert system. (**b**) Geometry of the 12-well membrane insert system. (**c**) Mesh of the 96-well membrane insert system. (**d**) Mesh of the 12-well membrane insert system. (**e**) Cross-section and parameters of the membrane insert system. Please click here to view a larger version of this figure.

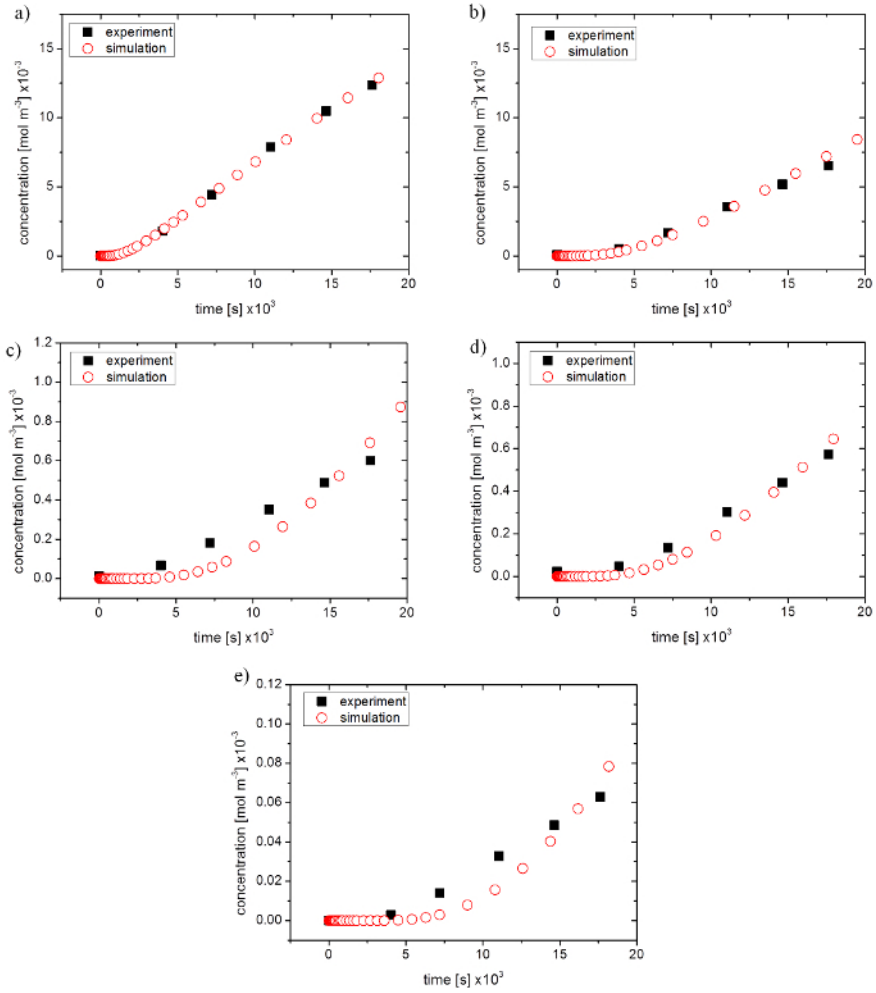

Figure 4: Comparison of the experimental data from a permeation experiment to the optimized simulation. (**a**) fluorescein sodium salt, (**b**) fluorescein isothiocyanate-dextran 4,000 mol wt., (**c**) 10,000 mol wt., (**d**) 20,000 mol wt., and (**e**) 40,000 mol wt. Please click here to view a larger version of this figure.

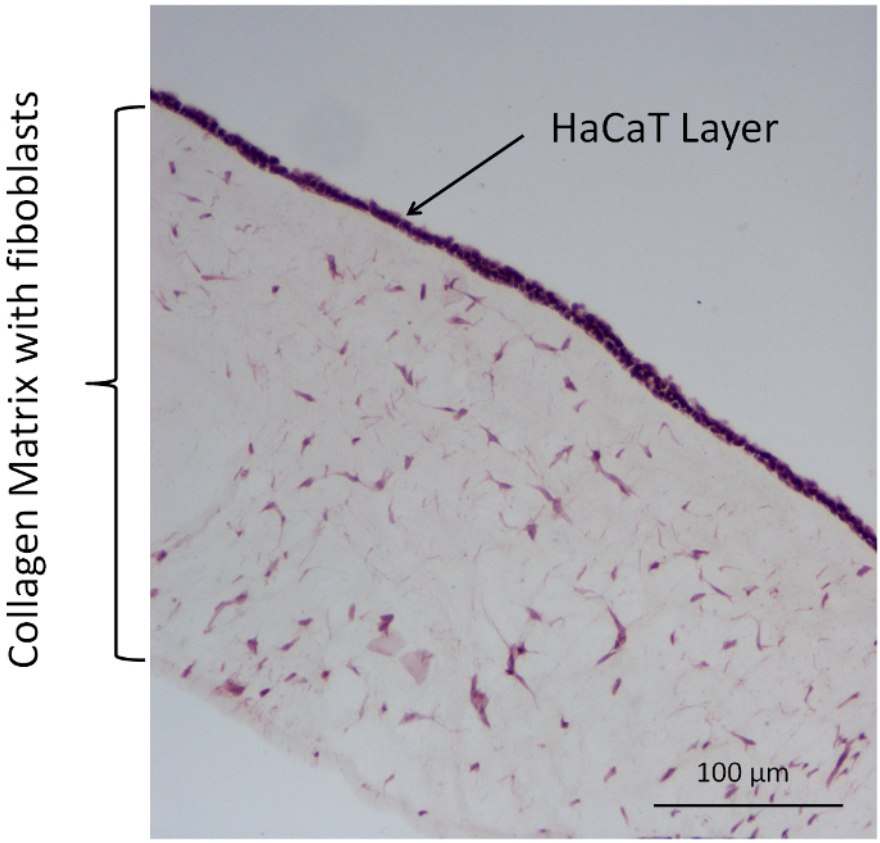

Figure 5: Representative HE staining of a collagen cell model (Collagen + Fibroblasts + HaCaT). Please click here to view a larger version of this figure.

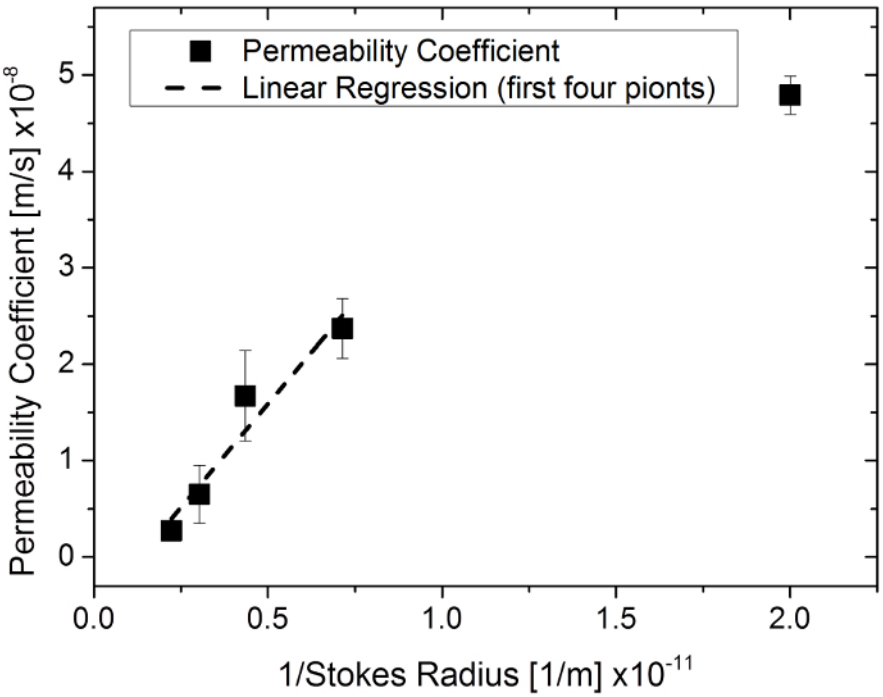

Figure 6: Permeability coefficient as a function of the 1/Stokes radius using fluorescein sodium salt and fluorescein isothiocyanate-dextran in a 96-well membrane insert system. Please click here to view a larger version of this figure.

Name	Experession for 96 well System in mm	Experssion for 12-well System in mm	Description
d_tran	5.65 [mm]	14.7 [mm]	Diameter of the well
d_a	4.26 [mm]	12.1 [mm]	Diameter of the Membrane
d_w	8.79 [mm]	21.97 [mm]	Diameter of the Acceptor
h_b	2 [mm]	2 [mm]	Heigh of the Barrier
h_sp	1 [mm]	1 [mm]	Distance between well and Bottom
h_a	4.73 [mm]	5.24 [mm]	High of the Acceptor
b	h_b/2	-	Immersion Depth
r	((d_a)^2+4*b^2)/(8*b)	-	Radius of Immersion Ball+
r_z	r+h_b	-	z-Position of the Immersion Ball+

Table 1: Geometry parameters for "Chemical Species Transport" simulation. +Only to be used for the simulation of agarose gel in 96 well membrane insert system.

Name	Expression	Value	Description
C_fl	0.1 [mg/ml]/376.28 [g/mol]	0.26576 mol/m^2	Concentration of Fl.So.
C_4	2 [mg/ml]/4000 [g/mol]	0.5 mol/m2	Concentration of FD 4.000
C_10	2 [mg/ml]/10000 [g/mol]	0.2 mol/m2	Concentration of FD 10.000
C_20	2 [mg/ml]/20000 [g/mol]	0.1 mol/m2	Concentration of FD 20.000
C_40	2 [mg/ml]/40000 [g/mol]	0.05 mol/m2	Concentration of FD 40.000
Dif_w	1e-9 [m^2/s]	1E-9m^2/s	Diffusion Coefficient of mixing water

Table 2: Physical parameters for "Chemical Species Transport" simulation.

Name	Expression	Description
C	Acceptor(c)	Definition of the acceptor concentration
D	D_search*1e-10	Factor change for D

Table 3: Parameters for "Optimization" simulation.

Permeate	Permeability coefficient (m/s)x10^{-8}	Diffusion coefficient (m/s^2)x10^{-10}	Stokes radius of permeate (m)x10^{-10}
Fl.So.	4.79 ± 0.20	1.94 ± 0.34	5
FD 4,000	2.37 ± 0.31	0.65 ± 0.12	14
FD10,000	1.67 ± 0.47	0.22 ± 0.02	23
FD 20,000	0.65 ± 0.30	0.29 ± 0.04	33
FD 40,000	0.27 ± 0.08	0.14 ± 0.02	45

Table 4: Permeability and diffusion coefficient of substances with different Stokes radius through 2% Agarose gel + membrane in a 96-well membrane insert system. (fluorescein sodium salt = Fl. So., fitc dextran = FD).

Model	Permeability coefficient (m/s)x10^{-8}	Diffusion coefficient (m/s^2)x10^{-10}
Col.	2.18 ± 0.29	1.22 ± 0.06
Col.+F.	1.77 ± 0.38	0.93 ± 0.12
Col.+H.	1.64 ± 0.40	0.96 ± 0.05
Col.+F.+H.	1.65 ± 0.18	0.88 ± 0.11

Table 5: Permeability and diffusion coefficient of fluorescein sodium salt through a collagen cell model in a 12-well membrane insert system (Col. = Collagen, F. = Fibroblast, H. = HaCaT).

Discussion

This study documents a method developed to quantify permeation through a tissue-construct engineered on a membrane. Permeation of substances with varying molecular sizes through agarose gel was first examined to test and validate the method and the corresponding simulation. It is well known that smaller molecules permeate faster through a matrix mesh (with the exception of the effect in gel filtration by permeability chromatography). Similar observations were made with size-exclusion experiments of substances through sclera[26], human epidermal membrane[27], human skin[17], and rat skin[28]. An inverse correlation between permeability coefficients and the corresponding Stokes radius (the radius of a hard sphere that moves with the same diffusion rate as the molecules described, usually smaller than the effective radius of the molecule) has been shown[26,28], and a similar relationship was observed in experiments with substances of different molecular sizes. By plotting the permeability coefficients over 1/Stokes radius, a linear correlation over the four groups with the smallest molecular size was found (R^2 = 0.93) (**Figure 6**). This indicates that simulated permeability coefficients with the method suggested are in a realistic range.

The error of 46.15% in the experiments is slightly larger than reported for permeability experiments with the Franz diffusion cell system[10]. One possible explanation could be the size distribution of fluorescein-isothiocyanate-dextran, which is discussed later.

The method described has important advantages compared with methods using the Franz diffusion cell system. Firstly, the setup is more compact; the experiments are executed directly in a membrane insert system, which has the scale of a commercial well plate (# 13 cm x 8.5 cm). This enables multiple samples to be run simultaneously, whereas a separate Franz diffusion cell is needed for each sample. Secondly, the permeability of a skin model can be directly measured in the membrane insert, where the cultivation takes place. Using Franz diffusion cells, the samples have to be taken out and mounted on the system, which is more cumbersome for small samples and is also more time-consuming.

Permeation experiments with collagen cell matrices showed that this method can be applied successfully to cell-seeded systems. The model presented here was verified for skin models; however, the method can be applied to other types of organic cell cultures, *e.g.*, kidney or liver.

In this study, a collagen-cell model was used in which the HaCaT cells completely covered the model surface (see **Figure 5**). This led to a reduction of permeability coefficient, demonstrating that the method is sensitive enough to distinguish the permeability coefficient between a collagen-cell model with and without a layer of HaCaT. Ideally, a skin model should build up a barrier, which approaches the epidermis of a real skin[29], and it is therefore important to verify the quality (*e.g.*, building of dermis, epidermis) of the skin model before actual use. The development of a skin model can be visualized with staining techniques and quantified from the detection of skin protein and collagen[30,31,32]. The permeability coefficient may also be an important factor for assessing the development of the skin model, but further experiments are required to confirm this. As previously mentioned, this method enables running multiple samples in parallel. It is also possible to take samples during the cultivation to measure permeability, and thereby observe the development of this parameter of the skin model.

It should be noted that permeability is measured through a gel/collagen-cell-model and a membrane simultaneously. The detected permeability coefficient is system-specific, whereby the results of different skin models can only be compared when using the same membrane insert. Furthermore, the skin model needs to cover the entire cultivation area in order to ensure that the test substance will permeate only through the model and not adjacent to it, which would induce errors in the permeability measured. Another aspect that should be considered in future experiments is the natural environment surrounding the skin. Normally, the temperature of the skin surface is lower in comparison to the inner region, which can influence permeation conditions.

In order to align lab experiments with computer simulations, a method which enables parameter optimization for applied simulation was presented. Simulations were found to coincide well with experimental data for substances with small molecular sizes. However, deviations between simulation and experimental data were observed for substances with larger molecular sizes. Large polysaccharide molecules can increase friction and slow down the diffusion process in a gel. This effect causes abnormal diffusion, which is a possible reason for the deviation between experimental and simulation values[33,34]. Another explanation might be the presence of smaller or larger particles in fluorescein-isothiocyanate-dextran. The manufacturer specifies the molecular weight of the substance as the mean size with a given range, which allows smaller and larger particles to be present. It is also unclear how dispersed these substances are, as the smaller particles permeate faster through the gel and the fluid channel. It is possible to extend the simulation to consider these diffusion and friction effects.

The permeability experiment and simulation were developed for use in a 2-OC. With the help of the simulation, this experimental method can be directly transferred to more sophisticated experimental setups. For example, the membrane insert system simulation can easily be transferred to the geometry of a 2-OC or to other systems with similar set-ups. This option of modulating the simulation can be used to support the design of future experiments. In addition, side effects such as evaporation, abnormal diffusion, and membrane effects can be integrated to enhance the simulation, thereby improving accuracy. The simulation program gives the opportunity to change or enhance the simulation equation, as well as to integrate other physical modules in order to investigate other aspects of skin model development. One example is the simulation of glucose consumption and lactate production in a collagen cell model.

A particularly interesting aspect in the testing of medical substances is how the substances are distributed in an organ-on-a-chip system. The simulation and permeability parameter my help to answer questions such as how fast a substance permeates into the system as well as which concentration will be available for other tissues in a multi-organ-chip. This method can support and enhance the development and testing of such organ-on-chip systems.

Disclosures

Uwe Marx is the CEO and a shareholder and Gerd Lindner is a shareholder of TissUse GmbH, a company manufacturing and commercializing the MOC technology. Other authors declare no conflict of interests regarding the publication of this paper.

Acknowledgements

This work was created with financial support from Deutsche Forschungsgemeinschaft (DFG) under grant No. PO413/12-1 and LA 1028/7-1.

References

1. Marx, U., *et al.* Human-on-a-chip developments: a translational cutting-edge alternative to systemic safety assessment and efficiency evaluation of substances in laboratory animals and man? *ATLA.* **40** (5), 235-257 (2012).
2. Maschmeyer, I., *et al.* Chip-based human liver-intestine and liver-skin co-cultures - A first step toward systemic repeated dose substance testing in vitro. *Eur J Pharm Biopharm.* **95**, 77-87 (2015).
3. Prunieras, M., Regnier, M., Woodley, D. Methods for Cultivation of Keratinocytes with an Air-Liquid Interface. *J Invest Dermatol.* **81** (1), 28-33 (1983).
4. Cannon, C. L., *et al.* New epidermal model for dermal irritancy testing. *Toxicol In Vitro.* **8** (4), 889-891 (1994).
5. Ackermann, K., *et al.* The Phenion Full-Thickness Skin Model for Percutaneous Absorption Testing. *Skin Pharmacol Physiol.* **23** (2), 105-112 (2010).
6. Netzlaff, F., *et al.* Permeability of the reconstructed human epidermis model Episkin in comparison to various human skin preparations. *Eur J Pharm Biopharm.* **66** (1), 127-134 (2007).
7. Bran, B., *et al.* A New Discriminative Criterion for the Development of Franz Diffusion Tests for Transdermal Pharmaceuticals. *J Pharm Sci.* **13** (2), 218-230 (2010).
8. Pineau, A., *et al.* In vitro study of percutaneous absorption of aluminum from antiperspirants through human skin in the Franz diffusion cell. *J Inorg Biochem.* **110**, 21-26 (2012).
9. Filon, F. L., *et al.* In vitro percutaneous absorption of cobalt. *Int Arch Occup Environ Health.* **77** (2), 85-89 (2004).
10. Ng, S.-F., *et al.*, Validation of a Static Franz Diffusion Cell System for In Vitro Permeation Studies. *AAPS PharmSciTech.* **11** (3), 1432-1441 (2010).
11. Bonferoni, M.C., *et al.* A Modified Franz Diffusion Cell for Simultaneous Assessment of Drug Release and Washability of Mucoadhesive Gels. *Pharm Dev Tecnol.* **4** (1), 45-53 (1999).
12. Seiffer, S., Oppermann, W. Systematic evaluation of FRAP experiments performed in a confocal laser scanning microscope. *J Microsc.* **220** (1), 20-30 (2005).
13. Pluen, A., *et al.* Diffusion of Macromolecules in Agarose Gels: Comparison of Linear and Globular Configurations. *Biophys J.* **77**, 542-552 (1999).
14. Cornelissen, L. H., *et al.* Diffusion measurements in epidermal tissues with fluorescent recovery after photobleaching. *Skin Res Technol.* **14** (4), 462-467 (2008).
15. Pirot, F., *et al.* Characterization of the permeability barrier of human skin in vivo. *PNAS.* **94** (4), 1562-1567 (1997).
16. Tetteh, J., *et al.* Local examination of skin diffusion using FTIR spectroscopic imaging and multivariate target factor analysis. *Anal Chim Acta.* **642** (1-2), 246-256 (2009).
17. Guldbrand, S., *et al.* Two-photon fluorescence correlation spectroscopy as a tool for measuring molecular diffusion within human skin. *Eur J Pharm Biopharm.* **84** (2), 430-436 (2013).
18. Kehe, K., *et al.* Tissue engineering with HaCaT cells and a fibroblast cell line. *Arch Dermatol Rech.* **291** (11), 600-605 (1999).
19. Veronike, M., *et al.* Epidermal Organization and Differentiation of HaCaT Keratinocytes in Organotypic Coculture with Human Dermal Fibroblasts. *J Invest Dermatol.* **112** (3), 343-353 (1999).
20. Moraes, C., *et al.* Organ-on-a-Chip: A Focus on Compartmentalized Mircodevices. *Ann Biomed Eng.* **40** (6), 1211-1227 (2012).
21. Huh, D., *et al.* From Three-Dimensional Cell Culture to Organs-on-Chips. *Trends Cell Biol.* **21** (12), 745-754 (2011).
22. Schimek, K., *et al.*, Integrating biological vasculature into a multi-organ-chip microsystem. *Lab Chip.* **13** (18), 3588 (2013).
23. Materne, E.-M., *et al.* The Multi-organ Chip - A Microfluidic Platform for Long-term Multi-tissue Coculture. *J Vis Exp.* (98) (2015).
24. Materne, E.-M., *et al.*, A multi-organ chip co-culture of neurospheres and liver equivalents for long-term substance testing. *J Biotechnology.* **205**, 36-46 (2015).
25. Materne, E.-M., Tonevitsky, A. G., Marx, U. Chip-based liver equivalents for toxicity testing - organotypicalness versus cost-efficient high throughput. *Lab Chip.* **13** (18), 3481 (2013).
26. Jayakrishna, A., *et al.* Diffusion of High Molecular Weight Compounds through Sclera. *IOVS.* **41** (5), 1181-1185, (2000).
27. Peck, K., *et al.* Hindered Diffusion of Polar Molecules Through and Effective Pore Radii Estimate of Intact and Ethanol. *Pharm Res.* **11** (9), 1309-1314 (1994).
28. Ogiso, T., *et al.* Mechanism of the Enhancement Effect of n-Octyl-β-D-thioglucoside on the Transdermal Penetration of Fluorescein Isothiocyanate-Labeled Dextrans and the Molecular Weight Dependence of Water-Soluble Penetrants through Stripped Skin. *J Pharm Sci.* **83** (12), 1676-1681 (1994).
29. Hadgraft, J. Skin, the final frontier. *Int J Pharm.* **224** (1-2), 1-18 (2001).
30. Asselineau, D., *et al.* Human Epidermis Reconstructed by Culture: Is It "Normal"? *J Invest Dermatol.* **86** (2), 181-186 (1986).
31. Casasco, A., *et al.* A. Cell proliferation and differentiation in a model of human skin equivalent. *Anat Rec.* **264** (3), 261-272 (2001).
32. Kehe, K., *et al.* Tissue engineering with HaCaT cells and a fibroblast cell line. *Arch Dermatol Res.* **291** (11), 600-605 (1999).
33. Laurent, T. C., *et al.* Diffusion of Dextran in Concentrated Solutions. *Eur J Biochem.* **68**, 95-102 (1976).
34. Metzler, R., Klafter, J. The random walk's guide to anomalous diffusion: A fractional dynamics approach. *Phys Rep.* **339** (1), 1-77 (2000).