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 $14 \times 10^{-10}$ 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. 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.

With respect to cultivation of a skin equivalent, the air-liquid interface (ALI) is an essential element for proper epidermal differentiation. 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, Phenion, and Episkin, 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, 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. 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. 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\textsuperscript{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\textsuperscript{15,16}. However, these or other imaging methods (e.g., two-photon fluorescence correlation spectroscopy\textsuperscript{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\textsuperscript{15}. 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\textsuperscript{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\textsuperscript{1,20,21}, specifically the 2-organ-chip (2-OC) developed commercially\textsuperscript{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\textsubscript{2}O (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 Hank's® 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\textsubscript{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\textsuperscript{2}) and change the medium every 2-3 days.
   2. Remove the medium from the cell culture flask (80% confluent) and wash twice with 10 mL (culture flask 75 cm\textsuperscript{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. Centrifuge the cell suspension at 120 x g, remove the supernatant, and resuspend the cells with 0.5 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\textsuperscript{5} cells/mL.
   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\textsuperscript{5} 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% CO₂) 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 mm²) 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 cm²) 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 10⁶ 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 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% CO₂) 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 red.
3. Add 125 μL of primary fibroblast cell suspension consisting of DMEM + 10% FCS + 0.5 x 10⁶ 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% CO₂) 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 of DMEM + 10% FCS for the receiver plate.
7. Incubate for 1 day at 37 °C and 5% CO₂.
8. Remove the medium from the surface and add a HaCaT cell suspension with 0.5 x 10⁶ cells/mL. The volume is the same as described 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 H₂O 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 H₂O 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.

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 (H₂O 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. 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 H₂O 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 m²/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:

   \[
   \frac{\partial c}{\partial t} + \nabla \cdot (D \nabla c) = R
   \]

   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 the 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/m²".

   **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".

   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/m².

   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):

   \[
   \frac{dC_A}{dt} = P \cdot A \cdot \frac{C_D}{V_A}
   \]

   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 \(C_D > C_A^{0.22}\).

   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.
2. **Example setup for a geometry**

   **NOTE:** Here is an example 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 \( \frac{d_w}{2} \) and height of \( h_{sp}+h_b+h_a \).
   2. Generate a cylinder 2 with radius of \( \frac{d_{tran}}{2} \), 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 "cy1" in the "Objects to add", activate "Object to subtract" and chose "cy2". The new volume is the geometry of the acceptor.
   4. Generate a cylinder 3 with radius of \( \frac{d_a}{2} \), 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 (sp1) from cylinder 3 (cy3). The new volume is called Difference 2.
   7. Generate a cylinder 4 with radius of \( \frac{d_a}{2} \), height of \( h_b+h_a \), and position \( z \) of \( h_{sp} \).
   8. Use the "Difference" option to subtract cylinder 4 (cy4) 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"). 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).
   12. Start the simulation with "compute" in the "Study 1".

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.
   3. Add an "Average 1" with right click on "Definitions" in section "Component Coupling" and type in the operator name "Acceptor".
   4. Add a second "Variables 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., \( C_b \) from Table 2).
   5. 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).
   6. 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., \( C_b \) from Table 2).
   7. 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 \( 2E-10 \text{ m}^2/\text{s} \).
   8. 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 "Value 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".
   9. 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 \( 1000 \).
   10. 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.
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 $5 \times 10^{-10}$ m up to $45 \times 10^{-10}$ 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 $0.08 \times 10^{-8}$ m/s and $0.47 \times 10^{-8}$ 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.](https://jove.com/doi/10.3772/jove.56412)
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     | Expression for 96 well System in mm | Expression 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/m² | 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 [m²/s] | 1E-9 m²/s | Diffusion Coefficient of mixing water |

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

| Name | Expression | Value       | 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)x10⁸ | Diffusion coefficient (m²/s)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                               |
| FD 10,000| 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 (m²/s)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 sclera26,28, human epidermal membrane27, human skin17, and rat skin28. 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 shown26,28, 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 (R² = 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. 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 skin17, 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. 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. 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.
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