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Lab-on-a-chip tool for the investigation of biological barriers

PhD Thesis

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List of publications

1. List of full papers directly related to the subject of the thesis

I. Walter FR, Valkai S., **Kincses A**, Petneházi A, Czeller T, Veszelka S, Ormos P, Deli MA, Dér A. A versatile lab-on-a-chip tool for modeling biological barriers. Sensors and Actuators B: Chemical. 2016;222:1209-1219. IF: 5.401

II. Kincses A, Santa-Maria AR, Walter FR, Dér L, Horányi N, Lipka DV, Valkai S, Deli MA, Dér A. A chip device to determine surface charge properties of confluent cell monolayers by measuring streaming potential. Lab. Chip. 2020;20(20):3792-3805. IF: 6.774

Cumulative impact factor of papers directly related to the subject of thesis: 12.175

2. List of other full papers

I. Kincses A, Toth-Boconadi R, Dér A. 2D measurement of ion currents associated to the signal transduction of the phototactic alga Chlamydomonas reinhardtii. J Photochem Photobiol B 2012;114:147-52. IF: 3.110

II. Sántha P, Veszelka S, Hoyk Z, Mészáros M, Walter FR, Tóth AE, Kiss L, **Kincses A**, Oláh Z, Seprényi G, Rákhely G, Dér A, Pákáski M, Kálmán J, Kittel Á, Deli MA. Restraint Stress-Induced Morphological Changes at the Blood-Brain Barrier in Adult Rats. Frontiers in molecular neuroscience. 2016; 8(88). IF: 5.076

III. Lázár V, Martins A, Spohn R, Daruka L, Grézal G, Fekete G, Számel M, Jangir PK, Kintses B, Csörgő B, Nyerges Á, Györkei Á, **Kincses A**, Dér A, Walter FR, Deli MA, Urbán E, Hegedűs Z, Olajos G, Méhi O, Bálint B, Nagy I, Martinek TA, Papp B, Pál C. Antibiotic-resistant bacteria show widespread collateral sensitivity to antimicrobial peptides. Nat Microbiol. 2018;3(6):718-731. IF=14.633

IV. Hoyk Z, Tóth ME, Lénárt N, Nagy D, Dukay B, Csefová A, Zvara Á, Seprényi G, **Kincses A**, Walter FR, Veszelka S, Vígh J, Barabási B, Harazin A, Kittel Á, Puskás LG, Penke B, Vígh L, Deli MA, Sántha M. Cerebrovascular Pathology in Hypertriglyceridemic APOB-100 Transgenic Mice. Front Cell Neurosci. 2018;12:380. IF=3.9

V. Harazin A, Bocsik A, Barna L, **Kincses A**, Váradi J, Fenyvesi F, Tubak V, Deli MA, Vecsernyés M. Protection of cultured brain endothelial cells from cytokine-induced damage by α-melanocyte stimulating hormone. PeerJ. 2018;15;6:e4774. IF=2.353

VI. Santa-Maria AR, Walter FR, Valkai S, Brás AR, Mészáros M, **Kincses A**, Klepe A, Gaspar D, Castanho MARB, Zimányi L, Dér A, Deli MA. Lidocaine turns the surface charge of biological membranes more positive and changes the permeability of blood-brain barrier culture models. Biochim Biophys Acta Biomembr. 2019;1861(9):1579-1591. IF=3.411

VII. Barna L, Walter FR, Harazin A, Bocsik A, **Kincses A**, Tubak V, Jósvay K, Zvara Á, Campos-Bedolla P, Deli MA. Simvastatin, edaravone and dexamethasone protect against kainate-induced brain endothelial cell damage. Fluids Barriers CNS. 2020;17(1):5. IF=4.47

VIII. Taneva SG, Krumova S, Bogár F, **Kincses A**, Stoichev S, Todinova S, Danailova A, Horváth J, Násztor Z, Kelemen L, Dér A. Insights into graphene oxide interaction with human serum albumin in isolated state and in blood plasma. Int J Biol Macromol. 2021;175:19-29. IF=5.162

IX. Santa-Maria AR, Walter FR, Figueiredo R, **Kincses A**, Vigh JP, Heymans M, Culot M, Winter P, Gosselet F, Dér A, Deli MA. Flow induces barrier and glycocalyx-related genes and negative surface charge in a lab-on-a-chip human blood-brain barrier model. J Cereb Blood Flow Metab. 2021;271678X21992638. IF=5.681

X. Dukay B, Walter FR, Vigh JP, Barabási B, Hajdu P, Balassa T, Migh E, **Kincses A**, Hoyk Z, Szögi T, Borbély E, Csoboz B, Horváth P, Fülöp L, Penke B, Vígh L, Deli MA, Sántha M, Tóth ME. Neuroinflammatory processes are augmented in mice overexpressing human heat-shock protein B1 following ethanol-induced brain injury. J Neuroinflammation. 2021 Jan 10;18(1):22. IF=5.793

Cumulative impact factor of other full papers: 53.589

Total cumulative impact factor: 65.764

Introduction

Epithelial outer, and endothelial inner barriers of the body are important defense systems to maintain homeostasis, and play a crucial role in drug absorption and transport. Culture models of biological barriers are important tools to study physiological functions, transport mechanisms, drug delivery and pathological processes. Tight intercellular junctions are fundamental features of epithelial and endothelial barriers *in vivo,* which are reflected in high electrical resistance and low passive permeability for hydrophilic and hydrophobic compounds. These physical and physico-chemical parameters describe the barrier integrity and function accurately. In more detail, the trans-endothelial/epithelial electric resistance (TEER) represents the paracellular ion mobility which gives information about the tightness of the intercellular junctions. The passive permeability of small, charged or electroneutral molecules describe the para- and transcellular pathways. Another important contributor to the barrier function could be the high negative surface charge of the cell monolayers, yet this is the least explored field of the physical properties of the biological barriers.

In the past 10 years, besides static models cultured on inserts, dynamic lab-on-a-chip (LOC)/organ-on-chip (OC) devices were developed to study cell-cell interactions, molecular pathways, pathological conditions and drug delivery in biological barriers. These models incorporate the use of fluid flow enabling the investigation of physiological-like functions such as receptor and mechanosensor expression, transport mechanisms, pathologies and drug delivery. LOC/OC devices became important tools since they provide controlled conditions for cellular signaling and external stimulus, and are able to track the development and changes in the barrier function.

Confluent monolayers of adherent epithelial or endothelial cells grown on culture inserts are widely used static models for intestinal, lung and blood-

brain barriers (BBB). The field of biochips modeling gut and blood-brain barriers has been rapidly evolving during the last fifteen years, while the focus of the devices being shifted according to the main interest of the corresponding study. Our aim was to develop a versatile LOC device that can be used for different kinds of biological barriers, and thus, for different kinds of approaches, as well. Also, the measurement of all crucial physical parameters, such as TEER and permeability assays, are included.

The negative surface charge of the cell layers is an important element of the defense system of barriers. A quantitative description of the surface electric properties of cell layers forming biological barriers is essential for the broader understanding of their function in physiological processes and diseases. The role of the negatively charged glycocalyx of the vascular endothelial barrier, for example, is well known in the protection of the cardiovascular system, and important in microbiological infections. Despite the recent boom in LOC devices, no biochip to determine the surface charge of intact cell layers forming biological barriers has been published, yet.

Goals of the thesis

The first aim of the study was to design and fabricate a versatile lab-on-a-chip device that can monitor all the crucial properties of biological barrier models. The structure is based on the commercially available culture inserts, so a top and bottom channel is separated by a porous culture membrane that supports the barrier-forming cells. The design should enable the mono- and co-culturing of different types of biological barriers. The trans-endohtelial/epithelial electric resistance can be measured with integrated, transparent gold electrodes and the top and bottom channel enable permeability assays. The whole surface of the culture membrane can be monitored by phase contrast microscopy. Immunohistochemistry can also be performed, and the cells on the removable culture membrane can be

investigated by fluorescent microscopy. In addition, tubes and pumps can easily be connected to the device, thus the introduction of periodic or constant fluid flow can mimic certain biological processes, for instance blood flow in veins.

The second goal was to improve the LOC device with the possibility of zeta potential measurement on cell monolayers. The strategy was to develop a streaming potential-based measurement technique, that can be used for determining the zeta potential. The transient signal of the streaming potential was planned to be recorded with a pair of Ag/AgCl electrodes, a voltage pre-amplifier and an oscilloscope. The results were ought to be compared to model simulations and laser-Doppler velocimetry method (LDv).

Materials and methods

LOC design and fabrication process

The device was formed by top and bottom channels, separated by a porous polyester (PET) membrane. The geometry of the channels enabled the measurement of TEER and the performance of permeability assays. The channels were fabricated from poly(dimethylsiloxane) (PDMS) by injection molding. The top and bottom sides of the channels were covered by plastic slides containing the integrated gold electrodes for TEER measurement. Luer-lock inlets were applied to provide easy access for the tubing. Ag/AgCl electrodes for the streaming potential measurement were placed in luer-lock connectors for the easy mounting on the inlets.

Zeta potential measurements: Detection of streaming potential

Development of streaming potential is a well-known electrokinetic phenomenon occurring in microfluidic channels. If the inner surface of the channel is covered with charges (intrinsic or adsorbed), it attracts counterions from the solution, and keeps them near the surface. Due to a balance of Coulomb attraction and Browninan motion, a diffuse double layer is formed

by the mobile ions and the fixed surface charges, the Gouy-Chapman layer (GCL). As a consequence, an electric potential gradient develops perpendicular to the membrane plane, screening the surface potential of the membrane across the GCL. If a fluid flow is applied in the channel, a major part of the counterion cloud of GCL, divided by a "slipping plane" to a moving part and a layer sticking the channel wall, will be grabbed by the solution under Poisseuille flow. The resulting flow of net charge along the channel represents an electric current called streaming current, and the accompanying streaming potential can be detected by an electrode pair separated alongside the channel. The streaming potential under stationary conditions is proportional to the surface potential of the shear plane called zeta potential, according to the Helmholtz-Smoluchowski equation. In this work, we measured a nonstationary (transient) streaming potential, in order to maximize the signal amplitude by applying high inlet flow rates. We provided both theoretical and experimental evidences that the amplitude of the transient signal is proportional to the zeta-potential at the surface, in this case, too.

Zeta potential measurements: Laser-Doppler velocimetry

LDv measures the electrophoretic mobility of charged particles with two collimated, monochromatic and coherent laser light beams, forming a set of straight fringes by interference. The moving particles go through the fringes and reflect light to a photodetector. The frequency of the reflected light's intensity fluctuation is proportional to the Doppler shift between the scattered and incident light, and the velocity of the particles is proportional to the Doppler shift. Using the Smoluchowski equation, the zeta potential ζ can be calculated.

Simulations

Model calculations were carried out on a flow channel by the COMSOL Multiphysics work package (Comsol Inc., USA) run on a personal computer, to describe time- and zeta potential dependence of the transient streaming

potential signal. The simulations were carried out by solving coupled differential equations of the Electrostatics, Transport of diluted species and Creeping flow work packages, using the Poisson approximation and the Nernst-Planck, and Navier-Stokes equations for the creeping flow of an incompressible fluid. The simulations were carried out in two steps: first, under no-flow conditions a stationary state was developed, while in the second step, a creeping flow was also introduced. The coupled differential equations were solved by the implicit method of Backward Differentiation Formula (BDF).

Cell cultures

To test the versatility of the barrier chip, both epithelial and endothelial monolayers were cultured and monitored under static and flow conditions, as was appropriate. Cell cultures were grown in a humidified, 37 °C incubator with 5 % CO_2 in both experimental setups. Caco-2 intestinal epithelial cells (ATCC, USA) were cultured under static conditions to model an epithelial barrier on the chip. Brain microvascular endothelial cell line hCMEC/D3 and primary rat brain endothelial cells were used as models of the blood-brain barrier on the chip.

Results and discussion

Design and operation of the device

The basic structure of the barrier device mimics that of the culture inserts: top and bottom channels separated by a porous PET membrane. The two parallel channels were made of PDMS. The arrangement of our channels enables a large overlapping area (ca. 1 cm^2), allowing a large surface and a big sampling size for *in vitro* permeability studies. The thin, transparent electrodes grant a special advantage to the chip, permitting a continuous visual monitoring of the cells by a microscope, above the entire membrane surface, during the full time span of the experiment.

We tested the impedance spectra of both transparent ITO and gold electrode pairs as possible candidates. In the low-frequency regime (between 1Hz and 1kHz) the electric double layer formed upon electrode polarization hinders the correct measurement of the ohmic resistance of the chip, while in the high-frequency regime (above 3 MHz), the reduced values of ionic mobilities limit conductivity. In between the two extremes, the sample resistance is nearly constant, but its value is an order of magnitude smaller with gold electrodes than with the ITO ones.

The Ag/AgCl zeta electrodes were inserted in luer connectors, so they could be mounted easily. A programmable syringe pump fed the cells during the growth period, while the TEER values were recorded and the monolayer was monitored with a phase contrast microscope, each day. After the cell monolayers reached about 90% confluency, a peristaltic pump was introduced for constant flow, to mimic the shear stress of the blood stream for 1 day.

The streaming potential was measured with the Ag/AgCl electrodes between the inlet and outlet sides of the top channel. For the recording, the flow was periodically stopped and restarted after equilibration of the ions close to the surface of the cell monolayer. This case is beyond the scope of the Helmholtz-Smoluchowsky equation establishing a linear relationship between the zeta and steady-state streaming potentials, so we presented experimental and theoretical evidence for the proportionality of the zeta potential and the amplitude of the transient streaming potential in our approach, as well.

The streaming potential feature

The streaming potential was measured in the device either on a test membrane or on cell monolayers, in the form of a transient potential difference evolving between the inlet and outlet electrodes, due to migration of ions from the vicinity of the negatively charged surface of the channel under flow conditions. The negative charge derives from the overwhelming anionic groups on the surface of the confluent cell monolayer due to the lipid

headgroups and the surface glycocalyx in the BBB experiments, or from the sulfate groups of the Nafion membrane in the control measurements. The electric double layer close to a charged surface has a different ion concentration compared to the solution. If flow is applied to the system, the mobile part of the GCL containing an excess number of positive counterions moves towards the outlet electrode, and temporarily increases the positive charge density in the larger volume of the socket of the electrode, giving rise to an increase in electric potential, as compared to the reference electrode. Model calculations and control experiments using the LDv method proved that the amplitude of this transient streaming potential signal is proportional to the zeta potential of the membrane surface.

Simulations

In order to give a theoretical background for the measured transient streaming potential signals, we carried out model calculations on a flow channel by the COMSOL Multiphysics work package. The dynamics of the system was modelled in two steps: 1) to establish stationary conditions without flow, first the system was let to equilibrate according to the Poisson-Botzmann-Nernst-Planck approximation, assuming electro-neutrality of the channel-fluid system; 2) in the second step, a creeping flow with a proportional average velocity was applied to the inlet of the channel, and the electric potential was measured on two probe planes placed in front of and behind the charged surface, along the long axis of the channel. In order to establish the connection between the simulated signal amplitudes and the zeta potential, the latter was swept two orders of magnitude, and the simulated time-evolution of the streaming potential functions was recorded. The dependence of the amplitudes of these curves as a function of the zeta potential shows a clear linear relationship, in full concert with the experiments.

Cell cultures

In order to illustrate the main features of our LOC device, it was used to model three different biological barriers with cell cultures: human intestinal epithelial and brain endothelial cell lines, as well as a triple primary co-culture BBB model, were tested for barrier function in the chip. The results for both barrier models were compared to conventionally used static culture insert models. We compared the models using the physical properties: TEER and passive permeability of fluorescently labeled molecules.

The Caco-2 intestinal epithelial cell line formed the tightest monolayer among the 3 models in the microdevice, with higher TEER and lower passive permeability values. Cell morphology, good resistance and permeability properties of Caco-2 cells in the new device were similar to data obtained on Transwell culture.

The human hCMEC/D3 brain endothelial cell line is a well-characterized, simplified *in vitro* model of the BBB. In the LOC device, after reaching almost full confluency, constant flow of the culture media was introduced to mimic the hydrodynamic effects of the blood flow. During the experiments, non-flow (static) and under-flow (dynamic) conditions were also tested in the device. The TEER results of the static mode showed good correlation with the culture inserts, while the dynamic had significantly higher resistance values. The permeability values of the static and dynamic modes showed the same pattern as the TEER for larger marker molecules. The permeability of the static device was lower compared to the inserts, but we could not use the exact same membrane, so the effect of membrane thickness on permeability coefficients cannot be excluded.

Our primary-cell-based triple co-culture BBB model was characterized for the first time in a miniaturized flow chip device. Co-culturing with glial cells and pericytes on Transwell inserts in the anatomical position have proved to be the most efficient in the induction of barrier properties in brain endothelial

cells; better than double cultures using only brain endothelial cell and glial cell or brain endothelial cell and pericyte. In this model, brain endothelial cells are not directly contacting pericytes or glial cells.

TEER values measured under static and dynamic conditions were not significantly different. These resistance values are lower than data obtained on culture inserts. The permeability for small marker molecule sodium fluorescein on the triple BBB model grown on cell culture inserts was higher compared to the static biochip model. There was no difference in the flux of the other two permeability markers after flow conditions, neither in the static chip compared to the dynamic model, nor in the chip models compared to the culture inserts. In our setup, the low-shear-stress exposure did not elevate the resistance of primary endothelial cells.

Effects of surface charge modifications and measurement of streaming potential on the brain endothelial cell line model hCMEC/D3

The main sources of the, usually negative, surface charge densities of cells are the lipid head groups of the plasma membrane, and the so-called glycocalyx, composed of highly negatively charged polysaccharide chains at the surface of the cells. The surface charge density of brain endothelial cells can be modified by both enzymatic digestion of the glycocalyx or cationic lipophilic molecules which are inserted to the plasma membrane.

We used two clinically relevant surface charge modulators. The antiarrhythmic intravenous drug, lidocaine incorporates into the plasma membrane of vascular endothelial cells. Neuraminidase, a glycoside-hydrolase enzyme, cleaves sialic acids and reduces the amount of negative charge on the glycocalyx, thus mimics glycocalyx shedding observed in sepsis.

The results clearly prove the feasibility of the new “zeta-feature” of the device, at the same time provide a calibration factor for the determination of the zeta potential of the cell layer. The 1 U/ml neuraminidase treatment

effectively altered the zeta potential and changed the pattern of the glycocalyx. The 1000 uM lidocaine did not change the viability of the cells while effectively intercorporate the plasma membrane of the cells and altered the zeta potential. It was also shown that the sensitivity of the technique is sufficient to measure changes of the surface charge properties of the BBB layer that was demonstrated to be linked to altered penetration of charged molecules and nanoparticles.

Conclusion

The first goal of the study was to develop a general-purpose device that can be used to investigate multiple types of biological barriers as mono-, double- or triple cultures monitoring the electric resistivity and molecular permeability of the (co-)cultured cells under no-flow or low-flow conditions, at the same time allowing microscopic visualization of the whole membrane surface. The miniaturized chip was successfully used to model two different biological barriers with three types of cell cultures.

The second goal was to develop a method to quantify the surface charge properties of cell monolayers. We successfully measured the streaming potential of a biological barrier culture model with the help of our versatile lab-on-a-chip device upgraded with two Ag/AgCl electrodes. The inclusion of the “zeta electrodes”, a voltage preamplifier and an oscilloscope in our set-up made it possible to successfully record signals describing the surface charge properties of brain endothelial cell monolayers, used as a barrier model in our experiments.