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M-ERA.NET nanoPD
DEVELOPMENT OF A NOVEL ORGANS-ON-A-CHIP PLATFORM FOR NANODRUG DELIVERY AND FUNCTIONALITY TESTING TO TREAT PARKINSON'S DISEASE
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FINAL REPORT

Organs on chip device

In the present project the chip device was based on the previously patented microfluidic and micro-electronic integrated biochip to monitor cell-culture-based barrier models (Walter et al. 2016, doi.org/10.1016/j.snb.2015.07.110). This device allows the co-culture of 2 or more types of cells; flow of culture medium; visualization of the entire cell layer by microscopy; real-time transcellular electrical resistance monitoring; permeability measurements. First a chip manufacturing protocol was established together with an individual registry system for each biochip to allow follow up, reproducibility and easy problem solving. The barrier chip device was modified as follows: (1) the previously used glass slides were changed to plastic slides with better properties for the attachment of the gold layers; (ii) the design of the mask for the gold electrodes (to measure transendothelial electrical resistance) was changed, this allowed better attachment of the PDMS based channels to the slides and prevented leakage of the culture medium; (iii) the previous PDMS inlets and outlets were changed to new types of luer inlets/outlets which provided better and easier attachment and removal of the flow tubes; (iv) for the assembly of the chip the synthetic glue was replaced by plastic screws along the two longer sides of the slides making the assembly/disassembly quicker and the device reusable. The new version of the chip device provided better conditions for the barrier experiments.

Brain endothelial cells forming the blood-brain barrier have a highly negative cell surface charge and in a previous project we demonstrated that this charge contributes to the transfer of nanoparticles across the barrier (Mészáros et al. 2018, doi: 10.1016/j.ejps.2018.07.042). We have also proved that a charged lipophilic therapeutic drug, lidocaine, by altering cell surface charge modifies blood-brain barrier properties and permeability of other charged lipophilic molecules (Santa-Maria et al. Biochim Biophys Acta Biomembr. 2019, 1861(9):1579-1591, doi: 10.1016/j.bbamem.2019.07.008, IF 3.411). Despite the importance of cell surface charge in the function of biological barriers, no chip device has been prepared to measure cell surface charge properties of confluent barrier cell monolayers. We successfully designed and fabricated a dynamic chip device to monitor transcellular electrical resistance, as well as streaming potential parallel to the surface of cell layers (Kincses et al. Lab Chip. 2020, 20(20):3792-3805, doi: 10.1039/d0lc00558d, IF: 6.799). We measured the streaming potential of a blood-brain barrier culture model with the help of our previously published chip device equipped with two Ag/AgCl electrodes, for the first time. Data obtained on the new chip device were verified by comparing streaming potential results measured in the chip and zeta potential results obtained by the commonly used laser-Doppler velocimetry method and model simulations. The new chip device was important to gain meaningful new information on how cell surface charge is linked to barrier function in both physiological and pathological conditions, highly relevant for the nanoPD project.

As part of the project, we wrote two invited review articles related to chip devices. One of them was focused on the methods, technical details and problems related to the measurement of transendothelial electrical resistance across the blood-brain barrier in vivo and in vitro, including chip devices (Vigh et al. Micromachines, 2021, 12(6):685, doi: 10.3390/mi12060685, IF: 3.523). The second paper was prepared together with our Taiwanese research partners in the nanoPD consortium on the use of sensors in blood-brain barrier-on-a-chip devices with an emphasis on current practice and future directions (Kincses et al. Biosensors, 2023, 13(3):357, doi: 10.3390/bios13030357, IF: 5.4).

Blood-brain barrier model

The microfluidic chip device developed by our group allows the study of blood-brain barrier properties in dynamic conditions. We studied a blood-brain barrier model, consisting of human endothelial cells derived from hematopoietic stem cells in co-culture with brain pericytes, in the chip device to study fluid flow on the regulation of endothelial, blood-brain barrier and glycocalyx-related genes and surface charge. The highly negatively charged endothelial surface glycocalyx functions as mechano-sensor detecting shear forces generated by blood flow on the luminal side of brain endothelial cells and contributes to the physical barrier of the blood-brain barrier. Despite the importance of glycocalyx in the regulation of blood-brain barrier permeability in physiological conditions and in diseases, the underlying mechanisms remained unclear. The MACE-seq gene expression profiling analysis showed differentially expressed endothelial, blood-brain barrier related and glycocalyx core protein genes after fluid flow induced shear stress, as well as enriched pathways for extracellular matrix molecules. We observed increased barrier properties, a higher intensity glycocalyx staining and a more negative surface charge of human brain endothelial cells in dynamic conditions. Our work was the first study to provide data on blood-brain barrier properties and glycocalyx of brain endothelial cells in a chip device under dynamic conditions and confirmed the importance of fluid flow for blood-brain barrier culture models (Santa-Maria et al. J Cereb Blood Flow Metab. 2021, 41(9):2201-2215, doi: 10.1177/0271678X21992638, IF: 6.960).

Since the properties and handling of the barrier models were key elements of the project, we have contributed to a critical review and guidelines on transport studies using in vitro blood-brain barrier models (Santa-Maria et al. Handb Exp Pharmacol. 2022, 273:187-204, doi: 10.1007/164_2020_394). An invited review was written on the importance of the brain endothelial surface charge, glycocalyx, and blood-brain barrier function, including nanoparticle internalization and transfer (Walter et al. Tissue Barriers. 2021, 9(3):1904773, doi: 10.1080/21688370.2021.1904773).

Nanodrug delivery systems

The nanoparticle targeting system developed previously by our team (Mészáros et al. 2018, doi: 10.1016/j.ejps.2018.07.042) was further characterized. We showed that targeting vesicular nanoparticles with both alanine and the tripeptide glutathione enhanced the delivery of protein cargo into cultured brain endothelial cells, brain pericytes, astrocytes and neurons. We demonstrated the ability of the targeted nanovesicles to deliver their cargo into astroglial cells after crossing the blood-brain barrier in vitro. These data indicate that dual-labeling of nanoparticles with alanine and glutathione can potentially be exploited to deliver drugs, even biopharmacons, across the blood-brain barrier and into multiple cell types in the brain (Porkoláb et al. Pharmaceutics. 2020, 12(7):635, doi: 10.3390/pharmaceutics12070635, IF: 6.321).

Our group have previously demonstrated that glutathione can be successfully used as a targeting ligand to increase the internalization of nanoparticles in brain endothelial cells. Docking of nanoparticles by targeting ligands on cell membranes is the first step for the initiation of cellular uptake. To investigate this initial docking step between glutathione and the membrane of living brain endothelial cells, we applied an innovative optical method recently developed by our colleagues at the Biophysical Institute of BRC. We described a microtool, with a task-specific geometry used as probe, actuated by multifocus optical tweezers to characterize the adhesion probability and strength of glutathione-coated surfaces to the plasma membrane of endothelial cells. The binding probability of the glutathione-coated surface and the adhesion force between the microtool and cell membrane were measured in a novel arrangement: cells were cultured on a vertical polymer wall and the mechanical forces were generated laterally, and at the same time perpendicularly to the plasma membrane. The adhesion force values were also determined with more conventional atomic force microscopy measurements using functionalized colloidal probes. The optical trapping-based method was found to be suitable to measure very low adhesion forces (≤ 20 pN) without high level of noise, which is characteristic for AFM measurements in this range. The functionalized microtools directed by holographic optical tweezers helped to characterize the adhesion step of

nanoparticles initiating transcytosis and will be useful to select novel ligands to target nanoparticles in the future (Fekete et al. ACS Appl Mater Interfaces. 2021, 13(33):39018-39029, doi: 10.1021/acsami.1c08454, IF: 10.383).

To develop drug delivery systems for the central nervous system other targeting ligands for nanosized drug carriers were also investigated. The research aim was to design a nanoscale drug delivery system for a more efficient transfer of donepezil across the blood-brain barrier. This anticholinergic drug is not only used clinically in the therapy of Alzheimer's disease, but also for mild cognitive impairment in Parkinson's disease. Rhodamine B-labeled solid lipid nanoparticles with donepezil cargo were prepared and targeted with apolipoprotein E (ApoE), a ligand of blood-brain barrier receptors (scavenger receptors, LDL receptor and LRPs). The nanoparticles were characterized by measurement of size, polydispersity index, zeta potential, thermal analysis, Fourier-transform infrared spectroscopy, in vitro release, and stability. Cytotoxicity of nanoparticles were investigated by a metabolic assay and impedance-based cell analysis. ApoE-targeting increased the uptake of lipid nanoparticles in cultured brain endothelial cells and neurons. The permeability of ApoE-targeted nanoparticles across a co-culture model of the blood-brain barrier was also elevated. Our data indicated that ApoE, which binds blood-brain barrier receptors, can potentially be exploited for successful brain targeting of solid lipid nanoparticles (Topal et al., Pharmaceutics, 2020, 13(1):38, doi: 10.3390/pharmaceutics13010038, IF: 6.321).

In cooperation with our Taiwanese partner in the nanoPD consortium (Dr. Jeng-Shiung Jan, National Cheng Kung University, Tainan) several types of targeted nanodrug delivery systems were investigated for blood-brain barrier transfer. From these, peptide targeted cyclodextrins and 3-armed polypeptide nanocarriers decorated with alanine and glutathione, seems to be the most promising. The peptide targeting a brain endothelial cell receptor was linked to cyclodextrins and complexed with adamantane labelled with the fluorescent dye sulforhodamine B. The construct showed no cellular toxicity. We measured a temperature dependent active uptake in brain endothelial cells and a high permeability across the blood-brain barrier model. Additional in vivo experiments in C. elegans and mice were performed in Taiwan. The investigations on the peptide targeted cyclodextrins were carried out with the cooperation of Cyclolab, a cyclodextrin SME company from Budapest. There is an ongoing discussion and negotiation process between the partners about the submission of a patent.

Regarding the 3-armed polypeptide nanocarriers, because these were investigated on integrated systems incorporating Parkinson’s disease and healthy brain organoids, the results are summarized in the “Integration” section.

An important part of the project was to investigate targeted and non-targeted magnetic nanoparticles on blood-brain barrier models. The literature is very controversial, with very few papers using appropriate blood-brain barrier models and well characterized magnetic fields. We designed a systematic study to explore the penetration of magnetic nanoparticles across a human blood-brain barrier model testing different magnetic field strength. We found that in contrast to literature data and our expectations 100 mT non-uniform magnetic field does not help the transfer of either untargeted or glutathione targeted dextran coated fluorescent magnetic nanocarriers, purchased from commercial sources, across the culture model of the blood-brain barrier. We analyzed the intracellular fate of the magnetic nanoparticles by transmission electron microscopy integrated with elemental analysis and found that these were trapped intracellularly, most probably in lysosomes (manuscript in preparation).

Integration: chip device, nanodrug delivery systems, blood-brain barrier models and brain organoids

With our partners in the nanoPD consortium from the University of Luxembourg we worked out the protocol of the co-culture of the human blood-brain barrier model with Parkinson’s disease and healthy midbrain organoids in a static model using culture inserts. We have redesigned the blood-brain barrier microfluidic chip to hold 3 midbrain organoids in the bottom channel. We successfully integrated the human blood-brain barrier model and the brain organoids in the chip device with culture medium flow in

the top channel mimicking the blood flow. The blood-brain barrier model integrity was well retained as evaluated by functional measurements (transendothelial electrical resistance, paracellular marker permeability) and the organoids showed characteristics similar to the static model optimized in the culture inserts (immunohistochemistry for neuronal and glial markers).

Partners at the University of Luxembourg led by Prof. Jens Schwamborn successfully demonstrated the integration of a microfluidic chip device for brain organoids with oxygen and dopamine sensors. With our partner from Taiwan (Dr. Hung-Yin Lin, National University of Kaohsiung) we completed experiments with molecularly imprinted polymer composite nanoparticles (MIPs) binding α-synuclein, a key protein in the pathogenesis of Parkinson's disease. We demonstrated that magnetic MIPs bind α-synuclein and this can be visualized by immunostaining and confocal microscopy, and brain endothelial cells tolerate well MIP incubation. These MIPs were also incorporated to electrochemical sensors and α-synuclein levels were measured from culture medium of brain organoids indicating their diagnostic potential. Most importantly, these MIPs were able to decrease the α-synuclein content of a transfected cell line indicating that these nanoparticles may also have therapeutic potential in synucleopathies, like Pakinson's disease (Lee et al. Cells. 2022; 11(16):2584. doi: 10.3390/cells11162584. IF: 6.0).

Since targeting is the most important step in the delivery of nanoparticles across biological barriers, we continued our studies on the investigation of specific ligand combinations to improve blood-brain barrier crossing. We decorated vesicular nanoparticles with the combination of solute carrier ligands ascorbic acid, leucine and the tripeptide glutathione. We measured elevated cellular uptake of the targeted nanoparticles as compared to untargeted nanovesicles. The cellular uptake was temperature and energy-dependent based on metabolic inhibition. The process was decreased by filipin and cytochalasin D, indicating that the internalization of nanoparticles was partially mediated by endocytosis. The uptake of the triple-targeted nanoparticles increased after shifting the negative zeta potential of endothelial cells to more positive with a cationic lipid or by enzymatic cleaving of the glycocalyx that suggests the role of surface charge in cellular penetration of these nanovesicles. We revealed that the targeted nanoparticles elevated plasma membrane fluidity, indicating the fusion of nanovesicles with endothelial cell membranes. When nanoparticles were examined in a complex system that contained brain organoids in the basal compartment, triple targeting promoted their penetration across the co-culture model of the blood-brain barrier and facilitated the delivery of the large biomolecule cargo into midbrain organoids. We could conclude that labeling nanoparticles with three different ligands of multiple transporters of brain endothelial cells can promote the transfer across the blood-brain barrier and brain delivery of molecules (Veszelka et al. Pharmaceutics. 2022, 14(1):86, doi: 10.3390/pharmaceutics14010086, IF: 5.4).

To our request, Dr. Jan and his co-workers has prepared 3-armed poly(l-glutamic acid) nanoparticles targeted with alanine and glutathione. We could prove using a non-vesicular nanoparticle that the combination of these molecules can also increase the internalization and permeability of a nanocarrier using a human co-culture model of the blood-brain barrier. Alanine and glutathione dual-targeted polypeptide nanoparticles showed good cytocompatibility and elevated cellular uptake in a time-dependent and active manner. The targeted polypeptide nanoparticles not only had a high permeability across the blood-brain barrier model, but could subsequently enter midbrain organoids derived from healthy and Parkinson's disease patient-specific stem cells. These results proved that poly(l-glutamic acid) nanoparticles can be used as nanocarriers for nervous system application and that the right combination of molecules that target cerebral endothelial cells, in this case alanine and glutathione, can facilitate drug delivery to the brain (Mészáros et al. Cells. 2023, 12(3):503, doi: 10.3390/cells12030503, IF: 6.0). Finally, we have demonstrated the higher uptake and penetration of the targeted polypeptide nanoparticle loaded with ibuprofen and dopamine on the human blood-brain barrier model using the chip device containing midbrain organoids (manuscript in preparation) and with this final integration step could reach the major research aim of the project.

The main funding source for the project was the present grant. Since many young researchers who participated in the work were recipients of different stipends and fellowships from several foundations and other sources (Bolyai research fellowship, UNKP, Kuffler, Richter, etc.) these additional supports contributing to their personal income were also acknowledged in the funding sections of the papers.