Scientific report on the exchange grant between 15 April - 15 July, 2010 entitled

"Protein Corona Role in Nanoparticle Trafficking in the Cerebral Nervous System"

Purpose of the visit

The passage of nanoparticles (NPs) to the central nervous system (CNS) is possible via the blood-brain barrier (BBB) and via the olfactory nerves. The BBB is comprised primarily of brain capillary endothelial cells which form tight junctions resulting in an exceptionally low permeability and penetration of substances from the systemic circulation into the CNS. Experimental evidence suggests that the initiation and promotion of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Pick's disease, are associated with oxidative stress and accumulation of high concentrations of metals (such as copper, aluminium, zinc, and especially iron) in brain regions associated with function loss and cell damage.

The aim of the visit was to establish and validate an *in-vitro* BBB model using immortalized human endothelial cells (hCMEC/D3), and to determine if engineered nanoparticles present a significant neuro-toxicological risk to humans (e.g. correlation between nanoparticle access to the brain with induction of oxidative stress and/or protein fibrillation). *In vitro* models of such a barrier have already been developed in the group of Prof. K. Dawson, where this project has been carried out, and can be used to assess the capability of NPs to cross physiological barrier and follow the localisation in time and space of NPs, in a quantitative and reproducible manner. This has been achieved by a combination of electron microscopy and fluorescence based techniques, including fluorescence microscopy and flow-cytometry. In particular particles of different sizes and surface composition were used in order to relate the eventual translocation behaviour to the different biological coronas that will be formed upon contact with serum. Ultimately, NPs with chemically bound proteins were used in order to manipulate the corona layer and to elucidate the protein-receptor interactions that are involved in the transport of NPs across the BBB model.

Description of the work carried out during the visit

In this project, an *in vitro* BBB model was established with an immortalized hCMEC/D3 cell line (human brain capillary endothelial cells) and used for the investigation of the ability of 50nm SiO_2 NPs, 40nm and 100nm polystyrene (PS) NPs trafficking through the barriers. The hCMEC/D3 cell line was developed in 2005 by immortalization of primary human BCEC through expression of hTERT and the SV40 large T antigen via a lentiviral vector system. hCMEC/D3 cells maintain most of the key characteristics of *in vivo* BBB and offer a good model for drug predictions or any other molecules entry into the CNS.

In recent years, some researchers have made a lot of effort to improve the hCMEC/D3 *in vitro* model to better mimic *in vivo* BBB function. As this cell line can differentiate into a monolayer in culture, it has been adopted this cell line's property by seeding onto a permeable filter, a so called „transwell" system which is made of an insert filter and an acceptor well (Fig.1). Once a confluent monolayer is formed, the apparent permeability of different NPs was calculated to evaluate the extent of substance crossing which is called „fluid fluxes".

Many different NPs in size and material were screened, also coating or adjusting the surface of NPs to manipulate biologically the behaviour of the nanomaterial (additionally bioactive molecules can be easily chemically-linked and/or physically-adsorbed to the NPs surface). Their intrinsic hydrophilicity and biocompatibility makes them perfect candidates for controlled drug-delivery applications.

The NPs used for the transport study were characterized by dynamic light scattering (DLS).

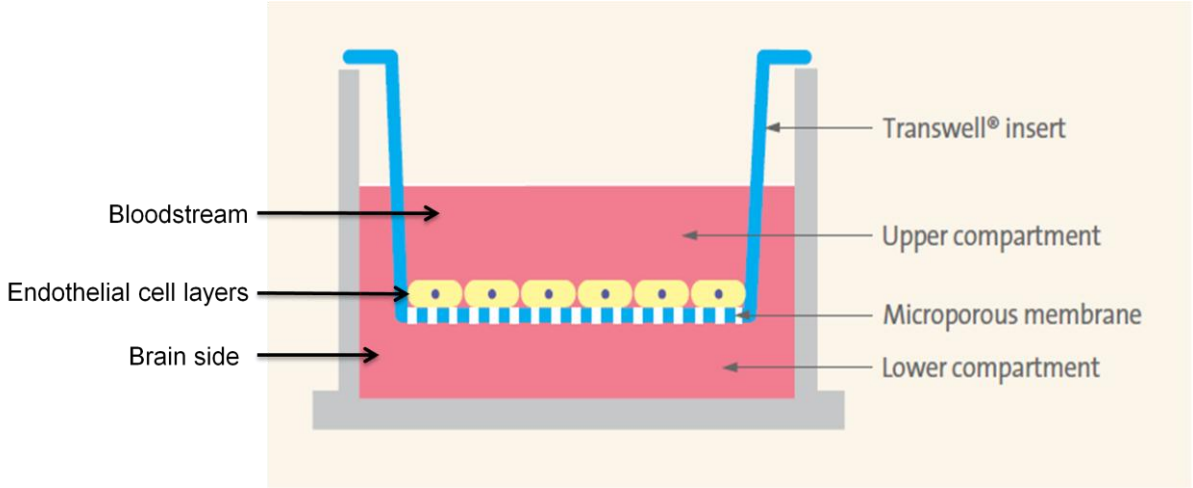


Fig.1 Transwell system

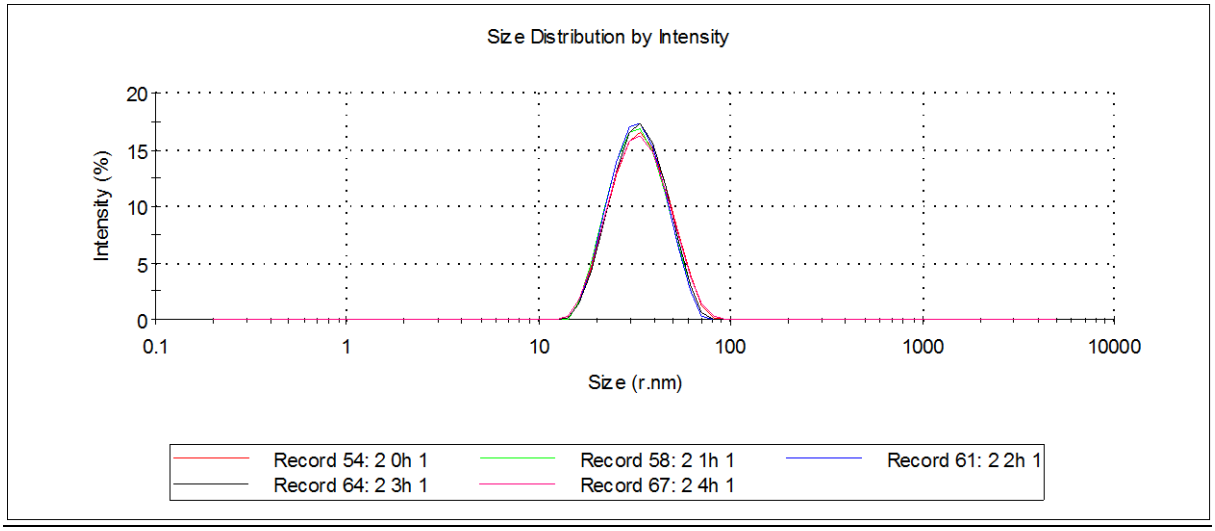
Description of the main results obtained

Dynamic Light Scattering (DLS)

Malvern Zetasizer NS was used to measure the size of NPs. The solution of particles was incubated inthe same medium used for the transport assay at 37°C in an orbital shaker over 4 hours and sampled each hour.

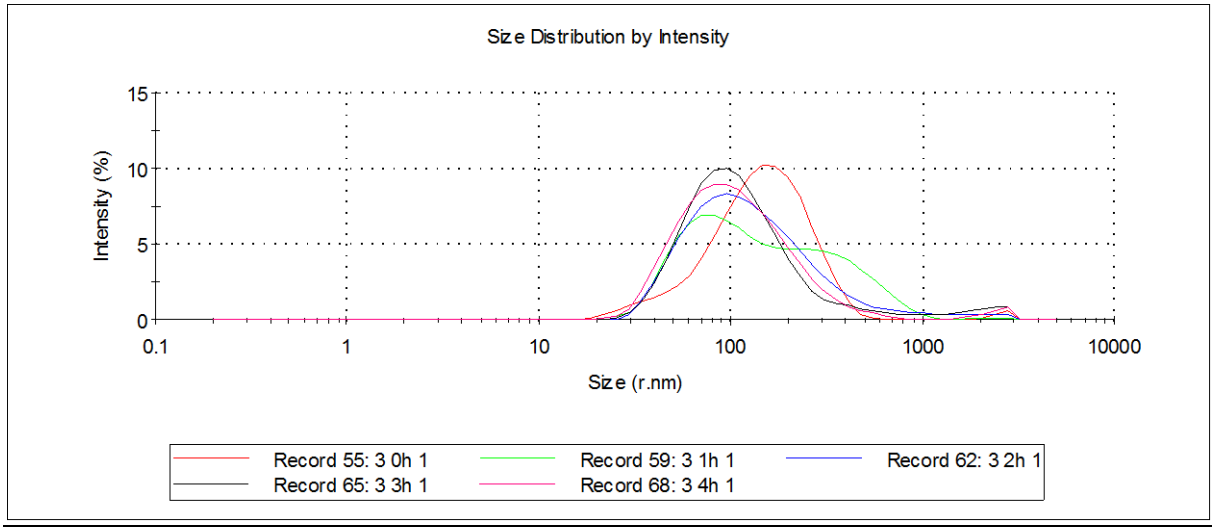
40nm PS-Carbox (0-1-2-3-4 h)

sample	Z aver (d.nm)	Peak 1 (nm)	% Intensity	PDI	WIDTH (nm)	Attn
0 h	31,85	35,45	100	0,095	11,75	7
1 h	30,75	34,41	100	0,100	10,88	6
2 h	30,87	34,07	100	0,087	10,89	6
3 h	31,33	34,75	100	0,094	10,84	6
4 h	31,76	35,24	100	0,146	11,95	6



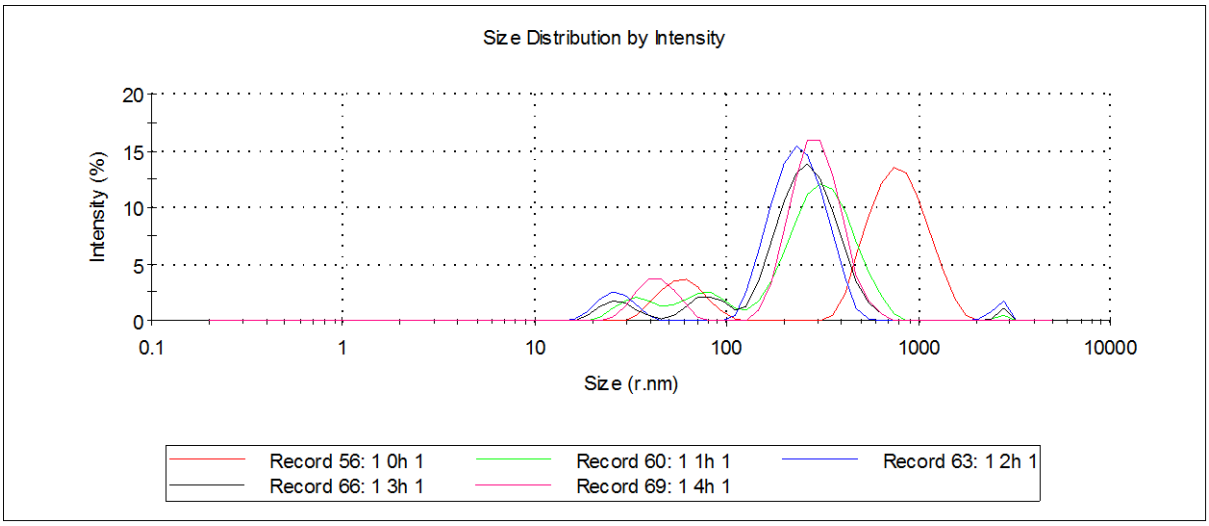
100nm PS-Carbox (0-1-2-3-4 h)

sample	Z aver (d.nm)	Peak 1 (nm)	% Intensity	PDI	WIDTH (nm)	Attn
0 h	122,4	158,3	98,9	0,337	85,83	6
1 h	107,7	199,2	99,5	0,378	178,1	6
2 h	100,7	165,1	97,7	0,333	170,7	6
3 h	93,71	131,7	95,5	0,316	117,8	6
4 h	90,26	124,7	98,0	0,293	91,89	6



50nm Si-plain (0-1-2-3-4 h)

sample	Z aver (d.nm)	Peak 1 (nm)	% Intensity	PDI	WIDTH (nm)	Attn
0 h	288,3	825,7	82,3	0,837	275,9	7
1 h	175,0	334,4	77,9	0,632	123.0	7
2 h	185,1	247,4	88,0	0,576	77,12	7
3 h	189,6	279,9	83,4	0,569	99,38	7
4 h	201,7	304,0	84,4	0,623	91,91	7



Transcellular epithelium resistance measurements and FITC-dextran studies

The *in vitro* BBB system was prepared on 12-well format transwell plates with collagen-I polyester filters (12 mm diameter; 0.4 and 3.0 μm pore size). Transport assays have been conducted 7-10 days after seeding.

The integrity of the BBB model was evaluated here by TEER (Transcellular Epithelium Resistance measurement). Replicates for each time points were 12 and resistance from blank wells was subtracted. The low TEER values reached are consistent with TEER values reported in the literature for this model.

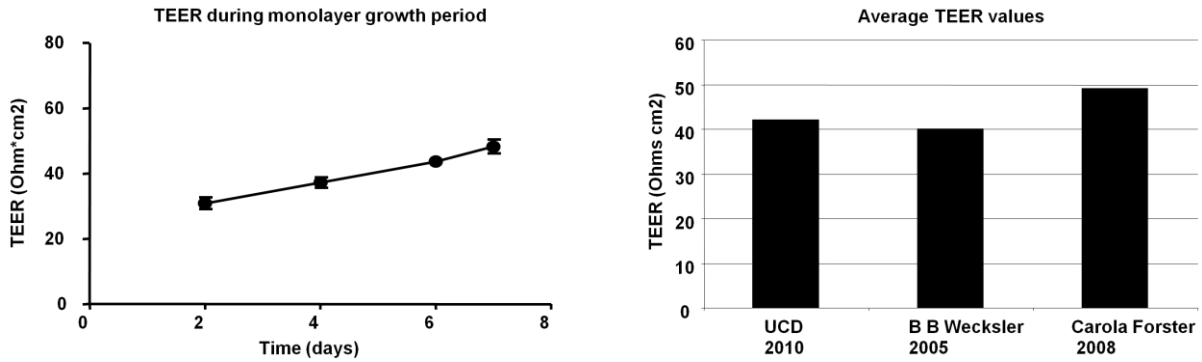
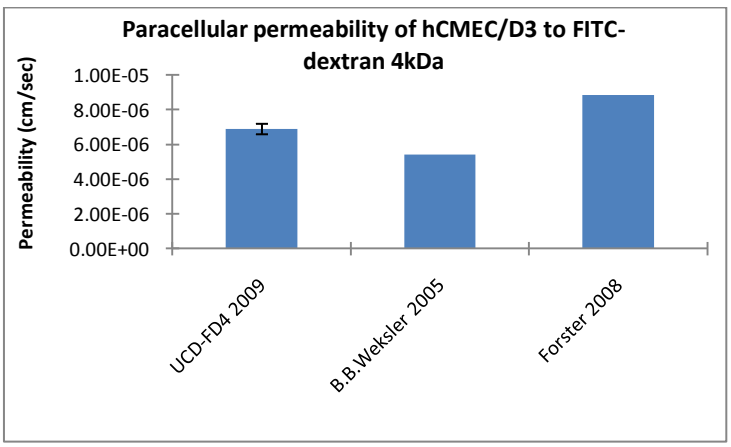


Fig.2 TEER values of the BBB *in vitro* model on 3.0μm pore-size filter.

We measure at the same time the permeability of the BBB to FITC Dextran-4kDa (FD4), a small molecule to evaluate the paracellular permeability, or „leakiness" of our model. Experimental permeability data were compared with published values of permeability coefficients for the same compounds. Here permeability values of FD4 from our barrier system were in the range from $5x10^{6}$ (reported by Weskler, 2005) to $8x10^{6}$ (reported by Forster, 2008). Therefore, the barrier is adequate tightness for the transport study.



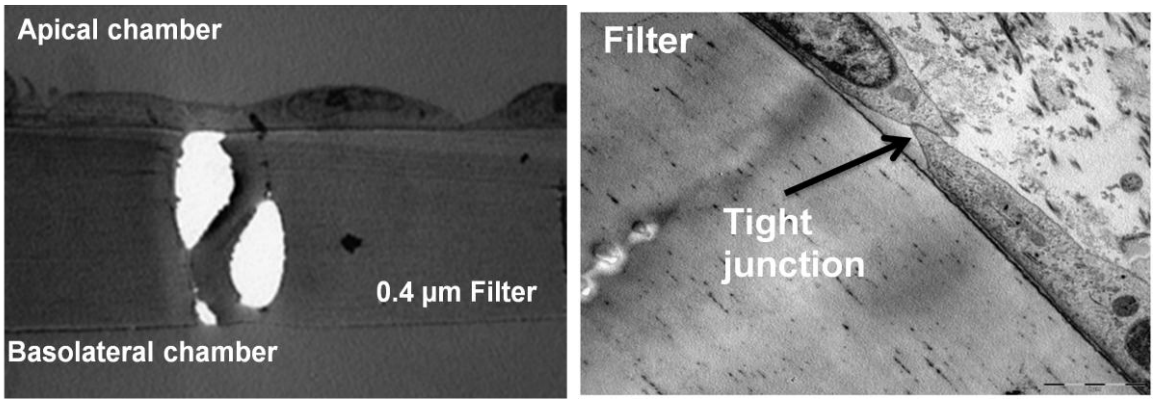
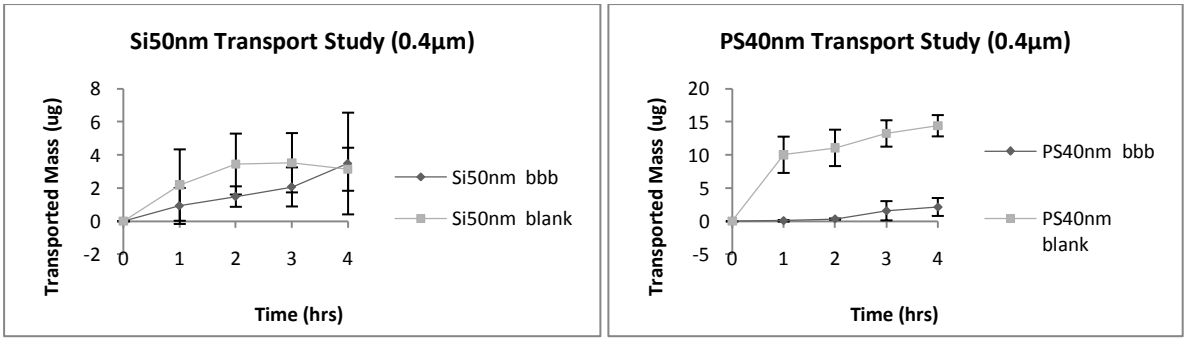


Fig. 3 Electron Microscopy images of the hCMEC/D3 monolayer on the 0.4 µm pore-size filter. The formation of tight junctions is confirmed.

Transport study of silica and polystyrene particles

50nm SiO_2, 40nm and 100nm PS particles were used to test their own permeability through BBB in time lapse studies. Each particle's concentration was 100 µg/ml. Time range is 4 hours which was split into 4 equal time points (1h, 2h, 3h and 4h) to plot particle fluxes to the basolateral side of the *in vitro* BBB. After 4-h incubation (37°C) 50nm SiO_2 and 40nm PS NPs all showed an increase in the number of transported mass through the barrier. By the end of 4 hours, 3.48± 3.0 µg SiO_2 particles and 2.15±1.3 µg 40nm PS particles were transported via BBB at 37°C using 0.4µm pore-size transwell.

Similarly to what has been done with the 0.4µm pore-size transwell, a transport study over 4 hours time has been performed on 3.0µm pore-size transwell using also 100nm PS particles. After 4-h incubation (37°C) 50nm SiO_2, 40nm and 100nm PS NPs all showed an increase in the number of transported mass through the barrier. By the end of 4 hours, 3.00± 0.12µg SiO_2 particles, 2.10±0.75µg 40nm PS particles and 3.10± 0.10 µg 100nm PS particles were transported via BBB at 37°C using 3.0µm pore-size transwell (Fig.4).



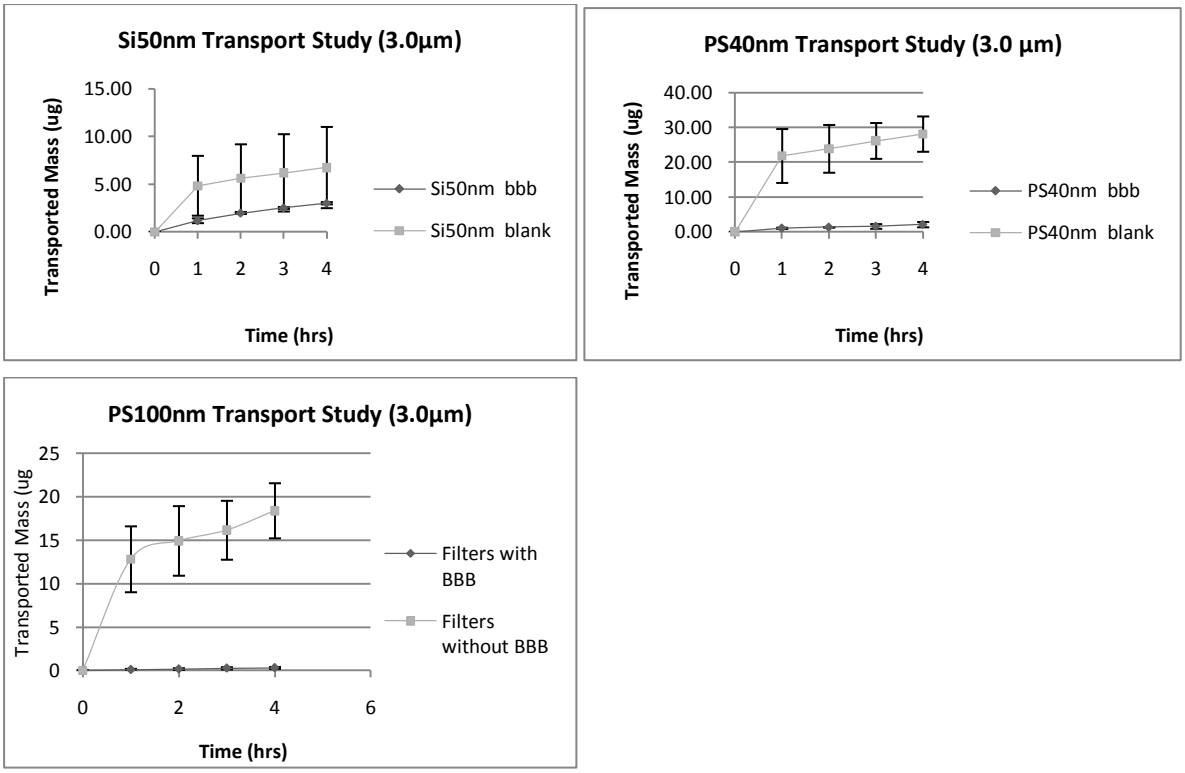


Fig.4 Different kinds of nanoparticles trafficking across the *in vitro* BBB-model using 0.4µm and 3.0µm pore-size transwell.

With the same concentration and same temperature (37°C), silica and polystyrene particles were dosed into the apical side of the *in vitro* BBB-model to compare their permeability. (Fig. 4 and fig. 5)

According to the transport and apparent permeability studies, 50nm SiO_2 particles has a dominant advantage in penetrating the BBB compared to the polystyrene particles.

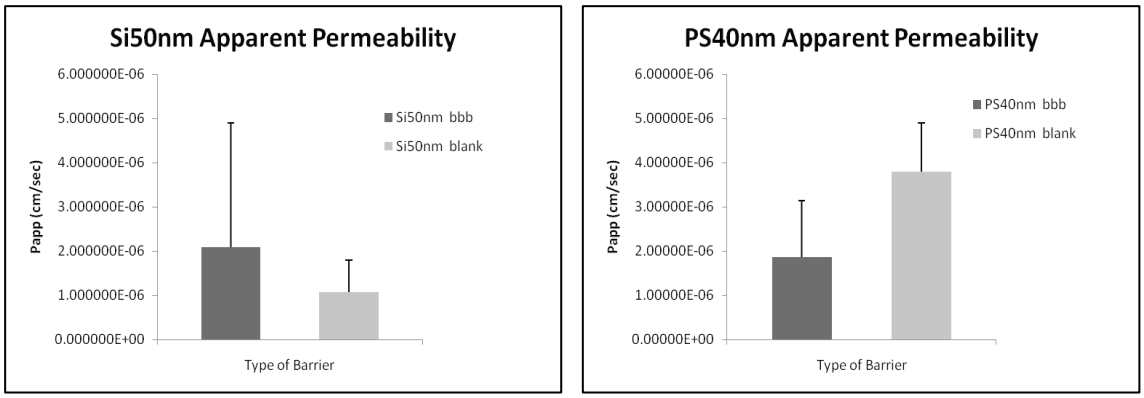


Fig. 5 Apparent permeability of different nanoparticles through *in vitro* BBB-model after 4 hours at 37°C using 0.4µm pore-size transwell.

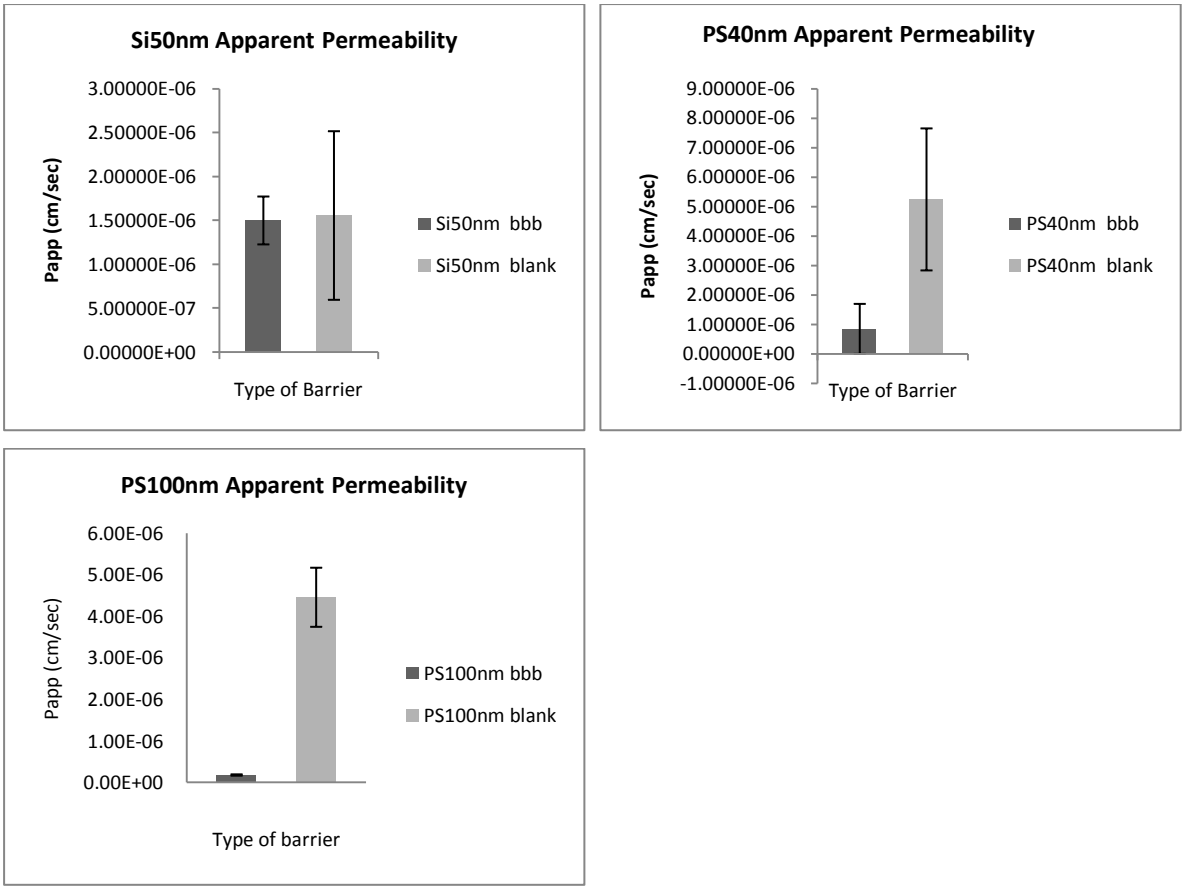


Fig. 6 Apparent permeability of different nanoparticles through *in vitro* BBB-model after 4 hours at 37°C using 3.0 μm pore-size transwell.

Studying active transport

Many researchers, including us, prefer to perform transport experiments in both directions across the cell monolayer. The ratio between the two permeability coefficients obtained, which is usually named the efflux ratio or uptake ratio, can then be used as a first indication of the involvement of an active transport process.

A bidirectional transport experiment has been conducted to investigate the affinity of silica and polystyrene NPs as substrates for an apically polarized efflux system. If the permeability of a compound in the basolateral (BL) to apical (AP) direction is significantly higher than that in the AP-to-BL direction, the compound may be a substrate for an efflux transporter. Rhodamine 123, a well known substrate for the apically polarized efflux transporter P-glycoprotein, has been used as positive control. The study has been performed on 3.0 μm pore-size transwell with silica 50nm and polystyrene 40nm NPs (Fig.7).

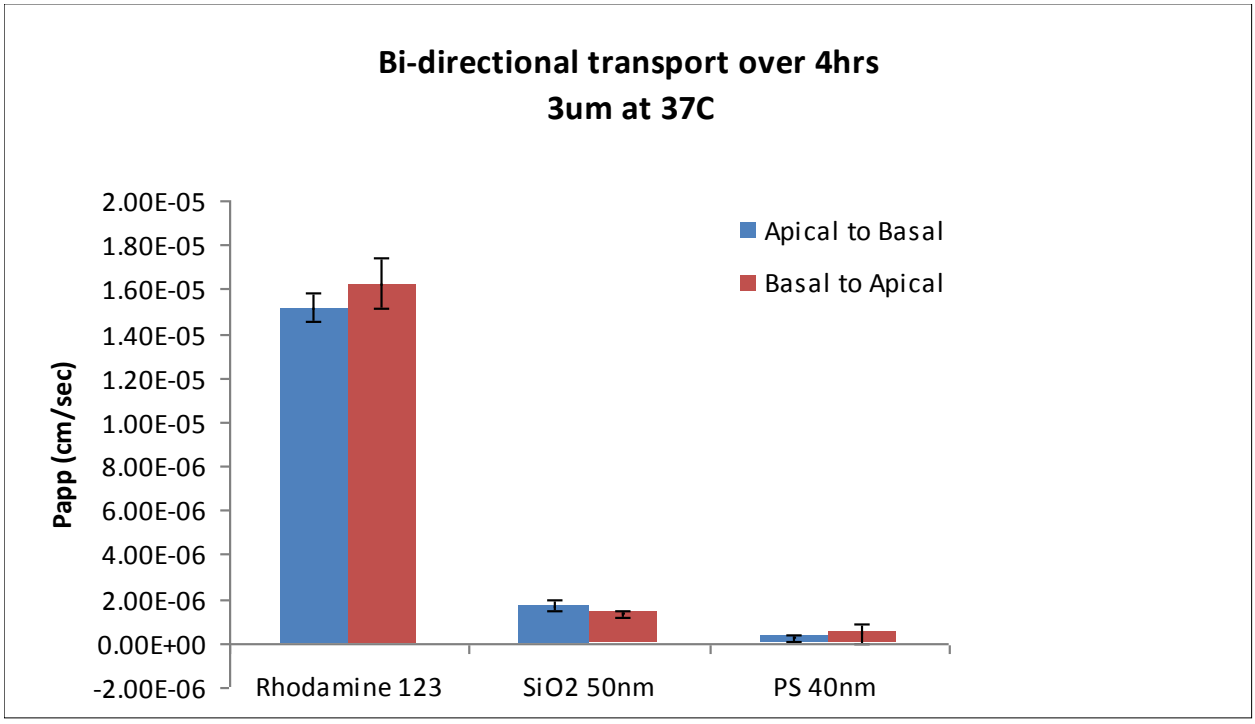


Fig. 7 Bi-directional transport over 4h time through hCMEC/D3 monolayer

Conclusions

In this project, an *in vitro* BBB model has been established using an immortalized hCMEC/D3 cell line and has been used to investigate the ability of silica 50nm, polystyrene 40nm and polystyrene 100nm nanoparticles trafficking through the cell monolayer. The barrier formed by hCMEC/D3 in 7 to 10 days culture has been found to maintain a very low transepithelial electrical resistance (TEER) value, which is consistent to the values being reported in literature for this model. Therefore, instead of TEER, it has been introduced FITC-dextran 4kDa molecule as barrier quality indicator of paracellular permeability. According to the results obtained and the values reported by other researchers, it has been concluded that the barrier quality it is good.

Undoubtedly, the choice of the porous membrane, on which cells grow, is a very important issue. First of all, good cell attachment should be guaranteed. Thus, the inserts, need to be coated with either collagen or collagen plus fibronectin to facilitate cell attachment. Besides of the aspect of cell attachment, another important fact is the permeability of the porous membrane support for the compound under investigation. The rate-limiting step for the transport process of different nanoparticles should be the cell layer and not the coated inserts, which should be a growth support for the monolayer rather than an obstacle for transport

processes. In case of passive diffusion, fluxes across inserts containing monolayer should be significantly smaller than those across the inserts without monolayer, indicating the presence of additional barrier functions due to the monolayer. To reduce the influence of the uncoated inserts, the diameter pore size has been increased from 0.4 to 3.0μm. No migration of cells into the pores has been reported. Additionally, at the control inserts equilibrium between apical and luminal compartment was reached within 20-30 min (results not shown here).

DLS results showed that SiO_2 50nm nanoparticle size has been severely impacted when the surrounding buffer is the assay medium used for the 4h transport study. 40nm and 100nm PS NPs did not show any relevant change in the size over 4h time at 37°C in assay medium. Basically de-ionized water can disperse silica particles properly (results not showed here) but adversely water cannot be allowed to be used in transport study because of the necessity of the barrier viability maintaining. Using assay medium, despite proteins, growth factors tend to be adsorbed onto the particle surface and increase its size.

For transport study, results showed that 50nm SiO_2 nanoparticles manifest a transport predominant in both fluxes increasing rate and penetrating permeability. Anyway the huge standard deviation indicates a low reproducibility of the results with silica nanoparticles compared to polystyrene values. It could be more applicable when we can focus 50nm SiO_2 in studies with resolving particle dispersion and aggregation problems.

Future work in collaboration with the host institution

Since the preliminary results seem rather interesting, it will be worth publishing the results in a highly ranked journal, although additional work is needed. Many different future directions have been discussed with Professor Kenneth Dawson and Dr. Iseult Lynch, due to the fact that I will be involved in a PhD programme by CBNI (Center for BioNano Interaction, UCD, Dublin, Ireland).

First of all the possible transport mechanisms through BBB will be investigated with unfunctionalized and functionalized nanoparticles with proteins of known function (i.e Si/PS nanoparticles chemically linked with transferrin, albumin and apolipoprotein E). Micro-array screen of BBB post-nanoparticle exposure will be taken in consideration and elucidation of gene regulation in order to investigate transport mechanism through hCMEC/D3 cells will be carried out as cluster regulated genes via signalling pathways/biological function post-nanoparticle exposure. Establish the role of protein corona properties of

nanoparticles pre- and post-transport through BBB will be one of the main aim in future and the same for the alteration of nanoparticle corona post-transport through BBB as indicator of cellular interaction between nanoparticle and cells.

Different techniques can be applied to achieve the very different goals of the project: confocal imaging will be useful to identify the role of transcytosis post-nanoparticle exposure (Caveolae and/or Clathrin-mediated transcytosis) and the co-localization of nanoparticles with the cellular organelles. Live cell imaging will also play a central role in the understanding of nanoparticles intracellular trafficking through the monolayer; while using Real-Time PCR a temporal gene expression modification over the 4h of transport study can be monitored, trying to understand cell behaviour and response after nanoparticles interaction.