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Nanoparticle Accumulation and Transcytosis in Brain Endothelial Cell Layers

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Abstract
The Blood-Brain Barrier (BBB) is a selective barrier, which controls and limits access to the central nervous system (CNS). The selectivity of the BBB relies on specialized characteristics of the endothelial cells that line the microvasculature, including the expression of intercellular tight junctions, which limit paracellular permeability. Several reports suggest that nanoparticles have a unique capacity to cross the BBB. However, direct evidence of nanoparticle transcytosis is difficult to obtain, and we found that typical transport studies present several limitations when applied to nanoparticles. In order to investigate the capacity of nanoparticles to access and transport across the BBB, several different nanomaterials, including silica, titania and albumin- or transferrin-conjugated gold nanoparticles of different sizes, were exposed to a human in vitro BBB model of endothelial hCMEC/D3 cells. Extensive transmission electron microscopy imaging was applied in order to describe nanoparticle endocytosis and typical intracellular localisation, as well as to look for evidence of eventual transcytosis. Our results show that all of the nanoparticles were internalised, to different extents, by the BBB model and accumulated along the endo-lysosomal pathway. Rare events suggestive of nanoparticle transcytosis were also observed for several of the tested materials.

Introduction
Access to the central nervous system (CNS) is strictly regulated by the blood-brain barrier (BBB), the main hindrance being constituted by a monolayer of endothelial cells which are in close contact with astrocytes. The BBB endothelial cells are characterized by high expression of tight junction proteins and selective pathways for controlled transport between circulating blood and the CNS. For neurodegenerative disease therapies and treatment of other diseases localized in the CNS, highly effective treatments demand efficient drug delivery to the brain. Such transport is strongly limited by the BBB, which exhibits minimal permeability to most drugs and neurotherapeutic agents. As a result, extensive research has been focused on the development of strategies to cross the BBB and deliver therapeutic agents efficiently in the CNS.

Within this context, nanotechnology applied to medicine has the potential to lead to significant advances in the diagnosis and treatment of diseases; as a result of their unique properties, such as size, surface energy, large surface area to volume ratio and the possibility of introducing a variety of surface modifications, engineered nanoparticles offer enormous promise as drug delivery vehicles specifically targeting a range of human diseases.
Several nanoparticles have been reported to accumulate in the CNS, thus suggesting their ability to cross the BBB.\textsuperscript{14,16} However the mechanisms still remain unclear and direct evidence of transcytosis \textit{in vivo} has not been generated as yet. These observations have raised safety concerns due to the increased exposure of organisms and humans to nanoparticulate matter.\textsuperscript{17,22} It is also known that nanoparticles can affect protein fibrillation rates.\textsuperscript{31} At the same time, these observations have also opened unique possibilities for using nanoparticles to understand transcytosis and gain access to the CNS.

Different strategies are currently under study in order to achieve nanoparticle transcytosis across the BBB. A common approach is to couple nanoparticles to targeting agents in order to actively seek receptor-mediated transcytosis. For instance, the transferrin receptor is abundant in brain capillary endothelium, and it has been reported that coupling nanoparticles to transferrin can aid nanoparticle penetration into the BBB through the transferrin receptor mediated pathway.\textsuperscript{16,24} Apolipoprotein E is an endogenous protein involved in the trafficking of naturally occurring LDL particles and when grafted on nanoparticles was reported to enhance BBB penetration,\textsuperscript{25-30} though not necessarily translocation.\textsuperscript{9} Similarly, cationised serum albumin has been reported to cross the BBB by absorptive-mediated transcytosis\textsuperscript{1} and could also be used to gain nanoparticle access to the brain. However, achieving targeting in realistic biological environments is not necessarily straightforward, because interactions with biomolecules in the medium may screen the targeting agent on the nanoparticle surface and thereby cause loss of specificity.\textsuperscript{32}

Another approach to improve nanoparticle transcytosis is to modify the nanoparticles themselves, such as modification of size, shape, and surface.\textsuperscript{33,34} By changing nanoparticle properties and design, the profile of proteins adsorbed to their surfaces from the surrounding environment, such as for instance blood serum, is also changed.\textsuperscript{35,36} It has been hypothesized that this layer (corona) of proteins on the nanoparticle surface affects much of the interactions with cells,\textsuperscript{37} and thus properties of the bare nanoparticle surface, such as charge, are less important for cellular interactions. Indeed initial evidence suggests that the capacity of nanoparticles to cross the BBB is connected to the nature of the proteins adsorbed on their surface, such as, for instance, apolipoproteins.\textsuperscript{15,25,26} Hence, by changing nanoparticle design, the corona that adsorbs on the nanoparticles could be used to achieve transcytosis across the BBB, and increase targeting.\textsuperscript{39}

In order to investigate the capacity of nanoparticles to cross the BBB and to test the efficiency of targeted nanoparticles to reach the brain, it is essential to be able to follow nanoparticle uptake in the BBB and to find evidence of transcytosis. \textit{In vitro} BBB models can be used in order to address some of the fundamental mechanisms involved in BBB regulation and also for nanoparticle screening and risk-assessment studies for human health. Recently, a human brain endothelial cell line, hCMEC/D3, has been used to establish a well characterised \textit{in vitro} BBB model.\textsuperscript{44} While the endothelial cells are not the sole structure of the BBB, they are believed to be the main permeability barrier.\textsuperscript{7} In cell culture, the hCMEC/D3 cell line maintains a contact-inhibited monolayer, exhibits robust proliferation in response to endothelial growth factors and grows indefinitely without phenotypic de-differentiation.\textsuperscript{45} Moreover, these cells express chemokine receptors in response to inflammatory cytokines, a series of tight junction proteins\textsuperscript{41} and multidrug resistant proteins, e.g., P-glycoprotein (P-gp).\textsuperscript{42} Thus, hCMEC/D3 BBB models are an excellent candidate for blood-brain barrier function and transport studies. This human BBB model has been already widely utilized to test the delivery of neurotherapeutic molecules\textsuperscript{43} and to study brain signalling mechanisms.\textsuperscript{44,45}

In this work, we studied the fate of various types of nano-sized materials accessing the blood brain barrier, and we used the hCMEC/D3 cells as an \textit{in vitro} BBB model for nanoparticle screening.\textsuperscript{46} Unmodified bare silica (SiO$_2$) nanoparticles of different sizes, anatase titanium dioxide (TiO$_2$) nanoparticles and gold (Au) nanoparticles of different sizes, conjugated with endogenous proteins (albumin or transferrin) were applied to this model in typical \textit{in vitro} concentrations (10-100 \textmu g/mL).\textsuperscript{47} Fluorescence based methods can be efficiently used to quantify the uptake of fluoroscently labeled nanoparticles by flow cytometry and/or fluorescence imaging and also to study nanoparticle
subcellular locations.\textsuperscript{48, 49} However, we found that for nanoparticle applications, classic transport studies by fluorescence using transwell systems presents several limits, and in some cases can produce unreliable results, due to technical limitations of nanoparticle fluorescence and nanoparticle adherence to the transwell filter pores. In order to overcome these limitations, Transmission Electron Microscopy (TEM) has been used to monitor the BBB model’s morphology in an effort to define nanoparticles’ subcellular locations within the BBB. In this way, we have investigated in a qualitative way typical nanoparticle locations from early interactions with the cell membrane and endocytosis, to intracellular trafficking and transcytosis. Thus, we provide an extensive gallery of images to follow the nanoparticles as they accumulate in the BBB and are trafficked intracellularly. Similar approaches can be used in future to study nanoparticle interactions with the BBB and overcome technical limitations of transwell system studies when applied to nanoparticles.

**Experimental**

**Materials**

Disposable culture plastic ware was purchased from Sigma (Sigma chemical co., St. Louis, Mo, USA), Corning Costar (Cambridge, MA, USA) and Becton-Dickinson (Boston, MA, USA). Cell culture medium EBM-2 and supplements [VEGF, IGF-1, EGF and basic FGF factors, hydrocortisone, ascorbate, gentamycin and 2 % fetal bovine serum (FBS)] were all purchased from Lonza (Bioscience). Lyophilized bovine plasma fibronectin, human serum albumin and tranferrin proteins were obtained from Biosciences. HEPES buffer was acquired from Sigma. Three different sizes (50 nm, 100 nm and 200 nm) of yellow green fluorescently labelled SiO\textsubscript{2} nanoparticles were purchased from G. Kisker, Biotech (www.kisker-biotech.com). Gold (III) chloride trihydrate (cat. no. 520918), sodium citrate tribasic dihydrate (cat. no. C7254), Sodium borohydride (cat. no. 480886), O-[2-(3-Mercaptopropionylamino)ethyl]-O′-methylpolyethylene glycol 5000 (“PEG-thiol 5k”) (cat. no. 11124), O-[2-(3-Mercaptopropionylamino)ethyl]-O′-methylpolyethylene glycol 20000 (“PEG-thiol 20k”), Human Serum Albumin A8763 and holo-Transferrin human T4132 were all purchased from Sigma-Aldrich and used without further modification. STO1 (anatase phase TiO\textsubscript{2} with nominal primary particle size 3-5nm) was obtained from Ishihara Sangyo Kaisha, Ltd, Japan.

**Nanoparticle Synthesis**

Gold (Au) nanoparticles of three sizes (5 nm, 12 nm and 25 nm) were synthesized in our laboratory. 5 nm Au nanoparticles were prepared following previously described methods.\textsuperscript{50} Briefly, 1ml of 50 mM AuCl\textsubscript{4} solution was added to 93.5 ml ultrapure water, and 6.5 ml of NaBH\textsubscript{4} solution (prepared by mixing 18.5 mg NaBH\textsubscript{4} in 9.5 ml water and 0.5 ml 1M NaOH). After 5 minutes mixing at room temperature, the temperature was increased to 100 °C for 5 minutes. 12 nm Au nanoparticles were prepared following the Turkevich and Frens method.\textsuperscript{51, 52} Gold (III) chloride trihydrate (12.5 mg dissolved in 1ml ultrapure water) was added to a boiling solution of trisodium citrate (50 mg in 149 ml ultrapure water). The reaction proceeded under reflux with rapid stirring for 30 minutes, resulting in a dark red coloured solution. 25 nm Au nanoparticles were prepared in a similar manner, but with altered Au/citrate ratio. In this case, 30 mg trisodium citrate and 20 mg Gold (III) chloride trihydrate were used. PEGylation of the gold was accomplished by addition of a 1 ml aqueous solution of PEG-thiol 20 k or 5 k to a rapidly stirring suspension of the prepared citrate-stabilized Au nanoparticles, to give a final thiol concentration of 5 µM. This mixture was then stirred continuously for 24 hours before washing by centrifugation, followed by resuspension in water (3 washes total). To prepare protein-functionalized Au nanoparticles, 10 ml of Au nanoparticle dispersion was added to 5 ml of albumin or transferrin protein solution in water (3 mg protein, which constitutes an excess, estimated to be more than 50 times higher than the amount needed to form a protein monolayer). This suspension was then incubated overnight at 4 °C. Finally, the particles were separated by centrifugation and resuspended in PBS. The centrifugation/resuspension cycle was
repeated 3 times to ensure removal of excess proteins. Particle concentration was estimated by surface plasmon absorbance reading.\textsuperscript{33}

**Anatase Titanium Dioxide Nanoparticle Dispersions**

The TiO\textsubscript{2} dispersion was prepared from STO1 following the method of Ramirez-Garcia et al.\textsuperscript{54} Dispersions in water of aggregates of around 180 nm (Polydispersity index 0.2) were obtained.

**Cell Culture**

The immortalized human capillary microvascular endothelial cells (hCMEC/D3)\textsuperscript{40} used for the experiments were maintained between passage 25 and 35. Cells were cultured in EBM-2 complete medium (growth medium) supplemented with VEGF, IGF-1, EGF, bFGF, 2.0 % FCS, ascorbate, gentamycin and hydrocortisone, as recommended by the manufacturer. To induce barrier differentiation, cells were grown in a growth-factor depleted medium (assay medium, consisting of EBM-2 supplemented with only bFGF, 2.5 % FCS, hydrocortisone, and 10 mM HEPES and gentamycine) as reported in literature.\textsuperscript{40, 55} Cells were grown in an incubator at 37 °C with 5 % CO\textsubscript{2}, 95 % air and saturated humidity. Cell medium was changed every 3 days.

**Transport Study**

Before seeding cells to prepare BBB monolayers, transwell membranes were coated with rat tail type I collagen and fibronectin overnight at 37 °C in a dry incubator. Then, hCMEC/D3 cells were seeded on the pre-coated transwell membranes (Polyester membrane, 12 well, pore size 0.4 μm, growth area 1.12 cm\textsuperscript{2}) at a density of 50,000 cells/cm\textsuperscript{2}. The assay medium was changed after 4 and 7 days and transport studies were performed between 7 to 10 days after seeding. Before each transport study, transwells were rinsed with pre-warmed assay medium, then 0.5 ml and 1.5 ml of fresh medium were added to the apical and basolateral chambers, respectively. Subsequently, barriers were equilibrated in assay medium on an orbital shaker at 100 rpm for 30-60 min.

For the transport studies with green fluorescently labelled SiO\textsubscript{2} nanoparticles (50 nm, 100 nm and 200 nm), at t=0 min, the apical medium was removed and immediately replaced with 0.5 ml 100 μg/ml SiO\textsubscript{2} nanoparticles. 100 μl aliquots were sampled from the basolateral chamber every hour for 4 hours and immediately replaced with 100 μl fresh assay medium. Fluorescence measurement of the sampled medium was conducted in a Varioskan flash (Thermo Scientific, USA) plate reader using 485 nm FITC channel for the excitation and reading the fluorescence emission at 514 nm. Serial dilutions of SiO\textsubscript{2} nanoparticles, in the range 0-100μg/ml in assay medium were prepared in order to obtain a calibration curve for nanoparticle fluorescence as a function of nanoparticle concentration. Linear regression was applied in order to define the correlation between fluorescence and concentration. This was used to determine the total mass of silica in the basal chamber, thus calculating the percentage of transport in respect to the mass initially applied to the apical chamber.

**Dynamic Light Scattering (DLS)**

DLS measurements were carried out using a Malvern Zetasizer Nano ZS90 (Worcestershire, UK). The nanoparticle dispersions were prepared by direct dilution in de-ionized water and assay medium containing 2 % Foetal Bovine Serum (FBS). Final nanoparticle concentrations were, respectively, 100 μg/ml for the SiO\textsubscript{2} and TiO\textsubscript{2} nanoparticles, and 50 μg/ml for the Au nanoparticles. The temperature was set at 27 °C for samples in water and 37 °C for the samples in cell culture medium. Dynamic light scattering (DLS) measurements were taken as the average of a minimum of three runs, each containing 11-sub measurements, with standard deviation.

**Transmission Electron Microscopy**

Transwells containing confluent hCMEC/D3 cell monolayers were fixed with glutaraldehyde (2.5 %, v/v) at room temperature for 1 hour in Sorensen's phosphate buffer, and postfixed in osmium tetroxide (1 %, w/v) in 0.05 M potassium phosphate buffer, also for 1 hour. The barrier samples were dehydrated by washes in 70 %, 90 % and 100 % ethanol respectively, before cells were
embedded in epoxy resin. Ultrathin sections (80 nm) were cut with a diamond knife (Diatome, US) on a Leica Microtome and further stained with 2 % uranyl acetate for 20 min and lead citrate for 10 min. Images were analyzed in a transmission electron microscope (TECNAI, FEI Company, Hillsboro, OR, USA) under a 120 kV electron beam.

Results and Discussion

Optimization of the \textit{in vitro} BBB model and common limitations for transport studies

hCMEC/D3 cells were used to prepare \textit{in vitro} BBB monolayers as described in the Methods. An endothelial cell monolayer was successfully obtained (See Supplementary Fig. S1) by culturing the cells on filters until confluency and tight junction formation (typically after 7-10 days), as assessed by tight junction protein staining, TEER value measurements (40-50 Ω·cm$^2$) and FITC dextran permeability studies, as previously described.\cite{46} In some cases, endothelial cells formed multiple layers on the transwell filters, rather than a monolayer (See Supplementary Fig. S2 for some examples), and this could signal the presence of areas which were not properly differentiated. We have minimized the formation of these areas with multiple layers by either thoroughly dispersing the cell suspension prior to their seeding, or by removing the non-adhering cells 6 hours after seeding, in order to achieve better control of the starting cell density and ensure optimal growth. In addition, multi-layer formation can also be overcome by using growth factor depleted medium during barrier formation as described previously.\cite{40,46}

In order to perform transport studies across similar \textit{in vitro} barrier models, transwell systems are often used. The transwell device features an upper donor side and a lower acceptor side, with the two compartments separated by a porous filter membrane of selected composition and pore size. When endothelial cells form a well differentiated monolayer on these filters, added compounds (or nanoparticles in this case) in the upper chamber are internalised by the cells and if transcytosis occurs, they pass through the pores of the filters and reach the lower chamber. By analyzing the ratio of a compound travelling through the model, we can rank the transport efficiency of different compounds.\cite{40,46} However, we have observed several limitations which hinder the study of nanoparticle transport through the endothelial cells in such transwell systems.

Firstly, transport is often quantified by using fluorescently labeled nanoparticles and assessing the fluorescence intensity in the basal chamber. It has been observed that in several cases fluorescently labeled nanoparticles retain a fraction of labile dye which can be difficult to remove by common cleaning procedures and which can leak once in contact with biological environments,\cite{48,56} generating false positive signals (see for instance Supplementary Fig. S3). Similarly, nanoparticle degradation in the medium or inside the cells could also lead to dye release which could be misinterpreted as nanoparticle transport.\cite{46} Simple SDS-PAGE (such as shown in Supplementary Fig. S4) can be used to separate nanoparticles from eventual labile free dye and to confirm whether the fluorescence recovered in the basal chamber is really due to the presence of transcytosed nanoparticles.

Secondly, nanoparticle agglomeration, which sometimes occurs in biological media as well as after nanoparticle internalisation, could impair transwell system transport studies since particle agglomerates may not be able to pass through the filter pores. Simple equilibration studies across blank filters (without cells) can be used as an indication of such problems, by adding the nanoparticle suspension to the apical chamber and measuring transport to the basal chamber. However, such studies cannot show agglomeration inside cells of potentially transcytosed nanoparticles.

Thirdly, we also found that even well mono-dispersed nanoparticles may adhere to the pores in various types of filters, and remain within the filter without reaching the lower basal chamber. See
for instance Supplementary Fig. S5, where we observed adhesion of SiO$_2$ nanoparticles to filters of different composition. Nanoparticle adsorption to the filters will obviously affect the quantification of the final transport ratios.

Because of these limitations of common fluorescence-based transport studies using transwell systems when applied to nanoparticles, we turned to Transmission Electron Microscopy (TEM) to clearly visualize the location of the nanoparticles in relation to the BBB model. Quantification of nanoparticle uptake by TEM requires specific methodologies such as for instance methods based on stereology. In this work, prior to any attempt at quantifying nanoparticle accumulation, we used TEM imaging to visualize and follow nanoparticle uptake and intracellular trafficking within the barrier and monitor eventual evidence of nanoparticle transcytosis. TEM is a powerful tool to analyze with higher resolution than can be achieved with optical methods materials or ultramicroscopic structures of biological organisms. Nanoparticles of materials with high electron density and well defined shapes can be easily visualized and distinguished from the cell background (and artifacts introduced by the staining) by TEM. TEM can also give enhanced subcellular details compared to other imaging techniques, thus allowing following nanoparticles as they accumulate and are trafficked within the barrier. The transwell filter plays an important role as a growth support for the development of the endothelial cell apical-basolateral polarity. The presence of the filter also facilitates sample embedding and ultramicrotomy in order to obtain transversal sections of the BBB layer by cutting across the filter membrane (see images in Supplementary Fig. S1).

**Uptake and Transcytosis of Plain SiO$_2$ Nanoparticles of Different Sizes.**

SiO$_2$ nanoparticles of different sizes are known to enter cells by active transport and were used here to test the blood-brain barrier model, in order to investigate the cellular interactions of the nanoparticles and look for potential evidence of transcytosis. Particle characterisation in assay medium containing 2% foetal bovine serum (FBS) indicated that 50 nm (nominal diameter) SiO$_2$ nanoparticles were partially agglomerated when exposed to cells (See Supplementary Table S1). This was probably caused by the low concentration of serum proteins in the assay medium. Good dispersions were obtained for the same nanoparticles in 10% FBS. TEM imaging of cells exposed to these particles also suggested the presence of large clusters of nanoparticles adhering to the cell membrane (see Supplementary Fig. S6). Consistent with this, uptake of single nanoparticles into vesicles was a rare event. See for instance the image in Fig. 1a, where a SiO$_2$ nanoparticle is entering a cell, possibly in a clathrin-coated pit. More commonly, cell protrusions around larger clusters of 50 nm SiO$_2$ nanoparticles were observed as shown in Fig. 1b, resembling what is observed in macropinocytosis.

Once inside the cells, in rare cases single particles were observed in intracellular vesicles along the endo-lysosomal pathway. See for instance Fig. 1c showing a nanoparticle in an endosomal vesicle. Most nanoparticles were found accumulated in large numbers inside membrane-bound vesicles, as in several examples in Fig. 1. As discussed above, poor nanoparticle dispersion in cell medium may explain these observations, even though we cannot exclude that these agglomerates were formed by the cells, upon trafficking of different nanoparticles to the same intracellular structures. Other typical intracellular locations included compartments of the endo-lysosomal pathway, such as late endosomes, multivesicular bodies and finally the lysosomes, as shown in Fig.1d-f. This is consistent with what is observed for SiO$_2$ nanoparticles and also many other nanomaterials in other cell types, where the final intracellular location is often the lysosomes. In addition to this, however, in few cases we also observed nanoparticles closer to the basolateral side of the BBB layers, or outside the cells (between the cell layer and the filter), close to invaginations of the basal cell membrane, suggestive of transcytosis. Some examples are shown in Fig. 1g-i.

The same study was performed with 100 nm (nominal diameter) SiO$_2$ nanoparticles (Fig. 2). Particle characterisation for this sample in 2% FBS assay medium was also indicative of partial agglomeration (Supplementary Table S1). After 4 hours of incubation with the endothelial cell
monolayer, we found that 100 nm SiO$_2$ nanoparticles could enter invaginated vesicles in the cell membrane as shown in Fig. 2a, which possibly indicates the formation of a clathrin-coated vesicle. Similar to what was observed for the 50 nm SiO$_2$ nanoparticles outside the cells were observed also for 100 nm SiO$_2$. Large numbers of nanoparticles accumulated into membrane-bound intracellular structures, including lysosomes, as shown in Fig. 2b-d. Evidence of transcytosis was found also for these larger SiO$_2$ nanoparticles, as shown in the examples in Fig. 2e-f. In particular, Fig. 2f shows a large cluster of nanoparticles outside the cells on the basal side of the layer (above the filter), suggesting that the large intracellular structures filled with the nanoparticles which we observed inside the cells (Fig. 2b-d) could possibly be released outside the cells. It is possible that these events could be captured only by TEM imaging, since it is unlikely that such large nanoparticle clusters, if irreversibly agglomerated, could transport through the transwell filter pores and reach the basal chamber.

Finally, the BBB layers were also exposed to 200 nm (nominal diameter) SiO$_2$ nanoparticles; in the sections of these samples, nanoparticles of diameter smaller than 200 nm were found, as well as several holes. This was already observed for TEM sections of cells exposed to large particles of similar sizes and is connected to the thickness of the ultramicrotomy sections, which is 80 nm, much smaller than the real particle diameter. Probably the diamond blade can in some cases cut through the nanoparticles (which, if not through the centre, generates sections of an apparent smaller diameter), but in some others this is not possible and can result in damage to the sections and presence of holes (previously occupied by nanoparticles). Overall, TEM imaging indicated that even particles of this size could enter the BBB layers. Fig. 3a shows 200 nm SiO$_2$ nanoparticles outside the cell, close to membrane invaginations, which – despite the particle size – appeared coated by clathrin. The typical size of clathrin-coated vesicles is normally considered ~100 nm in diameter. However, literature has shown that clathrin can assist also in the formation of larger structures.

In Fig. 3b, we also show a single nanoparticle engulfed in some membrane protrusion, possibly suggesting that cells can use multiple mechanisms for nanoparticle uptake. As also observed in cells exposed to smaller SiO$_2$ nanoparticles, the internalised nanoparticles were found in compartments of the endo-lysosomal pathway, such as endosomes, multivesicular bodies and lysosomes, as shown in Fig. 3c-d. Interestingly, we saw evidence of transcytosis also for these larger 200 nm SiO$_2$ nanoparticles, as shown in Fig. 3e-f. A vesicle docking to the border of the basal membrane was observed, close to a SiO$_2$ nanoparticle already outside the cell on the filter (Fig. 3e). Similarly, in Fig. 3f we observe some particles already outside the cells, inside the filter pores.

In summary, all the tested SiO$_2$ nanoparticles entered the BBB layers and were found inside the cells along the endo-lysosomal pathway. Uptake was higher and events which could suggest transcytosis were more frequent for the 50 nm particles than for the larger nanoparticles, although this could be connected to the different numbers of nanoparticles initially applied (the cells were exposed to the same mass of SiO$_2$ in all cases) and this conclusion is also complicated by the presence of partial agglomeration. It is important to stress that in all the cases reported, the cell monolayer structure close to the nanoparticles found outside the cells on the basolateral side was intact and we could not observe cell monolayer imperfections (as indeed we found in other examples, such as those shown in Supplementary Fig. S7). However, due to the thin (80 nm) EM sections it is impossible to exclude such imperfections in the orthogonal unsampled direction. Consequently, caution is warranted as to whether these images show nanoparticle export or transcytosis, respectively, from the cells. In any case, such events are at most rare.

Classic transport studies by fluorescence assessment of the medium in the basal chamber (as shown in Supplementary Fig. S8) indicated that for the 50 nm SiO$_2$ after 4 hours, 3.6 % of the initial mass reached the basal chamber. This value is higher than the 1.7 % and 1.1 % observed for the 100 nm and 200 nm SiO$_2$ nanoparticles respectively. However, as discussed above, nanoparticle transport ratios could be affected by particle loss due to nanoparticles adhering to the filter pore walls.
Uptake and Transcytosis of Human Serum Albumin and Transferrin Conjugated Au nanoparticles.

In order to further investigate the interactions of nanoparticles with the BBB layer, we also used Au nanoparticles of sizes between 5 and 25 nm (as determined by electron microscopy) to prepare protein-conjugated nanoparticles in order to investigate how smaller objects are processed by the BBB, and monitor eventual differences in uptake, intracellular trafficking, and transcytosis. The Au nanoparticles were conjugated with human serum albumin or transferrin to allow potential biological recognition, which, as discussed previously, may affect transcytosis\textsuperscript{16, 23, 53, 64} and nanoparticle behavior in general. (BBB cells were also treated with simple PEGylated Au for comparison; some images are shown in Supplementary Fig. S9.)

Nanoparticle characterization of the different conjugated particles (Supplementary Table S2) showed partial agglomeration in 2 % FBS assay medium. However, apart from the smallest particles, relatively good dispersions could be obtained.

Fig. 4 shows 25 nm (diameter in electron microscopy) albumin-conjugated Au nanoparticles exposed to the BBB model. A first observation is that nanoparticle uptake was rather low and only few particles could be observed inside the cells (note, however, the number concentration implied by the high density of gold). Fig. 4a shows a nanoparticle in the neck of a still open vesicle or caveola on the apical membrane. Inside the cells, particles were observed in what appears to be clathrin-coated vesicles (some examples are shown in Fig. 4). As described for the SiO\textsubscript{2} nanoparticles, Au nanoparticles were also observed in different endo-lysosomal structures, such as endosomes, multivesicular bodies and lysosomes, as shown in Fig.4c-e respectively. Again, some (very rare) examples of Au nanoparticles in the sub-endothelial space were observed, as shown in the example in Fig. 4f. Similar conclusions could be drawn for cells exposed to 12 nm albumin-conjugated Au nanoparticles (see Supplementary Fig. S10).

With the transferrin-Au nanoparticles, we noted very low levels of uptake, as observed also for albumin-Au nanoparticles. Fig. 5a shows a single nanoparticle, moving into the neck of a vesicle, which is still open, on the apical membrane. Similar to what was observed for the albumin-Au nanoparticles, transferrin-conjugated Au nanoparticles were also found to localise mostly with endosomal vesicles and lysosomes, as shown in Fig. 5b-d. Nevertheless, a few transferrin-conjugated Au nanoparticles were observed in the intercellular space between cells and on the basal side of the cells close to the filter, where we could also observe several other vesicles and invaginations, as shown in Fig. 5e-f respectively. This further supports the presence of transport processes at the basal side in this model. Similar conclusions could be drawn from the images of cells exposed to 5 and 12 nm (diameter in electron microscopy) transferrin-conjugated Au nanoparticles (examples are given in Supplementary Fig. S11-12).

Overall, all the functionalised Au nanoparticles, despite their functionalisation and smaller size, seemed to be processed along the endo-lysosomal pathways as also observed for the unfunctionalised SiO\textsubscript{2} particles of larger size. Furthermore, events of apparent transcytosis were very rare. Thus the surface modification and smaller size does not seem to improve the capacity of these nanoparticles to cross the BBB layer, at least superficially. More complex nanoparticle design and grafting strategies are probably necessary to ensure that conjugated molecules could be recognised by specific receptors and maintain their biological activity\textsuperscript{32, 42}.

Uptake and Transcytosis of TiO\textsubscript{2} Nanoparticles.

TiO\textsubscript{2} is one of most frequently used materials in commercial products, e.g., cosmetics and dental care\textsuperscript{66} and concerns regarding the effects of TiO\textsubscript{2} nanoparticles on human health, including neurological damage\textsuperscript{67, 68} have been raised. However, the fate of TiO\textsubscript{2} nanoparticles upon exposure
to the human BBB has not been clearly defined to date. We therefore used our model to investigate accumulation and potential transcytosis of these nanoparticles (Fig. 6).

TiO2 nanoparticles are well known to be very difficult to disperse as single objects. For this study, however, 5-10 nm anatase TiO2 nanoparticles were dispersed to a final size of around 180 nm (see Methods for details), using a dispersion protocol optimised in previous work. These small clusters were easily visualised as high-density objects under an electron beam. As shown in Fig. 6a, TiO2 nanoparticles were associated with the cell membrane and some were internalised inside vesicles (likely clathrin-coated pits). Typical intracellular locations included endosomes, multivesicular body and lysosomes, as shown in Fig. 6b-d respectively. This suggests that TiO2 nanoparticles were mainly trafficked along the endo-lysosomal pathway, as observed for all other materials investigated. For these nanoparticles, however, we could not see any evidence of trancytosis by TEM. This is in agreement with other TiO2 studies, where no evidence of TiO2 nanoparticle transcytosis was observed using an in vitro rat BBB co-culture model after 4 or 24 hours exposure (though after 5-day chronic exposure the same study reported transcytosis of TiO2 nanoparticles to the co-cultured glial cells).

Conclusions

Uptake and transcytosis of different nanosized materials were investigated in a qualitative way using TEM imaging, in an attempt to understand intracellular trafficking of nanoparticles within the BBB. For this purpose, we used the immortalized human hCEMC/D3 cells, which have been established previously as a robust in vitro human BBB model. Filter-based transwell systems are conventionally applied for transport studies across such layers, and have been extended to nanoparticle applications. However, we found that when using nanoparticles, filter membranes can exhibit several limitations, e.g. nanoparticle adherence to filter pores, poor nanoparticle equilibration through filters and potential dye leakage from the nanoparticles into the basal chamber. In order to overcome these limitations, we used TEM imaging to qualitatively follow nanoparticles inside and across the BBB monolayer. This allowed us to obtain a gallery of images of typical nanoparticle locations and final fate inside the cells.

We selected both unmodified engineered nanoparticles of different sizes and material, as well as protein-conjugated nanoparticles. We found that all tested nanomaterials entered the endothelial cell monolayer and accumulated along the endo-lysosomal pathway, as already observed for several other nanoparticles and cell types. For most materials investigated, very few nanoparticles were found outside the cells on the basal side of the BBB, suggesting rare events of transcytosis. The observation of such rare events was clearly favoured by the high doses applied in these in vitro studies. Thanks to the high resolution of TEM imaging, this constitutes, to our knowledge, the first direct evidence of such processes for nanoparticles in BBB models. We hasten to add, however, that such observations may be skewed by imperfections in the monolayer in the unsampled direction.

Nanoparticles of smaller sizes, such as the Au nanoparticles of sizes in the range of 5 to 25 nm, were processed by the BBB layer in much similar manner as what was observed for the nanoparticles of larger sizes. Neither the smaller size nor the conjugation with proteins reported to affect transcytosis, such as albumin and transferrin, seemed to favour the occurrence of transcytosis.

From a broader perspective, it is important to note that while several studies so far have been focused on defining the capacity of nanoparticles to cross the BBB in relation to nanosafety (as well as for nanomedicine applications), a general outcome of this study is that nanomaterials are capable to accumulate within the BBB. Considering that export is generally absent for nanoparticles accumulating in the lysosomes, it will be important to consider in future whether nanoparticle internalisation and lysosomal accumulation could affect the BBB itself, regardless of the capacity for transcytosis and access to the brain.
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Notes and References
Electronic Supplementary Information (ESI) available: Nanoparticle characterization in relevant media by Dynamic Light Scattering and SDS-PAGE. Transport study for silica nanoparticles across the BBB layer. Additional Electron Microscopy images of cells treated with the different nanoparticles investigated and details of the filters of the transwell systems. See DOI: 10.1039/c3nr02905k


Fig. 1 Uptake and transcytosis of 50 nm SiO$_2$ nanoparticles in hCMEC/D3 cell monolayers. Cells were exposed to 100 µg/ml nanoparticles in assay medium containing 2 % FBS for 4 hours. Arrows indicate nanoparticles. (a) A single SiO$_2$ nanoparticle entering a cell (possibly in a clathrin coated pit). (b) A cluster of SiO$_2$ nanoparticles entering a cell. (c) A SiO$_2$ nanoparticle in an endosomal vesicle and clusters of nanoparticles outside the cells. (d) SiO$_2$ nanoparticles in an endosome (E); (e) SiO$_2$ nanoparticles in a multivesicular body (MVB); (f) SiO$_2$ nanoparticles in a lysosome (L). (g) SiO$_2$ nanoparticles were found close to membrane invaginations between two cells, suggesting possible transcytosis or export. (h) A few SiO$_2$ nanoparticles were found beneath the basolateral cell membrane close to the filter membrane, also suggesting transcytosis. In the same image we also observe large numbers of SiO$_2$ nanoparticles accumulated into a multivesicular body (MVB) and single nanoparticles in other intracellular structures. (i) A large cluster of SiO$_2$ nanoparticles was observed between the BBB layer and the filter, with the cell basal membrane engulfed around it.
Fig. 2 Uptake and transcytosis of 100 nm SiO₂ nanoparticles in hCMEC/D3 cell monolayers. Cells were exposed to 100 µg/ml nanoparticles in 2 % FBS assay medium for 4 hours. Arrows indicate nanoparticles. (a) Some SiO₂ nanoparticles engaging with a cell membrane invagination, possibly entering the cells in a clathrin-coated pit. (b) A cytoplasmic vesicle full of SiO₂ nanoparticles (on the right: a single SiO₂ nanoparticle interacting with the cell membrane). (c) Internalised SiO₂ nanoparticles clustered in large vesicular structures and an endosome (E). (d) SiO₂ nanoparticles in lysosomes (L) and other large intracellular structures. (e) A number of SiO₂ nanoparticles were found outside the cells close to the basolateral membrane, suggestive of transcytosis. (f) A large cluster of SiO₂ nanoparticles outside the cells, below the basolateral membrane, also possibly indicating some final stage of transcytosis.
Fig. 3 Uptake and transcytosis of 200 nm SiO$_2$ nanoparticles in hCMEC/D3 cell monolayers. Cells were exposed to 100 µg/ml nanoparticles in 2 % FBS assay medium for 4 hours. Arrows indicate nanoparticles. (a) SiO$_2$ nanoparticles interacting with the cell membrane, possibly a clathrin-coated pit. (b) A single SiO$_2$ nanoparticle engulfed by the apical cell membrane. (c) SiO$_2$ nanoparticles in an endosome (E) and a lysosome (L). (d) SiO$_2$ nanoparticles in a multivesicular body (MVB) and lysosomes (L) in close proximity. (e) A few SiO$_2$ nanoparticles outside the cells above the filter, possibly released from a vesicle bound to the basal cell membrane. (f) A nanoparticle close to the basal side of cells, possibly indicating transcytosis, and SiO$_2$ nanoparticles entering the filter membrane pores.
Fig. 4 Uptake and transcytosis of 25 nm albumin conjugated Au nanoparticles in hCMEC/D3 cell monolayers. Cells were exposed to 50 µg/ml nanoparticles in 2 % FBS assay medium for 4 hours. Arrows indicate nanoparticles. (a) A single Au nanoparticle entering an endocytic vesicle (possibly a caveola). (b) A single Au nanoparticle in what appears to be a clathrin-coated pit. (c) A late endosome (E) filled with several Au nanoparticles. (d) Au nanoparticles in a multivesicular body (MVB). (e) A large number of Au nanoparticles outside the cells and a single Au nanoparticle in a lysosome (L) and some other intracellular vesicle. (f) A rare case was found of a single Au nanoparticle beneath the basal membrane of endothelial cell monolayer, possibly having undergone transcytosis.
Fig. 5 Uptake and transcytosis of 25 nm transferrin-conjugated Au nanoparticles in hCMEC/D3 cell monolayers. Cells were exposed to 50 µg/ml nanoparticles in 2 % FBS assay medium for 4 hours. Arrows indicate nanoparticles. (a) A single Au nanoparticle possibly entering a cell in a small vesicle, which is still open, on the apical membrane. (b) A single Au nanoparticle in a small cytosolic vesicle and (c) in an endosome (E). (d) Two Au nanoparticles in a lysosome (L). (e) A single Au nanoparticle in the intercellular space, close to a vesicle docking to the basal cell membrane. (f) A rare example of a free Au nanoparticle found outside the BBB monolayer on the filter surface, suggestive of potential transcytosis.
Fig. 6 Uptake and transcytosis of TiO$_2$ nanoparticles in hCMEC/D3 cell monolayers. Cells were exposed to 100 µg/ml nanoparticles in 2 % FBS assay medium for 4 hours. In these images the cell structures appear less visible because TiO$_2$ nanoparticles are very dense under the electron beam, hence the lower level of contrast in comparison to the cell background. Arrows indicate nanoparticles. (a) TiO$_2$ nanoparticles in membrane bound vesicles. (b) TiO$_2$ nanoparticles in an early endosome (E). (c) Large clusters of TiO$_2$ nanoparticles inside multivesicular bodies (MVB) and (d) TiO$_2$ nanoparticles in lysosomes (L).