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Effect of endothelial cell heterogeneity on nanoparticle uptake

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ABSTRACT

Endothelial cells exhibit distinct properties in morphology and functions in different organs that can be exploited for nanomedicine targeting. In this work, endothelial cells from different organs, i.e. brain, lung, liver, and kidney, were exposed to plain, carboxylated, and amino-modified silica. As expected, different protein coronas were formed on the different nanoparticle types and these changed when foetal bovine serum (FBS) or human serum were used. Uptake efficiencies differed strongly in the different endothelia, confirming that the cells retained some of their organ-specific differences. However, all endothelia showed higher uptake for the amino-modified silica in FBS, but, interestingly, this changed to the carboxylated silica when human serum was used, confirming that differences in the protein corona affect uptake preferences by cells. Thus, uptake rates of fluid phase markers and transferrin were determined in liver and brain endothelium to compare their endocytic activity. Overall, our results showed that endothelial cells of different organs have very different nanoparticle uptake efficiency, likely due to differences in receptor expression, affinity, and activity. A thorough characterization of phenotypic differences in the endothelia lining different organs is key to the development of targeted nanomedicine.

1. Introduction

Nano-sized materials hold tremendous potential as drug carriers, thanks to their ability to distribute within organisms and enter cells (Bareford and Swaan, 2007; Ferrari, 2005; Peer et al., 2007; Sahay et al., 2010). Although their use as carriers of therapeutic agents has been growing rapidly during the past decades, crucial questions still arise as to how nanoparticles can be effectively and selectively delivered to their target. In order to reach their target tissue, following administration into the bloodstream, nano-sized drug carriers, in most cases, first need to interact with and cross endothelial cell barriers. Due to the diversity in vascular channels and other associated differences – for instance, in hemodynamics and their embryonic origin — endothelial cells lining blood vessels of different organs exhibit very distinct properties in morphology and functions (Aird, 2012; Chi et al., 2003; Ribatti et al., 2002). Consequently, these differences provide a great opportunity to selectively target drug carriers to specific endothelial cell barriers (Ding et al., 2006; Kowalski et al., 2013; Muro et al., 2008; Simone et al., 2009).

To date, most efforts in nanomedicine targeting have been devoted to understanding the effect of different physicochemical properties of nanoparticles and the environment in which they are applied on their interaction with cells. Several nanoparticles properties such as the size (Chithrani et al., 2006; He et al., 2010; Rejman et al., 2004), shape (Chithrani et al., 2006), surface charge (Arvizo et al., 2010; He et al., 2010), and (core) materials (Georgieva et al., 2011), as well as environmental properties such as the protein composition of the biological fluids in which they are dispersed (Lesniak et al., 2012), pH (Shen et al., 2008), temperature, flow (Freese et al., 2017) and shear stress (Klingberg et al., 2015) have been shown to have a significant influence on nanoparticle-cell interactions. However, relatively less attention has been paid on differences in cellular properties, which can affect nanoparticle-cell interactions. For instance, we have previously shown that a specific cell type can show very different nanoparticle uptake behavior

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when it is developed into a polarized cell monolayer, i.e., a cell barrier, as opposed to confluent cells (Francia et al., 2018; Zuhorn et al., 2007). The development of cells into cell barriers reduced the gene expression levels of different protein markers and/or caused their relocation to the abluminal plasma membrane, resulting in lower uptake efficiency of luminally applied nanoparticles. It is known that polarized cell barriers express different uptake pathways and receptors on their apical and basal side, and this can affect nanoparticle uptake (Georgieva et al., 2011; Iversen et al., 2011; Wang et al., 2016). Similarly, because of their phenotypical heterogeneity, endothelial cells of different tissue origins are likely to show very different nanoparticle uptake behavior, and this could be exploited for nanomedicine targeting. As an example, a recent study showed that kidney glomerulus and blood–brain barrier had a distinct nanoparticle uptake behavior (Gromnicova et al., 2016). In addition to a different origin, heterogeneity induced by physiological stress can also lead to the expression of different proteins on endothelial cells, and these have been shown to be excellent targets for drug carriers (Ding et al., 2006; Kowalski et al., 2013, 2011).

The aim of this study was to investigate the cellular uptake behavior of nanoparticles on endothelial barriers generated from endothelial cells derived from different organs. We hypothesized that different endothelia would show preferential uptake for certain types of nanoparticles, and similar differences could be exploited for targeting. To test this hypothesis, four endothelial cell lines derived from different organs were chosen as endothelial cell models: hCMEC/D3 (brain), HPMEC-ST1.6R (lung), TRP3 (liver), and ciGENC (kidney). The selection of these organs was based on the high phenotypic differences between one another. In addition, the selected immortalized endothelial cell lines have been shown to be excellent models of the endothelia of the respective organ from which they were derived, since they retained their organ-specific properties *in vitro* (Krump-Konvalinkova et al., 2001; Parent et al., 2014; Satchell et al., 2006; Weksler et al., 2005). The exhibition of organ-specific characteristics of the cell lines *in vitro* was essential for this comparative study. Amorphous silica nanoparticles of 100 nm with three different surface functionalizations, plain, carboxylated and amino-modified, were used as representative model nanoparticles. It is known that surface properties affect corona formation on the nanoparticles in serum and this, in turn, affects nanoparticle recognition by cell receptors and uptake by cells (Caracciolo et al., 2017, 2013; Francia et al., 2019; Lara et al., 2017; Lundqvist et al., 2008; Ritz et al., 2015; Tenzer et al., 2013). We hypothesized that due to varying coronas, the different nanoparticles would naturally target specific organ endothelia. Therefore, cell culture conditions were optimized to develop endothelial cell barriers, and uptake kinetics of the various nanoparticles were compared in the organ-specific endothelia in order to determine differences in uptake behavior. Finally, the rate of endocytosis of fluid phase markers and transferrin in the blood brain barrier and the liver sinusoids were compared in order to identify potential differences which may account for different uptake efficiency of nanoparticles in these cells.

2. Methods

2.1. Cell culture

The immortalized human brain endothelial cell line, hCMEC/D3, was supplied by Pierre-Olivier Couraud (Weksler et al., 2005). Cells were cultured in standard cell culture flasks pre-coated with 0.1 mg/ml cold rat-tail collagen type-I (Corning, NY, USA) in an endothelial basal medium (EBM-2, LONZA, Allendale, NJ, USA) supplemented with 5% fetal bovine serum (FBS, Gibco Thermofisher Scientific, Landsmeer, Netherlands), 200 ng/ml bFGF (Peprotech, London, United Kingdom), 1 μg/ml hydrocortisone (Sigma-Aldrich, St Luis, USA), 1% chemically defined lipid concentrate (Thermofisher Scientific), and 10 mM HEPES (Thermofisher Scientific). The medium was refreshed every 2–3 days, and cells were cultured between passages 29–38. Cells were kept under standard conditions (37 °C, 5% CO_2).

The immortalized human pulmonary microvascular endothelial cell line, HPMEC-ST1.6R, was supplied by Ronald E. Unger (Krump-Konvalinkova et al., 2001). Cells were cultured in standard cell culture flasks pre-coated with 0.2% cold gelatin (Sigma-Aldrich) in an EBM-2 supplemented with an EGM-2 bullet kit (LONZA). The medium was refreshed every 2–3 days, and cells were kept under standard conditions (37 °C, 5% CO_2).

The immortalized human liver endothelial sinusoidal cell line, TRP3, was supplied by Birke Bartosch and Romain Parent (Parent et al., 2014). Cells were cultured in standard cell culture flasks pre-coated with 0.1% cold gelatin (Sigma-Aldrich) in an MCDB 131 medium (Gibco Thermofisher Scientific) supplemented with 20% FBS (Gibco Thermofisher Scientific), 10 mM glutamine (Thermofisher Scientific), 250 μg/ml cAMP (Sigma-Aldrich),1 μg/ml hydrocortisone (Sigma-Aldrich), and 50 μg/ml endothelial cell growth supplement (ECGS, Corning). The medium was refreshed every 2–3 days, and cells were kept under standard conditions (37°C, 5% CO_2).

The conditionally immortalized glomerular endothelial cell line, CiGENC, was supplied by Simon Satchell (Satchell et al., 2006). Cells were cultured in standard cell culture flasks pre-coated with 1 μg/cm^2 fibronectin (Corning) in an EBM-2MV supplemented with an EGM-2MV bullet kit (LONZA), with the exception of VEGF, which was not added to the medium. The medium was refreshed every 2–3 days. Cells were kept at 33°C with 5% CO_2 until they were 90% confluent.

2.2. Endothelial cell barrier formation

An endothelial cell barrier with each cell line was obtained by seeding 25,000 cells/cm^2 for HPMEC-ST1.6R, or 50,000 cells/cm^2 for the other cell lines, respectively, in a 24-well plate (Corning) pre-coated as described above. The cells were cultured for an additional three days for ciGENC and TRP3, or four days for hCMEC/D3 and HPMEC-ST1.6R, respectively, and kept under standard conditions (37°C, 5% CO_2). The medium was refreshed every two days.

2.3. Immunohistochemistry

Cell confluency and morphology were assessed by light microscopy (Olympus IX50). For immunohistochemistry, 25,000 cells/cm^2 for HPMEC-ST1.6R or 50,000 cells/cm^2 for the other cell lines, respectively, were seeded in a 24-well plate (Corning) on glass coverslips pre-coated as described above for each cell line. Three days after seeding for ciGENC and TRP3, or four days for hCMEC/D3 and HPMEC-ST1.6R, respectively, cells were fixed with a formaldehyde solution (4% v/v) for 15 min and then permeabilized with Triton X-100 (0.1% v/v) for 5 min. Then, cells were incubated with an antibody against the tight junction proteins zonula occludens-1 (ZO-1, Life technologies, NY, USA) and CD31 (also known as platelet endothelial cell adhesion molecule, PECAM1, Dako, Glostrup, Denmark) for 1 h at room temperature, followed by incubation with Alexa Fluor 488- (Life Technologies, NY, USA) and Cy5- labelled (Jackson Immuno Research Laboratories, Inc., PA, USA) secondary antibodies for 1 h. Nuclear staining was performed by incubating cells for 5 min with 0.2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). Afterwards, slides were mounted with Mowiol 4–88 mounting medium (EMD Chemical, Inc., CA, USA). Fluorescence imaging was performed using a Leica TCS SP8 confocal fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with a 405 nm laser for DAPI excitation, a 488 nm laser for Alexa Fluor 488, and 638 nm laser for Cy5. Images were processed using ImageJ software (http://www.fiji.sc). Brightness was adjusted to improve visualization.

2.4. Nanoparticle characterization

Green fluorescently labeled (maximum excitation and emission wavelength 485 and 510 nm, respectively) plain (non-functionalized,

SiO_2), amino-modified (SiO_2-NH_2), and carboxylated silica nanoparticles (SiO_2-COOH) of 100 nm were purchased from Micromod Partikeltechnologie GmbH (Rostock, Germany). Nanoparticle size distribution by dynamic light scattering (DLS) and zeta potential (ζ-potential) were measured using a Malvern Zetasizer Nano ZS (Malvern Instrument Ltd., Worcestershire, UK). Briefly, nanoparticles (100 μg/ml) were dispersed in PBS, dH_2O or cell culture medium supplemented with 5 mg/ml FBS (Gibco Thermofisher Scientific), corresponding to the standard 10% v/v cell culture medium, or the same amount of human serum (human serum from pooled donors, from TCS BioSciences Ltd Botolph Claydon, Buckingham, UK). Samples were measured at 20°C immediately, or after 24-hour incubation at 37°C using disposable capillary cells (Malvern). The results are the average of 5 runs of at least 3 measurements.

2.5. Nanoparticle uptake and flow cytometry analysis

Cell fluorescence intensity was used as a measurement of nanoparticle uptake on the endothelial cell barriers. Briefly, after developing cell barriers as described above, cells were exposed for 1, 3, 5, 24, and 26 h to 50 μg/ml SiO_2, SiO_2-NH_2, or SiO_2-COOH. Nanoparticles were dispersed at room temperature in cell culture medium containing 5 mg/ml FBS (Gibco Thermofisher Scientific) or human serum (TCS BioSciences). Cells were exposed to the freshly prepared nanoparticle dispersions immediately after mixing by replacing the cell culture medium. After exposure, in order to remove the excess of nanoparticles and reduce the presence of nanoparticles adhering outside the cell membrane which could interfere with uptake quantification, cells were washed once with cell culture medium supplemented with 10% FBS (Gibco Thermofisher Scientific) and twice with PBS. Afterwards, cells were detached using 0.05% trypsin-EDTA. Cell fluorescence was measured using a Cytoflex S Flow Cytometer (Beckman Coulter, Woerden, The Netherlands) with a 488 nm laser. Data were analyzed by Flowjo data analysis software (Flowjo, LLC). Dead cells and cell doublets were excluded from the plots by setting gates in the forward and side scattering double scatter plots. At least 15,000 cells were acquired per sample, and the median of the obtained cell fluorescence distribution calculated. For each exposure time, duplicate samples were made and their median cell fluorescence intensity is shown, together with their average. The results of an independent replicate experiment are shown in Supplementary Figs. S2-3 to confirm the trends observed.

2.6. Nanoparticle-corona formation and characterization

In order to examine the corona formed on the different nanoparticles, 1 mg/ml SiO_2, SiO_2-NH_2, or SiO_2-COOH of 100 nm size were dispersed in PBS containing 5 mg/ml FBS (Gibco Thermofisher Scientific) or human serum (TCS BioSciences) and incubated at 37°C under continuous shaking at 300 rpm for 1 h. After this, the dispersion was centrifuged for 1 h at 16,000 g in order to pellet the corona-coated nanoparticles. The pellet was washed in PBS and centrifuged again for 1 h at 16,000 g for a total of three centrifugations to remove the soft serum corona and excess free proteins in solutions and isolate hard corona-coated nanoparticles. The final amount of nanoparticles present in the pellet was quantified by measuring their fluorescence with a spectrofluorometer. Afterwards, 200 μg hard corona-coated nanoparticles were resuspended in gel loading buffer, boiled for 5 min at 95°C, and loaded onto 10% polyacrylamide gel for SDS-PAGE. After electrophoresis, the gel was incubated for 1 h with a solution containing 0.1% w/v Coomassie blue R-250 in a water : methanol : glacial acetic acid (5:4:1) solution and washed with milliQ water. Pictures were taken with a ChemiDoc XRS (Biorad, USA). After this, the intensity of selected bands was quantified using ImageJ software (http://www.fiji.sc) to evaluate differences in the isolated coronas.

2.7. mRNA expression of transferrin

The expression level of transferrin receptor genes TFR1 and TFR2 in brain and liver endothelium was determined by RT-PCR. TRP3 or hCMEC/D3 cells were cultured to form a cell barrier as described above. Then, total mRNA was isolated with a Maxwell instrument and Maxwell 16 LEV simplyRNA Cells Kit (Promega, Madison, WI, USA) according to the instructions provided by the manufacturer. Reverse transcription of mRNA into cDNA was performed with a Reverse Transcription System (Promega, Leiden, The Netherlands) in an Eppendorf Mastercycler gradient (the following cycle was used: 20°C for 10 min, 42°C for 30 min, 20°C for 12 min, 99°C for 5 min and 20°C for 5 min). The transcription levels were measured by quantitative real-time PCR (SensiMix SYBR kit, Bioline, Taunton, MA, USA) in an ABI7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) from cDNA (10 ng per sample). The Ct values were obtained with the SDS 2.4 software (Applied Biosystems). For each target, four replicates were used, and the average Ct value and its standard deviation were calculated. Results are expressed as fold-change of the averaged Ct values of TRP3 (Ct_{TRP3}) related to Ct values of hCMEC/D3 (Ct_{D3}) as follows:

$$\text{Fold change} = 2^{-(\text{Mean } Ct_{TRP3} - -\text{Mean } Ct_{D3})}.$$

2.8. Analysis of the rate of endocytosis

The rate of endocytosis in brain and liver endothelium was determined using dextran as a fluid-phase marker and transferrin as an example of a molecule that follows the route of receptor-mediated endocytosis. TRP3 and hCMEC/D3 cells were cultured to form a cell barrier as described above. Then, cells were incubated for different time periods with 250 μg/ml TRITC dextran 10 kDa (Life technologies, NY, USA) dispersed in cell culture medium or with 10 μg/ml Alexa Fluor 546 fluorescently labeled transferrin (Life Technologies, NY, USA) dispersed in serum-free medium. Prior to incubation with transferrin, cells were pre-incubated with serum-free medium for 20 min. After exposure, cells were harvested and analyzed by flow cytometry as described above with a 488 nm and 561 nm laser. At least 15,000 cells were acquired per sample, and the median of the obtained cell fluorescence distribution was calculated. For each exposure time, duplicate samples were made and their median cell fluorescence intensity is shown, together with their average. The results of an independent replicate experiment are shown in Supplementary Fig. S5 to confirm the trends observed.

2.9. Statistical analysis

For nanoparticle uptake and rate of endocytosis studies, linear regression two-tailed Student's *t*-test was used as a simple approximation to compare the uptake kinetics between different samples. For DLS results, an unpaired two-tailed Student's *t*-test was used to determine statistically significant differences in the average nanoparticle hydrodynamic diameter after dispersion in serum in comparison to the results in PBS. In addition, for qPCR, an unpaired two-tailed Student's *t*-test was used to determine statistically significant differences in expression levels in TRP3 cells in comparison to the results in hCMEC/D3. A p value < 0.01 or < 0.05 was considered statistically significant. Differences are not labelled if not significant.

3. Results

3.1. Endothelial cell barrier characterization

Prior to nanoparticle uptake studies, cell culture conditions were optimized to ensure a proper formation of cell barriers. Four

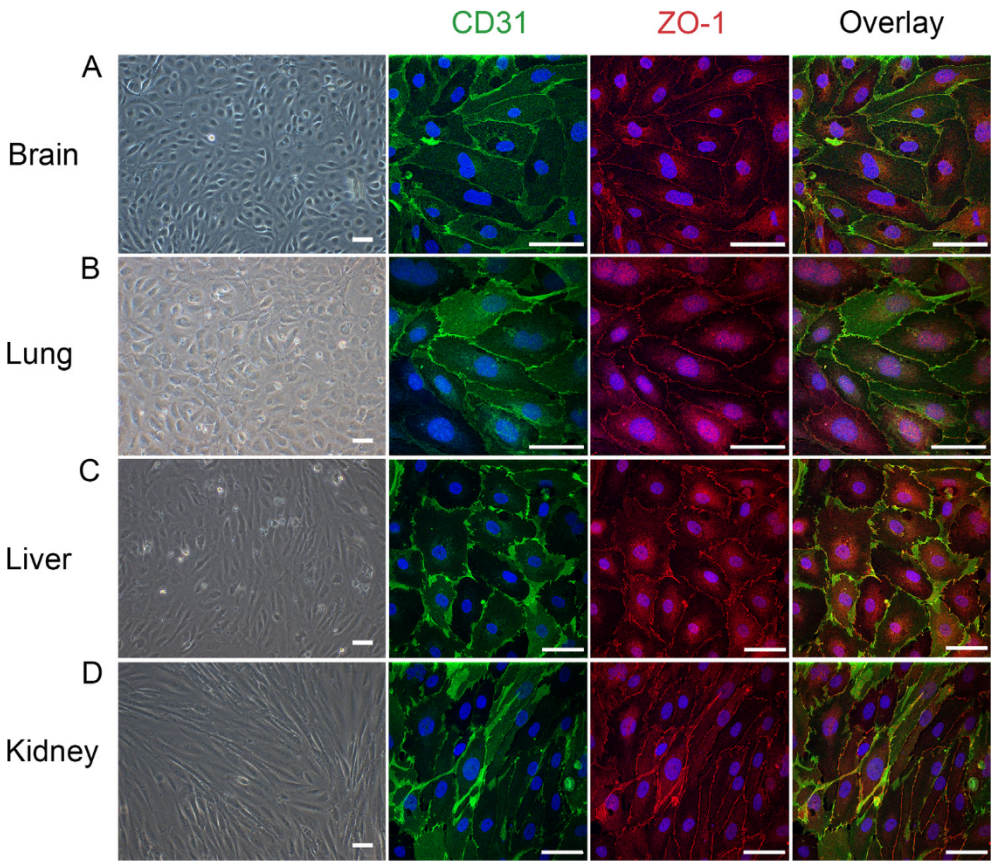

Fig. 1. Morphology, ZO-1, and CD31 expression in endothelial barriers derived from different organs. Endothelial barriers were prepared as described in the Methods. Left: light microscopy images of the endothelial barriers (scale bar: 50 μm). Right: confocal images of anti-ZO-1 (red) and anti-CD31 (green) immunostainings. Blue: DAPI stained nuclei (scale bar: 50 μm). (A) hCMEC/D3, (B) HPMEC-ST1.6R, (C) TRP3, and (D) ciGENC cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immortalized endothelial cell lines derived from different organs were selected as cell models: hCMEC/D3 (brain), HPMEC-ST1.6R (lung), TRP3 (liver), and ciGENC (kidney). To confirm cell barrier formation, the expression and cellular distribution of ZO-1, a tight junction protein, and CD31, an adhesion protein that is enriched at the basolateral surface of polarized endothelial cell monolayers, were assessed using confocal microscopy. As shown in Fig. 1A-D, cell monolayers derived from all four cell lines showed a lateral localization of ZO-1 and CD31, indicating the development of a cell barrier. Interestingly, each cell line also showed a unique cell shape and barrier morphology (Fig. 1A-D, left), which suggested that the different endothelia retained – at least in part – some of their organ-specific properties (Krump-Konvalinkova et al., 2001; Parent et al., 2014; Satchell et al., 2006; Weksler et al., 2005).

3.2. Nanoparticle physicochemical characterization

Green-labeled 100 nm silica nanoparticles with three different surface functionalizations (SiO_2, SiO_2-NH_2, and SiO_2-COOH) were selected as nanoparticle models with varying surface properties to form different coronas and test uptake preferences in the different endothelial barriers. For exposure to cells, the nanoparticles were dispersed in their respective cell culture media supplemented with 5 mg/ml FBs or human serum. Dynamic light scattering (DLS) was used to characterize the stability of the nanoparticle dispersions under these conditions. The results confirmed that stable dispersions were obtained for all nanoparticles (Table 1 and Fig. 2). The observed increase in hydrodynamic diameter of nanoparticles when incubated in medium containing FBS or human serum confirmed protein adsorption on the surface of nanoparticles and corona formation.

In addition to the hydrodynamic diameter, the zeta potential of the nanoparticles was determined. As expected, plain (SiO_2) and carboxylated (SiO_2-COOH) silica nanoparticles showed negative zeta potential when dispersed in water or PBS (Table 1). For the amino-modified silica nanoparticles (SiO_2-NH_2), a negative zeta potential in water and PBS was also observed. These nanoparticles show a positive zeta potential only at pH below 3.5 (data from the manufacturer). As expected (Lesniak et al., 2012; Monopoli et al., 2011), all the nanoparticles showed near-neutral zeta potential when incubated in a medium containing FBS or human serum, regardless of their surface functionalization (Table 1), as a consequence of protein adsorption and corona formation on the surface of nanoparticles. Despite the decrease in zeta potential absolute value towards neutrality, nanoparticle dispersions remained stable, likely due to the steric stabilization by the adsorbed proteins. SDS-PAGE was then used to identify the corona proteins on the different nanoparticles (Supplementary Fig. S1). Quantification of the intensity of selected bands confirmed that different coronas were formed, as expected due to the different functionalization (also in Supplementary Fig. S1). However, the banding pattern was relatively similar across the different samples.

3.3. Nanoparticle uptake by human endothelial barriers from different organs

We next investigated the cellular uptake kinetics of the silica nanoparticles in the endothelial cell barriers of different organs in order to determine whether there was preferential uptake of certain types of nanoparticles by specific endothelial cell types. In addition, we also investigated whether the uptake was different when the nanoparticles were dispersed in different types of serum, bovine or human. It is known that dispersions in different sera can lead to the formation of different coronas (Francia et al., 2019; Monopoli et al., 2011; Partikel et al., 2019). SDS-PAGE of the corona proteins isolated from the surface of the nanoparticles after incubation with either FBS or human serum shows differences in the banding patterns (molecular weight and intensity), confirming that different coronas were formed when

Table 1
Nanoparticle characterization by dynamic light scattering and zeta potential measurements. Z-average hydrodynamic diameter and polydispersity index (PDI), or average hydrodynamic diameter as obtained by dynamic light scattering (DLS) and zeta potential (mV) of 100 μg/ml 100 nm SiO_2, SiO_2-NH_2, and SiO_2-COOH in dH_2O, PBS, or EBM-2 basal medium supplemented with 5 mg/ml FBS or human serum (HS). Size distributions in supplemented basal medium were measured immediately, or after 24 h of incubation of the dispersions at 37 °C, 5% CO_2. Either cumulant or CONTIN analyses were performed for measurements in buffer and supplemented medium, respectively, to account for multimodal peaks which arise in supplemented medium because of co-detection of the excess free proteins in solution. The results are the average of 5 runs of at least 3 measurements. An unpaired student's *t*-test was used to determine statistically significant differences in comparison to the results in PBS. Statistically significant differences are indicated with an asterisk (*p < 0.05; ** p < 0.01; n = 3). All nanoparticles remained stable also after dispersion in the cell culture medium with serum and after 24 h of incubation in the conditions used for experiments with cells.

Sample	Medium	Diameter[1] (z-average, nm)	PDI[2]	Diameter[3] (nm)	ζ-potential (mV)
SiO_2	Water	111 ± 1	0.02 ± 0.02		−41 ± 1
	PBS	106 ± 2	0.03 ± 0.01		−14 ± 1
	EBM-2 + FBS	–	–	168 ± 3**	−8 ± 1**
	EBM-2 + HS	–	–	159 ± 12*	−6 ± 1**
	EBM-2 + FBS 24H	–	–	165 ± 9**	–
	EBM-2 + HS 24H	–	–	152 ± 7**	–
SiO_2-NH_2	Water	108 ± 1	0.01 ± 0.01		−27 ± 1
	PBS	107 ± 2	0.03 ± 0.01		−20 ± 2
	EBM-2 + FBS	–	–	178 ± 7**	−8 ± 1**
	EBM-2 + HS	–	–	197 ± 8**	−6 ± 1**
	EBM-2 + FBS 24H	–	–	169 ± 16*	–
	EBM-2 + HS 24H	–	–	182 ± 14*	–
SiO_2-COOH	Water	111 ± 2	0.03 ± 0.01		−33 ± 1
	PBS	105 ± 2	0.02 ± 0.02		−19 ± 1
	EBM-2 + FBS	–	–	173 ± 2**	−7 ± 1**
	EBM-2 + HS	–	–	167 ± 6**	−5 ± 1**
	EBM-2 + FBS 24H	–	–	160 ± 11*	–
	EBM-2 + HS 24H	–	–	157 ± 9**	–

[1] z-average hydrodynamic diameter extracted by cumulant analysis of the data.
[2] Polydispersity index (PDI) from cumulant fitting of the data.
[3] Average hydrodynamic diameter determined from CONTIN size distribution (the corresponding size distributions are shown in Fig. 2).

nanoparticles were dispersed in different sera (Supplementary Fig. S1).

Figs. 3 and 4 clearly show that nanoparticle uptake efficiency was different in each barrier culture, confirming that, even when cultured *in vitro,* these cells retain – at least in part – some of the different cellular properties of the organs from which they originated (Figs. 3 and 4 for experiments in bovine or human serum, respectively and Supplementary Fig. S2-3 for the results obtained in independent replicate experiments). When incubated in FBS, nanoparticles accumulated at the highest level in kidney and liver endothelium, followed by lung endothelium, and the lowest accumulation was observed in the cells from the blood–brain barrier (Fig. 3). Similar results were also observed when human serum was used, but with closer uptake levels in liver and lung endothelium, than in kidney (Fig. 4). In addition, the overall uptake efficiency for all nanoparticles in all barrier cultures was lower when nanoparticles were incubated in human serum, indicating that the serum source also clearly influenced uptake. Similar effects were previously observed (Francia et al., 2019; Salvati et al., 2013). After this, as a simple approximation, linear fit and statistical analysis were applied to compare the uptake kinetics of the different nanoparticles for each cell line (see Section 2.9 for details). No differences were observed between the different endothelial cell barriers concerning the relative uptake efficiencies of the different nanoparticles (Figs. 3 and 4). Specifically, statistical analysis confirmed that all cell lines showed higher uptake for SiO_2-NH_2 when dispersed in FBS (Fig. 3), while a higher uptake for SiO_2-COOH was observed when nanoparticles were dispersed in human serum. However, this latter was statistically significant only in the case of the brain endothelial cells (Fig. 4A). This indicated that changing corona composition by dispersion in different sera can affect nanoparticle uptake preference. Similar trends were observed in independent replicate experiments (Supplementary Fig. S2-3). Additionally, we performed equivalent studies using another nanoparticle type, namely liposomes of around 100 nm, and compared uptake of negatively-charged DOPG and zwitterionic DOPC liposomes in brain and liver endothelium, both in FBS and in human serum (Supplementary Fig. S4). The zwitterionic liposomes are known to adsorb lower amounts of protein in serum and

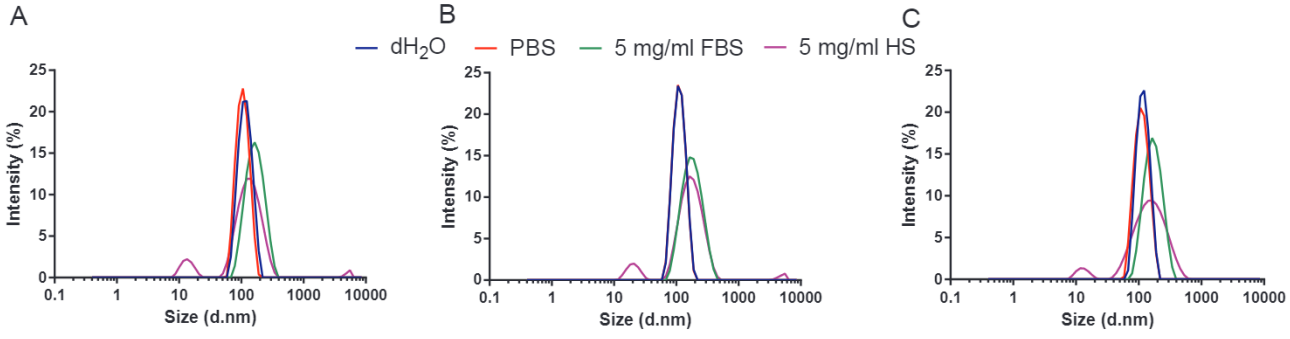

Fig. 2. Size distribution by intensity (diameter, d, nm) of 100 nm SiO_2 (A), SiO_2-NH_2 (B), and SiO_2-COOH (C) as obtained by dynamic light scattering (DLS). Silica nanoparticles (100 μg/ml) were dispersed in dH_2O, PBS, and EBM-2 cell culture medium supplemented with 5 mg/ml of FBS or human serum (HS). All nanoparticles remained stable after dispersion in the cell culture medium with serum.

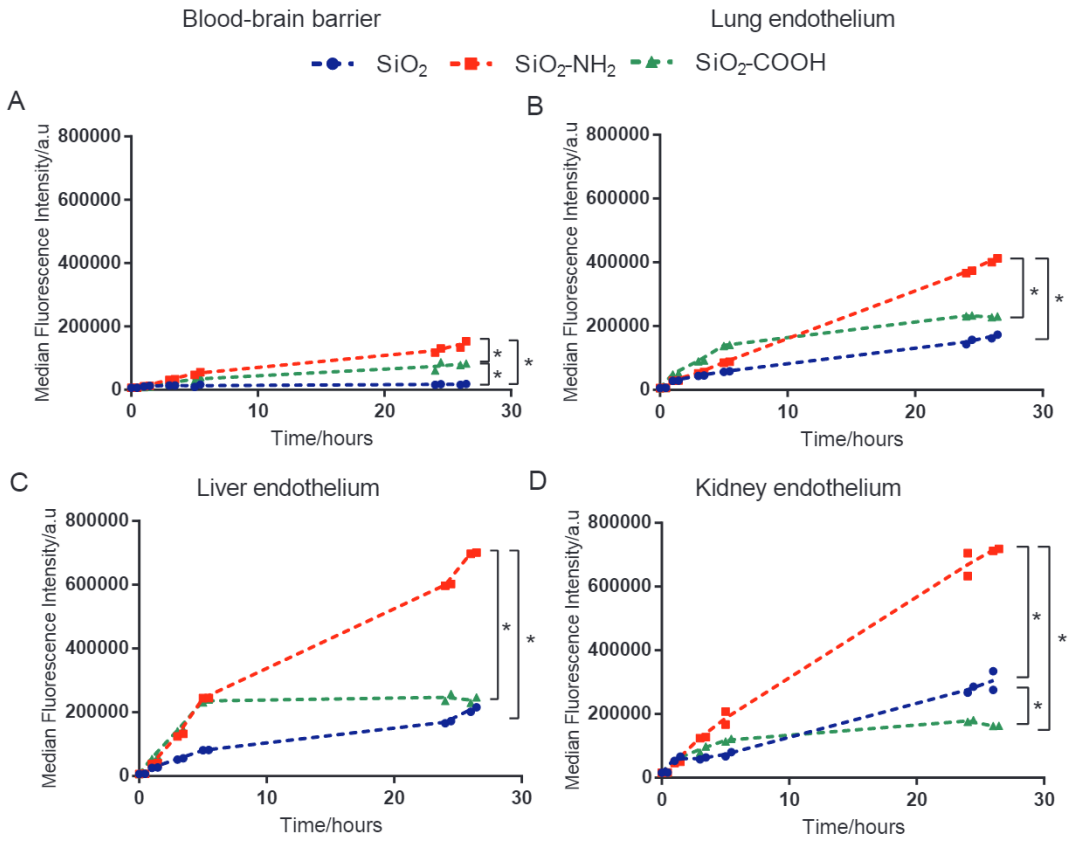

Fig. 3. Nanoparticle uptake levels in endothelial barriers derived from different organs in the presence of bovine serum. Median cell fluorescence intensity as obtained by flow cytometry of hCMEC/D3 (A), HPMEC-ST1.6R (B), TRP3 (C), and ciGENC (D) exposed to different nanoparticles. Endothelial barriers were prepared as described in the Methods and exposed to 50 μg/ml of 100 nm SiO_2, SiO_2-NH_2, or SiO_2-COOH in a cell culture medium supplemented with 5 mg/ml FBS for the indicated times. The median cell fluorescence intensity of two replicate samples is shown, together with a line that passes through their average. The results of an independent replicate experiment are shown in Supplementary Fig. S2. For each uptake kinetics, a linear regression two-tailed Student's *t*-test was applied to compare them (see Section 2.9 for details). Statistically significant differences are indicated with an asterisk. $p \leq 0.05$ was considered significant.

show lower uptake than charged ones (Yang et al., 2020). In line with this, we found that in both endothelia the uptake kinetics of the two formulations differed, and in all conditions, in the first hours of exposure, the negatively-charged DOPG liposomes showed significantly higher uptake in comparison to the zwitterionic DOPC liposomes, later converging to comparable levels (Supplementary Fig. S4). Additionally, as also observed with the silica nanoparticles, in all cases, uptake was higher when the liposomes were added to cells in medium with FBS than with HS, and uptake was higher in liver than in brain endothelium, the effect being more evident for liposomes dispersed in FBS.

3.4. Rate of endocytosis of brain and liver endothelium

As a final step, we investigated whether the observed differences in nanoparticle uptake efficiency between the different barrier cultures could be due to differences in their rate of endocytosis. For this purpose, we used brain and liver endothelium, which showed the lowest and highest nanoparticle uptake, respectively. The rate of endocytosis was determined by using FITC-dextran 10 kDa as a fluid-phase marker, and transferrin, as an example of a protein which is internalized via receptor-mediated endocytosis. Uptake kinetics were determined and linear fit applied to statistically compare the uptake rates (see Section 2.9 for details, Fig. 5 and Supplementary Fig. S5 for the results obtained in independent replicate experiments). As shown in Fig. 5A, there was no major difference in the uptake rate of FITC-dextran. However, the liver endothelium showed much higher transferrin uptake in comparison to brain endothelium (Fig. 5B). We then investigated whether this was due to a higher expression level of the transferrin receptor TFR1. Interestingly, as shown in Fig. 5C, we found that the liver endothelium had 2.5 times lower expression of TFR1 compared to the brain endothelium. However, the expression level of the secondary transferrin receptor TFR2 was shown to be three times higher in liver endothelium, possibly explaining the higher transferrin uptake in the liver. Overall, these results confirmed that different endothelial cells express cell receptors to different levels and show differences in uptake rates.

4. Discussion

The aim of this study was to investigate the effect of endothelial cell heterogeneity on the cellular uptake of nanoparticles. More specifically, we aimed to determine whether by changing nanoparticle properties, thus forming different coronas, nanoparticles could be targeted to specific organs. Previously, it has been shown that changing nanoparticle size, charge, or other similar parameters can affect nanoparticle distribution *in vivo*. This may be related to differences in the type of corona formed and may confer "natural targeting" to organ-specific endothelial cells (De Jong et al., 2008; He et al., 2010; Hirn et al., 2011). These effects could be exploited for nanomedicine targeting (Blanco et al., 2015). To determine whether this could be possible, we chose four unique organ-derived endothelial cell lines: hCMEC/D3 (blood–brain barrier), HPMEC-ST1.6R (lung microvasculature), TRP3 (liver sinusoid), and ciGENC (kidney glomerulus). These endothelial cell lines are well characterized immortalized cell lines and retain many of their organ-specific properties (Krump-Konvalinkova et al., 2001; Parent et al., 2014; Satchell et al., 2006; Weksler et al., 2005). Endothelial cell barriers were formed and exposed to silica nanoparticles

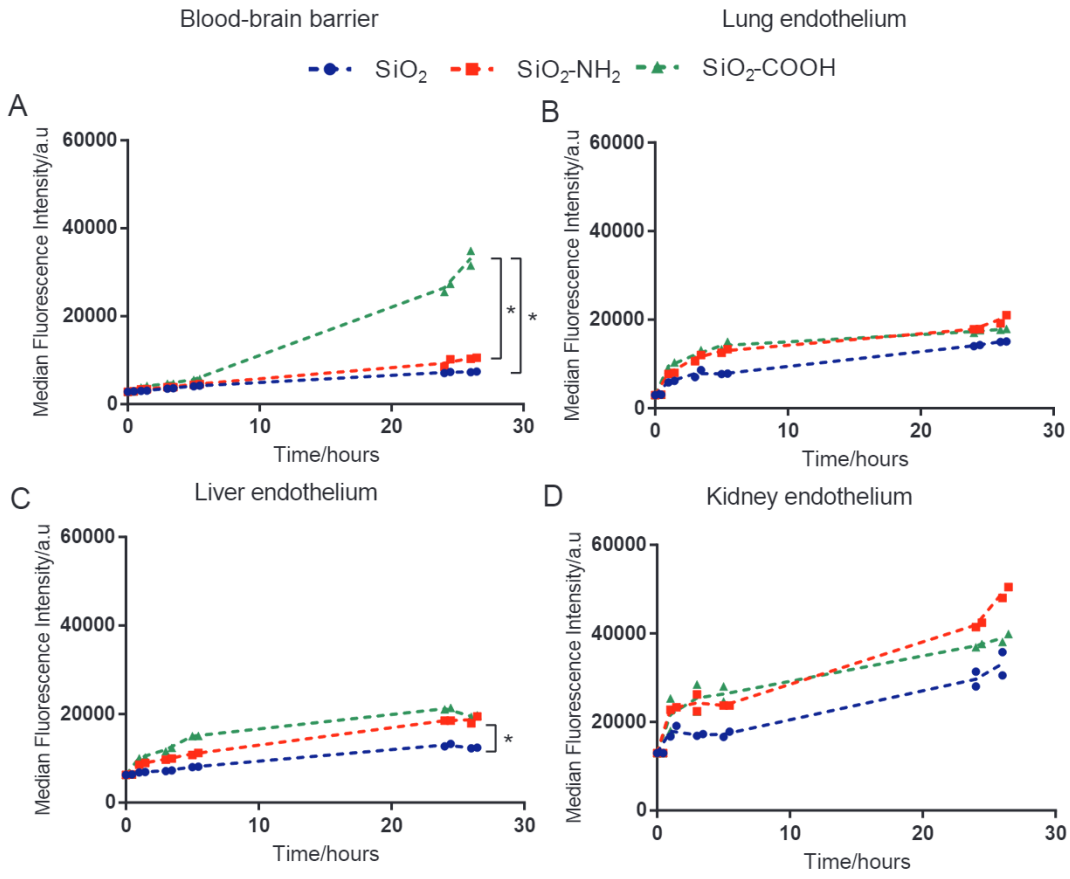

Fig. 4. Nanoparticle uptake levels in endothelial cells derived from different organs in the presence of human serum. Median cell fluorescence intensity as obtained by flow cytometry of hCMEC/D3 (A), HPMEC-ST1.6R (B), TRP3 (C), and ciGENC (D) exposed to different nanoparticles. Endothelial barriers were prepared as described in the Methods and exposed to 50 μg/ml 100 nm SiO_2, SiO_2-NH_2, or SiO_2-COOH in a cell culture medium supplemented with 5 mg/ml human serum for the indicated times. The median cell fluorescence intensity of two replicate samples is shown, together with a line that passes through their average. The results of an independent replicate experiment are shown in Supplementary Fig. S3. For each uptake kinetics, a linear regression two-tailed Student's *t*-test was applied to compare them (see Section 2.9 for details). Statistically significant differences are indicated with an asterisk. $p \leq 0.05$ was considered significant.

with three different surface functionalizations in the presence of bovine or human serum. Silica nanoparticles, in general, are well characterized and are known to form stable dispersions in cell medium supplemented with proteins (Lesniak et al., 2012; Monopoli et al., 2011; Shapero et al., 2011). Several studies have reported that the surface charge (among many other nanoparticle properties) affects corona formation and nanoparticle uptake into specific cell types (Arvizo et al., 2010; Chithrani et al., 2006; He et al., 2010; Rejman et al., 2004). Thus, here,

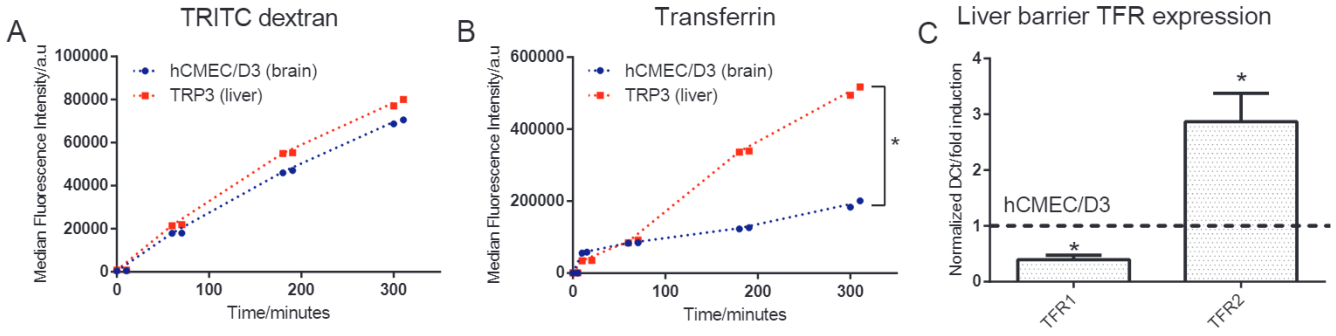

Fig. 5. Rates of endocytosis and expression levels of transferrin receptors in liver and brain endothelial barriers. Median cell fluorescence intensity as obtained by flow cytometry of hCMEC/D3 and TRP3 barriers exposed to different fluorescent molecules. Endothelial barriers were prepared as described in the Methods and exposed to 250 μg/ml TRITC dextran 10 kDa in cell culture medium (A) or 10 μg/ml Alexa Fluor546 fluorescently labeled transferrin (B) in serum-free medium for the indicated times. The median cell fluorescence intensity of two replicate samples is shown, together with a line that passes through their average. The results of an independent replicate experiment are shown in Supplementary Fig. S5. For each uptake kinetics, a linear regression two-tailed Student's *t*-test was applied to compare them (see Section 2.9 for details). Statistically significant differences are indicated with an asterisk. $p \leq 0.05$ was considered significant. C: expression levels of genes coding for the transferrin receptors TFR1 and TFR2 in TRP3 in comparison to hCMEC/D3. The results are average and standard deviation over four technical replicates of the fold-change in gene expression levels in TRP3 normalized to the expression levels in hCMEC/D3, calculated as detailed in the Methods (C). An unpaired two-tailed student's *t*-test was used to determine statistically significant difference compared to the expression level in hCMEC/D3 cells (see Section 2.9 for details). Statistically significant differences are indicated with an asterisk ($*p < 0.05$, $n = 4$).

silica nanoparticles with different surface functionalization were used as a model system to form different coronas and test potential differences in uptake preferences across the different cell types. As expected due to the different surface functionalization, quantification of the bands obtained by SDS-PAGE on the proteins recovered from the three nanoparticles confirmed that different coronas were formed (Supplementary Fig. S1).

Then, flow cytometry was used to measure nanoparticle uptake by cells. Protocols were optimized to reduce the eventual presence of extracellular nanoparticles adhering to the cell membrane (see Section 2.5 for details). Additionally, uptake was measured at multiple time points to determine uptake kinetics and compare their rate (this also allows to exclude eventual contribution of residual nanoparticles adhering outside cells (Lesniak et al., 2013)). As previously observed, uptake levels were much lower in human serum than in FBS, likely due to higher competition for cell receptors between the nanoparticles and free ligands in serum when human instead of bovine serum was used on the human cells, as well as differences in corona compostion in the different sera (Supplementary Fig. S1) (Francia et al., 2019). In all endothelia, the uptake levels of the different nanoparticle types were different. Thus, despite having the same size and similar zeta potential after dispersion in serum (Table 1 and Fig. 2), the three nanoparticles were taken up by cells at different levels, as expected because of their different functionalization and corona (Supplementary Fig. S1). However, the three nanoparticles were taken up by the various endothelial barrier models following similar trends: in fact, it was found that for all cell types, when the nanoparticles were dispersed in FBS, the uptake was higher for SiO_2-NH_2 (also confirmed by statistical analysis on the uptake rates, see Fig. 3). For nanoparticles in human serum, however, a higher uptake was observed for SiO_2-COOH and the effect was only statistically significant in the brain endothelium (Fig. 4). A possible interpretation of the similar uptake preferences among the different types of endothelial cells is that the degree of functionalization of these nanoparticles was not very high. It will be interesting to perform similar studies for nanoparticles with a higher degree of functionalization to study in detail how the degree of functionalization affects the nanoparticle corona, thus also uptake by cells. Nevertheless, uptake preferences changed dramatically when the nanoparticles were incubated with either FBS or human serum. It is known that corona proteins can mediate recognition and interaction of nanoparticles with cell receptors (Francia et al., 2020, 2019; Lara et al., 2018, 2017; Nel et al., 2009; Salvati et al., 2013). Thus, the different uptake preferences observed are likely due to differences in the protein corona composition on the nanoparticles when incubated with FBS or human serum (as indeed we show in Supplementary Fig. S1). This supports the overall hypothesis that tuning the corona composition on a nanoparticle (for instance by changing nanomaterial properties such as size, charge, composition or by pre-forming artificial coronas) can be used as a strategy to modulate uptake preferences in different cell types, thus potentially allowing a preferential targeting of nanoparticles to specific organs *in vivo*. Additionally the results from our studies have shown that even small changes in a single type of nanoparticle can affect its uptake in endothelial cells from different locations in the body. These studies indicate that much needs to be done to determine how size, shape, chemical composition etc. influence the uptake of nanoparticles. Nanoparticles of different size or material could also be used to form different coronas and test similar effects, as indeed we observed here using liposomes of different charge (Supplementary Fig. S4). Another important observation is that despite the similar uptake preferences observed by certain types of nanoparticles, nanoparticle uptake efficiency differed strongly in the different endothelial barrier models. Thus, in agreement with common observations for *in vivo* distribution studies with silica and other nanoparticles, kidney and liver endothelium showed a higher uptake, possibly related to the physiological role of these organs in excretion (De Jong et al., 2008; Gromnicova et al., 2016; Waegeneers et al., 2018; Wilhelm et al., 2016). In contrast, the blood–brain barrier, known to be the tightest of the endothelial barriers (Abbott et al., 2010; Ballabh et al., 2004), showed the lowest uptake efficiency for the nanoparticles. In agreement with this, lower nanoparticle uptake in the brain is also commonly observed *in vivo* (De Jong et al., 2008; Gromnicova et al., 2016; Semete et al., 2010). Similar results were observed by Gromnicova et al., who showed that the internalization of gold nanoparticles was significantly higher in kidney than in brain endothelium (Gromnicova et al., 2016). The differences in uptake efficiency could also be a reflection of differences in their rate of endocytosis. In line with this, we showed that while the rate of endocytosis of a fluid-phase marker (FITC-dextran 10 kDa) in liver and brain endothelium was comparable, the uptake of transferrin, here selected as an example to compare the rate of a receptor-mediated uptake in the two cell types, was much higher in the liver endothelium (Fig. 5). Since corona-coated nanoparticles are known to be internalized via active processes, and in several cases it has been shown that uptake occurs after interaction with cell receptors (Francia et al., 2020; Lara et al., 2018, 2017), the higher nanoparticle uptake in liver endothelium in comparison with brain endothelium is likely connected to similar differences in receptor activity among the different endothelia. Interestingly, the uptake rate of transferrin and the expression level of transferrin receptor TFR1 were inversely related between liver and brain endothelium. Since TFR1 is known to be the main receptor for transferrin, it is possible that despite the lower expression level in liver endothelium, this receptor is recycled faster in liver endothelium than in brain endothelium. In addition, the higher expression level of the secondary transferrin receptor TFR2 in liver endothelium could also contribute to the higher uptake of transferrin. These results suggest that differences in receptor expression and activity, rather than the basal rate of (fluid-phase) endocytosis, may potentially determine the differences observed in the uptake efficiency of nanoparticles. Thus, it would be interesting to identify the receptors involved in the uptake of these specific nanoparticles and compare their expression and activity in the different cell types. It is also important to note that in order to use the same protein content for corona formation, the nanoparticle dispersions were prepared with the same type and amount of serum for all endothelia, but using different basal media, as required for each cell type. Previous studies have shown that even when using the same serum and nanoparticles, the use of different cell culture media could affect protein corona formation, and, consequently, uptake by cells (Maiorano et al., 2010; Strojan et al., 2017). However, here, three out of the four endothelia (brain, lung and kidney endothelium) were cultured using the same basal media and only some supplements differed. Thus, the observed uptake differences were most likely due to the heterogeneity of each endothelium, rather than these subtle differences in the media. More importantly, the different endothelial barrier models showed differences in uptake efficiency, which reflected the different physiological functions of the various organs from which they originated. These results suggested that the *in vitro* models generated with the different cell lines retained a high degree of the distinctive features present in the endothelia of the organ type.

5. Conclusion

The results from the present study confirm that the heterogeneity of endothelial cells clearly influences the uptake of nanoparticles by different organs. Since most drugs lack specificity for endothelial cells, the ability to effectively target specific endothelial cells using nanoparticles could offer significant benefits for future clinical applications. Our findings highlight the importance of exploiting further endothelial cell heterogeneity for better design of targeted drug carriers. Identification of relevant receptors to enable selective uptake by specific endothelial cell types may provide valuable information for improving the design of such carriers. In addition to the genotypic and phenotypic profiling of endothelial cell types, the identification of relevant receptors for targeted nanomedicine may be deduced from the protein corona

composition of nanoparticles that show preferential interaction with the cell type of choice (Bewersdorff et al., 2017).

CRediT authorship contribution statement

Aldy Aliyandi: Conceptualization, Investigation, Formal analysis, Writing - original draft, Visualization. **Simon Satchell:** Resources, Writing - review & editing. **Ronald E. Unger:** Resources, Writing - review & editing. **Birke Bartosch:** Resources, Writing - review & editing. **Romain Parent:** Resources, Writing - review & editing. **Inge S. Zuhorn:** Conceptualization, Methodology, Writing - review & editing, Supervision. **Anna Salvati:** Conceptualization, Methodology, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2020.119699.

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