Supporting Information for

Uptake and transcytosis of functionalized superparamagnetic iron oxide nanoparticles in an *in vitro* blood brain barrier model

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Materials and Methods

Neutral red uptake assay for cytotoxicity. A neutral red in vitro toxicology assay kit (Sigma) was used to determine IONP-induced cytotoxicity. Specifically, hCMEC/D3 cells were seeded at 5×10^4 cells/mL to collagen I-coated 96 well plates and allowed to attach to the plate surfaces for 24 h. The cell growth medium was discarded and 100 µL of IONPs suspended in cell culture medium at different concentrations (0.5-100 µg/mL) was added. The cells were exposed to the IONPs for an additional 24 h before 0.033% of the neutral red dye was added. The plates were incubated with neutral red dye for an additional 3 h in 37° C and 5% CO_2 after which the growth medium with the dye was discarded, and the cells were washed 3 times with PBS buffer (pH 7.4, Sigma), followed by addition of 100 µL of neutral red dissolution buffer. The plates were mixed and kept at room temperature for 10 min to allow the cell-associated dye to dissolve. The dye absorbance was measured at 540 nm using Synergy2 plate reader (Biotek). Cellular viability was calculated using the following formula:

$$\%\,Viability = \frac{(Abs_{sample} - Abs_{blank}) * 100}{(Abs_{control} - Abs_{blank})},$$

where Abs_{sample} is absorbance of nanoparticle-exposed sample at 540 nm, Abs_{blank} absorbance of a blank well and $Abs_{control}$ absorbance of non-exposed cells.

Determination of generalized polarization (GP) in Laurdan assay. 64-bit TIFF images taken with confocal fluorescence microscope (Elyra, Zeiss) at 430-470 and 480-550 nm were analyzed using ImageJ (National Institute of Health) and a custom-written macro.[1] The generalized polarization (GP) values of the cell membranes can be calculated as:

$$GP = \frac{(I_{430-470} - I_{480-550})}{(I_{430-470} + I_{480-550})},$$

where *I* represents the Laurdan fluorescence intensity associated with a membrane pixel of interest in the ordered (430-470 nm) or disordered (480-550 nm) spectral channel.

Cross sectioning of cells grown on transwell membranes. hCMEC/D3 cells at 2×10^5 cells/mL were seeded to collagen I-treated transwell permeable supports (Corning, 6.5 mm diameter) with 3.0 µm pore size membranes that were placed into 24-well plates. For cross-sectioning, the cells were grown for 6 d, rinsed with PBS, fixed with 4% formaldehyde and soaked in a 30% sucrose solution overnight. The membrane was then frozen in the optimum cutting temperature (O.C.T.) formulation (Sakura Finetek) and sectioned to 3-5 µm slices using a cryomicrotome (Leica CM1860). The slices were mounted on poly-L-lysine coated glass slides, stained with haematoxylin (Sigma) for 4 min, thoroughly rinsed with running water, differentiated with 0.3% acid alcohol (99.7 mL 70% ethanol and 0.3 mL HCl), rinsed thoroughly with running water again, and finally stained with eosin (Sigma) for 2 min, dehydrated and mounted using DEPEX. The cross sections were imaged under bright-field (Nikon) using a 40× objective.

Staining of cells on transwell membranes for confocal microscopy. hCMEC/D3 cells grown on transwell membranes for 6 d (see above) were rinsed with PBS and fixed with 4% formaldehyde for 10 min, rinsed with PBS twice and incubated with 0.1% Triton X-100 for 5 min. The cells were blocked by incubating with 1% BSA for 30 min and after that 100 µg/mL Anti-VE cadherin antibody (Abcam) in 0.1% BSA was added to the cells for 1 h. Next, the cells were rinsed with PBS and incubated with 100 µg/mL Alexa Fluor 488-conjugated goat anti-Rabbit IgG secondary antibody (ThermoFisher) for 60 min. Subsequently, the cells were incubated with 0.16 µM rhodamine phalloidin (ThermoFisher) in PBS for 20 min and then stained with 0.2 µg/mL Hoechst 33342 (ThermoFisher) in PBS for 10 min. Images were taken using confocal fluorescence microscope (Zeiss Elyra) using 405 nm laser for Hoechst 33342, 488 nm laser for Alexa 488 IgG, 530 nm for rhodamine phalloidin, and a 63× water-immersion objective. 3D images were constructed from z-stacks measured with a step of 0.4 µm between individual layers.

Preparation of hCMEC/D3 cells for transmission electron microscopy

Chemicals for electron microscopy were of the following origins: glutaraldehyde (50% in H_2O) was purchased from Fluka, OsO_4 (4% in H_2O) from Sigma-Aldrich, and agarose and Propylene oxide (≥ 99.5%) from Sigma-Aldrich. Resin components: EPON 812, DDSA and DMP-30 were obtained from SERVA and MNA (~97%) was acquired from Fluka.

For electron microscopy, cells were prepared according to a modified protocol from Schrand et al.[2] hCMEC/D3 cell monolayers on 24-well transwell membranes that were exposed for 24 h to 50 µg/mL of IONPs were rinsed with cell culture medium and PBS to remove loosely bound NPs. The membranes were carefully separated from transwell inserts and placed to microcentrifuge tubes where the cell layer was fixed with 2.5 % glutaraldehyde (bought as 25% solution from Fluka) overnight at 4 °C. After fixation the cells were washed with 0.1 M phosphate buffer (pH 7.2) and treated with 1 % OsO_4 in phosphate buffer (Sigma-Aldrich) for 1 h at room temperature. The membranes were washed with DI water and wet membranes were cut to 2-3 mm slices under binoculars and placed to new microcentrifuge tubes. Cells on membrane slices were then dehydrated using graded ethanol solutions (50% ethanol for 5 min, 60% ethanol for 5 min, 70% ethanol for 10 min, 90% ethanol for 15 min and absolute ethanol for 20 min). The dehydrated samples were embedded in various strengths of EPON 812 resin (EPON 812, DDSA and DMP-30 were from SERVA, MNA (~97%) was from Fluka) (30% resin for 2 h, 50% resin overnight, 70% resin for 2 h and 100% resin at 37°C for 1 d and at 60°C for 3 d). Ultrathin sections were cut with a Leica EM UC7 ultramicrotome using a 45° diamond knife (Diatome, Hatfield, PA, USA). Samples were contrasted using 2% uranyl acetate in 50% ethanol solution for 20 min and in 0.2% lead citrate in 0.1 M sodium hydroxide solution for 20 min, as described in Bozzola and Russell.[3] Ultrathin slizes were visualized with TEM (Tecnai G2 Spirit BioTwin) at 120 kV. Elemental analysis of the viewed sections was conducted with SEM-FIB-EDX instrument (FEI Helios Nano-Lab 600, USA) using energy-dispersive X-ray spectroscopy (EDX) function (Oxford Instruments, UK) to confirm the internalization of IONPs.

Calculation of permeability of hCMEC/D3 cell monolayer for IONPs. Based on IONPs translocation through the cell layer on transwell membranes, the permeability was estimated by calculating the permeability coefficient (P_{app}):[4]

$$P_{app} = \frac{V}{A \times C_{apical}} \times \frac{\Delta C_{bas}}{\Delta t},$$

where V is the volume of medium in the basolaterial layer (cm^3), A is surface area of membrane (cm^2), C_{apical} is the concentration of IONPs in the apical layer, and ΔC_{bas} is the change in concentration during time Δt.

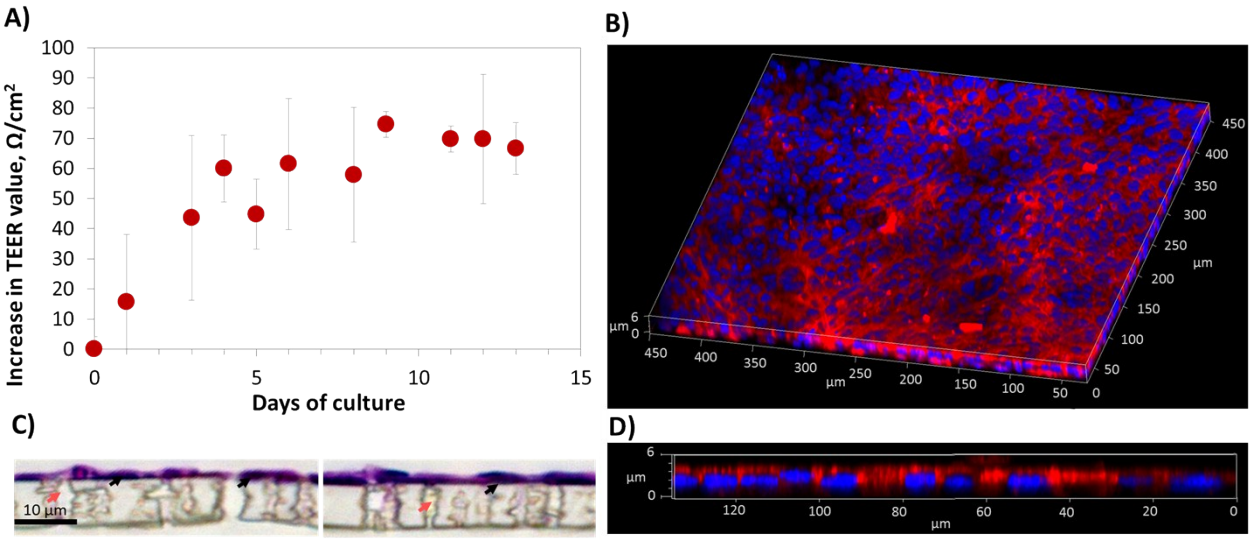

Fig. S1. **6 d grown layer of hCMEC/D3 cells layer on 3 µm pore size transwell membranes.** (A) Transepithelial resistance (TEER) of cell layer on 3 µm pore size polyester membranes over 13 days. Averages and standard deviations of 3-6 independent experiments are shown; (C) cross-section of 6 d monolayer; black arrows show cell nuclei and red arrows indicate ~3 µm membrane pores; (B) 3D image of 6 d cell layer on membrane; cell nuclei stained blue (Hoechst 3342) and cytoskeleton red (TRITC-phalloidin); (D) cross section of (B).

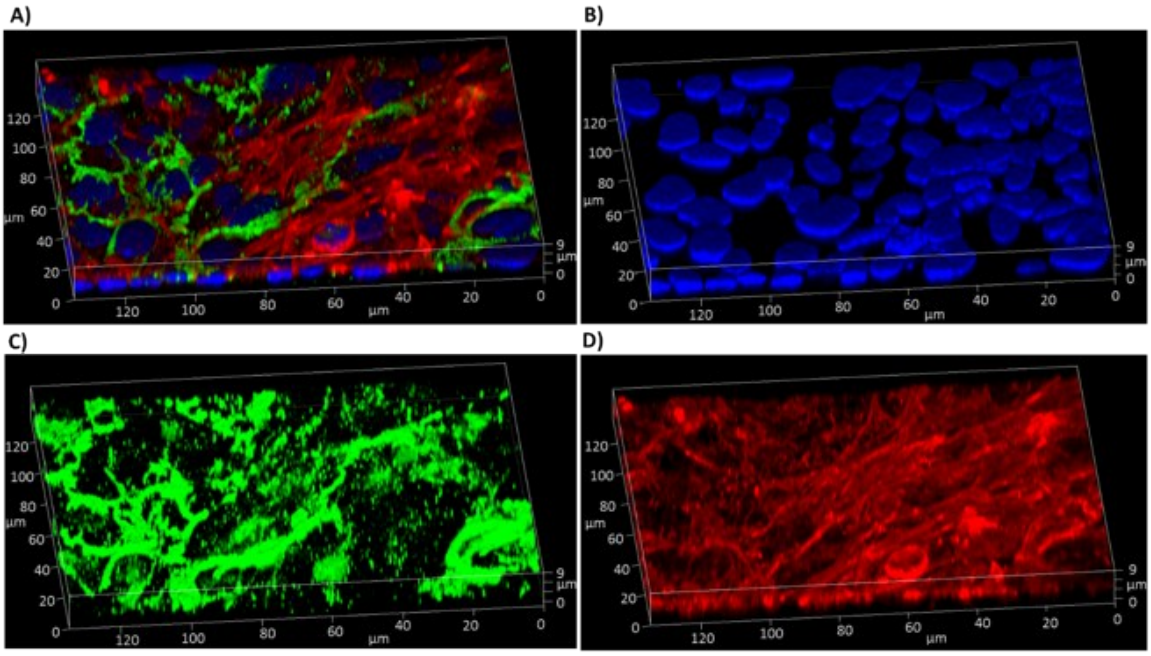

Fig. S2. Expression of tight junction in cells grown on transwell membranes. (B) Cell nuclei (Hoechst 33342 staining), (C) Tight junctions (staining with VE cadherin antibody and Alexa Fluor 488-conjugated secondary antibody), (D) Actin filaments (stained red with rhodamine phalloidin). (A) Overlay image of (B), (C) and (D).

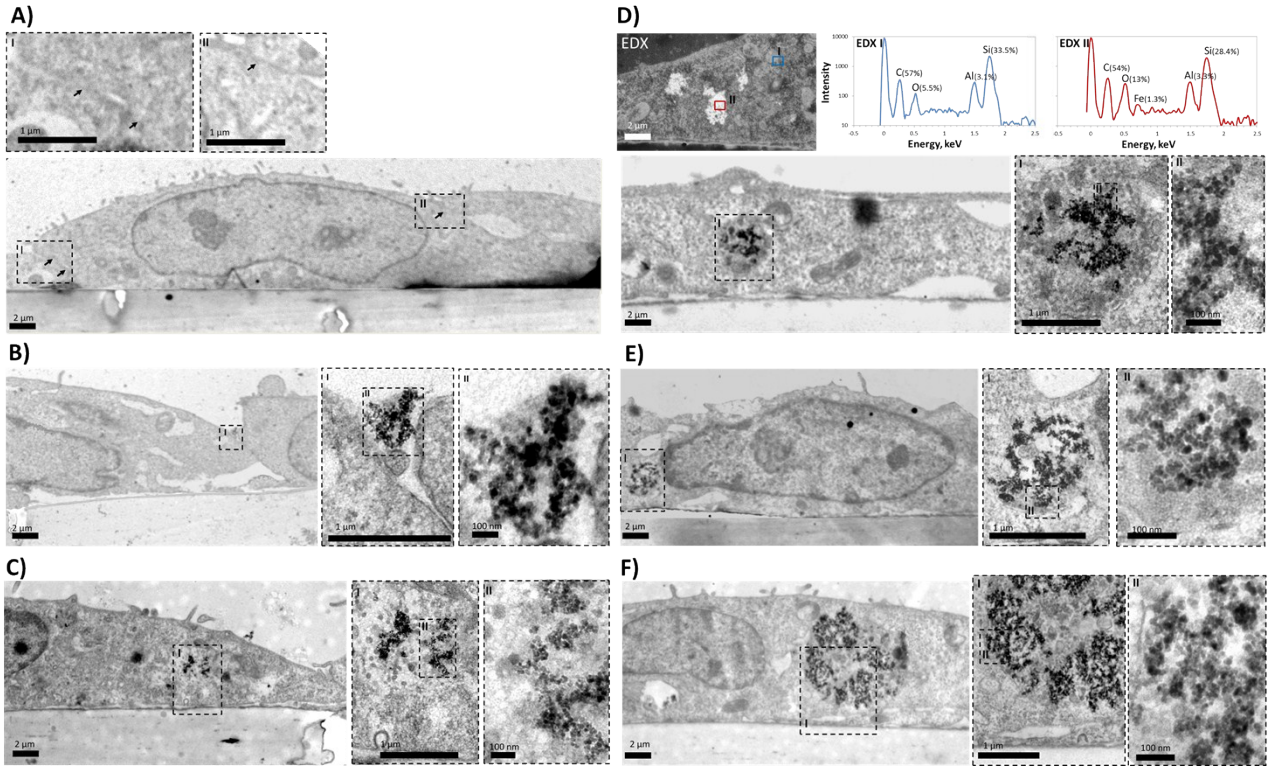

Fig. S3. Localization of IONPs in hCMEC/D3 cell layer on transwell membrane under TEM. (A) Non-exposed cells; I and II show insets of tight junctions (indicated with black arrows) between adjacent cells. Cells exposed for 24 h to 50 µg/mL of bare IONPs (B), IONP-PEG (C), IONP-PC (D), IONP PC:PEG 75:25 (E), or IONP PC:PEG 50:50 (F). Insets indicated with I show enlarged views of NP-containing cellular compartments; insets indicated with II show further magnified views of the NPs. Selected images showing early and late endocytotic vesicles that carry IONPs through hCMEC/D3 cells are shown in Fig. 6. In (D), EDX image of IONP-PC NPs in a cell is shown; the inset indicated with EDX I shows the EDX spectrum of a cellular region where no NPs were observed, while the inset EDX II represents the EDX spectrum of a region with NPs. Regions from which EDX I and II spectra were collected are indicated in the EDX image. In EDX I and EDX II, the % of O, C, Al, Si and Fe (only in EDX II) are shown. Scale bars are shown.

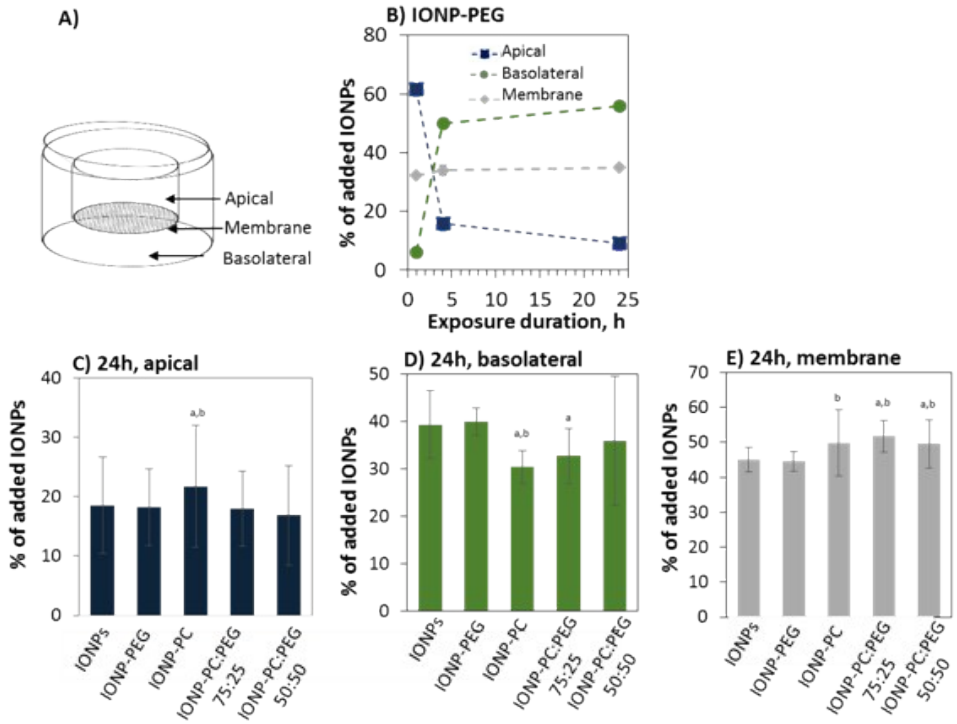

Fig. S4. Localization of IONPs in an empty transwell system. (A) Schematic representation of the transwell system. 50 μg/mL of different types of the IONPs was added to the apical layer of the transwell membrane and the Fe concentrations were measured in the (C) apical, (D) basolateral layer and (E) on membranes after 24 h. (B) Time-dependent localization of IONP-PEG. Means and standard deviations of three independent experiments are shown, [a] – significant ($p < 0.05$) difference between bare IONPs and grafted IONPs; [b] - significant ($p < 0.05$) difference between IONP-PEG and IONP-PC, IONP-PC:PEG 75:25 or IONP-PC:PEG 50:50.

References

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4. K. Vu, B. Weksler, I. Romero, P.-O.Couraud, A. Gelli, *Eukar Cell* 2009, **8**: 1803-1807.