Supporting Information

Correlating Corona Composition and Cell Uptake to Identify Proteins Affecting Nanoparticle Entry into Endothelial Cells

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Additional Materials and Methods

Control for free dye in the nanoparticles by SDS-PAGE

In order to exclude the presence of free fluorescent dye leaking from the nanoparticles which could confuse cell uptake results, 10 µl of the 100 and 200 nm plain silica nanoparticle stock was centrifuged at 16,000 g for 1 hour at 15 ˚C, the supernatant was discarded and the pellet resuspended in 10 µl distilled water. Then, the samples or the same volume of nanoparticle stock (without centrifugation) were diluted 4 times with sample-buffer 4x (0.25 M Tris-Hcl pH 6.8, 6.2% w/v DTT, 8% w/v SDS, 40% v/v Glycerol, 0.05% w/v bromophenol blue) and 10 µl were loaded on a 10% polyacrylamide gel for SDS-PAGE. After the gel was run, a fluorescence image of the gel was taken using a GBox (Syngene United Kingdom).

Control for protein separation during corona isolation

As an additional control, in order to exclude separation of proteins with the applied centrifugation settings, the corona formed on 1 ml 1 mg ml^{-1} plain SiO_2 of 100 and 200 nm in full (86 mg ml^{-1}) human plasma was prepared and isolated as described in the Methods and 0.8 ml full human plasma was centrifuged 4 times at 16,000 g, as for corona separation and each time resuspended in 0.8 ml PBS. After the 4th centrifugation the corona samples and the plasma were resuspended in 0.5 ml and 0.4 ml sample-buffer, respectively and the particle concentration was determined by fluorescence measurements (see Methods for details). Then, the volume corresponding to 300 µg silica and for the plasma sample the same volume were loaded on a 10% polyacrylamide gel for SDS-PAGE, stained and washed as described in the Methods and imaged using a GBox (Syngene United Kingdom).

Western blot

In order to confirm enrichment of HRG in the corona formed on the silica nanoparticles, the corona formed on 1 ml 1 mg ml^{-1} 200 nm plain, carboxylated and amino-modified SiO_2 in full (86 mg ml^{-1}) human plasma was prepared and isolated as described in the Methods. After the 4th centrifugation the pellet was resuspended in 30 µl PBS and the recovered proteins quantified using a Pierce BCA Protein Assay Kit (Thermofisher Scientific). Then, samples were diluted 1,000 times with PBS and after dilution 3: 1 with 4 x sample-buffer, 30 µl sample corresponding to roughly 11 ng corona proteins and the same amount of full human plasma were loaded onto a 10% polyacrylamide gel for SDS-PAGE. After the gel was run, the proteins were transferred onto a PVDF membrane (Roche) at 300 mA for 90 minutes on ice. The membrane was then incubated in a blocking buffer (20 mM Tris, pH 7.6, 1.5 M NaCl, 0.1% Tween-20, and 5% non-fat dry milk) for 2 hours at room temperature. Then the membrane was stained by incubation for 16 hours with a 1: 20,000 dilution of a rabbit polyclonal antibody against HRG (Protein tech, catalog 26252-1-AP) in blocking buffer at 4°C and for 2 hours in a 1: 2,000 dilution of a HRP labeled secondary antibody (Southern Biotech) in blocking buffer at room temperature. The signal was detected using ECL Prime Western Blotting reagents, GE Healthcare, United Kingdom. Pictures were taken with GBox (Syngene United Kingdom)

Uptake inhibition with chlorpromazine

In order to assess the role of clathrin-mediated endocytosis (CME) on nanoparticle uptake, TRP3 cells were treated with chlorpromazine prior to and during exposure to nanoparticles. Effects of chlorpromazine on cell viability were measured by MTT assay (see below for details). Briefly, TRP3 cells were seeded at a density of 50,000 cells cm^{-2} in a 24-well plate (Greiner) pre-coated with 0.1% cold gelatin as described in the Methods. Three days after seeding, cells were pre-

incubated for 10 minutes in serum-free medium with different concentrations of chlorpromazine hydrochloride. After the pre-incubation, cells were incubated for 4 hours with 30 μg ml^{-1} of freshly prepared hard corona-coated SiO_2-NH_2 200 nm in serum-free medium as described in the Methods, with or without the inhibitor. As control to assess the efficacy of the inhibitor, in parallel, cells were incubated for 10 minutes with 5 μg ml^{-1} Alexa Fluor 546 fluorescently labeled transferrin, or for 4 hours with 1 μg ml^{-1} fluorescently labeled low-density lipoprotein, Dil-LDL (Thermofisher Scientific) in serum-free medium. After exposure, cells were harvested and analyzed by flow cytometry as described in the Methods.

MTT assay

TRP3 cells viability after treatment with chlorpromazine was measured by MTT assay. Briefly, TRP3 cells were seeded at a density of 50,000 cells cm^{-2} in a 24-well plate (Greiner) pre-coated with 0.1% cold gelatin as described in the Methods. Three days after seeding, cells were incubated for 4 hours in serum-free medium with different concentrations of chlorpromazine hydrochloride. As a positive control, cells were incubated with 50 μg ml^{-1} amino-modified 50 nm polystyrene nanoparticles (Bang Laboratories, Sanbio BV, Uden, Netherlands) in serum-free medium. These nanoparticles are known to induce cell death due to their positive charge. After incubation, cells were washed with complete cell culture medium and were incubated with 0.5 mg ml^{-1} MTT solution (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) dissolved in complete cell culture medium. After the formation of blue precipitates (about 2 hours), cells were incubated under continuous shaking with DMSO in order to dissolve the precipitates. After 15 minutes, the absorbance of the solution was measured at 550 nm in a 96-well plate (Greiner) using UV-plate reader (Molecular Devices LLC., Sunnyvale CA, USA). The cell viability was expressed as a percentage of absorbance relative to untreated cells.

Plasmid transfection with AP180

The construct containing the RFP-tagged C-terminal of AP180 was kindly provided by Yvonne Vallis and Harvey T McMahon (University of Cambridge, UK). Briefly, TRP3 cells were seeded at a density of 50,000 cells per well in a 24-well plate (Greiner) on glass coverslips pre-coated with 0.1% cold gelatin as described in the Methods. Twenty-four hours after seeding, cells were transfected with 0.4 ng per well of plasmid DNA using 1.2 µl Fugene HD (Promega) as transfection reagent in complete cell culture medium. After 24 hours, cells were washed in serum-free medium and incubated for 15 minutes with 15 µg ml^{-1} Alexa Fluor 488 fluorescently labeled transferrin (Life Technologies, NY, USA) in serum free-medium, or for 24 hours with 50 µg ml^{-1} of hard corona-coated SiO_2-NH_2 200 nm in serum-free medium freshly prepared as described in the Methods, followed by nanoparticle removal and 4 hours incubation in nanoparticle-free complete cell culture medium (chase). Cells were then washed and prepared for immunohistochemistry as described below.

Confocal microscopy

Immunohistochemistry was performed in TRP3 cells plated on glass coverslips inserted in 24-well plates, and experiments were performed as described in the Methods. Then, cells were fixed with formaldehyde (4% v/v) for 15 minutes, followed by nuclear staining, which was performed by incubating cells for 5 minutes with 0.2 µg ml^{-1} 4',6-diamidino-2-phenylindole (DAPI). Afterwards, slides were mounted with Mowiol 4-88 mounting medium (EMD Chemical, Inc., CA, USA). Image acquisition 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 RFP. Images were processed using ImageJ software (http://www.fiji.sc).

Supporting Figures

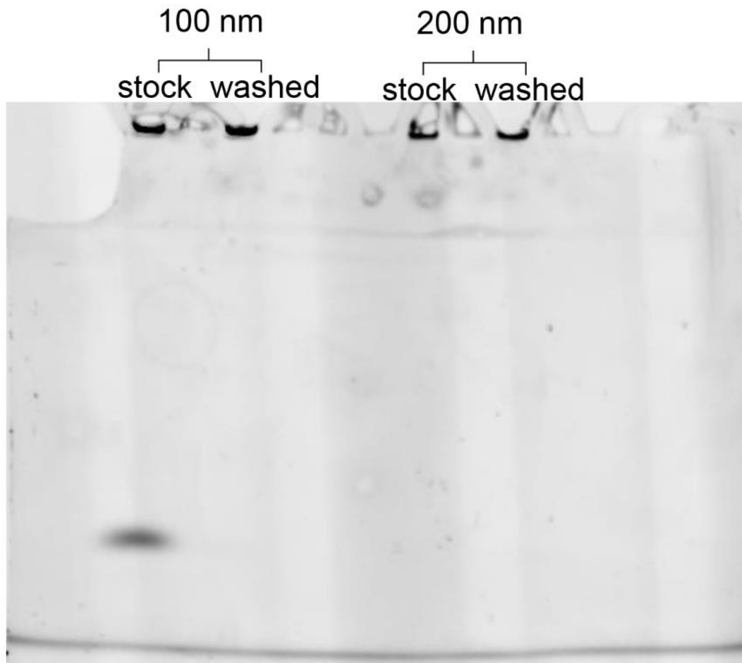

Figure S1. Control for free dye leaking from the nanoparticles. The figure shows a fluorescence image of a SDS-PAGE gel where silica nanoparticles were loaded in order to separate the nanoparticles inside the wells of the gel from eventual free dye running to the bottom of the gel. The 100 and 200 nm plain silica nanoparticles were loaded into the gel together with a sample of the same nanoparticles after a wash by centrifugation performed as described in the Additional Methods. The results showed that in all cases the nanoparticles remained stuck inside the wells of the gel, as expected because of their size. At the bottom of the gel, for the 200 nm nanoparticles no free fluorescent dye could be detected, while some fluorescence was visible for the 100 nm nanoparticle stock solution. However, this was removed after the sample was washed one time by centrifugation as performed to isolate corona-coated nanoparticles prior to exposure to cells. These results confirmed that when nanoparticles were added to cells after corona isolation no residual free dye was present, which could confuse fluorescence measurements for cell uptake studies.

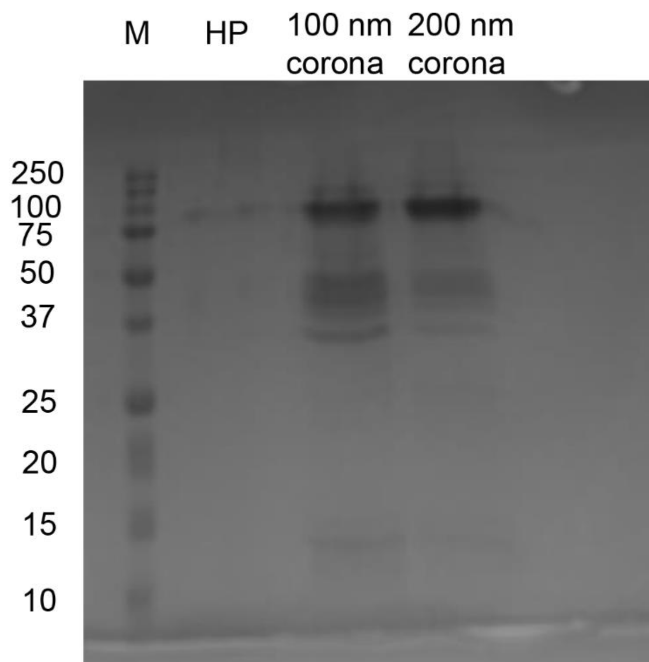

Figure S2. Control for protein separation during corona isolation. SDS-PAGE image of the proteins recovered from full human plasma (HP) and from 100 and 200 nm plain silica exposed to full human plasma after 4 centrifugation steps performed as for corona isolation for mass spectrometry. Briefly, a sample of full human plasma and samples of plain SiO_2 of 100 and 200 nm in full human plasma were centrifuged 4 times at 16,000 g, as for corona separation as described in the Additional Methods and the recovered pellets were loaded into a gel for SDS-PAGE. The results showed that while for the samples with nanoparticles many bands of corona proteins were detected, almost no proteins were present in a sample of full human plasma subjected to the same centrifugation procedure. This confirmed that the centrifugation settings used for corona isolation did not cause protein separation from full human plasma and only when nanoparticles were present many proteins could be recovered with the same procedure, due to corona formation and separation of the corona-coated nanoparticles. (M: molecular weight ladder).

(a)

Particle	Surface charge density (μmol/g)	PBS		Hard corona		ζ-potential (mV)	
		D (nm)	PDI	D (nm)	PDI	PBS	Hard corona
SiO_2 100 nm	-	101 ± 0.5	0.06 ± 0.03	146 ± 1.1	0.12 ± 0.04	-15 ± 1	-7 ± 2
SiO_2-NH_2 100 nm	1	100 ± 1.2	0.04 ± 0.01	140 ± 2.3	0.12 ± 0.03	-11 ± 1	-7 ± 1
SiO_2-COOH 100 nm	1	105 ± 0.5	0.02 ± 0.02	142 ± 2.9	0.12 ± 0.04	-15 ± 1	-7 ± 1
SiO_2 200 nm	-	165 ± 3.0	0.05 ± 0.03	192 ± 0.2	0.12 ± 0.04	-12 ± 1	-7 ± 1
SiO_2-NH_2 200 nm	4	192 ± 5.4	0.04 ± 0.01	235 ± 3.6	0.17 ± 0.03	-7 ± 1	-7 ± 1
SiO_2-COOH 200 nm	1	203 ± 1.1	0.05 ± 0.03	251 ± 0.5	0.08 ± 0.01	-13 ± 1	-7 ± 1

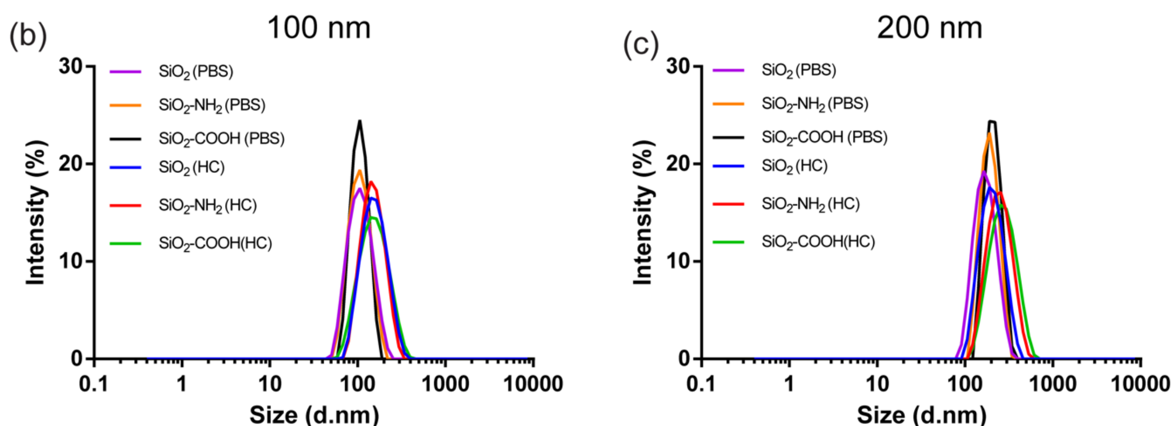

Figure S3. Physicochemical characterization of the nanoparticles and isolated corona-coated nanoparticle complexes formed on 100 nm and 200 nm SiO_2, SiO_2-NH_2, and SiO_2-COOH (50 μg ml^{-1} and 30 μg ml^{-1}, for 100 nm and 200 nm nanoparticles, respectively). Dynamic light scattering (DLS) of silica nanoparticles in PBS and the nanoparticle-corona complexes (hard corona, HC) formed in full human plasma (a-c). The size distributions (b and c) showed all dispersions remained stable in the conditions used for cell experiments. All the experiments in the manuscript were performed using these batches of nanoparticles. Only for the 200 nm SiO_2-NH_2 the results shown in Supporting Figures S5-6 and one of the three repeated experiments of Figure 5a and 5b were performed using a different batch. Dispersions of this batch of nanoparticles in PBS showed partial agglomeration and a zeta potential of +7 mV. Nevertheless, the results were highly reproducible (see results in Figure 5). D: diameter obtained by cumulant analysis of DLS data; PDI: polydispersity index. The surface charge density of all nanoparticles is also included, as obtained from the supplier.

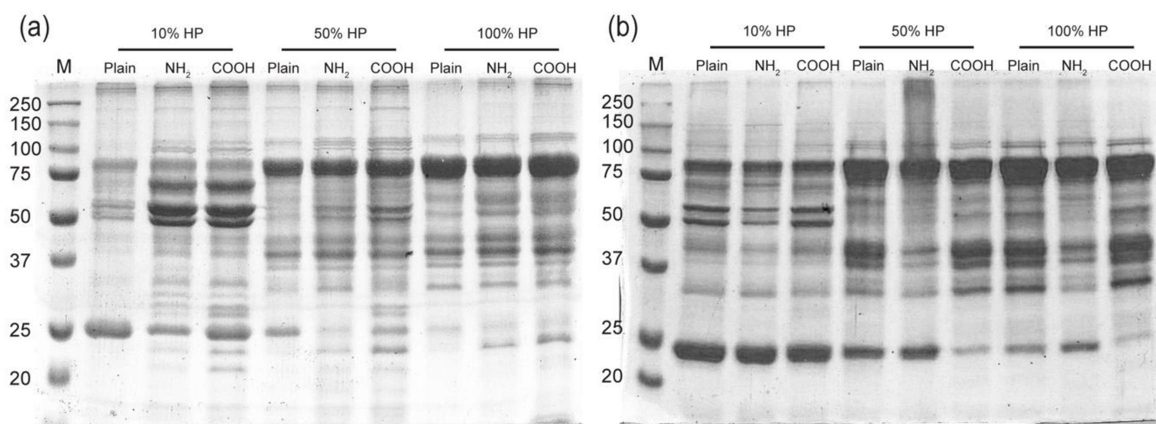

Figure S4. Identification of the corona proteins on 100 and 200 nm SiO_2 (plain), SiO_2-NH_2 (NH_2), and SiO_2-COOH (COOH) nanoparticles dispersed in human plasma. SDS-PAGE gel image of the proteins recovered on nanoparticle-corona complexes of 100 nm (a) or 200 nm (b) silica formed in different concentrations of human plasma. Nanoparticle-corona complexes were washed and centrifuged for a total of 4 times, after which the same amounts of nanoparticles were loaded onto 10% polyacrylamide gel. The gel shows that different bands were present in the corona formed on the different types of nanoparticles and also when human plasma concentration (HP) was varied. M: molecular weight size marker.

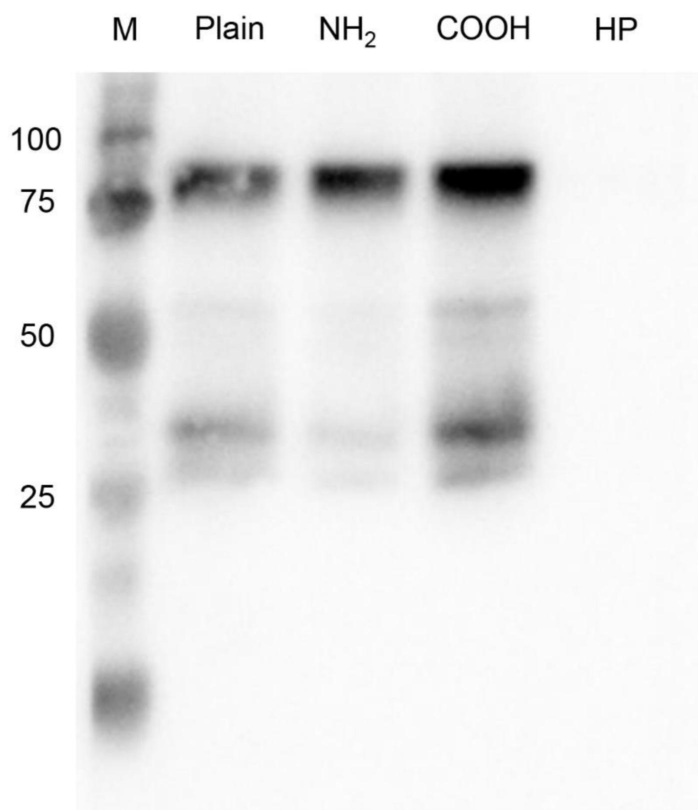

Figure S5. Western blot of histidine rich glycoprotein, HRG in full human plasma (HP) and in the corona formed on 200 nm plain, amino-modified and carboxylated SiO_2 in full human plasma (plain, NH_2, COOH, respectively). A western blot for HRG was performed for nanoparticle-corona complexes prepared and isolated as described in the Methods together with a sample with the same amount of full human plasma (see Additional Methods for details). The results showed that a strong band of HRG was visible at around 75KDa in the different corona samples, while at these concentrations, the HRG in full human plasma was below the limits of detection by WB (instead it could be detected by mass spectrometry, see full results in Supporting Information). This confirmed that as observed by mass spectrometry, corona formation led to a strong enrichment of low abundant proteins, in this case HRG. M: molecular weight size marker.

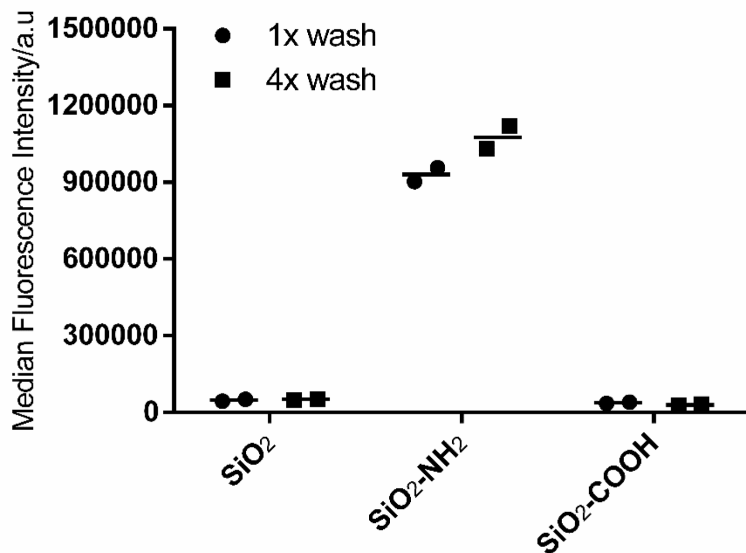

Figure S6. Effect of different washing procedures on nanoparticle uptake levels by cells. TRP3 liver endothelial cells were exposed for 24 hours to 30 µg ml^{-1} corona-coated SiO_2, SiO_2-NH_2, or SiO_2-COOH in serum free medium, isolated from full human plasma after 1 or 4 washes by centrifugation, performed as described in the Methods. Then, cell fluorescence was measured by flow cytometry. The results showed that in both cases higher uptake was observed for cells exposed to the amino-modified silica, thus the trend in uptake for the different nanoparticles was the same after the two different washing procedures. Because of this, for practical reasons, to reduce the time of sample preparation for cell uptake studies, cells were exposed to the corona-coated nanoparticles recovered after 1 wash, instead of 4 washes as performed for corona characterization by mass spectrometry. The results show the median cell fluorescence intensity of two replicate samples, together with their average, indicated by a line.

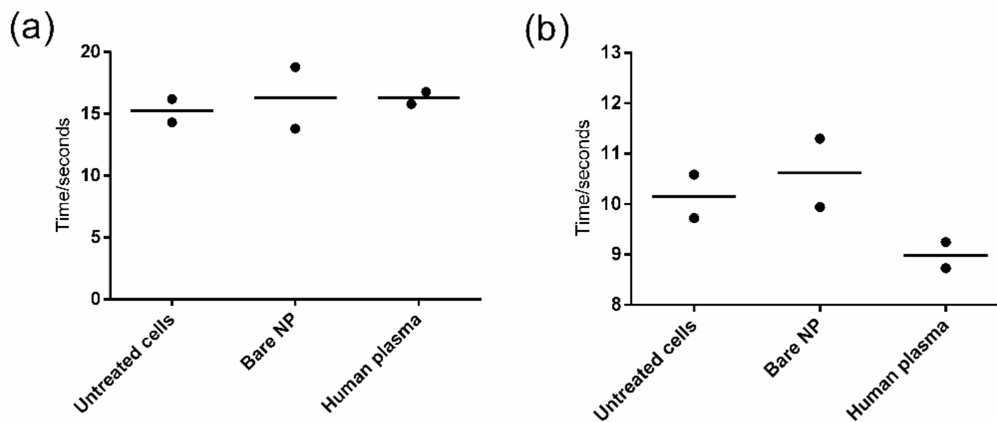

Figure S7. Cell viability after exposure to SiO_2-NH_2 200 nm. Liver (a) or brain (b) endothelial cells were exposed for 4 hours to 30 μg ml^{-1} bare or corona-coated nanoparticles, isolated from full human plasma as described in the Methods, in serum free medium. Cells were collected and resuspended in the same volume as described in the Methods, and then measured by flow cytometry using the same flow rate. The time required to acquire 15000 cells is shown to compare cell concentrations for the different samples, as an indication of cell viability after exposure to nanoparticles. The results of two replicate samples are shown, together with their average indicated with a line, and show that, for both untreated cells and cells exposed to the nanoparticles, the acquisition time that was needed to reach a cell count of 15000 cells was similar, indicating that no major change in cell number was observed after nanoparticle exposure.

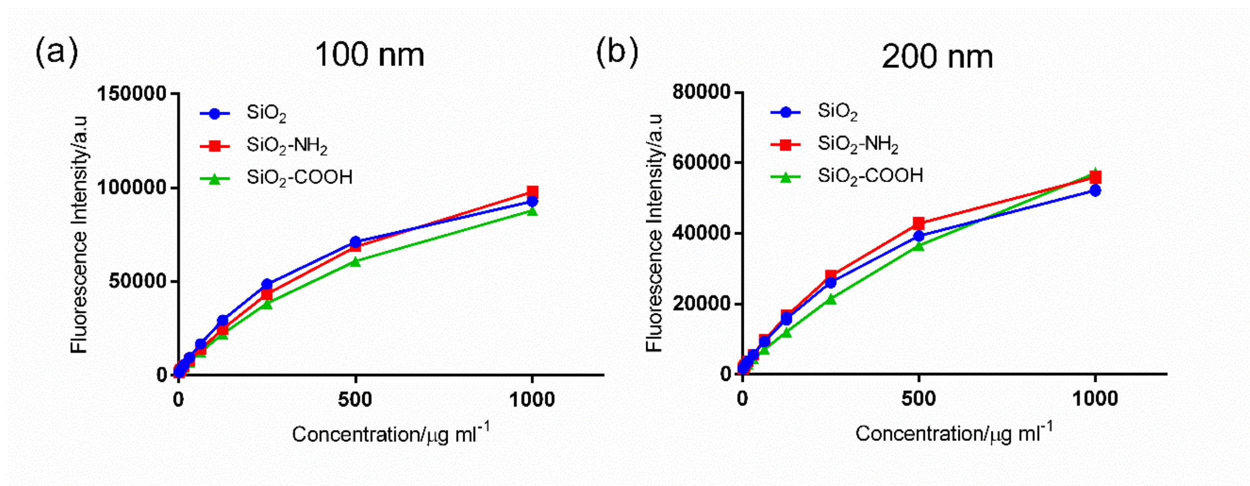

Figure S8. Fluorescence intensity of bare silica nanoparticles. SiO_2, SiO_2-NH_2, or SiO_2-COOH of 100 (a) and 200 nm (b) were dispersed in PBS at increasing concentrations, and their fluorescence was measured using a spectrofluorometer. The results show that the fluorescence intensity was comparable for silica nanoparticles of the same size, regardless of their functionalization.

Table S1. Correlation analysis between nanoparticle uptake in in brain, lung, liver and kidney endothelium after 24 hour exposure and the relative protein abundance of adsorbed corona proteins, performed as described in the Methods. *

Protein name	Entry short	r (brain)	r (lung)	r (liver)	r (kidney)
Alpha-1-antitrypsin	A1AT	0.8	0.8	0.7	0.8
Apolipoprotein A-I	APOA1	0.0	0.0	0.1	0.1
Apolipoprotein A-II	APOA2	0.1	0.1	0.1	0.1
Apolipoprotein B-100	APOB	0.1	0.1	0.1	0.1
Apolipoprotein C-I	APOC1	-0.1	-0.1	-0.1	-0.1
Apolipoprotein E	APOE	0.0	0.0	0.2	0.0
Haptoglobin	HPT	0.9	0.9	0.9	0.9
Histidine-rich glycoprotein	HRG	-0.3	-0.3	-0.5	-0.4
Immunoglobulin heavy constant gamma 1	IGHG1	0.9	0.9	0.8	0.8
Immunoglobulin heavy constant gamma 2	IGHG2	0.9	0.9	0.9	0.9
Immunoglobulin heavy constant gamma 3	IGHG3	0.9	0.9	0.9	0.9
Immunoglobulin heavy constant gamma 4	IGHG4	0.9	0.9	0.9	0.9
Immunoglobulin kappa constant	IGKC	0.7	0.7	0.5	0.6
Immunoglobulin lambda constant 2	IGLC2	-0.4	-0.4	-0.4	-0.3
Immunoglobulin lambda constant 3	IGLC3	-0.4	-0.4	-0.4	-0.3
Plasminogen	PLMN	-0.2	-0.2	-0.5	-0.3
Prothrombin	THRB	0.5	0.5	0.7	0.6
Serotransferrin	TRFE	0.9	0.9	0.9	0.9
Serum albumin	ALBU	0.9	0.9	0.8	0.9
Serum amyloid A-4 protein	SAA4	-0.3	-0.2	-0.1	-0.2

* Positive correlation coefficients (r) ≥ 0.6 are shaded in light gray, and negative correlation coefficients (r) ≤ 0.6 in dark gray.

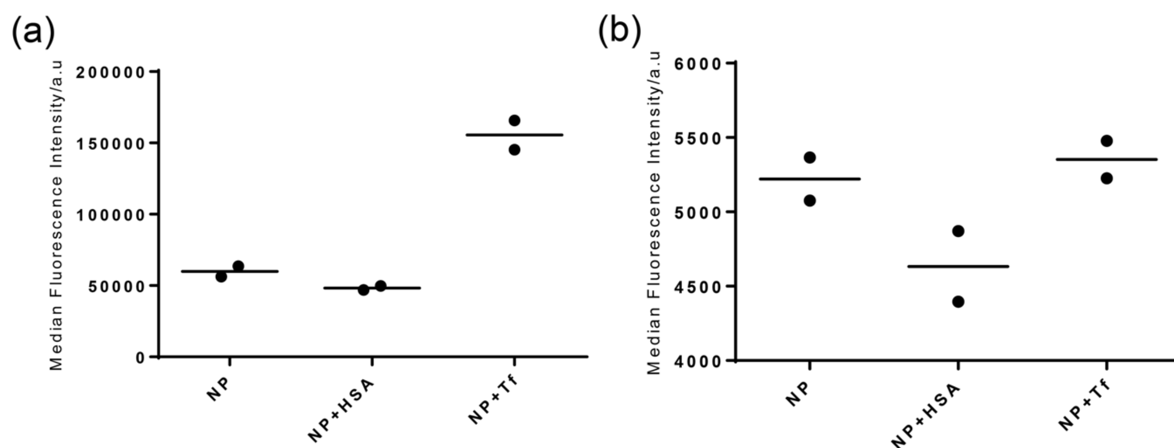

Figure S9. Competition study of nanoparticle-corona complexes with HSA and transferrin in liver (a) and brain endothelium (b). TRP3 and hCMEC/D3 cells were exposed for 4 hours to 30 µg ml^{-1} of 200 nm corona-coated SiO_2-NH_2 in serum free medium, isolated from full human plasma as described in the Methods, in the presence of 1 mg ml^{-1} HSA or transferrin. The results show the median cell fluorescence intensity of two replicate samples, together with their average indicated with a line .

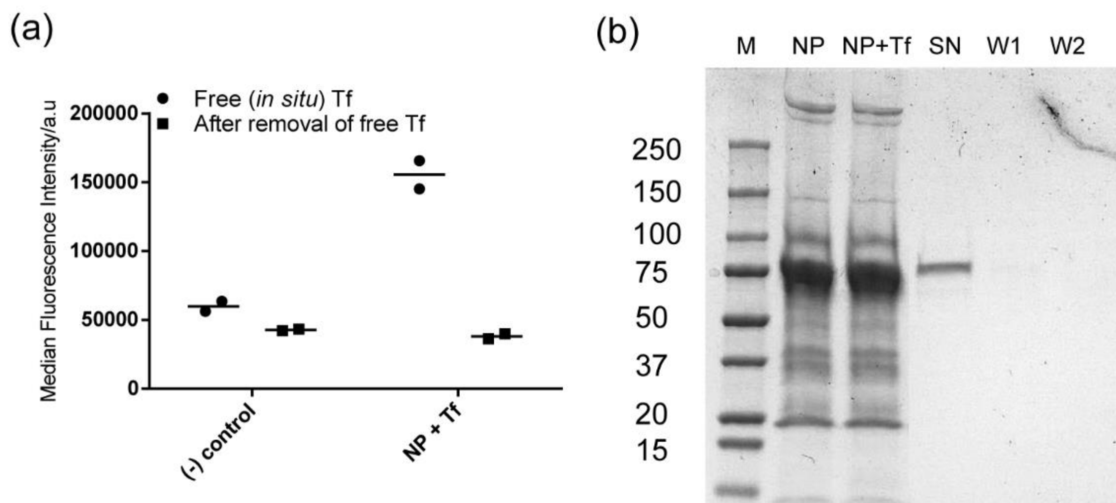

Figure S10. Effect of free transferrin on nanoparticle uptake in liver endothelium. (a) TRP3 liver cells were exposed for 4 hours to 30 µg ml^{-1} corona-coated nanoparticle complexes formed on 200 nm SiO_2-NH_2 in full human plasma in serum-free medium ("(-) control") or in the presence of 1 mg ml^{-1} human transferrin (NP+ Tf). Thus, the corona-coated nanoparticles were spin down a second time to remove the free transferrin and their uptake measured again ("After removal of free Tf"). The results show that the uptake of corona-coated nanoparticles increased when Tf was added, but the effect was lost after removal of free Tf in solution. (b) SDS-PAGE gel image of the proteins recovered on corona-coated nanoparticle complexes after incubation with free transferrin in serum-free medium. Nanoparticle-corona complexes were isolated as described in the Methods, then the same amounts of nanoparticles before and after addition of free transferrin (NP and NP+Tf, respectively) as well as the corresponding supernatant (SN) were loaded in a 10% polyacrylamide gel, and then washed by centrifugation two more times. The supernatants after the washing steps (W1 and W2) were also loaded to the gel. (M: molecular weight size marker; SN: supernatant after the first centrifugation; W1: supernatant after the first washing; W2; supernatant after the second washing.) The gel showed that the bands in the corona were comparable before and after exposure to free transferrin, suggesting that the higher uptake observed in the presence of free transferrin (a) was likely not caused by adsorption of additional transferrin on the nanoparticle surface.

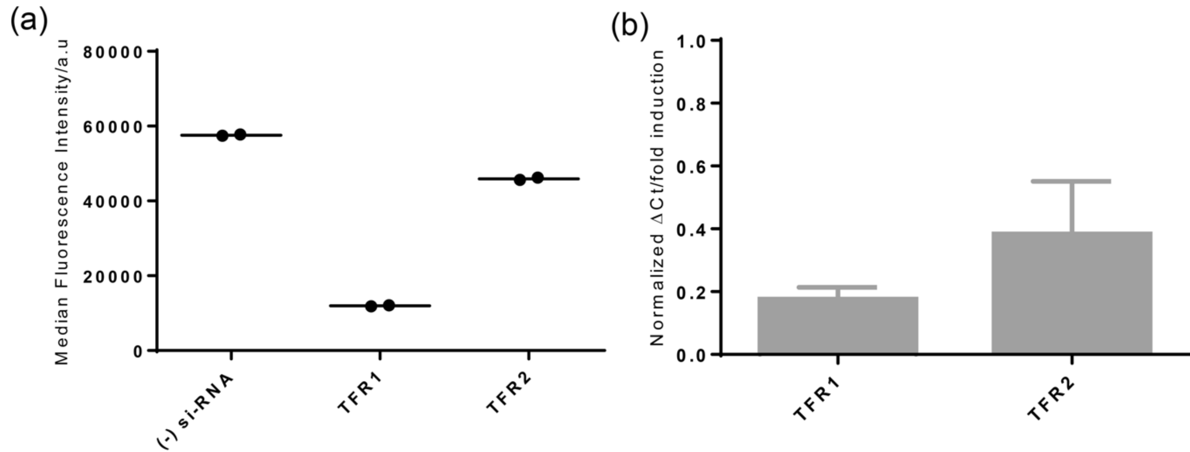

Figure S11 Silencing efficiency of TFR1 and TFR2 in liver endothelium. TRP3 cells were silenced for 72 hours for TFR1 and TFR2, and then exposed for 10 minutes to 10 μg ml^{-1} Alexa Fluor 546 fluorescently labeled transferrin in serum-free medium (a). The results of a representative experiment are given and show the median cell fluorescence intensity of two replicate samples, together with their average indicated with a line. The corresponding expression levels of TFR1 and TFR2 after silencing was determined by RT-qPCR (b). The results are the average and standard deviation over four replicate samples of the fold-change in gene expression levels in silenced cells compared to cells silenced with a scramble RNA as a negative control, calculated as detailed in the Methods. The results show that TFR1 was effectively silenced (b), and as a consequence of this, transferrin uptake was reduced of ~ 70% in TFR1 silenced cells (a).

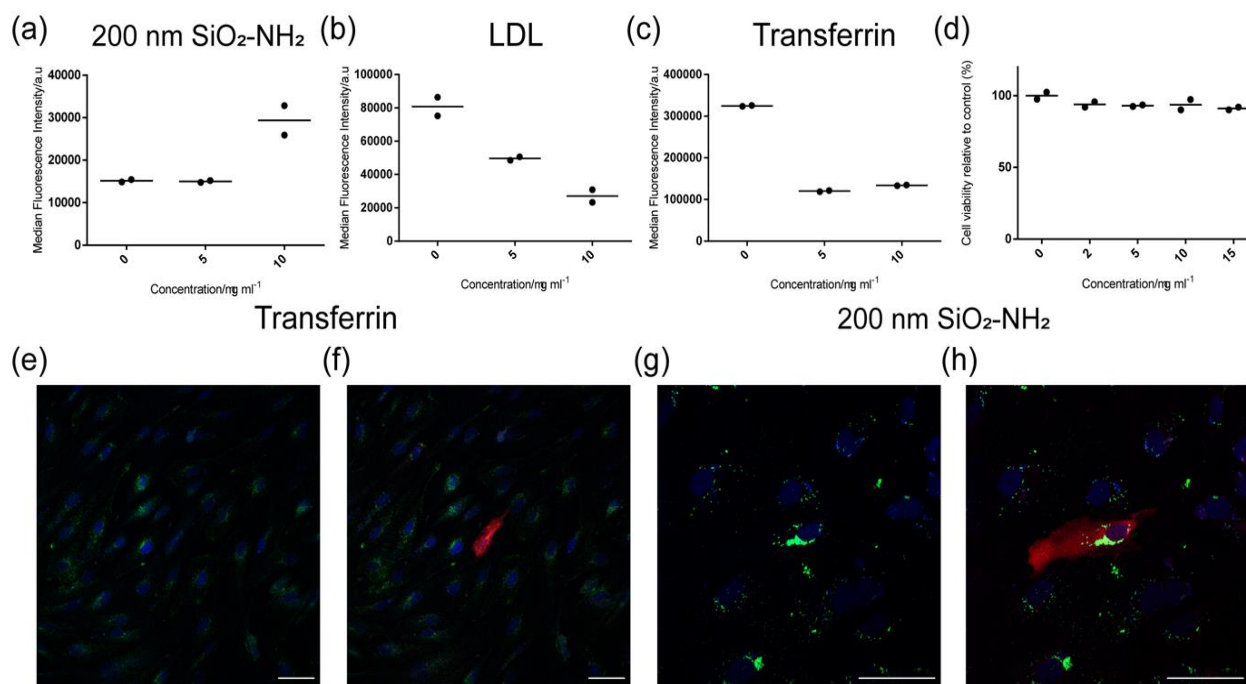

Figure S12. Effect of inhibition of clathrin-mediated endocytosis on nanoparticle uptake in liver endothelium. Briefly, TRP3 cells were exposed for 4 hours to 30 µg ml^{-1} nanoparticle-corona complexes of 200 nm SiO_2-NH_2 formed in full human plasma, as described in the Methods, in serum-free medium in standard conditions or in the presence of different concentrations of chlorpromazine (a). In order to confirm drug efficacy, TRP3 cells were exposed for 4 hours to 1 µg ml^{-1} fluorescently labeled low-density lipoprotein (Dil-LDL) (b), or for 10 minutes to 5 µg ml^{-1} Alexa Fluor 546 fluorescently labeled transferrin (c) in serum-free medium. Uptake was measured by flow cytometry, and the results show the median cell fluorescence intensity of two replicate samples, together with their average indicated with a line. (d) Cell viability assessed by MTT test on TRP3 cells exposed for 4 hours to different concentrations of chlorpromazine in complete cell culture medium to exclude drug toxicity at the concentrations tested. (e-h) Confocal fluorescence images of TRP3 transfected with a plasmid encoding a RFP tagged AP180 C-terminus, whose expression blocks clathrin-mediated endocytosis. After 24 hour transfection, cells were exposed for 15 minutes to 15 µg ml^{-1} Alexa Fluor 488 fluorescently labeled transferrin (e and f), or for 24 hours to 50 µg ml^{-1} of the nanoparticle-corona complexes in serum-free medium, followed by nanoparticle removal and 4 hours chase in nanoparticle-free medium (g and h). Blue: DAPI stained nuclei; red: RFP expression of transfected cells; green: transferrin (e and f) or nanoparticle-corona complexes (g and h). Scale bar: 50 µm. The same images without (e and g) and with (f and h) the red channel of the RFP tagged AP180 are shown. The results show that in cells expressing the AP180 terminus (red) no Tf uptake could be detected, confirming inhibition of clathrin mediated endocytosis (e and f), while the uptake of the corona-coated nanoparticles was higher (g and h).