

Supplementary Information

Influence of Protein Corona and Caveolae-Mediated Endocytosis on Nanoparticle Uptake and Transcytosis

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Preparation and characterization of PEGylated pNPs (pNP-PEG)

Commercially purchased carboxylate coated red fluorescent pNPs ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 580/605$ nm) of size 20, 40, 100 and 200 nm (F-8786, F-8793, F-8801, F-8763, FluoSpheres, Life Technologies, Massachusetts, USA) were PEGylated with 2 kDa methoxy-polyethylene glycol (PEG)-amine (JenKem Technology, Texas, USA) via conjugation with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Bangs Laboratories, Indiana, USA) and sulfo-N-hydroxysuccinimide (sulfo-NHS) (Sigma-Aldrich, Missouri, USA) chemistry¹. Briefly, the suspension buffer of the stock pNPs was first exchanged by dialyzing against 25 mM-PIPES buffer, pH 7.0, (Gold Biotechnologies, Missouri, USA) for 24 h at 4 °C in 10 kDa molecular weight cut-off dialysis cassettes (Slide-A-Lyzer dialysis cassettes, ThermoFisher Scientific, Massachusetts, USA).

Based on the information on the number of moles of negatively charged groups on the surface of each pNP provided by the commercial supplier, molar excesses of EDC, sulfo-NHS and 2 kDa PEG were prepared. The final molar ratios of negatively charged groups on stock pNPs: EDC: sulfo-NHS: 2 kDa PEG prepared were 1:20:25:25.

EDC and sulfo-NHS were first added to the pNPs in 25 mM PIPES and allowed to react for 10 mins at room temperature with gentle mixing before 2 kDa PEG was added to the reaction mixture. The reaction mixture was then sonicated for 2 mins and allowed to react for 1 h at room temperature with mixing. Finally, the reaction mixture dialyzed against 50-mM TRIS at pH 7.4 for 24 h at 4 °C in 10 kDa molecular weight cut-off dialysis cassettes (Slide-A-Lyzer dialysis cassettes, ThermoFisher Scientific, Massachusetts, USA) to remove excess reactants.

In order to verify the successful PEGylation of the commercially purchased pNPs, we characterized the intensity weighted mean hydrodynamic diameters (D_H) of the pNPs in ultra-pure water, 1% HCl (Sigma-Aldrich, Missouri, USA), and FBS (Gibco, Massachusetts, USA)

before and after PEGylation (Figure S1A and B). Due to their intrinsically negative zeta potential (Figure S1D), bare carboxylated pNPs introduced into 1% HCl aggregated, resulting in an increase in D_H (Figure S1A). On the other hand, no statistically significant increases in the D_H were observed for the pNPs-PEG after they were introduced into 1% HCl ($P < 0.05$) (Figure S1B). Similarly, we expected the formation of a protein corona on the surface of the bare carboxylated pNPs following incubation with FBS²⁻⁴, resulting in a consequential increase in measured D_H (Figure S1A). With the pNP-PEG however, no statistically significant increases in D_H was observed (Figure S1B). Taken together, our results reflect the colloidal stability expected with PEGylated NPs⁵⁻⁷, thus verifying the successful conjugation of 2 kDa PEG onto the surface of the different sized pNPs, which resulted in an increase in the mean D_H of all pNPs by ~ 14.5 nm (Figure S1C).

Additionally, we characterized the zeta potential of the different sized pNPs before and after the PEGylation reaction (Figure S1C). Through replacing negatively charged carboxylate groups on the surface of the pNPs with neutrally charged methoxy-PEG, we expected a reduction in the zeta potential of the pNPs following successful PEGylation. Interestingly however, significant reductions in zeta potential was observed only for 20 and 40 nm pNPs, with 100 and 200 nm pNPs showing no significant changes in the zeta potential following PEGylation (Figure S1D). We checked with the supplier of the commercially purchased bare carboxylated pNPs (Life Technologies, Massachusetts, USA) and found that this was a result of the presence of sulfate groups passivated onto the surface of the bare carboxylated pNPs in addition to the carboxylate groups. These sulfate groups were more abundant on larger pNPs due to their larger surface area and were not modified by the PEGylation reaction.

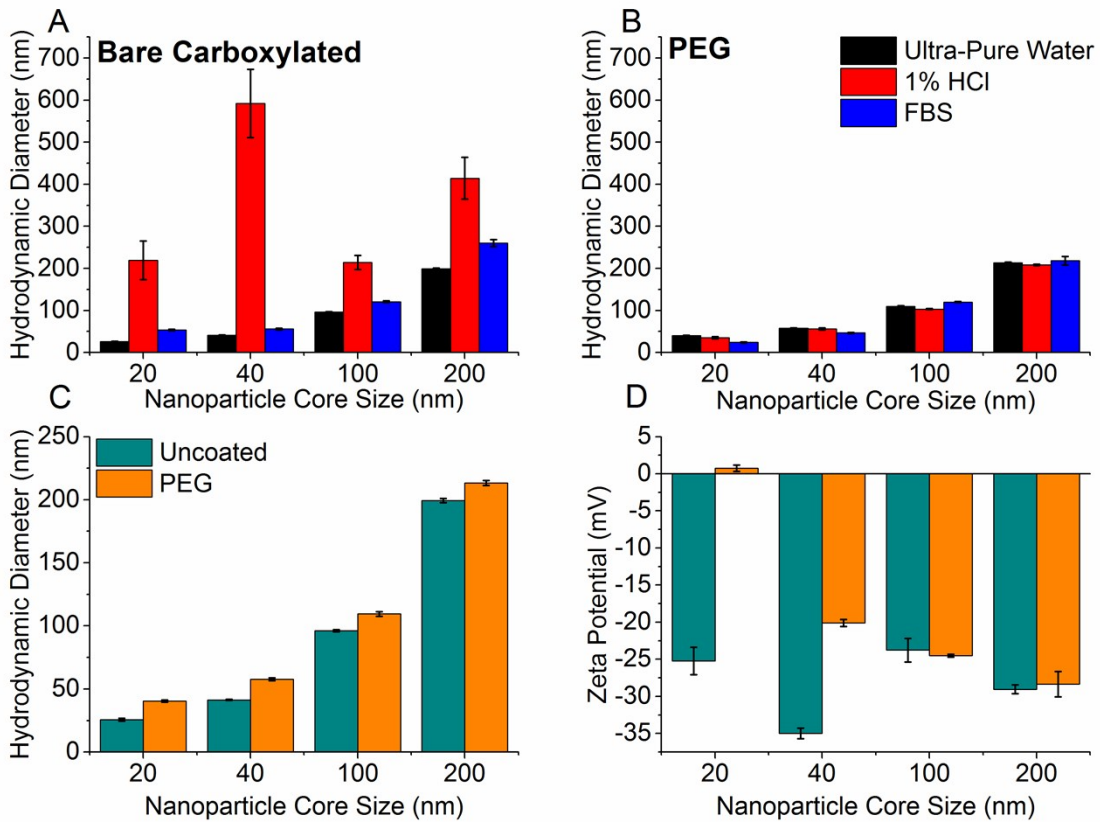


Figure S1. Hydrodynamic diameter (D_H) measurements of (A) bare carboxylated pNPs; and (B) pNPs PEGylated with 2 kDa PEG; in water, 1% HCl, and FBS. (C) D_H of the bare carboxylated pNPs and their PEGylated counterparts after successful chemical conjugation with 2 kDa PEG. (D) Zeta potential of the bare carboxylated pNPs and their PEGylated counterparts. In all measurements, $3 \leq n \leq 4$; where each sample, n , was measured 3 times.

Characterizing the permeability coefficients of dextran across the HUVEC monolayers after restricting paracellular permeability

Confluent HUVEC monolayers in the microfluidic devices were probed for their diffusional permeabilities (P_d) to Oregon Green 488 tagged 10 kDa ($\lambda_{ex}/\lambda_{em} = 501 / 526$ nm) and Texas Red tagged 70 kDa Dextran ($\lambda_{ex}/\lambda_{em} = 561$ nm/ 594 nm) (Life Technologies, Massachusetts, USA) in order to access the reproducibility of the P_d measurements with the

microfluidic based permeability assay that we had previously developed ². P+5 HUVECs were seeded into the cell channels of the microfluidic device (Figure 1A) and cultured to confluency with endothelial growth medium (EGM-2, Lonza, Basel, Switzerland) containing 0, 25, 50, or 100 $\mu\text{g}/\text{mL}$ of cell permeable cyclic adenosine monophosphate (pCPT-cAMP, Sigma-Aldrich, Missouri, USA). Confluent cells were allowed to stabilize for an additional day before the permeability measurements were conducted. Cell media was changed daily in all devices.

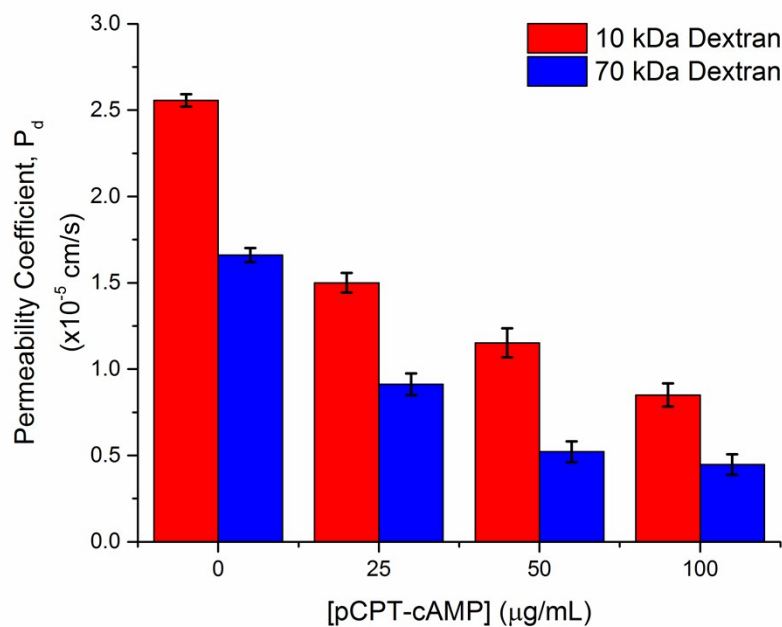


Figure S2. Diffusional permeability coefficients (P_d) of the HUVECs to the 10 and 70 kDa dextran following treatment with varying concentrations of pCPT-cAMP ($3 \leq N \leq 17$). In all cases, the HUVECs were observed to have a significantly higher P_d to 10 kDa dextran as opposed to the larger 70 kDa dextran. No statistically significant change in the P_d of 70 kDa dextran was observed between 50 and 100 $\mu\text{g}/\text{mL}$ pCPT-cAMP treated HUVECs. For 10 kDa dextran, no statistically significant differences in P_d was observed between 25 and 50 $\mu\text{g}/\text{mL}$ pCPT-cAMP treated HUVECs ($P < 0.05$).

The devices were then transferred onto the stage of an Olympus IX81 inverted microscope (Olympus, Tokyo, Japan) with an EMCCD camera (Andor iXon⁺ EMCCD camera, Andor, Belfast, Northern Ireland). 20 μ L of 25 μ g/mL of 10 or 70 kDa dextran re-constituted in EGM-2 was then added to each device and a 30 min time lapse with 30 s intervals was started to capture the diffusion of the fluorescent dextrans across the HUVEC monolayer. The P_d of the HUVECs to the 10 and 70 kDa dextrans was then calculated from the captured fluorescent images using a dedicated MATLAB programme and analysis method that we had previously developed².

Size selectivity was observed for all pCPT-cAMP concentrations tested, with 10 kDa dextrans showing a higher mean P_d as compared to the larger 70 kDa dextrans (Figure S2). The P_d values of the differentially treated HUVECs to 10 and 70 kDa dextrans were also largely similar to what had been reported previously (Figure S2)². These results support the reproducibility of the microfluidic based permeability assay that we had previously developed, where we had successfully demonstrated the ability to direct the movement of pNP-PC across the HUVECs towards the transcellular route with pCPT-cAMP treatment². As such, in order to limit access to the paracellular route and ensure that the transcellular route becomes the dominant mode of transendothelial transport for all pNPs used in this study, all P+5 HUVECs used in this study will be cultured with EGM-2 containing 100 μ g/mL pCPT-cAMP.

References

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