Protein Corona Formation Moderates the Release Kinetics of Ion Channel Antagonists from Transferrin-Functionalized Polymeric Nanoparticles.

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SUPPORTING FIGURES

S1. QUALITATIVE ASSESSMENT BY SDS-PAGE OF TRANSFERRIN (TF) POST-STRUCTURAL MODIFICATIONS PRIOR TO CONJUGATION TO NP
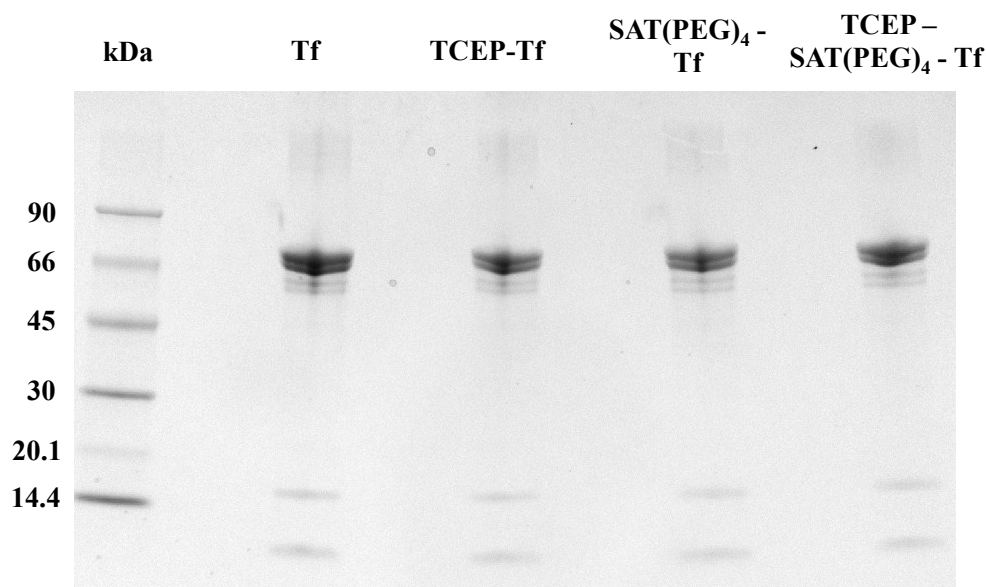

Figure S1. Qualitative assessment of Transferrin (Tf) post-modification by N-Succinimidyl S-acetyl (thiotetraethylene glycol) (SAT(PEG)$_4$) and tris (2-carboxyethyl) phosphine (TCEP) by sodium dodecyl sulphate acrylamide gel electrophoresis (SDS-PAGE).

Notes:

The Tf conjugation technique was adapted from the protocol established by Hristov, *et al.*[1] In brief, human holo-Tf (76 – 81 kDa) was covalently conjugated to amine-functionalized NP using hetero-bifunctional cross-linkers, Succinimidyl-([N-maleimidoproprionamido]-octyl ethylene glycol) ester (SM(PEG)$_8$) (Thermo Scientific™). While providing the availability of maleimide functional groups as reactive sites for Tf conjugation, modification with SM(PEG)$_8$ additionally enabled increased nanoparticle stability in highly ionic solutions such as blood.[2, 3] Prior to bioconjugation to SM(PEG)$_8$-modified NP *via* thiol coupling, the Tf molecules were subjected to modifications with N-Succinimidyl S-acetyl (thiotetraethylene glycol) (SAT(PEG)$_4$), in order to protect primary amine groups of the protein. Subsequently, modification with tris (2-carboxyethyl) phosphine (TCEP) promoted the reduction of the disulphide bridge on the Tf molecule in preparation for covalent conjugation to

SM(PEG)$_8$-modified NP. Rigorous step-wise evaluations of this 3-stage bioconjugation method had previously confirmed the capacity to successfully control and couple Tf to nanoparticles. Furthermore, *in vitro* receptor-binding and cellular uptake studies had corroborated the preservation of Tf antigenicity following conjugation to nanoparticles.[1, 4] Correspondingly, in this study, SDS-PAGE analysis of purified Tf post-modification, in comparison to unmodified native Tf, confirmed that the primary structure of the protein was preserved prior to NP functionalization as observed in Figure S1. A colorimetric protein quantification assay of increasing concentrations of Tf-NP suspensions showed proportionate increases in Tf concentrations, confirming the availability of approximately 27 μg of conjugated Tf per mg of Tf-NP (Figure S2).

S2. QUANTIFICATION OF FUNCTIONALIZED TRANSFERRIN ON TF-NP SUSPENSIONS BY MICRO BCA™ PROTEIN QUANTIFICATION ASSAY
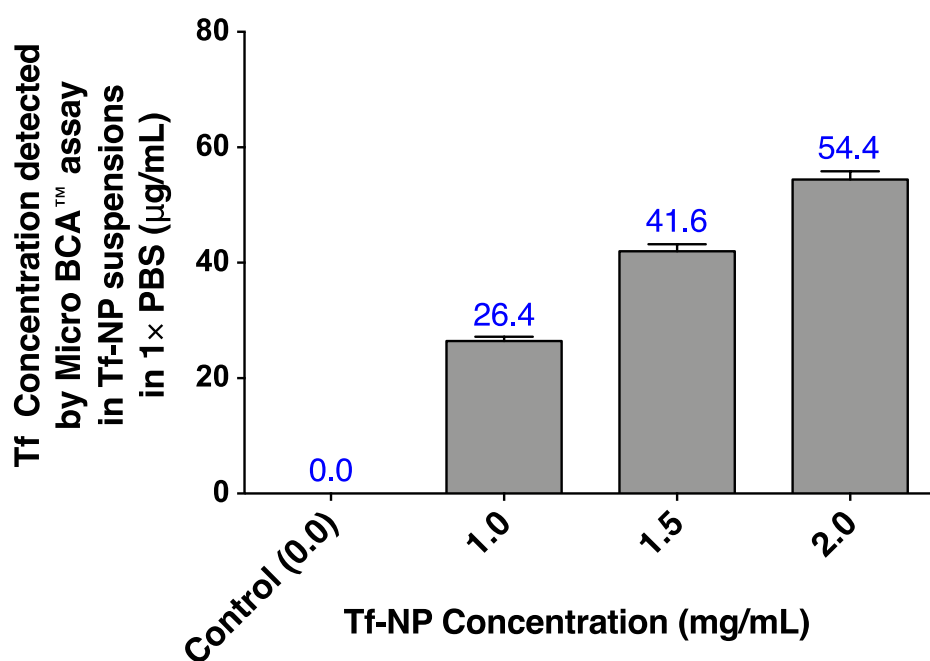

Figure S2. Micro BCA™ protein quantification assay confirms transferrin (Tf) functionalization on p(HEMA-ran-GMA)-based nanoparticles by assessing the amount of protein detected in varying concentrations of Tf-NP suspensions. (n = 3; ± SEM) Values depicted in blue above the bar charts represent mean Tf concentrations detected for respective Tf-NP suspension concentrations in 1× phosphate buffered saline (PBS).

S3. CYTOTOXICITY ASSESSMENT OF NANOPARTICLE VARIANTS IN PRIMARY MIXED CORTICAL CULTURES
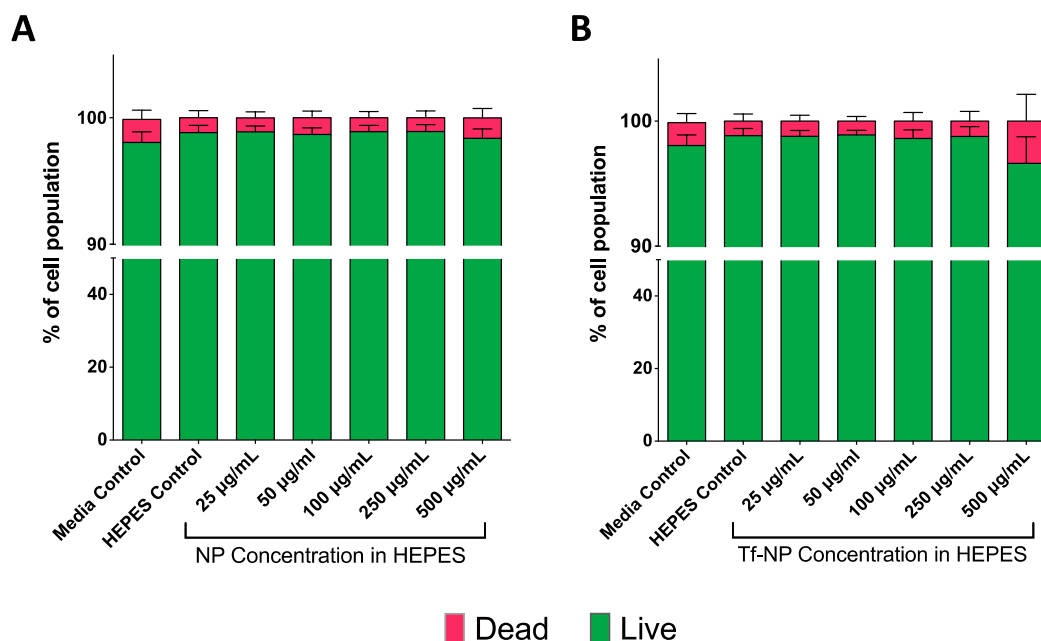

Figure S3. Cytotoxicity assessment of nanoparticle variants ± transferrin (Tf) functionalization (NP and Tf-NP) of varying concentrations by Live/Dead™ assay in primary mixed cortical cultures. Live and dead cells were quantified by fluorescence measurements under standardized fields of view (FOV; 293.703 × 293.703 μm) at 40× magnification using 3 biological replicates after incubation with specific nanoparticle variant concentrations made up in 50 mM HEPES buffer (pH 7.4). Plate reader was set to 494/517 nm (excitation/emission) for calcein AM (live cells) and 528/617 nm for EthD-1 (dead cells). Negative controls with 50 mM HEPES buffer and media controls were assessed for comparison. All samples were assessed in triplicates (n=3) and four standard FOV were analysed per technical replicate. The mean numbers of live and dead cells were quantified and expressed as percentages of the cell population within the FOV. Statistical analysis was conducted using two-way ANOVA with post-hoc analysis by Sidak's multiple comparisons test. (*p < 0.05)
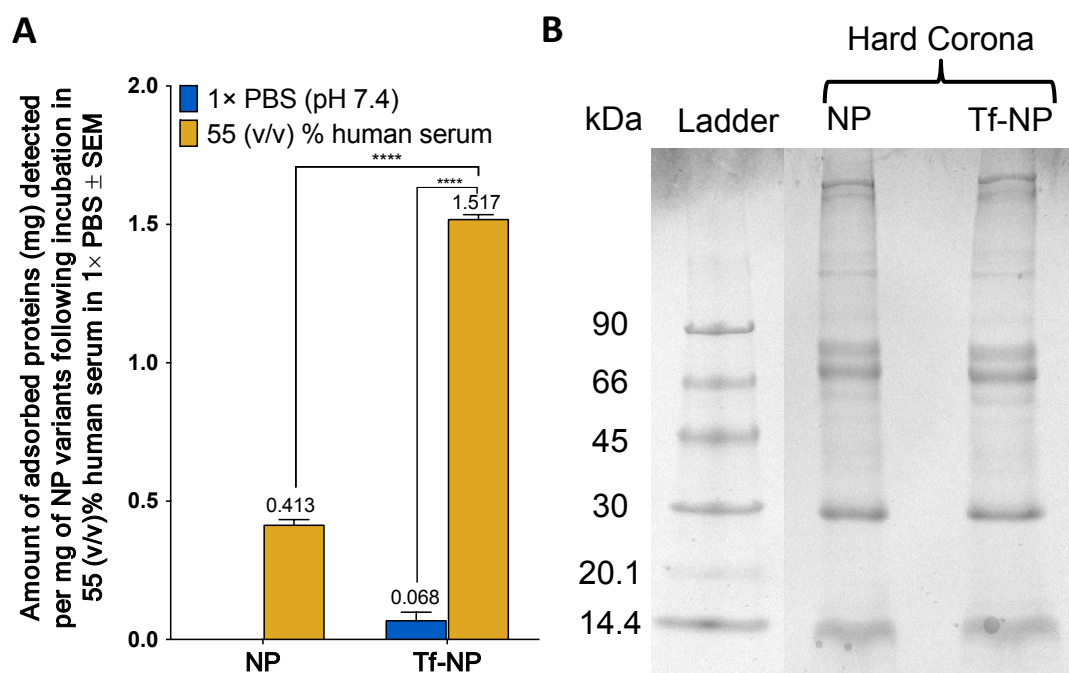
S4. PROTEIN CORONA ASSESSMENT OF TF-NP AND NP BY MICRO BCA™ PROTEIN QUANTIFICATION AND SDS-PAGE

A

B

Figure S4. Protein corona assessment on Cy5-labelled p(HEMA-ran-GMA) nanoparticles (NP) and transferrin-functionalized nanoparticles (Tf-NP). (A) Serum protein quantification of adsorbed hard corona (± standard error of measurement (SEM)) on nanoparticle variants by Micro BCA™ assay. Two-way ANOVA was conducted with post-hoc analysis by Tukey's multiple comparisons test (n = 3; *p < 0.05). (B) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separation of hard corona serum proteins eluted from NP and Tf-NP (10 µg protein loaded per sample well).
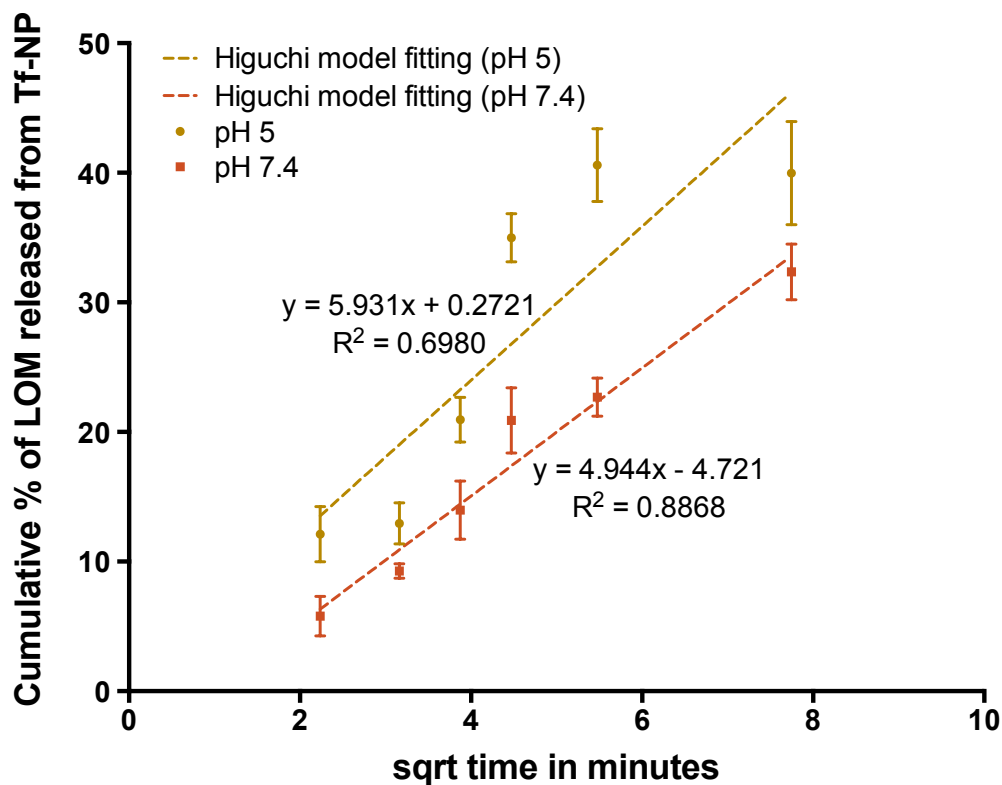
S5. Mathematical simulation of the release of LOM from Tf-NP with respect to specific pH environments, using the Higuchi Model

Figure S5. Comparison of the release kinetics of LOM from Tf-functionalized p(HEMA-ran-GMA) nanoparticles (Tf-NP) with respect to specific pH environments (pH 5 and 7.4) using mathematical simulation by the Higuchi Model.

SUPPORTING METHODS

- **SYNTHESIS OF P(HEMA-RAN-GMA) COPOLYMER BY ATOM-TRANSFER RADICAL POLYMERIZATION (ATRP)**

Glycidyl methacrylate (GMA) (12 mmol, 1.64 mL) and 2-hydroxyethyl methacrylate (HEMA) (28 mmol, 3.40 mL) were dissolved in methanol at monomer to solvent ratio of 1:3 and an alkyl halide initiator (ME-Br, 450 µL, 280.16 g/mol, 2 mmol), copper (I) bromide (CuBr, 143 mg, 1 mmol) and 2,2-bipyridine (bpy, 143 mg, 2 mmol) were added. The ME-Br initiator was synthesized according to protocols established by Bories-Azeau, *et al.*[7] The resulting solution was degassed 3× using a standard freeze/pump/thaw method. Polymerization was heated at 80°C under standard Schlenk conditions for 1 hour and the copolymer, p(HEMA-ran-GMA), product was collected by precipitation in excess diethyl ether and dried overnight under vacuum. The purified copolymer product was assessed by [1]H Nuclear Magnetic Resonance (NMR). The spectrum was measured using Bruker 500 MHz spectrometer, using deuterated methanol (CD$_3$OD) as solvent. All chemical shifts were referenced to the solvent peak for CD$_3$OD (δ3.31 ppm). Gel Permeation Chromatography (GPC) was used to determine the weighted average molecular weight ($\overline{M}_w$) and polydispersity index (PDI) of the polymer (Waters Styragel HR 3 7.8 x 300 mm column, Waters Styragel HR 4 7.8 × 300 mm column, 5 µm). Agilent Technologies 1100 Series GPC and Agilent GPC software were used for measurements and data analysis respectively. Measurements were taken using dimethylformamide (DMF) as the eluent at the flow rate of 0.7 mL/min at 40°C, and calibrated against poly (methyl methacrylate) (PMMA) standard. The synthesis and characterization of p(HEMA-ran-GMA) copolymer has been previously described by Kretzmann, *et al.*[8]

- **PROTEIN QUANTIFICATION**

Micro BCA™ Protein assay kit (Thermo Scientific™) was utilized in the study for (a) confirmation of Tf-conjugation on Cy5-*p*(HEMA-*ran*-GMA) nanoparticles and (b) quantification of adsorbed serum proteins on nanoparticle variants. The assay was performed according to the manufacturer's protocol. In brief, bovine serum

albumin (BSA) standards at known concentrations ranging from 50 μg/mL – 5 μg/mL, and test samples were loaded into individual wells of a 96-well plate. All standard and sample volumes were kept consistent at 150 μL and tested in triplicate. 150 μL of Micro BCA™ working reagent, as described by the manufacturer, was delivered into each well and the plate was incubated for 2 h at 37°C. After cooling to room temperature, the absorbance of all samples was measured at 562 nm on a plate reader (Perkin Elmer™ EnSpire® Multimode Plate Reader) to determine protein concentration.

a. Confirmation of Tf conjugation on Cy5-p(HEMA-ran-GMA) NPs

Tf-NP were dispersed in 1× PBS (pH 7.4) at concentrations ranging from 10 – 200 μg/mL. Control samples with NP were prepared in the same sample concentration range. Positive controls SAT(PEG)$_4$-modified Tf in known concentrations: 50 μg/mL – 5 μg/mL) were also tested.

b. Quantification of serum proteins adsorbed on nanoparticle variants (protein corona)

The nanoparticle variants (± Tf) were incubated in 55 % (v/v) human serum and protease inhibitor (1× cOmplete™, Mini EDTA-free Protease inhibitor Cocktail) in 1× PBS (pH 7.4) at 37°C for 30 minutes. The serum-incubated nanoparticle suspensions (1 mL) were loaded onto a sucrose cushion (0.7 M sucrose) and centrifuged at 20000× g for 30 minutes at 4°C. The nanoparticle pellet retrieved was then washed 3× with 1× PBS (pH 7.4) to remove serum proteins that are not tightly adsorbed on the surfaces of the nanoparticle variants and were immediately lyophilized. The dried samples were reconstituted in ~100 – 150 μL rehydration buffer made up of 8 M urea and 2 % (w/v) 3-((3-Cholamidopropyl)dimethylammonio)-1-propanesulfonate hydrate (CHAPS) and sonicated on a sonic water bath until the nanoparticles were well dispersed. The suspension was centrifuged at 20000× g for 30 minutes and the supernatant was retained for protein quantification by Micro BCA™ assay. 5 μL of the sample was diluted in sufficient 1× PBS (pH 7.4) for the protein quantification analysis.

- **GEL ELECTROPHORESIS (SDS-PAGE) ASSESSMENTS**

  5× SDS loading buffer (250 mM Tris-HCl pH 6.8; 10 % (w/v) SDS; 30 % (v/v) glycerol; 5 % (v/v) 2-mercaptoethanol; 0.02 % (w/v) bromophenol blue) was added to the appropriate volume of protein samples and heated for 5 minutes at 95°C. The samples were cooled and 10 μg of protein per sample were loaded into the wells of a precast 4 – 15 % polyacrylamide gel (Biorad). The loaded denatured protein samples were allowed to stack by running the gel at 50 V for 15 minutes. After which the samples were subjected to gel electrophoresis separation at a constant current of 15 mA. After SDS-PAGE, the gel was washed with MilliQ water and stained with Coomassie Brilliant Blue R-250 stain for 30 minutes and then destained overnight (acetic acid: methanol: water = 1:4:5) on a rocking platform at room temperature. The gels were imaged using the Biorad ChemiDoc MP system.

- **DRUG LOADING AND RELEASE ASSESSMENTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)**

  Known masses of lyophilized Tf-NP were backfilled with a combination of LOM, YM872 and oxATP. Solutions of the drugs were prepared accordingly:

  5 mg of LOM was dissolved in 50 μL of methanol, followed by dilution sterilized MilliQ water to make up a final drug concentration of 20 mg/mL. YM872 and oxATP were also each dissolved in sterilized MilliQ water to make up a final concentration of 20 mg/mL. Drug solutions were combined in water to provide a final concentration of 5 mg/mL of each drug, and 300 L of the solution was exposed to 1 mg of lyophilized Tf-NP in 1.5 mL Eppendorf tubes. Specifically, 75 μL of each drug's stock solution was combined and diluted with 125 μL of MilliQ water, which was then added to the lyophilized Tf-NP. The nanoparticles were briefly sonicated in the combined drug solution, and allowed to stand for 30 minutes at 4°C. The suspension was then centrifuged at 10000× g for 10 minutes at 4°C, and the supernatant was carefully removed from the pellet consisting of drug-loaded Tf-NP. It should be noted that the drug backfilling was performed within the hour prior to HPLC analyses.

HPLC coupled with UV/Vis detector was used to determine drug loading and the respective release profiles from Tf-NP. To assess drug release in the presence of serum proteins, drug-loaded Tf-NP were incubated in 55 % (v/v) human serum in 1× PBS at 37°C. At designated release assessment time points, 150 μL aliquots of the samples were immediately centrifuged through filter units (Amicon Ultra-4 Centrifugal Filter units NMWL 3 kDa) according to the manufacturer's specifications. The filtrate collected was then used for the detection of the drug concentration by HPLC at specific time points over 1 hour.

Detection of LOM and YM872:

Isocratic elution using a 69:31 mixture of acetonitrile (ACN) and 0.5 % (w/v) trifluoroacetic acid at 0.5 mL/min, monitoring the eluent at 210 nm. The retention time of LOM was at 4.7 minutes and YM872 at 2.9 minutes. Samples were run for 13 minutes and the integrated area of the peaks at the designated retentions times were used for the calculation of drug concentration. The limit of detection for LOM and YM872 in water at 210 nm was 0.1 μg/L.

Detection of oxATP:

Gradient elution using mobile phase consisting of a combination of 2 eluents, (A) ACN and (B) 0.1 M phosphate buffer pH 7 at the following varying flow rates: At t = 0 minute, 100 % B at 0.85 mL/min; 4 minutes, 95 % B at 0.8 mL/min; 8 min, 75 % B at 1 mL/min; 12 minutes, 70 % B at 1 mL/min. The detection of oxATP was monitored at 254 nm and the retention time was 5.4 minutes. Total run time per sample was 12 minutes.

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