Supplementary

Title: Blood-brain-barrier disruption dictates nanoparticle accumulation following experimental brain injury

Nanoparticle (NP) PEG conjugation

As presented in our previous study(23), carboxylated NPs were PEGylated using EDC/NHS chemistry. Briefly, mPEGamine 750 Da was mixed with 20 nm NPs (NH_2:COOH at 2:1 mole excess) whereas mPEGamine 2 kDa was mixed with 40 nm, 100 nm and 500 nm NPs; (NH_2:COOH at 5:1 mole excess). EDC/NHS (in MES buffer) was added to NP / PEG mixture (8 mM/4 mM for 20 nm and 200 mM/100 mM for other NPs) and HEPES buffer was added to obtain a final pH of 7.8 before incubating for 3 h at room temperature. Glycine (100 mM) was added to quench the reaction. Unbound PEG was removed via dialysis (20 kDa MW). PEGylated NPs were suspended in a 20 mM HEPES (pH 7.4). The concentration of each NP solution was determined with fluorescent standard curves generated from known concentrations of as-received Fluorospheres (FLUOstar Omega fluorescence plate reader; BMG Labtech, Ortenberg, Germany). Yields of NPs ranged between 40-60 %. A concentration of 13.3 mg/ml for each NP was used for all *in vivo* studies.

Nanoparticle characterization

The mean hydrodynamic diameter (number distribution) and zeta potential of NPs in 20 mM HEPES (pH 7.4) were measured pre- and post-PEGylation with a dynamic light scattering (DLS) device (Zetasizer Nano Malvern; Malvern, UK). For each NP, three measurements were made and the mean ± standard error of mean (s.e.m.)

was reported (supplementary).

Animals and Study Design

Mice were housed in a 14h light/10h dark cycle at a constant temperature (23°C ± 2° C) with food and water available *ad libitum* according to the Association for Assessment and Accreditation of Laboratory Animal Care International. All mice used in this study were group housed. Mice were acclimated to their environment following shipment for at least three days prior to any experiments. All animal studies were conducted in accordance with the guidelines established by the internal IACUC (Institutional Animal Care and Use Committee) and the NIH guidelines for the care and use of laboratory animals. Studies are reported following the ARRIVE (Animal Research: Reporting In Vivo experiments) guidelines(27). Randomization of animals was achieved by assigning animals to time points before the initiation of the study to ensure equal distribution across groups. A power analysis was performed to calculate group sizes based on preliminary data and previously published work from our group investigating nanoparticle accumulation following controlled cortical impact in the mouse(23). Data collection stopped at pre-determined final endpoints based on time post-injury for each animal.

Midline Fluid Percussion Injury (FPI)- Craniotomy:

Mice were subjected to midline fluid percussion injury (FPI) consistent with methods previously described (29-34). Group sizes are indicated in the results section and figure legends for individual studies. Mice were anesthetized using 5% isoflurane in 100% oxygen for five minutes and the head of the mouse was placed in a stereotaxic

frame with continuously delivered isoflurane at 2.5% via nosecone. While anesthetized, body temperature was maintained using a Deltaphase® isothermal heating pad (Braintree Scientific Inc., Braintree, MA). A midline incision was made exposing bregma and lambda, and fascia was removed from the surface of the skull. A trephine (3 mm outer diameter) was used for the craniotomy, centered on the sagittal suture between bregma and lambda without disruption of the dura. An injury cap prepared from the female portion of a Luer-Loc needle hub was fixed over the craniotomy using cyanoacrylate gel and methyl-methacrylate (Hygenic Corp., Akron, OH). The incision was sutured at the anterior and posterior edges and topical Lidocaine ointment was applied. The injury hub was closed using a Luer-Loc cap and mice were placed in a heated recovery cage and monitored until ambulatory before being returned to their home cage.

After injury induction, the injury hub was removed and the brain was inspected for uniform herniation and integrity of the dura. The dura was intact in all mice; none were excluded as technical failures. The incision was cleaned using saline and closed using sutures. Diffuse brain-injured mice had righting reflex recovery times greater than five minutes and a positive fencing response (29). Sham injured mice recovered a righting reflex within 20 seconds. After spontaneously righting, mice were placed in a heated recovery cage and monitored until ambulatory (approximately 5 to 15 additional minutes) before being returned to their home cage. Adequate measures were taken to minimize pain or discomfort(36).

Analysis of HRP and NP accumulation after mCHI/RmCHI

Tissue sections were incubated in PBS buffer for 20 mins at room temperature prior to use. The tissue sampling regions were directly under the impact, specifically, -1 mm to -3 mm Bregma (three sections per animal, three animals per cohort). For HRP analysis, freshly prepared DAB substrate solution (200 µl) was added to the tissue and incubated for ten mins at room temperature. Slides were then washed in PBS buffer three times (two mins each) and coversliped after adding a drop of aqueous mounting media. Sections were imaged using Slide Scanner (PathScan Enabler IV, Meyer Instruments, TX, USA).

For NP analysis, slides containing the frozen sections were incubated at room temperature for 20 mins in 1X PBS to rehydrate the tissue and remove OCT compound. Slides were coverslipped after adding one drop of fluorescent mounting media (Vectashield). The tissue sections sampling under the impact region, specifically, -1 mm to -3 mm Bregma (three sections per animal, three animals per cohort) were used. The whole brain sections were imaged with conventional epifluorescent microscopy at 10X objective.

Analysis/quantification of HRP extravasation after midline FPI

The tissue sections were incubated in PBS buffer for 20 mins at room temperature. Freshly prepared DAB substrate solution (200 µl) was added and incubated for ten mins at room temperature. Slides were then washed in PBS buffer three times (two mins each) and coverslipped after adding a drop of aqueous mounting media. Sections (three sections per animal, three animals per group) were imaged using Slide Scanner. Sections were located ~-1.65 mm Bregma. Quantitative analysis of

HRP staining was accomplished by defining two regions of interest (ROI) based on previously established HRP staining patterns for FPI: (1) cortex (includes primary somatosensory and primary, and secondary motor cortex) and (2) corpus callosum (17)((38), (S1). Specifically, ImageJ software (National institute of health, Bethesda, MD, USA) was used to draw ROIs manually and grid lines were used as a reference tool. The full brain scan images were rotated such that the midline was oriented at the center of the grid. Next, the horizontal line tangential to the ventral aspect of the corpus callosum was used to mark the maximum extent of ROI Next, the line just below the corpus callosum was used to mark the maximum extent of the ROI (Figure S1). The ROI for the corpus callosum was drawn spanning through the entire corpus callosum above the maximum extent line. The images were then thresholded to remove background using tissue sections from HRP injected naïve brain. Thresholded images were analyzed using ImageJ to obtain percent area of positive HRP staining. Pixel value higher than that of the threshold value was considered positive for HRP stain.

Quantification of NP accumulation after midline FPI

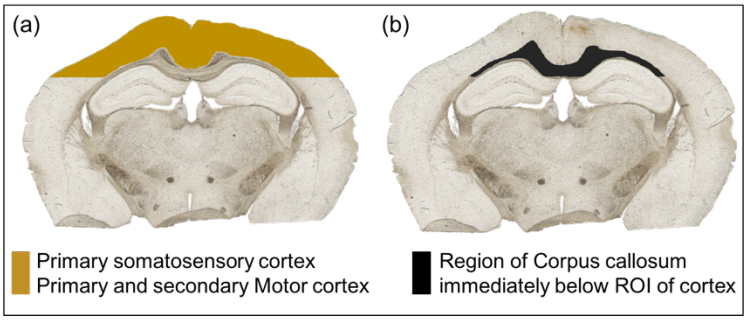
The brain tissue was processed and analyzed similar to our previous study(23). Briefly, slides were incubated at room temperature for 20 mins in 1X PBS to rehydrate the tissue and remove OCT compound. Coverslips were mounted on the section after adding one drop of fluorescent mounting media (Vectashield). The full brain sections (three sections per animal, three animals per group) were scanned using confocal microscopy (ZEISS LSM 800 with Airyscan, Carl Zeiss, CA, USA) at 10X magnification. Sections were located ~-1.65 mm Bregma. Scanning settings for each NP: 20 nm, 40 nm, 100 nm and 500 nm were $\lambda_{ex}/\lambda_{em}$=656 nm/683nm (600 V gain); $\lambda_{ex}/\lambda_{em}$=576 nm/607

nm (600 V gain); $\lambda_{ex}/\lambda_{em}$=357 nm/414 nm (640 V gain), and $\lambda_{ex}/\lambda_{em}$=503 nm/514 nm (600 V gain), respectively. Configuration settings were maintained constant for all the images collected. For brain sections, Z stacking was performed and total Z depth was 20 μm with a slice thickness of 5 μm. The Z stacks were converted to a single image by maximum projection tool using Zeiss software (Zen, Carl Zeiss, CA, USA). Quantitative analysis of NP accumulation was accomplished by defining two regions of interest (ROI) based on previously established HRP staining patterns for FPI: (1) cortex (includes primary somatosensory and primary, and secondary motor cortex) and (2) corpus callosum (17)((38), (Figure S1). Specifically, ImageJ software was used to draw ROIs manually and grid lines were used as a reference tool. The full brain scan images were rotated such that the midline was oriented at the center of the grid. Next, the horizontal line tangential to the ventral aspect of the corpus callosum was used to mark the maximum extent of ROI (Figure S1). The ROI for cortex was manually drawn spanning through the entire cortex above the maximum extent line. The ROI for corpus callosum was drawn spanning through the entire corpus callosum above the maximum extent line. The ROI images were thresholded to remove background fluorescence using tissue sections from NP injected naïve brain. The percent area of total positive NP pixels was calculated using ImageJ software. Pixel intensity value higher than that of the threshold value was considered positive for NP.

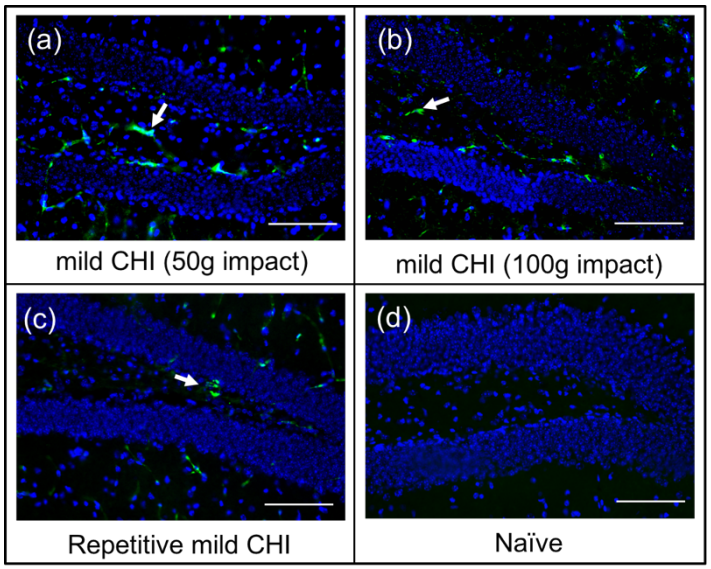
Immunohistochemical analysis for mCHI/RmCHI

Due to the complex pathophysiology of the mCHI and RmCHI models, we assessed the integrity of the BBB by the presence of intracerebral mouse IgG (immunoglobulin). A positive IgG stain within the brain parenchyma indicated the BBB

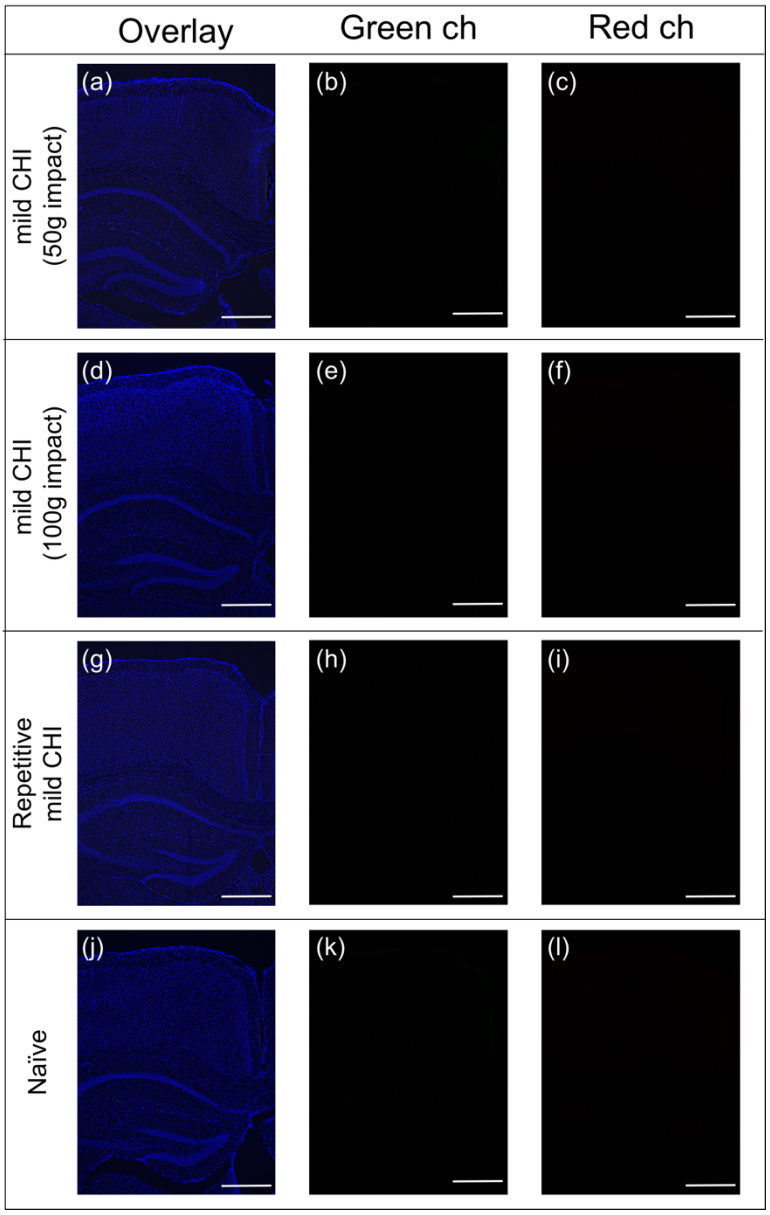
was compromised at some point after injury. Brain sections from the mCHI study with single and multiple CHI impacts were used. The sections (stored at -80°C) were incubated at room temperature for 20 mins before placing them in PBS bath and washed 3 times. The slides were incubated with a solution made up of anti-mouse IgG secondary antibody 488 (1:200) with 2% goat serum and 0.1% triton X-100 for 2h at room temperature in the dark. The sections were rinsed with PBS (4 times, 5 min each) before placing the coverslip with fluorescent mounting media (Vectashield). A conventional epifluorescent microscope (Leica DMI6000 B, Leica Microsystems, Wetzlar, Germany) was used to image the stained sections.



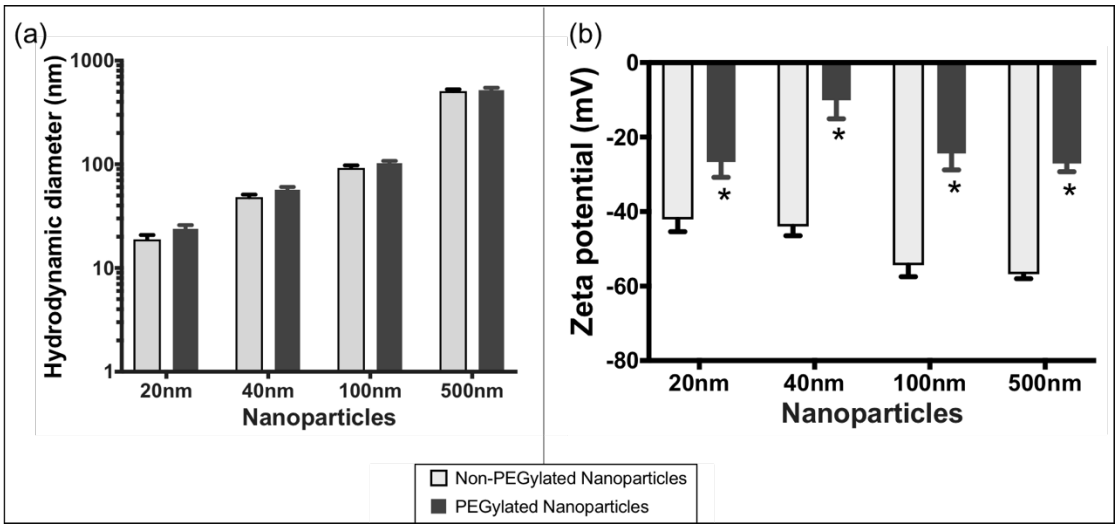
***Supplementary Figure S1**: **Region of interest for quantification of FPI cohorts for HRP staining and NP accumulation**. (a) Colored area shows the primary somatosensory, primary and secondary motor cortex. (b) Colored area shows the region of the corpus callosum immediately below the ROI of cortex.*



***Supplementary Figure S2**: **Immunostain for endogenous IgG after mild/repetitive mild CHI**. Positive staining of IgG shown in hippocampus (green, white arrows) and DAPI (blue) at 1h post mild CHI (50g) (a), mild CHI (100g) (b), and repetitive mild CHI (c). As a control, naïve tissue displays no IgG but only DAPI stain (d). Scale bar = 100 μm. Note: IgG stain was observed in cortex as well (data not shown).*



Supplementary Figure S3. Representative images from epifluorescent microscopy after mild/repetitive mild CHI*. No fluorescence is observed in green (λ_{ex} 488 nm) and red (λ_{ex} 555 nm) channels (ch) after mild 50g CHI ((a)-(c)), mild 100g CHI ((d)-(f)), repetitive mild CHI ((g)-(i)), and in control naïve groups ((j)-(l)). Overlay images were obtained by overlaying DAPI, green and red channel. Scale bar = 500 µm.*



Supplementary Figure S4. Characterization of NPs (**a**) Mean hydrodynamic diameter (number distribution) of non-PEGylated and PEGylated nanoparticles. (**b**) Zeta potential of non-PEGylated and PEGylated nanoparticles, *p < 0.05, One-way ANOVA, Sidak multiple comparisons. Error bars represent standard error of mean with n = 3 per group.

Nominal NP size (nm)	**Non-PEGylated NPs**		**PEGylated NPs**	
	Hydrodynamic diameter (nm)	**Zeta potential (mV)**	**Hydrodynamic diameter (nm)**	**Zeta potential (mV)**
20	18.9±1.9	-42.1±3.3	23.9±2.0	-26.7±4.1
40	48.2±2.9	-44.0±2.5	56.9±3.5	-10.1±5.0
100	92.2±5.3	-54.4±3.1	102.3±5.6	-24.4±4.4
500	507.3±20.1	-56.8±1.2	519.1±29.1	-27.1±2.2

Supplementary Table S1. PEGylated nanoparticle characterization: *The mean hydrodynamic diameter (number distribution) and zeta potential of PEGylated NP, mean±standard error of mean (n=3) with PDI <0.1. Measurements in 20 mM HEPES (pH 7.4).*

1	HRP - Cortex	Comparison	F (DFn, DFd)	P value	Significant
		Interaction	F (2, 12) = 7.76	P=0.0069	Yes
		Injured vs Sham	F (1, 12) = 29.02	P=0.0002	Yes
		Time points	F (2, 12) = 7.814	P=0.0067	Yes
2	HRP - Corpus callosum	Comparison	F (DFn, DFd)	P value	Significant
		Interaction	F (2, 12) = 3.928	P=0.0487	Yes
		Injured vs Sham	F (1, 12) = 3.824	P=0.0742	No
		Time points	F (2, 12) = 3.907	P=0.0493	Yes
NP - Cortex					
3	20 nm	Comparison	F (DFn, DFd)	P value	Significant
		Interaction	F (2, 12) = 17.71	P=0.0003	Yes
		Injured vs Sham	F (1, 12) = 27.49	P=0.0002	Yes
		Time points	F (2, 12) = 18.14	P=0.0002	Yes
4	40 nm	Comparison	F (DFn, DFd)	P value	Significant
		Interaction	F (2, 12) = 13.94	P=0.0007	Yes
		Injured vs Sham	F (1, 12) = 20.97	P=0.0006	Yes
		Time points	F (2, 12) = 14.72	P=0.0006	Yes
5	100 nm	Comparison	F (DFn, DFd)	P value	Significant
		Interaction	F (2, 12) = 5.373	P=0.0216	Yes
		Injured vs Sham	F (1, 12) = 10.47	P=0.0071	Yes
		Time points	F (2, 12) = 7.76	P=0.0069	Yes

6	500 nm	Comparison	F (DFn, DFd)	P value	Significant
		Interaction	F (2, 12) = 11.15	P=0.0018	Yes
		Injured vs Sham	F (1, 12) = 26.16	P=0.0003	Yes
		Time points	F (2, 12) = 12.23	P=0.0013	Yes
NP - Corpus callosum					
7	20 nm	Comparison	F(DFn, DFd)	P value	Significant
		Interaction	F (2, 12) = 0.4299	P=0.6602	No
		Injured vs Sham	F (1, 12) = 0.7643	P=0.3991	No
		Time points	F (2, 12) = 1.736	P=0.2176	No
8	40 nm	Comparison	F(DFn, DFd)	P value	Significant
		Interaction	F (2, 12) = 1.015	P=0.3915	No
		Injured vs Sham	F (1, 12) = 1.502	P=0.2438	No
		Time points	F (2, 12) = 1.081	P=0.3701	No
9	100 nm	Comparison	F (DFn, DFd)	P value	Significant
		Interaction	F (2, 12) = 3.555	P=0.0613	No
		Injured vs Sham	F (1, 12) = 2.893	P=0.1147	No
		Time points	F (2, 12) = 3.106	P=0.0818	No
10	500 nm	Comparison	F (DFn, DFd)	P value	Significant
		Interaction	F (2, 12) = 0.8051	P=0.4698	No
		Injured vs Sham	F (1, 12) = 1.491	P=0.2455	No
		Time points	F (2, 12) = 1	P=0.3965	No

Supplementary Table S2. Statistical analysis results: *Tabular results of two-way ANOVA for HRP extravasation in the cortex (row 1) and corpus callosum (row 2). The two-way ANOVA results of Nanoparticle accumulation in the cortex for 20 nm (row 3), 40 nm (row 4), 100 nm (row 5) and 500 nm (row 6) are displayed. The two-way ANOVA*

results for nanoparticle analysis in the corpus callosum for 20 nm (row 7), 40 nm (row 8), 100 nm (row 9) and 500 nm (row 10) are shown.