# Imaging early endothelial inflammation following stroke by core shell silica superparamagnetic glyconanoparticles that target selectin

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[¶] Equal contribution.

Supporting Information

## Contents

## 1. Synthesis of carbohydrate coated magnetic nanoparticles (MNPs)

The overall synthesis is depicted in Scheme 1.

Synthesis of carboxylic acid MNPs (**COOH@MNPs**)

The amine coated MNPs (**$NH_2$@MNPs**) were synthesized as previously described[1]. The **$NH_2$@MNPs** (50 mg, about 2 µmol of amino functionalized groups) were suspended in a solution of dichloromethane (DCM) and pyridine (Py) (DCM:Py; 1:1; 1 mL) in a 1.5 mL Eppendorf tube. Succinic anhydride (250 mg, 2.50 mmol) was added and the reaction mixture was sonicated at 22 °C for 30 min. The resulting mixture was stirred at 22 °C for another 18 h to afford the **COOH@MNPs**. **COOH@MNPs** were isolated by magnetic separation and purified by washing with dimethylformamide (DMF) (3 × 1 mL) to remove excess succinic anhydride.

Synthesis of *N*-hydroxysuccinimide activated MNPs (**NHS@MNPs**)

**COOH@MNPs** (50 mg, about 2 µmol of carboxyl groups) were suspended in DMF (1 mL) in a 1.5 mL Eppendorf tube. *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) (100 mg, 0.52 mmol) and *N*-hydroxysuccinimide (NHS) (100 mg, 0.87mmol) were then added. The reaction mixture was sonicated at 22 °C for 30 min. The resulting mixture was stirred at 22 °C for another 18 h to afford the **NHS@MNPs**. **NHS@MNPs** were isolated by magnetic separation and purified upon washing with DMF (3 × 1 mL) to remove excess EDC and NHS. After the reaction, the **NHS@MNPs** were immediately used for further coating with amino-modified carbohydrates or ethanolamine.

Synthesis of Lewis$^X$ MNPs (**LX@MNPs**), Sialyl Lewis$^X$ MNPs (**SX@MNPs**), and hydroxy MNPs (**HO@MNPs**)

**NHS@MNP**s (10 mg, about 0.4 µmol NHS groups) were suspended in DMF (1 mL) in a 1.5 mL Eppendorf tube and sonicated at 22 °C for 30 min. The MNPs were then incubated with amino-modified carbohydrates [Lewis$^X$ **1**[2] (2 mg; 3.25 µmol) or Sialyl Lewis$^X$ **2**[3] (2 mg; 2.20 µmol)] and triethylamine ($NEt_3$) (10 µL) at 22 °C for 48 h. After amide coupling, the DMF solvent was discarded by magnetic separation. Subsequently, DMF (1 mL) and ethanolamine (10 µL) were added to each sample and the reaction mixtures were allowed to stir at 22 °C for another 4 h. **LX@MNPs** and **SX@MNPs** were isolated by magnetic separation and purified by washing with DMF (3 × 1 mL) and double distilled water (3 × 1 mL) to remove traces of unreacted sugar or ethanolamine. A similar procedure was applied for the synthesis of **HO@MNPs** without the addition of amino-modified carbohydrates.

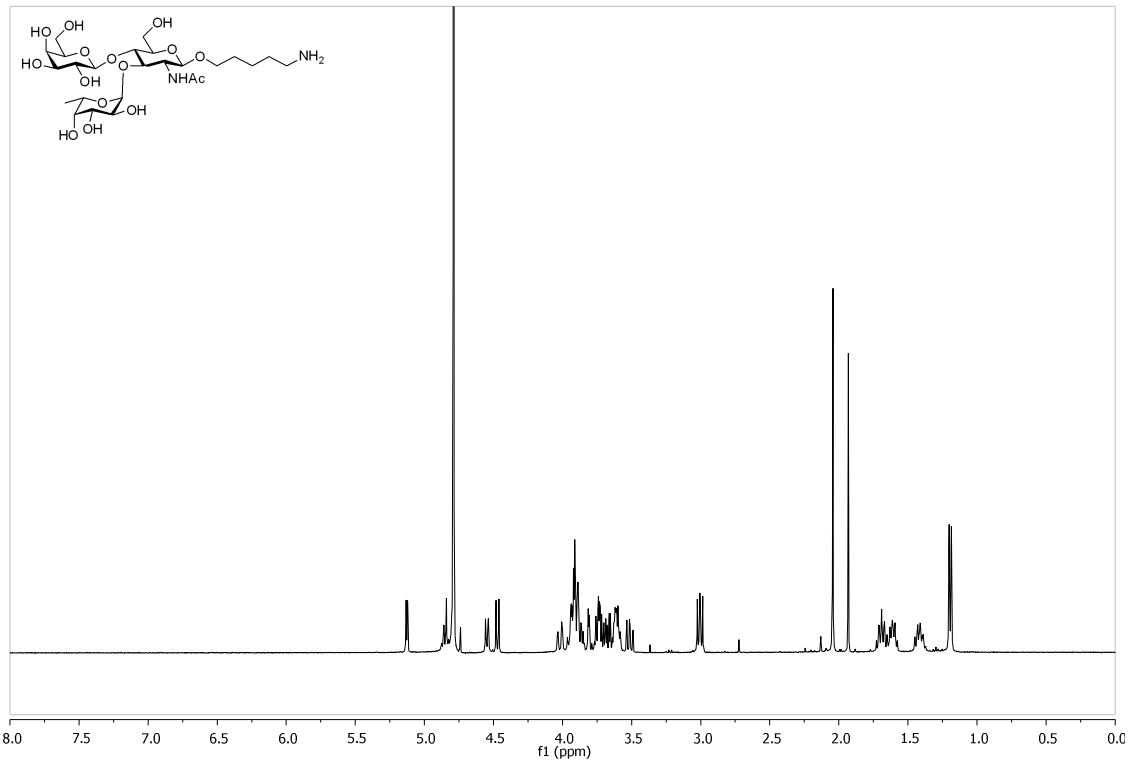


**Figure S1**. $^{1}$H-NMR of Lewis$^{X}$ (LX) **1**.

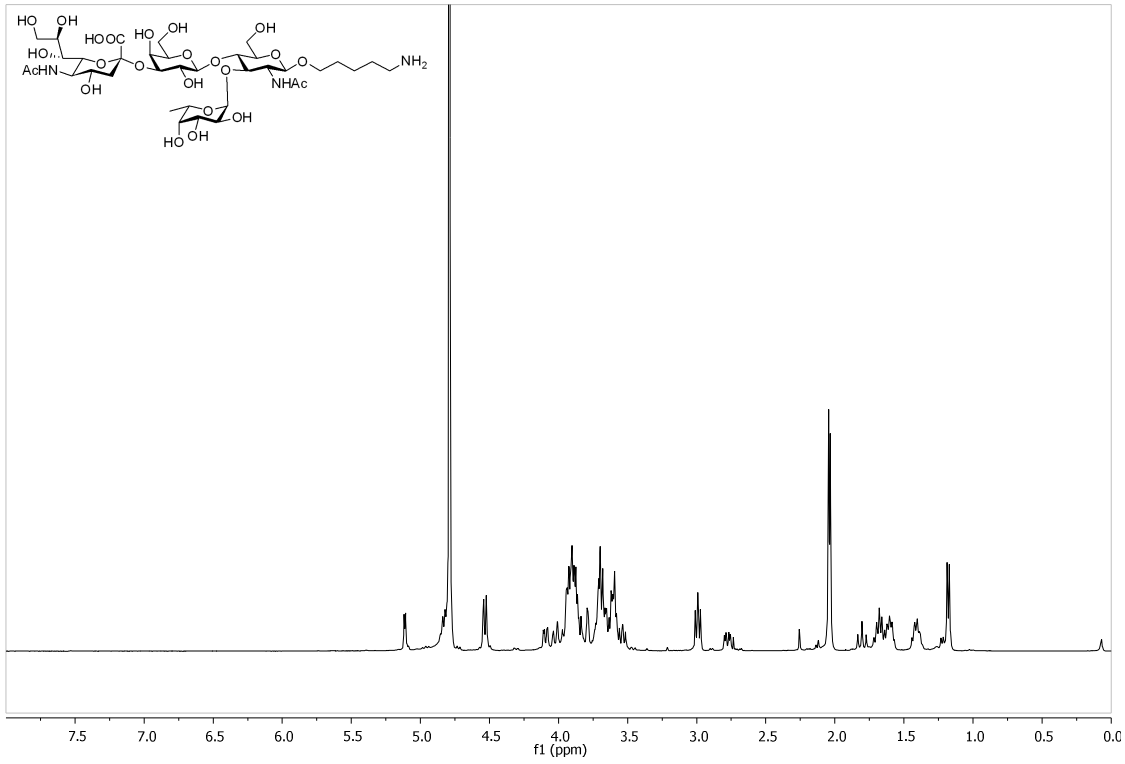


**Figure S2**. $^{1}$H-NMR of Sialyl Lewis$^{X}$ (SX) **2**.

## 2. TEM measurements

TEM measurements were performed on a Jeol JEM 2200-FS, operating at 200 kV. At high magnification, the in-column Ω-Filter was used to improve the contrast. The samples were prepared by immersion of grids of type R 1/4 (Cu coated with carbon film, Quantifoil GmbH) into a small volume (0.5 mL) of the sample and subsequent solvent evaporation in a dust protected atmosphere.

## 3. Surface charge determination (zeta-potential) and dynamic light scattering measurements

Zeta potential

A Malvern Zetasizer instrument was used to measure the electrophoretic mobility of nanoparticles. The Helmholtz-Smoluchowski equation was used to correlate the measured electrophoretic mobilities to the zeta potentials. Three replicates of each sample were measured six times at 25°C s, totalling 100 data acquisitions. Except for **$NH_2$@MNPs**, which were measured in double distilled-$H_2O$, the measurements of the other MNPs were carried out in phosphate buffered saline (PBS).

Dynamic light scattering (DLS) for hydrodynamic diameter

DLS measurements were carried out at a scattering angle of 173° with a Malvern Zeta Nanosizer working at 4-mW He–Ne laser (633 nm). The decay of the correlation function was fitted by the cumulant method.[4] The first cumulant provides the mean value of the diffusion relaxation rates, and the second cumulant the variance of the distribution or polydispersity index (PDI). The measurements were carried out in PBS buffer.

**Table S1.**

| | COOH@MNPs | HO@MNPs | LX@MNPs | SX@MNPs |
|---|---|---|---|---|
| Size (number, nm) | 44 | 78 | 58 | 59 |
| Size (intensity, nm) | 140 | 164 | 124 | 131 |
| PDI | 0.224 | 0.197 | 0.176 | 0.183 |

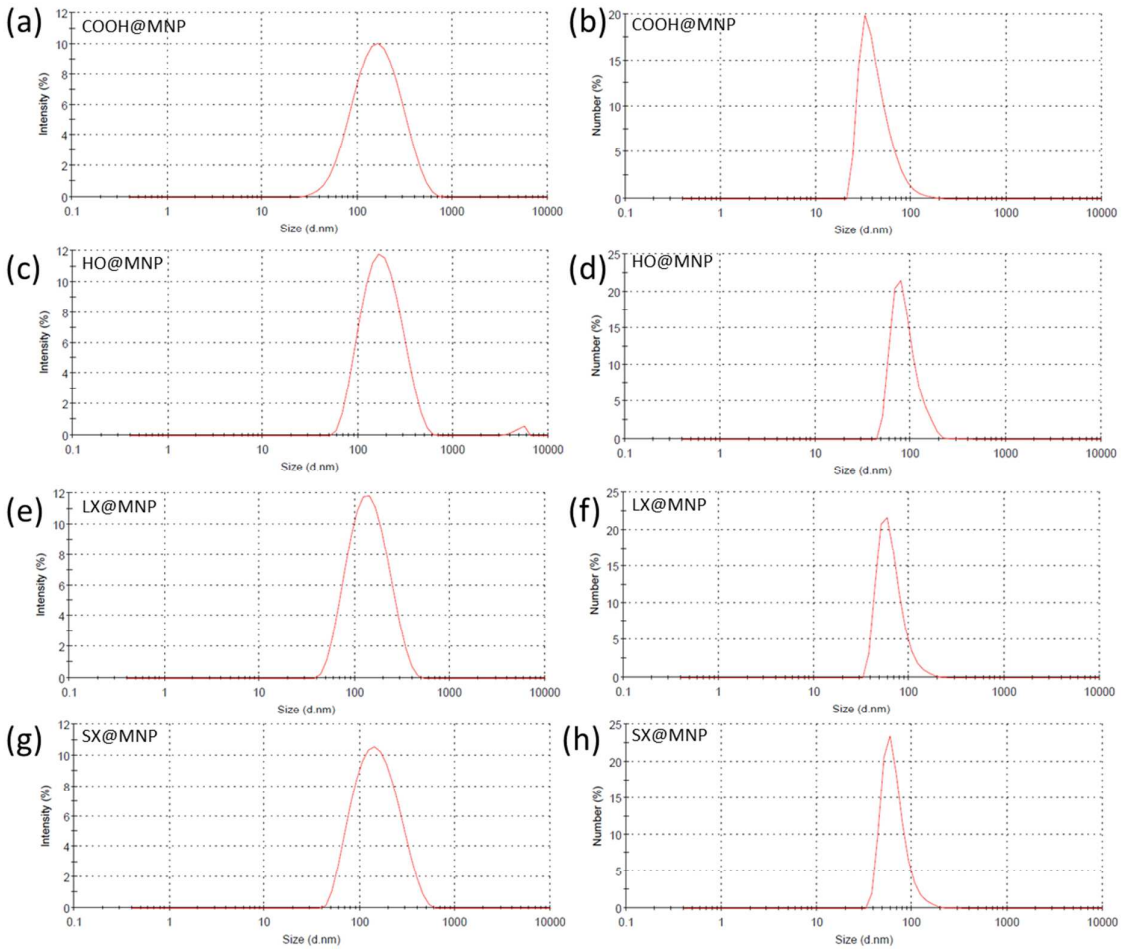


**Figure S3**. DLS hydrodynamic diameter measurements. The intensity of the hydrodynamic diameter of the particles is depicted in the left panel, whereas the number of the hydrodynamic diameter of the particles is depicted in the right panel.

## 4. Sialyl Lewis$^{X}$ estimation on SX@MNPs

A manufacturer's protocol was followed for the determination of sialic acid content of the modified MNPs using the SialicQ quantification kit (Sigma-Aldrich). The method selectively detects sialic acid incorporation levels. Quantitation was accomplished with an enzymatic method. The principle is shown in Figure S4. β-NADH oxidation can be accurately measured spectrophotometrically; Sialyl LewisX levels were approximately 10.5 ± 1.2 nmol•mg$^{-1}$.

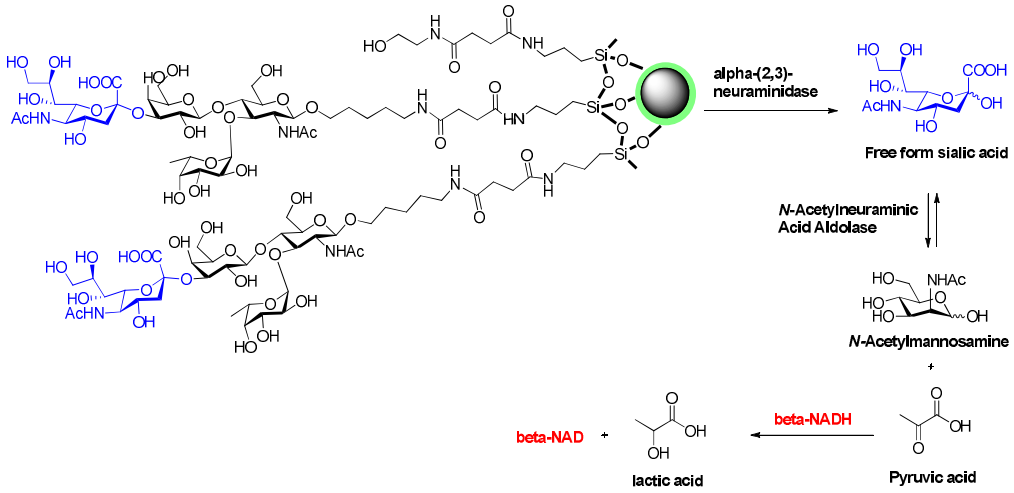


**Figure S4.** The reaction principle of SX estimation on **SX@MNPs**.

## 5. *in vitro* Binding Assay of MNPs to Rat E-Selectin

The solutions of different MNPs (10 uL, 50 mg/mL) were incubated with E-selection-human IgG-Fc chimera (200 uL, 500 ng/mL; assay buffer: 10 mM Tris, 150 mM $NaCl_2$, pH = 7.2). The reaction mixture was shaken at 37 $^{o}$C for 3 h, the solutions were placed in a magnetic stand, and washed three times with buffer (assay buffer with 0.2% Tween20 and 1% BSA). Anti-human IgG-HPR-conjugate (10,000 times dilute-Fc-specific in assay buffer with 1% BSA and 0.02% Tween 20) was added, and the resulting mixture was shaken at 37 $^{o}$C for 1 h. The MNPs were then washed three times with washing buffer. The tetramethylbenzidine (TMB) substrate mix (0.5 mL) was added, and the mixture was incubated at room temperature for 10 min. The resulting supernatant was taken to measure the UV absorbance at 655 nm.

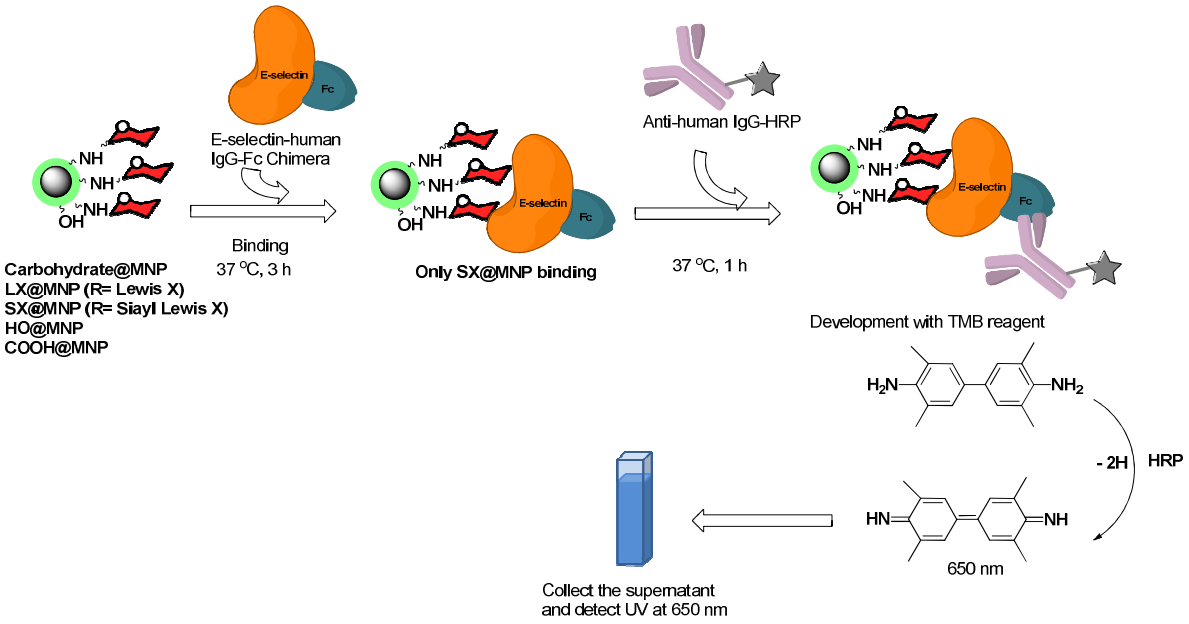


**Figure S5.** Schematic illustration of the E-selectin binding assay.

## 6. Anaesthesia and surgical procedures

All animal experiments were approved by the Landesamt für Gesundheit und Soziales Berlin under licence number G0200/07 and performed in accordance with the German Animal Welfare Act. Eighteen male C57/BL6 mice were housed in a temperature (22 °C ± 2 °C), humidity (55% ± 10%), and light (12/12 h light/dark cycle) controlled environment, and given *ad libitum* access to food and water.

Anaesthesia was achieved using isoflurane in a 70:30 nitrous oxide: oxygen mixture, and core body temperature was maintained at 37.2 ± 0.8 °C with automated heating blankets. Transient occlusion of the middle cerebral artery was performed using a modified intraluminal filament technique[5]. Mice were placed in a supine position, the neck was shaved, cleaned, and a midline incision was made. The left sternomastoid muscle was retracted and the carotid artery exposed and ligated. A small incision was made in the carotid artery and a 170 µm diameter filament (Doccol Corporation) was advanced up the internal carotid artery until resistance was felt. The animals were recovered and the filament was left in place for 60 min. Subsequently, the filament was withdrawn to induce reperfusion and the carotid artery permanently ligated. The incision was sutured, local anaesthetic was applied to the wound, and the animals were kept hydrated with physiological saline, and provided with wet diet.

## 7. MRI procedures and MNP administration

MRI experiments were conducted on a 7T Pharmascan® 70/16 (Bruker BioSpin, Ettlingen, Germany) equipped with actively shielded gradient coils (300 mT/m, rise time 80 µs). Radio frequency transmission was achieved with a 72 mm diameter quadrature resonator actively decoupled to a mouse head quadrature surface coil for signal reception. Anaesthesia was also achieved using isoflurane at approximately 4 h post MCAO. A 32 G dental needle attached to 1 m of polyethylene tubing was inserted into a lateral tail vein for administration of the MNPs during scanning. Body temperature and respiration rate were monitored with an MRI compatible system (Small Animal Instruments, Inc.).

The imaging protocol (FOV: 25 x 25 $mm^2$, matrix: 256 x 256, 12 contiguous 0.6 mm thick slices) consisted of a $T_2$ measurement using a multi slice multi echo (MSME) sequence (TR/TE: 3000/10.5 ms, 16 echoes, 9 min), a fast low angle shot (FLASH) $T_2$*-weighted sequence (TR/TE: 1000/7.2 ms, flip angle 30˚, 4 min), and a FLASH $T_1$-weighted sequence (TR/TE: 150/3.5 ms, flip angle 30˚, 4 min). The scan package was acquired approximately 4.5 h after MCAO. Subsequently, mice were randomized to receive 1000 µmol/kg one of the MNP formulations (n=6 per group). Following the injection, the scan package was repeated. The animals were recovered, and 24 h later the scan package was repeated for a final time. One animal in the **HO@MNP** and one from the **SX@MNP** treated group was excluded from the analysis due to lack of evidence of an ischemic lesion in the MR images at 24hrs.

## 8. Tissue preparation and histology

At the conclusion of the final imaging experiment, mice were euthanized with intraperitoneal chloral hydrate (4% in water) and perfused transcardially with physiological saline. The brains, livers, and spleens were removed and snap frozen in -40 ºC methylbutane. All tissue was sectioned to 20 µm using a cryostat (Leica Microsystems) and stained with Prussian blue to detect iron using the Accustain Iron Kit (Sigma Aldrich). Slides were placed in equal parts 4% potassium ferrocyanide ($K_4Fe(CN)_6$) and 1.2 mmol/L hydrochloric acid (HCl) for 10 min at room temperature. They were subsequently rinsed in de-ionized water and counterstained in 1% pararosaniline hydrochloride for 4 min. The slides were rinsed again, rapidly dehydrated in 70, 80, 96, and 100% alcohol, placed in xylene, and subsequently coverslipped.

## 9. Image analysis

Quantitative $T_2$ maps were fitted on a voxelwise basis using a monoexponential decay function in Paravision software (Bruker BioSpin). Subsequently, all data spatial dimensions were scaled by a factor of 10 and exported into FSL software (Analysis

Group, FMRIB, Oxford). The BET tool was used to segment the brain in the $T_2$ weighted images, and the process was completed manually. Subsequently, the segmented brain was co-registered using the FLIRT tool to a template brain (average of 10 different C57/BL6 mouse brains). The segmented, co-registered image was used to prepare three masks: the entire brain, the ischemic territory, and the mirrored contralateral region in ImageJ freeware (http://rsbweb.nih.gov/ij/). The co-registration transformation was applied to all the other data sets ($T_1$ and $T_2$*) and the masks were applied to extract the three volumes of interest (VOIs). The number of dark voxels was simply counted in each of the three VOIs for each animal. A rolling ball paradigm (radius of 10 pixels) was used to reduce the surface coil profile in the image and produce a thresholded image that excluded all of the dark voxels for a second count. Thus, the total number of dark voxels was estimated by subtracting the difference between the two measurements. All area measurements were corrected for the transformation that occurred during the co-registration process.

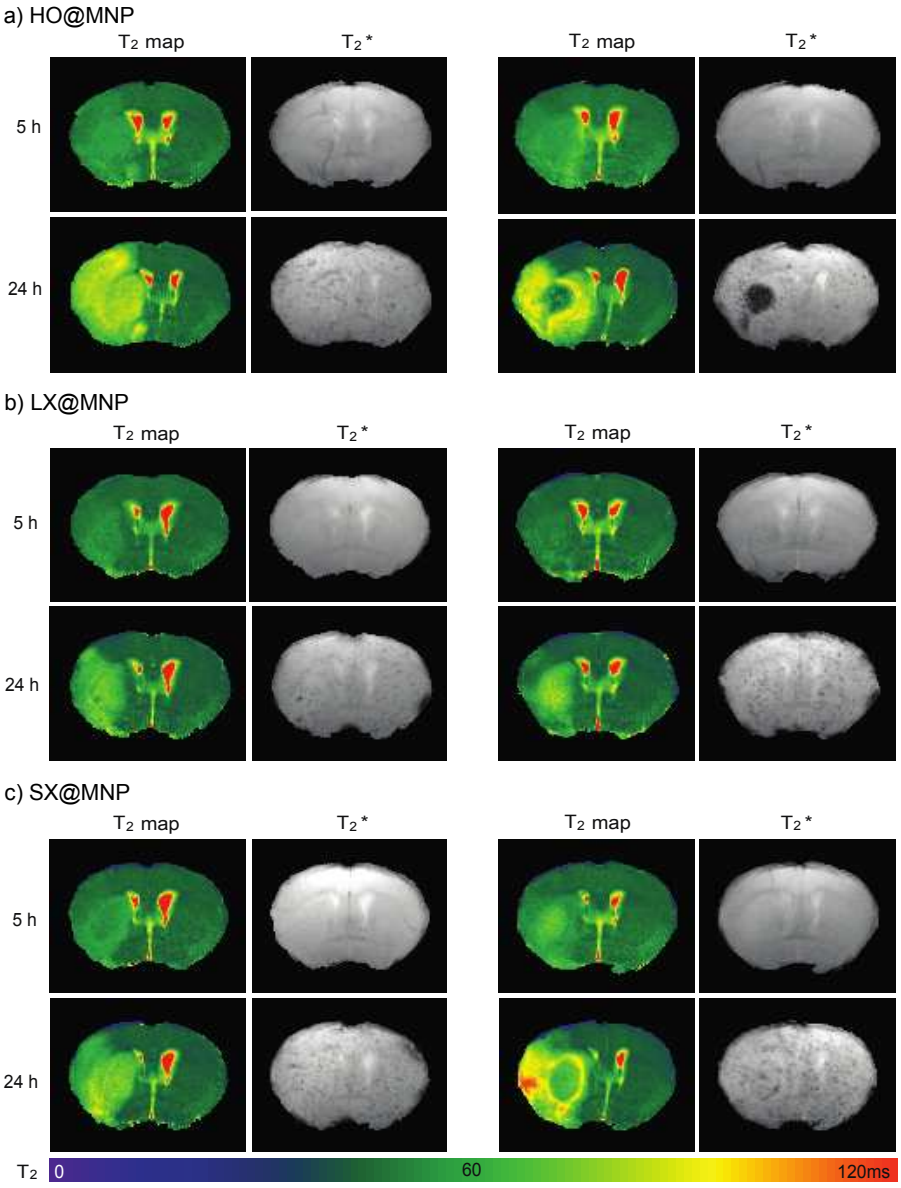


**Figure S6.** $T_2$ and $T_2$* weighted images at 5 and 24 h after MCAO in two animals that received **HO@MNPs** (a), **LX@MNPs** (b), or **SX@MNPs** (c). The animal with the smallest accumulation of MNPs in each group is depicted in the left panel, whereas the animal with the largest accumulation is depicted in the right panel. Note: the color scale bar corresponds to the $T_2$ values.

A three dimensional, transparent, surface projection of the brain, infarct, and MNP distribution from an animal in the **SX@MNP** treated group is depicted in Figure S7.

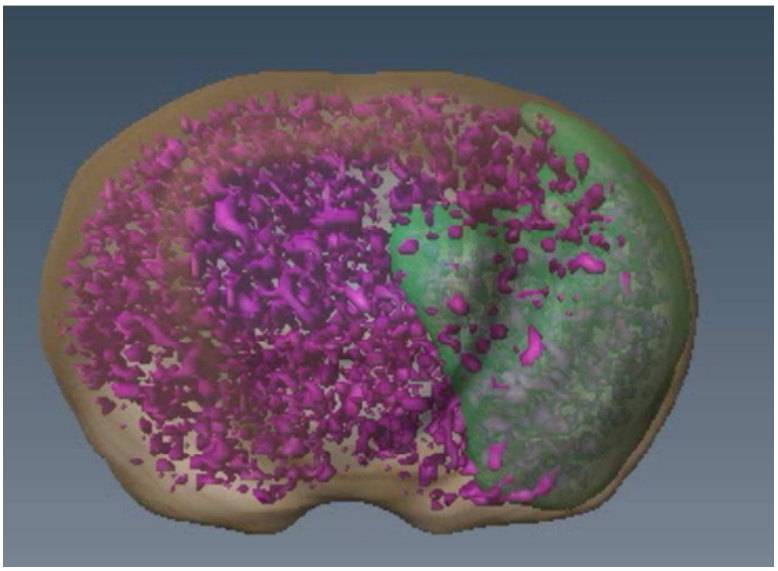

**Figure S7.** The surface projection of the brain (yellow), infarct (green), and MNPs (pink) is depicted at 24 h after MCAO in the animal with the median accumulation of **SX@MNPs**. There is also a 3D-movie in additional supporting information.

## 10. Statistical analysis

Results were expressed as group means ± standard deviation of the mean. Statistical analysis was performed using SPSS software and a p-value of less than 0.05 was chosen as the significance level for all analyses. Image analysis data was compared using one way analysis of variance (ANOVA). A Bonferroni correction was applied to all post-hoc pairwise multiple comparisons. Western blot data was compared using a mixed design ANOVA with side (ipsilateral and contralateral) as the within subject factor and timepoint (5hrs and 24hrs) as the between subject factor. Effect sizes were calculated for the omnibus tests (Omega, $\omega^2$) and for post-hoc comparisons (Cohen's d).

## 11. Western blot

An additional eleven animals were used to assess selectin (CD62E) expression using Western blots. Eight animals underwent 60 min of MCAO and three underwent the corresponding sham procedure. Four animals with MCAO were sacrificed at 5 h and the remaining animals at 24 h. Coronal slices (2 mm thick) within the middle of the ischemic tissue, or the corresponding contralateral hemisphere, were cut using a Brain Matrix and minced in 300 µL of ice cold TNN lysis buffer containing 50 mM Tris (pH 7.5), 1 mm DTT, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 10 mM $Na_4P_2O_7$, 2 mM $Na_3VO_4$, 100 mM NaF, and Roche Complete™ Protease Inhibitor Cocktail without EDTA. After lysis, whole brain homogenates were sonicated for 10 s with 10% amplitude on ice. Equal protein loading was achieved by protein concentration determination using the Bradford™

method and a NanoDrop™ 2000c spectrophotometer (Thermo Scientific). Equal protein loading was confirmed by anti-GAPDH immunoblotting (Millipore, MAB374, 1:7500) as a house keeping protein. 40 µg of protein for each sample were separated in Laemmli buffer after 5 min denaturation at 95° Celsius using 4-20% gradient gels (Bio-Rad Laboratories), transferred on 0.45 µm PVDF membrane for 50 min at 70 V, and blocked using 5% semidry skim milk. Immunodetection of E-selectin was carried out using rabbit anti-CD62E (abcam, ab18981; 1:500 in 3% Tris-BSA) or anti-GAPDH (in 3% Tris-BSA) and detected with horseradish peroxidase coupled donkey anti mouse or rabbit secondary antibodies (GE Healthcare; 1:5000). CD62E immunoreactive bands appeared at three distinct bands with individual electromobility shifts around 62 and at 72 to 75 kD indicative for post-translationally modified isoforms. GAPDH was detected around 36 kD. Immunoblots of all samples were run and detected in one digital image using chemiluminescence imaging (Vilber Lourmat, Fusion Fx7).

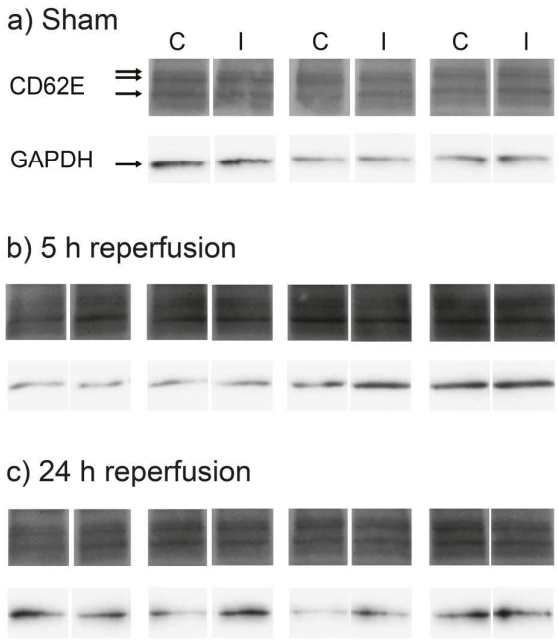


**Figure S8.** Immunblots of 11 mice after sham or MCAO surgery with either 5 or 24 h reperfusion period. CD62E expression in 2 mm slices within the center of the ischemic tissue was compared to the contralateral expression pattern. Molecular weight of E-selectin appeared around 67 kD with two post-translationally modified bands indicated by arrows which are probably glycosylated. Glyceraldehyd 3-phosphate dehydrogenase (GAPDH) served as a loading control.

Immunoblot images were quantified using Image J. First, the background was removed, then variability in protein loading was taken into account by calculating the ratio between CD62E and GAPDH intensity, and finally the ratios were expressed as increased CD62E fold

expression relative to the sham animals.

**Reference:**

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