Supporting Information

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Core Cross-Linked Polymeric Micelles for Specific Iron Delivery: Inducing Sterile Inflammation in Macrophages

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Results and Discussion

Polymer Synthesis and Characterization

Scheme S1. Polymerization scheme for azide-functionalized pSar\textsubscript{\textit{n}}-\textit{block}-pCys(SO\textsubscript{2}Et\textsubscript{\textit{m}}) (P1 to P3) copolypepti(o)ides.

Table S1. Characterization of pSar\textsubscript{\textit{n}}-\textit{block}-pCys(SO\textsubscript{2}Et\textsubscript{\textit{m}}) (P1 to P3) copolymers.

| polymer | end-group | \(X_n\) pSar\textsuperscript{[a]} | \(X_n\) pCys(SO\textsubscript{2}Et)\textsuperscript{[b]} | wt. % Cys(SO\textsubscript{2}Et) | \(M_n\) \textsuperscript{[c]} | \(\Theta\) \textsuperscript{[c]} |
|---------|-----------|----------------|----------------|----------------|----------------|----------------|
| P1      | Ac        | 225            | 31             | 27.5           | 31150          | 2.64           |
| P2      | N\textsubscript{3} | 200            | 17             | 18.9           | 31700          | 1.25           |
| P3      | N\textsubscript{3} | 170            | 27             | 30.4           | 35100          | 7.06           |

\textsuperscript{[a]} HFIP-GPC, relative to pSar standards. \textsuperscript{[b]} as determined by \textsuperscript{1}H-NMR. \textsuperscript{[c]} HFIP-GPC, relative to PMMA standards.
Figure S1. HFIP-GPC traces of P1-P3 (see Table 1). Note that secondary structures are not suppressed in the eluent (HFIP containing 3 gL\(^{-1}\) of CF\(_3\)COOK), and elution volumes may be influenced by the degree of secondary structure formation of the pCys(SO\(_2\)Et)\(_m\) block, as reported by previously.\(^{1-3}\)

Figure S2. Single-angle DLS of pSar-\(b\)-pCys(SO\(_2\)Et) block copolymers (P1 - P3) in DMSO (\(\beta = 18 \text{ g L}^{-1}\)) confirms the absence of larger structures but polymer species only.
Figure S3. $^1$H DOSY NMR spectrum of P1 (pSar$_{225}$-block-pCys(SO$_2$Et)$_{31}$) in DMSO-$d_6$.

Figure S4. $^1$H DOSY NMR spectrum of P2 (pSar$_{200}$-block-pCys(SO$_2$Et)$_{17}$) in DMSO-$d_6$. 
Figure S5. $^1$H DOSY NMR spectrum of P3 (pSar$_{170}$-block-pCys(SO$_2$Et)$_{27}$) in DMSO-$d_6$. 
Nanoparticle Characterization

Table S2. Characterization of core cross-linked polymeric micelles with and without embedded iron oxide nanoparticles.

| particle           | polymer | cross-linker      | yield | \(D_h\) [nm\textsuperscript{[a]}] | PDI    | wt.% Fe\textsubscript{2}O\textsubscript{3} [\textsuperscript{[b]}] | \(N_{Dye}\)\textsuperscript{[c]} |
|-------------------|---------|-------------------|-------|-----------------------------------|--------|--------------------------------------------------|------------------|
| \(\text{SPION-CCPM}_{Cy5}\) | P3      | Lipoic acid       | 22%   | 82                                | 0.163  | 33                                               | 16.5             |
| \(\text{SPION-CCPM}_{Cy5}\#2\) | P2      | Lipoic acid       | 36%   | 63                                | 0.122  | 42                                               | 4.1              |
| CCPMs\textsubscript{Cy5}       | P1      | N-3-Azidopropyl-liponamide | 46%   | 49                                | 0.131  | -                                                | 2.5              |

[a] determined by single-angle DLS. [b] Determined by TGA in O\textsubscript{2} atmosphere. [c] Determined by FCS.

Figure S6. Characterization of CCPM control particles. (A) DLS analysis shows core cross-linked polymeric micelles (CCPMS) with narrow dispersity. (B) CryoTEM confirmed the presence of nanoparticles with sizes well below 100 nm with spherical morphology. (C) HFIP GPC analysis confirmed successful cross-linking.
**Figure S7.** Multi-angle DLS shows no aggregation or increasing sizes for CCPM\textsuperscript{Cy5} after incubation in human plasma.
Figure S8. Characterization of SPION-CCPMs (A) ATR-FT-IR Spectroscopy of SPION-CCPMs, CCPMs, SPIONs and block copolymer pSar-b-pCys(SO$_2$Et). (B) UV-Vis spectroscopy of SPION-CCPM dispersions in water. Strong absorbance below $\lambda = 500$ nm refers to embedded iron oxide nanoparticles. Distinct absorbance of Cy5 can be detected for SPION-CCPM$^{Cy5}$ after dye conjugation and purification. (C) GPC-analysis in HFIP implies stable cross-linking and absence of residual unconjugated dye or polymer for SPION-CCPM$^{Cy5}$. The multimodal GPC-trace for polymer P3 is attributed to $\beta$-sheet induced aggregation (see Figure S1). (D) Zeta potential distribution. Slightly negative zeta-potentials were determined for both, SPION-CCPM$^{Cy5}$ and CCPM$^{Cy5}$, in 3 mM sodium chloride solution.
Figure S9. Characterization of SPION-CCPM<sup>Cy5</sup> #2 particles. (A) DLS analysis reveals SPION-CCPM<sup>Cy5</sup> #2 particles with narrow dispersity. (B) TGA analysis confirms higher iron oxide contents for SPION-CCPM<sup>Cy5</sup> #2 (42 wt.%) compared to SPION-CCPM<sup>Cy5</sup> (33 wt.%). (C) HFIP GPC analysis confirmed successful cross-linking and removal of unconjugated dye or polymer.

Figure S10. Fluorescence correlation spectroscopy. Normalized autocorrelation curves of Cy5-labelled SPION-CCPMs<sup>Cy5</sup> (red circles) and CCPMs<sup>Cy5</sup> (blue squares) measured in PBS buffer. The solid lines represent the corresponding fits with eq. 2 (main text). The fitting was done using single component (m = 1 in eq. 2) that confirms the absence of unconjugated dye.
Figure S11. Additional TEM images of SPION-CCPMs\textsuperscript{Cy5}.

Figure S12. TEM images of oleic acid coated SPIONs. No organized clusters of nanoparticles can be detected.
**Figure S13.** (A) Magnetization hysteresis loop recorded for SPION-CCPMs at 5K conforms superparamagnetic behavior. (B) Zero field cooling/field cooling curves revealed a blocking temperature of 42 K, confirming the presence of superparamagnetic iron oxide nanoparticles.[4]
**Figure S14.** Images of the magnetic response of SPION-CCPM\textsuperscript{Cys} dispersions in water. (upper images) In proximity of a permanent magnet, the meniscus of the dispersion changes immediately. (lower images) Slow accumulation of SPION-CCPM\textsuperscript{Cys} by magnetic force.

**Figure S15.** Image of the quadrupolar/dipolar ring-type magnet used for magnetic guidance experiments.\textsuperscript{[5]}
Magnetic Guidance in vivo

If a magnetic particle should be moved against a blood stream, the magnetic force, \( F_{\text{mag}} \), must overcome the hydrodynamic (Stokes) friction, \( F_{\text{fric}} \). A straight-forward calculation then gives

\[
F_{\text{mag}} > F_{\text{fric}} \tag{S1}
\]

\[
mG = gVMG > 6\pi\eta R_h v \tag{S2}
\]

Where \( m \) [Am\(^2\)] is the magnetic moment of the particle and \( G \) [T·m\(^{-1}\)] the magnetic field gradient. It is more useful to express \( m \) by a magnetization per mass \( M \) [Am\(^2\)·kg\(^{-1}\)] times its mass or density \( \rho \) [kg·m\(^{-3}\)] times particle volume \( V \) [m\(^3\)]. On the other side of the equation, the dynamic viscosity, \( \eta \) [Pa·s], of the surrounding liquid, its velocity, \( v \) [m·s\(^{-1}\)], relative to a sphere with hydrodynamic radius, \( R_h \) [m], is determining the friction. If, like in our case, a larger particle contains \( N \) spherical SPION centers of radius \( R \), this can be rearranged to find the necessary field gradient to counter the blood flow

\[
G > \frac{9\pi\eta R_h}{2\rho N R^3 M} \tag{S3}
\]

**Equation S3.** Approximation of the magnetic gradient required to direct magnetic particles in dispersion of a fluid in motion.

with \( v \) as the velocity of the blood flow (2·10\(^{-3}\) m·s\(^{-1}\) (zebrafish embryo)\(^{[6]}\), 0.15 m·s\(^{-1}\) (human)\(^{[7]}\), \( \eta \) as the dynamic viscosity of the blood (5·10\(^{-3}\) Pa·s (zebrafish embryo)\(^{[6]}\), 3.5·10\(^{-3}\) Pa·s (human)\(^{[8]}\), \( R_h \) as the hydrodynamic radius of the SPION-CCPM nanoparticle, \( \rho \) as the density of the nanoparticle (approx. 1500 kg·m\(^{-3}\) for SPION-CCPMs), \( R \) as the radius magnetic SPION core, and \( M \) as the saturation magnetization of the SPION nanoparticle (50 Am\(^2\)·kg\(^{-1}\) for 10 nm iron oxide nanoparticles, 74 Am\(^2\)·kg\(^{-1}\) for magnetite nanoparticles > 20 nm)\(^{[4]}\).

For SPION-CCPMs with \( R_h = 40 \) nm, each containing 5 SPIONs cores of \( R = 5 \) nm, the magnetic gradient needs to be larger than 3.84·10\(^7\) T·m\(^{-1}\) or 2.02·10\(^8\) T·m\(^{-1}\) to overcome the velocity of the blood flow and guide those nanoparticles in the vasculature of zebrafish embryos or humans. For SPION-CCPMs with increased dimension of the magnetic cores \( (R_h = 20 \) nm, \( R_{\text{SPION}} = 10 \) nm, \( N = 3 \) values slightly decrease to 2.70·10\(^6\) T·m\(^{-1}\) or 1.42·10\(^6\) T·m\(^{-1}\) for zebrafish embryos or humans, still by far extending the capabilities of the displayed magnet guidance system \( (G = 2.5 \) T·m\(^{-1}\))\(^{[5]}\).
Figure S16. Concentration dependent uptake of SPION-CCPMs<sub>Cy5</sub> and CCPMs<sub>Cy5</sub> in BMDMs. (A and B) Non-treated (NT) BMDMs, or BMDMs treated with increasing concentrations of SPION-CCPMs<sub>Cy5</sub> or CCPMs<sub>Cy5</sub> (red) for 24 hrs. (A) Internalization of nanoparticles was measured by FACS fluorescence detection (intensity of Cy5). (B) Representative images of BMDMs with and without nanoparticle treatment. Cells were stained with Iba1 (green), a cell surface marker for macrophages, and DAPI. Data reported as mean ± SEM, n = 3 independent experiments. One-way ANOVA (black): * p < 0.01, ** p < 0.001, *** p < 0.0001.
Figure S17. Uptake of SPION-CCPMs and CCPMs in primary murine Hepatocytes, LLCs and BMDMs. Cells were incubated with SPION-CCPMs or CCPMs. Amount of SPION-CCPMs added to cells was calculated based on iron concentration from the core and the amount of CCPMs was calculated to match the mass of CCPMs contained within SPION-CCPMs. (A) Representative images of primary hepatocytes, (B) Lewis Lung Cancer Cells (LLCs), and (C) BMDMs treated with SPION-CCPMsCy5 or CCPMsCy5 (red) for 24 hours. Quantification of nanoparticle signal within cells is below each respective cell type, whereby at least n = 30 cells was analyzed. Primary hepatocytes and LLCs were stained with Phalloidin or β-actin (green) and DAPI (blue). BMDMs were stained with Iba1 antibody (green). Data reported as n ± Standard Error of the Mean (SEM). One-way ANOVA: * p < 0.01, ** p < 0.001, *** p < 0.0001.
Figure S18. SPION-CCPMs and CCPMs are taken up by BMDMs after a 1-hour incubation. BMDMs were incubated with 20 μM SPION-CCPMs or CCPMs and fixed with 4% paraformaldehyde after one hour. Cells were stained with a macrophage marker, Iba1 (green), and DAPI (blue).
Figure S19. SPION-CCPMs do not cause cytotoxicity in BMDMs. Cells were incubated with 20 μM SPION-CCPMs, dose matched CCPMs, or 20 μM ferric ammonium citrate (FAC). Lactate dehydrogenase (LDH) quantities were measured in the supernatant of cell cultures at 490 nm wavelength after adding CytoTox 96© substrate (Promega). Values are represented as a percentage of the 0 hour condition at each time point. Data reported as n ± SEM. n = 3 independent experiments. One-way ANOVA: * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure S20. SPION-CCPMs and not CCPMs activate an inflammatory response in human macrophages. (A and B) Human peripheral monocytes were differentiated for 10 days using M-CSF to produce macrophages. Macrophages were incubated with 20 μM SPION-CCPMs, Feraheme, CCPMs, or 100 ng/mL lipopolysaccharide (LPS). After 24 hours, cells were harvested for FACS analysis to detect the cell surface markers CD80 and CD86 (A) or differential cytokine mRNA expression using qPCR (B). (B) Data show mean and SEM of mRNA expression compared to the non-treated (NT) condition and all samples were corrected for RPL19 mRNA expression. One-way ANOVA (black): * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
Figure S21. CD86 protein expression in macrophages following treatment with various cysteine dimers. Cells were incubated for 24 hours with 20 μM iron (SPION-CCPMs, heme or ferric ammonium citrate (FAC)), CCPMs, L-cysteine (L-Cys), S-ethylsulfonyl-L-cysteine (L-Cys(SO₂Et)), S-ethylsulfonyl-L-homocysteine (L-Hcy(SO₂Et)), and cell surface marker CD86 was measured using fluorescence detection by FACS. Values are represented as fold-change compared to non-treated (NT) condition. Data show mean and SEM, n = 2 independent experiments. One-way ANOVA (black): * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
Figure S22. SPION-CCPMs induce sterile inflammation in macrophages. (A-C) BMDMs were incubated with 100 ng/mL LPS, 20 μM FAC, 20 μM Heme, 20 μM SPION-CCPMs or CCPMs for 18 hours. Amount of SPION-CCPMs added to cells was calculated to 20 μM iron from the core and the amount of CCPMs added to cells was calculated to match the mass of CCPMs contained within SPION-CCPMs. The graphs show mean and SEM of mRNA expression compared to the non-treated (NT) condition and all samples were corrected for Rpl19 mRNA expression. One-way ANOVA (black): * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
Table S3. Antibodies used for Flow Cytometry

| Antibody | Fluorophore | Clone | Isotype | Manufacturer         |
|----------|-------------|-------|---------|----------------------|
| **Anti-mouse cell culture experimentation** |
| CD206    | Alexa Fluor 700 | MR6F3 | Rat IgG2b, κ | ThermoFisher         |
| CD38     | FITC        | 90    | Rat IgG2a, κ | BioLegend            |
| CD86     | Brilliant Violet 421 | GL-1 | Rat IgG2a, κ | BioLegend            |
| CD80     | Brilliant Violet 650 | 16-10A1 | Armenian Hamster IgG | BioLegend |
| MHC II   | PE-Cy7      | M5/114.15.2 | Rat IgG2b, κ | BioLegend            |
| **Anti-mouse in vivo experimentation** |
| CD45     | PerCP-Cy5.5 | 104   | N/A     | ThermoFisher         |
| Ly6G     | FITC        | 1A8   | N/A     | BioLegend            |
| Ly6C     | PE-Dazzle   | HK1.4 | N/A     | BioLegend            |
| F4/80    | BV605       | T45-2342 | N/A     | ThermoFisher         |
| CD11c    | PE          | N418  | N/A     | BioLegend            |
| Siglec-F | APC-Cy7     | E50-2440 | N/A     | ThermoFisher         |
| CD11b    | PerCP       | ICRF44 | N/A     | ThermoFisher         |
| CD64     | BV711       | X54-5/7.1 | N/A     | BioLegend            |
| CD80     | BV650       | 16-10A1 | Armenian Hamster IgG | BioLegend |
| CD71     | BV510       | RI7217 | Rat IgG2a, κ | BioLegend |
| MerTK    | BV421       | 108928 | Rat IgG2a | ThermoFisher        |
| **Anti-human** |
| CD80     | PE          | 2D10  | Mouse IgG1, κ | BioLegend |
| CD86     | Alexa Fluor 488 | IT2.2 | Mouse IgG2b, κ | BioLegend |
### Table S4. Primers for quantitative RT-PCR (mus musculus)

| Gene  | Sequence |
|-------|----------|
| Arg1  | Forward 5' AATCTGCATGGGCAACCTGT 3'  
|       | Reverse 5' GTCTACGCTCTCCAAGCCAA 3' |
| Cxcl10| Forward 5' ACGTGTGGAGATCATGGCAC 3'  
|       | Reverse 5' GTGCACTCCACATAGCCTT 3' |
| Fpn1  | Forward 5' TGTCAGGCACATTGTGGAGA 3'  
|       | Reverse 5' TCTTGCAAGCACTGTGTCAGGG 3' |
| Gstm1 | Forward 5' TCGGGTTCAAGAGCTGGGTT 3'  
|       | Reverse 5' TCTGTTCCCTAGGTCTGACATCA 3' |
| Ho-1  | Forward 5' AGGGTAAGACCGCTTCTCCT 3'  
|       | Reverse 5' CCAGGTAAGCTATGGTACTCCAGAA 3' |
| Il6   | Forward 5' GCTACCAAACTGGAATAAT 3'  
|       | Reverse 5' CGTCACAGGTACTCCAGAGAGAGG 3' |
| Il1β  | Forward 5' GCAACTTCTCTGAACTCAACT 3'  
|       | Reverse 5' ATCTTTGGGTCCGTCGAACCT 3' |
| Nos2  | Forward 5' TGGGATCTGTTCCAGAACTG 3'  
|       | Reverse 5' CAGGCGAACACAGCACTAC 3' |
| Nqo1  | Forward 5' AGGGTGTTGAATGTCGACTCACT 3'  
|       | Reverse 5' ATCTTTGGGTCCGTCGACTCACT 3' |
| Rpl19 | Forward 5' AGGGTGTTGAATGTCGACTCACT 3'  
|       | Reverse 5' ATCTTTGGGTCCGTCGACTCACT 3' |
| Slc7a11| Forward 5' CCCATGACCTCTCTGCTTCG 3'  
|        | Reverse 5' GCCAGCATAAAGCCCTCTCA 3' |
| Socs3 | Forward 5' CCGGCTCAGACCCAGGAC 3'  
|       | Reverse 5' GGAAGGTTCAGGTTTCTTG 3' |
| Tfr1  | Forward 5' CCCATGACCTCTCTGCTTCG 3'  
|       | Reverse 5' GCCAGCATAAAGCCCTCTCA 3' |
| Tnfa  | Forward 5' CCCATGACCTCTCTGCTTCG 3'  
|       | Reverse 5' GCCAGCATAAAGCCCTCTCA 3' |

### Table S5. Primers for quantitative RT-PCR (homo sapiens)

| Gene   | Sequence |
|--------|----------|
| IL6    | Forward 5' AAATTCGGTACATCCTCGGCAAGGA 3'  
|        | Reverse 5' GGAAGGTTCAGGTTTCTTGCTC 3' |
| IL1β   | Forward 5' CTGGCCAGTGAAATGATGCTG 3'  
|        | Reverse 5' GTGGGAGATCTGAGCTGGATGCTG 3' |
| RPL19  | Forward 5' TGGGCTCAGACCCAGGAC 3'  
|        | Reverse 5' GGAAGGTTCAGGTTTCTTGCTC 3' |
| TNFa   | Forward 5' ATGAGGACTGAAAGCATCCGTC 3'  
|        | Reverse 5' GGAAGGTTCAGGTTTCTTGCTC 3' |
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Appendix

$^1$H NMR Spectra

Figure S23. $^1$H NMR spectrum of P1 (pSar$_{225}$-block-pCys(SO$_2$Et)$_{31}$) in DMSO-$d_6$. 
Figure S24. $^1$H NMR spectrum of P2 (pSar$_{200}$-block-pCys(SO$_2$Et)$_{17}$) in DMSO-$d_6$.

Figure S25. $^1$H NMR spectrum of P3 (pSar$_{170}$-block-pCys(SO$_2$Et)$_{27}$) in DMSO-$d_6$.

Figure S26. $^1$H NMR spectrum N-3-azidopropyl liponamide in CDCl$_3$. 