# Supporting Information

## Assembly of chemically modified protein nanocages into 3D materials for the adsorption of uremic toxins

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**Supplementary Tables and Figures**

**General**

All chemicals were obtained from commercial sources and used without further purification. All solutions are prepared using ultrapure water (prepared with a Purelab Flex 2 system, resistivity 18.2 MΩ cm) and analytical grade reagents where possible unless indicated otherwise.

**Mutagenesis**

Introduction of cysteine anchor sites have been performed by multiple cycles of QuickChange™ site-directed mutagenesis using a two-step polymerase chain reaction (PCR) protocol.[32] The used primers for the different mutation sites are shown in Table S1. A mixture of 2.9 µL pET-22b(+) plasmid containing the gene of interest (7 ng $\mu L^{-1}$), 1 µL of 10 mM dNTP mix, 5 µL reaction buffer 10x (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl pH 8.8, 20 mM $MgSO_4$, 1% Triton® X-100, 1 mg $mL^{-1}$ nuclease -free bovine serum albumin (BSA)), 1 µL Pfu DNA polymerase (2.5 U $\mu L^{-1}$) and 38.1 µL ultrapure water. The mixture is split in half and 1 µL of forward or reverse primer (10 pmol $\mu L^{-1}$) is added to each tube. PCR thermocycler (Eppendorf Mastercycler Nexus PCR Cycler) is prepared by initial heating step for 30 s at 95°C. The first step of the PCR protocol is composed of 3 cycles of 30 s denaturation at 95°C, annealing for 1 min at 61°C followed by elongation for 6 min at 68°C. After the first 3 cycles, the separate mixtures with the forward and reverse primers are combined and the PCR is continued for 16 additional cycles with the same parameters as the first 3. Followed by a final elongation phase for 10 min at 68°C to finish the PCR. Digestion of parental plasmid is done by adding 1 µL DpnI (10 U $\mu L^{-1}$) and incubation overnight at 37°C. DpnI is deactivated by 20 min heating at 80°C and the mixture is purified using the NucleoSpin® Gel and PCR clean-up kit according to the manufacturer's instructions. *E. coli* DH5α calcium-competent cells were incubated with 200 ng of the purified plasmid for 30 min on ice, followed by a heat shock for 45 s at 42°C. Next, cells were incubated for 1 h in super optimal broth (SOB) media, centrifuged at 1000 g, resuspended in 100 µL media, plated on an LB agar plate, and incubated for 16 h at 37°C. A single colony was picked and incubated overnight at 37°C and 250 rpm in 5 mL sterile LB medium supplemented with 150 µg $mL^{-1}$ ampicillin. The next day, plasmids were extracted by NucleoSpin® Plasmid miniprep kit according to the manufacturer's instructions.

The sequence was confirmed by mixing 500 ng plasmid with 25 pmol T7 forward or reverse primer in a 10 µL solution and sent in for DNA sequencing (Eurofins Genomics). Plasmids with the desired mutations inside were then chosen as parental plasmids for further mutagenesis till all 5 mutations were present.

Table S1: Sequence of primers used for QuickChange™ PCR protocol.

| Mutation | Forward primer |
|---|---|
| C130A | 5'-C AAG AAC GAT CCG CAT CTG GCC GAT TTC ATC GAA ACC CAC-3' |
| K53C | 5'-T GTT GCA CTG AAG AAC TTT GCG TGT TAC TTT CTG CAT CAG TCC CAT G-3' |
| E64C | 5'- TTT CAT CAG TTT CTC GGC ATG ACA GCG TTC TTC ATG GGA CTG ATG-3' |
| K143C | 5'-AT CCC CGA GTT CTT TGA TCG CAC AGA CCT GTT CGT TCA GAT AGT G -3' |
| S178C | 5‘ - CAC ACT TTG GGT GAT TGC GAT AAT GAA TCG TAA CTC GAG CAC C - 3’ |

**Production and purification of ferritin cysteine variants**

Production of $Ftn^{(neg)}$ and cysteine bearing variants is identical to the production of $Ftn^{(neg)}$ previously published.[33]

First calcium-competent *E. coli* BL21-Gold (DE3) cells were thawed on ice for 10 min. Then 1 µL of 40 ng $\mu L^{-1}$ plasmid solution was added to the cells and the mixture was incubated on ice for 30 min. Heat shock was done by incubating the mixture at 42°C for 45 s followed by 2 min incubation of ice. The cells were suspended in 1 mL SOB media and incubated for 1 h at 37°C. Cells were centrifuged at 1000 g and 1 mL of the mixture is removed. The cell pellet is resuspended in the remaining solution and streaked out on an LB-agar plate suspended with 150 µg $mL^{-1}$ ampicillin and incubated at 37°C overnight.

To prepare precultures colonies of transformed *E. coli* BL21-Gold (DE3) cells (Agilent) were incubated overnight in 5 mL sterile LB-Miller medium supplemented with 150 µg $mL^{-1}$ sodium ampicillin at 37°C and 180 rpm.

Then 400 mL of Terrific broth (TB) medium supplemented with 150 µg $mL^{-1}$ was inoculated with 4 mL of the preculture. The cells were grown at 37°C and 180 rpm till an $OD_{600}$ of 0.6 was reached. Protein overexpression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.25 mM and the cells were incubated for an additional 48 h at 18°C. The cells were harvested via centrifugation at 4000 g. Pellets were stored at -20°C until further use.

Cells from 400 mL culture were resuspended in 20 mL buffer (50 mM Tris, pH 7.5, 0.3 M NaCl). Cell lysis was achieved by sonicating (60% amplitude) for six times 1 min on ice with 1 min break in between with a Vibra-Cell VCX-130 ultrasonic processor (Sonics). The resulting suspension was centrifuged at 14,000 g for 20 min to separate the cell debris from the soluble proteins. Denaturation of most of the *E.coli* proteins was achieved by heating the supernatant to 65°C for 10 min in a water bath. The denatured proteins were separated by centrifugation at 14,000 g for 15 min. Proteins left in the solution were precipitated with ammonium sulfate at a final concentration of 70% of its saturation concentration, followed by centrifugation at 14,000 g for 20 min. After rebuffering the pellet in 10 mL buffer (50 mM Tris, pH 7.5, 0.15 M NaCl) the ammonium sulfated precipitation was repeated. The resulting pellet was dissolved in 50 mL IEC loading buffer (50 mM Tris, pH 7.5, 0.15 M NaCl) and purified by ion-exchange chromatography (IEC) with a linear gradient from 0.15 to 1 M NaCl using a 5 mL HiTrap™ Q HP anion exchange column (Cytiva). All $Ftn^{(neg)}$-4xCys containing fractions were collected and concentrated to a final volume of 2 mL with a Sartorius Vivaspin® Turbo 15 (MWCO 30,000) filter unit. Finally, the sample is purified via gel filtration with a HiLoad 16/600 Superdex™ 200 pg column. All chromatography steps were performed on an Äkta pure system from Cytiva. All $Ftn^{(neg)}$-4xCys containing fractions were collected and stored at 4°C until further use.

**Functionalization with 2-Iodo-N-phenyl acetamide**

5 mg $Ftn^{(neg)}$-3xCys or $Ftn^{(neg)}$-4xCys is incubated for 4 h in disassembly buffer (10 mM phosphate; 50 mM NaCl, pH 2). After 3 ½ h 10 eq (with respect to each cysteine) Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Iris Biotech GmbH) is added to the solution from a 10 mg $mL^{-1}$ stock solution. Subsequently, the solution is filled up to 15 mL with reassembly buffer (50 M Tris, 50 mM NaCl, pH 7.6) and concentrated with a membrane filter (Sartorius Vivaspin Turbo 15; 30 kDa MWCO) to a final volume of 200 µL. Again 10 eq TCEP is added and the solution is filled up to a volume of 2 mL. The pH value is set to 7.6 with 1M NaOH or HCl. Afterwards 2 mL Ethanol containing 20 eq 2-Iodo-N-phenylacetamid (abcr GmbH) is added to the solution and the mixture is stirred at 300 rpm in the dark for 1 h. Next, the solution is filled up with reassembly buffer to a total volume of 30 mL. The protein reassembles overnight. Finally the solution is concentrated to 2 mL and purified via gel filtration on a HiLoad 16/600 Superdex™ 200 pg column. Protein containing fractions are collected and stored at 4°C for further use.

**Functionalization with 2-Bromo-N-decylacetamid**

The functionalization follows closely the protocol for 2-iodo-N-phenylacetamid. But during the functionalization reaction the protein/TCEP solution is not filled up to 2 mL but to 800 µL and then 3.2 mL Ethanol containing 40 eq 2-bromo-N-decylacetamid (Sigma-Aldrich) is added to the solution. All other steps are performed equal to the protocol of 2-iodo-N-phenylacetamide functionalization.

**Hanging-drop crystallization**

Crystallization of small amounts of protein or functionalized protein variants were performed via hanging drop vapor diffusion techniques. Reservoir solution (100 mM Tris, 500 mM MgOAc, pH 8.5) was prepared in a 24- well manual plate set. Drops were prepared on siliconized glass cover slides (Jena Bioscience) by mixing 2 µL reservoir solutions with 1 µL 50 mM Tris, 1 M NaCl, pH 7.5 buffer and 1 µL of respective ferritin variant. Plates were incubated at 25°C. After one day first crystals were visible.

**Batch crystallization**

For crystallization of larger amounts of $Ftn^{(neg)}$ and functionalized variants of $Ftn^{(neg)}$, a batch crystallization approach was employed based on a protocol from I. Rayment.[34] For a standard experiment, 250 µL of a 50 mM Tris 1 M NaCl pH 7.5 buffer was carefully mixed with equal amounts of $Ftn^{(neg)}$ stock solution with a concentration of 12 mg $mL^{-1}$ in a 50 mM Tris 0.3 M NaCl pH 7.5 buffer. Afterward 500 µL of the precipitant solution (133 mM Tris, 333 mM MgOAc, pH 8.5) is added dropwise under constant shaking. The mixture is kept at rest for 7 days at an ambient temperature of 20°C before crystals are fixated.

**Crystal fixation**

Increasing the stability of the crystals is needed for the adsorption experiments. For that purpose, they were fixated with a Sulfo-SMCC (sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, Sigma-Aldrich) crosslinker. The crystals are centrifuged down at 1000 g for 2 min. In a standard experiment with 3 mg crystals. The crystallization solution is removed till 246 µL are left and 64 µL of a freshly prepared 4.8 mg $mL^{-1}$ aqueous Sulfo-SMCC solution is added resulting in an end concentration of 1 mg $mL^{-1}$.The mixture is kept at rest for 4 h at room temperature. And subsequently filled up to 1 mL with ultrapure water, the crystals are spun down via centrifugation at 1500 g for 2 min. The supernatant is removed and the crystals are resuspended in ultrapure water afterwards. The procedure is repeated up to three times to wash the crystals from the residual cross-linking

agent. The material is stored at an ambient temperature of 20°C until further use. Crystals were photographed under a Leica S9D microscope with a FlexaCamC1.

For fixation with glutaraldehyde (Merck) to 1 mL of crystal solution containing 3 mg crystals, 50 µL of a 2.5% aqueous glutaraldehyde solution is added, resulting in an end concertation of 0,00119%. Crystals are incubated for 4 h and subsequently, three times washed with ultrapure water. The crystals are stored at 20°C till further use.

After the glutaraldehyde crosslinking, the crystals still dissolved in a 60 mg $mL^{-1}$. The stability could be increased by an additional fixation step. The procedure is repeated, but the crystals are only incubated for 10 min before washing. However, toxins adsorption assays revealed a significantly reduced adsorption capacity. We hypothesize that polymerization of the glutaraldehyde leads to blocking of the pores.

**Structure Determination and refinement**

Crystals, soaked for 30 s in cryo buffer containing 25% glycerol and the respective reservoir solution, were vitrified in liquid nitrogen. Diffraction data were collected at 100 K on P11 at the Deutsches Elektronen-Synchrotron DESY (support by the beamline staff at P11 is gratefully acknowledged). For structure determination, the CCP4 suite was used.[35] Data processing and scaling were done with XDS[36] and the structures were solved via molecular replacement with Phaser.[37] As input structure, an adapted version of the PDB ID 5JKK, with $Ftn^{(neg)}$-Cys mutations manually added with COOT,[38] was used. At some cysteine sites, additional electron density was observed. At this positions, a shortened version of the aliphatic ligand with only 3 carbon atoms of the aliphatic chain was introduced and covalently linked to the model using AceDRG.[39] The model was improved by iterative rounds of refinement and manual rebuilding using Refmac.[40] Metal ions and water molecules were placed based on the electron density. $Fe^{3+}$ ions were placed at the ferroxidase site. $Mg^{2+}$ ions coordinated by six water were placed in the threefold channel. The model were validated with the Molprobity server reaching a score of 0.9.[41] Crystallographic details are summarized in Table S8.

**ESI-MS measurements**

The protein sample were rebuffered to ultrapure water with an Amicon® Ultra 0.5 mL (MWCO 30,000 Da) centrifugal filter. The protein sample is filled up with ultrapure water to a volume of 500 µL, concentrated to roughly 20 µL and refilled to 500 µL. The rebuffering is repeated 5 times and the concentration is set between 0.15 and 0.2 mg $mL^{-1}$. The mass of the proteins is determined with electron-spray ionization time of flight mass spectrometry (Agilent 6224 ESI-TOF-MS). Measurement is done in positive mode with a mass range of m/z 110 – 3200, with a

rate of 1.03 spectra/s. The source temperature was set to 325 °C, the drying gas flow to 10 L min1, the nebulizer pressure to 15 psig and the capillary voltage to 4000 V. Data interpretation was performed using the software MestreNova.

**Bradford assay**

Bradford assay was used to determine concentration of proteins loaded with cargo molecules. For calibration $Ftn^{(neg)}$-4xCys solutions at 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 mg $mL^{-1}$ were prepared. Bradford solution (AppliChem) is filtered through a syringe filter (0.22 µm) and 750 µL solution is mixed with 15 µL sample or calibration solution. After 5 min incubation time absorbance at 595 nm is determined with an UV/Vis spectroscope (Agilent Cary 60 UV-Vis). Linear regression is done for the calibration row and the concentration of the unknown sample is calculated.

**TEM samples**

Uranylacetat stained protein samples were investigated by transmission electron microscopy. For negative staining copper grids, 200 mesh, covered with Formvar and carbon (Ted Pella, 01810), were incubated face down on a droplet of 10 µL protein solutions (concentration 0.3 mg $mL^{-1}$). Hereafter, the grid is washed 3 x in ultrapure water, followed by 1 wash and 1 incubation (45 s) step on 2% uranyl acetate drops. The remaining solution is blotted and the grids dried. All analysis were carried out with a Philips CM 300 UT at 100kV.

**DLS measurement**

Protein size in aqueous solution were determined with dynamic light scattering (DLS) techniques. Protein samples (4 mg $mL^{-1}$) in 50 mM Tris, 0.3 M NaCl, pH 7.5 were centrifuged at 20,000 g for 20 min. Subsequently the samples are measured on a Zetasizer Nano S from Malvern Instrument at a backscattering angle of 173°. All samples were measured at 20°C in disposable cuvettes (PMMA, semi-micro). The data were analyzed with Malvern Zetasizer Software v. 8.00.4813

**Toxin assay**

For determination of the uremic toxin adsorption capacity of the ferritin variants adsorption experiments were conducted. All solutions and samples were handled in glassware (Macherey-NAGEL Vials N9) since in initial experiments adsorption of the toxins on the polypropylene walls of the reaction tubes was observed.

At first, a stock solution of the desired toxin with a concentration of 50 µg $mL^{-1}$ for *p*CS and IS and 500 µg $mL^{-1}$ for PheAc is prepared in a buffered solution (50 mM Tris, 0.136 M NaCl pH 7.4). From the initial stock solutions, a calibration row is prepared at concentrations of 0.01,

0.05, 0.1, 0.2, 0.5, 0.7, and 1 μg $mL^{-1}$ for later determination of the absolute toxin concentrations in the samples.

The stock solutions were diluted further to reach final uremic toxin concentrations expected in a stage five CKD patient (41 mg $L^{-1}$ for *p*CS,[29] 44 mg $L^{-1}$ for IS,[11] and 474 mg $L^{-1}$ for PheAc[28]). The adsorbent is centrifuged down at 1500 g for 2 min and the complete supernatant is removed from the sample. 150 μL of the respective toxin solution is added and the crystals are incubated for 3 h at room temperature. Additionally, 150 μL of the toxin solution is incubated as a control. Three aliquots of 10 μL were removed from all samples and diluted 100 times in ultrapure water. Finally, crystals were washed with water, dried under vacuum, and weighed.

The uremic toxin concentration was quantified an an ultrahigh-performance liquid chromatography system (1290 infinity II UHPLC, Agilent) with a reversed phase C18 column (Zorbax Extend-C18, 2.1x50 mm, 1.8 μm particle size, Agilent) coupled to an electron-spray-ionization quadrupole-linear ion trap-mass spectrometer (QTRAP 5500, Sciex). All compounds were measured in positive mode with an Ionspray Voltage of -4500 V, Temperature 500°C, Ion Source Gas 1 and 2 50 psi, Curtain Gas 20 psi. MRM parameter for all compounds are listed in Table S2.

As solvent a mixture of HPLC grade water (LiChrosolv® Merck) and acetonitrile (LiChrosolv® Merck) both with addition of 0.1% formic acid (Honeywell Fluka) is used. Specific compositions at each step during the 15 min long chromatography program are summarized in Table S3. Before each sample incubated with the crystals, the control is measured. Chromatograms are evaluated with the Analyst® Instrument control and Data processing Software. Peaks are integrated and the toxin concentration is determined from the calibration row, which is remeasured every 20 samples. From the concentration difference between the control and the samples the amount of adsorbed uremic toxin is determined. The adsorption capacity is then determined by dividing the mass of the adsorbed toxins by the mass of the crystals. One challenge was the unspecific adsorption of the IS towards the vials of the reaction tubes made from polymers, which could be overcome by shifting to glass vials for the incubation and storage of the samples.

**Table S2.** MRM parameters

| | Q1 mass [Da] | Q3 mass [Da] | Collision energy [V] |
|---|---|---|---|
| **Indoxyl sulfate** | | | |
| Quantifier | 211.884 | 80.000 | -38.000 |
| Qualifier | 21.884 | 80.900 | -24.000 |
| **p-Cresyl sulfate** | | | |
| Quantifier | 186.852 | 106.900 | -32.000 |
| Qualifier | 186.852 | 79.900 | -24.000 |
| **Phenylacetic acid** | | | |
| Quantifier | 134.927 | 90.900 | -12.000 |
| Qualifier | 134.927 | 64.900 | -66.000 |

**Table S3.** Sequence of HPLC program

| Time [min] | $H_2O$ [%] | Acetonitrile [%] | Flow rate [mL $min^{-1}$] |
|---|---|---|---|
| 0.00 | 97 | 3 | 0.3 |
| 8.00 | 70 | 30 | 0.3 |
| 9.00 | 40 | 60 | 0.3 |
| 10.50 | 97 | 3 | 0.3 |

**Quantitative polymerase chain reaction (qPCR) analysis of mRNA expression in human aortic endothelial cells**

Human aortic endothelial cells (hAoECs) (Promocell) were cultivated in a Endothelial Cell Growth Medium MV (Promocell). Cells were seeded in 24 well plates (15 x $10^4$ cells/well) at

80% confluence and were incubated for 6 h with 100 ng $mL^{-1}$ lipopolysaccharides (LPS) or functionalized or unfunctionalized protein crystals. After incubation time the total RNA was extracted using RNAeasy mini kit (Qiagen). Reverse transcription was performed using 1 µg total RNA (600 ng), random hexamers, and Verso reverse transcriptase (Thermo Scientific) as per the manufacturer's instructions. For real-time PCR, gene expression levels were quantified using SYBR Green I dye chemistry on a LightCycler 480 system (Roche Applied Sciences). The following primers were used for relative quantification of targeted gene expression - for human TNF-alpha: forward primer 5′ -GCC CAG GCA GTC AGA TCA TCT-3′ , reverse primer 5′ -TTG AGG GTT TGC TAC AAC ATG G-3′ and for human beta-actin: forward primer 5′ -CAA CCG CGA GAA GAT GAC-3′ , reverse primer 5′ -GTC CAT CAC GAT GCC AGT-3′ . Data were represented as the mean level of gene expression relative to the expression of the reference gene (β-Actin).

**Platelet activation assay**

The platelets of three donors were isolated by centrifugation of citrate anticoagulated whole blood at 260 g for 15 min. Written consent was obtained from all participants (approval from ethics board with application number EK153-18). After a second centrifugation step, platelets were resuspended in Hepes buffer pH 6.6 (10 mmol $L^{-1}$ Hepes, 136 mmol $L^{-1}$ NaCl, 2.7 mmol $L^{-1}$ KCl, 2 mmol $L^{-1}$ MgCl2 and 5 mmol $L^{-1}$ glucose). Platelet suspensions were re-centrifuged in the presence of 1:15 acid citrate dextrose (ACD) and 0.1 U $mL^{-1}$ apyrase and subsequently resuspended into Hepes buffer pH 7.45 (10 mmol $L^{-1}$ Hepes, 136 mmol $L^{-1}$ NaCl, 2.7 mmol $L^{-1}$ KCl, 2 mmol $L^{-1}$ $MgCl_2$, 5 mmol $L^{-1}$ glucose). 15 x $10^6$ platelets were incubated for 15 min with 4 nmol $L^{-1}$ Thrombin in presence of 2 mmol $L^{-1}$ $CaCl_2$ or different protein crystals. The platelets were lysed with 4% SDS lysis buffer (200 mmol $L^{-1}$ Tris, 600 mmol $L^{-1}$ NaCl, 4% SDS) including EDTA-free Halt Protease Inhibitor Cocktail (1:10; Sigma-Aldrich) and Halt Phosphatase Inhibitor Cocktail (1:10; Sigma-Aldrich). Protein amount was quantified following the protocol for DC protein assay (Bio-Rad). An equal amount of protein from each sample was resolved by 10% SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature. Anti-phospho-Akt antibody (1:1000; Cell signaling Technology) and anti-tubulin (1:1000; Cell signaling Technology) were used as primary antibodies. The blots were incubated overnight at 4°C. A second anti-rabbit antibody (1:1000; Cell signaling Technology) was used and incubated for 1 h at room temperature. Immunoreactive bands were visualized via enhanced

chemiluminescence, and densitometry was performed using ´Quantity One software´ (Bio-Rad Laboratories).

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**Table S4: Sequence of Ftn$^{(neg)}$-3xCys and Ftn$^{(neg)}$4xCys.** Amino acid sequence of Ftn$^{(neg)}$ and respective cysteine containing variants.

**Ftn$^{(neg)}$ [33]**
TTASTSQVRQNYHQDSE**E**AINRQINLELYASYVYLSMSYYFDRDDVALKNFAKYFLH
QSHEEREHAEKLMKLQNQRGGRIFLQDIQKPD**E**DDWESGLNAME**E**AL**E**LEKNVNQS
LLELHKLATDKNDPHLCDFIETHYLNEQVKAIKELGDHVTNLRKMGAPESGLAEYLF
DKHTLGDSDNES

**Ftn$^{(neg)}$-3xCys**: K53C, E64C, C130A, K143C
TTASTSQVRQNYHQDSEEAINRQINLELYASYVYLSMSYYFDRDDVALKNFA**C**YFLH
QSHEER**C**HAEKLMKLQNQRGGRIFLQDIQKPDEDDWESGLNAMEEALELEKNVNQS
LLELHKLATDKNDPHLADFIETHYLNEQV**C**AIKELGDHVTNLRKMGAPESGLAEYLF
DKHTLGDSDNES

**Ftn$^{(neg)}$-4xCys**: K53C, E64C, C130A, K143C, S178C
TTASTSQVRQNYHQDSEEAINRQINLELYASYVYLSMSYYFDRDDVALKNFA**C**YFLH
QSHEER**C**HAEKLMKLQNQRGGRIFLQDIQKPDEDDWESGLNAMEEALELEKNVNQS
LLELHKLATDKNDPHLADFIETHYLNEQV**C**AIKELGDHVTNLRKMGAPESGLAEYLF
DKHTLGD**C**DNES

**Table S5. Solvent-accessible surface area**. The ratio of the sidechain to the average solvent-accessible surface area of X in the tripeptide Gly-X-Gly in an ensemble of 30 random conformations for the selected positions. Calculations were done with the webservice GETAREA*[42]* The calculations are based on the crystal structure (PDB ID: 5JKK). The position S178 is near the end of the ferritin subunit and because of the lacking electron density not modeled, therefore the accessible surface area could not be calculated on this position.

| Amino acid | Ratio |
|---|---|
| K53 | 78.1 |
| E64 | 65.7 |
| K143 | 53.2 |
| S178 | N.A |

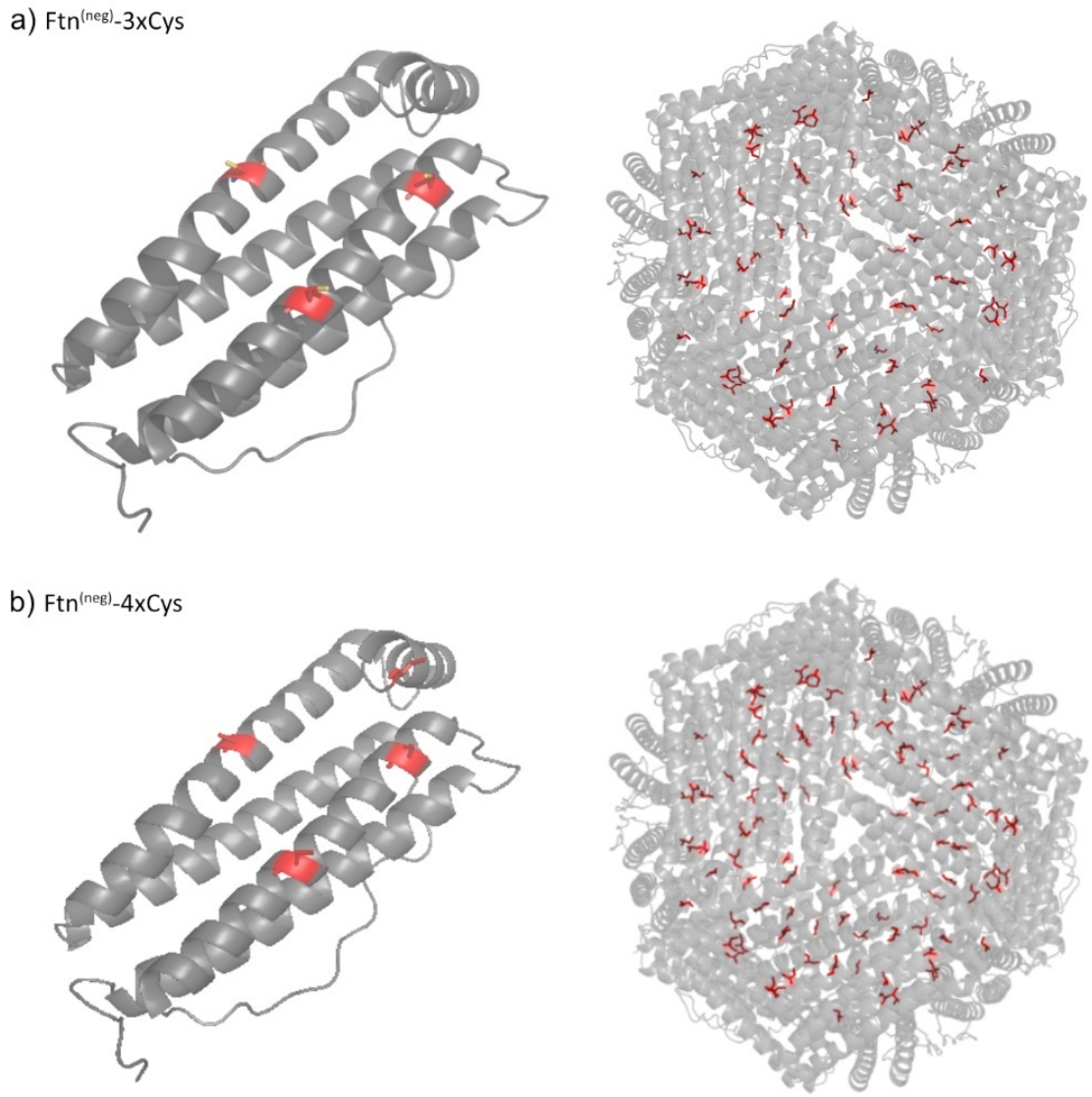


**Figure S1: Cysteine anchor sites in the Ferritin cage.** Ferritin subunit (left) and full cage (right) for a) Ftn(neg)-3xCys und b) Ftn(neg)-4xCys. The mutated positions are highlighted in red (Position 178 near the end of the E-helix is not present in the protein structure due to missing electron density, instead the last modeled amino acid at position 176 is highlighted).

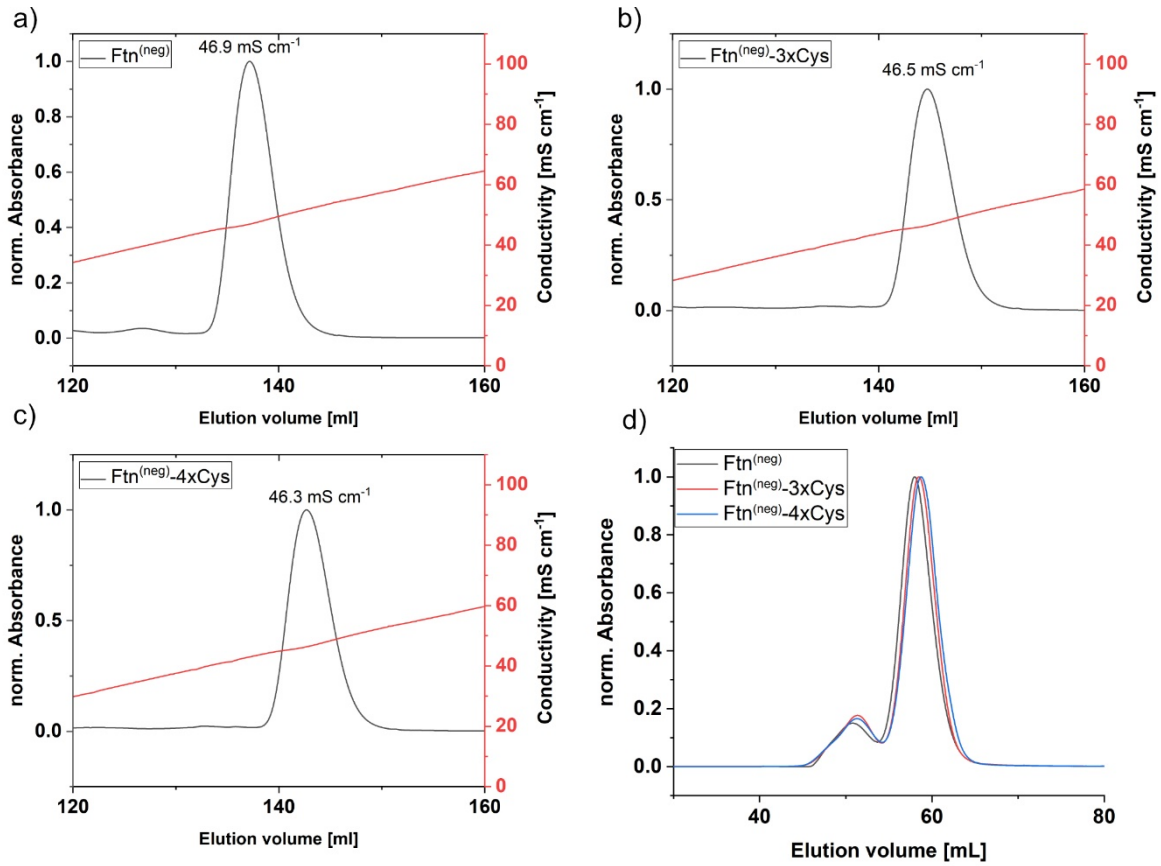


**Figure S2: Purification of Ftn$^{(neg)}$-3xCys and Ftn$^{(neg)}$-4xCys**: a) b) c) Ion-exchange chromatogram for the respective ferritin variant. All variants elute at comparable conductivity indicating that introduced mutations had no influence on the outer surface charge. d) Size-exclusion chromatogram of the respective variants. All proteins elute at the same elution volume indicating a fully assembled protein cage.

**Table S6: Comparison of molecular weight.** For variants without chemical modification, the expected mass was calculated based on the amino acid composition using the Expasy ProtParam tool.[43] Molecular weight of ferritin cysteine variants with chemical modifications is calculated by adding three times the molecular masses of the functionalization agents without the halogen atom to the molecular mass of the untreated proteins.

| Protein | $MW_{Theo.}$ [kDa ] | $MW_{Exp}$ [a] [kDa] |
|---|---|---|
| Ftn$^{(neg)}$ | 21.196 | 21.196 |
| Ftn$^{(neg)}$-3xCys | 21.088 | 21.088 |
| Ftn$^{(neg)}$-3xPhe | 21.487 | 21.487 |
| Ftn$^{(neg)}$-3xC10 | 21.680 | 21.680 |

[a] from ESI-MS measurements

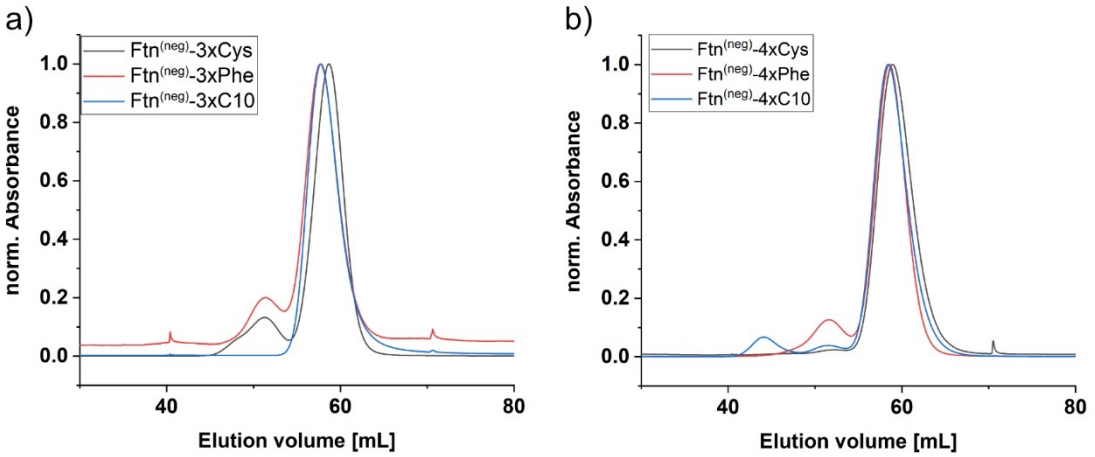


**Figure S3: Size-exclusion chromatography (SEC) of functionalized ferritin variants.** SEC of Ftn$^{(neg)}$-3xCys (a) and Ftn$^{(neg)}$-4xCys (b). In both cases only small derivations between the functionalized und unfunctionalized cages are observable. Therefore, the functionalization seems not to interfere with the assembly of the cage**.**

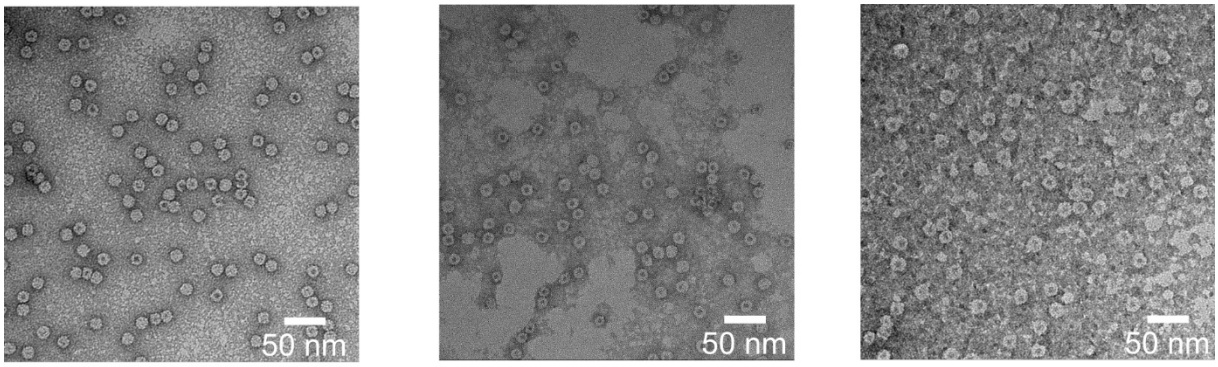


**Figure S4: TEM-Images of Ftn$^{(neg)}$-4xCys and functionalized variants.** In all cases characteristic cage geometries are present: The cages were reassembled after the functionalization reaction.

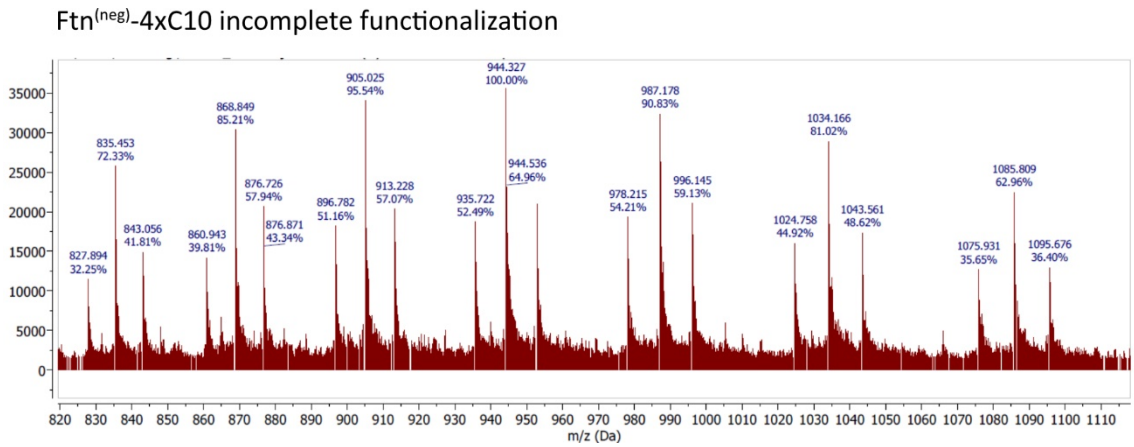

**Figure S5: ESI-MS spectrum for an unoptimized functionalization protocol.** Instead of a single signal for an ion with charge z, three signals can be observed. The respective calculated molecular masses fit to ferritin subunits with two to four aliphatic functionalization agents. Due to the unoptimized parameters, the reaction yields a population of ferritin subunits with different degrees of functionalization.

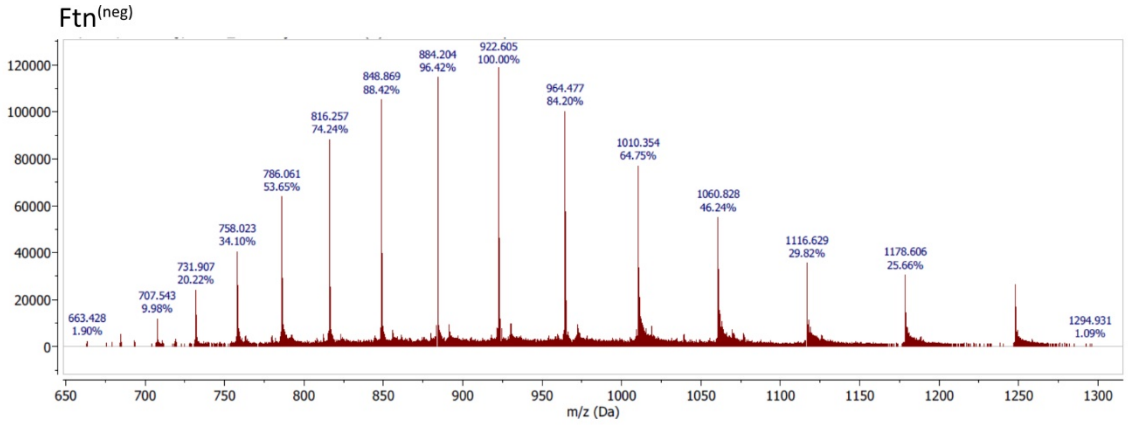


**Figure S6: ESI-MS spectrum of untreated Ftn$^{(neg)}$.** The mass is calculated using equation 1 and 2 to a value of 21.196 kDa. Comparison between the expected und measured mass is given in Table S7.

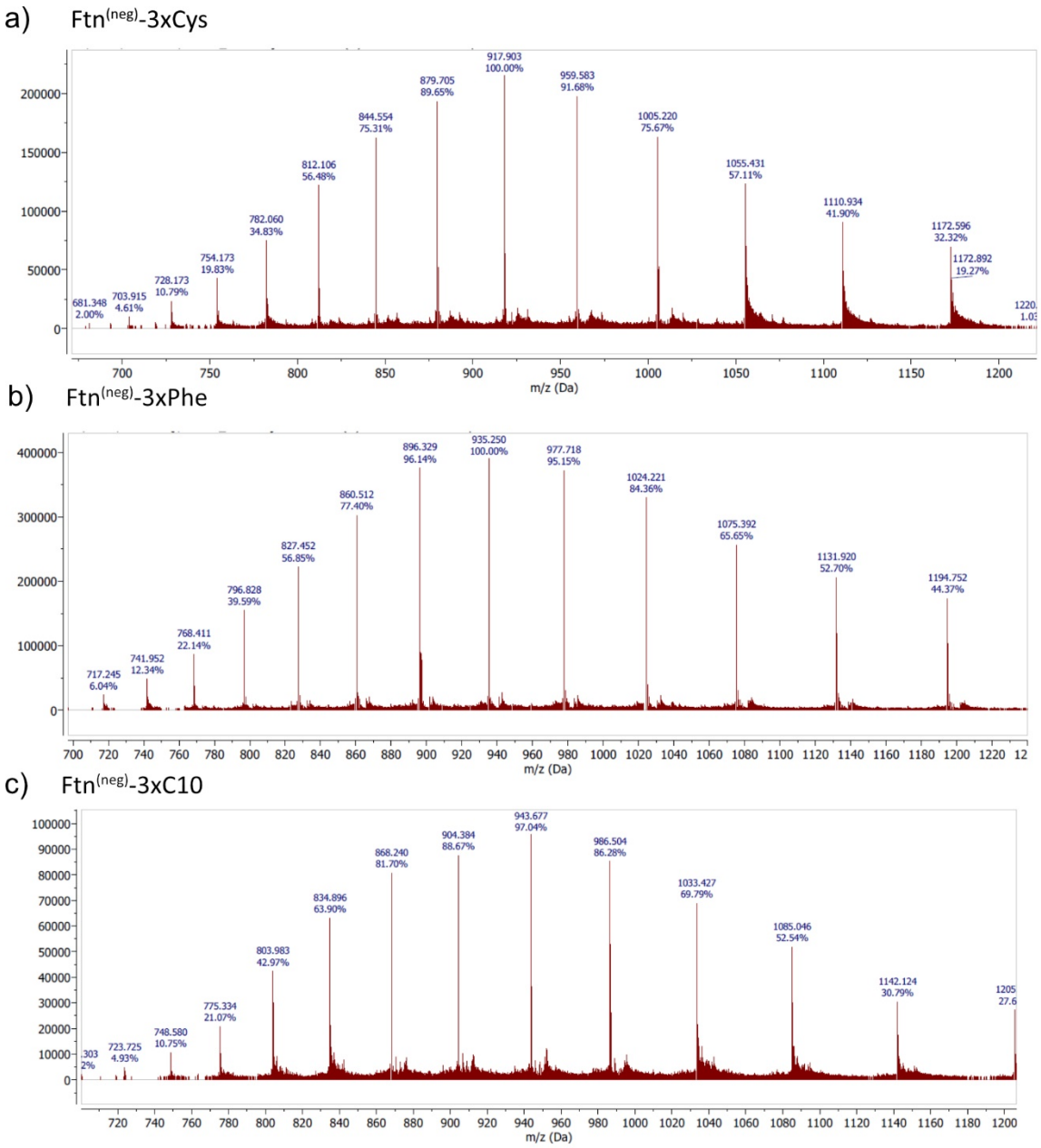


**Figure S7: ESI-MS spectrum of Ftn$^{(neg)}$-3xCys variants**. The mass is calculated using equation 1 and 2. Comparison between the expected und measured mass is given in Table S7.

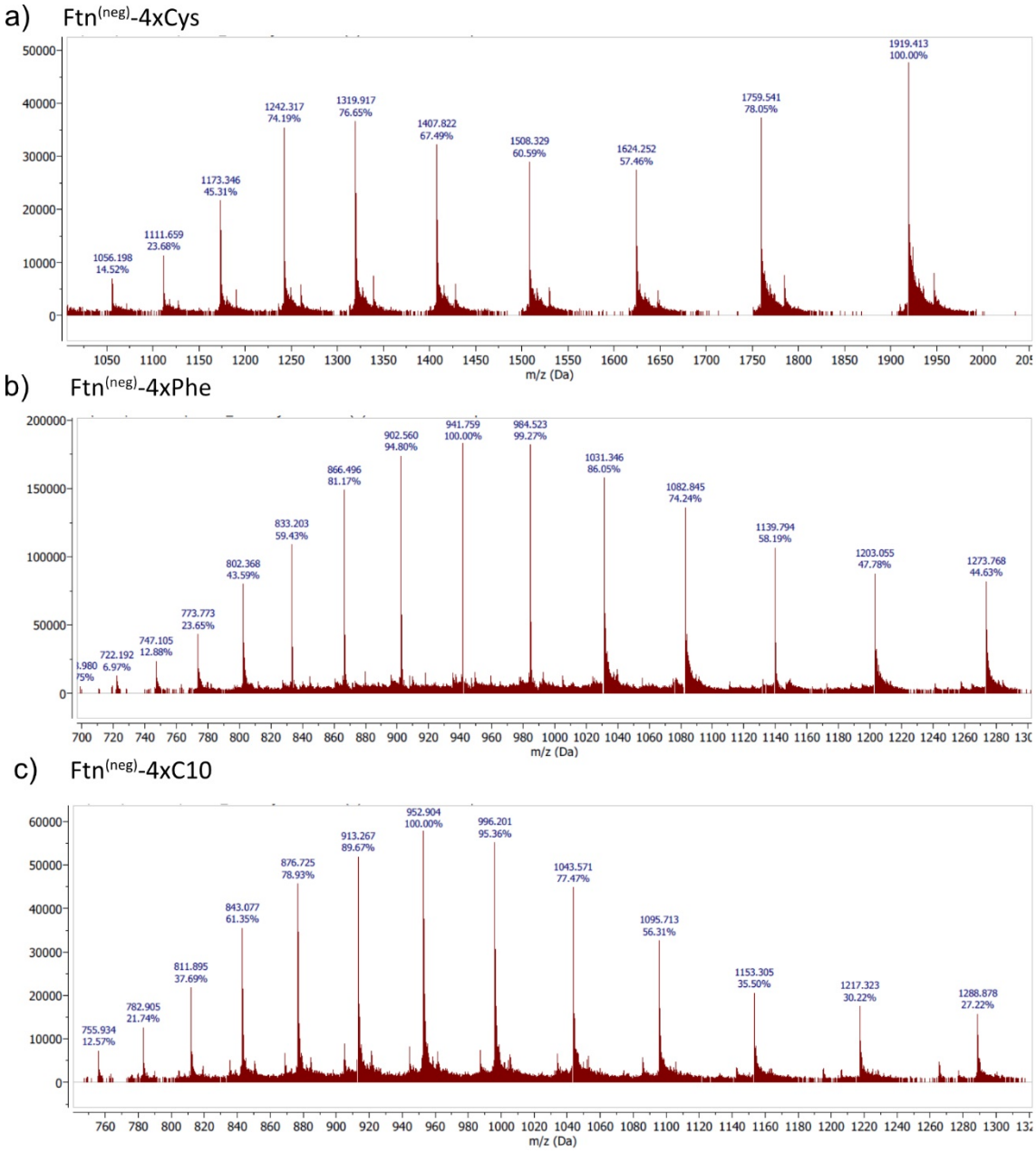


**Figure S8: ESI-MS spectrum of Ftn$^{(neg)}$-4xCys variants**. Comparison between the expected und measured mass is given in Table 1

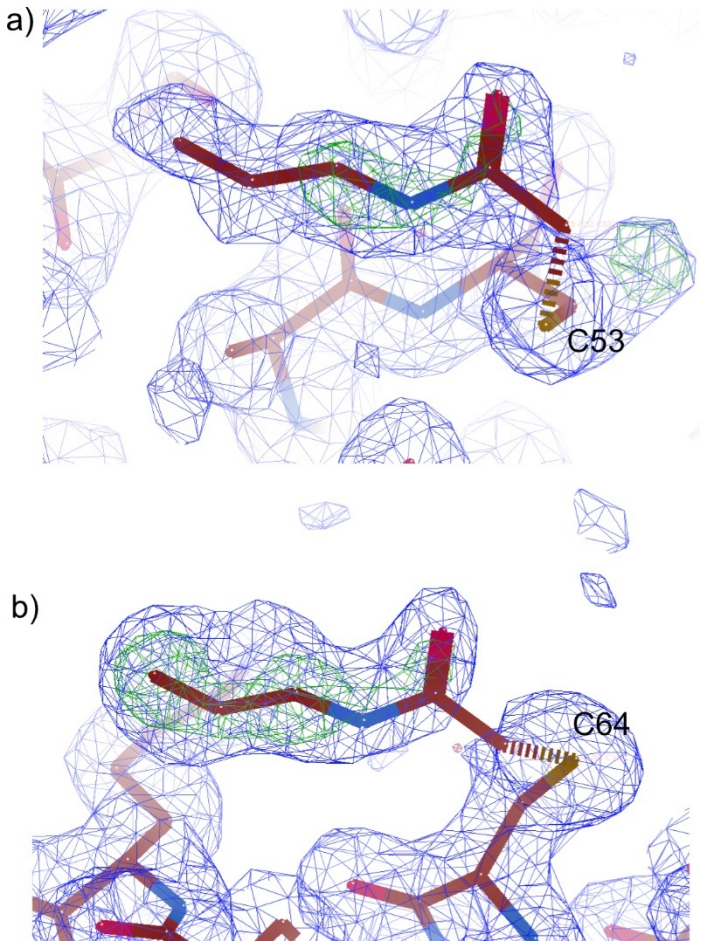


**Figure S9: Electron density omit maps for introduced material.** Electron density ($2F_O$-$F_C$ omit map, blue) and difference electron density ($F_O$-$F_C$, green) map for two functionalized cysteine residues with the aliphatic molecule. The electron density was only clearly visible at position 53 and 64. $2F_O$-$F_C$ map (blue): 1 rmsd, $F_O$-$F_C$ (green): 5 rmsd. Maps were calculated without ligand atoms**.**

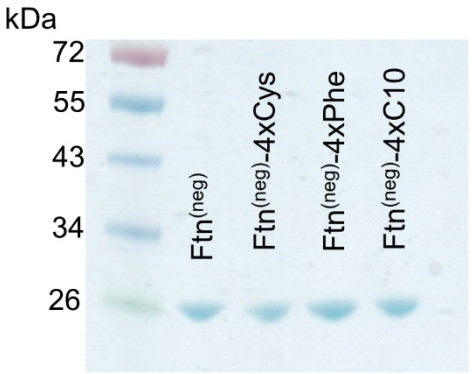


**Figure S10: SDS-PAGE of Ftn$^{(neg)}$-4xCys and functionalized variants.** The mass is in good agreement with the expected mass for the ferritin subunits. No difference in the masses of the different variants is observable, indicating that the mass difference is not large enough to be resolved with SDS-PAGE.

**Table S8:** Summary of crystallographic data. Data statistics and refinement details for the crystal structures obtained from crystals of Ftn$^{(neg)}$ functionalized with three C10 ligands.

| | $Ftn^{(neg)}$-3xC10 |
|---|---|
| **Data collection** | |
| Wavelength (Å) | 1.00 |
| Space group | *P*23 |
| Unit cell dimensions [a, (Å)] | 181.44 |
| Resolution range (Å) | 44.01 – 2.0 |
| Highest resolution shell (Å) | 2.072 – 2.0 |
| No. of observed reflections | 2 767 050 (272 472) |
| No. of unique reflections[a] | 133 407 (13 123) |
| Multiplicity | 20.7 (20.8) |
| Completeness (%) | 99.75 (98.91) |
| <I/σI> | 46.05 (17.04) |
| $R_{merge}$ (%) | 5.56 (17.1) |
| $R_{meas}$ (%) | 5.70 (17.5) |
| Wilson B-factor | 15.94 |
| **Refinement** | |
| $R_{work}$ (%) | 14.4 |
| $R_{free}$ (%) | 17.7 |
| No. atoms | 12512 |
| macromolecules | 11301 |
| ions/glycerol | 135 |
| water | 1076 |
| B-factor ($Å^2$) | 17.76 |
| macromolecules | 16.96 |
| ions/glycerol | 34.63 |
| water | 24.05 |
| R.m.s deviations | |
| bong lengths (Å) | 0.016 |
| bond angles (deg) | 1.84 |
| Ramachandran statistics (%) | |
| favoured | 98.38 |
| outliers | 0 |
| Molprobity score | 0.9 |
| PDB ID | 8AAV |

**[a] Value in parentheses indicates number of reflections used for $R_{free}$ calculation.**

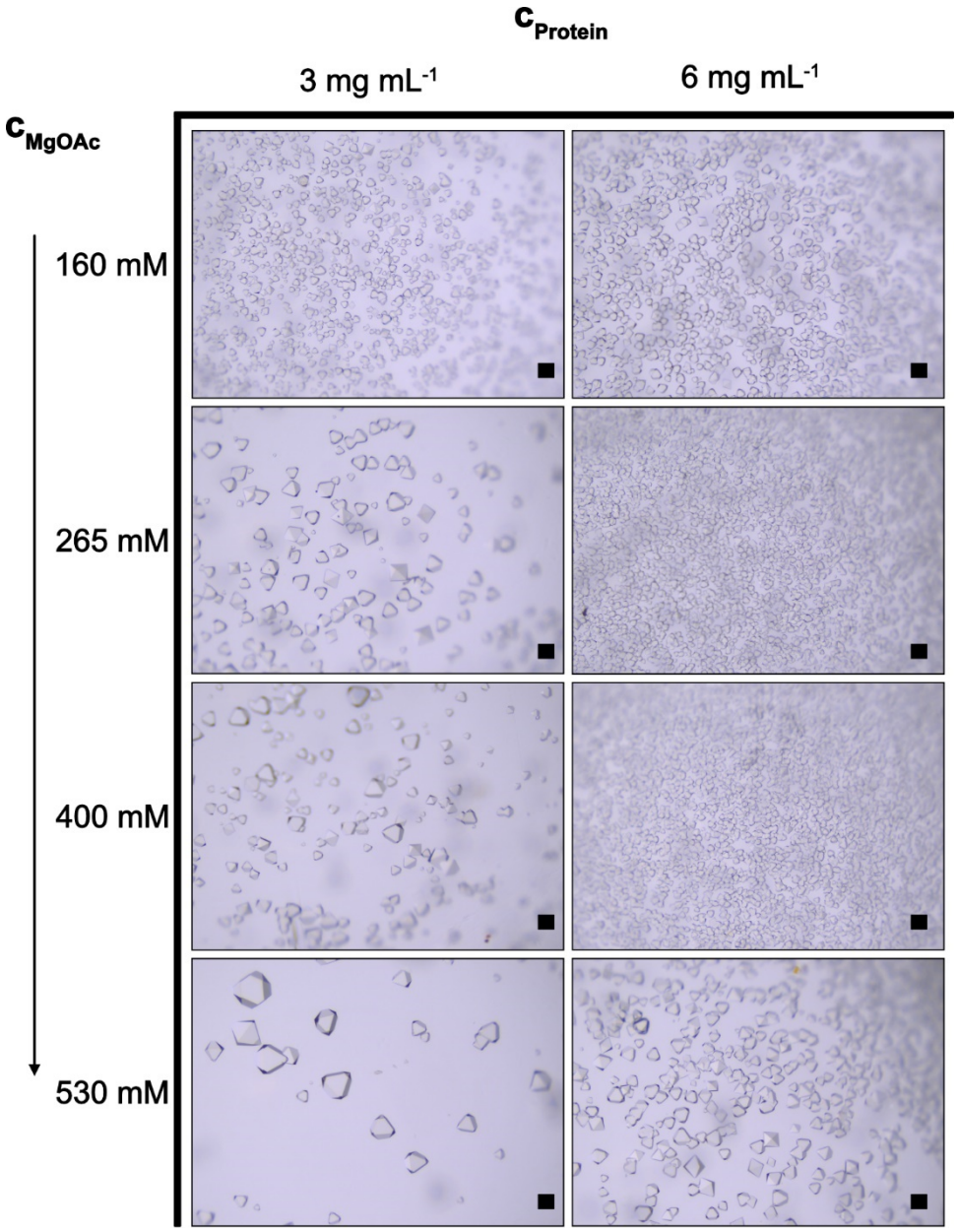


**Figure S11: Crystal size against precipitant and protein concentration.** Crystal size for Ftn$^{(neg)}$-4xPhe in dependency of precipitate and protein concentration. The size of the crystals can be increased by increasing the concentration of the precipitant MgOAc. The crystal size can be decreased by increasing the protein concentration. (Scale bar is equal to 200 µm**).**

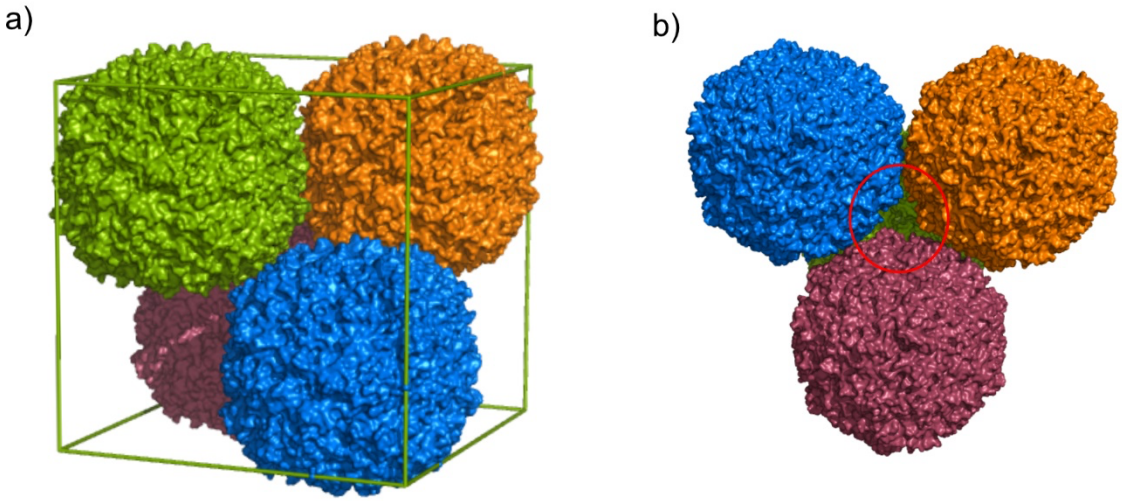


**Figure S12: Crystal structure of Ftn$^{(neg)}$**. a) Unit cell of Ftn$^{(neg)}$ crystals used as adsorbent (unit cell parameters: a = 188.44 Å) b) solvent channel (red circle) between three Ftn$^{(neg)}$ cages with a diameter of around 3 nm.

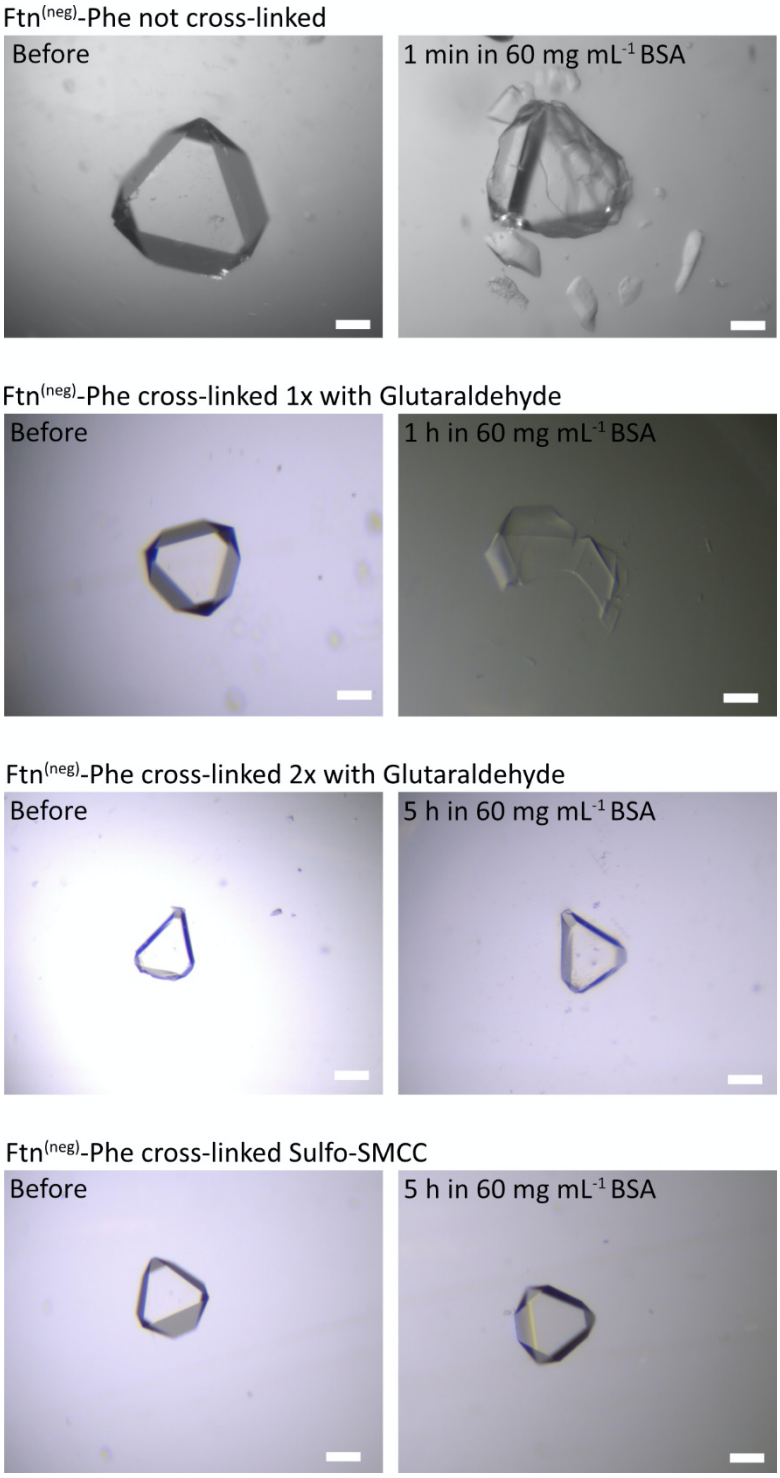


**Figure S13: Stability of Ftn$^{(neg)}$ crystals after different cross-linking procedures.** Crystals incubated in 60 mg mL$^{-1}$ BSA solution. Crystals without chemical fixation break and dissolve in a matter of minutes. A first crosslinking with glutaraldehyde increased the stability, but after one hour the crystal is already heavily damaged. Repeating the cross-linking procedure produces crystals that can withstand the protein solution for multiple hours, but the adsorption capacity is heavily diminished after the second cross-linking step. Crosslinking with the sulfo-SMCC cross-linker yields a crystal stable in the protein solution. Even after several days the crystal shows no damage**.**

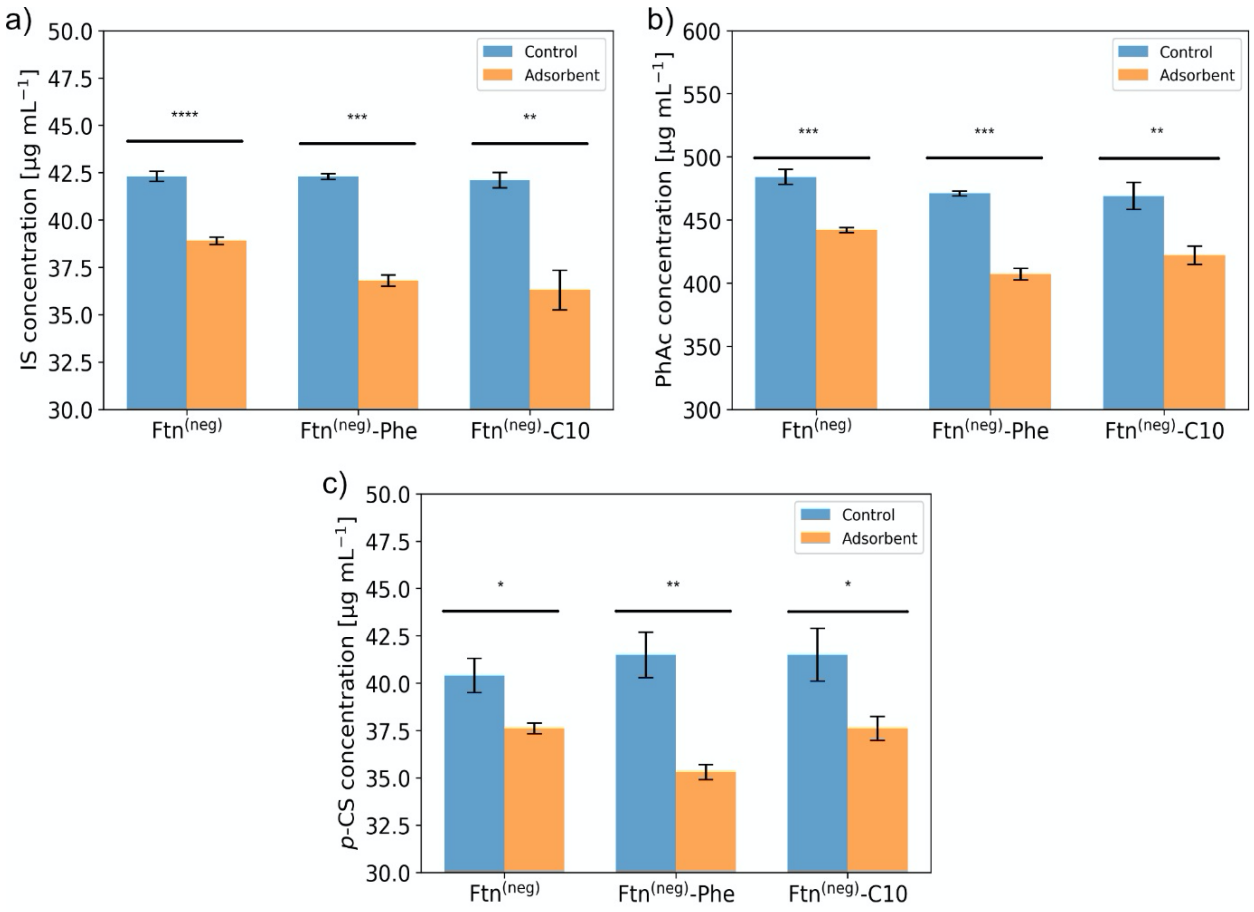


**Figure S14**. **PBUT concentration in adsorption assay.** PBUT concentration of a control sample and a sample treated with the respective adsorbent for a) indoxylsulfate, b) phenylacetic acid and c) *p*-cresyl sulfate. Data are shown as mean ± SEM for three measurements. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$ compared with the control based on two sample t-test.

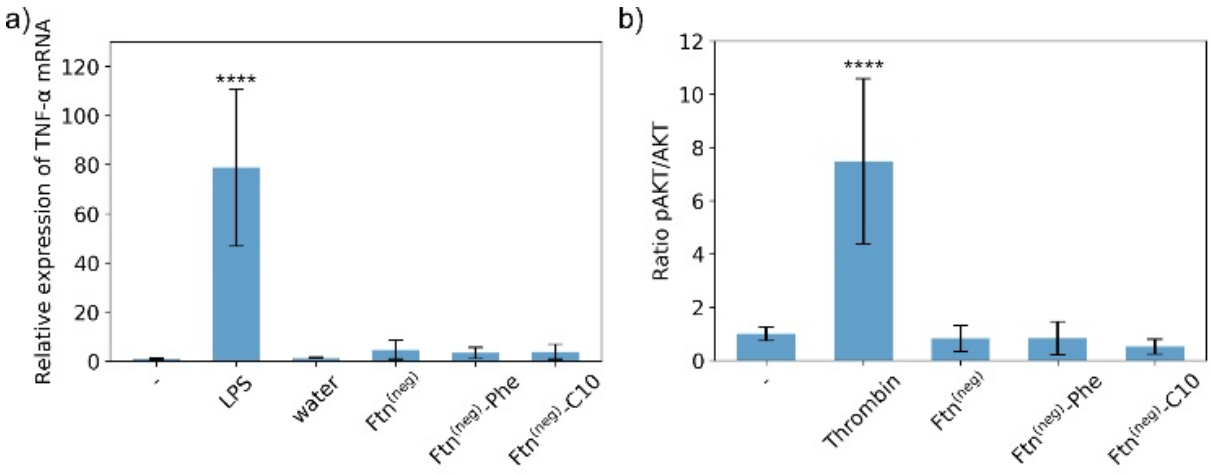


**Figure S15**. **Biocompatibility assays for sulfo-SMCC cross-linked crystals** a) Expression of TNFα mRNA of HAoEC cells incubated with crystalline protein material as a measure for endotoxin contaminations b) Ratio of pAKT to AKT in blood platelets incubated with the crystalline protein-based material as a measure for platelet activation.

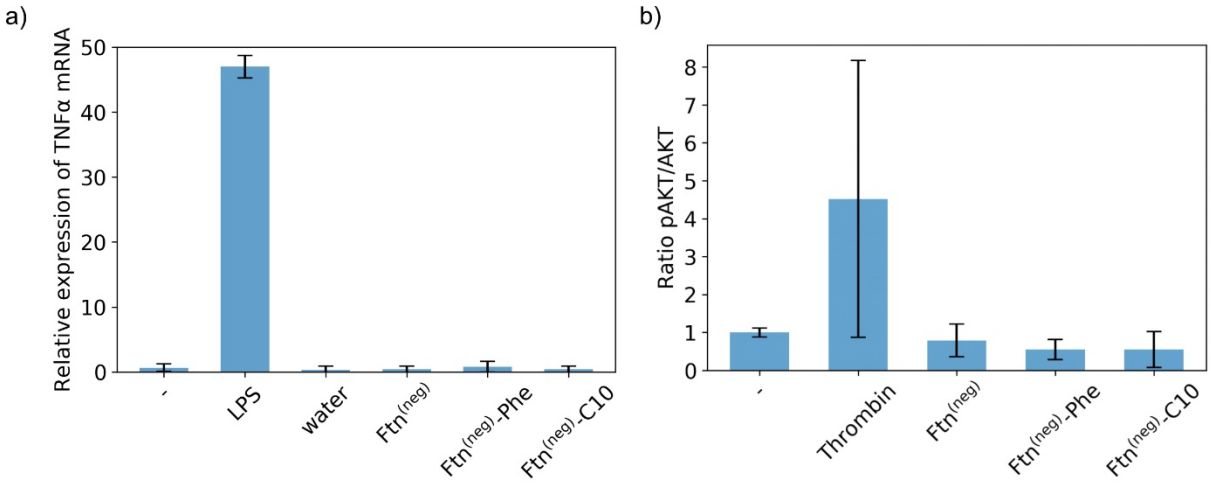


**Figure S16: Biocompatibility assays for glutaraldehyde cross-linked crystals.** a) Relative expression of TNFα mRNA for HAoEC (Endothelial cells) incubated for 6 h with the crosslinked crystal material or positive and negative control. No signs for endotoxin contamination in all samples. b) Ratio of AKT to phosphorylated AKT as measured from isolated platelets incubated for 15 min with the crystal material. No evidence for activation of platelets from the crystalline adsorber material.