Tuneable peptide cross-linked nanogels for enzymetriggered protein delivery

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Supporting information

Materials and methods

Materials

Acryloyl chloride, 4,4'-azobis(4-cyanovaleric acid) (VA501), bovine serum albumin (BSA, \geq 96%), Brij[®]35, Copper (II) sulfate pentahydrate (CuSO₄·H₂O, 99.999 % trace metal basis), Cy5azide (95 %), cyclopropyl amine, diethyl ether, 1,4 dioxane, anhydrous dichloromethane, EDTA (anhydrous, crystalline, BioReagent), Fmoc-protected amino acids, HEPES (\geq 99.5%), hydrochloric acid (ACS reagent, 37 %), imidazole (≥ 99 %), L-ascorbic acid 2-phosphate sesquimagnesiumsalt hydrate (ASAP, ≥95%), L-proline (≥ 99.0%), mesitylene, methanol (HPLC grade), N- isopropylacrylamide (NIPAM), poly(ethylene glycol) methyl ether (mPEG, average M_n 5000), 1-propanethiol, sodium bicarbonate (NaHCO₃, ACS reagent, \geq 99.7 %), Sepharose[®] 2B (60-200 μ m bead diameter), sodium carbonate (Na₂CO₃, ACS reagent, \geq 99.5 % dry basis), sodium chloride (NaCl, BioXtra, \geq 99.5%), (+)–sodium L-ascorbate, sodium azide (NaN₃, \geq 99.5 %), sodium phosphate dibasic (*Reagent Plus*[®] \geq 99.0%), sodium phosphate monobasic (*Reagent Plus*[®] \ge 99.0%), sulforyl chloride (SO₂Cl₂, 97 %), 1.0 M tetrabutylammonium fluoride solution in THF (TBAF), tetrahydrofuran (THF), tricine (\geq 99%), triethylamine (TEA), trifluoroacetic acid (TFA≥ 98 %), and triisopropylsilane (TIPS, 98 %), zinc chloride (ZnCl₂, 99.9999%) were purchased from Sigma Aldrich and used as-received without further purification. Acetonitrile (MeCN), deuterated chloroform (CDCl₃), deuterated water (D₂O), ethanol (EtOH), ethyl acetate (EtOAc) and magnesium sulphate (MgSO₄) were purchased from VWR. Acetyl chloride (≥ 98 %), Amicon Ultra-0.5 centrifugal filter units (MWCO: 100 kDa), and Human recombinant active MMP-7 were purchased from Merck Millipore and used asreceived. PD-10 Minitrap desalting columns (Sephadex G-25) were purchased from GE Healthcare Life Sciences. Oregon GreenTM 488 carboxylic acid succinimidyl ester, 6-isomer, and phosphate buffered saline (PBS, pH 7.4) were purchased from ThermoFisher. Dichloromethane (DCM), dithiothreitol (DTT), Fmoc-Rink Amide Resin (0.65 g/mmol), *N*,*N*diisopropylethylamine (DIPEA), *N*,*N*-dimethylformamide (DMF), *N*,*N*,*N'*,*N'*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) and piperidine (20 % in DMF solution) were purchased from AGTC Bioproducts and used as-received. Lacey carbon on Cu-200 mesh EM Grids and Holey Carbon on Cu-200 mesh EM Grids were purchased from Electron Microscopy Sciences.

Azobisisobutyronitrile (AIBN) was purchased from Sigma Aldrich and re-crystallized from hexane and methanol prior to use. Methacrylic acid (MAA, containing 250 ppm MEHQ as inhibitor) was purchased from Sigma Aldrich and the MEHQ inhibitor was removed prior to use through a column containing MEHQ inhibitor remover, which was purchased from Sigma Aldrich. TMSPMA was synthesised using a published protocol.¹

Copolymer synthesis

The RAFT agent 4-cyano-4-(propylthiocarbonothioylthio)pentanoic acid **1** was synthesised using a published protocol.² Propanethiol (0.3 g, 3.9 mmol) and potassium hydroxide (0.44 g, 7.79 mmol) were dissolved in a 1:1 mixture of acetone and water (20 mL). The reaction mixture was cooled in an ice bath prior to dropwise addition of carbon disulfide (285 μ L, 4.73 mmol) and the resulting reaction mixture was stirred for 2 hours at room temperature. Tosyl chloride (0.90 g, 4.73 mmol) was dissolved in acetone (5 mL) and then added dropwise. The reaction mixture was stirred for a further 1 hour, acidified to pH 2 and finally extracted with ethyl acetate (2 x 50 mL). The organic solution was concentrated under vacuum to ~25 mL, 4,4-azobis(4-cyanovaleric acid) (VA501, 2.2 g, 7.9 mmol) was added and the mixture stirred at 80 °C under reflux overnight. The crude was purified by column chromatography over silica using a mixture of ethyl acetate/hexane (1:3) + 1 % v/v of acetic acid as mobile phase to yield the final product as a yellow oil (0.88 g, 3.20 mmol, 82 % yield). Spectroscopic data matched those previously reported in the literature.²

¹H NMR (CDCl₃, 400 MHz): δ = 4.04-4.06 (br s, 1H, OH), 3.33-3.37 (t, 2H, CH₃CH₂CH₂S, *J* = 8 Hz), 2.69-2.73 (m, 2H, CH₂C<u>H₂</u>COOH), 2.54-2.58 (m, 1H, C<u>H₂CH₂COOH), 2.42-2.46 (m, 1H, CH₂CH₂COOH), 1.88 (s, 3H, C<u>H₃CCN), 1.77-1.82 (m, 2H, CH₃CH₂CH₂S), 1.03-1.07 (t, 3H, C<u>H₃CH₂CH₂S, *J* = 8 Hz) ppm.</u></u></u>

The Macro-RAFT agent **2** was synthesised as follows. The RAFT agent **1** (0.7 g, 2.53 mmol) was dissolved in DCM (25 mL). While stirring in an ice bath, PEG monomethyl ether (5 kDa, 5.54 g, 1.01 mmol), DMAP (69 mg, 0.57 mmol), and EDC.HCl (1.49 g, 7.58 mmol) were added, and the reaction mixture was stirred for 6h at 35 °C. After evaporation of DCM, the mixture was dissolved in MeOH / H₂O (1:1 v/v), dialysed (3.5 kDa membrane) against MeOH/H₂O (1:1 v/v), H₂O, and then freeze-dried to yield a pale-yellow powder (4.2 g, 0.8 mmol).

¹H NMR (CDCl₃, 400 MHz): δ = 4.26-4.29 (m, 2H, CH₂CH₂OC=O), 3.82-3.84 (m, 2H, CH₃OCH₂CH₂), 3.55-3.73 (m, 448 H, -(OCH₂CH₂)₁₁₃-O), 3.39 (s, 3H, CH₃(OCH₂CH₂)₁₁₃), 3.32-3.36 (t, 2H, CH₃CH₂C<u>H₂S</u>, *J* = 6 Hz), 2.65-2.70 (m, 2H, CH₂C<u>H₂COOH), 2.50-2.58 (m, 1H, CH₂CH₂COOH), 2.36-2.44 (m, 1H, CH₂CH₂COOH), 1.88 (s, 3H, CH₃CCN), 1.73-1.77 (m, 2H, CH₃CH₂CH₂S), 1.02-1.06 (t, 3H, CH₃CH₂CH₂S, *J* = 8 Hz) ppm.</u>

The macro-RAFT agent **2** (2 g, 0.38 mmol) was added in a 15 mL Schlenk tube equipped with a magnetic stirrer and a septum. MAA (0.327 g, 3.8 mmol), TMSPMA (1.88 g, 9.6 mmol) and 11.4 mL of 1,4 dioxane were charged to the Schlenk tube to a final monomer concentration of 1M. AIBN (6.24 mg, 0.038 mmol) and mesitylene (100 µL) were added to the reaction mixture as initiator and reference for calculating monomer conversion respectively. After 3 freeze-pump-thawing cycles, the reaction mixture was transferred to a pre-heated oil bath at 65 °C and stirred overnight. After 16 h, the reaction was quenched by introducing oxygen. MAA monomer conversion was calculated by ¹H NMR in CDCl₃ by comparing the integrals of the peak corresponding to the vinyl protons of MAA (δ = 6.17 and 5.62) at the start and at the end of the reaction, maintaining constant the integrals of the aromatic protons of mesitylene (δ = 6.80). The result was confirmed by comparing the integrals of the peak corresponding to the vinyl protons of MAA (δ = 6.17 and 5.62) at the start and at the end of the reaction, maintaining constant the integrals of the start and the end of the reactions maintaining constant the integrals of the three methyl groups of TMSPMA (δ = 0.18). Similarly, TMSPMA monomer conversion was calculated by ¹H NMR in CDCl₃ by comparing the integrals of the peak corresponding to CH₂ protons adjacent to the C-C triple bond (δ = 4.76) at the start and at the end of the reaction maintaining constant the integrals of the aromatic protons of mesitylene (δ = 6.80). The result was confirmed by comparing the integrals of the peak corresponding to CH₂ protons adjacent to the C-C triple bond (δ = 4.76) at the start and the end of the reactions maintaining constant the integrals of the three methyl groups of TMSPMA (δ = 0.18). The resulting diblock copolymer **3** was purified by precipitation into cold diethyl ether (200 mL) and dried under vacuum resulting in a yellow powder.

For synthesis of triblock copolymers $4a_{1-5}$ (PNIPAM), the reaction mixture was prepared by adding to the diblock copolymer **3**, NIPAM and methanol to a final monomer concentration of 1 or 1.5 M. The exact quantity of each compound is reported in **Table S1**. A 10 mg/mL stock solution of AIBN was prepared and added to the reaction mixture prior to 3 cycles of freezepump-thawing. The Schlenk tube containing the reaction mixture was then transferred to a pre-heated oil bath at 65 °C and stirred overnight. After 16 hours, the reaction was quenched by introducing oxygen. Monomer conversion was calculated by ¹H NMR. The resulting reaction mixture containing the final triblock copolymers $4a_{1-5}$ was used for the deprotection step without any further purification.

#	Copolymer 2	NIPAM	methanol	AIBN
4 a ₁	40.85 mg 4.32 · 10 ⁻³ mmol	226 mg 2 mmol	1.33 mL	$7.09 \cdot 10^{-2} \text{ mg}$ $4.32 \cdot 10^{-4} \text{ mmol}$
4 a ₂	95.17 mg 1.00 · 10 ⁻² mmol	453 mg 4 mmol	4 mL	1.64 · 10 ⁻¹ mg 1.00 · 10 ⁻³ mmol
4a₃	88.94 mg 9.41 · 10 ⁻³ mmol	226 mg 2 mmol	1.33 mL	1.52 10 ⁻¹ mg 9.41 · 10 ⁻⁴ mmol
4 a ₄	304 mg 3.22 · 10 ⁻² mmol	1.73 ·10 ³ mg 15.28 mmol	15.28 mL	5.29 · 10 ⁻¹ mg 3.22 10 ⁻³ mmol
4a₅	250 mg 2.67 · 10 ⁻² mmol	1.73 · 10 ³ mg 15.28 mmol	15.28 mL	$4.27 \cdot 10^{-1} \text{ mg}$ $2.60 \cdot 10^{-3} \text{ mmol}$

Table S1. Table indicating the exact quantities of compounds added for the NIPAM chain extension step of each final triblock copolymer.

NCPAM monomer was synthesised following a similar procedure reported by Maeda *et al.*³ Cyclopropyl amine (1.67 g, 2 mL, 29.4 mmol) was dissolved in 36 mL of anhydrous dichloromethane under N₂. Triethylamine (2.1 g, 3 mL, 21.5 mmol) was then added to the reaction mixture at 0 °C. Acryloyl chloride (1.77 g, 1.58 mL, 19.6 mmol) was diluted in 4 mL of anhydrous DCM under inert atmosphere and added dropwise maintaining the temperature at 0 °C. The resulting reaction mixture was finally stirred at 0 °C for 1 hour, slowly brought to room temperature and further stirred overnight. The reaction mixture was finally purified by column chromatography over silica using a mixture of dichloromethane/methanol (98:2) as mobile phase to yield the final product as a pale pink oil (1.69 g, 15.28 mmol, 78% yield).

Spectroscopic data matched those previously reported in the literature.³

¹H NMR (CDCl₃, 400 MHz): δ = 7.64 (br s, 1H, NH), 6.24-6.28 (dd, 1H, C<u>H₂</u>CHCO, *J* = 16, 2 Hz), 6.02- 6.09 (dd, 1H, C<u>H₂</u>CHCO, *J* = 16, 10 Hz), 5.58-5.61 (dd, 1H, CH₂C<u>H</u>CO, *J* = 10 Hz, 2 Hz), 2.75-2.81 (m, 1H, cyclopropyl C<u>H</u>), 0.77-0.81 (m, 2H, cyclopropyl C<u>H₂</u>), 0.52-0.56 (m, 2H, cyclopropyl C<u>H₂</u>) ppm;

¹³C NMR (CDCl₃, 400 MHz): δ = 166.98 (1C, <u>C</u>=O), 130.58 (1C, CH₂<u>C</u>HCO), 125.78 (1C, <u>C</u>H₂CHCO), 22.45 (1C, cyclopropyl <u>C</u>H), 6.22 (2C, cyclopropyl <u>C</u>H₂).

For synthesis of triblock copolymers **4b**₁₋₅ (PNCPAM), the reaction mixture was prepared by adding to the diblock copolymer **3**, NCPAM and methanol to a final monomer concentration of 1.5 M. The exact quantity of each compound is reported in **Table S2**. A 10 mg/mL stock solution of AIBN was prepared and added to the reaction mixture prior to 3 cycles of freeze-pump-thawing. The Schlenk tube containing the reaction mixture was then transferred to a pre-heated oil bath at 65 °C and stirred overnight. After 16 hours, the reaction was quenched by introducing oxygen. Monomer conversion was calculated by ¹H NMR. The resulting reaction mixture containing the final triblock copolymer **4b**₁₋₅ was used for the deprotection step without any further purification.

#	Copolymer 2	NCPAM	Methanol	AIBN	
4b ₁	$162.0 \ { m mg}$ $1.71 \cdot 10^{-2} \ { m mmol}$	333 mg 3 mmol	2 mL	2.81 · 10 ⁻¹ mg 1.71 · 10 ⁻³ mmol	
4b ₂	109.5 mg 1 .15∙ 10 ⁻² mmol	333 mg 3 mmol	2 mL	1.88 · 10 ⁻¹ mg 1.15 · 10 ⁻³ mmol	
4b ₃	109.5 mg 1.15 · 10 ⁻² mmol	333 mg 3 mmol	2 mL	3.77 · 10 ⁻¹ mg 2.30 · 10 ⁻³ mmol	
4b ₄	161.8 mg 1.7 · 10 ⁻² mmol	1.1∙ 10 ³ mg 10 mmol	10 mL	2.79 · 10 ⁻¹ mg 1.70 10 ⁻³ mmol	
4b ₅	57.1 mg 6.04 · 10 ⁻³ mmol	333 mg 3 mmol	2 mL	1.97 · 10 ⁻¹ mg 1.20 · 10 ⁻³ mmol	

Table S2. Table indicating the exact quantities of each compounds added for the NCPAM chain extension step of each final triblock copolymer.

For synthesis of triblock copolymers $4c_{1-3}$ (NIPAM/NCPAM), the reaction mixture was prepared by dissolving the diblock copolymer **3** together with NIPAM and NCPAM in methanol (overall concentration: 1.5 M). A 10 mg/mL stock solution of AIBN was prepared and added to the reaction mixture prior to 3 cycles of freeze-pump- thawing. The exact quantity of each compound is reported in **Table S3**. The Schlenk tube containing the reaction mixture was then transferred to a pre-heated oil bath at 65 °C and stirred overnight. After 16 hours, the reaction was quenched by introducing oxygen. Monomer conversion was calculated by ¹H NMR. The resulting reaction mixture containing the triblock copolymers $4c_{1-3}$ were used for the deprotection step without any further purification.

#	Copolymer 2	NIPAM	NCPAM	Methanol	AIBN
4c ₁	57.1 mg 6.04 · 10 ⁻³ mmol	271.5 mg 2.39 mmol	66.6 mg 5.99 · 10 ⁻¹ mmol	2 mL	$1.97 \cdot 10^{-1} \text{ mg}$ $1.19 \cdot 10^{-3} \text{ mmol}$
4c ₂	57.1 mg 6.04 [,] 10 ⁻³ mmol	166.7 mg 1.47 mmol	169.74 mg 1.52 mmol	2 mL	$1.97 \cdot 10^{-1} \text{ mg}$ $1.19 \cdot 10^{-3} \text{ mmol}$
4c ₃	57.1 mg 6.04 · 10 ⁻³ mmol	271.5 mg 2.39 mmol	66.6 mg 5.99 · 10 ⁻¹ mmol	2 mL	$1.97 \cdot 10^{-1} \text{ mg}$ $1.19 \cdot 10^{-3} \text{ mmol}$

Table S3. Table indicating the exact quantities of each compound added for the randomcopolymerisation of NIPAM and NCPAM.

A 1 M solution of TBAF in THF (2.1 molar equivalents respect to the number of moles of alkynes in the polymer) and acetic acid (2.1 molar equivalents respect to the number of moles of alkynes in the polymer) were added to the reaction mixture directly after the NIPAM/NCPAM chain extension step and stirred at r.t. The exact quantity of each compound is reported in **Table S4**. After 3 hours, the solvent was evaporated under vacuum and the dried reaction mixture was dissolved in cold water. An ice-bath was used to aid complete dissolution. The resulting final triblock copolymers were dialyzed against milli-Q[®] water and lyophilized to obtain a white powder. The final compound purity was assessed by ¹H NMR in D_2O .

#	Copolymer 3	Alkynes (19 per polymer chain)	TBAF (1 M in THF)	Acetic acid
5a₁	$4.32 \cdot 10^{-3}$ mmol	$8.2 \cdot 10^{-1}$ mmol	$1.72 \cdot 10^{-1} \text{mL}$ $1.72 \cdot 10^{-1} \text{mmol}$	$1.06 \cdot 10^{-2} \text{ mL}$ $1.72 \cdot 10^{-1} \text{ mmol}$
5a₂	$1.0 \cdot 10^{-2}$ mmol	$1.9 \cdot 10^{-1}$ mmol	$3.99 \cdot 10^{-1} \mathrm{mL}$ $3.99 \cdot 10^{-1} \mathrm{mmol}$	$2.45 \cdot 10^{-2} \text{ mL}$ $3.99 \cdot 10^{-1} \text{ mmol}$
5a₃	$9.41 \cdot 10^{-3}$ mmol	1.78 · 10 ⁻¹ mmol	$3.72 \cdot 10^{-1} \text{mL}$ $3.72 \cdot 10^{-1} \text{mmol}$	$2.1 \cdot 10^{-2} \text{ mL}$ $3.72 \cdot 10^{-1} \text{ mmol}$
5a₄	$3.22 \cdot 10^{-2}$ mmol	$6.11 \cdot 10^{-1} \text{ mmol}$	1.28 mL 1.28 mmol	7.6 · 10 ⁻² mL 1.28 mmol
5a₅	$2.67 \cdot 10^{-2}$ mmol	$5.07 \cdot 10^{-1}$ mmol	1.04 mL 1.04 mmol	6.1 · 10 ⁻² mL 1.04 mmol
5b₁	$1.71 \cdot 10^{-2}$ mmol	$3.24 \cdot 10^{-1}$ mmol	$6.8 \cdot 10^{-1} mL$ $6.8 \cdot 10^{-1} mmol$	$4 \cdot 10^{-2} mL$ 6.8 · 10 ⁻¹ mmol
5b ₂	$1.15 \cdot 10^{-2}$ mmol	$2.18 \cdot 10^{-1}$ mmol	$4.6 \cdot 10^{-1} mL$ $4.6 \cdot 10^{-1} mmol$	$2.72 \cdot 10^{-2} mL$ $4.6 \cdot 10^{-1} mmol$
5b ₃	$1.15 \cdot 10^{-2}$ mmol	$2.18 \cdot 10^{-1}$ mmol	$4.6 \cdot 10^{-1} mL$ $4.6 \cdot 10^{-1} mmol$	$2.72 \cdot 10^{-2} mL$ $4.6 \cdot 10^{-1} mmol$
5b₄	$1.70 \cdot 10^{-2}$ mmol	$3.24 \cdot 10^{-1} \text{mmol}$	$6.8 \cdot 10^{-1} mL$ $6.8 \cdot 10^{-1} mmol$	$4 \cdot 10^{-2} mL$ 6.8 · 10 ⁻¹ mmol
5b₅	$6.04 \cdot 10^{-3}$ mmol	$1.14 \cdot 10^{-1} \text{mmol}$	$2.4 \cdot 10^{-1} \mathrm{mL}$ $2.4 \cdot 10^{-1} \mathrm{mmol}$	$1.42 \cdot 10^{-2} mL$ 2.4 $\cdot 10^{-1} mmol$
5c₁	$6.04 \cdot 10^{-3}$ mmol	$1.14 \cdot 10^{-1} \text{mmol}$	$2.4 \cdot 10^{-1} \mathrm{mL}$ $2.4 \cdot 10^{-1} \mathrm{mmol}$	$1.42 \cdot 10^{-2} mL$ $2.4 \cdot 10^{-1} mmol$
5c₂	6.04 · 10 ⁻³ mmol	$1.14 \cdot 10^{-1}$ mmol	$2.4 \cdot 10^{-1} \text{mL}$ $2.4 \cdot 10^{-1} \text{mmol}$	$1.42 \cdot 10^{-2} mL$ $2.4 \cdot 10^{-1} mmol$
5c₃	6.04 · 10 ⁻³ mmol	$1.14 \cdot 10^{-1}$ mmol	$2.4 \cdot 10^{-1} \text{mL}$ $2.4 \cdot 10^{-1} \text{mmol}$	$1.42 \cdot 10^{-2} mL$ $2.4 \cdot 10^{-1} mmol$

Table S4. Table indicating the exact quantities of each compound added for the alkyne deprotection step of all the final triblock copolymers.

Polymer characterisation (NMR and GPC)

¹H NMR spectra were recorded on a Bruker AV 400 spectrometer operating at 400 MHz using deuterated solvents. The chemical shifts were calibrated against residual solvent signals and polymerisation conversion was calculated by ¹H NMR. Polymer molecular weight distributions were determined by gel permeation chromatography (GPC) over two PSS GRAM columns in

series. The instrument was calibrated using ten polystyrene calibration standards and water was used as a flow rate marker. The measurements were performed using DMF with 0.075 % (w/v) of LiBr and 2 % (v/v) of water as eluent with a flow rate of 0.8- 1.0 mL/min at 40 °C.

Synthesis of azide-bi-functionalised peptides

Sodium azide (1.60 g, 25 mmol) was suspended in MeCN (50 mL) at 0 °C. SO_2Cl_2 (3.34 g, 25 mmol) was added to the cold suspension over 2 minutes. The mixture was warmed to r.t. and stirred overnight. The solution was cooled again to 0 °C, imidazole (3.04 g, 25 mmol) added, and the reaction mixture was stirred for 4 hours at r.t. The reaction mixture was diluted with EtOAc (100 mL), washed with H₂O (2 × 50 mL) and NaHCO₃ solution (50 mL). The organic phase was dried over MgSO₄, filtered and dried *in vacuo*. A solution of HCl in MeOH was prepared by adding acetyl chloride (5 mL) in MeOH (20 mL). Methanolic HCl was then added dropwise to the dried organic phase to precipitate imidazole-1-sulfonyl azide hydrochloride. The precipitate was filtered under vacuum and the desired product was obtained as a white solid with a yield of 40%. Spectroscopic data matched those previously reported in the literature.⁴

¹H NMR (D₂O, 400 MHz): δ = 9.11 (m, 1H), 7.93 (m, 1H), 7.50 (m, 1H) ppm.

Fmoc-L-Lys(Boc)-OH (5 g, 10 mmol) was dissolved in DCM (100 mL). TFA (10 mL, 130 mmol) was added and the resulting solution was stirred for 2 hours at r.t. The solvent was removed *in vacuo* to obtain the product as a yellow oil without any further purification. A yield of 99% was obtained. Spectroscopic data matched those previously reported in the literature.⁵

¹H NMR (DMSO, 400 MHz): δ = 7.88-7.90 (d, 2H, *J* = 7.6 Hz), 7.61-7.63 (d, 2H, *J* = 8.0 Hz), 7.39-7.43 (m, 2H), 7.30-7.34 (m, 2H), 4.82 (br s, s, 2H), 4.27-4.33 (m, 2H), 4.20-4.25 (m, 1H), 3.89-3.94 (m, 1H), 2.74-2.78 (m, 2H), 1.66-1.75 (m, 1H), 1.56-1.62 (m, 1H), 1.46-1.55 (m, 2H) 1.33-1.38 (m, 2H) ppm.

Fmoc-L-Lys-OH (3.69 g, 10 mmol), Na₂CO₃ (3.18 g, 30 mmol), imidazole-1-sulfonyl azide hydrochloride (2.51 g, 12 mmol) and CuSO₄·5H₂O (25 mg, 0.1 mmol) were dissolved in methanol (125 mL). A few drops of water were added to aid dissolution. The resulting reaction

mixture was stirred overnight at r.t. and then the solvent removed *in vacuo*. The crude material was partitioned between water (50 mL) and EtOAc (50 mL) and acidified to pH 4 by dropwise addition of 37 % HCl. The organic phase was washed with water (50 mL) and brine (50 mL) and dried over MgSO₄. The solvent was removed *in vacuo* to obtain a pale-yellow oil with a yield of 75 %. Spectroscopic data matched those previously reported in the literature.⁴

¹H NMR (CDCl₃, 400 MHz): δ = 7.75-7.77 (d, 2H, *J* = 7.6 HZ), 7.59-7.60 (d, 2H, *J* = 7.2 Hz), 7.38-7.42 (m, 2H), 7.29-7.33 (m, 2H), 4.40-4.44 (m, 3H), 4.20-4.24 (t, 1H, *J* = 6.8 Hz), 3.26-3.29 (t, 2H, *J* = 6.4 Hz), 1.89-1.94 (m, 1H), 1.69-1.78 m, 1H), 1.60-1.63 (m, 2H), 1.39-1.48 (m, 2H).

The MMP-7-cleavable peptide (Figure S2) was synthesised by standard solid phase peptide synthesis (SPPS) on Rink amide resin (0.65 g/mol), using Fmoc-based peptide chemistry with HBTU activation as previously described in the literature.^{6,7} The synthesis was conducted on 1 mmol scale and after initial swelling of the resin with DCM, a sequence of deprotection and coupling steps was repeated for each amino acid of the peptide sequence of interest. For each coupling, the Fmoc-protecting group was removed with 20 % v/v piperidine in DMF solution followed by washing of the resin with DCM and DMF. After deprotection, an activated solution containing 4 molar excess of Fmoc- protected amino acid, 3.95 molar excess of HBTU and 6 molar excess of DIPEA in DMF was added to the resin. The coupling reaction mixture was shaken for two to three hours at r.t. before the resin was washed in vacuo with DCM and DMF. Coupling and Fmoc-deprotection steps were monitored by Kaiser test. For all sequences, Fmoc-L-Lys(N₃)-OH were introduced as the first and last amino acids in order to introduce an azide functionality at the N- and C- termini. Finally, the resin-bound peptide was cleaved and the respective side-chains deprotected using a cleavage cocktail containing 90 % v/v TFA, 5 % v/v H₂O, 3 % v/v TIPS and 2 % w/v DTT for three hours at r.t. The solution was collected by filtration and the resin washed thoroughly with DCM. After removing the solvent in vacuo, the desired peptide was precipitated in cold diethyl ether by centrifugation. Residual ether was removed under vacuum prior to peptide purification.

The peptide was purified using reverse phase HPLC under acidic conditions using a acetonitrile/water gradient at a flow rate of 15 mL/min, using a Shimadzu prominence system equipped with UV detector at 220 and 280 nm and a Phenomenex C18 Gemini NX column (5 μ m pore size, 110 Å particle size, 150 x 21.2 mm). **Table S5** reports details of the HPLC method

adopted for the purification of the peptide. The retention time of the MMP-7-cleavable peptide was 9.5 min.

Time (min)	0.1% TFA in MilliQ water	0.1% TFA in MeCN
1-3	95 %	5 %
4	60 %	40 %
5-11	30 %	70 %
12-13	0 %	100 %
14-16	95 %	5 %

Table S5. Details of HPLC acidic method used for the purification of the peptide of Scheme S1.

Finally, the purified peptides were lyophilised and analysed by liquid chromatography-mass spectrometry (LC-MS) on an Agilent 6130 Quadrupole coupled to an Agilent 1260 Infinity LC equipped with a 150 x 4.6 mm Phenomenex C18 Gemini NX column (5 μ m pore size and 100 Å particle size). Peptide LC-MS (m/z): Calcd for C₆₉H₁₂₉N₃₁O₁₅ [M+H]: 1005.6, Obsv: 1005.5. Calcd for [M/2+H]: 502.8, Obsv: 503.4.

Study of MMP-triggered peptide degradation by LC-MS

Peptide degradation by MMP-7

A 5 mg/mL solution of MMP-7-sensitive peptide (0.25 mg, 0.25 μ mol) was prepared in the enzyme buffer (50 mM tricine, 50 mM NaCl, 10 mM CaCl₂, 20 μ M ZnCl₂ and 0.05% w/v Brij[®]35 at pH 7.5). The solution was divided into 2 aliquots and incubated with 0.2 μ M MMP-7 or without enzyme. Final sample volume was 50 μ L. The samples were mixed overnight at 37 °C using an AccuThermTM microtube shaking incubator with 96 x 0.2 mL block.

LC-MS analysis

Following degradation, the solutions containing the peptide were diluted to 250 μ L with methanol and analysed by liquid chromatography-mass spectrometry (LC-MS) on an Agilent 6130 Quadrupole coupled to an Agilent 1260 Infinity LC equipped with a 150 x 4.6 mm

Phenomenex C18 Gemini NX column (5 μ m pore size and 100 Å particle size). Mass-to-charge ratios (m/z) of treated peptides were compared with expected fragments and those of initial peptides.

Optimised protocol for peptide cross-linked nanogel preparation

The desired block copolymer $(1.76 \cdot 10^{-2} \,\mu\text{mol})$ was dissolved at r.t. in 125 µL of PBS in a 1.5 mL Eppendorf tube and the resulting solution was heated up to a temperature T ≥ LCST_(Polymer) + 10°C, and mixed at 350 rpm for 10 minutes by using an AccuThermTM microtube shaking incubator with 40 x 1.5 mL block to induce nanogel self-assembly. Following self-assembly, a 15 mg/mL solution of the peptide cross-linker in PBS ($3.52 \cdot 10^{-1} \,\mu\text{mol}$) was added to the preformed nanogels, followed by addition of 75 mM (+)- sodium-L-ascorbate solution in PBS (70 µg, 4.6 µL, $3.52 \cdot 10^{-1} \,\mu\text{mol}$) and 40 mM Copper(II) sulfate pentahydrate in MilliQ water (88 µg, 8.8 µL, $3.52 \cdot 10^{-1} \,\mu\text{mol}$). The final reaction volume was adjusted with PBS to 166 µL to keep a final copolymer concentration of 6 mg/mL. The resulting reaction mixture was kept at the initial temperature and mixed with a speed of 350 rpm for 16 h to ensure complete cross-linking. After cross-linking, the reaction mixture was cooled down to r.t. and subsequently purified through Amicon Ultra-4 centrifugal filter units with an MWCO of 100 kDa (5000 g, 5 min) reconstituting the concentrated sample with fresh PBS and repeating this purification process 5 times.

Characterisation of nanogels by DLS

A Zetasizer Nano ZS (Malvern) was used for DLS measurements. For all the DLS measurements, the scattering angle was fixed at 173° and disposable micro cuvettes were used.

Study of temperature-triggered self-assembly behaviour

A 1 mg/mL solution of each block copolymer was prepared in PBS prior to analysis. Each measurement, consisting of 15 runs, was repeated three times at the desired temperature. Averaging the results from the three repeated measurements, an average size distribution and an average size value (number mean) was obtained. The temperature-responsive behaviour of the triblock copolymers and their temperature of cloud point (T_{cp}) was determined by DLS. Solutions of the final triblock copolymers (1mg/mL) in PBS were subjected to a 1 °C temperature ramp (from 25 to 60 °C and from 60 to 25 °C) with 5 min equilibration time between each temperature-controlled measurement. The reported derived count-rate (kcps) is proportional to the total intensity of the scattered light and therefore to the self-assembly of the copolymers. The inflection points of each curve represent the T_{cp} of the corresponding copolymer.

MMPs-triggered nanogel degradation

Following cross-linking, the nanogels were buffer exchanged from PBS to enzyme buffer (50 mM tricine, 50 mM NaCl, 10 mM CaCl₂, 20 μ M ZnCl₂ and 0.05 % w/v Brij at pH 7.5) using Amicon Ultra-0.5 mL centrifugal filter units with a MWCO of 100 kDa (5 x 5 min, 5500 g). For studying MMP-7-triggered degradation, 20 μ L-aliquots of peptide-cross-linked nanogels solution with a concentration of 6 mg/mL copolymer were incubated overnight with increasing concentrations of MMP-7 (0, 9 pM, 90 pM, 0.9 nM, 9 nM, 90 nM and 0.9 μ M) at 37 °C using an AccuThermTM microtube shaking incubator with 96 x 0.2 mL block. Final sample volume: 22 μ L. After 16 h-incubation, the samples were analysed by DLS at 25 °C to monitor degradation. Kinetics of degradation was studied by fixing the MMP-7 concentration to 90 nM. A 6 mg/mL solution of cross-linked nanogels (198 μ L) was incubated with 90 nM MMP-7 in enzyme buffer at 37 °C using AccuThermTM microtube shaking incubator. Final sample volume: 218 μ L. 70 μ L aliquots were collected every hour and analysed by DLS at 25 °C to monitor nanogel degradation over a period of 24 hours.

Nanogel stability in biological environment

5a₄-based cross-linked nanogel stability was tested in 5% v/v fetal bovine serum (FBS). The solution containing cross-linked nanogels was sterile filtered through a 0.45 μ m syringe filter in a laminar flow microbiological safety cabinet and mixed with FBS to yield a final nanogel concentration of 3 mg/mL. This solution was incubated at 37 °C. DLS measurements were performed over time by collecting 70 μ L of the sample under a laminar flow microbiological safety cabinet to maintain sterility. Each measurement, consisting of 15 runs, was repeated three times at 25 °C, resulting in an average size value (number mean) with standard deviation of the three repeated measurements.

Preparation of cargo-loaded cross-linked nanogels

Cargo labelling

The protein (BSA) was dissolved in 50 mM HEPES buffer, pH 8.5. The NHS-functionalized dye of (NHS-Oregon Green, 1.5 μmol) was dissolved in DMSO and added to the protein solution prior to stirring at room temperature overnight. The labelled-protein was then purified and buffer exchanged by using PD-10 minitrap desalting columns containing Sephadex G-25 resin and PBS as running buffeer. The labelled protein was subsequently concentrated to 10 mg/mL with Amicon Ultra 0.5 mL centrifugal filter units (MWCO: 10kDa).

Cargo loading and cross-linking

A 15 mg/mL solution of **5a**₄ block copolymer (66 μ L) in 25 mM phosphate buffer was mixed with a 10 mg/mL solution of OG-BSA (66 kDa, 35 μ L) in 25 mM phosphate buffer in a 1.5 mL Eppendorf tube. Volume was adjusted with 25 mM phosphate buffer to reach a final polymer concentration of 6 mg/mL. The resulting solution was heated up to 45 °C and mixed at 350 rpm for 10 minutes by using AccuThermTM microtube shaking incubator with 40 x 1.5 mL block to induce nanogel self-assembly and cargo loading. A 15 mg/mL solution of the peptide crosslinker in 25 mM phosphate buffer (0.35 μ mol) was then added to the pre-formed nanogels, followed by addition of 75mM (+)-sodium-L-ascorbate solution in 25 mM phosphate buffer (70 µg, 4.6 µL, 0.35 µmol) and 40 mM Copper(II) sulfate pentahydrate in MilliQ water (88 µg, 8.8 µL, 0.35 µmol). The resulting reaction mixture was kept at initial temperature and mixed with a speed of 350 rpm for 16 h. Finally, the cargo-loaded cross-linked nanogels were purified by using Sepharose 2B size exclusion chromatography in PBS and column fractions were concentrated back to initial concentrations (6 mg/mL) using Amicon Ultra-0.5 mL centrifugal filter units with a MWCO of 100 kDa.

Cryo-TEM sample preparation and imaging

Holey Carbon on Cu-200 mesh EM Grids were used for sample deposition and were glowdischarged on a Gatan SOLARIS plasma cleaner for 15 seconds with O_2/H_2 1:1. Block copolymer nanogel solutions with a concentration of 3-6 mg/mL in PBS were incubated in a block heater at 50 °C for 15 minutes. 4 µL of the pre-heated cryo-specimen solution was loaded onto the carbon side of the plasma- treated grid, which was incubated for 30 sec at 60 % humidity and 50 °C temperature using a Leica EM GP plunge-freezer. Following sample deposition, the grid was blotted twice for 1 sec using filter paper and immediately vitrified. The grids were then stored under liquid nitrogen until imaging. For imaging, grids were inserted in a Gatan 914 cryo-holder and images were collected using a JEOL 2100 Plus electron microscope under low dose conditions using Minimum Dose System software settings. Images were acquired using a Gatan Orius SC 1000 camera over 5 sec of exposure time. Magnification of 15, 25 and 30 k and a defocus of -5 and -10 µm were used. From the cryo-TEM pictures of each block copolymer, analysis of the size distribution was performed by manually measuring the size of about 100 nanogels using the line drawing tool in Fiji-Image J to obtain the diameters.

SANS sample preparation and measurements

Deuterated PBS was prepared by dissolving the appropriate amount of Gibco PBS tablets (manufacturer's composition: 10 mM sodium phosphates, 2.68 mM potassium chloride, 140 mM sodium chloride) (ThermoFisher Scientific) in D₂O. Solutions of copolymers were

prepared in deuterated PBS at a concentration of 6 mg/mL immediately prior to measurement to avoid any labile hydrogen/deuterium exchange. **5**c₁-based cross-linked nanogels were formed by cross-linking the corresponding self-assembled nanostructure with the MMP-7-specific peptide using the methodology reported above. It must be noted that in this case, both polymer and reagent solutions for the preparation of cross-linked nanogels were prepared in deuterated PBS to avoid incoherent scattering from H₂O and increase scattering contrast. Following cross-linking, the nanogels were purified through 100 kDa MWCO Amicon Ultra 0.5 mL centrifugal filter units (5500 rpm, 5 min x 5). For the measurements above the LCST, these solutions were incubated at 45 °C for 10 min in the beamline sample holder prior to measurements to ensure complete phase transition. Phase transitions of both **5**c₁-based self- assembled and cross-linked nanogels were followed real-time by performing a temperature trend from 25 to 45 °C equilibrating the sample for 5 min at each measured temperature (25, 32-38, 40, 42 and 45 °C).

All the measurements were carried out at the SANS2d beamline of the ISIS pulsed neutron source at the Rutherford Appleton Laboratory, Didcot, UK, using a sample changer and 1 mm path length quartz cuvette cells with sample volumes of 150-200 µL. The beamline was configured with L1 = L2 = 12 m, where L1 is the source to sample distance and L2 is the sample to detector distance, giving a scattering variable (Q) range of 0.001 to 1 Å⁻¹. Q is defined as Q = $2\pi \theta/\lambda$.

SANS data fitting

SANS data reduction was performed with MantidPlot and SasView v4.1. was used to fit the experimental data. The analysed block copolymers/nanogels were shown to have temperature- and cross-linking- dependent structural transition. Therefore, the analysis of their scattering behaviour required the use of several models of fitting to best represent the different temperature and solvent conditions. The fitting models were selected on the basis of the system information previously collected by DLS and cryo-TEM. All the fitted data were plotted using Prism 6. For all the graphs, since axes are logarithmic, only values greater than

zero can be plotted using Prism 6. For this reason, the down error bar that would go to a negative Y value are missing from some data points.

Some of the SANS data was fitted using a power law model. This is a shape-independent function where the scattering intensity I(q) is calculated as simple power law with a flat background using the following:

$$I(q) = \text{scale} \cdot q^{-m} + \text{background}$$

A scaling factor is used to dictate the intensity with a default value of 1. Scale in this model is not explicitly related to a volume fraction; m is the power law exponent; additionally, the model allows for a variable flat background to accommodate for any under- or oversubtraction, or the incoherent background from hydrogen. In polymer systems, a power law exponent $1 \le m \le 3$ typically indicates mass fractal structures ($q^{-5/3}$ for swollen chains, q^{-2} for Gaussian chains and q^{-3} for clustered networks) while a power law exponent $3 \le m \le 4$ characterises surface fractals (q^{-3} for rough surfaces and q^{-4} for smooth surfaces). This means that the model is indicative of the molecular configuration of polymer structures in different solvation conditions.

Other SANS data was fitted using a Guinier-Porod model.^{8,9} This is an empirical model that can be used to determine the size and dimensionality of a generalised Guinier/power law object including both spherical and non-spherical objects such as rods, platelets and intermediate shapes as long as the *q*-range collected is sufficient to cover the Guinier (overall shape of the scatterer) and Porod (internal/surface configuration of the scatterer) regimes.⁸ The scattering intensity I(q) results from the contributions of the two regimes as shown here:

$$I(q) = \begin{cases} \frac{G}{q^s} \exp\left[\frac{-q^2 R_g^2}{3-s}\right] & q \le q_1 \\ \frac{D}{q^m} & q \ge q_1 \end{cases}$$

q is the scattering vector, R_g is the radius of gyration, *s* is the dimension variable, *G* and *D* are the Guinier and Porod scale factors respectively, and *m* is the Porod exponent. The Guinier form is used for $q \le q_1$ and the Porod form is used for $q \ge q_1$ where q_1 is the value at which the slopes of the Guinier and Porod terms are connected. q_1 is calculated using the following equation:

$$q_1 = \frac{1}{R_g} \left(\frac{3d}{2}\right)^{1/2}$$

For 3D globular objects such as spheres s = 0. For a 2D symmetry such as rods s = 1 and for 1D symmetries such as lamellae and platelets s = 2. Therefore, a dimensionality parameter (3 - s) is defined and is 3 for spheres, 2 for rods and 1 for lamellae and platelets. Regarding the Porod exponent, $1 \le m \le 3$ indicates mass fractal ($q^{-5/3}$ for swollen chains, q^{-2} for Gaussian chains and q^{-3} for clustered networks) while $3 \le m \le 4$ characterises surface fractals such as spheres and cylinders (q^{-3} for rough surfaces and q^{-4} for smooth surfaces). The Guinier-Porod model is completely empirical and provides a shortcut for collecting useful information from the scattering of non-spherical objects and systems with complex phase behaviour rather than having to use multiple models for each phase structures.⁸

Some SANS data was fitted using a sphere model.¹⁰ The 1D scattering intensity I(q) is calculated from the following equation:

$$I(q) = \frac{scale}{V} \cdot \left[3V(\Delta \rho) \cdot \frac{\sin(qr) - qr\cos(qr)}{(qr)^3} \right]^2 + \text{ background}$$

were *scale* is a volume fraction, *V* is the volume of the scattering object, *r* is the sphere radius and background is the background level. $\Delta \rho$ is the difference between scattering length density of the scattering object *(sld)* and the scattering length density of the solvent *(sld_solvent)*.

Another model that was used for SANS data fitting was a Unified Power Rg model.^{9,11} This is a shape-independent model where an empirical multiple level unified Exponential/Power law fit method is employed. Similar to the Guinier- Porod model, this method, also known as Beaucage method, is able to approximate the scattering of many different types of objects including fractal clusters, random coils and ellipsoidal particles. This model works best for fractal objects characterised by a Porod exponent of 5/3 (polymer chains in good solvent conditions) and 3 (polymer chain network) and should not be used for describing surface fractals, as opposed to the Guinier-Porod model. The empirical fitting function is represented by the following equation:

$$I(q) = \text{background} + \sum_{i=1}^{N} \left[G_i \exp\left(-\frac{q^2 R_{gi}^2}{3}\right) + B_i \exp\left(-\frac{q^2 R_{g(i+1)}^2}{3}\right) \left(\frac{1}{q_i^*}\right)^{p_i} \right]$$

where

$$q_i^* = q \left[erf\left(\frac{qR_{gi}}{\sqrt{6}}\right) \right]^{-3}$$

 G_i and B_i are Guinier and Power law/Porod scale factors respectively, P_i is the Power law/Porod exponent and R_g is the radius of gyration. This model takes into account N possible structural levels and implies that each structural level (larger in size as the index *i* increases) is composed of the previous smaller level. Thus, four functions are included so that 1,2,3 and 4 levels can be used. Similarly to the Guinier-porod model, this empirical method enables a simplified analysis of complex systems by combining the Guinier form (low *q*) and the Porod form (high *q*) and proving a smooth transition between the two with the addition of the $\left[erf \left(\frac{qR_{gi}}{\sqrt{6}} \right) \right]^{-3}$ term. However, this might create artefacts that show as kinks in the fitted curve.

The last model used was the triaxial ellipsoid model. This model is based on an ellipse with three axes of different length. The three axes are named minor equatorial radius (R_a), major equatorial radius (R_b) and polar radius (R_c) with $R_a \le R_b \le R_c$.



Scheme S2. Ellipsoid schematic.

This model calculates the scattering intensity through the following equation:

$I(q) = scale/V \langle F^2(q) \rangle + background$

where the volume of the ellipsoid is $V = 4/3\pi R_a R_b R_c$ and $\langle ... \rangle$ is an average of all the possible orientations of the ellipsoid. The form factor calculated corresponds to:

$$I(q) = \frac{scale}{V} \int_0^1 \int_0^1 \Phi^2 \left(q R_a^2 cos^2 \left(\frac{\pi x}{2} \right) + q R_b^2 sin^2 \left(\frac{\pi y}{2} \right) (1 - y^2) + R_c^2 y^2 \right) + background$$

The radius of gyration for this system is calculated as $R_g^2 = (R_a R_b R_c)^2 / 5$. The contrast is defined as SLD(ellipsoid) – SLD(solvent).

Study of enzyme-triggered nanogel degradation and cargo release by FCS

Enzyme-triggered nanogel degradation for FCS

For the analysis of nanogel degradation by FCS, nanogels were first cross-linked and then labelled with Cy5-azide. Following cross-linking a 6 mg/mL solution of **5a**₄-based cross-linked nanogels (150 μ L), the solution was heated up to 45 °C. A 12 mM solution of Cy5-azide (10 μ L, 0.124 μ mo) was added together with 5mM (+)-sodium-L-ascorbate solution in PBS (23 μ g, 4.6 μ L, 0.11 μ mol) and 40 mM Copper(II) sulfate pentahydrate in MilliQ water (26 μ g, 8.8 μ L, 0.11 μ mol). The resulting reaction mixture was stirred at 45 °C overnight and then purified using Sepharose 2B size exclusion chromatography. The column fractions containing the labelled nanogels were collected and concentrated to initial volume using Amicon Ultra-0.5 mL centrifugal filter units with an MWCO of 100 kDa.

MMP-7-triggered cargo release for FCS study

The release of OG-BSA from the cross-linked nanogels was tested by FCS upon addition of MMP-7 and compared to diffusion-based release. Prior to enzyme degradation, the solution containing the loaded nanogels was buffer exchanged to enzyme buffer using Amicon Ultra-0.5 mL centrifugal filter units with a MWCO of 100 kDa (5 times x 5 min, 5500 g). For monitoring OG-BSA release, 6 mg/mL solutions containing OG-BSA loaded cross-linked nanogels (20 μ L) were incubated with increasing MMP-7 concentrations (no enzyme, 0.9 nM, 9 nM, 90 nM) and studied over time by incubating for 0, 0.5, 2, 6, 14, 18 and 27 h at 37 °C.

Sample incubation was performed using AccuThermTM microtube shaking incubator with 96 x 0.2 mL block. Final sample volumes: 22 μ L. The samples were then analysed by FCS as described below. For the study of OG-BSA release, OG-BSA loaded nanogel stock solution and free OG-BSA were used as controls and OG was used as reference for all the measurements. This experiment was performed with three independent particle samples (N = 3).

Fluorescence correlation spectroscopy (FCS)

FCS was conducted on a commercial LSM 780 (Carl Zeiss, Jena, Germany) equipped with an incubation chamber. All measurements were performed at 25 °C. The following lasers were used as excitation sources for the various dyes: Ar⁺ laser for 488nm (OG) and a HeNe-laser for 633 nm (Cy5), respectively. Appropriate filter sets were used to detect the fluorescence signal. A 40x C- Apochromat water immersion objective with a numeric aperture of 1.2 was used to focus the laser beam. For each sample, a 5 µL droplet was placed in an ibidi 8-well glass bottom plate and measurements were performed 200 µm above the glass. Free dye solutions were first used as standards to calibrate the beam waist for each laser (OG488: D = 4.1 x 10^{-6} cm²/s, Cy5: 3.6 x 10^{-6} cm²/s, all at 25 °C).¹² Before the measurement, stocks or incubated samples were diluted 10-fold in enzyme buffer (see above). Intensity traces of 30 x 5 s were recorded for each sample, which were subsequently autocorrelated and analysed. Autocorrelation curves were produced by ZEN software (Carl Zeiss, Jena, Germany) and the obtained curves were fitted using the PyCorrfit program 1.1.1.¹³ Data for all 30 curves are presented in most graphs, except for the autocorrelation curves, which are the average curves for the entire measurement time (150 s). OG-BSA release data was averaged across 3 independent particle samples (N = 3, n = 30, mean \pm SEM). To calculate the percentage of free cargo, measurements of free cargo were first fitted using one component fits ($G_{1comp}(\tau)$) to obtain the diffusion time (τ_1) and brightness for one cargo molecule (CPP1 in kHz). Then, loaded particle samples (with or without enzymes) were fitted with two component fits $(G_{2comp}(\tau))$ with one component fixed to pure cargo diffusion (τ_1) . Fraction of free cargo (F1) had to be adjusted with the accompanying decrease in number of cargos per nanogel, due to the non-proportional contribution of slow diffusing and bright particles.¹⁴ Across all the FCS

data shown in this paper (total of 1770 curves), 3 individual curves had to be excluded from the averages do to presence of aggregates or fit errors.¹³ A triplet fraction with a triplet time between 1-10 μ s was included where appropriate.

$$G_{1comp}(\tau) = \left(1 + \frac{T}{1 - T} e^{\frac{-\tau}{\tau_{trip}}}\right) * \frac{1}{N * \left(1 + \frac{\tau}{\tau_D}\right) * \sqrt{1 + \frac{\tau}{SP^2 \tau_D}}}$$
$$G_{2comp}(\tau) = \left(1 + \frac{T}{1 - T} e^{\frac{-\tau}{\tau_{trip}}}\right) * \frac{1}{N} * \left[\frac{F_1}{\left(1 + \frac{\tau}{\tau_1}\right) * \sqrt{1 + \frac{\tau}{SP^2 \tau_1}}} + \frac{1 - F_1}{\left(1 + \frac{\tau}{\tau_2}\right) * \sqrt{1 + \frac{\tau}{SP^2 \tau_2}}}\right]$$

$$CPP \ total = F1 * CPP1 + (1 - F1) * CPP2$$

cargo per particle = NR = CPP2/CPP1

% free cargo =
$$\left\{F1 + (1 - F1) * \left(1 - \frac{NR(t)}{NR(0)}\right)\right\} * 100$$

T is the triplet fraction with corresponding triplet time τ_{trip} , *N* is the effective number of diffusing particles in the confocal volume (N = n1 + n2), τ_D is the diffusion time (τ_1, τ_2 diffusion times of corresponding fractions), F_1 fraction of component with diffusion time τ_1 , and *SP* is the structural parameter defined as the ratio of height to width of the confocal volume (fixed to 5). CPP is counts per particle in kHz. NR(0) is the number of cargo per particle averaged for each independent particle sample across all time points that show no release. NR(t) is the number of cargo per particle at timepoint t. In the few cases when NR(t) was higher than NR(0) due to a large variability in measurements, NR(t)/NR(0) was set to 1.

The subsequent equation relates the x-y dimension of the confocal volume (ω_{xy}^2) to the diffusion coefficient (*D*). First, (ω_{xy}^2) was calibrated by using standard measurements of the free dyes, and then D was calculated for each sample using the obtained diffusion time (τ_D) :

$$D = \frac{\omega_{xy}^2}{4\tau_D}$$

Stokes-Einstein equation was then used to calculate hydrodynamic radii (R_h) using the obtained diffusion coefficients (D).

Cytocompatibility study with macrophages

Cytocompatibility was measured using RAW 264.7 cell line and MTS assay following the standard procedure BS ISO 19007:2018. Briefly, 15'000 RAW 264.7 cells/well were seeded in a 96-well plate using the following medium (DMEM-high glucose containing 10% v/v FBS and 1% v/v P/S) and incubated overnight. Fresh medium including nanogels or controls were added at different concentrations and incubated for another 24 h. A mixture of MTX (317 μ g/mL, Abcam) and PMS (7.3 μ g/mL, Sigma) in phenol-red free RPMI medium was added to each well and the absorbance was recorded at 490 nm after 1-2 h.

|--|

#	NI/NC ratio	DP (NMR)	<i>M</i> n (Da) (NMR)	<i>M</i> n (Da) (GPC)	Ð (<i>M</i> _w / <i>M</i> _n) (GPC)	D _H (nm) (DLS)			
	PNIPAM-based library								
5a₁	$NI_{1.0}NC_{0.0}$	190	29700	54600	1.16	71 ± 2			
5a₂	NI _{1.0} NC _{0.0}	236	34900	64700	1.17	64 ± 2			
5a₃	$NI_{1.0}NC_{0.0}$	373	50400	67900	1.28	149±6			
5a4	NI _{1.0} NC _{0.0}	421	55800	66600	1.41	74 ± 7			
5a₅	NI _{1.0} NC _{0.0}	466	60900	77800	1.48	150 ± 4			
			PNCPAN	1-based libr	ary				
5b1	$NI_{0.0}NC_{1.0}$	100	19300	29000	1.07	$\textbf{23} \pm \textbf{11}$			
5b2	$NI_{0.0}NC_{1.0}$	130	22600	34600	1.09	171 ± 248			
5b₃	NI _{0.0} NC _{1.0}	171	27200	48100	1.16	$\textbf{451} \pm \textbf{381}$			
5b4	NI _{0.0} NC _{1.0}	297	41200	68900	1.20	68 ± 4			
5b₅	$NI_{0.0}NC_{1.0}$	450	58200	73500	1.47	58 ± 2			

Table S6. Library of PEG-b-P(MAA-co-PMA)-b-PNIPAM and PEG-b-P(MAA-co-PMA)-b-PNCPAM triblock copolymer libraries. (DP is degree of polymerisation calculated by NMR corresponding to the number of NIPAM or NCPAM monomer units in the thermo-responsive block; M_n is the number average molecular weight calculated by GPC and NMR; D represents dispersity obtained by GPC; D_H in nm represent the hydrodynamic diameter of the resulting self-assembled nanostructures as measured by DLS (Number/%) above the LCST).

T (°C)	Fitting	Fitting parameters					
(C)	model	Power law exp	R _g (nm)	R _a (nm)	<i>R₀</i> (nm)	<i>R_c</i> (nm)	<i>R</i> (nm)
25	Power law	1.93 ± 0.02	_	-	-	—	—
32	Power law	1.90 ± 0.02	_	_	_	_	_
33	Power law	1.92 ± 0.02	_	_	_	_	_
34	Power law	1.94 ± 0.02	_	_	_	_	_
35	Unified power Rg	2.40 ± 0.04	52.0 ± 0.6	_	_	_	_
36	Triaxial ellipsoid	_	_	38.9 ± 0.3	64.0 ± 0.9	178 ± 35	_
37	Triaxial ellipsoid	_	_	41.5 ± 0.2	61.0 ± 0.7	158 ± 16	_
38	Triaxial ellipsoid	_	_	44.2 ± 0.2	60.7 ± 0.6	181 ± 30	_
40	Triaxial ellipsoid	_	_	46.2 ± 0.2	63.4 ± 0.6	149 ± 11	_
42	Triaxial ellipsoid	_	_	47.9 ± 0.2	68.7 ± 0.7	141 ± 8	_
45	Triaxial ellipsoid	_	_	48.2 ± 0.6	72.5 ± 1.8	107 ± 8	_
45	Sphere	_	_	_	_	_	55.2 ± 0.6

Table S7. Fitting parameters from SANS data of **5** c_1 block copolymer self-assembly shown in Figure 4a,b. Power law exp is the power law exponent for the SANS data fitted with either Power law or Unified power Rg models; R_g is the radius of gyration of the scattering object obtained for data fitted with the Unified power Rg model; R_a, R_b, R_c are the minor equatorial radius, major equatorial radius and polar radius respectively obtained from the triaxial ellipsoid fitting model; R the sphere radius determined by fitting the data with the sphere model.

T	Fitting	Fitting parameters					
(°C) m	model	Power law exp	<i>R_g</i> (nm)	R _a (nm)	<i>R_b</i> (nm)	R _c (nm)	<i>R</i> (nm)
32	Unified	2.31	76.4				
	power Rg	± 0.03	± 2.4	-	-	-	-
33	Unified	2.34	73.6				
	power Rg	± 0.03	± 2.3	_	-	-	_
34	Unified	2.38	74.6				
	power Rg	± 0.03	± 2.0	—	-	-	-
35	Unified	2.81	66.2				
	power Rg	± 0.03	± 1.8	—	-	-	-
36	Triaxial			22.5	39.3	1058	
	ellipsoid	-	-	± 0.2	± 1.0	± 346	_
37	Coboro	-					31.2
	sphere	-	-	—	-	-	± 0.7
38	Coboro	-					33.0
	sphere	-	-	—	-	-	± 0.7
40	Cabara						33.3
	sphere	-	-	-	_	-	± 0.7
42	Cabara						31.9
	Sphere	-	_	-	_	_	± 0.7

Table S8. Fitting parameters from SANS data of cross-linked **5** c_1 block copolymer shown in Figure 4c,d. Power law exp is the power law exponent for the SANS data fitted with either Power law or Unified power Rg models; R_g is the radius of gyration of the scattering object obtained for data fitted with the Unified power Rg model; R_a, R_b, R_c are the minor equatorial radius, major equatorial radius and polar radius respectively obtained from the triaxial ellipsoid fitting model; R is the sphere radius determined by fitting the data with sphere model.



Figure S1. Characterisation of PEG-P(MA-co-PMA)-P(NIPAM-co-NCPAM) copolymers. a) GPC traces of NIPAM- and NIPAM-co-NCPAM-based block copolymers and precursors 2 and 3 of Scheme 1. b) Plots showing the evolution of M_n (filled symbols) and dispersity (open symbols) versus degree of polymerisation (DP from NMR) for copolymers shown in a. c) DLS distributions corresponding to Figure 1b after one heating-cooling cycle, measured at 25 °C.



Figure S2. LC-MS characterisation of the MMP-7 cleavable peptide. a) Amino acid sequence and structure and MMP-7 cleavage site with the two fragments originating from the cleavage. b) LC-MS spectra of the peptide (top) compared to after overnight incubation at 37 °C with 2 μ M MMP-7 (bottom). Shaded (purple) lines indicate peak positions of peptide without enzyme incubation.



Figure S3. Cross-linked nanostructure morphology characterisation. *a)* Representative cryo-TEM image of **5c1** copolymer after cross-linking (scale bar: 100 nm). *b*) Size distribution histogram from representative cryo-TEM images.



Figure S4. DLS analysis of the MMP-7-triggered degradation of cross-linked $5a_4$ -based nanogels. a) Evolution of cross-linked nanostructure average size (number mean) after overnight incubation at 37 °C with increasing MMP-7 concentration (0-900 nM range). b) Evolution of normalised derived count rate for samples in a). Error bars are standard deviations (N = 1, n = 3 measurements).



Figure S5. Study of nanogel stability in 5% v/v FBS over time by DLS. a) Evolution of cross-linked $5a_4$ nanogel size (number mean) over time after incubation with FBS at different time points at 37 °C. b) Evolution of nanogel PDI over time after incubation with FBS at 37 °C. All measurements have been performed at 25 °C in order to be able to evaluate cross-linked nanogel stability in 5% v/v FBS. c)

Corresponding number and intensity distribution for NG stock (no FBS) and d) for NG in 5% v/v FBS (t = 0 h).



Figure S6. Normalised autocorrelation curves (coloured lines) and corresponding fits (black line) of the controls (Cy5-azide, polymer + free Cy5-amine, nanogels + free Cy5-amine, Cy5-polymer) used in the FCS analysis of MMP-7 triggered nanogel degradation shown in Figure 5c.



Figure S7. Study of MMP-7 triggered release by FCS. Normalised autocorrelation curves (coloured lines) and corresponding fits (black lines) from FCS data in enzyme buffer showing the overtime evolution of the loaded nanogel autocorrelation function upon incubation with different MMP-7 concentrations (no MMP-7, 0.9 nM, 9 nM and 90 nM). Light green represents free dye (OG) and dark green is OG-labelled BSA (OG-BSA); both are the same for all the four conditions. 0, 2, 6, 14 and 27 h time points are reported

for the samples incubated with no MMP-7. 0, 2, 14 and 27 h time points are reported for the samples incubated with 0.9 and 9 nM MMP-7. 0, 0.5, 2, 6, 14, 18 and 27 h time points are reported for the samples incubated with 9 nM MMP-7.



Figure S8. Cytocompatibility study of cross-linked $5a_4$ nanogels after incubation with macrophages (RAW 264.7 cells) for 24 h (N = 3, n = 3).

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