Supporting Information:

Systematic study of the structural parameters affecting the self-assembly of cyclic peptide-poly(ethylene glycol) conjugates

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Experimental procedure

Materials and instrumentation

Chemicals and solvents were purchased from Fisher Scientific, Sigma-Aldrich, Merck, Fluka, and Acros. Fmoc protected amino acids and O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) were obtained from Iris Biotech GmbH (Germany). Amine functional poly(ethylene glycol) (PEG) was obtained from RAPP Polymere (Tübingen, Germany). Synthesis of linear peptide was performed on a Prelude automated peptide synthesizer from Gyros Protein technologies AB Ltd. (Tuscon (AZ), USA). The synthesis of chain transfer agent N-hydroxysuccinimide-(propanoic acid)yl butyl trithiocarbonate (PABTC) was carried out according to literature procedure.[25] Poly ethylene glycol methyl ether acrylate (PEGA) and 4,4′-Azobis(4-cyanovaleric acid) (ACVA) were obtained from Sigma-Aldrich.

^{1}H-NMR spectra were measured using a Bruker DPX-300 or DPX-400 NMR spectrometer which operated at 300.13 and 400.05 MHz, respectively. The residual solvent peaks were used as internal references.

Size exclusion chromatography (SEC) was carried out on a Polymer Laboratories PL-GPC 50 Plus system using a PolarGel-M guard column (7.5 × 50 mm) followed by two PolarGel-M

columns (7.5 × 300 mm). DMF (0.1% LiBr) was used as eluent at 1.0 mL min^{-1} at 50 °C. Commercial narrow linear poly(methyl methacrylate) standards in range of 2.0 × 10^2−1.0 × 10^6 g mol^{-1} were used to calibrate the DMF SEC system.

Analyte samples were filtered through a nylon membrane with 0.22 μm pore size before injection. Respectively, experimental molar mass (M_n, SEC) and dispersity (Đ) values of synthesized polymers were determined by conventional calibration using Agilent GPC/SEC software.

Electrospray Ionisation (ESI) measurements were obtained using a Bruker MicroToF and the results analysed using Bruker Data Analysis. Samples were dissolved in methanol at a concentration of 1 μg mL^{-1}.

The data presented was carried out over two separate SANS experiments, one on SANS2D at the ISIS Pulsed Neutron Source (STFC Rutherford Appleton Laboratory, Didcot, U.K.),[1,2] and the other using D11 at the Institut Laue Langevin (ILL, Grenoble, France).[3]
Prior to measurement, each sample was dissolved in the respective deuterated solvent and placed in 1 or 2 mm quartz Hellma cuvettes. The scattering cross-section was measured over a Q-range of 0.004 - 0.7 $Å^{-1}$ (SANS2D), or 0.006 – 0.24 $Å^{-1}$ (D11), where Q is defined as:

$$Q = \frac{4\pi \sin\frac{\theta}{2}}{\lambda} \quad (1)$$

Here, θ is the scattered angle, and λ is the incident neutron wavelength.

Using SANS2D, a simultaneous Q-range of 0.0045 – 0.7 $Å^{-1}$ was achieved utilizing an incident wavelength range of 1.75 – 16.5 Å and employing an instrument set up of L1=L2=4m, with the 1 m^2 detector offset vertically 60 mm and sideways 100 mm. The beam diameter was 8 mm. Each raw scattering data set was corrected for the detector efficiencies, sample transmission and background scattering and converted to scattering cross-section data (∂Σ/∂Ω vs. Q) using the instrument-specific software.[4] These data were placed on an absolute scale (cm^{-1}) using the scattering from a standard sample (a solid blend of hydrogenous and perdeuterated polystyrene) in accordance with established procedures.[5]

Additional experiments were performed on the D11 instrument at the Institut Laue–Langevin, Grenoble (DOI: 10.5291/ILL-DATA.9-13-668). An incident wavelength of 8 Å coupled with a detector distances of 1.2 m, 8 m and 28 m were used to cover a Q-range of 2 x10^{-3} to 0.3 $Å^{-1}$. Data were recorded on a 2D ^{3}He detector. In all cases, they were radial averaged and corrected for transmission, detector efficiency and a background of pure D_2O. The resulting data were converted into a scattering cross-section (∂Σ/∂Ω vs. Q), and placed

on an absolute scale (I(Q)). Prior to any analysis, the incoherent background was subtracted off.

In addition, we have expressed the SANS results in terms of M_a using equation S1. For SANS the constant K is given by equation 2:

$$K_{SANS} = \frac{1}{N_A} \times \left(\frac{\rho_{\text{solute}} - \rho_{solvent}}{d}\right)^2 \quad (2)$$

where d is the density of the solution. ρ_{solute} is the scattering length density for the polymer and has been computed according to its chemical structure.
The obtained reduced data was analysed with the open access software SASfit.[6]

The best fits for the nanotubes were obtained from a form factor for a cylindrical micelle (CYL+CHAINS). SLD values were calculated using based on the molecular structure of each unimer, and the V_{brush} value was calculated by dividing the Mw of the polymer by Avogadro's number multiplied by the density. In all cases the Rcore value was fixed at 5 Å, representing the radius of the cyclic peptide itself. To determine the N_{agg}, the length of the tube calculated by the CYL+CHAINS model was divided by 4.7 Å, the distance between two cyclic peptide unimers.

*General procedure for the synthesis of linear peptides (**1**, **4**, **7**)*
All peptides were synthesised *via* fully automatic Solid Phase Peptide Synthesis (SPPS) in a DMF/DCM solvent system using Fmoc protected amino acids on a 2-Chlorotrityl resin (1.1 mmol g^{-1} loading capacity). The concentration of amino acids during coupling was 0.2 mol L^{-1} and HCTU/*N*-methyl morpholine (NMM) (0.2 mol L^{-1}/0.4 mol L^{-1}) was used as activation agent. The Fmoc deprotection was conducted using 20% piperidine in DMF. Cleavage of protected peptides from the resin was carried out using a 20% HFIP in DCM solution. The cleavage mixture was evaporated under reduced pressure and the linear peptide was dried under vacuum.
^{1}H-NMR (300 MHz, TFA-d, **1**): δ (ppm) = 8.08-8.00 (m, 3 H, Trp (C-CH-N), 7.53-7.15 (m, 12 H, Trp (aromatic protons)), 5.11-4.98 (m, 3 H, Trp (peptide backbone)), 4.60-4.34 (m, 4 H, (Leu peptide backbone), 4.16 (t, 1 H, Lys (peptide backbone, N-terminus)), 3.27-2.99 (m, 8 H, (Lys (CH_2-CH_2-NHBoc); Trp (CH_2))), 2.05-1.89 (m, 2 H, Lys (CH_2-CH_2-CH_2NR)), 1.83-1.70 (q, 2 H, Lys (CH_2-CH_2-CH_2)), 1.60-1.40 (m, 38 H, Boc (CH_3); Lys (CH-CH_2-CH_2)), 1.28-1.04 (m, Leu (CH_2)) , 1.03-0.78 (m, 4 H, Leu (CH_2-CH-(CH_3)$_2$), 0.78-0.46 (m, 24 H, Leu, CH_3).
ESI-ToF-MS (MeOH, **1**): calculated: 1633.8085 m/z (M(COOK)+K^+), found: 1633.8413 m/z.

*General Procedure for the cyclization of linear peptides (**2**, **5**, **8**)*

Linear peptide (**1**, 560 mg, 0.36 mmol) was dissolved in DMF (400 mL) and 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoro borate (DMTMM · BF_4) (141.6 mg, 0.43 mmol, 1,2 eq.) dissolved in DMF (10 mL) was added under stirring. The mixture was left to stir at room temperature for 7 d. The solvent was evaporated under reduced pressure and the residual peptide was suspended in MeOH (100 mL). The compound was isolated by centrifugation and washed with MeOH three times using the same procedure. The cyclic peptide was dried under vacuum and obtained as a white solid (**2**, 250 mg, 45%)

^{1}H-NMR (300 MHz, TFA-d, **2**): δ (ppm) 8.36-8.31 (m, 3 H, Trp (C-CH-N), 7.69-7.11 (m, 12 H, Trp (arom. protons)), 5.32-5.00 (m, 3 H, Trp (peptide backbone)), 4.83-4.40 (m, 5 H, (Lys/Leu peptide backbone), 3.36-2.80 (m, 8 H, (Lys (CH_2-CH_2-NHBoc); Trp (CH_2))), 1.92 -1.60 (m, 2 H, Lys (CH_2-CH_2-CH_2NR)), 1.60-1.36 (q, 2 H, Lys (CH_2-CH_2-CH_2)), 1.36-0.44 (m, 74 H, Boc (CH_3), Lys (CH-CH_2-CH_2) (m, Leu (CH_2), Leu (CH_2-CH-(CH_3)$_2$), (Leu, CH_3)).

ESI-ToF-MS (MeOH, **2**): calculated: 1561.8681 m/z ($M+Na^+$), found: 1561.8896 m/z.

*General procedure of the deprotection of cyclic peptides (**3**, **6**, **9**)*

Cyclic peptide (**2**, 200 mg, 0.13 mmol) was dissolved in a mixture of TFA (9 mL), Triisopropyl silane (TIPS) (0.5 mL) and water (0.5 mL) and stirred at room temperature for 2 h. The peptide was precipitated in diethyl ether (100 mL) and isolated by centrifugation. The compound was washed with diethyl ether (2 x 100 mL) and dried under vacuum to yield cyclic deprotected peptide (**3**, 140 mg, 95%)

^{1}H-NMR (300 MHz, TFA-d, **3**): δ (ppm) = 7.70-6.92 (m, 15 H, Trp (arom. protons)), 6.73-6.53 (s, 5 H (amine)), 5.18-4.95 (m, 3 H, Trp (peptide backbone)), 4.70-4.36 (m, 5 H, (Leu peptide backbone), 3.32-2.95 (m, 8 H, (Lys (CH_2-CH_2-NHBoc); Trp (CH_2))), 1.68-1.56 (m, 6 H, Lys (CH_2)), 1.48-1.04 ((6 H, m, Leu (CH_2)) , 1.04-0.53 (m, 28 H, Leu (CH_2-CH-(CH_3)$_2$), (m, 24 H, Leu, CH_3).

ESI-ToF-MS (MeOH, **3**): calculated: 1177.6323 m/z ($M+K^+$), found: 1177.6460 m/z.

*General procedure of the conjugation of cyclic peptides and polymers (**10**, **11**, **12**)*

For polymer conjugation, cyclic peptide (**3**, 50 mg, 0.05 mmol) was dissolved in DMF (1 mL) and NMM (11 mg, 0.11 mmol, 2.5 eq.) was added. The solution was stirred for 30 min at room temperature and NHS functionalized PEG (2,000 g mol^{-1}) (220 mg, 0.11 mmol. 2.5 eq.) was added to the mixture. After 3 d the reaction mixture was diluted to 25 mL using water and the conjugate was isolated using centrifuge filter tubes (Amicon, Ultracel – 10K). After freeze drying the product was obtained as a white powder (**10**, 139 mg, 41%).

SEC (DMF (0.1% LiBr), PMMA standard): M_n = 4800 g mol^{-1}, $Đ$ = 1.04.

*Polymerization of PEG-acrylate using a RAFT methodology (**13**)*

Chain transfer agent (NHS-PABTC), monomer ($PEGA_{480}$, 1 mol L^{-1}), initiator (ACVA) and solvent (DMSO) were introduced into a flask equipped with a magnetic stirrer and sealed with a rubber septum (see Table S1 for detailed conditions). The solution was degassed by bubbling nitrogen through it for 15 min, and then put in an oil bath at 70°C for the indicated time. Conversions were determined by ^{1}H-NMR.

Table S1: Conditions for the polymerization of PPEGA using RAFT polymerization.

Sample	Composition	Time (h)	$\frac{[M]_0}{[CTA]_0}$	$\frac{[CTA]_0}{[ACVA]_0}$	Conversion (%)
13	$PPEGA_{10}$	18	12	100	82

*Conjugation of brush copolymers to cyclic peptides (**14**)*

Cyclic peptide and polymer (3 eq.) were solubilized in DMSO (1.5 mL). NMM (6 eq.) was added and the reaction mixture was left to stir at room temperature for 5 days. After the reaction, DMSO was removed using a stream of N_2 and the conjugates were dissolved in water and purified from the excess polymer using a centrifugal ultrafiltration unit with a molecular weight cut off of 30 kDa (Amicon® Ultra centrifugal filter). The isolated conjugates were freeze-dried.

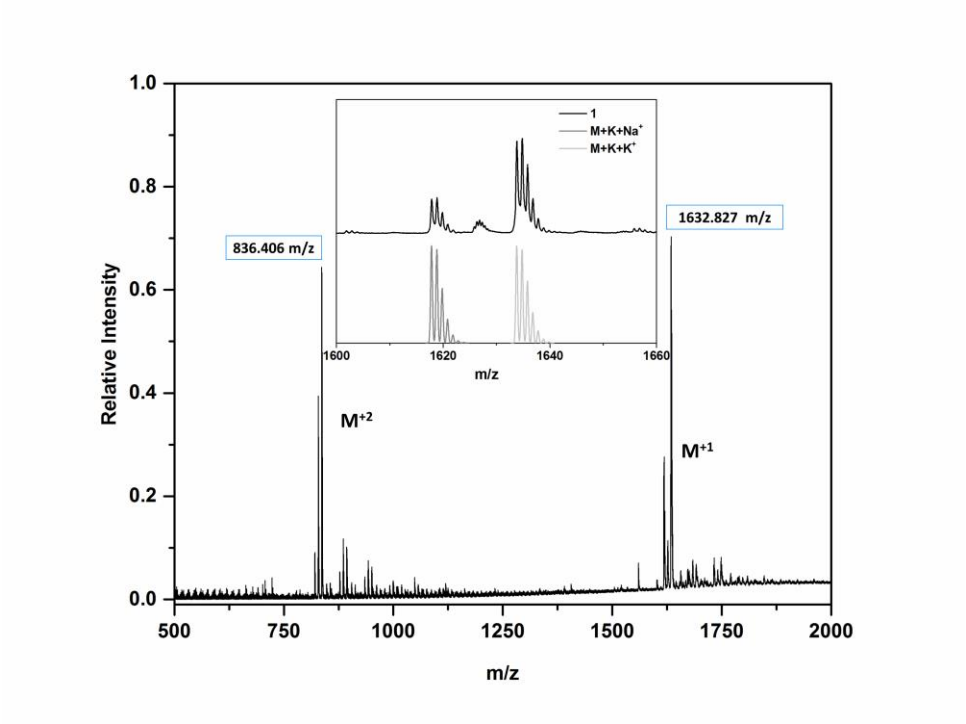

Figure S1: ESI-MS of linear peptide **1**.

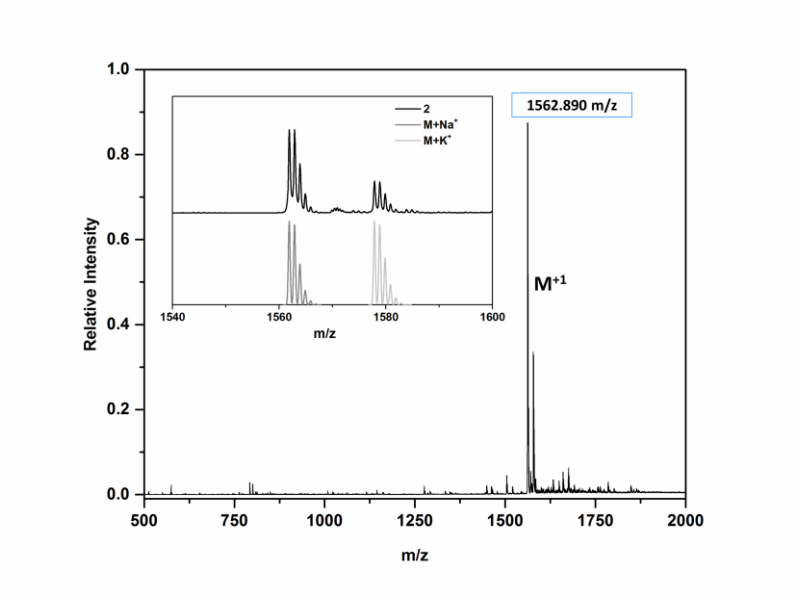

Figure S2: ESI-MS of cyclic peptide **2**.

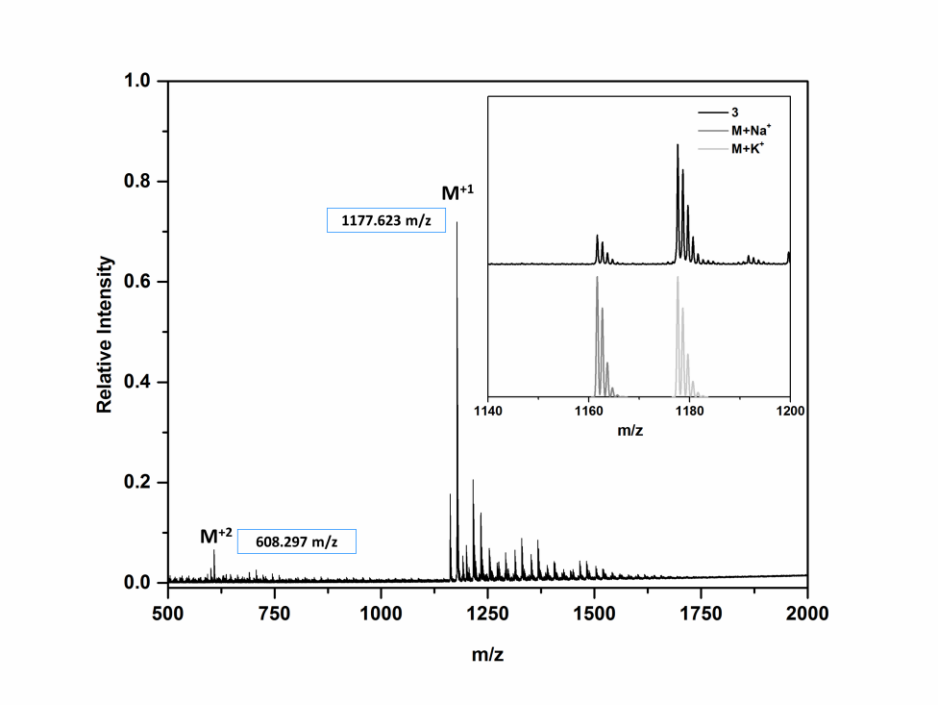

Figure S3: ESI-MS of cyclic deprotected peptide **3**.

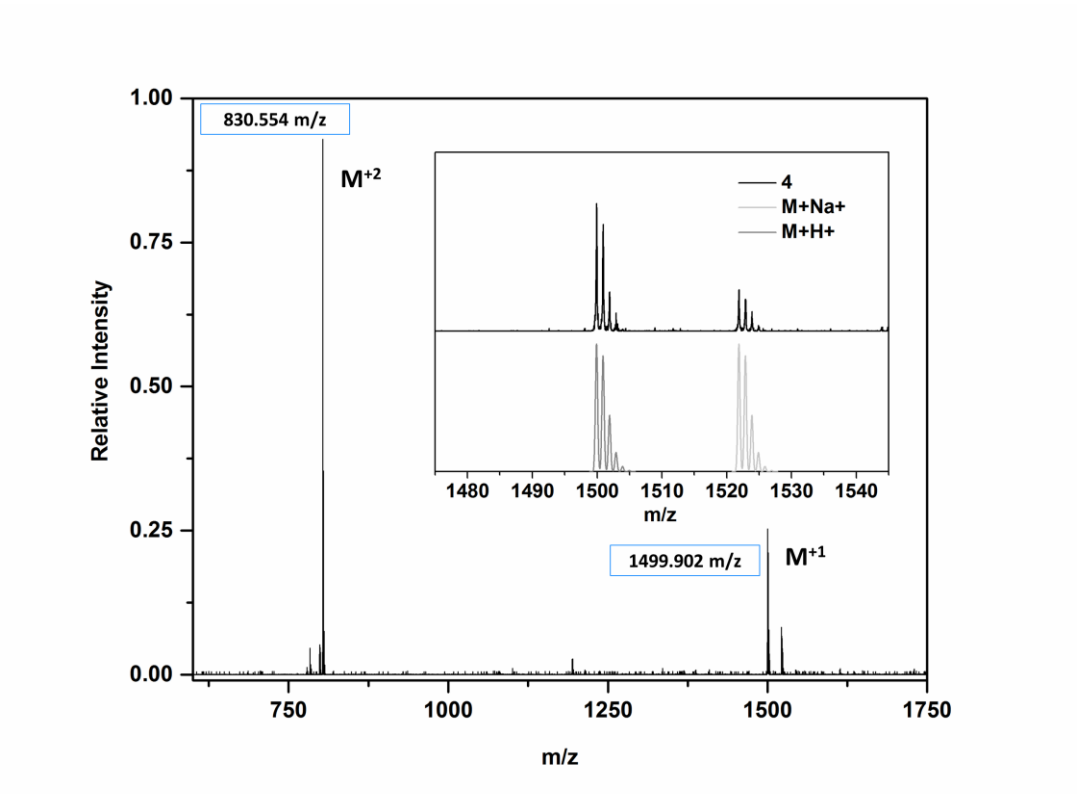

Figure S4: ESI-MS of linear peptide **4**.

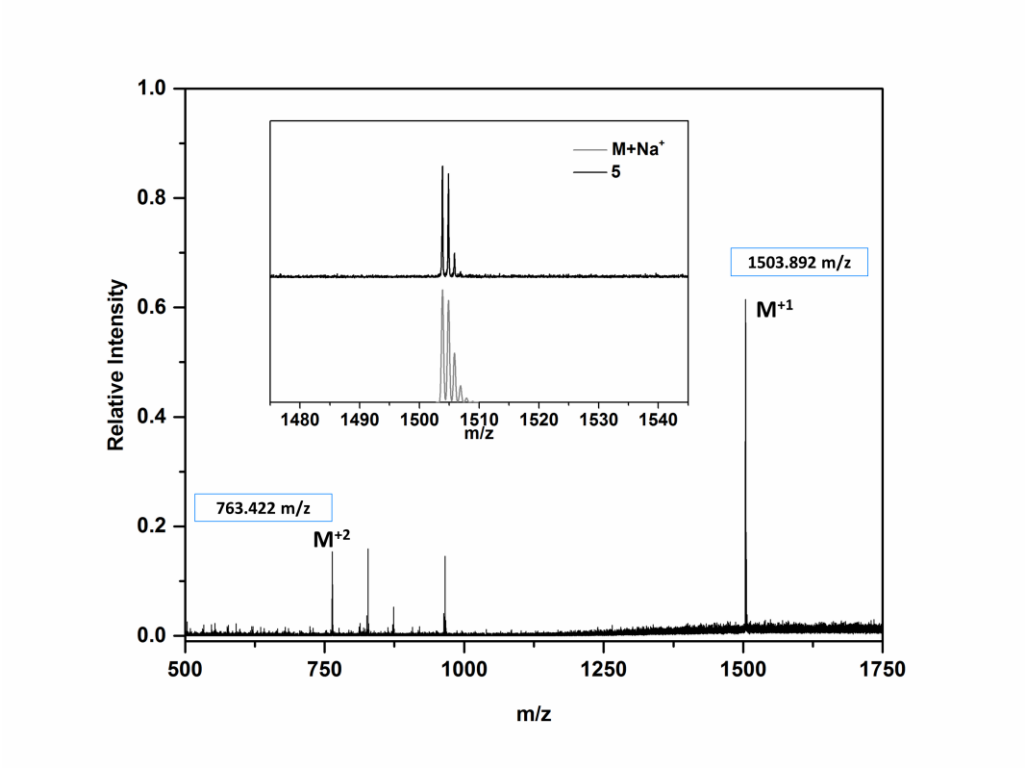

Figure S5: ESI-MS of cyclic peptide **5**.

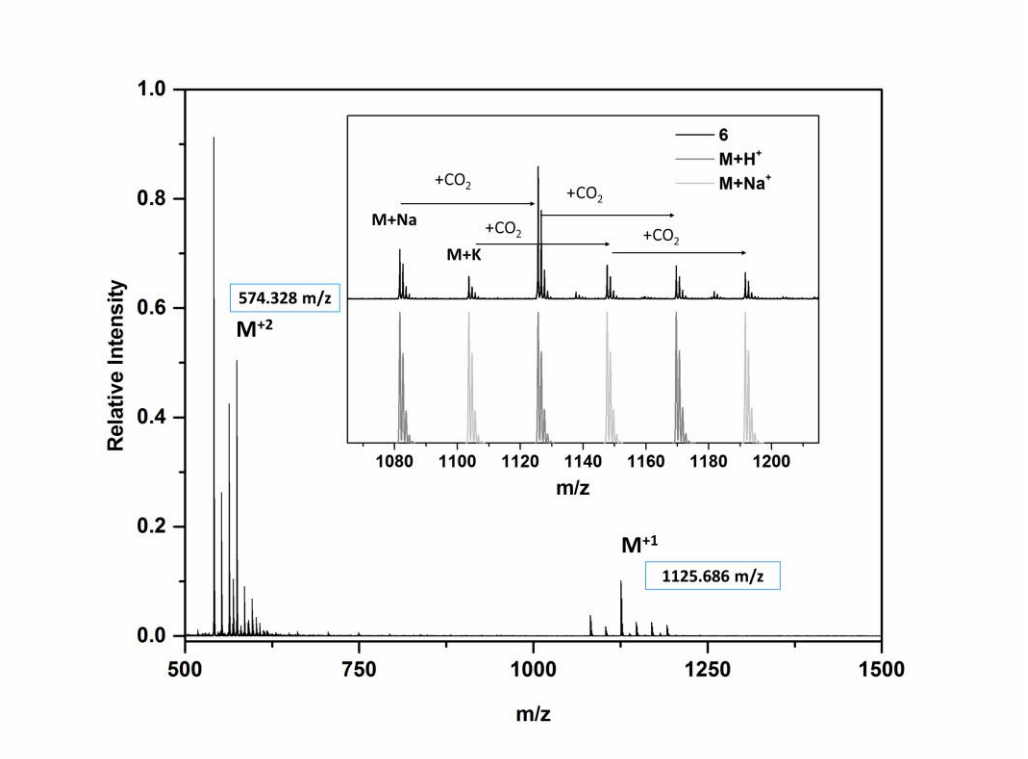

Figure S6: ESI-MS of cyclic deprotected peptide **6**.

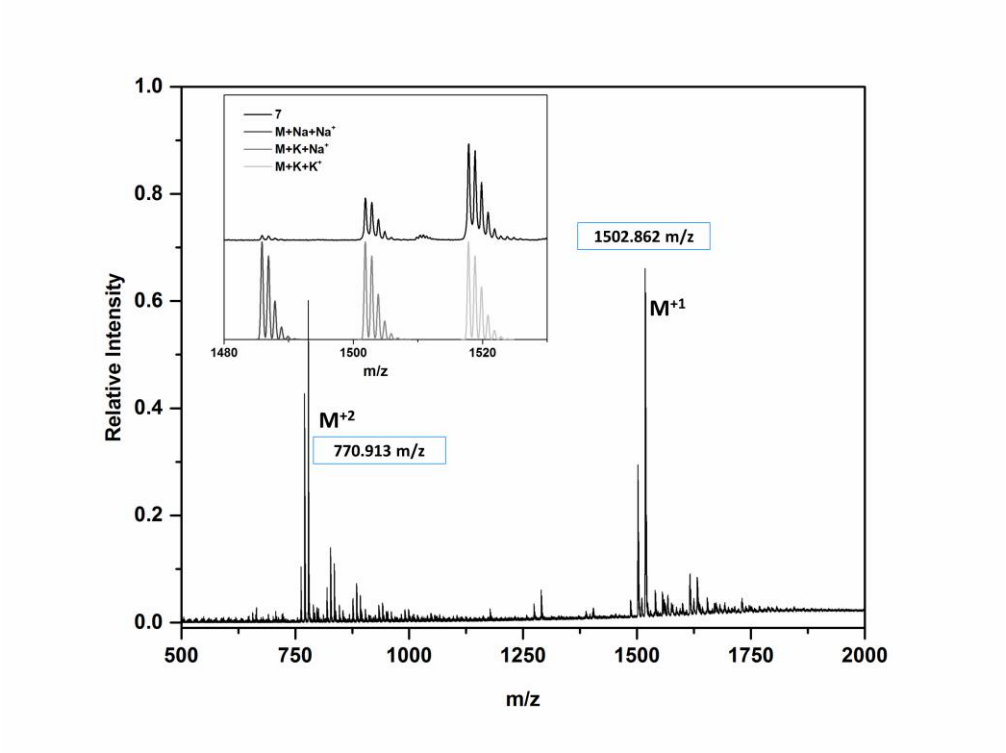

Figure S7: ESI-MS of linear peptide **7**.

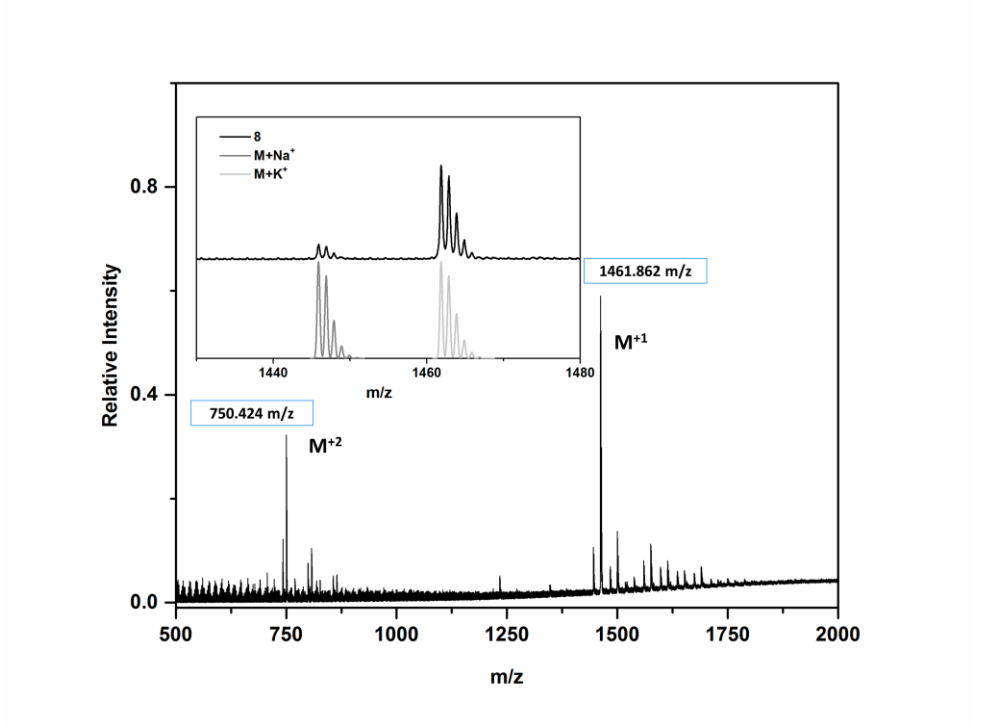

Figure S8: ESI-MS of cyclic peptide **8**.

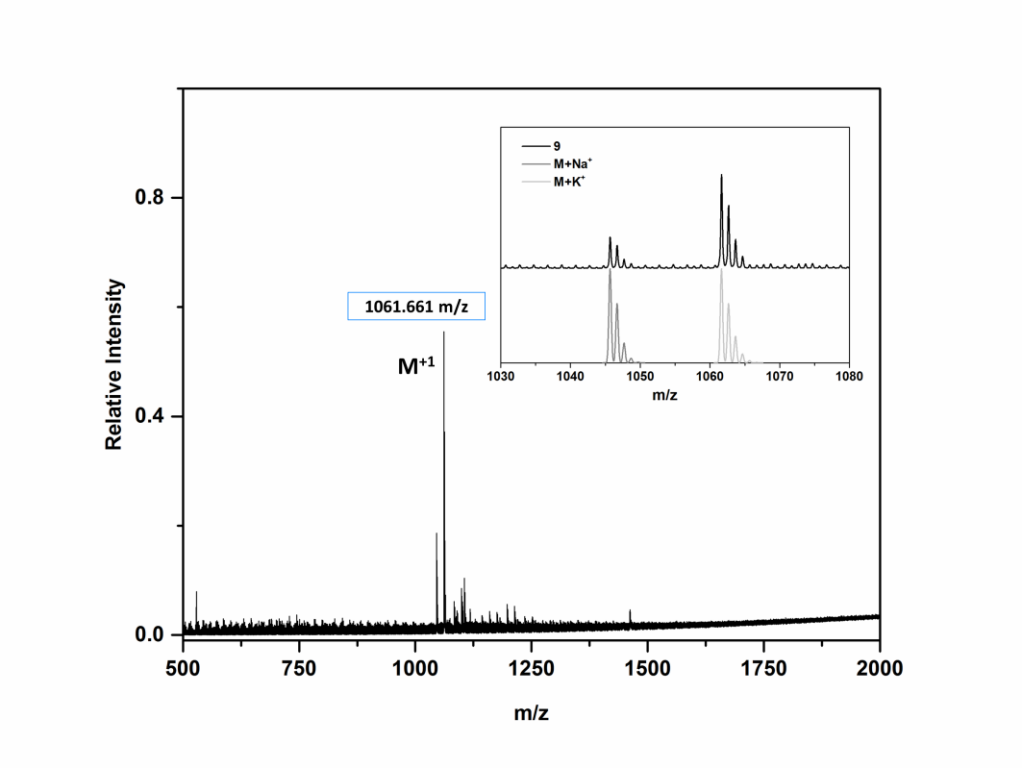

Figure S9: ESI-MS of cyclic deprotected peptide **9**.

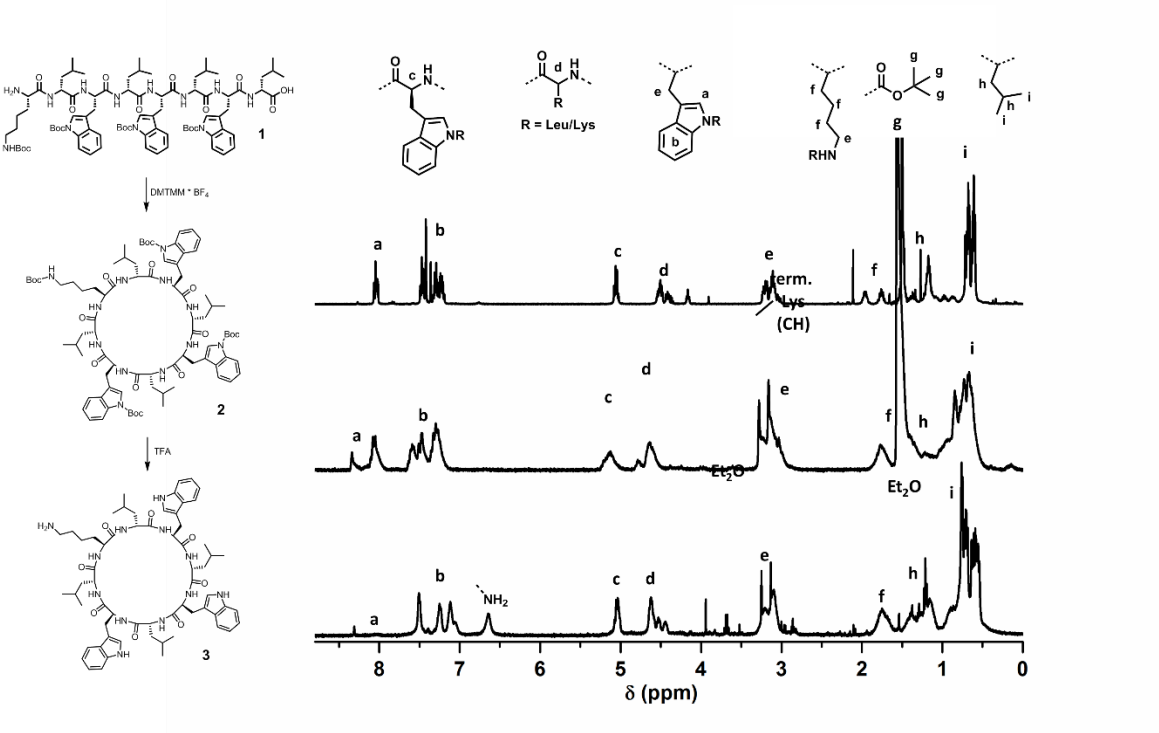

Figure S10: ^{1}H-NMR (TFA-d) spectra of linear, cyclic and deprotected peptide with one attachment site.

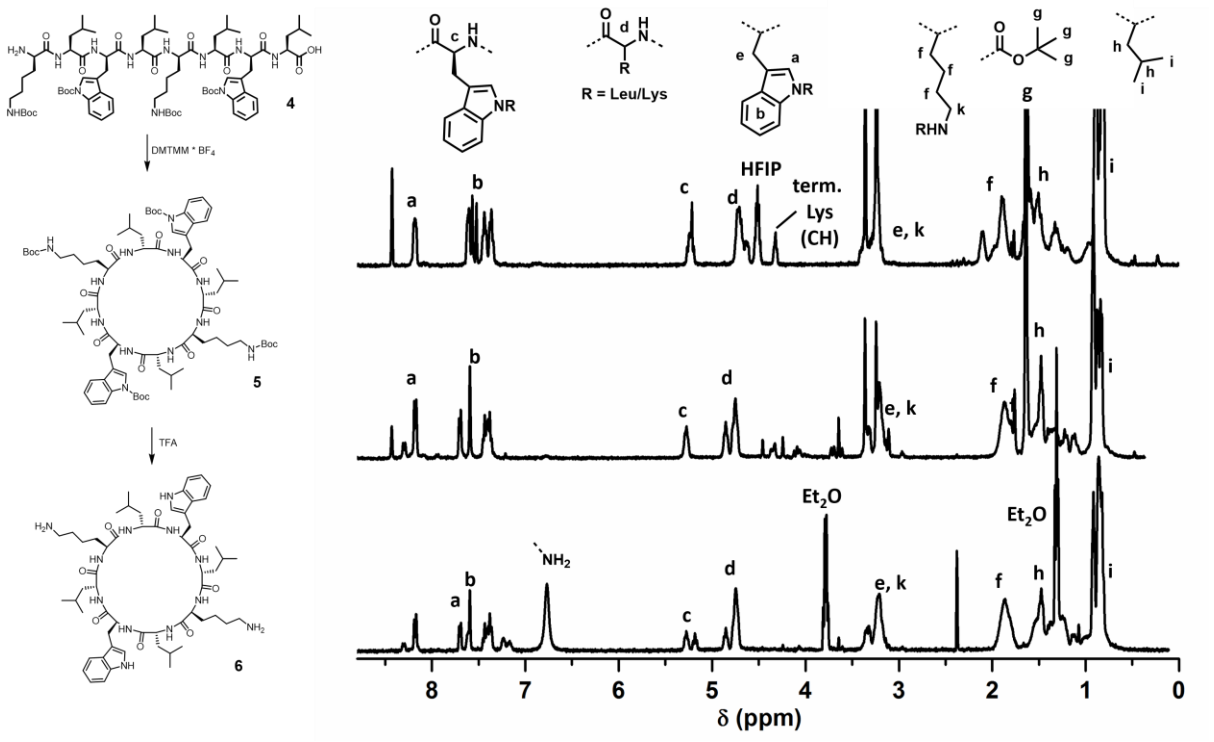

Figure S11: ^{1}H-NMR (TFA-d) spectra of linear, cyclic and deprotected peptide with two attachment sites.

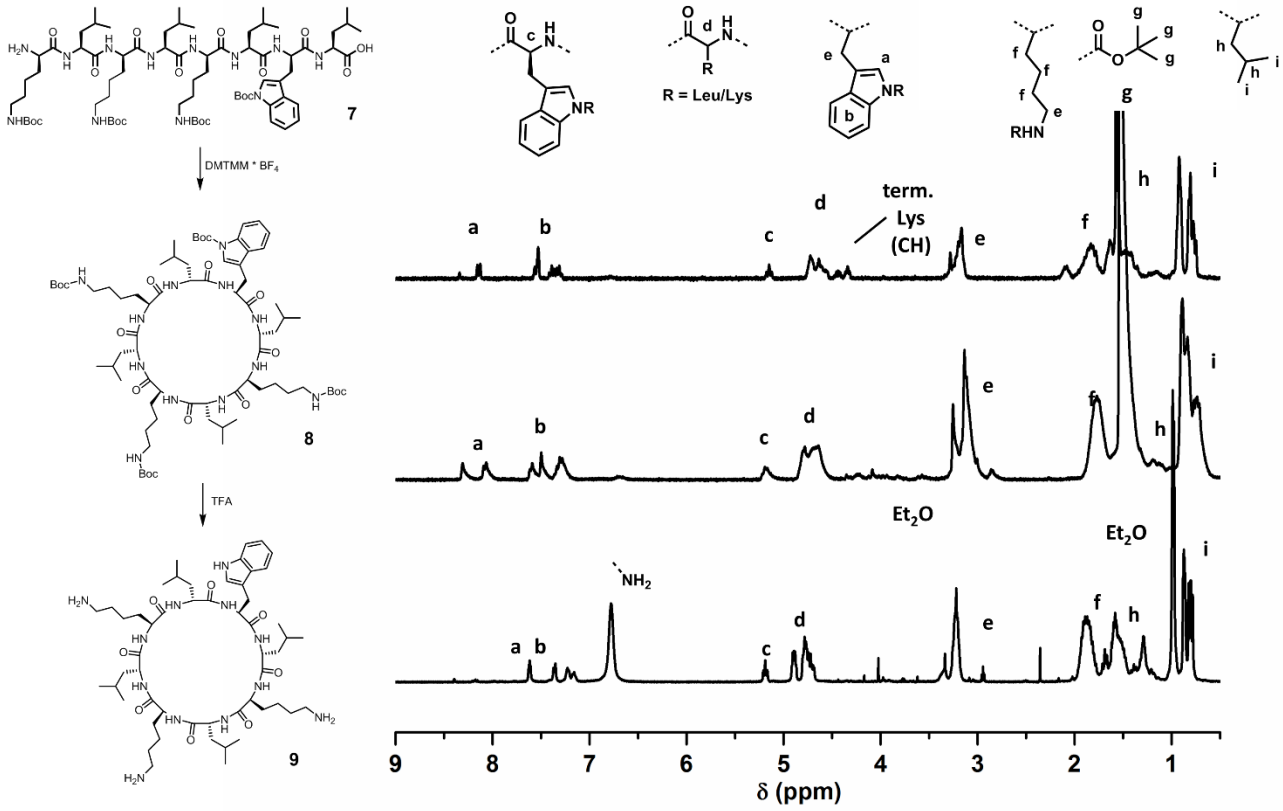

Figure S12: ^{1}H-NMR (TFA-d) spectra of linear, cyclic and deprotected peptide with three attachment sites.

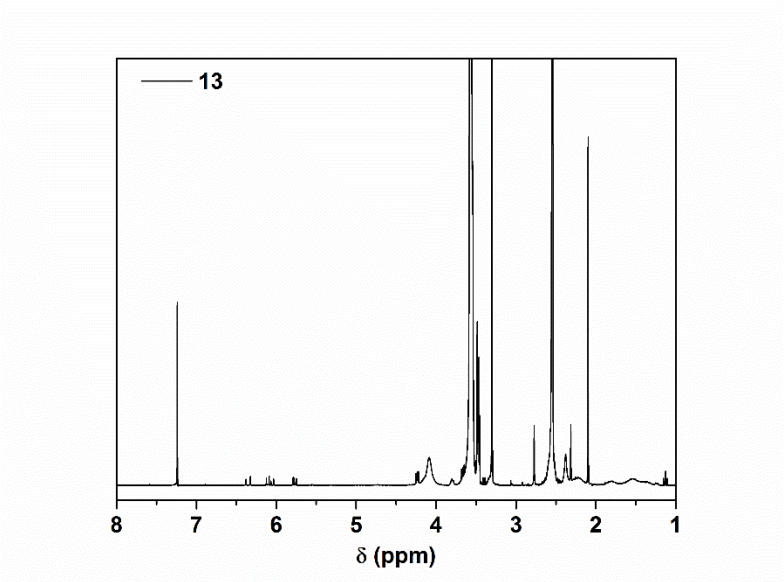

Figure S13: ^{1}H-NMR spectra of PPEGA brush copolymer in $CDCl_3$.

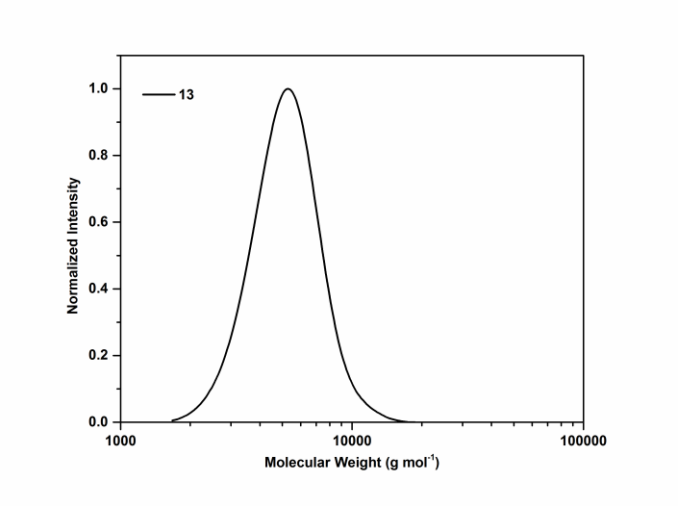

Figure S14: GPC trace of PPEGA brush copolymer using a DMF (0.1% LiBr) eluent at 1.0 mL min^{-1} at 50 °C. Commercial narrow linear poly(methyl methacrylate) were used as standards (2.0×10^{2}−1.0×10^{6} g mol^{-1})

Table S2: GPC-characterization data of PPEGA and cyclic peptide brush conjugates

Sample	**Composition**	$M_{n,\,th}$ (g mol^{-1})	M_n (g mol^{-1})	$Đ$

13	$PPEGA_{10}$	5100	4800	1.12
14	$CP\text{-}(PPEGA_{10})_2$	11200	10800	1.12

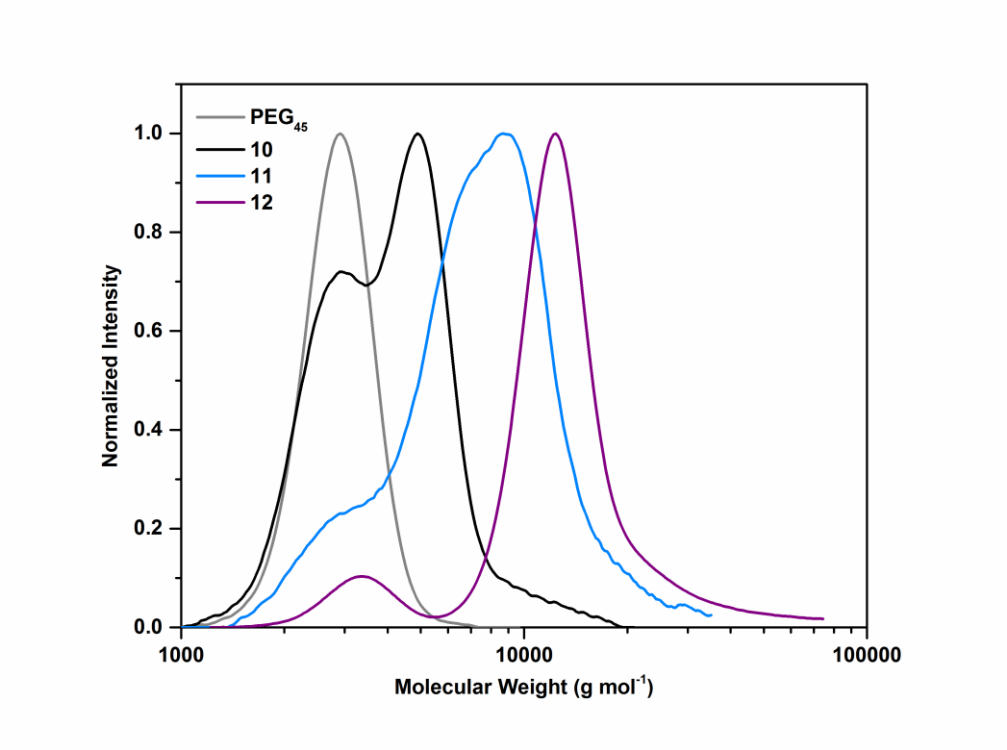

Figure S15: GPC analysis of PEG and PEG-peptide conjugates with a varying number of arms after 3 d of reaction from the reaction mixture (PMMA standard).

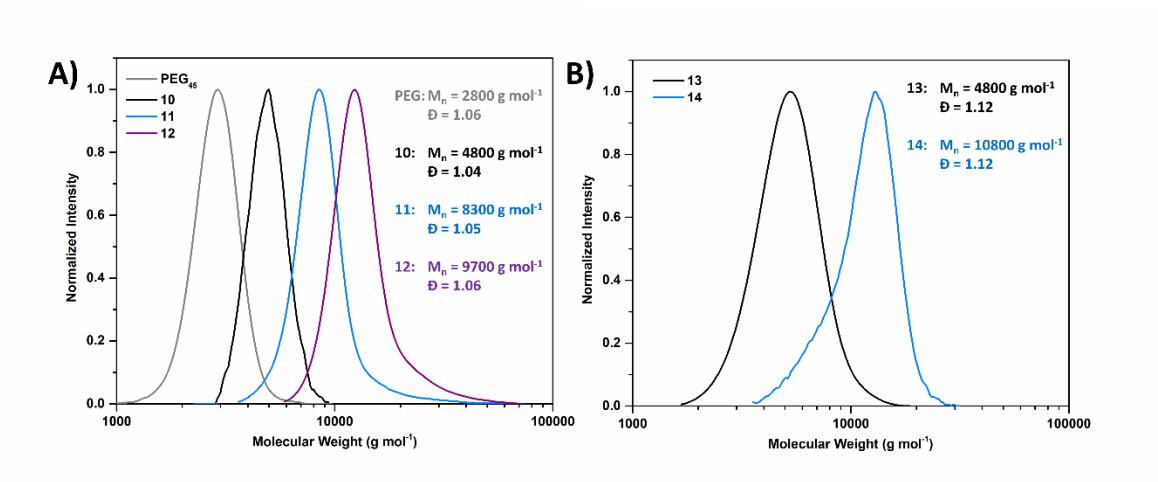

Figure S16: SEC analysis of A) linear PEG and CP-PEG conjugates having one, two, or three linear polymer arms (**10** – **12**); B) PPEGA-brush copolymers (**13**) and PPEGA-peptide conjugates (**14**). All measurements were carried out in a DMF/LiBr solvent system using a PMMA calibration. The poor resolution of compound **10** is a result of its high tendency to stack and associated low solubility in the eluent.

Table S3: Description of fitting parameters and results obtained for scattering of two-armed PPEGA (**11**) as well as linear PEG conjugate-cyclic peptide (**14**) in D_2O.

Sample		**11**	**14**
Concentration (g mL^{-1})		5	10
Model		Hairy cylinder*	Comb
Instrument		D11	D11
Hairy cylinder	**R (Å)**	5	-
	nagg	0.0325	-
	Vbrush (cm^3)	2700	-
	eta core (Å^{-2})	9.12E-7	-
	eta brush (Å^{-2})	6.22E-7	-
	eta Solvent (Å^{-2})	6.35E-6	-
	Rg (Å)	26.1	-
	d	1	-
	H (Å)	2000	-
	N	0.0865	-
Comb	**f**	-	60.85876442
	RgE (Å)	-	92
	Mw (g mol^{-1})	-	34000
	Rg (Å)	-	81.27208481
	Chi^2	4.5	1.5

Table S4: Description of fitting parameters and results obtained for scattering of a one-armed PEG conjugate-cyclic peptide (**10**) in different solvents.

10		**DMF**	**DMSO**	**D_2O**	**DCM**	**THF**
Concentration (g mL^{-1})		5	5	5	5	5
Model		Gaussian chain + extended Guinier	Gaussian chain + extended Guinier	Hairy cylinder	Hairy cylinder	Hairy cylinder + extended Guinier
Instrument		D11	D11	D11	D11	D11
Hairy cylinder	**R_{core} (Å)**	-	-	5	5	5
	nagg	-	-	0.0325	0.00567	0.00248
	Vbrush (cm^3)	-	-	2700	2700	2700
	eta core ($Å^{-2}$)	-	-	9.12E-7	9.12E-7	9.12E-07
	eta brush ($Å^{-2}$)	-	-	6.22E-7	6.22E-7	6.22E-07
	eta Solvent ($Å^{-2}$)	-	-	6.35E-6	2.56E-6	5.77E-06
	Rg (Å)	-	-	26.1	19.5	20.6
	d	-	-	1	1	1
	H (Å)	-	-	2000	177	239
	N	-	-	0.0865	0.09	0.05
Gaussian coil	**Rg (Å)**	28	21	-	-	-
	Mw (g mol^{-1})	5400	3600	-	-	-
	Vp (cm^3)	7.47508E-21	4.98339E-21	-	-	-
Extended Guinier	**I0**	0.00786	0.0167	-	-	0.0203
	α	2	2	-	-	2
	Rg	24.8	97.7	-	-	29.7
	N	0.00101	0.000144	-	-	0.246
	Chi^2	1.5	1.2	4.5	1.8	2

Table S5: Description of fitting parameters and results obtained for scattering of a two-armed PEG conjugate-cyclic peptide (**11**) in different solvents.

11		**DMF**	**DMSO**	**D_2O**	**DCM**	**THF**	**Toluene**
Concentration (g mL^{-1})		3.5	2.5	5	5	5	5
Model		Gaussian chain	Gaussian chain	Hairy cylinder + extended Guinier	Hairy cylinder	Hairy cylinder + extended Guinier	Hairy cylinder
Instrument		SANS2D	SANS2D	D11	D11	SANS2D	D11
Hairy cylinder	**R_{core} (Å)**	-	-	5	5	5	5
	nagg	-	-	0.0437	0.00849	0.0083	0.0203
	Vbrush (cm^3)	-	-	2700	2700	2700	2700
	eta core ($Å^{-2}$)	-	-	8.21E-07	8.21E-07	8.21E-07	8.21E-07
	eta brush ($Å^{-2}$)	-	-	6.22E-07	6.22E-07	6.22E-07	6.22E-07
	eta Solvant ($Å^{-2}$)	-	-	6.35E-06	2.56E-06	5.77E-06	4.57E-06
	Rg (Å)	-	-	18.5	18.21	19.2	19.1
	d	-	-	1	1	1	1
	H (Å)	-	-	106	94	2000	2000
	N	-	-	0.0136	0.76452	0.308	0.123
Gaussian coil	**Rg (Å)**	35	35	-	-	-	-
	Mw (g mol^{-1})	6500	3500	-	-	-	-
	Vp (cm^3)	1.08E-20	5.81E-21	-	-	-	-
Extended Guinier	**I0**	-	-	0.078	-	0.0785	-
	α	-	-	2	-	2	-
	Rg	-	-	165	-	17.2	-
	N	-	-	0.0000538	-	0.000394	-
	Chi^2	1	22	3.1	1.7	3.5	1.7

Table S6: Description of fitting parameters and results obtained for scattering of a three-armed PEG conjugate-cyclic peptide in different solvents

12		DMF	DMSO	D_2O	DCM	THF	Toluene
Concentration ($g\ mL^{-1}$)		5	5	5	5	5	5
Model		Gaussian chain	Gaussian chain + extended Guinier	Hairy cylinder + extended Guinier	Hairy cylinder	Hairy cylinder	Hairy cylinder + extended Guinier
Instrument		D11	D11	D11	D11	D11	D11
Hairy cylinder	**R_{core} (Å)**	-	-	5	5	5	5
	nagg	-	-	0.00563	0.00557	0.025	0.0195
	Vbrush (cm^3)	-	-	2700	2700	2700	2700
	eta core ($Å^{-2}$)	-	-	7.15E-07	7.15E-07	7.17E-07	7.17E-06
	eta brush ($Å^{-2}$)	-	-	6.22E-07	6.22E-07	6.22E-07	6.22E-06
	eta Solvant ($Å^{-2}$)	-	-	6.35E-06	2.56E-06	5.77E-06	4.57E-06
	Rg (Å)	-	-	12.2	21.5	16.8	16.6
	d	-	-	1	1	1	1
	H (Å)	-	-	66.3	143	592	2000
	N	-	-	0.0231	0.088	0.507	0.115
Gaussian coil	**Rg (Å)**	28	33	-	-	-	-
	Mw ($g\ mol^{-1}$)	5400	8500	-	-	-	-
	Vp (cm^3)	7.47508E-21	1.17663E-20	-	-	-	-
Extended Guinier	**I0**	-	0.00947	16.536	-	-	0.111
	α	-	2	2	-	-	2
	Rg	-	90.2	177	-	-	69.7
	N	-	0.000117	0.000106	-	-	0.00227
	Chi^2	1.6	1.5	1.5	2	6.4	1.8

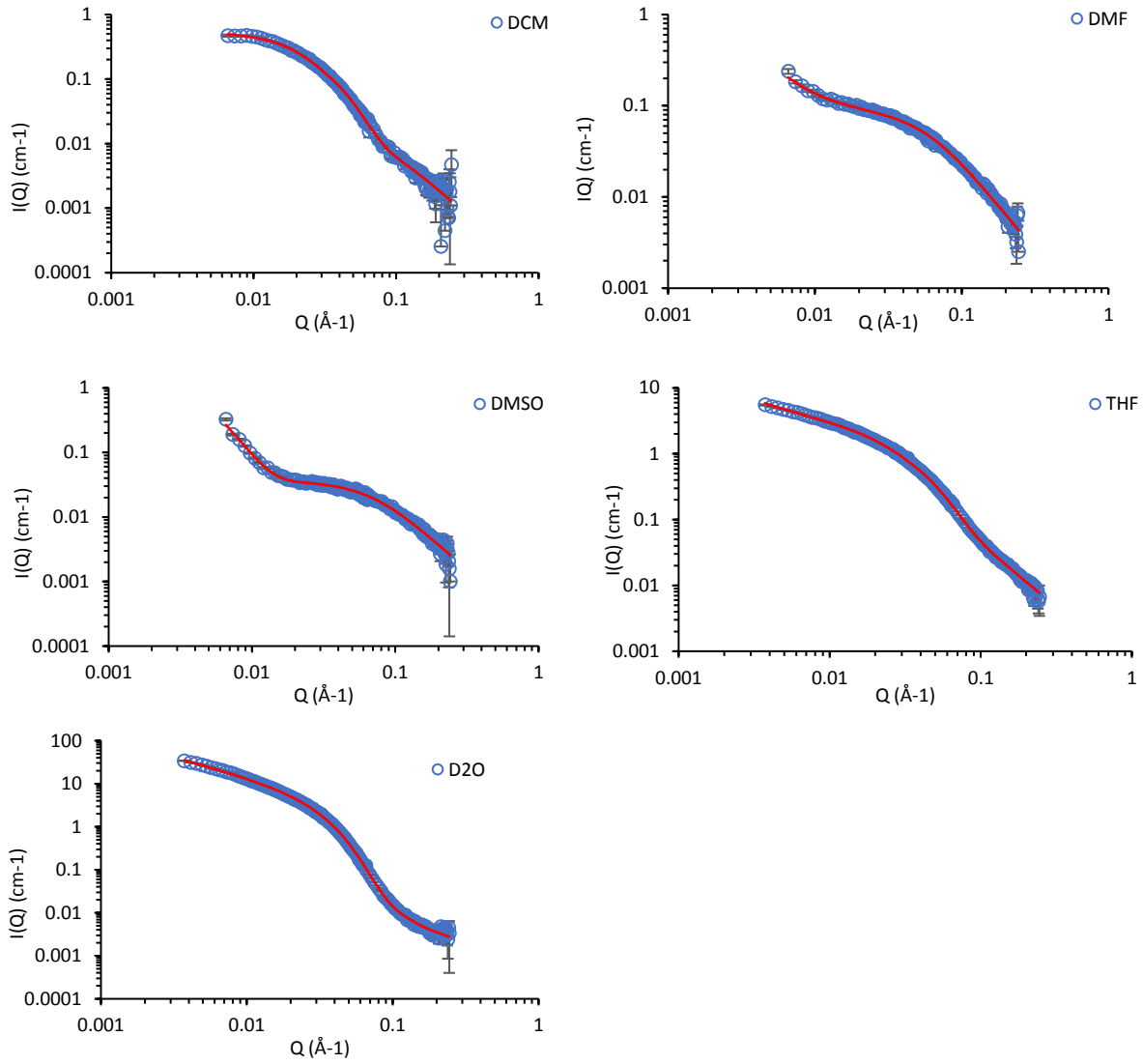

Figure S17: SANS scattering profiles for a PEG-conjugated one-armed cyclic peptide in various solvents.

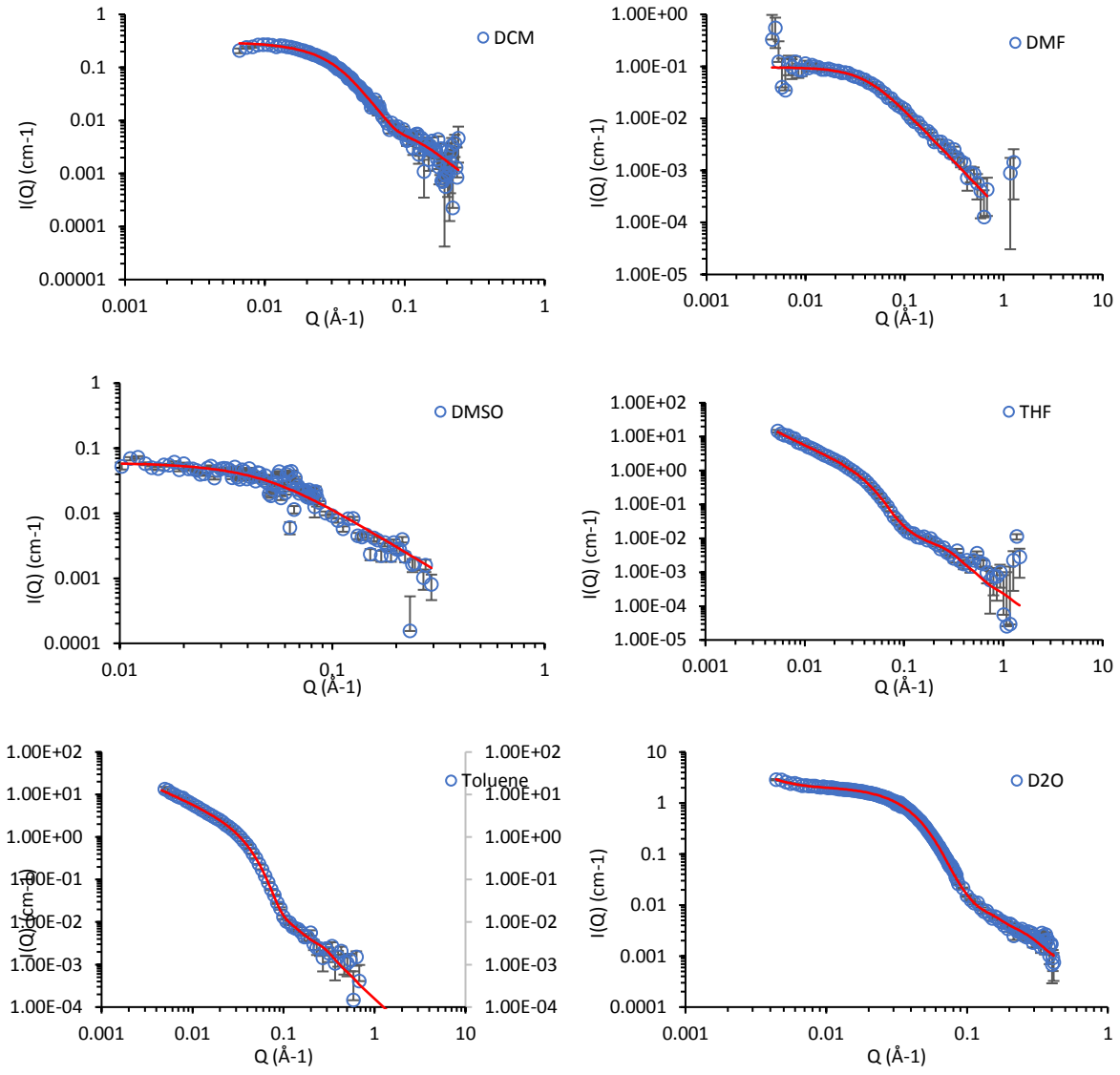

Figure S18: SANS scattering profiles for a PEG-conjugated two-armed cyclic peptide in various solvents.

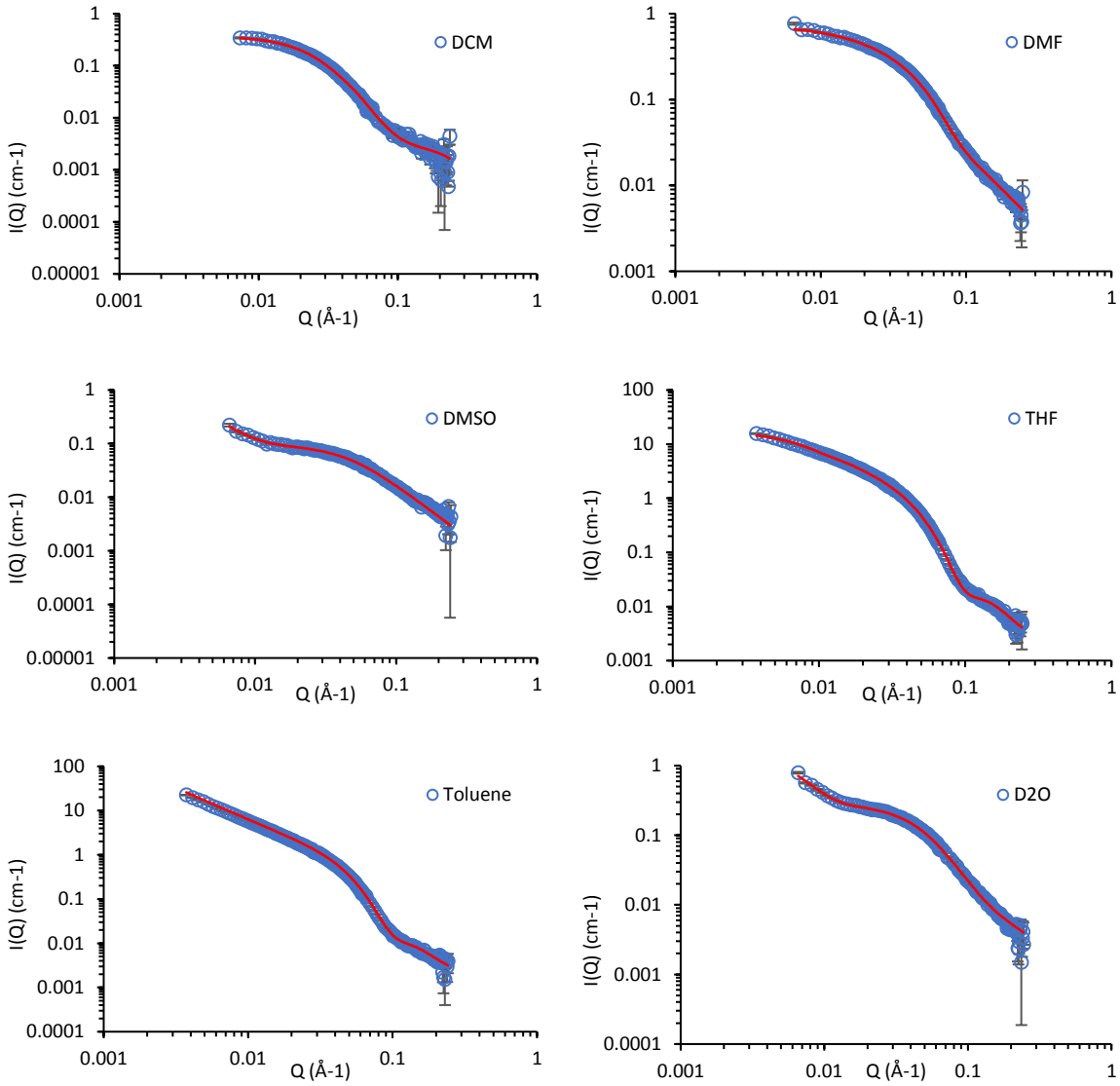

Figure S19: SANS scattering profiles for a PEG-conjugated three-armed cyclic peptide in various deuterated solvents.

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