Supplementary

Chemicals

Commercially available chemicals were purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA), Merck KGaA (Darmstadt, Hesse, Germany), GL Biochem (Shanghai, China). (9-Fluorenylmethyloxycarbonyl)amino-4-azido-butanoic acid (β-homo-azidoalanine) was purchased from Iris Biotech Gmbh (Marktredwitz, Germany). $^{i}Pr_2EtN$ was distilled from CaH; DMF was stored over 4 Å molecular sieves.

Mass spectrometry

Mass spectra were acquired using an Agilent 1100 MSD SL ion trap mass spectrometer (Agilent Technologies, Santa Clara, California, USA).

High performance liquid chromatography

Preparative High-Performance Liquid Chromatography (HPLC) was performed using an Agilent 1200 series HPLC system. Samples were injected onto a reverse-phase preparative (C18, 300 Å, 5 μm, 10 mm × 250 mm) column and detected at wavelengths of 214 nm and 280 nm. Analytical HPLC was performed using an Agilent 1100 series HPLC system. The samples were injected onto a reverse-phase analytical (C18, 300 Å, 5 μm, 4.6 mm × 150 mm) column and detected at a wavelength of 214 nm.

Synthesis

N-acetylated functional lipidated β3-peptides

The peptides were synthesised on a 0.3 mmol scale using standard Fmoc chemistry on Wang resin (1 mmol/g loading). The resin was swollen in DMF (3 mL) and then soaked in Fmoc-protected β-homo-lysine (2.1 eq. to resin loading), dissolved in DMF (3 mL) along with HATU (2 eq. to resin loading), DMAP (10 mol %) and DIPEA (3 eq. to resin loading), overnight with gentle agitation. The resin was thoroughly washed with DMF (3×3 mL) and the Fmoc protecting group on the amino acid was removed by soaking the resin twice in 20 % piperidine in DMF (3 mL) for 15 minutes each. The resin was washed with DMF (3×3 mL), soaked in (R)-N-Fmoc α-aspartic acid (allyloxycarbonyl)-aminoethyl amide (The synthesis of this novel alloc-protected β-amino acid is published [1]) (2.1 eq. to resin loading), dissolved in DMF (3 mL) along with HATU (2 eq. to resin loading), and DIPEA (3 eq. to resin loading), for 2 hours. β-peptide elongation cycle was then repeated to add β-homo-azidoalanine to the sequence. After removing the terminal Fmoc protecting group on the peptide, the resin was treated with a solution of 10 % v/v acetic anhydride and 2.5 % v/v DIPEA in DMF (3 mL) for 30 minutes to afford an acetyl-capped N-terminus. The resin was washed with DMF (2×3 mL), CH_2Cl_2 (2×3

mL), Et_2O (2×3 mL), air dried for 10 minutes, and transferred to a 15 mL vial for further manipulation.

Lipidation

To facilitate attachment of the desired aliphatic chain, reduction of the azidoalanine residue was performed on solid support. The resin (0.3 mmol) was swollen in DMF (2 mL) and then soaked in a solution of dithiothreitol (1.23 g, 8 mmol) in DMF (3 mL) and DIPEA (700 µL, 4 mmol), overnight at room temperature with gentle agitation. The resin was washed with DMF (2×3 mL), CH_2Cl_2 (2×3 mL), Et_2O (2×3 mL) and air dried for 10 minutes. The resin was swollen in DMF (2 mL), and the reduction cycle was repeated with a fresh batch of dithiothreitol (1.23 g, 8 mmol) in DMF (3 mL) and DIPEA (700 µL, 4 mmol). The resin was washed with DMF (2×3 mL), then soaked in palmitic acid (2 eq. to resin loading), dissolved in DMF (5 mL) along with HATU (2 eq. to resin loading), and DIPEA (3 eq. to resin loading), for 2 hours. The resin was subsequently washed with DMF (2×3 mL), CH_2Cl_2 (2×3 mL), Et_2O (2×3 mL), air dried for 10 minutes, and transferred to a 15 mL vial for further manipulation.

β3-peptide functionalisation with α-peptide epitopes

Functionalisation of the β^3-peptide backbone with α-peptide on solid support was preceded by the selective cleavage of the allyloxycarbonyl substituent. To a 50 mL vial was added $CHCl_3$ (15 mL) which was rigorously degassed by bubbling a stream of argon. A portion of the degassed $CHCl_3$ (~2 mL) was then used to swell the resin. $PhSiH_3$ (700 µL) was added to the remaining $CHCl_3$ (~10 mL) whilst still bubbling with a stream of argon. $Pd(PPh_3)_4$ (575 mg, 0.5 mmol) was then added and the mixture was shaken gently until a homogeneous solution was achieved. The resin was then soaked in the $Pd(PPh_3)_4$ solution for 2 hours, with gentle agitation, and washed with CH_2Cl_2 (3×3 mL) and DMF (3×3 mL) to remove the catalyst.

The resin was soaked in Fmoc-protected α-amino acid (3.1 eq. to resin loading), dissolved in DMF (3 mL) along with HATU (3 eq. to resin loading), and DIPEA (4.5 eq. to resin loading), for an hour. The resin was thoroughly washed with DMF (3×3 mL) and the Fmoc protecting group on the amino acid was removed by soaking the resin twice in 20 % piperidine in DMF (3 mL) for 15 minutes each. α-Peptide elongation cycle was then repeated until the sequence was complete, with double coupling for α-arginine.

After removing the terminal Fmoc protecting group on the peptide, the resin was treated with a solution of 10 % v/v acetic anhydride and 2.5 % v/v DIPEA in DMF (3 mL) for 30 minutes to afford an acetyl-capped N-terminus. The resin was washed with DMF (2×3 mL), CH_2Cl_2 (2×3 mL), Et_2O (2×3 mL), air dried for 10 minutes and transferred to a 15 mL vial for cleavage.

Cleavage of functional lipidated β3-peptide from resin

Cleavage was performed on resin (0.3 mmol), by treating the resin with a cleavage solution (10 mL) comprising of H_2O (2.5 % v/v), triisopropylsilane (2.5 % v/v) and 3,6-dioxa-1,8-octanedithiol (0.5 % v/v) in CF_3COOH, for 3 hours. CF_3COOH was then evaporated under a stream of N_2, and the peptide was precipitated by addition of Et_2O (50 mL). The precipitate was filtered and lyophilised for purification.

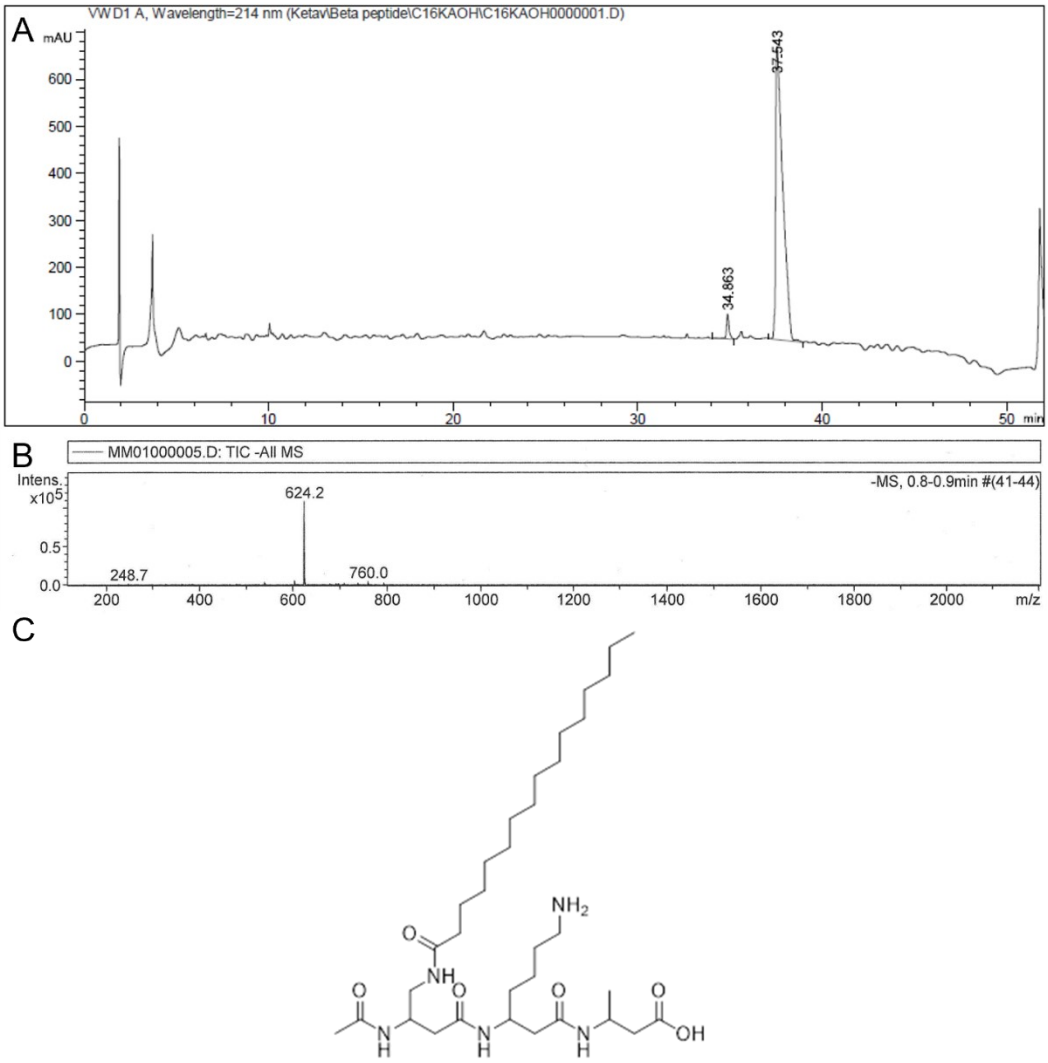
Lactonisation

This was performed for peptides that had β-homoserine as the C terminal amino acid residue. The crude lyophilised peptide (Supp. 3) was dissolved in CF_3COOH (10 mL). After 30 mins, the acid was evaporated by bubbling a stream of N_2 and the neat residue (without addition or dilution with H_2O) lyophilised immediately.

Purification of functional lipidated β3-peptides

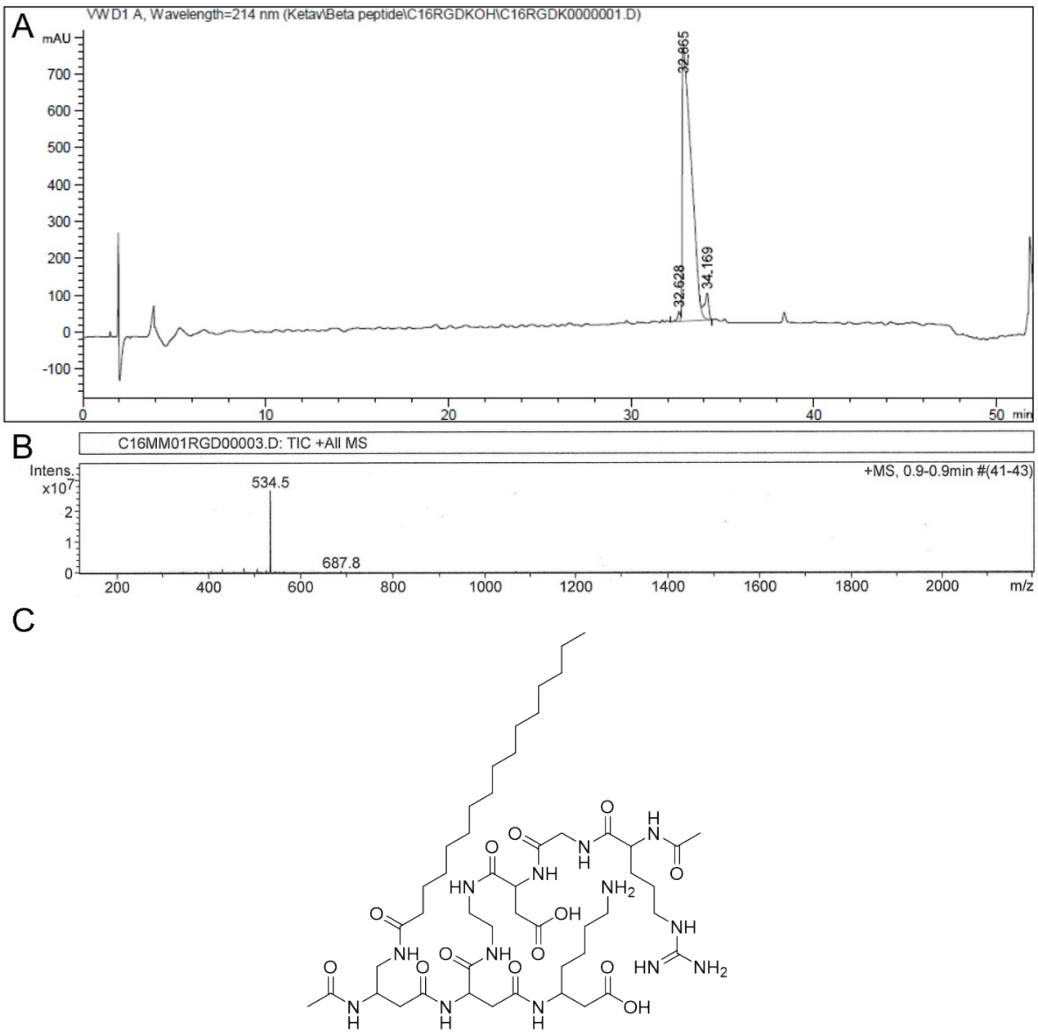
The peptide was redissolved in 50 % aqueous CH_3CN (5 mL) and purified by injecting the sample onto a reverse-phase preparative column, eluted over a 55 min gradient from 20 to 85 % solvent B, (solvent A: 0.1 % CF_3COOH/H_2O; solvent B: 0.1 % CF_3COOH/CH_3CN) with a flow rate of 5 mL/min. The fractions were collected and analysed for purity by injecting the samples onto a reverse-phase analytical column, eluted over a 45 min gradient from 0 to 70 % solvent B, (solvent A: 0.1 % CF_3COOH/H_2O; solvent B: 0.1 % CF_3COOH/CH_3CN) with a flow rate of 1 mL/min (Supp. 1, Supp. 2, Supp. 4).

HPLC showed purity greater than 95% for all peptides. Negative ion mode MS showed the correct mass for N-acetyl (C16) lipidated β^3-Tripeptide (m/z [-] 624.2), N-acetyl (C16) lipidated β^3-Tripeptide-RGD (m/z [2-] 534.5), N-acetyl (14) lipidated β^3-Tetrapeptide-acid (m/z [-] 697.2) and N-acetyl (14) lipidated β^3-Tetrapeptide-lactone (m/z [-] 681.2).



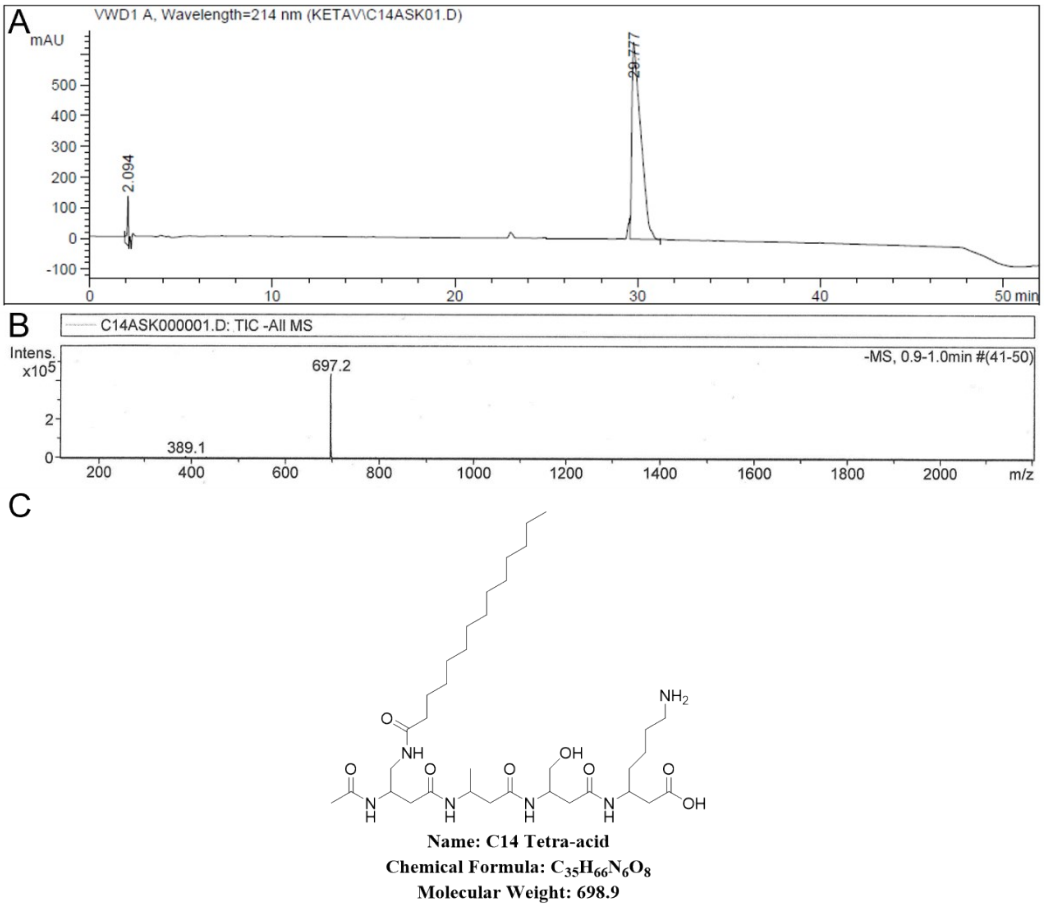
Name: C16 Tri-acid
Chemical Formula: $C_{33}H_{63}N_5O_6$
Molecular Weight: 625.9

Supp. 1 Characterisation of N-acetyl (C16) lipidated β3-Tripeptide (C16-Tripeptide). A) HPLC chromatogram. HPLC retention time was 37.5min with a solvent gradient of 0-70% acetonitrile over 45min. B) LC-MS in negative ion mode validated the peptide mass (m/z [-] 624.2) and purity. C) Chemical structure of N-acetyl (C16) lipidated β3-Tripeptide.

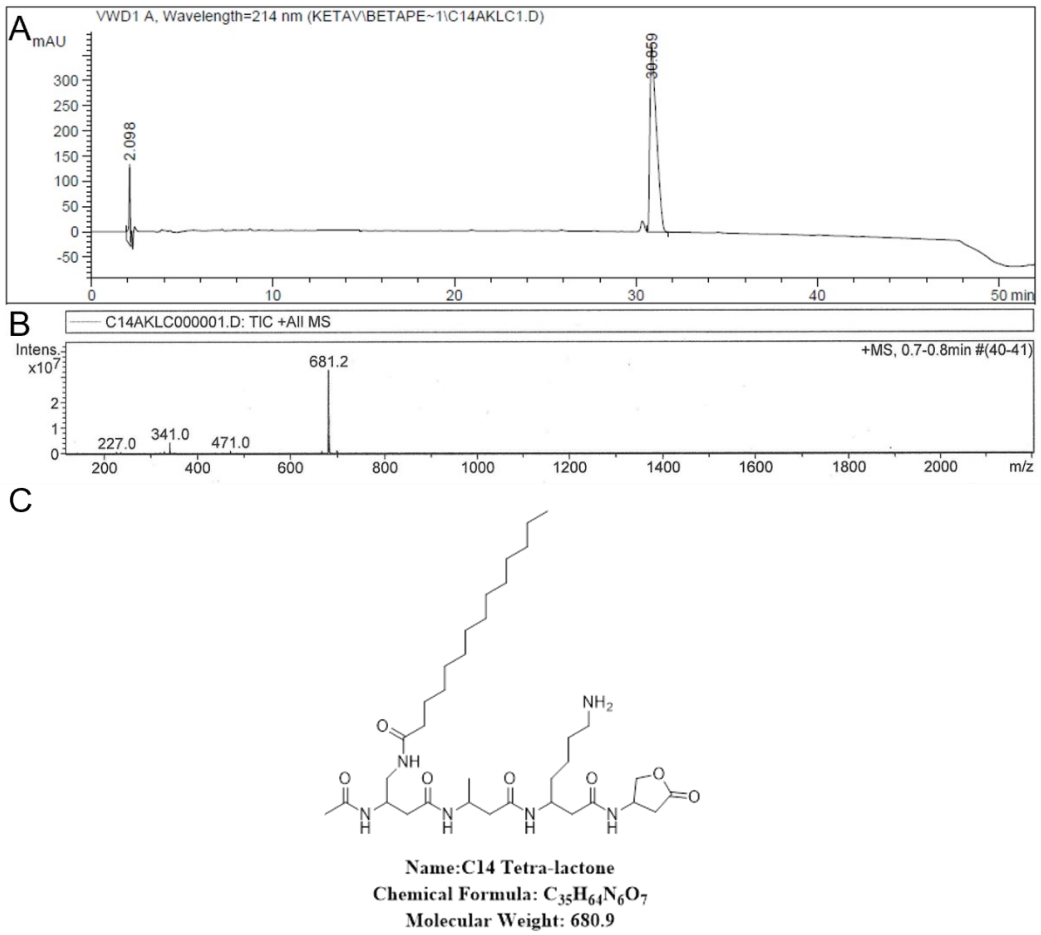


Name: C16 Tri-RGD-acid
Chemical Formula: $C_{49}H_{89}N_{13}O_{13}$
Molecular Weight: 1068.3

Supp. 2 Characterisation of N-acetyl (C16) lipidated β3-Tripeptide-RGD (Tripeptide-RGD). A) HPLC chromatogram. HPLC retention time was 32.86min with a solvent gradient of 0-70% acetonitrile over 45min. B) LC-MS in negative ion mode validated the peptide mass (m/z [2-] 534.5) and purity. C) Chemical structure of N-acetyl (C16) lipidated β3-Tripeptide-RGD (Tripeptide-RGD).



Supp. 3 Characterisation of N-acetyl (C14) lipidated β3-Tetra-acid (Tetrapeptide-acid). A) HPLC chromatogram. HPLC retention time was 29.77min with a solvent gradient of 0-70% acetonitrile over 45min. B) LC-MS in negative ion mode validated the peptide mass (m/z [-] 697.2) and purity. C) Chemical structure of N-acetyl (C14) lipidated β3-Tetra-acid (Tetrapeptide-acid).



Supp. 4 Characterisation of N-acetyl (C14) lipidated β3-Tetra-lactone (Tetrapeptide). A) HPLC chromatogram. HPLC retention time was 30.86min with a solvent gradient of 0-70% acetonitrile over 45min. B) LC-MS in negative ion mode validated the peptide mass (m/z [-] 681.2) and purity. C) Chemical structure of N-acetyl (C14) lipidated β3-Tetra-lactone (Tetrapeptide).

Image Analysis: Fibre Width

Fibre width was extracted from SEMs (5000x magnification) using the following ImageJ/FIJI macro

```
run("Set Scale...", "distance=1 known=1 pixel=1 unit=pixels");

setAutoThreshold("Default dark");

//run("Threshold...");

//setThreshold(123, 255);

name = getTitle()

setOption("BlackBackground", true);

run("Convert to Mask");
```

```
rename("Thresholded");

run("Duplicate...", " ");

run("Distance Map");

rename("DM_"+name);

selectWindow("Thresholded");

run("Duplicate...", "title=Skeleton");

run("Skeletonize");

run("Create Selection");

run("Set Measurements...", "area mean min median stack display add redirect=DM_"+name+" decimal=3");

run("Measure");

run("Close All");
```

This approach returns the mean and median distance to the peak (px) of fibres in a field of view. Mean fibre width of the sample was calculated by 2*(mean distance to the peak (nm)) for each image. Minimum 12 images per sample were averaged to produce one replicate, n=10.

Artefacts produced by each method (Conventional chemical fixation for SEM (CF-SEM), HPF for Cryo-SEM (HPF-CryoSEM), and HPF and dehydration for SEM (HPF-SEM)

Etching artefacts – To remove bulk water from HPF-CryoSEM samples temperature was ramped from -140˚C to -105 at 5˚C/min, then held at -105˚C for 60 minutes. Ramping faster and etching at -100˚C for 30 minutes resulted in “popping” artefacts, while etching at -105˚C for 10 minutes was insufficient. We also observed sputtering artefacts on some samples.

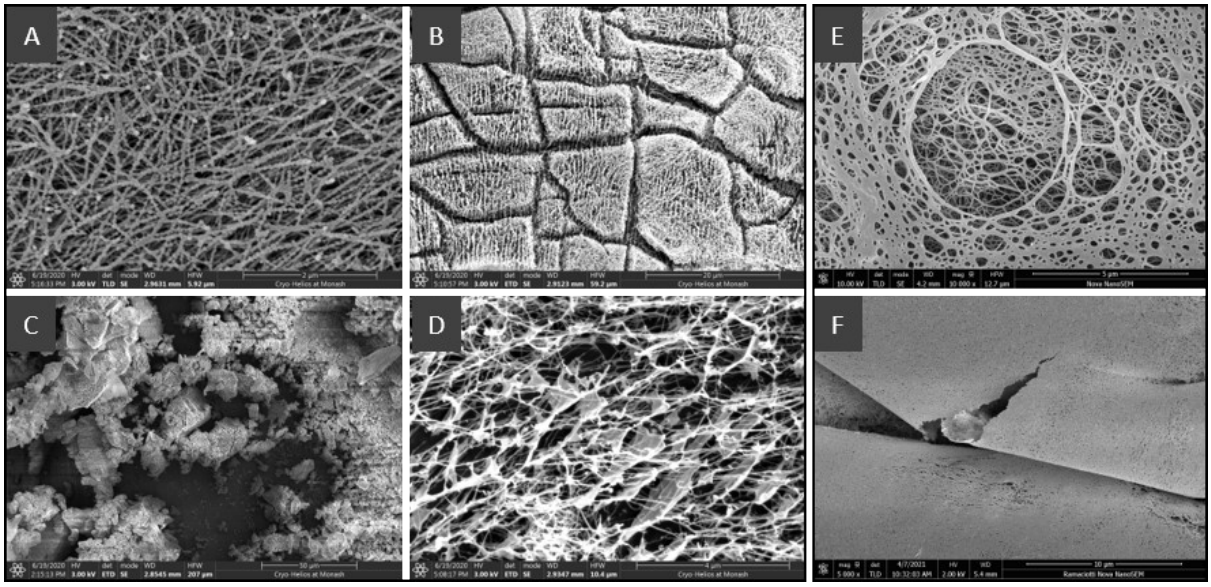
Crush/coalesce artefacts – In some dried samples regions of the sample surface appear to be distorted, which results in extremely dense mesh as the fibres appear to have coalesced. We hypothesise this artefact occurs due to handling the fragile samples during dehydration or critical point drying.

Optimisation of etching and sputtering for HPF-CryoSEM

Samples etched at -105˚C for 10min and sputtered with 4.8nm gold/palladium were insufficiently etched.

Samples etched at -100˚C for 30min and sputtered with 5.2nm gold/palladium were etched too rapidly and appeared to “explode” from the sample holder (Supp. 5, C).

Samples ramped from -140˚C to -105˚C at 5˚C/min, then etched at -105˚C for 1h and sputtered with 9.3nm gold/palladium were sufficiently etched without obvious damage to the sample, and in one region there were sputtering artefacts (Supp. 5, A).



Supp. 5 Artefacts typical of each preparation method. A-D HPF-CryoSEM A) Sputtering artefact. B) Expansion cracks. C) Explosive expansion. D) Residual water. E-F HPF-SEM and CF-SEM. E) Surface crush/coalesce. F) Surface collapse.

Development of HPF methods

High pressure freezing is usually performed with one deep freezer hat to hold the sample and a flat cap to cover the top, with the goal of the sample adhering to the flat cap and detaching from the deeper hat to expose the sample for freeze-fracturing. We initially trialled different coatings to encourage sample release from the deep hat, including graphene painting, carbon sputtering and aerosol lubricant spray, but could not achieve reproducible sample release.

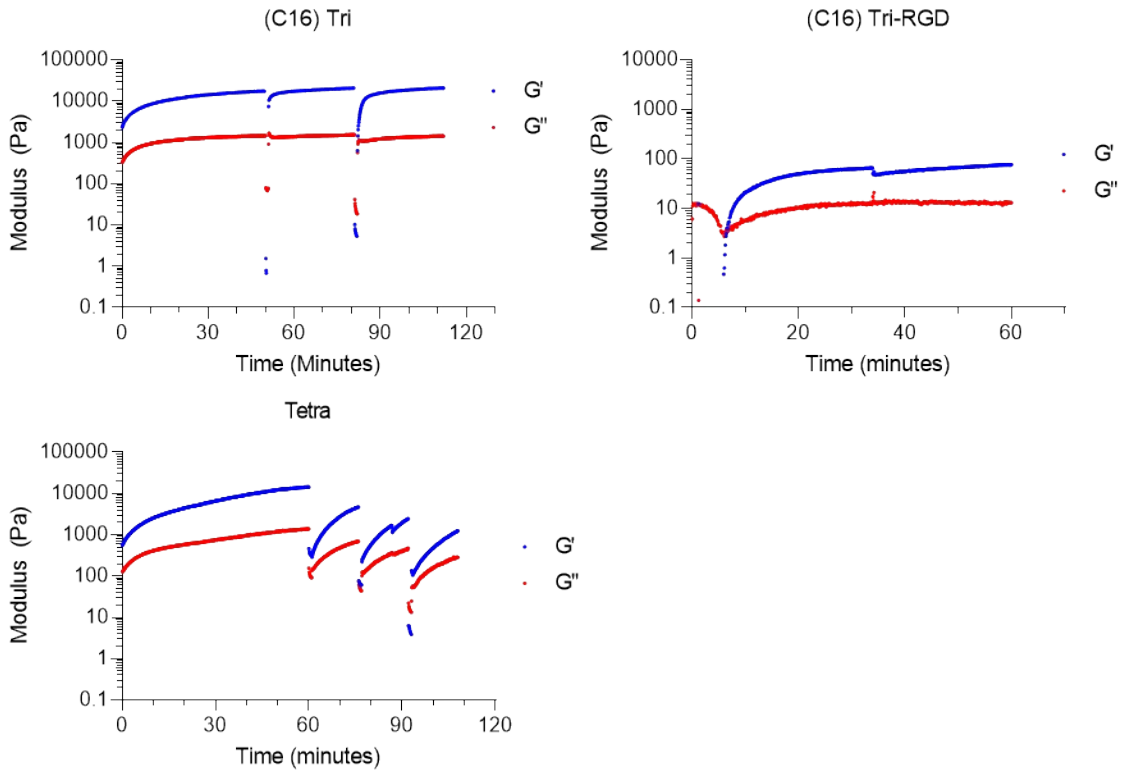
This led us to develop a new approach, where two uncoated freezer hats of equal depth were used to contain the sample so that upon separating the two halves there were identical.

After automatic freeze substitution (AFS) we noticed that many samples had detached from the freezer hats. We suspect this is due to the shallow depth of the sample holder, and in future a coating could be introduced to improve the stickiness.

Rheological Characterisation

Dried peptide samples were prepared ahead of time to make 20µL aliquots of 30mM or 15mM hydrogel solution. Rheological studies were conducted using an Anton Paar rheometer (Physica MCR 501) with an 8 mm parallel plate. The temperature was maintained at 37°C for all experiments. Hydrogel was prepared as previously described and immediately pipetted on the bottom plate, top plate was lowered and the sample was trimmed before completely covering the sample/air interface with paraffin oil to prevent drying. Experiments were immediately performed with a plate gap of 0.1 mm. The gelation of the peptide over time was examined for 60 minutes, unless noted otherwise in the text, and the storage (G', elastic) and loss (G", viscous) moduli were reported as a function of time.

The self-healing properties of the hydrogel were examined by its ability to recover after network disruption under high strain. The network of the stable hydrogel was disrupted under high strain (100% unless otherwise noted) for 1 min and the recovery of hydrogel properties was assessed by returning the strain to its standard value (1%).



Supp. 6 Time sweeps and recovery after 100% strain for 1min of 30mM C16-Tripeptide and 15mM C14-Tetrapeptide. Time sweep of 30mM C16-Tripeptide-RGD.

References

1. K. Kulkarni, S. Motamed, N. Habila, P. Perlmutter, J.S. Forsythe, M.I. Aguilar, and M.P. Del Borgo, *Orthogonal strategy for the synthesis of dual-functionalised beta(3)-peptide based hydrogels.* Chem Commun (Camb), 2016. **52**(34): p. 5844-7.