Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information for

Enzyme-triggered Gelation: Targeting Proteases with Internal Cleavage Sites

Steven C. Bremmer,[a,b] Anne J. McNeil[a] and Matthew B. Soellner[b]

[a]Department of Chemistry and Macromolecular Science and Engineering Program

[b]Departments of Chemistry and Medicinal Chemistry

University of Michigan, 930 North University Avenue,

Ann Arbor, Michigan, 48109-1055

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I. Materials

Fmoc-protected L-amino acids were purchased from Advanced ChemTech. 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N*,*N*,*N*',*N*'-tetramethyl-O-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate (HATU), trifluoroacetic acid (TFA) and Rink Amide AM resin were purchased from ChemImpex. All solvents were used as received; N-methyl-2-pyrrolidinone (NMP) was purchased from Advanced ChemTech, diisopropylethylamine (DIEA) from Fisher, dimethylsulfoxide (DMSO) from EMD and piperidine from Sigma-Aldrich. Aminopeptidase M (AP-M), matrix metalloproteinase 9 (MMP-9) and prostate specific antigen (PSA) were purchased from Calbiochem. PEG_4-OH was synthesized via a modified literature protocol.[1]

Reaction Buffer

All enzyme-triggered gelations were performed in a buffer consisting of 50 mM HEPES, 200 mM NaCl and 10 mM $CaCl_2$ at a pH = 7.5.

Aminopeptidase M Buffer Exchange

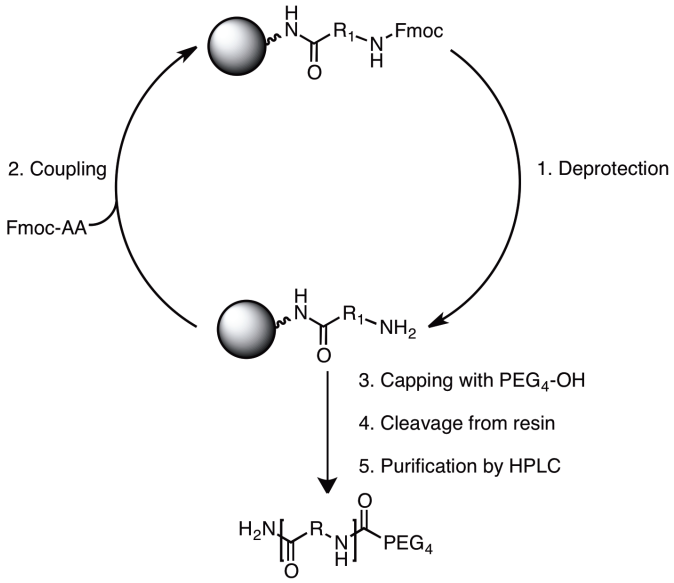
Aminopeptidase M (AP-M) is supplied as an $(NH_4)_2SO_4$ suspension (0.06 U/μL). An aliquot (150 μL) was diluted with Reaction Buffer (300 μL) and applied to a Millipore Microcon YM-10 centrifugal filter and spun for 20 min @ 10,000x g-force to concentrate the aminopeptidase. The remaining solution (~ 150 μL) was again diluted with Reaction Buffer (300 μL) and spun for an additional 20 min @ 10,000x g-force. This procedure was repeated 4 additional times to remove excess $(NH_4)_2SO_4$. The final solution was concentrated to ~150 μL and kept frozen at -80 °C.

Note that one unit of Aminopeptidase-M is defined as the amount of enzyme that will hydrolyze 1 μmol of Leu-pNA per min at 37 ºC, pH 7.2.

II. General Experimental

Peptide Synthesis and Purification

Peptides were synthesized on a 0.1 mmol scale using standard solid-phase Fmoc chemistry on rink amide resin.[2]



1. Deprotection

The Fmoc protecting group was removed by stirring the functionalized polystyrene (PS) beads in a solution of 20 vol% piperidine in NMP (6 mL) for at least 10 min at rt. The resin was then washed with NMP (4 x 4 mL) to remove the by-products.

2. Coupling

The Fmoc amino acid (0.5 mmol) and HBTU (0.5 mmol) were dissolved in NMP (4 mL) containing DIEA (287 mM). The solution was added to the Rink Amide AM resin (0.1 mmol) in a 12 mL cartridge and mixed on a rocking plate for at least 30 min at rt. The Kaiser test was used to monitor the progress of reaction.[3] After the reaction was complete, the resin was washed with NMP (4 x 4 mL).

Special Coupling Reaction for PABA Insertion

After coupling *p*-aminobenzoic acid to the resin and subsequent deprotection, a solution containing the next amino acid (1 mmol) and HATU (1 mmol) was added. The reaction was heated to 75 °C with a CEM Discover® microwave synthesizer for at least 1 h. The reaction progress was monitored using the Kaiser test.[3]

3. Capping with PEG_4-OH

PEG_4-OH (0.5 mmol) and HBTU (0.5 mmol) were dissolved in NMP (4 mL) containing DIEA (287 mM). This solution was added to the Rink Amide AM resin (0.1 mmol) in a 12 mL cartridge and the mixture was shaken for at least 30 min at rt. The reaction progress was monitored using the Kaiser test.[3] After the reaction was complete, the resin was washed with NMP (4 x 4 mL).

4. Cleavage from resin

The peptides were cleaved from the resin by incubating with a 5 mL solution of TFA/H_2O/iPr_3SiH (95/2.5/2.5 vol ratio) for at least 2 h at rt. The peptides were precipitated by adding cold Et_2O (40 mL) and collected by centrifugation. The precipitates were then dissolved in DMSO (2 mL) for HPLC purification.

5. Purification by HPLC

The peptides were purified using an HPLC with a Waters© Xbridge Prep C18 column (19 x 250 mm) using a linear gradient of CH_3CN (5-60%) in H_2O with 0.1 vol% TFA at 20 mL/min. Fractions containing the product were combined and lyophilized.

Procedure for Enzyme-triggered Gelation

The peptide was first dissolved in DMSO (100 mg/mL). An aliquot (22.5 μL) of this peptide solution was added to a 2 mL glass vial. Reaction Buffer (267.5 μL) containing ~1 U of aminopeptidase M was then added. Protease (10 μL) was then added to this solution and the time was recorded. The reaction was periodically monitored using the stable-to-inversion test to check for gel formation.

Analytical HPLC-MS Measurements

Aliquots (5 μL) of each reaction were added to a quench solution of CH_3CN/H_2O (100 μL, 50/50 v/v with 0.1 vol% HCO_2H). An aliquot (10 μL) of this solution was injected into a Waters© Xbridge C18 column (2.1 x 100 mm) and eluted using a linear gradient of CH_3CN (5-95%) in H_2O with HCO_2H (0.1 vol%) over 15 min at a flow rate of 0.3 mL/min. The nominal mass for each peak was determined using a Waters© Micromass ZQ mass spectrometer.

Rheology

Rheological measurements were performed on a TA Instruments AR2000ex rheometer using 20 mm parallel plates. A pre-formed gel was loaded onto the Peltier plate at rt. The gap was then fixed at 450 μm. The sample was pre-sheared under a stress of 0.1 Pa for 30 s before conducting the frequency sweep and oscillating stress sweep experiments. All measurements were repeated 2-3 times to ensure reproducibility. The frequency sweep experiment was performed under 0.1 Pa stress with a frequency range from 0.628 rad/s to 628 rad/s (i.e., 0.1 Hz–100 Hz). The oscillating stress sweep experiment was

performed at 1 Hz, with a stress range from 0.06 Pa to 153 Pa.

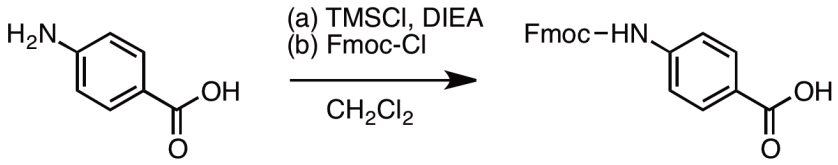
High Resolution Mass Spec Measurements

The high-resolution mass spec analysis was performed on a Micromass LCT Time-of-Flight mass spectrometer with electrospray.

NMR spectroscopy

^{1}H and ^{13}C NMR spectra were acquired on a Varian MR 400 operating at 400 and 125 MHz, respectively. For ^{1}H and ^{13}C NMR spectra, the chemical shift data are reported in units of δ (ppm) relative to tetramethylsilane and referenced with residual solvent. Multiplicities are reported as follows: singlet (s), doublet (d), triplet (t), broad resonance (br) and multiplet (m).

III. Synthesis and Characterization of Fmoc-PABA-OH



S1

4-Aminobenzoic acid (1.8 g, 13 mmol) was suspended in CH_2Cl_2 (30 mL) in a two-neck 250 mL round-bottom flask fitted with a water-jacketed condenser and a stir bar under argon. To the suspension was added TMSCl (3.6 mL, 28 mmol) and DIEA (5.9 mL, 34 mmol). The reaction was refluxed for 3 h and resulted in a pink solution. The solution was then cooled in an ice-water bath and Fmoc-Cl (3.9 g, 15 mmol) was added. Then the reaction was warmed to rt and stirred for 16 h. MeOH (90 mL) was then added with vigorous stirring, resulting in a white precipitate. The precipitate was filtered and washed with MeOH (4 x 50 mL) and dried by rotary evaporation to yield 3.1 g (66% yield).

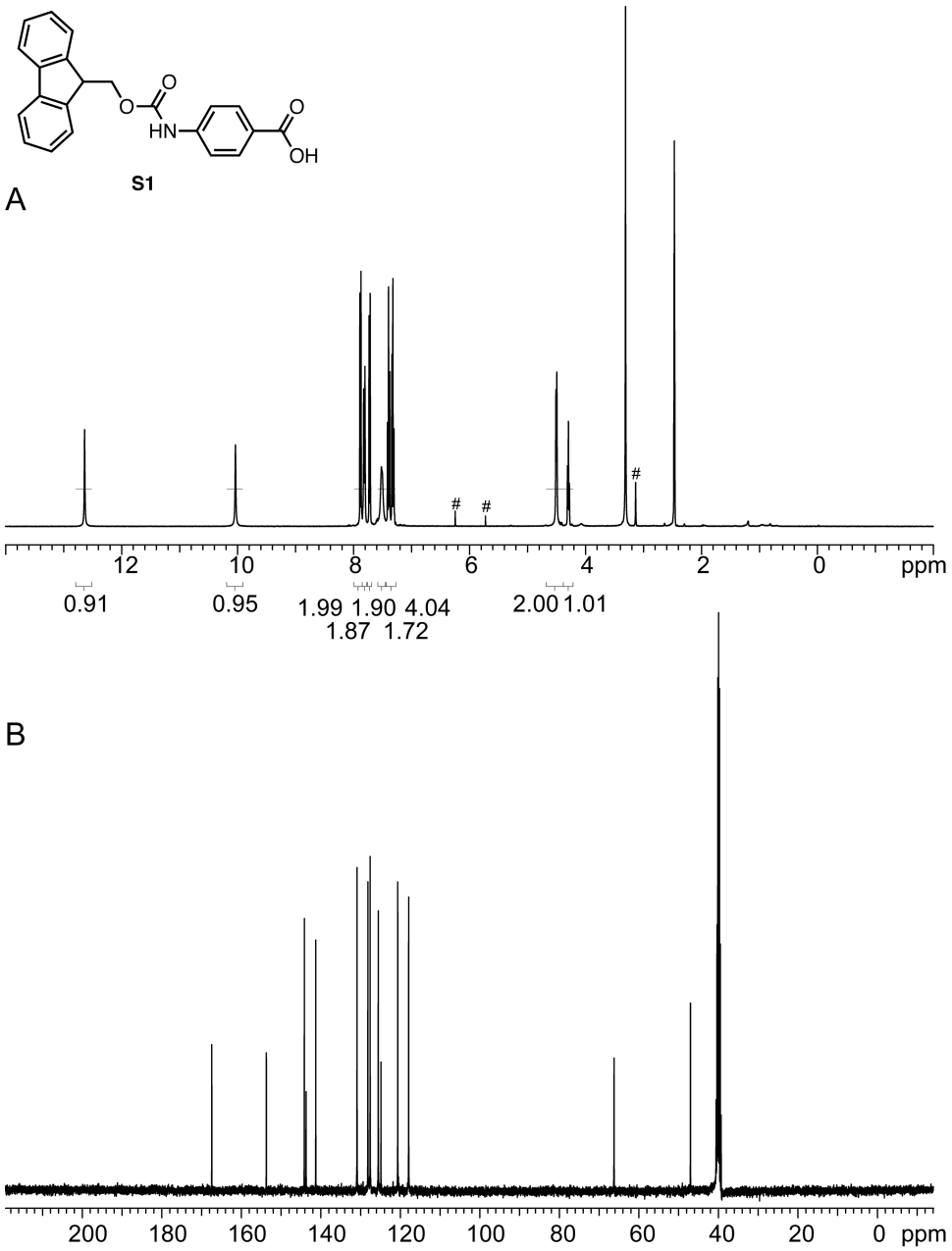
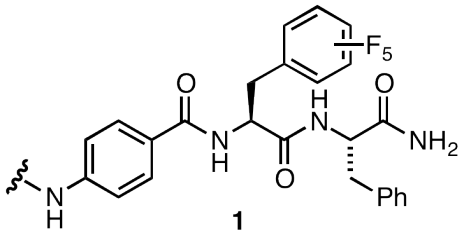


Figure S1. (A) ^{1}H and (B) ^{13}C NMR spectra of Fmoc-PABA-OH. ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) 12.64 (s, 1H), 10.04 (s, 1H), 7.88 (d, J = 7.4 Hz, 2H), 7.81 (d, J = 8.6 Hz, 2H), 7.72 (d, J = 7.2 Hz, 2H), 7.52 (br, 2H), 7.36 (m, 4H), 4.50 (d, J = 6.5 Hz, 2H) 4.29 (t, J = 6.5 Hz, 1H). ^{13}C {^{1}H} NMR (DMSO-d_6, 125 MHz): δ (ppm) 167.41, 153.69, 144.13, 143.69, 141.26, 130.88, 128.15, 127.58, 125.53, 124.85, 120.63, 117.88, 66.22, 47.00. $^{\#}$Denotes an unknown impurity.

IV. Peptide Characterization Data

Note that SF = PEG_4–RPSP–⦚
and **1** =



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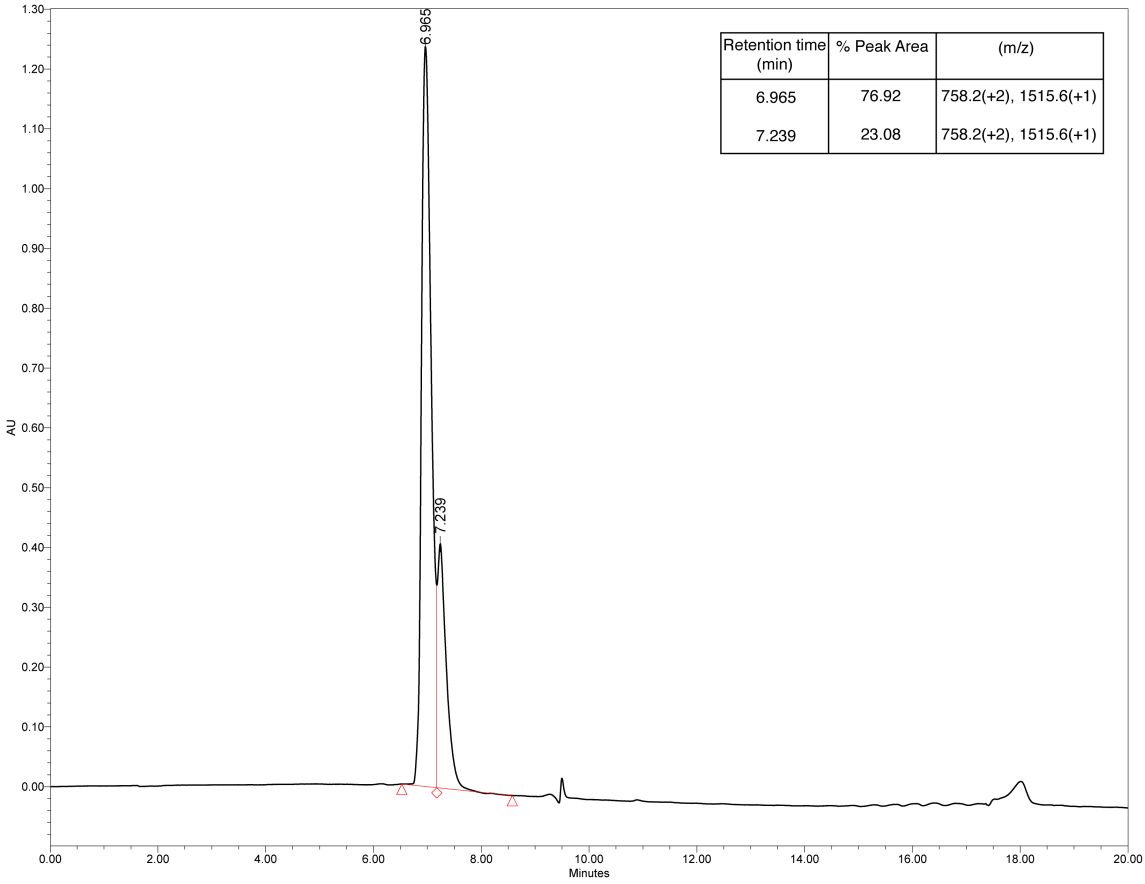
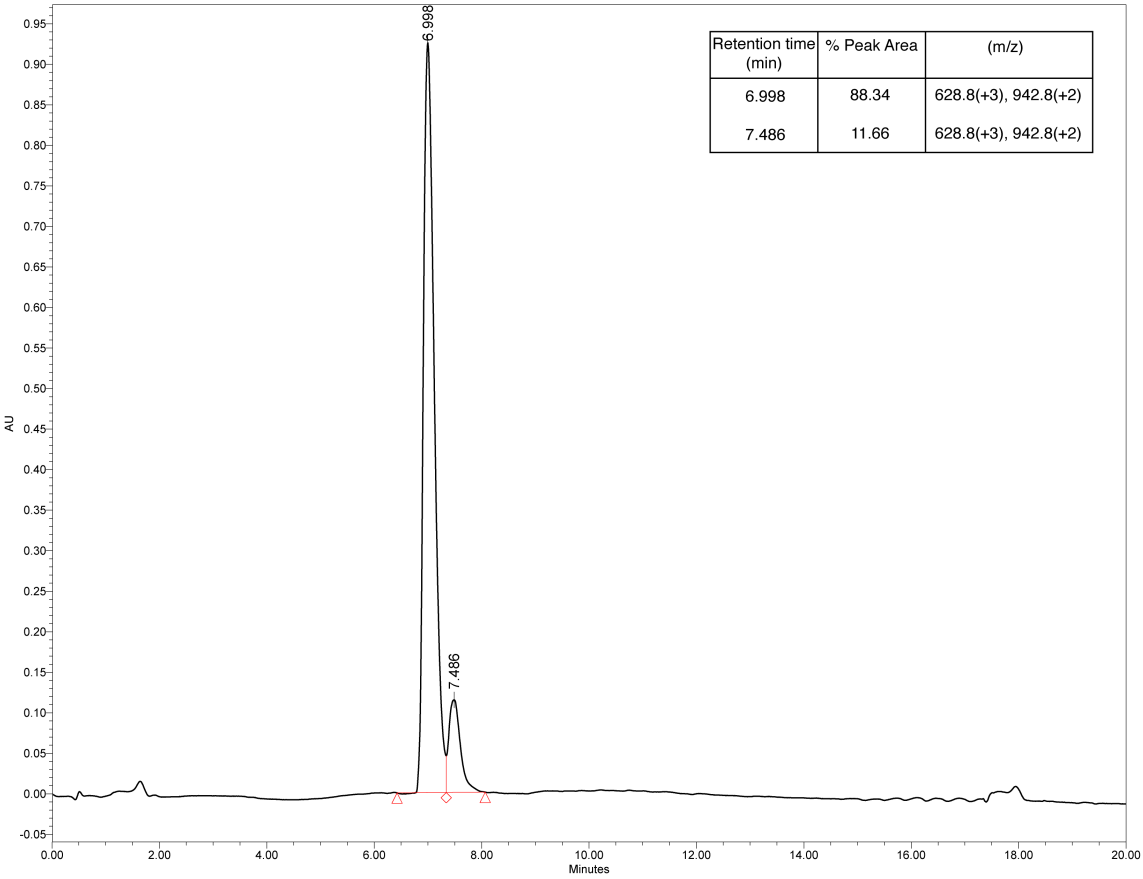


Retention time (min)	% Peak Area	(m/z)
6.965	76.92	758.2(+2), 1515.6(+1)
7.239	23.08	758.2(+2), 1515.6(+1)

Figure S3. Analytical LC-MS trace for SF–GPKG–**1**. The peptide eluted at 6.965 and 7.239 min (aggregated peptide) and is greater than 99% pure based on the absorbance at 280 nm. ESI-MS was used to determine the exact mass of the molecule. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{69}H_{95}F_5N_{16}O_{17}$, 1515.7054; found, 1515.7051.



Retention time (min)	% Peak Area	(m/z)
6.998	88.34	628.8(+3), 942.8(+2)
7.486	11.66	628.8(+3), 942.8(+2)

Figure S4. Analytical LC-MS trace for SF–GPKG–LKGA–**1**. The peptide eluted at 6.998 min and 7.486 min (aggregated peptide) and is greater than 99% pure based on the absorbance at 280 nm. ESI-MS was used to determine the exact mass of the molecule. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{86}H_{126}F_5N_{21}O_{21}$, 1884.9430; found, 1884.9415.

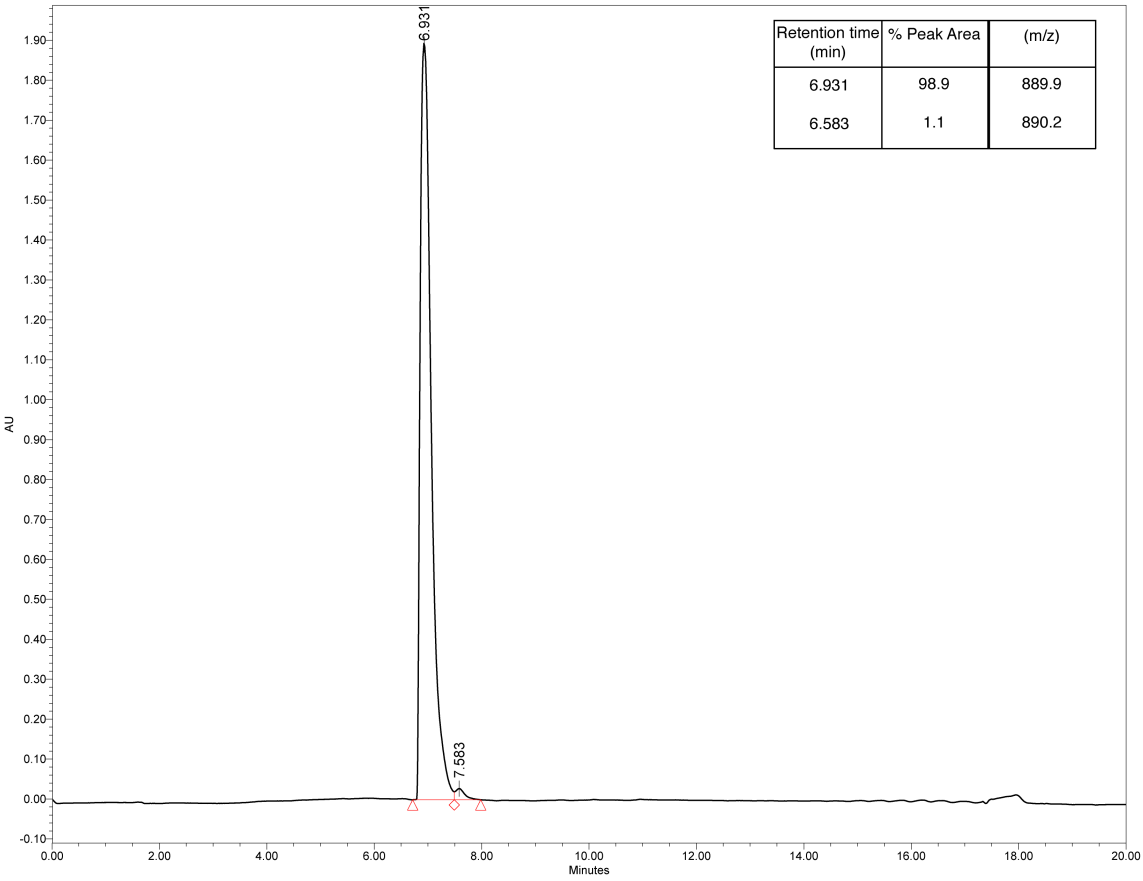
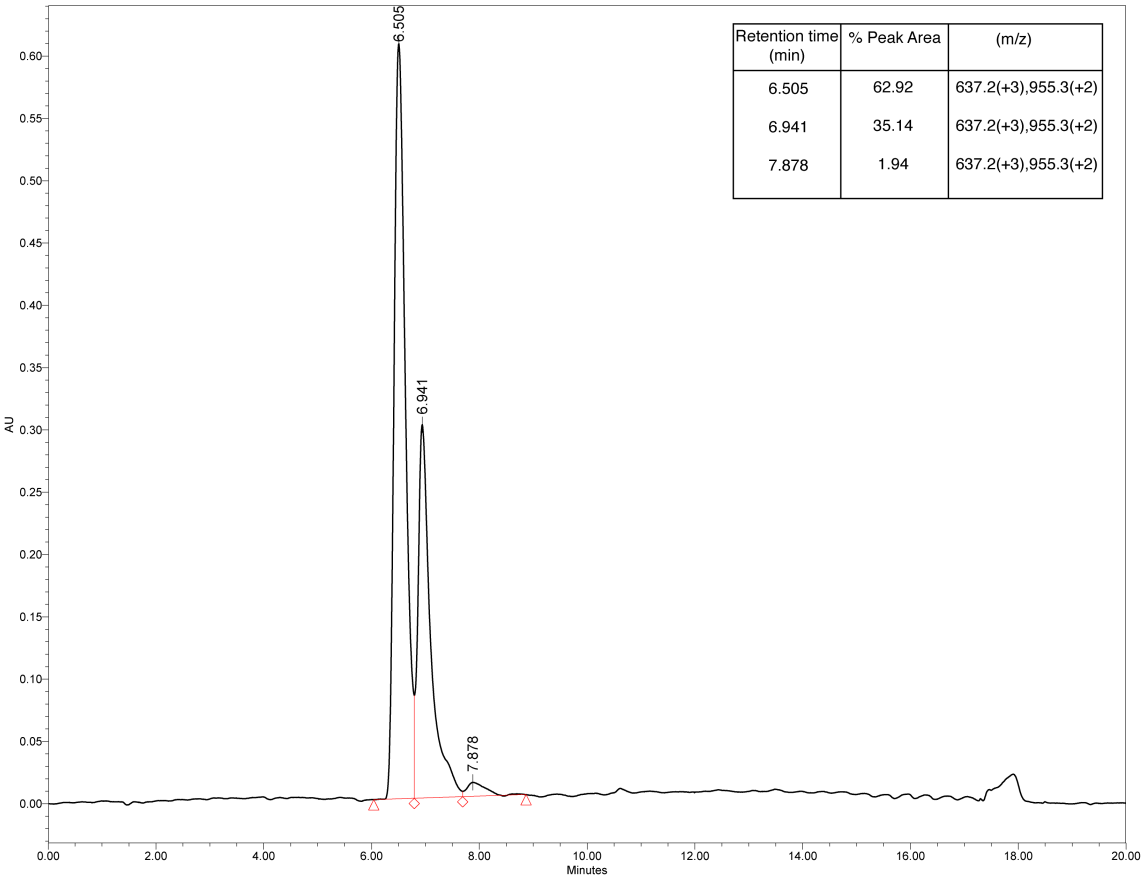
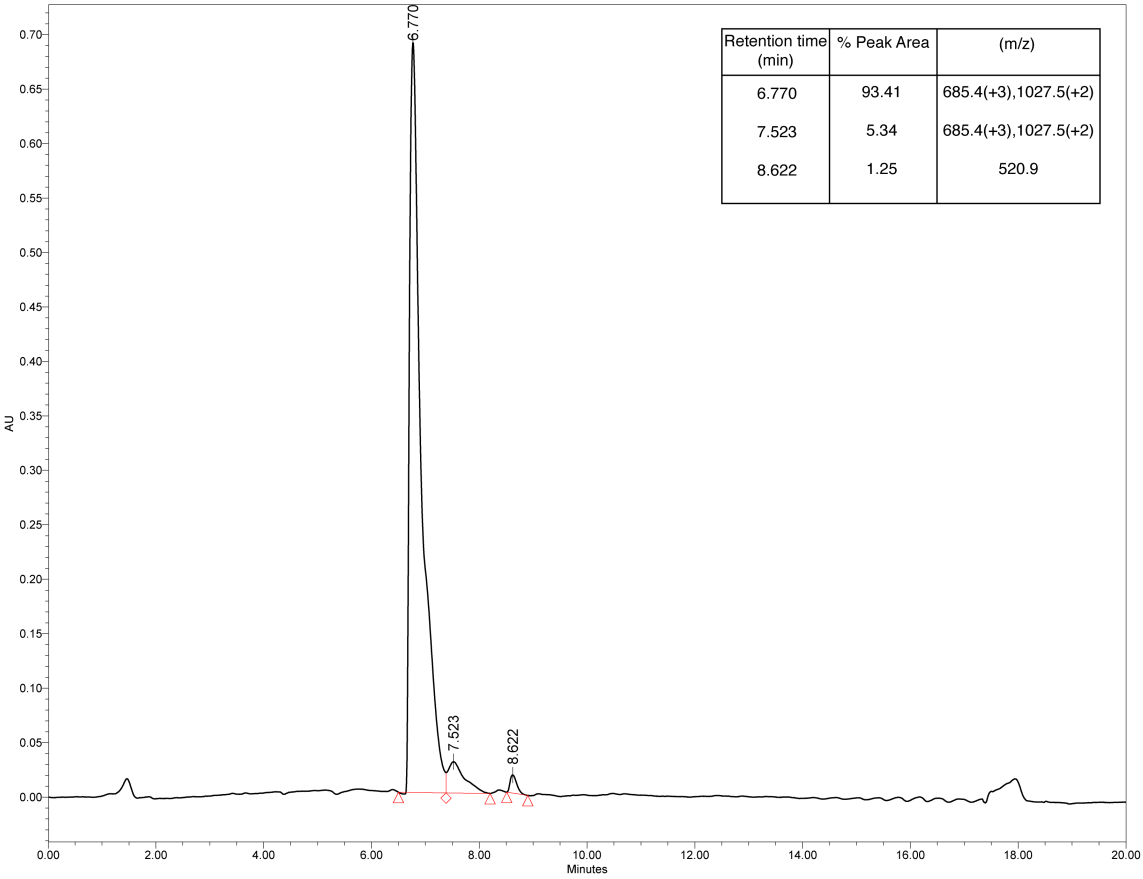


Figure S5. Analytical LC-MS trace for LKGA–**1**. The peptide eluted at 6.931 min and 7.583 min (aggregated peptide) and is greater than 99% pure based on the absorbance at 280 nm. ESI-MS was used to determine the exact mass of the molecule. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{42}H_{52}F_5N_9O_7$, 890.3983; found, 890.3976.



Retention time (min)	% Peak Area	(m/z)
6.505	62.92	637.2(+3),955.3(+2)
6.941	35.14	637.2(+3),955.3(+2)
7.878	1.94	637.2(+3),955.3(+2)

Figure S6. Analytical LC-MS trace for SF–HSAKFY–**1**. The peptide eluted at 6.505, 6.941 (aggregated peptide) and 7.878 min (aggregated peptide) and is greater than 99% pure based on the absorbance at 280 nm. ESI-MS was used to determine the exact mass of the molecule. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{90}H_{117}F_5N_{20}O_{21}$, 1908.8622; found, 1908.8659.



Retention time (min)	% Peak Area	(m/z)
6.770	93.41	685.4(+3),1027.5(+2)
7.523	5.34	685.4(+3),1027.5(+2)
8.622	1.25	520.9

Figure S7. Analytical LC-MS trace for SF–HSAKFY–SG–**1**. The peptide eluted at 6.770 and 7.523 min (aggregated peptide) with a minor impurity at 8.622 min and is 98% pure based on the absorbance at 280 nm. ESI-MS was used to determine the exact mass of the molecule. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{95}H_{125}F_5N_{22}O_{24}$, 2052.9157; found, 2052.9205.

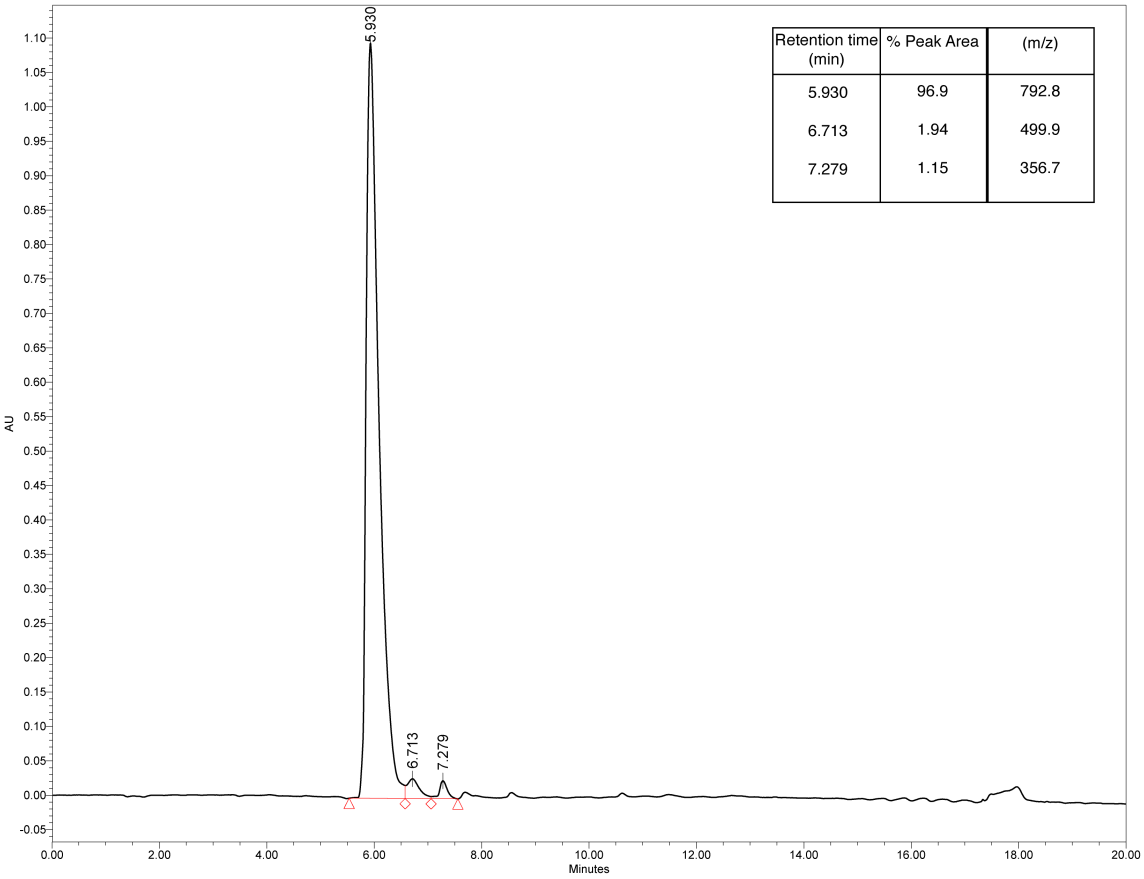


Figure S8. Analytical LC-MS trace for SGK–**1**. The peptide eluted at 5.930 min with minor impurities at 6.713 and 7.279 min and is 97% pure based on the absorbance at 280 nm. ESI-MS was used to determine the exact mass of the molecule. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{36}H_{41}F_5N_8O_7$, 793.3091; found, 793.3092.

V. MMP-9-triggered gelation

Experimental Details

To a 2 mL glass vial was added SF–GPKG–**1** (22.5 μL of a 100 mg/mL DMSO stock solution). The solution was then diluted with Reaction Buffer (262.5 μL). The reaction was started by addition of MMP-9 (50 nM, 10 μL of a 1.5 μM stock).

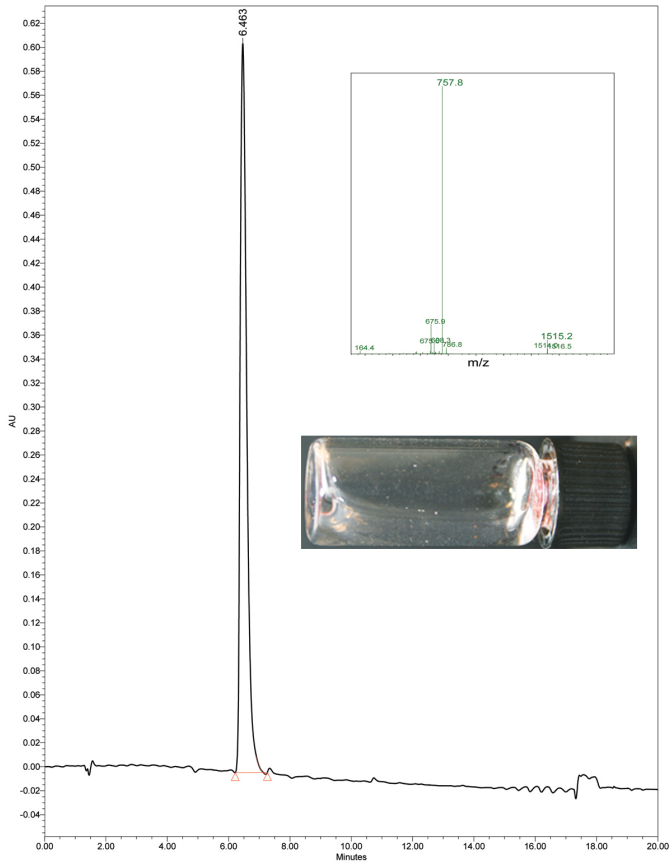


Figure S9. Results from incubating SF–GPKG–**1** with MMP-9 (50 nM) in Reaction Buffer. The LC-MS trace was acquired after 24 h incubation and exhibited only one peak corresponding to unreacted SF–GPKG–**1** (inset shows m/z data). The reaction mixture remains a solution after 24 h (see vial).

Experimental Details

To a 0.5 mL eppendorf tube, SF–GPKG–**1** or SF–GPKG–LKGA–**1** (5 μL of a 100 mg/mL DMSO stock solution) was added. Each solution was then diluted with 0.3 U of Aminopeptidase M in Reaction Buffer (70 μL). The reaction was started by addition of MMP-9 (50 nM, 2.5 μL of a 1.5 μM stock solution).

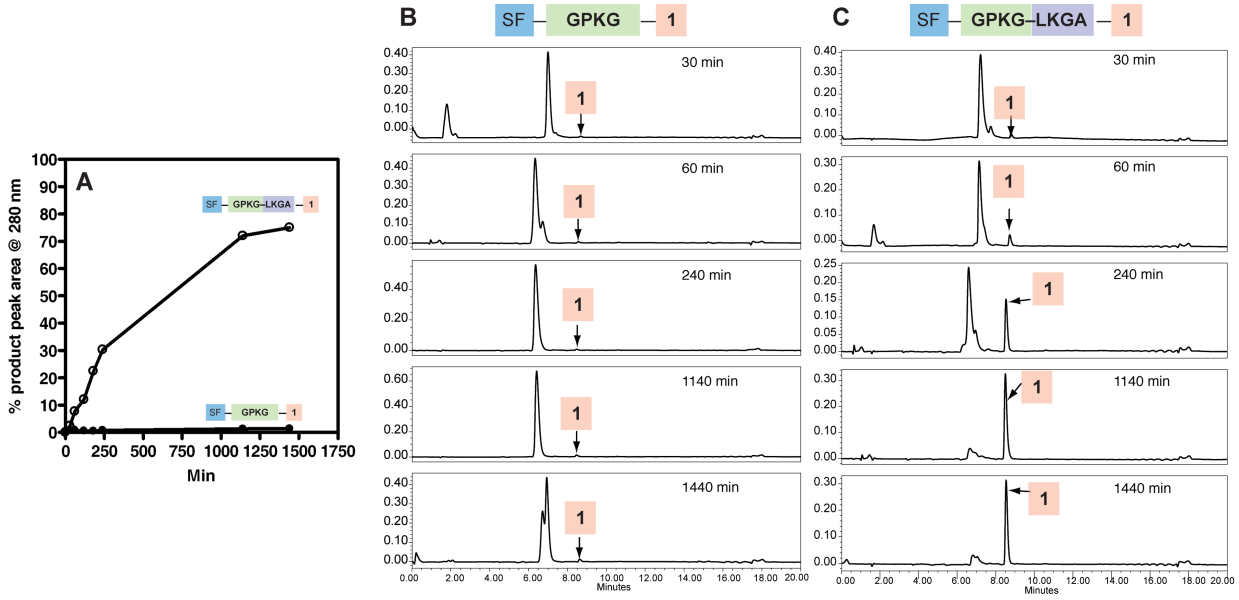


Figure S10. (A) Plot of absorbance (% total peak area at 280 nm from **1**) versus time for the reaction of MMP-9 (50 nM) with SF–GPKG–**1** (●) and SF–GPKG–LKGA–**1** (○). (B) Raw LC-MS traces used to determine conversion for SF–GPKG–**1** and SF–GPKG–LKGA–**1**.

Experimental Details

To a 2 mL vial containing SF–GPKG–LKGA–**1** (22.5 μL of a 100 mg/mL DMSO stock solution) was added 0.3 U of Aminopeptidase M in Reaction Buffer (267.5 μL). The reaction was started by addition of MMP-9 (50 nM, 10 μL of a 1.5 μM stock). [Note that no gel was observed after 24 h when 10 nM MMP-9 was used.]

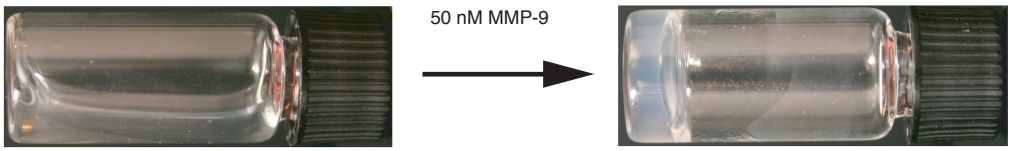


Figure S11. Gel formation is observed after 24 h when SF–GPKG–LKGA–**1** is treated with MMP-9 and Aminopeptidase M in Reaction Buffer.

Experimental Details

To a plastic cap containing SF–GPKG–LKGA–**1** (37.5 μL of a 100 mg/mL DMSO stock solution) was added 1 U of Aminopeptidase M in Reaction Buffer (445.5 μL). The reaction was started by adding MMP-9 (50 nM, 17 μL of a 1.5 μM stock). After 24 h, the gel was removed from the cap and placed in the rheometer for analysis.

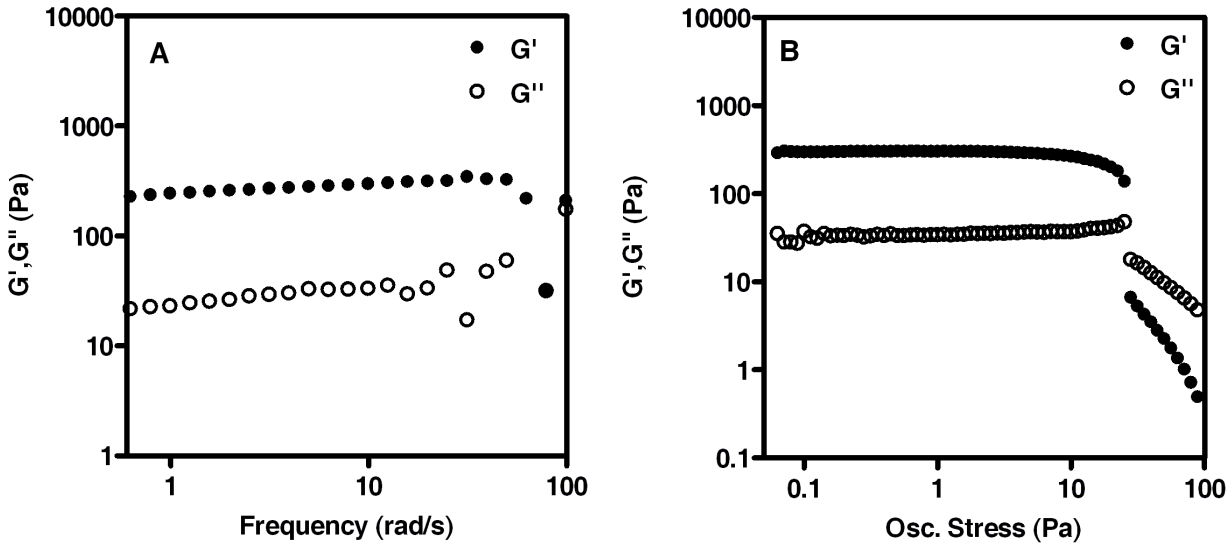


Figure S12. Plot of elastic modulus (G′, ●) and loss modulus (G″, ○) as a function of (A) frequency and (B) oscillatory stress.

VI. PSA-triggered gelation

Experimental Details

To a 0.5 mL eppendorf tube was added SF–HSAKFY–**1** or SF–HSAKFY–SG–**1** (5 μL of a 100 mg/mL DMSO stock solution). Each reaction was then diluted with 0.3 U of Aminopeptidase M in Reaction Buffer (70 μL). The reaction was started by addition of PSA (2 μM, 2.5 μL of a 65 μM stock).

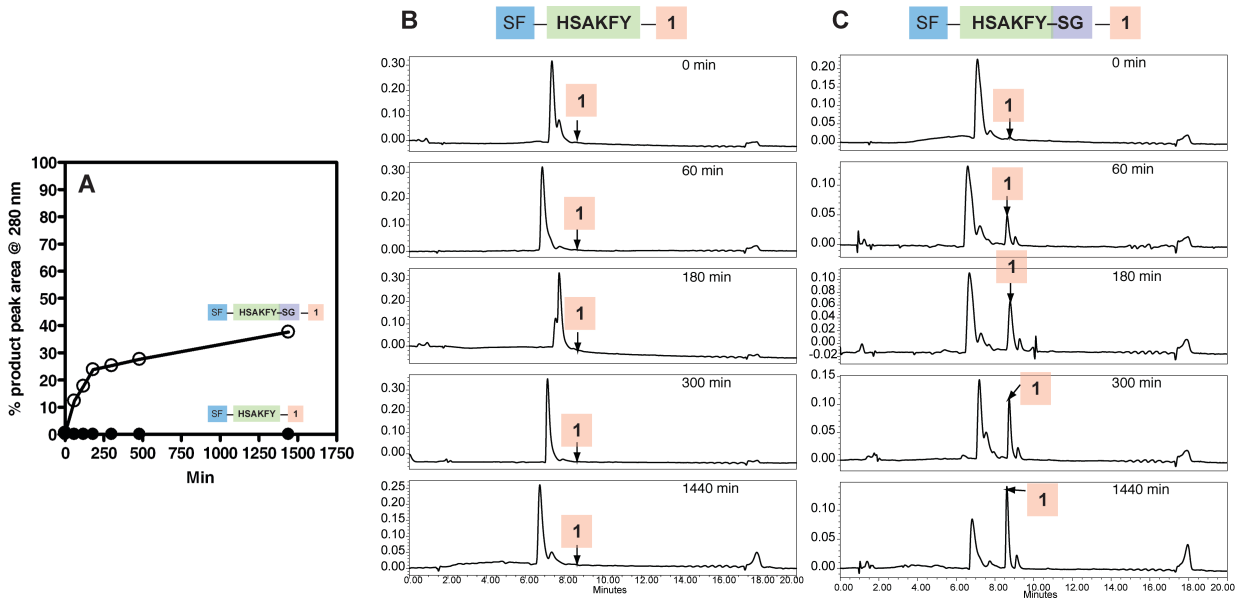
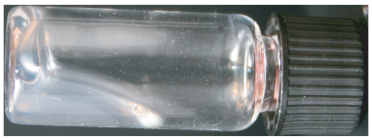


Figure S13. (A) Plot of absorbance (% total peak area at 280 nm from **1**) versus time for the reaction of PSA with SF–HSAKFY–**1** (●) and SF–HSAKFY–SG–**1** (○). Raw LC-MS traces used to determine conversion with (B) SF–HSAKFY–**1** and (C) SF–HSAKFY–SG–**1**.

Experimental Details

SF–HSAKFY–SG–**1** (22.5 μL of a 100 mg/mL DMSO stock solution) was added into two different 2 mL glass vials. Each solution was then diluted with 1 U of Aminopeptidase M in Reaction Buffer (270 μL). In the control vial, Reaction Buffer was added (7.5 μL). In the other vial, the reaction was started by adding PSA (1.6 μM, 7.5 μL of a 65 μM stock solution).



24 h
– PSA
+ aminopeptidase

24 h
+ PSA
+ aminopeptidase

Figure S14. Gel formation was observed within 24 h when SF–HSAKFY–SG–**1** was treated with PSA (1.6 μM) and Aminopeptidase M (1 U) in Reaction buffer (right). No gel is observed in the control vial (left).

Experimental Details

In three separate 2 mL glass vials was added SF–HSAKFY–SG–**1** (15 μL of a 100 mg/mL DMSO stock solution) and SGK–**1** (2.25 μL of a 100 mg/mL DMSO stock solution). Each solution was then diluted with 1 U of Aminopeptidase M in reaction buffer (275.25 μL). The reaction was started by adding PSA (1.6 μM, 7.5 μL of a 65 μM stock solution). In the control vials, an equivalent volume of Reaction Buffer was added instead of either PSA or Aminopeptidase M. [Note that gelation was observed within 5 h when 2.0 μM PSA was used.]

7.5 h
+PSA
+ aminopeptidase

7.5 h
+PSA
– aminopeptidase

7.5 h
– PSA
+ aminopeptidase

Figure S15. Gel formation is observed within 7.5 h when a solution containing both SF–HSAKFY–SG–**1** and SGK–**1** is treated with PSA (1.6 μM) and Aminopeptidase M (1 U) in Reaction Buffer (left). No gels were observed when either Aminopeptidase M (middle) or PSA (right) is absent.

Experimental Details

To a plastic cap containing SF–HSAKFY–SG–1 (37.5 μL of a 100 mg/mL DMSO stock solution) was added 0.6 U of Aminopeptidase M in Reaction Buffer (453.6 μL). The reaction is started by addition of 1.6 μM PSA (8.9 μL of a 90 μM stock solution). After 24 h of incubation, the gel was removed from the cap and placed in the rheometer for analysis.

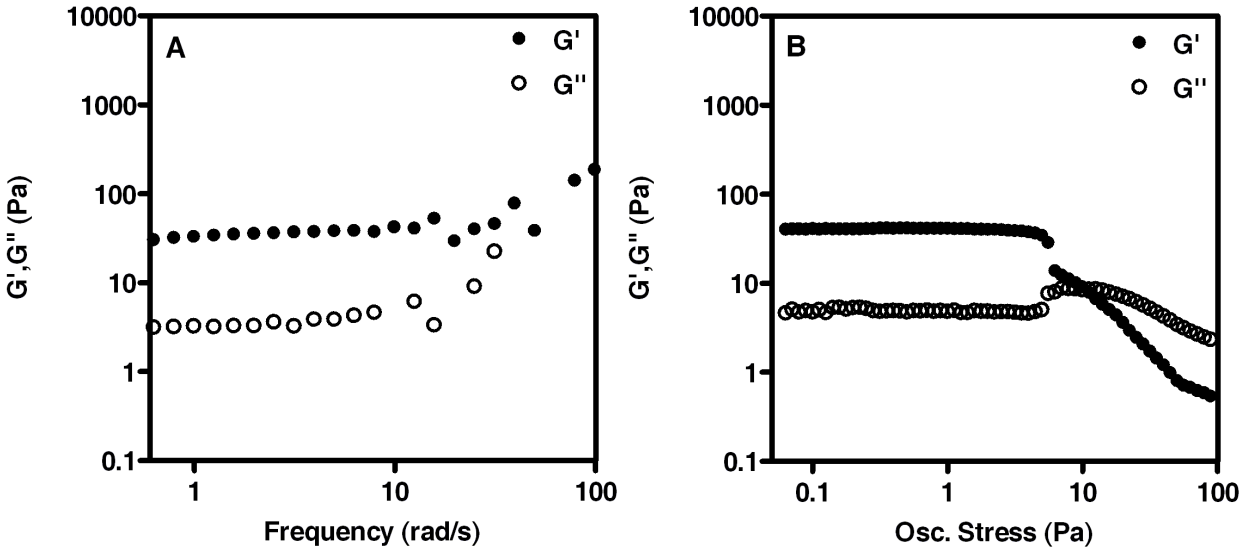


Figure S16. Plot of elastic modulus (G′, ●) and loss modulus (G″, ○) as a function of (A) frequency and (B) oscillatory stress.

VII. Characterization of Aminopeptidase M activity

Experimental Details

Aminopeptidase M (0.6 U) was incubated with LKGA–**1** (7.5 μL of 100 mg/mL DMSO stock solution) in Reaction Buffer (92.5 μL) for 3 d at rt. Aliquots were taken and analyzed by LC-MS to determine the conversion of LKGA–**1** to **1**.

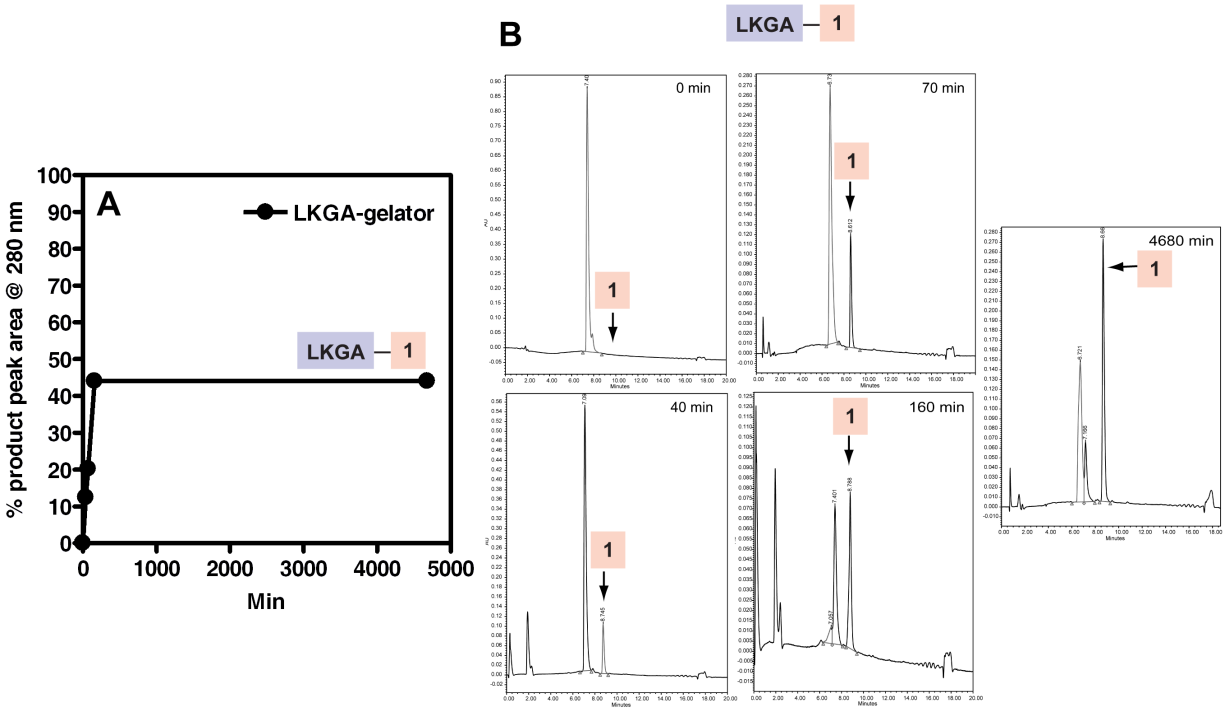


Figure S17. Plot of absorbance (% total peak area at 280 nm from **1**) versus time for the reaction of Aminopeptidase M with LKGA–**1** (●).

VIII. References

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