Supplementary information

MMP-9 responsive supramolecular peptide amphiphiles for slow release of doxorubicin

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Materials and methods

Materials

All reagents were purchased from Sigma Aldrich and used without further purification unless differently stated. Peptide grade N,N-Dimethylformamide (DMF) was obtained by Rathburn chemicals. The preloaded Wang resins were purchased from Merck (Novabiochem). Monomeric MMP-9 proenzyme from human neutrophils (as a solution in 50mM TRIS, containing 200mM sodium chloride, 5mM calcium chloride, 1 μM zinc chloride, 0.05% Brij-35 and 0.05% sodium azide) and MMP-9 catalytic domain (as a solution in 50mM TRIS, containing 5mM calcium chloride, 300mM sodium chloride, 20μM zinc chloride, 0.5% Brij-35 and 30% glycerol, at pH 7.5) were acquired from Enzo Life Sciences.

High-performance liquid chromatography (HPLC) analyses of peptides were performed using Dionex P680 HPLC system equipped with a Macherey-Nagel C18 column of 250 mm length, 4.6 mm internal diameter and 5 mm particle size equipped with UV-Vis detector. The gradient: (Solvent A: 0.1% TFA in water; Solvent B: 0.1% TFA acetonitrile) 20-80% B was utilised, with each run lasting a total of 46 minutes using a flow rate of 1 mL min^{-1} and detection wavelengths set at 214 nm and 254 nm using the UV-Vis detector. For separation of peptides **1a** and **1b** a 30-50% B gradient was utilised with each run lasting a total of 70 minutes using a flow rate of 1 mL min^{-1} and detection wavelengths set at 214 nm and 254 nm using the UV-Vis detector.

Liquid chromatography-mass spectrometry (LC-MS) was used to confirm peptide molecular weights. All analyses were carried out on a reverse-phase 15 cm Kinetex C18, 150 x 4.6 mm, 5 micron column. The LC-MS instrument was an Agilent 1200 Series HPLC, coupled to an Agilent 6130 Dual source MS detector. The gradient: (Solvent C: 5 mM ammonium acetate in water; Solvent D: 5 mM ammonium acetate in acetonitrile) 0-3 min 5% D, 3-17 min 5-100% D, 17-27 min 100% D, 27-33 min 100-5% D, and 33-36 min 5% D was used in all analyses; the flow rate was set at 1 mL min^{-1} and detection wavelengths at 214 nm. Mass detection was set to analyse in Scan mode with electrospray ionisation (MM-ES+APCI).

Atomic force microscopy (AFM) was performed using a Veeco diINNOVA Scanning Probe Microscope (VEECO/BRUKER). The samples were placed on a trimmed and freshly cleaved mica sheet (G250-2 Mica sheets 1" x 1" x 0.006"; Agar Scientific Ltd, Essex, UK) attached to an AFM support stub and left to air-dry overnight in a dust-free environment, prior to imaging. All the images were obtained in tapping mode. The resolution of the scans is 512 x 512 pixels. Typical scanning parameters are the following: tapping frequency 308 kHz, integral and proportional gains 0.3 and 0.5, respectively, set point 0.5–0.8 V and scanning speed 1.0 Hz. The images were analysed using NanoScope Analysis software Version 1.40.

Transmission electron microscopy (TEM) imaging was performed at the University of Glasgow Electron Microscopy facility, using a LEO 912 energy filtering transmission electron microscope operating at 120kV fitted with 14 bit/2 K Proscan CCD camera. Carbon-coated copper grids (200 mesh) were glow discharged in air for 30 s. The support film was touched onto the gel surface for 3 s and blotted down using filter paper. Negative stain (20 ml, 1% aqueous methylamine vanadate obtained from Nanovan; Nanoprobes) was applied and the mixture blotted again using filter paper to remove excess. The dried specimens were then imaged.

Fluorescence emission spectra for determination of critical aggregation concentration were measured on a Jasco FP-6500 spectrofluorometer. The ANS probe was excited at 360 nm and the data collected in the range between 370 and 700 nm. The excitation and emission bandwidths were both set to 5 nm. Fluorescence emission spectra for determination of critical micelle concentration were measured on an X Fluor Safire 2 fluorescence plate reader (version: V 4.62n). The pyrene probe was excited at 338 nm and the emission data were collected in the range between 360 to 410 nm. The emission wavelength step size was set to 2 nm and the excitation and emission bandwidths to 5 nm each. Fluorescence emission spectra for determination of doxorubicin release from micellar aggregates and after enzyme treatment was measured on an X Fluor Safire 2 fluorescence plate reader (version: V 4.62n) at the Beatson Cancer Research Institute (Glasgow). Doxorubicin was excited at 480 nm and the emission intensity at 596 nm was monitored over time. The doxorubicin release form **1b** gel was monitored using Jasco FP-6500 spectrofluorometer using the same parameters mentioned above.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR measurements were performed at the Department of Physics, University of Strathclyde. Spectra were recorded on a Bruker Vertex 70 spectrometer, averaging 25 scans per sample at a resolution of 1 cm^{-1}. Samples were sandwiched between two 2 mm CaF_2 windows separated with a 50 μm polytetrafluoroethylene (PTFE) spacer.

Dynamic Light Scattering (DLS)

DLS was performed at the Department of Chemical Engineering, University of Strathclyde. DLS measurements were carried out by using an ALV (ALV, GMBH, Germany) spectrophotometer using vertically polarized He-Ne laser light (25 mW with wavelength of 632.8 nm) with an avalanche photodiode detector. The DLS measurements were carried out at angle of 90° at 25 °C. Intensity autocorrelation functions were recorded and analysed by means of the cumulant method in order to determine the intensity weighted diffusion coefficients D and the average hydrodynamic radius R_H by using the Stokes-Einstein equation, $R_H = kBT/6\pi \eta D$, where kB is the Boltzmann constant, T is the absolute temperature and η is the solvent viscosity at the given temperature.

Rheology

To assess the mechanical properties of the **1b** hydrogel, dynamic frequency sweep experiments were carried out on a strain-controlled rheometer (Bohlin C-CVO) using a parallel-plate geometry (20 mm) with a 0.25 cm gap. The temperature of the sample stage was maintained at 25°C using an integrated temperature controller. To minimize solvent evaporation and to keep the sample hydrated a solvent trap was used and the atmosphere within was kept saturated. To ensure the measurements were made in the linear viscoelastic regime, an amplitude sweep was performed and the results showed no variation in elastic modulus (G') and viscous modulus (G'') up to a strain of 1%. The dynamic modulus of gels was measured as a frequency function, where the frequency sweeps were carried out between 0.1 and 10 Hz.

Solid phase peptide synthesis (SPPS) procedure

All peptide sequences where prepared using the standard Fmoc SPPS on Wang resin pre-loaded with the first amino acid. The chain growth was performed with a three-fold excess of

amino acid over the resin in DMF, using N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU) and N,N-Diisopropylethylamine (DIPEA) as activating and coupling reagents in 1:2 ratio respectively relative to the amino acid. Fmoc removal was carried out with 20% piperidine in DMF. The cleavage of the peptides from the resin was achieved using a cleavage cocktail: 95% TFA, 2.5 % triisopropylsilane (TIS), and 2.5 % water. The crude peptide was precipitated and washed in cold diethyl ether and subsequently dissolved in water to allow further purification. For the dissolution of hydrophobic sequences minimal amount of acetonitrile was used to facilitate the dissolution of any non-dissolved material. The samples were then characterised by reverse phase high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LCMS) to determine the purity and M_w of the product. The peptides were purified by Almac Sciences (Elvingston Science Centre, Gladsmuir, East Lothian, Edinburgh, EH33 1EH, Scotland).

Characterisation of peptides produced by SPPS

1a peptide sequence: PhAc-FFAGLDD. HPLC (20-80% Solvent B, retention time = 22.17 min); HPLC (30-50% Solvent B, retention time = 32.3 min). LCMS: LC (5-100% Solvent D, retention time = 10.74 min), MS (mass calculated: $[M+H]^+$= 902.39, mass observed: $[M-H]^-$= 900.30)

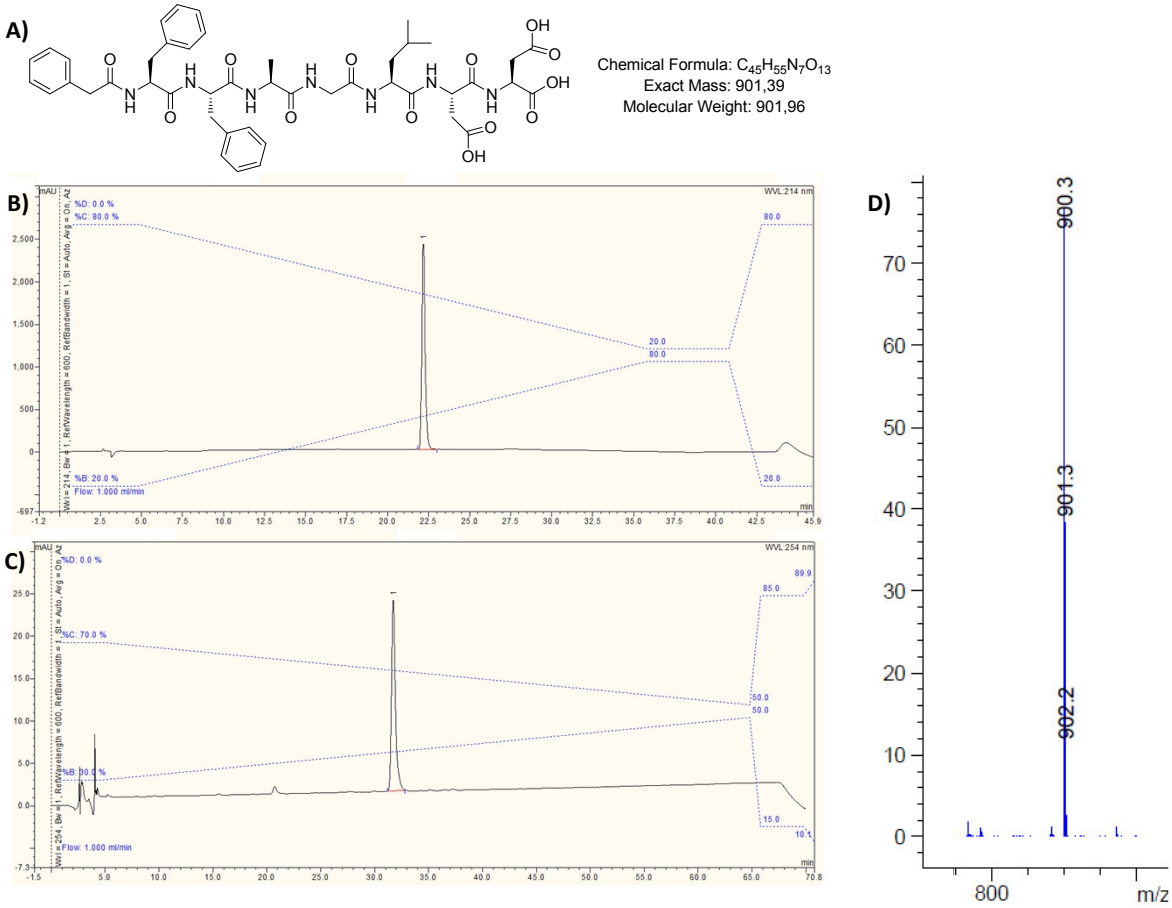


Figure S1. A) Chemical structure of PhAc-FFAGLDD (**1a**); B) HPLC chromatogram for **1a** (20-80% solvent B gradient, detection wavelength = 214 nm); C) HPLC chromatogram for **1a** (30-50% solvent B gradient, detection wavelength = 254 nm); D) Fragment of MS spectra showing the negative ion of **1a** detected.

1b peptide sequence: PhAc-FFAG. HPLC (20-80% Solvent B, retention time = 22.17 min); HPLC (30-50% Solvent B, retention time = 27.7 min). LCMS: LC (5-100% Solvent D, retention time = 11.20 min), MS (mass calculated: $[M+H]^+ = 559.25$, mass observed: $[M+H]^+ = 559.20$)

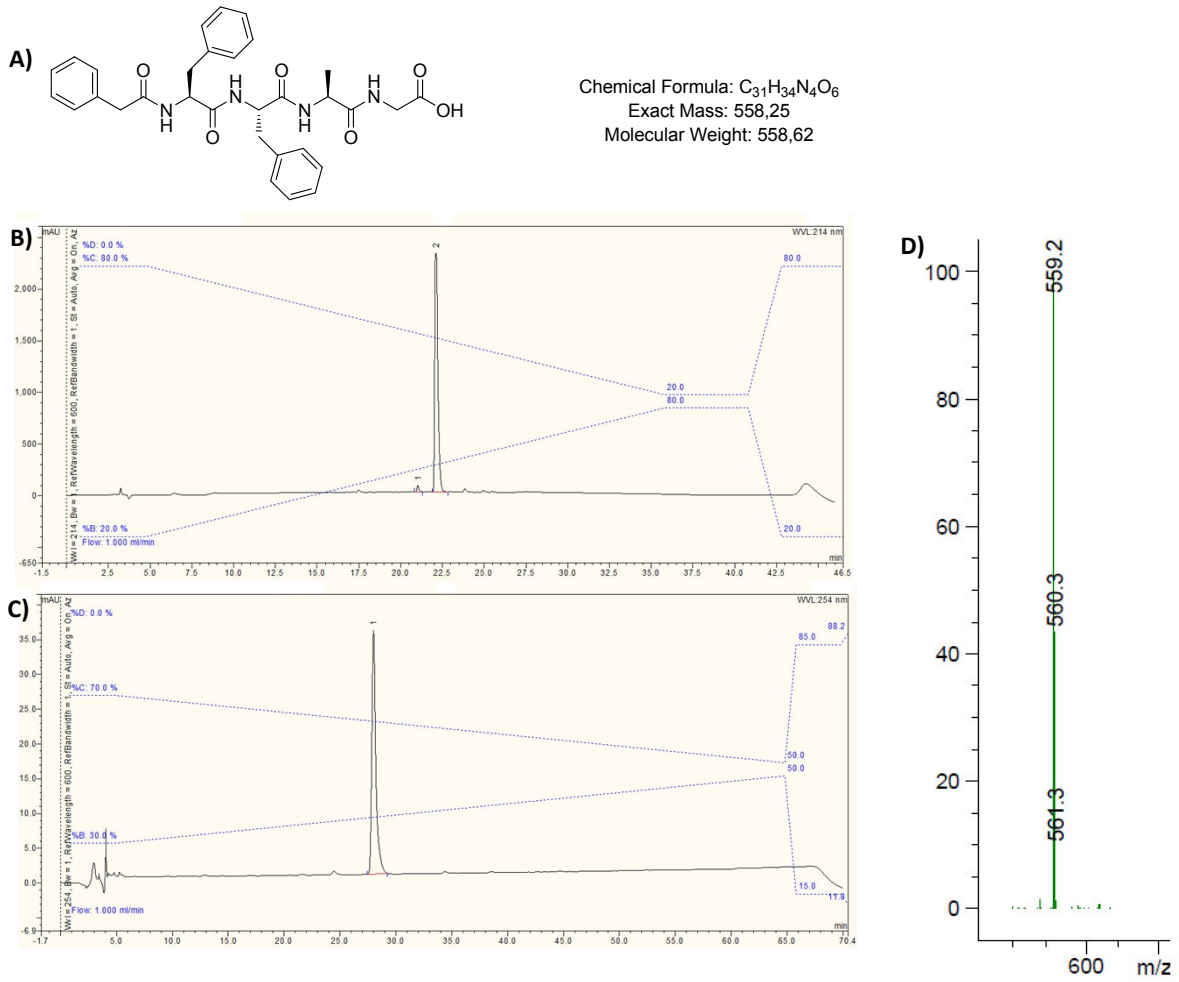


Figure S2. A) Chemical structure of PhAc-FFAG (**1b**); B) HPLC chromatogram for **1b** (20-80% solvent B gradient, detection wavelength = 214 nm); C) HPLC chromatogram for **1b** (30-50% solvent B gradient, detection wavelength = 254 nm); D) Fragment of MS spectra showing the positive ion of **1b** detected.

Gel preparation

Samples were prepared by suspending the peptide powder (PheAc-FFAG) to a total peptide concentration of 10, 20 and 30 mM in DI water. NaOH solution (0.5 M) was drop wise added to reach complete dissolution of the peptide (pH 9-10) followed by vortexing and sonication until full dissolution. HCl (0.5 M) was then drop wise added to neutralize solution's basicity (pH 6.8.-7.4) followed by vortex and sonication to trigger gelation. Immediately after that, the solution was allowed to gel on the bench. All characterizations were performed after 24 hours unless otherwise mentioned.

DLS results

Autocorrelation functions were recorded for peptide solutions of **1a** and **1b** at various concentrations (5 mM, 2.5 mM, 1.25 mM, 0.625 mM) and are shown in the figure below.

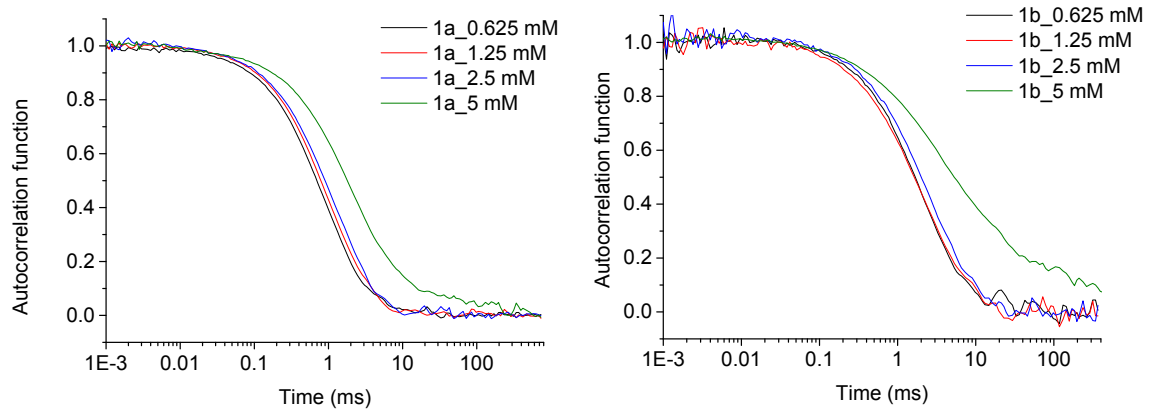
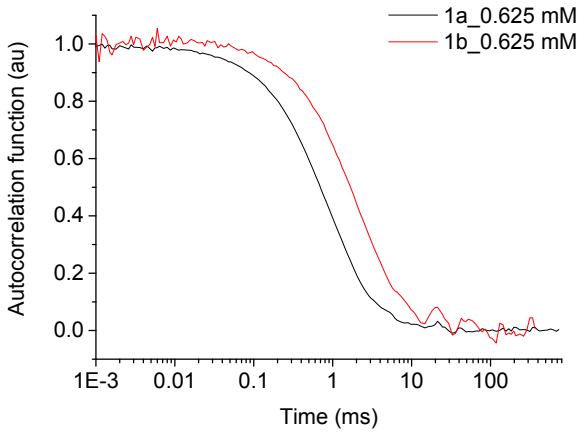


Figure S3. Autocorrelation functions of peptide solutions **1a** and **1b** at different concentrations showing that the size of aggregates further increases at concentrations of $\geq$ 5 mM for both peptides and it is stable in the 0.625-2.5 mM range.

Table 1. R_H values for peptide solutions of **1a** and **1b** at various concentrations.

Peptide	1a		1b	
Concentration (mM)	R_H (nm)	sd	R_H (nm)	sd
5	328	26	639	48
2.5	205	18	392	52
1.25	167	5	307	28
0.625	165	3	358	17

For further analysis 0.625 mM samples were chosen. The diffusion coefficient was calculated and the size difference between the micellar aggregates (**1a**) and fibres (**1b**) at 0.625 mM are shown in the graph and table below.



Peptide	1a	1b
D (m^2s^{-1})	$1.5x10^{-12}$	$6.8x10^{-13}$
R_h (nm)	165	358

Figure S4. Autocorrelation functions of peptides **1a** and **1b** at the concentration of 0.625 mM indicating a difference in decay rates for micellar aggregates and fibres.

Determination of Critical Aggregation Concentration (CAC) and Critical Micelle Concentration (CMC)

Critical aggregation concentration measurement was carried out for PhAc-FFAGLDD and PhAc-FFAG using 8-Anilino-1-naphthalenesulphonic acid (ANS) as a fluorescence probe.[1] Varying concentrations (5 mM, 2.5 mM, 1.25 mM, 0.625 mM, 0.312 mM, 0.156 mM and 0.078 mM) of each peptide were used. A 0.01 M ANS stock solution was made in methanol. 1 µL of the ANS stock solution was added to 1 mL aqueous solution of peptides (to a final probe concentration of $1x\ 10^{-5}$ M) and the fluorescence emission measured immediately after mixing. The ANS fluorescence was monitored at room temperature using an excitation wavelength of 360 nm. Fluorescence emission was monitored over a range of 370 nm to 700 nm. The CAC calculation method was adapted from literature.[2] CAC was calculated by plotting the absorption of ANS at 470 nm (at the emission maximum) against the log of peptide concentration. The two trendline equations from each graph were set as equal and from there the value of *x* at the turning point, corresponding to the log concentration, was calculated. By solving 10^x the CAC in mM is obtained. The fluorescence data for the two peptides investigated are presented in the figure below.

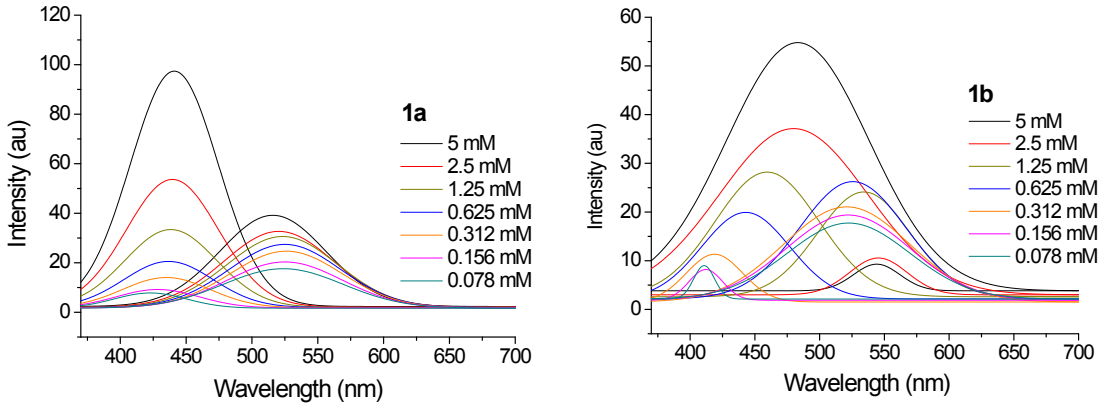


Figure S5. A) Fluorescence intensities of the ANS probe for different concentrations of each peptide: **1a** and **1b** (λ_{ex} = 360 nm).

Critical micelle concentration measurement was carried out for PhAc-FFAGLDD using pyrene as a fluorescent probe following a previously reported method.[3] Varying concentrations (5 mM, 2.5 mM, 1.25 mM, 0.625 mM, 0.312 mM, 0.156 mM and 0.078 mM) of each peptide were used. A stock solution of 2.5 mM was made in methanol, which was then diluted 20 times. 5 µL of the diluted solution of pyrene was added to each of the seven different concentrations of peptide solutions (to reach the final probe concentration of 6.25 x 10^{-6} M). These samples were then analysed using excitation at 338 nm and fluorescence emission was monitored over a range of 360 nm to 410 nm. CMC was obtained by plotting the ratio the first peak (λ_{max}=372 nm) and the third peak (λ_{max}=384 nm) against concentration.

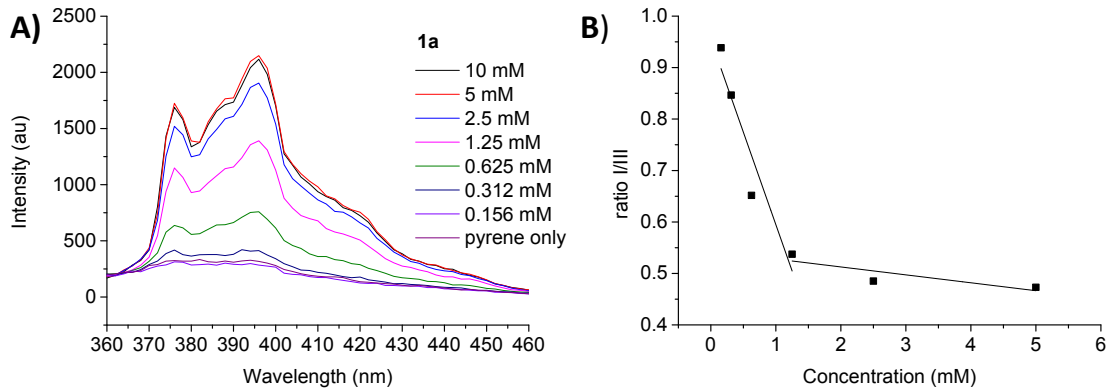


Figure S6. A) Fluorescence intensities of pyrene for different concentrations of **1a** (λ_{ex} = 295 nm). B) The ratio of the first (λ_{em}=372 nm) and the third peak (λ_{em}=384 nm) in the emission spectra of pyrene plotted against peptide concentration for peptide surfactant **1a**.

MMP-9 activation

The enzyme was activated by incubation with 2 mM p-aminophenyl mercuric acetate (APMA). A 10 mM stock solution of APMA was made in 0.1M NaOH. This stock was diluted 5x to give a final APMA concentration of 2mM. 1:1 volume ratio (MMP-9:APMA) were used for activation that was performed at 37°C for 2 h. When the catalytic domain was used no activation was required.

Digestion of substrates by MMP-9

The digestion of the peptides was carried out at 37°C in PBS for 96 h at a volume of 1 mL for peptides only and in a well plate at a final volume of 100 µL for peptides loaded with doxorubicin.

A 10 mM stock solution of each peptide was made by dissolving the peptide in PBS buffer (pH=7.4). The peptide solution was diluted to a final concentration of 5 mM and MMP-9 was added to give a final enzyme concentration of 50 ng/mL. The reaction was then incubated at 37°C for the desired time.

The products of enzyme cleavage were analysed by HPLC and MALDI:

PhAc-FFAGLDD (**1a**) converted to PhAc-FFAGL (**1c**) after 96 h: HPLC (30-50% Solvent B, retention time = 21.6 min). MS (mass calculated: $[M+H]^+$= 671.39, mass observed: $[M+Na]^+$= 694.38).

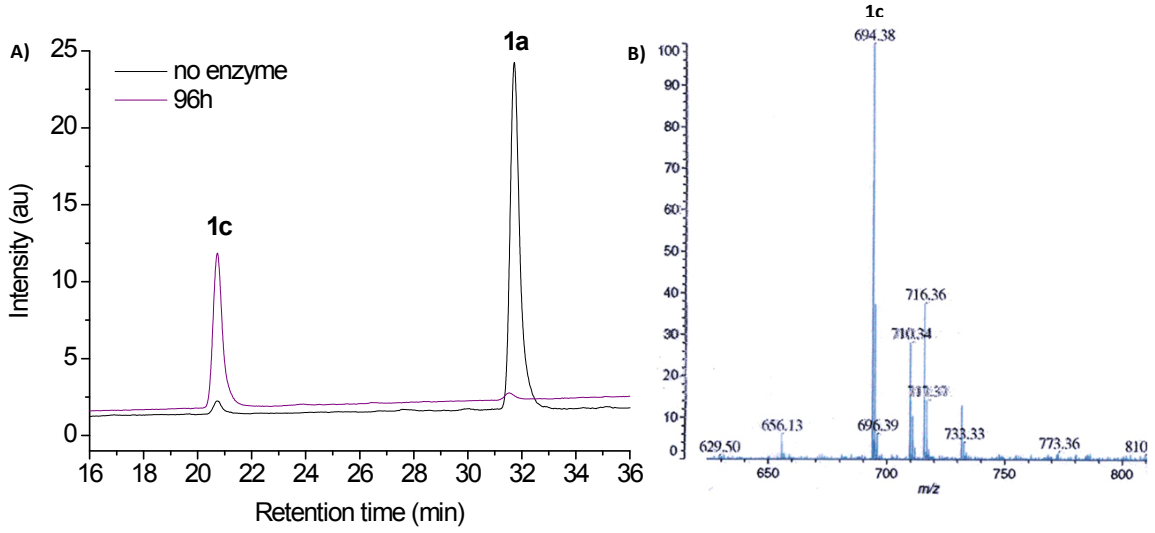


Figure S7. A) HPLC chromatogram showing complete conversion of **1a** (PhAc-FFAGLDD) to **1c** (PhAc-FFAGL) after 96 h MMP-9 treatment HPLC chromatogram for **1b** (30-50% solvent B

gradient, detection wavelength = 254 nm). B) MALDI spectra recorded after enzyme treatment showing the sodium adduct of **1c** detected.

Physical entrapment of doxorubicin

Doxorubicin was solubilized in DMSO by sonication and a 1mM stock solution was made and subsequently diluted into the suspension of peptide micelles (final concentration of doxorubicin: 5 µM). The samples were then sonicated to allow doxorubicin diffusion into the hydrophobic core of the micelles.

Emission dependence of doxorubicin on solvent polarity

A 1mM stock of doxorubicin in DMSO was diluted in solvents with different polarities: ethanol, methanol, DMSO and water[4] to a final concentration 5µM. The doxorubicin fluorescent emission was recorded (λ_{ex} = 480 nm) and the results presented below.

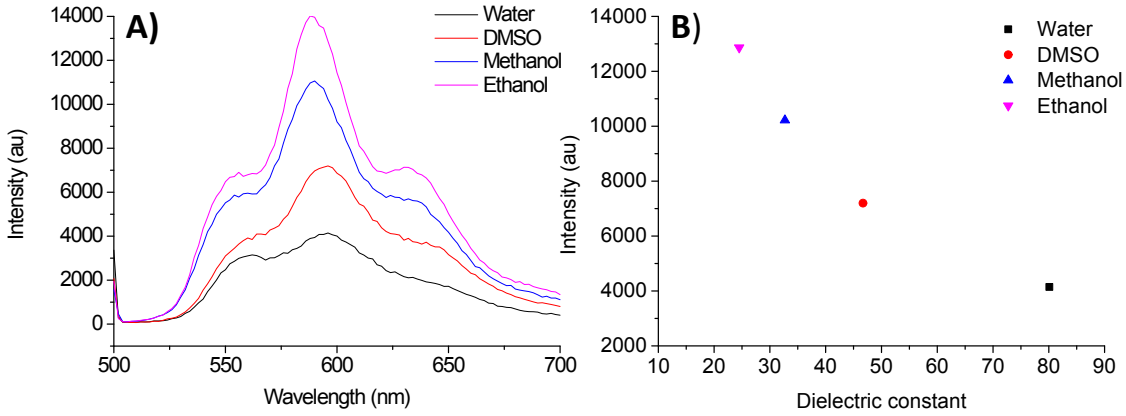


Figure S8. A) Fluorescent emission spectroscopy of doxorubicin in solvents with different polarities (i.e. dielectric constant): ethanol, methanol, DMSO and water. B) Maximum doxorubicin emission intensity (596 nm) plotted against the dielectric constant of the examined solvents.

A decrease in solvent polarity resulted in an increase in fluorescence intensity. This result is in agreement with the fluorescence results obtained for doxorubicin loaded into peptides. When doxorubicin is loaded into the micelles thus in more hydrophobic environment compared to water (free doxorubicin) the fluorescence intensity increases.

References

(1) Roy, S.; Dasgupta, A.; Das, P. K. *Langmuir* **2007**, *23*, 11769.
(2) Welsh, D. J.; Posocco, P.; Pricl, S.; Smith, D. K. *Org. Biomol. Chem.* **2013**, *11*, 3177.
(3) Sadownik, J. W.; Leckie, J.; Ulijn, R. V. *Chem. Commun.* **2011**, *47*, 728.

(4) Birchall, L. S.; Roy, S.; Jayawarna, V.; Hughes, M.; Irvine, E.; Okorogheye, G. T.; Saudi, N.; De Santis, E.; Tuttle, T.; Edwards, A. A. *Chemical Science* **2011**, *2*, 1349.