Electronic Supplementary Information

An Enzyme-Responsive Controlled Release System Based on a Dual-Functional Peptide

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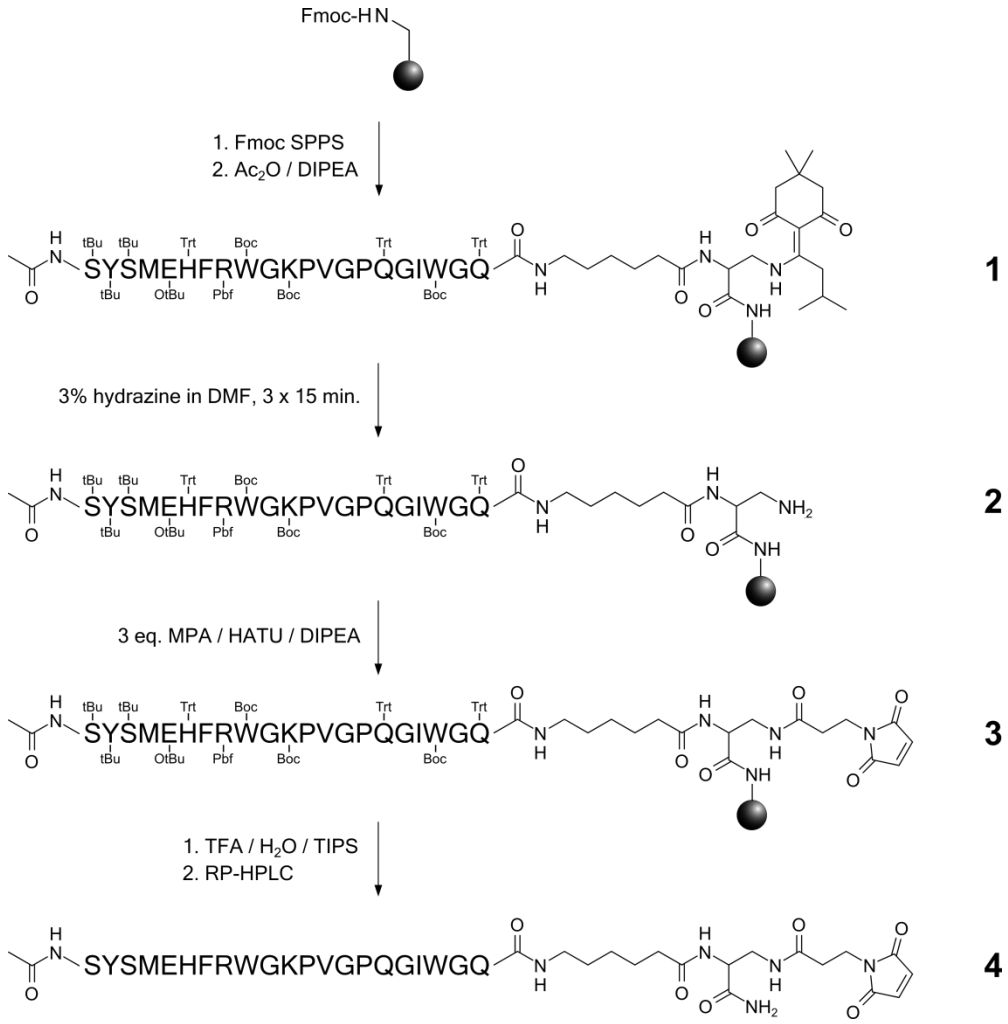
1 Synthesis and characterisation of dual-function peptide (DFP)

Peptide synthesis was performed on a Liberty microwave peptide synthesiser (CEM Corporation, U.S.A.) based on standard 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS) chemistry.[1] TentaGel® MB RAM MB 250 230 (Rapp Polymere GmbH, Germany) with the capacity of 0.2-0.3 mmol/g was used as the resin. Fmoc-protected amino acid derivatives were purchased from GL Biochem Ltd (Shanghai, China). Other solvents and chemicals used include: 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) from GL Biochem Ltd, China; triisopropylsilane (TIPS), piperazine , N,N-diisopropylethylamine (DIPEA), 2,2-ethylenedioxy diethanethiol (DODT), 1-methyl-2-pryrrolidinone and hydrazine monohydrate from Sigma Aldrich, U.S.A., acetic anhydride (Ac_2O) and N,N-dimethylformamide (DMF) from Ajax Finechem, Australia; ethanol, diethyl ether, formic acid (FA), trifluoroacetic acid (TFA) and dichloromethane (DCM) from Merck (U.S.A.). All solvents were analytical reagent grade (AR) or above.

The sequence SYSMEHFRWGKPVGPQGIWGQ (DFP, i.e. Pep 3, see Table 1 in main text) consists of two parts: an α-MSH peptide sequence SYSMEHFRWGKPV (Pep 1) and a collagenase-cleavable peptide (CCP) sequence GPQGIWGQ (Pep 2). Fmoc-ε-Acp-OH (GL Biochem, China) and Fmoc-Dpr(ivDde)-OH (Novabiochem, Switzerland) were introduced during the automatic SPPS to derivatise the C-terminus of Pep 3. The N-terminus was acetylated for 20 min using Ac_2O/DIPEA/DMF (1:1:23, v/v/v), followed by three resin washes with DCM and then 3 washes with DMF. The ivDde protection group was removed

using 3% (w/v) hydrazine monohydrate/DMF (three times, each time using a 15 min reaction) to expose the free amine group of diaminopropionic acid, which was then acylated with 3-maleimido-propionic acid (MPA) (BACHEM, Switzerland) using HATU/DIPEA. Finally, the whole molecule DFP-Acp-Dpr-MPA (Pep 4) was cleaved from the resin using TFA. Scheme S1 shows the detailed procedure for synthesising Pep 4. The solution containing crude Pep 4 was concentrated to ~ 2 mL under a nitrogen stream and the crude peptide was precipitated by adding cold diethyl ether followed by centrifugation. The solid material was washed once with cold diethyl ether and dissolved in MilliQ water (resistivity of $\geq$ 18.2 MΩ.cm) prior to purification.

The crude Pep 4 was purified by reversed phase high performance liquid chromatography (RP-HPLC) using an Agilent 1200 series HPLC system (Agilent Technologies, U.S.A.). Purification was carried out on a C18 reversed phase column (Phenomenex Kinetex 5 u, 150 × 21.2 mm) with a linear gradient of 0-60% buffer B [buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in acetonitrile] over 60 min and a flow rate of 5 mL/min. The purified peptide was characterised by RP-HPLC using an Agilent Eclipse XDB-C18 column (150 × 4.6 mm) with a linear gradient of 0-60% buffer B [buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in acetonitrile] over 30 min and electrospray ionisation time-of-flight (ESI-TOF) mass spectrometry (MS) using an Agilent 6200 Series ESI-TOF spectrometer. The purity of Pep 4 was determined to be more than 92% (Fig. S1); the mass of Pep 4 was calculated to be 2838.35 Da based on the multiply-charged series in the spectra (Fig. S2), while the expected mass is 2839.18 Da.



Scheme S1: The synthesis of Pep 4 (DFP-Acp-Dpr-MPA). The DFP (i.e. Pep 3) was synthesised using microwave-assisted Fmoc SPPS chemistry, with a C-terminal Dpr(ivDde) residue separated by an Acp spacer. The N-terminus was then acetylated to give 1. Treatment with hydrazine to selectively cleave the ivDde protecting group was performed to liberate the β-amino group of Dpr to give 2. The free amino group was then acylated with MPA to give 3. Finally, cleavage off the solid support and global deprotection of the peptide was achieved with a TFA cocktail. The isolated peptide was then purified by RP-HPLC to give 4. Abbreviations: Fmoc = 9-fluorenylmethyloxycarbonyl; Ac_2O = acetic anhydride; DIPEA = diisopropylethylamine; tBu = tert.-butyl; OtBu = O-tert.-butyl; Trt = trityl; Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Boc = tert.-butyloxycarbonyl; MPA = maleimidopropionic acid; HATU = 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; TFA = trifluoroacetic acid; TIPS = triisopropylsilane; RP-HPLC = reversed-phase high performance liquid chromatography."

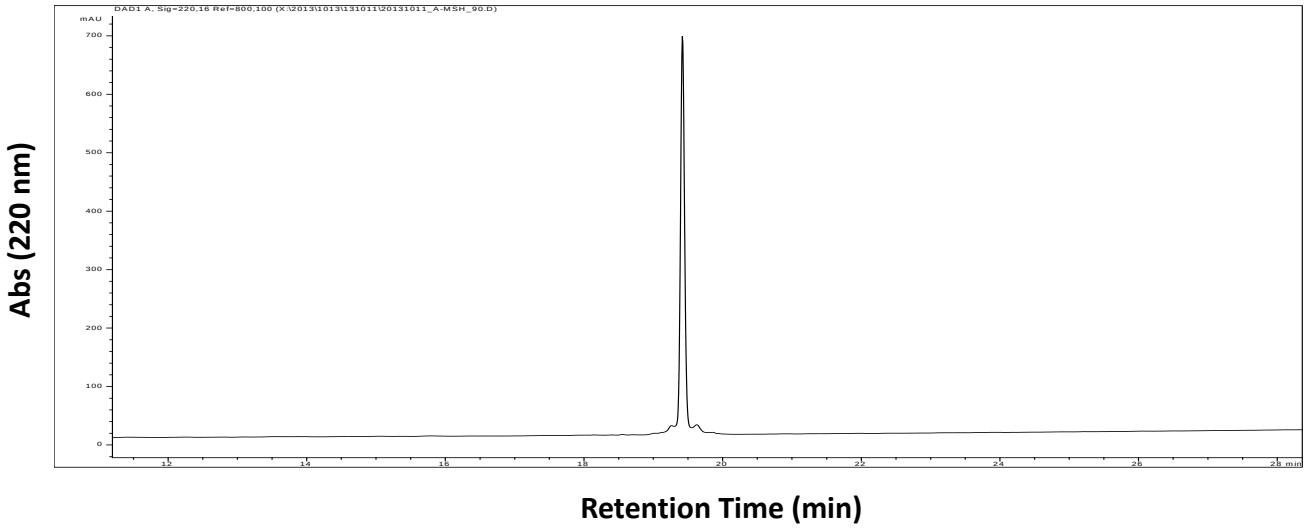


Fig. S1: HPLC trace of purified Pep 4, where Pep 4 eluted as a single peak.

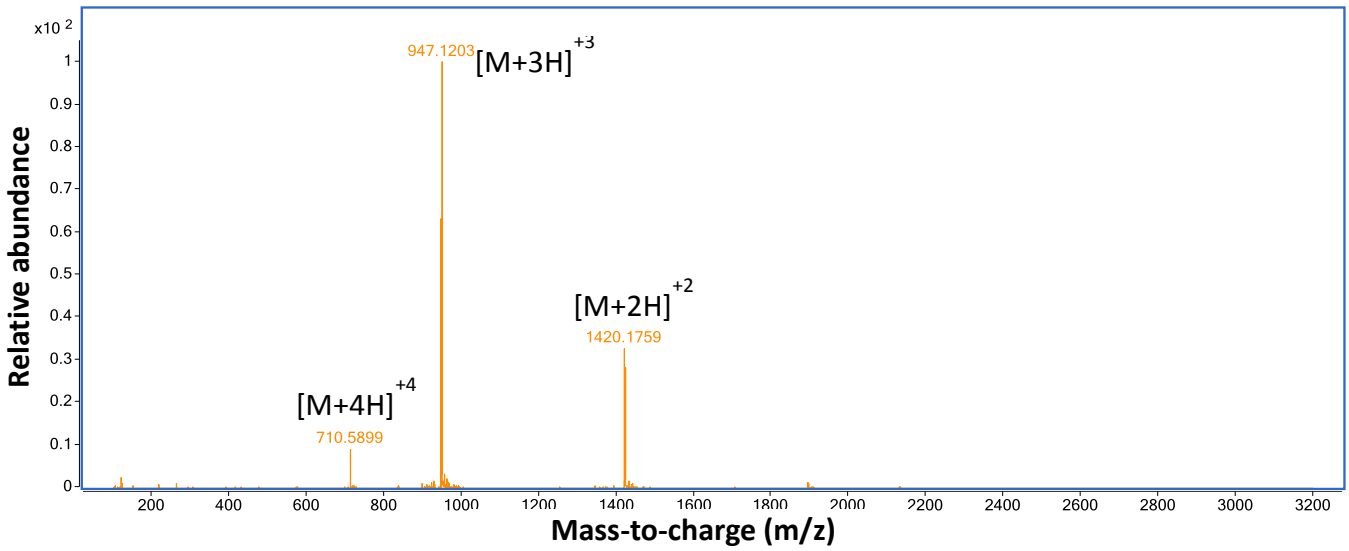


Fig. S2: ESI-TOF-MS of purified Pep 4. The observed *m/z* includes 1420.2 ($[M+2H]^{+2}$), 947.1 ($[M+3H]^{+3}$) and 710.6 ($[M+4H]^{+4}$); the *m/z* after deconvolution is 2839.4 ($[M+H]^{+}$), corresponding to a calculated mass of 2838.4 Da for Pep 4. The expected mass for Pep 4 is 2839.2 Da.

2 In solution cleavage of peptide via collagenase

The collagenase (Col) mediated cleavage of Pep 4 was first examined in solution, where collagenase type 4 (Worthington, U.S.A.) was used to test the specificity of the cleavage. This enzyme was obtained from the culture filtrate of *Clostridium histolyticum* and contains at least 7 different proteases of varying molecular weight (MW) 68-130 kDa.[2]

Col of different concentrations (20 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL or 300 µg/mL) was incubated with Pep 4 (100 µg/mL) at 25 °C for 10 min in a 1 mL solution of 10 mM $CaCl_2$. After incubation the solution was analysed using HPLC/MS (Agilent 1200 series HPLC system with Agilent 6200 Series ESI-TOF spectrometer). Specifically a SGE Protecol C18 HQ203 column (100 × 2.1 mm) was used with a linear gradient of 0-60% buffer B [buffer A: 0.1% FA in water; buffer B: 0.1% FA in 95% acetonitrile and 5% water] over 30 min. Elution peaks were monitored by measuring absorbance at 280 nm (Fig. S3). MS was used to determine the mass of peptide present in each peak within the HPLC profile (Fig. S4). The data was representative of two independent analyses.

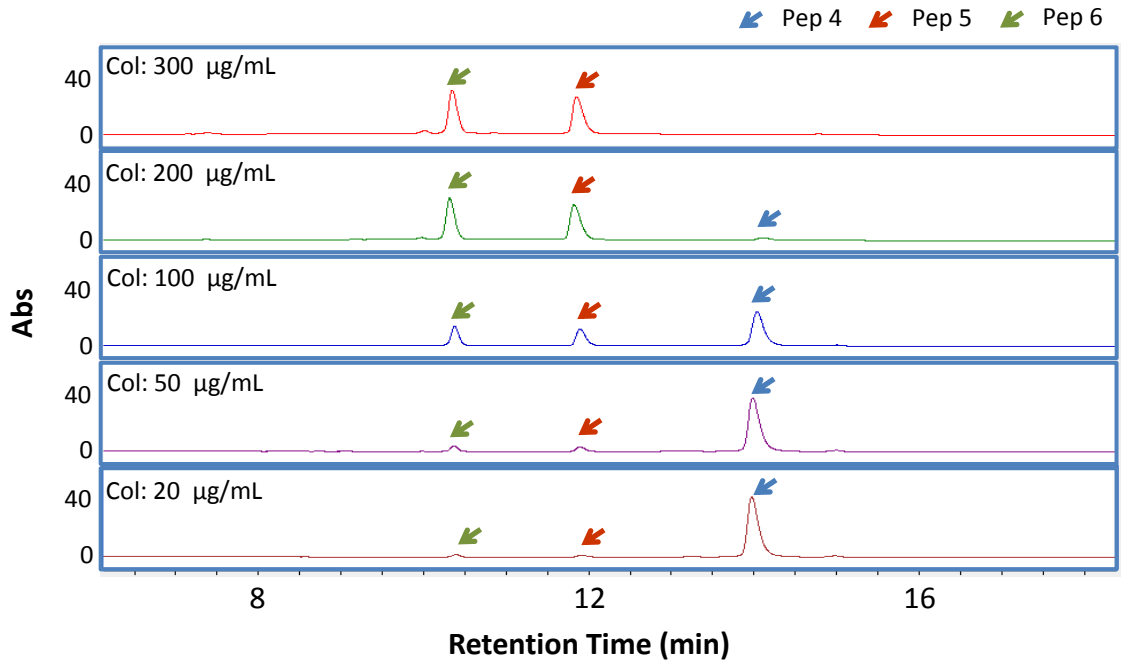


Fig. S3: HPLC profiles the peptide solution after Col (20-300 µg/mL) mediated in-solutions cleavage of Pep 4 for 10 min measured by absorbance at 280 nm.

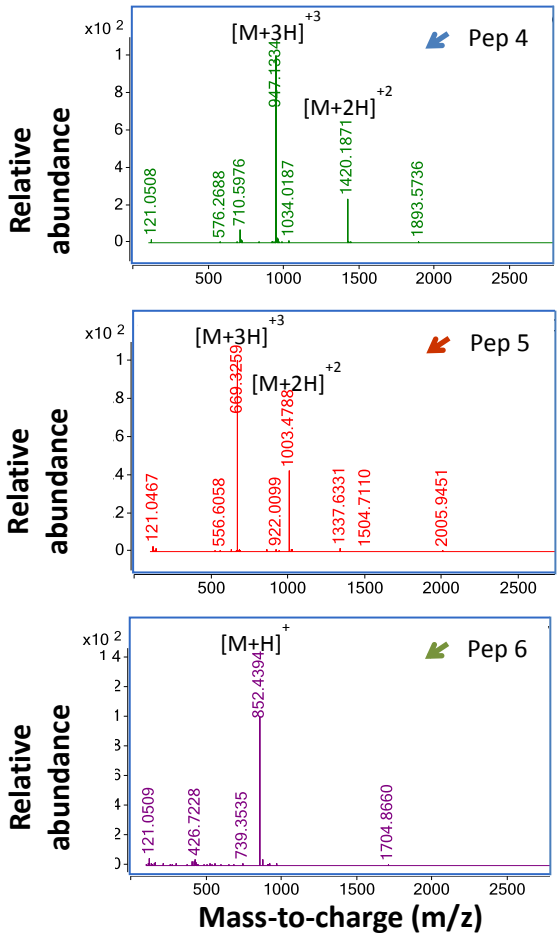


Fig. S4: ESI-TOF-MS of three peptides (Pep 4, Pep 5 and Pep 6) corresponding to the three peaks shown in Fig. S3. The observed *m/z* includes 947.1 ($[M+3H]^{+3}$) and 1420.2 ($[M+2H]^{+2}$) for Pep 4, 669.3 ($[M+3H]^{+3}$) and 1003.5 ($[M+2H]^{+2}$) for Pep 5, 852.4 ($[M+H]^{+}$) for Pep 6. The data corresponds to a calculated mass of 2838.4 Da for Pep 4, 2005.0 Da for Pep 5 and 851.4 Da for Pep 6. The expected mass of Pep 4, Pep 5 and Pep 6 are 2839.2 Da, 2005.2 Da and 852.0 Da.

3 Fabrication and surface functionalisation of MCF

The MCF silica materials were synthesised based on a reported method,[3, 4] using Pluronic P123 (poly(ethylene oxide)$_{20}$-poly(propylene oxide)$_{70}$-poly(ethylene oxide)$_{20}$, BASF, U.S.A.) as a structure directing agent and 1,3,5-trimethylbenzene (TMB, Acros, 99%) as an organic swelling agent, with a TMB/P123 ratio of 2.5 w/w. Briefly, 4 g of P123 was dissolved in 150 mL of HCl (1.6 M, made from 37% (w/w) solution, Merck, Australia). Thereafter the solution was heated to 37-40 °C followed by the addition of the desired amount of TMB and 46 mg of NH_4F. The resulting solution was stirred for 30 min. Tetraethyl orthosilicate (TEOS) (8.8 g) was then added followed by stirring for 20 h. The resulting mixture was transferred to a polytetrafluoroethylene (PTFE) coated autoclave and aged at 140 °C for 24 h. After cooling, the mixture was filtered with filter paper on a Buchner funnel and allowed to dry for 2 days. Finally, the resulting powders were calcined at 500 °C in air for 8 h to remove the organic template. The MCF samples were stored in a desiccator prior to use.

The sulfhydryl functionalisation of the MCF surface was carried out using reported procedures.[5-8] Briefly, 1 g of calcined MCF powder was suspended in 100 mL of MilliQ water and boiled in a round bottom flask under reflux for 3 h. The mixture was cooled in a fume cupboard, filtered using filter paper and a Buchner funnel and dried, all at room temperature. The powder was then suspended in 100 mL of methanol (AR, ≥ 99.8%, Ajax Finechem, Australia) and boiled under reflux for 2 h, in order to remove excessive water. The mixture was filtered using filter paper on a Buchner funnel and vacuum dried in a desiccator. The powder was then boiled under reflux in 100 mL of dry toluene (Ajax Finechem, Australia) and 5 mL of (3-mercaptopropyl) trimethoxysilane (MPTS, 95%, Sigma-Aldrich, Australia) for 24 h, filtered using filter paper on a Buchner funnel and dried at room temperature. To remove any unbound organosilane in the powder, soxhlet extraction was carried out for 20 h using 100 mL of an azeotrope of dichloromethane and diethyl ether (AR, ≥ 99.5%, Merck, Australia) with a volumetric ratio of 0.56:0.44. After extraction, the sample was vacuum filtered and stored in a desiccator. The resulting product is referred to as MCF-SH.

4 Characterisation of MCF and MCF-SH

4.1 Nitrogen adsorption-desorption isotherms

The pore characteristics of MCF and MCF-SH were studied by recording nitrogen adsorption-desorption isotherms (Fig. S5) at 77 K using a Micromeritics ASAP 2010 gas sorption analyser. The calcined MCF samples were degassed at 150 ℃ for 6 h prior to the analysis. The Brunauer-Emmett-Teller surface area (S_{BET}) was estimated from nitrogen adsorption data recorded in the relative vapour pressure range from 0.05 to 0.2. The total pore volume (V_{pore}) of the MCF materials was estimated from the amount of gas adsorbed at a relative vapour pressure of ~0.99. Pore diameters were calculated by the BDB-FHH method.[3] The BDB-FHH method allows the determination of spherical cell size from the adsorption isotherm and the window or pore entry size from the desorption isotherm (Fig. S6).

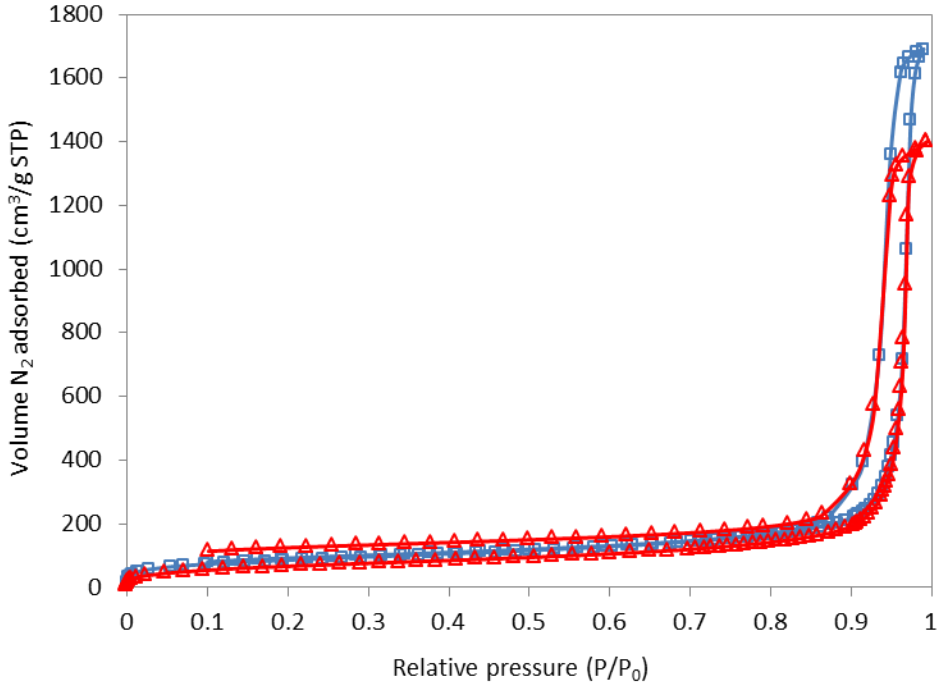


Fig. S5: Nitrogen sorption isotherms of the MCF (□) and the functionalised MCF-SH (Δ) samples.

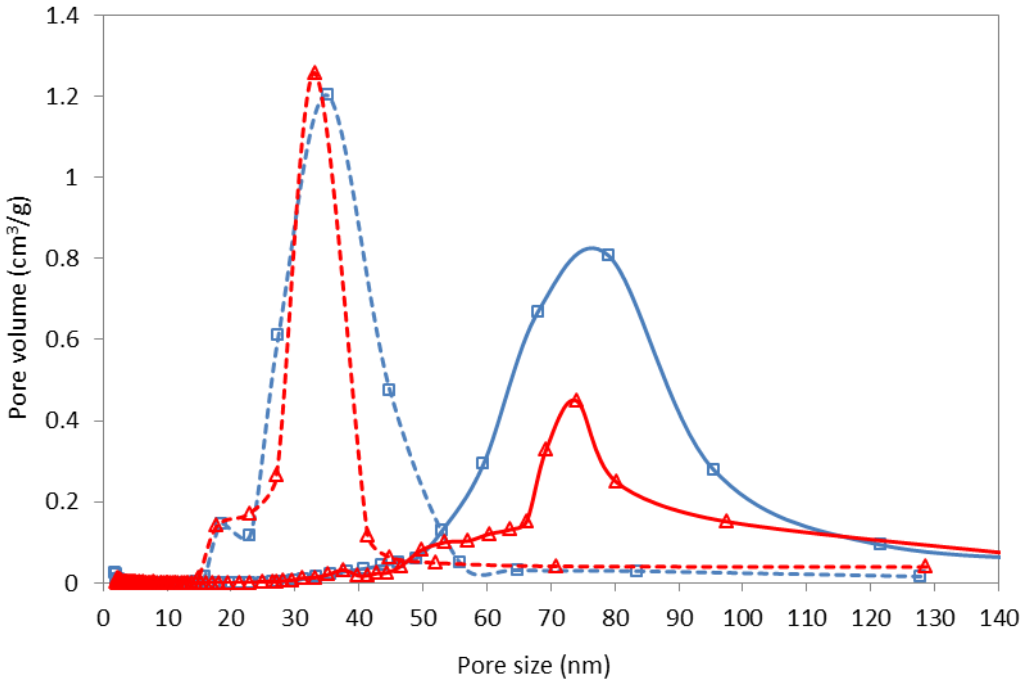


Fig. S6: BdB-FHH pore size distributions of the cells (——) and windows (- - -) of the MCF (☐) and functionalised MCF-SH (Δ) samples.

4.2 Morphology of MCF-SH

The morphology and powder size of MCF-SH materials were characterised using a FEI Quanta 200 Field Emission Gun Scanning Electron Microscope (SEM) operated at 12.5-20 kV (Fig. S7). The samples were gold sputter-coated prior to imaging; this process creates a thin film coating of ~ 5 nm in thickness.

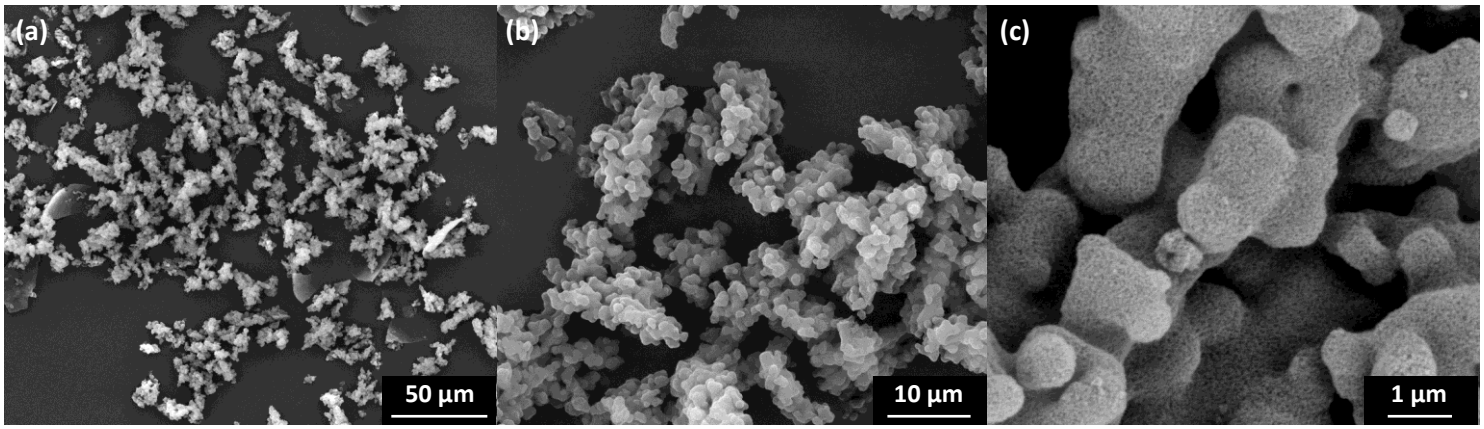


Fig. S7: Scanning electron microscopy images of MCF-SH samples. The scale bars are 50 µm, 10 µm and 1 µm in length for (a), (b) and (c) respectively.

4.3 Thermogravimetric analysis

The thermal behavior of MCF and MCF-SH samples was measured using thermogravimetric analysis (TGA) with a Pyris 1 Perkin-Elmer TGA under an air atmosphere at temperatures from 25 °C to 800 °C at a heating rate of 5 °C/min. The representative TGA profiles are shown in Fig. S8. Before the temperature increased to about 130 °C both samples experienced same weight loss due to the loss of water molecules. The MCF-SH material exhibited a greater weight loss above ~ 210 °C, corresponding to the release and decomposition of mercaptopropyl groups.[9, 10] A mass loss of 2.53% or 2.57% during heating between 210-350 °C was observed for two separate experiments using the MCF-SH material. This was evaluated to correspond to a thiol content of 339 ± 4 µmol (n=2) per gram of MCF-SH.

4.4 Ellman's reagent tests

An Ellman's reagent test was used to determine the concentration of free thiol groups on MCF-SH surface. A sample of 0.5 mg of MCF-SH material and 0.5 mg of MCF material (as the negative control) were separately treated with 5,5'-dithio-*bis*(2-nitrobenzoic acid) (Thermo Scientific, U.S.A.) in a reaction solution of 2.8 mL following the protocol provided by Thermo Scientific. A positive test for thiols generated a yellow solution and the thiol content was quantified by measuring the absorbance at 412 nm (ε = 14150 M^{-1} cm^{-1}).[11] The test for the MCF-SH material was repeated three times, giving an estimate of free thiol groups of 320 ± 11 µmol thiol/g MCF (n=3).

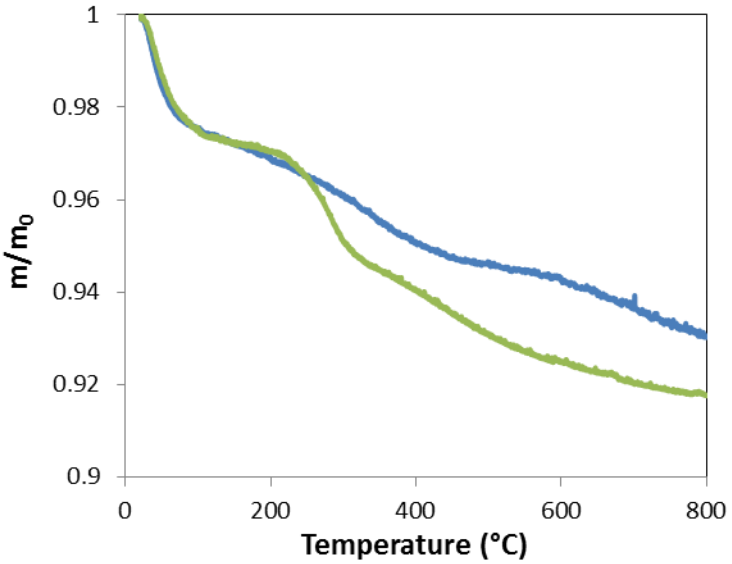
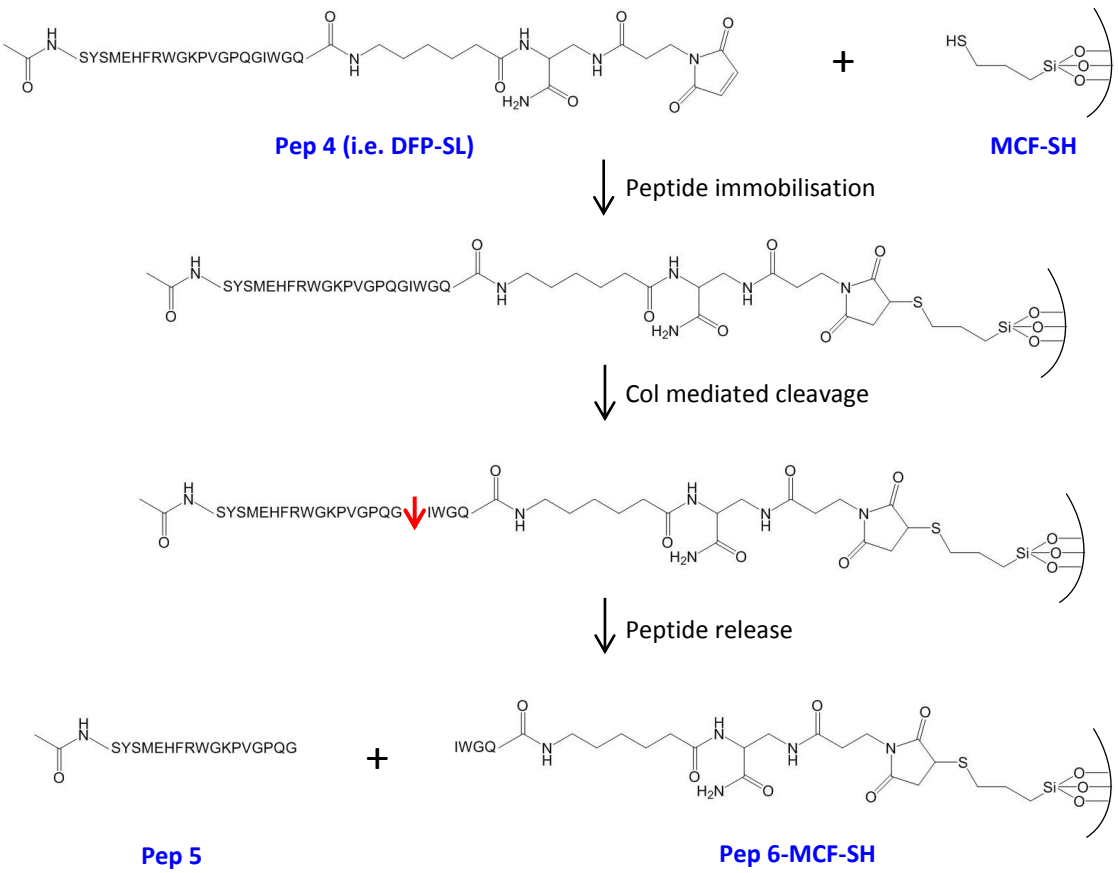


Fig. S8: Thermogravimetric analysis of the MCF (—) and functionalised MCF-SH (—) samples. Two separate analyses for the MCF-SH material indicated a mass loss of 2.53% and 2.57% during heating between 210-350 °C. This data corresponds to an thiol content of 339 ± 4 µmol per gram of MCF-SH (n=2).

5 Immobilisation of Pep 4 onto MCF-SH material

Scheme S2 showed the detailed reaction steps in Fig. 1 in the main text, showing the thiol-maleimide ligation between the thiolated MCF and the functionalised DFP (Pep 4) and the cleavage of Pep 4 at the site indicated by an arrow to release Pep 5. Pep 6 was retained on the surface of the MCF-SH materials after cleavage.



Scheme S2: A detailed schematic illustration of the reaction steps in Fig. 1 in the main text, showing the thiol-maleimide ligation between the thiolated MCF and the functionalised DFP (Pep 4) and the cleavage of Pep 4 at the site indicated by an arrow to release Pep 5. Pep 6 was retained on the surface of the MCF-SH materials after cleavage.

To quantify the mass of Pep 4 loaded onto the MCF-SH material surface, a NanoDrop 2000C UV-vis spectrophotometer (Thermo Fisher Scientific, U.S.A.) was used to record the peptide absorbance at 280 nm. Pep 4 has two tryptophan residues and one tyrosine residue (see Table 1 in main text). Each tryptophan contributes 5500 M^{-1} cm^{-1} and the tyrosine contributes 1490 M^{-1} cm^{-1} to the extinction coefficient (ε) at 280 nm.[12] Therefore the ε_{280} of Pep 4 is 12490 M^{-1} cm^{-1}. Theoretically a maximum of 339 μmol (i.e. 962 mg) of Pep 4 could be loaded onto 1 g of the MCF-SH material, provided that all thiol groups on MCF-SH are occupied (see TGA data in part 4.3). To evaluate the actual Pep 4 loading, 0.2 mg of MCF-SH (with a maximum theoretical loading of 192 μg Pep 4) was incubated with a ~5 fold excess of Pep 4 (1.11 mg) in 1 mL water at room temperature overnight. Five repeated tests were carried out for the Pep 4 immobilisation. After immobilisation, the MCF-SH sample was centrifuged at 10000 g for 10 minutes and the supernatant containing unloaded Pep 4 collected. The pellet of MCF-SH material was washed with water 3 times and after each wash the sample was centrifuged and the supernatant collected. The total volume of the collected supernatant was 3.6 mL.

The concentration of Pep 4 in the collected supernatant was quantified by measuring the absorbance at 280 nm, in order to calculate the mass of Pep 4 in the supernatant and thus the mass of Pep 4 immobilised on the MCF-SH material. According to the Lambert-Beer law $A_{280} = \varepsilon_{280} C l$, where l is the light path length (1 cm in this study) and ε_{280} is 12490 M^{-1} cm^{-1}, the concentration (C) of Pep 4 in the supernatant was found to be 103 ± 3 μmol/L (n=5). The average mass of Pep 4 in the 3.6 mL of collected solution was therefore 1.05 mg, indicating that 60 μg (i.e. 0.02 μmol) of Pep 4 was loaded on the 0.2 mg of MCF-SH. Compared to the maximum theoretical loading of 192 μg of Pep 4 per 0.2 mg of MCF-SH, approximately 31% of thiol groups on the MCF-SH material were found to be occupied by Pep 4 under these conditions. We have compared the Pep 4 loading capacity of the MCF materials to a published work by Schlossbauer et al.[13] in which the functionalised mesoporous silica SBA-15 had a peptide loading capacity of 0.4 μmol of melittin in 1 mg of SBA-15, that is, 0.02 μmol of melittin in 0.2 mg of SBA-15. Both peptide loading capacities are of the same order of magnitude; the slightly lower capacity of our MCF system may be caused by many factors, for example, the configuration and charge of different peptides, the silica porosity.

6 Release of peptide from MCF-SH via collagenase

To study the release of the therapeutic peptide (Pep 5) from the MCF-SH material, Col (180 μg) was added to a 0.6 mL suspension, which contained 0.2 mg of MCF-SH loaded with 60 μg of Pep 4. The concentration of Col and Pep 4 is thus 300 μg/mL and 100 μg/mL respectively, corresponding to a ratio of 3:1 (w:w) of Col to Pep 4. Different cleavage conditions were also tested by reducing the amount of Col and maintaining the amount of Pep 4 in the reaction to allow for a ratio of Col to Pep 4 of 2:1 1:1, 1:2 or 1:5. After Col mediated cleavage, the collected supernatant was first analysed by measuring the A280 absorbance to quantify the concentration of peptide present in the supernatant. Pep 5 (i.e. the peptide expected to be released) has one tryptophan residue and one tyrosine residue. Therefore the ε280 of Pep 5 is 6990 M^{-1} cm^{-1}; this value was used to estimate the concentration (*C*) of total peptide present in the solution for different cleavage conditions. The use of this absorptivity coefficient provides a conservative estimate, as any smaller peptide fragments would have a lower absorptivity coefficient.

The collected supernantant was analysed by HPLC-MS (Agilent 1200 series HPLC system with Agilent 6200 Series ESI-TOF spectrometer). Specifically a SGE Protecol C18 HQ203 column (100 × 2.1 mm) with a linear gradient was applied as described above. Fig. S9a shows the HPLC profile of the supernatant containing Pep 5 released from the MCF-SH material after Col mediated cleavage for 10 min. Under these conditions of the highest Col:Pep 4 ratio (3:1) the peak of Pep 5 was small. MS was used to confirm the mass of the peptide in the peak containing Pep 5 (Fig. S9b).

For the supernatant obtained after 10 min of cleavage using a lower concentration of Col (100 μg/mL), the HPLC profile (Fig. 4 in main text, i.e. the third HPLC profile in Fig. S9a) shows that there were two main peaks in addition to the desired Pep 5. Fig. S10 shows the mass spectra of the three main peaks. The mass of the peptides corresponding to the two unexpected peaks (Peak 1 and Peak 2) confirmed that one is a peptide with the sequence WGKPVGPQG and the other is a peptide with the sequence SYSMEHFR (Table S1). It was also confirmed by MS that the Peak 3 corresponds to Pep 5, which is the desired product for enzyme release.

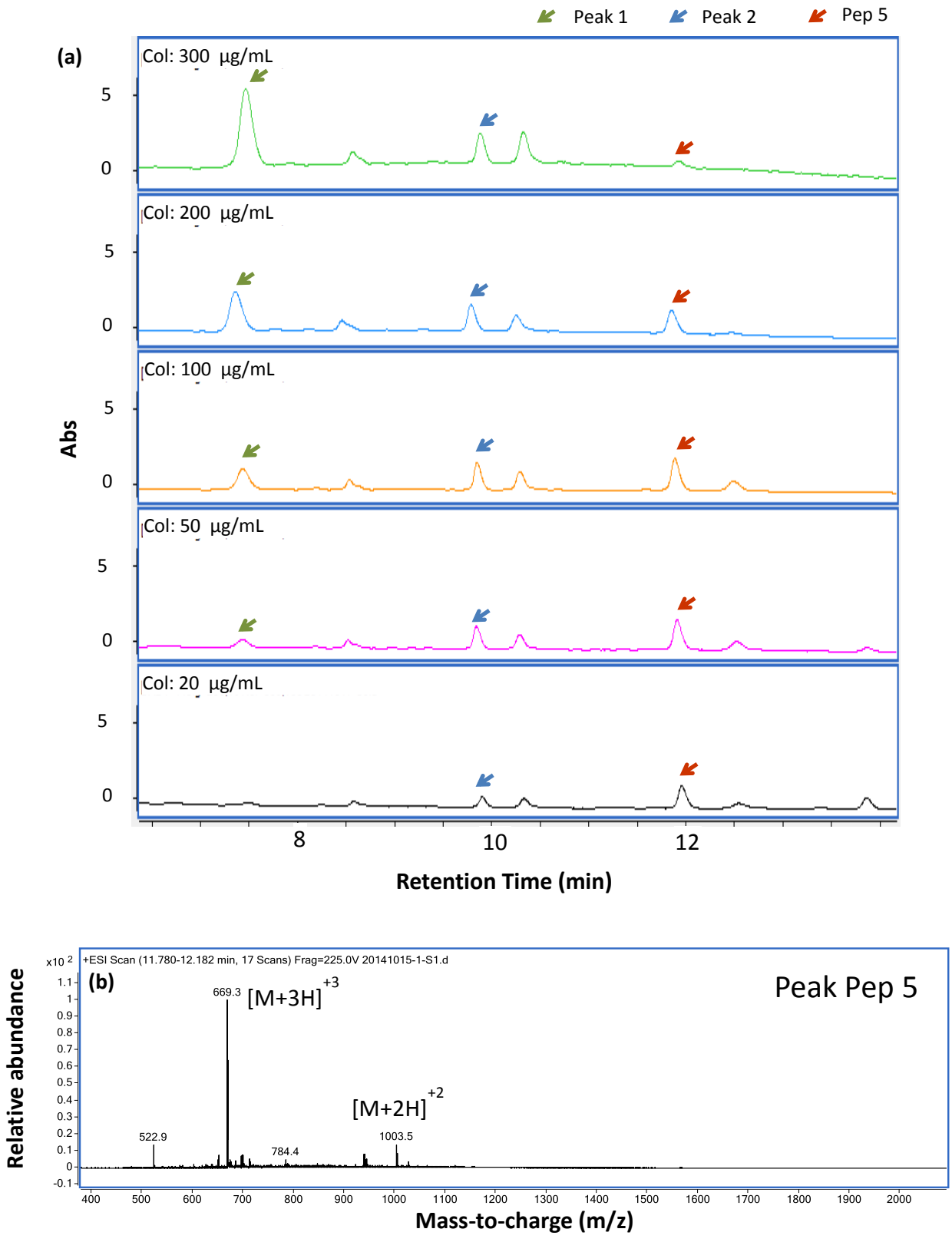


Fig. S9: HPLC-MS analysis of the supernatant containing peptides released from 0.2 mg of MCF-SH material immobilised with 60 µg of Pep 4, after cleavage mediated by Col (20-300 µg/mL) for 10 min. (a) peptides found in the HPLC profile of the cleaved samples, including Pep 5 which is indicated by red arrow and other peptide fragments corresponding to Peak 1 and Peak 2 which are indicated by green and blue arrow. The mass spectra in (b) confirms the presence of Pep 5 in the peak identified in (a). The observed *m/z* includes 669.3 ($[M+3H]^{+3}$) and 1003.5 ($[M+2H]^{+2}$) for Pep 5, corresponding to a calculated mass of 2005.0 Da for Pep 5. The expected mass of Pep 5 is 2005.2 Da.

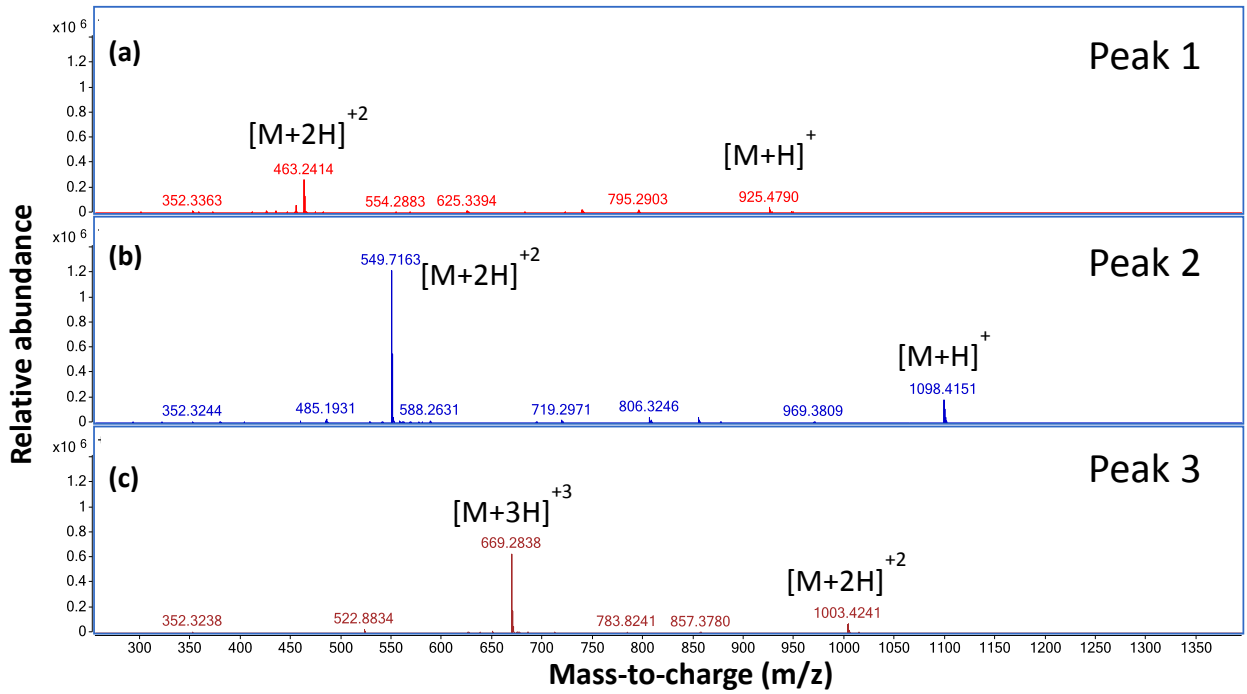


Fig. S10: Mass spectra of the peptides corresponding to the three main peaks shown in Fig. 4 for in meso cleavage of Pep 4. Peak 3 corresponds to the expected product Pep 5 and peak 1 and 2 are the two peptides corresponding to the two additional peaks within the HPLC spectra of Fig. 4 in the main text and Fig. S9a. The observed *m/z* includes 463.2 ($[M+2H]^{+2}$) and 925.5 ($[M+H]^{+}$) for Peak 1 peptide, 549.7 ($[M+2H]^{+2}$) and 1098.4 ($[M+H]^{+}$) for Peak 2 peptide, 669.3 ($[M+3H]^{+3}$) and 1003.4 ($[M+2H]^{+2}$) for Peak 3 peptide (i.e. the expected product Pep 5). The data corresponds to a calculated mass of 924.5 Da, 1097.4 Da and 2004.9 Da for Peak 1, Peak 2 and Peak 3 peptides respectively.

Table S1 Prediction of the possible cleavage sites within Pep 4 based on the mass of released peptide found in peaks 1, 2 and 3 of Fig. 4 in the main text and Fig. S10.

Peak	MW (Da)	Released peptide	Predicted cleavage site	Possible enzymes
Peak 1	925.1	**WGKPVGPQG**	SYSMEHFR↓**WGKPVGPQG**↓IWGQ	↓ point of cleavage by clostripain ↓ point of cleavage by collagenase
Peak 2	1098.2	**SYSMEHFR**	**SYSMEHFR**↓WGKPVGPQGIWGQ	
Peak 3	2005.2	**SYSMEHFRWGKPVGPQG**	**SYSMEHFRWGKPVGPQG**↓IWGQ	

7 Prediction of collagenase transport within the pores of MCF-SH

There are several different collangenases present in the Worthing type 4 bacterial collagenase from *Clostridium histolyticum*, ranging from 68 kDa to 130 kDa. Among them is collagenase G (ColG, ~120 kDa), for which a structural model has been determined.[14] The structure shows a distance of 11.5 nm along the longest axis, which was used as an estimate of the hydrodynamic diameter of ColG to predict the diffusion of collagenase inside the pores of the MCF-SH. The actual hydrodynamic diameter may be larger than 11.5 nm, as the crystal structure does not include the collagen binding domain. Equation (1)[15] was used to predict the time (*t*) for the enzyme to diffuse from the surface to the centre of the MCF particles:

$$t = \frac{\epsilon R^2}{D_p} \qquad \text{Eq. (1)}$$

where ϵ is the voidage of the MCF material, R is the MCF average particle radius, D_p is the enzyme diffusion coefficient in the pores of the MCF. The MCF has an irregular shape and different sizes, as shown in Fig. S7 by SEM. A radius of 5 μm was used as R here, which is a conservative estimate as the primary particles forming the MCF particles are generally less than 1 μm; the predicted diffusion time for the enzyme is therefore likely to be at the higher end of the range of possible diffusion times.

Equation (2) was used to calculate ϵ to use in equation (1):

$$\epsilon = \frac{v_p}{v_{total}} = \frac{v_p}{v_p + \frac{m_s}{\rho_s}} \qquad \text{Eq. (2)}$$

where v_p is the specific pore volume (measured as 2.62 cm^3) of MCF of 1 g, v_{total} is the total volume of the MCF particle (including the silica framework and pores), ρ_s is the density of the silica framework (2.2 g/cm^3), m_s is the mass (1 g) of silica framework. The voidage, ϵ, is calculated to be 85% here.

The Stokes-Einstein equation (eq. (3))[16] and the empirical Renkin equation (eq. (4))[17, 18] were then used to calculate the diffusivity in the bulk, D_0, and the lower hindered diffusivity expected within the pores of the MCF material, D_p:

$$D_0 = \frac{kT}{3\pi\mu d} \qquad \text{Eq. (3)}$$

$$\frac{D_p}{D_0} = \left(1 - \frac{R_e}{R_p}\right)^2 \times \left[1 - 2.10\left(\frac{R_e}{R_p}\right) + 2.09\left(\frac{R_e}{R_p}\right)^3 - 0.95\left(\frac{R_e}{R_p}\right)^5\right] \qquad \text{Eq. (4)}$$

where in eq. (3), k is the Boltzmann's constant, T is the absolute temperature, μ is the viscosity of the suspending fluid (here we used the viscosity of water, since the buffer for cleavage containing 10 mM $CaCl_2$ is expected to have a similar viscosity) and d is the diameter of enzyme that is diffusing (i.e. 11.5 nm). D_0 is calculated to be 3.8×10^{-11} m^2/s here; in eq. (4) R_e is the radius of the enzyme (5.75 nm), R_p is the radius of the window of MCF cells (here we used the window radius instead of the pore radius, because the smaller windows are the main constraint on enzyme transport compared to the bigger pores). The Renkin equation is based on diffusion in cylindrical channels[18] and therefore approximates the pores as cylinders with a diameter equal to the window size, this results in a lower diffusion coefficeint D_p. D_p is calculated to be 5.7×10^{-12} m^2/s here and is about seven times less than D_0. This confirms that the enzyme diffusion in the pores is hindered compared to the diffusion in bulk solution.

Using the above four equations the time for enzyme diffusion t in equation (1) is calculated as ~ 4 s. The enzymatic cleavage was allowed to occur for 10 minutes for in-meso cleavage, which therefore provided sufficient time for collagenase diffusion from the suface to the centre of MCF particles in order to achieve cleavage of the Pep 4 within the MCF pores.

8 Calculation of the mass of released Pep 5 from MCF-SH

It was shown that for the cleavage of the immobilised Pep 4 in MCF, in addition to the desired Pep 5, other peptides were also released by cleavage at amide bonds at places different to the expected G-I bond (where G and I stand for glycine and isoleucine amino acids, respectively). To quantify the extent of cleavage at the desired site, the percentage of Pep 5 released from the MCF material was determined for reactions with different Col:Pep 4 ratios, based on the HPLC peak area of Pep 5 determined from in-meso cleavage expressed as a percentage of total peak area measured at A280 nm.

A calibration curve for the quantities of Pep 5 in solution as a function of peak area (Fig. S11) was used to determine the amount of Pep 5 present. The total peak area was the sum of the peak area of each peptide shown in the HPLC profile assessed at 280 nm (Fig. S9a). The mass of total peptide released from 0.2 mg MCF predicted using this method (Fig. S12), is consistent with the analysis in Fig. 3 in the main text and part 6 in the ESI.

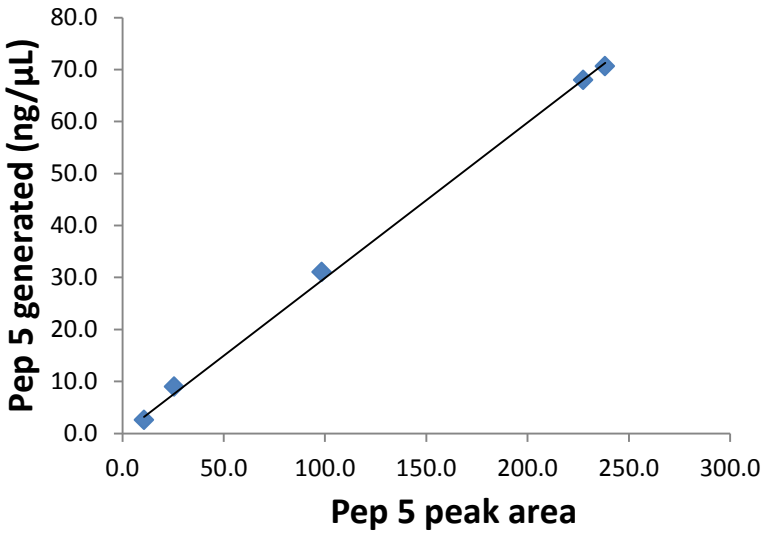


Fig. S11 The linear relationalship between the concentration of Pep 5 and the HPLC peak area of Pep 5 for in solution cleavage (Concentration of Pep 5 = 0.2992 × Pep 5 peak area). The concentration of Pep 5 was calculated based on the molecular weight ratio of Pep 4/Pep 5 and the concentration of Pep 4 that has been cleaved in the reaction solution.

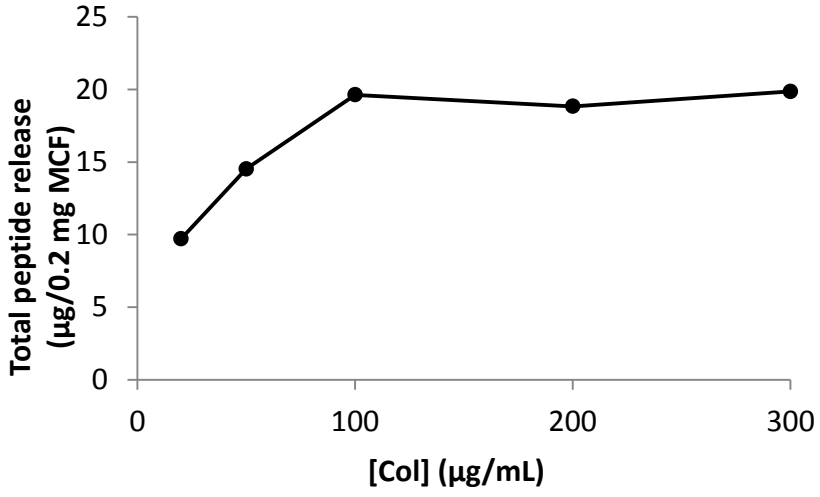


Fig.S12 The mass of peptide released from 0.2 mg of MCF-SH material within 10 min. Analysis is based on the mass of Pep 5 released from 0.2 mg of MCF materials (Fig. 5 a in the main text) and the percentage of Pep 5 in total released peptide (Fig. 5b in the main text).

9 SDS-PAGE of collagenase

In order to check the impurity of the Worthing type 4 collagenase, the preparation was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a pre-cast gel (Bolt 4-12% Bis-Tris Plus Gel) from Life Technologies, Australia. A 20 µL sample of collagenase was prepared by mixing 13 µL of Col solution (1 mg/mL), 2 µL Novex Bolt sample reduing agent (10X, Life Technologies) and 5 µL Novex Bolt LDS sample buffer (4X, Life Technologies). The sample was then boiled at 100 °C for 5 min. The protein molecular weight standard was 8 µL of Precision PlusProtein All Blue Prestained Protein standard (BioRad). Gel electrophoresis confirmed the presence of at least four different collagenases up to to 130 kDa in size, the gel also contained three extra bands below 50 kDa (Fig.S13). Two of these bands are consistent with the molecular weight of the heavy chain (~ 43 kDa) and light chain (~ 12.5 kDa) of clostripain, based on the studies by Gilles et al.;[19] the other band could not be assigned to any specific enzyme. Band intensity was assessed using the Gels Plot Lane function in the ImageJ software. Approximately 9% of the protein present in the collagenase mixture was estimated to be clostripain. Our in-meso substrate cleavage data is also consistent with the presence of clostripain, which can cleave the peptide amide bond in different locations to collagenase, as discussed in the main text.

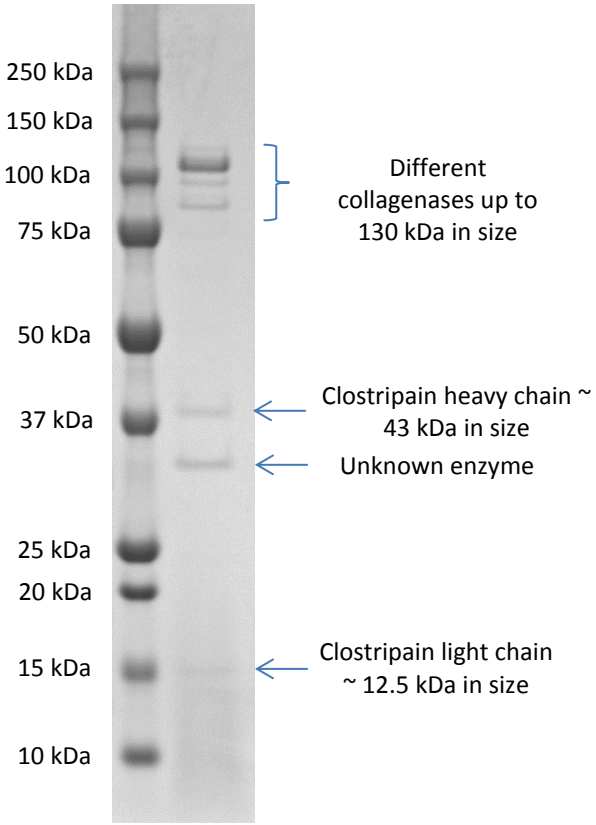


Fig. S13: SDS-PAGE of Worthing type 4 collagenase in a precast 4-12% gel, sample in denaturing and reducing conditions. Left lane: molecular weight standard; right lane: Worthing collagenase, a mixture of various collagenases (68 -130 kDa, note that there is no band corresponding to the 68 kDa collagenase, which may be due to a low content of this enzyme), clostripain (composed of a heavy and a light chain) and other unknown enzyme(s), as pointed by different arrows.

10 Distance of the cleavage site from silica surface

The distance between the enzyme cleavage site and the MCF surface is an important consideration when evaluating the performance of in-meso enzyme cleavage, as it will affect the spacial constraints imposed on each of the enzymes.

The Acp, Dpr, MPA of the spacer in Pep 4 and mercaptopropyl on the MCF surface together provide a distance of ~ 28 Å between DFP (i.e. Pep 3) and the MCF surface. For the cleavage mediated by Col, the cleavage site is at the G-I amide bond; therefore, the length of ~ 28 Å and the length of the residues IWGQ within Pep 4 combine to form a distance of ~ 42 Å between the Col cleavage site and the surface, where the length of IWGQ peptide segment is calculated by assuming the alpha carbons of adjacent amino acid residues of a peptide chain are at a distance of 3.6 Å when the peptide is fully extended.[20] For the cleavage mediated by clostripain, the cleavage site is at the R-W amide bond, therefore, the length of ~ 28 Å and the length of the residues WGKPVGPQG within Pep 4 combine to form a distance of ~ 75 Å between the clostripain cleavage site and the surface. These calculations assume that the DFP is completely unfolded, which provided an estimate of relative distances for clostripain and collagenase. Previously the α-MSH peptide has been shown to be predominantly random coil with far-UV circular dichroism.[21] Structural predictions using a peptide structure prediction server PEP-FOLD showed a predominant alpha-helix and random coil structure for Pep 3.[22] This structure may alter the relative space of the two cleavage sites. Any structural differences between the peptide in solution or on the MCF surface could also alter the relative distance.

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