Electronic Supplementary Information

Unusual Confinement Property of a Water Insoluble Small Peptide Hydrogel

Nilotpal Singha, Arpita Srivastava, Bapan Pramanik, Sahnawaz Ahmed, Payel Dowari, Sumit Chowdhury, Basab Kanti Das, Ananya Debnath* and Debapratim Das*

Experimental Section

General: Rink amide MBHA resin, Fmoc-Cys(Trt)OH, Fmoc-Lys(Boc)-OH, HBTU and HOBT were procured from GL Biochem, China. Lipases from *Candida Rugosa* and *Chromobacterium Viscosum*, Trypsin, urea, Rhodamine B, TCEP, 1-Pyrenebutyric acid and Triethylsilane (TES), DTT, GSH, p-Nitrophenylacetate (PNPA), HPLC-grade dimethyl formamide (DMF) and Trifluoroacetic acid (TFA), dichloromethane (DCM), and acetonitrile (ACN) were purchased from. To prepare samples, Milli-Q water with a conductivity of less than 2 mScm^{-1} was used. Chromato-graphic purifications were performed on a Luna 5 μm (C18, 250x10 mm) column (Phenomenex) whereas, ana-lytical HPLC were performed on a Luna 5 μm (C18, 250 × 4.6 mm) column (Phenomenex) using a Dionex Ultimate 3000 HPLC. UV-Visible spectra were recorded on a PerkinElmer Lambda 750 spectrometer, while fluorescence measurements were performed on a Fluoromax 4 (Horiba) spectrophotometer. Standard 10 mm-path quartz cuvettes were used for all spectroscopic measurements. ^{1}H NMR, ^{13}C NMR were recorded with a Bruker Ascend 400 MHz (Bruker, Coventry, UK) spectrometer and referenced to deuterated solvents. ESI-MS were performed with a Q-Tof Micro Quadrupole mass spectrophotometer (Micromass). FETEM images were taken using JEOL JEM-2100F microscope. Atomic Force Microscope images were taken on Nanosurf Flex-Axiom (Nanosurf, Switzerland). Circular Dichroism (CD) experiment was performed by using Jasco J-1500 spectropolarimeter.

Preparation of Hydrogel: To prepare the hydrogel, appropriate amount of **PyKC** was added in required volume of 20 mM Tris-HCl buffer at pH 8 (to attain a concentration of 1 wt%) and shaken to completely dissolve the solid. The solution was kept undisturbed at room temperature for 12 h to get the self-supporting hydrogel. Unless otherwise mentioned, all the studies were performed with 1 wt% hydrogel at room temperature.

Rheology: The viscoelastic properties of the hydrogel were characterized using rheometer (Anton-Paar MCR 102) equipped with a 20 mm parallel plate (with 0.3 mm zero gap) measuring system at 25 °C. A strain sweep test was performed first to identify the linear viscoelastic region (LVR) over a range from 0.01 to 100 % strain at a fixed oscillatory frequency of 1 rad/s. The LVR can be defined as, where strain has no impact upon G' and G''. Further, the mechanical strength of the gel was determined from oscillatory test i.e. frequency sweep, which was carried out under an appropriate strain (γ = 0.1 %) selected from the LVR with the frequency ranging from 0.1 to 1000 rad/s at 25 °C.

For time dependent gelation studies, the experiment was set up at constant strain of 1% and constant frequency of 5 Hz. The whole experiment was done at 25 °C. Since the overall experiment time was ~ 14h, measurement of one sample for such a long period resulted in erroneous observation due to evaporation effect. A 5 mL hydrogel was prepared in 1 wt% concentration and samples were taken from the hydrogel at different time interval. Each sample analysed for one hour. The plot was obtained by combining the G' and G" values with time.

NMR Studies: The ^{1}H NMR and ^{13}C NMR studies for characterization purpose were performed in DMSO-d6. The role of hydrogen bonding in gelation was explained from ^{1}H NMR experiment in DMSO-d$_6$-water complex system at varying percentages of water.

Water exchange experiment:

A) **From hydrogel to bulk water:** 750 μL solution of **PyKC** (1 wt%) in water was prepared and 200 μL of it was distributed in three centrifuge tubes and allowed to incubate for 24h to form hydrogel. In a separate experiment, 1.5 mL of fresh D$_2$O, NaOD (in D$_2$O) and DCl (in D$_2$O) solvents were taken and 2 mg of glycine was dissolved in each of these solvents. From these solutions, 500 mL were taken in NMR tubes and the ^{1}H NMR spectra were recorded, a) of these samles, b) after adding 5 μL of water and c) after adding another 95 μL of water (total of 100 μL of water). From the remaining deuterated solvents, 400 mL were added to the centrifuge tubes containing the hydrogels (200 mL each as prepared before) to wash the surface of the hydrogel and after centrifugation, the solvents were discarded. The remaining of the glycine containing deuterated solvents (600 μL) were added to the centrifuge tubes containing hydrogels and sealed. The tubes were incubated at room temperature while

shaking at 100 rpm. After 1day of incubation, the samples were centrifuged and 500 µL of the supernatant solvents were taken and ^{1}H NMR spectra were recorded. The solvents were again added back to their respective centrifuge tubes and incubated. The procedure was repeated to get the spectra after 3 and 7 days of incubation.

For the analyses of the data, the peak area ratio of glycine-CH_2 peaks (A$_{Gly}$) and residual water peaks (A$_{Residual}$) were considered. The increase/change in this ratio after incubation of hydrogel samples was considered to calculate the amount of water (H$_2$O) exchanged from the hydrogel samples. In order to get the values for 100% exchange, the peak ratios of samples with external water (5 µL and 100 µL) were considered. Unfortunately, with 100 mL water, no ratio could be calculated and thus the ratio corresponding to the 5 mL water samples were extrapolated by multiplying it with 40 (as 200 µL of gels were present.

B) **From bulk water to hydrogel:** In a typical experiment, 2 mg of glycine was dissolved in 1 mL of D$_2$O and the ^{1}H NMR of the sample was recorded. A 1 wt% hydrogel of **PyKC** was then prepared in 100 µL of the D$_2$O solution. The hydrogel was suspended in 500 µL and shaken at 100 rpm at room temperature. After 7 days of incubation, the sample was centrifuged and the supernatant bulk water was removed. The remaining hydrogel was washed with the glycine containing D$_2$O solution two times (200 µL). The remaining D$_2$O solution was then added to the hydrogel followed by 1 mg of TCEP to break the hydrogel. This sample was used for ^{1}H NMR recording.
Similar to the previous experiment, the peak area ratio of glycine-CH_2 peaks (A$_{Gly}$) and residual water peaks (A$_{Residual}$) were considered to calculate the extent of exchange.

Dye exchange experiments:

A) **From hydrogel to bulk water:** 200µL of **PyKC** hydrogels were prepared in solutions containing 6 different dyes. These gels were suspended in 1 mL of water while shaking at 100 rpm. At different time intervals, aliquots were taken and equal volume of water was added to the bulk water. The absorbance spectra of the aliquots were recorded. After 7 days, the hydrogel was dissolved using TCEP and the absorption spectra were recorded. To calculate the cumulative % release, the absorption (at the λ_{max} of the corresponding dye) of the original dye solution was compared with the values obtained at different time interval.

B) **From hydrogel to bulk water:** 200µL of **PyKC** hydrogels were prepared in water and suspended in aqueous solutions of methylene blue or perylene (containing 1% acetonitrile) while shaking at 100 rpm. After 7 days, the gels were centrifuged and washed twice with water before suspending them again in measured volumes of water or water containing 1% acetonitrile. The gels were dissolved with the help of TCEP and the absorption spectra were recorded. To compare, the original dye solutions were treated with PyKC and TCEP (maintaining the concentration at par with the final gel-dissolved solutions) and the absorption spectra were recorded.

Determination of Sol−Gel Transition Temperature (Tg): T_g was determined using standard ball dropping method. A small steel ball was placed on top of the hydrogel sample (equal volume). The samples were placed in a water bath and the bath was heated at a rate of 0.5 °C/min. The temperature at which the steel ball drops to the bottom was noted as T_g. The experiments were performed in triplicate and the results were obtained within ± 0.5 °C.

FETEM: 5 µL of the samples were casted on carbon coated copper grid (300 mesh Cu grid with thick carbon film from Pacific Grid Tech, USA) and allowed to air dry for 10 minutes and then the excess sample was bloated with a tissue paper. The grid was then allowed to air dry for 1 day.

PXRD: Two samples were analyzed by PXRD, a) the gelator in monomeric state, which was prepared by dissolving in hexafluoroisopropanol (HFIP) followed by drying under ambient condition, and b) a thin film of the gel was prepared on the surface and dried. The samples were analysed on a Bruker D2 Phaser X-ray diffractometer (30 kV, 10 mA). The Bragg peak λ was extracted from the XRD data and the layer thickness d could be obtained according to the Bragg equation $d = \lambda/2\sin\theta$, $\lambda = 0.15405$ nm.

N₂ Adsorption/Desorption: N_2 adsorption/desorption isotherms were obtained by using a Quantachrome Autosorb 1-C surface area analyzer at 77 K. Typically, a matured hydrogel was lyophilized for 48 h to get the solid xerogel and placed into the instrument for the analysis. Pore size distribution was calculated employing the NLDFT (nonlocalized density functional theory) model.

Dissolution Study: In a typical experiment, 500 μL of a 1 wt% hydrogel of **PyKC** (in 20 mM Tris buffer, pH 8) was added to 10 mL of the bulk solvent/solution and the sample was incubated with slow shaking (100 rpm) at room temperature. Aliquots of the bulk solvent/solution was taken time to time and replaced with the same amounts of fresh bulk solvent/solution to keep the overall volume intact. The aliquots were diluted with the same bulk solvent/solution before recording their absorption. Absorbance spectra of freshly prepared solutions of **PyKC**-dimer in the respective bulk solvent/solution were recorded to get the calibration curve to determine the concentrations of **PyKC**-dimer in the aliquots. The % dissolutions were calculated using these calibration curves following the cumulative absorbance at λ_{max} (352 nm). Moreover, to verify that the 100% values obtained from extrapolating the calibration curves were not affected due to aggregation of the molecules at that concentration, we have dissolved exactly the same amount of the gelator (used to create 500 ml gel) in 10 mL of water and allowed it to form the dimer by incubating the solution for 24 h and the absorbance values at λ_{max} (352 nm) were recorded. To our satisfaction, this value matches with the values obtained from the calibration curve. For clarity of understanding, 5% dissolution means that only 5% of the gelator was found in the bulk solvent. Here the values were represented in terms of **PyKC** and not its dimers.

For temperature dependent study, every sample was incubated at a precisely temperature controlled water bath at the respective temperature for 1 h followed by incubation at to room temperature (30 mins). Aliquots were withdrawn from bulk solvent and analyzed. Analyses beyond 70 ° C could not be performed as the melting temperature of the hydrogel was found to be 75 °C. All experiments were performed in triplicate.

Circular Dichroism (CD): The CD spectra of all the samples were recorded at room temperature. The data were collected at 1 nm intervals with 2 nm band width. All measurements were done in 0.2 cm path length cuvette with 400 μL sample volume. Each CD profile is an average of 3 scans of the same sample collected at a scan rate 100 nm min⁻¹, with a proper baseline correction from the respective solvents. In a typical experiment, three samples were prepared and analyzed by CD.

a) **PyKC** (1 wt%) solution was prepared by dissolving appropriate amount of **PyKC** in 20 mM Tris buffer (pH 8) containing 400 μg mL⁻¹ of the respective enzyme and 100 μL portions of the solution were incubated separately at room temperature for 12 h to form the hydrogel where the enzyme was encapsulated. The gel was kept at room temperature for seven days and then re-dissolved in 400 μL of Tris buffer containing 7 mg mL⁻¹ of TCEP. The CD spectra of this solution was recorded.

b) The CD spectra of native enzyme solution maintaining the enzyme concentration as in the previous case were recorded in presence and absence of TCEP (Importantly, the presence of TCEP did not impart any noticeable change in the conformation of the enzymes).

c) CD spectra of a re-dissolved **PyKC** hydrogel (maintaining the amount of **PyKC**, buffer and TCEP as in case of sample (a)) without the enzyme was also recorded and any contribution from this spectrum was subtracted from the CD spectra of sample (a). The spectra obtained from sample (b) and (a-c) were compared to find any change in the tertiary structure of the enzyme. These experiments were performed five times to confirm the obtained results.

Enzyme activity studies: For enzyme activity, 1 wt% **PyKC** solutions were prepared in 20 mM Tris buffer (pH 8) containing the enzyme (70 units/mL). From these solutions, 100 μL portions were taken in 2 mL centrifuge tubes and incubated at room temperature for 12h to form hydrogels. Similar samples maintaining the buffer and enzyme concentrations were prepared without the gelator. To the hydrogel samples, 1.5 mL of bulk water were added and shaken slowly to suspend the gels in water. Both series of samples were incubated at room temperature and at different time intervals, a pair of gel trapped enzyme and free enzyme sample were taken for analyses. For the gel trapped enzyme samples, the gels

were centrifuged and washed with water three times before releasing the enzyme with the help of 0.3 mL of 25 mM TCEP solution and then diluting the sample with appropriate amounts of phosphate buffer (pH 7.2). Free enzyme samples were diluted with phosphate buffer to maintain the overall enzyme concentration as in case of gel-trapped samples. The enzyme activity was measured by adding 10 μL of 30 mM substrate (PNPA) to these solutions and monitoring the change in absorbance at 400 nm. The loss in activities were calculated with respect to the initial activity of the free enzyme sample at 0h. For temperature dependent study, the samples were prepared in glass vials and incubated in a precisely controlled water bath maintaining a particular temperature for 1h. After the incubation period, the samples were allowed to come back to room temperature (30 mins) before doing all the treatments and activity measurements as mentioned before. For the effect of denaturants only the activity of CR-Lipase was monitored. a) MeOH and Urea: hydrogels were incubated in bulk MeOH or 6 M urea solutions. Samples at different times were centrifuged and washed three times with water before analyzing the activity following similar procedure as mentioned before. For free enzyme activities, 10 μL of either MeOH or 6 M urea solutions were added to the enzyme solutions before measuring the activities. In both cases, the enzyme showed no activity at all. b) Effect of pH: hydrogel samples containing the enzyme were incubated in buffer solutions of different pH (pH3: citrate buffer; pH5: acetate buffer; pH 7: phosphate buffer; pH9: Tris buffer; $NaHCO_3$/NaOH; all 20 mM) for 6 h before centrifugation and washing with water three times. The enzyme activities were then studied following the protocol mentioned before. For free enzyme, the activities were measured in the mentioned buffers.

Electronic Structure Calculations and Molecular Dynamics Simulations: For Folded (C-1, Figure S17) and open (C-2, Figure S17) conformations of **PyKC**-dimer were obtained using ChemDraw and used as the initial configurations for the geometry optimization using DFTB+ package[1]. We used density functional tight binding (DFTB-D3)[2-4] method to get the molecular structure of the **PyKC**-dimer. DFTB uses minimal atom-centered Slater-type all-valence basis sets and an approximate Hamiltonian matrix based on DFT energy equation. The electrostatic interactions between the partial charges of the molecule were evaluated considering self-consistent charges (SCC). Dispersion corrections were included to consider van der Waals and π−π stacking interactions. C, H, N element pair interactions were obtained from standard DFTB parameters (http://www.dftb.org). Earlier DFTB has been found useful to calculate the stacking energy for pyrene based systems[5, 6]. The total energy of the system in DFTB[7] is written as,

$$E_{tot} = E_{BS}[n0] + E_{coul}[\delta n] + E_{rep}(R),$$ (1)

where, $E_{BS}[n0]$ is the band structure energy term, $E_{coul}[\delta n]$ is the energy due to charge transfer and $E_{rep}(R)$ is the repulsion energy. Dimers of open and folded conformers of **PyKC** are optimized without and with dispersion correction terms. Convergence in the geometry optimization was achieved within 20000 steps. Both the optimized folded and open **PyKC**-dimers (from C-1 and C-2) were separately stacked in such a manner that the pyrene rings remain parallel. The optimized geometries of both open and folded conformers are shown in figure S28 in SI in absence and presence of dispersion corrections. The energies of the optimized folded and open dimers were presented in table S1 of SI. The binding energies of the stacked conformers are calculated using the following equation,[8]

$$\Delta E_b = E_s - 2E_d,$$ (2)

where, ΔE_b is the binding energy, E_s is the energy of the stacked configuration and E_d is the dimer energy. The folded conformer was found to have stronger binding energy (table S1 in SI) and chosen as the initial conformation of the **PyKC** dimer to perform all-atom molecular dynamics (MD) simulations using GROMACS v4.6.5.[9-11]

Since **PyKC** forms hydrogel at or above 1 wt% concentration, (a) one dimer, (b) two dimers, (c) 60 dimers of geometry optimized **PyKC** each at a concentration of 43.4 wt%, were simulated in presence of SPC/E[12] water model. The bonded parameters of the folded **PyKC**-dimer were obtained by Automatic topology builder (ATB).[13] Non-bonded parameters were obtained from GROMOS54a7[14] force-field. For (a), one **PyKC** dimer was inserted in a box of length 4.16 nm in each xyz direction followed by the random insertion of 75 water molecules. The system was energy minimized using the steepest descent algorithm.[15] Temperature of the system was maintained at 300 K by velocity rescale thermostat[16] in an NVT ensemble for 100 ps. Next a 42.5 ns NPT simulation was performed for equilibration using Parinello Rahman barostat[17, 18] for pressure coupling with a coupling constant of 1

ps. The pressure of the system was held at 1 bar. A time step of 2 fs was used for the simulation and trajectories were collected at every 10 ps and periodic boundary conditions were applied in all 3 directions. The non-bonded interactions were cut-off at a distance of 1 nm. The columbic interactions were determined by Particle mesh Ewald (PME) method.[19]   Last 5.5 ns of the total run-length is analyzed for all calculations performed. Systems (b) and (c) were simulated with the same set of parameters. To compare the dynamics of water near **PyKC** with the dynamics of bulk water (BW), a box of bulk SPC/E[12] water molecules is simulated for 2 ns with a time step of 2 fs in an NPT ensemble. Temperature is coupled at 300 K using Nose-Hoover thermostat[20] and Parinello Rahman[17, 18] barostat is used to maintain the constant pressure of 1 bar. This is followed by an NVT run of 5 ns with a time step of 0.4 fs. The system is coupled to the velocity rescale thermostat[15] with a coupling constant of 2 ps. Non-bonded interactions were cut-off at 0.9 nm. The box length was 1.87 nm in each xyz direction. The last 50 ps of the run were analyzed to calculate the dynamics of BW.

Synthesis of the peptides:

The peptides were prepared using solid phase peptide synthesis technique employing Rink-amide MBHA resin as the solid support. Sequence elongation at the N-terminus was performed by coupling the appropriate Fmoc protected amino acids (4 equiv.) under standard conditions employing HBTU (4 equiv.) and DIPEA (8 equiv.) as coupling reagents in presence of HOBT (4 equiv.) in DMF. Fmoc deprotection was achieved by treating the resin bound peptide with 20% piperidine in DMF. The peptide was cleaved from the resin using a cocktail of 95% TFA - 4% dichloromethane - 1% triethylsilane. Precipitation from dry ether followed by purification using semi-prep HPLC (acetonitrile/water system as the eluent) and lyophilisation provided the pure peptides. All the control molecules were prepared following similar protocol.
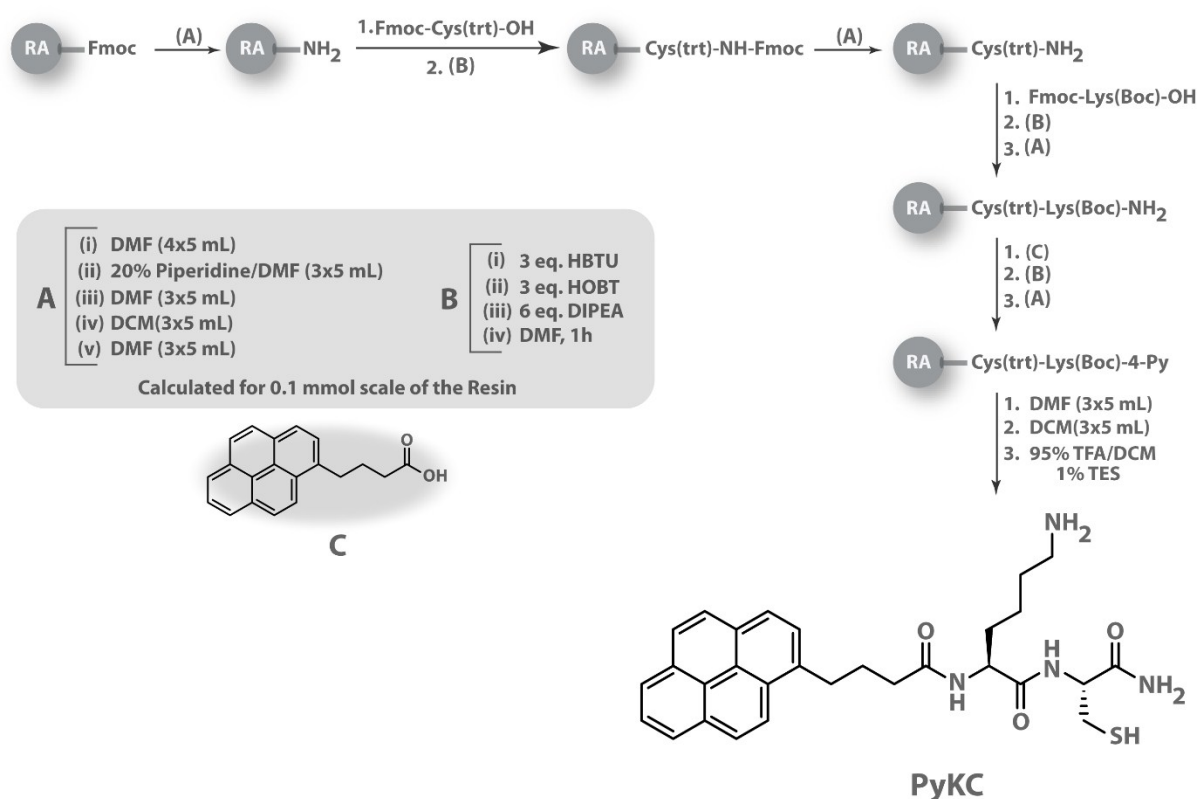
Scheme 1: Synthetic route for **PyKC.**

Characterization of synthesized peptides:

PyKC:

¹HNMR (DMSO-d$_6$, 400 MHz): δ/ppm = 8.39 (d, J = 9.3 Hz, 1H), 8.28 (m, 2H), 8.23 (m, 2H), 8.14 (d, J = 2.0 Hz, 2H), 8.07 (t, J = 7.6 Hz, 1H), 7.97 (t, J = 8.2 Hz, 2H), 7.66 (s, 3H), 7.29 (s, 1H), 7.20 (s, 1H), 4.39 – 4.21 (m, 2H), 2.90 – 2.66 (m, 4H), 2.29 (m, 3H), 2.03 (p, J = 7.3 Hz, 2H), 1.69 (m, 1H), 1.55 (m, 3H), 1.35 (d, J = 35.3 Hz, 2H). **¹³C NMR** (100 MHz, DMSO-d$_6$): δ/ppm = 172.96, 172.31, 171.87, 137.06, 131.36, 130.90, 129.78, 128.06, 127.93, 127.70, 126.99, 126.63, 125.42, 125.26, 124.03, 55.06, 53.19, 39.18, 35.29, 32.71, 31.44, 28.00, 27.10, 26.58, 22.82. **ESI-MS** calcd. for [M+H]$^+$, C$_{29}$H$_{34}$N$_4$O$_3$S: 519.24, found: 519.24 (m/z). **HPLC:** The peak of **PyKC** arrived at 12.8 min retention time when eluted with gradient started from 5% to finish at 100% of acetonitrile in water in a 20 min program.

Pep-2:

¹H NMR (400 MHz, DMSO-d$_6$): δ/ppm= 8.92 (d, J = 7.5 Hz, 1H), 8.54 (d, J = 9.3 Hz, 1H), 8.42 (s, 1H), 8.36 (dd, J = 7.7, 4.1 Hz, 3H), 8.31 – 8.21 (m, 3H), 8.20 (d, J = 7.9 Hz, 1H), 8.14 (m, 2H), 7.70 (s, 3H), 7.53 (s, 1H), 7.29 (s, 1H), 4.68 – 4.58 (m, 1H), 4.46 (m, 1H), 2.93 (dd, J = 13.5, 5.0 Hz, 1H), 2.88 – 2.79 (m, 3H), 1.85 (s, 2H), 1.72 – 1.48 (m, 2H), 1.54 (s, 2H). **HPLC:** The peak of **Pep-2** arrived at 10.1 min retention time eluted with a gradient started from 5% to finish at 100% of acetonitrile in water in a 20 min program.

Pep-3:

¹H NMR (400 MHz, DMSO-d$_6$): δ/ppm = 8.39 (d, J = 9.3 Hz, 1H), 8.31 – 8.01 (m, 9H), 7.95 (d, J = 7.8 Hz, 1H), 7.69 (s, 3H), 4.38 (m, 2H), 2.90 (dd, J = 13.7, 4.6 Hz, 1H), 2.77 (m, 3H), 2.32 (t, J = 7.2 Hz, 2H), 2.02 (m, 2H), 1.73 – 1.62 (m, 1H), 1.54 (m, 3H), 1.35 (m, 2H). **HPLC:** The peak of **Pep-3** arrived at 11.85 min retention time when eluted with a gradient started from 5% to finish at 100% of acetonitrile in water in a 20 min program.

Pep-4:

¹H NMR (400 MHz, DMSO-d$_6$): δ/ppm = 8.40 (d, J = 9.3 Hz, 1H), 8.33 – 7.99 (m, 8H), 7.96 (d, J = 7.8 Hz, 1H), 7.63 (s, 3H), 7.31 (d, J = 11.9 Hz, 1H), 7.10 (d, J = 14.2 Hz, 1H), 4.40 (td, J = 12.1, 11.2, 7.3 Hz, 1H), 4.16 (m, 1H), 2.82 (s, 1H), 2.73 (s, 3H), 2.42 (s, 1H), 2.33 (s, 2H), 2.40 – 2.26 (m, 0H), 2.07 – 1.95 (m, 1H), 1.70 (d, J = 9.7 Hz, 1H), 1.58 – 1.46 (m, 0H), 1.32 (s, 2H), 1.29 (d, J = 7.5 Hz, 0H). **HPLC:** The peak of **Pep-4** arrived at 11 min retention time when eluted with a gradient started from 5% to finish at 100% of acetonitrile in water in a 20 min program.

Characterization of Py^{D}KC (Pep-5):

¹H NMR (400 MHz, DMSO-d$_6$): δ/ppm = 8.37 (dd, J = 9.3, 5.7 Hz, 1H), 8.28 (dt, J = 3.8, 1.9 Hz, 1H), 8.29 – 8.10 (m, 6H), 8.13 – 8.01 (m, 1H), 7.94 (dd, J = 7.8, 5.5 Hz, 1H), 7.67 (s, 3H), 7.42 (d, J = 8.7 Hz, 1H), 7.26 (s, 1H), 4.36 – 4.18 (m, 1H), 2.89 (m, 1H), 2.77 – 2.64 (m, 3H), 2.31 (dd, J = 9.2, 5.2 Hz,

2H), 2.22 (t, J = 8.3 Hz, 1H), 2.02 (m, 2H), 1.66 (dd, J = 13.1, 6.2 Hz, 1H), 1.55 (h, J = 7.5, 6.2 Hz, 4H), 1.37 (d, J = 7.6 Hz, 1H), 1.34 (d, J = 7.7 Hz, 1H). **HPLC:** The peak of **Pep-5** arrived at 10.76 min retention time when eluted with a gradient started from 5% to finish at 100% of acetonitrile in water in a 20 min program.

Pep-6:

¹H NMR (400 MHz, DMSO-d$_6$): δ/ppm = 8.37 (m, 1H), 8.31 – 8.17 (m, 5H), 8.15 (m, 3H), 8.13 – 8.02 (m, 1H), 7.95 (d, J = 7.8 Hz, 1H), 7.67 (s, 3H), 7.41 (s, 1H), 7.26 (s, 1H), 4.38 – 4.18 (m, 2H), 2.89 (m, 1H), 2.74 (s, 3H), 2.32 (t, J = 7.3 Hz, 2H), 2.30 (s, 1H), 2.22 (t, J = 8.4 Hz, 1H), 2.01 (t, J = 7.5 Hz, 2H), 1.67 (s, 1H), 1.55 (m, 3H), 1.36 (m, 7.6 Hz, 2H). **HPLC:** The peak of **Pep-6** arrived at 10.9 min retention time when eluted with a gradient started from 5% to finish at 100% of acetonitrile in water in a 20 min program.

PyKC-dimer:

The disulphide linked dimer of **PyKC** was prepared by incubating a 0.01 wt% solution of **PyKC** in water for 24 h followed by lyophilisation and purity check by analytical HPLC and ESI-MS. No further purification was needed and the yield was more than 99.5%.

ESI-MS calcd. for [M+H]⁺, $C_{58}H_{66}N_8O_6S_2$: 1035.4580, found: 1035.4574, and 518.2320 [M²⁺]. **HPLC:** The peak for **PyKC-dimer** arrived at 18.9 min retention time when eluted with a gradient started from 5% to reach 30% after 5 min and continued to reach 100% at 40 min.
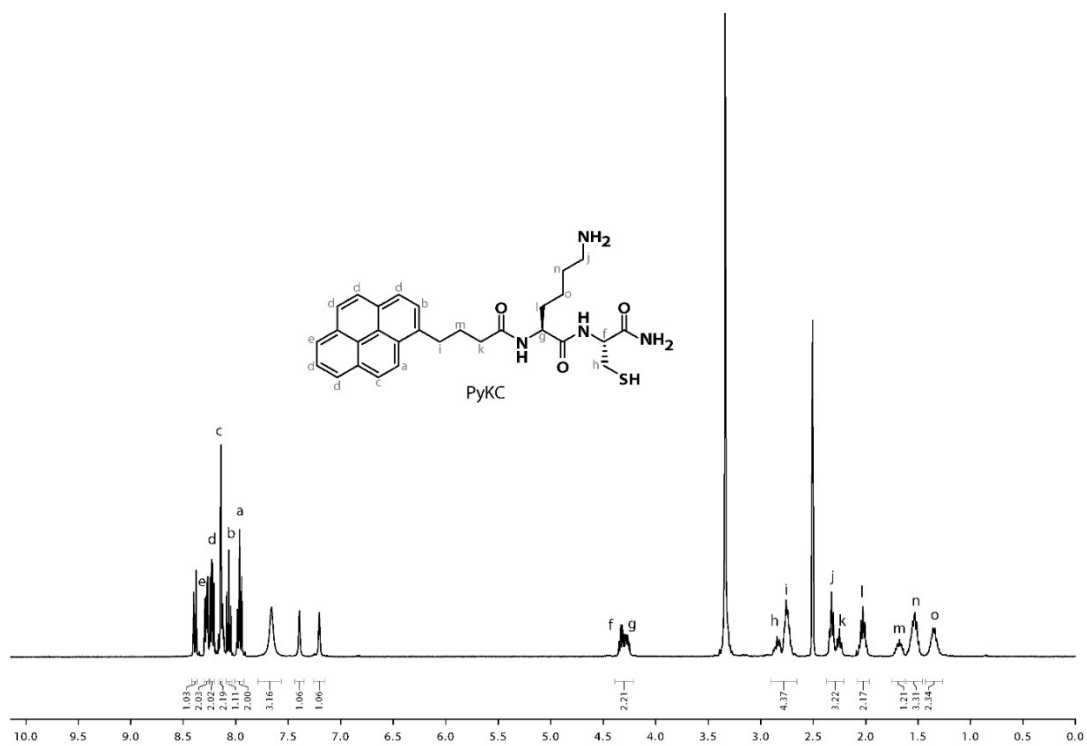
Figure S1. ^{1}H NMR spectra of **PyKC** in DMSO-d$_6$.
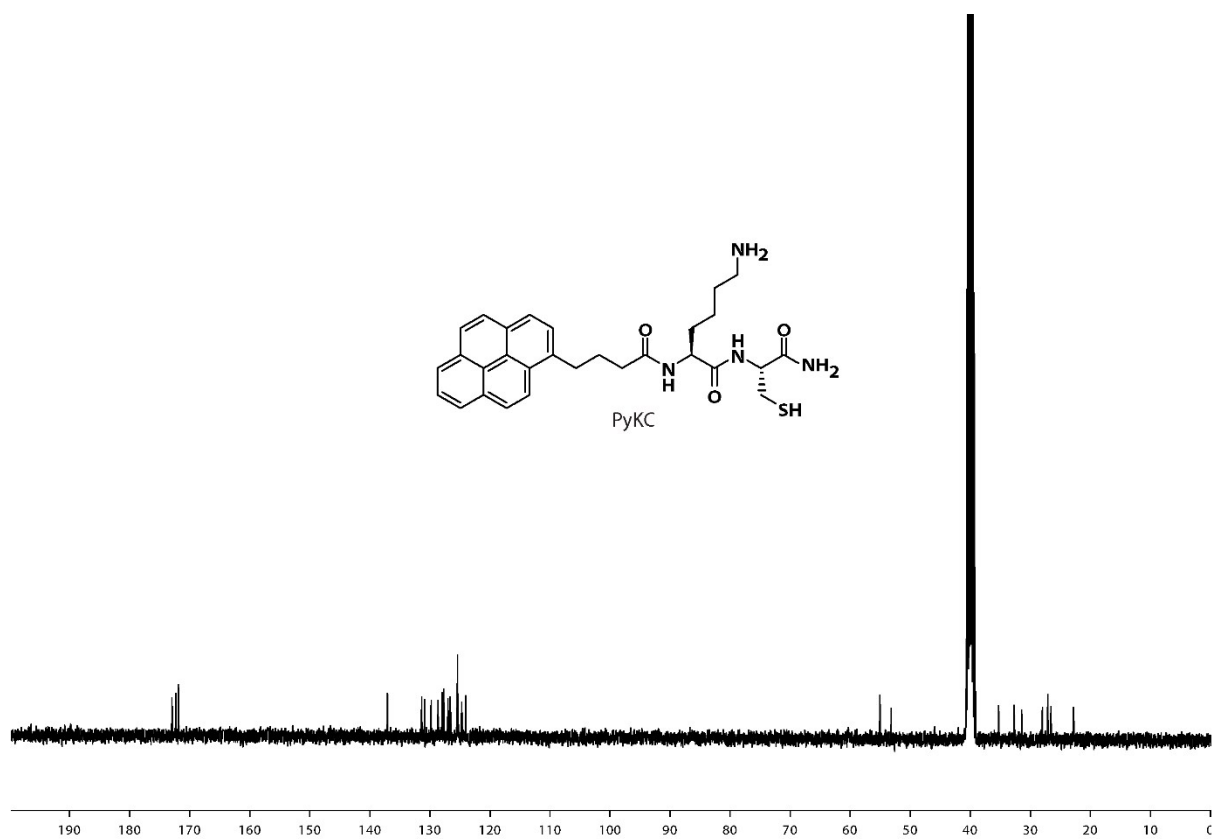
Figure S2. ^{13}C NMR spectra of **PyKC** in DMSO-d$_6$.
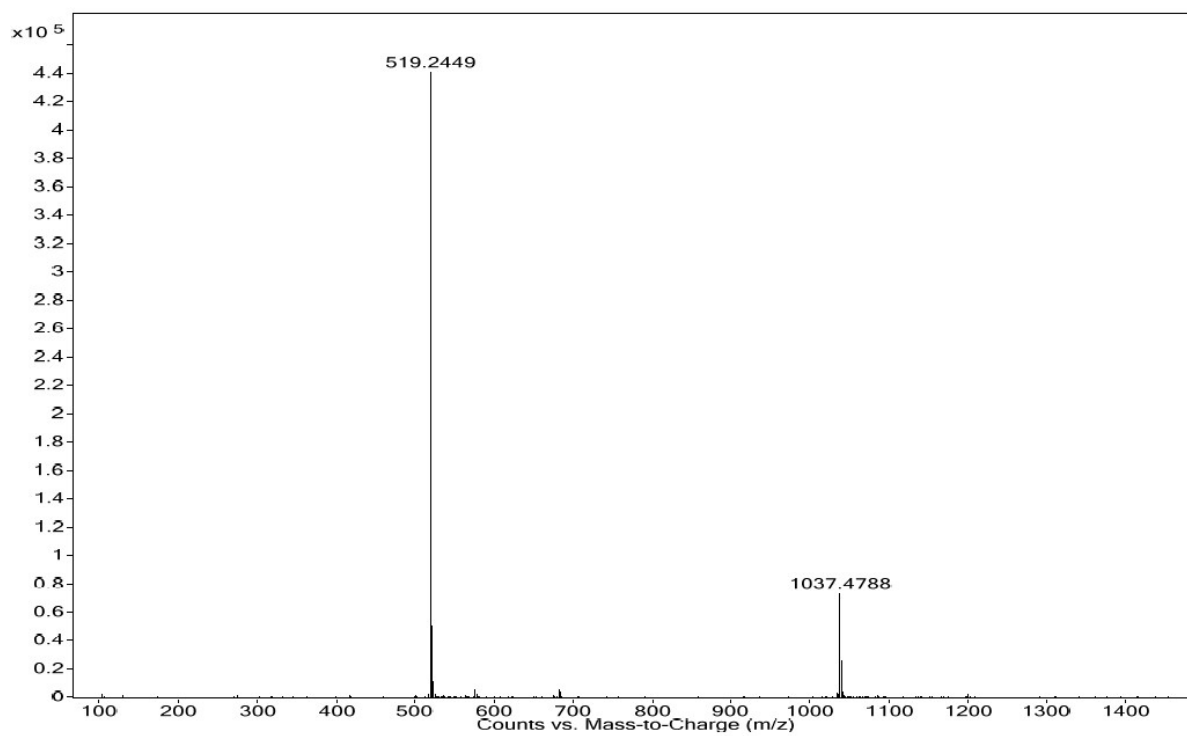
Figure S3. ESI-MS of **PyKC**.
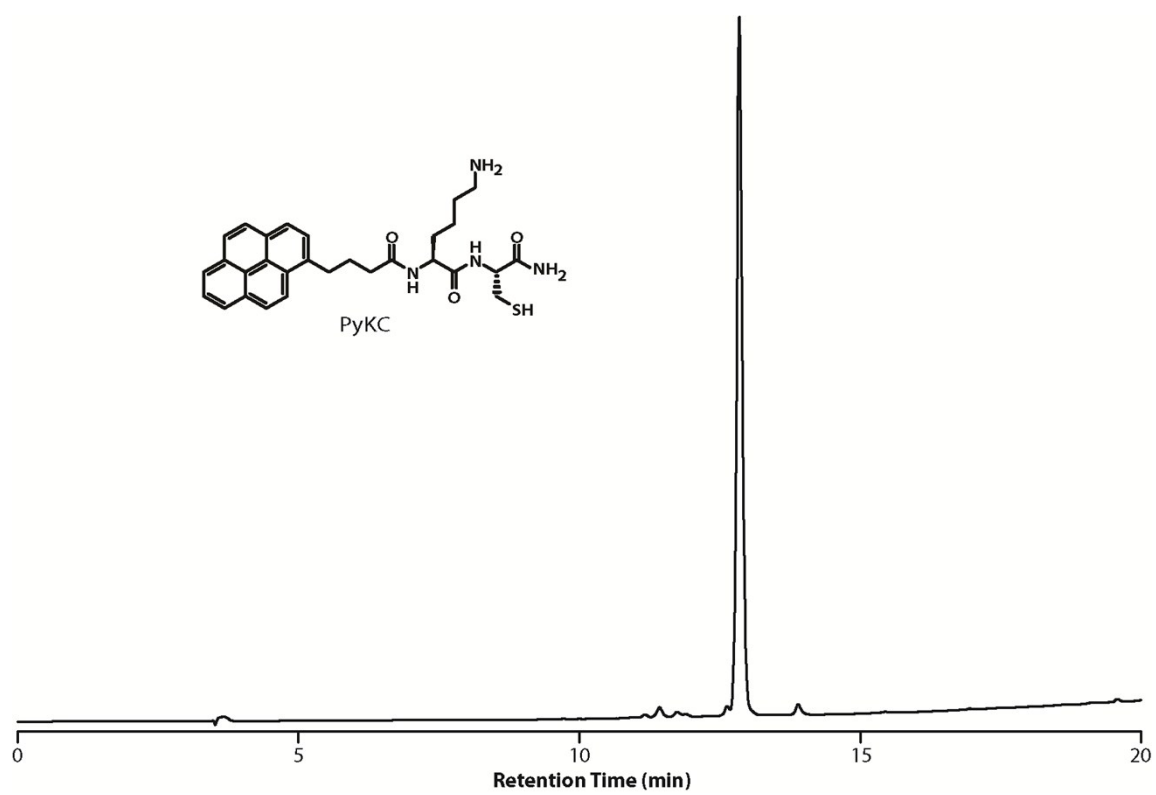
Figure S4. Chromatogram of **PyKC**.
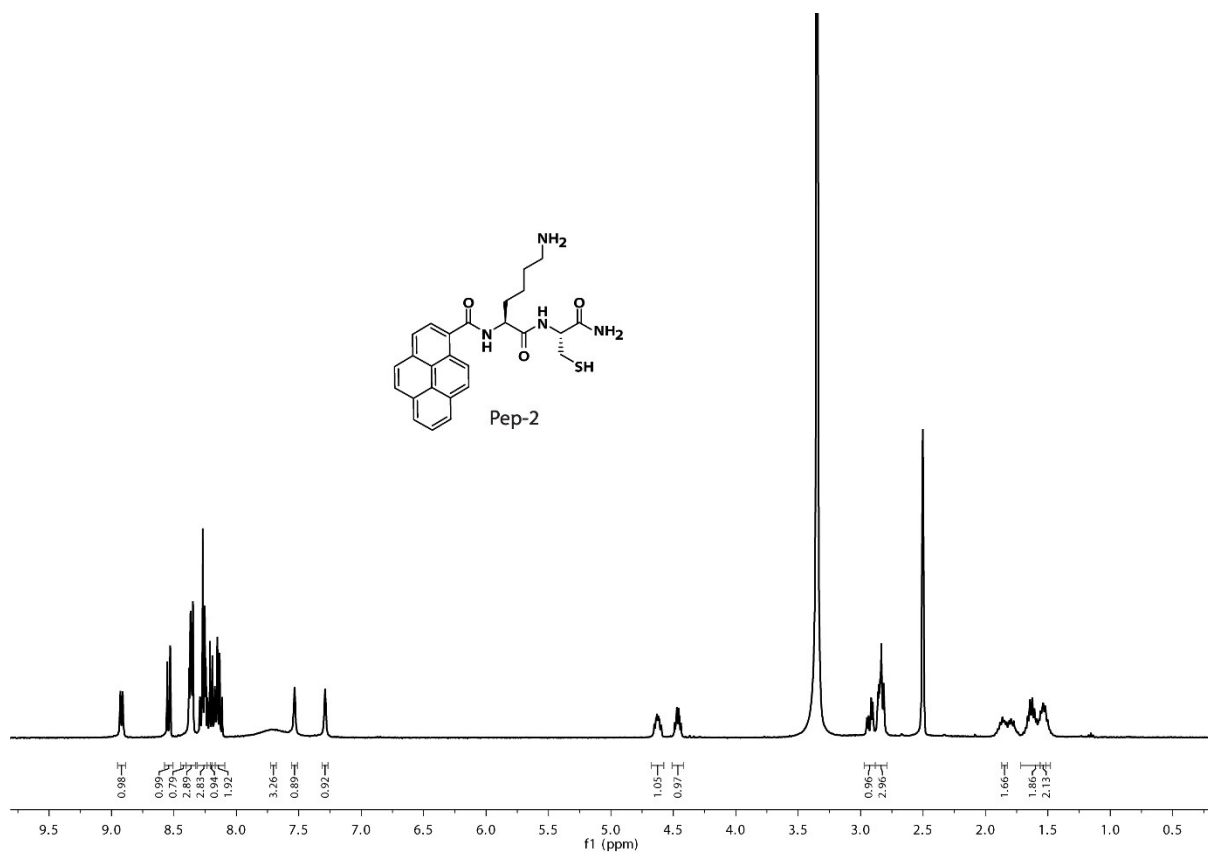
Figure S5. ^{1}H NMR spectra of **Pep-2** in DMSO-d$_6$.
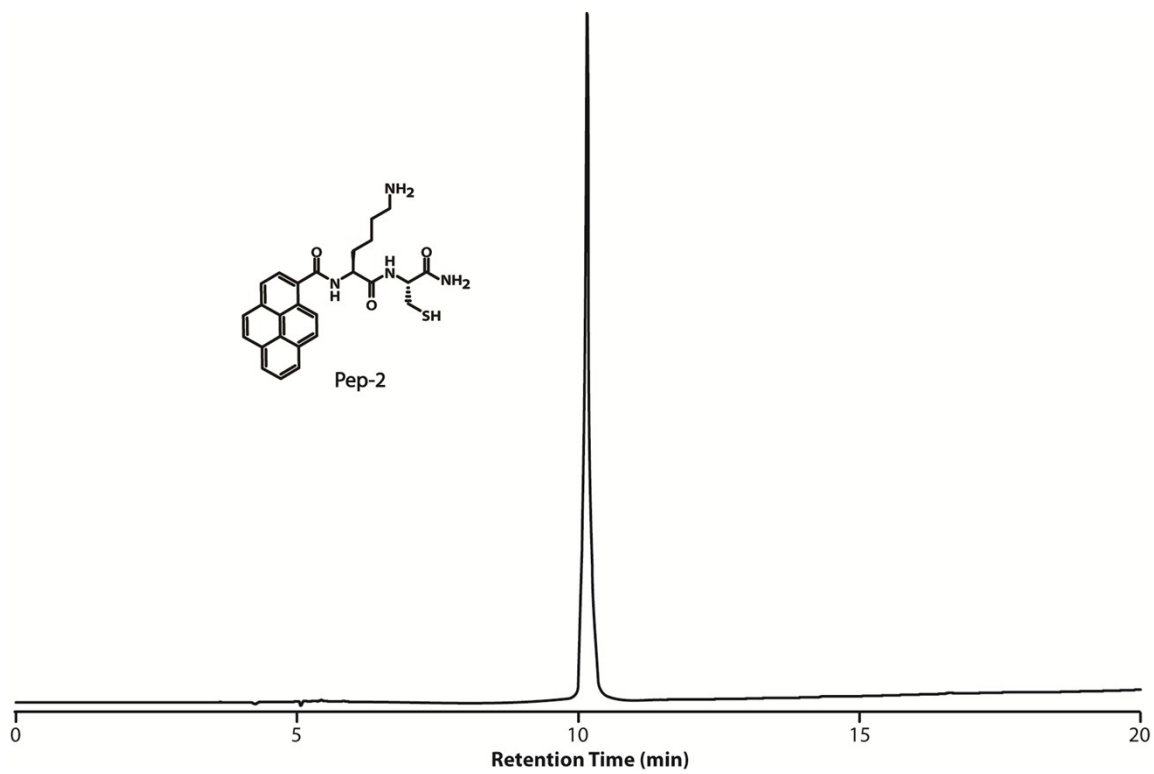
Figure S6. Chromatogram of **Pep-2**.
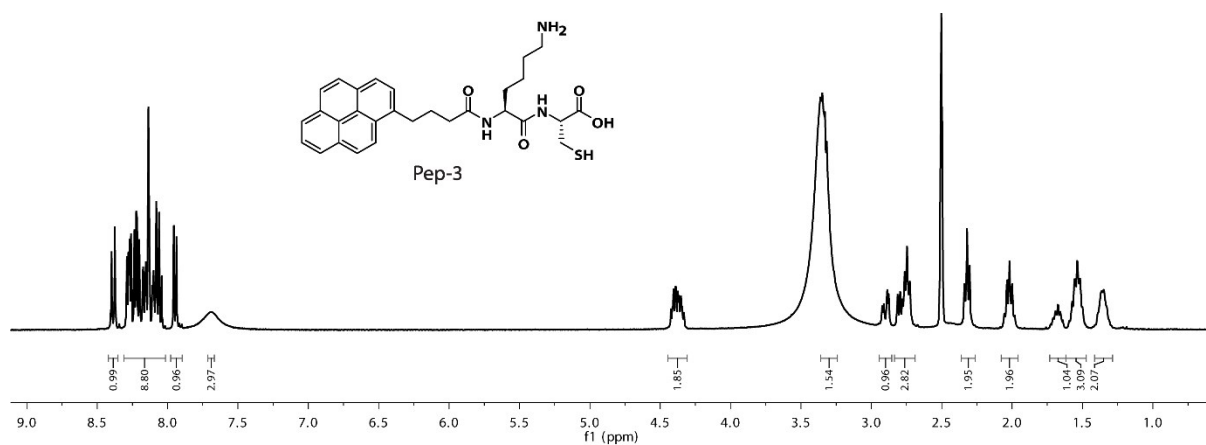
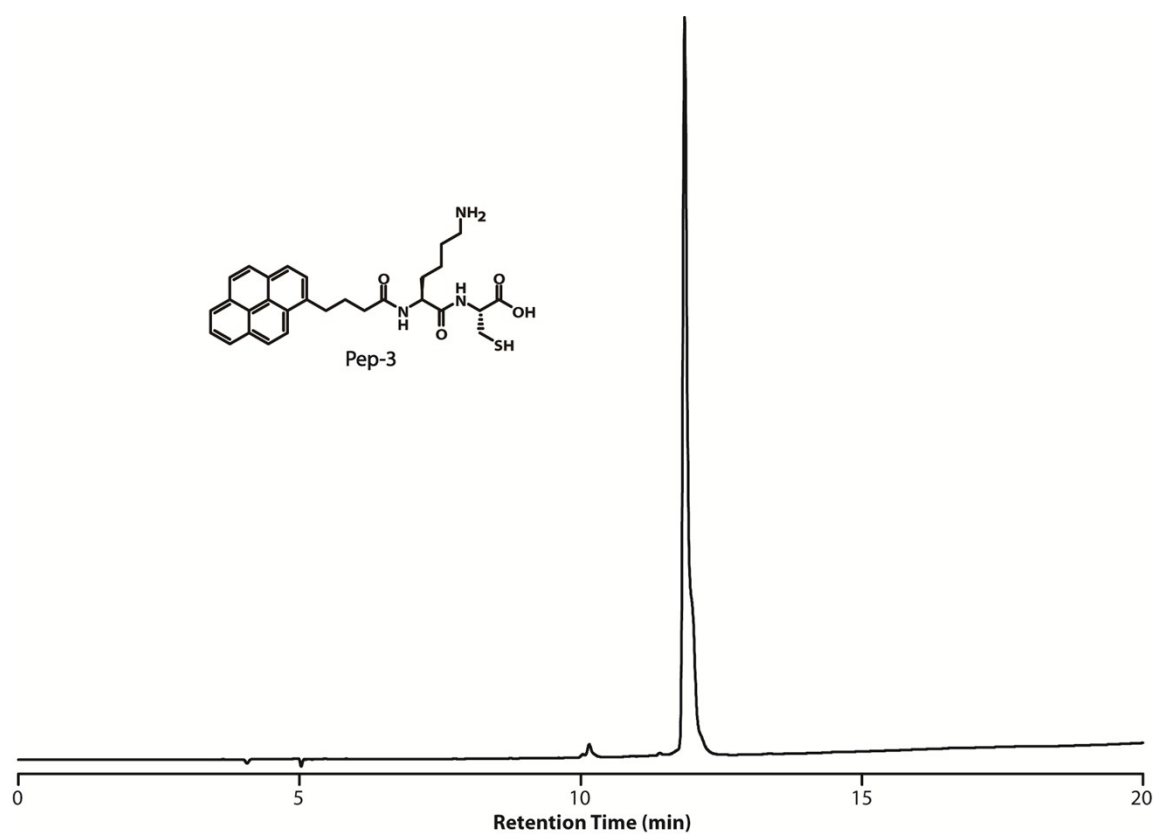**Figure S7.** ^{1}H NMR spectra of **Pep-3** in DMSO-d_6.
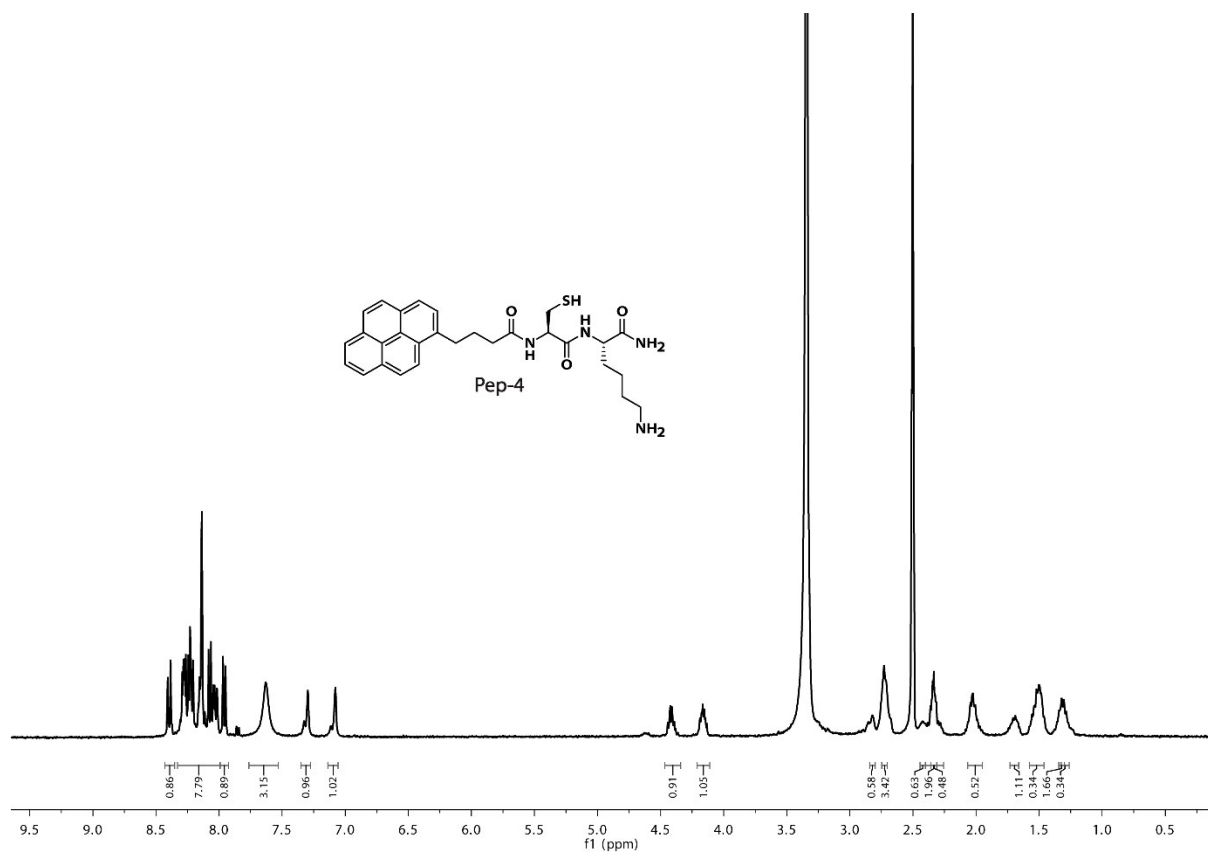

Figure S8. Chromatogram of **Pep-3**.
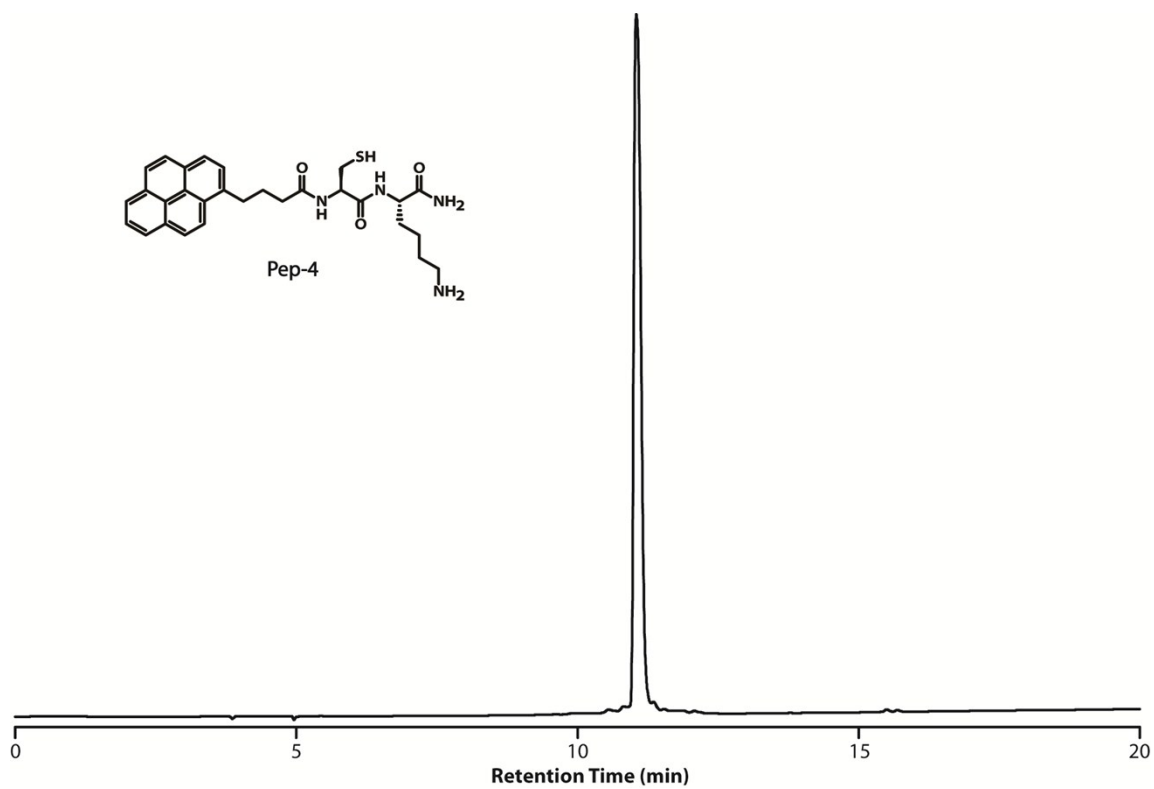
Figure S9. ^{1}H NMR spectra of **Pep-4** in DMSO-d$_6$.

Figure S10. Chromatogram of **Pep-4**.
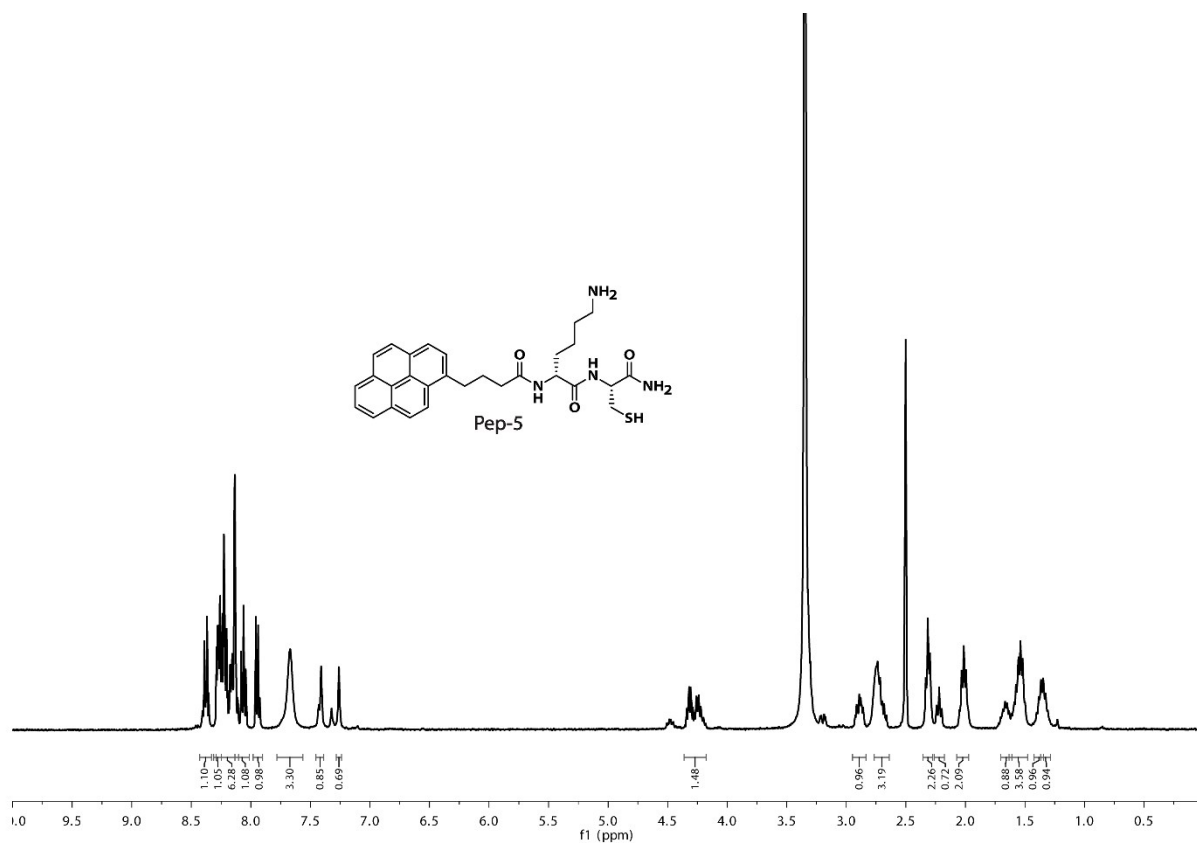
Figure S11. ^{1}H NMR spectra of **Pep-5** in DMSO-d$_6$.
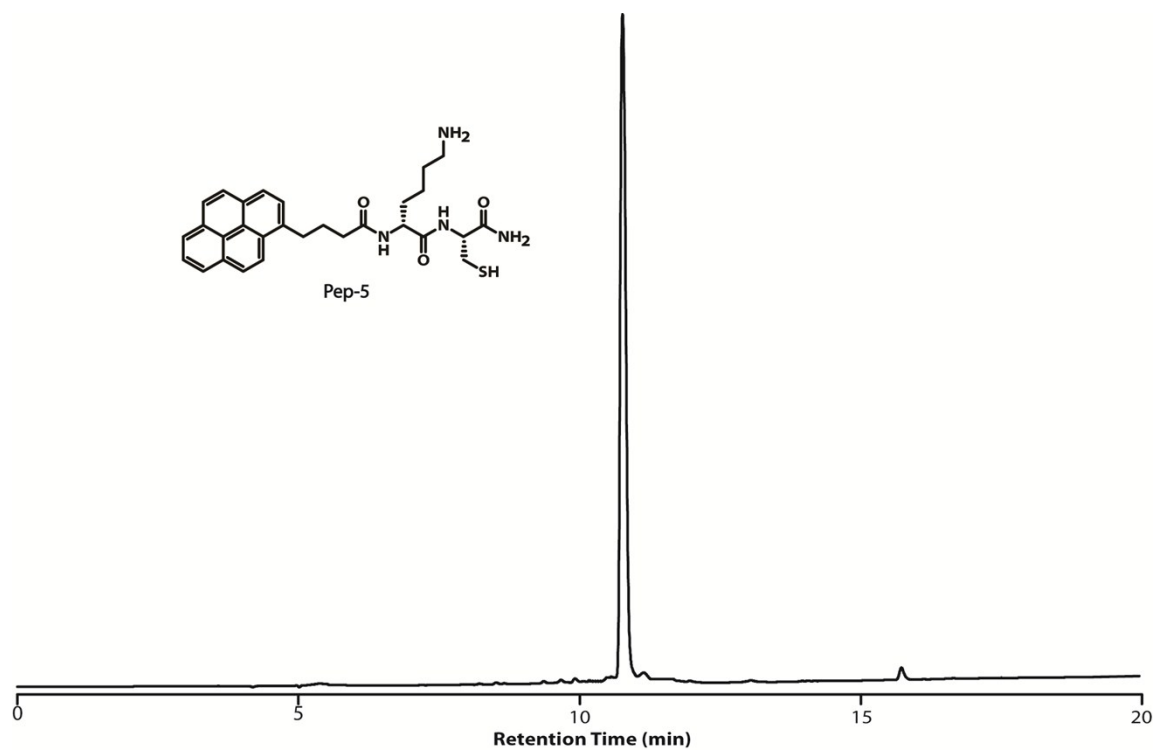
Figure S12. Chromatogram of **Pep-5**.
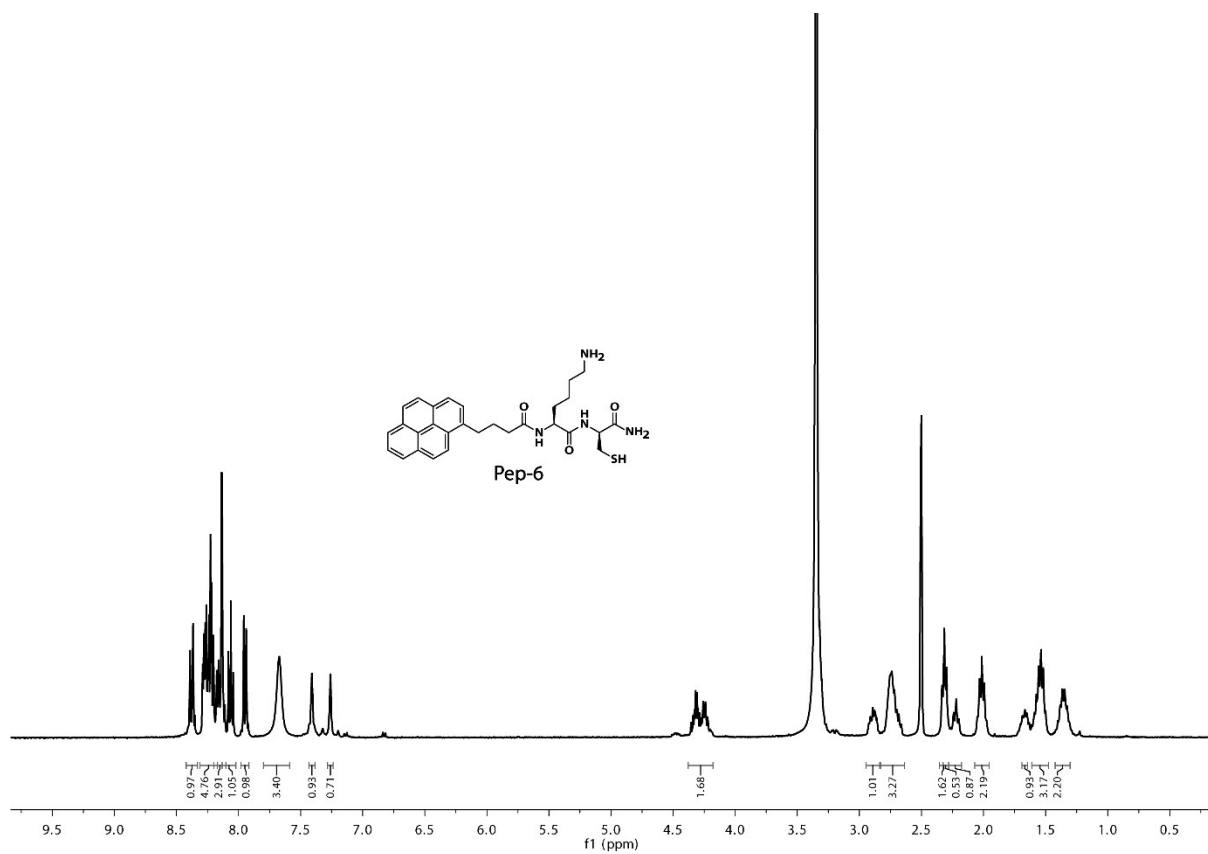
Figure S13. ^{1}H NMR spectra of **Pep-6** in DMSO-d$_6$.
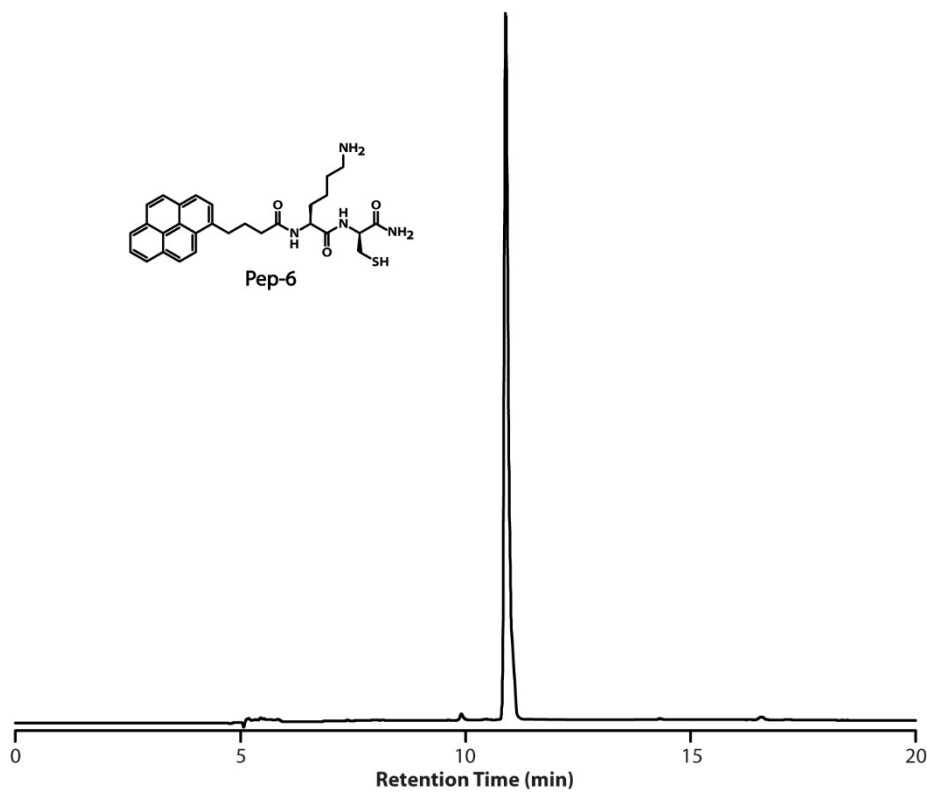
Figure S14. Chromatogram of **Pep-6**.
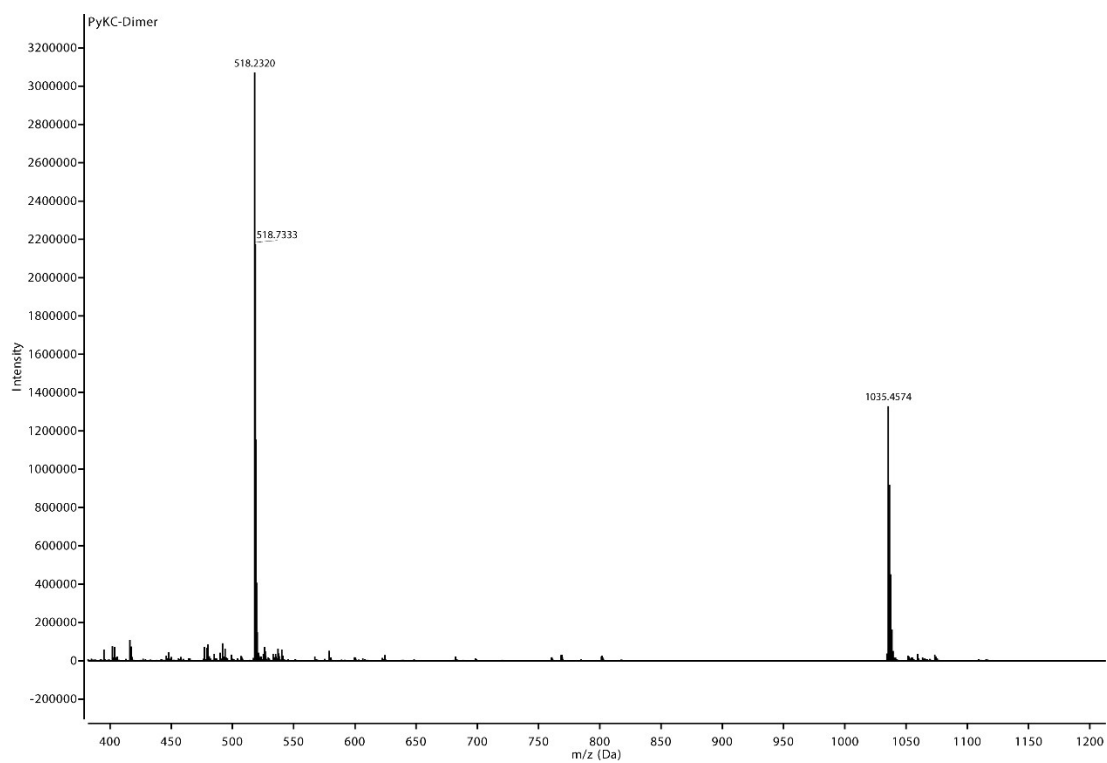
Figure S15. ESI-MS of **PyKC**-dimer.
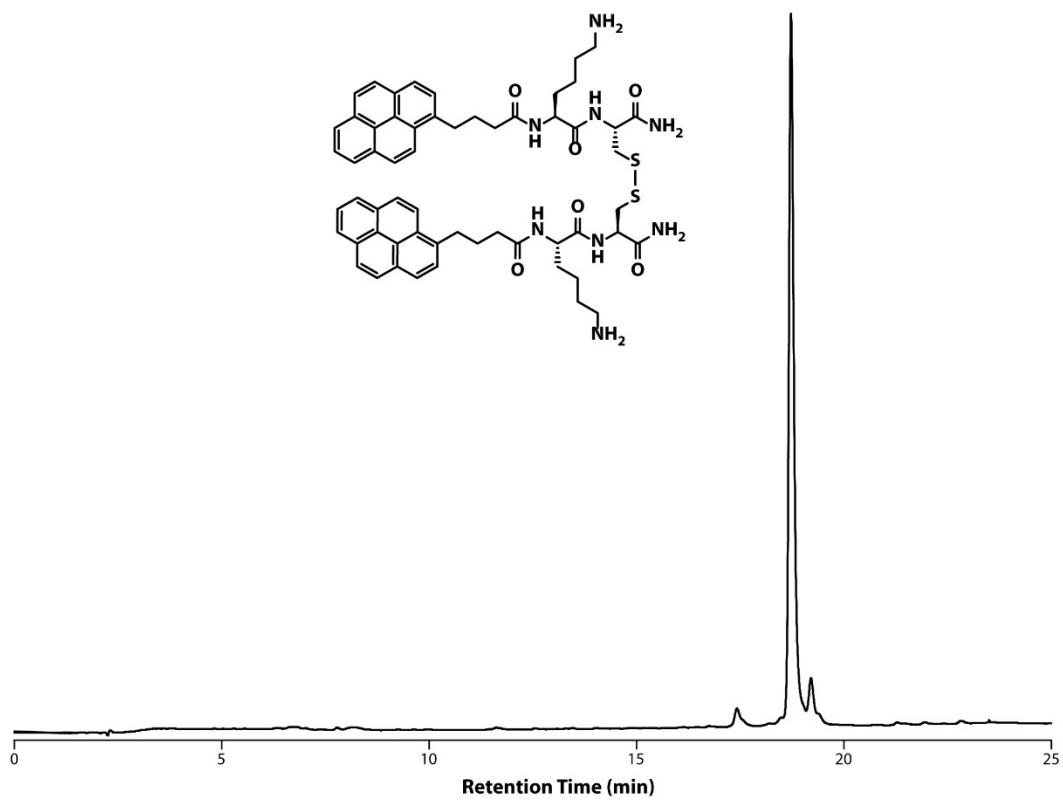
Figure S16. Chromatogram of **PyKC**-dime

C-1   C-2
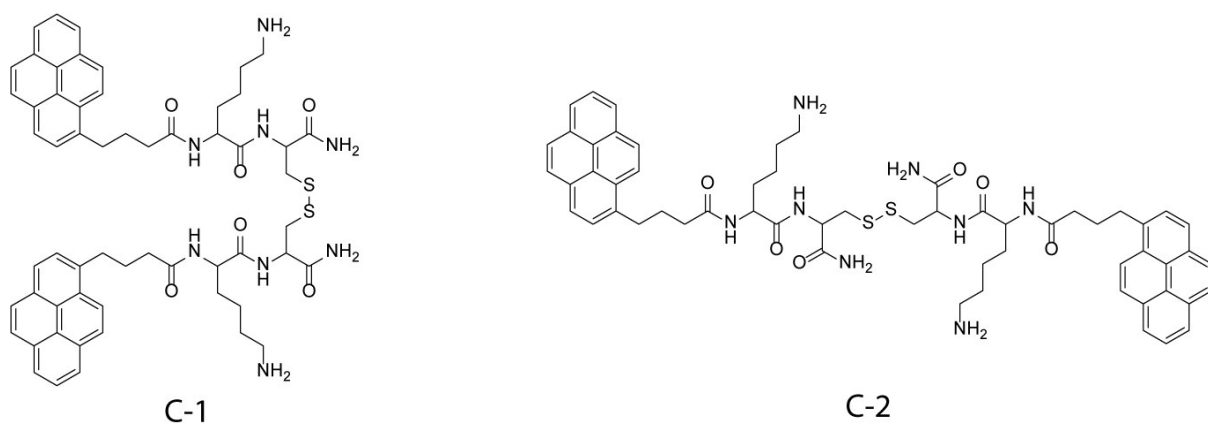
Figure S17. Two different conformers drawn in ChemDraw which were used as the initial configurations for the geometry optimization.

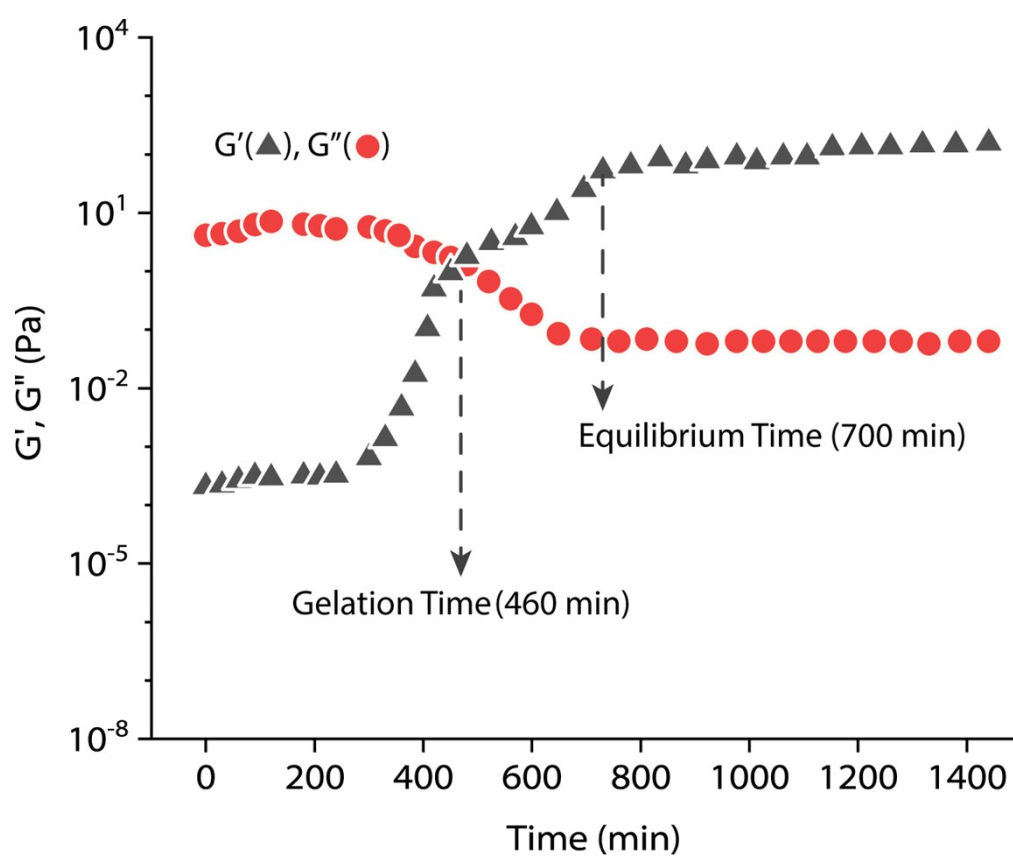**Figure S18.** Time dependent rheology of a 1 wt% **PyKC** hydrogel showing the sol to gel transition and time required to reach equilibrium.
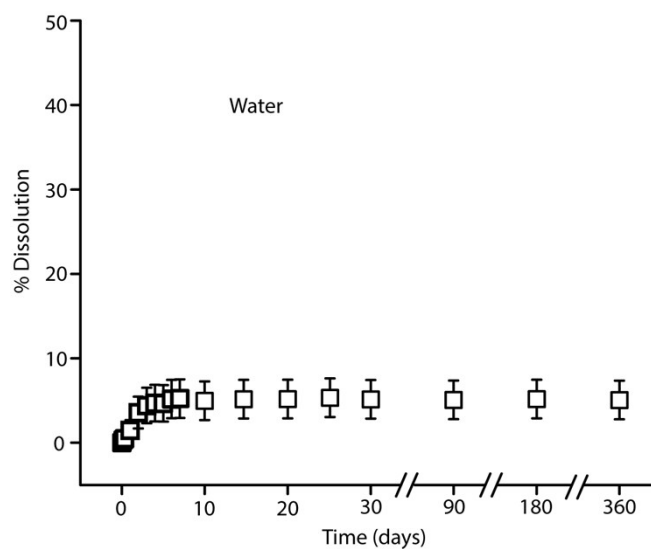
Figure S19. % Dissolution of the 1 wt% hydrogel formed by **PyKC** (in 20 mM Tris buffer, pH 8) when dispersed in bulk water.
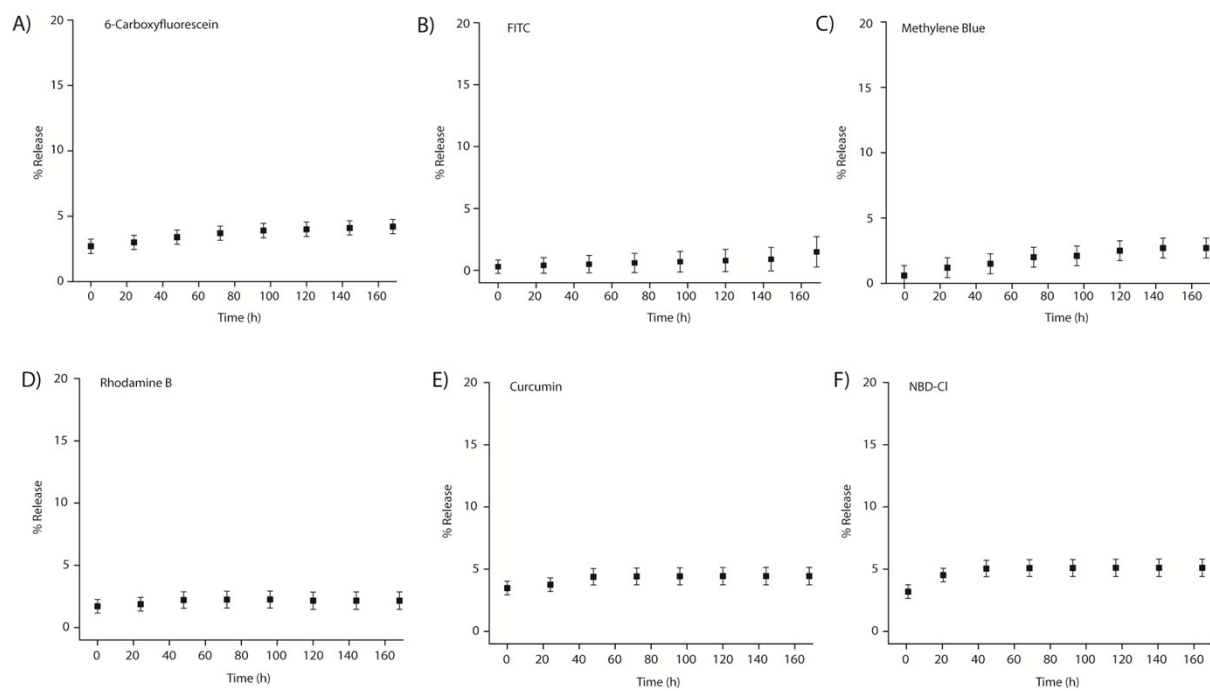

Figure S20. Time dependent % release of entrapped dyes inside1 wt% hydrogel formed by **PyKC** (in 20 mM Tris buffer, pH 8 containing the respective dyes) when dispersed in bulk water.
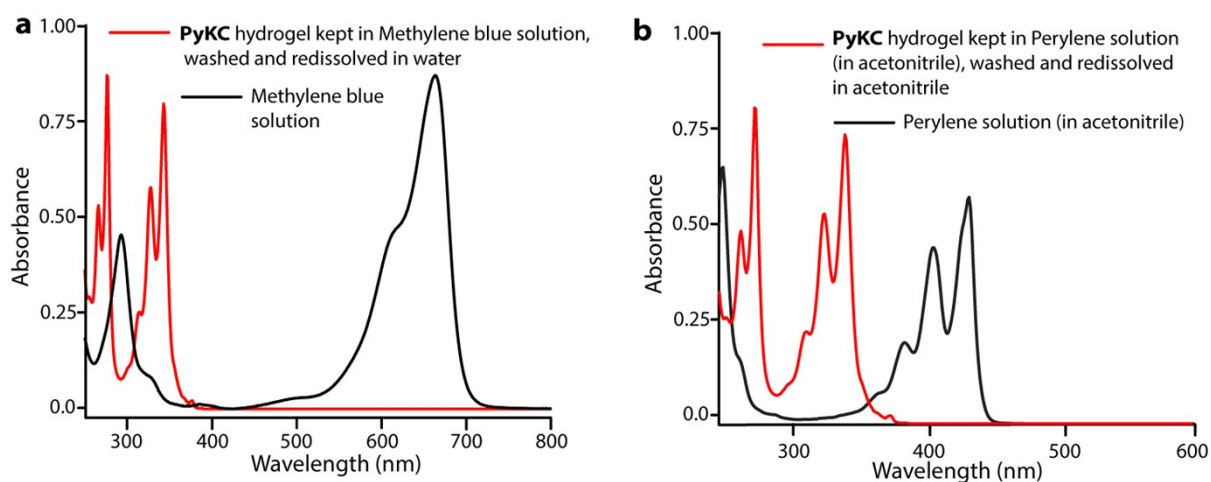
Figure S21. A small portion of **PyKC** hydrogel was dispersed in solutions of methylene blue (in water) and perylene (in acetonitrile) and incubated at room temperature. After seven days, the gels were centrifuged, washed and dissolved in water or acetonitrile with the help of TCEP and the absorption spectra of the solutions were recorded to see any incorporation of the dyes. The absorption spectra of the gel from (a) methylene blue test and perylene test (b) along with the absorption spectra of methylene blue and perylene.
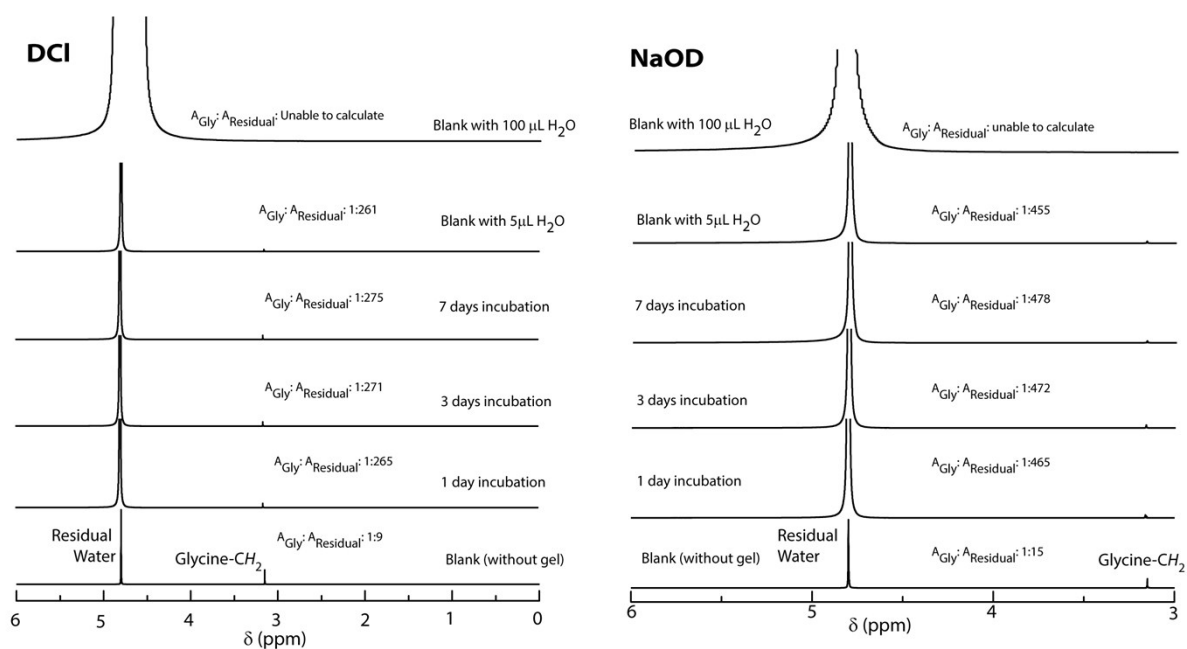

Figure S22. ^{1}H NMR spectra of DCl and NaOD (in D_2O) containing glycine before and after incubating **PyKC** hydrogel inside the solvent for different time period to determine the extent of exchange of water of gelation.
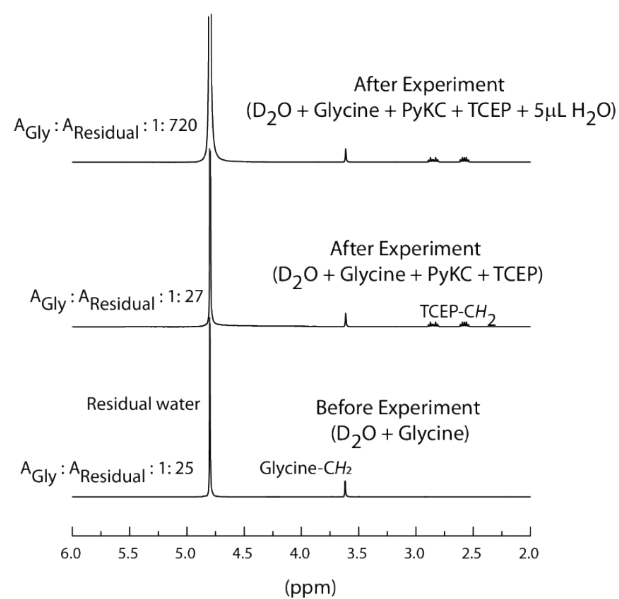
Figure S23. ^{1}H NMR spectra of samples from water-exchange experiment from bulk water to the hydrogel.
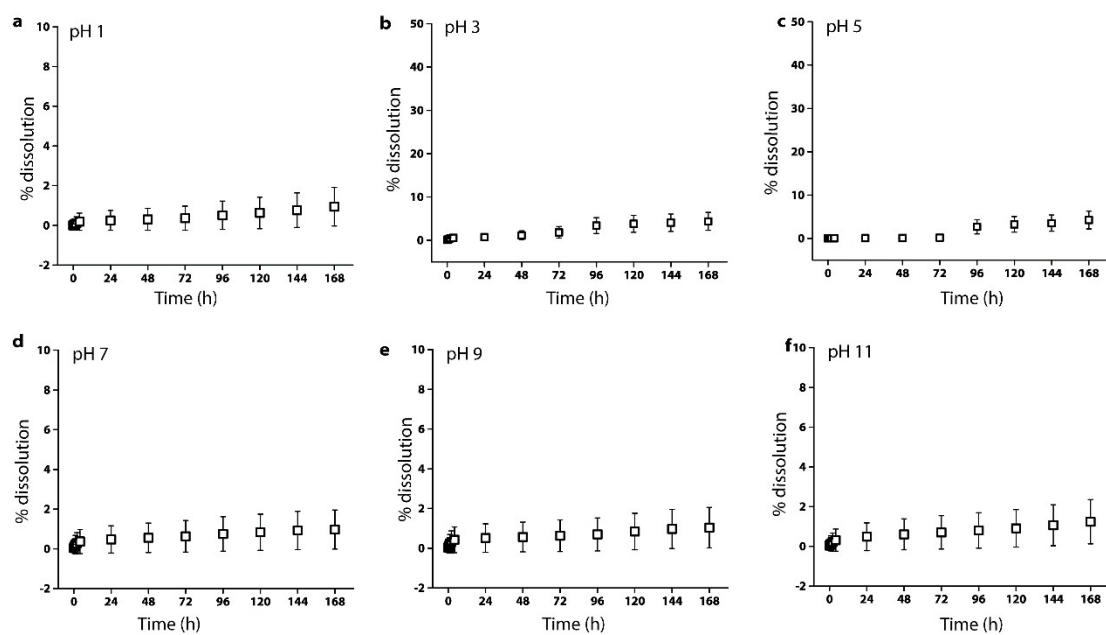

Figure S24. % Dissolution with time for 500 μl of 1 wt% hydrogel of **PyKC** when dispersed in 10 mL of different buffers and incubated at room temperature
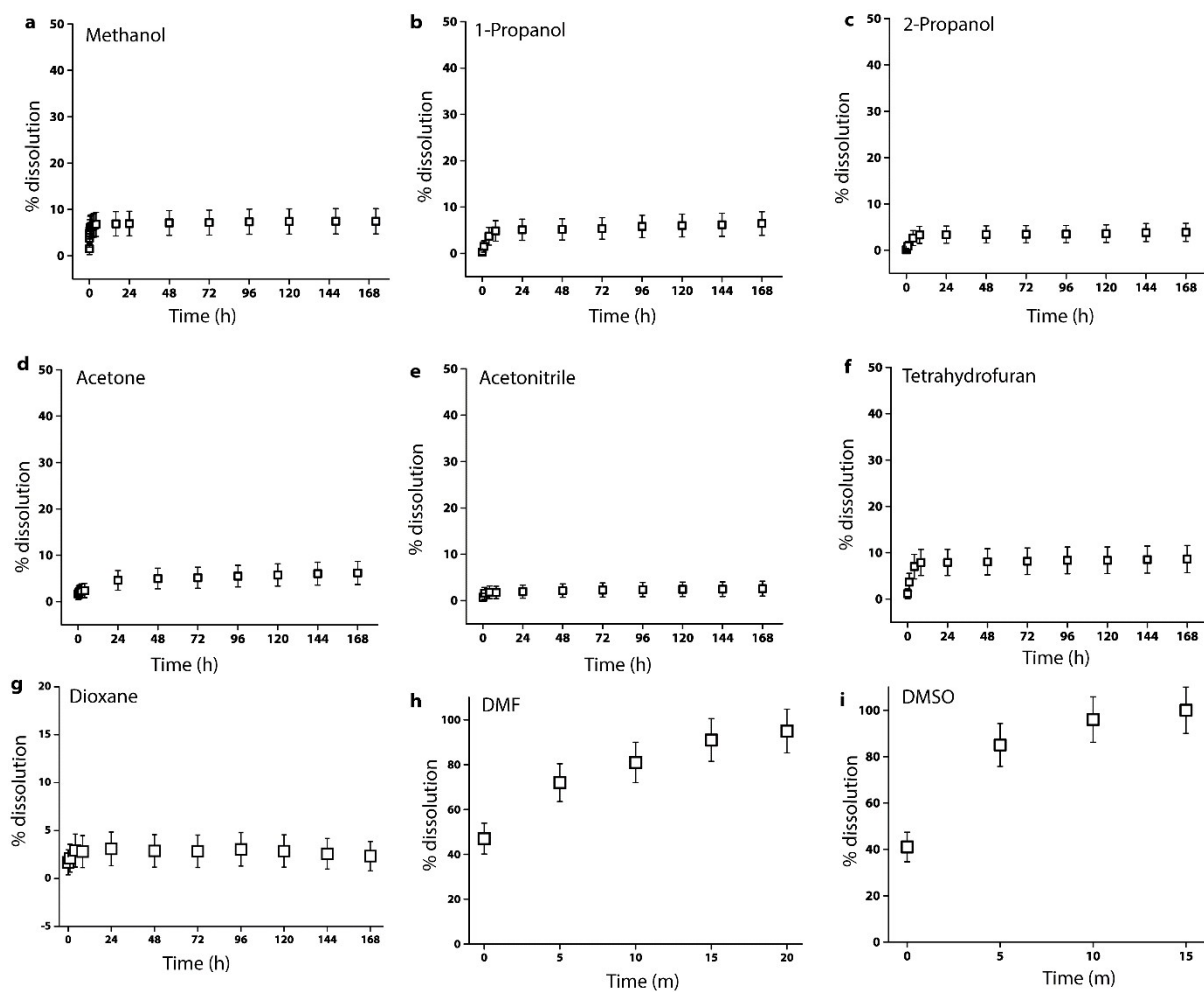
Figure S25. .% Dissolution with time for 500 μL of 1 wt% hydrogel of **PyKC** when dispersed in 10 mL of different organic solvents and incubated at room temperature.
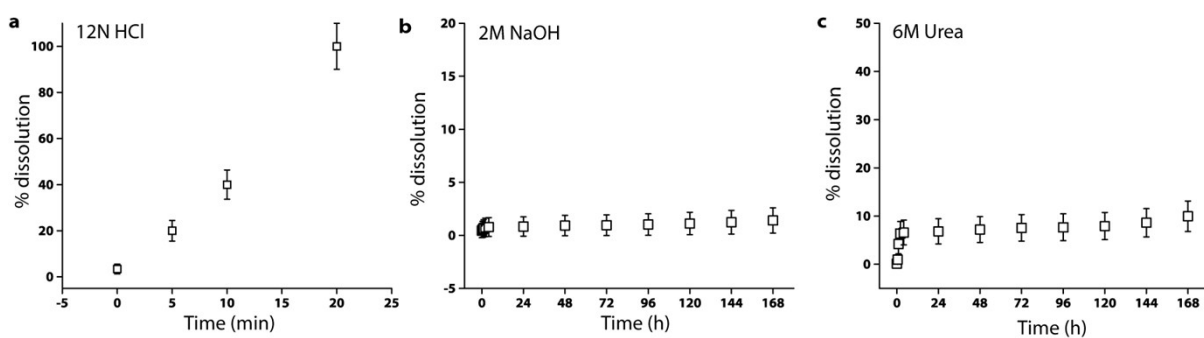

Figure S26. % dissolution with time for 500 μl of 1 wt% hydrogel of **PyKC** when dispersed in 10 mL of aqueous **a**, 12 N HCl; **b**, 2 M NaOH and **c**, 6 M urea solutions and incubated at room temperature.
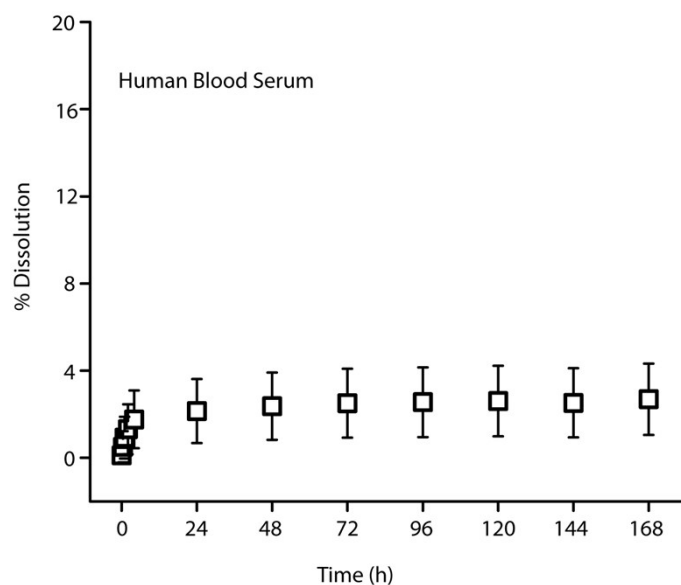
Figure S27. % Dissolution with time for 100 µl of 1 wt% hydrogel of **PyKC** when dispersed in 2 mL of human blood serum and incubated at room temperature.
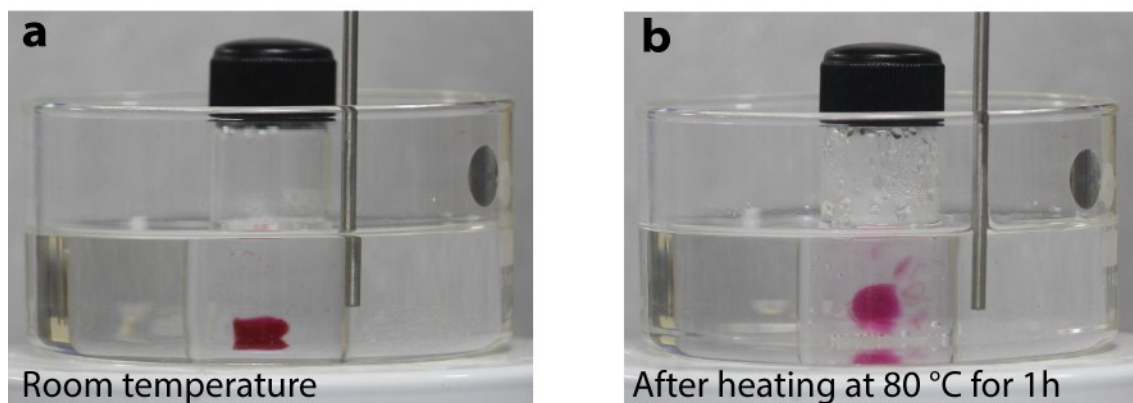
Figure S28. Photographs of a 1 wt% hydrogel of **PyKC** (containing Rhodamine B) before and after heating at 80 °C for 1 h.
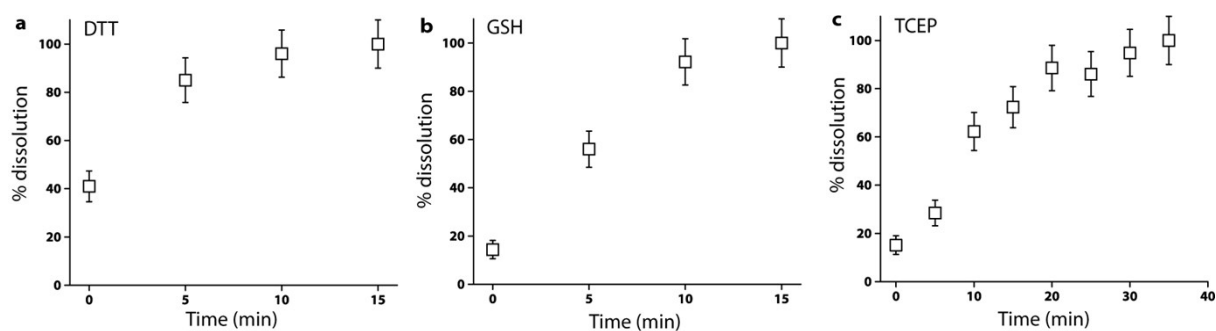
Figure S29. % Dissolution with time for 500 µl of 1 wt% hydrogel of **PyKC** when dispersed in 10 mL of aqueous solutions of different disulphide bond breaking agents and incubated at room temperature.
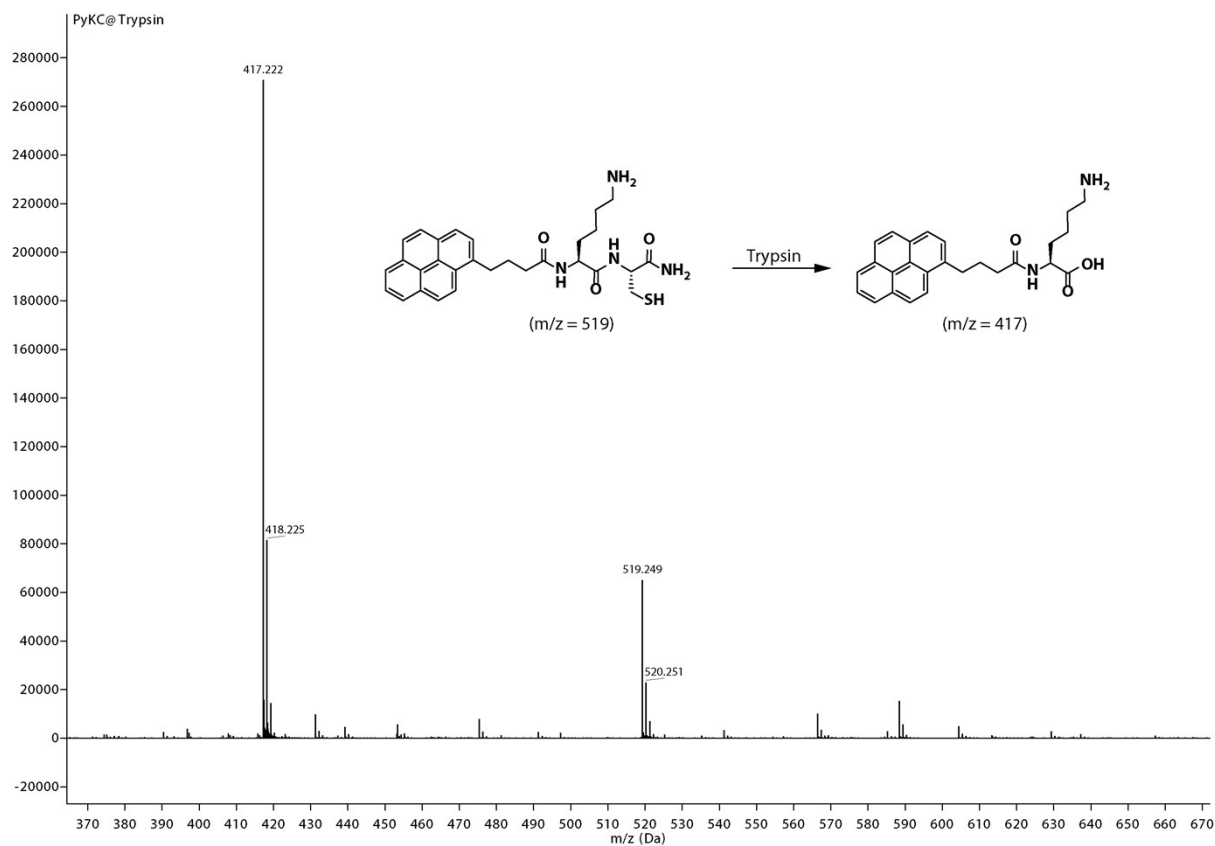
Figure S30. ESI-MS spectrum of a trypsin treated hydrogel of **PyKC**.
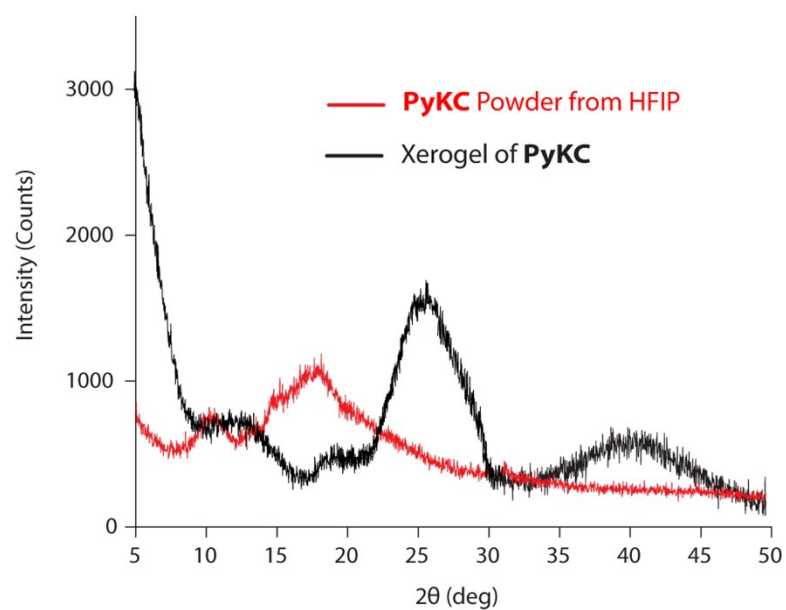
Figure S31. PXRD profiles of xerogel 1wt% hydrogel of **PyKC** in 20 mM Tris buffer pH 8 and dried powder of **PyKC** from HFIP solution. All experiments were carried out at room temperature.
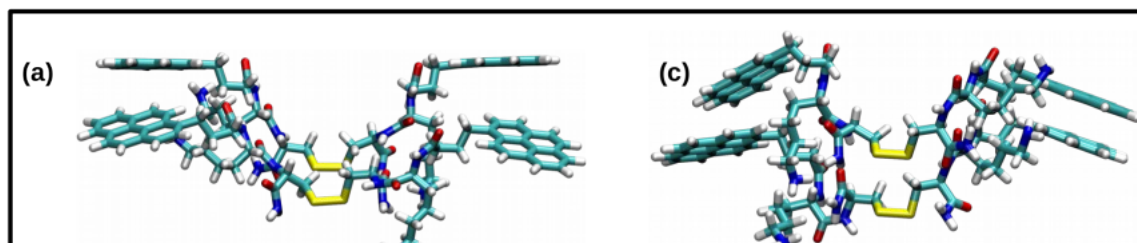
Figure S32. Snapshot of optimized geometries of **PyKC** dimers in open and folded states, (a) Geometry optimized stacked open chain dimer without dispersion correction (b) Geometry optimized stacked folded dimer without dispersion correction, (c) Geometry optimized stacked open chain dimer with dispersion correction and (d) Geometry optimized stacked folded dimer with dispersion correction.
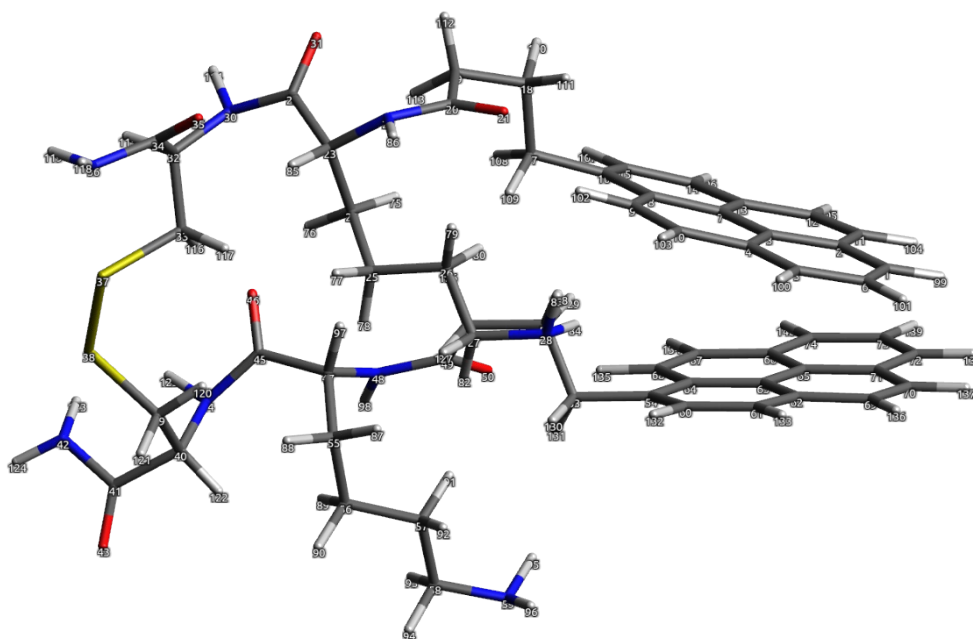
Figure S33. Snapshot of geometry optimized folded **PyKC** dimer with atom labels.
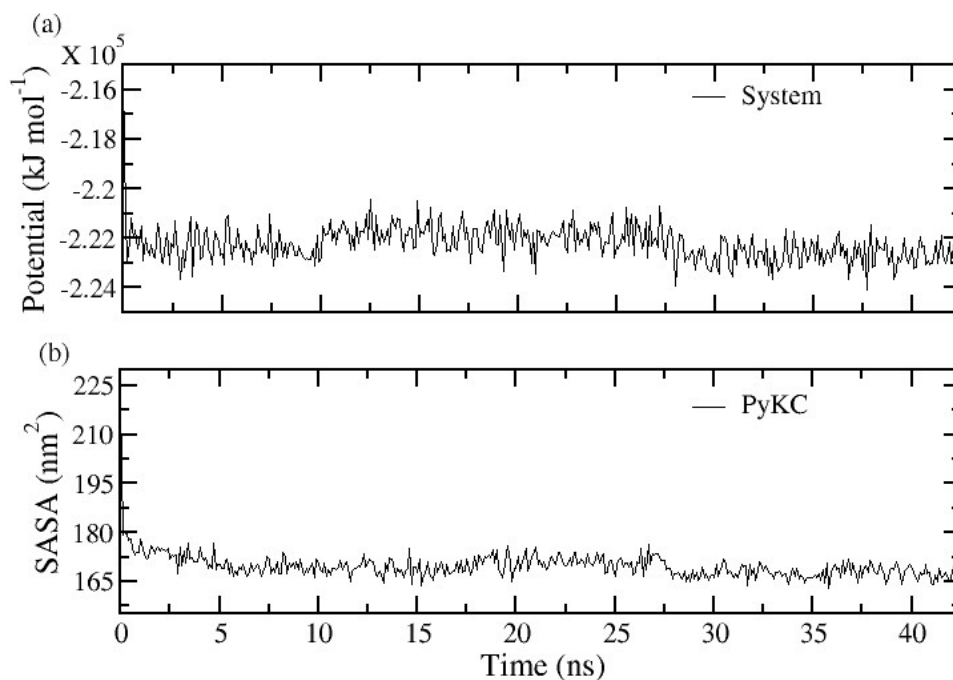
Figure S34. Evolution of potential energy and solvent accessible surface area (SASA) to monitor the equilibration of the system.
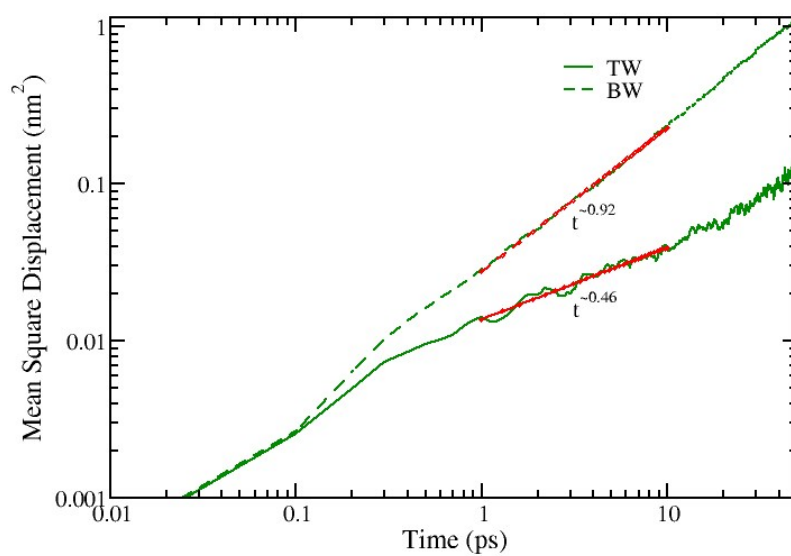
Figure S35. Translational mean square displacement (MSD) of the trapped water (TW) and bulk water (BW). TW follows a sub-diffusive behavior and BW follows diffusive behavior showing slow dynamics of trapped water (TW).
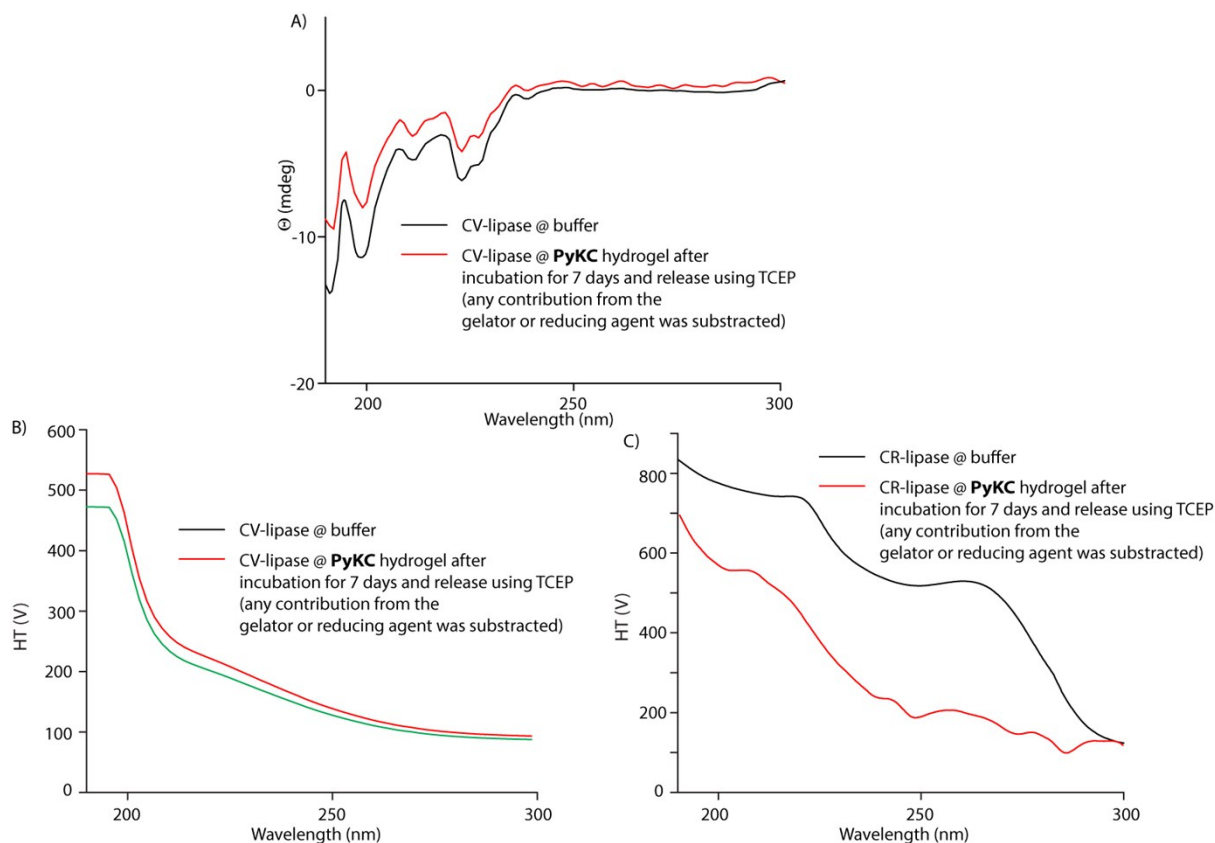
Figure S36. A) Circular dichroism spectra of CV-Lipase in free and gel-trapped conditions to identify any denaturation due to encapsulation and incubation at room temperature for 7 days. B-C) HT curves corresponding to the CD graphs presented in (B) Figure S35A and (C) Figure 8B of the main manuscript.
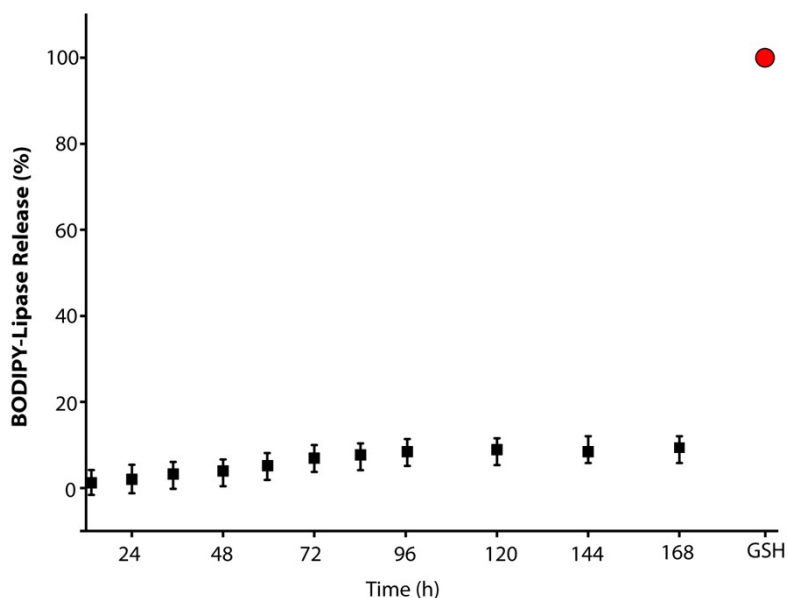
Figure S37. Time dependent % release of entrapped BODIPY-Lipase inside1 wt% hydrogel formed by **PyKC** (in 20 mM Tris buffer, pH 8 containing the respective dyes) when dispersed in bulk water.
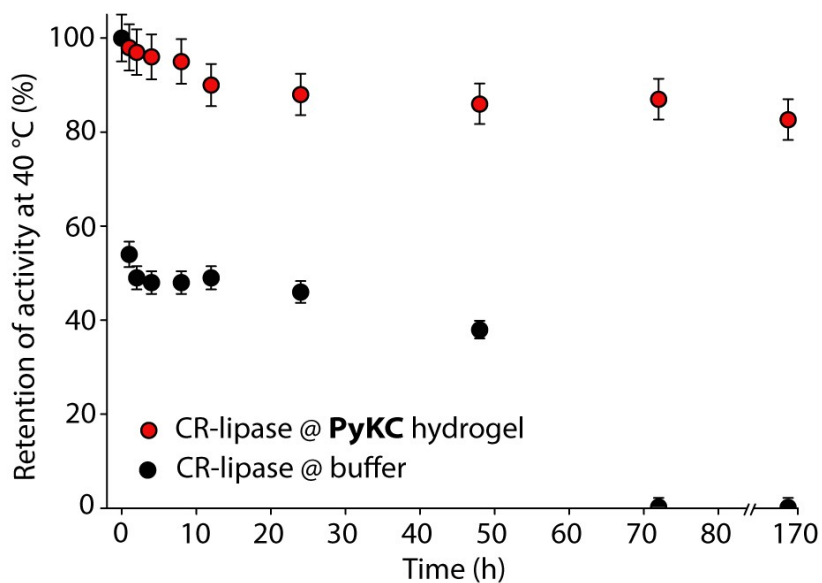
Figure S38. Retention of activity (%) at different time interval by gel-trapped and free CR-lipase when incubated at 40 °C.

Table S1. Energy of the geometry optimized configurations for both open and folded PyKC dimers from DFTB calculations. The folded conformer has stronger stacking energy. The inter-planar distance is in good agreement with PXRD results (figure S26) and indicates the presence of ▢▢▢▢stacking.

Configuration	Energy (Hartree)				Stacking energy (ΔEb)	Inter-planar distance (A^0)
	Monomer		Dimer			
	Dispersion	Total	Dispersion	Total	Kcal/mol	
(a) Open	-0.255	-168.811	-0.571	-337.714	-57.92	2.96 (H-type)
(b) folded	-0.275	-168.807	-0.607	-337.740	-78.50	2.44 (T-type)

Table S2. Co-ordinates of geometry optimized folded **PyKC** dimer.

1	-14.96449970	3.89450661	-0.89986452
2	-13.51051887	1.98866368	0.25745794
3	-10.93598244	2.48712940	0.87081960
4	-9.87214238	4.89946454	0.32332495
5	-11.36760946	6.73643556	-0.88900660
6	-13.88546051	6.22773251	-1.48616885
7	-9.43040960	0.58335400	2.04128811
8	-6.90276915	1.13322014	2.80644727
9	-5.91015899	3.61798357	2.32112393
10	-7.31734051	5.39871322	1.08285398
11	-14.54585178	-0.45742920	0.80460409
12	-13.07217548	-2.32575681	1.81173969
13	-0.48019603	-1.86267406	2.46350958
14	-8.95139635	-3.75178540	3.54237544
15	-6.48354250	-3.20673841	4.28245445
16	-5.44783603	-0.78783565	3.97662113
17	-2.81192506	-0.28177531	4.88572141
18	-2.74774785	1.16473538	7.36877698
19	-0.10067368	2.10687506	7.92773819
20	0.41470215	4.58841601	6.59858397
21	-1.31273577	6.02465429	5.99736587
22	2.89541784	5.41116188	6.24320464
23	4.98647815	3.79124024	5.49824741
24	4.24516192	2.12025666	3.24562614
25	3.89182992	3.66838773	0.84585862
26	1.26856586	4.80674031	0.63242290
27	0.61865793	5.57799020	-2.06564317
28	-1.97187047	6.40524110	-2.31105645
29	5.98564004	2.24731539	7.71950046
30	8.17003207	0.77712821	7.42762672
31	4.84445349	2.08194430	9.72407291
32	10.47243734	1.43476769	6.08762908
33	10.40450396	0.89255509	3.24356783
34	11.28904629	4.18966971	6.34817342
35	9.89768205	5.95276098	6.84063579
36	13.80036620	4.56457650	5.60100353
37	13.55604511	1.44801648	1.80426088
38	14.03027338	-1.61187969	-0.65594209
39	11.79510474	-0.96485777	-3.27763757
40	10.87553089	-3.44637228	-4.44927035
41	13.26671544	-4.77186872	-5.36490882
42	14.89699970	-5.56276048	-3.42841514
43	13.91619738	-4.79996858	-7.56638344
44	9.52926415	-4.97786397	-2.59904456
45	7.53101753	-4.06618577	-1.12152193
46	7.48720546	-4.67327420	1.11460347
47	5.23029622	-2.65546881	-2.17550926
48	3.20984102	-4.51260416	-2.23656170
49	0.67186070	-3.91603209	-1.98988790
50	-0.93709042	-5.52994485	-2.43807629
51	0.05553022	-1.27945593	-1.07018761
52	-2.70706232	-0.63195803	-1.37263148
53	-3.45690355	-0.49086443	-4.13828772
54	-6.25044081	-0.12041952	-4.40460035
55	5.43129148	-1.21543502	-4.68764224
56	5.07732021	-2.86909171	-7.01654609
57	2.35238252	-2.87769433	-7.90516489
58	1.92259392	-4.63994513	-10.14301521
59	-0.69785872	-4.71325778	-10.90406978
60	-7.20431670	2.24845046	-5.10081768
61	-9.79121693	2.64654649	-5.40293094
62	-11.51132214	0.65917116	-5.00104953
63	-10.58257156	-1.73670283	-4.19129176
64	-7.93476445	-2.13682947	-3.89047630
65	-12.31795360	-3.73160478	-3.66818051
66	-11.41124255	-6.10318190	-2.77415626
67	-8.73805005	-6.46600470	-2.51116640
68	-7.06355864	-4.57974439	-3.07675077
69	-14.17968980	0.98453949	-5.37423819

70	-15.83909675	-0.94027831	-4.90176101
71	-14.96451318	-3.34279789	-3.99101064
72	-16.65050210	-5.30742216	-3.37666899
73	-15.74323801	-7.61493523	-2.47588251
74	-13.15588469	-8.02531866	-2.18453138
75	2.48308919	1.08919462	3.68213874
76	5.69839383	0.65276663	2.95110857
77	5.34685425	5.16769321	0.74257078
78	4.23670537	2.44240184	-0.81599951
79	1.07964135	6.45579584	1.89411059
80	-0.14300095	3.41706844	1.29817291
81	1.91492893	7.10594809	-2.69659739
82	0.93991016	3.95259935	-3.36046748
83	-2.36496195	7.85764313	-1.07632503
84	-3.21437135	4.94737265	-1.95378722
85	6.53385242	5.07740133	4.87568465
86	2.96662991	7.17749996	5.41716337
87	3.96248717	0.27302176	-4.68831715
88	7.23740154	-0.18034547	-4.76288673
89	5.69966294	-4.82563863	-6.61242801
90	6.30109645	-2.17509379	-8.56176432
91	1.09430959	-3.44212867	-6.33681314
92	1.79203568	-0.93151049	-8.42646295
93	2.54976705	-6.57787830	-9.63829019
94	3.10582637	-4.02709266	-11.76507961
95	-1.82898773	-5.38001097	-9.46751230
96	-1.33221515	-2.94991348	-11.42725882
97	4.75896139	-1.24998355	-0.67609914
98	3.61468726	-6.24341715	-3.03185043
99	-16.95321237	3.51887143	-1.36399423
100	-10.54478739	8.58860263	-1.33932350
101	-15.03340690	7.68418132	-2.41916511
102	-4.00017492	4.10916727	2.97573461
103	-6.51752369	7.27908393	0.70720373
104	-16.53637026	-0.82189144	0.33874107
105	-13.86253200	-4.21655872	2.14748995
106	-9.72379283	-5.65810524	3.82153172
107	-5.31679034	-4.69999071	5.13154316
108	-1.78464247	-2.08930142	5.10856123
109	-1.75745724	0.82266128	3.45614415
110	-3.42481277	-0.06439457	8.91449679
111	-4.04164880	2.80076526	7.26650392
112	0.11630623	2.46394441	9.97397272
113	1.31902310	0.66036324	7.44139031
114	8.52496918	-0.25260709	9.04785638
115	11.98486710	0.22639039	6.92163905
116	9.80727413	-1.07336764	2.88371987
117	9.04377586	2.15966821	2.30225136
118	14.38204680	6.41783401	5.59948688
119	15.11254531	3.35220971	6.35894000
120	10.18549733	0.10607897	-2.49840130
121	12.69807678	0.21689882	-4.73807615
122	9.65821002	-3.04538619	-6.11643825
123	14.14266960	-6.66588039	-2.02230530
124	16.60836032	-6.23334574	-4.05861300
125	10.72974335	-6.03183828	-1.47021784
126	0.61762241	-1.14713140	0.94045932
127	1.20837305	0.12233773	-2.10028938
128	-3.10069040	1.21027332	-0.46763828
129	-3.87437798	-2.04414280	-0.37662372
130	-2.42749066	1.08375300	-5.05350871
131	-2.86298776	-2.24489608	-5.11054365
132	-5.87866466	3.81370329	-5.43001009
133	-10.49871388	4.51810454	-5.95533165
134	-8.04536332	-8.31168197	-1.85860031
135	-5.02436722	-4.94009635	-2.88984717
136	-14.87013919	2.82461679	-6.04514587
137	-17.87905473	-0.65940589	-5.17128341
138	-18.69294619	-5.00598945	-3.59429658
139	-17.08190974	-9.12805786	-1.99929953
140	-12.46186471	-9.85472575	-1.48967640

References

1.	B. Aradi, B. Hourahine and T. Frauenheim, *J. Phys. Chem. A*, 2007, **111**, 5678-5684.
2.	P. Koskinen and V. Mäkinen, *Comput. Mater. Sci.*, 2009, **47**, 237-253.
3.	S. Grimme, J. Antony, S. Ehrlich and H. Krieg, *J. Chem. Phys.* , 2010, **132**, 154104.
4.	S. Grimme, S. Ehrlich and L. Goerigk, *J Comput Chem.*, 2011, **32**, 1456-1465.
5.	S. Nénon and B. Champagne, *J. Chem. Phys.*, 2013, **138**, 204107.
6.	J. Guo, Y. Xu, S. Jin, L. Chen, T. Kaji, Y. Honsho, M. A. Addicoat, J. Kim, A. Saeki, H. Ihee, S. Seki, S. Irle, M. Hiramoto, J. Gao and D. Jiang, *Nat. Comm.*, 2013, **4**, 2736.
7.	N. Goldman, S. Goverapet Srinivasan, S. Hamel, L. E. Fried, M. Gaus and M. Elstner, *J. Phys. Chem. C* 2013, **117**, 7885-7894.
8.	D. Vijay and G. N. Sastry, *Chem. Phys. Lett.* , 2010, **485**, 235-242.
9.	H. J. C. Berendsen, D. Vanderspoel and R. Vandrunen, *Comput Phys Commun*, 1995, **91**, 43-56.
10.	E. Lindahl, B. Hess and D. van der Spoel, *J. Mol. Model.*, 2001, **7**, 306-317.
11.	B. Hess, C. Kutzner, D. van der Spoel and E. Lindahl, *J. Chem. Theory Comput.*, 2008, **4**, 435-447.
12.	P. Mark and L. Nilsson, *J. Phys. Chem. A* 2001, **105**, 9954-9960.
13.	A. K. Malde, L. Zuo, M. Breeze, M. Stroet, D. Poger, P. C. Nair, C. Oostenbrink and A. E. Mark, *J. Chem. Theory Comput.*, 2011, **7**, 4026-4037.
14.	N. Schmid, A. P. Eichenberger, A. Choutko, S. Riniker, M. Winger, A. E. Mark and W. F. van Gunsteren, *Eur. Biophys. J.* , 2011, **40**, 843.
15.	A. R. Leach, *Molecular modelling: principles and applications*, Pearson Education Limited: Essex, England, 2001.
16.	G. Bussi, D. Donadio and M. Parrinello, *J. Chem. Phys.*, 2007, **126**, 014101.
17.	M. Parrinello and A. Rahman, *J. Appl. Phys.* , 1981, **52**, 7182-7190.
18.	M. L. Klein, *Mol. Phys.* , 1983, **50**, 1055-1076.
19.	T. Darden, D. York and L. Pedersen, *J. Chem. Phys.*, 1993, **98**, 10089-10092.
20.	D. J. Evans and B. L. Holian, *J. Chem. Phys.* , 1985, **83**, 4069-4074.