Supporting information

Hydrophobicity as a tool for programming sequential mesophase transitions of enzyme responsive polymeric systems

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Instrumentation and Materials

Instrumentation

^{1}H/^{13}C NMR: Spectra were recorded on Bruker Avance I and Avance III 400 MHz/100 MHz spectrometers as indicated. Chemical shifts are reported in ppm and referenced to the solvent. **SEC:** All measurements were recorded on Viscotek GPCmax by Malvern using a refractive index detector and PEG standards (purchased from Sigma-Aldrich) were used for calibration. **DLS**: All measurements were recorded on a Corduan Technology VASCOγ particle size analyzer. **Fluorescence spectra:** All measurements were recorded on a TECAN Infinite M200Pro device. **HPLC:** All measurements were recorded on a Waters Alliance e2695 separations module equipped with a Waters 2998 photodiode array detector. All solvents were purchased from Bio-Lab Chemicals and were used as received. All solvents are HPLC grade. **Spectrophotometer:** All measurments were recorded on an Agilent Cary 60 UV-Vis spectrophotometer. **Rheometer:** Rheological measurements were performed using a TA instruments AR-G2 controlled- stress rheometer. **TEM**: Images were taken by a JEM-1400PlusTEM at 80 kV. **SEM**: Images were taken by a JCM-6000PLUS NeoScope Benchtop SEM.

Materials

Poly (Ethylene Glycol) methyl ether (M_n=5kDa) and Poly (Ethylene Glycol) (M_n=10kDa), 2,2-dimethoxy-2-phenylacetophenone (DMPA, 99%), allyl bromide, propargyl bromide (80% in toluene), 4-dimethylaminopyridine (4-DMAP, 99%), 4-Nitrophenol (99.5%), N,N'-dicyclohexylcarbodiimide (DCC, 99%), 2-Mercaptoethanol, triphenylmethyl chloride, hexanoic acid, heptanoic acid, octanoic acid, triethylsilane, trifluoroacetic acid and Sephadex® LH20 were purchased from Sigma-Aldrich. Cysteamine hydrochloride (98%), potassium hydroxide and Diisopropylethylamine (DIPEA) were purchased from Merck.. 3,5 dihydroxy benzoic acid was purchased from Apollo Scientific Ltd. Anhydrous potassium carbonate (K_2CO_3) was purchased from J. T. Baker. Anhydrous Na_2SO_4 (granular, 10-60 mesh) was purchased from Macron. Silica Gel 60Å, 0.040-0.063mm, Sodium Hydroxide and all solvents were purchased from Bio-Lab and were used as received. Deuterated solvents for NMR were purchased from Cambridge Isotope Laboratories (CIL), Inc.

Synthesis and characterization of amphiphilic polymers

Synthesis of DBA amphiphilic hybrids

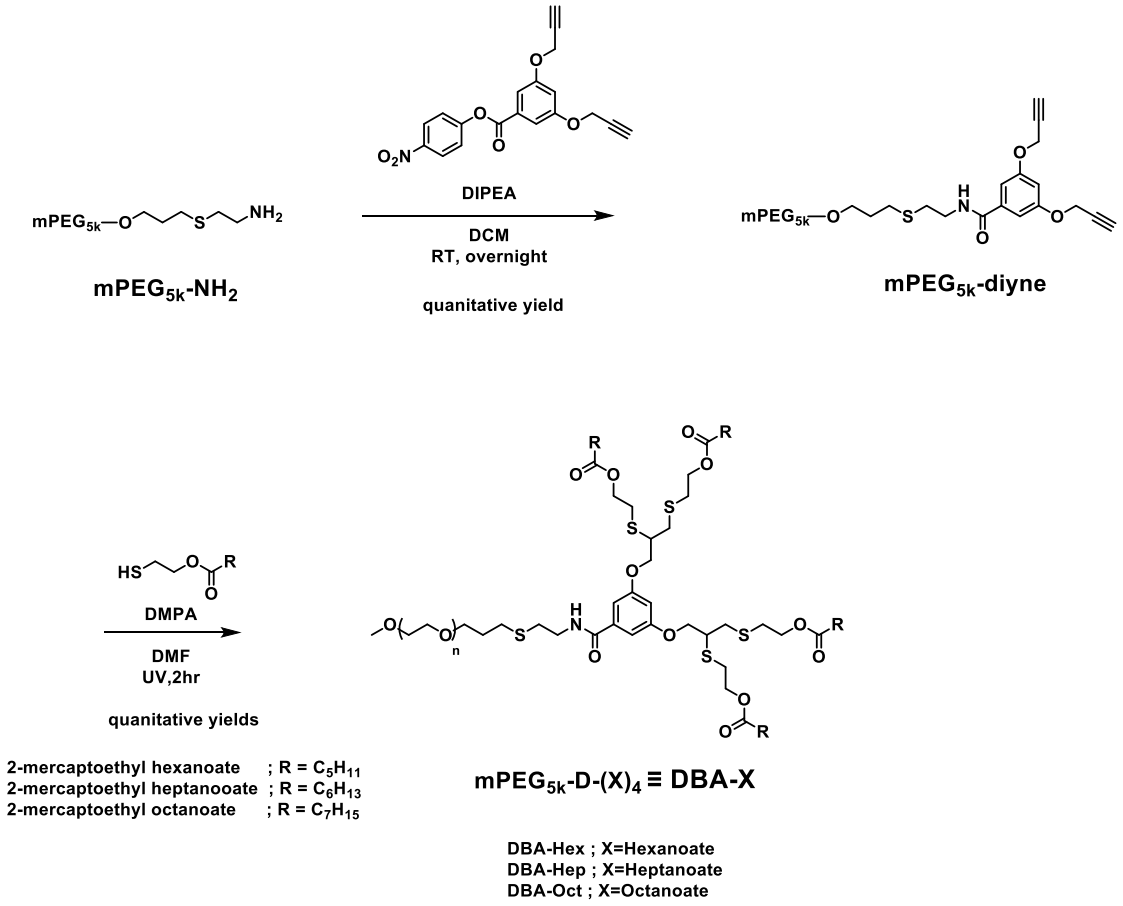


Figure S1. Synthetic Route for $mPEG_{5k}$-D-$(CX)_4$ amphiphilic hybrids.

*$mPEG_{5k}$-NH_2 and mPEG5k-diyne were synthesized as previously reported[1] and the spectroscopic characterization correlated well with these reports.

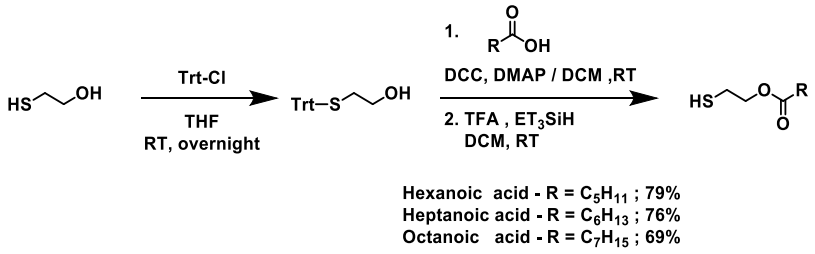


Figure S2. Synthesis of thiol functionalized enzymatically cleavable end-groups.

*2-(tritylthio)ethan-1-ol was synthesized as previously reported[2] and the spectroscopic characterization correlated well with these reports.

Preparation of thiol functionalized enzymatically cleavable end-groups was done by the general procedure published before[2]: 2-Mercaptoethanol was masked with trityl group, followed by Steglich esterification reaction with specific acid – Hexanoic, Heptanoic and Octanoic. In the last step, the trityl protecting group was removed by treating it with trifluoroacetic acid (TFA) in the presence of triethyl silane (Et_3SiH) as a scavenging agent for triphenylmethyl carbocation.

2-mercaptoethyl hexanoate: 2-(tritylthio)ethan-1-ol (2 gr, 6.24 mmol), Hexanoic acid (800 mg, 6.86 mmol) DMAP (230 mg, 1.87 mmol) and DCC (1.4 gr, 6.86 mmol) were dissolved in DCM (20 mL) and stirred at RT for 1hr. Crude was filtered, then TFA (5 mL) and Et3SiH (1.3 mL) were added. Reaction was stirred for few minutes at RT, then evaporated and dried under high vacuum. Product was purified by silica column (40:60 Hex:DCM, TLC plates were stained with $KMnO_4$). Product was obtained as colorless oil in 79% yield (830 mg).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.17 (t, *J* = 6.6 Hz, 2H, -C***H***$_2$-O-CO-), 2.73 (dt, *J* = 8.4, 6.6 Hz, 2H, -CH_2-C***H***$_2$-SH), 2.31 (t, *J* = 7.5 Hz, 2H, -C***H***$_2$-COO-), 1.68 – 1.56 (m, 2H, -C***H***$_2$-CH_2-COO-), 1.47 (t, *J* = 8.5 Hz, 1H, -CH_2-S***H***), 1.36 – 1.23 (m, 4H, -C***H***$_2$-C***H***$_2$-CH_3), 0.91 – 0.85 (m, 3H, -CH_2-C***H***$_3$). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 173.5 , 65.6 , 34.2 , 31.3 , 24.7 , 23.4 , 22.4 , 13.9. ss

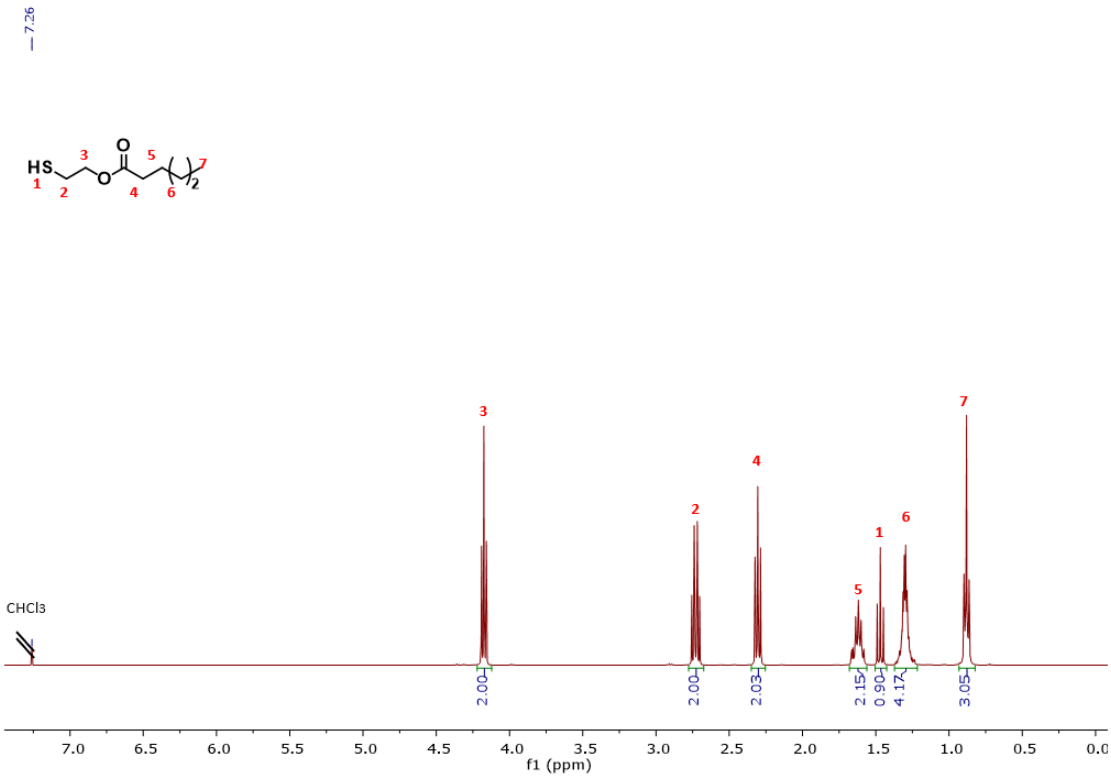


Figure S3. ^{1}H-NMR spectrum of 2-mercaptoethyl hexanoate in $CDCl_3$.

2-mercaptoethyl heptanoate: 2-(tritylthio)ethan-1-ol (2 gr, 6.24 mmol), Heptanoic acid (895 mg, 6.86 mmol) DMAP (230 mg, 1.87 mmol) and DCC (1.4 gr, 6.86 mmol) were dissolved in DCM (20 mL) and stirred at RT for 1hr. Crude was filtered, then TFA (5 mL) and Et3SiH (1.3 mL) were added. Reaction was stirred for few minutes at RT, then evaporated and dried under high vacuum. Product was purified by silica column (40:60 Hex:DCM, TLC plates were stained with $KMnO_4$). Product was obtained as colorless oil in 76% yield (912 mg).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.19 (t, *J* = 6.6 Hz, 2H, -C***H***$_2$-O-CO-), 2.74 (dt, *J* = 8.5, 6.6 Hz, 2H, -CH$_2$-C***H***$_2$-SH), 2.32 (t, *J* = 7.5 Hz, 2H, -C***H***$_2$-COO-), 1.69 – 1.54 (m, 2H, -C***H***$_2$-CH$_2$-COO-), 1.48 (t, *J* = 8.5 Hz, 1H, -CH$_2$-S***H***), 1.39 – 1.20 (m, 6H, -C***H***$_2$-C***H***$_2$-C***H***$_2$-CH$_3$), 0.95 – 0.85 (m, 3H, -CH$_2$-C***H***$_3$). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 173.5 , 65.5 , 34.2 , 31.5 , 28.8 , 24.9 , 23.4 , 22.5 , 14.0.

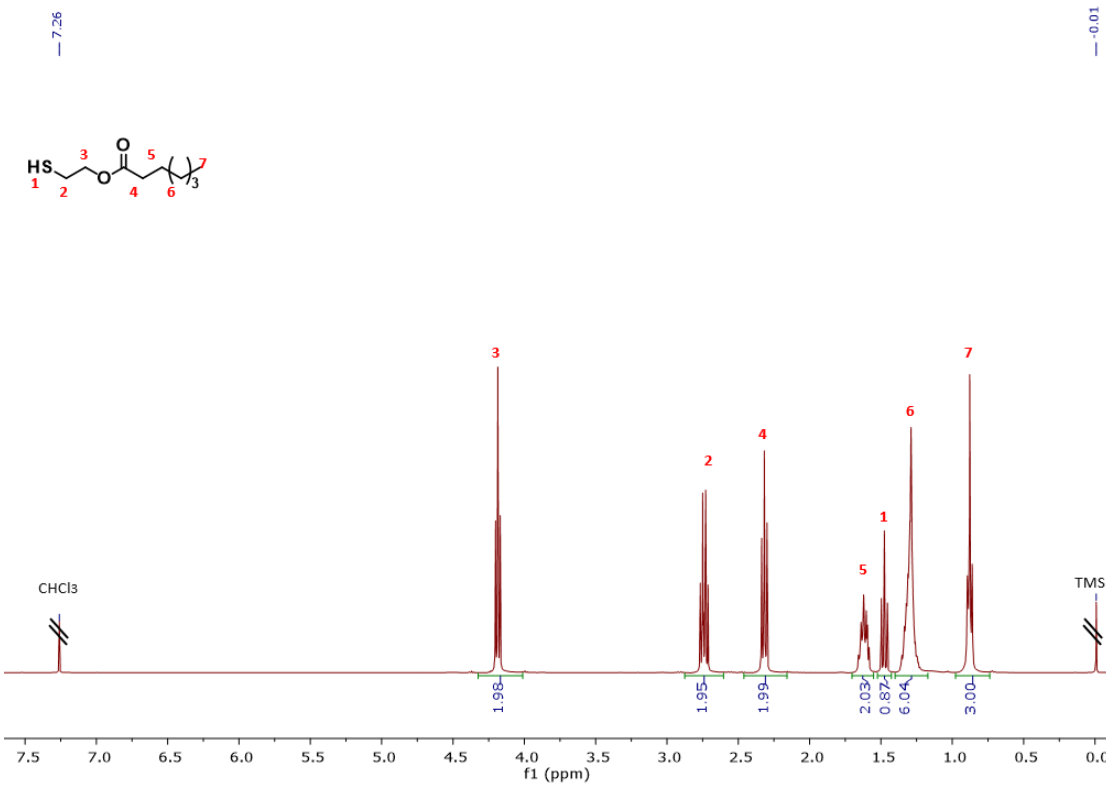


Figure S4. ^{1}H-NMR spectrum of 2-mercaptoethyl heptanoate in $CDCl_3$.

2-mercaptoethyl octanoate: 2-(tritylthio)ethan-1-ol (2 gr, 6.24 mmol), Octanoic acid (990 mg, 6.86 mmol) DMAP (230 mg, 1.87 mmol) and DCC (1.4 gr, 6.86 mmol) were dissolved in DCM (20 mL) and stirred at RT for 1hr. Crude was filtered, then TFA (5 mL) and Et3SiH (1.3 mL) were added. Reaction was stirred for few minutes at RT, then evaporated and dried under high vacuum. Product was purified by silica column (40:60 Hex:DCM, TLC plates were stained with $KMnO_4$). Product was obtained as colorless oil in 69% yield (877 mg).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.18 (t, *J* = 6.6 Hz, 2H, -C***H***$_2$-O-CO-), 2.74 (dt, *J* = 8.5, 6.6 Hz, 2H, -CH$_2$-C***H***$_2$-SH), 2.31 (t, *J* = 7.5 Hz, 2H, -C***H***$_2$-COO-), 1.68 – 1.56 (m, 2H, -C***H***$_2$-CH$_2$-COO-), 1.47 (t, *J* = 8.5 Hz, 1H, -CH$_2$-S***H***), 1.36 – 1.20 (m, 8H, -C***H***$_2$-C***H***$_2$-C***H***$_2$-C***H***$_2$-CH$_3$), 0.90 – 0.84 (m, 3H, -CH$_2$-C***H***$_3$). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 173.5 , 65.5 , 34.2 , 31.7 29.1 , 29.0 , 25.0 , 23.4 , 22.6 , 14.1.

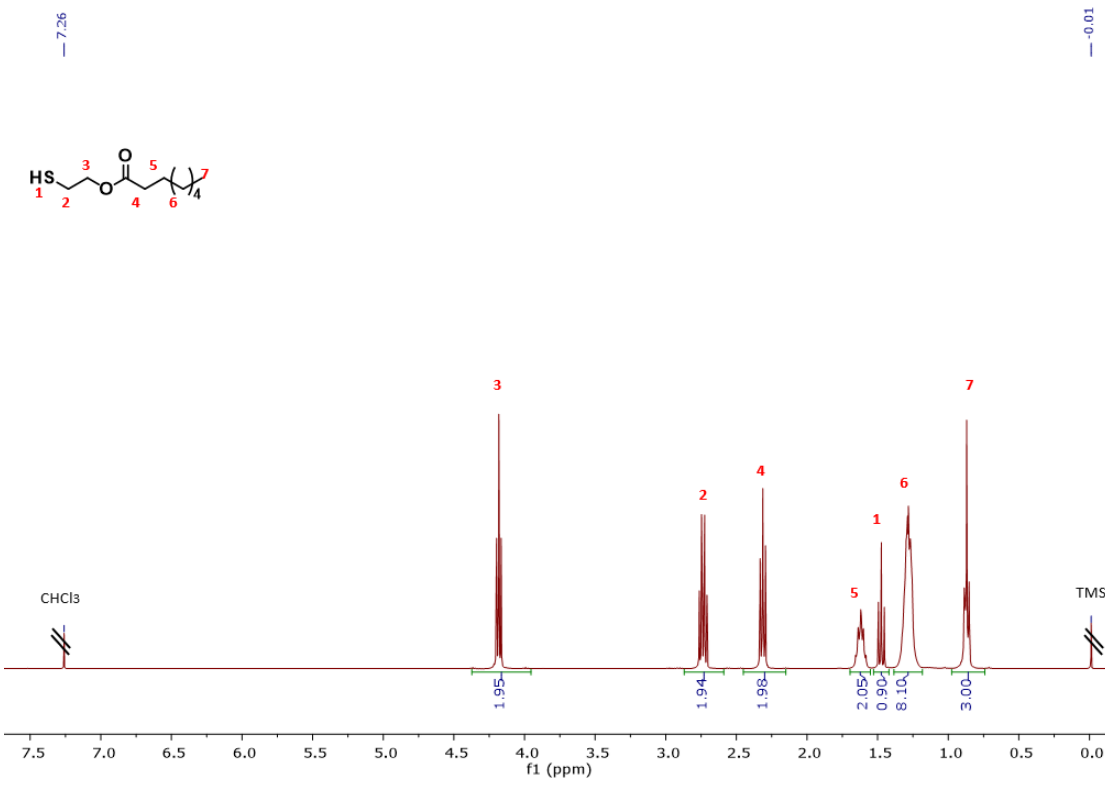


Figure S5. ^{1}H-NMR spectrum of 2-mercaptoethyl octanoate in $CDCl_3$.

General procedure for thiol-yne reaction with of $mPEG_{5k}$-diyne with aliphatic thiols of various lengths:

$mPEG_{5k}$-diyne (1 eq), the relevant thiol (40 eq) and DMPA (0.4 eq; 1 mol% with respect to the thiol) were dissolved in DMF (0.5 mL per 100 mg of hybrid). The solution was purged with nitrogen for 20 minutes and then stirred under UV light (365 nm) for 2 hours. Then, the reaction mixture was loaded as-is on a MeOH-based LH20 (Sephadex®) size exclusion column. Fractions that contained the product (identified by UV light and/or coloring with iodine) were unified, the organic solvents were evaporated to dryness and the white solid was dried under high vacuum.

DBA-Hex: $mPEG_{5k}$-diyne (200 mg, 0.037 mmol), 2-mercaptoethyl hexanoate (264 mg, 1.49 mmol) and DMPA (4 mg, 0.015 mmol) were reacted in DMF according to the general procedure. The product was obtained as a white solid in quantitative yield (214 mg).

^{1}H NMR (400 MHz, Chloroform-d) δ 6.97 (d, J = 2.2 Hz, 2H, Ar-***H***), 6.93 (t, J = 5.8 Hz, 1H, -CH_2-N***H***-CO-Ar-), 6.59 (t, J = 2.2 Hz, 1H, Ar-***H***), 4.29 – 4.13 (m, 12H, -C***H***$_2$-O-Ar + -C***H***$_2$-O-

CO-), 3.82-3.40 (m, PEG backbone), 3.36 (s, 3H, C***H***$_3$-O-PEG), 3.19 (p, J = 6.1 Hz, 2H, -C***H***-S-), 3.05 – 2.70 (m, 12H, -CH$_2$-C***H***$_2$-S-), 2.63 (t, J = 7.2 Hz, 2H, -CH$_2$-C***H***$_2$-S-), 2.29 (td, J = 7.5, 2.1 Hz, 8H, -C***H***$_2$-COO-), 1.86 (p, J = 6.8 Hz, 2H, -C***H***$_2$-CH$_2$-S-), 1.66-1.53 (m, 8H, -C***H***$_2$-CH$_2$-COO-), 1.36 – 1.19 (m, 16H, -CH$_2$-C***H***$_2$-C***H***$_2$-CH$_2$- + -C***H***$_2$-CH$_3$), 0.87 (t, J = 7.2 Hz, 12H, -CH$_2$-C***H***$_3$).

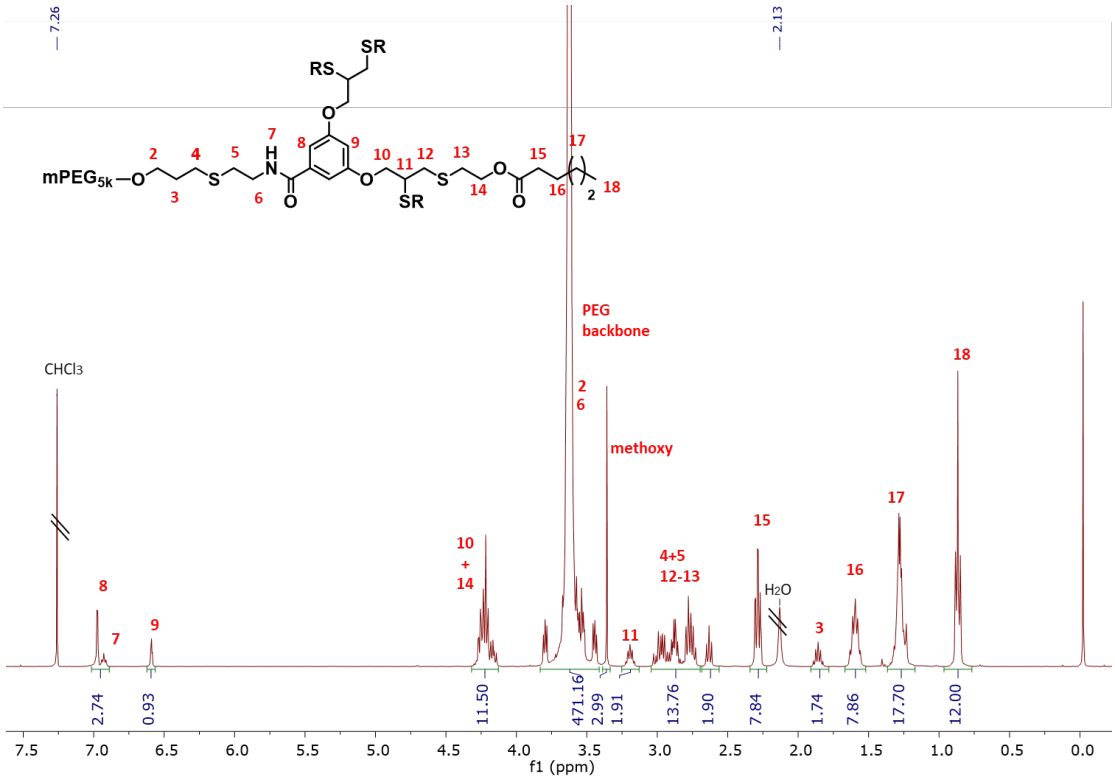


Figure S6. ^{1}H-NMR spectrum of DBA-Hex in $CDCl_3$.

<u>DBA-Hep:</u> mPEG$_{5k}$-diyne (200 mg, 0.037 mmol), 2-mercaptoethyl heptanoate (284 mg, 1.49 mmol) and DMPA (4 mg, 0.015 mmol) were reacted in DMF according to the general procedure. The product was obtained as a white solid in quantitative yield (218 mg).

^{1}H NMR (400 MHz, Chloroform-d) δ 6.98 (d, J = 2.2 Hz, 2H, Ar-***H***), 6.93 (t, J = 5.8 Hz, 1H, -CH$_2$-N***H***-CO-Ar-), 6.59 (t, J = 2.2 Hz, 1H, Ar-***H***), 4.33 – 4.08 (m, 12H, -C***H***$_2$-O-Ar + -C***H***$_2$-O-CO-), 3.85-3.40 (m, PEG backbone), 3.36 (s, 3H, C***H***$_3$-O-PEG), 3.19 (p, J = 6.0 Hz, 2H, -C***H***-S-), 3.10 – 2.68 (m, 12H, -CH$_2$-C***H***$_2$-S-), 2.63 (t, J = 7.2 Hz, 2H, -CH$_2$-C***H***$_2$-S-), 2.29 (td, J = 7.5, 2.1 Hz, 8H, -C***H***$_2$-COO-), 1.86 (p, J = 6.4 Hz, 2H, -C***H***$_2$-CH$_2$-S-), 1.66-1.53 (m, 8H, -C***H***$_2$-CH$_2$-COO-), 1.36 – 1.17 (m, 24H, -CH$_2$-C***H***$_2$-C***H***$_2$-CH$_2$- + -C***H***$_2$-CH$_3$), 0.87 (t, J = 7.2 Hz, 12H, -CH$_2$-C***H***$_3$).

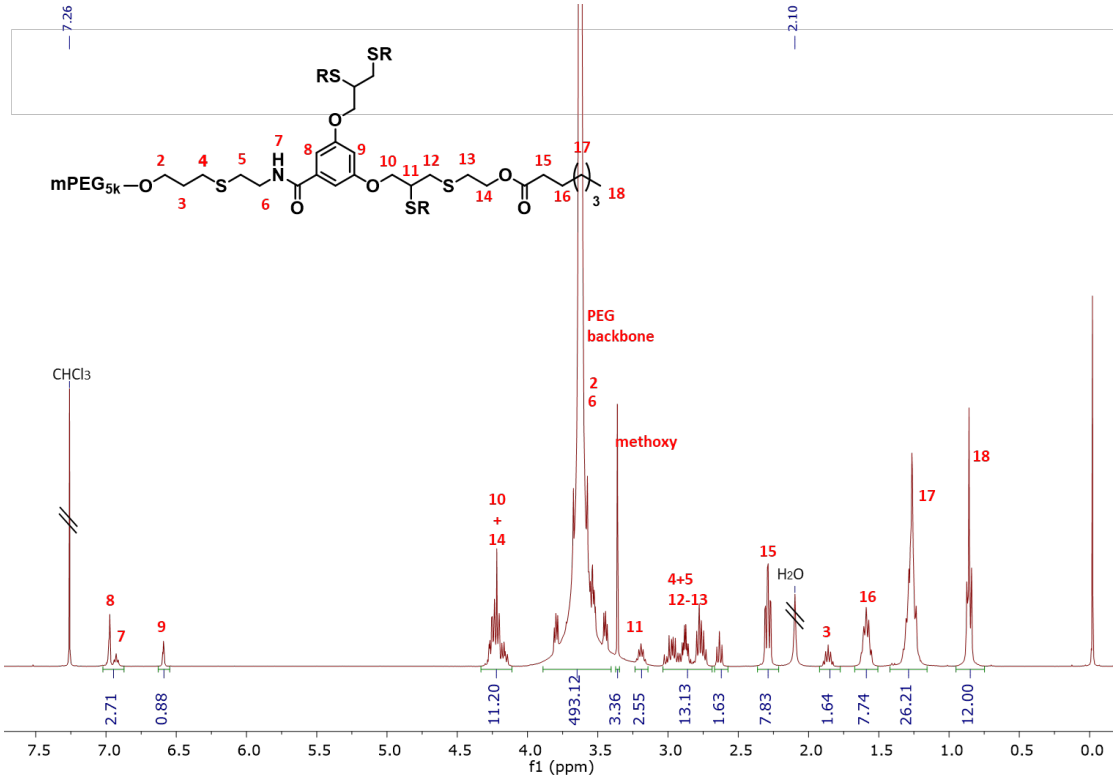


Figure S7. ^{1}H-NMR spectrum of DBA-Hep in $CDCl_3$.

DBA-Oct: $mPEG_{5k}$-diyne (200 mg, 0.037 mmol), 2-mercaptoethyl octanoate (306 mg, 1.49 mmol) and DMPA (4 mg, 0.015 mmol) were reacted in DMF according to the general procedure. The product was obtained as a white solid in quantitative yield (215 mg).

^{1}H NMR (400 MHz, Chloroform-d) δ 6.98 (d, J = 2.2 Hz, 2H, Ar-***H***), 6.93 (t, J = 5.8 Hz, 1H, -CH_2-N***H***-CO-Ar-), 6.59 (t, J = 2.2 Hz, 1H, Ar-***H***), 4.31 – 4.13 (m, 12H, -C***H***$_2$-O-Ar + -C***H***$_2$-O-CO-), 3.84-3.41 (m, PEG backbone), 3.36 (s, 3H, C***H***$_3$-O-PEG), 3.19 (p, J = 6.0 Hz, 2H, -C***H***-S-), 3.10 – 2.68 (m, 12H, -CH_2-C***H***$_2$-S-), 2.64 (t, J = 7.2 Hz, 2H, -CH_2-C***H***$_2$-S-), 2.29 (td, J = 7.5, 2.1 Hz, 8H, -C***H***$_2$-COO-), 1.87 (p, J = 6.4 Hz, 2H, -C***H***$_2$-CH_2-S-), 1.64-1.53 (m, 8H, -C***H***$_2$-CH_2-COO-), 1.34 – 1.16 (m, 32H, -CH_2-C***H***$_2$-C***H***$_2$-CH_2- + -C***H***$_2$-CH_3), 0.86 (t, J = 7.2 Hz, 12H, -CH_2-C***H***$_3$).

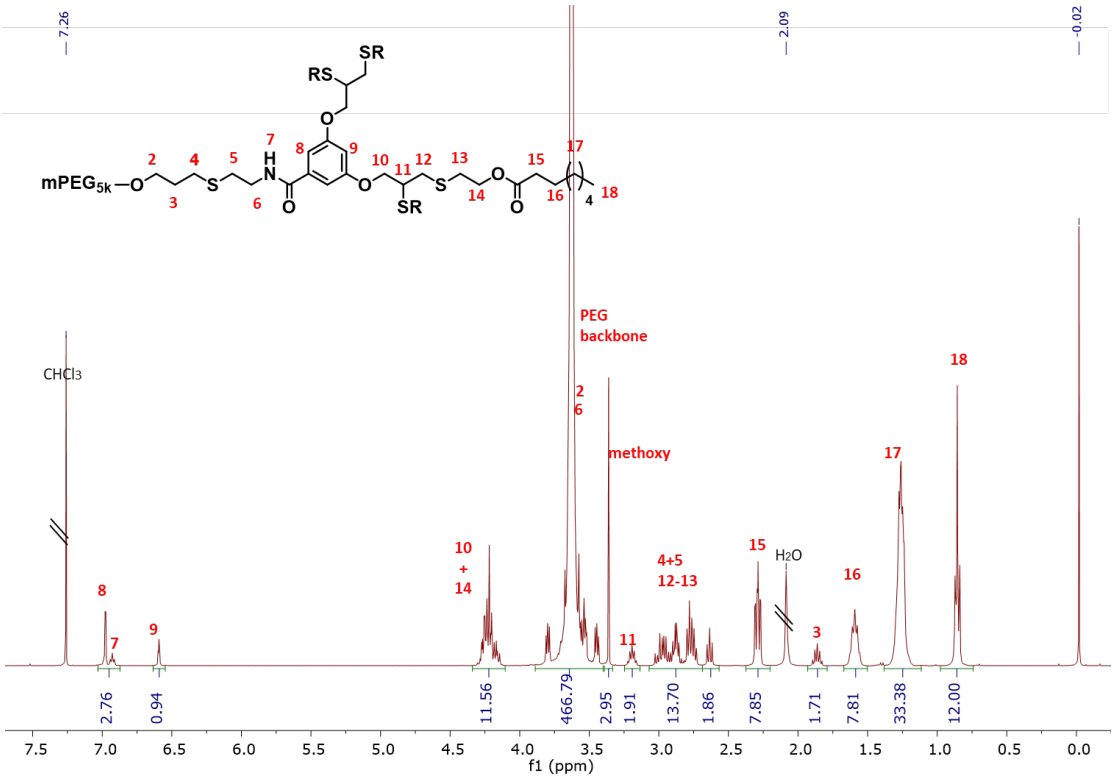


Figure S8. ^{1}H-NMR spectrum of DBA-Oct in $CDCl_3$.

Synthesis of TBA-Oct amphiphilic hybrid

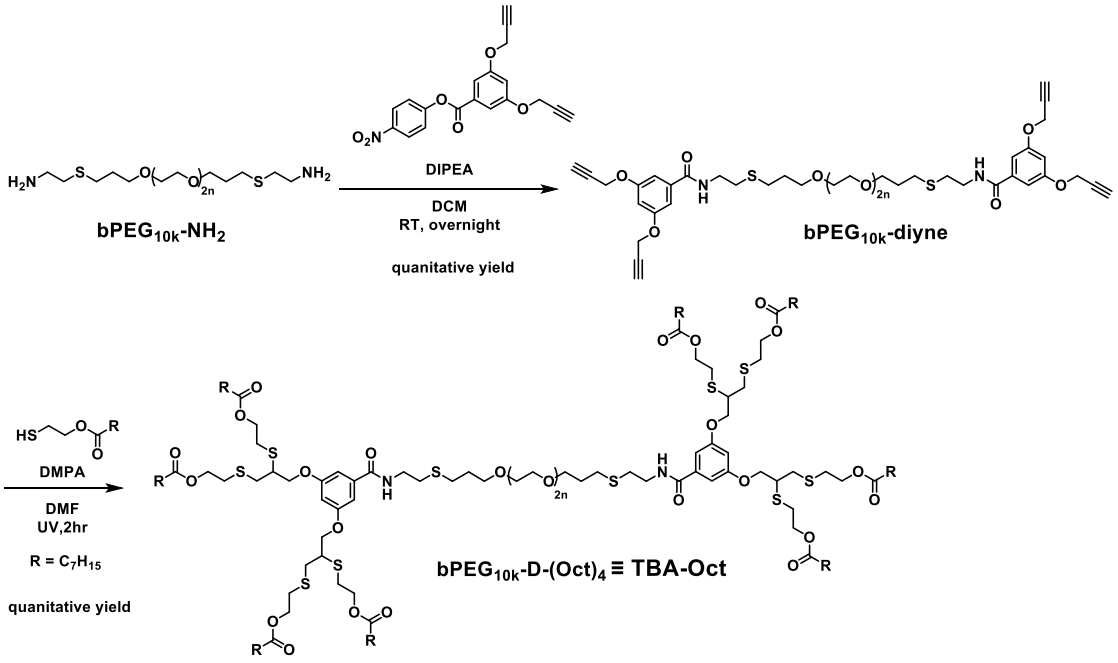


Figure S9. Synthetic Route for $bPEG_{10k}$-D-$(Oct)_4$ amphiphilic hybrid.

*$bPEG_{10k}$-NH_2 and $bPEG_{10k}$-diyne were synthesized as previously reported[2] and the spectroscopic characterization correlated well with these reports.

TBA-Oct: $bPEG_{10k}$-diyne (200 mg, 0.019 mmol), 2-mercaptoethyl octanoate (306 mg, 1.49 mmol) and DMPA (4 mg, 0.015 mmol) were dissolved in DMF (1 mL). The solution was purged with nitrogen for 20 minutes and then stirred under UV light (365 nm) for 2 hours. Then, the reaction mixture was loaded as-is on a MeOH-based LH20 (Sephadex®) size exclusion column. Fractions that contained the product (identified by UV light and/or coloring with iodine) were unified, the organic solvents were evaporated to dryness and the white solid was dried under high vacuum. The product was obtained as a white solid in quantitative yield (227 mg).

^{1}H NMR (400 MHz, Chloroform-d) δ 6.97 (d, J = 2.2 Hz, 4H, Ar-***H***), 6.93 (t, J = 5.8 Hz, 2H, -CH_2-N***H***-CO-Ar-), 6.59 (t, J = 2.2 Hz, 2H, Ar-***H***), 4.32 – 4.09 (m, 24H, -C***H***$_2$-O-Ar + -C***H***$_2$-O-CO-), 3.83-3.41 (m, PEG backbone), 3.19 (p, J = 6.1 Hz, 4H, -C***H***-S-), 3.05 – 2.70 (m, 24H, -CH_2-C***H***$_2$-S-), 2.63 (t, J = 7.2 Hz, 4H, -CH_2-C***H***$_2$-S-), 2.28 (td, J = 7.5, 2.1 Hz, 16H, -C***H***$_2$-COO-), 1.86 (p, J = 6.6 Hz, 4H, -C***H***$_2$-CH_2-S-), 1.63-1.52 (m, 16H, -C***H***$_2$-CH_2-COO-), 1.34 – 1.16 (m, 64H, -CH_2-C***H***$_2$-C***H***$_2$-CH_2- + -C***H***$_2$-CH_3), 0.85 (t, J = 6.8 Hz, 24H, -CH_2-C***H***$_3$).

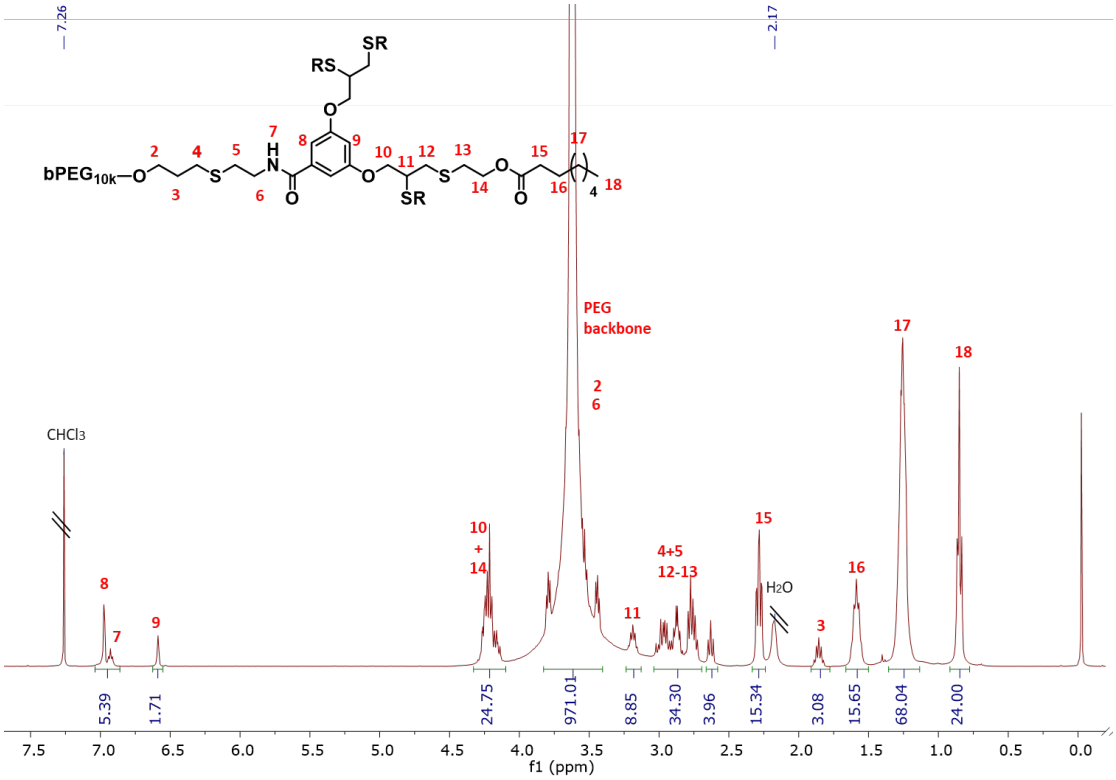


Figure S10. ^{1}H-NMR spectrum of TBA-Oct in $CDCl_3$.

Size exclusion chromatography (SEC)

Instrument method:

Instrument: Malvern Viscotek GPCmax

Columns: 2xPSS GRAM 1000Å

Column temperature: 50°C

Flow rate: 0.5 mL/min

Injection time: 60 min

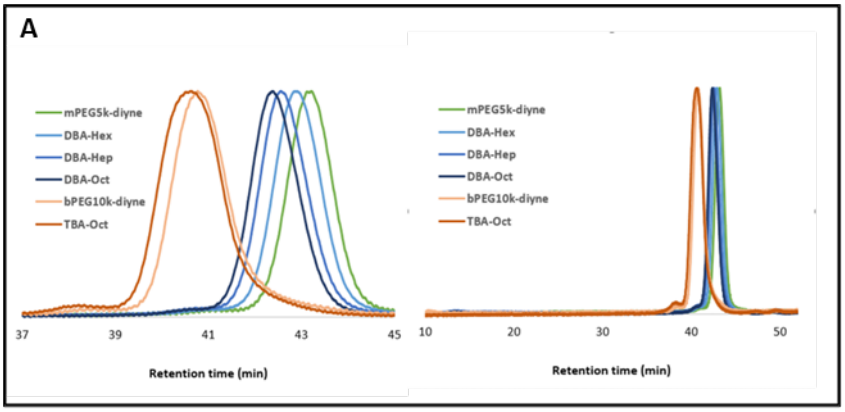
Injection volume: 50 µL from a 10 mg/mL sample

Diluent + mobile phase: DMF + 25mM NH_4Ac

Needle wash: DMF

Detector: Viscotek VE3580 RI detector

Sample preparation: The amphiphiles were directly dissolved in the diluent to give a final concentration of 10 mg/mL and filtered with 0.45 µm PTFE syringe filter.



B

Name	Calculated Mn	Đ
$mPEG_{5k}$-diyne	5.4	1.05
DBA-Hex	6.0	1.05
DBA-Hep	6.6	1.05
DBA-Oct	7.0	1.05
$bPEG_{10k}$-diyne	11.5	1.08
TBA-Oct	12.5	1.04

Figure S11. A) SEC traces overlay of $mPEG_{5k}$-diyne and DBA amphiphiles as well as $bPEG_{10k}$-diyne and TBA-Oct. Both complete and zoomed in chromatograms are displayed. B) Theoretical MW (based on NMR analysis, where commercially purchased $mPEG_{5k}$-OH and $bPEG_{10k}$-OH were used as references for 5 and 10kDa polymers, respectively), calculated Mn and Đ values of different amphiphiles.

Characterization of co-assembled micellar structures

Critical micelles' concentration (CMC)

General procedure of measurement:

Preparation of diluent:

Nile Red stock solution (0.88 mg/mL in ethanol) was diluted into a phosphate buffer saline (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl; pH 7.4) to afford a final concentration of 1.25 μM.

Preparation and measurement of samples:

The relevant DBC and TBC polymers were dissolved together in DCM, at a weight ratio of 3:2, respectively. The organic solvent was evaporated under reduced pressure to afford a formation of thin film and the mixture was left to dry under high vacuum. The amphiphiles mixture was dissolved in the diluent to give a final concentration of 500 μM. The solution was vortexed vigorously until the amphiphile completely dissolved and further sonicated for 15 minutes in an ultrasonic bath. The solutions were consecutively diluted by a factor of 1.5 with the diluent to afford a series of 24 samples for each amphiphile. 150 μL of each sample was loaded onto a 96 well plate and a fluorescence emission scan was performed for each well. To determine the hybrid's CMC – the maximum emission of Nile Red (at about 630 nm) was plotted as a function of the amphiphile's concentration. This procedure was repeated thrice for each amphiphiles mixture, and mean value is reported as the CMC value and the standard deviation as measurement error.

Similarly, a DBA-only micellar solutions were prepared, and CMC measurements were conducted.

Instrument method:

Instrument: TECAN Infinite M200Pro

Excitation: 550 nm

Emission intensity scan: 580-800 nm

Step: 2 nm

Number of flashes: 15

Gain: 100

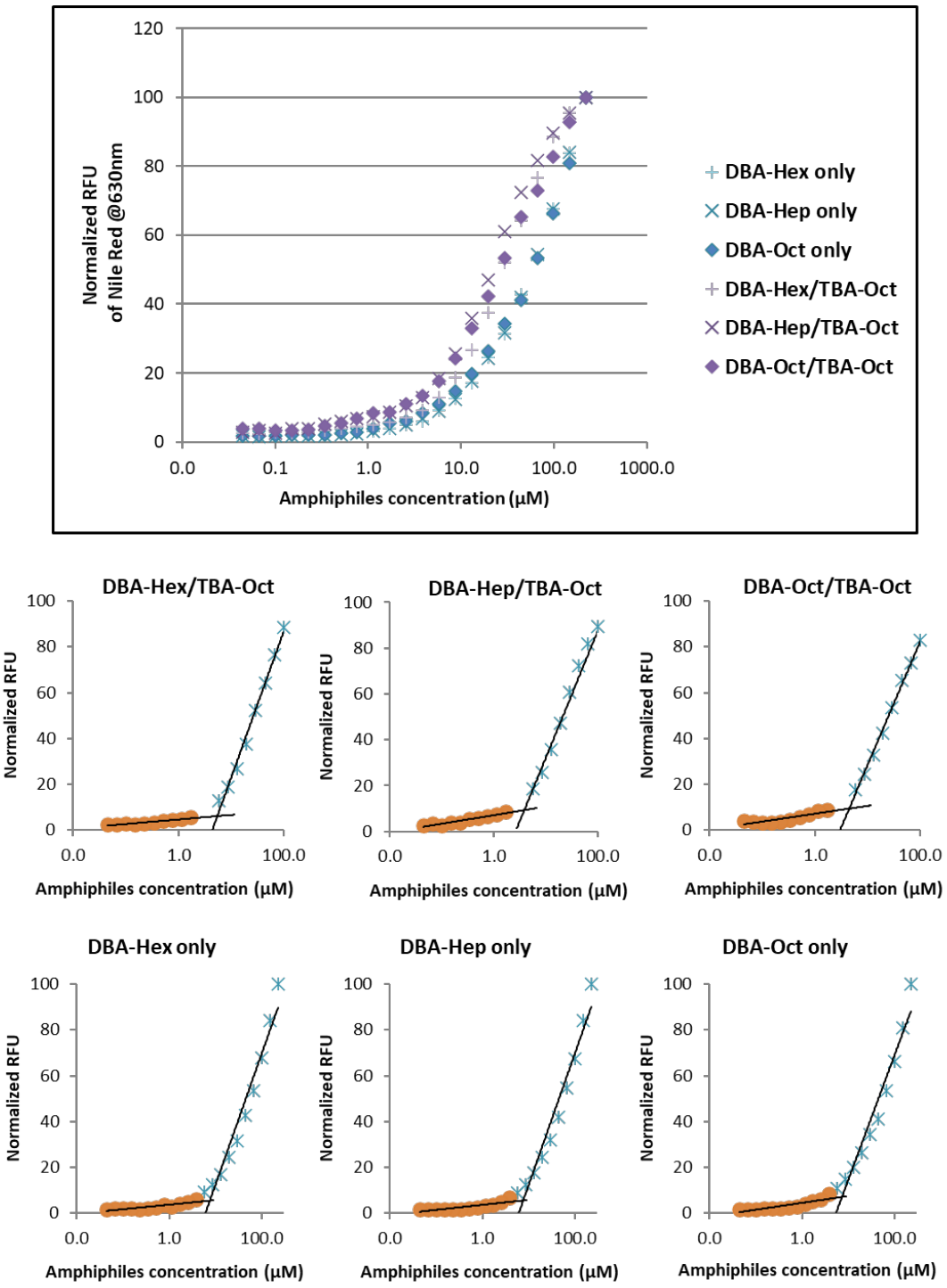


Figure S12. Overlay of CMC measurements of TBA and DBA co-assembled micellar mixtures [DBA/TBA weigh ratio 3:2], as well as DBA-only micelles.

CMC values for these mixtures were found to be 5±1, 4±1 and 4±1 µM for the mixtures containing DBA-Hex, -Hep and -Oct, respectively; and 8±1, 8±1 and 7±1 µM for the micelles composed solely from DBA-Hex, -Hep and -Oct, respectively.

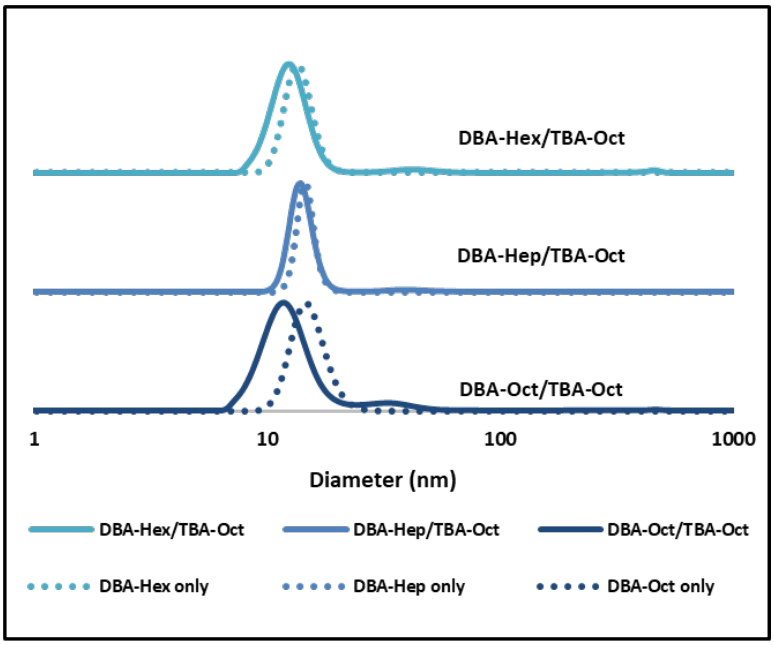
Dynamic light scattering

Sample preparation and measurements:

The co-assembled micellar solutions were prepared as mentioned in the 'Enzymatically induced mesophase transition' section and filtered through a 0.22 μm nylon syringe filter. Measurements were performed at t=0. In addition, after the DBA amphiphiles were enzymatically degraded and the TBA-based hydrogel was formed, the outer solutions were measured as well.

For the DBA-only micelles, a direct dissolution of the amphiphiles in PBS in the same concentration mentioned above, followed by vortex and sonication, achieved the micellar solution. These micellar solutions were filtered through a 0.22 μm nylon syringe filter and measured.

A

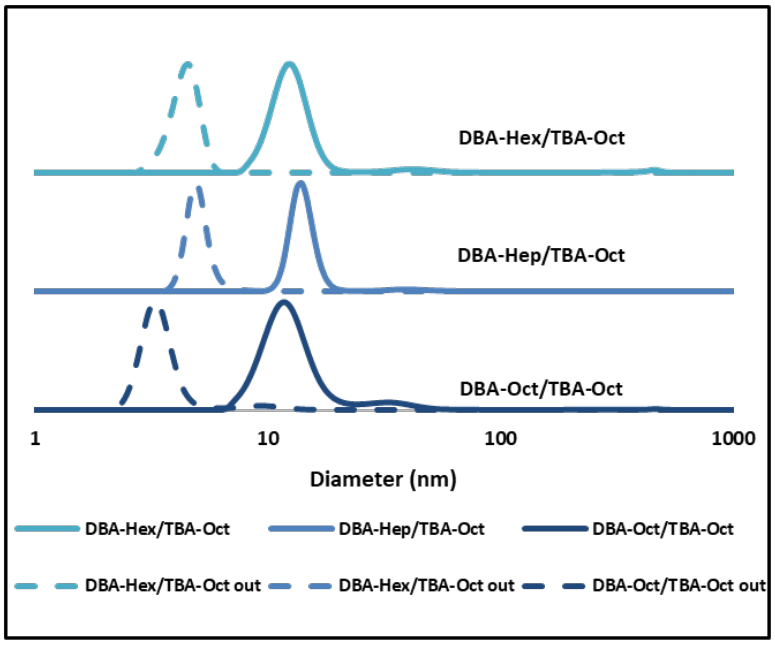


B

Mixture	Diameter (nm)
DBA-Hex/TBA-Oct	12±2
DBA-Hep/TBA-Oct	14±2
DBA-Oct/TBA-Oct	12±3
DBA-Hex only	14±2
DBA-Hep only	15±1
DBA-Oct only	15±3

Figure S13. **A)** Size measurements overlay of co-assembled amphiphiles mixtures (full line) alongside the comparable DBA-only micelles (dotted line). **B)** Analyzed D_H values of the solution.

A



B

	Diameter (nm)	
Mixture	**t=0**	**outer solution (after hydrogel formation)**
DBA-Hex/TBA-Oct	12±2	4±1
DBA-Hep/TBA-Oct	14±2	5±1
DBA-Oct/TBA-Oct	12±3	3±1

Figure S14. **A)** Size measurements overlay of co-assembled amphiphiles mixtures at t=0 (full line) and of the upper aqueous solution after enzymatic hydrolysis of DBA amphiphiles and hydrogel formation and precipitation (dashed line). **B)** Analyzed D_H values of the solution.

TEM imaging

Sample preparation:

30 µL of the co-assembled micellar solutions (prepared as mentioned above) were deposited onto carbon coated copper grids. The excessive solvent of the droplet was wiped away using a solvent-absorbing filter paper after 1 minute and the sample grids were left to dry in air at RT for 8 hours. Then, grids were inspected in transmission electron microscope (TEM), operated at 80 kV (JEM-1400Plus).

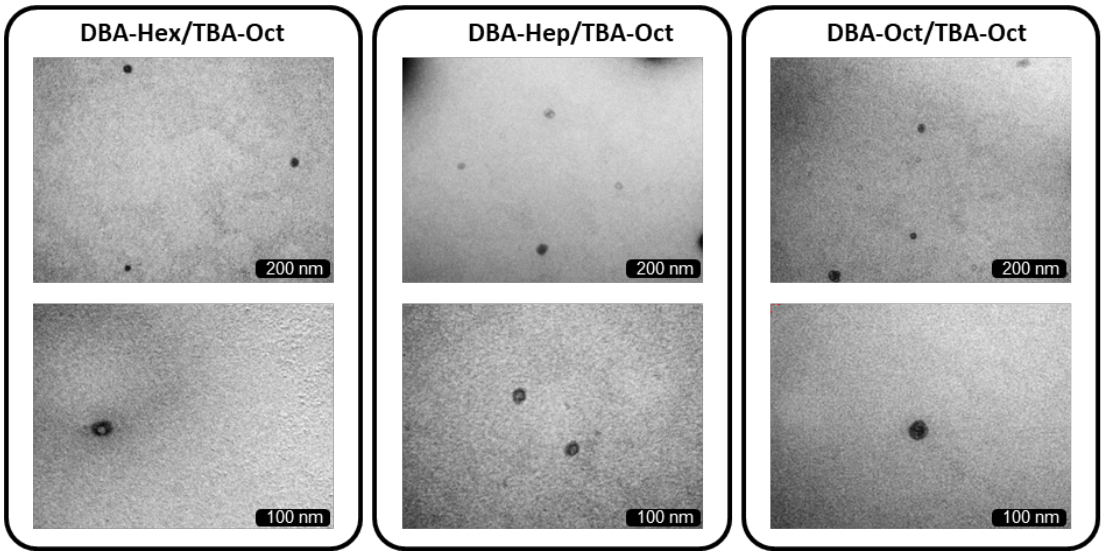


Figure S15. TEM images of the micellar solution of co-assembled micellar systems. The top row shows zoomed-out images (scale bar: 200 nm), and the bottom row shows zoomed-in images (scale bar: 100 nm).

Enzymatically induced mesophase transition

General protocol for the preparation of co-assembled micellar solution

The relevant DBC and TBC polymers were dissolved together in DCM, at a weight ratio of 3:2, respectively. The organic solvent was evaporated under reduced pressure to afford a formation of thin film and the mixture was left to dry under high vacuum. Then, the mixture was re-dissolved in phosphate buffer saline (PBS, pH=7.4) to yield a final concentration of 10.5 mg/mL and 7.0 mg/mL of DBC and TBC amphiphiles, respectively. The aqueous solutions were vortexed and sonicated for 15 minutes until full solubility was obtained.

Experimental procedure

1 mL of the micellar solution was transferred into an HPLC vial. PLE was added to yield a final concentration of 0.7 μM and degradation was followed at 37°C by monitoring the area under the peak of the parent amphiphile and hydrolyzed polymer by HPLC at 297 nm. Each experiment was conducted thrice; the reported values in each time point are the mean value, and the standard deviation is the error. (Results shown in main text, Figures 2 and 4).

As for the spectroscopy analysis of microgel suspension formation, samples were prepared and treated with PLE as mentioned above and were placed into plastic cuvettes at 37°C. The suspension formation was measured by monitoring the change in absorbance at 600 nm. (Results shown in main text, Figures 2 and 4).

Instrument method for HPLC:

Instrument: Waters Alliance e2695

Column: Aeris WIDEPORE, C4, 3.6 μm, 150x4.6 mm

Column temperature: 30°C

Sample temperature: 37°C

Solution A: 0.1% $HClO_4$:ACN 95:5 v/v

Solution B: 0.1% $HClO_4$:ACN 5:95 v/v

Solution C: ACN

Flow rate: 1 mL/min

Injection volume: 30 μL

Seal wash: H_2O:MeOH 90:10 v/v

Needle wash: MeOH

Detector: Waters 2998 photodiode array detector

Sampling rate: 2 points/sec

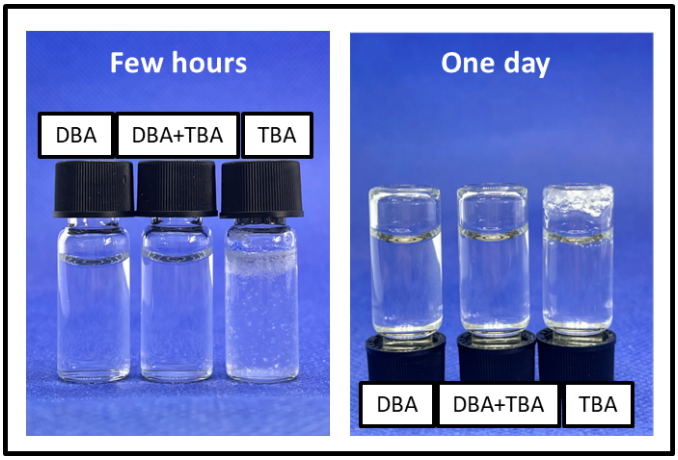


Figure S16. Images of vials containing (left to right) DBA-Hex, DBA-Hex/TBA-Oct formulation and TBA-Oct after few hours and one day of incubation at 37°C ([DBA]=10.5 mg/mL; [TBA]=7.0 mg/mL).

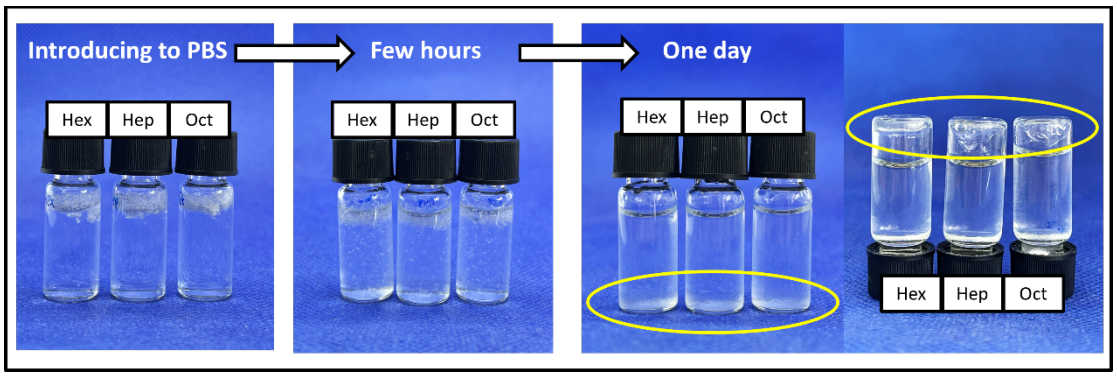


Figure S17. Images of vials containing TBA-Oct (7 mg/mL) and hexanoic, heptanoic, and octanoic acids at the same concentrations formed through DBAs' enzymatic degradation in situ (7 mmol). The images show the formation of TBA-based hydrogel over time, transitioning from suspended solids to hydrogel particle suspension and finally to settled hydrogel.

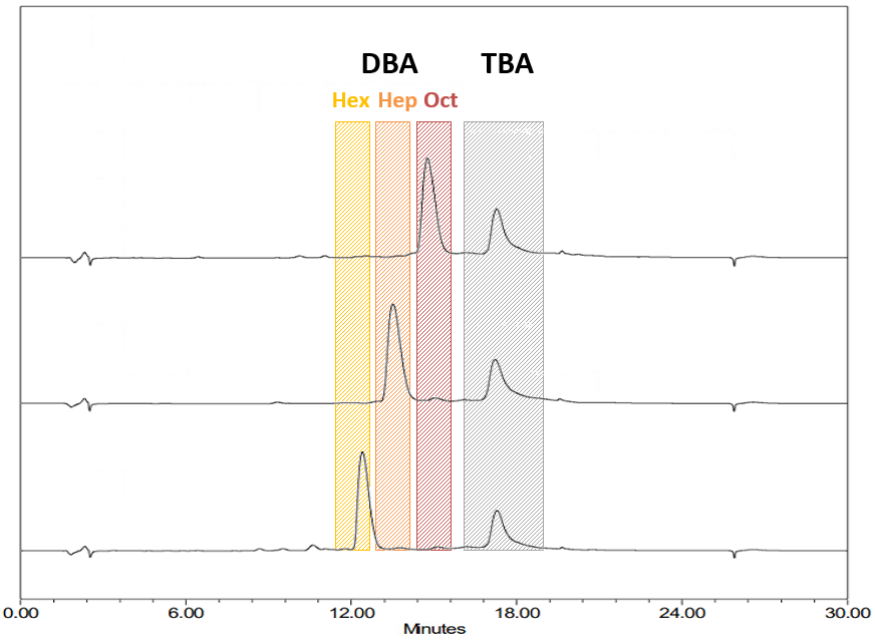


Figure S18. HPLC chromatogram overlay of DBA/TBA mixed micellar systems at t=0.

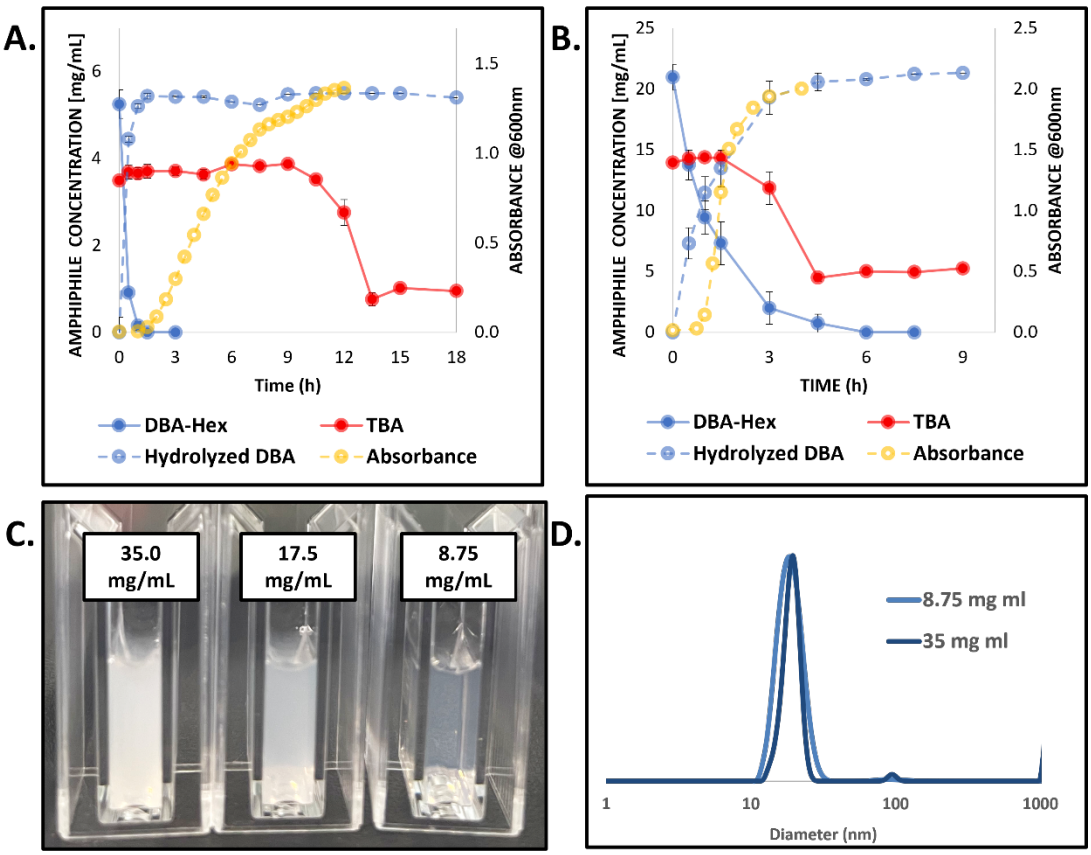


Figure S19. Enzymatic degradation and induced gelation of the DBA-Hex/TBA-Oct mixed-micellar system at different concentrations: (A+B) Overlay of HPLC-analyzed kinetic data for the enzymatic degradation of DBA (blue), accompanied by TBA peak disappearance (red), and a change in the absorbance (yellow), indicating the occurrence of the enzymatically induced sequential mesophase transitions, for formulation concentrations of (A) 8.75 and (B) 35 mg/mL. (C) Photos of the experimental cuvettes after 2 hours of incubation with the activating enzyme for formulation concentrations of 8.75, 17.5, and 35 mg/mL (from right to left). (D) Size measurements overlay of DBA-Hex/TBA-Oct co-assembled amphiphile mixtures at 8.75 and 35 mg/mL (D_H = 18±4 nm).

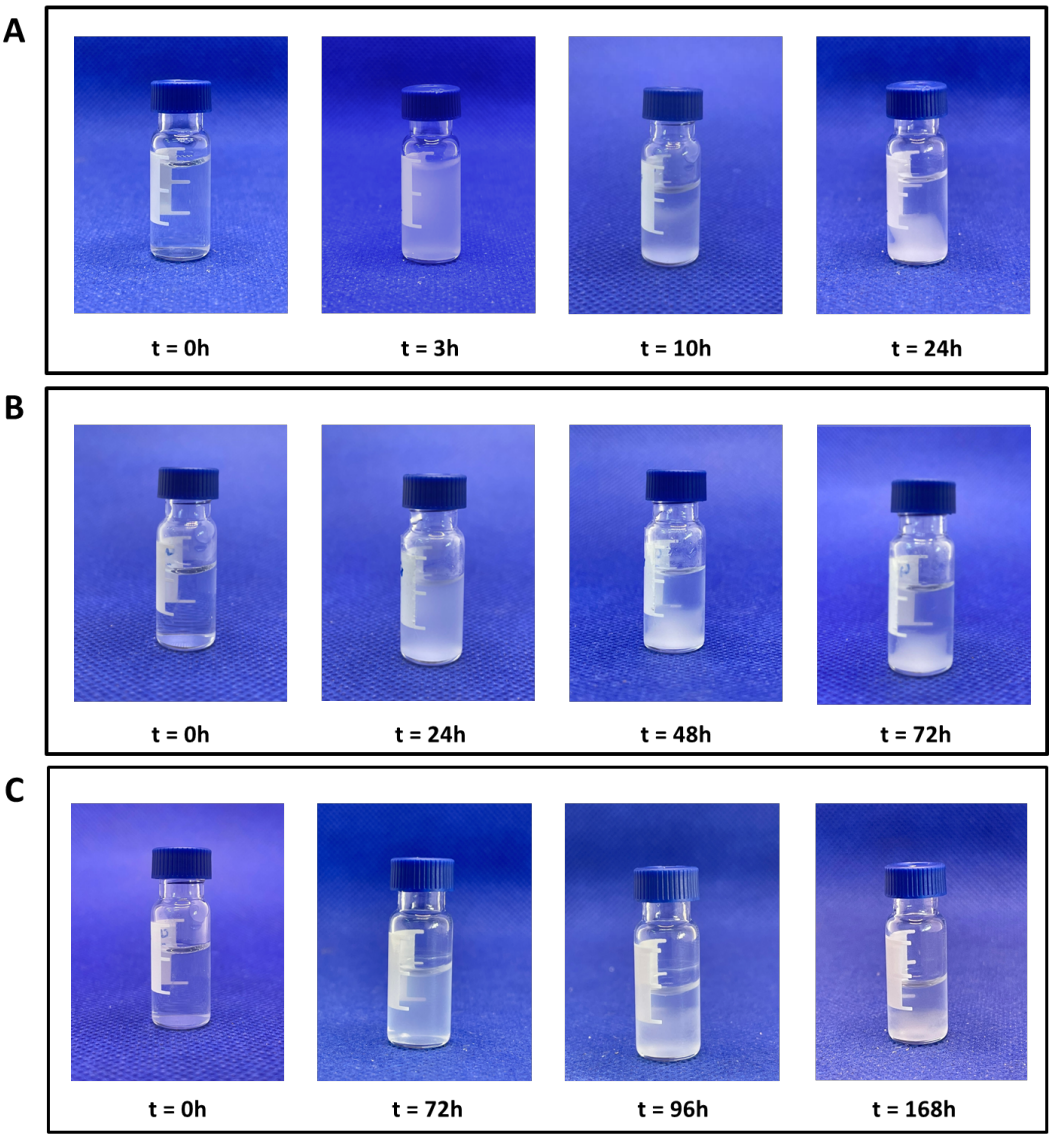


Figure S20. Experimental HPLC vials of A) DBA-Hex/TBA-Oct B) DBA-Hep/TBA-Oct and C) DBA-Oct/TBA-Oct systems at different time-points.

Characterization of the formed hydrogels

Hydrogel composition

The aqueous solution above the hydrogel was removed and the remaining hydrogel was washed 3 times with PBS and then dissolved in acetonitrile and injected into HPLC for analysis of its' molecular composition.

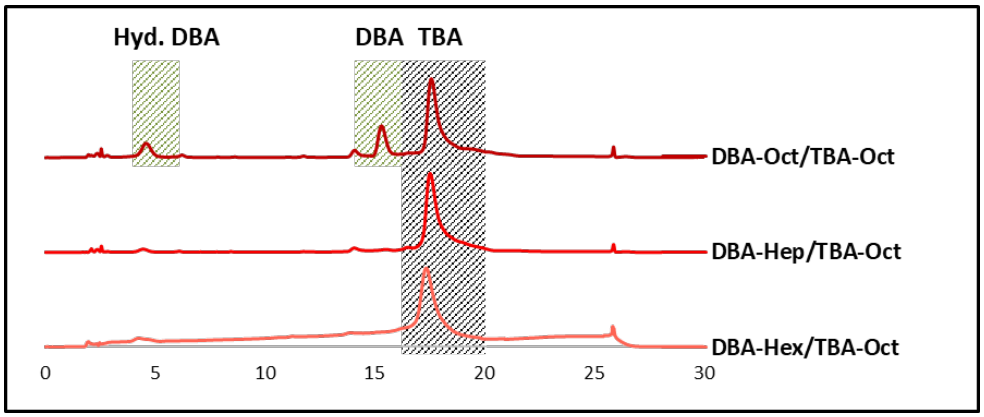


Figure S21. HPLC results of the hydrogels composition, after adding acetonitrile to the gel formed upon the degradation of the DBA in the mixed-micellar systems.

Rheology measurements

Rheological measurements were performed using a controlled- stress rheometer (AR-G2, TA instruments, USA). An 8 mm diameter flat-plate geometry with a rough surface was used for the study. The viscous elastic region was determined by strain sweep from 0.01 to 100% strain at 1Hz frequency at 25°C, with a gap size of 0.9 mm.

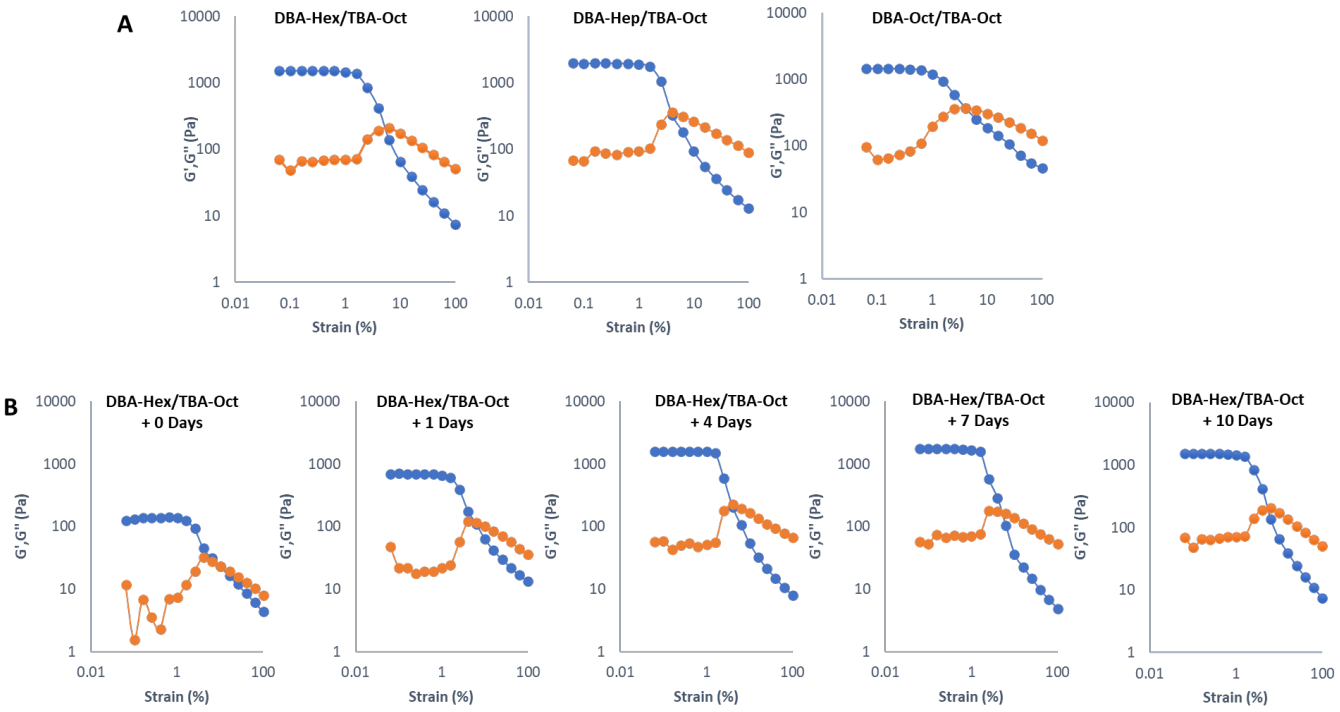


Figure S22. Amplitude sweep tests of the hydrogels obtained from (a) DBA/TBA mixed-micellar systems, after hydrogels' aging period (b) DBA-Hex/TBA-Oct mixed-micellar system, at 0, 1, 4, 7 and 10 days after the hydrogel was settled.
Storage modulus (G') presented in blue lines and loss modulus (G'') in orange lines.

SEM measurements

Hydrogels of three formulation were prepared in situ as described before for the general protocol. After aging period of one week, the hydrogels were lyophilized, gold coated and measured.

All images were taken using a JCM-6000PLUS NeoScope Benchtop scanning electron microscope in high vacuum, 15kV.

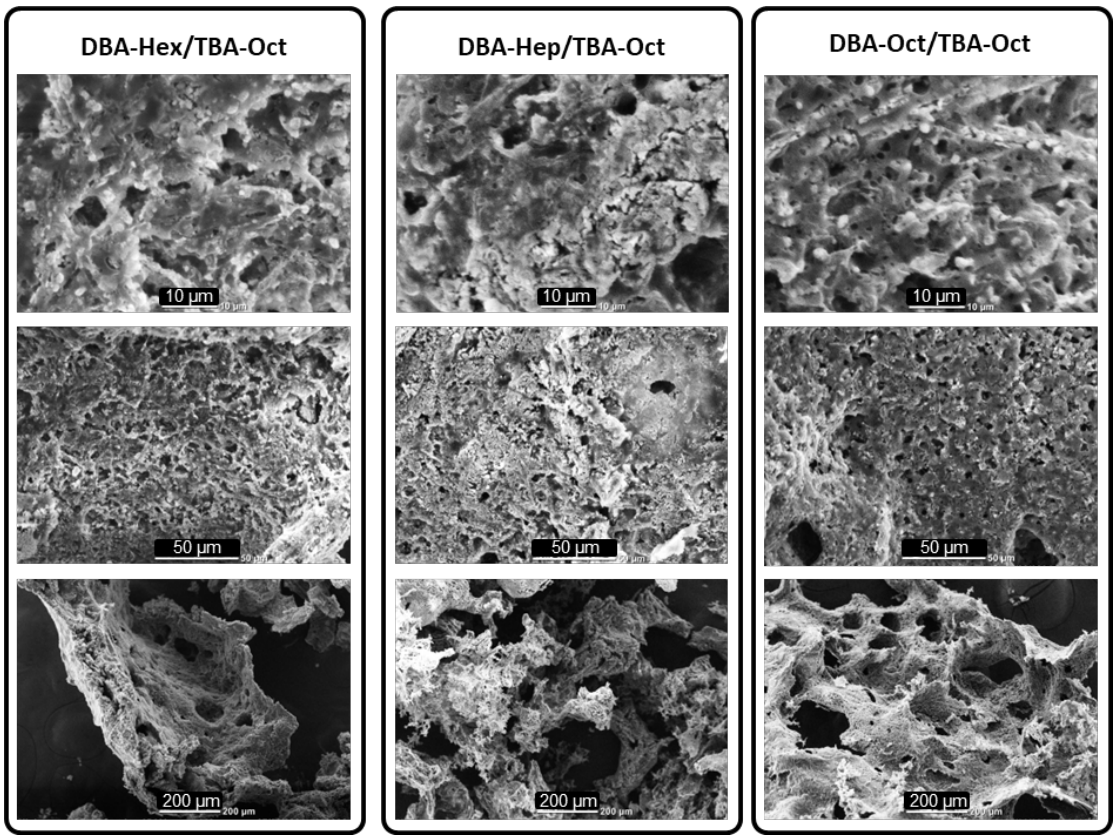


Figure S23. SEM images of the lyophilized hydrogels obtained from DBA/TBA mixed-micellar systems.

Hydrogel degradation

To study the stability of the hydrogel against enzymatic degradation, the hydrogels generated from DBA-Hex/TBA-Oct formulations were incubated for an additional two months under the experimental conditions ([PLE] = 0.7 μM, pH 7.4, 37°C). No visual disassembly was observed during this period. Subsequently, the aqueous supernatant was analyzed by HPLC to assess whether further degradation of the TBA amphiphiles had occurred.

To induce the enzymatic degradation of these hydrogels, BSA (Bovine serum albumin) and an excess of PLE enzyme (20-fold greater than the experimental conditions mentioned earlier) were added. New hydrogels were prepared as described above, and after allowing them to settle, the solution above the hydrogel was removed, and the remaining hydrogel was washed once with PBS. A solution containing 1 mL of 50 mg/mL BSA and 14 μM PLE in PBS was added. The vial was kept at 37°C and monitored over two months. The same protocol was followed for hydrogels containing only BSA or only PLE, aiming to determine if the desired degradation occurred under those conditions as well. After two months, once the hydrogels had visually fully degraded, the aqueous solutions were lyophilized, and BSA and/or PLE were neutralized and precipitated by adding 1 mL of acetonitrile. The diluted sample was then centrifuged and injected into the HPLC for compositional analysis of the degraded gel.

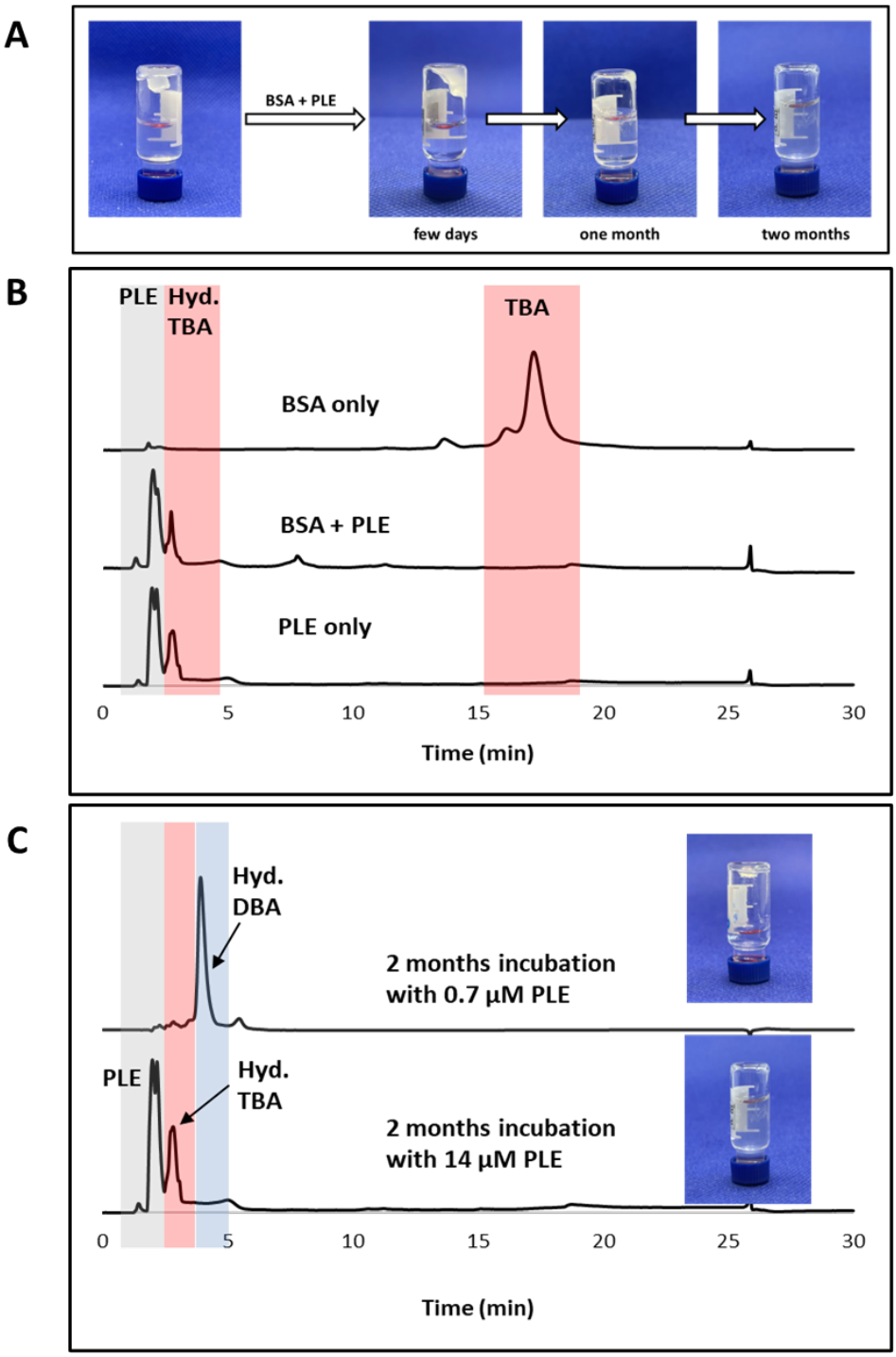


Figure S24. A) Photos of the vial containing hydrogel with BSA and PLE over two months, indicating the hydrogel transformation into soluble hydrolyzed polymers. B) HPLC overlay of the transitioned solutions after two months of incubation with either BSA, excess of PLE, or the combination of both. C) HPLC analysis of the aqueous supernatant upon two months of incubation with 0.7 and 14 µM of PLE.

References

(1) Harnoy, A. J.; Buzhor, M.; Tirosh, E.; Shaharabani, R.; Beck, R.; Amir, R. J. Modular Synthetic Approach for Adjusting the Disassembly Rates of Enzyme-Responsive Polymeric Micelles. *Biomacromolecules* **2017**, *18* (4), 1218–1228.

(2) Edelstein-Pardo, N.; Molco, M.; Rathee, P.; Koren, G.; Tevet, S.; Ziv-Sharabani, S.; Beck, R.; Amir, R. J.; Sitt, A. Anisotropic Microparticles through Periodic Autofragmentation of Amphiphilic Triblock Copolymer Microfibers *Chemistry of Materials* **2022** *34* (14), 6367-6377.