Supplementary Information

Thermoreversible and Injectable ABC Polypeptoid Hydrogels: Controlling the Hydrogel Properties through Molecular Design

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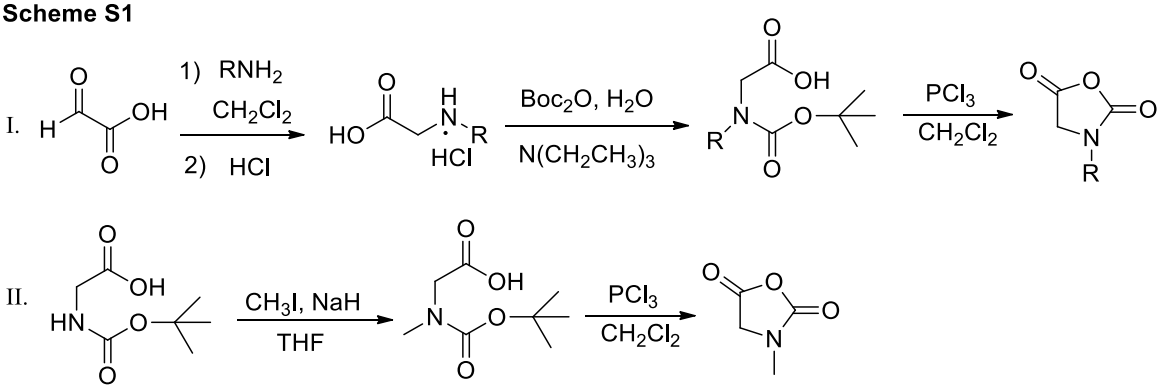
Experimental Section

General considerations. All chemicals used were purchased from Sigma-Aldrich and used as received unless otherwise noted. THF, CH_2Cl_2, and acetonitrile used in polymerization reaction were purified by passing through alumina columns under argon. ^1H and ^{13}C $\{^1H\}$ NMR were recorded on a Bruker AV-400 Nanobay spectrometer, and the chemical shifts in parts per million (ppm) were referenced to protio impurities of $CDCl_3$ and CD_2Cl_2. SEC analyses were performed using an Agilent 1200 system (Agilent 1200 series degasser, isocratic pump, auto sampler and column heater) equipped with three Phenomenex 5 μm, 300 × 7.8 mm columns, a Wyatt OptilabrEX differential refractive index (DRI) detector with a 690 nm light source, and a Wyatt DAWN EOS multiangle light scattering (MALS) detector (GaAs 30mW laser at λ = 690 nm). DMF with 0.1M LiBr was used as the eluent at a flow rate of 0.5 mL min^{-1}. The column and detector temperature was 25 °C. The standard used was twenty three pauci-disperse polystyrene standards (590 g·mol^{-1}-1472 kg·mol^{-1} MW, Polymer Laboratories, Inc.). All data analysis was performed using Wyatt Astra V 5.3 software.

Monomer synthesis. All the monomers were synthesized by adapting reported procedures.[1] *N*-alkyl *N*-carboxyanhydrides (R-NCAs) with allyl, butyl, octyl, decyl, and methoxyethyl, methoxyethoxyethyl side chains were synthesized via synthetic routes (I), whereas Me-NCA was synthesized via route II (Scheme S1).

Scheme S1



Polymer synthesis. All the ABC triblock copolypeptoids were synthesized by primary amine-initiated ring-opening polymerization of the corresponding *N*-substituted *N*-carboxyany-drides (R-NCAs) in a sequential manner. A representative procedure for the synthesis of $A_{98}M_{98}D_{18}$ was presented. In the glovebox, Al-NCA (136.3 mg, 0.97 mmol, $[M_1]_0$=0.4 M) was dissolved in anhydrous acetonitrile. Stock solution of benzyl amine (104 μL, 92.7 mM, $[M_1]$: $[BnNH_2]_0$=100:1) was added. The reaction mixture was heated at 50 °C for 24 h to reach a complete conversion of polymerization. Acetonitrile solution of Me-NCA (2.4 mL, 0.4 M, 0.97 mmol, $[M_1]_0$:$[M_2]_0$: $[BnNH_2]_0$=100:100:1) was added to the above mixture and allowed to stir at room temperature for another 24 h to reach a full conversion. Acetonitrile solution of De-NCA (475 μL, 0.4 M, 0.19 mmol, $[M_1]_0$:$[M_2]_0$:$[M_3]_0$:$[BnNH_2]_0$=100:100:20:1) was added and stirred at room temperature for an additional 24 h. The polymer was precipitated out by the addition of excess THF. The polymer was collected by filtration and washed with ample THF and hexane followed by drying under vacuum (178 mg, 89%). The composition of the polymer was determined by ^1H NMR spectroscopy using end-group analysis. The number average degree of

polymerization (DP_n) of each block was calculated based on the integration ratios of characteristic proton peaks due to individual block. $DP_n(A)=(5 \times$ integration of –CH=)/(1 $\times$ integration of C_6H_5); $DP_n(M)=(5 \times$ integration of $^aCH_3)/(3 \times$ integration of C_6H_5); $DP_n(D)=(5 \times$ integration of $^bCH_3)/(3 \times$ integration of C_6H_5) (*a.* methyl protons of M block, b. methyl protons of D block).

Hydrogel preparation. The 5 wt% aqueous solution of triblock copolypeptoids was prepared by "thin-film hydration", as reported by Zhou *et al*.[19] Briefly, a known amount of dry polymer was dissolved in CH_2Cl_2, followed by evaporation of the solvent under a stream of N_2 overnight to form a thin film on the wall of the vial. The thin film was further dried under vacuum for 1 d. Nanopure water was added, and the solution was stirred at room temperature for 3 d before further characterization. 1 wt% and 2.5 wt% aqueous solutions were prepared by direct dilution of the concentrated solution (5 wt%).

Rheological measurements. Rheological study was conducted using a TA AR 2000ex rheometer. A parallel-plate geometry of 40 mm diameter was used. In each experiment, approximately 0.3 mL of the sample was loaded between the plates with a gap of 200 μm. The metallic plate covers were then put on the plates that were sealed with highly viscous oil. Dynamic storage modulus (G') and loss modulus (G'') were measured by oscillatory shear experiments. Dynamic strain sweep experiments were conducted at a frequency of 10 rad/s at 25 °C and 45 °C to determine the linear viscoelastic regime. Dynamic frequency sweep measurements were conducted within the linear viscoelastic regime with angular frequency from 0.1 to 100 rad/s. Temperature sweep measurements were conducted from 15 °C to 60 °C at a heating rate of 1 °C/min. Below the critical gelation temperature, the solution was measured at a strain of 50 %, and above the gelation temperature at a strain of 0.75 %. The storage moduli (G') show some fluctuation after the sol-gel transition. This could be due to the structural reorganization within the gels.

Dynamic light scattering measurement of dilute solution. An aqueous solution of polymers (0.5 wt% in nanopure water) was filtered through a 0.22 μm filter. All the DLS measurements were conducted using Malvern Zetasizer Nano-zs (Zen3600). The He-Ne laser operating at 633 nm was utilized, and scattered light intensity was detected at an external angle of 173 °C using non-invasive backscatter (NIBS) technology. Data from three measurements with 12 scans for each measurement was recorded. At each temperature, the sample was equilibrated for 3 min.

Temperature dependent ^{1}H NMR experiments of polymer solution. The $A_{92}M_{94}D_{12}$ triblock copolypeptoid was dissolved in CD_2Cl_2 and D_2O at 5 wt% respectively. In CD_2Cl_2, the ^{1}H spectrum was obtained at 25 °C, whereas the spectra were collected at 25, 37 and 60 °C with increasing temperature in D_2O.

TEM/CryoTEM sample preparation and analysis. FEI Vitrobot was used for the sample preparation of cryo-TEM experiment. 5 μL aqueous solution of ABC copolymer (1 wt%) was applied to a 300 mesh lacey carbon coated TEM grid. Double side blotting to the grid for 2 seconds leaves a thin film on the grid. The grid then was quickly plunged into liquid ethane chilled by liquid nitrogen. The vitrified sample grid

was loaded in a single tilt liquid nitrogen cryo transfer holder, and was then inserted to FEI G2 F30 Tecnal TEM operated at 120keV, with a FEI digital camera and analyzed using FEI Digital Micrograph software. The grids for the regular TEM was prepared by adding 5 µL polymer aqueous solution (0.2 wt%) onto the 300 mesh carbon grid followed by blotting with a filter paper and drying at room temperature. The grids then were stained with uranyl acetate for 1 min.

Cryo-SEM sample preparation and analysis. Aqueous Polymer solution of 5 wt% $A_{92}M_{94}D_{12}$ (~40 µL)was pipetted on top of a metal rivet and was heated up by a hot plate for gel formation, indicated by the color change of the liquid drop from clear to white. The gel sample was then plunged into liquid nitrogen, followed by fracturing at -130 °C using a flat-edge cold knife. The solvent was sublimated at -95 °C for 5 min and the sample was sputtered with a platinum-palladium composite at 10 mA for 88 seconds before imaging. The vitrified sample was imaged on a Hitachi S-4800 field emission scanning electron microscope using cryo-mode operated at a voltage of 3 kV.

Cloud point measurement of ABC triblock copolypeptoid solutions. Cloud point measurements of aqueous solutions of both poly(*N*-allyl glycine) homopolymer (A) and $A_{98}M_{98}D_{18}$ triblock copolymers (1 wt%) were conducted using Varian Cary 50 Bio-UV-vis spectrophotometer equipped with a Thermo/Neslab RTE-7 refrigerated bath circulator for temperature control. The experimental temperature range is 15-60 °C and all UV-vis absorptions were referenced against distilled water. The temperature at 50 % UV-vis transmittance (λ = 450nm) is defined as the cloud point.

Protein encapsulation study. 10 µL of 0.14 mg/mL HRP in PBS buffer was added to 100 µL of aqueous polymer solution (5 wt%) and well mixed. The mixture was incubated at 37 °C in an oil bath for different time (1 h, 4 h, 7 h, 24 h, and 7 d). After each time period, the mixture was cooled down to room temperature and diluted with PBS buffer using 1.0 ml volumetric flask, which was further used for the kinetic study. To a disposable cuvette, 3 ml of PBS buffer, 20 µL of 0.1 M Guaiacol in PBS buffer and 40 µL of the above diluted solution was added. 20 µL of 0.1 M H_2O_2 in PBS buffer was quickly injected to the above mixture and well mixed. The cuvette was immediately monitored at room temperature every 40 seconds by a Varian Cary 50 Bio-UV-Vis spectrophotometer at a wavelength range of 200-600 nm. All UV-Vis absorptions were referenced against PBS buffer. For comparison, the enzymatic activity of two sets of controls was measured using the same method described above. One control involves incubation of HRP in PBS buffer at 37 °C for different duration (1, 4, 7, 24 h and 7 d). The other control is the as-received HRP whose enzymatic activity was directly measured in PBS at 25 °C.

Adult stem cells isolation and culture. Liposuction aspirates from subcutaneous adipose tissue were obtained from three healthy adult subjects (male = 1 and females = 2) undergoing elective procedures. All tissues were obtained with informed consent under a clinical protocol reviewed and approved by the Institutional Review Board at the LSU Pennington Biomedical Research Center and used under an exempted protocol at LSU A&M College. Isolation of hASC was performed as published.[2] Passage 2 of each individual was used for *in vitro* hASC evaluation on tissue culture treated plastic or on scaffold of hydrogel. In both cases, hASC were cultured in stromal (control - DMEM, 10 % FBS, and 1 % triple antibiotic solution) media

with media maintenance performed three times a week. For the live control, stem cells were cultured in plain media. For the dead control, stem cells were cultured in media and then introduced to 70 % ethanol.

Extract cytotoxicity Assessment. The extract cytotoxicity study was modified based on FDA protocol.[3] The hydrogel samples (0.1g) were incubated on an orbital shaker with 5 mL stromal medium at 37 °C and 200rpm/min for 7 days. The extractives were filtered (0.22 µm pore size) and pipetted (100 µL/well) into a 96-well plate previously sub-cultured with hASC (2,500 cells/well) and incubated in a CO_2 incubator at 37 °C containing 5 % CO_2 for 24 hours. 10 µL alamarBlue® reagent to each well and re-incubated at 37 °C in 5 % CO_2 for 2 h. The fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 595 nm using a fluorescence plate reader. The tissue culture treated plastic 96-well plate served as a control substrate.

hASC loading on ABC hydrogel and culture. The ABC hydrogel was filtered through a 0.22 µm filter at 20 °C and 100 µL aliquots was mixed with 1×10^4 cells/µL for total volume of 10 µL. The cells and hydrogel mixture was transferred to a 96 well plate afterwards. 100 µL stromal medium was added to each well after the cells/hydrogel mixture was solidified at 37 °C.

***In vitro* hASCs viability on scaffolds with alamarBlue® stain**. The viability of cells within ABC hydrogel in stromal media was determined after 3 days using an alamarBlue® metabolic activity assay. The cells/hydrogel mixture were removed from culture, washed three times in PBS, and incubated with 10 % alamarBlue® in Hank's balanced salt solution (HBSS) without phenol red (pH 7) for 90 min. Aliquots (100 µL) of alamarBlue®/HBSS were placed in a 96-well plate in triplicate, and the fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 595 nm using a fluorescence plate reader.

***In vitro* quantification of DNA on scaffolds**. Total DNA content was used to determine the number of cells on each scaffold as previously described.[4] After the scaffolds were minced by a scalpel and the DNA was digested with 0.5 mL 0.5 mg/mL proteinase K (Sigma-Aldrich) at 56 °C overnight, aliquots (50 µL) were mixed with equal volumes of 0.1 g/mL PicoGreen dye solution (Invitrogen) in 96-well plates. Samples were then excited at 480 nm with a plate reader (Wallac 1420 multilabel hts counter). Scaffolds without cells were used as negative controls.

In vitro hASCs Viability on polymer solution with alamarBlue® Stain.The copolymer was dissolved in stem cell media (40 % v/v). The solution was used as a stock solution to dilute and receive concentration values of 20 %, 10 %, 5 %, 2 %, and 1 % copolymer in media. Stem cells were seeded in a 96-well plate at a density of 40,000 cells per well and incubated for 24 h at 37 °C. Culture media (95% Dulbecco's Modified Eagle Medium, 4.5 % fetal bovine serum, 0.5 % penicillin, streptomycin, and amphotericin) was subsequently replaced with serum-free media. Polypeptoids were then added at 40, 20, 10, 5, 2, and 1 % (v/v) per well. Cells were incubated for 4 h before the medium was replaced with serum-containing media. Cell viability was assessed using the standard alamarBlue® cell viability assay 24 h later. Following 4 h of alamarBlue uptake, fluorescence was collected at an excitation wavelength of 530 nm and an emission wavelength of 595 nm using fluorescence plate reader.

Quantitative real-time polymerase chain reaction (QPCR). Quantitative real-time polymerase chain reaction (QPCR) was utilized to examine the effects of the hydrogel on human adult stem cell (hASC) differentiation. hASC were cultured in 100 µL of hydrogel (5 wt% of $A_{92}M_{94}D_{12}$ in medium) and stromal media (Dulbecco's minimal essential medium (DMEM), 10 % fetal bovine serum (FBS), and 1 % antibiotic solution (penicillin, streptomycin, fungizone)) at 37 °C and 5 % CO_2 in a humidified atmosphere. Following incubation, RNA was extracted with TRI Reagent® (Sigma) according to the manufacturer's instructions. The isolated RNA was then used to perform QPCR with iScript™ One-Step RT-PCR Kit with SYBR®Green (Bio-Rad) using a MiniOpticon™ Real-Time PCR Detection System (Bio-Rad). Expression of the genes *Col2a1* and *ANGPT1* was used to quantify the hydrogel's ability to induce chondrogenic and angiogenic differentiation, respectively. QPCR samples were normalized against the house keeping gene *18s rRNA* and relative to separate hASC cultures maintained in chondrogenic growth medium (StemPro® Chondrogenesis Differentiation Kit; Life Technologies) and endothelial growth medium (EGM™-2 BulletKit™; Lonza) with SingleQuots™ (Lonza) containing vascular endothelial growth factor (VEGF), human basic fibroblast growth factor (hFGF-b), epidermal growth factor, insulin-like growth factor-1, heparin, ascorbic acid, and 5 % FBS.

Statistical analysis. The results of alamarBlue, PicoGreen and QPCR analysis were reported as mean ± standard deviation. Data was evaluated with one or two way analysis of variance (ANOVA), and analyzed by Tukey's minimum significant difference (MSD) post hoc test for pairwise comparisons of main effects. A *P*-value<0.05 was considered significant for all comparisons.[5]

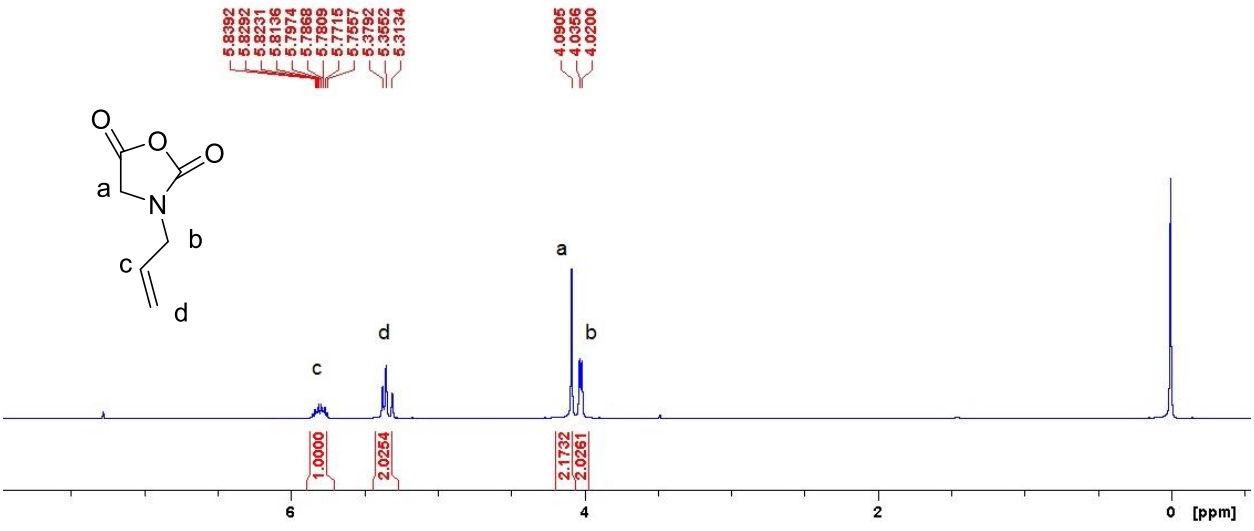


Figure S1. ^{1}H NMR spectrum of allyl-NCA in $CDCl_3$.

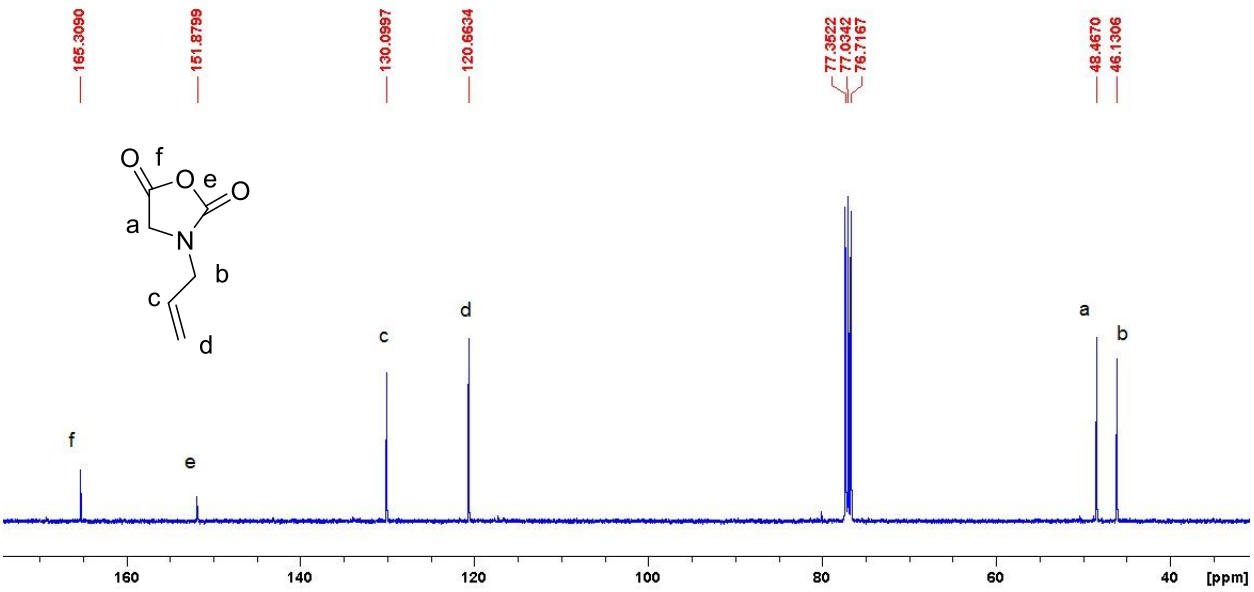


Figure S2. $^{13}C\{^{1}H\}$ NMR spectrum of allyl-NCA in $CDCl_3$.

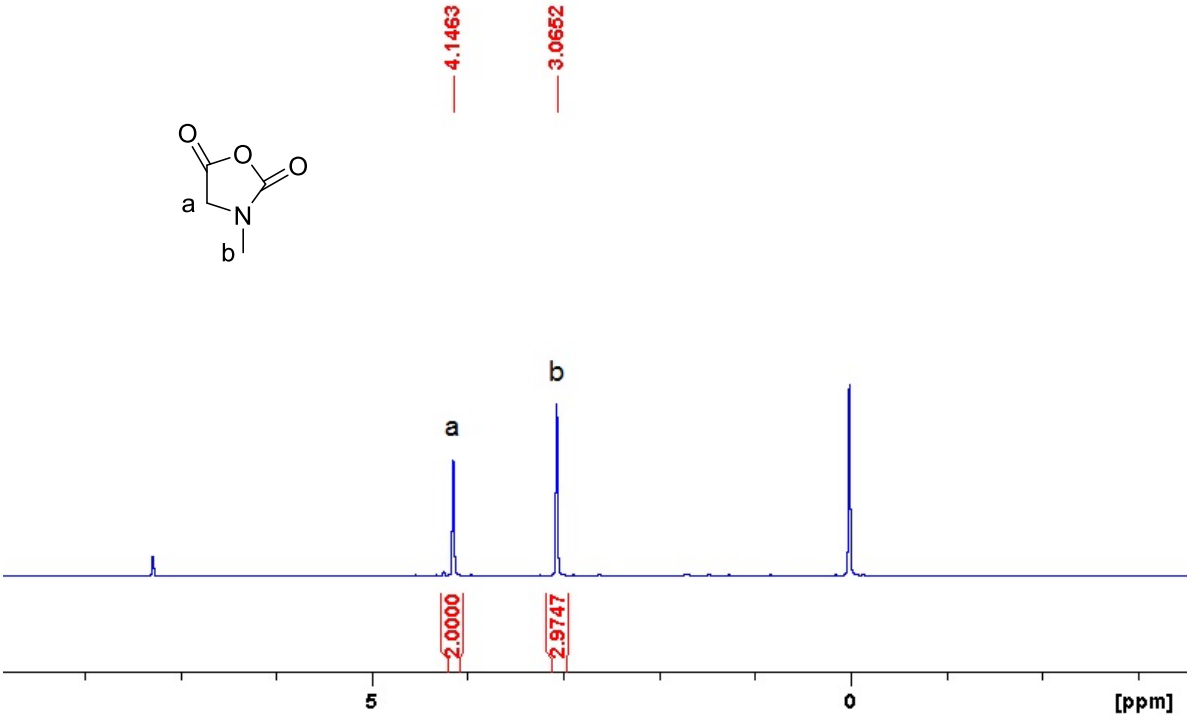


Figure S3. ^{1}H NMR spectrum of Methyl-NCA in $CDCl_3$.

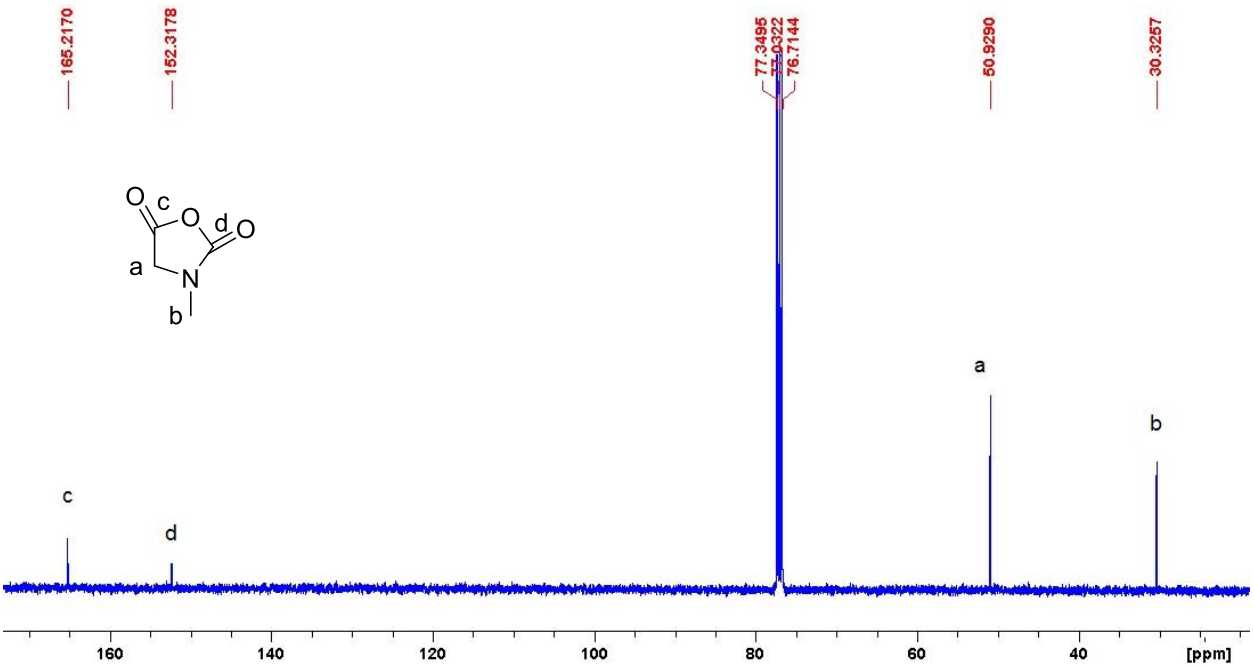


Figure S4. $^{13}C\{^{1}H\}$ NMR spectrum of Methyl-NCA in $CDCl_3$.

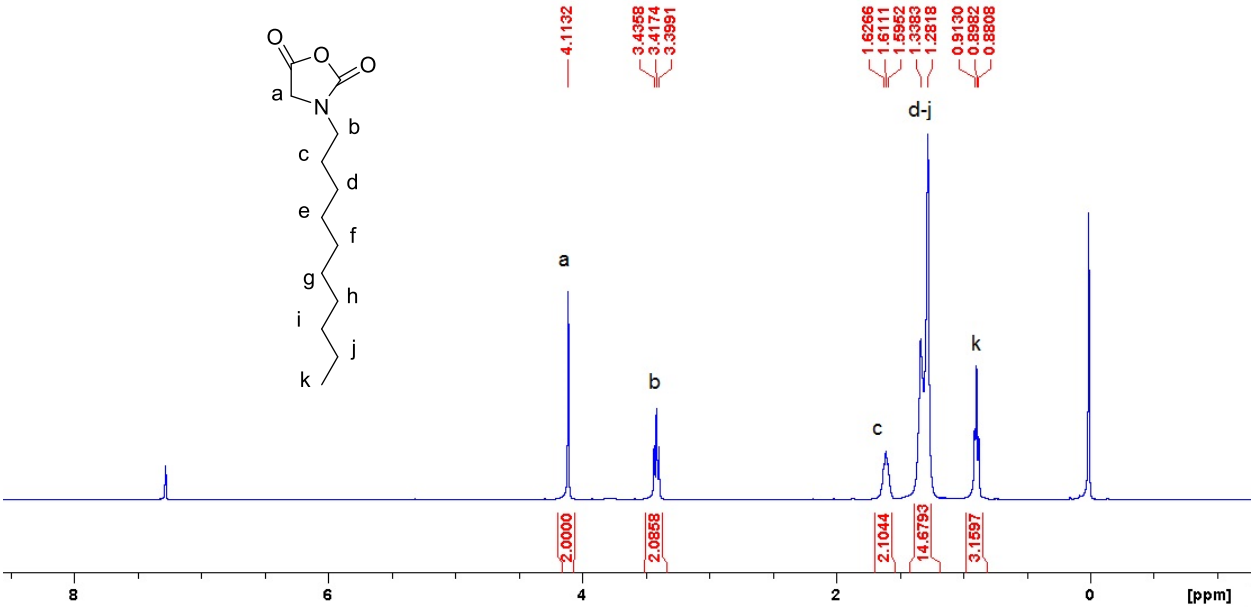


Figure S5. ^{1}H NMR spectrum of Decyl-NCA in $CDCl_3$.

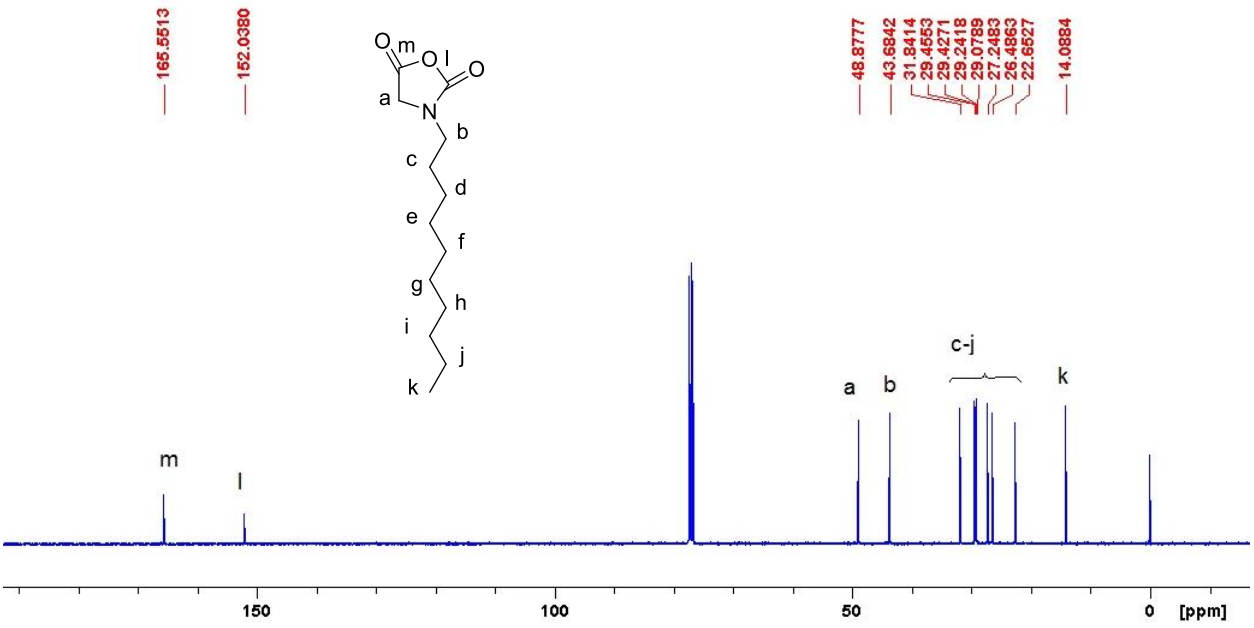


Figure S6. $^{13}C\{^{1}H\}$ NMR spectrum of Decyl-NCA in $CDCl_3$.

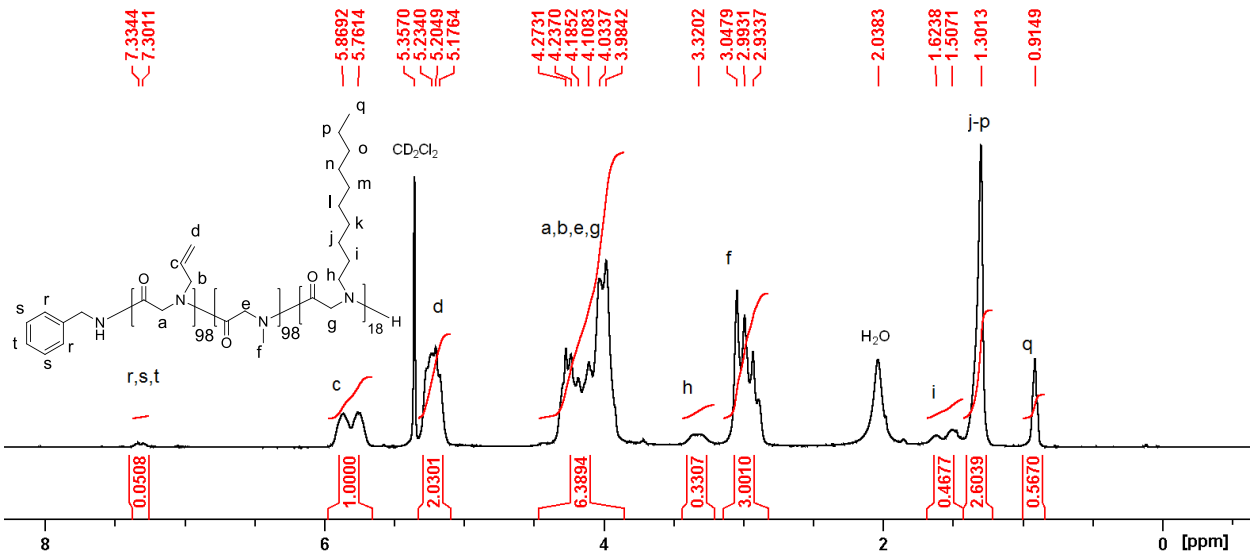


Figure S7. ^{1}H NMR spectrum of the $A_{98}M_{98}D_{18}$ triblock copolypeptoid (Entry 1, Table 1) in CD_2Cl_2.

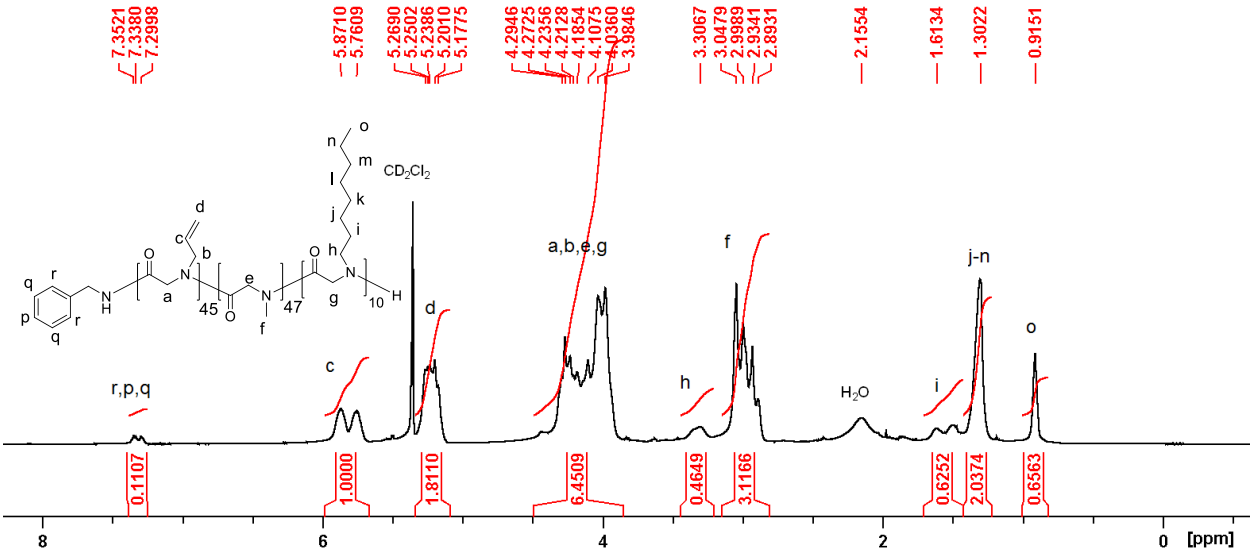


Figure S8. ^{1}H NMR spectrum of the $A_{45}M_{47}O_{10}$ triblock copolypeptoid (Entry 7, Table 1) in CD_2Cl_2

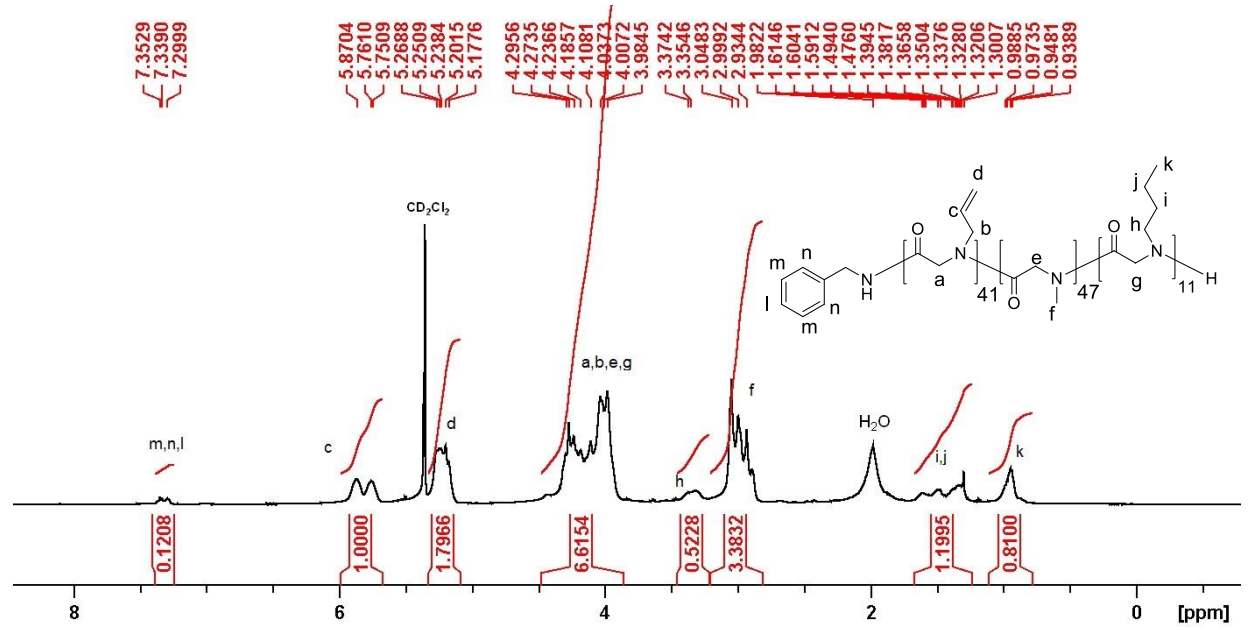


Figure S9. ^{1}H NMR spectrum of the $A_{41}M_{47}B_{11}$ triblock copolypeptoid (Entry 8, Table 1) in CD_2Cl_2.

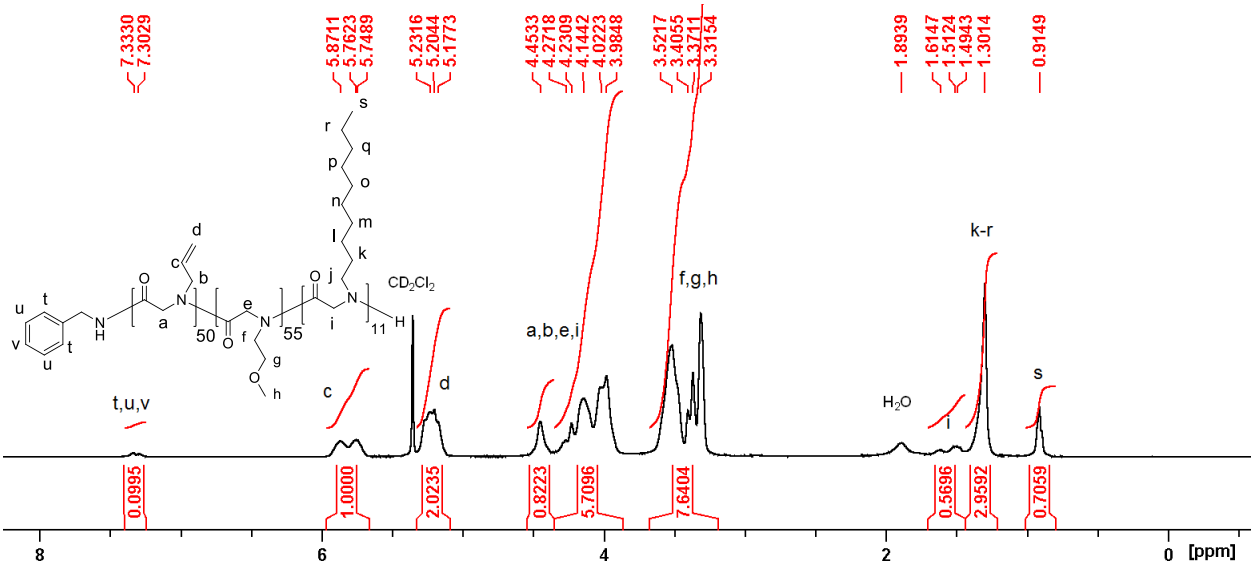


Figure S10. ^{1}H NMR spectrum of the $A_{50}m_{55}D_{11}$ triblock copolypeptoid (Entry 9, Table 1) in CD_2Cl_2.

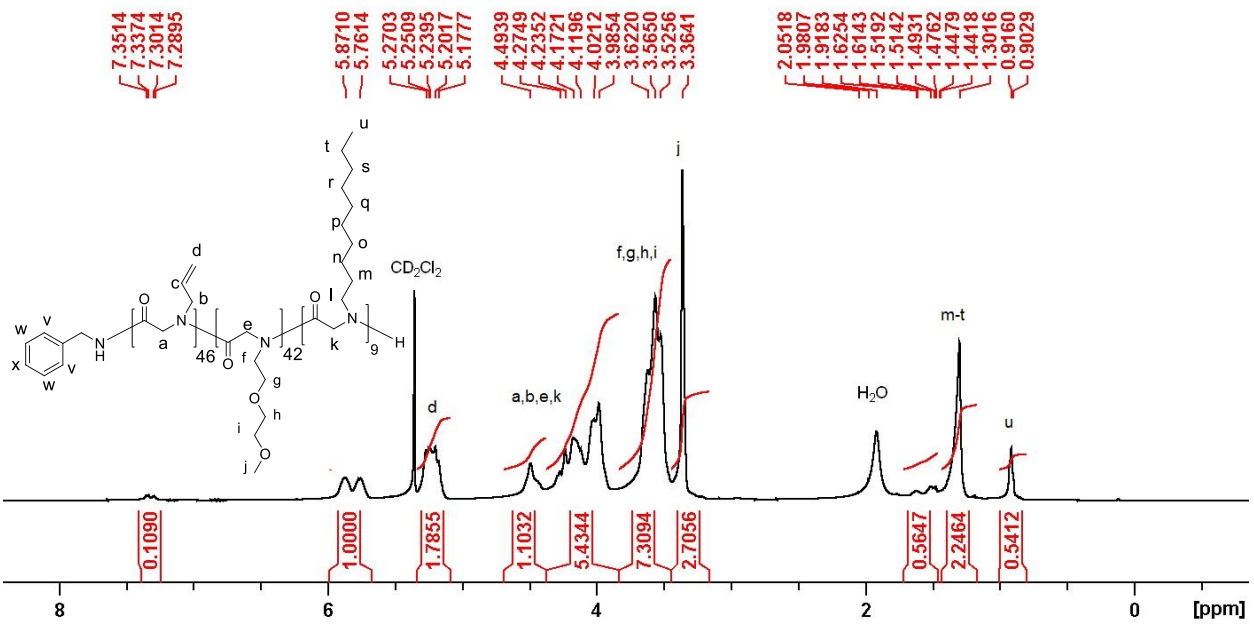


Figure S11. ^{1}H NMR spectrum of the $A_{46}d_{42}D_{9}$ triblock copolypeptoid (Entry 10, Table 1) in CD_2Cl_2.

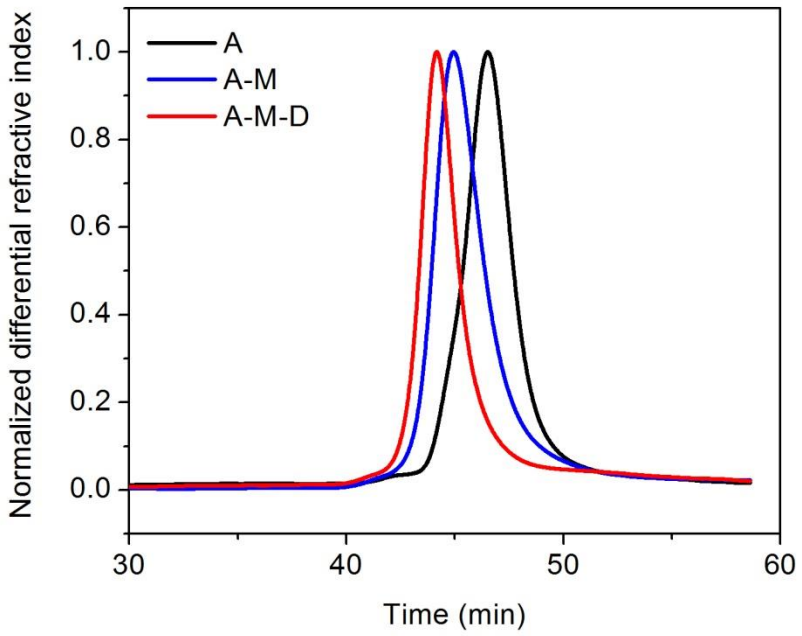


Figure S12. Representative SEC chromatograms showing the successful enchainment for the synthesis of the ABC triblock copolypeptoid ($A_{98}M_{98}D_{18}$, Entry 1, Table 1).

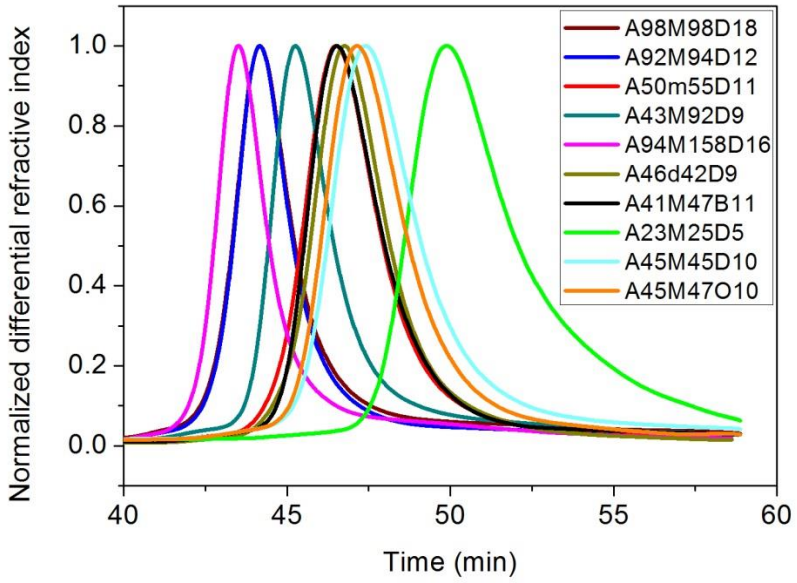


Figure S13. Representative SEC chromatograms of all ABC triblock copolypeptoids (Entry 1-10, Table 1).

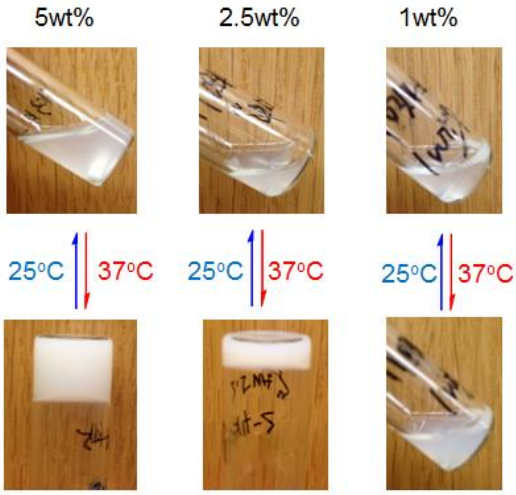
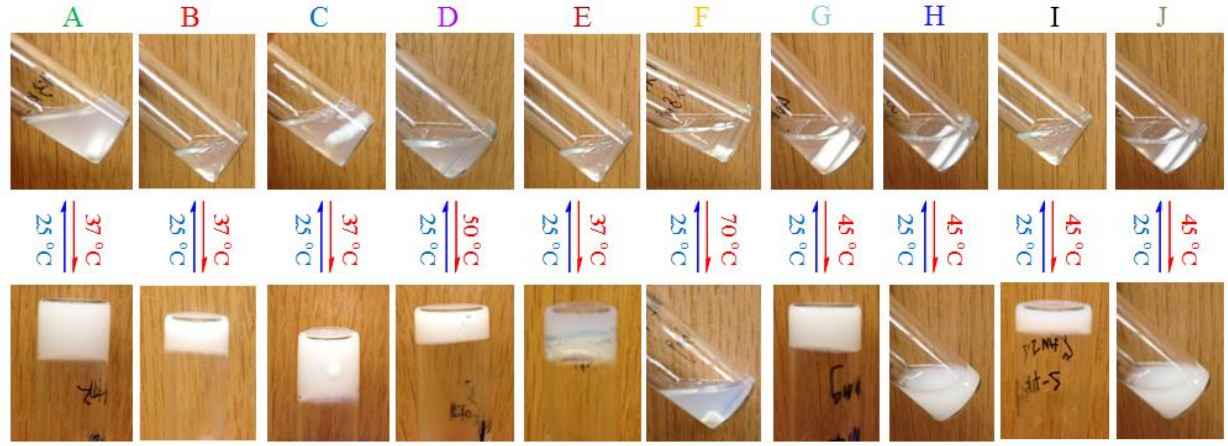


Figure S14. Optical images showing the thermoreversible gelation of $A_{98}M_{98}$ D_{18} at different concentration in DI water.



A. $A_{98}M_{98}D_{18}$. B. $A_{92}M_{94}D_{12}$. C. $A_{94}M_{158}D_{16}$. D. $A_{43}M_{92}D_{9}$. E. $A_{45}M_{45}D_{10}$.
F. $A_{23}M_{25}D_{5}$. G. $A_{45}M_{47}O_{10}$. H. $A_{41}M_{47}B_{11}$. I. $A_{50}m_{55}D_{11}$. J. $A_{46}d_{42}D_{9}$

Figure S15. Optical images showing the thermoreversible gelation of ABC triblock copolymers at 5 wt% in DI water.

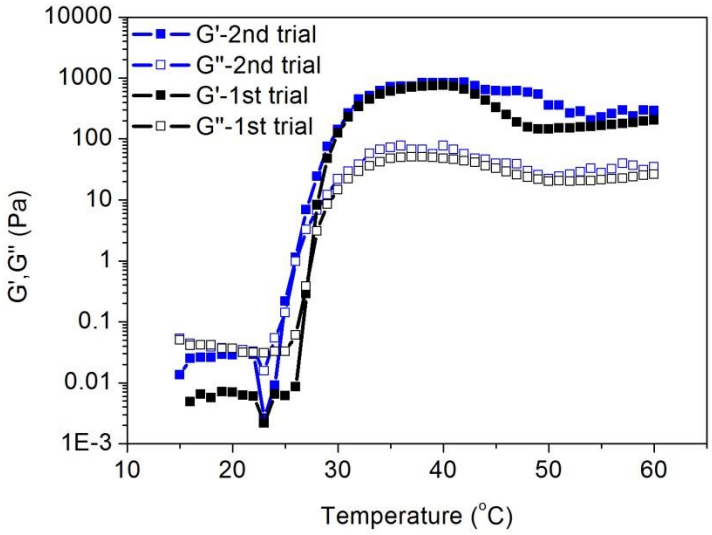


Figure S16. Plots of storage (G', filled symbols) and loss moduli (G", open symbols) versus temperature for the $A_{92}M_{94}D_{12}$ (5 wt%): 1st trial (G', ■ ; G", □) and 2nd trial (G', ■ ; G", □).

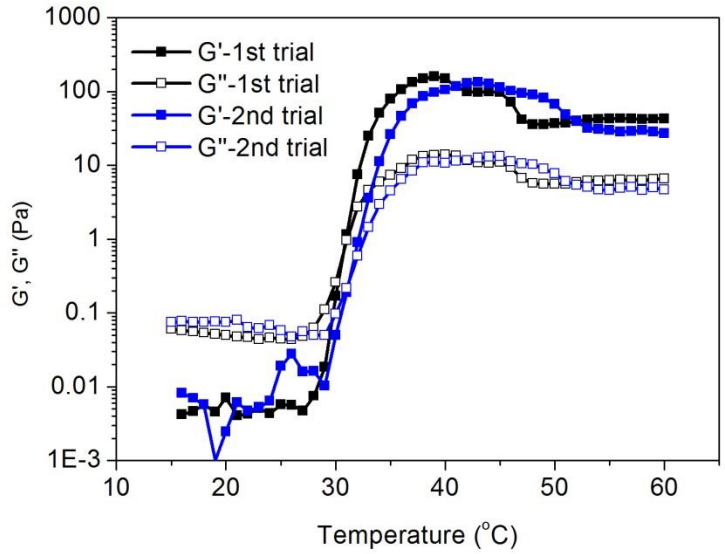


Figure S17. Plots of storage (G', filled symbols) and loss moduli (G", open symbols) versus temperature for the $A_{45}M_{45}D_{10}$ (5 wt%): 1st trial (G', ■ ; G", □) and 2nd trial (G', ■ ; G", □).

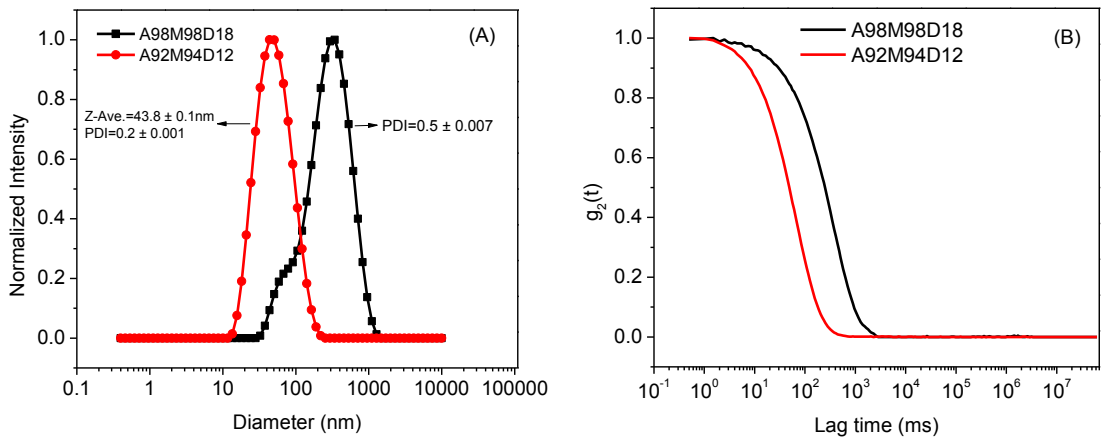


Figure S18. (A) Hydrodynamic size distribution of the $A_{92}M_{94}D_{12}$ and $A_{98}M_{98}D_{18}$ micelles (0.5 wt% in nanopure water) at room temperature (below the T_{gel}) and (B) the corresponding correlograms.

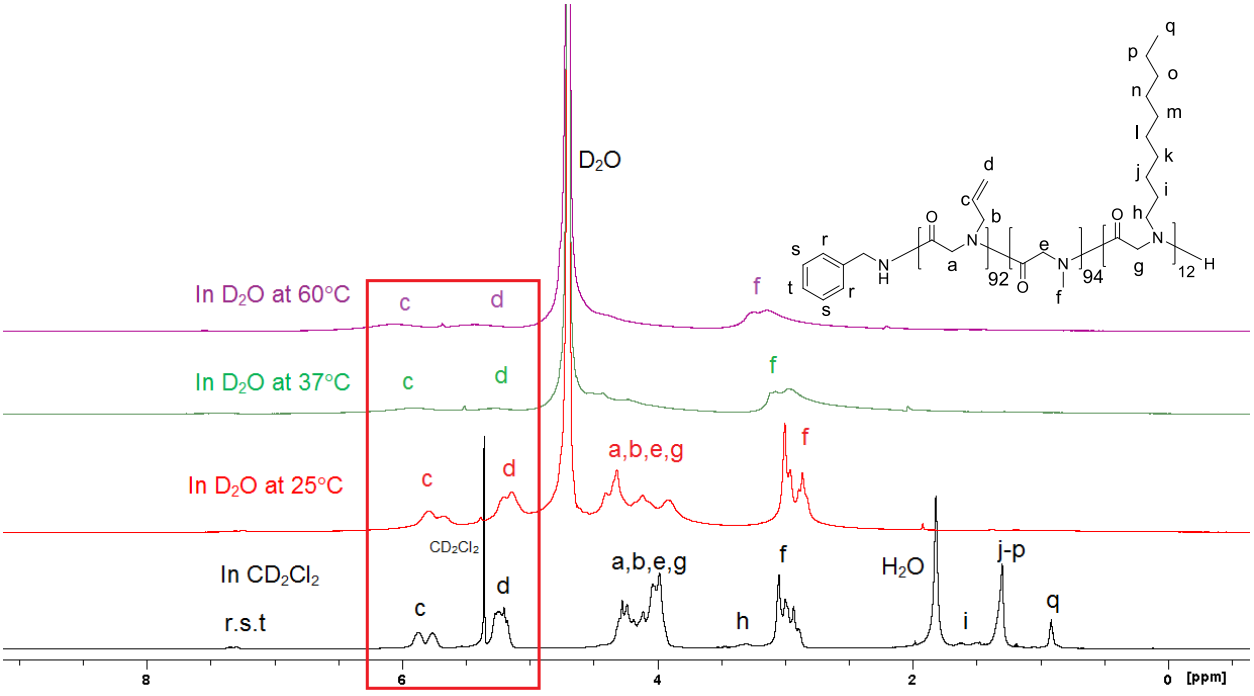


Figure S19. ^{1}H NMR spectra of $A_{92}M_{94}D_{12}$ in solvent CD_2Cl_2 and D_2O at different temperature (from below to above the T_{gel}).

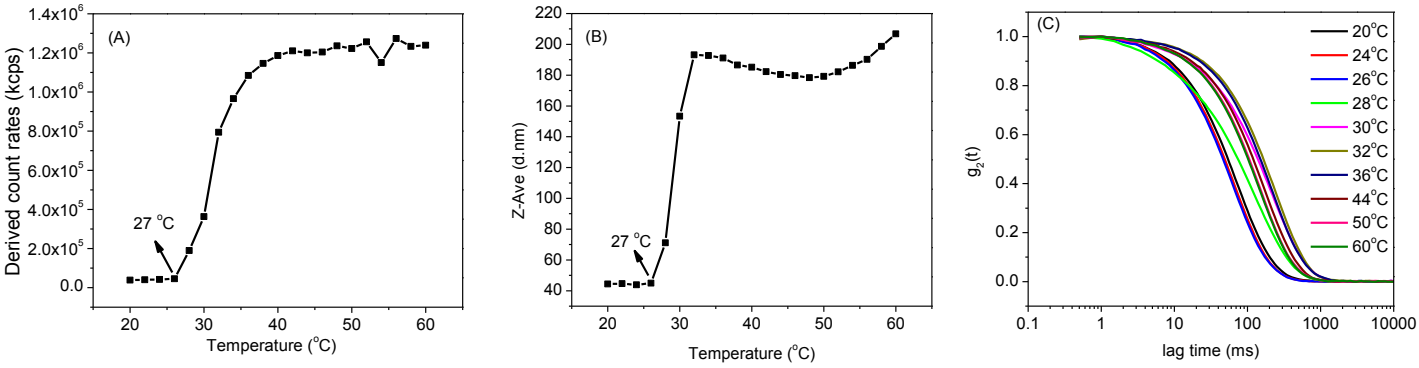


Figure S20. (A) Plots of derived count rate and (B) Z-averaged diameter versus temperature for the $A_{92}M_{94}D_{12}$ micellar solution (0.5 wt% in water) together with (C) the corresponding correlograms.

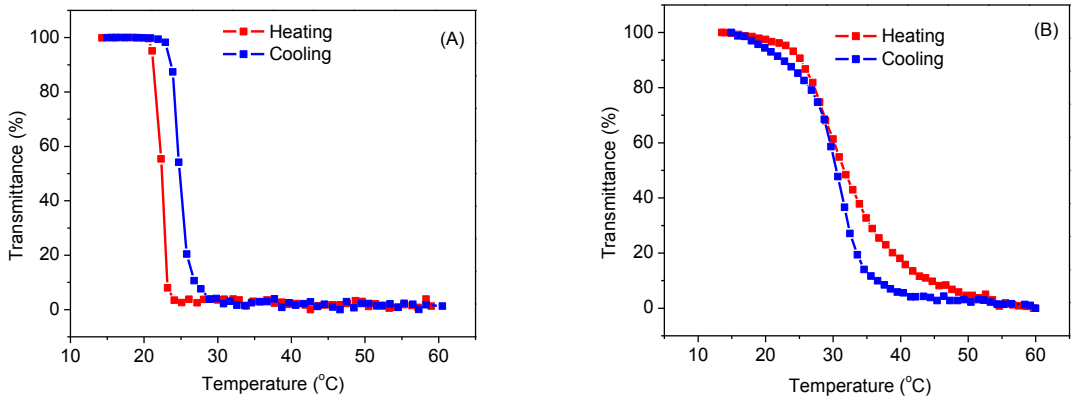
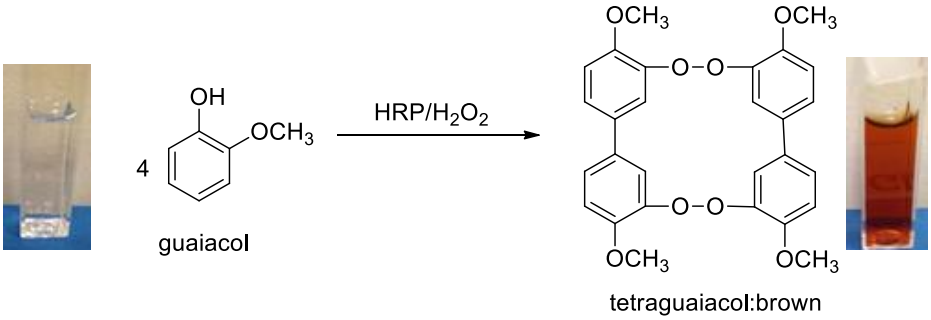


Figure S21. (A) Plots of transmittance at λ = 450 nm versus temperature for the aqueous solutions of homopolymer A_{105} and (B) the $A_{98}M_{98}D_{18}$ triblock copolypeptoid (both samples: 1 wt% in DI water).

Scheme S2. Catalytic Reaction of Guaiacol with HRP and H_2O_2.



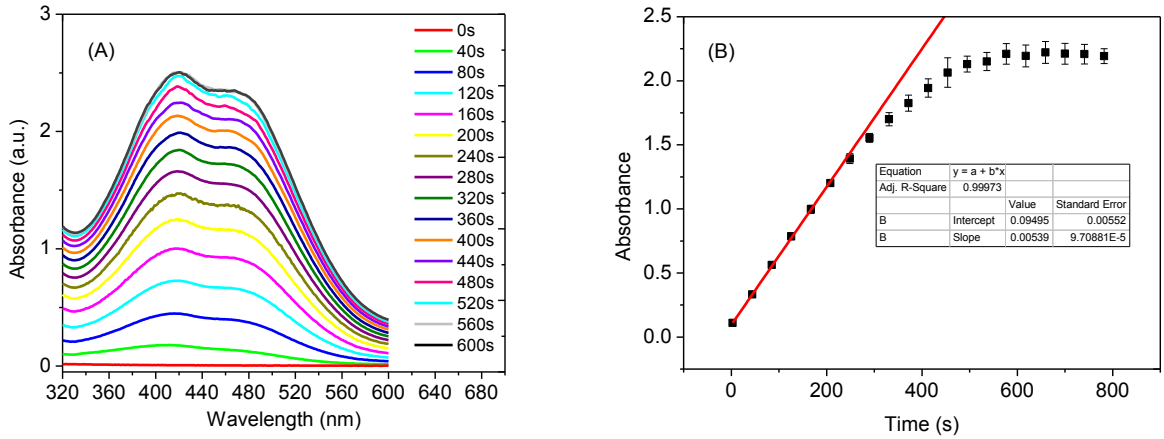


Figure S22. (A) Representative UV-Vis spectra of the enzymatic reaction involving Guaiacol, HRP and H_2O_2 showing the absorbance increase over time after incubation of HRP in the $A_{92}M_{94}D_{12}$ hydrogel for 24 h; (B) plot of absorbance at λ = 470nm versus time.

Table S1. Systematic study of HRP enzyme activity upon incubation in hydrogel for different time.

Enzyme	Entry	dA/dt (s^{-1}) [d]	Enzyme Activity (umol/min) [e]	Average Enzyme Activity (umol/min) [f]	Specific Enzyme Activity (umol/min/mg) [g]
1h-in gel[a]	1	0.0054	0.187	0.185±0.002	329.71±3.6
	2	0.0053	0.183		
	3	0.0053	0.183		
1h-no gel[b]	1	0.0054	0.187	0.185±0.004	329.71±7.1
	2	0.0054	0.187		
	3	0.0052	0.180		
4h-in gel[a]	1	0.0055	0.190	0.186±0.004	331.7±7.1
	2	0.0053	0.183		
	3	0.0053	0.183		
4h-no gel[b]	1	0.0053	0.183	0.182±0.002	325.5±3.6
	2	0.0053	0.183		
	3	0.0052	0.180		
7h-in gel[a]	1	0.0055	0.190	0.188±0.002	335.9±3.6
	2	0.0054	0.187		
	3	0.0054	0.187		
7h-no gel[b]	1	0.0054	0.187	0.183±0.003	327.6±6.2
	2	0.0053	0.1837		
	3	0.0052	0.180		
24h-in gel[a]	1	0.0054	0.187	0.186±0.005	331.7±9.4
	2	0.0055	0.190		
	3	0.0052	0.180		
24h-no gel[b]	1	0.0054	0.187	0.187±0.003	333.8±6.2
	2	0.0053	0.183		
	3	0.0055	0.190		
7d-in gel[a]	1	0.0047	0.166	0.167±0.002	298.8±3.6
	2	0.0050	0.173		
	3	0.0048	0.163		
7d-no gel[b]	1	0.0048	0.166	0.167±0.005	298.8±9.4
	2	0.0049	0.170		
	3	0.0048	0.166		
control[c]	1	0.0053	0.183	0.185±0.002	329.7±3.6
	2	0.0054	0.187		
	3	0.0053	0.183		

[a.] The enzyme is incubated in hydrogel at 37 ^{o}C; [b.] the enzyme is incubated in water at 37 ^{o}C; [c.] the enzyme is used as received without any further treatment; [d.] the slope of the

plot (absorbance at 470 nm versus time); [e.] enzyme activity (μmol/min) = $\frac{dA}{dt} \times \frac{V(L)}{\varepsilon b} \times 60$ (ε = 5200 $M^{-1} \cdot cm^{-1}$, b = 1 cm); [f.] enzyme activity by taking the average of three measurements; [g.] specific enzyme activity (μmol/min/mg) = enzyme activity/mass of enzyme used.

References and Notes

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