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Design and synthesis of non-ionic copolypeptide hydrogels with reversible thermoresponsive and tunable physical properties

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Abstract

Polypeptide based formulations that undergo liquid to hydrogel transitions upon change in temperature have become desirable targets since they can be mixed with cells or injected into tissues as liquids, and subsequently transform into rigid scaffolds or depots. Such materials have been challenging to prepare using synthetic polypeptides, especially when reversible gelation and tunable physical properties are desired. Here, we designed and prepared new non-ionic diblock copolypeptide hydrogels (DCH) containing hydrophilic poly(γ-[2-(2-methoxyethoxy)ethyl]-*rac*-glutamate) and hydrophobic poly(L-leucine) segments named DCH_{EO}, and also further incorporated copolypeptide domains into DCH_{EO} to yield unprecedented thermoresponsive DCH, named DCH_T. Although previous attempts to prepare non-ionic hydrogels composed solely of synthetic polypeptides have been unsuccessful, our designs yielded materials with highly reversible thermal transitions and tunable properties. Non-ionic, thermoresponsive DCH_T were found to support the viability of suspended mesenchymal stem cells *in vitro*, and were able to dissolve and provide prolonged release of both hydrophilic and hydrophobic molecules. The versatility of these materials was further demonstrated by the independent molecular tuning of DCH_T liquid viscosity at room temperature, DCH_T hydrogel stiffness at elevated temperature, as well as the DCH_T liquid to hydrogel transition temperature itself.

Key words: hydrogel, polypeptide, block copolymer, thermoresponsive

Introduction

Synthetic hydrogels have received much attention in recent years due to their inherent similarities to biological materials such as extracellular matrix.[1,2] In order to successfully mimic the properties of complex natural materials, and for optimization of properties for potential applications in regenerative medicine and therapeutic delivery, it is desirable that synthetic hydrogels replicate both the structural properties and chemical functionality found in natural hydrogels. These features may include stimulus responsive mechanical properties as well as the incorporation of bioactive and biodegradable components.[3] Polypeptides are excellent candidates for synthesis of biomimetic hydrogels since they are enzymatically degradable, can include a wide range of chemical functionality, and they adopt ordered conformations that can drive structure formation and also respond to stimuli.[4] In particular, polypeptide formulations that undergo liquid to hydrogel transitions upon change in temperature have become desirable targets since they can be mixed with cells or injected into tissues as liquids, and subsequently transform into rigid scaffolds or depots.

Previous examples of thermoresponsive hydrogels based solely on polypeptides have all been created from protein polymers produced biosynthetically in microorganisms. In 1998, Tirrell reported protein based triblock copolymers that formed hydrogels via coiled-coil assembly of terminal α-helical domains, where these domains could be reversibly disassembled to yield liquids at elevated temperatures.[5] Triblock protein copolymers were also prepared using elastin mimetic domains designed so that the central domain is water-soluble and the terminal domains have solubility that changes with temperature.[6,7] Due to the thermoresponsive behavior of the terminal domains, these materials undergo reversible liquid to hydrogel transitions as temperature is increased. Gelation in both designs occurs mainly due to temperature dependent interchain assembly driven primarily via hydrophobic interactions, which allows good reversibility. Replication of these properties in synthetic polypeptide hydrogels is desirable since it may allow more facile tunability of physical properties, which is of particular importance for *in vivo* applications where synthetic materials avoid the potential drawbacks of immunogenicity and pathogen contamination that can occur with proteins isolated from mammalian sources.

The preparation of thermoresponsive hydrogels based solely on synthetic polypeptides has been challenging, and has only been demonstrated using small, sequence-specific peptides under certain conditions.[8] The closest polypeptide containing examples are based on hybrid block copolymers, where a nondegradable segment, usually polyethylene oxide, PEO, is connected to a polypeptide segment.[3,9–12] Hydrogel formation is driven by dehydration of the PEO segments as temperature increased, as well as use of short polypeptide segments able to transition to β-sheet rich conformations at elevated temperatures. The β-sheet structures stabilize hydrogel formation via interchain H-bonding, but also lead to irreversibility of the liquid to hydrogel transition.[3,12,13] These materials typically show transitions over broad temperature ranges and transition temperatures that vary greatly with sample concentration.[3,9,10,12] Recently, Li developed ethylene oxide side-chain modified polypeptides that are themselves thermoresponsive, and found that these can also form thermoresponsive hydrogels as diblock copolymers with PEO.[13,14] These materials formed weak hydrogels at 20 ºC that increased in stiffness at elevated temperatures due to PEO dehydration and formation of β-sheet structures. Although much progress has been made in this area, there are currently no reversible, thermoresponsive hydrogels based solely on synthetic polypeptide components.

For many years, our lab has prepared and studied synthetic block copolypeptide hydrogels (DCH), which are soft hydrogels composed of discrete ionic and hydrophobic polypeptide segments that can be adjusted to allow a high level of control over gel stiffness, gel porosity, gel functionality and media stability.[15–18] DCH are physically associated gels composed of branched and tangled self-assembled fibrils that can be deformed by applied stress and injected through small-bore cannulae, after which they rapidly re-assemble into rigid gel networks.[16] When injected into mouse central nervous system (CNS) tissues, ionic DCH self-assemble into discrete, well formed deposits of rigid gel networks *in vivo* that integrate well with host CNS tissue, causing no detectable toxicity or adverse inflammatory reaction, and are fully degraded after several months *in vivo*.[19] We also reported both *in vitro* and *in vivo* evidence that ionic DCH can serve as depots for sustained local release of both hydrophilic and hydrophobic effector molecules for investigative and potential therapeutic applications in the CNS.[20,21] Ionic DCH have many advantageous properties as biomimetic hydrogels, yet lack thermoresponsive properties

that would be beneficial for supporting the survival of suspended cells and for use as vehicles for cell transplantation.

The goal of the present study was to extend the capabilities of DCH in two ways by developing DCH that are non-ionic and that also incorporate thermoresponsive behavior. Initial experiments using our traditional ionic DCH compositions, $K_{180}L_{20}$ and $E_{180}L_{20}$, showed that these highly charged DCH were prohibitively cytotoxic to cell suspensions *in vitro* (*vide infra*). We realized that DCH needed to be redesigned to improve their cell compatibility, and focused our efforts first on the development of non-ionic DCH, named DCH_{EO}, since non-ionic polymers and hydrogels are well known to be less toxic to cells *in vitro*[1,22]. We also report on the design and successful incorporation of α-helical copolypeptide domains into DCH_{EO} that give rise to thermoresponsive DCH, named DCH_T. These new α-helical segments in DCH_T were found to provide excellent tunability of physical properties and allow completely reversible liquid to hydrogel transitions between room temperature and body temperature in a variety of media. DCH_T were also found to successfully support survival of encapsulated cells *in vitro*.

Experimental Section

Materials and instrumentation Tetrahydrofuran (THF), hexanes, and methylene chloride were dried by purging with nitrogen and passage through activated alumina columns prior to use. $Co(PMe_3)_4$ and amino acid N-carboxyanhydride (NCA) monomers were prepared according to literature procedures.[16,23] All other chemicals were purchased from commercial suppliers and used without further purification unless otherwise noted. Thin-layer chromatography (TLC) was conducted with EMD gel 60 F254 precoated plates (0.25 mm) and visualized using a combination of UV, anisaldehyde, and phosphomolybdic acid staining. Selecto silica gel 60 (particle size 0.032– 0.063 mm) was used for flash column chromatography. Fourier Transform Infrared (FTIR) measurements were taken on a Perkin Elmer RX1 FTIR spectrophotometer calibrated using polystyrene film. ^{1}H NMR spectra were acquired on a Bruker ARX 400 spectrometer. Tandem gel permeation chromatography/light scattering (GPC/LS) was performed at 60 ^{o}C using an SSI Accuflow Series III pump equipped with Wyatt DAWN EOS light scattering and Optilab rEX refractive index detectors. Separations were achieved using 10^5, 10^4, and 10^3 Å

Phenomenex Phenogel 5 μm columns at 60 ºC with 0.1 M LiBr in DMF as eluent and sample concentrations of 5 mg/mL. Pyrogen free deionized water (DI) was obtained from a Millipore Milli-Q Biocel A10 purification unit.

General polypeptide synthesis DCH_T and DCH_{EO} samples were designed with average total lengths of around 200 residues, and contained either poly(L-leucine), L, poly(γ-[2-(2-methoxyethoxy)ethyl]-L-glutamate-stat-L-leucine), (E^{P2}/L), or poly(γ-(2-methoxyethyl)-L-glutamate-stat-γ-[2-(2-methoxyethoxy)ethyl]-L-glutamate-*stat*-L-leucine), (E^{P1}/E^{P2}/L), as the hydrophobic domain, and poly(γ-[2-(2-methoxyethoxy)ethyl]-*rac*-glutamate), (*rac*-E^{P2}), as the hydrophilic domain (Fig. 1). DCH_T and DCH_{EO} were synthesized by sequential copolymerization of desired NCA monomers using the transition metal initiator $Co(PMe_3)_4$ in THF according to published procedures[16]. All copolymerization reactions were performed in a dinitrogen filled glovebox using anhydrous solvents. Isolated yields of the purified copolymers ranged between 90% and 95%. Relative copolypeptide compositions (Fig. 1) were determined using ^{1}H NMR and were found to be within 1% of predicted values. Chain lengths of initial poly(*rac*-E^{P2}) segments were determined using end-capping analysis (see Table S1, Fig. S1)[24].

Representative synthesis of a DCH_T sample: (rac-E^{P2})$_{180}$($E^{P2}_{0.5}$/$L_{0.5}$)$_{30}$ In a dinitrogen filled glovebox, a solution of $Co(PMe_3)_4$ (22 mg, 0.059 mmol) in THF (20 mg/ml) was rapidly added, via syringe, to a solution of *rac*-E^{P2} NCA (1.0 g, 3.6 mmol) in THF (50 mg/ml). The reaction was stirred at 20 ºC and polymerization progress was monitored by FTIR. Polymerization reactions were generally completed within 4 hours. Immediately upon polymerization completion, an aliquot was removed for end-capping analysis to determine the length of the poly(*rac*-E^{P2}) segment.[24] A solution of a 1:1 molar mixture of L-leucine NCA (47.7 mg, 0.31 mmol) and L-E^{P2} NCA (83.3 mg, 0.31 mmol) in THF (50 mg/ml) was then added to the active poly(*rac*-E^{P2}) segment, and the reaction was monitored by FTIR. Copolymerization was generally complete within another 2 hours. After complete NCA consumption, the reaction was removed from the glovebox and diethyl ether (30 ml) was then added to precipitate the copolymer. The product was isolated by centrifugation and was washed with ether twice more before re-suspending in water.

The copolymer was dispersed in DI water and then placed in a dialysis bag (MWCO = 2000 Da) and dialyzed exhaustively against DI water for four days in a sterile container. Lyophilization of the resulting clear suspension gave 720 mg poly(*rac*-E^{P2})$_{180}$-*block*-poly(L-E^{P2}-*stat*-L-leucine)$_{30}$, (*rac*-E^{P2})$_{180}$($E^{P2}_{0.5}$/$L_{0.5}$)$_{30}$, (90% yield) as a fluffy white solid. ^{1}H NMR analysis of this material was used to determine the actual copolymer composition, which was found to be (*rac*-E^{P2})$_{180}$($E^{P2}_{0.5}$/$L_{0.5}$)$_{28}$ (see Table S2).

Circular Dichroism Studies Circular dichroism spectra (190–250 nm) were recorded in a quartz cuvette of 0.1 cm path length. All spectra were recorded as an average of 3 scans. The spectra are reported in units of molar ellipticity [θ] (deg·cm^2·dmol^{-1}). The formula used for calculating molar ellipticity, [θ], was [θ] = (θ x 100 x M_W)/(c x l) where θ is the experimental ellipticity in millidegrees, M_W is the average molecular weight of a residue in the thermoresponsive segment in g/mol, c is the ($E^{P2}_{0.5}$/$L_{0.5}$)$_{30}$ polypeptide segment concentration in mg/mL; and l is the cuvette pathlength in cm. The percent α-helical content of the polypeptides was calculated using the formula % α-helix = (-[θ]$_{222nm}$ +3000)/39000) where [θ]$_{222nm}$ is the measured molar ellipticity at 222 nm.[25]

Rheology measurements DCH$_T$ were prepared by rehydrating freeze-dried samples in aqueous phosphate buffered saline pH = 7.4 (PBS). Rheological measurements (dynamic) were performed in triplicate on a strain controlled Reometrics fluids spectrometer RFS II in a cone-plate geometry with diameter of 25 mm and cone angle of 0.02 rad similar to previously described.[15] Strain was set at 10% and the sweep frequency was 1 rad/s. For each sample small-deformation linearity was checked before performing oscillatory measurements. Temperature was increased 0.4 ^{o}C every minute during temperature sweep measurements.

Gelation temperature (T_G) measurements 0.5 ml of 3 wt% DCH$_T$ samples in PBS composed of different hydrophobic segments, or mixed with different amounts of DCH$_{EO}$, were injected into separate 1 cm diameter, flat-bottomed glass vials. All samples were then immersed in a large water bath and equilibrated at 25 ºC. The temperature of the water bath was increased at a rate of 0.5 ºC per min, and the vials were repeatedly inverted to test if

gelation (loss of sample flow) had occurred. Once gelation was observed, the bath temperature was defined as the gelation temperature (T_G) for that sample. All measurements were performed in triplicate.

Solubility studies on DCH_T using thermal cycling 3 wt% **9+1** (1:1) in DI water was transferred into a sealed 1 ml glass vial and then heated slowly to 90 ºC in a water bath to assess copolypeptide solubility as a function of temperature. After incubating at 90 ºC for 5 min, the vial was then placed into a 4 ºC refrigerator to slowly cool the hydrogel, and was left in the refrigerator for 30 min. This heating-cooling cycle was performed 10 times, and copolypeptide solubility was checked each half-cycle. After all the heating-cooling cycles, the vial was incubated in a 90 ºC oven for two days. Under all these conditions, the copolypeptide solution or hydrogel remained clear with no evidence of copolypeptide precipitation.

Release of hydrophilic molecules from $K_{180}L_{20}$ and DCH_T 0.5 ml of $K_{180}L_{20}$ or DCH_T in PBS containing a test protein (chicken egg white lysozyme (Sigma), chicken egg white ovalbumin (Sigma) or immunoglobin G from bovine serum (Sigma)) was injected into a dialysis cassette (Thermo Scientific) with a membrane molecular weight cut-off of 100 kDa for lysozyme, or 200 kDa for ovalbumin and IgG. The dialysate was PBS for all release studies. The dialysis cassettes containing protein loaded $K_{180}L_{20}$ or DCH_T were warmed to 37 ^{o}C before immersing into 37 ^{o}C dialysate (200 ml in an amber bottle) without stirring. Aliquots of dialysate (1 ml each) were sampled for analysis after 0, 1, 2, 4, 6, 8, 10, 12, and 24 hours, and the dialysate was stirred briefly for one minute immediately prior to removal of aliquots. The concentrations of test proteins in dialyzate samples were determined by measurement of UV absorption and use of calibration curves for each protein. UV spectra were measured using a Perkin Elmer LambdaEZ210 (λ = 280 nm). All measurements were performed in triplicate and average values reported. Protein release data were fitted using methods described previously.[26]

Anionic lysozyme and neutral lysozyme were prepared by literature procedures where lysine residues in native, cationic chicken egg white lysozyme (30 mg/ml in phosphate buffer at pH 8) were reacted with large excesses (more than 10 equivalents per lysine residue) of succinic anhydride or acetic anhydride, respectively.[27,28] The reaction pH was kept between 8 and 9 by adding aliquots of 0.1 M NaOH until the pH remained constant. The solutions of modified lysozyme were then separately dialyzed against PBS for four days and then freeze-dried to obtain materials for subsequent studies. The modified lysozymes were both soluble at 10% in PBS buffer and showed no signs of aggregation.

Loading and release of hydrophobic molecules from DCH_T Cholesterol (Sigma, 386.65 g/mol) was used as a model hydrophobic test molecule for *in vitro* release studies. Cholesterol stock solutions were prepared by dissolving directly in 90 % ethanol (3.5 mg/ml). DCH_T was dispersed at 15 mg/ml in 90 % ethanol to yield a clear fluid. The DCH_T and cholesterol samples in ethanol were mixed together in the desired proportions and then transferred to 1 cm diameter, flat-bottomed vials. After evaporating the ethanol and water under vacuum to dryness, homogeneous transparent films formed on the bottom of the vials. Either DI water or PBS was added to rehydrate the film, which yielded transparent viscous solutions at room temperature containing the desired amount of cholesterol (up to 1.5 wt%).

0.5 ml of DCH_T prepared at different concentrations in PBS buffer that contained cholesterol at different concentrations were injected into dialysis cassettes (Thermo Scientific) with membrane molecular weight cut-offs of 20 kDa. The dialysis cassettes with cholesterol loaded DCH_T were warmed to 37 ^oC before being immersed into 37 ^oC dialysate. The dialysate was PBS that contained 10 mg/ml BSA to help capture the released hydrophobic molecules. Aliquots of dialysate (1 ml each) were sampled for analysis after 0, 1, 2, 4, 8, 12, 24 and 48 hours. After 2 days, the time interval between sampling was adjusted according to the release kinetics of each sample. The concentrations of cholesterol in dialyzate samples were measured using a calibrated gas chromatography (GC) method.[21]

The cholesterol was extracted from an aliquot of dialysate with toluene (0.5 ml x3), and its concentration was quantified using a Shimdazu 6890N gas chromatographic system with a DB-17 capillary column (30 m x 0.250 mm x 0.15 mm, Agilent Technologies Inc.,

CA, USA). The DB-17 column has mid-polarity and is suitable for analysis of free steroids. One microliter (1.0 μl) of analyte in toluene was injected into GC system with split/splitless injector and flame ionization detector. The inlet temperature was 250 ºC and the split ratio was 10:1. The carrier gas was helium at 2.5 ml/min constant flow. The oven was programmed to start at 250 ºC, hold for 5 min, followed by increasing temperature at a rate of 5 ºC/min up to 260 ºC, and then hold again for 8 min. The total time for GC analysis was 15 min. The detector was set at 300 ºC with 200 ml/min air flow, 80 ml/min hydrogen flow, and 40 ml/min helium makeup flow. All measurements were performed in triplicate and average values reported.

Cells for in vitro evaluations of DCH cytotoxicity The cytotoxicity of the different DCH was evaluated using HeLa cells and murine bone-marrow mesenchymal stem cells (MSCs). The HeLa cell line was maintained using standard tissue culture protocols.[29] MSCs were isolated from 6- to 8-week-old wild type mice.[30] Briefly, marrow cells, flushed out of tibias and femurs, were plated in 75 cm^2 tissue culture flasks at the concentration of 0.3 to 0.4 x 10^6 cells/cm^2 using Murine Mesencult as medium (Stem Cell Technologies, Vancouver, British Columbia, Canada). Cells were cultured in plastic plates as adherent cells and kept in a humidified 5% CO_2 incubator at 37 °C, refreshing medium every 3 days until cells reached 80% confluence. For cytotoxicity evaluations, HeLa cells or MSCs were mixed into 2%, 3% or 4% DCH, (either $K_{180}L_{20}$ or $E_{180}L_{20}$ prepared as described previously[19]), or in serum-free medium containing DMEM/F12 (Invitrogen) and seeded onto wells of a 96-well tissue culture plates at a density of 8×10^4 cells/cm^2. Cells were cultured in a 37 ^oC humidified atmosphere with 5% CO_2, and at desired time points, cell viability was measured using the MTS assay.

In vitro cell viability assay The viability of cells maintained under different conditions *in vitro* was quantified using the Cell Titer 96 Aqueous Nonradioactive Cell Proliferation Assay (MTS assay) (Promega, Madison WI) with triplicates measured for each condition.[29] For cells cultured in 96 well plates, the culture plates were centrifuged briefly and the cell culture medium was aspirated. Fresh medium containing 20% MTS solution was then

added to the cells, which were then transferred to a humidified 5% CO_2 incubator at 37°C for 1 hour. Absorbance at 490 nm (A490) was measured for each well using an Infinite F200 plate reader (Tecan Systems Inc., San Jose, CA, USA). The background absorbance was read at 700 nm (A700) and subtracted from A490. The relative survival of the cells was quantified by taking the ratio of the (A490–A700) values and comparing between the experimental and control cells.

Three dimensional culture of MSCs in DCH_T Primary MSCs, prepared as described above, were dissociated and re-suspended at a final concentration of 2×10^5 cells/ml in either serum-free culture medium (DMEM/F12, Invitrogen) or in 2 wt% or 3 wt% DCH_T prepared in DMEM/F12. Re-suspended MSCs were transferred to wells of 96-well cell culture plates and cultured in a 37 ^oC humidified atmosphere with 5% CO_2 for 24h. Control cells were submerged in media at 37 ºC and incubated for 24h. At desired time points, MSC were harvested and cell viability determined using the MTS assay. Fresh medium containing 20% MTS was then added to the cells. The cells were returned to the CO_2 incubator for 1 hour and then the absorbance at 490 nm (A490) was measured for each well using an Infinite F200 plate reader (Tecan Systems Inc., San Jose, CA, USA). The background absorbance was read at 700 nm (A700) and subtracted from A490. The relative survival of the cells was quantified by taking the ratio of the (A490–A700) values and comparing between the experimental and control cells.

Results and Discussion

Rationale and design of non-ionic DCH To evaluate the potential of DCH as cell carriers and vehicles for transplantation, we first examined cell suspensions prepared in conventional ionic DCH and maintained *in vitro*. We used our well characterized cationic and anionic DCH, $K_{180}L_{20}$ and $E_{180}L_{20}$ respectively, which previous studies had shown were well tolerated and caused no detectable cell damage or toxicity when injected as circumscribed deposits into the CNS *in vivo*.[19,21,26] In spite of the lack of toxicity of ionic DCH deposits *in vivo*, we found that both $K_{180}L_{20}$ and $E_{180}L_{20}$ exhibited pronounced and rapid toxicity towards fully immersed suspensions of HeLa cells (see Figure S1). Compared with cells incubated in standard culture media, survival of cells suspended in

ionic DCH declined steadily and rapidly over time (see Figure S1). Toxicity also increased with increasing concentration of ionic DCH (see Figure S1). The cationic DCH, $K_{180}L_{20}$, was substantially more toxic than the anionic DCH, $E_{180}L_{20}$ (see Figure S1). These results were not entirely surprising based on previous observations by ourselves and others showing *in vitro* cytotoxicity of highly charged ionic polypeptides.[31,32] A likely explanation for the contrasting observations with ionic DCH *in vivo* and *in vitro,* is that *in vivo*, DCH form discrete deposits that interface with host cells only along borders, whereas in cell suspensions *in vitro*, cells are fully immersed in and encapsulated by DCH. This notion is consistent with observations that poly(lysine) is a non-toxic and preferred adhesive substrate commonly used for preparation of two dimensional neural cell cultures, whereas immersion of such cultures in solutions with high concentrations of soluble poly(lysine) results in high toxicity.[33] In work on other polypeptide assemblies, such as with vesicles and micelles, we have found that replacement of cationic hydrophilic segments with non-ionic hydrophilic segments resulted in materials with minimal *in vitro* cytotoxicity.[29,34,35] We therefore set about redesigning DCH to improve their cell compatibility by focusing our efforts on the development of non-ionic DCH.

Based on previous results,[29,34–36] we recognized that a critical requirement of non-ionic DCH design strategy would be that candidate non-ionic hydrophilic segments possess disordered chain conformations in order to promote hydrogel formation. We have shown previously that use of ordered hydrophilic segments (i.e. α-helical) in polypeptide assemblies generally results in formation of rigid sheets that will not form hydrogels.[36] With this in mind, we chose to utilize poly(γ-[2-(2-methoxyethoxy)ethyl]-*rac*-glutamate), (*rac*-E^{P2}), as a hydrophilic segment to prepare non-ionic DCH (Figure 1), since this polypeptide is non-ionic, highly water soluble, and possesses a disordered chain conformation due to its racemic residue composition.[23]

We prepared diblock copolypeptides containing hydrophilic (*rac*-E^{P2}) and hydrophobic poly(L-leucine), L, segments with compositions similar to those of ionic DCH, e.g. $(rac\text{-}E^{P2})_{180}L_x$, x = 20, 30, or 40, and designated this family of copolymers as DCH_{EO} (Figure 1, see Tables S1 and S2, Figure S2). These samples were found to form hydrogels upon direct dissolution in aqueous media at low concentrations, greater than *ca.* 2-3 wt%, and were found to possess gel stiffness (storage modulus, G′) that increased with

hydrophobic L segment chain lengths, similar to ionic DCH (see Table S3). These results show that non-ionic poly(*rac*-E^{P2}) is a good surrogate for polyelectrolyte K or E segments, and that the molecular design criteria used for tuning properties of ionic DCH also apply to formation and tuning of non-ionic DCH_{EO}.[18] These features allows the straightforward preparation of non-ionic DCH with tunable properties that should have improved cell compatibility (*vide infra*), and also retain the desirable characteristics of ionic DCH, such as injectability, tunable rigidity and porosity.

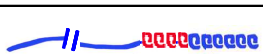
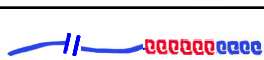
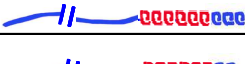
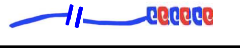
Sample number	Sample notation	Polymer composition	Polymer structure at 25°C	3% at 25°C	3% at 40°C
1	DCH_{EO}	$(rac\text{-}E^{P2})_{180}L_{30}$		Gel	Gel
2	/	$(rac\text{-}E^{P2})_{180}E^{P2}{}_{30}L_{20}$		Liquid	Liquid
3	/	$(rac\text{-}E^{P2})_{180}E^{P2}{}_{20}L_{30}$		Gel	Gel
4	/	$(rac\text{-}E^{P2})_{180}E^{P2}{}_{10}L_{30}$		Gel	Gel
5	/	$(rac\text{-}E^{P2})_{180}L_{20}E^{P2}{}_{30}$		Liquid	Precipitate
6	/	$(rac\text{-}E^{P2})_{180}L_{30}E^{P2}{}_{20}$		Gel	Precipitate
7	/	$(rac\text{-}E^{P2})_{180}L_{30}E^{P2}{}_{10}$		Gel	Gel
8	/	$(rac\text{-}E^{P2})_{180}(E^{P2}{}_{0.5}/L_{0.5})_{20}$		Liquid	Liquid
9	DCH_T	$(rac\text{-}E^{P2})_{180}(E^{P2}{}_{0.5}/L_{0.5})_{30}$		Liquid	Gel
10	DCH_T	$(rac\text{-}E^{P2})_{180}(E^{P2}{}_{0.5}/L_{0.5})_{40}$		Liquid	Gel
11	DCH_T	$(rac\text{-}E^{P2})_{180}(E^{P1}{}_{0.1}/E^{P2}{}_{0.4}/L_{0.5})_{30}$		Liquid	Gel
12	DCH_T	$(rac\text{-}E^{P2})_{180}(E^{P1}{}_{0.2}/E^{P2}{}_{0.3}/L_{0.5})_{30}$		Liquid	Gel
13	DCH_T	$(rac\text{-}E^{P2})_{180}(E^{P2}{}_{0.5}/A_{0.5})_{40}$		Liquid	Gel
14	DCH_T	$(rac\text{-}E^{P2})_{180}(E^{P2}{}_{0.5}/A_{0.5})_{50}$		Liquid	Gel

Figure 1. Schematic representations, structures, and properties of non-ionic DCH compositions. Amphiphilic, non-ionic DCH samples were designed with average total lengths of *ca.* 210 to 230 residues, and contained a *rac*-E^{P2} hydrophilic domain (blue) with a disordered conformation, as well as an α-helical hydrophobic (red) or thermoresponsive (red and blue) domain. Only the samples with thermoresponsive domains composed of statistical sequences of thermoresponsive (E^{P2} or E^{P1}/E^{P2}) and hydrophobic (L or A) residues were found to form DCH_T. *rac*-E^{P2} = poly(γ-[2-(2-methoxyethoxy)ethyl]-*rac*-glutamate); E^{P1} = poly(γ-(2-methoxyethyl)-L-glutamate); E^{P2} = poly(γ-[2-(2-

methoxyethoxy)ethyl]-L-glutamate); L = poly(L-leucine); A = poly(L-alanine). DCH_{EO} = non-ionic, oligoethylene oxide based diblock copolypeptide hydrogel; DCH_T = non-ionic, thermoresponsive diblock copolypeptide that is liquid below *ca.* 30 °C and forms a hydrogel at temperatures greater than *ca.* 30 °C; Liquid = sample flows when inverted; Gel = sample does not flow when inverted; Precipitate = sample is insoluble in aqueous media.

Rationale and design of thermoresponsive DCH While DCH_{EO} are expected to be more cell compatible compared to ionic DCH, they do not improve upon injectability of the hydrogels into tissues. We have demonstrated that many DCH formulations are readily injected into CNS tissues through small bore cannulae. However, there are physical limitations on injectability where DCH that are very stiff can be difficult to extrude through small bore needles due to the high stresses required. The injection of materials as liquids that can then transform into hydrogels *in vivo* is a strategy that has been used to overcome this issue,[1,37,38] and would simplify the injection of DCH with increased stiffness. Such materials would also provide advantages in the encapsulation of live cells for grafting into tissues, due to the ease of dispersing cells within a fluid as opposed to a hydrogel. One way to obtain such properties is with a thermoresponsive formulation that would exist as a viscous liquid at ambient temperature (below *ca*. 30 ºC), yet would transform into a rigid hydrogel at physiological temperatures (above *ca*. 30 ºC). If such a formulation could also retain the other desirable characteristics of DCH_{EO}, it would be potentially valuable for encapsulation and delivery of molecules and cells.

Enantiomerically pure poly(γ-[2-(2-methoxyethoxy)ethyl]-L-glutamate), E^{P2}, is known to be α-helical and this ordered conformation gives this polymer temperature sensitive solubility (i.e. a lower critical solution temperature) in water, where it is soluble at ambient temperature and forms polymer-rich precipitates at temperatures above *ca*. 37 ºC.[23,39] We recognized E^{P2} could be a suitable component for use in development of thermoresponsive DCH, and so explored triblock copolypeptide designs where E^{P2} residues were incorporated as additional segments either before or after the hydrophobic L segments of DCH_{EO} type copolypeptides (Figure 1). When dispersed in aqueous media, none of these triblock copolypeptide designs displayed the desired thermoresponsive hydrogel formation characteristics, and instead either formed hydrogels or liquids that did not respond to

temperature changes (Figure. 1 samples **2**-**4**, **7**), or formed precipitates when temperature was increased to 40 ºC (Figure 1 samples **5**, **6**).

In order to better combine the properties of thermosensitive E^{P2} residues and permanently hydrophobic L residues, we redesigned the copolypeptides as diblock copolymers where equimolar E^{P2} and L residues were combined together in statistical sequences as single thermoresponsive segments, e.g. poly(γ-[2-(2-methoxyethoxy)ethyl]-*rac*-glutamate)-*block*-poly(γ-[2-(2-methoxyethoxy)ethyl]-L-glutamate-*stat*-L-leucine), $(rac\text{-}E^{P2})_{180}(E^{P2}/L)_x$ x = 20, 30, or 40, (Figure 1 samples **8**-**10**). With thermoresponsive segments 30 residues or longer this design proved successful and gave materials that, when dispersed in aqueous media, flow as liquids at 25 ºC, and rapidly form rigid hydrogels at 40 ºC (Figure 2, see Figure S3). As with other DCH, the properties of these thermoresponsive hydrogels, designated as DCH_T, are readily tuned (*vide infra*) by straightforward synthesis of samples with different hydrophobic segment lengths (Figure 1 samples **9**,**10**), or with different amino acid compositions (Figure 1 samples **11**,**12**). All DCH_T samples in Figure 1 formed completely transparent liquids and hydrogels over a temperature range of 4 to 90 ºC when dispersed in aqueous media, and all transitions between liquid and hydrogel states were fully reversible over many heating and cooling cycles. Such reversibility has not been observed in other synthetic polypeptide containing thermoresponsive hydrogels and is likely due to the stable α-helical conformations of the thermoresponsive domains in our samples (see Figure S4). Also, in either liquid or gel state, all DCH_T (3 wt %) could be injected through 30 Ga needles without difficulty (*vide infra*), similar to other DCH.

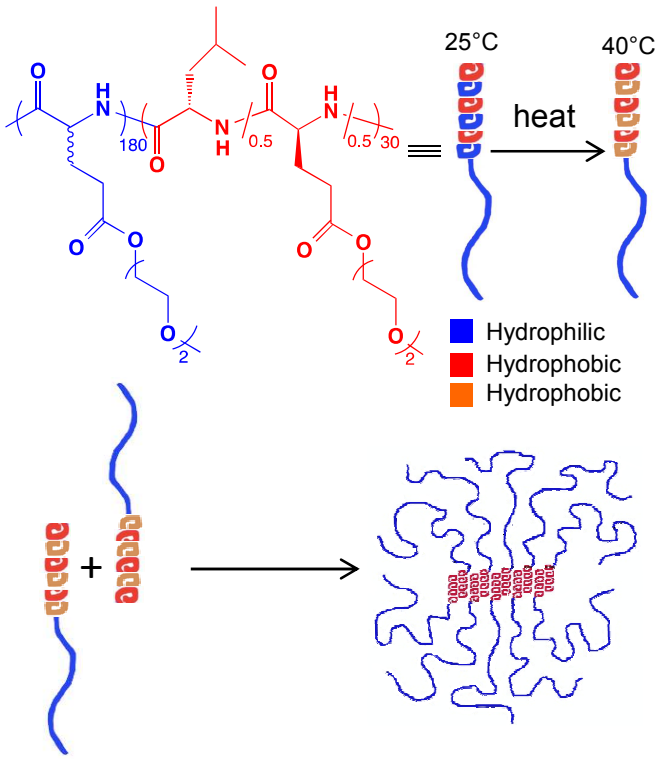


Figure 2. Schematic representation, structure, and thermoresponsive gelation process for DCH$_T$ 9. E^{P2} containing segments in **9** are hydrophilic at <37 °C (blue) and are hydrophobic at >37 °C (orange). In aqueous solutions at >37 °C, the hydrophobic thermoresponsive segments in **9** associate to form assemblies that give 3D hydrogel networks with hydrophilic *rac*-E^{P2} segments exposed.

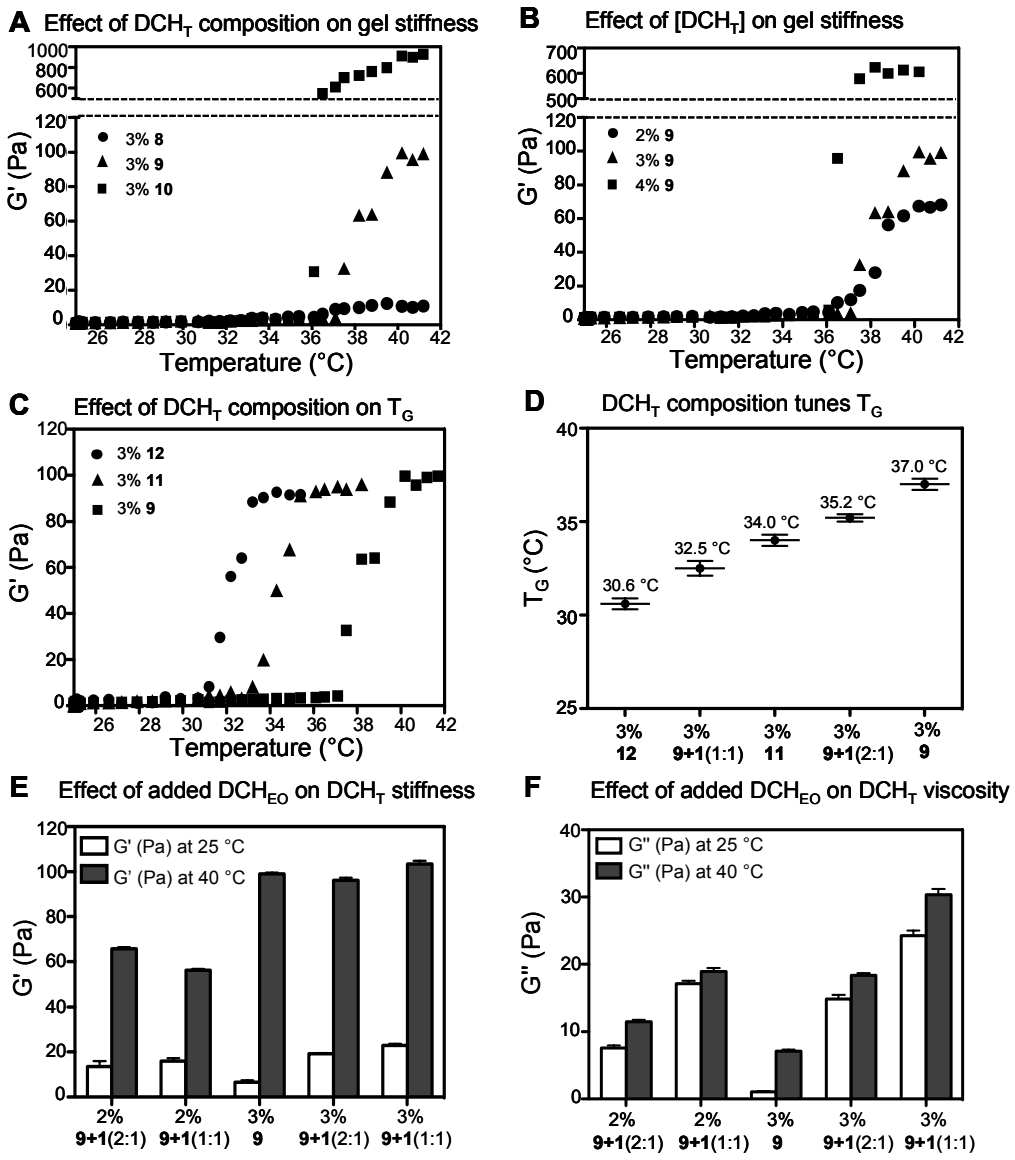


Figure 3. Tuning physical properties of non-ionic DCH$_T$. (A) Effects of DCH$_T$ with different thermoresponsive segment lengths in PBS buffer on sample stiffness in PBS buffer (storage modulus, G′) as temperature was increased (0.4 °C/min). (B) Effects of different concentrations of DCH$_T$ **9** in PBS buffer on sample stiffness as temperature was increased (0.4 °C/min). (C) Effects of different DCH$_T$ compositions in PBS buffer on sample stiffness as temperature was increased (0.4 °C/min). (D) Fine adjustment of gelation temperature (T$_G$) using different compositions or formulations of DCH$_T$ in PBS. (E) Stiffness (G′) at 25 °C and 40 °C for different compositions, formulations, and concentrations of DCH$_T$ in PBS. (F) Viscosity (loss modulus, G″) at 25 °C and 40 °C for

different compositions, formulations, and concentrations of DCH_T in PBS. All G′ and G″ values measured at 1 Hz. Pa = Pascal units.

Tuning properties of DCH_T As noted previously, an advantage of DCH as synthetic biomaterials is the ease with which their physical properties, such as hydrogel stiffness (G′) and porosity, can be predictably, and often independently, adjusted. In DCH_T, hydrogel stiffness is readily adjusted by variation of the length of the thermoresponsive segments (Figure 3A), where increasing segment length results in increased stiffness above the gelation temperature (T_G). Alternatively, adjustment of DCH_T concentration can also be used to tune gel stiffness above T_G (Figure 3B), and also modify hydrogel porosity. These characteristics of DCH_T are similar to properties observed with other ionic DCH,[18] indicating that DCH_T are able to incorporate thermoresponsive properties while retaining the other features of DCH. We have also observed that DCH_T are able to respond quickly to temperature changes, with rapid formation of rigid hydrogels occurring within seconds of heating above T_G.

In addition to the tuning of properties described above, another important parameter to adjust in DCH_T samples is their transition temperature T_G. For use in biomaterial applications *in vivo*, it is desirable to fine-tune T_G to both control the rate of gel formation and for use in tissues at different temperatures. The temperature of internal tissues in animals will be close to 37 ºC, however, exposed tissues, as would be encountered during a surgery may be at temperatures significantly lower than 37 ºC. We observed that the T_G of 3 wt % DCH_T sample **9** was *ca*. 37 ºC in DI water, which may not result in complete gel formation in tissues at or below that temperature. In order to adjust T_G in DCH_T, we designed new samples where some of the E^{P2} residues in the thermoresponsive segments in DCH_T were replaced with γ-(2-methoxyethyl)-L-glutamate, E^{P1}, residues (Figure 1 samples **11**,**12**).

The number of ethylene glycol repeats in the side-chains of thermoresponsive polymers is known to affect their thermoresponsive temperatures (i.e. cloud point temperatures),[37–39] with transition temperatures generally lower in samples with fewer ethylene glycol repeats. Hence, incorporation of increasing amounts of E^{P1} residues into DCH_T was expected to result in progressively lower T_G values, which was observed in

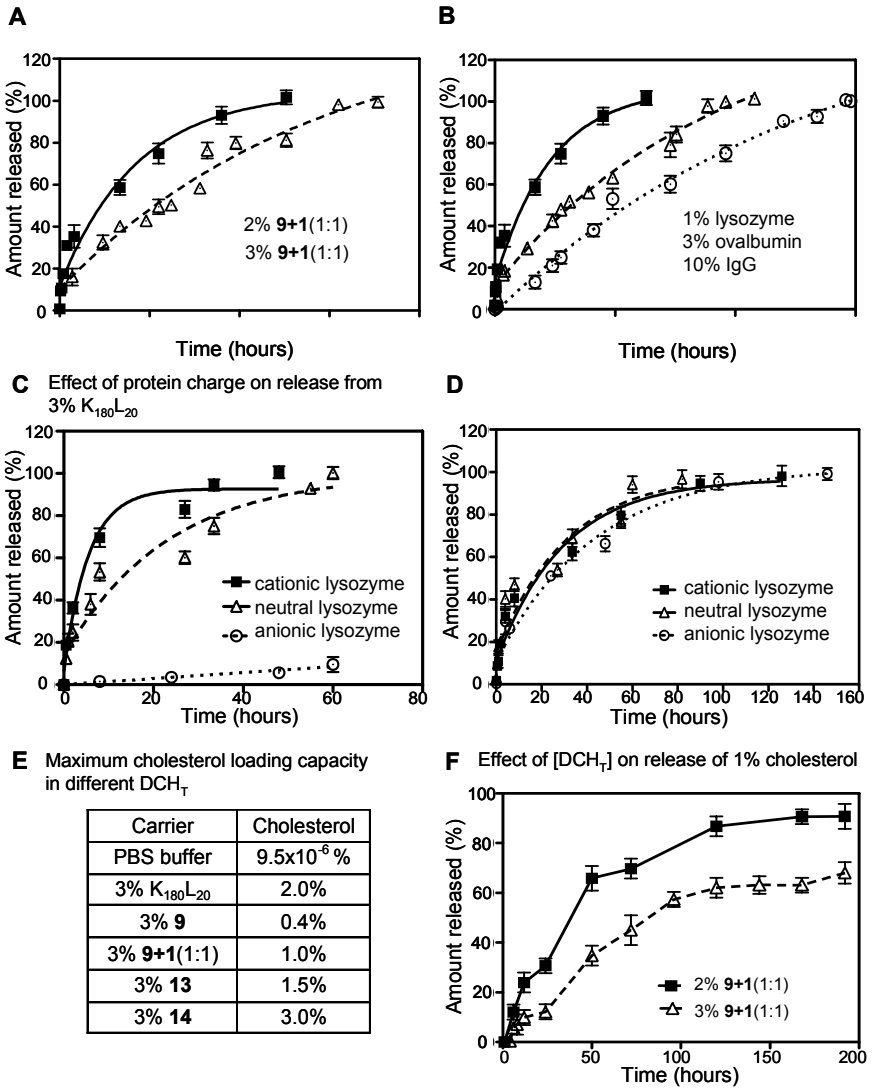
samples **9**, **11**, and **12** where E^{P1} content increased from 0 to 20 to 40% of the total E^{P1} and E^{P2} present, respectively (Figure 3C, 3D). While 40% substitution of E^{P1} for E^{P2} in sample **12** resulted in a significant decrease in T_G (30.6 ºC) compared to **9** (37.0 ºC), the effect of this substitution on DCH_T stiffness (G′) above T_G was minimal (Figure 3C). These results show that T_G in DCH_T can be adjusted essentially independently of hydrogel stiffness. An alternative method to adjust T_G in DCH_T was also developed, where thermoresponsive DCH_T **9** was physically mixed in different proportions in aqueous media with non-thermoresponsive DCH_{EO} **1**. These copolypeptide blends were found to possess lower T_G compared to **9**, with T_G decreasing as the fraction of more hydrophobic **1** was increased (Figure 3D). This approach utilizes the increased hydrophobicity of DCH_{EO} compared to DCH_T to decrease T_G in the blends, and is advantageous in that T_G can be finely tuned by simply varying the mixing ratio of pre-made **9** and **1**. Together, both methods described above provide ample means to fine-tune T_G to desired values over a broad temperature range useful for *in vivo* applications (Figure 3D).

The formation of blends containing DCH_{EO} and DCH_T also affects other physical properties of the resulting materials, in particular their viscosities, which can be gauged by their loss moduli (G″). To study these physical changes in more detail, we compared storage modulus or stiffness (G′) between **9** and its blends with **1** at 25 ºC and 40 ºC at 3 wt % total DCH concentration (Figure 3E). These data show that while G′ of the samples does not vary significantly above T_G, the G′ of the blends are different from **9** in the viscous fluid state at 25 ºC, due to the incorporation of **1**, which associates strongly at this temperature. DCH_T loss moduli (G″), which are more sensitive to the liquid (i.e. viscous) properties of the samples, show even greater differences between **9** and its blends with **1** (Figure 3F). At both 25 ºC and 40 ºC, the G″ values of the blends of **9** and **1** are much greater that those for pure **9**, indicating significantly higher viscosity in the blends, especially at 25 ºC. Similar data for the blends at 2 wt % total DCH concentration (Figure 3E, 3F) show these trends persist at different sample concentrations and thus allow precise and independent tuning of DCH_T viscosity at 25 ºC, as well as stiffness above T_G. It is also worth noting that, contrary to most PEO-polypeptide thermoresponsive hydrogels, T_G in our samples vary only slightly with sample concentration (see Figure S5), which allows near independent tuning of hydrogel physical properties and T_G. Overall, blending of

increasing amounts of **1** with **9** resulted in DCH_T with lower T_G, as well as increased viscosity at 25 ºC. Increasing DCH_T viscosity at ambient temperature can be beneficial for preparation of stable suspensions of cells or micro particles within DCH_T below their T_G (*vide infra*), which is not possible in pure **9** since it has a viscosity similar to water in the liquid state.

Loading and release of cargos in DCH_T We have previously shown that hydrophilic and/or hydrophobic molecules can be loaded into ionic DCH and then released over time either *in vitro* or *in vivo*.[21,26] Here, we studied the ability of DCH_T to perform similar functions, evaluated how adjustment of DCH_T or cargo properties affected release, and compared the results with those described earlier for ionic $K_{180}L_{20}$ DCH. To examine the *in vitro* release dynamics of a protein from DCH_T, we initially used the model protein lysozyme (m.w. 14.7 kDa) dissolved in different concentrations of an optimized DCH_T formulation composed of **9** blended with **1** in an equimolar ratio, i.e. **9**+**1** (1:1) (Figure 4A). Samples were prepared in PBS buffer, placed into dialysis cassettes and warmed to 37 ºC to trigger gelation, and then suspended in a large reservoir of PBS at 37 ºC. PBS media was used instead of water for these experiments to better mimic physiological conditions. Sampling of the dialyzate at different time intervals allowed measurement of released lysozyme concentrations over time, and these data were fit as described previously.[26]

Different DCH_T concentrations were compared to examine how alteration of DCH_T G′ and G″, which vary in relation to concentration, affect release dynamics. Similar to results for ionic $K_{180}L_{20}$, increased DCH_T concentrations resulted in slower lysozyme release from the hydrogels (Figure 4A). The effect of protein size on rate of release was also examined by use of soluble model proteins ovalbumin (m.w. *ca*. 45 kDa) and immunoglobulin G (m.w. *ca.* 150 kDa) in addition to lysozyme. These three proteins were each dissolved at equimolar concentrations into DCH_T **9**+**1** (1:1) and their release monitored over time (Figure 4B). Protein release from DCH_T was found to become slower as protein size increased, as would be consistent with release of different sized molecules from a hydrogel mesh of fixed porosity. These results show that DCH_T concentration, as well as protein size, can both significantly affect release rates of proteins from DCH_T formulations.



Carrier	Cholesterol
PBS buffer	9.5×10^{-6} %
3% $K_{180}L_{20}$	2.0%
3% **9**	0.4%
3% **9+1**(1:1)	1.0%
3% **13**	1.5%
3% **14**	3.0%

Figure 4. Hydrophilic or hydrophobic molecule release kinetics from DCH_T *in vitro*. (A) Effects of optimized DCH_T **9**+**1** (1:1) concentration on release of model protein 1% lysozyme. (B) Effect of molecular weight of model proteins (all at the same molar concentration) on their release from 2% DCH_T **9**+**1** (1:1). (C) Effect of different overall charge on proteins, modeled using native and chemically modified lysozymes, on their release from cationic 3% $K_{180}L_{20}$. (D) Effect of different overall charge on proteins, modeled using native and chemically modified lysozymes, on their release from 2% DCH_T **9**+**1** (1:1). (E) Data showing the effect of different compositions or formulations of DCH_T on their maximum cholesterol loading capacity. (F) Effects of DCH_T **9**+**1** (1:1) concentration on release of model hydrophobic molecule cholesterol. All release studies conducted in PBS buffer at 37 °C.

Since charged proteins are expected to interact strongly with ionic DCH (i.e. $K_{180}L_{20}$) via polyion complexation,[26] we also studied how overall protein charge affected their rate of release from DCH_T. In this study, proteins of similar size, but different overall charge at pH 7 were prepared by chemical modification of lysozyme. We converted the majority of cationic ammonium groups in native "cationic" lysozyme (isoelectric point (pI) of 11.4) to either anionic carboxylate or neutral acetamide groups, by reaction with excess succinic anhydride or acetic anhydride, respectively, using well characterized methods.[27,28] The carboxylate modification yields an "anionic" lysozyme (reported pI 4.5),[27] and acetamide modification yields a "neutral" lysozyme (reported pI 10),[28] both of which were found to have good water solubility in PBS media with no signs of aggregation over time. The different overall charges present on these cationic, anionic, and neutral test proteins were found to strongly affect their rates of release from cationic $K_{180}L_{20}$ DCH (Figure 4C). The slow release of the anionic protein and the fast release of the cationic protein can be explained by polyionic interactions with these highly cationic polyelectrolyte DCH.[26] With non-ionic DCH_T, the overall charges of the test proteins had no significant effect on their rates of release (Figure 4D), with cationic, anionic and neutral lysozymes all releasing at essentially the same rates. These data show that with non-ionic DCH_T, rates of protein release are predominantly controlled by gel porosity and protein molecular weight, and their non-ionic nature allows release rates to be decoupled from overall charges present on different cargos.

The ability to dissolve and release hydrophobic therapeutic molecules is an attractive feature of traditional DCH that utilizes the hydrophobic segments of the polypeptides as reservoirs for these compounds.[21] However, DCH_T differ from other DCH since they incorporate hydrophilic E^{P1} and E^{P2} components in their thermoresponsive domains that only become hydrophobic above T_G. As such, we wanted to test if DCH_T were also able to dissolve and provide prolonged release of hydrophobic molecules similar to our previously studied ionic DCH.[21] We have shown that cholesterol is a suitable test molecule for such studies, and now found that cholesterol is readily dissolved in 3 wt. % DCH_T **9**+**1** (1:1) up to concentrations of 1.0 wt. % in DI water, which is lower than that found for $K_{180}L_{20}$ DCH (Figure 4E). It is particularly noteworthy that the amount of cholesterol solubilized by DCH_T is substantially greater than the amount soluble in water

alone, and it is solubilized both in the hydrogel state at 37 ºC, as well as in the liquid state at 20 ºC.

To increase the hydrophobic cargo loading capacity of DCH_T, we designed additional DCH_T compositions based on L-alanine, A, as opposed to L-leucine, L, residues, e.g. poly(γ-[2-(2-methoxyethoxy)ethyl]-*rac*-glutamate)-*block*-poly(γ-[2-(2-methoxyethoxy)ethyl]-L-glutamate-stat-L-alanine), $(rac\text{-}E^{P2})_{180}(E^{P2}/A)_x$ x = 40 or 50 (Figure 1 samples **13**,**14**). These samples were prepared since we have shown that A based hydrophobic segments in DCH are more effective than L segments for solubilization of hydrophobic molecules.[21] Samples **13** and **14** were both found to behave as DCH_T that are liquids at 25 ºC and transform to rigid hydrogels at *ca*. 37 ºC. With samples **13** and **14** at 3 wt. % in DI water, we found that cholesterol is readily dissolved up to concentrations of 1.5 and 3.0 wt. %, respectively (Figure 4E). These loading capacities are comparable with those previously found for ionic DCH, and show that DCH_T can be tuned to retain advantageous capacity to dissolve hydrophobic molecules while also incorporating thermoresponsive properties.

Once dissolved in DCH_T **9**+**1** (1:1), cholesterol (1.0 wt. %) was found to be released over time when the hydrogels were placed in constant volume dialysis cassettes and dialyzed against PBS buffer containing BSA at 37 ºC, which is above the T_G for these samples. BSA was added to the dialyzate to capture released cholesterol, which would otherwise precipitate in the aqueous media.[21] Cholesterol release from different concentration DCH_T **9**+**1** (1:1) samples was monitored over time, and in all cases *ca.* 65-90% of the loaded cholesterol was released into the dialysate over a period of days, with the remainder still encapsulated in the DCH (Figure 4F). Similar to results previously obtained for ionic DCH,[21] increasing the DCH_T carrier concentration resulted in slower release of cholesterol, which can be advantageous for avoiding a "burst" type release of cargo. Overall, these data show that the many adjustable parameters available within DCH_T can be used to predictably tune both loading capacity and release profiles of hydrophobic molecules in these hydrogels.

Cell compatibility of DCH_T To test the suitability of DCH_T for use as a cell carrier, we conducted experiments to test the viability or murine bone-marrow mesenchymal stem cells (MSCs) suspended in cell culture medium with DCH_T as

compared to cell culture medium alone over 24 hours *in vitro* at 37 ºC. MSCs were suspended in either $K_{180}L_{20}$ DCH or DCH_T **9**+**1** (1:1) in culture medium (Figure 5A) and incubated at 37 ºC, which is above the T_G for this DCH_T. After 24 hours, cell viability was measured using the MTS assay relative to MSC viability in media only (Figure 5B). Cationic $K_{180}L_{20}$ DCH was highly toxic to fully immersed and suspended MSCs, consistent with earlier studies using HeLa cells (see Figure S1) and with the properties of cationic polymers in general.[31,32] In contrast, both 2% and 3% DCH_T **9**+**1** (1:1) samples displayed no significant cytotoxicity (Figure 5B) after 24 hours. Together our findings indicate that DCH_T may be useful cell carriers and vehicles for transplantation, particularly in situations where requiring simultaneous delivery of hydrophilic and or hydrophobic bioactive molecules.

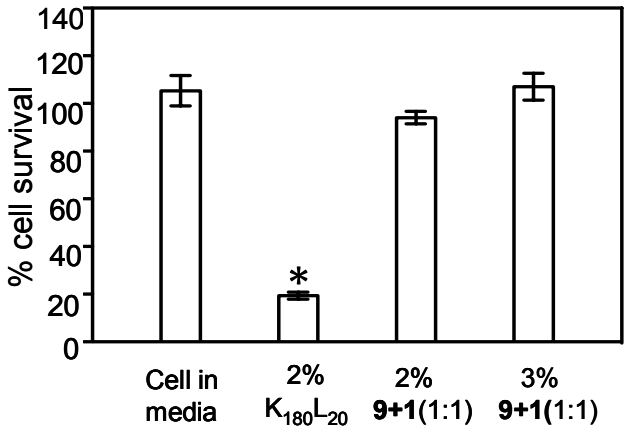


Figure 5. Viability of MSCs suspended in DCH_T *in vitro*. Viability of MSCs did not significantly change after culturing for 24 hours in cell culture media or in DCH_T **9**+**1** (1:1), whereas viability was significantly reduced to less than 20% after incubation in cationic 2% $K_{180}L_{20}$. * $p<0.001$ (ANOVA with post-hoc Newman-Keuls) (n=3 cultures).

Conclusions

In this study, we designed and developed non-ionic DCH, named DCH_{EO}, and thermoresponsive DCH, named DCH_T, and showed that these new DCH are useful tools for encapsulation and release of molecules and culture of cells *in vitro*. A critical design element for successful formation of DCH_{EO} was the use of a racemic, non-ionic hydrophilic segment, i.e. (*rac*-E^{P2}), that possesses a disordered chain conformation in water, which allows the block copolypeptides to assemble into structures to yield the desired hydrogels.[18]

We also incorporated thermoresponsive elements into DCH_{EO} to yield DCH_T, which were designed to undergo liquid to hydrogel transitions at predetermined temperatures between ambient room temperature at 22 ºC and body temperature at 37 ºC. A key innovation in successful development of DCH_T was our use of statistical copolymerization of E^{P2} residues with helicogenic hydrophobic residues, e.g. leucine or alanine, to prepare thermoresponsive segments that adopted stable α-helical conformations. Our DCH_T design takes full advantage of the α-helical thermoresponsive segments, which allow reversible hydrogel formation as well as facile tunability of different physical characteristics. *In vitro* experiments showed that DCH_T retained the many advantageous features of our original ionic DCH, such as injectability, tunable stiffness and viscosity, as well as the ability to load and provide prolonged release of both hydrophilic and hydrophobic molecules.[18,19,21,26] In addition, non-ionic DCH_T exhibited good cytocompatibility by supporting the survival of suspended MSCs *in vitro*. Our findings show that our design of non-ionic DCH_T yields numerous advantageous properties for experimental investigations and potential therapeutic strategies.

Acknowledgements

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Supporting Information Available.

Compositional data for all new compounds, circular dichroism spectra, and additional physical data. Supporting information for this article is available on the WWW under http://pubs.acs.org or from the author.

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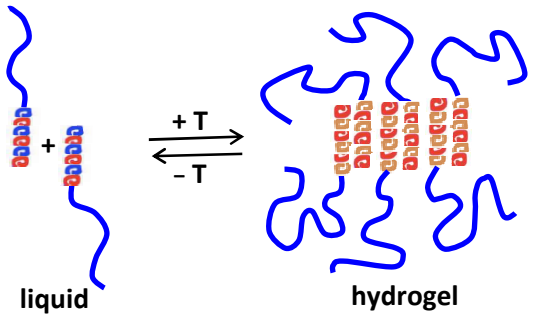
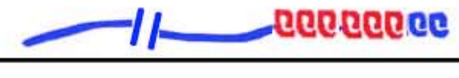
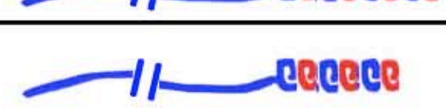
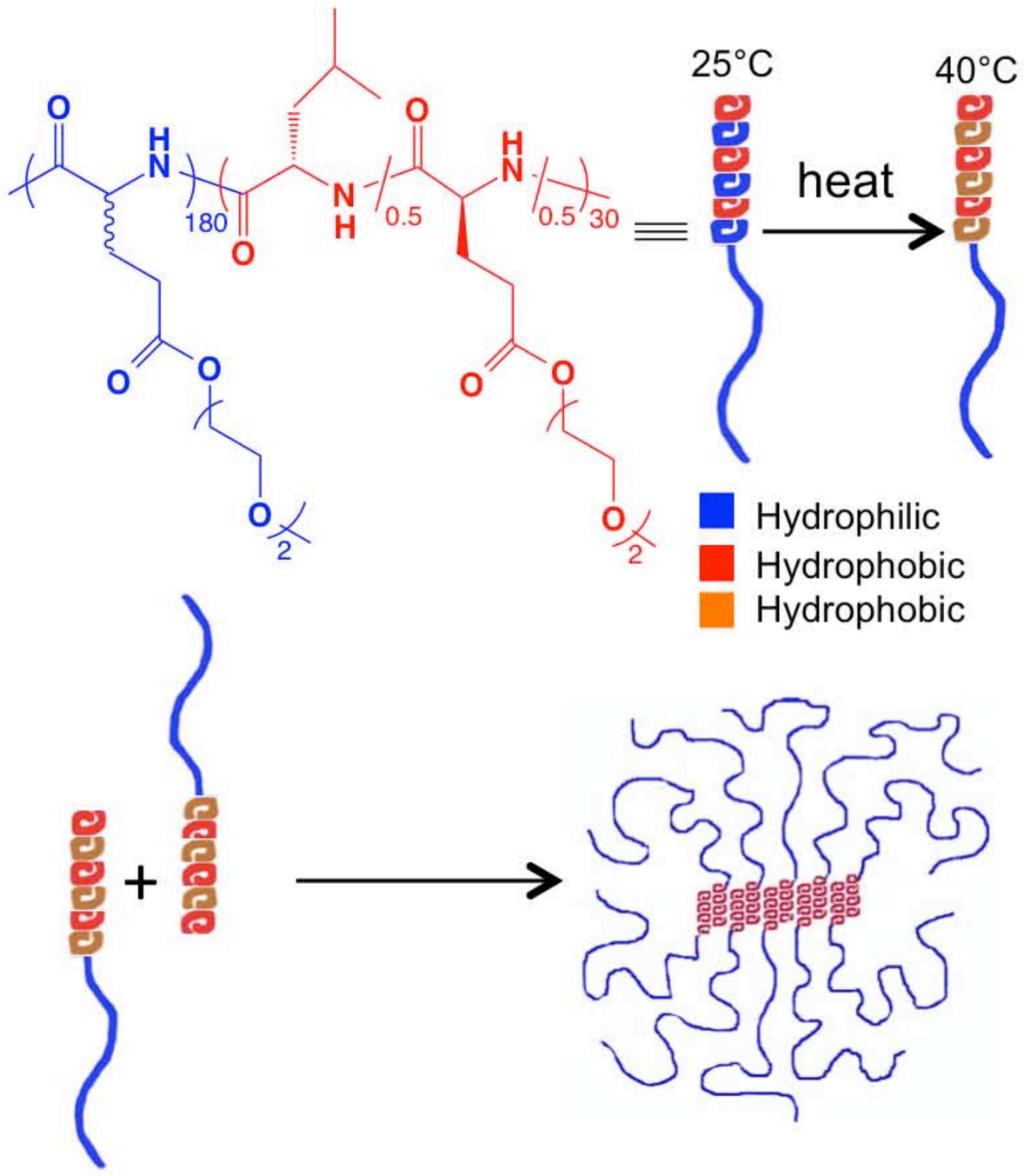
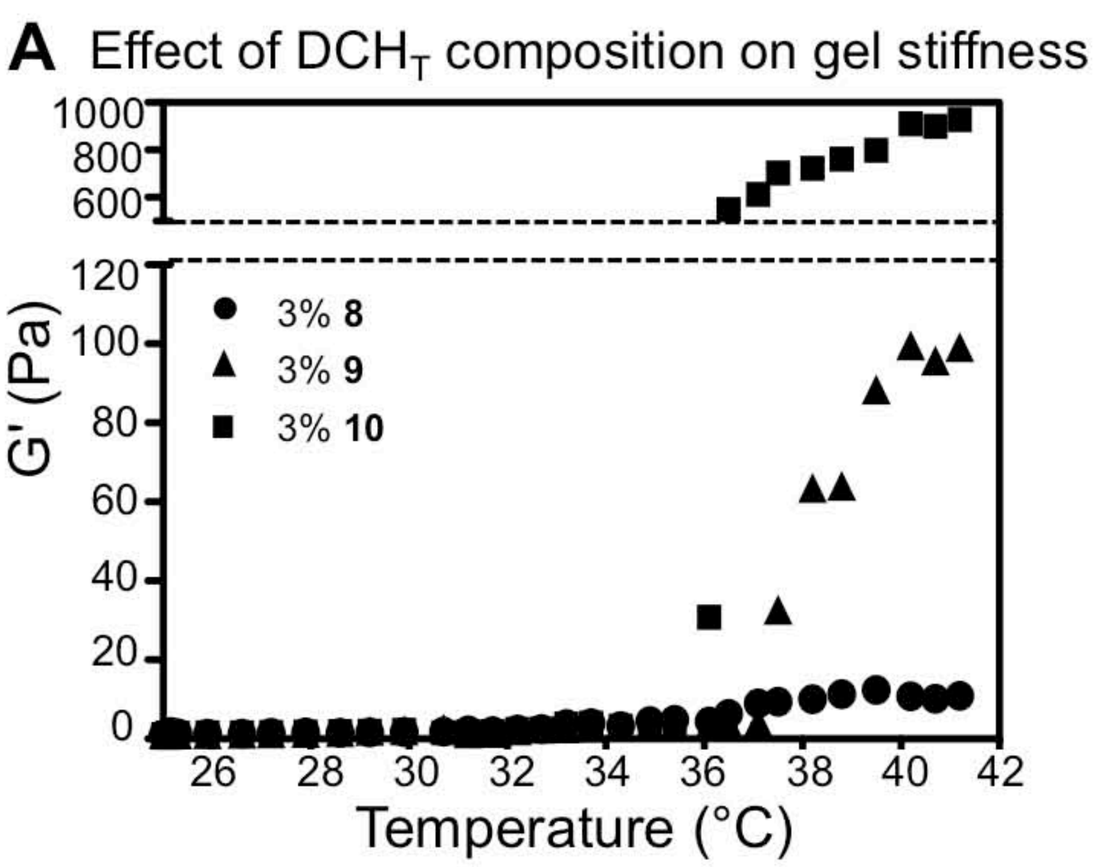
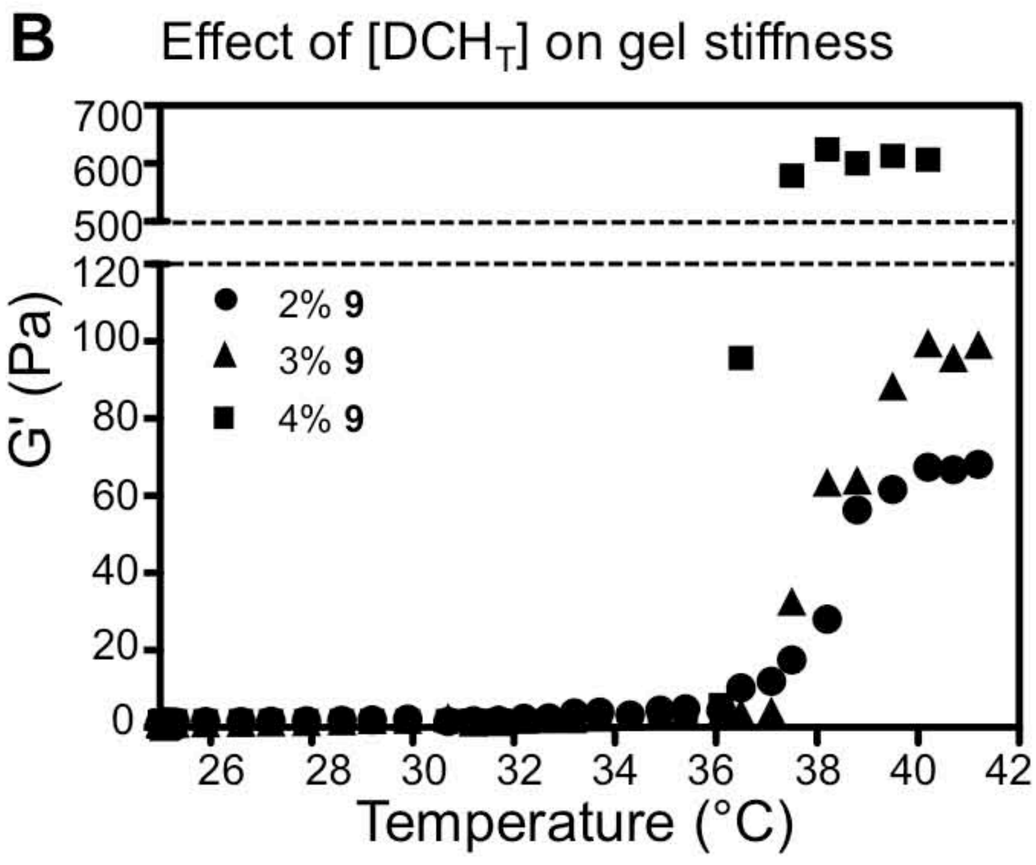
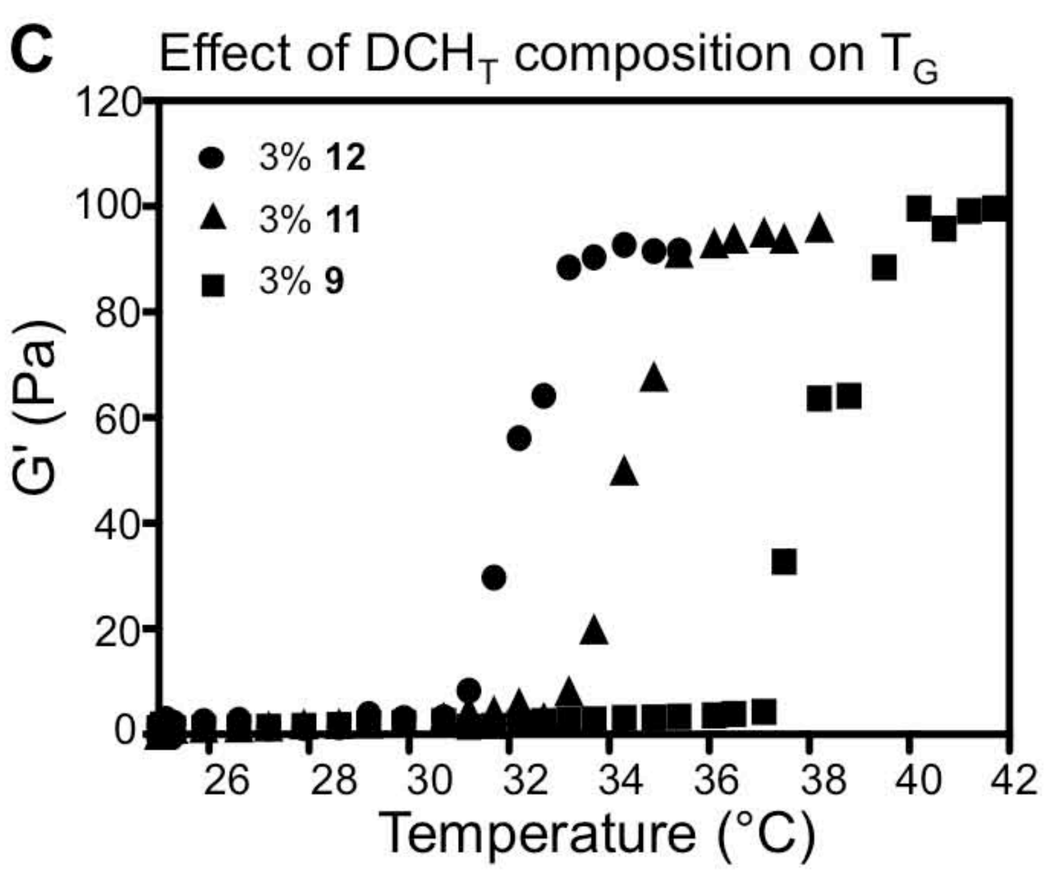


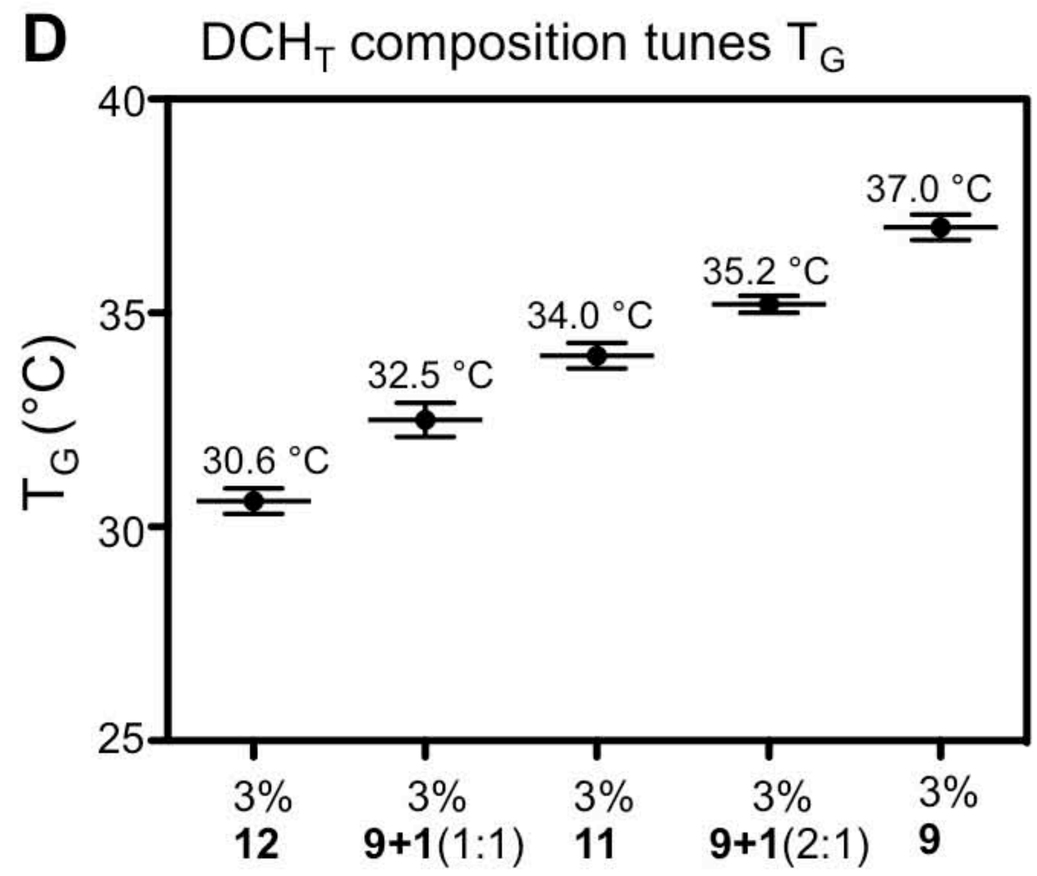
Sample number	Sample notation	Polymer composition	Polymer structure at 25°C	3% at 25°C	3% at 40°C
1	DCH_{EO}	$(rac\text{-}E^{P2})_{180}L_{30}$		Gel	Gel
2	/	$(rac\text{-}E^{P2})_{180}E^{P2}{}_{30}L_{20}$		Liquid	Liquid
3	/	$(rac\text{-}E^{P2})_{180}E^{P2}{}_{20}L_{30}$		Gel	Gel
4	/	$(rac\text{-}E^{P2})_{180}E^{P2}{}_{10}L_{30}$		Gel	Gel
5	/	$(rac\text{-}E^{P2})_{180}L_{20}E^{P2}{}_{30}$		Liquid	Precipitate
6	/	$(rac\text{-}E^{P2})_{180}L_{30}E^{P2}{}_{20}$		Gel	Precipitate
7	/	$(rac\text{-}E^{P2})_{180}L_{30}E^{P2}{}_{10}$		Gel	Gel
8	/	$(rac\text{-}E^{P2})_{180}(E^{P2}{}_{0.5}/L_{0.5})_{20}$		Liquid	Liquid
9	DCH_T	$(rac\text{-}E^{P2})_{180}(E^{P2}{}_{0.5}/L_{0.5})_{30}$		Liquid	Gel
10	DCH_T	$(rac\text{-}E^{P2})_{180}(E^{P2}{}_{0.5}/L_{0.5})_{40}$		Liquid	Gel
11	DCH_T	$(rac\text{-}E^{P2})_{180}(E^{P1}{}_{0.1}/E^{P2}{}_{0.4}/L_{0.5})_{30}$		Liquid	Gel
12	DCH_T	$(rac\text{-}E^{P2})_{180}(E^{P1}{}_{0.2}/E^{P2}{}_{0.3}/L_{0.5})_{30}$		Liquid	Gel
13	DCH_T	$(rac\text{-}E^{P2})_{180}(E^{P2}{}_{0.5}/A_{0.5})_{40}$		Liquid	Gel
14	DCH_T	$(rac\text{-}E^{P2})_{180}(E^{P2}{}_{0.5}/A_{0.5})_{50}$		Liquid	Gel

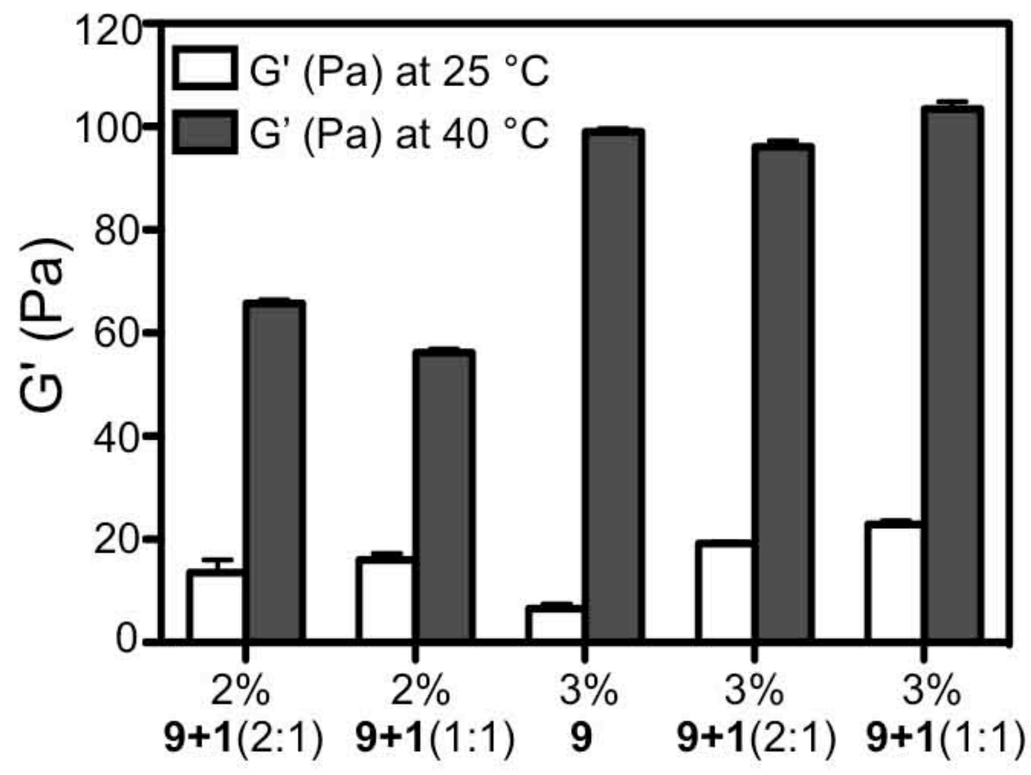

25°C
40°C
heat
O
HN
180
O
NH
0.5
O
HN
0.5
30
O
O
O
2
O
O
O
2
Hydrophilic
Hydrophobic
Hydrophobic
+

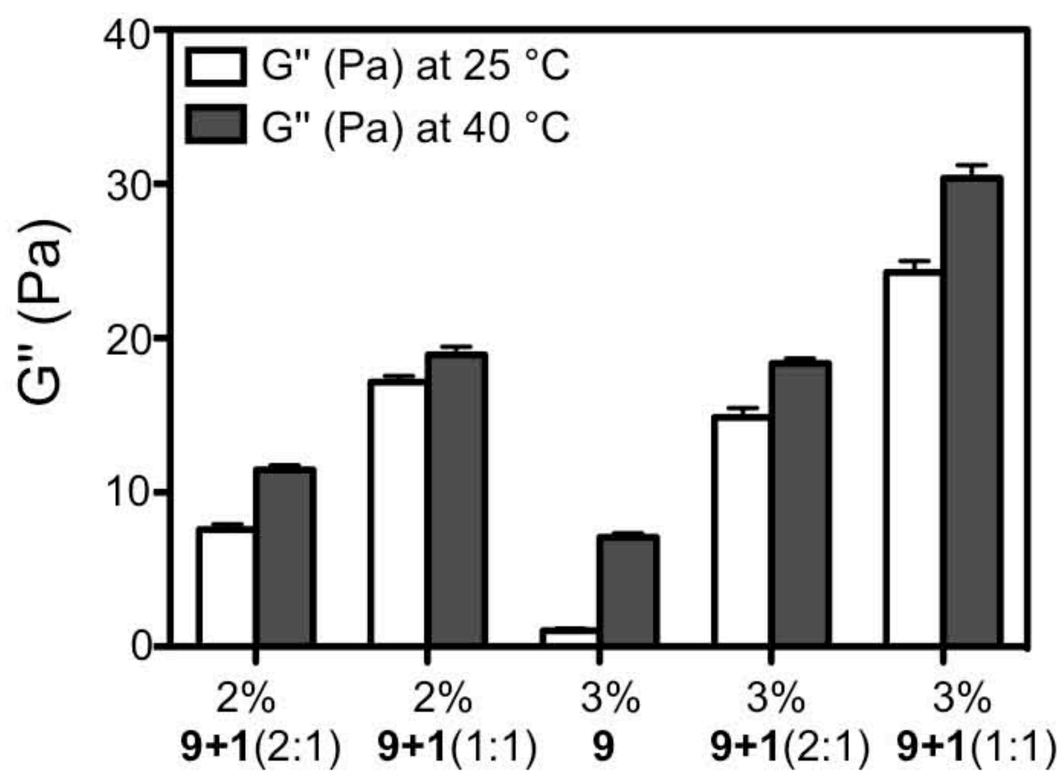

A
Effect of DCH$_T$ composition on gel stiffness
G' (Pa)
3% 8
3% 9
3% 10
Temperature (°C)

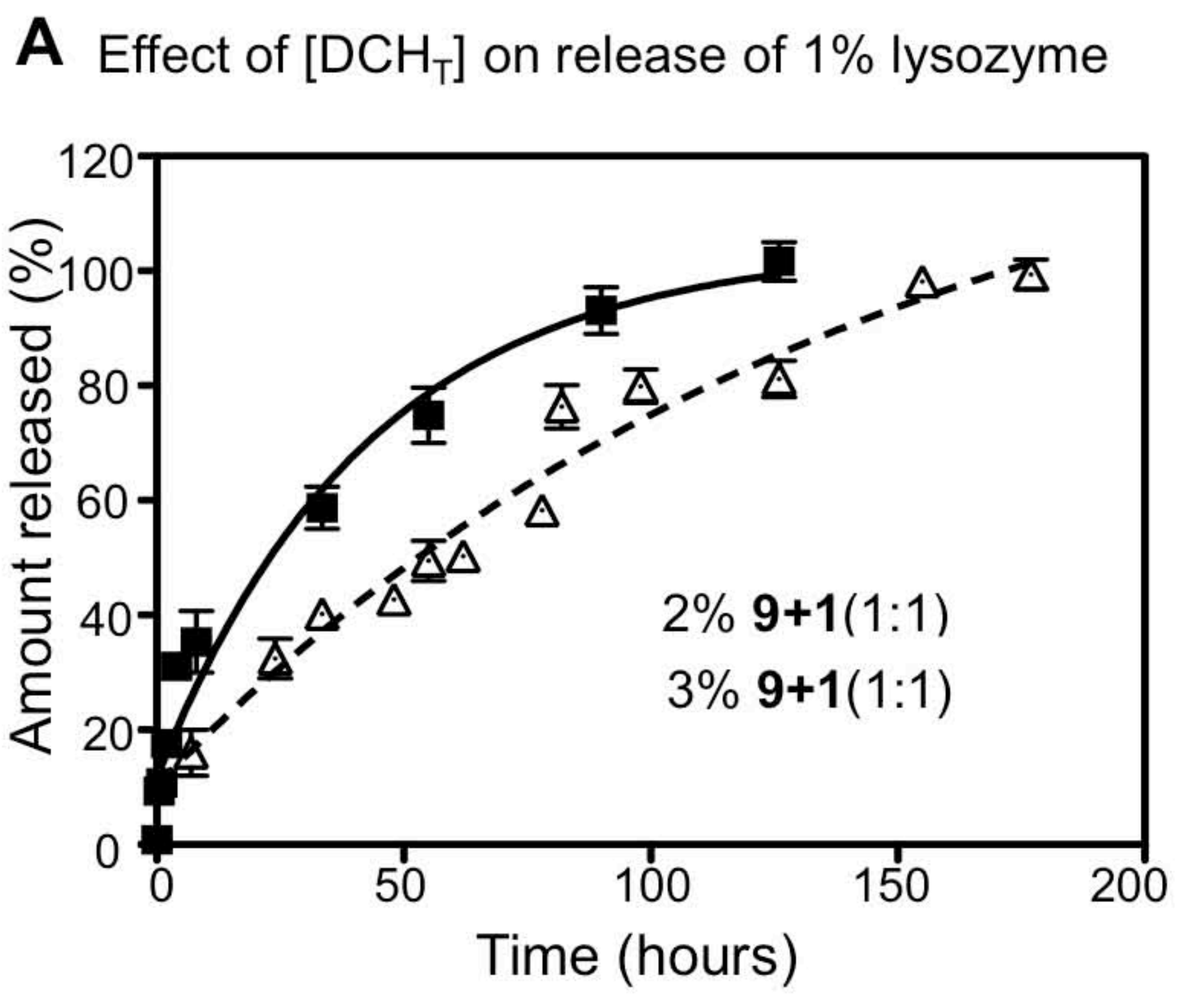

B
Effect of [DCH$_T$] on gel stiffness
G' (Pa)
2% 9
3% 9
4% 9
Temperature (°C)

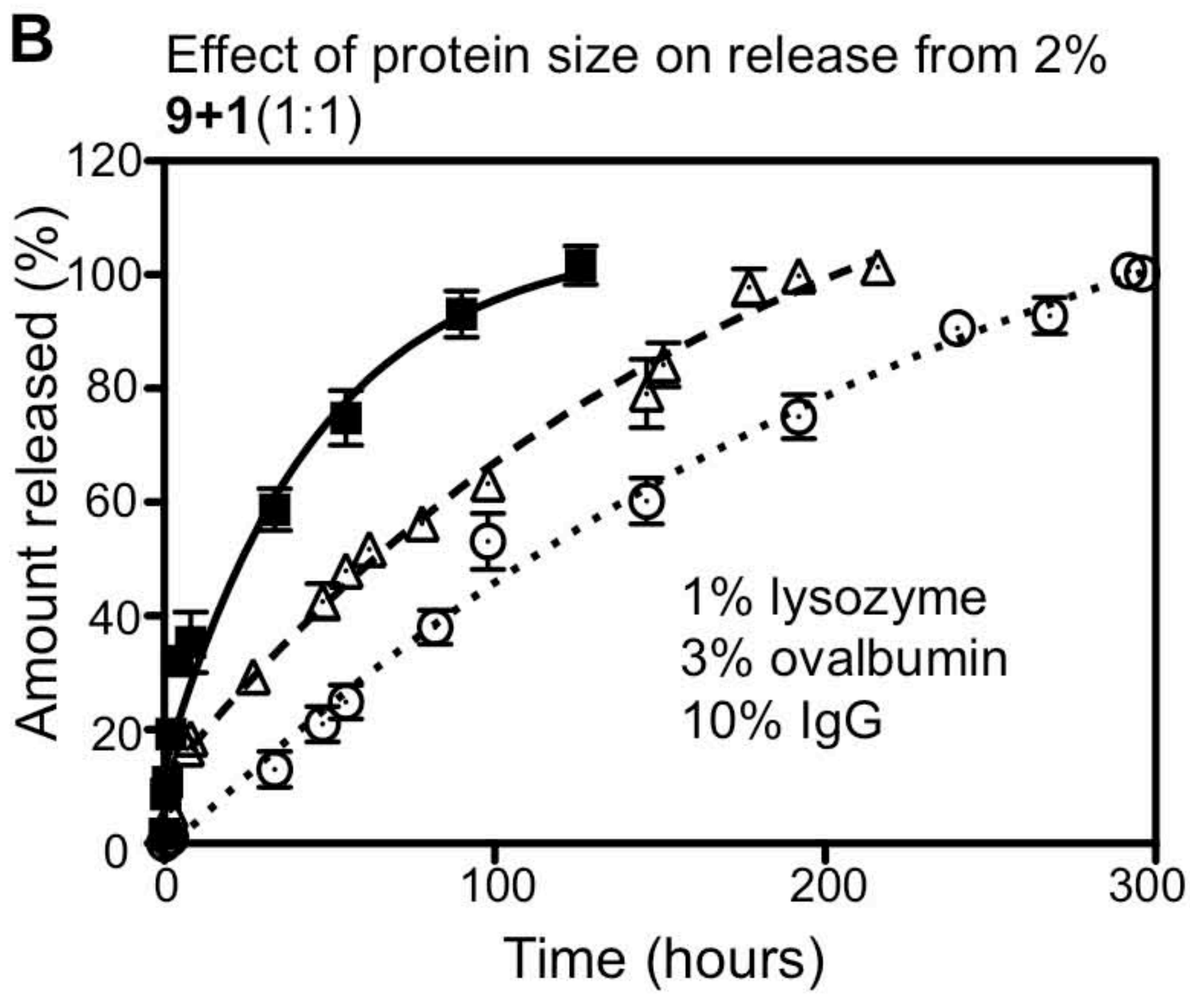

C
Effect of DCH$_T$ composition on T$_G$
G' (Pa)
3% 12
3% 11
3% 9
Temperature (°C)

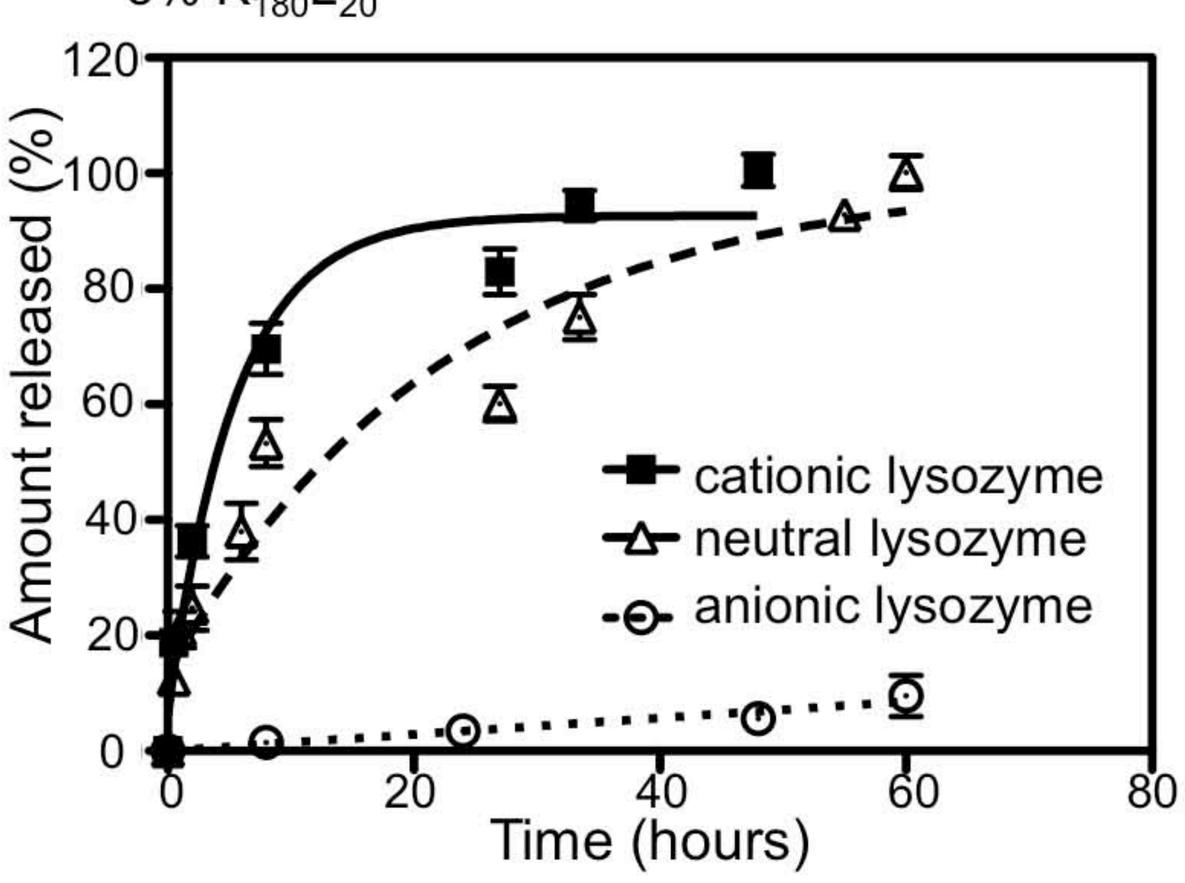

D
DCH$_T$ composition tunes T$_G$
T$_G$ (°C)
30.6 °C
32.5 °C
34.0 °C
35.2 °C
37.0 °C
3% 12
3% 9+1(1:1)
3% 11
3% 9+1(2:1)
3% 9

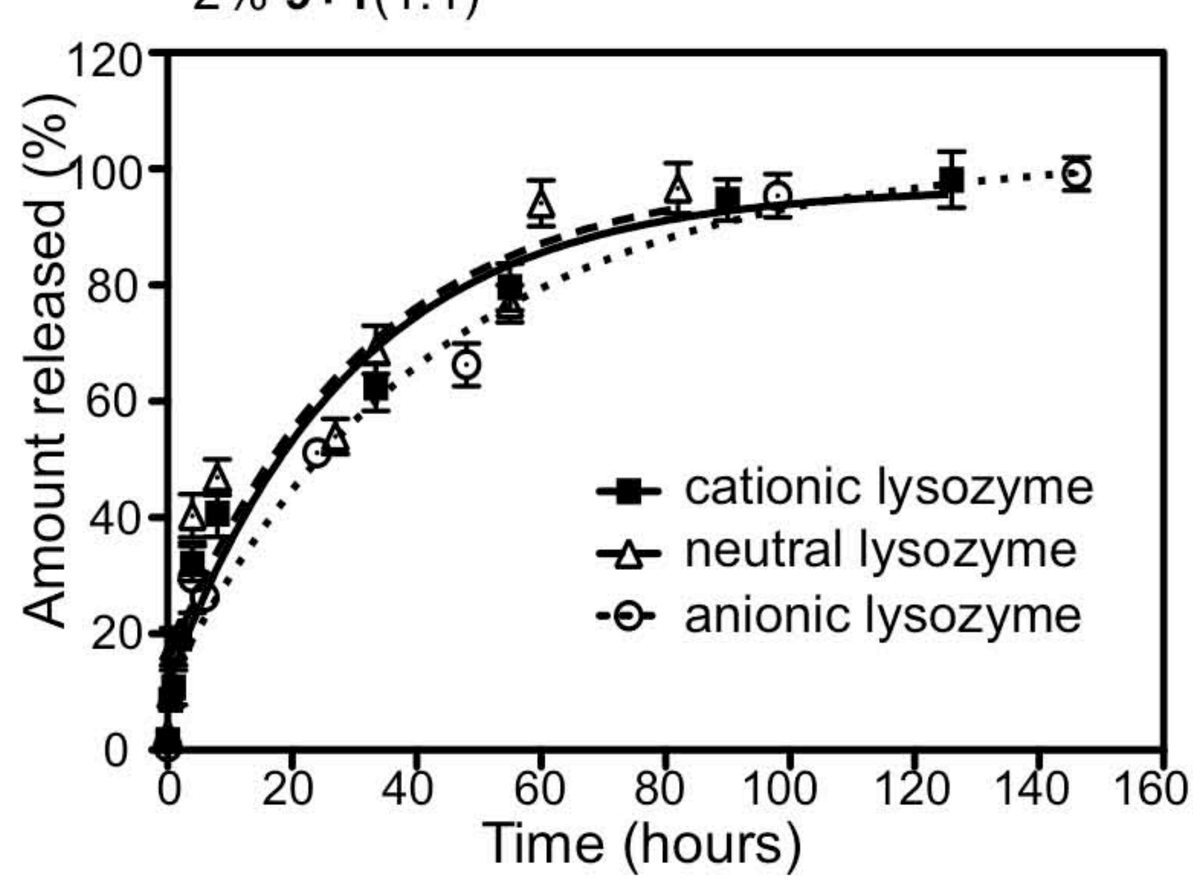

E
Effect of added DCH$_{EO}$ on DCH$_T$ stiffness
G' (Pa)
G' (Pa) at 25 °C
G' (Pa) at 40 °C
2% 9+1(2:1)
2% 9+1(1:1)
3% 9
3% 9+1(2:1)
3% 9+1(1:1)


F
Effect of added DCH$_{EO}$ on DCH$_T$ viscosity
G'' (Pa)
G'' (Pa) at 25 °C
G'' (Pa) at 40 °C
2% 9+1(2:1)
2% 9+1(1:1)
3% 9
3% 9+1(2:1)
3% 9+1(1:1)



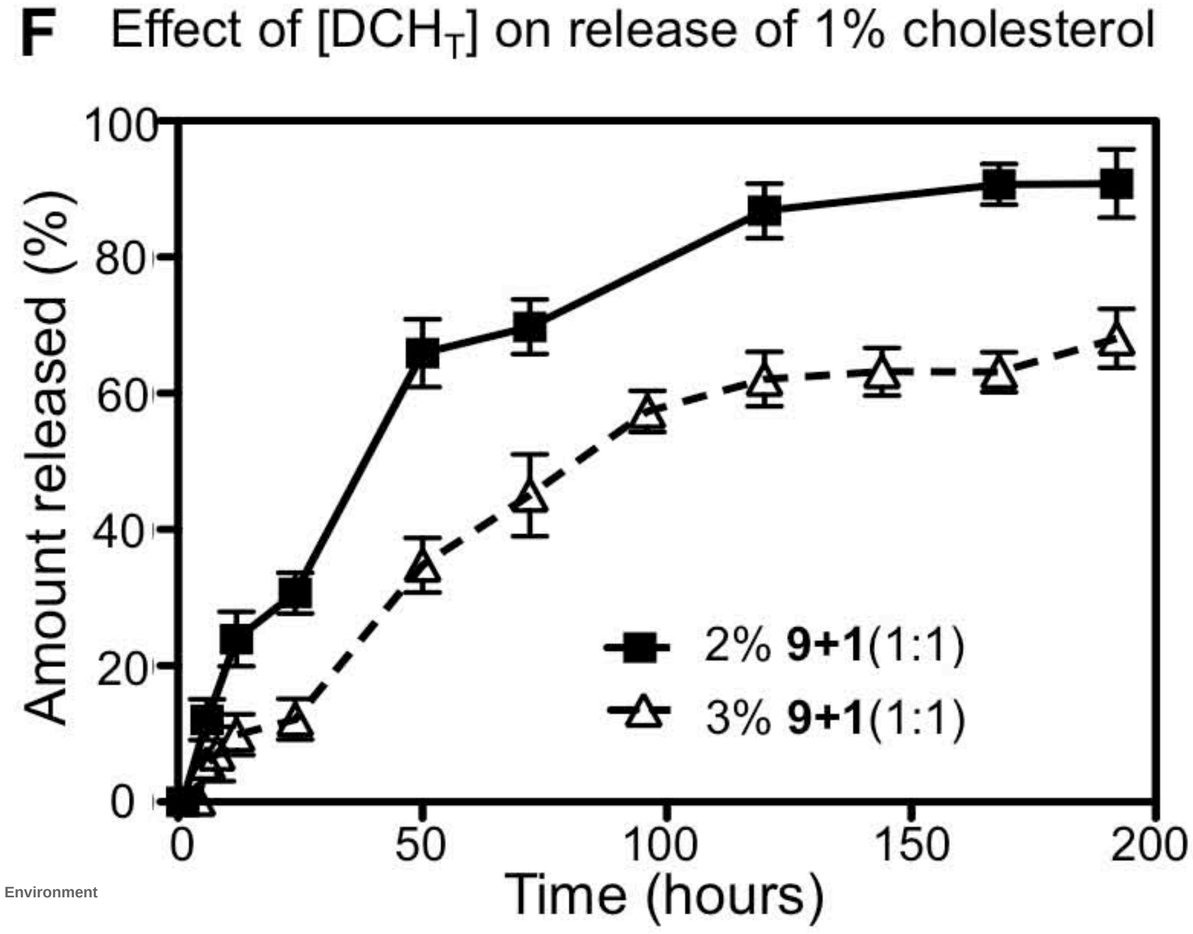


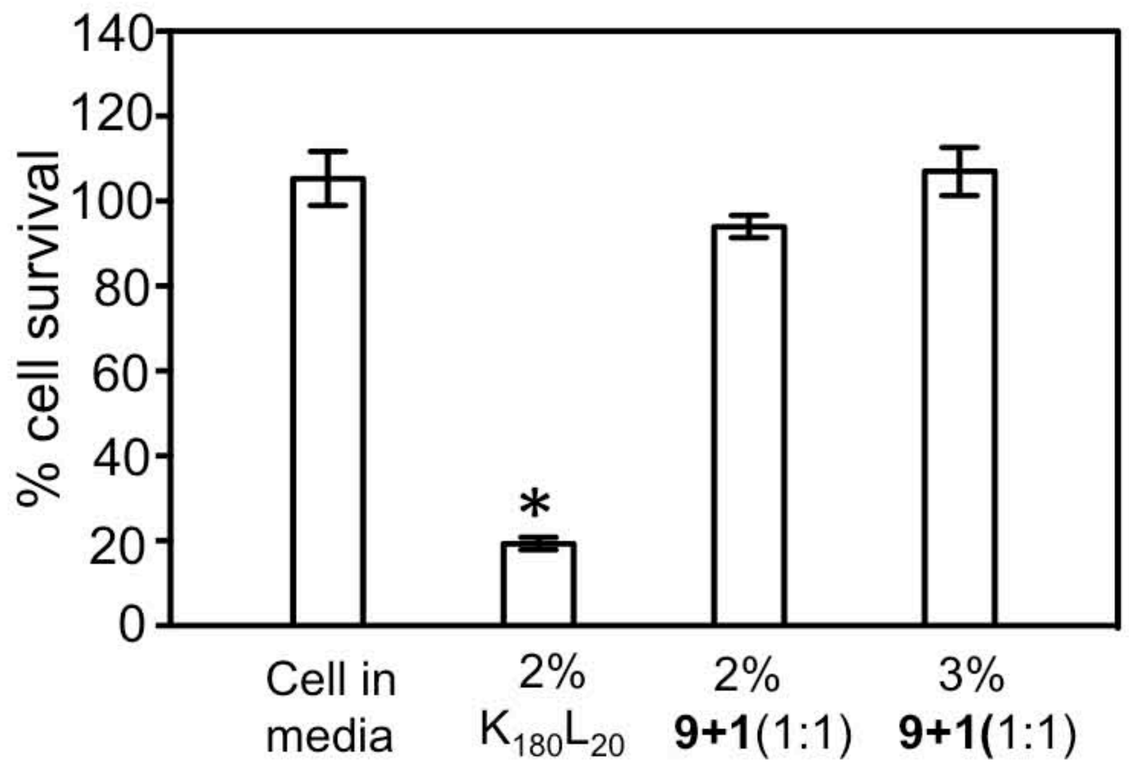


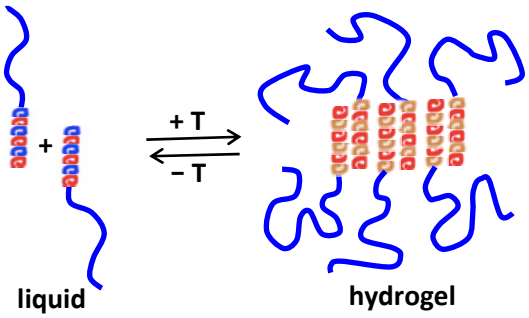


E Maximum cholesterol loading capacity in different DCH$_T$

Carrier	Cholesterol
PBS buffer	9.5x10^{-6} %
3% K$_{180}$L$_{20}$	2.0%
3% **9**	0.4%
3% **9+1**(1:1)	1.0%
3% **13**	1.5%
3% **14**	3.0%




140
120
100
80
60
40
20
0
% cell survival
*
Cell in media
2% $K_{180}L_{20}$
2% **9+1**(1:1)
3% **9+1(**1:1)



TOC