Polymer Chemistry

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ARTICLE

A Coacervate-forming Biodegradable Polyester with Elevated LCST based on Bis-(2-methoxyethyl)amine

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Recently, we reported a new class of biodegradable, thermoresponsive polyesters (TR-PEs) inspired by polyacrylamides and elastin-like proteins (ELPs). The polyesters exhibit tuneable cloud point temperatures (T_{cp}) and thermoresponsive coacervation in aqueous solution as shown via UV–vis spectroscopy, ^{1}H NMR, and DLS. However, the T_{cp} of all TR-PEs remained low (<15 °C), and higher thermoresponsivity would be beneficial for many applications. This study examines the synthesis, polymerization, and analysis of a new TR-PE bearing a more hydrophilic pendant group, bis-2-methoxyethylamine (bMoEtA). The resulting TR-PE, **TR-bMoEtAPE**, displays a threefold increase in T_{cp} (*ca.* 50 °C) that is affected by solution (DI water vs. phosphate buffered saline), concentration (1-40 mg mL^{-1}) molecular weight (20 – 130 kDa), and cosolutes (Hofmeister salts and urea). The T_{cp} and T_g of random **TR-bMoEtAPE** copolymers can be tuned via comonomer feed. Variable temperature ^{1}H NMR indicated a cooperative coacervation mechanism above T_{cp}, further reinforced by DLS measurements. As evidenced by UV–vis and SEC analysis, **TR-bMoEtAPE** underwent rapid degradation over a period of 7 days in DI water and PBS. Finally, cytotoxicity studies suggested that **TR-bMoEtAPE** is non-cytotoxic even at high concentrations (*ca.* 1000 μg mL^{-1}). The increased T_{cp} and tunability suggests **TR-bMoEtAPE** as a potential candidate for future functionalized TR-PE therapeutic-delivery systems.

Introduction

Recently, our lab reported the modular synthesis of a variety of *N*-functionalized diol monomers to generate 'peptide-like' polyesters in order to address the current lack of hydrophilic, synthetic peptidomimetic biomaterials.[1] Inspired by the structural similarity between 'peptide-like' polyesters and common thermoresponsive polymers such as poly(acrylamide)s (PAMs) and elastin-like peptides (ELPs), we then developed thermoresponsive polyesters (TR-PEs) which contain inherent biodegradability not commonly found in thermoresponsive biomaterials.[2]

Interestingly, TR-PEs did not undergo complete and efficient dehydration when brought above their respective Lower Critical Solution Temperatures (LCSTs), but instead exhibited a liquid-liquid phase separation to form stable polymer-rich coacervates. Generally, the term "coacervate" has been applied to any phase-separated solution in which a particular component presents itself richly in one phase, and poorly in another.[3-5] Coacervates are found extensively in nature, such as in the adhesive mechanism used by mussels[6,7] and in protective tube cement created by sandcastle worms,[8,9] and have been studied extensively as drug delivery agents.[10,11] Typically, coacervates are formed by the addition of a small molecule or colloid to a polyelectrolyte, or by the mixing of two oppositely changed polyelectrolytes (termed "complex coacervates" by Bungenberg de Jong and Kruyt[12]). Compared to complex coacervates, far fewer examples of thermoresponsive coacervates exist in literature. As compared to the vast majority of thermoresponsive polymers which undergo efficient dehydration and a liquid-solid phase transition above their LCST, coacervate-type thermoresponsive polymers undergo minimal conformational change above their LCST, limiting possible damage to sensitive biomolecules.[13,14] This makes coacervate-type thermoresponsive polymers particularly attractive for next-generation biomedical applications such as protein purification,[15] drug delivery,[16] and tissue engineering.[17,18] However, the cloud point temperature (T_{cp}, often used as an approximation of the LCST[19-22]) of all TR-PE homopolymers we previously reported remained in the low range of 0–15.8 °C, limiting their possible biomedical applications especially if polymerized with more hydrophobic comonomers.

In this work, we describe our efforts to synthesize a new biodegradable, coacervate forming TR-PE with increased LCST and explore its physical and thermal properties in detail. Ideally, the LCST should be significantly above body

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temperature (37 °C) so that the temperature-induced phase transition can be easily tuned by copolymerization. Our previous studies suggested that a hydrophilic tertiary amine would likely increase the LCST of the system. In order to achieve this, we were inspired by the rarely studied poly(alkoxyacrylamide) (PAOM), poly(*N,N*-bis(2-methoxyethyl)acrylamide) (LCST *ca.* 54 °C).[23,24] Bis-2-methoxyethylamine (**bMoEtA**) is a tertiary amine containing two alkoxy groups and was chosen as a starting material to generate a hydrophilic diol monomer. The resulting high M_n TR-PE, **TR-bMoEtAPE**, displayed a threefold increase in LCST as compared to the previous TR-PEs as well as a decrease in T_g. Thermoreversibility was observed to be nearly constant over a series of cycles and was affected by polyester concentration, molecular weight, cosolutes, and comonomer feed ratio. **TR-bMoEtAPE** exhibited similar coacervation behaviour as observed with previous TR-PEs and hydrolytically degraded over a period of seven days. Additionally, preliminary in vivo studies indicated that **TR-bMoEtAPE** was non-toxic even at high concentrations.

Experimental section

Materials

Ethyl succinyl chloride (98%), isopropylamine (iPrA, 99%), triethylamine (Et_3N, 98%), diethanolamine (DEA, 99%), sodium chloride (NaCl, 99%), sodium nitrate ($NaNO_3$, 98%), sodium bromide (NaBr, 97%), sodium iodide (NaI, 98%), sodium fluoride (NaF, 99%), sodium sulfate (Na_2SO_4, 99%), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \bullet H_2O$, 98%), sodium thiosulfate pentahydrate ($Na_2S_2O_3 \bullet 5H_2O$ 99%), and urea (98%) were purchased from Acros Organic and used as received. Succinic acid (99%) was purchased from Acros Organic and recrystallized from water before use. Bis(2-methoxyethyl)amine (bMoEtA**,** 98%), was purchased from TCI America and used as received. *N,N'*-diisopropylcarbodiimide (DIC, 99%) was purchased from Oakwood Chemical and used as received. N^1,N^1-bis(2-hydroxyethyl)-N^4-isopropylsuccinamide (**iPrADEA**) was synthesized according to previous literature procedures.[2] 4-(dimethylamino) pyridinium 4-toluene sulfonate (DPTS) was prepared according to literature methods.[25,26] Reagent grade dichloromethane (CH_2Cl_2) was purchased from Thermo Fisher Scientific and dried by distilling over anhydrous CaH_2. Methanol (MeOH) was used as received from Thermo Fisher Scientific. Silica gel (40-63 µm, 230 x 400 mesh) for flash chromatography was purchased from Sorbent Technologies, Inc. Dialysis tubing (regenerated cellulose, MWCO 3500 Da) and Promega CellTiter-Blue® cell viability assay were obtained from Thermo Fisher Scientific. Deionized water (DI H_2O) was used to prepare polymer solutions unless otherwise stated.

Characterization

All ^1H and ^{13}C NMR spectra in $CDCl_3$ of the monomers and polyesters were recorded on either a Varian Mercury 300 MHz or 500 MHz spectrometer. Chemical shifts were recorded in ppm (δ) relative to solvent signals. Variable temperature ^1H NMR spectra in D_2O were recorded on a Varian INOVA 400 MHz spectrometer with 5 min equilibrations at each temperature. Glass transition temperatures (T_g) of the polymers were determined using a TA Q2000 DSC with a liquid N_2 cooling unit and a heating/cooling rate of 10 °C min^{-1}. Polymer molecular weights were analysed on a TOSOH EcoSec HLC-8320 SEC equipped with a refractive index detector (RI) and UV detector. Separation occurred over two PSS Gram Analytical SEC Columns in series using 25 mM LiBr in DMF as eluent at a flow rate of 0.8 mL min^{-1}. The column and detector temperatures were maintained at 50 °C. Molecular weights were obtained relative to PMMA standards using the RI signal.

Coacervate analysis

To probe coacervation polyester concentration, aqueous polyester solutions (15 mg mL^{-1}) were prepared and left at 4 °C overnight. The solutions were then placed in a static 65 °C water bath for 30 min. Incubated samples were centrifuged at 3600 RPM for 5 minutes to achieve rapid phase separation. The heating/centrifugation process was repeated three times to ensure complete coacervation formation. The polyester-dilute supernatant was then carefully removed with a glass pipette leaving the polyester-rich coacervate phase at the bottom of the vial. The resulting coacervate was then weighed and lyophilized. The coacervate polyester concentration was determined using the ratio of the dried polyester weight to the weight of the coacervate as the average of three separate samples.

Cloud point measurements

Turbidity measurements were carried out on a Shimadzu UV-1800 UV–vis spectrophotometer equipped with a Shimadzu S-1700 thermoelectric single cell holder in a 1 cm quartz cell. Deionized water was used as a reference. Polymer solutions (10 mg mL^{-1} unless otherwise noted) were prepared in DI water and left at 4 °C overnight to ensure complete dissolution and equilibration. Solutions were equilibrated below the cloud point temperature (T_{cp}) until no change in transmittance was observed. Transmittance was recorded as a function of temperature at 1 °C min^{-1} and a fixed 500 nm wavelength. The T_{cp} for each experiment was defined as the temperature at which the transmittance was 50%.

Light Scattering

Dynamic Light Scattering (DLS) measurements of aqueous homopolymer solutions (0.5 mg mL^{-1}) were performed on a Brookhaven Inc. Laser light scattering spectrometer equipped with a temperature controlled and a solid state laser (λ = 532 nm, detection angle: 90°). The solutions were filtered through a 0.45 µm PVDF filter prior to measurements. The solutions were equilibrated for 30 min at each temperature. An intensity-intensity time correlation function was measured by means of a multichannel digital correlator, which was then processed using the CONTIN method to obtain the average hydrodynamic radius of the particles in solution. Increasing the temperature far above the T_{cp} over the long duration of the

experiment resulted in very turbid solutions that did not allow the laser of the Brookhaven Goniometer to fully transmit, as such experiments were only carried out while accurate scattering data could be obtained.

Degradation analysis

Aqueous polyester solutions (10.0 mg mL^{-1} in DI water) were placed in a static 65 °C incubator and removed at various time points. The polyester-dilute supernatant was then carefully removed with a glass pipette leaving the polyester-rich coacervate phase at the bottom of the vial. The resulting coacervate was rinsed with DI water, and lyophilized. The molecular weight of the dried coacervate was analysed via GPC.

Cytotoxicity studies

The effect of polymer on the viability of mammalian cells was performed using NIH-3T3 mouse embryonic fibroblasts. NIH-3T3 cells were cultured using growth medium composed of DMEM supplemented with 10% foetal bovine serum (Hyclone) and 1% penicillin-streptomycin (10,000 U mL^{-1}, Thermo Fisher Scientific) at 37 °C in a 5% CO_2 environment. For the experiment, the cells were expanded to *ca.* 75-85% confluence, harvested, and seeded into a tissue culture 96 well plate at a density of *ca.* 5,000 cells cm^{-2}. After allowing cells to adhere and grow overnight, the medium was replaced with polymer-supplemented medium. The growth media containing polymer was prepared by performing a serial dilution of a concentrated polymer stock solution (10 mg mL^{-1}) to make multiple concentrations of the polymer solution in PBS. Then, using the stock solution and the serially diluted solutions of polymer in PBS, growth media supplemented with 10% of the solutions were prepared. CellTiter-Blue® cell viability assay (n=6) was performed after allowing the cells to incubate for one day in the polymer supplemented media. The assay was performed by aspirating the medium from each well, rinsing the cells with warm PBS solution, and then adding growth medium supplemented with 20% of CellTiter-Blue® reagent. The well plate was incubated for 1 hr followed by fluorescence measurement at 560 nm/590 nm for each well on a Biotek Synergy 2 multimode microplate reader.

Synthesis of ethyl 4-(bis(2-methoxyethyl)amino)-4-oxobutanoate (bMoEtSA, Compound 1)

The ethoxy succinamide precursor was synthesized according to modified literature procedures.[1,27] A solution of bMoEtA (3.0 mL, 20.3 mmol, 1 eq.) and Et_3N (2.89 mL, 20.7 mmol, 1.02 eq.) in dry CH_2Cl_2 (30 mL) was cooled to 0 °C and purged with N_2 for 15 min with magnetic stirring. Ethyl succinyl chloride (2.89 mL, 20.3 mmol, 1 eq.) was added dropwise and the reaction became an opaque white solution. The reaction was brought to room-temperature and allowed to stir for 1 h under N_2. The solution was then added to DI water and extracted (3 x 40 mL CH_2Cl_2). The product was dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford the pure product as a pale oil (4.78 g, 18.3 mmol, 90%). The product was characterized via NMR. ^{1}H NMR (300 MHz; $CDCl_3$): δ 1.25 (t, J = 7.1 Hz, 3H), 2.73-2.60 (m, 4H), 3.32 (d, J = 6.9 Hz, 6H), 3.57-3.50 (m, 9H), 4.13 (q, J = 7.1 Hz, 2H). ^{13}C NMR (126 MHz; $CDCl_3$): δ 14.13, 28.00, 29.45, 46.43, 48.65, 58.69, 58.98, 60.35, 70.66, 71.10, 171.70, 173.14.

Synthesis of N^1,N^1-bis(2-hydroxyethyl)-N^4,N^4-bis(2-methoxyethyl)succinamide (bMoEtDEA, Compound 2)

A solution of **1** (3.89 g, 16.6 mmol, 1 eq.) and DEA (3.49 g, 33.2 mmol, 2 eq.) was allowed to heat at 80 °C overnight under magnetic stirring and reduced pressure. The crude compound was analysed via TLC (15% MeOH in DCM, ninhydrin). A small amount of unreacted ethoxy amide (R_f *ca.* 0.56) was observed along with the desired compound (R_f *ca.* 0.40). The crude mixture was purified via silica gel flash chromatography (10-20% MeOH in CH_2Cl_2). The product was dried under reduced pressure to afford pure monomer as a pale oil (4.25 g, 13.3 mmol, 80%). The product was characterized via NMR. ^{1}H NMR (300 MHz; $CDCl_3$): δ 2.71-2.67 (m, 2H), 2.87-2.83 (m, 2H), 3.32 (d, J = 10.1 Hz, 6H), 3.61-3.44 (m, 12H), 3.83 (dt, J = 9.0, 4.6 Hz, 4H); ^{13}C NMR (126 MHz; $CDCl_3$): δ 14.13, 28.00, 29.45, 46.43, 48.65, 58.69, 58.98, 60.35, 70.66, 71.10, 171.70, 173.14.

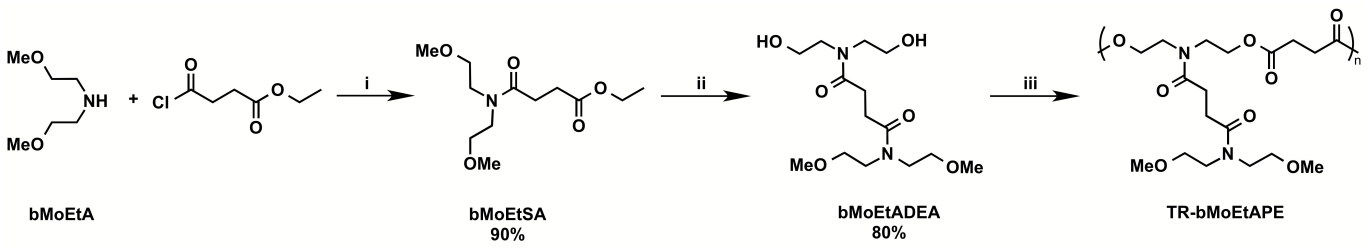
General Polymerization conditions

A solution of **2** (1.33 g, 4.56 mmol, 1 equiv.), succinic acid (538 mg, 4.56 mmol, 1 equiv.), and DPTS (533 mg, 1.82 mmol, 0.4 equiv.) in dry CH_2Cl_2 (4.5 mL, 1 mL per 100 mg succinic acid) was purged with N_2 for 15 min with magnetic stirring. The mixture was briefly heated to reflux to homogenize the solution. The reaction was cooled to 0 °C and DIC (2.2 mL, 13.7 mmol, 3 eq.) was added dropwise. The reaction was allowed to come to room-temperature and stir for 48 h under N_2. The crude reaction mixture was diluted with CH_2Cl_2 and the diisopropyl urea by-product was filtered off. The dilute mixture was then concentrated under reduced pressure, solvated in a minimal amount of MeOH, and purified via dialysis against MeOH for 24 h with solvent changes at 3 h, 6 h, and 16 h. The dialysis mixture was dried under reduced pressure to obtain pure polyester as a white amorphous solid. The resultant polyester was characterized by SEC, DSC, TGA, and NMR. Recovered = 1.12 g, M_n = 113.5 kDa, $Đ_M$ = 1.58, T_g = -20 °C, T_{deg} = 315 °C. ^{1}H NMR (300 MHz; $CDCl_3$): δ 2.66 (dq, J = 20.0, 6.2 Hz, 8H), 3.31 (d, J = 7.0 Hz, 6H), 3.70-3.47 (m, 12H), 4.22 (dt, J = 13.5, 6.3 Hz, 4H).

Results and discussion

Synthesis and characterization of monomer and polyesters

As previously reported, *N*,*N*-bis(hydroxyethyl) *N*-(alkyl)succinamide (HESA) monomers were used to synthesize TR-PEs with a maximum T_{cp} of 15.8 °C resulting from the overall hydrophobic-hydrophilic balance of the polyesters. The design of a more hydrophilic monomer was required in order to increase T_{cp} to more biologically relevant temperatures. It was seen that in these polyesters, TR-PEs containing tertiary amides exhibited a higher T_{cp} than those based on secondary



Scheme 1. Synthetic route for the preparation of TR-bMoEtAPE. Reagents and conditions: (i) Et_3N, CH_2Cl_2, 0 °C to room-temperature, 1 h. (ii) DEA, neat, 80 °C, vacuum, 16 h. (iii) SA, DIC, DPTS, CH_2Cl_2, 0 °C to room-temperature, 48 h.

amides, a trend not always observed in PAMs. Generally, amide hydrophilicity follows 3° < 2° < 1°. It is commonly accepted that increasing the overall polarity of a thermoresponsive polymer will increase the LCST. However, synthesizing monomers containing polar hydroxyl, amine, primary amide, and/or carboxylic acid functionality was decided against as the nucleophilic group would likely increase the degradation rate of the polyester backbone. Additionally, such a strategy would also require the use of protection-deprotection chemistry to prevent side reactions during the polymerization. Instead, the tertiary alkoxyamine bMoEtA, a structural analogue of diethylamine (starting amine for **TR-dEtAPE**, T_{cp} = 11.8 °C) bearing two methoxy groups, was chosen as a starting material. It was hypothesized that the introduction of polar oxygen atoms would further increase the hydrophilic nature of the monomer, thereby shifting the hydrophobic/hydrophilic balance of the polyester and increasing the overall LCST. bMoEtA was reacted with succinyl chloride to generate an *N*-(alkoxy)succinamide ester intermediate (**bMoEtSA**, 92%), which was then heated in the presence of diethanolamine and purified via silica flash chromatography to yield **bMoEtADEA** monomer (80%). By performing this reaction under vacuum, displaced ethanol from the transamidation was removed, driving the reaction forward and increasing the yield as compared to the previous TR-PE monomer synthesis.

As demonstrated by Stupp, Meyer, and others,[28-35] carbodiimide-mediated coupling has been shown to be an effective means of synthesizing high molecular weight polyesters at room-temperature using a simple procedure (Scheme 1). In a typical polymerization, the **bMoEtADEA** diol succinic acid, and DPTS were taken into anhydrous CH_2Cl_2. The reaction was cooled to 0 °C and DIC was added. The mixture was then allowed to stir for 24–48 h at room-temperature, with longer reaction times generally leading to higher molecular weight polymers. A majority of diisopropyl urea by-product was then filtered off. As the monomers, oligomers, polymer, DPTS catalyst, and diisopropyl urea exhibited similar solubility in common solvents, purification by dialysis against MeOH was used to obtain pure polymers as tacky, low T_g elastomeric materials (65% polymer recovery). NMR confirmed purity of **TR-bMoEtAPE** (Fig. 1).

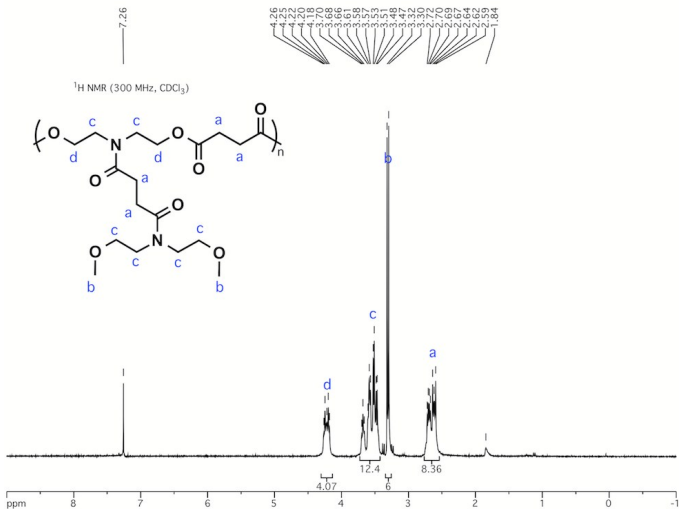


Fig. 1 ^{1}H NMR spectra of TR-bMoEtAPE (300 MHz, $CDCl_3$).

Thermoresponsive behaviour

As previously mentioned, above the LCST most thermoresponsive polymers undergo a relatively efficient dehydration, leading to a solid-liquid phase separation which is easily observed via DSC. However, certain polymers such as ELPs, poly(phosphoester)s (PPEs), and polar poly(acrylamides) (PAMs) and poly(pyrrolidone)s (PVPs) exhibit an inefficient dehydration above their LCST.[36-42] The resulting liquid-liquid phase separation results in the formation of a polymer-rich coacervate phase and polymer-poor aqueous phase. Thermally induced inefficient dehydration is often unobservable via DSC, so T_{cp} was used to quantify the approximate LCST.

Using temperature controlled UV–vis, the sharp (occurring over *ca.* 3.5 °C) thermal response of **TR-bMoEtAPE** (132 kDa) was explored. As can be seen in Fig. 2A, $T_{cp,\ heat}$ was observed at 48.2 °C and $T_{cp,\ cool}$ was observed at 39.6 °C. Hydrogen bonding in thermoresponsive polymers have been shown to be a primary cause of thermal hysteresis (i.e., the difference between $T_{cp,\ heat}$ and $T_{cp,\ cool}$) as intra- and intermolecular hydrogen bonds form between the dehydrated polymer chains, making resolvation difficult.[43] Although **TR-bMoEtAPE** contains only hydrogen bond accepting groups, a thermal hysteresis of 8.6 °C was observed. This value is greater than that of the previously reported TR-PEs and is likely due to the significant amount of water present in the semi-dehydrated coacervate phase. The water molecules likely form non-covalent intra- and intermolecular hydrogen bonded bridges between the many oxygen and nitrogen atoms present in polymer chains, making rehydration more difficult. A similar mechanism has been proposed for thermoresponsive PVPs.[44]

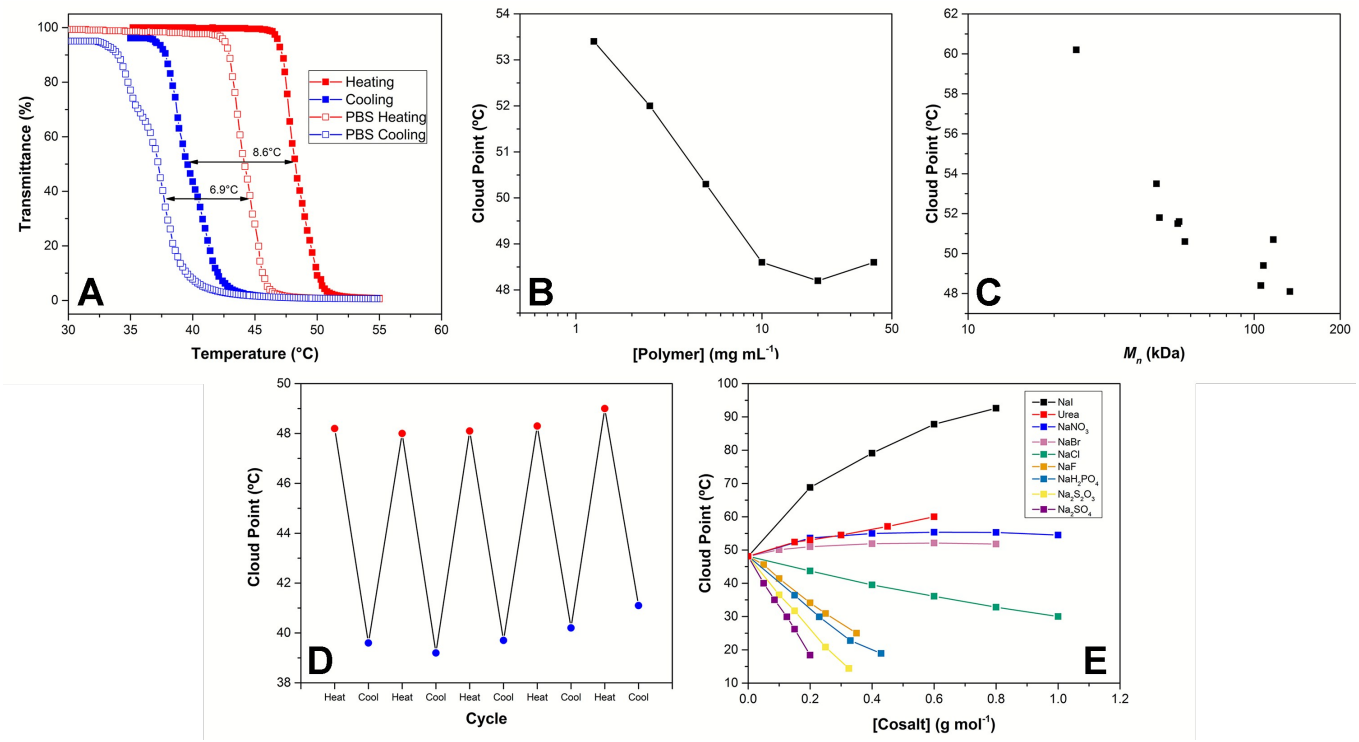


Fig. 2 T_{cp} investigations of TR-bMoEtAPE (132 kDa) using UV–vis (500 nm, 1 °C min^{-1} heat/cool, 10 mg mL^{-1} PE solution unless otherwise noted) (A) UV–vis spectra of TR-bMoEtAPE (132 kDa) showing hysteresis of initial heating and cooling cycles. (B) The effect of TR-bMoEtAPE concentration on T_{cp}. (C) The effect of molecular weight on T_{cp}. (D) Thermal reversibility of TR- bMoEtAPE over six cycles. (E) The effect of urea and various Hofmeister anions on T_c

Effects of concentration

The effect of polymer concentration on T_{cp} varies for different materials and should be understood when T_{cp} is to be used as an approximation for LCST. Typically, the T_{cp} of PAMs are not significantly affected by concentration, whereas poly(alkyloxazoline)s (PAOxs) and ELPs are much more dependent.[21] Likewise, previous TR-PEs were shown to be highly dependent on solution polymer concentration.[2] As the solution polymer concentration increases, more polymer is available to form aggregates. This decreases the time required for dehydrated polymer globules to come together and form aggregates large enough to scatter light at the desired UV–vis wavelength. As expected, increasing TR-bMoEtAPE concentration resulted in an earlier onset of T_{cp} ranging from 48.6 °C to 53.4 °C (Fig. 2B). Similar to previous TR-PEs, above 10 mg mL^{-1} the **TR-bMoEtAPE** concentration had little effect on T_{cp} and so this was chosen as the standard concentration for UV-Vis T_{cp} measurements.

Effect of molecular weight

Since polymer molecular weight can influence solution behaviour, samples of **TR-bMoEtAPE** with varying molecular weights were prepared. As shown in Fig. 2C, the T_{cp} of **TR-bMoEtAPE** displays a logarithmic dependence on molecular weight, with low molecular weight samples exhibiting the highest T_{cp} similar to what was observed to previous TR-PEs.[2] This is likely due to the polyester chain ends, likely polar hydroxyl or carboxylic acid groups, having a greater influence on the overall hydrophilicity of **TR-bMoEtAPE** resulting in an increased T_{cp}. Additionally, higher molecular weight **TR-bMoEtAPEs** likely exhibit increased hydrophobic polymer-polymer interactions, resulting in a decreased T_{cp}.

Thermal cycle testing

Thermal cycling showed reversible transitions between coacervate and water-soluble states with clear hysteresis between phase transitions. As seen in Fig. 2D, the T_{cp} remained relatively constant after several cycles. The $T_{cp,\ heat}$ increased from 48.2 °C to 49.0 °C, the $T_{cp,\ cool}$ increased from 39.6 °C to 41.1 °C, and the thermal hysteresis decreased from 8.6 °C to 7.9 °C. These data indicate that the temperature-induced phase transition of **TR-bMoEtAPE** is relatively stable and that the polymer does not undergo significant degradation, crosslinking, or removal from solution during the experimental timeframe.

Effects of additives

Since the first report in 1888,[45] Hofmeister salts have been widely studied for their influence on aqueous biological processes such as enzyme activity,[46-49] protein-protein interactions,[50,51] and protein stability.[52-54] Recently, increasing attention has been paid to the interaction of Hofmeister salts on synthetic water-soluble materials, especially thermoresponsive polymers.[55,56] Anions within the Hofmeister series are generally classified according to their ability to salt-out (kosmotropes, "disorder maker") or salt-in (chaotropes, "order-maker") a macromolecule in a solution. The LCST of

thermoresponsive polymers can be modulated by Hofmeister ions: chaotropic salts increase the LCST, while kosmotropic salts decrease the LCST.[55,56]

While previous theories to explain this behaviour dealt with the effects of Hofmeister ions on bulk water properties, Cremer and co-workers put forth a possible mechanism for both poly(isopropylacrylaminde) (PNIPAM) and ELPs which involve the ions interacting directly with the polymers in three possible ways: destabilization of hydrogen bonding between water and polar groups through directed ionization of water by the anion (salting-out), an increased hydrophobic effect as salt is added to the solution (salting-out), and by direct binding of the anion to polar groups (salting-in).[57,58] The effect of these interactions depends on ion size and charge density and generally follows the trend: $CO_3^{2-} > SO_4^{2-} > S_2O_3^{2-} > H_2PO_4^{2-} > F^- > Cl^- > Br^- > I^- > ClO_4^- > SCN^-$, with CO_3^{2-} acting as the strongest kosmotrope (supress LCST) and SCN^- acting as the strongest chaotrope (raise LCST).

To this end, the effects of Hofmeister sodium salts on **TR-bMoEtAPE** was explored. As shown in Fig. 2E, the NaI, $NaNO_3$, and NaBr were shown to have a chaotropic effect, stabilizing the polymer in solution at elevated temperature. The chaotropic effect followed the expected relationship with Hofmeister anions, with NaI showing the greatest ability to salt-in **TR-bMoEtAPE**. Likewise, the kosmotropes NaCl, NaF, NaH_2PO_4, $Na_2S_2O_3$, and Na_2SO_4 were likewise shown to decrease the stability of hydrated **TR-bMoEtAPE** chains in solution, resulting in a lowered T_{cp} related to the kosmotropic strength of the anion. The only Hofmeister salt investigated in our previous studies, NaCl, was shown to have a greater effect on lowering T_{cp} for **TR-bMoEtAPE** than was observed for earlier TR-PEs. This is likely due to the hydrogen bonds between water and the ether groups in **TR-bMoEtAPE** being more easily disrupted by NaCl than those between water and amides.[37] No T_{cp} was observed for Na_2CO_3 and $NaClO_4$ at any concentration, possibly as a consequence of rapid hydrolytic degradation of the polyester in the basic solution.

The effect of urea on the thermoresponsive properties of **TR-bMoEtAPE** was also explored. Previously, the T_{cp} of TR-PEs were shown to increase with increasing solution concentrations of urea, indicating that urea stabilized the solvated chains. A similar magnitude T_{cp} increase is seen for **TR-bMoEtAPE** with increasing solution concentration of urea. The increase in T_{cp} with urea for TR-PEs is comparable to that observed with ELPs, but opposite of the T_{cp} depression reported for PNIPAM.[59] This is especially interesting since PNIPAM is often used as a model for investigating the cold denaturation of proteins and the effects that additives play in that process. Recently, Feng and co-workers used urea-polymer Nuclear Overhauser Effect (NOE) measurements of PNIPAM and poly(diethylacrylamide) (PDEAM) to show that the type of amide as well as the size of hydrophobic domains determine whether or not urea will stabilize the solvated chain and increase T_{cp}, (PDEAM), or further dehydrate the polymer globule and decrease T_{cp} (PNIPAM).[60] Although similar factors likely affect the stabilizing behaviour urea demonstrates for **TR-bMoEtAPE** chains, the more complex structure of the

Table 1 Characterization of TR-(bMoEtA-*r*-iPrA)PE Copolyesters

bMoEtA : iPrA	M_n[a] (kDa)	T_{cp}[b] (°C)	T_g[c] (°C)
1.0 : 0	45.6	53.4	-7.51
0.75 : 0.25	59.1	39.8	-3.85
0.50 : 0.50	61.8	27.2	-0.45
0.25 : 0.75	61.1	13.3	7.31
0 : 1.0	56.5	7.3	10.0

[a]Determined by DMF SEC relative to PMMA standards. [b]Defined as 50% transmission during temperature controlled UV–vis analysis. [c]Determined by DSC.

polymer as compared to the PAMs makes direct comparison difficult. It is likely that the many predominantly hydrogen bond accepting groups (tertiary amides, ethers) of **TR-bMoEtAPE** results in solvating monovalent urea-polymer interactions as opposed to desolvating divalent interactions (i.e., ones where urea acts as a bridge between intra- and intermolecular hydrogen bonding sites). Our lab is currently exploring the use of UV–vis and NOE measurements to better understand urea-TR-PE interactions.

Copolymerization Results

The LCST of a thermoresponsive polymer is affected by the overall hydrophobic-hydrophilic balance and as such can be tuned by copolymerization of monomers with differing hydrophilicity. This is especially true for random copolymers, even those that form coacervates.[13,61] The LCST of non-interacting copolymers is always between the LCST of the respective homopolymers can be fitted using the formula:[62]

$$T_{cp,co} = \mu_1 T_{cp,1} + \mu_2 T_{cp,2} \quad (1)$$

where μ_n is the mole fraction of each monomer ($\mu_1 + \mu_2 = 1$), $T_{cp,\,n}$ is the T_{cp} of the corresponding homopolymer, and $T_{cp,\,co}$ is the calculated copolymer T_{cp}. As previously reported, the T_{cp} of TR-PE copolymers of similar molecular weight could be tuned

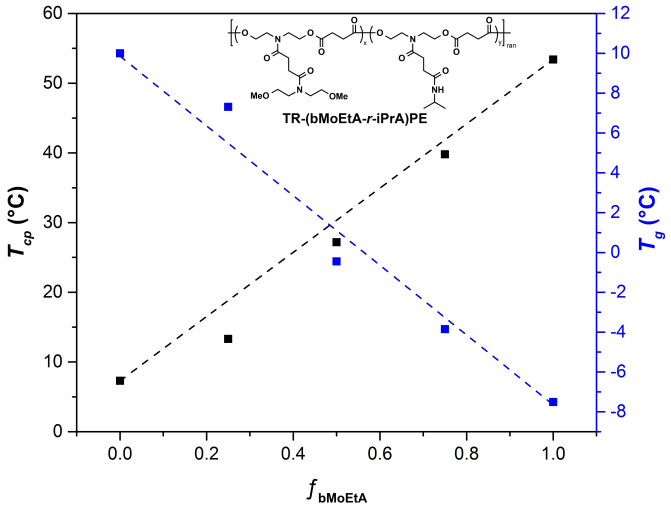


Fig. 3 Effect of copolyester composition on T_{cp} and T_g for a series of TR-(bMoEtA-*r*-iPrA) polyesters. Dashed lines represents theoretical values using the Fox equation (blue) and best fit (black).

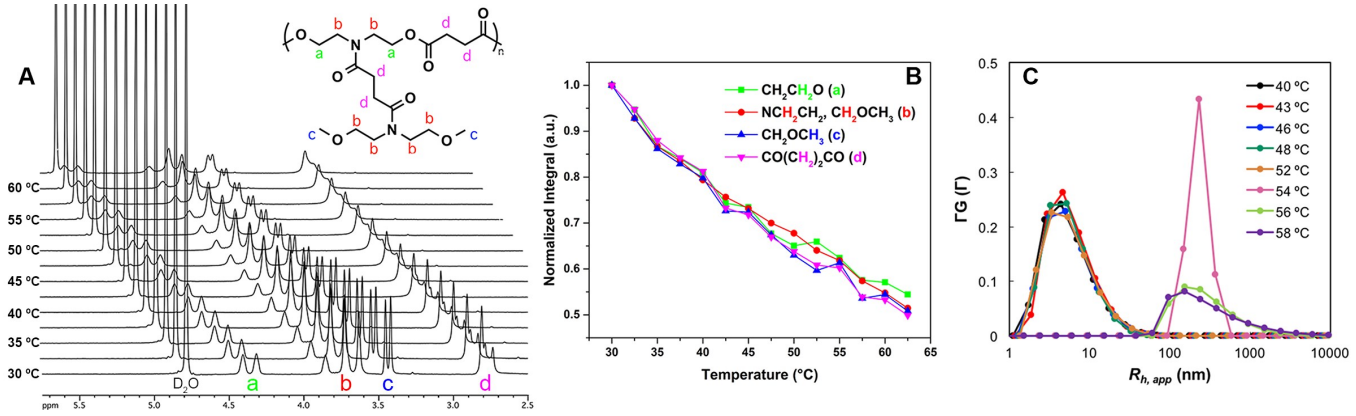


Fig. 4 (A) Variable temperature ^{1}H NMR spectra of TR-bMoEtAPE (400 MHz, D_2O, T_{cp} = 48.1 °C) above and below cloud point, (B) normalized proton integral signals, and (C) CONTIN analysis of the DLS data of TR-bMoEtAPE (T_{cp} = 53.5 °C) above and below the cloud point.

in a linear fashion from 7.8 °C to 15.9 °C based on monomer feed ratio. It was hypothesized that a similar effect would be seen for copolymers of similar molecular weight presenting bMoEtA and isopropylamine-based iPrA (**TR-iPrAPE** T_{cp} = 7.8 °C) pendant groups. A linear correlation between T_{cp} and monomer feed was expected. As shown in Fig. 3, increasing the content of more hydrophilic **bMoEtADEA** increased the overall hydrophilicity of the polymer, thereby increasing the T_{cp} with good agreement to the predicted values.

Interestingly, the T_g of the copolyesters was seen to be inversely correlated to the cloud point temperature. The T_g decreased with increasing bMoEtA content and appeared to follow the Fox equation:

$$\frac{1}{T_g} = \frac{n_1}{T_{g,1}} + \frac{n_2}{T_{g,2}} \quad (2)$$

where n is the mole fraction of each monomer in the copolymer (in the traditional Fox equation, n is the weight fraction of each polymer in a miscible blend) and T_g is the glass transition temperature of the corresponding homopolymer (Fig. 3,). The correlation is likely due to a number of factors. With increasing amounts of bMoEtA in copolyester composition, a decrease in hydrogen bond donating secondary iPrA amides decreases polymer-polymer interactions resulting in a reduction of T_g. Additionally, the T_g is likely reduced with increasing amounts of flexible bMoEtA methoxy branches as the polymer free volume increases and disrupts packing. This represents significant increase in overall thermal and physical tunability for the TR-PE system, as the range of T_{cp} could be tuned theoretically in the range of 0–55 °C. Furthermore, the T_g of the TR-PEs eliminates the possibility of forming glassy material above the LCST, a drawback for many thermoresponsive polymers such as PNIPAM (T_g *ca.* 140 °C).

Coacervate Analysis

It was predicted that **TR-bMoEtAPE** would form polymer-rich coacervates above the LCST due to their structural similarities to previous TR-PEs. Centrifugation of **TR-bMoEtAPE** solutions above the T_{cp} resulted in the formation of a dense, transparent liquid phase, not a precipitate, indicating a coacervation-type dehydration. Variable temperature ^{1}H NMR was used to further verify the coacervation response. As seen in Fig. 4A, peaks corresponding to the backbone and pendant protons decreased with increasing temperature. The disappearance of an individual signal typically occurs when local dehydration occurs, resulting in a lack of segmental mobility and can give insight as to the mechanism of temperature-induced phase transition.[63,64] However, even above the T_{cp}, no proton signals completely disappeared. This suggested that the polymer was still flexible and well hydratedabove the LCST, indicative of coacervate-type demixing. From the normalized proton integral signals (Fig. 4B), the mechanism of dehydration was partially explored similar to that for other thermoresponsive polymer systems.[63,65] Between 30–62.5 °C, the signals corresponding to the protons of the pendant OCH_3, backbone CH_2O, and those adjacent to the amide and carbonyl groups exhibited relatively constant reduction in signal, indicating a disruption in hydrogen bonding between those protons and D_2O. However, even far above the T_{cp} at 62.5 °C, all four signals remained in the range of 50-55% of the original value. These data suggest that the various domains of **TR-bMoEtAPE** dehydrate in a cooperative manner above the LCST, leading to coacervation as polymer chains are brought together by hydrophobic and polar interactions. Cooperative dehydration above the LCST is further reinforced by the sharp T_{cp} transition range (*ca.* 3.5 °C).

The polymer concentration of **TR-bMoEtAPE** coacervates was determined to be 36.3%. This is lower than the previously investigated TR-PEs (44.2–65.4%), where it was observed that T_{cp} was inversely correlated to the coacervate concentration of polymer. Above the LCST, the more hydrophilic **TR-bMoEtAPE** likely undergoes an even less efficient dehydration than previous TR-PEs, resulting in increased water content and decreased polymer content in the coacervate.

The sizes of **TR-bMoEtAPE** coacervates at different temperatures was examined using DLS. A lower molecular weight **TR-bMoEtAPE** (45.6 kDa, T_{cp} = 53.5 °C) was used in order to eliminate any molecular weight effect when making comparisons to previous TR-PEs. Generally, previous TR-PE coacervate size (102–226 nm) was seen to increase with T_{cp} and coacervate water content. As shown in Fig. 4C, below LCST the polymers exist primarily as solvated unimers with $R_{h,\,app}$ = *ca.* 8 nm. As the solution is heated above the LCST, the $R_{h,\,app}$

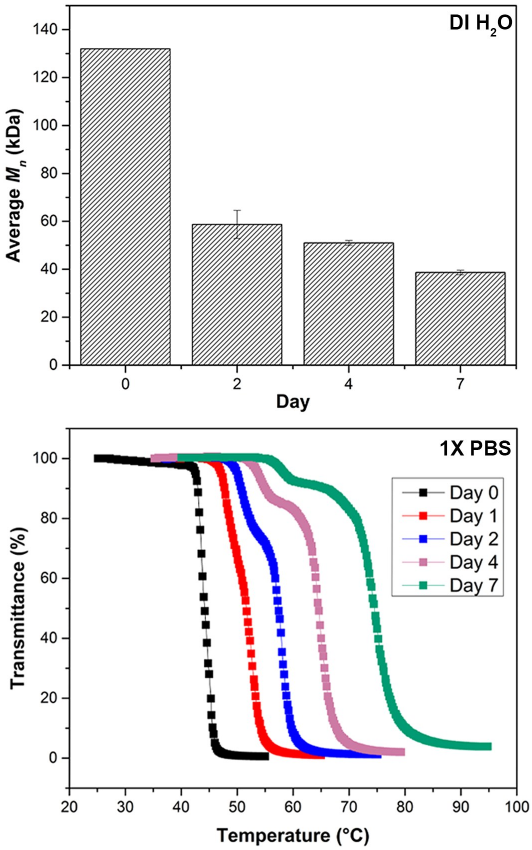


Fig. 5 Top: Hydrolytic degradation via SEC of TR-bMoEtAPE incubated 37 °C in DI water; n = 3. Bottom: hydrolytic degradation via UV–vis of TR-bMoEtAPE incubated at 37 °C in 1X PBS buffer.

increases to 231 nm at 54 °C as initial coacervates form. The observed LCST from DLS matches relatively well with the T_{cp}. Interestingly, the more hydrophilic **TR-bMoEtAPE** coacervates were in the same size range as the previously reported pyrrolidine-based **TR-PyrAPE** (56.5 kDa, T_{cp} = 15.8 °C, 226 nm, polymer coacervate concentration = 44.2%) coacervates despite the increased coacervate water content.

Polymer Degradation

Previous work in our lab has shown that polyesters based on succinic acid and *N*-substituted diols degrade relatively quickly. At 37 °C in DI water, the 'peptide-like' polyester p(mAla) degraded to 66% of the starting M_n after 7 days,[1] while the more hydrophilic TR-PE polyester **TR-dEtAPE** degraded to 30% of the original M_n.[2] As shown in Fig 5, under the same conditions **TR-bMoEtAPE** was observed to degrade from 132 kDa to an average of 38.7 kDa, or 29.3% of the original M_n. The magnitude of degradation was similar to that of other TR-PEs.

It has been shown that lower molecular weight TR-PEs have higher T_{cp} and hence polymer degradation can be monitored by change in T_{cp}. A solution of **TR-bMoEtAPE** in PBS buffer was incubated at 37 °C to explore degradation under biological conditions. Over a period of 7 days, a clear shift in T_{cp} from 44.2 °C to 74.5 °C was observed, with the

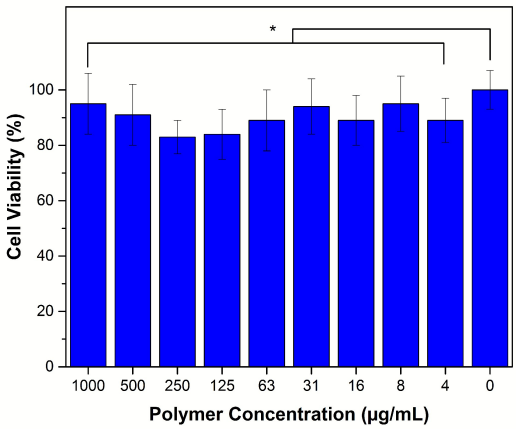


Fig 6. Cell viability of TR-bMoEtAPE against NIH 3T3 cells, 1 day; n = 6.

transmittance curve at longer degradation times beginning to show a two-step transmittance transition. As the T_{cp} of TR-PEs exhibit a significant dependence on both concentration and molecular weight below 20 kDa, the observed change in transmittance suggests the presence of highly variable molecular weight degradation products whose temperature-induced dehydration are independent of one another.[66] The multi-step transmittance likely occurs as follows: below the LCST, all **TR-bMoEtAPE** chains are soluble. As the temperature is raised, the higher molecular weight chains begin to dehydrate first. The hydrophobic dehydrated chains begin to aggregate only with other high molecular weight dehydrated chains, not with hydrated lower molecular weight chains. The dehydrated aggregated high molecular weight chains begin to scatter light and cause initial drop in transmittance. The magnitude of the transmittance drop is thus indicative of both the molecular weight of the dehydrated chains and total number of chains of that molecular weight. The second drop occurs as the more soluble lower molecular weight chains are brought above their LCST, resulting in a drop to near-zero transmittance as nearly all chains in solution, regardless of molecular weight, are dehydrated, aggregated, and scattering light.

Cell Viability

Since **TR-bMoEtAPE** was designed as a possible biomaterial, cell viability studies were used to probe possible toxicity. **TR-bMoEtAPE** was incorporated into cell growth medium at various concentrations and added to proliferating NIH 3T3 mouse embryonic fibroblast cells. After 1 day of growth in the presence of **TR-bMoEtAPE**, cell viability was probed. As shown in Fig 6, even at high concentrations (1 mg mL^{-1}) of **TR-bMoEAPE**, the cell viability was not statistically different (one-way ANOVA Tukey's test, F(9,49)=0.46, p=0.8909) between solutions containing polymer and the non-inoculated control sample. The implied non-cytotoxicity of **TR-bMoEtAPE** is a good starting point to investigate its potential for biomedical applications.

Conclusions

In this work, we have expanded the previously described TR-PE system through the synthesis of a hydrophilic monomer based on **bMoEtA**. The resultant polyester, **TR-bMoEtAPE**, displays a sharp and highly reversible T_{cp} around 50 °C, a three-fold increase as compared to our previous TR-PEs. In addition, TR-bMoEtAPE has low T_g, displays fast hydrolytic degradation and shows coacervation-type dehydration behaviour. Variable temperature NMR indicates temperature-induced dehydration is a cooperative event. Moreover, the thermal and physical properties are highly tuneable and predictable via copolymerization with other TR-PE monomers. Preliminary cell viability experiments suggest that **TR-bMoEtAPE** is non-cytotoxic even at high concentrations. The above features collectively represent significant advantages for several biomaterial applications. Most notably, the degradable nature of the polyester enables its use for *in-vivo* applications. The ability to tune the T_{cp} by copolymerization enables tailoring the onset of coacervate behaviour. In addition, the incomplete dehydration in the coacervate phase can provide a protective environment for sensitive encapsulated biomolecules. These features present the potential of using such thermoresponsive polyesters as matrices for fabricating releasable cell sheets, as injectable depots and carriers for therapeutics, especially sensitive protein biologics.

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Notes and references

‡ Additional NMR spectra of monomers and polymers, as well as SEC degradation curves, are included in the supporting information.

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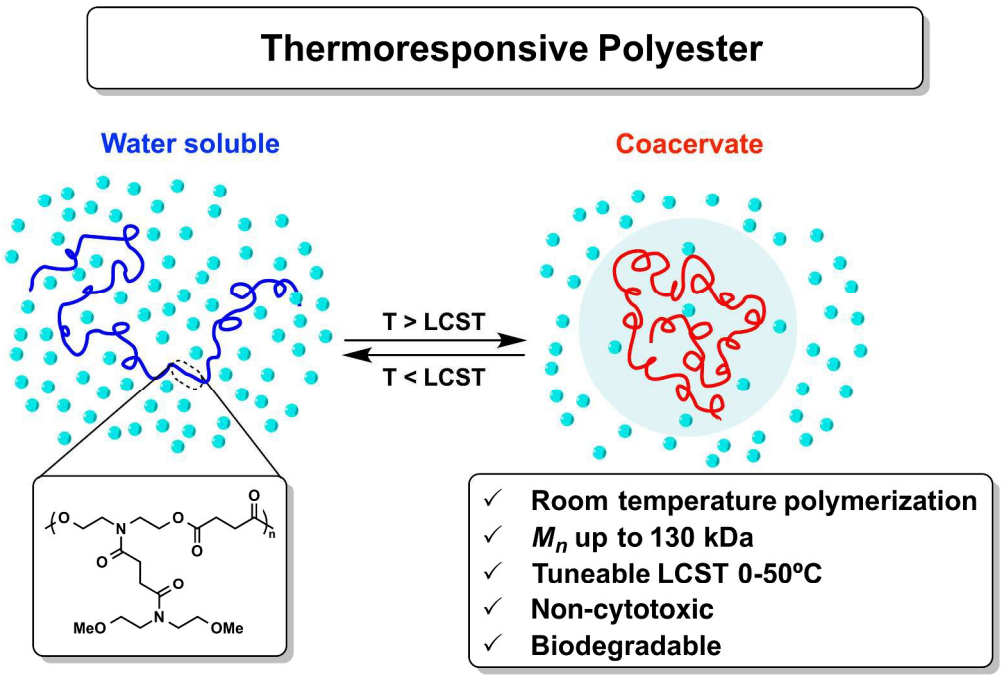

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