8-17-2020

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Citation of this paper:

Rabiee Kenaree, Amir; Sirianni, Quinton E. A.; Classen, Kyle; and Gillies, Elizabeth R., "Thermoresponsive Self-Immolative Polyglyoxylamides." (2020). *Chemistry Publications*. 186.
<https://ir.lib.uwo.ca/chempub/186>

Thermo-Responsive Self-Immulative Polyglyoxylamides

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Abstract

Thermo-responsive polymers with lower critical solution temperatures (LCSTs) are of significant interest for a wide range of applications from sensors to drug delivery vehicles. However, the most widely investigated LCST polymers have non-degradable backbones, limiting their applications *in vivo* or in the environment. Described here are thermo-responsive polymers based on a self-immolative polyglyoxylamide (PGAM) backbone. Poly(ethyl glyoxylate) was amidated with six different alkoxyalkyl amines to afford the corresponding PGAMs and their cloud point temperatures (T_{cps}) were studied in water and buffer. Selected examples with promising thermo-responsive behavior were also studied in cell culture media and their aggregation behaviour was investigated using dynamic light scattering (DLS). The T_{cps} were effectively tuned by varying the pendent functional groups. These polymers depolymerized end-to-end following the cleavage of end-caps from their termini. The structures and aggregation behavior of the polymers influenced

their rates of depolymerization, and in turn the depolymerization influenced their T_{cp} . Cell culture experiments indicated that the polymers exhibited low toxicity to C2C12 mouse myoblast cells. This interplay between LCST and depolymerization behaviour, combined with low toxicity, make this new class of polymers of particular interest for biomedical applications.

Keywords

Self-immolative, depolymerization, cloud point, thermo-responsive

Introduction

Thermo-responsive polymers,¹⁻² which exhibit lower critical solution temperature (LCST) behaviour, have attracted significant attention due to their potential for fabricating smart materials including actuators,³ plasmonic sensors,⁴ batteries,⁵ drug delivery vehicles,⁶ and scaffolds for tissue engineering.⁷ For example, poly(*N*-isopropylacrylamide) (PNIPAM) is the most extensively studied thermo-responsive polymer, with a cloud point temperature (T_{cp}) of *ca.* 32 °C in water (Figure 1a).⁸⁻¹⁰ Thermo-responsive poly[oligo(ethylene glycol) (meth)acrylate]s [POEG(M)As, Figure 1b] have also been widely explored.¹¹⁻¹³ They are promising candidates for biological applications as their pendent groups are based on oligo(ethylene glycol) and the low toxicity of ethylene glycol-based polymers is well established.¹⁴ POEGMAs exhibit reversible transitions in different environments and their T_{cps} can be synthetically tuned. For example, for methacrylate-based backbones, increasing the number of ethylene glycol units in the pendent groups from 2 to *ca.* 10 increased the T_{cp} from 28 to 90 °C.¹²

Figure 1. Thermo-responsive polymers: (a) PNIPAM and (b) POEG(M)A. (c) General chemical structure of PGAMs and their depolymerization to glyoxylamide hydrates following end-cap cleavage (Init = polymerization initiator; EC = end-cap).

While PNIPAM and POEGMAs exhibit thermo-responsive behaviour near the physiological temperature of 37 °C, they have fully carbon-carbon backbones, so they are not considered biodegradable *in vivo* and would also be expected to degrade slowly in the environment. To address this challenge, degradable polymers exhibiting LCST behavior have also been explored. For example, degradable ester linkages were incorporated into the backbones of POEGMAs through copolymerization reactions using 5,6-benzo-2-methylene-1,3-dioxepane, and their degradation into smaller fragments in KOH solution and in the presence of lipases was demonstrated.¹⁵ Reducible disulfide linkages were incorporated into PNIPAM through the polycondensation of telechelic PNIPAM that was prepared by reversible addition-fragmentation chain transfer polymerization.¹⁶ Elastin-like peptides, which undergo temperature-dependent aggregation and enzymatic degradation have also been investigated.¹⁷ Moieties imparting thermo-responsivity have been introduced as pendent groups to degradable backbones such as polyesters¹⁸⁻

¹⁹ and chitosan.²⁰ Furthermore, polyacetals with pH-sensitive degradation behavior and tunable T_{cp} values were synthesized from diols and divinyl ethers based on oligo(ethylene glycol).²¹ However, in all of these examples, the polymers degraded by multiple random backbone cleavage events, resulting in their gradual breakdown into lower molar mass polymers. In addition, very few studies have investigated the effects of degradation on T_{cp} and vice versa.^{16, 18-19}

Self-immolative polymers (SIPs) are a recently developed class of degradable polymers, which undergo end-to-end depolymerization when their end-caps are removed by stimuli such as enzymes, light, changes in pH, or other specific chemical species.²²⁻²³ The most investigated backbones thus far include polycarbamates,²⁴⁻²⁵ poly(benzyl ether)s,²⁶ polyphthalaldehydes,²⁷⁻²⁸ and polyglyoxylates.²⁹ SIPs have been investigated for their potential as sensors,³⁰ drug delivery vehicles,³¹ patterned devices,³² recyclable plastics³³ and composites.³⁴ Despite their unique degradation pathways relative to conventional degradable and stimuli-responsive polymers, to the best of our knowledge, thermo-responsive SIPs have not yet been developed and studied. Described here is the synthesis of a series of self-immolative polyglyoxylamides (PGAMs) with tunable structures and LCST behaviour, the measurement of their T_{cps} under different conditions, and studies of their degradation behavior. PGAMS depolymerize through the sequential fragmentation of terminal hemiacetals after end-cap cleavage, a reaction that propagates down the entire polymer backbone (Figure 1c). We demonstrate that the structure and T_{cp} affect the rate of end-cap cleavage and depolymerization, and that the depolymerization also affects T_{cp} . The polymers are also shown to exhibit low cytotoxicity, demonstrating their potential for further exploration in biomedical applications.

Experimental

Polymer nomenclature. The polymers were named as **P(Pendent group)-End-cap**, where **P** denotes polymer. Pendent groups are abbreviated as: **MeMEG** = methoxy mono(ethylene glycol); **MeDEG** = methoxy di(ethylene glycol); **EtMEG** = ethoxy mono(ethylene glycol); **EtDEG** = ethoxy di(ethylene glycol); **PrMEG** = propoxy mono(ethylene glycol); **MeMPG** = methoxy mono(propylene glycol). End-caps are abbreviated as: **Trit** = triphenylmethyl; **Bom** = benzyloxymethyl.

General materials. All reactions and manipulations were carried out under a nitrogen atmosphere using standard Schlenk techniques unless otherwise stated. All reagents were used as received unless otherwise stated. Ethyl glyoxylate solution (*ca.* 50% in toluene) was obtained from Alfa Aesar and purified according to a previously published procedure.³⁵ Triphenylmethyl end-capped poly(ethyl glyoxylate) (**PEtG-Trit**) was synthesized as previously reported.³⁶ Citric acid was obtained from Alfa Aesar. NaOH was obtained from Fisher Scientific. Phosphate buffered saline (PBS, pH 7.4) was prepared from sachets of premixed salts (SKU No. P38135, Sigma-Aldrich) according to the manufacturer's instructions. Ultrapure water was obtained from a Barnstead EASYpure II system. Chloromethyl benzyl ether (technical, *ca.* 60%), LiBr, *n*-BuLi solution (2.5 M in hexanes), 2-methoxyethylamine (99%), 3-methoxypropylamine (99%), 2-(2-ethoxyethoxy)ethanamine ($\geq 95\%$), and 2-(2-methoxyethoxy)ethanamine ($\geq 95\%$), and all cell culture reagents were obtained from Sigma-Aldrich. 2-Ethoxyethylamine (95–98%) and 2-propoxyethylamine (95–98%) were purchased from Aurora Fine Chemicals LLC. 1,4-Dioxane, acetone, methanol, and chromatography-grade *N,N*-dimethylformamide (DMF) were obtained

from Caledon Laboratories. NEt_3 was purchased from Fisher Scientific and distilled over CaH_2 . Toluene was purchased from Caledon Laboratories and distilled over Na/benzophenone.

General procedures. For nuclear magnetic resonance (NMR) spectroscopy, D_2O and CDCl_3 were purchased from Sigma-Aldrich and used as received. ^1H and ^{13}C NMR spectra were recorded using a 400 MHz Bruker AvIII HD 400 instrument and referenced to residual CHCl_3 (7.26 ppm), HOD (4.79 ppm) or CDCl_3 (77.2 ppm). Fourier transform-infrared (FT-IR) spectra were recorded using a PerkinElmer Spectrum Two FT-IR spectrometer with an attenuated total reflectance (ATR) attachment and a single reflection diamond. Size-exclusion chromatography (SEC) was performed on an instrument equipped with a Waters 515 HPLC pump with a Waters In-Line Degasser AF, two PLgel mixed D 5 μm (300×1.5 mm) columns connected to a corresponding PLgel guard column, and a Wyatt Optilab Rex RI detector. Polymer solutions (at a concentration of *ca.* 5 mg/mL) in DMF containing LiBr (10 mM) and NEt_3 (1% v/v) were filtered (using 0.2 μm polytetrafluoroethylene syringe filters) before they were injected (using a 50 μL loop) and run at a flow rate of 1 mL/min for 30 min at 85 $^\circ\text{C}$ in the same solvent as an eluent. Molar masses were determined by comparison to poly(methyl methacrylate) (PMMA) standards purchased from Viscotek.

Synthesis of PEtG-Bom. In a Schlenk flask, freshly distilled toluene (20 mL) and an *n*-BuLi solution (200 μL of 2.5 M in hexanes, 0.50 mmol, 1.0 equiv) were combined at room temperature and vigorously stirred for 3 min. The flask was then instantly charged with freshly distilled ethyl glyoxylate (5.0 mL, 50 mmol, 100 equiv.) and stirred for another 10 min before cooling the solution to -20 $^\circ\text{C}$ and stirring at that temperature for 20 min. Then, freshly distilled NEt_3 (0.30 mL, 2.2 mmol, 4.4 equiv.) was added and the resulting solution was stirred for 10 min before the addition of chloromethyl benzyl ether (0.30 mL of 60%, *ca.* 1.3 mmol, *ca.* 2.6 equiv.). The

resulting mixture was stirred for another 3 h, at $-20\text{ }^{\circ}\text{C}$, then it was allowed to gradually reach $20\text{ }^{\circ}\text{C}$, over 16 h. Concentration of the polymerization mixture *in vacuo* at $45\text{ }^{\circ}\text{C}$ gave crude residue. The residue was dissolved in CH_2Cl_2 (10 mL) and slowly added to a vigorously stirring methanol/water mixture (4/1; 250 mL). The flask was then sealed and transferred into a $-20\text{ }^{\circ}\text{C}$ freezer where it was kept for 16 h before decanting the solvent and drying *in vacuo* the resulting purified residue. Yield = 3.9 g, 76%. ^1H NMR (CDCl_3 , 400 MHz): δ 7.31 (br s, 5 H), 5.46–5.76 (m, 110 H), 4.86–5.04 (m, 2 H), 4.21 (br s, 224 H), 1.28 (br s, 346 H), 0.87 (br s, 3 H). $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3 , 100 MHz): δ 165.0–166.9, 128.5, 128.0, 90.8–94.4, 62.2, 14.0. FT-IR: 2990, 1750 cm^{-1} . SEC (DMF, PMMA): $M_n = 8.9\text{ kg/mol}$, $M_w = 13.3\text{ kg/mol}$, $D = 1.5$.

Synthesis of P(MeMEG)-Trit and representative PGAM synthesis procedure (details for additional polymers are in the supporting information). In air, a pressure tube (25 mL) was charged with **PEtG-Trit** (270 mg of polymer, 2.6 mmol of ester, 1.0 equiv.), 2-methoxyethanamine (900 mg, 12 mmol, 4.6 equiv.), and 1,4-dioxane (3.0 mL) before it was sealed and heated for 40 h at $70\text{ }^{\circ}\text{C}$. The crude mixture was dialyzed against acetone (1.0 L) using a 6–8 kg/mol molecular weight cut-off membrane (Spectra/Por, regenerated cellulose) for 40 h (solvent was changed once after 16 h). The PGAM solution was then concentrated and the resulting residues were dried *in vacuo* for 16 h. Yield = 200 mg, 58%. ^1H NMR (CDCl_3 , 400 MHz): δ 7.59–8.70 (m, 1 H), 5.74 (br s, 1 H), 3.05–3.71 (m, 7 H). $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3 , 100 MHz): δ 166.5–167.9, 129.0, 128.0, 127.7, 96.7, 58.7, 39.4. FT-IR: 3290, 3080, 2990, 2930, 2890, 2830, 1670, 1540 cm^{-1} . SEC (DMF, PMMA): $M_n = 12.0\text{ kg/mol}$, $M_w = 20.0\text{ kg/mol}$, $D = 1.8$.

NMR depolymerization studies. Polymer samples (10.0 mg/mL) were dissolved in D_2O or buffers made from D_2O (deuterated PBS or 0.1 M pH 3.0 citrate buffered D_2O) and incubated at ambient temperature ($20\text{ }^{\circ}\text{C}$) for 13 days. ^1H NMR spectra were obtained periodically at either 25

°C [P(EtDEG)-Trit, P(MeMPG)-Trit, P(EtDEG)-Bom, P(MeMPG)-Bom] or 5 °C [P(EtMEG)-Trit, P(EtMEG)-Bom]. The degradation percent values were calculated by comparison of the intensity of the backbone methine peak (CH) of polymers (broad peak at *ca.* 5.5 ppm) and that of the CH peak of the resulting hydrate depolymerization product (sharp peak at *ca.* 5.1 ppm).

***T_{cp}* measurements.** The measurements were obtained using a Varian UV-Vis Cary 300 instrument equipped with a temperature controller unit. Polymer solutions (10.0 mg/mL) were prepared by dissolving the polymers in water, buffer, or Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) (at 4 °C), then filtering through 0.2 µm Nylon syringe filters. Then, they were placed inside low-volume quartz cuvettes and their absorbance values, at 600 nm, were recorded after every 1 °C temperature change while they were being gradually heated (1 °C/min) to maximum temperatures and subsequently cooled (1 °C/min) to the initial temperature (see Figures S42–S64 and S86–S89 for the specific temperature ranges used). The *T_{cp}* was taken as the temperature at which 50% of the initial transmittance was observed. For polymers where *T_{cp}* was observed, the solution was diluted 2-fold to 5.00 mg/mL and the *T_{cp}* measurement was repeated. The dilution and measurement steps were repeated down to 1.25 mg/mL. Three runs were performed on P(MeMPG)-Bom at 10.0 mg/mL to determine the reproducibility of the *T_{cp}* measurements.

Dynamic light scattering (DLS). DLS measurements were obtained using a Malvern Zetasizer Nano ZS instrument equipped with a 633 nm laser and at a scattering angle of 173°. The position and attenuation of the light source were fixed between measurements to allow for direct comparisons of the measured count rates. Polymer solutions (1.25 mg/mL) were prepared by dissolving the polymers in PBS (at 4 °C), then filtering through 0.2 µm Nylon syringe filters and

placing them inside low volume polystyrene cuvettes. For monitoring over temperature ranges, measurements were taken every 1 °C, with a 2 minute equilibration time at each temperature before the measurement was taken. For monitoring over time, the instrument was set to the T_{cp} of the polymer that was previously determined by DLS monitoring of the polymer solution over temperature. The polymer solution was kept below its T_{cp} and was quickly inserted into the instrument and measured in 100 s intervals, with no additional delays between measurements.

Cell metabolic activity assays. C2C12 mouse myoblast cells were thawed and cultured as previously described.³⁷ The culture media consisted of DMEM (500 mL) supplemented with 10 mL of penicillin-streptomycin (1000 units/mL), 5 mL of L-glutamine (200 mM) and 50 mL of FBS. The cells were then seeded in a Nunclon 96-well U bottom transparent polystyrol plate to obtain approximately 10,000 cells/well in 100 μ L of media and were allowed to adhere to the plate in a 5% CO₂ incubator at 37 °C for 24 h. The growth media was then aspirated from the cells and replaced with either solutions of sodium dodecyl sulfate (SDS) in the cell culture media at concentrations of 0.20, 0.15, 0.10, or 0.050 mg/mL, which were used as positive controls, serial 2-fold dilutions of polymer in culture media ranging from 1.0 mg/mL to 7.8 μ g/mL, or fresh media as a negative control. The cells were then incubated at 37 °C (5% CO₂) for 24 h. The media was again aspirated and replaced with 110 μ L of fresh media containing 0.5 mg/mL (3-(4,5-dimethylthiazol-2-yl)- 2,5- diphenyltetrazolium bromide) (MTT). After 4 h of incubation (37 °C, 5% CO₂), the MTT solution was carefully aspirated and the purple crystals were dissolved by the addition of 50 μ L of spectroscopic grade dimethyl sulfoxide (DMSO). After shaking (1 s, 2 mm amp, 654 rpm), the absorbance of the wells at 540 nm was read using an M1000-Pro plate reader (Tecan). The absorbance of wells prepared in the same way but without cells was subtracted as a background and the metabolic activity was calculated relative to the negative control. No

metabolic activity was detected for cells exposed to the highest concentrations of SDS, confirming the sensitivity of the assay.

Results and Discussion

Polymer synthesis. To synthesize the target thermo-responsive PGAMs, PEtGs with either a pH-sensitive triphenylmethyl (trityl) end-cap (**PEtG-Trit**) or stable benzyloxymethyl (Bom) end-cap (**PEtG-Bom**) were first prepared by *n*-BuLi initiated polymerization of ethyl glyoxylate in toluene at $-20\text{ }^{\circ}\text{C}$,³⁵ followed by end-capping with either trityl chloride³⁶ or benzyl chloromethyl ether respectively (Scheme 1). The polymers were characterized by ^1H and ^{13}C NMR spectroscopy, FT-IR spectroscopy, and SEC in DMF relative to PMMA standards. **PEtG-Trit** had a number average molar mass (M_n) of 8.8 kg/mol, degree of polymerization (DP_n) of *ca.* 86, and dispersity (D) of 1.5, while **PEtG-Bom** had an M_n of 8.9 kg/mol, DP_n of *ca.* 87, and D of 1.5. Next, to obtain a library of PGAMs with different structures and T_{cp} values, the pendent ester groups on **PEtG-Trit** and **PEtG-Bom** were reacted with different alkoxyalkyl amines (Scheme 1). These amidation reactions were performed in 1,4-dioxane solutions of amine and PEtG at 60–70 $^{\circ}\text{C}$ for 16 h (shorter, less sterically hindered amines) to 72 h (longer, more sterically hindered amines). The resulting PGAMs were isolated in 47–94% yield. NMR spectroscopy showed complete disappearance of the peaks corresponding to the pendent ethyl ester groups (Figures S1–S26) and FT-IR spectra showed disappearance of the carbonyl absorption bands of the starting ester groups (1750 cm^{-1}) and appearance of peaks at *ca.* 1670 cm^{-1} corresponding to the resulting amides (Figures S27–S39). SEC showed that the DP_n and D values for the PGAMs remained similar to those of the starting polymers, confirming that the amidation reactions did not substantially affect the polymer backbones (Table 1, Figures S40–S41).

Table 1. Molar mass and DP_n data obtained from SEC and T_{cp} values determined by turbidimetry for the polymers (data for **P(PrMEG)-Trit**, and **P(PrMEG)-Bom** are in the supporting information). ND = not detected ($T_{cp} > 80$ °C). NM = not measured because no T_{cp} was detected at the higher concentration or in the case of culture media (CM) because only selected polymers were evaluated.

	P(MeMEG)-Trit	P(MeMEG)-Bom	P(MeDEG)-Trit	P(MeDEG)-Bom	P(EtMEG)-Trit	P(EtMEG)-Bom	P(EtDEG)-Trit	P(EtDEG)-Bom	P(MeMPG)-Trit	P(MeMPG)-Bom
M_n (kg/mol)	12.0	14.6	18.0	14.7	12.6	14.0	16.0	17.4	12.4	13.7
M_w (kg/mol)	20.0	23.1	30.0	27.0	23.5	20.8	31.0	26.4	21.7	19.6
$\bar{D}$	1.8	1.6	1.7	1.8	1.9	1.5	1.9	1.5	1.8	1.4
DP_n	92	111	103	84	87	96	85	92	85	94
T_{cp} , water, 10.0 mg/mL (°C)	66	ND	ND	ND	14	38	45	49	33	45
T_{cp} , water, 5.0 mg/mL (°C)	ND	ND	NM	NM	17	41	48	52	36	46
T_{cp} , water, 2.5 mg/mL (°C)	NM	ND	NM	NM	20	ND	ND	58	39	47
T_{cp} , water, 1.25 mg/mL (°C)	NM	ND	NM	NM	24	ND	ND	ND	43	48
T_{cp} , PBS, 10.0 mg/mL (°C)	52	ND	65	ND	12	26	39	41	28	42
T_{cp} , PBS, 5.0 mg/mL (°C)	64	NM	ND	NM	16	25	41	41	32	43
T_{cp} , PBS, 2.5 mg/mL (°C)	ND	NM	NM	NM	19	28	42	42	36	44
T_{cp} , PBS, 1.25 mg/mL (°C)	NM	NM	NM	NM	22	31	44	44	40	44
T_{cp} , CM, 10.0 mg/mL (°C)	NM	NM	NM	NM	NM	23	NM	40	NM	40

Cloud point measurements. The T_{cp} values for the polymers were first measured in water and in PBS, to examine the effects of biologically relevant salt concentrations. The polymer solutions were prepared at 10.0 mg/mL concentration, filtered at 4 °C, then their transmittance at 600 nm as a function of temperature was measured at a heating rate of 1 °C/min. The temperature corresponding to 50% transmittance was taken as the T_{cp} . Three runs performed on one polymer under the same conditions indicated a standard deviation of less than 1 °C on the T_{cp} values (Figure S62).

Previously, POEGMA with di(ethylene glycol) (DEG) pendent groups had a T_{cp} of *ca.* 28 °C at 3.0 mg/mL in water.¹² In contrast, **P(MeDEG)-Trit** had a T_{cp} of greater than 80 °C in water and 65 °C in PBS at 10.0 mg/mL (Table 1). The higher T_{cp} of the PGAM likely arises from its higher hydrophilicity, as the backbone acetal oxygens and pendent amides can participate in hydrogen bonding with water. The lower T_{cp} in PBS than in water is common for thermo-responsive polymers and has been attributed to the salting-out effect.³⁸ **P(MeDEG)-Bom** did not have a T_{cp} below 80 °C in either water or PBS, indicating that the trityl end-cap played a role in the overall hydrophobicity of **P(MeDEG)-Trit**, lowering its T_{cp} .

To lower the T_{cp} values into a more biologically relevant range, PGAMs with mono(ethylene glycol) (MEG) pendent groups were investigated next. In PBS at 10.0 mg/mL, **P(MeMEG)-Trit** had a T_{cp} of 52 °C, compared 65 °C for **P(MeDEG)-Trit**, showing the effect of shortening the pendent oligo(ethylene glycol) chain. Lowering the concentration of **P(MeMEG)-Trit** in PBS to 5.0 mg/mL resulted in an increase in T_{cp} to 64 °C. Decreasing the polymer concentration has been found previously to increase the T_{cp} of some thermo-responsive polymers, which may be due in part to the slower aggregation of polymer chains in more dilute solutions.³⁹

In addition, **P(MeMEG)-Trit** had a T_{cp} of 66 °C in water. However, **P(MeMEG)-Bom** did not have a detectable T_{cp} in either water or PBS.

To further lower the hydrophilicity of the pendent groups and consequently T_{cp} , **P(EtMEG)-Trit**, **P(EtMEG)-Bom**, **P(PrMEG)-Trit**, and **P(PrMEG)-Bom** with longer ethyl and propyl hydrophobic tails, were examined. **P(PrMEG)-Trit** and **P(PrMEG)-Bom** were so hydrophobic that they did not dissolve in water at 4 °C. In contrast, **P(EtMEG)-Trit** had T_{cps} of 12 and 14 °C in PBS and water respectively at 10.0 mg/mL. These values increased to 22 and 24 °C respectively as the polymer concentration was decreased to 1.25 mg/mL. **P(EtMEG)-Bom** demonstrated similar behaviour but with higher T_{cps} of 26–31 °C in PBS due to the end-cap effect. The fact that **P(EtMEG)** has a T_{cp} below the physiological temperature of 37 °C across a range of concentrations and with different end-caps is particularly interesting for biomedical applications. For example, thermo-responsive polymers can be used to produce injectable formulations that exist as soluble polymers at low temperature (e.g., in the fridge), but spontaneously gel through aggregation *in vivo*.⁴⁰

Further tuning of T_{cp} was achieved through the use of DEG in combination with an ethyl tail in **P(EtDEG)-Trit** and **P(EtDEG)-Bom**. The presence of an additional ethylene glycol unit in the pendent groups made the DEG analogues more hydrophilic, leading to T_{cps} of 39 and 41 °C in PBS and 45 and 49 °C in water at 10.0 mg/mL for **P(EtDEG)-Trit** and **P(EtDEG)-Bom** respectively, about 30 °C higher than their corresponding MEG analogues **P(EtMEG)-Trit** and **P(EtMEG)-Bom**. Interestingly, these DEG polymers showed little sensitivity to the identity of the end-cap or to concentration in PBS, with **P(EtDEG)-Trit** and **P(EtDEG)-Bom** both having T_{cps} of 44 °C at 1.25 mg/mL. This property is particularly useful as it suggests that different end-caps can be used to enable triggering of degradation by different stimuli. In addition, the polymers

should retain their thermo-responsiveness upon dilution. Furthermore, the presence of a T_{cp} just above physiological temperature should make these polymers useful for applications such as thermally-triggered drug release, which could be induced either through direct heating or magnetic hyperthermia.⁶

Mono(propylene glycol) (MPG) pendent groups with methyl tails were also investigated. The corresponding polymers, **P(MeMPG)-Trit** and **P(MeMPG)-Bom**, had T_{cp} values of 28 and 42 °C respectively in PBS and 33 and 45 °C in water respectively at 10.0 mg/mL. The variation in the T_{cp} values across the different media was relatively minimal but a substantial end-cap effect was observed for these polymers. In terms of concentration dependence, **P(MeMPG)-Trit** exhibited concentration dependent T_{cp} values, which increased to 40 °C in PBS and 43 °C in water at 1.25 mg/mL. However, the T_{cp} values of **P(MeMPG)-Bom** were relatively insensitive to concentration, increasing from 42 to 44 °C in PBS and from 45 to 48 °C in water as the concentration was decreased from 10.0 to 1.25 mg/mL. Though more pronounced for the MPG derivatives, the Bom end-capped PGAMs tended to exhibit less concentration dependence than the trityl end-capped PGAMs overall. As the trityl group can be considered as a highly hydrophobic moiety, concentration dependent intermolecular interactions may be important in the early phases of aggregation for the trityl series. It should also be noted that the methoxy-MPG pendent group is a structural isomer of the ethoxy-MEG pendent group. This structural change resulted in *ca.* 18 °C increase of the T_{cp} values for **P(MeMPG)-Trit** compared to **P(EtMEG)-Trit** across different concentrations and conditions. This result can likely be attributed to the higher dipole moment and polarity of the methoxy-MPG substituents, which is in agreement with the higher boiling point of methoxypropylamine (117 °C) compared to ethoxyethylamine (105 °C).

Finally, the thermo-responsive behaviour of **P(EtMEG)-Bom**, **P(EtDEG)-Bom**, and **P(MeMPG)-Bom** in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (FBS) was investigated, to understand how the polymers would behave in cell culture media containing proteins. These polymers were selected as they exhibited T_{cp} s close to ambient and physiological temperatures. For each polymer, the T_{cp} was within 1–3 °C of that measured in PBS, showing a minimal effect of culture media components such as proteins (from FBS), inorganic salts, amino acids, glucose, and vitamins. In addition, each polymer exhibited a reversible transition with negligible (*ca.* 1 °C) hysteresis (Figures 2a, S63, S64). These properties are favourable, as they indicate that the polymers should exhibit relatively predictable thermo-responsive behaviour. In contrast, T_{cp} of PNIPAM copolymers were previously found to be highly sensitive to the presence of serum proteins.⁴¹

Figure 2. (a) Thermo-responsive behaviour of **P(MeMPG)-Bom** in water, pH 7.4 PBS, and cell culture media containing FBS, showing dependence on the solvent/media. Minimal hysteresis was observed in the presence of FBS; (b) Thermo-responsive behaviour of **P(MeMPG)-Bom** in pH 3.0 citrate buffer at different points showing minimal change in the T_{cp} ; (c) Thermo-responsive behaviour of **P(MeMPG)-Trit** in pH 3.0 citrate buffer at different points showing an increase in the T_{cp} as the polymer degrades.

DLS. To further understand the aggregation behaviour of the polymers below, at, and above their cloud points, six of the synthesized polymers with cloud points closest to ambient and physiological temperature [**P(EtMEG)-Trit**, **P(EtMEG)-Bom**, **P(EtDEG)-Trit**, **P(EtDEG)-Bom**, **P(MeMPG)-Trit**, **P(MeMPG)-Bom**] were investigated using DLS. First, solutions of these polymers in PBS (1.25 mg/mL) were monitored for changes in the Z average diameters and mean scattering count rates as the temperature was increased from below to above the T_{cp} . Below the T_{cp} , the solutions comprised mainly molecularly dissolved polymers, as indicated by diameters well below 100 nm (Figure S65). However, some polymers including **P(EtDEG)-Trit** and **P(EtDEG)-Bom** exhibited some tendency to aggregate below the T_{cp} , as indicated by an increase the scattering count rate, likely due to their amphiphilic structures. It is possible that this tendency to aggregate explains the lower concentration and end-cap dependence of these polymers compared to the other derivatives. At temperatures very similar to the T_{cp} values measured in the turbidimetry experiments for each polymer, there was a rapid increase in diameter to micro-sized aggregates and corresponding increase in the count rate. When the solutions were incubated over time at the T_{cp} , again initially dissolved polymers and nanoscale assemblies were observed (Figure 3, S66). Over 200–800 s these transformed first to larger nano-sized aggregates, and then to micron-sized aggregates.

Figure 3. (a) Z average diameter and mean count rate of **P(EtDEG)-Bom** at 43 °C in PBS (1.25 mg/mL) over time. The solution temperature was the T_{cp} of the polymer solution as determined previously by DLS (Figure S65); (b) The intensity distribution of diameters in the solution at different time points showing the conversion of dissolved polymers and nanoscale assemblies into large micron-sized aggregates over time.

Depolymerization. Depolymerization of the PGAMs was examined to understand how their thermo-responsive properties would impact their degradation rates and how their degradation would affect their T_{cp} values. Again, we chose **P(EtMEG)-Trit**, **P(EtDEG)-Trit**, and **P(MeMPG)-Trit** and their non-stimuli-responsive analogues **P(EtMEG)-Bom**, **P(EtDEG)-Bom**, and **P(MeMPG)-Bom** as they had T_{cp} values closest to ambient and physiological temperatures. 10.0 mg/mL solutions of the polymers in D₂O, deuterated PBS (pH 7.4), and deuterated citrate buffer (pH 3.0) were placed in NMR tubes, which were then sealed and stored at 20 °C. pH 7.4 was selected to mimic neutral physiological conditions. pH 3.0 was selected to achieve sufficient responsiveness of the trityl end-cap, while mimicking physiological environments such as the stomach. Their depolymerization behaviour was monitored *via* ¹H NMR spectroscopy at different time intervals by integrating the peaks associated with the methine (CH) proton of the hydrate depolymerization product (Figure 1) at *ca.* 5.1 ppm and the methine proton of the PGAM backbone at *ca.* 5.5 ppm. As **P(EtMEG)-Trit** and **P(EtMEG)-Bom** were insoluble in the aqueous media at room temperature ($T_{cps} < 20$ °C) their spectra were obtained at 5 °C to ensure dissolution of both the polymer and degradation products for accurate quantification of the degradation. The spectra for the other systems were obtained at the standard instrument operating temperature of 25 °C.

In D₂O (Figure 4a), **P(EtDEG)-Trit** and **P(MeMPG)-Trit** degraded most rapidly, with *ca.* 15% depolymerization after 13 days. This depolymerization occurs as a result of gradual cleavage

of the trityl end-cap, even under neutral conditions.³⁶ On the other hand, **P(EtMEG)-Trit** depolymerized more slowly, with only 7% degradation over the same time period. Under the degradation conditions (10.0 mg/mL, 20 °C), **P(EtMEG)-Trit** would be in an aggregated state which may slow degradation, as we and other groups have found the depolymerization of self-immolative polymers to be slower in the solid state compared to solution.^{29, 42-43} All of the control polymers **P(EtMEG)-Bom**, **P(EtDEG)-Bom**, and **P(MeMPG)-Bom** exhibited less than 1% depolymerization in D₂O over 13 days, showing that the backbone is inherently stable under these conditions, and that depolymerization of the trityl end-capped polymers can indeed be attributed to end-cap cleavage followed by end-to-end depolymerization rather than random backbone cleavage. Very similar results were obtained in pH 7.4 PBS (Figure 4b), except that, like **P(EtMEG)-Trit** (above its T_{cp}), **P(EtDEG)-Trit** was also slower than **P(MeMPG)-Trit** despite being below its T_{cp} (39 °C). This result may arise from the tendency of **P(EtDEG)-Trit** to self-assemble, even below its T_{cp} (Figure S65), which would make the trityl end-cap less accessible for hydrolytic cleavage.

Figure 4. Degradation of **P(EtMEG)-Trit**, **P(EtMEG)-Bom**, **P(EtDEG)-Trit**, **P(EtDEG)-Bom**, **P(MeMPG)-Trit**, and **P(MeMPG)-Bom** in (a) D₂O, (b) deuterated pH 7.4 PBS, and (c) deuterated pH 3.0 (0.1 M) citrate buffer, calculated by ¹H NMR spectroscopy at different time intervals. All degradation studies were performed at 20 °C, while spectra for **P(EtDEG)** and **P(MeMPG)**

polymers were obtained at 25 °C and those for **P(EtMEG)** polymers were obtained at 5 °C due to their T_{cp} s being less than 25 °C. The depolymerization rate depended on the medium and polymer structure.

In pH 3.0 citrate buffer, the depolymerization rate was faster for all of the trityl end-capped polymers compared to the other conditions (Figure 4c). This result can be attributed to the sensitivity of the trityl end-cap to the acid stimulus. Consistent with the other conditions, **P(MeMPG)-Trit** degraded most rapidly, with about 70% depolymerization over 13 days. **P(EtMEG)-Trit** and **P(EtDEG)-Trit** degraded at similar rates, with about 30% depolymerization over 13 days. These behaviours can be rationalized in the same manner as for the pH 7.4 results. While these rates of depolymerization are relatively slow due to the limited pH-sensitivity of the trityl group, the rate of trityl end-cap cleavage can be increased by the introduction of electron-donating substituents on the phenyl rings. All of the Bom end-capped polymers exhibited negligible degradation over 13 days, showing that the backbone is very stable, even at pH 3.0. Overall, the end-cap, pendent group structure, and T_{cp} of the polymer influence the depolymerization rate.

We previously found that the rate of PGAM depolymerization was limited by the rate of end-cap cleavage.³⁶ Depolymerization was fast following end-cap cleavage and partially depolymerized polymers were not observed by SEC. The current depolymerization kinetics data for the trityl end-capped polymers fit well with a pseudo-first-order kinetics model. This analysis suggests that end-cap cleavage is indeed the rate limiting step for these polymers as well, since for self-immolative polymers that undergo slow depolymerization after end-cap cleavage, the kinetics are instead pseudo-zero-order in the early phases of depolymerization.⁴⁴ Comparison of the

pseudo-first-order rate constants (k) confirmed the qualitative trends and indicated that depolymerization was four to seven-fold faster at pH 3.0 than at pH 7.4 and two to seven-fold faster than in D₂O (Table 2).

Table 2. Pseudo-first-order rate constants (k , s⁻¹) for the depolymerization of **P(EtMEG)-Trit**, **P(EtDEG)-Trit**, and **P(MeMPG)-Trit** in D₂O, deuterated PBS, and deuterated pH 3.0 citrate buffer.

	P(EtMEG)-Trit	P(EtDEG)-Trit	P(MeMPG)-Trit
D₂O	5.6×10^{-8}	1.5×10^{-7}	1.4×10^{-7}
PBS	7.3×10^{-8}	3.9×10^{-8}	1.4×10^{-7}
pH 3.0 Citrate Buffer	3.1×10^{-7}	2.8×10^{-7}	1.0×10^{-6}

The effect of depolymerization on T_{cp} was also studied by performing turbidimetry measurements for **P(EtMEG)-Trit**, **P(EtMEG)-Bom**, **P(EtDEG)-Trit**, **P(EtDEG)-Bom**, **P(MeMPG)-Trit**, and **P(MeMPG)-Bom** over time in pH 3.0 citrate buffer (10.0 mg/mL). In each case, the Bom end-capped polymers exhibited less than 3 °C change in T_{cp} over 15 days (Figures 2b, S86, S88). In contrast, substantial changes in T_{cp} of 9–13 °C were observed for the trityl end-capped PGAMs (Figure 2c, S87, S89). For example, **P(MeMPG)-Trit** exhibited an increase in T_{cp} from 30 to 40 °C over 15 days. This finding, combined with the observed rate-limiting end-cap cleavage and concentration dependence of T_{cp} for **P(MeMPG)-Trit** (i.e., increase in T_{cp} from 28–40 °C as the concentration was decreased from 10.0 to 1.25 mg/mL in PBS), indicates that the

increase in T_{cp} over time can likely be attributed to a decrease in polymer concentration as depolymerization occurred. This interpretation differs from that of previous studies involving the random backbone cleavage of polyesters and PNIPAM containing disulfide linkages, where a decrease in T_{cp} was attributed primarily to decreasing polymer chain length as random backbone cleavages occurred.^{16, 18-19} It also suggests that the T_{cp} can therefore be modulated according to the rate of end-cap cleavage, which is a key difference between self-immolative polymers and conventional backbone-degradable polymers.

In vitro cytotoxicity studies. MTT assays were performed to provide an indication of the cytotoxicities of the polymers. C2C12 mouse myoblast cells were used as they are a common cell line for *in vitro* work. The polymers were incubated with the cells for 24 h prior to performing the assay. Six different polymers were evaluated to determine the effects of the pendent groups and the end-caps on cytotoxicity. Comparing **P(MeMEG)-Trit** and **P(EtMEG)-Trit**, both polymers exhibited high cell metabolic activity (> 75%) at concentrations up to 0.25 mg/mL (Figure 5a). However, the more hydrophobic polymer **P(EtMEG)-Trit** with the ethyl tail was less toxic at higher concentrations than the analogue with the methyl tail. **P(EtMEG)-Trit** was initially dissolved and diluted in the cell culture media at 4 °C, due to its low T_{cp} . However, it would be expected to aggregate during cell culture at 37 °C, so may interact less with cells and be taken up to a different extent than the soluble analogue. **P(MeDEG)-Trit** and **P(EtDEG)-Trit** followed the same trend as the MEG analogues, with the more hydrophobic polymer with the ethyl tails being less toxic, likely because its T_{cp} is very close to the incubation temperature of 37 °C (Figure 5b). The effect of the end-cap was also examined by comparing **P(MeMPG)-Trit** and **P(MeMPG)-Bom** (Figure 5c). No significant end-cap effects were observed. Overall, the polymers exhibited low cytotoxicity, suggesting their potential for biomedical applications.

Figure 5. Cell metabolic activity (relative to control), as measured by MTT assays, as a function of polymer concentration: (a) **P(MeMEG)-Trit** vs **P(EtMEG)-Trit**, (b) **P(MeMEG)-Trit** vs **P(EtDEG)-Trit**, (c) **P(MeMPG)-Trit** vs **P(MeMPG)-Bom** as measured by MTT assays on C2C12 cells following a 24 h incubation.

Conclusions

Thermo-responsive PGAMs were readily synthesized through the amidation of PEGs and their LCST behaviour was tuned through the introduction of different pendent alkoxyalkyl amines. PGAMs with T_{cp} values just below ambient temperature and just above physiological temperature were obtained, demonstrating the promise for these polymers in applications such as injectable hydrogels and drug delivery vehicles, where aggregation above T_{cp} could be used to induce gelation or drug release. The influence of the end-cap and polymer concentration on T_{cp} depended on the particular structure of the pendent group. The trityl end-capped polymers degraded more rapidly than the Bom end-capped polymers, showing that degradation occurred selectively through an end-cap cleavage and end-to-end depolymerization process under all of the evaluated conditions. It was found that both the structure and T_{cp} of the polymers influenced their depolymerization rates and that depolymerization led to an increase in T_{cp} . Furthermore, the polymers exhibiting the most interesting T_{cp} values, near physiological and ambient temperature,

exhibited low cytotoxicity, demonstrating their promise for biomedical and other applications. While the polymers in the current work underwent relatively slow end-cap cleavage and consequently slow depolymerization, the advantage of SIPs is that the end-cap can be readily substituted to afford responsiveness to different stimuli and to tune the rate of depolymerization, without changing the polymer backbone.

Associated Content

Supporting Information

Additional experimental procedures and characterization data for the polymers, ^1H NMR, ^{13}C NMR, and FT-IR spectra, size-exclusion chromatograms, turbidimetry curves, DLS studies, depolymerization studies, depolymerization kinetics plots.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

This work was funded by the Natural Sciences and Engineering Research Council of Canada (E. W. R. Steacie Memorial Fellowship 2017-507348 and Discovery Grant 2016-04636). QEAS thanks the Government of Ontario for an Ontario Graduate Scholarship.

Acknowledgements

We thank Aneta Borecki for help with the cell toxicity studies.

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