Thermoresponsive Polymer-Antibiotic Conjugates based on Gradient Copolymers of 2-Oxazoline and 2-Oxazine

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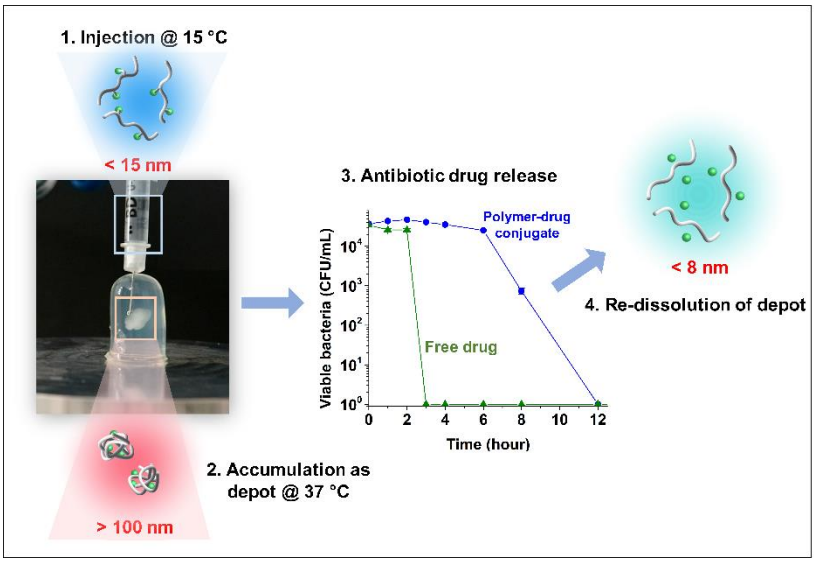
Abstract

A polymer-antibiotic conjugate with thermoresponsive properties near body temperature is presented. The backbone polymer is a copolymer of 2-*n*-propyl-2-oxazine (PropOzi) and methoxycarbonylethyl-2-oxazoline (C_2MestOx) which is conjugated with the broad-spectrum antibiotic, cefazolin, ia modification of the methyl ester group of C_2MestOx. The resulting polymer-antibiotic conjugate has a cloud point temperature near body temperature meaning it can form a homogenous solution if cooled but when injected into a skin-mimic at 37 °C forms a drug depot precipitate. Cleavage of the ester linker leads to quantitative release of the pristine cefazolin (with some antibiotic degradation observed) and redissolution of the polymer. When *E. coli* were treated with polymer-antibiotic total clearance is observed within 12 hours. The power or this approach is the potential for localized antibiotic delivery, for example, at a specific tissue site or into infected phagocytic cells.



Graphical Abstract

Highlights

- synthesis of a novel poly(2-oxazoline/oxazine) containing conjugated antibiotic

- cloud point temperature can be tuned to body temperature by balancing monomer ratio and drug content

- depot forming properties and released cefazolin maintains potency against *E. coli*.

1. Introduction

Polymer-drug conjugation is an effective strategy for making controlled drug delivery systems with clinical relevance.[1-3] Compared to free drugs, polymer-drug conjugates have distinct features, such as reduced toxicity, increased solubility, enhanced bioavailability and biocompatibility, and controlled mechanism of delivery.[2, 4] Moreover, the chemical architecture of polymer-drug conjugates can be varied based on the choice of polymer backbone and conjugation functionality, while labile linkers and targeting groups can improve the pharmacokinetics.[1, 5]

A range of therapeutic polymer-drug conjugates based on poly(*N*-(2-hydroxypropyl) methacrylamide) (PHPMA), poly(glutamic acid) (PGA) and poly(ethylene glycol) (PEG) have reached clinical trials.[6] Despite the promise of these systems, the polymers have largely fixed physicochemical properties and limited tailorability. Another class of polymers recently introduced for the design of polymer-drug conjugates are the poly(2-oxazoline)s (PAOx), which are biocompatible, display minimal immunogenicity and have excellent potential for customization. Over the last decade, PAOx-drug conjugates have been studied with several drug molecules, including rotigotine,[7, 8] doxorubicin,[9, 10] benazepril,[11] and penicillin.[12] The PAOx-rotigotine conjugate is a sustained-release formulation that successfully passed a preliminary Phase 1A human clinical trial for treatment of Parkinson's disease.[7]

Thermoresponsive properties may also be designed into polymer-drug conjugates to improve control over the release profile of drugs.[13] Recently, we synthesized thermoresponsive PAOx-benazepril conjugates and reported their ability to form an insoluble polymer-drug depot at body temperature which redissolves upon drug release.[11] The mechanism of this system relies on the hydrophobicity of the conjugated drug leading to dehydration of the polymer backbone and improved stability of hydrolyzable linker above the cloud point temperature (T_{cp}). The release kinetics are governed by cleavage of the linker group which is slow at first due to the aggregated, dehydrated phase of the polymer-drug conjugate but accelerates as the T_{cp} drops and the hydration of the system increases. This leads to burst-free, constant, and complete drug release over time, and in some cases auto-acceleration of drug release. This approach has the advantage

over other commonly used drug delivery devices (e.g., hydrogels and soluble polymer-drug conjugates) that typically exhibit plateau regions in the final stages of release leading to a trickle of drug.

One particularly appealing class of payloads for a drug delivery system with complete release without exhibiting a plateau are antibiotics due to the concerns over antibiotic resistance from under-dosing. PAOx has previously been used as a carrier for penicillin via end group conjugation by Schmidt *et al*.[12] to improve the stability and potency of the tethered antibiotic. However, PAOx has not been reported for release of conjugated antibiotics. A potentially useful system is a thermoreponsive PAOx-antibiotic conjugate with a cleavable linker which forms a precipitate after injection for local delivery of antibiotics, as opposed to traditional oral or intravenous antibiotic administration. The phase change into a precipitate could have an immobilizing effect within tissue thereby forming a depot for local treatment of infections, or the precipitate could be used to attract bacterially infected phagocytic cells known for their ability to take up nanoparticles.[14]

In this study, we have chosen the broad-spectrum antibiotic, cefazolin, and conjugated it to copolymers of 2-*n*-propyl-2-oxazine (PropOzi) and methoxycarbonylethyl-2-oxazoline (C_2MestOx). Similar to PAOx, poly(2-oxazine)s can also be prepared by living cationic ring-opening polymerization and have exceptional hydration properties, protein absorption, and bioinertness.[15-19] Recently it was reported that the statistical copolymerization of 2-oxazolines and 2-oxazines leads to the formation of defined gradient copolymers, whereby the 2-oxazine monomer is incorporated faster than the 2-oxazolines.[20] Two types of ester-containing linkers between the polymer backbone and the cefazolin were compared in this study as it is known that the hydrolytic cleavage of ester linker groups is greatly influenced by the adjacent chemical groups and the stability of the products following cleavage.[21] This has been demonstrated by Bernhard *et al*.[22] using poly(2-ethyl-*co*-C_3MestOx) copolymers with three different ester linkages, obtained by the esterification between the acid functional group in salicylic acid and three hydroxyl groups (primary, secondary, and tertiary alcohols) on the side chain of the polymer, and showed the significant influence of modular ester chemistry on the kinetics of drug release. Furthermore, the release of the drug from the

poly(2-ethyl-2-oxazoline)-rotigotine conjugate is also facilitated by the presence of a beta-triazole ester group. In our study, C_2MestOx was amidated with either 2-aminoethanol or *m*-hydroxybenzylamine to give hydroxyl and phenol functional groups, respectively, as handles for conjugation of cefazolin. The copolymerization with PropOzi allowed for further control over the hydrophilic-hydrophobic balance of the polymer and the resulting LCST behavior. The influence of the polymer-drug conjugate structure on the thermoresponsive behavior was studied together with the dependence of drug release on the type of linker.

2. Experimental

2.1. Materials

The following chemicals were used as-received: butyronitrile (>99%, TCI chemicals), 3-amino-1-propanol (>99%, TCI chemicals), zinc acetate dihydrate (≥98%, Sigma Aldrich), succinic anhydride (>98%, Sigma Aldrich), thionyl chloride ($SOCl_2$) (>98%, Sigma-Aldrich), 2-chloroethylamine hydrochloride (98%, Sigma Aldrich), sodium methoxide (>99.8%, UNIVAR®), sodium carbonate (99.5%, UNIVAR®), aminoethanol (98%, TCI chemicals), 3-(aminomethyl)phenol (97%, Sigma Aldrich), 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) (98%, Sigma-Aldrich), 4-(dimethylamino)pyridine (DMAP) (≥99%, Sigma-Aldrich), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) (98%, Sigma-Aldrich), 1-hydroxybenzotriazole hydrate (HOBt) (>97%, Sigma Aldrich), 2-amino-2-(hydroxymethyl)propane-1,3-diol (TRIS) (98%, Sigma-Aldrich), and cefazolin sodium salt (>98%, TCI chemicals).

2.2. Characterization

Nuclear magnetic resonance (NMR) spectroscopy: 1D ^{1}H-NMR spectra (Avance 600 MHz Bruker spectrometer) were recorded using deuterated chloroform or DMSO as the solvent. Diffusion-ordered spectroscopy (DOSY) experiments based on ^{1}H-NMR spectroscopy were acquired at 298 K on a 400 MHz Bruker UltraShield spectrometer with a Quattro Nucleus Probe. DOSY measurements were performed with

gradient intensity linearly sampled from 2% at 0.96 G $^{-1}$ to 95% at 45.7 G/cm. The number of gradient steps was set to 32. The DOSY NMR spectra were processed in MestReNova with a Bayesian method and 1 resolution factor and 2 repeats.

Fourier-transform infrared (FT-IR) spectroscopy: FT-IR spectra were acquired on a Bruker Alpha-P FT-IR spectrometer fitted with a diamond attenuated total reflectance (ATR) accessory with the range of 400 cm^{-1} to 4000 cm^{-1}.

Determination of cloud point temperature: The T_{cp} were determined by turbidity measurements using a UV-vis spectrophotometer (Cary 60, Agilent) equipped with recirculating cooler chiller (FL300, Julabo). Polymers were prepared as 5 mg/mL solutions in phosphate buffered saline (PBS, pH 7.4) and the transmittance monitored at 600 nm. The T_{cp} were defined as 50% transmittance during the heating ramp.

Particle size determination: Dynamic light scattering (DLS) was performed using a Zetasizer NanoZS Instrument (ZEN 3600, Malvern Instruments, UK) equipped with a 4 mW He-Ne laser (l_0 = 633 nm) and non-invasive backscattering detection at a scattering angle of 173°.

Polymerization kinetic studies: Gas chromatography (GC) was used to determine monomer consumption and performed on an Agilent Technologies 7890A system equipped with an Agilent Technologies 7693 autosampler and an Agilent Technologies HP-5 column of 15 m length and 0.32 mm diameter. A FID detector was used and the inlet was set to 250 °C with a split injection ratio of 100:1 (120 mL/min). Hydrogen was used as the carrier gas at a flow rate of 1.2 mL/min. The oven temperature was set to 60 °C for 3 mins and increased to 270 °C at 20 °C/min.

Molecular weight determination: Size-exclusion chromatography (SEC) was measured on an Agilent 1260 infinity HPLC system equipped with an inline degasser, a diode array detector, a refractive index detector, a UV detector and a temperature controller set to 25 °C. A series of 300 x 8 mm 30 Å, 100 Å, and 1000 Å mixed D Polymer Standards Service (PSS) GRAM columns were used with a PSS GRAM pre-column. The mobile phase was *N*,*N*-dimethylacetamide (DMAc) with 0.8% lithium bromide at a flow rate

of 1 mL/min. The average molecular weights and molecular weight distribution (*Đ*) were calculated against poly(methyl methacrylate) (PMMA) standards from PSS.

2.3. Synthesis

2.3.1. Synthesis of P(POzi-C$_2$MestOx) copolymers

The synthetic routes and characterization of the PropOzi and C_2MestOx monomers are described in detail in the supporting information (**Scheme S1, S2** and **Figure S1)**. After successful synthesis of the two monomers, two polymerization mixtures containing PropOzi, C_2MestOx, and MeOTs were prepared with the monomer concentration of 4 M in acetonitrile. These polymerization mixtures were heated at 120 °C for 24 hours for the kinetic studies. For scale-up they were heated at 80 °C for 6 days to reach conversions of ≥98% by ^{1}H NMR spectroscopy. After cooling the mixtures to room temperature, the polymerizations were terminated with dry piperidine (1.5 eq. relative to MeOTs) and stirred for 3 hours. The polymers were precipitated in cold diethyl ether and lyophilized.

P(POzi$_{50}$-C$_2$MestOx$_{50}$) C_2MestOx (0.83 mL, 6.0 mmol, 50 eq.), PropOzi (0.79 mL, 6.0 mmol, 50 eq.), and MeOTs (18.1 µL, 0.12 mmol, 1 eq.) were used for polymerization. ^{1}H NMR (DMSO-*d6*) (**Figure 2b**): 3.56 (159H, OC$\underline{H}_3$), 3.50-3.10 (400H, NC$\underline{H}_2$), 2.63-2.1 (300H, (C=O)C$\underline{H}_2$C$\underline{H}_2$), 1.86 (102H, NCH$_2$C$\underline{H}_2$CH$_2$), 1.56-1.44 (100H, C$\underline{H}_2$CH$_3$), 0.86 (150H, C$\underline{H}_3$).

P(POzi$_{30}$-C$_2$MestOx$_{70}$) C_2MestOx (1.16 mL, 8.4 mmol, 70 eq.), PropOzi (0.47 mL, 3.6 mmol, 30 eq.) and MeOTs (18.1 µL, 0.12 mmol, 1 eq.) were used for polymerization. ^{1}H NMR (DMSO-*d6*) (**Figure S2**): 3.56 (209H, OC$\underline{H}_3$), 3.50-3.10 (400H, NC$\underline{H}_2$), 2.63-2.1 (340H, (C=O)C$\underline{H}_2$C$\underline{H}_2$), 1.86 (59H, NCH$_2$C$\underline{H}_2$CH$_2$), 1.56-1.44 (63H, C$\underline{H}_2$CH$_3$), 0.86 (90H, C$\underline{H}_3$).

2.3.2. Hydroxyl functionalization of polymer by amidation

P(POzi-C_2MestOx) copolymers and TBD were dissolved in acetonitrile, followed by the addition of amine compounds to the polymer solutions then stirred overnight at 80 °C. The polymers were precipitated in the cold diethyl ether and dialyzed against distilled water for 3 days, then lyophilized.

POzi-Et-OH (polymer containing hydroxyl ethyl group)

P($POzi_{50}$-C_2MestOx_{50}) (300 mg, 0.02 mmol), 2-aminoethanol (0.37 g, 6 mmol, 6 eq. to carbonyl group), TBD (69.5 mg 0.5 mmol, 0.5 eq. to carbonyl group), and 10 mL of acetonitrile. ^{1}H NMR (DMSO-*d6*) (**Figure 2b**): 7.80 (30H, CONH), 3.68-3.14 (400H, CH_2), 3.08 (85H, $NHCH_2CH_2OH$), 2.75-2.07 (300H, $(C=O)CH_2CH_2$), 1.82-1.53 (99H, $NCH_2CH_2CH_2$), 1.49 (100H, CH_2CH_3), 0.84 (150H, CH_3).

POzi-*m*Bz-OH (polymers containing phenol group)

P($POzi_{30}$-C_2MestOx_{70}) (300 mg, 0.02 mmol), TBD (55.68 mg, 0.4 mmol, 0.5 eq. to carbonyl group), 3-hydroxylbenzylamine (49.26 mg, 0.4 mmol, 0.5 eq. to carbonyl group), and 10 mL of acetonitrile were used for amidation. ^{1}H NMR of POzi-*m*Bz-OH (DMSO-*d6*) (**Figure S3**): 9.93 (3H, OH), 8.27 (5H, CONH), 7.03 (7H, aromatic CH), 6.62 (21H, aromatic CH), 4.14 (16H, CH_2-aromatic), 3.55 (185H, OCH_3), 3.50-3.10 (400H, CH_2), 3.11 (20H, CH_2), 2.75-1.32 (340H, $(C=O)CH_2CH_2$), 1.84 (16H, $CH_2(C=O)NH$), 1.80-1.56 (59H, $NCH_2CH_2CH_2$), 1.50 (64H CH_2CH_3), 0.86 (90H, CH_3).

2.3.3. Cefazolin conjugation by esterification (POzi-CZ)

Cefazolin acid (72 mg, 0.16 mmol, 12 eq. relative to polymer ~~hydroxyl/phenol group~~) was placed in a 1 mL mixture of DCM and DMF (90:10 %v/v), followed by the addition of EDC (41.84 mg, 0.2 mmol, 15.6 eq. relative to polymer ~~hydroxyl group~~) and HOBt (27.02 mg, 0.2 mmol 15.6 eq. relative to polymer ~~hydroxyl group/phenol~~) at 0 °C, and stirred for 2 h. The polymers containing hydroxyl/phenol groups (200 mg, 0.013 mmol, 1 eq.) and DMAP (19.55 mg, 0.16 mmol, 12 eq. relative to polymer ~~hydroxyl group~~) were then added and the final mixture was stirred for 24 hours at room temperature. The polymers were

precipitated in cold diethyl ether. The obtained polymers were re-dissolved in a small portion of DMF then precipitated in tetrahydrofuran (THF), which was repeated three times. No free cefazolin was detected by HPLC. The obtained polymer was lyophilized. ^{1}H NMR of POzi-Et-CZ (DMSO-*d6*) (**Figure 2b**): 9.60 (5H, C<u>H</u>), 9.38 (5H, N<u>H</u>), 6.95 (28H, CON<u>H</u>), 5.39 (10H, C<u>H</u>), 5.20 (8H, C<u>H</u>$_2$), 4.23 (16H, CH$_2$), 3.75-3.15 (400H, C<u>H</u>$_2$), 3.10 (85H, NHC<u>H</u>$_2$CH$_2$), 2.77-2.11 (300H, (C=O)C<u>H</u>$_2$C<u>H</u>$_2$), 1.94-1.57 (89H, NCH$_2$C<u>H</u>$_2$CH$_2$), 1.50 (97H, C<u>H</u>$_2$CH$_3$), 0.87 (150H, CH$_3$). ^{1}H NMR of POzi-*m*Bz-CZ (DMSO-*d6*) (**Figure S4**): 9.53 (3H, C<u>H</u>), 9.36 (3H, N<u>H</u>), 8.27 (3H, CON<u>H</u>), 7.41-6.21 (24H, C<u>H</u>$_2$-aromatic), 5.50-5.08 (14H, C<u>H</u>$_2$), 4.57 (6H, CH$_2$), 4.24 (6H, NH-CH$_2$-aromatic), 4.41 (13H, CH$_2$), 3.75-3.15 (400H, C<u>H</u>$_2$), 2.75-1.32 (340H, (C=O)C<u>H</u>$_2$C<u>H</u>$_2$), 1.71 (65H, NCH$_2$C<u>H</u>$_2$CH$_2$), 1.49 (64H C<u>H</u>$_2$CH$_3$), 0.86 (90H, C<u>H</u>$_3$).

2.4. Drug release studies

The two POzi-CZ polymers were dissolved at concentrations of 5 mg/mL in PBS adjusted to pH 8.5 with sodium hydroxide. The polymer solutions were incubated at 37 °C, and 10 μL of buffer was taken periodically and diluted in 1 mL of DI water. The amount of released drug was quantified by High Performance Liquid Chromatography (HPLC) (Agilent 1100 Series) equipped with an InertSustain™ C18, 250 x 4.6 mm, 5 μm particle size HPLC column. The mobile phase was 60 vol% methanol in ultrapure water with 0.1% trifluoroacetic acid (≥99%, Sigma Aldrich) with a flow rate of 1 mL/min and an injection volume of 100 μL. Released cefazolin was detected at 275 nm.

2.5. Minimum Inhibitory Concentration (MIC) assay

Drug release prior to MIC assay: POzi-Et-CZ was suspended in Mueller Hinton broth (MHB) (pH 8.5) to give a calculated cefazolin concentration of 512 μg/mL. POzi-Et-OH acted as template control and was matched by mass to POzi-Et-CZ. A solution of free cefazolin (512 μg/mL) in MHB (pH 8.5) was prepared and acted as an antibiotic stability control. These mixtures were incubated at 37 °C, 200 rpm for 24 hours. The MICs for the mixtures containing POzi-Et-CZ, POzi-Et-OH or cefazolin, were determined against *Escherichia coli* MG1655 by the broth microdilution method, in accordance with the 2015 (M07-A10)

Clinical and Laboratory Standards Institute (CLSI). In a 96-well plate, eleven two-fold serial dilutions of each compound were prepared to a final volume of 100 µL in MHB (pH 8.5) medium. At the time of inoculation, 5×10^5 bacterial cells, prepared from fresh overnight MHB (pH 8.5) cultures, were added to each well. The MIC for a compound was defined as the lowest concentration that prevented visible bacterial growth after 24 hours of static incubation at 37 °C. MIC values were obtained from 3 biological replicates.

2.6. Time-Kill Assay

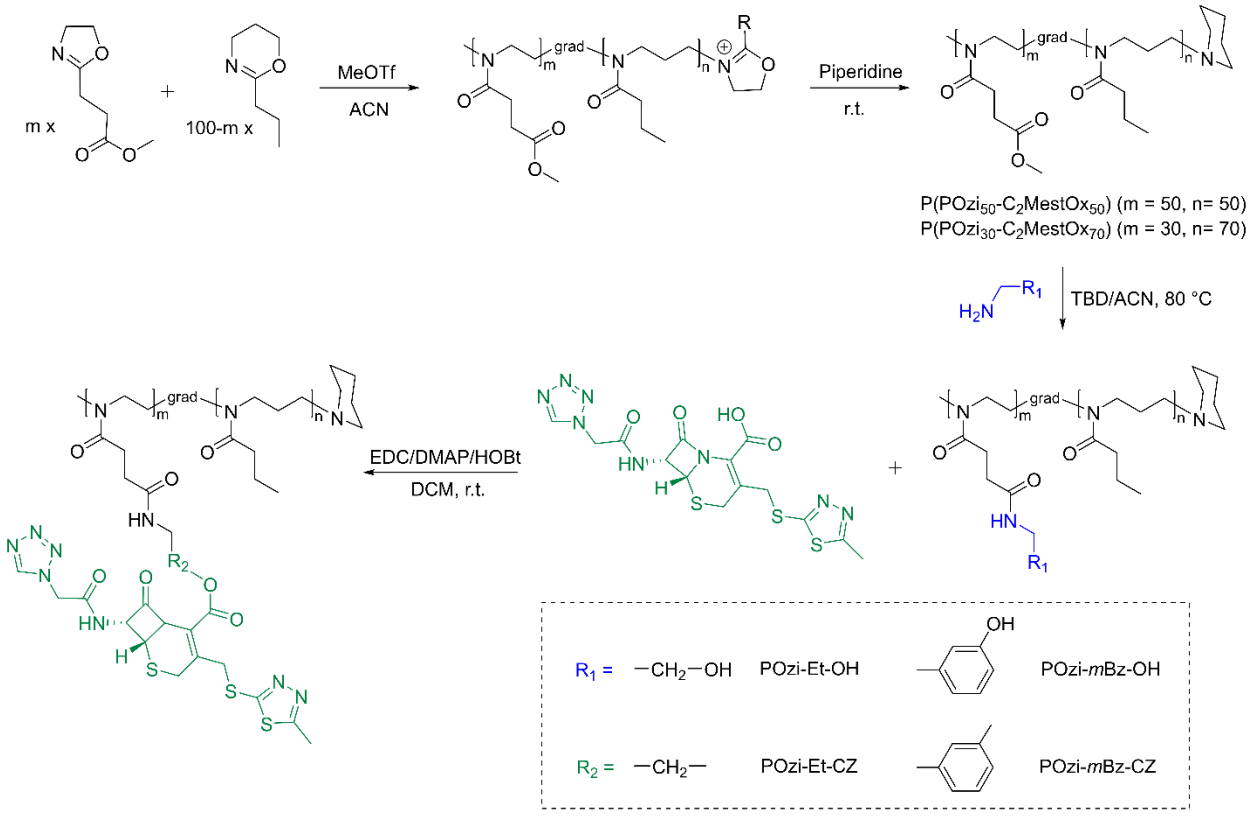
POzi-Et-CZ and cefazolin were studied at a concentration of 64 µg/mL (cefazolin), and POzi-Et-OH was studied at a mass equivalent to the drug concentration of 64 µg/mL. A starting inoculum was prepared by diluting an overnight culture of *E. coli* MG1655 in MHB (pH 8.5) to a concentration of 5×10^4 Colony Forming Units (CFU)/mL. This inoculum (4 mL) was added to individual tubes, containing either POzi-Et-CZ, cefazolin, or POzi-Et-OH, to give the desired final concentration (64 µg/mL or equivalent). Three tubes without drugs were inoculated as growth controls. Tubes were incubated at 37 °C, 200 rpm in a shaking incubator for a total duration of 24 hours. At predetermined time points (0, 1, 2, 3, 4, 6, 8, 12, 24 hours), samples were removed (50 µL), serially diluted in PBS, and plated onto LB agar plates. Plates were incubated at 37 °C for 18 hours and then enumerated for CFU/mL counts. The mean CFU/mL for each sample was determined from three biological replicates.

3. Results and discussion

3.1. Kinetics of PropOzi and C_2MestOx copolymerization

A series of polymers with different monomer ratios were prepared by copolymerizing PropOzi and C_2MestOx by cationic ring-opening polymerization (CROP) at 120 °C with methyl *p*-toluenesulfonate as the initiator and piperidine as the terminator (**Scheme 1**). The complete consumption of monomers was confirmed by disappearance of the signals from both monomers in the ^{1}H-NMR spectra. The kinetics of the

polymerizations with both 50:50 and 30:70 PropOzi-C_2MestOx monomer ratios showed faster incorporation of PropOzi compared to C_2MestOx which was attributed to the higher nucleophilicity of the 2-oxazine monomer compared to the 2-oxazoline monomer (**Figure 1a** and **Figure S5**).[20] Note that this enhancement in rate due to nucleophilicity outweighs the carboxyl-cation interactions which give C_2MestOx faster polymerization rates than other aliphatic 2-oxazolines.[23] The faster incorporation of PropOzi resulted in the formation of amphiphilic "quasi" di-block gradient copolymers with PropOzi as the hydrophobic block and C_2MestOx as the hydrophilic block. Similar observations have been reported for the copolymerization of 2-methyl-2-oxazine (MeOzi) with 2-*n*-propyl-2-oxazoline (PropOx) or 2-butyl-2-oxazoline (ButOx) where MeOzi showed much faster propagation than the other two 2-oxazoline monomers resulting in gradient copolymers.[20]



Scheme 1. Synthesis of P(POzi-C_2MestOx) copolymers from 2-methoxycarbonylethyl-2-oxazoline (C_2MestOx) and 2-*n*-propyl-2-oxazine (PropOzi) monomers by CROP with termination using piperidine followed by TBD-catalysed amidation of P(POzi-C_2MestOx) copolymers with 2-aminoethanol and *m*-benzylamine. Esterification was used to conjugate cefazolin (shown in green) to the polymers containing hydroxyl/phenol groups.

Subsequently, a series of copolymers of PropOzi and C_2MestOx was prepared with different monomer ratios to study the effect of composition on the LCST behavior by measuring their respective T_{cp}'s (**Table S1**). The relationship between the mol% of C_2MestOx and T_{cp} (**Figure 1b**) shows an increase in T_{cp} with increasing proportion of C_2MestOx relative to PropOzi, as expected due to the increase in hydrophilicity. Based on this screening experiment two compositions, namely P($POzi_{50}$-$C_2MestOx_{50}$) and P($POzi_{30}$-$C_2MestOx_{70}$), with T_{cp} closest to body temperature, were selected for further scale-up. Due to the known chain transfer side reactions of PAOx at 120 °C leading to a high molecular weight shoulder in the SEC,[24] a lower temperature of 80 °C and longer reaction time (6 days) was used to suppress the side reactions in the scale-up of P($POzi_{50}$-$C_2MestOx_{50}$) and P($POzi_{30}$-$C_2MestOx_{70}$) resulting in the absence of high molecular weight shoulders in the SEC (**Figure S6**). The ^{1}H NMR spectra of the products from the scaled-up P($POzi_{50}$-$C_2MestOx_{50}$) and P($POzi_{30}$-$C_2MestOx_{70}$) syntheses confirmed the consumption of the 2-oxazoline and 2-oxazine monomers based on loss of peaks at 4.2 ppm and 3.8 ppm, respectively, and the presence of the polymer backbone methylene group at 3.5 ppm (**Figure 2b** and **Figure S2**). The calculated C_2MestOx content of 49 mol% for P($POzi_{50}$-$C_2MestOx_{50}$) and 69 mol%, for P($POzi_{30}$-$C_2MestOx_{70}$) also confirmed the successful synthesis.

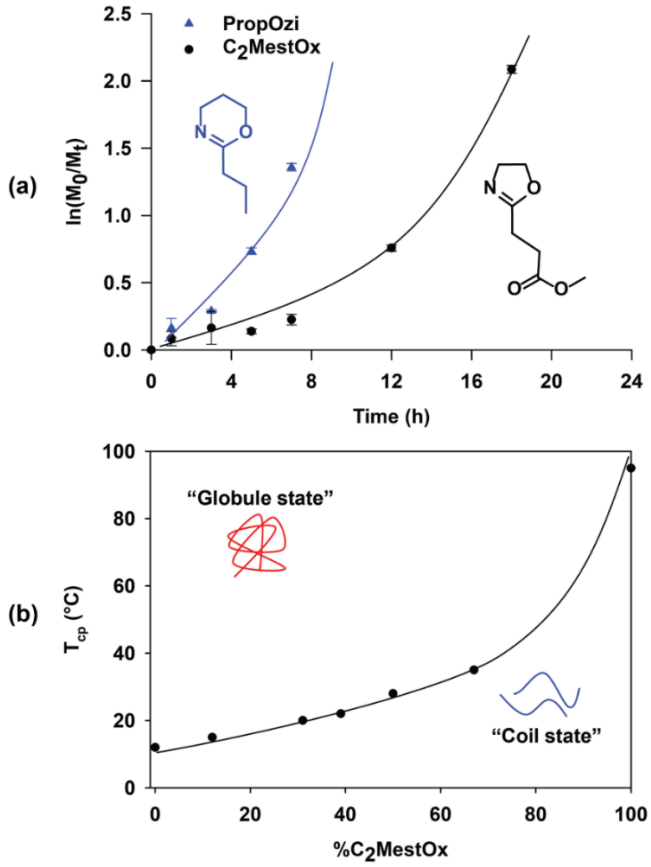


Figure 1. (a) Kinetics of polymerization for the PropOzi and C_2MestOx monomers. The polymerization was carried out at 120 °C with ratio of [PropOzi]:[C_2MestOx]:MeOTs=50:50:1 and monomer concentration of 4 M in acetonitrile. (b) T_{cp} of P(POzi-C_2MestOx) copolymer as a function of C_2MestOx content. Polymer concentration was 5 mg/mL in PBS. The lines are to aid the eye only.

3.2. Introduction of hydroxyl/phenol groups by amidation

The synthesized copolymers were then used as the precursors for the amidation and conjugation of cefazolin. To introduce hydroxyl/phenol groups to the side chains, TBD-catalyzed amidation with two different amines was performed (**Scheme 1**) using a similar method as reported by Van Guyse *et al.*.[25] To compensate for shifts in T_{cp} upon modification (thereby maintaining T_{cp} near body temperature) the low T_{cp} polymer was used with the hydrophilic amine and the high T_{cp} polymer was used with the hydrophobic amine. That is, P(POzi$_{50}$-C_2MestOx$_{50}$) was modified with 2-aminoethanol and P(POzi$_{30}$-C_2MestOx$_{70}$) was

used with *meta*-hydroxyl benzylamine. ^{1}H NMR and FT-IR spectra were used to follow the reaction with 2-aminoethanol (to form the product designated POzi-Et-OH) to confirm complete conversion of the methyl ester to a hydroxyl group, based on the disappearance of the methyl ester peaks at 3.6 ppm and 1730 cm^{-1}, respectively (**Figure 2b, S7**). For incorporation of the *m*-hydroxyl benzylamine, the reaction was less efficient and only 6 mol% of phenol groups were incorporated into the polymer designated POzi-*m*Bz-OH (**Figure S3**). **Table 1** summarizes the compositions, molecular weights and dispersities of the copolymers before and after the amidation reactions.

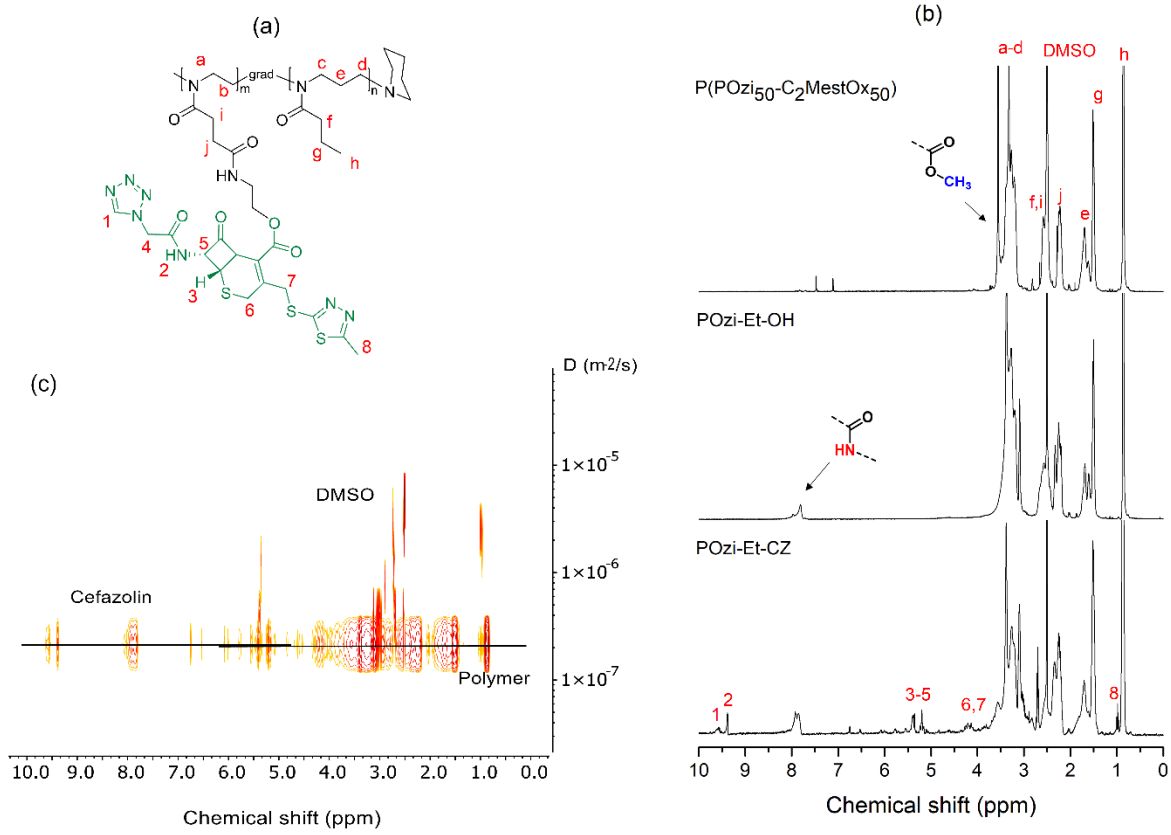


Figure 2. (a) Chemical structure of POzi-Et-CZ, (b) ^{1}H-NMR spectra of P(POzi$_{50}$-C$_2$MestOx$_{50}$), POzi-Et-OH, and POzi-CZ, and (c) DOSY map of POzi-Et-CZ in DMSO-*d6* with a polymer concentration of 10 mg/mL.

Table 1. Composition and drug content of copolymers and conjugates

Polymer	PropOzi[a] (mol%)	C_2MestOx[a] (mol%)	Hydroxyl / phenol[a] (mol%)	M_n[b] (kDa)	($Đ$)[b]	Cefazolin[c] (wt%)
P(POzi$_{50}$-C_2MestOx$_{50}$)	51	49	-	11.8	1.24	-
P(POzi$_{30}$-C_2MestOx$_{70}$)	31	69	-	11.0	1.28	-
POzi-Et-OH	51	-	49	8.4	1.31	-
POzi-*m*Bz-OH	31	63	6	13.6	1.36	-
POzi-Et-CZ	51	-	45[d]	11.4	1.35	8.7
POzi-*m*Bz-CZ	31	62	3[d]	15.7	1.61	7.3

[a] obtained from ^{1}H NMR spectra
[b] obtained by SEC
[c] determined by UV-vis spectroscopy
[d] based on the cefazolin content and the theoretical molecular weight (M_n) of POzi-CZ

3.3. Cefazolin conjugation by esterification

The antibiotic cefazolin was conjugated to the side chains of the copolymers by esterification. Firstly, the free acid of cefazolin was prepared from the carboxylate by acidification resulting in a shift of the ^{1}H NMR spectra peaks (**Figure S8)** and a reduction in water solubility, as expected for the free acid form of the drug. The HPLC retention times and peak integrals before and after acidification were similar suggesting that there was no measurable degradation during acidification (**Figure S8**).

Conjugation of cefazolin was carried out with the polymers containing either hydroxyl or phenol pendant groups by DMAP-catalysed esterification (**Scheme 1**). Since the conversion of the methyl ester in P(POzi$_{50}$-C_2MestOx$_{50}$) to hydroxyl groups (49 mol%) was more efficient than conversion of the methyl ester in P(POzi$_{30}$-C_2MestOx$_{70}$) to phenol groups (6 mol%), the amount of cefazolin added to the conjugation reaction was varied relative to the amount of alcohol groups to obtain similar final cefazolin loading for both systems. In both cases 12 mol% cefazolin (relative to polymer) was added to each system – for the hydroxyl conjugation this was a small amount relative to the hydroxyl groups, whereas for the phenol conjugation it represented an excess. As a result ~~After conjugation, unreacted cefazolin and other impurities were removed by repeated dissolution and precipitation until no free cefazolin was detected by HPLC.~~ the

amount of drug, by weight, conjugated to POzi-Et-CZ and POzi-*m*Bz-CZ was 8.7 and 7.3 wt%, respectively, as determined by UV-vis spectroscopy. ~~These amounts correspond to conjugation yields of approximately 35% – this low conversion was perhaps due to steric hindrance near the hydroxyl/phenol groups from the gradient polymeric structure, or potentially from cleaving during purification.~~ The ^{1}H NMR spectra (**Figure 2b** and **Figure S4**) revealed peaks at 9.5 and 9.4 ppm for the tetrazole and the secondary amine, and peaks at 5.0-6.0 ppm for β-lactam of cefazolin. Similarly, the ester absorbance of all POzi-CZ conjugates at 1730-1770 cm^{-1} was observed in the FT-IR spectra (representative spectrum shown in **Figure S7**). To further confirm successful conjugation of cefazolin, diffusion ordered spectroscopy (DOSY) was used to compare diffusion coefficients of the cefazolin and the polymers under different pulsed field gradient intensity, as shown in **Figure 2c** for POzi-Et-CZ and **Figure S9** for POzi-*m*Bz-CZ. The diffusion coefficients of the cefazolin and the polymer were the same, confirming the successful conjugation of cefazolin to the polymer.

The SEC of the POzi-Et-OH revealed a lower apparent molecular weight than P(POzi$_{50}$-C$_2$MestOx$_{50}$) as shown in **Figure 3a**. This is likely due to the hydroxyl group in the side chains that decrease the hydrodynamic volume of the polymer by increasing the intramolecular hydrogen bonds. Conversely, the hydrodynamic volumes of the two polymers, POzi-Et-CZ and POzi-*m*Bz-CZ, increased upon conjugation of cefazolin due to the larger molar mass of the the drug. The large increase in dispersity of the CZ-conjugated polymers may be ascribed to not only side reactions from the multiple modification steps (amidation and esterification), but also from the increased irregularity between individual polymer chains resulting from variations in the number of drug-molecules that are attached.

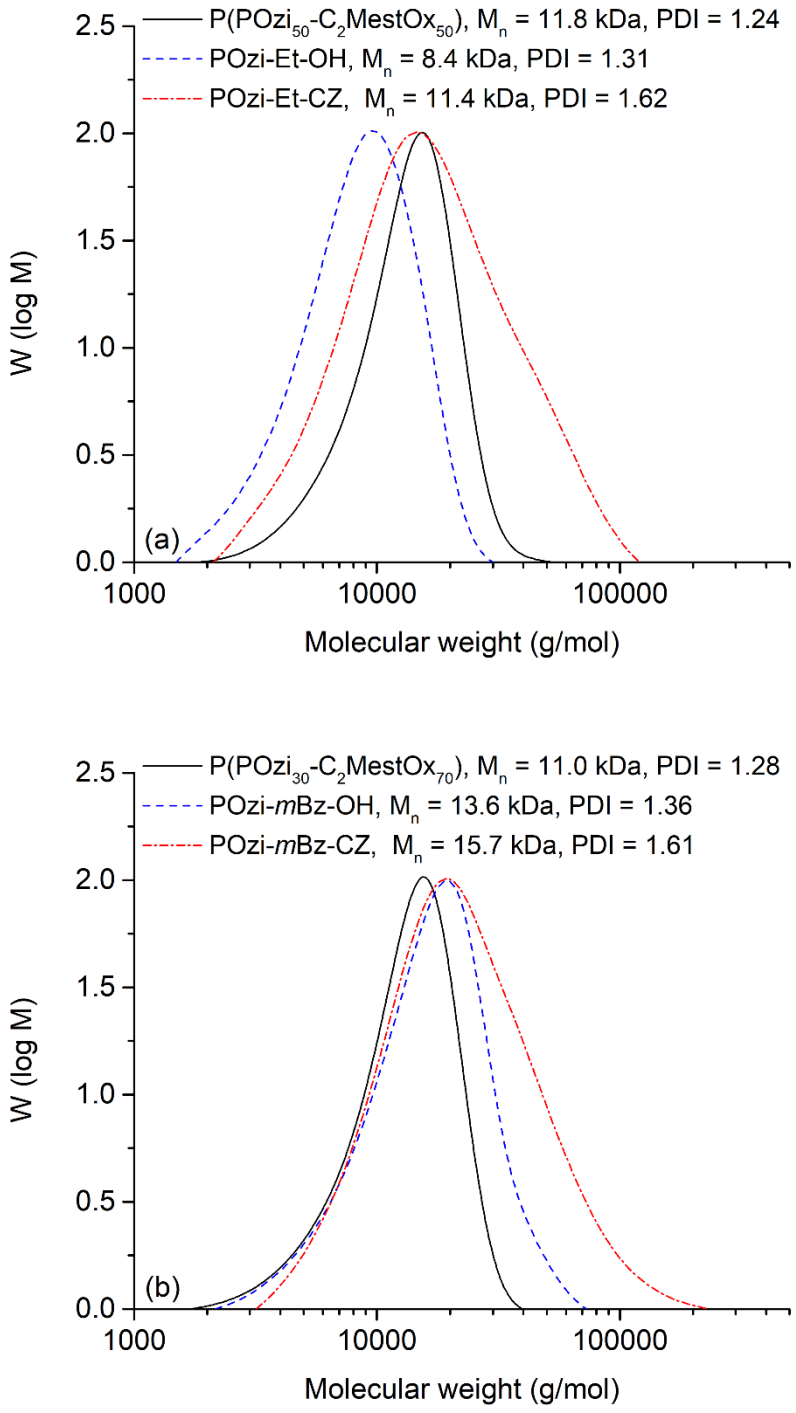


Figure 3. Molecular weight distributions of a series of copolymers, amidated polymers, and conjugates with (a) ethyl and (b) *m*-benzyl linkages determined by DMAc-SEC containing 5 mM LiBr.

3.4. LCST behavior of the copolymers

The turbidity of the polymer solutions in PBS was studied by UV-vis spectroscopy to determine the LCST behavior. For the unmodified polymers, P($POzi_{50}$-$C_2MestOx_{50}$) and P($POzi_{30}$-$C_2MestOx_{70}$), the T_{cp} values were 26.8 °C and 37.9 °C, respectively (**Figure 4a**), which is higher than the reported T_{cp} of the PPropOzi homopolymer (T_{cp} = 18 °C)[26] due to incorporation of the more hydrophilic C_2MestOx repeat units.

After amidation, the two POzi-OH (Et and *m*Bz) copolymers were completely soluble in water below 40 °C (at 5 mg/mL). After conjugation of cefazolin, however, both polymers exhibited coil-to-globule phase transitions below body temperature due to the hydrophobic contribution from the drug.

As illustrated in **Figure 4b**, conceptually a cooled solution of the polymer-drug conjugates can be rendered insoluble when injected into the body at 37 °C, thereby forming a drug depot immobilized in the injection site tissue. Upon subsequent partial cleavage of the drug, the polymer can be re-dissolved for the rapid elimination of the drug-free polymer.

To test the drug depot concept, a POzi-Et-CZ solution with a concentration of 5 mg/mL was prepared and cooled to refrigeration temperature (below the T_{cp}) followed by injection of the homogeneous solution through a 29 G needle (184 µm inner diameter) into a skin-mimic (crosslinked gelatin methacryloyl (GelMA)) at 37 °C with similar water content to that of skin. Using this protocol, the solution of the polymer-drug conjugate could successfully be injected into the model tissue without blocking the needle, followed by immediate precipitation inside the skin-mimic (**Figure 4c and Movie SI1**).

It is important to note that in order to design a polymer-drug conjugate system with the desired LCST behavior, a series of iterative experiments were needed. We know from a previous study that the amount of drug conjugated to the polymer can be used to tune the T_{cp}, for instance, a 5% change in loading of a hydrophobic drug similar to cefazolin can lead to a *ca.* 10 °C shift in T_{cp}.[11] A similar result was found here, for instance, when a high amount of cefazolin was conjugated, the polymers were insoluble in water. Similarly, no T_{cp}, or T_{cp} higher than body temperature were obtained when only a small amount of drug

was conjugated. Hence, it was only after experimenting with the amount of drug loading that the two polymers, POzi-CZ (Et and *m*Bz), with optimal drug loading showed useful LCST behavior with T_{cp} (heating cycle) in the range 18-20 °C (**Figure 4a**).

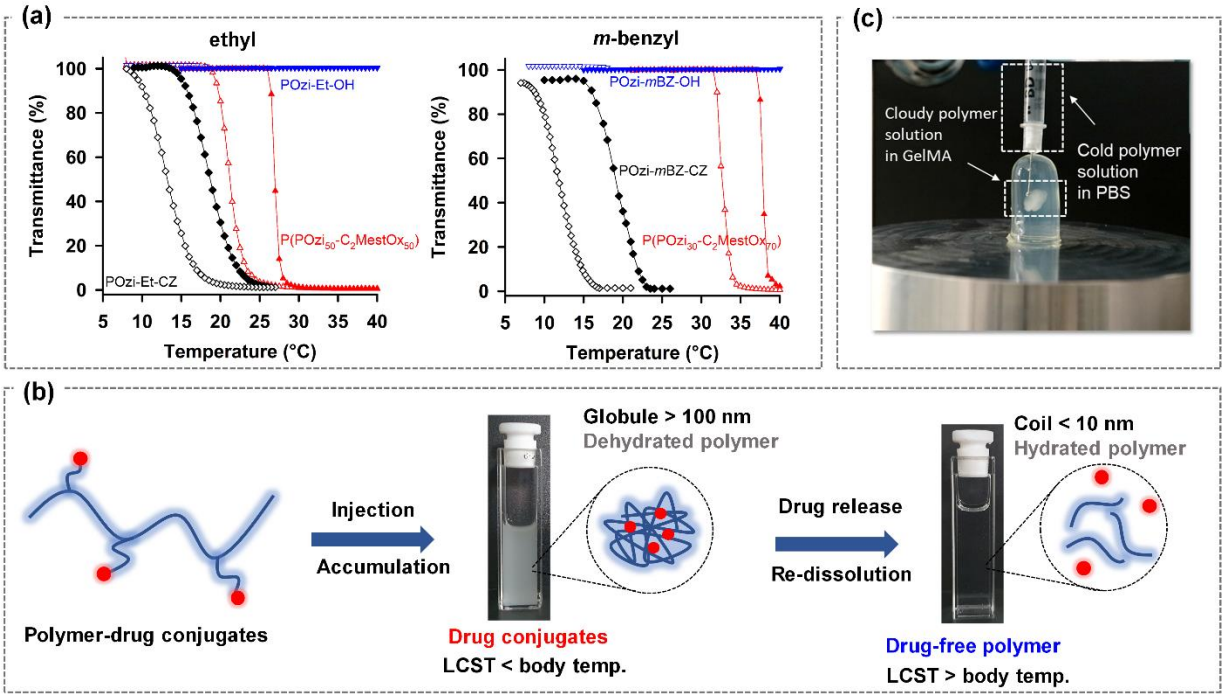


Figure 4. (a) Turbidity versus temperature for a series of copolymers when heating (filled symbol) and cooling (empty symbol) with a polymer concentration of 5 mg/mL in PBS (left) ethyl linkage and (right) *m*-benzyl linkage. (b) Illustration of polymer-drug conjugates and drug-free polymers in a solution at body temperature. (c) The image of polymer-drug conjugate solution injected into a GelMA skin-mimic at 37 °C (also see Movie SI1).

3.5. Size determination of polymers in solution

To further investigate the aggregation behavior of the polymer-drug conjugates, DLS measurements were performed at 15 °C (potential injection temperature) and 37 °C (body temperature). For a concentration of 5 mg/mL at 37 °C, the P($POzi_{50}$-$C_2MestOx_{50}$) showed aggregation with a globule size of 3-6 µm. Conversely at 15 °C, the polymer was completely soluble with a typical size of 7 ± 3 nm indicative of individual polymer chains (**Figure 5** and **Figure S10**), similar to previous reports for PAOx

copolymers.[20, 27] By lowering the concentration to 0.5 mg/mL the globules were slightly smaller at 37 °C (1.4±0.4 µm), revealing that the aggregation depends on the concentration of polymer.

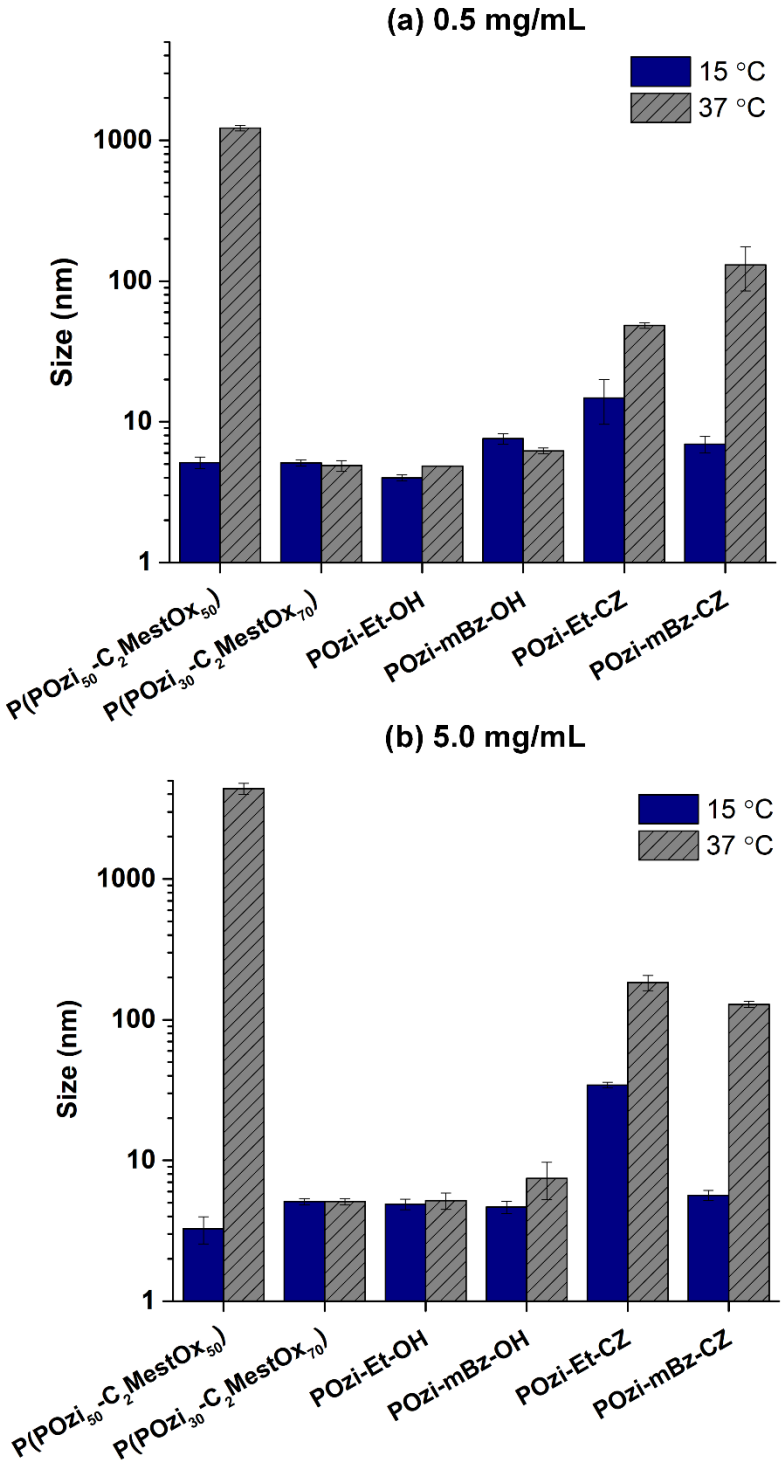


Figure 5. Size measurement of a series of copolymers at 15 and 37 °C, determined by DLS. Polymer concentration = (a) 0.5 mg/mL and (b) 5.0 mg/mL in PBS (pH 7.4). Mean ± standard error (n=3).

The sizes of P($POzi_{30}$-C_2MestOx_{70}), POzi-Et-OH and POzi-*m*Bz-OH were 5-8 nm at both 15 °C and 37 °C (**Figure 5a**) in agreement with the T_{cp} measurements that revealed water-solubility up to at least 40 °C. This result highlights the potential for elimination of the polymers after drug release from the depot following injection into the body.[28-30]

Importantly, the two drug-conjugated systems, POzi-CZ (Et and mBz), demonstrated different particle sizes at high and low temperatures by DLS. At 37 °C particles of ~~the two temperatures examined of~~ *ca.* 100 nm were observed for both systems (for 5.0 mg/mL). At 10 °C (data not shown) no particles were observed which is consistent with the 100% transmittance observed for the T_{cp} experiments (**Figure 4a**). At 15 °C the particles were measured to be < 40 nm for the drug conjugated polymers (Figure 5). These values were determined by cooling the polymer solution to 15 °C leading to the presence of the particles due to the hysteresis in the LCST transitions as also shown in Figure 4, where low transmittance was observed upon cooling the samples to 15 °C.

To further investigate the lower concentration limit for which aggregation occurs, the UV absorbance of the solubility probe[31] 1,6-diphenyl-1,3,5-hexatriene was performed in PBS buffer (pH 7.3) at body temperature. It was shown that a break in the absorbance versus concentration occurred in the range 0.04-0.05 mg/mL for the two drug conjugated systems (**Figure S11**) indicating the critical aggregation concentration (CAC) at 37 °C, also implying that this is the lower limit in concentration for the aggregation upon injection phenomenon to be active.

3.6. Release of free drug from polymer-drug conjugates

The dynamic phase transition behavior of the polymers used here would be expected to influence the release of the drug due to changes in hydration, thus impacting the rate of hydrolysis of the linker between

the polymer and drug. To test this, solutions of the two polymer-drug conjugates, POzi-Et-CZ and POzi-*m*Bz-CZ were prepared at a concentration of 5 mg/mL and the drug release was studied at 37 °C and pH of 8.5 – this pH was chosen as an accelerated drug release model compensating for absence of esterases that would be found in human tissue.[22, 32] Because of the long release times (up to 48 hrs) some degradation of the cefazolin was observed. Cefazolin is known to be unstable under various conditions, including basic conditions[33,34] By measuring the degradation of cefazolin at pH 8.5 over time (**Figure S12**) the degraded cefazolin could be compensated for in the release plots by adding the degraded amount to the measured amount (equations can be found in the supporting information). As shown in **Figure 6a**, quantification of the released cefazolin by HPLC revealed that the release-rate was close to zero-order or minor accelerated drug release in the first 8 hours, which may be ascribed to the enhanced re-hydration of the polymer-drug conjugates upon release of the drug. The kinetics of release are also expressed as percent release per hour in **Figure 6b**. This highlights the small initial burst followed by a slight acceleration in rate up to 8 hours, after which the release rate drops ~~to *ca.* <1% per hour~~. Curiously, the POzi-Et-CZ system reached full drug release at 48 hrs whereas the POzi-*m*Bz-CZ system did display some of the undesirable slow ~~trickle~~ release, although more frequent sampling would be required between 20 and 48 hrs to fully investigate the release kinetics in this region.

Both **Figure 6a** and **b** highlight the slightly faster drug release from POzi-*m*Bz-CZ compared to POzi-Et-CZ in the first 8 hours. Since the rate of drug release is supposedly dictated by both the physical re-hydration polymer-drug conjugates and the stability of the ester bond, it is difficult to conclude which factor is most influential, however we hypothesize that the slightly faster release of cefazolin from POzi-*m*Bz-CZ, at least initially, could be due to the aryl-ester being more labile than the aliphatic-ester. This can be attributed to the lower electron density of the aryl-ester compared to the aliphatic ester at the hydrolytic active site leading to faster degradation as explained by the Hammett equation.[35, 36] Similar results by the groups of Stayton and Convertine were observed for the conjugation of the antibiotic ciprofloxacin on a

hydrophilic scaffold using aliphatic- and aromatic-esters, also showing that the drug release was faster when the aryl-ester was used as a linker.[37-39]

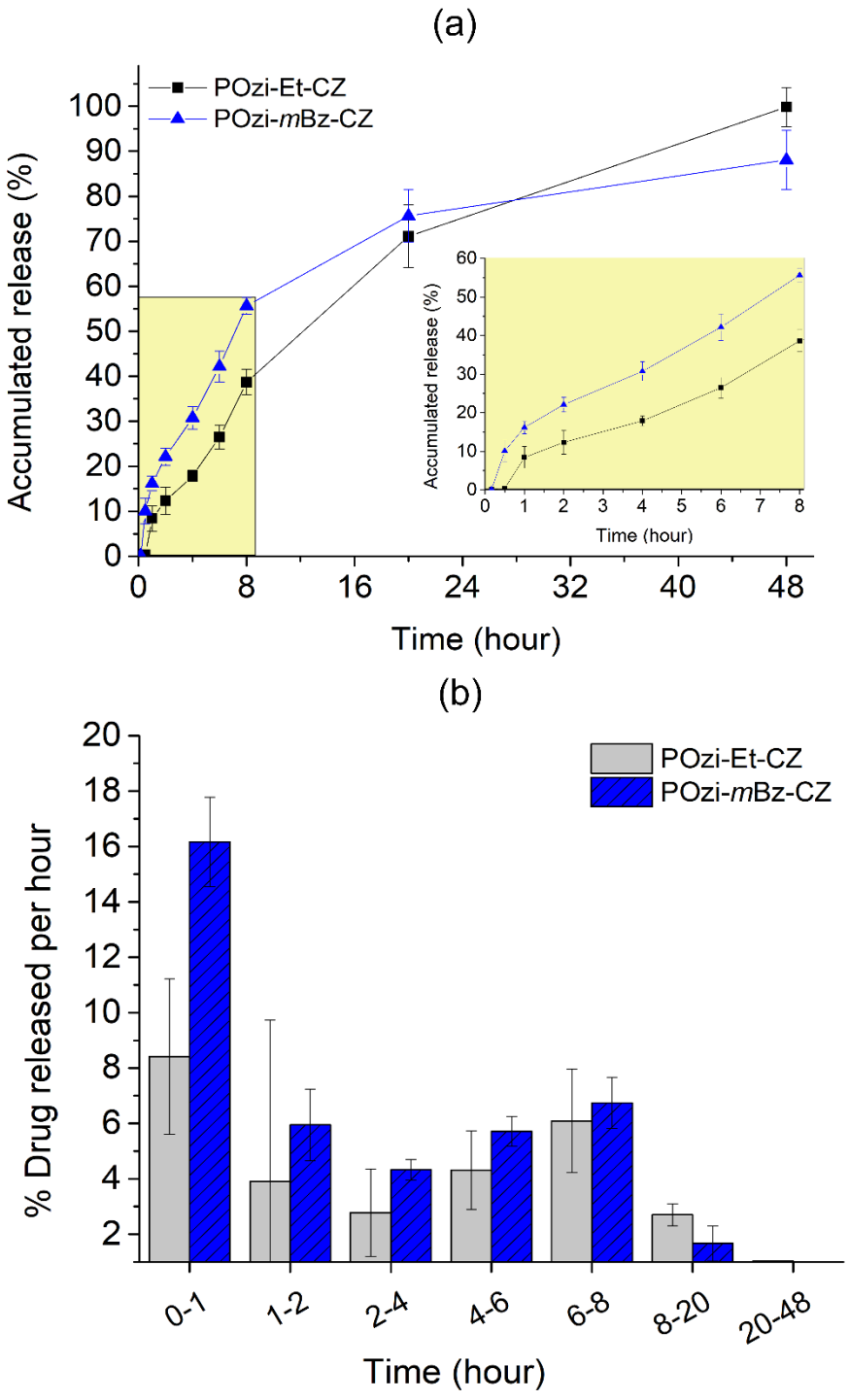


Figure 6. (a) The drug release profiles of POzi-Et-CZ and POzi-*m*Bz-CZ at pH 8.5 and 37 °C. The accumulated release is expressed as a percentage of the total drug conjugated to the polymers, (b) percentage drug release per hour over different periods for POzi-Et-CZ and POzi-*m*Bz-*CZ*. Mean ± standard error (n=3).

3.7. Antibiotic activity of polymer-released cefazolin

Recent clinical studies have recommended the use of cefazolin for treatment of *E. coli* infections in circumstances where a high concentration of drug can be achieved at the infection site to avoid antibiotic resistance.[40, 41] Hence, to test our thermoresponsive cefazolin conjugates in a relevant scenario *E. coli* were treated with the drug loaded polymer, together with POzi-Et-OH and no polymer as controls. Considering the kinetics of cefazolin release of POzi-Et-CZ was similar (albeit slightly faster initially) to that of POzi-*m*Bz-CZ (**Figure 7**) only the former was used.

Firstly, the MIC of cefazolin and polymers towards *E. coli* was determined in Mueller Hinton broth (MHB) with Tris Buffer (50 mM) at pH 8.5 to be consistent with the pH conditions of the drug release study. Cefazolin, POzi-Et-OH, and POzi-Et-CZ were dissolved (or suspended in the case of POzi-Et-CZ as this is above the T_{cp}) in media to achieve concentration ranges between 512-2 μg/mL then incubated for 24 hours at 37 °C with *E. coli*. The resulting MICs were 8 μg/mL for cefazolin and 32 μg/mL for POzi-Et-CZ. The polymer without cefazolin (POzi-Et-OH) showed no antibacterial activity (MIC > 512 μg/mL).

Based on the MIC results, a concentration of 64 μg/mL cefazolin (≈ 0.74 mg/mL of polymer) was used in time-kill assays. Both the growth control and the cefazolin-free polymer (POzi-Et-OH) showed consistent *E. coli* growth over 24 hours (**Figure 7**), in agreement with the findings above that the polymer alone has no anti-bacterial activity. This is also consistent with previous studies with mammalian cells which showed that non-ionic PAOx copolymers do not have any structural features to cause them to be toxic.[42] Conversely, for *E. coli* treated with cefazolin, there was a dramatic decrease in the number of viable bacteria recovered after 3 hours, as expected for a bolus dose of this antibiotic. *E. coli* treatment with the POzi-Et-CZ conjugate prevented the increase in bacterial numbers seen in the untreated growth control,

with recovered bacterial numbers remaining stable for 6 hours. Following that, an increase of released cefazolin between 6 and 12 hours led to a reduction in viable bacteria and total clearance by 12 hours, consistent with the release profile in **Figure 6**.

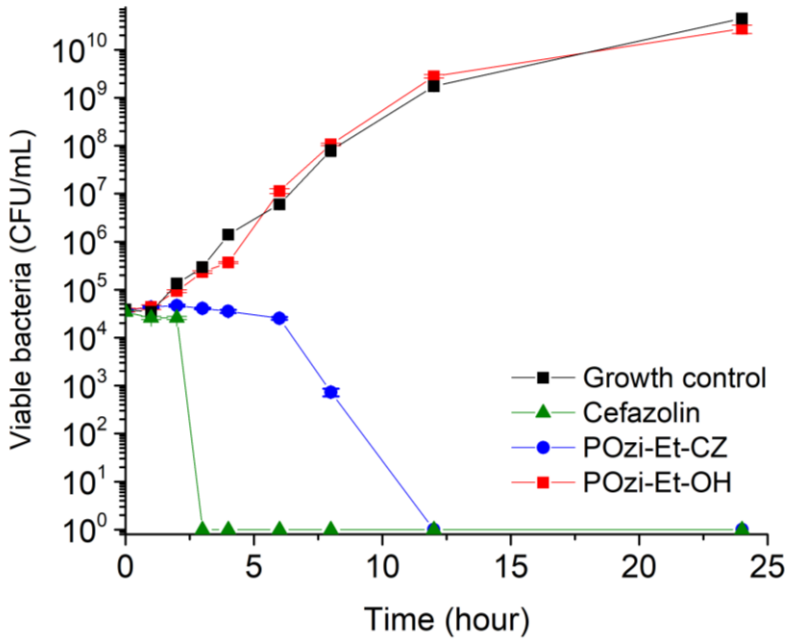


Figure 7. *E. coli* time-kill assay for polymers and cefazolin at 37 °C, pH 8.5 with the drug concentration set at 64 μg/mL either as free drug or as the equivalent amount of drug loaded to the polymer. Mean ± standard deviation (n=3).

4. Conclusions

Thermoresponsive POzi-cefazolin conjugates with two different ester linkages (ethyl and *m*-benzyl) between the drug and the polymer chain were synthesized. The thermoresponsive behavior of the polymers was highly dependent on their structures and their hydrophobicity ~~of polymers~~. The hydroxyl-functionalized polymers were water-soluble with a size of 5-8 nm at 37 °C while the polymer-drug conjugates aggregated at 37 °C due to their LCST behavior. This meant the polymer-drug conjugate could be cooled to below the T_{cp} and injected through a narrow-gauge needle as a homogeneous solution into a

skin-mimic at body temperature where it collapses to form a depot. The drug release of the polymer-drug conjugates showed zero-order or minor accelerated release due to the dynamic change of hydrophilicity upon drug release. Furthermore, the polymer-drug conjugates were effective in killing *E. coli* indicating the release of active cefazolin. The results suggest these polymers may be useful as injectable and thermoresponsive materials for applications where constant and complete elution of an antibiotic from a dynamically soluble depot is desirable. Studies investigating the toxicity and safety of these materials both in their soluble and precipitated forms using animal models are part of future work.

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