Poly(2-ethyl-2-oxazoline) Conjugates with Salicylic Acid via Degradable Modular Ester Linkages

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ABSTRACT.

Conjugation of drugs to polymers is a widely used approach to gain control over the release of therapeutics. In this contribution, salicylic acid, a multipurpose model drug, is conjugated to the biocompatible poly(2-ethyl-2-oxazoline) (PEtOx). The drug is attached to the side chains of a polymer carrier trough a hydrolytically cleavable ester linker, via a sequential post-polymerization modification. The chemical modulation of this ester, *i.e.,* obtained from primary or secondary alcohol, is demonstrated to greatly influence the ester hydrolysis rate. This crucial parameter allows to tune the *in vitro* kinetics of the sustained drug release for periods over a month in PBS. The synthetic accessibility of the cleavable linker together with the modularity of the drug release rate offered by this approach, highlights the utility of this class of polymers in the field of long-lasting drug delivery systems for persistent and chronic disease treatment.

KEYWORDS. Polymer-drug conjugates, polymer therapeutics, poly(2-alkyl-2-oxazoline)s, salicylic Acid.

1. INTRODUCTION.

Controlled release of drugs is one of the key features for the next generation of effective therapeutics.[1] An ideal system for persistent and chronic disease treatment delivers the therapeutic agent at a constant rate over the treatment period, maintaining the drug concentration within the therapeutic window, thereby reducing the total amount of drug required, as well as side effects. A long-term controlled drug release system also offers the possibility to inject or implant it directly within a specific diseased tissue, thereby limiting off-target effects, increasing potency and avoiding multiple injections. It is particularly relevant for the treatment of persistent and chronic disorders such as infection, inflammation or treatment of pain.[2,3]

Polymeric drug delivery systems have been widely developed for this purpose, and numerous systems are clinically applied.[4] They can be obtained by physical incorporation or formulation of the drug into a polymeric material (*e.g.,* hydrogel), or a (partially) polymeric nanoparticle (polymeric micelles, PEG-stabilized liposomes). Polymer-drug conjugates (PDC) in which drug molecules are covalently bound to polymeric systems via biodegradable linkers, are another promising approach to gain better control over the release profile. The clinical application of polymer drug conjugates for this purpose is increasing due to attractive features such as improved solubility of the conjugated drugs, prolonged circulation, reduced immunogenicity, controlled release, reduced toxicity and targeted delivery.[5] The choice of linker chemistry can provide specificity from a trigger (*e.g.,* pH, enzymes), increased the drug stability and allow for spatial and kinetic control of the drug release.[6] So far, many PDC have been developed, mostly based on the poly(ethylene oxide) (PEO) and poly(2-hydroxylpropyl methacrylamide) (PHPMA) polymeric scaffolds.[5,7–9]

As a promising alternative, poly(2-alkyl-2-oxazoline)s (PAOx) are a class of polymers of growing interest.[10–16] Especially, poly(2-methyl-2-oxazoline) (PMeOx) and poly(2-ethyl-2-oxazoline) (PEtOx) possess desirable features for use in drug delivery systems, as they are generally safe at therapeutic doses, biocompatible, highly soluble in water and resistant toward degradation. Furthermore, they can be prepared in a controlled and straightforward manner via cationic ring-opening polymerization (CROP) of functional 2-oxazoline monomers, yielding well-defined polymers with narrow dispersity and with control over side chain and chain-end functionality.[17–22] This chemical versatility creates many opportunities for the development of PAOx-based PDC. However, the reports of PAOx-drug conjugates are still relatively limited, opening the way for further studies.[15] Even though drugs, including Ara-C,[23] ciprofloxacin,[24] penicillin,[25] and doxorubicin,[26] have been linked to PAOx using end-chain functionalization strategies, it is more promising to introduce functional groups and drugs in the side-chains, which allows for higher drug loading. This approach has been strongly developed by Moreadith and coworkers at Serina therapeutics to conjugate multiple rotigonine (a dopamine agonist) molecules to the side-chain of PEtOx through a cleavable ester linker, introduced via CuAAc click chemistry.[27–29] The resulting system (SER214) was recently tested in humans revealing that the once-a-week subcutaneous administration of SER-214 leads to steady blood levels of drug, due to slow cleavage of the ester linker. These extended *in-vivo* pharmacokinetic profiles indicate excellent effectivity in the treatment of Parkinson's disease symptoms. Related PEtOx PDC with phenolic drugs, including buprenorphine, dexanabinol, cannabidiol, Δ9-tetrahydrocannabinol, and cannabigerol, have been reported by Serina Therapeutics.[29] The corresponding PDC all show slow release kinetics due to ester cleavage combined with a "core-shell" behavior of the polymer which delay the exposure of the drug to the external media and

the hydrolytic cleavage. PAOx-drug conjugates with side-chain loading of the drug have also been developed by doxorubicin conjugation via a hydrazone linker,[30] or by irinotecan metabolite[31] or pterostilbene[32] conjugation via ester linkers.

Aiming for tunable control over the drug release rate, we studied the influence of the chemical nature of the ester linker in PAOx-based PDC. In contrast to the approaches mentioned above based on hydroxyl-containing drugs, we conjugated carboxylic acid-containing drugs to PEtOx having alcohol groups in the side chains. In this fashion, the chemical nature of the ester, *i.e.* based on a primary or secondary alcohol-based, and consequently the hydrolysis rate can be modulated by varying the chemical structure of the alcohol that is attached to the polymer. This follows our previous work centred on conjugation, *via* thiol-ene chemistry, of the carboxylic acid drug benazepril to a PAOx hydrogel network though a primary alcohol based ester linkage.[33] Other groups have conjugated carboxylic acid drugs to polymeric carriers via linkers based on an ester bond, but examples are mostly based on esters constructed from primary alcohols,[34–37] and only few examples have been reported based on phenols[38] or secondary alcohols.[39]

Within this work, we further open up possibilities to conjugate a wider variety of other drugs that contain carboxylic acid groups to the polymer trough a tunable linker. Salicylic acid (SA) was chosen as model drug as it perfectly fits our objectives for this study: it is relatively inexpensive, usually administered on a regular and recurring basis for long-term treatment and it contains a carboxylic acid group suitable for our conjugation approach. Beyond this classic use as an anti-pain agent (in the form of prodrug, i.e. aspirin) or for topical skincare, SA has shown potential as an analgesic, antipyretic, or anti-inflammatory agent,[40,41] and also to treat cancer,[42] arthritis,[43] fungal infections,[44] or diabetes.[45] However, its low half-life and stability in the body can induce toxicity and side-effects, such as the risk of gastrointestinal bleeding during repeated oral

consumption,[46] and are major concerns that need to be addressed in order to use it as a potent drug in new fields of application. Studies have already been reported to reach controlled release of SA by conjugating or incorporating it to various polymeric scaffolds, such as poly(anhydride ester)s,[47,48] polyesters,[49] polypropylene,[50] polyacrylamide,[51,52] polyacrylic esters,[53] and poly(2-hydroxyethyl methacrylate).[54] However, most of these polymer carriers are hydrophobic, thereby limiting liquid form administration.

This study aims to further develop PDC to increase their potential applicability: 1) their solubility is sometimes limited, 2) their release rate over time is not always controlled. In this work, we report the successful conjugation of SA to PEtOx (Figure 1). The use of the water soluble and biocompatible PAOx carrier combined with tunable ester linkers allow to overcome the abovementioned challenges. Overall, this work will demonstrate that the chemical versatility and post-modification opportunities of the PAOx platform offer large potential for the preparation of a sustained release drug delivery system, potentially useful for chronic and recurrent disease treatment trough soluble delivery forms.

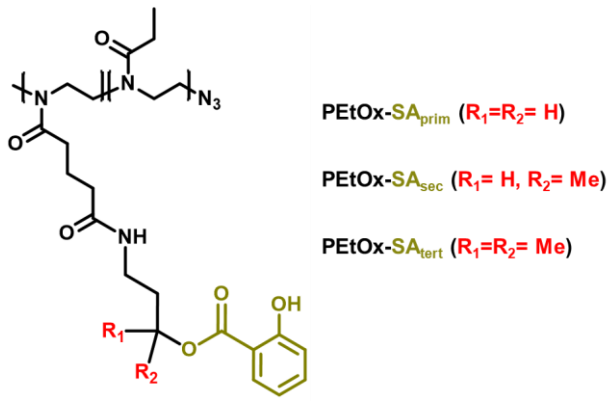


Figure 1. *Structure of the studied PEtOx-Salicylic Acid conjugates*

2. EXPERIMENTAL SECTION

2.1. Materials.

2-Ethyl-2-oxazoline (EtOx) was kindly donated by Polymer Chemistry Innovation and was distilled over BaO before use. 2-Methoxycarbonylpropyl-2-oxazoline (C3-MestOx) was synthesized according to literature,[55] and distilled over BaO before use. Methyl *p*-toluenesulfonate (MeOTs) was obtained from Sigma-Aldrich and was distilled from CaH_2 prior to use. Acetonitrile (Sigma-Aldrich) was purified over aluminum oxide using a solvent purification system from J.C. Meyer. Salicylic acid *N*-hydroxysuccinimide ester was prepared according to literature and purified by silica gel column chromatography (AcOEt/Hexane) prior to use.[56] All other chemicals, including 2-ethanolamine, 1-amino-2-propanol, 1-amino-2-methylpropan-2-ol, 1,5,7-Triazabicyclo[4.4.0]dec-5-ene (TBD), 4-dimethylaminopyridine (DMAP), potassium carbonate, triethylamine, imidazole (Im), ethanol, *iso*propanol, *tert*-butanol, were purchased from TCI Europe or Sigma Aldrich and were used as received. Dialysis membranes (regenerated cellulose - 3.5 kDa cutoff) were acquired from Roth. Lipophilic Sephadex LH20 for preparative gel filtration chromatography was purchased from Merck. Acid, base and salts for buffers solutions preparation, *i.e.,* PBS tablets, citric acid, dibasic sodium phosphate, boric acid, sodium hydroxide, were purchased from Sigma Aldrich.

2.2. Methods.

Nuclear magnetic resonance (^{1}H-NMR) spectra were measured at room temperature with a Bruker Advance MSL 400 MHz or 300 MHz NMR spectrometer. All chemical shifts are given in parts per million (δ, ppm) relative to tetramethylsilane. Deuterated solvents, such as chloroform-d, and dimethylsulfoxide-d_6, were purchased from Eurisotop. Size exclusion chromatography (SEC) was performed on an Agilent 1260-series HPLC system equipped with a 1260 online degasser, a 1260 ISO-pump, a 1260 automatic liquid sampler (ALS), a thermostatted

column compartment (TCC) set at 50 °C equipped with two PLgel 5 µm mixed-D columns (7.5 mm × 300 mm) and a precolumn in series, a 1260 diode array detector (DAD) and a 1260 refractive index detector (RID). Distilled *N*,*N*-dimethyl acetamide (DMA) containing 50 mM of LiCl was used at eluant at a flow rate of 0.5 mL min^{-1}. Number-averaged molar mass values (M_n) and dispersity ($Đ$) values are calculated against narrow dispersity PEtOx standards that were prepared in house. Infrared (IR) spectra were measured on a PerkinElmer 1600 series FTIR spectrometer and are reported in wavenumber (cm^{-1}). Lyophilization was performed on a Martin Christ freeze-dryer, model Alpha 2–4 LSC plus. High-performance liquid chromatography (HPLC) was performed on a Agilent 1200 Series HPLC instrument equipped with a 1200 DAD detector, a TCC set at 35 °C, and a Kinetex C18 (5u, 100 Å, 150 x 4.60 mm) column from Phenomenex. The flow rate was 4.5 mL/min and the column was eluted with a gradient from 100% H_2O with 0.1% TFA to 100% acetonitrile in 10 min, then with 100% acetonitrile for 5 min. Solvents used were HPLC grade and obtained from Sigma Aldrich. Ultrapure deionized water (Milli Q) was prepared with a resistivity less than 18.2 MΩ x cm using an Arium 611 from Sartorius with the Sartopore® 2 150 (0.45 + 0.2 µm pore size) cartridge filter.

2.3. Procedures

Synthesis of $PEtOx_{94}$-co-$C3PMestOx_6$.

EtOx (6.82 mL, 67.68 mmol), C3MestOx (0.754 mL, 4.32 mmol), MeOTs (0.108 mL, 0.72 mmol) and acetonitrile (10.42 mL) were introduced in a 20 mL microwave vial in a glove box. The polymerization was performed in the Biotage microwave for 12 min at 140 °C. Then, sodium azide (140 mg, 2.16 mmol) was added to the mixture, which was stirred at room temperature overnight to terminate the polymerization. The polymer was precipitated in tenfold

excess of cold diethyl ether, and isolated by decantation and drying. It was then dissolved in water and dialyzed with 3.5 kDa cutoff tubes against Milli-Q water which was replaced three times, and freeze-dried to obtain the final polymer as a white powder. The product was finally dried in a vacuum oven for several hours.

Yield = 92%. ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm)= 0.85-1.05 (m, 282H), 1.72 (m, 12H), 2.15-2.40 (m, 212H), 3.15-3.55 (m, 400H), 3.57 (s, 18H). SEC (DMA): M_n = 9.4 kDa, $Đ$ = 1.16.

Synthesis of PEtOx-co-C3MestOx-OH$_{prim}$ and PEtOx-co-C3MestOx-OH$_{sec}$

PEtOx-co-C3MestOx (1 g, 0.096 mmol) was dissolved in 5 mL of acetonitrile and 2-ethanolamine (0.207 mL, 3.465 mmol, 6 eq. per COOMe), 1-amino-2-propanol (0.267 ml, 3.465 mmol, 6 eq. per COOMe) or 1-amino-2-methylpropan-2-ol (0.646 mL, 6.930 mmol, 12 eq. per COOMe) was added to the mixture, followed by addition of TBD (40.2 mg, 0.287 mmol, 0.5 eq. per COOMe or 80.4 mg, 0.574 mmol, 0.5 eq. per COOMe). The reaction was performed at 40°C (50°C for 1-amino-2-methylpropan-2-ol) for 24h. After cooling down to room temperature, the mixture was added to tenfold excess of cold diethyl ether to precipitate the polymer, and the resulting solid was isolated by filtration. It was dissolved in water and dialyzed three times against Milli-Q water with 3.5 kDa cutoff tubes and then freeze-dried to obtain the final polymer. The white powder was dried in a vacuum oven for several hours.

PEtOx-co-C3MestOx-OH$_{prim}$: Yield = 82%. ^{1}H NMR (300 MHz, DMSO-d_6): δ_H 0.85-1.05 (m, 282H), 1.68 (m, 12H), 2.07 (m, 12H) 2.15-2.40 (m, 200H), 3.08 (m, 12H), 3.15-3.55 (m, 412H), 4.61 (m, 6H), 7.74 (s, 6H). SEC (DMA): M_n = 10.9 kDa, $Đ$ = 1.16.

PEtOx-co-C3MestOx-OH_{sec}: Yield = 78%. ^{1}H NMR (300 MHz, DMSO-d_6): δ_H 0.85-1.05 (m, 300H), 1.69 (m, 12H), 2.07 (m, 12H) 2.15-2.40 (m, 200H), 2.97 (m, 12H), 3.15-3.55 (m, 406H), 3.57 (s, 12H), 4.62 (m, 6H), 7.72 (s, 6H). SEC (DMA): M_n = 11.1 kDa, $Đ$ = 1.17.

PEtOx-co-C3MestOx-OH_{tert}: Yield = 79 %. ^{1}H NMR (300 MHz, DMSO-d_6): δ_H 0.85-1.02 (m, 282H), 1.03 (s), 2.07 (m, 12H) 2.15-2.40 (m, 200H), 3.05 (m), 3.15-3.55 (m, 400H), 5.41 (m), 7.65 (s). SEC (DMA): M_n = 10.8 kDa, $Đ$ = 1.18

Synthesis of PEtOx-co-C3MestOx-SA_{prim} and PEtOx-co-C3MestOx-SA_{sec}

PEtOx-co-C3MestOx-OH_{prim} or PEtOx-co-C3MestOx-OH_{sec} (0.200 g, 0.019 mmol) was dissolved in chloroform (1 mL), and DMAP (42 mg, 0.343 mmol, 3 eq. per OH) followed by addition of salicylic acid NHS-ester (40 mg, 0.171 mmol, 1.5 eq. per OH). The reaction was conducted at room temperature for 24 h. The chloroform was evaporated, and a small amount of methanol was added. The mixture was subjected to LH20 preparative SEC. The desired fractions were collected and concentered under vacuum. The polymers were then dissolved in water and freeze dried to obtain the final products as powders, which were further dried in the vacuum oven.

PEtOx-co-C3MestOx-SA_{prim}: Yield = 91%. ^{1}H NMR (300 MHz, DMSO-d_6): δ_H 0.80-1.10 (m, 282H), 1.67 (m, 12H), 2.10 (m, 12H), 2.15-2.50 (m, 200H), 3.20-3.60 (m, 412H), 4.30 (t, ^3J= 5.4 Hz, 12H), 6.94 (m, 24H), 7.51 (t, ^3J= 7.9 Hz, 12H), 7.83 (d, ^3J= 7.94 Hz, 12H), 8.06 (s, 12H), 10.46 (m, 12H). SEC (DMA): M_n = 11.5 kDa, $Đ$ = 1.21.

PEtOx-co-C3MestOx-SA_{sec}: Yield = 79%. ^{1}H NMR (300 MHz, DMSO-d_6): δ_H 0.80-1.15 (m, 282H),1.25 (m, 18H), 1.70 (m, 12H), 2.10 (m, 12H), 2.15-2.50 (m, 200H), 3.20-3.60 (m, 412H),

5.11 (m, 6H), 6.95 (m, 24H), 7.50 (t, ^3J= 7.9 Hz, 12H), 7.81 (d, ^3J= 7.94 Hz, 12H), 8.04 (s, 12H), 10.54 (m, 12H). SEC (DMA): M_n = 11.4 kDa, $Đ$ = 1.21.

Drug release measurements.

The samples were dried in a vacuum oven at 40 °C for 24 h before use. The polymer conjugates (1 mg) were dissolved in 1 mL of the appropriate buffer, respectively citrate-phosphate buffer (0.1 M, pH 3.0 or pH 5.0), HCl/KCl (0.1 M, pH 1.0), PBS (0.15 M, pH 7.4) or sodium borate buffer (0.1 M, pH 8.5) and incubated at 37°C. In predetermined time intervals, the samples were taken and analyzed by HPLC (detection UV 310 nm). The percentage of release was determined by comparison of the initial SA peak and the peak at the different intervals of time. Calibration curves have been established for SA solutions in each buffer prior to measurement.

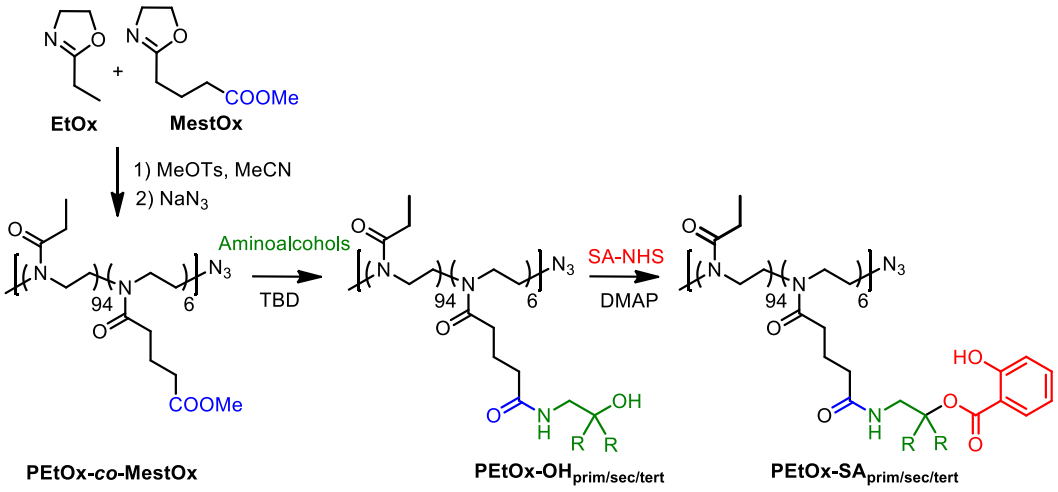
MTT assay

DC2.4 cells were seeded in 96-well plates at a density of 20,000 cells in160 µL per well and incubated at 37 °C. Each well was pulsed with 40 µL of samples, PBS (negative control) or DMSO (positive control). After overnight incubation at 37 °C, 20 µL of MTT reagent (5 mg/mL in PBS) was added to each well and incubated for 2 h at 37 °C. The formed formazan crystals were dissolved by addition of 100 µL DMSO and incubated 10 min. Quantification was done by measuring the absorbance at 590 nm using a microplate reader (n =6, data shown as mean + SD).

3. RESULTS AND DISCUSSION

3.1. Synthesis.

The synthetic approach used to prepare the polymer-drug conjugates is depicted in Scheme 1. The functional monomer C3MestOx was prepared as previously reported.[55] Subsequent statistical copolymerization of C3MestOx and EtOx by living cationic ring-opening co-polymerization (CROP) in acetonitrile at 140°C under microwave irradiation yielded the PEtOx-co-C3MestOx copolymer with side-chain methyl ester group as narrow dispersity polymers. The living polymerization is terminated by the addition of sodium azide, leading to an azide chain-end group. This end-group is suitable for potential further functionalization, albeit this was not further exploited in this work. A low percentage of functional side chains (6% COOMe targeted, 6.4% obtained, Table 1) was introduced in order to keep the final PDC (*i.e.,* after conjugation of the drug) fully water-soluble to suppress aggregation or micelle formation, that can potentially occur in solution and affect the drug release. The full water-solubility of the PDC will allow us to truly assess the impact of the linker during release measurements.



Scheme 1. *Synthetic pathway for the preparation of the PEtOx-salicylic acid conjugates (R= H or Me; SA-NHS is the NHS-ester of siacylic acid).*

The post-polymerization modification of the PEtOx-C3MestOx copolymer to introduce the OH functionality is performed by direct organocatalyzed amidation of the pendant methyl ester group using 5 mol% per ester group of triazabicyclodecene (TBD) as catalyst and an excess (6 equivalents) of the aminoalcohols, *i.e.,* aminoethanol, aminopropan-2-ol and 1-amino-2-methylpropan-2-ol. This method, previously developed in our group,[57–59] allows a straightforward introduction of various functional handles, and was found to be perfectly compatible with the reactants of the current study. The full conversion of the methyl ester group to amide is evidenced by FTIR spectroscopy (Figure 2A). A higher temperature (50 °C), together with more equivalents of TBD (10 mol%) and more equivalents of amine (12 eq.) were required with 1-amino-2-methylpropan-2-ol as the nucleophile. The lower reactivity observed is probably due to the presence of the sterically hindered proximal quaternary carbon center. The ^{1}H NMR spectra in Figure 2B confirm the complete removal of methyl ester group (peak at 3.55 ppm) and the appearance of the representative peaks for the amidation products at higher ppm values (4.5-5.5 ppm for OH and 7.5-8 ppm for NH). The degree of functionalization was determined from the spectra (Figure 2B and Figure S11-13; Table 1). This percentage is rather low (0.5%) for PEtOx-SA_{tert}, while it is around 4-5% for PEtOx-$OH_{prim/sec}$. However, this should be taken with caution as values are obtained from integration of polar exchangeable protons (-OH).

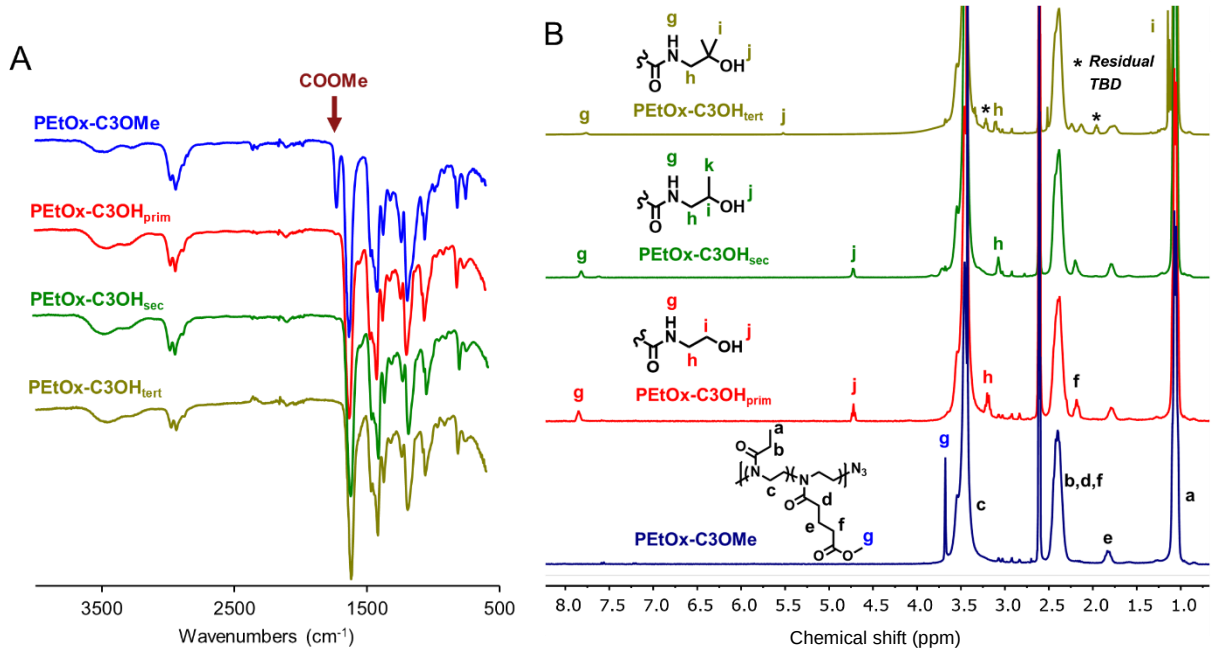


Figure 2. *Stacked FTIR spectra (A) and ^{1}H-NMR spectra (DMSO-d_6) (B) of the starting PEtOx-co-MestOx together with the products after amidation with the different aminoalcohols.*

To conjugate SA to the polymer, it was first converted to the activated NHS ester as described previously,[56] yielding SA-NHS. Before conjugating SA to the polymer, we optimized the conditions for the acylation of alcohols with SA-NHS. 4-Dimethylaminopyridine (DMAP) was found to be preferable over the other investigated bases, as it leads to higher reaction efficiency (Figure S1.A). Model reactions of SA-NHS with ethanol, *iso*propanol and *tert*-butanol (Figure S1.B), revealed that the reaction is significantly affected by the substitution degree of the alcohol as very low quantities of the *tert*-butyl ester were detected even after longer reaction time.

The optimized coupling conditions were subsequently applied to react SA-NHS with the OH containing polymers in the presence of DMAP as a catalyst. The unreacted free drug could be completely removed by preparative SEC, as confirmed by the absence of any low molar mass peaks on the SEC eluogram (Figure S5). The presence of the SA on the polymer was confirmed

by the broad and low-intensity signals in the aromatic region in the ^{1}H-NMR spectrum of PEtOx-SA$_{prim}$ (Figure 4B). The mass loading of SA was found to be 6.7 % in case of PEtOx-SA$_{prim}$ and 4.2 % in the case of PEtOx-SA$_{sec}$ (Table 1, see SI for calculation and figure S16-S17). This is consistent with the lower reactivity observed for the secondary alcohol in the model reactions. The slower conjugation of SA-NHS to the PEtOx-OH$_{sec}$ was also evidenced when monitoring the conjugation reaction by SEC with a UV-detection (Figure S4). Unfortunately, it was impossible to conjugate SA to the polymer with the tertiary alcohol-containing side-chain despite several attempts. Even after a long period (6 days), with large excess of SA-NHS (5-10 eq.), at higher temperature (60°C), no clear SA peaks were found in the ^{1}H NMR spectrum, and the reaction led to high dispersity polymers (Table S1, Figure S6). Again, this is also consistent with the poor reactivity observed during model reactions.

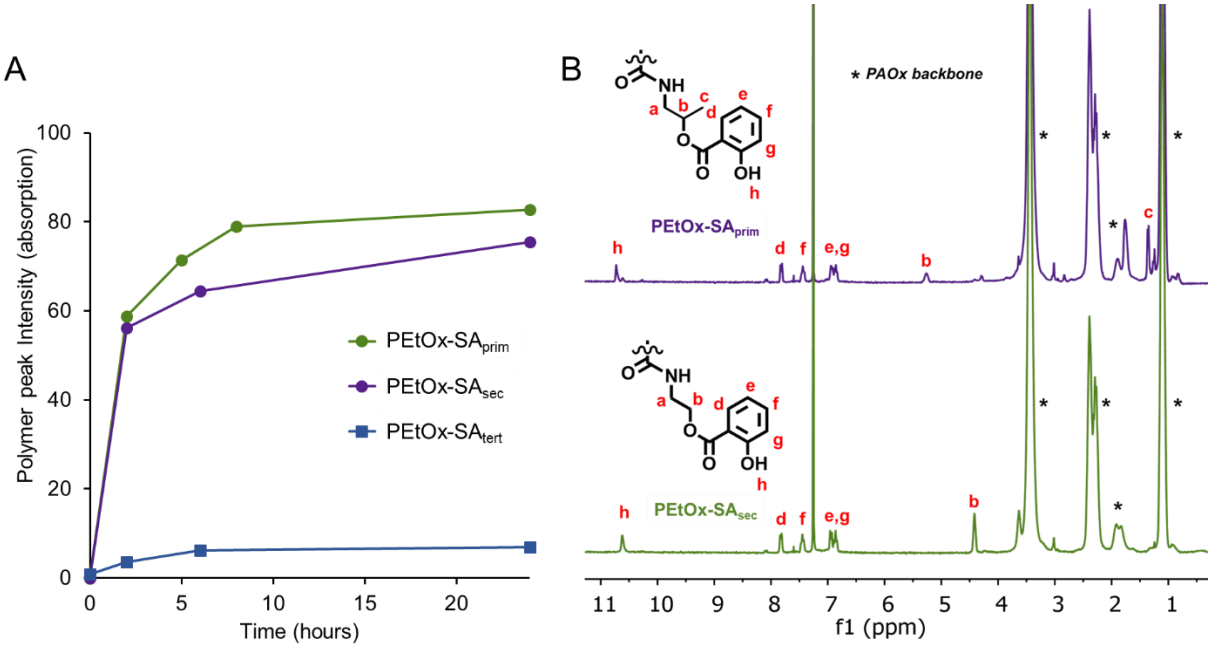


Figure 3. *Kinetic curves of PAOx-SA conjugates detected by SEC (300 nm detection) (A) and ^{1}H-NMR spectra ($CDCl_3$) of the PEtOx-SA$_{prim}$ and PEtOx-SAs$_{ec}$ conjugates (B).*

For the PEtOx-SA conjugates based on the primary and secondary alcohols, the narrow dispersity is maintained during the synthetic sequence, resulting in PDC of $Đ < 1.2$ (Figure 4 and Table 1). The step-by-step modification of the polymer side groups only has a minor influence on the retention time of the SEC curves, indicating that the polymers have similar hydrodynamic sizes in the eluent.

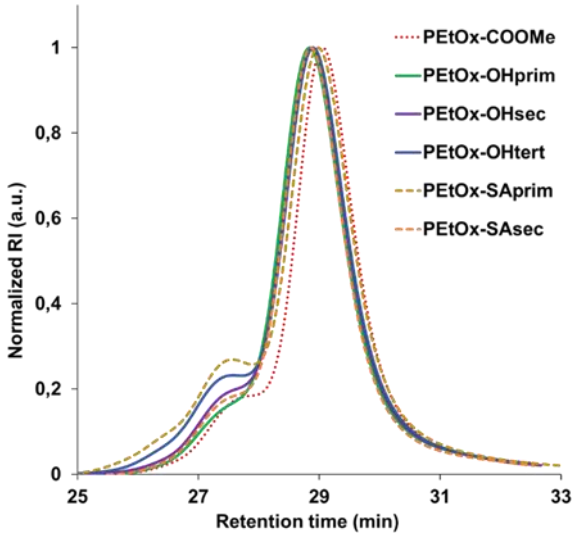


Figure 4. *Stacked SEC traces of the different polymers (SEC DMA – 50 mM LiCl)*

Table 1. *SEC data, percentage of functional group and mass percentage of drug (drug loading - for the polymer-drug conjugates). [a] Determined from SEC DMA – 50 mM LiCl, against PEtOx standart calibration; [b] calculated from the ^{1}H-NMR integrations, [c] calculated from the ^{1}H-NMR integration and SEC Mw (see SI for calculation)*

Polymer	SEC[a]		% functional group[b] (mol %)	Drug loading[c] (mass %)
	M_n (kDa)	Đ		
PEtOx-MestOx	10.2	1.12	6.4 % COOMe	-
PEtOx-OH_{prim}	10.9	1.16	4.5 % OH	-
PEtOx-OH_{sec}	11.1	1.17	4.5 % OH	-
PEtOx-OH_{tert}	10.8	1.18	0.5 % OH	-
PEtOx-SA_{prim}	11.5	1.21	-	6.7 % SA
PEtOx-SA_{sec}	11.4	1.21	-	4.2 % SA

3.2. In vitro drug release behavior.

The *in vitro* drug release of SA from the PEtOx-SA conjugates was investigated by incubating the polymers at 37°C in different buffers, including basic, acidic and neutral media. The quantification of SA release was performed either by SEC or by HPLC (see SI). The release profiles, expressed as cumulative percentage of drug release, have been determined by HPLC, from both conjugates and at various time scale (Figure 5A). As expected, the release rate was found to be strongly dependent on the pH of the solution and the chemical nature of the ester linker. Both conjugates show negligible drug release in acidic media (< 1% after 1 day at pH 1). It was necessary to heat up the solutions to 70°C to observe significant release of the drug after 24 hours at this pH value (Table S2). This observation is consistent with the fact that benzoyl groups are more stable than other acyl-groups, as acetyl groups, for instance, which are usually cleaved faster in those conditions.[60] Nonetheless, both functional groups are prone to quick degradation in basic media and the SA release was relatively fast at pH 8.5 for each conjugate. This is consistent with the base-catalyzed ester-hydrolysis mechanism that progresses through the nucleophilic addition of a hydroxyl ion to the carbonyl ester group. For the conjugate based on the primary alcohol (PEtOx-SA_{prim}), 80% of SA was released after 24 h, and full release was observed at around 48 h (Figure 5B). The quantitative release was confirmed by the SEC measurements, as the UV-absorption of the polymer is lost after full SA-release (Figure S8). In comparison, only 40% of SA was released after one day for the PEtOx-SA_{sec} conjugate and full ester cleavage required 4 days at pH 8.5.

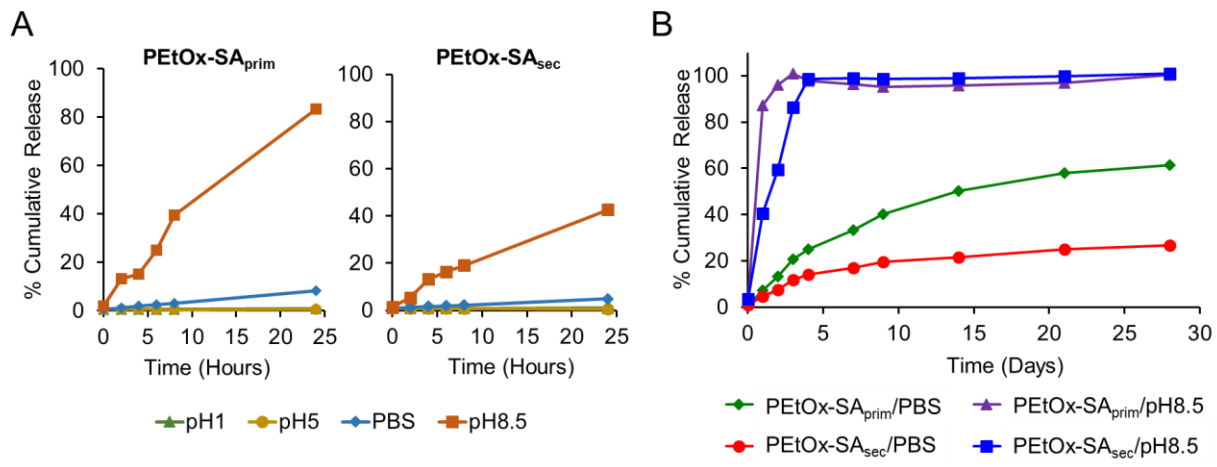


Figure 5. *Cumulative release of salicylic acid measured by HPLC within 1 day for the conjugates incubated at different pH (A) and measured within 1 month for the conjugates incubated in PBS or in pH 8.5 buffers (B). Temperature of the buffers was maintained at 37°C. Lines are guide for the eye.*

Subsequently, the SA release was investigated at pH 7.4 (PBS), a pH mimicking the blood plasma. Both conjugates show a slow release of the drug from the polymer carrier, without any burst effects. The measurements were performed over a period of one month, which is not sufficient to observe the full release in this media. Nonetheless, the trend observed for basic pH is also found in PBS, as the conjugation of SA to a secondary alcohol (PEtOx-SA_{sec}) results in a slower release compared to the conjugate based on the primary alcohol. As a general trend, the release rate of SA from the conjugate based on a primary alcohol is about two times faster than the release rate from the conjugate based on the secondary alcohol, whatever the media (PBS or pH= 8.5). The release profiles were also investigated in the presence of esterase (from porcine liver), to mimic the *in vivo* conditions (Figure S11). The enzyme activity in PBS slightly increased the SA release rate from PEtOx-SA_{prim}, while it only had a minor effect on the conjugate based on a secondary alcohol (*i.e.,* for PEtOx-SA_{sec}), presumably because of sterical effects, indicating the potential to further modulate the *in vivo* release by tuning the ester linker.

3.3. In vitro Cytotoxocity.

To further highlight the utility of the biocompatible PAOx structure for controlled release of SA, the non-cytotoxic character of the polymeric carriers was evaluated by in vitro cytotoxicity experiments on DC2.4 cells. Figure 6 shows the cell viabilities for the three polymers (PEtOx-$OH_{prim/sec/tert}$) and for two concentrations (0.2 mg/L and 1 mg/mL) after incubating for 48 h. The polymers are nontoxic at 0.2 mg/mL and shows no major toxicity at 1 mg/ mL, although PEtOx-OH_{sec} show a slight toxicity at 1 mg/ml, but still statistically hovering around the ISO norm of 70%.[61,62]

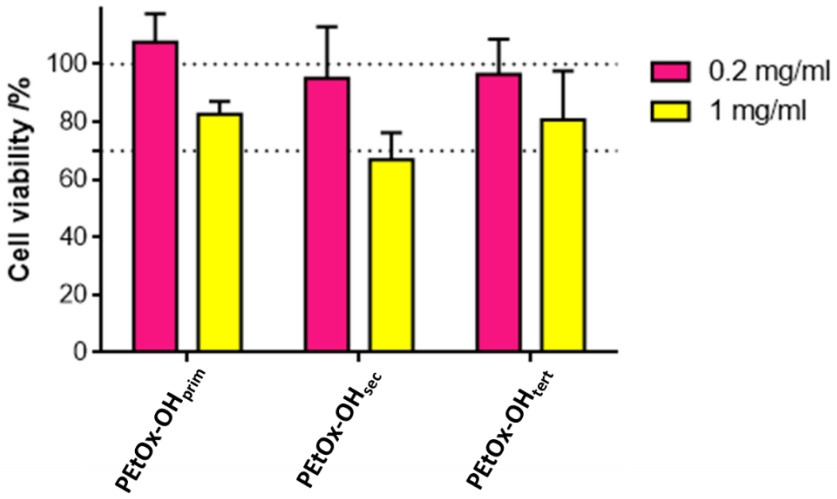


Figure 6. *Cytotoxicity studies by MTT assay for DC2.4 cells after incubation with various concentrations of the three different polymers carriers.*

4. CONCLUSIONS

To summarize, an original synthetic approach was developed to prepare PEtOx-SA conjugates with hydrolytically cleavable ester linkers. Starting from a PEtOx precursor with alcohol groups

in the side chains with different substitution degree permits to modulate this ester linker and consequently the release profile of the drug. The linker effect on the release profile was evidenced by *in vitro* drug release experiments at different pH values. The conjugates prepared from a primary alcohol, PEtOx-SA_{prim}, showed a twice faster SA release in PBS and at pH 8.8, compared to the conjugate based on a secondary alcohol, highlighting the possibility to tune the release rate. Both conjugates deliver the drug in PBS over a period exceeding one month, which is demanded for treatment of chronic diseases. Furthermore, cytotoxicity experiments with the polymeric hydroxylated precursors proved the non-toxicity of the drug carriers. Overall, the results demonstrate that the biocompatibility, chemical versatility and post-polymerization modification possibilities of the PAOx platform offer large potential for preparation of drug delivery system with tunable release rates. As outlined by the degradation properties in aqueous media, this approach represents an important tool for applications in biotechnology and drug delivery, as it offer possibilitis for liquid form administration with controllable delivery rate. Beyond SA that we have chosen as a model drug, it is applicable to other potent drugs used in the context of persistent and chronic diseases, especially Non-Steroidal Anti-Inflammatory Drugs (NSAIDS) such as ibuprofen, naproxen, ketoprofen, diclofenac and indomethacin, as many of them contain carboxylic acid groups, and their ester prodrugs have proved to be efficient.

ASSOCIATED CONTENT

Supporting Information. Additional experiments, procedures and characterization data are described in the Supporting Information

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Author Contributions

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

Notes

Conflict of interest: RH is one of the founders of Avroxa BVBA that commercializes poly(2-oxazoline)s as Ultroxa® and is listed as inventor on patents WO2013103297A1 and WO2019224356A1 that cover the side-chain amidation that is used in this work. JVG is also listed as inventor on patent WO2019224356A1. The other authors have no conflicts to declare.

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REFERENCES

(1) Fenton, O. S.; Olafson, K. N.; Pillai, P. S.; Mitchell, M. J.; Langer, R. Advances in Biomaterials for Drug Delivery. *Adv. Mater.* **2018**, *30* (29), 1705328.
(2) Feng, X.; Chen, Y. Drug Delivery Targets and Systems for Targeted Treatment of Rheumatoid Arthritis. *J. Drug Target.* **2018**, *26* (10), 845–857.

(3) Martin, C.; De Baerdemaeker, A.; Poelaert, J.; Madder, A.; Hoogenboom, R.; Ballet, S. Controlled-Release of Opioids for Improved Pain Management. *Mater. Today* **2016**, *19* (9), 491–502.
(4) Allen, T. M. Drug Delivery Systems: Entering the Mainstream. *Science* **2004**, *303* (5665), 1818–1822.
(5) Ekladious, I.; Colson, Y. L.; Grinstaff, M. W. Polymer–Drug Conjugate Therapeutics: Advances, Insights and Prospects. *Nat. Rev. Drug Discov.* **2018**, *18*, 273-294.
(6) Seidi, F.; Jenjob, R.; Crespy, D. Designing Smart Polymer Conjugates for Controlled Release of Payloads. *Chem. Rev.* **2018**, *118* (7), 3965–4036.
(7) Banerjee, S. S.; Aher, N.; Patil, R.; Khandare, J. Poly(Ethylene Glycol)-Prodrug Conjugates: Concept, Design, and Applications. *J. Drug Deliv.* **2012**, *2012*, 1–17.
(8) Kopeček, J.; Kopečková, P. HPMA Copolymers: Origins, Early Developments, Present, and Future☆. *Adv. Drug Deliv. Rev.* **2010**, *62* (2), 122–149.
(9) Tucker, B. S.; Sumerlin, B. S. Poly(N-(2-Hydroxypropyl) Methacrylamide)-Based Nanotherapeutics. *Polym. Chem.* **2014**, *5* (5), 1566–1572.
(10) Lorson, T.; Lübtow, M. M.; Wegener, E.; Haider, M. S.; Borova, S.; Nahm, D.; Jordan, R.; Sokolski-Papkov, M.; Kabanov, A. V.; Luxenhofer, R. Poly(2-Oxazoline)s Based Biomaterials: A Comprehensive and Critical Update. *Biomaterials* **2018**, *178*, 204–280.
(11) de la Rosa, V. R. Poly(2-Oxazoline)s as Materials for Biomedical Applications. *J. Mater. Sci.: Mater. Med.* **2014**, *25* (5), 1211–1225.
(12) Luxenhofer, R.; Han, Y.; Schulz, A.; Tong, J.; He, Z.; Kabanov, A. V.; Jordan, R. Poly(2-Oxazoline)s as Polymer Therapeutics. *Macromol. Rapid Commun.* **2012**, *33* (19), 1613–1631.
(13) Adams, N.; Schubert, U. S. Poly(2-Oxazolines) in Biological and Biomedical Application Contexts. *Adv. Drug Deliv. Rev.* **2007**, *59* (15), 1504–1520.
(14) Sedlacek, O.; Monnery, B. D.; Filippov, S. K.; Hoogenboom, R.; Hruby, M. Poly(2-Oxazoline)s - Are They More Advantageous for Biomedical Applications Than Other Polymers? *Macromol. Rapid Commun.* **2012**, *33* (19), 1648–1662.
(15) Sedlacek, O.; Hoogenboom, R. Drug Delivery Systems Based on Poly(2-Oxazoline)s and Poly(2-Oxazine)s. *Adv. Therap.* **2020**, *3* (1), 1900168.
(16) Kronek, J.; Paulovicova, E.; Paulovicova, L.; Kronekova, Z.; Lusto, J. Biocompatibility and Immunocompatibility Assessment of Poly(2-Oxazolines). In *Practical Applications in Biomedical Engineering*; Andrade, A., Ed.; InTech, 2013.
(17) Glassner, M.; Vergaelen, M.; Hoogenboom, R. Poly(2-Oxazoline)s: A Comprehensive Overview of Polymer Structures and Their Physical Properties: Poly(2-Oxazoline)s. *Polym. Int.* **2018**, *67* (1), 32–45.
(18) Verbraeken, B.; Monnery, B. D.; Lava, K.; Hoogenboom, R. The Chemistry of Poly(2-Oxazoline)s. *Eur. Polym. J.* **2017**, *88*, 451–469.
(19) Delaittre, G. Telechelic Poly(2-Oxazoline)s. *Eur. Polym. J.* **2019**, *121*, 109281.
(20) Drain, B. A.; Becer, C. R. Synthetic Approaches on Conjugation of Poly(2-Oxazoline)s with Vinyl Based Polymers. *Eur. Polym. J.* **2019**, *119*, 344–351.
(21) Pizzi, D.; Humphries, J.; Morrow, J. P.; Fletcher, N. L.; Bell, C. A.; Thurecht, K. J.; Kempe, K. Poly(2-Oxazoline) Macromonomers as Building Blocks for Functional and Biocompatible Polymer Architectures. *Eur. Polym. J.* **2019**, *121*, 109258.
(22) Rossegger, E.; Schenk, V.; Wiesbrock, F. Design Strategies for Functionalized Poly(2-Oxazoline)s and Derived Materials. *Polymers* **2013**, *5* (3), 956–1011.

(23) Mero, A.; Pasut, G.; Via, L. D.; Fijten, M. W. M.; Schubert, U. S.; Hoogenboom, R.; Veronese, F. M. Synthesis and Characterization of Poly(2-Ethyl 2-Oxazoline)-Conjugates with Proteins and Drugs: Suitable Alternatives to PEG-Conjugates? *J. Control. Release* **2008**, *125* (2), 87–95.
(24) Schmidt, M.; Harmuth, S.; Barth, E. R.; Wurm, E.; Fobbe, R.; Sickmann, A.; Krumm, C.; Tiller, J. C. Conjugation of Ciprofloxacin with Poly(2-Oxazoline)s and Polyethylene Glycol via End Groups. *Bioconjug. Chem.* **2015**, *26* (9), 1950–1962.
(25) Schmidt, M.; Bast, L. K.; Lanfer, F.; Richter, L.; Hennes, E.; Seymen, R.; Krumm, C.; Tiller, J. C. Poly(2-Oxazoline)–Antibiotic Conjugates with Penicillins. *Bioconjug. Chem.* **2017**, *28* (9), 2440–2451.
(26) Li, J.; Zhou, Y.; Li, C.; Wang, D.; Gao, Y.; Zhang, C.; Zhao, L.; Li, Y.; Liu, Y.; Li, X. Poly(2-Ethyl-2-Oxazoline)–Doxorubicin Conjugate-Based Dual Endosomal PH-Sensitive Micelles with Enhanced Antitumor Efficacy. *Bioconjug. Chem.* **2015**, *26* (1), 110–119.
(27) Moreadith, R. W.; Viegas, T. X.; Bentley, M. D.; Harris, J. M.; Fang, Z.; Yoon, K.; Dizman, B.; Weimer, R.; Rae, B. P.; Li, X.; Rader, C.; Standaert, D.; Olanow, W. Clinical Development of a Poly(2-Oxazoline) (POZ) Polymer Therapeutic for the Treatment of Parkinson's Disease – Proof of Concept of POZ as a Versatile Polymer Platform for Drug Development in Multiple Therapeutic Indications. *Eur. Polym. J.* **2017**, *88*, 524–552.
(28) Eskow Jaunarajs, K. L.; Standaert, D. G.; Viegas, T. X.; Bentley, M. D.; Fang, Z.; Dizman, B.; Yoon, K.; Weimer, R.; Ravenscroft, P.; Johnston, T. H.; Hill, M. P.; Brotchie, J. M.; Moreadith, R. W. Rotigotine Polyoxazoline Conjugate SER-214 Provides Robust and Sustained Antiparkinsonian Benefit: Continuous Dopaminergic Tone with Poz-Rotigotine. *Mov. Disord.* **2013**, *28* (12), 1675–1682.
(29) Harris, J. M.; Bentley, M. D.; Moreadith, R. W.; Viegas, T. X.; Fang, Z.; Yoon, K.; Weimer, R.; Dizman, B.; Nordstierna, L. Tuning Drug Release from Polyoxazoline-Drug Conjugates. *Eur. Polym. J.* **2019**, *120*, 109241.
(30) Sedlacek, O.; Monnery, B. D.; Mattova, J.; Kucka, J.; Panek, J.; Janouskova, O.; Hocherl, A.; Verbraeken, B.; Vergaelen, M.; Zadinova, M.; Hoogenboom, R.; Hruby, M. Poly(2-Ethyl-2-Oxazoline) Conjugates with Doxorubicin for Cancer Therapy: In Vitro and in Vivo Evaluation and Direct Comparison to Poly[N-(2-Hydroxypropyl)Methacrylamide] Analogues. *Biomaterials* **2017**, *146*, 1–12.
(31) Salmanpour, M.; Yousefi, G.; Samani, S. M.; Mohammadi, S.; Anbardar, M. H.; Tamaddon, A. Nanoparticulate Delivery of Irinotecan Active Metabolite (SN38) in Murine Colorectal Carcinoma through Conjugation to Poly (2-Ethyl 2-Oxazoline)-b-Poly (L-Glutamic Acid) Double Hydrophilic Copolymer. *Eur. J. Pharm. Sci.* **2019**, *136*, 104941.
(32) Romio, M.; Morgese, G.; Trachsel, L.; Babity, S.; Paradisi, C.; Brambilla, D.; Benetti, E. M. Poly(2-Oxazoline)–Pterostilbene Block Copolymer Nanoparticles for Dual-Anticancer Drug Delivery. *Biomacromolecules* **2018**, *19* (1), 103–111.
(33) Park, J.-R.; Van Guyse, J. F. R.; Podevyn, A.; Bolle, E. C. L.; Bock, N.; Linde, E.; Celina, M.; Hoogenboom, R.; Dargaville, T. R. Influence of Side-Chain Length on Long-Term Release Kinetics from Poly(2-Oxazoline)-Drug Conjugate Networks. *Eur. Polym. J.* **2019**, *120*, 109217.
(34) Mauri, E.; Rossetti, A.; Mozetic, P.; Schiavon, C.; Sacchetti, A.; Rainer, A.; Rossi, F. Ester Coupling of Ibuprofen in Hydrogel Matrix: A Facile One-Step Strategy for

Controlled Anti-Inflammatory Drug Release. *Eur. J. Pharm. Biopharm.* **2020**, *146*, 143–149.
(35) Al-Khoury, H.; Espinosa-Cano, E.; Aguilar, M. R.; Román, J. S.; Syrowatka, F.; Schmidt, G.; Groth, T. Anti-Inflammatory Surface Coatings Based on Polyelectrolyte Multilayers of Heparin and Polycationic Nanoparticles of Naproxen-Bearing Polymeric Drugs. *Biomacromolecules* **2019**, *20* (10), 4015–4025.
(36) Wei, J.; Shi, J.; Zhang, J.; He, G.; Pan, J.; He, J.; Zhou, R.; Guo, L.; Ouyang, L. Design, Synthesis and Biological Evaluation of Enzymatically Cleavable NSAIDs Prodrugs Derived from Self-Immolative Dendritic Scaffolds for the Treatment of Inflammatory Diseases. *Bioorg. Med. Chem.* **2013**, *21* (14), 4192–4200.
(37) Sumer Bolu, B.; Manavoglu Gecici, E.; Sanyal, R. Combretastatin A-4 Conjugated Antiangiogenic Micellar Drug Delivery Systems Using Dendron–Polymer Conjugates. *Mol. Pharm.* **2016**, *13* (5), 1482–1490.
(38) Sulistio, A.; Reyes-Ortega, F.; D'Souza, A. M.; Ng, S. M. Y.; Valade, D.; Quinn, J. F.; Donohue, A. C.; Mansfeld, F.; Blencowe, A.; Qiao, G.; Prankerd, R.; Quirk, S.; Whittaker, M. R.; Davis, T. P.; Tait, R. J. Precise Control of Drug Loading and Release of an NSAID–Polymer Conjugate for Long Term Osteoarthritis Intra-Articular Drug Delivery. *J. Mater. Chem. B* **2017**, *5* (31), 6221–6226.
(39) Rosario-Meléndez, R.; Yu, W.; Uhrich, K. E. Biodegradable Polyesters Containing Ibuprofen and Naproxen As Pendant Groups. *Biomacromolecules* **2013**, *14* (10), 3542–3548.
(40) Wu, K. K. Aspirin and Salicylate: An Old Remedy With a New Twist. *Circulation* **2000**, *102* (17), 2022–2023.
(41) Amann, R.; Peskar, B. A. Anti-Inflammatory Effects of Aspirin and Sodium Salicylate. *Eur. J. Pharm.* **2002**, *447* (1), 1–9.
(42) Bastiaannet, E.; Sampieri, K.; Dekkers, O. M.; de Craen, A. J. M.; van Herk-Sukel, M. P. P.; Lemmens, V.; van den Broek, C. B. M.; Coebergh, J. W.; Herings, R. M. C.; van de Velde, C. J. H.; Fodde, R.; Liefers, G. J. Use of Aspirin Postdiagnosis Improves Survival for Colon Cancer Patients. *Br. J. Cancer* **2012**, *106* (9), 1564–1570.
(43) Yamazaki, R.; Kusunoki, N.; Matsuzaki, T.; Hashimoto, S.; Kawai, S. Aspirin and Sodium Salicylate Inhibit Proliferation and Induce Apoptosis in Rheumatoid Synovial Cells. *J. Pharm. Pharm.* **2002**, *54* (12), 1675–1679.
(44) Amborabé, B.-E.; Fleurat-Lessard, P.; Chollet, J.-F.; Roblin, G. Antifungal Effects of Salicylic Acid and Other Benzoic Acid Derivatives towards Eutypa Lata: Structure–Activity Relationship. *Plant Phys. Biochem.* **2002**, *40* (12), 1051–1060.
(45) Hundal, R. S.; Petersen, K. F.; Mayerson, A. B.; Randhawa, P. S.; Inzucchi, S.; Shoelson, S. E.; Shulman, G. I. Mechanism by Which High-Dose Aspirin Improves Glucose Metabolism in Type 2 Diabetes. *J. Clin. Invest.* **2002**, *109* (10), 1321–1326.
(46) Huang, E. S.; Strate, L. L.; Ho, W. W.; Lee, S. S.; Chan, A. T. A Prospective Study of Aspirin Use and the Risk of Gastrointestinal Bleeding in Men. *PLoS ONE* **2010**, *5* (12), e15721.
(47) Erdmann, L.; Uhrich, K. E. Synthesis and Degradation Characteristics of Salicylic Acid-Derived Poly(Anhydride-Esters). *Biomaterials* **2000**, *21* (19), 1941–1946.
(48) Bryers, J. D.; Jarvis, R. A.; Lebo, J.; Prudencio, A.; Kyriakides, T. R.; Uhrich, K. Biodegradation of Poly(Anhydride-Esters) into Non-Steroidal Anti-Inflammatory Drugs

and Their Effect on Pseudomonas Aeruginosa Biofilms in Vitro and on the Foreign-Body Response in Vivo. *Biomaterials* **2006**, *27* (29), 5039–5048.
(49) Dasgupta, Q.; Chatterjee, K.; Madras, G. Controlled Release of Salicylic Acid from Biodegradable Cross-Linked Polyesters. *Mol. Pharm.* **2015**, *12* (9), 3479–3489.
(50) Marisol Arteaga-Luna, M.; Hugo Pino-Ramos, V.; Magaña, H.; Bucio, E. Polymeric Pro-Drug Sutures for Potential Local Release of Salicylic Acid. *Int. J. Polym. Mater. Polym. Biomater.* **2018**, *67* (13), 792–799.
(51) Niamlang, S.; Sirivat, A. Electrically Controlled Release of Salicylic Acid from Poly(p-Phenylene Vinylene)/Polyacrylamide Hydrogels. *Int. J. Pharm.* **2009**, *371* (1–2), 126–133.
(52) Demirdirek, B.; Uhrich, K. E. Physically Crosslinked Salicylate-Based Poly (*N* -Isopropylacrylamide- *Co* -Acrylic Acid) Hydrogels for Protein Delivery. *J. Bioact. Compat. Polym.* **2018**, *33* (2), 224–236.
(53) Demirdirek, B.; Uhrich, K. E. Novel Salicylic Acid-Based Chemically Crosslinked PH-Sensitive Hydrogels as Potential Drug Delivery Systems. *Int. J. Pharm.* **2017**, *528* (1–2), 406–415.
(54) Jantas, R.; Herczyńska, L. Preparation and Characterization of the Poly(2-Hydroxyethyl Methacrylate)–Salicylic Acid Conjugate. *Polym. Bull.* **2010**, *64* (5), 459–469.
(55) Bouten, P. J. M.; Hertsen, D.; Vergaelen, M.; Monnery, B. D.; Boerman, M. A.; Goossens, H.; Catak, S.; van Hest, J. C. M.; Van Speybroeck, V.; Hoogenboom, R. Accelerated Living Cationic Ring-Opening Polymerization of a Methyl Ester Functionalized 2-Oxazoline Monomer. *Polym. Chem.* **2015**, *6* (4), 514–518.
(56) Hayne, D. J.; White, J. M.; McLean, C. A.; Villemagne, V. L.; Barnham, K. J.; Donnelly, P. S. Synthesis of Oxorhenium(V) and Oxotechnetium(V) Complexes That Bind to Amyloid-β Plaques. *Inorg. Chem.* **2016**, *55* (16), 7944–7953.
(57) Mees, M. A.; Hoogenboom, R. Functional Poly(2-Oxazoline)s by Direct Amidation of Methyl Ester Side Chains. *Macromolecules* **2015**, *48* (11), 3531–3538.
(58) Van Guyse, J. F. R.; Mees, M. A.; Vergaelen, M.; Baert, M.; Verbraeken, B.; Martens, P. J.; Hoogenboom, R. Amidation of Methyl Ester Side Chain Bearing Poly(2-Oxazoline)s with Tyramine: A Quest for a Selective and Quantitative Approach. *Polym. Chem.* **2019**, *10* (8), 954–962.
(59) Van Guyse, J. F. R.; Xu, X.; Hoogenboom, R. Acyl Guanidine Functional Poly(2-oxazoline)s as Reactive Intermediates and Stimuli-responsive Materials. *J. Polym. Sci. Part A: Polym. Chem.* **2019**, *57* (24), 2616–2624.
(60) Gordon, M.; Miller, J. G.; Day, A. R. Effect of Structure on Reactivity.1 I. Ammonolysis of Esters with Special Reference to the Electron Release Effects of Alkyl and Aryl Groups. *J. Am. Chem. Soc.* **1948**, *70* (5), 1946–1953.
(61) Osibote, E.; Noah, N.; Sadik, O.; McGee, D.; Ogunlesi, M. Electrochemical Sensors, MTT and Immunofluorescence Assays for Monitoring the Proliferation Effects of Cissus Populnea Extracts on Sertoli Cells. *Reprod. Biol. Endocrin.* **2011**, *9* (1), 65.
(62) Tai, K.-K.; Pham, L.; Truong, D. D. Idebenone Induces Apoptotic Cell Death in the Human Dopaminergic Neuroblastoma SHSY-5Y Cells. *Neurotox. Res.* **2011**, *20* (4), 321.

Graphical abstract: