Bioconjugate Chemistry

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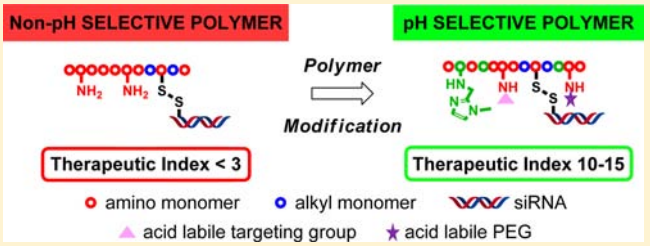
Improving the In Vivo Therapeutic Index of siRNA Polymer Conjugates through Increasing pH Responsiveness

Erin N. Guidry,*[,†] Julie Farand,[†] Arash Soheili,[†] Craig A. Parish,[†] Nancy J. Kevin,[†] Brenda Pipik,[†] Kathleen B. Calati,[†] Nori Ikemoto,[†] Jacob H. Waldman,[†] Andrew H. Latham,[⊥] Bonnie J. Howell,[§] Anthony Leone,[⊥] Robert M. Garbaccio,[‡] Stephanie E. Barrett,[‡] Rubina Giare Parmar,[‡] Quang T. Truong,[†] Bing Mao,[∥] Ian W. Davies,[†] Steven L. Colletti,[‡] and Laura Sepp-Lorenzino[§]

[†]Department of Process Chemistry and [∥]Department of Analytical Chemistry, Merck & Co. Inc., , Rahway, New Jersey, United States
[‡]Department of Medicinal Chemistry, [§]Department of RNA Biology, and [⊥]Department of Pharmaceutical Sciences, Merck & Co. Inc., West Point, Pennsylvania, United States

Supporting Information

ABSTRACT: Polymer based carriers that aid in endosomal escape have proven to be efficacious siRNA delivery agents in vitro and in vivo; however, most suffer from cytotoxicity due in part to a lack of selectivity for endosomal versus cell membrane lysis. For polymer based carriers to move beyond the laboratory and into the clinic, it is critical to find carriers that are not only efficacious, but also have margins that are clinically relevant. In this paper we report three distinct categories of polymer conjugates that improve the selectivity of endosomal membrane lysis by relying on the change in pH associated with endosomal trafficking, including incorporation of low pK_a heterocycles, acid cleavable amino side chains, or carboxylic acid pH sensitive charge switches. Additionally, we determine the therapeutic index of our polymer conjugates in vivo and demonstrate that the incorporation of pH responsive elements dramatically expands the therapeutic index to 10−15, beyond that of the therapeutic index (less than 3), for polymer conjugates previously reported.



INTRODUCTION

Small interfering RNA (siRNA)[1−3] has attracted considerable attention as an emerging technology for the treatment of human disease due to its potency, specificity, duration, and ability to engage virtually all disease relevant targets at the genetic level, including those that are difficult to address through traditional small molecule therapies.[4−6] Successful systemic delivery of siRNA into cells depends on the ability of the delivery vehicle to transport the genetic material to the target cell of interest and access the cytosolic RNA-induced silencing complex (RISC).[7] Trafficking of siRNA via an endosome requires that the delivery vehicle also assist in the escape of the siRNA from that organelle. Polymer based delivery vehicles have proven to be effective siRNA delivery agents, offering small sizes compared to lipid nanoparticle technologies for improved tissue penetration (diameters <50 nm), and providing a high degree of chemical control over the structure of the conjugate.[8−16] Various polymer-based carriers have been developed for this purpose, but most suffer from toxicity derived from the carrier components, limiting the practical application of such systems.[17−20,29]

Recently, several reports describing pH responsive polymer conjugate delivery systems for siRNA have been reported that demonstrate in vitro and in vivo mRNA knockdown (KD).[8,21−29] The design employed in these pH responsive polymer conjugates consists of a membrane active, cationic, amphiphilic polymer in which the cationic functionality of the polymer is reversibly functionalized, masking the membranolytic activity of the polymer conjugate in systemic circulation until it reaches the acidic environment of the endosome. While our group[21−25] and others[8,27,29,55] have proven this to be an effective strategy for achieving efficacy in vivo and these conjugates are nontoxic at therapeutic doses, no studies have been reported that directly address the therapeutic margin of these polymer conjugates by examining the toxicity of these polymer conjugates at doses beyond the therapeutic minimum. For polymer conjugates to move beyond the laboratory and into the clinic, it is critical to find polymer conjugates that are not only efficacious, but also have margins that are clinically relevant. In this report, we determine the therapeutic index (TI) of cationic amphiphilic dynamic polymer conjugates. We also present three distinct strategies that dramatically expand the TI beyond that of polymer conjugates previously reported[8,21−25,29,55] as part of our effort to achieve clinically relevant therapeutic margins.

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EXPERIMENTAL PROCEDURES

Materials. All polymer and polymer conjugate purifications were performed using the Millipore Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-3 Membrane (UFC900324). Filter units were dialyzed with 0.1 M NaOH (1 cycle, 15 mL) followed by rinsing with Milli-Q water immediately prior to use to remove the glycerol preservative per the manufacturer's instructions. Polymer **1**, unmodified poly(vinyl ether) terpolymer (15:4:1 amine:butyl:octadecyl) was synthesized according to the literature procedure and a single batch of polymer was used for all polymer modifications.[31] Carboxydimethylmaleic anhydride-poly(ethylene glycol) (CDM-PEG; MW = 680 g/mol) and carboxydimethylmaleic anhydride-*N*-acetylgalactosamine (CDM-NAG) were synthesized according to a literature procedure.[8] The active siRNA targeting Apolipoprotein B17 (ApoB, sequence shown below) was used in the in vivo experiments. The siRNA with an irrelevant sequence (Low Hex 9) was used as a control (sequence shown below).

ApoB.

5′-amil-iB-CUUU*AA*C*AA*UUCCU*GAAA*U**TsT**-iB-3′
3′-Us*UGAAAUUG*UU*AAGGA*CUs<u>UsUsA</u>-5′
<u>AUGC</u> − Ribose; amil = hexyl amino linker; iB − Inverted deoxy abasic; UC − 2′ Fluoro; **AGT** − 2′ Deoxy; AGU − 2′ OCH_3; UsA − phosphorothioate linkage

Low Hex 9.

5′-amil-iB-CUAGCUGGACACGUCGAUATsT-iB-3′
3′-UsUGAUCGACCUGUGCAGCUAU-5′
amil − hexyl amino linker; iB - Inverted deoxy abasic; CU − 2′-Fluoro (F); AGT − 2′-Deoxy; UGA − 2′-Methoxy (OMe); AU − Ribose; s − phosphorothioate linkage

Methods. *General Procedure for the Post-Synthetic Reductive Amination of Polymer 1.* The synthesis of 15% 2-imidazole modified polymer **2** is used as an illustrative example. In a typical experiment, 2-imidazole carboxaldehyde (0.384 g, 3.99 mmol) was charged to a 250 mL round-bottom flask fitted with a reflux condenser. Unmodified polymer **1** (4.74 g) dissolved in anhydrous MeOH (34.5 mL, 137.5 mg/mL) was subsequently charged to the flask containing the aldehyde. The reaction mixture was heated to 50 °C under nitrogen for 12 h. The reaction mixture was then allowed to cool to room temperature followed by portion-wise addition of solid sodium borohydride (2.013 g, 53.2 mmol). The reaction mixture was stirred at room temperature for 2 h and was subsequently diluted with Milli-Q water and dialyzed against Milli-Q water (3 cycles, 15 mL water per cycle). The polymer was removed from the dialysis unit and lyophilized to yield a pale yellow viscous oil. Analysis of characteristic resonances in the ^{1}H NMR of each modified polymer was used to assess the level of modification (Supporting Information).

General Procedure for the Post-Synthetic Modification of Polymer 1 with Acetal and Ketal Units. Acetal and ketal side chain units with monotrifluoroacetamide protected amines were prepared as described in a literature procedure.[41] The synthesis of 20% acetal modified polymer **25** is used as an illustrative example. To a solution of monoprotected amine (0.023 g, 0.10 mmol) was added triethylamine (0.032 mL, 0.23 mmol) followed by solid *p*-nitro-chloroformate (0.015 g, 0.08 mmol). A precipitate formed and the reaction was stirred for one hour at room temperature. A solution of polymer **1** (0.052 g, 0.003 mmol) in 1 mL THF and DMAP (5 mg) was added to the reaction. After stirring for 4 h at 50 °C, the reaction mixture was dialyzed against MeOH. The resulting solution was concentrated in vacuo providing a pale yellow oil (0.064 g). The modified polymer was dissolved in 1.5 mL THF and to it was added 1.5 mL 0.2 N NaOH. This reaction mixture was stirred at room temperature for two days and then dialyzed against MeOH. The modified polymer was concentrated in vacuo to provide a yellow oil (0.049 g). Analysis of characteristic resonances in the ^{1}H NMR of each modified polymer was used to assess the level of acetal or ketal modification (Supporting Information).

General Procedure for the Post-Synthetic Modification of Polymer 1 with Acetic Acid. The synthesis of 25% acetic acid modified polymer **33** is used as an illustrative example. Polymer **1** (3.21 g, 0.208 mmol) was dissolved in water (80.2 mL, 40 mg/mL) in a 250 mL round-bottom flask. To this was added a solution of iodoacetic acid (1.255 g, 6.75 mmol) in 25.1 mL dimethylsulfoxide (50 mg/mL). After stirring for 3 days at room temperature, the reaction mixture was diluted with Milli-Q water and dialyzed against Milli-Q water (3 cycles, 15 mL water per cycle). The polymer was removed from the dialysis unit and lyophilized to yield a pale yellow viscous oil. Analysis of characteristic resonances in the ^{1}H NMR of each modified polymer was used to assess the level of modification (Supporting Information).

Polymer Characterization. ^{1}H spectra were recorded on a Bruker spectrometer (400 and 500 MHz) as noted, and are internally referenced to residual protio solvent signals. Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad multiplet), integration, and coupling constant (Hz). ^{1}H NMR spectra were in full accordance with the expected structures. A detailed report describing the determination of the ratio of the monomer components and level of modification can be found in the Supporting Information section.

Polymer molecular weight determination for phthalimide protected polymers was performed on an Agilent 1100 high performance liquid chromatograph (HPLC) coupled with a Wyatt miniDAWN TREOS (3 angle multi angle light scattering (MALS) system) and a Wyatt Optilab T-rEX (refractive index detector). Chromatography was performed using two size exclusion chromatographic columns in tandem, Waters Styragel HR3 Column, 5 μm, 7.8 × 300 mm (THF) and Waters Styragel HR4E Column, 5 μm, 7.8 × 300 mm (THF) with 100% THF as mobile phase at a flow rate of 1.0 mL/min. The temperature of the column was set at 25 °C and the UV detection wavelength was 260 nm. The polymer sample was dissolved in THF at 1−10 mg/mL and 0.5 mg material was injected. Instrument normalization and calibration was performed using 2−40K polystyrene standards with a PDI of less than 1.1 (Polymer Laboratories). No calibration standards were used in determination of molecular weights or polydispersities. The d*n*/d*c* values were obtained for each injection assuming 100% mass elution from the columns. These values were also independently verified by measuring the d*n*/d*c* using a Wyatt Optilab T-rEX refractometer. The data was collected and processed using Wyatt Astra software.

Molecular weights of deprotected polymer and small molecule postsynthetically modified polymer were not measured directly. Molecular weight values were calculated by

adjusting the corresponding protected polymer molecular weight based on the mass loss due to removal of the protecting group along with any mass gain due to functionalization.

General Procedure for the Preparation of Polymer Conjugates. Step 1. Activation of the siRNA. ApoB siRNA (1 g, 0.0714 mmol) was dissolved in 0.1 M sodium bicarbonate buffer (20 mL, 50 mg/mL) in a vial with magnetic stir bar and cooled to 0−5 °C in an ice water bath. In a separate vial, *N*-succinimidyl-*S*-acetylthioacetate (SATA, 83 mg, 0.357 mmol, 5 equiv) was dissolved in 0.78 mL DMSO. The SATA solution was added over 1 min and the clear, colorless reaction mixture was stirred at 0−5 °C for 2 h. After 2 h, the reaction mixture was sampled and analyzed by HPLC for completion of the conjugation. If greater than 5% siRNA remained unreacted, another charge of SATA in DMSO (2.0 equiv) was added and the reaction aged at 0−5 °C for completion of the SATA conjugation. The reaction mixture was purified by dialysis using Milli-Q water until HPLC indicated removal of *N*-hydroxysuccinimide, and *N*-succinimidyl-*S*-acetylthioacetate. The dialyzed solution was lyophilized to afford the product, SATA-modified ApoB siRNA, as a white fluffy solid.

Step 2. Activation of the Polymer. Polymer (1.2 g) and 100 mM sterile TRIS buffer pH 9 (120 mL, 10 mg/mL) were added to a 1 L sterile plastic bottle and stirred at room temperature to dissolve the polymer. To this solution was added 4-succinimidyloxycarbonyl-methyl-[a]-(2-pyridyldithio) toluene (SMPT) as a 1 mg/mL solution in DMSO (18 mg, 1800 uL) corresponding to 1.5 wt % with respect to the polymer weight. The solution was stirred for 1 h at room temperature to generate activated polymer. The reaction was monitored for consumption of SMPT by HPLC.

Step 3. Polymer−siRNA Conjugation. All polymer−siRNA conjugations were performed at a 4.8:1 weight ratio of polymer to siRNA. The activated polymer was further diluted using 100 mM sterile TRIS buffer at pH 9 (496 mL), followed by the addition of SATA-modified siRNA as a solution in water (250 mg, 32.4 mg/mL, 7.7 uL). This reaction mixture was aged for 4 h at room temperature to generate the siRNA−polymer product. The reaction was monitored by HPLC for release of 2-thiopyridine.

Step 4. Masking of Polymer Conjugates. In a separate 1 L sterile plastic bottle, solid CDM-NAG (5.5 g) and CDM-PEG (2.85 g) were added. The siRNA−polymer conjugate solution was transferred by pouring into the plastic bottle containing CDM-NAG and CDM-PEG solids. The mixture was stirred for 2 min to dissolve all solids and then transferred by pouring into the original plastic bottle which contained the siRNA−polymer conjugate. The reaction was stirred for 1 h to generate the final polymer conjugate product. The pH of the final solution was monitored to ensure the pH was between 8 and 9 throughout the conjugation process.

Step 5. Purification. Purification for Mouse In Vivo Studies. Centrifugal dialysis filter units were used to purify masked polymer conjugate formulations of unincorporated components. Polymer conjugates were diluted to a volume of 15 mL with pH 9.0 100 mM TRIS buffer and dialyzed using the centrifugal dialysis filters for 3 cycles. The polymer conjugates were concentrated to a range of 0.4−2.0 mg/mL of siRNA and stored at −20 °C until use.

Purification for Rat In Vivo Studies. Tangential flow filtration (TFF) was used to purify masked polymer conjugate formulations of unincorporated components. The TFF filter material was made of 3K MW cutoff polyethersulfone (PES). The processing parameters, including but not limited to feed pressure, retentate pressure, crossflow rate, and filtrate flux were set to allow reproducibility from batch to batch and linear scaling of the process. Using the difiltration mode of TFF, the reaction impurities were filtered out into the permeate, while the retained polymer conjugate was rinsed with buffer. After TFF, the final product was concentrated to 0.4−2.0 mg/mL of siRNA and sterile filtered using a 0.2 μm PES syringe filter and stored at −20 °C until use.

Polymer Conjugate Characterization. Conjugation Efficiency and siRNA Concentration. Free RNA duplex as well as free RNA duplex-dimer (disulfide) were determined by aqueous SEC using a GE Healthsciences Superdex 75HR 10/300 column (UV at a wavelength of 260 nm). The mobile phase was composed of 100 mM Tris with 2 M NaCl, pH 8.4. Total RNA (both free and bound) was determined by using inductively coupled plasma (ICP) spectroscopy. Since the RNA is the only phosphorus containing species in the formulations, determining the total phosphorus content can be used to directly determine the total RNA concentration. Once the free RNA (duplex and duplex-dimer) and total RNA is determined, the amount of RNA conjugated to the polymer can be calculated (i.e., conjugation efficiency). All siRNA−polymer conjugates used in generating mouse or rat in vivo data had a conjugation efficiency of greater than 75%.

Masking Efficiency. Total concentrations of CDM-NAG and CDM-PEG were determined using reverse-phase HPLC (UV at a wavelength of 260 nm) with mobile phases of 0.1% TFA in water and 0.1% TFA in 70/30 methanol:acetonitrile. Rapid demasking of the polymer after injection onto the column allows quantitation of CDMs with the polymer removed using a C18 guard column to prevent chromatographic interference. Free (i.e., unbound) CDM-NAG and CDM-PEG are analyzed by first filtering through a 10K centrifuge filter followed by analysis of the permeate using the same reverse-phase HPLC method. Masking efficiency can be calculated by determining the total bound siRNA, CDM-NAG, and CDM-PEG. The polymer molecular weight in combination with the total amines available for conjugation is then used with the bound ligands to calculate masking efficiency. Masking efficiencies were between 40% and 65% for all siRNA−polymer conjugates used in generating mouse or rat in vivo data.

In Vivo Efficacy Evaluation. CD1 mice or Sprague−Dawley rats were tail vein injected with the siRNA containing polymer conjugates at a specified dose (mg/kg) in a volume of 0.2 mL (mice) or 5.6 mL/kg at a rate of 3.0 mL/min (rat), 100 mM TRIS/9% glucose, pH 9, vehicle. Forty-eight hours (mice) or 5 days post-dose (rats), rodents were sacrificed and liver tissue samples were immediately preserved in RNALater (Ambion). Preserved liver tissue was homogenized and total RNA isolated using a Qiagen bead mill and the Qiagen miRNA-Easy RNA isolation kit following the manufacturer's instructions. Liver ApoB mRNA levels were determined by quantitative RT-PCR. Message was amplified from purified RNA utilizing primers against the mouse ApoB mRNA (Applied Biosystems Cat. No. Mm01545156_m1). The PCR reaction was run on an ABI 7500 instrument with a 96-well Fast Block. The ApoB mRNA level is normalized to the housekeeping PPIB and GAPDH mRNA. PPIB and GAPDH mRNA levels were determined by RT-PCR using a commercial probe set (Applied Biosytems Cat. No. Mm00478295_m1 and Mm4352339E_m1). Results are expressed as a ratio of ApoB mRNA/PPIB/GAPDH mRNA.

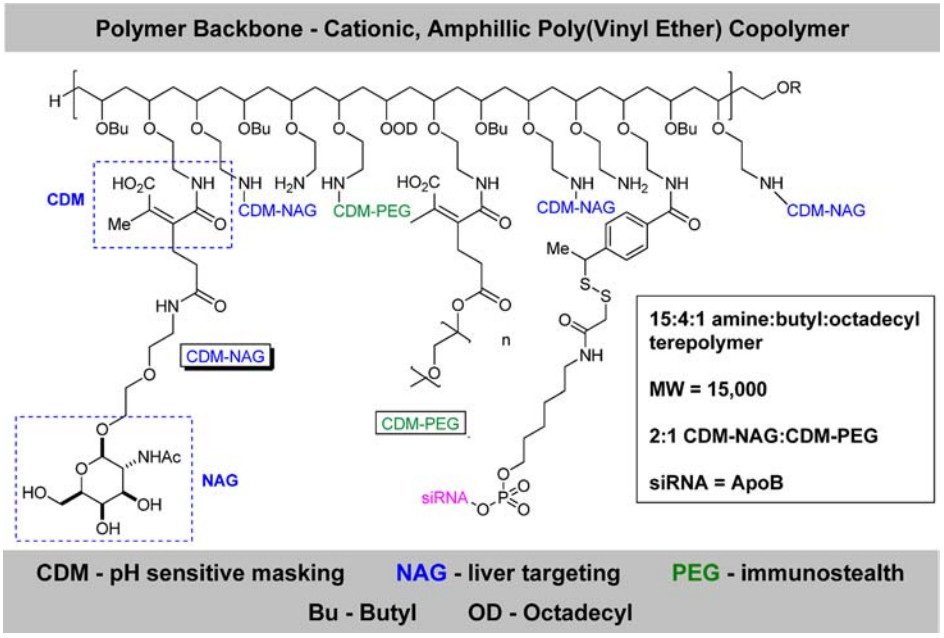

Figure 1. Graphical representation of dynamic polymer conjugate **1′**.

All mRNA knockdown data is expressed relative to the vehicle control.

In Vivo Evaluation of Alanine Aminotransferase (ALT) Levels in Rat. Blood and tissues are collected for assessment of pharmacokinetics, pharmacodynamics, efficacy, and toxicity. ALT levels were measured using the Siemens Advia ALT methodology on the Siemens Advia 1800 instrument according to the manufacturer's specifications.

RESULTS AND DISCUSSION

Therapeutic Index Evaluation—Dynamic Polymer Conjugate. We synthesized polymer conjugate **1′** (polymer **1** provides polymer conjugate **1′**), which comprises a cationic, amphiphilic vinyl ether terpolymer (15:4:1 amine:butyl:octadecyl), conjugated to its Apolipoprotein B (ApoB) siRNA payload through a cytosolically labile disulfide bond, as described in our work[31] and that reported by Rozema et al. (Figure 1).[8] *N*-Acetylgalactosamine (NAG),[32−34] a hepatocyte specific targeting ligand for the asialogylcoprotein receptor (ASGPR), and PEG were conjugated to primary amines of the polymer backbone through an endosomally labile, carboxy dimethylmaleic (CDM) linkage,[35] which under acidic conditions cleaves to reveal the primary amines of the polymer, generating a membrane active agent. A dose ranging experiment was conducted to determine the TI of unmodified polymer conjugate **1′**. Female Sprague−Dawley rats were dosed i.v. with a solution of polymer conjugate **1′** via tail vein injection. Toxicity of the polymer conjugate was assessed by monitoring rats for lethalities within 5 days of treatment and monitoring the clinical biochemistry of the serum, particularly the liver enzyme alanine aminotransferase (ALT) levels, as these have been shown to rise dramatically in the case of acute liver damage. Livers were harvested 5 days post-dose and assayed by RT-qPCR for ApoB mRNA levels relative to the mRNA levels of housekeeping genes PPIB and GAPDH. TI was used to normalize potency versus toxicity regardless of dose. The TI was defined as the margin between the minimally efficacious dose (MED), which we have designated as the dose that achieves 50% mRNA KD, and the highest nontoxic dose, which we have designated as the highest dose at which no lethalities are observed in rats, our primary safety species.

Robust mRNA KD (58%, Table 1) was achieved with polymer conjugate **1′** at an siRNA dose of 1 mpk (mg/kg) with no adverse physical effects observed in any animals (0/10 lethalities) and with no elevation in liver enzyme levels (ALT) or other abnormal serum biochemistry levels. At a dose of 3 mpk, one lethality was observed (1/10 lethalities) and modest elevations in ALT (less than 3-fold) were observed. At a higher dose (6 mpk), a significant number of lethalities were observed (9/10 animals) along with a 7-fold elevation in the ALT level for the remaining animal in the dosing group. While it is clear that dynamic polymer conjugate **1′** is capable of providing robust mRNA KD and no toxicity at therapeutic doses, the TI between a minimally efficacious dose and a nontoxic dose is quite small, less than 3-fold.

Table 1. Summary of In Vivo Rat Data for Unmodified Polymer Conjugate 1′

polymer conjugate	compound description	dose	% mRNA KD	lethalities	ALT fold change relative to buffer	TI
X	buffer control	0	0	0/7	NA	NA
1′	unmodified polymer	0.6	17	0/10	1.5	<3
		1	58	0/10	1.5	
		3	91	1/10	2.5	
		6	93	9/10	7	

NAG targeted dynamic polymer conjugates are taken into hepatocytes via the ASGPR through receptor mediated endocytosis.[32−34] The endosomal pH decreases from ∼7.4 to 5.5−5.0 during endosomal trafficking and eventual lysosomal fusion.[36] While CDM masking is sufficient to mitigate polycation toxicity in systemic circulation, it is not able to protect liver tissue from unmasked polymers after endosomal processing, as is evident by the small margins observed for polymer conjugate **1′**. We were interested in exploring other

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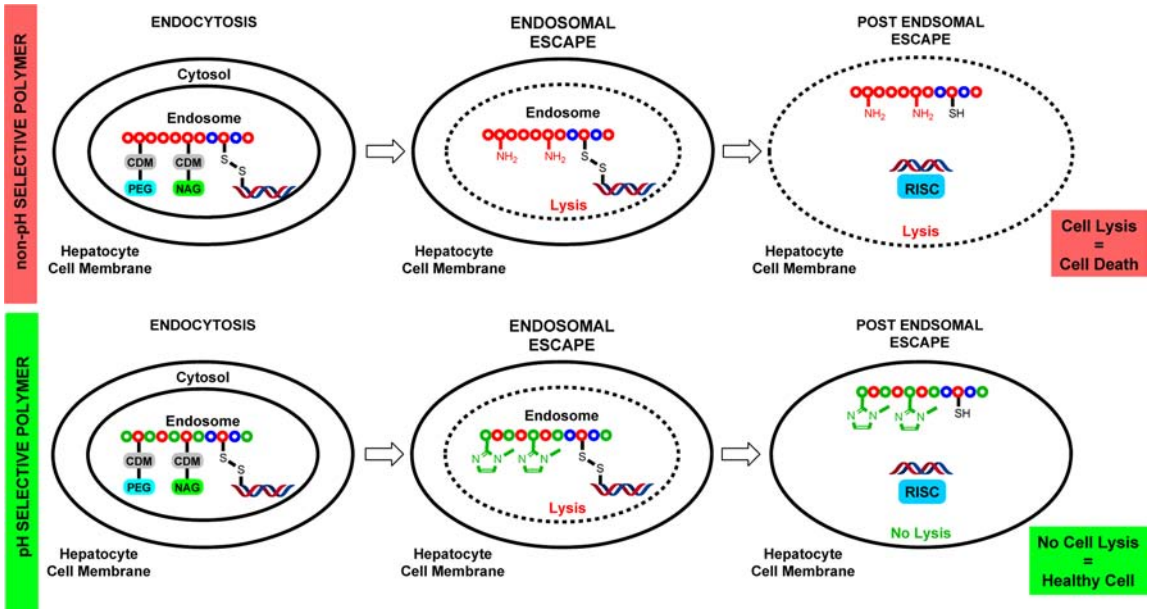
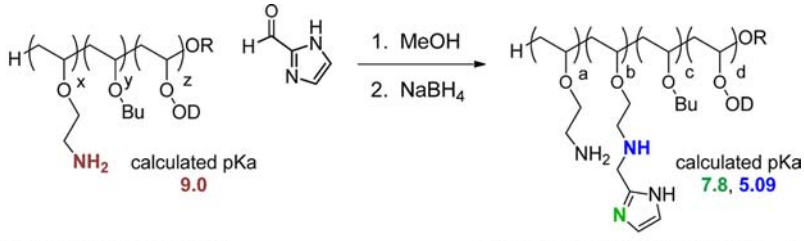


Figure 2. Representation of endosomal escape and post endosomal escape for pH-selective and non-pH-selective polymers.

Scheme 1. Reductive Amination of Unmodified Polymer 1 with 2-Imidazole Carboxaldehyde to Generate 2-Imidazole Modified Polymers



methods of directly adjusting the pH responsiveness of our polymers, utilizing the drop in endosomal pH as a trigger for increasing the selectivity of polymer-based lysis, thus focusing the activity of the polymer on endosomal membrane disruption versus hepatocyte cell membrane disruption (Figure 2). Reported herein are three strategies to improve endosomal membrane lysis selectivity. The first strategy, a pK_a modulation strategy, focuses on generating pH responsive lysis through replacement of high pK_a primary amines with lower pK_a nitrogen-containing heterocycles. The second strategy utilizes acid cleavable linkers that are attached to high pK_a primary amines, but are then removed from the polymer backbone in the lower pH environment of the endosome, thus reducing the overall cationic charge of the polymer. The third strategy is directed at reverse charging of the polymer, focusing on creating pH responsiveness through incorporation of low pK_a carboxylic acids that act as selective charge switches—altering the charge of the overall polymer in a pH dependent fashion. All strategies utilize post-polymerization modification to incorporate pH responsive elements, which not only allows for a high level of chemical diversity, but also fixes many of the variables that can be difficult to control in a nonliving cationic polymerization, such as monomer ratio, molecular weight, tacticity, and regiochemistry. Given our ultimate goal of finding polymer conjugates with clinically relevant animal margins, we chose to examine these strategies in vivo, focusing on improvements in the TI as the marker of success.

pK_a Modulation Through Heterocyclic Incorporation. Based on the high pK_a of the pendant primary amine functionality (pK_a ~ 9), we concluded that the polymer would be cationic and lytic at both physiological pH and endosomal pH once demasking of the CDM functional group had occurred. One hypothesis we wished to explore was whether the replacement of this high pK_a functionality in the polymer backbone with lower pK_a moieties would improve selectivity for endosomal membrane lysis under lower pH conditions versus cell membrane lysis at physiological pH. After receptor mediated endocytosis, the pH drops from pH 7.4 to approximately pH 5 as the endosomal compartments mature to lysosomes. Functionalities with pK_a's below 7.4, but above 5, were targeted as candidates to improve selectivity for endosomal membrane lysis. In order to hold constant all other polymer variables including monomer ratio, tacticity, molecular weight, and regiochemistry, we chose post-polymerization modification to incorporate low pK_a functionality through reductive amination, converting the high pK_a primary amines to lower pK_a heterocyclic containing secondary amines.

The imidazole functionality was selected for initial evaluation based on its calculated pK_a (5.1), structural similarity to the heterocycle contained in the amino acid histidine, and

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successful usage of this functional group in polymer-mediated delivery of DNA or siRNA in the literature.[37−39] Unmodified polymer **1** was functionalized with 2-imidazole-carboxaldehyde through reductive amination (Scheme 1). The level of modification could be controlled by the stoichiometric charge of aldehyde relative to polymer during the reductive amination reaction. Levels of modification for each polymer discussed here are reported as a molar percentage of the total amines in the original polymer. Initially, low and moderate levels of modification were examined and polymers **2** and **3**, with 15% and 29% of 2-imidazole modification, respectively, were prepared. Polymers **2** and **3** were formulated as ApoB siRNA polymer conjugates **2′** and **3′** employing the same procedure and ratios of components as used for unmodified polymer conjugate **1′**. Mice were chosen as a resource sparing model to initially evaluate efficacy in vivo prior to evaluation of TI in rats. CD1 mice were dosed i.v. through tail vein injection with unmodified polymer conjugate **1′** and 2-imidazole modified polymer conjugates **2′** and **3′**. Livers were harvested 48 h post-dose and ApoB mRNA levels were quantitated using RT-qPCR.

As can be seen in Table 2, 2-imidazole containing polymer conjugates **2′** and **3′** showed robust mRNA KD (81% and 93%, respectively) at 1 mpk in mice with levels of mRNA KD that were significantly higher than that observed for unmodified polymer conjugate **1′** (34%). 2-Imidazole containing polymer conjugates **2′** and **3′** were next evaluated in rats at 0.6 and 6 mpk to establish the TI, monitoring for adverse physical effects, liver enzyme levels, and mRNA KD. Both polymer conjugates showed comparable mRNA KD at 0.6 mpk (Table 3), but polymer conjugate **3′**, containing the higher level of 2-imidazole incorporation (29%), showed a dramatic improvement in its toxicity profile as is evident by fewer lethalities in the 6 mpk dosing group (7/9 vs 1/10, respectively) and the relative ALT levels (24-fold and 3-fold, respectively).

Encouraged by the increase in TI observed by incorporation of 29% 2-imidazole, we sought to examine other related heterocyclic functional groups to see if this trend was general or specific to only 2-imidazole (Table 2). We also examined several polymer conjugates containing polymers modified with nonheterocyclic functional groups, such as variously substituted benzyl modifications, that presumably would not lower polymer pK_a (Table 2). Again, we first examined these modifications in mice to determine if their efficacy warranted further examination of TI in rat.

Most heterocyclic modifications (**4′−15′**, Table 2) were well tolerated from a potency standpoint, and showed some efficacy at low dose. All lipophilic, non-pK_a lowering modifications (**16′−19′**, Table 2) showed an increase in potency (48−84% KD @ 0.6 mpk) versus unmodified polymer conjugate **1′** (34% KD @ 1 mpk). Based on these mouse efficacy studies, the 1-methyl-2-imidazole and 1-methyl-3-pyrazole polymer conjugates stood out as being some of the most potent of the heterocyclic polymer conjugates examined (95% KD @ 1 mpk, and 32% KD @ 0.6 mpk) and were selected for further TI evaluation in rats. In addition, the benzyl modified polymer conjugate was also dosed in rats as a control for our pK_a hypothesis. A 30−50% modification level was selected for evaluation of TI in rats based on modification level data from the 2-imidazole rat study, and polymer conjugates **20′−25′** were evaluated for TI in rats. The 30% 1-methyl-2-imidazole modified polymer conjugate **20′** was found to have robust KD at 0.6 mpk (48%, Table 4), higher than either the unmodified polymer conjugate **1′** (17%) or the 30% 2-imidazole polymer conjugate **3′** (31%). Dosing at 6 mpk with this conjugate led to no lethalities, no observable adverse physical effects, and no increases in liver enzyme levels, thus expanding the TI from less than 3 for the unmodified polymer conjugate **1′** to greater than 10. The 30% 1-methyl-2-imidazole polymer conjugate **20′** was also dosed at 9 mpk; however, lethalities were observed at this dose (3/5, Table 4). Polymer conjugate **21′**, containing 50% 1-

Table 2. Mouse in Vivo Efficacy for Polymer Conjugates 2′−19′

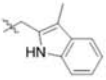
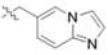
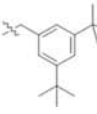
Polymer Conjugate	Modification	Compound Description	Dose (mpk)	% mRNA KD
1'	none	unmodified polymer	1	34
2'		15% 2-imidazole	1	81
3'		29% 2-imidazole	1	93
4'		15% 1-methyl-2-imidazole	1	95
5'		15% 4-imidazole	1	80
6'		19% 4-methyl-5-imidazole	1	82
7'		14% 1-ethyl-2-imidazole	1	61
8'		12% 1,5-dimethyl-4-imidazole	1	60
9'		15% 1-methyl-3-pyrazole	0.6	32
10'		17% 1-methyl-1H-pyrazole-5	0.6	47
11'		17% 4-phenyl-thiazole	0.6	37
12'		17% 3-methyl-indole-2	0.6	22
13'		12% 2-pyridyl	0.6	5.7
14'		14% 6-imidazole-[1,5]-pyridyl	0.6	43
15'		12% 8-imidazole-[1,2]-pyridine	0.6	28
16'		30% benzyl	0.6	75
17'		22% 2,4- methyl-benzyl	0.6	86
18'		23% 3,5-methyl-benzyl	0.6	77
19'		26% 3,5-bis-t-butyl-benzyl	0.6	48

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Table 3. Rat in Vivo Data for 2-Imidazole Containing Polymer Conjugates 2′ and 3′

polymer conjugate	compound description	dose (mpk)	% mRNA KD	lethalities	ALT fold change relative to buffer	TI
X	buffer control	0	0	0/5	NA	NA
2′	15%	0.6	49	0/9	0.7	<10
	2-imidazole	6	95	7/9	24.0	
3′	31%	0.6	31	0/5	1.1	<10
	2-imidazole	6	94	1/10	3.6	

methyl-2-imidazole modified polymer was also examined for TI in rats; however, further increasing the level of modification did not improve the TI and a decrease in potency was observed (17% KD, Table 4) along with a slight increase in toxicity versus polymer conjugate **21**′, containing 30% 1-methyl-2-imidazole. Polymer conjugate **22**′, consisting of polymer modified with 50% 1-methyl-3-pyrazole showed 38% KD in rat at 0.6 mpk, similar to the level of KD observed in rats for the 30% 1-methyl-2-imidazole modified polymer conjugate **20**′ (48%, Table 4) and a decrease in lethality level at 9 mpk (1/5 vs 3/5, respectively, Table 4). Polymer conjugate **23**′, consisting of polymer modified with 32% benzyl, was efficacious at 0.6 mpk (45% KD, Table 4), but showed lethalities at 3 mpk (4/5) even higher than that observed with unmodified polymer conjugate **1**′ (1/10 @ 3 mpk, 9/10 @ 6 mpk, Table 1) and resulting in no improvement in TI.

In order to further confirm that the improvement in TI was associated with pK_a lowering, polymer conjugates **24**′ and **25**′ were synthesized containing a 2-imidazole functional group incorporated through an amidation rather than a reductive amination (Scheme 2). In this way, the contribution of the imidazole moiety to the improved toxicity profile could be separated from that derived from the introduction of the secondary amines generated through reductive amination. Polymer conjugates **24**′ and **25**′ showed more modest, but measurable, KD at 1 mpk (31% and 49%, respectively, Table 4) as compared to polymer conjugates **2**′ and **3**′ (49% and 31%, respectively, Table 3). However, toxicity was diminished compared to that of unmodified polymer conjugate **1**′ (59% KD @ 1 mpk, 9/10 at 6 mpk) with a substantial decrease in lethalities observed for the 15% 2-imidazole amide modified polymer conjugate **24**′ (1/10) and no lethalities observed at 6 mpk for the 30% 2-imidazole amide modified polymer conjugate **25**′ (Table 4).

Acid Cleavable Amino Side Chains. The acid cleavable linker strategy relies on the maintenance of the primary amine content of the original polymer, while at the same time providing for a mechanism whereby the polymer can lose a portion of that amine content in a cellular environment with a lower pH. Intracellular cleavage would then reduce the overall cationic nature of the polymer, leading to an improved toxicity profile and an increased TI of the corresponding polymer conjugate. We chose to examine acid labile acetal and ketal linkers based on their lability at acidic pHs, the tuneability available by variation of the R group, with acetals (R = H) being more acid stable than ketals (R = CH_3) and their successful usage in polymer mediated siRNA and DNA delivery.[40−42,54] While dimethyl ketal polymer linkages were relatively labile with a half-life of approximately 1 h at pH 5/37 °C, the methyl acetal moieties were significantly more stable and hydrolyzed only 5−10% over 2 days under similar conditions. More rapid methyl acetal hydrolysis ($t_{1/2}$ ∼ 1 h) was observed at pH 3 and 37 °C. This range of linker stability allowed us to probe variable impacts of a cleavable amine side chain strategy. We again chose a post-polymerization modification strategy to incorporate primary amine containing acetal and ketal side chains through urea bond formation with the primary amines in the polymer (Scheme 3). As mentioned previously, this approach eliminated the variability that is inherent in polymer synthesis

Table 4. Rat in Vivo Data for Polymer Conjugates 20′−25′

polymer conjugate	compound description	dose (mpk)	% mRNA KD	lethalities	ALT fold change relative to buffer	TI
X	Buffer Control	0	0	0/5	NA	NA
20′	30% 1-methyl-2-imidazole	0.6	48	0/5	0.9	10−15
		6	92	0/5	1	
		9	93	3/5	1.2	
21′	50% 1-methyl-2-imidazole	0.6	17	0/5	1.3	<10
		6	94	2/5	1	
		9	95	4/5	1.7	
22′	50% 1-methyl-3-pyrazole	0.6	38	0/5	1.5	5−15
		3	94	0/5	1.7	
		9	94	1/5	1.5	
23′	32% benzyl	0.6	45	0/5	1.4	<5
		3	93	4/5	2	
		9	all dead	5/5	all dead	
24′	12% 2-imidzole amide	0.6	4	0/5	1	5−10
		1	31	0/5	1	
		3	88	0/5	1	
		6	89	1/10	3.4	
		12	99	4/5	13.1	
25′	25% 2-imidazole amide	0.6	28	1/5	1.1	<6
		1	49	1/5	1	
		3	84	0/5	1	
		6	91	0/5	1.1	

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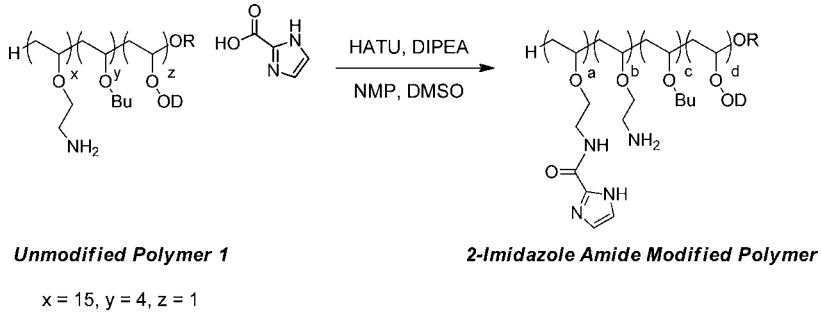
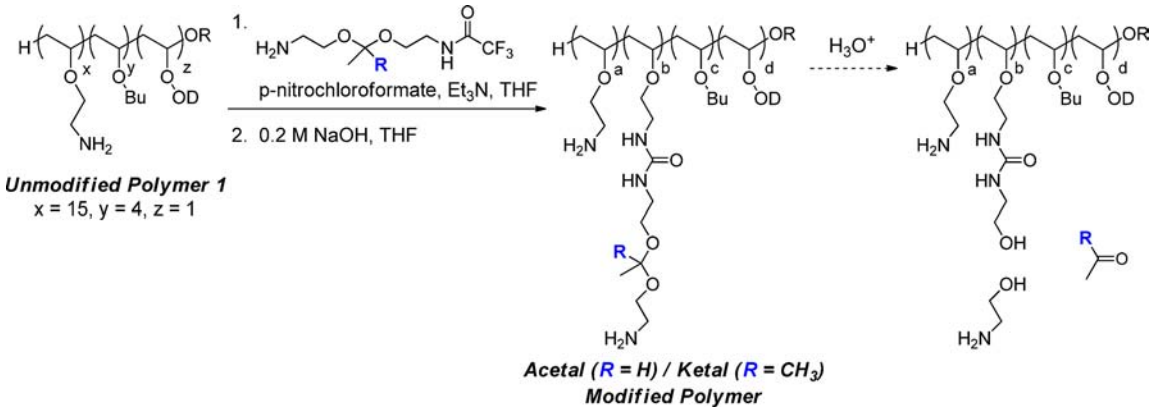
Scheme 2. Amidation of Unmodified Polymer 1 with 2-Imidazole Carboxylic Acid to Generate 2-Imidazole Amide Modified Polymers



Scheme 3. Modification of Unmodified Polymer 1 with Acetal (R = H)/Ketal (R = CH_3) Side Chains to Generate Polymers Containing an Acid Cleavable Amine Side Chain



and provided an approach that would focus specifically on the impact of the acid cleavable linker.

Unmodified polymer **1** was functionalized with a monotrifluoroacetamide protected acetal or ketal side chain using *p*-nitrochloroformate (Scheme 3). The trifluoroacetamide protecting group was subsequently removed under basic conditions to yield the acetal or ketal modified polymers **26**–**30**. The level of modification could be controlled by the relative charge of the monoprotected side chain as compared with the polymer during the urea forming reaction. Levels of modification are reported as a molar percentage of total amines in the polymer modified through post-polymerization modification. Initially, a range of levels of modification were examined for both ketal and acetal modified polymers. Acetal or ketal modified polymers **26**–**30** were formulated as ApoB siRNA polymer conjugates **26′**–**30′** using the same procedure that was used for unmodified polymer conjugate **1′**.

Acetal modified polymer conjugates **26′** and **27′** showed an improvement in mRNA KD at 1 mpk (97% and 70%, respectively, Table 5) as compared with unmodified polymer conjugate **1′** (34% KD). However, a higher level of modification led to a decrease in mRNA KD. Ketal modified polymer conjugates **28′**–**30′** showed comparable levels of KD at 1 mpk (48%, 38%, 32%, respectively, Table 5) as compared to unmodified polymer conjugate **1′** (32%); however, they were significantly less potent than acetal modified polymer conjugates **26′** and **27′**. The lower potency of the ketal modified polymers can be attributed to rapid cleavage of these side chains under acidic conditions. Only slow release of the methyl acetal is observed in an acidic environment, and this relatively stable side chain is able to improve overall polymer conjugate potency.

Table 5. Mouse in Vivo Efficacy Data for Acetal and Ketal Modified Polymer Conjugates 26′–30′

polymer conjugate	modification	compound description	dose (mpk)	% mRNA KD
1′	none	PVE	1	34
26′	R = H	20% acetal	1	97
27′	R = H	40% acetal	1	70
28′	R = CH_3	30% ketal	1	48
29′	R = CH_3	50% ketal	1	38
30′	R = CH_3	80% ketal	1	32

In order to assess the TI of polymer conjugates containing acetal and ketal modifications, polymer conjugates **27′** and **28′** were examined at a range of doses in rats. The 40% acetal modified polymer conjugate **27′** showed a modest increase in KD at 1 mpk (73%, Table 6) as compared to the unmodified polymer conjugate **1′** (59%, Table 1). Additionally, toxicity was diminished compared to unmodified polymer conjugate **1′** (9/10 lethalities, 7-fold increase in ALT @ 6 mpk) with no lethalities observed for the 40% acetal modified polymer conjugate **27′** (0/5) at 6 mpk and only a modest 3-fold elevation in ALT (Table 6), thus expanding the TI to greater than 6-fold through acetal incorporation. The 30% ketal modified polymer conjugate **28′** showed a 3-fold loss in potency (5% KD @ 1 mpk, 80% KD at 3 mpk, Table 6); however, the toxicity was also diminished compared to the unmodified polymer conjugate (0/5 lethalities @ 6 mpk vs 9/10 respectively). Despite the improvement in toxicity, it was unclear whether there was an improvement in TI for this

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Table 6. Rat in Vivo Data for Acetal and Ketal Modified Polymer Conjugates 27′ and 28′

polymer conjugate	compound description	dose (mpk)	% mRNA KD	lethalities	ALT fold change relative to buffer	TI
X	buffer control	0	0	0/7	NA	NA
27′	40% acetal	1	73	0/5	1.4	≥6
		3	96	0/5	1.8	
		6	96	0/5	2.9	
28′	30% ketal	1	5	0/5	1.5	≥2
		3	80	0/5	1.2	
		6	98	0/5	1.6	

conjugate since there was a concomitant loss of overall potency with this ketal modification. Since the ketal side chain is highly labile, both the decreased potency and toxicity are likely due to the premature hydrolysis of the ketal side chains, which results in an overall decrease in polymer charge and loss of activity.

pH Selective Reverse Charging. The pH selective reverse charging strategy focuses on creating pH responsiveness through incorporation of low pK_a carboxylic acids. These modifications can act as a pH selective charge switch, altering the charge of the overall polymer in a pH dependent fashion. This strategy has been applied in the successful biocompatible delivery of siRNA.[43−53] Alkylation of the high pK_a primary amines in the polymer backbone with a low pK_a carboxylic acid results in a highly cationic polymer while located in the acidic endosomal compartment, where the carboxylic acids will exist primarily in the protonated state. Upon entering the relatively more basic environment of the cytosol, these carboxylic acids would be deprotonated to the carboxylate form, providing additional negative charge that would reduce the overall charge and lead to a decrease in overall positive charge with more zwitterionic character. This pH selective transition in overall polymer charge is hypothesized to improve selectivity for endosomal membrane lysis versus cell membrane lysis. We chose post-polymerization modification to incorporate carboxylic acid functionality through alkylation with iodo-acetic acid (Scheme 4). Unmodified polymer **1** was functionalized with iodo-acetic acid under basic aqueous conditions generating a mixture of secondary and tertiary amines through either mono- or dialkylation. The ratio levels of modification are reported as a molar percentage of total amines in the polymer modified through post-polymerization modification. Initially, a range of acetic acid modification, from 7% to 33% was examined. Acetic acid modified polymers **31**−**34** were formulated as ApoB siRNA polymer conjugates **31**′−**34**′ using the same procedure as that used for unmodified polymer conjugate **1**′.

Table 7. Mouse in Vivo Data for Acetic Acid Modified Polymer Conjugates 31′−34′

polymer conjugate	compound description	mono/di alkylation	dose (mpk)	% mRNA KD
1′	PVE	NA	1	34
31′	7% acetic acid	4/1	1	56
32′	10% acetic acid	4/1	1	78
33′	25% acetic acid	2/1	1	68
34′	33% acetic acid	2/1	1	93

Acetic acid modified polymer conjugates **31**′−**34**′ showed an improvement in KD at 1 mpk (Table 7) as compared to unmodified polymer conjugate **1**′, with up to 33% modification showing robust KD at 1 mpk. Again, the slight increase in lipophilicity of the carboxylic acid under the acidic endosomal conditions may explain the increase in potency observed for the modified polymer conjugates.

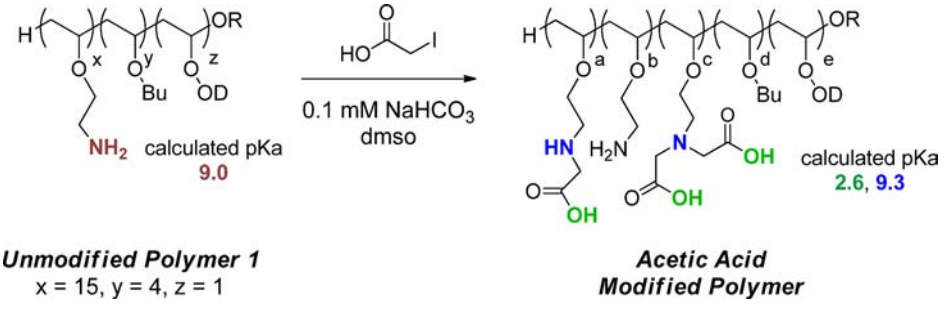
In order to assess the TI of polymer conjugates containing the acetic acid modification, polymer conjugate **34**′ was examined at a range of doses in rat. The 33% acetic acid modified polymer conjugate **34**′ showed comparable KD at 0.6 mpk (44%, Table 9) as compared to the unmodified polymer conjugate **1**′ (59%, Table 1, 1 mpk). Additionally, toxicity was also diminished compared to unmodified polymer conjugate **1**′ (9/10 lethalities @ 6 mpk) with no lethalities observed for the 30% acetic acid modified polymer conjugate **34**′ (0/5) at 6 mpk and no elevation in ALT observed (Table 8), thus expanding the TI to greater than 6 through incorporation of the acetic acid functionality.

Table 8. Rat in Vivo Data for Acetic Acid Modified Polymer Conjugate 34′

polymer conjugate	compound description	dose (mpk)	% mRNA KD	lethalities	ALT fold change relative to buffer	TI
X	buffer control	0	0	0/7	NA	NA
34′	33% acetic acid	0.6	34	0/5	1.5	≥6
		3	88	1/5	1.3	
		6	94	0/10	1.5	

Comparison of In Vivo TI of Polymer Conjugates. While it is clear that dynamic polymer conjugate **1**′ is capable of providing robust mRNA KD, the TI between a minimally efficacious dose and a nontoxic dose is quite small, less than 3-fold. The high pK_a of the primary amines of polymer **1** provide for membrane lysis under a wide pH range, leading to undesired cell membrane lysis in addition to the desired

Scheme 4. Modification of Unmodified Polymer 1 with Iodo-Acetic Acid to Generate Acetic Acid Modified Polymer



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endosomal membrane lysis, resulting in toxicity and poor TI. From the rat in vivo data, it is apparent that the TI of polymer conjugates can be dramatically expanded by manipulation of the overall cationic charge at physiological pH through replacement of high pK_a primary amines with low pK_a heterocycles, acid labile amino side chains, or pH sensitive charge switching carboxylic acids.

The decrease in toxicity and improvement in TI observed through incorporation of low pK_a heterocycles into polymer conjugates is general, and the introduction of a range of structurally diverse heterocyclic functional groups improves the toxicity versus the unmodified polymer conjugate **1′** with a TI of 10−15 observed for the 30% 1-methyl-2-imidazole modified polymer conjugate **20′** and a TI of 5−15 for the 50% 1-methyl-3-pyrazole modified polymer conjugate **22′** (Table 9).

Table 9. Comparison of in Vivo TI for Polymer Conjugates

polymer conjugate	compound description	MED (mpk)	highest nonlethal dose	lowest lethal dose	TI
1′	unmodified polymer	1	1	3	<3
20′	30% 1-methyl-2-imidazole	0.6	6	9	10−15
25′	25% 2-imidazole amide	1	6	NA	≥6
23′	32% benzyl	0.6	0.6	3	<6
22′	50% 1-methyl-3-pyrazole	0.6	3	9	5−15
27′	40% acetal	1	6	NA	≥6
28′	30% ketal	3	6	NA	≥2
34′	33% acetic acid	1	6	NA	≥6

Additionally, low pK_a heterocycles may be incorporated through multiple chemistries, including amidation or reductive amination, with improvements in TI being observed for polymer conjugates containing either type of linkage, and a TI of greater than 6 observed for polymer conjugate **25′**, modified with 25% 2-imidazole amide. The fact that there is no loss in potency and an improvement in the toxicity profile for heterocycle-containing polymers is likely due to a similar overall cationic charge at low endosomal pH but a diminished cationic charge at higher physiological pH, thus rendering the polymers similarly lytic at endosomal pH, but less lytic at physiological pH. The higher toxicity and loss of TI resulting from benzyl modification as compared to various heterocycles is likely due to an increase in cationic charge at physiological pH due to a lack of amine pK_a reduction resulting from benzyl modification. Thus, the 32% benzyl modified polymer conjugate **23′** is cationic at both physiological and endosomal pHs leading to good potency, but with a relatively high level of toxicity, resulting in diminished margins.

The decrease in toxicity observed through incorporation of acid labile amine side chains appears general, and the introduction of either ketal or acetal acid-labile linkages improves the toxicity profile versus the unmodified polymer with a TI of greater than 6 observed for the 30% acetal modified polymer conjugate **27′** (Table 9). The fact that ketal-containing polymers provide decreased potency but with no associated toxicity is likely due to the rapid loss of amine content from the polymer, suggesting that high amine content is required for potency, but also contributes to toxicity. The higher potency observed for acetal versus ketal containing polymer conjugates is likely due to the increased stability of the acetal versus the ketal linkage, which allows for less rapid loss of amine content from the polymer. The improved toxicity profile of the acetal polymer conjugates is likely due to the slow release of the amine side chain, thereby reducing the cationic character of the residual polymer in vivo. While methyl acetal hydrolysis was apparently slow in comparison to the overall time course of the in vivo studies, the lower toxicity of this polymer in rats is possibly attributed to the fact that this linkage is being hydrolyzed more rapidly under physiological conditions as compared with the hydrolysis observed during our in vitro analyses.

The decrease in toxicity observed through incorporation of low pK_a carboxylic acid charge switches improves the toxicity versus the unmodified polymer polymer conjugate **1′** with a TI of greater than 6 observed for the 30% acetic acid modified polymer conjugate **34′** (Table 9). The fact that acetic acid-modified polymers provide similar potency but with no associated toxicity is likely due to protonation of the negatively charged carboxylate while present in the low pH environment of the endosome, leading to a cationic charge at endosomal pH, but an overall decrease in cationic charge at physiological pH.

CONCLUSIONS

We have demonstrated three distinct post-polymerization modification strategies to improve the in vivo therapeutic margin of siRNA polymer conjugates by decreasing conjugate toxicity through incorporation of pH responsive elements. This effect of improving pH responsiveness appears general with a diversity of approaches, such as modification with acetic acid, incorporation of low pK_a heterocycles, and inclusion of acid cleavable acetal linkers, leading to improved pH selectivity and a decrease in toxicity in vivo. It was also demonstrated that this effect is specific to functionalities that improve pH selectivity, with certain lipophilic modifications such as benzyl showing no improvement in TI and an increase in toxicity. These approaches to lowering polymer toxicity while maintaining, if not improving, potency provide a pathway for the development of delivery vehicles that are able to safely and effectively deliver siRNAs for therapeutic use.

ASSOCIATED CONTENT

Supporting Information

Additional spectra and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: erin_guidry@merck.com, Phone: + 1-908-740-5415, Fax: +1-908-740-6136.

Notes

The authors declare no competing financial interest.

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