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Novel Endosomolytic Poly(amido amine) Polymer Conjugates for Systemic Delivery of siRNA to Hepatocytes in Rodents and Nonhuman Primates

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Supporting Information

ABSTRACT: The application of small interfering (si)RNAs as potential therapeutic agents requires safe and effective methods for their delivery to the cytoplasm of the target cells and tissues. Recent studies have shown significant progress in the development of targeting reagents that facilitate the recognition of, and siRNA delivery to, specific cell types. Among recently reported delivery approaches, polymers with amphipathic properties have been used to enable endosome escape and cytosolic delivery. Here, we describe a linear amphipathic poly(amido amine) polymer conjugate system for the efficient siRNA delivery *in vitro* and *in vivo*. This polymer contains a novel amine bearing bis-acrylamide monomer designed for increasing amine density, which resulted in substantial improvement in liver uptake and RNAi activity compared to our previously reported poly(amido amine disulfide) polymer.[1] The activity for this liver targeted delivery system was demonstrated in rodents and nonhuman primates.

INTRODUCTION

RNA interference is an evolutionary conserved pathway for double-stranded RNA to silence gene expression in a sequence-specific manner.[2] Successful demonstration using synthetic small interference RNA (siRNA) to suppress endogenous genes provides great potential for siRNAs as therapeutics.[3] To harness this potential, safe and effective delivery of siRNA to target tissue and cell types is critical. Many barriers exist for delivery, including enzymatic degradation of the oligonucleotide, rapid clearance, cellular uptake, endosomal escape, and tolerability of the delivery vehicle after systemic administration.[4]

A multitude of polymer-based delivery systems have been developed to overcome the above mentioned barriers to delivery.[5] Most of these systems rely on cationic charges to encapsulate or form complexes with the negatively charged siRNA. This strategy protects siRNA from degradation, and improves cell uptake. However, the resulting polyplexes are toxic due to the cationic charges interacting with serum proteins or cellular components after systemic administration. Further, they may not be stable enough to survive systemic circulation and their composition may change by either shedding their unstable components or exchanging with biological components such as lipoproteins. The aggregation in blood can also cause rapid clearance by the RES system.

Recently, several polymer−conjugate systems have been described.[6−9] These systems use a cleavable covalent bond between siRNA and polymers to keep them intact in circulation and release the siRNA in the appropriate cellular compartments once internalized into the cells. The cationic charges are masked by poly(ethylene glycol) (PEG) and targeting ligand, e.g., *N*-acetylgalactosamine(GalNAc) with pH labile linkers, therefore minimizing systemic toxicity. Once in endosome where pH becomes acidic, the masking groups are expected to be cleaved to release the membrane lytic polymer for enhancing endosome escape. Despite the great potential of this conjugate strategy, tolerability is a major concern due to the non-degradable nature of these systems.

In our previous publication,[1] we discussed the combination of novel biodegradable poly(amido amine) disulfide (PAADS) polymer with acid labile masking groups for targeting and stealth that led to an effective siRNA delivery system with demonstrated mRNA knockdown *in vivo* in mice. Incorporation of disulfides using cystamine bis-acrylamide (Figure 1A-I) in the PAADS polymer backbone makes the polymer biodegradable but limits the number of cationic charges (number of

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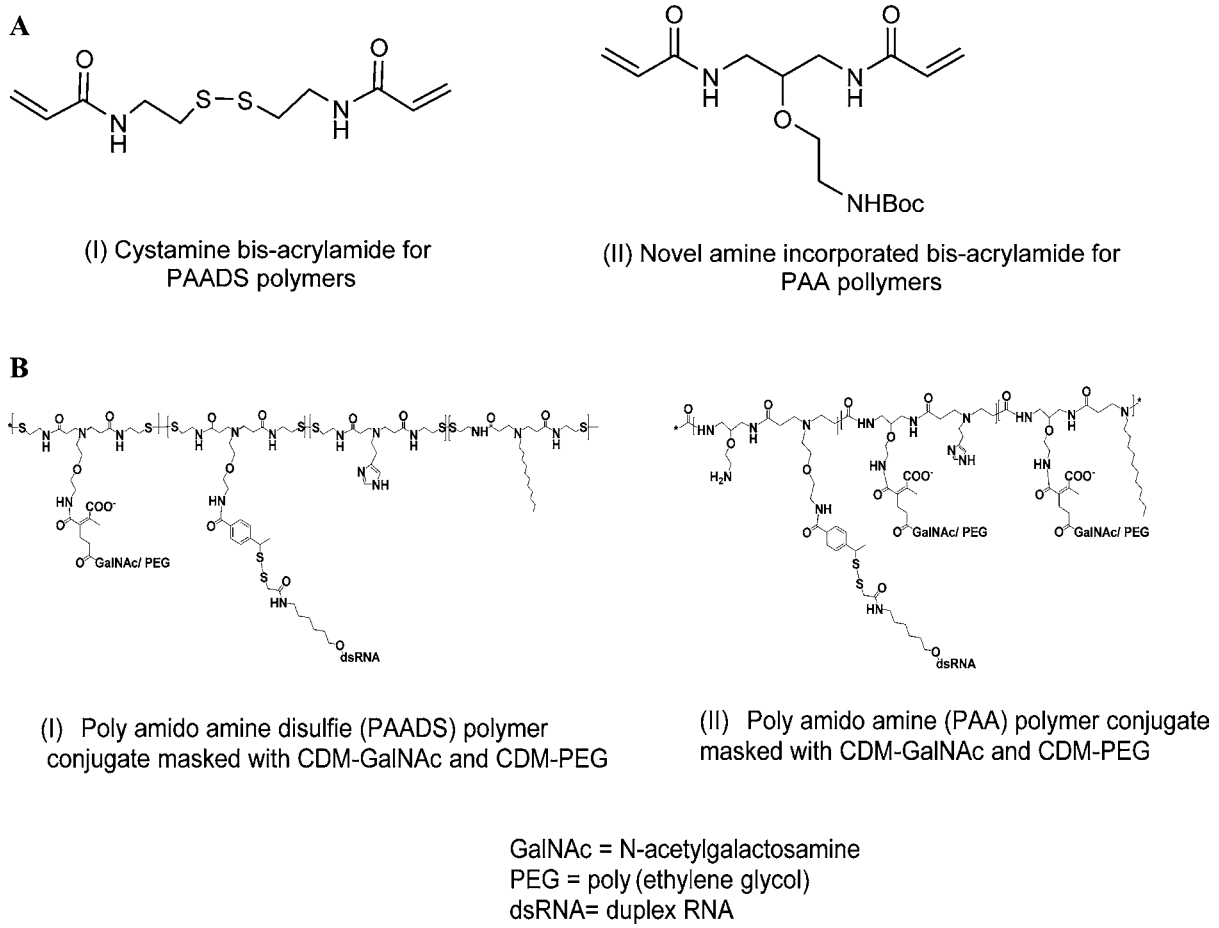

Figure 1. A. Monomers for PAADS and PAA polymers. B. PAADS and PAA polymer conjugates.

amines), potentially restricting cellular uptake and the endosomal escape. The smaller number of amines per polymer chain is believed to lead to less GalNAc targeting density resulting in compromised liver uptake. To further enhance the *in vivo* activity of these polymer conjugates, a new bis-acrylamide monomer containing an amine side chain (Figure 1A-II) was designed to improve the amine density and liver uptake. In this paper, we have described a novel poly(amido amine) polymer (PAA) that incorporates an amine-bearing bis-acrylamide monomer for effective systemic delivery of siRNA to hepatocytes in rodents and nonhuman primates.

■ RESULTS AND DISCUSSION

In our previous publication,[1] we discussed the combination of novel biodegradable PAADS polymer with acid labile masking groups for GalNAc and poly(ethylene glycol) led to an effective siRNA delivery system with demonstrated mRNA knockdown *in vivo* in mice. However, siRNA liver levels were very low (below 0.001 nmol/g) with PAADS polymer conjugates. The lower siRNA liver levels with PAADS polymer conjugates might be because of insufficient targeting of asiloglycoprotein receptors in liver hepatocytes with CDM-GalNAc, as a smaller number of pendant amines are available in the polymer chain for masking with targeting groups. It is well-known in the literature that the efficient targeting of asiloglycoprotein receptor in liver hepatocytes can be achieved by a cluster of GalNAc ligands. Therefore, the density of these ligands is important for liver uptake. PAADS polymers are synthesized by Michael addition of cystamine bis-acrylamide with amines. Cystamine bis-acrylamide monomer (Figure 1A-I), being a long linear structure, affects the amine density of the polymer. Our hypothesis is that the polymer conjugate efficacy can be further enhanced by increasing the amine density of polymer, thereby enhancing the targeting of polymer conjugates. To prove this hypothesis, novel amine incorporated bis-acrylamide (*tert*-butyl (2-{[1,3-bis(prop-2-enoylamino)propan-2-yl]oxy}ethyl)-carbamate) was designed, synthesized (Figure 1A-II), and substituted with cystamine bis-acrylamide (Figure 1A-I) for Michael addition polymerization. Our strategy was to first completely replace cystamine bis-acrylamide with novel amine incorporated bis-acrylamide to maximize the amine density and later introduce some cystamine bis-acrylamide to increase the biodegradability of the polymer. These polymers were tested as polymer conjugates (Figure 1B-II) in mice to test the *in vivo* activity and compared with PAADS polymer conjugates.

Synthesis of Polymer. Poly(amido amine) polymers were synthesized via step-growth polymerization using a Michael addition reaction as previously reported.[11] The stoichiometric ratio of amine to novel amine (protected with Boc group) incorporated bis-acrylamide was kept at 1:1 for all the polymers (Figure 2B). The number-average molecular weight (M_n) of the polymers was determined to be in the range from 6000 to 15 000 Da with a polydispersity index of 1.9 to 3. Monomer incorporation ratio was determined by NMR integration (see Supporting Information (SI)).

As discussed in our previous paper,[1] three components (amine:imidazole:dodecyl) were chosen for PAADS polymer synthesis. Amines were used for the purpose of enhancing

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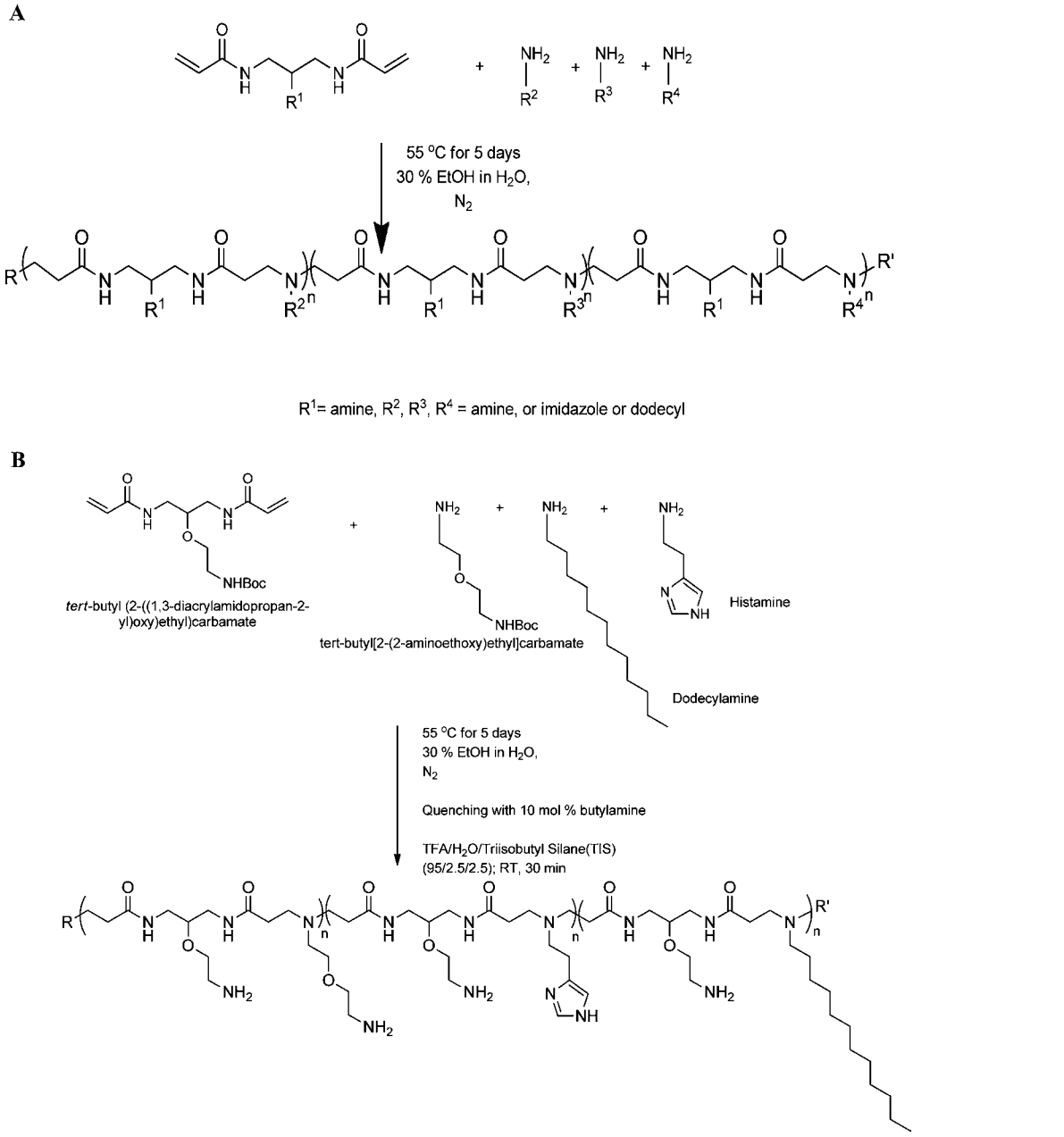

Figure 2. A. Polymer synthesis via Michael addition. B. Polymer synthesis.

Table 1. Polymers with Different Monomer Ratios and M_n of Polymer

polymer	aminoethoxy [bis-acrylamide]	2-(2-aminoethoxy)ethyl [amine]	2-(1*H*-imidazol-4-yl)ethyl [imidazole]	dodecyl [dodecyl]	M_n = kDa (PDI)
Polymer 1	100	40	30	30	13.7 (2.1)
Polymer 2	100	30	50	20	12.2 (2.5)
Polymer 3	100	30	20	50	9.8 (2.1)
Polymer 4	100	0	20	80	6.1 (2.0)
Polymer 5	100	70	0	30	6.8 (1.9)
Polymer 6	100	20	80	0	11.0 (2.0)
Polymer 7	100	40	60	0	8.5 (2.3)
Polymer 8	100	100	0	0	6.0 (2.0)

interactions with membranes and will be masked with acid-labile CDM groups, hydrophobic dodecyl groups were introduced to provide amphipathic properties to the polymers, and imidazole groups were used for increasing the endosomal lytic behavior. The imidazole group, as reported in the literature,[12] may assist in the endosomal escape through the proton sponge effect and in fusing the lipid bilayers through ionic interactions in a mildly acidic environment. As reported in our earlier publication,[1] screening of the different components at different ratios identified two lead PAADS polymers that

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gave good *in vitro* and *in vivo* activity. These PAADS polymers consist of amine:imidazole:dodecyl components at ratios 30:50:20 and 70:0:30. Multiple PAA polymers were synthesized using the same three components (amine:imidazole:dodecyl) with ratios similar to the ones used for PAADS polymers (Table 1).

These polymers were tested in red blood cell hemolysis assay to test the lytic behavior. The increase in lytic behavior was observed with increase in dodecyl contents in the polymer. As shown in Table 2, polymer 4 is most lytic. Polymer 8 is not lytic probably because of the absence of both hydrophobic group (dodecyl) and imidazole group.

Table 2. Red Blood Cell Hemolysis Data of Polymers

polymer	red blood cell hemolysis—EC_{50} @ pH 5.4 (mg/mL)	red blood cell hemolysis—EC_{50} @ pH 7.4(mg/mL)
Polymer 1	0.025	0.020
Polymer 2	0.036	0.048
Polymer 3	0.010	0.011
Polymer 4	0.003	0.003
Polymer 5	0.012	0.012
Polymer 6	Not active	Not active
Polymer 8	Not active	Not active

Polymer Conjugate Synthesis. Polymer conjugates (polymer conjugated with siRNA and masked with CDM) were prepared as previously described.[7] Polymers were conjugated with Apo B targeted siRNA at different polymer:-siRNA weight ratios. Here we have used two active Apo lipoprotein B siRNA's and one irrelevant control inactive siRNA. Active siRNA 1 is a literature siRNA[7,10] and active siRNA 2 is Merck's proprietary siRNA. In our previous paper,[1] active siRNA1 was used for Apo lipoprotein silencing. Polymer−siRNA conjugates were then masked with CDM-GalNAc and CDM-PEG using 3:1 molar ratios, respectively. Conjugation efficiency of siRNA to masked polymer was determined by size exclusive column (SEC) chromatography and was in general higher than 80% (see SI). Masking efficiency of amines by CDMs was determined to be 50−70% (see SI). Reversible masking of the amines was incorporated as an attempt to reduce systemic toxicity under physiological conditions. pH responsive hydrolysis restores the membrane lytic activity in the acidic environment of the endosome. Thus, pH responsive behavior of these masked polymer conjugates was also evaluated in the RBC lysis assay. Robust hemolysis was observed at pH 5.4 but not at pH 7.4 as shown in Figure 3, presumably due to the accelerated demasking of amines on the polymer at acidic pH. All the polymer conjugates were tested in RBC hemolysis assay, and all of them showed similar behavior, i.e., robust hydrolysis at pH 5.4.

In this manuscript, each polymer conjugate (polymer conjugated with siRNA and masked with CDMs) is assigned different ID = (P) Polymer # (1 to 15) − siRNA (AR1 or AR2 or CR) − polymer to siRNA ratio (4, 6, 11) − siRNA dose (1, 3, 6 mg/kg dose): Active siRNA 1(AR1) = literature Apo B sequence; Active siRNA 2 (AR2) = Apo B sequence; Control siRNA (CR) = Irrelevant control sequence.

Mouse Data. Polymer 1 and polymer 2 were conjugated with both active siRNA 1 and active siRNA 2. Polymer conjugates P-1-AR1-4-6/P-1-AR1-4-3 and P-1-AR2-4-6/P-1-AR2-4-3 showed similar mRNA knockdown. Similarly, P-2-AR1-6-6/P-2-AR1-6-3 and P-2-AR2-6-6/P-2-AR2-6-3 gave

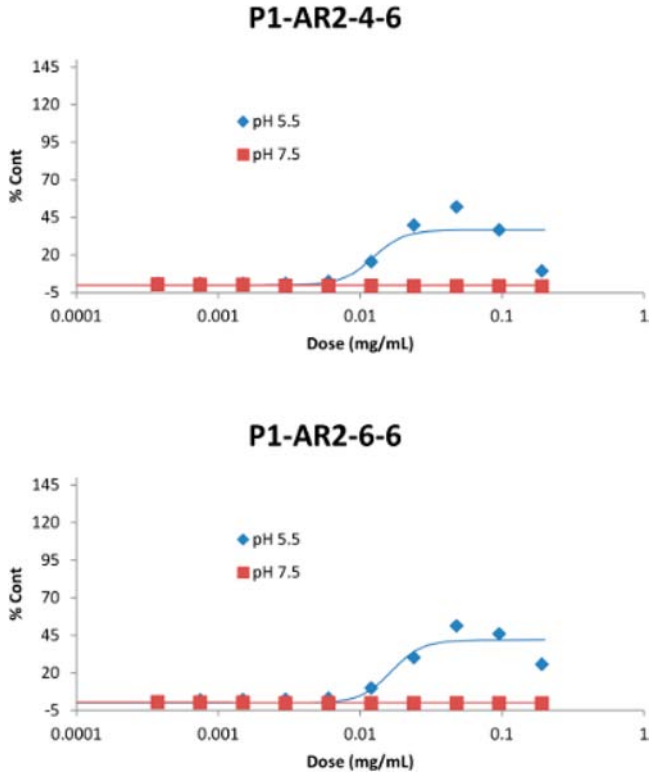

Figure 3. Red blood cell hemolysis data of polymer conjugates.

more than 90% knockdown (Figure 4), thus indicating that both active siRNA 1 and active siRNA 2 have similar mRNA knockdown activity *in vivo*.

Polymer 1 was formulated with siRNA at 4:1 as well as at 8:1 polymer:siRNA weight ratio to see the effect of polymer on the *in vivo* efficacy. As shown in Figure 4, P-1-AR1-4-3 showed 60% knockdown, whereas P-1-AR1-8-3 showed more than 75% knockdown, suggesting that increasing the amount of polymer increases the knockdown. Polymer 1 was also formulated at 6:1 polymer:siRNA ratio with active siRNA2 resulting in P-1-AR2-6-3, which showed the activity similar to P-1-AR1-8-3. Higher polymer:siRNA ratio may led to higher toxicity; therefore, 6:1 polymer:siRNA ratio was chosen for further studies. To stack-rank the polymers based on their efficacy, polymer conjugates were also prepared at 4:1 polymer:siRNA ratio.

Polymer conjugate P1-C-4-6, prepared by conjugating control siRNA, did not show any activity, proving that these polymer conjugates show siRNA sequence specific activity. Polymer 2 formulated at 6:1 polymer:siRNA ratio gave maximum knockdown (>90%) with conjugates P-2-AR2-6-6, P-2-AR2-6-3, P-2-AR2-6-1. Polymer 3 was formulated at 4:1 and 6:1 polymer:siRNA ratio. P3-AR1-4-6 and P3-AR1-6-6 were less active than polymer 2 conjugates P2-AR1-4-6 and P2-AR1-6-6. Polymer 4 has the highest dodecyl moiety in it. It was difficult to formulate polymer 4 at 4:1 and 6:1 polymer:siRNA ratio. This polymer was then formulated at 11:1 polymer:-siRNA ratio. P4-AR2-11-6 conjugate showed only 50% mRNA knockdown.

Polymer 5 is without an imidazole group. This polymer was formulated with siRNA at 6:1 and 11:1 ratio. P-5-AR2-6-6 and P-5-AR2-11-6 were less active than P2-AR2-6-6. Polymer 6 and polymer 7 do not contain hydrophobic dodecyl group. All the conjugates of polymer 6 and polymer 7 were less active than polymer 2 conjugates. Polymer 8 consists of only amines,

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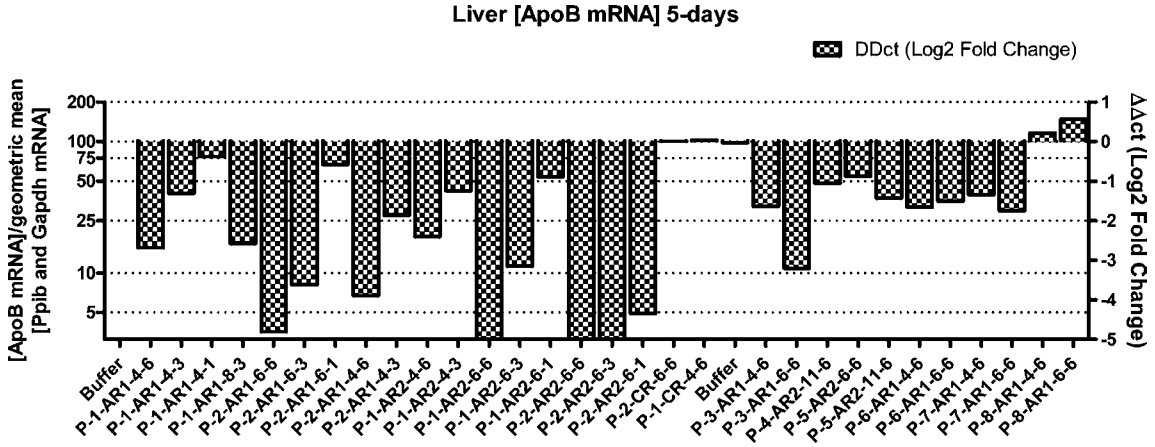

Figure 4. mRNA expression in mouse liver.

without dodecyl and imidazole groups. This polymer conjugate P-8-AR1-6-6 was not active *in vivo*.

As shown in Table 3, all these polymer conjugates were simultaneously tested in *in vitro* bDNA assay. All the polymer conjugates were active in *in vitro* assay except polymer 8 conjugate. Toxicity was observed for polymer conjugate P4-AR2-11-6 in MTS assay. This may be because of higher polymer:siRNA 11:1 ratio, as it was difficult to formulate this polymer 4 at 6:1 polymer:siRNA ratio.

Table 3. bDNA and MTS *in Vitro* Assay

ID = Polymer # - siRNA# -ratio of polymer to siRNA -mg/kg dose	bDNA (nM)	MTS (nM)
P-1-AR1-6-6	126	>300
P-2-AR1-6-6	112	>300
P-3-AR1-6-6	135	>300
P-4-AR2-11-6	11	13
P-5-AR2-6-6	148	>300
P-6-AR1-6-6	53	>300
P-7-AR1-6-6	59	>300
P-8-AR1-6-6	300	300

These results suggest that amine density, imidazole, and hydrophobic components play an important role to improve the cytosolic delivery properties of a polymer. Polymer 1 and polymer 2 consist of all three components and showed better *in vivo* activity as compared to polymers 4, 5, 6, and 7 containing only two of these components. Polymer 3 also contains all three components but this polymer contains a higher ratio of dodecyl showing that increasing the % of hydrophobic components affects *in vivo* activity. Polymer 8 with amine only monomer did not show any *in vivo* activity.

P2-AR2-6 PAA polymer conjugate showed >90% Apo B knockdown at 1 mg/kg dose, whereas lead PAADS conjugate showed 80% knockdown at 3 mg/kg dose showing that increasing the amine density does increase the *in vivo* efficacy.

siRNA liver levels for these new polymer conjugates were also tested and compared with PAADS polymer conjugates discussed in our previous paper.[1] As shown in Figure 5, new poly(amido amine) polymer conjugates have 1000 times higher (1−0.1 nmol/g) siRNA concentration as compared to poly(amido amine disulfide) polymer conjugates (0.001−0.0001 nmol/g). It is interesting to observe that though PAADS polymer conjugates have low siRNA liver levels, they still give good *in vivo* activity at 3 mg/kg dose, suggesting that PAADS polymer conjugates might work by a different mechanism. PAADS polymer conjugates form 80−100 nm particles, whereas PAA polymer conjugates do not form any particles. The bigger particle size of PAADS polymer conjugates might be a contributing factor for enhanced *in vivo* activity even with low siRNA liver levels.

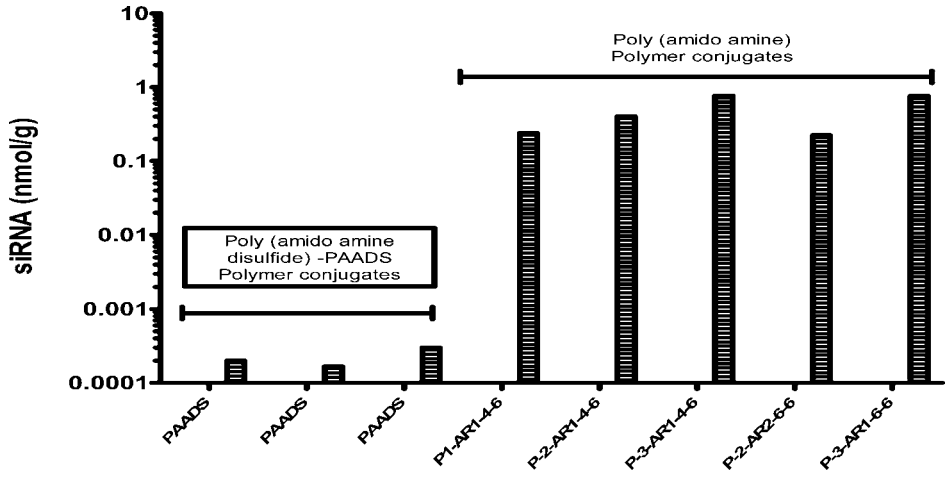

Figure 5. siRNA liver levels: comparison of PAADS vs PAA polymer conjugates.

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Rat and Nonhuman Primate Data. Polymer conjugates of eight polymers were tested at different polymer:siRNA ratios in mice. Based on mice mRNA knockdown data, polymer conjugate P2-AR2-6 (Polymer 2 conjugated with AR2 siRNA with 6:1 polymer:siRNA ratio masked with CDM-GalNAc and CDM-PEG) is most potent. For species translation, this conjugate was also tested in rats and nonhuman primates.

As shown in Figure 6A, dose response was observed with polymer conjugate P2-A2-6 @ 6, 3, and 1 mg/kg of siRNA

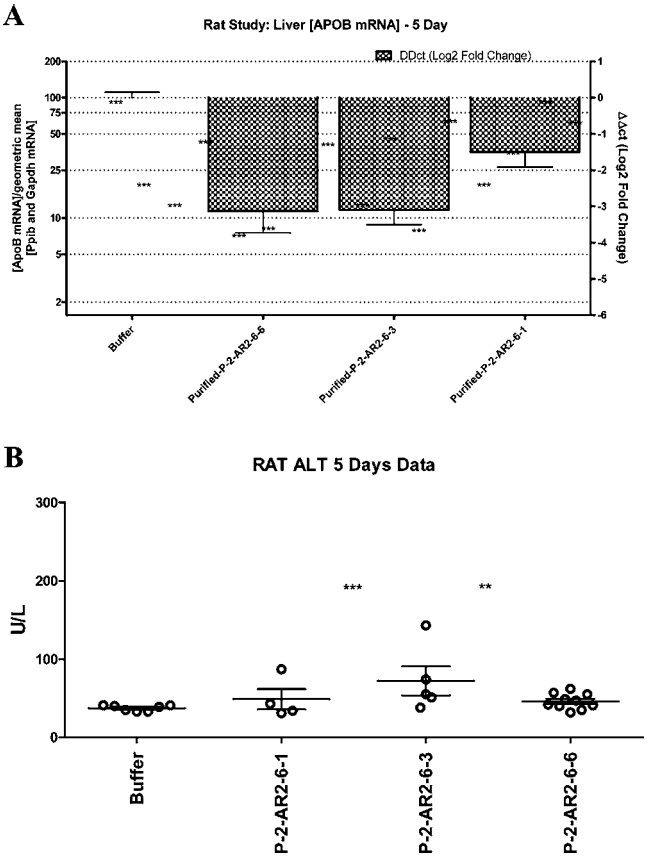

Figure 6. A. mRNA knockdown data in rats. B. Alanine aminotransferase (ALT) levels in rats.

dose. The toxicity of these polymer conjugates was assessed by measuring serum levels of alanine aminotransferase (ALT) after 5 days postdose. As shown in Figure 6B, slight elevations in ALT levels were observed at day 5 in individual animals at 1 and 3 mg/kg but not at 6 mg/kg dose. As ALT levels were measured 5 days after dosing, it is possible that ALT elevations occurred acutely, but were resolved by 5 days later.

Polymer conjugate P2-AR2-6 was dosed intravenously to nonhuman primates at 3 mg/kg, with the efficacy determined by measuring the Apo lipoprotein B levels in the serum. As shown in Figure 7, there is 50−70% reduction in Apo lipoprotein levels with P2-AR2-6 conjugate, showing the efficacy of this polymer conjugate in nonhuman primates. The toxicity produced by these polymers is predominantly liver toxicity, so alanine aminotransferase (ALT) is used as a marker for liver toxicity. ALT levels in the serum rise following the loss of membrane integrity upon death of hepatocytes that normally contain intracellular ALT. The toxicity of these systems was assessed by measuring serum levels of ALT out to 32 days postdose. This polymer conjugate showed 8−10-fold increase in ALT on the 9th day which returns to normal on the 32nd day in nonhuman primates. Therefore, there is a concern with liver toxicity when using these polymer conjugates.

Radiolabeling Studies with PAA Polymer Conjugates. Rodent and nonhuman primate data showed that PAA conjugates (Figure 2B-II) are efficacious. As PAA polymer conjugates do not have a biodegradable disulfide bond in the backbone, it might be less biodegradable than PAADS polymer conjugates. To understand the biodegradability and biodistribution of PAA polymer conjugates, ^{14}C-radiolabeled P-2-AR2-6 (see SI for synthesis) was dosed in rats.

The 1 mg/kg dose of ^{14}C-radiolabeled P-2-AR2-6 polymer conjugate generated maximum Apo B mRNA knockdown in liver of approximately 85% 5 days postdose (data not shown). The high degree of activity was driven by the high concentration of polymer in the liver, with nearly 70% of the injected ^{14}C-polymer in the liver by 2 h postdose (Figure 8A). In contrast, approximately 3% of the injected ^{14}C-polymer was found in the kidneys and only 0.4% of the injected ^{14}C-polymer was found in the spleen, highlighting the fact that the P-2-AR2-6 polymer conjugate predominantly distributes into liver. It can also be seen from Figure 8A that the amount of ^{14}C-polymer in the liver continually decreases over time, with approximately 30% of injected polymer in the liver 5 days postdose and only 20% of injected polymer in the liver 14 days postdose. One likely explanation for the decrease in polymer-associated radioactivity in the liver is that the polymer is being excreted from the body. The levels of ^{14}C were determined in the urine and feces as shown in Figure 8B. It can be seen that roughly 7% of the injected ^{14}C-polymer was excreted in the urine within 24 h postdose, and very little additional polymer was eliminated in the urine over the next 6 days. The amount of ^{14}C-polymer in the feces, on the other hand, continually increased over time reaching a total of approximately 40% of the injected ^{14}C-polymer being cumulatively excreted in the feces. So, it appears that most of the polymer is distributed to target tissues within the first 24 h, with nearly 7% of the injected ^{14}C-polymer excreted in the urine during that period. It is likely that this fraction of the polymer initially excreted in the urine is intact polymer, which is consistent with other siRNA delivery polymers that have been found intact in the urine.[13] At 24 h postdose, there is approximately 3% of injected ^{14}C-polymer in the kidneys and that drops by less than 0.5% of injected dose over the next few days (data not shown), which is consistent with the less than 0.5% of injected ^{14}C-polymer that is excreted in the urine (after the first 24 h). It seems that there may be a very low level of continued excretion of intact polymer in the urine following the initial burst of renal elimination. Nearly 60% of the injected ^{14}C-polymer is still in the liver 24 h postdose, and the level drops to roughly 30% of the injected ^{14}C-polymer by 5 days postdose. The 30% of dose cleared from the liver almost perfectly matches the 30% of the injected ^{14}C-polymer excreted in the feces over the same time range, which seems to support a hypothesis of intact polymer eliminated from the hepatocytes into the bile and then into the feces. Last, the level of ^{14}C-polymer in the spleen remains fairly constant near 0.4% of injected ^{14}C-polymer over the two week span (data not shown) due to the fact that the spleen cannot directly expel material into the urine or the feces. If the polymer was degrading, it would be expected that small metabolites could be released from the spleen into blood circulation where they would be renally eliminated. Thus, it would be expected that if the polymer was degrading there would be loss of material from

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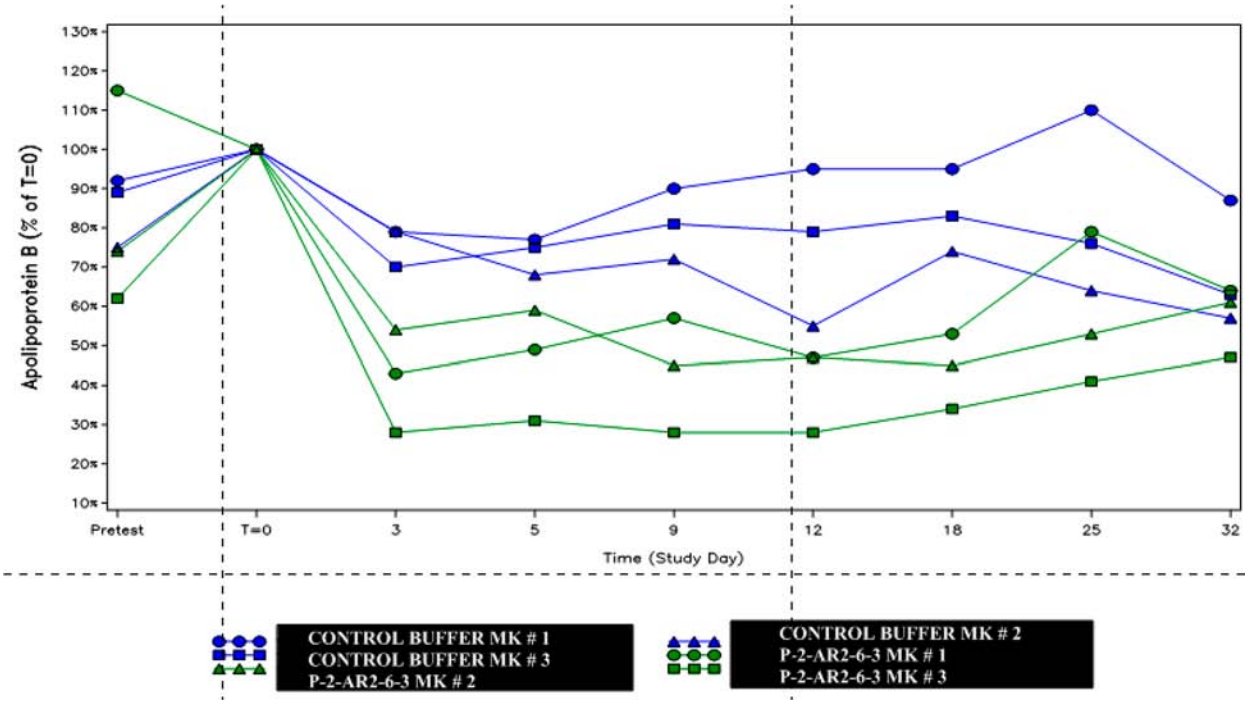

Figure 7. Apo lipoprotein reduction in nonhuman primates with polymer conjugate dosed at 3 mg/kg P2-AR2-6-3.

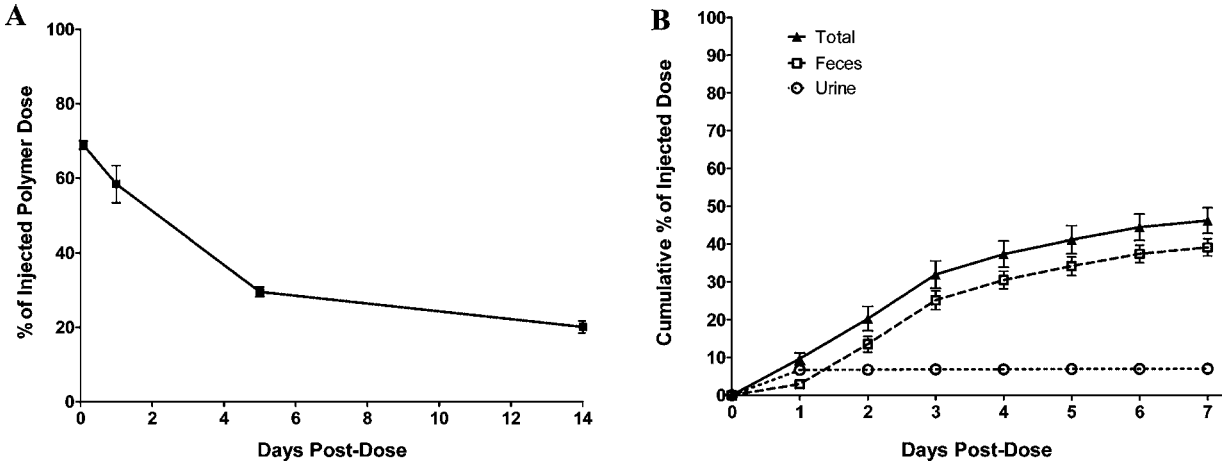

Figure 8. (A) The percent of injected ^{14}C-polymer dose in liver over time. (B) The cumulative percent of injected polymer dose excreted in the urine and feces over time. The total excretion of ^{14}C-P-2-AR2-6 polymer is also shown as the sum of excretion in the urine and feces. With n = 3 rats per group, the data are shown as mean ± SEM.

Table 4. PAA Polymers with Disulfide Incorporation

polymer #	aminoethoxy [bis-acrylamide]	cystamine [bis-acrylamide]	2-(2-aminoethoxy)ethyl [amine]	2-(1*H*-imidazol-4-yl)ethyl [imidazole]	dodecyl [dodecyl]	M_n = kDa (PDI)
Polymer 9	95	5	30	50	20	10.2 (2.6)
Polymer 10	90	10	30	50	20	11.4 (2.5)
Polymer 11	83	17	30	50	20	9.9 (2.6)
Polymer 12	76	24	30	50	20	11.6 (2.5)
Polymer 13	71	29	30	50	20	10.5 (2.6)
Polymer 14	67	33	30	50	20	15.0 (1.8)
Polymer 15	50	50	30	50	20	11.6 (2.0)

the spleen, faster loss of material from both liver and kidney, and much greater elimination in the urine. Taken together, the data seem to point toward the conclusion that the polymer is not degrading *in vivo*, and that significant amounts of the polymer remain in the body even 2 weeks postdose.

Introduction of Disulfide in PAA Polymer Backbone. PAA conjugates showed that increasing the amine density increased the siRNA liver levels as well as increasing mRNA knockdown of Apo lipoproteins. Concomitant to this an increase in liver enzyme ALT in monkeys was also observed.

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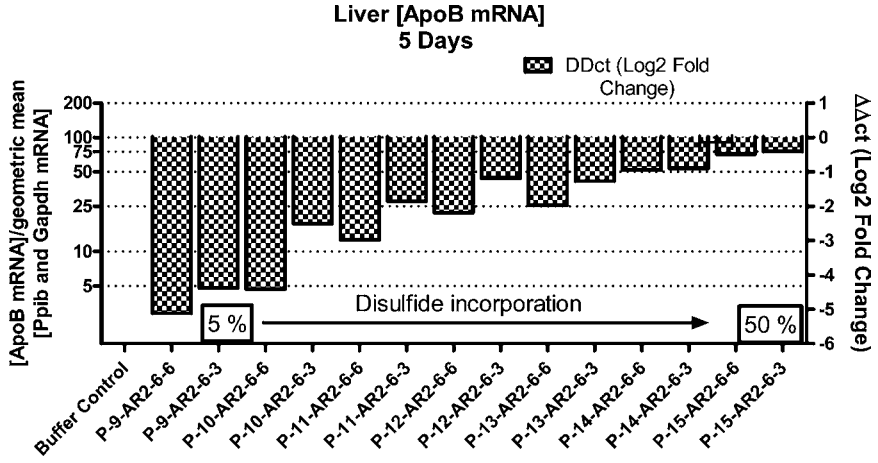

Figure 9. Mouse mRNA knockdown data of disulfide incorporated polymer conjugates.

Radiolabeling study also showed that this polymer is not biodegradable. Reduction in toxicity could be achieved by making this polymer conjugate more biodegradable by introduction of disulfide bonds in the polymer backbone. As shown in Table 4, seven polymers were synthesized by incorporating 5−50% of cystamine bis-acrylamide in lead polymer 2.

Polymer conjugates of polymers 9−15 were prepared as discussed previously at 6:1 polymer: siRNA ratio, masked with CDMs, and dosed at 6 mg/kg and 3 mg/kg in mice for mRNA knockdown study.

As shown in Figure 9, with the increase in disulfide contents in the polymer backbone, the decrease in mRNA knockdown was observed. Decrease in siRNA liver levels were also observed with increase in disulfide contents (Figure 10), suggesting that decreasing the amine contents effect the efficacy of polymer.

■ CONCLUSION

In conclusion, PAA polymer conjugates mediated very efficient delivery of siRNA to liver resulting in significant gene knockdown in mice, rats, and nonhuman primates. PAA polymers were prepared from novel amine incorporated bis-acrylamide that was designed to increase the amine density of previously synthesized[1] PAADS polymer, thereby increasing the targeting of polymer conjugate. Radiolabeling study shows that the PAA polymer is not degradable *in vivo*, and that significant amounts of the polymer remain in the body even 2 weeks postdose. Replacing this novel amine incorporated bis-acrylamide up to 50% with more biodegradable cystamine bis-acrylamide decreased the *in vivo* activity of polymer conjugate. Efforts to further improve the *in vivo* efficacy and margin for these PAA polymers by synthesizing novel amine and disulfide incorporated monomer will be reported in due course.

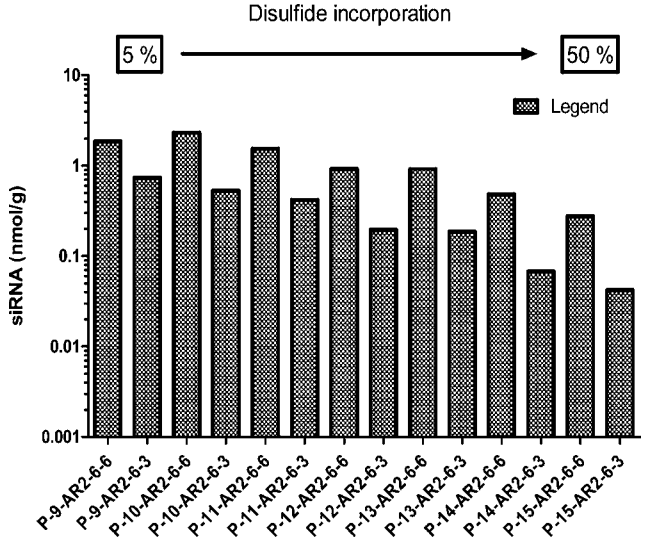

Figure 10. siRNA liver levels with disulfide incorporated polymer conjugates.

■ EXPERIMENTAL SECTION

Materials. Cystamine bis-acrylamide, *N*-(*tert*-butoxycarbonyl)-1,4-butanediamine, *N*-(*tert*-butoxycarbonyl)-1,6-hexanediamine, *N*-(*tert*-butoxycarbonyl)-1,8-octanediamine, butyl amine, dodecyl amine, trifluoroacetic acid (TFA), and triisopropylsilane (TIS) were purchased from Sigma. Carboxydimethylmaleic anhydride-poly(ethylene glycol) (CDM-PEG; MW= 680 g/mol) and carboxydimethylmaleic anhydride-*N*-acetylgalactosamine (CDM-GalNAc) were synthesized according to the literature procedure.[7] Novel amine incorporated bis-acrylamide monomer (*tert*-butyl (2-{[1,3-bis(prop-2-enoylamino)propan-2-yl]oxy}ethyl)carbamate) was synthesized in our laboratory using the procedure mentioned in the Supporting Information. Active siRNA1 used here is discussed in the literature.[7,10] Active siRNA2 for targeting apolipoprotein B is shown below:

Passenger Strand: [6amiL][iB][omeC][omeU][omeU]-[omeU][fluA][fluA][omeC][fluA][fluA][omeU][omeU]-[omeC][omeC][omeU][fluG][fluA][fluA][fluA][omeU]-[dTs]dT[iB].

Guide Strand: [rAs][rUs][rUs][omeU][omeC][fluA][fluG]-[fluG][fluA][fluA][omeU][omeU][fluG][fluU][omeUA]-[fluA][fluA][fluA][fluG][omeUs][omeU].

An siRNA with an irrelevant sequence was used as control (sequence shown below):

Passenger Strand: [6amiL][iB][fluC][fluU][dA][dG][fluC]-[fluU][dG][dG][dA][fluC][dA][fluC][dG][fluU][fluC][dG]-[dA][fluU][dA][dTs]dT[iB].

Guide Strand: UAU[fluC][omeG][omeA][fluC][omeG]-[fluU][omeG][fluU][fluC][fluC]A[omeG][fluC][fluU]-[omeA][omeG][omeUs][omeU].

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As used herein, ome = 2′ methoxy; flu = 2′ fluoro; click = 2′ propagyl; iB = inverted abasic; "s" subscript = phosphorothioate; and r = 2′ ribo; 6amil = *n*-hexylamino.

Polymer Synthesis. Poly(amido amine) "PAA" polymers were synthesized by polyaddition of primary amines to *tert*-butyl (2-{[1,3-bis(prop-2-enoylamino)propan-2-yl]oxy}ethyl)-carbamate (Figure 2A).

In a typical experiment, *tert*-butyl (2-{[1,3-bis(prop-2-enoylamino)propan-2-yl]oxy}ethyl)carbamate (1 equiv: 0.58 mmol, 200 mg), *tert*-butyl [2-(2-aminoethoxy)ethyl]carbamate (0.4 equiv: 0.23 mmol, 47.8 mg), histamine (0.3 equiv: 0.17 mmol, 19.5 mg), and dodecylamine (0.3 equiv: 0.17 mmol, 32.57 mg) were added to a reaction flask (Figure 1B). The solvent mixture (30% ethanol in water 1.2 mL; 0.5 M) was added to the reaction flask and the reaction mixture was degassed. Polymerization was carried out in the dark at 55 °C under nitrogen atmosphere. The reaction mixture became homogeneous in less than 15 min at 55 °C. The reaction was allowed to proceed for 5 days. Subsequently, 10−20 mol % of butyl amine in ethanol was added to the reaction mixture to consume any unreacted acrylamide groups. The resulting mixture was heated overnight (15−20 h) at 65 °C under N_2. The reaction mixture was cooled to room temperature and polymer solution was precipitated in 100 mL diethyl ether, centrifuged, decanted, and the residue flushed with N_2. The deprotection of Boc-amine was carried out by dissolving protected polymer in 2 mL of TFA/TIS (triisopropylsilane)/H_2O 95/2.5/2.5 solution for 30−60 min at ice−bath temperature. The reaction mixture was allowed to warm up to room temperature. The crude polymer was precipitated out again in 100 mL diethyl ether, centrifuged, decanted, and the residue flushed with N_2. The residue was dissolved in 10 mL DI water and the pH was adjusted to pH 7 with 1 N NaOH solution. The polymer solution was then transferred in to 2K dialysis bag and dialyzed with Milli-Q water for 24−48 h and then lyophilized.

^1H and ^{13}C NMR spectra were recorded on Varian spectrometer operating at 500 MHz. ^1H NMR spectra were in full accordance with the expected structures. No signals were present in the region between 5 and 7 ppm, corresponding to the acrylamide group, indicating that these polymers have capped end groups. Feed ratios of different monomers in the polymers were confirmed by NMR. All NMR spectra were taken in deuterated methanol.

All these PAA polymers contain 30−40 primary amines (for masking) per polymer chain, whereas PAADS polymers contain around 8−10 amines per polymer chain. Number of amines is calculated using molecular weight of polymer and monomer incorporation ratios of amine:imidazole:dodecyl obtained by NMR integration.

Polymer Conjugate Synthesis. Amine terminated siRNA (1 g, 0.07 mmol) was dissolved in 0.1 M sodium bicarbonate buffer (20 mL, 50 mg/mL) in a separate 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, 0.35 mmol, 5 equiv) was dissolved in 0.8 mL of DMSO. The SATA solution was added over 1 min and the clear, colorless reaction mixture was stirred at 0−5 °C. After 2 h, the reaction mixture was sampled and analyzed by UPLC or HPLC (Dionex DNApac) for completion of the reaction. Additional SATA can be added to effect complete conversion of the oligonucleotide (<5% remaining unreacted). The reaction mixture was purified by tangential flow filtration (TFF) using water (∼2 L). The retentate was lyophilized to give a white solid. The recovery was ∼95% and the purity was greater than 70% by UPLC.

PAA polymer (64 mg, 95 wt % purity) was dissolved in 2.1 mL of 100 mM TRIS buffer. The solution was mixed until the polymer was completely dissolved and 96 μL of a solution of 4-succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio) toluene (SMPT) in DMSO (1 mg/100 μL) was added (corresponding to 1.5 wt % with respect to the polymer weight). The activated polymer was diluted with 23 mL of 100 mM TRIS, 5% glucose buffer at pH 9, resulting in a final polymer concentration of ∼2.5 mg/mL. Next, 10.73 mg of SATA modified oligonucleotide was added to the activated polymer solution and allowed to react at room temperature for 1 h until the final masking step. *In situ*, the primary amine on the polymer is assumed to deprotect the SATA modified siRNA to produce the free thiol siRNA, which can then react with the SMPT-modified polymer. In a separate vial, 148 mg (311 μmol) of carboxydimethylmaleic anhydride-*N*-acetylgalactosamine (CDM-GalNAc) and 76.5 mg (108 μmol) of carboxydimethylmaleic anhydride poly(ethylene glycol) (CDM-PEG) were weighed out. The siRNA−polymer conjugate solution was then transferred into the vial containing CDM-GalNAc and CDM-PEG and the resulting solution was stirred at room temperature for 10 min. The final pH of the polymer conjugate solution was 8.5 (see the Supporting Information for polymer conjugate preparation scheme). The diafiltration mode of TFF was used to purify polymer conjugate formulations of unincorporated components and to exchange buffer to a pharmaceutically acceptable formulation vehicle. The TFF filter material was made of regenerated cellulose and the selection of molecular weight cutoff for these membranes was done with efficiency of purification and retention of polymer conjugate in mind. The processing parameters included feed pressure, retentate pressure, crossflow rate, and filtrate flux were set to allow reproducibility from batch to batch and linear scaling of the process. 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.

Red Blood Cell Hemolysis Analysis. Human blood was collected in 10 mL EDTA Vacutainer tubes. A small aliquot was assessed for evidence of hemolysis by centrifugation at 15000 RCF for 2 min and nonhemolyzed samples were carried forward into the assay. Red blood cells (RBCs) were washed three times in either 150 mM sodium chloride (NaCl)/20 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 5.4, or 150 mM NaCl/20 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), pH 7.5 by centrifuging at 1700*g* for 3 min and resuspending in the same buffer to yield a 10× dilution. RBCs were then diluted in appropriate pH buffer to yield approximately 108 cells in suspension. A 10× stock concentration of each test agent was prepared and a 10 point, 2-fold dilution was performed in appropriate pH buffers. Diluted test agents were added to the RBCs in appropriate pH buffers in Costar 3797 round-bottom 96 well plates. Solutions were mixed 6 to 8 times and the microtiter plate was covered with a low evaporation lid and incubated in a 37 °C warm room or incubator for 30 min to induce hemolysis. The plate was then centrifuged at 1700*g* for 5 min and 150 μL supernatants were transferred to a Costar 3632 clear bottom 96 well plate. Hemoglobin absorbance was read at 541 nm using a Tecan Safire plate reader and percent hemolysis was calculated assuming 100% lysis to be measured by the hemoglobin released by RBCs in 1% Triton X-100.

***In Vitro* HepG2 Gene Silencing and Toxicity (bDNA/MTS Assay).** HepG2 cells were plated in 96-well microtiter plates at 6000 cells/well and incubated overnight at 37 °C to allow cell adherence. A 10 times stock of PCs (polymer conjugates) were prepared in media and 20 μL 10× PC was added to 180 μL media already in wells resulting in 1× final treatment and a 300−0 nM 10-point half log titration, based on siRNA concentration. Cells were incubated with PCs in 37 °C CO_2 incubator for 24−72 h. MTS Toxicity Assay was performed on 24−72 h treated cells and cytotoxicity was assessed by Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega #G3581, Madison, WI). 40 μL MTS Solution was added incubated in 37 °C CO_2 incubator 1 h, absorbance at 490 nm was read on Tecan Safire. Cells were then washed 3× in PBS and 150 μL/well bDNA DLM Lysis Buffer (Panomics "Quantigene" 1.0 bDNA kit #QG0002, Fremont, CA) was added. The plate was then incubated at 37 °C in warm room for 30 min. Lysates were removed and frozen at −70 °C overnight. The next day, all cell lysates were thawed at RT and 20 μL of each lysate was removed and used for determination of total protein using a Micro BCA Protein Assay kit (Pierce #23235, through Thermo Scientific, Rockford, IL).

Absorbance was measured on a Tecan Safire: Wavelength = 562 nM, Plate = Costar96 ft. Number of Reads = 100, Time between Reads = 5. 50 μL of each lysate was also used to determine mRNA expression levels in cells treated with ApoB siRNA.

ApoB mRNA knockdown was determined using a Quantigene 1.0 bDNA Assay (Panomics # QG0002 Lot # 51CW36, Fremont, CA), a kit designed to quantitate RNA using a set of targetspecific oligonucleotide probes.

***In Vivo* Studies.** All animal studies were conducted following protocols in accordance with the Institutional Animal Care and Use Committee (IACUC) at NIRC and Merck, which adhere to the regulations outlined in the USDA Animal Welfare Act. For studies in mice, CD1 animals (n = 4, each group) were tail vein injected with polymer conjugates at a dose of 3 mg/kg in a volume of 0.2 mL, 10 mM TAPS 5% glucose buffer, pH 9, vehicle. Mice were sacrificed 48 h postdose and 3 mm liver tissue biopsies were immediately preserved in RNALater (Ambion). For evaluation in rats, female Sprague−Dawley were tail vein injected with polymer conjugates at doses of 1, 3, or 6 mg/kg in 2.0 mL vehicle, and blood and liver tissues were collected 5 d postdose, as described above. Preserved rodent 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 reverse transcription polymerase chain reaction (RT-PCR). Message was amplified from purified RNA utilizing primers against the mouse ApoB mRNA (Applied Biosystems Cat. No. Mm01545156_m1). A PCR reaction was run on an ABI 7500 instrument with a 96-well Fast Block. ApoB mRNA level is normalized to the housekeeping PPIB mRNA and GAPDH. PPIB and GAPDH mRNA levels were determined by RT-PCR using a commercial probe set (Applied Biosytems Cat. No. Mm00478295_m1 and Mm4352339E_m1). For studies in the nonhuman primates, rhesus monkeys were dosed via a timed intravenous infusion (12 mL/min) into the saphenous vein. At indicated time points, 2 mL blood samples were collected from alert animals into serum separator vacutainers and processed for quantification of ApoB protein and ALT. Apo B was measured using Roche Tina-quant Apolipoprotein B immunoturbidometric assay, Roche Modular Analyzer (Roche Diagnostics Corporation, Indianapolis, IN). Anti-apolipoprotein B antibodies react with the antigen in the sample to form antigen/antibody complexes which, following agglutination, are measured turbidimetrically. ALT was measured using Roche ALT IFCC without pyridoxal phosphate activation (Roche Diagnostics Corporation, Indianapolis, IN). NADH is oxidized to NAD+. The rate of the photometrically determined NADH decrease is directly proportional to the rate of formation of pyruvate and thus the ALT activity.

Additional methods are available in Supporting Information.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Monomer synthesis; ^{1}H NMR spectrum of polymer; gel permeation chromatography of polymer; preparation of polymer conjugates; polymer conjugate analysis; radiolabeled polymer synthesis, analysis, and purification. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.

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