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Triplex Hybridization of siRNA with Bifacial Glycopolymer Nucleic Acid Enables Hepatocyte-Targeted Silencing

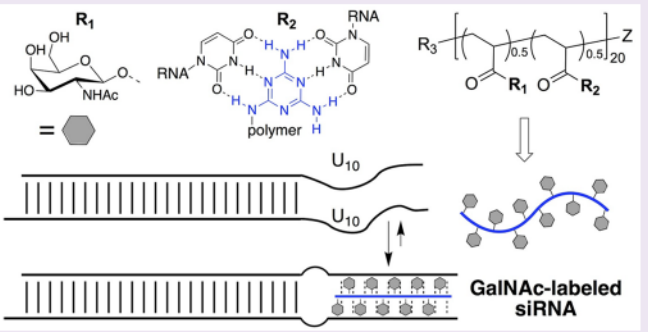
Xin Xia,[‡,§] Zhun Zhou,[†,§] Chris DeSantis,[†] John J. Rossi,[‡] and Dennis Bong*[,†]

[†]Department of Chemistry and Biochemistry, The Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210, United States

[‡]Department of Molecular and Cellular Biology, Beckman Research Institute of City of Hope, Duarte, California 91010, United States

Supporting Information

ABSTRACT: Herein, we describe a versatile non-covalent strategy for packaging nucleic acid cargo with targeting modalities, based on triplex hybridization of oligo-uridylate RNA with bifacial polymer nucleic acid (bP$_o$NA). Polyacrylate bP$_o$NA was prepared and side chain-functionalized with *N*-acetylgalactosamine (GalNAc), which is known to enable delivery to hepatocytes and liver *via* binding to the asialoglycoprotein receptor (ASGPR). Polymer binding resulted in successful delivery of both native and synthetically modified siRNAs to HepG2 cells in culture, yielding in low nanomolar IC$_{50}$ silencing of the endogenous ApoB target, in line with observations of expected Dicer processing of the polymer−siRNA targeting complex. Indeed, *in vitro* Dicer treatment of the polymer complex indicated that triplex hybridization does not impede RNA processing and release from the polymer. The complex itself elicited a quiescent immunostimulation profile relative to free RNA in a cytokine screen, setting the stage for a preliminary *in vivo* study in a high-calorie-diet mouse model. Gratifyingly, we observed significant ApoB silencing in a preliminary animal study, validating bP$_o$NA as an *in vivo* carrier platform for systemic siRNA delivery. Thus, this new siRNA carrier platform exhibits generally useful function and is accessible through scalable synthesis. In addition to its utility as a carrier, the triplex-hybridizing synthetic platform could be useful for optimization screens of siRNA sequences using the identical polymer carriers, thus alleviating the need for covalent ligand modification of each RNA substrate.



Triplex hybridization of two oligothymidylate (T) or oligouridylate (U) domains with one strand of a melamine-displaying macromolecule appears to be largely backbone independent.[1−5] We have previously designed and characterized a family of melamine-displaying peptides generally called bifacial peptide nucleic acids (bPNAs) and found that oligo-T/U targeting can be accomplished with non-native backbones, including polyacrylates, which we denote bifacial *polymer* nucleic acid (bP$_o$NA). This family of triplex-forming molecules relies on the artificial melamine base to bind two equivalents of thymine or uracil (T/U) to form a base triple,[6] which integrates well within duplex structures containing a TT or UU mismatch site.[7] The ability to triplex hybridize with unstructured oligo T/U domains enables bPNAs to provide a functional counterpoint to other types of PNA backbones[8−10] and nucleic acid mimics that use artificial bases[11−14] that target the duplex hybridization interface and pre-formed duplex structures. Thus, triplex hybridization can elicit structure−function turn-on[15] while also targeting ligand placement to single-stranded oligo T/U domains that may be either terminal or internal within the nucleic acid fold. Herein, we have placed oligo-U domains at the 3′ and 5′ termini of an siRNA duplex and exploited triplex hybridization with bifacial polymer nucleic acid to non-covalently install liver-targeting GalNAc moieties along the hybrid stem. The highly heterogeneous polyacrylate backbones displaying melamine can triplex hybridize efficiently with native bases[16] and nucleic acids and could thus help bridge native and artificial architectures, uniting nucleic acid biotechnology[17] with new materials.[18] Polyacrylate analogues of nucleic acids[19,20] and other backbones formed through uncontrolled polymerization have been reported,[21−23] including polyester, polyvinyl, polyamide, and others,[24] though nucleic acid hybridization with all carbon backbones is generally inefficient.[25,26] In contrast, bifacial polymer nucleic acid (bP$_o$NA), derived from low polydispersity polyacrylates *via* controlled radical polymerization (RAFT),[27] can engage cooperatively with oligo-T/U tracts with good thermal stability and nanomolar range affinity *via* a biomimetic triazine,[28,29] base-triple interface[3] that forms on mixing, compatible with

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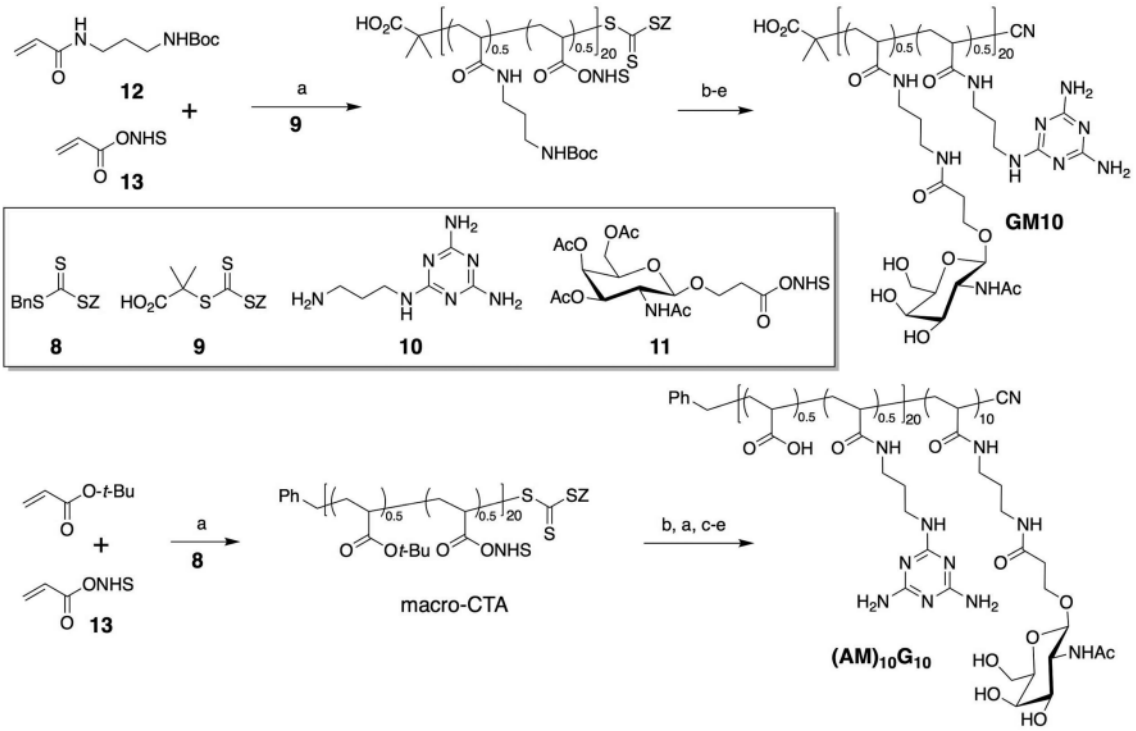
Scheme 1. Synthetic Approach to GalNAc-bP$_o$NAs[a]



[a](Top) RAFT polymerization of monomers **12** and **13** with chain transfer agent (CTA) **9** (Z = $C_{12}H_{25}$) produces base polymer for GM10 with PDI = 1.10. (Bottom) Diblock bP$_o$NA $(AM)_{10}G_{10}$ synthesis uses acrylates shown with CTA **8** (Z = $CH_2CH_2CO_2H$) to produce macro-CTA intermediate (PDI = 1.09), which is then reacted further to produce diblock base polymer (PDI = 1.19). Reagents and conditions: (a) AIBN, DMF, 70 °C; (b) **10**, Et_3N, DMSO; (c) TFA; (d) **11**, Et_3N, DMSO; (e) NaOMe, MeOH.

both polymer nanoparticle assembly as well as native RNA folding and function.[3] It is likely that the native-like hybridization of these polyacrylates with DNA and RNA derives largely from advances in controlled radical polymerization methods such as RAFT, which produces polyacrylates with narrow polydispersity. However, uncontrolled backbone stereo- and regiochemistry in the polymerization theoretically yields millions of backbone variants for a 20mer synthesis. Despite this considerable heterogeneity, triazine-base recognition enables functional integration of synthetic polymer architectures with DNA and RNA, provided the backbone is separated from the base triple with a linker of sufficient length (~3 sp^3-hybridized atoms) connecting the acrylamide carbonyl with triazine.[3] We have previously demonstrated that triplex hybridization of siRNA duplexes with bifacial polymer nucleic acid can be used to display cell-surface targeting sterol ligands that enabled a significant 40% luciferase silencing in a HeLa cell line engineered to express luciferase, similar to that observed with covalent siRNA lipidation.[30] Polymer carriers[32] for nucleic acid delivery[33,34] have been the subject of intense study for many years, and glycosylation has been shown to effectively direct DNA[35−37] and RNA[31] cargo to liver and hepatocytes. We hypothesized that triplex hybridization of glycosylated bP$_o$NA polymer with siRNA would allow nucleic acid loading and liver targeting functions to be integrated into a single, net neutral polymer platform, without covalent nucleic acid modification[38,39] or the use of cationic components.[33,40] This would afford greater control over charge-tuning of the delivery platform, with the option of introducing cationic sites as necessary.[33,41] Indeed, effective luciferase silencing by cholesterol-bP$_o$NA siRNA complexes in HeLa cells, in addition to the considerable literature precedent for GalNAc targeting, augured well for GalNAc-bP$_o$NA as a hepatocyte delivery vehicle for siRNA.[3] Herein, we describe siRNA silencing of the endogenous target ApoB in human hepatocytes (HepG2) using an RNA delivery platform derived from bP$_o$NA densely functionalized with *N*-acetylgalactosamine (GalNAc), an established ligand for liver and liver cell targeting.[31] We set out to test the extent to which non-covalent ligand display on siRNA duplexes could match RNA systems covalently modified with targeting ligands. In particular, we were interested to test this approach in a non-engineered mammalian cell line against a native target. Alnylam Pharmaceuticals has demonstrated that mature siRNA duplexes covalently modified on the sense strand with a triantennary display of *N*-acetylgalactosamine (GalNAc) exhibit low nanomolar IC_{50} for siRNA silencing of ApoB.[42] ApoB is a major protein component of low-density lipoprotein (LDL), natively expressed in the liver and hepatocyte-derived cells in culture, and considered to be a target for therapeutic silencing in heart disease. Delivery of siRNA to hepatocytes and systemic delivery to the liver is efficiently mediated by GalNAc targeting of the asialoglycoprotein receptor (ASGPR), which is highly expressed on the cell surface.[31] In the work described herein, we applied our bP$_o$NA hybridization strategy to GalNAc targeting of ApoB in a hepatocyte-derived cell line (HepG2) and a preliminary animal study using heart disease mouse model in which ApoB levels are elevated. These results are encouraging for future expanded studies using the bP$_o$NA platform for *in vivo* siRNA delivery.

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Table 1. ApoB100-Targeting siRNAs[a]

siRNA	sense/antisense	ApoB100-targeting sequence
U10	(S)	5′-*GGAAUCUUAUAUUUGAUCCAAAUACCUUUUUUUUUU-3′
	(AS)	5′-UUUUUUUUUUCCUAUUUGGACRCAAAUAUAAGAUUCCCU-3′
U10-Mod2	(S)	5′-*GgAaUcUuAUaUuUgAuCCAAAUAccuuuuuuuuTT-3′
	(AS)	5′-uuuuuuuuuu ccUAUUUGGAuCaAaUAuAaGaUuCccu-3′
J-turn	(S)	5′-*GGAAUCUUAUAUUUGAUCCAACC-$(U)_{10}(C)_{10}(U)_{10}$-3′
	(AS)	5′-UUGGAUCAAAUAUAAGAUUCCCU-3′

[a]Oligonucleotide domains used in bP$_o$NA binding are shown in bold. Annotations indicating modified nucleotides (N) are as follows: *N = 5′ phosphate; *n* = 2′-methoxy; *N* = deoxyribose.

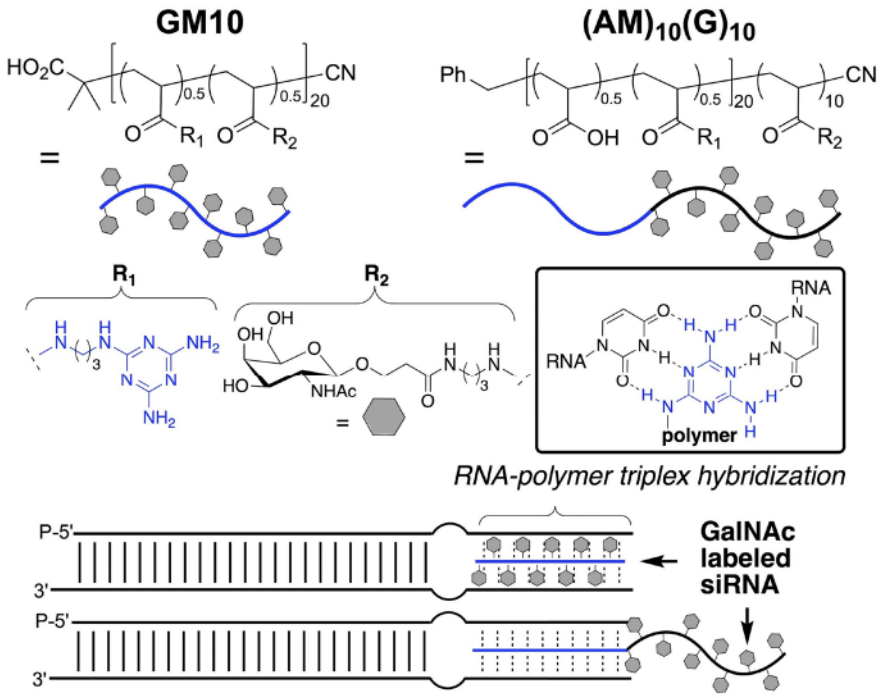

Figure 1. (Top) Structure of mixed block and diblock bifacial polymer nucleic acids, GM10 and $(AM)_{10}G10$. Melamine base (R_1), hepatocyte-targeting GalNAc ligand (R_2), and base-triple motif (boxed) are indicated. (Bottom) Triplex hybridization of siRNA duplexes with the polyacrylates results in non-covalent labeling with GalNAc.

RESULTS AND DISCUSSION

Design and Synthesis of Liver-Targeted bP$_o$NA siRNA Carriers. Prior work from Alnylam indicated the importance of presenting GalNAc on the siRNA duplex in a cluster of at least three. We set out to test two polymer scaffolds that presented GalNAc on monomer side chains either in a polymer block fused to the triplex-forming polyacrylate domain in a "diblock" format, or integrated with the triplex domain, in a random co-monomer format. In both designs, approximately 8−10 GalNAc moieties are incorporated along the polymer backbone, with the diblock format utilizing a denser presentation. These polymer architectures were accessed using standard RAFT polymerization methods and post-polymerization modification (Scheme 1).

Synthesis of a GalNAc-displaying bP$_o$NA carriers was accomplished using commercially available and readily prepared monomers *tert*-butyl acrylate, Boc-protected amino-propyl acrylamide **12**, and *N*-hydroxysuccinimide (NHS) ester acrylate **13** (Scheme 1). Additionally, trithiocarbonyl chain transfer agents (CTAs) **8** and **9** were prepared according to literature procedures.[27,43] The similar reactivity of these monomers allowed convenient preparation of the random mixed block base polymer through RAFT co-polymerization of NHS-ester acrylate and Boc-protected acrylamide at 1:1 stoichiometry using CTA **9**, reaching degree of polymerization (DP) ∼20 with reasonably low polydispersity (PDI = 1.10). CTA **9** was chosen for its dodecyl chain Z-group, which facilitates polymer precipitation and purification. This hydrophobic base polymer was prepared on multigram scale, purified *via* precipitation, and analyzed by ^{1}H NMR and GPC to assess DP and PDI (Supporting Information) prior to further functionalization to enable use of organic solvents and polymer standards in GPC and NMR analysis. Similarly, the diblock base polymer was prepared by RAFT polymerization using CTA **8** with *tert*-butyl acrylate and NHS-acrylate **13** to produce a 20mer mixed block (PDI = 1.09); the choice of CTA **8** is primarily one of convenience, as the diblock base polymer can be efficiently precipitated without additional hydrophobicity to purify the macro-CTA from unreacted monomers (Scheme 1). The macro-CTA retains a reactive end group for RAFT polymerization, enabling growth of the second 8−10mer block using monomer **12** from the macro-CTA

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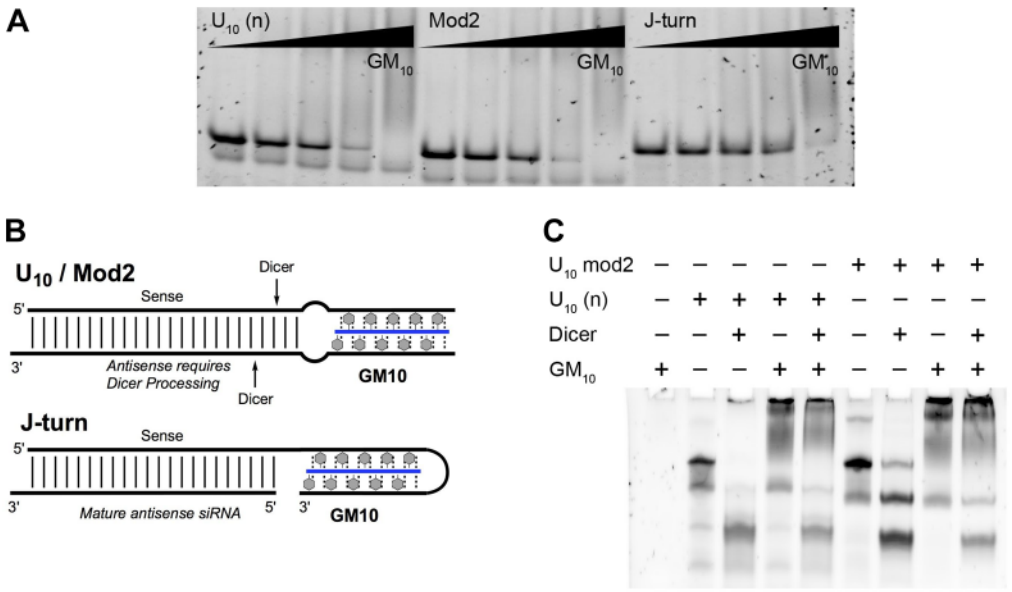

Figure 2. (A) Native EMSA of siRNAs as indicated at 50 nM in each lane with increasing quantity of bP$_o$NA GM10 (0, 37.5, 75, 150, and 300 nM). Polymer complexation reduces staining efficiency. (B) Illustration of the expected bP$_o$NA complexes formed with the indicated siRNA duplexes, with expected Dicer cleavage sites. (C) Denaturing gels of siRNAs complexed with GM10 and treated with Dicer, indicating release of processed siRNA from the complex. Gels were stained with SYBR-gold.

intermediate. The resulting fully protected, hydrophobic diblock base polymer (PDI = 1.14) was then characterized by ^{1}H NMR and GPC; like the mixed block and the macro-CTA, a low PDI was observed as expected for a controlled polymerization (Supporting Information). Following polymerization, both base polymers were reacted to completion at their NHS-ester sites with **10** to install the melamine side chain. Acid cleavage of the polymer Boc-protected amines from **12** (concomitant with the *tert*-butyl ester of the diblock base polymer) yielded polyamine salts that were acylated with **11**, an NHS-ester derivative of GalNAc.[42] This post-polymerization modification procedure furnished both mixed block and diblock polymers that we denoted GM10 and (AM)$_{10}$G10, respectively, that feature acrylamide-displayed GalNAc targeting ligand (G), melamine (M), and acrylic acid (A) in the diblock glycopolymer.

Evaluation of siRNA-bP$_o$NA Complex Processing and Silencing in HepG2 Cells. We designed an ApoB siRNA duplex we called U10 (Table 1) with an siRNA duplex region targeted to ApoB100 mRNA and blunt-ended 3′ (sense) and 5′ (antisense) U$_{10}$ tracts. The sequence U10-Mod2, which incorporates many synthetic modifications known to enhance *in vivo* stability and reduce immunogenicity,[42] was also studied. As the modified domains are nuclease-resistant, native nucleotides were used at the predicted Dicer cleavage site to facilitate processing. Both mixed block GM10 and diblock (AM)$_{10}$G10 glycopolymers were designed to triplex hybridize with the unstructured U$_{10}$ RNA tails in these duplexes (Figure 1). Notably, both polymer-siRNA complexes require intracellular Dicer processing to generate mature siRNA. Additionally, we designed an siRNA duplex with mature ApoB100 antisense RNA (not requiring Dicer processing) hybridized with a sense strand with a 32 nt overhang at the 3′ end containing a U$_{10}$C$_{10}$U$_{10}$ sequence that is known to form a hairpin triplex hybrid with bP$_o$NA,[3] which we called J-turn siRNA (Table 1, Figure 2). Polymer complexation of these siRNAs by GM10 was evaluated *via* gel, which revealed GM10 concentration dependent RNA binding (Figure 2) as judged by disappearance of the siRNA band; these soluble complexes did not detectably scatter light in DLS measurements, suggesting the absence of further assembly into particles. As previously observed, triplex hybridization reduces the efficacy of RNA staining due to dye displacement resulting in loss of the band. By this measure, the siRNAs were completely complexed by the uncharged polyacrylate GM10 at 3:1 mol ratio.

Release of RNA from the polymer complex was expected to be facilitated by Dicer processing, which was designed to cleave mature siRNA from the triplex hybrid domain. Additionally, it has been reported that Dicer processing can facilitate loading of the RISC complex, resulting in increased silencing efficiency;[44] however, it was unclear whether the polymer complex would interfere with processing as prior studies had shown bPNA triplex hybrids to block the action of processive enzymes such as exonuclease.[45] It was confirmed that bP$_o$NA polymer complexation to siRNA duplexes also blocks exo-T degradation (Supporting Information), leaving open the question of siRNA susceptibility to endonucleases such as Dicer. To test the extent to which the polymer complex would be accepted as a substrate, we performed an *in vitro* Dicer processing assay on GM10 complexes with U10 and U10-Mod2 (Figure 2). Interestingly, though the bP$_o$NA-RNA complex does not migrate cleanly as a single band, presumably due to backbone heterogeneity and decreased negative charge density, Dicer treatment releases the expected RNA fragments from the complex. Overall, the GM10 complexes with both native and modified RNA were effectively processed to release mature siRNA.

With processing assured, we treated HepG2 cells in culture with bP$_o$NA polymer at increasing levels to evaluate potential cellular toxicity, focusing on the simplest glycopolymer, GM10; indeed, cell viability was maintained up to the micromolar GM10 polymer concentration regime while HepG2 cells exhibited more significant sensitivity to lipofectamine (Figure

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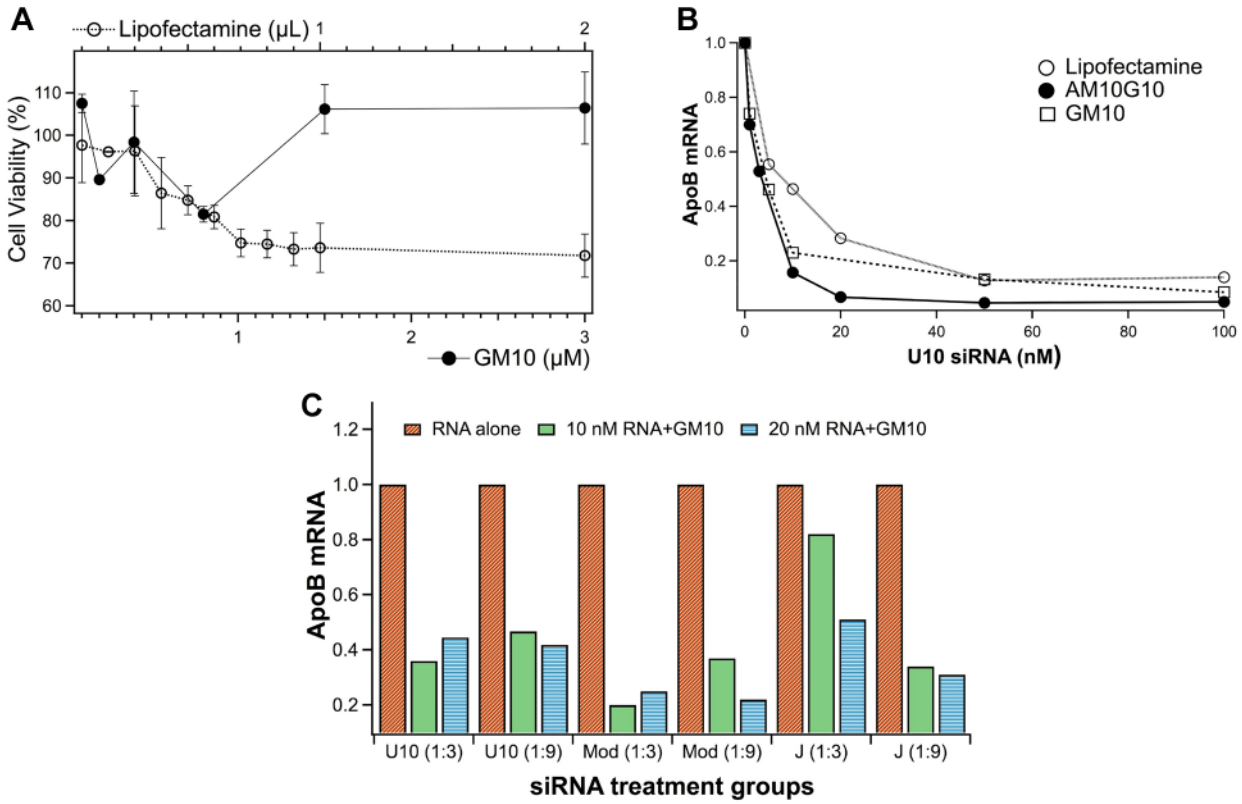

Figure 3. (A) HepG2 cell viability on treatment with bP$_o$NA GM10 or lipofectamine solution, as determined by MTS assay. (B) Representative ApoB silencing data from HepG2 cells using U10 siRNA and the carriers indicated in buffer, normalized against housekeeping gene GADPH. Approximate IC_{50} values for both AM10G10 and GM10 are ∼5 nM. Additional data are available in the Supporting Information. (C) ApoB silencing in HepG2 cells in full media (with FBS), treated with 10 and 20 nM siRNAs (U10, Mod U10, and J-turn) and GM10 mole ratio as indicated.

3A). This cell viability guided design of dose-dependent ApoB100 silencing studies in HepG2 cells with GM10 and $(AM)_{10}G10$ complexes with siRNA, carried out in buffer without FBS (Figure 3B). ApoB knockdown was assessed by RT-PCR as previously reported. Gratifyingly, an efficient knockdown was observed with both glycopolymer hybrid Dicer substrates, with an IC_{50} of ∼5 nM (Figure 3B), with no silencing observed in the absence of polymer carrier. These silencing efficiencies against a native target in a non-engineered human cell line validate the use of bifacial polymer nucleic acids as siRNA carriers and are in line with the low- to sub-nanomolar IC_{50} results from covalent modification previously reported for siRNA duplexes modified with triantennary GalNAc on the sense strand.[42] Silencing of ApoB in hepatocytes was further tested in full media to evaluate the tolerance of this bP$_o$NA carrier strategy under more general conditions, focusing on the simplest carrier to prepare, GM10. Based on the initial silencing parameters, two siRNA concentrations were studied (10 and 20 nM) at two different ratios of siRNA to GM10 polymer carrier (1:3 and 1:9) with each of the three siRNA scaffolds—U10, U10-Mod2, and J-turn (Figure 3C). All conditions resulted in silencing, demonstrating compatibility of the carrier with full media conditions, while siRNA alone did induce silencing as previously observed. Notably, all siRNA complexes, including J-turn, gave similar results, with modest differences between the two ratios. Given efficacy of the cell-based silencing, we focused our studies on the U10 and U10-Mod2 system with the GM10 carrier.

To bolster the functional profile of the GM10 carrier found with hepatocytes in culture, we assessed immunogenic response of the polymer-bound siRNA complex. Donor samples of human primary blood monocyte cells (PBMCs) were collected for evaluation of IFN-α and IFN-γ cytokine response to challenge from GM10 polymer and siRNA complexes, using standard ELISA assays. A strong cytokine response was observed with unmodified RNA complexed to lipofectamine for both IFN-α and IFN-γ assays, indicating typical assay sensitivity (Figure 4). While the substitution of modified RNA for native in the lipofectamine complex resulted in a greatly diminished IFN-α response as expected,[46] a significant IFN-γ response remained for this sample. In contrast, GM10-siRNA complexes did not elicit significant cytokine response for either the native or modified siRNA, even with increasing polymer/siRNA dose. The weak cytokine response to GM10 polymer complexed RNA relative to lipofectamine complexed RNA is suggestive of the benefits of using a neutral charge carrier rather than a cationic lipid carrier. A muted response was observed for both lipofectamine and GM10 polymer carriers, as well as both native and unmodified RNA alone. Notably, the lack of immunogenic response to GM10 alone and GM10-RNA complexes with both native and modified siRNA is supportive of a favorable *in vivo* carrier profile and sets the stage for a preliminary proof-of-concept animal study.

Preliminary animal studies were carried out to examine ApoB silencing in normal diet, healthy mice *via* IV injection of siRNA/polymer complexes. These treatment groups, with

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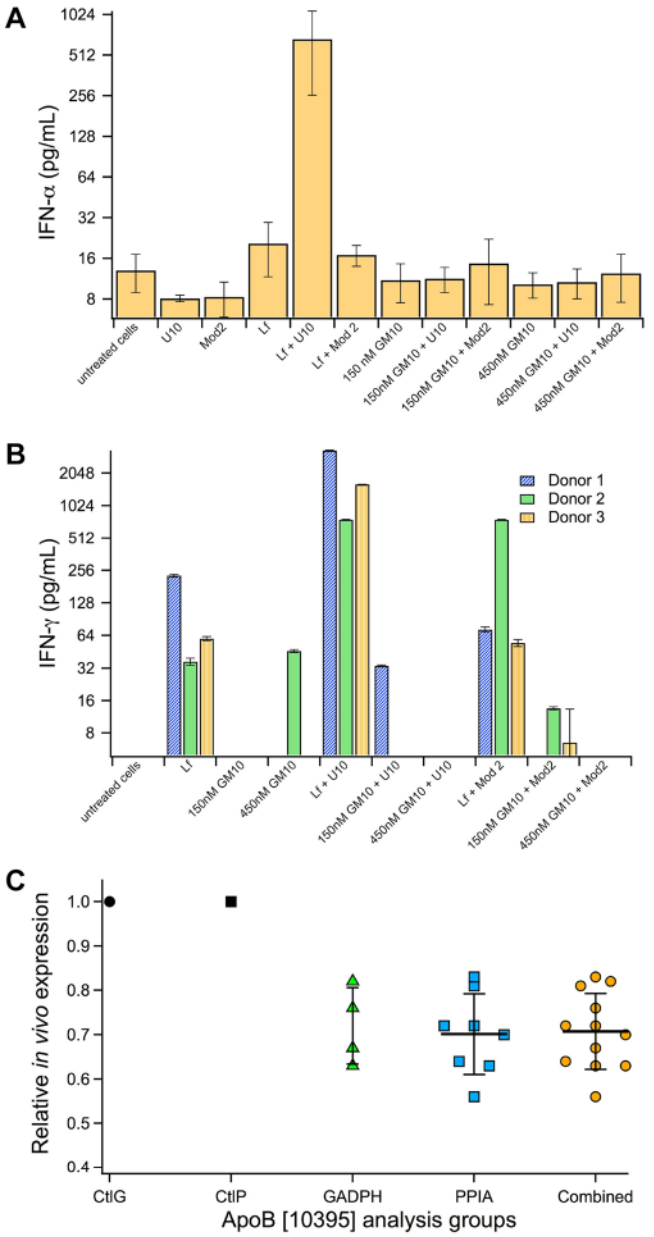

Figure 4. (A, B) Cytokine production from human peripheral blood monocytes (PBMCs) as stimulated by lipofectamine (Lf), polymer (GM10), siRNA (U10, U10 Mod2), and combinations of carrier and RNA as indicated. siRNA concentration is constant at 50 nM. Cytokines measured by ELISA targeting (A) IFN-α and (B) IFN-δ, with variation in donor responses indicated in (B). (C) *In vivo* ApoB silencing data from a limited mouse study using a single gene locus (ApoB 10395) and two housekeeping genes (GADPH and PPIA) to normalize data, as indicated separately and combined. Each data point represents the average of triplicated normalized data from a single mouse, with the standard deviation of each group indicated.

native U10 siRNA complexed with GM10, as well as a 1 mg/kg dosage trial using U10-Mod2 complexed with GM10 did not result in detectable *in vivo* silencing of ApoB100 following analysis of liver samples (data not shown). We hypothesized that an increase in dosage of the more stable, synthetically modified siRNA (Mod2), in an ApoB-focused animal model would yield better results for our proof-of-principle study. We thus designed a limited trial with mice using a set of C57BL/6J male mice conditioned on a high fat diet to provide a heart disease model with elevated ApoB levels.[47−49] Further, we switched from IV to subcutaneous injection, which has been broadly successful for other GalNAc platforms.[31] Gratifyingly, successful silencing was observed in an *in vivo* study with 5 mg/kg subcutaneous dosing, repeated each day for 5 days, followed by euthanasia and liver harvest. Three liver tissue samples from each animal were used to obtain total RNA for RT-qPCR normalized using housekeeping genes GADPH or PPIA (Figure 4). Notably, this limited animal study revealed a significant ApoB silencing of ~30% (Figure 4). While the *in vivo* silencing is moderate, this experiment validates the GalNAc-decorated bifacial polymer nucleic acid GM10 scaffold as a viable siRNA delivery platform. Silencing optimization through dose and treatment schedule variation as well as examination of gender as a variable will be pursued in a larger, follow-up animal study. However, it is clear from these preliminary findings that the siRNA-GM10 complex survives systemic *in vivo* delivery *via* subcutaneous injection and can penetrate liver tissue effectively to significantly silence an endogenous target in an animal model.

Conclusions. Overall, these results provide a straightforward synthetic protocol to prepare GalNAc-displaying bP$_o$NA scaffolds for targeting siRNA to hepatocytes in culture and the liver *in vivo*. While both mixed block and diblock polymer designs appear to be effective in eliciting a silencing response against a native target in human cell lines, we have focused our attention primarily on the simpler and lower molecular weight mixed block polymer carrier design, GM10. Robust ApoB silencing in cell culture and *in vitro* Dicer cleavage/RNA release results indicates that bifacial polymer nucleic acid hybridization does not interfere with native processing. Further, ligand display may be intimately integrated into the bP$_o$NA backbone with nominal impact on silencing in the synthetically accessible mixed block design of GM10. This approach uniquely employs non-covalent triplex hybridization to install the GalNAc targeting ligands, and the silencing outcomes in cell culture compare well to prior liver-targeting siRNA platforms that utilize covalent linkage of GalNAc to RNA. Non-covalent ligand display using the bP$_o$NA approach presents potential advantages in screening of siRNA sequences for optimization of silencing, obviating the need for more complex synthetic manipulations on each siRNA duplex. Further, the demonstrated compatibility of bP$_o$NA-RNA hybrids with all stages of RNA silencing, from *in vivo* delivery to Dicer processing, highlights the possibility of integrating siRNA with other polymer architectures useful as carriers, potentially including nanoparticles. We anticipate that the demonstrated versatility of the bP$_o$NA platform combined with the scalable synthetic accessibility of polyacrylates will enable its use in a range of delivery applications, utilizing GalNAc targeting as well as other established ligands.[50]

MATERIALS AND METHODS

General. Acrylate monomers other than NHS acrylate were purchased from Sigma-Aldrich. 2,2′-Azobis(2-methylpropionitrile) was purchased from Sigma-Aldrich and was recrystallized from methanol prior to use. Oligonucleotides were purchased from Integrated DNA technologies (Coralville, IA). ^{1}H NMR spectra were recorded either in $CDCl_3$, D_2O, or DMSO-d_6 on a Bruker Avance 400 MHz. ^{1}H NMR spectra are referenced to internal standard tetramethylsilane (TMS, $\delta = 0.00$), $CHCl_3$ (7.27 ppm), D_2O (4.78 ppm) or DMSO-d_6 (2.49 ppm). DLS measurements were performed using a Malvern Nano series Zetasizer. CTAs 8 and 9, amine 10, and monomers 12 and 13 were prepared according to

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literature procedures.[7,43,51] GalNAc derivatives including active ester **11** were prepared as previously reported[52] and reacted in post-polymerization modification as previously described.[3]

Representative Polymerization Protocol: Mixed Block Base Polymer. *N*-Hydroxysuccinimidyl acrylate **13** (0. 676 g, 4 mmol) and *tert*-butyl (3-acrylamidopropyl)carbamate **12** (0.912 g, 4 mmol) were dissolved in degassed dimethylformamide (8 mL) in a sealed round-bottom flask, followed by addition of CTA **9**, 2-(((dodecylthio)-carbonothioyl)thio)-2-methylpropanoic acid (80 mg, 0.22 mmol). The resulting mixture was heated to 70 °C under nitrogen and polymerization initiated with 2,2′-azobis(2-methylpropionitrile) (3.6 mg, 22 μmol). Reaction progress was followed by ^{1}H NMR spectroscopy. As reaction times will vary based on concentration and preparation, ^{1}H NMR monitoring was used to stop the reaction at approximately 65% conversion (typically 1−3 h), the reaction was cooled, vented to air, and concentrated under vacuum. The residue was dissolved in 15 mL of dichloromethane and added dropwise to 100 mL of ice-cooled diethyl ether to precipitate polymer as a yellow solid. This dissolution/precipitation process was repeated three or four times to completely remove unreacted monomers. Resulting polymer was dried under vacuum, yielding a faintly yellow powder (1.01 g, 64%). Polymer was characterized by ^{1}H NMR and gel permeation chromatography (GPC) using polystyrene standards.

Representative Post-polymerization Modification Reactions. Base polymers containing both NHS ester **13** and Boc-protected amine **12** were reacted as follows: polymer equivalent of ∼0.60 mmol NHS ester was dissolved in 1 mL of anhydrous DMSO, followed by **10** (0.84 mmol) and triethylamine (0.84 mmol). The resulting reaction mixture was stirred at RT for 24 h. The reaction was cooled and the desired product precipitated in H_2O. Polymer was then dissolved in neat TFA and reacted for 1 h. TFA was removed by vacuum and the residue dissolved in minimum methanol and precipitated in 10 mL of cold diethyl ether at least three times. The polymer salt was dissolved in 1 mL of DMSO and treated with triethylamine (0.84 mmol) and acetyl-protected GalNAc NHS ester **11** (1.68 mmol) for 12 h. The glycopolymer conjugate product was precipitated in acetone, and acetyl protection hydrolyzed in NaOMe in methanol for 30 min. Methanol was removed by vacuum, and the residue was dissolved in water and neutralized with 1 M sodium phosphate monobasic solution. The aqueous solution (around 1 mL) was dialyzed against 1 L of ddH_2O for 72 h (MWCO 3000), changing reservoir ddH_2O every 12 h. The solution in the dialysis bag was lyophilized to yield a white powder.

Cell Viability. Viability was assessed using the CellTiter 96 AQ_{ueous} One Solution cell proliferation colorimetric assay based on MTS reaction (Promega). Freshly isolated peripheral blood mononuclear cells (PBMCs) were seeded onto a 96-well tissue culture plate to reach final conditions of 100 μL per well at a density of 10^6 cells/mL. Cells were treated with siRNA/carrier samples or equal amount of DPBS as described and incubated for 72 h in humidified tissue culture incubator (5% CO_2). Following incubation, 20 μL of MTS working solution ess added into each well, and incubation continued for an additional 3 h. Colorimetric reading was then performed with two wavelength settings (490/570 nm) to assess cell viability according to product protocol.

Dicer Cleavage Assays. Recombinant Dicer was purchased from Genlantis for *in vitro* cleavage analysis. RNA at 100 nM was incubated with 1 unit of recombinant Dicer for 18 h at 37 °C, with or without GM10 polymer (300 nM) following the manufacturer's protocol. The reactions were sampled and analyzed on a native 4−20% gradient acrylamide gel and stained with SYBR-gold (ThermoFisher).

Cell Culture Silencing Studies. HepG2 cells were acquired from the American Type Culture Collection (ATCC), cultured on Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS, penicillin, and streptomycin and passaged at 60% confluency with trypsin/EDTA. HepG2 cells were seeded the day before treatment with siRNA/vehicle at a density of 2×10^5 cells/mL on a 24-well culture plate. On the day of treatment, plated cells were washed two times with DPBS, and fresh culture medium with or without FBS was added. Delivery samples were prepared just prior to treatment (siRNA:polymer = 1:3) and incubated at RT for 10 min before adding to plated cells with fresh culture medium. In the absence of FBS, cells were washed with DPBS 6 h post-treatment to minimize cell death, followed by regular culture for 48 h before total RNA extraction. For treatment in full media (with FBS), no washing step was required; cells were incubated with delivery samples for 48 h before total RNA extraction. To isolate RNA, cultured cells were homogenized with Trizol (ThermoFisher) and extracted with chloroform to isolate crude RNA from the aqueous layers. Total RNA was sequentially precipitated from the aqueous fraction with isopropanol and 70% ethanol. Purified RNAs were stored in 70% ethanol at −20 °C until analysis.

RT-qPCR. Concentrations of isolated total RNA samples (see above) obtained from cell culture or liver tissue were determined by UV−vis spectrometry. Reverse transcription was performed with 10 unit/μL of M-MuLV reverse transcriptase (New England Biolabs) and 200 nM gene-specific reverse primers for both target gene and the housekeeping genes to obtain cDNA. Freshly synthesized cDNAs were heat-treated at 65 °C for 20 min and directly subjected to qPCR analysis with Luna Universal qPCR Master Mix (New England Biolabs).

Cytokine Assays. Fresh human peripheral blood mononuclear cells (PBMCs) from three healthy donors were isolated individually using Sepmate-50 (StemCell Tech) and Histopaque-1077 (Sigma). Red blood cells were removed using Red Blood Cell Lysis buffer (Sigma). Isolated PBMCs were counted and seeded into a 96-well tissue culture plate to arrive at 100 μL per well at a density of 10^6 cells/mL. PBMCs were treated with RNA/carrier samples in triplicate as described, and 50 μL of supernatant was collected for analysis from each well after 12 h of incubation in the humidified tissue culture incubator (5% CO_2). Levels of IFN-α (ThermoFisher) and IFN-γ (BD Biosciences) were analyzed by ELISA using the manufacturer's protocols.

***In Vivo* Studies.** A group of five male C57BL/6J mice were placed on a high-fat diet for >6 weeks to reach an average weight of 45 g—a significant weight increase over normal diet (20 g). Mice were treated subcutaneously with a 200 μL injection volume daily for 5 days, with either a 5 mg/kg siRNA dose (1.5 equiv of polymer GM10) or buffer control. Subcutaneous injections of siRNA and polymer complex were made into the loose skin over the neck and shoulder of each mouse. The Mod2 siRNA was annealed at 65 °C for 10 min and then allowed to cool to RT. Polymer GM10 (1.5 equiv) was annealed to the duplex at RT before injection. Mice were euthanized 5 days after the last injection. For each mouse, three pieces of liver tissue were collected through aseptic necropsy procedures, homogenized in Trizol (10×), and analyzed for silencing outcomes using ApoB100 gene locus 10395. Total RNA was extracted from each liver tissue, and RT-qPCR (see above) was performed to confirm the knockdown efficiency, with GAPDH and HPRT as housekeeping control.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.9b00273.

GPC, NMR spectra, and silencing experiments (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: bong.6@osu.edu.

ORCID

Dennis Bong: 0000-0003-3778-9183

Author Contributions

§These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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