Published in final edited form as:

ACS Chem Biol. 2019 June 21; 14(6): 1310–1318. doi:10.1021/acscchembio.9b00273.

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

Xin Xia^{‡,§}, Zhun Zhou^{†,§}, Chris DeSantis[†], John J. Rossi[‡], 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

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_oNA). Polyacrylate bP_oNA 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 IC₅₀ 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_oNA 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.

Graphical Abstract

*Corresponding Author: bong.6@osu.edu.

[§]These authors contributed equally to this work.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:10.1021/acscchem-bio.9b00273. GPC, NMR spectra, and silencing experiments (PDF)

The authors declare no competing financial interest.

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 nonnative backbones, including polyacrylates, which we denote bifacial *polymer* nucleic acid (bP_oNA). 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-on¹⁵ 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 bases¹⁶ and nucleic acids and could thus help bridge native and artificial architectures, uniting nucleic acid biotechnology¹⁷ 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_oNA), 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 interface³ that forms on mixing, compatible with 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 sp³-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 carriers³² 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 RNA³¹ cargo to liver and hepatocytes. We hypothesized that triplex hybridization of glycosylated bP_oNA 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_oNA siRNA complexes in HeLa cells, in addition to the considerable literature precedent for GalNAc targeting, augured well for GalNAc-bP_oNA 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_oNA 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 IC₅₀ 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_oNA 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_oNA platform for *in vivo* siRNA delivery.

RESULTS AND DISCUSSION

Design and Synthesis of Liver-Targeted bP_oNA 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 bP₀NA 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 ¹H 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 intermediate. The resulting fully protected, hydrophobic diblock base polymer (PDI = 1.14) was then characterized by ¹H 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)₁₀G10, respectively, that feature acrylamide-displayed GalNAc targeting ligand (G), melamine (M), and acrylic acid (A) in the diblock glycopolymer.

Evaluation of siRNA-bP₀NA 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) U₁₀ 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)₁₀G10 glycopolymers were designed to triplex hybridize with the unstructured U₁₀ 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 U₁₀C₁₀U₁₀ sequence that is known to form a hairpin triplex hybrid with bP₀NA,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_oNA 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_oNA-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_oNA 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 3A). This cell viability guided design of dose-dependent ApoB100 silencing studies in HepG2 cells with GM10 and (AM)₁₀G10 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 IC₅₀ 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 subnanomolar IC₅₀ 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_oNA 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 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_oNA 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_oNA 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_oNA 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_oNA-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_oNA 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). ¹H NMR spectra were recorded either in CDCl₃, D₂O, or DMSO-*d*₆ on a Bruker Avance 400 MHz. ¹H NMR spectra are referenced to internal standard tetramethylsilane (TMS, δ = 0.00), CHCl₃ (7.27 ppm), D₂O (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 literature procedures.^{7,43,51} GalNAc derivatives including active ester **11** were prepared as previously reported⁵² 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 μ mol). Reaction progress was followed by ¹H NMR spectroscopy. As reaction times will vary based on concentration and preparation, ¹H 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 ^1H 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 dd H_2O for 72 h (MWCO 3000), changing reservoir dd H_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 AQueous 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 was 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 $^\circ\text{C}$, 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 $100 \mu\text{L}$ per well at a density of 10^6 cells/mL. PBMCs were treated with RNA/carrier samples in triplicate as described, and $50 \mu\text{L}$ 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 $200 \mu\text{L}$ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported in part by funding from the National Institutes of Health (GM111995-01A1 to D.B., AI42554 and AI29329 to J.R.), NASA (GRT00044810 to D.B.), and NSF (DMR 1802432 to D.B.).

REFERENCES

- (1). Mao J, and Bong D (2015) Synthesis of DNA-Binding Peptoids. *Synlett* 26, 1581–1585.
- (2). Mao J, DeSantis C, and Bong D (2017) Small Molecule Recognition Triggers Secondary and Tertiary Interactions in DNA Folding and Hammerhead Ribozyme Catalysis. *J. Am. Chem. Soc* 139, 9815–9818. [PubMed: 28691825]
- (3). Zhou Z, Xia X, and Bong D (2015) Synthetic Polymer Hybridization with DNA and RNA Directs Nanoparticle Loading, Silencing Delivery, and Aptamer Function. *J. Am. Chem. Soc* 137, 8920–8923. [PubMed: 26138550]
- (4). Zeng Y, Pratumyot Y, Piao X, and Bong D (2012) Discrete assembly of synthetic peptide-DNA triplex structures from polyvalent melamine-thymine bifacial recognition. *J. Am. Chem. Soc* 134, 832–835. [PubMed: 22201288]
- (5). Piao X, Xia X, and Bong D (2013) Bifacial Peptide Nucleic Acid Directs Cooperative Folding and Assembly of Binary, Ternary, and Quaternary DNA Complexes. *Biochemistry* 52, 6313–6323. [PubMed: 23964711]
- (6). Lange RFM, Beijer FH, Sijbesma RP, Hooft RWW, Kooijman H, Spek AL, Kroon J, and Meijer EW (1997) Crystal Engineering of Melamine-Imide Complexes; Tuning the Stoichiometry by Steric Hindrance of the Imide Carbonyl Groups. *Angew. Chem., Int. Ed. Engl* 36, 969–971.
- (7). Arambula JF, Ramisetty SR, Baranger AM, and Zimmerman SC (2009) A simple ligand that selectively targets CUG trinucleotide repeats and inhibits MBNL protein binding. *Proc. Natl. Acad. Sci. U. S. A* 106, 16068–16073. [PubMed: 19805260]
- (8). Vilaivan T (2015) Pyrrolidinyl PNA with α/β -Dipeptide Backbone: From Development to Applications. *Acc. Chem. Res* 48, 1645–1656. [PubMed: 26022340]
- (9). Sahu B, Sacui I, Rapireddy S, Zanotti KJ, Bahal R, Armitage BA, and Ly DH (2011) Synthesis and characterization of conformationally preorganized, (R)-diethylene glycol-containing γ -peptide nucleic acids with superior hybridization properties and water solubility. *J. Org. Chem* 76, 5614–5627. [PubMed: 21619025]
- (10). Uhlmann E, Peyman A, Breipohl G, and Will DW (1998) PNA: Synthetic Polyamide Nucleic Acids with Unusual Binding Properties. *Angew. Chem. Int. Ed* 37, 2796–2823.
- (11). Biondi E, and Benner SA (2018) Artificially Expanded Genetic Information Systems for New Aptamer Technologies. *Biomedicines* 6, 53.
- (12). Hoshika S, Singh I, Switzer C, Molt R, Leal NA, Kim M-J, Kim M-S, Kim H-J, Georgiadis MM, and Benner SA (2018) “Skinny” and “Fat” DNA: Two New Double Helices. *J. Am. Chem. Soc* 140, 11655–11660. [PubMed: 30148365]
- (13). Muse O, Zengeya T, Mwaura J, Hnedzko D, McGee DW, Grewer CT, and Rozners E (2013) Sequence selective recognition of double-stranded RNA at physiologically relevant conditions using PNA-peptide conjugates. *ACS Chem. Biol* 8, 1683–1686. [PubMed: 23721369]

- (14). Hnedzko D, McGee DW, Karamitas YA, and Rozners E (2017) Sequence-selective recognition of double-stranded RNA and enhanced cellular uptake of cationic nucleobase and backbone-modified peptide nucleic acids. *RNA* 23, 58–69. [PubMed: 27742909]
- (15). Xia X, Piao X, and Bong D (2014) Bifacial peptide nucleic acid as an allosteric switch for aptamer and ribozyme function. *J. Am. Chem. Soc* 136, 7265–7268. [PubMed: 24796374]
- (16). Zhou Z, and Bong D (2013) Small-Molecule/Polymer Recognition Triggers Aqueous-Phase Assembly and Encapsulation. *Langmuir* 29, 144–150. [PubMed: 23205819]
- (17). Burnett JC, and Rossi JJ (2012) RNA-Based Therapeutics: Current Progress and Future Prospects. *Chem. Biol* 19, 60–71. [PubMed: 22284355]
- (18). Roh YH, Ruiz RCH, Peng S, Lee JB, and Luo D (2011) Engineering DNA-based functional materials. *Chem. Soc. Rev* 40, 5730–5744. [PubMed: 21858293]
- (19). Jones AS, and Taylor N (1967) Synthetic analogues of polynucleotides: interaction of a polymeric adenine derivative with DNA. *Nature* 215, 505–506. [PubMed: 6057907]
- (20). Cassidy F, and Jones AS (1966) Synthetic analogues of polynucleotides—I. The synthesis and properties of copolymers of 5'-o-acrylylthymidine and 5'-o-acrylyluridine with acrylamide. *Eur. Polym. J* 2, 319–328.
- (21). Jones AS (1979) Synthetic analogues of nucleic acids—a review. *Int. J. Biol. Macromol* 1, 194–207.
- (22). Boguslawski S, Olson PE, and Mertes MP (1976) Mechanism of stimulation of in vitro protein synthesis by some copolymers of styrene, vinyluracil, and vinyladenine with maleic acid and acrylic acid. *Biochemistry* 15, 3536–3540. [PubMed: 782518]
- (23). Gait MJ, Jones AS, Jones MD, Shepherd MJ, and Walker RT (1979) Synthetic analogues of polynucleotides. Part 15. The synthesis and properties of poly (5'-amino-3'-O-carboxymethyl-2', 5'-dideoxy-erythro-pentonucleosides) containing 3'(O)' 5'(C) acetamidate linkages. *J. Chem. Soc., Perkin Trans 1*, 1389–1394.
- (24). Eschenmoser A (1999) Chemical etiology of nucleic acid structure. *Science* 284, 2118–2124. [PubMed: 10381870]
- (25). Kaye H (1970) Nucleic acid analogs. The interaction of poly-9-vinyladenine with polyuridylic acid. *J. Am. Chem. Soc* 92, 5777–5779.
- (26). Lo PK, and Sleiman HF (2008) Synthesis and Molecular Recognition of Conjugated Polymer with DNA-Mimetic Properties. *Macromolecules* 41, 5590–5603.
- (27). Keddie DJ, Moad G, Rizzardo E, and Thang SH (2012) RAFT Agent Design and Synthesis. *Macromolecules* 45, 5321–5342.
- (28). Mittapalli GK, Reddy KR, Xiong H, Munoz O, Han B, De Riccardis F, Krishnamurthy R, and Eschenmoser A (2007) Mapping the landscape of potentially primordial informational oligomers: oligodipeptides and oligodipeptoids tagged with triazines as recognition elements. *Angew. Chem., Int. Ed* 46, 2470–2477.
- (29). Vysabhattar R, and Ganesh KN (2008) Cyanuric peptide nucleic acid: synthesis and DNA complexation properties. *Tetrahedron Lett.* 49, 1314–1318.
- (30). Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, et al. (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 432, 173–178. [PubMed: 15538359]
- (31). Huang Y (2017) Preclinical and Clinical Advances of GalNAc-Decorated Nucleic Acid Therapeutics. *Mol. Ther.–Nucleic Acids* 6, 116–132. [PubMed: 28325278]
- (32). Gaspar R, and Duncan R (2009) Polymeric carriers: preclinical safety and the regulatory implications for design and development of polymer therapeutics. *Adv. Drug Delivery Rev* 61, 1220–1231.
- (33). Nguyen J, and Szoka FC (2012) Nucleic Acid Delivery: The Missing Pieces of the Puzzle? *Acc. Chem. Res* 45, 1153–1162. [PubMed: 22428908]
- (34). Wagner E (2012) Polymers for siRNA Delivery: Inspired by Viruses to be Targeted, Dynamic, and Precise. *Acc. Chem. Res* 45, 1005–1013. [PubMed: 22191535]
- (35). Thapa B, Kumar P, Zeng H, and Narain R (2015) Asialoglycoprotein Receptor-Mediated Gene Delivery to Hepatocytes Using Galactosylated Polymers. *Biomacromolecules* 16, 3008–3020. [PubMed: 26258607]

- (36). Hashida M, Nishikawa M, Yamashita F, and Takakura Y (2001) Cell-specific delivery of genes with glycosylated carriers. *Adv. Drug Delivery Rev* 52, 187–196.
- (37). Dhande YK, Wagh BS, Hall BC, Sprouse D, Hackett PB, and Reineke TM (2016) N-Acetylgalactosamine Block-co-Polycations Form Stable Polyplexes with Plasmids and Promote Liver-Targeted Delivery. *Biomacromolecules* 17, 830–840. [PubMed: 26854615]
- (38). Peterson AM, and Heemstra JM (2015) Controlling self-assembly of DNA-polymer conjugates for applications in imaging and drug delivery. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol* 7, 282–297. [PubMed: 25327363]
- (39). Kedracki D, Chekini M, Maroni P, Schlaad H, and Nardin C (2014) Synthesis and self-assembly of a DNA molecular brush. *Biomacromolecules* 15, 3375–3382. [PubMed: 25105945]
- (40). Zhang S, Zhao B, Jiang H, Wang B, and Ma B (2007) Cationic lipids and polymers mediated vectors for delivery of siRNA. *J. Controlled Release* 123, 1–10.
- (41). Grijalvo S, Avmo A, and Eritja R (2014) Oligonucleotide delivery: a patent review (2010 – 2013). *Expert Opin. Ther. Pat* 24, 801–819. [PubMed: 24798406]
- (42). Nair JK, Willoughby JLS, Chan A, Charisse K, Alam MR, Wang Q, Hoekstra M, Kandasamy P, Kel'in AV, Milstein S, Taneja N, O'Shea J, Shaikh S, Zhang L, van der Sluis RJ, Jung ME, Akinc A, Hutabarat R, Kuchimanchi S, Fitzgerald K, Zimmermann T, van Berkel TJC, Maier MA, Rajeev KG, and Manoharan M (2014) Multivalent N-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. *J. Am. Chem. Soc* 136, 16958–16961. [PubMed: 25434769]
- (43). Thang SH, Chong YK, Mayadunne RTA, Moad G, and Rizzardo E (1999) A novel synthesis of functional dithioesters, dithiocarbamates, xanthates and trithiocarbonates. *Tetrahedron Lett.* 40, 2435–2438.
- (44). Kim D-H, Behlke MA, Rose SD, Chang M-S, Choi S, and Rossi JJ (2005) Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat. Biotechnol* 23, 222–226. [PubMed: 15619617]
- (45). Xia X, Piao X, Fredrick K, and Bong D (2014) Bifacial PNA complexation inhibits enzymatic access to DNA and RNA. *ChemBioChem* 15, 31–36. [PubMed: 24259287]
- (46). Kauffman KJ, Mir FF, Jhunjhunwala S, Kaczmarek JC, Hurtado JE, Yang JH, Webber MJ, Kowalski PS, Heartlein MW, DeRosa F, and Anderson DG (2016) Efficacy and immunogenicity of unmodified and pseudouridine-modified mRNA delivered systemically with lipid nanoparticles in vivo. *Biomaterials* 109, 78–87. [PubMed: 27680591]
- (47). Wang C-Y, and Liao JK (2012) A mouse model of diet-induced obesity and insulin resistance. *Methods Mol. Biol* 821, 421433.
- (48). Farese RV, Ruland SL, Flynn LM, Stokowski RP, and Young SG (1995) Knockout of the mouse apolipoprotein B gene results in embryonic lethality in homozygotes and protection against diet-induced hypercholesterolemia in heterozygotes. *Proc. Natl. Acad. Sci. U. S. A* 92, 1774–1778. [PubMed: 7878058]
- (49). Morrison JR, Pászty C, Stevens ME, Hughes SD, Forte T, Scott J, and Rubin EM (1996) Apolipoprotein B RNA editing enzyme-deficient mice are viable despite alterations in lipoprotein metabolism. *Proc. Natl. Acad. Sci. U. S. A* 93, 7154–7159. [PubMed: 8692961]
- (50). Srinivasarao M, and Low PS (2017) Ligand-Targeted Drug Delivery. *Chem. Rev* 117, 12133–12164. [PubMed: 28898067]
- (51). Marsden DM, Nicholson RL, Ladlow M, and Spring DR (2009) 3D small-molecule microarrays. *Chem. Commun.* 7107–7109.
- (52). Prakash TP, Graham MJ, Yu J, Carty R, Low A, Chappell A, Schmidt K, Zhao C, Aghajan M, Murray HF, Riney S, Booten SL, Murray SF, Gaus H, Crosby J, Lima WF, Guo S, Monia BP, Swayze EE, and Seth PP (2014) Targeted delivery of antisense oligonucleotides to hepatocytes using triantennary N-acetyl galactosamine improves potency 10-fold in mice. *Nucleic Acids Res.* 42, 8796–8807. [PubMed: 24992960]

Figure 1. (Top) Structure of mixed block and diblock bifacial polymer nucleic acids, GM10 and (AM)₁₀G10. Melamine base (R₁), hepatocyte-targeting GalNAc ligand (R₂), and base-triple motif (boxed) are indicated. (Bottom) Triplex hybridization of siRNA duplexes with the polyacrylates results in non-covalent labeling with GalNAc.

Figure 2.

(A) Native EMSA of siRNAs as indicated at 50 nM in each lane with increasing quantity of bP₀NA GM10 (0, 37.5, 75, 150, and 300 nM). Polymer complexation reduces staining efficiency. (B) Illustration of the expected bP₀NA 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.

Figure 3.

(A) HepG2 cell viability on treatment with bP₀NA 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 IC₅₀ 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.

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.

Scheme 1. Synthetic Approach to GalNAc-bP₀NAs^a

^a(Top) RAFT polymerization of monomers **12** and **13** with chain transfer agent (CTA) **9** (Z = C₁₂H₂₅) produces base polymer for GM10 with PDI = 1.10. (Bottom) Diblock bP₀NA (AM)₁₀G₁₀ synthesis uses acrylates shown with CTA **8** (Z = CH₂CH₂CO₂H) 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**, Et₃N, DMSO; (c) TFA; (d) **11**, Et₃N, DMSO; (e) NaOMe, MeOH.

Table 1.

ApoB100-Targeting siRNAs^a

siRNA	sense/antisense	ApoB100-targeting sequence
U10	(S)	5'-* GGAAUCUUAU/AUUUGAUCCAAAUACCUUUUUUUUUU-3'
	(AS)	5'-UUUUUUUUUUCCU/AUUUGGACRCAAAUAAAGAUUCCCU-3'
U10-Mod2	(S)	5'-*GgAaUcUuAUuUgAuCCAAAUAcc <u>uuuuuuuu</u> 7T-3'
	(AS)	5'- <u>uuuuuuuuuu</u> ccUAUUUGGAuCaAaUAuAaGaUuCc <u>uu</u> -3'
J-turn	(S)	5'-*GGAAUCUUAU/AUUUGAUCCCAACC-(U) ₁₀ (C) ₁₀ (U) ₁₀ -3'
	(AS)	5'-UUGGAUCAAU/AUAAAGAUUCCCU-3'

^a Oligonucleotide domains used in bPONA binding are shown in bold. Annotations indicating modified nucleotides (N) are as follows:

* N = 5' phosphate; n = 2'-methoxy; N = deoxyribose.