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High-Throughput Chemical Modification of Oligonucleotides for Systematic Structure–Activity Relationship Evaluation

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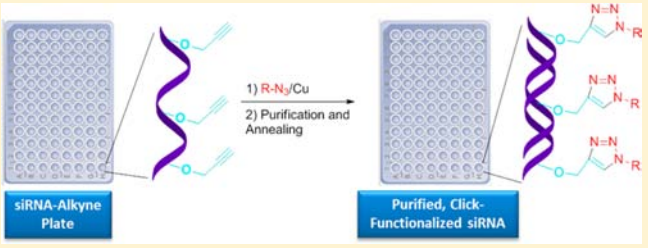
ABSTRACT: Chemical modification of siRNA is achieved in a high-throughput manner (96-well plate format) by copper catalyzed azide–alkyne cycloadditions. This transformation can be performed in one synthetic operation at up to four positions with complete specificity, good yield, and acceptable purity. As demonstrated here, this approach extends the current synthetic options for oligonucleotide modifications and simultaneously facilitates the systematic, rapid biological evaluation of modified siRNA.



INTRODUCTION

Pharmaceutical application of siRNA requires both passenger and guide strand chemical modifications to improve desirable qualities such as in vivo stability, potency, toxicity, and deliverability.[1−13] Despite tremendous strides in the field, the clinical application of siRNA is limited and thus necessitates improved chemical and biological approaches to address these challenges.[14−24] Years of research have also made it apparent that structural modifications that improve one property may be detrimental to other desired properties and no single positional modification stands out as a universal solution. As a result, researchers continue to explore modifications on various components of nucleosides (bases, ribose ring, and phosphate backbone) and continue to optimize physicochemical and biochemical properties of siRNA with a desire to realize their therapeutic capacity as gene silencing agents (Figure 1).[14−24]

In recent years, various groups have reported improved delivery and activity of oligonucleotides via conjugation/complexation of various molecular entities like PEG, polyamines, peptides, and lipid nanoparticles.[26−34] These advances have made it clear that improved methods for rapid evaluation of analogs is warranted. As successfully demonstrated with small molecules, chemistry amenable to high throughput screening (HTS) would greatly expidite both oligonucleotide discovery and optimization. Currently, intensive structure–activity relationship studies via multistep, nucleoside building block modifications are synthetically challenging and time-consuming, making high-throughput synthesis difficult and uneconomical.[35−39] A viable alternative that avoids these complications involves postsynthetic high throughput chemical modifications of oligonucleotides that is operationally simple, avoids the use of excess reagents, and uses established purification protocols.

One potential solution to this challenge is the use of the "click" reaction, a highly efficient method for installation of chemical diversity. This well established [3 + 2] cycloaddition reaction has been applied to a wide variety of molecules with a diverse array of physical properties: small molecules, antibodies, oligonucleotides, and proteins.[40−43] We therefore chose to focus on the use of click chemistry to achieve our HTS goal, with particular focus placed on application at the 2′ position of the oligonucleotide (Figure 2).

Modification of the 2′-position of ribose is implicated in improved siRNA properties including reduced off-target effects, immunogenicity, and nuclease resistance compared to native RNA.[44−52] It is important to note that ligand assisted copper catalyzed click chemistry of oligonucleotides under microwave conditions and cyclooctyne-strain–release click conjugations have been reported.[53,54] This previous work serves as an excellent starting point for our studies. In particular, the creation of a methodology that would allow for the fast and efficient introduction and evaluation of a library of desirable substrates[55−63] on bases (A, C, G, U) in a high throughput manner. Herein we have addressed existing challenges and expanded the scope of postsynthetic modifications by developing a high yielding Cu(I)-catalyzed click[64−67] methodology which does not require use of ligand or a strained alkyne appendage. Furthermore, this approach is amenable to HTS, enables rapid exploration of the structure–activity relationships, and fits into the well-established oligosynthesis protocols used in many laboratories.

RESULTS AND DISCUSSION

Click Optimization and Scope. Multiwell oligonucleotide synthesis is a well-established technique for library generation. As such, we wanted to develop a system which would fit into this workstream so as to minimize unecessary manipulations and incorporation of additional instruments. We opted to focus

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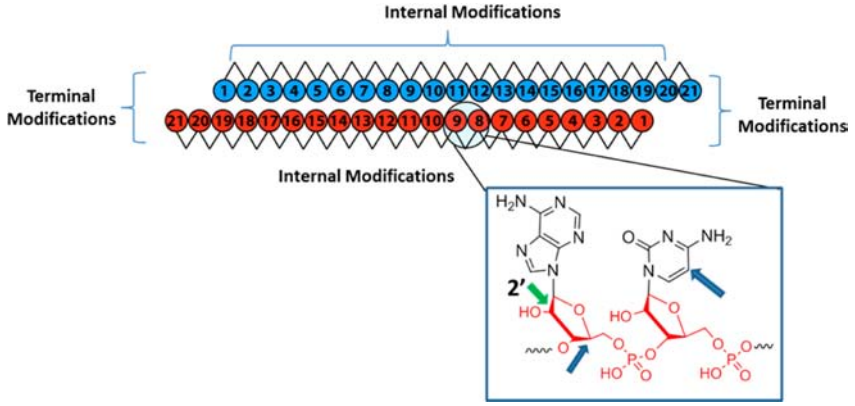

Figure 1. Modification options for siRNA include both terminal and internal positions.[25] This work focuses on modifications at the 2′ position of the ribose.

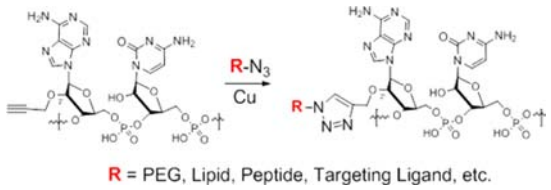

Figure 2. Example of an oligonucleotide 2′ position click modification.

on modifying the siRNA after cleavage from the resin, but prior to protecting group removal which serves as a useful handle for purification. The representative process is illustrated in Scheme 1. All four canonical 2′-*O*-propargyl nucleosides, generalized as **1**, were incorporated into RNA using oligonucleotide synthesis via phosphoramidite chemistry.[68,69] The oligonucleotides were released from the solid support with concurrent removal of the nucleobase amide and cyanoethyl phosphate protecting groups using 40% aqueous $MeNH_2$. The crude products were lyophilized to remove volatiles and leave the click precursor single strand. Peg-24 azide substrate **a** was selected for click development and optimization studies (Figure 3). After investigating a number of catalyst systems and reaction conditions, we were gratified to find that the cycloaddition reaction proceeded with excellent conversion (>95%) using $CuBr{\cdot}Me_2S$ as catalyst in DMAc:H_2O to afford modified RNA strand **3**. Removal of the 2′-O-TBS groups was then effected by treatment with aqueous NH_4F.[70] Subsequent purification of RNA strand **4** over C18-cartridges allowed for removal of failure sequences, protecting group byproducts and removal of residual copper. This process gave **5** with residual copper <0.1 ppm as ascertained by ICP-MS analysis. Annealing with the complementary RNA strand affords the siRNA duplex as free-flowing white powder after lyophilization.

With proof of concept demonstrated, we sought to explore the scope and robustness of this process in a multiwell format on three test substrates (Figure 3). In particular, we sought to understand what types of substrates could be tolerated and the extent to which they could be incorporated multiple times. In addition to chemical validation, we sought to explore what impact these type of modifications would have on biological activity—information that has, up to now, not been obtained for these subtrates and oligonucleotides.

We first examined the positional impact ("walkthrough") of polydisperse PEG substrate **b** on the click reaction using an siRNA complementary to SSB291, a ubiquitously expressed gene useful for siRNA proof of concept studies. Using the conditions outlined above, we were pleased to find that the click reaction at all 18 positions individually resulted in good conversion and yield; i.e., the reaction tolerated the PEG moiety, even on the more sterically encumbered internal positions of the oligonucleotide. A similar walkthrough was done on the guide strand of a Luciferase-80 siRNA, this time with azide subtrates **c** and **d**. Much like the Peg substrate, both of these substrates cleanly incorporated into the oligonucleotide. The impact on multiple substitutions was evaluated as well, this time with the well-defined PEG moiety $N_3{-}PEG_{24}{-}OH$ (**a**) as this PEG would make characterization more straightforward. Again, we were pleased to see that upward of four modifications were tolerated. It should be noted that higher-molecular-weight PEG azides and peptide azides also worked and will be reported at a later time.[71]

Biological Evaluation of Click Modified Oligonucleotides. To highlight the utility of this approach, we investigated the effect of chemical modifications performed above on the biological activity of both the Sjögren syndrome antigen B gene (SSB291)[72] and luciferase[73] (Figure 4a–d). For the SSB291 siRNA with MeO-PEG2000 modifications, SAR was established at 0.1 nM where several oligomers exhibited either markedly improved (positions 1 and 2), equal (positions 12–16), or lessened (positions 11 and 12) gene silencing activity relative to unmodified siRNA (Figure 4a). For ease of analysis we then selected monodisperse azido-PEG_{24}-OH to install at multiple positions along the oligomer (Figure 4b). The relative levels of SSB291 mRNA were reduced at 0.1 nM siRNA concentrations; however, in general multiple substitutions did attenuate the overall activity of the siRNA with the following trend: unmodified > 2 position click > 3 position, 4 position click. These PEG modifications highlight the scope of this methodology and may also allow for modulation of stability and plasma exposure of siRNAs.

Cells stably expressing Luc80 were transfected with either unmodified Luc80 siRNAs or benzyl-modified Luc80 siRNAs. As illustrated in Figure 4c, incorporation of 2′-benzyl-modification at nucleotides 3, 5, 18, and 19 afforded oligomers with gene-silencing activity similar to that of unmodified RNA. In comparison, modification of the 2′-position at nucleotides 1, 2, 4, 6–14, 16, and 17 led to reduction in RNAi activity. Incorporation of a single benzyl-modification at position 15 of the guide strand of siRNA led to a 10% increase of RNAi activity compared to unmodified RNA at 0.1 nM siRNA. The incorporation of a single benzyl modification at position 15 in

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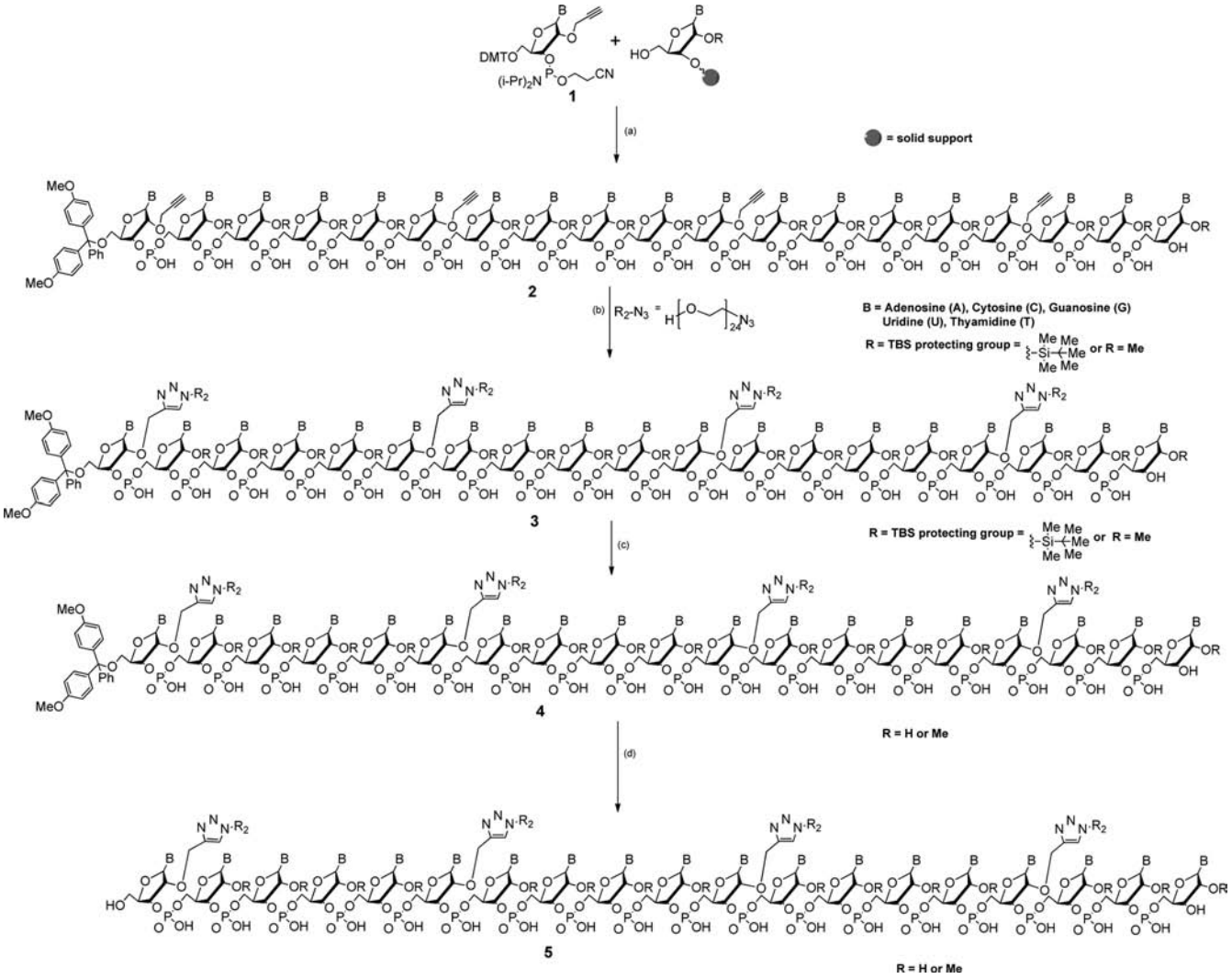
Scheme 1. Process for Post-Synthetic Chemical Modification of RNA[a]



[a]Exemplified for ribo-guanidine adenine at position 4, ribo-uridine at positions 9, 14 and ribo- adenine at position 19. Conditions: (a) RNA synthesis incorporating 2′-*O*-propargyl ribonucleotides, 40% aqueous $MeNH_2$, 45 min, 35 °C, lyophilization; (b) N_3PEG_{24}—OH, CuBr·Me_2S, DMAc:H_2O (75:25), rt to 50 °C; (c) NH_4F; (d) C18 cartridge purification/deprotection.

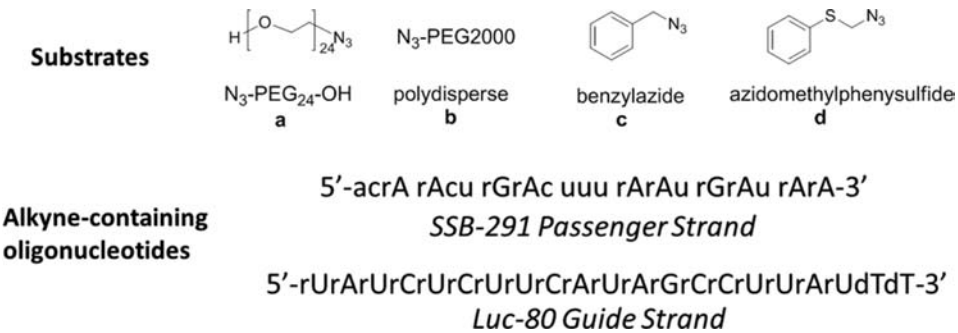


Figure 3. Azide and alkyne substrates. Abbreviations: rA = ribo-adenosine; rC = ribo-cytidine; rG = ribo-guanosine; rU = ribo-uridine; dT = deoxythymidine; a = 2′-OMe-adenosine; c = 2′-OMe cytidine; u = 2′-OMe-uridine. The numbering scheme for the specific nucleotide positional click modifications begins at the 5′ position of both the SSB291 and Luc80 oligonucleotide strands.

the guide strand of Luc80 also translated into a 3.5-fold increase in the measured EC_{50} of RNAi potency and exhibited 15% increased duration of action as measured in vitro at day 5 after transfection (data not shown). Parallel experiments were conducted on Luc80 sequence that was modified with azidomethyl phenyl sulfide (Figure 4d). Despite the relatively small difference in size between the phenyl sulfide (**d**) and benzyl (**c**), statistically significant differences in activity were observed at different positions demonstrating the utility of this method—rapid evaluation of substitutional differences. To

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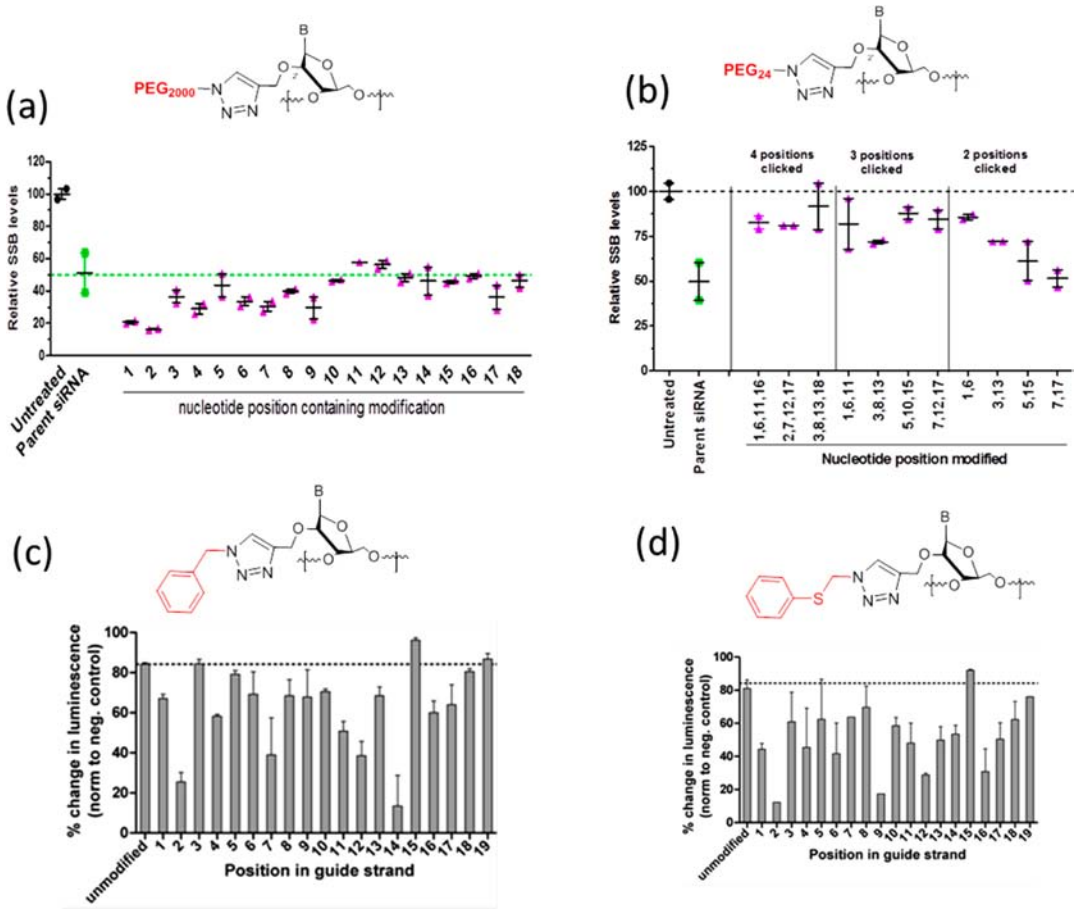

Figure 4. (a) In vitro assay of SSB-291 siRNA with triazole-PEG2000 conjugated passenger strand at single nucleotide positions, [0.1 nM siRNA]; (b) In vitro assay of SSB-291 siRNA with triazole-PEG_{24}-OH conjugated passenger strand at multiple nucleotide positions, [0.1 nM siRNA]; (c) position-dependent mRNA knockdown for luciferase(80) at 1 nM [siRNA] modified with benzyl-triazole group; (d) position-dependent mRNA knockdown for luciferase (80) at 1 nM [siRNA] modified with mercapto benzyl-triazole group. Cell viability was unaffected when transfected with modified siRNAs as compared to unmodified siRNAs. The numbering scheme for the specific nucleotide positional click modifications begin at the 5′ position of both the SSB291 and Luc80 oligonucleotide strands (see Figure 3).

Table 1. Benzyl Azide Clicked Luc-80 Guide Strand[a]

Plate position	Seq (5'-3')	Modified position From 5'
A1	rUrArUrCrUrCrUrUBnclickrCrArUrArGrCrCrUrUrArUdTdT*	9
A2	rUrArUrCrUrCrUrBnclickrUrCrArUrArGrCrCrUrUrArUdTdT**	8
A3	rUrArUrCrUrCBnclickrUrUrCrArUrArGrCrCrUrUrArUdTdT**	7
A4	rUrArUrCrUBnclickrCrUrUrCrArUrArGrCrCrUrUrArUdTdT*	6
A5	rUrArUrCBnclickrUrCrUrUrCrArUrArGrCrCrUrUrArUdTdT*	5
A6	rUrArUBnclickrCrUrCrUrUrCrArUrArGrCrCrUrUrArUdTdT**	4
A7	rUrABnclickrUrCrUrCrUrUrCrArUrArGrCrCrUrUrArUdTdT**	3
A8	rUBnclickrArUrCrUrCrUrUrCrArUrArGrCrCrUrUrArUdTdT**	2
A9	BnclickrUrArUrCrUrCrUrUrCrArUrArGrCrCrUrUrArUdTdT**	1
A10	rUrArUrCrUrCrUrUrCrArUrArGrCrCrUrUrABnclickrUdTdT**	19
A11	rUrArUrCrUrCrUrUrCrArUrArGrCrCrUrUBnclickrArUdTdT*	18
A12	rUrArUrCrUrCrUrUrCrArUrArGrCrCrUBnclickrUrArUdTdT**	17
B1	rUrArUrCrUrCrUrUrCrArUrArGrCrCBnclickrUrUrArUdTdT**	16
B2	rUrArUrCrUrCrUrUrCrArUrArGrCBnclickrCrUrUrArUdTdT**	15
B3	rUrArUrCrUrCrUrUrCrArUrArGBnclickrCrCrUrUrArUdTdT**	14
B4	rUrArUrCrUrCrUrUrCrArUrABnclickrGrCrCrUrUrArUdTdT*	13
B5	rUrArUrCrUrCrUrUrCrArUBnclickrArGrCrCrUrUrArUdTdT**	12
B6	rUrArUrCrUrCrUrUrCrABnclickrUrArGrCrCrUrUrArUdTdT**	11
B7	rUrArUrCrUrCrUrUrCBnclickrArUrArGrCrCrUrUrArUdTdT**	10

[a]Molecular formula = $C_{206}H_{255}N_{65}O_{150}P_{20}$, MW (calculated) = 6661 (obtained). * = M-2 = 6659, ** = M-1 = 6660. BnclickrA = ribo-adenosine clicked with benzyl azide at the 2′-position. BnclickrC = ribo-cytosine clicked with benzyl azide at the 2′-position.

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Table 2. Azidomethylphenyl Sulfide Clicked Luc-80 Guide Strand[a]

Plate position	Seq (5'-3')	Modified position From 5'
C1	rUrArUrCrUrCrUrU PhsclickrC rArUrArGrCrCrUrUrArUdTdT***	9
C2	rUrArUrCrUrCrUr PhsclickrU rCrArUrArGrCrCrUrUrArUdTdT**	8
C3	rUrArUrCrUrC PhsclickrU rUrCrArUrArGrCrCrUrUrArUdTdT**	7
C4	rUrArUrCrU PhsclickrC rUrUrCrArUrArGrCrCrUrUrArUdTdT**	6
C5	rUrArUrC PhsclickrU rCrUrUrCrArUrArGrCrCrUrUrArUdTdT**	5
C6	rUrArU PhsclickrC rUrCrUrUrCrArUrArGrCrCrUrUrArUdTdT*	4
C7	rUrA PhsclickrU rCrUrCrUrUrCrArUrArGrCrCrUrUrArUdTdT**	3
C8	rU PhsclickrA rUrCrUrCrUrUrCrArUrArGrCrCrUrUrArUdTdT**	2
C9	PhsclickrU rArUrCrUrCrUrUrCrArUrArGrCrCrUrUrArUdTdT**	1
C10	rUrArUrCrUrCrUrUrCrArUrArGrCrCrUrUrA PhsclickrU dTdT**	19
C11	rUrArUrCrUrCrUrUrCrArUrArGrCrCrUrU PhsclickrA rUdTdT*	18
C12	rUrArUrCrUrCrUrUrCrArUrArGrCrCrU PhsclickrU rArUdTdT*	17
D1	rUrArUrCrUrCrUrUrCrArUrArGrCrC PhsclickrU rUrArUdTdT**	16
D2	rUrArUrCrUrCrUrUrCrArUrArGrC PhsclickrC rUrUrArUdTdT**	15
D3	rUrArUrCrUrCrUrUrCrArUrArG PhsclickrC rCrUrUrArUdTdT**	14
D4	rUrArUrCrUrCrUrUrCrArUrA PhsclickrG rCrCrUrUrArUdTdT**	13
D5	rUrArUrCrUrCrUrUrCrArU PhsclickrA rGrCrCrUrUrArUdTdT**	12
D6	rUrArUrCrUrCrUrUrCrA PhsclickrU rArGrCrCrUrUrArUdTdT**	11
D7	rUrArUrCrUrCrUrUrC PhsclickrA rUrArGrCrCrUrUrArUdTdT**	10

[a]Molecular formula = $C_{206}H_{255}N_{65}O_{150}P_{20}S$, MW (calculated) = 6693 (obtained). * = M-2 = 6691, ** = M-1 = 6692, *** = M = 6693. Abbreviations: PhsclickrA = ribo-adenosine clicked with azidomethylphenyl sulfide at the 2′-position. PhsclickrC = riibo-cytosine clicked with azidomethylphenyl sulfide at the 2′-position, PhsclickrG = ribo-guanidine clicked with azidomethylphenyl sulfide at the 2′-position, PhsclickrU = ribo-uridine clicked with azidomethylphenyl sulfide at the 2′-position.

Table 3. SSB291 (Sense or Passenger) Strand Multi-Clicked with Azido-dPEG 24-Alcohol[a]

Plate position	Seq (5'-3')	Modified positions From 5'
A1	pegclickAcA AcpegclickU GAc upegclickUu AAu pegclickGuA A	1,6,11,16
A2	apegclickrCA Acu pegclickGAc uupegclickU AAu GpegclickUA A	2,7,12,17
A3	acpegclickA Acu GpegclickAc uuu pegclickAAu Gu pegclickA A	3,8,13,18
A4	pegclickAcA AcpegclickU GAc upegclickUu AAu GuA A	1,6,11
A5	acpegclickA Acu GpegclickAc uuu pegclickAAu GuA A	3,8,13
A6	acA ApegclickCu GAc pegclickUuu AApegclickU GuA A	5, 10, 15
A7	acA Acu pegclickGAc uupegclickU AAu GpegclickUA A	7,12,17
A8	pegclickAcA AcpegclickU GAc uuu AAu GuA A	1,6
A9	acpegclickA Acu GAc uuu pegclickAAu GuA A	3,13
A10	acA ApegclickCu GAc uuu AApegclickU GuA A	5, 15
A11	acA Acu pegclickGAc uuu AAu GpegclickUA A	7,17

[a]Abbreviations: pegclickA = Peg-24-OH clicked on 2′-*O*-propargyl rA, pegclickC = Peg-24-OH clicked on 2′-*O*-propargyl rC, pegclickG = Peg-24-OH clicked on 2′-*O*-propargyl rG, pegclickU = Peg-24-OH clicked on 2′-*O*-propargyl rU. A1−A3 = quadruple clicked with PEG-24-OH, A4−A7 = triple clicked with PEG-24-OH, A8−A10 = bis clicked with PEG-24-OH.

highlight, while benzyl substitution was tolerated at position 9, phenyl sulfide activity was not tolerated. In contrast, position 14 was tolerated with phenyl sulfide but not tolerated with benzyl substitutions. While we ideally are searching for modifications which improve activity against the unmodified siRNA, these experiments demonstrate that every position of the siRNA will give different activity depending on the substitution, and as such, this method is well suited to exploring this variability.

SUMMARY AND CONCLUSIONS

We have developed a high yielding and operationally simple strategy for high-throughput postsynthetic chemical modification of RNA molecules that is readily amenable to all canonical bases. Our approach was effective for generating both single and multiple conjugations, and allowed for rapid identification of nucleotide positions that tolerate small and large modifications. This protocol offers many advantages in terms of throughput and flexibility relative to traditional approaches used to generate 2′-modified RNAs and avoids the requirement for myriad novel nucleoside phosphoramidites to perform SAR studies. Our results suggest that similar modification strategies should allow expeditious production and identification of RNA molecules that exhibit potent and persistent RNAi activity with improved plasma exposure and cell permeability.

MATERIALS AND METHODS

Phosphoramidites. Phosphoramidites used in the synthesis of RNA sequences were obtained from commercial sources.[74] The following chemicals used for preparing reagents for oligoribonucleotide synthesis were purchased from Sigma-Aldrich, and were used without further purification: acetonitrile, acetic anhydride, azidomethylphenyl sulfide (95%), citric acid, $CuBr{\cdot}SMe_2$, trichloroacetic acid, dichloromethane, *N,N*-dimethylacetamide, DMSO, hexafluoroisopropanol, H_2O, imidazole, iodine, 2,6-lutidine, methanol, methylamine, *N*-methylimidazole, ammonium fluoride, pyridine, and triethylamine. Azido-dPEG 24-alcohol was purchased from Quanta. Benzyl azide (94%) was purchased from Alfa-Aesar. 5-Ethylthio-1*H*-tetrazole was purchased from American International Chemicals (AIC).

Table 4. LC/MS of SSB291 (Sense or Passenger) Strand Multi-Clicked with Azido-dPEG 24-Alcohol

entry	molecular formula	MW (calcd.)	MW (obtained)
A1	$C_{393}H_{634}N_{82}O_{225}P_{18}$	10665	10666
A2	$C_{393}H_{634}N_{82}O_{225}P_{18}$	10665	10666
A3	$C_{396}H_{640}N_{82}O_{225}P_{18}$	10707	10708
A4	$C_{342}H_{535}N_{79}O_{201}P_{18}$	9527	9526
A5	$C_{345}H_{541}N_{79}O_{201}P_{18}$	9569	9570
A6	$C_{342}H_{535}N_{79}O_{201}P_{18}$	9527	9526
A7	$C_{343}H_{537}N_{79}O_{201}P_{18}$	9541	9542
A8	$C_{292}H_{438}N_{76}O_{177}P_{18}$	8403	8403
A9	$C_{294}H_{442}N_{76}O_{177}P_{18}$	8431	8431
A10	$C_{292}H_{438}N_{76}O_{177}P_{18}$	8403	8402
A11	$C_{293}H_{440}N_{76}O_{177}P_{18}$	8417	8417

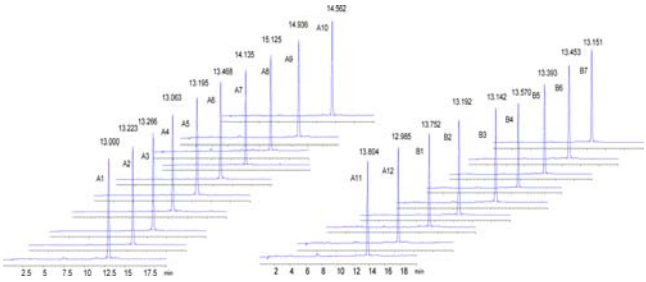

Figure 5. RP HPLC analysis of C-18 purified Luc-80 sequences modified via copper catalyzed click reaction with benzyl azide (Table 1, A1−B7). Purity of modified guide strands is >98A% by RP HPLC.

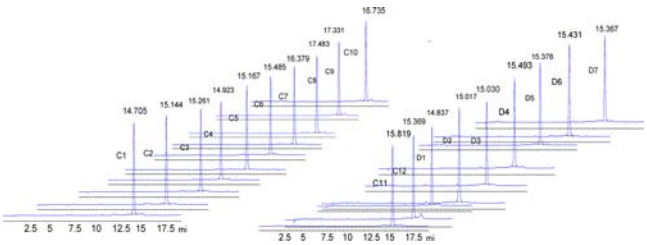

Figure 6. RP HPLC analysis of C-18 purified Luc-80 sequences modified via copper catalyzed click reaction with azidomethylphenylsulfide (Table 2, C1−D7). Purity of modified guide strands is >98 A% by RP HPLC.

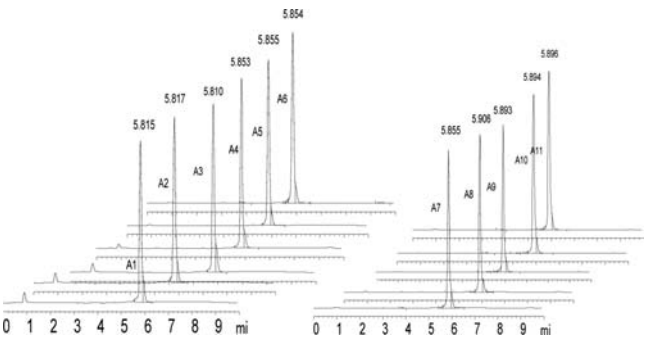

Figure 7. SAX chromatogram analysis of SSB-291 passenger sequence multi-clicked with azido-dPEG 24 alcohol (Table 3, entries A1−A11). Purity of modified passenger strands is >98% by SAX HPLC.

Succinyl long chain alkylamine controlled-pore glass (Succ-LCAA-CPG) functionalized with deoxy-thymidine and riboadenosine were obtained from Prime Synthesis and used directly. Synthesis columns were packed with dT CPG (75 μmol/g, 500 nmol, ~6.7 mg) for Luc-80 guide strand synthesis and rA CPG (72 μmol/g, 500 nmol, ~6.9 mg) for SSB-291 passenger strand synthesis. Triethylammonium acetate buffer was purchased from EMD chemicals.

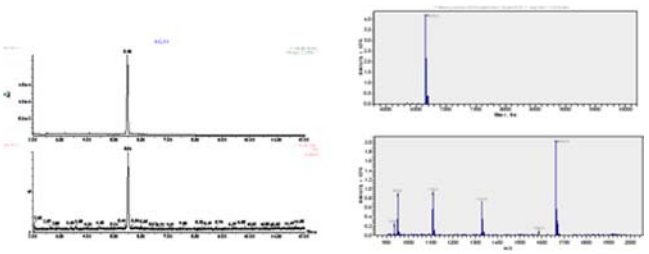

Figure 8. UPLC-MS analysis of a C-18 purified Luc-80 guide representative sequence clicked with benzyl azide (Table 1, entry A11). MW calcd for $C_{206}H_{255}N_{65}O_{150}P_{20}$ = 6661, obtained = 6659.

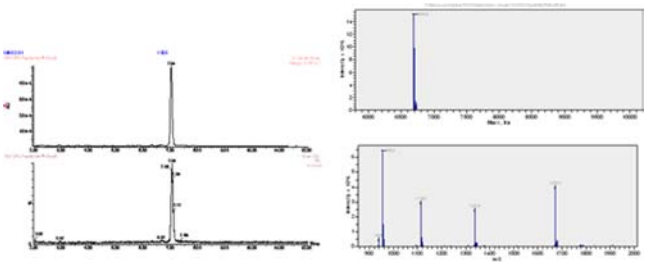

Figure 9. UPLC-MS analysis of a C-18 purified Luc-80 guide representative sequence clicked with azidomethylphenylsulfide (Table 2, entry D5). MW calcd for $C_{206}H_{255}N_{65}O_{150}P_{20}S$ = 6693, obtained = 6693.

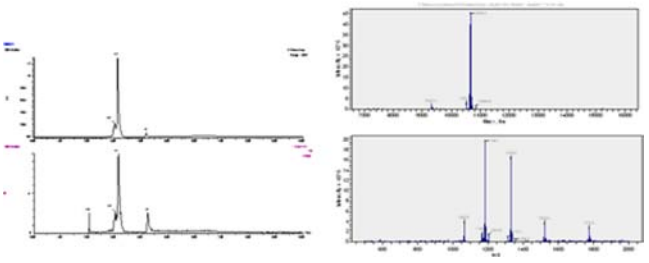

Figure 10. UPLC-MS analysis of a C-18 purified SSB-291 passenger sequence quadruple clicked with azido-dPEG 24 alcohol (Table 3, entry A1). MW calcd for $C_{393}H_{634}N_{82}O_{225}P_{18}$ = 10665, obtained = 10665.

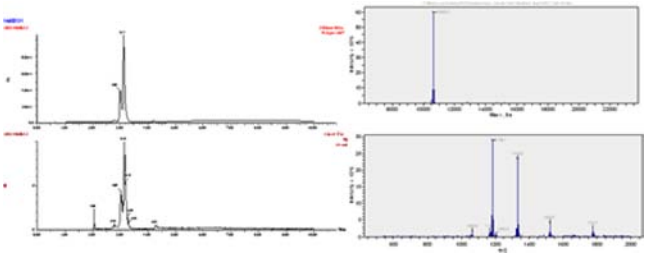

Figure 11. UPLC-MS analysis of a C-18 purified SSB-291 passenger sequence quadruple clicked with azido-dPEG 24 alcohol (Table 3, entry A2). MW calcd for $C_{393}H_{634}N_{82}O_{225}P_{18}$ = 10665, obtained = 10665.

Solid-Phase Synthesis and Annealing of Oligoribonucleotides. Succinyl long chain alkylamine controlled-pore glass (Succ-LCAA-CPG) solid support functionalized with the base

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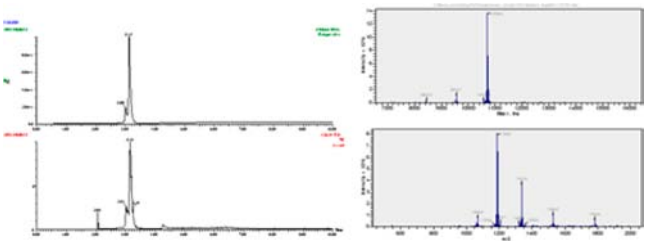

Figure 12. UPLC-MS analysis of a C-18 purified SSB-291 passenger sequence quadruple clicked with azido-dPEG 24 alcohol (Table 3, entry A3). MW calcd for $C_{396}H_{640}N_{82}O_{225}P_{18}$ = 10707, obtained = 10708.

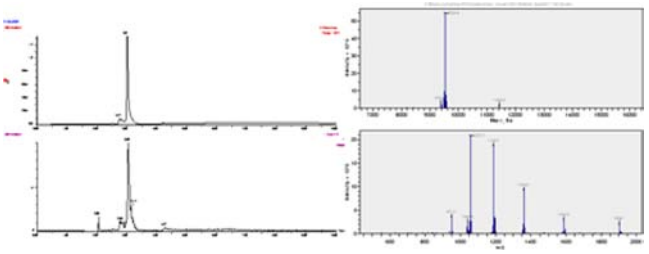

Figure 13. UPLC-MS analysis of a C-18 purified SSB-291 passenger sequence triple clicked with azido-dPEG 24 alcohol (Table 3, entry A4). MW calcd for $C_{342}H_{535}N_{79}O_{201}P_{18}$ = 9527, obtained = 9526.

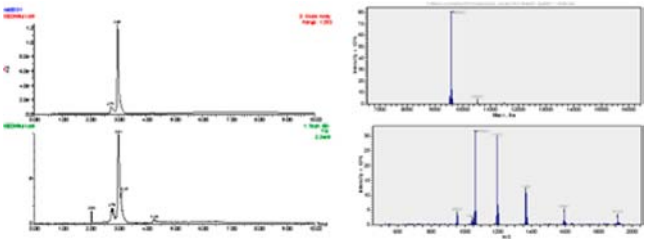

Figure 14. UPLC-MS analysis of a C-18 purified SSB-291 passenger sequence triple clicked with azido-dPEG 24 alcohol (Table 3, entry A5). MW calcd for $C_{345}H_{541}N_{79}O_{201}P_{18}$ = 9569, obtained = 9570.

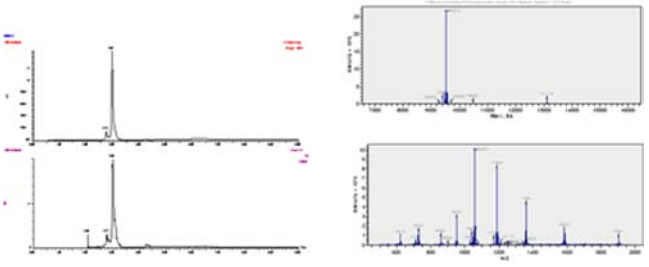

Figure 15. UPLC-MS analysis of a C-18 purified SSB-291 passenger sequence triple clicked with azido-dPEG 24 alcohol (Table 3, entry A6). MW calcd for $C_{342}H_{535}N_{79}O_{201}P_{18}$ = 9527, obtained = 9527.

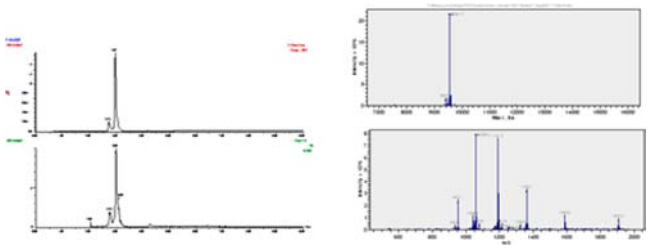

Figure 16. UPLC-MS analysis of a C-18 purified SSB-291 passenger sequence bis clicked with azido-dPEG 24 alcohol (Table 3, entry A7). MW calcd for $C_{343}H_{537}N_{79}O_{201}P_{18}$ = 9541, obtained = 9542.

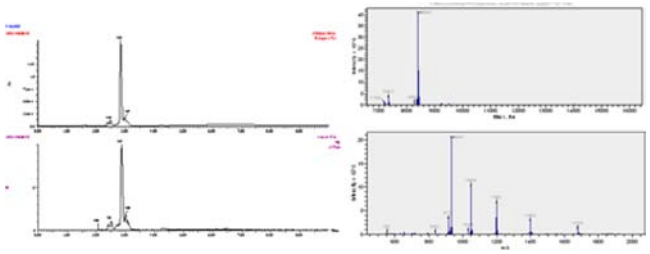

Figure 17. UPLC-MS analysis of a C-18 purified SSB-291 passenger sequence bis clicked with azido-dPEG 24 alcohol (Table 3, entry A8). MW calcd for $C_{292}H_{438}N_{76}O_{177}P_{18}$ = 8403, obtained = 8403.

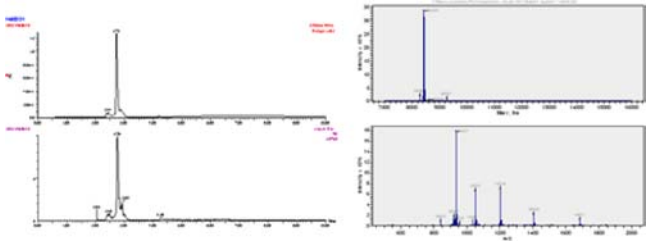

Figure 18. UPLC-MS analysis of a C-18 purified SSB-291 passenger sequence bis clicked with azido-dPEG 24 alcohol (Table 3, entry A9). MW calcd for $C_{294}H_{442}N_{76}O_{177}P_{18}$ = 8431, obtained = 8431.

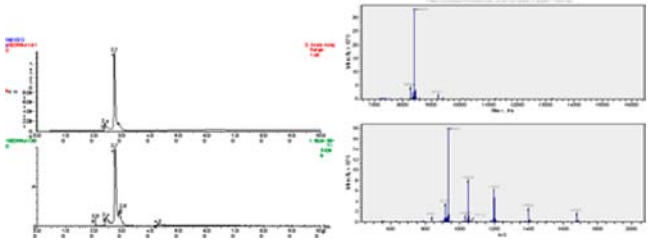

Figure 19. UPLC-MS analysis of a C-18 purified SSB-291 passenger sequence bis clicked with azido-dPEG 24 alcohol (Table 3, entry A10). MW calcd for $C_{292}H_{438}N_{76}O_{177}P_{18}$ = 8403, obtained = 8402.

at position 3′ (500 nmol) was placed in a 96 well reaction plate and RNA sequences were synthesized on MerMade-192 (BioAutomation) oligonucleotide synthesizer.[75] The automated synthesis protocol involved iterative construction via four synthetic steps: (a) detritylation of nucleoside on solid support, (b) activation of phosphoramidite followed by coupling, (c) oxidation of the phosphite, followed by (d) capping of any unreacted 5′-OH species.[76] Once the synthesis of the desired sequence was completed, cleavage from CPG support and deprotection of phosphate and nucleobases was performed using methylamine (40% aq). Lyophilization gave RNA oligomers as oils that were used directly for subsequent click modifications. Final desilylation was done using aqueous NH_4F.[70]After deprotection of 2′-O-TBDMS ethers crude oligomers were purified using C-18 cartridges (Orochem).[77] Purified oligoribonucleotides were assayed via RP HPLC (Figures 5, 6), SAX HPLC (Figure 7), and LC-MS (Figures 8−20). siRNA Duplexes were assembled by mixing equimolar amounts of complementary oligoribonucleotides in 20% aqueous acetonitrile (Figures 21, 22).[78]

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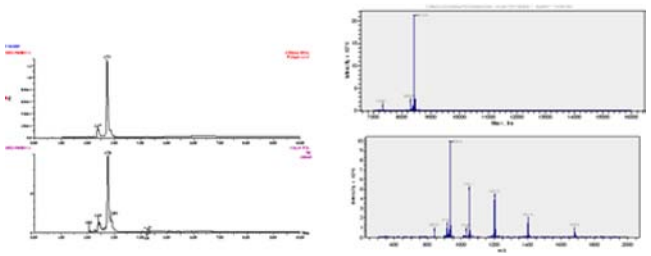

Figure 20. UPLC-MS analysis of a C-18 purified SSB-291 passenger sequence bis clicked with azido-dPEG 24 alcohol (Table 3, entry A11). MW calcd for $C_{293}H_{440}N_{76}O_{177}P_{18}$ = 8417, obtained = 8417. Note: modified strands are of high purity (>98A% by RP & SAX HPLC). No corresponding mass was observed by LC/MS for smaller peaks (Figures 8–20).

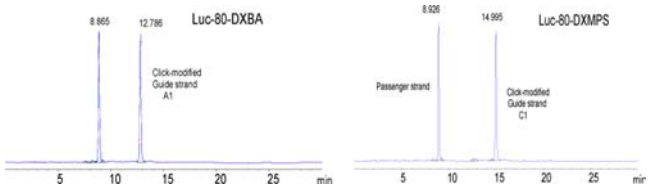

Figure 21. RP HPLC of typical duplex. Luc80-DXBA: duplex of Luc-80 guide strand (A1) with Luc-80 passenger strand and Luc80-DXMPS: duplex of guide strand (C1) with Luc-80 passenger strand.

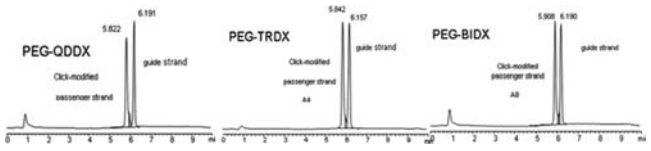

Figure 22. SAX chromatogram analysis of a typical duplex prepared from qudruple clicked SSB-291 passenger sequence and SSB-291 guide sequence (PEG-QDDX), triple clicked (TRDX), and bis-clicked (BIDX).

Protocol for High-Throughput Click Reaction (96 Well Plate Format). Parallel synthesis of 19 21-mer oligoribonucleotides (SSB291 sequences) was carried out on a CPG solid support (rA, 63 μmol/g, 7.9 mg, 500 nmol) in a 96 well plate format. After the synthesis was complete, synthesis tips were treated with 40% aqueous $MeNH_2$ (2 × 100 μL) and aged at rt (2 × 4 min). The CPG support was then washed with DMSO (2 × 150 μL) and product collected into a 96 well deep well plate (1.75 mL capacity). The deep well plate with TBS-protected RNA oligomers in DMSO/40% aqueous methylamine was covered with a cap mat and aged at 37 °C (200 rpm) over 45 min for complete deprotection of phosphate and nucleobases. The reaction plate was then subjected to overnight lyophilization. To lyophilized RNA (500 nmol) in 2 mL deep well plate were added parylene coated stir bars (1.67 × 2.01 × 4.80 mm). DMAC:H_2O (75/25, 50 μL) was added to individual wells followed by benzyl azide (0.1 M, 12.5 μL, 2.5 equiv) and CuBr·SMe_2 (100 μL, 4 mM in DMSO or DMAC, ~0.2 equiv/click site). Reaction mixture was aged in at 50 °C over 3 h (800 rpm). The deep well plate was then cooled to rt and treated with NH_4F (2.7 M, 25 μL) and aged at 65 °C over 1 h. The plate was then cooled to rt. To the crude RNAs was added aqueous NaCl (1 M, 600 μL), and samples were loaded onto a C-18 column for purification. On column detritylation was performed with 1% aqueous TFA (aged 10 min after wetting column). The purified RNAs were eluted with 20% acetonitrile in water (1.2 mL) and then subjected to LC-MS and RP-HPLC analysis. Lyophilization afforded the desired click modified siRNA as a white solid (2 mg, ~38% isolated yield, 96–98 A% SAX-HPLC). Note: Isolated yield is calculated based on starting monomer used for nucleotide assembly.

RP HPLC Method. Sample preparation: 10 μL of final eluent from C-18 purification is diluted to 300 μL with 20% ACN in H_2O. HPLC conditions: Eclipse X-bridge-Phenyl DB-C8, 4.6 × 150 mm column. Flow rate = 1.5 mL/min. Temperature = 65 °C, injection volume = 10 μL, UV detector at 260 nm. Gradient Method: eluent A. 200 mM TEAA in DI water. B = 200 mM TEAA in ACN. Run time = 20–30 min, post time = 5 min. % B, 0 min = 8, 20 min = 17, 28 min = 25, 30 min = 25.

SAX HPLC Methods. Sample preparation: 10 μL of reaction mixture is diluted to 300 μL with 20% ACN in H_2O. HPLC conditions: Dionex DNApac PA-100; 4 × 250 mm column. Flow rate = 1.3 mL/min. Temperature = 80 °C, injection volume = 10 μL, UV detector at 260 nm. Gradient Method: eluent A. 10 mM $NaClO_4$ and 10 mM tris HCl in EtOH/H_2O (pH = 6.1). B = 300 mM $NaClO_4$ and 10 mM tris HCl in EtOH/H_2O (pH = 6.1). Run time = 10 min, post time = 5 min. % B, 0 min = 5, 4 min = 30, 5 min = 90, 5.2 min = 100, 5.6 min = 8 0, 7 min = 100, 8 min = 0, 10 min = 0.

UPLC-MS Method. UPLC-SQD instrument from Waters was used. Sample preparation: 10 μL of reaction mixture was diluted to 300 μL with 20% ACN in H_2O. HPLC conditions: Acquity BEH C18, 2.1 × 50 mm column. Particle size = 1.7 μm. Flow rate = 0.3 mL/min. Column temperature = 65 °C, injection volume = 1 μL, UV detector at 260 nm. Gradient method: Eluent A = 400 mM HFIP + 16.3 mM TEA/Eluent B = MeOH. Run time = 12 min, post time = 5 min. % B, 0 min = 10, 5 min = 18, 10 min = 22, 12 min = 25, 15 min = 10. A guard column (C-18, OPTI-LYNX, 3 mm) was used to remove salts from crude reaction mixture. MS acquisition = Scan Range: 400–2000 amu. Ionization = ESI. Data = continuum. Scan duration = 0.5 s. Time = 0–20 min. Deconvolution method: Range = 4000–10 000; Resolution = 1.00 Da/Channel; Damage model = Uniform Gaussian. Width at half height = 1.7 Da. Minimum intensity ratios: left = 30%, right = 35%.

Protocol for Knockdown Analysis. Luciferase stable expressed HeLa-Luc cells were plated in 96-well plates at density of 4500 cells per well in 100 μL DMEM media without antibiotics 24 h prior to transfection. siRNA transfection was performed using the RNAiMAX (Invitrogen): Briefly, 0.05 μM siRNA was mixed with Opti-MEMmedia and RNAiMAX and incubated at rt for 15 min. The mixture was then added to the cells. The final siRNA concentration was 1 nM. Cell plates for all time points were transfected at the same time with a medium change at 6 h post-transfection into 100 μL of fresh completed DMEM (DMEM + 10% FBS + Pen/strep). In vitro duration was determined by the luciferase expression post-transfection at 4 time points: day 1, day 2, day 5, and day 7. Addition medium changes were performed at day 2 and day 5 into 100 μL of fresh completed DMEM (DMEM + 10% FBS + Penn/strep). Luciferase levels were determined using the Bright-Glo Luminescence Assay (Promega) and measuring the wells on an Envison instrument (PerkinElmer) according to manufacturer's instructions. To reduce the variation caused by cell viability and cell plating process, the cell viability of the same treatment wells was measured using CellTiter-fluor kit (Promega) according to manufacturer's instructions. This assay

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measured the conserved and constitutive protease activity within live cells and therefore served as a marker of cell viability, using a fluorogenic, cell-permeable peptide substrate (glycyl-phenylalanyl-aminofluorocoumarin; GF-AFC). The fluorescence was measured on the Envision using exciton filter at 405 nm and emission filter at 510 nm. The luciferase expression was normalized to cell viability. The log of this number was calculated to determine the luciferase protein that was degraded (knockdown). A nontargeting siRNA was subtracted from this value to account for nonspecific background.

Walkthrough Click Modified Luc-80 Guide sequences (Tables 1 and 2). Click Modified SSB-291 Passenger Sequences (Tables 3 and 4). RP HPLC Profile of C-18 Purified Luc-80 Guide Sequences. See Figure 5.

RP HPLC of C-18 Purified Luc-80 Guide Sequences. See Figure 6.

SAX HPLC Analysis for a C-18 Purified SSB-291 Passenger Strands. See Figure 7.

Typical UPLC/MS Analysis for a Guide Strand (A1). See Figure 8.

Typical UPLC-MS Analysis for a Guide Strand (C1). See Figure 9.

Passenger Strand (A1) Quadruple-Clicked with Azido-dPEG 24 Alcohol. See Figure 10.

Passenger Strand (A2) Quadruple-Clicked with Azido-dPEG 24 Alcohol. See Figure 11.

Passenger Strand (A3) Quadruple-Clicked with Azido-dPEG 24 Alcohol. See Figure 12.

Passenger Strand (A4) Quadruple-Clicked with Azido-dPEG 24 Alcohol. See Figure 13.

Passenger Strand (A5) Triple-Clicked with Azido-dPEG 24 Alcohol. See Figure 14.

Passenger Strand (A6) Triple-Clicked with Azido-dPEG 24 Alcohol. See Figure 15.

Passenger Strand (A7) Triple-Clicked with Azido-dPEG 24 Alcohol. See Figure 16.

Passenger Strand (A8) Bis-Clicked with Azido-dPEG 24 Alcohol. See Figure 17.

Passenger Strand (A9) Bis-Clicked with Azido-dPEG 24 Alcohol. See Figure 18.

Passenger Strand (A10) Bis-Clicked with Azido-dPEG 24 Alcohol. See Figure 19.

Passenger Strand (A11) Bis-Clicked with Azido-dPEG 24 Alcohol. See Figure 20.

RP HPLC Analysis of Representative Double-Stranded RNA (Luc-80). See Figure 21.

SAX HPLC Analysis for a Double-Stranded siRNA. See Figure 22.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Retting, G. R., and Behlke, M. A. (2012) Progress toward *in vivo* use of siRNAs-II. *Mol. Ther. 20*, 483−512.

(2) Horter, J. A. H., and Walter, N. G. (2007) Chemical modification resolves the asymmetry of siRNA strand degradation in human blood serum. *RNA 13*, 1887−1893.

(3) Ge, Q., Dallas, A., and Lives, H. (2010) Effects of Chemical modification on the potency, serum stability,and immunostimulatory properties of short shRNAs. *RNA 16*, 118−130.

(4) Ren, Y., Hauert, S., Lo, J. H., and Bhatia, S. N. (2012) Identification and characterization of receptor-specific peptides for siRNA delivery. *Nano 6*, 8620−8631.

(5) Kubo, T., Yanagihara, K., Takei, Y., Mihara, K., Morita, Y., and Seyama, T. (2011) Palmitic acid-conjugated 21-nucleotide siRNA enhances gene-silencing activity. *Mol. Pharmaceutics 8*, 2193−2203.

(6) Averick, S., Paredes, E., Dey, S. K., Snyder, K. M., Tapinos, N., Matyjaszewski, K., and Das, S. R. (2013) Autotransfering short intefering RNA through facile covalent polymer escorts. *J. Am. Chem. Soc. 135*, 12508−2511.

(7) Lee, S. H., Chung, B. H., Park, T. G., Nam, Y. S., and Mok, A. H. (2012) Small-interfering RNA (siRNA)-based functional micro- and nanostructures for efficient and selective gene silencing. *Acc. Chem. Res. 45*, 1014−1025.

(8) Kow, S. C., McCarroll, J. M., Valade, D., Boyer, C., Dwarte, T., Davis, T. P., Kavallaris, M., and Bulmus, V. (2011) Dicer-labile PEG conjugates for siRNA delivery. *Biomacromolecules 12*, 4301−4310.

(9) Dominiska, M., and Dykxhoorn, D. M. (2010) Breaking down the barriers: siRNA delivery and endosome escape. *J. Cell. Sci. 123*, 1183−1189.

(10) Watts, J. K., Deleavey, G. F., and Damha, M. J. (2008) Chemically modified siRNA: tools and applications. *Drug Discovery Today 13*, 842−855.

(11) Yameen, B., Choi, W. I., Vilos, C., Swami, A., Shi, J., and Farokhzad, O. C. (2014) Insight into nanoparticle cellular uptake and intracellular targeting. *J. Controlled Release,*.

(12) (a) Fire, A. Z. (2007) Gene silencing by double-stranded RNA. *Angew. Chem., Int. Ed. 46*, 6966−6984. (b) Mello, C. C. (2007) Return to the RNAi world: rethinking gene expression and evolution. *Angew. Chem., Int. Ed. 46*, 6985−6994. (n) Gaynor, J. W., Campbell, B. J., and Cosstick, R. (2010) RNA interference: a chemist's perspective. *Chem. Soc. Rev. 39*, 4169−4184.

(13) Baker, M. (2010) RNA interference: Homing in on delivery. *Nature 464*, 1225−1228.

(14) Cho, I. S., Kim, J., Lim, D. H., Ahn, H.-C., Kim, H., Lee, K.-B., and Lee, Y. S. (2008) Improved serum stability and biophysical properties of siRNAs following chemical modifications. *Biotechnol. Lett. 30*, 1901−1908.

(15) Manoharan, M. (2004) RNA interference and chemically modified small interfereing RNAs. *Curr. Opin. Chem. Biol. 8*, 570−579.

(16) Hammond, S. M., Caudy, A. A., and Hannon, G. J. (2001) Post-transcriptional gene silencing by double-stranded RNA. *Nat. Rev. Genet. 2*, 110−119.

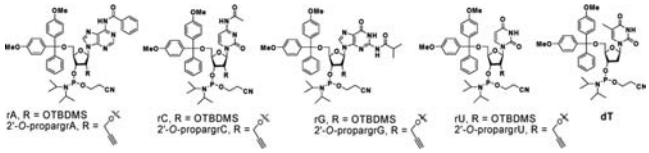
(17) Bell, N. M., and Micklefield, J. (2009) Chemical modification of oligonucleotides for therapeutic, bioanalytical and other applications. *ChemBioChem 10*, 2691−2703.

(18) Youn, P., Chen, Y., and Furgeson, D. Y. (2014) A myristoylated cell-penetrating peptide bearing a transferrin receptor-targeting sequence for neuro-targeted siRNA delivery. *Mol. Pharmaceutics 11*, 486−495.

(19) Yu, D., Penddergraff, H., Liu, J., Kordasiewicz, H. B., Clevealand, D. W., Swayze, E. E., Lima, W. F., Crooke, S. T., Parakash, T. P., and Corey, D. R. (2012) Single-stranded RNAs use RNAi to potently and

allele-selectively inhibit mutant huntingtin expression. *Cell 150*, 895−908.
(20) Abe, N., Abe, H., Nagai, C., Harada, M., Hatakeyama, H., Harashima, H., Ohshiro, T., Nishihara, M., Furukawa, K., Maeda, M., Tsuneda, S., and Ito, Y. (2011) Synthesis, structure, and biological activity of dumbbell-shaped nanocircular RNAs for RNA interference. *Bioconjugate Chem. 22*, 2082−2092.
(21) Phelps, K. J., Ibarra-Soza, J. M., Tran, K., Fisher, A. J., and Beal, P. A. (2014) Click modification of RNA at adenosine: structure and reactivity of 7-ethynyl- and 7-triazolyl-8-aza-7-deazaadenosine in RNA. *Chem. Biol. 9*, 1780−1787.
(22) Kenski, D., Butora, G., Willingham, A. T., Copper, A. J., Fu, W., Qi, N., Soriano, F., Davies, I. W., and Flanagan, W. M. (2012) siRNA-optimized modifications for enhanced in vivo activity. *Mol. Ther.—Nucleic Acids 1*, 1−7.
(23) Broering, R., Real, C., John, M. J., Jahn-Hoffmann, K., Ickenstein, L. M., Kleinehr, K., Paul, A., Gibbert, K., Dittmer, U., Gerken, G., and Schlaak, J. F. (2013) Chemical modifications on siRNAs avoid Toll-likereceptor- mediated activation of the hepatic immune system *in vivo* and *in vitro*. *Int. Immunol. 26*, 35−46.
(24) Peacock, H., Fucini, R. V., Jayalath, P., Ibarra-Soza, J. M., Haringsma, H. J., Flanagan, W. M., Willingham, A., and Beal, P. A. (2011) Nucleobase and ribose modifications control immunostimulation by a microRNA-122-mimetic RNA. *J. Am. Chem. Soc. 133*, 9200−9203.
(25) Yuan, Y., and Wang, W. (2014) Oligonucleotide post synthetic-modifications. In *Comprehensive Organic Synthesis*, 2nd ed., Volume *9*, in print.
(26) Forbes, D. C., and Peppas, N. A. (2014) Polycationic nanoparticles for siRNA delivery: comparing ARGET ATRP abd UV-initiated formulations. *Nano 8*, 2908−2917.
(27) Lu, Z.-X., Liu, L.-T., and Qi, X.-R. (2011) Development of small interfering RNA delivery system using PEI-PEG-APRPG polymer for antiangiogenic vascular endothelial growth factor tumor-targeted therapy. *Int. J. Nanomed. 6*, 1661−1673.
(28) Iverson, F., Yang, C., Dagnaes-Hansen, F., Schaffert, D. H., Kjems, J., and Gao, S. (2013) Optimized siRNA-PEG conjugates for extended blood circulation and reduced urine excretion in mice. *Theranostics 3*, 201−209.
(29) Oishi, M., Nagasaki, Y., Itaka, K., Nishiyama, N., and Kataoka, K. (2005) Lactosylated polyethylene glycol-siRNA conjugate through acid-labile beta-thiopropionate linkage to construct pH-sensitive polyion complex micelles achieving enhanced gene silencing in hepatoma cells. *J. Am. Chem. Soc. 127*, 1624−1625.
(30) Deng, Z. J., Morton, S. W., Ben-Akiva, E., Dreaden, E. C., Shopsowitz, K., and Hammond, P. T. (2013) Layer-by-layer nanoparticles for systemic codelivery of an anticancer drug and siRNA for potential triple-negative breast cancer treatment. *Nano 7*, 9571−9584.
(31) Gujrati, M., Malamas, A., Shin, T., Jin, E., Sun, Y., and Lu, Z.-R. (2014) Multifunctional cationic lipid-based nanoparticles facilitate endosomal escape and reduction-triggered cytosolic siRNA release. *Mol. Pharmaceutics 11*, 2734−2744.
(32) Patil, S. P., Yi, J. W., Bang, E.-K., Jeon, E. M., and Kim, B. H. (2011) Synthesis and efficient siRNA delivery of polyamineconjugated cationic nucleoside lipids. Med. *Chem. Commun. 2*, 505−508.
(33) Draz, M. S., Fang, B. A., Zhang, P., Hu, Z., Gu, S., Weng, K. C., Gray, J. W., and Chen, F. F. (2014) Nanoparticle-mediated systemic delivery of siRNA for treatment of cancers and viral infections. *Thranostics 4*, 872−892.
(34) Park, J. W., Bang, E.-K., Mi, E., and Kim, B. H. (2012) Complexation and conjugation approaches to evaluate siRNA delivery using cationic, hydrophobic and amphiphilic peptides. *Org. Biomol. Chem. 10*, 96−102.
(35) Butora, G., Kenski, D. M., Cooper, A. J., Fu, W., Qi, N., Li, J., Flanagan, W. M., and Davies, I. W. (2011) Nucleoside optimization for RNAi: a high-throughput platform. *J. Am. Chem. Soc. 133*, 16766−16769.
(36) Brennan, L., Cosstick, R., O'Neil, I. A., and Aerschot, A. V. (2007) Syntheses of 2′-C-amidoalkyl and 2′-C-cyanoalkyl containing oligodeoxyribonucleotides and assessment of their hybridization affinity for complementary DNA and RNA. *Tetrahedron 63*, 577−585.
(37) Griffey, R. H., Monia, B. P., Cummins, L. L., Freier, S., Greig, M. J., Guinosso, C. J., Lesnik, E., Manalili, S. M., Mohan, V., Owens, S., Ross, B. R., Sasmor, H., Wancewicz, E., Weiler, K., Wheeler, P. D., and Cook, P. D. (1996) 2′-*O*-aminopropyl ribonucleotides: a zwitteronic modification that enhances the exonuclease resistance and biological activity of antisense oligonucleotides. *J. Med. Chem. 39*, 5100−5109.
(38) Lönnberg, H. (2009) Solid-phase synthesis of oligonucleotide conjugates useful for delivery and targeting of potential nucleic acid therapeutics. *Bioconjugate Chem. 20*, 1065−1094.
(39) Sing, Y., Murat, P., and Defrancq, E. (2010) Recent developments in oligonucleotide conjugation. *Chem. Soc. Rev. 39*, 2054−2070.
(40) New, K., and Brechbiel, M. W. (2009) Growing applications of "click chemistry" for bioconjugation in contemporary biomedical research. *Cancer Biother. Radiopharm. 24*, 289−301.
(41) Simon, M., Zangemeister-W, U., and Plückthun, A. (2012) Facile double-functionalization of designed ankyrin repeat proteins using click and thiol chemistries. *Bioconjugate Chem. 23*, 279−286.
(42) Hein, C. D., Liu, X.-M., and Wang, D. (2008) Click chemistry, a powerful tool for pharmaceutical sciences. *Pharm. Res. 25*, 2216−2230. (d) Kolb, H. C., and Sharpless, K. B. (2003) The growing impact of click chemistry on drug discovery. *Drug Discovery Today 8*, 1128−1136.
(43) Amblard, F., Cho, J. H., and Schinazi, R. F. (2009) Cu(I)-catalyzed Husigen azide-alkyne 1,3-dipolar cycloaddition reaction in nucleoside, nucleotide and oligonucleotide chemistry. *Chem. Rev. 109*, 4207−4220.
(44) Sioud, M. (2006) Innate sensing of self and non-self RNAs by Toll-like receptors. *Trends Mol. Med. 12*, 167−176.
(45) Jackson, A. L., Burchard, J., Leake, D., Reynolds, A., Schelter, J., Guo, J., Johnson, J. M., Karpilow, J., Nichols, K., Marshall, W., Khvorova, A., and Linsley, P. S. (2006) Position-specifc chemical modification of siRNAs reduces "off-target" transcript silencing. *RNA 12*, 1197−1205.
(46) Koller, E., Propp, S., Murray, H., Lima, W., Bhat, B., Prakash, T. P., Allerson, C. R., Swayze, E. E., Marcusson, E. G., and Dean, N. M. (2006) Competition for RISC binding predicts *in vitro* potency of siRNA. *Nucleic Acids Res. 34*, 4467−4476.
(47) Shukla, S., Sumaria, C. S., and Pradeepkumar, P. I. (2010) Exploring chemical modifications for siRNA therapeutics: a structural and functional outlook. *ChemMedChem 5*, 328−349.
(48) Allerson, C. R., Sioufi, N., Jarres, R., Prakash, T. P., Naik, N., Berdeja, A., Wanders, L., Griffey, R. H., Swayze, E. E., and Bhat, B. (2005) Fully 2′-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering RNA. *J. Med. Chem. 48*, 901−904.
(49) Hamm, S., Latz, E., Hangel, D., Müeller, T., Yu, P., Golenbock, D., Sparwasser, T., Wagner, H., and Bauer, S. (2010) Alternating 2′-*O*-ribose methylation is a universal approach for generating non-stimulatory siRNA by acting as TLR7 antagonist. *Immunobiology 215*, 559−569.
(50) Eberle, F., Giessler, K., Deck, C., Heeg, K., Peter, M., Richert, C., and Dalpke, A. H. J. (2008) Modifications in siRNA that separate immunostimulation from RNA interference. *Immunology 180*, 3229−3237.
(51) Tluk, S., Jurk, M., Forsbach, A., Weeratna, R., Samulowitz, U., Krieg, A. M., Bauer, S., and Vollmer, J. (2009) Sequences derived from self-RNA containing certain natural modifications act as suppressors of RNA-mediated inflammatory immune responses. *Int. Immunol. 21*, 607−619.
(52) Sioud, M., Furset, G., and Cekaite, L. (2007) Suppression of immunostimulatory siRNA-driven innate immune activation by 2′-modified RNAs. *Biochem. Biophys. Res. Commun. 361*, 122−126.
(53) Yamada, T., Peng, C. G., Matsuda, S., Addepalli, H., Jayaprakash, K. N., Alam, M. R., Mills, K., Maier, M. A., Charisse, K., Sekine, M.,

Manoharan, M., and Rajeev, K. G. (2011) Versatile site-specific conjugation of small molecules to siRNA using click chemistry. *J. Org. Chem. 76*, 1198−1211.
(54) Jayaprakash, K. N., Peng, C. G., Butler, D., Varghese, J. P., Maier, M. A., Rajeev, K. G., and Manoharan, M. (2010) Non-nucleoside building blocks for copper-assisted and copper-free click chemistry for the efficient synthesis of RNA conjugates. *Org. Lett. 12*, 5410−5413.
(55) Zewge, D., and Gosselin, F. (2013) Post-synthetic chemical modifications of RNA at the 2′-position of the ribose ring via "click" chemistry, US Patent No 20130116419 A1.
(56) Wllibald, J., Harder, J., Sparrer, K., Conzelmann, K.-K., and Carell, T. (2012) Click-modified anandamide siRNA enables delivery and gene silencing in neuronal and immune cells. *J. Am. Chem. Soc. 134*, 12330−12333.
(57) Yu, S. S., Lau, C. M., Barham, W. J., Onishko, H. M., Nelson, C. E., Li, H., Smith, C. A., Yull, F. E., Duvall, C. L., and Giorgio, T. D. (2013) Macrophage-specific RNA interference targeting via "click", mannosylated polymeric micelles. *Mol. Pharmaceutics 10*, 975−987.
(58) Pujari, S. S., Leonard, P., and Seela, F. (2014) Oligonucleotides with "clickable" sugar residues: synthesis, duplex stability, and terminal versus central interstrand cross-linking of 2′-O-propargylated 2-aminoadenosine with a bifunctional azide. *J. Org. Chem. 79*, 4423−4437.
(59) O'Mahony, A. M., Ogier, J., Desgranges, S., Cryan, J. F., Darcy, R., and O'Driscoll, C. M. (2012) A click chemistry route to 2-functionalised PEGylated and cationic *β*-cyclodextrins: co-formulation opportunities for siRNA delivery. *Org. Biomol. Chem. 10*, 4954−4960.
(60) El-Sagheer, A. H., and Brown, T. (2010) New strategy for the synthesis of chemically modified RNA constructs exemplified by hairpin and hammerhead ribozymes. *Proc. Natl. Acad. Sci. U. S. A. 107*, 15329−15334.
(61) Peacock, H., Fostvedt, E., and Beal, P. (2010) Minor-groove-modulating adenosine replacements control protein binding and RNAi activity in siRNAs. *ACS Chem. Biol. 5*, 1115−1124.
(62) Grajkowski, A., Cieslak, J., Gapeev, A., Schindler, C., and Beaucage, S. L. (2010) Convenient synthesis of a propargylated cyclic (3′−5′) diguanylic acid and its ″click″ conjugation to a biotinylated azide. *Bioconjugate Chem. 21*, 2147−2152.
(63) Paredes, E., and Das, S. R. (2011) Click chemistry for rapid labeling and ligation of RNA. *ChemBioChem 12*, 125−131.
(64) Jao, C. Y., and Salic, A. (2008) Exploring RNA transcription and turnover in vivo by using click chemistry. *Proc. Natl. Acad. Sci. U.S.A. 105*, 15779−15784.
(65) Kolb, H. C., Finn, M. G., and Sharpless, K. B. (2001) Click chemistry: diverse chemical function from a few good reactions. *Angew. Chem., Int. Ed. 40*, 2004−2021.
(66) Tornøe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem. 67*, 3057−3064.
(67) Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective ligation of azides and terminal alkynes. *Angew. Chem., Int. Ed. 41*, 2596−2599.
(68) Beaucage, S. L., and Caruthers, M. H. (1981) Deoxynucleoside phosphoramidites: a new class of key intermediates for deoxypolynucleotide synt. *Tetrahedron Lett. 22*, 1859−1862.
(69) Wincott, F. E. (2000) Strategies for oligoribonucleotide synthesis according to the phosphoramidite method. *Curr. Protoc. Nucleic Acid Chem.*, 3.5.1−3.5.12.
(70) Zewge, D., Gosselin, F., Sidler, R., DiMichele, L., and Cvetovich, R. J. (2010) A safe and practical procedure for global deprotection of oligoribonucleotides. *J. Org. Chem. 75*, 5305−5307.
(71) Colletti, S. L., Gosselin, F., Jadhav, V. R., Shaw, A. W., Tellers, D. M., Tucker, T. J., Yuan, Y., and Zewge, D. *Novel single chemical entities and methods for delivery of oligonucleotides*, US Patent No 201/0253168 A1, Sept 26, 2013.
(72) Campochiaro, P. A. (2006) Potential applications for RNAi to probe pathogenesis and develop new treatments for ocular disorders. *Gene Ther. 13*, 559−562.
(73) Wei, J., Jones, J., Kang, J., Card, A., Krimm, M., Hancock, P., Pei, Y., Ason, B., Payson, E., Dubinina, N., Cancilla, M., Stroh, M., Burchard, J., Sachs, A. B., Hochman, J. H., Flanagan, W. M., and Kuklin, N. A. (2011) RNA-induced silencing complex-bound small interfering RNA is a determinant of RNA interference-mediated gene silencing in mice. *Mol. Pharmacol. 79*, 953−963.
(74) *2′-O*-propargyl phosphoramidites used for high throughput screening: Note: In addition to being essential scafolds for [3 + 2]



cycloaddition reactions (copper catalyzed click reactions), propargyl groups were selected due to their stability under basic, acidic, and oxidation conditions required for nucleotide assembly.
(75) Rayner, S., Brignac, S., Bumeister, R., Belosludtsev, Y., Ward, T., Grant, O., O'Brien, K., Evans, G. A., and Garner, H. R. (1998) MerMade: an oligodeoxyribonucleotide synthesizer for high throughput oligonucleotide production in dual 96-well plates. *Genome Res. 8*, 741−747.
(76) Beaucage, S. L., and Caruthers, M. H. (2000) Synthetic strategies and parameters involved in the synthesis of oligodeoxyribonucleotides according to the phosphoramidite method. *Curr. Protoc. Nucleic Acid Chem.*, 3.3.1−3.3.20.
(77) Semenyuk, A., Ahnfelt, M., Nilsson, C. E., Hao, X. Y., Földesi, A., Kao, Y.-S., Chen, H.-H., Kao, W−C., and Peck, K. (2006) Cartridge-based high-throughput purification of oligonucleotides for reliable oligonucleotide arrays. *Anal. Biochem. 356*, 132−141.
(78) Lyophilized duplexes are stable for up to one year at −20 °C (HPLC, LC/MS studies). In this study biological evaluations were performed with in two to four weeks of synthesis and duplexing of modified oligonucleotides.