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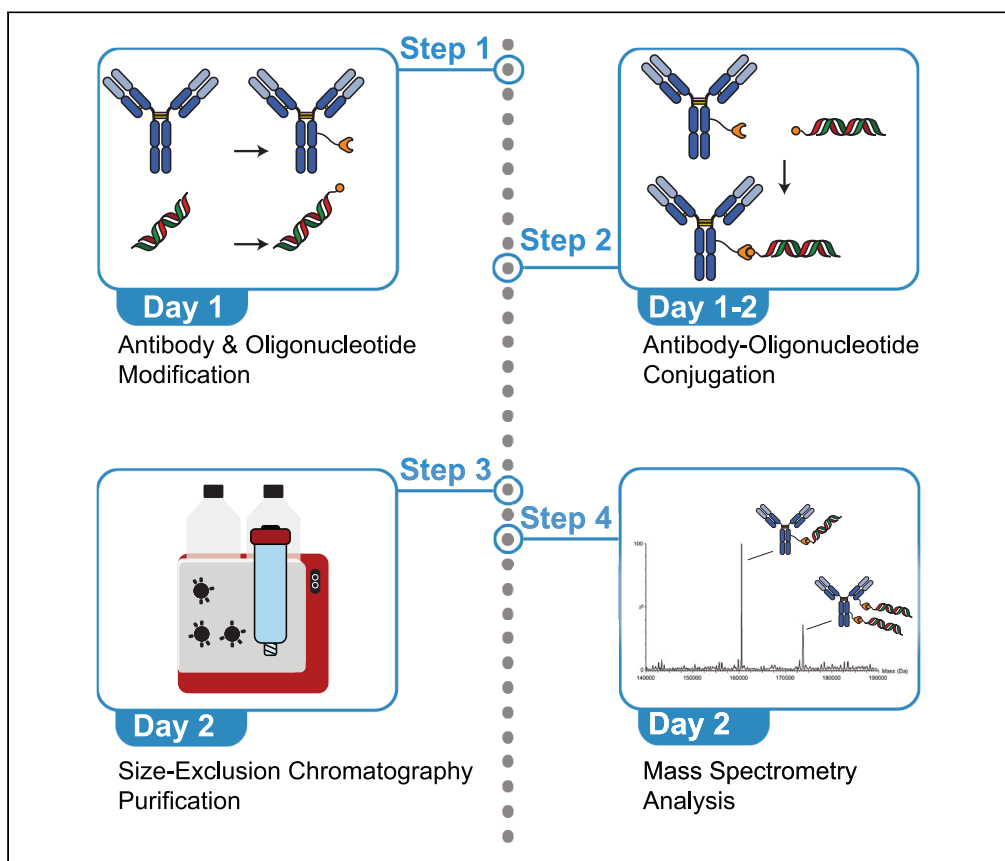
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Antibody-oligonucleotide conjugates (AOCs) are a fast-expanding modality for targeted delivery of therapeutic oligonucleotides to tissues. Here, we present a protocol to generate, purify, and analyze AOCs from off-the-shelf antibodies. We describe steps to conjugate single/double-stranded oligonucleotides bearing amine handles to linkers and, then, to antibodies using well-established chemistry. In addition, we provide details regarding the purification techniques and analytical methods suitable for AOC. This protocol can be applied for several purposes where AOC is a modality of interest.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Straightforward method to conjugate amino-modified oligonucleotides to linkers

Steps for hybridizing linker-oligonucleotide to its complementary strand

Fast and reproducible lysine conjugation of azide groups to off-the-shelf antibodies

State-of-the-art purification and analysis methods of antibody-siRNA conjugates

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Protocol

Protocol to generate, purify, and analyze antibody-oligonucleotide conjugates from off-the-shelf antibodies

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SUMMARY

Antibody-oligonucleotide conjugates (AOCs) are a fast-expanding modality for targeted delivery of therapeutic oligonucleotides to tissues. Here, we present a protocol to generate, purify, and analyze AOCs from off-the-shelf antibodies. We describe steps to conjugate single/double-stranded oligonucleotides bearing amine handles to linkers and, then, to antibodies using well-established chemistry. In addition, we provide details regarding the purification techniques and analytical methods suitable for AOC. This protocol can be applied for several purposes where AOC is a modality of interest. For complete details on the use and execution of this protocol, please refer to Rady et al.¹

BEFORE YOU BEGIN

Therapeutic oligonucleotides are a class of single or double stranded nucleic acids that are used to modulate the expression of a wide range of proteins involved in numerous diseases.² Among the different modalities that have been developed, the most preeminent ones are mRNA, antisense oligonucleotide (ASO) and small interfering RNA (siRNA).³ While mRNAs are administered to induce the expression of a target protein, ASOs and siRNAs induce its knockdown. From a mechanistic point of view, the last two can specifically decrease the expression of a target protein by either blocking or degrading its coding mRNA by complementary association.⁴ The capacity of siRNA and ASO to virtually target any mRNA demonstrates their high versatility and ability to access so-called “undrugable” targets.⁵

While being promising therapeutic agents, these molecules are extremely hydrophilic and negatively charged, leading to very poor membrane-permeability.⁶ To facilitate their delivery to the site of action, encapsulation inside lipidic and cationic nanoparticles or active targeting of chemically-stabilized oligonucleotides via conjugation to tri-GalNAc are the current gold-standard methods.⁷ However, these two technologies are limited to targeting the liver, and expanding to other organs is highly challenging, thus dampening the clinical uses of therapeutic



oligonucleotides.^{8,9} To expand the field of application to non-hepatic diseases, conjugation of these molecules to monoclonal antibodies has been envisioned as a viable approach^{10,11} that recently reached clinical trials.¹²

Here, we describe a protocol to generate and analyze antibody-siRNA conjugates, although this method is also applicable for antibody-ASO conjugates. This protocol consists of four major steps: (1) modification of single stranded RNA with a bicyclo[6.1.0]nonyne (BCN)-bearing linker, (2) hybridization with the complementary strand, (3) pre-functionalization of the antibody with an azide moiety via lysine conjugation,¹³ and (4) conjugation of siRNA to antibody via strain-promoted alkyne-azide cycloaddition (SPAAC). Furthermore, we briefly discuss the analytical characterization of the obtained conjugates.

Preparation of the oligonucleotide upon arrival

⌚ Timing: 5 min

A complete list of reagents and equipment can be found in the “[key resources table](#)” and “[materials and equipment](#)”.

⚠ **CRITICAL:** All the steps involving oligonucleotide, especially RNA, must be performed under a biosafety cabinet cleaned with RNaseZap (Fisher Scientific, cat. 10708345) to minimize nuclease-mediated degradation of the oligonucleotide.

1. Prior to opening any vial containing the oligonucleotide, centrifugate the vial for 1 min at 4000 g.
2. Dissolve the oligonucleotide with DEPC-treated water to an estimated concentration of 1 mM.
3. Take 1 μ L of this solution and add 9 μ L of DEPC-treated water to prepare a solution at 100 μ M.
4. Measure the absorbance of the latter solution at 260 nm and divide it by the molar extinction coefficient (provided by manufacturer) to determine the actual molar concentration of your solution.

⚠ **CRITICAL:** Due to the chemistry used in this protocol (relying on amide coupling), the use of amine-containing buffers, such as Tris or amino-acid based, must be avoided.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
EphA2 antibody (5 g/L)	Internal (Sanofi)	N/A
Chemicals, peptides, and recombinant proteins		
IgZERO (EndoS enzyme)	Genovis	A0-IZ1-010
IonHance ammonium acetate pH 6.8 concentrate	Waters	186009705
BCN-PEG6-PFP	Synthesized in-house	N/A
Boric acid	Merck	B0394-100G
Sodium tetraborate decahydrate	Merck	S9640-500G
DEPC-treated water	Merck	693520-1L
Lithium perchlorate	Merck	205281-100G
Triethylammonium acetate buffer, HPLC-grade	Merck	69372-1L
HPLC-grade water	Merck	270733-2.5L
HPLC-grade acetonitrile	Merck	34851-2.5L
LC-MS-grade water	Merck	1153332500
Tris base	Merck	T1503-500G
Sodium chloride	Merck	S9888-1KG
Anhydrous DMSO	Merck	276855-100ML

(Continued on next page)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Continued		
Critical commercial assays		
BCA protein assay kit	Thermo Fisher Scientific	23225
DPBS 1×, no calcium, no magnesium	Thermo Fisher Scientific	14190144
Deposited data		
Targeted delivery of immune-stimulating bispecific RNA, inducing apoptosis and anti-tumor immunity in cancer cells	https://www.cell.com/iscience/fulltext/S2589-0042(24)00289-X	N/A
Oligonucleotides		
siPLK1 (sense strand, (5'ppp)-AGAUCACCCUCCUAAAUA)	Eurogentec	N/A
siPLK1 (antisense strand, UAUUAAGGAGGGUGAUCU)	Eurogentec	N/A
Software and algorithms		
Unicorn 7.3	Cytiva	https://www.cytivalifesciences.com/en/us/shop/chromatography/software/unicorn-7-p-05649
Empower 2	Waters	https://www.waters.com/waters/library.htm?locale=en_US&lid=1529008
UNIFI	Waters	https://www.waters.com/waters/en_US/UNIFI-Scientific-Information-System/nav.htm?cid=134801359
Other		
NanoDrop One/One ^c microvolume UV-vis spectrophotometer	Thermo Fisher Scientific	N/A
Bio-Spin P-30 gel filtration spin columns	Bio-Rad	7326232
Corning Costar Spin-X centrifuge tube filters	Thermo Fisher Scientific	10310361
Äkta Pure chromatography system	Cytiva	N/A
Superdex 200 increase 10/300 GL column	Cytiva	28990944
Hypersil GOLD C18 selectivity HPLC column, 3 μm, 2.1 mm × 100 mm	Thermo Fisher Scientific	25003-102130
ACQUITY UPLC protein BEH, 200 Å, 1.7 μm, 2.1 mm × 150 mm	Waters	186008471
DNAPac PA200, 8 μm, 9 mm × 250 mm	Thermo Fisher Scientific	063421
SYNAPT G2 HDMS mass spectrometer	Waters	N/A
Invitrogen RNaseZap	Fisher Scientific	10708345
Megafuge 16R centrifuge	Thermo Fisher Scientific	75004270
TX-400 4 × 400 mL swinging bucket rotor, ClickSeal biocontainment lids	Thermo Fisher Scientific	75003656
Microliter 30 × 2 mL fixed angle rotor	Thermo Fisher Scientific	75003652

MATERIALS AND EQUIPMENT

Preparation of the reaction buffer

- Prepare the Reaction Buffer Part A and Reaction Buffer Part B as explained in Table 1 and in Table 2.
- Adjust the pH of the Reaction Buffer part A with Reaction Buffer part B until a pH meter indicates pH 8.0.
- Store the buffer at +4°C and discard after 2 months.

Preparation of the precipitation buffer

- Prepare the Precipitation Buffer as explained in Table 3. No need to adjust the pH.

Table 1. Recipe of Reaction Buffer Part A

0.1 M Borate	Final concentration	Reagent	Amount
	0.1 M	Sodium tetraborate decahydrate	1.90 g
	N/A	DEPC-treated water	50 mL

Table 2. Recipe of Reaction Buffer Part B

0.1 M Boric acid	Final concentration	Reagent	Amount
	0.1 M	Boric acid	0.31 g
	N/A	DEPC-treated water	50 mL

- Store the buffer at room temperature and discard after 2 months.

△ **CRITICAL:** Handle lithium perchlorate with gloves, and wash hands thoroughly after use with water. Keep away from any flame or hot surfaces.

Method 1: RP-HPLC

The method shown in [Table 4](#) is used to analyze and purify oligonucleotide-linker conjugates.

Preparation of RP-HPLC buffer A

- Prepare the RP-HPLC Buffer A as explained in [Table 5](#). No need to adjust the pH.
- Store the buffer at room temperature and discard after 2 months.

Preparation of RP-HPLC buffer B

- Prepare the RP-HPLC Buffer B as explained in [Table 6](#). No need to adjust the pH.
- Store the buffer at room temperature and discard after 2 months.

Method 2: Preparative SEC

Antibody-siRNA conjugates were purified by ÄKTA pure system (Cytiva) using the procedure shown in [Table 7](#). Prior to the purification, the column was sanitized by eluting 0.5 M NaOH for 2 Column Volume (CV), followed by eluting DPBS 1× for 2 CV to reduce endotoxin levels as much as possible. A 1.0 mL injection loop was used to inject the conjugates.

Method 3: Native SEC-ESI-MS analysis

The identity and the degree of conjugation of antibody-siRNA conjugates was determined by SEC-ESI-MS using the procedure shown in [Table 8](#). Thoroughly wash the system with LC-MS-grade water after use to remove remaining salts.

Preparation of SEC-MS buffer

- Prepare the SEC-MS Buffer as explained in [Table 9](#). No need to adjust the pH.
- Store the buffer at +4°C and discard after 1 month.

Method 4: SAX-HPLC

The degree of conjugation can be determined by SAX-HPLC using the procedure shown in [Table 10](#).

Preparation of SAX-HPLC buffer A

- Prepare the SAX-HPLC Buffer A as explained in [Table 11](#).
- Adjust the pH using 0.1 M HCl and 0.1 M NaOH to pH 8.0.
- Store the buffer at room temperature and discard after 2 months.

Table 3. Recipe of Precipitation Buffer

3.0 M lithium perchlorate	Final concentration	Reagent	Amount
	3.0 M	Lithium perchlorate	15.96 g
	N/A	DEPC-treated water	50 mL

Table 4. System information and method for RP-HPLC

System information	Column	Hypersil Gold C18, 3 μ m, 2.1 \times 100 mm
	Column temperature	25°C
	Flow rate	0.8 mL/min
	Detection wavelength	260 and 280 nm
	Loading buffer	Any, the column will desalt the sample
	Injection volume	100 μ L at 10 μ M
	Buffer A	RP-HPLC Buffer A
	Buffer B	RP-HPLC Buffer B
Method	Time	Buffer composition
	0.00 min	0% B
	11.00 min	75% B
	11.10 min	100% B
	13.10 min	100% B
	13.60 min	0% B
	15.00 min	0% B

Preparation of SAX-HPLC buffer B

- Prepare the SAX-HPLC Buffer B as explained in [Table 12](#).
- Adjust the pH using 0.1 M HCl and 0.1 M NaOH to pH 8.0.
- Store the buffer at room temperature and discard after 2 months.

STEP-BY-STEP METHOD DETAILS

Part 1: Conjugation of a linker to amine-modified oligonucleotide

⌚ Timing: 6 h

This step enables the preparation of BCN-linker-oligonucleotide conjugates for further conjugation on antibodies using amine-reactive linkers.

1. Conjugation of amino-modified oligonucleotides (example on 100 nmol scale, [Figure 1](#)).
 - a. In a standard sterile 1.5 mL plastic tube, dilute 100 nmol of oligonucleotide (1 mM in water, 100 μ L) with 3 volumes of Reaction Buffer (300 μ L).

⚠ CRITICAL: This step is to obtain a slightly basic pH to make sure the primary amine is reactive, but not too basic to limit 2'OH-mediated phosphodiester cleavage of RNA ([Figure 2](#)). We thus highly suggest confirming the pH at this stage using a pH meter or pH paper.

- b. Add DMSO until 20% v/v (100 μ L).
- c. Prepare a solution of a heterobifunctional amine-reactive and strained-alkyne-containing linker at 100 mM in anhydrous DMSO. In this example, we have used a home-made activated ester, bicyclo[6.1.0]nonyne-PEG₆-PFP linker (BCN-PEG₆-PFP, [Figure 1](#)). Alternatively, any commercially available activated ester linkers bearing a strained-alkyne end-group (e.g., DBCO-PEG₄-TFP, Thermo Fisher Scientific, cat. C20039) would work with similar conditions.

⚠ CRITICAL: Anhydrous DMSO (e.g. Merck, cat. 276855-100ML) must be used, especially for storage, to limit linker hydrolysis.

Table 5. Recipe of RP-HPLC Buffer A

0.1 M TEAAc in water	Final concentration	Reagent	Amount
	0.1 M	1.0 M Triethylammonium acetate buffer	200 mL
	N/A	HPLC-grade water	1.8 L

Table 6. Recipe of RP-HPLC Buffer B

0.1 M TEAAc in acetonitrile	Final concentration	Reagent	Amount
	0.1 M	1.0 M Triethylammonium acetate buffer	200 mL
	N/A	HPLC-grade acetonitrile	1.8 L

d. Add the linker to the oligonucleotide solution (20 equiv., 2 μ mol, 20 μ L) and incubate the reaction at 20°C for up to 4 h, monitored by RP-HPLC every 30 min (see [Method 1](#)). The reaction should be stopped in all cases after 4 h maximum to limit RNA hydrolysis. We recommend analyzing the starting oligonucleotide as a reference to compare with the conjugated material.

Note: If using *N*-hydroxysuccinimide (NHS) esters instead of TFP (tetrafluorophenyl) or PFP (pentafluorophenyl) esters, an instant precipitation of the NHS can be observed. This will not interfere with the reaction.

Note: If using a more activated amine-reactive function, such as activated carbonate, the amount of linker used in the reaction should be increased to 50 equiv. to compensate for the linker hydrolysis. The amount of DMSO can also be doubled to compensate for any low solubility of the linker.

- e. After completion of the reaction, add 10% v/v of Precipitation Buffer (52 μ L).
- f. Directly after addition of the precipitation buffer, add 3 volumes of cold acetone (kept at -20°C , 1716 μ L) to the reaction at room temperature, then vortex the sample for 10 s. An instant precipitation of the oligonucleotide should be observed.
- g. Leave the reaction mixture at -20°C for 30 min to maximize the precipitation of the oligonucleotide.
- h. Centrifuge the mixture at 15000 g at $+4^{\circ}\text{C}$ for 5 min and discard the supernatant by pipetting while avoiding aspirating the pellet.
- i. Repeat steps 1.f to 1.h one more time and dry the pellets under a nitrogen stream or using a speedvac.

Pause point: If the reader cannot proceed with the following steps straight away due to a lack of time, we recommend to do a pause now by storing this intermediate dry at -20°C overnight and proceed with the next steps the following day.

- j. Resuspend the linker-oligonucleotide conjugate to approximately 250 μ M with DEPC-treated water (400 μ L).
PAUSE POINT: If the reaction did not reach completion after 4 h (as monitored by RP-HPLC), the oligonucleotide must be purified by RP-HPLC after the precipitation step using [Method 1](#).
- k. Confirm the actual concentration of the oligonucleotide by measuring the absorbance at 260 nm using a UV-Vis spectrometer (e.g., Nanodrop) and dividing it by the molar extinction coefficient at 260 nm of the oligonucleotide-linker conjugate.

Table 7. System information and method for SEC

System information	Column	Superdex 200 Increase 10/300 GL
	Flow rate	0.75 mL/min
	Detection wavelength	280 nm
	Loading buffer	Any, the column will desalt the sample
	Injection volume	Whole sample, maximum 500 μ L
	Buffer A	DPBS 1x, pH 7.4
Method	Time	Buffer composition
	0.00 min	100% A
	25.00 min	100% A

Table 8. System information and method for SEC-ESI-MS analysis

System information	Column	Protein BEH SEC, 1.7 μm, 200 Å, 2.1 × 150 mm
	Column temperature	15°C
	Flow rate	0.1 mL/min
	Ionization mode	ESI+
	Capillary voltage	3 kV
	Cone voltage	180 V
	Pressure	6 mbar
	Scan time	1.5 s
	m/z range	1000–10000
	Detection wavelength	214 and 280 nm
	Loading buffer	Any, the column will desalt the sample
	Injection volume	10 μL at 5 μM
	Buffer A	SEC-MS Buffer
Method	Time	Buffer composition
	0.00 min	100% A
	6.00 min	100% A

△ **CRITICAL:** Some linkers also absorb at 260 nm, their extinction coefficient must be considered for the determination of the concentration. If the linker does not absorb at 260 nm, the molar extinction coefficient of the starting oligonucleotide should be used. If the linker absorbs at 260 nm, then the molar extinction coefficient of the oligonucleotide-linker conjugate can be calculated using the following formula:

$$e_{\text{Oligo-linker}}^{260 \text{ nm}} (M^{-1} \cdot \text{cm}^{-1}) = e_{\text{Oligo}}^{260 \text{ nm}} (M^{-1} \cdot \text{cm}^{-1}) + e_{\text{Linker}}^{260 \text{ nm}} (M^{-1} \cdot \text{cm}^{-1})$$

1. Check the purity using RP-HPLC (see [Method 1](#)).
2. Hybridization of linker-oligonucleotide to its complementary strand (example on 20 nmol).

Note: This step can be skipped if the reader wants to prepare antibody-ASO conjugates.

- a. Add the complementary oligonucleotide strand (1.5 equiv., 30 nmol, 120 μL, 250 μM in water) to the solution of linker-oligonucleotide strand (1 equiv., 20 nmol, 80 μL, 250 μM in water).
- b. Incubate the solution at the melting point of both strands (in our case, 65°C) for 5 min, then slowly cool down the temperature to 25°C over 15 min by unplugging the heater.

Note: No need to purify at this stage, as the excess of complementary strand will be discarded during the final purification step by size-exclusion chromatography. While we have never studied the stability of this intermediate, we recommend to not store it and to proceed with the next steps straight away.

Part 2: Preparation of antibody-azide conjugates

⌚ Timing: 1 h

This step enables the preparation of antibody-azide conjugates.

Table 9. Recipe for SEC-MS buffer

	Final concentration	Reagent	Amount
0.1 M Ammonium acetate buffer pH 6.8	0.1 M	IonHance Ammonium Acetate pH 6.8 Concentrate	100 mL
	N/A	LC-MS-grade water	900 mL

Table 10. System information and method for SAX-HPLC

System information	Column	DNAPAc PA200, 8 μ m, 9 \times 250 mm
	Column temperature	25°C
	Flow rate	2.5 mL/min
	Detection wavelength	260 and 280 nm
	Loading buffer	Sample must be diluted with pure water
	Injection volume	100 μ L at 5 μ M
	Buffer A	SAX-HPLC Buffer A
	Buffer B	SAX-HPLC Buffer B
Method	Time	Buffer composition
	0.00 min	12% B
	1.00 min	12% B
	17.00 min	72% B
	18.00 min	100% B
	20.00 min	100% B
	20.10 min	12% B
	22.10 min	12% B

3. Preparation of antibody-azide conjugates (example on 1 mg of antibody, [Figure 3](#)).
 - a. Prepare a solution of a heterobifunctional amine-reactive and azide-containing linker at 20 mM in anhydrous DMSO. In this example, we have used a home-made acyl fluoride, 4-azido-benzoyl fluoride (ABF, [Figure 3](#)).¹³ Alternatively, other amine-reactive linkers bearing an azide group (e.g., azido-PEG₄-NHS, Thermo Fisher Scientific, cat. 26130) would work under classical lysine conjugation conditions (not described herein).¹⁴

△ CRITICAL: Anhydrous DMSO must be used to limit hydrolysis of the amine-reactive group. However, we found out that the half-life of ABF in DMSO is extremely short (< 30 min), thus the stock solution cannot be stored and must be discarded after use. For this reason, a freshly prepared solution must be used each time.

- b. Add ABF (3.0 equiv., 20.25 nmol, 1.0 μ L, 20 mM in DMSO) to the antibody (1.0 equiv., 6.75 nmol, 1.0 mg, 200 μ L, 5 g/L stock in PBS 1 \times).
- c. Incubate for 30 min at 37°C in the dark.
- d. Purify the antibody-azide conjugates using gel filtration spin columns, pre-equilibrated with PBS 1 \times according to [manufacturer's protocol](#).
- e. Filter the product using centrifuge tube filters (0.22 μ m, 1 min, 15 000 g) to remove potential bacteria.
- f. Determine the concentration of the antibody by measuring the absorbance at 280 nm using a UV-Vis spectrometer (e.g., Nanodrop) and dividing it by the extinction coefficient at 280 nm of the antibody.
- g. For mass spectrometry analyses, deglycosylate 25 μ g of conjugate using EndoS enzyme according to [manufacturer's instructions](#). We recommend analyzing the starting antibody as a reference to compare with the conjugated material.
- h. This intermediate can be stored at +4°C for up to a month.

Note: Antibody-azide conjugates can be analyzed by either denaturing or native LC-ESI-MS analysis, but as an intermediate for antibody-siRNA conjugates (that must be analyzed by

Table 11. Recipe for SAX-HPLC Buffer A

50 mM Tris, 10% MeCN, pH 8.0	Final concentration	Reagent	Amount
	50 mM	Tris base	12.10 g
	N/A	HPLC-grade water	1.8 L
	N/A	HPLC-grade acetonitrile	200 mL

Table 12. Recipe for SAX-HPLC Buffer B

50 mM Tris, 1.0 M NaCl, 10% MeCN, pH 8.0	Final concentration	Reagent	Amount
	50 mM	Tris base	12.10 g
	1.0 M	Sodium chloride	116.8 g
	N/A	HPLC-grade water	1.8 L
	N/A	HPLC-grade acetonitrile	200 mL

native SEC-ESI-MS), we highly recommend analyzing antibody-azide conjugates by native MS (see [Method 2](#)) as well for consistency.

Part 3: Preparation of antibody-oligonucleotide conjugates

⌚ Timing: 5 min, then 24 h of incubation, then 2 h

This step enables the preparation of antibody-oligonucleotide conjugates.

4. Conjugation of oligonucleotide to antibodies using Strain-Promoted Alkyne-Azide Cycloaddition (SPAAC, example on 1 mg of antibody-azide conjugate, [Figure 4](#)).
 - a. Add the linker-containing double-stranded oligonucleotide (3.0 equiv., 20.25 nmol, 202.7 μ L, 100 μ M in water) to the antibody-azide conjugate (1.0 equiv., 6.75 nmol, 1 mg, 100 μ L, 10 g/L in PBS 1 \times).
 - b. Incubate for 24 h at 25°C.
 - c. Purify the reaction mixture by size-exclusion chromatography (SEC, see [Method 3](#)) by injecting the whole solution of conjugated material. We recommend analyzing the starting antibody as a reference to compare with the conjugated material.

⚠ CRITICAL: Do not purify using spin/gravity gel filtration columns nor centrifugal concentrators, as these are not efficient to fully eliminate the excess siRNAs.

Note: Multiple peaks can be observed by SEC ([Figure 5](#)), all the peaks with short retention times (9–12 min) are different degree of conjugation of the antibody-siRNA conjugate and all these peaks should be collected and merged. The following peak (12–14 min) is the unconjugated antibody and the last peaks (>15 min, not shown) are the excess oligonucleotides. To avoid having material that can later interfere with bioassays, only the antibody-siRNA-corresponding peaks should be collected.

- d. Concentrate the fractions containing the product by centrifugation (50 kDa MWCO, 12 min, 15 000 g).
- e. Filter the product using centrifuge tube filters (0.22 μ m, 1 min, 15 000 g) prior to any cell-based bioassay to remove potential bacterial contamination.
- f. Determine the protein concentration by a BCA assay according to [manufacturer's protocol](#).
- g. For mass spectrometry analyses, deglycosylate 25 μ g of conjugate using EndoS enzyme according to [manufacturer's instructions](#) and analyze the samples by native SEC-ESI-MS (see [Method 2](#)) to determine the degree of conjugation and the identity of the molecule.
- h. Store this conjugate at -80°C for long-term storage, and at $+4^{\circ}\text{C}$ for immediate use.

⚠ CRITICAL: Unlike the antibody-azide conjugate, a native SEC-MS method must be used as antibody-siRNA conjugates cannot be observed by any denaturing mass spectrometry method due to denaturation of the double-stranded oligonucleotide.

Note: Mass spectrometry can underestimate the average degree of conjugation of these constructs. Indeed, the added negative charges from the siRNA will impact the overall

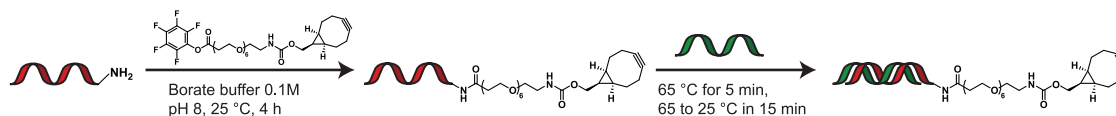


Figure 1. Conjugation of amine-modified oligonucleotide using BCN-PEG6-PFP linker and hybridization with complementary sequence

charge of the conjugate, dramatically decreasing the observed signal of high degrees of conjugation (>3) in positive mode. As an orthogonal method, we recommend performing SAX-HPLC analysis (see [Method 4](#)).

- i. Dilute 10 μg of antibody-siRNA conjugate with pure water to 0.1 g/L and analyze the sample by SAX-HPLC (see [Method 4](#)). We recommend analyzing the starting antibody and the siRNA-linker conjugate as well as references.

Note: By SAX-HPLC, the injection peak is generally the unconjugated antibody (which counts in the calculation), and the following peaks corresponds to the different degree of conjugation. As the conjugate become more negatively charged, an increased degree of conjugation will elute with an increased retention time. The average degree of conjugation can be calculated using the areas under the curves (AUC) of each peak at 280 nm and the following formula:

$$\text{Average Degree of Conjugation} = \frac{\sum_{i=0}^n \text{AUC}_i \times i}{\sum_{i=0}^n \text{AUC}_i}$$

EXPECTED OUTCOMES

For the oligonucleotide-BCN conjugate synthesis, full conversion by RP-HPLC should be expected, and a yield between 55 and 85% should be obtained, with an average yield of 70%.

The average degree of conjugation for antibody-azide conjugates should be around 2.5 and 3.0 by mass spectrometry and yields >90% with no aggregation observed by SEC.

The average degree of conjugation for antibody-siRNA conjugates should be between 1.0 and 1.5 by native mass spectrometry ([Figure 6](#)), with complete absence of unconjugated antibody. Yields of SEC-purified conjugates were between 40% and 60%.

Note: We have observed that the hybridization of the siRNA must be performed before the conjugation step to the antibody to maximize the amount of double-stranded conjugate

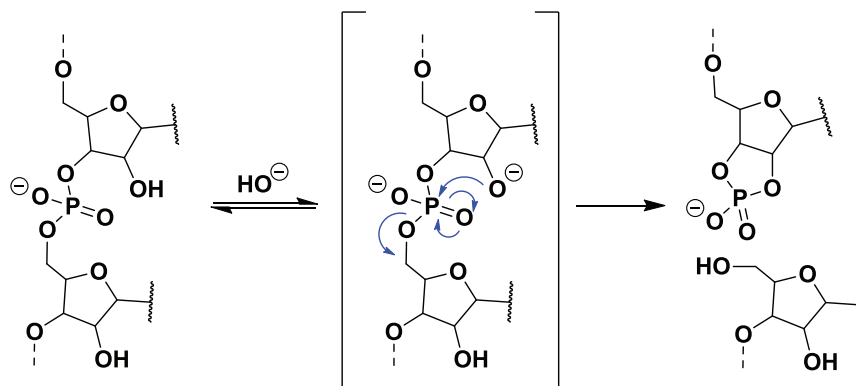


Figure 2. Mechanism of pH-mediated cleavage of RNA molecules

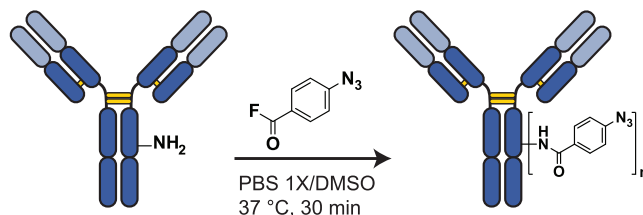


Figure 3. Lysine conjugation of antibody using ABF

(Figure 6A). If the hybridization is performed after conjugation on the antibody, some single-stranded conjugates will be observed by mass spectrometry (Figure 6B) and will not be biologically active.

LIMITATIONS

The pH of the solution during oligonucleotide conjugation is critical. At pH > 8.5, RNA can be degraded, and similarly, a too high pH will hydrolyze the activated species faster than its reaction with the oligonucleotide.

Depending on the linker's structure, a too long reaction time can have a dramatic effect on the oligonucleotide conjugate. For example, maleimides tend to slowly hydrolyze over time, leading to non-reactive linker-oligonucleotide conjugates.

We have observed that the successfulness of silencing experiments using antibody-siRNA conjugates was dependent on the antibody used and the target antigen. More specifically, one should use fast internalizing antibodies targeting an antigen that do not recycle.

Generally, a low degree of conjugation is preferred for antibody-oligonucleotide conjugates, as the added negative charges from oligonucleotides "anionize" the antibody, overall decreasing its pI. This will significantly impact the biophysical properties of the construct, such as decrease in cell-permeability, tissue retention and faster clearance.¹⁵

TROUBLESHOOTING

Problem 1

Reaction with the oligonucleotide and the linker is not starting or is not complete (refer to step 1.d).

Potential solution

- Make sure that the pH of your reaction mixture is not too high and not too low (should be between 7.4 and 8.0).
- Use only dry DMSO to solubilize the linker.

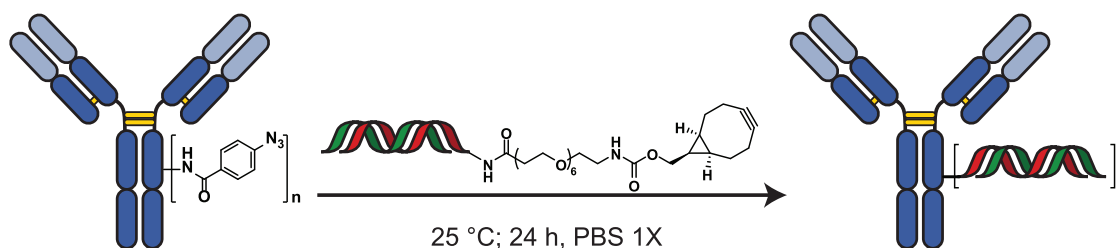


Figure 4. Conjugation of BCN-siRNA to antibody-azide to generate antibody-siRNA conjugates

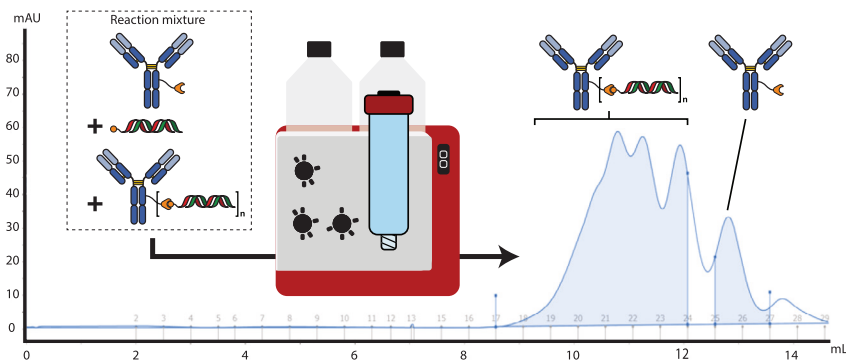


Figure 5. Representative purification SEC profile obtained after conjugation of siRNA to antibody

Problem 2

Oligonucleotide doesn't precipitate upon LiClO_4 /acetone treatment (refer to step 1.f).

Potential solution

- Make sure the oligonucleotide's concentration is high enough ($>100 \mu\text{M}$).
- Only use acetone that has been stored at -20°C to maximize precipitation.
- Alternatively, even though less effective with small oligonucleotides, use sodium acetate instead of lithium perchlorate, and ethanol instead of acetone.

Problem 3

Yields are lower than expected after oligonucleotide conjugation with the linker.

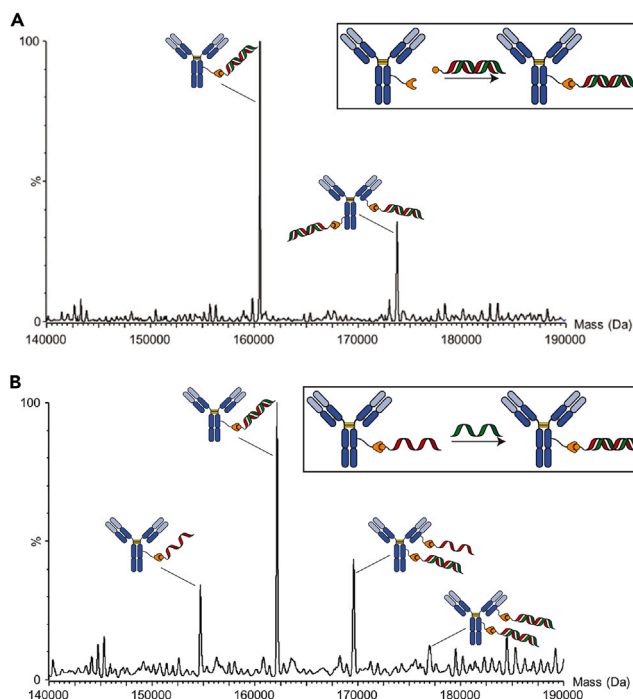


Figure 6. Comparison of SEC-MS profile obtained when hybridization is performed before or after antibody conjugation

(A) Representative mass spectrum of antibody-siRNA conjugates.

(B) Representative mass spectrum of antibody-siRNA conjugates if the hybridization step is performed after the conjugation to the antibody.

Potential solution

- Work under a safety cabinet to minimize nuclease-mediated degradation of the oligonucleotide.
- Thoroughly wash the safety cabinet with RNaseZap and 20% ethanol before handling oligonucleotides.
- Make sure the reaction reaches completion by monitoring regularly with RP-HPLC before purifying the conjugate.
- Do not let the oligonucleotide react more than 4 h if working with RNA, as prolonged incubation at slightly basic pH leads to base-mediated RNA cleavage.

Problem 4

A precipitate can be seen when reacting to the oligonucleotide with the antibody (refer to step 4.a).

Potential solution

- This is because the antibody and the oligonucleotide are forming non-covalent complexes instead of reacting with each other. This is generally observed when a high excess of oligonucleotide has been added to the antibody, or that a longer oligonucleotide has been used.
- Adding salt (10% v/v DPBS 10×) can help resolubilize the reactants.

Problem 5

Reaction between antibody and oligonucleotide is not complete.

Potential solution

- Make sure you have a high-purity linker-oligonucleotide conjugate, with an active chemical handle. The use of any commercially available fluorophore-strained alkyne can be used as a control reaction.
- Make sure the antibody contains the desired number of azide moieties before starting the conjugation with the oligonucleotide.

Problem 6

Some single-stranded oligonucleotide can be observed on the antibody by LC-MS (refer to step 4.g).

Potential solution

- Carefully follow the hybridization protocol, the temperature needs to cool down slowly.
- Add more equivalents of complementary strand during the hybridization step. The excess will be separated by the size exclusion chromatography step.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tony Rady (rady.tony@gmail.com).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Tony Rady (rady.tony@gmail.com).

Materials availability

This study did not generate new unique reagents. While we will not provide any reagents, the organic syntheses protocols to produce ABF and BCN-PEG₆-PFP have been published.

Data and code availability

This study did not generate/analyze either datasets or codes.

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AUTHOR CONTRIBUTIONS

T.R. wrote the protocol, the troubleshooting part, the introduction, the outcomes, and the limitations. J.M. wrote the introduction and the material and equipment setup part of the manuscript. V.L. made the figures and suggested modifications to the manuscript. T.R., V.L., and J.M. performed the conjugation experiments described herein independently. S.E. performed mass spectrometry analyses. S.C., G.C., N.B., and A.W. supervised the original study. T.R. supervised this manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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