Assembly of Bleomycin Saccharide-Decorated Spherical Nucleic Acids

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ABSTRACT: Glyco-decorated spherical nucleic acids (SNAs) may be attractive delivery vehicles, emphasizing the sugar-specific effect on the outer sphere of the construct and at the same time hiding unfavorable distribution properties of the loaded oligonucleotides. As examples of such nanoparticles, tripodal sugar constituents of bleomycin were synthesized and conjugated with a fluorescence-labeled antisense oligonucleotide (AONARV7). Successive copper(I)-catalyzed azide-alkyne and strain-promoted alkyne-nitrone cycloadditions (SPANC) were utilized for the synthesis. Then, the glyco-AONARV7 conjugates were hybridized with complementary strands of a C60-based molecular spherical nucleic acid (i.e., a hybridization-mediated carrier). The formation and stability of these assembled glyco-decorated SNAs were evaluated by polyacrylamide gel electrophoresis (PAGE), UV melting profile analysis, and time-resolved fluorescence spectroscopy. Association constants were extracted from time-resolved fluorescence data. Preliminary cellular uptake experiments of the glyco-AONARV7 conjugates (120 nM solutions) and of the corresponding glyco-decorated SNAs (10 nM solutions) with human prostate cancer cells (PC3) showed an efficient uptake in each case. A marked variation in intracellular distribution was observed.

INTRODUCTION

Spherical nucleic acids (SNAs) are nanostructures consisting of an appropriate core (gold, silica, liposomes, proteins) and a densely packed layer of oligonucleotides.1−9 Compared to the poor cellular delivery of linear oligonucleotides,10−13 SNAs are efficiently taken up by cells via class A scavenger receptor-mediated endocytosis.14,15 Furthermore, SNAs are more resistant toward nuclease degradation16 and they elicit low innate immune response.17 In addition to these beneficial properties of naked SNAs, the radial formulation may be utilized together with the covalent conjugation strategy.18 The effect of potential cell/tissue-specific ligands may be emphasized on the outer sphere, which at the same time hide unfavorable distribution properties of the loaded oligonucleotide content. This approach may be particularly useful for weakly interacting ligands, the sufficient cell-targeted delivery potential of which requires multivalent binding to the cell membrane receptors. Carbohydrate−lectin binding is a typical example of such multivalent interaction.19

The present article describes the assembly of glyco-decorated and hybridization-mediated SNAs, which consisted of bleomycin (BLM) saccharides as a decoration sphere and a known splice-switching oligonucleotide (AONARV7) payload that is known to suppress prostate tumor cell survival by inhibiting the mRNA synthesis of AR-V7 (an androgen receptor variant).20 These nanoparticles may be classified as hybrid structures between fullerene C60-based molecular SNAs...
Scheme 1. Synthesis of Azide-Modified Carbamoyl Mannose and Bleomycin Disaccharide Precursors

Reagents and conditions: (i) Ph2SO, Tf2O, 2,4,6-tri-tert-butyl pyrimidine, 2-(2-azidoethoxy)ethanol, CH2Cl2, −60 °C to room temperature (rt); (ii) 0.4 N NH3 in tetrahydrofuran (THF), CH2Cl2; (iii) 2-(2-azidoethoxy)ethanol, TMSOTf, 0 °C.

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and “glyco-superballs” that have gained marked interest as virus-like synthetic macromolecules.21–24 Bleomycin saccharides (i.e., 2-O-(3-carbamoyl-α-D-mannopyranosyl)-L-gulose and 3-carbamoyl-α-D-mannose) are constituents of bleomycin (BLM), which is a glycopeptoid-derived natural compound used in clinics as an antitumor agent against squamous cell carcinomas and malignant lymphomas.35–38 The peptoid moiety of bleomycin is responsible for the cytotoxicity (oxidative cleavage of DNA and RNA),31,32 whereas the tumor cell selectivity derives from the disaccharide moiety.39–41 The reason for the selective uptake by tumor cells is still not fully understood, but data suggest that bleomycins are transported into cells by cell surface receptors involved in glucose transport, which is upregulated in tumor cells.41 The position and substitution of the carbamoyl group in the disaccharide are important for the uptake by cancer cells, and 3-carbamoyl-L-gulopyranosyl units are taken up by cells more efficiently, indicating that the binding of bleomycin disaccharide to cell surface receptors is multivalent.40,42,43 Hence, the bleomycin saccharides may be potential targeting agents that would deliver oligonucleotide payloads to cancer cells,44 but the efficient enough targeting may need high multivalency.

For the assembly of the glyco-decorated SNAs, azide-modified bleomycin disaccharide precursors (3α, 3β, and 5, Scheme 1) were synthesized and attached to an aldehyde-functionalized branching unit (6, Scheme 2) by Cu(1)-catalyzed 1,3-dipolar cycloaddition (“click” reaction) to obtain trivalent bleomycin disaccharide and carbamoyl mannose clusters (8, 10α, and 10β, Scheme 2). The aldehyde moiety of the clusters was converted into the reactive nitrone functional group using 5'-dibenzo-bicyclo-octyne (DBCO)-modified 2'-O-methylated oligoribonucleotide AONARV7 (ON1, Scheme 3) by strain-promoted alkyn-nitrene cycloaddition (SPANC).45 The conjugates were additionally labeled with a fluorescent dye (AF488, ON5–ON7, Scheme 3). A C60-based SNA46 bearing DNA strands with complementary sequence to glyco-AONARV7 conjugates (1:12 stoichiometry) to obtain the glyco-decorated SNAs (SNA2–SNA4, Scheme 4). The formation and stability of these glyco-decorated SNAs were evaluated by polyacrylamide gel electrophoresis (PAGE), UV melting profile analysis, and time-resolved fluorescence spectroscopy. The preliminary uptake of the glycolucostar–oligonucleotide conjugates ON5–ON7 and the corresponding glyco-decorated SNAs (SNA2–4) to human prostate cancer cells (PC3) was evaluated by confocal microscopy.

**RESULTS AND DISCUSSION**

**Synthesis of Azide-Modified Bleomycin Disaccharide Precursors.** For the synthesis of azide-modified bleomycin disaccharide precursors 3α and 3β (Scheme 1), the previously reported 2-O-[2,4,6-tri-O-acetyl-3-O-(p-nitrophenylformyl)-α-D-mannopyranosyl]-3,4-di-O-benzoyl-6-O-acetyl-L-gulopyra-
were then converted in situ aldehyde moieties of the resulting compounds by treatment with chloride. The resulting nitrone-modified carbamoyl mannose by treatment with 2-(2-azidoethoxy)ethanol in the presence of ON7 tri- and α-fluoromethanesulfonic anhydride, diphenyl sulfoxide, and tert-butyl pyridine. Compound α was obtained as pure α-fluoromethanesulfonate. 

**Synthesis of the Bleomycin Saccharide-Oligonucleotide Conjugates.** Prior to conjugation with AON<sub>ARV7</sub> the glyoclusters were globally deprotected by 7N ammonia in methanol (Scheme 3). The authenticity of the crude products (11, 12α, and 12β) was verified by MS, and their applicability for strain-promoted alkyne-nitrone cycloaddition (SPANC) was confirmed with a commercially available dibenzocyclooctyne (DBCO)-modified rhodamine dye. After this successful small-molecule trial (see compounds 13, 14α, and 14β in the Supporting Information), 5′-DBCO- and 3′-amino-modified 2′-O-methylated phosphorothioate oligoribonucleotide AON<sub>ARV7</sub> (ON1) was synthesized by an automated synthesizer and exposed to SPANC with 11, 12α, and 12β (2 equiv each vs ON1, overnight at rt). The product mixtures were purified by RP-HPLC (Scheme 3A) to yield the glyocluster–AON<sub>ARV7</sub> conjugates ON2, ON3, and ON4 in 17, 11, and 15% isolated yields, respectively. The resulting glyocluster–AON<sub>ARV7</sub> conjugates ON2–ON4 were then labeled with Alexa (AF488) fluorescent dye NHS ester using a well-established procedure to yield the labeled conjugates ON5, ON6, and ON7 in ca. 20% isolated yields.

"The oligonucleotides are 2′-OMe-ribonucleotide phosphorothioates. (A) Crude RP-HPLC profile of ON2, (B) RP-HPLC profile of purified ON7. Reagents and conditions: (i) 7N NH<sub>3</sub> in MeOH, 2 days at rt; (ii) ON1 + 11, 12α, or 12β (2 equiv), H<sub>2</sub>O, overnight at rt; (iii) AF488 N-hydroxysuccinimide ester (20 equiv in dimethyl sulfoxide, DMSO), 0.1 M sodium borate (aq, pH 8.5), overnight at room temperature. RP-HPLC conditions: (A) an analytical C-18 column (250 × 4.6 mm, 5 μm), detection at λ = 260 nm, gradient elution (0–25 min) from 0 to 50% MeCN in 0.1 M aqueous triethylammonium acetate, flow rate 1.0 mL min<sup>−1</sup>; (B) same as A, except gradient elution (0–25 min) from 5 to 95% MeCN in 0.1 M aqueous triethylammonium acetate.
authenticity of the products was verified by MS (orbitrap) spectroscopy.

**SNA Synthesis.** Fullerene C$_{60}$-based SNA1 with twelve 2′-deoxy oligoribonucleotide sequences, complementary to AON$_{dRV7}$, was synthesized using strain-promoted alkyne-azide cycloaddition (SPAAC) and following the procedure reported previously by our group.$^{49}$ Due to the solubility issues, the azide-functionalized C$_{60}$ core $^{15,46,49}$ was first monosubstituted using a substoichiometric amount (0.3 equiv) of bicyclo[6,1,0]nonyne (BCN)-modified oligonucleotide (ON9) in DMSO (Scheme 4). The monosubstituted product (16) was then exposed to an excess of the same oligonucleotide in

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**Scheme 4. SNA Synthesis and Hybridization of AF488-Labeled Glycocuster–Oligonucleotide Conjugates ON5–ON7 with Complementary SNA1**

**(A) Crude RP-HPLC profile of SNA1.** (B) RP-HPLC profile of purified SNA1. (C) Electrospray ionization mass spectrometry (MS-ESI) of SNA1 (a spectrometer equipped with a hybrid quadrupole orbitrap and nano-ESI ionization). (D) Polyacrylamide gel electrophoresis (PAGE) of SNAs 1–5. Conditions: (i) ON9 (0.3 equiv/compound 15), DMSO/H$_2$O 9:1 v/v, overnight at room temperature; (ii) ON9 (1.2 equiv/azide arm of 16), 1.5 M NaCl (aq), 3 days at room temperature; (A and B) An analytical RP-HPLC column Phenomenex, Aeris 3.6 μm WIDEPORE XB-C18 200 Å, 150 × 4.6 mm, linear gradient from 5 to 45% MeCN in 50 mmol L$^{-1}$ triethylammonium acetate over 30 min, a flow rate of 1.0 mL min$^{-1}$, detection at 260 nm; (D) for conditions, see the Experimental Section.
aqueous medium and high salt concentration. This two-step process alleviated the solubility issues and yielded a more homogeneous product. The homogeneity of SNA1 was confirmed by RP-HPLC and PAGE, and its authenticity was confirmed by MS (a spectrometer equipped with a hybrid quadrupole orbitrap and nano-ESI ionization; Scheme 4A–D). The particle size of SNA1 (in 100 μL aqueous 10 mmol L⁻¹ phosphate-buffered saline (PBS), 1.1 mmol L⁻¹ M KCl, 0.154 mol L⁻¹ NaCl, pH 7) was evaluated by dynamic light scattering (DLS) that showed a hydrodynamic diameter of 10.6 ± 0.2 nm.

**Formation and Stability of Hybridization-Mediated SNAs 2–5.** On the gel electrophoresis, SNA1 alone resulted in a distinct and relatively sharp band (Scheme 4D). A trace of a faster eluting side product was also observed (may refer to an 11-armed SNA), but the overall purity of SNA1 after single RP-HPLC purification proved high. Mixtures of SNA1 with ON5–8 [12 equiv, samples prepared in phosphate-buffered saline (PBS) at pH 7.4] resulted in slower eluting broad bands on the gel representing the formation of the hybridization-mediated SNAs: SNA2–SNA5. We also evaluated the particle size of the hybridization-mediated SNAs by DLS in 100 μL of aqueous 10 mmol L⁻¹ PBS, 1.1 mmol L⁻¹ M KCl, 0.154 mol L⁻¹ NaCl, pH 7.4. Hydrodynamic diameters with relatively large error limits were obtained: SNAs 5: 11.5 ± 1.1 nm, SNAs 2–4: 13.8 ± 1.7 nm (no marked difference between the different glyco decorations).

The UV thermal melting temperatures were next measured using 0.083 μmol L⁻¹ SNA1 and 12 equiv (1.0 μmol L⁻¹) of ON5–8 in 10 mmol L⁻¹ sodium cacodylate buffer (pH 7.0) with 0.1 mol L⁻¹ NaCl (Table 1). The UV melting curves showed inflection points at 56–57 °C. Compared to nonconjugated oligonucleotide ON8, the glycocluster moieties of ON5–7 decreased the melting temperature of the SNA/oligonucleotide duplex slightly: 1.1–1.6 °C.

The hybridization between SNA1 and the AF488-labeled oligonucleotides ON5–8 was studied in more detail by fluorescence spectroscopy. The fluorescence properties of the ON5–8 and SNA1 complexes are presented in Table S3. The presence of the glycocluster moieties in ON5–7 clearly influenced the spectral properties of AF488. For ON6–7, the absorption maximum was shifted 3 nm to the red compared with that of ON5 and ON8. Thus, it seems that AF488 interacts with α- and β-bleomycin moieties in the ground state. The fluorescence intensity increased during complexation (Figures S62 and S63), but so did the absorbance as the amount of AF488 in the samples increased. Determining the change in absorption for the 50 μL samples especially at low oligonucleotide amounts resulted in large errors. However, the fluorescence lifetimes do not depend on the dye concentration and thus the association constants were determined from the time-resolved data. For all glycocluster derivatives ON5–7, the fluorescence decay curves were one-exponential, whereas for ON8, they were two-exponential (Figure S64). In the presence of SNA1, the decays of ON5–7 became two-exponential due to the complex formation with SNA1. The average fluorescence lifetime (τ) (Table S2) decreased during complexation for all oligonucleotides (Figure S65). For ON8 and ON5, the decrease was due to the increase in the proportion of the short-living component, whereas for ON6–7, both the lifetime and the proportion of the short-living component changed during complexation. The association constants were determined by plotting the (τ) as a function of inverse oligonucleotide concentration (Figure S65). For carbamoyl mannosyl conjugate ON5, the association constant was nearly equal to that obtained for ON8, whereas for α- and β-bleomycin disaccharide conjugates ON6 and ON7, the association constant was about half of that for ON5 and ON8 (Table 2). For all of the oligonucleotides, the complexation seemed to be complete at a 12:1 oligonucleotide/SNA ratio. For the association constants determined from the time-resolved data, only ratios 4–12:1 or 8–12:1 could be used. This could indicate that the oligonucleotides first bind to different parts of the SNA. Only when the oligonucleotides start to bind to the adjacent strands of already filled positions, the AF488 fluorescence is quenched, and this can be observed as the decrease in the (τ). For ON6–7, the quenching starts at higher ratios than for ON8 and ON5, due to the stronger interaction of AF488 with ON6–7 sugar moieties.

**Cell Uptake Studies.** The cell uptake studies of all of ON5–8 (120 nM) and the corresponding SNAs 2–5 (10 nM, i.e., the total oligonucleotide concentration was the same in each experiment) were carried out with PC3 cells, and the obtained data were compared with the untreated PC3 cells as controls. The incubation time of ON5–ON8 and SNAs 2–5 with the PC3 cells was about 4 h, after which the cells were labeled and fixed for the confocal and wide-field microscopy examination. Figure 1 presents, as an example, the obtained cell uptake data of ON5 and SNA2, and the others are shown in Figures S67–S69. Based on these data, it is clearly shown that ON5–8 and the corresponding SNAs are taken up by the PC3. It also could be stated that the oligonucleotides with the SNA1 carrier are taken up by the cells more effectively than those without the carrier. In this primarily synthetic technical report, PC3 cell line was selected for the preliminary cell uptake study. A more detailed mechanistic study of the internalization and intracellular trafficking in 22Rv1 prostate

Table 1. UV Thermal Melting Temperatures of the SNA1/ AONARV7 Complexes

|            | Tm/°C |
|------------|-------|
| SNA1 + 12 equiv ON8 | 57.8 ± 0.9 |
| SNA1 + 12 equiv ON5 | 56.2 ± 0.6 (−1.6) |
| SNA1 + 12 equiv ON6 | 56.3 ± 0.6 (−1.5) |
| SNA1 + 12 equiv ON7 | 56.7 ± 1.2 (−1.1) |

“Conditions: 0.083 μmol L⁻¹ SNA + 12 equiv (1.0 μmol L⁻¹) of the AONARV7 conjugates ON5–8, 10 mmol L⁻¹ sodium cacodylate (pH 7.0), 0.1 mol L⁻¹ NaCl in H2O. Detection wavelength 260 nm. ΔTm values in parentheses are compared to SNA + 12 equiv ON8.”

Table 2. Association Constants for the SNA1/AONARV7 Complexes

|            | Kassoc/M⁻¹ (equivalents of ON/SNA) |
|------------|-----------------------------------|
| ON8        | 5.18 × 10⁶ (4–12)                 |
| ON5        | 4.64 × 10⁶ (4–12)                 |
| ON6        | 2.05 × 10⁶ (8–12)                 |
| ON7        | 2.24 × 10⁶ (8–12)                 |

“Conditions: 0.1 μM SNA1 in Dulbecco’s phosphate-buffered saline (DPBS) was titrated with 10 μM ON5–ON8 solutions. Δν343 nm and decays were monitored at 500–700 nm. Numbers in parentheses refer to oligonucleotide-SNA ratios used to extract the association constants.”
cancer cells (overexpressing AR-V7), together with PNT2 health prostate cells as controls, is underway in our laboratory.

### CONCLUSIONS

Tripodal clusters of bleomycin disaccharide and carbamoyl mannose were synthesized and conjugated with an antisense oligonucleotide. The oligonucleotide conjugates were labeled with a fluorescent dye and hybridized with complementary strands of a C60-based molecular SNA to form SNA structures surrounded by a carbohydrate sphere. The formation and stability of these hybridization-mediated glyco-decorated SNAs were evaluated by PAGE, UV melting profile analysis, and time-resolved fluorescence spectroscopy. The melting temperatures (ca. 56 °C) and the association constants (2.1 × 10^6 L mol⁻¹), extracted from the time-resolved fluorescence spectroscopy, confirmed that the complex formation of these hybridization-mediated SNAs was favored under physiological conditions. Preliminary cell uptake experiments showed that the glyco-oligonucleotide conjugates (120 nM solutions) and the corresponding glyco-decorated SNAs (10 nM solutions) were efficiently taken up by prostate cancer cells (PC3). The results indicated that the glyco-decorated SNAs were taken up by the cells more efficiently than the corresponding glyco-oligonucleotide conjugates. Furthermore, variation in the intracellular distribution was noticed. A more detailed mechanistic study of internalization and intracellular trafficking is currently underway in our laboratory.

### EXPERIMENTAL SECTION

**General Remarks.** RP-HPLC analysis and purification of the oligonucleotides and oligonucleotide conjugates were performed using Thermo ODS Hypersil C18 (250 × 4.6 mm, 5 μm) analytical column. The mass spectra were recorded using either an MS (orbitrap) or MS electrospray ionization time-of-flight (ESI-TOF) spectrometer. The isolated yields of the oligonucleotide conjugates were determined according to their UV absorbance at 260 nm and 495 nm (AF488-labeled oligonucleotides).

2-(2-Azidoethoxy)ethyl-2-O-{2,4,6-tri-O-acetyl-3-O-[p-nitrophenyl]formyl}]-α-L-gulopyranoside (2α) and 2-(2-Azidoethoxy)ethyl-2-O-{2,4,6-tri-O-acetyl-3-O-[p-nitrophenyl]formyl}]-β-L-gulopyranoside (2β). Trifluoromethanesulfonic anhydride (76 μL, 0.45 mmol) was added to a suspension of compound 1 (280 mg, 0.32 mmol), diphenyl sulfoxide (180 mg, 0.90 mmol), 2,4,6-tri-tert-butyl pyrimidine (240 mg, 2.7 mmol), and 4 Å molecular sieves in dichloromethane (10 mL) at −60 °C under argon. The reaction mixture was stirred at this temperature for 5 min and then at −40 °C for 1.5 h. 2-(2-Azidoethoxy)ethanol

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Figure 1. Uptake of AF488-labeled carbamoyl mannose conjugate ON5 and SNA2 by PC3 cells (Note: the scale bar is 20 μm). The cells were incubated (37 °C with 5% CO2) with 120 nM oligonucleotide and 10 nM SNA concentrations or in PBS (controls) for 4 h. After incubation, the cells were washed three times with PBS and fixed using 4% paraformaldehyde solution (diluted in PBS). The intracellular delivery of oligonucleotides and SNAs was quantified via wide-field microscopy on a Nikon Eclipse Ti2-E microscope using Alexa 488 (475 nm) and 4,6-diamidino-2-phenylindole (DAPI) (395 nm) channels and normalized based on the intensity of the untreated control cell.
(84 mg, 0.64 mmol) was added at −40 °C. The solution was stirred at this temperature for 1 h, and then the temperature was allowed to increase slowly to room temperature for 3 h. The reaction was quenched with excess triethylamine and filtered through a Celite bed, and the bed was washed with dichloromethane (20 mL). The filtrate was washed sequentially with saturated aqueous sodium bicarbonate solution (50 mL) and saturated aqueous sodium chloride solution (50 mL). The organic layer was dried over sodium sulfate and concentrated, and the residue was purified by silica gel flash column chromatography (30% EtOAc-toluene) to afford 2a (150 mg, 48%) and 2b (100 mg, 32%) as colorless syrups.

2a: 1H NMR (500 MHz, CDCl3) δ 2.01 (s, 3 H, COCH3), 2.07 (s, 3 H, COCH3), 2.11 (s, 3 H, COCH3), 2.14 (s, 3 H, COCH3), 3.20–3.30 (m, 2 H, CH2N3), 3.54–3.58 (m, 1 H, OCH2), 3.66–3.75 (m, 3 H, OCH2), 3.79–3.84 (m, 1 H, OCH2), 4.01–4.06 (m, 1 H, OCH2), 4.09–4.13 (m, 2 H, H-5’, H-6), 4.20–4.24 (m, 2 H, H-2, H-6’), 4.26–4.33 (m, 2 H, H-6’), 4.73 (m, 1 H, H-4’), 5.05 (dd, J = 3.5, 10.0 Hz, 1 H, H-3’), 5.10 (d, J = 4.0 Hz, 1 H, H-1), 5.15 (d, J = 1.5 Hz, 1 H, H-1’), 5.33 (t, J = 10.0 Hz, 1 H, H-4’), 5.38 (dd, J = 1.5, 3.0 Hz, 1 H, H-1), 2.44 (d, J = 3.0 Hz, 1 H, H-4), 5.70 (t, J = 3.5 Hz, 1 H, H-3), 7.31–7.34 (m, 2 H, PNP), 7.47–7.50 (m, 4 H, Bz), 7.57–7.64 (m, 2 H, Bz), 8.06–8.07 (m, 2 H, Bz), 8.13–8.17 (m, 2 H, Bz), 8.24–8.27 (m, 2 H, PNP); 13C NMR (125 MHz, CDCl3) δ 20.72, 20.74, 20.8, 50.6, 62.3, 64.2, 65.6, 66.0, 68.2, 68.4, 69.10, 69.14, 70.3, 71.0, 74.2, 97.1 (C-1'), Jc-H-1 = 174.4 Hz), 97.4 (C-1, Jc-H-1 = 170.1 Hz), 121.9, 125.2, 128.3, 128.7, 128.9, 129.5, 129.7, 129.9, 130.3, 133.4, 134.5, 145.5, 151.2, 155.3, 165.0, 165.4, 169.6, 169.8, 170.5, 170.6; HRMS calcd for C38H45N4O19Na (M + Na)+: 897.2654, found: 897.2642.

2b: 1H NMR (500 MHz, CDCl3) δ 2.03 (s, 3 H, COCH3), 2.08 (s, 3 H, COCH3), 2.11 (s, 3 H, COCH3), 2.16 (s, 3 H, COCH3), 3.24–3.33 (m, 2 H, CH2N3), 3.60–3.74 (m, 10 H, OCH2), 3.83–3.87 (m, 1 H, OCH2), 4.03–4.07 (m, 2 H, H-2 and OCH2), 4.20 (dd, J = 2.5, 12.5 Hz, 1 H, H-5’), 4.23–4.32 (m, 4 H, H-6 and H-6’), 4.41 (m, 1 H, H-5’), 4.47 (m, 1 H, H-5), 4.97 (d, J = 7.5 Hz, 1 H, H-1), 5.04 (dd, J = 3.5, 10.0 Hz, 1 H, H-3’), 5.11 (d, J = 1.0 Hz, 1 H, H-1), 5.13 (d, J = 2.0, 3.5 Hz, 1 H, H-1’), 5.34 (t, J = 1.0 Hz, 1 H, H-1’), 5.39 (dd, J = 1.5, 4.0 Hz, 1 H, H-4), 5.40 (t, J = 3.5 Hz, 1 H, H-3), 7.29–7.34 (m, 2 H, PNP), 7.46–7.50 (m, 4 H, Bz), 7.60–7.65 (m, 2 H, Bz), 8.07–8.08 (m, 4 H, Bz), 8.24–8.29 (m, 2 H, PNP); 13C NMR (125 MHz, CDCl3) δ 20.85, 20.89, 20.9, 20.94, 50.6, 61.9, 62.2, 65.5, 66.5, 68.2, 68.6, 68.7, 69.0, 70.1, 70.8, 70.9, 71.4, 74.8, 95.4 (C-1'), Jc-H-1 = 175.7 Hz), 99.5 (C-1, Jc-H-1’ = 163.2 Hz), 122.0, 125.3, 128.7, 128.8, 129.0, 130.1, 130.2, 132.0, 133.9, 134.0, 145.6, 151.5, 155.5, 165.2, 165.3, 166.9, 170.7, 170.0, 170.9.

(2-Azidoethoxy)ethyl-2-O-(2,4,6-tri-O-acetyl-3-O-carbamoyl-α-D-mannopyranosyl)-3,4-di-O-benzoyl-6-O-acetyl-β-L-gulopyranoside (3a). A solution of compound 4 (370 mg, 0.64 mmol) and 2-(2-azidoethoxy)ethanol (100 mg, 0.77 mmol) in dry CH3Cl2 (5 mL) was treated with TMSOTf (230 µL, 1.3 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 10 min and then poured into a two-phase solution of EtOAc (25 mL) and saturated aqueous NaHCO3 (50 mL) with vigorous stirring. The organic layer was washed with saturated aqueous NaCl, dried over anhydrous Na2SO4, and concentrated in vacuo. Flash chromatography over silica gel using 50% EtOAc-hexane gave 5 (220 mg, 75%) as a syrup; 1H NMR (500 MHz, CDCl3) δ 2.05 (s, 3 H, COCH3), 2.09 (s, 3 H, COCH3), 2.14 (s, 3 H, COCH3), 2.38 (m, 3 H, CH2N3), 3.65–3.66 (m, 5 H, OCH2), 3.81 (m, 1 H, OCH2), 4.07–4.10 (m, 2 H, H-5 and H-6), 4.28 (dd, 1 H, J = 5.0, 12.5 Hz, H-6), 4.84 (bs, 2 H, NH2), 4.88 (s, 1 H, H-1), 5.25–5.27 (m, 3 H, H-2, H-3 and H-4); 13C NMR (125 MHz, CDCl3) δ 20.8, 21.0, 50.7, 62.4, 66.2, 67.3, 68.4, 69.9, 70.0, 70.2, 97.7 (Jc-H-1 = 174.5 Hz), 155.4, 170.0, 170.7; HRMS calcd for C53H62N4O13K (M + K)+: 814.2357, found: 814.2355.

Synthesis of Nitrene-Modified Trivalent Clusters of Carbamoyl Mannose and Bleomycin Disaccharide (Compounds 8, 10a, and 10b). Compounds 5 (21 mg, 45...
pounds ligation was procedure described for compound ∼ 5.1 Hz, 12.3 Hz, H-6), 4.47 (b, 12H), 4.76 (s, 3 H, H-1), 5.13 (b, 12H), 5.17 (m, 6 H, J = 3.5 Hz, Ph), 7.68 (s, 1 H, J = 8.9 Hz, Ph), 7.68 (s, 1 H, CHN), 7.83 (s, 3 H, triazol), 8.14 (d, 2 H, J = 8.9 Hz, Ph), 13C NMR (125 MHz, CD3OD) δ 44.8, 50.0, 52.1, 62.3, 63.8, 66.3, 66.5, 66.9, 68.5, 69.0, 69.3, 69.7, 69.8, 97.5, 114.2, 122.9, 124.3, 131.3, 138.3, 14.5, 156.7, 161.4, 170.1, 170.3, 171.0; 1H/13C/15N/14N/14S S34, found 1770.63. The NMR spectra of compound 8 are presented in Figures S34–S37.

Compounds 10α and 10β were synthesized following the same procedure described for compound 8. The NMR spectra of compounds 10α and 10β are presented in Figures S38–S56. 10α: 8.2 mg (2.7 μmol, 32% overall yield from 6). HRMS (ESI-TOF): m/z C20H16N12O12K2 2+ [(M + H + K)/2]2+ requires 1533.9763, found 1533.9757. 10β: 6.1 mg (2.0 μmol, 24% overall yield from 6). HRMS (ESI-TOF): m/z C20H16N12O12K2 2+ [(M + H + K)/2]2+ requires 1533.9763, found 1533.9710.

Global Deprotection of Glycoclusters (Removal of Acetyl and Benzoyl Protections to Yield Compounds 11, 12α, and 12β). Compound 8 (5.0 mg, 2.8 μmol) was dissolved in 7 N NH3 in MeOH (200 μL). The reaction mixture was stirred for 2 days at room temperature and evaporated to dryness. HRMS (ESI-TOF) confirmed the global deprotection and formation of compound 11. The crude product was dissolved in water and used as such for strain-promoted alkyne-nitrene cycloaddition (SPANC) reactions. Compounds 12α and 12β were synthesized following the same procedure described for compound 11. The applicability of the nitrene-modified glycoclusters 11, 12α, and 12β for SPANC ligation was first tested by labeling the glycoclusters with dibenzyclooctyne-PEG4-5/6-carboxyhdamine dye (compounds 13, 14α, and 14β, Supporting Information). 11: HRMS (ESI-TOF): m/z C20H18N12NaO12 2+ [(M + Na)+] requires 1414.55, found 1414.56. 12α: HRMS (ESI-TOF): m/z C20H16N12O12 2+ [(M + 2 H)/2]2+ requires 939.8653, found 939.8671. 12β: HRMS (ESI-TOF): m/z C18H11N13O4 2+ [(M + 2 H)/2]2+ requires 939.8653, found 939.8680.

Synthesis of the AF488-Labeled Glycocluster—Oligonucleotide Conjugates ON5–7. The DBCO-modified oligonucleotide ON1 was synthesized on a 1.0 μmol scale using an automatic DNA/RNA synthesizer and commercially available (2-dimethoxytrityloxymethyl-6-fluorenylmethoxycarbonylamino-hexane-1-succinoyl) long-chain alkylamino-CPG solid support. Commercially available building blocks of 2′-Ome nucleoside phosphoramidites and 5′-DBCO-triethylene-glycol phosphoramidite were used for the chain elongation. The oligonucleotide was a full phosphorothioate except for one phosphodiester bond connecting the 5′-DBCO-triethylene-glycol unit to the oligonucleotide. To prevent loss of DBCO in the 5′-DBCO-triethylene-glycol phosphoramidite coupling step, (1S)-(−)-(10-camphorsulfonfyl)-oxaziridine was used as an oxidizer, as recommended by the manufacturer. The oligonucleotide was released from the support by the usual ammonolysis protocol for DBCO-modified oligonucleotides (concentrated ammonium hydroxide, 2 h at 65 °C), homogenized by RP-HPLC, and lyophilized. The DBCO-oligonucleotide was then dissolved in water and incubated with aqueous solutions of nitrene-modified glycoclusters 11, 12α, and 12β (2 equiv) overnight at room temperature to give glycocluster—oligonucleotide conjugates. The resulting glycocluster—oligonucleotide conjugates ON2—ON4 were homogenized by RP-HPLC (Scheme 3A) and lyophilized to dryness. The isolated yields 11, 12α, and 12β in 15% of the conjugates ON2, ON3, and ON4, in this order, were determined according to UV absorbance at λ = 260 nm. The authenticity of the products was verified by MS (ESI-TOF) spectroscopy, and they were then exposed to the AF488 labeling, following an established protocol: The glycocluster—oligonucleotide conjugates were dissolved in 0.1 M sodium borate (aq, pH 8.5), AF488 NHS ester (purchased from Lumiprobe, 20 equiv in DMSO) was added, and the reaction mixtures were incubated overnight at room temperature. The resulting AF488-labeled glycocluster—oligonucleotide conjugates were purified by RP-HPLC and lyophilized to dryness to yield ON5—ON7 in ca. 20% yields. The homogeneity of the products was confirmed by RP-HPLC, and the authenticity of the products was verified by MS (orbitrap) spectroscopy (Scheme 3B, Figures S57–S60, and Table S1).

SNA Synthesis. To a solution of C60 Buckminsterfullerene core 15 (60 nmol in 65 μL DMSO), BCN-modified oligonucleotide ON9 (20 nmol in 10 μL H2O) was added. The reaction mixture was gently shaken overnight at room temperature and the monosubstituted product was purified by RP-HPLC (Figure S61, an analytical C-18 column (250 × 4.6 mm, 5 μm), detection at λ = 260 nm, gradient elution (0–20 min) from 40 to 100% MeCN in 50 mM aqueous triethylammonium acetate, flow rate 1.0 mL min−1). The product fractions were collected, lyophilized to dryness, and the authenticity of the monosubstituted compound 16 was verified by MS (ESI-TOF, Figure S61). The isolated yield of the product (9.6 nmol, 48%) was determined by UV absorbance at 260 nm. The monosubstituted product 16 (7.7 nmol) was mixed with ON9 (110 nmol, 1.2 equiv of ON9/azide arm) in 1.5 M aqueous NaCl solution, and the reaction mixture was gently shaken for 72 h at room temperature. The resulting SNA1 was purified by RP-HPLC (Scheme 4A, an analytical C-18 column Phenomenex, Aeras 3.6 μm WIDEPORE XB-C18 200 A, 150 × 4.6 mm, detection
at $\lambda = 260$ nm, gradient elution (0−30 min) from 5 to 45% MeCN in 50 mM aqueous triethylammonium acetate, flow rate 1.0 mL min$^{-1}$). The product fractions were collected and lyophilized to dryness. The isolated yield of the product (4.0 nmol, 52%) was determined by UV absorbance at 260 nm. The homogeneity of SNA1 was confirmed by RP-HPLC, size exclusion chromatography (SEC), and PAGE, and the authenticity of SNA1 was confirmed by MS (a spectrometer equipped with a hybrid quadrupole orbitrap and nano-ESI ionization; Scheme 4A−D).

**UV Thermal Melting Studies.** The thermal melting curves of the hybridized SNA/oligonucleotide complexes were measured at 260 nm with a PerkinElmer Lambda 35 UV−vis spectrometer equipped with a multiple cell holder and a Peltier temperature controller. Additionally, an internal thermometer was used. The temperature was changed between 10 and 80 °C at the rate of 0.5 °C min$^{-1}$. Each $T_m$ value was determined from the maximum of the first derivative of the melting curve (average of three heating and three cooling curves). The measurements were performed using 0.083 μmol L$^{-1}$ SNA and 12 equiv (1.0 μmol L$^{-1}$) of the oligonucleotide in 10 mmol L$^{-1}$ sodium cacodylate buffer (pH 7.0) with 0.1 mol L$^{-1}$ NaCl.

**PAGE Analysis of SNAs.** Native 6% Tris base, boric acid, ethylenediaminetetraacetic acid (EDTA), and acrylamide (TBE) gel were used for confirming the purity of SNA1 and the hybridization of the glycocluster-modified oligonucleotides ONS−7 with the complementary SNA. A pre-cast gel cover (10 cm × 10 cm in size, Thermo Fisher Scientific) was fixed into a vertical electrophoresis chamber, and the running buffer (90 mM Tris, 90 mM borate, and 2 mM EDTA, 8.3 pH) was filled into the chamber. SNA samples were prepared by mixing 5 μL of 0.05 μM SNA1 in phosphate-buffered saline (PBS), pH 7.4, with 5 μL TBE sample buffer. Hybridized SNA/oligonucleotide samples were prepared by adding 12 equiv of oligonucleotides ONS−8 in the mixture. The SNA samples and a DNA ladder (100 bp, note: the ladder is just used to confirm the quality and comparability of the runs, and it cannot be used for size evaluation of the SNAs) were loaded and electrophoresed at 200 V constant (45 mA) for approximately 30 min. After completion of electrophoresis, gel was removed from the chamber and the SNA bands were monitored after staining by SYBR Green Gold Nucleic Acid Stain (Thermo Fisher Scientific).

**DLS Experiments.** The size of the SNAs was measured at room temperature using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., U.K.). Settings and conditions for the measurements were: material Protein (RI: 1.450; Absorption: 0.001), dispersant water (Viscosity: 0.8872 cP; RI: 1.330) temperature at 20 °C, and equilibration time was 60 s. Each sample (10 μg SNA in 100 μL aqueous 10 mmol L$^{-1}$ PBS, 1.1 mmol L$^{-1}$ KCl, 0.154 mol L$^{-1}$ NaCl, pH 7.4) was measured three times.

**Spectroscopic Measurements.** The binding constants were measured by stepwise addition of ONS−8 to SNA1 solution starting with an ON:SNA ratio of 2:1 and finishing at a 14:1 ratio. DPBS was used as the solvent. The fluorescence and excitation spectra were recorded with an FLS-1000 spectrophotometer (Edinburgh Instruments, U.K.). The fluorescence spectra were corrected according to the wavelength sensitivity of the detector and the excitation source intensity.

Time-resolved fluorescence was measured using a time-correlated single photon counting (TCSPPC) system (PicoQuant GmbH, Chaussee, Germany) consisting of a PicoHarp 300 controller and a PDL 800-B driver. The samples were excited with the pulsed diode laser head LDH-P-C-485 at 483 nm at a time resolution of 130 ps. The signals were detected with a microchannel plate photomultiplier tube (Hamamatsu R2809U). The influence of the scattered excitation light was reduced with a cutoff filter (transmission > 490 nm) in front of the monitoring monochromator. Fluorescence decays were collected with a constant accumulation time in the 500−570 nm wavelength range with steps of 10−20 nm. The instrumental response function (IRF) was measured separately, and the decays were deconvoluted and fitted globally by applying the iterative least-squares method to the sum of 2 exponents (eq 1).

$$ I(t) = \sum_i a_i e^{-\tau_i/t_i} $$

(1)

In this eq 1, $\tau_i$ is the global lifetime and $a_i$ is the local amplitude (preexponential factor). The average lifetimes $\langle \tau \rangle$ were calculated using eq 2

$$ \langle \tau \rangle = \frac{\sum_i a_i \tau_i}{\sum_i a_i} $$

(2)

**Materials for Cell Uptake Studies.** All cell reagents were purchased via CityLab Helsinki/Turku/Finland. RPMI 1640 Medium (Gibco, catalog no: 21870034). 10× DPBS (DPBS, 10×, no calcium, no magnesium, catalog number 1400-067) was diluted to autoclaved Milli-Q water to make 1× DPBS (osmolality in 10X: 2630−3000 mOsm kg$^{-1}$, after dilution osmolality in 1X: 263−300 mOsm kg$^{-1}$). TrypLE Express enzyme 1X without phenol red (Gibco, catalog number 12604012). PBS (Lonza, catalog number:BE17-S16F) 6.7 mM (PO$_4$), 50 mM, pH 7.4 without calcium, magnesium, and phenol red. Live dead kit (Invitrogen LIVE/DEAD Viability/ Cytotoxicity Kit, for mammalian cells catalog number: L3224). Paraformaldehyde (4%) was obtained from the University of Helsinki. Fetal bovine serum (FBS) (Gibco, catalog no: 10270-106), Antibiotic, penicillin−streptomycin (Gibco, catalog no: 15140-122). DAPI + mountant [Prolong Diamond antifade mountant with DAPI (Invitrogen by Thermo Fisher Scientific)]; 8-well chamber slides [Nunc Lab-Tek 2 Chamber slide, 8-well (Thermo Scientific)].

**Cell Culture.** PC3 cells were purchased from American Tissue Culture Collection (ATCC) and grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin−streptomycin. During subculturing, the cells were washed with DPBS and detached from the T25 flask using TrypLe Express. After aliquoting the cells to new flasks (in 1:3−1:6 ratio, v/v), the cell culture was maintained at 37 °C with 5% CO$_2$. For incoming cell uptake experiments, the cells were plated in eight-well chamber slides at 30% confluency 24 h prior to the treatment. Cell passage numbers during the assays in question ranged from p46 to p53. During subculturing, the cells were examined through a Nikon TMS inverted microscope using 10X PH1 objective.

**Cell Uptake Experiments.** The PC3 cell uptake studies of the oligonucleotides and SNAs were compared to untreated PC3 cells as control. For the experiments, the cells were seeded at 30% confluency in an eight-well chamber slide [Nunc Lab-Tek 2 Chamber slide, 8-well (Thermo Scientific)] 24 h before...
experiments. With oligonucleotides and SNAs, the used cell confluency was about 90%. The cell uptake studies were carried out at the concentration of 120 nM of oligonucleotides and 10 nM SNA. After that, the cells were incubated (37 °C with 5% CO₂) with the studied compounds or in PBS (controls) for 4 h. After uptake, the cells were washed three times with PBS, each wash taking 5 min. The cells were then fixed using 4% paraformaldehyde solution (diluted in PBS). The intracellular delivery of oligonucleotides and SNAs was quantified via wide-field microscopy on a Nikon Eclipse Ti2-E microscope using the Alexa 488 (475 nm) and DAPI (395 nm) channels and normalized based on the intensity of the untreated control cells.

Confocal and Wide-Field Microscopy. PC3 cells were cultured on the eight-well chamber slides and incubated for 24 h before being treated with the appropriate oligonucleotide formulation (final oligonucleotide concentration = 120 nM). The treated cells were then fixed using 4% paraformaldehyde solution (PFA) and washed three times with PBS. The well was removed, and the nuclei of the cells were stained and mounted on the slides using Prolong Diamond antifade mountant with DAPI. Imaging of the cells was performed using a Nikon Eclipse Ti2-E wide-field inverted microscope using Nikon NIS Elements AR 5.11.01 64-bit acquisition software. The objective, used as dry, was a 20x Nikon CFI Plan Apo Lambda 0.75 NA with a 1 mm working distance. Excitation wavelengths were for DAPI channel: 395/25 nm and emission wavelength: 435/26 nm (DAPI sPx), and for Alexa 488 channel: 475/28 nm and emission wavelength: 515/30 nm (GFP sPx). Images were acquired using a Hamamatsu ORCA-Fire camera with a pixel size of 6.5 μm. Images were 12-bit with pixel dimensions 600 × 600 px. Wide-field microscopy was carried out with a Nikon Eclipse Ti2-E wide-field microscope. Imaging was performed with Nikon NIS Elements 4.11 acquisition software, and the picture format of the images is 2048 × 2044 px. The picture format of cropped images is 600 × 600 px. The images were edited with the Fiji-ImageJ software (2.1.0) by adjusting minimum and maximum, brightness and contrast, sharpening, adding scale bar, and adding text bar. During subculturing, the cells were examined through a Nikon TMS inverted microscope with a 10× Ph1 objective.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.1c00539.
NMR data of the carbohydrates synthesized, experimental details for small-molecule trials of the SPANC conjugates, additional fluorescence data, and cell uptake images (PDF)

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Notes
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