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# Quantitative Fluorine NMR To Determine Carbohydrate Density on Glyconanomaterials Synthesized from Perfluorophenyl Azide-Functionalized Silica Nanoparticles by Click Reaction

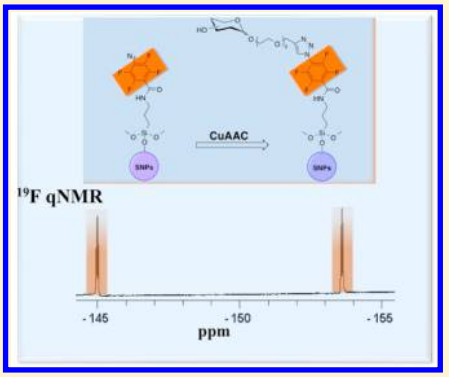
Na Kong,[†] Juan Zhou,[†] JaeHyeung Park,[‡] Sheng Xie,[†] Olof Ramström,*[,†] and Mingdi Yan*[,†,‡]

[†]Department of Chemistry, KTH - Royal Institute of Technology, Teknikringen 30, S-10044 Stockholm, Sweden

[‡]Department of Chemistry, University of Massachusetts Lowell, 1 University Ave., Lowell, Massachusetts 01854, United States

Supporting Information

**ABSTRACT:** A quantitative fluorine NMR ($^{19}$F qNMR) method was developed to determine the carbohydrate density on glyconanomaterials. Mannose (Man)- and galactose (Gal)-conjugated silica nanoparticles (SNPs) were synthesized from perfluorophenyl azide (PFPA)-functionalized SNPs and propargylated Man or Gal by copper-catalyzed azide−alkyne cycloaddition (click reaction). After treating PFPA-SNPs or Man-SNPs with hydrofluoric acid followed by lyophilization, the remaining residues were directly subjected to $^{19}$F NMR analysis. The density of PFPA on PFPA-SNP was determined to be $7.7 \pm 0.2 \times 10^{-16}$ nmol/nm$^2$ and Man on Man-SNP to be $6.4 \pm 0.2 \times 10^{-16}$ nmol/nm$^2$ giving a yield of ~83% for the click coupling reaction. The apparent dissociation constant ($K_d$) of Man-SNPs with fluorescein isothiocyanate (FITC)-concanavalin A (Con A) was determined using a fluorescence competition assay to be $0.289 \pm 0.003$ $\mu$M, which represents more than 3 orders of magnitude affinity increase compared to free Man with Con A.




Glycoconjugates are key structural components of the cell membrane and extracellular matrix and serve a variety of functional roles in cell-mediated processes as well as infection by pathogens.[1] Glycans have relatively low affinity toward glycan-binding entities, and as such, nature takes advantage of the multivalent effect where multiple copies of glycans cluster together to interact with lectins in a cooperative manner to greatly enhance the binding affinity. Glyconanomaterials use nanomaterials as scaffolds and, by conjugating multiple copies of glycans, amplify their affinity toward glycan-binding lectins. A variety of glyconanomaterials have been developed, such as carbohydrate-coated gold nanoparticles, carbon nanomaterials, quantum dots, silica nanoparticles, etc., and have found utilities in for example biosensing, imaging, and therapeutics.[2−10]

The binding affinity of the glyconanomaterials with their receptors is the most important feature that dictates the performance of the glyconanomaterials in these applications. In order to calculate the affinity constant, the carbohydrate density on the nanomaterials must be determined. However, quantitative analysis of carbohydrate density on glyconanomaterials is challenging. Commonly used analytical techniques include thermogravimetric analysis (TGA)[11−13] and anthrone/phenol-sulfuric acid assay.[10,14−23] TGA measures the weight loss of the sample as it is heated, and it does not provide the structural information on the ligand. In addition, organic components other than the carbohydrate, impurities, and solvents entrapped in the particles will all contribute to the weight loss, thus complicating the data analysis. The anthrone- or phenol-sulfuric acid method is a colorimetric assay based on the reaction between carbohydrates and the anthrone/phenol reagent to form colored complexes that can be quantified with absorption spectrophotometry.[24] The assay has high specificity toward carbohydrates and derivatives, and is relatively easy to perform. However, the method suffers from low reproducibility. The anthrone-sulfuric acid reagent is unstable and must be freshly prepared before the assay. Also, the temperature needs to be carefully controlled to ensure adequate color development.[25]

Nuclear magnetic resonance (NMR) spectroscopy is a widely used technique that gives precise structural information on organic compounds. qNMR, NMR used for quantitative analysis, has been applied in the analysis of pharmaceutics, natural products, and metabolites,[26−35] providing absolute and accurate information on structure and composition. Among the nuclei used for NMR, the $^{19}$F nucleus is 100% naturally abundant, and no isotope enrichment is therefore needed, thus giving high NMR sensitivity. The absolute sensitivity of $^{19}$F is ~4700 times higher than $^{13}$C and only ~12% lower than $^{1}$H. Furthermore, $^{19}$F has negligible background interference, and the spectra are relatively simple. Quantitative $^{19}$F NMR ($^{19}$F qNMR) has found important uses in quantification of fluorinated compounds in complex matrices,[36−38] in polymers grafted on nanoparticles,[39] and for the quantitative measurement of enzymatic activity by determining released fluorinated groups from nanomaterials.[40−44] There has been no report on

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applying $^{19}$F qNMR for the analysis of carbohydrate density on glyconanomaterials.

Here, we report that $^{19}$F qNMR can be used to quantitatively determine the density of carbohydrate ligands on glyconanomaterials. Perfluorophenyl azide (PFPA)-functionalized silica nanoparticles (SNPs) were used as a model system based on the following considerations: (1) SNPs can be readily prepared in large quantity using simple protocols; (2) the presence of F atoms on PFPA allows the quantification of ligands by $^{19}$F NMR; (3) PFPA is a versatile functional group[45,46] that has been used as photocoupling agent to conjugate a wide range of entities, including carbohydrates, to flat surfaces[47−56] and nanomaterials.[15−17,22,23,57−65] Here, we explore the reactivity of PFPA in the copper-catalyzed azide−alkyne cycloaddition (CuAAC) for the synthesis of glyconanomaterials. $^{19}$F qNMR was employed to quantitatively determine the carbohydrate ligand density and the conjugation yield. The result was furthermore used to determine the apparent dissociation constant ($K_d$) of carbohydrate-presenting SNPs with lectins by a fluorescence competition binding assay.

## EXPERIMENTAL SECTION

**Synthesis.** *2-(2-(2-(Prop-2-ynyloxy)ethoxy)ethoxy)ethanol (**1**).* This compound was synthesized according to a previous procedure.[56] Triethylene glycol (22.3 mL, 167 mmol) and potassium hydroxide (3.7 g, 61.7 mmol) were added to a flask and stirred at 40 °C for half an hour under nitrogen atmosphere. Propargyl bromide (6 mL, 55.7 mmol) was subsequently added. The resulting mixture was heated at 60 °C for 3 h. Water (50 mL) was added to dilute the reaction mixture, and 1 M hydrochloric acid was used to acidify the solution to pH 1. The obtained mixture was extracted with EtOAc (3 × 100 mL), and brine (2 × 100 mL) was used to wash the combined organic phase. The resulting solution was then dried over $MgSO_4$ and evaporated to give **1** as a yellow oil, which was purified by flash column chromatography using hexanes/EtOAc (2:3 v/v, 2.52 g, 24%). $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 4.19 (d, 2 H, $J$ = 2.2 Hz, CHCC$H_2$O), 3.72−3.64 (m, 10 H, OC$H_2$), 3.58 (t, 2 H, $J$ = 4.4 Hz, C$H_2$OH), 2.56 (s, 1 H, O$H$), 2.43 (t, 1 H, $J$ = 2.3 Hz, C$H$CCH$_2$). $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 79.6, 74.8, 72.6, 70.7, 70.4, 70.3, 69.2, 61.8, 58.5.

*2-[2-(2-Propargyloxyethoxy)ethoxy]ethanol-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside (**4a**).*[66] Penta-*O*-acetate-α-D-mannose (**3a**) was synthesized following a previously reported method.[58,67] Compound **3a** (1 g, 2.56 mmol) and **1** (720 mg, 3.84 mmol) in dry dichloromethane (DCM, 30 mL) was stirred at 0 °C, and $BF_3{\cdot}Et_2O$ (1.82 g, 12.8 mmol) was added dropwise. After stirring at r.t. overnight under nitrogen atmosphere, ice water (30 mL) was poured into the solution, and DCM (3 × 50 mL) was used to extract the resulting product. Saturated $NaHCO_3$ and brine was subsequently applied to wash the organic extract, which was then dried over $MgSO_4$. Finally, the DCM was evaporated under reduced pressure, and the resulting product was purified by column chromatography using hexane/EtOAc (2:1 to 1:1 v/v) to give compound **4a** as a pale yellow oil (763 mg, 57%). $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 5.15 (dd, 1 H, $J$ = 10.0 and 3.3 Hz, H-3), 5.14−5.10 (m, 2 H, H-4, 2), 4.73 (d, 1 H, $J$ = 1.5 Hz, H-1), 4.15 (dd, 1 H, $J$ = 12.6 and 5.0 Hz, H-6b), 4.05 (d, 2 H, $J$ = 2.24, C$H_2$CCH), 3.98−3.91 (m, 2 H, H-6a, H-5), 3.70−3.64 (m, 1 H, OC$H_2$), 3.57−3.44 (m, 11 H, OC$H_2$), 2.36 (t, 1 H, $J$ = 2.3 Hz, CH$_2$CC$H$), 2.01 (s, 3 H, OAc), 1.95 (s, 3 H, OAc), 1.90 (s, 3 H, OAc), 1.84 (s, 3 H, OAc). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ 170.2, 169.6, 169.5, 169.4, 97.3, 79.5, 75.5, 70.37, 70.3, 70.1, 69.7, 69.2, 68.8, 68.1, 67.0, 65.7, 62.1, 58.0, 20.7, 20.6, 20.4, 20.3.

*2-[2-(2-Propargyloxyethoxy)ethoxy]ethanol-α-D-mannopyranoside (**5a**).*[66] Compound **4a** (653 mg, 1.26 mmol) and NaOMe (136 mg, 2.52 mmol) were dissolved in dry MeOH (5 mL) stirring under nitrogen at r.t. for 4 h. The resulting mixture was neutralized by addition of Amberlyst 15 $H^+$ resin and filtered. The filtrate was evaporated, and the crude product was purified by flash column chromatography using solvent system $CH_2Cl_2/CH_3OH$ (6:1 v/v) to give compound **5a** as a yellowish oil (280 mg, 67%). $^1$H NMR (500 MHz, $D_2O$): $\delta$ 4.86 (s, 1 H, H-1), 4.28 (d, 2 H, $J$ = 2.3 Hz, C$H_2$CCH), 3.94 (dd, 1 H, $J$ = 3.0 and 1.5 Hz, H-2), 3.86 (m, 2 H, OC$H_2$), 3.79 (dd, 1 H, $J$ = 3.0 and 1.5 Hz, H-3), 3.74−3.61 (m, 14 H, H-4, 5, 6a, 6b, OC$H_2$), 2.87 (t, 1 H, $J$ = 2.3 Hz, CH$_2$CC$H$). $^{13}$C NMR (125 MHz, $D_2O$): $\delta$ 99.9, 79.2, 75.9, 72.7, 70.5, 69.9, 69.6, 69.5, 69.5, 69.4, 68.6, 66.7, 66.4, 60.9, 57.9. MS (ESI, MeOH): $m/z$ = 373.27 $[M + Na]^+$.

*2-[2-(2-Propargyloxyethoxy)ethoxy]ethanol-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (**4b**).*[66] The synthesis was carried out as described for **4a** starting from commercially available β-D-(+)-galactose pentaacetate **3b** and compound **1** to give **4b** as a pale yellow oil (yield 60%). $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 5.22 (d, 1 H, $J$ = 3.18 Hz, H-4), 5.03 (m, 1 H, H-2), 4.86 (dd, 1 H, $J$ = 10.4 and 3.3 Hz, H-3), 4.45 (d, 1 H, $J$ = 7.9 Hz, H-1), 4.04 (d, 2 H, $J$ = 2.3 Hz, C$H_2$CCH), 4.01−3.92 (m, 2 H, H-6a, H-6b), 3.82−3.76 (m, 2 H, H-5, OC$H_2$), 3.64−3.43 (m, 1 H, OC$H_2$), 2.36 (t, 1 H, $J$ = 2.25 Hz, CH$_2$CC$H$), 1.99 (s, 3 H, OAc), 1.91 (s, 3 H, OAc), 1.89 (s, 3 H, OAc), 1.82 (s, 3 H, OAc). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ 170.1, 170.0, 169.8, 169.2, 100.9, 79.5, 74.6, 70.6, 70.4, 70.3, 70.3, 70.1, 70.1, 68.8, 68.7, 68.6, 66.9, 61.08, 58.1, 20.5 20.4, 20.4, 20.3.

*2-[2-(2-Propargyloxyethoxy)ethoxy]ethanol-β-D-galactopyranoside (**5b**).*[66] The compound was prepared following the same procedure as compound **5a** to give **5b** as a yellowish oil (yield 75%). $^1$H NMR (400 MHz, $D_2O$): $\delta$ 4.38 (d, 1 H, $J$ = 7.6 Hz, H-1), 4.21 (d, 2 H, $J$ = 2.1 Hz, C$H_2$CCH), 4.0 (m, 1H, H-5), 3.88 (d, 1H, $J$ = 3.2 Hz, H-4), 3.83−3.5 (m, 16 H, H-2, H-3, H-6a, H-6b, OC$H_2$), 2.86 (t, 1 H, $J$ = 2.2 Hz, CH$_2$CC$H$). $^{13}$C NMR (100 MHz, $D_2O$): $\delta$ 102.8, 79.2, 75.9, 75.1, 72.6, 70.7, 69.7, 69.5, 69.4, 69.3, 68.6, 60.9, 57.9, 48.8. MS (ESI, MeOH): $m/z$ = 373.27 $[M + Na]^+$.

*4-Azido-N-butyl-2,3,5,6-tetrafluorobenzamide (**6**).*[68] PFPA-NHS (100 mg, 0.30 mmol), TEA (30 mg, 0.30 mmol), and propylamine (19.4 mg, 0.33 mmol) were dissolved in DCM (15 mL) and stirred at r.t. for 4 h. The resulting mixture was diluted with water and extracted twice with DCM. 1 M hydrochloric acid and brine were used to wash the combined organic phase, respectively. The extract was then dried over $MgSO_4$ and evaporated under reduced pressure. The resulting product was purified by flash column chromatography using hexane/EtOAc (3:1, v/v) to yield compound **6** as white crystals (69 mg, 83%). $^1$H NMR (400 MHz, methanol-$d_4$): $\delta$ 3.35 (dd, 2 H, $J$ = 6.79 and 13.17 Hz, NHC$H_2$), 1.63 (m, 2 H, CH$_3$C$H_2$), 0.91 (t, 3H, $J$ = 7.17 Hz, CH$_2$C$H_3$). $^{13}$C NMR (100 MHz, methanol-$d_4$): $\delta$ 160.1, 146.6, 143.9, 143.4, 110.4, 42.8, 23.4, 11.6. $^{19}$F NMR (376 MHz, methanol-$d_4$): $\delta$ 145.04, 153.64. HRMS-ESI: Calcd for $C_{10}H_8F_4N_4O_{10}$ $[M + H]^+$: 277.0707, obtained 277.0703; FT-IR: 672.3, 687.3, 730.9, 768.5, 819.5, 867.1, 888.7, 933.7, 978.7, 1016.2, 1154.5, 1220.5, 1271.7,





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1367.9, 1407.1, 1476.2, 1551.4, 1650.7, 1740.8, 2129.2, 2868.4, 2931.6, 2972.2, 3021.6, 3080.4, 3278.7, 3461.4 $cm^{-1}$.

*Compound 7.* To an acetone solution (2 mL) of compound **6** (15 mg, 0.054 mmol), a solution of **5a** (28 mg, 0.08 mmol) in $H_2O$ (1 mL) was added. An aqueous solution of sodium ascorbate (2.1 mg, 0.011 mmol) and $CuSO_4{\cdot}5H_2O$ (2.7 mg, 0.011 mmol) in $H_2O$ (1 mL) was subsequently added. The mixture was stirred at r.t. overnight and evaporated to give the crude product. The crude product was purified by column chromatography using $CH_2Cl_2$/methanol (7:1, v/v) to yield compound **7** as a yellow oil (25 mg, 74%). $^1H$ NMR (400 MHz, methanol-$d_4$): $\delta$ 8.46 (s, 1 H, triazole-H), 4.80 (s, 1 H, H-1), 4.21 (s, 1 H, $CH_2CNN$), 3.83−3.59 (m, 19 H, 6 × $OCH_2$, $CH_2CNN$, H-2, H-3, H-4, H-5, H-6a, 6b), 3.40 (m, 2 H, $NHCH_2$), $\delta$ 1.67 (m, 2 H, $CH_3CH_2$), 1.02 (t, 3H, $J$ = 7.42 Hz, $CH_2CH_3$). $^{13}C$ NMR (100 MHz, methanol-$d_4$): $\delta$ 159.2, 146.7, 141.7, 127.7, 119.8, 101.7, 162.4, 136.2, 129.6, 127.9, 101.8, 80.6, 75.9, 75.1, 74.8, 74.6, 73.7, 72.6, 72.1, 71.6, 71.4, 71.1, 70.1, 68.6, 67.7, 64.8, 62.9, 62.2, 59.0, 42.9, 23.4, 11.6. $^{19}F$ NMR (376 MHz, methanol-$d_4$): $\delta$ 142.97, 148.30. ESI-HRMS: Calcd for $C_{25}H_{34}F_4N_4O_{10}$ $[M + H]^+$: 627.2284, obtained 627.2284; FT-IR: 891.9, 988.1, 1032.9, 1059.1, 1095.3, 1137.2, 1221.3, 1365.6, 1443.8, 1494.8, 1666.2, 1738.1, 2968.3, 2929.3, 3023.1, 3425.2 $cm^{-1}$.

**Synthesis of SNPs.** Ammonium hydroxide (28%, 15 mL) was dissolved in ethanol (250 mL) in a three-necked round-bottom flask at 30 °C. A solution of TEOS (10 mL) in ethanol (10 mL) was added dropwise into the flask, and the resulting mixture stirred for 24 h. The reaction mixture was centrifuged at 7500 rpm for 20 min, and the sediments were washed with ethanol. The washing was repeated three times, and the resulting silica nanoparticles were dried under vacuum at 50 °C.

**Synthesis of PFPA-Functionalized SNPs (PFPA-SNPs).** A mixture of PFPA-silane (219 mg, 0.5 mmol) and SNPs (400 mg) in dry toluene (10 mL) was stirred at 70 °C for 16 h. The obtained nanoparticles were collected by centrifugation and washed two times with toluene and ethanol (7500 rpm, 20 min), respectively. The obtained PFPA-SNPs were dried under vacuum.

**Conjugation of Carbohydrates to PFPA-SNPs by CuAAC.** To an acetone suspension (2 mL) of PFPA-SNPs (63 mg), an aqueous solution (1 mL) of **5a** or **5b** (100 mg, 0.28 mmol) was added, followed by a solution of sodium ascorbate (5.5 mg, 0.028 mmol) and $CuSO_4{\cdot}5H_2O$ (7.0 mg, 0.028 mmol) in $H_2O$ (1 mL). The reaction mixture was stirred at r.t. overnight. Membrane dialysis in water was used to remove excess reagents. The particles were collected by centrifugation of the suspension at 11 000 rpm for 20 min and washed sequentially with $H_2O$ twice (11 000 rpm, 20 min) and acetone once (11 000 rpm, 20 min). The precipitate was finally dried under reduced pressure to give mannose-presenting (Man-SNPs) or galactose-presenting (Gal-SNPs) (∼50 mg).

**Determination of Ligand Density by $^{19}F$ qNMR.** PFPA-SNPs (50 mg) or Man-SNPs (46 mg) were treated with 3 mL of 5% HF aqueous solution at r.t. for 1 h (Caution: extreme care should be taken when handling hydrofluoric acid as it is highly toxic to skin). The resulting mixture was lyophilized with an in-line trap containing solid CaO to remove excess HF and other volatiles. The residual was then dissolved in methanol-$d_4$ together with methyl pentafluorobenzoate as the internal standard for $^{19}F$ NMR analysis. $^{19}F$ NMR spectra were recorded on a Bruker Ascend TM 400 instrument at 376 MHz equipped with 5 mm PABBO BB probe. Acquisitions were performed in 5 mm NMR tubes at 298 K, with acquisition time (AQ) of 0.734 s followed by a relaxation delay (D1) of 1 s for complete longitudinal relaxation (T1). The number of scans (NS) was 128 for model compounds and 256 for particle samples. The spectra were obtained by 90° pulses over a spectral width (SW) of 237.1418 ppm. The transmitter offset (o1p) and time domain (TD) were −99.999 ppm and 131 072, respectively. For the post acquisition processing, a line broadening for em (LB) of 1.00 Hz and a Gaussian factor (GB) of 0.05 were used. Phase correction and integration were manually carried out, and baseline correction was performed under the fifth order polynomial. The ligand density $\rho_L$ (nmol/nm$^2$) of PFPA on PFPA-SNP or Man on Man-SNP was calculated according to

$$\rho_L = \frac{I_L n_S d_P \rho_P}{12 I_S m_P} \quad (1)$$

where $I_L$ and $I_S$ are the NMR integrals of the ligand and standard, respectively, $n_s$ is the molar amount of the standard, $d_P$ is the particle diameter, $\rho_P$ is the particle density (2.2 g/cm$^3$), and $m_P$ is the initial weight of nanoparticle sample used (see the Supporting Information for calculation).

In the case of the PFPA-SNPs (50 mg), methyl pentafluorobenzoate standard (0.0136 mmol) together with the product after lyophilization were dissolved in 0.6 mL of methanol-$d_4$. The amount of sample used depends on the size of the nanoparticle and sensitivity of the NMR instrument. The smaller the particle and the higher the sensitivity of the instrument, the less the sample needed. The peak integration of F-2,6 at −145.0 ppm from the PFPA-SNPs was used as $I_L$. The peak integration of F-4 at −152.3 ppm from methyl pentafluorobenzoate was used as $I_S$. The experiment was repeated twice with samples from the same batch, and the results were averaged to obtain the PFPA density on PFPA-SNP.

For the Man-SNPs (46 mg), methyl pentafluorobenzoate (0.007 mmol) was added to the lyophilized products in 0.5 mL of methanol-$d_4$. The peak integration of F-2,6 at −143.0 ppm from the Man-SNPs was used as $I_L$. The peak integration of F-4 at −152.3 ppm from methyl pentafluorobenzoate was used as $I_S$. The experiment was repeated twice with samples from the same batch, and the results were averaged to obtain the density of Man on Man-SNP.

**Determination of Ligand Density by TGA.** For TGA analysis, the samples were dried for 3 days in a vacuum oven. Measurements were carried out under nitrogen at a constant heating rate of 20 °C/min from 20 to 800 °C. Two sets of each analysis were carried out. The percent weight difference (%) before and after functionalization, together with the percent weight (%) after functionalization at 800 °C, was used for ligand density calculation. The ligand density $\rho_L$ (nmol/nm$^2$) of PFPA on PFPA-SNP or Man on Man-SNP was calculated according to

$$\rho_L = \frac{(P_b - P_a) d_P \rho_P}{6 P_a M} \quad (2)$$

where $P$ is the percent weight (%) of the SNPs before ($P_b$) and after ($P_a$) ligand coupling at 800 °C, $d_P$ is the particle diameter, $\rho_P$ is the particle density (2.2 g/cm$^3$), and $M$ is the molecular weight of the organic residue of the particles (see the Supporting Information for calculation).



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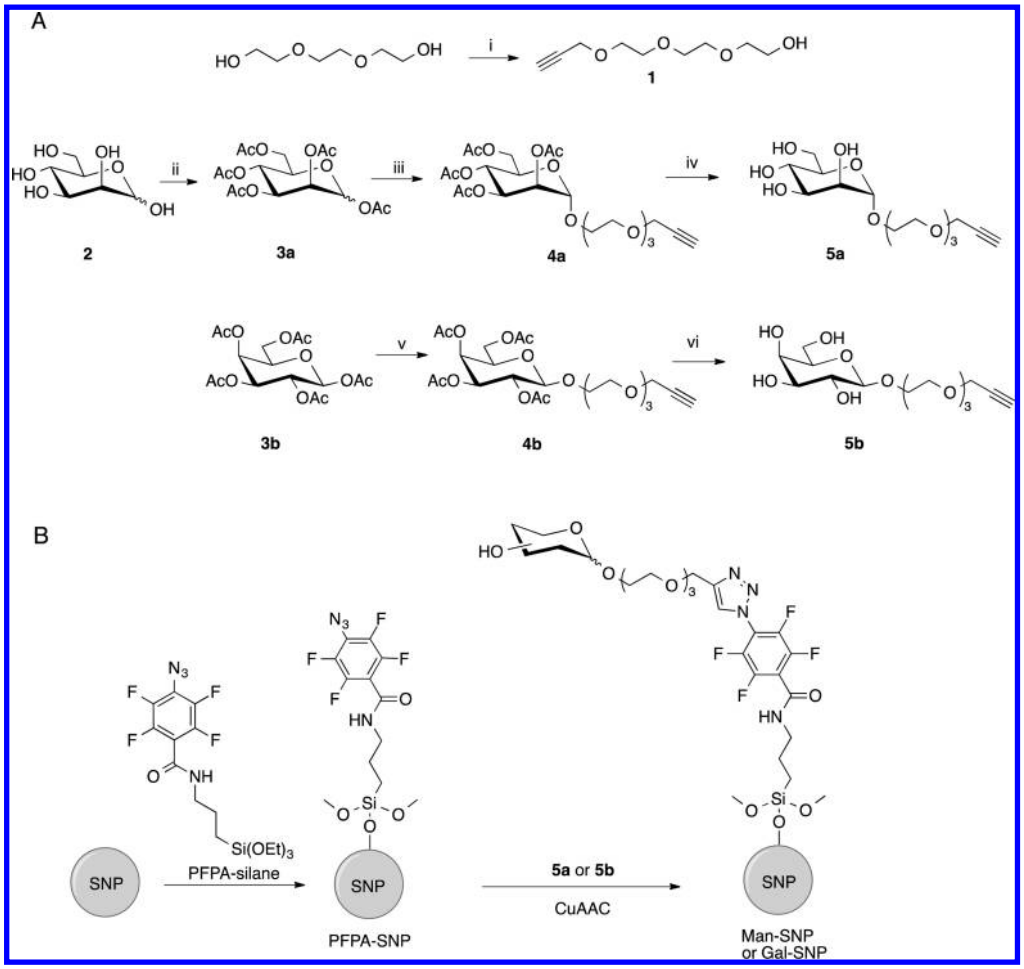
**Scheme 1. (A) Synthesis of Propargylated Carbohydrate;[a] (B) Synthesis of PFPA-SNPs and Conjugation of Carbohydrate by the Click Reaction**




[a](i) KOH, propargyl bromide, 60 °C, 3 h; (ii) $Ac_2O$, $I_2$, 0−25 °C, 1 h; (iii, v) **1**, $BF_3{\cdot}Et_2O$, DCM, 0−25 °C, 24 h; (iv, vi) NaOMe, MeOH, 4 h.

For the PFPA density on PFPA-SNP, $P_a$ is the percent weight (%) of the PFPA-SNPs, and $P_b$ is percent weight (%) of the SNPs. For the Man density on Man-SNP, $P_a$ is the percent weight (%) of Man-SNPs, and $P_b$ is the percent weight (%) of PFPA-SNPs. The experiment was repeated twice with samples from the same batch, and the results were averaged.

**Lectin Binding Analysis.** The binding affinity and selectivity of the carbohydrate-conjugated SNPs were tested using fluorescein isothiocyanate (FITC)-concanavalin A (Con A) and FITC-Ricinus Communis Agglutin I (RCA I). Man-SNPs and Gal-SNPs (3.0 mg) were incubated in a solution of BSA (3 wt %) in pH 7.4 PBS buffer containing 0.05% Tween (2.0 mL, 10 mM) for 30 min, respectively, and centrifuged. The nanoparticles were then dispersed in the PBS buffer without BSA for another 20 min and centrifuged. The particles were subsequently incubated in the FITC-Con A buffer solution (2.5 mL, 10 μg/mL) containing $MnCl_2$ (1.0 mM) and $CaCl_2$ (1.0 mM) for 1 h or FITC-RCA I buffer solution (2.5 mL, 10 μg/mL) for 4 h under ambient conditions while shaking. The mixtures were centrifuged to separate unbound FITC-lectins in the supernatant, and the fluorescence intensity of the unbound lectins was measured using a fluorescence spectrophotometer. For fluorescence microscopy studies, the collected nanoparticles were washed with water and centrifuged 3 times (11 000 rpm, 20 min) and dried under vacuum.

**Fluorescence Competition Binding Assay.** All solutions were prepared in PBS (pH 7.4) buffer containing $MnCl_2$ (1 mM) and $CaCl_2$ (1 mM). To a Man-SNP suspension (1.8 mL) of varying concentrations ($1 \times 10^{-6}$−12 mg/mL), Man (6.7 mM, 0.1 mL) and FITC-Con A (109 μg/mL, 0.1 mL) were added, resulting in a total of 2 mL of solution. The mixture was shaken at r.t. for 1 h, and the particles were collected by centrifugation at 10 000 rpm for 15 min. The supernatants were subjected to analysis by spectrofluorimetry (ex = 495 nm), and the emissions at 516 nm were recorded. Three sets of data at each concentration were collected, and the mean intensity values were used. The apparent dissociation constant $K_d$ was estimated by the Cheng-Prusoff equation:

$$K_d = \frac{IC_{50}}{1 + \frac{[M]}{K_D}} \quad (3)$$

where $IC_{50}$ is the ligand concentration displaying 50% of specific binding, $[M]$ is the concentration of free Man, $K_D$ is the dissociation constant of free Man with Con A, and $K_d$ is the dissociation constant of Man-SNPs with FITC-Con A.

## RESULTS AND DISCUSSION

Stöber SNPs having particle sizes of 87 ± 8.0 nm (Figure S1a) were synthesized and functionalized with PFPA-silane (Scheme





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1) following a previously reported protocol to give PFPA-SNPs.[52] The azide absorption signal at 2134 $cm^{-1}$ was clearly observed on the PFPA-SNPs, as well as the amide C═O absorption band at 1639 $cm^{-1}$ (Figure 1), indicating successful

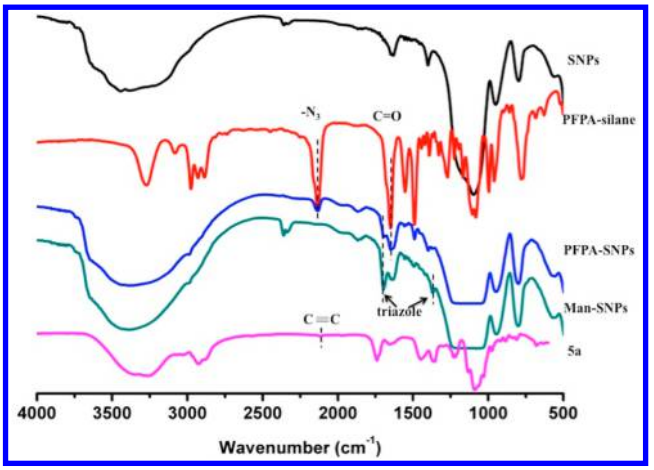



**Figure 1.** FT-IR spectra of SNPs, PFPA-silane, PFPA-SNPs, Man-SNPs, and propargylated mannose **5a**.

functionalization of the SNPs with PFPA-silane. Man or Gal was next conjugated to the PFPA-SNPs by stirring an acetone solution of PFPA-SNPs and propargylated carbohydrate (**5a** or **5b**) together with sodium ascorbate and $CuSO_4$ in water at room temperature (r.t.) overnight (Scheme 1). Excess reagents were removed by membrane dialysis and centrifugation. The FT-IR spectrum of the product showed the disappearance of the characteristic azide signal at 2134 $cm^{-1}$ and the terminal alkyne absorption band of propargylated mannose **5a** at 2114 $cm^{-1}$, together with the appearance of triazole bands at 1697 and 1377 $cm^{-1}$ on the Man-SNPs (Figure 1).

The presence of F atoms on the particles allows for accurate analysis of ligand density by $^{19}F$ qNMR. To accomplish this, the PFPA-containing nanoparticles were treated with hydrofluoric acid. Silica is readily dissolved in HF, which allows for the release of organic ligands that can be analyzed by NMR.[69,70] The PFPA-SNPs or Man-SNPs were treated with 5% HF aqueous solution at r.t. for 1 h, and the volatiles were removed by lyophilization into a CaO trap. Because F is only present in PFPA and as silicon fluorides in our system, no interference from other organic compounds are possible, and the reaction mixture can be analyzed directly without the need for further purification. Thus, the residual solids after lyophilization were dissolved in methanol-$d_4$ and subjected to $^{19}F$ NMR spectroscopy using methyl pentafluorobenzoate as the internal standard (Figure 2, marked as "S"). Methyl pentafluorobenzoate gave three F signals at −141.3 (F-2,6), −152.3 (F-4), and −163.9 (F-3,5) ppm. F-4 at 152.3 ppm was an isolated peak without any interference and was therefore used as the reference in density calculation. For the PFPA-SNPs, two sets of F signals were observed at −145.0 and −153.6 ppm (Figure 2A, marked as "∗"). These are assigned to F-2,6 and F-3,5 in PFPA since PFPA-silane gave exactly the same peaks (Figure 2B). Furthermore, these peaks did not change position when PFPA-silane was treated with HF (Figure S3), indicating the high stability of PFPA toward HF. Subsequently, the amount of PFPA on the PFPA-SNPs was quantified from integration of the PFPA signals from the PFPA-SNPs and that of the internal

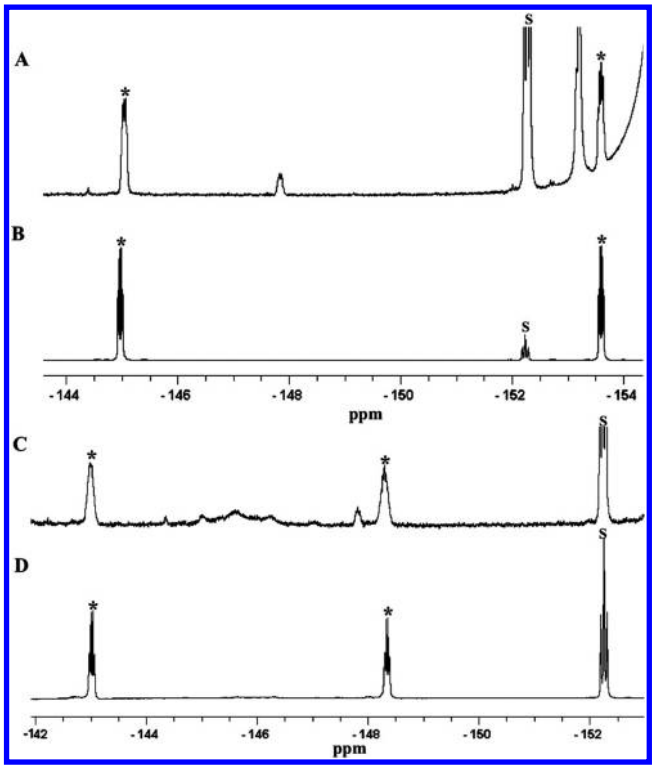
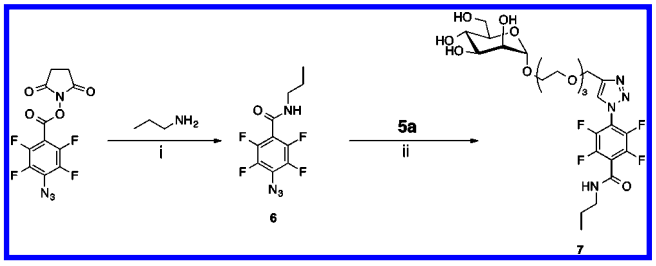



**Figure 2.** $^{19}F$ NMR spectra of (A) PFPA-SNPs after treatment with aqueous HF, (B) PFPA-silane, (C) Man-SNPs after treatment with aqueous HF, (D) compound **7**. Peak (F-4) from methyl pentafluorobenzoate standard is marked as "S". Signals F-2,6 and F-3,5 from the perfluoroaryl entities are marked as "∗".

standard eq 1. A density of 7.7 ± 0.2 × $10^{-16}$ nmol/$nm^2$ (PFPA/PFPA-SNP) was obtained.

After the click reaction of the PFPA-SNPs with propargylated Man **5a**, the signals from the PFPA-SNPs disappeared almost completely. Instead, two new sets of peaks at −143.0 and −148.3 ppm appeared in the $^{19}F$ NMR spectrum of the Man-SNPs after HF treatment (Figure 2C, marked as "∗"). These peaks matched those from the model compound **7** synthesized by cycloaddition of PFPA-amide **6** and **5a** (Scheme 2), which

**Scheme 2. Synthesis of Model Compound 7 by CuAAC**[a]




[a](i) TEA, DCM, r.t., 4 h, 83%; (ii) sodium ascorbate, $CuSO_4 \cdot 5H_2O$, acetone/$H_2O$ = 1:1, r.t., overnight.

showed the same sets of F peaks at −143.0 ppm (F-2,6) and −148.3 ppm (F-3,5, Figure 2D). These results supported the successful conjugation of Man to PFPA-SNPs and also show the high stability of the triazole moiety toward HF. The density of Man ligand on Man-SNP was calculated in the same manner from peak integration of the methyl pentafluorobenzoate standard and the Man-SNPs (eq 1) to be 6.4 ± 0.2 × $10^{-16}$





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nmol/nm$^2$ (Man/Man-SNP). From this and the density of PFPA determined previously ($7.7 \pm 0.2 \times 10^{-16}$ nmol/nm$^2$), a coupling yield of 83% was obtained for the click reaction of **5a** to PFPA-SNPs. The yield is reflected in the trace amount of unreacted PFPA-SNPs (−145.0 ppm) and unidentified side products (−145 to −146 ppm, Figure 2C) in the $^{19}$F NMR spectra.

We also measured the carbohydrate density by TGA to evaluate the TGA protocol (Figure 3). To minimize the

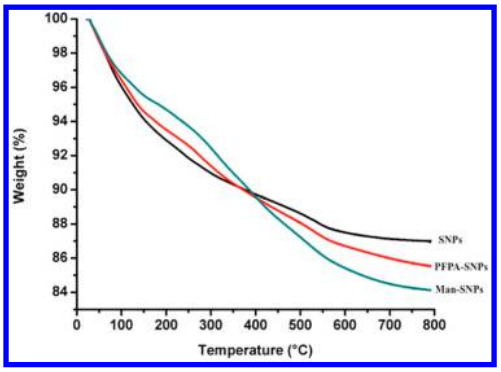


**Figure 3.** Typical TGA graphs of SNPs, PFPA-SNPs, and Man-SNPs.

influence of volatiles, samples were dried under vacuum at room temperature for 3 days before TGA measurements. The weight loss difference and the percent weight of PFPA-SNPs at 800 °C were used to estimate the density of PFPA on PFPA-SNPs according to eq 2, which gave $15.2 \pm 4.1 \times 10^{-16}$ nmol/nm$^2$. Similarly, a density of $13.0 \pm 3.4 \times 10^{-16}$ nmol/nm$^2$ was obtained for Man on Man-SNP by the weight loss difference between Man-SNPs and PFPA-SNPs. These results were higher than those obtained from $^{19}$F qNMR. The error margins were also larger in the case of TGA (Table 1). Since TGA measures

**Table 1. Ligand Density Measured by $^{19}$F qNMR and TGA**

| method | PFPA/PFPA-SNP ($\times 10^{-16}$ nmol/nm$^2$) | Man/Man-SNP ($\times 10^{-16}$ nmol/nm$^2$) |
|---|---|---|
| $^{19}$F qNMR | 7.7 ± 0.2 | 6.4 ± 0.2 |
| TGA | 15.2 ± 4.1 | 13.0 ± 3.4 |

weight loss of the material when it is heated up to high temperature, any materials that decompose and/or vaporize at elevated temperatures, such as solvents and organic contaminants, will be included in the weight loss. The higher results from TGA could thus potentially be caused by residual organic contaminants trapped inside the particles, resulting in higher weight loss of the particles.

The binding affinity of the carbohydrate-presenting SNPs was evaluated by treating the Man-SNPs or Gal-SNPs with FITC-labeled concanavalin A (FITC-Con A) and *Ricinus communis* agglutinin I (FITC-RCA I). Con A, a homotetramer lectin at neutral pH, selectively binds to cognate structures, such as α-D-mannopyranosides and α-D-glucopyranosides.[71] When Man-SNPs (Figure 4A) were incubated with FITC-Con A, agglomeration was observed, resulting from cross-linking of Man-SNPs by tetrameric Con A (Figures 4B and S1c). The fluorescence intensity of the FITC-Con A solution (Figure 5A, I) decreased drastically after incubation with Man-SNPs for 1 h (Figure 5A, II). In contrast, when Gal-SNPs (Figure 4C) were treated with FITC-Con A in the same manner, a very small

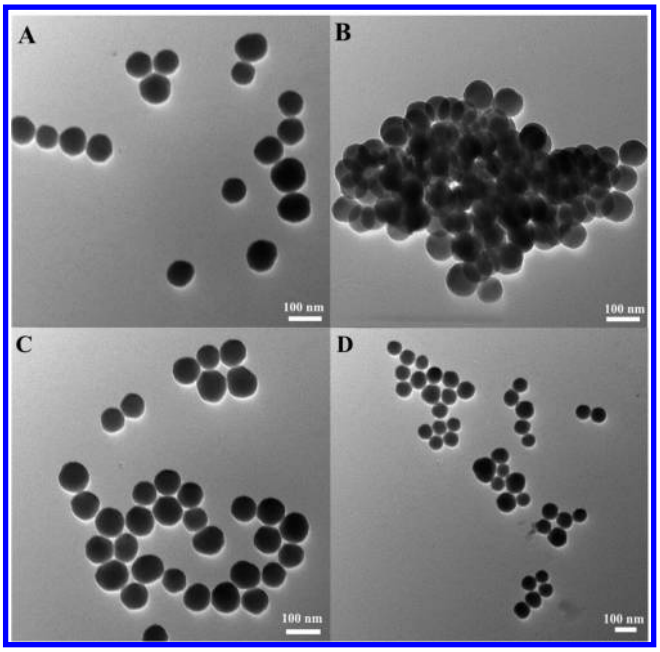


**Figure 4.** TEM images of Man-SNPs (A) before and (B) after incubation with FITC-Con A; Gal-SNPs (C) before and (D) after incubation with FITC-Con A.

decrease in fluorescence intensity was observed (Figures 4D and 5A, III). FITC-RCA I, a D-galactose-binding lectin,[72] was also applied to further investigate binding selectivity and specificity. As shown in Figure 5B, the fluorescence intensity of FITC-RCA I solution (I) decreased upon incubating with the Gal-SNPs (III). For the Man-SNPs, however, the fluorescence intensity of FITC-RCA I decreased only slightly (II), likely due to the nonspecific adsorption of protein with nanoparticles. In addition, bright fluorescence was observed for Man-SNPs/FITC-Con A agglomerates and Gal-SNPs/FITC-RCA I agglomerates (Figure S6). These results indicated that the carbohydrate ligands on the nanoparticles retained their binding affinity and selectivity.

With the ligand density accurately measured by $^{19}$F qNMR, a fluorescence competition assay previously developed in our laboratory was used to quantitatively determine the binding affinity of the glyconanoparticles with the lectins using FITC-Con A together with free Man as the competing ligand (Scheme S1).[21,22,57] Man-SNPs of varying concentrations and free Man at a fixed concentration were incubated with FITC-Con A for 1 h. The Man-SNPs/FITC-Con A agglomerates were sedimented by centrifugation, and unbound FITC-Con A and the Man/FITC-Con A complex remained in the supernatant (Figure 6A). The fluorescence intensity of the supernatant at 516 nm was plotted against the logarithmic concentration of the Man ligand on the Man-SNPs (Figure 6B). The $IC_{50}$ value obtained from the dose−response curve was subsequently used to calculate the apparent dissociation constant $K_d$ according to the Cheng-Prusoff eq (eq 3). The $K_d$ value, 0.289 ± 0.003 μM, represents more than 3 orders of magnitude higher affinity between the Man-SNPs and FITC-Con A compared to the free Man with Con A ($K_D$ = 470 μM).[73]

## CONCLUSIONS

We demonstrate that $^{19}$F qNMR can be used to efficiently determine the ligand density on fluorine-containing nano-




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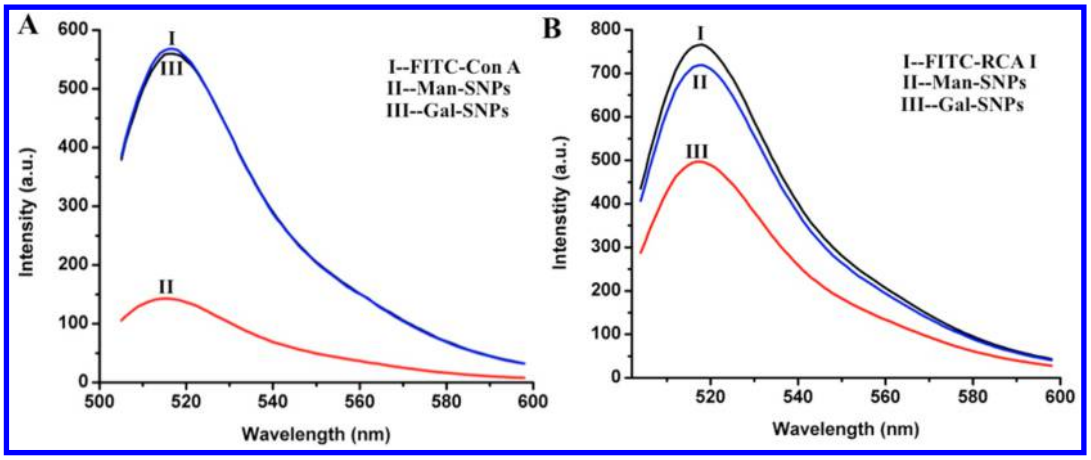


**Figure 5.** Fluorescence intensities of (A) FITC-Con A and (B) FITC-RCA I before (I) and after treatment with Man-SNPs (II) and Gal-SNPs (III).

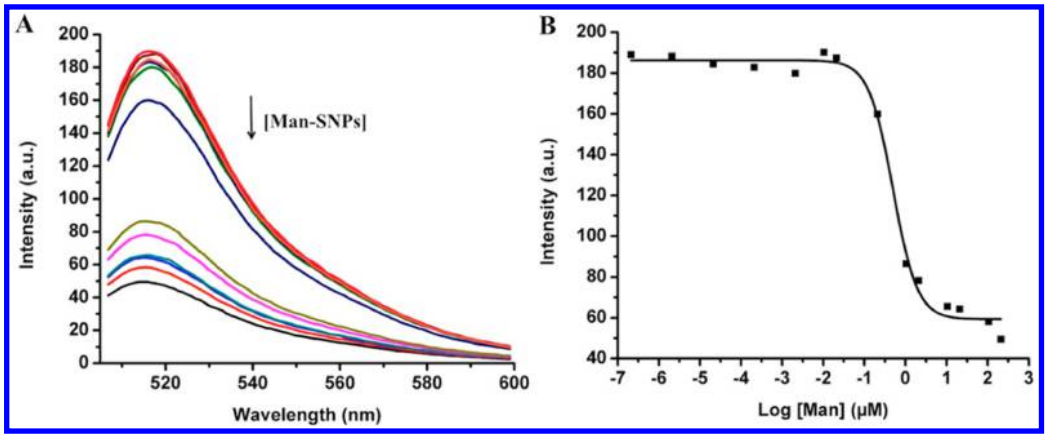


**Figure 6.** (A) Fluorescence spectra of the unbound FITC-Con A in the supernatant after treatment with increasing concentrations of Man-SNPs. (B) Fluorescence intensity vs log [Man], where [Man] is the concentration of Man from Man-SNPs determined by $^{19}$F qNMR.

particles. The quantification was accomplished without separating individual components from the mixture. In this work, we used PFPA-SNPs and conjugated carbohydrates using the CuAAC click reaction. The density of PFPA on PFPA-SNP and of Man on Man-SNP was successfully determined by $^{19}$F qNMR. The coupling yield for the synthesis of Man-SNPs by the click reaction showed reasonably high coupling efficiency. The PFPA click chemistry broadens the application of PFPA and adds to the collection of carbohydrate conjugation methods for the synthesis of glyconanomaterials. Using the ligand density measured by $^{19}$F qNMR, the apparent dissociation constant of Man-SNPs with FITC-Con A could be determined by a fluorescence competition assay. The developed $^{19}$F qNMR spectroscopy represents a new method for the quantitative analysis of glyconanomaterials. Because $^{19}$F qNMR relies on the structural information and the composition can be quantified, it measures density far more accurately than TGA or the anthrone/phenol-sulfuric acid assay. Furthermore, we believe that these conditions are amenable for other carbohydrate structures including large sugars. This is a significant advantage of the current method that no additional treatment is needed as long as the triazole linkage is robust, which proved to be true as shown by our experimental data.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b02507.

Nanoparticle characterization, titration experiment of PFPA-silane with HF, characterization of compound **7**, and fluorescence microscopy images (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Authors

*E-mail: ramstrom@kth.se.

*E-mail: Mingdi_Yan@uml.edu.

### Notes

The authors declare no competing financial interest.

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