Electronic Supplementary Material (ESI) for Nanoscale Horizons.

Electronic supplementary information

Simple engineering of hybrid cellulose nanocrystal-gold nanoparticles results in a functional glyconanomaterial with biomolecular recognition properties

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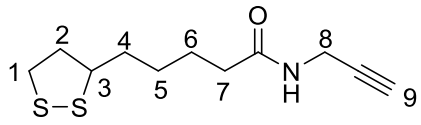

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Materials and methods

All reagents, whose synthesis is not described, were commercially available and were used without any further purification, if not specified otherwise. Cellulose nanocrystals were purchased from CelluForce® (product name CelluForce NCC^{TM}). Concavalin A (ConA) was purchased from Merk Millipore. NMR spectra were recorded on Varian Inova 400, Mercury plus 400 and Gemini 200 instruments. ESI-MS were recorded on LC-MS LCQ Fleet ThermoFisher Scientific. UV–vis spectra were recorded on a Varian Cary 4000 UV–vis spectrophotometer using a 1 cm cell. Fluorescence spectra were registered on a Jasco FP750 spectrofluorimeter using 1.0 cm cell. Powder X-ray diffraction (XRD) measurements were carried out on loosely packed powdered samples using a Bruker D8 Advance diffractometer equipped with a Cu Kα radiation (λ = 1.54178 Å) and operating in θ–θ Bragg Brentano geometry at 40 kV and 40 mA. The measurements were carried out in the 15-90 º 2θ range with a step size of 0.03 º and collection time of 1 s. Lattice parameters, a, and the mean crystallite diameters, d_{XRD}, were evaluated using the TOPAS® software (Bruker) using the method of fundamental parameter approach based on the Scherrer equation, considering a cubic space group Fm-3m. Elemental analyses were performed on a Vario MICRO cube instrument (Elementar).

ELISA solid-phase assays

The CNC-AuNPs glycoconjugates were diluted in PBS to final concentrations of 400 µg/mL, then 50 µL were used to coat NUNC MaxiSorp wells at room temperature for 4h. After washing with PBS (2×200 µL), 1% BSA (protease-free, Roche) in PBS was used as blocking agent (100 µL at room temperature for 30 min). Wells were discarded and subsequently 50 µL of the tested C-type lectins-Fc constructs solution in PBS (at 2.0 µg/mL or by titrating the lectins concentration from 10 µg/mL to 0.04 µg/mL with 0.5% BSA) were added to the wells and incubated for 1 h at room temperature. After washing with PBS (2×200 µL), 70 µL of 0.8 µg/mL of goat anti-human IgG-horse radish peroxidase (HRP, Invitrogen) were added and incubated for 30min at room temperature. After washing with PBS (2×200 µL), finally 100 µL of TMB (3,3',5,5'-Tetramethylbenzidine) solution (Sigma-Aldrich) were added. The chromogenic reaction was stopped after approximately 10 min with 50 µL of 0.8 M H_2SO_4, and the lectins binding was measured at an optical density (OD) of 450 nm. For the "quantitative" experiments data have been normalized over the binding to the positive controls set as 100% of binding. Error bars indicate standard deviations of between the mean values from three independent experiments performed in duplicate. One asterisk indicates a *p*-value <0.05; ns: not significant. Experimental data were analyzed by multiple t-test and the statistical significance was determined by the Holm-Sidak method, with alpha=5.000%, using GraphPad prism 6.

X-ray diffraction (XRD) measurements

Powder X-ray diffraction (XRD) measurements were carried out on loosely packed powdered samples using a Bruker D8 Advance diffractometer equipped with a Cu Kα radiation (λ = 1.54178 Å) and operating in θ–θ Bragg Brentano geometry at 40 kV and 40 mA. The measurements were carried out in the 10-80 º 2θ range with a step size of 0.03 º and collection time of 1 s. Lattice parameters and the mean crystallite diameters, dXRD, were evaluated using the TOPAS® software (Bruker) using the method of fundamental parameter approach based on the Scherrer equation, considering a cubic space group Fm-3m for gold and a monoclinic space group P21 for CNC.

Transmission Electron Microscopy (TEM)

TEM sample of CNC-Au-LA **1** was carefully sonicated for 1 h in water before dropping it onto a lacey carbon copper grid and left to dry overnight. The sample grid was analyzed by a ZEISS LIBRA200FE. EDX (Energy Dispersive X-ray Analysis) was performed in Scanning-TEM mode by an Oxford probe and an X-stream-2 module.

Inductive coupled plasma atomic emission spectroscopy (ICP-AES)

The determination of Au content in the samples was performed in triplicate by using a Varian 720-ES Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES). An accurately weighted amount of each sample (1-2 mg) was treated with a microwave-assisted digestion (CEM MARS Xpress) using 500 µL mL of suprapure HNO_3 obtained by sub-boiling distillation, 250 µL of suprapure HCl (Merck) and 250 µL of H_2O_2 (for ultratrace analysis, Sigma Aldrich). Each sample was thus diluted to 10 mL with Ultrapure water (UHQ), spiked with 0.5 ppm of Ge used as an internal standard, and analyzed. Calibration standards were prepared by gravimetric serial dilution from a commercial stock standard solution of gold at 1000 mg L^{-1}. The analytic wavelength used for Au determination was 242.794 nm whereas for Ge we used the line at 209.426 nm. The operating conditions were optimized in order to obtain the maximum signal intensity, and between each sample, a rinse solution constituted of 2% v/v HNO_3 was used to avoid memory effects.

Cryo-transmission electron microscopy (Cryo-TEM) sample preparation

Samples were prepared as follow: 10 µL of a 0.05% w/w suspension in MilliQ water of the CNC-AuNPs conjugate were diluted in 30 µL of the selected buffer (TMS or TRIS), then 10 µL of a 0.5 mg/mL solution of the chimera lectin (in PBS + 1 mM $CaCl_2$) were added. The mixture was kept at room temperature for 5' then ~3µL of sample were applied onto freshly glow-discharged grids (Quantifoil R2.2 Cu Mesh 300, Germany) kept at 100% humidity and 10 °C. Using a dedicated device, such as the Vitrobot Mark IV (Thermo Fisher Scientific™), the grids were then blotted with filter paper for 2 sec and quickly plunge frozen in liquid ethane in order to allow the formation of a thin (ideally ≤200nm) layer of amorphous ice with the samples embedded within. Micrographs were recorded at the Florence Center for Electron Nanoscopy (FloCEN, c/o Department of Chemistry "Ugo Schiff", University of Firenze, Italy) using a Thermo Fisher Scientific™ Glacios™ microscope equipped with X-FEG high-brightness gun at 200 keV and a Falcon 3 camera (Thermo Scientific™) operated in linear mode. Nominal magnification was set at 36000x for a calibrated pixel size of 0.4 nm. Total dose of ~4800 e^-/nm^2 was spread over 2.5 sec of exposure time with defocus value -6.0 µm. Micrographs were recorded via the EPU control software (Thermo Fisher Scientific™).

Turbidimetry assay

Turbidimetry measurements were performed in triplicate using a 96 well plate and a biorad plate reader. ConA was dissolved at the concentration of 20μM in 10 mM HEPES (pH 7.40 ± 0.01), 1 mM $MnCl_2$ and CNC-Au-Man **2** were dispersed at the concentration of 0.1% in MilliQ water. Then the two solutions were mixed in the 96-wells plate (Greiner bio-one, PS, F-bottom clear, non-binding) to obtain 200 µL 10 µM of ConA and 0.01% dispersion of **2** using 10 mM HEPES (pH 7.40 ± 0.01), 1 mM $MnCl_2$ for the dilution. The absorbance at 490 nm was read each minute for 30 minutes. In a second assay to determine the amount of ConA required to observe the agglutination of nanoparticles, CNC-Au-Man **2** 0.01% were titrated with ConA in the range of concentration from 0 to 11.3 µM measuring the absorbance at 490 nm after 60 minutes of incubation at room temperature.

Synthesis of LA 5

To a stirred solution of lipoic acid (2 g, 9.7 mmol) in dry dichloromethane (25 mL), carbonyl diimidazole (2.36 g, 14.6 mmol) and *N*-methylmorpholine (1.47 g, 14.5 mmol) were added. The mixture was stirred for 30' at room temperature, then propargylamine (806 µL, 12.6 mmol) was added dropwise and the mixture stirred overnight. The reaction mixture was diluted with 100 mL of dichloromethane and washed with water (10 x 10 mL) and brine (1 x 10 mL). The organic phase was dried over sodium sulfate, filtered and the solvent removed under vacuum. The product was recovered after filtration on silica gel (eluent dichloromethane:methanol 30:1) affording 2.2 g of pale yellow solid (93 % yield). ^{1}H-NMR (400 MHz, $CDCl_3$): 5.59 (bs, 1 H, NH), 4.05 (dd, *J* = 2.4 Hz, *J* = 5.2 Hz, 2 H, H-8), 3.59 – 3.53 (m, 1 H, H-3), 3.20 – 3.07 (m, 2 H, H-1), 2.49 – 2.41 (m, 1 H, H-2_a), 2.23 – 2.18 (m, 2 H, H-7 and H-9), 1.94 – 1.86 (m, 1 H, H-2_b), 1.72 – 1.62 (m, 4 H, H-6 and H-4), 1.51 – 1.42 (m, 2H, H-5). 1H NMR (400 MHz, DMSO-d6) δ: 8.20 (bs, 1H, NH), 3.79 (ddd, J = 18.1 Hz, J = 5.7 Hz, J = 2.5 Hz, 2H, H-8), 3.62 – 3.65 (m, , 1H, H-3), 3.23 – 2.95 (m, 2H, H-1), 2.43 – 2.35 (m , 1H, H-2a), 2.08 – 2.04 (m, 2H, H-7, H-9), 1.92 – 1.76 (m, 1H, H-2b), 1.72 – 1.58 (m, 1H, H-6a), 1.58 – 1.42 (m, 3H, H-4, H-6b), 1.40 – 1.21 (m, 2H, H-5). ESI-MS (m/z) calculated for $C_{11}H_{17}NOS_2Na^+$ $[M+Na]^+$ 266.06, found 266.00. Reported data agree with literature.[1,2]

Synthesis of the CNC-Au-LA 1

A stirred solution of tetrachloroauric acid trihydrate 80 mg (0.23 mmol) in MilliQ water (23 mL) was diluted with methanol (205 mL), then 230 mg of CNC were added, and the mixture was vigorously stirred for 30'. Then, a solution of LA **5** (172 mg, 0.71 mmol) in methanol (1.5 mL) and sodium borohydride (196 mg, 37.8 mmol) were added sequentially and the mixture stirred for 2 h at r.t.. Then, the reaction mixture was dialyzed for 24 h *vs* MilliQ water and freeze dried to afford 392 mg of CNC-Au-LA **1**. The solid was suspended in dichloromethane (30 mL) and centrifuged (8000 rpm, 10 minutes), then the supernatant was removed, and the process repeated for two times. Then, the solid was washed with methanol (2 x 30 mL) and with water (1 x 30 mL). Finally, the solid was dispersed in water (1.0 mg\mL) dialyzed *vs* MilliQ water and freeze dried. ICP-AES: Au 9.18 % w/w. Elemental analysis C 31.38 %, H 5.26 %, N 1.02 % and S 4.86 %. Loading of **5**: 17.6 % w/w (based on N content in the elemental analysis).

Synthesis of the CNC-Au-Man 2

To a stirred solution of CNC-Au-LA **1** (30 mg, 0.022 mmol of LA) in MilliQ water (3 mL) the Man-PEG-N_3 **6** (15 mg, 0.044 mmol) was added and the mixture stirred for 30' at r.t.. Then copper sulfate (1.4 mg) and sodium ascorbate (1.2 mg) were added, and the mixture stirred for 2 h at

r.t.. Then, an additional batch of catalyst was added (1.4 mg of copper sulfate and 1.2 mg of sodium ascorbate) and the reaction mixture stirred for additional 12 h at r.t.. Then, the reaction mixture was dialyzed *vs* MilliQ water for 24 h and freeze dried to afford 29 mg of CNC-Au-Man **2**. ICP-AES: Au 9.5% w/w. Elemental analysis C 36.06 %, H 5.39 %, N 1.60 % and S 4.96 %. Loading of **6**: 5.21 % w/w (based on N content in the elemental analysis).

Synthesis of CNC-Au-Fuc 3

To a stirred solution of CNC-Au-LA **1** (67 mg, 0.048 mmol of LA) in MilliQ water (6.7 mL) the Fuc-PEG-N_3 **7** (31 mg, 0.096 mmol) was added and the mixture stirred for 30', then copper sulfate (2.8 mg) and sodium ascorbate (2.4 mg) were added, and the mixture stirred for additional 2 h at r.t.. Then, an additional batch of catalyst was added (2.8 mg of copper sulfate and 2.4 mg of sodium ascorbate) and the mixture stirred for 12 h at r.t.. Then, the reaction mixture was dialyzed *vs* milliQ water for 24 h and freeze dried to afford 27 mg of CNC-Au-Fuc **3**. ICP-AES: Au 10.7% w/w. Elemental analysis C 35.23 %, H 4.55 %, N 1.58 % and S 5.7 %. Loading of **7**: 4.25 % w/w (based on N content in the elemental analysis).

Synthesis of the CNC-Au-Man 2a

To a stirred solution of CNC-Au-LA **1** (60 mg, 0.044 mmol of LA) in MilliQ water (3 mL) the Man-PEG-N_3 **6** (6.5 mg, 0.020 mmol) was added, and the mixture stirred for 30', then copper sulfate (1.4 mg) and sodium ascorbate (1.2 mg) were added, and the mixture stirred for 2 h at r.t.. Then, an additional batch of catalyst was added (1.4 mg of copper sulfate and 1.2 mg of sodium ascorbate) and the mixture stirred for 12 h at r.t.. Then, the reaction mixture was dialyzed *vs* MilliQ water for 24 h and freeze dried to afford 30.7 mg of CNC-Au-Man **2a**. ICP-AES: Au content 44.6 µg/mg. ICP-AES: Au 9.7%. Elemental analysis C 35.66 %, H 5.38 %, N 1.46 % and S 5.26 %. Loading of **6**: 3.55 % w/w (based on N content in the elemental analysis).

Synthesis of the CNC-Au-Man-BODIPY 4.

To a stirred solution of CNC-Au-Man **2a** (20 mg) in dimethylformamide (2 mL) the BODIPY **8** (7.1 mg, 0.013 mmol) was added and the mixture stirred for 30', then copper sulfate (0.9 mg) and sodium ascorbate (0.8 mg) were added and the mixture stirred for additional 2 h at r.t.. Then, an additional batch of catalyst was added (0.9 mg of copper sulfate and 0.8 mg of sodium ascorbate) and the mixture stirred for 12 h at r.t.. The mixture was filtered over a 0.2 µm nylon membrane and the solid washed with methanol until colorless solution was obtained. Then the solid was dispersed in MilliQ water (1 mg/mL), dialyzed *vs* MilliQ water for 24 h and freeze dried to afford 19 mg of CNC-Au-Man-BODIPY **4**. ICP-AES Au 9.8 % w/w. Elemental analysis C 37.09 %, H 5.54 %, N 1.71 % and S 6.17 %. Loading of BODIPY **8**: 0.38 % w/w (based on B content in the elemental analysis).

Confocal microscopy measurements

The lyophilized CNC-AuNPs conjugates were first resuspended in DMSO to 1.0 mg/ml and sonicated vigorously for 10 minutes on an ultrasonic bath (Branson 2510). Just prior to imaging, this stock solution was freshly diluted 100-times with DMSO to a final concentration 10 µg/ml, and 25 µl of this sample was transferred into an 18-well Ibidi µ-slide with a 1.5H glass bottom. Imaging of the CNC-Au-Man-BODIPY **4** was performed on a laser-scanning microscope (Abberior Instruments) built around an IX83 Olympus microscope with a 60x water-immersion objective (UPLSAPO60, NA 1.2). A 120-ps pulsed laser at the wavelength of 640 nm was used for excitation, tuned to an average power of around 6 µW at the sample (20 µM for photostability

measurements). After passing through a 1.1 AU pinhole and an emission filter with the transmission band of 650–720 nm, the light was detected by an avalanche photodiode (APD). Images were acquired using the Imspector software with the following settings: field-of-view 50 µm (15 µm for lifetime measurements), pixel size 50 nm, pixel dwell-time 20 µs (200 µs for lifetime measurements). For fluorescence lifetime measurements, the signal from the APD was streamed to the time-correlated single-photon-counting unit (TCSPC; Becker&Hickl SPC150), which records the arrival time of each photon after the excitation pulse (*i.e.* microtime, τ). The fluorescence lifetime was determined by fitting a mono-exponential decay curve to the histogram of photon arrival times of each 550 x 550 nm area in the image. The mean lifetime of that area was color-coded in the same manner for both samples to enable easier comparison of their lifetimes. For the photostability comparison, LA-4 murine lung epithelial cells in an 8-wells Ibidi µ-slide were cultured as described in reference[3]. Cells were labelled with the fluorescent lipid analogue Atto647N-DPPE (AttoTec) at the concentration of 0.7 µM for 10 min, and then washed several times with PBS. Photobleaching was determined by repeated imaging of the same field of view. The mean intensity of each frame in the scan was determined and normalized to the first frame. This was repeated at different sites of the sample. Runs that contained obvious artefacts (e.g. focus drift, large aggregates, and noticeable movement of the sample) were discarded.

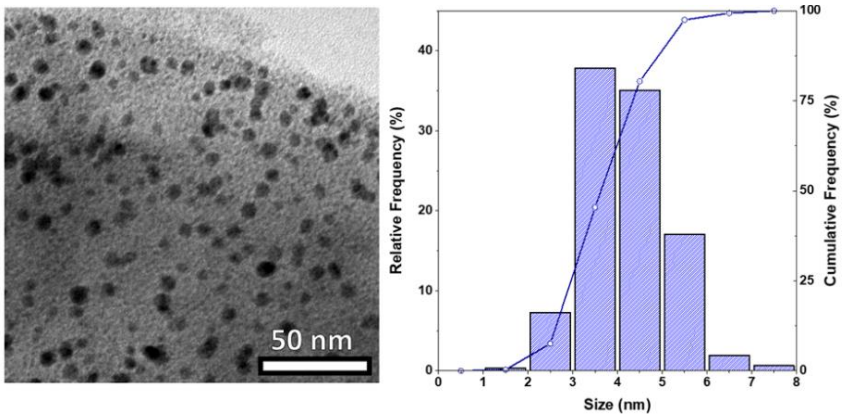

Figure S1. Representative TEM micrograph of the CNC-Au-LA **1** and related size distribution.

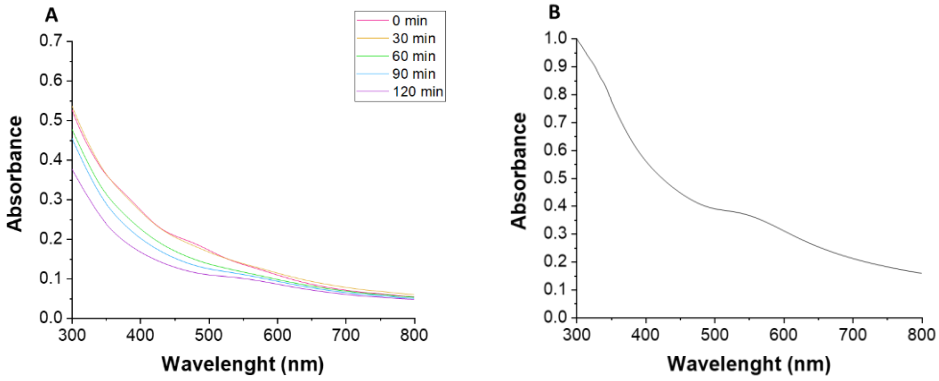

Figure S2. UV-Vis Spectra of: **A**) the progression of the reaction for the preparation of the CNC-Au-LA **1** over the time (0-120 min); **B**) CNC-Au-LA **1** dispersion after lyophilization (0.1 mg/mL in water).

Table S1. Functionalization expressed as % w/w based on: [a] nitrogen content from Elemental Analysis (E.A.); [b]sulfur content from E.A.; [c]boron content based on Inductive coupled plasma atomic emission spectroscopy (ICP-AES).

Glyconanomaterials	B % (ICP-AES)	N % (E.A.)	Headgroups loading % w/w
Sulfated pristine CNC (CelluForce NCC™)	< 0.001	0.01	---------
CNC-Au-LA **1**	< 0.001	1.02	17.6[a], 16.1[b]
CNC-Au-Man **2**	0.009	1.60	5.21 [a]
CNC-Au-Fuc **3**	<0.001	1.58	4.25[a]
CNC-Au-Man **2a**	0.027	1.46	3.55[a]
CNC-Au-Man-BODIPY **4**	0.034	1.71	0.38[c] of **8**

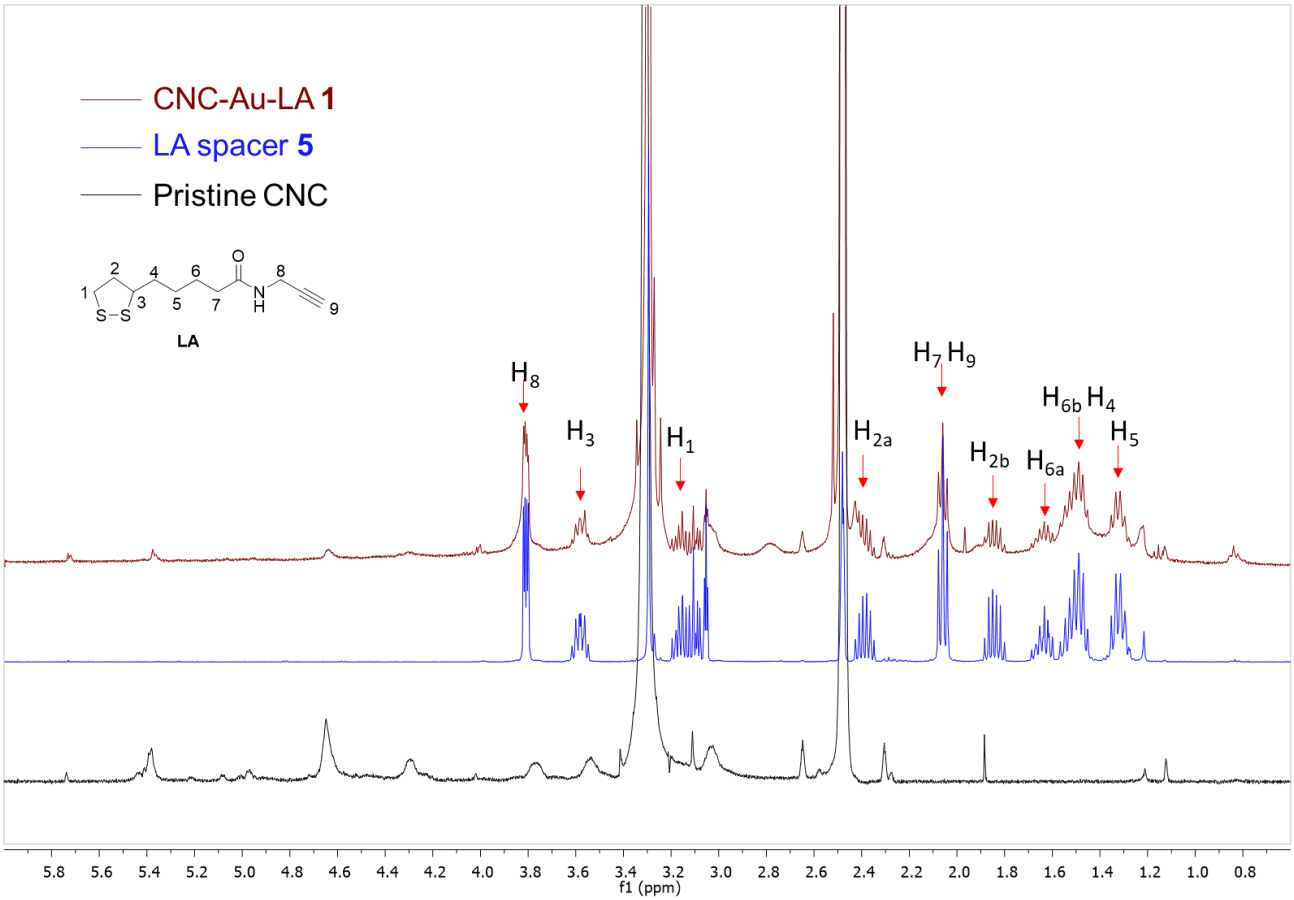

Figure S3. [1]H-NMR spectra (400MHz) of the CNC-Au-LA **1** (dispersion in DMSO-d6, red), the LA spacer **5** (DMSO-d6, blue), and the sulfated pristine CNC (dispersion in DMSO-d6, black).

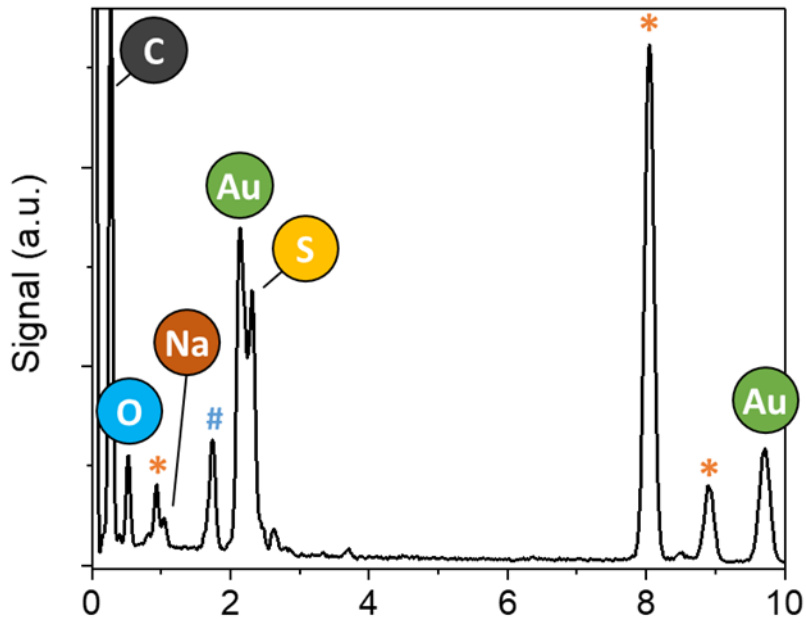

Figure S4. Representative EDX spectra collected in STEM mode of the CNC-Au-LA **1**. Signals labeled with # and * are indexed as silicon and copper respectively and are contaminations from the lacey carbon Cu TEM grid itself. The spectra show the co-presence of Au and S along with C, O and a small signal of Na.

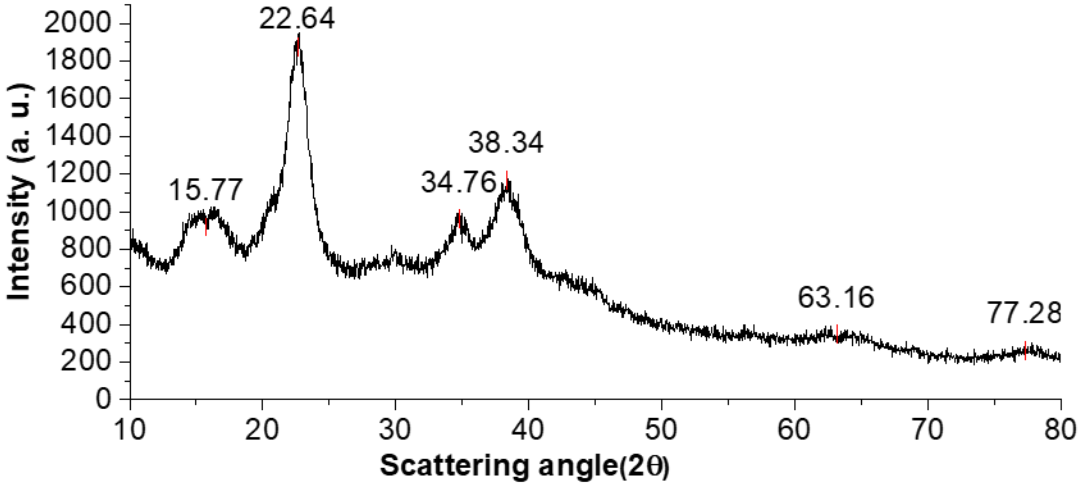

Figure S5. XRD pattern of the CNC-Au-LA **1.**

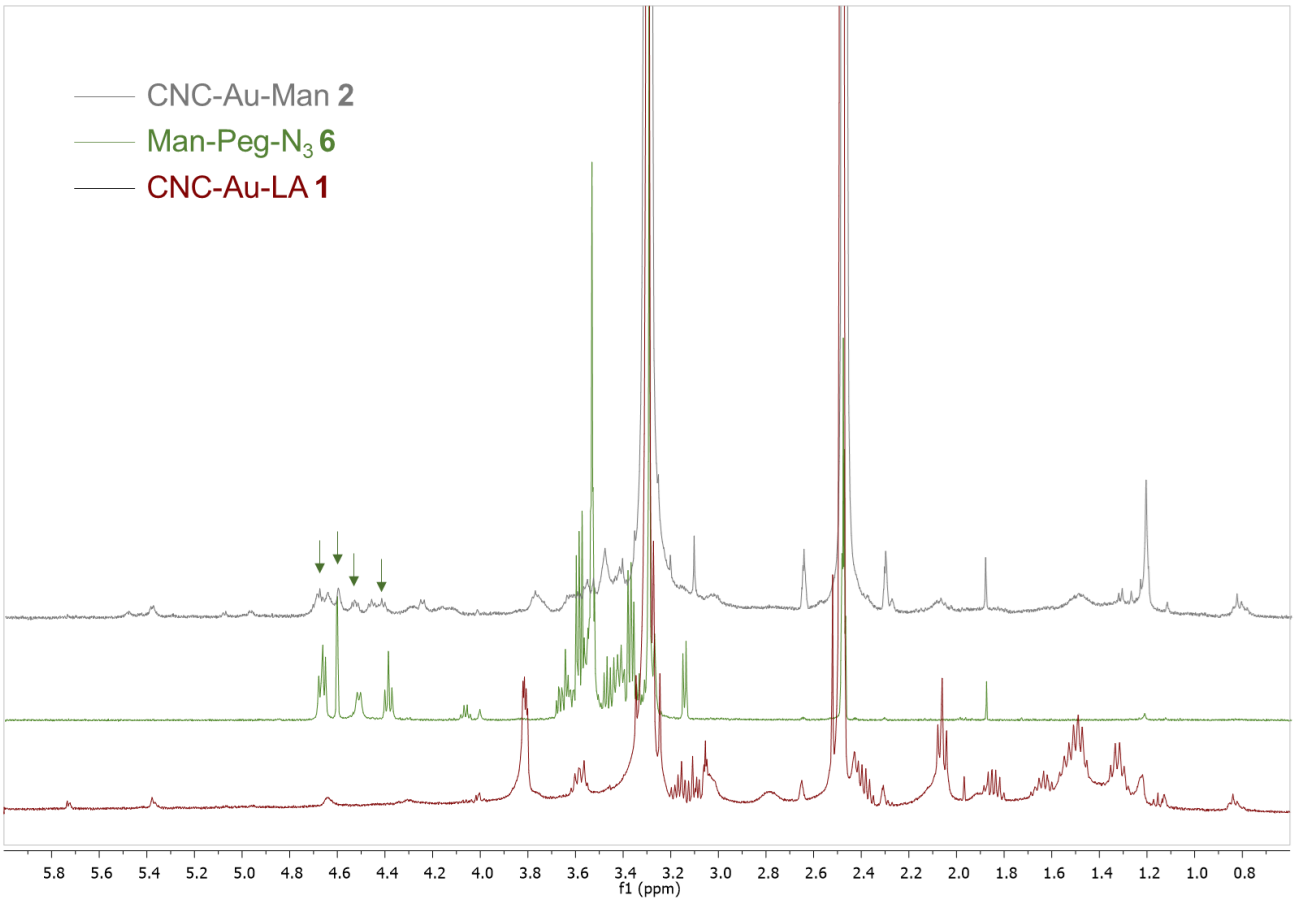

Figure S6. ^{1}H-NMR spectra (400 MHz) of the CNC-Au-Man **2** (dispersion in DMSO-d6, grey), the Man-PEG-N_3 **6** (DMSO-d6, green), and the CNC-Au-LA **1** (dispersion in DMSO-d6, red).

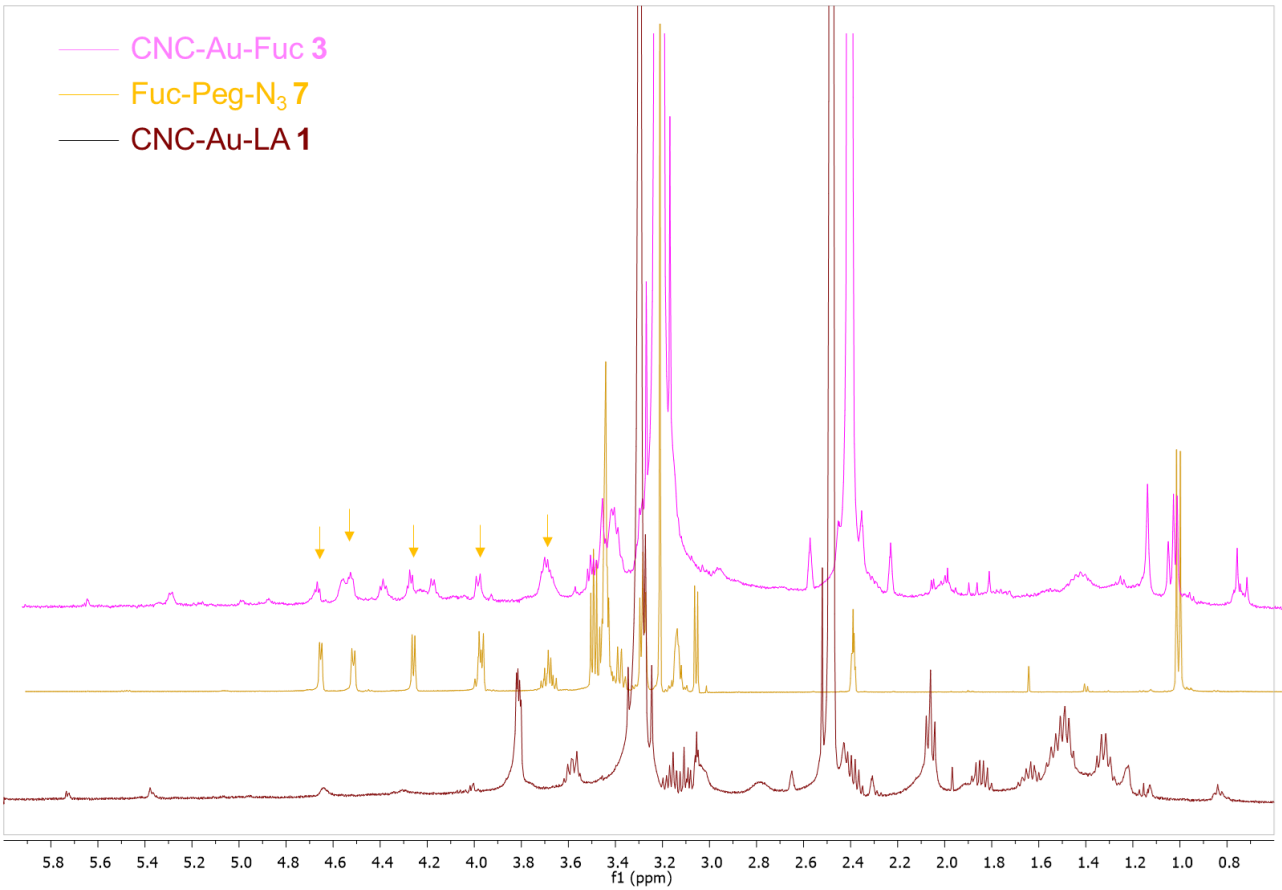

Figure S7. ^{1}H-NMR spectra (400 MHz) of the CNC-Au-Fuc **3** (dispersion in DMSO-d6, pink), the Fuc-PEG-N_3 **7** (DMSO-d6, orange) and the CNC-Au-LA **1** (dispersion in DMSO-d6, brown).

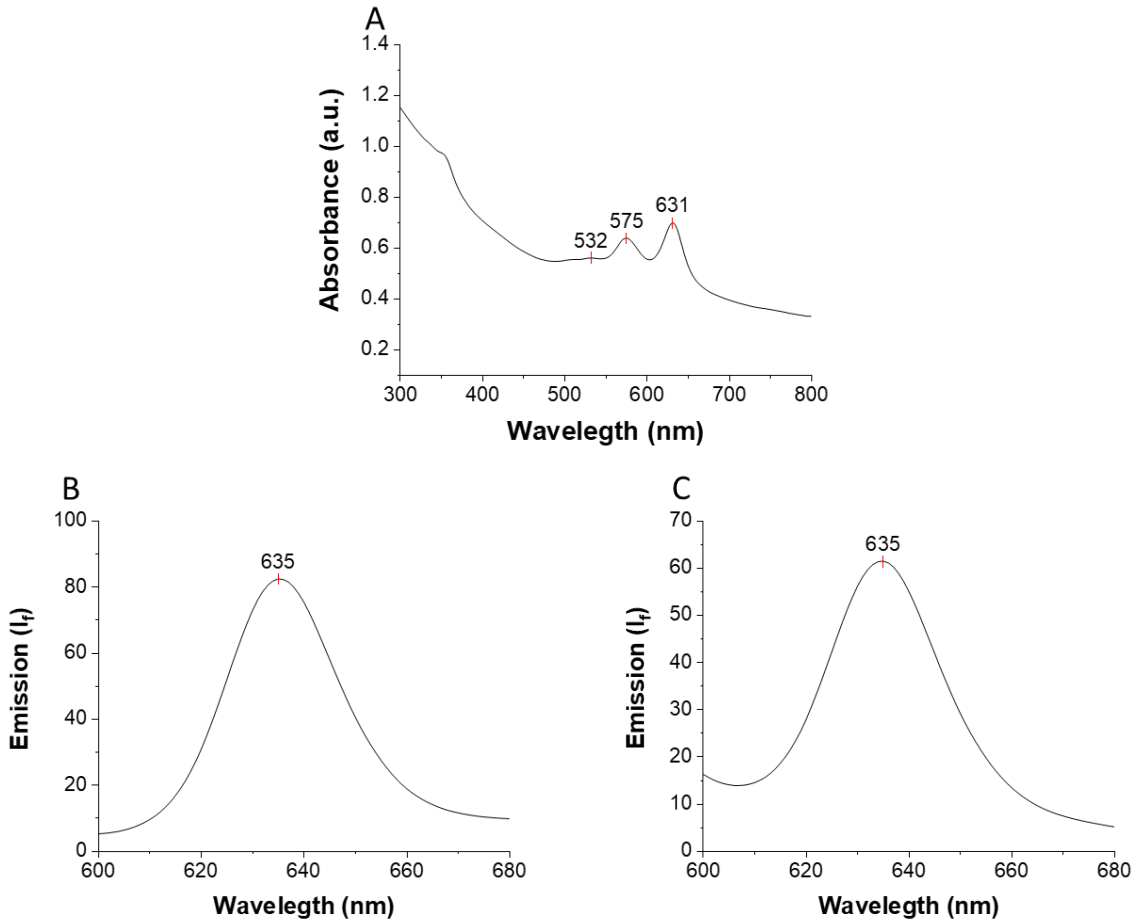

Figure S8. A) UV-Vis absorbance spectrum of the CNC-Au-Man-BODIPY **4** (DMSO, 1.0 mg/mL), **B**) and **C**) fluorescence emission spectra of CNC-Au-Man-BODIPY **4** (DMSO, 1.0 mg/mL) after excitation at λ = 380 (**B**) and 580 nm (**C**) respectively.

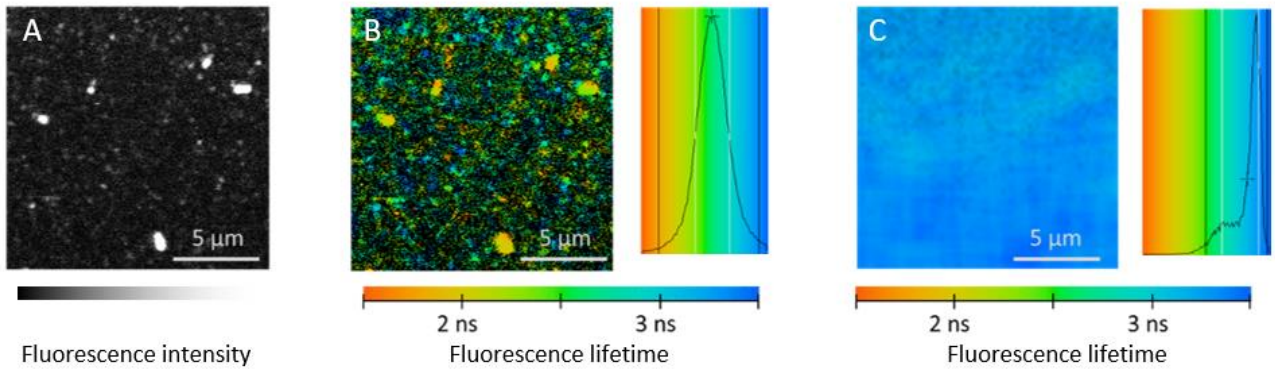

Figure S9. A) Fluorescence intensity of CNC-Au-Man-BODIPY **4** in DMSO on a glass substrate. B) The same image colored by the local fluorescence lifetime (color scale below), with the corresponding histogram of lifetimes across the image (right panel). C) Lifetime-colored image of unbound BODIPY **8** (2 µM in DMSO). CNC-Au-Man-BODIPY **4** shows a shorter lifetime (2.0–2.7 ns) than BODIPY **8** (approx. 3.2 ns).

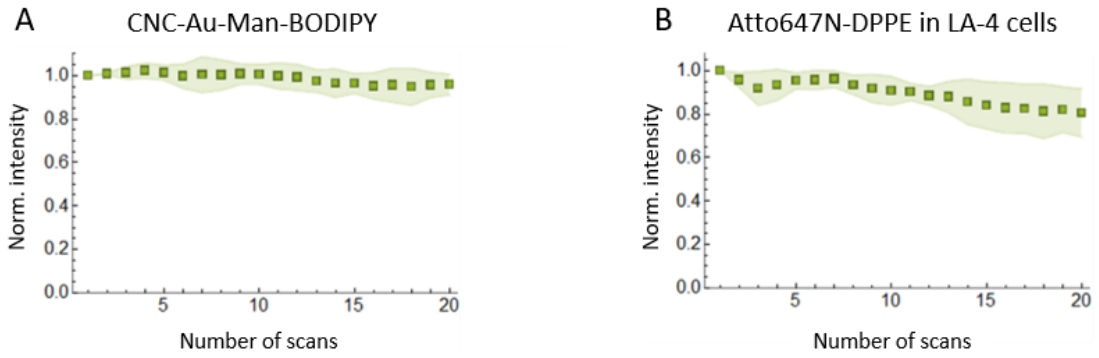

Figure S10. Comparison of the photobleaching induced by continuous confocal imaging of a chosen area for A) CNC-Au-Man-BODIPY **4** in DMSO and B) a commercial fluorophore Atto647N-DPPE in the membranes of LA-4 epithelial cells. The squares represent the mean value of 2–5 experiments, and the shaded area the standard deviation of the repeats.

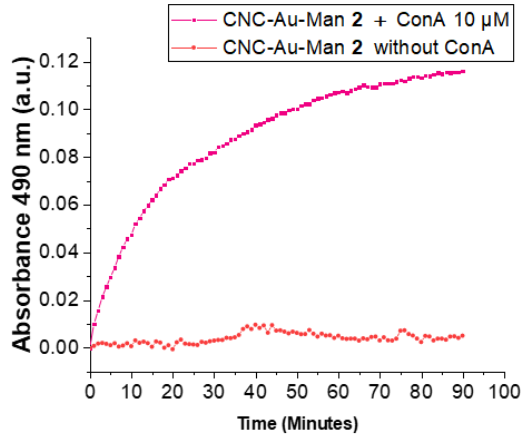

Figure S11. Turbidimetry analysis, absorbance (λ = 490 nm) changes over the time of a dispersion of CNC-Au-Man **2** 0.01% w/w before and after addition of ConA 10 µM in HEPES (10 mM, pH 7.40 ± 0.01) + 1 mM $MnCl_2$.

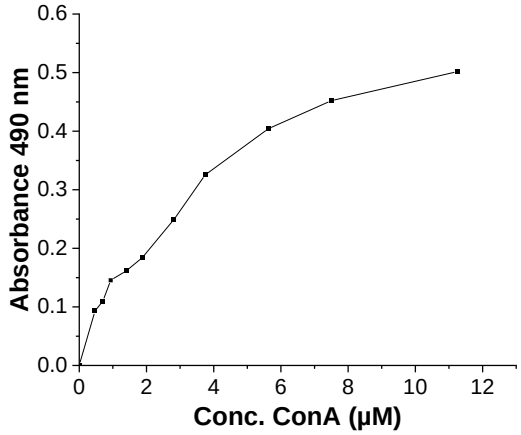

Figure S12. Turbidimetry assay of a dispersion of the CNC-Au-Man **2** (0.01% w/w) treated with increasing concentrations of the ConA (0 - 11.3 µM) at λ = 490 nm, in HEPES (10 mM, pH 7.40 ± 0.01) + 1 mM $MnCl_2$ after 60 minutes of incubation at room temperature.

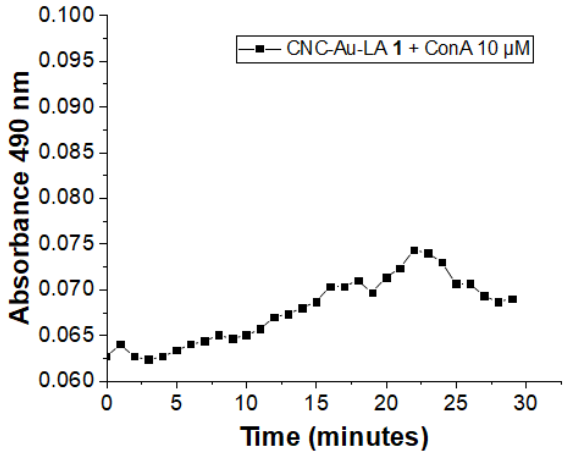

Figure S13. Turbidimetry analysis, absorbance (λ = 490 nm) changes over the time of CNC-Au-LA **1** (0.01% w/w in H_2O) with ConA 10 µM in HEPES (10 mM, pH 7.40 ± 0.01) + 1 mM $MnCl_2$.

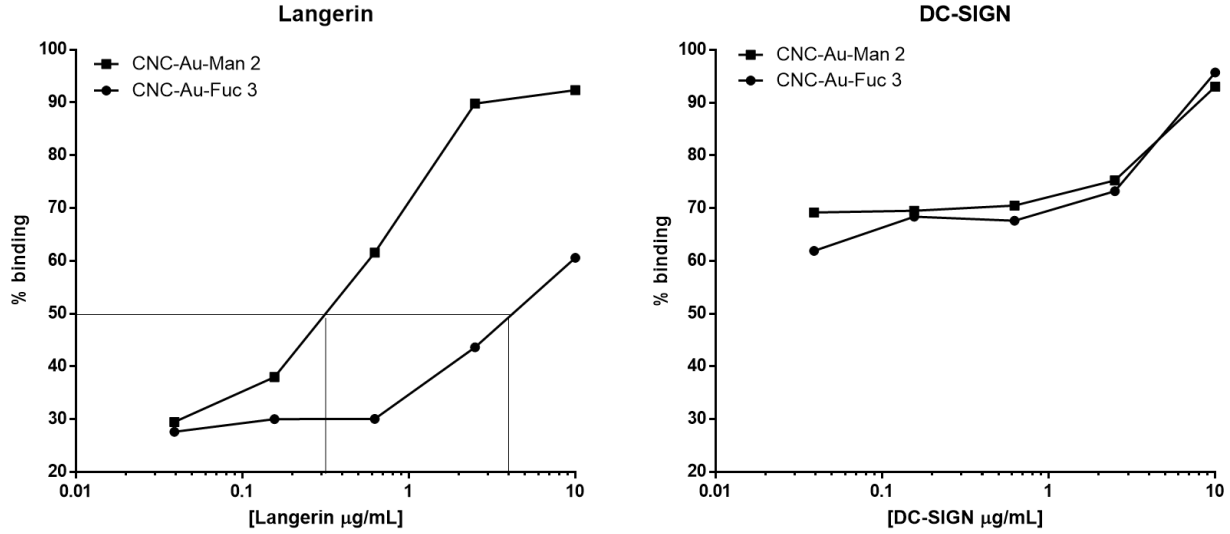

Figure S14. Binding of CNC-AuNPs conjugates CNC-Au-Man **2** and CNC-Au-Fuc **3** to different concentrations of the C-type lectins-Fc constructs of langerin (chimera langerin-Fc lectin) and DC-SIGN (Ig fusion protein, DC-SIGN-Fc) and measured by ELISA. Wells were coated with the corresponding glyconanomaterials **2**-**3** and the binding of lectins-Fc was measured as Optical density at 450 nm. Data are expressed as "% of binding" and have been normalized over the binding to the positive controls set as 100% of binding.

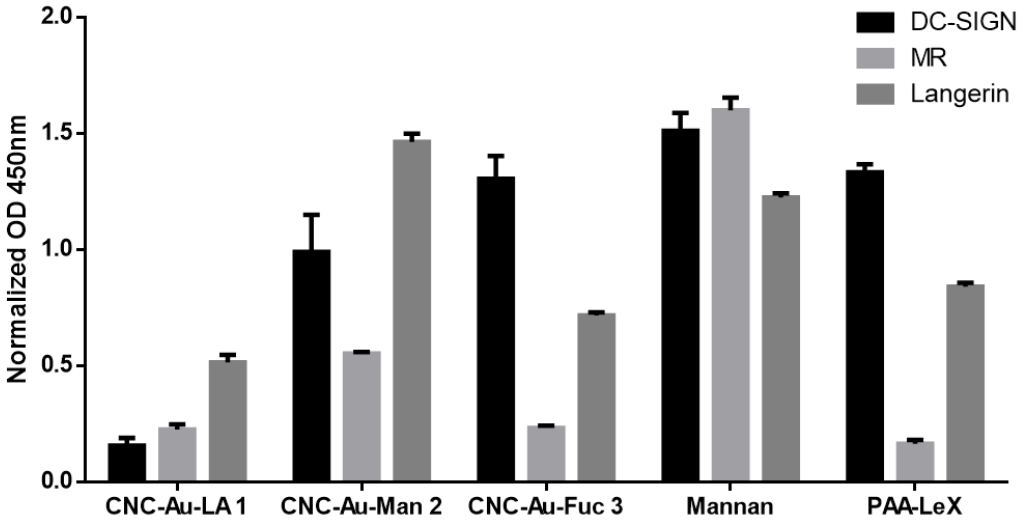

Figure S15. Binding of CNC-AuNPs conjugates CNC-Au-Man **2** and CNC-Au-Fuc **3** and of the unfunctionalized CNC-Au-LA **1** to C-type lectins-Fc constructs of DC-SIGN (Ig fusion protein, DC-SIGN-Fc), langerin (chimera langerin-Fc lectin) and Mannose Receptor (MR) measured by ELISA. Wells were coated with the corresponding glyconanomaterials **1**-**3** and the binding of lectins-Fc was measured as Optical density (OD) read at 450 nm. Data were normalized against BSA-coated wells used as blocking agent. Error bars indicate standard deviations between the mean values from three independent experiments performed in duplicate.

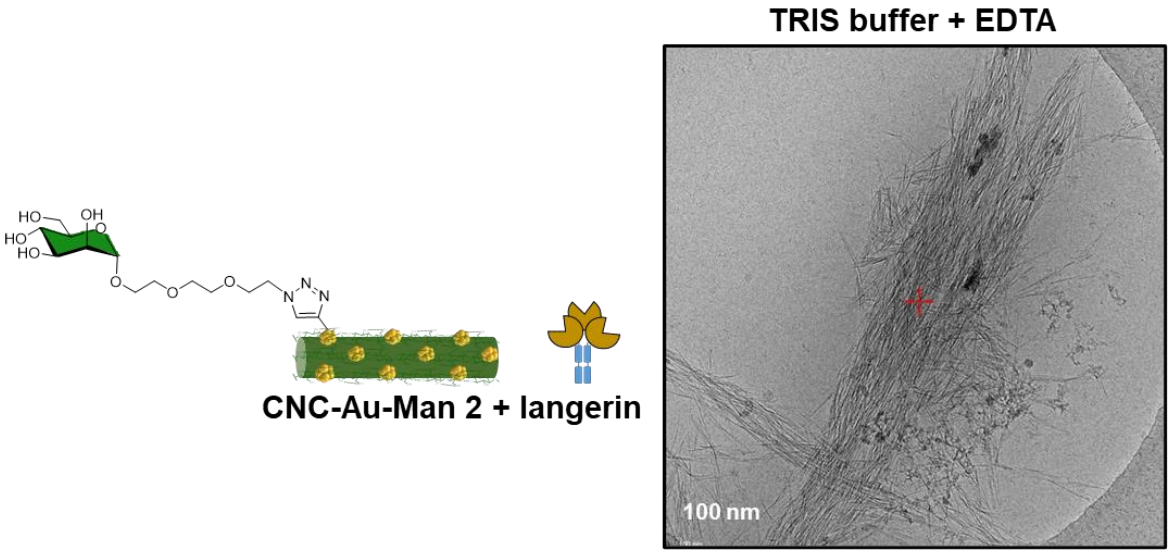

Figure S16. Cryo-TEM images of a dispersion of CNC-Au-Man **2** (0.01% w/w in H_2O) treated with the C-type lectin-Fc construct of langerin (0.1 mg/mL) in: 20 mM TRIS pH = 8, 150 mM NaCl + 4.0 mM EDTA solution. Scale bar 100 nm.

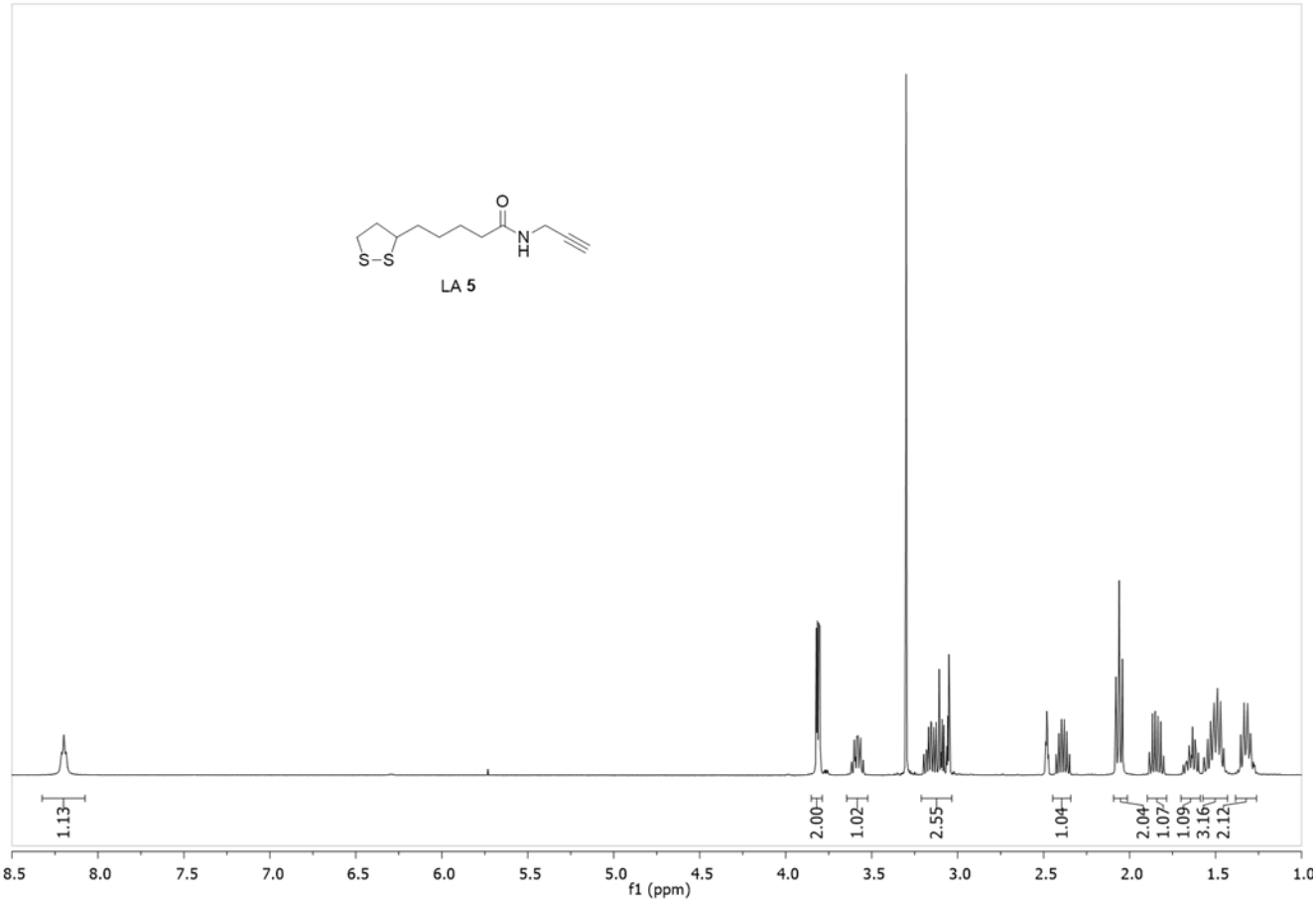

Figure S17. 1H-NMR spectrum (400MHz, DMSO-d6) of the LA ligand **5.**

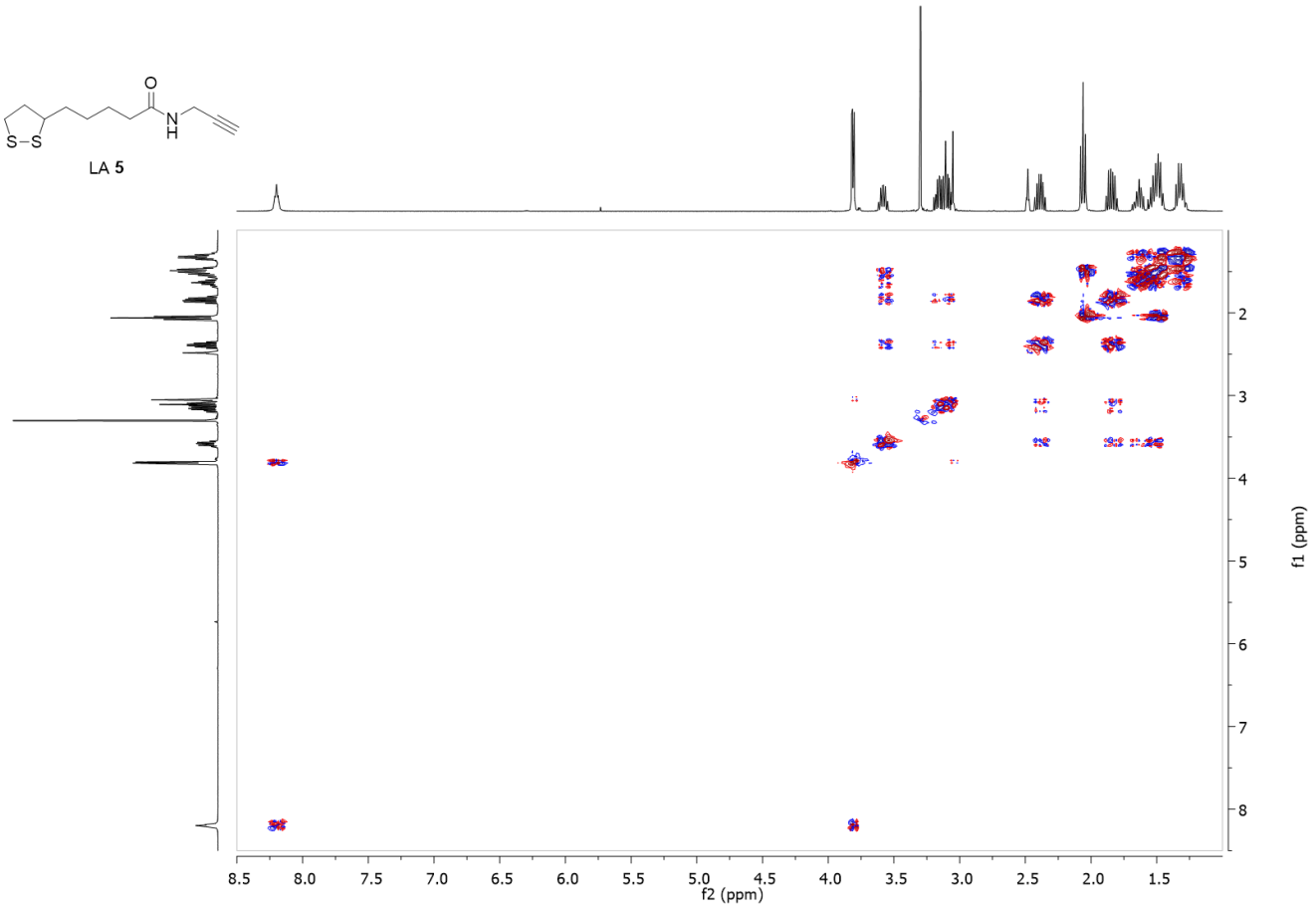

Figure S18. gCOSY-NMR spectrum (400 MHz DMSO-d6) of the LA ligand **5**.

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