Supplementary Information

Glycosylated Carbon Nanodots as Multivalent Blockers of Lectin-Driven Viral Entry: Structural Insights and Antiviral Performance

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1. General Methods

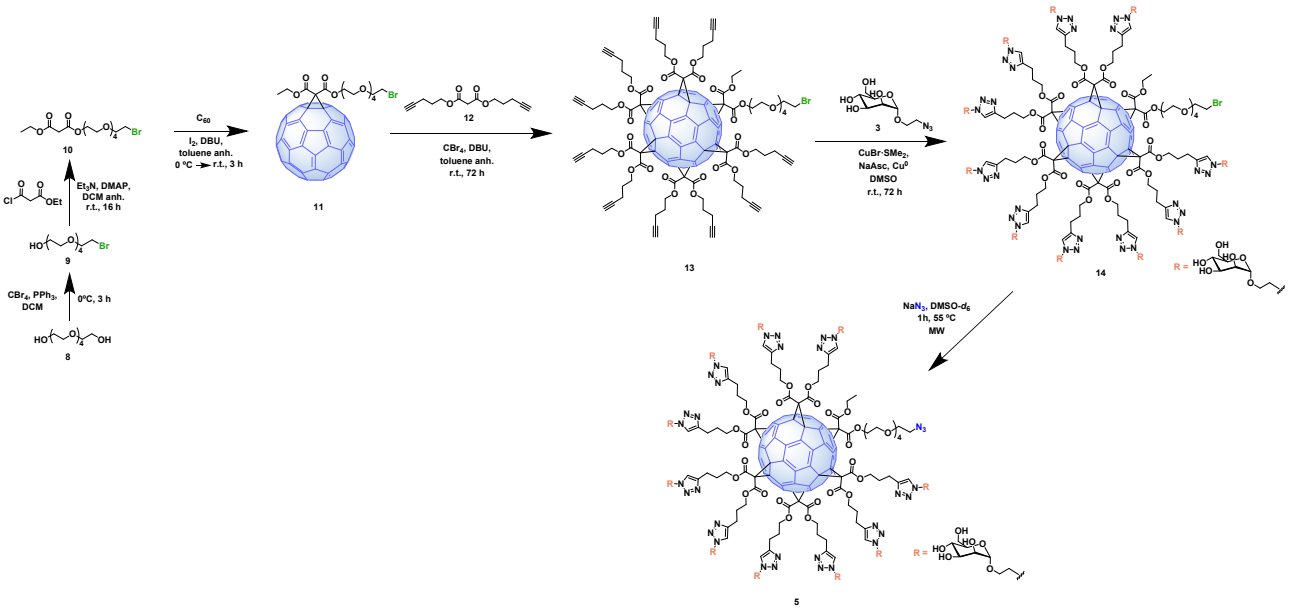
Materials

Reagents and solvents were obtained from commercial suppliers and used without further purification. Compound **3** was prepared according to previously reported procedures.[1] All work-up and purification procedures were carried out with HPLC-grade solvents in air. Microwave reactions were performed in an Anton-Paar Monowave 300 microwave reactor. Flash chromatography was performed using Scharlab silica gel (230-400 mesh, 0.040-0.063 mm) and for gel filtration Sephadex G-25 or G-50 were purchased from Cytiva. Analytical thin layer chromatography (TLC) was performed using E. Merck silica gel 60 F254 precoated plates (0.25 mm). The developed chromatogram was analyzed by UV lamp (λ = 254 nm) charring with potassium permanganate as development reagent.

Instruments

^{1}H and **^{13}C NMR** spectra were performed on a Bruker AVIIIHD 300MHz BACS-60 (1H: 300 MHz, 13C: 75 MHz) and Bruker AVIII 700MHz (1H: 700 MHz, 13C:176 MHz) at 298 K using partially deuterated solvents as internal standards. Coupling constants (*J*) are expressed in Hertz (Hz) and chemical shifts (δ) in parts per million (ppm) relative to the solvent. **FTIR** spectra (cm^{-1}) were carried out in a Spectrum 3™ FT-IR spectrometer using a spectral range of 4000-400 cm-1, with a resolution of 1 cm^{-1}, and in pellets of dispersed samples of the corresponding materials in dried KBr. **TGA** analyses were carried out under nitrogen in a TA-TGA-Q500 apparatus. The sample (~0,5 mg) was introduced inside a platinum crucible and equilibrated at 90°C followed by a 10°C min^{-1} ramp between 90 and 1000°C. **XPS** analyses were carried out using a SPECS GmbH (PHOIBOS 150 9MCD) spectrometer operating in the constant analyzer energy mode. A non-monochromatic aluminium X-ray source (1486.61 eV) was used with a power of 200 W and voltage of 12 kV. Pass energies of 75 and 25 eV were used for acquiring both survey and high-resolution spectra, respectively. Survey data were acquired from kinetic energies of 1487 - 400 eV with an energy step of 1 eV and 100 ms dwell time per point. The high-resolution scans were taken around the emission lines of interest with 0.1 eV steps and 100 ms dwell time per point. SpecsLab Version 2.48 software was used for spectrometer control and data handling. The semi-quantitative analyses were performed from the C1s (284.6 eV) signal. The samples were introduced as pellets of 8 mm diameter. **TEM** micrographs were obtained using a JEOL 2100 microscope, operating at 120 kV and a JEOL 1400 microscope, operating at 200 kV. The samples were dispersed in water with a few drops of ethanol (to facilitate the dispersion of the samples), sonicated for 10 minutes and the resulting suspension was dropped onto a holey carbon copper grid (200 mesh), and the solvent was allowed to evaporate. **AFM** images were acquired under ambient conditions using SPM Nanoscope IIIa multimode working on tapping mode with a TESPSS tip (Veeco) at a working frequency of ~235 kHz. The samples were prepared by drop casting from DMSO solutions on a freshly cleaved mica surface and were dried under ambient conditions for 24 hours. Hydrodynamic diameter measurements were acquired on a Dynamic Light Scattering (**DLS**) Zetasizer Nano ZS Zen 3600 (Malvern), working with an He-Ne laser operating at λ = 633 nm. The samples were recorded with a scattering angle of 173°. Measurements were made in a 1 cm path-length round quartz cell maintained at 298 K. Solution samples in water were filtered through nylon Acrodisc syringe filters (Pall Life Sciences) with 0.2-μm pore size. **UV-Vis** absorption spectra were performed in a Shimadzu UV-3600 UV-VIS-NIR Spectrophotometer, using 1 cm pathlength quartz cells at 298 K. **Emission** spectra were recorded in a FluoTime 300 fluorescence spectrometer using 1 cm pathlength quartz cells at 298 K.

2. Synthetic Procedures



Scheme S1. *Synthetic pathway to obtain glycofullerene-N_3* ***5****.*

Synthesis of ***CNDs-1****:*

Citric acid (0.750 g) and urea (0.750 g) were dissolved in 2.5 mL of deionized water, introduced into a hydrothermal reactor and heated to a constant pressure of 10 bar for 1 h. A dark blue-green solution was obtained, which showed a strong blue fluorescence. This solution was poured into a 250 mL Erlenmeyer flask and introduced in an oven for 48 hours at 80°C. The resulting solid was suspended and washed with acetone using a 0.1 µm Omnipore membrane to remove possible polymeric organic by-products. Then, the solid was diluted with water and dialyzed (2 kDa membrane) against deionized water for 2 days. Finally, the content of the dialysis bags was concentrated to obtain **CNDs-1** (176 mg) as a black powder.

Synthesis of ***CNDs-2****:*

$SOCl_2$ (3 mL) was added to CNDs-**1** (60 mg) and the mixture was refluxed at 70°C for 3 h under argon atmosphere. After this time, excess $SOCl_2$ was evaporated using a stream of argon and 4-pentyn-1-ol (60 mg, 0.713 mmol) dissolved in dry DMSO (2 mL) was added over the activated CNDs. The solution was heated under reflux at 120°C for 24 hours. The reaction mixture was poured over cold water and filtered through a 0.1 µm Omnipore membrane. The obtained solid was washed several times with cold water until the filtrate was colourless. Finally, it was washed with MeOH, DCM and acetone, obtaining **CNDs-2** (45 mg) as a black solid.

Synthesis of ***CNDs-4****:*

A mixture of **CNDs-2** (10 mg), mannose-N_3 **3** (40 mg), $CuBr{\cdot}S(CH_3)_2$ (16 mg) and sodium ascorbate (50 mg) were dissolved in the minimum possible amount of DMSO (1.5 mL), in the presence of a metallic copper wire. The reaction mixture was deoxygenated with a stream of argon and left with

vigorous stirring under argon atmosphere for 72 h. Next, Quadrasil® Mercaptopropyl was added, the mixture was stirred for 30 min and filtered. The crude was purified by size exclusion chromatography (Sephadex G:50, H_2O/MeOH 9:1). The first fraction was collected and freeze-dried to obtain **CNDs-4** (8 mg) as a brown solid.

*Synthesis of **CNDs-6**:*

A mixture of **CNDs-2** (10 mg), glycofullerene-N_3 **5** (50 mg), CuBr·$S(CH_3)_2$ (16 mg) and sodium ascorbate (50 mg) were dissolved in the minimum possible amount of DMSO (1.5 mL), in the presence of a metallic copper wire. The reaction mixture was deoxygenated with a stream of argon and left with vigorous stirring under argon atmosphere for 72 h. Next, Quadrasil® Mercaptopropyl was added, the mixture was stirred for 30 min and filtered. The crude was purified by size exclusion chromatography (Sephadex G:50, H_2O/MeOH 9:1). The first fraction was collected and freeze-dried to obtain **CNDs-6** (31 mg) as a brown solid.

*Synthesis of **MCNDs-7**:*

Citric acid (0.750 g), urea (0.750 g) and mannose (0.750 mg) were dissolved in 2.5 mL of deionized water, introduced into a hydrothermal reactor, and heated to a constant pressure of 10 bar for 1 h. A dark blue-green solution was obtained, which showed a strong blue fluorescence. This solution was poured into an Erlenmeyer flask and kept in an oven for 48 hours at 80ºC. The obtained solid was suspended and washed with acetone using a 0.1 µm Omnipore membrane to remove possible polymeric organic by-products. Then, the solid was diluted with water and dialyzed (2 kDa membrane) against deionized water for 7 days. Finally, the content of the dialysis bags was concentrated to obtain **MCNDs-7** (378 mg) as a black powder.

3. Characterization Techniques

Thermogravimetric analysis (TGA)

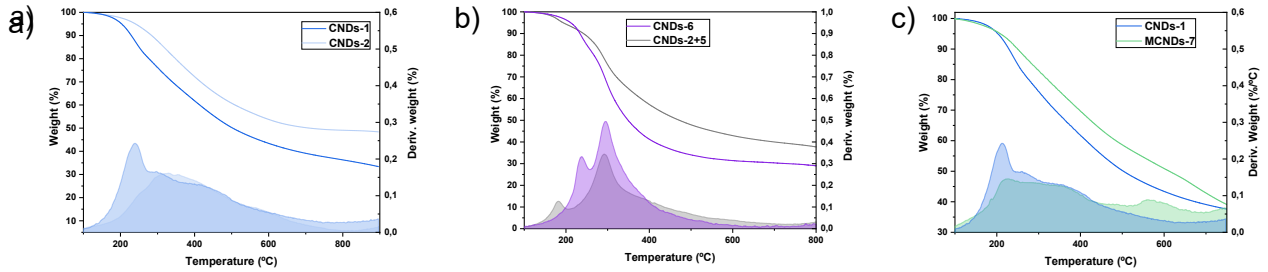


Fig. S1. *TGA analysis and first derivate under inert conditions of a) **CNDs-1** and **CNDs-2**; b) **CNDs-6** and a mixture of **5** and **CNDs-2**; c) **CNDs-1** and **MCNDs-7**.*

NMR spectra

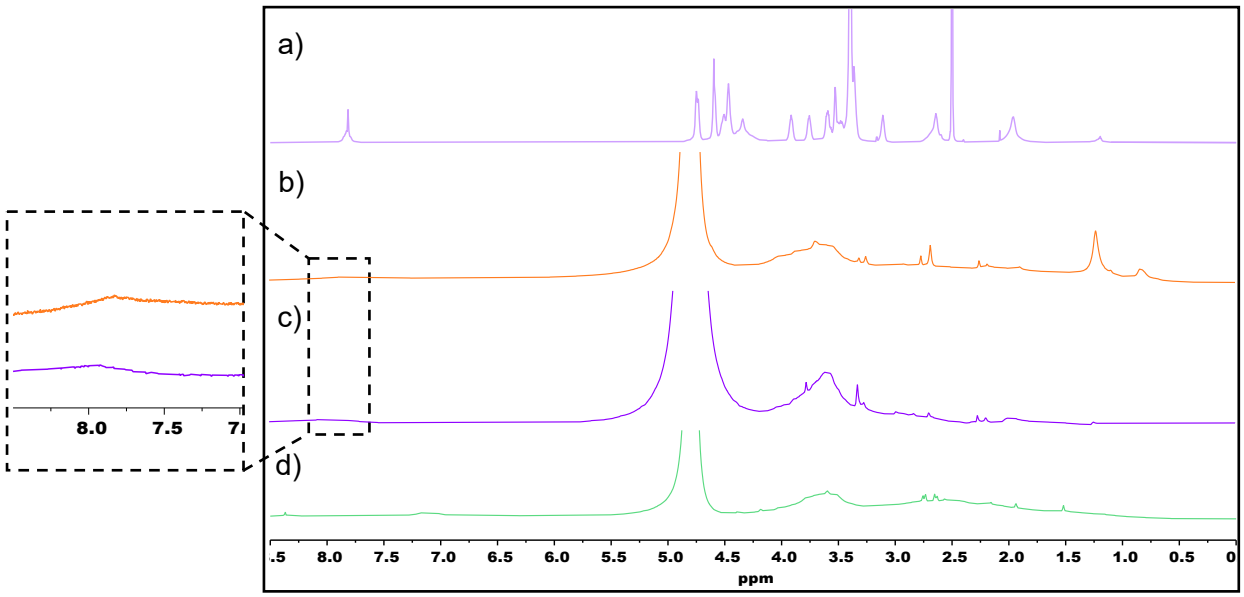


Fig. S2. *^{1}H-NMR spectra (700 MHz) of a)* ***5*** *(DMSO-d_6), b)* ***CNDs-4*** *(D_2O), c)* ***CNDs-6*** *(D_2O) and d)* ***MCNDs-7*** *(D_2O).*

X-ray photoelectron spectroscopy (XPS)

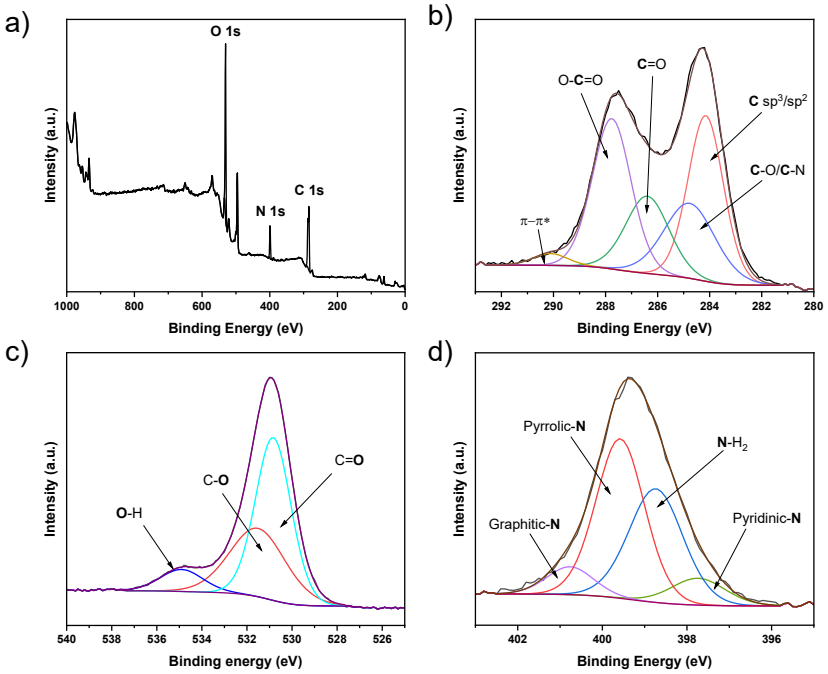


Fig. S3. a) XPS survey spectra; XPS high resolution spectra of b) C1s, c) O1s; d) N1s of **CNDs-1**.

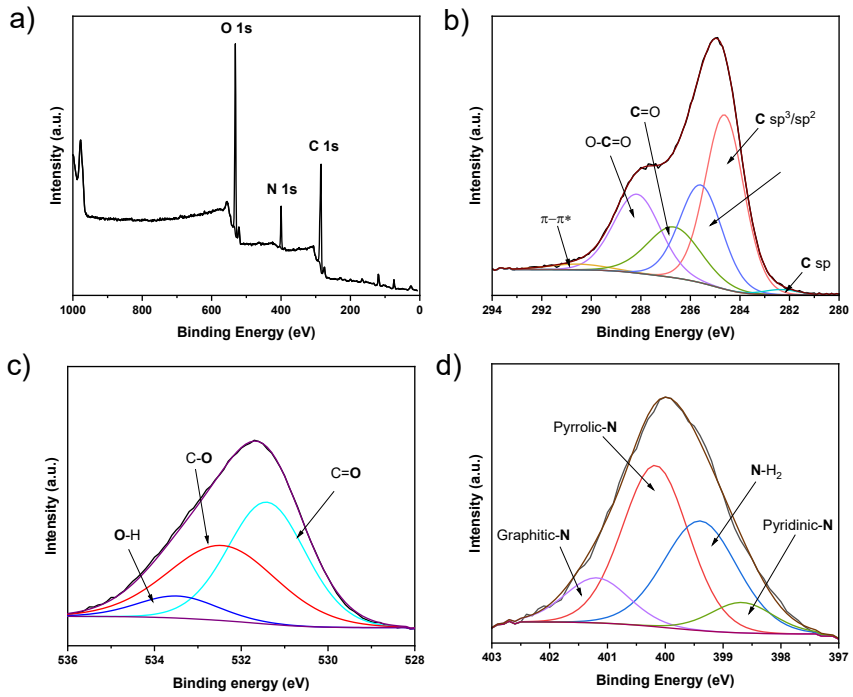


Fig. S4. *a) XPS survey spectra; XPS high resolution spectra of b) C1s, c) O1s; d) N1s of* ***CNDs-2****.*

Table S1. *XPS relative abundance and position for all materials.*

Sample	**C 1s** % (position eV)	**N 1s** % (position eV)	**O 1s** % (position eV)
CNDs-1	60.5 (284.6)	10.0 (399.6)	29.5 (530.6)
CNDs-2	63.2 (284.6)	9.0 (399.6)	27.8 (531.6)
CNDs-4	68.2 (284.6)	5.5 (399.6)	26.3 (531.6)
CNDs-6	60.9 (284.6)	7.0 (398.6)	32.1 (530.6)
MCNDs-7	69.5 (284.6)	11.0 (399.60)	19.5 (531.6)

Transmission electron microscopy (TEM)

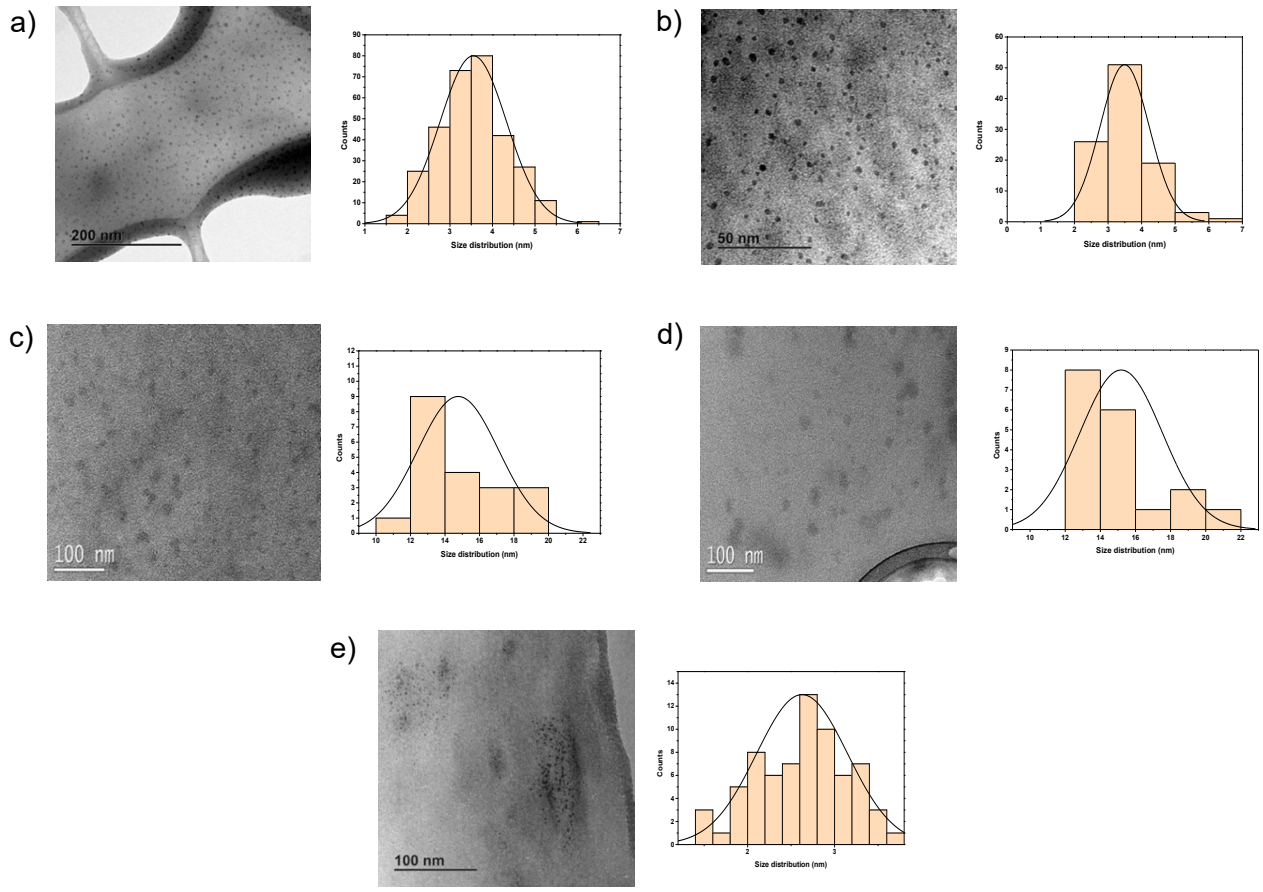


Fig. S5. *Representative TEM image and size histogram with a curve fit of the data using a Gaussian model of a)* ***CNDs-1****, average size: 3.5 ± 0.8 nm; b)* ***CNDs-2****, average size: 3.5 ± 0.7 nm; c)* ***CNDs-4****, average size: 14.7 ± 2.3 nm; d)* ***CNDs-6****, average size: 15.2 ± 2.3 nm; e)* ***MCNDs-7****; average size:2.6 ± 0.5 nm.*

Atomic Force Microscopy (AFM)

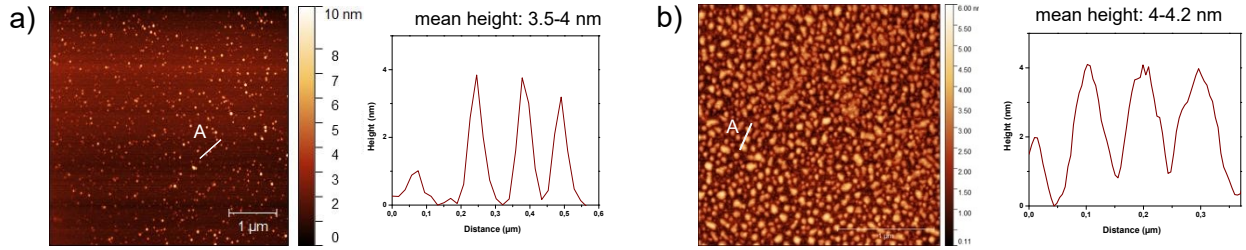


Fig. S6*. AFM image and height profile of a)* ***CNDs-1*** *and b)* ***CNDs-2****.*

DLS analysis

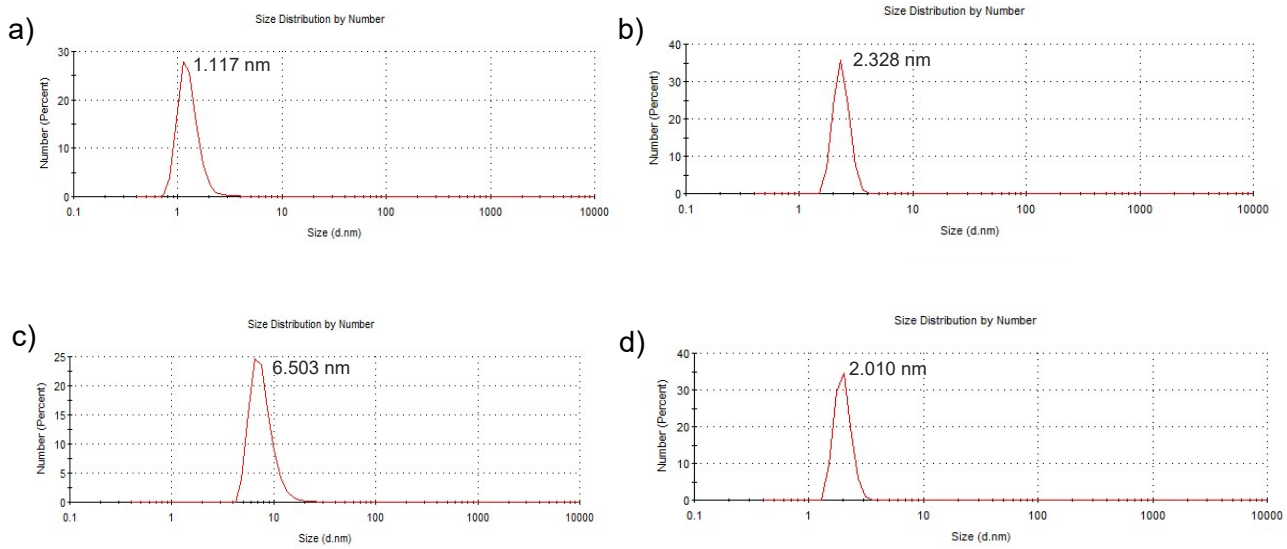


Fig. S7. *DLS number size histograms of aqueous solutions of a)* ***CNDs-1;*** *b)* ***CNDs-4****; c)* ***CNDs-6*** *and d)* ***MCNDs-7****.*

Electronic spectroscopy

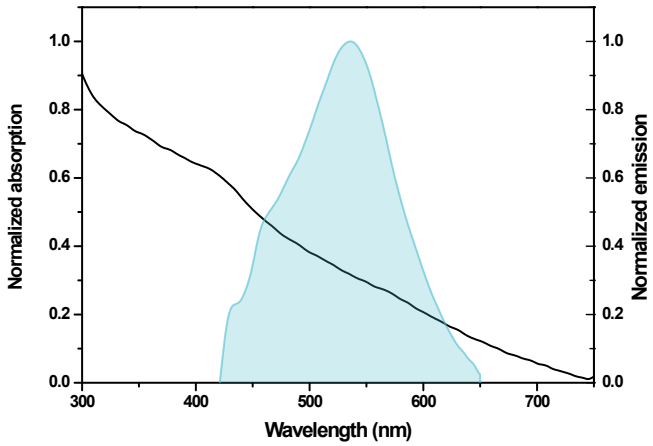


Fig. S8. *Normalized absorption and emission (λ_{exc}=335 nm) spectra of* ***CNDs-2*** *in DMSO.*

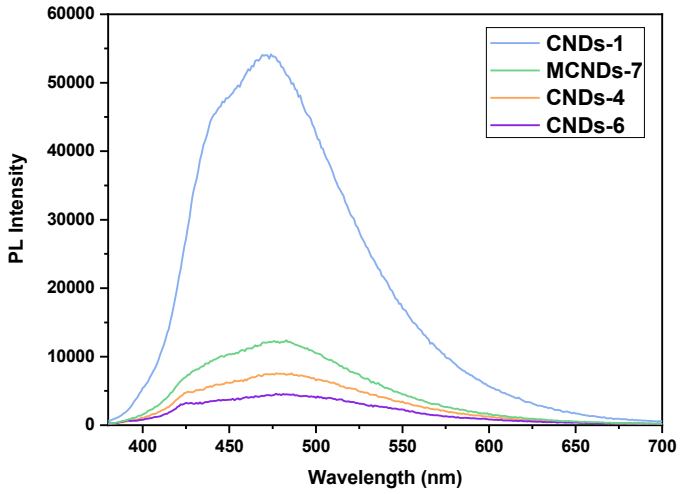


Fig. S9. *Comparison of emission spectra of* ***CNDs-1*** *(blue),* ***MCNDs-7*** *(green),* ***CNDs-4*** *(orange) and* ***CNDs-6*** *(purple) in H_2O (λ_{exc}=335 nm).*

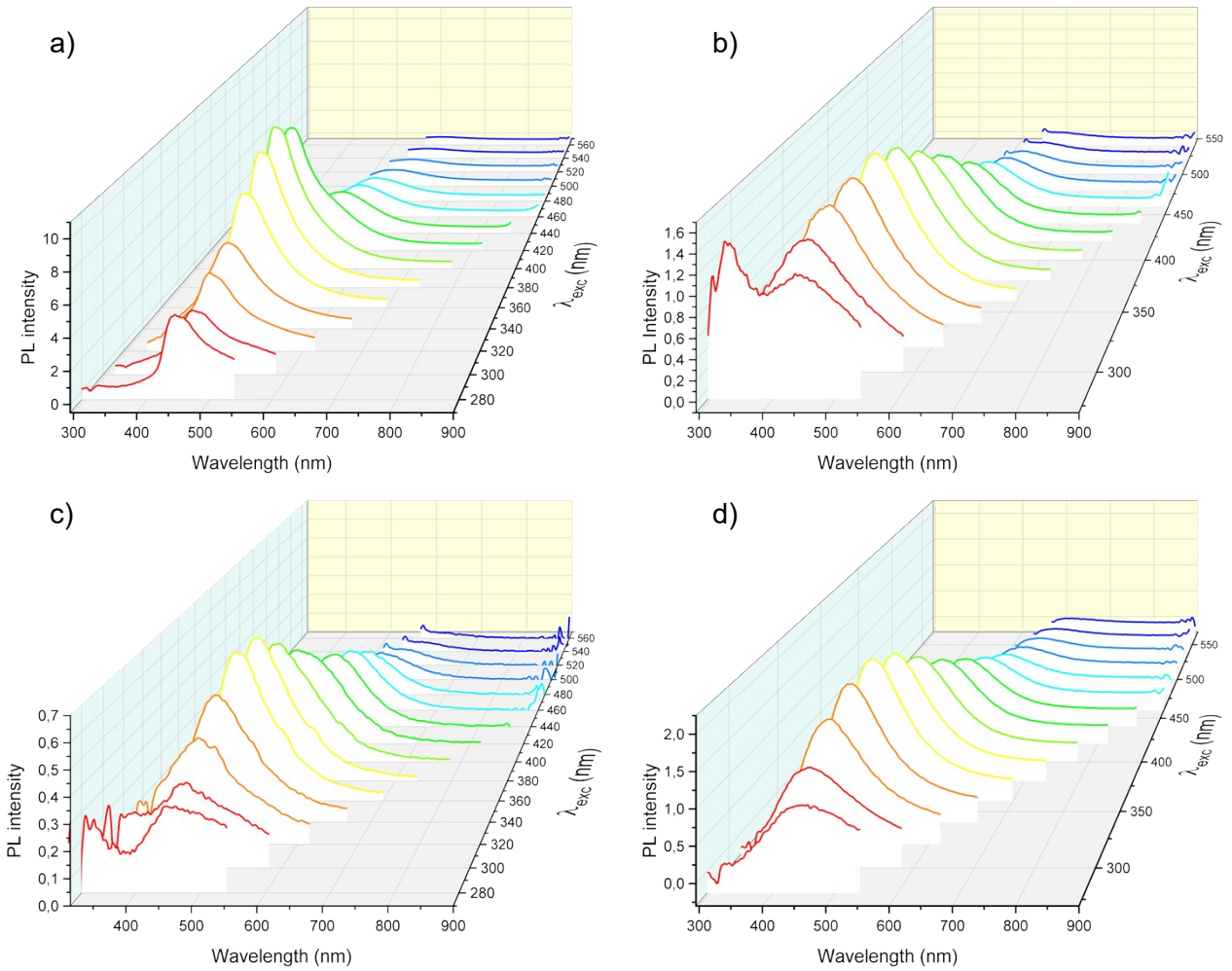


Fig. S10. *Excitation-dependent fluorescence emission spectra of a)* ***CNDs-1****; b)* ***CNDs-4****; c)* ***CNDs-6****; d)* ***MCNDs-7*** *in H_2O.*

Quantum yields

The fluorescence quantum yields of aqueous solutions (purged with Ar) of the materials were measured at 25 ºC and calculated by comparison with the corresponding emission spectrum of quinine sulfate in H_2SO_4 0.5 M ($\Phi_{fluo\ ref}$= 0.55) following the formula:

$$\phi_{fluo} = \phi_{fluo\ ref} \frac{I \ \ 1 - 10^{-A_{ref}} \ n^2}{I_{ref} \ \ 1 - 10^{-A} \ n_{ref}^2}$$

where Φ_{fluo} is the fluorescence quantum yield of the sample, I is the integrated area of the emission intensity under the spectral curve, A is the absorbance at the excitation wavelength, and n is the refractive index of H_2O at 25ºC (1.3325).

Table S2. *Quantum yields of all materials measured at their corresponding excitation wavelength.*

Sample	λ_{exc} (nm)	Φ_{fluo} (%)
CNDs-1	370	1.4
CNDs-4	340	0.3
CNDs-6	340	0.3
MCNDs-7	370	0.3

Anthrone Method: determination of mannose content

Determination of mannose content in glyco-CNDs was carried out by a previously reported procedure.[2]

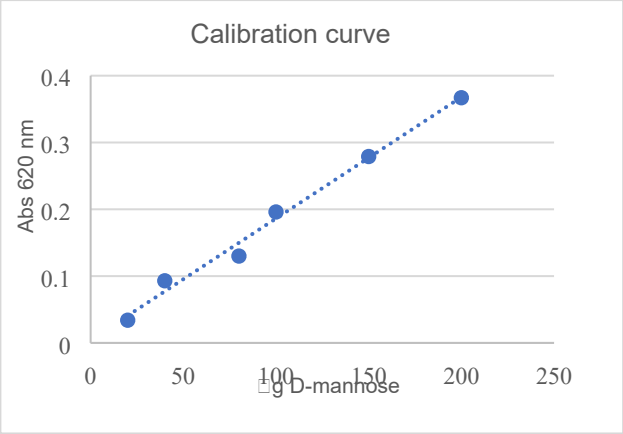


Fig. S11. *D-mannose calibration curve.*

Table S3. *Mannose content determined for all glyco-CNDs.*

Sample	Abs	μg D-mannose (for 0.6 mg of material)
CNDs-4	0.226	123.05
CNDs-6	0.101	53.61
MCNDs-7	0.410	225.27

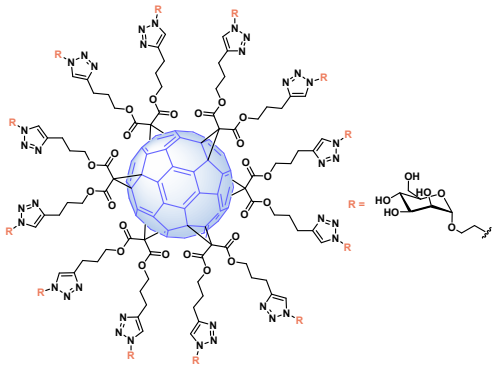


Fig. S12. *Chemical structure of compound* ***ManC$_{60}$****.*

4. Biological Assays

Culture cells

Baby hamster kidney cells (BHK-21/WI-2, Kerafast # EH1011) and African Green Monkey Cell Line (VeroE6) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 25 µg/mL gentamycin and 2 mM L-glutamine.

Jurkat cells (CD4+ T-lymphocyte cell line) stably expressing DC-SIGN or L-SIGN lectins[3] were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 25 µg/mL gentamycin and 2 mM L-glutamine. Cells were maintained at 37ºC in an environment with 5% CO_2.

Cytotoxicity analysis of compounds

VeroE6 ($2x10^4$) cells were seeded in a 96-well plate and incubated with DMEM containing each compound at concentrations ranging from 0 to 500 µg/ml. After 48 hours, cell viability was measured by CellTiter-Glo Luminescent Cell Viability Assay (Promega). Cell viability was reported as the percentage of luminescence in treated cells relative to non-treated cells. Non-toxic working concentrations (over 70% cell viability) was used to test the activities of these compounds on SARS-CoV2-pseudotyped infection.

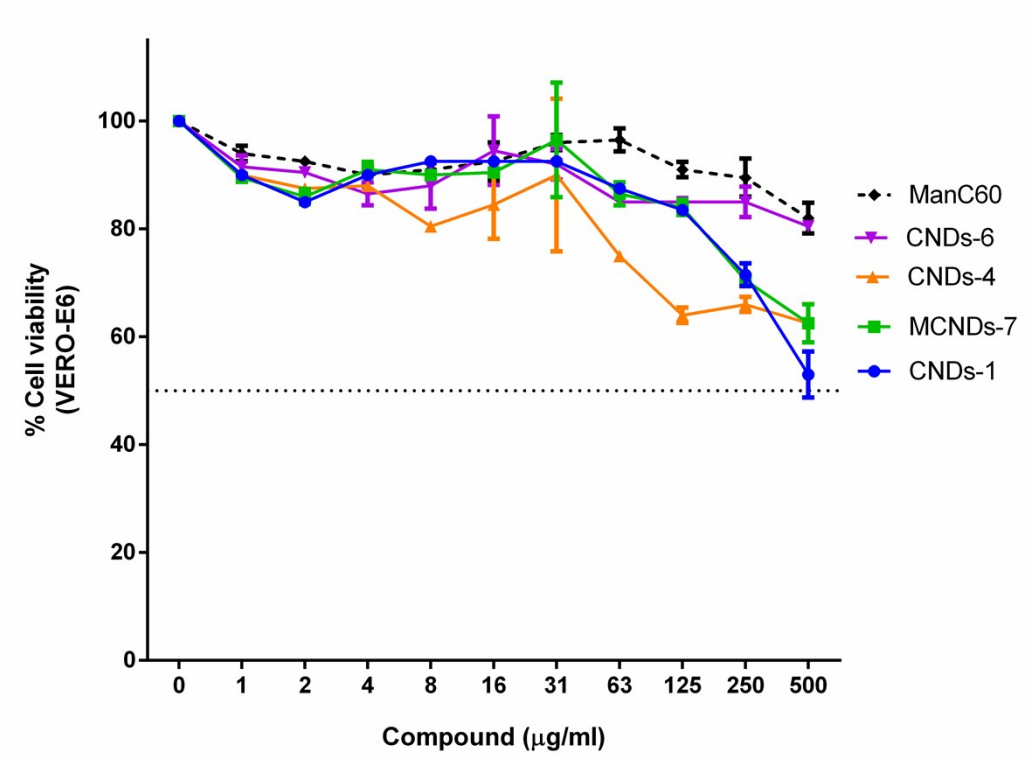


Fig. S13. *Evaluation of the cytotoxic activity of CNDs in Vero E6 cells. Cytotoxic effect of each compound was measured and presented as the percentage of viability of VERO cells after 48 h culture in the presence of different concentrations of each CNDs. Symbols on the graph represent the mean +/- SEM with N=3.*

SARS-CoV-2 pseudotyped VSV production

VSV-G pseudotyped replication deficient rVSV-luc recombinant viruses were produced following previously published protocol.[4] To generate SARS-CoV-2 pseudotyped vesicular stomatitis virus, BHK-21/WI-2, (Kerafast) were seeded in 10-cm dishes. Next day with 80% confluency, cells were transfected with a plasmid encoding for SARS-CoV-2 S-glycoprotein 614G using Lipofectamine 3000 (Thermo Fisher Scientific, Madrid, Spain). One day post-transfection, cells were infected with VSV (G*ΔG-luciferase) (Kerafast) at an MOI of 3 infectious units/cell. Viral inoculum was washed off after one hour and cells were incubated for another day at 37ºC. Pseudotyped particles were harvested 20 h post-inoculation, clarified from cellular debris by centrifugation and stored at -80ºC. Infectious titers were estimated as tissue culture infectious dose per mL by limiting dilution of the SARS-CoV2 rVSV-luc-containing supernatants on Vero E6 cells. Luciferase activity was determined by luciferase assay (Steady-Glo® Luciferase Assay System, Promega) in a GloMax® Navigator Microplate Luminometer (Promega).

Trans-Infection experiments

Jurkat cells expressing the receptor DC-SIGN or L-SIGN on its surface (1.5×10^5) were preincubated for 2 h at room temperature in rotation in the presence or absence of the CNDs (10 or 100 µg/ml) and supernatants containing rVSV-SARS-CoV2 recombinant particles. After washing DC/L-SIGN positive cells to remove not bounded viral particles, they were added to Vero cells for 24 hours and assayed for luciferase activity.

Infection values are presented as the percentage of trans infection with respect of the control (no compound added).

Statistical analysis

Graphs were created using GraphPad Prism v6.0 with N=3 and error bars corresponding to the standard errors of the mean.

Table S4. Trans infection mediated by Jurkat-DC/L-SIGN cells of SARS-CoV2 pseudotypes in presence of 10 or 100 μg/mL of each compound.

J-DC-SIGN-gfp	rVSV-SARS-CoV2-luc	J-L-SIGN-gfp	rVSV-SARS-CoV2-luc
$ManC_{60}$ (10 μg/mL)	68	**$ManC_{60}$ (10 μg/mL)**	72.3
$ManC_{60}$ (100 μg/mL)	27.3	**$ManC_{60}$ (100 μg/mL)**	49
CNDs-1 (10 μg/mL)	116	**CNDs-1 (10 μg/mL)**	83
CNDs-1 (100 μg/mL)	106.5	**CNDs-1 (100 μg/mL)**	99.3
CNDs-4 (10 μg/mL)	1.5	**CNDs-4 (10 μg/mL)**	49.5
CNDs-4 (100 μg/mL)	0	**CNDs-4 (100 μg/mL)**	10
CNDs-6 (10 μg/mL)	4	**CNDs-6 (10 μg/mL)**	40.5
CNDs-6 (100 μg/mL)	0.5	**CNDs-6 (100 μg/mL)**	7
MCNDs-7 (10 μg/mL)	2	**MCNDs-7 (10 μg/mL)**	30.5
MCNDs-7 (100 μg/mL)	0	**MCNDs-7 (100 μg/mL)**	14.5
Manano (25 μM)	6	**Manano (25 μM)**	4

Confocal laser scanning microscopy

WT, DC-SIGN- and LC-SIGN-expressing Jurkat cells were maintained in RPMI 1640 supplemented with 10% heating activated FBS, 25 μg/mL gentamycin and 2 mM L-glutamine. For internalization experiments, 300,000 cells in 100 μL were incubated for different times (0, 30, 60 and 120 min) at 37 ºC in the presence of **CNDs-4**, **CNDs-6** and **MCNDs-7** at 1 mg/mL concentration. After incubation, cells were washed three times in phosphate-buffered saline (PBS) and seeded in a four-chamber LabTeck® (C6807, Sigma-Aldrich) for imaging (200 μL final volume). The LabTeck® chamber was mounted on a stage of a Nikon Ti-E inverted microscope equipped with a Nikon C2 confocal scanning module, a 488 nm continuous laser, an emission dichroic 525/50 band pass filter and a Nikon Plan Apo λ 100 × 1.45 oil ∞/0.17 WD 0.13 immersion objective. Images were collected with Nikon NIS-Elements software and processed with ImageJ. Internalization ratios were calculated by normalizing the average fluorescence intensity (FI) of each condition to that of WT Jurkat cells treated under identical conditions. For all conditions, *6* independent images were acquired, each containing an average of *15* cells per field of view.

5. References

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