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Supporting Information

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Supporting Information

for

Multivalent *Manno*-Glyconanoparticles Inhibit DC-SIGN-Mediated HIV-1 *trans*-Infection of Human T-Cells

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1. Synthesis and Characterization of Products

1.1. General Methods: l chemicals were purchased as reagent grade from Sigma-Aldrich, except chloroauric acid (Strem Chemicals), and used without further purification. Reactions were monitored by thin layer chromatography (TLC) with silica gel 60 F_{254} aluminium sheets (Merck) and visualized under UV (254 nm) and/or staining with acidic ceric ammonium molybdate (water solution), acidic *p*-anisaldehyde or ninhydrin (ethanol solutions). Size-exclusion column chromatography was performed on Sephadex^{TM} LH-20 (GE Healthcare). Flash column chromatography (FCC) was performed on silica gel 60 (0.063-0.200 mm, Merck). UV-Vis spectra were carried out with a Beckman Coulter DU 800 spectrometer. Infrared spectra (IR) were recorded from 4000 to 750 cm^{-1} with a JASCO FT/IR 410 model spectrometer: solids were pressed into a KBr plate and oils were subjected to attenuated total reflection (ATR). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE spectrometer at 500 MHz and 125 MHz, respectively. Chemical shifts (δ) are given in ppm relative to the residual signal of the solvent used. Coupling constants (*J*) are reported in Hz. Splitting patterns are described by using the following abbreviations: br, broad; s, singlet; d, doublet; t, triplet; m, multiplet. High resolution mass spectra (HR-MS) were obtained using the MALDI technique with a 4700 Proteomics Analyzer (Applied Biosystems) with MALDI-TOF-TOF configuration. Samples of the products were dissolved in water, 2,5-dihidroxybenzoic acid (DHB) was used as a matrix, cesium iodide was added to favour the ionization process, and polyethylenglycol was used as internal reference. For transmission electron microscopy (TEM) examinations, a single drop (10 μL) of the aqueous solution (ca. 0.1 mg/mL in milliQ water) of the gold glyconanoparticles (GNPs) was placed onto a copper grid coated with a carbon film (Electron Microscopy Sciences). The grid was left to dry in air for several hours at room temperature. TEM analysis was carried out in a Philips JEOL JEM-2100F working at 200 kV. A detailed description of the preparation and characterization of the neoglycoconjugates **1**-**10** and their intermediates, as well as all *manno*-GNPs is the object of a full paper (manuscript submitted for publication).

1.2. Synthesis of Glycoconjugates: Manno-conjugates **6**-**10** were obtained by thiourea coupling of aminoethyl mannosides **1**-**5** (respectively) with isothiocyanate linker **11**. Wong's procedure[S1] was used for the synthesis of mannosides **1**-**5** inserting a two carbon atoms aliphatic linker.

*Isothiocyanate linker **11***. Bromotrichloromethane (330 µL, 3.34 mmol, 1.5 equiv) was added to a stirred solution of linker 23-thioacetyl-3,6,9,12-tetraoxatricosan-1-ol[S2] (942 mg, 2.23 mmol, 1 equiv), triphenylphosphine (877.3 mg, 3.34 mmol, 1.5 equiv) and NaN_3 (290.0 mg, 4.46 mmol, 2 equiv) in DMF (10 mL). The reaction mixture was first stirred 30 minutes at room temperature and then 30 minutes at 40 °C. The reaction mixture was diluted with Et_2O (20 mL), washed with water (2 x 30 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure. The resulting residue was dissolved in toluene (5 mL). Carbon disulfide (2.63 mL, 44.6 mmol, 20 equiv) and a supplementary amount of triphenylphosphine (877.3 mg, 3.34 mmol, 1.5 equiv) were then added. The reaction mixture was stirred at 50 °C for 4 hours, concentrated under reduced pressure and purified by FCC (gradient Hexane to Hexane/EtOAc 95:5) to afford **11** as a colourless oil (748 mg, 1.61 mmol, 72%). R_f: 0.71 (EtOAc/MeOH 95:5); ^{1}H NMR ($CDCl_3$): δ 3.69-3.55 (m, 16H), 3.44 (t, *J*=7.0 Hz, 2H; $CH_2CH_2CH_2O$), 2.86 (t, *J*=7.5 Hz, 2H; CH_2SAc), 2.32 (s, 3H; SAc), 1.60-1.52 (m, 4H), 1.36-1.24 (m, 14H); ^{13}C NMR ($CDCl_3$): δ 195.7 (*C*=O), 131.9 (N*C*S), 71.3, 70.6, 70.55, 70.51, 70.45, 69.9, 69.2, 45.1 (CH_2NCS), 30.5 (CH_3CO), 29.5, 29.40, 29.37, 29.35, 29.31, 29.0, 28.96, 28.66, 25.9; IR (ATR): ν 2113, 2186 cm^{-1}; HR-MS calcd. for $C_{22}H_{41}NO_5S_2Na^+$ $[M+Na]^+$ 486.2318, found 486.2329.

General procedure for thiourea coupling of aminoethyl-oligomannosides and subsequent deprotection of the corresponding glycoconjugates. A solution of isothiocyanate linker **11** (0.12 M, 2 equiv) in H_2O/^{i}PrOH/CH_3CN 1:1:1 was added to a solution of aminoethyl oligomannoside **1**-**5** (0.09 M, 1 equiv) and the pH was adjusted to 8–9 with triethylamine. The solution stirred for 3–5 h at room temperature. The solvent was removed under reduced pressure and the crude was triturated with Et_2O to get rid of the excess of the linker. The residue was treated with sodium methoxide (1 equiv, 1 N in MeOH). The resulting mixture was stirred for 2 h at room temperature and then neutralised with HCl 0.1 N. Purification by Sephadex™ LH-20 and lyophilization afforded the glycoconjugate **6**-**10**.

*Tetramannose conjugate **8***. Aminoethyl tetramannoside **3** (62.0 mg, 0.0874 mmol) and linker **11** (81.0 mg, 0.1748 mmol) gave tetramannoside **8** (80.0 mg, 0.071 mmol, 81%) as a white solid after Sephadex™ LH-20 (MeOH/H_2O 9:1) and lyophilisation. R_f: 0.81 (^{i}PrOH/NH_3 1:1); ^{1}H NMR (D_2O): δ 5.38 (s, 1H), 5.32 (s, 1H), 5.07 (s, 1H), 4.86 (s, 1H), 4.15-3.59 (m, 44H), 3.51 (br t, 2H; $OCH_2CH_2CH_2$), 2.73 (t, *J*=7.1 Hz, 2H; CH_2S), 1.78-1.56 (m, 4H), 1.49-1.28 (m, 14H). ^{13}C NMR (D_2O): δ *C*=S signal unde-

tected, 102.2, 100.62 (overlapped), 99.7, 78.4, 78.1, 73.3, 73.23, 73.17, 72.9, 71.2, 70.3, 70.0, 69.9, 69.7, 69.2, 66.9, 66.8, 65.9, 61.0, 60.9, 60.7, 43.7 (br t, 2C; 2 x *C*H_2NH), 39.0, 34.1, 29.7, 29.6, 29.5, 29.3, 28.5, 26.1; IR (KBr): ν 3361 (br), 2926, 2856, 1646, 1556, 1459, 1352, 1296, 1131, 1058; HR-MS calcd. for $C_{46}H_{86}N_2O_{25}S_2$-Na^+ 1153.4859, found 1153.4879.

1.3. Synthesis of Manno-*Glyconanoparticles*

General procedure for the preparation of glyconanoparticles (GNPs). A solution of disulfide **6**-**10** (0.012 M, 3 equiv) in methanol or, a mixture of disulfide **6**-**10** in a different ratio (50:50 or 10:90) with glucose conjugate **GlcC$_5$S** (0.012 M, 3 equiv) in methanol was added to a solution of tetrachloroauric acid (0.025 M, 1 equiv) in water. An aqueous solution of $NaBH_4$ (1 M, 22 equiv) was then added in four portions, with rapid shaking. The black suspension formed was shaken for additional 2 h and the supernatant was then removed and analysed (see below). The residue was dissolved in the minimum amount of NANOPURE water and purified by dialysis. This solution was loaded into 5–10 cm segments of SnakeSkin® pleated dialysis tubing (Pierce, 3500 MWCO), placed in a 3 L beaker of water, and stirred slowly, recharging with distilled fresh water every 3–4 h over the course of 72 h. The solution in the mem-brane was lyophilised to afford the GNP. ^{1}H NMR spectra of the glycoconjugates mixture used for the GNP synthesis and of the products recovered from the supernatant after GNP formation were recorded. The ratio of the ligands in the GNPs was thus confirmed through integration of the anomeric protons of the mannoside with respect to the anomeric protons of the glucoside. The particle size distribution of the gold nanoparticles was evaluated from several TEM micrographs by means of an automatic image analyser. The average diameter and number of gold atoms of the GNPs was assigned according to a previous work. [S3] Average molecular formula of the nanoparticles was calculated, on the basis of the average diameter obtained by TEM, and confirmed by elemental analysis.

D-50. Reaction of a 1:1 mixture of **4** (22.9 mg, 0.028 mmol) and **GlcC$_5$S** (8.0 mg, 0.028 mmol) with $HAuCl_4$ (756 µL, 0.025 M) and $NaBH_4$ (415 µL, 1 N) gave **D-50** (9.4 mg) as a brown powder. TEM (average diameter and number of gold atoms): 1.3 ± 0.4 nm, 79 (Figure S1); ^{1}H NMR (D_2O): δ 5.14 (s, 1H; H-1), 5.05 (s, 1H; H-1'), 4.96-4.37 (br m, 1H; H-1 glucose), 4.09 (s, 1H; H-2'), 4.04-3.21 (m, 41H), 2.78 (br m; C*H$_2$*S), 1.89-0.81 (m, 24H) (Figure S3); IR (KBr): ν 3425 (broad), 2923, 2852, 1635,

1380, 1063 (Figure S5, left); UV-Vis (H_2O, 0.1 mg/mL): surface plasmon band not observed (Figure S6, left); elemental analysis calcd (%) for $(C_{34}H_{65}N_2O_{15}S_2)_{22}$-$(C_{11}H_{21}O_6S)_{22}Au_{79}$ (39482): C 30.12, H 4.83, N 1.56, S 5.36; found: C 29.93, H 4.88, N 1.80, S 5.93.

Te-50. Reaction of a 1:1 mixture of **5** (16.9 mg, 0.0149 mmol) and **GlcC$_5$S** (4.23 mg, 0.0149 mmol) with aqueous $HAuCl_4$ (398 µL, 0.025 M) and $NaBH_4$ (218 µL, 1 N) gave **Te-50** (6.0 mg) as a brown powder. TEM (average diameter and number of gold atoms): 1.9 ± 0.5 nm (Figure S2), 225; ^{1}H NMR (D_2O): δ 5.38 (brs, 1H), 5.33 (brs, 1H), 5.08 (s, 1H), 4.86 (s, 1H), 4.47 (d, *J*=7.5 Hz, 1H; H-1 glucose), 4.24-3.31 (m, 55H), 1.81-1.13 (m, 24H) (Figure S4); IR (KBr): ν 3360 (broad), 2922, 2852, 1648, 1583, 1384, 1130, 1059 (Figure S5, right); UV-Vis (H_2O, 0.1 mg/mL): λ= 520 nm (surface plasmon band) (Figure S6, right); elemental analysis calcd (%) for $(C_{46}H_{85}N_2O_{25}S_2)_{56}(C_{11}H_{21}O_6S)_{56}Au_{225}$ (123369): C 31.08, H 4.85, N 1.27, S 4.37; found: C 31.23, H 5.09, N 1.94. S 4.63.

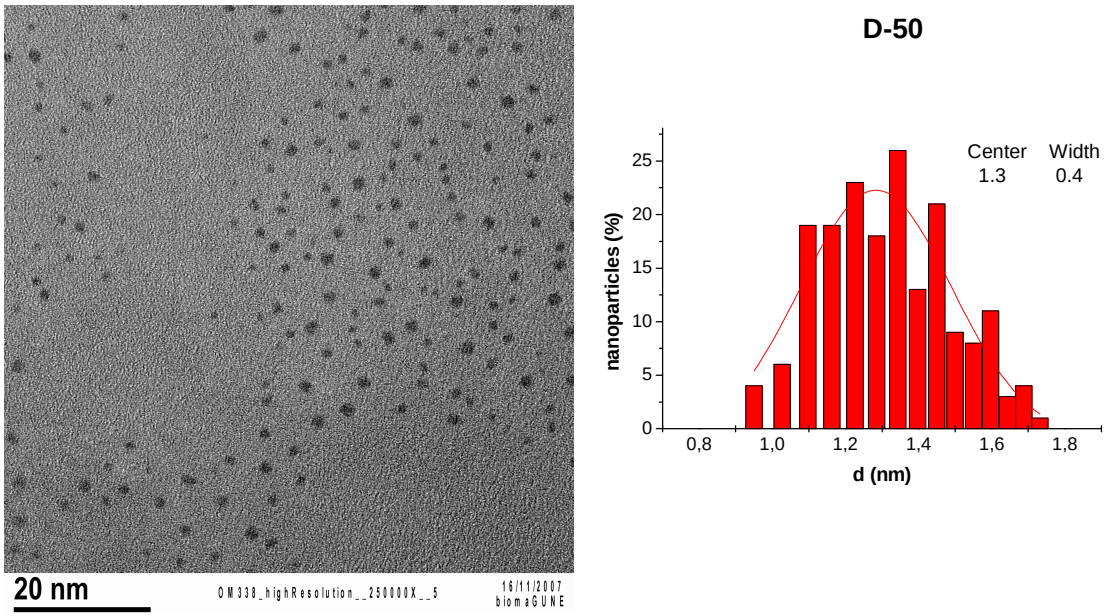


Figure S1. TEM micrographs (left) and size distribution diagram (right) of **D-50**.

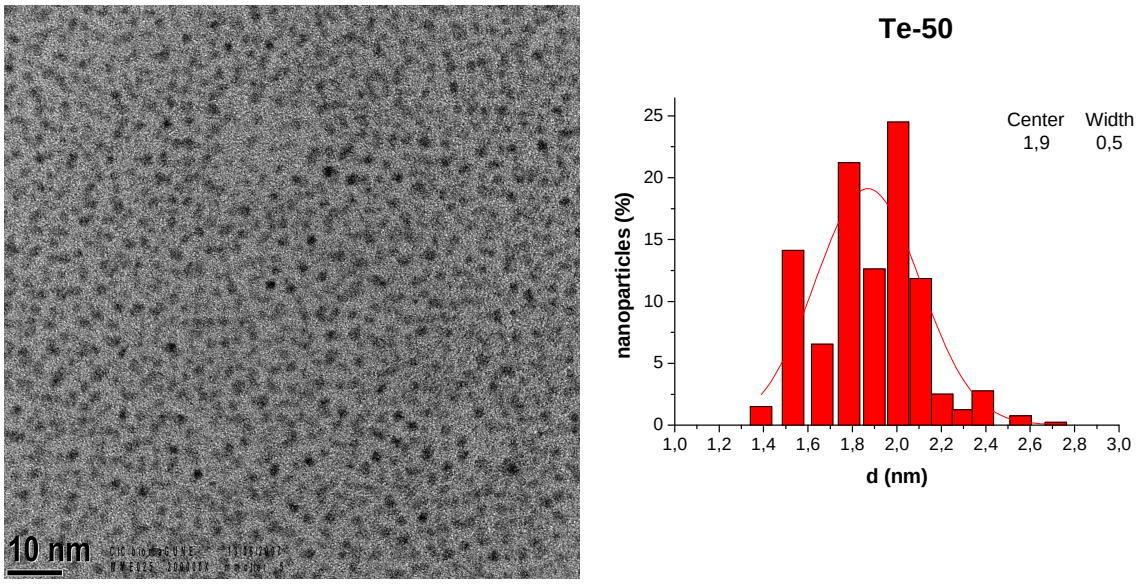


Figure S2. TEM micrographs (left) and size distribution diagram (right) of **Te-50**.

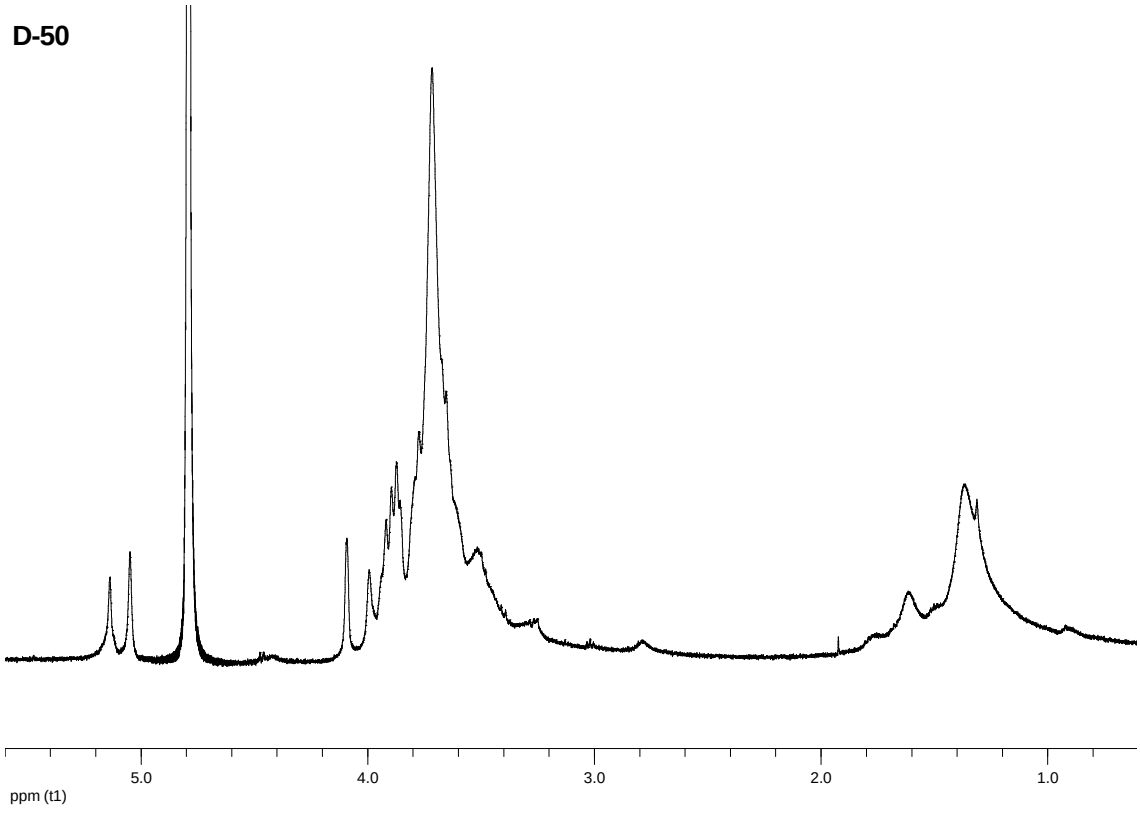


Figure S3. ^{1}H NMR spectrum (500 MHz, D_2O) of **D-50**.

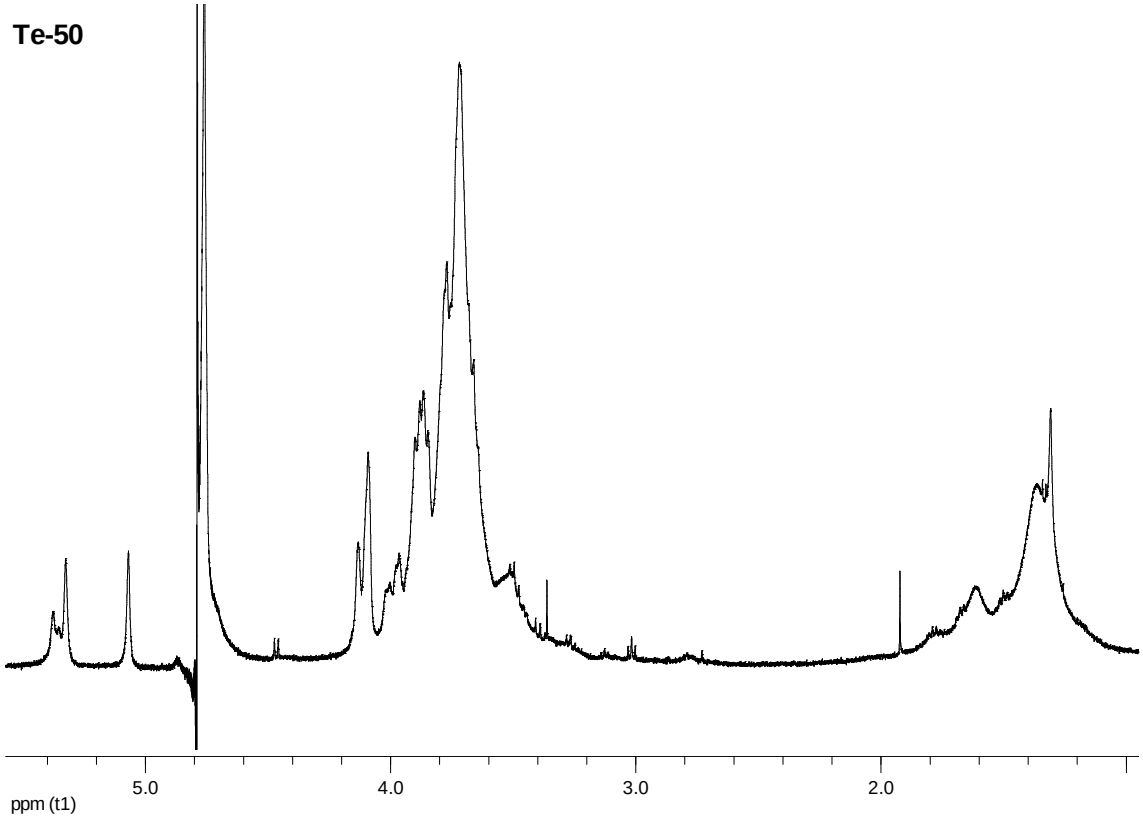


Figure S4. ^{1}H NMR spectrum (500 MHz, D_2O, water suppression) of **Te-50**.

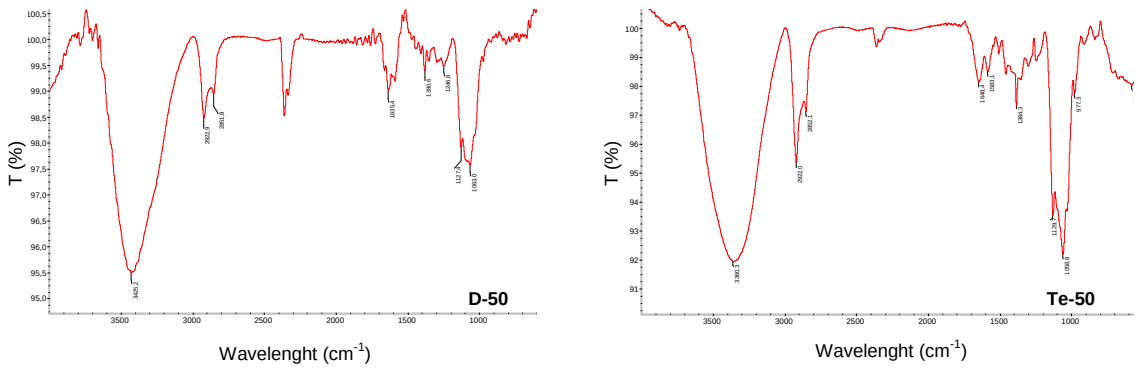


Figure S5. IR spectrum of **D-50** (left) and **Te-50** (right).

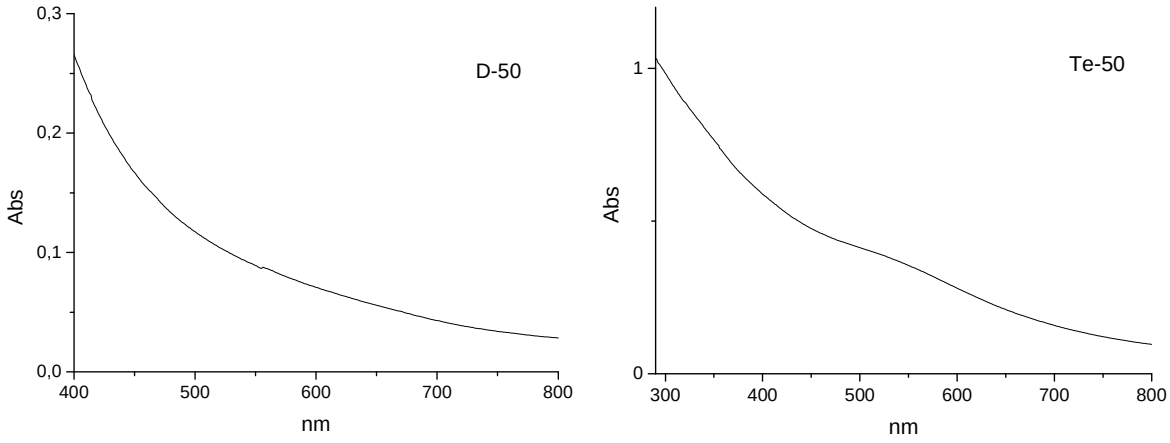


Figure S6. UV-Vis spectra of **D-50** (left) and **Te-50** (right) at 0.1 mg/mL in H_2O

2. Cellular assays

2.1. Cells and Virus General Methods

Cell culture. Raji cells were kindly provided by Dr. Toraño (Instituto de Salud Carlos III, Madrid, Spain) and Raji DC-SIGN+ cells were kindly provided by Dr. Arenzana-Seisdedos (Institut Pasteur, Paris, France). Both cell lines were cultured in RPMI 1640 medium containing 10% (*v/v*) fetal bovine serum, 2 mM L-glutamine, penicillin (50 IU/mL) and streptomycin (50 μg/mL; all Whittaker M.A. Bio-Products). 293 T cells[S4] were cultured in DMEM medium containing 10% (*v/v*) fetal bovine serum, 2 mM L-glutamine, penicillin (50 IU/mL) and streptomycin (50 μg/mL; all Whittaker M.A. Bio-Products). 293T cells were cultured at 37 ºC in a 5% CO_2 humidified atmosphere and splinted twice a week.

Preparation of PBMCs from blood. PBMCs were obtained from buffy coats from healthy donors. Briefly PBMCs were harvested from buffy coats by centrifugation over Lymphoprep (Sigma-Aldrich) gradient according to standard procedures, stimulated with IL-2 (300 IU/mL, Chiron), and PHA (5 μg/ml) and incubated at 37 ºC in 5% humidified CO_2 for 48 h.

Plasmids. The vector pNL4.3-luciferase was generated by replacing the *nef* gene by the luciferase reporter gene in the HIV-1 proviral clone pNL4.3. The pNL4.3-Renilla (X4 tropic) was generated by replacing the *nef* gene on the proviral clone (pNL4.3) by the Renilla reporter gene. The pJR Renilla (R5 tropic) plasmid was generated by cloning the env gene of HIV-1 JR_{CSF} on the pNL4.3-Renilla plasmid.[S5] The vector

pNL4.3.Luc.R$^-$E$^-$ has been previously described[S6,S7] (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH). The pcDNA$_3$-VSV plasmid contains the cDNA encoding the vesicular stomatitis virus (VSV) G protein.

Production of recombinant viruses. Infectious supernatants were obtained from calcium phosphate transfection on 293T cells of full-length provirus constructs, pNL4.3-Renilla or pJR-Renilla. Supernatants were collected after 48 h and frozen until use. Viral supernatants were subjected to p24 antigen quantification (INNO-TEST^{TM} HIV antigen mAb, Innogenetics, Belgium). In order to assure viral infectivity, supernatants were also subjected to a previous infection assay in the absence of any treatment, infecting the target cells with increasing amounts (p24 antigen). Briefly, preactivated PBMCs were infected for 2 h with different amounts of p24 antigen, extensively washed and left in culture for 48 h. Afterwards, cells were lysed and Renilla-luciferase activity was measured in cell lysates using a luminometer (Berthold Detection Systems).

Flow cytometry. Raji DC-SIGN+ and Raji cells were analysed using forward-versus-side scatter dot plots after single colour immunophenotyping with anti-DC-SIGN and anti-CD4. Background staining was assessed with the appropriate isotype- and fluorochrome-matched control mAb and subtracted.

2.2. **trans-*Infection Assay***: Raji or Raji DC-SIGN+ cells (10^5 cells/well) were incubated with GNPs for one hour prior to addition of either R5 or X4 tropic recombinant viruses (JR-Renilla or NL4.3-Renilla, respectively; both 200 ng p24/well) and left for 2 h at 37 ℃ for efficient adsorption. Afterwards, cells were washed extensively with PBS, followed by the addition of preactivated PBMCs (10^5/well). Viral replication after *trans*-infection was followed by measuring RLU activity in cell lysates. Briefly, cell cultures were harvested and lysed after 48 hours and sample activity was measured by following the “Renilla luciferase assay system” (Promega) manufacturer procedures. Relative luminescence units (RLUs) were obtained in a luminometer (Berthold Detection Systems) after the addition of substrate to cells extracts. To rule out possible interferences of DC-SIGN independent mechanisms all the experiments were performed in parallel with non expressing Raji cells as control.

Statistical analysis. Inhibitory Concentrations 50 (IC_{50}) were calculated with Graph-Pad Prism Software (Sigmoidal dose-response analysis). Results are representative of at least three independent experiments (Figure S8)

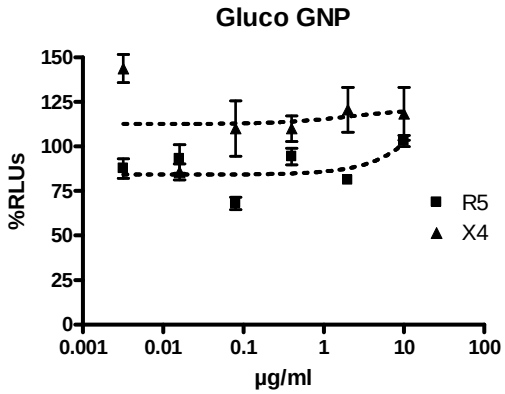
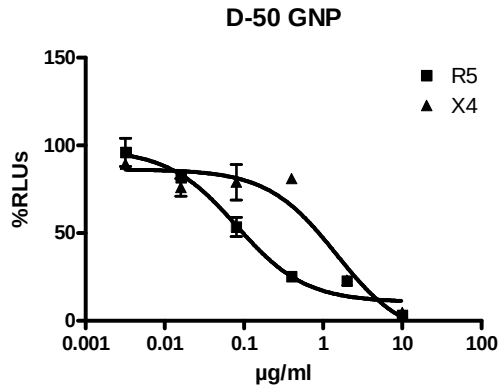
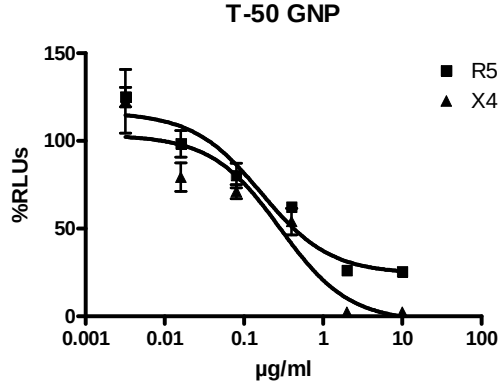


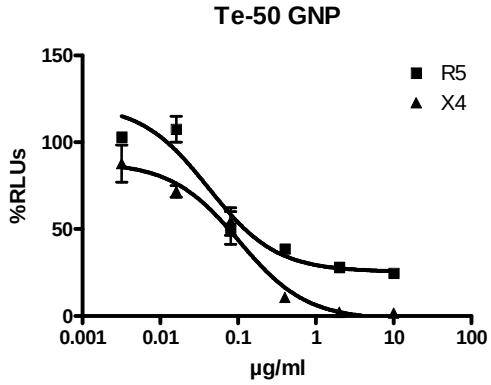
Figure S7. Concentration-response curves of anti-HIV activity of ***gluco*-GNP** against R5 JR-Renilla and X4 NL4.3-Renilla infection of PBMCs. Data do not converge to sigmoidal dose-response fitting as reported by GraphPad Prism Software.



	R5	X4
Sigmoidal dose-response		
Best-fit values		
BOTTOM	10,64	-10,43
TOP	97,75	86,35
LOG IC50	-1,094	0,1671
IC50	**0,08056**	**1,469**
Std. Error		
BOTTOM	3,25	10,76
TOP	5,34	4,231
LOG IC50	**0,1277**	**0,1835**
95% Confidence Intervals		
BOTTOM	3.562 to 17.73	-33.67 to 12.82
TOP	86.12 to 109.4	77.21 to 95.49
LOG IC50	-1.372 to -0.8155	-0.2292 to 0.5634
IC50	**0.04244 to 0.1529**	**0.5899 to 3.659**
Goodness of Fit		
R^2	**0,9564**	**0,9148**

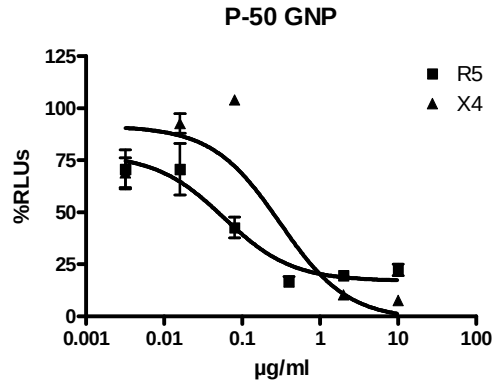


	R5	X4
Sigmoidal dose-response		
Best-fit values		
BOTTOM	24,2	-3,049
TOP	116,3	103,4
LOG IC50	-0,7798	-0,5426
IC50	**0,166**	**0,2867**
Std. Error		
BOTTOM	5,558	10,74
TOP	5,733	8,898
LOG IC50	**0,1572**	**0,2349**
95% Confidence Intervals		
BOTTOM	12.36 to 36.05	-25.93 to 19.83
TOP	104.1 to 128.5	84.41 to 122.3
LOG IC50	-1.115 to -0.4447	-1.043 to -0.04197
IC50	**0.07675 to 0.3591**	**0.09051 to 0.9079**
Goodness of Fit		
R^2	**0,9174**	**0,8313**

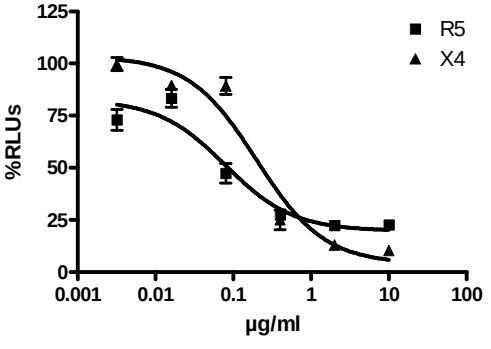


	R5	X4
Sigmoidal dose-response		
Best-fit values		
BOTTOM	25,46	-2,154
TOP	121,9	88,42
LOG IC50	-1,381	-0,9888
IC50	**0,04161**	**0,1026**
Std. Error		
BOTTOM	4,218	4,294
TOP	11,91	5,367
LOG IC50	**0,18**	**0,1355**
95% Confidence Intervals		
BOTTOM	16.27 to 34.65	-11.31 to 6.997
TOP	95.91 to 147.8	76.98 to 99.86
LOG IC50	-1.773 to -0.9886	-1.277 to -0.7001
IC50	**0.01686 to 0.1027**	**0.05279 to 0.1995**
Goodness of Fit		
R²	**0,8992**	**0,9367**

Figure S8. Concentration-response curves of anti-HIV activity of *manno*-GNPs **D-50, T-50** and **Te-50** against R5 JR-Renilla and X4 NL4.3-Renilla infection of PBMCs used to calculate IC_{50} values (left) and data analysis (right) obtained with GraphPad Prism Software.



	R5	X4
Sigmoidal dose-response		
Best-fit values		
BOTTOM	17,06	-1,538
TOP	77,74	91,48
LOG IC50	-1,233	-0,5148
IC50	**0,0585**	**0,3056**
Std. Error		
BOTTOM	4,725	11,43
TOP	7,516	9,228
LOG IC50	**0,2514**	**0,2823**
95% Confidence Intervals		
BOTTOM	6.991 to 27.13	-25.90 to 22.82
TOP	61.72 to 93.76	71.82 to 111.2
LOG IC50	-1.768 to -0.6972	-1.116 to 0.08674
IC50	**0.01704 to 0.2008**	**0.07651 to 1.221**
Goodness of Fit		
R²	**0,8128**	0,7732



	R5	X4
Sigmoidal dose-response		
Best-fit values		
BOTTOM	19,72	3,981
TOP	82,73	103,5
LOG IC50	-1,101	-0,7004
IC50	**0,07924**	**0,1994**
Std. Error		
BOTTOM	3,489	5,191
TOP	4,857	5,277
LOG IC50	**0,1668**	**0,1316**
95% Confidence Intervals		
BOTTOM	12.29 to 27.16	-7.155 to 15.12
TOP	72.38 to 93.09	92.18 to 114.8
LOG IC50	-1.456 to -0.7457	-0.9827 to -0.4181
IC50	**0.03496 to 0.1796**	**0.1041 to 0.3819**
Goodness of Fit		
R²	**0,9069**	**0,941**

Figure S8. Concentration-response curves of anti-HIV activity of *manno*-GNPs **P-50** and **H-50** against R5 JR-Renilla and X4 NL4.3-Renilla infection of PBMCs used to calculate IC_{50} values (left) and data analysis (right) obtained with GraphPad Prism Software.

2.3. Cell Viability Assay: The cell viability test was performed in Raji DC-SIGN+ or in Raji DC-SIGN+ plus preactivated PBMCs using the same protocol described for the Raji DC-SIGN mediated *trans*-infection assay, but in the absence of viral supernatants. After 48 h CellTiter-Glo reagent (CellTiter-Glo® Luminescent Cell Viability Assay, Promega) was added to cell cultures and viability was quantified in a luminometer (Figure S9).

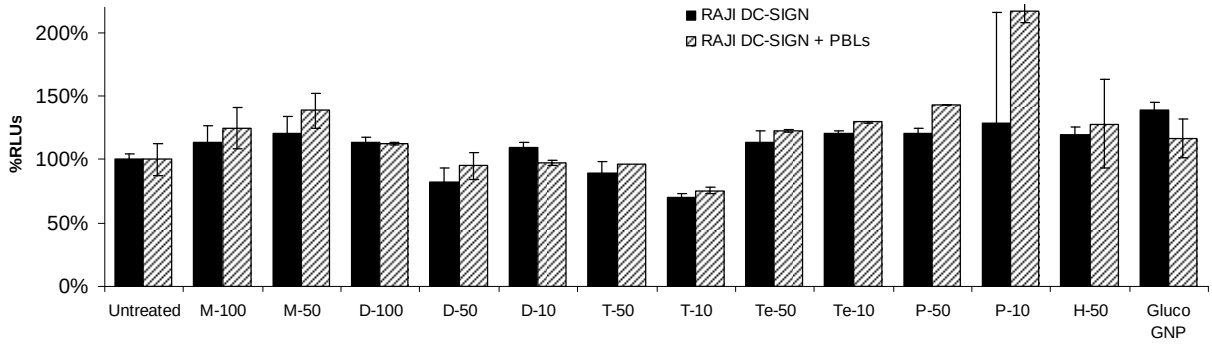


Figure S9. Evaluation of cell viability in the presence of *manno*-GNPs at 100 µg/mL; Raji DC-SIGN (black bars) or of Raji DC-SIGN+PBMCs (striped bars).

References

[S1] H. K. Lee, C. N. Scanlan, C. Y. Huang, A. Y. Chang, D. A. Calarese, R. A. Dwek, P. M. Rudd, D. R. Burton, I. A. Wilson, C. H. Wong, *Angew. Chem.* **2004***, 116,* 1018-1021; *Angew. Chem. Int. Ed. Engl.* **2004**, *43*, 1000-1003.

[S2] C. Pale-Grosdemange, E. S. Simon, K. L. Prime, G. M. Whitesides, *J. Am. Chem. Soc.* **1991**, *113*, 12-20.

[S3] M. J. Hostetler, J. E. Wingate, C.-J. Zhong, J. E. Harris, R. W. Vachet, M. R. Clark, J. D. Londono, S. J. Green, J. J. Stokes, G. D. Wignall, G. L. Glish, M. D. Porter, N. D. Evans, R. W. Murray, *Langmuir* **1998**, *14*, 17-30.

[S4] R. B. DuBridge, P. Tang, H. C. Hsia, P. M. Leong, J. H. Miller, M. P. Calos, *Mol. Cell. Biol*.**1987**, *7*, 379-387.

[S5] J. Garcia-Perez, S. Sanchez-Palomino, M. Perez-Olmeda, B. Fernandez, J. Alcami, *J. Med. Virol*. **2007**, *79*, 127-37.

[S6] R. I. Connor, B. K. Chen, S. Choe, N. R. Landau, *Virology* **1995**, *206*, 935-944.

[S7] J. He, S. Choe, R. Walker, P. Di Marzio, D. O. Morgan, N. R. Landau, *J. Virol.* **1995**, *69*, 6705-6711.