Synthesis of giant globular multivalent glycofullerenes as potent inhibitors in a model of Ebola virus infection

Antonio Muñoz,[§] David Sigwalt,[¥,£] Beatriz M. Illescas,[§] Joanna Luczkowiak,[‡] Laura Rodríguez,[§] Iwona Nierengarten,[¥] Michel Holler,[¥] Jean-Serge Remy,[£] Kevin Buffet,[¶] Stéphane P. Vincent,[¶] Javier Rojo,[†,*] Rafael Delgado,[‡,*] Jean-François Nierengarten,[¥,*] Nazario Martín[§,#,*]

[§]*Departamento de Química Orgánica, Facultad de Química, Universidad Complutense, 28040 Madrid, Spain, e-mail: nazmar@quim.ucm.es;* [†] *Glycosystems Laboratory, Instituto de Investigaciones Químicas (IIQ), CSIC – Universidad de Sevilla, Av. Américo Vespucio 49, Seville 41092 Spain. Tel: + 34 954489568; FAX +34 954460165; e-mail: javier.rojo@iiq.csic.es;* [£] *Laboratory V-SAT (CAMB UMR 7199, CNRS), Labex Medalis, Université de Strasbourg, 74 Route du Rhin, 67401 Illkirch-Graffenstaden, France;* [¶] *University of Namur (FUNDP), Département de Chimie, Laboratoire de Chimie Bio-Organique, rue de Bruxelles 61, B-5000 Namur, Belgium;* [‡]*Laboratorio de Microbiología Molecular, Instituto de Investigación Hospital 12 de Octubre (imas12) 28041 Madrid, Spain, e-mail: rafael.delgado@salud.madrid.org;* [¥] *Laboratoire de Chimie des Matériaux Moléculaires, Université de Strasbourg et CNRS (UMR 7509), Ecole Européenne de Chimie, Polymères et Matériaux, 25 rue Becquerel, 67087 Strasbourg, France, e-mail: nierengarten@unistra.fr;* [#] *IMDEA-Nanoscience, Campus Cantoblanco, 28049 Madrid, Spain.*

Abstract

The inhibition of pathogens entry through the blockade of DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin) receptor at early stages of infection is an efficient strategy for testing new antiviral agents. Although a variety of models have been reported, one of the main problems is achieving adequate size and multivalency to mimic natural systems such as viruses or other pathogens. In this regard, hexakis-adducts of [60]fullerene allow a full control of size and multivalency while maintaining a globular shape. For this purpose,tridecafullerenes decorated with 120 peripheral carbohydrate subunits have been efficiently synthesized from hexakis-adducts of [60]fullerene in one synthetic step by using the copper-catalyzed azide–alkyne cycloaddition (CuAAC) click-chemistry methodology. These so-called 'superballs' decorated with 120 mono-saccharides (mannose or galactose) moieties are water soluble, allowing the study of their biological properties. An infection assay has been employed to test the ability of these compounds to inhibit the infection of cells by an artificial Ebola virus. The results obtained in these experiments show that the 'superballs' are potent inhibitors of cell infection by this artificial Ebola virus with IC50s in the sub-nanomolar range. This implies more than a three-orders-of-magnitude increase of activity in comparison to the hexakis-adduct containing just 12 mannoses and only 18-fold less activity than virus-like glycodendrinanoparticles with diameters of roughly 32 nm displaying up to 1620 glycans.

Multivalency is a general and efficient tool used by nature for achieving strong interactions in a reversible manner. At the molecular level, multivalent interactions have the advantage of enhancing drastically the binding between molecules when compared with the monovalent binding.[1]

A remarkable example from nature where multivalency plays a significant role is the interaction between viruses and bacteria with their respective host cells. In particular, DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) receptor is one of the most important pathogen recognition receptors. This lectin efficiently recognizes in a multivalent manner saccharides containing mannoses and fucoses from glycoproteins.[2] In this regard, it is well established that protein−carbohydrate interactions are a key issue in a variety of biological processes, but since the affinity of simple glycans for their respective receptors is often weak, multivalent interactions typically occur. An important open challenge of research nowadays is the better understanding and practical use of multivalency. Actually, in a broader sense, glycobiology is currently a field of research where chemically inspired approaches and strategies are producing significant advances.[3]

It is well-known, however, that some viruses are able to escape from processing by the immune defense by using DC-SIGN as an entry point to infect the cell. Therefore, the inhibition of pathogens entry by blocking this receptor at the early stages of infection represents a valuable strategy for the design of new antiviral agents. In order to address this challenge, a variety of different multivalent scaffold architectures have been synthesized, all of them endowed with multiple carbohydrates.[4] Design of these glyco-conjugates typically requires a multivalent central scaffold or core covalently connected to the carbohydrate epitopes decorating the periphery. Thus, glyco-clusters showing the carbohydrate units directly connected to the core, glyco-dendrimers connected through a dendritic structure and glyco-polymers involving a polymeric backbone have intensively been investigated in recent years.[5, 6, 7, 8, 9]

Fullerenes also have been employed as a biocompatible scaffold for the multivalent presentation of ligands, given the possibility of multiple functionalization on their convex surface. In particular, hexakis-adducts of [60]fullerene with a T_h-symmetrical octahedral addition pattern have a unique three-dimensional structure which allows the introduction of six one-type or mixed-types addends.[10, 11] Such derivatives can be obtained in one synthetic step by the addition of malonates to C_{60}, but it has been somehow limited by the low yields resulting with bigger malonates, as the reaction is very sensitive to steric effects.[12] We have recently developed a procedure based on a click-chemistry approach which provides hexakis-adducts with twelve

alkyne or azide terminal groups in high yields from simple malonates.[13, 14] These hexakis-adducts can be easily and efficiently functionalized by using the copper-catalyzed azide–alkyne cycloaddition (CuAAC) reaction allowing the introduction of twelve functional groups simultaneously in a regioselective and efficient way. Since these hexakis-adducts show an octahedral arrangement of equally separated addends located on the [60]fullerene periphery in a globular topology, they constitute a very attractive platform for the study of multivalent interactions with lectins, with important advantages such as the better biocompatibility of the carbon central core, the globular geometry of the functional groups and the ease and versatile chemical functionalization.

Thus, we have recently reported the syntheses of [60]fullerene hexakis-adducts endowed with different carbohydrate units in the periphery. Depending upon the starting sugar derivatives employed (monomer, dimer or trimer) 12, 24 and up to 36 monosaccharides have been introduced on the periphery of the fullerene central core in a straightforward manner.[14, 15, 16, 17]

Remarkably, some of the aforementioned glycofullerenes have proven to show interesting bio-medical applications.[18] Thus, a significant multivalent effect has been observed in the inhibition profile of a [60]fullerene hexakis-adduct endowed with 12 iminosugar units towards different glycosidases.[16] On the other hand, fullerene hexakis-adducts bearing 12 mannoses on the periphery behave as inhibitors of FimH, a bacterial adhesin.[19] Furthermore, studies carried out on hexakis-adducts of [60]fullerene endowed with 12, 24 and 36 mannoses have shown a multivalent effect in the interaction with Concanavalin A,[15] and acting as efficient inhibitors of cell infection by Ebola pseudotyped viral particles.[20] These preliminary studies reveal that fullerenes are adequate platforms for the multivalent presentation of carbohydrates, thus paving the way to the covalent linkage of a wide variety of different bio-active molecules in a multivalent manner and a singular and less-explored globular topology.

In this work, we have synthesized unprecedented and giant molecules showing a globular topology and formed by hexakis-adduct of hexakis adducts of [60]fullerene, carrying out the fastest dendrimeric growth reported up to now, with the introduction of 120 sugar units in one synthetic step by using the highly efficient CuAAC click-chemistry methodology. The synthesis of hexakis-adducts of hexakis-adducts of [60]fullerene with an octahedral addition pattern has previously been reported. However, as macrocyclic bis-malonates were used, this procedure allows the synthesis of heptafullerenes with tunable properties.[21, 21 22, 23] In contrast, our click-chemistry approach yields tridecafullerenes in which the central [60]fullerene is covalently connected to twelve [60]fullerenes, each of them endowed, in turn, with ten monosaccharides.

To confirm the proposed structures, we have also carried out a thorough structural study involving a variety of techniques (FTIR, ^{1}H NMR, ^{13}C-NMR, DLS, TEM, XPS). Remarkably, biological studies reveal that the new giant glycofullerenes exhibit a very strong inhibition of cell infection by Ebola pseudotyped viral particles with IC_{50} values in the sub-nanomolar range.

Synthesis

The synthesis of the glycofullerene superballs has been carried out as depicted in Figures 1-3. Although these molecules (**17a-c**) may appear difficult to obtain at a first glance, mainly due to their size and molecular complexity, their synthesis is straightforward and, most importantly, easily reproducible affording the final compounds in good overall yields. Furthermore, as discussed below, purification of the samples including the required removal of copper for further biological studies are carried out easily since these compounds (**17a,b**), after column filtration, are precipitated in the reaction medium and pure samples showing highly reproducible biological essays are obtained.

With the aim of studying the effect of the size and/or steric congestion of these superballs on the biological properties, two different and complementary synthetic strategies have been simultaneously developed to obtain final products with different spacers in between the central fullerene core and the peripheral carbohydrate substituted fullerene appendages. In both cases, the synthesis relies on the grafting of clickable $A_{10}B$ macromonomers onto a compact C_{60} hexa-adduct scaffold bearing twelve terminal alkyne units. The preparation of macromonomer **9** is depicted in Figure 1. Esterification of alcohol **1** with ethylmalonyl chloride followed by reaction of the resulting malonate (**2**) with C_{60} under Bingel conditions gave methanofullerene **3**. Subsequent treatment of **3** with an excess of malonate **4**, CBr_4, and DBU in *o*-dichorobenzene (ODCB) gave building block **5** to which mannose derivative **6** was clicked. Importantly, the TMS-protected alkyne unit is not reactive under these conditions and intermediate **7** was thus obtained in a good yield. Finally, compound **7** was desilylated *in situ* with tetrabutylammonium fluoride (TBAF) to generate the corresponding terminal alkyne and reaction with a large excess of diazide **8** provided the desired macromonomer **9** in 87% yield.

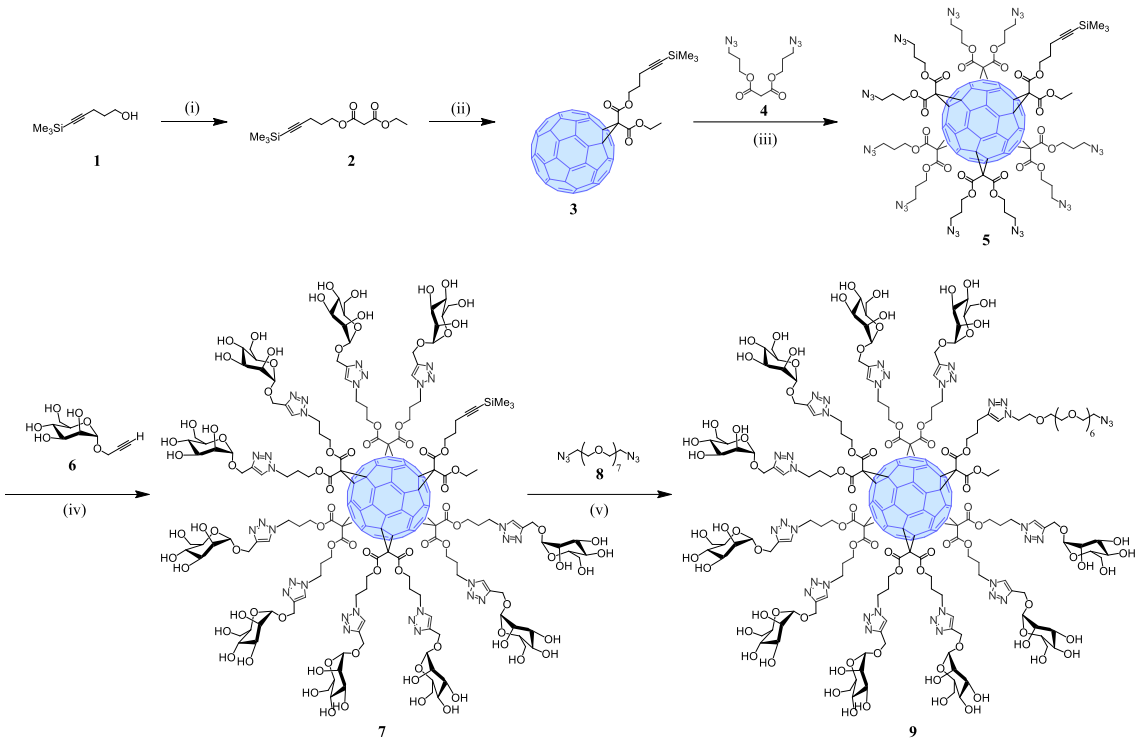

Figure 1. Synthesis of azidofullerene derivative 9. *Reagents and conditions*. (i) ethylmalonyl chloride, pyridine, CH_2Cl_2, 0 to 25°C, 1 h (99%); (ii) C_{60}, I_2, DBU, 25 °C, Tol, 16 h (49%); (iii) CBr_4, DBU, 25 °C, ODCB, 72 h (67%); (iv) $CuSO_4.5H_2O$, sodium ascorbate, THF/H_2O, 100°C (MW), 2 h (91%); (v) **8** (19 equiv.), $CuSO_4.5H_2O$, sodium ascorbate, TBAF, THF/H_2O, 80°C (MW), 1.5 h (87%).

The synthesis of macromonomers **15a-b** starts from monoadduct **10** resulting from the Bingel nucleophilic cyclopropanation of C_{60} with 6-bromohexyl ethyl malonate (Figure 2).[25] To obtain the [5:1]- hexaadduct **12**, a tenfold excess amount of di(pent-4-yn-1-yl) malonate and a ~50-fold excess of carbon tetrabromide were added in the presence of DBU as the base. Flash chromatography purification provided hexaadduct **12** as a red solid. Different carbohydrate azides (mannose, galactose) were then linked to C_{60} by the CuAAC reaction, employing $CuBr{\cdot}S(CH_3)_2$ as the catalyst and sodium ascorbate as the reducing agent in the presence of a piece of metallic Cu, to yield derivatives **14a-b**. The nucleophilic substitution of bromine by azide was carried out with an excess of sodium azide under microwave heating, thus giving versatile building blocks **15a-b**.

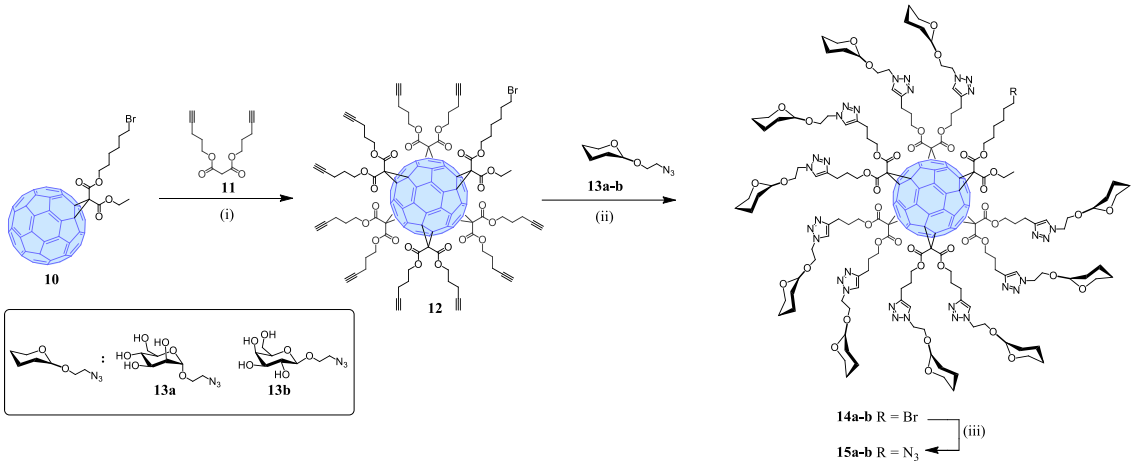

Figure 2. Synthetic pathway to azidofullerene derivatives 15a-b. *Reagents and conditions*. (i) di(pent-4-yn-1-yl) malonate, CBr_4, DBU, 20 °C, Tol, 72 h (49%); (ii) 2-azidoethyl α-D-mannopyranoside for **14a** (or 2-azidoethyl α-D-galactopyranoside for **14b**), CuBr·$S(CH_3)_2$, sodium ascorbate, Cu^0, DMSO, 72 h (**14a**: 86%; **14b**: 86%); (iii) NaN_3, 70 °C (MW), DMSO, 3 h (**15a**: 84%; **15b**: 81%).

The azide-containing macromonomers **9** and **14a-b** were then clicked to symmetric alkyne derivative **16**[14] under CuAAC conditions. Superballs **17a-c** substituted with up to 120 monosaccharide units were thus obtained with yields over 70% (Figure 3). Remarkably, derivatives **17a-b** have been obtained in only three synthetic steps from easily accessible reactives and avoiding the use of protecting groups.

Although the three tridecafullerenes **17a-c** contain the same number of monosaccharides, compound **17c** has a larger spacer between the central fullerene moiety and the peripheral carbohydrate functionalized fullerenes. This longer spacer affects the flexibility and the size of the compound and can favor the accessibility and availability of the carbohydrate ligands to interact with the receptor, which could have an important influence on the biological activity.[20]

It is important to note, however, that the aforementioned synthetic approaches allow synthesizing fullerene derivatives from malonates endowed with alkyl chains of variable length through a first Bingel cyclopropanation and a subsequent CuAAC reaction. This synthetic strategy affords hexakis-adducts with long chains connecting the fullerenes and carbohydrates since their direct preparation from suitably functionalized malonates bearing long chains typically occur with remarkably low yields.[10]

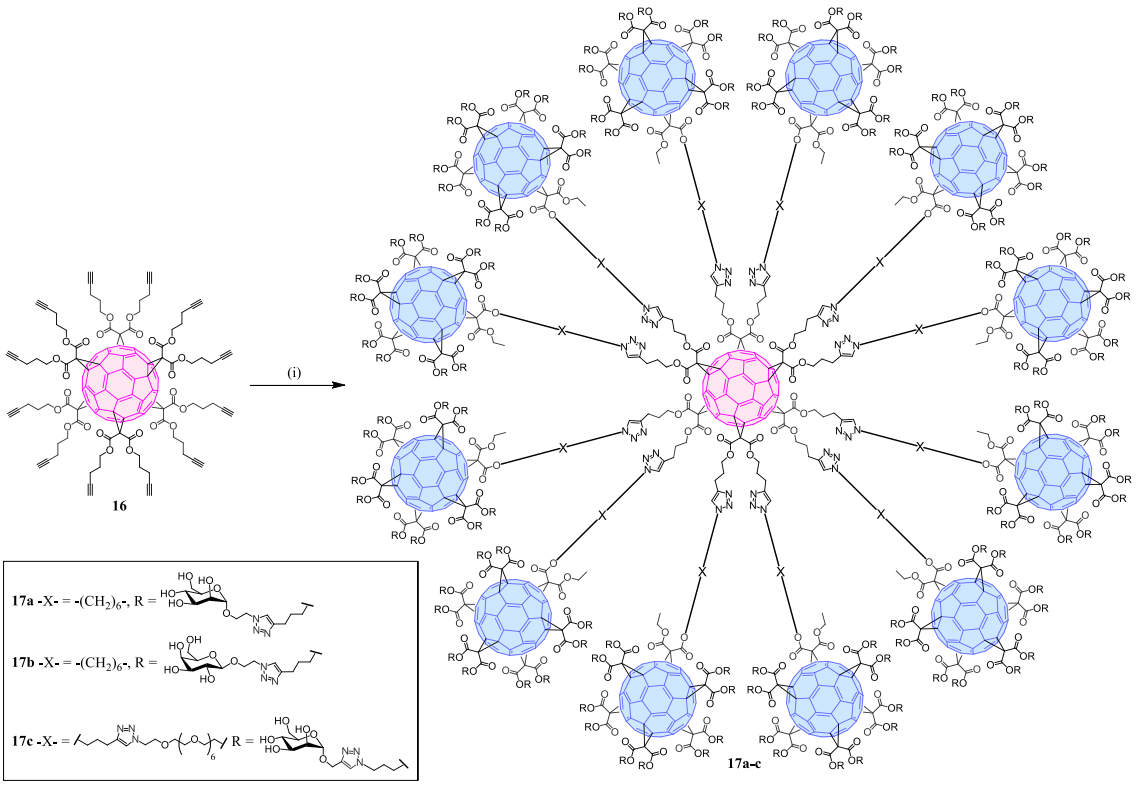

Figure 3. Synthetic scheme for tridecafullerenes 17a-c. *Reagents and conditions*. For compounds **17a-b**: (i) **15a-b**, CuBr·S(CH_3)$_2$, sodium ascorbate, Cu^0, DMSO, 25°C, 48 h [**17a** (from **15a**): 73%; **17b** (from **15b**): 79%]. For compound **17c**: (i) **9**, $CuSO_4.5H_2O$, sodium ascorbate, THF/H_2O, 80°C (MW), 2 h (76%).

Characterization of these superballs was carried out by standard spectroscopic techniques. Thus, FTIR spectra do not show the presence of neither azide group (typical signal observed at ~2097 cm^{-1}) nor alkyne (~ 2117 cm^{-1}) (See the Supplementary Information). The molecular ion peak of these compounds could not be detected. Actually the transfer of such high molecular weight glycoclusters in the gas phase during the MALDI-TOF MS analysis is very difficult. Moreover, both the sugars and the fullerene hexaadduct moieties give rise to high level of fragmentation.[26] The unambiguous structural characterization of **17a-c** was, however, greatly facilitated by their high symmetry. Indeed, ^{13}C NMR spectroscopy was particularly helpful for the characterization of hexakis-adducts of [60]fullerene, as only two signals are usually observed for the sp^2 carbons of C_{60}, evidencing the octahedral symmetry of the fullerene core. As a typical example, the ^{13}C NMR spectrum of compound **17a** is depicted in Figure 4. In this case, only two sp^2 carbons are observed in the spectra (δ ~ 145.4 and 141.6), where we can also distinguish the two different kinds of triazole rings present in the derivative (at δ ~ 146.9 and 123.3 the carbons of the outer triazole rings and at δ ~ 146.4 and 122.9 the carbons of the inner triazole rings). In

addition, only one signal is detected for all the carbonyl groups (δ ~ 163.8) and the sp^3 carbons of the C_{60} (δ ~ 69.3), while the malonate bridgehead carbons present in the structure are observed at δ ~ 45.2.

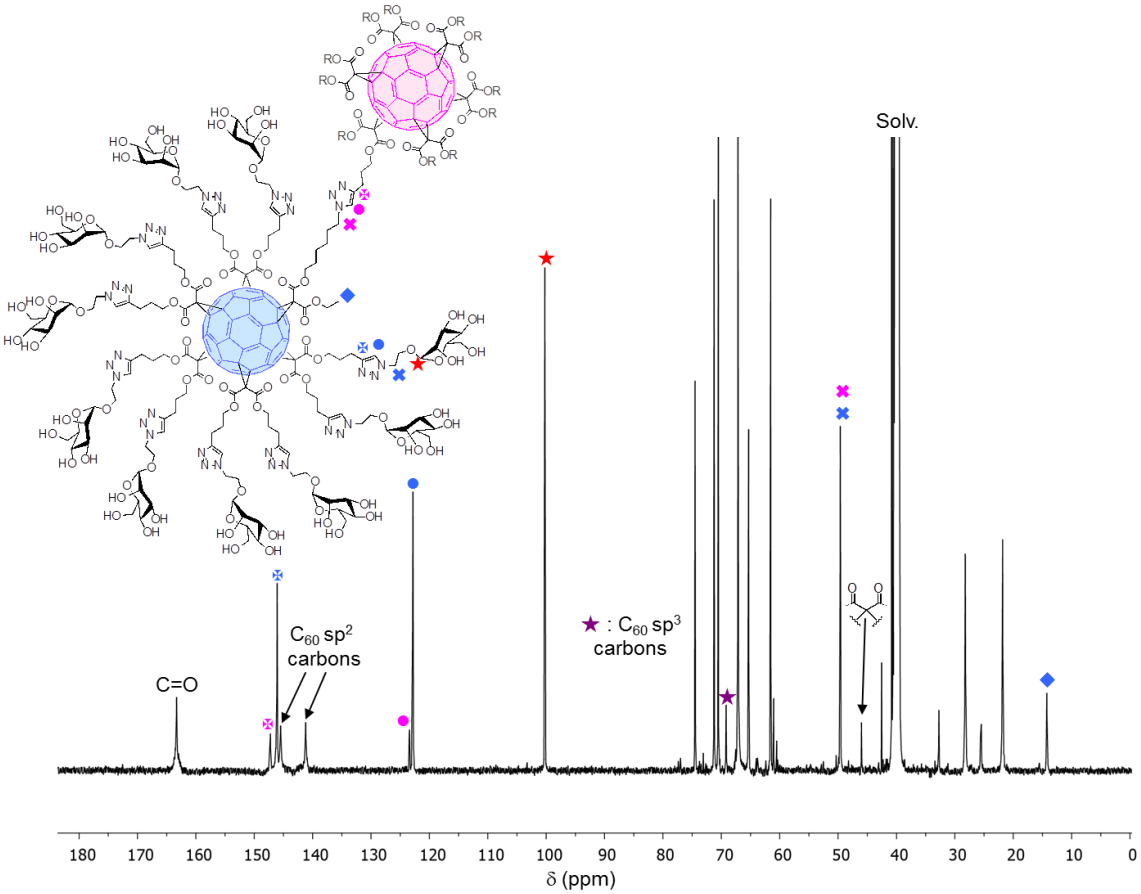

Figure 4. ^{13}C NMR spectrum of tridecafullerene 17a in DMSO-d_6. Assignment of the most representative signals is depicted.

Additional characterization was achieved by DLS analysis (H_2O, 0.01 mg/mL and 0.1 mg/mL), where, regardless the concentration used, we have found two or three main size distributions for **17a-c** (Supplementary Figure 1). This is compatible with a weakly cooperative aggregation of **17a-c** in water. The first, around 5-6 nm, must correspond to only one molecule, while the second, at ~120-150 nm and third, ≥200 nm, show the aggregation of several molecules. In DMSO (0.1 mg/mL) (Supplementary Figure 2), although most of the molecules show no aggregation, the presence of aggregates of different sizes and, especially, very large aggregates, is also detected. The tendency to form aggregates was also confirmed by the broadening of the ^{13}C NMR spectrum recorded in D_2O when compared to the one recorded in DMSO-d_6 (see Supplementary Information).

The TEM images of freshly prepared samples reveal the presence of small spherical particles, corresponding to a few or even just one molecule (~4 nm), independently of whether the concentration is 0.01 mg/mL or 0.1 mg/mL, in good agreement with the experimental findings in DLS analyses (Figure 5 and Supplemntary Figure 3).

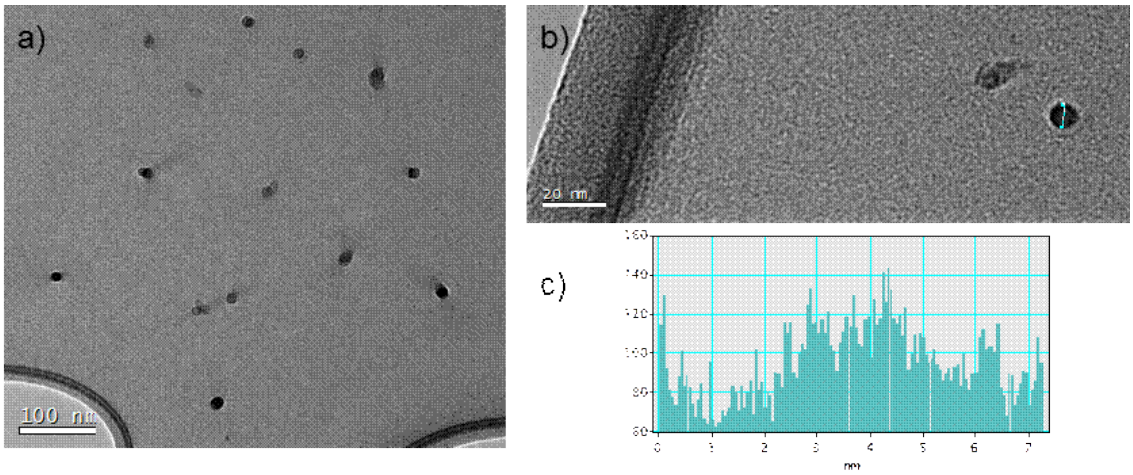

Figure 5. TEM images of tridecafullerene 17a. a) TEM images of compound **17a** upon deposition of a 0.01 mg/mL solution in H_2O. b) Detail of a particle corresponding apparently to one molecule. c) Width profile of the particle shown in b).

X-ray photoelectron spectroscopy (XPS) was additionally employed to confirm the composition of the sugar balls. This surface technique allows the identification of the atoms present on the molecule together with their chemical state and their relative abundance. The survey spectra of superball **17a** (Supplementary Figure 4) displays the C 1s, O 1s and N 1s features as expected, with no additional spectroscopy signatures of possible impurities. Moreover, the high resolution N 1s core level spectrum (Supplementary Figure 4, inset right) was composed of two different components with a 1:2 ratio of the integrated areas, the one located at 400.3 eV is related to one nitrogen atom of the triazole ring (N-**N**-N) and the other centered at 398.8 eV is attributed to the other two nitrogen atoms attached to carbon atoms (C-**N**).[27] The absence of a well resolved peak around 405.0 eV demonstrates the lack of the electron-deficient nitrogen of the azide group in the final compound.[28, 29] The composition of compounds **17b** and **17c** has also been ascertained by their respective XPS analyses which showed the presence of the expected elements, according to their relative abundance (see Supplementary Figure 4 and Supplementary Table 1).

Biological studies

A number of molecules including DC-SIGN have been proposed as receptors for Ebola virus, [30, 31, 32] although DC-SIGN is not the main receptor in case of Ebola virus, it is thought to play a significant role in the cell entrance of this infectious agent in significant cell populations such as dendritic cells,[30, 33] thus facilitating early viral dissemination. Therefore, DC-SIGN can function as a good model of studying the first steps of pathogenesis of Ebola virus and screening the antiviral strategies based on DC-SIGN targeting compounds for prevention and treatment purposes. DC-SIGN recognizes mannosylated and fucosylated oligosaccharides presented in a multivalent manner on the surface of several pathogen envelope glycoproteins. Thus the preparation of multivalent carbohydrate systems is necessary for the efficient interaction with this receptor as well as for the effective competition with the natural ligands. In this study we have evaluated the inhibitory effect of giant globular multivalent glycofullerenes in the experiment of direct infection of Jurkat DC-SIGN$^+$ with pseudotyped viral particles presenting Ebola virus glycoprotein GP1. These globular multivalent systems are water soluble and show no cytotoxicity in cell lines allowing the study of their potential biological function in preventing viral infection. All multivalent compounds were checked for the possibility of blocking DC-SIGN receptor in 6 independent experiments. The results of blocking DC-SIGN receptor by different compounds were shown as a function of concentration. The 50% of inhibition of the infection was calculated with the 95% confidence intervals. As a control, infection with DC-SIGN-independent VSV glycoprotein-pseudotyped lentiviral particles was performed in the same conditions.

The results obtained in the infection experiment revealed the dependence of the inhibition effect on mannoses. Compound **17b** displaying 120 galactoses, as expected, was not able to inhibit the infection process mediated by DC-SIGN. Compounds envisaged with 120 mannose-based residues (**17a** and **17c**) showed very strong antiviral activity at picomolar to nanomolar concentrations. Compound **17a** could effectively block Ebola virus infection at low nanomolar concentrations with the IC_{50} of 20.375 nM (95%CI = 14.63 – 28.37 nM). Compound **17c** was almost one order of magnitude more potent at inhibiting the infection process showing the IC_{50} of 667 pM (95%CI = 411.1 pM – 1.08 nM) (Figure 6).

Previous inhibition studies using the same infection model and fullerenes displaying up to 36 mannoses show relative inhibitor potency (RIP) values at least two orders of magnitude smaller.[19] Moreover, huge virus-like particles (VLP) with a radius of 16 nm and up to 1640 mannoses[33] were 18 fold less potent than compound **17c** described in this work (see Supplementary Table 2). These results have confirmed the efficiency of these systems to interact

with DC-SIGN and to compete with Ebola virus glycoprotein-pseudotyped particles during their entry into target cells."

The cytotoxic effect of multivalent glycofullerenes was verified by a cell proliferation assay using the Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega). Notably, there was not any appreciable cytotoxic effect of compounds **17a-c** at the concentration used in the infection experiments (see Figure S5).

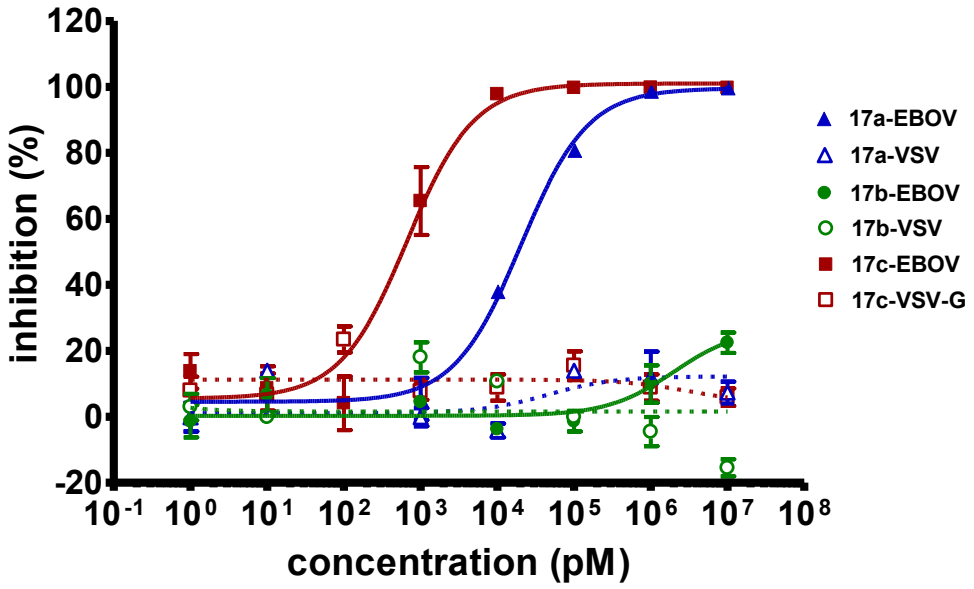

Figure 6. Biological study of tridecafullerenes (17a-c). Inhibition of infection with EBOV or VSV GP-pseudotyped lentiviral particles of Jurkat DC-SIGN$^+$ cells using **17a** (blue), **17b** (green) and **17c** (red). In the cis-infection experiments 2.5x10^{5}Jurkat DC-SIGN$^+$ were challenged with 5000 TCID of recombinant lentiviral particles. Results represent the mean of 6 independent experiments +/- SEM.

Conclusions

In summary, we have synthesized for the first time giant globular multivalent glycofullerenes in which the central C_{60} core is covalently connected to twelve hexakis-adducts of C_{60}, thus forming the first tridecafullerenes reported so far. Since each peripheral fullerene is endowed with ten monosaccharides, a total of 120 carbohydrates are decorating the periphery of the supermolecule which represents the fastest dendrimer growth ever reported, affording molecular weights as high as 56 KDa.

The synthesis of non-symmetric hexakis-adducts (**7**, **9**, **14a,b**, **15a,b**) as well as the formed tridecafullerenes (**17a-c**) have been accomplished by using CuAAC click-chemistry reactions in an efficient manner. In this way, very sophisticated molecular ensembles have been efficiently produced in a minimum of synthetic steps and their apparent structural complexity is not a limitation for their applications. Despite the high molecular weights, the new molecules have been characterized by standard spectroscopic techniques (FTIR, ^{1}H NMR, ^{13}C NMR) as well as by DLS, XPS and TEM. Interestingly, NMR spectroscopy unambiguously reveals the high degree of symmetry (T_h) in hexakis-adducts.

Tridecafullerenes are soluble in water and, therefore, they have been tested as globular multivalent systems to inhibit lectin-mediated viral infection processes in cellular assays. In particular, compounds **17a-c** have been used to test their ability to inhibit the infection of cells by an artificial Ebola virus. Remarkably, tridecafullerenes efficiently block Ebola virus infection in the range of sub-nanomolar concentrations. These values surpass in three orders of magnitude (two if the number of mannoses is considered) to that of hexakis-adducts endowed with 12 mannoses.

The aforementioned results reveal fullerenes as a very appealing platform for the study of multivalent interactions with significant advantages such as their biocompatibility and globular presentation. Furthermore, the compounds now reported pave the way to the introduction of dendritic dimers and trimers of monosaccharides as well as the use of disaccharides to significantly improve the scope of the biological applications of tridecafullerenes.

METHODS

Production of recombinant viruses

Recombinant viruses were produced in 293T cells. The viral construction was pseudotyped with Zaire Ebola virus (ZEBOV) envelope glycoprotein (GP) or vesicular stomatitis virus envelope GP (VSV-G) and expressed luciferase as a reporter of the infection.[34] One day (18-24 h) before transfection, 5 x 10^6 293T were seeded onto 10 cm plates. Cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 25 mg Gentamycin, 2 mM L-glutamine. Few minutes before transfection, the medium on transfection plates was changed to 9 ml DMEM and chloroquine was added to 25µM final concentration. Transfection reaction with all reagents at room temperature (RT) was prepared in 15 ml tubes: 183 µl of 2M $CaCl_2$, 500 ng of EBOV-GP or 2 µg of VSV-G, 21 µg of pNL4-3 luc[35], 1300 µl of H_2O. Next, 1.5 ml of 2xHBS (Hepes Buffer

Saline) pH 7.00 was added quickly to the tubes and bubbled for 30 seconds. HBS/DNA solution was gently dropped onto medium. After 8 hours of incubation at 37º C with 5% CO_2, medium on transfection plates was changed to 10 ml DMEM and once again one day after transfection to 7 ml DMEM. Transfection supernatants were harvested after 48 h, centrifuged at 1200 rpm for 10 minutes at RT to remove cell debris, and stored frozen at -80° C.[33, 36]

Ebola virus infection experiments

Infection was performed on Jurkat cells (T-lymphocyte cell line) expressing receptor DC-SIGN on its surface. Since Ebola virus does not infect T-lymphocytes, its entry is absolutely dependent on DC-SIGN for infection of Jurkat cells.[30, 37]

Jurkat DC-SIGN$^+$ cells (2.5×10^5) were plated into each well of 96-well plate. Cells were incubated at RT for 20 minutes with the carbohydrate-based compounds and then challenged with 5000 TCID (Tissue Culture Infective Dose) of recombinant viruses. After 48 h of incubation cells were washed twice with PBS and assayed with the Luficerase Assay System (Promega, Madison, WI).

The range of concentrations tested for compounds **17a-c** was 1 pM – 10 µM. As a control, experiment of infection with VSV-G pseudoviruses was performed in the same conditions. Infection with VSV-G is independent of the presence of DC-SIGN receptor.

Statistical analysis

The values of percentage of inhibition of the infection presented on the graph correspond to the mean of 6 independent experiments with error bars corresponding to the standard errors of the mean. The IC_{50}s values were estimated using GraphPad Prism v6.0 with a 95% confidence interval and settings for normalize dose-response curves.

Cytotoxicity assay

The Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) was used. Jurkat DC-SIGN+ (5 x 105 cells/well) were seeded into wells of 96-well plate and left cultured in the presence of different concentrations of glycofullerenes for the time of infection assay. After 48 h, the proliferation cell assay was performed. Briefly, the 2 ml MTS solution was mixed with 100 µl of PMS solution. The mix MTS/PMS in a volume of 20 µl was pipetted into each well of the 96-well assay plate containing 100 µl of cells in culture medium. The plate was incubated for 2 h at 37º C in a humidified 5% CO_2 atmosphere. After incubation time, the absorbance at 490 nm was recorded using an ELISA plate reader. As a control of toxicity, the viability of cells in the presence of 0.1% Triton-X100 was measured. The results of the assay were presented as the percentage

of cells viability, which was calculated from the absorbance at 490 nm as compared to absorbance shown by cells incubated without addition of glycofullerenes representing 100 % of cells viability.

Acknowledgements

Financial support by the European Research Council (ERC-2012-ADG_20120216 (Chirallcarbon), ITN-2008-213592 (CARMUSYS)), Ministerio de Economía y Competitividad (MINECO) of Spain (projects CTQ2011-24652, CTQ2011-23410 and CTQ2012-31914), the Comunidad Autónoma de Madrid (PHOTOCARBON project S2013/MIT-2841), Instituto de Salud Carlos III (ISCIII) (FIS PI1101580 and FIS1400708), the *Agence National de la Recherche* (ANR, *Programme Blanc 2011*, Sweet60s), the International Center for Frontier Research in Chemistry and LabEx "Chimie des Systèmes Complexes" is acknowledged. NM thanks to Alexander von Humboldt Foundation. SV and KB thank FNRS (FRIA fellowship).

Author contributions

A. M., D. S., I. N., M. H. and K. B. carried out the synthesis and characterization of all new derivatives. L. R. and J.-S. R. realized and analyzed the DLS and TEM. L. R. realized the XPS analyses and contributed in the writing of the paper. J. L. and R. D. realized the biological and cytotoxicity studies. B. M. I., S. P. V., J. R., R. D., J.-F. N. and N. M. designed the project, supervised the work, discussed the data and wrote the manuscript.

References:

1. Mammen M, Choi S-K, Whitesides GM. Polyvalent Interactions in Biological Systems: Implications for Design and Use of Multivalent Ligands and Inhibitors. *Angew Chem Int Ed* 1998, **37**(20)**:** 2754-2794.

2. Guo Y, Feinberg H, Conroy E, Mitchell DA, Alvarez R, Blixt O*, et al.* Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. *Nat Struct Mol Biol* 2004, **11**(7)**:** 591-598.

3. Imperiali B. The Chemistry–Glycobiology Frontier. *J Am Chem Soc* 2012, **134**(43)**:** 17835-17839.

4. Fasting C, Schalley CA, Weber M, Seitz O, Hecht S, Koksch B*, et al.* Multivalency as a Chemical Organization and Action Principle. *Angew Chem Int Ed* 2012, **51**(42)**:** 10472-10498.

5. Imberty A, Chabre YM, Roy R. Glycomimetics and Glycodendrimers as High Affinity Microbial Anti-adhesins. *Chem Eur J* 2008, **14**(25)**:** 7490-7499.

6. Roy R, eacute, author_in_Japanese. A Decade of Glycodendrimer Chemistry. *Trends in Glycoscience and Glycotechnology* 2003, **15**(85)**:** 291-310.

7. Roy R, Baek M-G. Glycodendrimers: novel glycotope isosteres unmasking sugar coding. Case study with T-antigen markers from breast cancer MUC1 glycoprotein. *Reviews in Molecular Biotechnology* 2002, **90**(3–4)**:** 291-309.

8. Chabre YM, Roy R. Chapter 6 - Design and Creativity in Synthesis of Multivalent Neoglycoconjugates. In: Derek H (ed). *Advances in Carbohydrate Chemistry and Biochemistry*, vol. Volume 63. Academic Press, 2010, pp 165-393.

9. Cecioni S, Imberty A, Vidal S. Glycomimetics versus Multivalent Glycoconjugates for the Design of High Affinity Lectin Ligands. *Chem Rev* 2015, **115**(1)**:** 525-561.

10. Hirsch A, Vostrowsky O. C60 Hexakisadducts with an Octahedral Addition Pattern − A New Structure Motif in Organic Chemistry. *Eur J Org Chem* 2001, **2001**(5)**:** 829-848.

11. Lamparth I, Maichle–Mössmer C, Hirsch A. Reversible Template-Directed Activation of Equatorial Double Bonds of the Fullerene Framework: Regioselective Direct Synthesis, Crystal Structure, and Aromatic Properties of Th-C66(COOEt)12. *Angew Chem Int Ed* 1995, **34**(15)**:** 1607-1609.

12. Hirsch A. Principles of Fullerene Reactivity. In: Hirsch A (ed). *Fullerenes and Related Structures*, vol. 199. Springer Berlin Heidelberg, 1999, pp 1-65.

13. Iehl J, Pereira de Freitas R, Delavaux-Nicot B, Nierengarten J-F. Click chemistry for the efficient preparation of functionalized [60]fullerene hexakis-adducts. *Chem Commun* 2008(21)**:** 2450-2452.

14. Nierengarten J-F, Iehl J, Oerthel V, Holler M, Illescas BM, Munoz A*, et al.* Fullerene sugar balls. *Chem Commun* 2010, **46**(22)**:** 3860-3862.

15. Sánchez-Navarro M, Muñoz A, Illescas BM, Rojo J, Martín N. [60]Fullerene as Multivalent Scaffold: Efficient Molecular Recognition of Globular Glycofullerenes by Concanavalin A. *Chem Eur J* 2011, **17**(3)**:** 766-769.

16. Rísquez-Cuadro R, García Fernández JM, Nierengarten J-F, Ortiz Mellet C. Fullerene-sp2-Iminosugar Balls as Multimodal Ligands for Lectins and Glycosidases: A Mechanistic Hypothesis for the Inhibitory Multivalent Effect. *Chem Eur J* 2013, **19**(49)**:** 16791-16803.

17. Cecioni S, Oerthel V, Iehl J, Holler M, Goyard D, Praly J-P*, et al.* Synthesis of Dodecavalent Fullerene-Based Glycoclusters and Evaluation of Their Binding Properties towards a Bacterial Lectin. *Chem Eur J* 2011, **17**(11)**:** 3252-3261.

18. Nierengarten I, Nierengarten J-F. Fullerene Sugar Balls: A New Class of Biologically Active Fullerene Derivatives. *Chemistry – An Asian Journal* 2014, **9**(6)**:** 1436-1444.

19. Durka M, Buffet K, Iehl J, Holler M, Nierengarten J-F, Taganna J*, et al.* The functional valency of dodecamannosylated fullerenes with Escherichia coli FimH-towards novel bacterial antiadhesives. *Chem Commun* 2011, **47**(4)**:** 1321-1323.

20. Luczkowiak J, Muñoz A, Sánchez-Navarro M, Ribeiro-Viana R, Ginieis A, Illescas BM*, et al.* Glycofullerenes Inhibit Viral Infection. *Biomacromolecules* 2013, **14**(2)**:** 431-437.

21. Hirsch et al. have also reported the synthesis of hexakis-adducts of hexakis-adducts of [60]fullerene with an octahedral addition pattern. However, as they employ macrocyclic bis-malonates, this procedure allows the synthesis of heptafullerenes with tunable properties (see ref. 21 and 22 below). In contrast, our click-chemistry approach yields tridecafullerenes in which the central [60]fullerene is covalently connected to twelve [60]fullerenes, each of them endowed, in turn, with ten monosaccharides.

22. Hörmann F, Hirsch A. Giant Fullerene Polyelectrolytes Composed of C60 Building Blocks with an Octahedral Addition Pattern and Discovery of a New Cyclopropanation Reaction Involving Dibromomalonates. *Chem Eur J* 2013, **19**(9)**:** 3188-3197.

23. Wasserthal LK, Kratzer A, Hirsch A. Sequential Fullerenylation of Bis-malonates – Efficient Access to Oligoclusters with Different Fullerene Building Blocks. *Eur J Org Chem* 2013, **2013**(12)**:** 2355-2361.

24. Balbinot D, Atalick S, Guldi DM, Hatzimarinaki M, Hirsch A, Jux N. Electrostatic Assemblies of Fullerene−Porphyrin Hybrids: Toward Long-Lived Charge Separation. *J Phys Chem B* 2003, **107**(48)**:** 13273-13279.

25. Wessendorf F, Gnichwitz J-F, Sarova GH, Hager K, Hartnagel U, Guldi DM*, et al.* Implementation of a Hamiliton-Receptor-Based Hydrogen-Bonding Motif toward a New Electron Donor−Acceptor Prototype: Electron versus Energy Transfer. *J Am Chem Soc* 2007, **129**(51)**:** 16057-16071.

26. Durka M, Buffet K, Iehl J, Holler M, Nierengarten J-F, Vincent SP. The Inhibition of Liposaccharide Heptosyltransferase WaaC with Multivalent Glycosylated Fullerenes: A New Mode of Glycosyltransferase Inhibition. *Chem Eur J* 2012, **18**(2)**:** 641-651.

27. Ciampi S, Böcking T, Kilian KA, James M, Harper JB, Gooding JJ. Functionalization of Acetylene-Terminated Monolayers on Si(100) Surfaces: A Click Chemistry Approach. *Langmuir* 2007, **23**(18)**:** 9320-9329.

28. Collman JP, Devaraj NK, Eberspacher TPA, Chidsey CED. Mixed Azide-Terminated Monolayers: A Platform for Modifying Electrode Surfaces. *Langmuir* 2006, **22**(6)**:** 2457-2464.

29. Devaraj NK, Decreau RA, Ebina W, Collman JP, Chidsey CED. Rate of Interfacial Electron Transfer through the 1,2,3-Triazole Linkage. *J Phys Chem B* 2006, **110**(32)**:** 15955-15962.

30. Alvarez CP, Lasala F, Carrillo J, Muniz O, Corbi AL, Delgado R. C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. *J Virol* 2002, **76**(13)**:** 6841-6844.

31. Kondratowicz AS, Lennemann NJ, Sinn PL, Davey RA, Hunt CL, Moller-Tank S*, et al.* T-cell immunoglobulin and mucin domain 1 (TIM-1) is a receptor for Zaire Ebolavirus and Lake Victoria Marburgvirus. *Proceedings of the National Academy of Sciences* 2011, **108**(20)**:** 8426-8431.

32. Carette JE, Raaben M, Wong AC, Herbert AS, Obernosterer G, Mulherkar N*, et al.* Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature* 2011, **477**(7364)**:** 340-343.

33. Ribeiro-Viana R, Sánchez-Navarro M, Luczkowiak J, Koeppe JR, Delgado R, Rojo J*, et al.* Virus-like glycodendrinanoparticles displaying quasi-equivalent nested polyvalency upon glycoprotein platforms potently block viral infection. *Nat Commun* 2012, **3:** 1303.

34. Yang SL, Delgado R, King SR, Woffendin C, Barker CS, Yang ZY*, et al.* Generation of retroviral vector for clinical studies using transient transfection. *Hum Gene Ther* 1999, **10**(1)**:** 123-132.

35. Connor RI, Chen BK, Choe S, Landau NR. Vpr is required for efficient replication of Human-Immuno-Deficiency-Virus Type-1 in mononuclear phagocytes. *Virology* 1995, **206**(2)**:** 935-944.

36. Luczkowiak J, Sattin S, Sutkeviciute I, Reina JJ, Sanchez-Navarro M, Thepaut M*, et al.* Pseudosaccharide Functionalized Dendrimers as Potent Inhibitors of DC-SIGN Dependent Ebola Pseudotyped Viral Infection. *Bioconjugate chemistry* 2011, **22**(7)**:** 1354-1365.

37. Lasala F, Arce E, Otero JR, Rojo J, Delgado R. Mannosyl glycodendritic structure inhibits DC-SIGN-mediated Ebola virus infection in cis and in trans. *Antimicrob Agents Chemother* 2003, **47**(12)**:** 3970-3972.