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

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## Abstract

The use of multivalent carbohydrate compounds to block cell-surface lectin receptors is a promising strategy for inhibiting the entry of pathogens into cells and could lead to the discovery of novel antiviral agents. One of the main problems with this approach, however, is the difficulty in making compounds with adequate size and multivalency to mimic natural systems such as viruses. Hexakis-adducts of [60]fullerene are a useful building block in this regard because they maintain a globular shape while allowing control over size and multivalency. Here we report water-soluble tridecafullerenes decorated with 120 peripheral carbohydrate subunits, so-called 'superballs', that can be efficiently synthesized from hexakis-adducts of [60]fullerene in one step by using copper-catalyzed azide–alkyne cycloaddition (CuAAC) click-chemistry. Infection assays show that these 'superballs' are potent inhibitors of cell infection by an artificial Ebola virus with  $IC_{50}$ s in the sub-nanomolar range.

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 monovalent binding.<sup>1</sup>

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.<sup>2</sup> 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.<sup>3</sup>

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, inhibiting the entry of pathogens 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.<sup>4</sup> 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 in which the carbohydrate units are directly connected to the core, glyco-dendrimers connected through a dendritic structure, and glyco-polymers involving a polymeric backbone have been intensively investigated in recent years.<sup>5-9</sup>

Fullerenes have also 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-type addends.<sup>10,11</sup> Such derivatives can be obtained in one synthetic step by the addition of malonates to  $C_{60}$ , but this approach has been limited by the low yields that result when larger malonates are used because the reaction is very sensitive to steric effects.<sup>12</sup> 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.<sup>13,14</sup> 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.

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.<sup>14-17</sup>

Some of the aforementioned glycofullerenes have shown interesting bio-medical applications.<sup>18</sup> 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.<sup>16</sup> On the other hand, fullerene hexakis-adducts bearing 12 mannoses on the periphery behave as inhibitors of FimH, a bacterial adhesin.<sup>19</sup> 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,<sup>15</sup> and act as efficient inhibitors of cell infection by Ebola pseudotyped viral particles.<sup>20</sup> 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 molecules with a globular topology formed by an 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. This previous example used macrocyclic bis-malonates and enabled the synthesis of heptafullerenes with tunable properties.<sup>21-23</sup> 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 carried out a thorough structural study involving a variety of techniques (Infrared,  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectroscopy; dynamic light scattering (DLS), transmission electron microscopy (TEM) and X-ray photoelectron spectroscopy (XPS)). Biological studies reveal that the new giant glycofullerenes exhibit a very strong inhibition of cell infection by Ebola pseudotyped viral particles with  $\text{IC}_{50}$  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 because after column filtration these compounds (**17a,b**) are precipitated in the reaction medium and pure samples showing highly reproducible biological assays 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 inbetween the central fullerene core and the peripheral carbohydrate-substituted fullerene appendages. In both cases, the synthesis relies on the grafting of clickable  $\text{A}_{10}\text{B}$  macromonomers onto a compact  $\text{C}_{60}$  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  $\text{C}_{60}$  under Bingel conditions gave methanofullerene **3**. Subsequent treatment of **3** with an excess of malonate **4**,  $\text{CBr}_4$ , and DBU in *o*-dichlorobenzene (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.

The synthesis of macromonomers **15a-b** starts from monoadduct **10** resulting from the Bingel nucleophilic cyclopropanation of  $\text{C}_{60}$  with 6-bromohexyl ethyl malonate (Figure 2).<sup>24</sup> 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<sub>60</sub> by the CuAAC reaction, employing CuBr·S(CH<sub>3</sub>)<sub>2</sub> 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**.

The azide-containing macromonomers **9** and **14a-b** were then clicked to symmetric alkyne derivative **16**<sup>14</sup> under CuAAC conditions. Superballs **17a-c** substituted with up to 120 monosaccharide units were thus obtained with yields over 70% (Figure 3). Derivatives **17a-b** were 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 favour the accessibility and availability of the carbohydrate ligands to interact with the receptor, which could have an important influence on the biological activity.<sup>20</sup>

It is important to note, however, that the aforementioned synthetic approaches enables fullerene derivatives to be synthesized from malonates endowed with alkyl chains of variable length through a Bingel cyclopropanation followed by 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 low yields.<sup>10</sup>

Characterization of these superballs was carried out by standard spectroscopic techniques. Thus, FTIR spectra do not show the presence of either azide groups (typical signal observed at ~2097 cm<sup>-1</sup>) or alkynes (~ 2117 cm<sup>-1</sup>) (See the Supplementary Information). The molecular ion peak of these compounds could not be detected but it should be noted that the transfer of such high molecular weight glycoclusters into the gas phase during MALDI-TOF MS analysis is very difficult. Moreover, both the sugars and the fullerene hexaadduct moieties give rise to a high level of fragmentation.<sup>25</sup> The unambiguous structural characterization of **17a-c** was, however, greatly facilitated by their high symmetry. Indeed, <sup>13</sup>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<sup>2</sup> carbons of C<sub>60</sub>, providing evidence for the octahedral symmetry of the fullerene core. As a typical example, the <sup>13</sup>C NMR spectrum of compound **17a** is depicted in Figure 4. In this case, only two sp<sup>2</sup> carbons are observed in the spectra (δ ~ 145.4

and 141.6 ppm), where we can also distinguish the two different kinds of triazole rings present in the compound (at  $\delta \sim 146.9$  and 123.3 ppm for the carbons of the outer triazole rings and at  $\delta \sim 146.4$  and 122.9 ppm for the carbons of the inner triazole rings). In addition, only one signal is detected for all the carbonyl groups ( $\delta \sim 163.8$  ppm) and the  $sp^3$  carbons of the  $C_{60}$  ( $\delta \sim 69.3$  ppm), while the malonate bridgehead carbons present in the structure are observed at  $\delta \sim 45.2$  ppm.

Additional characterization was achieved by DLS analysis ( $H_2O$ , 0.01 mg/mL and 0.1 mg/mL), where, regardless the concentration used, we found two or three main size distributions for **17a-c** (Supplementary Figure 1). This is compatible with a weak aggregation of **17a-c** in water. The first, around 5-6 nm, must correspond to only one molecule, while the second, at  $\sim 120$ -150 nm and third,  $\geq 200$  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 ( $\sim 4$  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 Supplementary Figure 3).

X-ray photoelectron spectroscopy (XPS) was additionally used to confirm the composition of the sugar balls. This surface technique enables 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).<sup>26</sup> 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.<sup>27,28</sup> 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 the Ebola virus<sup>29-31</sup>. 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,<sup>29,32</sup> thus facilitating early viral dissemination. Therefore, DC-SIGN can function as a good model for (i) studying the first steps of pathogenesis of Ebola virus and (ii) 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 cells expressing the surface receptor DC-SIGN (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 interval (CI). As a control, infection with DC-SIGN-independent VSV glycoprotein-pseudotyped lentiviral particles<sup>29</sup> 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 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 an IC<sub>50</sub> of 20.37 nM (95%CI = 14.63 – 28.37 nM). Compound **17c** was

almost one order of magnitude more potent at inhibiting the infection process with an  $IC_{50}$  of 667 pM (95%CI = 411 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.<sup>19</sup> Moreover, huge virus-like particles (VLP) with a radius of 16 nm and up to 1640 mannoses<sup>32</sup> were 18-fold less potent than compound **17c** described in this work (see Table 1). 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 supplementary Figure 6).

## Conclusions

In summary, we have synthesized 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. Each peripheral fullerene is endowed with ten monosaccharides and so a total of 120 carbohydrates decorate the periphery of each molecule. This 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 tridecafullerenes (**17a-c**) have been accomplished efficiently by using CuAAC click-chemistry reactions. In this way, very sophisticated molecular ensembles have been 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,  $^1H$  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. These tridecafullerenes have been found to efficiently block Ebola virus infection in the range of sub-nanomolar concentrations. These values surpass by three orders of magnitude (two if the number of mannoses is considered) those exhibited by hexakis-adducts endowed with 12 mannoses.

The aforementioned results reveal fullerenes to be appealing platforms for the study of multivalent interactions particularly because of their biocompatibility and globular presentation. Furthermore, the compounds reported here 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.<sup>29,33</sup> One day (18-24 h) before transfection,  $5 \times 10^6$  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 25 $\mu$ M final concentration. Transfection reaction with all reagents at room temperature (RT) was prepared in 15 ml tubes: 183  $\mu$ l of 2M CaCl<sub>2</sub>, 500 ng of EBOV-GP or 2  $\mu$ g of VSV-G, 21  $\mu$ g of pNL4-3 luc<sup>34</sup>, 1300  $\mu$ l of H<sub>2</sub>O. 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 37 $^{\circ}$  C with 5% CO<sub>2</sub>, 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 $^{\circ}$  C.<sup>32,33,35</sup>

### **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.<sup>29,36</sup>

Jurkat-DC-SIGN cells ( $2.5 \times 10^5$ ) 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 Luciferase Assay System (Promega, Madison, WI).

The range of concentrations tested for compounds **17a-c** was 1 pM – 10  $\mu$ M. 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<sub>50</sub> 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 ( $5 \times 10^5$  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  $\mu$ l of PMS solution. The mix MTS/PMS in a volume of 20  $\mu$ l was pipetted into each well of the 96-well assay plate containing 100  $\mu$ l of cells in culture medium. The plate was incubated for 2 h at 37<sup>o</sup> C in a humidified 5% CO<sub>2</sub> 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.

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#### 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 to 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.

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## Figure Captions

**Figure 1. Synthesis of azide substituted glycofullerene 9 appended with a long linker and mannose as the carbohydrate unit.** The figure describes the 5 linear steps required to prepare compound 9 in a 26% overall yield. Reagents and conditions: (i) ethylmalonyl chloride, pyridine, CH<sub>2</sub>Cl<sub>2</sub>, 0 to 25°C, 1 h (99%); (ii) C<sub>60</sub>, I<sub>2</sub>, 1,8-Diazabicycloundec-7-ene (DBU), 25 °C, PhMe, 16 h (49%); (iii) CBr<sub>4</sub>, DBU, 25 °C, *o*-dichlorobenzene (ODCB), 72 h (67%); (iv) CuSO<sub>4</sub>·5H<sub>2</sub>O, sodium ascorbate, THF/H<sub>2</sub>O, 100°C (MW), 2 h (91%); (v) **8** (19 equiv.), CuSO<sub>4</sub>·5H<sub>2</sub>O, sodium ascorbate, Tetrabutylammonium fluoride (TBAF), Tetrahydrofurane/H<sub>2</sub>O, 80°C (Microwave), 1.5 h (87%).

**Figure 2. Synthetic pathway to azide substituted glycofullerenes 15a-b with a short spacer. Mannose and galactose are employed as carbohydrates.** The peripheral glycofullerene units 15a-b have been prepared as described in the figure using the Bingel conditions and the CuAAC click chemistry to conjugate the carbohydrate moieties. Reagents and conditions: (i) di(pent-4-yn-1-yl) malonate, CBr<sub>4</sub>, 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<sub>3</sub>)<sub>2</sub>, sodium ascorbate, Cu<sup>0</sup>, dimethylsulfoxide (DMSO), 72 h (**14a**: 86%; **14b**: 86%); (iii) NaN<sub>3</sub>, 70 °C (MW), DMSO, 3 h (**15a**: 84%; **15b**: 81%).

**Figure 3. Synthesis of the tridecafullerenes 17a-c using the copper-catalyzed azide–alkyne cycloaddition (CuAAC) click-chemistry methodology.** The figure describes the conjugation of the core fullerene 16 endowed with 12 alkyne groups and the peripheral fullerenes 9 and 15a-b by click chemistry. Reagents and conditions: For compounds **17a-b**: (i) **15a-b**, CuBr·S(CH<sub>3</sub>)<sub>2</sub>, sodium ascorbate, Cu<sup>0</sup>, DMSO, 25°C, 48 h [**17a** (from **15a**): 73%; **17b** (from **15b**): 79%]. For compound **17c**: (i) **9**, CuSO<sub>4</sub>·5H<sub>2</sub>O, sodium ascorbate, THF/H<sub>2</sub>O, 80°C (MW), 2 h (76%).

**Figure 4. <sup>13</sup>C NMR spectrum of tridecafullerene 17a in DMSO-*d*<sub>6</sub>.** Assignment of the most representative signals is depicted. This spectrum is in full agreement with its *T*-symmetrical structure and allows to confirm the complete functionalization of the alkyne moieties in precursor **16**, as the signals corresponding to the sp carbons are not present anymore.

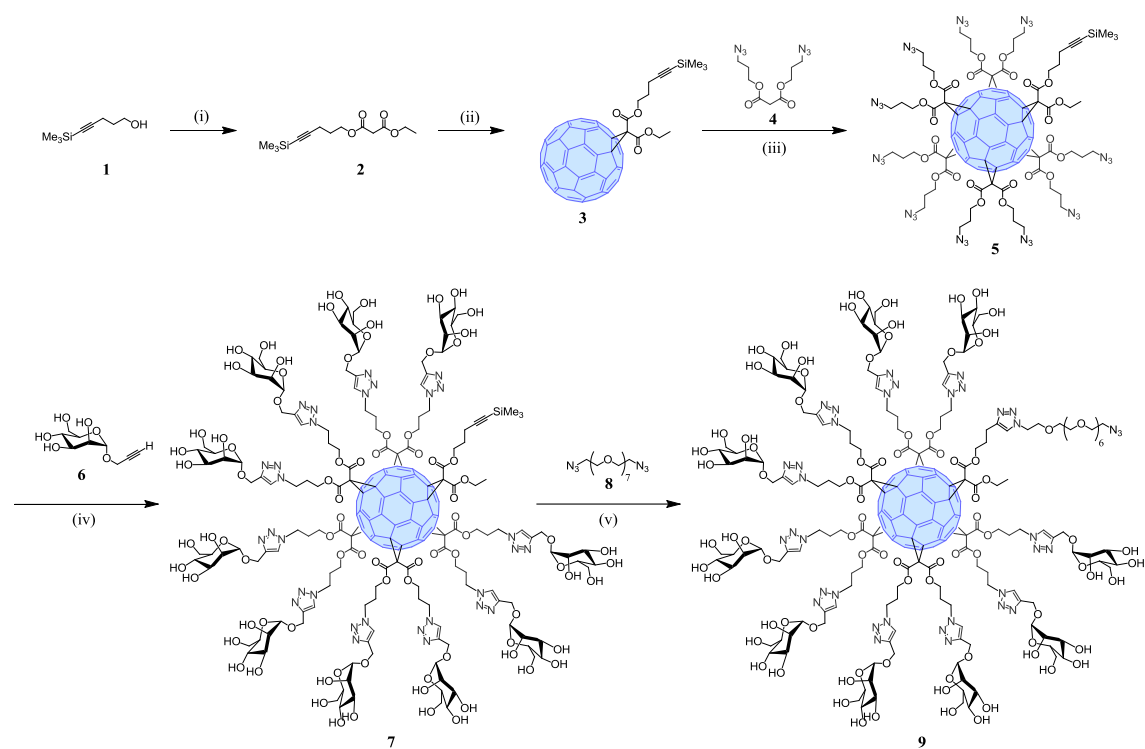


**Figure 5. TEM images of tridecafullerene 17a.** These images show small spherical particles with a diameter of around 4 nm corresponding to a single molecule. a) TEM images of compound **17a** upon deposition of a 0.01 mg/mL solution in H<sub>2</sub>O. b) Detail of a particle corresponding apparently to one molecule. c) Width profile of the particle shown in b) which has a diameter of ~ 4 nm in accordance with the DLS data.

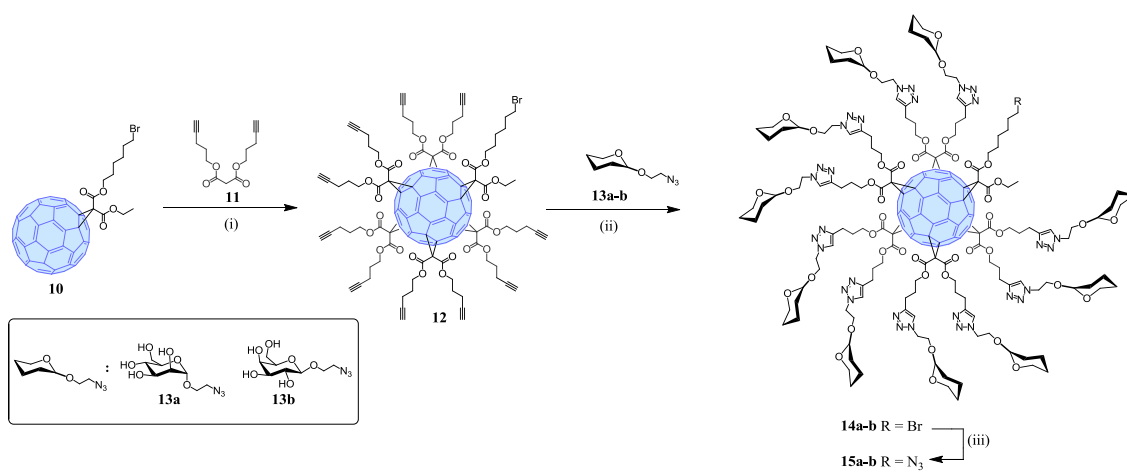
**Figure 6. Biological study of tridecafullerenes (17a-c).** The graphic shows the inhibition of infection with EBOV or VSV GP-pseudotyped lentiviral particles of Jurkat DC-SIGN<sup>+</sup> cells using **17a** (blue), **17b** (green) and **17c** (red). In the cis-infection experiments 2.5x10<sup>5</sup> Jurkat DC-SIGN<sup>+</sup> were challenged with 5000 Tissue Culture Infective Dose (TCID) of recombinant lentiviral particles. Results represent the mean of 6 independent experiments +/- SEM. Compounds 17a and 17c show a strong inhibitory activity when EBOV GP pseudotyped lentiviral particles are used. No inhibitory activity is detected when VSV GP pseudotyped particles (control) are used as infective agent. Compound 17b endowed with galactoses as carbohydrate units does not show inhibitory activity because is not able to block DC-SIGN receptor.

**Table 1.** Comparative of IC<sub>50</sub> values and relative inhibitory potency of different mannosylated multivalent compounds in inhibition studies using pseudotyped Ebola virus particles. The table shows the data for the new compounds 17a and 17c described in comparison with those carbohydrate multivalent systems previously reported by us.

**Figure 1.**



**Figure 2.**



**Figure 3.**

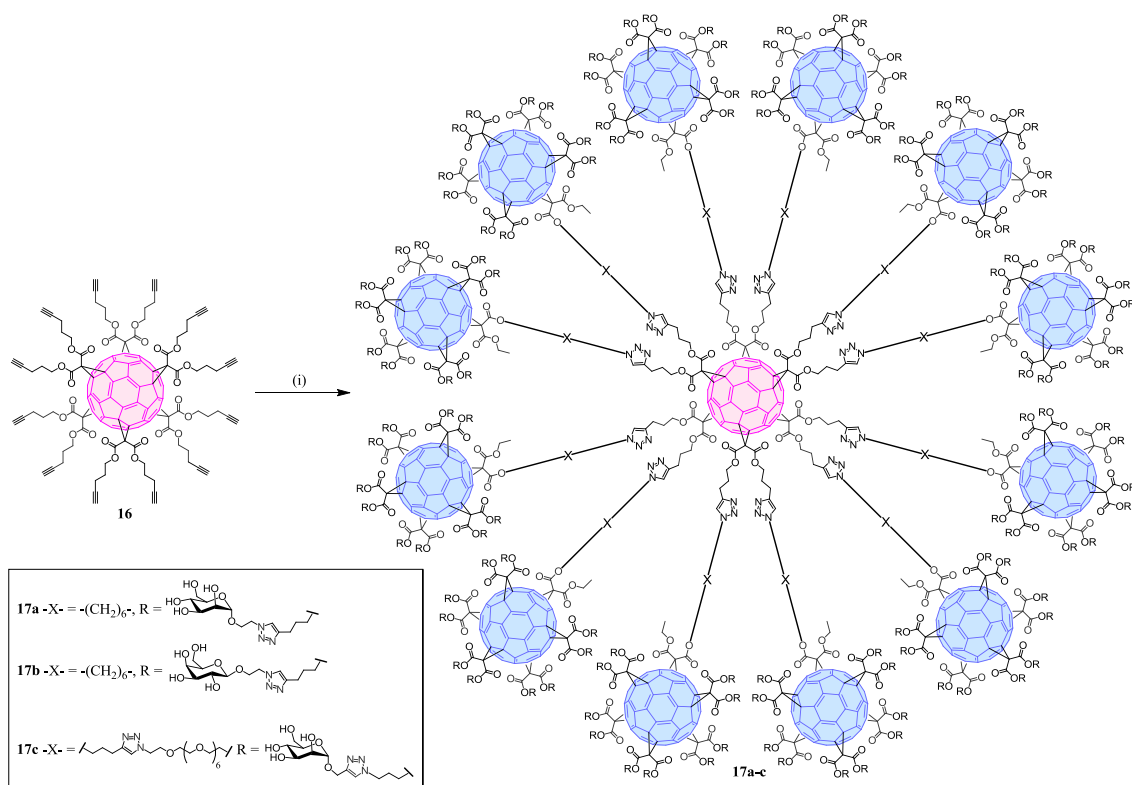


Figure 4.

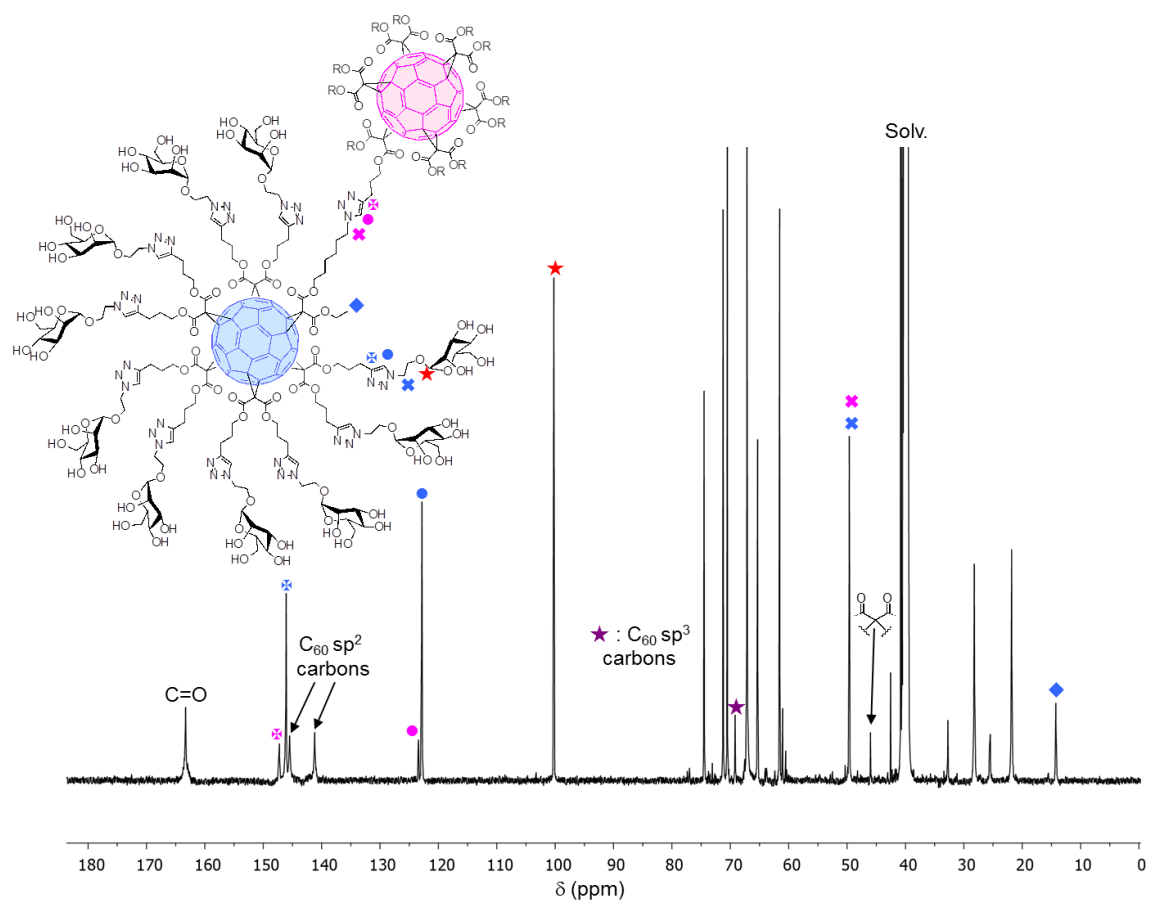


Figure 5.

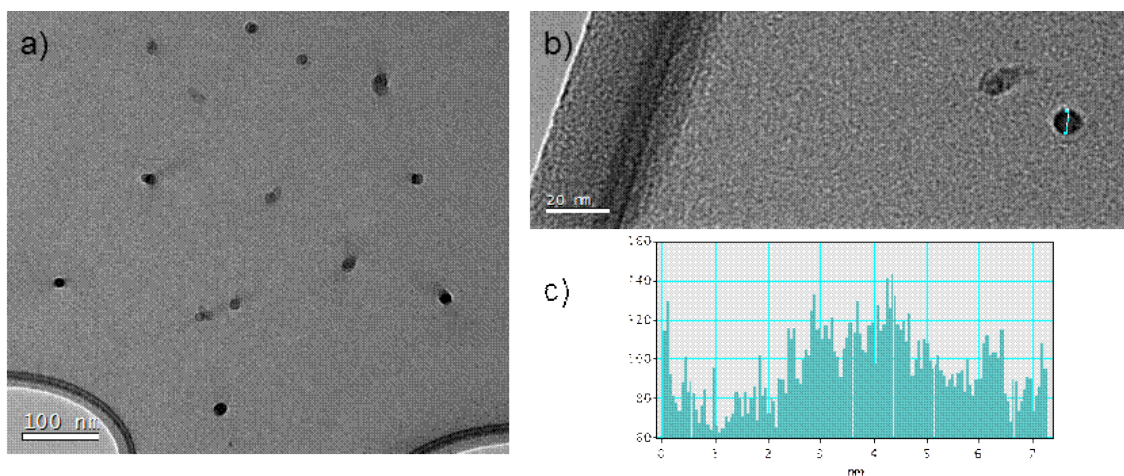


Figure 6.

