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UNIVERSITÀ DEGLI STUDI DI TORINO

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1 Auto-associative heparin nanoassemblies: a biomimetic platform
2 against the heparan-sulfate-dependent viruses HSV-1, HSV-2,
3 HPV-16 and RSV

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20

Abstract

21 A new, simple and green method was developed for the manufacturing of heparin nanoassemblies
22 active against the heparan-sulfate-dependent viruses HSV-1, HSV-2, HPV-16 and RSV. These
23 nanoassemblies were obtained by the auto-association of *O*-palmitoyl-heparin and α -cyclodextrin
24 in water. The synthesized *O*-palmitoyl-heparin derivatives mixed with α -cyclodextrin resulted in
25 the formation of crystalline hexagonal nanoassemblies as observed by transmission electron
26 microscopy. The nanoassembly mean hydrodynamic diameters were modulated from 340 to
27 659 nm depending on the type and the initial concentration of *O*-palmitoyl-heparin or α -
28 cyclodextrin. The antiviral activity of the nanoassemblies was not affected by the concentration of
29 the components. However, the method of the synthesis of *O*-palmitoyl-heparin affected the
30 antiviral activity of the formulations. We showed that reduced antiviral activity is correlated to
31 lower sulfation degree and anticoagulant activity.

32 **Keywords:** Nanoassemblies, α -cyclodextrin, glycosaminoglycan, HSV, HPV, heparin.

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1. Introduction

35 The first step in the infection of mucosal surfaces by viruses involves their attachment to cellular
36 receptors exposed on the surface of epithelial cells. In many instances, virus–cell interaction is
37 mediated by cell surface heparan sulfate proteoglycans (HSPGs) [1,2]. These negatively charged
38 molecules are a core protein linked to glycosaminoglycan (GAG) chains of unbranched sulfated
39 polysaccharides known as heparan sulfates (HS). HS are structurally related to heparin except that
40 heparin has higher level of sulfation and higher content of iduronic acid [3,4].

41 The interaction between the viruses and HSPGs occurs between the basic amino acid residues of
42 viral proteins and the negatively charged sulfated/carboxyl groups of the GAG chains. For this
43 reason heparin and other GAGs can competitively interfere with virus attachment to cells. Many
44 viruses exploit HSPGs as attachment receptors, namely the herpes simplex virus type 1 and type 2
45 (HSV-1 and HSV-2), the human papilloma virus (HPV) and respiratory syncytial virus (RSV) [5-
46 7].

47 So far, GAGs have been explored as potential candidate in the prevention of viral infections [8,9].
48 Accumulated data from the literature indicates that the inhibitory effect of heparin and HS was
49 demonstrated on HSV by acting on its earliest phase [10,11], while the binding of HPV-like
50 particles to cells has been shown to be inhibited by heparin [12]. HS has proved to play an
51 important role in the prevention of HPV infections [13].

52 Surprisingly, although numerous research works were already described in the literature on the
53 activity of GAGs against viral mucosal infections, there is a clear gap concerning the design of
54 efficient locally-administrated formulations. Besides the prevention of the infection, the
55 formulation of GAGs as a drug delivery system able to target the viruses, to load antiviral drugs

56 and to control their release over time represents an interesting strategy against viral mucosal
57 infections. However, although the large number of publications in the field of drug delivery
58 systems, current nanotechnologies have, unfortunately, important limitations due to the
59 complexity of the processes used to their manufacturing and thus the difficulty for the scaling-up
60 of their production to pharmaceutical companies. Furthermore, manufacture processes require the
61 use of toxic solvents (acetone and ethanol for flash nanoprecipitation), surfactants, polymerization
62 initiators and extremely reactive monomers (anionic and radical emulsion polymerization).
63 Expensive techniques must be employed to completely remove the solvents and the surfactants at
64 the end of the preparation process. Solvent and surfactant traces may persist and constitute a
65 drawback for the medical applications of these systems. While monomers present in the
66 polymerization medium could interact with the drug leading to its instability and the formation of
67 toxic products.

68 In this context, Bouchemal's group has designed an innovative drug delivery system composed of
69 nanoassemblies spontaneously formed in aqueous medium without using surfactants, pH
70 modification and without heating or purification steps [14]. In this process, nanoassemblies were
71 obtained by mixing a hydrophobically-modified polysaccharide and an α -cyclodextrin (α -CD).

72 The aim of the present work is to use this process to obtain new GAG-based nanoassemblies and
73 to evaluate their ability to inhibit viral attachment to cells. We took advantage of structural
74 similarities between heparin and cell surface HS and evaluated the antiviral activity of heparin-
75 based formulations. The preparation of heparin nanoassemblies was achieved by the self-
76 association in aqueous media of *O*-palmitoyl-heparin (OPH) and α -CD. This biomimetic barrier
77 could act like a "trap" able to specifically catch up the viruses and avoid their attachment to the
78 cells. The effect of the chemical modification of OPH on the antiviral activity was evaluated

79 against HSV-1, HSV-2, RSV and the high-risk type of HPV (HPV-16) involved in cervical
80 cancers. Rotavirus, a HSPG-independent virus was used as control.

81 2. Materials

82 Heparin sodium salt from porcine intestinal mucosa 500 kU, palmitoyl chloride, anhydrous
83 pyridine, sodium chloride and sodium acetate were from Sigma (Saint-Quentin Fallavier, France).
84 Acetone was from Carlo Erba (Val de Reuil, France). α -CD was from Cyclolab (Budapest,
85 Hungary). Anhydrous dimethylformamide (DMF), anhydrous dichloromethane (DCM),
86 diethylether, ethanol, methanol were from VWR (Fontenay sous-bois, France).

87 **Cells.** African green monkey fibroblastoid kidney cells (Vero) (ATCC CCL-81) , human epithelial
88 cells Hep-2 (ATCC CCL-23), A549 (ATCC CCL-185) and african green monkey kidney epithelial
89 (MA-104) cells (ATCC CRL-2378.1) were grown as monolayers in Eagle's minimal essential
90 medium (MEM) (Gibco/BRL, Gaithersburg, MD) supplemented with 10 % heat inactivated fetal
91 calf serum and 1 % antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin,
92 Germany). The 293TT cell line, derived from human embryonic kidney cells transformed with the
93 simian virus 40 (SV40) large T antigen, was cultured in Dulbecco's modified Eagle's medium
94 (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with heat-inactivated 10 % fetal calf
95 serum (FCS; Gibco- BRL), Glutamax-I 1 % (Invitrogen, Carlsbad, CA) and nonessential amino
96 acids 1 % (Sigma Aldrich, Steinheim, Germany). 293TT cells allow high levels of protein to be
97 expressed from vectors containing the SV40 origin due to over replication of the expression
98 plasmid [15].

99 **Viruses.** Clinical isolates of HSV-1 and HSV-2 were kindly provided by Prof. M. Pistello,
100 University of Pisa, Italy. HSV-1 and HSV-2 strains were propagated and titrated by plaque assay
101 on Vero cells. RSV strain A2 (ATCC VR-1540) was propagated in Hep-2 and titrated by the

102 indirect immunoperoxidase staining procedure using an RSV monoclonal antibody (Ab35958;
103 Abcam, Cambridge, United Kingdom) as described previously [16]. Human rotavirus strain Wa
104 (ATCC VR-2018) was activated with 5 µg/mL of porcine pancreatic trypsin type IX (Sigma, St.
105 Louis, Mo.) for 30 minutes at 37 °C and propagated in MA104 cells by using MEM containing
106 0.5 µg of trypsin per mL as described previously [17]. Virus stocks were maintained frozen (-
107 80 °C).

108 **HPV PsV production.** Plasmids and 293TT cells used for pseudovirus (PsV) production were
109 kindly provided by John Schiller (National Cancer Institute, Bethesda, MD). Detailed protocols
110 and plasmid maps for this study can be seen at <http://home.ccr.cancer.gov/lco/default.asp>. HPV16
111 PsVs were produced according to previously described methods [18]. Briefly, 293TT cells were
112 transfected with a plasmid, named p16LLw, expressing the papillomavirus major and minor
113 capsid proteins (L1 and L2, respectively), together with a reporter plasmid expressing the secreted
114 alkaline phosphatase (SEAP), named pYSEAP. Capsids were allowed to mature overnight in cell
115 lysate; the clarified supernatant was then loaded on top of a density gradient of 27 to 33 to 39 %
116 Optiprep (Sigma-Aldrich, St. Louis, MO) at room temperature for 3 h. The material was
117 centrifuged at 28000 rpm for 16 h at 4 °C in an SW41.1 rotor (Beckman Coulter, Inc., Fullerton,
118 CA) and then collected by bottom puncture of the tubes. Fractions were inspected for purity in
119 10% sodium dodecyl sulfate (SDS)–Tris–glycine gels, titrated on 293TT cells to test for
120 infectivity by SEAP detection, and then pooled and frozen at -80 °C until needed. The L1 protein
121 content of PsV stocks was determined by comparison with bovine serum albumin standards in
122 Coomassie-stained SDS-polyacrylamide gels.

123

124

3. Methods

125 3.1. Preparation of *O*-palmitoyl heparin. Two methods for the esterification of
126 heparin were used:

127 *Method 1. Synthesis of OPH-1:* Heparin (1 g) was suspended into 11 mL of anhydrous DCM and
128 heated at 60 °C under magnetic stirring. Then, anhydrous pyridine (5 mL) was added followed by
129 palmitoyl chloride (2.5 g) dissolved in 6 mL anhydrous DMF under continuous magnetic stirring
130 at 60 °C during 2 h and 1 h at room temperature. Then, 100 mL of cold ethanol (at 4 °C) was
131 added. The precipitate was collected and washed with 100 mL of ethanol, then with 100 mL of
132 diethylether using a Buchner filter. The solid materials were dried under vacuum at room
133 temperature.

134 *Method 2. Synthesis of OPH-2:* Heparin (2 g) was added to 10 mL of anhydrous DCM and
135 palmitoyl chloride (2.5 g) under continuous magnetic stirring at room temperature during 72 h.
136 Then, 20 mL of a solution of 10 % of sodium acetate in methanol was added. The precipitate was
137 collected and washed with 100 mL of methanol then with 100 mL of acetone using a Buchner
138 filter. The solid materials were dried under vacuum at room temperature. The ester was then
139 purified by dissolution in 10 mL of water and progressive addition of NaCl until the concentration
140 reaches 10 %. After the addition of 20 mL of methanol, the precipitate formed was collected and
141 washed with methanol and acetone and dried under vacuum at room temperature.

142 3.2. Chemical characterization of *O*-palmitoyl heparin. The synthesized
143 heparins were then characterized by using Attenuated total reflectance-Fourier transform infrared
144 (ATR-FTIR) spectroscopy. Infrared spectra were obtained with an ATR-IR spectrometer (FT/IR-

145 4100, JASCO) operating at 4 cm⁻¹ resolution. Fifty scans were accumulated in each run and
146 referred to air. The ATR sampling device utilized a diamond internal reflection element embedded
147 into a ZnSe support/focusing element in a single reflection configuration. The resultant spectra
148 over the range of 4000–400 cm⁻¹ was analyzed using the IR Protein Secondary Structure Analysis
149 program (JASCO Co).

150 The total amount of carbon and sulfur in esterified heparins and native heparin was determined by
151 elemental analysis using an Analyzer LECO SC144 (Service central d'analyse du CNRS,
152 Vernaison, France). Samples of 20 mg were burned at 1350 °C over oxygen flux and the detection
153 of SO₂ was performed by infrared measurements.

154 The degree of substitution of each derivative was evaluated from the determination of the
155 percentage of carbon in comparison with native heparin.

$$156 \quad \mathbf{DS} = \left(\frac{\mathbf{C\%}}{6} \right)_{\mathbf{OPH}} - \left(\frac{\mathbf{C\%}}{6} \right)_{\mathbf{Heparin}} \quad \text{Eq.1}$$

157 2.3. Preparation and physico-chemical characterization of nanoassemblies

158 *Preparation of the nanoassemblies.* Nanoassembly suspensions were prepared by mixing a
159 suspension of OPH and a solution of α-CD at room temperature under magnetic stirring during
160 72 h. The effect of the variation of each component on the size of the nanoassemblies was then
161 studied.

162 *Size measurements.* The hydrodynamic diameter of the nanoassemblies was determined at 25 °C
163 by quasi-elastic light scattering using a Zetasizer Nanoseries Nano-ZS (Malvern Instruments,
164 France). The scattered angle was fixed at 173° and 30 μL of each sample was diluted in 1 mL of
165 MilliQ[®] water.

166 *Zeta potential determination.*

167 Zeta potential of nanoassemblies was measured using Zetasizer Nanoseries (Malvern Instruments
168 Ltd. UK). The dilution of the suspensions (1:33 (v/v)) was performed in NaCl (1 mM). Each
169 experiment was replicated three times.

170 *Transmission Electron Microscopy.* The TEM images were obtained using the transmission
171 electron microscope of 60 kV Jeol 1400 (Imagif, Gif sur Yvette, France). For this, 1 μ L of the
172 nanoassembly suspension was diluted in 29 μ L of MilliQ[®] water. Then, 3 μ L of this dilution are
173 placed on a grid. After 5 minutes of drying, the grid is inserted into the microscope to view the
174 sample.

175 **2.4. Anticoagulant activity evaluation.** The anticoagulant activity of the
176 nanoassemblies was in vitro evaluated by the measurement of anti-Xa activity with the Sta-
177 Rotachrom Heparin assay (Diagnostica-Stago) in a pool of normal human plasma provided by
178 Cryocheck company. Plasma will be supplemented with the nanoassemblies, and after 5 minutes,
179 anti-FXa activity was measured following the manufacturer's recommendations. The results were
180 compared to the native heparin [19].

181 **2.5. Cell viability assay.** Cell viability was measured by the MTS [3-(4,5-dimethylthiazol-
182 2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. Confluent cell
183 cultures seeded in 96-well plates were incubated with different concentrations of nanoassemblies
184 (calculated on the heparin content) in triplicate under the same experimental conditions described
185 for the antiviral assays. Cell viability was determined by the CellTiter 96 Proliferation Assay Kit
186 (Promega, Madison, WI,USA) according to the manufacturer's instructions. Absorbances were
187 measured using a Microplate Reader (Model 680, BIORAD) at 490 nm. The effect on cell
188 viability at different concentrations of nanoassemblies was expressed as a percentage, by

189 comparing absorbances of treated cells with the ones of cells incubated with culture medium
190 alone. The 50 % cytotoxic concentrations (CC_{50}) and 95 % confidence intervals (CIs) were
191 determined using Prism software (Graph-Pad Software, San Diego, CA).

192 2.6. In vitro antiviral assays

193 *HSV inhibition assays.* The effect of nanoassemblies on HSV infection was evaluated by a plaque
194 reduction assay. Vero cells were preplated 24 h in advance in 24-well plates at a density of $10 \times$
195 10^4 cells. Increasing concentrations of nanoassemblies (calculated on the heparin content) were
196 incubated with HSV-1 or HSV-2 (MOI 0.0003 pfu/cell) at 37 °C for one hour and then the
197 mixtures were added to cells. Following virus adsorption (2 h at 37 °C), the virus inoculum was
198 removed, the cells were washed and then overlaid with a medium containing 1.2 %
199 methylcellulose (Sigma). After 24 h (HSV-2) or 48 h (HSV-1) of incubation at 37 °C, cells were
200 fixed and stained with 0.1 % crystal violet in 20 % ethanol and viral plaques were counted. The
201 concentration producing 50 % reduction in plaque formation (IC_{50}) was determined using the
202 Prism software by comparing drug-treated and untreated wells.

203 *HPV inhibition assays.* 293TT cells were preplated 24 h in advance in 96-well tissue culture-
204 treated flat bottom plates at a density of 20000 cells/well in 100 μ L of neutralization buffer
205 (DMEM without phenol red, 10 % heat-inactivated fetal bovine serum, 1 % glutamate, 1 %
206 nonessential aminoacids, 1 % penicillin-streptomycin-fungizone, and 10 mM HEPES). Diluted
207 PsV stocks (80 μ L/well) were placed on 96-well non treated sterile, polystyrene plates (Nalge-
208 Nunc, Roskilde, Denmark), combined with 20 μ L of serially diluted nanoassemblies, and placed
209 for 1 h at 37°C. The 100- μ L PsV-compound mixture was transferred onto the preplated cells and
210 incubated for 72 h. The final concentration of PsV was approximately 1 ng/mL L1. After
211 incubation, 25 μ L of supernatant was harvested. The SEAP content in the supernatant was

212 determined using a Great Escape SEAP Chemiluminescence Kit (BD Clon- tech, Mountain View,
213 CA) as directed by the manufacturer.

214 *RSV inhibition assay.* Nanoassemblies were serially diluted and incubated with virus (MOI 0.01)
215 for one hour at 37 °C. Then the mixture was added to A549 cells grown as monolayers in a 96-
216 well plate to allow the viral adsorption for 3 h at room temperature; the monolayers were then
217 washed and overlaid with 1.2 % methylcellulose medium. Three days post-infection, cells were
218 fixed with cold methanol and acetone for 1 min and subjected to RSV-specific immunostaining.
219 Immunostained plaques were counted, and the percent inhibition of virus infectivity was
220 determined by comparing the number of plaques in treated wells with the number in untreated
221 control wells.

222 *Rotavirus inhibition assay.* Assays of inhibition of rotavirus infectivity were carried out with
223 confluent MA104 cell monolayers plated in 96-well trays. Virus infectivity was activated with
224 5 µg of porcine trypsin (Sigma)/mL for 30 minutes at 37 °C. Activated virus (MOI 0.02 pfu/cell)
225 was incubated for one hour at 37 °C in presence of different concentrations of nanoassemblies.
226 The virus-nanoassembly mixtures were added on cells for one hour at 37 °C and then the cells
227 were washed and fresh medium was added. After 16 h, cells were fixed with cold acetone-
228 methanol (50:50), and viral titers were determined by indirect immunostaining by using the
229 monoclonal antibody mab0036 (specific for human 41 kDa inner capsid protein - VP6 - of
230 Rotavirus) purchased from Covalab (Villeurbanne, France) and the UltraTech HRP Streptavidin-
231 Biotin Detection System (Beckman Coulter).

232 **Data analysis.** All results are presented as the mean values from three independent experiments.
233 The IC50 values for inhibition curves were calculated by regression analysis using the program
234 GraphPad Prism version 4.0 (GraphPad Software, San Diego, California, U.S.A.) to fit a variable

235 slope-sigmoidal dose–response curve. A selectivity index (SI) was calculated by dividing the CC_{50}
236 by the IC_{50} value.

237 3. Results and discussion

238 The heparan-sulfate dependent viruses HSV-1, HSV-2, HPV-16 and RSV interact with HSPGs
239 receptors on the epithelial cells facilitating thus their initial attachment and subsequent cellular
240 entry and infection. In the present work a new approach was proposed to design locally-
241 administrated HS-mimetic formulations able to specifically target the viruses and avoid their
242 attachment to the mucosal surfaces. HS is distinguished from the closely related GAG heparin by
243 its lower degree of sulfation, higher degree of *N*-acetylation compared with the *N*-sulfation of
244 glucosamine residues, and the predominance of glucuronic acid rather than iduronic acid [20].
245 Taking advantage of the similarities between heparin and HS, heparin was used in this work as a
246 GAG model for investigating the ability of heparin nanoassemblies to inhibit HS-dependent
247 viruses. Heparin nanoassemblies were obtained by a new, simple and green method based on the
248 self-association of heparin grafted with palmitic acid residues and α -CD in water without using
249 surfactants, pH modification and without heating or purification steps.

250 *O*-palmitoyl-heparin was obtained using two different methods as indicated in Figure 1. ATR-IR
251 spectroscopy was used to reveal the grafting of palmitic acid on heparin (Figure 2). In comparison
252 with native heparin, the spectrum of OPH-1 showed two additional small peaks at 2929 cm^{-1} and
253 1733 cm^{-1} . The first band was attributed to the stretching vibrations of C-H bounds of $-\text{CH}_2-$ and
254 $-\text{CH}_3$ groups of palmitic acid grafted on OPH-1, while the second band was due to ester function.

255 The presence of alkyl chains of palmitic acid on heparin was better revealed in the infrared
256 spectrum of OPH-2 because higher degree of substitution obtained with this component as
257 indicated in Table 1. Indeed, additional bands around $2848\text{-}2957\text{ cm}^{-1}$ due to the stretching

258 vibrations of C-H bounds of $-\text{CH}_2-$ and $-\text{CH}_3$ groups were clearly observed. Infrared absorption
259 of OPH-2 showed three bands from 1636 cm^{-1} to 1793 cm^{-1} corresponding to carbonyl groups of
260 the ester function. This allowed to ascertain that palmitoyl moieties are covalently bound to
261 heparin. Whatever the synthesis method, a vibration peak around 1225 cm^{-1} was observed and was
262 attributed to the sulfate the S=O stretches.

263 The synthesized *O*-palmitoyl-heparin derivatives were then used to prepare heparin
264 nanoassemblies by mixing OPH-1 or OPH-2 with α -CD in water under moderate magnetic
265 stirring. The association of *O*-palmitoyl-heparin and α -CD results in the formation of well-
266 structured hexagonal-shaped nanoassemblies as showed by TEM observations (Figure 3). This
267 kind of hexagonal shape has never been reported in the literature yet for heparin particles. Usually,
268 spherical morphologies were obtained as reported for heparin/chitosan nanoparticles prepared by
269 polyelectrolyte complexation [21] and deoxycholic acid-heparin amphiphilic conjugates [22].

270 The mechanism leading to the formation of the nanoassemblies by the auto-association of *O*-
271 palmitoyl-heparin and α -CD is different from the one described by previous works. The
272 explanation of the well-organized and hexagonal shape of the particles observed by TEM comes
273 from the understanding of the mechanism of the interaction between alkyl chains and α -CD.
274 Hexagonal crystalline structures have already been observed for short-chain compounds forming
275 an inclusion complex of one alkyl per two α -CD [23-25]. Shaped as a hollow truncated cone, CDs
276 are cyclic oligosaccharides of six D-(+) glucopyranose all in chair conformation. The inclusion
277 phenomena are the result of weak interactions involving both hydrophobic and hydrophilic parts
278 of CDs such as hydrogen bonds, electrostatic interactions and van der Waals forces. In comparison
279 with other CDs such as β -CD and γ -CD, α -CD had the smallest cavity (4.9 \AA internal diameter and
280 7.9 \AA depth). The width of the hydrophobic part of the guest has to be lower than 4.5 \AA to permit
281 the formation of a stable inclusion compound with α -CD. This was the case of lipids such as

282 | palmitic acid; the cross-section does not exceed 4.5 Å in the zig-zag conformation. This has been
283 shown by the crystallographic study of the α -CD/12-dodecanoic acid complex [26]. In a more
284 recent work, high-resolution neutron diffraction was used for the characterization of the highly
285 hydrated α -CD/1-undecanol inclusion complex [27]. In a preceding paper, calorimetric data for
286 the interaction of linear carboxylic acids with α -CD reported that by increasing length of the alkyl
287 chains, the association constants increased. When the alkyl chain is not long enough (as for C6)
288 interactions are so weak that association does not occur [28]. The interaction between palmitic
289 acid and α -CD is thus the driving force for the formation of the nanoassemblies. A simple mixture
290 of native heparin with α -CD in aqueous media did not give any nanoassembly formation because
291 heparin does not form any inclusion complex with α -CD (data not shown). Hexagonal plate
292 habitus of the crystallites seen in Figure 3 strongly suggest a molecular arrangement of the
293 nanoassemblies in a triclinic lattice [24] in agreement with α -CD dimeric arrangement [23]. The
294 α -CD/lipid complexes are known to crystallize spontaneously in which the hydrophobic residue of
295 the lipid molecule is not in a disordered conformation state. This kind of interaction is typical of
296 α -CD. It is indeed not the case of β -CD with a large ring allowing the reorientation of aliphatic
297 chains and thus leading to higher mobility and disorder inside the host cavity [29].

298 The effect of *O*-palmitoyl-heparin concentration on the nanoassembly size was first studied for
299 OPH-1. The concentration of α -CD was kept constant (10 wt%) while the concentration of the
300 polysaccharide was progressively decreased from 1 to 0.25 wt%. At fixed concentration of α -CD,
301 nanoassembly size increased from (344 ± 105) nm to (659 ± 260) nm when the concentration of
302 OPH-1 was decreased from 1 to 0.25 wt% (Table 2). Furthermore, the nanoassemblies obtained at
303 low OPH-1 concentration are highly polydisperse in comparison with the ones obtained at high
304 OPH-1 concentration. One hypothesis to explain these results is that more than one OPH-1 is
305 involved in nanoassembly formation.

306 The antiviral activity evaluation of nanoassemblies showed that, as expected, HSV-2 infection of
307 cells was more efficiently inhibited than that of HSV-1. The half-maximal inhibitory
308 concentrations are between 0.86 and 2.19 $\mu\text{g}/\text{mL}$ against HSV-1 infection and between 0.42 and
309 1.42 $\mu\text{g}/\text{mL}$ against HSV-2 infection. Previous works in the literature have also reported that
310 HSV-2 infection of cells was more efficiently inhibited than that of HSV-1 by polyanionic
311 substances such as heparin but also dextran sulphate, agar inhibitors and chondroitin sulphate B
312 [30]. Native heparin and heparin nanoassemblies did not inhibit Rotavirus infection since the cell
313 attachment and entry of Rotavirus depends on several integrins but not on HSPGs [31]. Whatever
314 the formulation, heparin nanoassemblies did not affect cell viability and the CC_{50} values were
315 higher than 300 $\mu\text{g}/\text{mL}$ in all the tested cell lines indicating that the inhibitory activity does not
316 have any consequence on cytotoxicity.

317 The antiviral activity of OPH-1 was improved in comparison with heparin even without $\alpha\text{-CD}$. As
318 we can see from Table 2, without $\alpha\text{-CD}$ the IC_{50} values against HSV-1, HSV-2 and RSV were
319 lower for OPH-1 in comparison with native heparin. The grafting of palmitic acid on the
320 hydrophilic heparin results on its hydrophobization. Previous works have reported that lipid
321 conjugates were able to increase the interaction of the molecule with biological membranes due to
322 their lipophilicity and resemblance to lipids in biological membranes [32].

323 The amphiphilic nature of OPH partly explains the higher antiviral activity obtained in comparison
324 with native heparin. The presence of $\alpha\text{-CD}$ will further decrease the IC_{50} for HSV-1, HSV-2 and
325 HPV-16. This increase of the antiviral activity was not due to the $\alpha\text{-CD}$ itself because it did not
326 have any antiviral effect (data not shown). However, the presence of $\alpha\text{-CD}$ leads to the formation
327 of hexagonal-shaped heparin nanoassemblies. It is likely to postulate that this hexagonal geometry
328 is favourable for the interaction of the nanoassemblies with the viruses. Previous research works

329 conducted on other types of nanoparticles have reported that the particle geometry influenced their
330 interaction with cells, their subcellular trafficking and distribution in organs [33-36].

331 For the next experiments, the concentration of hydrophobically-modified heparin (OPH-2) was
332 fixed at 1 wt%. The effect of α -CD concentration on nanoassembly size was investigated by
333 progressively decreasing α -CD concentration from 10 wt% to 2.5 wt%. Table 3 depicts the
334 physicochemical properties and shows that nanoassembly size decreased from (410 ± 24) nm to
335 (340 ± 19) nm for α -CD concentrations of 10 wt% and 2.5 wt% respectively.

336 Generally speaking, the antiviral activities obtained with nanoassemblies composed of OPH-1
337 were better than the ones of OPH-2. There is a growing body of evidence that heparan sulphate
338 proteoglycans act as receptors for HSV-1, HSV-2, HPV-16 and RSV. So it is expected that the
339 antiviral activity decreased by reducing the level of sulphation. Elemental analysis of heparin and
340 the two *O*-palmitoyl-heparin derivatives showed that the total amount of sulphur was decreased
341 from 9.1 for heparin to 8.9 and 8.1 for OPH-1 and OPH-2 respectively (Table 1). The negative
342 charge of heparin results from *N*-sulfation, *O*-sulfation and the presence of carboxylic groups.
343 Particularly, *N*-sulfations and 6-*O*-sulfations of heparin play a key role in its interaction with HS-
344 dependent viruses. The presence of sulfate groups in heparin structure results in its anticoagulant
345 activity. Specifically, this property is due to the pentasaccharide section in heparin that has high
346 affinity for antithrombin III. Higher sulfation of OPH-1 results in lower zeta potential of the
347 nanoassemblies in comparison with OPH-2 (Tables 2 and 3). These differences between OPH-1
348 and OPH-2 nanoassemblies could be a consequence of the partial desulfation of OPH-2 in acidic
349 medium due to hydrochloric acid release upon the reaction between palmitoyl chloride and
350 heparin. The synthesis of OPH-1 was conducted during shorter time than for OPH-2 and in the
351 presence of pyridine which is a basic heterocyclic organic compound. With hydrochloric acid
352 pyridine forms a crystalline hydrochloride salt.

353 Even if the domains involved in viral binding are independent on the anticoagulant activity of
354 heparin [37], non-specific desulfation of heparin results in lower anticoagulant and antiviral
355 activities (Figure 4). So far, the anticoagulant activity of heparin is closely related to its *N*-sulfated
356 and 6-*O*-sulfated contents [37]. The desulfation of heparin at these sites decreased or even
357 abolished the antiviral activity of heparin. The sulfation of polysaccharides initially without
358 anticoagulant activity such as pullulan, allowed to obtain pullulan sulfates with potent
359 anticoagulant activity reaching the efficacy of heparin [38].

360 4. Conclusions

361 Novel heparin nanoassemblies active against four HSPGs-dependent viruses HSV-1, HSV-2,
362 HPV-16 and RSV were designed by a new simple and green method. The nanoassemblies were
363 formed by the auto-association of *O*-palmitoyl heparin with α -CD. Two hydrophobically-modified
364 heparins (OPH-1 and OPH-2) were obtained by using two different esterification methods.
365 Whatever the chemical method used, the observed hexagonal-shaped nanoassemblies strongly
366 suggest a crystal-like arrangement. Nanoassemblies with higher level of sulfation obtained with
367 OPH-1 exerted a stronger antiviral activity in comparison with OPH-2. These encouraging results
368 warrant further investigations of these nanoassemblies as drug delivery systems able to target the
369 HS-dependent viruses.

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Figure caption:

Figure 1. Synthetic scheme of the two methods for the grafting of palmitic acid on heparin.

Figure 2. ATR-IR spectra of OPH-1 and OPH-2 and comparison with native heparin.

Figure 3. TEM observations of Hep2 (**A**) and Hep6 (**B**).

Figure 4. Anticoagulant activity of heparin nanoassemblies Hep2 and Hep6 composed of 1 wt% of OPH-1 and OPH-2 respectively and 10 wt% of α -CD. The results were compared to the anticoagulant activity of native heparin. The anticoagulant activity was expressed by U/mg of heparin or *O*-palmitoyl-heparin.

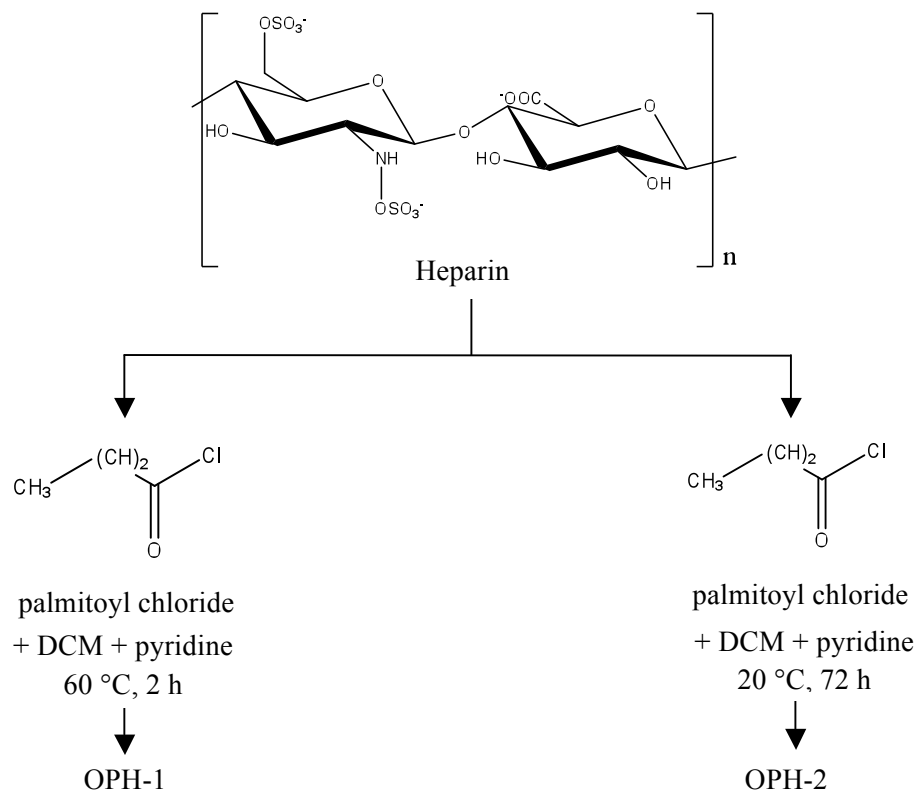


Figure 1:

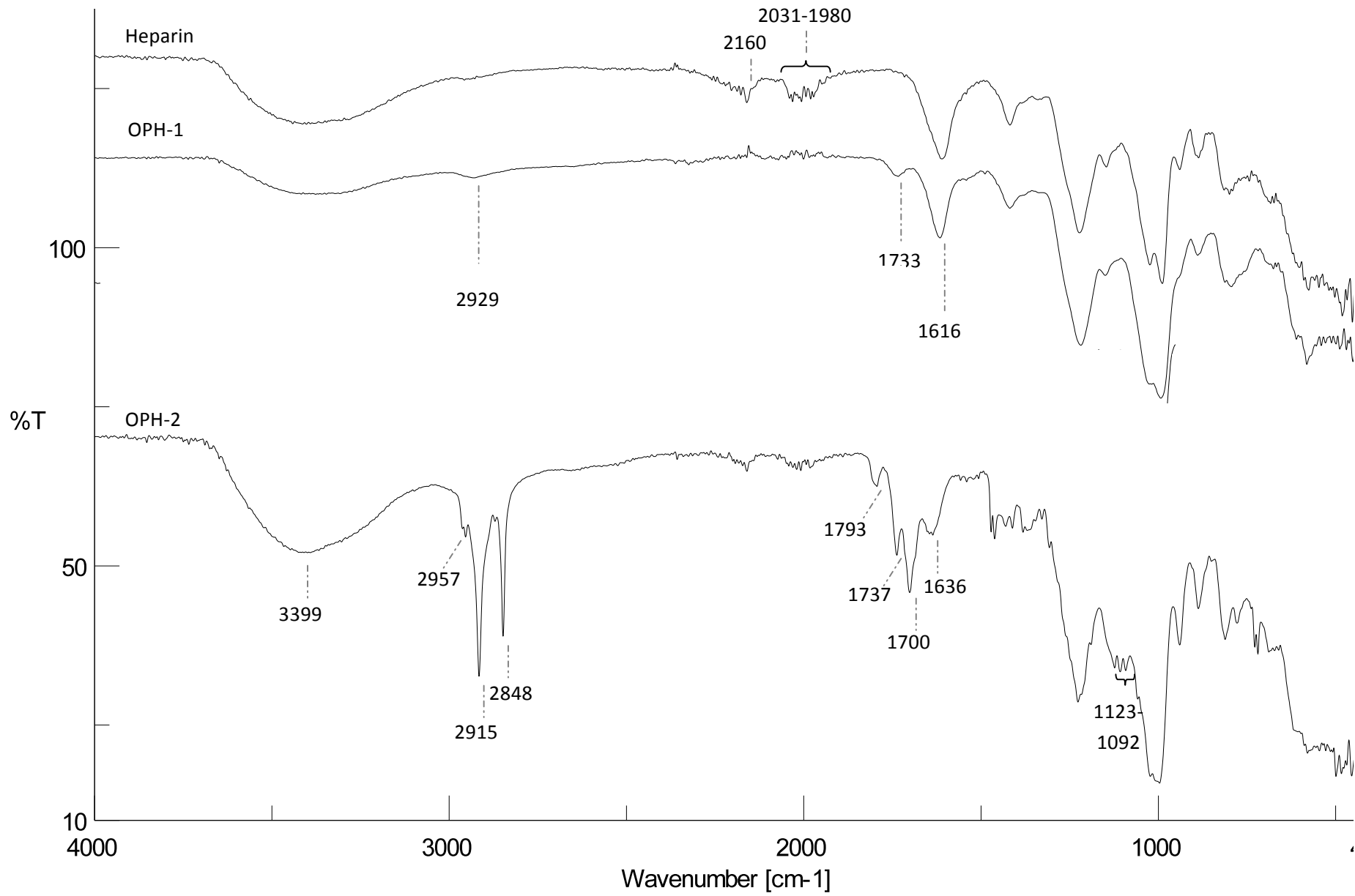


Figure 2.

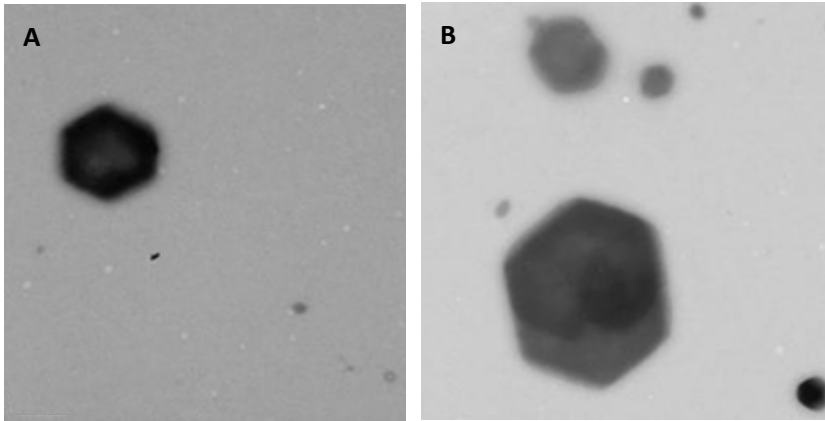


Figure 3.

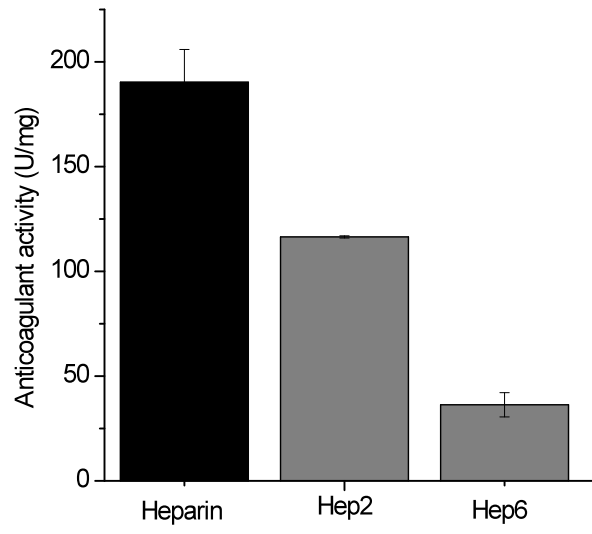


Figure 4.

Table 1: Total carbon and sulfur quantification evaluated by elemental analysis into *O*-palmitoyl-heparins (OPH-1 and OPH-2) in comparison with native heparin.

Polymer	Total C in OPH (% w/w)	Total C in OPH (% mol)^a	DS^b (%)	Total S in OPH (% w/w)
Heparin	19.0	3.1	-	9.1
OPH-1	21.8	3.6	0.5	8.9
OPH-2	33.7	5.6	2.5	8.1

^aTotal carbon in OPH conjugates was calculated from the equation $C\%6$

^bThe degree of substitution was calculated from the equation Eq.1.

Table 2. Effect of OPH-1 concentration on assembly D_h and their antiviral activities against HSV-1, HSV-2, HPV16-SEAP PsV and RSV. The content of α -CD was kept constant, while OPH-1 was progressively decreased from 1 to 0.25 wt%. Rotavirus was used as a control. [Polydispersity indexes were lower than 0.10, indicating a relatively homogenous size distribution.](#)

Polymer	Assembly code	OPH-1 (wt%)	α -CD (wt%)	D_h (nm)	ξ (mV)	virus	IC ₅₀ μ g/mL (95% C.I.)	CC ₅₀ (μ g/mL)	SI	
Heparin	-	-	-	-	-	HSV-1	2.19 (0.97 - 3.56)	> 300	> 136.98	
						HSV-2	1.42 (0.23 - 2.55)	> 300	> 211.26	
						HPV-16	2.01 (1.12 - 3.88)	> 300	> 149.25	
						RSV	2.11 (1.69 - 3.43)	> 300	> 142.18	
						Rotavirus	- ^b	> 300		
OPH-1	Hep1	1	0	- ^a		HSV-1	1.62 (0.51 - 2.66)	> 300	> 185.18	
						HSV-2	0.62 (0.13 - 1.78)	> 300	> 483.87	
						HPV-16	2.88 (1.81 - 4.21)	> 300	> 104.16	
						RSV	0.94 (0.47 - 1.87)	> 300	> 319.14	
						Rotavirus	- ^b	> 300		
	Hep2	1	10	344 \pm 105		-59 \pm 1	HSV-1	1.07 (0.13 - 2.07)	> 300	> 280.37
							HSV-2	0.51 (0.08 - 1.92)	> 300	> 588.23
							HPV-16	1.25 (0.33 - 2.04)	> 300	> 240.00
							RSV	1.00 (0.46 - 2.17)	> 300	> 300.00
							Rotavirus	- ^b	> 300	
	Hep3	0.5	10	344 \pm 128		-58 \pm 1	HSV-1	0.99 (0.21 - 1.99)	> 300	> 303.03
							HSV-2	0.66 (0.09 - 2.10)	> 300	> 454.54
							HPV-16	1.76 (0.69 - 4.11)	> 300	> 170.45
							RSV	0.99 (0.47 - 2.10)	> 300	> 303.03
							Rotavirus	- ^b	> 300	
	Hep4	0.25	10	659 \pm 260		-62 \pm 2	HSV-1	0.86 (0.32 - 2.05)	> 300	> 348.83
							HSV-2	0.42 (0.24 - 1.68)	> 300	> 714.28
							HPV-16	2.20 (1.03 - 5.43)	> 300	> 136.36
							RSV	1.01 (0.51 - 1.99)	> 300	> 297.02
							Rotavirus	- ^b	> 300	

^a -, no formation of the assemblies.

^b -, the compound was non inhibitory at a dose of $\leq 100 \mu$ g/mL.

Table 3. Effect of α -CD concentration on assembly D_h and their antiviral activities against HSV-1, HSV-2, HPV16-SEAP PsV and RSV. The concentration of OPH-2 was kept constant, while α -CD concentration was progressively decreased from 10 wt% to 2.5 wt%. Rotavirus was used as a negative control. [Polydispersity indexes were lower than 0.10, indicating a relatively homogenous size distribution.](#)

Polymer	Assembly code	OPH-1 (wt%)	α -CD (wt%)	D_h (nm)	ξ (mV)	virus	IC ₅₀ μ g/mL (95% C.I.)	CC ₅₀ μ g/mL	SI
Heparin	-	-				HSV-1	2.19 (0.97 - 3.56)	> 300	> 136.98
						HSV-2	1.42 (0.23 - 2.55)	> 300	> 211.26
						HPV-16	2.01 (1.12 - 3.88)	> 300	> 149.25
						RSV	2.11 (1.69 - 3.43)	> 300	> 142.18
						Rotavirus	-	> 300	
OPH-2	Hep6	1	10	340 \pm 19	-52 \pm 1	HSV-1	5.55 (3.27- 10.34)	> 300	> 54.05
						HSV-2	2.52 (1.06 - 6.59)	> 300	> 119.04
						HPV-16	n.c. ^a	> 300	
						RSV	5.41 (2.83 - 10.33)	> 300	> 55.45
						Rotavirus	- ^b	> 300	
	Hep7	1	5	355 \pm 11	-53 \pm 1	HSV-1	4.09 (2.89 - 9.27)	> 300	> 73.34
						HSV-2	2.39 (0.41 - 8.03)	> 300	> 125.52
						HPV-16	n.c.	> 300	
						RSV	2.45 (1.59 - 3.78)	> 300	> 122.44
						Rotavirus	-	> 300	
	Hep8	1	2.5	410 \pm 24	-54 \pm 4	HSV-1	5.03 (2.54 - 8.29)	> 300	> 59.64
						HSV-2	2.54 (1.31 - 4.81)	> 300	> 118.11
						HPV-16	2.50 (1.33 - 3.46)	> 300	> 120.00
						RSV	1.15 (0.65 - 2.04)	> 300	> 260.86
						Rotavirus	-	> 300	

^a n.c., not calculable.

^b-, the compound was non inhibitory at a dose of \leq 100 μ g/mL.