

Low molecular weight heparin-vectorized β -cyclodextrin nanostructures

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ABSTRACT

We have in our hand three different nanostructures **3**, **6** and **7** (Fig. 3, 4 and 5) built up by β -cyclodextrin and low molecular weight heparin (LMWH) skeleton, characterized by covalent bonds (amine or amide) between the 6-monoamino β -cyclodextrin **1** and the LMWH moieties **2**, **4** and **5**, in different positions of the heparin chain (Fig. 1 and 2, for convenience we report only the part of the heparin chain, including the sugar unit directly involved in the new bond formation). **3** is characterised by an amido group linkage between the NH_2 group of **1** and the carboxylic group of one of the uronic acid units, present in the unmodified heparin chain of **2**. The nanostructure **6** is characterised by a secondary amino group linkage between the NH_2 group of **1** and the position 2 or 3 of a desulphated modified uronic acid of the adduct **4**. **7** is characterised by a secondary amino group linkage between the NH_2 group of **1** and the terminal 2,5-anhydromannose unit of the adduct **5**. The three different nanostructures **3**, **6** and **7** sum in one adduct the structures of the cyclodextrin and the heparin, which alone have the well-known biological properties. In this context, we are looking for them new biological properties and activities, induced by the synergy of the cyclodextrin and the heparin alone.

Keywords: nanostructures, cyclodextrin, LMWH.

1 INTRODUCTION

Cyclodextrins (CDs) are a family of naturally occurring, water-soluble oligosaccharides forming a bucket-shaped macrocycle and made up of α -(+)-glucopyranose units, 6 (α -CD), 7 (β -CD) and 8 (γ -CD) units. They are produced on an industrial scale and widely used to solubilise in water lipophilic drugs, encapsulated inside the same polarity cavity [1]. Heparin (UH), Mw of 15-20 kDa, is a naturally occurring heterogeneous glycosaminoglycan, with the most clinical relevance as anticoagulant, mainly consisting of repeating units of variously sulphated hexuronic (D-glucuronic or L-iduronic acid) and D-glucosamine. Low molecular weight heparins (4-5 kDa), obtained by different depolymerisation methods of UF, produce a more predictable anticoagulant response than UH which reflect

their better bioavailability, longer half-life, and dose independent clearance [2].

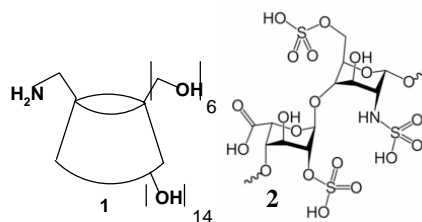


Figure 1: Formulas of CD-6-NH₂ **1** and LMWH **2**.

2 RESULTS

Mono-6-amino- β -cyclodextrin **1** was synthesized from commercial β -cyclodextrin. Two different LMWH were prepared, **2** (Mw= 6092 Da), by UH γ ray irradiation depolymerisation, and **5** (Mw= 3265 Da), by UH nitrous acid depolymerisation, associated with glucosamine conversion into 2,5-anhydromannose terminal residue. **1** was submitted to condensation with **2** affording the amido-condensated nanostructure **3**. **2** was also desulfated to the epoxyde derivative **4**, that was condensed with **1** to give the amino-condensated nanostructures **6**. **5** was directly condensed with **1** to afford the nanostructure **7**.

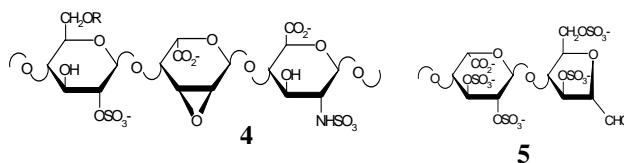


Figure 2: Formulas of **4** and **5**.

2.1 Starting Materials **1**, **2**, **4** and **5**

Mono-6-amino- β -cyclodextrin **1** was obtained from mono-tosyl- β -cyclodextrin and the subsequent azido derivative [3]. **2** (Mw around 6092 Da, SEC/TDA analysis) was prepared by γ ray irradiation depolymerisation [4]. **4**

was obtained in alkali: in details, reaction condition has been chosen in order to obtain an average of one epoxyde ring *per* heparin chain [5]. **5** was every time freshly prepared by depolymerisation induced by aqueous nitrous acid [6].

2.2 Nanostructure 3 by Amidation of 2

The condensation between **1** and **2** was attempted in dimethylformamide (DMF) by two carbodiimides, N⁷-(3-dimethylaminopropyl)-N-ethyl-carbodiimide hydrochloride (EDC) and N,N⁷-dicyclohexyl-carbodiimide (DCC). EDC led to the isolation of the adduct **3**, whose structure was tentatively shown in Fig. 3: due to the heterogeneity of the heparin chain and to the presence of different uronic acids (gluco- or ido-), different amide bonds can be formed on the heparin chain. In turn, DCC did not work. EDC was also used in water. **1** was used in both the solvents (30 ml) at the same concentration (300 mg, $8.8 \cdot 10^{-3}$ M), together with **2** (300 mg, $2 \cdot 10^{-3}$ M) and EDC (300 mg, $1.04 \cdot 10^{-2}$ M). The reaction in DMF was heterogeneous. In both cases, the reaction mixtures were stirred at room temperature for 24 hrs, then concentrated to 4/5 ml under vacuum and precipitated in abs EtOH (ca 15 ml). The precipitates were eluted on CM Sephadex™C-25 column, to eliminate the excess of **1**. The eluted samples were submitted to SEC/TDA analyses to determine their average Mw (Table 1). The average Mw of both the derivatives in water and in DMF suggest the formation of adducts CD/LMWH 1:1. The sample **3**, obtained from reaction in DMF, was analyzed by mono ¹H and bidimensional (COSY and HSQC) NMR spectroscopy in order to find evidence of the new amide bond and to define as well as possible the structures. Even if **3** was not fractionated in the different components, we have evidence of the amide bond formation, as, in the NMR HSQC in Fig 6, the CH₂ signals and the H-4/C-4 of the amino-CD disappear and it seems possible to follow all the signals of the glucose CD ring involved in the amide bond. On the base of the SEC/TDA analysis, the crude yields of the reactions in water and in DMF seem of the same order same of magnitude, around 32% on the heparin conversion.

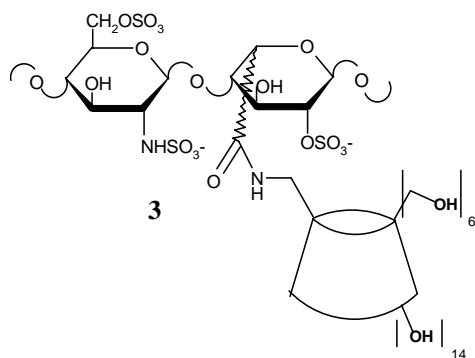


Figure 3: Formula of **3**.

2.3 Nanostructure 6 by Epoxyde Ring Opening of 4

1 and **4** were linked by epoxyde ring opening reaction, affording the adduct **6**. The one step reaction led to the glycoconjugate **6** in water solvent (1.0 ml) in 24 hrs at 70°C. From the reaction between **1** (100 mg, $88.5 \cdot 10^{-3}$ M) and **4** (70 mg, $11.6 \cdot 10^{-3}$ M) we collected crude reaction mixture made of the conjugate **6** and of the heparin alone, coming from the water epoxyde ring opening. The crude reaction mixture was eluted on CM Sephadex™ C-25 column and QAE Sephadex™ A-25, in order to eliminate both the excess of **1** and the no bound desulphated-LMWH, respectively. The eluted sample was eventually desalted through TSK-GEL resin. After desalting procedure, **6** was submitted to SEC/TDA analyses to determine its average Mw (Table 1). The average Mw suggests the formation of adducts CD/LMWH 1:1. **6** was also analyzed by ¹H and HSQC NMR spectroscopy, in order to find evidence of the new secondary amine bond between **4** and **1** and to define as well as possible the structures. Until now, it is not yet possible to say in which position (C-2 or C-3) of the uronic acid (gluco- or ido-) the linkage occurs. In the NMR spectrum of nanoconjugate **6** (Fig. 7), the signals H-4 and H-6 of the 6-amino-glucopyranose ring of cyclodextrin alone **1** are absent. The reaction yield was around 25% respect to starting product **4**.

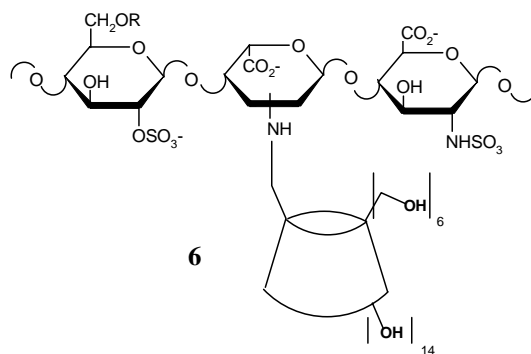


Figure 4: Formula of **6**.

2.4 Nanostructure 7 by Red. Amination of 5

The compounds **1** and **5** were linked by reductive amination affording the sample **7**. We were able to further fractionate **7** into three components **7a**, **7b** and **7c**, depending on their Mw. **7** was prepared in water (2.5 ml) starting from CD **1** (100 mg, $35.2 \cdot 10^{-3}$ M) and **5** (35 mg, $5.6 \cdot 10^{-3}$ M) at pH 5.5 in the presence of NaBH₃CN (5.5 mg, $5.6 \cdot 10^{-3}$ M) as reducing agent; the reaction was stirred at room temperature for 24 hrs in the dark. The crude reaction mixture was purified with CM Sephadex™ C-25 column able to retain the excess of **1**. After the purification, QAE

Sephadex™ A-25 was used to fractionate the eluted sample by ionic strength, eluent made by NaCl gradient, 0.5, 0.75, 1.0 M. The corresponding three fractions of different LMWH chain length/negative charge (**7a**: 29.3 mg, **7b**: 11.6 mg, **7c**) were eventually desalted with TSK-GEL resin. From the NMR spectra (¹H, HSQC) the fraction **7a**, eluted with the lower ionic strength, is made by the shortest heparin chain, and it was also possible to assign the position of the atoms involved in amine bond between **1** and **5**, (Fig. 8). The HSQC spectra of the fraction **7b** and **7c** increase in complexity, depending on heparin chain length. SEC-TDA analyses confirm these results (Table 1). It is impossible to calculate each condensation yield for **7** fractions, because **5** was not submitted to the same fraction treatment, before coupling with **1**.

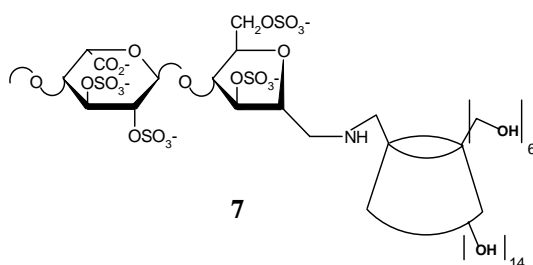


Figure 5: Formula of **7**

3 CHARACTERIZATION

Modern and sophisticated techniques were successfully applied to characterize the obtained compounds. All compounds were characterised by high resolution NMR spectroscopy (500 MHz) in deuterium oxide at 30°C. Proton and carbon signals of each spectrum were assigned using homonuclear (COSY and TOCSY) and heteronuclear (HSQC) experiments. Size exclusion chromatography (SEC), associated with triple detector array (TDA, simultaneous action of three on-line detectors: light scattering detectors, refractometer and viscometer) is a modern high-level analytic equipment to determine the size of small polymeric molecules [7].

| Product | Mn | Mw | Mp |
|---------------------------|------|------|------|
| 2 | 5653 | 6092 | 5519 |
| 3 _(DMF) | 7028 | 7740 | 7250 |
| 3 _(H2O) | 6152 | 7180 | 6351 |
| 5 | 2498 | 3265 | 6398 |
| 6 | 7152 | 7484 | 6963 |
| 7b | 3997 | 4220 | 3785 |
| 7c | 4548 | 5140 | 4926 |

Tab 1: SEC/TDA experiments; Mn: Mass numeral; Mw: Average weight; Mp: Mass peak; **7b**: eluted from QAE column with NaCl solution 0.75 M; **7c**: eluted with NaCl solution 1.0 M.

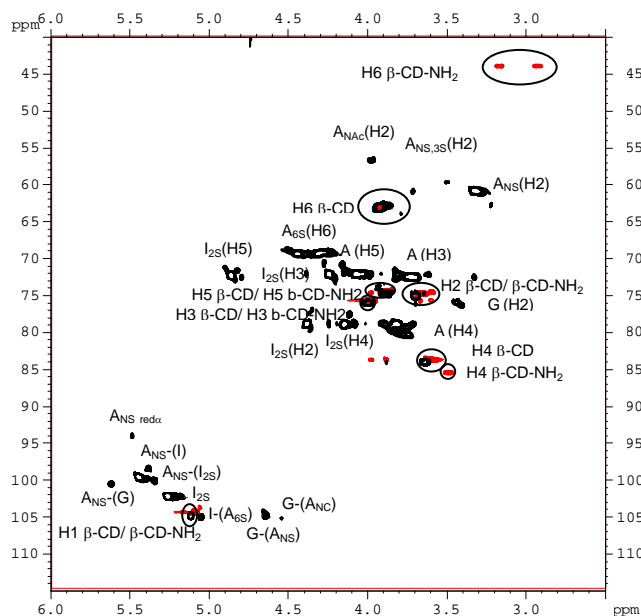


Fig. 6: HSQC spectrum; • compound **1**, • compound **3**; A: glucosamine; AN_S: glucosamine N-sulphated; I: iduronic acid, G: glucuronic acid

In bidimensional spectrum (HSQC) of **3** (Fig. 6) it was possible to follow disappearance of CD-CH₂-NH₂ signals (3.11-2.87 ppm, 44 ppm) and H-4 β-CD-NH₂ (3.44 ppm, 85.5 ppm), diagnostic of the amino-glucopyranose ring (red signals).

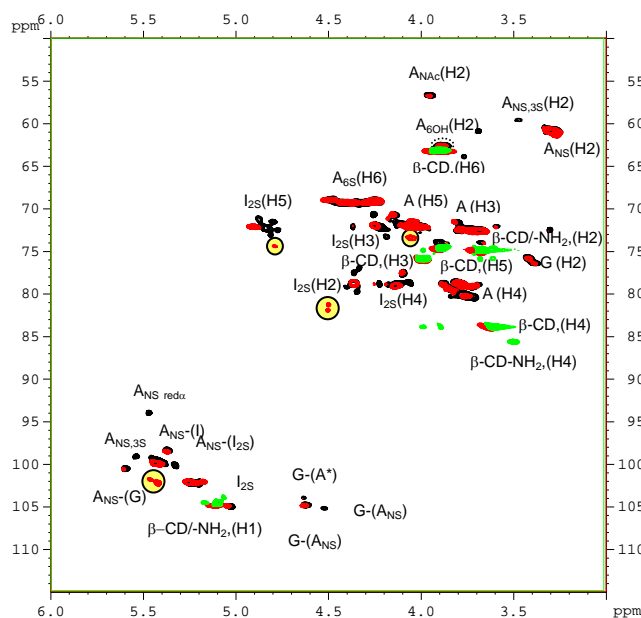


Fig. 7: HSQC spectrum; • compound **1**, • compound **2**, • compound **6**

HSQC spectra shown in Fig. 7 indicate clearly the presence of new crosspeaks signals belonging to

compound **6**, even if not yet assigned (yellow circles). Further, it is possible to note the absence of H-4 (3.44 ppm, 85.5 ppm) and H-6 (3.11-2.87 ppm, 44 ppm), assigned to the 6-aminoglucose ring of **1**.

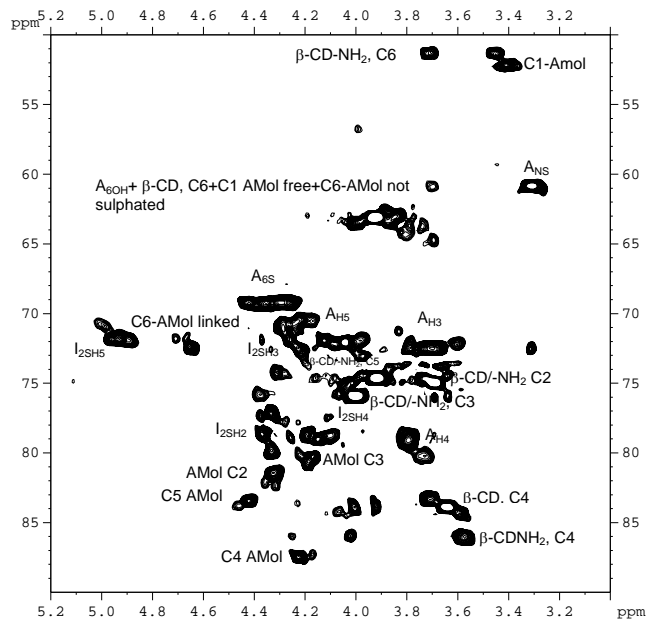


Fig. 8: HSQC spectrum of the product **7a**; Amol: 2,5-anhydro-mannose residue

Bidimensional spectrum, shown in Fig. 8, of the simplest reductive amination compound **7a** evidences with no doubt, the presence of typical signals of this product: the atom 6 of **1** moves from 3.11-2.87 ppm/44 ppm (free amine) to 3.68-3.41 ppm/51.3 ppm (bridging amine) and the atom 1 of **5** moves from 4.03-3.80 ppm/63.8-62.1 ppm (free anhydro-mannitole) to 3.38 ppm/52.2 ppm (bridging 2,5-anhydro-mannose). NMR spectra of the compounds **7b** and **7c** are more complex.

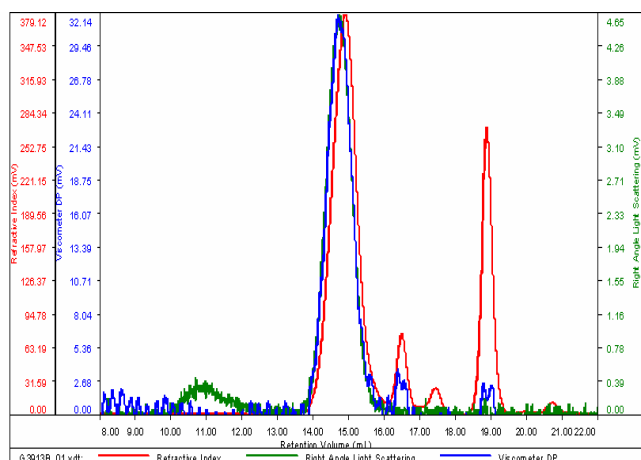


Fig. 9: SEC/TDA chromatogram of LMWH

Fig. 9 shows an example of SEC/TDA chromatogram with the three on-line detectors, here reported with different colours (red: refractive index, green: right angle light scattering, blue: viscosimeter). In Table 1 we reported the SEC/TDA analyses of all the nanostructures.

4 CONCLUSION

The synthesis of several cyclodextrin nanostructures, with β -CD bonded in different positions of various LMWHs, was successfully performed, as demonstrated by NMR spectroscopy and SEC/TDA analysis. The differences in the nanostructures will allow us to work up biological assays to test new biological properties and activities, induced both by the synergy of the cyclodextrin and the heparin alone, and by the different positions of the linkage between CD and the heparin chain.

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