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Note

Sepharose-bound, highly sulfated glycosaminoglycans can capture HIV-1 from culture medium

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Abstract—In the search for new strategies against HIV-1 and on the basis of a number of previous studies reporting on the capacity of certain polyanionic compounds to influence the replication of HIV-1, we prepared a few chemically oversulfated dermatan and chondroitin sulfates. Four of these compounds and two samples of heparin were bound to activated Sepharose through either their carboxylic groups, or their aldehydic groups, or their deacetylated primary amino groups. Some of these so-derivatised resins, packed into columns, proved able to remove HIV-1 IIIB, a laboratory adapted strain, and one clinical primary isolate from an AIDS patient, from infected cell culture medium. The resins bind the virus very tightly and could be useful for capturing the virus from infected fluids.

Keywords: Chondroitin sulfate; Dermatan sulfate; Heparin; Oversulfated glycosaminoglycans; HIV-1

Dermatan sulfate (DS), chondroitin sulfate (CS) and heparin (HP) (Fig. 1), three glycosaminoglycan sulfates (GAGS), interfere, like other polyanions, with the in vitro replication of Human Immunodeficiency Virus type 1 (HIV-1),[1–4] most probably by interacting with the enveloped virus pericapsidic proteins, producing strong inhibition as well as stimulation, with an intensity of the effects paralleling high charge density, high MW, high concentration, being higher with DS and HP than with CS,[1–4] depending also on the type of virus, type of cell culture and cell batch used in the in vitro experiments.[5–7]

In consideration of their hypothesised ability to adhere to HIV-1 pericapsidic proteins, we planned to immobilise a few chemically oversulfated GAGS onto an insoluble matrix such as Sepharose, as a potential tool for removing virus particles from infected fluids, and, to this aim, two natural DS (**1** and **4**, Table 1) and one CS (**8**) were oversulfated using established procedures[8,9] in order to increase their affinity towards the virus particles. The origin, molecular mass, sulfation index (SO_3H/CO_2H ratio) and specific optical rotation of parent GAGS and their derivatives are reported in Table 1.

DS samples **1** and **4**, from porcine mucosa, mainly contain GalNAc-4–SO_3H, thus presenting many (GalNAc) O-6 primary hydroxyls and were oversulfated under milder conditions, with SO_3–TEA,[8] the expected regioselectivity of oversulfation of the available groups being in the order (GalNAc) O-6, uronic acid (IdoA) O-2/O-3, (GalNAc) O-4.[10] CS sample **8**, of shark cartilage origin, is a CS type C,[11] and therefore mainly contains GalNAc-6–SO_3H (65%), is rich in (GalNAc) O-4 secondary hydroxyls, and required a more drastic treatment with $ClSO_3H$ to be oversulfated,[9] but his latter

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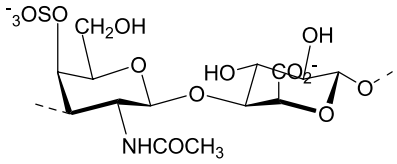
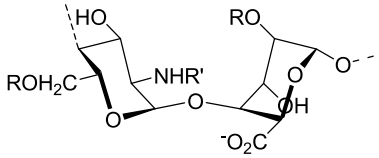


→ 3)-β-D-GalNAc*p*-4-OSO_3^--(1→ 4)-α-L-IdA*p*-(1→

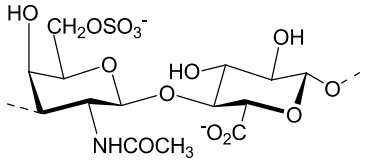
Dermatan sulfate



→ 4)-α-D-GlcN*p*-(1→ 4)-α-L-IdA*p*-(1→

Heparin

Variants: R = SO_3^- or H, R' = SO_3^- or Ac



→ 3)-β-D-GalNAc*p*-6-OSO_3^--(1→ 4)-β-D-GlcA*p*-(1 →

Chondroitin 6-sulfate

Figure 1. The most representative disaccharide units in Dermatan Sulfate, Heparin, Chondroitin 6-sulfate.

Table 1. Chemical characterisation of GAGS

Compound	Type[a]	Origin[b]	Molecular mass (kDa)	SI[c]	$[\alpha]_D^{20}$ (°)
1	DS	PM	32	1.10	−57.5
2	DS	**1**	32	2.27	−51.0
3	DS	**1**	30	2.81	−49.5
4	DS	PM	19	1.04	−68.5
5	DS	**4**	n.d.[d]	n.d.[d]	n.d.[d]
6	DS	**5**	11	2.89	n.d.[d]
7	DS	**6**	10	2.70	−51.0
8	CS	SC	12	1.02	−15.0
9	CS	**8**	n.d.[d]	n.d.[d]	n.d.[d]
10	CS	**9**	6	2.2	n.d.[d]
11	CS	**10**	6	2.0	−14.0
12	HP	BM	11	2.2	n.d.[d]
13	HP	**12**	10	2.1	n.d.[d]

[a] DS, dermatan sulfate; CS, chondroitin sulfate; HP, heparin.
[b] PM, porcine mucosa; SC shark cartilage; BM, bovine mucosa.
[c] SI, sulfation index (SO_3H/CO_2H molar ratio).
[d] Not determined.

treatment can give place to relevant depolymerisation. DS **1** was transformed into **2** and **3**, DS **4** into **4a** and CS **8** into **8a** (data not shown) accordingly (Table 1).

Sepharose, that is cross-linked agarose, which as such has no charge, is resistant to microbial attacks, and is stable in aqueous media, was chosen as an insoluble matrix to which the oversulfated GAGS should be linked through either their uronate carboxylic groups, or their polysaccharidic reducing ends, or their deacetylated *N*-acetylglycosamine amino groups. Oversulfated DS **4a** and CS **8a** were then submitted to *N*-deacetylation (with hydrazine/hydrazine sulfate)[12] but with poor yields (<50%, data not shown), a different strategy was thus adopted, performing *N*-deacetylation before oversulfation and obtaining products **5** and **9** with *N*-acetyl removal >90%. The analogous deacetylation of HP **12** produced **13**. The deacetylated DS **5** and CS **9** were oversulfated with SO_3–TEA[8] and with $ClSO_3H$,[9] respectively, giving **6** and **10**. These sulfation reactions take place preferentially at the hydroxyl groups, however, the *N*-deacetylated and oversulfated **6** and **10** were selectively *N*-desulfated with HCl giving **7** and **11**, respectively, the small reduction of the SO_3H/CO_2H ratio likely indicating the removal of a few *N*-sulfate groups from the ($GalNH_2$) C-2. The reaction caused only a modest MW decrease (Table 1).

The influence of the GAGS on the replication of HIV-1 IIIB, one of the most widely used HIV-1 laboratory adapted strains, grown in C8166 T cell line, and of one clinical primary isolate, Is 13, taken from one AIDS patient and grown in peripheral blood lymphocytes (PBL) from healthy donors, was then studied as indicative of the ability of interaction, having first assessed the lack of cytotoxicity of all the compounds towards C8166 T cells and of **2**, **3**, **12** towards PBL cells,[5] at a concentration of 100 μg/mL. The GAGS were tested at 0.01–10 μg/mL on HIV-1 IIIB strain in C8166 T cells; compound **3**, characterised by a DS-type structure, a high sulfation index (SI: 2.81) and a high MW (ca. 30 kDa) proved a potent inhibitor (EC_{50} 0.06 μg/mL) of the viral replication (Table 2), other effective inhibitors (EC_{50} 0.5–1 μg/mL) being **2**, **5**, **6**, **7** of DS-type, and **12**, **13** of HP-type, with MW ranging from 11 to 30 kDa. DS-type **1** and **4**, and CS-type **8**–**11** resulted all practically inactive (EC_{50} > 10 μg/mL), having either a low SI or a low MW, glucuroate CS derivatives being in general less active than iduronate DS derivatives with the same IS and MW, because the iduronate residues of DS are more flexible that the glucuronate residues of CS.[13] Rather different effects were previously obtained by the action of **2**, **3** and **12** (10 μg/mL) on the replication of HIV-1 Is 13, which was little or not inhibited in PBL and highly stimulated in macrophages.[5]

GAGS-Sepharose type I **A**, **B**, **C** resins were then prepared[14] by coupling the carboxyl groups of the oversulfated GAGS **2**, **3** and **12**, all very active in vitro on HIV-1 replication (Table 2 and Ref. 5), with the aminohexyl groups of AH-Sepharose 4B, with the aid of a water-soluble carbodiimide (Table 2); GAGS-Sepharose type II

Table 2. Characterisation of GAGS-Sepharose gels and their capacity of retention of HIV-1 IIIB and Is 13

Gel	Code	GAGS[a]	MW[a]	SI[a]	Bound GAGS (mg/mL)	HIV-1 binding[b] (%)			HIV-1 recovery[c] (%)
						IIIB[d]	IIIB[e]	Is 13[e]	IIIB[e]
Type I	**A**	DS **2**	32	2.27	1.1	85	84	>95	18
	B	DS **3**	30	2.81	2.4	96	83	>95	15
	C	HP **12**	11	2.2	3.4	76	82	>95	11
Type II	**D**	DS **2**	32	2.27	1.3	82	90	>95	2
	E	DS **3**	30	2.81	2.9	88	94	>95	3
	F	HP **12**	11	2.2	1.9	75	71	>95	2
Type III	**G**	DS **7**	6	2.0	1.6	<5	n.d.[f]	n.d.[f]	n.d.[f]
	H	CS **11**	10	2.7	1.8	<5	n.d.[f]	n.d.[f]	n.d.[f]
	I	HP **13**	10	2.1	1.0	<5	n.d.[f]	n.d.[f]	n.d.[f]

[a] From Table 1.
[b] Calculated as the ratio between the amount of p24 retained by the gels and the amount of p24 applied onto the column ×100. Average of a double (±15%) for HIV-1 IIIB and of twice a double determination for Is 13.
[c] Calculated as the ratio between the amount of p24 eluted with spermine solution and the amount of p24 retained by the columns ×100. Average of two determinations (±15%).
[d] Flow loading.
[e] Stationary loading.
[f] Not determined.

D, **E**, **F** resins were prepared from the same GAGS, via a reductive amination,[15] with sodium cyanoborohydride, of the condensation product between the terminal hemiacetal function of the polysaccharides and the aminohexyl groups of AH-Sepharose-4B; GAGS-Sepharose type III **G**, **H**, **I** resins were prepared by coupling DS-type **7**, CS-type **11** and HP-type **13**, through their free amino groups, to CNBr-activated Sepharose 4B.[16] The amount of GAGS bound to the resins was determined colorimetrically by the toluidine blue assay[17] and ranged between 1 and 3 mg/mL resin gel (Table 2).

All nine types of modified gels were packed into plastic columns, which were used to capture HIV-1 IIIB from a fluid medium. The original stock of the two viral strains was diluted up to a viral load corresponding to 500 pg/mL of HIV-1 p24 antigen, more than four times higher than the concentration usually found in the biological fluids of AIDS patients,[18] and close to the upper limit of the linearity range for p24 standard assay determination, thus allowing for direct titration, without any further dilutions, potential source of error.

HIV-1 IIIB culture medium was percolated through the columns and the retention rate was calculated as the difference between the p24 loaded onto the column and the total amount recovered in the collected fractions. Resins **A**–**F**, derivatised with the compounds most reactive in vitro (**2**, **3**, **12**), gave the best results, proving very efficient, thus supporting the hypothesised mechanism of virus capture. With this respect, there is no apparent significance in the different performance of type I resins **A**–**C**, obtained by amidation, and type II resins **D**–**F**, obtained by reductive amination (Table 2). Furthermore, all three type III resins **G**–**I** proved practically unable to capture HIV-1 IIIB (Table 2), although bound GAGS DS **7** (**G**) and HP **13** (**I**) proved rather active viral inhibitors, and the amount of bound GAGS was in the same range as in **A**–**F**. The incapacity to capture the virus is thus to be apparently attributed either to the way of binding used in such cases, or to differences between the two matrixes AH-Sepharose and CNBr-activated Sepharose as such. What is known is that the electrophylic groups of CNBr-activated Sepharose are imidocarbonates, which are formed bridging two adjacent hydroxyls of the same polysaccharide or two different polysaccharide chains.[19] A number of different products form upon coupling, most of which are probably isourea derivatives, *N*-substituted imidocarbonates, *N*-substituted carbamates.[20]

The ability of **A**–**F** to capture both HIV-1 IIIB and Is 13 was tested under static conditions, by letting the virus stay 30 min in the column before starting the elution, and confirmed the results above obtained for IIIB strain under dynamic conditions; in particular, the primary isolate Is 13 was apparently completely removed from the fluid (Table 2).

With the aim of recovering HIV-1 IIIB bound to the resins **A**–**F** under static conditions, the columns were extensively washed, although p24 had already disappeared from the eluate (i.e., <5 pg/mL), and were then eluted with a spermine solution as a potential competitor for the binding of the GAGS to the virus. The collected fractions were titrated for the presence of viral p24, having verified no interference by spermine present in the samples; only a small part of the bound virus was recovered in this way, with a minor recovery from type II resins than from type I (Table 2).

In summary, oversulfated GAGS, bound to insoluble matrixes, can capture cell-free HIV-1 from infected cell culture medium very efficiently. The IIIB laboratory strain was 70–95% retained by some of the resins,

depending on the GAGS, and only small amounts could be then eluted back; the clinical isolate 13, taken from an AIDS patient and therefore more representative of HIV-1 really harboured in infected patients, was totally removed from the infected fluid.

1. Experimental

1.1. Chemistry

Natural DS, CS and HP sodium salts (Table 1), supplied by Opocrin SpA, were transformed into triethylammonium salts by SCX chromatography in order to increase their solubility in DMF. DS-type derivatives (equivalent to 1 g of the original sodium salt) were oversulfated by treatment with adducts of SO_3–TEA in a 3:1 (**1** to give **2**) or 7:1 (**1** to give **3**, **4** to give **4a**, **5** to give **6**) excess by weight, for 5 h in DMF (3.5 mL/g of SO_3–TEA added), at rt, the suspension was then treated with water, adjusted to pH 6.5 with NaOH, then either added with ethanol saturated in sodium acetate and filtered, or lyophilised, and the solid products were dissolved in water, dialysed and precipitated with MeOH,[8] giving ca. 75% recovery. CS-type samples were oversulfated by dissolving their triethylammonium salt (equivalent to 1 g of the original sodium salt) in the minimum volume of DMF, adding chlorosulfonic acid 6:1 excess by weight, heating for 1 h, at 50 °C (**8** to give **8a**, **9** to give **10**), neutralising with methanolic sodium acetate, precipitating the products with cold acetone, filtering, redissolving raw oversulfated CS in water, dialysing and then precipitating with MeOH,[9] to give ca. 60% recovery. ^{1}H NMR showed that in DS, upon sulfation, the intensity of the signals at 4.4 ppm of protons bound to C-6 of GalNAc residues increases; furthermore, ^{13}C NMR showed that the signal of C-6 of GalNAc moved from 62 to 69 ppm; sulfation at O-2 of IdoA moved the vicinal C-1 signal from 104.5 to 101.5 ppm; in CS sulfation at O-2 GlcA moved the vicinal C-1 signal from 106.5 to 103.0 ppm, in accordance with what previously reported.[21–23] *N*-Deacetylation (**4** to give **5**, **8** to give **9**, **12** to give **13**) was obtained via hydrazinolysis of *N*-acetylglycosamine residues, by dissolving the samples (2 g) in 40 mL of anhydrous hydrazine in the presence of 1% (w/v) hydrazine sulfate, heating for 5 h at 100 °C,[12] evaporating the liquid phase under reduced pressure, adding toluene to promote the removal of hydrazine, taking up the residue with water, neutralising with 1 M HCl, dialysing against distilled water and lyophilising, to give ca. 70% recovery. The acetyl removal produced a decrease of the ^{1}H NMR signal at ca. 2.2 ppm; ^{13}C NMR showed the loss of the acetyl methyl at ca. 25 ppm, and the shift of the C-2 signal from ca. 54 to ca. 50 ppm, in accordance with what previously reported.[24]

N-Desulfation (**6** to give **7**, **10** to give **11**) was obtained by controlled acid hydrolysis,[25] dissolving the samples (1 g) in 9 mL water and heating at 60 °C, adding then 0.4 mL of concentrated HCl and water to a final volume of 10 mL, leaving for 4 h, diluting with water, adjusting to pH 6.0 with NaOH 2 N, precipitating the product with MeOH, to give ca. 90% recovery. ^{13}C NMR confirmed the selectivity of *N*-desulfation, since the signals of the sulfated alcohol carbons remained practically unaltered.

The molecular masses were determined by SEC-HPLC using a polynomial third order curve calculated on appropriate MW standards.[26] The sulfation index (SO_3H/CO_2H) was determined by potentiometric titration in water, so obtaining clear flexes.[27] The *O*-6, *O*-4 sulfate group distribution in CS type C was assessed by digestion with chondroitinase ABC (EC 4.2.2.5), followed by SAX-HPLC, as previously described.[27] Oversulfated polysaccharides are instead not accepted by the enzyme, so that the position of their sulfate groups was assessed by ^{1}H and ^{13}C NMR[21–23] on intact GAGS. The absence of contamination by heparin in DS and CS samples was determined as previously described.[3]

1.2. Coupling of oversulfated GAGS to AH- and CNBr-Sepharose 4B

GAGS-Sepharose type I. One gram of suction-dried AH-Sepharose 4B (Sigma–Aldrich) was added to 2.5 mL of an aqueous solution containing 9 μmol of the polysaccharide, calculated on the basis of MW as determined by GPC, adjusting the pH of the suspension to 4.75 with 0.1 M HCl, then 1 mL of a 3% solution (w/v) of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) was added to the suspension with continuous stirring and the pH was readjusted to 4.75. When the pH remained constant for 15 min a second millilitre of EDC solution was added and the pH regulated again, repeating the process with a third millilitre of EDC solution and keeping the mixture overnight at 4 °C,[14] the reaction mixture was then filtered and the residue was washed with 4 M NaCl. To remove free amino groups remaining in the gel, the mixture was first washed with water and then suspended in 1 mL of 0.2 M sodium acetate and 0.5 mL of Ac_2O,[14] after an incubation of 30 min at 0 °C another 0.5 mL of Ac_2O was added, the suspension was incubated for 30 min at room temperature and then washed with water, 0.1 M NaOH, water and 4 M NaCl. GAGS-Sepharose type II. One gram of suction-dried AH-Sepharose 4B (Sigma–Aldrich) suspended in 3 mL of 0.2 M phosphate buffer (pH 7.0) containing 9 μmol of polysaccharide, on the basis of MW as determined by GPC, and 3 mg of $NaCNBH_3$, was incubated at room temperature for 3 days with horizontal shaking,[15] then the gel was washed with water and the amino groups remaining on the gel were acetylated as

described above. GAGS-Sepharose type III. Three millilitres of swollen gel of CNBr-activated Sepharose 4B (Sigma–Aldrich), were mixed with 6 mL of 0.1 M bicarbonate buffer (pH 9.0) containing 0.5 M NaCl and 9 μmol of the polysaccharide, on the basis of MW as determined by GPC. The reaction must be carried on at such a pH that the amino groups are not protonated, however, the reaction efficacy declines at pH > 10.[16] The suspension was kept at room temperature for 2 h with end over end shaking; residual reactive groups of the gel remaining after coupling were blocked by adding ethanolamine 1 M at pH 8.0; after 2 h, the gel was washed with sodium acetate (0.1 M, pH 4) and coupling buffer, each containing 0.5 M NaCl. All derivatised matrixes were stored at 4 °C in 4 M NaCl. The amount of polysaccharides bound to the matrixes is reported in Table 2 and was determined colorimetrically by the toluidine blue assay.[17]

1.3. Virology

The stock of lymphotropic HIV-1 IIIB strain was obtained from the supernatant of chronically infected cell line H9/IIIB[28] and the stock of X4 HIV-1 Is 13 was obtained by growing the clinical primary isolate in peripheral blood lymphocytes from healthy donors.[5] The culture medium was then diluted with PBS (Phosphate Buffered Saline, pH 7.4, Sigma), to a viral load corresponding to 500 pg/mL of HIV-1 p24 antigen (Vironostika HIV-1, Antigen Microelisa System, BioMerieux, Boxtel, NL).

For the evaluation of cell toxicity the replication of the host cells used for the tests proved unaffected by the presence of the GAGS at 100 μg/mL, by incubation for 4 days, in triplicate. The cell viability was assessed by the MTT assay[29] (data not shown). The activity of the GAGS on HIV-1 IIIB replication was evaluated in C8166 T cells (5×10^5 cells/mL/well) suspended in RPMI, 10% Foetal Calf Serum containing the compounds. After 30 min of incubation at 37 °C, in 5% CO_2, in the presence of the compounds, HIV-1 was added and the cultures were re-incubated. The infection was carried out by exposing the cells to the equivalent of 20 ng of p24 of the virus, as previously described.[3] After 2 h, the medium was removed and the cells were repeatedly washed in order to eliminate the unbound viral particles, then re-suspended in fresh culture medium containing the GAGS and incubated. After 4 days, the extent of viral replication was determined by measuring the concentration of viral p24 in the supernatant obtained by centrifugation. The compounds were tested in triplicate at the concentrations of 0.01, 0.1, 1 and 10 μg/mL. The antiviral activity is expressed as EC_{50}, the concentration inhibiting 50% viral replication, extrapolated from the experimental results (Table 2). The percent inhibition was calculated with respect to untreated, infected cultures. EC_{50}s as average of three determination with e.s.d. in parentheses: **1**, **4**, **8**, **9**, **10**, **11** > 10 μg/mL, **5** 5 (1.5) μg/mL, **6**, **7** 1 (0.3) μg/mL, **2** 0.6 (0.2) μg/mL, **12**, **13** 0.5 (0.3) μg/mL, **3** 0.05 (0.04) μg/mL. The effects of **2**, **3** and **12**, at the concentration of 10 μg/mL, on the replication of HIV-1 Is 13 were previously reported.[5]

For the viral retention assay with continuous load, 2 mL of each GAG-linked Sepharose were placed into the plastic column (7 × 1 cm), in double, and equilibrated with PBS. The columns were run at 0.5 mL/min. Four millilitres of a supernatant HIV-1 IIIB solution in PBS, corresponding to 500 pg/mL of p24 antigen, were loaded continuously onto the columns and allowed to run into them by gravity. The columns were then washed with 8 mL of PBS and 1 mL fractions were collected. The concentration of p24 was determined in every millilitre eluted from the column. The virus started appearing from the 3rd fraction, slowly decreased from the 5th to 10th, and was always undetectable in the 11th and 12th fraction. The p24 concentration was determined in every fraction. The retention rate was calculated by the evaluation of the p24 loaded onto the column and the total amount recovered in the collected fractions.

For the viral retention assay with stationary load, 2 mL of the HIV-1 IIIB or Is 13 supernatant solution in PBS, corresponding to 500 pg/mL of p24 antigen, were applied onto the columns, in double for IIIB and twice in double for Is 13, and let stay for 30 min at room temperature. Then it was adopted the same procedure as for the continuous load.

The elution of the virus particles retained by the gel was attempted by eluting with 4 mL of a spermine solution (1 mg/mL, pH 7.4). The concentration of p24 was determined in all the eluted fractions. The recovery rate was calculated by the ratio between the amount of p24 eluted in the spermine solution and the amount of p24 bound to the columns.

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