Nondegradative Sulfation of Polysaccharides. Synthesis and Structure Characterization of Biologically Active Heparan Sulfate Mimetics

Dulce Papy-Garcia,[†,‡] Véronique Barbier-Chassefière,[†] Vincent Rouet,[‡] Marie-Emmanuelle Kerros,[†] Cécile Klochendler,[‡] Marie-Claude Tournaire,[‡] Denis Barritault,[†,‡] Jean-Pierre Caruelle,[‡] and Emmanuel Petit*[,†]

Société OTR³ SAS; 4, rue Française, 75001 Paris, France, and Laboratoire de Recherche sur la Croissance, la Réparation et la Régénération Tissulaires (CRRET), CNRS UMR 7149, Université Paris 12-Val de Marne, 61 Avenue du Général de Gaulle, 94010 Créteil Cedex, France

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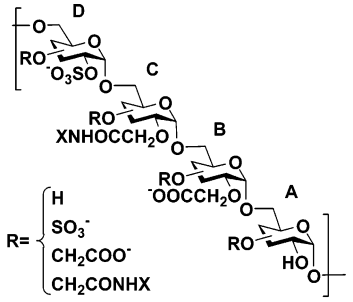
ABSTRACT: Reproducible and nondegradative preparation of sulfated molecules exhibiting diverse biological properties requires caring out sulfation reactions under ready controlled mild conditions. Although, to date, sulfur trioxide is the most used sulfation agent, its highly acid character has brought its use in association with different nitrogen bases as sulfur trioxide−nitrogen base complexes and/or by introduction of basic solvents as pyridine. We have applied sulfur trioxide and other sulfation agents including protocols for the synthesis of biologically active sulfated polysaccharides and demonstrated that these agents provoke cleavage of glycosidic bonds and other acid labile functions as amides, esters and even ethers. These facts prompted us to develop new reaction conditions for a general and nondestructive sulfation protocol. Our approach consists of the introduction of 2-methyl-2-butene as an acid scavenger of neutral character. Application of the method leads to an efficient, reproducible, and controlled synthesis of acid labile dextran derivatives well-known to be active in tissue repair and recently proposed for example as a new therapeutic agent for prion diseases. A novel ^{1}H NMR structural analysis of this kind of macromolecules is presented. This method offers a new advance for more efficient synthesis of biologically active sulfated macromolecules.

Introduction

Many components of biological origin are polyanionic macromolecules which interactions are of great importance in several biological processes. Synthetic polyanions similar to those naturally occurring have been the subject of considerable interest, for instance, as therapeutic substitutes of natural products or in connection with the mechanistic studies of their biological reactions.[1−4] Heparan sulfate (HS), a member of the glycosaminoglycan (GAG) family of macromolecules, stands out as a key player in the regulatory network of the cell. Its highly variable structure, owing to the polymorphed nature of the differently sulfated sequences within its chains, makes of HS a family of related molecules exerting a large diversity of biological activities.[5−7]

It has been reported that a family of dextran-based molecules, structurally related to HS by means of their polysaccharidic backbone containing sulfate, carboxylate and hydroxyl functionalities (Chart 1), possess the ability to stimulate tissue repair.[8−10] Certain heparan sulfate mimetics (HM), also called RGTAs (for regenerating agents), acted as functional mimetics of HS in terms of stabilizers, protectors, and potentiators of endogenously released heparin-binding growth factors (HBGF).[11−14] We have recently found that HM bind to vascular endothelial growth factor (VEGF), a specific angiogenic growth factor, and potentiate its angiogenic activity both in vitro and in vivo (unpublished results). Moreover, other HM have been shown to potently inhibit the replication and the endocytosis of the scrapie prion protein.[15,16] From their potential pharmaceutical significance, HM type macromolecules constitute an attractive chemical approach for the synthesis of therapeutic and prophylactic agents. Previous reports considering the synthesis of HM type molecules have appointed two basic synthetic approaches differing notably on the sulfation system, one based on the use of chlorosulfonic acid in a heterogeneous reaction mixture[17,18] and the second on the use of sulfur trioxide (SO_3) complexes in a homogeneous reaction.[19] It is known that, because of its highly acidic nature, chlorosulfonic acid presents multiple and nonreproducible side reaction effects such

Chart 1. General Structure of HM[a]



OTR4120 composition : A<1%, B=32%, C=0%, and D=67%

[a] According to the nature of the group present at the position 2 (C2) of the glucosidic unit, four differently substituted units (A, B, C, and D) can be present in HM polymers. Identification of each unit on the polysaccharide is possible by 200 MHz ^{1}H NMR analysis as showed in Figure 1. For an easier representation these units were here arranged in an arbitrary combination. In addition, R represents the proportion of substituted group in the global C3 and C4 positions arranged to set the global ds of each group. The OTR4120 composition, as stated by titrimetry, elemental analyses and 1H RMN (200 MHz) studies, is given.

* Corresponding author to whom correspondence and requests for reprints should be addressed. Telephone: (33) 870724453. Fax: (33) 145171816. E-mail: e.petit@univ-paris12.fr.
[†] Société OTR³ SAS.
[‡] Université Paris 12-Val de Marne.

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as cleavage of labile functional groups, presence of hazardous and nonreproducible sulfation rates and patterns along the insoluble polysaccharide chain, and backbone degradation.[20−23] To partially limit the highly acidic character of chlorosulfonic acid, pyridine has been employed as the reaction solvent, making the reaction mixture homogeneous but keeping an important degree of depolymerization accompanied by a real difficulty for solvent elimination, which in part resulted from the introduction of pyridinium groups at the reducing end groups of the polysaccharides.[22,23]

In the other hand, sulfur trioxide or its nitrogen base complexes have been well-established agents for sulfation of alcohol or amine bearing molecules in aprotic solvents such as pyridine, dimethyl sulfoxide (DMSO), or dimethylformamide (DMF). However, even when the reaction is conducted by adding the sulfating reagent to a tetrabutylammonium salt of a polysaccharide in solution, their use induces cleavage of acid labile groups, as amides or esters, and partial depolymerization.[19,24] Moreover and in addition to a covalent introduction of the base at the reducing end of the sugar chain, ionic complexation of charged amino species with the sulfated polysaccharides may considerably affect its physicochemical or biological properties.[24−27] A SO_3−DMF complex has also been widely employed, but its instability and highly acidic character implies the use of pyridine as the solvent of choice.

In this report, we describe a nitrogen base-free sulfation protocol which avoids depolymerization reactions and cleavage of acid labile groups by the introduction of an acid scavenger of neutral nature, 2-methyl-2-butene (2M2B). The presence of this low molecular weight compound during the sulfation reaction eludes the use of amines in the SO_3 complex and the use of pyridine as solvent. HM were produced in a highly efficient manner with a high degree of purity with nondegradation of the macromolecular chain nor partial hydrolysis of other functionalities as carboxymethyl ethers, esters and amides, observed by using other techniques. The degree of sulfation was easily controlled by the reactive extent. Reproducibility of the method was validated by the synthesis of a particular compound called OTR4120 and considered as a functional mimetic of heparin or HS. OTR4120 was structurally characterized by the global degree of substitution (ds) of the different substituted groups in the polymer, and more fine structure was determined by high-resolution ^{1}H RMN spectroscopy. Biological properties of different batches of OTR4120 were performed in accordance with heparin properties by a VEGF binding assay and by a in vitro mitogenic assays.

Application of this method to sulfation of low and high molecular weight molecules, in particular of glycosidic character, offers a new approach for more efficient synthesis of biologically active sulfated compounds.

Experimental Section

Dextran 40 USP was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). 2M2B, bovine serum albumin, heparin, SO_3−pyridine, SO_3−trimethylamine (SO_3−Me_3N), SO_3−triethylamine (SO_3−Et_3N), and SO_3−dimethylformamide (SO_3−DMF) complexes and all other chemicals were from Sigma-Aldrich (Saint Quentin Fallavier, France). Human recombinant $VEGF_{165}$ was expressed in Sf9 insect cells and purified by cation exchange and heparin-affinity chromatography, as previously reported.[28] BCA protein assay kit and Iodobeads were purchased from Perbio Science France (Bezons, France). DMEM culture medium was from Invitrogene (France). Pyrogen free water was obtained by a Milli-Q system dotted with a Pyrogard D ultrafiltration cartridge (Millipore SAS, France). Water and products qualities were systematically controlled by the endotoxine-detecting test Limulus Amebocyte Lysate QCL-1000 (Bio-Whittaker, France). All aqueous solutions were prepared using this water.

Product Purification and Structure Characterization. Product purification was systematically achieved by tangential ultrafiltration on a 1000 normal-molecular-weight cutoff (NMWCO) regenerated cellulose membrane (Pellicon2, 0.5 m^2, Millipore, MA) against 5 L of NaCl 1 M and then 20 L of Milli-Q water. The resulting concentrated solution was freeze-dried. Pure dry products were homogenized to obtain a fine powder by a Universal mill A10 IKA (IKA-WERKE GMBH & CO. KG, Germany). ^{1}H NMR spectra were recorded with a 200 MHz Bruker spectrometer and with a 600 MHz Varian spectrometer from samples in D_2O using residual H_2O peak as a standard (4.805 ppm). Absolute determination of molecular weights and size distributions were performed on polysaccharide solutions by a size exclusion chromatography (SEC) eluted in 0.1 M $LiNO_3$ coupled to a multiangle laser light-scattering photometer (MALLS; Dawn DSP−F, Wyatt Technology, Santa Barbara, CA) connected in series to a differential refractive index detector (RI, ERC 7515A, Erma Cr. Inc., France). An TSK Gel G3000 PWXL (TosoHaas, Cambridge, U.K.) column was used for polysaccharide analysis. Degrees of substitution (ds), defined as the number of substituted carboxymethyl (dsCM), carboxymethyl amide (dsX), and sulfate (dsS) groups, contained in global glucosidic units, were determined by resolving the equation system stated below.

$$\mathrm{dsCM} = \frac{(162T_1) + (M_w\mathrm{X} \times T_1 \times \mathrm{dsCM}_1)}{10^3 + (M_w\mathrm{X} \times T_1) - (80T_1) - (102T_2)}$$

$$\mathrm{dsS} = \frac{(162T_2) + (M_w\mathrm{X} \times T_2 \times \mathrm{dsCM}_1)}{10^3 + (M_w\mathrm{X} \times T_1) - (80T_1) - (102T_2)}$$

$$\mathrm{dsX} = \mathrm{dsCM}_1 - \mathrm{dsCM}$$

where T_1 and T_2 represent, respectively, the carboxylate and the sulfate contents (mequiv/g) obtained from potentiometric 0.2 M NaOH titration of protonated samples performed with a Metrohm 682 Titroprocessor. dsCM_1 represents dsCM before amidation and M_wX corresponds to the molecular weight of the carboxymethyl amide. dsS and dsX were confirmed by elemental analysis of sulfur and nitrogen contents (Service de Microanalyse, Gif sur Yvette, France) by using equations below in where % S and % N represent, respectively, the sulfur and nitrogen content on the pure sample.

$$\%\,\mathrm{S} = \frac{32 \times \mathrm{dsS}}{162 + (80 \times \mathrm{dsCM}) + (M_w\mathrm{X} \times \mathrm{dsX}) + (102 \times \mathrm{dsS})} \times 100$$

$$\%\,\mathrm{N} = \frac{14 \times \mathrm{dsX}}{162 + (80 \times \mathrm{dsCM}) + (M_w\mathrm{X} \times \mathrm{dsX}) + (102 \times \mathrm{dsS})} \times 100$$

Statement of depolymerization was achieved by the reducing sugars quantification assay as described below.

Synthesis of Carboxymethyl Dextran (CMD). Dextran 40 USP (300 g, 1.85 mol of glucose) was dissolved in 1460 mL of water, and separately, 592 g of NaOH (14.3 mol) was dissolved in 590 mL of water. Both solutions were cooled to 4 °C. The NaOH solution was slowly poured into the dextran solution under stirring and controlling temperature not to exceed 15 °C. The reaction mixture was stirred for 20 min and then allowed to cool at 4 °C. Monochloroacetic acid (613 g, 6.5 mol) was added in small portions with controlling reaction temperature <20 °C, and then the reaction mixture was stirred at 50 °C during 40 min. The reaction was then quenched by slow addition of acetic acid to pH 7. The final product was

Table 1. Comparative Structural Data for HM Type Polysaccharides Synthesized by Different Sulfation Agents or Reaction Conditions[d]

entry	sulfation agent	reaction conditions	dsCM[a]	dsS[a]	reducing sugars[a] (nmol glu/mg)
1[b]			0.56		1.03
2	$ClSO_3H$	1 equiv/22 °C	0.41	0.22	11.04
3	$ClSO_3H$	2 equiv/22 °C	0.37	0.35	12.71
4	$ClSO_3H$	2 equiv/4 °C	0.50	0.15	4.04
5	SO_3-Me_3N	2 equiv/30 °C	0.49	0.05	1.98
6	SO_3-Et_3N	2 equiv/30 °C	0.29	0.40	2.61
7	SO_3−pyridine	2 equiv/30 °C	0.44	0.17	1.84
8	SO_3−DMF	2 equiv/30 °C	0.37	0.23	1.57
9	SO_3−DMF/2M2B[c]	2 equiv/30 °C	0.56	0.42	0.82
10	SO_3−DMF/2M2B	2.5 equiv/30 °C	0.57	0.89	0.77
11	SO_3−DMF/2M2B	3 equiv/30 °C	0.57	1.00	0.71
12	SO_3−DMF/2M2B	5 equiv/30 °C	0.55	1.20	0.50
13	SO_3−DMF/2M2B	8 equiv/30 °C	0.56	1.40	0.35
14	SO_3−DMF/2M2B	10 equiv/30 °C	0.53	1.41	0.38

[a] Each sample was analyzed at least 3 times with a coefficient of variation inferior to 8% for any of the performed analysis. [b] Starting material: CMD (ds = 0.56). [c] 2M2B implied reactions were carried out in a 2M2B/DMF/formamide ratio of 1:4:1, which guarantees a 1.5−8 molar excess of 2M2B vs the sulfation agent without difference in reaction efficacy and smoothness. [d] Reaction time was 2 h in every case.

Table 2. Composition Data of Different Batches of OTR4120 Prepared by SO_3−DMF (5 equiv) in the Presence of 2M2B/DMF/Formamide (1:4:1) at 30 °C for 2 h

product batch	dsCM (cv)[a]	dsS (cv)	reducing sugars (nmol glu/mg) (cv)	$\overline{M_w}$[b] (cv)	$\overline{M_n}$[c] (cv)	$\overline{DPn}$[d] (cv)
CMD	0.56		1.03	54 000	39 000	187
OTR4120−1	0.55 (4.8)	1.20 (0.6)	0.50 (1.0)	86 000 (1.2)	60 000 (0.4)	183 (1.1)
OTR4120−2	0.49 (6.7)	1.17 (3.1)	0.49 (3.0)	81 000 (4.7)	57 000 (6.4)	178 (3.8)
OTR4120−3	0.58 (10.5)	1.23 (1.9)	0.55 (8.9)	87 000 (2.4)	62 000 (2.9)	186 (0.5)
OTR4120−4	0.48 (8.6)	1.22 (1.0)	0.48 (5.0)	86 000 (1.2)	62 000 (2.9)	191 (3.2)
median[e]	0.53	1.21	0.51	85 000	60 000	185

[a] cv: coefficient of variation, defined as the percent relative standard deviation from the four reaction median. Each product was analyzed 3 times. [b] $\overline{M_w}$: average molecular weight in daltons. [c] $\overline{M_n}$: number-average molecular weight in daltons. [d] $\overline{DP_n}$: degree of polymerization. [e] Median from the four D120 batches.

purified by tangential ultrafiltration of the resulting aqueous solution using a 1000 or 10000, when specified, molecular-weight cutoff membrane, followed by freeze-drying as described above.

For preparation of products with higher carboxymethyl content the same procedure was repeated two or three times in order to obtained desired dsCM.

Amidation Reactions. CMD (5 g, dsCM = 1.1, 21.5 mmol of COO^-) was dissolved in 136 mL of water, and then 71 mL of acetone was added. The temperature was kept at 40 °C. To activate the carboxylic functions, 5.3 g of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (21.5 mmol) in 20 mL of acetone was added, and the reaction mixture was stirred for 20 min at 40 °C. The final solvent was composed by a ratio 60/40 water and acetone. Then, 2.5 mL of isobutylamine (21.5 mmol) or the desired amine was added and the pH was adjusted to 7 by HCl 4 M. The reaction was stirred at 40 °C overnight. The final product was purified by tangential ultrafiltration of the resulting aqueous solution followed by freeze-drying as described above.

Chlorosulfonic Acid Mediated Synthesis of Sulfated Polysaccharides. CMD (5.0 g, dsCM = 0.56, 24.3 mmol of glucose) was suspended in 200 mL of dichloromethane. 1.6 mL of chlorosulfonic acid (24.3 mmol) was slowly added and the reaction mixture was stirred at room temperature for 2 h. After filtration of the resulting suspension through a ceramic filter (porosity 4), the resulting powdered material was washed twice with 100 mL of dichloromethane, three times with a 1:1 mixture of dichloromethane/dioxane (100 mL), and once with 100 mL of dioxane. The resulting material was immediately dissolved in 200 mL of a 5% aqueous solution of $NaHCO_3$ and the pH controlled so that it was not acid. The final product was obtained by tangential ultrafiltration followed by freeze-drying as described earlier in the Experimental Section.

SO_3-Complexes Mediated Synthesis of Sulfated Polysaccharides in the Absence of 2M2B. The next protocol was used for SO_3-Me_3N, SO_3-Et_3N, SO_3−pyridine, and SO_3−DMF complexes. CMD (5.0 g, dsCM = 0.56, 24.3 mmol of glucose) was dissolved in 200 mL of formamide and stirred to complete dissolution. A SO_3−pyridine complex (7.7 g, 48.6 mmol) was rapidly added, and the reaction mixture was stirred at 30 °C for 2 h. The reaction was then quenched by slowly pouring into 200 mL of a 5% aqueous solution of $NaHCO_3$ under stirring with continuous pH control. The final product was obtained by tangential ultrafiltration of the resulting aqueous solution followed by freeze-drying as described above.

SO_3−DMF Mediated Synthesis of Sulfated Polysaccharides in the Presence of 2M2B. An aqueous 10 g/L solution of CMD (5.0 g, dsCM = 0.56, 24.3 mmol of glucose) was protonated with an acidified sulfonic resin (Amberlite IR120, Sigma) and dried as previously to give CMD−H^+. The resulting product was dissolved in 40 mL of formamide and 160 mL of DMF. After complete dissolution, 40 mL of 2M2B (26.5 g, 378.6 mmol) was slowly added. A SO_3−DMF complex (7.4 g, 48.6 mmol) was rapidly added, and the reaction mixture was stirred at 30 °C for 2 h. The reaction was quenched by slowly pouring it into 200 mL of $NaHCO_3$ and the final product was purified by tangential ultrafiltration followed by freeze-drying as described above. Amidated products were not protonated before sulfation.

Reducing Sugars Quantification. Polysaccharides reducing sugar quantification was performed by a modified bicinchoninate protocol as follows. Reagent A was prepared by dissolving 6.35 g of $NaHCO_3$ and 2.42 g of Na_2CO_3 in 90 mL of water. This solution was used to dissolve 194.2 mg of 4,4′-dicarboxy-2,2′-biquinoline and completed to 100 mL. Reagent B was prepared by dissolving 124.8 mg of $CuSO_4 \cdot 5\ H_2O$ and 126.2 mg of L-serine in 100 mL of water. To 100 μL of each sample solution was added 250 μL of the A/B mixed reagent (1:1 v/v), and it was incubated at 100 °C during 15 min. After cooling for 10 min at 20 °C, 200 μL of the resulting solution was deposed in an ELISA type 96 wells plate. Absorbance

Table 3. SO_3−DMF Mediated Sulfation of Different Polysaccharides in the Presence of 2M2B[c]

entry	starting material polymer[a] [scale, g]	composition: starting material dsCM	starting material dsX	product dsCM	product dsX	product dsS
1	CMD[a] [10]	0.56		0.56		1.18
2	CMD [24]	0.56		0.55		1.17
3	CMC[a] [4.5]	0.60		0.59		1.19
4	CMD−isobutyl amide[b] [2]	1.03	0.17	1.03	0.17	0.36
5	CMD−ethylhexylamide[b] [2.5]	1.00	0.20	1.02	0.18	0.47
6	CMD−phenylalanine[b] *tert*-butylester [2]	0.88	0.32	0.89	0.31	0.38
7	CMD−benzylamide[b] [3]	0.76	0.44	0.80	0.40	0.39

[a] Abbreviations: CM, carboxymethyl ether; D, dextran; C, cellulose. [b] Products were prepared from a CMD containing a dsCM = 1.2 before the amidation reaction. [c] Reaction time was 2 h in every case.

Table 4. Chemical Shifts and Peak Assignment of OTR4120 Anomeric Protons (600 MHz, D_2O)

unit code	chemical shift	% of the anomeric H surface	glucose unit carbon (C) substitution[a] C2	C3	C4
a	5.032	<0.2	OH	SO_3 or CM[b]	SO_3 or CM[b]
b	5.115	4.7	CM	OH	OH
c	5.153	15.2	CM	OH	CM
d	5.241	12.0	CM	OH	SO_3
e	5.284	17.6	SO_3	OH	OH
f	5.329	31.2	SO_3	OH	SO_3
g	5.376	12.7	SO_3	CM	OH
h	5.425	6.7	SO_3	SO_3	OH
i	5.475	<0.2	SO_3	SO_3	SO_3 or CM[b]

[a] Abbreviations: C, carbon; CM, carboxymethyl ether. [b] Any of the two substitutions may be allowed.

Table 5. Thrombin Time Evaluation of Synthetic OTR4120 and Heparin

polysaccharide	concentration (μg/mL)	thrombin time (s)
OTR4120	100	>300
	50	>300
	10	105.0
	5	27.0
	2.5	20.4
	1	17.3
	0	14.9
heparin	2.5	>300

readings were done at 540 nm in a multiwell plate reader (Labosystems, France). A calibration curve was prepared using glucose samples as external standards (10, 25, 50, and 100 nmol). The amount of reducing sugar on each sample was correlated to its mass and results were expressed in nmol of reducing sugar/mg of sample. Repeated samples of native dextran (0.5, 0.75, and 1.0 mg/mL; 60 nmol of reducing sugar/mg) were used as control on each experiment.

Dermal Fibroblast Proliferation Assay. Primary cultures of fibroblast from mice skin (P4−P6) were grown in DMEM medium supplemented with 10% foetal calf serum at 37 °C in a humidified atmosphere with 5% CO_2. Cells were plated in 24-well tissue culture plates (Costar, France) at an initial density of 12000 cells/well. The antiproliferative effect of each batch of HM was evaluated by counting the cells in Malassez blade each 2 days during 10 days in the presence of increasing concentrations of product (0−100 μg/mL). Each experiment was performed in triplicate. Results are presented as the percentage of growth inhibition for the dose of 100 μg/mL of each different OTR4120 batch.

Binding of ^{125}I—VEGF$_{165}$ to Coupled Heparin—BSA. Heparin was covalently linked to BSA as described previously.[29] Final protein content was determined by the BCA method as indicated by the fabricant. Human recombinant VEGF radio-iodination was carried out by the iodobeads method as indicated by the fabricant, and the iodinated GF was used at a final concentration of 200000 cpm/mL (specific activity = 200000 cpm/ng) in a PBS buffer. Each well of an ELISA type 96 wells plate was coated with 100 μL of a 2 μg/mL heparin−BSA solution (Tris−HCl 50 mM, EDTA 12.7 mM, pH 7.4) overnight at 4 °C. After three washings with PBS containing 0.05% Tween-20 (washing buffer), wells were saturated with 3% BSA in PBS for 1 h. Then, 100 μL of ^{125}I−VEGF$_{165}$ supplemented or not with heparin or with each batch of HM at increasing concentrations (0−100 μg/mL) was added to each well. After an overnight incubation at 4 °C, wells were washed five times with washing buffer, 100 μL of 0.2 M NaOH was added, and the radioactivity in each well was measured in a γ-counter (1275 minigamma counter, LKB Wallac). Results were analyzed according to the Scatchard procedure by fitting to a logistic curve using the software Ligand.[30] All experiments were carried out in triplicate.

Anticoagulant activity assay. The anticoagulant activity of the Polymer OTR4120 was determined by measuring the thrombin time (TT), which reflects the amount of time it takes to form a clot when thrombin is added to a sample of plasma. Briefly, 100 μL of plasma was added to 100 μL of saline buffer and the mixture was prewarmed to 37 °C for 3 mn. To this mixture was added 100 μL of human α-thrombin solution (10 U/mL in saline) supplemented by either OTR4120 or heparin at the concentration stated in Table 5.

Results and Discussion

Comparative Syntheses of Sulfated HM Polysaccharides by Different Methods. To compare the efficiency and smoothness of typical techniques used for sulfation of hydroxyl bearing macromolecules, a batch of carboxymethyl dextran (CMD) with a dsCM of 0.56 was prepared as indicated in the Experimental Section. This product was used as the starting point for the synthesis of several HM. Table 1 summarizes reaction conditions and analytical data obtained from products prepared with chlorosulfonic acid and with different SO_3 complexes. These two families of reagents were studied since they represent the most commonly used sulfation agents. Products dsS reflected the reaction efficiency under the given conditions. dsCM was taken as a criterion to evaluate the reaction smoothness, the original dsCM of 0.56 will decrease if reaction conditions cause cleavage of carboxymethyl groups. We parallelly evaluated the integrity of the macromolecular chain by determination of the reducing sugar content. It is important to point out that, during sulfation, all hemiacetalic reducing functions can be sulfated, resulting in a reducing sugar content inferior to that of the starting unsulfated material. Thus, an increase in this value from that of the original CMD (1.0 nmol of equivalent glucose by mg of product) indicates that new reducing sugars were generated as a result of glycosidic bond cleavage. Our results show that sulfation reactions using chlorosulfonic acid causes depolymerization as stated by the reducing sugar content of products (Table 1, entries 2−4). This degradation increases by increas-

ing the amount of reagent used (Table 1, entries 2 and 3) and decreased by decreasing the reaction temperature (Table 1, entries 3 and 4). It should be noted that use of chlorosulfonic acid causes severe depolymerization even at low temperature (Table 1, entry 4). Chlorosulfonic acid has been largely reported for the synthesis of HM related products with dichloromethane as a solvent.[17−19,21] However, this method results destructive and drastically non reproducible, the later may be due to the heterogeneous character of the reaction mixture in which the dextran derivative keeps insoluble. It is well-known that sulfated polysaccharides prepared by sulfation techniques involving the use of sulfuric or chlorosulfonic acids results in products which are impure, discolored, and possibly toxic as a consequence of a large depolymerization even at temperatures below 10 °C.[22,23]

Although SO_3 complexes have improved the preparation of HM type sulfated polysaccharides, our results show that their use carries partial deetherification and depolymerization side reactions, although in lower degree than the sulfonic acid (Table 1, entries 5−8). It is interesting to remark that among the polymers prepared with SO_3 complexes, the one treated by the SO_3−Et_3N was the more sulfated but also the one which followed the bigger fragmentation (Table 1, entry 6). Similar data was obtained with the polysaccharide's tetrabutylammonium salt as starting material (results not shown). These results are in agreement with previous reported SO_3−pyridine mediated synthesis of HM-like polymers in where a significant and not reproducible decrease on the polymer size is observed after sulfation.[19,32] Moreover, in our hands, pyridine absorbance could not be removed from polymers treated by the SO_3−pyridine complex even after elution through an Amberlite IR 120 H^+ column (50 mL). It can been explained from the found that when a polysaccharide in solution is reacted with a SO_3−nitrogen base complex or in pyridine as a solvent, a covalently bound nitrogen can accordingly be introduced at the reducing end of the polysaccharide.[22,24] Noncovalently bound pyridine elimination from the final product was extremely difficult requiring elution through an Amberlite IR 120 H^+ and several additional tangential ultrafiltration cycles. The same difficulty for solvent elimination was observed when DMSO was used.

We have avoided deetherification and depolymerization side reactions as well as use of nitrogen bases during the sulfation protocol by introducing a small excess of 2M2B, a volatile molecule (boiling point = 38 °C) of neutral character, in to the reaction medium with SO_3−DMF as the sulfation agent. 2M2B has previously been reported by Nishizawa et al.[33] as an acid scavenger able to trap acidic protons resulting from the glycosylation of protected sugars. Entries 9 and 10 of Table 1 show that in the presence of 2M2B sulfation takes place efficiently with no increase of the reducing sugar content and remaining dsCM stable. Under these conditions, a 2-fold excess of the volatile 2M2B over the sulfation agent was enough to protect the polysaccharide from acid degradation and deetherification. 2M2B was used between 2- and 10-fold excess with equal results.

Control of the Sulfation Degree on HM, Reproducibility, and Application. The sulfation reaction may be carried out to produce any desired sulfur content. Sulfation of CMD by different amounts of SO_3−DMF in the presence of 2M2B is reported in Table 1 (entries 9−14). Results show that product dsS increased with the amount SO_3−DMF to a maximum dsS of 1.41 reached with a 8−10 molar excess of sulfating agent over the starting CMD (Table 1, entries 13, 14). dsCM remained stable, and as expected, reducing sugars quantification decreased with increasing sulfation degree.

From our interest in HS mimetics, we have focused our attention to one specific compound, OTR4120, which mimics the structure of heparin at least in that concerning the polysaccharide character of its backbone and the global content of carboxylate and sulfate groups (dsCM = 0.5, dsS = 1.2). Table 2 shows the reproducibility (n = 4) of the CMD sulfation reaction in the presence of 2M2B. dsCM and dsS were constant in the four reactions as stated by the obtained coefficient of variation (cv) to the median value. The reducing sugar values were consistent with the maximum accepted variations from the median value fixed to 10%.

On HPLC (SEC-MALLS/RI) analysis of the OTR4120 products, the calculated degree of polymerizations (DP_n) did not vary from that of the starting CMD. As expected, the obtained molecular weights increased but kept reproducible between the different product batches. Together with the nonincreasement of reducing sugar content, these results indicate a nondegradation of the polymeric chain under 2M2B sulfation conditions. It is interesting to remark that the HPLC profile of products afforded variant peak symmetries depending on the cutoff of the ultrafiltration membrane used for CMD purification. When 1000 Da cutoff membranes are used, HPLC peaks are nonsymmetric, reflecting a β-elimination mediated cleavage of dextran under strong basic conditions. However, when 10000 Da cutoff membranes are used, peaks are perfectly symmetric since low molecular weight entities are eliminated (results not shown). Nevertheless, it is known that the peak symmetries will also depend on the column type used for achieving separation.

We have applied the sulfation technique to the synthesis of other HM type polymers bearing ester and amide groups as model functionalities susceptible to be cleaved during sulfation (for biological interest of these compounds see.[8,9,11,13,16] We have also modified the nature of the sugar backbone and the reaction scale. Our results are summarized in Table 3. *tert*-Butyl ester moieties and amides functions were perfectly stable under the reaction conditions (Table 3, entries 4−7). In all cases, terminal reducing end groups were stable and nondegradation was detected during sulfation. Carboxymethyl cellulose showed the same rate of reactivity than dextran derivatives with 5 equiv de SO_3−DMF (Table 3, entry 3). Increasing the reaction scale to 5-fold did not affect the rate, efficiency, and mildness of reaction (Table 3, entries 1 and 2). Our results clearly show that, in the presence of 2M2B, we are able to control the desired sulfate content on the resulting polysaccharide, that the reactions conditions are mild enough to keep intact labile groups as esters and amides and that the integrity of the polysaccharidic chain remains intact.

OTR4120 Structure Determination. HS are a family of related molecules which large diversity of biological activities exerted by differently sulfated sugar sequences. Although a vast number of biological activities have been conferred to different molecules belonging to the sulfated dextran family of compounds, no real

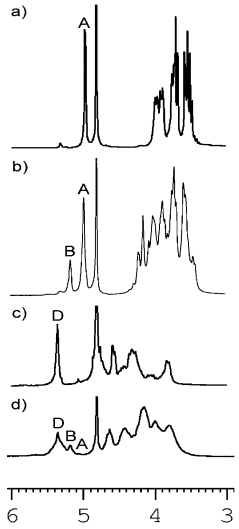


Figure 1. ^{1}H NMR (200 MHz, D_2O) spectra of (a) dextran, (b) carboxymethyl dextran, (c) dextran sulfate, and (d) carboxymethylated sulfated dextran OTR4120. The effect of the sulfate or carboxymethyl substitution at carbon 2 (C2) is clearly observed at the anomeric proton (H1) region. Anomeric signals of glucosidic units bearing different substitutions at C2 are indicated as A (OH in C2), B (CM in C2), and D (SO_3 in C2), as represented in Chart 1. Other glucosidic protons (H2−H6) are globaly shifted to the low field.

efforts have been devoted to their structure characterization and only a global ds determination of the different substituents has been referenced. Therefore, special attention was directed to improve structure characterization of dextran based HMs. In this report, we have focused our efforts to the preparation of OTR4120. Figure 1 shows the ^{1}H NMR (200 MHz, D_2O) spectra of dextran, CMD, carboxymethylated sulfated product OTR4120, as well as a noncarboxymethylated totally sulfated dextran. In this figure we note the effect of carboxymethyl and sulfate substitutions on the dextran ^{1}H NMR spectrum. When CM or sulfate substitutions are present in the glycosidic unit, dextran anomeric peak (5.01 ppm) is shifted through the low field (5.1−5.5 ppm). We have assigned the three detected anomeric peaks as follows: 5.01 ppm, OH in C2 (A); 5.18 ppm, CM in C2 (B); 5.36 ppm, SO_3 in C2 (D). This assignment was confirmed by ^{1}H RMN simulations using the ACD/labs HNMR v7.04 predictor program (Toronto, Canada). The anomeric peak's split is the result of the C2 substitution effect (β-effect), characteristic of glucopyranosyl compounds.[34] Thus, differential integration of the three possible anomeric peaks allows determination of the relative abundance of the glucosidic units A (<1%), B (32%), and D (67%) as represented in Chart 1. ^{1}H NMR (600 MHz, D_2O) spectrum of OTR4120 (Figure 2) was used for approximate determination of C3 and C4 substitution. Table 4 resumes the individual chemical shift for each anomeric peak, their relative integration value, and their respective C2, C3, and C4 substitutions. This assignment was performed, first, from the C2 substitution pattern determined by 200 MHz ^{1}H NMR followed by fine analysis of the 600 MHz spectrum combined with ^{1}H NMR simulations using the ACD/labs HNMR predictor program. Thus, the first minor peaks between 4.987 and 5.032 ppm correspond to the glycosidic units bearing a OH at C2. Previous studies using ^{1}H NMR (1D and 2D), ^{13}C NMR spectroscopy and Monte Carlo simulations have shown C2 as the most reactive position during carboxymethylation of dextran ($k_2 = 1.2 \times 10^3$ L^2/(mol^2

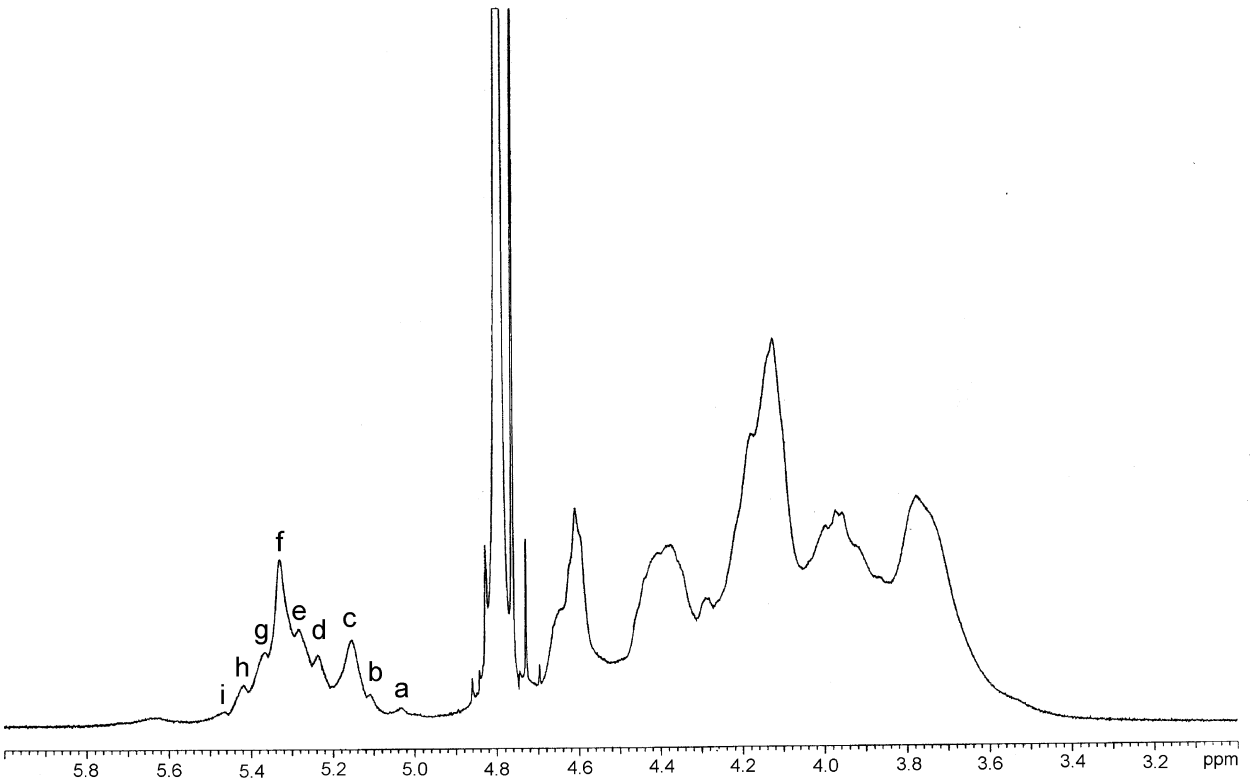
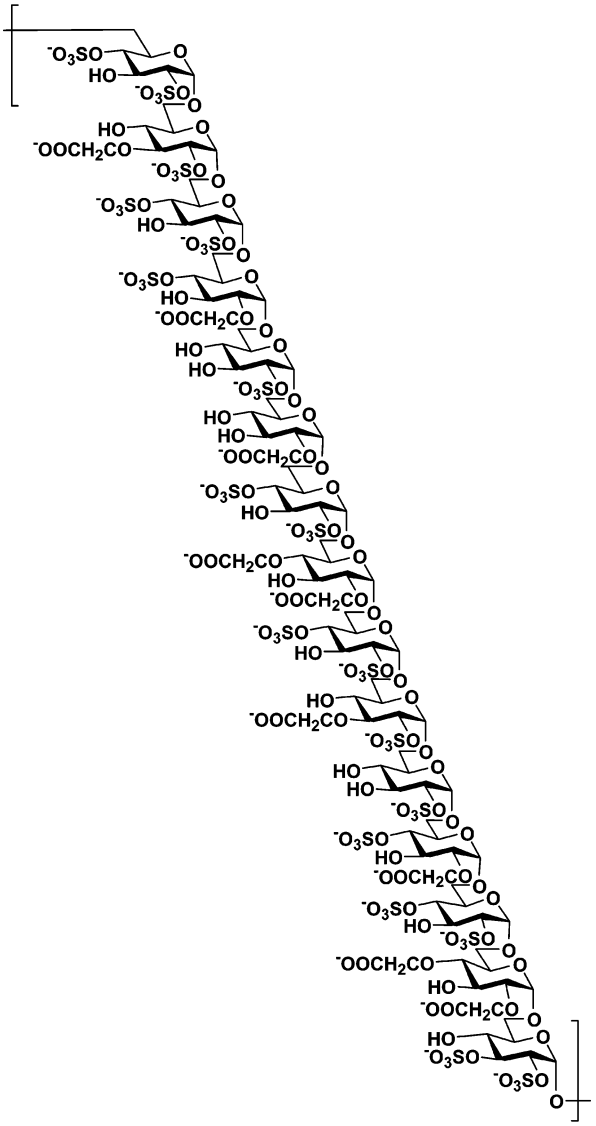


Figure 2. 600 MHz ^{1}H NMR spectrum of OTR4120 in D_2O. The anomeric protons (H1) from all represented glucosidic units are located between 4.9 and 5.5 ppm. Relative abundance of peaks denoted a−i, as detailed in Table 4, was calculated from the anomeric proton individual peak height. This assignment was confirmed for each unit by ^{1}H NMR simulations using the ACD/labs H NMR v7.04 predictor program.

Chart 2. Potential Sulfation Pattern of the Polymer OTR4120 Represented in a 15 Units Fragment[a]



[a] This pattern was obtained from the constitutive sugar units stated in Table 4. The seven mainly represented units were arranged by two Borland combinatorial analyses to give the sequence FGFDEBFCFGEDFCH. These programs place each unit in an order that respects the relative abundances of each glucosidic unit calculated from 600 MHz ^{1}H NMR analyses. A size of 15 units was found to be the minimal size able to respect the NMR calculated relative abundances.

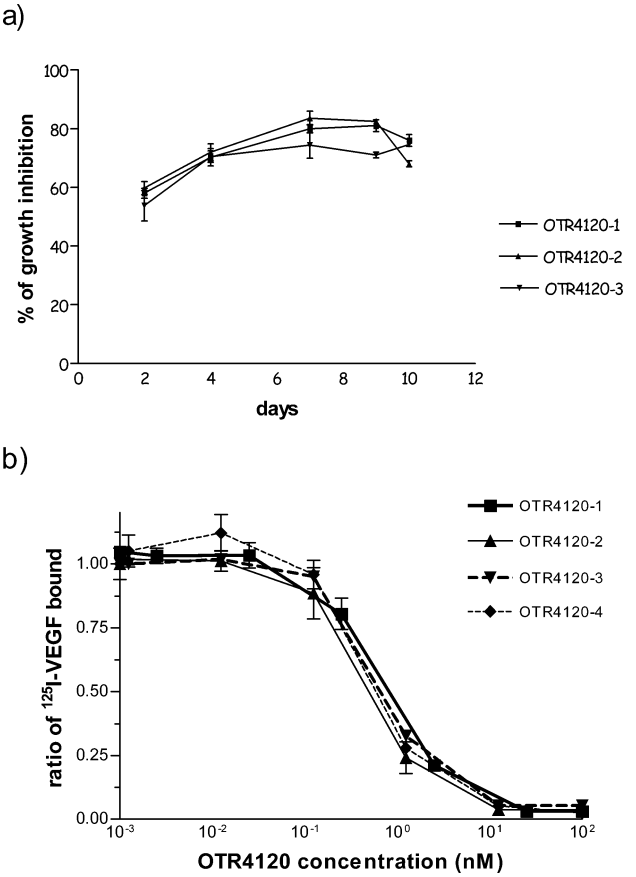


Figure 3. Reproducibility of the biological activities of the different batches of OTR4120. (a) Proliferation of dermal fibroblasts. The anti-proliferative effect was evaluated by counting cultured cells exposed to 100 μg/mL of the sulfated polysaccharide during 10 days. (b) Avidity of OTR4120 for ^{125}I−VEGF$_{165}$. Binding of the sulfated polymer was evaluated by a competitive assay with heparin. BSA-heparin was coated in an ELISA plate and a mixture of ^{125}I−VEGF$_{165}$ and OTR4120 at different concentrations was added. Residual radioactivity on the plate was evaluated after washing. Each value represents the mean (±SD) of three independent experiments each performed in triplicate.

min^{-1})) followed by C4 ($k_4 = 0.8 \times 10^3$ L^2/(mol^2 min^{-1})).[35] Our medium resolution ^{1}H NMR studies, together with the determination of the total ds confirmed this fact. Thus, we can assume that when a substituted group is present at C2, C4 will became sterically more accessible than C3 for a second substitution. C3 remains the less substituted position. Units not represented in Table 4 were considered of negligible relative abundance.

A combinatorial analysis of these data was performed by using two algorithms written in Borland C++. The first, determine distances between the all different carboxymethylated units from the percentage of anomeric abundance (Table 4, units B, C, D, and G; units A and I are neglected). Carboxymethylated units are then placed along a backbone of a imposed size. The algorithm give different sequences and their respective units composition. After performing a number of simulations with different backbone sizes, a chain of 15 units was retained since it was the minimal size which respected at most the original unit abundances. As in heparin, a carboxylate is then located every two sugar units. The second algorithm works on the chosen sequences and places remaining sulfated units by calculating distances from their relative abundances. Thus, we have determined two possible 15 units sequences for OTR4120: **FGFDEBFCFGEDFCH** (Chart 2) and **FDFGEBFCFDEGFCH**. These structural approaches might be at the base of fine structure−function studies and should be of great importance for minimal active fine structure modeling of oligomeric compounds with potential biological activities.

OTR4120 Biological Activity. To validate the functional reproducibility of the different synthetic batches of OTR4120 (Table 2), we have studied their dose−response effects on cultured dermal fibroblasts growth and their avidity for VEGF$_{165}$ bound onto heparin. Figure 3a shows the inhibitory effect of each polymer batch on the growth of fibroblasts in primary cultures. All assayed compounds showed the same inhibitory activity on fibroblast proliferation. This biological effect is in agreement with previous reports demonstrating that, as heparin, HM-like products interact closely with TGF-β1, a HBGF that inhibits proliferation of mesen-

chymal cells.[36] We have shown that OTR4120 interacts with VEGF, a specific angiogenic HBGF. To compare the avidity of $VEGF_{165}$ for the different OTR4120 batches, we performed displacement experiments of $^{125}I-VEGF_{165}$ bound onto immobilized heparin−BSA. Figure 3b shows displacement results obtained by the different batches of OTR4120. The IC_{50} of OTR4120 (20 ng/mL) was 10-fold lower than IC_{50} of heparin (not shown). This interaction, essentially mediated through sulfate and carboxylate residues, enhances VEGF binding to its heparin-sensitive receptors (submitted results). The biological activities of the four compounds in this model were equivalent from one batch to another confirming the reproducibility of the synthetic method.

In the other hand, considering the potential anticoagulant activity of sulfated polysaccharides, we were interested to determine the anticoagulant activity of the sulfated polymer OTR4120. Table 5 reports the value of the thrombin time determined at different OTR4120 concentrations. Although the polymer shows a thrombin time of 20.4 s at 2.5 μg/mL, it is interesting to remark that, at the same concentration, heparin showed a thrombin time superior to 300 s.

Conclusions

We have developed a nondestructive synthetic alternative for controlled and smooth preparation of sulfated polysaccharides or other molecules bearing hydroxyl or amine groups in the absence of any aliphatic or aromatic amine as acid scavenger or as solvent. The technique makes use of SO_3−DMF complex as sulfation agent in association to an acid scavenger of neutral character, 2M2B, which presence in reaction medium avoids depolymerization and cleavage of acid labile groups as amides, esters and ethers. The possible intermediate on this reaction might be a carbocation which is stabilized by sulfate groups and subsequently quenched during neutralization. 2M2B is a volatile molecule which allows its easy elimination from the reaction medium avoiding contamination of the final product. The degree of sulfation of the reaction is controllable, reproducible and in correlation with biological activities of different batches of the same compound studied in two different assays. Structure characterization of a biologically active HM polysaccharide, OTR4120, could be assessed by even medium resolution ^1H NMR spectroscopy (200 MHz) indicating that finest structure−function studies of this kind of charged polysaccharides can readily be performed in order to better understand their specific HS mimicking sequences and action mechanisms. We are currently working in this area.

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