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Synthesis of New Regioselectively Sulfated Hyaluronans for Biomedical Application

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Summary: Sulfated glycosaminoglycans (GAGs) display various biological effects which are strongly influenced by the degree of sulfation and the position of sulfate groups within the polymer. Hyaluronan, a non-sulfated GAG, represents a readily accessible educt to synthesize structural analogues of sulfated GAGs mimicking their biological activity. Different strategies were developed and evaluated to synthesize hyaluronan sulfates with a free primary hydroxyl group at C-6' and sulfated secondary hydroxyl groups. Applying selective desulfation methods of high-sulfated hyaluronan by means of silylating agents, products regioselectively desulfated at the primary C-6' but also partly the C-4' position were obtained. A pathway using benzoyl ester protecting groups to block the primary hydroxyl function of Hya during the sulfation resulted in a high-sulfated product, functionalized only at the secondary hydroxyl groups.

Keywords: biomaterials; esterification; hyaluronan; regioselective; sulfation

Introduction

Sulfated glycosaminoglycans (GAGs) like heparan, dermatan or chondroitin sulfate are biocompatible and biodegradable polysaccharides that possess various biomedical activities. They can act anticoagulant, antithrombogenic and antiviral, and are clinically used e.g. as injectable anticoagulants (heparin) or dietary supplements for the treatment of osteoarthritis (chondroitin sulfate).[1–5] Besides sulfated GAGs are under investigation for drug delivery and tissue engineering, their ability to sequester and stabilize bone growth-promoting molecules like bone morphogenetic proteins offers a promising approach to generate GAG-based implant coatings promoting the ingrowth of such implants into the surrounding tissue.[6–8]

It is well known that the biomedical activities of sulfated GAGs are strongly influenced not only by the degree of sulfation but also the position of the sulfate groups within the disaccharide repeating unit.[3,4,9,10] In naturally sulfated GAGs the sulfation pattern may vary, but it is given for the particular polymer and cannot easily be changed. Hence a directed introduction of sulfate groups into a non-sulfated backbone represents an effective way to obtain polymers with an adjusted degree of sulfation and a reproducible, regioselective functionalization pattern.

Hyaluronan (Hya, Figure 1) consists of linear disaccharide repeating units of β-D-glucuronic acid and *N*-acetyl-β-D-glucosamine residues linked at the 1,4 and 1,3 positions, respectively. It is the only non-sulfated GAG and it can be produced biosynthetically in large scale.[11] Therefore and due to its cytocompatibility and immunoneutrality it represents an appropriate starting material to synthesize designed sulfated GAGs for biomedical applications.

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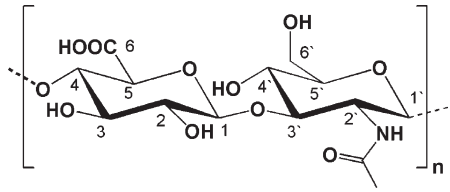


Figure 1.
Hyaluronan (Hya).

Herein we describe synthetic routes to obtain regioselectively sulfated Hya derivatives with both, an adjusted degree of sulfation and a predetermined distribution of sulfate groups within the disaccharide repeating unit starting from Hya sodium salt. The procedures developed allow the synthesis of a palette of variously sulfated hyaluronans for a detailed investigation of the correlation between degree and pattern of sulfation and the biomedical properties of sulfated GAGs.

Experimental Part

Materials

Hya (from *Streptococcus*, $M_W = 1 * 10^6$ gmol^{-1}) was purchased from Aqua-Biochem (Dessau, Germany). All chemicals and solvents were obtained from Fluka (Buchs, Switzerland) and Roth (Karlsruhe, Germany), respectively, in analytical reagent grade and used without further purification.

Analytical Instrumentation

NMR spectra were recorded in D_2O at 343 K on a Bruker Advance 400 MHz spectrometer. IR spectra were obtained on a FT-IR-Spectrometer *FTS 175* (BIO RAD, Krefeld, Germany) applying KBr technique. The average degree of sulfate groups (DS_S) per disaccharide repeating unit of the Hya derivatives was determined by estimation of the sulfur content using an automatic elemental analyzer (*CHNS-932*, Leco, Moenchengladbach, Germany). The degree of benzoylation (DS_B) was determined by integration of the ^{1}H-NMR signals and by the C/N ratio in the elemental analysis, respectively. Gel permeation chromatography (GPC) analysis was performed using the following system: Jasco PU 980 pump, Postnova Analytics PN 3000 (15°) laser light scattering detector (LLS), a Jasco RID-1531 refraction detector (RI), and Suprema-Gel 10 μm - 100 Å, 10 μm – 1,000 Å and 20 μm – 30,000 Å columns. The eluent was PBS-Puffer (P3813, Sigma-Aldrich) and the flow rate was 0.5 and 0.8 ml/min, respectively. The system was calibrated with standard pullulan (PSS Mainz, Germany) samples.

Synthesis of High-Sulfated Hyaluronan (sHya-3.1)

This derivative was prepared as previously described.[5,12] Briefly, the Hya sodium salt was transferred into the corresponding tetrabutylammonium (TBA) salt and afterwards reacted with a 20-fold molar excess of SO_3/DMF at room temperature. The resulting polymer has a DS_S of 3.1. M_w (LLS) = 49,300; D (RI) = 1.7.

Preparation of Hyaluronan Sulfate Tributylammonium Salt (Tr-sHya-3.1)

1 g sHya-3.1 (1.39 mmol) was dissolved in 50 ml H_2O until a clear solution was formed. Then 1 g DOWEX™ (tributylammonium form) was added and the suspension was stirred over night. The crude product was filtered, lyophilized, dried under vacuum and used without further purification.

Preparation of Hyaluronan Sulfate Pyridinium Salt (Py-sHya-3.1)

1 g sHya-3.1 (1.39 mmol) was dissolved in 100 ml H_2O and stirred with 10 g DOWEX™ ion exchanger (H-form) to form the free acid. Then the solution was filtered, 100 ml pyridine were added and the solvents were removed under reduced pressure. The resulting oil was dissolved in 100 ml pyridine and evaporated to form a white solid. This crude product was dried in high vacuum and used without further purification.

Table 1.
Desulfation of sHya-3.1 tributylammonium and pyridinium salt, respectively.

starting material	reagent	T °C	t h	DS_S (product)
Tr-sHya-3.1	MSTFA	60	4	2.7
Tr-sHya-3.1	MSTFA	60	24	2.2
Tr-sHya-3.1	MSTFA	80	4	2.5
Py-sHya-3.1	MSTFA	80	24	1.5
Tr-sHya-3.1	BTSA	60	4	2.9
Tr-sHya-3.1	BTSA	60	24	2.4
Tr-sHya-3.1	BTSA	80	4	2.6
Tr-sHya-3.1	BTSA	80	24	2.2
Py-sHya-3.1	BTSA	80	24	1.6

Desulfation of Tr-sHya-3.1 and Py-sHya-3.1, Respectively (sHya-A)

0.5 g of Tr-sHya-3.1 and Py-sHya-3.1 (0.57 and 0.65 mmol), respectively were dissolved in 50 ml dry DMSO under N_2. After 1 h the respective desulfation agent was added in an 80-fold molar excess, the solution was heated to the reaction temperature and stirred for the given time (see Table 1). Then 50 ml H_2O were added and the mixture was stirred at the reaction temperature for another 30 minutes. After cooling to room temperature the product was purified by dialysis against distilled water, lyophilized and dried in vacuum. IR (KBr): 3484 (OH, CO-NH), 2961, 2869 (2 * CH), 1645 (C = O), 1416, 1383, 1260 (SO_2), 823 cm^{-1}. ^{13}C-NMR (D_2O, 323 K): 175.3–174.2 (C = O, C-6), 102.8 (C-1), 100.8 (C-1'), 79.2–70.6 (C-3', C-4, C-5, C-5', C-3, C-2, C-4'), 69.4 (C-4', desulfated), 68.3 (C-6', sulfated), 61.8 (C-6', desulfated), 56.1 (C-2'), 23.6 ppm (NH-$COCH_3$).

Benzoylation of TBA-Hya (TBA-Bz-Hya)

0.5 g TBA-Hya (0.81 mmol) were suspended in a mixture of 80 ml dry DMF and 20 ml dry pyridine and stirred over night at room temperature. Then 0.57 g (4.03 mmol) benzoyl chloride were added and the suspension was stirred for 24 h at ambient temperature. The product was precipitated in 1 l H_2O and washed several times with H_2O and CH_2Cl_2. After drying in high vacuum 0.39 g of a polymer with $DS_B = 1.2$ could be obtained. IR (KBr): 3476 (OH, CO-NH), 2965 (CH), 1722, 1659 (2 * C = O), 1602 (Ar), 716 cm-1 (Ar).

Sulfation and Deprotection of TBA-Bz-Hya (sHya-B)

0.5 g (0.67 mmol) TBA-Bz-Hya were suspended in 100 ml dry DMF and stirred over night. Then 1.58 g (10.35 mmol) SO_3/DMF in 3 ml dry DMF were added and the solution was stirred at room temperature for 1 h. The reaction mixture was then poured into 1 l acetone which was adjusted to pH = 10 with ethanolic NaOH solution before. After stirring for 20 minutes the mixture was neutralized with 1N HCl whereas the product precipitated. The crude product was washed several times with acetone, dissolved in 100 ml of saturated K_2CO_3 solution and stirred over night. Purification was performed by dialysis against distilled water. Lyophilization of the solution and drying of the resulting fleece under vacuum gave 0.49 g of sHya-B with $DS_S = 2.6$. IR (KBr): 3473 (OH, CO-NH), 2949 (CH), 1648, 1619 (2 * C = O), 1407, 1379, 1259 (SO_2), 1063, 818 cm-1. ^{13}C-NMR (D_2O, 323 K): 175.3 (C = O), 174.3 (C-6), 103.5 (C-1), 101.1 (C-1'), 79.4–69.1 (C-3', C-4, C-5, C-5', C-3, C-2, C-4'), 61.8 (C-6'), 56.1 (C-2'), 23.0 ppm (NH-$COCH_3$). M_w (LLS) = 44,800; D (RI) = 1.9.

Results and Discussion

The sulfation of Hya is well examined and can be controlled to obtain desired degrees of sulfation (DS_S) by means of varying reagents and reaction conditions.[13–16,5] For the synthesis of high-sulfated hyaluronan (sHya), SO_3/DMF complex is the reagent of choice, however for derivatives with a lower DS_S less potent sulfation agents like SO_3/pyridine complex are more suitable for an accurate control of the extent of sulfation. Previous studies prove that the primary OH-group at C-6' is favoured during the sulfation reaction and is completely substituted even at a DS_S of marginal over 1.[14]

Here we focussed our research on potential strategies to obtain Hya sulfates with the opposite regioselectivity, namely derivatives with a free primary OH-group at C-6' and sulfated secondary hydroxyl groups.

One possible approach to Hya sulfates with a free primary hydroxyl group is represented by the regioselective desulfation of high-sulfated Hya at C-6'. This desulfation reaction has already been described for heparin and other naturally sulfated polysaccharides and performed most frequently by means of silylating agents like *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) or *N*,*O*-bis(trimethylsilyl)acetamide (BTSA).[17–21] Scheme 1 shows the proposed mechanism for this reaction.

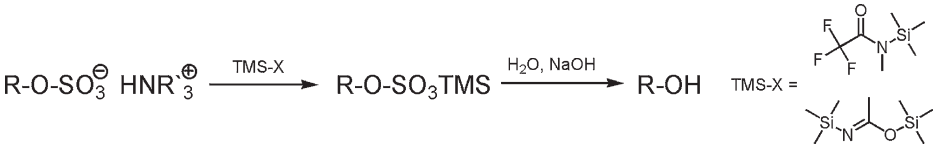
To investigate if similar procedures can be applied to sHya it was first necessary to synthesize a high-sulfated Hya derivative. This sulfation of Hya was performed with SO_3/DMF complex as previously described[12] and resulted in polymers with an average degree of sulfation of $DS_S = 3.1$ (sHya-3.1). During the reaction the molecular weight of the polysaccharide decreases from about 1 Mio g/mol to ca. 50,000 g/mol, which corresponds with data already published before.[22,23]

Since the desulfation with silylating agents seems to require either the pyridinium or the tributylammonium salt of the sulfated polymer[18,17] we transferred sHya-3.1 into both forms (Py-sHya-3.1 and Tr-sHya-3.1, respectively). These substances were then reacted with MSTFA and BTSA, respectively with varying reaction conditions and the obtained products were characterized via elemental analysis and NMR. The results are summarized in Table 1.

The DS_S of the Tr-sHya-3.1 decreases from $DS_S = 3.1$ to 2.9 after 4 h at 60°C with BTSA and to 2.7 with MSTFA as desulfation agent. After 24 h reaction time the number of sulfate groups is reduced to $DS_S = 2.4$ and 2.2, respectively. Based on these results MSTFA seems to be the more effective desulfation agent. Comparing the tributylammonium salts with the pyridinium ones we find that Py-sHya-3.1 is more reactive than Tr-sHya-3.1. Combining Py-sHya-3.1 as starting material with MSTFA as desulfation agent a decrease of the DS_S up to 50% is possible. The M_w-values of the desulfated derivatives did not show a significant change compared to the starting sHya-3.1.

Analyzing the ^{13}C-NMR-spectra to examine the regioselectivity of the reaction we find that after 4 h with BTSA at 60°C one new small resonance at 61.8 ppm appears that can be assigned to the desulfated primary carbon C-6', while a strong peak of the sulfated C-6' at 68.3 ppm is still present in the spectrum (see Figure 2B). This correlates with the determined decrease of the degree of sulfation of $\Delta DS_S = 0.2$. So indeed the primary OH-group reacts first. After 24 h reaction time already more than 40% of the primary sulfate groups are cleaved (see Figure 2C). However there is another new signal appearing at 69.4 ppm which can be assigned to the desulfated C-4'. This means that the sulfate groups at C-4' react before the primary ones are completely cleaved.

A similar effect can be observed with Py-sHya-3.1 as starting material and MSTFA as desulfation agent. Also at different reaction temperatures this sequence of reactivity does not change. This means that under the chosen reaction conditions the desulfation of high-sulfated



Scheme 1.
Desulfation with silylating agents.[18]

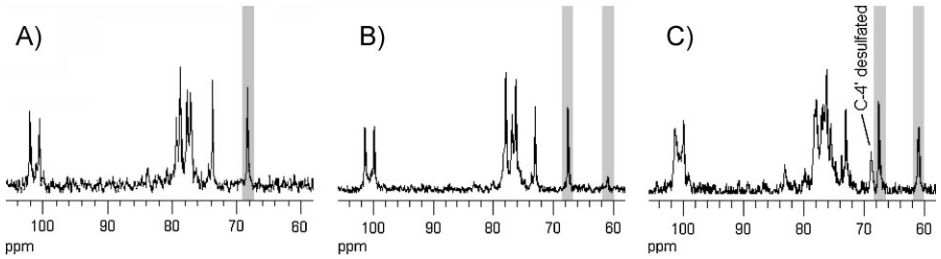


Figure 2.
Detail of the ^{13}C-NMR-spectra of sHya-3.1 (A), desulfated Tr-sHya-3.1 with BTSA as desulfation reagent at 60°C after 4 (B) and 24 (C) hours reaction time, respectively. Grey marked: C-6' sulfated (68.3 ppm) and desulfated (61.8 ppm); (B) at 69.4 ppm: C-4' desulfated.

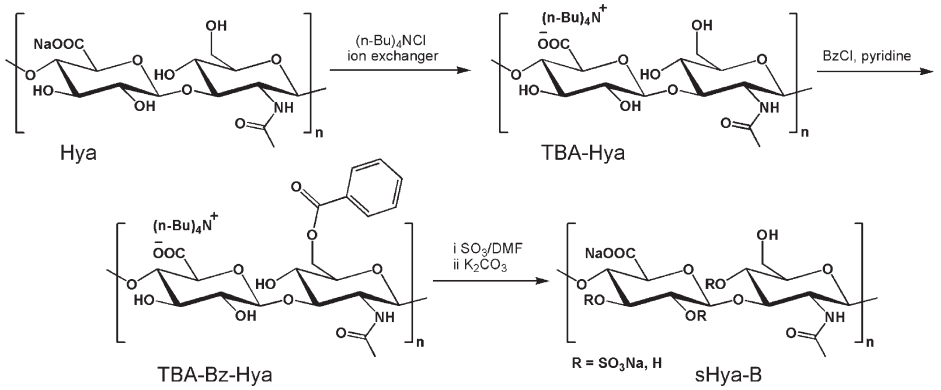
Hya does not represent an adequate way to obtain Hya derivatives with a free primary and highly sulfated secondary hydroxyl groups, but results in selectively OH-6' and OH-4' desulfated products.

A second pathway to synthesize high-sulfated Hya with a free OH-6' is to react Hya with a protecting agent prior sulfation and cleave this protecting group subsequently. Due to its stability under acidic conditions as they occur during the sulfation reaction and its steric demand, the benzoyl group seems to be most appropriate to protect the primary OH-group at C-6'.

In order to increase the solubility of Hya for the subsequent benzoylation reaction, it was transformed into its tetrabutylammonium salt (TBA-Hya) by stirring the corresponding sodium salt with ion exchange resin[12] (see Scheme 2).

The benzoylation of TBA-Hya was performed with benzoyl chloride in dry DMF and pyridine at room temperature. With a 5-molar excess of benzoyl chloride and 24 hours reaction time a degree of benzoylation of 1.2 could be obtained. Sulfation was carried out as previously described with SO_3/DMF in DMF.[12,5] The cleavage of the benzoyl group could then be performed during the workup procedure by stirring of the crude product under slight basic conditions. The degree of sulfation of the final product sHya-B was determined to be $DS_S = 2.6$ via elemental analysis. The M_w-value of this derivative is with 44,800 g/mol in the same range as other sulfated hyaluronic acid derivatives.[22]

To prove the regioselectivity of the sulfation in sHya-B ^{13}C-NMR is very useful (see Figure 3).



Scheme 2.
Pathway for the synthesis of sHya-B with a free primary OH-6' using benzoyl ester as protecting group.

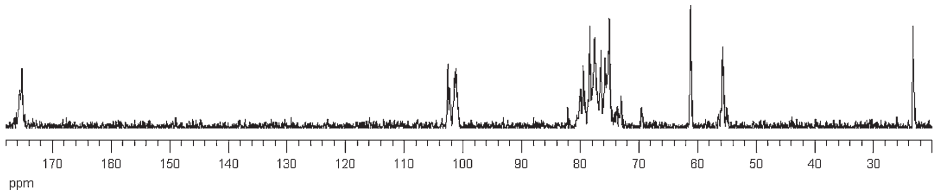


Figure 3.
^{13}C-NMR spectrum of sHya-B ($DS_S = 2.6$) with a free primary OH-group, signal at 61.8 ppm (C-6').

As shown in Figure 3 only one signal for the primary carbon C-6' can be found at 61.8 ppm, which corresponds to the non-sulfated C-6'. C-4' seems only partially sulfated, since there is a small peak at 69.4 ppm, where the non-sulfated carbon can be found. The signals for the sugar ring carbons between 79.4 and 69.1 ppm are shifted closer, which is typical for high-sulfated Hya derivatives. The absence of signals for aromatic carbon atoms in the spectrum confirms the complete removal of the benzoyl protecting group after sulfation. Thus the spectrum proves that sHya-B has indeed a complete free primary OH-group and is highly sulfated, so the pathway using protecting groups yields in the desired product.

Conclusion

The sulfation of hyaluronan usually leads to a product in which the primary OH-group is preferentially substituted. As it is known from other naturally occurring sulfated polysaccharides, an opposite sulfation pattern where sulfate groups are mainly located at the secondary hydroxyl function of the sugar repeating unit may result in considerable changes of the biological activity of those biopolymers. In addition, with regard to the structural similarity of Hya with other sulfated GAGs, there exist a special interest in regioselective sulfation pathways resulting in Hya derivatives predominantly sulfated at the secondary hydroxyl groups. For this purpose different pathways were developed and evaluated, namely the desulfation of high-sulfated Hya by means of silylating agents and a protecting group strategy to block the most reactive primary OH-group of Hya during the sulfation. The desulfation of sHya with a $DS_S = 3.1$ by means of silylating agent yields in products with selectively desulfated C-6' but also at C-4'. Using benzoyl ester protecting groups during the sulfation, a high-sulfated Hya with a completely free C-6' position exclusively substituted at the secondary hydroxyl groups (C-2, C-3, C-4') can easily be synthesized.

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