Original article

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DEVELOPMENT OF BIOACTIVE CELLULOSE SULFATES FOR BIOMEDICAL APPLICATIONS

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Cellulose is the most abundant biomolecules on earth. Chemical derivatives of cellulose have found multitude of application in industrial and biotechnological applications. Cellulose sulfates is a class of water-soluble derivatives that has been employed in industrial application, but not yet in medicine. Here derivatives of different degree of sulfation of anhydroglucose unit (AGU) of cellulose have been studied toward anticoagulant effects and modulating effects of growth factors with heparin-binding domains like fibroblast growth factor 2 (FGF-2). The results show that CS of higher sulfation degree have an anti-coagulant activity comparable to that of heparin with cooperative action to anti-thrombin III that inhibits thrombin and Factor Xa activity making CS interesting for anticoagulant coating of blood-contacting medical devices. Furthermore, the studies show that CS with comparable sulfation degree to heparin have a promoting activity on the mitogenic effect of FGF-2 shown in cell culture studies that indicate their application as coatings of implant materials or component of tissue engineering scaffolds in the area of traumatology and regenerative medicine.

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Key words: *cellulose sulfates, sulfation degree, anticoagulation, thrombin, growth factors, FGF-2*

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Introduction

Glycans have diverse functions in the organism. They are relevant for not only maintaining of normal functions of cells and tissue, but also important in wound healing or pathological processes, like bacterial or viral infections, growth and metastasis of tumors (1). An important subgroup of glycans are glycosaminoglycans (GAG), which comprise specific dimeric repeating units that usually consist of a uronic acid connected to a N-acetylglucosamine or N-acetylgalactosamine sugar. Important representatives of GAG are hyaluronic acid, chondroitin sulfate, heparan sulfate and heparin. It is interesting to note that the degree of sulfation is highest in heparin, followed by heparan sulfate and other sulfated GAG, while hyaluronic acid is the only non-sulfated GAG (1, 2). Heparan sulfate and heparin are structurally quite similar, but differ mainly in their degree of sulfation, molecular weight and occurrence in the organism (3). Because of its high number of sulfate and carboxyl groups, heparin is the glycosaminoglycan with the highest negative charge density (see Figure 1). Heparin is com-posed of disaccharide-subunits that are either D-glucuronic acid (10 %) bound by a β-(1→4) glycosidic bond or L-iduronic acid (90%) bound by an α-(1→4) glycosidic bond to N-Acetyl-D-glucosamine. The typical structure is usually the trisulfated disaccharide. Sulfate groups can be located at the 2-O position of the iduronic or glucuronic acid, as well as the 3-O and 6-O position of the glucosamine. The amino group of the glucosamine on the other hand can be either substituted by an acetyl or sulfate group. The average molecular weight of a heparin molecule is about 15 kDa (4). By comparison, he-paran sulfate has a higher average molecular weight (around 30 kDa) and a lower degree of sulfation. Heparan sulfate chains frequently contain domains with long sequences of either high or low sulfation degree. Heparin shows such inhomogeneities in its degree of sulfation as well, but to a lesser extent in comparison to heparan sulfate (5). The synthesis of heparin happens mainly in mast cells and basophil

granulocytes. Heparin shows a multitude of functions, such as the binding of histamine and the control of the activity of different proteases (6). Heparin that has been released also inhibits the interactions of blood platelets with collagen and the binding of von Willebrand factors to them. In addition, it can also bind low density lipoprotein (LPL). Such bound constructs can subsequently be taken up and degraded by macrophages8. However, the well-known property of heparin is its ability to bind to anti-thrombin III (ATIII), which enhances ATIII affinity to thrombin and other activated clotting factors of the coagulation cascade, which is the basis of its use as anticoagulant (6).

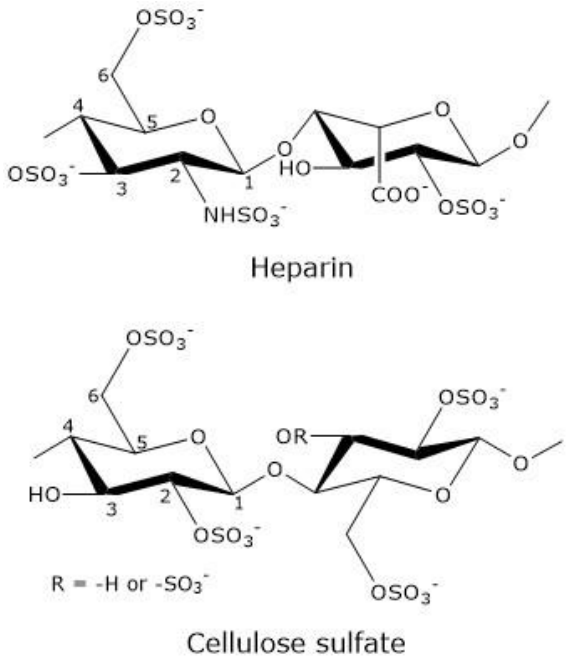

Figure 1. Chemical structure of heparin and cellulose sulfate

On the other hand, heparan sulfate proteoglycans (HSPG) are localised on the surface of many cells and represent components of the extracellular matrix. The most known representatives of cell membrane localised HSPG are syndecans and glypicans (3). For example, syndecan-1 und -3 carry chondroitin sulfate proximal to the cell membrane, while heparan sulfate (HS) is found at the distal part of proteoglycans. Conversely, syndecan-2 and -4 are decorated exclusively with heparan sulfates. Because of their cytoplasmic domain, syndecans can transmit signals from the extracellular area to the inner part of the cell. This includes the binding of ligands to the HS chains, followed by oligomerisation of syndecans, which triggers the activation of signalling proteins like kinases in the cytoplasm (1). Many functions of the HSPG are also related to the regulation of the activity of chemokines, growth factors glycoproteins of the extracellular matrix (ECM) in which they can function as co-receptors. By presenting growth factors as co-receptors for receptor-tyrosine kinases they can contribute to signal transduction processes (9). The HS chains are directly involved in the formation of the receptor-ligand complex, as described for the fibroblast growth factor FGF-2 and can influence the mitogenic effect of cytokines (10). HSPG can also store growth factors outside the cell as component of ECM and essentially function as a reservoir. The release can be caused either through a change in the degree of sulfation through locally expressed sulfatases (11), through pro-teolysis of the protein backbone or through the HS chain fission caused by heparanases. The binding of heparin or HS to regulatory proteins occurs through heparin binding sites, which are generally located at the outside of proteins. Those are mostly rift-like domains with a high amount of positively charged amino acids like lysine or arginine (4).

The inhibition of blood coagulation through heparin is an effect that has been used in hospitals for a long time (4). Heparin is also used to make the surface of biomaterials like catheters, tube systems or dialysis membranes more compatible to blood (12). This can reduce the need for a systemic application of heparin, since that can cause side effects like the aggregation of thrombocytes, an increased tendency for bleeding or a retarded healing process of bone tissue and an increased risk for osteoporosis (13, 14). Besides these well-known risks, there are further disadvantages in the use of natural GAG´s

since the extraction of them out of animal tissue is time-consuming and carries further risk of infections and immunological reactions during their clinical application. The bioactivity of heparin is additionally very dependent on its biological origin (species, organs), which is related to differences in degree of sulfation and substitution pattern (4). Lastly, the use of natural GAG´s in the clinical field opens the possibility to contaminate heparin with highly sulfated chondroitin sulfate falsely claimed with criminal intent as pure heparin, which was fatal for some patients in US (15). Because of these challenges, it would be highly desirable to synthesize biocompatible polymers, which can replace heparin showing less variance in their biological activity with no risk of transmission of diseases and hence better safety.

Cellulose is one of the most abundant polysaccharides that exists in nature. It is a main component of the cell wall of plants, shows a high molecular weight, but is non-soluble in water and most organic solvents. In comparison to other natural polysaccharides like hemicellulose or pectin, cellulose is a non-branched polymer (16). It is composed of D-glucose units, which are connected via β-(1→4) glycosidic bonds. The hydroxyl groups at the C2, C3 and C6 atoms of the anhydroglucose unit (AGU) can be chemically functionalized to synthesize many widespread polymers such as e.g. carboxymethylcellulose, which are used in the paper industry, food technology and partly in medical applications (17). The sulfation of cellulose has a long tradition and leads to water soluble products with many different application possibilities (18). The main structure of cellulose sulfate (CS) is shown in Figure 1. It is obvious that sulfation of cellulose leads to derivatives that have similarities to the highly N-acetyl glucosamine unit of heparin. Hence, it seems to be reasonable to assume that CS of higher sulfation degree might be also effective in inhibition of blood coagulation and being effective in supporting the activity of growth factors that possess heparin-binding domains. This article presents the effect of CS on coagulation and the mitogenic activity of the fibroblast growth factor 2 (FGF-2) showing that sulfation degree has an effect on both phenomena.

Materials and methods

Synthesis of cellulose derivatives

Synthesis and chemical analysis of cellulose derivatives have been presented in more detail by us (28, 29) and will not be described here in detail. The cellulose sulfates were named later in the result section according to the degree of substitution with sulfate (DS) as CS X.

Analytical methods

The DS of the cellulose derivatives obtained by different sulfation methods was characterized by elemental analysis and 13C-NMR spectroscopy. The substituent distribution within the AGU was assessed from the 13C-NMR spectrum of the cellulose derivatives dissolved in D_2O by integrating the signal areas and comparing those of the substituted position to those of the appropriate non-substituted one.

Study on anticoagulant activity of cellulose sulfates

Collection and preparation of blood

Blood was drawn from healthy human volunteers, who had no medication for at least 10 days. Blood was anticoagulated with sodium citrate (3.8 g/100 ml). The blood was centrifuged at 2000g for 20 min. The supernatant cell free plasma was separated. Plasma samples from 10 different donors were pooled, aliquoted and snap frozen at -80 °C. For experimental work, plasma was thawed at 37 °C and used within 2 h.

Measurement of clotting times

Thrombin time (TT) was measured using thrombin (Behring Werke, Germany). Partial thromboplastin time (PTT) was estimated using a commercial test kit (Boehringer Mannheim, Germany). Measurements were carried out with a coagulometer KC 4A (Amelung, Germany). Cellulose derivatives were dissolved in TRIS buffer, pH 7.4. 100 µl pooled plasma were mixed with 50 µl cellulose derivative solution and incubated for 1min (TT) or 3 min (PTT), respectively. TT was measured after addition of 100 µl thrombin solution (0.3 IU/ml). PTT was estimated after addition of 100 µl kaolin-cephalic solution, followed by the addition of 100 µl 25mM $CaCl_2$ solution. After the addition of activator the time needed for clotting was measured. If samples did not clot within 10 min it was observed that no clotting occurred afterwards. Therefore, measurements were stopped after 10 min and those samples denoted as non-clottable (n.c.). To still obtain visible data points in the graphs these values were set at a clotting time of 600 s. However, it should be kept in mind that these data represent conditions under which the plasma did not clot at all.

Inactivation of thrombin and factor Xa

The anticoagulant potential of CS was tested in addition by their ability to support the inactivation of thrombin and factor Xa in the presence of antithrombin III. This was possible by the development of amidolytic assays for thrombin and factor Xa in separate investigations. Cellulose derivatives or reference substances were dissolved in 50mM Tris-HCl, 175mM NaCl, 10mM EDTA, and 0.5 mg/ml human serum albumin (24). The thrombin assay was carried out mixing 50 µl AT III (activity 0.265 pkat/ml) with 200 µl thrombin (activity 0.53 nkat/ml), and 50 µl of the test substance. After 5 min incubation at 37 °C 200 µl chromogenic substrate S-2238 (0.22 mM) was added and the mixture was incubated for 2 min. The conversion of the chromogenic substrate was stopped by the addition of 200 µl acetic acid (20% v/v). The optical density

was measured at 405 nm in 96 well plates with a plate reader (Anthos 2001, Austria). A standard curve was obtained under identical conditions for thrombin activities from 0 up to 1.053 nkat/ml and used for the calculation of residual thrombin activity from the measured OD.

The factor Xa assay was performed using 200 µl of factor Xa solution (activity 1.06 nkat/ml), 50 µl AT III solution (activity 0.265 pkat/ml), 50 µl test solution and 200 µl chromogenic substrate S-2222 (0.22 mM). The experiment was carried out in the same manner as the thrombin assay. Residual factor Xa activities were calculated from a standard curve. Thrombin, factor Xa, AT III, and the chromogenic substrates S-2238, and S-2222 were supplied by Chromogenix, Sweden.

Studies on mitogenic activity of cellulose sulfates

Estimation of binding growth factor FGF-2 to cellulose sulfates

The binding affinity of the synthesized CS to the growth factor FGF-2 (b-FGF) was performed with a competition assay using heparin agarose beads (Fluka, Biochemica). 25 ng of b-FGF obtained from InVitrogen were mixed with heparin agarose beads and PBS and agitated for 30 min at 200 rpm at RT to allow the binding of growth factors to the beads. The unbound growth factor was removed by two times washing with PBS. For the release of the growth factor from the beads, cellulose derivatives or heparin (control) were added to the mixture and agitated for 30 min at 200 rpm at RT. After centrifugation the supernatants with the polysaccharides and the released b-FGF were applied to cellulose nitrate membrane in a slot-blot apparatus. A primary antibody against FGF-2 (Sigma, Germany) and a horseradish peroxidase labelled secondary antibody (Dianova, Germany) were applied to the membrane to label bound growth factors. Detection was performed with ECL plus chemiluminescence kit and a CCD camera (Raytest, Diana 2). The quantification of the signals was done by ImageJ.

Cell culture

3T3-L1 fibroblast cells obtained from ATCC (Manassas, USA) were cultured in flasks (75 cm^2, Greiner bio-one, Frickenhausen, Germany) in Dulbecco´s modified Eagle medium (DMEM, Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom AG) and 1% penicillin-streptomycin-fungizone (PSF, Promocell, Heidelberg, Germany) in a 37 °C humidified atmosphere of 5% CO_2 and 95% air. Cells were harvested by treatment with trypsin/EDTA (Biochrom AG). Trypsinization was stopped by addition of FBS and cells were washed twice with DMEM.

Investigation of mitogenic effects of cellulose derivatives on 3T3-L1 fibroblasts

3T3-L1 fibroblast cells were seeded at a density of 10.000 cells/well in black 96 well plates (Greiner bio-one) in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin-fungizone and cultured for 24. After washing the plates with DMEM only, the cellulose derivatives or heparin were applied to the cells in DMEM without FBS at a concentration range of 1 µg to 1000 µg/ml for 48 h in the presence or absence of 10 ng/ml FGF2. The proliferation was measured based on the DNA content using the Quant -iTTM PicoGreen dsDNA quantification assay (Invitrogen, Karlsruhe, Germany). The fluorescent intensity was measured with an excitation wavelength of 485 nm and an emission wavelength of 520 nm by the plate reader Fluostar Optima. The proliferation was expressed as a ratio to the control wells with 10 ng/ml FGF2. All experiments were carried out with six wells per sample and dilution from which means and standard deviation were calculated.

Results and discussion

Studies on anticoagulant activity of cellulose sulfates

The inhibition of blood coagulation through cellulose sulfates

The sulfation of partially substituted cellulose acetates has been described elsewhere in more detail (19). The total content of sulfur was determined through elemental analysis while the distribution of the substituents was determined through quantitative 13C-NMR spectroscopy. The synthesized CS are listed in Table 1 and sorted by degree of sulfation or substitution (DS) and the distribution of the substituents. Figure 1 shows the typical structure of sulfated cellulloses. It is visible that sulfation degree DSs reached from low of about 0.25 to relatively high of 1.35, which is lower than that of heparin. The latter can have two sulfation sites at the N-acetylglucosamine unit with sulfation at C2, C3 and C6 position, while the uronic acid may be substituted at C2 position with a sulfate group (see Figure 1). Hence, the overall sulfation degree can have maximum around 2.0.

The effect of CS on blood coagulation was determined using citrate plasma and commercial test kits for measurements of thrombin time (TT) and partial thromboplastin time (PTT). Figures 2A and B show the results of the TT and PTT coagulation time measurements. It can be seen that an increase in the degree of sulfation of CS leads to an increase in the coagulation times. In addition the results imply that the inhibition of coagulation increases in the case of TT and PTT, if the degree of sulfation is increased in C2 position, which can be shown by comparing the samples CS1.33 and

CS1.35 that have almost the same degree of sulfation, but at different substitution site. (see Table 1). This is especially obvious in the case of the determination of TT, since an increase of the DS at C6 and a decrease at C2 position shows a shorter time of coagulation in comparison to a sample that has a similar DS but a higher sulfation in C2 position. At a CS concentration of 25 µg/mL with a DS ≥ 0.95 for TT measurements and a DS ≥ 1.15 for PTT measurements the blood clotting was completely inhibited, which demonstrates the potential of these cellulose derivative to be used as anti-coagulants for modification of blood-contacting materials surfaces like membranes for haemodialysis , blood linings, etc.

Table 1. Degree of sulfation (DStotal) and distribution of the sulfate groups in the derivatives in the determiantion of coagulation inhibition

Cellulose sulfate (CS)	DS_{total} Elemental analysis	DS_{total} ^{13}C-NMR	Substitution pattern of sulfates*		
			C2	C3	C6
CS 0.26	0.35	0.25	0.17	0.08	0
CS 0.95	0.80	0.95	0.55	0.20	0.20
CS 1.14	1.10	1.14	0.74	0.09	0.31
CS 1.33	1.40	1.33	0.76	0.10	0.47
CS 1.35	1.07	1.35	0.67	0.33	0.35

* DS values of the sulfate groups at the C2, C3 and C6 position were determined via 13C-NMR spectroscopy

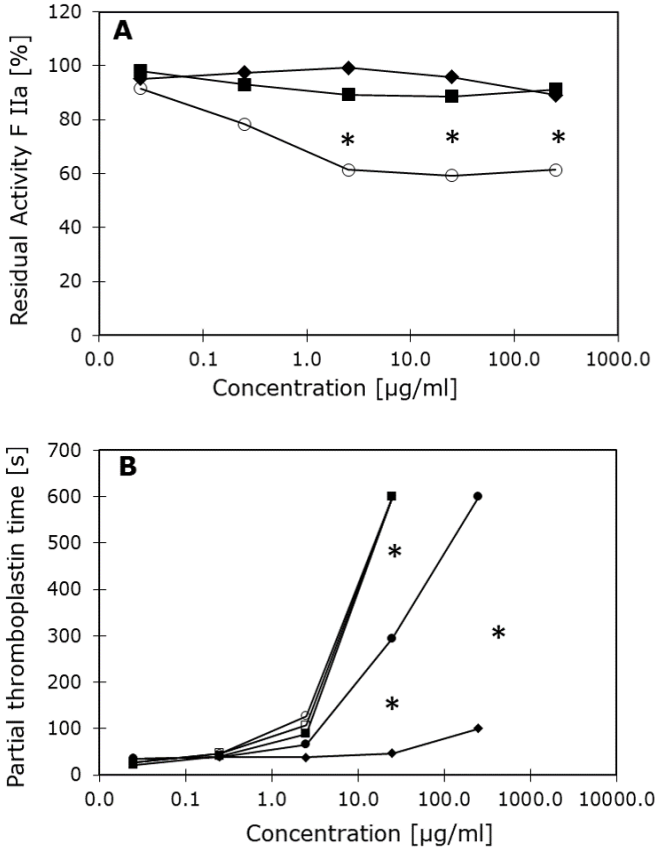

Figure 2. Thrombin time (A) and partial thromboplastin time (B) of citrate in the presence of cellulose sulfates (concentration range: 0.025 µg/mL to 2.5 mg/mL). (♦) – CS 0.26; (●) – CS 0.95; (□) - CS 1.14; (❍) - CS 1.33; (■) - CS 1.35. Asterisks in the figures show significant deviation (p <= 0.05).

Next, we were interested to find out more about the underlying mechanism of inhibition of coagulation regarding the activity of anti-thrombin III (AT III) in presence of CS toward factor IIa

(thrombin, F IIa) and Factor Xa inhibition. It is known that for the inhibition of factor Xa shorter heparin sequences with specific patterns of sulfation are relevant because of the formation of a binary complex between AT III and heparin that inhibits F Xa. For F IIa inhibition on the other hand, longer heparin sequences play a role because they form a ternary complex in which heparin forms a quasi-catalytic surface for the interaction of AT III and F IIa (3, 4). In analysing in which way the regio-selective derivatisation inhibited the blood clotting, some clues were already present that the inhibition of thrombin was stronger, as seen in the TT measurements in comparison to PTT measurements. Because of this, tests with factor IIa and Xa were performed in the presence of AT III and selected CS with high and low degree of DSs. For this a single factor test with chromogenic substances for the specific serine proteinases F IIa (Thrombin) and F Xa was developed (19). Figure 3A shows that CS 0.26 showed no inhibiting effect against thrombin, since the residual activity of thrombin was roughly 100% even at CS concentrations of 2.5 mg/mL. Conversely sulfated cellulose with a DSs 1.33 (CS1.33) demonstrated an inhibition of about 40% even at concentrations as low as 2.5 µg/mL, which points also to the importance of overall sulfation degree. Indeed, also sites of sulfation seemed to be important since compared to CS1.33, the derivative CS1.35 showed an inhibition of about 10%, only. This surprising result can be partially explained with the different substitution pattern of both samples. As already shown in Table 1, the sample CS1.35 has a higher degree of substitution on position C3, which leads to a lower substitution on position C2 and C6. Although a C3 sulfation is critical for an anti-thrombogenic effect (especially for heparin) (20, 21), the case seems different for cellulose sulfates because of their different structure and the β-(1→4) glycosidic bond. Here it seems that a higher sulfation in C2 and C6 position is more beneficial in terms of the inhibition of thrombin. The existence of sulfate groups on the C2 position of the iduronic acid of the heparin is important for the chain conformation and results in a high affinity for anti-thrombin III (20, 21), which might be supporting the anticoagulant activity of the cellulose-2-6 sulfates towards thrombin, too. However, the results of F Xa assay are looking different (Figure 3B). Here both derivatives with a high DS show a significant inhibition of factor Xa at higher concentration ranges starting at 50 µg/mL compared to the low sulfated CS0.26. In summary, these investigation show that cellulose sulfates with a high DS in position C2 and C6 show an effectiveness analogous to heparin regarding the inhibition of blood coagulation, especially towards the inhibition of thrombin.

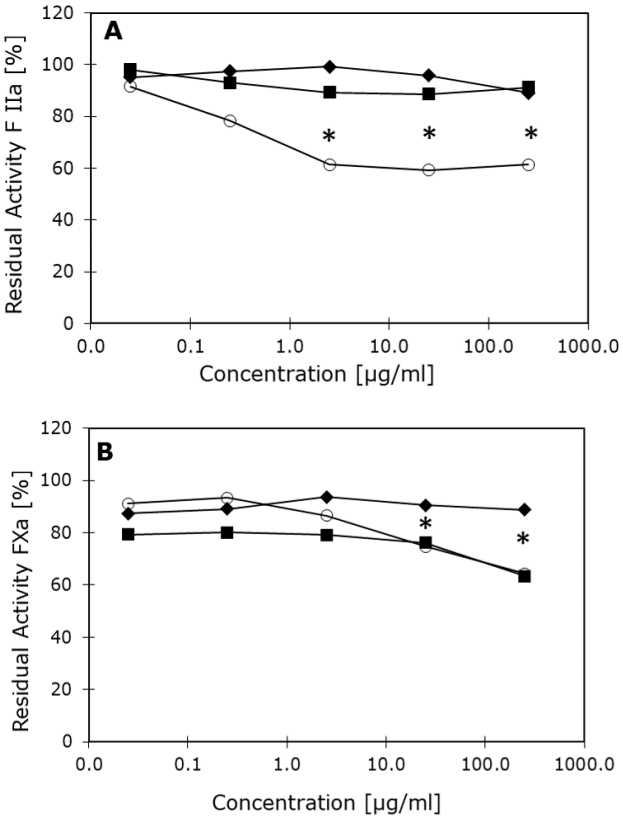

Figure 3 A and B. Residual activity of thrombin (F IIa, A) and factor Xa (F Xa, B) after the addition of chosen cellulose sulfates with different degrees of derivatisation.
(♦) - CS 0.26; (❍) - CS 1.33; (■) - CS 1.35.
Asterisks in the figures show significant deviation ($p <= 0.05$).

Influence of the degree of derivatisation and regioselectivity of cellulose sulfates on the binding and activity of the fibroblast growth factor FGF-2

Cellulose sulfates were synthesized through acetosulfation or direct sulfation by which a wide range of derivatisation degrees was achieved. Table 2 gives an overview on the synthesized cellulose sulfates. The distinctive feature of this sulfation procedure is that there was no detectable derivatisation at C3 position of the AGU. The details of this sulation procedure can be found in the previous work published by Peschel et al (22).

Table 2. Degree of sulfation (DStotal) and distribution of the sulfate groups in the derivatives in the determiantion of the activity of the growth factor FGF-2

Cellulose sulfate (CS)	DS_S (^{13}C-NMR)*			
	C6	C2	C3	DS_{total}
CS 0.39	0.36	0.03	0	0.39
CS 0.58	0.52	0.06	0	0.58
CS 0.66	0.60	0.06	0	0.66
CS 0.92	0.77	0.15	0	0.92
CS 1.57	1.0	0.57	0	1.57
CS 1.69	1.0	0.69	0	1.69
CS 1.80	1.0	0.80	n.d.	1.80
CS 1.94	1.0	0.94	n.d.	1.94

* DS-values of sulfate groups at the C2- C3 and C6 position were determined 13C-NMR spectroscopy. n.d. – not determined

Binding of the fibroblast growth factor FGF-2 to cellulose sulfates

Growth factors like FGF-2 are presented to their corresponding receptor tyrosine kinases on the cell surface through proteoglycans like syndecan, which is decorated with heparan sulfate side chains followed by the activation of cellular kinases that induce cell proliferation (4, 5, 9). Because of this, the binding affinity of FGF-2 to CS was determine in a competitive approach. Figure 4 shows the DS of the derivatives, whereas the light gray and dark gray bars illustrate the derivatization in C2 and C6 position and the observed binding of FDF-2 in comparison to heparin as a control (100%). It can be seen that in comparison to heparin, cellulose sulfates with a DS ≤ 0.92 allow no significant binding of FGF-2. Only for samples with a DS ≥ 1.57 an increase in the growth factor binding correlating with an increase in the DS of up to 60% compared to heparin could be verified. An increase of ca. 40% ($p < 0.05$) was found between the samples of CS1.94 and CS1.57.

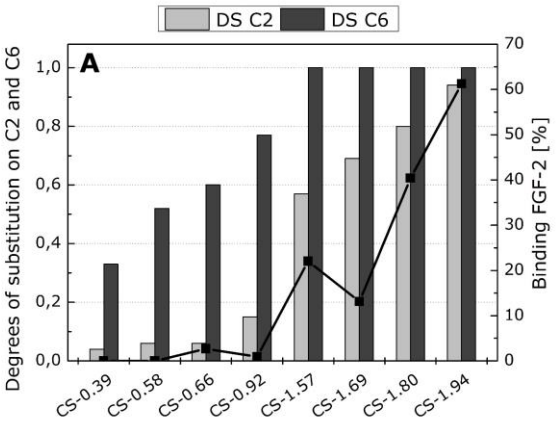

Figure 4. Binding of FGF-2 to sulfated cellulose in dependence of the degree of sulfation in position C2 (light grey columns) and C6 (dark grey columns) compared to the binding to heparin (100%). The binding of FGF-2 is plotted against the degree of sulfation. Values represent the average ± standard deviation (n = 4).

The binding affinity is an important indicator for the biological efficacy of the cellulose sulfates since the binding of growth factors through GAG´s often correlates with the in vitro measured biological activity (23). Here a significant binding of the cellulose sulfates to FGF-2 could only be verified for samples with a DS ≥ 1.00 in C6 position of the AGU, which apparently correlates with an increasing DS in 2-O position. Research of other authors could show that none of the hydrogen bonds that develop during the binding of heparin to FGF-2 are realized with the 6-O sulfate groups, while the sulfation of the 2-O or the 2-N position is of some importance for this bond (24). This might be the case for samples with a DS > 1.5 that express also stronger binding of FGF-2.

Determination of the effect of cellulose sulfates on FGF-2 induced proliferation of mouse fibroblasts

To determine the influence of FGF-2 induced proliferation through CS, a cell culture comprised of embryonic mouse fibroblasts (cell line 3T3-L1) was used, analogous to works of other authors (25). For this, fibroblasts were incubated in a culture medium without serum with 10 ng/mL FGF-2 and the cellulose derivatives for 48 hours. An additional incubation was done with heparin as a control group and cell growth was determined with a DNA quantification assay.

Mitogenic activity of cellulose sulfates at a concentration of 1 mg/mL

The results in Figure 5 show that all samples with a DS ≤ 0.58 inhibited the proliferation of cells. Starting with a DS ≥ 0.66 a stimulation of FGF-2-induced proliferation was observed. With an increasing degree of sulfation the mitogenic effect of the cellulose sulfates was increased too. For the derivative with the highest degree of sulfation CS 1.94 a proliferation of 160% was detected, compared with 10 ng/mL FGF-2 used here as a control.

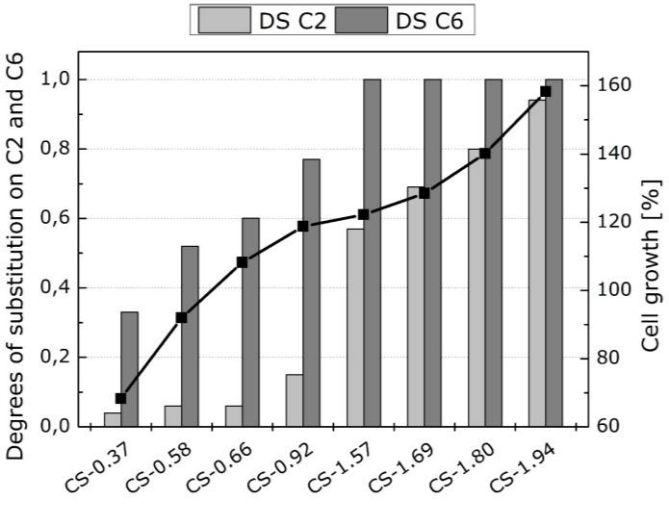

Figure 5. Comparison of fibroblast growth after the addition of FGF-2 (10 ng/mL) and 1 mg/mL cellulose sulfates (CS) with different degrees of sulfation (CS 0.37-CS 1.94).
The proliferation was determined via the content of DNA (average ± standard deviation, n = 5).

In general the proliferation correlates with the binding affinity of the growth factor, which means an increase in the sulfation results in a stronger FGF-2 induced mitogenic activity. Derivatives with a DS ≤ 1.57 that showed no binding of FGF-2 could also lead to an increased proliferation. The reason for this could be based on the high affinity of FGF-2 to heparin, so that those derivatives in the competitive binding assay (Figure 4) could not bind the growth factor. Without the heparin however, the binding of FGF-2 was possible in the cellular assay, which is then visible by the increased FGF-2 activity expressed by cell growth. Similar to these results, Kunou et al. could prove an increase of FGF-1 induced proliferation through dextran sulfate with a DS of ca 1.026.

Dependence of mitogenic activity of cellulose sulfates on the concentration in comparison to heparin

In addition to degree of sulfation and position of the sulfate groups, the concentration of heparin analogous CS also influences the activity of growth factors. In studies of other authors, concentrations of heparin analogous substances in a range of less than 1 µg/mL could already increase the FGF-2 activity. Because of this the analysis of the mitogenic activity of cellulose sulfates was performed in a

concentration range of 1 µg/mL up to 1 mg/mL. The mitogenic activity was compared with the fibroblast growth in the presence of 10 ng/mL FGF-2 as a control group. In the following experiments, cellulose sulfates with a DS of ≥ 0.92 and heparin were used. In Figure 6A and 6B, a strong dependence of the proliferation on the concentration of the samples is visible. At a concentration of 1 mg/mL with the exception of CS-0.92 all cellulose sulfates showed a proliferation of 3T3 cells that was comparable to that of heparin. In a concentration range of 1 – 500 µg/mL a stepwise concentration-dependent increase in proliferation of 3T3 fibroblasts was visible from low- to high-sulfated derivatives.

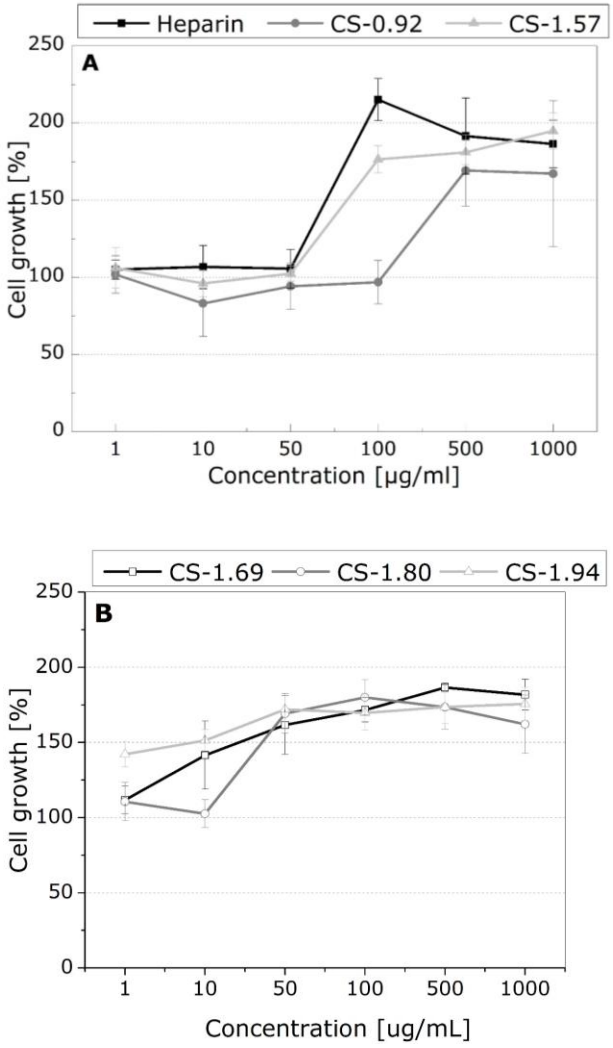

Figure 6. Comparison of proliferation in dependence of the concentration and degree of sulfation of cellulose sulfates (CS) and heparin. 3T3-L1 fibroblasts were incubated with 10 ng/mL FGF-2 and 1 µg/mL bis 1 mg/mL of the derivatives for 48 hours. The proliferation was determined through the DNA content with Pico green.
(A) Heparin and medium sulfated CS, (B) high sulfated CS (average ± standard deviation, n = 5).

The results of these investigations show that with an increasing degree of sulfation lesser amounts of cellulose sulfate were needed to enhance the FGF-2 induced proliferation. It is important to note that highly sulfated celluloses are needed in much lower concentrations than heparin to promote cell proliferation. Since these also show an increased sulfation in the C2 position, this correlates with an increased affinity of FGF-2 to these cellulose sulfates. Heparin binding growth factors like FGF-2 show special often slit like domains that are rich in basic and partly also hydrophobic amino acids and connect to the charged chains of helically-ordered heparin as stretched chains (27). One cause for the comparable or superior activity of the cellulose sulfates (compared to heparin) could be their high degree of sulfation. The heparin that was used in this experiment possesses a DS of 1.3, which is below the DS of some CS used in these studies (22). Nevertheless an important role for the interactions between heparin and FGF-2 and the interaction with the FGF-2 receptor on the cell surface is related to

the carboxy group in C6 position of the iduronic acid (20). In the case of a relatively high sulfation in C2 and C6 position, the β-(1→4) glycosidic bond of AGU in cellulose should lead to a homogeneous charge density on both sides of the chain, which could facilitate the binding of FGF-2 to cellulose sulfates through Coulomb interactions and hydrogen bonds from remaining hydroxyl groupof AGU. The increase in the mitogenic activity of FGF-2 in combination with CS can be traced back to two causes. Growth factors like FGF-2 have a relatively short half-life period also *in vitro* because of the rapid proteolytic fission of the protein. *In vitro* experiments have shown that the stability of FGF-2 against proteases can be increased in the presence of heparin or highly sulfated celluloses because of the interaction between polysaccharide and growth factor (28). On the other hand the high DS could (like heparin) lead to the formation of a FGF-2 – CS – FGF receptor-complex, which leads to an activation of the mitogen activated protein kinases (MAPK/ERK). The research of other authors have shown that especially a high sulfation of heparin in C6 position of the glucosamine monomer plays an important role for this effect (29). Because of this and the β-(1→4) glycosidic bond a higher sulfation in the C6 and C2 position of the AGU of cellulose could be advantageous, which in the end could lead to an enhanced growth of cells in the presence of FGF-2 and higher sulfated celluloses

Conclusion

In this study cellulose sulfate were synthesized that show a bioactivity that rivals or even surpasses that of heparin. It is obvious that a complete sulfation in C6 position and a higher sulfation in C2 position are very important for the biological activity of CS both in anti-coagulation but also promoting mitogenic activity of the heparin-binding growth factors FGF-2. This was evident in the inactivation of thrombin, which is due to a specific interaction with anti-thrombin III that plays a significant role in the inhibition of blood coagulation. Although the activity of higher sulfated celluloses suggests a medical application, a direct systemic use by intravenous injection to inhibit blood clotting like heparin is not advisable due to reasons of product safety. On the other hand, immobilization of cellulose sulfates on the surface of medical devices like catheters and tube systems, which have contact to blood could be an interesting alternative to heparin to increase the blood compatibility of biomaterials (30). Aside from the described inhibition of blood coagulation, CS can also bind different growth factors and influence their activity. The here described stimulating effect on the growth factor FGF-2, which has a mitogenic and angiogenic effects on cells and tissue, and recent studies showing that CS have modulating effects other growth factors like the bone morphogenic protein (BMP-2) suggest applications as bioactive coatings on surfaces of implants in the field of tissue engineering (31,32).

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RAZVOJ BIOAKTIVNIH CELULOZNIH SULFATA ZA BIOMEDICINSKU PRIMENU

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Celuloza je najobilnija biomolekula na zemlji. Hemijski derivati celuloze pronašli su mnoštvo primjena u industrijskim i biotehnološkim primjenama. Celulozni sulfati su klasa derivata rastvorljivih u vodi koji su korišteni u industrijskoj primeni, ali još ne u medicini. Ovde su derivati različitog stepena sulfacije jedinice anhidroglukoze (AGU) celuloze proučavani prema antikoagulacijskim efektima i modulacionim efektima faktora rasta sa domenima koji vežu heparin poput faktora rasta fibroblasta 2 (FGF-2). Rezultati pokazuju da CS višeg stepena sulfacije ima antikoagulantnu aktivnost uporedivu sa heparinom sa kooperativnim delovanjem na antitrombin III koji inhibira aktivnost trombina i faktora Ksa što CS čini zanimljivim za antikoagulantno prekrivanje medicinskih uređaja koji dodiruju krv. Dalje, studije pokazuju da CS sa stepenom sulfacije sa heparinom ima promotivnu aktivnost na mitogeni efekat FGF-2 prikazan u ispitivanjima ćelijske kulture koji ukazuju na njihovu primenu kao obloga materijala za implantate ili komponente tkivnih inženjerskih skela u području traumatologije i regenerativna medicina.

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Ključne reči: *celulozni sulfati, stepen sulfacije, antikoagulacija, trombin, faktori rasta, FGF-2*