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Concepts for improved regioselective placement of *O*-sulfo, *N*-sulfo, *N*-acetyl, and *N*-carboxymethyl groups in chitosan derivatives☆

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Abstract

In the present paper a new strategy has been studied to introduce solely or in combination *N*-sulfo, *O*-sulfo, *N*-acetyl, and *N*-carboxymethyl groups into chitosan with highest possible regioselectivity and completeness and defined distribution along the polymer chain. The aim was to generate compounds having lowest toxicity for determining the pharmacological structure–function relationships among different backbone structures and differently arranged functional groups compared to those of heparin and heparan sulfate. The water-soluble starting material, chitosan, with a degree of acetylation (DA) of 0.14 and a molecular weight of 29 kD, allows one to apply most of the known reactions of chitosan as well as some reactions of heparin chemistry successfully and with improved regioselectivity and completeness. On the other hand, a number of these reactions were not successful by application to water-soluble high-molecular-weight chitosan (DA 0.45 and 150 kD). The starting material showed statistical *N*-acetyl (*N*-Ac) distribution along the polymer chain according to the rules of Bernoulli, with highest abundance of the GlcNAc–GlcNAc diad along with a lower abundance of triads, tetrads, and pentads. The space between the *N*-Ac groups was filled up in homogeneous reactions by *N*-sulfo and/or *N*-carboxymethyl groups, which also resulted in a Bernoulli statistical distribution. The N-substitution reaction showed highest regioselectivity and completeness with up to three combined different functional groups. The regioselectivity of the 3-*O*-sulfo groups was improved by regioselective 6-desulfation of nearly completely sulfated 3,6-di-*O*-sulfochitosan. By means of desulfation reactions, all of the possible intermediate sulfated products are possible. 6-*O*-Sulfo groups can also be introduced with highest regioselectivity and completeness, and a number of partially 6-desulfated products are possible.

Keywords: Chitosan; Regioselective reactions; Heparin; Sulfation; Desulfation; Statistical distributed repeating units

1. Introduction

Various natural polysaccharides with amino sugar repeating units in the backbone and with different regioselective arranged *N*- and *O*-sulfo, *N*-acetyl and carboxyl groups are known and belong to the class of glycosaminoglycans, type II. These substances are known to have important athrombogenic,

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antithrombogenic, antilipemic, antiarteriosclerotic, antiproliferative, and antiviral pharmacological properties. Furthermore, they play an important role in wound healing, tumor growth, and angiogenesis, depending on the type of functional groups, their amount, and their regioselective arrangement.[1–8] Some of these substances are difficult to isolate in sufficient amounts, and, therefore, synthetic methods are required and have been appropriately developed. Stepwise oligosaccharide synthesis using protecting groups allows one to prepare derivatives consisting of up to ten sugar repeating units.[9] Up to now a number of regioselective reactions with high selectivity have been developed to modify heparin and domain structures of heparan sulfates with β-(1→4) and α-(1→4) backbone structures. Methods for synthesizing regioselective derivatives are well established.[10–17] However, it was realized that chitosan with its different backbone [β-(1→4)] compared to that of heparin could be a good, inexpensive source of starting material for preparing derivatives with groups arranged analogously to those of heparin or heparan sulfates. These compounds would be expected to have different properties relative to their different β-(1→4) backbone structures. The regioselective reactions with chitosan described herein are not as well established as those of heparin for which no systematic protection group strategy is used. The main reason for this is the fact that chitosans from different sources showing different molecular weights and degrees of acetylation are used, which sometimes causes solubility problems and limits the application of known literature methods.

In the present paper we have applied some regioselective reactions of heparin with reaction conditions adjusted to chitosan to improve the regioselectivity of the above-mentioned groups. Furthermore we selected known reactions of chitosan from the literature and searched for new methods that would result in high regioselectivity of the derivatives.[18–26] We have extended some of the recently published regioselective reactions of chitosan[27] in developing our concepts, which are outlined as follows:

1. The starting material is a commercially available water-insoluble chitosan with a molecular weight of 150 kD and a DA of 0.28. An acetylated chitosan with DA 0.45 becomes water-soluble.
2. The molecular weight of 150 kD is to be reduced by acidic hydrolysis to a water-soluble 29 kD chitosan, DA 0.14, which has a number of advantages over a high-molecular-weight water-soluble chitosan.
3. The low-molecular-weight sulfated chitosan derivatives are known to be less toxic than the high-molecular-weight derivatives.[21]
4. The low-molecular-weight derivatives will result in lower viscosity of the solutions and, therefore, a higher resolution of the signals in ^{13}C NMR analysis.
5. The selected molecular weight (29 kD) of the chitosan derivatives is twice that of commercial heparin and lower than that of endothelial-cell heparan sulfate. It is therefore deemed more useful for structure–function studies than high-molecular-weight chitosan.
6. Whenever possible we will select reactions that do not require the use of protecting groups so that only in a few cases will protecting groups be needed.
7. Low-molecular-weight chitosan will allow the successful application of most known regioselective reactions, whereas the use of high-molecular-weight water-soluble chitosan results in very few successful reactions.
8. Most reactions are to be carried out in homogeneous media, resulting in a statistical distribution of functional groups along the polymer chain. In contrast, heterogeneous reactions are known to result in block structures that cause solubility problems.
9. The difference in reactivity between amino and hydroxyl groups will be used in the absence of protecting groups to attain complete regioselectivity.
10. In a few cases, solubility in organic solvents will be improved by using intermediate solubilizing groups.
11. The sequence of reactions will be chosen in such way that most of the reactions

can be carried out in homogeneous solution and that labile functional groups such as *N*-sulfo will be introduced at the end of the sequence. ^{13}C NMR spectra of the reaction products will be used to monitor the success of the modifications.

2. Experimental

Starting materials.—The starting material, poly-β-(1→4)-D-glucopyranosamine), for all the experimental work was shrimp and crab chitosan (origin *Chionecetes japonicas*) from Fluka Chemical Co. (EG no. 2223112, product no. 22741) with low-molecular-weight of 150 kD and a degree of acetylation (DA) of 0.28. Insoluble components $\leq 1\%$, viscosity 100 mPa (1% in 1% AcOH, 20 °C). All other certifications are described under the product number. Other chemicals and solvents were purchased either from Fluka or Aldrich Chemical Co. All chemicals and reagents, unless otherwise specified, were not purified, dried or pretreated. Solvents [pyridine and *N*,*N*-dimethylformamide (DMF)] were dried over CaH_2 and subsequently distilled in vacuum.

Analytical methods.—Dialysis tubing (Visking) with a pore size 15–20 Å from Serva were used to purify the water-soluble chitosan derivatives. NMR spectra of chitosan derivatives were measured with a Bruker AC 300 spectrometer in D_2O, 1% CD_3COOD, or Me_2SO-d_6. The sulfation pattern and degree of sulfation was calculated by the 'triangle method' from Casu et al.[28] All infrared (IR) spectra were measured in KBr pellets with an IMPACT 400 FTIR spectrometer from Nicolet.

Determination of molecular weight.—Molecular weights of water-soluble chitosan derivatives were determined by HPLC–GPC on a Dionex DX 500 system using a Bio Rad SEC 400 column. The detection was carried out with a UV detector at 206 nm. The elution was carried out with a 0.02 mol/L NaH_2PO_4 and 0.15 mol/L NaCl buffer, pH 5.8. Calibration of the column was carried out by the use of pullulan standards with molecular masses between 5.8 and 850 kD.

Partial hydrolysis of chitosan.—Milled chitosan (2 g) was dissolved in 0.1 N HCl (150 mL) and refluxed for 24 h under a N_2 atmosphere. After cooling to rt, the solution was adjusted to pH 5.8 with 2 N NaOH. The solution was dialyzed against deionized water and freeze dried. Yield: 80%. ^{13}C NMR (1% CD_3COOD in D_2O): δ 104.2 (C-1–NAc), 101.6 (C-1), 80.2 (C-4), 77.6 (C-5), 73.9 (C-3), 63.2 (C-6), 59.0 (C-2).

Partial N-acetylation of chitosan (DA 0.14).—Partially hydrolyzed chitosan (1 g, 6.0 mmol) was dissolved in 1% AcOH (100 mL). After addition of MeOH (100 mL) to the solution, Ac_2O (0.605 mL, 6.42 mmol) dissolved in MeOH (30 mL) was added. The mixture was stirred at rt for 40 min. The product was precipitated by pouring the mixture into a mixture of MeOH (140 mL) and 25% NH_3 (60 mL). The product was filtered off, washed with MeOH and Et_2O, and dried overnight in vacuum at rt.[24] Yield: 82%. IR (KBr): 1654 cm^{-1} ν(C=O) amide I, 1562 cm^{-1} ν(N–H) amide II. ^{13}C NMR: δ 101.9 (C-1–NAc), 98.4 (C-1), 77.7 (C-4), 75.7 (C-5), 72.0 (C-3), 61.1 (C-6), 56.4 (C-2).

N-carboxymethylation of partially N-*acetylated chitosan.*—Partially hydrolyzed and partially *N*-acetylated chitosan (1 g, 6.0 mmol) was suspended in water (50 mL). After addition of glyoxylic acid monohydrate (0.57 g, 6.19 mmol), the chitosan derivative dissolved within the next 45 min. The solution was adjusted to pH 12 by addition of 2 N NaOH. A solution of 0.4 g of sodium cyanoborohydride dissolved in water was added, and the mixture was stirred for 45 min. The product was precipitated in EtOH (400 mL), filtered, washed with EtOH and dried overnight in vacuo at rt (a variation of Ref. 26). Yield: 90%. IR (KBr): 1635 cm^{-1} ν(C=O) carboxylic acid, 1530 cm^{-1} ν(N–H) 2° amine. ^{13}C NMR (D_2O): δ 176.4 (COOH), 174.9 (C=O), 101.8 (C-1–NCM), 98.2 (C-1), 77.6 (C-4), 75.4 (C-5), 71.9 (C-3), 61.2 (C-6), 56.3 (C-2).

N-sulfation of partially N-*acetylated chitosans.*—Partially hydrolyzed and partially *N*-acetylated chitosan (300 mg, 1.8 mmol) was dissolved in water (50 mL). To this viscous

solution were added Na_2CO_3 (635 mg, 3.3 equiv) and $SO_3{\cdot}NMe_3$ complex (1.3 g, 5 equiv). After stirring for 18 h at 65 °C under a N_2 atmosphere, the solution was dialyzed against deionized water for 24 h, against 25 mM NaOH for 4 h, against deionized water for 3 days, and it was then freeze dried.[23] Yield: 85%. IR (KBr): 1237 cm^{-1} ν_{as}(O=S=O), 1032 cm^{-1} ν_s(O=S=O). ^{13}C NMR (D_2O): δ 175.0 (C=O), 104.6 (C-1–NS), 104.0 (C-1–NAc), 81.7 (C-4), 77.6 (C-5), 75.8 (C-3), 63.0 (C-6), 62.5 (C-2–NS), 58.0 (C-2–NAc).

Synthesis of 2-phthalimido-chitosan (DA 0.14).—Partially hydrolyzed chitosan (1 g, 6.0 mmol) was dissolved in 1% AcOH (100 mL). After addition of MeOH (100 mL), the chitosan was reprecipitated in 4% $NaHCO_3$ solution (100 mL) and stirred for 2 h at rt. The precipitate was filtered and subsequently washed with MeOH. After drying for 15 min on a Buchner funnel, the product was suspended in dry DMF (40 mL) and stirred for 16 h at rt. It was again filtered off and resuspended in dry DMF for 1 h. This procedure was repeated a third time. The DMF–wet chitosan powder was added to dry DMF(25 mL). After addition of phthalic anhydride (1.84 g, 12.42 mmol) and ethylene glycol (0.69 mL, 12.42 mmol), the mixture was stirred for 2 h at 130 °C under a N_2 atmosphere. After cooling, the transparent yellow solution was poured into ice-cold water (200 mL). The precipitate was filtered off, washed with water, resuspended in EtOH, filtered off again, and washed subsequently with EtOH and Et_2O. The product was dried overnight in vacuo at rt (a variation of Ref. 29). Yield: 87%. IR (KBr): 1770–1705 cm^{-1} ν(C=O) phthalimide. ^{13}C NMR (Me_2SO-d_6): δ 167.7 (C=O, phthalimide), 123.1–134.5 (ring, phthalimide), 100.9 (C-1), 97.3 (C-1), 79.3 (C-4), 74.5 (C-5), 71.9 (C-3), 59.6 (C-6), 56.4 (C-2).

*Synthesis of 3,6-di-*O*-sulfochitosan.*—2-Phthalimidochitosan (250 mg, DA 0.14, 0.86 mmol) was dissolved in dry DMF (50 mL) by stirring overnight. To this clear solution a second solution of $SO_3{\cdot}$pyridine (1.37 g, 8.62 mmol) in dry DMF (10 mL) was added dropwise (1:10 = molar ratio repeating unit:$SO_3{\cdot}$pyridine). Within the first 10 min the product started to precipitate, and the reaction mixture was stirred at rt overnight. Afterwards, the mixture was poured into ice-cold water (70 mL), yielding a clear solution that was neutralized by 2 N NaOH. This solution was dialyzed against 0.2% $NaHCO_3$ solution for 4 h, then against deionized water.

To eliminate the phthalimido group, the product (210 mg, 0.46 mmol) was dissolved in deionized water (30 mL), and hydrazine hydrate (1.13 mL, 35.6 mmol) was added. The solution was heated to 70 °C for 16 h. Afterwards water (50 mL) was added, and the solution was evaporated nearly to dryness. This was repeated twice to eliminate the remaining hydrazine. The volume of the solution was brought to 50 mL by adding deionized water; afterwards, it was dialyzed and freeze dried. Yield: 74%, $DS_{sulfate} = 1.76$. IR (KBr): 1232 cm^{-1} ν_{as}(O=S=O), 1027 cm^{-1} ν_s(O=S=O). ^{13}C NMR (D_2O): δ 103.3 (C-1–NAc), 99.2 (C-1), 81.3 (C-4), 78.4 (C-5), 75.5 (C-3–S), 73.2 (C-3), 69.6 (C-6–S), 63.0 (C-6), 58.0 (C-2).

*Synthesis of 3-*O*-sulfochitosan by 6-desulfation*[14] *of 3,6-di-*O*-sulfochitosan (DA 0.14).*—A solution of 3,6-di-*O*-sulfochitosan (DA 0.14, 335 mg, 1.09 mmol) in water (10 mL) was passed through a column (2.5 × 20 cm) of Amberlite IR-120 (H^+, 20–50 mesh) cation-exchange resin. The eluate was adjusted to pH 6.0 by addition of pyridine and then lyophilized. The pyridinium salt of the sulfated chitosan derivative was dissolved in dry pyridine (35 mL), and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (6.7 mL, 36.2 mmol) was added. After stirring for 2 h at 110 °C, the solution was added to ice water (35 mL). After dialysis for 24 h, the solution was adjusted to pH 10 by 0.1 N NaOH. The solution was again dialyzed for 3 days, then freeze-dried. Yield: 81%, $DS_{sulfate} = 1.0$. IR (KBr): 1230 cm^{-1} ν_{as}(O=S=O), 1043 cm^{-1} ν_s(O=S=O). ^{13}C NMR (D_2O): δ 81.3 (C-4), 75.8 (C-5), 73.6 (C-3–S), 72.0 (C-3), 69.7 (C-6–S), 63.6 (C-6), 58.1 (C-2).

*Synthesis of 3-*O*-sulfochitosan by 6-desulfation*[15] *of 3,6-di-*O*-sulfochitosan (DA 0.14).*—A solution of 3,6-di-*O*-sulfochitosan (DA 0.14, 450 mg, 1.47 mmol) in water (20 mL) was passed through a column (2.5 × 20 cm) of Amberlite IR-120 (H^+, 20–50 mesh)

cation-exchange resin. The eluate was adjusted to pH 6.0 by addition of pyridine and then lyophilized. The pyridinium salt of the sulfated chitosan derivative was dissolved in a mixture of *N*-methylpyrrolidinone (36 mL) and water (4 mL). The yellow solution was stirred for 24 h at 90 °C under a N_2 atmosphere. After dilution with water (40 mL), the pH was adjusted to 9 by 2 N NaOH. The solution was then dialyzed and freeze-dried. Yield: 55%, $DS_{sulfate} = 0.62$. IR (KBr): 1227 cm^{-1} ν_{as}(O=S=O), 1015 cm^{-1} ν_s(O=S=O). ^{13}C NMR (D_2O): δ 104.4 (C-1–NAc), 100.1 (C-1), 81.6 (C-4), 78.0 (C-5), 76.0 (C-3-S), 73.3 (C-3), 69.6 (C-6–S), 63.3 (C-6), 58.8 (C-2).

Synthesis of 6-O-*sulfochitosan (DA 0.14).*—Partially hydrolyzed chitosan (DA 0.14, 500 mg, 3.0 mmol) was dissolved in 2% formic acid (50 mL). To this solution 1 M $CuSO_4 \cdot 5 H_2O$ (8.3 mL, 8.3 mmol) was added dropwise at rt. After stirring for 16 h, the resulting precipitate was filtered off and washed subsequently with water, acetone, and Et_2O. This precipitate was then dispersed in dry DMF (30 mL) for 16 h, filtered off again, and redispersed in dry DMF (30 mL). The SO_3·pyridine complex (1.9 g, 12.0 mmol) dissolved in dry DMF (15 mL) was added dropwise at 0–2 °C (1:4 = molar ratio repeating units:SO_3·pyridine) and stirred for 1 h. Afterwards the mixture was stirred for 16 h at 50 °C under a N_2 atmosphere. The solution was adjusted to pH 8 by addition of satd aq $NaHCO_3$. After addition of water (100 mL), the solution was dialyzed for 3 days. The solution was passed through an Amberlite IRC 718 column to remove the copper from the complex. The eluate was freeze-dried.[19] Yield: 85%, $DS_{sulfate} = 1.0$ IR (KBr): 1240 cm^{-1} ν_{as}(O=S=O), 1030 cm^{-1} ν_s(O=S=O). ^{13}C NMR (D_2O): δ 104.2 (C-1), 85.3 (C-4), 75.8 (C-5, C-3), 69.6 (C-6–S), 59.2 (C-2).

Synthesis of N,-*sulfo*-6-O-*sulfochitosan (DA 0.14).*—6-*O*-Sulfochitosan (250 mg, 1.0 mmol) was regioselectively *N*-sulfonated by the method described above. Yield: 92%, $DS_{sulfate} = 1.57$. IR (KBr): 1240 cm^{-1} ν_{as}(O=S=O), 1030 cm^{-1} ν_s(O=S=O). ^{13}C NMR (D_2O): δ 104.8 (C-1–NS), 104.1 (C-1–NAc), 80.6 (C-4), 75.8 (C-5), 75.1 (C-3), 69.3 (C-6–S), 63.0 (C-6), 62.4 (C-2–NS), 58.6 (C-2–NAc).

Synthesis of N-*carboxymethyl*-6-O-*sulfochitosan (DA 0.14).*—6-*O*-Sulfochitosan (250 mg, 1.0 mmol) was regioselectively *N*-carboxymethylated by the method described above. Yield: 67%; $DS_{sulfate} = 1.0$. IR (KBr): 1632 cm^{-1} ν(C=O) acid, 1530 δ (N–H) 2° amine, 1248 cm^{-1} ν_{as}(O=S=O), 1027 cm^{-1} ν_s(O=S=O), 810 cm^{-1} ν(S–O). ^{13}C NMR (D_2O): δ 103.7 (C-1–NAc), 99.3 (C-1–NCM), 78.4 (C-4), 76.0 (C-5), 75.5 (C-3), 69.6 (C-6–S), 57.9 (C-2–NAc–NCM).

3. Results

The starting material was a commercially available (Fluka), water-insoluble chitosan derivative with a degree of acetylation (DA) of 0.28 and a molecular weight of 150 isolated from shrimps and crabs (source *C. japonicas*). N-Acetylation with acetic anhydride in methanol in homogeneous solution according to the method of Lee et al.[24] resulted in a water-soluble chitosan (DA 0.45). The distribution of the NAc groups along the polymer chain was measured by a specific cleavage reaction of the main chain at free amino groups with HNO_2 causing ring closure reaction to 2,5-anhydro-D-mannose, according to the methods of Shively and Conrad[30] and of Sashiwa et al.[31] (Fig. 1).

The resulting oligomers were separated by gel-permeation chromatography, and the sequence of the GlcNAc block structures was determined experimentally as well as theoretically.[31]

A good correlation was demonstrated between the calculated and experimental data. The most frequent sequences were found to be diblocked GlcNAc structures along the polymer backbone, while the less frequently encountered sequences were *N*-acetylated tri-, tetra- and pentasaccharides. These are statistically distributed in GlcN repeating units and follow the rules of a Bernoullian statistics according to the method of Sashiwa et al.[31]

For development of regioselective reactions such as N- and O-sulfonation and acetylation, as well as carboxymethylation at the N of chitosan, with highest possible regioselectivity according to our concepts described above, we

hydrolyzed a 150 kD chitosan in homogeneous medium with HCl. The result was a water-soluble 29 kD chitosan (DA 0.14) at pH $\leq$ 6 that we used as a starting material (**1**) for preparing all the products shown in Table 2. The distribution of the NAc groups along the polymer chain was comparable to the 150 kD chitosan (DA 0.45) and also roughly followed the rules of Bernoullian statistics[31] according to the method of Sashiwa et al.[31] These results are not shown.

At first we made use of the differential reactivity between amino groups and hydroxyl groups of chitosan to study the regioselectivity at the N-position without using protecting groups. The reaction products were analyzed by IR spectroscopy, and the number of functional groups was determined by ^{13}C NMR spectroscopy using the triangle method of Casu et al.[28]

N-Acetylation.—In some cases the *N*-acetylchitosan (DA 0.14) was additionally *N*-acetylated with acetic anhydride in methanol in homogeneous solution (**3** and **7**) according to the method of Lee et al.[24] Each desired NAc content up to 50% can be regioselectively introduced without any side reactions. N-Acetylation showed highest regioselectivity. No reactions occurred at the OH-groups, as shown by the lack of the ester carbonyl vibration at 1740 cm^{-1}. In the ^{13}C NMR spectrum there was observed two C-1 signals, with one signal at 101.9 ppm for the *N*-acetylated C-2 and second signal at 98.4 ppm for the C-2 with an unsubstituted amino group.

N-Carboxymethylation.—Introduction of *N*-carboxymethyl groups into chitosan (DA 0.14, 29 kD) according to the method of Muzzarelli et al.[26] is highly regioselective (ex. **7**, see Table 2), even in the presence of 6-OSO_3^- **5**. There is a specific reaction at the C-2 amino group without influence of the C-6–OSO_3^- and without reaction at C-3. The reaction can be carried out quantitatively up to an overall substituted amino DS of nearly 1.0. The free nonacetylated amino groups react almost completely (100%) and exclusively when using the Muzzarelli method.[26] The ^{13}C NMR spectrum shows two carbonyl signals at 176.4 ppm (carboxyl) and at 174.9 ppm (acetyl). The IR spectrum shows a new signal at 1530 cm^{-1} that can be assigned to a deformation vibration of a secondary amine, which indicates no disubstitition of carboxymethyl groups at the N-position occurs. The space between the statistically distributed NAc groups along the polymer chain can be filled up nearly quantitatively by *N*-carboxymethyl groups.

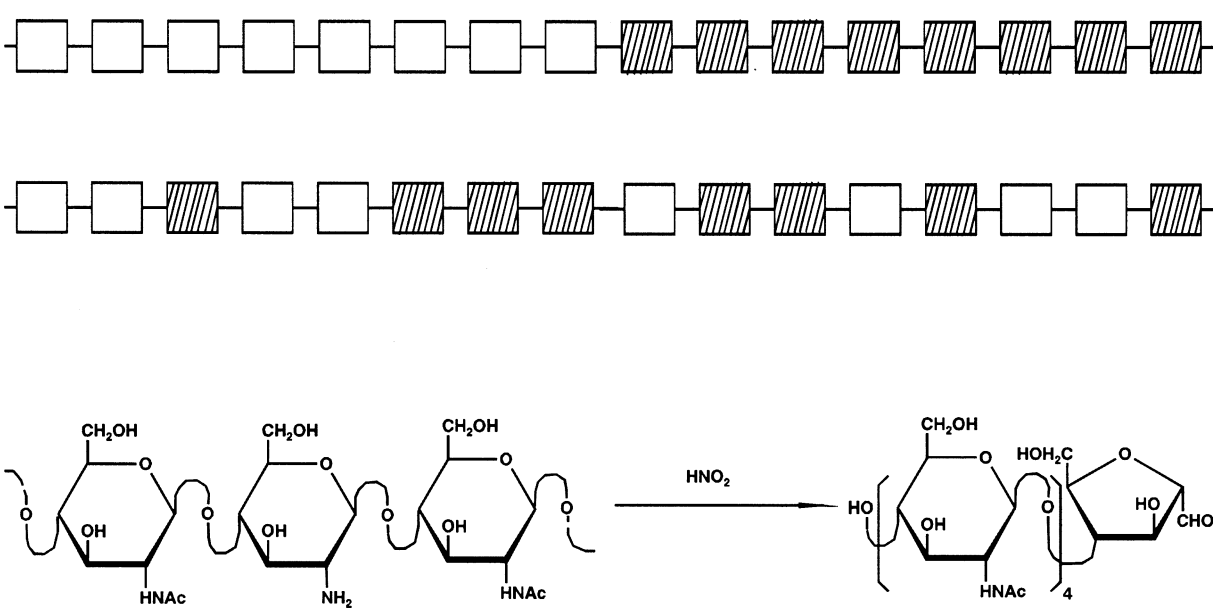
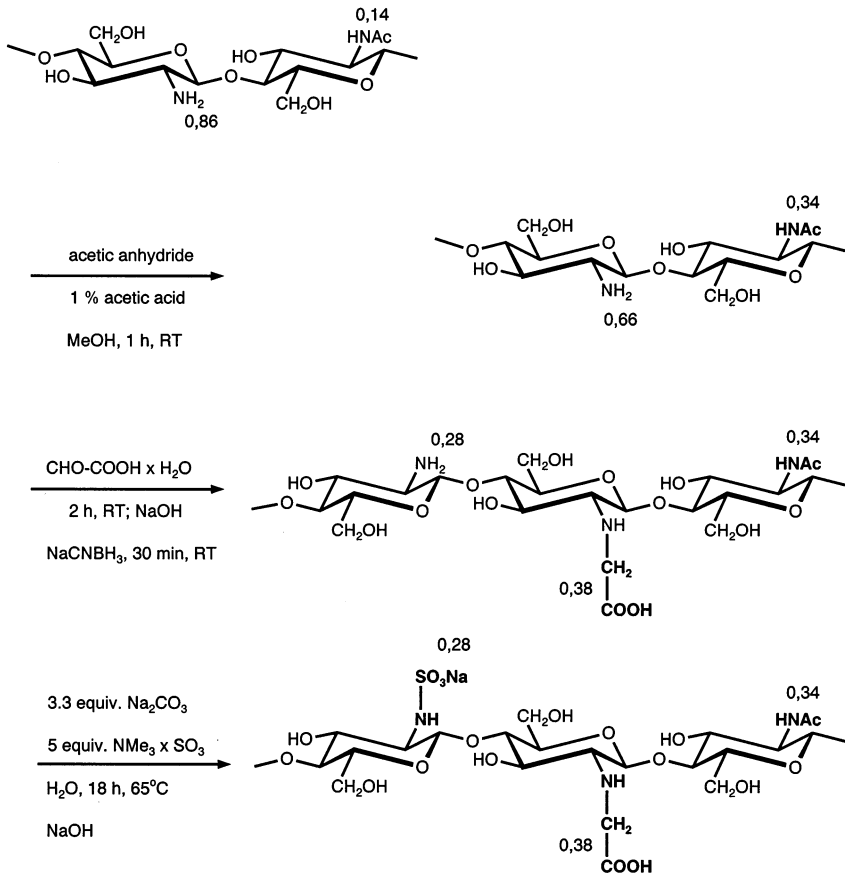


Fig. 1. Schematic presentation of partially acetylated chitosan with block structures (top) and statistically distributed GlcNAc units (bottom). The reductive deamination of chitosan by HNO_2 cleaves the polysaccharide chains at nonacetylated GlcN units with formation of 2,5-anhydromannose units by ring contraction.



Scheme 1. Scheme for regioselective modification of chitosan or 6-sulfated chitosan at the free amino groups resulting in up to three different combined functional groups at the nitrogen along the polymer chain.

N-Sulfonation.—N-Sulfonation of chitosan (DA 0.14) or 6-*O*-sulfochitosan in homogeneous aqueous solution using the trimethylamine·SO_3 complex according to Holme and Perlin[23] resulted in almost complete *N*-acetyl-*N*-sulfochitosan (0.14 NAc + 0.86 DSNS) without sulfation at C-3 and without a decrease of the original NAc content of 0.14 (**2**). However, about 0.29 of the *O*-sulfo groups were lost at the 6-O-position (**6**). Noteworthy is the large upfield shift of the C-2 *N*-sulfo signal at 4.5 ppm in the ^{13}C NMR spectrum relative to the C-2 *N*-acetyl signal at 58.0 ppm. The IR spectrum of both substances showed the asymmetric and the symmetric valence vibrations of the O=S=O group at about 1235 and 1035 cm^{-1}, which can be observed in all sulfated chitosan derivatives. The distribution of NAc and NSO_3 groups along the polymer chain is comparable as shown by Bernoullian statistics described above.

The differential reactivity of amino and hydroxyl groups can be used successfully as shown above to achieve the highest possible regioselectivity as well as completeness for all three N-substituted products without using protecting groups (**7**, Scheme 1). In contrast to this, the regioselective modification of the OH groups in the 6-position can be accomplished only by either (1) intermediate protection of the amino group by protonating in acidic in medium[18] or (2) by making use of the reactivity differences between primary OH groups in the 6-position and secondary OH groups in the 3-position by way of systematic permanent protecting groups. Another possibility is the use of a chelating reagent for intermediate protection of primary amino groups and C-3 OH groups in one step[19]. 3-*O*-Sulfochitosan may be synthesized using intermediate protecting groups for amino groups and preparing 6-*O*- and 3-*O*-disulfated chitosan derivatives, which then can be regioselectively 6-desulfated afterwards with regioselective

desulfation reactions taken from heparin chemistry.[14] For all these known reactions, literature examples have been evaluated. We have excluded possible reactions using more than one protecting group for preparing 3- or 6-*O*-sulfochitosan derivatives.

6-O-Sulfonation.—Focher et al.[19] described a method for regioselective 6-O-sulfonation of chitosan. These workers used a copper complex for simultaneous intermediate protection of the amino groups at C-2 and the OH group in the 3-position of the saccharide backbone. Application of this method to chitosan (DA 0.14) was highly regioselective and complete without changes in NAc content and without reaction at C-3 (**4**), as can be seen in Table 1

Table 1
Comparsion of calculated data of oligomer fractions according to Sashiwa et al.[31] and experimentally determined data (Fig. 2) [a]

Oligomer (n)	Weight % of oligomers (W_n)	
	Calculated according to Bernoullian statistics	Experimental
1	27.1	28.3
2	27.5	37.9
3	19.3	17.6
4	11.8	9.2
5	6.7	7.0

[a] $W_n = n \times (1-\mathrm{DA})^2 \times \mathrm{DA}^{n-1} \times M_n \times (n \times M_0)^{-1}$. N, number of residues in oligomers; DA, degree of acetylation; M_n, molecular weight of nth oligomer; M_0, average molecular weight of a unit in partially acetylated chitosan.

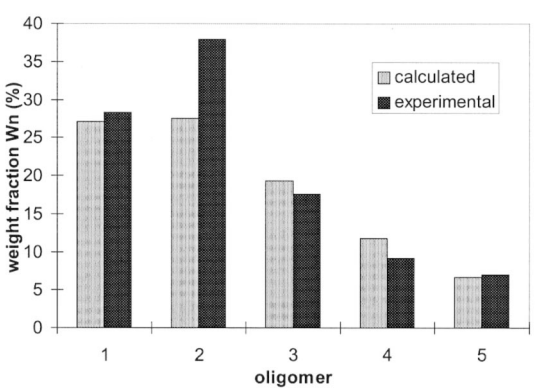


Fig. 2. Comparison of calculated[31] and experimental data of GlcNAc diad, triad, tetrad and pentad with experimental data after HNO_2 degradation. The formed oligosaccharides were separated and quantified by gel-chromatography on Bio-Gel P2 using a UV detector at 206 nm. The frequency of one to five GlcNAc repeating units follow Bernoulli statistics.

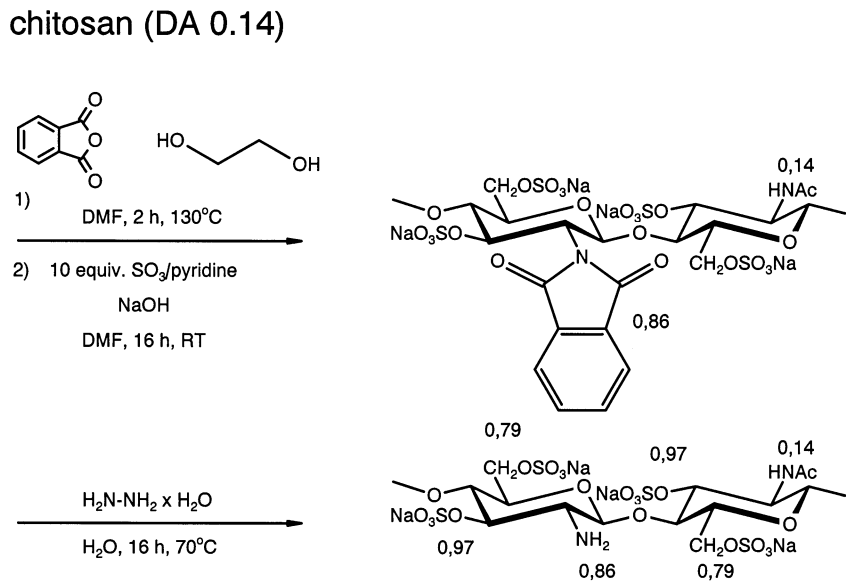
(Fig. 2). The success of the complete 6-O-sulfonation can be observed by the lack of a C-6–OH signal in the ^{13}C NMR spectrum at about 62 ppm, with the new signal of the sulfated C-6 shifted downfield to 69.6 ppm. Another 6-O-sulfonation reaction reported in the literature[18] could not be reproduced.

3,6-Di-O-sulfonation.—Because chitosan (DA 0.14) or carboxymethylated chitosan (0.86) is insoluble in DMF, we used a phthalimido group as the intermediate protecting group to solubilize the polymer according to a method of Nishimura et al.[29] (Scheme 2). The IR spectrum showed two typical signals for the phthalimide carbonyl vibration at 1770 and 1705 cm^{-1}, whereas the lack of signals at 1745 and 1230 cm^{-1} were good indicators for the absence of *O*-acyl byproducts.

The 3,6-O-disulfonation was carried out with the SO_3·pyridine complex, which when followed by deprotecting the amino group with hydrazine hydrate, resulted in a highly sulfated chitosan derivative with a sulfate DS at C-3 of 0.97 and at C-6 of 0.79. This was carried out without decrease of the original NAc content of 0.14 (**10**) and was a nearly complete reaction at C-3, whereas C-6 was only substituted to 79%, which is not important, because 6-O-desulfonation follows. The ^{13}C NMR signal at 73.2 ppm for the C-3–OH is nearly absent, while there is a larger signal for the C-6–OH at 63.0 ppm. The lack of signals between 120 and 140 ppm, as well as the lack of signals in the IR spectrum at 1770 and 1705 cm^{-1}, revealed the success of the removal of the phthalimido group by hydrazine hydrate.

3-O-Sulfonation.—In heparin chemistry there is a known sulfatotransfer reaction for specific 3-O-sulfonation from glucosamine-*N*-sulfonate units, which is not applicable to chitosan.[16] Therefore we began to completely sulfate both the 3- and 6-position of the glucosamine moiety, and then we tried to use specific 6-O-desulfonation reactions from heparin chemistry.[14,15]

6-O-Desulfonation.—Solvolytic 6-O-desulfonation reaction of the pyridinium salt of 3,6-di-O-sulfochitosan in an *N*-methylpyrrolidinone–water mixture as used in heparin chemistry[15] was applied to reduce the 6-*O*-



Scheme 2. 3,6-Di-O-sulfonation of *N*-carboxymethylated chitosan using the *N*-phthalimido group as an intermediate solubilizing group. The group is completely removed by hydrazine hydrate with minimal effect on the sulfate content at C-6.

sulfo content. This reaction reduces the 6-*O*-sulfo content from 0.79 to 0.15 (**9**) as can be observed via ^{13}C NMR spectroscopy by the signals at 69.6 and 63.3 ppm, but the selectivity is not as high as with heparin because nearly half of the 3-*O*-sulfo groups were also removed. Therefore, we adapted the method of Takano et al.[14] for specific 6-O-desulfonation of heparin with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide in pyridine to our derivative (**8**). The result was the same pronounced decrease in 6-*O*-sulfo content from 0.79 to 0.15, but with high regioselectivity. Only 15% of the 3-*O*-sulfo groups were lost (**8**). The degree of acetylation remained constant at DA 0.14.

4. Discussion

The regioselective reactions originating from chitosan and heparin chemistry, which serve to introduce additional *N*-acetyl, *N*-carboxymethyl, *N*-sulfo, and 6-*O*-sulfo groups with highest possible regioselectivity at the *N*- as well as in the 6-O-position, could be successfully applied to a water-soluble chitosan starting material with a degree of acetylation of 0.14 and an average-molecular weight of 29 kD. All reactions for introducing *N*-carboxymethyl, *N*-sulfo, and additional *N*-acetyl groups in homogeneous medium were highly regioselective and without any side reactions. This is because of the known difference in reactivity between hydroxyl and primary amino groups. When two or three of the above-mentioned groups at the N-position are introduced in succession, a reaction up to nearly a total DS of 1 can be reached. Although the detection limit of the ^{13}C NMR method is a good one, it has taken into account that variations in analytical results amount to 1–2%. This has neither been mentioned nor calculated in the results shown in Table 1. However, we used an additional analytical method (ninhydrin) to estimate the completeness of substitution reactions [sulfation (**6**) and/or carboxymethylation (**5**,**7**)] at primary amino groups in the 2-position of chitosan. The amount of unreacted aminogroups was 1–2% (**5**,**6**,**7**). The results are in very good agreement between the two different independent methods of analysis. Muzzarelli and co-workers were able to synthesize with other starting materials either *N*-carboxymethylated and *N*-acetylated or *N*-carboxymethylated and *N*-sulfonated chitosan derivatives with a total DS at the nitrogen of 1.0.[22] The statistical distribution of GlcNAc repeating units in our starting material, mainly according to Bernoulli along the polymer chain, may also be observed with the

N-sulfonation reaction in the second step. The distribution of GlcN–CH_2–COOH along the polymer chain directly combined with GlcNAc cannot be detected with specific HNO_2 degradation of the main chain (**5**). However, a Bernoulli distribution of GlcN–CH_2–COOH along the chain can also be expected, because the gaps of GlcNAc in statistically arranged Bernoulli block structures will be either partially or nearly completely filled up. Furthermore, the reaction that was carried out in homogeneous medium, also supports formation of a statistical distribution of Bernoulli block structures. The block structures are constant in the statistical average. Chitosan with DA 0.45 consists mainly of diad (GlcNAc–GlcNAc) and triad (GlcNAc–GlcNAc–GlcNAc) in highest abundance; a DA of 0.14 showed smaller blocks, diads of GlcNAc–GlcNAc in highest abundance.

N-Disubstituted *N*-carboxymethyl derivatives[32] were not needed for our concept and were not found (no indication by ^{13}C NMR), because we used in a two-step reaction selected specific reaction conditions with 1.4:1 (respectively others) stoichiometric ratios of glyoxylic acid to GlcN. In the second step, alkaline reduction conditions with cyanoborohydride in a 1:1 ratio to initial glyoxylic acid, which was based on the procedure of Muzzarelli et al.,[26] with small variations, was employed. These selected two-step conditions are different to those of the modified one-step method of Muzzarelli in which there is the exactly needed 1:1 ratio of glyoxylic acid to the amino group of chitosan and parallel reduction in the acidic region with 1.5 excess reduction medium to the glyoxylic acid. It has been reported that this modified one-step Muzzarelli method produces the expected *N*-carboxymethylchitosan as well as the unavoidable disubstituted *N*-carboxymethylchitosan.[32] Under special circumstances with a ratio of glyoxylic acid to primary amine higher than 1:1, partially insoluble reaction products were also formed,[32] which may be a typical indication for additional crosslinking reactions in a one-step reaction in the acidic region. However, in organic chemistry[40,41] in homogeneous solution with the two-step reaction disubstitution products have not been reported with 1:1 stoichiometric ratio of primary amines with aldehydes via an aldimine reaction, followed in the next step by a complete reduction. But disubstitution products may be possible either when two-step reactions take place in series or in a one-step reaction if aldehyde remains under simultaneous reaction conditions when the monocarbomethylated product may then react for a second time. The results of our selected two-step reaction conditions indicate that reaction took place without formation of disubstituted products, which is also in agreement with Muzzarelli et al.[26] The excess ratio of glyoxylic acid to amine in the first step seems not to be critical, because we used in the second step an equivalent of sodium borohydride to glyoxylic acid for getting products under optimal alkaline conditions for complete reduction without producing disubstitution products. Excess glyoxylic acid in the first step was used by us to get the highest possible completeness of the reaction with chitosan. We did not find insoluble reaction products in the second step reaction, which has been observed during acidic reduction with the modified one-step Muzzarelli method when a higher ratio of glyoxylic acid to amine than 1:1 was used.[32] Although a 1:1 stoichiometric ratio of glyoxylic acid to glucosamine has been used with the one-step Muzzarelli method, it is unavoidable that all reactions are parallel, and therefore, part of the intermediate formed—the monocarboxylated product—reacts a second time. The disubstitution products may additionally react at the methylene group by possible formation of carbenium ions, which then may react with another monosubstituted carboxymethylchitosan to give a crosslinked product. This reaction seems to be possible only in the one-step reaction, because Muzzarelli reported partially insoluble products. This crosslinking reaction seems not to be possible in the two-step alkaline reaction described. In conclusion, with the described (and used) selected two-step reaction with the separate aldimine reaction and the alkaline reduction step even under variable excesses of glyoxylic acid to amine, the disubstitution products as well as crosslinking can be excluded. Another second possible reaction of a secondary amine with aldehyde via a

hydrogenolysis pathway[41] will be not discussed because this reaction is only relevant for the one-step reaction and not the two-step reaction used by us. Other N-disubstituted reaction products such as –Ac or combined –Ac, $-CH_2COOH$ or –Ac and $-SO_3$ seem not to be likely and were not found (no indication by NMR) because of steric effects.

In order to get derivatives with special biological properties, it is important to know besides the defined, regioselectively arranged functional groups of a repeating unit, the individual frequency and distribution of the di- and triad as well the tetrad and pentad along the polymer chain. This is why we used a starting material with a defined GlcNAc diad repeating structure with highest abundance, and a triad and tetrad, as well as pentad, with lower abundance and with the gaps filled nearly completely by *N*-sulfo and/or *N*-carboxymethyl groups. The sequence of reactions was chosen in such way that possibly all reactions could be done in homogeneous solution and that labile functional groups as *N*-sulfo are introduced at the end of the sequence (**2**,**3**,**6**,**7**, Table 2).

6-O-Sulfonation according to the method of Focher et al.[19] was of highest possible regioselectivity, and the distribution is uniform along the polymer chain corresponding to DS 1. Nishimura obtained via his concept only a 6-*O*-sulfo content of 0.87.[29] On the other hand, it was the only case where we used an insoluble intermediate, the copper complex of chitosan, but this does not have any influence on the homogenous distribution of $6\text{-}OSO_3$ when a DS of 1 will be reached. Desulfonation reactions of pyridinium salts of 6-*O*-sulfochitosan derivatives in aprotic organic solvent–water mixtures[15] or according to the method of Takano[14] allows us to synthesize all desired partially 6-*O*-sulfo derivatives. The distribution of partially 6-desulfated groups along the polymer chain may also follow Bernoullian statistics. Regioselective 3-O-sulfonation may be reached using protecting groups used in cellulose[33] or in chitin chemistry.[29] However, the reactions are complex and should lead to complete removal of the protecting groups without splitting of functional sulfo groups. No complete regioselective 3-*O*-sulfocellulose is known;[27] also, no complete 3-*O*-sulfochitosan is known.[29] Therefore we decided to prepare a regioselective modified 3,6-di-*O*-sulfochitosan (**10**) with nearly complete 3-sulfation and a lower 6-sulfation. In this case, splitting of labile $6\text{-}OSO_3$ during removal of the *N*-phthalimido group does have not any consequences, because in our concept 6-O-desulfonation follows. This can be achieved only when chitosan is rendered DMF soluble for the sulfonation reaction using an intermediate *N*-phthalimido protecting group that can be completely deblocked by hydrazine hydrate. The result is a 97% 3-sulfated and 79% 6-sulfated chitosan. Solvolytic 6-desulfation from heparin chemistry[15] or better using Takano's reagent *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide[14] leads to DS 0.15 at the C-6-position and a DS 0.85 at the C-3-position (**8**). The regioselectivity of 3-O-sulfonation of chitosan may be improved by variation of the reaction conditions.

In summary, the concept described herein for sulfation and desulfation reactions leads to defined chitosan derivatives with high regioselectivity and a high degree of completeness, as well with tailor-made partially substituted repeating units which may be mostly distributed according to Bernoullian statistics along the polymer chain. Such defined derivatives are very important for studying their pharmacological properties. Complete reaction with highest regioselectivity in one position of chitosan with only one reagent leads to homopolymer structures. On the other hand, when two regioselective reagents with highest possible regioselectivity for two different positions, N and O, one with 100% completeness and the other with lower completeness is used, a defined copolymer structure will be found (**4**,**5**). It will be especially important to know if the different backbone structure and its regioselective arranged functional groups, as well as the repeating units of chitosan compared to those of heparin derivatives, give rise to different pharmacological properties. For our concept it was especially important to characterize at least the frequency of the repeating units and their abundance (GlcNAc diad, triad, tetrad, and pentad) of the starting

Table 2
Introduction of functional groups (–NAc, $–NSO_3^-$, $–N–CH_2–COOH$, $6\text{-}OSO_3^-$, and $3\text{-}OSO_3^-$) solely or in combination, with different amounts (DS) and different regioselectivity into the β-(1→4) glucosamine repeating units and regular distribution of functional groups along the polymer chain or at least statistical distribution according to Bernoulli [a]

No.	Sequence of reaction	DS of glucosamine repeating units					% Regio-selectivity at N	% or Relative regioselectivity at		Distribution along the polymer chain		Methods
		NAc	NSO_3^-	$N–CH_2–COOH$	$6\text{-}OSO_3^-$	$3\text{-}OSO_3^-$		6-O	3-O	Regular	Bernoulli at N	
1	a	0.14									NAc	
2	a, d	0.14	0.86				100				NAc, NSO_3	23
3	a, b, d	0.39	0.61				100				NAc, NSO_3	24, 23
4	a, e	0.14			1.0			100		$6\text{-}OSO_3^-$	NAc	19
5	a, e, c	0.14		0.86	1.0			100		$6\text{-}OSO_3^-$	NAc, $N–CH_2–COOH$	19, 26
6	a, e, d	0.14	0.86		0.71		100	high			NAc, NSO_3	19, 23
7	a, b, c, d	0.34	0.28	0.38			100				NAc, NSO_3, $N–CH_2–COOH$	24, 26, 23
8	a, f, g	0.14			0.15	0.85			high		NAc	29, 14
9	a, f, g	0.14			0.15	0.47			medium		NAc	29, 15
10	a, f	0.14			0.79	0.97					NAc	29

[a] The reaction sequence is as follows: (a) starting material; (b) additional acetylation; (c) carboxymethylation; (d) N-sulfonation; (e) 6-O-sulfonation; (f) 6-O + 3-O-disulfonation; (g) 6-O-desulfonation.

chitosan material, the HCl degradation product, before selected regioselective reactions are carried out. Our theoretical calculations and experimental results of gel chromatography are in very good agreement for estimating the frequency of statistical distributed one to five repeating units of GlcNAc structures according to Bernoulli statistics. Sashiwa et al. found also less than six randomly distributed *N*-acetylated repeating units with partial deacetylated chitin under homogeneous alkaline conditions.[31] They suggested that this procedure should be one of the methods for estimating the degree of deacetylation of deacetylatet chitin. For that we used a very well-known specific main-chain cleavage reaction (HNO_2), which has also been established to estimate the block structure of heparan sulfate.[34,35] Further knowledge about specific main-chain cleavage reactions and methods for elucidation of the primary structure[38] of heparan sulfate,[34,35] as well as their analytical estimation, is needed to transfer the knowledge to chitin derivatives for characterizing more precisely the sequence of the repeating units along the polymer chains of sulfated and carboxymethylated chitosan. Further sequence analysis with enzymes such as lysozyme and chitinases with different cleavage patterns may support our results or give more information about defined structure. Lysozyme releases GlcNAc sequences,[39] whereas chitinases recognize GlcNAc residues.[39] Related enzymes, which were found to be intermediate or less efficient catalysts, could also be tested.[37] Additional quantitative estimation of different substituted GlcNAc derivatives or sequences released from the polymer chain is needed which gives information about their composition and frequency. This is also possible with permethylated products. Also NMR methods may be used in addition for estimation of diad, triad etc. of repeating units of the polymer chain[36] to support our results.

All types of derivatives, their regioselectivity, and the distribution of the functional groups along the polymer chain are listed in Table 2.

The molecular weight of chitosan derivatives is nearly twice as high as commercially available high-molecular-weight heparin, but 1/3 lower than that of endothelial cell-surface heparan sulfate.[5] This makes it possible to compare properties and study structure–function relationships. By variation of NAc content, it is possible to simulate that of heparin and heparan sulfate.[5] That is true also with *N*-sulfo, 3-*O*-, and 6-*O*-sulfo as well as with *N*-carboxymethyl content. If the described regioselective reactions were done with a 150 kD chitosan (DA 0.28) as a starting material, O-sulfonation according to Hirano et al.[20] would not be possible. The O-sulfonation according to Wolfrom and Shen Han[21] was incomplete; also, the reaction according to Muzzarelli et al.[22] was not possible. Even the 6-O-sulfonation reactions according to Focher et al.[19] and to Naggi et al.[18] would not be possible. This was the same in the case of N-sulfonation according to the method of Holme and Perlin.[23] Only the N-carboxymethylation of Muzzarelli et al.[26] and the N-acetylation of Lee et al.[24] were successful with both chitosan derivatives (29 and 150 kD). On the other hand, except the O-sulfation reactions of Wolfrom et al.[21] and Muzzarelli et al.[22] that have not been tested, all other reactions described above were successful with the low-molecular-weight chitosan. Muzzarelli described his problems concerning the reproducibility of his own N-carboxymethylation reaction of chitosan from Antarctic krill and with chitosan from prawn, shrimp, and crab.[32] The N-carboxymethylation of chitosan from krill gave water-soluble derivatives, whereas the same reaction under the same conditions with the other above-mentioned chitosans gave mostly insoluble derivatives. Newer results of Muzzarelli and co-workers[37] offer another way to shorten the polymer chain of chitosan of different natural origins by enzymatic digestion with or papain in order to obtain water-soluble starting materials. But further work is needed to get the knowledge about the type of distribution of GlcNAc repeating units along the polymer chain, as well as the protocol for further reducing the molecular weight to use this as another starting product for our concept. Homogenous alkaline degradation of chitosan resulting in water-soluble derivatives with diads and triads in high frequencies of GlcNAc as

estimated by NMR methods could be another staring material for our concept when the needed low-molecular-weight product is reached.[36] The concept of Nishimura et al. using *N*-phathaloylchitosan as a key starting material and additionally a sterically hindered trityl group introduced through systematic protection strategy under homogenous conditions was not further developed by us because the result was only medium substitution degrees of sulfation 3S (0.44), 6S (0.87).[29] Therefore, we decided in all cases not to use systematic protection strategy. Only in one case we used *N*-phthaloylchitosan as an intermediate solubilizing group for getting a high degree of 3-sulfation. The disadvantage of trityl groups is the required strongly acidic deprotecting conditions which reduces labile sulfo groups. Furthermore, tailor-made intermediate substitution products are easier to synthesize without using systematic protection group strategy.

The relative-molecular weight of the synthesized chitosan derivatives were measured by gel chromatography and compared with pullulan standards. The molecular weight was expressed by the chain length and by the number of monosaccharide repeating units. However, we observed with increasing sulfation degree a drastic increase in hydrodynamic volume and thus an increase in relative-molecular weight of the derivatives, compared to that of pullulans. Therefore, a comparison with pullulans is only correct with low-sulfated derivatives. Because there are no other high-sulfated polysaccharides commercially available as molecular weight standards, we decline to present the results for high-sulfated chitosan derivatives.

For regioselective reactions with heparin such as 6-O-desulfonation according to Takano et al.[14] or Baumann et al.,[15] no significant chain degradation was observed. This might be also true in the case of applying these reactions to chitosan derivatives. Also other described regioselective reactions of chitosan seem to be not so drastic that the original chain length would be expected to decrease significantly. Although we used mostly diverse single, known literature reaction conditions for preparing regioselectively modified chitosan derivatives, many factors serve to influence the type of reaction products. These include the variation of the molecular weight, the sequence of the reactions, the combination of the reaction, the viscosity of the products, the stoichiometry of the reagents to the repeating units, the time, the temperature, the solubility and the type of functional groups, and the type of starting product, as well as distribution along the main chain and homogenous reaction conditions. Therefore, most of the reactions products were new indicated by intensity and characteristic of NMR peaks which may be shifted by the influence of the type of individual groups or neighboring group effects.

These studies will be continued with 6-deoxy-aminocellulose and regioselective introduction of analogous functional groups. At the end of the study, the goal is to show differences in biological properties between the different backbone structures of chitosan and cellulose, compared to those of heparin and heparan sulfate, with well defined regioselectively arranged functional groups compared to heparin and heparan sulfates.

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