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(54) Title: PREPARATION OF NANOFIBRES FROM POLYSACCHARIDES AND MIXTURES THEREOF WITH
POLYVINYL ALCOHOL

(57) Abstract: The invention relates to a method of preparation of nanomaterials-based nanofibres and scaffolds by means of an
electrospinning method, made from polysaccharides and the mixtures thereof with polyvinyl alcohol in an aqueous solution or a
water/ethanol solution in the presence of a surfactant.



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Preparation of Nanofibres from Polysaccharides and Mixtures thereof with Polyvinyl Alcohol

Field of the Invention

This invention relates to nanomaterials-based nanofibres and scaffolds made from polysaccharides and the physical mixtures thereof with polyvinyl alcohol.

State of the Art

Polysaccharides are biopolymers composed of monosaccharides. The scientists focus on several polysaccharides differing in the type of the monosaccharide forming the main chain, and therefore, differing in their chemical and physical properties as well.

β -(1,3) glucans consisting of solely glucose molecules represent a large group of polysaccharides. This type of glucans can be found in the cell walls of some yeasts, lower and higher fungi.

β -(1,3) glucans include also a neutral polysaccharide called schizophyllan (Fig. 1) produced by the fungus *Schizophyllum commune*. It is a polysaccharide the main chain of which is constituted by D-glucose molecules linked by the β -(1,3)-glycosidic bonds, the side chains being linked thereto by the β -(1,6)-glycosidic bond. The side chains consist of one to two glucose molecules. The molecular weight of the individual chains is about hundreds of thousands g/mol. The individual chains combine in a solution in such a way as to produce a highly stable triple-helix the molecular weight of which is of an order of 10^6 g/mol. Schizophyllan forms totally transparent highly viscous solutions.

Schizophyllan is a biologically active polysaccharide that is able to activate the immune system cells after binding to specific receptors.

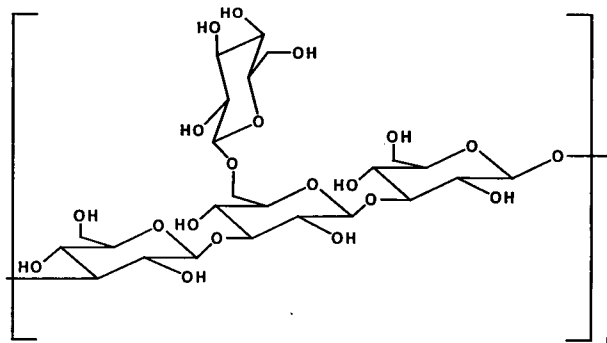


Fig. 1 Schizophyllan

Glucomannan is mostly composed of mannopyranose units forming a highly crosslinked structure. The basic scaffold is composed of mannopyranose units linked by the α -(1,6)-bond. The side chain is composed of mannopyranose units linked to the main scaffold by the α -(1,2)-bond. There are more or less randomly located glucopyranose units on this crosslinked chain. The mannose and the glucose are present in a ratio 8,4 : 1ⁱ (Fig. 2).

Glucomannan isolated from *Candida utilis* is a biologically active polysaccharide as well that is able to activate the immune system cells. It has also been found to increase the number of blood cells at the patients treated by radiotherapy and to be able to activate hematopoiesis and to protect against the potential lethal doses of radiation exposure.

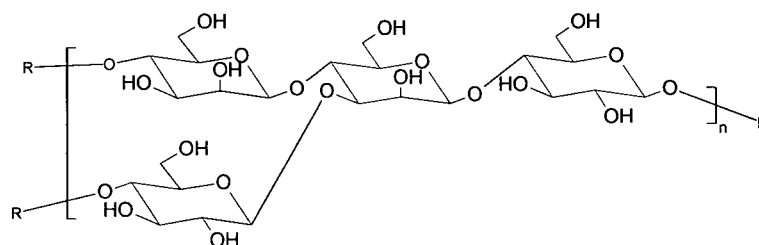


Fig. 2 Glucomannan

Another biologically important polysaccharide belonging to the group of glycosaminoglycans is the hyaluronic acid. The basic unit of hyaluronan is a dimer composed of two monosaccharides, which are D-glucuronic acid and N-acetyl-D-glucosamineⁱⁱ. The monosaccharides in the basic unit are linked by the β -(1,3)-glycosidic bond, the dimers are linked to each other by the β -(1,4)-glycosidic bond. The hyaluronic acid forms linear chains having a high molecular weight from several thousands to millions g/mol (Fig. 3).

Hyaluronic acid may be found in the synovial fluidⁱⁱⁱ of joints and in the extracellular matrix of higher animals, especially in the connective tissues^{iv}. As an active non-sulphate glycosaminoglycan, hyaluronan binds to the extracellular matrix proteins and to the cellular surface which plays an important role in the regulation of many cell processes, in the embryonic development^v, in the cancer development^{vi} and in inflammations^{vii} and the like.

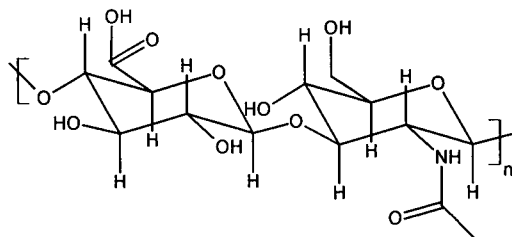


Fig. 3 Hyaluronic acid

The chemistry of nanofibres, fabrics, membranes and scaffolds made of biodegradable and biocompatible substances originated out of the necessity of tissue engineering. Scaffolds based on nanofibres and fabric made of fibres should ideally provide for a 3-D cultivation pad for cultivation of the respective cells, thanks to their mechanical characteristics enable an implantation of such scaffold into the repaired tissue, ensure its incorporation into this tissue and then gradually degrade in such a way that no trace thereof can be found there.

The production of nanofibres and nanofabrics from polysaccharides based on a combination of electrostatic force and electrospinning has several advantages^{viii}. One of them, a quite essential one, is the fact that the amount of substances required for the scaffold production is reducing compared to the conventional membrane production method.

In the preparation of nanofibres from biopolymers by the electrospinning method, several requirements must be taken into account. The main requirements on the polymer solution are: viscosity, surface voltage, polymer concentration, molecular weight of the polymer and molecular weight distribution. The first experiments mentioned in literature^{viii} describe the use of hyaluronic acid having the molecular weight of 3 MDa in a solution of 2 - 3% by weight, the injection was performed at the rate of 30 - 50 µl/min and the air blow rate of 30 - 150 SCFH (standard cubic feet per hour). The voltage of the electric field was 30 - 45 kV. The distance between the electrodes may be modified - the distance of 9.5 cm is often used. Solutions of the following compounds may be used as solvents: chloroform, THF, acetic acid and formic acid, ethanol, 2-propanol, dimethylformamide and dimethylacetamide etc. Other materials, such as poly(acrylonitril) 2 - 14 % by weight in DMF; poly(urethane) 1-15 % by weight, poly(glycolide-co-lactide) 10 - 40 % by weight in DMF, poly(caprolactam), poly(amide); collagen; poly propylene; poly ethylene; poly (vinyl phenol), may be fibrillated as well.

A nanofibre structure defined by a net of one or more fibres containing one or more biomolecules and a substrate is described in the U.S. patent No. 20050095695^{ix}. At least one of the molecules is a lipid, monosaccharide, polysaccharide, amino acid, nucleotide, nucleic acid and hybrid molecules of said compounds. The lipids include cholesterol, the saccharides include cellulose, chitosan and hyaluronan. Beside these compounds the nanofibres contain other possible alcohol, aldehyde, amine, carboxy or sulphhydryl groups.

The size of the nanofibres formed is comprised within the range of 50 to 1000 nanometres. The polymeric materials include esters, polyethylene and biodegradable and

biocompatible aliphatic polyesters, such as ϵ -caprolactam, poly-lactide-co-glycolide and copolymers thereof. The fibres developed may be used in biological applications which comprise cell cultures, tissue cultures and in tissue engineering applications. The electrospinning process uses the electric field for controlling the polymer generation and storage. The electric potential creates a charge instability which ejects the polymer out of the tip having a needle-like shape. A polymer having the concentration of 1 - 15 % by weight in THF was used for fibrillation. The voltage on the tip was 18 kV^{ix}.

Materials consisting of one to three polymers in the form of nanofibres and other biomolecules linked to the fibres by cross-link elements (various enzymes and the like) are protected by the US patent^x. Therefore, the nanofibres contain at least one polymer, preferably two. The polymers may be present in a ratio 1:20, 20:1, 10:1, 1:10, 1:5, 5:1, 1:4 and 4:1. The suitable polymers soluble in organic solutions include: polyacrylonitril, polyamide, polyester, polystyrene, polyvinyl chloride, cellulose derivatives. The water-soluble polymers include e.g. polyacrylic acid, polyvinyl alcohol, polyethylene oxide, polyaniline etc. The biomolecules may be linked to the fibres directly or via cross-link elements which may be done before, during or after the production of nanofibres. The biomolecules include, among others, proteins, polypeptides, enzymes, hormones, antigens, nucleic acids, polysaccharides.

In some cases the polymer fibres may be cross-linked so as to be reinforced. This further leads to modifications in their solubility in water and also in various organic solvents, in some cases they may become insoluble. The cross-linking agents include isocyanates and their derivatives.

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Summary of the Invention

The subject-matter of the invention consists in finding a method of preparation of nanofibres from polysaccharides, particularly from hyaluronan, schizophyllan and glucomannan and mixtures thereof with polyvinyl alcohol. The fibres prepared from polysaccharides and mixtures thereof with polyvinyl alcohol are water-soluble. In case of fibres prepared from a pure polysaccharide, the fibres are totally decomposable in the organism, in case of fibres prepared from mixtures of polysaccharides and polyvinyl alcohol, the polyvinyl alcoholic part is inert with respect to the enzyme decomposition. The materials prepared this way are suitable for wounds covering. The advantage of adding polyvinyl alcohol is a significant reduction of pure polysaccharide consumption.

The method of fibre preparation mentioned in this patent application permits the use of hyaluronan and other polysaccharides in a wide range of molecular weights and at the same time it permits a great variability of polysaccharide concentration. The fibrillation process takes place at a room temperature and a standard pressure from an aqueous medium.

The fibrillation takes place in an electrostatic field where a bunch of fibres collected on a collector covered with a support fabric is picked up from the solution by an electrostatic force. This configuration facilitates the collection of fibres and at the same time of fabrics and after the sterilisation, it may serve together with a nanofibre layer directly as a covering material. Thanks to the fact that the whole process is performed from water being the solvent, it does not induce increased demands on the device, preparation of solutions or labour safety.

The nanofibres made of pure polysaccharides (hyaluronan, schizophyllan, glucomannan and others) having various molecular weights are prepared from the water solutions thereof, having a concentration within the range of 1 - 10 % by weight. Various surfactants are added to the solution, such as dermally acceptable surfactants, e.g. octyl phenol ethoxylate (TRITON X 100), sodium dodecyl sulphate (SDS), sodium bis-2-ethylhexylsulphosuccinate (SPOLION 8), and pharmaceutically acceptable surfactants, e.g. polyoxyethylene sorbitan monolaurate (TWEEN 20), polyoxyethylene sorbitan monooleate (TWEEN 80), glyceryl monostearate (IMWITOR 191) and others, for reducing the surface voltage of the polysaccharide solutions. The ratio of the polysaccharide to the surfactant varies within the range of 10/1 to 10/0.02 polysaccharide/surfactant. The solutions are homogenized long enough, in various ways. The voltage used in the process is 10 - 60 kV.

For the fibrillation of the mixture of polyvinyl alcohol and polysaccharides (hyaluronan, schizophyllan, glucomannan and others), aqueous solutions of polyvinyl alcohol having a molecular weight about 100 000 g/mol within the concentration range of 7 - 12 % by weight are prepared. The solutions of polysaccharides are prepared, having various molecular weights within the concentration range of 1 - 10 % by weight. The solution of the polysaccharide is mixed with the solution of polyvinyl alcohol in a ratio polysaccharide/polyvinyl alcohol 5/1 to 1/5. The solution mixture is homogenized in a suitable way. The voltage used at the fibrillation is 10 - 60 kV.

10 Preferred Embodiments

Example 1

Hyaluronic acid and TWEEN 20

Aqueous solutions of hyaluronic acid (HA) having the molecular weight of 1 690 000 g/mol and 310 000 g/mol and the concentration of 0.5; 1.0; 1.5 and 2 % by weight were prepared. The solutions were homogenized for 30 minutes. Solutions of the surfactant TWEEN 20, having the concentration of 1 % by weight and 5 % by weight were prepared. Fig. 4 represents a scanning electron microscope photo where thick fibres of the support fabric with a net of hyaluronic acid deposited thereon, accentuated by beads of hyaluronic acid, can be observed.

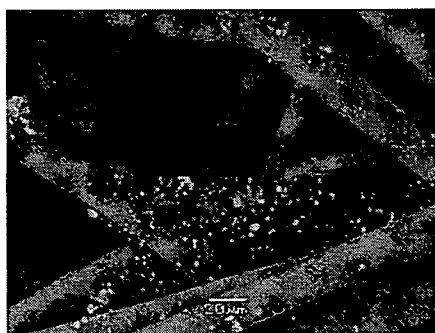


Fig. 4 SEM – 20 ml; 1 % by weight of hyaluronic acid (1.69 MDa) and 1 ml of 5 % by weight TWEEN 20

Example 2

Hyaluronic acid and SPOLION 8

An aqueous solution of hyaluronic acid (HA) having the molecular weight of 1 690 000 g/mol and the concentration of 1.0 % by weight was prepared. The solution was homogenized for 60 minutes. As a surfactant, a concentrated SPOLION 8 was used. Fig. 5 represents a scanning electron microscope photo where beside the thick fibres of the support fabric there is a net of hyaluronic acid fibres, accentuated by beads of hyaluronic acid.

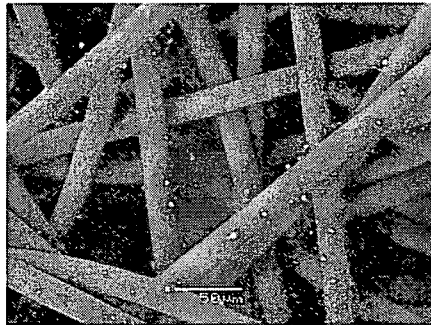


Fig. 5 SEM – 5 ml; 1 % by weight of hyaluronic acid (1,69 MDa) and 10 μ l of SPOLION 8

Example 3

Hyaluronic acid and TWEEN 80

A solution of hyaluronic acid (HA) having the molecular weight of 1 690 000 g/mol and the concentration of 1.0 % by weight in a mixture of ethanol/water 1/9 was prepared. The solution was homogenized for 60 minutes. As a surfactant, a concentrated TWEEN 80 was used. Fig. 6 represents a scanning electron microscope photo, again with a net of hyaluronic acid nanofibres covering the thick fibres of the support fabric.

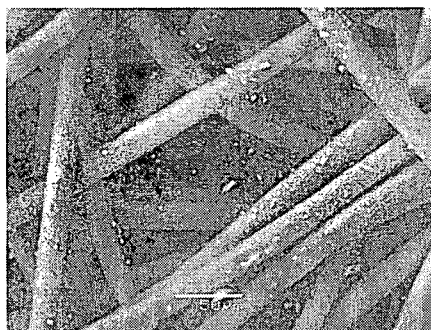


Fig. 6 SEM –5 ml; 1 % by weight of hyaluronic acid (1,69 MDa) and 10 μ l of TWEEN 80

Example 4

Hyaluronic acid and polyvinyl alcohol

An aqueous solution containing 3 % by weight of hyaluronic acid having the molecular weight of 310 kDa. The solution was homogenized for 1 hour. A 9 % solution of polyvinyl alcohol was prepared. The solutions were mixed in a ratio of PVA/HA 5/1, 4/2 and 3/3, were homogenized for 30 minutes and then were fibrillated. Fig. 7 represents a scanning electron microscope photo where a thick net of nanofibres of the mixture of PVA and HA covers the support fibres. The surface voltage and conductivity data and the course of the fibrillation are indicated in Table 1.

Table 1 Mixtures of PVA/HA and the experiment results

	PVA parts	HA parts	Voltage kV	The distance of the collector mm	Process Y/N	Hairs Y/N	Ray division mm
1	5	1	41,8	110	A	N	20
2	4	2	45,6	110	A	N	15
3	3	3	50,4	110	A	N	15

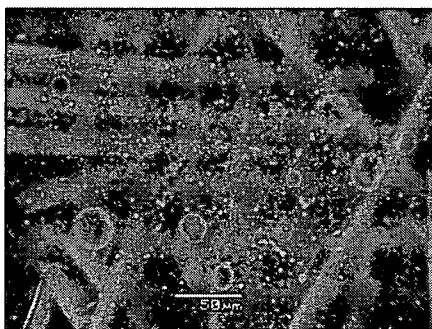


Fig. 7 SEM – 3 % by weight of hyaluronic acid (310 kDa) and 9 % by weight of polyvinyl alcohol 1/5

Example 5

Schizophyllan and polyvinyl alcohol

220 mg of schizophyllan (SPG) having the molecular weight of 100 kDa was dissolved in 20 ml of demineralized water at the room temperature which produced a

1 % solution. The solution was homogenized for 1 hour. A 9 % solution of polyvinyl alcohol (PVA) was prepared.

The solutions were mixed in a ratio of PVA/SPG 5/1, 4/2 and 3/3, were homogenized for 15 minutes and then were fibrillated. Fig. 8 represents a scanning electron microscope photo where beside the thick fibres of the support fabric there are also nanofibres of schizophyllan and PVA. The surface voltage and conductivity data and the course of the fibrillation are indicated in Table 2.

Table 2 Mixtures of PVA/SPG and the experiment results

	PVA parts	SPG parts	Voltage kV	The distance of the collector mm	Process Y/N	Hairs Y/N	Ray division mm
1	5	1	27,4	110	A	N	20
2	4	2	27,4	110	A	N	10
3	3	3	24,2	110	A	N	10



Fig. 8 SEM – 1 % by weight of schizophyllan and 9 % by weight of polyvinyl alcohol 2/4

Example 6

Glucomannan and polyvinyl alcohol

220 mg of glucomannan (GM) having the molecular weight of 100 kDa was dissolved in 20 ml of demineralized water at the room temperature which produced a 1 % solution. The solution was homogenized for 1 hour. A 9 % solution of polyvinyl alcohol (PVA) was prepared.

The solutions were mixed in a ratio of PVA/GM 5/1, 4/2 and 3/3, were homogenized for 15 minutes and then were fibrillated. Fig. 9 represents a scanning electron microscope photo where thick fibres of the support fabric with a net of glukomannan and PVA deposited thereon can be observed. The surface voltage and conductivity data and the course of the fibrillation are indicated in Table 3.

Table 3 Mixtures of PVA/GM and the experiment results

	PVA parts	GM parts	Voltage kV	The distance of the collectors mm	Process Y/N	Hairs Y/N	Ray division mm
1	5	1	33,1	110	A	N	10
2	4	2	26,5	110	A	N	10
3	3	3	30,5	110	A	N	10

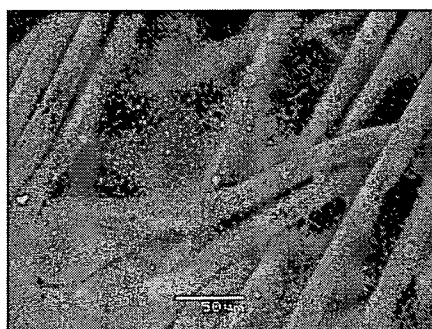


Fig. 9 SEM – 1 % by weight of glukomannan and 9 % by weight of polyvinyl alcohol 2/4

Example 7

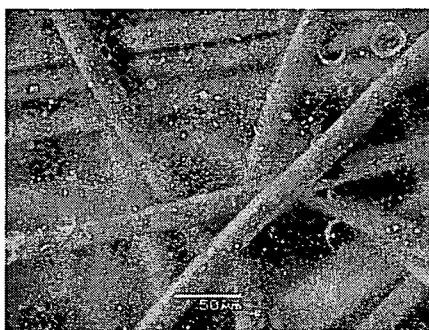
Schizophyllan 3 % and polyvinyl alcohol

A corresponding amount of schizophyllan was dissolved in 20 ml of demineralized water at the room temperature which produced a 3 % solution. The solution was homogenized for 1 hour. A 9 % solution of polyvinyl alcohol (PVA) was prepared.

The solutions were mixed in a ratio of PVA/SPG 5/1, 4/2 and 3/3, were homogenized for 15 minutes and then were fibrillated. Fig. 10 represents a scanning electron microscope photo where a thick net of nanofibres of the mixture of PVA and SPG covers the support fibres. The surface voltage and conductivity data and the course of the fibrillation are indicated in Table 4.

Table 4 Mixtures of PVA/SPG and the experiment results

	PVA parts	SPG parts	Voltage kV	The distance of the collectors mm	Process Y/N	Hairs Y/N	Ray division mm
1	5	1	28,6	110	A	N	15
2	4	2	39,2	110	A	A	10
3	3	3	28,9	110	A	N	10

**Fig. 10** SEM – 3 % by weight of schizophyllan and 9 % by weight of polyvinyl alcohol 3/3

Example 8

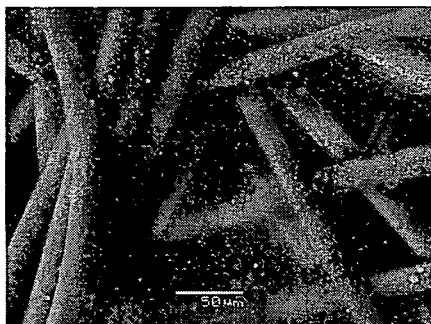
10 Glucomannan 3 % and polyvinyl alcohol

A corresponding amount of glucomannan was dissolved in 20 ml of demineralized water at the room temperature. The solution was homogenized for 1 hour. A 9 % solution of polyvinyl alcohol (PVA) was prepared.

15 The solutions were mixed in a ratio of PVA/GM 5/1, 4/2 and 3/3, were homogenized for 15 minutes and then were fibrillated. Fig. 11 represents a scanning electron microscope photo, again with a net of nanofibres of glucomannan and PVA covering the thick fibres of the support fabric. The surface voltage and conductivity data and the course of the fibrillation are indicated in Table 5.

Table 5 Mixtures of PVA/GM - experiment results

	PVA parts	GM parts	Voltage kV	The distance of the collectors mm	Process Y/N	Hairs Y/N	Ray division mm
1	5	1	23,7	110	A	N	10
2	4	2	26,0	110	A	N	10
3	3	3	28,6	110	A	N	5

**Fig. 11** SEM – 3 % by weight of glucomannan and 9 % by weight of polyvinyl alcohol 1/5

CLAIMS

1. A method of preparation of nanofibres from polysaccharides by means of an electrospinning method, characterized by that polysaccharides or a mixture thereof with polyvinyl alcohol in an aqueous solution or a water/ethanol solution, and a surfactant are used for the preparation.
2. The method of preparation of nanofibres according to claim 1, wherein the polysaccharide is hyaluronic acid or a pharmacologically acceptable salt thereof having the molecular weight within the range of 10 000 - 3 500 000 g/mol and/or schizophyllan having the molecular weight within the range of 10 000 - 2 000 000 g/mol and/or glucomannan having the molecular weight within the range of 10 000 - 500 000 g/mol.
3. The method of preparation of nanofibres according to claim 1, wherein the polyvinyl alcohol has the molecular weight within the range of 60 000 - 120 000 g/mol.
4. The method of preparation of nanofibres according to claim 1, wherein the surfactants are pharmacologically and dermally acceptable cation, anion and non-ionic surfactants.
5. The method of preparation of nanofibres according to claim 1, wherein the polysaccharides are present in the solution within the concentration range of 1 - 10 % by weight, preferably 1 - 5 % by weight.
6. The method of preparation of nanofibres according to claim 1, wherein the polyvinyl alcohol is present in the solution within the concentration range of 7 - 12 % by weight, preferably 8 - 10 % by weight.
7. The method of preparation of nanofibres according to claim 1, wherein the surfactants are present in the solution within the concentration range of 0.05 - 5 % by weight.
8. The method of preparation of nanofibres according to claim 1, wherein the volume ratio of the polyvinyl alcohol to polysaccharide is 5/1 to 1/5, preferably 4/2 to 2/4.
9. The method of preparation of nanofibres according to claim 1, wherein the surface voltage during the electrospinning process is within the range of 10 - 60 kV, preferably 20 - 50 kV.
10. A use of nanofibres made of polysaccharides prepared by means of the method according to claim 1 for therapeutic and pharmaceutical purposes.