

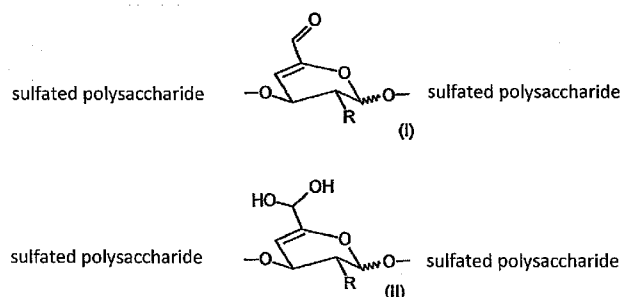


- (51) **International Patent Classification:**
C08B 37/00 (2006.01) **C08G 81/00** (2006.01)
A61K 31/726 (2006.01) **C08J 3/24** (2006.01)
- (21) **International Application Number:**
PCT/CZ2016/000071
- (22) **International Filing Date:**
24 June 2016 (24.06.2016)
- (25) **Filing Language:** Czech
- (26) **Publication Language:** English
- (30) **Priority Data:**
PV 2015-445 26 June 2015 (26.06.2015) CZ
- (71) **Applicant:** CONTIPRO A.S. [CZ/CZ]; Dolní Dobrouč 401, 561 02 Dolní Dobrouč (CZ).
- (72) **Inventors:** BOBULA, Tomas; Hajova 4, 059 21 Svit (SK). BUFFA, Radovan; Kukorelliho 1495/2, 066 01 Humenne (SK). VAGNEROVA, Hana; Dolni Cermna 102, 561 53 Dolni Cermna (CZ). SULAKOVA, Romana; T.G. Masaryka 174, 562 01 Usti nad Orlici (CZ). WOLFOVA, Lucie; Kollarova 11, 746 01 Opava (CZ). KOHUTOVA, Lenka; Hlavni 1378, 739 11 Frydlant nad Ostravici (CZ). MORAVCOVA, Veronika; Podmesti 123, 561 51 Letohrad (CZ). ZIDEK, Ondrej; 17. listopadu 843, 570 01 Litomysl (CZ). PROCHAZKOVA, Pavlina; Vezky 34, 751 19 Vlkos (CZ). VELEBNY, Vladimir; Sadova 1466, 564 01 Zamberk (CZ).
- (74) **Agent:** LUNZAROVA, Lucie; Kania, Sedlak, Smola, Mendlovo Namesti 1a, 603 00 Brno (CZ).
- (81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) **Designated States** (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) **Title:** DERIVATIVES OF SULFATED POLYSACCHARIDES, METHOD OF PREPARATION, MODIFICATION AND USE THEREOF



(57) **Abstract:** The invention relates to the preparation and the use of α,β -unsaturated aldehydes in the structure of sulfated polysaccharides. It concerns the derivatives with a conjugated double bond in the 4th and 5th positions of the galactopyranose part situated in the 6th position with respect to the aldehyde, according to the general structural formula (I) or its hydrated form according to the general structural formula (II). The preparation of these derivatives derives from sulfated polysaccharides containing a galactopyranose ring sulfated in the 4th position that is bound in the polymer chain via $\alpha(1\rightarrow3)$ or $\beta(1\rightarrow3)$ O-glycosidic bond. In the described solution, the sulfated polysaccharides undergo a regio- and chemoselective oxidation to form C6-saturated aldehyde, which, via a direct elimination of the sulfate group, provides the α,β -unsaturated derivative according to the general formula (I) or (II). The described solution is technically advantageous, because it leads directly to α,β -unsaturated aldehydes, without any elimination agents, higher temperature, or isolation of intermediates during the synthesis. The conjugation in the structure of α,β -unsaturated aldehyde allows, under physiological conditions, to bind a wide variety of biocompatible amines in the structure of the sulfated polysaccharides. The proposed method allows to prepare materials suitable for pH-responsive drug delivery systems, or for the preparation of scaffolds in tissue engineering or regenerative medicine. Formulae for the abstract (I), (II) above, where R is OH, O-SO₂-ONa, or NH-Ac.

Derivatives of sulfated polysaccharides, method of preparation, modification and use thereof

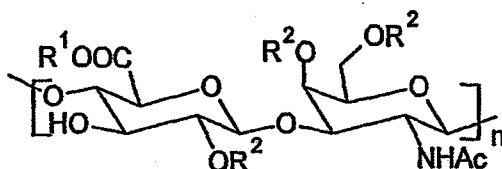
Field of the Invention

The invention relates to derivatives of sulfated polysaccharides having a conjugated double bond in the 4th and 5th position of the galactopyranose ring situated in the 6th position with respect to the aldehyde. Further, the invention relates to the method of preparation, modification and use thereof.

Background of the Invention

Glycosaminoglycans are linear polysaccharides consisting of aminohexose and uronic acid, except of keratin sulfate. They form a large part of intracellular matrix of connective tissue, in particular cartilage, ligaments and tendons. Sulfated polysaccharides, e.g. chondroitin sulfate or dermatan sulfate, are also, besides hyaluronic acid, important examples of glycosaminoglycans.

Chondroitin sulfate is a linear, sulfated, and negatively charged glycosaminoglycan composed of recurrent monomer units of *N*-acetyl-D-galactosamine and D-glucuronic acid attached to each other via $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ *O*-glycosidic bonds (the structural formula of chondroitin sulfate see below).



where

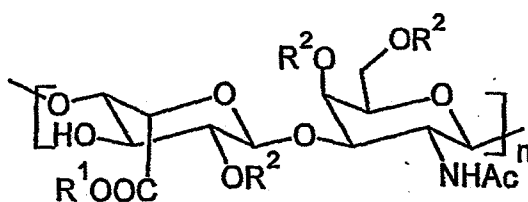
R^1 is H or Na,

R^2 is H, $-\text{SO}_2\text{-OH}$, or $-\text{SO}_2\text{-ONa}$

Chondroitin sulfate derives from animal connective tissues, where it binds to proteins and thus forms a part of proteoglycans. The sulfation of chondroitin is realized by means of sulfotransferases in various positions and of various kinds. The unique sulfation pattern of particular positions in the polymer chain encodes the specific biological activity of chondroitin sulfate. Chondroitin sulfate is an important building component of cartilage in

joints, conferring them the compression resistance and restoring the balance of the joint lubricant composition (Baeurle S. A., Kiselev M. G., Makarova E. S., Nogovitsin E. A. 2009. *Polymer* 50: 1805). Together with glucosamine, chondroitin sulfate is used as nutritional supplement for treating or prevention of osteoarthritis in humans (e.g. Flextor®, Advance Nutraceuticals, Ltd.) or animals (e.g. Geloren^{dog}®, Contipro Pharma, Ltd.). From the pharmaceutical point of view, chondroitin sulfate is considered to be a drug with delayed response for pain control at degenerative diseases of joints (Aubry-Rozier B. 2012. *Revue Médicale Suisse* 14: 571).

Dermatan sulfate is linear, sulfated, and negatively charged glycosaminoglycan composed of repeating monomer units of *N*-acetyl-D-galactosamine and L-iduronic acid attached to each other via $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ *O*-glycosidic bonds (the structural formula of dermatan sulfate see below).



where

R^1 is H or Na,

R^2 is H, $-\text{SO}_2\text{-OH}$, or $-\text{SO}_2\text{-ONa}$

Dermatan sulfate differs from chondroitin sulfate by the presence of L-iduronic acid, which is a C5 epimer of D-glucuronic acid. The inverse configuration of iduronic acid allows a better flexibility of dermatan sulfate chains and ensures their specific interaction of glycosamine - glycoprotein in the surrounding area. These interactions contribute to the regulation of several cell processes, such as migration, proliferation, differentiation, or angiogenesis. The transformation of chondroitin sulfate into dermatan sulfate is provided by means of three enzymes: dermatan sulfate epimerase 1 (DS-epi1), dermatan sulfate epimerase 2 (DS-epi2), and dermatan 4-*O*-sulfotransferase (D4ST1). The epimerisation reaction of glucuronic acid into iduronic acid, together with the way of the sulfation, is not random but specifically

enzymatically controlled, which results in encoding the information concerning the function of the construed glycosaminoglycan (Thelin M., et al. 2013. *FEBS Journal* 280: 2431).

Carrageenans are a group of linearly sulfated polysaccharides obtained by the extraction of red marine algae. Galactose and its 3,6-anhydroderivative, that are associated to each other via $\alpha(1\rightarrow3)$ or $\beta(1\rightarrow4)$ *O*-glycosidic bonds, are their basic building units. There are three main types of carrageenan, which differ in their degree of sulfation and water solubility. Kappa-carrageenan has one sulfate per dimer and forms rigid gels in water. Iota-carrageenan comprises two sulfates and forms soft gels, whereas lambda-carrageenan with three sulfates does not exhibit gel forming properties. Carrageenan is an alternative of animal gelatine for vegetarians and vegans. It is used for thickening and stabilization of food products and as an emulsifier in pharmaceutical and textile industry.

Oxidation of glycosaminoglycans

Thanks to their functional diversity, the polysaccharides can be oxidized in various positions (Cumpstey I., 2013. *ISRN Organic Chemistry*, 1). In the case of glycosaminoglycans there are three ways of oxidation. In the first one, the primary hydroxyl is oxidized to form a carboxylic acid. The combination of TEMPO/NaClO is used for the oxidation the most often (Jiang B., et al. 2000. *Carbohydrate Research* 327: 455; Huang L. et al. 2006. *Chemistry*, 12: 5264). Due to the steric bulkiness of TEMPO, this method is regioselective for primary hydroxyls only.

On the contrary, the second way leads to the oxidation of secondary hydroxyls to form diketone compounds. In this case, as the oxidation agents the oxides of transition metals based on Cr(VI) (Hassan R., et al. 2013. *Carbohydrate Polymers*, 92: 2321) or Mn (VII) (Gobouri A. A., et al. 2013. *International Journal of Sciences*, 2:1; Zaafarany I. A., et al. 2013. *Journal of Materials Science Research*, 2: 23) are used.

The third type of oxidation is based on periodate (IO_4^-) oxidation which also attacks secondary hydroxyl groups but simultaneously the pyranose ring breaks (Dawlee S. et al. 2005. *Biomacromolecules*, 6: 2040; Liang Y., et al. 2011. *Colloids and Surfaces B: Biointerfaces*, 82: 1; Xu Y., et al. 2012. *Carbohydrate Polymers*, 87: 1589). During the oxidation, dialdehyde forms first and then it is further oxidized to dicarboxylic acid.

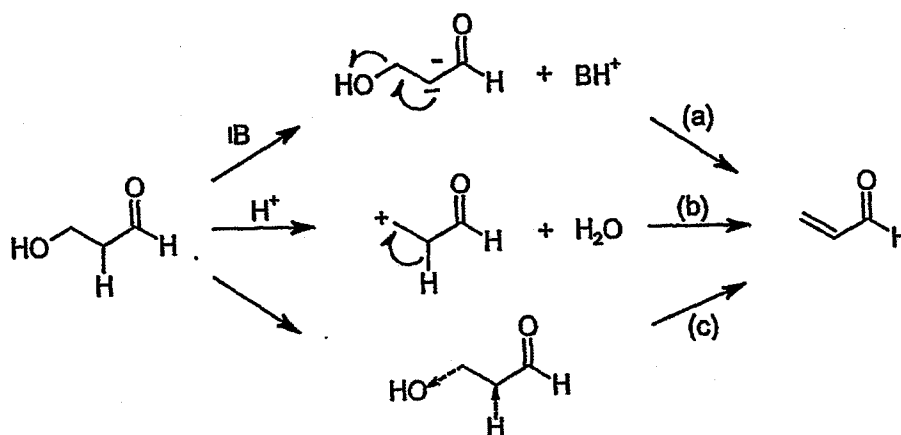
All afore mentioned ways of the oxidation have several drawbacks. In case of the oxidation with the use of TEMPO/NaClO, the formation of polyuronic acid is favoured instead of the desired C6-aldehyde. The reaction conditions for the aldehyde level need to be optimized, as it was demonstrated in the case of hyaluronic acid (Buffa R., et al., WO2011069475, Šedová P., et al., 2013. *Carbohydrate Research*, 371: 8). In addition, a higher content of carboxylic groups in the polymer significantly influences the conformation, interaction, and recognizing the polysaccharide with the biological surrounding (Zou X. H., et al. 2009. *Acta Biomaterialia*, 5: 1588).

Even though a chemoselective course of the reaction can be achieved periodate oxidation, this way is not preferred due to the dramatic decrease of the molecular weight of the polymer and irreversible cleavage of the pyranose ring, which results in the loss of the native character of the polysaccharide.

As regards the use of the oxidation agents derived from the transition metal oxides, the oxidized polysaccharides cannot be used for biomedical applications because of their high toxicity (Normandin L., et al. 2002. *Metabolic Brain Disease*, 17: 375; Katz S. A., et al. 2006. *Journal of Applied Toxicology*, 13: 217).

Dehydration reactions of oxidized derivatives of polysaccharides

The presence of an aldehyde in the polysaccharide structure results in an acid character of the hydrogen atom in the adjacent α -position. This hydrogen becomes easily accessible under basic conditions for elimination reactions to form a carbanion, which is stabilized by the conjugation with the adjacent aldehyde and thus displaces the leaving group in the β -position (way a, Scheme 1). The elimination can proceed also under acid conditions, where the activation of the leaving group occurs first to form a carbanion in the β -position (way b, Scheme 1). In the reaction mixture, the carbanion is neutralized with a free electron pair in the α -position. The third possible way can be performed without the addition of a base or an acid, using a simultaneous elimination of a molecule (way c, Scheme 1).



Scheme 1. Elimination reaction in the aldehyde structure: (a) formation of a carbanion by the treatment with a base, mechanism E1cb (b) formation of a carbanion by the treatment with an acid, mechanism E1 (c) simultaneous elimination, mechanism E2.

- 5 A targeted dehydration of aldehyde of hyaluronan in the 6th position in the glucosamine ring was described in the patent (Buffa et al.: CZ304512). The authors describe the preparation of α,β -unsaturated aldehyde of hyaluronan and its use in cross-linking reactions. The disclosed synthesis involves the use of sterically voluminous organic bases (e.g. diisopropylamine, trimethylamine), inorganic bases, e.g. $\text{Ca}(\text{OH})_2$ in the mixture of water-organic solvent of the
- 10 type of DMSO, sulfolane in the ratio of 3/1 to 1/2 under higher temperatures of 50 – 60 °C. The dehydration is also performed in solid state by heating the polymer to 50 – 100 °C for 4 – 5 days. The authors describe the oxidation and dehydration of hyaluronic acid in two steps and they do not describe the direct dehydration during the oxidation step. This solution has an important drawback of two steps synthesis and the use of inappropriate reaction conditions in
- 15 the presence of caustic (corrosive) elimination agents, presence of an organic solvent, necessity of an elevated temperature, and a long reaction time. All these parameters cause the synthesis to be more expensive and more complicated from the technological point of view (e.g. the corrosion of production apparatus, difficult purification of the product, higher price of dipolar aprotic solvents such as DMSO, sulfolane, and elimination agents such as Et_3N and
- 20 DIPEA, a high consumption of energy and cooling water, a higher risk of dangerous residues in the product, the product biocompatibility at risk, a higher rate of polymer degradation due to the basic environment and higher temperature). The said drawbacks of the synthesis of α,β -unsaturated aldehyde of HA in CZ304512 are, according to this invention, successfully overcome, as the synthesis proceeds in one pot without the necessity to isolate the

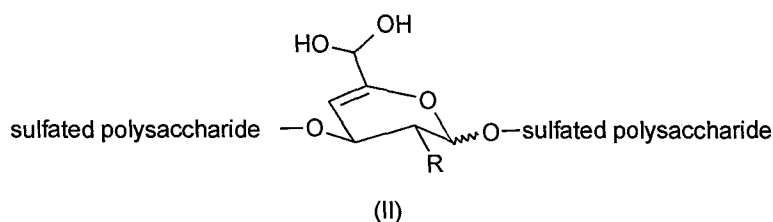
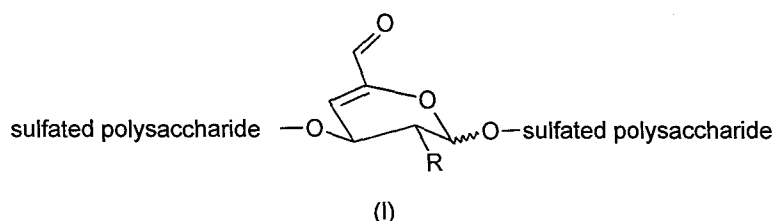
intermediate product in the form of a saturated C6-aldehyde, without adding the elimination agent, without adding the organic solvent, under room temperature and with the reaction times in the order of hours.

5 *Cross-linking reaction of oxidized polysaccharides*

The introduction of an aldehyde into the polysaccharide structure allows an additional modification of the polymer chain with the aid of nucleophilic addition. Several patent documents describing the binding of amines to aldehydes are known. A typical exemplary reaction for glycosaminoglycans is the reaction of dialdehyde formed by the oxidation with
10 periodate with various low molecular (amines, hydrazides, alkoxyamines, semicarbazides) or polymeric N-nucleophiles (gelatine, chitosan), or S-nucleophiles (thiols, aminothiols) to prepare biocompatible hydrogels (Dawlee S., et al. 2005. *Biomacromolecules*, 6: 2040; Weng L., et al. 2008. *Journal of Biomedical Materials Research part A*, 85: 352, Bergman K., et al.: WO2009/108100, Hilborn J., et al.: WO2010/138074). The cross-linking of aldehyde of
15 hyaluronic acid prepared with the use of Dess-Martin periodinane or with the use of the combination of TEMPO/NaClO with various amines was described in patent documents (Buffa R., et al.: WO2011069474; Buffa R., et al.: WO2011069475). α,β -Unsaturated aldehyde of hyaluronic acid was prepared by the dehydration of C6-aldehyde in *N*-acetyl-D-glucosamine subunit (Buffa R., et al: CZ304512). In addition to oxidized derivatives of
20 hyaluronic acid, the authors describe also its use in reactions with aliphatic, aromatic amines having an optional content of N, S, or O atoms. However, they are prepared under high temperatures and with the use of corrosive elimination agents, which is considerably unfavourable for maintaining their biological activity due to their possible denaturation and the presence of byproducts. Further, the cross-linking reactions of α,β -unsaturated aldehyde
25 of hyaluronic acid with deacetylated polysaccharides as a multifunctional amino linker are mentioned to illustrate the advantages of the conjugation of the aldehyde from the polysaccharide influencing the rheological properties of the prepared hydrogels. However, the hydrogels prepared in this way do not show satisfactory mechanical properties, especially as far as the hydrogel rigidity is concerned.

Summary of the Invention

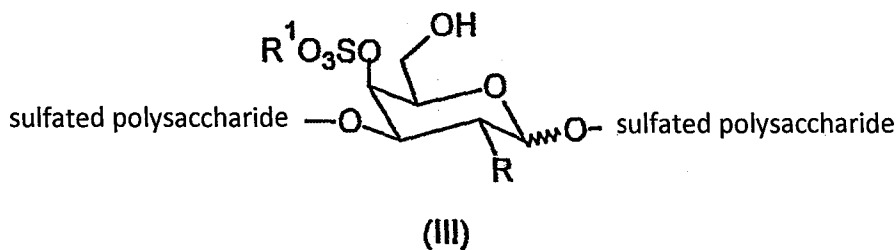
The aim of the invention is the preparation of sulfated polysaccharides under mild reaction conditions, in a shorter time, and without the use of undesirable impurities of elimination agents or organic solvents. This method prevents the significant degradation and loss of biological properties of sulfated polysaccharides, which are important for tissue engineering, regenerative medicine, or biomedical applications. The object of the invention consists in the derivatives of sulfated polysaccharides having, as a part of their polymer chain, at least one galactopyranose ring modified according to the general formula I or II, said ring containing a double bond in the 4th and 5th position with a conjugated aldehyde or its hydrated form respectively (general formula II)



where

R is OH, O-SO₂-OH, O-SO₂-ONa, or NH-C(O)-CH₃.

A necessary condition is the use of sulfated polysaccharides containing at least one galactopyranose ring in their chain, this ring being sulfated in the 4th position, and simultaneously bound in the chain via α (1→3) or β (1→3) O-glycosidic bond according to the general structural formula III.



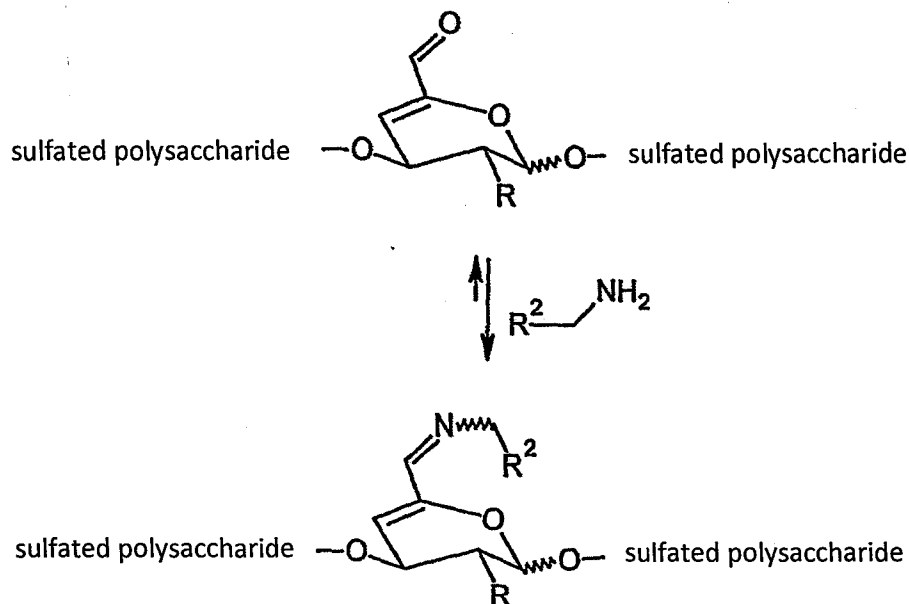
where

R is OH, O-SO₂-OH, O-SO₂-ONa, or NH-C(O)-CH₃

R¹ is H, or Na.

- 5 The polysaccharide is preferably selected from the group comprising chondroitin sulfate, dermatan sulfate, carrageenan, and their pharmaceutically acceptable derivatives and/or salts, and its molecular weight is preferably in the range of 1×10^3 to 5×10^4 g.mol⁻¹, and the degree of the substitution in the range of 1 to 40%, preferably 10 to 25%. In the formula I or II respectively, the term “degree of substitution” refers to the degree of the modification to an
- 10 unsaturated aldehyde, or its hydrated form respectively.

This solution enables to stabilize the conjugates of sulfated polysaccharides with amines by means of the multiple bond from the aldehyde, so a substantially wider range of amines can be bound more stably on the polysaccharides modified in this way (Scheme 2), under physiological conditions.



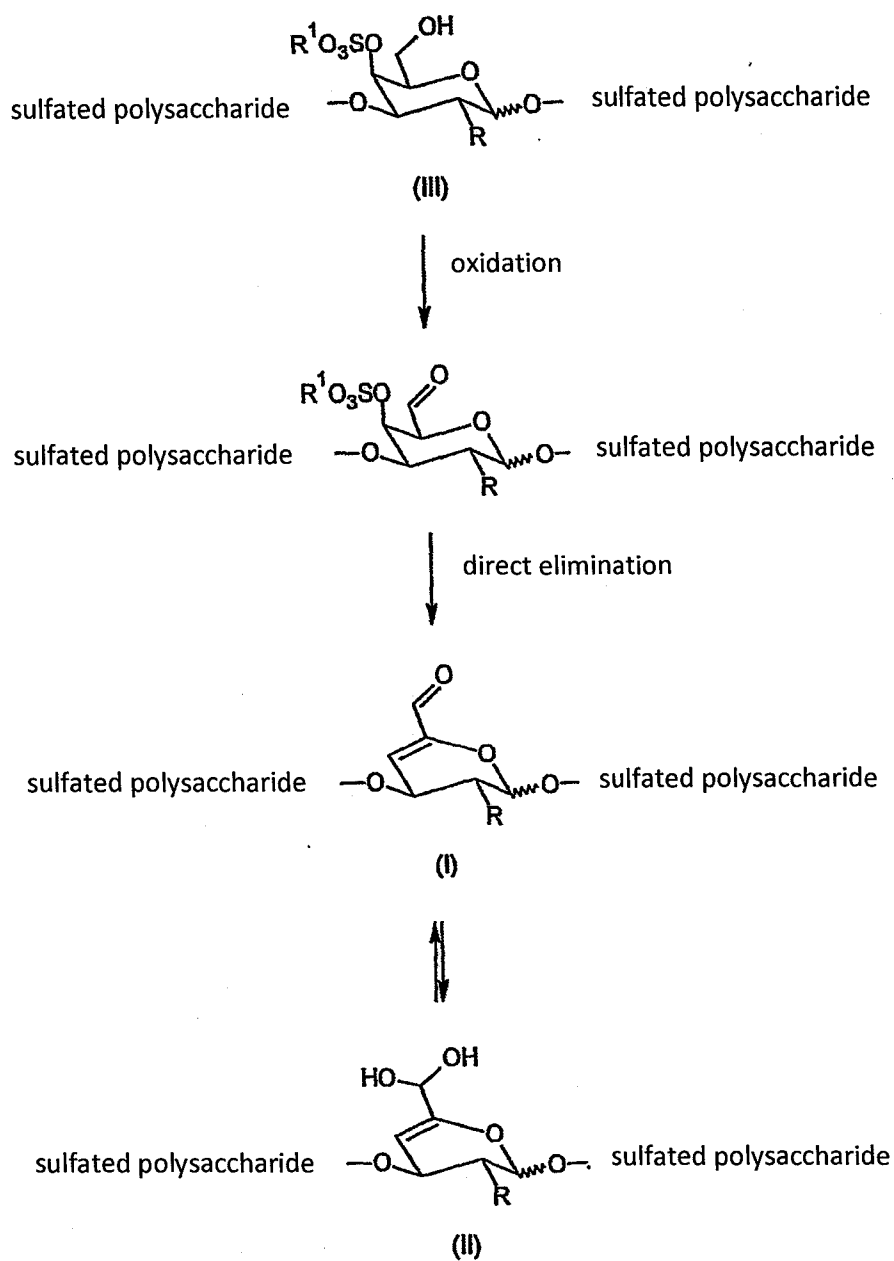
where

R is OH, O-SO₂-OH, O-SO₂-ONa, or NH-C(O)CH₃

R² is alkyl, aryl, hetaryl.

- 5 Scheme 2. Binding of amine to α,β -unsaturated aldehyde of sulfated polysaccharide.

Further, the invention relates to the method of preparation of a derivative of the general structural formula I or II, where the sulfated polysaccharide, which is water soluble in its native form and contains, in its structure, at least one galactopyranose unit sulfated in the 4th position, this unit being bound in the polymer chain via α (1→3) or β (1→3) *O*-glycosidic bond, is first oxidized to an aldehyde in the 6th position and immediately after the oxidation in the current reaction mixture provides an α,β -unsaturated aldehyde via the direct elimination (Scheme 3).



where

R is OH, O-SO₂-OH, O-SO₂-ONa, or NH-C(O)CH₃

R¹ is H or Na.

- 5 Scheme 3. Method of preparation of an α,β -unsaturated aldehyde in the structure of the sulfated polysaccharide.

The selective oxidation of the primary hydroxyl group in the 6th position of galactopyranose can be realized e.g. by means of the oxidation system of 2,2,6,6-tetramethyl-1-piperidinyloxy radical R³-TEMPO/NaClO, where R³ is hydrogen or N-acetyl, in water or an aqueous solution of inorganic salts. Preferably, this step proceeds in water, under the temperature of 5 to 25 °C, more preferably 5 to 10 °C, the molar amount of NaClO is within the range of 0.1 to 2.0 equivalent, and the molar amount of R³ -TEMPO is within the range of 0.01 to 0.2 equivalent, with respect to a sulfated polysaccharide dimer. The molecular weight of the initial sulfated polysaccharide is within the range of 1×10^4 to 5×10^6 g.mol⁻¹, and it must contain galactopyranose units sulfated in the 4th position, and it is bound via α (1→3) or β (1→3) O-glycosidic bonds in the polymer chain. Preferably, the initial sulfated polysaccharide is chondroitin sulfate, dermatan sulfate, carrageenan, or their pharmaceutically acceptable derivative and/or salt. The aqueous solution of salts may be e.g. an aqueous solution comprising an alkaline metal salt and/or a buffer, e.g. PBS.

The elimination reaction of the oxidized and sulfated polysaccharide proceeds immediately after the oxidative stage in the same reaction mixture without the need of adding an elimination agent, especially an acid or a base, an organic solvent, or without elevation of the reaction temperature, and without the isolation of the saturated C6-aldehyde in galactopyranose subunit being sulfated in 4th position. The elimination reaction proceeds in water or in aqueous solutions of inorganic salts (e.g. alkali metals salts), or buffers (e.g. PBS) at the temperature of 5 – 25 °C and there is no need of an additional reaction time. Further, the elimination stage is proportional to the achieved oxidation stage in the reaction mixture. The method of preparation of the α,β -unsaturated aldehyde integrates two reaction steps (oxidation and elimination) into one pot without the isolation of the intermediate from the oxidation stage. The oxidation results in an α,β -unsaturated aldehyde instead of a saturated C6-aldehyde in the structure of sulfated polysaccharide. In comparison to the method of the preparation of an α,β -unsaturated aldehyde of hyaluronic acid (Buffa R et al.: CZ304512), the presented approach is different and definitely advantageous in the parameters listed in Table 1.

Table 1: Differences in the preparation of α,β -unsaturated aldehydes in comparison with the prior art

Parameter	CZ304512	Invention
Polysaccharide type	Hyaluronic acid	Sulfated polysaccharides
Number of steps of the synthesis	2	1
Elimination agent	yes (10-15 equivalents)	no
Solvent	water-organic solvent mixture	water
Reaction temperature	50-100 °C	Room temperature – 5-25°C
Reaction time	2-5 days	1-2 h
Degree of substitution	5-7%	20-25%

Table 1 above clearly shows that the method of preparation of sulfated polysaccharide

- 5 derivatives according to the invention results directly in the formation of α,β -unsaturated aldehydes instead of their saturated analogues. Another difference of the presented method of the invention consists in that it is not applicable to the polysaccharides mentioned in the prior art, because the presence of the sulfate is necessary for the process. Other advantages address to the reaction proceeding exclusively in water, without the need of addition of any organic
- 10 solvent or any elimination agent. Furthermore, the reaction proceeds at room temperature (20 – 25 °C) with short reaction times (1 – 2 h) without isolation of the saturated C6-aldehyde. The above mentioned method results in derivatives of sulfated derivatives having the general formulae I and/or II, DS within the range of 20 to 25%. The method of their preparation is technologically interesting and substantially more preferable from the temporal and cost point
- 15 of view in comparison with the known methods.

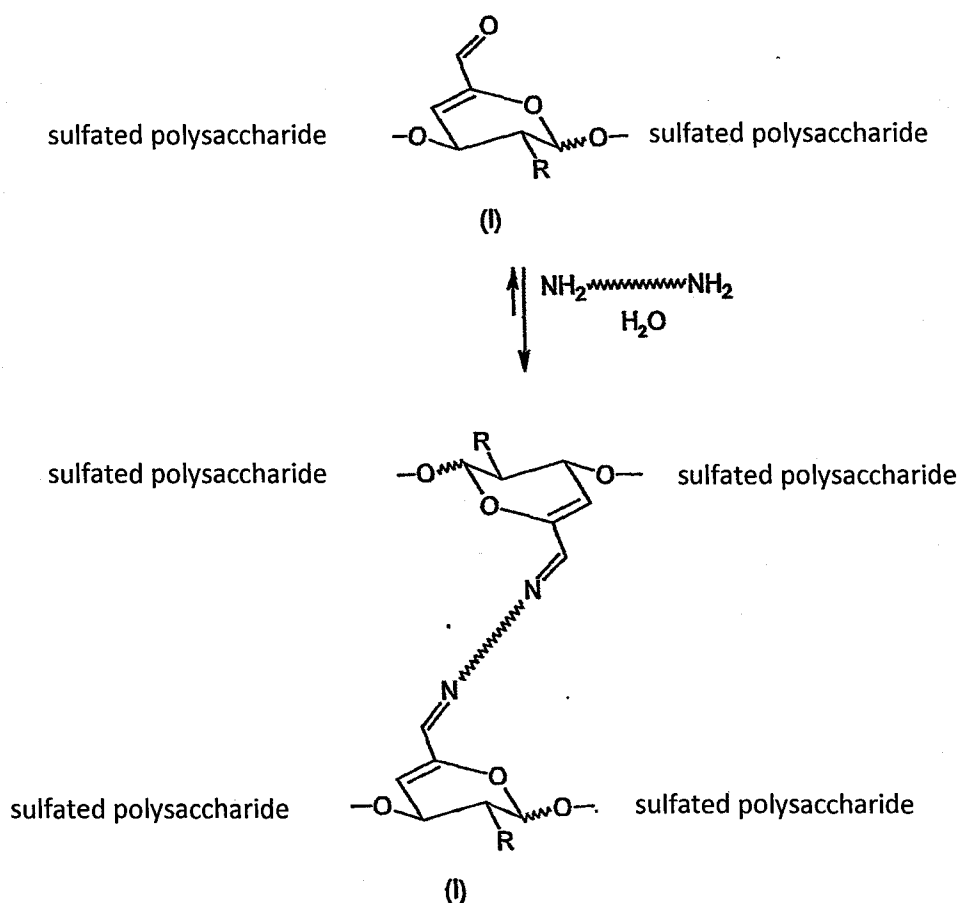
Considering the chemical modification, the α,β -unsaturated aldehyde in the sulfated polysaccharides can be used mainly for condensation reactions with various N-nucleophiles. The aldehyde, in the role of the reactive electrophilic centre, maintains its stability and reactivity also in water, which can be preferably used for the mentioned binding (conjugation)

20 of biocompatible amines with the derivatives of the general formulae I and II. The term “amine” is well known to a person skilled in the art, and it can represent, without any

limitation, alkyl amine, aryl amine, hetaryl amine, amino acid, peptide, or polymer with a free amino group. The latter can be directly incorporated into the polymer or is bound via a suitable linker that can be linear or branched, optionally containing N, S, or O atoms. The term "polymer with an amino group" is understood to be a deacetylated polysaccharide, protein, peptide, or another biopolymer or biocompatible synthetic polymer.

Thus, the invention further relates to the method of modification of the derivative of the general formula I or II, where the derivative reacts with an amine of the general formula R^2-NH_2 , where R^2 is alkyl, aryl, hetero aryl, linear or branched C_1 to C_{30} chain, optionally containing N, S, or O atoms. The amine is preferably a biologically active amine, particularly amino acid or peptide, or a biologically acceptable polymer containing a free amino group, where this amino group is an inherent part of the polymer (e.g. gelatine, chitosan, deacetylated hyaluronic acid, deacetylated chondroitin sulfate, etc.) or is bound to the polymer via a linker containing amino, hydrazine, hydrazide, amino alkoxy, hydroxyl, carboxyl, thiol group or any combination thereof. The molar amount of amine can be, preferably, within the range of 0.05 - 3 equivalents with respect to a dimer of the sulfated polysaccharide. The binding of amine can proceed in water, phosphate buffer, or water-organic solvent system at the temperature in the range of 20 to 60 °C for 10 minutes to 150 h. A suitable organic solvent is understood to be a water miscible alcohol, preferably isopropanol or ethanol, and water miscible aprotic solvents, preferably dimethyl sulfoxide, wherein their content in the reaction mixture does not exceed 50 % (v/v). The reaction with amine can be preferably performed under physiological conditions (pH = 7.4 and T = 37 °C). Besides amines, the reaction proceeds also with other N-nucleophiles containing an amino group in their structure, such as hydrazines, hydroxyl amines, hydrazides, semicarbazides, or thiosemicarbazides. In case of a reaction with monofunctional N-nucleophiles, they are bound to the polymer, wherein the use of bi- and polyfunctional N-nucleophiles provides crosslinking of polymer chains, i.e. the formation of hydrogels. Depending on the type of the N-nucleophile used, its amount with respect to the ratio of the binding sites, polymer structure, solution concentration, degree of substitution, and molecular weight of the polymer, cross-linked polymers with wide range of viscoelastic and mechanical properties can be prepared exactly according to the needs of the intended applications in tissue engineering or regenerative medicine. In some specific cases, the reaction of the derivative of the invention with an amine can proceed within the whole range of pH, wherein in other cases the pH value is important for the reaction. A person skilled in the art can recognize it in advance, or determine by means of routine measurements.

The intended applications are meant to be mainly the preparations of scaffolds as bioactive and biodegradable support materials imitating the extracellular matrix. These materials can serve as the carriers for cells or biologically active substances, cell attractants, as the carrier medium for cell delivery to the site of a tissue defect, as a tissue filler, an adequate tissue substitute, or a protective barrier. Other demands put on the functional scaffolds comprise ensuring a suitable chemical and physiological environment for the cell proliferation and differentiation, transport of nutrients and waste products of cell metabolism. Depending on the way of the scaffold application, it is possible to prepare injection scaffolds from the cross-linked sulfated polysaccharides in the form of gel forming solutions, wherein the scaffold and the new tissue form *in vivo*, or solid scaffolds, which are implanted to an organism after the cell cultivation and formation of the new tissue *in vitro*. Furthermore, the proper choice of parameters of the cross-linking reaction (concentration and binding sites ratio) enables to achieve short times of gelation, in the order of seconds (see Example 30), which can be preferably used for the gelation *in situ* at the presence of a biological material, the so-called the cell encapsulation. The cross-linking reaction is illustrated in the Scheme 4:



where

R is OH, O-SO₂-OH, O-SO₂-ONa or NH-C(O)CH₃

Scheme 4. Cross-linking of a sulfated polysaccharide by means of an α,β -unsaturated aldehyde and a diamine.

- 5 The higher stability of the bond of an amine with an α,β -unsaturated aldehyde comparing to a conventional saturated aldehyde is ensured by means of the conjugation of the aldehyde with the adjacent double bond. Thus, more stable and better cross-linked materials based on sulfated polysaccharides can be prepared, as was shown by the example of the non-sulfated polysaccharide of hyaluronic acid (Buffa R., et al: CZ304512).
- 10 The crosslinking is performed by reacting the derivative with a water soluble biocompatible bi- and polyfunctional N-nucleophile selected from the group comprising alkyl amines, aryl amines, heteroalkyl amines, hetaryl amines, amino acids, peptides, polymers with free amino group, hydrazines, hydroxyl amines, hydrazides, semicarbazides, or thiosemicarbazides, wherein the crosslinking of the derivative proceeds. The preferred nucleophiles comprise
- 15 hydrazides, dihydrazides, deacetylated polysaccharides, or alkoxyamines. The reaction can preferably proceed in phosphate buffer.

However, the comparative analysis of mechanical properties (Young's modulus of elasticity in compression, elastic limit in compression, and deformation rate of the crosslinked gels) proved a higher density of the gels prepared from the oxidized chondroitin sulfate (see

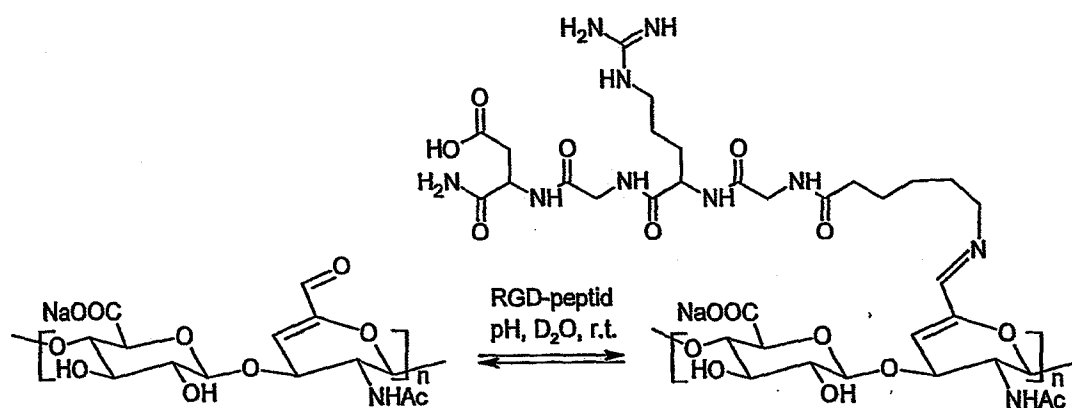
20 Example 31 of the invention) in comparison with the gels based on the oxidized hyaluronan. The higher rigidity of gels reflects the higher crosslink density in the structure of the polysaccharide, and thus a better volume and shape stability of the crosslinked material is ensured. In addition, better crosslinked materials show less changes in mechanical properties in the course of time and thus meet the needs put on the functional cell scaffold. In this case,

25 more effective crosslinking can be achieved by means of a higher degree of substitution of the α,β -unsaturated aldehyde in the structure of the sulfated polysaccharide (see Table 1 above), which is one of the important advantages of the invention in comparison with the prior art.

The second advantage of more efficiently crosslinked gels consists in a lower rate of swelling in a physiological medium. This can be preferably used for scaffolds in tissue engineering

30 where controlled material performance in a living organism at contact with the tissue is desired, without dramatic changes of their mechanical properties or shape or volume.

The third advantage of the higher degree of substitution of the α,β -unsaturated aldehyde in the structure of the sulfated polysaccharide is the possibility of binding a higher amount of e.g. a biologically active amine. In this way, a higher concentration of the biologically active substance can be achieved at the site of the effect for applications of support systems, for which the described invention can be also preferably used. Furthermore, the proposed method enables binding of a broader variety of biologically active amines (e.g. amino acids, peptides) that can be naturally released in their native (active) form. It was found repetitively in several examples (butylamine, lysine, RGD peptide) that at lower pH the bond amine - α,β -unsaturated aldehyde is less stable (Scheme 5), so the prepared conjugates can be preferably used for drug delivery systems based on pH-responsive biomaterials.



pH	DS imine %
4.22	0
7.22	4
10.49	20

Scheme 5. Stability of imine of α,β -unsaturated aldehyde chondroitin sulfate (DS = 20 %) with RGD peptide (aminohexane acid-Gly-Arg-Gly-Asp-NH₂) in water.

More particularly, this stability of an imine based on alkyl, aryl, or hetaryl amine in water can be used as follows: when the conjugate (imine), formed by a biologically active amine (e.g. drug, antiseptic preparation, peptide, amino acid etc.) and polysaccharide (carrier) being stable under moderately basic conditions, is incorporated into the target site of an organism, whose pH is different (neutral or moderately acid), this conjugate is decomposed and the biologically active substance is released in this site.

It was proved that the α,β -unsaturated aldehyde itself in the chondroitin sulfate structure is not cytotoxic (see Example 32 according to the invention), so the conjugates and the crosslinked products of α,β -unsaturated aldehydes of the formula I or II with biocompatible amines are suitable for targeted applications in biomedicine and tissue engineering. These substances are supposed not to influence negatively the cell viability, not to induce an immune reaction in the organism, to be enzymatically degradable, whereas the products of their degradation are biocompatible as well. Thus the derivatives of the formulae I or II can be used for the preparation of supports of biologically active substances in the field of cosmetics or pharmacy, or as the supports of biologically active substances with the controlled release by means of the change of pH. With regard to the reaction fluently proceeding under physiological conditions and with biocompatible initial materials, the crosslinked products of sulfated polysaccharides can be also considered to be a promising material for cell scaffolds in tissue engineering or regenerative medicine, where they can be preferably used for incorporating the cells and their subsequent cultivation. The method described in this invention can be easily realized in the industry, because it is neither expensive, nor time consuming. This is because of the combination of the two steps into one pot without the need of isolation an intermediate. Another advantage presents the absence of toxic, corrosive, or expensive chemicals in the role of an elimination agent, as well as the absence of an organic solvent, as the reaction proceeds exclusively in water. The reaction times are short; and, moreover, the reaction proceeds at room temperatures. The final products are isolated by precipitation with alcohols or inorganic salt solutions without any harmful effect on the environment. In addition, relatively high degrees of substitution (20 – 25 %) are achieved by the proposed method under substantially milder conditions than in (Buffa R., et al: CZ304512, see Table 1 above).

The sulfated polysaccharides modified by the described method of the invention are suitable as precursors for conjugation or crosslinking reactions with various N-nucleophiles resulting in biocompatible materials suitable for biomedical applications, tissue engineering, and

regenerative medicine. More particularly, the derivatives prepared by the method of the invention can be used as supports of biologically active substances with their controlled release with the use of changing the pH value, in the field of cosmetics and pharmacy. The derivatives modified by the method of the invention can be used as biocompatible materials for biomedical applications and formation of scaffolds for tissue engineering, or for regenerative medicine.

Brief Description of Drawings:

Fig. 1 illustrates the formation of a hydrogel based on oxidized chondroitin sulfate with adipic dihydrazide (Example 21): (a) solution of α,β -unsaturated aldehyde of chondroitin sulfate in PBS, (b) gelation of the solution after the addition of adipic dihydrazide solution in PBS, (c) the hydrogel after 1 h in PBS (pH = 7.4, c = 0.9% w/v), (d) the use of an illustrative form for the preparation of the hydrogel, (e) the use of a defined form for the preparation of the hydrogel, (f) a detailed view of a segment of the hydrogel.

Fig. 2 Photographs of the lyophilized hydrogel based on the oxidized chondroitin sulfate with adipic dihydrazide taken by the scanning electron microscope: (a) a cross section, magnification 200x, detection of secondary electrons, (b) an enlarged section of the porous structure of the hydrogel with the measured diameter.

Fig. 3 illustrates the results of the cell viability tests of 3T3 fibroblasts in α,β -unsaturated aldehyde of chondroitin sulfate ($M_w = 4 \times 10^4$ g/mol, DS = 20 %). The activation vs inhibition curve, expressed in %, with respect to the control in time T = 0 h (100%). Evaluation by means of MTT method, six repetitions.

Fig. 4 Kinetics of the gelation in Example 30, with the determination of the gel point ($T_g = 97$ s, $T = 45$ °C). A graphical representation of the dependence of elastic (G') and viscous (G'') modulus in time.

Examples:

The term “equivalent” (eq), as used herein, relates to the dimer unit of the sulfated polysaccharide, if not stated otherwise. The percentage is expressed as weight percentage, if not stated otherwise. The molecular weight of the initial chondroitin sulfate (the source:

Sigma Aldrich, Ltd., Prague, CZ) is the weight-average molecular weight within the range of 4×10^4 to 5×10^4 g.mol⁻¹.

The ratio of chondroitin-4-sulfate (type A) and chondroitin-6-sulfate (type C) was 3:2. The material was isolated from an animal material.

- 5 The sodium salt of dermatan sulfate (chondroitin sulfate B sodium salt) of the solubility of 5 mg/ml in water was purchased from Sigma Aldrich. The material was isolated from an animal material.

Lambda carrageenan of the solubility of 10 mg/ml in water was purchased from Sigma Aldrich and was isolated from sea algae without gelation properties in the native form.

- 10 The degree of substitution of α,β -unsaturated aldehyde in the structure of the sulfated polysaccharide was determined according to the following calculation:

DS = the degree of substitution of α,β -unsaturated aldehyde = $100 \% \cdot (\text{the molar amount of the modified dimer of the sulfated polysaccharide} / (\text{the molar amount of all dimers of the sulfated polysaccharide}))$

- 15 The degree of substitution of the amination reaction in the structure of the sulfated polysaccharide was determined according to the following calculation:

DS = the degree of substitution for amination = $100 \% \cdot (\text{the molar amount of the modified dimer of the sulfated polysaccharide} / (\text{the molar amount of all dimers of the sulfated polysaccharide}))$

- 20 FT-IR spectra were measured within the range of $4000 - 400 \text{ cm}^{-1}$ in KBr, by means of the spectrometer Nicolet 6700 FTIR. UV-VIS spectra were measured by means of the apparatus Shimadzu UV-2401PC within the range of 200-600 nm and processed by means of the UV Probe software, version 2,00.

- 25 The gelation kinetics was determined by means of the apparatus AR-G2 and TA Analysis was used as the evaluation software. The gel point (T_g) was determined from the time dependence of the elastic and viscous modulus.

The mechanical properties of the selected gels were measured by the compression test by means of the apparatus Instron 3433 and evaluated by means of Bluehill software. The

determined parameters for each sample were as follows: Young's modulus for compression, compressive strength, deformation at ultimate strength, and tenacity.

The surface morphology of the lyophilized materials was analysed by means of the electron microscope Zeiss Ultra Plus.

- 5 Deacetylated hyaluronic acid was prepared by deacetylation with hydrazine according to Buffa R., et al CZ304512.

Aminopropoxyle and hydrazine derivative of hylauronic acid were prepared by the reductive amination according to Buffa R., et al.: WO2011069474.

- 10 List of abbreviations

TEMPO – 2,2,6,6-tetramethyl-1-piperidinyloxy radical

4-AcNH-TEMPO – TEMPO with an acetamide group in the 4th position

PBS – phosphate buffered saline

RGD peptide – peptide of the sequence of aminohexane acid-Gly-Arg-Gly-Asp-NH₂

- 15 AMK – amino acid

IPA – isopropylalcohol

DMSO – dimethylsulfoxid

Example 1

- 20 Preparation of α,β -unsaturated aldehyde of chondroitin sulfate

Method 1: An aqueous solution of sodium hypochlorite (0.8 eq, 11 % of active chlorine) was gradually added into a 2% aqueous solution of chondroitin sulfate (200 mg, $M_w = 4.5 \times 10^4$ g.mol⁻¹) cooled to 5 °C, containing disodium phosphate dodecahydrate (2.2 eq), sodium bromide (0.8 eq) and 4-AcNH-TEMPO (0.01%) . The mixture was stirred for 2 h at 5 °C.

- 25 Then ethanol (10 eq) was added to the reaction which was stirred for an additional hour at the room temperature.

The product was isolated by precipitation with IPA and analysed by NMR.

DS = 23% (determined by NMR), $M_w = 2.1 \times 10^4$ g.mol⁻¹ (determined by SEC MALLS)

Method 2: An aqueous solution of sodium hypochlorite (0.8 eq, 11 % of active chlorine) was gradually added into a 2% aqueous solution of chondroitin sulfate (200 mg, $M_w = 4.5 \times 10^4$ g.mol⁻¹) cooled to 5 °C, containing sodium bromide (0.8 wq) and 4-AcNH-TEMPO (0.01%). The mixture was stirred for 2 h at 5 °C. Then ethanol (10 eq) was added to the reaction which was stirred for an additional hour at the room temperature. The product was isolated by precipitation with IPA and analysed by NMR.

DS = 20% (determined by NMR)

Spectral analysis of α,β -unsaturated aldehyde of chondroitin sulfate: NMR ¹H (500 MHz, D₂O, δ ppm): 2.02 (3H, Ac-NH-, bs), 4.31 (1H, H₂, bs), 4.49 (1H, H₃, bs), 5.20 (1H, H₁, bs), 6.34 (1H, H₄, bs), 9.21 (1H, H₆, bs);

NMR ¹H-¹H COSY (D₂O), crosspeaks, δ ppm: 4.31-4.49, 4.31-5.20, 4.49-6.34; NMR ¹H-¹³C HSQC

(D₂O), crosspeaks, δ ppm: 2.02-25.1, 4.31-51.0, 4.49-73.1, 5.20-98.6, 6.34-122.0, 9.21-189.0;

NMR DOSY (D₂O), log D ((2.02, Ac-NH-), (4.31, H₂), (4.49, H₃), (5.20, H₁), (6.34, H₄), (9.21, H₆)) ~ -10.3 m²s⁻¹, log D (4.72, H₂O) ~ -8.6 m²s⁻¹;

IR (KBr, cm⁻¹): 1725, 1650 (ν C=O st), 1615, 1663 (ν C=C st);

UV/Vis (0,1%, H₂O); $\lambda_{\max 1,2}$ (C β =C α -C=O) = 254 nm ($\pi \rightarrow \pi^*$), 300-350 (n $\rightarrow \pi^*$).

Example 2

Preparation of α,β -unsaturated aldehyde of chondroitin sulfate

An aqueous solution of sodium hypochlorite (0.4 eq, 11 % of active chlorine) was gradually added into a 2% aqueous solution of chondroitin sulfate (200 mg, $M_w = 4.5 \times 10^4$ g.mol⁻¹) cooled to 5 °C, containing disodium phosphate dodecahydrate (2.2 eq), sodium bromide (0.4 eq) and 4-AcNH-TEMPO (0.01%). The mixture was stirred for 2 hours at 5 °C. Then ethanol (10 eq) was added to the reaction which was stirred for an additional hour at the room temperature. The product was isolated by precipitation with IPA and analysed by NMR.

DS = 2% (determined by NMR), $M_w = 2.8 \times 10^4 \text{ g.mol}^{-1}$ (determined by SEC MALLS). The structural analysis of the product is presented in Example 1.

Example 3

5 Preparation of α,β -unsaturated aldehyde of chondroitin sulfate

An aqueous solution of sodium hypochlorite (1 eq, 11 % of active chlorine) was gradually added into a 2% aqueous solution of chondroitin sulfate (200 mg, $M_w = 4.5 \times 10^4 \text{ g.mol}^{-1}$) cooled to 5 °C, containing disodium phosphate dodecahydrate (2.2 eq), sodium bromide (1 eq) and 4-AcNH-TEMPO (0.01%). The mixture was stirred for 2 hours at 5 °C. Then ethanol
10 (10 eq) was added to the reaction which was stirred for an additional hour at the room temperature. The product was isolated by precipitation with IPA and analysed by NMR.

DS = 21% (determined by NMR), $M_w = 2.0 \times 10^4 \text{ g.mol}^{-1}$ (determined by SEC MALLS). The structural analysis of the product is presented in Example 1.

15 Example 4

Preparation of α,β -unsaturated aldehyde of chondroitin sulfate

An aqueous solution of sodium hypochlorite (2 eq, 11 % of active chlorine) was gradually added into a 2% aqueous solution of chondroitin sulfate (200 mg, $M_w = 4.5 \times 10^4 \text{ g.mol}^{-1}$) cooled to 5 °C, containing disodium phosphate dodecahydrate (2.2 eq), sodium bromide (2
20 eq) and 4-AcNH-TEMPO (0.01%). The mixture was stirred for 2 hours at 5 °C. Then ethanol (10 eq) was added to the reaction which was stirred for an additional hour at the room temperature. The product was isolated by precipitation with IPA and analysed by NMR.

DS = 21% (determined by NMR), $M_w = 1.8 \times 10^4 \text{ g.mol}^{-1}$ (determined by SEC MALLS). The structural analysis of the product is presented in Example 1.

25

Example 5

Preparation of α,β -unsaturated aldehyde of dermatan sulfate

An aqueous solution of sodium hypochlorite (0.8 eq, 11 % of active chlorine) was gradually added into a 2% aqueous solution of dermatan sulfate (200 mg, 0.42 mol) cooled to 5 °C, containing disodium phosphate dodecahydrate (2.2 eq), sodium bromide (0.8 eq) and 4-AcNH-TEMPO (0.01%). The mixture was stirred for 2 hours at 5 °C. Then ethanol (10 eq) was added to the reaction which was stirred for an additional hour at the room temperature. The product was isolated by precipitation with IPA and analysed by NMR.

DS = 20% (determined by NMR)

Spectral analysis of α,β -unsaturated aldehyde of dermatan sulfate: NMR ^1H (500 MHz, D_2O , δ ppm): 2.01 (3H, Ac-NH-, bs), 6.30 (1H, H4, bs), 9.20 (1H, H6, bs).

Example 6

Preparation of α,β -unsaturated aldehyde of carrageenan

An aqueous solution of sodium hypochlorite (0.8 eq, 11 % of active chlorine) was gradually added into a 1% aqueous solution of carrageenan (200 mg, 0.31 mol) cooled to 10 °C, containing disodium phosphate dodecahydrate (2.2 eq), sodium bromide (0.8 eq) and 4-AcNH-TEMPO (0.01%). The mixture was stirred for 2 hours at 10 °C. Then ethanol (10 eq) was added to the reaction which was stirred for an additional hour at the room temperature. The product was isolated by precipitation with IPA and analysed by NMR.

DS = 10% (determined by NMR)

Spectral analysis of α,β -unsaturated aldehyde of carrageenan: NMR ^1H (500 MHz, D_2O , δ ppm): 6.30 (1H, H4, bs), 9.20 (1H, H6, bs).

Example 7

Binding of hydrazine to α,β -unsaturated aldehyde of chondroitin sulfate

Hydrazine hydrate (2 eq) was added to a 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, $\text{Mw} = 2.1 \times 10^4 \text{ g.mol}^{-1}$) in D_2O . The reaction was stirred for 24 h at the room temperature. The product was analysed in the form of a crude reaction mixture.

DS = 20% (determined by NMR)

NMR ^1H (500 MHz, D_2O , δ ppm): 5.40 (1H, $-\underline{\text{CH}}=\text{C}-\text{CH}=\text{N}-$, bs), 7.38 (1H, $-\text{CH}=\text{C}-\underline{\text{CH}}=\text{N}-$, bs)

5 Example 8

Binding of butylamine to α,β -unsaturated aldehyde of chondroitin sulfate

Method 1: Butylamine (0.2 eq) was added to a 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, $\text{Mw} = 2.1 \times 10^4 \text{ g.mol}^{-1}$) in D_2O . The reaction was stirred for 24 h at the room temperature and pH = 11.20. The product was analysed in the form of a crude reaction mixture.

DS = 20 % (determined by NMR)

NMR ^1H (500 MHz, D_2O , δ ppm): 5.67 (1H, $-\underline{\text{CH}}=\text{C}-\text{CH}=\text{N}-$, bs), 7.74 (1H, $-\text{CH}=\text{C}-\underline{\text{CH}}=\text{N}-$, bs)

Method 2: Deuterated acetic acid (14.5 μL) was added to a NMR sample of method 1. The measured pH was 4.10 and the sample was then analysed by NMR.

DS = 0% (determined by NMR)

Example 9

Binding of butylamine to α,β -unsaturated aldehyde of chondroitin sulfate

Butylamine (0.2 eq) was added to a 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, $\text{Mw} = 2.1 \times 10^4 \text{ g.mol}^{-1}$) in deuterated PBS. The reaction was stirred for 24 h at the room temperature and pH = 7.30. The product was analysed in the form of a crude reaction mixture.

DS = 0% (determined by NMR)

Example 10

Binding of hexane-1,6-diamine to α,β -unsaturated aldehyde of chondroitin sulfate

Hexane-1,6-diamine (0.5 eq) was added to a 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, Mw = 2.1×10^4 g.mol⁻¹) in D₂O. The reaction was stirred for 24 h at the room temperature and pH = 11.60. The product was analysed in the form of a crude reaction mixture.

DS = 20% (determined by NMR)

NMR ¹H (500 MHz, D₂O, δ ppm): 5.68 (1H, -CH=C-CH=N-, bs), 7.74 (1H, -CH=C-CH=N-, bs)

Example 11

Binding of propoxyamine to α,β -unsaturated aldehyde of chondroitin sulfate

Propoxyamine hydrochloride (0.5 eq) was added to a 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, Mw = 2.1×10^4 g.mol⁻¹) in D₂O. The reaction was stirred for 24 h at the room temperature and pH = 3.90. The product was analysed in the form of a crude reaction mixture.

DS = 20% (determined by NMR)

NMR ¹H (500 MHz, D₂O, δ ppm): 5.57 and 6.88 (1H, -CH=C-CH=N-, bs), 7.52 and 7.70 (1H, -CH=C-CH=N-, bs)

Example 12

Binding of lysine to α,β -unsaturated aldehyde of chondroitin sulfate

Lysine hydrochloride (0.5 eq) was added to a 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, Mw = 2.1×10^4 g.mol⁻¹) in deuterated PBS. The reaction was stirred for 24 h at the room temperature and pH = 7.46. The product was analysed in the form of a crude reaction mixture.

DS = 4% (determined by NMR)

NMR ^1H (500 MHz, D_2O , δ ppm): 5,69-5,75 (1H, $-\underline{\text{CH}}=\text{C}-\text{CH}=\text{N}-$, bs), 7,70-7,75 (1H, $-\text{CH}=\text{C}\underline{\text{CH}}=\text{N}-$, bs)

5 Example 13

Binding of lysine to α,β -unsaturated aldehyde of chondroitin sulfate

Lysine hydrochloride (0.5 eq) was added to a 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, $\text{Mw} = 2.1 \times 10^4 \text{ g.mol}^{-1}$) in D_2O . pH of the reaction was adjusted to 8.40 by adding sodium bicarbonate (2 eq). The reaction was stirred for 24 h at the room temperature. The product was analysed in the form of a crude reaction mixture.

DS = 7% (determined by NMR)

The structural analysis is presented in Example 12.

Example 14

15 Binding of RGD peptide to α,β -unsaturated aldehyde of chondroitin sulfate

RGD peptide (0.2 eq, sequence Ahx-Gly-Arg-GlyAsp-NH₂) was added to 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, $\text{Mw} = 2.1 \times 10^4 \text{ g.mol}^{-1}$) in D_2O . The reaction was stirred for 24 h at the room temperature and pH = 4.22. The product was analysed in the form of a crude reaction mixture.

20 DS = 0% (determined by NMR)

Example 15

Binding of RGD peptide to α,β -unsaturated aldehyde of chondroitin sulfate

RGD peptide (0.2 eq, sequence Ahx-Gly-Arg-GlyAsp-NH₂) was added to a 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, $\text{Mw} = 2.1 \times 10^4 \text{ g.mol}^{-1}$) in

deuterated PBS. The reaction was stirred for 24 h at the room temperature and pH = 7.22. The product was analysed in the form of a crude reaction mixture.

DS = 4% (determined by NMR)

NMR ^1H (500 MHz, D_2O , δ ppm): 5.68 (1H, $-\underline{\text{CH}}=\text{C}-\text{CH}=\text{N}-$, bs), 7.74 (1H, $-\text{CH}=\text{C}-\underline{\text{CH}}=\text{N}-$, bs)

Example 16

Binding of RGD peptide to α,β -unsaturated aldehyde of chondroitin sulfate

RGD peptide (0.2 eq, sequence Ahx-Gly-Arg-GlyAsp-NH₂) was added to a 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, Mw = 2.1×10^4 g.mol⁻¹) in D_2O . pH of the reaction was adjusted to 10.49 by adding sodium bicarbonate (2 eq). The reaction was stirred for 24 h at the room temperature. The product was analysed in the form of a crude reaction mixture.

DS = 20% (determined by NMR)

The structural analysis is presented in the Example 15.

Example 17

Binding of aniline to α,β -unsaturated aldehyde of chondroitin sulfate

Aniline (0.3 eq) was added to a 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, Mw = 2.1×10^4 g.mol⁻¹) in D_2O . pH of the reaction was adjusted to 4.22 by adding deuterated acetic acid (8.8 μL). The reaction was stirred for 24 h at the room temperature. The product was analysed in the form of a crude reaction mixture.

DS = 0% (determined by NMR)

Example 18

Binding of aniline to α,β -unsaturated aldehyde of chondroitin sulfate

Aniline (0.3 eq) was added to a 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, $M_w = 2.1 \times 10^4$ g.mol⁻¹) in deuterated PBS. The reaction was stirred for 24 h at the room temperature and pH = 7.42. The product was analysed in the form of a crude reaction mixture.

5 DS = 5% (determined by NMR)

NMR ¹H (500 MHz, D₂O, δ ppm): 5.93 (1H, -CH=C-CH=N-, bs), 8.03 (1H, -CH=C-CH=N-, bs)

Example 19

10 Binding of aniline to α,β -unsaturated aldehyde of chondroitin sulfate

Aniline (0.3 eq) was added to a 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, $M_w = 2.1 \times 10^4$ g.mol⁻¹) in D₂O. pH of the reaction was adjusted to 10.73 by adding sodium carbonate. The reaction was stirred for 24 h at the room temperature. The product was analysed in the form of a crude reaction mixture.

15 DS = 25% (determined by NMR)

The structural analysis is presented in the Example 18.

Example 20

Binding of dihydrazide adipate to α,β -unsaturated aldehyde of chondroitin sulfate

20 Dihydrazide adipate (3 eq) was added to a 2% solution of α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, $M_w = 2.1 \times 10^4$ g.mol⁻¹) in D₂O. The reaction was stirred for 24 h at the room temperature and pH = 7.50. The product was analysed in the form of a crude reaction mixture.

DS = 20% (determined by NMR)

25 NMR ¹H (500 MHz, D₂O, δ ppm): 1.64 (4H, DHA_{2,3}, bs), 2.04 (3H, Ac-NH-, bs), 2.34 (4H, DHA_{1,4}, bs), 4.28 (1H, H₂, bs), 4.36 (1H, H₃, bs), 5.20 (1H, H₁, bs), 5.62 (1H, H_{4cis}, bs), 5.68 (1H, H_{4trans}, bs), 7.52-7.48 (1H, H_{6cis}, bs), 7.61 (1H, H_{6trans}, bs);

NMR ^1H - ^1H COSY (D_2O), crosspeaks, δ ppm: 1.64-2.34, 4.28-5.20, 4.36-5.68; NMR ^1H - ^{13}C HSQC

(D_2O), crosspeaks, δ ppm: 1.64-24.9, 2.34-34.1, 4.28-51.0, 4.36-73.6, 5.20-98.8, 5.68-111.3, 7.61-148.5;

- 5 NMR DOSY (D_2O), log D ((2.04, Ac-NH-), (4.28, H2), (4.36, H3), (5.20, H1), (5.62 and 5.68,

H4cis/trans), (7.52 and 7.68, H6cis/trans)) $\sim -10.4 \text{ m}^2\text{s}^{-1}$, log D (4.72, H_2O) $\sim -8.6 \text{ m}^2\text{s}^{-1}$;

IR (KBr, cm^{-1}): 1640-1650 (ν -C=N- st);

UV/Vis (0.1%, H_2O); $\lambda_{\text{max}1,2}$ (-C=N-) = 280 nm ($\pi \rightarrow \pi^*$).

10

Example 21

Crosslinking of α,β -unsaturated aldehyde of chondroitin sulfate with dihydrazide adipate

Dihydrazide adipate (0.12 eq, binding sites ratio 1:1) in PBS was added to an 8% solution of α,β -unsaturated aldehyde of chondroitin sulfate (40 mg, DS = 23 %, $M_w = 2.1 \times 10^4 \text{ g.mol}^{-1}$) in

- 15 PBS (pH = 7.40, c = 0.9% w/v). After the addition of the solution of dihydrazide adipate, the gelation occurred in time ($T_g = 34 \text{ s}$). The elastic gel was photographed (Fig. 1), lyophilized, and analyzed by SEM (Fig. 2).

Young's modulus of elasticity in compression = $9 \times 10^3 \text{ Pa}$

Ultimate strength in compression = $64 \times 10^3 \text{ Pa}$

- 20 Deformation at ultimate strength = 64 %

Tenacity = 3668 J.m^{-3}

Example 22

Crosslinking of α,β -unsaturated aldehyde of dermatan sulfate with dihydrazide adipate

- 25 Dihydrazide adipate (0.1 eq, binding sites ratio 1:1) in PBS was added to an 8% solution of α,β -unsaturated aldehyde of dermatan sulfate (40 mg, DS = 20 %, $M_w \leq 40 \text{ kDa}$) in PBS (pH = 7.40, c = 0.9% w/v). After the addition of the solution of dihydrazide adipate, the gelation proceeded.

Example 23

Crosslinking of α,β -unsaturated aldehyde of carrageenan with dihydrazide adipate

40 μL of dihydrazide adipate (0.05 eq) in PBS was added to an 8% solution of α,β -unsaturated aldehyde of carrageenan (40 mg, DS = 10 %, $M_w \leq 50 \text{ kDa}$) in PBS (pH = 7.40, c = 0.9% w/v) . After the addition of the solution of dihydrazide adipate, the viscosity increased.

Example 24

Crosslinking of α,β -unsaturated aldehyde of chondroitin sulfate with hydrazide derivative of hyaluronic acid

A 4% solution of hydrazide derivative of hyaluronic acid (9.2 mg, DS = 25%, $M_w = 138 \times 10^3 \text{ g.mol}^{-1}$) in PBS (pH = 7.4, c = 0.9% w/v) was added to a 4% solution of α,β -unsaturated aldehyde of chondroitin sulfate (10 mg, DS = 23 %, $M_w = 2.1 \times 10^4 \text{ g.mol}^{-1}$) in PBS. After mixing the solutions, the gelation occurred in time ($T_g = 109 \text{ s}$).

Young's modulus of elasticity in compression = $6 \times 10^3 \text{ Pa}$

Ultimate strength in compression = $840 \times 10^3 \text{ Pa}$

Deformation at ultimate strength = 96 %

Tenacity = 11978 J.m^{-3}

Example 25

Crosslinking of α,β -unsaturated aldehyde of chondroitin sulfate with deacetylated hyaluronic acid

A 2% solution of deacetylated hyaluronic acid (2 eq, DS = 11%, $M_w = 116 \text{ kDa}$, binding sites ratio 1:1) in PBS (pH = 7.4, c = 0.9% w/v) was added to a 4% solution of α,β -unsaturated aldehyde of chondroitin sulfate (10 mg, DS = 23 %, $M_w = 2.1 \times 10^4 \text{ g.mol}^{-1}$) in PBS (pH = 7.40, c = 0.9% w/v). The reaction mixture was stirred at the room temperature, wherein an increase in viscosity was observed after 0.5 h, and an elastic gel was formed after 1 h.

Young's modulus of elasticity in compression = $3 \times 10^3 \text{ Pa}$

Ultimate strength in compression = 395×10^3 Pa

Deformation at ultimate strength = 95 %

Tenacity = 14670 J.m^{-3}

5 Example 26

Preparation of the acid form of chondroitin sulfate

A 1% solution of chondroitin sulfate (500 mg, 1.1 mmol) in distilled water was prepared. The solution was cooled to 5 °C and 1.2 ml of Amberlite IR 120 Na (H⁺) catex was added. The reaction mixture was stirred for 24 h at 5 °C. The catex was filtered off, the product was
10 frozen and lyophilized. Its solubility in DMSO was tested and found satisfactory.

Example 27

Preparation of deacetylated chondroitin sulfate

A 1% solution of acid form of chondroitin sulfate was prepared (200 mg, 0.44 mmol, Mw ≤
15 40 kDa) in DMSO. The solution was degassed by a stream of nitrogen. 10.6 mL of hydrazine hydrate and 3 eqs of hydrazine sulfate were added. The reaction mixture was stirred for 24 h at 60 °C under nitrogen. Then NaHCO₃ was added to the reaction mixture. The product was isolated by precipitation with IPA.

DS = 10% (determined by NMR), Mw = $1.8 \times 10^4 \text{ g.mol}^{-1}$ (determined by SEC/MALLS)

20 NMR ¹H (500 MHz, 1% NaOD v D₂O, δ ppm): 3.01 (1H, -CH=C-CH=N-, bs) HSQC (500 MHz, D₂O, δ ppm): crosspeak: 3.42-52.2 ppm

Example 28

25 Crosslinking of α,β-unsaturated aldehyde of chondroitin sulfate with deacetylated chondroitin sulfate

An 8% solution of deacetylated chondroitin sulfate (2 eq, DS = 10%, Mw = $1.8 \times 10^4 \text{ g.mol}^{-1}$, binding sites ratio = 1/0.85) in PBS (pH = 7.4, c = 0.9% w/v) was added to an 8% solution of

α,β -unsaturated aldehyde of chondroitin sulfate (20 mg, DS = 23 %, $M_w = 2.1 \times 10^4$ g.mol⁻¹) in PBS (pH = 7.40, c = 0,9% w/v). The reaction mixture was stirred at the room temperature, wherein an increase in viscosity was observed after 0.5 h, and an elastic gel was formed after 3 h.

- 5 Young's modulus of elasticity in compression = 3×10^3 Pa
 Ultimate strength in compression = 774×10^3 Pa
 Deformation at ultimate strength = 95 %
 Tenacity = 16489 J.m⁻³

10 Example 29

Crosslinking of α,β -unsaturated aldehyde of chondroitin sulfate with propoxyamine

A PBS solution of propoxyamine hydrochloride (0.12 eq, binding sites ratio = 1/1) was added to a 10% solution of α,β -unsaturated aldehyde of chondroitin sulfate (50 mg, DS = 23 %, $M_w = 2.1 \times 10^4$ g.mol⁻¹) in PBS (pH = 7.40, c = 0,9% w/v), wherein a gel was formed in time ($T_g =$
 15 110 s).

Young's modulus of elasticity in compression = 8×10^3 Pa
 Ultimate strength in compression = 74×10^3 Pa
 Deformation at ultimate strength = 65 %
 Tenacity = 3768 J.m⁻³

20 Example 30

Measurement of gelation kinetics of crosslinking reaction of α,β -unsaturated aldehyde of chondroitin sulfate with aminopropoxyl derivative of hyaluronic acid

15 The measurement of gelation kinetics was performed by means of a 4% sample of α,β -
 25 unsaturated aldehyde of chondroitin sulfate (10 mg, DS = 23%, $M_w = 2.1 \times 10^4$ g.mol⁻¹) in PBS (pH = 7.40, c = 0.9% w/v) with a 4% solution of aminopropoxyl derivative of hyaluronic acid (1 eq, DS = 25%, $M_w = 66$ kDa) in PBS (pH = 7.4, c = 0.9% w/v). The gelation time, i.e. the stage, when the first macroscopic gel network was formed, was determined to be ($T_g = 97$ s,
 30 Fig. 4).

Example 31

Comparison of mechanical properties of hydrogels based on the crosslinked α,β -unsaturated aldehyde of chondroitin sulfate and α,β -unsaturated aldehyde of hyaluronan

- 5 Solution 1: 4% solution of α,β -unsaturated aldehyde of chondroitin sulfate (DS = 23%, $M_w = 2.1 \times 10^4 \text{ g.mol}^{-1}$, Example 1) in PBS (pH = 7.40, c = 0.9% w/v).

Solution 2: 4% solution of α,β -unsaturated aldehyde of hyaluronic acid (DS = 7%, $M_w = 2.5 \times 10^4 \text{ g.mol}^{-1}$) in PBS (pH = 7.40, c = 0.9% w/v).

- 10 Solution 3: 4% solution of aminopropoxyl derivative of hyaluronic acid (DS = 25%, $M_w = 66 \text{ kDa}$) in PBS (pH = 7.40, c = 0.9% w/v).

Hydrogels were prepared from the said solutions by mixing their equivalent volume ratios in the following combinations: solution 1 + solution 3 (sample A) and solution 2 + solution 3 (sample B). Samples A and B were left to mature at the room temperature for 3 hours. Then the mechanical properties of the materials were measured, namely Young's modulus of elasticity in compression, ultimate strength in compression, and deformation at ultimate strength (Table 2).

Table 2: Comparison of the mechanical properties of the hydrogels based on crosslinked α,β -unsaturated aldehydes derived from chondroitin sulfate and hyaluronic acid

Sample	Young's modulus of elasticity in compression (kPa)	ultimate strength in compression (kPa)	Elongation in ultimate strength (%)
A	11	30	52
B	7	58	76

The measured data indicate the advantages of the use of the material of a higher degree of substitution in the chondroitin sulfate derivative (sample A), because the hydrogels prepared from this derivative have a higher rigidity and show a lower deformation rate in comparison with the hyaluronic acid derivative (sample B). As the samples were of the same molecular

weight and were analysed under the same conditions, this fact seems to be the consequence of a higher crosslink density and it directly correlates, while maintaining the same molecular weight, with the higher degree of substitution of the α,β -unsaturated aldehyde in the structure of the modified polysaccharide.

5

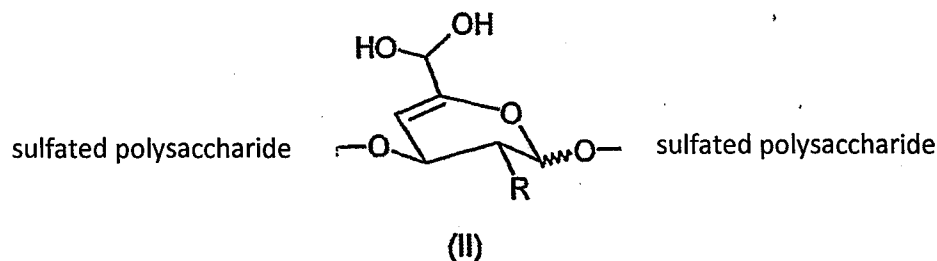
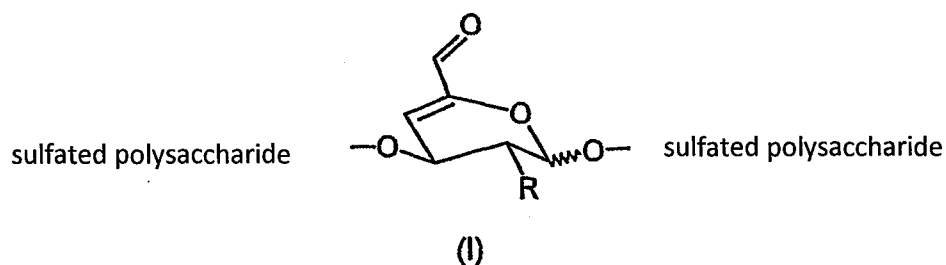
Example 32

Viability tests of 3T3 fibroblasts in the presence of α,β -unsaturated aldehyde of chondroitin sulfate

The tested substance, α,β -unsaturated aldehyde of chondroitin sulfate (DS = 20%, Mw = 40 kDa), was dissolved in a complete 3T3 medium. The solution was filtered via a 0.22 μm filter. The final test concentrations of the tested solution were 10, 100, 500, and 1000 $\mu\text{g.mL}^{-1}$. 3T3 cells of the density of 3000 cells per a well were inoculated into 96-well plates. Before the treatment, the cells were cultured for 24 hours in a complete medium. The cell viability was evaluated by spectrophotometry by means of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide (MTT method) in the intervals of 0, 24, 48, 72 hours. The whole experiment was supplemented with a set of non-treated controls and blank samples. The measured optical density data were converted into percentage formulation related to the control in the time T0 hours (the ratio of the optical density of the treated sample with respect to the optical density of the non-treated control T0, multiplied by 100) and the standard error of the mean (SEM) was calculated. The results of the test are graphically presented at Fig. 3.

CLAIMS

1. A derivative of a sulfated polysaccharide, modified with a double bond in the 4th and 5th position of the galactosamine ring in a conjugation with an aldehyde in the 6th position according to the general formula I or next to its hydrated form according to the general formula II

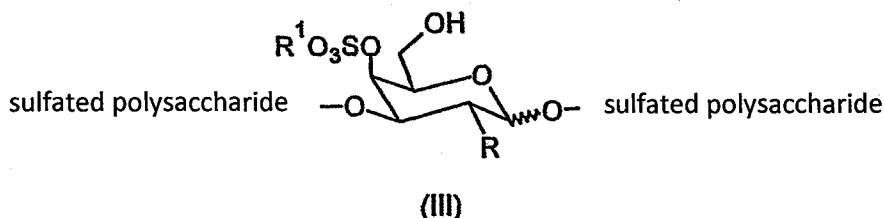


where

R is OH, O-SO₂-OH, O-SO₂-ONa or NH-Ac.

2. The derivative of a sulfated polysaccharide according to claim 1, **characterized in that** the polysaccharide is selected from the group comprising chondroitin sulfate, dermatan sulfate, carrageenan, and their pharmaceutically acceptable derivatives and/or salts.
3. The derivative of a sulfated polysaccharide according to claim 1 or 2, **characterized in that** the polysaccharide has the molecular weight within the range of 1×10^3 to 5×10^4 g.mol⁻¹ and the degree of substitution within the range of 1 to 40 %, preferably 10 to 25 %.
4. A method of preparation of the derivatives of sulfated polysaccharides as defined in claims 1 to 3, **characterized in that** the sulfated polysaccharide, that is soluble in water in its native form and that contains at least one galactopyranose unit sulfated in

the 4th position which is bound via $\alpha(1\rightarrow3)$ or $\beta(1\rightarrow3)$ *O*-glycosidic bond in the polymer chain, according to the general structural formula (III)



where

R is OH, O-SO₂-OH, O-SO₂-ONa or NH-C(O)CH₃, R¹ is H or Na, is oxidized to an aldehyde in the 6th position, and immediately after the oxidation the reaction mixture is subjected to elimination in the same reaction mixture to form the derivative of the sulfated polysaccharide defined in claims 1 to 3.

5. The method of preparation according to claim 4, **characterized in that** a sulfated polysaccharide of the molecular weight within the range of 1×10^4 to 5×10^6 g.mol⁻¹ is used.
6. The method of preparation according to claims 4 or 5, **characterized in that** a sulfated polysaccharide selected from the group comprising chondroitin sulfate, dermatan sulfate, carrageenan, and their pharmaceutically acceptable derivatives and/or salts is used.
7. The method of preparation according to any of claims 4 to 6, **characterized in that** the oxidation is performed by means of the system R³-TEMPO/NaClO, where R³ is hydrogen or N-acetyl, in water or in an aqueous solution of inorganic salts, at 5 to 25 °C.
8. The method of preparation according to claim 7, **characterized in that** the molar amount of R³-TEMPO is 0.01 to 0.2 equivalent and the molar amount of NaClO is within the range of 0.1 to 2.0 equivalents with respect to a dimer of the sulfated polysaccharide.

9. The method of preparation according to claim 7 or 8, **characterized in that** the aqueous solution of inorganic salts is an aqueous solution containing an alkaline metal salt and/or a buffer, e.g. the phosphate buffer.
- 5 10. The method of preparation according to any of claims 4 to 9, **characterized in that** the elimination proceeds at 5 to 25 °C, and without the isolation of the saturated C6-aldehyde in the galactopyranose subunit sulfated in the 4th position.
- 10 11. A method of modification of the derivative defined in any of claims 1 to 3, **characterized in that** the derivative reacts with an amine according to the general formula R^2-NH_2 , where R^2 is an alkyl, aryl, hetaryl, linear, or branched C_1 to C_{30} chain, optionally containing N, S, or O atoms.
- 15 12. The method of modification according to claim 11, **characterized in that** the amine is a biologically active amine, mainly an amino acid or a peptide.
- 20 13. The method of modification of the derivative according to claim 11, **characterized in that** the derivative reacts with an amine which is a biologically acceptable polymer with a free amino group.
- 25 14. The method of modification according to claim 13, **characterized in that** the amino group is a direct part of the polymer selected from the group comprising gelatine, chitosan, deacetylated hyaluronic acid, and deacetylated chondroitin sulfate.
- 30 15. The method of modification according to claim 13, **characterized in that** the amino group is bound to the polymer via a linker containing an amino, hydrazine, hydrazide, aminoalkoxy, hydroxyl, carboxyl, thiol group, or any combination thereof.
- 35 16. The method of modification according to any of claims 11 to 15, **characterized in that** the amount of the amine, amino acid, peptide, or free amino groups of the polymer is within the range of 0.05 to 3.0 equivalents with respect to a dimer of the sulfated polysaccharide.
17. The method of modification according to any of claims 11 to 16, **characterized in that** the reaction with the amine, amino acid, peptide, or polymer containing a free

amino group proceeds in water, phosphate buffer, or water-organic solvent system, at the temperature within the range of 20 to 60 °C, for 10 min to 150 h.

18. The method of modification according to claim 17, **characterized in that** the organic solvent is selected from the group comprising water miscible alcohols, in particular isopropanol or ethanol, and water miscible polar aprotic solvents, in particular dimethyl sulfoxide, wherein the water content in the mixture is at least 50% vol.

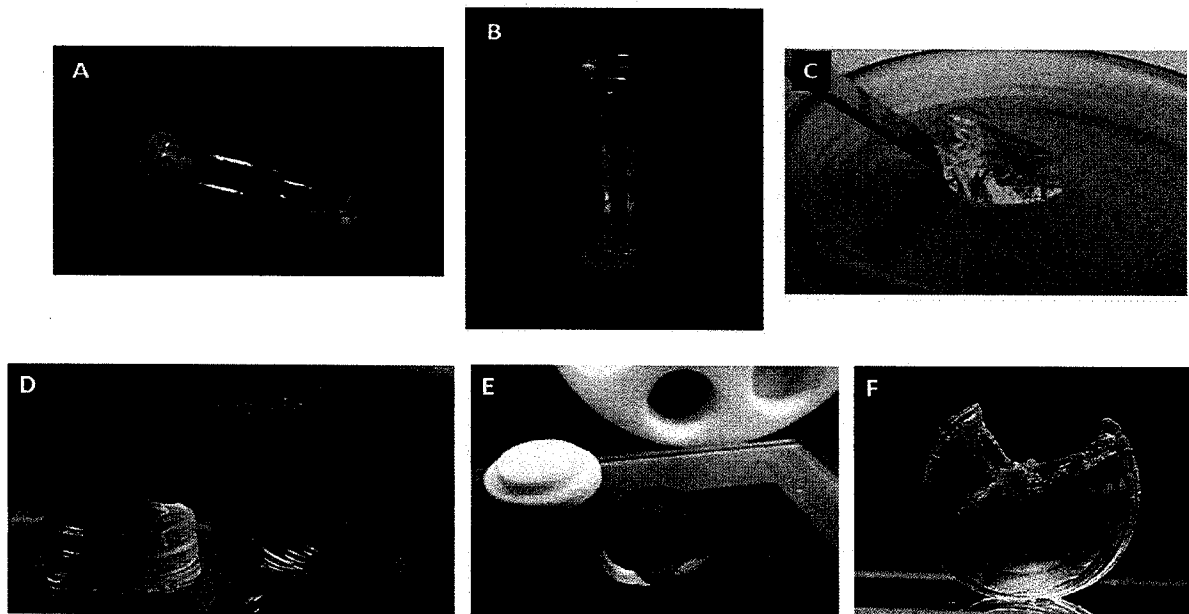
19. The method of modification of the derivative according to any of claims 1 to 3, **characterized in that** the derivative reacts with a water soluble biocompatible bi-and polyfunctional N-nucleophile selected from the group comprising alkyl amines, aryl amines, heteroalkyl amines, hetaryl amines, amino acids, peptides, polymers with a free amino group, hydrazines, hydroxylamines, hydrazides, semicarbazides, or thiosemicarbazides, wherein crosslinking of the derivative occurs.

20. The method of modification according to claim 19, **characterized in that** the N-nucleophile is hydrazide, deacetylated polysaccharide, or alkoxyamine, and in that the reaction proceeds in phosphate buffer.

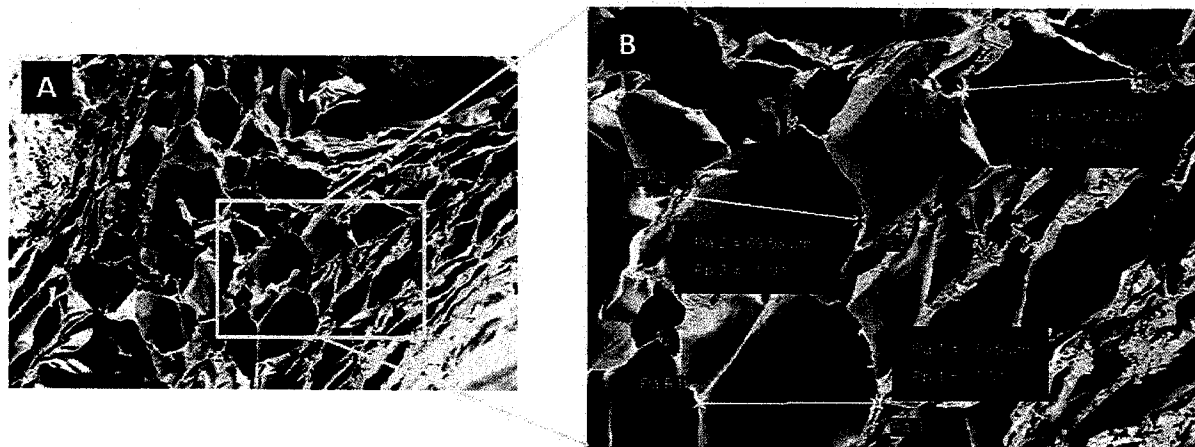
21. The use of the derivatives defined in any of the claims 1 to 3 as the carriers of biologically active substances with their controlled release by means of the change of pH value, in the field of cosmetics and pharmacy.

22. The use of the derivatives modified by the method as defined in any of claims 11 to 20 as biocompatible materials for biomedical applications and formation of scaffolds for tissue engineering, or for regenerative medicine.

1/2

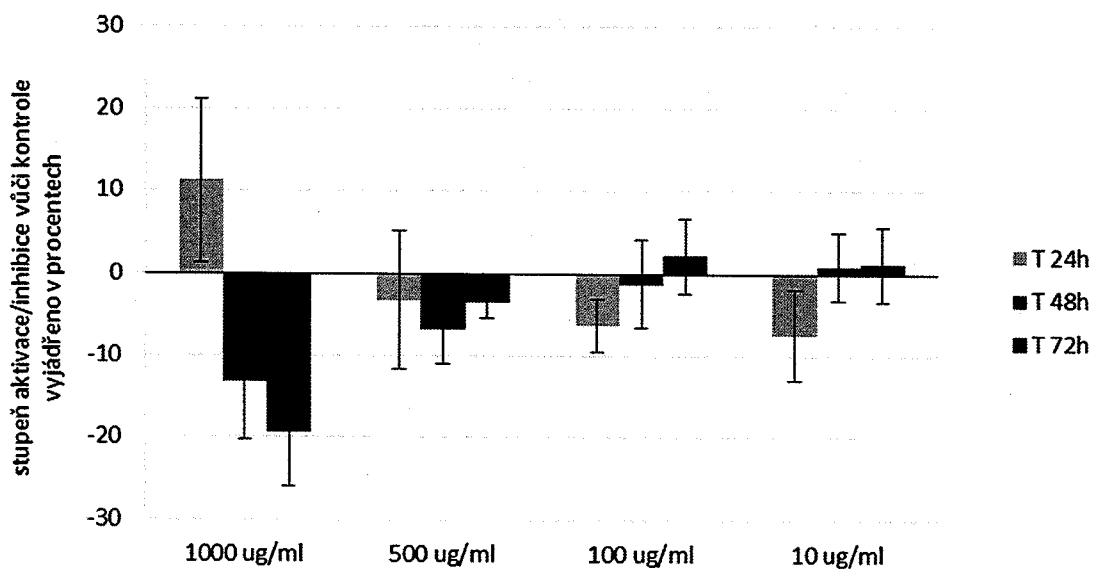


Obr. 1

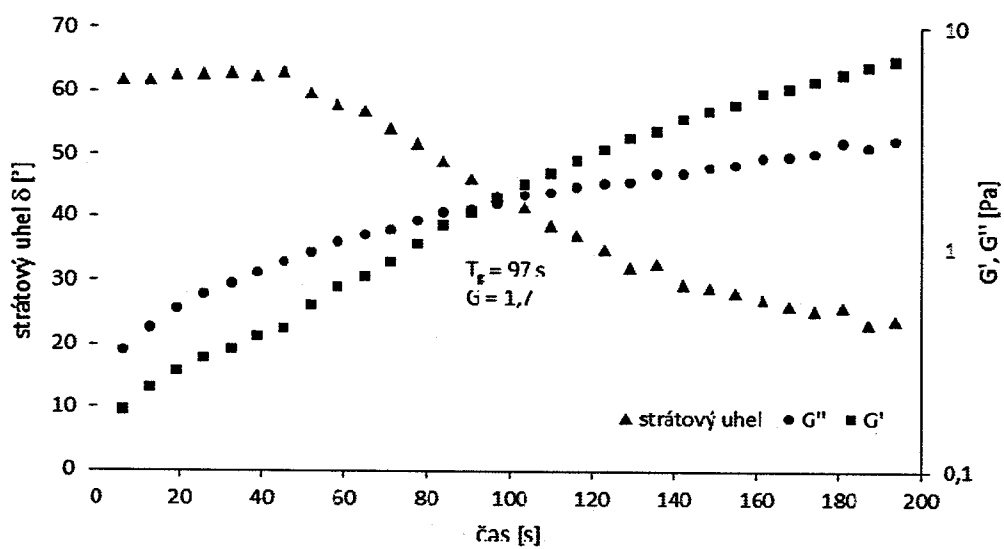


Obr. 2

2/2



Obr. 3



Obr. 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2016/000071

A. CLASSIFICATION OF SUBJECT MATTER

INV. C08B37/00 A61K31/726 C08G81/00 C08J3/24
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C08B A61K C08G C08J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EP0-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2014/023272 A1 (CONTIPRO BIOTECH SRO [CZ]) 13 February 2014 (2014-02-13) cited in the application pages 2,3,7,8 -----	1-22



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

30 September 2016

Date of mailing of the international search report

10/10/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Friedrich, Christof

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/CZ2016/000071

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014023272 A1	13-02-2014	CZ 304512 B6	11-06-2014
		EP 2882779 A1	17-06-2015
		JP 2015525820 A	07-09-2015
		KR 20150040966 A	15-04-2015
		US 2015175717 A1	25-06-2015
		WO 2014023272 A1	13-02-2014
