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(54) Benævnelse: **Oxideret derivat af hyaluronansyre, en fremgangsmåde til fremstilling deraf og en fremgangsmåde til modifikation deraf**

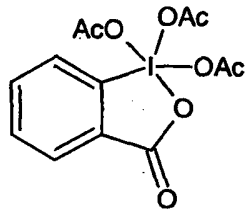
(56) Fremdragne publikationer:
JIANG B ET AL: "Study on TEMPO-mediated selective oxidation of hyaluronan and the effects of salt on the reaction kinetics", CARBOHYDRATE RESEARCH, PERGAMON, GB, vol. 327, no. 4, 7 August 2000 (2000-08-07), pages 455-461, XP004213357, ISSN: 0008-6215, DOI: DOI:10.1016/S0008-6215(00)00059-8
ANGELIN ET AL.: "Direct, Mild, and Selective Synthesis of Unprotected Dialdo-Glycosides", EUR. J. ORG. CHEM., 1 January 2006 (2006-01-01), pages 4323-4326, XP002637227, DOI: 10.1002/ejoc.200600288 cited in the application
CORNWELL ET AL: "A one-step synthesis of cyclodextrin monoaldehydes", TETRAHEDRON LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 36, no. 46, 13 November 1995 (1995-11-13), pages 8371-8374, XP005270626, ISSN: 0040-4039, DOI: DOI:10.1016/0040-4039(95)01808-U cited in the application
DING B ET AL: "TEMPO-mediated selective oxidation of substituted polysaccharides-an efficient approach for the determination of the degree of substitution at C-6", CARBOHYDRATE RESEARCH, PERGAMON, GB, vol. 343, no. 18, 8 December 2008 (2008-12-08), pages 3112-3116, XP025625802, ISSN: 0008-6215, DOI: DOI:10.1016/J.CARRES.2008.09.005 [retrieved on 2008-09-10]

Fortsættes ...

Description

Fieldof the Invention

[0001] The present invention relates to a method of preparation of a new hyaluronic acid derivative containing an aldehydic group -CH=O instead of the primary hydroxyl group -CH_2-OH. The oxidation can be performed by use of DMP (Dess-Martin Periodinane) agent in polar aprotic solvents,

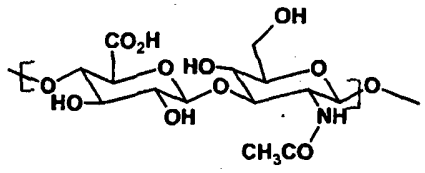


DMP Dess-Martin periodinane

where the solvent includes for example dimethylsulfoxide.

Background of the Invention

[0002] Hyaluronic acid is an important polysaccharide, composed of two repeating units of β--(1,3)-D-glucuronic acid and β-(1,4)-*N*-acetyl-D-glucosamine. The molecular weight, depending on the method its isolation and on the source material, is within the range from 5.10^4 to 5.10^6 $g.mol^{-1}$. Hyaluronic acid or its sodium salt hyaluronan, is an essential component of connective tissues, synovial fluid of joints, and plays an important role in biological processes such as hydratation, organization of proteoglycans, cells differentiation, proliferation and angiogenesis. Hyaluronic acid is a considerably hydrophilic polysaccharide that is water-soluble in the form of its salt within the whole range of pH.



Scheme 1 Hyaluronic Acid

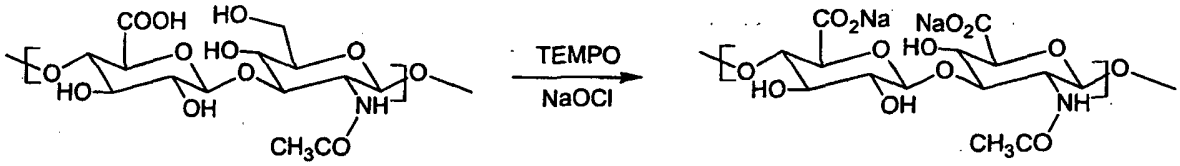
Oxidation of hyaluronic acid

[0003] Oxidation of polysaccharides is a process in which the oxidation degree of the polysaccharide functional groups is changing. Most frequently, the carboxylic acids or aldehydes are formed, which can dramatically change the properties of the polysaccharide. In most cases, the reaction is performed by use of agents containing atoms in higher oxidation degrees.

[0004] The method of selective oxidation of saccharides on the primary hydroxyl group, described in Angelino, European Journal of Organic Chemistry 2006, 19, 4323 - 4326, the system of 2,2,6,6-tetramethyl-1-piperidinyloxyl radical TEMPO / TCC in DMF at the temperature of 0°C was used, with the corresponding aldehyde as the main product.

[0005] The oxidation of cyclodextrin to monoaldehyde was described by Cornwell, Tetrahedron Letters 1995, 36 (46), 8371 - 8374. The oxidation was performed by adding Dess-Martin periodinane (DMP) in DMSO or in DMF at the temperature of 20°C as an oxidizing agent.

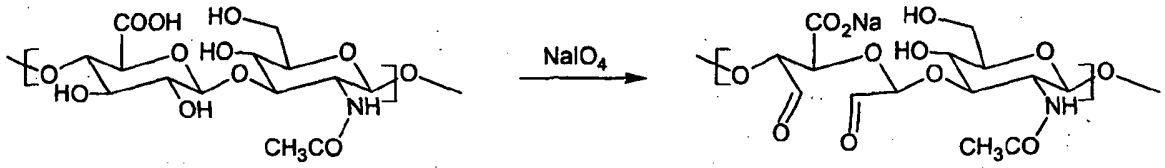
[0006] 2,2,6,6-tetramethyl-1-piperidinyloxyl radical (TEMPO)- and NaOCl-mediated oxidation of the primary hydroxyl group of hyaluronan to a carboxylic acid was performed at pH 10.2 and at the temperature of 0°C (Scheme 2) (Carbohydr Res 2000, 327 (4), 455-61).



Scheme 2 Oxidation to a carboxylic acid

Analogous to other polysaccharides, a high regioselectivity and slight degradation of the polymer were observed. An increase of the concentration of the salt (NaBr, NaCl, Na_2SO_4) in the solution caused a decrease in the oxidation rate.

[0007] Oxidation of hyaluronan by use of TEMPO/NaClO system was described in the patent application WO 02/18448 A2. The authors also dealt with interactions of percarboxylated polysaccharides, while forming biological complexes.



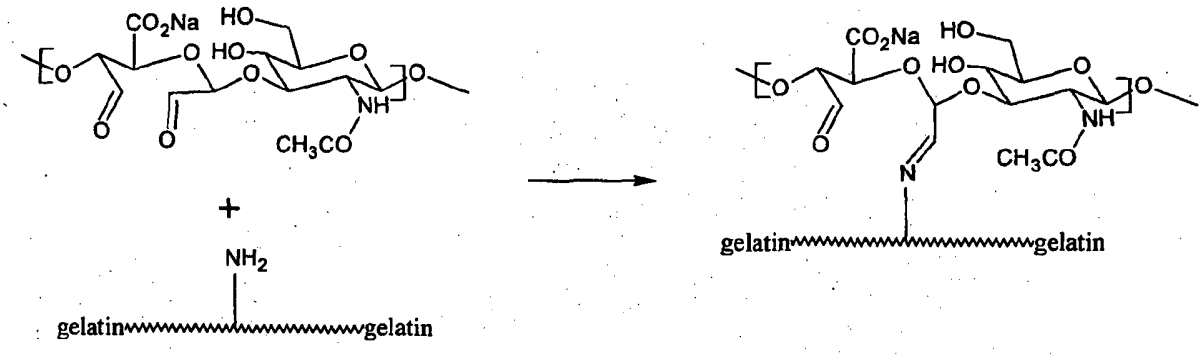
Scheme 3 Oxidation to a dialdehyde

The rate of oxidation of HA and other polysaccharides by use of sodium periodate was studied by Scott et al. (Scheme 3) (Histochemie 1969, 19(2), 155-61). The factors such as the chain length, substitution, polymer configuration and temperature were studied and quantified. The use of $NaIO_4$ for an oxidation of hyaluronan was also disclosed in the patents Nos. US 6 683 064 and US 6 953 784.

[0008] Model reactions of HA analogues with low molecular weight in a physiological buffer were studied (Carbohydr Res 1999, 321, (3-4), 228-34). Oxidation products of the glucuronic and glucosamine parts were identified by GC-MS analysis. The results also suggest that the oxidation is performed primarily on the glucuronic part, while the meso-tartaric acid is the main product and may be used as a biomarker of the hyaluronan oxidation.

3.4.2 Use of an oxidized HA in cross-linking reactions

[0009] The use of an oxidized HA for the preparation of cross-linked hydrogels was described by Weng et al. (Scheme 4), J Biomed Mater Res A 2008, 85 (2), 352-65. Two precursors were used in this case: a partially oxidized hyaluronan and gelatin:

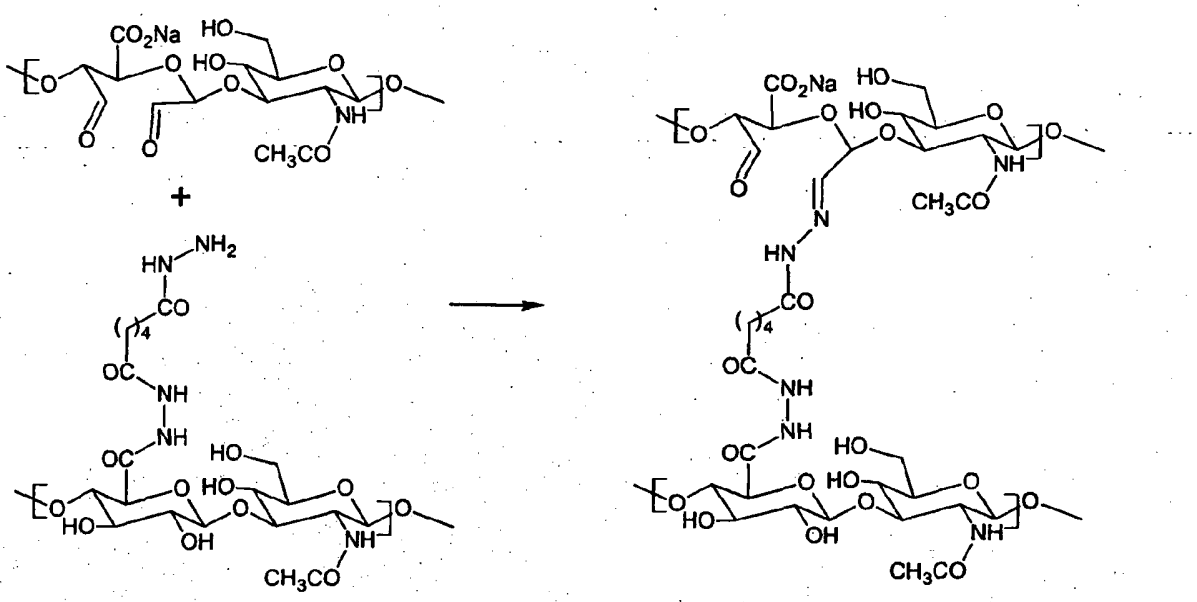


Scheme 4 The cross-linking reaction of the oxidized hyaluronan and gelatin

The physico-chemical properties of the resulting hydrogels have been elucidated by instrumental analyses FT-IR, SEM (scanning electron microscopy) and rheometry. Increasing the oxidation degree of the hyaluronan lead to a corresponding increase of hydrogels compatibility and decrease of water absorption capacity. Dermal fibroblasts were used to study the cell-hydrogel interactions. Both the hydrogels and their degradation products are biocompatible, as proved by the long-term cell viability assay. When cultured with cells, the hydrogel underwent a degradation within 4 weeks, with an obvious loss of cohesiveness. The good biocompatibility and biodegradability was further demonstrated in mice sub-dermal implantations. Lastly, *in vitro* and *in vivo* depositions of extracellular matrix in hydrogels were demonstrated by SEM analysis.

[0010] The method of preparation of cross-linked HA from an oxidized hyaluronan and gelatin by a water-in-oil-emulsion method, where a 3-dimensional hydrogel is formed in the absence of any external cross-linker, was described in the publication of Weng et al., Biomaterials 2008, 29, (31), 4149-56. In this work, incorporation of model drugs into the hydrogel structure (encapsulation) and their releasing through macrophages were studied by HPLC.

[0011] The preparation of elastic hydrogels by coupling the HA oxidized to HA-aldehyde by means of sodium periodate and the HA modified with adipic acid dihydrazide, was described by Sahiner et al., (Scheme 5), J. Biomater. Sci. Polym. Ed 2008, 19 (2), 223-43.

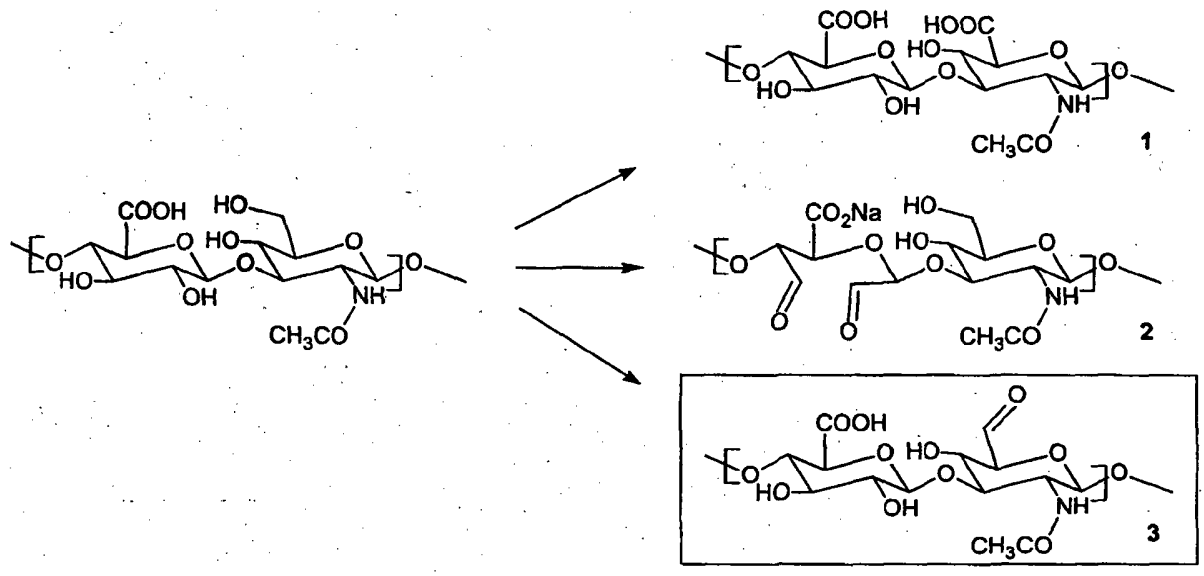


Scheme 5 Preparation of the cross-linked hyaluronan

The resulting derivatives did not have any observable effect on the proliferation of the cultured fibroblasts, as shown by a MTT assay.

Summary of the Invention

[0012] The present invention relates to a method of selective oxidation of the primary hydroxyl group of hyaluronic acid in position 6 of the glucosamine part of the polysaccharide to aldehyde. The reaction is performed in an aprotic environment by use of Dess-Martin periodinane DMP as an oxidizing agent. The presented procedure is original in that it introduces an aldehydic group to the position 6 of the hyaluronan glucosamine part (scheme 6, structure **3**). Approaches published up to date have introduced either an aldehydic group to the position 2 and 3 of the hyaluronan glucuronic part, while opening the saccharide ring (scheme 6, structure 2), or a carboxyl group to the position 6 of the hyaluronan glucosamine part (scheme 6, structure **1**).



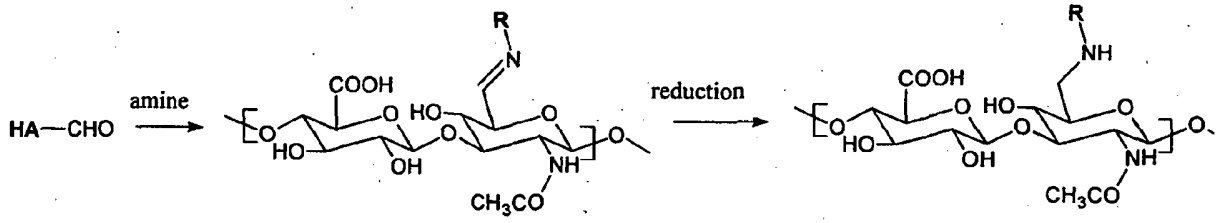
Scheme 6 Hyaluronan oxidation, reaction products

[0013] The method according to the invention is more advantageous in that the respective oxidation product (structure **3**, scheme 6) maintains the structure of conjugated saccharide rings. Ring opening in the product oxidized to dialdehyde (structure **2**, scheme 6) gives rise to the chain linearity "breakage" and, therefore, a significant change of the polysaccharide 3-dimensional structure compared to the non-modified hyaluronan. Although in the product oxidized to a carboxylic acid (structure 1, scheme 6) the chain linearity "breakage" does not occur, the carboxyl group does not enable such various modification (binding) possibilities like the aldehydic group. As the carboxyl group is a component of the non-modified polysaccharide already, the oxidation to the structure **1** (scheme 6) brings only an increase of the polysaccharide polarity, not a development of new quality utilizable for binding new substitutes.

[0014] It is known that an aldehydic group with a bound alkyl group exists in water in the so-called geminal diol form HA-CH$(OH)_2$, which reacts with nucleophiles similarly as aldehydes do. In aqueous solutions, more than 95% of the hyaluronan oxidized in the position 6 of the glucosamine part (product **3,** scheme 6) exists in the form of a geminal diol, as demonstrated by NMR spectroscopy.

[0015] In the method according to the present invention, the hyaluronic acid is dissolved in polar aprotic solvents, e.g. DMSO, then an oxidizing agent is added and the mixture is stirred at the temperature of 10 to 50°C, preferably at 20°C, for at least 5 minutes, preferably for 1 to 150 hours, more preferably for at least 10 hours.

[0016] The prepared oxidized hyaluronan can be used for binding of compounds containing for example amino group. The binding can be realized either in an imine form or after the reduction in an amine form (reductive amination) (scheme 7):



Scheme 7 Binding of amines to the oxidized hyaluronan

[0017] Both degrees of this modification are performed in an aqueous solution, the reduction is performed by means of $NaBH_3CN$. Both degrees of the reaction described in scheme 7 can be performed in one step.

[0018] The modification of the hyaluronic acid derivative can be performed by a reaction of the oxidized derivative with an amine of the general formula H_2N-R or with a hyaluronan substituted by an -R-NH_2 group, wherein R is an alkyl, linear or branched chain C1- C30, optionally containing aromatic or heteroaromatic groups. This amine can be an

alkylamine, e.g. butylamine or hexanediamine, amino acid, peptide or polysaccharide containing a free amino group. In case of using a diamine or compounds containing three or more amino groups, cross-linked hyaluronan derivatives can be prepared. The cross-linked derivatives can be prepared also by the reaction of an aldehyde with a hyaluronan substituted by an aminoalkyl group HA-alkyl-NH_2.

Detailed description of the preferred embodiments of the invention

[0019]

DS = substitution degree = 100% * the molar amount of the bound substitute / the molar amount of all the polysaccharide dimers.

The term "equivalent (eq)" as used herein, refers to a hyaluronic acid dimer, unless otherwise indicated: The percentages as used herein mean a percentage by weight, unless otherwise indicated..
The molecular weight of the starting hyaluronan (source: CPN spol. s r.o., Dolni Dobrouc, CZ) was determined by the SEC-MALLS assay.

Example 1 Oxidation of the hyaluronic acid by DMP.

[0020] DMP solution (0.2 eq) in DMSO (1 ml) was added to a 1% solution of hyaluronic acid (0.1g, 20 kDa) in DMSO. The mixture was stirred for 24 hours at the temperature of 20°C. The solution was then diluted to 0.1% and dialyzed against the mixture (0.1% NaCl, 0.1% $NaHCO_3$) 3 x 5 litres (once per day) and against distilled water 7 x 5 liters (twice per day). The resulting solution was evaporated and analyzed.
DS 10 % (determined by NMR)

^{1}H NMR(D_2O)	δ 5.26 (s, 1H, polymer-C*H*$(OH)_2$)
HSQC (D_2O)	cross signal 5.26 ppm (^{1}H) - 90 ppm (^{13}C) (polymer-*CH*$(OH)_2$)
FT-IR (KBr)	1740 cm^{-1} -CH=O

Example 2 Oxidation of the hyaluronic acid by DMP.

[0021] DMP solution (0.2 eq) in DMSO (1 ml) was added to a 0.5% solution of hyaluronic acid sodium salt (0.1g, 600 kDa) in DMSO. The mixture was stirred for 24 hours at the temperature of 20°C. The solution was then diluted to 0.1% and dialyzed against the mixture (0.1 % NaCl, 0.1 % $NaHCO_3$) 3 x 5 liters (once per day) and against distilled water 7 x 5 liters (twice per day). The resulting solution was evaporated and analyzed.
DS 10 % (determined by NMR, for details see Example 1)

Example 3 Oxidation of the hyaluronic acid by DMP.

[0022] DMP (1 eq) solution in DMSO (1 ml) was added to a 1% solution of hyaluronic acid (0.1g, 20 kDa) in DMSO. The mixture was stirred for 24 hours at the temperature of 20°C. The solution was then diluted to 0.1 % and dialyzed against the mixture (0.1% NaCl, 0.1% $NaHCO_3$) 3 x 5 liters (once per day) and against distilled water 7 x 5 liters (twice per day). The resulting solution was evaporated and analyzed.
DS 50 % (determined by NMR, for details see Example 1)

Example 4 Oxidation of the hyaluronic acid by DMP.

[0023] DMP solution (1 eq) in DMSO (1 ml) was added to a 1% solution of hyaluronic acid (0.1g, 20 kDa) in DMSO. The mixture was stirred for 1 hour at the temperature of 20°C. The solution was then diluted to 0.1 % and dialyzed against mixture (0.1 % NaCl, 0.1 % $NaHCO_3$) 3 x 5 liters (once per day) and against distilled water 7 x 5 liters (twice per day). The resulting solution was evaporated and analyzed.
US 30 % (determined by NMR, for details see Example 1)

Example 5 Oxidation of the hyaluronic acid by DMP.

[0024] DMP solution (0.2 eq) in DMSO (1 ml) was added to a 1% solution of hyaluronic acid (0.1 g, 20 kDa) in DMSO. The mixture was stirred for 1 hour at the temperature of 50°C. The solution was then diluted to 0.1% and dialyzed against the mixture (0.1 NaCl, 0.1% $NaHCO_3$) 3 x 5 liters (once per day) and against distilled water 7 x 5 liters (twice per day). The resulting solution was evaporated and analyzed.
DS 10 % (determined by NMR, for details see Example 1)

Example 6 Reaction of the oxidized hyaluronan with an amine

[0025] An aqueous solution of oxidized hyaluronic acid (1%) (0.1 g, oxidation degree DS=30 %, Example 4) was mixed with butylamine (0.4 eq). The mixture was stirred for 24 hours at the temperature of 20°C. The solution was then precipitated by a double amount of acetone and 0.1 ml of a saturated aqueous solution of NaCl, filtrated and dried in vacuum. The resulting yellow material was then analyzed.

UV-VIS 328nm, $n \rightarrow \pi^*$ transition -CH=N-

Example 7 Reaction of the oxidized hyaluronan with butylamine and the subsequent reduction

[0026] An aqueous solution of oxidized hyaluronic acid (1%) (0.1 g, oxidation degree DS=50 %, Example 3) was mixed with butylamine (0.4 eq). The mixture was stirred for 24 hours at the temperature of 20°C. The solution was then mixed with $NaBH_3CN$ (3 eq) in 0.5 ml of water. The mixture was stirred for 24 hours at the temperature of 20°C. The solution was then diluted to 0.1% and dialyzed against the mixture (0.1 % NaCl, 0.1 % $NaHCO_3$) 3 x 5 liters (once per day) and against distilled water 7 x 5 liters (twice per day). The resulting solution was evaporated and analyzed.
DS 35 % (determined by NMR)

^{1}H NMR (D_2O)	δ 3.05 (m, 2H, polymer-CH_2-NH-CH_2-), 1.60 (m, 2H, polymer-CH_2--NH-CH_2-CH_2-), 1.35 (m, 2H, polymer-CH_2NH-CH_2-CH_2-CH_2-), 0.85 (m, 3H, -CH_2-CH_3)
DOSY NMR (D_2O)	logD (0.85 ppm, -CH_2-CH_3) ~ -10.3 m^2/s
	logD (1.35 ppm, polymer-CH_2-NH-CH_2-CH_2-CH_2-)~-10.3 m^2/s
	logD (1.60 ppm, polymer-CH_2-NH-CH_2-CH_2-)~-10.3 m^2/s
	logD (3.05 ppm, polymer-CH_2-NH-CH_2-)~-10.3 m^2/s
	logD (2.03 ppm, CH_3-CO-NH-polymer)~-10.3 m^2/s
	logD (H_2O)~-8.6 m^2/s

Example 8 Reaction of the oxidized hyaluronan with a diamine and the subsequent reduction

[0027] An aqueous solution of the oxidized hyaluronic acid (1%) (0.1 g, oxidation degree DS=50%, Example 3) was mixed with hexanediamine (0.4 eq). The mixture was stirred for 24 hours at the temperature of 20°C. The solution was then mixed with $NaBH_3CN$ (3 eq) in 0.5 ml of water. The mixture was stirred for 24 hours at the temperature of 20°C. The solution was then diluted to 0.1% and dialyzed against the mixture (0.1% NaCl, 0.1% $NaHCO_3$) 3 x 5 liters (once per day) and against distilled water 7 x 5 liters (twice per day). The resulting solution was evaporated and analyzed.
DS 35 % (determined by NMR)

^{1}HNMR(D_2O)	δ 3.12 (m, 2H, polymer-CH_2-NH-CH_2-), 3.02 (m, 2H, -CH_2-NH_2), 1.7 (m, 4H, -NH-CH_2-CH_2-CH_2-CH_2-CH_2), 1.45 (m, 4H, -NH-CH_2--CH_2-CH_2-CH_2-CH_2-)
DOSY NMR (D_2O)	logD (1.45 ppm, -NH-CH_2-CH_2-CH_2-CH_2-CH_2-)~-10.5 m^2/s
	logD (1.7 ppm, -NH-CH_2-CH_2-CH_2-CH_2-CH_2-)~-10.5 m^2/s
	logD (3.02 ppm, -CH_2-NH_2)~-10.5 m^2/s
	logD (2.03 ppm, CH_3-CO-NH-polymer)~-10.5 m^2/s
	logD (H_2O)~-8.7 m^2/s

Example 9 Reaction of oxidized hyaluronan with amino-hyaluronan

[0028] An aqueous solution of the oxidized hyaluronic acid (1%) (0.1 g, oxidation degree DS=30 %, Example 4) was mixed with a 1% aqueous solution of hyaluronan derivative substituted by hexanediamine (1 eq, DS=35 %, Example 8) at the temperature of 20°C. The insoluble compact gummy precipitate, obtained after several minutes, was mechanically ground to small pieces that were filtrated and dried under reduced pressure.
FT-IR (KBr) 1700 cm^{-1}

Example 10 Reductive amination of the oxidized hyaluronan with lysine

[0029] An aqueous solution of the oxidized hyaluronic acid (1%) (0.1 g, oxidation degree DS=30 %, Example 4) was mixed with lysine (0.3 eq). The mixture was stirred for 24 hours at the temperature of 20°C. The solution was then mixed with $NaBH_3CN$ (3 eq) in 0.5 ml of water. The mixture was stirred for 24 hours at the temperature of 20°C. The solution was diluted to 0.1% and dialyzed against the mixture (0.1% NaCl, 0.1% $NaHCO_3$) 3 x 5 liters (once per day) and against distilled water 7 x 5 liters (twice per day). The resulting solution was evaporated and analyzed.
DS 25 % (determined by NMR).
^{1}H, HSQC, DOSY NMR (2% NaOD/D_2O): δ 1.33 (m, 2H, -CH-CH_2-C*H*$_2$-), 1.48 (m, 2H, -CH-CH_2-CH_2-C*H*$_2$-), 1.55 (m, 1H, -CH-C*H*H-), 1.63 (m, 1H, -CH--CH*H*-), 2.62 (m, 2H, -CH-CH_2-CH_2-CH_2-C*H*$_2$-), 2.65 (m, 1H, polymer-C*H*H-NH-), 2.99 (m, 1H, polymer-CH*H*-NH-), 3.16 (m, 1H, -C*H*-CK_2-).

Example 11 Reductive amination of the oxidized hyaluronan with lysine

[0030] An aqueous solution of the oxidized hyaluronic acid (1%) (0.1 g, oxidation degree DS=30 %, Example 4) was mixed with lysine (20 eq). The mixture was stirred for 24 hours at the temperature of 20°C. The solution was then mixed with $NaBH_3CN$ (10 eq) in 0.5 ml of water. The mixture was stirred for 24 hours at the temperature of 20°C. The solution was diluted to 0.1 % and dialyzed against the mixture (0.1 % NaCl, 0.1 % $NaHCO_3$) 3 x 5 liters (once per day) and against distilled water 7 x 5 liters (twice per day). The resulting solution was then evaporated and analyzed.
DS 28 % (determined by NMR, Example 10)

Example 12 Reductive amination of the oxidized hyaluronan with serine

[0031] An aqueous solution of the oxidized hyaluronic acid (1%) (0.1 g, oxidation degree DS=30 %, Example 4) was mixed with serine (0.3 eq). The mixture was stirred for 1 min at the temperature of 20°C. The solution was then mixed with $NaBH_3CN$ (3 eq) in 0.5 ml of water. The mixture was stirred for 24 hours at the temperature of 20°C. The solution was diluted to 0.1% and dialyzed against the mixture (0.1% NaCl, 0.1% $NaHCO_3$) 3 x 5 liters (once per day) and against distilled water 7 x 5 liters (twice per day). The resulting solution was evaporated and analyzed.
DS 26 % (determined by NMR)
^{1}H, HSQC, DOSY NMR (2% NaOD/D_2O): δ 2.74 (m, 1H, polymer-C*H*H-NH-), 3.08 (m, 1H, polymer-CH*H*-NH-), 3.21. (m, 1H, -C*H*-CH_2-OH), 3.72 (m, 1H, -CH-C*H*H-OH), 3.78 (m, 1H, -CH-CH*H*-OH).

Example 13 Reductive amination of the oxidized hyaluronan with arginine

[0032] An aqueous solution of the oxidized hyaluronic acid (1%) (0.1 g, oxidation degree DS=30 %, Example 4) was mixed with arginine (0.3 eq). The mixture was stirred for 100 hours at the temperature of 20°C. The solution was then mixed with $NaBH_3CN$ (3 eq) in 0.5 ml of water. The mixture was stirred for 24 hours at the temperature of 20°C. The solution was diluted to 0.1 % and dialyzed against the mixture (0.1 % NaCl, 0.1 % $NaHC0_3$) 3 x 5 liters (once per day) and against distilled water 7 x 5 liters (twice per day). The resulting solution was evaporated and analyzed.
DS 23 % (determined by NMR)
^{1}H, HSQC, DOSY NMR (2% NaOD/D_2O): δ 1.61 (m, 2H, -CH-CH_2-C*H*$_2$-), 1.63 (m, 1H, -CH-CH*H*-), 1.70 (m, 1H, -CH-CH*H*-), 2.65 (m, 1H, polymer-C*H*H--NH-), 3.01 (m, 1H, polymer-CH*H*-NH-), 3.13 (m, 1H, -C*H*-CH_2-), 3.21 (m, 2H, -CH-CH_2-CH_2-C*H*$_2$-).

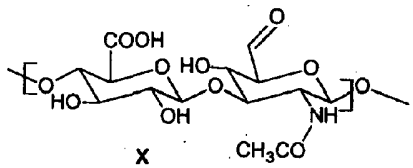
Example 14 Reductive amination of the oxidized hyaluronan with pentapeptide PAL-KTTKS (palmytoyl-Lys-Thr-Thr-Lys-Ser)

[0033] A solution of the oxidized hyaluronic acid (1%) (0.1 g, oxidation degree DS=10 %, Example 1) in a water/isopropanol system 2/1 was mixed with a 1% solution of substituted pentapeptide PAL-KTTKS (0.05 eq) in isopropyl alcohol. The mixture was stirred for 24 hours at the temperature of 20°C. The solution was then mixed with $NaBH_3CN$ (3 eq) in

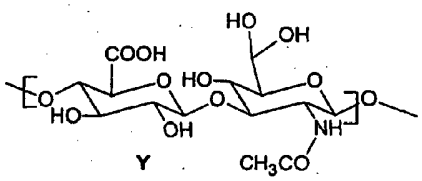
0.5 ml of water. The mixture was stirred for 24 hours at the temperature of 20°C. The solution was diluted to 0.1 % and dialyzed against the mixture (0.1 % NaCl, 0.1 % $NaHCO_3$) 3 x 5 liters (once per day) and against distilled water 7 x 5 liters (twice per day). The resulting solution was evaporated and analyzed.
DS 8 % (determined by NMR)
^1H, HSQC, DOSY NMR (2% NaOD/D_2O): δ 1.61 (m, 2H, -CH-CH_2-CH_2-), 1.63 (m, 1H, -CH-CH*H*), 1.70 (m, 1H, -CH-CH*H*-), 2.65 (m, 1H, polymer-C*H*H--NH-), 3.01 (m, 1H, polymer-CH*H*-NH-), 3.13 (m, 1H, -C*H*-CH_2-), 3.21 (m, 2H, -CH-CH_2-CH_2-CH_2-).

KRAV

1. Hyaluronsyrederivat, oxideret ved glucosamindelens position 6 til et aldehyd ifølge formlen **X**, og dets hydrerede form, den såkaldte geminaldiol ifølge formlen **Y**:



X



Y

2. Fremgangsmåde til fremstilling af hyaluronsyrederivatet ifølge krav 1, hvori hyaluronsyren reagerer med Dess-Martin periodinan (DMP) i en polær aprotisk solvent, særligt i dimethylsulfoxid.

3. Fremgangsmåde til fremstilling af hyaluronsyrederivatet ifølge krav 2, hvori hyaluronsyren er i formen af en frie syre eller et salt.

4. Fremgangsmåde til fremstilling af hyaluronsyrederivatet ifølge ethvert af kravene 2 eller 3, hvori hyaluronsyrens molekylevægt er inden for intervallet fra $1 \cdot 10^4$ til $5 \cdot 10^6$ g·mol^{-1}.

5. Fremgangsmåde til fremstilling af hyaluronsyrederivatet ifølge ethvert af kravene 2 til 4, hvori hyaluronsyrens reaktion med Dess-Martin periodinan (DMP) udføres ved temperaturen inden for intervallet fra 10 til 50 °C, fortrinsvis ved 20 °C, i 5 minutter til 150 timer, fortrinsvis i mindst 10 timer.

6. Fremgangsmåde til fremstilling af hyaluronsyrederivatet ifølge ethvert af kravene 2 til 5, hvori DMP er til stede i en mængde inden for intervallet fra 0,05 til 1 ækvivalent i forhold til hyaluronsyredimeren.

7. Fremgangsmåde til modifikation af hyaluronsyrederivatet fremstillet ved anvendelse af fremgangsmåden ifølge ethvert af kravene 2 til 6, hvori det oxiderede hyaluronsyrederivat reagerer med en amin med den generelle formel H_2N-R eller med en hyaluronan substitueret med en –R-NH_2 gruppe, hvori R er en alkyl, lineær eller forgrenet kæde C_1 – C_{30}, valgfrit indeholdende aromatiske eller heteroaromatiske grupper.

8. Fremgangsmåde til modifikation ifølge krav 7, hvori det oxiderede hyaluronansyrederivat reagerer med en aminosyre.

9. Fremgangsmåde til modifikation ifølge krav 7, hvori det oxiderede hyaluronansyrederivat reagerer med et peptid.

10. Fremgangsmåde til modifikation ifølge krav 7, hvori det oxiderede hyaluronansyrederivat reagerer med et polysaccharid indeholdende en fri aminogruppe.

11. Fremgangsmåde til modifikation ifølge ethvert af kravene 7 til 10, hvori mængden af aminen, aminosyren, peptidet eller polysaccharidet er inden for intervallet fra 0,05 til 10 ækvivalenter i forhold til hyaluronandimeren.

12. Fremgangsmåde til modifikation ifølge ethvert af kravene 7 til 11, hvori det oxiderede hyaluronsyrederivats reaktion med aminen, aminosyren, peptidet eller polysaccharidet udføres i vand eller i et vand-organisk solventsystem ved temperaturen inden for intervallet fra 0 til 80°C, fortrinsvis ved 20°C, i 1 minut til 24 timer, fortrinsvis i 1 time.

13. Fremgangsmåde til modifikation ifølge krav 12, hvori det oxiderede hyaluronsyrederivats reaktion med aminen, aminosyren, peptidet eller polysaccharidet udføres i tilstedeværelsen af $NaBH_3CN$ som et reduktionsmiddel, der tilsættes reaktionsblandingen i tiden 0 til 100 timer efter tilsætningen af aminen, aminosyren, peptidet eller poysaccharidet.

14. Fremgangsmåde til modifikation ifølge krav 13, hvori mængden af $NaBH_3CN$ som reduktionsmidlet er inden for intervallet fra 0 til 20 molrækvivalenter i forhold til molarmængden af aldehydet eller geminaldiolen.

15. Fremgangsmåde til modifikation ifølge ethvert af kravene 12 til 14, hvori den organiske solvent er valgt fra gruppen omfattende vandblandbare alkoholer, særligt isopropanol eller ethanol, og vandblandbare polære aprote solventer, særligt dimethylsulfoxid.

16. Fremgangsmåde til modifikation ifølge ethvert af kravene 12 til 15, hvori mængden af vand er mindst 50 % (v/v) i forhold til hele opløsningens volumen.