(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 December 2016 (22.12.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/202314 A1

(51) International Patent Classification:
*C08B 37/08* (2006.01) *C08B 37/00* (2006.01)
*C08B 15/00* (2006.01) *A61K 47/48* (2006.01)

(21) International Application Number:
PCT/CZ2016/000065

(22) International Filing Date:
14 June 2016 (14.06.2016)

(25) Filing Language: Czech

(26) Publication Language: English

(30) Priority Data:
PV 2015-398 15 June 2015 (15.06.2015) CZ

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(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).

**Declarations under Rule 4.17**:

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

**Published**:

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

WO 2016/202314 A1

(54) Title: METHOD OF CROSSLINKING OF POLYSACCHARIDES USING PHOTOREMOVABLE PROTECTING GROUPS

(57) Abstract: The invention discloses a method of preparation of crosslinked materials based on polysaccharides using electromagnetic radiation in an aqueous solution containing a polysaccharide with a bound carbamate photoremovable protecting group (PPG with group - NH-CO-O-) and a polysaccharide containing an aldehyde group -CHO. The crosslinking process itself is carried out by means of a condensation reaction of the photochemically released amino group (-$NH_2$) with the aldehyde group (-CHO) forming a bond of imine type (-N=CH-). Both processes proceed simultaneously and they can be performed under physiological conditions. The advantage of the suggested solution is the temporal and spatial control of crosslinking that allows the preparation of advanced materials for tissue engineering where the crosslink density and thus the mechanical properties in the material structure can be tailored.

# Method of crosslinking of polysaccharides using photoremovable protecting groups

## Field of invention

The invention relates to polysaccharide crosslinking using photoremovable protecting groups.

## Background of the invention

Hydrogels represent physically or chemically crosslinked polymer structures which are able to absorb large amounts of water without their dissolution in the aqueous solution. Regarding the suitable rheological parameters, the hydrogels with their properties resemble living tissues. Hydrogels are used in form of scaffolds in replacements, or tissues regeneration in case of a tissue damage. The cell organization, cell proliferation or morphogenesis determination may be controlled through hydrogels. At the same time, the hydrogels represent a suitable energy source for the cells. These insoluble three dimensional nets enable the immobilization of biologically active agents (amino acids, peptides, drugs, enzymes, grow factors etc.) and their following controlled release in the desired concentration, time and space. Out of building components of hydrogels, biopolymers are preferred to synthetic polymers, especially where the final application aims at the area of tissue engineering or regenerative medicine and a high biocompatibility of the tested material has to be ensured (Slaughter V. B., Khurshid S. S., Fisher O. Z., Khademhosseini, Peppas, N. A. 2009. *Adv Mater* 21: 3307). Polysaccharides are suitable polymers thanks to their easy availability, a relatively low price, excellent biocompatibility, useful mechanical properties and a manifold structural or functional variability. The most often used polysaccharides for pharmaceutical and biomedical applications are:

Hyaluronic acid (HA) is a natural heteropolysaccharide of glycosaminoglycan kind, formed with D-glucuronic and *N*-acetyl-D-glucosamine subunit, mutually bound through β(1-3) and β(1-4) *O*-glycosidic bond. HA naturally appears in many connective tissues, synovial fluid, aqueous humour, skin and cartilages (Smeds K. A., Pfister-Serres A., Miki G., Dastqheib K., Inoue M., Hatchell D. L., Grinstaff M. W. 2001. *J Biomed Mater Res* 54: 115). Thanks to its biocompatibility, HA is utilized in biomedicine, nutrition, cosmetic and pharmaceutical industry.

Chondroitin sulfate (CS) is a glycosaminoglycan composed of sulfated N-acetylgalactosamine and D-glucuronic acid that is in the greatest amount present in the extracellular matrix of cartilage. CS participates in the articular metabolism and is used as a therapeutic means against degenerative arthritis. As food supplements (e. g. Hyalgel) it plays an important role

in prevention of osteoarthrosis (Bottegoni C., Muzzarelli R. A. A., Giovannini F., Busilacchi A., Gigante A. 2014: *CarbPol* 109: 126).

Chitosan (CH) is a cationic homopolysaccharide prepared by deacetylation of chitin and is extracted from exoskeleton of sea crustaceans. As CH comes from a natural, renewable nontoxic and biodegradable source, it is regarded as an ecologically acceptable product. Its quality and properties are dependent on its purity and the degree of deacetylation (usually in the range 70-95%), further on the molecular weight and also on the crystallinity. CH is usually used as a hypocholesterolemic and bacteriostatic preparation, drug vehicle or material for cell scaffold formation (Pasqui D., De Cagna M., Barbucci, R. 2012. *Polymers* 4: 1517).

Sodium carboxymethyl cellulose (CMCNa) is a hydrophilic cellulose derivative produced by alkylation of swollen cellulose (homopolymer of β-D-glucopyranose) with chloroacetic acid under basic conditions. CMCNa, in combination with various drugs or optionally co-excipients, in the form of medical devices (gauze, bandage, wound dressings) is used in the therapy of skin diseases. It is applied in treating of diabetic foot, skin ulcers, post-operative surgery wound, in toxic epidermal necrolysis and also as skin implants (Pasqui D., De Cagna M., Barbucci, R. 2012. *Polymers* 4: 1517).

Polysaccharides in their native form do not form hydrogels. For this reason, an additional modification of their physical properties is required. It is mainly the decrease of solubility and increase of stability in aqueous solution. One option is a chemical modification, through which the polarity of the polysaccharide chain is decreased e. g. by blocking of the carboxyl group resulting in ester formation (US4851521, US4965353) or by hydrophobization of the polar hydroxyl groups (WO1996/035720, WO2010/105582, US3720662).

The second option is a chemical crosslinking within the polysaccharide structure. The most used reactions leading to a chemical crosslinking include polymerization (Burdick J. A., Chung c., Jia X., Randolph M. A., Langer R. 2005. *Biomacromolecules* 6: 386), condensation reactions (WO2008014787, WO2009/108100, WO2011/069474), dimerization reactions (EP0554898B1, EP0763754A2, US006025444), cycloaddition reactions (CZ304072), optionally enzymatic reactions (CZ303879). Oxidative reactions of polysaccharides according to WO2011/069474 and WO2011/069475 may be used for the synthesis of polysaccharide precursors that are suitable for additional chemical modifications including crosslinking reactions. Dehydration reaction of these precursors was thus used for the preparation of α,β-unsaturated analogues (CZ304512). Deacetylation of polysaccharides according to US7345117 is used for the preparation of polyamino derivatives required e. g. for nucleophilic addition.

However, classical chemical crosslinking also has several important and indisputable drawbacks, i. e. uncontrollable propagation of chemical reaction, insufficient chemoselectivity, using of crosslinking agents and the necessity of an additional purification of the final products. The combination of the classical chemical polysaccharide crosslinking with the use of photoreactive linkers can successfully overcome the above limitations. The photoreactive linkers contain photoremovable protecting groups (PPG) built in their structure. The preparation of monofunctional photoremovable carbamate linkers can be carried out according to (Figueiredo R. M., Fröhlich R., Christmann M. 2006 *J OrgChem* 71: 4147) or (Werner T., Barrett A. G. M. 2006 *J OrgChem* 71:4302 or Furuta, T., Hirayma Y., Iwamura M. 2001. *OrgLett* 3: 1809) by a reaction of an excess of a bifunctional aminolinker with an acylation agent carrying PPG.

One example of the application of PPG is substrate masking from recognition in biological system *in vitro* or *in vivo*, the so-called triggering of the biological response to the presence of a specific agent. These masked substrates are called caged molecules and in case of the used PPG, the term caging groups (CG) is used. CG help mainly in biotechnology and cellular biology as their photocleavage takes place under mild conditions, rapidly, precisely and can be excellently controlled in time and space. The CG applications fall in the area of photolithographic creation of complex peptides, oligonucleotides or in the area of biologically active compounds release in cells or tissues (US2002/0016472).

Another practical example of PPG can be a chemical reaction of two involved functional groups that does not proceed as long as one of them is masked with a photoremovable protecting group (PPG). After the PPG removal, the original reactive group is restored and it reacts with the other participating group in the reaction mixture. The advantage of the two-step process, the installation and cleavage of PPG, thus enables the control of the chemical reaction course. If the substrate in the reaction mixture is masked (protected), the chemical reaction does not proceed. If the substrate is regenerated (released) in the reaction mixture, the chemical reaction proceeds. The amount or the concentration of the masked and released substrate can be determined by using the source of electromagnetic radiation, both in the time aspect (on-off switch, light impulse), and in the space aspect (focused light, laser, use of a photomask etc.). Another advantage of the photocleavage is that it can be reliably applied where other approaches of introduction of protecting groups fail. It applies for example for pH-sensitive or thermosensitive substrates, biomaterials and in *in vitro* or *in vivo* applications. The approach disclosed here thus enables to control the qualitative parameters (crosslink accuracy and density), as well as the quantity parameters (the whole volume vs part of the

sample) of the crosslinked material. For this reason the final crosslinked product can reach from viscous solutions, through soft, to elastic gels.

The term photochemically controlled chemical reaction can represent not only a conjugation reaction or a reaction leading to immobilization or, in the contrary, to the release of the substrate from the carrier structure. This approach can also be applied to the formation of crosslinked polymer structures through the crosslinking reaction with the masked substrate, which is the subject matter of this patent document.

In the literature, there are more practical examples of PPGs that undergo photolysis (Green T. W. & Wuts P. G. M., 1999, John Wiley, *3rd edition*). Photolysis (chemical cleavage) of chemical bonds in these groups is the result of the light quantum – photon absorption by the substrate molecule. Photochemical cleavage of the protecting group can be accomplished by a direct chromophore excitation after the absorption of a single photon with the desired energy or by a multiphoton absorption followed by an electron transfer to the protection group (US210/0207078). In case of ammines, the introduced protecting groups are carbamate functional groups. The most used PPGs are alkoxy, or alternatively nitro derivatives of aromatic alcohols (Klán P., Šolomek T., Bochet Ch. G., Blanc A, Givens R., Rubina M., Popik V., Kostikov A., Wirz J. 2013*: ChemRev* 113: 119; US2008/0009630, and also heteroaromatics of coumarin, quinoline, xanthan or thioxanthone type (US2002/0016472). The application of carbamate PPGs falls mainly in the area of combinatorial peptide synthesis or nucleic acids synthesis (Piggot A. M. & Karuzo P. 2005. *Tetr Lett* 46: 8241). Some more patent documents exist (US2013309706A1, US20008028630A1, US20060216324A1), that use photolysis for the surface modification of polymer materials, controlled release of a biologically active compound controlled or, in the contrary, its covalent immobilization to the polymer structure. However, the use of PPGs for the controlled polysaccharide crosslinking has not been published yet. Presumably, the reason is a combination of multiple factors including for example an insufficient molar absorption coefficient of the chosen PPG for the desired wavelength range, a low quantum yield of the photolysis, a slow substrate release, a low stability and hydrophobic character of the PPG, a formation of potentially toxic and absorbing disintegrative photolysis products, their consecutive competitive reaction with the released substrate or the biological material.

## Summary of the invention

The present invention provides a method for performing crosslinking reactions in polysaccharide solutions that is based on the photochemical control of chemical crosslinking

process with the use of carbamate PPG. The term photochemical control represents the photochemical cleavage of the carbamate bond (-NH-CO-O-) forming the respective amino group (-$HN_2$) using the electromagnetic radiation. The term chemically crosslinking process represents a condensation reaction of the released amino group with an aldehyde group forming an imino group (-N=CH-). Both simultaneously running processes can be performed under physiologically acceptable conditions.

The advantage of the suggested solution in comparison to methods of polysaccharide crosslinking used up to now is the temporal and spatial control over the course of crosslinking, which enables the preparation of advanced materials for tissue engineering where it is possible to influence the crosslink density and thus also the mechanic properties in the material structures. The photochemical control is very advantageous in the cases when it is desirable to regulate the cell growth in a given environment, which is essential e. g. for biomaterials designated for the reparation of neural tissues (Perale G. et. al 2011. *ACS Chem. Neursci.* 2: 336) or in the production of injectable hydrogels in an effort to minimalize the impact of classical invasive surgery (Pasqui D., De Cagna M., Barbucci R. 2012. *Polymers* 4: 1517).

With the combination of the classical chemical crosslinking with the photoreactive polysaccharide derivatives it is possible to achieve the advantages of temporal control of crosslinking reaction progress in such a way that the reaction proceeds only when the respective material is irradiated with the electromagnetic radiation. Under normal conditions, the crosslinking reaction proceeds until running out of the starting material, which is undesired in the cases when specific properties of the crosslinked product are desired, such as material tenacity, pore size, permeability or biodegradability. Also the spatial control over the reaction progress in the form of a proper photomask or focused light ensures local proceeding of the crosslinking reaction in the reaction mixture. A typical example is the photolitographic approach of hydrogel forming, which uses the light for the transfer of a geometrical photomask pattern to a light sensitive substrate (Khetan S., Burdick J. A. (2010). *Biomaterials*, 31: 8228).

Another advantage of the PPG introduction in the polysaccharide structure is the chemospecific course of photolysis and secondly also the crosslinking reaction. The light of desired energy excites only those PPGs which photolytically generate reactive sites for the following crosslinking reaction in exactly defined sites in the polysaccharide polymer structure. Further, the photolysis and the crosslinking reactions proceed under physiological conditions without the requirement of an additional crosslinking agent, organic solvent or

isolation of the final crosslinked products that form gels in an aqueous environment, have an enhanced hydrolytic stability, show sorption properties and ensure retention of liquids and the present agents. The application of these crosslinked polysaccharides belong to the area of tissue engineering, regenerative medicine or biomedical applications in the form of scaffolds, implants or drug carriers.

The carbamate derivatives of polysaccharides according to the present invention are understood to be derivatives that possess a carbamate PPG built in their structure either directly or via a proper linker derived from diamine, amino alcohol, dihydrazide, amino acid, alkoxyamine, eventually via a linker with a combination of the following groups: -OH, $-NH_2$, $-O-NH_2$, -COOH, $-CONHNH_2$, $-NH-NH_2$, -SH.

It is further defined that the carbamate PPG group is derived from an aromatic or heteroaromatic alcohol that shows absorption of electromagnetic radiation within the range 320-400 nm, preferably 330-370 nm.

The carbamate PPG is photolysed (cleaved photochemically) during the radiation with electromagnetic light to an aromatic alcohol, carbon dioxide and a compound with the released amine or hydrazide group. This amine or hydrazide group interacts with an aldehyde group of the other (unsubstituted) polysaccharide forming an imine or hydrazone group. Both the first and the second polysaccharide (polysaccharide 1 and polysaccharide 2) can be of identical or different structure of the hyaluronan, chondroitin sulfate or cellulose type, eventually pharmaceutically acceptable derivatives and/or salts thereof. The crosslinking among the polysaccharide derivatives occurs through condensation reactions. The carbamate PPG photolysis demands the presence of water and it proceeds based on and only after the material irradiation with electromagnetic radiation. The photolysis further proceeds simultaneously with the crosslinking reaction and can be performed under physiological conditions or in the presence of other additives (organic, inorganic salts or buffers).

The present invention therefore discloses a method of crosslinking reaction realization in aqueous polysaccharide solutions that is controlled photochemically. Due to the photochemical control the presence of carbamate photoremovable group (PPG) is necessary, as the carbamate group protects the amino group ($-NH_2$) of the polysaccharide derivative from an early or undesirable reaction with the aldehyde group of the other polysaccharide that is present in the reaction mixture. If the amino group is not protected, an uncontrollable reaction occurs without any way to influence the course thereof.

If the protecting PPG group is present, the reaction between the amino group and the aldehyde group occurs in the aqueous reaction mixture only when the reaction mixture is subjected to

the electromagnetic radiation, preferably UVA within the range of wavelengths from 320 to 400 nm. This enables the temporal control of the reaction, for example with the radiation source switch, or shading of the reaction mixture. The crosslinking density grows with the increasing radiation time, see Fig. 1, Examples 8 and 9. The spatial control of the reaction for example with a photomask or a light beam occurs only in the irradiated places, see Fig. 2.

The present invention particularly relates to a method of preparation of crosslinked polysaccharide materials according to the general formula (I)

polysaccharide1-$R^1$-N=CH-polysaccharide2 (I),

where polysaccharide1 and polysaccharide2 are identical or different polysaccharides and $R^1$ is $C_1$-$C_{30}$ alkyl residue, $C_1$-$C_{30}$ alkylaryl residue or $C_1$-$C_{30}$ alkylheteroaryl residue, optionally containing one or more identical or different heteroatoms selected from the group comprising N, O, S. The method is performed in the following way: an aqueous solution of the aldehyde of polysaccharide2 according to the general formula III

polysaccharide2-CH=O (III),

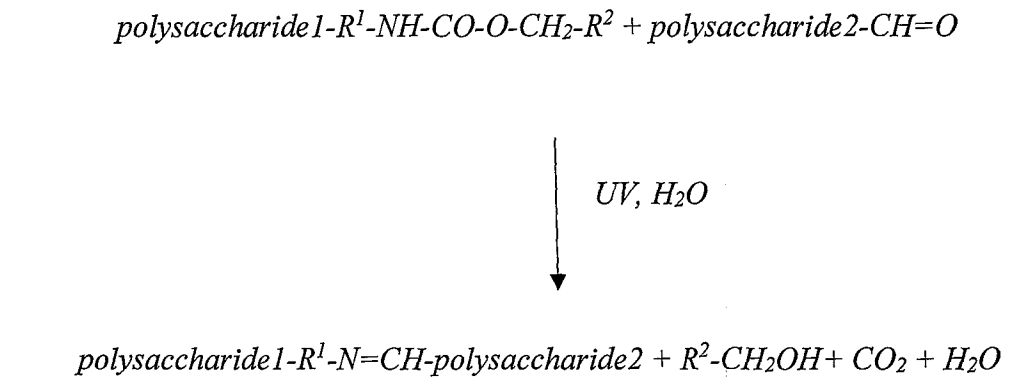
where the substitution degree of aldehyde in polysaccharide2 is within the range of 1 to 50 %, is added to an aqueous solution of polysaccharide1 substituted with amine group modified by a photoremovable group according to the general formula (II)

polysaccharide1-$R^1$-NH-CO-O-$CH_2$-$R^2$ (II),

where $R^1$ is defined above; $R^2$ is an aromatic system and where the substitution degree of carbamate is within the range of 1 to 10%.

The reaction mixture is subjected to an electromagnetic radiation and at the same time deoxygenation takes place of the mixture.

The reaction can be expressed with the general scheme 1:

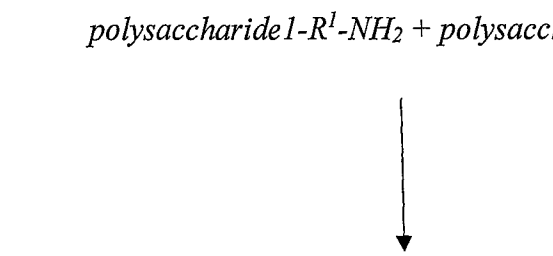



Scheme 1

Scheme 1 actually includes two simultaneously proceeding reactions, the photolysis of PPG in polysaccharide 1 and the condensation/crosslinking reaction of the amine of polysaccharide 1 with the aldehyde of polysaccharide 2:


$$polysaccharide1\text{-}R^1\text{-}NH_2 + R^2\text{-}CH_2OH + CO_2$$

Scheme 1a – Photolysis




$$polysaccharide1\text{-}R^1\text{-}N{=}CH\text{-}polysaccharide2 + H_2O$$

Scheme 1b – Crosslinking

The nitrogen or NH group in the formulae (I) and (II) is indicated in addition, although it is a part of the group $R^1$. Also the group CH in the formula (I) or CH=O in the formula (III) is indicated in addition, although it is a part of polysaccharide2. Those skilled in the art will understand that it is done only for a better understanding and lucidity of the reaction and the reaction substrates.

As presented above, the degree of substitution of PPG in polysaccharide 1 is within the range of 1 to 10 %, preferably 3 to 10%, and its molecular weight is 10 to 400 kDa, preferably 20 to 300 kDa, more preferably 20 to 100 kDa. The degree of polysaccharide2 substitution to an aldehyde is within the range of 1 to 50 %, preferably 3 to 25% and its molecular weight is 10

to 800 kDa, preferably 50 to 250 kDa. The preferred polysaccharides include for example hyaluronan, chondroitin sulfate, cellulose and pharmaceutically acceptable derivatives and/or salts thereof.

$R^1$ is preferably selected from a group comprising dihydrazide adipate and hexamethylenediamine and $R^2$ is preferably a condensed aromatic system, more preferably selected from a group comprising pyrene, anthracene, phenanthrene, perylene, anthraquinone, coumarin and substitution derivatives thereof, that may contain atoms C, H, O, S, N in their structure and show absorption of electromagnetic radiation, most preferably $R^2$ is pyrene.

The weight ratio of polysaccharide 1 to polysaccharide 2 is preferably within the range 1:2 to 2:1. Aqueous solutions of polysaccharides 1 and 2 may further contain water soluble agents selected from a group comprising inorganic salts or buffers, preferably phosphate buffer, whereas pH of the solution is within the range of 6.5 to 7.5, preferably 7.0.

The reaction mixture prepared by the method described in the present invention is subjected to the electromagnetic radiation for 0.25 to 2 hours, preferably 0.5 to 1 hour, at the temperature of 10 to 50 °C, preferably 20 to 35 °C, whereas the electromagnetic radiation used has the wavelength within the range of 320-400 nm, preferably 330-370 nm. As was stated above, the advantage of the invention is that the reaction can be temporal controlled using an electromagnetic radiation source switch or a pulse source of electromagnetic radiation or shading of the reaction mixture. The present invention further also allows the spatial control of the reaction using a photomask, the focused electromagnetic radiation or a beam of electromagnetic radiation.

The material produced according to the present invention can be used in the field of tissue engineering or regenerative medicine in the form of scaffolds, fillers or in the field of biomedicine in the form of drug carriers based on photosensitive materials with the controlled release of the biologically active agent.

**Detailed description of drawings.**

Fig. 1 represents the use of upside-down method for determination of gelation in the reaction mixture. (a) Solution of two-component reaction mixture (Pmoc-DHA-HA and HA-aldehyde) before photolysis. (b) Hydrogel of the crosslinked product (HA-DHA-HA) after photolysis. (c) Hydrogel of crosslinked product (HA-DHA-HA) after 1 h in PBS (pH = 7.4, c = 0.9%, w/v).

Fig. 2 illustrates the spatial control of the crosslinking reaction (Pmoc-DHA-HA and HA-aldehyde) using a photomask in the shape of a semi-circle on 50 % of the surface of the

reaction mixture. (a) the reaction mixture after photolysis, (b) the reaction mixture after 15 min in PBS (pH = 7.4, c = 0.9% w/v) and decanting of PBS solution, (c) the reaction mixture after 15 min in PBS (pH = 7.4, c = 0.9% w/v) and adding new portion of PBS.

Fig. 3 shows microscopic photographs of freeze-dried samples. (a): the hydrogel surface (100x), (b) the cross-section of the hydrogel (100x), (c) the cross-section of the hydrogel after 1 h in PBS (pH = 7.4, c = 0.9% w/v).

**Examples**

The term equivalent (eqv) used herein relates to a disaccharide of hyaluronic acid, disaccharide of chondroitin sulfate or monosaccharide of sodium carboxymethyl cellulose, if not stated otherwise. Percentage is used as mass percentage, if not stated otherwise.

The molecular weight of the initial hyaluronic acid (source: Contipro Pharma a.s., Dolní Dobrouč, CZ) is the average molecular weight within the range of $10^4$ to $10^6$ g.mol$^{-1}$ and was determined by SEC-MALLS.

The molecular weight of the initial chondroitin sulfate (source: Sigma-Aldrich s.r.o., Prague, CZ) is the average molecular weight within the range of 4 x $10^4$ to 5 x $10^4$ Da or g.mol$^{-1}$ and was determined with the method SEC-MALLS. The ratio of chondroitin-4-sulfate (C4S) and chondroitin-6-sulfate (C6S) was 2:3. The material was isolated from an animal material.

The molecular weight of the initial sodium carboxymethyl cellulose (source: Sigma-Aldrich s.r.o., Prague, CZ) is the average molecular weight within the range of 22 x $10^4$ to 25 x $10^4$ g.mol$^{-1}$ and was determined with SEC-MALLS. The degree of alkylation with the carboxymethyl group was 70%.

The degree of substitution or modification in the structure of glycosaminoglycans was determined by means of the following calculation:

DS = substitution degree = 100 % * (the molar amount of the bound substituent or modified disaccharide)/(the molar amount of all disaccharides)

The degree of modification in the structure of sodium carboxymethyl cellulose was determined by means of the following calculation:

DS = substitution degree = 100 % * (the molar amount of the bound substituent or modified monosaccharide)/(the molar amount of all monosaccharides)

PPG = photoremovable protection group

DHA = dihydrazide adipate

HMD = 1,6-hexamethylenediamine

Pmoc = pyren-1-ylmethoxycarbonyl

UVA = near ultraviolet radiation within the range of wavelengths 320-400 nm, emitted by longwave ultraviolet source Black-Ray mercury spot lamp, model B-100A (UVP) with declared λmax = 365 nm.

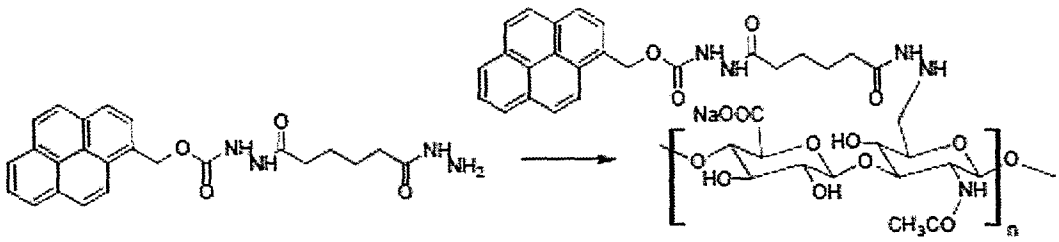
The surface morphology of the freeze-dried gels was analyzed with scanning electron microscope Zeiss Ultra Plus.

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

Oxidation of polysaccharides was performed according to Buffa R, et al.: WO2011069474 and WO2011069475.

**Example 1. Preparation of Pmoc-dihydrazide adipate hyaluronan (Pmoc-DHA-HA)**

HA aldehyde (100 mg, 0.265 mmol, DS = 43%, Mw = 1.35 x $10^5$ g/mol) was dissolved in 5 mL of distilled water (solution I). Pmoc-DHA (54 mg, 0.126 mmol) was dissolved in 5 mL of DMSO (solution II). Both solutions were mixed and reacted for 24 h at room temperature. In the second step $PicBH_3$ (81 mg, 0.754 mmol) was added. The reaction mixture was stirred for 48 h at room temperature. The product was precipitated with IPA.



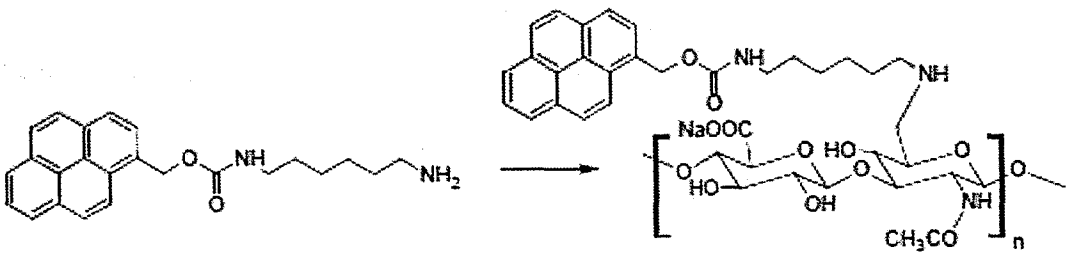
DS = 10%, Mw = 0.34x$10^5$ g/mol, isolated yield 85%

| | |
|---|---|
| $^1$H NMR ($D_2O$) | δ 1.60 (bs, 4H); 2.21 (bs, 2H); 2.25 (bs, 2H); 2.98 (bs, 1H,polymer-$N_{6a}$); 3.26 (bs, 1H, polymer-$N_{6b}$); 5.89 (s, 2H, -$\underline{CH_2}$-pyr); 7.98 – 8.41 (m, 9$H_{Ar}$) ppm |
| H-H COSY ($D_2O$) cross-peak | δ 1.60 – 2.21; 1.60 – 2.25; 2.98 – 3.26 ppm |
| HSQC ($D_2O$) cross-peak | δ 1.60 ($^1$H) – 24.6 ($^{13}$C); 2.21 ($^1$H) – 33.0 ($^{13}$C); 2.25 ($^1$H) – 33.1($^{13}$C); 2.98 ($^1$H) – 50.0 ($^{13}$C); 3.26 ($^1$H) – 50.0 ($^{13}$C); 5.89 ($^1$H) -64.3 ($^{13}$C); 7.98 ($^1$H) – 124.2 ($^{13}$C); 8.05 ($^1$H) – 125.3 ($^{13}$C); 8.30 ($^1$H) – 129.6 ($^{13}$C); 8.41 ($^1$H) – 131.2 ($^{13}$C) ppm |
| DOSY NMR ($D_2O$) | log D (1.60 ppm, 2x-$CH_2$-linker) ~ -10.70 $m^2/s$ |

| | |
|---|---|
| | log D (2.03 ppm, Me-CO-NH-polymer) ~ -10.70 $m^2/s$ |
| | log D (2.21 ppm, -$\underline{CH_2}$-CONHNH$_2$) ~ -10.70 $m^2/s$ |
| | log D (2.25 ppm, -$\underline{CH_2}$-CONHNH-polymer) ~ -10.70 $m^2/s$ |
| | log D (2.98 ppm, polymer-$N_{6a}$) ~ -10,70 $m^2/s$ |
| | log D (3.26 ppm, polymer-$N_{6b}$) ~ -10,70 $m^2/s$ |
| | log D (5.89 ppm, -$\underline{CH_2}$-pyr) ~ -10.70 $m^2/s$ |
| | log D (7.98 - 8,41 ppm, -$CH_2$-$\underline{pyr}$) ~ -10.70 $m^2/s$ |
| | log D (4.72 ppm, $H_2O$) ~ -8.6 $m^2/s$ |
| UV/Vis (0,01 %, $H_2O$) | $\lambda_{max1,2}$ = 350, 329 nm |

**Example 2. Preparation of Pmoc-hexamethylene diamine hyaluronan (Pmoc-HMD-HA)**

HA aldehyde (100 mg, 0.265 mmol, DS = 10%, Mw = 1.92 x $10^5$ g/mol) was dissolved in 5 mL of distilled water (solution I). Pmoc-HMD (19 mg, 0.05 mmol) was dissolved in 5 mL of DMSO (solution II). Both solutions were mixed and reacted for 24 h at room temperature. In the second step $PicBH_3$ (81 mg, 0.754 mmol) was added. The reaction mixture was stirred for 48 hours at room temperature. The product was obtained by precipitation with IPA.



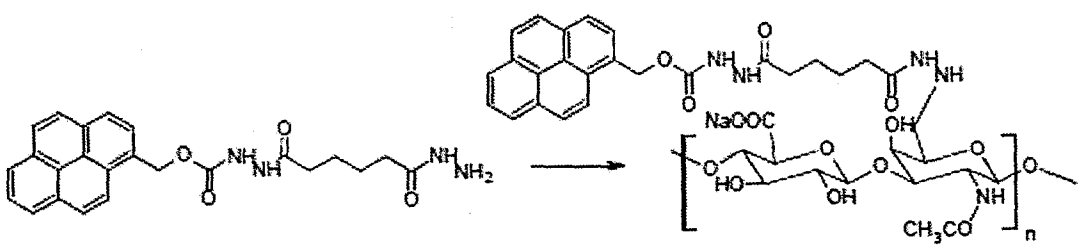

DS = 7%, Mw = 1.92 x$10^5$ g/mol, isolated yield 71%.

| | |
|---|---|
| $^1$H NMR ($D_2O$) | δ 1.34 (bs, 4H); 1.45 (bs, 2H); 1.66 (bs; 2H; 2H); 3.05 (bs; 2H; -$\underline{CH_2}$-NHCO-pyr); 3.15 (bs; 2H; -$CH_2$-NH-polymer); 3.26 (bs; 1H; polymer-$N_{6a}$); 3.48 (bs; 1H; polymer-$N_{6b}$); 5.83 (bs, 2H, -$CH_2$-pyr), 8.00 – 8.45 (m, 9$H_{Ar}$) ppm |
| H-H COSY ($D_2O$) cross-peak | δ 1.34 – 1.45; 1.34 – 1.66; 1.66 – 3.05; 1.45 – 3.15; 3.26 – 3.48 ppm |

| | |
|---|---|
| HSQC ($D_2O$) cross-peak | δ 1.34 ($^1H$) – 26.3 ($^{13}C$); 1.45 ($^1H$) – 28.7 ($^{13}C$); 1.66 ($^1H$) – 26.1 ($^{13}C$); 3.05 ($^1H$) – 48.2 ($^{13}C$); 3.15 ($^1H$) – 41.3 ($^{13}C$); 3.26 ($^1H$) – 48.5 ($^{13}C$); 3.48 ($^1H$) – 48.5 ($^{13}C$); 5.83 ($^1H$) – 64.3 ($^{13}C$); 8.00 ($^1H$) – 124.2 ($^{13}C$); 8.09 ($^1H$) – 125.7 ($^{13}C$); 8.26 ($^1H$) – 130.1 ($^{13}C$); 8.45 ($^1H$) – 131.7 ($^{13}C$) ppm |
| DOSY NMR ($D_2O$) | log D (1.34 ppm, 2x-$CH_2$-linker) ~ -10.60 $m^2/s$<br>log D (1.45 ppm, -$CH_2$-linker) ~ -10.60 $m^2/s$<br>log D (1.66 ppm, -$CH_2$-linker) ~ -10.60 $m^2/s$<br>log D (2.03 ppm, Me-CO-NH-polymer) ~ -10.60 $m^2/s$<br>log D (3.05 ppm, -$CH_2$-NHCO-) ~ -10.60 $m^2/s$<br>log D (3.15 ppm, -$CH_2$-NH-polymer) ~ -10.60$m^2/s$<br>log D (3.26 ppm, polymer-$N_{6a}$) ~ -10.60 $m^2/s$<br>log D (3.48 ppm, polymer-$N_{6b}$) ~ -10.60 $m^2/s$<br>log D (5.83 ppm, -$CH_2$-pyr) ~ -10.60 $m^2/s$<br>log D (8.00 – 8.45 ppm, $H_{Ar}$) ~ -10.60 $m^2/s$<br>log D (4.72 ppm, $H_2O$) ~ -8.6 $m^2/s$ |
| UV/Vis (0.01 %, $H_2O$) | $\lambda_{max1,2}$ = 348, 330 nm |

**Example 3. Preparation of Pmoc-dihydrazide adipate chondroitin sulfate (Pmoc-DHA-CS)**

CS aldehyde (50 mg, 0.10 mmol, DS = 14%, Mw = 3.0-4.0 x $10^5$ g/mol) was dissolved in 2.5 mL of distilled water (solution I). Pmoc-DHA (8.7 mg, 0.02 mmol, 0.2 eqv.) was dissolved in 2.5 mL of DMSO (solution II). Both solutions were mixed and reacted for 24 h at room temperature. In the second step $PicBH_3$ (32 mg, 0.3 mmol, 3 eqv.) was added. The reaction mixture was stirred for 48 h at room temperature. The product was obtained with precipitation with IPA.

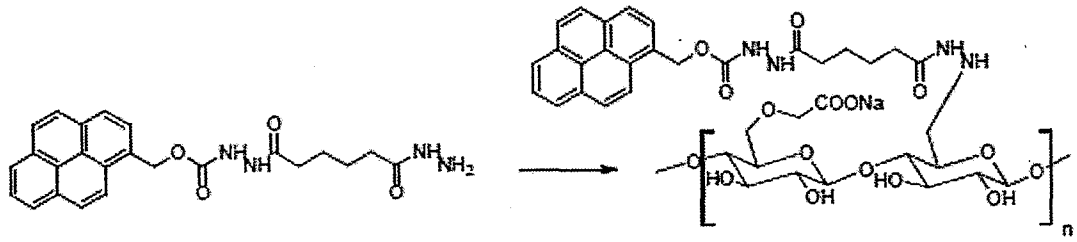


DS = 5-6%, Mw = 3.0-4.0 x$10^5$ g/mol, isolated yield 84%

| | |
|---|---|
| $^1$H NMR ($D_2O$) | δ 1.66 (bs, 4H); 2.25-2.32 (m, 4H); 3.00 (bs, 1H, polymer-$N_{6a}$); 3.25 (bs, 1H, polymer-$N_{6b}$); 5.89 (bs, 2H, -$CH_2$-pyr); 8.15 – 8.38 (m, 9$H_{Ar}$) ppm |
| H-H COSY ($D_2O$) cross-peak | δ 1.66 – 2.25; 1.66- 2.32; 3.00 – 3.25 ppm |
| HSQC ($D_2O$) cross-peak | δ 1.66 ($^1$H) – 25.0 ($^{13}$C); 2.25 ($^1$H) – 31.2 ($^{13}$C); 2.32 ($^1$H) – 32.8 ($^{13}$C); 3.00 ($^1$H) – 50.6 ($^{13}$C); 3.25 ($^1$H) – 50.6 ($^{13}$C); 5.89 ($^1$H) – 64.6 ($^{13}$C); 8.16 ($^1$H) – 124.8 ($^{13}$C); 8.38 ($^1$H) – 125.6 ($^{13}$C); 8.30 ($^1$H) – 129.6 ($^{13}$C) ppm |
| DOSY NMR ($D_2O$) | log D (1.66 ppm, 2x-$CH_2$-linker) ~ -10.50 $m^2/s$<br>log D (2.04 ppm, Me-CO-NH-polymer) ~ -10.50 $m^2/s$<br>log D (2.25-2.32 ppm, -$CH_2$-CONHN$H_2$,-$CH_2$-CONHNHpolymer) ~ -10.50 $m^2/s$<br>log D (3.00 ppm, polymer-$N_{6a}$) ~ -10.50 $m^2/s$<br>log D (3.25 ppm, polymer-$N_{6b}$) ~ -10.50 $m^2/s$<br>log D (5.89 ppm, -$CH_2$-pyr) ~ -10.50 $m^2/s$<br>log D (8.15 – 8.38 ppm, -CH2-pyr) ~ -10.50 $m^2/s$<br>log D (4.72 ppm, $H_2O$) ~ -8.6 $m^2/s$ |
| UV/Vis (0.01%, $H_2O$) | $\lambda_{max1,2}$ = 343, 328 nm |

**Example 4. Preparation of Pmoc-dihydrazide adipate of sodium carboxymethyl cellulose (Pmoc-DHA-CMCNa)**

CMCNa aldehyde (100 mg, 0.45 mmol, DS = 4-5%, Mw = 8.2 x $10^5$ g/mol) was dissolved in 5 mL of distilled water (solution I). Pmoc-DHA (19.4 mg, 0.045 mmol, 0.1 eqv.) was dissolved in 5 mL of DMSO (solution II). Both solutions were mixed and reacted for 24 h at room temperature. In the second step $PicBH_3$ (144 mg, 1.345 mmol, 3 eqv.) was added. The reaction mixture was stirred for 48 h at room temperature. The product was obtained by precipitation with IPA.



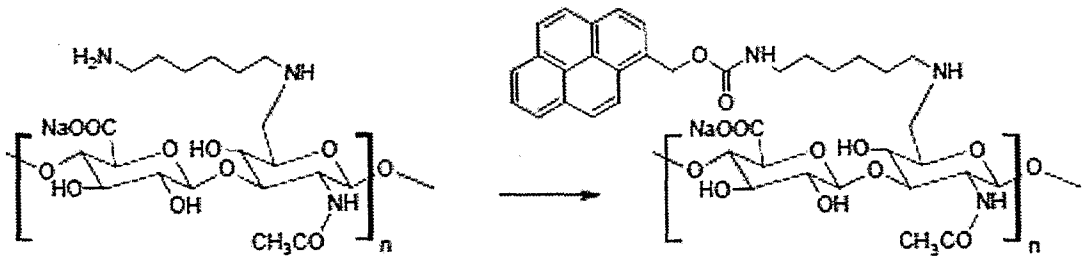
DS = 2%, Mw = 0.80 x$10^5$ g/mol, isolated yield 88%

| | |
|---|---|
| $^1$H NMR ($D_2O$) | δ 1.60-1.65 (bs, 4H); 2.22 (bs, 2H); 2.38 (bs, 2H); 3.00 (bs, 1H,polymer-$H_{6a}$); 3.37 (bs, 1H, polymer-$H_{6b}$); 5.84-5.87 (bs, 2H, -$CH_2$-pyr); 8.05 – 8.33 (m, 9$H_{Ar}$) ppm |
| H-H COSY ($D_2O$) cross-peak | δ 1.60 – 2.22; 1.65-2.38; 3.00 – 3.37 ppm |
| HSQC ($D_2O$) cross-peak | δ 1.60-1.65 ($^1$H) – 25.3 ($^{13}$C); 2.22 ($^1$H) – 33.6 ($^{13}$C); 2.38 ($^1$H) – 32.7 ($^{13}$C); 3.00 ($^1$H) – 50.1 ($^{13}$C); 3.37 ($^1$H) – 51.3 ($^{13}$C); 5.84-5.87 ($^1$H) – 64.2 ($^{13}$C); 8.05 ($^1$H) – 123.5 ($^{13}$C); 8.30 ($^1$H) -125.1($^{13}$C); 8.33 ($^1$H) – 129.4 ($^{13}$C) ppm |
| DOSY NMR ($D_2O$) | log D (1.60-1.65 ppm, 2x-$CH_2$-linker) ~ -10.60 $m^2/s$<br>log D (2.22-2.38 ppm, -$CH_2$-CONHN$H_2$,-$CH_2$-CONHNHpolymer)~ -10.60 $m^2/s$<br>log D (3.00 ppm, polymer-$N_{6a}$) ~ -10.60 $m^2/s$<br>log D (3.37 ppm, polymer-$N_{6b}$) ~ -10.60 $m^2/s$<br>log D (4.55-4.61 ppm, H1aH1'-polymer) ~ -10,60 m2/s<br>log D (5,84-5,87 ppm, -$CH_2$-pyr) ~ -10.60 $m^2/s$<br>log D (8.05 – 8.33 ppm, -$CH_2$-pyr) ~ -10.60 $m^2/s$<br>log D (4.72 ppm, $H_2O$) ~ -8.6 $m^2/s$ |
| FT-IR (KBr) | C=O st 1750-1680 $cm^{-1}$ (carbamate)<br>N-CO-O st as 1270-1210 $cm^{-1}$ (carbamate)<br>st sy 1050-850 $cm^{-1}$ (carbamate) |
| UV/Vis (0,01%, $H_2O$) | $\lambda_{max1,2}$ = 344, 329 nm |

**Example 5. Preparation of Pmoc-HMD-HA**

Pmoc-1-*H*-imidazole carboxylate (326 mg, 1 mmol) dissolved in 20 mL THF was added to 20 mL of an aqueous solution of HMD-HA (200 mg, 0.5 mmol, DS = 36%) and the reaction

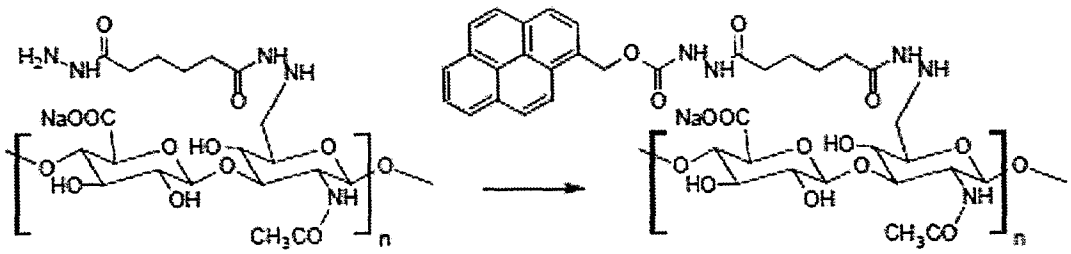
mixture was stirred for 24 h at room temperature. The product (DS = 8%, Y = 40%) was obtained by precipitation with IPA.



The structural analysis of the product is shown in Example 2.

**Example 6. Preparation of Pmoc-DHA-HA**

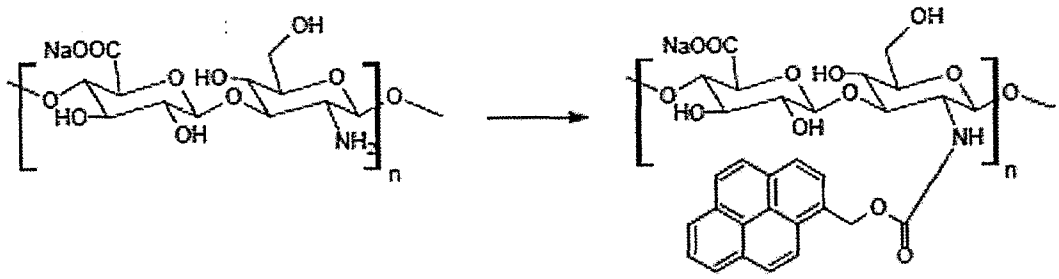
Pmoc-1-*H*-imidazole carboxylate (326 mg, 1 mmol) dissolved in 20 mL THF was added to 20 mL of an aqueous solution of DHA-HA (200 mg, 0.5 mmol, DS = 25%) and the reaction mixture was stirred for 24 h at room temperature. The product (DS = 6%, Y = 45%) was obtained by precipitation with IPA.



The structural analysis of the product is shown in Example 1.

**Example 7. Preparation of Pmoc-deacetylated hyaluronan (Pmoc-DEA-HA)**

Pmoc-1-*H*-imidazole carboxylate (326 mg, 1 mmol) dissolved in 20 mL of THF was added to 20 mL of an aqueous solution DEA-HA (200 mg, 0.5 mmol, DS = 32%, Mw = 0.37 x $10^5$ g/mol) and the reaction mixture was stirred for 24 h at 40 °C. The product was obtained by precipitation with IPA.



DS = 7%, isolated yield 35%

| | | |
|---|---|---|
| $^1$H NMR ($D_2O$) | δ 3.70 (bs, 1H, N2); 5.86 (bs, 2H, -$CH_2$-pyr); 8.10 – 8.35 (m, 9H, pyr) ppm |
| HSQC (D2O) cross-peak | δ 3.70 ($^1$H) – 56.3 ($^{13}$C); 5.86 ($^1$H) – 63.90 ($^{13}$C); 8.10 ($^1$H) - 124.0 ($^{13}$C); 8.20 ($^1$H) - 125.4 ($^{13}$C); 8.30 ($^1$H) - 129.0 ($^{13}$C); 8.35 ($^1$H) - 131.8 ($^{13}$C) ppm |
| UV/Vis (0.01 %, $H_2O$) | $\lambda_{max1,2}$ = 348, 329 nm |

**Example 8. Photolysis of Pmoc-DHA-HA in the presence of HA-aldehyde and crosslinking**

Method 1: Pmoc-DHA-HA (10 mg, 0.025 mmol, DS = 10%, Mw = 2.64 x $10^5$ g/mol) was dissolved in 2 mL of $D_2O$ in a quartz flask. HA-aldehyde (10 mg, 0.025 mmol, DS = 11%, Mw = 0.66 x $10^5$ g/mol) was added. The sample was deoxygenated with a stream of nitrogen and was irradiated for 1 h in UVA under $N_2$ at 25 °C, pH = 7 while stirred, while samples were taken in 15 minute intervals for $^1$H NMR analysis. The increase of the crosslink density (δ = 7.49 ppm, HA-CH=N-HA) was monitored at particular time intervals (15/30/45/60 min) at the level (18/31/66/85%), respectively.

**Example 9. Photolysis of Pmoc-DHA-HA in the presence of α,β-unsaturated HA-aldehyde and crosslinking**

Method 1: Pmoc-DHA-HA (10 mg, 0.025 mmol, DS = 10%, Mw = 2.64 x $10^5$ g/mol) was dissolved in 2 mL of $D_2O$ in a quartz flask. α,β-Unsaturated HA-aldehyde (10 mg, 0.025 mmol, DS = 5%, Mw = 0.68 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of $N_2$ and irradiated for 1 h in UVA under $N_2$ at 25 °C, pH = 7 while stirred, while

samples for $^1$H NMR analysis were taken in 15 minutes intervals. The increase of the crosslink density (δ = 7.58 ppm (H6) and 5.60 ppm (H4), HA-CH=N-HA) was monitored at particular time intervals (15/30/45/60 min) at the level (20/32/48/75%), respectively.

**Example 10. Photolysis of Pmoc-DHA-HA in the presence of saturated HA-aldehyde and crosslinking.**

Method 1: Pmoc-DHA-HA (10 mg, 0.025 mmol, DS = 10%, Mw = 2.64 x $10^5$ g/mol) was dissolved in 2 mL $D_2O$ in quartz flask. HA-aldehyde (10 mg, 0.025 mmol, DS = 11%, Mw = 0.66 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of $N_2$ and was irradiated for 1 h in UVA under $N_2$ at 25 °C, pH = 7 while stirred whereas aliquots for $^1$H NMR analysis were withdrawn in 15 minute intervals. After 60 min UV exposition 85% of hydrazone was formed (δ = 7.49 ppm, HA-CH=N-HA).

Method 2: Pmoc-DHA-HA (10 mg, 0.025 mmol, DS = 10%, Mw = 2.64 x $10^5$ g/mol) was dissolved in 2 mL of $D_2O$ in a quartz flask. HA-aldehyde (10 mg, 0.025 mmol, DS = 45%, Mw = 0.35 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and irradiated for 1 h in UVA under $N_2$ at 25 °C, pH = 7 while stirred, wherein samples for $^1$H HMR analysis were taken in 15 minute intervals. After 60 min of UV exposition, 95% of hydrazone was formed (δ = 7.49 ppm, HA-CH=N-HA).

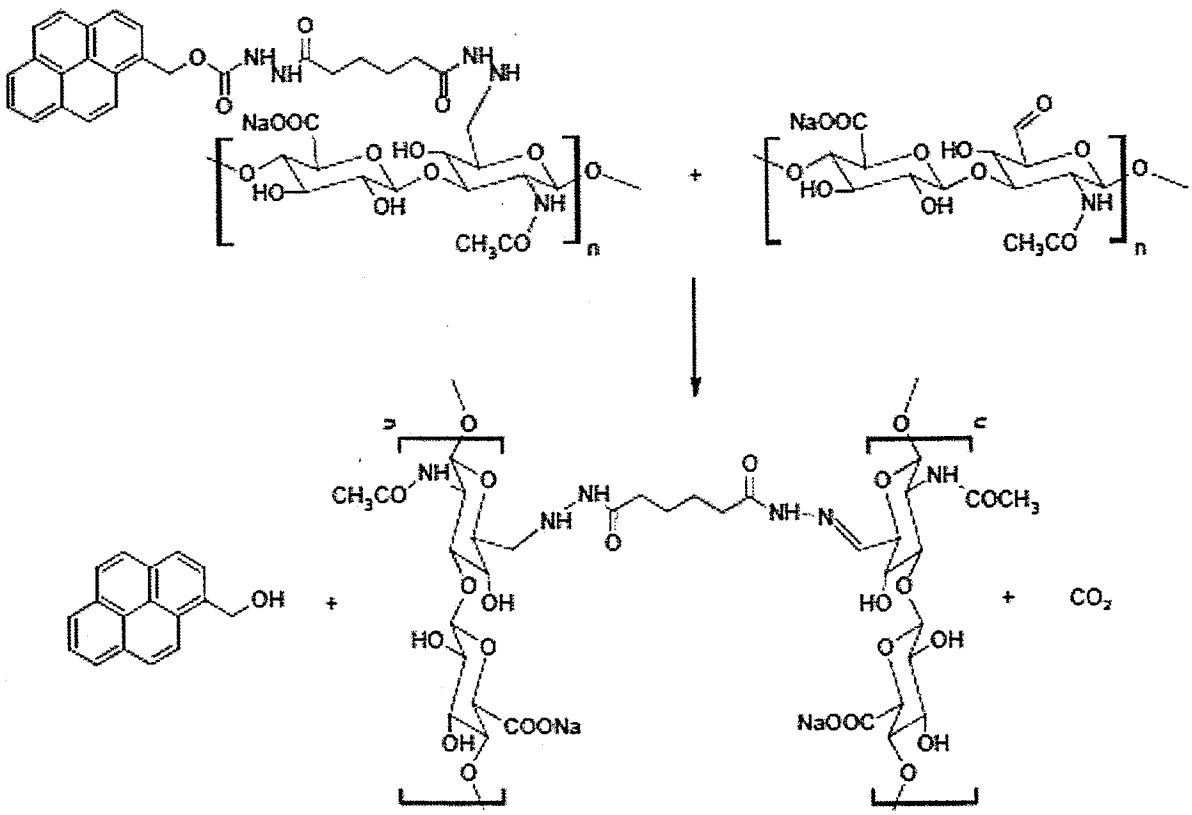
Method 3: Pmoc-DHA-HA (10 mg, 0.025 mmol, DS = 10%, Mw = 2.64 x $10^5$ g/mol) was dissolved in 2 mL of PBS (c = 0.9%, pH = 7.4) in a quartz flask. HA-aldehyde (10 mg, 0.025 mmol, DS = 11%, Mw = 5.10 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and irradiated for 1 h in UVA under $N_2$ at 37 °C, pH = 7 while stirred. After 1 h of UVA exposition, the viscosity of the solution increased.

Method 4: Pmoc-DHA-HA (10 mg, 0.025 mmol, DS = 10%, Mw = 2.64 x $10^5$ g/mol) was dissolved in 2 mL of PBS (c = 0.9%, pH = 7.4) in a quartz flask. HA-aldehyde (10 mg, 0.025 mmol, DS = 11%, Mw = 5.1 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and was irradiated for 1 h in UVA, under $N_2$ at 50 °C, at 25 °C, pH = 7 while stirred. After 1 h of UVA exposition, the viscosity of the solution increased.

Method 5: Pmoc-DHA-HA (10 mg, 0.025 mmol; DS = 10%, Mw = 2.64 x $10^5$ g/mol) was dissolved in 3 mL of PBS (c = 0.9%, pH = 7.4) in a quartz flask. HA-aldehyde (20 mg, 0.050 mmol, DS = 11%, Mw = 5.10 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and was irradiated for 1 h in UVA, under $N_2$, at 25 °C, pH = 7 while stirred. After 1 h of UVA exposition, a gel was formed.

Method 6: Pmoc-DHA-HA (10 mg, 0.025 mmol, DS = 10%, Mw = 2.64 x $10^5$ g/mol) was dissolved in 3 mL of PBS (c = 0.9%, pH = 7.4) in a quartz flask. HA-aldehyde (20 mg, 0.050 mmol, DS = 11%, Mw = 5.10 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and was irradiated for 0.25 h in UVA, under $N_2$, at 25 °C, pH = 6.5 while stirred. After 1 h of UVA exposition, the viscosity of the solution increased.

Method 7: Pmoc-DHA-HA (10 mg, 0.025 mmol, DS = 10%, Mw = 2.64 x $10^5$ g/mol) was dissolved in 3 mL of PBS (c = 0.9%, pH = 7.4) in a quartz flask. HA-aldehyde (20 mg, 0.050 mmol, DS = 11%, Mw = 5.10 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and was irradiated for 2 h in UVA, under $N_2$ at 25 °C, pH = 7.5 while stirred. After 2 h of UVA exposition, a gel was formed.



DS = 3%, hydrazone group

| | |
|---|---|
| $^1$H NMR ($D_2O$) | δ 7.49 (bs, 1H, -N=CH-) ppm |
| HSQC ($D_2O$) cross-peak | δ 7.49 ($^1$H) – 146.6 ($^{13}$C) ppm |
| DOSY NMR ($D_2O$) | log D (2.04 ppm, Ac-NH-polymer) ~ -11.5 $m^2/s$ |
| | log D (7.49 ppm, -N=CH-) ~ -11.5 $m^2/s$ |
| | log D (4.75 ppm, $H_2O$) ~ -8.6 $m^2/s$ |

**Example 11. Photolysis of Pmoc-DHA-HA in the presence of α,β-unsaturated HA-aldehyde and crosslinking**

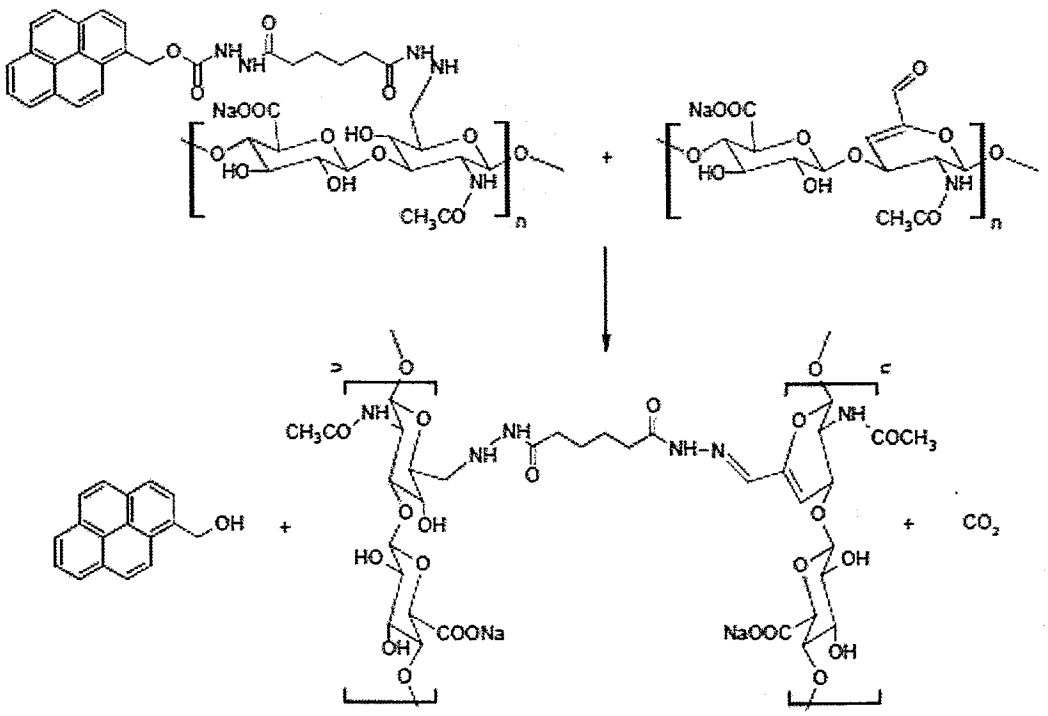
Method 1: Pmoc-DHA-HA (10 mg, 0.025 mmol, DS = 10%, Mw = 2.64 x $10^5$ g/mol) was dissolved in 2 mL of $D_2O$ in a quartz flask. α,β-Unsaturated HA-aldehyde (10 mg, 0.025 mmol, DS = 5%, Mw = 0.68 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and was irradiated for 1 h in UVA, under $N_2$, at 25 °C, pH = 7 while stirred, wherein samples for $^1$H NMR analysis were taken in 15 minute intervals. After 60 min

of UVA exposition, 75% of hydrazone (δ = 7.58 ppm (H6) and 5.60 ppm (H4), HA-CH=N-HA) was formed.

Method 2: Pmoc-DHA-HA (10 mg, 0.025 mmol, DS = 10%, Mw = 2.64 x $10^5$ g/mol) was dissolved in 2 mL of PBS (0.9%, pH = 7.4) in a quartz flask. α,β-unsaturated HA-aldehyde (10 mg, 0.025 mmol, DS = 4 %, Mw = 2.05 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and was irradiated for 1 h in UVA, under $N_2$, at 25 °C, pH = 7 while stirred. After 1 h exposition, the viscosity of the solution increased.

Method 3: Pmoc-DHA-HA (10 mg, 0.025 mmol, DS = 10%, Mw = 2.64 x $10^5$ g/mol) was dissolved in 3 mL of PBS (0.9%, pH = 7.4) in a quartz flask. α,β-unsaturated HA-aldehyde (20 mg, 0.05 mmol, DS = 4%, Mw = 2.05 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and was irradiated for 1 h in UVA, under $N_2$, at 25 °C, pH = 7 while stirred. After 1 h of UVA, the viscosity of the solution increased.

Method 4: Pmoc-DHA-HA (20 mg, 0.05 mmol, DS = 10%, Mw = 2.64 x $10^5$ g/mol) was dissolved in 3 mL of PBS (0.9%, pH = 7.4) in a quartz flask. α,β-unsaturated HA-aldehyde (10 mg, 0.025 mmol, DS = 4%, Mw = 2.05 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and was irradiated for 1 h in UVA, under $N_2$, at 25 °C, pH = 7 while stirred. After 1 h of UVA, the viscosity of the solution increased.



DS = 3%, hydrazone group

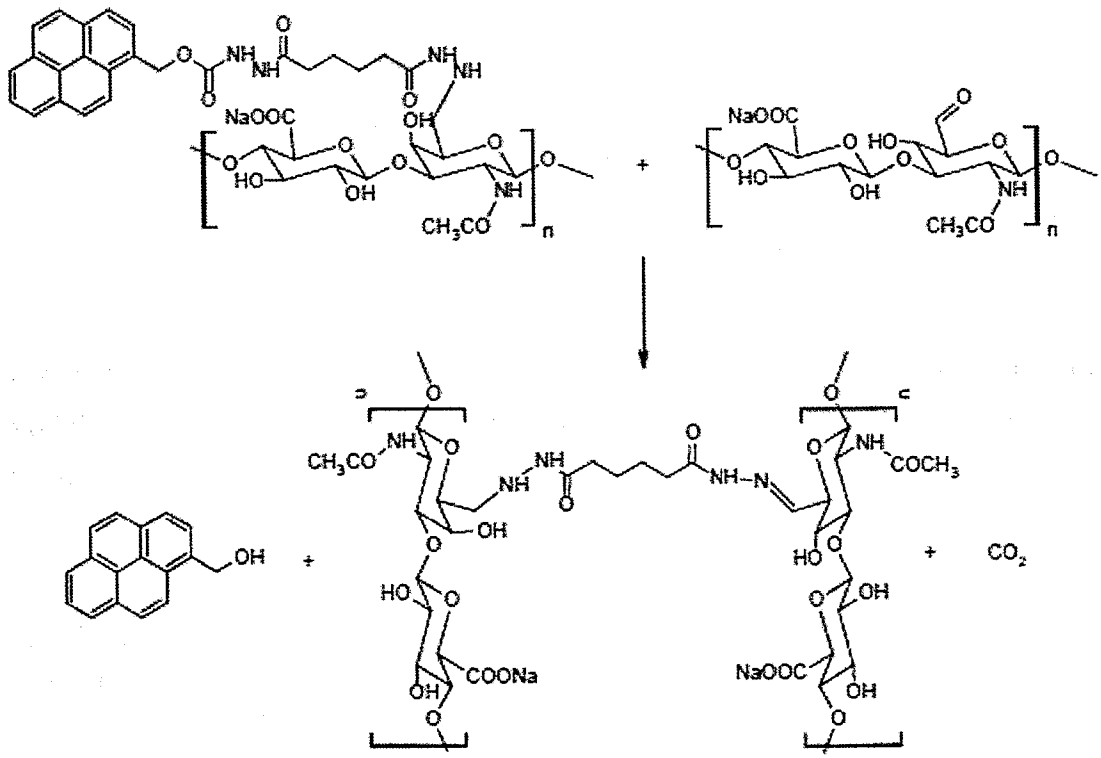
| | |
|---|---|
| $^1$H NMR ($D_2O$) | δ 7.58 (bs, 1H, -N=CH-); 5.60 (bs, 1H, -CH=C-) ppm |
| HSQC ($D_2O$) cross-peak | δ 7.58 ($^1$H) – 147.3 ($^{13}$C); 5.60 ($^1$H) – 110.30 ($^{13}$C) ppm |
| DOSY NMR ($D_2O$) | log D (2.04 ppm, Ac-NH-polymer) ~ -11.2 $m^2/s$ |
| | log D (5.60 ppm, -CH=C-) ~ -11.2 $m^2/s$ |
| | log D (7.58 ppm, -N=CH-) ~ -11.2 $m^2/s$ |
| | log D (4.75 ppm, $H_2O$) ~ -8.6 $m^2/s$ |

**Example 12. Photolysis of Pmoc-DHA-CS in the presence of saturated HA-aldehyde and crosslinking**

Method 1: Pmoc-DHA-CS (10 mg, 0.020 mmol, DS = 5%, Mw = 2-4 x $10^4$ g/mol) was dissolved in 1 mL of $D_2O$ in a quartz flask. HA-aldehyde (8 mg, 0.020 mmol, DS = 33%, Mw = 0.40 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and was irradiated for 1 h in UVA, under $N_2$, at 25 °C, pH = 7 while stirred. After 60 min of UVA exposition, 100% of hydrazone (δ= 7.60 ppm, HA-CH=N-DHA-CS) was formed.

Method 2: Pmoc-DHA-CS (10 mg, 0.020 mmol, DS = 5%, Mw = 2-4 x $10^4$ g/mol) was dissolved in 1 mL of PBS (c = 0.9%, pH = 7.4) in a quartz flask. HA aldehyde (8 mg, 0.025

mmol, DS = 33%, Mw = 0.40 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and was irradiated for 1 h in UVA, under $N_2$, at 25 °C, pH = 7 while stirred. After 1 h of UVA exposition, 70% of hydrazone was formed.



DS = 5%, hydrazone group

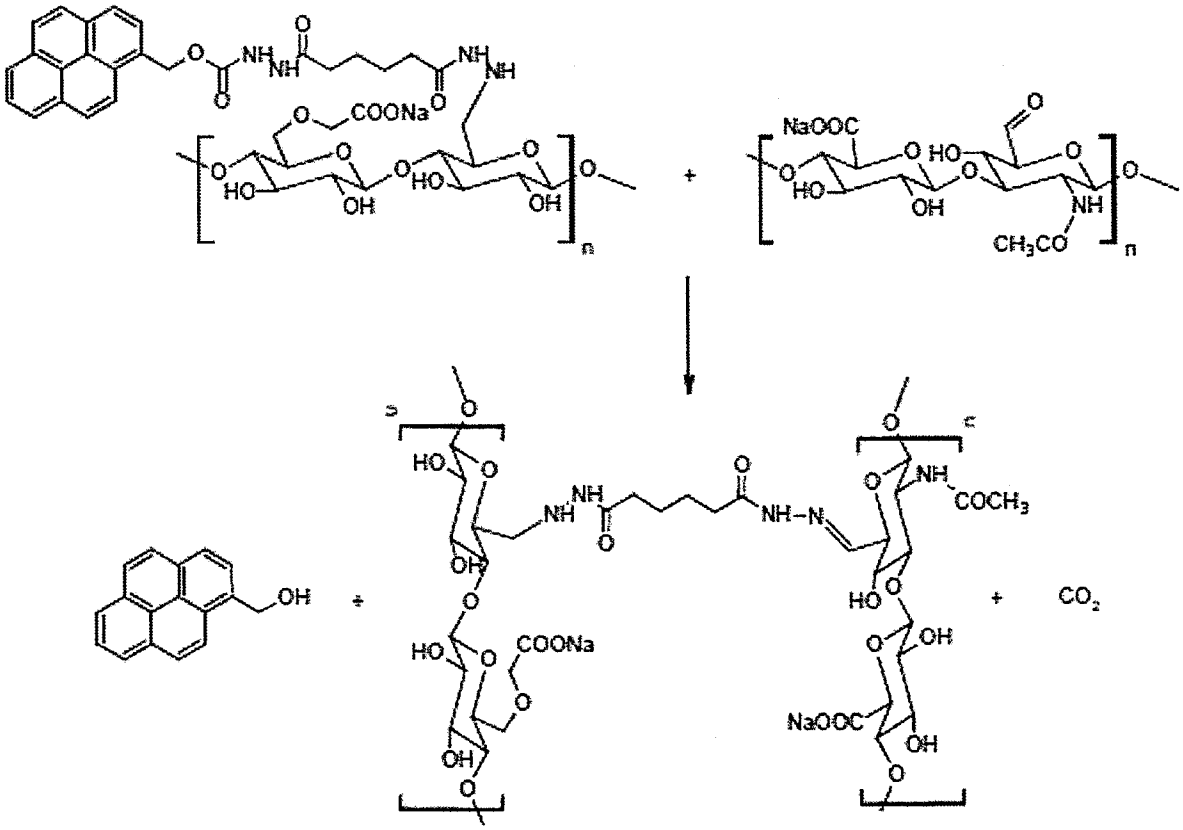
| | |
|---|---|
| $^1$H NMR ($D_2O$) | δ 7.60 (bs, 1H, -N=CH-) ppm |
| HSQC ($D_2O$) cross-peak | δ 7.60 ($^1$H) – 145.0 ($^{13}$C) ppm |
| DOSY NMR ($D_2O$) | log D (2.04 ppm, Ac-NH-polymer) ~ -11.2 $m^2/s$ |
| | log D (7.60 ppm, -N=CH-) ~ -11.2 $m^2/s$ |
| | log D (4.75 ppm, $H_2O$) ~ -8.6 $m^2/s$ |

**Example 13. Photolysis of Pmoc-DHA-CMCNa in the presence of saturated HA aldehyde and crosslinking**

Method 1: Pmoc-DHA-CMCNa (10 mg, 0.038 mmol, DS = 3-4%, Mw = 6-8 x $10^4$ g/mol) was dissolved in 1 mL of $D_2O$ in a quartz flask. HA-aldehyde (15 mg, 0.038 mmol, DS = 33%, Mw = 0.40 x $10^5$ g/mol). The sample was deoxygenated in the stream of nitrogen and

was irradiated for 1 h in UVA, under $N_2$, at 25 °C, pH = 7. After 60 min of UVA exposition, 100% of hydrazone (δ= 7.55 and 7.60 ppm, HA-CH=N-DHA-CMC) was formed.

Method 2: Pmoc-DHA-CMCNa (10 mg, 0.038 mmol, DS = 3-4%, Mw = 6-8 x $10^4$ g/mol) was dissolved in 1 mL of PBS (c = 0.9%, pH = 7.4) in a quartz flask. HA-aldehyde (15 mg, 0.038 mmol, DS = 33%, Mw = 0.40 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and was irradiated for 1 h in UVA, under $N_2$, at 25 °C, pH = 7 while stirred. After 1 h of UVA exposition, 90% of hydrazone was formed.

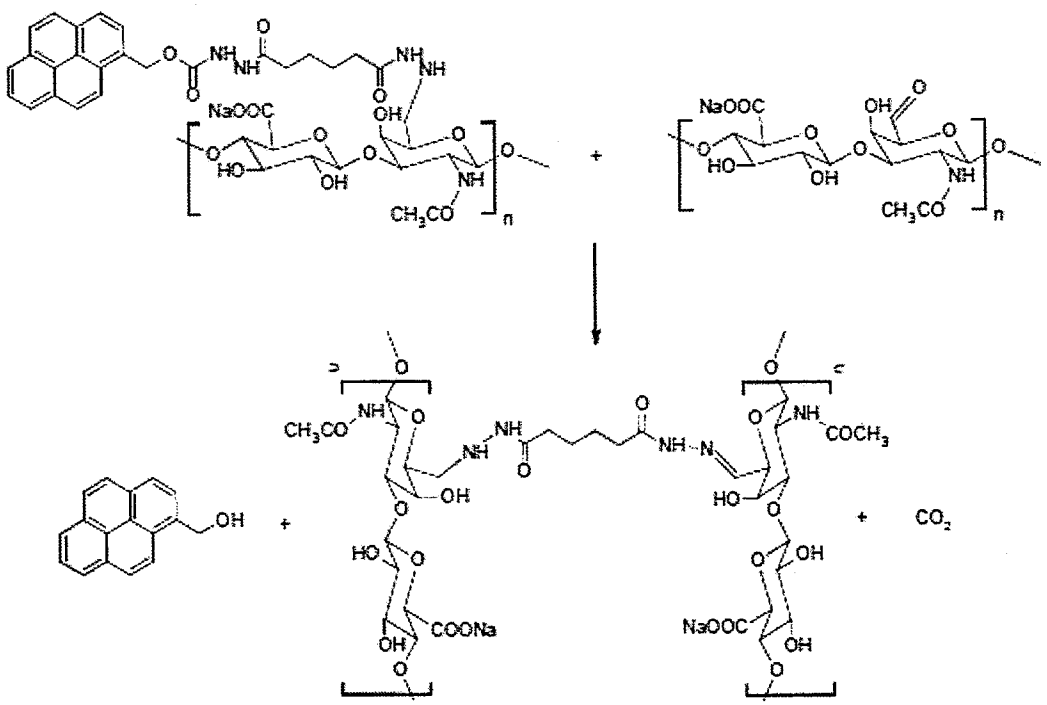


DS = 4%, hydrazone group

| | |
|---|---|
| $^1$H NMR ($D_2O$) | δ 7.55 and 7.60 (bs, 1H, -N=CH-) ppm |
| HSQC ($D_2O$) cross-peak | δ 7.55 ($^1$H) – 148.2 ($^{13}$C); 7.60 ($^1$H) – 148.2 ($^{13}$C); ppm |
| DOSY NMR ($D_2O$) | log D (2.04 ppm, Ac-NH-HA) ~ -11.4 $m^2/s$ |
| | log D (4.55 - 4,60 ppm, H1aH1'-CMCNa) ~ -11.4 $m^2/s$ |
| | log D (7.55 and 7.60 ppm, -N=CH-) ~ -11.4 $m^2/s$ |
| | log D (4.75 ppm, $H_2O$) ~ -8.6 $m^2/s$ |

**Example 14 Photolysis of Pmoc-DHA-CS in the presence of saturated CS-aldehyde and crosslinking**

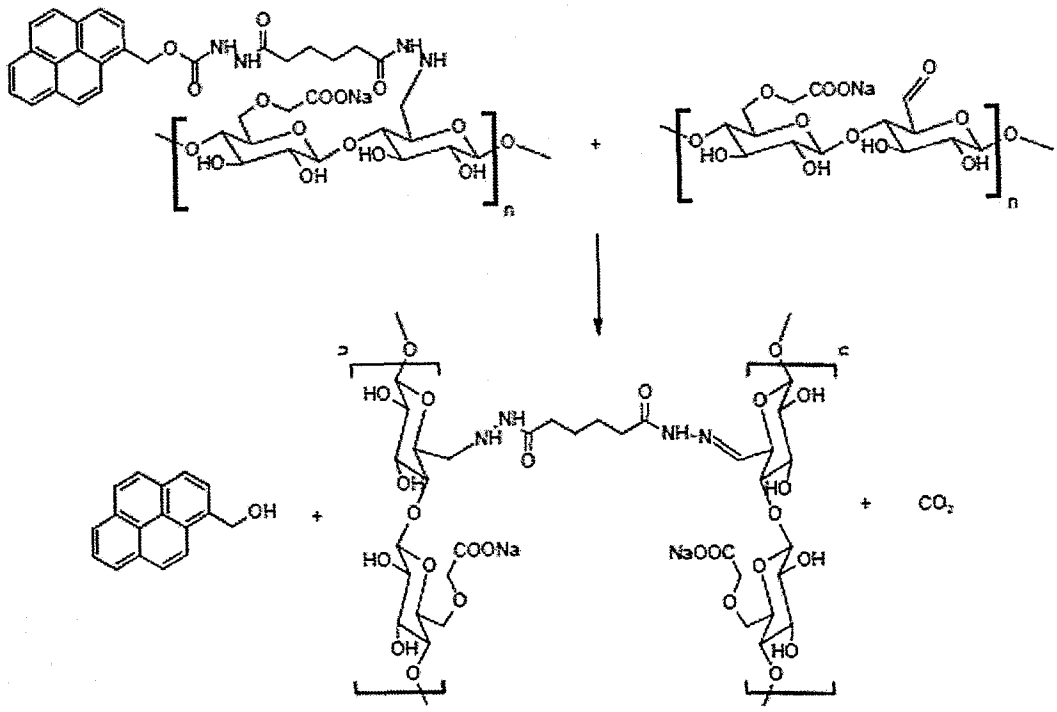
Pmoc-DHA-CS (10 mg, 0.020 mmol, DS = 5%, Mw = 2-4 x $10^4$ g/mol) was dissolved in 1 mL of PBS (c = 0.9%, pH = 7.4) in a quartz flask. CS (10 mg, 0.02 mmol, DS = 5%) was added. The sample was deoxygenated in the stream of nitrogen and was irradiated for 1 h in UVA, under $N_2$ at 25 °C, pH = 7, while stirred. After 60 min of UVA exposition, the viscosity increased.



$^1$H NMR ($D_2O$) δ 7.55-7.60 (bs, 1H, -N=CH-) ppm

**Example 15. Photolysis of Pmoc-DHA-CMCNa in the presence of CMCNa-aldehyde and crosslinking**

Pmoc-DHA-CMCNa (10 mg, 0.038 mmol, DS = 3-4%, Mw = 0.60-0.80 x $10^5$ g/mol) was dissolved in 1 mL of PBS (c = 0.9%, pH = 7.4) in a quartz flask. CMCNa-aldehyde (9 mg, 0.038 mmol, DS = 3-4%, Mw = 0.6 x $10^5$ g/mol) was added. The sample was deoxygenated in the stream of nitrogen and was irradiated for 1 h in UVA, under $N_2$, at 25 °C, pH = 7 while stirred. After 60 min of UVA exposition, the viscosity increased.



$^1$H NMR ($D_2O$) δ 7.55-7.60 (bs, 1H, -N=CH-) ppm

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# CLAIMS

1. A method of preparation of crosslinked polysaccharide materials according to the general formula (I)

polysaccharide1-$R^1$-N=CH-polysaccharide2 (I)

where polysaccharide1 and polysaccharide2 are identical or different polysaccharides and $R^1$ is $C_1$-$C_{30}$ alkyl residue, $C_1$-$C_{30}$ alkylaryl residue or $C_1$-$C_{30}$ alkylheteroaryl residue, optionally containing one or more identical or different heteroatoms selected from the group comprising N, O, S, **characterized in that**
an aqueous solution of aldehyde of polysaccharide 2 according to the general formula III

polysaccharide2-CH=O (III),

where the substitution degree of aldehyde in polysaccharide2 is within the range of 1 to 50 %, is added to an aqueous solution of polysaccharide 1 substituted on the site of amino group by a photoremovable group, according to the general formula II

polysaccharide1-$R^1$-NH-CO-O-$CH_2$-$R^2$ (II),

where $R^1$ is defined above; $R^2$ is an aromatic system, and where the substitution degree of carbamate in polysaccharide 1 is within the range of 1 to 10 %,
and the formed mixture is subjected to electromagnetic radiation and deoxygenation simultaneously.

2. The method of preparation according to claim 1, **characterized in that** the substitution degree of carbamate in polysaccharide 1 is within the range of 3 to 10% and its molecular weight is 10 to 400 kDa, preferably 20 to 300, more preferably 20 to 100 kDa.

3. The method of preparation according to claim 1 or 2, **characterized in that** the substitution degree of aldehyde in polysaccharide 2 is within the range of 3 to 25% and its molecular mass is 10 to 800 kDa, preferably 50 to 250 kDa.

4. The method of preparation according to any of the preceding claims, **characterized in that** polysaccharide 1 and polysaccharide 2 are selected from the group comprising hyaluronan, chondroitin sulfate, cellulose and pharmaceutically acceptable derivatives and/or salts thereof.

5. The method of preparation according to any of the preceding claims, **characterized in that** $R^1$ is selected from the group comprising adipic acid dihydrazide and hexamethylene diamine.

6. The method of preparation according to any of the preceding claims, **characterized in that** $R^2$ is selected from the group comprising pyrene, anthracene, phenanthrene, perylene, anthraquinone, coumarin and substitution derivatives thereof, that can contain atoms C, H, O, S, N in their structure and that exhibit absorption of electromagnetic radiation.

7. The method of preparation according to any of the preceding claims, **characterized in that** the weight ratio of polysaccharide 1 to polysaccharide 2 is within the range of 1:2 to 2:1.

8. The method of preparation according to any of the preceding claims, **characterized in that** the mixture is subjected to electromagnetic radiation for 0.25 to 2 hours, preferably 0.5 to 1 hour, at the temperature of 10 to 50 °C, preferably 25 to 35 °C.

9. The method of preparation according to any of the preceding claims, **characterized in that** the aqueous solutions of polysaccharides 1 and 2 further contain water soluble agents selected from the group comprising inorganic salts, or buffers, preferably phosphate buffer, wherein pH of the solution is within the range of 6.5 to 7.5, preferably 7.0.

10. The method of preparation according to any of the preceding claims, **characterized in that** electromagnetic radiation of wavelength 320-400 nm, preferably 330-370 nm is used.

11. The method of preparation according to any of the preceding claims, **characterized in that** the reaction is temporal controlled by means of a switch of the electromagnetic radiation source or by means of a pulse source of electromagnetic radiation or by means of shading of the reaction.

12. The method of preparation according to any of the preceding claims, **characterized in that** the reaction is spatially controlled by means of a photomask, focused electromagnetic radiation or a beam of electromagnetic radiation.

13. Material prepared by the method defined in any of claims 1-12 for use in the area of tissue engineering, regenerative medicine in the form of scaffolds, fillers or in drug delivery.

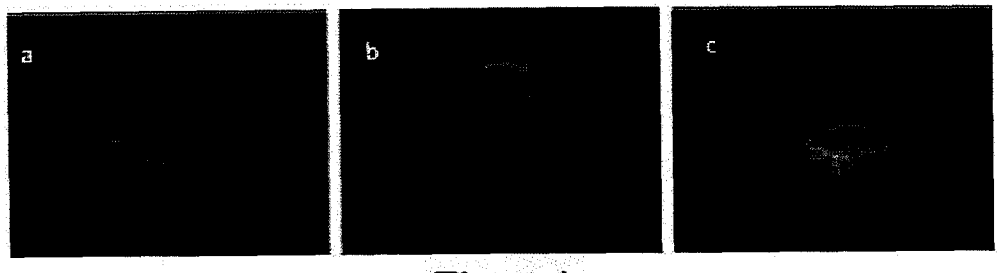


Figure 1

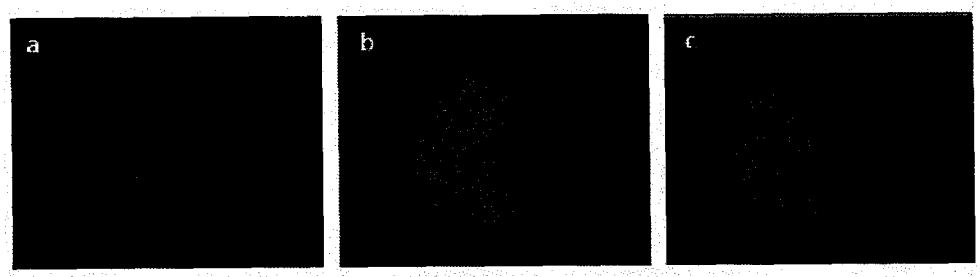


Figure 2

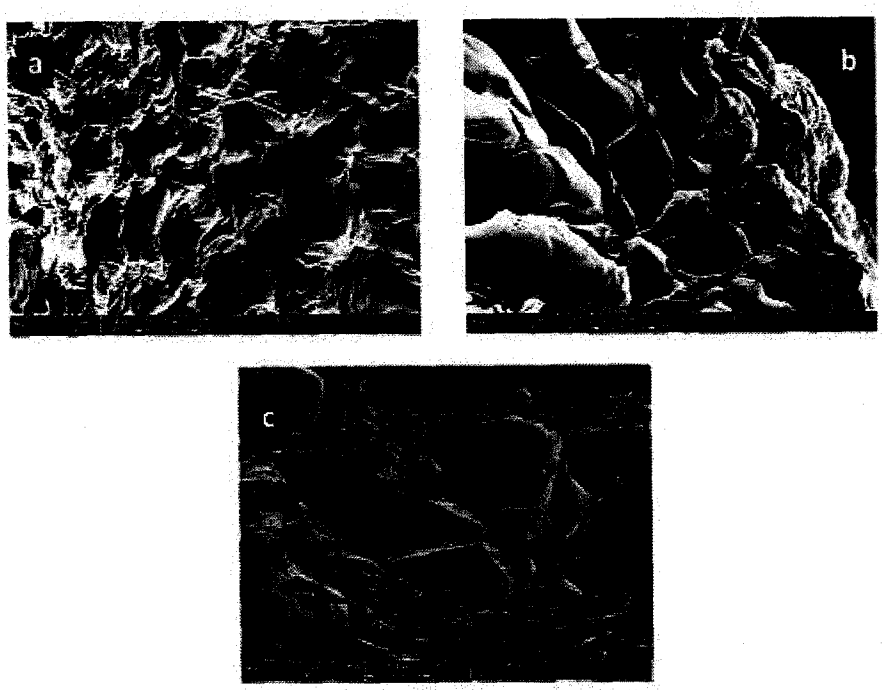


Figure 3

# INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2016/000065

A. CLASSIFICATION OF SUBJECT MATTER
INV. C08B37/08 C08B15/00 C08B37/00 A61K47/48
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

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)
EPO-Internal, BIOSIS, COMPENDEX, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| A | Scheme 5; example 17 | 1-12 |
| | ----- | |
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| A | the whole document | 1-12 |
| | ----- | |
| | -/-- | |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"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 | Date of mailing of the international search report |
|---|---|
| 22 September 2016 | 30/09/2016 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Vaccaro, Eleonora |

# INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2016/000065

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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International application No
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| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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