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α,β-Unsaturated aldehyde of hyaluronan—Synthesis, analysis and applications

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ABSTRACT

Hyaluronic acid (HA) modified with an aldehyde group (HA-CHO or HA-aldehyde) has been extensively used for various biomedical applications. The main advantage of the aldehyde moieties is the ability to react with a wide range of amino compounds under physiological conditions. Reactions of aldehydes with primary amines in water are reversible and equilibrium is thoroughly shifted towards starting aldehyde and amine. This work presents an unique modification of HA: α,β-unsaturated aldehyde of HA (4,5-anhydro-6(GlcNAc)-oxo HA or ΔHA-CHO), which allows the primary amines to be attached to HA more effectively in comparison to the saturated HA-CHO. Higher hydrolytic stability is caused by the conjugation of imine with an adjacent —C=C— double bond. Two strategies for the preparation of unsaturated HA-aldehyde were developed and chemical structures were studied in details. Cross-linked materials prepared from this precursor are biocompatible and suitable for applications in drug delivery and regenerative medicine.

1. Introduction

The development of new biomaterials for drug delivery and tissue engineering showed a significant progress in last decade. Many of them have been derived from natural components of the extracellular matrix. One of the most common and promising is hyaluronic acid (HA), a linear polysaccharide consisting of alternating β-1,4-linked units of β-1,3-linked glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc). HA is present in high concentration in extracellular matrix, articular cartilage, and synovial fluid and is also involved in cell mobility and cell differentiation or the regulation of wound healing (Balazs, 2009; Aya & Stern, 2014). Injection of HA into osteoarthritic joints significantly improves joint function (Muzzarelli, Greco, Busilacchi, Sollazzo & Gigante, 2012). Thanks to its biodegradability and biocompatibility, HA can be considered as an attractive building block for new biomaterials (Mortisen, Peroglio, Alini, & Eglin, 2011).

HA derivatives are often used in biomedical applications as precursors for crosslinked materials or as substrates for drug delivery systems. One of the most versatile precursors is based on the preparation of HA-aldehyde and subsequent conjugation with biologically active *N*-nucleophiles (Mero, Pasqualin, Campisi, Renier, & Pasut, 2013) or with polyamine to form hydrogels (Li, Fu & Zhang, 2014). The main advantage of the aldehydic groups is their ability to react with a wide range of the amino compounds even under the physiological conditions (Emoto et al., 2014). Direct oxidation of a macromolecular chain of HA is based either on cleavage of a vicinal diol with sodium periodate in the positions 2 and 3 of GlcNAc (Kuo, 2005; Wang, Oommen, Yan, & Warghese, 2013), or on the oxidation of the position 6 of GlcNAc with TEMPO/NaClO (Buffa et al., 2011; Šedova et al., 2013) eventually with Dess-Martin periodate (Buffa, Kettou, Pospíšilová, Berková & Velebný, 2011; Šedova et al., 2013). The main advantage of the oxidation in the position 6 of GlcNAc are intact saccharide rings of a polymeric chain while the oxidation with sodium periodate produces a less rigid (semi-broken) polymeric structure. Another way how to introduce an aldehyde group to the HA chain is an insertion of a linker containing a vicinal diol, which can be easily transformed to an aldehydic moiety by a selective oxidation with sodium periodate (Ossipov, Kootala,

Abbreviations: 3T3, standard fibroblast cell line; COSY, correlation spectroscopy; DMAP, 4-dimethylaminopyridine; DOSY, diffusion ordered spectroscopy; DS, degree of substitution; FTIR, Fourier transform infrared spectroscopy; HA, hyaluronan; h, hour; HSQC, heteronuclear singlequantum coherence; IPA, isopropyl alcohol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Mw, molecular weight; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; SEC-MALLS, size exclusion chromatography — multi angle laser lightscattering; TEA, tri-ethylamine; UV/vis, ultraviolet and visible spectroscopy; δ, chemical shift.

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Yi, Yang & Hilborn, 2013). The main application of HA-aldehydes is for biocompatible hydrogels where in vivo crosslinking reactions are needed. This process can be performed either with encapsulation of active compounds and cells (Emoto et al., 2014; Li, Fu et al., 2014) or without encapsulation in which the hydrogel itself offers specific biological functions e. g. prevention of postoperative adhesions (Li et al., 2014). The microfibres of HA-aldehyde were also fabricated and treated with dihydrazides as crosslinking agents. The surface was chemically crosslinked leading to hydrazone functionalized microfibers with decreased solubility in water compared to the microfibers prepared from a native HA (Bět'ák et al., 2014). Anyway, all state of art oxidation procedures lead to a saturated aldehyde of HA without any conjugated double bond.

The aim of this work was to study a new type of HA-aldehyde with a conjugated —C=C— double bond in the positions 4 and 5 of GlcNAc, its biocompatibility and its ability to attach a wide range of amino compounds under mild conditions. The structural analysis of final materials was performed by advanced NMR techniques, SEC-MALLS analysis and UV–vis spectroscopy. Mechanical properties of cross-linked hydrogels were also measured and compared with known materials.

2. Materials and methods

2.1. Chemicals and reagents

Hyaluronan sodium salt was provided by Contipro Biotech Ltd., Dolní Dobrouč, Czech Republic, D_2O (99.8%, CortecNet), DMSO-d_6 (99.9%, CortecNet) were used as obtained. DIPEA — *N,N*-diisopropyl-*N*-ethylamine (99%), DMSO — dimethyl sulfoxide (99.96%), pyridine (99.96% p.a.), AcOH — acetic acid (99.8% p.a.), $NaHCO_3$ (99.8% p.a.) and Na_2CO_3 (99.8% p.a.) were obtained from Lachner. DMF — dimethylformamide (99.8%), arginine (98%), lysine (98%), 1,6-hexamethylenediamine (98%) were purchased from Sigma–Aldrich. Et_3N — triethylamine (99% p.a.) was obtained from Penta and 1-butylamine (98%), KH_2PO_4 (99.6%), $Na_2HPO_4{\cdot}12H_2O$ (99%) and $Na_3PO_4{\cdot}12H_2O$ (98%) from FLUKA. PBS – 80 mg NaCl, 2 mg KCl, 28.5 mg $Na_2HPO_4{\cdot}12H_2O$, 2 mg KH_2PO_4 in 10 ml H_2O. Deionized water was used in all experiments.

2.2. NMR spectroscopy

Solution-state NMR spectroscopy was carried out on a Bruker Avance III. All of the spectra were acquired and elaborated by Bruker 2.1 Topspin software. About 10 mg of sample was dissolved in D_2O, DMSO-d_6 or $CDCl_3$ (0.8 ml) and transferred into 5 mm NMR quartz tubes. HSQC NMR spectra were acquired using gradient-pulse sequence and 1k data points, 3 kHz spectral width in f2, 80scans per increment, 256 increments, and heteronuclear scalar coupling C–H set at 145 Hz. DOSY NMR experiments were performed using a stimulated echo pulse sequence with bipolar gradients (2.5 ms) and water gate 3–9–19 pulse train with gradients, diffusion time 0.8 s, and 2.0 ms sine-shaped pulses with 32.030 G cm^{-1}.

2.2.1. Degree of substitution (DS) determination

4,5-Anhydro-6(GlcNAc)-oxo HA: The content of α,β-unsaturated aldehyde was determined by ^1H NMR spectroscopy. The samples were dissolved in D_2O. DS was defined as a molar ratio (integral) of proton at 6.32 ppm in a double bond and three protons of a methyl group in GlcNAc at 2.0 ppm multiplied by 100.

Deacetylated HA: The content of deacetylated HA was determined by ^1H NMR spectroscopy. Samples were dissolved in 1% NaOD in D_2O. DS was defined as a molar ratio (integral) of proton at 2.75 ppm in glucosamine unit and two anomeric protons at 4.40–4.59 ppm multiplied by 100.

2.3. Molecular weight (Mw) determination

Molecular weight of HA was assigned using SEC-MALLS. Samples were dissolved overnight in a mobile phase (0.1 M $NaH_2PO_4.2H_2O$ with 0.05% NaN_3, pH 7.4) with concentration between 1 and 20 mg ml^{-1}. The chromatographic system consisted of a LC-10ADVP Shimadzu HPLC pump, SIL-10AF autosampler, CTO-10AVP column oven, SCL-10AVP system controller, DGU-14A degasser, RID-10A refractive index detector, SPD-10AVVP UV–vis detector (all from Shimadzu), and miniDAWN TREOS light scattering photometer (Wyatt Technology Corporation). Data acquisition and Mw calculations were performed using ASTRA software (version 5.3.4, Wyatt Technology Corporation, USA). The specific refractive index increment of 0.155 ml g^{-1} was used for HA.

2.4. UV/vis spectroscopy

UV/vis spectroscopy was performed on a Shimadzu UV-2401PC spectrometer in a range of 200–600 nm. UV spectra were processed by software UV Probe version 2.00.

2.5. Preparation of HA-aldehyde

Regioselective oxidation of HA in position 6 of glucosamine part was performed according to previously described procedure (Šedova et al., 2013). Briefly, 1 g of sodium hyaluronate was dissolved in 100 ml of demineralized water containing 5 equivalents of sodium hydrogen phosphate. TEMPO (0.01 eq.) of the catalyst was added, the mixture was stirred briefly and 0.5 equivalent of sodium hypochlorite was added at 5 °C. The reaction mixture was stirred for 2 h at this temperature. A final product was isolated by dialysis (cut off 12 kg/mol) against demineralized water and freeze-dried.

2.5.1. Preparation of 4,5-anhydro-6(GlcNAc)-oxo hyaluronan (unsaturated HA-aldehyde)

To a 3% solution of HA-aldehyde (1 g, DS = 12%, Mw 798 kg/mol) in water 70 ml of DMSO and DIPEA (5 eq.) were added. The mixture was stirred for 72 h at 40 °C. The product was isolated by the precipitation with a solvent mixture of isopropanol/hexane (5/2) and dried in vacuum to give a white powder with DS = 5% and Mw = 110 kg/mol.

NMR ^1H (500 MHz, D_2O, δ ppm): 9.24 (s, 1H, —CH=O), 6.32 (m, 1H, —CH=C—CH=O)

NMR ^1H–^{13}C HSQC (D_2O): crosspeaks, δ ppm: 9.24H–189C, 6.32H–121C.

NMR ^1H–^1H COSY (D_2O) crosspeaks, δ ppm: 9.24 no cross signal, 6.32H–4.52H.

NMR DOSY (D_2O), δ ppm: log D (9.24, 6.32, 2.01 (CH_3—CO—NH-polymer), ~−9.8 m^2 s^{-1}, log D (4.72, H_2O) ~−8.2 m^2 s^{-1}.

UV–vis (H_2O) 252 nm, π–π^* transition of —CH=CH—CHO double bond.

SEC-MALLS: Mw = 110 kg/mol.

2.5.2. Preparation of 4,5-anhydro-6(GlcNAc)-oxo hyaluronan (unsaturated HA-aldehyde) — solvent free procedure.

HA-aldehyde (0.1 g, DS = 10%, Mw = 10 kg/mol) was heated in a powder form in closed Erlenmeyer flask under nitrogen for 2 days at 90°C (Table 1, entry 17) giving α,β-unsaturated HA-aldehyde, DS = 2% determined by NMR (details in Chapter 2.5.1.) SEC-MALLS: Mw = 58 kg/mol