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Conjugates of modified hyaluronic acid with amino compounds for biomedical applications

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ABSTRACT

Hyaluronic acid (HA) modified with an aldehyde group (HA-CHO) has enormous potential for the covalent attachment of the amino compounds and for the crosslinking of HA with bis or multi-functional amino linkers under physiological conditions. Modification of HA-CHO to its α,β-unsaturated analogue (ΔHA-CHO) generally increases the stability of the reversible imino-attachment due to conjugation of the imine moiety with the adjacent −C═C− double bound. Conjugation of a wide range of structurally different amines including the amines with enhanced biological activity were studied in detail. Results showed that the stabilities of the final conjugates ΔHA−CH═N−R significantly depend on the chemical structure of the amine and on the pH value.

1. Introduction

Development of the new materials for drug delivery and tissue engineering showed a quick progress in last years. One of the most popular is hyaluronic acid (HA), a natural component of the extracellular matrix, articular cartilage and synovial fluid. This polymer is a linear glycosaminoglycan consisting of alternating β-1,4-linked units of β-1,3-linked glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc). HA derivatives are often used as precursors for drug delivery systems or as substrates for preparation of crosslinked materials. One of the most versatile derivative is HA-aldehyde which can efficiently react with *N*-nucleophiles (Mero, Pasqualin, Campisi, Renier, & Pasut, 2013) even under physiological conditions (Emoto et al., 2014). Reactivity of *N*-nucleophiles with HA-aldehyde extensively depends on the character of the atom X connected to the terminal amino group of the *N*-nucleophile (NH_2-X- …). If X is carbon, stability of the corresponding imino-conjugate is extremely low. If X is oxygen or nitrogen, stability is significantly improved due to stabilizing conjugation between the imine double bond and the adjacent atom bearing a lone electron pair (Buffa et al., 2014).

In our previous paper (Buffa et al., 2015) we reported synthesis, analysis and basic chemical and physical characteristics of 4,5-anhydro-6(GlcNAc)-oxo HA (ΔHA-CHO). This biocompatible polymer can be prepared by the oxidation of the hydroxyl group in the position 6 of GlcNAc with TEMPO/NaClO (Buffa, Kettou, Pospíšilová, Huerta-Angeles et al., 2011; Šedova et al., 2013) or if higher degrees of modification are needed, Dess-Martin periodate in DMSO can be used as an oxidation agent (Buffa, Kettou, Pospíšilová, Berková, & Velebný, 2011; Šedova et al., 2013). Standard oxidation of HA with sodium periodate (Sahiner, Jha, Nguyen, & Jia, 2008) produces a less rigid polymer (saccharide cycle is opened), which cannot be subsequently dehydrated without any degradation of the polymer. On the other hand, the oxidation of HA in the position 6 of GlcNAc maintains compact saccharide cycle and involves mild and regioselective dehydration in positions 4 and 5.

Modification of HA is often focused on the development of drug delivery systems applied in various biomedical applications (Drobnik 1991). It was proved that hydrolytically cleavable covalent conjugation of an active compound with HA can dramatically improve solubility, absorption and efficacy of the drug (Vercruysse & Prestwich 1998). Furthermore, many cancer cells showed increased concentration of HA receptors and this fact can be used for an effective design of anticancer drug- delivery systems (Luo, Ziebell, & Prestwich, 2000). Variety of active molecules have been covalently conjugated on HA via irreversible ester bond which can be enzymatically hydrolyse in vivo (Schante, Zuber, Herlin, & Vandamme, 2011). Another type of pH-responsible

Abbreviations: COSY, correlation spectroscopy; DIPEA, diisopropylethylamine; DS, degree of substitution; DMSO, dimethylsulfoxide; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; HA, hyaluronan; h, hour; HSQC, hetero nuclear single quantum coherence; IPA, isopropyl alcohol; Mw, molecular weight; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; SEC-MALLS, size exclusion chromatography – multi angle laser light scattering; TEA, triethylamine; UV/vis, ultraviolet and visible spectroscopy; δ, chemical shift

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materials contains functional groups like −COOH or $-NH_2$ which are able to change their polarity depending on the pH value. Repulsion or attraction between charged atoms can transform the final 3D composition and release encapsulated active compounds. These materials have been succesfully applied in the field of pH-controled drug delivery systems (Sheng, Palaniswamy, & Kam, 2008). Another approach is based on stability of the specific chemical bond where the most common connections are imines (−CH═N−) or alfa,beta-unsaturated amides (−NH−CO−CH═CH−). Both are less stable in acidic condition and this capability was successfully tested in the field of anticancer materials (Kakinoki, Kaneo, Ikeda, Tanaka, & Fujita, 2008).

The aim of this work was to study imino-conjugates of ΔHA-CHO with variety of structurally different amines. As it was expected, hydrolytic stability of the final imino-conjugates depended on the chemical structure of the amines and on the pH value of the system. Series of amines including biologically active compounds were irreversibly attached on ΔHA-CHO and their pH-dependent stability were studied in detail. The structural analysis of the final materials was performed by advanced NMR techniques and UV–vis spectroscopy.

2. Materials and methods

2.1. Chemicals and reagents

Hyaluronic acid 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.), $NaHCO_3$ (99.8% p.a.) and Na_2CO_3 (99.8% p.a.) were obtained from Lachner. Et_3N – triethylamine (99% p.a.) was obtained from Penta and 1-butylamine (98%), KH_2PO_4 (99.6%), $Na_2HPO_4.12H_2O$ (99%) and $Na_3PO_4.12H_2O$ (98%) from FLUKA. 6-aminohexanoic acid, 4-bromoaniline, 4-nitroaniline, 4-toluensulfonylhydrazide, adenine, benzhydrazide, benzocaine, D-glucosamine, dihydrazideadipate, Doxorubicin, Dopamine, Histamine, Hydralazine, hydrazinium sulfate, hydroxylamine, hydroxylamine-O-sulfonic acid, phenylhydrazine, propane-1,3-bisoxyamine, Serotonine, Sulfanilamide and Thiamine were purchased from Sigma Aldrich. PBS − 80 mg NaCl, 2 mg KCl, 28.5 mg $Na_2HPO_4.12H_2O$, 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 the spectra were acquired and elaborated by Bruker 2.1 Topspin software. 5–10 mg of sample was dissolved in D_2O or DMSO-d_6 (0.8 mL) and transferred into 5 mm NMR quartz tubes. HSQC NMR spectra were acquired using edited 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.

2.2.1. Degree of substitution (DS) determination

4,5-Anhydro-6(GlcNAc)-imino HA (ΔHA−CH═N−R): The content of imino conjugates of ΔHA-CHO was determined by ^{1}H NMR spectroscopy. The samples were dissolved in D_2O. DS was defined as a molar ratio (integral) of imino proton −N═CH− in the area between 7.20–8.30 ppm and three protons of a methyl group of GlcNAc at 1.97 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 $NaH_2PO_4{\cdot}2H_2O$ 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 Cary 100 Conc UV–vis spectrophotometer, software Varian.

2.5. Preparation of HA-aldehyde

Oxidation of HA on primary hydroxy group was performed according to previously described procedures (Šedova et al., 2013) where HA-CHO is formed as a main product and HA-COOH is a minor product.

Procedure 1: 1 g of sodium hyaluronate (Mw = 750 kDa) was dissolved in 100 mL of demineralized water containing 3 equivalents of sodium hydrogen phosphate. Aminoxy radical TEMPO was added in catalytic amount (0.01 eq.) and then 0.5 equivalent of sodium hypochlorite was slowly dropped at 5 °C. The reaction mixture was stirred for 1 h at this temperature. A final product was isolated by dialysis (Cut off 12 kDa) against demineralized water and freeze-dried. DS of HA-CHO is 11%, DS of HA-COOH is 8%, Mw = 264 kDa.

Procedure 2: Sodium hyaluronate, converted to the acidic form by ion exchange using H^+catex according to literature (Haxaire, Maréchal, Milas, & Rinaudo, 2003) and isolated by freeze-drying, was dissolved in anhydrous dimethyl sulfoxide overnight to form a 1% (w/v) solution. Dess-Martin periodinane (0.7 eq.) was slowly added and the solution was stirred at room temperature for 24 h. The final mixture was diluted with demineralized water to double the volume. The product was isolated by dialysis (Cut off 6–8 kDa) against the mixture of 0.1% NaCl, and 0.1% $NaHCO_3$ (3 × 5L) and demineralized water (8 × 5L). DS of HA-CHO is 24%, DS of HA-COOH is 12%, Mw = 369 kDa.

2.6. Preparation of 4,5-anhydro-6(GlcNAc)-oxo hyaluronan (ΔHA-CHO)

ΔHA-CHO was synthetized according to previously described procedure (Buffa et al., 2015). Briefly, to a 3% solution of HA-aldehyde (1 g, DS = 11 or 24 %, Chapter 2.5, Procedures 1 and 2) 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 isopropanol/hexane in the volume ratio 5/2 and dried in vacuum to give a white powder with DS = 7% and Mw = 50 kDa or DS 15% and Mw = 207 kDa.

2.7. General procedure for conjugation of amines to ΔHA-CHO

Amine (1.5 eq. of hydrazide, hydrazine, oxyamine, alkyl or arylamine, eq. related to the molar amount of the aldehyde moieties) was added to the 1% solution of ΔHA-CHO (DS = 7 or 15%) in 0.9% solution of NaCl in water and the pH value was adjusted by adding of 0.01 M aqueous solution of hydrochloric acid or sodium hydroxide. The mixture was then stirred at room temperature 1 h. If D_2O was used as a solvent, the crude mixture was directly measured by NMR spectroscopy. Otherwise the final product was isolated by the precipitation with isopropanol and dried in vacuum. NMR spectra were measured also after 2 and 5 h and in all cases (at pH 5–9) the DS is the same as the DS measured after 1 h. It means that the equilibrium aldehyde ↔ hemiaminal ↔ imine ↔ aminal (Scheme 1) is established earlier than in 1 h.

The precise values of DS and the chemical shifts in NMR analysis depended on the structure of the amines and on the pH value. Data are described in Chapters 3.1–3.5.