Carbohydrate Polymers 137 (2016) 255–263

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Synthesis of photo-crosslinkable hyaluronan with tailored degree of substitution suitable for production of water resistant nanofibers

Gloria Huerta-Angeles*, Martina Brandejsová, Kateřina Knotková, Martina Hermannová, Martina Moravcová, Daniela Šmejkalová, Vladimír Velebný

Contipro Pharma a.s., Dolni Dobrouč 401, 561 02, Dolni Dobrouč, Czech Republic

ARTICLE INFO

Article history:
Received 15 July 2015
Received in revised form 9 October 2015
Accepted 24 October 2015
Available online 2 November 2015

Keywords:
Hyaluronic acid
Chemical modification
Nanofibers
Photocross-linking

ABSTRACT

In this work, hyaluronan (HA) was grafted by a novel and an efficient mixed anhydrides methodology with (hetero)-aryl and aliphatic acrylic moieties suitable for cross-linking. A precise control of stoichiometry was achieved. Derivatives with degree of substitution (DS) below 20% did not show self-crosslinking. Due to mild reaction conditions, a negligible degradation of the polysaccharide was obtained. The influence of the feed components on the reaction efficiency and DS were studied up to 200 g/batch. The structure of the modified HA was characterized by Infrared Spectroscopy, Nuclear Magnetic Resonance, SEC-MALS and chromatographic analyses. Enzymatic degradation of derivatives was performed and isolated dimers demonstrated to be non-cytotoxic. The feasibility of the grafted HA for electrospinning with subsequent photo-crosslinking to avoid nanofibers water dissolution was demonstrated. The biocompatibility of the material, its degradation products, and the formation of honeycomb porous structures also proved the potential of the material for future *in vivo* applications.

1. Introduction

Nanofiber scaffold formulations are widely used in various fields such as tissue engineering, drug delivery, cosmetics or wound dressing (Garg, Rath, & Goyal, 2015). Biopolymers are the most suitable materials that can be used for these formulations, because they meet several requirements such as biodegradability and biocompatibility (Zhao, Liu, Li, Lin, & Wang, 2015). Among them, hyaluronic acid (HA) or hyaluronan, a biopolymer consisting of D-glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc), has found a wide range of applications in medicine due to a positive influence during wound healing and the angiogenic character of its degradation products (Uppal, Ramaswamy, Arnold, Goodband, & Wang, 2011).

The electrospinning of HA in the absence of surfactants or toxic reagents as additives is still challenging due to the inherent properties of the polysaccharide, *e.g.* low electrical conductivity or high viscosity of solutions. Sodium hydroxide (NaOH) in *N*, *N*-dimethyl formamide (DMF) was used to prepare HA nanofibers (Kim, Chung, & Park, 2008). However, NaOH seriously degrades the HA backbone and this procedure cannot be recommended (Brenner, Schiffman, Thompson, Toth, & Schauer, 2012). Cocamidopropyl betaine, a known skin allergen was employed for the same purpose (Uppal et al., 2011). Unfortunately, the high toxicity of solvents and additives used for nanofiber production is still not considered a risk (Pelipenko, Kocbek, & Kristl, 2015). The main drawbacks of nanofibers made of unmodified HA are fast water solubilization, poor mechanical stability and rapid degradation (Pelipenko et al., 2015). To overcome these drawbacks, physical or chemical cross-linking could be applied.

Physical cross-linking may cause the formation of imperfect nanofibers (Sinha-Ray, Khansari, Yarin, & Pourdeyhimi, 2012). Chemical cross-linking can be carried out *in situ* using an external reagent (Tang, Saquing, Harding, & Khan, 2009), which can be electrospinned together with the polysaccharide but requires an additional process of purification. Cross-linking with external reagents after the electrospinning process was demonstrated to be poor (Arnal-Pastor, Martinez Ramos, Perez Garnes, Monleon Pradas, & Valles Lluch, 2013). Photo cross-linking could be applied after electrospinning but it requires a suitably modified polymer backbone (Liu, Bolger, Cahill, & McGuinness, 2009; Zeng, Hou, Wendorff, & Greiner, 2005).

A major challenge of industrial development is the modification of HA avoiding the use of toxic reagents in the chemical processes due to the difficulties for complete elimination from the final product toward pharma applications (Maeda, Miao, Simmons, Dordick, & Linhardt, 2014). Additionally, the processing of the derivative is also important. For nanofibers processing, some reagents

* Corresponding author. Tel.: +420 465 519 573; fax: +420 465 543 793.
E-mail address: huerta-angeles@contipro.com (G. Huerta-Angeles).

http://dx.doi.org/10.1016/j.carbpol.2015.10.077
0144-8617/

might cause undesired intramolecular cross-linking and induce structural defects (Saska et al., 2012). In terms of HA, the synthesis of HA derivatives suitable for chemical cross-linking was previously reported by means of 1,1′-carbonyldiimidazole (CDI) activation, for the grafting of lipoic (Picotti et al., 2013) or ferulic acid to HA (Cappelli et al., 2014). Unfortunately, the process becomes inconvenient in high scale due to the use of formamide as reaction solvent, which is corrosive and toxic. Another work discusses the synthesis of HA-derivatives useful for photo cross-linking by HA grafting with glycidyl methacrylate (Oudshoorn, Rissmann, Bouwstra, & Hennink, 2007). Unfortunately, (i) the reaction was carried out in dimethylsulfoxide (DMSO) for 48 h at 50 °C and (ii) the polysaccharide must be converted into a more apolar form (tetrabutylammonium salt) to become soluble in organic solvent before the chemical modification. The conversion generates low yields and produces toxic degradation products (Smejkalová et al., 2012; Yuan et al., 2015). Additionally, such conversion leads to significant HA chain fragmentation, and loss of material, resulting in poor reproducibility and low yields. The methacrylation of HA was also carried out in water at high temperature (Baier Leach, Bivens, Patrick, & Schmidt, 2003). Unfortunately, the polysaccharide-backbone undergoes rapid depolymerization when exposed to high temperature (Lowry & Beavers, 1994). A recently published work using phase transfer synthesis for acrylation, reported to be effective only for low molecular weight HA (Becher, Moller, & Schnabelrauch, 2013). And thus the functionalization with photo-reactive moieties has not been produced with a precise control of stoichiometry (Wang, Messman, Mays, & Baskaran, 2010).

Because of the above-mentioned disadvantages of the reported methods, alternative ways of synthesis of photo-cross-linkable HA must be developed. In a previous work, we have demonstrated the use of 2, 4, 6 trichlorobenzoyl chloride for the hydrophobization of HA (Huerta-Angeles, Bobek, Přikopová, Šmejkalová, & Velebný, 2014). The aim of the present study is to find out whether 2, 4, 6 trichlorobenzoyl chloride and derivatives are useful reagents to activate α, β-unsaturated carboxylic acids. The chemical structure of α,β-unsaturated carboxylic acids and reaction parameters that might influence the reaction were systematically studied. Since the final product was intended for biomedical applications, the purity of the product was also evaluated. The feasibility of novel HA derivatives for electrospinning and finally cross-linking was studied.

2. Experimental

2.1. Materials

Hyaluronan of Mw 87,000 and 115,000 g/mol and polydispersity 1.5 were provided by Contipro Pharma (Dolní Dobrouč, Czech Republic). Several HA batches of similar properties were used to ensure the reproducibility. Weight-average molecular weight (Mw) and polydispersity (P) of native HA was determined before the chemical modification was carried out. Tetrahydrofuran (THF), isopropanol (IPA), triethylamine (TEA), and NaCl were obtained from Lach-ner (Czech Republic). Methacrylic acid (ME), 3-(2-furyl)acrylic acid (FU) 3-(2-thienyl)acrylic acid (THIO), 4-imidazole-acrylic acid (Im), caffeic acid (CA), hydrocaffeic acid (HC), 2, 4, 6-trichlorobenzoyl chloride (TCBC), benzoyl chloride (BC), isobutyryl chloride (IBC), 4-methoxy -benzoyl chloride (MBC), 4-nitro-benzoyl chloride (NBC), 4-dimethylaminopyridine (DMAP), chlorpromazine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium lauryl sulfate (SDS) and polyethylene oxide (PEO, 400,000 g/mol) were commercially available products from Sigma-Aldrich.

2.2. Methods

2.2.1. Nuclear magnetic resonance spectroscopy

10 mg of derivatives were solubilized in 800 μl of D_2O. ^1H and ^{13}C experiments were carried out on a BRUKER Avance™ III 500 MHz operating at a ^1H frequency of 500.25 MHz. The ^1H and ^{13}C chemical shift were referenced to 3-(trimethylsilyl)propionic acid sodium salt (TSPA). ^1H–^{13}C HSQC spectra were acquired using gradient pulse sequences and 2 kHz data points, 80 scans per increment, 256 increments, and heteronuclear scalar coupling C—H set at 145 Hz DOSY (diffusion ordered spectra) were obtained using stimulated echo pulse sequence with bipolar gradients (STEBPGP). The degree of substitution (DS) (average number of modified HA dimers per 100 HA dimers expressed in percentage) was determined from ^1H NMR by the sum of three —CH protons (located at 6.8, 6.7 and 6.4 ppm), in respect to the integration of signal at $\delta = 2.1$ ppm in GlcNAc (—N—CO—CH_3) set to 100 (See example, Fig. S1).

2.2.2. FT-IR spectra

FT-IR spectra were recorded with a FT-IR-8400S Shimadzu spectrometer. Samples were studied as KBr pellets (1%) in anhydrous KBr. Spectra (32 scans) were recorded using 3600 cm^{-1} width (between 400 and 4000 cm^{-1}), and 2 cm^{-1} resolution.

2.2.3. SEC-MALS

The chromatographic system consisted of Waters Alliance HPLC system, two 7.5 mm PL aquagel OH-60 and PL aquagel OH-40 columns connected in series (Agilent Technologies), chromatographic detectors included a DAWN EOS multi-angle laser light scattering detector and an Optilab rEX differential refractometer (both from Wyatt Technology, Santa Barbara, CA, USA). Injection volume was 100 μL of 0.015–1% (w/v) of modified HA solution. The mobile phase was 100 mM sodium phosphate buffer pH 7.4, containing 0.02% of NaN_3 at the flow rate of 0.8 mL/min. A refractive index increment (d*n*/d*c*) of 0.155 mL g^{-1} was used for calculation of Mw and polydispersity (P) (Podzimek, Hermannová, Bilerová, Bezaková, & Velebný, 2010). The samples were measured three independent times. Data acquisition and processing were performed using the Wyatt Technology Corporation ASTRA software, Version 5.3.4.20.

2.2.4. Determination of loss on drying and acid-insoluble ash in modified HA samples

To determine the high purity of semi-production batches ash and organic matter were determined by methods described in European pharmacopeia (ASTM D2974). For the determination of loss on drying, the sample (1.0 g) is placed in the oven at 105 °C for 5 h. The sample is weighed again after cooling to room temperature and the difference of the weights is calculated. For determination of total ash, a known quantity of sample is weighed in a tared crucible, ignited at 550 °C, and kept there until free from carbon. For determination of total ash, the sample is boiled with diluted HCl for 5 min, the insoluble matter is collected on a suitable ash-less filter, ignited at 800 ± 25 °C, cooled down and weighed again. Results are resumed on Table 1.

2.2.5. Rheological analyses

A TA Instruments AR-G2 rheometer was used to measure the dynamic viscosity of the HA solutions in the range of shear rate 0.01–5000 s^{-1}. Stainless steel cone/plate (diameter 60 angle 1°) was used under steady-state mode. The sample temperature was maintained at 25 °C using a temperature control system. Solutions of the derivatives and HA were prepared at concentrations of 6%(w/w) and transferred to the rheometer. The obtained data are resumed in Table 2.