Carbohydrate Polymers 152 (2016) 815–824

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Linolenic acid grafted hyaluronan: Process development, structural characterization, biological assessing, and stability studies

Gloria Huerta-Angeles[a,*], Martina Brandejsová[a], Jaromír Kulhánek[a], Vojtěch Pavlík[a,b], Daniela Šmejkalová[a], Hana Vágnerová[a], Vladimír Velebný[a]

[a] Contipro Pharma a.s., Dolni Dobrouč 401, 561 02 Dolní Dobrouč, Czech Republic
[b] Department of Dermatology, Third Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

ARTICLE INFO

Article history:
Received 9 May 2016
Received in revised form 7 July 2016
Accepted 8 July 2016
Available online 9 July 2016

Chemical compounds studied in this article:
Linolenic acid (PubChem CID: 5280934)
Hyaluronic acid (PubChem CID: 24728612)

Keywords:
Hyaluronic acid
α-Linolenic acid
Lipid oxidation
Degradation
Stability
Encapsulation

ABSTRACT

In this study, hyaluronan (HA) was grafted with alpha-linolenic acid (αLNA) by benzoyl mixed anhydrides methodology, which allowed the derivatization of HA under mild reaction conditions. The reaction was optimized and transferred from laboratory to semi-scale production. The derivative revealed an unexpected cytotoxicity after oven drying and storage at 40 °C. For this reason, the storage conditions of sodium linolenyl hyaluronate (αLNA-HA) were optimized in order to preserve the beneficial effect of the derivative. Oven, spray dried and lyophilized samples were prepared and stored at −20 °C, 4 °C and 25 °C up to 6 months. A comprehensive material characterization including stability study of the derivative, as well as evaluation of possible changes on chemical structure and presence of peroxidation products were studied by Nuclear magnetic resonance (NMR), Fourier transform infrared spectroscopy (FTIR), gas chromatography–mass spectrometry (GC–MS), thermogravimetric analysis (TGA) and complemented with assessment of *in vitro* viability on mouse fibroblasts NIH-3T3. The most stable αLNA-HA derivative was obtained after spray drying and storage at ambient temperature under inert atmosphere. The choice of inert atmosphere is recommended to suppress oxidation of αLNA supporting the positive influence of the derivative on cell viability. The encapsulation of hydrophobic drugs of αLNA-HA were also demonstrated.

1. Introduction

Over the last decade, amphiphilic polymers have become prominent in the area of research due to its applications in regenerative medicine, cancer diagnosis and drug delivery (Palao-Suay, Gómez-Mascaraque, Aguilar, Vázquez-Lasa, & Román, 2016). For that reason, new strategies for their design and upscale should be revised. Amphiphilic polymers can be prepared by chemical conjugation of fatty acids and polymers (Silva et al., 2015). One of the most interesting fatty acids is α-linolenic acid (αLNA), an omega-3 fatty acid that exerts various beneficial physiological effects. The presence of several conjugated double bonds in αLNA might interact with aromatic compounds *via* π–π orbital overlapping and improve encapsulation efficiency of self-assembled nanocarriers (Heard et al., 2005). Unfortunately, αLNA is sensitive to environmental factors such as heat, oxygen, metal ions and humidity, which induce lipid peroxidation. Thus, the challenge is to attach covalently αLNA to a polymer under mild conditions so that, the structure and function of the fatty acid are still preserved. The conjugation of αLNA to poly(ethylene glycol)-*block*-poly(e-caprolactone) at low temperature was demonstrated and used for improvement of curcumin delivery (Song, Zhu, Liu, Yang, & Feng, 2014). Unfortunately, the use of synthetic polymers for the development of nanocarriers suffers from several drawbacks, especially regarding biocompatibility and biodegradability (Kang, Opatz, Landfester, & Wurm, 2015). Natural polymers such as chitosan have been also used for the fabrication of polymeric micelles based on conjugation of linoleic and oleic acids (Bonferoni et al., 2014). However, chitosan based nanoparticles are hepatotoxic (Loh, Yeoh, Saunders, & Lim, 2010). Thus, not all of the natural polymers can be considered as safe materials. Fully biodegradable biopolymers are always preferred for therapeutic applications. Among them, hyaluronic acid (HA) or hyaluronan, a natural polysaccharide composed of D-glucuronic acid and N-acetyl-D-glucosamine is ubiquitous in the human body, therefore, it is considered an attractive building block for the development of biocompatible materials (Kesharwani, Banerjee, Padhye, Sarkar, & Iyer, 2015). Amphiphilic

* Corresponding author.
E-mail addresses: huerta-angeles@contipro.com, huertang77@yahoo.com (G. Huerta-Angeles).

http://dx.doi.org/10.1016/j.carbpol.2016.07.030
0144-8617/

HA was produced by coupling oleic and linoleic acids to the polysaccharide combining high temperature under microwave irradiation (Calce, Mercurio, Leone, Saviano, & De Luca, 2016). Unfortunately, these conditions cannot be used for the covalent attachment of the sensitive αLNA, which quickly oxidizes at temperatures higher than 50 °C (Yang, Cao, Chen, & Chen, 2009) and decompose in reactive products (volatiles) of low molecular weight (Berdeaux et al., 2012), which are potentially cytotoxic (Yamauchi et al., 2008). For this reason, stability studies and biology assessments are recommended to guarantee the safety of any polymer after modification (Olejnik, Goscianska, Zielinska, & Nowak, 2015).

In a recent work, the synthesis of amphiphilic HA was mediated by 2, 4, 6 trichlorobenzoyl chloride (TBC) for the attachment of fatty acids to HA under mild conditions (Huerta-Angeles, Bobek, Přikopová, Šmejkalová, & Velebný, 2014). Similar amphiphilic HA derivatives were found to self-assemble in polymeric micelles useful as drug delivery systems (Šmejkalová et al., 2014). However, TBC is not easily available and is not a suitable reagent for industrial scale due to its high cost. Therefore, the objectives of the current work were to (i) replace TBC with a more appropriate activator and at the same time prove the reaction feasibility to prepare αLNA-HA, (ii) upscale the reaction process while ensuring batch to batch reproducibility, and (iii) demonstrate fatty acid stability towards oxidation during reaction, purification, drying and storage. The structural elucidation of αLNA-HA was performed by Nuclear Magnetic Resonance (NMR), SEC-MALS, Gas chromatography (GC–MS) and thermogravimetry (TGA) to characterize purity of the derivative and resistance towards degradation during 6 months storage. Lipid peroxidation was monitored as well as any possible change of *in vitro* cytotoxicity. The potential application of this derivative for drug encapsulation will be also demonstrated.

2. Materials and methods

2.1. Materials

Sodium hyaluronate (15,000 g mol^{-1}) was provided by Contipro Pharma (Dolní Dobrouč, Czech Republic). Tetrahydrofurane (THF, 99.5%), isopropanol (IPA, 99.7%), triethylamine (TEA, 98%) were purchased from Lach-Ner (Czech Republic). Benzoyl chloride (BC, 99%), 2,4,6-trichlorobenzoyl chloride (TCC, 97%) and 1-phenylhydrazine (PH, 97%) were purchased from Sigma–Aldrich. For chromatographic analysis, analytical grade solvents were used CHROMASOLV®. 4-Dimethylaminopyridine (DMAP, ≥99%) was purchased from Merck. Alfa-Linolenic acid (αLNA, 80%) is a commercial product of the company Finetech (UK). Deuterium oxide (D_2O) and isopropanol (IPA-d_8) were purchased from CortecNet. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and ethidium homodimer were purchased from Life Technologies.

2.2. Preparation of alpha linolenic acid-grafted HA

25.0 g (62.5 mmol, 15,000 g mol^{-1}) of sodium hyaluronate was dissolved in water (500 mL). After dissolution, the reagents were added to the reaction in the following order: 26.2 mL of triethylamine (TEA, 188 mmol), 0.38 g of 4-dimethylaminopyridine (DMAP, 3.13 mmol). In a separate flask, 7.0 g (25 mmol) of αLNA reacted with 3.5 g (25 mmol) of (substituted) benzoyl chloride in IPA (250 mL) in the presence of 26.2 mL (188 mmol) of TEA forming a mixed anhydride. After 30 min at room temperature, the solution was added to the solution containing HA and allowed to react for 2 h at 25 °C. The product was purified by precipitation using 250 mL of absolute isopropanol (IPA) and saturated solution of sodium chloride. The white precipitate was collected by decantation, and washed again with IPA/water solution (85% v/v, 4 × 250 mL) and finally with IPA (3 × 250 mL). The degree of substitution (DS, in mol% *i.e.*, moles of fatty acid to moles of HA dimer.) was obtained from ^{1}H NMR, setting the integral of HA anomeric proton signals (δ = 4.3–4.6 ppm) equal to the total number of dimers (67 dimers) and reading the integral value at δ = 0.9 ppm corresponding to the terminal $-CH_3$ of fatty acid (Fig. 1).

2.2.1. Drying methods and yield calculation

For the oven-drying process, the samples were placed in open petri-dishes, and oven-dried at 60 °C in a Binder FDL 115 universal oven for 24 h. For freeze-drying, a 5% (w/v) solution of the derivative in distilled water was prepared. The solutions were frozen at −20 °C for 5 h and then dried in vacuum for 48 h. For the spray-drying, the isolated product was solubilized in water to have a final concentration of 1.5% (w/v) and dried in Büchi Mini Spray Drier B-290, (inlet temperature: 190 °C; outlet temperature 90 °C, solution feed rate: 10 mL/min, atomization air flow rate of 0.5 kg/h in a spray chamber size 165 mm/600 mm). After drying, experimental yields (Y) were determined for the samples, using the modified and non-modified dimers, using a formula described before (Huerta-Angeles et al., 2014).

2.3. Long-term stability studies

Stability studies of αLNA-HA were performed after the process was completely optimized using five independent batches. Samples were dried in oven, lyophilized or spray-dried and analyzed. A set of αLNA-HA samples were packed in 5 g pouches with an inner lining of polyethylene film. Pouches were welded to become airtight and closed to avoid as much as possible the presence of air (A). Samples were submitted to long term accelerated storage at 40 ± 2 °C/75 ± 5% of relative humidity (RH) and in 25 ± 2° C 40% RH ± 5% in validated climate chambers (Binder, Germany) according to ICH Q1A(R), guide of industry.

A second set of samples was used for evaluation of storage temperature by incubation at −20 ± 3 °C (for later storage of samples in freezer), 5 ± 3 °C (refrigerator), or 25 ± 3 °C (room temperature) in the presence of air.

A third set of samples was used to evaluate the effect of non-oxidative conditions (Johnson & Decker, 2015). The samples were packed by using rubber stopper vials, flushed with nitrogen and sealed. Samples were kept at −20 ± 3 °C, 5 ± 3 °C, or 25 ± 3 °C.

2.4. NMR spectroscopy

Samples were prepared by dissolving αLNA-HA (10 mg/mL) in 750 uL of D_2O containing 3-(trimethylsilyl)-propionate-d_4 (TSP-d_4) as internal standard. NMR spectra were acquired at BRUKER Avance III 500 MHz operating at a ^{1}H frequency of 500.25 MHz. DOSY (diffusion ordered spectra) were obtained using a stimulated echo pulse sequence with bipolar gradients (STEBPGP).

2.5. Scanning electronic microscope (SEM)

Morphological changes of the HA-αLNA after drying by the three techniques were observed using an ultra PLUS sequential scanning electronic microscope (SEM) (ZEISS Germany). A Denton Vacuum desk-sputtering machine was utilized to coat the samples for 10 s with gold.

2.6. Thermogravimetric analysis (TGA)

Thermogravimetric measurements were carried out on a TGA Q500 (TA Instruments). 5 mg of sample was heated at a heating rate of 10 °C min^{-1} under nitrogen atmosphere (up to 600 °C), followed