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Hyaluronic acid lipoate: Synthesis and physicochemical properties

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ABSTRACT

The synthesis and physicochemical characterisation of mixed lipoic and formic esters of hyaluronan (Lipohyal) are presented in this paper. The synthesis was conducted by activating lipoic acid with 1,1′-carbonyldiimidazole to obtain lipoyl imidazolide, which reacted with hyaluronan (HA) in formamide under basic conditions.

This procedure allows researchers to modulate easily the degree of substitution over a range of 0.05–1.8.

Radical scavenger properties were analysed by UV–vis spectroscopy, where improved performance was demonstrated for Lipohyal with respect to the HA row material and lipoic acid. The chemical modification also causes HA to show an improved resistance to hyaluronidase digestion.

These findings show that Lipohyal is a highly interesting derivative for applications in the tricological and dermo-cosmetic field and as an anti-aging ingredient.

Moreover, Lipohyal can be easily crosslinked by UV irradiation, resulting in an innovative hydrogel with distinctive viscoelastic properties that is suitable as both a dermal-filler and as an intra-articular medical device.

1. Introduction

Hyaluronan (HA) is an unbranched glycosaminoglycan with a disaccharidic repeating unit composed of D-glucuronic acid and D-*N*-acetylglucosamine linked through alternating β-1-4 and β-1-3 glycosidic bonds.

HA is a polysaccharide that is present in all vertebrates as a main constituent of the extracellular matrix. HA plays a fundamental role in many physiological functions, including joint lubrication, tissue hydration, cell adhesion and differentiation (Laurent, 1989).

Metabolic studies have shown that the half-life of a hyaluronan macromolecule varies from 2 to 3 weeks in synovial fluid to a few minutes in blood. In vivo it exists as a polyanion and produces highly viscous solutions, due to its extended conformation.

The high capacity of HA for water retention and high viscoelasticity cause it to be suitable for various cosmetic and medical applications, such as a moisturising agents and anti-aging ingredients in cosmetics or as a biomaterial in medical devices for the treatment of osteoarthritis and ophthalmic pathologies (Kuo, 2006). To improve these properties, a long list of chemical modifications have been made on the HA backbone over the last several decades (Schanté, Zuber, Herlin, & Vandamme, 2011).

α-Lipoic acid (or thioctic acid) is a natural molecule; it was isolated in mammalian livers and acts as an essential cofactor for many enzymatic reactions, including the conversion of pyruvate to acetyl-CoA in the Krebs cycle (Zimmer, 1997). Lipoic acid is a potent antioxidant that prevents the symptoms associated with vitamin C and vitamin E deficiency, and it is also a powerful scavenger for reactive species, including free radicals such as hydroperoxides, superoxides, and peroxynitrites (Bilska & Wlodek, 2005).

The purpose for chemically conjugating lipoic acid and HA is to obtain a synergic combination of the properties of the two components.

2. Experimental

2.1. Materials

HA (HySilk®) with a molecular weight (MW) of 350 kDa was provided by CPN spol. Sro (Dolni Dobrouc, The Czech Republic).

LA (purity > 99%, racemic mixture) was provided by Giellepi Chemicals Spa (Milan, Italy).

CDI (purity 98%) was obtained from Fluorochem Limited (Hadfield, United Kingdom).

Abbreviations: BTH, bovine testicular hyaluronidase endo; CDI, 1,1′-carbonyldiimidazole; DMAc, *N*,*N*-dimethylacetamide; DMAP, 4-dimethylaminopyridine; DMSO, dimethyl sulphoxide; DOSY, diffusion ordered spectroscopy; DS, degree of substitution; FA, formamide; FTIR–ATR, Fourier transform infrared–attenuated total reflectance (ATR) spectroscopy; HA, hyaluronic acid (hyaluronan); LA, α-lipoic acid (1,2-dithiolane-3-pentanoic acid); Lip-Im, lipoyl imidazolide; Lipohyal, hyaluronic acid lipoate (lipoic acid ester of hyaluronan); MR, molar ratio; MW, molecular weight; NMR, nuclear magnetic resonance spectroscopy; TB, trypan blue; UV–vis, ultraviolet–visible spectroscopy.

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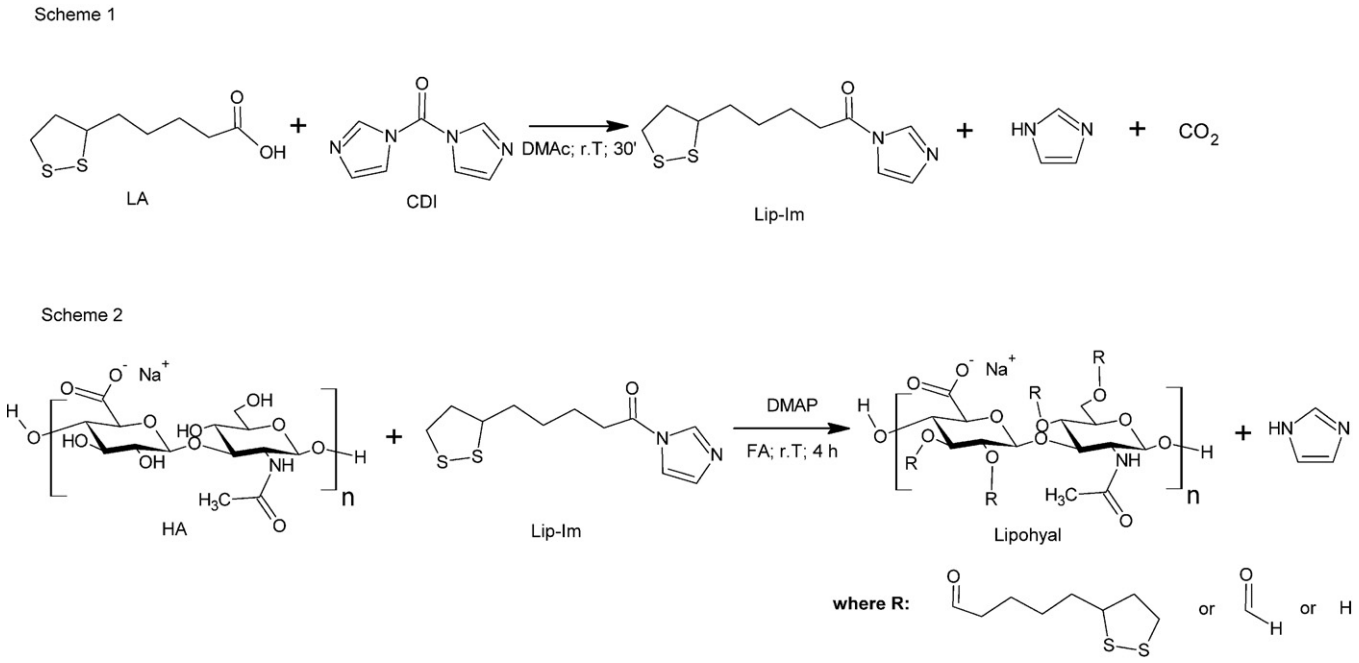

Fig. 1. Reaction schemes of the synthesis of Lipohyal. Scheme 1: synthesis of lipoyl imidazolide (Lip-Im) by addition of CDI. Scheme 2: modification of HA by reaction with Lip-Im in FA with DMAP.

Bovine testicular hyaluronidase endo 2140 U/mg (BTH; EC 3.2.1.35) was purchased from Sigma–Aldrich (Saint Louis, MO, United States).

All other chemicals were purchased from Sigma–Aldrich and were used without further purification.

The water used for solution preparation and dialysis was purified to a resistivity of 18 MΩ cm in an ultrapure-water WP4100 apparatus (SMEG Instruments, Guastalla, Italy).

2.2. Synthesis of Lipohyal

Lipoic acid (LA) was dissolved in *N*,*N*-dimethylacetamide (DMAc) at a concentration of 20% (w/v) at room temperature and activated by the addition of 1,1′-carbonyldiimidazole (CDI) to produce lipoyl imidazolide (Lip-Im), as reported in Fig. 1, Scheme 1 (molar ratio 1:1; reaction time 30 min) (Liebert, Hussain, Tahir, & Heinze, 2006).

The synthesis of Lipohyal was carried out by dissolving hyaluronic acid sodium salt (HA) in formamide (FA) at a concentration of 5% (w/v) (95 °C; 1 h); the solution was cooled at room temperature, and Lip-Im was added to react with HA under basic conditions (DMAP), as reported in Fig. 1, Scheme 2. After 4 h of reaction time with stirring, the crude was neutralised with KH_2PO_4, purified by dialysis and recovered by freeze-drying.

2.3. Structural analysis

The structure characterisation of Lipohyal was performed using FTIR–ATR and NMR, and the degree of substitution (DS) was measured by NMR.

The FTIR–ATR spectra for Lipohyal were measured to confirm the expected formation of the lipoate ester bond. IR spectra were obtained using a Varian FT-660 spectrometer equipped with a diamond crystal GladiATR accessory (Pike Technologies).

NMR spectra were obtained using a Bruker Avance 400 MHz spectrometer equipped with a 5 mm multinuclear probe with a z gradient. The analyses were performed on D_2O solutions of Lipohyal that were approximately 1% (w/w) at 300 K.

2.4. Radical scavenger analysis

The radical scavenger properties associated with Lipohyal were tested in comparison to the HA row material and the lipoic acid alone. •OH free radicals were generated using a Fenton reaction (H_2O_2 0.1 mM + Fe^{2+} 0.05 mM; pH 4.0 by addition of diluted acetic acid).

The amount of •OH was measured indirectly using spectrophotometry, where a standard solution of trypan blue (TB), 0.031 mM, was treated with the Fenton reagent at 25 °C for 30 min. Other identical solutions were added with increasing amounts of HA, Lipohyal and lipoic acid in concentrations ranging from 0.04 to 6.0 mM. The UV absorbance at 588 nm can be correlated to the mixture of the oxidised and the reduced forms of TB, where A_b is the absorbance of the fully oxidised TB form, A_0 is the absorbance of the fully reduced form and A_s is the sample absorbance.

The antioxidant potential of each sample (S(%)) can be evaluated according to Eq. (1) (Liu, Liu, Wang, Du, & Chen, 2007).

$$S\ (\%) = \frac{A_s - A_b}{A_0 - A_b} \times 100 \quad (1)$$

The absorbance measurements were obtained with a Varian Cary 50-scan spectrophotometer using a quartz square spectrophotometer cuvette (10 mm optical path length).

2.5. Enzymatic degradation analysis

1% (w/w) polymeric solutions of Lipohyal and HA in 30 mM acetate buffer, pH = 5.5, respectively, were treated with bovine testicular hyaluronidase endo (BTH) to induce de-polymerisation. The kinetics of the enzymatic digestion was recorded by measuring the solution viscosity directly on an Anton Paar MCR 301 rheometer. Next, 1.5 ml of polymer solution (1%, w/w) was added with the enzyme solution to yield an enzyme to polymer ratio 1:200 (w/w). After a 30 s of mixing, the solution was transferred onto the plates of the rheometer (diameter: 50 mm; cone–plate system; angle: 1°) and the temperature was fixed at 37 °C. The dynamic viscosity was stored every 60 s over 2 h of measurement.