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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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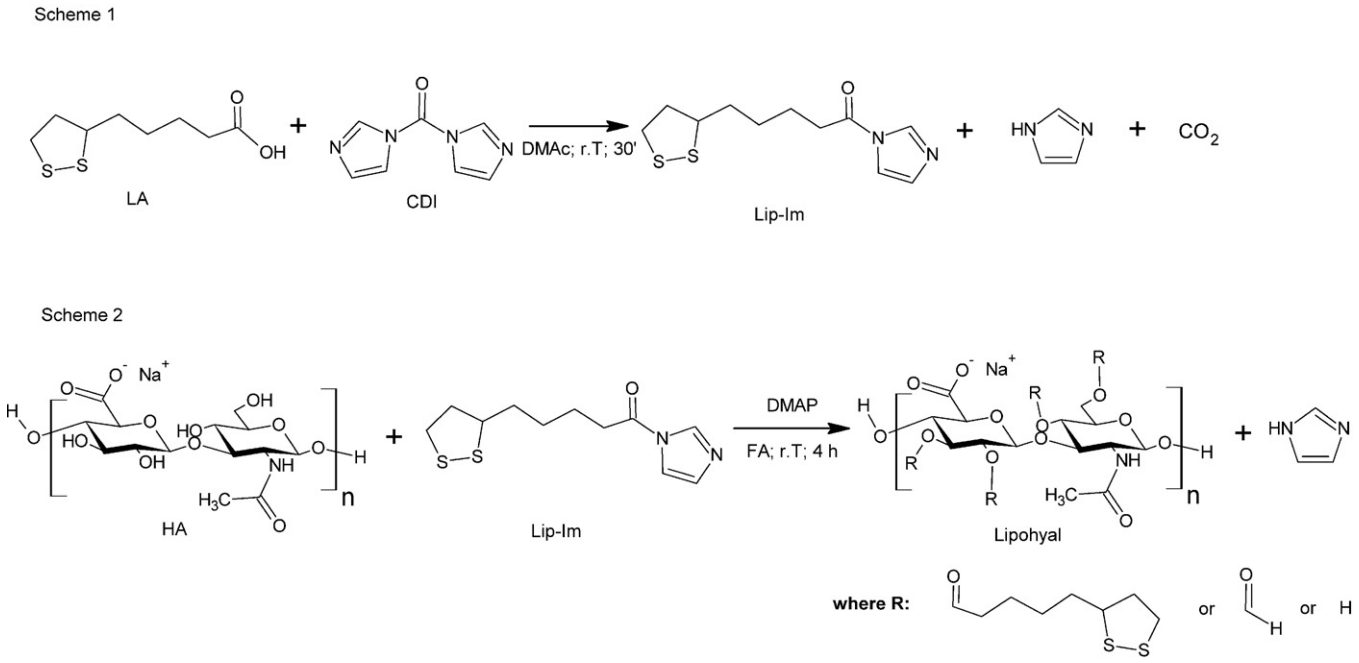

Fig. 1. Reaction schemes of the synthesis of Lipohyal. Scheme 1: synthesis of lipoyl imidazolide (Lip-Im) by addition of CDI. Scheme 2: modification of HA by reaction with Lip-Im in FA with DMAP.

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.

Table 1
Reaction conditions and DS of Lipohyal derivatives.

Sample name	Lip-Im/HA MR[a]	DMAP/HA MR[a]	DS[b] lipoic ester	DS[b] formic ester	Product recovery [%][d]
Lipohyal-01	0.5	1.0	0.17	0.01	92
Lipohyal-02	0.75	1.5	0.30	0.01	95
Lipohyal-03	1.0	2.0	0.46	0.02	98
Lipohyal-04[c]	3.0	3.0	1.8	0.07	–

[a] Molar ratio (MR): mole of reagent per mole of disaccharidic repeating unit.
[b] DS determined by means of ^{1}H NMR spectroscopy in D_2O–NaOD solution after ester hydrolysis.
[c] Reaction time: 20 h.
[d] Product recovery [%] = (weight of Lipohyal/weight of HA substrate) × 100.

2.6. UV crosslinking

Photo-crosslinking could be achieved through 1 h of UV irradiation (λ = 254 nm; power = 30 W; distance 20 cm) using 10 ml of 1.5%, 1.0% and 0.7% (w/w) Lipohyal solutions, respectively, in de-ionised water inside a Petri dish (7-cm diameter).

2.7. Rheological analysis

Rheological analysis of the photo-crosslinked hydrogels was performed using an Anton Paar MCR 301 rheometer equipped with parallel plates (diameter: 25 mm; sandblasted) with the temperature fixed at 25 °C.

3. Results and discussion

3.1. Synthesis of Lipohyal

A simple synthetic pathway has been developed to obtain the chemical derivatisation of HA. This method involves common reagents and mild reaction conditions in organic media and allows researchers to obtain both linear and crosslinked hyaluronan derivatives. In particular, mixed lipoic and formic esters of hyaluronic acid are described in this study. (A preliminary account was discussed previously in a Patent application (Picotti, Bosco, Stucchi, & Fabbian, 2010).) Formic esters are formed by formic acid, which arise from the decomposition of formamide during the dissolution step of hyaluronan.

HA is modified by introducing randomly lipoic and formic residues on the hydroxyl functions by an ester bond. Thus, hydrophilic and lipophilic properties can be modulated depending on the substitution degree (DS).

The synthetic procedure allows one to modulate the DS by changing the quantity of activated lipoic acid and that of the base, as shown in Table 1.

In all of the investigated cases reported in Table 1, over 90% (w/w) of the polymer is recovered.

3.2. Structure determination

In the FTIR–ATR spectra of Lipohyal, several carbonyl bands are observed over 1724–1561 cm^{-1}. The band at 1724 cm^{-1} is typical of the ester groups (Trombino et al., 2008) and its intensity increases with the degree of substitution (see Fig. 2); however, all other bands are typical of the hyaluronic acid sodium salt (Gilli, Kacuráková, Mathlouthi, Navarini, & Paoletti, 1994).

Standard ^{1}H NMR analysis depicts an extensive overlapping of signals belonging to HA and lipoic acid. To demonstrate the covalent linking of lipoic residue on the polymer, a diffusion ordered spectroscopy (DOSY) analysis can be performed (see Fig. 3). In DOSY spectra, all of the signals arising from rapidly diffusing molecules (that are not linked to the polymer) are suppressed as an effect of the gradient pulses. In Lipohyal DOSY, the formic and the lipoic ^{1}H signals show unchanged intensity with respect to HA ^{1}H signals, and this observation indicates that these residues have the same diffusion coefficient.

To make a quantitative determination of DS, the Lipohyal samples were dissolved in D_2O–NaOD solution to induce ester hydrolysis inside the NMR tube and produce higher-resolution signals.

3.3. Radical scavenger properties

The antioxidant potential of the Lipohyal-02, HA and lipoic acid, respectively, (S (%)) can be evaluated according to Eq. (1), and these values are reported in Fig. 4 as a function of the sample concentration.

Lipohyal scavenged •OH free radicals more efficiently than the HA row material (Šoltés et al., 2006) and lipoic acid alone (Packer, Witt, & Tritschler, 1995) at any tested concentration. More specifically, in this experiment, 50% of the free radicals can be quenched using a 1.50 mM HA solution or a 0.4 mM lipoic acid solution; the same result is obtained with Lipohyal at 0.26 mM concentration, and this result is indicative of higher activity.

3.4. Enzymatic degradation resistance

BTH is an endo-glycanohydrolase (EC 3.2.1.35) that degrades HA by randomly cleaving a β-1,4-glycosidic bond.

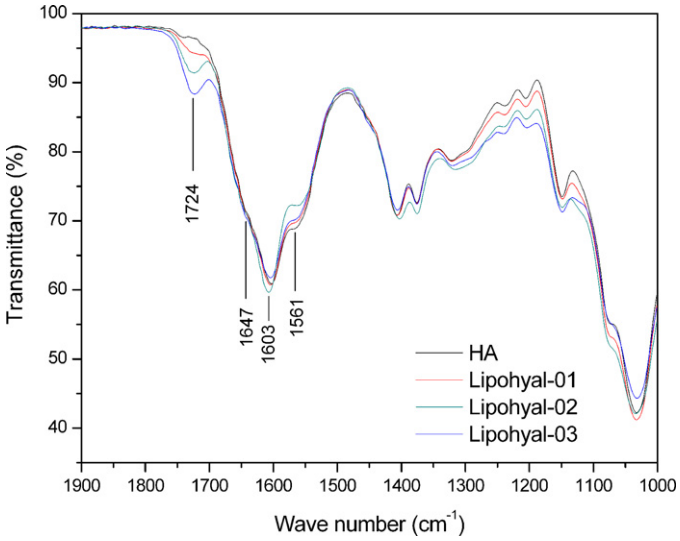

Fig. 2. FTIR–ATR spectra of HA and Lipohyal with increasing DS values. The following frequencies are marked: 1724 cm^{-1} C=O stretching of lipoic and formic esters; 1647 cm^{-1} amide I band of acetamido group; 1603 cm^{-1} C=O stretching of glucuronic acid sodium salt and 1561 cm^{-1} amide II band.

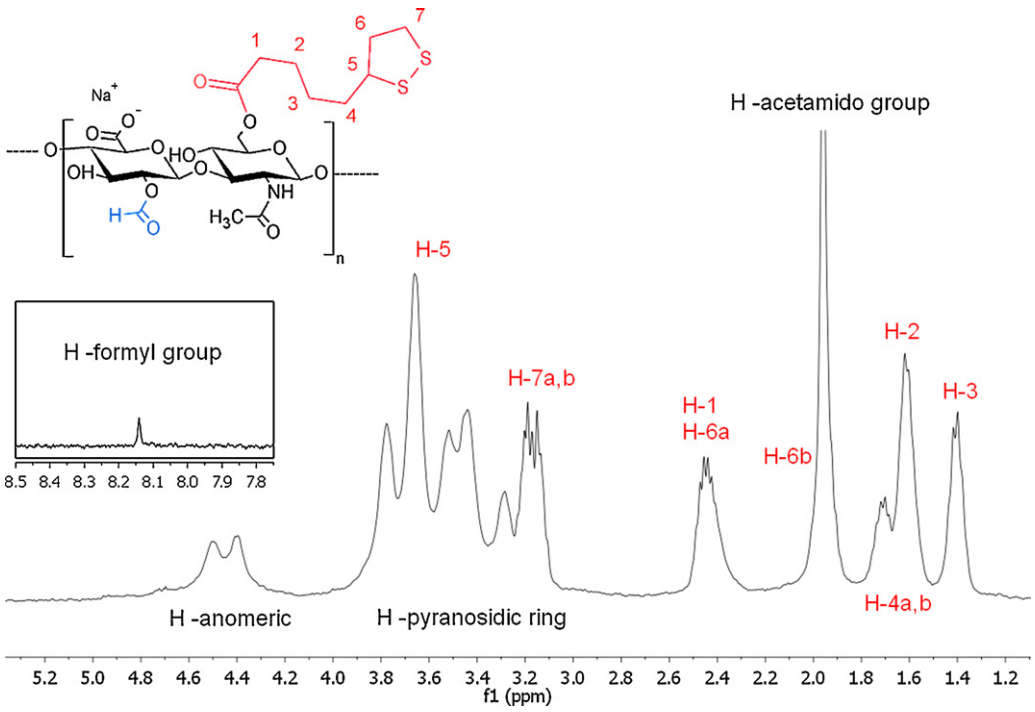

Fig. 3. ^{1}H DOSY–NMR spectrum of Lipohyal-02 (DS = 0.30) in D_2O.

Highsmith, Garvin, and Chipman (1975) reported that hyaluronidase exhibits a notably long binding site that links to a region of HA not shorter then a hexasaccharide. More recent publications confirm that shorter HA oligomers are not linked to the enzyme and are not hydrolysed further (Takagaki, Nakamura, Izumi, Saitoh, & Endo, 1994). The longer the HA fragment, the higher the association constant to the enzyme (Cramer, Bailey, Bailey, & Miller, 1994), and this finding confirms the need for high regularity in the primary structure to match the protein binding groove.

The kinetics of Lipohyal-02 was tracked by measuring the solution viscosity using a rheometer, as described in Section 2.5 and reported in Fig. 5. The kinetics associated with the enzymatic digestion of HA was also reported as a comparison.

The presence of lipoate ester residue along the linear HA chains increases the enzymatic degradation resistance. In fact, Lipohyal-02 shows a lower degradation rate than HA does.

Because the enzymatic binding is specific, the HA chain must exhibit a regular structural pattern; if this regularity is broken by the presence of a substitution, the binding affinity to the enzymatic active site is not efficient, and the overall degradation rate is reduced. The presence of substituents protect HA from hyaluronidase hydrolysis, as the substituted HA structure does not match with the enzyme, or at least, the overall binding constant is decreased. The lipoic and formic substituents are randomly distributed elements along the HA, and the statistical occurrence of a non-substituted, regular hexasaccharide element decreases when DS increases.

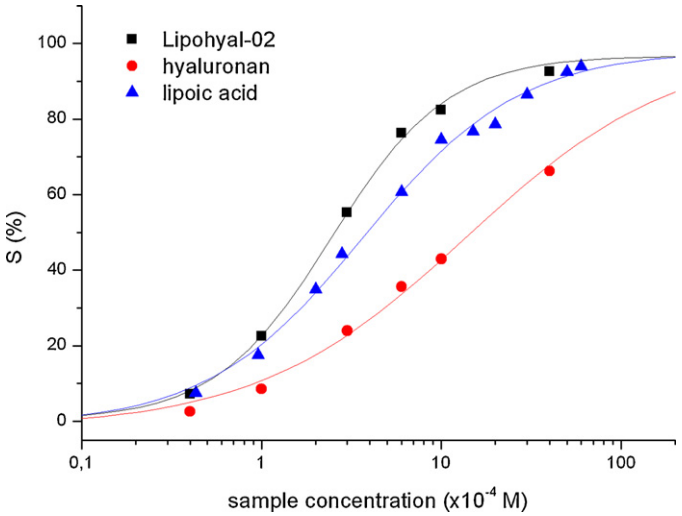

Fig. 4. Diagram of antioxidant potential of Lipohyal-02, HA and lipoic acid. *S* (%) is reported as a function of the sample concentration.

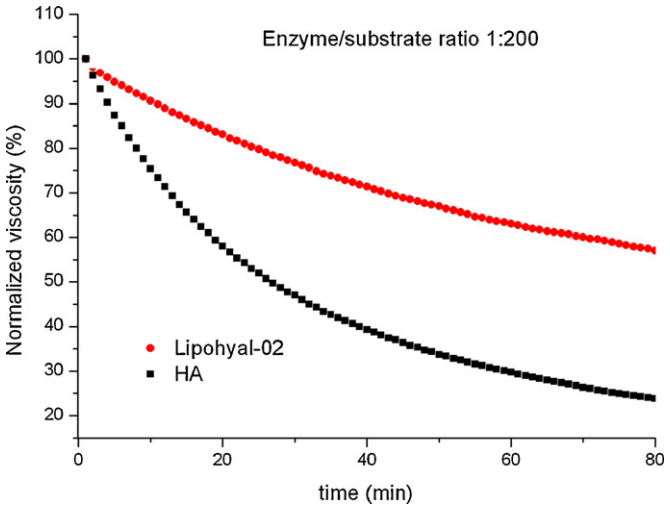

Fig. 5. Kinetics of the enzymatic degradation of Lipohyal-02 and HA (enzyme/substrate ratio 1:200) in acetate buffer solution at 37 °C. To make an easier comparison between the curves, the viscosity was reported as percentage of the viscosity value at t_0.

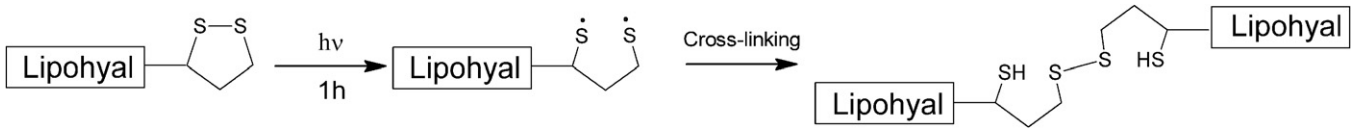

Fig. 6. Reaction scheme of the Lipohyal photo-crosslinking process.

3.5. UV crosslinking

Lipohyal-02 solutions could be easily crosslinked by UV irradiation, as reported in the experimental section, to obtain an innovative hydrogel with rheological properties that can be described through rheological characterisation.

Biewenga, Haenen, and Bast (1997) reported that lipoic acid undergoes a reversible opening of the 5-member ring upon UV irradiation, and that the resulting thiol groups react to form polymers. If this cross-reaction involves lipoic ester units belonging to different HA chains, a three dimensional network is produced, leading to the formation of a hydrogel. To demonstrate the existence of disulphide linking bridges, an excess of dithiothreitol (DTT) was added to photo-crosslinked Lipohyal. Full dissolution of the hydrogel was observed. During this experiment, ester linkages between lipoic groups and HA were not hydrolysed, as shown by NMR analysis (spectra not shown). DTT is a specific reagent for the reduction of disulphide to thiol groups (Lees & Whitesides, 1993). Thus, the photo-crosslinking process is depicted in Fig. 6.

The physical mixture of HA (1.3%, w/w) and lipoic acid (0.2%, w/w) does not exhibit any increase in viscosity upon UV irradiation (see Fig. 7a).

3.6. Rheological properties

Fig. 7a shows the dependence of the viscosity (flow curve) upon the shear stress variation. The viscosity of crosslinked Lipohyal, after UV irradiation is several orders of magnitude higher than that of viscous liquids, such as the corresponding Lipohyal solution, before UV irradiation or the physical mixture of HA row material and lipoic acid.

Fig. 7b shows the mechanical spectra for both solutions and the crosslinked hydrogels registered inside the linear viscoelastic field.

Typically, in a solution (HA and linear Lipohyal), the viscous modulus (G'') is greater than the elastic modulus (G'), but in the typical gel profile (UV-crosslinked Lipohyal), the elastic modulus (G') overcomes the viscous modulus throughout the entire oscillation frequency range and is essentially constant.

In Table 2, the rheological parameters of photo-crosslinked gels obtained from solutions at different polymer concentrations are reported. These data can be compared with the corresponding data measured on two HA-based hydrogels already present in the market as medical devices for intra-articular viscosupplementation: Durolane® by Q-Med and Sinvisc® (HYLAN G-F 20) by Genzyme.

The tailored viscosity of the final gels can be obtained upon modulation of the polymer concentration in solution before UV irradiation. Weak gels can be obtained from dilute solutions (approximately 0.7%), and stronger gels are formed from more concentrated solutions (approximately 1.5%) whose storage modulus grows up to the typical values of other well-known biomaterials, such as Durolane® and Sinvisc® (Ågerup, Berg, & Åkermark, 2005).

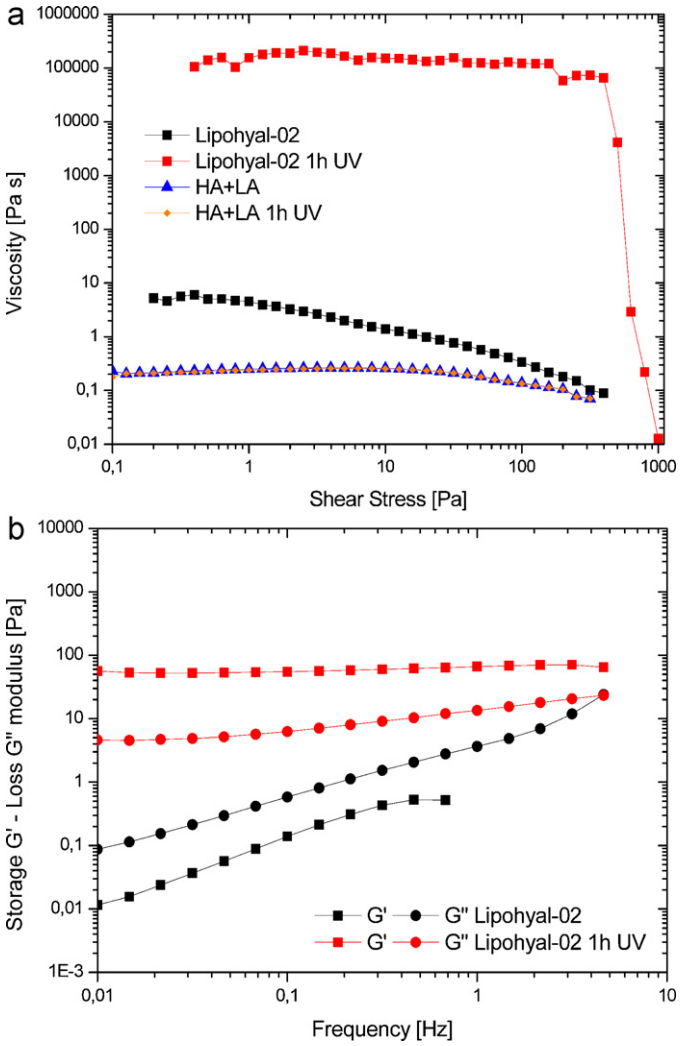

Fig. 7. Rheological properties of Lipohyal-02 before and after UV irradiation: (a) flow curves; (b) mechanical spectra.

Table 2
Rheological parameters of UV-crosslinked Lipohyal hydrogels and two commercial available crosslinked hyaluronic acid hydrogels.

Sample name	Viscosity[a] η^0 [Pa s]	G'[b] [Pa]	G''[b] [Pa]
Lipohyal-02 1.5% (w/w) 1 h UV	150,000	55.1	6.26
Lipohyal-02 1.0% (w/w) 1 h UV	68,000	26.4	4.18
Lipohyal-02 0.7% (w/w) 1 h UV	7000	1.28	0.62
Durolane®	95,000	316	45.6
Synvisc®	1600	45.5	22.0

[a] Viscosity at plateau.
[b] Storage modulus (G') and loss modulus (G'') measured at 0.1 Hz frequency.

4. Conclusion

Lipoic acid is a biocompatible, biologically active molecule that is degraded rapidly by metabolic processes in vivo. The conjugation to hyaluronic acid is designed also to modulate the release of lipoic acid and control its uptake. However, the presence of lipoic residues on the HA chains prolongs the natural turnover rate of the polymer (Fraser, Laurent, & Laurent, 1997), due to both enzymatic and free-radical degradation.

The resulting hyaluronan lipoate-formate (Lipohyal) is freely soluble in water, stable and biocompatible.

The radical scavenger properties were tested by UV–vis spectroscopy, where Lipophal showed enhanced performance compared to HA and lipoic acid.

As a result of these beneficial properties, Lipohyal can be used in topical compositions with a moisturising, elasticising, anti-aging profile or as adjuvant for the treatment of skin lesions, such as inflammations, ulcers, wounds, dermatitis, and skin hyperthermia caused by radiation.

Moreover, the ability to bind thiol groups, such as those present on keratin, suggests that Lipohyal may have potential applications in hair care.

Additionally, Lipohyal can be easily crosslinked by UV irradiation, resulting in an innovative hydrogel with distinctive viscoelastic properties suggesting applications as a dermal-filler and as an intra-articular medical device.

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