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Hyaluronan derivatives: Alkyl chain length boosts viscoelastic behavior to depolymerization

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ABSTRACT

Five amide derivatives of Hyaluronic Acid (HA) were synthesized with C8, C12, C15, C16 and C18 linear alkyl-amines. These polymers (Hyadd) were tested against thermal, oxidative and hyaluronidase degradation by means of rheological experiments and SEC analysis and compared to non-modified HA. First of all, no free hexadecylamine was detected in the treated samples, meaning that under these stressing conditions only cleavage of glycosidic bonds occurs. Then, viscoelastic properties were assessed during thermal degradation and their variation as a function of time was expressed by means of a decay constant $k_{G'}$: while no significant difference in the decrease rate was observed between Hyadd-C8 and Hyadd-C12, a marked stabilization of viscoelastic properties during thermal treatment was detected for Hyadd-C15, Hyadd-C16 and Hyadd-C18. On the other hand, no difference was observed between the MW decrease rate (k_{MW} decay constant) of HA and Hyadd-C12 to-C18; the depolymerization takes place on the backbone of the polymers independently whether they are derivatized or not, but longer alkyl chains lead to higher viscoelasticity in the depolymerized products. Finally, both oxidative and enzymatic degradation were carried out analyzing the changes in elastic modulus and in dynamic viscosity: once again, the amide side chain came out with similar behavior to chemical cross-linked HA (HBC) and with improved performances respect to linear HA in terms of preservation of viscoelasticity after chain depolymerization.

1. Introduction

Osteoarthritis (OA) is a multifactorial or injury-induced joint degeneration affecting large segments of the population and involving pathological changes in all joint tissues, including cartilage degradation and synovitis, leading to significal disability and impaired quality of life (Goldring and Goldring, 2007). Viscosupplementation of synovial fluid with Hyaluronic Acid (HA) has been demonstrated to be an efficacious treatment strategy for OA (Bellamy et al., 2005). HA is a natural heteropolysaccaride consisting of alternating residues of D-glucuronic acid and N-acetyl-D-glucosamine; it is found in human synovial fluid with a MW from 3 to 6 MDa and a concentration of approximately 2.4–4.0 mg/ml (Balazs, 2009). The intra-articular management of OA is currently based on the injection of mid-to-high MW HA, however unmodified HA has a short residence time ranging from 24 to 48 h (Balazs, 2009) due to the action of the enzyme hyaluronidase and free radicals. This limitation has been boosting the research in products based on chemically cross-linked HA (Durolane®, Ågerup, Berg, & Åkermark, 2005; Synvisc®, Frampton, 2010) and on chemically modified linear HA (Hymovis®, Bellini & Topai, 1998; Finelli, Chiessi, Galesso, Renier, & Paradossi, 2011).

In recent years, partially hydrophobic HA has been attracting interest for the development of new biocompatible materials: Pelletier et al. (2001) synthesized a series of HA derivatives partially grafted with long alkyl chains (C12 and C18) through ester bond, as potentially useful material in cartilage repair. Creuzet, Kadi, Rinaudo, and Auzély-Velty (2006) synthesized several amphiphilic derivatives of HA bearing an adipic-dihydrazine linker with pendant alkyl chains from C8 to C16, as thickeners or gelling polymers in cosmetic or biomedical domains in controlled drug delivery.

Bulpitt and Aeschlimann (1999) synthesized a series of biocompatible and biodegradable HA derivatives by coupling of primary or secondary amines to the carboxyl group of the glucuronic acid moiety using an active ester intermediate (e.g. obtained via EDC/HOBt, pH 6.8) in aqueous buffer.

In the field of viscosupplementation, Hyadd4® was successfully proposed by Fidia Farmaceutici. Hyadd4® is a water soluble HA derivative where the polysaccharide backbone is grafted with hexadecyl side chains through amide bonds. Despite the low degree of substitution, the hydrophobic interactions give a stable hydrogel at polymer concentration higher than 3 g/L (Borzachiello, Mayol,

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Schiavinato, & Ambrosio, 2009) which enhances the viscoelasticity of synovial fluid and may serve as an effective lubricant; moreover, a reduction of vascularity, intimal hyperplasia and an increase of endogenous high molecular weight (HMW) HA synthesis by synovial fibroblast was recently reported in an ovine meniscetomy model of OA (Smith et al., 2008).

Although several studies described the physical and biological effect of partially hydrophobic HA (Bulpitt & Aeschlimann, 1999; Creuzet et al., 2006; Pelletier et al., 2001; Prestwich, 2011), to the best of our knowledge the chemical properties of this modified polymer during in vivo depolymerization are not fully known, yet.

In the first part of our work we verified the stability of amide bond between HA and alkyl chain under well known conditions (Stern, Kogan, Ledrzejas, & Šoltés, 2007) as thermal, enzymatic and oxidative degradation. In the second part we studied the viscoelastic behavior of the partially hydrophobic HAs under thermal treatment and the influence of different alkyl chains. This investigation was performed on a series of Hyadd polymers synthesized at the same derivatization degree with C8, C12, C15, C16 and C18 alkyl chain. The kinetics of depolymerization were characterized by TDA-Viscotek analysis and the data obtained were correlated with the rheological measurements. Finally the viscoelastic profile during thermal depolymerization was compared with those obtained in the presence of reactive oxygen species (ROS) (La Gatta, De Rosa, Marzaioli, Busico, & Schiraldi, 2010; Šoltés et al., 2007) and hyaluronidase (Menzel & Farr, 1998; Stern, 2003) that physiologically are responsible for the reduction of synovial fluid viscosity in OA.

2. Experimental

2.1. Materials

Hyaluronic Acid Sodium Salt (HA) of different MW (700 kDa and 1.2 MDa) was provided by Fidia Farmaceutici S.p.A. MWs were determined by means of Viscotek TDA Max (Malvern Instruments) analysis.

Hyaluronic Acid Tetrabutylammonium Salt (HATBA), obtained from 700 kDa HA after resin-catalyzed ion exchange, was used as a freeze-dried powder for the synthesis of the alkylamide derivatives of HA (Hyadd, see Section 2.2). Methanesulfonic acid, 1,1-carbonyldiimidazole, octylamine, dodecylamine, pentadecylamine, hexadecylamine, octadecylamine, o-phthaldialdehyde (OPA), 1,4-Butanediol diglycidyl ether (BDDE), Hyaluronidase (608 units/mg; EC 3.2.1.35) were supplied by Sigma and used without further purification. Dimethylsulfoxide (DMSO), methanol (MeOH), ethanol (EtOH), phosphate-buffered solution (PBS), $CuCl_2$ and ascorbic acid were Carlo Erba, RPE grade products. DMSO-d_6 was provided by Merck; DOWEX M-31 Resin was supplied by Dow chemical. MilliQ water was used throughout this study.

2.2. Synthesis of Hyadd polymers

The synthesis of a similar hexadecyl derivative of Hyaluronic Acid, Hyadd®4, has been reported elsewhere (Bellini & Topai, 1998; Finelli, Chiessi, Galesso, Renier, & Paradossi, 2009).

Following the same scheme for the synthesis of each derivative tested in this work, 1.35 g of HATBA (Tetrabutylammonium Salt of HA) was dissolved at room temperature in 150 ml of DMSO, then a catalytic amount of methanesulfonic acid and 46 mg of 1,1-carbonyldiimidazole were added slowly. 211 mg of octylamine for Hyadd-C8, 302 mg of dodecylamine for Hyadd-C12, 371 mg of pentadecylamine for Hyadd-C15, 393 mg of hexadecylamine for Hyadd-C16 and 482 mg of octadecylamine for Hyadd-C18 was then respectively added and the amidation reaction was performed at 42 °C overnight while stirring. A saturated aqueous NaCl solution (35 ml) was added and the product was recovered by pouring 220 ml of EtOH into the solution and filtering the precipitate. The powder was then washed several times using a mixture of EtOH and water and finally dried in an oven. Following this procedure, about 900 mg of each substituted HA were obtained. The degree of substitution was determined by ^{1}H NMR analysis performed in DMSO-d_6 on a Bruker Advance spectrometer operating at 300 MHz. All the Hyadd polymers were found to be derivatized at 7.5 ± 0.6% mol/mol. In detail the signals used for the determination were: ^{1}H NMR (DMSO-d_6) δ 1.9–1.7 (s, 3H, HA-NHCO·CH_3), 0.9–0.8 (t, 3H, HA-NH$(CH_2)_n$·CH_3).

HA (700 kDa) and each Hyadd polymer were respectively prepared in phosphate buffered saline (PBS, pH 6.9) at concentration of 8 mg/ml in glass tubes with screw caps.

2.3. Free hexadecylamine assessment after thermal, enzymatic and oxidative degradation

Thermal degradation: 1 g of Hyadd-C16 gel (8 mg/ml) was diluted with 1 ml of PBS and heated in a block heater (Stuart) in glass tubes with screw caps at 105 °C, for 9 h.

Enzymatic degradation: 1 g of Hyadd-C16 gel was incubated for 3 h at 37 °C in a bath with 1 ml of hyaluronidase PBS solution (final concentration 300 U).

Oxidative degradation: 1 g of Hyadd-C16 gel was incubated for 3 h at 37 °C in a bath with 1 ml of oxidizing PBS solution (final concentration: $CuCl_2$ 80 μM and ascorbic acid 80 mM).

The absence of free hexadecylamine as an indicator of the amide bond resistance to degradation was investigated by re-suspending 1 g of each sample with 9 ml of MeOH for 30 min, in order to extract non-bound amine. The collected supernatant was then treated with OPA for amine derivatization and the hexadecylamine-OPA product was quantified using the HPLC/fluorimetry method described below.

A HPLC system Perkin Elmer series 200 was used, equipped with a Luminescent Spectr. LS30 detector and a Versapack C18 10 μm 25 cm column; eluting conditions: MeOH/H_2O (95/5) mobile phase, 0.3 ml/min flow rate. The fluorimetric detector was set at an excitation wavelength of 330 nm and an emission wavelength of 440 nm.

2.4. Hyaluronan BDDE cross-linked (HBC) polymer synthesis

47 μl of 1,4-Butanediol diglycidyl ether (BDDE) was dissolved in 7 ml of 0.25 M NaOH and added to 1 g of HA^-Na^+ (700 kDa) for HBC 10% synthesis. The reaction was carried out for 2 h at room temperature, and then by heating for 2 h at 45 °C. Finally, the solution was neutralized with 0.1 M HCl to a pH of approximately 7 and rehydrated in saline reaching a final HA concentration of 22 mg/ml. The final product is a transparent gel which has been characterized by TDA Viscotek elsewhere (Guarise, Pavan, Pirrone, & Renier, 2012).

2.5. Rheological experiments

The rheological experiments were performed by means of a cone-plate Thermo Haake Mars II Rheometer at 25 °C equipped with a cone of 6 cm of diameter and an angle of 1°. All the samples analyzed were processed with Haake Rheowin Job Manager 4.0 software and data collected were processed using Origin 8SR4 and Microsoft Excel.

Oscillatory Rheometry – thermal degradation: each Hyadd polymer sample was heated in a block heater (Stuart) at 105 °C, at different timepoints samples were taken out and cooled in a ice bath for 5 min and about 1 g of each gel sample (8 mg/ml) was analyzed directly in the viscometer. The G' (elastic modulus) and G'' (viscous modulus) were measured (in Pa) from 0.07 to 90.0 rad/s at fixed strain value of 10% (an initial strain sweep with an oscillatory

shear strain of increasing amplitude, γ, at the constant frequency of $\omega = 6.28$ rad/s was applied to determine the region of linear response of the sample: at 10% the viscoelastic range is linear).

Oscillatory Rheometry – oxidative degradation: 2 g of HA gel (1.2 MDa; 15 mg/ml in PBS), 2 g of HBC gel (22 mg/ml in saline) and 2 g of each Hyadd polymer (8 mg/ml) sample were added with 25 μl of ascorbic acid (32.8 mM) and with 25 μl of $CuCl_2$ (32.8 μM). Each sample was accurately mixed (with a 2-syringe system) for 1 min. About 1 g of each gel sample was analyzed by means of the rheometer. G' and G'' were measured (in Pa) at constant frequency of 0.628 rad/s s^{-1} at fixed strain value of 10% for 1800 s.

Rotational Rheometry – oxidative degradation: 2 g of HA gel (1.2 MDa; 15 mg/ml in PBS), 2 g of HBC gel (22 mg/ml in saline) and 2 g of each Hyadd polymer (8 mg/ml) sample were added with 25 μl of ascorbic acid (32.8 mM) and with 25 μl of $CuCl_2$ (32.8 μM). Each sample was accurately mixed (with a 2-syringe system) for 1 min. About 1 g of each gel sample was analyzed by means of the rheometer. The dynamic viscosity was measured (in Pa s) at 0.5 s^{-1} in controlled rotation for 1800 s (a setup experiment was performed on Hyadd-C16 gel 8 mg/ml from 0 to 5 s^{-1} in order to select the frequency giving the higher sensibility).

Rotational Rheometry – enzymatic degradation: The conditions described above were used to assess HA (1.2 MDa; 15 mg/ml in PBS) and Hyadd-C16 gels after hyaluronidase treatment. 2 g of each gel was added with growing concentrations of the enzyme (0; 136.8; 206.7; 273.6 U/ml); after 2 min of incubation, 1 g of each sample was tested for dynamic viscosity.

2.6. Polymer characterization using size exclusion chromatography (SEC)

All the HA and Hyadd polymers were analyzed (at 0.4 mg/ml) by means of the Viscotek TDA Max 302 system, equipped with a triple detector (RI, LALS-RALS and Differential Viscometer). A Viscogel GMH_{HR}-M column was eluted at 40 °C with DMSO with LiCl 0.1% (w/v) at a flow rate of 0.3 ml/min; an injection loop of 100 μl was used. 0.5 g of each sample (8 mg/ml) was pre-treated with Dowex resin and brought to final volume of 10 ml with DMSO, mixed overnight at RT and finally filtered on 0.45 μm RC syringe filters. All the acquired chromatograms were processed with OmniSec 4.5 software using a refractive index increment (dn/dc) of 0.054 for HA samples and 0.0443 for Hyadd samples. dn/dc values in DMSO were calculated at 5 different concentrations (0.15; 0.3; 0.5; 0.65; 0.8 mg/ml) of Hyadd-C16 and at 2 concentrations (0.3 and 0.5 mg/ml) of HA.

3. Results

3.1. Resistance of amide bond to thermal, enzymatic and oxidative degradation

When injected into the intra-articular environment, hyaluronan is subjected to chemical (Šoltés et al., 2007) and enzymatic degradation (Stern, 2003). Hyadd (HA derivatives alkylated at the carboxylic group of D-glucuronic acid unit with a series of alkyl chains: C8, C12, C15, C16 and C18) preparations were made in glass tubes by dissolution in PBS at a concentration of 8 mg/ml. First of all we checked the stability of the amide bond (Fig. 1a) in a Hyadd-C16 model. The Hyadd-C16 preparation was treated separately with hyaluronidase, oxidizing agents ($CuCl_2$/ascorbic acid system) and thermal heating, and the amount of free hexadecylamine was determined before and after the treatments through the method described in Section 2.3. A decrease in viscosity was observed by rheological measurements, while no free hexadecylamine was detected by HPLC analysis. Thermal, enzymatic and oxidative treatments cause degradation only through the hydrolysis of glycosidic bonds in the HA backbone, while the amide bond stability in the intra-articular environment is assured.

3.2. Hyadd depolymerization: role of the hydrophobicity during thermal hydrolysis

Rheological assessments of alkylated HA were recently performed: Finelli et al. (2009) described the correlation between the concentration and the network pore size in Hyadd4 (HA hexadecylamine) hydrogels, whereas Creuzet et al. (2006) studied the role of alkyl chain length in the HA derivatives solubility and the effect on the rheological properties; however, the behavior of alkyl-HA during depolymerization has not been thoroughly investigated, yet.

A set of Hyadd preparations were made in glass tubes mixing for about 1 h at RT the alkylated HA (C8, C12, C15, C16 and C18) in PBS at a 8 mg/ml concentration. All the samples were treated at 105 °C and analyzed by rheological and TDA-Viscotek experiments as previously described (Sections 2.4 and 2.5) at different timepoints. No rheological measurements were performed on HA because of its poor viscoelastic properties at 8 mg/ml.

Fig. 2 shows the data obtained for two samples, Hyadd-C12 and Hyadd-C16, plotting the elastic (G') and viscous (G'') modulus at 0.628 rad/s versus the hydrolysis time. The first part of the plot is not reported because the Hyadd samples gave an inhomogeneous gel.

For the Hyadd-C16 gel G' is always higher than G'', even after 5.5 h of heating; on the other hand, the G'' curve for Hyadd-C12 crosses over the G' curve after 1 h. In general, the longer the alkyl chain, the higher the energy required to obtain a conformational rearrangement that gives a homogeneous gel. The highest value of elastic modulus, at the same amidation degree, is directly related to the length of the alkyl chain; however the low gel homogeneity in the first part of heating makes it difficult to define the exact maximum in G', therefore the measured G' values were interpolated with an exponential decay function: $dG'/dt = -G'/k_{G'}$. The decay constant $k_{G'}$ defines with good approximation the variation of the polymer viscoelastic properties during thermal degradation. Since a relevant difference of the viscoelasticity decrease rate was observed between shorter-chain and longer-chain alkyl-amide derivatives,

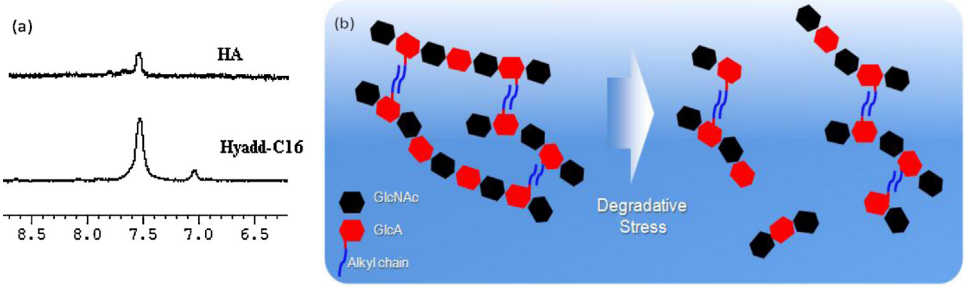

Fig. 1. (a) Overlapped ^{1}H NMR spectra in DMSO-d_6 of linear HA (upside) and Hyadd-C16 polymer (downside). Signal at 7.0 ppm represents the amidic proton of alkyl chain of Hyadd-C16, signals at 7.5 represent the amidic proton of N-Acetyl Glucosamine. (b) Scheme of alkylated HA degradation.

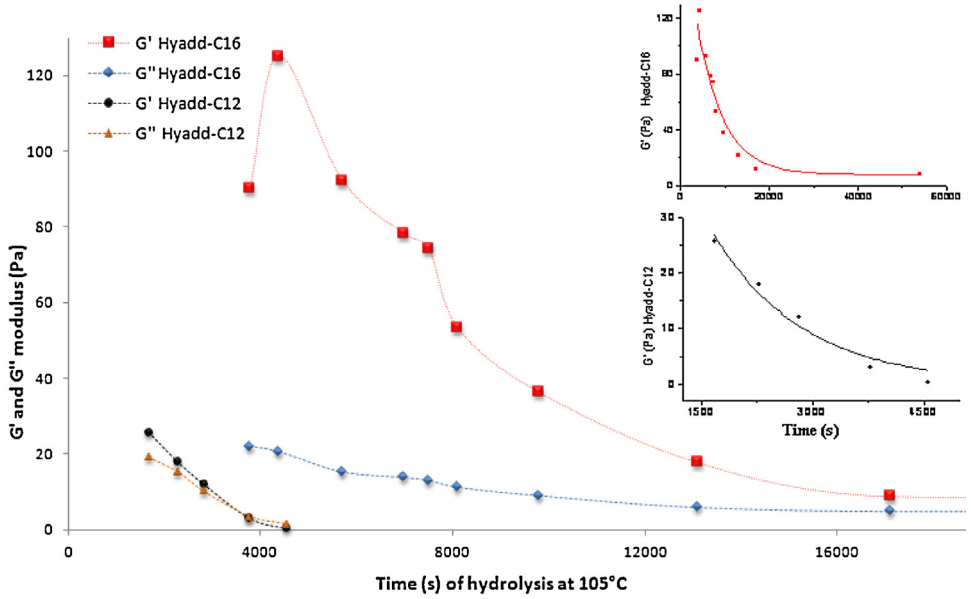

Fig. 2. (a) G' and G'' modulus at 0.628 rad/s vs. hydrolysis time. (b) Interpolation of G' data with an exponential decay function, for Hyadd-C16 (upside) and Hyadd-C12 (downside) gel.

we tried to evaluate $k_{G'}$ as a function of the hydrophobic chain length in the Hyadd family. A sigmoidal curve actually fits best the correlation between $k_{G'}$ and the amide chain length: a shorter chain (C8 and C12) implies a fast decay of G' as consequence of heating, whilst an exponential growth of $k_{G'}$ is observed shifting to C15 but no other significant variations are noticed raising the number of carbon atoms to 16 and 18.

The same samples were used for MW analysis in order to clarify whether the $k_{G'}$ variation was solely driven by the global strength of the hydrophobic interactions formed between side chains or it was also influenced by a different backbone hydrolysis rate.

The GPC analysis of HA is generally performed in aqueous buffer (Tømmeraas & Melander, 2008), but the Hyadd polymers generate a gel in water. In this work the HA and Hyadd polymers were solubilized in DMSO and analyzed in TDA-Viscotek using DMSO as mobile phase. The Hyadd polymers in powder are not immediately solvated in DMSO but need an initial heating to be homogenized and solvated, this explains why time zero MW for Hyadd polymers has not been measured (Fig. 3a). No depolymerization was observed after the sample preparation in DMSO: the HA polymer measurements at time zero in water (without treatment) and in DMSO give a comparable MW value (within a 10% error; data not shown).

The k_{MW}, expressed as exponential decay function of MW vs. the hydrolysis time, does not show any relevant difference due to the presence or the length of the alkyl chain in the HA backbone. The rate of depolymerization of Hyadd polymers is therefore not influenced by the amide chain length; clearly the $k_{G'}$ variations measured during thermal treatment are only due to the strength of cooperative hydrophobic interactions.

3.3. *Effect of alkyl chain on oxidative and enzymatic degradation*

Fast HA degradation in the joints is caused by the degradative action of ROS and hyaluronidase (Bastow et al., 2008). Šoltés et al. (2006) extensively described the degradative action of ROS on hyaluronan through rotational viscometry. Using the same approach, we verified if the variation of viscoelastic properties described under thermal depolymerization of the Hyadd derivatives was reproduced under free radical degradation.

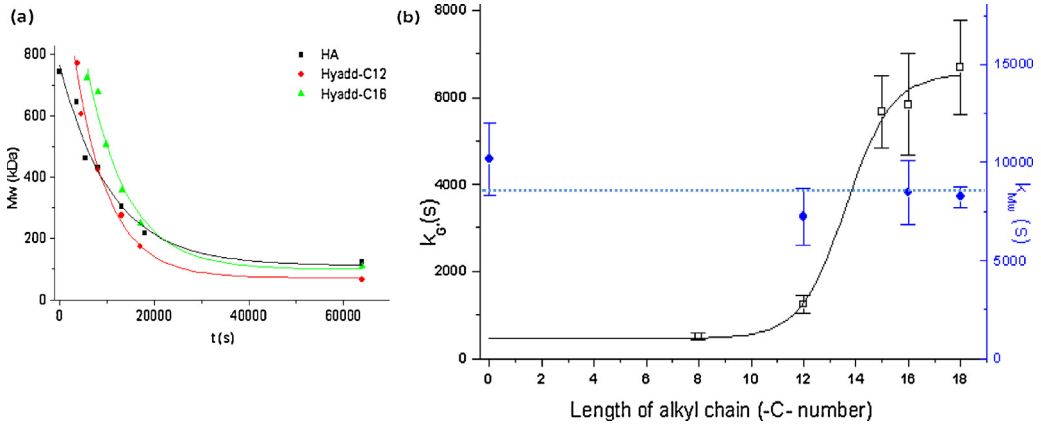

Fig. 3. (a) Interpolation of MW data (by TDA Viscotek) with an exponential decay function, for HA (black line), Hyadd-C16 (green line) and Hyadd-C12 (red line) gel. (b) Constants of exponential decay functions: $k_{G'}$ (black line) and k_{MW} (blue line) plotted versus the number of carbon atoms of alkyl chain functionalizing HA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

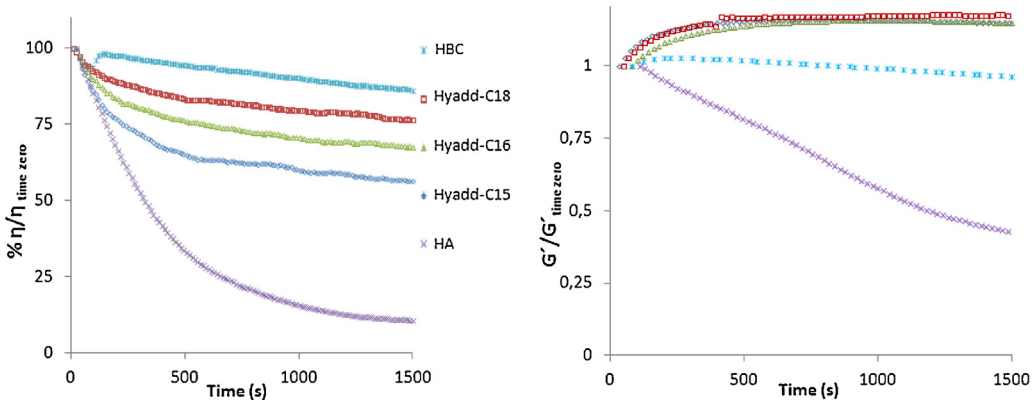

Fig. 4. (a) Normalized dynamic viscosity (% $\eta/\eta_{\text{time zero}}$) of HA (1.2 MDa, 15 mg/ml), HBC (25 mg/ml) and of Hyadd-C15, -C16, -C18 (8 mg/ml) vs. time during oxidative stress. (b) Elastic modulus ($G'/G'_{\text{time zero}}$) at 0.628 rad/s of HA (1.2 MDa, 15 mg/ml), HBC (25 mg/ml) and of Hyadd-C15, -C16, -C18 (8 mg/ml) vs. time during oxidative stress.

Fig. 4a shows the viscosity decrease of HA and Hyadd gels (8 mg/ml) during the action of an oxidative system consisting of 0.4 μM $CuCl_2$ and 0.4 mM ascorbic acid. Data obtained for HA 8 mg/ml, Hyadd-C8 and -C12 were not reported because the low initial dynamic viscosity does not allow for an accurate measurement of its decrease. In order to obtain a reference sample for an unmodified HA, a gel with comparable initial dynamic viscosity was thus prepared dissolving 1.2 MDa MW HA at 15 mg/ml in PBS. For Hyadd-C15, -C16 and -C18 the presence of long alkyl chains guarantees the maintaining of viscoelastic properties under oxidative stress, whilst their absence in unmodified HA results in a continuous and complete drop of the hydrogel viscoelastic properties. In detail, as already seen for the thermal hydrolysis (Section 3.2), the formation of hydrophobic interactions between chains from -C15 to -C18 engenders a comparable rate of dynamic viscosity decrease in the first 5 min, then once again it slows down hugely the loss of viscosity caused by depolymerization. The results obtained can be compared to the loss of viscosity observed for a chemical cross-linked HA (HBC, see Section 2.4) at higher concentration (25 mg/ml of HA).

The same experiment was performed recording the elastic modulus (at 0.628 rad/s): Fig. 4b shows a comparable resistance of viscoelastic properties to ROS between these physical hydrogels (Hyadd-C15, -C16 and -C18) and a chemical hydrogel (HBC).

Finally, in order to confirm the stronger resistance of the Hyadd polymers to degradation in comparison with unmodified HA, we assessed the enzymatic degradation rate of Hyadd-C16 (8 mg/ml) and the native polysaccharide (1.2 MDa at 15 mg/ml) at increasing hyaluronidase concentration. Fig. 5 shows how the rheological properties of the HA hydrogel are affected by the presence of the enzyme even at the lowest concentrations tested, while a quite large amount of hyaluronidase is needed to get a detectable decrease in dynamic viscosity of Hyadd-C16.

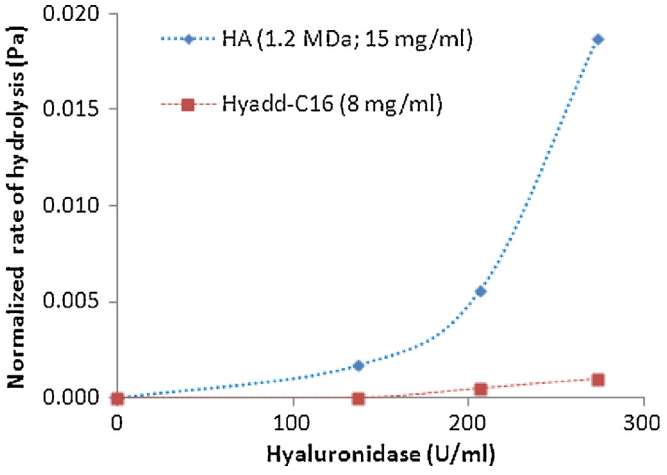

Fig. 5. Normalized rate of hydrolysis plotted against the amount of hyaluronidase. The rate of hydrolysis was obtained from the normalized angular coefficient of the initial (time zero) linear regression of dynamic viscosity (Pa s at 0.5 s^{-1}) vs. time (s) after enzyme incubation for 2 min.

4. Conclusions

Unmodified Hyaluronan has been widely used for viscosupplementation in OA; its short residence time in the joint is due to the extremely fast degradation occurring by hyaluronidase and oxidative stress. We synthesized a set of partial amide derivatives of HA, named Hyadd polymers, bearing C8, C12, C15, C16 and C18 *n*-alkyl moieties. We demonstrated that these derivatives are readily depolymerised under thermal, oxidative and enzymatic stress with a rate comparable with that observed in unmodified HA, but nevertheless, thanks to the cooperative hydrophobic interactions they maintain their viscoelastic properties which are fundamental for the treatment of joint degeneration. Furthermore, the longer the alkyl chain, the slower the drop in rheological properties, with the best performances obtained with the -C15, -C16 and -C18 derivatives, a behavior comparable to covalent cross-linked HA (HBC). We also checked that amide side chain is affected neither by hyaluronidase attack, nor by thermal and oxidative treatment. This multi-perspective study shows how viscoelastic properties of HA-alkyl derivatives can be preserved by hydrophobic interactions at the same level of covalent bonds under physiological conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.04.090.

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