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Synthesis of N-alanyl-hyaluronamide with high degree of substitution for enhanced resistance to hyaluronidase-mediated digestion

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ABSTRACT

In order to increase the resistance of hyaluronic acid towards enzymatic digestion, we prepared N-alanyl-hyaluronamide derivatives using different amidation methods performed either in water, water/acetonitrile mixture or anhydrous dimethylformamide. Our results indicate that amidation of hyaluronic acid in an anhydrous solvent is effective and led to a degree of substitution of the carboxylic groups up to 100%. We also demonstrated the N-alanyl-hyaluronamides present enhanced resistance towards enzymatic digestion while forming solutions with similar viscosities than solutions of hyaluronic acids of similar lengths.

1. Introduction

Hyaluronic acid (HA), also called hyaluronan (Meyer & Palmer, 1934), is a high molecular weight polymer with repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid, linked by alternating glycosidic bonds β-(1,4) and β-(1,3) (Fig. 1) (Weissmann & Meyer, 1954). It is a major component of the extracellular matrix of vertebrates and is found in high concentrations in synovial fluid, vitreous body and skin (Fraser, Laurent, & Laurent, 1997). Its main function is to serve as a lubricant, shock absorber and matrix (Baumann, 2004; Laurent, Laurent, & Fraser, 1995), properties which are strongly related to HA's ability to form shear-dependant and highly viscous solutions (Laurent & Fraser, 1992). HA can be easily obtained in large quantity from animal tissues such as rooster comb or more recently from recombinant bacteria (Shiedlin et al., 2004). This has allowed extensive use of HA and gels of HA for visco-supplementation by injection into joints to treat arthritis, for ophthalmic surgery and for tissue augmentation in cosmetic surgery (Kogan, Soltés, Stern, & Gemeiner, 2007). More recent applications include drug delivery and wound healing (Chen & Abatangelo, 1999; Esposito, Menegatti, & Cortesi, 2005). However, repeated injections of HA are often necessary to compensate for its naturally occurring degradation (Stern, Kogan, Jedrzejas, & Šoltés, 2007). The hyaluronidase enzymes and the CD44 (Cluster of Differentiation-44) cell surface receptors are responsible for HA degradation (Aruffo, Stamenkovic, Melnick, Underhill, & Seed, 1990). Molecular modeling based on crystallographic studies indicated that the recognition sites of the Hyal-2 enzymes and CD44 receptors are the carboxylate groups of HA (Banerji et al., 2007). Its chemical modification may hence diminish enzymatic recognition and enhances the stability of HA towards hyaluronidase-mediated hydrolysis.

In here, we evaluated the effect of grafting alanine onto HA to mask the HA backbone for enzymatic recognition by hyaluronidase while maintaining the overall anionic density of the polymer. For integral masking of the HA, the ideal is to obtain the highest achievable degree of modification and we therefore assayed various amidation methods. The effect of the chemical process on the physicochemical behavior of the Ala-HA and stability to hyaluronidase digestion was also reported.

2. Materials and methods

2.1. Materials

1-Ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC), N-hydroxysuccinimide (NHS), L-alanine ethyl ester HCl (AlaOEt-HCl), sodium hydroxide (NaOH), tetrabutylammonium (TBA) hydroxide solution (~40% or 1.5 M in water), 2-chloro-1-methylpyridinium iodide (CMPI), triethylamine, acetonitrile, N-methylmorpholine (NMM), 2-chloro-dimethoxy-1,3,5-triazine (CDMT) were pur-

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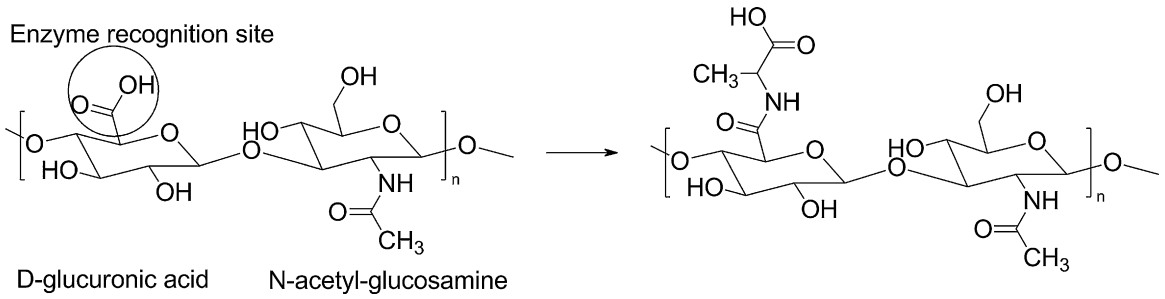

Fig. 1. Chemical structure of hyaluronic acid and N-alanyl-hyaluronamide with a degree of substitution of 100%.

chased from Sigma–Aldrich and used without purification except for ion exchange resin Dowex 50WX8-200 which was previously washed with water and ethanol. Hyaluronic acid (HA) sodium salt was isolated from *Streptococcus equi* and was given at a molecular weight of 1.58 MDa. Hyaluronidase from bovine testes Type I-S had a given activity of 850 units/mg units. Purified water was obtained with an Arium 661 Sartorius system (Goettingen, Germany). ^{1}H NMR spectra were obtained using a 400 MHz Bruker spectrometer. Lyophilization was performed using a Christ Alpha 2-4 LSC Lyophilizer at 0.2 mbar at 25 °C during 22 h then at 0.09 mbar at 25 °C during 2 h. Products were previously frozen by immersion into liquid nitrogen during 15 min. Visking dialysis membranes of 12–14,000 Da were purchased from Medicell International Ltd. (London).

2.2. Purification of the reaction mixtures

Each mixture was dialyzed 24 h against water (3 L), 2 h against 0.05 M NaOH (3 L), 40 h against 0.1 M NaCl (3 L), 8 h against 25% ethanol solution and finally 72 h against water (3 L). The solution was then lyophilized to obtain the various HA-alanine derivatives. For the reaction with EDC, the dialysis against ethanol solution was omitted.

^{1}H NMR (D_2O) δ ppm: 4.43 (m, 2H, O–CH–O), 3.88–3.11 (m, 12H, C–CH–O), 1.97 (s, 3H, CO–CH_3–N), 1.38–1.23 (m, 3H, C–CH_3–CO).

2.3. Synthesis of HA-alanine in aqueous solvent with EDC

Adapted from Bulpitt and Aeschlimann (1999). The sodium salt of HA (264 mg, 0.66 mmol) was dissolved in water (87 mL) for 1 h before addition of L-alanine ethyl ester (AlaOEt-HCl (811 mg, 5.28 mmol)). The pH was adjusted to 7.5 using 1 M NaOH. To this solution was added a freshly prepared solution of NHS (304 mg, 2.64 mmol) and EDC (506 mg, 2.64 mmol) in water (1 mL). The pH was again adjusted to 7.5 using 1 M NaOH and the reaction was allowed to proceed overnight under constant magnetic stirring (approximately 300 rpm). After purification (see procedure above), the HA-alanine was obtained as a white powder (229 mg, 86% yield). The same reaction was performed with the followings quantities: EDC (1012 mg, 5.28 mmol, 8 equiv.), Ala-HCl (1622 mg, 10.56 mmol), and NHS (607.6 mg, 5.28 mmol). For this reaction, 235 mg (88% yield) of HA-alanine was recovered.

2.4. Synthesis of HA-alanine using CDMT in water/acetonitrile

Adapted from Bergman, Elvingson, Hilborn, Svensk, and Bowden (2007). The sodium salt of HA (264 mg, 0.66 mmol) was first dissolved in water (52 mL) before addition of acetonitrile (35 mL). The solution was cooled in an ice bath and CDMT (362 mg, 2 mmol), was then added. One hour later, AlaOEt-HCl (460 mg, 3 mmol) then NMM (280 μL, 3 mmol) were added and the reaction was allowed to proceed overnight under constant stirring (approximately 300 rpm). Excess amine were removed by incubation with ion exchange resins (Dowex-H^+, 2 g then Dowex-Na^+, 2 g, 1 h each), The solution containing the HA was then purified as described above to give the HA-alanine (213 mg, 76% yield) as a white powder.

2.5. Synthesis of HA-alanine in anhydrous DMF

Adapted from Magnani, Rappuoli, Lamponi, and Barbucci (2000). HA was first prepared as a tetrabutylammonium (TBA) salt to allow its solubilization in DMF. The sodium salt of HA (2 g, 5.14 mmol) was dissolved in water at a concentration of 5 mg/mL. Dowex 50WX8-200, a strong acid exchange resin was added slowly until a measured pH of 2.5. The resin was filtered off and the solution containing the HA (acid form) was treated, dropwise with tetrabutylammonium hydroxide solution (40% m/m) until pH 9–10. The solution was then lyophilized to give the HA-TBA (2.1 g). HA-TBA salt (400 mg, 0.66 mmol) was dissolved in anhydrous DMF (80 mL) under argon for 6 h. The mixture was then ice-cooled and treated sequentially with AlaOEt-HCl (460 mg, 3 mmol), CMPI (511 mg, 2 mmol) and triethylamine (700 μL, 5 mmol). The reaction was allowed to warm to room temperature and stirred under argon overnight. The solution was then brought to 0 °C for dilution with water (80 mL) and purified as described above to obtain after lyophilization HA-alanine (264 mg, 89% yield) as a white powder. The same reaction was performed using: (1) HA-TBA (400 mg, 0.66 mmol), AlaOEt-HCl (154 mg, 1 mmol), CMPI (204 mg, 0.8 mmol), triethylammine (256 μL, 1.8 mmol) to obtain 279 mg of HA-alanine (94% yield), (2) HA-TBA (400 mg, 0.66 mmol) AlaOEt-HCl (92 mg, 0.6 mmol), CMPI (102 mg, 0.4 mmol), triethylammine (140 μL, 1 mmol) to obtain 237 mg of HA-alanine (83% yield).

2.6. Detection of amines

Adapted from Romberg et al. (2005). HA-alanine samples (100 μL, 5 mM solution in water) were mixed with 100 μL of 1 M acetate buffer (pH 5.5) and 200 μL of ninhydrin solution (0.2 g ninhydrin and 0.03 g hydrindantin dissolved in 7.5 mL 2-methoxyethanol and 2.5 mL acetate buffer pH 5.5). The solutions were heated at 100 °C for 15 min, cooled to room temperature and the volume adjusted to 1 mL by addition of 50% ethanol for absorbance measurement at 570 nm. Control solutions were prepared with 0.1 and 0.5 mM L-alanine ethyl ester in water.

2.7. Evaluation of polymer size

Size exclusion chromatography was performed using an Ultimate 3000 system from Dionex (Sunnyvale, CA, USA) and 3 columns Shodex OH-pak 1802.5Q, 1804HQ and 1806HQ (Showa Denko America, New York, USA) of 30 cm in series at 30 °C with a flow rate of 0.5 mL/min. The samples were dissolved overnight includ-

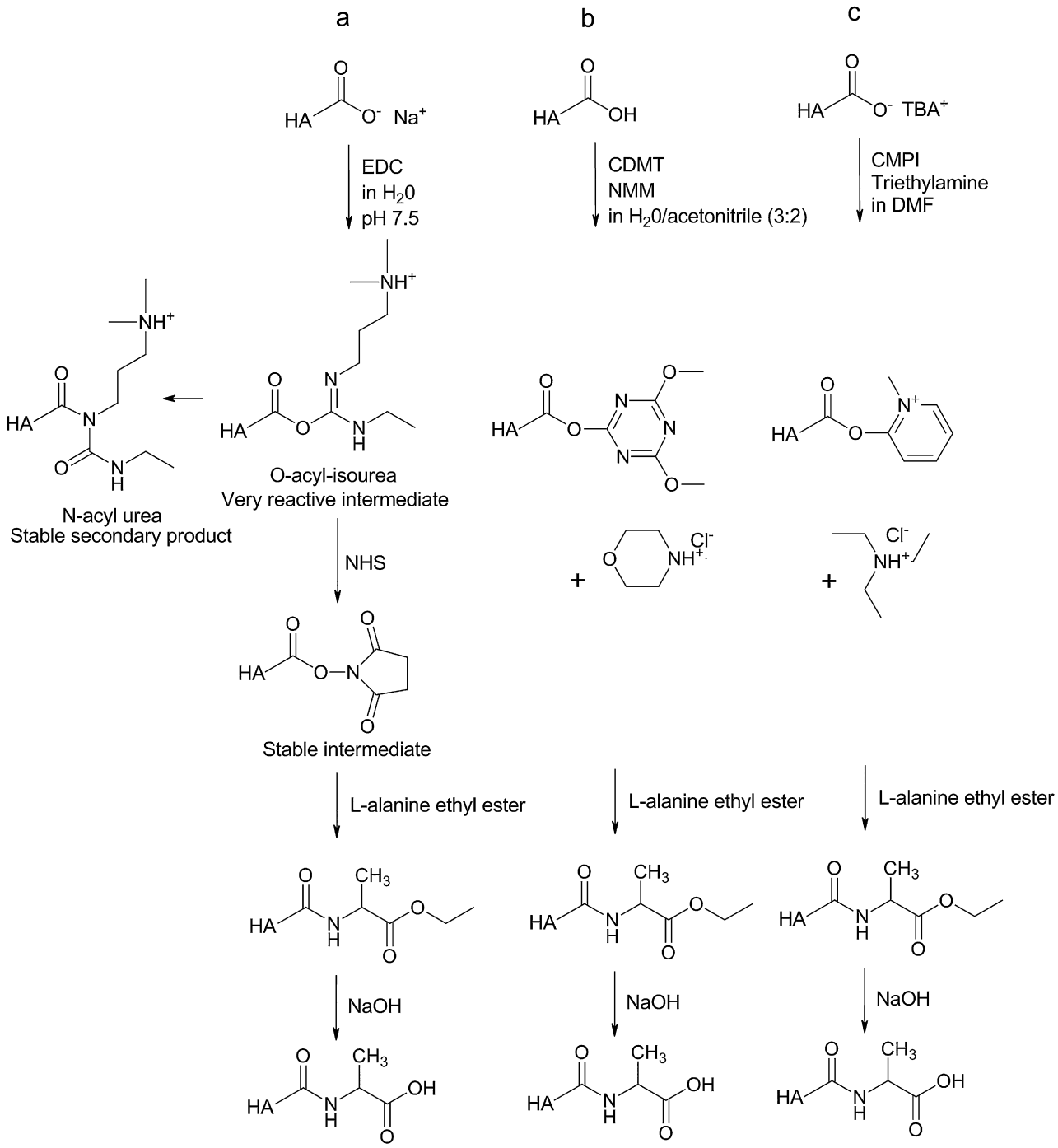

Fig. 2. Synthetic routes to N-alanyl hyaluronamide.

ing 6 h under mechanical stirring in an aqueous solution containing 0.1 M $NaNO_3$ at a concentration of 1.2–1.5 mg/mL depending on the sample. Prior to analysis, each sample was filtered through a 0.45 μm hydrophilic PTFE Milex-LCR filter (Millipore, Bedford, MA, USA). The injection volume was 100–120 μL depending on the sample concentration. MALS detection was performed continuously on the column eluate with a Dawn Heleos II light scattering detector in series with an Optilab rEX differential refractometer (both from Wyatt Technology, Santa Barbara, CA, USA) with a wavelength of 658 nm. Data were analyzed using Astra software 5.3.4 and first order Zimm fits. The dn/dC refractive index increments were experimentally determined using the same refractometer and solvent conditions as for SEC/MALS for native hyaluronic acid ($dn/dC = 0.149$ mL/g) as well as for HA-alanine with 100% grafting ratio ($dn/dC = 0.143$ mL/g).

2.8. Rheology

Dynamic viscosity measurements were performed in steady shear mode with a cone plate rheometer Rheostress RS100 (Haake Technik GmbH, Vreden, Germany) equipped with a Rheowin software (version Pro 2.93). The cone used had a diameter of 60 mm and an angle of 1°. The temperature was maintained at 25 °C with a TC Peltier thermostatic system. All samples were dissolved in water

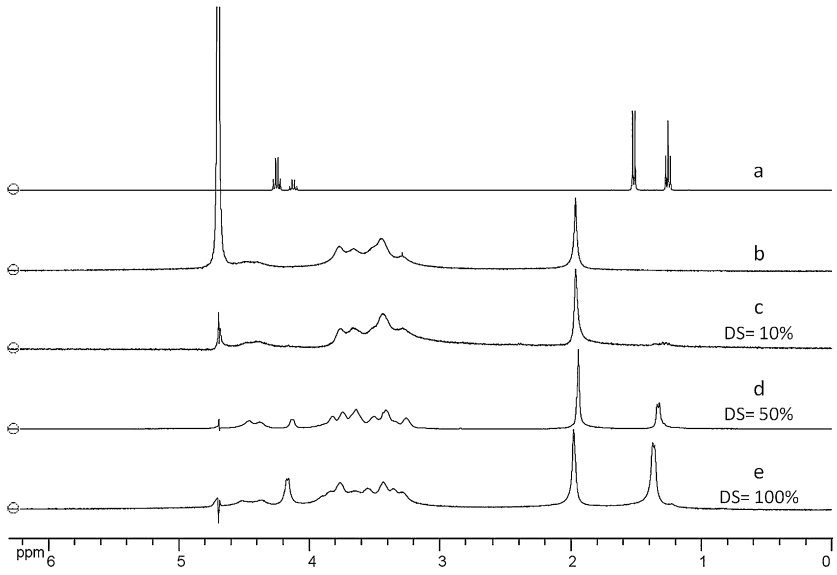

Fig. 3. Comparative analysis of ^{1}H NMR spectrum of the various N-alanyl-hyaluronamide with the highest possible degree of substitution that was achieved using: EDC in aqueous phase (spectrum c), CDMT in water/CH_3CN (spectrum d) and CPMI in DMF (spectrum e). Spectra (a) and (b) correspond to alanine ethyl ester and the native HA respectively.

at least 24 h before use at the same concentration of 2 mg/mL for comparison reasons. Measurements were made with a preset shear stress ramp to evaluate the corresponding shear rate.

2.9. Enzymatic degradation of Ala-HA

The method used was adapted from Muckenschnabel, Bernhardt, Spruss, Dietl, and Buschauer (1998) using the Morgan-Elson colored reaction. 100 μL of 5 mM Ala-HA solution, 100 μL of purified water and 50 μL of phosphate buffer were incubated with 25 μL of 4.10^6 IU/mL hyaluronidase solution at 37 °C during 1–6 h. The enzymatic reaction was stopped by adding 50 μL of tetraborate solution and heating the tubes in boiling water for 5 min. After cooling in ice for 2 min, 750 μL of dimethylamino benzaldehyde (DMAB) (2 g of DMAB of dissolved in 2.5 mL HCl and 47.5 mL glacial acetic acid immediately before use) solution were added to each tube. After 60 min incubation, the absorbance at 586 nm was measured.

3. Results and discussion

3.1. Synthesis of alanine-hyaluronic acid (Ala-HA) conjugate

There are almost innumerous methods to couple amines to carboxylic acid and a few have been adapted to HA. Activation of the HA carboxylic acid with EDC is one of the most popular methods (Bulpitt & Aeschlimann, 1999; Danishefsky & Siskovic, 1971), because it can be done in water, the native solvent for HA. EDC first reacts to the carboxylic acid to form a highly reactive O-acyl isourea intermediate (Fig. 2). This specie then either reacts with a nucleophile, namely the amine but also competitive water, or rearranges into a stable N-acyl urea, which impedes the reaction. The reaction is quite subtle as the optimal pH is different for each step. Indeed, the carboxylic acid activation by EDC is best performed in an acidic environment (pH 4.75) (Kuo, Swann, & Prestwich, 1991), whereas the nucleophilic attack by the amine is best done at high pH, when the amine is unprotonated. The compromise is therefore not easy to define, especially for amines with high pKa values and stabilization by formation of succinimidyl ester of HA showed to be helpful (Bulpitt & Aeschlimann, 1999). We therefore adapted the procedure and incubated the HA (1.58 MDa) in water with excess of AlanineOEt and EDC (8 and 4 equiv., respectively) in the presence of N-hydroxysuccinimide (4 equiv.) at pH 7.5. After one night, the mixture was dialysed to remove low molecular weight molecules. We found out convenient to deprotect the carboxylic acid of alanine during dialysis by incubating the dialysis tubes in 50 mM NaOH for 2 h at 20 °C. The duration of this step was carefully monitored since strong alkaline conditions are reported to give rise to HA fragmentation (Tokita & Okamoto, 1995). A 2 h treatment at 20 °C with a 50 mM NaOH solution was enough to provide a NMR spectrum devoid of peaks corresponding to ethyl groups, confirming an effective deprotection. Finally we assayed the purity of the polymer after dialysis by verifying the absence of free amines (unreacted amino-acids) with the ninhydrine colorimetric test. The final yield was 88% and the degree of substitution (DS) was calculated from the NMR spectrum (Fig. 3c) at 10%. Doubling the equivalents of both EDC and amine improved only marginally the DS from 10 to 13%. These results are in accordance with previous results of the literature indicating that amidation of HA with EDC and NHS in water led to relatively low DS values (Crescenzi, Francescangeli, Taglienti, Capitani, & Mannina, 2003; Kurisawa, Chung, Yang, Gao, & Uyama, 2005; Young, Cheng, Tsou, Liu, & Wang, 2004).

We next evaluated a more recently described condensation method which is performed in water/acetonitrile using 2-chloro-dimethoxy-1,3,5-triazine (CDMT) as the reagent (Bergman et al., 2007). The method appeared interesting because it avoided use of NHS and pH monitoring. At first, we performed the reaction exactly as described by preparing HA in its acid form (Bergman et al., 2007). However, in our hands, lyophilization of the acidic form of HA preceding the amidation reaction provoked important fragmentation of HA, a property that was already noticed by others (Doherty, Hughes, Kim, Mainwaring, & Charman, 1994; Tokita, Ohshima, & Okamoto, 1997). We therefore modified the reaction procedure to avoid handling of the HA in its acidic form and started directly from the commercial HA sodium salt. The HA was then reacted with CDMT to form a HA-dimethoxytriazine intermediate and the release of HCl was trapped with N-methylmorpholine (NMM). Deplacement of the dimethoxyhydroxytriazine by nucleophilic attack of the alanine ethyl ester amine led to N-alanyl ethylester-hyaluronamide (Fig. 2b). Deprotection of the ester and

Table 1
Summary of the preparation methods of Ala-HA.

Starting salt	Conditions	Yield (%)	DS (% relative to HA)
HA-Na	8 equiv. EDC, NHS, H_2O, overnight	88	13
HA-Na	3 equiv. CDMT, H_2O, CH3CN, NMM, overnight	76	50
HA-TBA	3 equiv. CMPI, DMF, triethylamine, overnight	89	100

purification of the polymer were carried out as well during the dialysis. After lyophilization, the final yield was 76%. NMR analysis (Fig. 3d) shows the expected N-alanyl hyaluronamide with a DS of 50%.

Although this method was more effective than EDC (DS around 50% versus 13%), our aim was to obtain a complete substitution of the HA carboxylic acid. We therefore assayed a third amidation which is performed in anhydrous conditions. Magnani et al. (2000) described the use of CMPI for cross linking HA in DMF. To ensure solvatation in anhydrous DMF, HA was firstly converted into a tetrabutylammonium (TBA) salt and condensed with alanine ethyl ester using a slight excess of CMPI (1.2 equiv.) and in the presence of triethylamine for neutralization of the released HCl and HI (Fig. 2). Purification and concomitant deprotection afforded a N-alanyl hyaluronamide with 100% substitution as calculated from the NMR spectrum (Fig. 3e). The effectiveness of this reaction (100% of HA substitution was observed using only 1.2 of activator equivalents) was remarkable and allowed us to obtain very easily Ala-HA derivative with lower DS by simple adjustment of the CMPI to HA stoichiometry (Table 1).

3.2. Physicochemical properties of the N-alanyl-hyaluronamide

We previously prepared Ala-HA with different DS from 10 to 100% using various synthetic methods. It was previously indicated that HA is very sensitive to fragmentation (Bergman et al., 2007; Pelletier, Hubert, Lapicque, Payan, & Dellacherie, 2000). We therefore studied the effect of the various synthetic methods on the HA molecular weights distribution. Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) was used to measure the molecular weight distribution of the polymers. For accuracy and to have access to the real molecular weights, we determined experimentally the refractive index increment (dn/dC) of N-alanyl-100-HA in the size exclusion elution phase (0.1 M $NaNO_3$ solution) and found a value of 0.143 mL/g. The dn/dC of HA was also measured in the same buffer at 0.149 mL/g, a value within the expected range (0.150 mL/g in NaCl (Mendichi, Schieroni, Grassi, & Re, 1998), 0.167 mL/g in PBS (Hokputsa, Jumel, Alexander, & Harding, 2003), 0.142 mL/g $NaNO_3$ (Moon, Shin, Lee, Hwang, & Cho, 2008)).

The results from these experiments are reported in Table 2. For clarity, the various N-alanyl-hyaluronamide were abbreviated as AlaXHA, *X* being the DS in percentage. The measured molecular weight of the commercial HA had almost the expected average molecular weight (measured at 1.26 MDa with a given one at 1.58 MDa). As seen, the molecular weights of Ala-HA are lower than the native HA indicating that the chemical and purification processes produced damages to the starting polymer. The least degradation was observed for Ala13HA obtained using EDC. Modification of HA in DMF with CMPI led to slightly more HA fragmentation. The lowest molecular weight values were obtained for Ala-HA that was prepared using CMPI in organic solvent.

Table 2
Molecular weights (M_w), polydispersity index (PDI) and fragmentation of modified HA.

	M_w (g/mol)	PDI (M_w/M_n)	Fragmentation ratio[a]
Native HA	1,261,000	1.29	1
Ala13HA – EDC	351,500	1.65	3.6
Ala10HA – CMPI	229,800	1.73	5.5
Ala40HA – CMPI	283,500	1.72	4.4
Ala67HA – CMPI	218,000	1.74	5.8
Ala100HA – CMPI	288,900	1.68	4.4
Ala50HA – CDMT	313,400	1.77	4.0

[a] Relative to the theoretical value calculated using the native HA M_w and the DS.

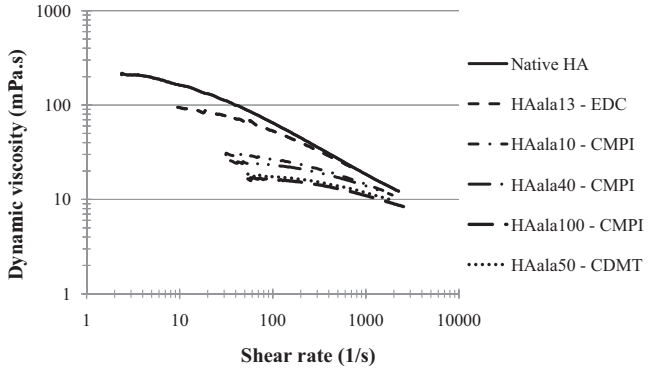

Fig. 4. Measurement of polymer viscosity. Each polymer was dissolved at 2 mg/mL in purified water.

We next evaluated the contribution of the chemical modification on the viscosity of the polymer in aqueous solution at concentration of 2 mg/mL. Fig. 4 shows the dynamic viscosity profile of AlaHA in function of shear stress. The viscosity profiles of all HA-ala derivatives are lower than the native HA, which is in line with the lowered molecular weight values measured by SEC-MALS. HAala13 obtained using EDC which had the highest molecular weight, also showed the highest viscosity compared to the other derivatives. However, for all polymers, the decrease in viscosity at higher shear rates shows that the shear thinning properties of native HA is preserved even after L-alanine grafting.

We finally assayed the effect of the modification on the susceptibility of Ala-HA towards enzymatic digestion. Ala-HAs of various DS (2 mM in PBS) were incubated in the presence of hyaluronidase at 37 °C (Muckenschnabel et al., 1998). The reaction mixtures were

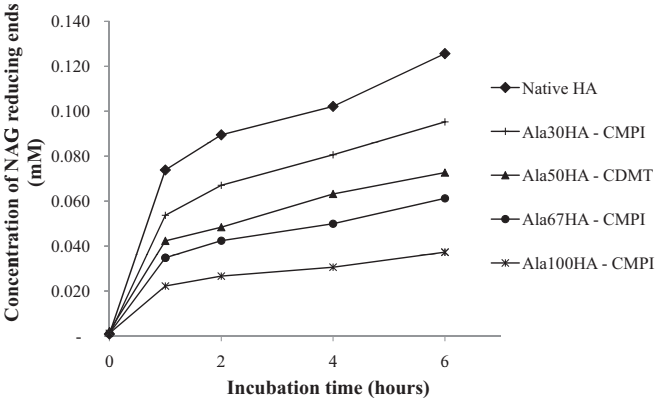

Fig. 5. Enzymatic degradation profiles for the various alanyl-hyaluronamide. Each polymer solution (2 mM) was incubated with hyaluronidase 4.10^6 IU/mL in PBS at 37 °C. Fragmented HAs were detected by colorimetric dosage of NAG reducing ends.

then stopped at different times and the N-acetyl glucosamine reducing ends were quantified using the Morgan-Elson colorimetric reaction. Results show alanine modification to diminish enzymatic degradation according to the grafting degree (Fig. 5).

4. Conclusion

In the present study, we prepared Ala-HA using different chemical methods and showed that alanine can be condensed to carboxylic acid of HA at will and at degrees of substitution up to 100%. The various chemical processes did induce HA fragmentation but to still acceptable levels. Moreover, we also demonstrated that Ala-HAs present enhanced resistance towards enzymatic digestion while forming solutions with shear-thinning properties. These results suggest that Ala-HAs and similar aminoacid-HA derivatives may become interesting for viscosupplementation purposes.

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