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Improvement of hyaluronic acid enzymatic stability by the grafting of amino-acids

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ABSTRACT

Injection of hyaluronic acid (HA)-based hydrogels has proven to provide many therapeutic benefits. To increase the stability of HA-based products against enzymatic digestion, we modified hyaluronic acid by grafting various amino acids on its carboxylic group and then evaluated the enzymatic stability of the various conjugates in presence of a hyaluronidase. Our results showed that all amino acid-modified HA polymers were more resistant to degradation compared to the native HA albeit with variation according to the amino acids. Amino acids with carboxylate groups such as aspartic acid or with hydroxyl functions (threonine, serine or tyrosine) conferred a particularly strong resistance to HA towards enzymatic digestion. The HA-amino acid products were then cross-linked with butanediol diglycidyl ether (BDDE). The swelling properties of the formed hydrogels appeared close to native HA whereas the increased resistance towards hyaluronidase digestion remained. These results suggest that amino acid-modified HA derivatives can become promising material for viscosupplementation or drug delivery.

1. Introduction

Hyaluronic acid (HA), a naturally occurring polymer, is today of major interest as a biomaterial for many medical applications. As a high molecular weight polysaccharide consisting of linear repeating units of D-glucuronic acid and N-acetylglucosamine, it has unique viscoelastic and rheological properties which make it a valuable component of many tissues in the body. As an answer to clinical signs of aging caused by lowered HA contents, HA supplementation by injection into joints and dermis has been used for many years (Kogan, Soltés, Stern, & Gemeiner, 2007). Obstacles to the treatment by HA injection are its enzymatic degradation by hyaluronidases in the body and as well as its dilution away from the injection site. It is known that the half-life of HA after injection in skin and joints is no longer than 24 h (Brown, Laurent, & Fraser, 1991). Many chemical modifications and other techniques have been extensively described to prolong HA's residence after administration in the body (Schanté, Zuber, Herlin, & Vandamme, 2011a). HA crosslinking is a suitable method to produce hydrogels with longer residence time in the tissues (Collins & Birkinshaw, 2007; Tomihata & Ikada, 1997a). To yet prolong the residence time of crosslinked HA hydrogels, an additional modification was studied, consisting in grafting on HA's carboxylic groups a small and natural molecule which would impact the least its valuable physicochemical properties and yet hinder its enzymatic degradation. A previous study showed that the grafting of an amino acid, L-alanine, onto HA, enabled to considerably decrease its enzymatic degradation (Schanté, Zuber, Herlin, & Vandamme, 2011b).

To further study the effect of amino acid grafting onto HA and subsequent crosslinking, the present article describes the synthesis of HA derivatives obtained by grafting a wide range of amino acids comprising various functional groups (Fig. 1) and further cross-linking with butanediol diglycidyl ether (BDDE). The *in vitro* stability of the novel HA derivatives was assessed as a function of the nature of the grafted amino acid and its degree of substitution. The cross-linked HA-amino acid derivatives (HA-aa) were studied to produce hydrogels for future therapeutic applications. The enzymatic degradation profile and physical characteristics were compared to the native HA hydrogels.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich. HA sodium salt, hyaluronidase from bovine testis, amino acids in ethyl

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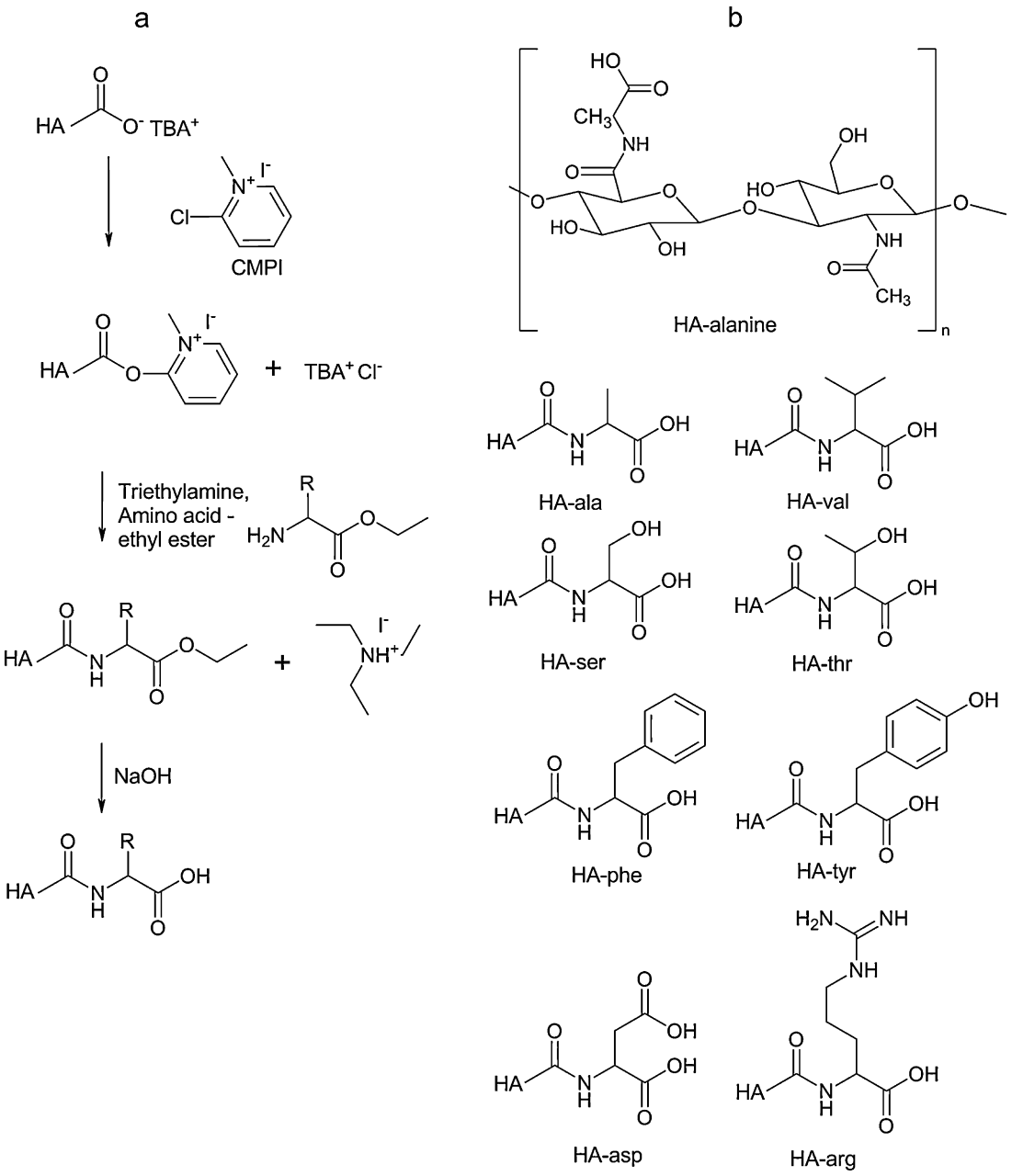

Fig. 1. (a) Synthesis of HA-amino acid by amidation with CMPI in DMF and (b) chemical structure of conjugated HA-amino acids.

ester HCl form, sodium hydroxide (NaOH), tetrabutylammonium (TBA) hydroxide solution (~40% or 1.5 M in water), 2-chloro-1-methylpyridinium iodide (CMPI), triethylamine, butanediol diglycidyl ether (BDDE) and acetic acid were used without purification. Ion exchange resin Dowex 50WX8-200 was previously washed with water and ethanol. HA sodium salt was isolated from *Streptococcus equi* and was given at a molecular weight of 1.58×10^6 Da. Purified water was obtained with an Arium 661 Sartorius system (Goettingen, Germany). ^{1}H NMR spectra were obtained using a 400 MHz Bruker spectrometer. Samples (8–10 mg) were previously dissolved in D_2O (650 μL). 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. Grafting of an amino acid on HA

HA sodium salt was previously converted to its tetrabutylammonium (TBA) salt to allow its solubilization in the organic solvent. 2 g (5.14 mmol) of HA sodium salt was solubilized in water at a concentration of 5 mg/mL. Dowex 50WX8-200, a strong acid exchange resin was added to the solution until reaching pH 2.5. The solution was then filtered to remove the resin and a 40% tetrabutylammonium hydroxide solution was added dropwise until pH 9–10. The solution was then lyophilized and 2.11 g of the HA-TBA salt was recovered.

The grafting method used is derived from Magnani, Rappuoli, Lamponi, and Barbucci (2000). HA-TBA salt (400 mg, 0.66 mmol) obtained previously was dissolved in anhydrous DMF (80 mL) under argon for 6 h. After cooling the solution with an ice bath, amino acid ethyl ester HCl (1 mmol), CMPI (204.4 mg, 0.8 mmol) and triethylamine (256 μL, 1.8 mmol) were added to the solution.

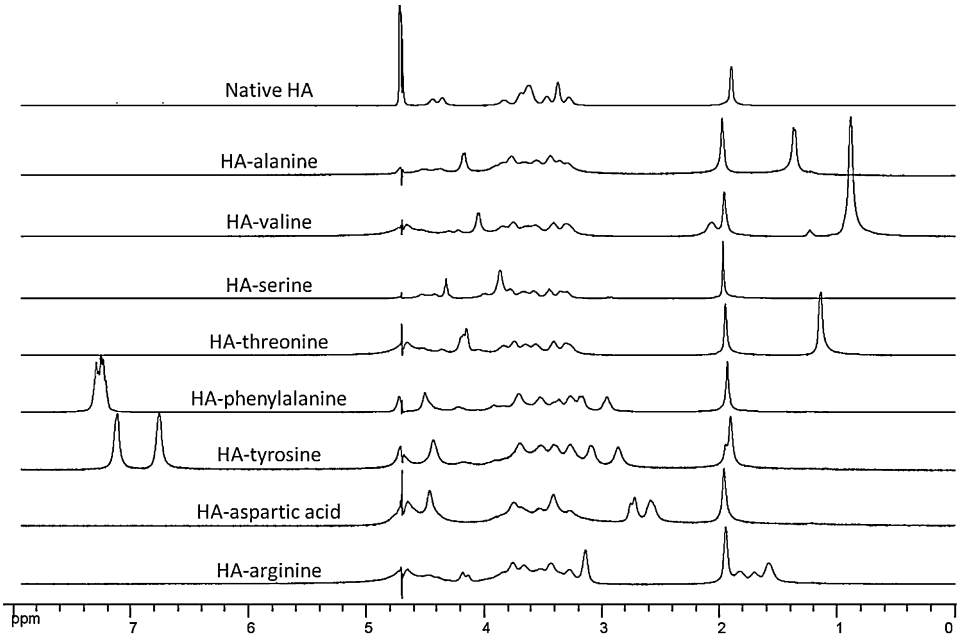

Fig. 2. NMR spectra of native HA and HA-amino acid derivatives.

The reaction was allowed to warm at room temperature and stirred under argon overnight. The solution was then brought to 0 °C for dilution with water (80 mL). 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-amino acid derivatives (HA-aa). ^{1}H NMR spectra are shown in Fig. 2.

2.3. Ninhydrin assay

The ninhydrin test was adapted from the method described by Romberg et al. (2005) HA-aa samples (100 μL, 5 mM solution in water) were mixed with acetate buffer (100 μL, 1 M, 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 was 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 amino acid in water.

2.4. Enzymatic degradation of HA-aa derivatives

The method used was adapted from Muckenschnabel, Bernhardt, Spruss, Dietl, and Buschauer (1998) using the Morgan-Elson colored reaction. HA-aa solutions (100 μL) at a concentration of 5 mM, water (100 μL) and phosphate buffer (50 μL) were incubated with 4×10^6 IU/mL hyaluronidase solution (25 μL) at 37 °C during 1–6 h. The enzymatic reaction was stopped by adding tetraborate solution (50 μL) and heating the tubes in boiling water for 5 min. After cooling in ice for 2 min, 750 μL of dimethylamino benzaldehyde (DMAB) solution (2 g of DMAB dissolved in 2.5 mL HCl and 47.5 mL glacial acetic acid prepared immediately before use) were added to each tube. After 60 min incubation, the absorbance at 586 nm was measured.

2.5. Rheology

Dynamic rheology experiments 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 at least 24 h before use at a concentration of 2 mg/mL. Measurements were made with a preset shear stress ramp to evaluate the corresponding shear rate.

2.6. 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, NY, USA) of 30 cm in series at 30 °C with a flow rate of 0.5 mL/min. The samples were dissolved overnight including 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 (dn/dC = 0.149 mL/g).

2.7. Crosslinking of the HA-aa derivatives

Crosslinking was performed in acidic conditions according to the method described by De Belder and Malson (1986). HA-aa (80 mg, 0.2 mmol) obtained previously was dissolved in water (800 μL) overnight. Butanediol diglycidyl ether (BDDE) (60 μL, 0.163 mmol, 0.82 equiv.) was mixed with glacial acetic acid (30 μL) and added to the HA solution. After immediate mixing with a vortex mixer, the tubes were centrifuged at 2500 rpm for 4 min and incubated at 60 °C during 4 h. After the crosslinking reactions, the resulting gels were purified by dialysis. Water (25 mL) was added to the gels

which were then transferred to dialysis membranes. Dialysis was performed against purified water during 72 h after which the gel was emptied out of the membrane onto a small strainer to eliminate the surrounding excess water and weighed. The swelling ratio was calculated by dividing the weight of the final swollen gel by the weight of the starting dry solid content (80 mg for each in this case). Experiments were made in triplicates and the results were analyzed by one-way ANOVA and the Student–Newman–Heuls multiple comparison test ($p = 0.05$) in order to determine the statistical significance of the differences observed.

2.8. Enzymatic degradation of HA-aa crosslinked hydrogels

The same method used as for the HA-aa polymers described above (Section 2.4) but with the crosslinked hydrogels. The gels were previously crushed and homogenized and then a stoichiometric amount was precisely weighed and adjusted with water to obtain a final concentration of 2.5 mg/mL in each test tube.

3. Results and discussion

3.1. Synthesis of HA-aa derivatives

Many chemical reactions have been reported for HA amidation and were previously reviewed (Schanté et al., 2011a). A previous study showed that, by using 2-chloro-1-methylpyridinium iodide (CMPI) in anhydrous conditions, the highest grafting ratios could be obtained compared to other methods, with only a slight excess of reagents for the grafting of L-alanine onto the carboxylic groups of HA (Schanté et al., 2011b). This method is derived from Magnani et al. (2000), who used CMPI for HA crosslinking with diamines (Magnani et al., 2000). In the present study, CMPI was used as the activator to graft the amino acids onto the carboxyl groups of HA. The sodium salt of HA was first converted into a tetrabutylammonium (TBA) salt to allow its solubilization in dimethylformamide (DMF) and condensed with the ethyl ester protected form of each amino acid using a slight excess of CMPI (1.2 equiv.) and triethylamine to neutralize the released HI (Fig. 1a).

Various amino acids comprising a wide range of functional groups (Fig. 1b) were grafted using the same synthetic route, the same reagent quantities with the objective to obtain the same grafting ratio of 100% for a most accurate comparison. Valine was grafted to evaluate the effect of isopropyl, a more important aliphatic group than methyl, whereas serine and threonine were grafted to evaluate the effect of a hydroxyl group. Phenylalanine and tyrosine both contain an aromatic group. Aspartic acid contains a second carboxylic acid group which adds a negative charge and the guanidinium group of arginine adds a positive charge to the polymer.

After incubation overnight, the reaction mixtures were all dialyzed against 100 mM NaCl solution followed by 25% ethanol solution to remove ionic and hydrophobic impurities. The carboxylic acid groups of the amino acids were deprotected by dialysis against 50 mM NaOH for 2 h at 20 °C. This was enough to provide a NMR spectrum devoid of peaks corresponding to ethyl groups, confirming an effective deprotection. Degrees of substitution (DS) of 100% for all derivatives were successfully obtained as calculated from the NMR spectra (Fig. 2). After lyophilization, a white powder was recovered for all HA-aa derivatives except for HA-serine, which had a light yellow color. Finally, the purity of the polymer after dialysis was confirmed by the absence of unreacted amino acids, assayed with the ninhydrine colorimetric test.

3.2. Rheology and molecular weight measurements

The rheological behavior of the HA-aa derivatives was evaluated by measuring their dynamic viscosity in steady shear mode at 25 °C in water. For comparison reasons, all amino acids were grafted using the same reaction conditions with 100% degree of substitution. The viscosity of all HA-aa derivatives is clearly dependant on the grafted amino acid (Fig. 3). The shear-thinning property of native HA is preserved for HA-tyr, HA-ala and HA-asp, which was one of our objectives. In addition, HA-tyr exhibited the highest viscosities of all followed by HA-ala and HA-asp. All other derivatives showed lower viscosities and no shear-thinning properties. HA-phe had the lowest viscosities of all. This variation could be due to mainly two factors: a difference in chain length, or a difference in inter and/or intramolecular interactions caused by the functional groups of the grafted amino acids. If not due to a lowered molecular weight, the lower viscosity of the derivatives could be caused by the disruption of the hydrogen bonds possibly by steric hindrance of the amino acids or by the formation of intramolecular interactions which tighten the molecular clusters. The higher viscosity of other derivative solutions could by contrast be caused by the formation of intermolecular interactions, giving rise to networks more resistant to shear stress.

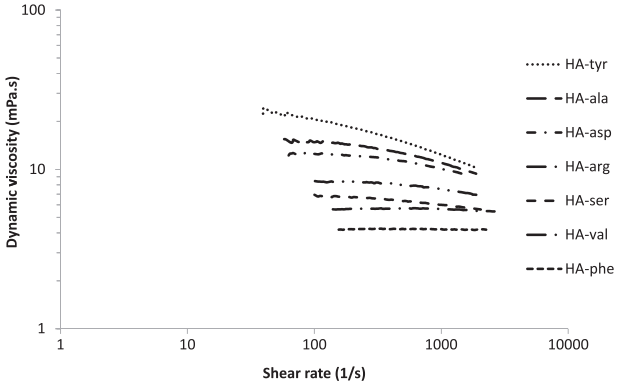

Fig. 3. Dynamic viscosity of HA-aa derivatives.

In order to evaluate the effect of the grafted amino acid on the molecular weight of the resulting derivative, two derivatives, HA-ala and HA-ser, were analyzed with SEC-MALS. These derivatives were chosen as their side chain is not hydrophobic or positively charged and the measurements could be performed in the same aqueous solvent as the one used commonly for native HA, namely water containing 0.1 M $NaNO_3$. The measurements of HA-ala grafted with 100% DS gave a molecular weight of 288,900 g/mol and a polydispersity index (PDI) of 1.68. For HA-ser with 100% DS, the molecular weight was 458,000 g/mol and the PDI was 3.86. The high PDI value obtained for HA-serine explains the low viscosity profile seen in Fig. 3 even though the molecular weight is higher. Indeed, the presence of low molecular weight HA fragments considerably lowers the viscosity of HA solutions. The reason for the difference in molecular weight between HA-ala and HA-ser, despite the same protocol used for their synthesis, is unknown but suggests that all derivatives were not subjected to the same fragmentation of the native HA backbone during the successive reaction steps depending on the nature of the amino acid. It is therefore not possible to predict the characteristics of HA-aa derivatives in terms of viscosity and molecular weight. The results obtained show tyrosine as the best candidate for further development as the viscosity profile of the resulting polymer is the highest.

3.3. Enzymatic degradation of the HA-aa derivatives

The effect of the amino acids on the susceptibility of HA-aa derivatives towards enzymatic digestion was evaluated using an *in vitro* enzymatic degradation assay derived from Muckenschnabel et al. (1998). The HA-aa derivatives with 100% DS (2 mM in PBS)

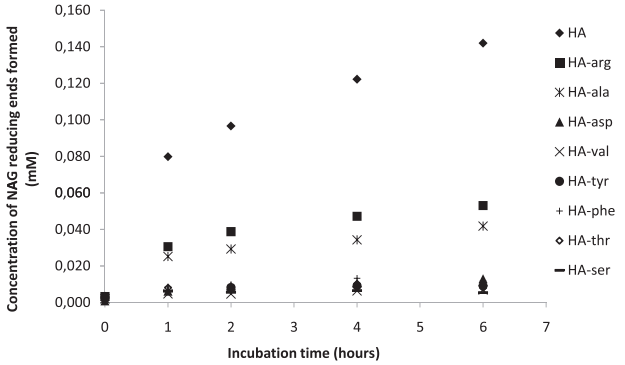

Fig. 4. Enzymatic degradation profiles of native HA and of HA grafted with amino acids.

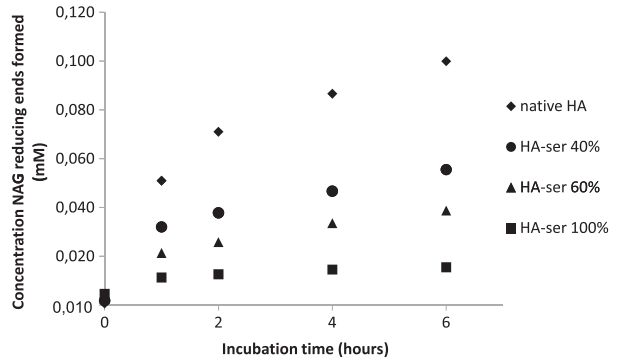

Fig. 5. Enzymatic profile of native HA and HA-serine with different degrees of substitution.

were incubated in the presence of hyaluronidase at 37 °C. The enzymatic reactions were stopped at different times by the addition of alkaline tetraborate solution and the N-acetyl glucosamine reducing ends were quantified using the Morgan-Elson colorimetric reaction. The degradation profiles of the derivatives were compared with native HA subjected to the same conditions (Fig. 4). All HA-aa derivatives showed considerably lowered enzymatic degradation compared to the native HA. HA-arg and HA-ala were the most degraded of the derivatives, suggesting that the positive charge of arginine did not improve the efficacy compared to HA-ala. All other HA-aa derivatives grafted with aspartic acid, valine, tyrosine, phenylalanine, threonine and serine showed almost no degradation. Even though the mechanism it unknown, it can be assumed that the hydroxyl, aromatic and aliphatic groups and negative charges enhance the inhibitory effect of amino acid grafting.

In addition, the results show that the viscosity of the HA-aa derivatives does not influence the enzymatic degradation. Indeed, HA-tyr and HA-phe, which have the highest gap between their viscosity values, show a similar enzymatic degradation profile. This also proves that the lowered enzymatic degradation is not due to the decrease in viscosity of the derivatives.

Since serine provided a strong protection for HA towards hyaluronidase digestion, it was grafted at different grafting ratios by adjusting the proportions of reagents during the amidation reaction. The resulting HA-ser derivatives with 40%, 60% and 100% DS were subjected to the same enzymatic degradation assay. The results presented in Fig. 5 show that the decrease of the enzymatic degradation is proportional to the grafting ratio of the amino acid. These findings are consistent with results reported previously (Eng, Caplan, Preul, & Panitch, 2010; Ibrahim, Kang, & Ramamurthi, 2010; Tomihata & Ikada, 1997b) and confirm the beneficial effect of the carboxyl protection of HA with amino acids.

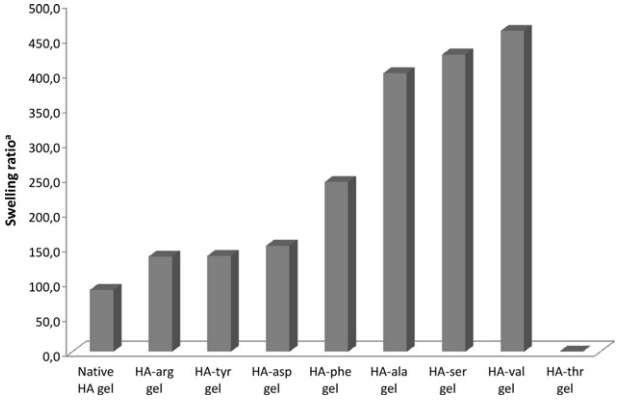

Fig. 6. Swelling ratios of different HA-amino acid crosslinked hydrogels ([a]: in g of water per mg of polymer).

3.4. Crosslinking of the HA-aa into hydrogels

To study the effect of the grafted amino acids on the formation of HA hydrogels, the HA-aa derivatives were crosslinked with a commonly used bisepoxide crosslinker, butanediol diglycidyl ether (BDDE) (Sall & Férard, 2007). The reaction was performed as described in a patent by De Belder and Malson (1986) in slightly acidic conditions. The formation of HA hydrogels with BDDE was also described in the literature in strongly alkaline conditions (pH > 12) (Malson & Lindqvist, 1986). This option was however left out to avoid additional HA chain fragmentation known to occur at high pH values (Tokita & Okamoto, 1995). Instead, the milder reaction performed in water with a small amount of acetic acid allowed to obtain water insoluble hydrogels, with the exception of HA-threonine. The derivatives (100% DS of amino acid) were crosslinked with 0.82 equiv. of BDDE, the theoretical crosslinking degree therefore being 82%. The real crosslinking density could not be measured accurately using NMR-HRMAS techniques as the peaks were too broad and no integration of the signals could be made (data not shown). The swelling ratio obtained for the native HA hydrogel is within the range of values previously reported (De Belder & Malson, 1986; Malson & Lindqvist, 1986). All HA-aa crosslinked hydrogels have higher swelling ratios than the native HA hydrogel when subjected to the same reaction conditions, suggesting a lower crosslinking density (Fig. 6). Indeed, studies have shown that the swelling ratio of hydrogels decreases when the crosslinking degree increases until reaching a saturation point (Magnani et al., 2000; Tomihata & Ikada, 1997b, 1997c). It can therefore be assumed that the grafted amino acids hindered the crosslinking reaction, most likely due to steric hindrance. For hydrogels formed from HA-ala, HA-val and HA-ser, some viscous solution remained in the dialysis tube indicating that the crosslinking was not complete. For HA-thr, no gel was formed and only viscous solution was recovered. The aliphatic groups and the hydroxyl groups thus did not appear favorable for the crosslinking reaction.

To synthesize hydrogels with various amino acid DS values, HA-tyr was selected for its high viscosity and its swelling ratio close to the native HA hydrogel in order to evaluate the influence of the amino acid grafting ratio on the swelling degree of the resulting hydrogels. The swelling ratios of hydrogels obtained from HA-tyr with 40%, 70% and 100% substituted carboxyl groups showed to slightly increase with the substitution degree (Fig. 7). Hydrogels from HA-tyr 100% were the only ones significantly different than native HA (Student's test, $p = 0.05$).

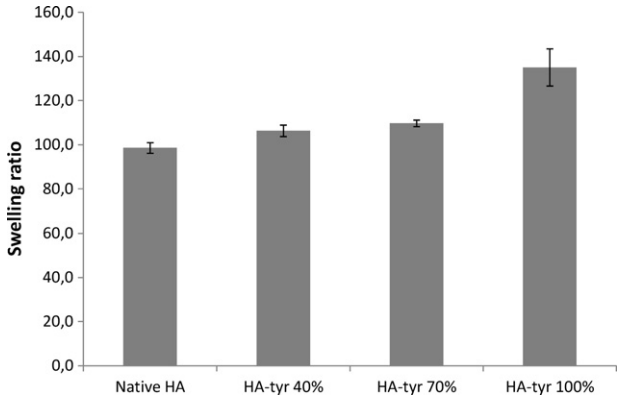

Fig. 7. Swelling ratios of HA-tyrosine crosslinked hydrogels with different DS ([a]: in g of water per mg of polymer).

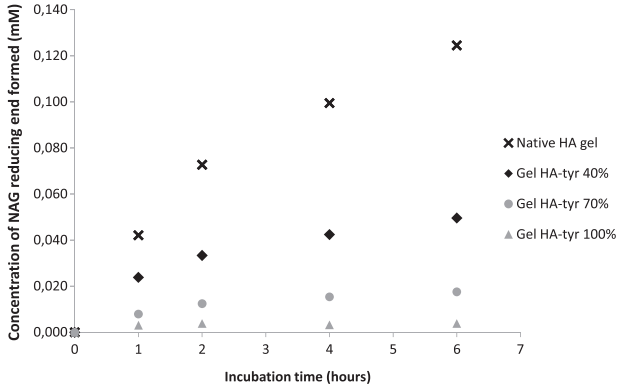

Fig. 8. Enzymatic degradation profile of cross-linked native HA gel and cross-linked HA-tyrosine gels with different tyrosine substitution degrees.

3.5. Enzymatic degradation of HA-aa crosslinked gels

To assess the efficacy of the amino-acid grafting after crosslinking of the HA-aa derivatives, these were subjected to enzymatic digestion by hyaluronidase as described above and the degradation was further quantified with the Morgan-Elson assay. The HA-tyr hydrogels with different grafting ratios of tyrosine were chosen for further analysis with this method. The gels were previously crushed and homogenized using a Poter instrument and a stoichiometric amount was precisely weighed in each test tube and adjusted with water to obtain a final concentration of 2.5 mg/mL. Fig. 8 points out that the hydrogels made from HA-tyrosine derivatives showed a lower enzymatic degradation than the native HA hydrogels synthesized in the same conditions. Crosslinking of the HA derivatives into hydrogels therefore preserved the beneficial effect of reducing their sensitivity to hyaluronidases enzymes. In addition, such as for their precursory HA-aa derivatives, the enzymatic degradation of crosslinked HA-aa hydrogels is dependent on the substitution degree.

4. Conclusion

In the present study, we synthesized new HA derivatives by grafting various amino acids onto its carboxylic groups. The amidation method used was performed in organic solvent with CMPI which allowed obtaining high grafting yields. All HA-aa derivatives grafted with 100% DS showed a significantly increased resistance to enzymatic digestion by hyaluronidases compared to the native HA. Amongst the most effective conjugates, HA-tyrosine showed the most favorable characteristics in terms of viscosity. Crosslinking of the HA-aa derivatives was achieved by using BDDE as the crosslinking agent in a slightly acidic media. The nature of the amino acid side chain however showed to greatly influence the crosslinking reaction in terms of swelling properties. HA-tyrosine hydrogels showed swelling degrees closest to the native HA gel. The crosslinked HA-tyrosine hydrogels also exhibited a higher resistance to hyaluronidase digestion compared to the hydrogels obtained from native HA in the same conditions, showing that the efficacy of amino acid grafting onto HA is conserved after crosslinking. We therefore successfully synthesized crosslinked HA hydrogels derivatives with higher resistance to hyaluronidases enzymes. These new derivatives are promising materials to be used for many biomedical applications, such as hydrogels for viscosupplementation, ophthalmic devices or drug delivery vehicles.

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