

New graft copolymers of hyaluronic acid and polylactic acid: Synthesis and characterization

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Abstract

New graft copolymers have been synthesized, using hyaluronic acid (HA) as a hydrophilic backbone and polylactic acid (PLA) as an aliphatic polyester in order to obtain new polymeric derivatives of HA able to hydrophobically associate in an aqueous medium. Hyaluronic acid with low molecular weight was made soluble in organic solvent by transformation to its tetrabutylammonium (TBA) salt. Using the HA–TBA derivative, the reaction was performed in dimethylsulfoxide adding as a reagent the *N*-hydroxysuccinimide derivative of PLA. Two HA–PLA graft copolymers have been synthesized and characterized by FT-IR, ¹H NMR spectroscopy and gel permeation chromatography. The interaction between these samples and an aqueous medium has been evaluated by rheological measurements. The obtained data evidenced that both HA–PLA graft copolymers show in aqueous medium a clear tendency for hydrophobic interaction dependent on the amount of PLA chains linked to the HA backbone.

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1. Introduction

The opportunity to modify natural polysaccharides such as cellulose, chitosan and hyaluronic acid to obtain amphiphilic derivatives and to change their solubility in different solvents, has recently received increasing interest (Pelletier, Hubert, Lapicque, Payan, & Dellacherie, 2000; Teramoto & Nishio, 2003; Wu et al., 2005). Hyaluronic acid is a natural glycosaminoglycan widely used in pharmaceutical field as a constituent of scaffolds for tissue engineering or to prepare polymeric drug delivery systems (Band, 1998). Moreover HA derivatives forming high viscosity solutions, have been used for the relief of pain in patients affected by osteoarthritis (Hamburger, Lakhanpal, Mooar, & Oster, 2003). As a scaffold constituent, HA lacks mechanical properties; in effect it forms biomaterials with low resistance to stress and degradation. Several chemical derivatives of HA have

been proposed with modified water solubility, capable to being transformed into biomaterials with good mechanical and hydrolytical resistance (Aigner et al., 1998; Benedetti et al., 1993; Dausse et al., 2003; Della Valle & Romeo, 1989; Pelletier et al., 2000).

Polylactic acid (PLA) can be a profitable pendant chain to increase the lipophilic property of a water soluble polymer. PLA is a well-known synthetic polymer, approved by FDA and used for various biomedical applications due to its biocompatibility and biodegradability. For example, Dainippon InK and Chemicals have produced a new lactide-cellulose graft copolymer thus making the polysaccharide soluble in dichloromethane (Ebato & Murakami, 1994; Ishikura & Matsumoto, 1986).

In this work we describe the synthesis and characterization of new graft copolymers prepared by linking polylactic acid to hyaluronic acid. Two HA–PLA derivatives have been obtained of different degree of substitution: HA–PLA 1.5 mol% (a) and HA–PLA 7.8 mol% (b) (expressed as percentage of moles of PLA chains relative

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to moles of HA repeating units). The first sample is freely water soluble, even in the presence of NaCl and it shows a high tendency for hydrophobic association. The second sample forms in water, even at very low concentration, gel-like dispersions but it is soluble in dimethylsulfoxide/water mixtures 90:10% (v/v). Both these samples have been characterized by FT-IR and ^1H NMR spectroscopy and rheological measurements.

2. Experimental

2.1. Materials

Hyaluronic acid sodium salt (HA) with high molecular weight (HA_{HMW} , M_w 1000 kDa) was a generous gift from SIFI (Catania, Italy). RESOMER R 202 D,L-poly(lactic acid) (PLA) (M_w 8000 Da) was purchased from Bida-chem-Boeringher Ingelheim (Milano, Italy). *N,N'*-Dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS) and tetrabutylammonium hydroxide (TBA-OH) were purchased from Sigma Aldrich (Milano, Italy). Ion exchange cationic Resin Dowex 50 W \times 8-200, anhydrous dimethylsulfoxide (DMSO) and diethylamine (DEA) were from Fluka (Milano, Italy).

2.2. Methods

^1H NMR spectra were obtained with a Bruker AC-250 instrument.

FT-IR spectra were recorded as pellets in KBr in the range 4000–400 cm^{-1} using a Perkin-Elmer 1720 Fourier Transform Spectrophotometer with a resolution of 1 cm^{-1} ; each spectrum was recorded after 100 scan.

SEC analysis was performed using a multidetector SEC system equipped with a Water 600 pump, a Water 410 Refractive Index Detector and a UNIVERSAL column from Water (particle size 5 μm). The calibration curve was determined using standards of HA purchased from Hyalose (USA). The conditions of elution were 200 mM phosphate buffer (pH 6.5)/MeOH 90:10 (v/v) (Shu, Liu, Luo, Roberts, & Prestwich, 2002), and a flow rate 0.6 ml/min, 35 $^\circ\text{C}$. Each experiment has been performed in triplicate and the results are in agreement within $\pm 2\%$ standard error.

Rheological measurements for dilute solutions, were carried out at 37 ± 0.01 $^\circ\text{C}$ using a Ubbelohde microviscometer (0.53 mm of diameter) equipped with an AVS 440 automatic viscosity measuring from Schott. Each experiment has been performed in triplicate and the results are in agreement within $\pm 3\%$ standard error.

Rheological measurements for concentrated solutions with high viscosity were carried using a Brookfield rotatory viscometer model DV-III equipped with a small sample adapter using a SC4-16 cylindrical spindle. The temperature was controlled using a Neslab RTE-110 thermostat and maintained at 37 ± 0.01 $^\circ\text{C}$. Each experiment has been performed in triplicate and the results are in agreement within $\pm 2\%$ standard error.

2.3. Synthesis of HA-PLA graft copolymers

HA with low molecular weight (HA_{LMW}) was obtained starting from HA with high molecular weight (HA_{HMW} ; M_w 1000 kDa) by a treatment with concentrated HCl solution as reported by Shu et al. (2002). The molecular weight of HA_{LMW} was determined by SEC analysis and found to be 266 kDa.

HA_{LMW} was made soluble in organic solvents by transformation to its tetrabutylammonium (TBA) salt. In particular, HA-TBA was obtained after acidification of the carboxylic groups of HA using a strong acidic ion exchange resin Dowex 50 W \times 8-200 and subsequent neutralization with tetrabutylammonium hydroxide (TBA-OH). HA-TBA was recovered after freeze-drying, then characterized by ^1H NMR that confirmed the presence of TBA with a % degree of substitution close to 100 mol%. In particular, ^1H NMR spectrum (D_2O) of HA-TBA showed: δ 0.97 (m, 12 H, $\text{N}^+(\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3)_4$); δ 1.40 (m, 8 H, $\text{N}^+(\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3)_4$); δ 1.64 (m, 8 H, $\text{N}^+(\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3)_4$); δ 3.82 (m, 8 H, $\text{N}^+(\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3)_4$); δ 2.04 (s, 3H, $-\text{NH}-\text{CO}-\text{CH}_3$).

The synthesis of the *N*-hydroxysuccinimide (NHS) derivative of PLA (i.e. PLA-NHS) was performed similarly to the synthesis of the *N*-hydroxysuccinimide derivative of poly(lactic-co-glycolic acid) (PLGA) reported elsewhere (Yoo & Park, 2004). ^1H NMR of PLA-NHS (CDCl_3) showed: δ 1.5 and δ 1.6 (d, 3H, $-\text{O}-\text{CO}-\text{CH}(\text{CH}_3)-\text{OH}$; d, 3H, $-\text{O}-\text{CO}-\text{CH}(\text{CH}_3)-\text{O}-$), δ 2.80 (m, 4H, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{CO}-$); δ 4.3 and δ 5.2 (m, 1H, $-\text{O}-\text{CO}-\text{CH}(\text{CH}_3)-\text{OH}$; m, 1H, $-\text{O}-\text{CO}-\text{CH}(\text{CH}_3)-\text{O}-$).

The synthesis of HA-PLA derivatives was carried out as follows: 600 mg of HA-TBA were solubilized in 48 ml of anhydrous DMSO then 576 μl of DEA, as a catalyst, were added. A suitable amount of PLA-NHS (solubilized in 6 ml of anhydrous DMSO) was added according to $X = 0.4$ or $X = 1$, being X equal to moles of PLA-NHS/moles of HA repeating units. The PLA-NHS solution was added drop by drop to the HA-TBA solution in about 1 h. The reaction was carried out under argon at 40 $^\circ\text{C}$ for 24 h. After this time the TBA was exchanged with Na^+ using a cationic exchange resin Dowex 50 W \times 8-200. ^1H NMR analysis showed the absence of the peaks attributable to the butyl chain of TBA thus confirming the complete exchange with Na^+ . Finally the product was precipitated in acetone and washed in the same solvent several times to remove unreacted PLA-NHS. The obtained HA-PLA graft copolymers have been named HA-PLA (a) and HA-PLA (b), prepared with $X = 0.4$ and $X = 1$, respectively. Both these samples have been characterized by FT-IR and ^1H NMR analyses to confirm the grafting of PLA to HA.

FT-IR spectra (KBr) of HA-PLA (a) and HA-PLA (b) graft copolymers showed a broad band centred at 3450 cm^{-1} (ν_{as} OH + ν_{as} NH of HA), bands at 1757 (ν_{as} COO of PLA), 1623 (amide I of HA), 1456 (δ_{as} CH_3 of

PLA), 1382 (δ_s CH₃ of PLA), 1189 (ν_s C–O–C ester group of PLA), 1089, 1048 (ν C–O alcoholic and ether of HA) cm⁻¹.

¹H NMR of HA–PLA (a) spectrum (D₂O) showed: δ 1.57 and δ 1.68 (2d, –O–CO–CH(CH₃)–O– of PLA), δ 2.12 (s, 3H–NH–CO–CH₃ of HA), δ 5.40 ppm (m, –O–CO–CH(CH₃)– of PLA).

¹H NMR of HA–PLA (b) spectrum (DMSO-*d*₆/D₂O 90:10) showed: δ 1.25 and δ 1.45 (2d, –O–CO–CH(CH₃)–O– of PLA); δ 1.85 (s, 3H, –NH–CO–CH₃ of HA), δ 5.1 ppm (m, –O–CO–CH(CH₃)– of PLA).

The %degree of substitution (DS) has been calculated as:

$$\%DS \text{ of HA-PLA} = (\text{moles PLA chains/moles of HA} \times \text{repeating units}) \times 100.$$

For HA–PLA (a) the degree of substitution was calculated by comparing the integral of the two peaks related to protons attributed to methyl groups of PLA chain at δ 1.57–1.68, with the integral related to protons at δ 2.12 attributed to NHCOCH₃ belonging to *N*-acetylglucosamine residue of HA. The degree of substitution of HA–PLA (a) resulted to be 1.5 mol%.

For HA–PLA (b) the degree of substitution was calculated in a similar manner by comparing the integral of the peaks related to methyl groups of PLA at δ 1.25–1.45 with the integral related to protons at δ 1.85 attributed to NHCOCH₃ belonging to *N*-acetylglucosamine residue of HA. The degree of substitution of HA–PLA (b) resulted to be 7.8 mol%.

2.4. Rheological measurements

The determination of intrinsic viscosity has been performed for the samples HA_{LMW}, HA–PLA (a) and HA_{control} (i.e. a sample obtained from HA_{LMW} transformed to HA–TBA and treated under the same conditions employed to prepare HA–PLA but in the absence of PLA). Each sample has been dissolved in a NaCl 0.15 M aqueous solution to reduce electrostatic repulsions (Cowman & Matsuoka, 2005). The investigated range of polymer concentration was from 0.125 to 1 mg/ml. This range has been chosen because it is known that for HA with a molecular weight ranging from 47 to 3500 kDa a linear correlation between the reduced viscosity and the concentration occurs (Hokptusa, Jumel, Alexander, & Harding, 2003). In these conditions, the rheological behaviour can be described by the Huggins equation (Huggins, 1942):

$$\eta_{\text{red}} = [\eta] + k_H [\eta]^2 c,$$

where, η_{red} (ml/g) is the reduced viscosity, $[\eta]$ (ml/g) is the intrinsic viscosity, k_H is the Huggins coefficient and c is the concentration of the sample (g/ml).

Besides, aqueous solutions (10 mg/ml) of HA_{HMW}, HA_{LMW}, HA_{control}, HA–PLA (a) or HA–PLA (b) have been prepared and their rheological behaviour has been

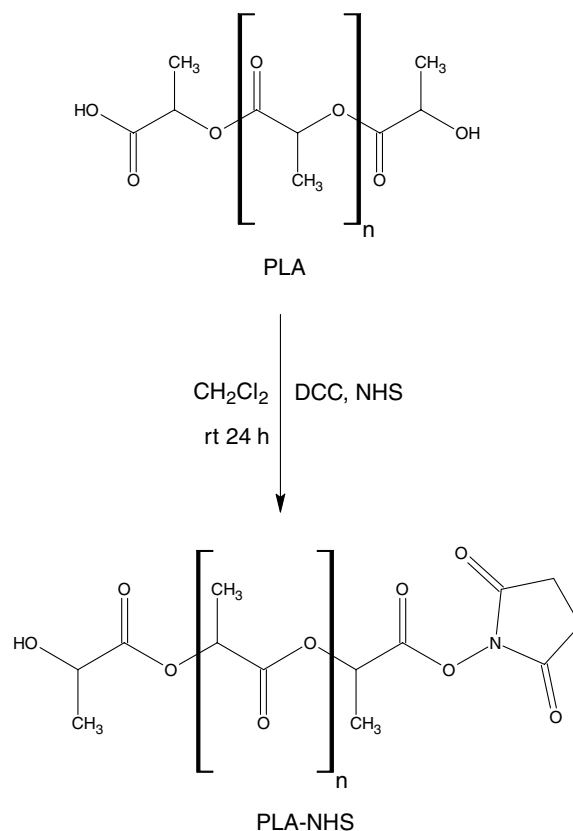
evaluated by using a rotatory viscometer with a shear rate ranging from 2.9 to 73.3 s⁻¹ corresponding to the angular speed range 10–250 rpm.

3. Results and discussion

3.1. Synthesis of HA–PLA graft copolymers

The objective of this work was the synthesis of new HA–PLA graft copolymers showing profitable physico-chemical properties. For example, the grafting of PLA to HA could allow to obtain copolymers able to form highly viscous solutions potentially suitable for biomedical applications. In addition, since PLA is a lipophilic polymer, its covalent linkage to a water soluble polysaccharide, like HA, could allow to obtain amphiphilic derivatives able to form hydrophobically associated hydrogels. Finally, since PLA is a biocompatible and biodegradable polymer, like HA, its grafting to HA allows biomaterials to be obtained that maintain these properties and that could be used for various applications in tissue engineering and pharmaceutical fields.

In order to allow the grafting of PLA to HA, it was necessary to increase the reactivity of carboxylic group of PLA by preparing its *N*-hydroxysuccinimide derivative (PLA–NHS) (see Scheme 1) and to transform the water soluble sodium salt of HA to the corresponding tetrabutylammonium salt (HA–TBA) (see Section 2).



Scheme 1. Reaction of functionalization of polylactic acid (PLA) with *N*-hydroxysuccinimide (NHS).

The reaction between HA–TBA and PLA–NHS was carried out in dimethylsulfoxide at 40 °C in the presence of diethylamine (DEA) as a catalyst for 24 h (see Scheme 2).

Two different HA–PLA graft copolymers have been prepared by varying the amount of PLA–NHS in the reaction medium ($X = 0.4$ and $X = 1$, see Section 2).

The samples obtained, named HA–PLA (a) (with $X = 0.4$) and HA–PLA (b) (with $X = 1$), have been characterized by FT-IR and ^1H NMR that confirmed the occurrence of substitution of HA with PLA.

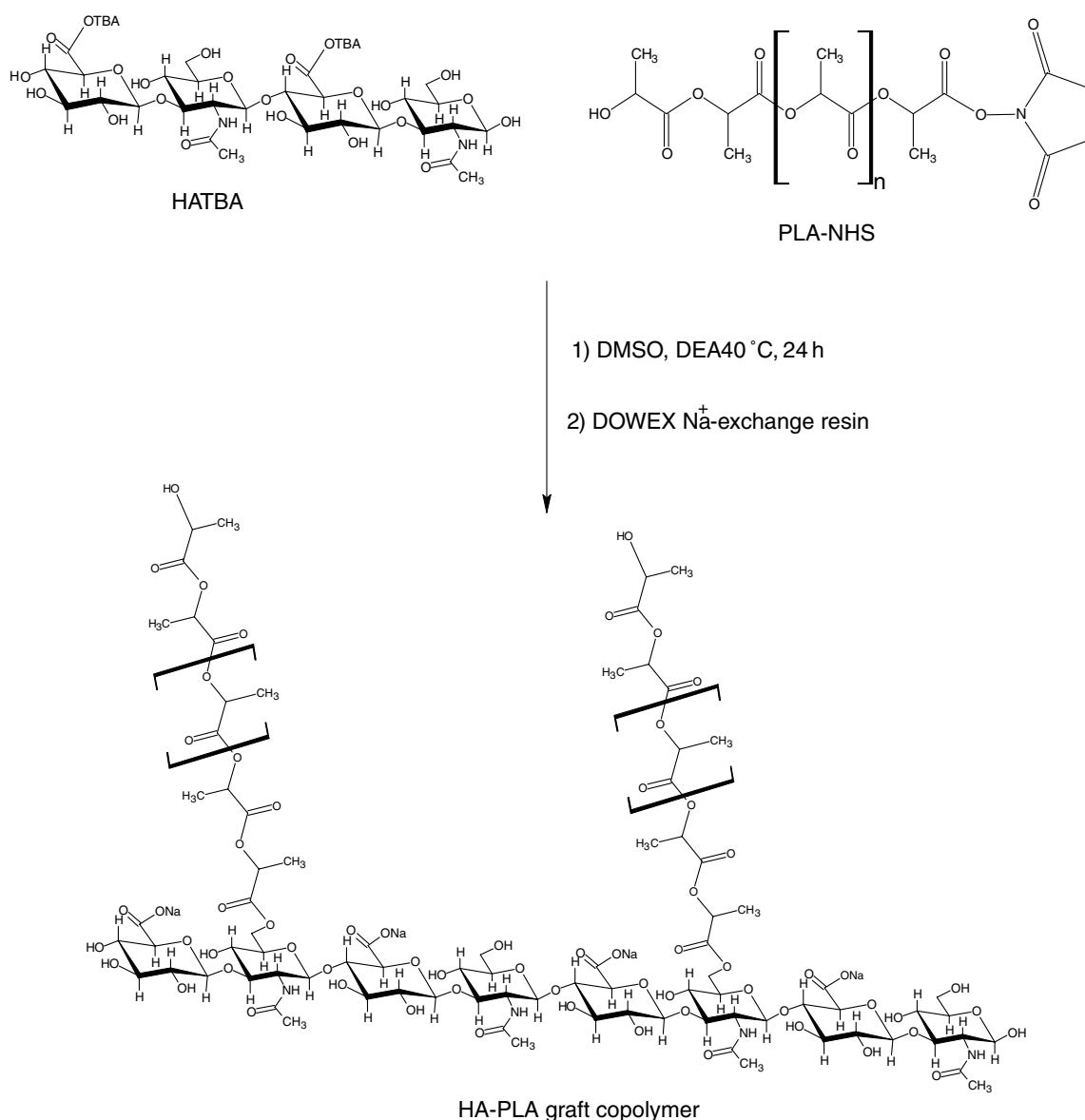
As an example, Fig. 1 shows the FT-IR spectrum of HA–PLA (a) sample in comparison with the spectrum of HA_{LMW} and PLA.

Fig. 2 reports as an example the ^1H NMR spectrum of HA–PLA (b) sample.

3.2. SEC analysis

Size Exclusion Chromatography (SEC) analysis was carried out to determine the molecular weight of HA–PLA (a) graft copolymer in comparison with starting HA_{LMW} . Since as reported by Pelletier et al. (2000), hyaluronic acid is sensitive to degradation after acidification using an acidic resin, we have also determined the molecular weight of the $\text{HA}_{\text{control}}$ (see Section 2.4). In this manner we show the effect of the acidic resin on the molecular weight of starting HA (HA_{LMW}). Table 1 reports the molecular weights of HA–PLA (a), HA_{LMW} and $\text{HA}_{\text{control}}$.

It is evident that the molecular weight of $\text{HA}_{\text{control}}$ is slightly smaller than that of HA_{LMW} , probably because of a slight degradation occurring during the elution in the Dowex column. Whereas, the molecular weight of



Scheme 2. Schematic representation of the reaction between HA–TBA and PLA–NHS to give HA–PLA graft copolymer.

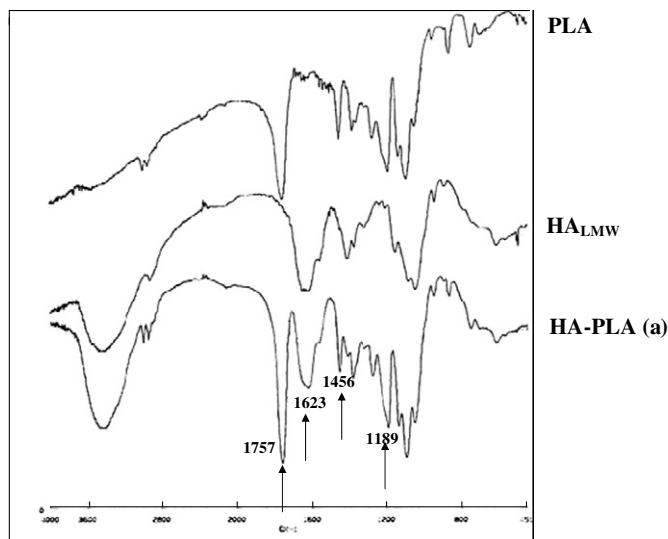


Fig. 1. FT-IR spectra of PLA, HA_{LMW} and HA-PLA (a) graft copolymer. The arrows indicate the significative functional groups in HA-PLA (a) graft copolymer.

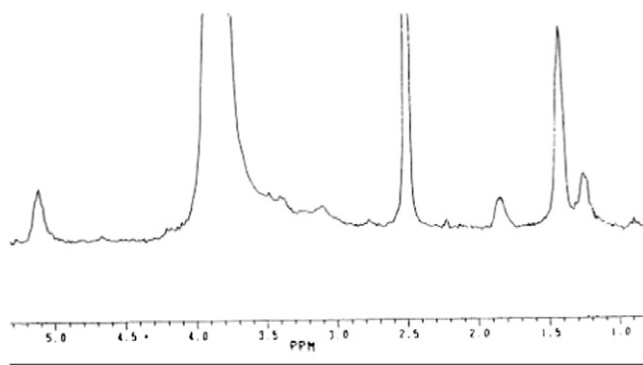


Fig. 2. ¹H NMR (DMSO-*d*₆/D₂O 90:10) of HA-PLA (b).

Table 1

Values of molecular weight (M_w), polydispersity index (M_w/M_n), intrinsic viscosity $[\eta]$ and Huggins coefficient (k_H) for HA_{LMW}, HA_{control} and HA-PLA (a) graft copolymer

	M_w (kDa)	M_w/M_n	$[\eta]$ (ml/g)	k_H
HA _{LMW}	265.7	1.78	255.5	0.46
HA _{control}	251.3	1.86	193.3	0.58
HA-PLA (a)	309.3	1.99	97	4.24

HA-PLA (a) was significantly higher than that of HA_{LMW} and HA_{control} thus proving the successful substitution of HA with PLA chains.

3.3. Rheological measurements

It is well known that intrinsic viscosity, $[\eta]$, of a macromolecule is a measure of its hydrodynamic volume in solution, and therefore it is a reflection of its size and affinity towards a solvent. The value of $[\eta]$ of HA-PLA (a) graft copolymer, has been obtained from η_{red} when extrapolated

to zero concentration, as it can be observed from the linear Huggins plot in Fig. 3.

As a comparison, we have also determined the value of intrinsic viscosity of HA_{LMW} and HA_{control}.

As it can be observed from values reported in the Table 1, the intrinsic viscosity of HA_{control} is smaller than that of starting HA_{LMW}. This result, in agreement with the values of molecular weights, is probably due to the small degradation of HA_{control} that occurs during the acidification step in the Dowex column. HA-PLA (a) shows the lowest value of intrinsic viscosity even if its molecular weight is the greatest. Therefore, this result can be attributed to the hydrophobic interactions between the polylactic chains in the aqueous medium. On the other hand these hydrophobic interactions are facilitated in the presence of NaCl 0.15 M thus causing a conformation of HA-PLA (a) more compact than that of HA_{LMW}. Obviously these interactions are more pronounced by increasing the substitution degree of HA with hydrophobic chains. In fact a degree of substitution 7.8 mol% results in a dramatic decrease in solubility of the sample HA-PLA (b) in aqueous solution containing NaCl 0.15 M. For this reason, it was not possible to determine, in these conditions, the value of intrinsic viscosity of HA-PLA (b) sample.

Another interesting parameter is the value of the Huggins coefficient (k_H) that gives a direct measure of the polymer-solvent interaction. A small value of the Huggins coefficient (≤ 0.5) belongs to a polymer with a good interaction with the solvent. A higher k_H value would mean that the interactions between the polymeric chains are predominant in comparison with the interactions between the polymer and the solvent (Hokptusa et al., 2003).

As it can be observed from data reported in Table 1, both HA_{LMW} and HA_{control} show small and similar values

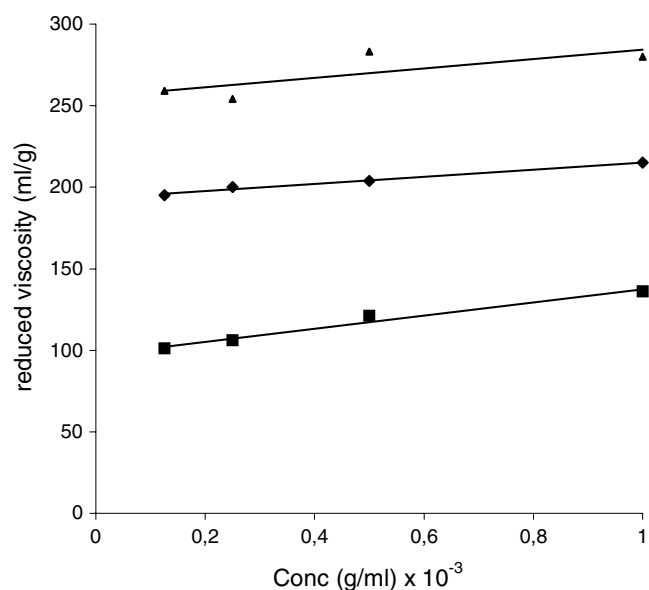


Fig. 3. Reduced viscosity values as a function of concentration of HA_{LMW}(▲), HA_{control}(◆) or HA-PLA (a) (■) in NaCl 0.15 M aqueous solution.

of k_H , i.e. a good interaction between these polymers and the aqueous medium occurs. On the contrary, HA–PLA (a) shows a high value of k_H thus indicating that this graft copolymer has a reduced affinity towards the aqueous medium due to the presence of hydrophobic interactions between the polylactic chains.

Besides, HA–PLA (a) and HA–PLA (b) have been dissolved in twice distilled water in order to obtain a concentration of 10 mg/ml (see Section 2). As a comparison, we have also evaluated the rheological behaviour of aqueous solutions of HA_{LMW}, HA_{control} and HA_{HMW} at the same concentration (10 mg/ml). Rheological data for all the investigated samples are reported in Fig. 4 as viscosity versus shear rate. However, since in these conditions the rheological behaviour of HA_{control} resulted similar to that of HA_{LMW}, to simplify the figure, the values of HA_{control} are not here reported.

It is evident that HA_{LMW} shows a viscosity very low (0.028–0.032 Pa*s) that does not depend on the shear rate in the range 2.9–73.3 s⁻¹, i.e. this sample shows, in the investigated conditions, a Newtonian behaviour. This result is generally observed for solutions of polysaccharides with a low molecular weight, as reported by other authors (Pelletier et al., 2001).

A different behaviour has been found for HA–PLA (a) and HA–PLA (b) graft copolymers that show a pronounced increase in viscosity at low shear rate, i.e. 70 and 100 times, respectively, higher than HA_{LMW}. This behaviour is probably due to the combination of hydrophobic interactions between polylactic chains and hydrogen bonds between HA chains. The former type of interaction decreases, whereas the latter one increases, the value of viscosity. Evidently in concentrated solutions,

the interactions between HA chains prevail over hydrophobic interactions thus causing the formation of a very entangled physical network which shows a high viscosity. However, by increasing the applied stress and, as a consequence, the value of shear rate, a shear-thinning trend is observed. This phenomenon is due to the disentanglement of the polymer chains associated with the breakdown of hydrophobic interactions thus resulting in a pseudoplastic behaviour of HA–PLA (a) and HA–PLA (b) samples.

Finally it is evident that, in the investigated range of shear rate, the values of viscosities of HA–PLA (b) are very similar to those of an aqueous solution of hyaluronic acid with high molecular weight (HA_{HMW}, M_w 1000 kDa) at the same concentration (10 mg/ml).

Therefore, we have demonstrated that the grafting of PLA to HA is a simple and suitable method to increase viscosity of starting HA. This result is very important since it is well known that several applications of hyaluronic acid in biomedical field depend on its rheological properties. For example aqueous solutions of HA are able to reduce postoperative adhesion following abdominal surgery. HA is also used as a surgical aid especially for the replacement of the vitreous humour, in ophthalmic procedures, and synovial fluid. HA is essential for lubrication of joints to prevent friction and its solutions are also used for viscosupplementation in the treatment of osteoarthritis (Dougados, 2000). It is reasonable to suppose that HA–PLA graft copolymers, due to their peculiar rheological behaviour, could be employed for the same uses.

4. Conclusion

New graft copolymers of hyaluronic acid (HA) with polylactic acid (PLA) have been prepared and characterized. The behaviour in aqueous medium of these HA–PLA derivatives depends on the amount of PLA chains linked to HA. In fact, HA–PLA derivative with a low degree of substitution (1.5 mol%) shows in aqueous medium a clear tendency for formation of coils more compact than HA, probably due to a strong hydrophobic interaction of the PLA chains, as evidenced by viscosity studies in dilute solutions. On the other hand, HA–PLA with a higher degree of substitution (7.8 mol%) shows a tremendous decrease in affinity towards the aqueous medium. In fact, it is insoluble in aqueous solution with strong ionic strength, gives rise to gel-like dispersions in water and results to be soluble in dimethylsulfoxide. Moreover, the rheological study in concentrated aqueous solutions, revealed that the substitution of HA with PLA increases the viscosity value producing solutions with a shear-thinning behaviour similar to that of HA with a high molecular weight (1000 kDa) and potentially useful for viscosupplementation and other biomedical uses. Besides, with the consideration that PLA is a lipophilic polymer, HA–PLA graft copolymers could be a new class of biomaterials, able to produce hydrophobically associated hydrogels suitable for tissue engineering or drug delivery applications. Finally

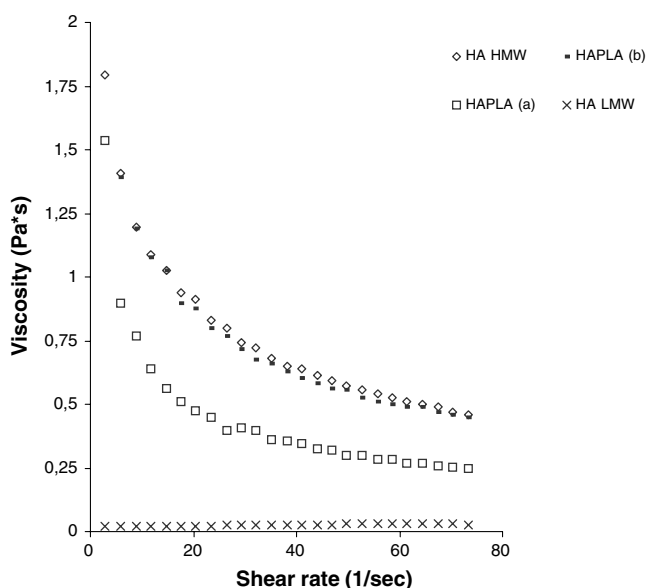


Fig. 4. Viscosity values as a function of shear rate of aqueous solutions containing 10 mg/ml of HA_{LMW} (×), HA_{HMW} (◊), HA–PLA (a) (□) or HA–PLA (b) (◊).

the increased solubility in organic solvents could be exploited to increase the chemical versatility of HA.

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