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Synthesis Of Novel Graft Copolymers Of Hyaluronan, Polyethyleneglycol And Polylactic Acid

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ABSTRACT

New amphiphilic derivatives of hyaluronic acid(HA) have been synthesized by grafting of both hydrophobic and hydrophilic branches, such as polylactic(PLA) and polyethylene glycol(PEG) chains. Graft copolymers so obtained, named PEG-g-HA-g-PLA have been characterized by spectral analysis that allowed to determine the grafting degree in PLA and PEG chains. Self assembling properties evaluated in aqueous media resulted to be dependent on the molar amount of PLA and PEG linked to HA. Further studies are in progress to evaluate other physicochemical properties of these materials and their biomedical and pharmaceutical applications.

KEYWORDS

Hyaluronic acid;
Polylactic acid;
Polyethylenglycol;
Graft copolymers;
Self assembling properties.

INTRODUCTION

Hyaluronic acid(HA) is a natural biocompatible and biodegradable polysaccharide consisting of alternating molecules of N-acetylglucosamine and glucuronic acid bound together by β1-3 and β1-4 glucosidic bonds. HA is produced by many types of cells and extruded into the extracellular space where it interacts with other constituents of the extra cellular matrix(ECM) to supportive and protective structure around the cells. HA binds to proteins in the ECM and on cell surface; this interaction mediates signalling cascades for cell motility, cell proliferation, morphogenesis, cancer metastasis and inflammation[1]. Besides its biocompatibility, the physicochemical properties of HA contribute also to made it suitable in drug delivery field, tissue engineering and viscosupplementation in treating of osteoarthritis. However pharmaceutical or biomedical systems based on HA alone lack suitable mechanical resistance and do not carry on a prolonged activity because of a rapid chemical and enzymatic degradation(by hyaluronidase (HAase)[2]. Further more they are too hydrophilic to favour cellular adhesion and differentiation, if an

application as scaffolds for tissue engineering is envisaged.

For these reasons, there is a great interest in developing hyaluronic acid based materials, able to show better mechanical and elastic properties and a greater resistance to chemical and enzymatic hydro lysis, thus performing a prolonged action in the place of application still maintaining the biodegrada bility and biocompatibility. At this aim, it has been proposed to crosslink HA with various molecules such as bisepoxides, formaldehyde, divinylsulfone or adipic dihydrazide[3-5]. The crosslinking can be also performed by using macromolecules as crosslinkers, such as collagen or gelatin, or synthetic polymers such as polylysine or α, β-polyaspartylhydrazide[6]. It is also possible to chemically modify HA with hydro phobic groups to change its solubility in aqueous or organic media and so obtaining derivatives proposed as biomaterials with good mechanical and hydro lytical resistance.

In a previous work we have reported the synthesis and characterization of new graft copolymers prepared by linking polylactic acid(PLA) to HA[7]. These derivatives, named HA-PLA, spontaneously self assemble in water forming colloidal aggregates or highly viscous gel-like dispersions in dependence on the molar substitution in PLA. However, when these derivatives are in contact with physiological saline buffer because of the increased ionic strength, they precipitate spontaneously forming microgel dispersions. The purpose of this study was to produce new copolymers of HA-PLA whose self assembling properties could be easily controlled in physiological solutions by avoiding the formation of microgel dispersion. For this reason new amphiphilic derivatives of hyaluronic acid have been synthesized by a suitable balance between the amount of hydrophobic chains of polylactic acid and hydrophilic polyethylene glycol (PEG) chains.

EXPERIMENTAL

All reagents were of analytical grade, unless otherwise stated.

RESOMER R 202 D, L-polylactic acid(PLA)(Mw 8000 Da) was purchased from Bidachem-Boeringher Ingelheim(Italy). PEG amino O-(2-aminoethyl)-o′-methyl-polyethylene glycol 5000(PEG-NH_2) and ion exchange cationic Resin Dowex 50 W×8-200 were purchased from Fluka(Italy).N,N'-dicyclohexyl carbodiimide(DCC), N-hydroxysuccinimide(NHS) and tetrabutylammonium hydroxide(TBA-OH) were purchased from Sigma Aldrich(Italy). HA with a low weight-average molecular weight was prepared by acidic degradation as reported by X.Z.Shu et al.[8] starting from a HA sodium salt, MW 1500 kDa that has been a generous gift from SIFI(Italy).

Weight-average molecular weight of HA was determined by SEC system equipped with a pump system, a Universal column(particle size 5m) and a 410 differential refractometer(DRI)as a concentration detector, all from Waters(USA). The molecular weight was evaluated by using HA standards(range 100-800 kDa) from Hyalose(USA), 200mM phosphate buffer(pH 6.5):MeOH 90:10(v/v) as a mobile phase, 36±0.1^{0}C and flow rate 0.6ml/min and resulted to be 222 kDa(M_w/M_n=1.85).

^{1}H-NMR spectra were obtained with a Brucker AC-250 instrument.

Step 1 : Synthesis of HA-TBA-*g*-PLA graft copolymer

The synthesis of tetrabutylammonium(TBA) salt of HA(i.e.HA-TBA) and the synthesis of N-hydroxysuccinimide(NHS) derivative of PLA(i.e. PLA-NHS) have been already reported[7]. To prepare HA-TBA-g-PLA copolymer with grafting degree in PLA equal to 3.7mol%, we have employed the procedure elsewhere described[7], but without the purification by the cationic exchange resin Dowex 50W×8-200. The presence of TBA in the graft copolymer allows the solubilization in an organic solvent to perform the step 2.

Step 2 : Synthesis of copolymers

HA-TBA-g-PLA(150mg) produced in the step 1 was dissolved in anhydrous dimethyl sulfoxide(12 ml) under argon. Then suitable amounts of PEG-NH_2 dissolved in dimethyl sulfoxide and DCC and NHS as activators, have been added in order to have a value of X=0.25 or 0.5 and a value of Y=0.25 or 0.5 being

X=moles of PEG-NH_2 /moles of HA-TBA-g-PLA repeating units

Y=moles of DCC(or NHS)/moles of PEG-NH_2

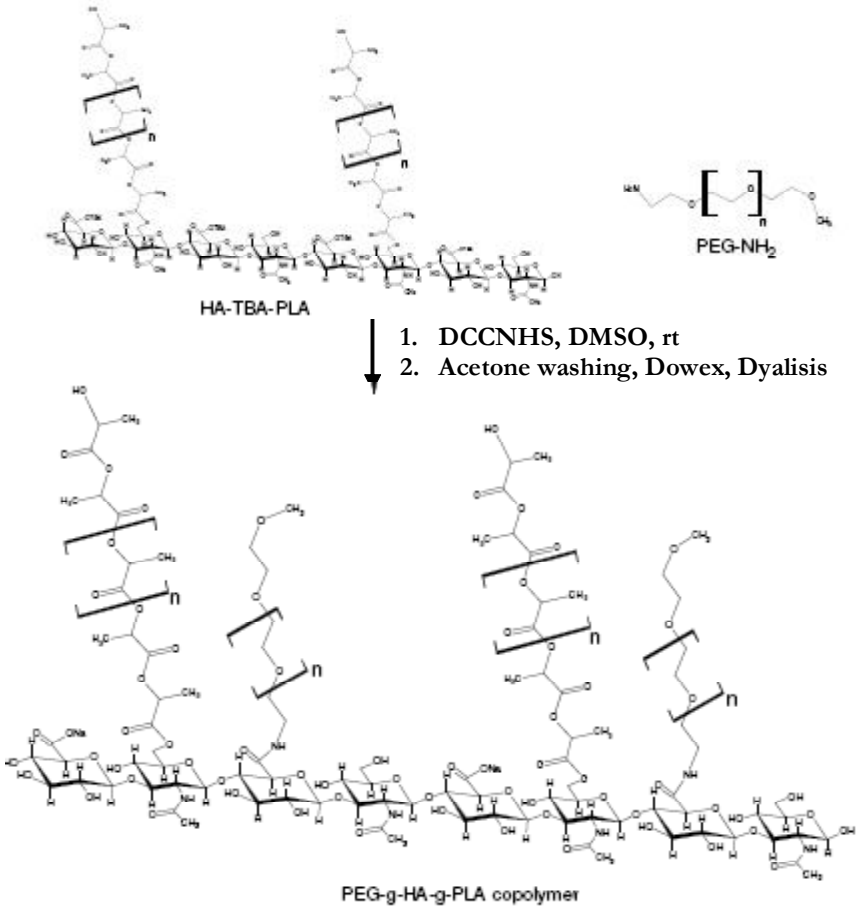
After 24h at room temperature, each reaction mixture was maintained at 5^0C for 10min, then filtered to remove DCC and precipitated in diethyl ether. Each solid product was recovered by filtration and washed several times with acetone. The sample was dissolved in dimethyl sulfoxide and this solution was purified by a cationic exchange resin Dowex 50W×8-200 to remove TBA. Finally the recovered solution was further purified by dialysis against distilled water by using Spectra/por tubing with a cut-off of 3500 Da then it was freeze-dried. The obtained graft copolymers have been named PEG-g-HA-g-PLA (a) or PEG-g-HA-g-PLA (b), prepared with X=0.25, Y=0.25 or X=0.5, Y=0.5 respectively.

^{1}H-NMR(THF-d_8/D_2O1/1) δppm 1.7 and 1.8 (2d, 3H, -O-CO-CH(CH_3)-O-of PLA), 2.1(s, 3H, -NH-CO-CH_3 of HA), 4.0(m, 4H, -CH_2-CH_2-of PEG), 5.40(m, 1H, -O-CO-CH(CH_3)-of PLA).

The grafting degree in PEG chains was calculated by comparing the integral of the peaks related to protons attributed to -CH_2-CH_2- of PEG at δ4.0 with the integral related to protons at δ2.1 attributed to -$NHCOCH_3$ belonging to N-acetylglucosamine residue of HA. The degree of substitution of PEG-g-HA-g-PLA (a) and (b) resulted to be 3 and 5mol% respectively.

Determination of critical aggregation concentration

A stock solution of pyrene 6×10^{-2} M was prepared in acetone and stored at 5^0C until utilization. The pyrene solution was diluted with distilled water to a concentration of 12×10^{-7} and distilled for 1 hour to remove the organic solvent. PEG-g-HA-g-PLA (a) or PEG-g-HA-g-PLA (b) were solubilized in distilled water in a range of concentration 2×10^{-4}-2.4mg/ml. Equal volumes of Pyrene and PEG-g-



SCHEME 1: Synthesis of PEG-g-HA-g-PLA copolymer

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TABLE 1 : Values of yield(mg), grafting degree (GD, mol%) in PLA or PEG chains, critical aggregation concentration (CAC; mg/ml) for PEG-g-HA-g-PLA(a) and PEG-g-HA-g-PLA(b) copolymers

Sample	Yield (mg)	Gd in pla chain (mol%)	Gd in peg chain (mol%)	Cac (g/ml)*
PEG-g-HA -g-PLA(a)	180	3.7	3	0.5
PEG-g-HA -g-PLA(b)	195	3.7	5	1.0

HA-g-PLA (a) or PEG-g-HA-g-PLA (b) solutions were mixed and left one night before the analysis. The I_1/I_3 ratio of the intensities of the first and the third peaks of fluorescence spectrum of pyrene was used to detect the formation of hydrophobic microdomains resulting from the association of the amphyphilic PEG-g-HA-g-PLA (a) or PEG-g-HA-g-PLA (b) derivatives.

RESULTS AND DISCUSSION

The reaction to obtain novel graft copolymers between hyaluronic acid(HA), polylactic acid(PLA) and polyethylene glycol(PEG) has been successful performed in two steps. In the first one, the grafting of PLA into HA tetrabutyl ammonium salt(HA-TBA) has been performed thus obtaining a derivative named HA-TBA-g-PLA soluble in dimethyl sulfoxide(unlike starting HA) having a grafting degree in PLA equal to 3.7 mol%. In the second one, HA-TBA-g-PLA reacted with two different amounts of PEG-NH_2 in the presence of two different amounts of DCC and NHS(see experimental) to obtain the PEG-g-HA-g-PLA derivatives(see SCHEME 1).

In particular PEG-g-HA-g-PLA (a) and PEG-g-HA-g-PLA (b) have been synthesized, purified and characterized by ^{1}H-NMR analysis to determine the value of grafting degree (GD) reported in TABLE 1.

It is evident that both the graft copolymers have been obtained with a high yield whereas the grafting degree in PEG chains is dependent on the amount of employed reagents (PEG-NH_2, DCC and NHS). In addition, we have also evaluated the self assembling ability of these graft copolymers by determining the values of critical aggregation concentration(CAC) reported in TABLE 1.

The obtained results demonstrate that both PEG-g-HA-g-PLA (a) and PEG-g-HA-g-PLA (b) are able to form aggregates at a concentration value greater than that of starting HA-g-PLA copolymer whose CAC was of 0.25mg/ml being these copolymer less hydrophobic than HA-g-PLA copolymer. However these aggregates of PEG-g-HA-g-PLA (a) and PEG-g-HA-g-PLA (b) copolymers do not give rise to a microgel dispersion when in contact with a saline buffer, unlike aggregates based on starting HA-g-PLA copolymer. Further studies are in progress to study the effect of different grafting degrees in PLA and PEG chains on physicochemical properties of this new family of auto-assembling graft copolymers and to investigate their use in biomedical and pharmaceutical field.

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