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Conjugation of hyaluronan to proteins

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ABSTRACT

Polymer conjugation has been widely exploited to prolong half-life and reduce immunogenicity of therapeutic proteins. Here, the potentials of hyaluronic acid (HA) have been investigated by studying the conjugates with two model enzymes, trypsin and RNase A, and with insulin. As the direct coupling of proteins to the HA's carboxylic groups can cause cross-linking problems, a hyaluronan-aldehyde derivative has been synthesized for *N*-terminal site-selective conjugation. HA conjugation, termed HAylation, preserved the activities of enzymes and their thermal stabilities. Insulin HAylation was studied by preparing two conjugates with different peptide loadings (32% and 17%, w/w). Noticeably, the conjugate with the lower loading showed the greater effect on blood glucose level. The 17% HA-insulin conjugate showed a lowering effect on blood glucose level for up to 6 h, while free insulin exhausted its action after 1 h. This study highlights the potentials of hyaluronan-aldehyde for protein delivery.

1. Introduction

Covalent attachment of PEG, PEGylation, has become one of the most exploited approaches for the delivery of therapeutic proteins (Harris & Chess, 2003; Pasut & Veronese, 2007). The advantages achieved by PEGylation can be summarized as follows: (i) remarkable *in vivo* half-life prolongation (Pasut & Veronese, 2012), (ii) elimination or reduction of protein immunogenicity (Mero & Veronese, 2008), and (iii) increased stability (Veronese et al., 2007). The effectiveness and potentials of this technique are demonstrated by several protein conjugates already on the market, such as Neulasta, Pegasys, Mircera, Cimzia and others. On the other hand, PEG presents some weaknesses that might restrict its full exploitation; nevertheless it is still considered the polymer of choice. A known limit is its non-biodegradability, which was easily overcome by using polymers with molecular weights below the kidney excretion threshold. Furthermore, a recent concern comes from some studies reporting cases of development of specific anti-PEG antibodies. These were detected in the serum of patients treated with PEG-asparaginase (Armstrong, Hempel, Koling, & Chan, 2007) and PEG-uricase (Sherman, Saifer, & Perez-Ruiz, 2008). Up to now, these events occurred only when the polymer was coupled to highly immunogenic and large proteins. Although the frequency of such reports is still limited, there is an unmet need of developing other polymers than PEG, offering a valid alternative that can also overcome the patent constrains of PEGylation.

Recent studies in this direction have investigated the potentials of new polymers such as hydroxy-ethyl-starch (Besheer, Hertel, Kressler, Mäder, & Pietzsch, 2009), polyoxazoline (Mero et al., 2008; Mero, Fang, Pasut, Veronese, & Viegas, 2012; Viegas et al., 2011), dextrin (Ferguson and Duncan, 2009) and polysialic acid (Gregoriadis, Jain, Papaioannou, & Laing, 2005). A forward step in protein conjugation might occur by the use of biodegradable polymers. Such polymers can prevent protein limitations such as aggregation and fast kidney clearance, problems also solved by using non-biodegradable polymers, but at the same time they can avoid the risks of polymer accumulation in the body. Furthermore, polymer degradation allows for the recovery, at least in part, of protein activity that is usually reduced after polymer conjugation (Duncan, Gilbert, Carbajo, & Vicent, 2008; Hardwicke et al., 2008).

Biodegradable polysaccharides, such as dextran, alginate, insulin, and hyaluronic acid, have been widely investigated as protein carriers. In the first studies, the conjugation strategies were based on random couplings between the protein's amino groups and either the carboxylic groups of the polymer, if present, or aldehyde groups generated by periodate oxidation of the polymeric backbone (Torchilin, Maksimenko, & Mazaev, 1988). The main limitations of these approaches were undesired cross-linking, low homogeneity of conjugates and potential formation of soluble aggregates, which yielded difficulties in terms of characterization, batch-to-batch reproducibility and immunogenicity.

Recently, we have investigated the use of hyaluronic acid as carrier of therapeutic proteins (D'Este, Pasut, Renier, & Rosato,

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2010). Hyaluronic acid (HA) is a natural polysaccharide largely present in the vitreous humor and in cartilages where it has a key structural role in the organization of the cartilage extracellular matrix. HA is also unique in having a relatively simple linear structure of repeating non-sulfated disaccharide units composed of D-glucuronic acid and *N*-acetyl-D-glucosamine (Almond, 2007). Among the several therapeutic applications, the polymer is currently in clinical use in arthritis as a viscosupplement for the joints. Furthermore, it is widely used in topical formulations for wound healing and in several injectable products for cosmetic applications (Balazs & Laurent, 1998; Laurent & Fraser, 1992; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012; Palumbo et al., 2012). Consequently, its clinical safety is well established. To date, the potentialities of HA in drug delivery have been investigated as carrier of anti-tumoral and anti-inflammatory drugs (Banzato et al., 2008; Homma et al., 2009; Pitarresi et al., 2010; Saravanakumar et al., 2010). In the last two cases, the presence of several carboxylic groups is an advantage for the delivery of small drugs because it ensures a high loading. In contrast, as aforementioned, an approach of site selective conjugation is needed for protein delivery to avoid cross-linking.

A *N*-terminal selective conjugation of interferon alpha to HA has been proposed by conducting the coupling at acidic pH (5.5) with a hyaluronan-aldehyde derivative (Yang et al., 2011). The aldehyde groups were obtained by oxidation of the polysaccharide backbone with sodium periodate, generating two aldehyde groups for each oxidized unit. This chemistry might present difficulties due to the formation of two close aldehyde groups (*e.g.* RO—CH(R′)—CHO and RO—CH(OR′)—CHO, where R and R′ are the HA backbone) with a different reactivity and it would be challenging to determine which one is involved in the protein coupling, or if both will react it is difficult to determine to what extent.

In this paper, we describe the development of a new hyaluronan-aldehyde derivative as suitable platform for the *N*-terminal selective conjugation of proteins. The method is based on the introduction of short pendant chains of 4-aminobutyraldehyde on the HA backbone. The butyraldehyde spacer was chosen to ensure the maximum stability of the aldehyde groups and to offer a unique pathway of conjugation, simplifying the characterization of the conjugates. Currently, the new hyaluronan-aldehyde derivative provides a new technology platform for protein conjugation and several conjugates are under investigation (D'Este et al., 2010).

The potentialities of this new hyaluronan-aldehyde derivative for HA conjugation to proteins (HAylation) were evaluated on two model proteins, RNase A and trypsin, and on a polypeptide of pharmaceutical interest, insulin. Conjugates were characterized and their residual activities were determined *in vivo* and *in vitro*.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salts (HA, 200 kDa) were from Fidia Farmaceutici s.p.a. (Abano Terme, Italy). Ribonuclease A, trypsin, bovine insulin, casein and testicular hyaluronidase, methanesulfonic acid (CH_3SO_3H), 1,1′-carbonyldiimidazole, 4-aminobutyraldehyde diethyl acetal, sodium cyanoborohydride ($NaBH_3CN$), cytidine 2′,3′-cyclic monophosphate monosodium salt (CCM), N_α-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME), triethylamine, ethanolamine, trifluoroacetic acid, copper(II) sulfate pentahydrate 4% (w/v), bicinchonicic acid solution, streptozotocin, D_2O, acetonitrile, ethanol, acetone, glacial acetic acid, hydrochloridric acid (HCl), dimethylsulfoxide (DMSO) and salts of analytical grade were from Sigma–Aldrich Co. (Germany).

2.2. Analytical methods

Spectrophotometer analysis was performed with a Lambda 25 UV-Vis Perkin-Elmer instrument (Northwolk, CT, USA). ^{1}H NMR spectroscopy was performed with Bruker 300 MHz spectrometer and NMR data were processed using the program Topspin (Bruker GmbH, Karlsruhe, Germany). Shimadzu analytical HPLC system was used with Zorbax GF-250 (Agilent Technologies, Palo Alto, CA; 4.6 mm × 250 mm or 9.4 mm × 250 mm; 4 μm) size exclusion chromatography (SEC) columns for analytical characterization and purification. Centrifugation was carried out with Hettich Zentrifugen mod. MIKRO 200 for small volume and Centrikon T-42K, Kontron Company, for large volume solution. The freeze-drying was performed with freeze-Hetossic Heto Lab Equipment. The dialysis Amicon® Ultra-15 membranes have been provided by Millipore (Billerica, MA, USA) and the dialysis tubing by Del Chimica Scientific Glassware (Naples, Italy). Aqueous and organic solutions were evaporated with Rotavapor mod. R II BÜCHI (Switzerland).

Glucose level determination was tested by OneTouch® II assay kit (LifeScan Inc., Johnson & Johnson, Milpitas, USA).

2.3. Synthesis of HA-acetal

HA sodium salt (100 mg, 0.25 mmol) was dissolved in 10 mL of DMSO with CH_3SO_3H (81 μL, 1.25 mmol). After complete dissolution, about 1–2 h, 1,1′-carbonyldiimidazole (203 mg, 1.25 mmol) was added, followed after 1 h by the addition of 4-aminobutyraldehyde diethyl acetal (0.25 mmol). Triethylamine was used, if necessary, to raise the pH to 8.0. The reaction mixture was stirred at 40 °C for 12 h and then 1 mL of NaCl saturated solution was added drop-wise, till the organic solution became opalescent. After 30 min of mixing, the polymer was precipitated slowly by adding 20 mL of chilly ethanol. The precipitate was washed with ethanol/H_2O solutions at an increasing percentage of ethanol (70:30, 80:20, 90:10, v/v) and filtered after each step. In order to better purify the product from unreacted amine, the dried polymer was dissolved in water and extensively dialyzed against demineralized water before lyophilization. The derivatization degree was determined by ^{1}H NMR.

HA-acetal (D_2O, σ ppm): 1.10 (t, 6H, —CH(OCH_2—CH_3)$_2$, acetal moiety), 1.85 (s, 3H, —NHCO—CH_3, HA), 3.2–3.8 (m, GlcA, GlcNAc methylene H, methane-H, HA).

2.4. Synthesis of random HA-RNase A and HA-trypsin (rHA-RNase A and rHA-trypsin) conjugates

The conjugation of RNase A and trypsin were performed using the same procedure, as an example the reaction of rHA-RNase A is here reported. HA-acetal (11 mg, 1.09 μmol; degree of acetal modification 4 mol%) was dissolved in 3 mL of 25 mM H_3PO_4 pH 2.1 and stirred for 1 h at 60 °C. After cooling to room temperature, the pH value of the solution was raised to 8.0 with 0.1 M NaOH. RNase A (3 mg, 0.22 μmol), previously dissolved in 1 mL of 0.1 M Na_2HPO_4 buffer pH 8.0, was added. The reaction mixture was diluted to a final protein concentration of 0.5 mg/mL with 0.1 M Na_2HPO_4 buffer pH 8.0. After 1 h, $NaBH_3CN$ (0.69 mg, 10.95 μmol), dissolved in 10 μL of 0.1 M Na_2HPO_4 buffer pH 8.0, was added and the reaction was stirred at room temperature for 48 h. Then, 5 equivalent of glycine, with respect to each equivalent of aldehyde in the starting HA, were added to the reaction mixture.

The reactions were analyzed by SEC with an analytical Zorbax GF-250 column (250 mm × 4.6 mm; 5 μm) eluted with 20 mM Na_2HPO_4, 130 mM NaCl pH 7.0, at a flow rate of 0.3 mL/min. The UV-Vis detector was settled at 280 nm. The purification was carried out with a semi-preparative GF-250 (250 mm × 9.4 mm) eluted with the same buffer used above at the flow rate of 1 mL/min.

The purified conjugate was concentrated to 10 mL and the purity was confirmed by analytical SEC. The products were extensively dialyzed against demineralized water and lyophilized. The total protein content was determined by the average of the results obtained by bicinchonicic acid solution colorimetric assay (Smith et al., 1985) and $Abs_{0.1\%}$ at 280 nm.

rHA-trypsin reaction mixture was prepared by using 10.72 mg (1.07 μmol) of HA-acetal, 5 mg of trypsin (0.21 μmol) and 0.67 mg of $NaBH_3CN$ (10.73 μmol). The reaction was carried out at 4 °C to prevent the autolysis of the enzyme.

2.5. Synthesis of N-terminal HA-RNase A and HA-trypsin (HA-N^{ter}-RNase A and HA-N^{ter}trypsin) conjugates

In order to selectively conjugate HA-acetal to the model enzymes at the *N*-terminal amino group, the conjugation was carried out as above reported except for the pH of the solution of the coupling reaction mixture that was 6.0 instead of 8.0. The reaction was then stopped and purified as reported above.

2.6. Determination of RNase A enzymatic activity

Free or HA conjugated RNase A samples (100 μL at 0.1 mg/mL protein concentration) were added to 1 mL of 0.15 mg/mL of CCM solution (0.46 mM) dissolved in 0.1 M Tris/acetate buffer pH 7.0. The hydrolysis of the substrate was evaluated by measuring the increase of absorbance at 247 nm every 5 min. The enzymatic activity was averaged between three experiments.

2.7. Determination of trypsin enzymatic activity

Two substrates were used for the evaluation of trypsin activity: (i) casein, a high molecular weight substrate for the determination of proteolytic activity and (ii) N_α-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME·HCl), a low molecular weight substrate for the determination of esterase activity. In the first case, the hydrolysis rate was evaluated by determining the absorbance of the digested peptides in solution after acid protein precipitation, according to Zwilling and Neurath (1981). In the second case, the method proposed by Hummel (1959) was used.

Briefly, casein substrate: in 2 mL test tubes, 500 μL of 0.1 M Tris/HCl pH 8.0 and 400 μL of 1% casein solution (dissolved in the same buffer) were added. To this solution 100 μL of free or HA conjugated trypsin samples (40–100 μg/mL protein concentration) were added. After 15 min at 30 °C, 500 μL of a 5% trichloroacetic acid solution were added. The precipitate was pelleted by centrifugation at 5000 × *g* for 5 min. The absorbance of the supernatant was determined at 280 nm. The enzymatic activity was averaged between three experiments.

TAME substrate: in 2 mL test tubes, 500 μL of 40 mM Tris, 10 mM $CaCl_2$, pH 8.1 and 500 μL of 1.04 mM TAME·HCl solution, in the same buffer, were added. The hydrolysis of the substrate was evaluated by monitoring the absorbance at 247 nm over 5 min after the addition of 50 μL of free or HA conjugated trypsin samples (0.1 mg/mL protein concentration). The enzymatic activity was averaged between three experiments.

2.8. Thermal stability of HA-conjugates

For the determination of thermal stability, RNase A, trypsin, HA-N^{ter}-RNase A and HA-N^{ter}-trypsin were separately incubated for 24 h at 37 °C in PBS solution. At 6 h and 24 h of incubation, 20 μL of each solution was withdrawn to measure the residual enzymatic activity as above reported.

2.9. Digestion of HA-N^{ter}-RNase A and HA-N^{ter}-trypsin conjugates by hyaluronidase

Hyaluronidase from bovine testes was added to a solution of HA-protein conjugate at 0.1 mg/mL (protein concentration) in 0.1 M Na_2HPO_4 buffer pH 7 at the enzyme/HA molar ratio of 1/25 (w/w). The solution was incubated for 24 h at 37 °C. At predetermined time points, a sample of 20 μL was analyzed by SEC under the same conditions reported above.

2.10. Synthesis of N-terminal HA-insulin conjugates

Insulin (INS) was coupled to two HA-acetal derivatives with different degrees of activation, 4 and 21 mol%, this yielding two conjugates: HA-N^{ter}-INS 1 and HA-N^{ter}-INS 2, respectively. For the preparation of HA-N^{ter}-INS 1, after activation of HA-acetal (8.81 mg, 0.87 μmol; degree of acetal modification 4 mol%) as above reported, the pH value of the solution was raised to 6.0 and bovine INS (1 mg, 0.17 μmol), dissolved in 0.35 mL of DMSO, was added. After 1 h, $NaBH_3CN$ (0.11 mg, 174 μmol) was added. For the preparation of HA-N^{ter}-INS 2, 1.66 mg of HA-acetal (0.87 μmol; degree of acetal modification 21 mol%), 1 mg of bovine INS (0.17 μmol) and 0.11 mg $NaBH_3CN$ (174 μmol) it was used. Purification and protein content were carried out as reported for the preparation of HA-enzymes.

2.11. Erythrocyte compatibility study

One milliliter of freshly collected heparinized rat blood was centrifuged at 4000 × *g* for 10 min. The precipitated erythrocytes were washed three times with PBS buffer. The red blood cells (RBCs) were resuspended in PBS buffer and diluted to have an optical density in the 0.6–0.7 range at 670 nm. This stock erythrocyte suspension was freshly prepared and used within 48 h. For the compatibility experiment, 300 μL of the stock suspension were added to 300 μL of PBS containing HA-N^{ter}-INS 1 or HA-N^{ter}-INS 2 at protein concentrations ranging from 0.5 to 3 mg/mL. The samples were incubated at 37 °C for 60 min under constant shaking. After centrifugation at 4000 × *g* for 10 min, the released hemoglobin was determined by measuring the absorbance at 414 nm. The experiment was performed also with HA sodium salts, using a polymer concentration range comparable to the HA content in the experiment with HA-N^{ter}-INS conjugates. Complete hemolysis, used as a positive control, was achieved using a 1% (v/v) solution of Triton X-100 in water, a surfactant known to lyse RBCs. The RBC-PBS solution was used as the negative control. The percentage RBC lyses was calculated according the following formula:

$$\%\,\text{lysis} = \left[\frac{A_{\text{sample}} - A_{\text{blank}}}{A_{100\%\,\text{lysis}} - A_{\text{blank}}}\right] \times 100$$

where A_{sample} is the absorbance value of the hemoglobin released from the RBCs treated with the polymer or the conjugate solution; A_{blank} is the absorbance value of the hemoglobin released from the RBCs treated with PBS buffer; and $A_{100\%\,\text{lysis}}$ is the absorbance value of the hemoglobin released from RBCs treated with 1% Triton X-100.

2.12. Ethics statement

All animal procedures were approved by the Ethic Committee of the University of Padova and the Italian Health Ministry, and all animals received care according to the DLGS 116/92 and in compliance with the "Guide for the Care and Use of Laboratory Animals".

2.13. Hypoglycemic potency of HA-N^{ter}-INS 1 and HA-N^{ter}-INS 2 in rats

Type I diabetes was induced in rats with streptozotocin (Akbarzadeh et al., 2007; Junod et al., 1967). Fifteen adult Sprague-Dawley male rats (250–300 g, 75–90 days old) received a dose of 60 mg/kg of streptozotocin in 10 mM citrate, 0.15 M NaCl, pH 4.5 by intraperitoneal injection (i.p.). Diabetes was induced in 12 animals within 3 days, owing to beta cells destruction.

Animals were randomly divided in four groups and injected subcutaneously with insulin, HA-N^{ter}-INS 1, HA-N^{ter}-INS 2 (0.135 units per rats) or PBS. At predetermined interval time points glucose levels (mg/dL) in blood samples were measured using OneTouch® II assay kit.

2.14. Labeling of HA-N^{ter}-INS 1 to FITC

To investigate the systemic presence of HA-N^{ter}-INS 1 in the body, the conjugate was labeled with fluorescein isothiocyanate (FITC). The FITC dye solution (1.39 mM) in DMSO was added to the solution of HA-N^{ter}-INS 1 (4 molar ratio to protein molecule) in PBS buffer, pH 8.0. The conjugation reaction was performed at room temperature for 48 h with mild stirring. The HA-N^{ter}-INS 1-FITC conjugate was purified from the unreacted dye using gel filtration chromatography (G-50) and then dialyzed *vs* 10 mM PBS. The degree of substitution was calculated by measuring the absorbance at 280 nm and 494 nm. HA-N^{ter}-INS 1-FITC conjugate was injected subcutaneously to Sprague-Dawley rats (6 animals). The dose was 0.55 nmol INS equiv. corresponding to 0.67 nmol dye. At predetermined interval time points, blood samples (100 μL) were taken from the tail vein of each animal. For each time point, the rats were anesthetized with 5% isoflurane gas (mixed with O_2) in enclosed cages. The samples were centrifuged and the plasma analyzed by size exclusion chromatography as reported above using a fluorescence detector settled at 494 nm.

3. Results

3.1. Synthesis and characterization of HA-acetal

In this study, the aldehyde groups were grafted on the HA backbone by exploiting a new strategy that allowed the preservation of HA backbone oppositely to the widely used procedure of polysaccharide backbone oxidation with periodate. In this case, desired degree of aldehyde modification were achieved by coupling few HA carboxylic groups with 4-aminobutyraldehyde diethyl acetal. The acetal spacer was firstly chosen because it contains a protected aldehyde functionality that avoids the stability issue of aldehyde groups during long-term storage. In fact, the HA-acetal derivative can be easily deprotected in the active form just before in a protein conjugation reaction. Secondly, the butyraldehyde group is less reactive than other aliphatic aldehydes, namely propyl or ethyl aldehydes (Hu & Sebald, 2011), thus allowing a better selectivity of the coupling reaction toward the protein *N*-terminus. In order to control the degree of HA modification, different molar excesses of reagents, polymer concentration and solvents were investigated. A proper acetal derivatization was obtained carrying out the reaction in DMSO using the conditions reported in Section 2. Even if the sodium salt of HA is only partially soluble in this solvent, the addition of CH_3SO_3H allowed a complete dissolution of the polymer in 1–2 h. HA-acetal was prepared at two different degrees of derivatization. The exact degree of modification was calculated by ^{1}H NMR spectroscopy, this offering a precise control over the reagents ratio in the following protein conjugation reaction. The comparison of the integral value of acetal group (1.10 ppm) with the integral value of HA acetyl group (1.85 ppm) demonstrated that the two polymer batches were activated at 4 and 21 mol% (Fig. 1).

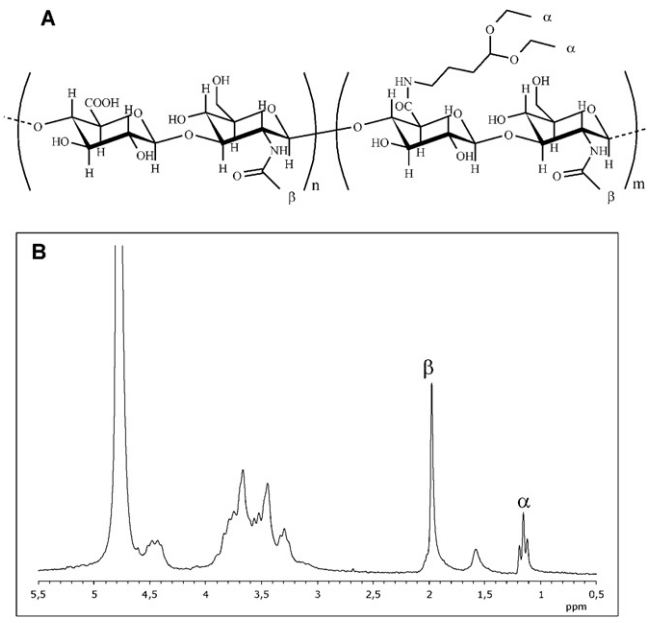

Fig. 1. Structure and characterization of HA-acetal derivative. (A) Chemical structure of HA-acetal derivative. (B) ^{1}H NMR of HA-acetal derivative in D_2O: 1.10 ppm, t (6H, —CH(OCH$_2$—C$\underline{H}_3$)$_2$, 4-aminobutyraldehyde diethyl acetal), 1.85 ppm, s (3H, —NHCO—C$\underline{H}_3$, HA), 3.2–3.8 ppm, m (GlcA, GlcNAc methylene H, methane-H, HA).

3.2. Synthesis and characterization of HA-enzyme conjugates

Acetal groups of the HA derivative were activated to aldehyde groups by a mild acid hydrolysis. The hyaluronan-aldehyde derivative was conjugated to model enzymes at two pH values, 8.0 for random coupling at the level of lysines and 6.0 for selective protein *N*-terminus coupling. Random conjugation was performed for an indirect evaluation of the steric hindrance and the flexibility of HA on the protein surface.

The conjugation reactions were monitored by SEC. In Fig. 2A, it is reported as an example the time course of the conjugation of HA-N^{ter}-trypsin. The disappearance over the time of the peak of free trypsin (t_R = 10.5 min) corresponded to the appearance of a new peak (t_R = 6.13 min), in agreement with the formation of HA-N^{ter}-trypsin conjugate that has a high hydrodynamic volume. The purified conjugate was investigated by SEC to ensure the elimination of unreacted enzyme (Fig. 2B). The same SEC protocols were applied to the other conjugates. The purification was considered satisfactory when the peak of the starting protein (t_R around 10 min) had disappeared or was lower than 1% (Fig. S1). Protein contents in the conjugates, shown in Table 1, were averaged from very similar data obtained by bicinchonicic acid solution assay and absorbance at 280 nm on the basis of protein molar extinction. It was verified that HA did not affected both determinations.

3.3. Measurements of HA-RNase A activity and thermal stability

The determination of RNase A activity in the corresponding conjugates, with the low molecular weight substrate, CCM, displayed a high enzymatic activity retention (Table 1). As expected, rHA-RNase A had a lower activity with respect to HA-N^{ter}-RNase A. This difference is due to the polymer chains that is a steric obstacle for the substrate recognition with the active site, an effect becoming more evident with the increase of the number of HA chains coupled per protein unit.

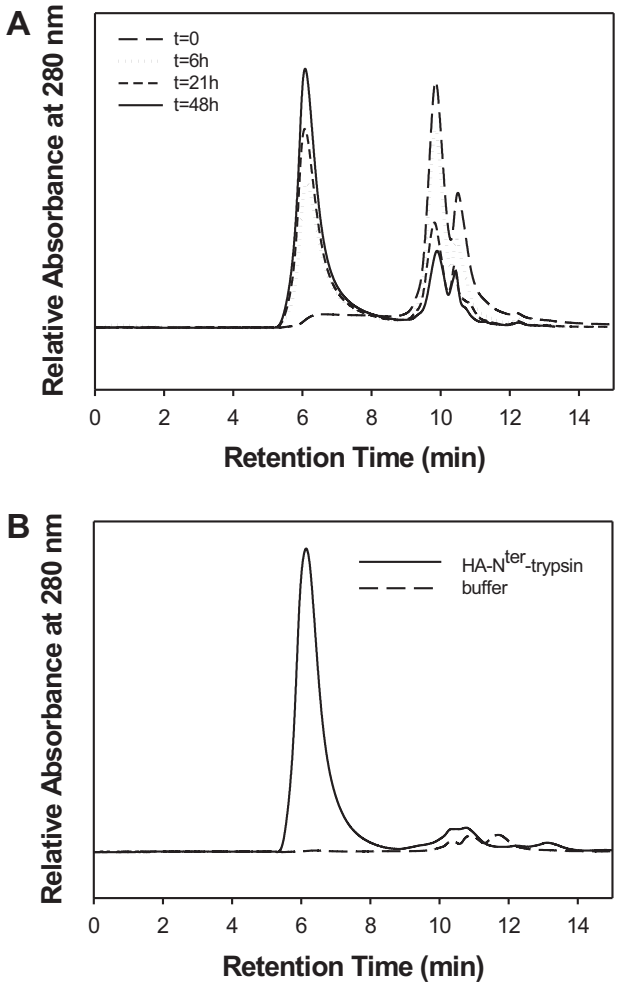

Fig. 2. Conjugation of HA-acetal to trypsin. (A) Conjugation reaction of HA-N^{ter}-trypsin at times of 0, 6, 21 and 48 h. (B) Elution profiles of HA-N^{ter}-trypsin conjugate after purification. The low peak eluting at 10–12 min is not referred to free trypsin but it is due to the Tris buffer used to dissolve the sample as shown in the elution profile of buffer used as control. The analyses were carried out with size exclusion chromatography accordingly to the conditions reported in Section 2.

The thermal stability investigation, evaluated by monitoring the residual enzymatic activity over time at 37 °C, showed that both RNase A and HA-N^{ter}-RNase A retained well the enzymatic activity by losing only 10% of the initial activity after 24 h of incubation (Fig. 3A).

3.4. Measurements of HA-trypsin activity and thermal stability

The residual activities of rHA-trypsin and HA-N^{ter}-trypsin have been evaluated on low and high molecular weight substrates, TAME·HCl and casein, respectively. As expected, also in these cases the two conjugates showed slightly different enzymatic activities on the basis of the method of coupling. The residual proteolytic activity on casein of rHA-trypsin and HA-N^{ter}-trypsin were reduced at 53% and 61%, while both conjugates displayed a better retention of esterolytic activity, 72% and 86%, respectively (Table 1). These results indicate that the polymer does not completely hinder the approach of even large substrates to the catalytic site, probably thanks to its backbone flexibility and hydrophilicity.

In the case of HA-N^{ter}-trypsin and trypsin, the residual activity over time during incubation at 37 °C decreased faster than for RNase A. Trypsin was quickly inactivated, showing 20% of residual esterolytic activity and 10% of proteolytic activity after just 6 h. The enzymatic activity decrease of the conjugate over time was slower than the free trypsin, as shown by the results after 6 h of incubation. In particular, the esterolytic and proteolytic activities were 59% and 34%, respectively, of that of trypsin at time zero (Fig. 3B and C). This demonstrates HAylation markedly increased enzyme stability reducing the typical autolysis process, an effect seen also after trypsin conjugation with other polymers (Gaertner & Puigserver, 1992).

3.5. Treatment of HA-N^{ter}-RNase A and HA-N^{ter}-trypsin by hyaluronidase

HA is a biodegradable polymer and this represents a relevant advantage over the most used polymer in this field, PEG, which is non biodegradable (Pasut et al., 2008). It was therefore important to verify if hyaluronidase, the enzyme involved in HA degradation *in vivo*, was still able to degrade HA after its conjugation to a protein The SEC analysis showed that the conjugates' molecular weight was reduced in a time depended manner. In fact, the conjugates were eluted as a main peak at 6 min. After incubation with hyaluronidase, these peaks disappeared and new peaks with higher retention times, and consequently smaller molecular weights appeared over time (Fig. 4).

3.6. Synthesis and characterization of HA-Insulin (HA-INS) conjugates

After testing and optimizing the conjugation of HA to model enzymes, the HA-acetal derivative was used to prepare a potential therapeutic conjugate with insulin. In this case two HA-acetal

Table 1
Properties of HA-enzyme conjugates. Values are mean ± S.D ($n = 3$).

Sample	Protein content (%, w/w)	Free protein (%)	Yield of reaction (%, w/w)	Activity (%)
RNase A	100		–	100
rHA-RNase A	21.4	<LOD	33.5	70.3 ± 3.05
HA-N^{ter}-RNase A	21.2	<LOD	48.5	82.9 ± 2.74
Trypsin	100		–	100 (LMWS)[a] 100 (HMWS)[b]
rHA-trypsin	8.9	<LOD	21.4	72.0 ± 2.74 (LMWS)[a] 53.0 ± 6.46 (HMWS)[b]
HA-N^{ter}-trypsin	14.8	<LOD	41.4	85.6 ± 4.34 (LMWS)[a] 61.3 ± 6.10 (HMWS)[b]

LOD = 0.01 mg/mL.
[a] Activity evaluated against Low Molecular Weight Substrate.
[b] Activity evaluated against High Molecular Weight Substrate.

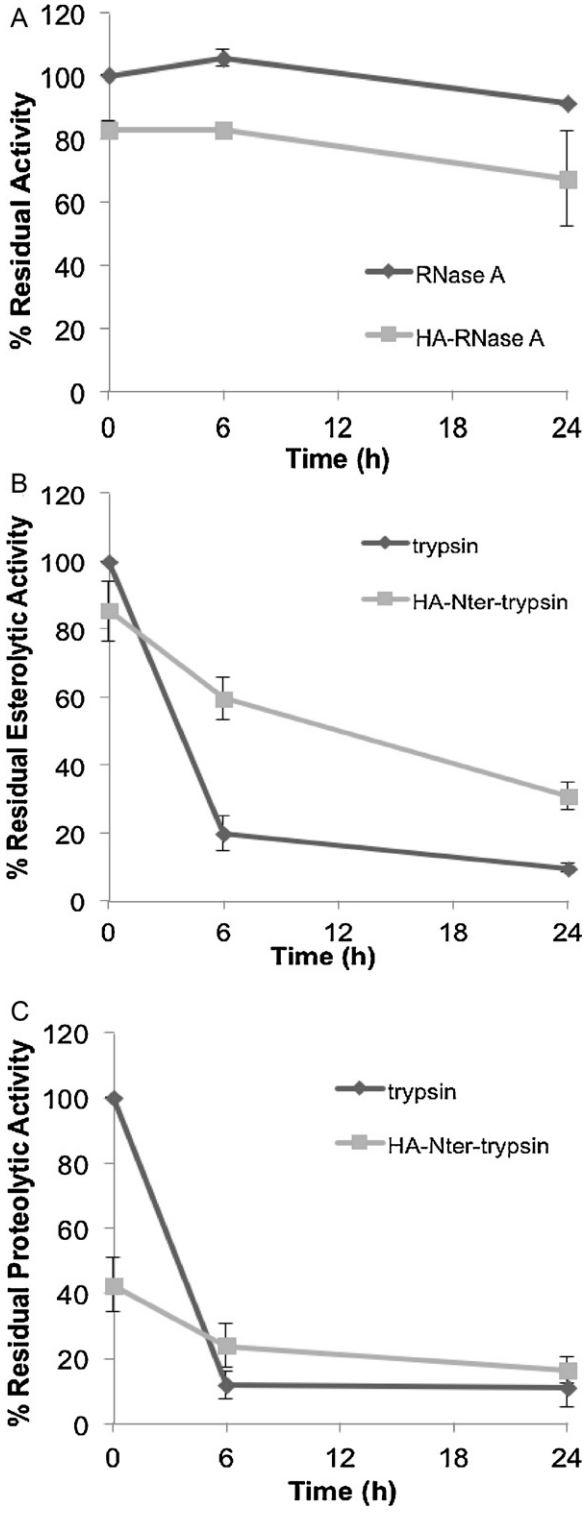

Fig. 3. Time-dependent RNase A, trypsin and HA conjugates after incubation in Tris/acetate buffer pH 7.0. The residual activity was determined after 0, 6 and 24 h of incubation. For RNase A and HA-N^{ter}-RNase A against the substrate CCM (panel A). For trypsin and HA-N^{ter}-trypsin against TAME (esterolysis) (panel B) and casein (proteolysis) (panel C). Results are presented as mean ± S.D (n = 3).

batches, differing in the degree of acetal substitution (4% and 21%), were used to also investigate the effect of protein loading.

The SEC elution profiles of purified products showed a main peak corresponding to the conjugates without the presence of detectable amounts of free insulin (Fig. 5). As reported in Table 2,

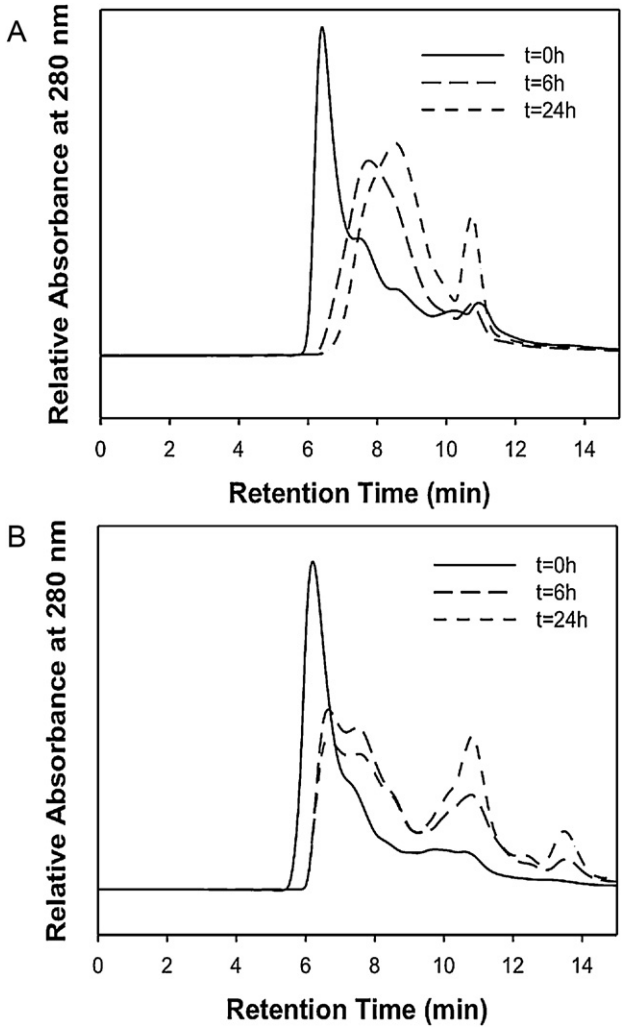

Fig. 4. Investigation of HA conjugates biodegradability by treatment with hyaluronidase. HA-N^{ter}-RNase A (panel A) and HA-N^{ter}-trypsin (panel B) degradation by hyaluronidase (t = 0, 6 and 24 h). The elution conditions are reported in Section 2.

the percentages of aldehyde groups in the HA backbone correlates, although not proportionally, with the insulin loading in the final conjugates, 17.2% and 32% (w/w) for HA-N^{ter}-INS 1 and HA-N^{ter}-INS 2, respectively, thus suggesting a steric hindrance between the polypeptide chains.

3.7. Erythrocyte compatibility study

Before testing the activity of HA-INS conjugates *in vivo*, the effect of the polymer and HA-INS conjugates on the integrity of the erythrocyte membrane was investigated to evaluate the blood compatibility of the compounds. The percent of cell lysis, as reported in Fig. S2, showed that both conjugates were non-hemolytic at the concentration used for the *in vivo* study. A partial lysis was only shown by HA-N^{ter}-INS 2 (<20% lysis) but it was for a concentration 100 times higher than that used with the animals. The starting

Table 2
Properties of HA-INS conjugates.

Sample	Protein content (%, w/w)	Free protein (%)	Yield of reaction (%, w/w)
HA-N^{ter}-INS 1	17.2	<LOD	9.6
HA-N^{ter}-INS 2	32.0	<LOD	18.0

LOD = 0.01 mg/mL.

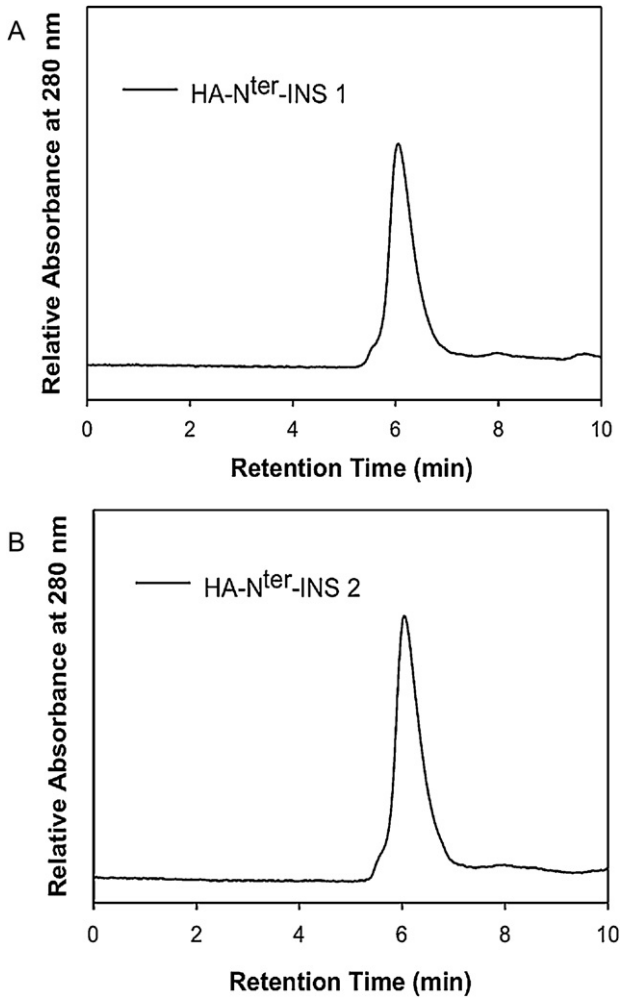

Fig. 5. Characterization of HA-INS conjugates. Elution profiles of purified HA-INS conjugates in size exclusion chromatography. The elution conditions are reported in Section 2.

HA and the insulin conjugate prepared with the HA-acetal at 4% activation degree showed negligible hemolytic activities.

3.8. *In vivo* evaluation of HA-INS activity

In order to study *in vivo* the residual activity of HA-INS conjugates, their glucose lowering effect was tested in rats. Type I diabetes was induced with streptozotocin in Sprague-Dawley rats, showing after the treatment an average basal blood glucose titer of >200 mg/dL compared to 60–70 mg/dL in normal rats. To each diabetic rat group was subcutaneously administered a single bolus of HA-N^{ter}-INS 1, HA-N^{ter}-INS 2, insulin or PBS (control). The glucose blood level for all groups was monitored over time and up to 24 h. The insulin equiv. dose was 0.135 IU per animal to avoid more severe hypoglycemic events, observed at higher doses. The experimental results are shown in Fig. 6. The lowering effect on glucose blood level of insulin was observed at 1 h post-injection, but the glucose titer returned already to the initial value after 2 h. Interestingly, HA-N^{ter}-INS 1 showed a slower but prolonged onset of the lowering effect, in fact the glucose titer reached the minimum concentration at 6 h and then progressively returned to the initial value. In order to determine the fate of this conjugate after *s.c.* injection, HA-N^{ter}-INS 1 was labeled with FITC. The study demonstrated that shortly after injection the high molecular weight conjugate reached the blood stream and later conjugate fractions

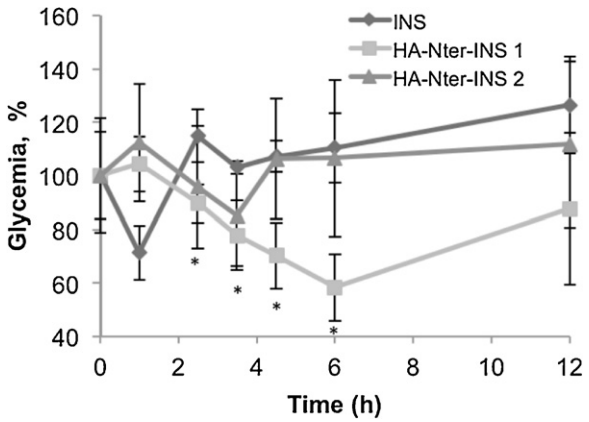

Fig. 6. Pharmacodynamic of HA-INS conjugates. Blood glucose levels in streptozocin-induced diabetic Sprague-Dawley rats after the injection of bovine insulin, HA-N^{ter}-INS 1, HA-N^{ter}-INS 2. Results are presented as mean ± s.e.m. (n = 3–5). Significance: $^{*}p < 0.05$ *vs* insulin.

with a decreased molecular weight were also detectable, but free insulin is not released even after 24 h post injection (Fig. S3). Therefore, the prolonged activity of HA-N^{ter}-INS 1 is a combination of the increased molecular weight of the conjugate and the prolonged release of conjugate fractions at a lower molecular weight from the site of injection, by the action of hyaluronidase.

Unexpectedly, HA-N^{ter}-INS 2 showed no significant effect on glucose titer. Probably the higher insulin loading of HA-N^{ter}-INS 2 led to a steric entanglement affecting the receptor/protein recognition.

The prolonged and enhanced hypoglycemic effect of the HA-N^{ter}-INS 1 conjugate, compared to free insulin, revealed that HAylation is able to modify the PK profile of proteins by increasing their half-lives and behaving as a depot system. All these findings encourage the use of HAylation technology for the delivery of proteins of pharmaceutical interest.

4. Conclusions

HAylation performed with this new HA-acetal derivative has demonstrated a great potential in the field of protein delivery. The obtainment of a stable and easily characterizable HA derivative, suitable for site selective protein conjugation, was a prerequisite for the full exploitation of the biodegradability advantage of HA over the most known polymer in the field of protein conjugation, PEG. The biodegradability of HA backbone, preserved after protein conjugation, should represent one of the main strengths of HA because it allows for the release of protein conjugates with short HA oligomers *in vivo*, thus allowing at least a partial recovery of protein activity. On the other hand, it should be highlighted that HAylation might not be suitable for immunogenic proteins because, for the same reason reported above, it would not offer complete shielding of immunogenic sites over the time.

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.2012.11.090.

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