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A new hyaluronic acid pH sensitive derivative obtained by ATRP for potential oral administration of proteins

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ABSTRACT

Atom transfer radical polymerization (ATRP) has been successfully employed to obtain a new derivative of hyaluronic acid (HA) able to change its solubility as a function of external pH and then to be potentially useful for intestinal release of bioactive molecules, included enzymes and proteins.

In particular, a macroinitiator has been prepared by linking 2-bromo-2-methypropionic acid (BMP) to the amino groups of ethylenediamino derivative of tetrabutyl ammonium salt of HA (HA-TBA-EDA). This macroinititor, named HA-TBA-EDA-BMP has been used for the ATRP of sodium methacrylate (MANa) using a complex of Cu(I) and 2,2′-bipyridyl (Byp) as a catalyst.

The resulting copolymer, named HA-EDA-BMP-MANa, has been characterized by ^{1}H NMR and size exclusion chromatography (SEC) analyses. A turbidimetric analysis has showed its pH sensitive behavior, being insoluble in simulated gastric fluid but soluble when pH increases more than 2.5. To confirm the ability of HA-EDA-BMP-MANa in protecting peptides or proteins from denaturation in acidic medium, α-chymotrypsin has been chosen as a model of protein molecule and its activity has been evaluated after entrapment into HA-EDA-BMP-MANa chains and treatment under simulated gastric conditions. Finally, cell compatibility has been evaluated by performing a MTS assay on murine dermal fibroblasts cultured with HA-EDA-BMP-MANa solutions.

1. Introduction

pH sensitive drug delivery systems represent a smart way for drug administration since they are able to release loaded molecules in response to pH gradient that exists between different sites of the body. For instance, pH gradients between normal tissues and some pathological sites, between the extracellular environment and some cellular compartments and along the gastrointestinal (GI) tract are well characterized (Felber et al., 2012).

In particular the pH difference between the stomach (pH 1–2 about) and the small intestine, jejunum and ileum (pH 6–7.5 about) requires the use of pH sensitive carriers capable to protect, if necessary, bioactive molecules from acid degradation and to release them in the intestine in their active form.

Especially peptides, proteins and nucleic acids easily undergo inactivation in the stomach; this represents a serious issue since these bioactive molecules are receiving an increasing interest for the treatment of several pathologies, thanks also to the increased availability of novel therapeutic molecules through the recombinant DNA technology (Yun et al., 2013). On the other hand, oral delivery is still the preferred route of drug administration since it offers to patients less pain, greater convenience, higher compliance, and reduced risk of cross-infection and needlestick injuries (Florence and Jani, 1993; Chen and Langer, 1998).

Starting from pH sensitive polymers, gastro-resistant drug delivery systems have been produced in the form of hydrogels (Pitarresi et al., 2008), nanoparticles (Licciardi et al., 2013) and microparticles (Licciardi et al., 2012). They can be also used as coating of tablets or capsules.

The main characteristic of gastro-resistant polymers is the presence of carboxyl groups able to allow drug release as a function of their deprotonation pH dependent. pH sensitive polymers can be natural, like alginate or synthetic, like poly(methacrylic acid) derivatives, as well as semisynthetic, like hydroxypropyl methylcellulose phthalate.

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Among natural polymers, hyaluronic acid (HA) possesses very interesting biological properties indeed it has been extensively employed for the production of drug delivery systems (Pitarresi et al., 2007, 2010) and scaffolds for tissue engineering (Ji et al., 2006). Being HA a linear polysaccharide consisting of alternating disaccharide units of α-1,4-D-glucuronic acid and β-1,3-N-acetyl-D-glucosamine, it contains one carboxylic group per repetitive unit then it shows some pH sensitivity in aqueous environment, especially when it is crosslinked to produce drug delivery systems like hydrogels and micro or nanoparticles. As example, recently HA nanoparticles have been employed for the oral administration of insulin (Han et al., 2012). Also the pH responsive behavior of hyaluronic acid hydrogels has been reported for photocrosslinked networks based on HA for the release of thrombin (Pitarresi et al., 2004).

However, unlike other pH sensitive polymers, for example Eudragit®, HA does not show a pronounced difference in solubility in aqueous media at different pH; this means that when pH is 1–2 (simulated gastric fluid) no precipitation of HA occurs. For this reason HA cannot be exploited as gastro-resistant coating for solid pharmaceutical forms.

With the aim to increase the number of carboxyl groups of natural HA, thus obtaining an appropriate derivative capable of changing its solubility in an aqueous environment as a function of pH, we have performed atom transfer radical polymerization (ATRP) of sodium methacrylate into a macroinitiator based on an amino functionalized HA derivative.

Recently we have demonstrated that it is possible to produce HA based "macroinitiators" by functionalizing HA or its ethylenediamino derivative (HA-EDA) with 2-bromo-2-methylpropionic acid (BMP). These "multi functional macroinitiators" have been employed for the ATRP of different molecules carrying vinyl portions to allow the polymerization of poly(ethylene glycol) methacrylate (PEGMA), butyl methacrylate (BUTMA) and N-isopropylamide (NIPAM) on their side chains (Pitarresi et al., 2013). Obtained results demonstrated that ATRP is more efficient starting from the amino funzionalized HA, for this reason, in this work we have used HA-EDA as initial material to perform ATRP of sodium methacrylate.

The resulting copolymer, named HA-EDA-BMP-MANa, has been characterized by ^{1}H NMR and size exclusion chromatography (SEC) ant its pH sensible behavior has been demonstrated by a turbidimetric analysis. α-Chymotrypsin has been chosen as a model of protein molecule, to verify the ability of HA-EDA-BMP-MANa to protect proteins from degradation in acidic medium, like simulated gastric fluid and an in vitro biological assay has been performed to evaluate its cell compatibility.

2. Experimental

2.1. Materials and methods

All reagents were of analytical grade unless otherwise stated.

α-Chymotrypsin from bovine pancreas, N-benzoyl-L-tyrosine ethyl ester (BTEE), acetone, methanol, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), bis(4-nitrophenyl) carbonate (4-NPBC), tetrabutylammonium hydroxide (TBA-OH), diethylamine (DEA), 2-bromo-2-methylpropionic acid (BMP), sodium methacrylate (MANa), copper bromide [Cu(I)Br], 2,2′-bipyridyl (Bpy), deuterium oxide (D_2O), were purchased from Sigma–Aldrich.

Hyaluronic acid (HA) having a low weight-average molecular weight was prepared by acidic degradation as reported by Shu et al. (2002) starting from a biotechnological HA sodium salt, Mw 1500 kDa that has been a generous gift from Novagenit s.r.l. (Italy). Briefly, 2 g of HA were dissolved in 200 ml of twice distilled water and the solution was kept in an orbital shaker incubator at 37 °C overnight.

After this time, 4 ml of HCl 37% (w/v) were added and the solution was stirred with a blade stirrer for 5 min at 350 rpm. The solution was kept in orbital shaker incubator at 37 °C for other 24 h then the pH was adjusted to 7 with NaOH 1 N. The so obtained solution was dialyzed against water for 5 days and the product was recovered by freeze drying.

Since HA is not soluble in organic solvents, the tetrabutylammonium salt of HA (HA-TBA) was produced as described in our previous work (Palumbo et al., 2006).

^{1}H NMR spectra were obtained with a Brucker AC-300 instrument and by using D_2O or D_2O/NaOD as a solvent.

2.2. Synthesis of hyaluronic acid-ethylenediamine (HA-TBA-EDA) derivative

HA-TBA-EDA was synthesized as reported in our previous work (Palumbo et al., 2012).

Briefly, 500 mg of HA-TBA was dissolved in 45 ml of anhydrous DMSO then a suitable amount of 4-NPBC, dissolved in 3 ml of DMSO, was added dropwise to the HA-TBA solution at 40 °C (molar ratio between 4-NPBC and HA-TBA equal to 0.75). The solution was left at the same temperature for 4 h. After this time, ethylenediamine (EDA) (molar ratio equal to 10 respect to the moles of 4-NPBC) was added and the solution was left at 40 °C for 3 h. The obtained HA-EDA-TBA derivative was precipitated in an excess of diethyl ether then washed 4 times in acetone and dried under vacuum. For ^{1}H NMR characterization, to obtain a spectrum as clear as possible, TBA was eliminated from the final product as follows: sample was dissolved in water in the presence of 500 μl of NaCl saturated aqueous solution, dialyzed against NaCl 5% (w/v) solution for 2 days and then against water for other 2 days. The product was recovered by freeze drying.

^{1}H NMR spectrum of HA-EDA derivative in D_2O showed principal peaks at δ 1.9 (-NH-CO-**CH_3** of HA), δ 3.1 (-CO-NH-CH_2-**CH_2**-NH_2 of EDA), δ 3.3–3.8 (pyranosyl **CH** of HA).

The derivatization degree in terms of moles of EDA linked to HA (DD_{EDA}%), calculated by ^{1}H NMR analysis, was 60 ± 5 mol%.

2.3. Synthesis of HA-TBA-EDA-BMP macroinitiator

HA-TBA-EDA-BMP macroinitiator was synthesized according to a procedure reported in our previous work (Pitarresi et al., 2013). BMP carboxyl groups were activated with EDC and NHS in DMSO at 37 °C overnight, by using a molar ratio between activating agents and BMP equal to 1.2. The obtained solution was added to a 1% (w/v) solution of HA-TBA-EDA in anhydrous DMSO, in the presence of DEA as a catalyst (molar ratio between DEA and amino groups of HA-TBA-EDA equal to 1). The molar ratio between activated BMP and HA-TBA-EDA amino groups was 1.8.

The reaction was carried out for 24 h at 40 °C, the product was isolated by precipitation in acetone, purified by several washings in the same solvent and recovered after drying under vacuum, then characterized by ^{1}H NMR analysis.

To obtain a spectrum as clear as possible, TBA was eliminated from the final product (HA-TBA-EDA-BMP) as described in the previous paragraph.

^{1}H NMR spectrum of HA-EDA-BMP derivative in D_2O showed principal peaks at δ 1.8 (-C(**CH_3**)$_2$-Br of BMP), δ 1.9 (-NH-CO-**CH_3** of HA), δ 3.1 (-CO-NH-CH_2-**CH_2**-NH_2 of EDA), δ 3.3–3.8 (pyranosyl **CH** of HA).

2.4. Synthesis of HA-EDA-BMP-MANa copolymer through ATRP

50 mg of HA-TBA-EDA-BMP were dissolved in 5 ml of twice distilled water, then MANa was added in order to obtain a molar ratio between vinyl monomer and BMP present in the macroinitiator equal to 100.

The resultant solution was kept under Argon bubbling, until the catalyst solution (obtained by dissolving Bpy and CuBr with a molar ratio of 4:1 in anhydrous DMF) was added. The molar ratio between CuBr and BMP groups present in HA-TBA-EDA-BMP was 2.

The reaction solution was sealed under Argon atmosphere for 24 h at 37 °C in orbital shaker incubator. Reaction was then stopped by keeping reaction mixture in contact with air oxygen until the complete oxidation of copper. The reaction mixture was dialyzed (12–14 kDa cut-off) against NaCl 5% (w/v) solution for 2 days and then against water for other 2 days, to eliminate the TBA, Cu^{++}, Bpy and the unreacted MANa. The product was recovered by freeze drying and characterized by ^{1}H NMR analysis and size exclusion chromatography (SEC). The final yield ranged from 130% to 145% compared to the initial weight of the macroinitiator.

^{1}H NMR spectrum of HA-EDA-BMP-MANa derivative in D_2O/NaOD showed principal peaks at δ 0.6 (-C(**CH_3**)$_2$-Br of BMP; -CH_2-C(**CH_3**)- of MANa), δ 1.3 (-**CH_2**-C(CH_3)- of MANa), δ 1.6 (-NH-CO-**CH_3** of HA), δ 2.8–3.6 (pyranosyl **CH** of HA).

2.5. Turbidimetric analysis

In order to determine pH value that causes precipitation of HA-EDA-BMP-MANa, a turbidimetric analysis has been performed on solutions of HA-EDA-BMP-MANa dissolved in buffer solutions with different pH values.

In particular, 900 μl of buffer solution at different pH values (7.4, 5.5, 4.5, 3.5 and 2.5) were added to 100 μl of a solution of HA-EDA-BMP-MANa 1% (w/v) in ultrafiltered water. The transmittance of the resulting mixtures was read at the wavelength of 500 nm using a spectrophotometer UV-2401PC SHIMADZU. The analysis has been performed in triplicate.

2.6. SEC characterization

Weight-average molecular weight (Mw) and polydispersity index (Mw/Mn) of starting HA-EDA, and its derivatives here prepared were determined by a SEC apparatus equipped with a pump system (Waters 600, Mildford, MA, USA), a Universal column (particle size 5 μm) and a 410 differential refractometer (DRI) as a concentration detector (Waters 2410, Mildford, MA, USA).

Employed conditions were: 200 mM phosphate buffer (pH 6.5):MeOH 90:10 (v/v) as a mobile phase, 36 ± 0.1 °C, flow rate 0.6 ml/min. The calibration curve was determined by using Pullulan standards from Hyalose (USA) as reported by Ferguson et al. (2010).

In order to determine whether the treatment at pH 1 causes polymer degradation, a SEC analysis has been also performed on HA-EDA-BMP-MANa after 2 h of incubation at 37 °C in HCl 0.1 N. The precipitated product was recovered by centrifugation, freeze-dried and dissolved in the mobile phase for SEC analysis. HA-EDA has been used as a control; it has been dissolved in HCl 0.1 N and incubated for 2 h at 37 °C. After this time, the pH has been increased to 5, the product has been freeze-dried and dissolved in the mobile phase for SEC analysis. The analysis has been performed in triplicate.

2.7. Incorporation and activity evaluation of α-chymotrypsin

α-Chymotrypsin has been dissolved in TRIS buffer at pH 7.8 at a concentration of 5 mg/ml, 450 μl of the obtained solution was used to dissolve 18 mg of HA-EDA-BMP-MANa or HA-EDA (weight ratio between copolymer and enzyme 8:1) at 37 °C under gentle stirring. The obtained copolymer/enzyme solution was freeze-dried.

The freeze-dried solid was treated with in 30 ml of HCl 0.1 M (pH 1) for 2 h at 37 °C (simulated gastric conditions), recovered by filtration and dissolved in 30 ml of HCl 0.001 M (pH 3.0). The activity of α-chymotrypsin has been determined by using BTEE as a substrate following supplier instruction. In particular, 100 μl of the obtained solution was mixed with 1.4 ml of BTEE solution (obtained by dissolving 37 mg of BTEE in 63.4 ml of methanol and diluting to a volume of 100 ml with ultra filtered water), 1.42 ml of TRIS pH 7.8 and 80 μl of a 2 mM of $CaCl_2$ solution.

Free freeze-dried α-chymotrypsin treated at pH 7.8 for 2 h has been used as a positive control, whereas free freeze-dried α-chymotrypsin treated at pH 1 for 2 h has been used as a negative control.

As the assay with BTEE allows to determine only the amount of active enzyme, to evaluate the total amount of protein entrapped by HA-EDA-BMP-MANa, a HPLC-SEC analysis has been performed by using a HPLC instrument Agilent 1100 equipped with a UV detector and a SEC column Phenogel, 200 mM phosphate buffer (pH 6.5):MeOH 90:10 (v/v) as a mobile phase, 36 ± 0.1 °C, flow rate 0.6 ml/min and a λ of 281 nm. α-Chymotrypsin dissolved in TRIS buffer at pH 7.8 has been used for calibration curve (range of concentration 0.25–1.5 mg/ml; $y = 2411.7x - 182.32$; $R^2 = 0.9938$).

2.8. Isolation of murine dermal fibroblasts and cell compatibility studies

Rat fibroblasts (wistar rats) have been isolated as follows: dermis has been accurately shaved, cut into 1 cm^2 pieces and immersed in sterile PBS with 1% (v/v) of penicillin/streptomycin solution. Specimens have been treated with an aqueous cold ethanol solution (70%, v/v) for 2 min, washed several times with sterile PBS and reduced in small pieces with a scalpel. So obtained pieces have been incubated with a Dispase II solution (2.5 U ml^{-1}) for 1 h, then the epidermis has been separated from the dermis with the use of forceps.

Dermis pieces have been kept in the bottom of T-75 culture flask for 1 h prior to add DMEM supplemented with FBS (10%, v/v), penicillin–streptomycin solution (1%, v/v), glutamine solution (1%, v/v) and amphotericin B solution (0.25%, v/v).

Specimens have been cultured for 2 weeks by changing the culture medium every two days until fibroblasts migrate from the dermis to the culture flask. The so obtained cells have been cultured from passage 1 to 7.

For cell compatibility studies, HA-EDA-BMP-MANa has been sterilized as a powder by UV irradiation for 1 h by using a 125 watt UV-lamp and dissolved in supplemented DMEM at 0.1 and 0.5 mg/ml. Cells (1×10^5) have been seeded in each well of a 96 well plate for 24 h and then the medium has been changed with each HA-EDA-BMP-MANa solution (0.1 or 0.5 mg/ml). Cell viability has been evaluated after 24 and 48 h of contact with the polymer through MTS test by following the supplier instructions. Cell morphology has been investigated by optical microscope Motic AE21.

Cells incubated in supplemented DMEM in the absence of polymer, have been used as a positive control. Each experiment has been performed in triplicate.

3. Results and discussion

3.1. Synthesis of HA-TBA-EDA-BMP macroinitiator

Following a procedure patented by our research group, we have performed the derivatization of primary hydroxyl groups of HA (in the form of TBA salt) with ethylenediamine (EDA) (Giammona

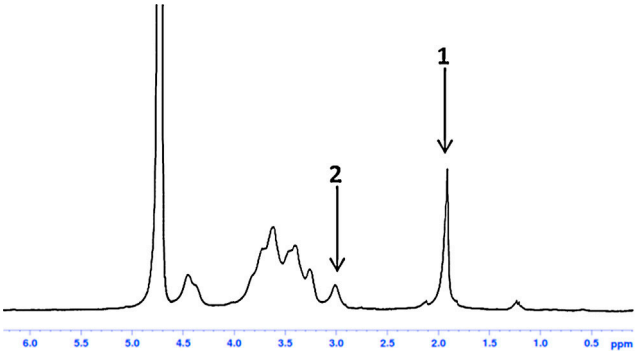


Fig. 1. ^{1}H NMR spectrum of HA-EDA in D_2O.

et al., 2010) and we have already shown the importance, in terms of increased reactivity for nucleophilic attack reactions, of including free amino groups in HA (Palumbo et al., 2012).

Now, the nucleophilic character of primary amino groups of EDA linked to HA (greater than primary hydroxyl groups of native polysaccharide) has been exploited to link an alkyl halide bearing molecule, like BMP, able to trigger, in the presence of an appropriate catalyst system, ATRP process of sodium methacrylate (MANa).

In a previous work, by using a molar ratio between EDA and repeating units of HA-TBA equal to 0.50, we have obtained a HA-EDA derivative with a functionalization in EDA of 50 mol% that subsequently has been derivatized with BMP (45 ± 5 mol%) (Pitarresi et al., 2013).

Here, to increase the amount of EDA linked to HA-EDA and in order to increase also the amount of BMP linked to EDA groups, we have chosen a molar ratio between EDA and repeating units of HA-TBA equal to 0.75 (see Section 2).

Fig. 1 shows the ^{1}H NMR spectrum of HA-EDA in D_2O which allowed to calculate the degree of functionalization in EDA.

In particular, comparing the integral of the peak at δ 1.9 (arrow 1) assigned to protons of $-CH_3$ belonging to N-acetyl-D-glucosamine portion of HA with the integral of the peak at δ 3.1 (arrow 2) assigned to one $-CH_2-$ belonging to EDA residues linked to HA, it was possible calculate a functionalization degree in EDA linked to HA equal to 60 ± 5 mol%.

This copolymer, as TBA salt, has been used for the reaction with BMP in order to prepare the macroinitiator.

First, BMP carboxyl groups have been activated with EDC and NHS in DMSO at 37 °C overnight, by using a molar ratio between activating agents and BMP equal to 1.2. Then, the obtained solution has been added to a 1% (w/v) solution of HA-TBA-EDA in anhydrous DMSO, in the presence of DEA as a catalyst; the molar ratio between activated BMP and HA-TBA-EDA amino groups has been 1.8.

After appropriate purification, the copolymer HA-EDA-BMP has been characterized by ^{1}H NMR analysis (see Fig. 2) that, thanks to the appearance of the peak at δ 1.8 (arrow 3) assigned to protons of $-CH_3$ belonging to BMP, allowed to calculate a functionalization in BMP equal to 55 ± 5 mol%. This means that almost all free amino groups of starting HA-EDA have been functionalized with BMP thus obtaining an optimal macroinitiator to be used for ATRP process.

3.2. Synthesis of HA-EDA-BMP-MANa copolymer through ATRP

In order to introduce carboxyl pendant groups on HA chains to confer a pH sensitive behavior, HA-TBA-EDA-BMP has been used as multifunctional macroinitiator for the polymerization of MANa via ATRP. The molar ratio between MANa and alkyl bromide moieties in HA-TBA-EDA-BMP has been 100.

ATRP has been performed in aqueous environment by using a DMF solution of Bpy and CuBr (molar ratio of 4:1) as a catalyst. The molar ratio between CuBr and BMP groups present in HA-TBA-EDA-BMP was set to 2. The catalyst allows the homolytic cleavage of the C-Br bond in BMP linked to HA thus producing a free radical able to trigger the polymerization of MANa. After 24 h, reaction has been

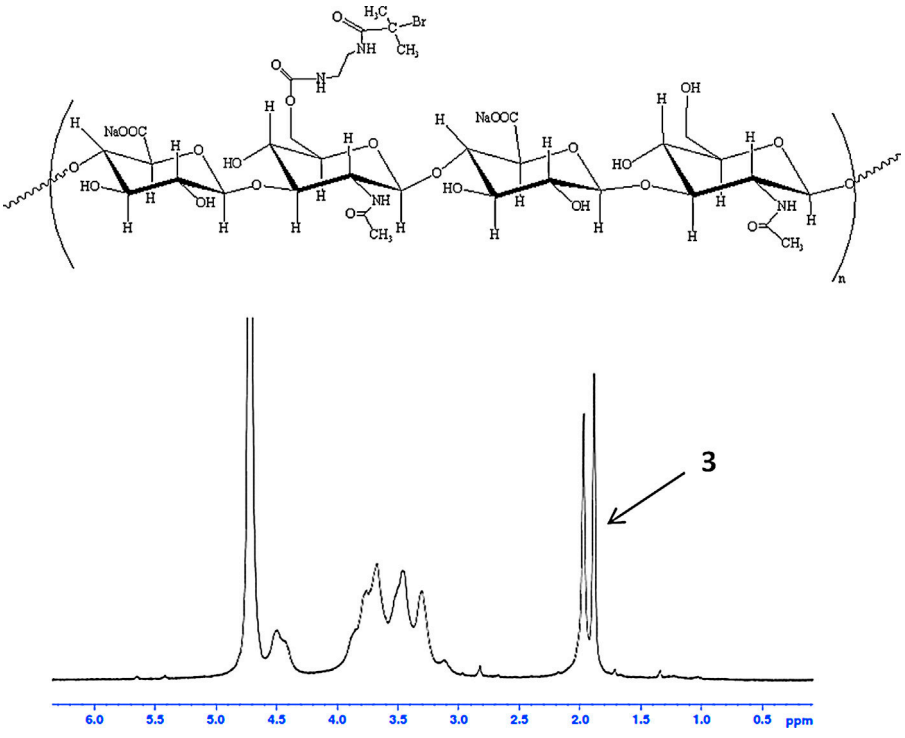


Fig. 2. ^{1}H NMR spectrum of HA-EDA-BMP in D_2O.

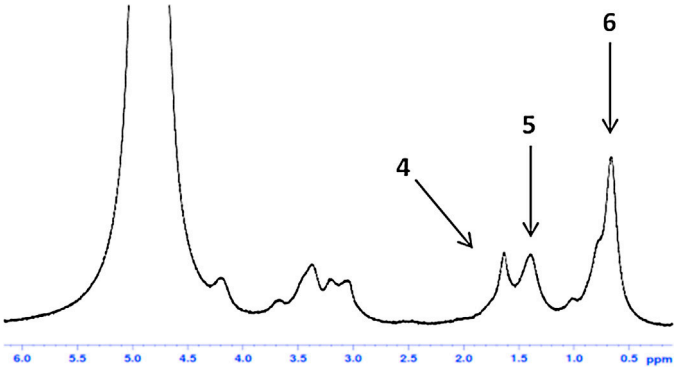


Fig. 3. ^{1}H NMR spectrum of HA-EDA-BMP-MANa in D_2O/NaDO.

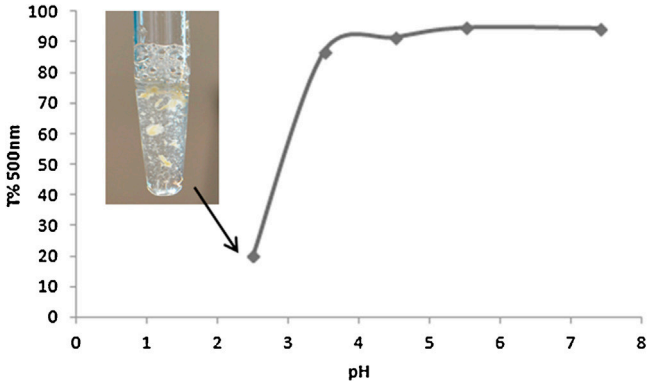


Fig. 5. Values of % of transmittance (*T*%) at 500 nm as a function of the pH of containing HA-EDA-BMP-MANa aqueous solutions.

stopped by simple contact with air oxygen until complete oxidation of copper. The purification of the obtained product has been performed by dialysis against NaCl solution to eliminate the TBA and then against water to eliminate the unreacted sodium methacrylate and exhausted catalyst.

The obtained product, named HA-EDA-BMP-MANa, has been characterized by ^{1}H NMR analysis to determine derivatization degree in MANa linked to starting macroinitiator (DD_{MANa} %) (Fig. 3).

Since at the concentration usually employed for NMR analysis in D_2O, HA-EDA-BMP-MANa gives rise to a very viscous solution, in order to obtain a spectrum easily interpretable, 0.01 N NaOD in D_2O has been used as a solvent. We have observed that this solvent allows to obtain a solution less viscous and therefore more easily usable for the analysis, even if peaks undergo a shift toward higher fields. Indeed, as it can be seen in Fig. 3, the peak assigned to protons of -NH-CO-**CH_3** of HA appears at δ 1.6 (arrow 4) instead of δ 1.9. There is also the appearance of a peak at δ 0.6 (arrow 6) attributable to -C(**CH_3**)$_2$-Br of BMP and -CH_2-C(**CH_3**)- of MANa, that shift their signals after the polymerization process (Pitarresi et al., 2013). Finally, there is the appearance of a peak at δ 1.3 (arrow 5) attributable to -**CH_2**-C(CH_3)- of MANa formed after cleavage of the double bond during polymerization process.

By comparing the integrals of these peaks, it was possible to calculate the derivatization degree in MANa molecules linked to HA-EDA-BMP that resulted to be 125 ± 2 mol%.

The extension of polymerization process of MANa has been expressed by means of the average polymerization number, "*n*" calculated as:

$$n\,\text{average} = \text{derivatization degree in methacrylic acid} / \text{derivatization degree in BMP}$$

Since as discussed in the previous paragraph the derivatization in BMP molecules was 55 ± 5 mol%, the "*n*" average in the final product was about 2.27; this means that the 55% of the repetitive units in HA-EDA-BMP-MANa contains three carboxyl groups (one native and two in side chains due to ATRP of MANa) instead of one (see Fig. 4).

3.3. Turbidimetric analysis

Turbidimetric analysis of HA-EDA-BMP-MANa aqueous solutions with pH ranging from 7.4 to 2.5 has been used to demonstrate the aggregation that occurs in acidic medium as a consequence of protonation of carboxyl groups present in the copolymer. This aggregation causes a precipitation of HA-EDA-BMP-MANa with formation of solid particles able to decrease the transmittance of the sample irradiated at 500 nm.

Fig. 5 shows the trend of the transmittance as a function of the pH of containing HA-EDA-BMP-MANa aqueous solutions.

How it is possible to observe, the transmittance is close to 100% for pH between 7.4 and 3 because the polymer is completely dissolved while when pH value is lowered below 3, transmittance begins to decrease simultaneously with the precipitation of HA-EDA-BMP-MANa. This precipitation is complete at pH lower than 2.5 when the formation of coarse particles produces a suspension (see image in Fig. 5). Therefore HA-EDA-BMP-MANa exhibits a solubility pH dependent and the observed change in solubility is reversible, indeed by raising the pH of the suspension to a value higher than 2.5, the polymer is again soluble.

This pH sensitive behavior suggests a potential use of HA-EDA-BMP-MANa as an innovative gastro-resistant excipient being able to combine the physicochemical properties of typical gastro-resistant polymers with the biological peculiarities of hyaluronic acid.

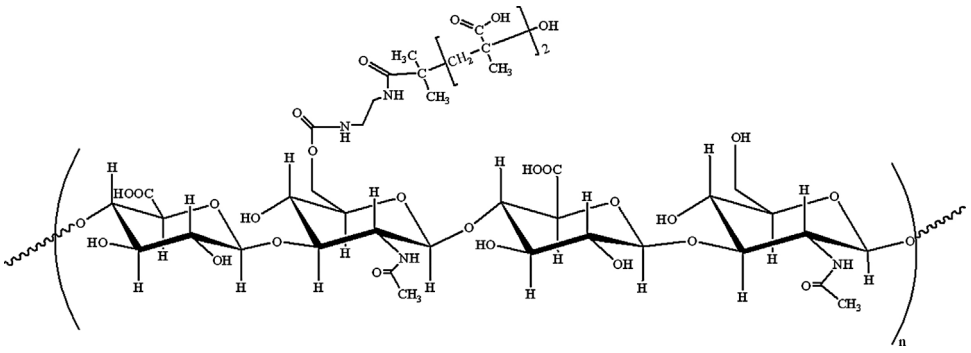


Fig. 4. Chemical structure of HA-EDA-BMP-MANa.

Table 1
Values of molecular weight (Mw) and polydispersity index (PDI), determined by SEC chromatography, for HA-EDA and its obtained derivatives.

Sample	Mw (kDa)	PDI
HA-EDA	260	1.8
HA-EDA-BMP	279	1.3
HA-EDA-BMP-MANa	341	1.5
HA-EDA-BMP-MANa after 2 h at pH 1	335	1.4
HA-EDA after 2 h at pH 1	195	1.6

3.4. SEC characterization

SEC chromatography has been performed on all prepared HA-EDA derivatives, to evaluate their molecular weight (Mw) and polydispersity index (PDI). The values are reported in Table 1.

As expected, the molecular weight of HA-EDA-BMP-MANa is greater than that of the other derivatives, thus confirming the success of ATRP reaction. It is also interesting to observe that the treatment at pH 1 for 2 h causes a decrease in molecular weight of HA-EDA, indeed being soluble in this condition, its chains are exposed to acid degradation. On the contrary HA-EDA-BMP-MANa undergoes only a slight reduction in molecular weight; this result suggests that the precipitation of HA-EDA-BMP-MANa at low pH value, protects it from acidic degradation.

3.5. Incorporation and activity evaluation of α-chymotrypsin

In order to determine if HA-EDA-BMP-MANa derivative can be exploited for the incorporation and protection of bioactive molecules that usually are inactivated in the gastric environment, α-chymotrypsin has been chosen as a model of a protein molecule. It is a proteolytic enzyme, degraded in acidic medium, indeed it is physiologically secreted by the pancreas into the small intestine and its optimal pH activity is between 7 and 9.

First as a negative control, we have evaluated the activity of free α-chymotrypsin in HCl 0.1 N at 37 °C as a function of time and how it is possible to observe in Fig. 6, in simulated gastric environment, there is a drastic decrease in its activity that comes almost zero after 2 h, i.e. the average time of residence in the stomach.

Therefore, our aim has been to investigate if at low pH values, HA-EDA-BMP-MANa is able to retain the enzyme protecting it from the acid degradation, thus preserving its activity.

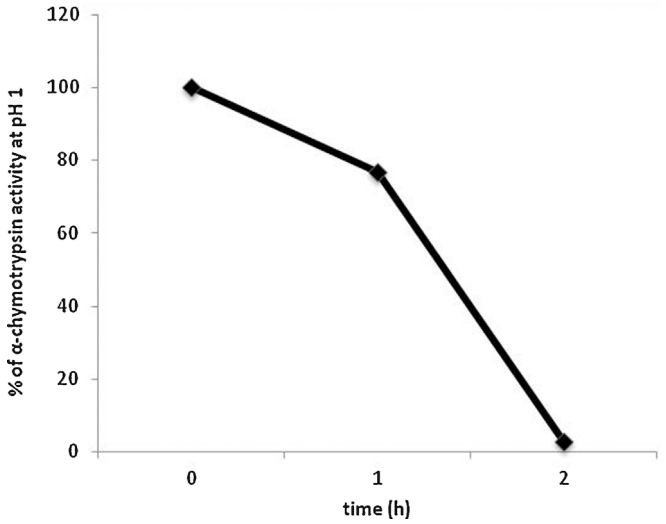


Fig. 6. Percentage of activity of free α-chymotrypsin at pH 1 and 37 °C as a function of time.

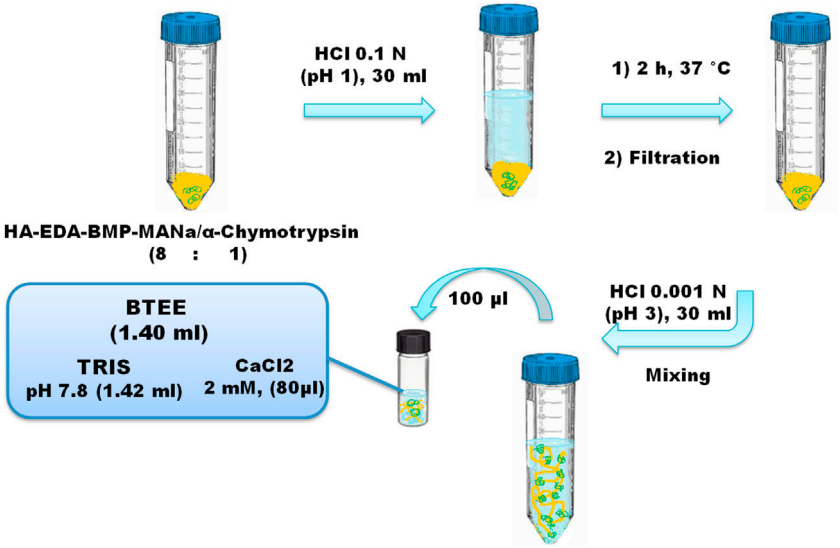
For this reason, α-chymotrypsin has been dissolved in TRIS buffer pH 7.8 and this solution has been used to dissolve HA-EDA-BMP-MANa; the resulting solution has been freeze-dried to obtain a solid that can be stored at −20 °C until use.

To mimic the gastric conditions, the freeze-dried polymer/enzyme solid has been kept in HCl 0.1 N for 2 h at 37 °C under gentle stirring, then to evaluate if the potentially incorporated enzyme is still active, the solid has been recovered by filtration, dissolved in HCl 0.001 N and tested at pH 7.8 through BTEE assay, chosen as a specific substrate for α-chymotrypsin (see Scheme 1).

By evaluating the increase in absorbance at 256 nm of a solution containing the enzyme and the substrate BTEE, as a function of time, it is possible to determine the amount of active α-chymotrypsin expressed as active units per ml.

Fig. 7 shows the percentage of enzymatic activity determined by BTEE assay.

How it is possible to observe, the activity of the enzyme entrapped by HA-EDA-BMP-MANa is 79 ± 4% respect to the positive control, i.e. a solution of free α-chymotrypsin incubated for 2 h at



Scheme 1. Schematic representation of procedure adopted to determine by BTEE assay the activity of α-chymotrypsin in the presence of HA-EDA-BMP-MANa after treatment in simulated gastric conditions.

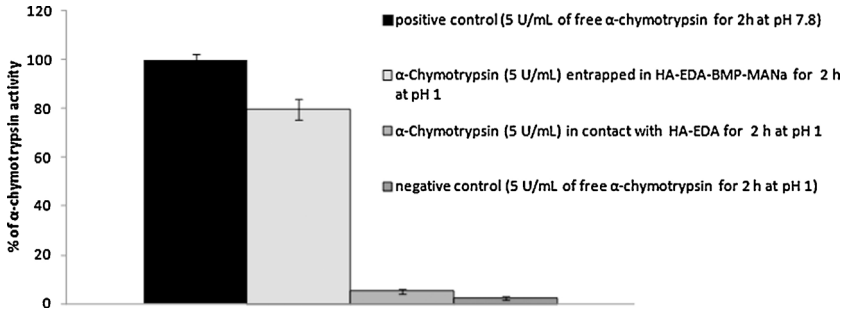


Fig. 7. Percentage of activity of α-chymotrypsin in the presence of HA-EDA-BMP-MANa or HA-EDA after treatment at pH 1 for 2 h. Positive and negative controls are also shown.

pH 7.8, whereas enzyme alone (negative control) or in the presence of HA-EDA and incubated for 2 h at pH 1 is almost inactive.

Since enzyme activity in the presence of HA-EDA-BMP-MANa is less than 100%, it could be supposed that α-chymotrypsin has lost part of its activity or that an amount of enzyme is released in the medium during the incubation at pH 1. On the other hand, the assay with BTEE allows to evaluate only the amount of active enzyme, and not the total amount of protein entrapped by HA-EDA-BMP-MANa. Therefore, in order to determine the total amount of enzyme that remains entrapped by HA-EDA-BMP-MANa after 2 h at pH 1, a HPLC-SEC analysis has been performed (see Section 2). Since the value found by HPLC-SEC analysis corresponds to 78% of starting amount of enzyme, according to the value obtained with BTEE assay, this result confirms that all entrapped enzyme maintains its activity thanks to the protection due to HA-EDA-BMP-MANa.

3.6. Cell compatibility studies

Cell compatibility has been evaluated by performing a MTS assay on murine dermal fibroblasts, chosen as model cells, in contact with HA-EDA-BMP-MANa. In particular, HA-EDA-BMP-MANa has been kept in direct contact for 24 and 48 h with cells by dissolving it in the culture medium at a concentration equal to 0.1 and 0.5 mg/ml. Results shown in Fig. 8A shows that cell viability is not influenced by the presence of HA-EDA-BMP-MANa for both investigated polymer concentrations and contact times, thus demonstrating that chemical modifications of HA, i.e. the derivatization with EDA, BMP and

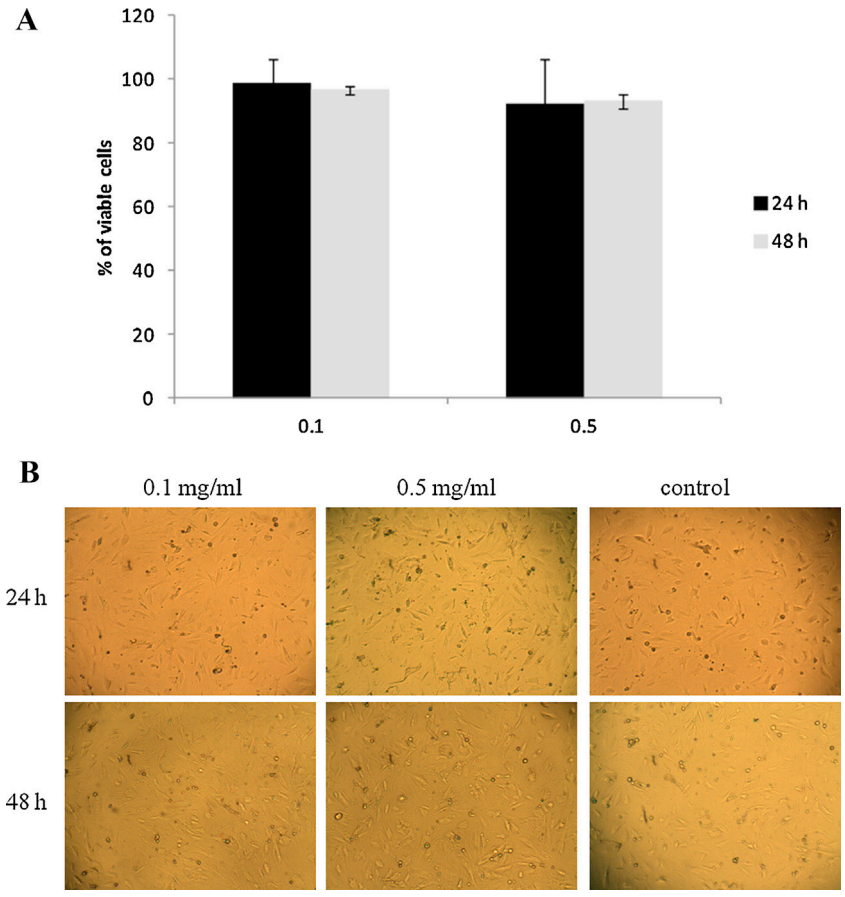


Fig. 8. Viability, expressed as percentage of viable cells compared to the positive control (absorbance read at 492 nm, MTS assay) (A) and optical images (B) of murine dermal fibroblasts after 24 and 48 h of contact with a solution of HA-EDA-BMP-MANa at 0.1 and 0.5 mg/ml.

final ATRP of MANa have not modified cell compatibility of starting polysaccharide.

Besides viability, also cell morphology does not undergo alteration indeed it is evident the spindle like shape of fibroblasts like the positive control (see images in Fig. 8B).

4. Conclusions

A new pH sensitive derivative of hyaluronic acid has been produced by ATRP. By exploiting free amino groups of HA-EDA, through a simple and reproducible chemistry, we have obtained a macroinitiator (HA-EDA-BMP) able to trigger the polymerization of sodium methacrylate (MANa) via ATRP process.

The obtained HA-EDA-BMP-MANa showed a pronounced pH sensitive behavior, indeed thanks to the presence of carboxyl groups introduced with MANa, it is able to precipitate in a reversible manner at pH below 2.5. This property has been exploited for the incorporation of α-chymotrypsin, chosen as an example of protein molecule inactivated in acid medium.

We have demonstrated that HA-EDA-BMP-MANa is able to entrap the enzyme and to preserve its activity in simulated gastric conditions. It has also been observed that HA-EDA-BMP-MANa does not affect the viability of murine dermal fibroblasts, chosen as model cells, thus demonstrating the cell compatibility of the polymer itself.

Thanks to these characteristics, HA-EDA-BMP-MANa appears as an interesting material for the production of micro or nano oral drug delivery systems that could combine pH sensitivity with biological properties of HA, like interaction with CD44 receptors over expressed by colon tumoral cells.

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