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New hyaluronic acid based brush copolymers synthesized by atom transfer radical polymerization

Giovanna Pitarresi[a,b], Calogero Fiorica[a], Mariano Licciardi[a], Fabio Salvatore Palumbo[a], Gaetano Giammona[a,c,*]

[a] Dipartimento di Scienze e Tecnologie Molecolari e Biomolecolari (STEMBIO), Sezione di Chimica e Tecnologie Farmaceutiche, Università degli Studi di Palermo, Via Archirafi, 32, 90123, Palermo, Italy
[b] IBIM-CNR, Via Ugo La Malfa 153, 90146 Palermo, Italy
[c] Institute of Biophysics, Italian National Research Council, Via Ugo La Malfa 153, 90146 Palermo, Italy

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ABSTRACT

In this work, an efficient method for the synthesis of hyaluronic acid based brush copolymers using atom transfer radical polymerization (ATRP) has been reported. At first, two different hyaluronic acid (HA) based macroinitiators have been prepared and then they have been used for the polymerization via ATRP of hydrophilic or hydrophobic molecules carrying vinyl portions.

In particular, by linking 2-bromo-2-methylpropionic acid (BMP) to the primary hydroxyl groups of tetrabutyl ammonium salt of HA (HA–TBA) or to amino groups of the ethylenediamino derivative of HA–TBA (HA–TBA–EDA), two macroinitiators (HA–TBA–BMP and HA–TBA–EDA–BMP) have been obtained. Then they have been used for the ATRP of poly(ethylene glycol) methacrylate (PEGMA), butyl methacrylate (BUTMA) or N-isopropylacrylamide (NIPAM) using a complex of Cu(I) and 2,2′-Bipyridyl (Bpy), as a catalyst.

Both macroinitiators and final copolymers, named as HA–BMP–pPEGMA, HA–BMP–pBUTMA, HA–BMP–pNIPAM, HA–EDA–BMP–pPEGMA, HA–EDA–BMP–pBUTMA and HA–EDA–BMP–pNIPAM, have been characterized by spectroscopic analysis and size exclusion chromatography to confirm the success of the polymerization process.

1. Introduction

Hyaluronic acid (HA) is a naturally occurring linear polysaccharide consisting of alternating disaccharide units of α-1,4-D-glucuronic acid and β-1,3-N-acetyl-D-glucosamine (Knudson & Knudson, 2001) that can be found in connective tissues such as umbilical cord, synovial fluid, vitreous, etc. (Laurent, 1970).

As a major component of the extracellular matrix (ECM), HA has been recognized as an important molecule to control cellular differentiation and proliferation as well as the inflammatory response and cellular motility (Fraser,Laurent, & Laurent, 1997;Turley, 1989).

Thanks to its peculiar properties, in the last decades, HA derivatives have been extensively employed for the production of drug delivery systems (Pitarresi et al., 2010; Pitarresi, Craparo, Palumbo, Carlisi, & Giammona, 2007) and scaffolds for tissue engineering (Ji et al., 2006; Palumbo et al., 2012).

However, the pronounced hydrophilic character of HA and its polyanionic nature do not promote cell attachment and subsequent tissue formation (Shu, Liu, Palumbo, & Prestwich, 2003), therefore there is the necessity to modify its chemical structure with molecules able to increase affinity toward cells thus allowing their adhesion and differentiation in the case of tissue engineering. On the other hand, the derivatization of HA with lipophilic molecules can produce amphiphilic derivatives able to self assemble in aqueous medium and to entrap poorly water soluble drugs, thus allowing their administration and modified release in physiological fluids.

These chemical modifications have been mostly performed on HA through grafting techniques and, in despite of several works published in this field by employing “grafting to” technique, the control of molecular weight (MW) and polydispersity index (PDI) is still a major issue that need to be addressed since these two parameters affect the chemical, physical and biological properties of the final product.

Atom Transfer Radical Polymerization (ATRP), discovered by Matyjaszewski and Sawamoto in 1995 (Wang & Matyjaszewski,

* Corresponding author at: Dipartimento di Scienze e Tecnologie Molecolari e Biomolecolari, Sezione di Chimica e Tecnologie Farmaceutiche,Università degli Studi di Palermo, Via Archirafi 32, 90123, Palermo, Italy. Tel.: +39 09123891928; fax: +39 09123891960.
E-mail address: gaetano.giammona@unipa.it (G. Giammona).

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1995), is a versatile controlled radical polymerization (CRP) process. It enables a precise control of MW, PDI and functionality (Coessens, Pintauer, & Matyjaszewski, 2001).

In this technique a transition metal complexed by an appropriate ligand is used as a catalyst for the reaction between an alkyl halide initiator and a vinyl monomer. The reaction can be carried out in a variety of solvents and conditions, including water at room temperature. ATRP has been exploited as homogeneous/heterogeneous solution polymerization technique, as well as "growing from" polymerization technique for molecule in solution, surfaces, proteins, organic and inorganic materials (Cavallaro, Licciardi, Di Stefano, Pitarresi, & Giammona, 2009;Pyun & Matyjaszewski, 2001).

Because ATRP can provide site-specific grafting to a variety of surfaces (essentially any surface containing an ATRP initiator), it is a useful method for preparing polymer-grafted materials (Siegwart & Matyjaszewski, 2012).

Already other research groups have worked in the production of polysaccharide macroinitiators to be employed in the ATRP process. For example, Meng et al. developed a cellulose based macroinitiator for the ATRP process of methyl methacrylate and styrene (Meng et al., 2009).

The aim of this work was to demonstrate that it is possible to employ ATRP as "growing from" technique to obtain HA derivatives with a narrow and controlled molecular weight distribution, both characteristics very important for a potential biomedical or pharmaceutical use of these materials.

The synthesis has been performed by using two subsequent steps. In the first step, two macroinitiators have been obtained by the conjugation of a proper number of 2-bromo-2-methylpropionic acid (BMP) to hyaluronic acid (as tetrabutyl ammonium salt: HA–TBA) or to ethylenediamino derivative of HA (HA–TBA–EDA). In the second step, HA–TBA–BMP and HA–TBA–EDA–BMP copolymers have been used as "multi-functional macroinitiators" for the polymerization via ATRP of poly(ethylene glycol) methacrylate (PEGMA), butyl methacrylate (BUTMA) or N-isopropylacrylamide (NIPAM) chosen as model molecules carrying vinyl groups affordable to the polymerization process and with hydrophilic (for PEGMA) or hydrophobic (for BUTMA and NIPAM) properties. Spectroscopic analysis and size exclusion chromatography have been used to verify the success of ATRP process in the production of new brush copolymers named as HA–BMP–pPEGMA, HA–BMP–pBUTMA, HA–BMP–pNIPAM, HA–EDA–BMP–pPEGMA, HA–EDA–BMP–pBUTMA and HA–EDA–BMP–pNIPAM that could be used as starting materials for preparing drug delivery systems or scaffolds for tissue engineering.

2. Experimental

2.1. Materials and methods

All reagents were of analytical grade unless otherwise stated.

Dimethylsufoxide (DMSO), acetone, 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), poly(ethylene glycol) methacrylate (PEGMA) with a molecular weight of 360 Da, butyl methacrylate (BUTMA), N-isopropylacrylamide (NIPAM), copper bromide [Cu(I)Br], 2,2′-bipyridyl (Bpy), deuterium oxide (D_2O), dimethyl sulfoxide-d_6 (DMSO d_6), N,N-dimethylformamide-d_7 ($DMFd_7$) and chloroform-d ($CDCl_3$) 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. (Shu, Liu, Luo, Roberts, & Prestwich, 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 1N. 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, Pitarresi, Mandracchia, Tripodo, & Giammona, 2006).

Hydroquinone, the stabilizing agent used in the commercial available PEGMA and BUTMA, was eliminated through basic activated aluminum oxide (Fluka) column.

Weight–average molecular weight (Mw) and polydispersity index (Mw/Mn) of starting HA 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 (pH6.5):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. (Ferguson, Alshame, & Thomas, 2010).

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

^1H NMR and Heteronuclear Multiple Quantum Coherence (HMQC) spectra were obtained by dissolving the samples in D_2O, $CDCl_3$ or mixture D_2O/$DMSOd_6$ or D_2O/$DMFd_7$ with a Brucker AC-300 instrument.

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 121 mg 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 0.50). 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 acetone then washed in the same solvent and dried under vacuum. The derivatization degree in terms of moles of EDA linked to HA (DD_{EDA}%), calculated by ^1H NMR analysis, was 50 mol%.

2.3. Synthesis of HA-based macroinitiators

The synthesis of HA–TBA–BMP and HA–TBA–EDA–BMP macroinitiators was performed as follows: 30 mg BMP were dissolved in anhydrous DMSO and, in order to activate the carboxyl group, 20 mg of EDC and 11.5 mg of NHS were added (molar ratio between EDC/NHS and BMP equal to 1). The obtained solution was stirred at 37 °C overnight then added to a 1% (w/v) solution of HA–TBA or HA–TBA–EDA in anhydrous DMSO, in the presence of DEA as a catalyst (molar ratio between DEA and primary hydroxyl groups of HA–TBA or amino groups of HA–TBA–EDA equal to 1). It was used a molar ratio between BMP–NHS and primary hydroxyl

groups of HA–TBA or amino groups of HA–TBA–EDA equal to 1.2. The reaction was carried out for 24 h at 40 °C, the products were isolated by precipitation in acetone, purified by several washings in the same solvent and recovered after drying under vacuum, then characterized by FT-IR analysis.

For ^{1}H NMR characterization of the obtained macroinitiators, in order to obtain easily interpretable spectra, TBA was eliminated from the final products as follow: samples were 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 products were recovered by freeze drying.

2.4. ATRP of BUTMA, PEGMA and NIPAM using HA–TBA–BMP or HA–TBA–EDA–BMP as macroinitiators

50 mg of HA–TBA–BMP or HA–TBA–EDA–BMP were dissolved in 5 ml of twice distilled water, then PEGMA, BUTMA or NIPAM was added in order to obtain a molar ratio between vinyl monomer and BMP present in the macroinitiator equal to 25.

The resultant solution was kept under Argon bubbling, until the catalyst solution was added. This has been obtained by dissolving Bpy and CuBr (molar ratio of 4:1) in anhydrous DMF. The molar ratio between CuBr and BMP groups present in each macroinitiator was set to 1.2.

The reaction solutions were 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 added dropwise into acetone and the resulting solid residue was washed five times in acetone and dried under vacuum. To eliminate the TBA from the final products, samples were 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 products were recovered by freeze drying and characterized by HMQC analysis and size exclusion chromatography (SEC).

The yield for the products obtained starting from HA–TBA–BMB macroinitiator ranged from 90 to 100% while the yield for the products obtained starting from HA–TBA–EDA–BMP macroinitiator ranged from 110 to 150%.

2.5. Degradation of HA brush copolymer and ^{1}H NMR analysis of side chains obtained after ATRP

For the degradation of HA backbone, copolymers obtained through ATRP were treated with a NaOH 2 N solution for 24 h at 37 °C.

The side chains were extracted in dichloromethane by a separating funnel and the organic solvent was removed by under vacuum evaporation. The products extracted were dissolved in D_2O or ($CDCl_3$) (for butyl methacrylate) in order to be analyzed by ^{1}H NMR.

Every time the extracted products were compared to the corresponding starting monomer employed in the ATRP.

3. Results and discussion

3.1. Synthesis of hyaluronic acid-ethylenediamine (HA–TBA–EDA) derivative and production of HA-based macroinitiators

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) in organic solvent (DMSO). This reaction has been carried out in two steps: (1) activation of primary hydroxyl groups of HA–TBA in DMSO by using bis(4-nitrophenyl) carbonate (4-NPBC) as a carbonating agent; (2) reaction between activated hydroxyl groups of HA–TBA with EDA through a nucleophilic substitution (Giammona, Palumbo, & Pitarresi, 2010).

In a previous work (Palumbo et al., 2012), we have demonstrated that this reaction is almost quantitative, indeed the degree of derivatization in EDA corresponds to the starting amount of 4-NPBC activated hydroxyl groups. Therefore, since in this work it has been used a molar ratio between 4-NPBC and HA–TBA equal to 0.5, the degree of derivatization in EDA linked to HA, resulted to be 50 mol%.

The possibility to introduce amino functionalities to HA is very important, because their nucleophilic character (greater than that of hydroxyl groups) can be exploited to synthesize other HA derivatives when a nucleophilic substitution with an appropriate reagent is involved. For example, this amino derivatization can be useful for producing HA based macroinitiators able to trigger, in the presence of an appropriate catalyst system, ATRP process of different vinyl monomers.

Therefore, in order to produce HA based macroinitiators, in this paper we have exploited the reaction of nucleophilic substitution of HA–TBA primary hydroxyl groups or HA–TBA–EDA amino groups toward the N-hydroxysuccinimide activated 2-Bromo-2-methylpropionic acid (BMP–NHS) (see experimental). In this way it was possible to produce two different HA based derivatives (HA–TBA–BMP and HA–TBA–EDA–BMP), that, thanks to the presence of alkyl bromide functional groups, can act as macroinitiators to trigger the ATRP process.

The synthesis of HA–TBA–BMP and HA–TBA–EDA–BMP macroinitiators has been performed in DMSO in two steps: (1) activation of carboxyl group of BMP with EDC and NHS, by using a molar ratio between EDC (or NHS) and BMP equal to 1; (2) addition of the organic solution of BMP–NHS to a 1% w/v solution of HA–TBA or HA–TBA–EDA, in the presence of DEA as a catalyst. The molar ratio between DEA and primary hydroxyl groups of HA–TBA or amino groups of HA–TBA–EDA was equal to 1.

Scheme 1 shows the synthesis of these HA-based macroinitiators.

Both macroinitiators, after appropriate purification by several washings in acetone and drying under vacuum, have been characterized by spectroscopic analysis and size exclusion chromatography.

Fig. 1 shows FT-IR spectrum of HA–TBA–BMP in comparison with that of HA–TBA, that confirms the success of reaction between HA–TBA and activated BMP. Indeed the peak at 1734 cm^{-1} in the spectrum of the macroinitiator HA–TBA–BMP is attributable to the

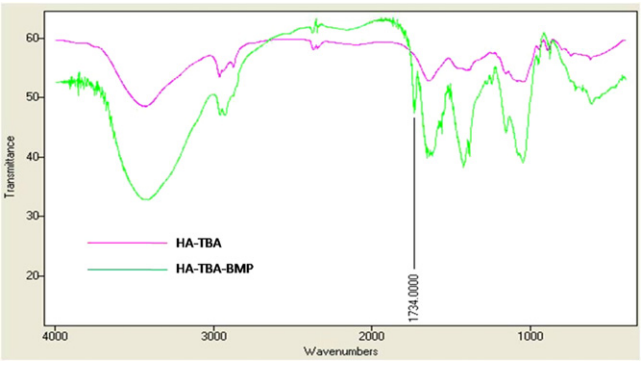
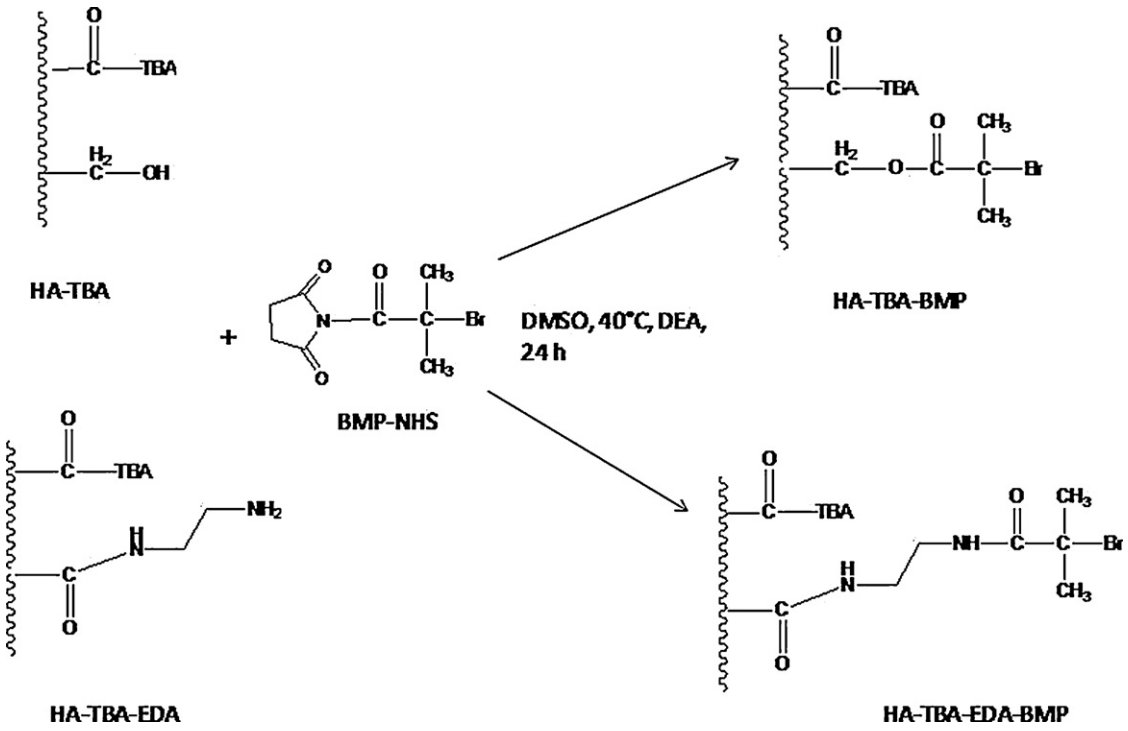


Fig. 1. FT-IR spectra of HA–TBA and HA–TBA–BMP.



Scheme 1. Schematic representation of the synthesis of HA-based macroinitiators.

ester bond formed after the coupling reaction (see chemical structure in Fig. 2).

Fig. 2 shows the chemical structure and the ^{1}H NMR spectrum of HA–BMP macroinitiator (TBA has been eliminated to give a more simple spectrum).

The derivatization degree % in BMP linked to HA–TBA (DD_{BMP}%) has been determined by

^{1}H NMR in D_2O comparing the integral of the peak at δ 1.8 assigned to protons of $-CH_3$ belonging to BMP residues linked HA (A) with the integral of the peak at δ 1.9 of acetamido group of N-acetyl-D-glucosamine (B). The (DD_{BMP}%) was equal to 18 ± 2 mol%.

With regard to HA–TBA–EDA–BMP, the FT-IR analysis was not diagnostic since the peak of new amide linkage (formed between HA–TBA–EDA and BMP, see chemical structure in Fig. 3) was indistinguishable from the amide bond of N-acetyl-D-glucosamine portion of HA.

However the ^{1}H NMR spectrum of HA–TBA–EDA–BMP showed the appearance of a peak at

δ 1.8 attributable to $-CH_3$ protons of BMP residues; this peak has been used to calculate the degree of derivatization in BMP (see Fig. 3). This value resulted to be 45 ± 5 mol%, therefore, considering that the degree of functionalization in EDA linked to HA was equal to 50 mol%, the derivatization in BMP corresponds almost to 100% of functionalization of free amino groups present in HA–TBA–EDA.

Moreover, how it is possible to observe in Fig. 3, the peak at δ 3.1 (see arrow) in the spectrum of HA–EDA, assigned to methylene groups of ethylenediamine, disappears in the spectrum

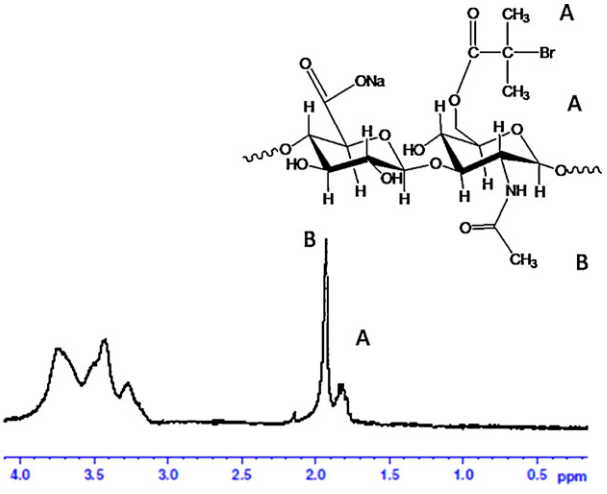


Fig. 2. Chemical structure and ^{1}H NMR spectrum (in D_2O) of HA–BMP macroinitiator.

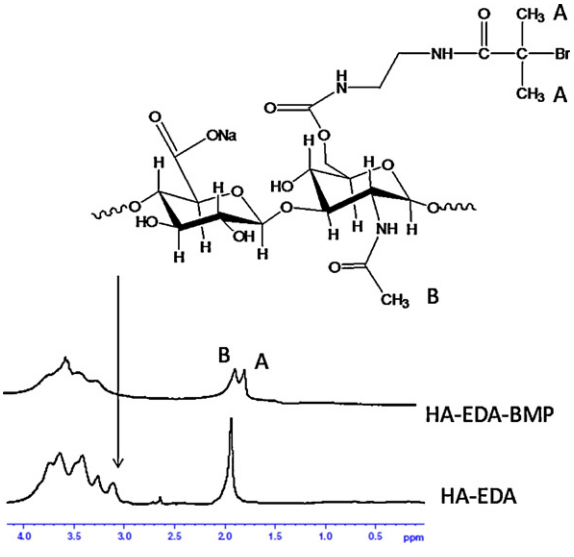
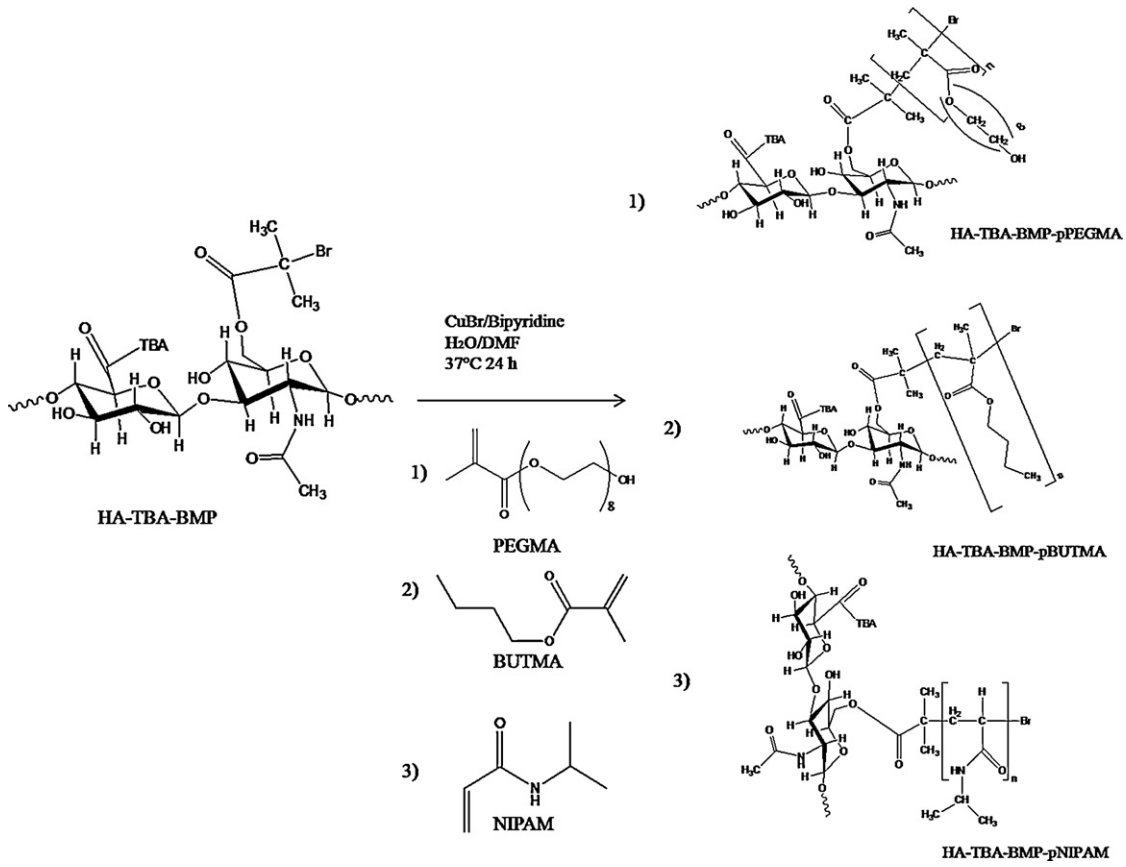


Fig. 3. Chemical structure and ^{1}H NMR spectrum(in D_2O) of HA–EDA–BMP macroinitiator and ^{1}H NMR spectrum (in D_2O) of HA–EDA.



Scheme 2. ATRP procedure for the production of HA brush copolymers from HA–TBA–BMP and different vinyl monomers.

of HA–EDA–BMP because of the shift and overlapping with pyranosyl HA protons. This shift suggests that almost all free amino groups in HA–EDA–TBA have been functionalized with BMP.

Therefore, as expected, obtained results showed that the reactivity of free amino groups in HA–TBA–EDA, higher than that of primary hydroxyl groups in HA–TBA, allows the production of a HA derivative with a greater amount in BMP residues that can act as initiators for ATRP process.

3.2. ATRP using HA–TBA–BMP or HA–TBA–EDA–BMP as macroinitiators

Both HA–TBA–BMP or HA–TBA–EDA–BMP have been used as "multifunctional macroinitiators" for the polymerization in the side chain, via ATRP, of hydrophilic molecules, such as poly(ethylene glycol) methacrylate (PEGMA) with a molecular weight of 360 Da, or hydrophobic monomers such as butyl methacrylate (BUTMA) and N-isopropylacrylamide (NIPAM). ATRP of PEGMA and NIPAM, by using other macroinitiators, has been already carried out in aqueous environment or complex aqueous environment (Mauricio et al., 2011; Zhang et al., 2011) while no example has been reported for ATRP of BUTMA in this medium.

The molar ratio between PEGMA, BUTMA or NIPAM and alkyl bromide moieties in HA–TBA–BMP was set to 25, this value represents the theoretical number of monomers that could polymerize for each mole of BMP present in the side chain of the macroinitiator.

ATPR was performed at 37 °C for 24 h in twice distilled water, under Argon bubbling and 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 each macroinitiator was set to 1.2. The reaction is stopped by simple contact with air oxygen until the complete oxidation of copper occurs. Each product has been recovered by freeze drying, after appropriate purification (see experimental).

Scheme 2 shows the synthetic procedure for the production of HA brush copolymers obtained starting from HA–TBA–BMP and different vinyl monomers.

All samples, named as HA–BMP–pBUTMA, HA–BMP–pNIPAM and HA–BMP–pPEGMA have been characterized by ^{1}H NMR analysis to determine derivatization degree in vinyl monomers (VM) linked to starting macroinitiator (DD_{VM} %).

Fig. 4 shows as an example the HMQC analysis of HA–BMP–pPEGMA where it is possible to notice that, in the ^{1}H NMR spectrum, compared to the HA–BMP one (see Fig. 2), there is a shifting of the signal attributable to protons of $-CH_3$ (A) of the BMP moieties from δ 1.8 to δ 0.8. This shifting is attributable to the changes in the molecule after the polymerization process.

In addition there is a perfect match between peaks in ^{1}H NMR and those in ^{13}C NMR.

The DD_{VM} % has been calculated by comparing the signals (^{1}H NMR) reported in Table 1 for each linked monomer with the signal at δ 1.9 of acetamido group of N-acetyl-D-glucosamine.

Moreover, Fig. 4 also shows the SEC chromatogram of HA–BMP–pPEGMA compared with that the starting macroinitiator; it is evident that, according to the increase in molecular weight, the elution time of the ATRP product is lower than that of HA–BMP.

In the case of HA–BMP–pBUTMA or HA–BMP–pNIPAM, the ^{1}H NMR signals attributable to the methacrylic portion of BUTMA or to the isopropylic portion of NIPAM are overlapped to the peak of BMP methyl groups. For this reason to calculate the DD_{VM} %, the contribution of the BMP six protons was subtracted from the integration of total peak.

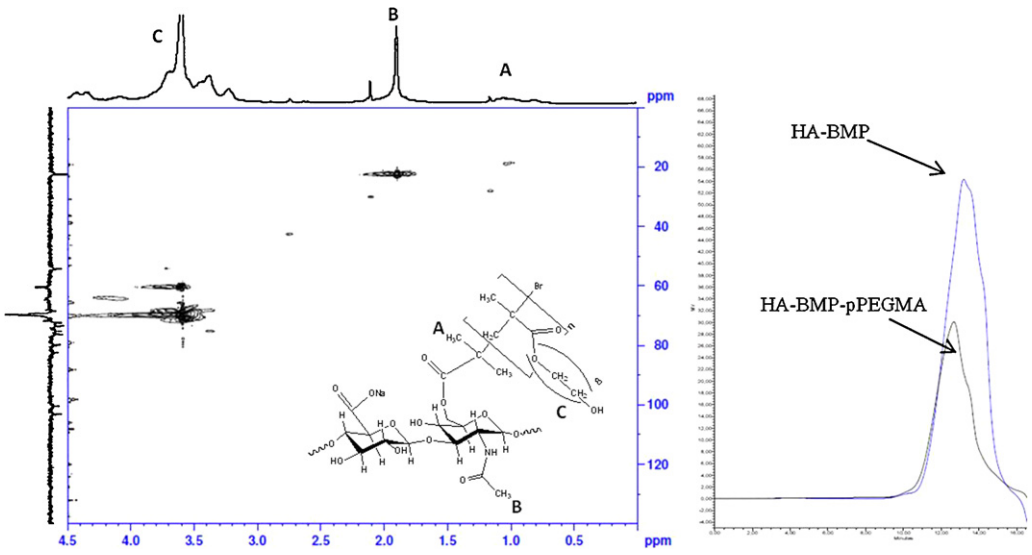


Fig. 4. HMQC analysis (in D_2O) of HA–BMP–pPEGMAand SEC chromatograms of HA–BMP–pPEGMA and HA–BMP.

Table 1
DD_{VM} % and n average, calculated by ^1H NMR, of ATRP products obtained employing HA–TBA–BMP as a macroinitiator and PEGMA, BUTMA or NIPAM as vinyl molecules.

Sample	δ of linked molecule peak	DD_{VM}%	*n* average	Solvent
HA–BMP–pPEGMA	3.6 $-CH_2-$ of PEG	35%	1.94	D_2O
HA–BMP–pBUTMA	1.2 $-CH_3$ of BUTMA	30%	1.66	D_2O/DMSO d_6
HA–BMP–pNIPAM	1.2 $-CH_3$ of NIPAM	38%	2.11	D_2O/DMF d_7

The extension of polymerization process of vinyl monomers was expressed by means of the average polymerization number, "*n*" calculated as:

$$n \text{ average} = \frac{\text{derivatization degree in vinyl monomers}}{\text{derivatization degree in BMP}}$$

Data reported in Table 1, show that by employing HA–TBA–BMP as a macroinitiator, ATRP process of investigated vinyl monomers is scarce. Indeed, the analysis of "*n* average" values shows that in all cases, less than two monomers polymerize from each bromide residue in the polymer side chain. This means that polymerization reaction switches off sooner after the linkage of the first vinyl molecule.

Differently, better results have been obtained by employing HA–TBA–EDA–BMP as a starting macroinitiator.

Scheme 3 shows the synthetic procedure for the production of HA–EDA brush copolymers obtained starting from HA–TBA–EDA–BMP and different vinyl monomers.

For all obtained samples, DD_{VM} % has been calculated by comparing the signals reported in Table 2 with the signal at δ 1.9 of acetamido group of N-acetyl-D-glucosamine.

It is evident that, when HA–TBA–EDA–BMP is used as a macroinitiator, for all employed monomers, the DD_{VM}% is greater than that found when HA–TBA–BMP was used and the value of n average is almost doubled, thus suggesting that in this case, ATRP process was more efficient.

Fig. 5 shows as an example the HMQC analysis of HA–EDA–BMP–pNIPAM where it is possible to see, in the ^1H NMR spectrum, a peak at δ 1.2 (A) attributable to $-CH_3$ isopropyl groups of NIPAM and to BMP methyl groups.

Also, in the SEC chromatogram reported in Fig. 5 for HA–EDA–BMP–pNIPAM in comparison with that of HA–EDA–BMP, the peak shifting confirms the increase in the molecular weight after ATRP process thus meaning that the polymerization process takes place.

Again, for this product there is a perfect match between peaks in ^1H NMR and those in ^{13}C NMR.

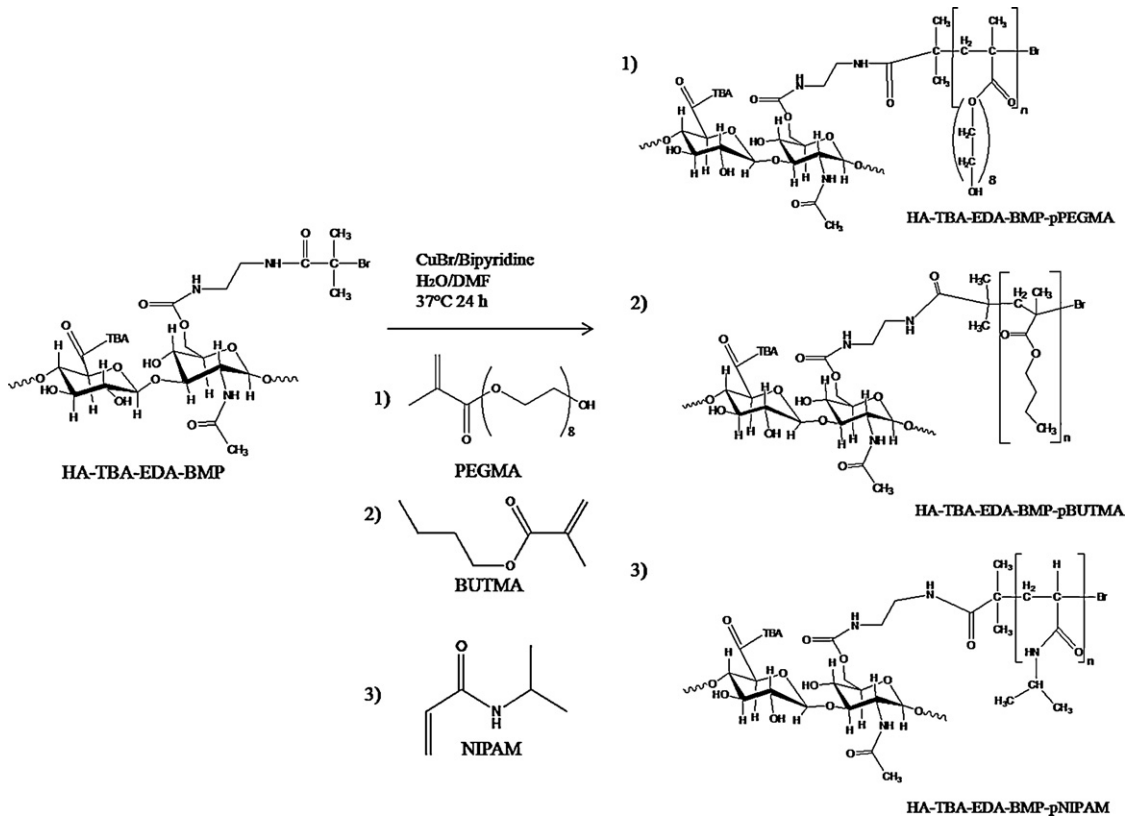
The differences observed using two different macroinitiators could be explained taking into account the HA radical scavenger property. Indeed, as reported by Sakurai et al. (Sakurai et al., 1997), in physiological conditions HA reacts and eliminates free radicals. Probably, for HA–TBA–BMP macroinitiator, the proximity between the polysaccharide chain and the radical, produced by the homolytic rupture of the alkyl bromide bond, inhibits the reactivity toward vinyl monomers, while for HA–EDA–TBA–BMP macroinitiator, ethylenediamino groups could act as spacers, thus determining a greater reactivity of the radical and the major propagation of the polymerization process.

A gel permeation chromatography has been performed for all obtained samples to evaluate their molecular weight and polydispersity index (PDI). Values are reported in Table 3.

As expected, the molecular weight increases after the ATRP reaction, in according with the different molecular weight of vinyl monomers used for the reaction, their derivatization degree and polymerization extension. In any case, it is evident that ATRP on HA

Table 2
DD_{VM} % and n average, calculated by ^1H NMR, of ATRP products obtained employing HA–TBA–EDA–BMP as a macroinitiator and PEGMA, BUTMA or NIPAM as vinyl molecules.

Sample	δ of linked molecule peak	DD_{VM}%	*n* average	Solvent
HA–EDA–BMP–pPEGMA	3.6 $-CH_2-$ of PEG	145%	3.62	D_2O
HA–EDA–BMP–pB0UTMA	1.2 $-CH_3$ of BUTMA	130%	3.25	D_2O/DMSO d_6
HA–EDA–BMP–pNIPAM	1.2 $-CH_3$ of NIPAM	163%	4.08	D_2O/DMF d_7



Scheme 3. ATRP procedure for the production of HA–EDA brush copolymers from HA–TBA–EDA–BMP and different vinyl monomers.

based macroinitiators allowed to obtain brush copolymers with a narrow polydispersity index.

3.3. Degradation of HA brush copolymer and ^{1}H NMR analysis of side chains obtained after ATRP

As a further confirmation of the occurrence of ATRP process, the obtained products were degraded in order to isolate the side chains grown on macroinitiators (HA–TBA–BMP or HA–TBA–EDA–BMP).

By treating ATRP products with a solution of NaOH 2N for 24 h at 37 °C, the degradation of the polysaccharide portion occurs together with the breaking of the ester or amide bond between HA or HA–EDA and BMP moieties that bring the ATRP products.

The extraction with dichloromethane allows to recover only the chains linked to the initial macroinitiator that were analyzed by ^{1}H NMR analysis and compared to the corresponding monomer employed in the ATRP. Figs. 6, 7 and 8 report as examples

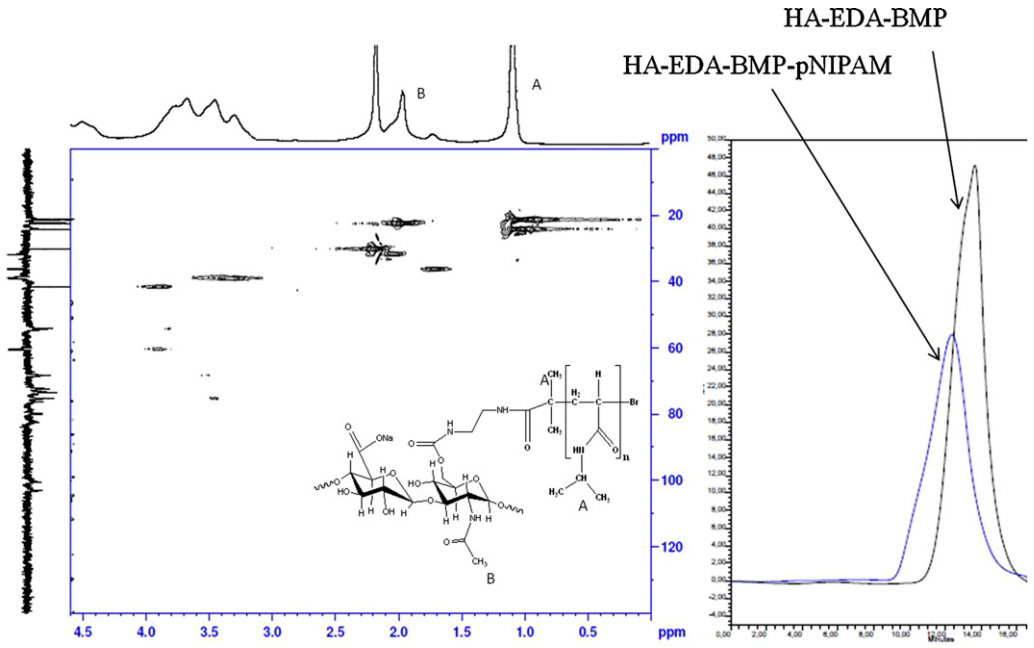


Fig. 5. HMQC analysis(in D_2O/$DMFd_7$) of HA-EDA-BMP-pNIPAM and SEC chromatograms of HA–EDA–BMP–pNIPAM and HA–EDA–BMP.

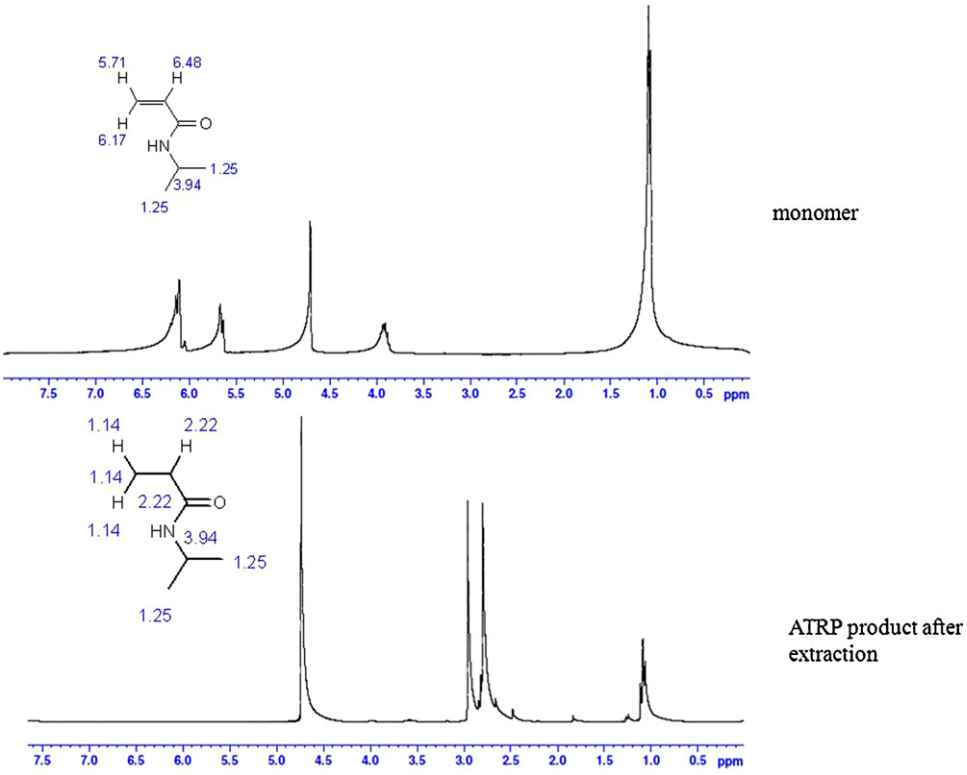


Fig. 6. ^{1}H NMR analysis (inD_2O) of pNIPAM recovered from HA–EDA–BMP–pNIPAM.

spectra obtained after degradation of HA–EDA–BMP–pNIPAM, HA–EDA–BMP–pPEGMA and HA–EDA–BMP–pBUTMA, respectively, compared with corresponding starting monomer.

It is possible to observe that in the ^{1}H NMR spectrum of each extracted product there are the same peaks of the starting monomer except for peaks of vinyl protons that disappear once that the double bond is broken in the ATRP process.

The obtained results both with HMQC analysis of synthesized products and with ^{1}H NMR analysis of products recovered after degradation of HA backbone, confirm that ATRP process has been successful.

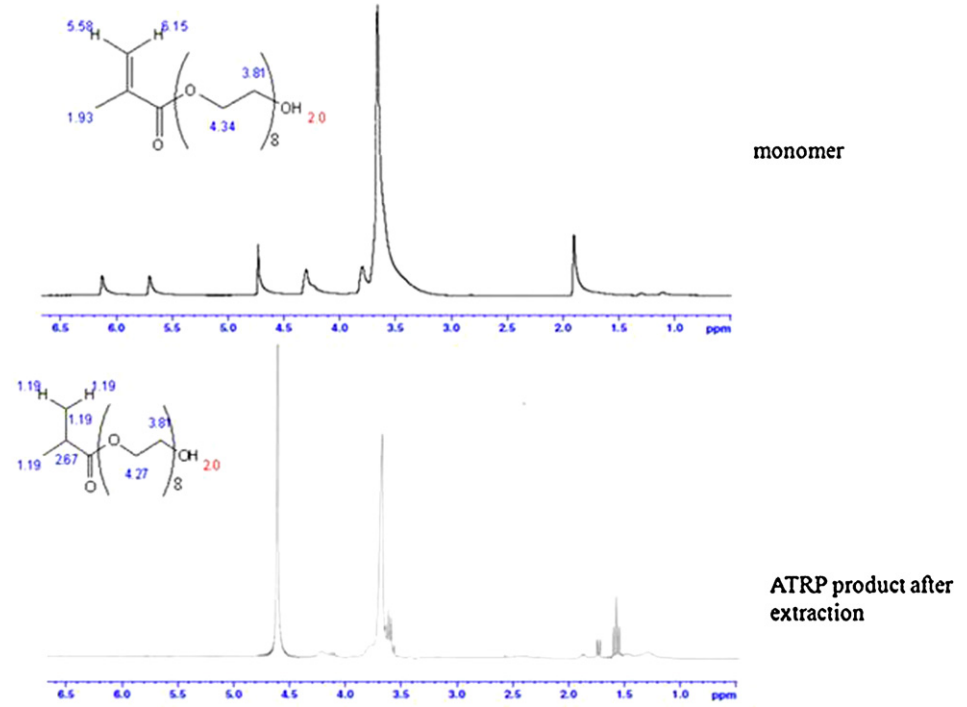


Fig. 7. ^{1}H NMR analysis (inD_2O)of pPEGMA recovered from HA–EDA–BMP–pPEGMA.

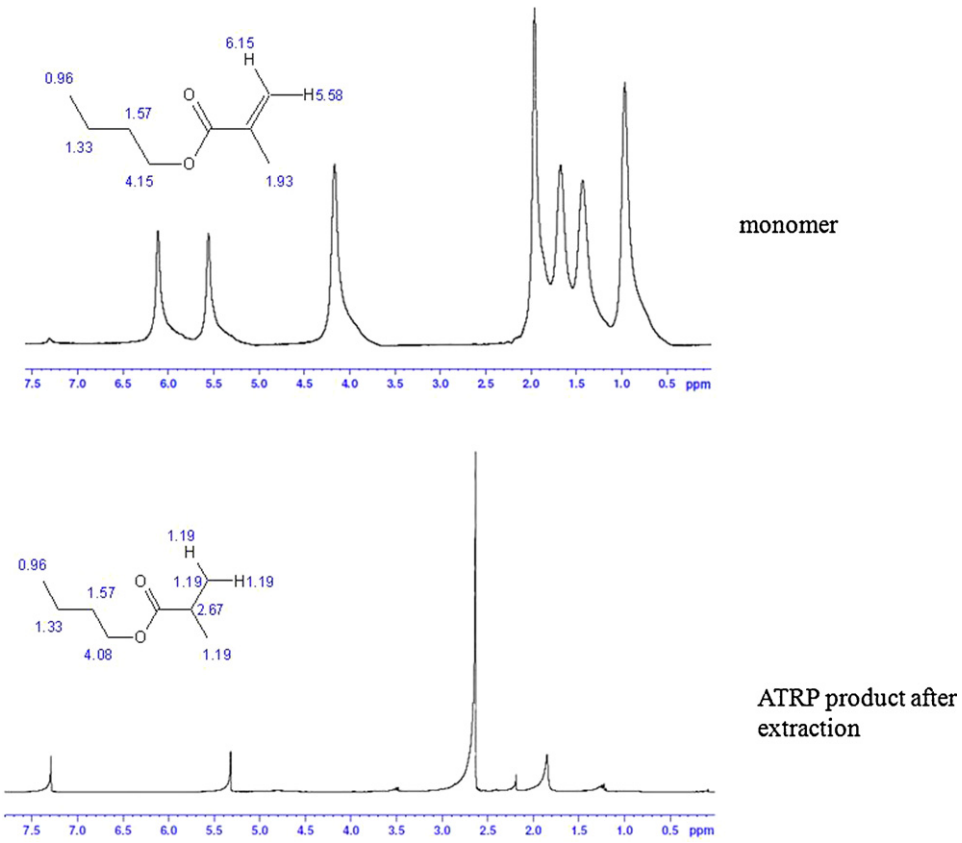


Fig. 8. ^{1}H NMR analysis (in$CDCl_3$) of pBUTMA recovered from HA–EDA–BMP–pBUTMA.

Table 3
Molecular weights and polydispersity index (PDI) of HA based macroinitiators (HA–BMP and HA–EDA–BMP) and ATRP products by using PEGMA, BUTMA or NIPAM as vinyl molecules.

Sample	MW (Da)	PDI
HA(lmw)	229,351	1.9
HA–BMP	231,324	1.36
HA–BMP–pPEGMA	319,551	1.43
HA–BMP–pBUTMA	260,150	1.45
HA–BMP–pNIPAM	336,729	1.33
HA–EDA–BMP	214,635	1.32
HA–EDA–BMP–pPEGMA	370,982	1.37
HA–EDA–BMP–pBUTMA	321,043	1.39
HA–EDA–BMP–pNIPAM	396,786	1.4

4. Conclusions

In the present work we have demonstrated that ATRP can represent a useful synthetic procedure to prepare HA based brush copolymers. At this aim HA–TBA and HA–TBA–EDA (i.e. the ethylenediamino derivative of HA) have been functionalized with 2-Bromo-2-methylpropionic acid (BMP), to produce two new macroinitiators (HA–TBA–BMP and HA–TBA–EDA–BMP) useful to perform ATRP of different molecules carrying vinyl portions. In particular, ATRP has been used to allow the polymerization of poly(ethylene glycol) methacrylate (PEGMA), butyl methacrylate (BUTMA) and N-isopropylamide (NIPAM) on the side chains of these multi functional macroinitiators. The results suggest that when HA–TBA–BMP is used, even if obtained products (HA–BMP–pPEGMA, HA–BMP–pBUTMA and HA–BMP–pNIPAM) have a narrow polydispersity index, ATRP process is scarcely efficient since the grade of polymerization is rather low. On the contrary, using HA–EDA–TBA–BMP, ATRP process was more efficient, indeed for samples HA–EDA–BMP–pPEGMA, HA–EDA–BMP–pBUTMA and HA–EDA–BMP–pNIPAM, the value of the average polymerization number doubled. Therefore by choosing the appropriate macroinitiator it is possible to control the molecular weight and the length of branches of HA brush copolymers.

In our opinion, this “growing from” synthetic procedure could represent an interesting alternative to the classical grafting reactions in producing HA derivatives potentially useful in the production of nano- or microsystems for drug delivery as well hydrogels and scaffolds for tissue engineering purposes. Further studies will be performed to complete the physicochemical and biological characterization of this new family of HA based copolymers as well as to exploit their potential in biomedical and pharmaceutical field.

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