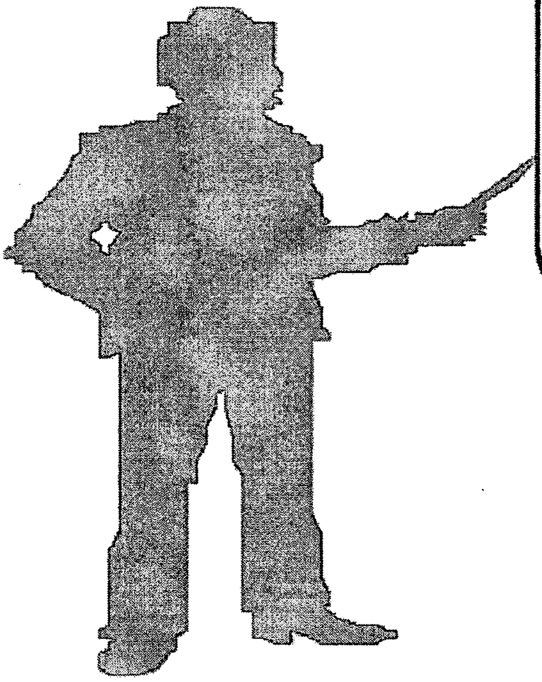
Chapter II



Synthesis of Polypeptide-block-Polysaccharide Copolymer

2.1. Introduction

Synthesis of amphiphilic block copolymers, which consist of hydrophilic and hydrophobic segments, and their self-assembly properties in aqueous solutions are emerging area in material sciences (Alli et al., 2006; Chen et al., 2006; Dimitrov et al., 2006; Jang et al., 2006; Jung and Yoshida, 2006; Lei and Fan, 2006; Trimaille et al., 2006). Structure of amphiphilic block copolymers in aqueous media can be divided into three classes depending on the nature of the hydrophilic block. There are uncharged blocks such as poly(ethylene oxide) (PEO) - also referred to as poly(ethylene glycol) (PEG), positively charged blocks such as quaternized poly(2- or 4-vinylpyridine), poly(ethyleneimine) or poly(L-lysine), and negatively charged ones such as poly(acrylic acid) (PAA), poly(styrene sulfonate) (PSS), or poly(L-glutamic acid) (PGA) (Riess, 2003).

In the present work, hyaluronan (HYA) and poly(γ-benzyl L-glutamate) (PBLG) were used as hydrophilic (anionic) and hydrophobic blocks respectively to synthesize an amphiphilic block copolymer structure with self-assembly properties. Hyaluronic acid (hyaluronan, HYA or HA), a (β-1,4)-linked D-glucuronic acid and (β-1,3) N-acetyl-D-glucosamine polysaccharide (Figure 2.1), is a nonsulfated glycosaminoglycan (GAG) found in the extracellular matrix (ECM) and has multiple functional group for chemical conjugation (Pouyani and Prestwich 1994; Prestwich et al., 1998; Vercruysse and Prestwich 1998). Since HYA and its derivatives show excellent biocompatibility and biodegradability, they have been widely used as temporal scaffolds for tissue engineering and drug delivery devices for many therapeutic applications (Gerecht et al., 2007; Kurisawa et al., 2005; Bulpitt and Aeschilimann 1999; Larsen et al., 1991).

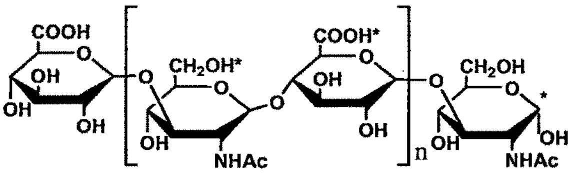


Figure 2.1 Sodium Hyaluronan (Repeating disaccharides) (β1-3 D-*N*-acetylglucosamine, β1-4 D-glucuronic acid). The asterisk (*) indicates possible sites for chemistry.

An important milestone in hyaluronan modification chemistry was the recognition that hydrazides with typical pKa values between 2 and 4, would retain their nucleophilicity at pH 4.75 and would couple efficiently to carbodiimide-activated glucuronic acid residues of hyaluronan (Figure 2.2) (Pouyani et al., 1994). The use of dihydrazide compounds, such as adipic dihydrazide (ADH), provided multiple pendant hydrazide groups for further derivatization with drugs, biochemical probes, and for cross-linking agents. For example, HA-Taxol® bioconjugates were synthesized by the conjugation of HA-ADH and ester activated Taxol® (Luo et al., 2000).

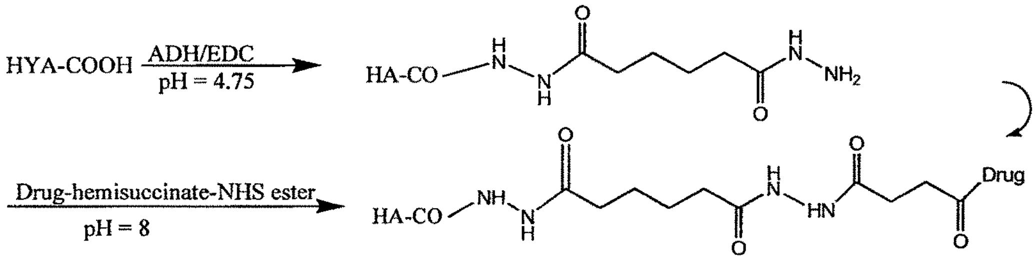


Figure 2.2 Bishydrazide modification of hyaluronan. EDC stands for 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

Hyaluronan-grafted copolymers have been prepared using reducing end of hyaluronan. The ε-amino groups of poly(L-lysine) (PLL) were covalently-coupled by reductive amination using sodium cyanoborohydride to obtain the resulting comb-type copolymers (PLL-*graft*-HA) (Asayama et al., 1998).

Poly(α-amino acid)s (PAAs) (Figure 2.3) are well known as very important synthetic biodegradable materials. They are usually synthesized by means of ring-opening polymerization of α-amino acid N-carboxyanhydrides (NCA) initiated by primary amine (Goshen et al., 1995). They could be degraded in biological environments because of their polypeptide backbone. Due to their low immunogenicity, good biocompatibility and excellent mechanical properties, they have been widely used for biomedical applications (Rypáček 1998; Iwata et al., 1998; Fukuoka et al., 2004).

Poly(γ-benzyl L-glutamate) (PBLG) is one example of poly(α-amino acid) which has been used as drug carrier and gene carrier (Sugimoto et al., 2004; Tian et al., 2005). However, because of the hydrophobic character and slow degradation, the application of PBLG is still limited in drug delivery. Chemical modification of PBLG by a second component such as PEG (Floudas et al., 2003), PEI (Tian et al., 2005), polyester (Deng et al., 2004), other poly(α-amino acid) (Hanski et al., 2006), or poly [2-(dimethylamino)ethyl methacrylate] (PDMAEMA) (Agut et al., 2007) to get block, graft or hyper-branched copolymers was an important way to improve PBLG's properties.

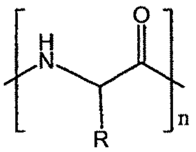


Figure 2.3 General structure of poly(α-amino acids) (PAAs).

In the past decade, the advancement and discovery of new synthetic approaches have opened the way for modification of polymer and preparation of complex structures. In 2001, Sharpless and colleague introduced the concept of 'click chemistry' as a set of powerful, highly reliable and selective reactions for the rapid synthesis and assembly of specially designed building blocks (Kolb and Sharpless 2003; Kolb et al., 2001). The Huisgen 1,3-dipolar cycloaddition reaction between alkynes and azides catalyzed by copper I, also known as the 'click chemistry' (Figure 2.4), represents an important contribution for the synthesis of block copolymer structures. This reaction combines high efficiency (usually above 95%) with a high tolerance of functional groups and solvents, as well as moderate reaction temperatures (25–70^{0}C) (Binder et al., 2007).

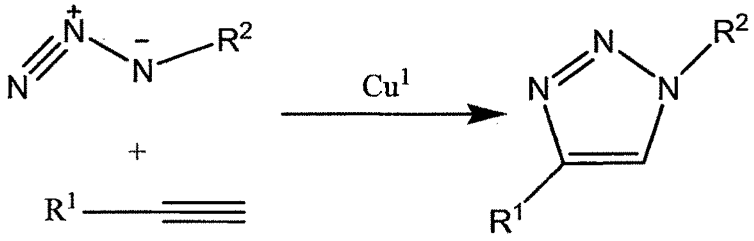


Figure 2.4 Cu^I-catalyzed azide–alkyne coupling.

The resulting heterocyclic 1,2,3-triazole structures are very interesting moieties in medicinal chemistry since they may act as rigid covalent linking units. Two additional reasons for the popularity of this cycloaddition are azides and terminal alkynes which are easy to obtain and that are extremely stable in standard conditions (Kolb and Sharpless 2003; Brase et al., 2005). Figure 2.5 represents proposed full mechanism of the alkyne–azide click reaction.

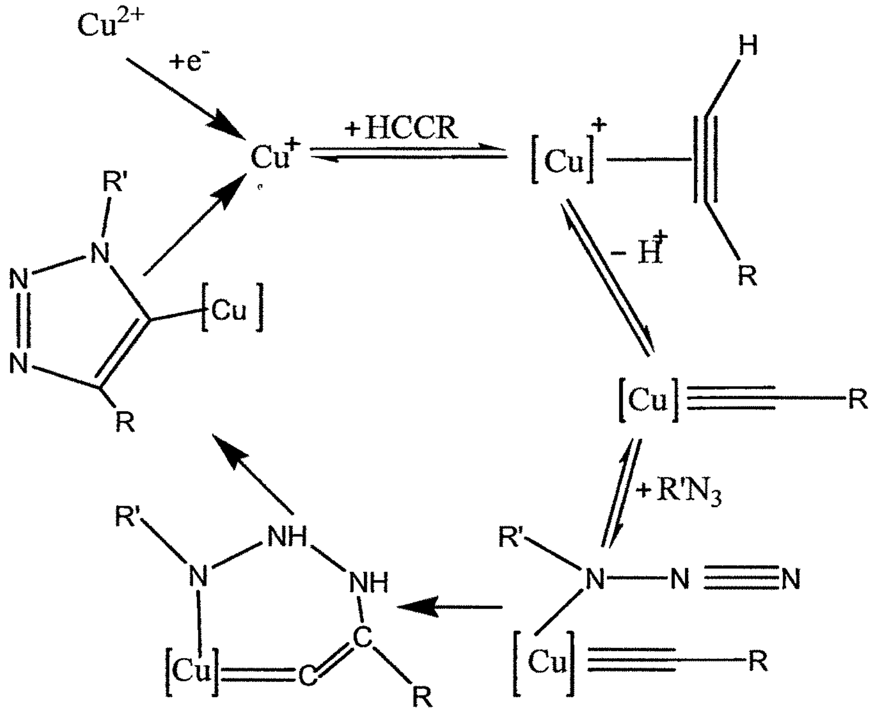


Figure 2.5 The alkyne–azide click reaction; [Cu] represents either mononuclear or dinuclear copper (I) complex fragments. (Nolte et al., 2007)

In this chapter, we have synthesized poly(γ-benzyl L-glutamate)-*block*-hyaluronan (PBLG-*b*-HYA) copolymer by click chemistry. All polymers and block copolymers have been characterized by spectroscopy (infra-red, ^{1}H-NMR), size exclusion chromatography (SEC) and differential scanning calorimetry (DSC).

2.2. Materials

Sodium hyaluronate (Mw = 5140 g/mol, Mn=3650 g/mol, LIFECORE, USA), potassium bromide (Aldrich), sodium acetate (Aldrich), acetic acid (Fluka),, propargylamine (Aldrich), sodium cyanoborohydride (Aldrich), 3-chloropropylamine hydrochloride (Aldrich), sodium azide (Aldrich), sodium sulfate (Aldrich), potassium hydroxide (Aldrich), γ-Benzyl-L-glutamate *N*-carboxyanhydride (Bz-L-GluNCA) (Isochem), N,N,N',N',N''-Pentamethyldiethylenetriamine (PMDETA, Aldrich) copper bromide (Aldrich) was used as received.

2.3. Synthesis of α-alkyne-HYA (HYACCH) by reductive amination

Conventional reductive amination was chosen to couple reducing end of hyaluronan with propargylamine, using sodium cyanoborohydride ($NaBH_3CN$) as a reducing agent. The reaction was carried out in acetate buffer at room temperature. In detail, sodium hyaluronan (2.2 g, 0.60 mmol) was solubilized in acetate buffer pH=5.6 at 2% w/v. 2.82 mL of propargylamine (~75 equivalents) was added under magnetic stirring. Then 2.77 g of $NaBH_3CN$ (44 mmol, ~75 equivalents) were added and the mixture was stirred for 5 days at 50°C. Subsequently, the mixture was concentrated under vacuum, precipitated in cold methanol (400 mL), collected by centrifugation and washed with cold methanol (100 mL) to remove excess of propargylamine and sodium cyanoborohydride. Then the product was dried under vacuum. The reaction is quantitative (~100% yield).

2.4. Acid treatment of α-alkyne-HYA (HYACCH)

Synthesized α-alkyne-HYA (HYACCH) was treated with hydrochloric acid (HCl) to get its acid form. The HYACCH (2.15 g) was solubilized in milli-Q water (6 mL) and pH was adjusted to 2.0 by adding 1M HCl solution. Acidic solution of HYACCH was precipitated drop wise in cold ethanol (400 mL). Precipitate was collected through centrifugation and dried in a vacuum dryer. Yield*: 1.75 g (~81%). *Yield (in wt %) is based on the mass of HYACCH before and after treatment.

2.5. Synthesis of 1-azido-3-aminopropane

This compound was synthesized following an already published procedure (Carboni et al., 1993). A solution of 3-chloropropylamine hydrochloride (4 g, 30.8 mmol) and sodium azide (6 g, 92.3 mmol, 3equiv.) in water (30 mL) was heated at 80°C for 15 h. After that most of the water was removed by distillation under vacuum, the reaction mixture was cooled in an ice bath. Diethyl ether (50 mL) and then KOH pellets (4 g) were added with controlling the temperature below 10°C. After separation of the organic phase, the aqueous layer was further extracted with diethyl ether (2 x 20 mL). The combined organic layers were dried over Na_2SO4 and concentrated to give oil which was purified by distillation. Yield: 2.3 g (~75%).

2.6. Synthesis of α-azido PBLG (PBLG-N_3)

This functionalized polypeptide was synthesized as per the reported procedure (Schatz et al., 2009; Agut et al., 2008; Agut et al., 2007). The Bz-L-GluNCA (6g, 22.81 mmol) was weighed in a glove box under pure argon, introduced in a flame-dried Schlenk, and dissolved in 60 mL of anhydrous DMF. The solution was stirred for 10 min, and 1-azido-3-aminopropane (57 μL, 570 μmol, density = 1.003 g/mL) was added with a nitrogen purged syringe. The solution was stirred for 40h under vacuum at room temperature. The polymer was recovered by precipitation in diethylether and dried under high vacuum, analyzed by SEC in DMF (LiBr, 60 °C), IR and ^{1}H-NMR. Molar mass was determined by ^{1}H-NMR (Figure 2.6) using the following equations where I_a, I_h, M_{BLG} and M_{init} are, respectively, the peak intensity of methylene protons a (initiator), the peak intensity of methylene protons h (α-azido PBLG), the molar mass of the BLG monomer unit and the molar mass of 1-azido-3-aminopropane initiator.

$$M_{n,PBLG} = \frac{I_h M_{BLG}}{I_a} + M_{init.}$$

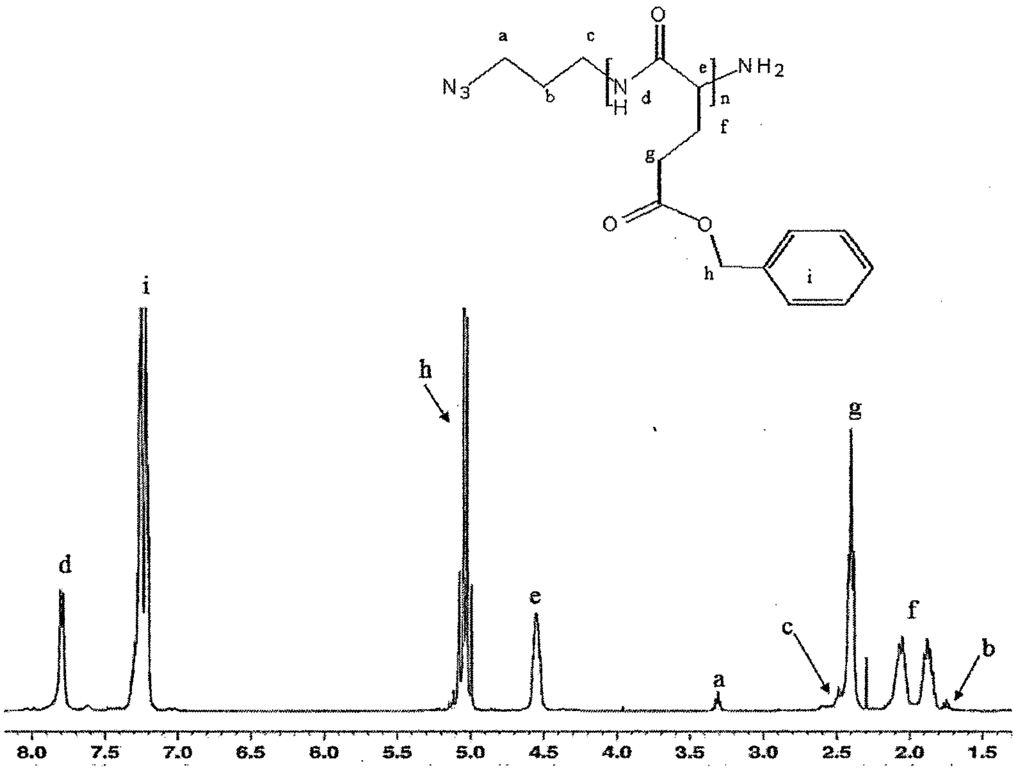


Figure 2.6 ^{1}H-NMR spectrum of PBLG-N_3 in $CDCl_3$ + 15%TFA.

2.7. Synthesis of HYA-*b*-PBLG block Co-polymer by Huisgen's 1,3-Dipolar Cycloaddition ("Click" Reaction)

Huisgen's 1,3-dipolar cycloaddition (click reaction) was performed in DMSO at 60°C using CuBr as catalyst and pentamethyldiethylenetriamine (PMDETA) as ligand. After 48h of reaction, click chemistry afforded the targeted PBLG-*b*-HYA block copolymers as illustrated in Scheme 2.1. Experimentally, N_3-PBLG (1.05 g, 210 μmol, 1 equiv) and α-alkyne-HYA (2.1 g, 410 μmol, 2 equiv) were solubilized in a flask containing 70 mL of anhydrous DMSO. The mixture was stirred for 20 min and PMDETA (87.04 μL, 420 μmol, 2 equiv) was added through sterile syringe under nitrogen environment. The mixture was degassed by three freeze-thaw cycles and added in another Schlenk containing CuBr (60 mg, 420 μmol, 2 equiv) via nitrogen-purged syringe. The Schlenk flask was placed in an oil bath regulated at 60°C for 2 days under static nitrogen pressure. The excess of HYA was removed from the block copolymers by extensive dialysis (MWCO 50kDa, 6Spectra/Por® membrane) against water at pH 3.5 to keep the polysaccharide in its acidic form. The block copolymer which progressively precipitates during dialysis was collected through centrifugation and dried under vacuum. The copolymer was analyzed by ^{1}H-NMR and IR spectroscopy.

2.8. Characterization

2.8.1. Fourier transform infrared spectroscopy

FT-IR spectra were recorded on pellets in KBr in the range 4000–500 cm^{-1} using a Perkin-Elmer 1720 Fourier Transform Spectrophotometer with a resolution of 1cm^{-1}. Each spectrum was recorded from 100 scans.

2.8.2. ^{1}H-NMR

^{1}H-NMR spectra were recorded in the respective deuterated solvent (D_2O, d_6-DMSO, $CDCl_3$) at 2 wt% using a Bruker AC 400 spectrometer with 1 second relaxation mode and 64 scans.

2.8.3. *Size exclusion chromatography measurement*

The PBLG molar masses were determined by size exclusion chromatography (SEC) equipped with two PLgel Mixed-C columns (7.5 × 300 mm) and one PLgel 5μm guard column (7.5 × 50 mm), a refractive index detector (Jasco 1530-RI), a UV detector (Jasco 875-UV) and with dimethylformamide (DMF) as eluent (0.8 mL/min) at 60°C in the presence of LiBr (1g/L). SEC was calibrated using linear polystyrene samples. HYA molar mass was determined by size exclusion chromatography (SEC) equipped with two PL aquagel-OH columns (Polymer Laboratories), a refractive index detector (Jasco 880-PU), and with 0.1M Acetate buffer (pH = 5.6) as eluent (0.8 mL/min) at 25°C and was calibrated using pullulan samples.

2.8.4. *Differential scanning calorimetry measurement*

The thermal properties of PBLG-N_3 were determined by DSC measurements in a Shimadzu 50 differential scanning calorimeter. Hermetic pans with 5mg dried sample were heated from 0°C to 200°C at 10°C/min and then cooled to 0°C at 5 °C/min under nitrogen atmosphere.

2.9. Results and Discussion

2.9.1. *The α-Alkyne-HYA by Reductive amination*

Hyaluronic acid (HYA) is a glycosaminoglycan with a repeating disaccharide consisting of two glucose derivatives, D-glucuronic acid and *N*-acetyl D-glucosamine. The glycosidic linkages are $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$. Disaccharide protons were identified by ^{1}H-NMR spectroscopy in D_2O (Figure 2.8). Hyaluronic acid has a reducing end as many polysaccharides, meaning that the last unit of the polysaccharide structure has an aldehyde function in equilibrium with a hemiacetal one. Molecular characterizations of HYA polymer were performed by static light scattering (SLS) and SEC in acetate buffer (pH=5.6). SLS provided a weight-average molecular weight (Mw) of 5100 g/mol, which is in good agreement with Mw = 5140 g/mol provided by the supplier of HYA (Lifecore US). Thanks to the polydispersity index given by SEC analysis, PDI = 1.4 (Figure 2.7), the number-average molecular weight has been derived, Mn = 3670 g/mol. This value is in good agreement too with the one deduced from NMR analysis (Mn = 3790 g/mol) and corresponds to a degree of polymerization DP=10 (the molar mass of a repeat unit of HYA = 379 g/mol).

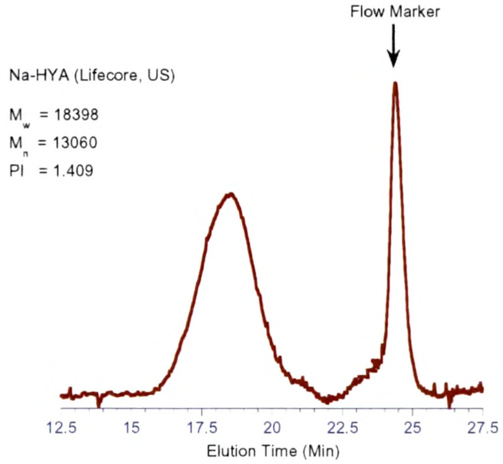


Figure 2.7 SEC of Sodium Hyaluronate (Lifecore) in acetate buffer at pH 5.6 using pullulan as an internal standard.

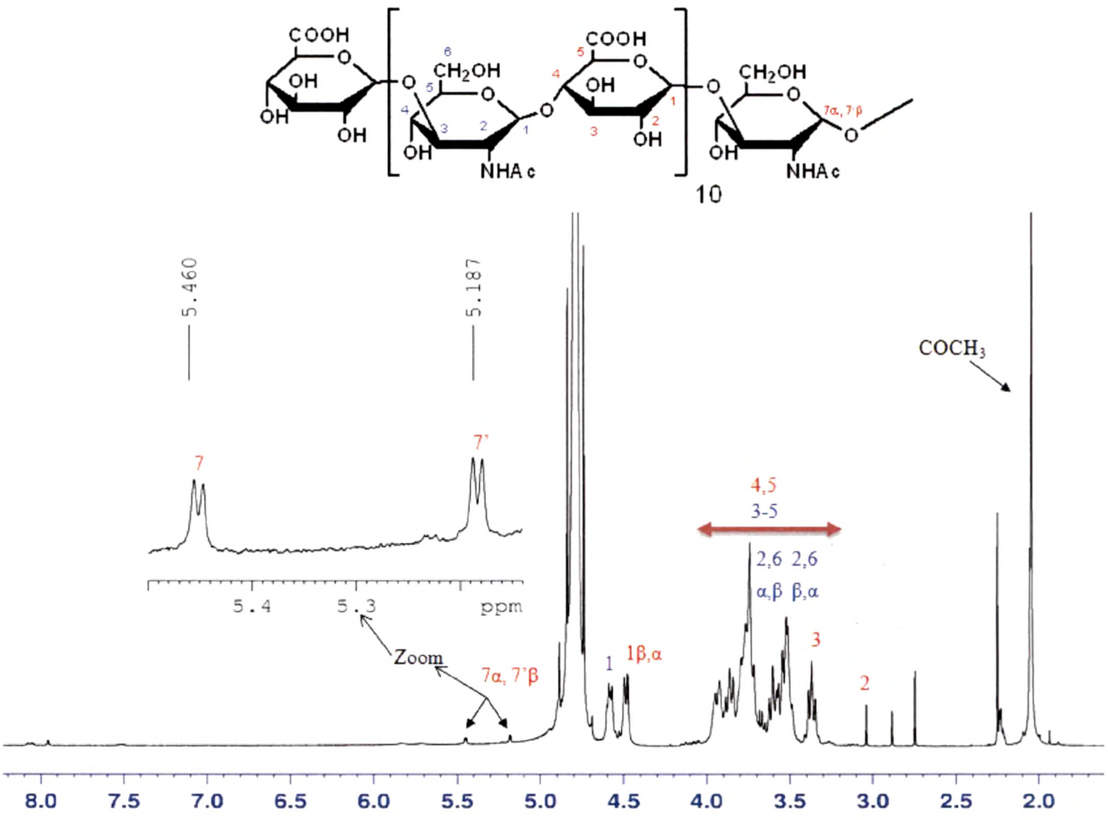
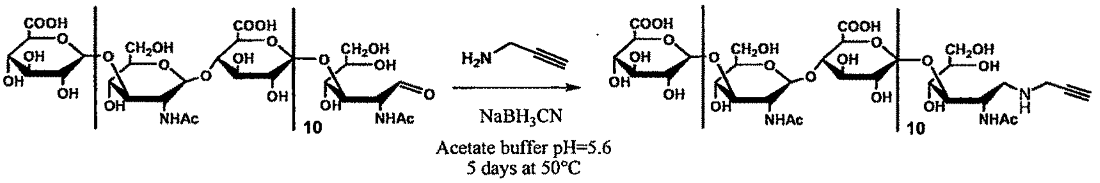


Figure 2.8 ^{1}H-NMR Spectrum of Na-Hyaluronate in D_2O.

NMR spectroscopy confirmed the presence of a reducing end. Indeed, anomeric proton peak of the reducing end were observed at δ =5.2 ppm and δ = 5.5 ppm while non-anomeric proton peaks were observed between δ = 3.00 ppm and δ = 4.7ppm. Methyl protons of acetamide groups (CH_3-CO-NH-) were identified at 2.08ppm (Figure 2.8) (Palumbo et al., 2006; Crescenzi et al., 2003; Zhang and James 2005). In scheme 2.1, the coupling reaction is illustrated for hyaluronan and propargylamine. Each polysaccharide chain contains only one aldehyde end group (in equilibrium with a hemiacetal function). Consequently, one mole of hyaluronan can react with one mole of propargylamine, resulting in an alkyne end-functionalized hyaluronan. In addition, working with a large excess of propargylamine ensures that the equilibrium is shifted towards the formation of aldehyde (Chatelier's law).



Scheme: 2.1. Preparation of a-alkyne-HYA* (HYACCH) *by reductive amination

Functionalized hyaluronan was isolated as a white powder by precipitating concentrated reaction medium in cold methanol and dried in a vacuum dryer. The full characterization was achieved by SEC, ^{1}H-NMR and IR spectroscopy. Reaction yield was 100 wt % which indicates a full conversion of hyaluronan in its alkyne end-functionalized form (HYACCH). IR spectra (Figure 2.9) of the dried alkyne functionalized hyaluronan was recorded and compared with IR spectra of native hyaluronan (Huang et al., 2007; Alkrad et al., 2003; Palumbo et al., 2006).

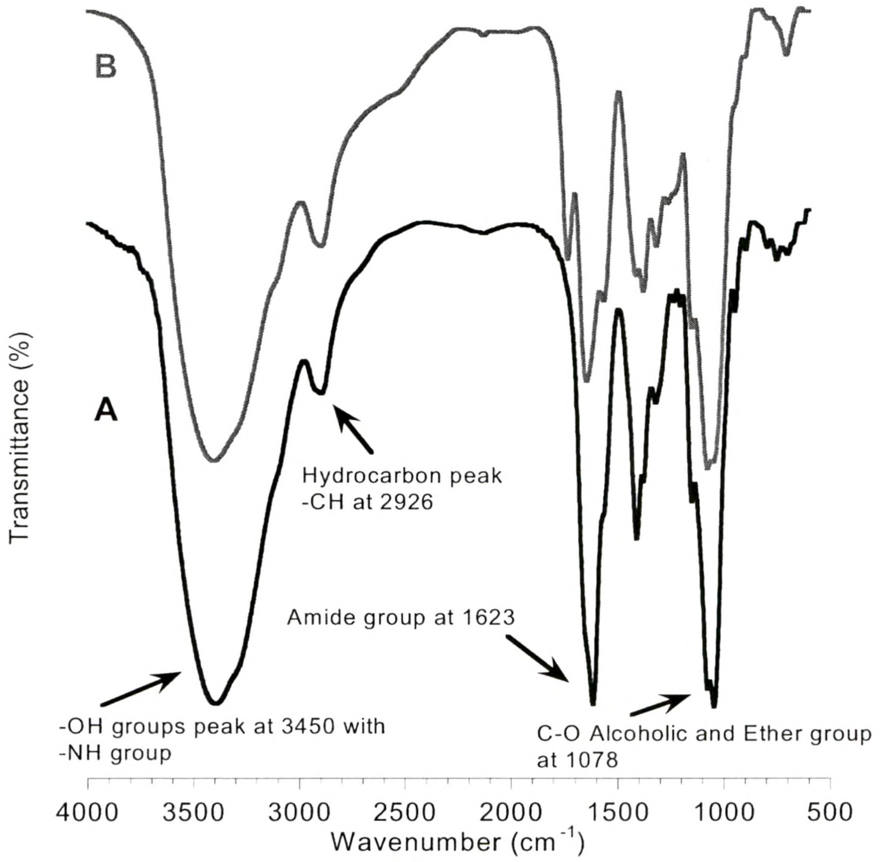


Figure 2.9 IR spectra of [A] HYACCH [B] acid HYACCH.

Figure 2.10 (A, B) shows the ^{1}H-NMR spectra of alkyne functionalized hyaluronan and it acid form. We identified all characteristic peaks of sodium hyaluronan excepted at δ = 5.2 ppm and δ = 5.5 ppm that are characteristic peaks of α, β anomeric protons. Therefore, the disappearance of anomeric proton peaks confirmed the functionalization of hyaluronan. In addition, IR spectra (Figure 2.9 A, B) and ^{1}H-NMR (Figure 2.10 A, B) shows no difference between HYACCH before and after acid treatment. As acid HYACCH was soluble in the DMSO at 60°C, it means that further chemistry can be performed using DMSO as solvent.

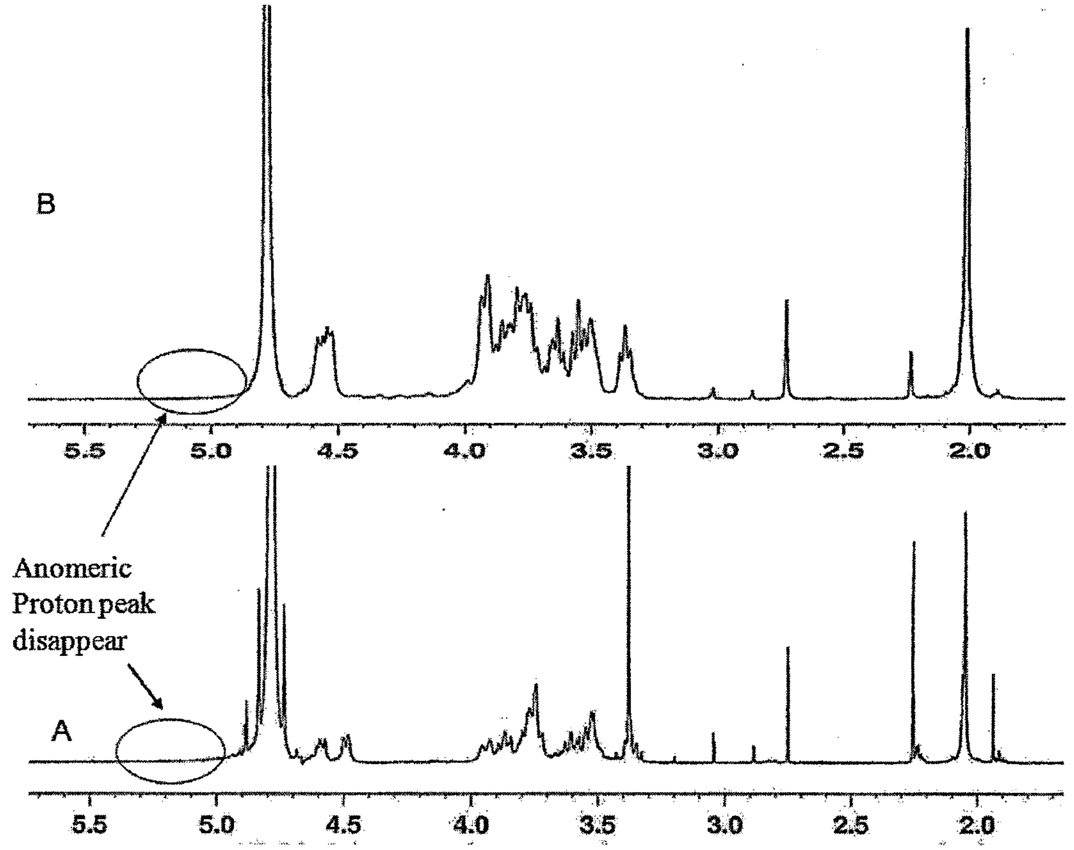
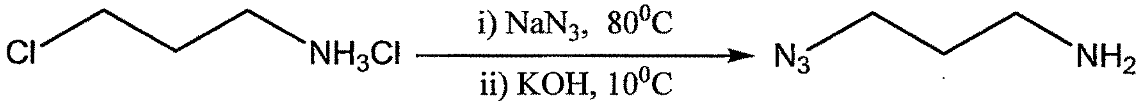


Figure 2.10 ^{1}H-NMR Spectrum [A] of HYACCH and [B] acid HYACCH in D_2O.

2.9.2. *The α-Azido PBLG (PBLG-N_3)*

1-azido-3-aminopropane synthesis strategy is described in Scheme 2.2. The compound was obtained in good yields (~75%) and its purity was checked by ^{1}H-NMR (Figure 2.11 A) and ^{13}C-NMR (Figure 2.11 B) spectroscopy. The sharp peak obtained near 2100cm^{-1} in the IR spectra (Figure 2.12) confirmed the presence of the azide function (-N_3).



Scheme: 2.2. Synthesis of 1-azido-3-aminopropane

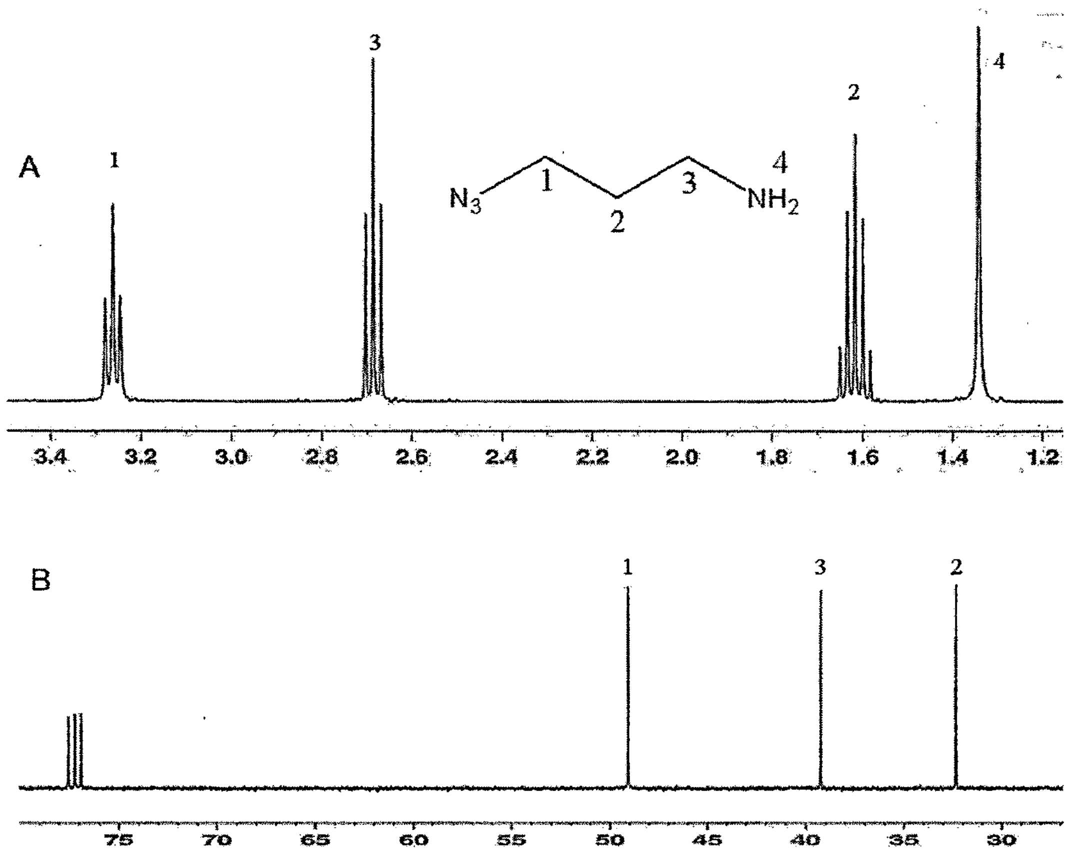


Figure 2.11 [A] ^{1}H-NMR spectrum and [B] ^{13}C NMR spectrum of 1-Azido-3-aminopropane in $CDCl_3$.

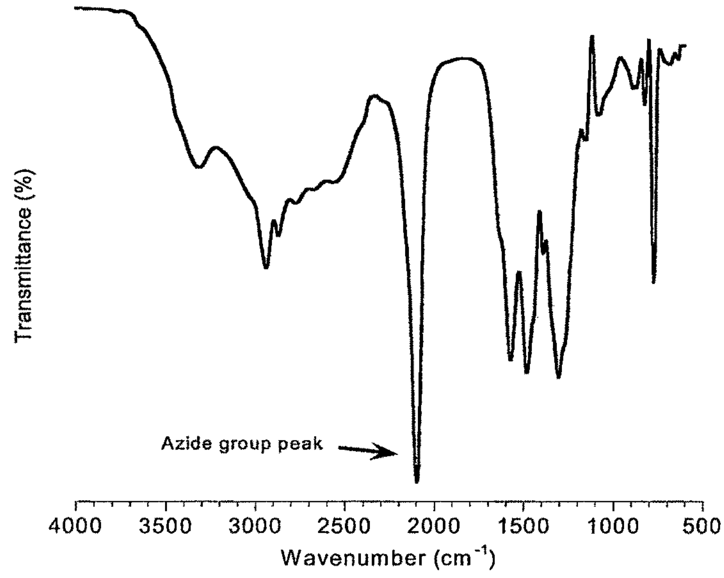
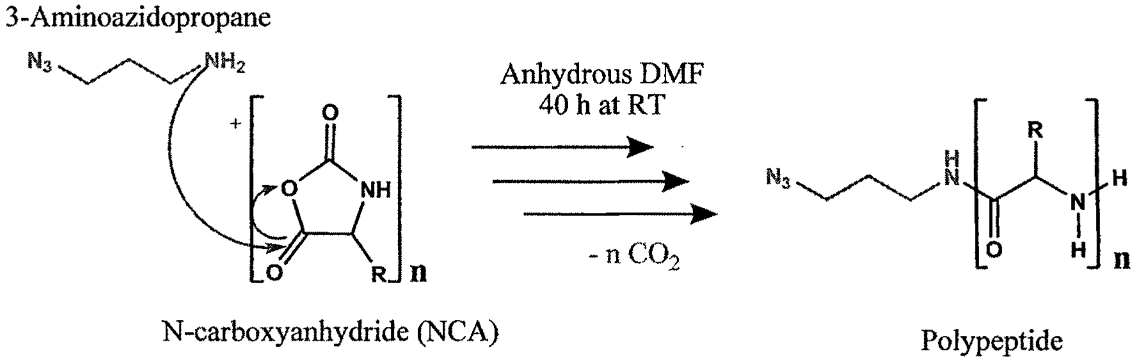


Figure 2.12 IR spectrum of 1-Azido-3-aminopropane.

The α-azido-PBLG was obtained by ring opening polymerization (ROP) of Bz-L-GluNCA using 1-azido-3-aminopropane as initiator. The scheme 2.3 represents the synthesis of PBLG-N_3. This compound was fully characterized by DSC, SEC, FTIR and ^{1}H-NMR spectroscopy.



Scheme: 2.3- Synthesis of a-azido poly(γ-benzyl L-glutamate) (N_3-PBLG)

FTIR analysis confirms the formation of α-azido-PBLG (Figure 2.13). Indeed it is known that this polypeptide preferentially adopts a α-helix conformation. Here, amide I and amide II peaks are located at 1656 and 1548cm^{-1} which is in agreement with a helical conformation. For a polypeptide in a β-sheet conformation, the amide I band and the amide II band are located at 1630 and 1536cm^{-1}, respectively (Fontaine et al., 2001). Moreover, a band at 1260cm^{-1} corresponding to the amide III band of α-helix conformation in polypeptide was also observed on figure 2.13 (Komoto et al., 1976). In addition the band at 2097cm^{-1} confirms the functionalization of PBLG by an azide group.

The molar mass was accurately determined from ^{1}H-NMR spectroscopy (Experimental Section) (Figure 2.6). Indeed, a characteristic proton of the chain end arising from α-functionalized initiator was clearly identified in the ^{1}H-NMR spectra of PBLG-N_3 in $CDCl_3$ + 15%TFA. For instance, the methylene protons adjacent to the azido function were observed at $\delta = 3.3$ ppm. Relative integration of this peak compared to that characteristic of protons of the PBLG main chain, e.g. aromatic protons at $\delta = 5.0$ ppm (Figure 2.6), allowed us to determine the molar mass of polymer in $CDCl_3$ in the presence of 15% of trifluoroacetic acid. A number-average degree of polymerization (

$\overline{DP_n}$) of 23 was derived from this NMR analysis. We also determined the polydispersity index= 1.1 by SEC (Figure 2.14).

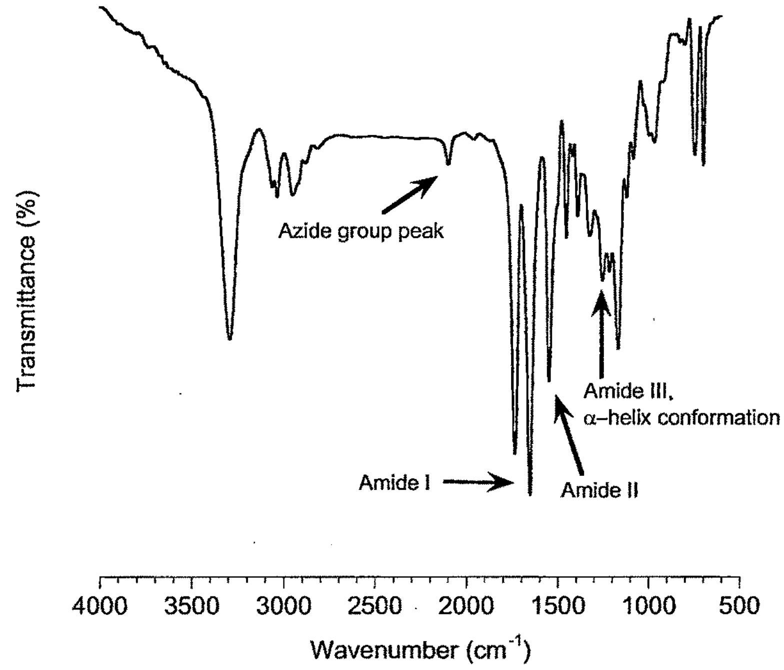


Figure 2.13 IR spectra of PBLG-N_3.

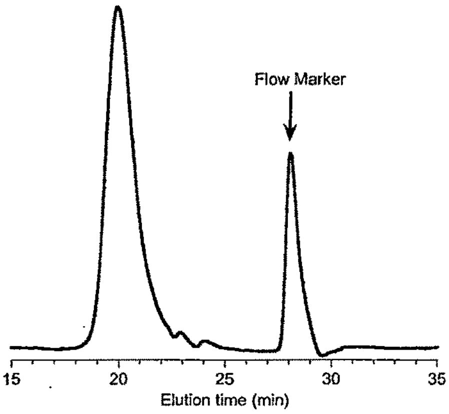


Figure 2.14 SEC analysis of PBLG-N_3 at 60°C in DMF in the presence of LiBr.

DSC analysis of PBLG-N_3 displays a first-order transition at 110^{0}C due to the transformation from a 7_2 to a 18_5 α-helical conformation. This transformation is irreversible as depicted by the absence of this feature in the cooling and second heating trace (Watanabe and Uematsu 1984). In addition, the heating and cooling DSC traces reveal a glass temperature at 15.12°C (Figure 2.15) (Papadopoulos et al., 2004).

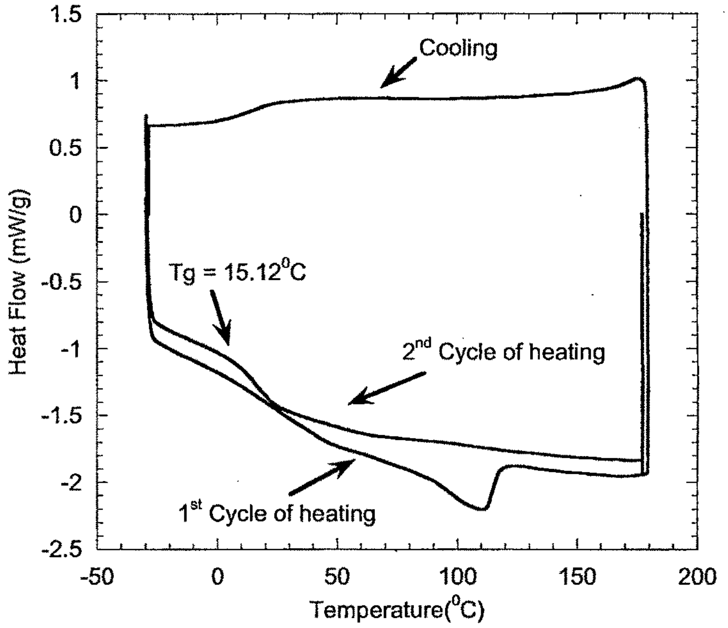
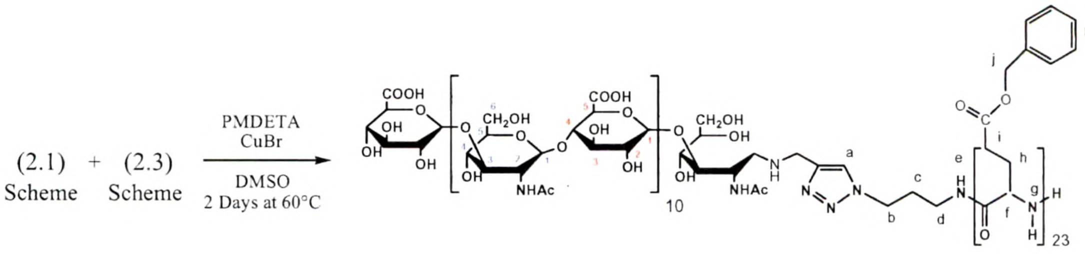


Figure 2.15 Thermal analysis of PBLG-N_3.

2.9.3. Click Reaction

Huisgen's 1, 3-dipolar cycloaddition (click reaction) was performed between the α-azide end-functionalized PBLG and α-alkyne-HYA acid (HYACCH acid). Scheme 2.4 represents the click reaction between PBLG-N_3 and HYACCH acid. The structure of the block copolymer was determined by ^{1}H-NMR and IR analysis. The peak of the triazole ring was found at 7.6 ppm (Figure 2.16) which indicates the coupling between both blocks by click reaction. This is also supported by data obtained by IR analysis (Figure 2.17) that shows the full disappearance of azide peak at 2090 cm^{-1} (Lin et al., 2008, Agut et al., 2007). In addition, the IR analysis of the block copolymer is very close to the mixture of both homopolymers which also confirms the success of the synthesis.



Scheme: 2.4. Synthesis of HYA-b-PBLG block copolymer by Huisgen's 1,3-dipolar cycloaddition (click reaction)

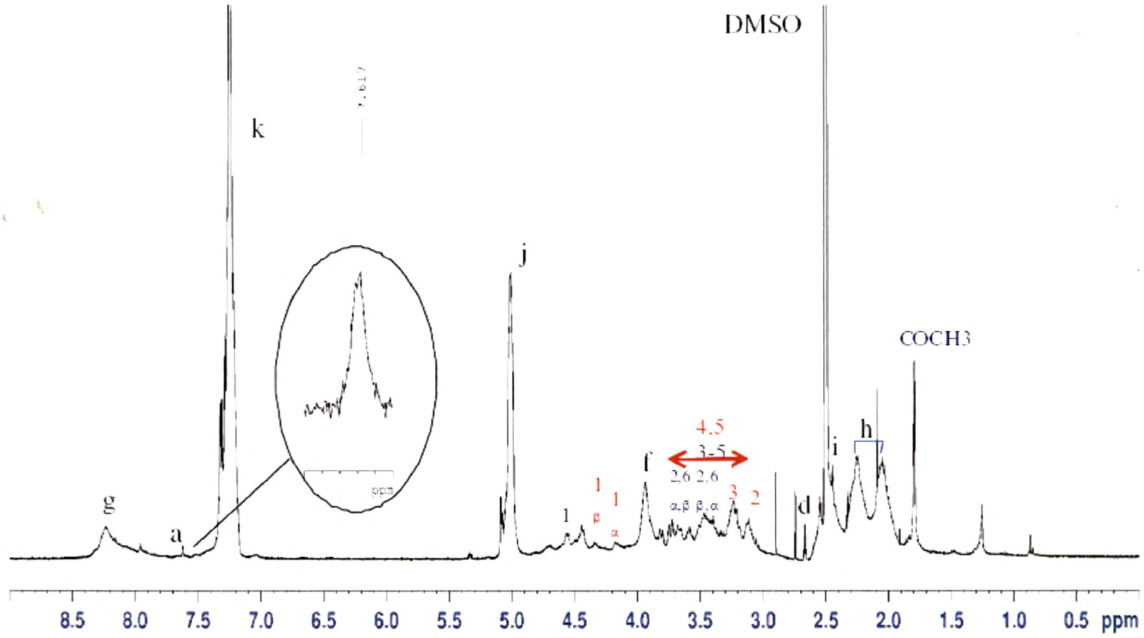


Figure 2.16 ^{1}H-NMR spectra of PBLG-*b*-HYA in *d_6*-DMSO at 60^{0}C.

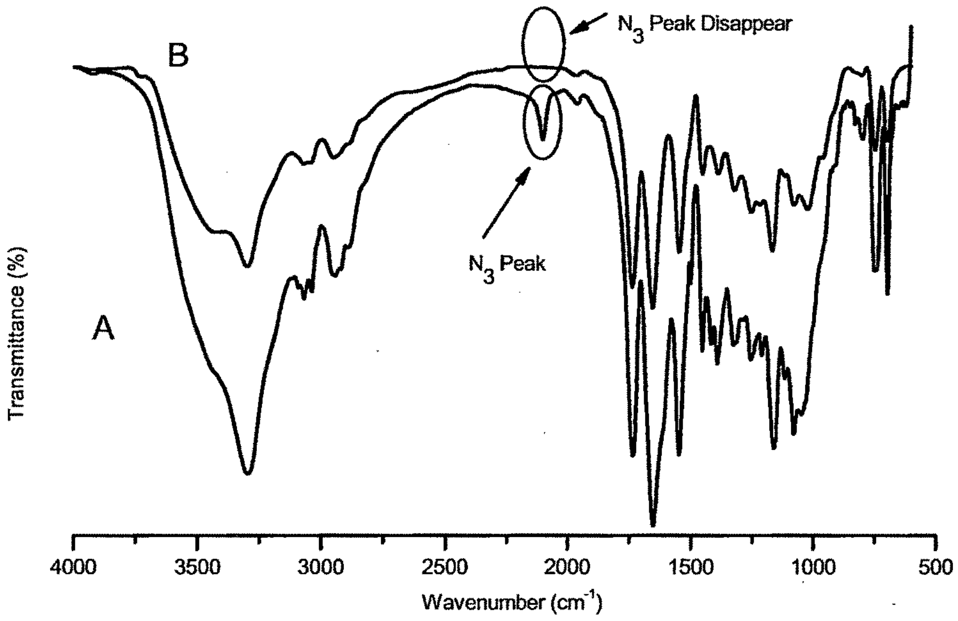


Figure 2.17 IR spectra of (A) mixtures of homopolymers (B) PBLG$_{23}$-*b*-HYA$_{10}$ copolymer.

Based on the analysis by SEC, NMR and static light scattering performed on the homopolymers, and from the NMR results obtained for the block copolymer we could define the chemical composition of the block copolymer (Table 2.1).

Table 2.1 Molecular characteristics of the block copolymer

M_n Hyaluronic acid g/mol	M_n PBLG g/mol	hydrophilic weight fraction (f)
3650	5000	42%

2.10. Conclusion

We have synthesized and characterized a biocompatible polypeptide-*block*-polysaccharide copolymer, namely a poly(γ-benzyl L-glutamate)-*block*-hyaluronan copolymer (PBLG-*b*-HYA). The presence of a covalent link between the two blocks has been confirmed by ^{1}H-NMR and IR analysis. Based on the static light scattering, SEC, and ^{1}H-NMR analysis of each block and PBLG-*b*-HYA copolymer, we are able to provide the right composition of the block copolymer, namely $PBLG_{23}$-*b*-HYA_{10}.

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