

Synthesis and characterization of thermo-responsive polyethers

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Tiivistelmä – Referat – Abstract <p>Hyaluronic acid (HA) hydrogels are interesting biomaterials for drug delivery and tissue engineering applications. Glycidyl ether derivatives have gained much interests due to their thermo-responsive properties. Thermo-responsive random copolymers of glycidyl methyl ether (GME) and epoxyhexane (EH) were synthesized. Once their properties were studied, they were grafted onto hyaluronic acid to obtain gelation at temperatures above the phase transition temperature of poly (GME-EH).</p> <p>PGME is a water-soluble polymer at low temperatures, but phase separates at 57.3oC. The transition temperature of PGME is too high to be utilized in medical applications. Thus a hydrophobic monomer EH was used to decrease the transition temperature of PGME via copolymerization. Several samples of random copolymers poly (GME-EH) were successfully synthesized by anionic ring opening polymerization (AROP). The transition temperature of copolymers was characterized by NMR, turbidimetry and micro-calorimetry respectively to study the phase transition behavior.</p> <p>Tetraoctylammonium bromide was used as initiator resulting with a bromide as the end group. Bromide was substituted by azide group to be used in click chemistry reaction with alkyne-functional HA. The reaction of the azide group on the end of copolymer chain was detected by FT-IR spectroscopy. Grafting was achieved by click chemistry following copper-catalyzed azide-alkyne cycloaddition (CuAAC) procedure.</p> <p>Rheology was used to study the gelation of the final product: thermo-responsive hyaluronic acid hydrogel. However, for different reasons the final product failed to form a gel.</p>			
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Abbreviation

1,2-epoxyhexane	EH
Anionic ring opening polymerization	AROP
Critical micellisation temperature	CMT
Cu (I) catalyzed Azide Alkyne Cycloaddition reaction	CuAAC
Di (ethylene glycol) methyl ether methacrylate	DEGMA
Dichloromethane	DCM
Differential Scanning Calorimetry	DSC
Dimethyl sulfoxide	DMSO
Dimethylformamide	DMF
Ethoxyethyl glycidyl ether	EEGE
Ethyl glycidyl ether	EGE
Ethylene oxide	EO
Ethylene-diamine-tetraacetic acid	EDTA
Fourier-transform Infrared Spectroscopy	FT-IR
Gel permeation chromatography	GPC
Glycidyl methyl ether	GME
Hyaluronic acid	HA
Lower critical solution temperature	LCST
Micro Differential Scanning Calorimetry	Micro-DSC
N,N,N',N'',N'''-penta-methyl-diethylene-triamine	PMDETA
N,N-diisopropyl ethanolamine glycidyl ether	DEGE
Nuclear Magnetic Resonance Spectroscopy	NMR
Oligo (ethylene glycol) methacrylate	OEGMA
Poly (2-glucosyloxyethyl methacrylate)	PGEMA
Poly (allyl glycidyl ether)	PAGE
Poly (Ethylene oxide)	PEO
Poly (N-isopropylacrylamide)	PNIPAM
Poly (Propylene oxide)	PPO
Polyacrylic acid	PAA

Polydispersity index	PDI
Propargyl glycidyl ether	PGE
Ring opening polymerization	ROP
Tetraoctylammonium bromide	NOct ₄ Br
Triisobutylaluminum	i-Bu ₃ Al
Tetrahydrofuran	THF
Upper critical solution temperature	UCST

I. Literature Part

1. Introduction

Thermo-responsive hydrogels gain much attention as biomaterials for instance in drug delivery and tissue engineering. [1, 2]

A popular thermo-responsive polymer is for example poly(N-isopropylacrylamide) (PNIPAM).[3] This material shows lower critical solution temperature (LCST) in water and the phase transition temperatures are hardly influenced by changes in concentration, pH, or ionic strength. Therefore it is a good candidates to be used as thermo-responsive biomaterials in biological environments.[4] Controlled polymerization methods are utilized for producing this polymers for instance by ionic or controlled radical polymerization.[5-7]

PGME used in this study is biocompatible and water soluble, and has similar properties compared to PNIPAM.[8] However it gained less attention by chemists in the past due to the low reactivity compared to other glycidyl ether derivatives. However, a monomer activation method AROP found to be a good approach.[9] It would be a good attempt for adventuring new biomaterial now.

Hyaluronic acid (HA) is widely investigated for medical application due to its biodegradation and biocompatibility. For example, crosslinked HA-based composite hydrogels have been used as sustained release drug delivery systems.[10] Furthermore, HA is a natural component of eye tissue which could serve as a potential drug delivery system for many ocular diseases. [11]

The main purpose of the study was to produce a HA-graft-poly(GME) based hydrogel, which can be used as thermo-responsive biomaterial in a range of applications.

2. Thermo-responsive Polymers

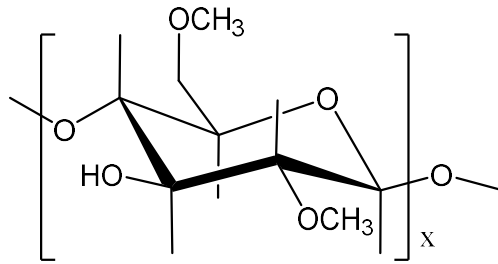
2.1 General review of Thermo-responsive Polymers

Stimuli-responsive polymers are a class of functional polymers with large variety of applications.[2, 12] Stimuli-responsive polymers can have phase transition triggered by for instance the change of temperature, pH, concentration of CO₂ and light, especially in aqueous solution.[13]

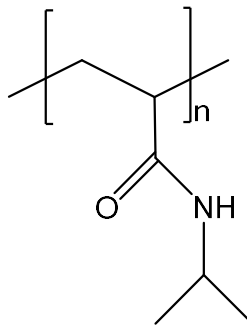
Stimuli-responsive polymers are used in controlled release technologies and drug delivery systems. Many of these polymers are able to carry drug molecules, encapsulated for example in self-assembled polymeric micelles and hydrogels.[14] Compared to the normal medical therapy using free anti-cancer agent, they may have significantly higher effectiveness and lower cytotoxicity to healthy cells since drugs can be surrounded by polymers and targeted to the target cell.[15]

There are several methods that can drive the response of polymers, but polymers exhibiting temperature responsiveness are the most studied among stimuli-responsive polymers, since changing temperature is relatively easier than that of pH, redox and concentration of CO₂. Furthermore, human body temperature is stable compared to the pH, which varies greatly in different organs and tissues.

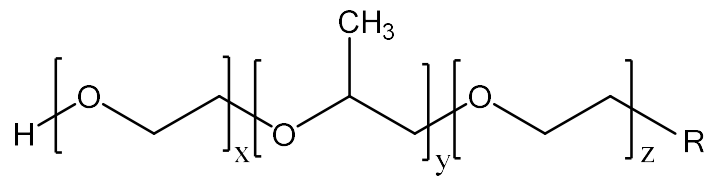
Plenty of polymers are thermo-responsive. The following *Figure.1* shows some examples, first category is natural polymers for example methylcellulose.[13] PNIPAM, poly(ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide) (PEO/PPO/PEO) block copolymers are the examples of synthetic polymers.[13]



Methylcellulose(MC)



Poly N-isopropylacrylamide



PEO-PPO-PEO triblock copolymers (or random copolymer)

Figure.1 Examples of natural and synthetic thermo-responsive polymers

2.2 Principle of Thermo-responsive Polymers

The temperature, at which the polymer solution phase separates, is called critical solution temperature (CST). As it shows below in *Figure.2*, temperature leads to the formation of phase transitions with different polymer concentrations and LCST is the minimum temperature of the coexistence curve of two phases in the phase diagram.

In contrast, a polymer with upper critical solution temperature (UCST) shows the opposite tendency. UCST is the maximum temperature of the coexistence curve. It is insoluble below UCST and when the temperature rises above UCST, it would be soluble.

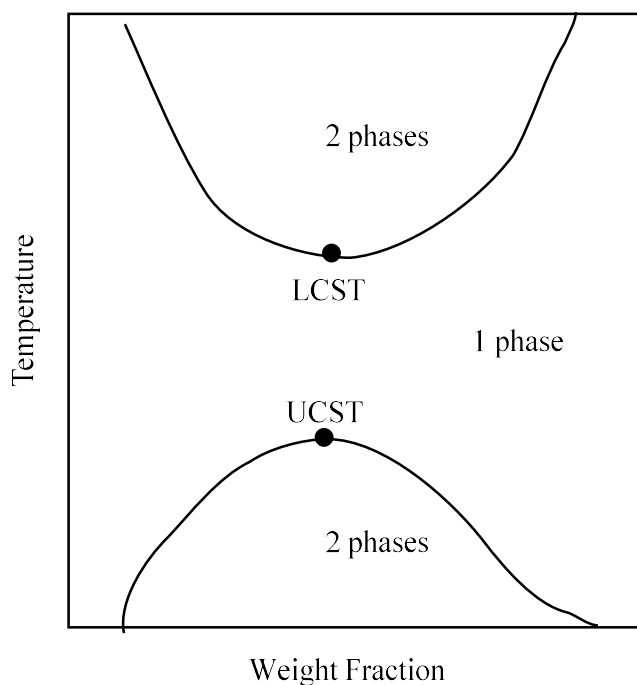


Figure.2 Phase diagram for binary mixtures exhibiting LCST or UCST

Figure.3 shows the polymers with lower critical solution temperature (LCST) are soluble and the polymer chains are relaxed and conformational extended coils in micro perspective below the LCST. Above the LCST, the coils collapse and the polymers become insoluble.[3]

Interaction forces can be the main mechanism that explain the phenomenon of phase transition. There are three kinds of interactions in polymer solution: polymer and polymer, polymer and solvent molecular, solvent molecules themselves. Below the LCST, hydrogen bonding between polymer and water molecule make it dissolve. After heating, interaction between polymer and solvent molecule decreases and intramolecular interactions increases. The liberation of solvent molecules gives rise to the entropy increasing.[16] The type of the forces among the intramolecular interactions could be Van-der Waals forces, hydrophobic interaction and hydrogen bonding. [17]

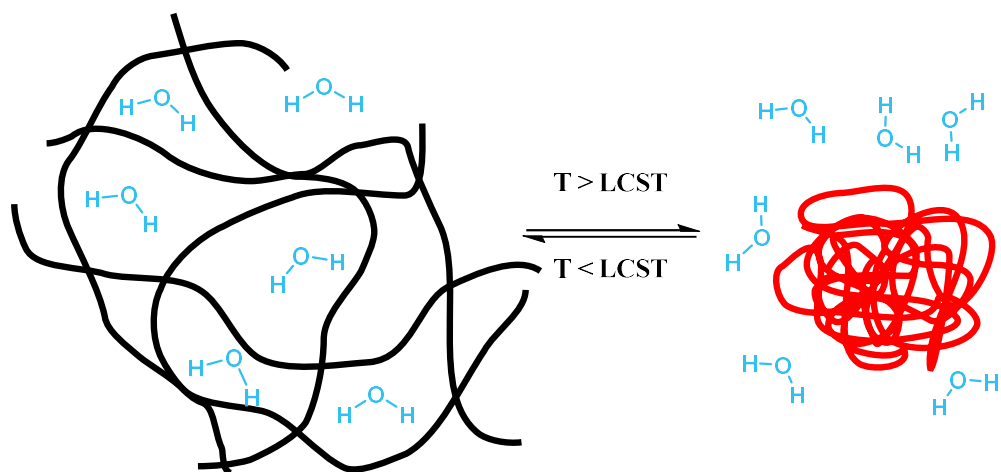


Figure.3 Behavior of LCST-type polymers in solution

2.3 Applications of Thermo-responsive Polymers

Thermo-responsive hydrogels are liquids at certain temperature, and form a gel after being adjusted to another temperature. Polymers with LCST have been more studied for producing such hydrogels than those with UCST, especially in drug delivery systems.

Since the polymers are intended for use in the human body, they should respond close to body temperature.[13] From this point of view, there are a lot of research on PNIPAM which has LCST at 32°C, leading to rapid phase transition and high volumetric collapse.[18] PNIPAM has been applied in the field of drug delivery, tissue engineering and in vitro cell cultures and in the maintenance of specific cell properties. [19]

PNIPAM is mostly produced by radical polymerization. In order to get more possible functional applications in various fields, copolymerization and grafting reactions are utilized. For example poly(N-isopropylacrylamide-co-butylmethacrylate) (structure see *Figure.3*) was used as a liposome carrier for delivery of doxorubicin, showing enhanced drug release in response to temperature fluctuation. In addition, *Poly (N-isopropylacrylamide-co-butylmethacrylate)* is recommended because of lower cytotoxicity. [20]

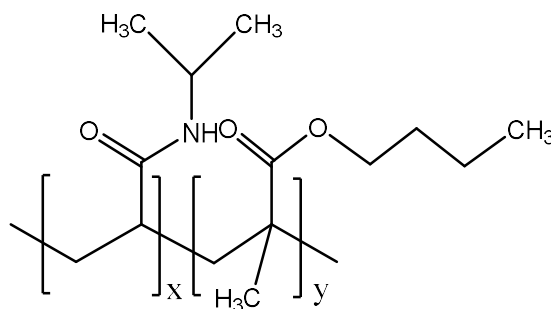


Figure.3 Structure of Poly (N-isopropylacrylamide-co-butylmethacrylate)

2.3.1 Thermo-responsive Block copolymers

Thermo-responsive block copolymers can self-assemble into polymeric micelles, which can be useful for drug delivery and release. Another used strategy is to use tri-block polymers consisting of hydrophobic cores that can encapsulate drug molecules and thermo-responsive hydrophilic block shells that are soluble. For example, Poloxamers, also known as Pluronic® produced by BASF, are composed of a central hydrophobic chain of poly(propylene oxide) flanked by two hydrophilic chains of poly(ethylene oxide). [13] The following *Figure.4* shows the mechanism of PEO-PPO-PEO micellisation. The PPO block chains are soluble when the temperatures is below the critical micellisation temperature (CMT) of the copolymer. The aggregation number and volume fraction of the micelles as well as the hard-sphere (PPO block) increased with increasing temperature. When volume fraction (ϕ_m) >0.53 with high enough concentration, PEO-PPO-PEO block copolymer can form gel. [21] It can be utilized as drug delivery system with drug molecules encapsulated in the hydrophobic core of the micelles as well as sustained drug release system in some case that the drugs in the core would be released slowly consequently. [22, 23]

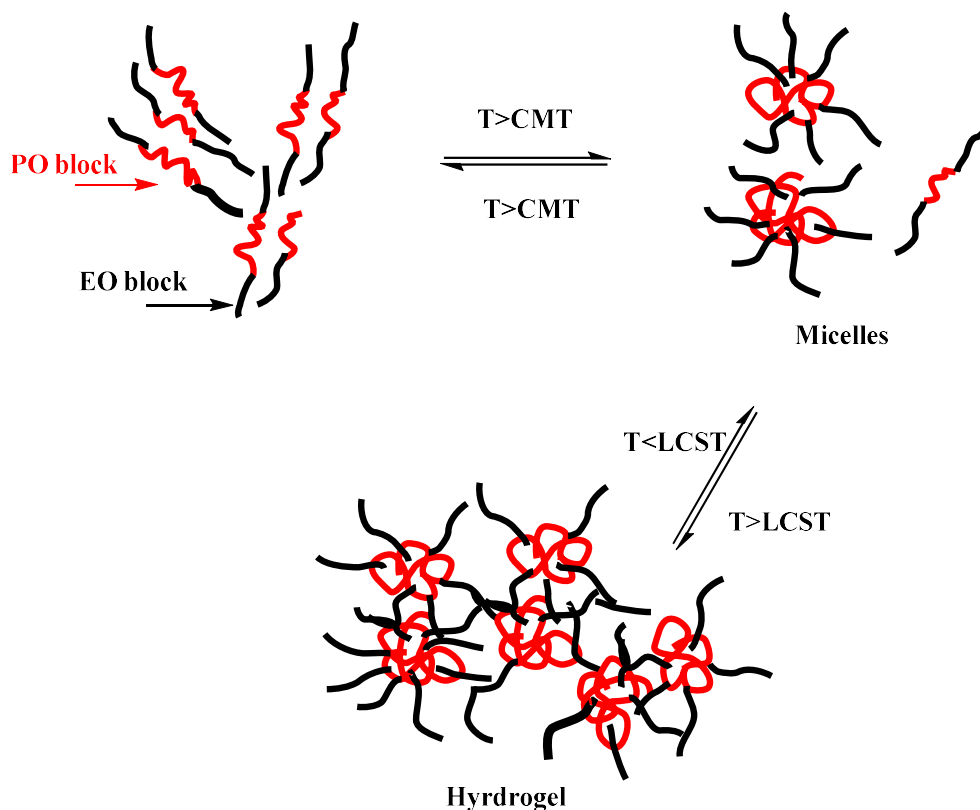


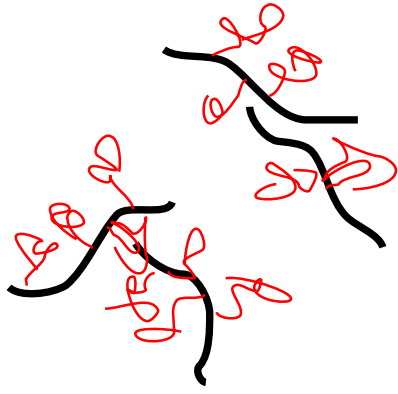
Figure.4 Mechanism of Thermo-responsive PEO-PPO-PEO copolymer

2.3.2 Thermo-responsive polymer grafted polysaccharides

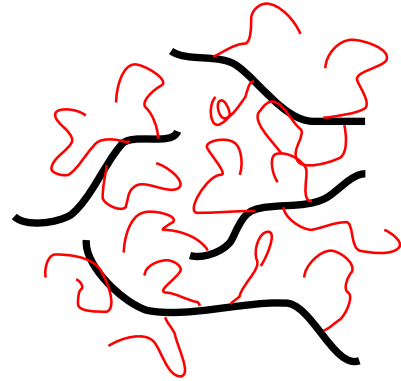
There are plenty of studies on the polysaccharides grafting by polymers with LCST which can produce hydrogel used in bio-applications. For example, PNIPAM grafted dextran hydrogel, HA-g-poly (DEGMA-co-OEGMA) [24-26] and also what has been produced in this experiment, poly (GME-co-EH)-grafted HA. These products are soluble below the LCST of the synthetic polymers. At temperatures above the LCST, the polymer grafts would be hydrophobic and the hydrophobic interaction between the grafts would lead to micellisation or gelation that can be applied in drug delivery system.[27]

Some drugs combine with this kind of hydrogel are able to be released slowly and with constant rate. The diameter and interaction of particle and size and properties of corresponding hydrogel network are rather important since if the size of hydrogel network (matrix) unit is much bigger than drug diameter, the elongation of drug releasing cannot be achieved.[13]

Figure.5 shows that intra-chain entanglements give rise to the aggregation of grafting chains. In addition, the balance of intra- and inter-chain entanglements plays an important role on gel network formation. If inter-chain entanglements are favored, the volume of gel network shrinks. The volume will not change, if there is a balance between inter-and intra-chains entanglements.



A:aggregation
(mainly intra-chains entanglements)



B:gel network
(balance of intra- and inter-chain entanglements)



C:gel network
(mainly inter-chain entanglements)

Figure.5 Inter-or intra-chain entanglements effect on micelle and gel network

3. Hyaluronic Acid

Hyaluronic acid (HA) is a natural polysaccharide composed of N-acetyl glucosamine and D-glucuronic acid as a repeating units. It is widely found in connective tissue, epithelial tissue, and vitreous of the eye tissue as well as the component of extracellular matrix. [28]

3.1 Hyaluronic acid bio-applications

Since HA is a component of synovial fluid, it can be used in clinical practice for treating osteoarthritis. The lack of joint lubricant causes osteoarthritis and the extra injectable HA can improve the condition. In addition, HA may reduce the symptoms of knee osteoarthritis by various mechanisms such as inhibition of chondrodegradative enzymes and synthesis of chondrocytes. [29, 30]

Grafting with different polymers on the polysaccharide chains is an efficient approach to functionalize them. Two examples are shown in *Figure.6*. Polyacrylic acid (PAA) grafted HA (HA-g-PAA) shows a slower degradation than that of unmodified HA since grafted PAA generates steric hindrance so that can prolong the time HA-g-PAA stay in human body for more medical applications. In addition, hydrogen bonding was disturbed which lead to the decreases of viscosity and make injection easier. PAA was detached by hydrolysis and enzymatic degradation by lipase and it has been already tested subcutaneously and intraperitoneally. It is supposed to be used in drug delivery in the peritoneum. HA cannot form the insoluble calcium salt itself [31] while the binding between grafted PAA and Ca^{2+} will form insoluble salt immediately. From the micro-view, PAA changes from a random coil to a compact spherical shape like pearl-necklace. In addition, it has been assumed that if the HA was partially modified by PAA, few hydrophobic functional polymer grafts will make it possible to form gel-like insoluble salts.[32]

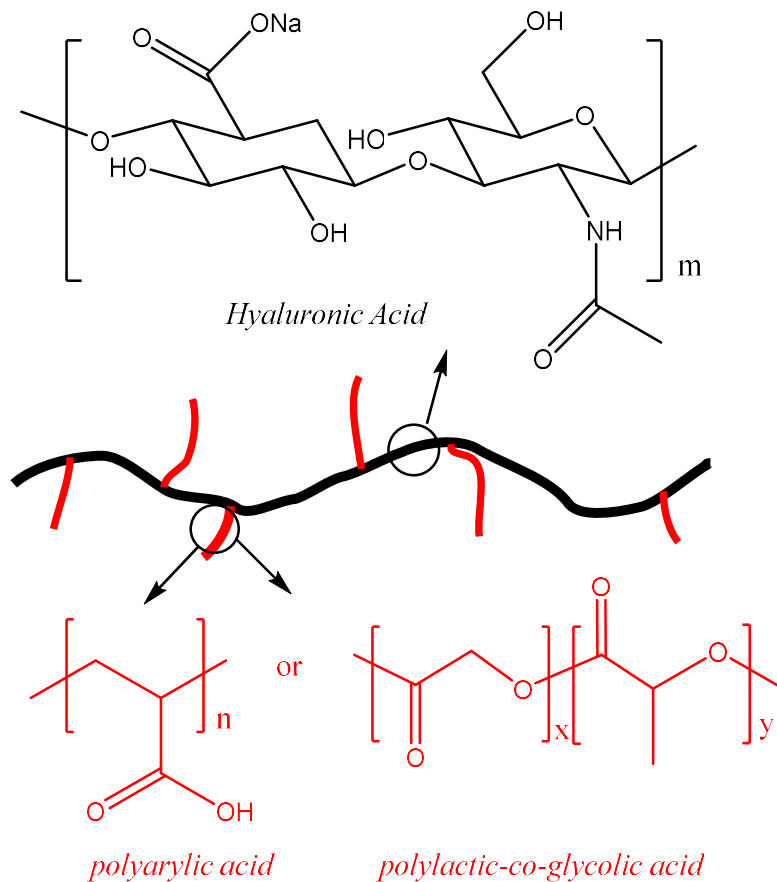


Figure.6 Examples of functional polymers grafted Hyaluronic Acid

Moreover, functional HA-graft copolymers have been reported for controlled release and targeted drug delivery systems for instance in anti-cancer therapy.[15] [28] Hyaluronic acid-cysteamine-poly(lactic-co-glycolic acid) (HA-SS-PLGA) was produced via HA backbone grafted by PLGA with coupling reaction in water-oil-water nano-emulsion. The formed micelles were able to encapsulate common chemotherapy drugs like doxorubicin (DOX) and cyclophosphamide (CYC) and to deliver them directly to the cancer stem cell. As a result, it does less harm to the healthy body tissue and present better effectiveness.[33]

3.2 Click chemistry in Hyaluronic acid bio-applications

The “Click Chemistry” was first introduced by Dr. Sharpless’s group in 1999[34] and later defined as a group of reactions that are modular, wide in scope, with high yields, simple reaction conditions, and easy purification.[35, 36] Briefly there are reliable one-step coupling reactions based on functional groups between two molecules A and B with the advantage of being highly selective as well as water and oxygen tolerant.[35, 37] A scheme can be seen from *Figure. 7*. It has already been used in many pharmacological applications.

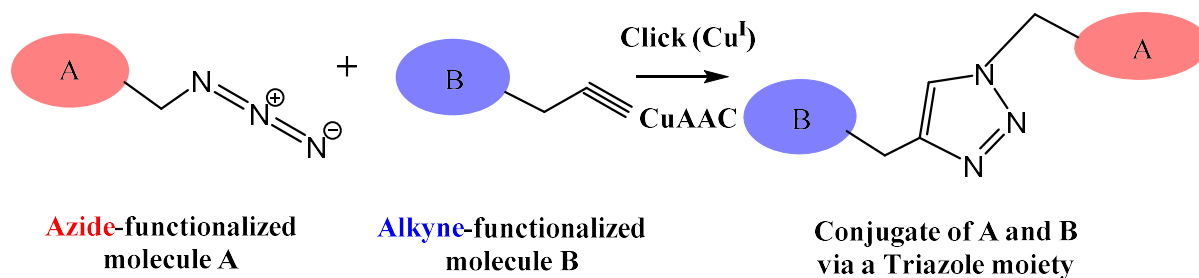


Figure.7 Scheme of Azide-Alkyne click chemistry reaction

The most common example of click reactions is the Cu (I) catalyzed Azide-Alkyne Cycloaddition reaction (CuAAC), which is also utilized in this study. There are also some other reactions, such as thiol based reactions, which have also been widely used.[38] In this case, HA chains have been pretreated to be alkyne-functionalized.[39] The grafting polymer has an azide head group and can be “clicked” on the HA chains with the reaction between alkyne and azide.

4. Ring Opening Polymerization

Among cyclic ethers, three-membered ring epoxides (with the few exceptions of four-membered ring oxetanes) are the only ones that can be polymerized by an anionic or a related nucleophilic polymerization mechanism. Other cyclic ethers with more carbon members can be polymerized by a cationic or electrophilic ring-opening mechanism. [40]

Ring opening polymerization (ROP) is the most important synthesis method for polyethers. 1,2-alkylene oxide and glycidyl ether with different substituent can be polymerized via ROP and especially anionic ring opening polymerization (AROP). [41]

Ring opening polymerization systems often require totally dry system, active catalyst to enable strict control. As for anionic ring opening polymerization, no hydroxyl group can exist in the system otherwise the chain transfer will occur which cause termination of polymerization.

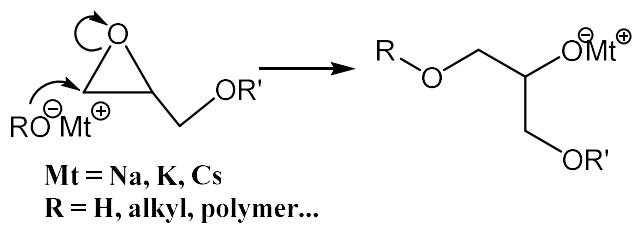
4.1 Conventional Anionic Ring Opening Polymerization

Conventional AROP is widely used in the precise polymerization, when narrow polydispersity and high molar mass are required, the mechanism of nucleophilic attack of propagating chain end to monomer can be learnt easily. Polymerization of poly (glycidyl ether) is shown as an example, the process of conventional AROP can be divided into several steps as most polymerization procedures which is illustrated in the following *Figure.8*. It involves initiation, propagation and termination (chain transfer).[8, 42]

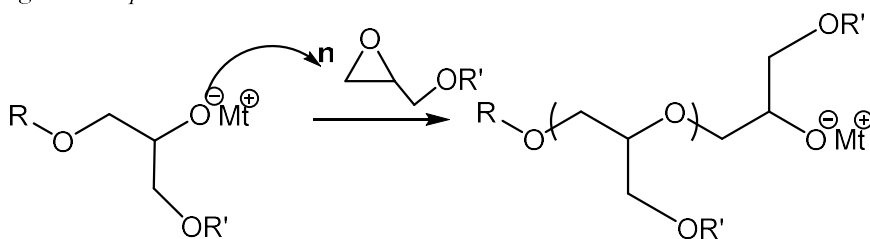
Nucleophilic initiators can be alkoxides for instance. Monomers like EO or other three membered cyclic ether have an electrophilic carbon atom according to the highly electron-withdrawing neighbor oxygen atom. The initiation occurs by the nucleophilic attack of initiator to the electrophilic carbon atom and a new propagating intermediate is formed. The new nucleophilic species will attack the carbon atom in another monomer and repeat this procedure until the monomers are used up.

The termination is always achieved by the chain transfer reaction instead of the theoretical termination step, though it is shown below as well. As a result, this chain transfer reaction give rise to the broader polydispersity and some oligomers.[8]

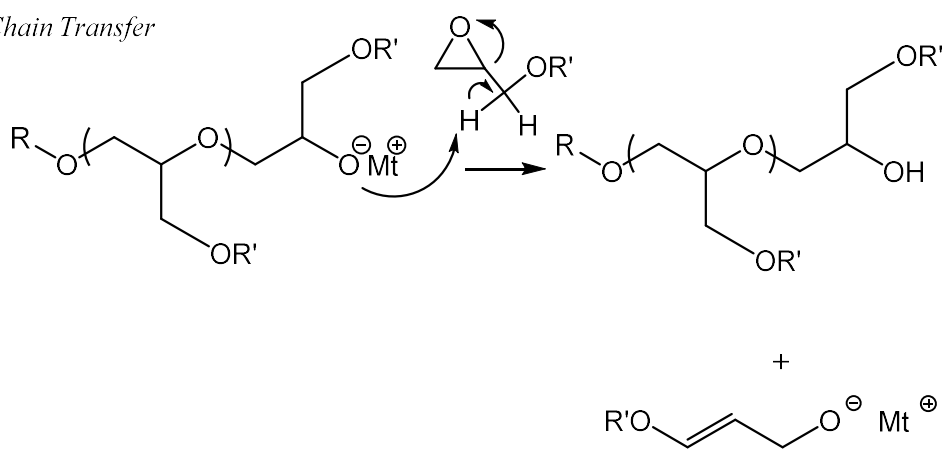
Initiation Step



Propagation Step



Chain Transfer



Termination Step

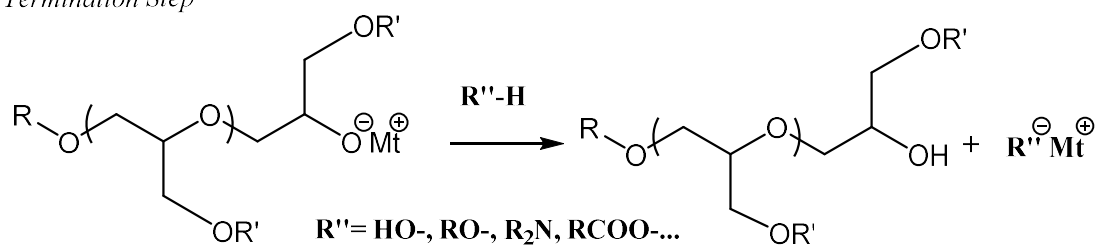


Figure.8 Mechanism of poly (glycidyl ether) produced by conventional AROP

4.2 Monomer Activated Anionic Ring Opening Polymerization

Conventional AROP always yields broad polydispersity of polymers, because of side reactions such as chain transfer in alkali metal anionic polymerization. In addition, copolymerization of different functional epoxide monomers via conventional AROP was shown to give gradient comonomer composition in copolymers.[8] As a result, monomer activated AROP first reported by Billouard in 2004 [43] has gained lots of interest presently. It has been already used in many ring opening copolymerizations including copolymers of glycidyl ether derivatives. [8]

Tetraalkylammonium or tetraalkylphosphonium halides (onium salts) replace the alkali metal initiators and triisobutylaluminum ($i\text{-Bu}_3\text{Al}$) is used as activator. The aluminate species ensures propagation in the AlR_3 /onium systems as it selectively activates the monomer.[40] As 1 equivalent of AlR_3 forms a complex with the initiator, more AlR_3 ($\text{AlR}_3/\text{initiator} > 1$) is needed to activate the monomer[42]. The proper ratio depends on the employed monomers and target degree of polymerization.[8, 9, 42]

Here is the mechanism of AROP of glycidyl ether derivatives (*Figure.9*).

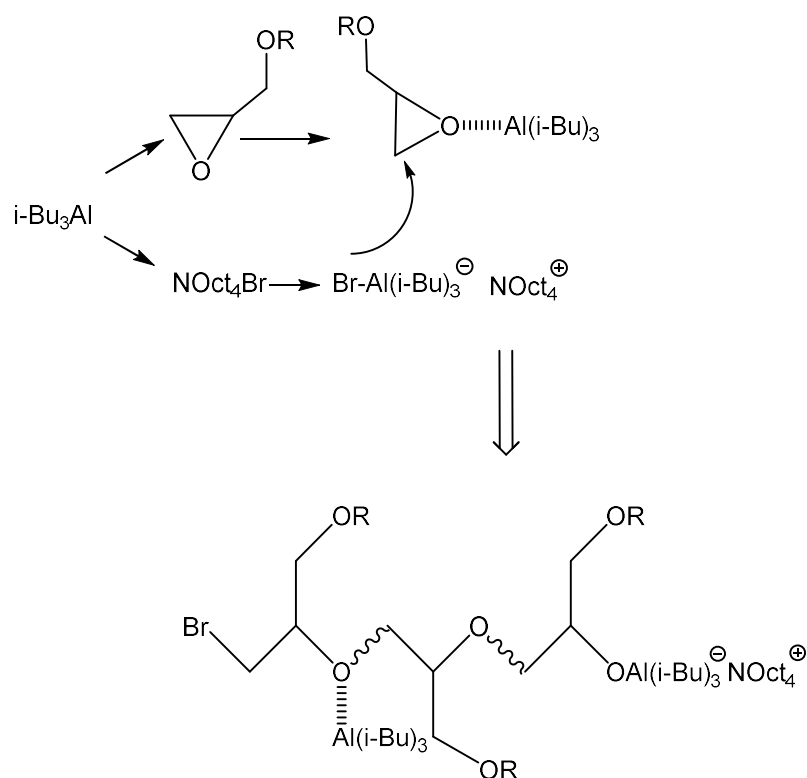


Figure.9 Mechanism of onium salts activated AROP of poly (glycidyl ether)

As for the drawback, metal element in the triisobutylaluminum may cause contamination to environment. There are still some challenges for developing environmental friendly system design. A good example has been reported that metal-free phosphazene base $t\text{-BuP}_4$ catalyst used in Glycidyl Phenyl Ether (GPE) polymerization.[44]

5. Glycidyl Methyl Ether based copolymer

Glycidyl methyl ether is a type of functional epoxide monomer that polymerizes to give a polyethylene oxide backbone with pendant methyl ether groups.[42, 45] Different glycidyl ethers have been studied, such as poly(allyl glycidyl ether) (PAGE) and propargyl glycidyl ether (PGE) [27, 46]. Some of them were block copolymerized to form micelles while some were random copolymerized.[8, 27] The scheme and of copolymerization and the structure of poly(GME-EH) are shown in the *Figure. 10*.

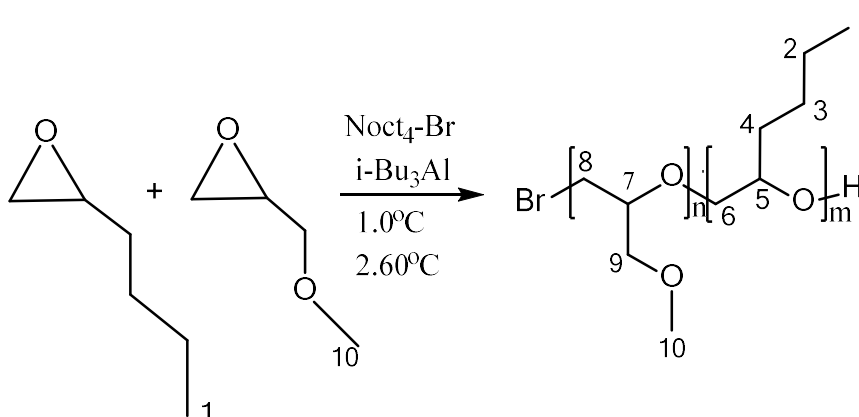


Figure.10 Scheme of copolymerization and structure of poly(GME-EH)

Glycidyl methyl ether (GME) is a kind of commercial and low-cost glycidyl ether polymer with highly biocompatibility.[8] It gains a lot of attractions due to the advantage of remarkable solubility in water in room temperature as well. It has been used for protein repellent coatings and drug delivery systems. [9] The poly (GME) exhibit thermo-responsive property with LCST at 57.7°C .[8] However for biomedical applications a transition temperature closer to body temperature would be favorable. Unlike block copolymerization which is normally applied in production of self-assembled functional polymers, random copolymerization is a general approach to adjust the phase transition temperature to a desired target temperature for designated applications.[8] Random copolymers form thermo-responsive aggregates when they reach the CST, while block copolymers form micelles. [47]

The *Figure.11* below shows the differences between these two copolymers when they are heated above LCST. The copolymerization strategies will be mentioned in the following section.

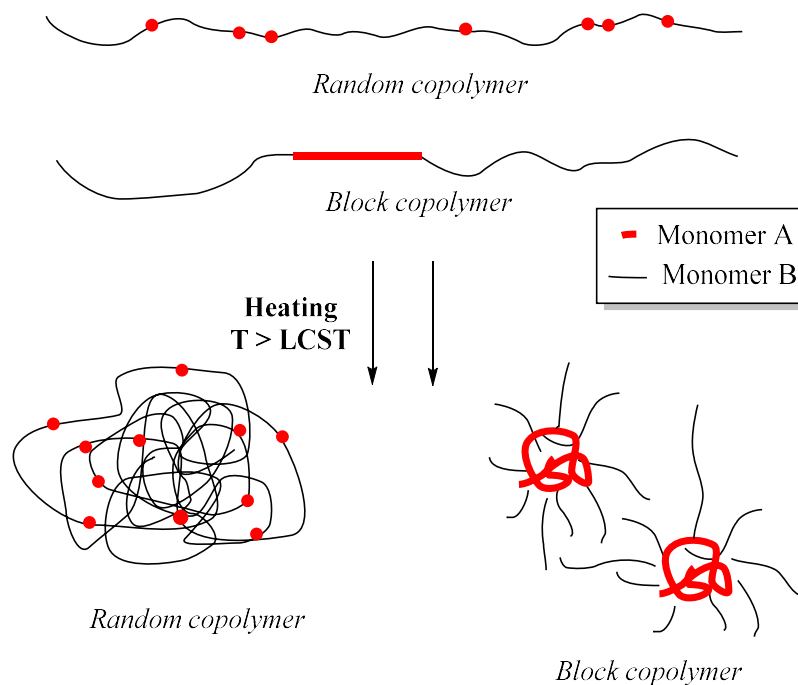
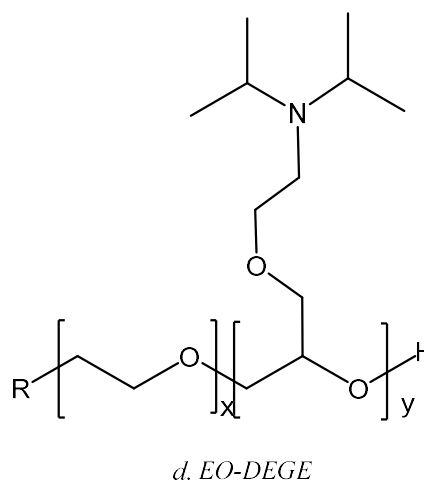
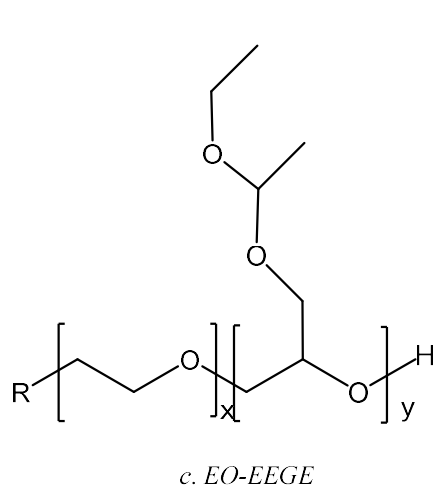
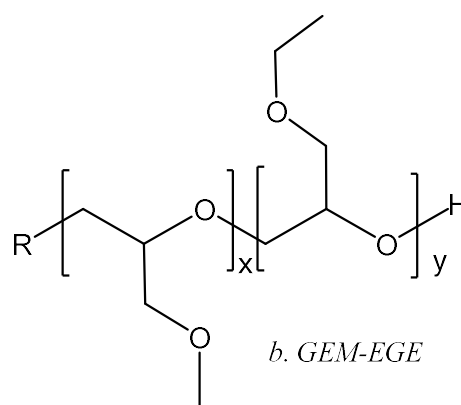
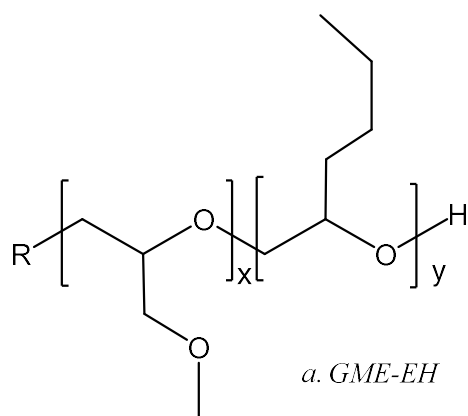


Figure.11 The different behavior of Random copolymer and Block copolymer at $T > LCST$

Since 1,2-epoxy hexane (EH) has low water-solubility,[48] it can be used for copolymerization and adjust the LCST of GME based copolymer, the structure see *Figure. 12 a*. Unlike ethyl glycidyl ether (EGE), which was also used for copolymerizing with GME (*Figure. 12 b*) by Heinen et al., [8] EH has longer alkyl group which gives more hydrophobic property than EGE with alkyl ether substitution so that it could be more efficient and less EH may be needed to adjust the LCST of GME based random poly(GME-co-EH). Therefore EH is a comparably better attempt.

As for the reactivity ratio in copolymerization, the previous researches offer some references. Ethoxyethyl glycidyl ether (EEGE) and ethylene oxide (EO) and copolymer (*Figure. 12 c*) was synthesized by Herzberger et al. [47] and they found that reactivity ratios of EEGE and EO are close to $r = 1$. Reactivity ratio describes how much monomer A and B eager to polymerize with themselves or copolymerize with another monomer. If the reactivity r_1r_2 approaches one, each radical has no preference, random copolymer will be achieved. Ethylene oxide (EO) and N,N-diisopropyl ethanolamine glycidyl ether (DEGE)

copolymer poly (EO-co-DEGE) (*Figure. 12 d*) as well as allyl glycidyl ether (AGE) and DEGE copolymer poly (AGE-co-DEGE) (*Figure. 12 e*) were produced by Lee et al. [49], which are both random copolymer with reactivity ratios close to 1 and $r_{DEGE} * r_{EO} \approx 1$. In addition, the bulky substituents hardly show the influence on the reactivity ratio of poly glycidyl ether. [47, 49, 50] Although EH has longer alkyl substituents compare to EO and reactivity ratios is unknown, random copolymerization could be tried and later NMR examination would give the results.



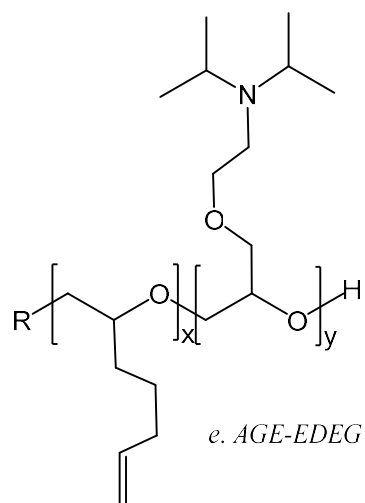


Figure.12 The structures of several glycidyl ether derivatives copolymer

II. Experimental

1. The aim of research

The aim of the research was to produce the thermo-responsive hydrogel of HA (hyaluronic acid) grafted with GME (Glycidyl methyl ether)-EH (Epoxy hexane) random copolymer shown in *Figure.13*.

The thermo-responsive hydrogel was aimed to be used as drug delivery material with biocompatibility and the phase transfer temperature of main component GME (about 60 °C) was to be adjusted to around body temperature by copolymerization with hydrophobic EH monomer. According to the thermo-responsive property of the copolymer poly-(GME-EH), the copolymer chains should collapse when they are heated above phase transition temperature, and inter-and intra-chain forces between grafted copolymers would lead to gelation of the Hyaluronic acid based product. The structures of product and the gelation processing can be seen from the following *Figure.13*.

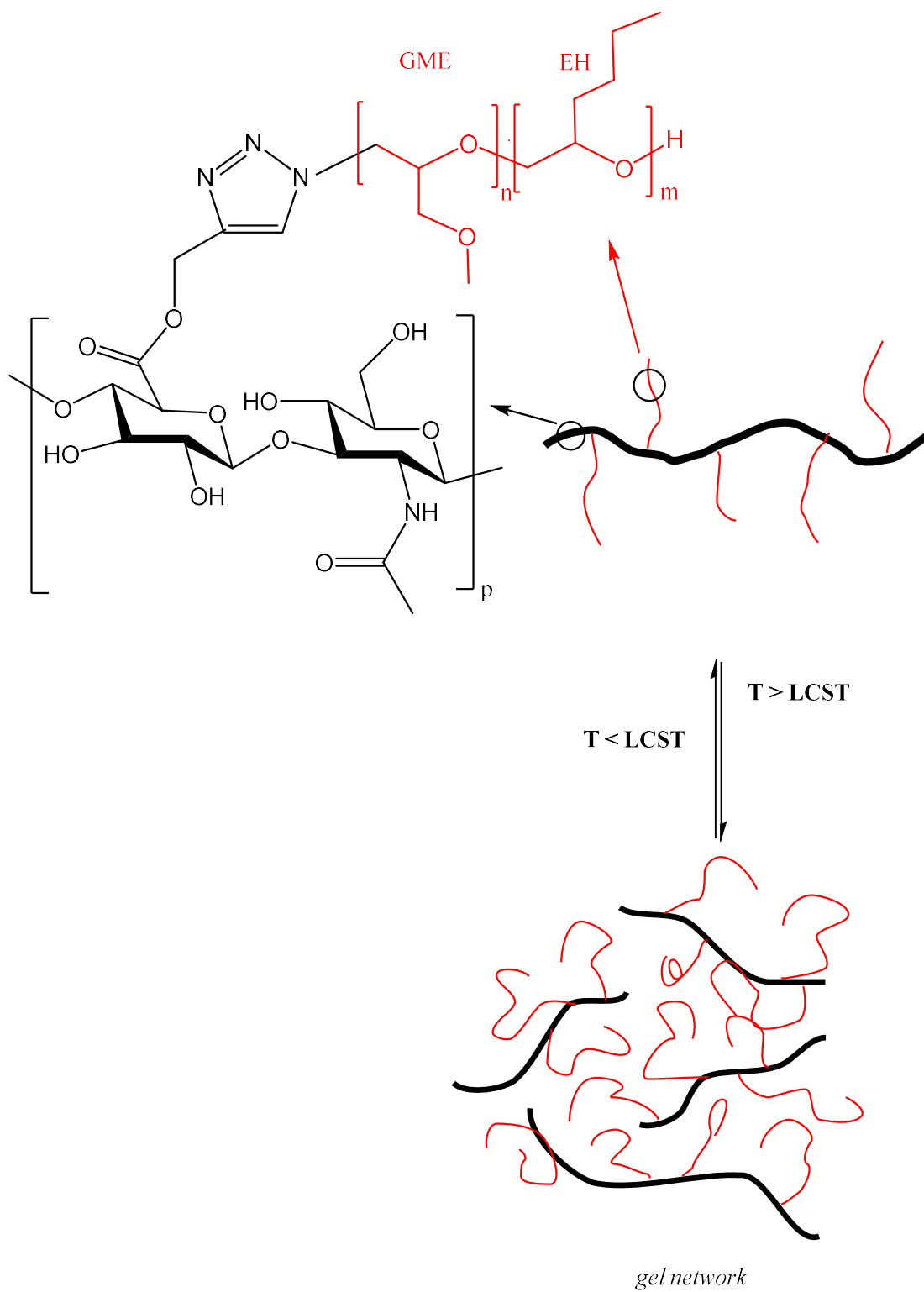


Figure.13 Intended structure of final product and process of gelation

2. Materials

Glycidyl methyl ether (GME, >85%, TCI, M=88.11) and 1,2-epoxyhexane (EH, >96%, TCI M=100.16) need to be purified in following steps: Molecular sieves (\AA 3) equal to ~10 weight % of the monomer were dried in 350 °C oven overnight then cooled down to room temperature in a desiccator and moved to a round bottomed flask. Monomer was refluxed with calcium hydride overnight and distilled under water pump vacuum. The purified monomers were stored with 3 \AA sieves (10 wt%) in the flask in the freezer until use.

Toluene was used as the solvent for initiator and it was firstly purified by refluxing with calcium hydride overnight then distilled (boiling point=110°C) under argon. The solvent was stored over 3 \AA sieves (10 wt%) at room temperature and DMF was stored with 4 \AA sieves (10 wt%) at room temperature.

Water used in all syntheses had been distilled. Chloroform, dichloromethane (DCM) and methanol were obtained from Fisher Scientific and acetone, dimethyl sulfoxide (DMSO), hexane and tetrahydrofuran (THF) from Sigma Aldrich. All the solvents used for synthesis were of HPLC grade and used as received.

NMR solvents were obtained from Euriso-Top. Purity of deuterated chloroform was 99.80 % D and of deuterium oxide was 99.96 % D.

Tetraoctylammonium bromide (NOct₄Br, Sigma Aldrich, 98%) was used as initiator which was dried in vacuum then solubilized into dried toluene with the concentration of 0.3mol/L and stored in the fridge.

Triisobutylaluminum (*i*-Bu₃Al, Sigma Aldrich, 25wt% 1.0M in toluene), *N,N,N',N'',N''*-pentamethyldiethylenetriamine (PMDETA), sodium azide (Sigma Aldrich, >99%), ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich).

Hyaluronic acid propargyl ester (DS=17%; M(RU)=404.03g/mol, *c*≈3g/L) were used as received.

Flasks were silanized by following steps: A 25 ml 2-necked flask and stir bar were carefully washed, rinsed with acetone and dried in oven. The stir bar was inserted into the flask and both were cooled to room temperature. The flask was fully filled with dichloromethane, then 8 drops of triethylamine (98.0 %, Fluka Analytical) were added, followed by 10 drops of dichlorodimethylsilane (99.5 %). The flasks with solution were left at room temperature under stirring overnight. The solution was discarded, the flasks rinsed with dichloromethane 3 times and dried in an oven until needed.

3. Synthesis

3.1 Synthesis of Poly-(GME-EH) with -Br-end group

Thermo-responsive copolymer poly-(GME-EH) was synthesized using tetraoctylammonium bromide as initiator and triisobutylaluminum as catalyst with various ratios of GME and EH monomers. The polymerization scheme is showed above as *Figure.10*.

The silanized flasks used for anionic polymerization were flame dried, filled with argon and weighted in room temperature. GME (84.4%-90.9% in feed), EH (9.1%-15.6% in feed) and toluene (total monomer concentration 4 mol/L) were added by syringe sequentially. The amount of initiator solution (Noct₄-Br in dried toluene) and catalyst (triisobutylaluminum) was adjusted to different targeting DP with the fixed ratio 1/5.

$$n_{(GME)+n_{(EH)}} / n_{(Noct4-Br)} = DP_{theo}$$

The flask was cooled in the ice bath to 0°C and Noct₄-Br and triisobutylaluminum were added drop by drop sequentially under argon. The reaction mixture was stirred at 0 °C for 30 min, then transferred into an oil bath at 60°C. The mixture was stirred overnight (18h-22.5h). To stop the reaction, 1 ml methanol was added into the flask for termination. The completion of the reaction was confirmed by ¹H NMR by disappearance of the epoxide ring protons between 3.30 and 2.50 ppm. The flask was washed by chloroform. The product mixed with chloroform was dropped in to cool diethyl ether and residual triisobutylaluminum was precipitated and filtered off. Rotary evaporator was used for removing solvent (DCM, chloroform and methanol). A yellowish viscous product was obtained and stored in the freezer. The yield was close to quantitative in all cases.

To remove residual initiator salt, the polymer was precipitated in pentane. Therefore, the polymer was dissolved in dichloromethane and added to 350ml pentane under stirring. The pure polymer was collected by centrifugation (Sigma Laboratory Centrifuge, 2K1SC). Since the precipitation was difficult to control, most of products were lost. The average yield was less than 20% but pure polymers were shown obtained by NMR.

3.2 Substitution of bromide end group

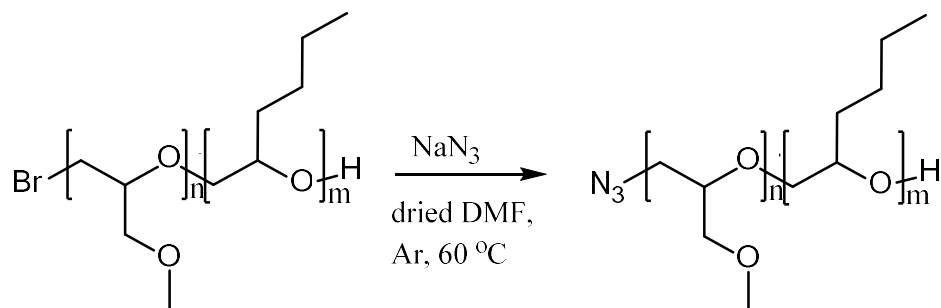


Figure.14 Substitution of bromide end group

The substitution reaction can be seen above in *Figure.14*. Copolymer poly-(GME-EH) (pol3 457.2 mg) was dried in vacuum to remove water before the substitution reaction. The poly-(GME-EH) was added in a dried clean flask under argon. DMF (4.3 ml) as solvent was injected into the flask by syringe and sodium azide (18.9 mg, 6eq. compared to the amount of bromide) was added. The flask was immersed into an oil bath at 60°C and stirred overnight. DMF was removed by rotary evaporation (70°C , 50kpa) and the residue was diluted with dichloromethane and water. NaCl was added into the two-phase solution. The organic phase was extracted 3 times with aqueous NaCl and collected. Organic phase then was dried with anhydrous magnesium sulfate, filtered and evaporated to obtain colorless oil-like product. The yield was 434.4mg (91.2%).

3.3 Grafting polymer with HA by Click Chemistry

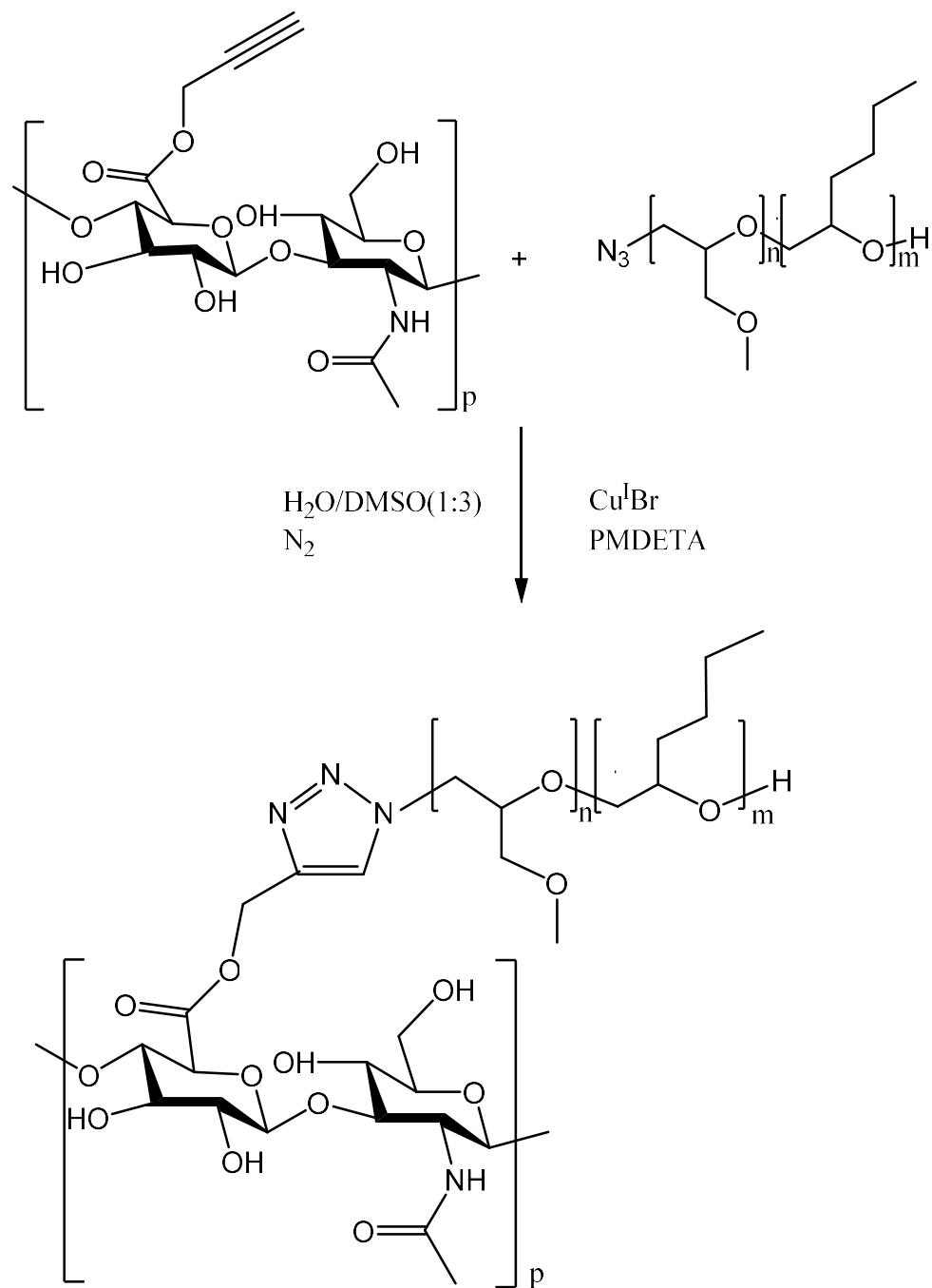


Figure.15 Scheme of grafting reaction by click chemistry

The scheme of click chemistry can be seen in *Figure.15*. HA-propargyl ester (27.6 mg, DS=17%; M (RU) =404.03g/mol, $c \approx 3$ g/L) was weighted in the dried clean flask. Copolymer poly-(GME-EH) (Pol3 313.7 mg) was dissolved in 2.5ml water first. The solution was added to HA and the mixture was cooled in ice bath. 7.5ml DMSO was added drop by drop under stirring. The mixture was degassed by 3 freeze-pump-thaw cycles. Copper (I) bromide (3.5 mg, 2.0eq, M=143.45g/mol) in 1 ml DMSO was added into the solution. PMDETA (0.5 μ L, 0.2eq, M=173.30g/mol, ρ =0.83g/ml) was added via automatic pipette under Argon. The reaction mixture was stirred over 48 hours at room temperature under Argon and protected from light. Resulting solution was dialyzed against 0.01mM EDTA_{aq}, 0.1M NaCl_{aq} and water for 2.5 hours and 3 times respectively, following by lyophilization to give the final product. The yield was 55.8 mg (16.3%).

4. Characterization

4.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

¹H-NMR and ¹³C-NMR spectra were recorded by the Bruker Avance III. Chemical shifts (δ) are given in ppm. ¹H-NMR was used to determine the purity of monomers, conversions of the reactions, LCST and compositions of copolymers.

4.2 Gel permeation chromatography (GPC)

In addition, the molar mass and PDI of copolymer poly-(GME-EH) were determined by the GPC using THF as the eluent containing 1% toluene and molar masses calculated against polystyrene standards (Scientific Polymer Products, Inc.). Separation was achieved by Waters Styragel Guard Column, Styragel HR1, HR2, and HR4 columns. HA-grafted poly-(GME-EH) were measured in 0.1 M aqueous sodium nitrate (NaNO₃) containing 3% acetonitrile and molar masses calculated against polyethylene oxide standards (PSS Polymer Standards Service). The column set consisted of a TOSOH Guard column PWXL, TSK gel G3000 PWXL, G5000 PWXL, and G6000 PWXL.[51]

4.3 Fourier-transform Infrared (FT-IR) Spectroscopy

IR spectra were recorded by the PerkinElmer Spectrum One ATR-spectrometer at room temperature. Spectra were used to determine the structural features of the products for example presence of azide-groups.

4.4 Turbidimetry

Phase transfer of the thermo-responsive block was determined by the JASCO J-8145 CD-spectrometer.

The turbidity recorded in distilled and deuterated water with sample concentration of 10g/L, heating from 5°C to 80°C and back to 5 °C with a heating rate of 1 °C/min.

4.5 Micro-Calorimetry

Micro-DSC were recorded by the MicroCal DSC Instruments with VPViewer 2000 DSC software. The Spectra were used to determine the phase transition temperature of the thermo-responsive polymers with high thermo-sensitivity.

The sample with concentration of 10g/L were placed in instrument pans and equilibrated at 5°C for 30 minutes before heating it from 5°C to 95°C and cooled to 5°C with different heating rate at 90K/ h, 60K/ h, 45K/ h, 30K/ h respectively. The first test with heating rate of 90K/ h was repeated twice to ensure the accuracy.

4.6 Rheology

The sample used for final product rheology test was dissolved in distilled water with concentration of 100g/L. TA Advanced Rheometer-2000 was used for temperature and frequency sweeps. The sample was heated from 20°C to 40°C with the heating rate of 1°C/min at a strain of 1% respectively.

III. Results and discussion

1. Synthesis of Poly-(GME-EH) with -Br-end group

The poly-(GME-EH) random copolymers were synthesized by anionic polymerization with the molar feed of GME from 84.4% to 90.9 %. Several copolymer samples were produced to test the proper phase transition temperature around body temperature (35°C-40°C). The first polymerization (Pol1) failed since the initiator tetrabutyl azide did not dissolve in the toluene solvent and showed obvious phase separation. The initiator was changed to tetraoctylammonium bromide, which can be dissolved in toluene. Pol2 was produced by low conversion (about 50%) with the low initiator / catalyst ratio of 2. The proper ratio was 5 since excess *i*-Bu₃Al was needed to form complex to activate the monomer to the growing chain. [52] Sequences of different monomer feed in Poly-(GME-EH) copolymer were obtained with a conversion of 100% as it shows in Table.1.

Toluene was forgotten to be added in Pol3 and Pol4 accidentally. Pol5 contained residual initiator salt since the solution was turbid below phase transition temperature and the residue was removed by precipitation which has been proven to be efficient.[8] The Pol5 was pure afterwards but the yield was quite low. The GPC data of Pol5 was unfortunately missing since there was no sample left before GPC examination.

The missing Pol6, Pol7 and Pol8 in table 1 failed to polymerize because of the invalid initiator solution. The long storage time made initiator form crystals in the toluene and rather hard to dissolve again which gave rise to the failure.

The molecular weight distributions were measured with GPC calibrated with polystyrene standards. As PS has larger hydrodynamic volume in THF compare to poly (GME-EH). GPC gives smaller than the real molecular weights.[53] The PDI can however be compared between different samples. GPC of poly(GME-co-EO) by Müller et al. [9] however used PEG as the standard which has much more similar structure with poly(GME-EH) and should give more realistic values.

The molecular weights of Pol3 and Pol4 were much lower than the theoretical values. Also the PDI of Pol3 and Pol4 were 1.60 and 1.57, respectively, which are not expected for a supposedly controlled polymerization. The reason is that the solvent was forgotten to be added so that the monomer concentration of Pol3 and Pol4 were quite high, representing 40.5 mol/L and 41.8mol/L respectively. After monomer concentration was corrected back to 4 mol/L, the molecular weights of Pol9 and Pol10 were closer to the theoretical one and the PDI was narrower than Pol3 and Pol4. A high concentration of monomer gives rise to an increasing polymerization rate and a gel-like high viscosity solution formed immediately after catalyst was added and the polymerization was not controlled. Diluted monomer solution bring better control over polymerization. [54] [8]

Table 1 shows that Pol3 and Pol4 have similar GME content corresponding to the monomer feed. However, in Pol5, Pol9 and Pol10, GME content in the copolymers are much lower than that in their monomer feeds. The only difference can be found is the monomer concentration of the feed mixture for these two groups of copolymers. Pol5, Pol9 and Pol10 were made with monomer concentration of 4 mol/L. Almost no reports show the trend like this: the conversion of polymerization is nearly 100%, but glycidyl ether content in copolymer is much lower than that in feeds. By contrast, other reported glycidyl ether copolymers did not have such deviation.[8, 9, 50, 55] There is no proper explanation to be found for this result at the moment and it should be solved out in future study. However the mistakes by operation can be considered as a possibility.

Table 1: Sequence of Poly-(GME-EH) copolymers with different GME composition

Name	GME feed mol%	GME NMR mol%	C _{feed} / mol /L	t/ h	Yield / mg	DP _{theo}	Mn _{theo} / g / mol	Mn _{GPC} / g / mol	PDI
Pol3	84,4	82,0	40,5	22,5	808,5	322	29 000	17800	1,60
Pol4	90,9	88,2	41,8	18	1715,2	325	28700	18300	1,57
Pol5	83,9	76,0	4,00	18,5	1109,0	98	8800	-	-
Pol9	86,6	80,7	4,00	20	2000,2	101	9100	6100	1,16
Pol10	87,0	73,9	4,00	20	630,6	197	17000	12500	1,33

The success of copolymerization was further demonstrated by comparison of ¹H-NMR data of GME, EH and Poly(GME-EH)(*Figure. 16*). The protons from monomers and copolymer are numbered. The protons from epoxy hexane marked as 2, 3, 4 in the scheme correspond to the broadened signals between 1.18-1.57 ppm in *Figure. 16*. The signals marked as 5-10 at 3.35-3.75 ppm appeared in the Poly-(GME-EH) spectra. Methyl group on number 10 in GME and Poly-(GME-EH) spectra showed sharp peak at 3.35-3.40 ppm. The protons of the methyl group of epoxy-hexane were barely influenced by polymerization and showed sharp signals in both EH and Poly-(GME-EH) spectra at 0.9 ppm. In addition, measurement conversion samples showed 100% of conversion.

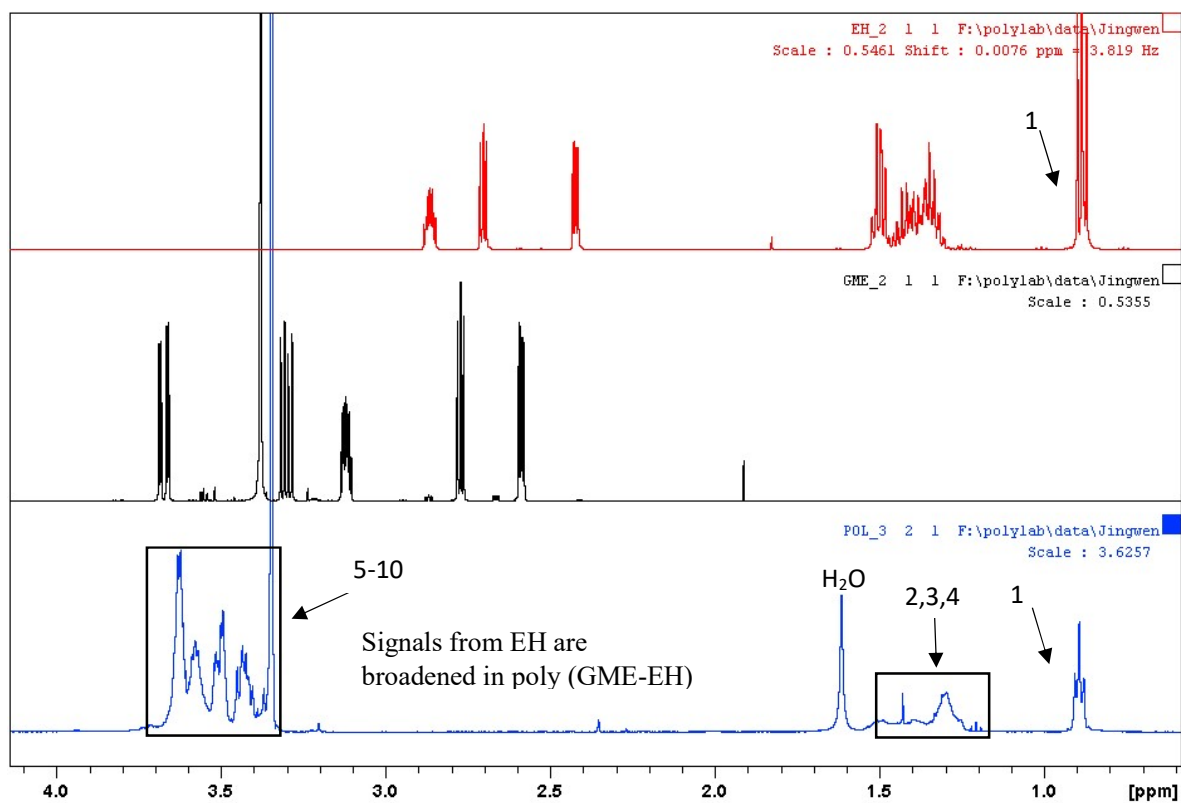
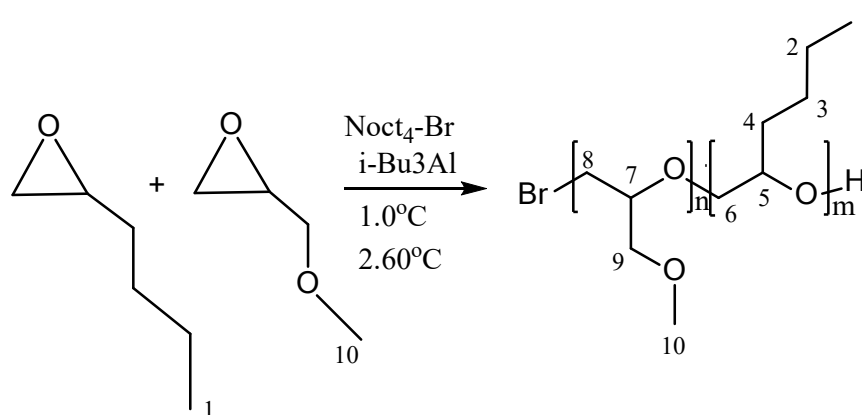


Figure.16 comparison of ¹H-NMR data of EH (top), GME (middle) and GME-EH-Pol (bottom)

2. Phase transition temperature determination

The phase transition temperature of Poly-(GME-EH)s was measured by three methods, turbidity measurements, microcalorimetry and variable temperature NMR.

2.1 Phase transition temperature determined via NMR

The sample Pol3 was used for phase transition temperature measurement by NMR. The sample was heated from 1°C to 55°C with rate of 1°C/ min. *Figure.17* shows the NMR spectrum of Pol3 measured during the heating process.

According to the assignment in *Figure.17*, the methyl group 1 from EH is visible in D₂O and remains unchanged with increasing temperature, which means the structure of copolymer is not blocky but rather random. Also the EH does not influence so much on copolymer conformation except phase transition temperature. In addition, the amount of EH in the copolymer is small which make the change even more invisible. The weakening and broadening peak 5-10 indicate the conformational change of copolymer chain, with incomplete collapse.

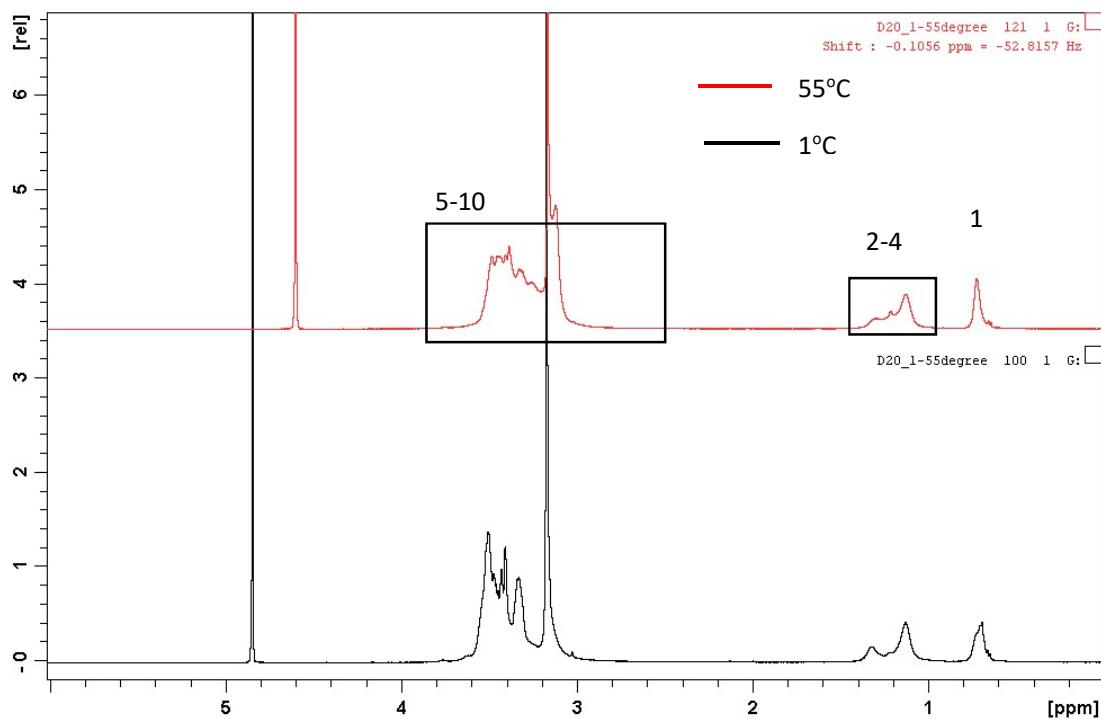


Figure. 17 NMR spectrum of Pol3 measured at 1°C and 55°C

The ether methyl group of GME, marked as No.10 shows obvious changes as shown in *Figure.18*. The sharp peak in 3.281 ppm transfers gradually to 3.218 ppm and it broadens in shape at 55°C after heating. This shows that the copolymer chain collapses when the solution reaches the phase transition temperature and the side group (ether methyl group of GME) collapse so that the signal decreases and shifts to lower ppm since the chemical environment changed. The transferring is however gradually without obvious change between any two temperature lines. It means that there is no sharp transition.

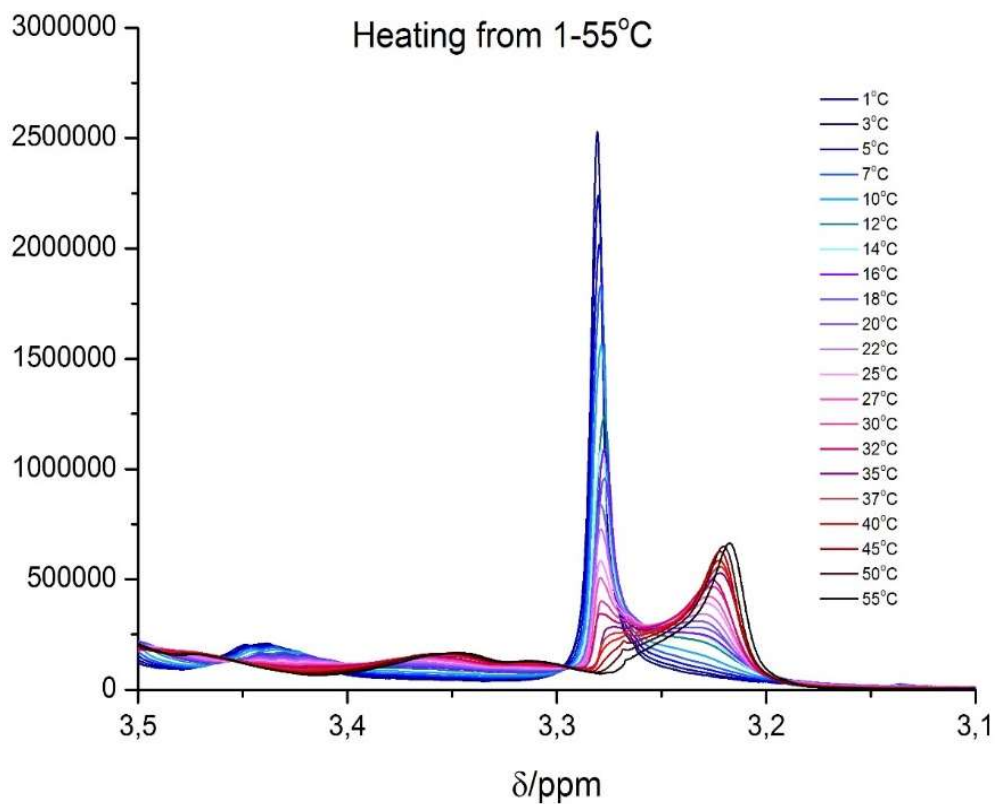


Figure.18 Detailed NMR spectrum of ether methyl group in Pol3 under heating procedure from 1°C to 55°C

As it can be seen in *Figure.19*, D₂O peaks were integrated as 1 in both 1°C and 55°C spectrum. The peaks of ether methyl group was found to transfer and broaden after heating from 1°C to 55°C. The integration at the peak of ether methyl group increased from 2.429 to 3.161 which illustrated the broadening so that the collapse of copolymer chain was determined.

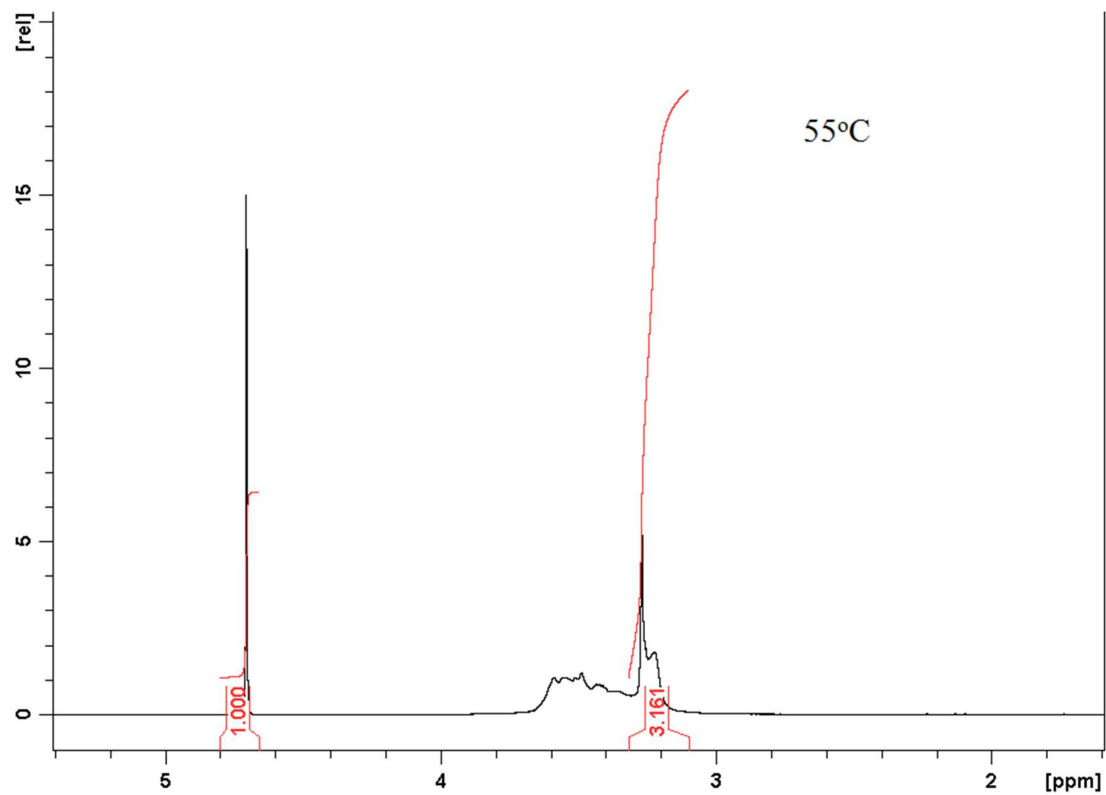
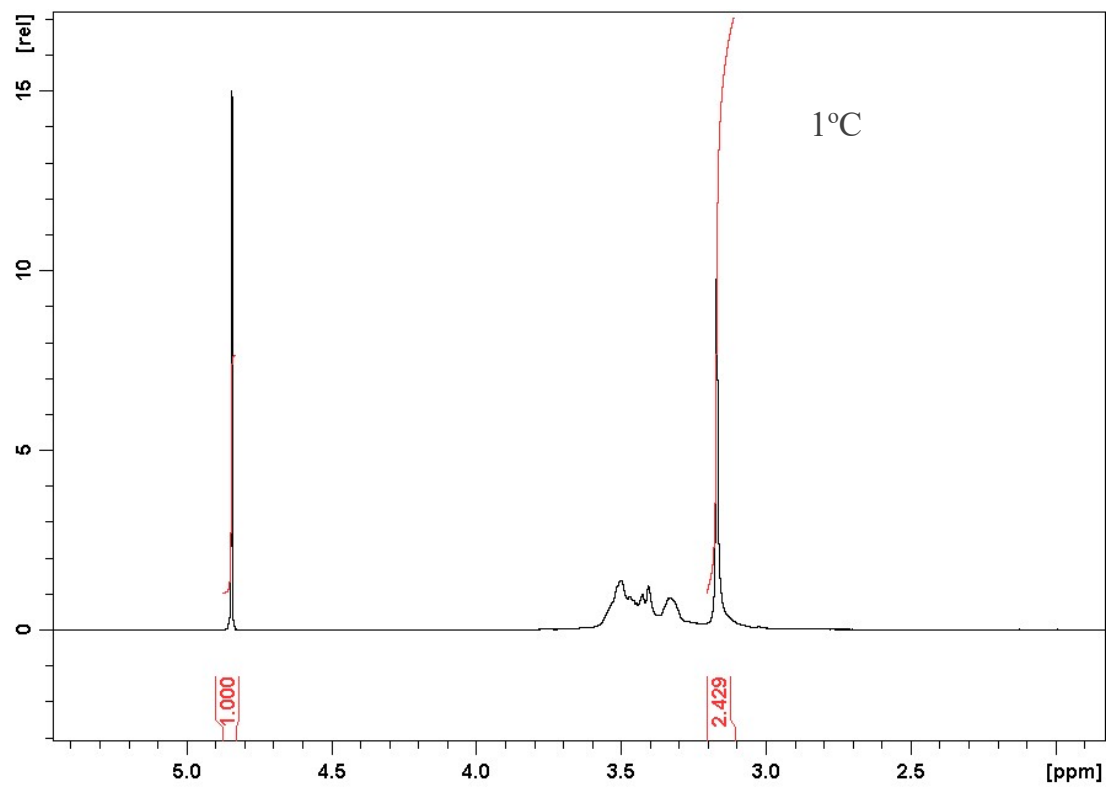


Figure.19 Integrated NMR spectrum of ether methyl group in Pol3 of 1°C and 55°C

Since the transition was too weak to observe directly from *Figure.18*. *Figure.20* below shows the value of signals at 3.281ppm divided by that at 3.218ppm. The ratio decreases with the temperature increasing and the curve showed a sudden turn at around 14°C which indicates the copolymer chain started to collapse. The transition temperature of 14°C obtained by this method is very different from the temperature obtained from two methods that would be discussed below.

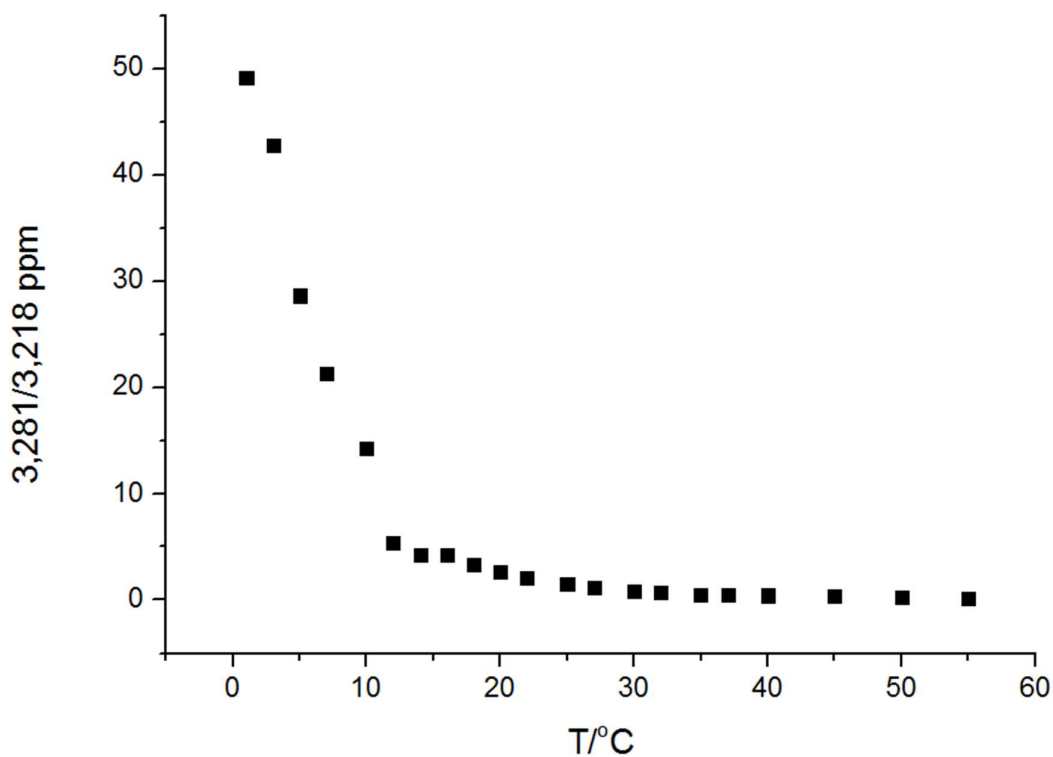


Figure.20 The ratio of signal intensity at 3.281ppm divided by that at 3.218ppm

2.2 Phase transition temperature determined via turbidity measurements

The turbidity measurements of Pol3 and Pol4 are shown in *Figure.21*. The samples were heated from 5°C to 80°C, back to 5 °C and repeating the heating procedure with a heating rate of 1 °C/min. The phase transition temperature of Poly(GME) is hardly influenced by the molecular weight.[56] While the phase transition temperature is strongly affected by the GME-content of the copolymer. [55, 57]

The *Table.2* shows the phase transition temperature response to the GME content and the turbidity curves are plotted in the *Figure.21*. The dotted line represented Pol4 with higher GME content (88.2%) than the Pol3 (solid line) with 82.0% of GME in copolymer. The phase transition temperature of Pol3 was 28 °C, which was lower than that of Pol4 (49°C) as expected. The phase transition temperature of Poly-(GME-EH) depends on the amount of GME in the copolymer and higher percentage of GME gave rise to the higher phase transition temperature. The hydrophobic comonomer EH was aimed to adjust the LCST of the copolymer with random structure. Such dependency on the composition of the comonomer feed was also found in random copolymers of various of vinylbenzylethers as comonomers with different phase transition temperatures produced by Weiss et al. [58] as well as random copolymers of 2-(2-methoxyethoxy)ethyl methacrylate (MEO2MA) and oligo(ethylene glycol) methacrylate (OEGMA) produced by Lutz et al. [59]. According to random copolymer poly (GME-EGE) reported by Heinen et al. [8], ethyl glycidyl ether was used for copolymerization instead of EH. The copolymer with 1:3 (GME: EGE) showed phase transition temperature at 16°C and the phase transition temperature was 45°C with 3:1 (GME: EGE) . Compared to the phase transition temperature of 28 °C given by 82% GME content in this experiment, more EGE is needed to decrease the phase transition temperature than EH. This EH has more hydrophobic alkyl substitute compared with EGE with ether group. Thus the more hydrophobic the comonomer is, more efficient it would be.

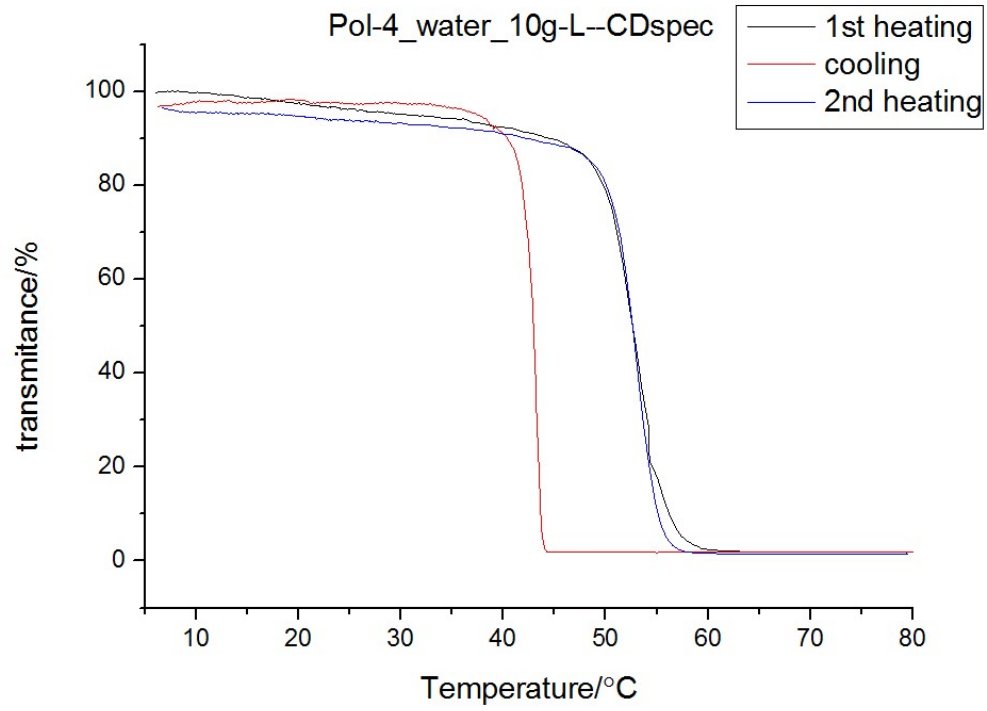
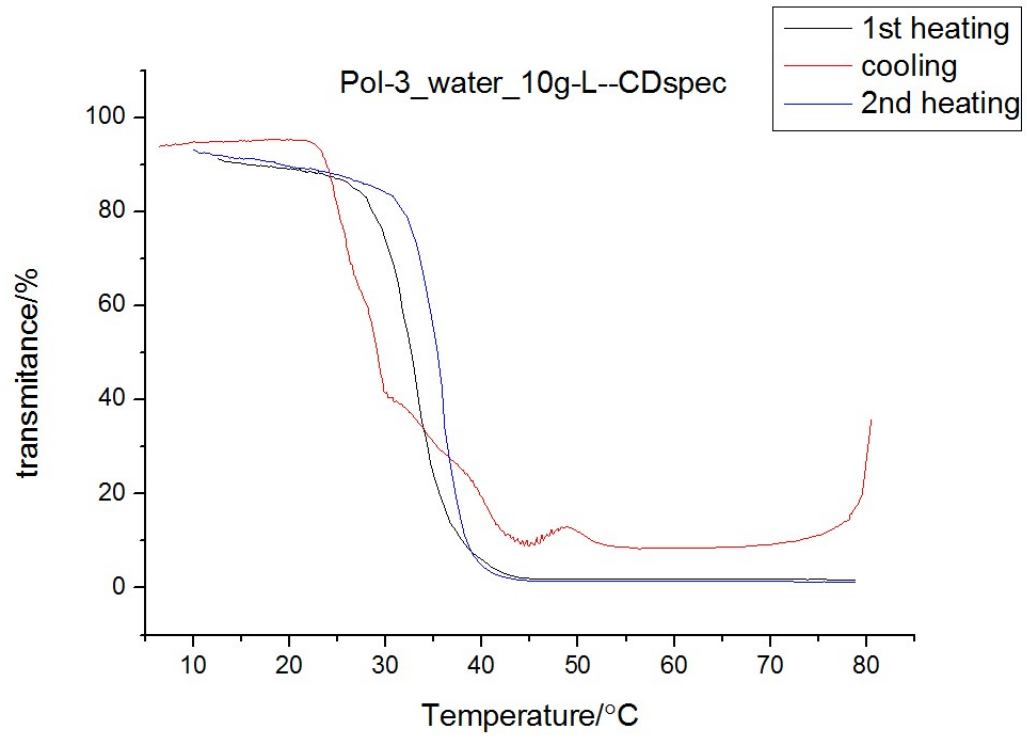


Figure.21 Turbidity of Pol3 and Pol4 measured by CD-Spectrometer with 10g/L in water

Hysteresis was observed when heating (black and blue line) and cooling (red line) curves were compared. The process is reversible since the second heating and first heating curve have similar shape, but the system reacts slowly on cooling process. Normally intramolecular hydrogen bonding of polymers in the collapsed state cause the hysteresis phenomenon, for instance in PNIPAM.[59] The similar Poly (GME-co-EGE) copolymers show almost no hysteresis within one heating-cooling cycle. [57] From *Figure.22* it can be seen that Pol 4 with higher GME content shows greater hysteresis phenomenon. The reason is still unknown that why the result was different to the previous reports.

<i>Table.2 GME content in polymer and their phase transition temperature</i>	Pol3	Pol4	PGME
Percentage of GME	82.0%	88.2%	100%
Phase transition temperature	28°C	49°C	57.7°C[8]

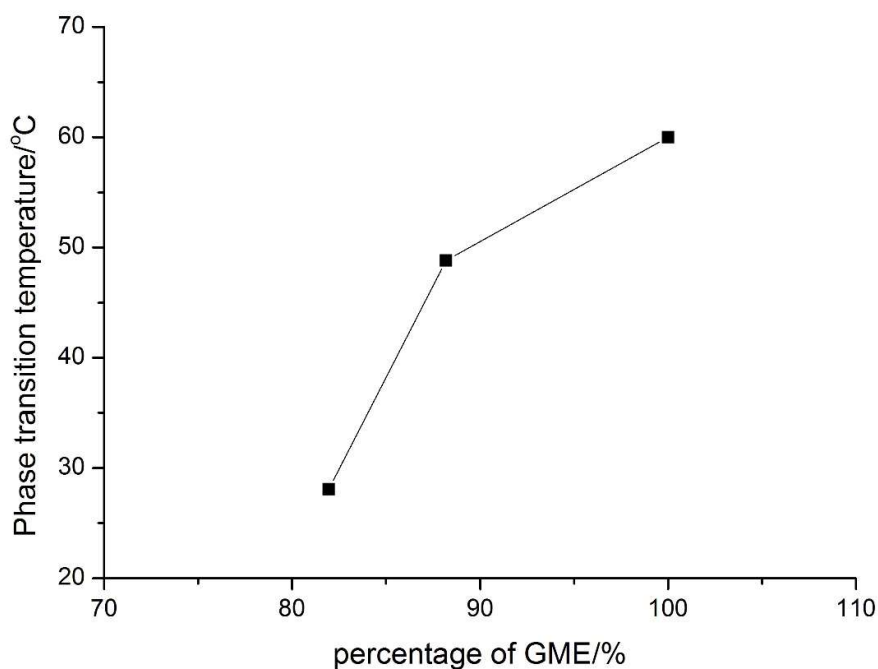


Figure.22 Variation of phase transition temperature influenced by the percentage of GME

However, compared to the transition temperature of Pol3 obtained from NMR which is 14°C, the phase transition temperature from turbidity measurements was much higher at 28°C. According to the NMR spectrum, the copolymer does not collapse completely and the transition is not sharp. This thermo-responsive copolymer is thus quite different compared to the other materials in literature.

2.3 Phase transition temperature determined via calorimetry

Micro-DSC was used for examination on Pol3, based on the measurement of differences in heat capacity between a sample and a reference cell. The reference samples were water and deuterated water. The concentration of Pol3 solution was 10g/L, the same as in the turbidity measurements. Since deuterated water was used in NMR test, it was also used in Micro-DSC examination in order to exclude the big difference between turbidity and NMR results. From the curves, a visible but weak transition was obtained at around 38°C. In addition, lower heating rate make transition happen earlier since the polymer chains had longer response time when absorbing the heat from environment. It can be seen from *Figure.24* that deuterated solvent hardly influence the phase transition temperature of Pol3. It shows a similar curve shape compared to the *Figure.23*. The curve with lower heating rate also shows slightly lower phase transition temperature and higher enthalpy of transition, the same as that in water.

However, the peak at phase transition temperature of Pol3 in deuterated water is more visible than that in water which means higher enthalpy during phase transition. The explanation for the phenomenon is different polymer–solvent interactions caused by the isotopic effect. Heavier deuterium water gives stronger hydrogen bonding than normal water.[60] More energy needed when D₂O molecules leave the polymer chains during the phase transition. Moreover the phase transition temperature in D₂O is lower than in H₂O at around 36°C. There is another report that poly(2-iso-propyl-2-oxazoline) tested by sensitive DSC also shows similar trend.[16]

Some previous reports about PNIPAM give opposite result, the LCST of PNIPAM in D₂O is about 0.7°C higher than in H₂O.[61] This effect only perceptible when concentration is high enough.[62] Thus, it cannot be the resolution in this case. The explanation of slightly lower phase transition temperature in D₂O remains unanswered.

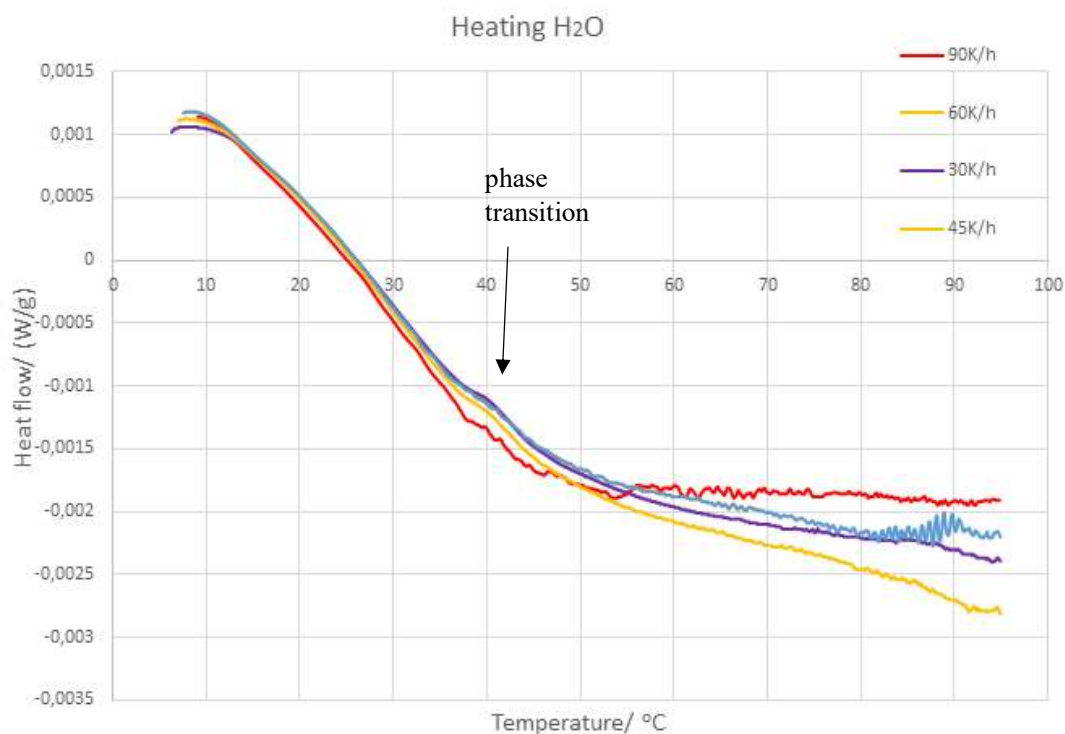


Figure.23 Pol3 phase transition temperature measurement by Micro-DSC with different heating rates (in water with 10g/L)

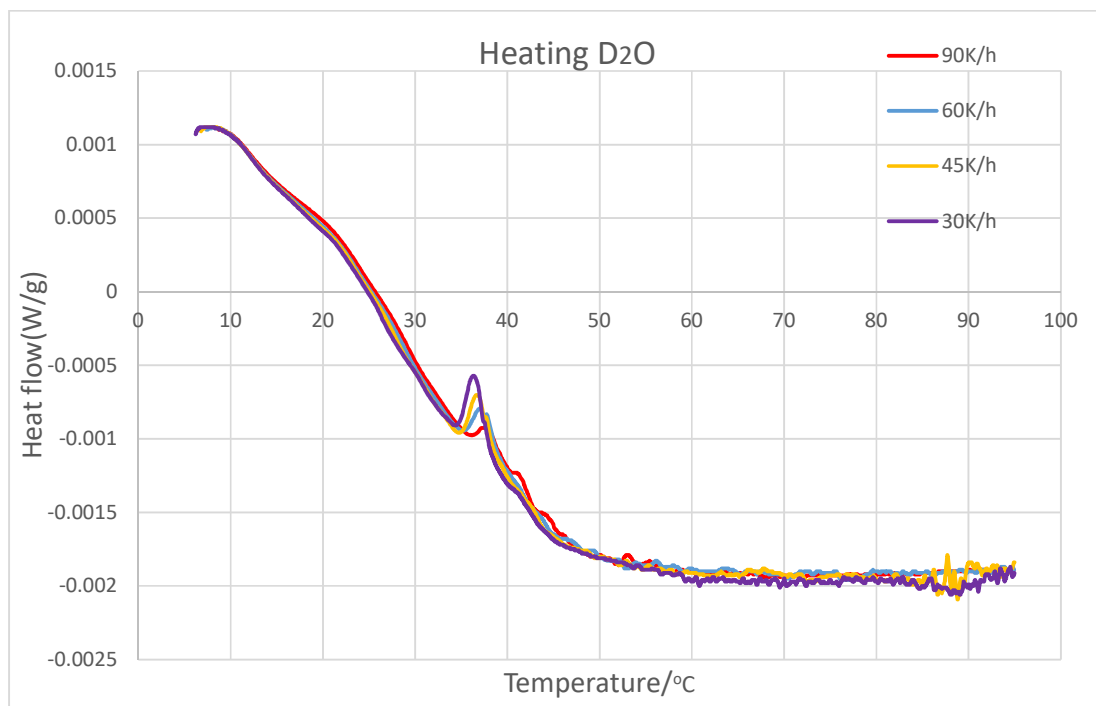


Figure.24 Pol3 phase transition temperature measurement by Micro-DSC with different heating rates (in deuterated water with 10g/L)

The results obtained from above three examined methods are different. The phase transition temperature tested by NMR was the lowest at around 14°C next to the one tested by turbidity at 28 °C, and Micro-DSC at 36°C to 38°C. It has been reported that pre-aggregation of polymer segments can be observed by NMR spectroscopy well before a macroscopic aggregation or visible phase transition is detected by turbidimetry test. [17]

The results obtained by turbidity test and Micro-DSC have less difference compared to that from NMR and the change of turbidity is visible so that they are trustable. The phase transition temperature is around 28-35°C which is suitable for the biomedical application that can be continued with substitution to hydrogel production.

3. Substitution of bromide

The bromide in copolymer of poly-(GME-EH) was substituted by azide from sodium azide. Substituted Pol3 and Pol5 was examined by FTIR as it shows in *Figure.25*. There was no peak at 2100 cm^{-1} in Pol3, which represents the azide group. Pol5 was tested as well and the peak at 2100 cm^{-1} demonstrated the existence of azide group. The same synthetic procedures were used in Pol3 and Pol5 substitution reactions so the reason why the azide group in Pol3 was invisible may be that Pol3 has three times larger DP than Pol5 (see *Table.1*). The polymer chain was so long that the amount of end azide group was tiny and therefore invisible in FTIR. Unfortunately the following click reaction cannot be proven due to the unknown azide group.

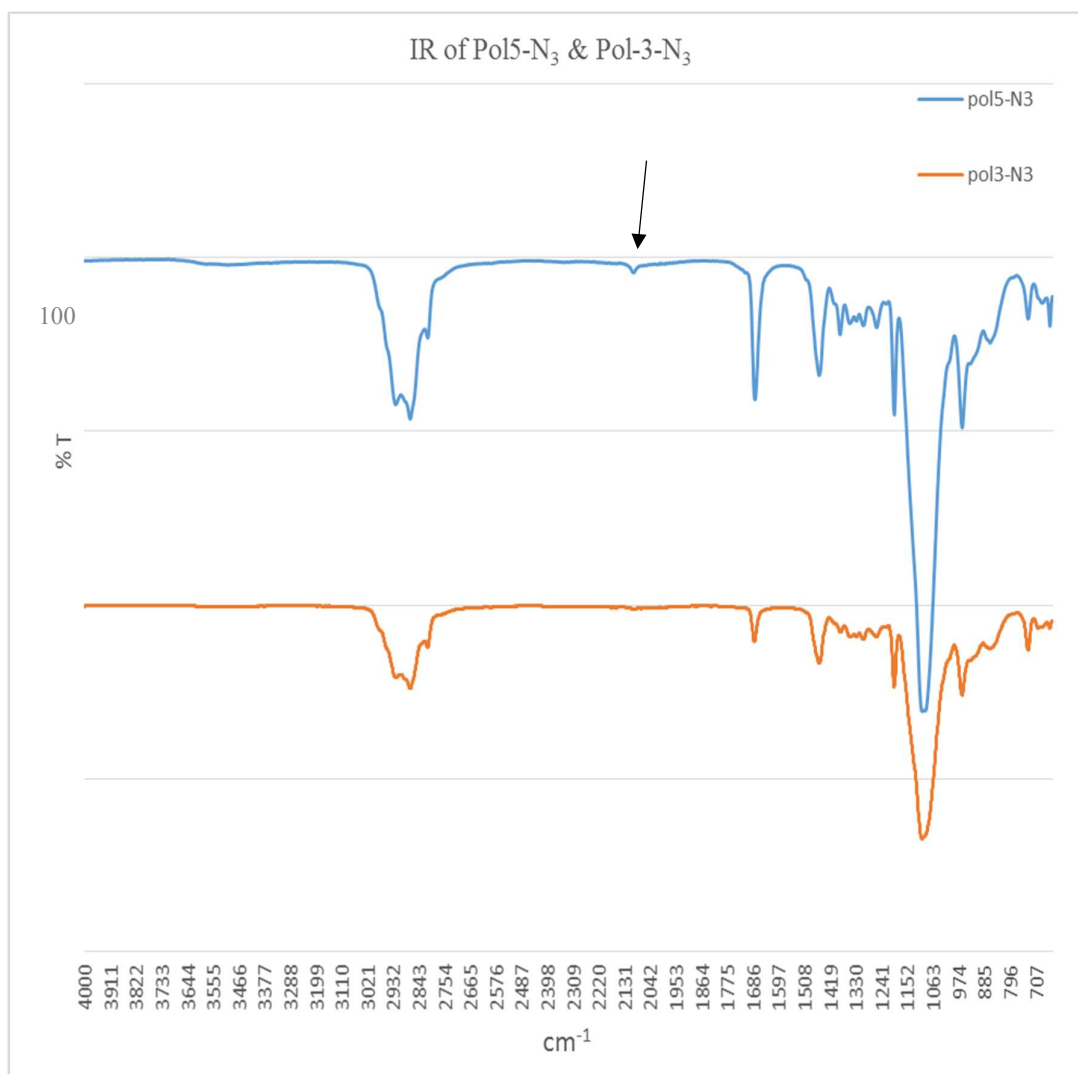


Figure.25 IR spectrum of azide substituted Pol3 and Pol5

4. Grafting of Hyaluronic acid

Pol3 was used for HA grafting by click chemistry. The alkyne group of HA-propargyl ester reacted with the azide group on Pol3 in presence of Copper (I).

Pol3, HA and HA-Pol3 clicked NMR spectrum are compared in *Figure.26*. The NMR shows signals of both components (HA and PGME-EH). However, it cannot be proven whether the dialysis purification was successful or not. Thus the success of the coupling reaction could not be determined.

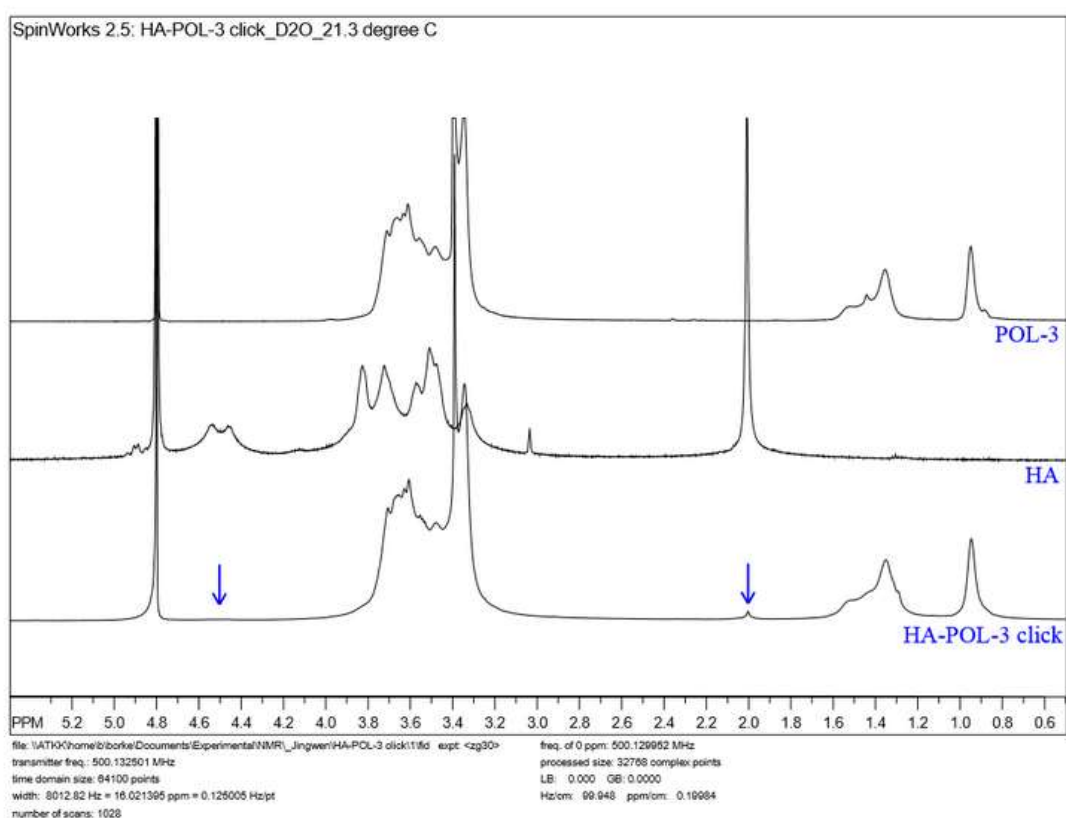


Figure.26 Comparison of NMR spectra of Pol3, HA and HA-Pol3

The gel forming phenomenon was expected when HA-Pol3 was heated above LSCT, but there was no viscosity increase during rheology measurement. Temperature sweep test by rheometer was conducted from 20°C to 40°C with hate rate of 1°C/min. The results of temperature dependent sweep test are shown in *Figure 27*. The storage modulus G' and loss modulus G'' decreased when the temperature increase, and storage modulus G' curve falls faster than loss modulus G'' which means that the viscosity of HA-Pol3 decrease in this process. As a result, no gel forming phenomenon was observed in the temperature sweep experiment. [63]

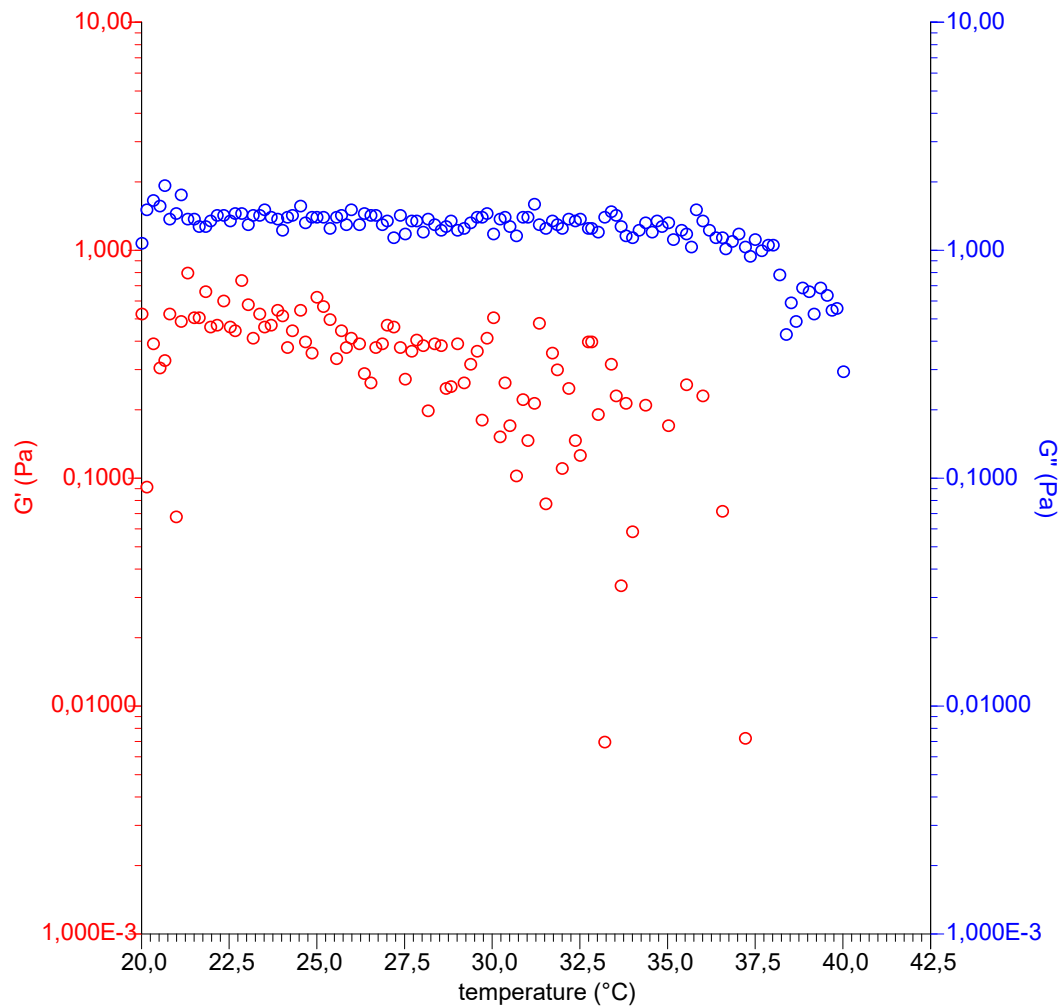


Figure.27 Temperature dependent sweep test by rheometer

Frequency sweep test by rheometer was performed at 40°C with 1% strain. The value of storage modulus G' is lower than loss modulus G'' in low frequency, and both of G' and G'' increase with increasing frequency, while G' increases faster than G'' . There is an intersection during frequency sweep test. The curves follow the typical behavior of unlinked polymers that G' is bigger than G'' before they crossover and G' is smaller than G'' after that. The frequency sweep test also gives the result that gel formation failed.

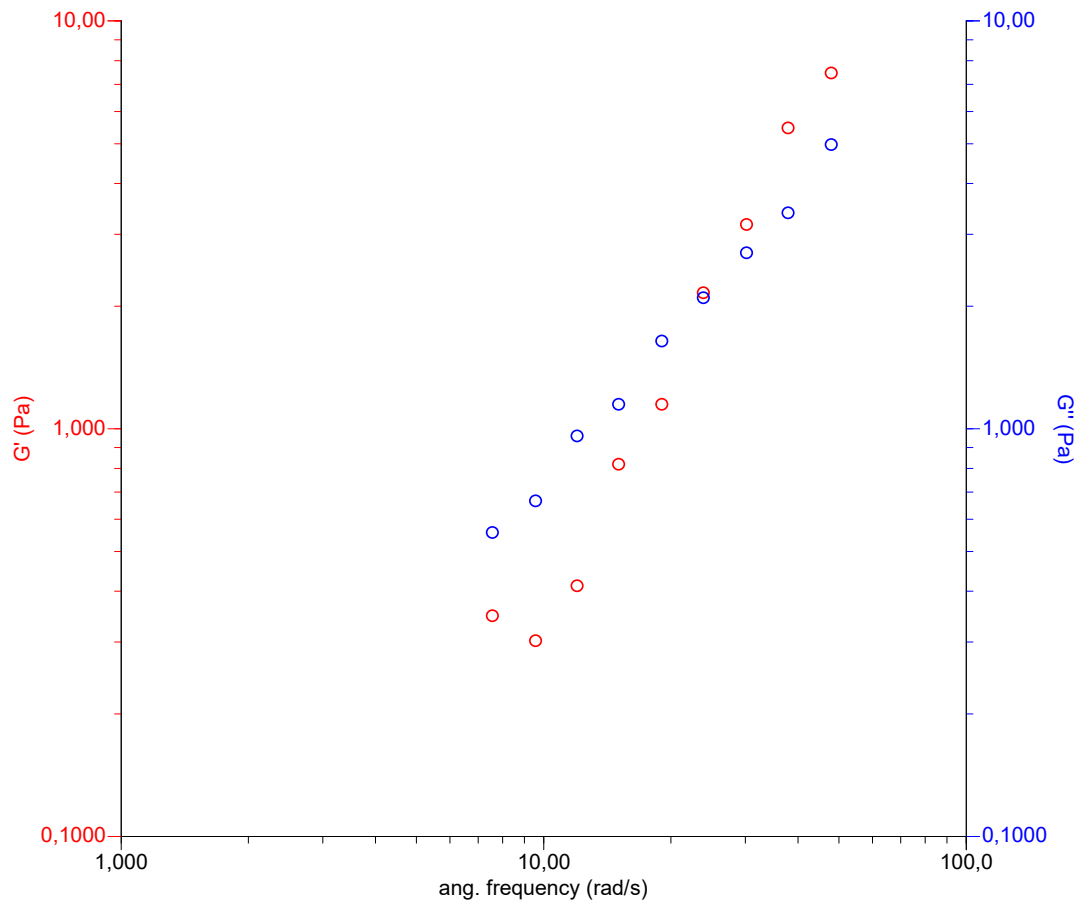


Figure.28 Frequency dependent sweep test by rheometer at 40 °C

The failure of gelation is most likely due to the failure of click chemistry. The chosen sample, Pol3, does not necessarily have azide functionalities and the PDI is broad due to absence of solvent during polymerization. As was mentioned above, whether the dialysis process is successful is unknown, so that the copolymer maybe not be grafted on the hyaluronic acid chains, but mixed together instead. Thus, no gel forming during the heating due to the unqualified composite structure. On the other hand, rigid polymer chains, such as HA, give rise to highly efficient coupling since alkyne moieties are exposed in the reaction mixture and can easier react with azide groups. [36] The poly-(GME-EH) copolymer chain however is a flexible coil with only one azide head group, which would weaken the reaction efficiency. Moreover, the NMR spectrum shows a continuous chain collapse during the heating process instead of a sharp turn like PNIPAM. The polymer could be partially collapsed during the coupling reaction even though the reaction temperature (room temperature) is under the LCST tested by turbidimetry. Other reasons for the failure of gelation could be low grafting density or the incomplete collapse of poly-(GEM-EH) leading to the inter-chain hydrophobic interactions not being strong enough to form a gel.

IV. Conclusion

The aim of the research was to obtain a hydrogel based on hyaluronic acid, grafted with thermo-responsive copolymer, poly (GME-EH). Anionic ring opening polymerization was successfully used in GME and EH random copolymerization. The purification required for removing the catalyst residue by precipitation and collecting via centrifugation gave low yield. The broad dispersity of products (Pol3 and Pol4) due to the absence of solvent in the synthesis indicate the significance of the solvent. Moreover, the choice of broad sample Pol3 might be one of the aspects that lead to final failure.

The difference between phase transition temperatures tested by various methods are quite large. It has been reported that the differences do exist due to the different mechanism and measuring scales. For example, NMR spectrometer gives insights of thermo-responsive behavior at molecular level. However turbidimetry is based on visible transmittance and thus macroscopic changes. Several measuring methods should be utilized based on different angles and scales to give a more complete picture of the thermo-responsive behavior of polymers.

Click chemistry in this experiment could not be proven directly. The failure of gelation in the following step can be associated with the non-grafted polymer or low grafting density or with the uncomplete collapse of PGME and resulting weak hydrophobic inter-chain interactions.

Future research

Since one of the possible reason lead to failure is the poor copolymer sample. More series of poly (GME-EH) with different chain length and better PDI would be polymerized. More studies on the grafting reaction with HA should be done. Various length of P(GME-EH) and grafting density on HA could be tried and characterize the products properly to find out if the gelation is possible.

Furthermore, other hydrophobic monomers for example ally glycidyl ether can be chosen for adjusting LCST of PGME and same characterization method should be utilized. It is interesting to observe a new monomer which has similar property as EH even better than that. There are some possibilities that a new hydrophobic monomer has similar reactivity ratio as GME which give random copolymer and adjust the LCST efficiently. Even give more obvious collapse during phase transition which may lead to easier gelation after being grafted on hyaluronic acid chains.

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