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Original article

SYNTHESIS OF IN SITU FORMING HYDROGELS OF PNIPAAm-PHPMA-PNIPAAm TRIBLOCK-COPOLYMERS USING RAFT POLYMERIZATION AND THEIR EVALUATION AS NEW THERMOSENSITIVE DELIVERY SYSTEM AGENT

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Abstract

Temperature responsive triblock copolymers of poly(N-isopropylacrylamide)-poly(N-(-2-hydroxypropyl)methacrylamide)-poly(N-isopropylacrylamide)(PNIPAAm-PHPMA-PNIPAAm) have been prepared in two steps via reversible addition fragmentation chain transfer (RAFT) polymerization using carboxyl terminated trithiocarbonate (S,S'-Bis(α,α'-dimethyl-α''-acetic acid)-trithiocarbonate) as the RAFT agent. The result showed that the copolymerization of PNIPAAm and PHPMA was of living character, and the corresponding copolymer produced had temperature responsive properties in water. While the block length of the hydrophilic middle block was kept constant, two different lengths of PNIPAAm blocks were used in the synthesis of triblock copolymers. The thermo-sensitive aggregation of polymers in water was studied using LCST (lower critical solution temperature) and sol-gel-sol change. The copolymers formed gels in situ under physiological condition. LCST increased as the PNIPAAm content decreased due to decrease in hydrophobicity of triblock-copolymer. All rheological measurements were performed using temperature controlled oscillating rheometer. The dynamic viscoelastic properties of the polymer solutions; storage modulus (G') and loss modulus (G'') were recorded. Storage modulus of polymer increased with increasing temperature as common in viscoelastic hydrogels. Results indicated that the hydrophobic/ hydrophilic balance achieved by varying the amount of comonomers used during synthesis was an important parameter in controlling the transition temperature of macromers in solution and stability of resultant gels.

Key words: *Reversible addition fragmentation chain transfer (RAFT), Thermo-sensitive hydrogels, Poly(N-isopropylmethacrylamide), poly(N-(2-hydroxypropyl)methacrylamide), Triblock-copolymer.*

Dönüşümlü Ekleme ve Kırılma Zincir Transfer (Raft) Polimerizasyon Yöntemi Kullanılarak in-situ Oluşan Poli(N-isopropilakrilamit)-Poli(N-(2-Hidroksipropil)Metakrilamit)- Poli(N-isopropilakrilamit) Triblok-Kopolimer Hidrojellerinin Sentezlenmesi ve Isıya Duyarlı Taşıyıcı Sistem Ajanı Olarak Değerlendirilmesi

Poli(N-isopropilakrilamit)-poli(N-(2-hidroksipropil)metakrilamit)-poli(N-isopropilakrilamit) (PNIPAAm-PHPMA-PNIPAAm) polimerlerinden oluşan ısıya duarlı triblok-ko-polimeri, karboksil sonlanmalı tritiyokarbonatın (S,S'-Bis(α,α'-dimethyl-α''-acetic acid)-trithiocarbonate) RAFT ajanı olarak kullanıldığı Dönüşümlü Ekleme ve Kırılma Zincir Reaksiyon (Reversible Addition Fragmentation Chain Transfer = RAFT) polimerizasyonu kullanılarak iki basamakta sentezlenmiştir. Poli(N-isopropilakrilamit)(PNIPAAm) ve poli(N-(2-hidroksipropil) metakrilamit) (PHPMA) kopolimerizasyonu yaşayan bir polimerizasyondur ve sonuçta sentezlenen kopolimer suda sıcaklığa duyarlı özellik gösterir. Kopolimer ortasında bulunan hidrofilik orta blok sabit tutularak iki farklı uzunlukta PNIPAAm blok içeren triblok-ko-polimer sentezlenmeye çalışılmıştır. Sentezlenen triblok-kopolimerin, ısıya duyarlı

olarak suda gösterdiği agregasyon Düşük Kritik Çözelti Sıcaklığı (Low critical solution temperaturte =LCST) ve sol-jel-sol donüşüm metodları kullanılarak test edilmiştir. Kopolimerler fizyolojik koşullarda in situ olarak jel oluşturmaktadır. LCST, PNIPAAm içeriğinin azalması ve sonuçta triblok-kopolimerdeki hidrofobik özelliklerin azalması nedeniyle artmıştır. Tüm reolojik ölçümler termostatlı osilasyonlu reometre kullanılarak tespit edilmiştir. Polimer çözeltisinin dinamik moduli olarak bilinen depolama modulusu (G') ve kayıp modulusu (G'') tespit edilmiştir. Polimerlerin depolama modulusu sıcaklık artıkça artmıştır. Sonuçta sentez aşamasında kullanılan komonomerlerin relatif miktarlari değiştirilerek kopolimerin hidrofilik/hidrofobik dengesini ayarlanabilmekte ve bu durum makromerin hem çözeltideki geçiş sıcaklığını kontrol etmekte hemde oluşan jelin stabilitesini etkilemektedir.

Anahtar kelimeler: *Dönüşümlü ekleme ve kırılma zincir transferi (RAFT), Sıcaklığa hassas hidrojeller, Poli(N-isopropilakrilamit)(PNIPAAm)*

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INTRODUCTION

Controlled drug delivery systems, which are intended to deliver drugs at predetermined rates for predetermined periods of time, have been used to overcome the shortcomings of conventional drug formulations. It would be most desirable if the drugs could be administrated in a manner that precisely matches physiological needs at proper times and/or proper site. Also it would be highly beneficial if the active agents were delivered by a system that sensed the signal, and then acted to release the right amount of drug in response (1, 2). Hydrogels have been used extensively in the development of the smart drug delivery systems. A hydrogel is a network of hydrophilic polymers that can swell in water and hold a large amount of water while maintaining the structure. Three-dimensional networks are formed by chemical or physical crosslinking (covalent bonds, hydrogen bonds, van der Waals interactions or physical entanglements) [3-5]. Hydrogels which undergo reversible volume phase transition or sol-gel phase transition in response to the external physical or chemical stimuli are known as *Stimuli Sensitive, Environment Sensitive or Responsive Hydrogels*. Among these stimuli: Temperature, electric fields, solvent composition, light, pressure, ultrasound, magnetic fields, pH, ions and specific molecular recognition (such as glucose recognition) can be given as examples. The hydrogels that exhibit a sol-gel phase transition in response to external stimuli provide a simple and safe method of preparing in-situ forming gels, leading to potential applications of *ocular systems* which have bioavailability problems due to their short stay in the eye. In situ forming hydrogels give to the active moiety chance to contact longer time with cornea and conjunctiva resulted in better bioavailability [6-9].

Temperature sensitive hydrogels are probably the most commonly studied class of environmentally sensitive polymer system in drug delivery research. The common characteristic of temperature sensitive polymer is the presence of hydrophobic groups, such as methyl, ethyl, and propyl groups. Of many temperature sensitive polymers, poly(N-isopropylacrylamide) (PNIPAAm) is probably the most extensively used. Copolymers of NIPAAm can also be made using other monomers. Certain types of block copolymers made of poly-(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) also possess an inverse temperature sensitive property. Because of their LCST at around the body temperature, they have been used widely in the development of controlled drug delivery systems based on the sol-gel phase conversion at the body temperature [10-12]. Hydrogels made of LCST polymers shrink as the temperature increases above LCST. This type of swelling behavior is known as inverse (or negative) temperature dependence. The inverse temperature dependent hydrogels are made of polymer chains that either possess moderately hydrophobic groups (if too hydrophobic, the polymer chains would not dissolve in water at all) or contain a mixture of hydrophilic and hydrophobic

segments. At lower temperatures, hydrogen bonding between hydrophilic segments of the polymer chain and water molecules are dominates, leading to enhanced dissolution in water. As the temperature increases, however, hydrophobic interactions among hydrophobic segments become stronger. The net result is shrinking of the hydrogels due to inter-polymer chain association through hydrophobic interactions. In general, as the polymer chain contains more hydrophobic constituent, LCST becomes lower. The LCST can be changed by adjusting the ratio of hydrophilic and hydrophobic segment of the polymer (13, 14). If the polymer chains in hydrogels are not covalently cross linked, temperature sensitive hydrogels may undergo sol-gel phase transitions, instead of swelling-shrinking transitions (15, 16).

Traditional methods of hydrogel synthesis include cross linking copolymerization, cross linking of reactive polymer precursors, and cross linking via polymer-polymer reaction. These methods of hydrogel synthesis were limited in the control of their detailed structure due to side reactions the networks contain cycles, unreacted pendant groups, and entanglements (3). Developments in controlled living radical polymerization, such as reversible addition-fragmentation chain transfer (RAFT) polymerization has aimed to control of their detailed structure (chain length, sequence, and 3D-structure), prevent side reactions and unreacted pendant groups, enhanced mechanical properties, fast response to the external stimulus, and control drug release by changing the gel structure in response to environmental stimuli (17- 19). It has been shown to be acceptable to the controlled polymerization of a wide range of monomers, under many different conditions to yield materials with predetermined molecular weight distributions (MWD), and advanced architectures (Figure 1) (20-24).

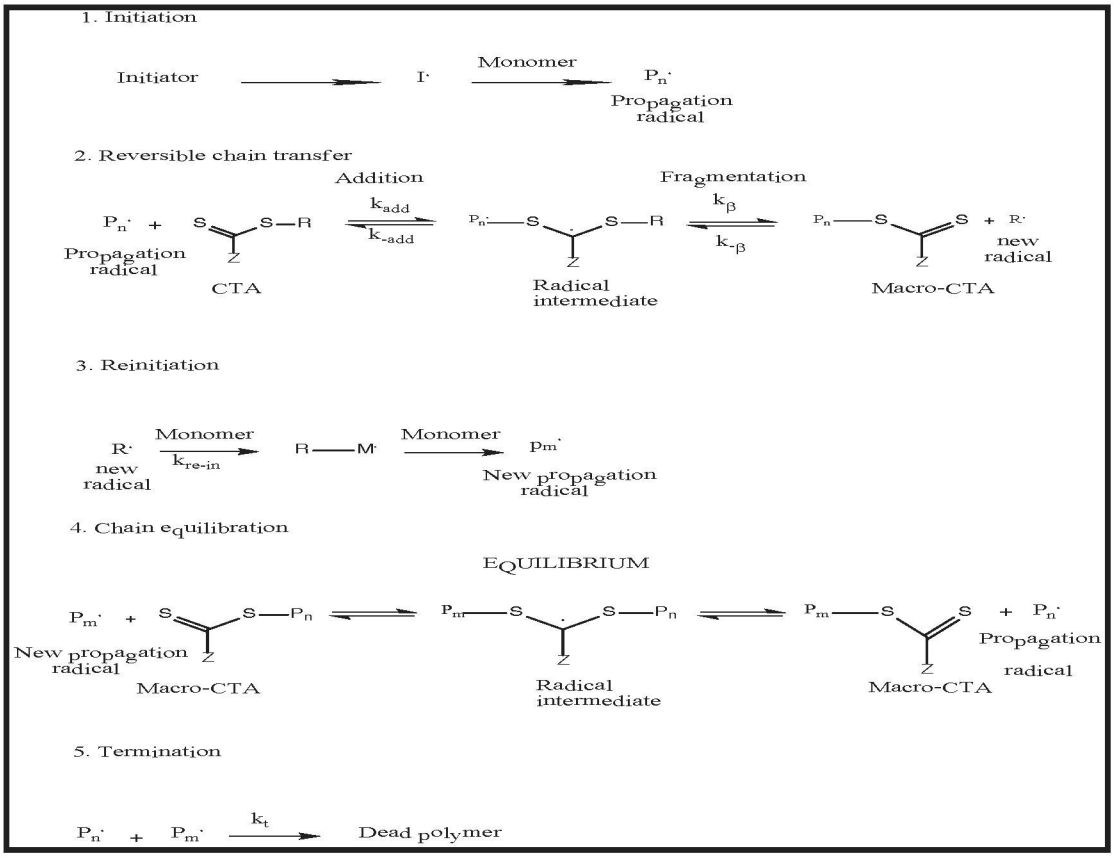

Figure 1. Reversible addition-fragmentation chain transfer (RAFT) polymerization [16-18].

In our study we tried to synthesize new thermosensitive PNIPAAM-PHPMA-PNIPAAM triblock-copolymer via RAFT polymerization. This stimuli sensitive hyrogel was thought to use as a vehicle in ocular application. Ocular drug delivery is one of the most interesting and challenging endeavors facing the pharmaceutical scientist since both systemic and local treatments can be achieved. The anatomy, physiology and biochemistry of the eye render this organ exquisitely impervious to foreign substances. The primitive ophthalmic solutions (are still given top priority by formulators since they are relatively simple to prepare, filter and sterilise) suspensions and ointment dosage forms are clearly no longer sufficient to combat some present diseases. There is urgent need to develop ocular drug delivery systems which provide controlled release for the treatment of chronic diseases, and increase patient's and doctor's convenience to reduce the dosing frequency and invasive treatment. More efficient ocular delivery systems that have been still under evaluation aim at enhancing the drug bioavailability by providing prolonged/sustained delivery to the eye. The absorption of drugs in the eye is severely limited by some protective mechanisms that ensure the proper functioning of the eye such as, drainage of the instilled solutions, lacrimation and tear turnover, lower contact time, and metabolism (25). Thus, extensive investigation has been dedicated to prolonging the retention time of medications on the eye surface and to the improvement of transcorneal penetration of traditional and of novel therapeutic agents such as protein and peptide drugs. For the treatment of the anterior segment of the eye, various droppable products to prolong the retention time on the ocular surface have been introduced in the market. Among these, liquid vehicles undergo a viscosity increase upon instillation in the eye, thus favoring precorneal retention. Such a change in viscosity can be triggered by a change in pH, electrolyte and most commonly temperature composition as described fully above. Recent developments showed that there has been an increasing interest for termosensitive polymers used in ocular drug delivery. Methylcellulose (MC) has a lower critical solution temperature (LCST) at approximately 50 °C (26, 27), and sol-gel phase transition occurs. Since the temperature of ocular surface is 33–37 °C (26,27), LCST needs to be lower to gel MC solution at ocular surface quickly after instillation as eye-drops. In general, high concentration of electrolytes leads to salting-out and gelation of MC (26). Wakamoto Pharmaceutical Co., Ltd. (Tokyo, Japan) has developed temperature-responsive eye-drops formulated timolol maleate for glaucoma therapy (Rysmon® TG) available in Japan, using combinations of MC, sodium citrate and polyethylene glycol, which can act by lowering LCST of MC (26,27).

However adjustment of LCST near to the ocular surface is thought to be easier with PNIPAAm than with other polymers since its unique phase transitation at the eye temperature, that is why we used PNIPAAm as thermosensitive polymer. To achieve reasonable LCST temperature we tried the combine PNIPAAm and PHPMA to get triblock-co-polymer. PNIPAAm is a well-known temperature sensitive polymer and exhibits a phase transition in water and demonstrates a lower critical solution temperature (LCST) at around 32-35°C. The distinctive properties of the PNIPAAm polymer are attributed to its pendant isopropylamide groups, which induced a change in the hydrophilic-hydrophobic balance as the temperature changes. At temperatures below the LCST, the hydrophilic moieties (-CONH-) may interact with water molecules through hydrogen bonding, which leads to water up-take by the polymer. However, as the external temperature increases, the hydrogen-bonding interactions become weakened or destroyed. And thus, the hydrophobic interactions among the hydrophobic moieties ($-CH(CH_3)_2$) grow to be strong, which induces the freeing of the entrapped water molecules from the network. When the temperature reaches or is above the LCST, the hydrophobic interactions become dominant. Combined with water release, polymer chains collapse abruptly and the phase separation of the PNIPAAm hydrogel system occurs. Owing to the thermally responsive coil-to-globule transition at a lower critical solution temperature (LCST), it looks promising to use the thermosensitive PNIPAAm hyrdogel for biomedical and biotechnological applications as controlled delivery devices (28-31). Both PNIPAAm and

PHMPA are biocompatible and nontoxic polymers. Chen and Cheng, 2008 (32), fabricated functionalized temperature sensitive copolymers of hyaluronic acid-g-chitosan-PNIPAAm injectable cell carrier for tissue engineering of articular cartilage and meniscus. They showed positive prolifereation behaviours of chondrocytes and meniscus. Further they test the cytotoxicity of copolymer hydrogel using live/dead assay kit to discriminate between live and dead cells. After 7days of cultivation with copolymer almost all chondrocytes are alive suggesting that no harmful molecules were released from the copolymer. On the other hand PHPMA as a vehicle in drug delivery has been well studied over the past few decades. Extensive research performed with poly[N-(2-hydroxypropyl) methacrylamide] (polyHPMA) and HPMAcopolymers has emphasized their suitability as carriers for drug delivery. PHPMA is a water-soluble, nonimmunogenic, synthetic polymer, which does not activate the complement system by either the classical or the alternate pathway. Further, PHPMA copolymers containing IgG have demonstrated reduced immunogenicity, probably by preventing associations between the immunogenic substance and mediators of immune response,or by direct interpolation with T cells. PHPMA homopolymer and its copolymers exhibit desirable pharmacokinetics, such as sustained retention due to increased MW, without long term toxicities invivo. It is biocompatible polymer, and bioconjugates with PHPMA are often used in vehicles for site-specific gene and drug delivery (33-35). RAFT polymerization of HPMA has been extensively studied in the field of controlled drug delivery systems (36-39). Here in this study our aim to use biocompatible PHPMA in the synthesis of triblock-co-polymer is to control both hydrophilic/ hydrophobic balance and pull the LCST of triblock-co-polymer near to body temperature. In the literature Hang and Pan, 2006 (40), prepared temperature sensitive biotinylated diblock-copolymer of PNIPAAm and water soluble PHPMA via one step RAFT polymerization to show conjugates of biomolecules with polymers combine the properties of components and may lead to unique properties. However, *to our knowledge it was the first time* we tried to synthesize thermosensitive PNIPAAM-PHPMA-PNIPAAM triblock-copolymers to get a vehicle which may be used as a model agent for the active moieties (eg. Peptide/proteins) to deliver them in solution form but immediately after they contact with eye at 33-37^{o}C they form proper gels. The release studies of triblock-copolymers have been left for the further evaluation, since Kopecek et al. (33-35) studied the release pattern of PHPMA and Zhang et al (46) studied the release pattern of PNIMAAm individually and achieved successful results for sustained drug delivery systems. As described above the key feature of liquid ocular system is to enhance the contact time of the delivery system into the eye surface. So our first aim was to synthesize the stimuli sensitive triblock-copolymer to get a reasonable delivery vehicle and evaluate its characteristic. As the polymer chain contains more hydrophobic constituent LCST starts to decrease and LCST can be changed according to change in hydrophilic/hydrophobic segment ratio (41, 42). The study was found to be promising for the further evaluation of release of biomolecules from triblock-co-polymer as a new thermo-sensitive polymer which can be used as a pharmaceutical excipient in modified ocular drug delivery.

MATERIALS AND METHODS

Materials

N-isopropylacrylamide (NIPAAm, 97%, Aldrich) was recrystallized from hexane/toluene (3/1,v/v). N,N-dimethylformamide (DMF, Fischer Scientific, Spectranalyzed). 2,2'-Azobis(isobutyronitrile) (AIBN, 98%, Sigma-Aldrich) was recrystallized from ethanol and dried at room temperature under vacuum. HPMA was synthesized according to a literature procedure reported by Kopecek et al. (43,44). S,S'-Bis(α,α'-dimethyl-α''-acetic acid)-trithiocarbonate (CTA; Chain transfer agent) was synthesized according to the literature procedure (45,46). All other chemicals were reagent grade unless otherwise noted.

Methods

2.2.1. Calibration curves of polymer standards and characterization of polymers

To determine molar mass distribution of polymers, 50 µl aliquot of standard samples of Poly(methylmethacrylate) (PMMA standards, Polyscience Inc., 27 kD, 74 kD, 350 kD) were injected to GPC (at a concentration of 5mg/ml, DMF as an eluent, 0.5 ml/min flow rate, 60°C column temperature, Polymer Labs PL gel 5µm Mixed-C 300x7.5mm GPC column, Agilent 1100 series, Model G1312A, USA) equipped with Optilab DSP Interferometric Refractometer (Wyatt Technology, Software; DNDC for windows 5.20, λ= 690 nm). Molecular weights and profiles of PNIPAAm homopolymers and PNIPAAm-PHPMA-PNIPAAm triblock-co-polymers were calculated from the calibration curves of known weight and narrow PDI of PMMA polymer standards.

Polymerization kinetics

Polymerization kinetics of NIPAAm was conducted at 60°C under a nitrogen atmosphere, with an initial monomer concentration ($[M]_o$) of 2.5 M, employing s,s'-Bis(α,α'-dimethyl-α''-acetic acid) trithiocarbonate as the CTA (*Chain transfer agent*) and AIBN as the primary radical source. The initial monomer to CTA ratio ($[M]_0/[CTA]_0$) was 175 and CTA to initiator ratio ($[CTA]_0/[I]_0$) was 3/1. NIPAAm (0.5g, 4,42mmol), CTA (7,15mg, 0.025mmol) AIBN (1,35mg, 0.0083mmol) and DMF were sealed in a glass tube with rubber septum, equipped with a magnetic stir bar. The solution was purged with nitrogen, and the reaction vial was placed in a preheated oil bath at 60°C. Polimerization kinetics and absolute molecular weights were determined by extracting aliquots (0.2ml) from the polymerization solution at predetermined time intervals which were immediately quenched in liquid nitrogen. Polymerization aliquots were analyzed directly by GPC using DMF as an eluent.

Standard curve of NIPAAm for monomer conversion during polymerization

To make calibration curve of NIPAAm, first of all stock solution of NIPAAm was prepared in DDI water (Exactly 4mg of NIPAAm was weighed and dissolved in 2ml of DDI water in volumetric flask). Then to achieve 12.5µg/ml, 25µg/ml, 50µg/ml, 100µg/ml, and 200 µg/ml concentrations required dilutions were made with DDI water. Five sample of NIPAAm were injected to the HPLC (mobile phase DDI water + 0.1% THA) then calibration curve of "NIPAAm concentration vs. Peak area" was plotted according to HPLC profiles.

Theoretical molecular weight equation of polymers:

$$M_{n,th} = \text{Conv.}(\%)\left(\frac{[M]_0}{[CTA]}\right)M_m + M_{CTA}$$

Equation 1: Theoretical molecular weight equation during RAFT polymerization (17, 18).

The molecular weight of polymer when using RAFT equation can be calculated theoretically by assuming that the efficiency of the trithiocarbonate is 100%. Mn,th ; The theoretical molecular weight of polymer, $[M]_0$; Initial monomer concentration, $[CTA]_0$; Initial CTA concentration, M_m; Molecular weight of monomer,M_{CTA} ; Molecular weight of CTA. Conv. (%); percent conversion. All calculations were done according to this formulation

Synthesis of PNIPAAm

Polymerization of NIPAAm was conducted at 60°C under a nitrogen atmosphere, with an initial monomer concentration ($[M]_o$) of 2.5 M, employing s,s'-Bis(α,α'-dimethyl-α''-acetic acid)trithiocarbonate as the CTA (*Chain transfer agent*) and AIBN as the primary radical source, and DMF were sealed in a glass tube with rubber septum, equipped with a magnetic stir bar. The solution was purged with nitrogen for 30min., and the reaction vials were placed in a preheated oil bath at 60°C. The polymerizations were quenched by exposing the solution to air.

The solutions were concentrated under vacuum, and the polymers were precipitated into cold ether, and dried under vacuum at room temperature for 24h. For further polymerization of triblock-co-polymer PNIPAAM was dialyzed from dialysis tubing (Spectra/Por molecular porous membrane, 6-8 kD) (Table 1). Dialysis was performed to get rid of any harmful monomer or byproduct occurred during the polymerization.

Table 1. Monomer, CTA and Initiator amounts for the synthesis of PNIPAAm 1 and 2

Theoretical molecular weight	Monomer (NIPAAm)	W_{CTA}	W_I	[CTA]/[I]:	$[M]_o$
PNIPAAm 1					
10 kD	500mg (4.42mmol)	14.50mg (0.0514mmol)	2.80mg (0.0171mmol)	3	2.5M
PNIPAAm 2					
50 kD	500mg (4.42mmol)	2.82mg (0.01mmol)	0.546mg (0.0033mmol)	3	2.5M

Synthesis of Triblock-co-polymer

To further demonstrate the retention of the trithiocarbonate end groups and the living nature of this polymerization, a macroCTA of PNIPAAm was used. This macroCTA was subsequently chain extended with additional HPMA monomer under experimental conditions identical to those reported above for the homopolymerization to get triblock-co-polymers $PNIPAAm_{(5000)}$-$PHPMA_{(50000)}$-$PNIPAAm_{(5000)}$ and $PNIPAAm_{(25000)}$-$PHPMA_{(50000)}$-$PNIPAAm_{(25000)}$. The polymerizations were quenched by exposing the solution to air. The solutions were concentrated under evaporator, and the polymers were precipitated into cold ether, and dried under vacuo at room temperature for 24h. Obtained polymers were dialyzed from dialysis tubing (Spectra/Por molecular porous membrane, 6-8 kD) (Table 2, Figure 2). Dialysis was performed to get rid of any harmful monomer or byproduct occurred during the polymerization.

Table 2. Polymer and monomer amount for Triblock-co-polymer 1 and 2

Triblock-co-polymer-1				
	PNIPAAm-1		**HPMA**	**AIBN**
(Theo Mw)	(*Exp Mw) (PDI)	100 mg	417mg	0.455mg
10 kD	12 kD (1.22)	(0.885mmol)	(2.91 mmol)	(0.0027mmol)
Triblock-co-polymer-2				
	PNIPAAm-2		**HPMA**	**AIBN**
(Theo Mw)	(*Exp Mw) (PDI)	200 mg	200 mg	0.223mg
50 kD	53 kD (1.33)	(1.77mmol)	(1.40 mmol)	(0.0014mmol)

DMF as a solvent, reaction temperature 60°C, *experimental molecular weight, PDI: polydispersity

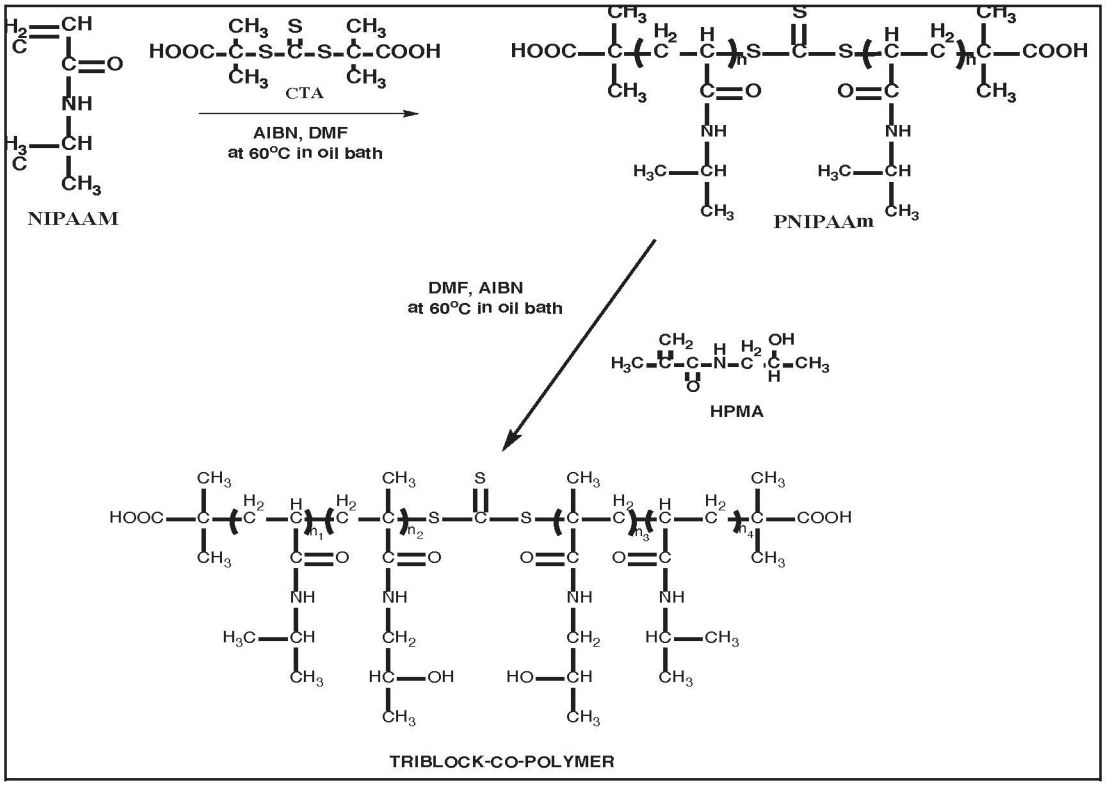

Figure 2. Synthesis of Triblock-co-polymers

Purification of Triblock-co-polymers

FPLC equipped with Superose 6 preparative (Size Exclusion Chromatography, AKTA FPLC Pharmacia; GE Healthcare, USA) column was used to purify the Triblock-co-polymer. Separation was performed according to the different elution times of homopolymer and triblock-co-polymer. 25mg of each polymer dissolved in PBS solution and injected to FPLC. After the separation finished polymer solutions in PBS buffer were dialyzed from dialysis tubing (Spectra/Por molecularporous membrane, 6-8 kD) to get rid of azide salt then freeze dried 24h. Obtained polymers first weighed and then injected to the GPC system.

Characterization of the LCST transition

The LCST transition of each polymer was determined from the dependence of the absorbance change at 500 nm on temperature. The UV–vis spectrophotometer (BIO-TEK Synergy HT spectrophotometer, USA) coupled with a temperature controller was used. The polymer solution (2%, w/v) was prepared in distilled water and put into a 96-well plate. The plate then placed into the spectrophotometer and LCST measurement was performed. The heating rate was 1° C every 1 min. LCST transition performed as written in literature (15).

Solution-Gelation-Solution transition

Polymer solutions (20%, w/v) in DDI water were prepared and stayed overnight at 4°C, and pipetted (300µl) into glass vials that were capped airtight. The vials were placed in a water bath at 37°C and analyzed after 2 hours. Later the precipitated polymers in vials put at 4°C for 2 hour and observed Sol-Gel-Sol transition macroscopically.

Rheological characterization

All rheological measurements were performed on a thermostatted oscillating rheometer (Advanced Rheometers AL550, TA Instruments, USA) equipped with a 20mm aluminum plate (TA Instruments, Serial 983990, USA). Polymers were dissolved at the concentration of 15, 17.5, and 25% w/v for PNIPAAm-1, 17.5, and 25% w/v for PNIPAAm-2, and 25% w/v for both Triblock-co-polymer-1 and 2 respectively, in DDI water and kept in a 4°C until all the polymers were dissolved. The storage modulus (G') and loss modulus (G'') values were recorded using the TA Rheology Advantage software at a gap size of 700μm and an oscillation stress of 10 Pas. Temperature sweep was performed from 10 to 60^{0}C with a heating rate of 1°C.min^{-1}. The sol-gel transition temperature was defined as the temperature at which the sharp change in the storage modulus (G') and the loss modulus (G'').

Temperature dependent swelling ratio

For the temperature induced swelling study, polymers were equilibrated in DDI water (20% w/v) at a temperature of 4^{0}C. The samples were allowed to swell in deionized water for at least 24h at 37^{0}C by a thermostated water bath. The gravimetric method was employed to study the polymer's swelling ratio. After immersion in deionized water at a predetermined temperature (37^0), the hydrogels were removed from water bath and blotted with a wet filter paper to remove excess water on the hydrogel surface and then weighted until constant weight was reached. After this weight measurement, the hydrogel was re-equilibrated in distilled water at a predetermined temperature again and its wet weight was determined thereafter. The average values among three measurements were taken for each sample, and the swelling ratio was calculated as follows,

$$\text{Swelling ratio} = \frac{W_s - W_d}{W_d}$$

Where W_s was the weight of the wet hydrogel after reaching equilibrium at a predetermined temperature and W_d was the dry weight of hydrogel. The data were expressed as mean ± S.D. (n=3).

RESULT AND DISCUSSION

Calibration curve of polymer standards

The calibration curves were obtained using known molecular weight and narrow polydispersity (PDI) of PMMA polymer standards. Different molecular weight of polymer standard gave different GPC profile with different retention time (Figure 3). PMMA polymer standards were used to get calibration curve since the calculated molecular weights of triblock-copolymers were in the range of molecular weight range of PMMA polymer standards. When we look the GPC profiles of polymer standards, peaks shifted to the left with the increasing molecular weight. It is clearly seen from Figure 3 that peaks of PMMA polymer standards is quite narrow and GPC column gave all the peaks with a very narrow time period, which was because of different interaction of polymer standards with packaging material of column when DMF used as an eluent. Calibration curve was obtained according to Retention times vs. Log(Mw) graph. When we look the regression squares of calibration graph it is close to one, which means quiet linear (Figure 4).

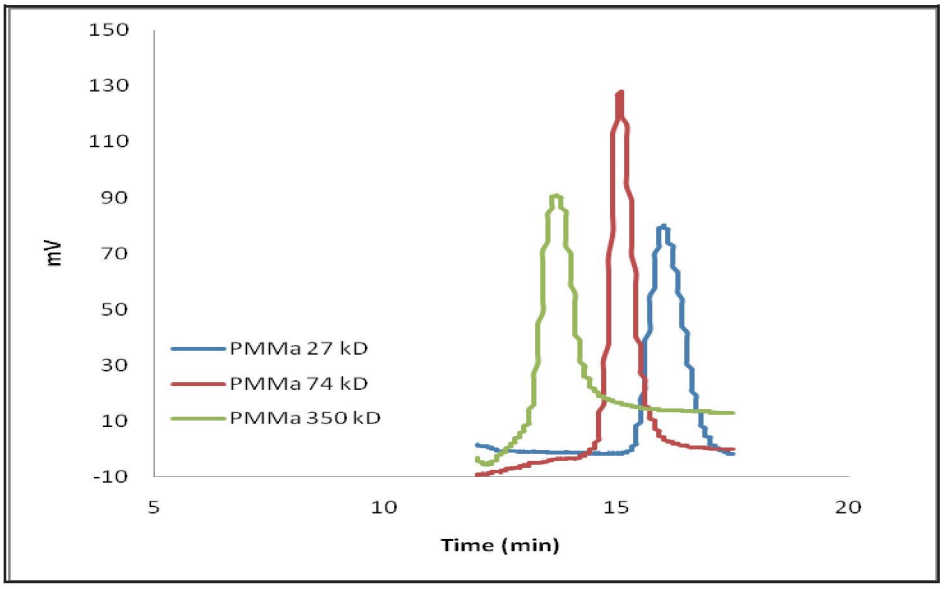

Figure 3. GPC profiles of PMMA 27kD; 74kD; and 350kD

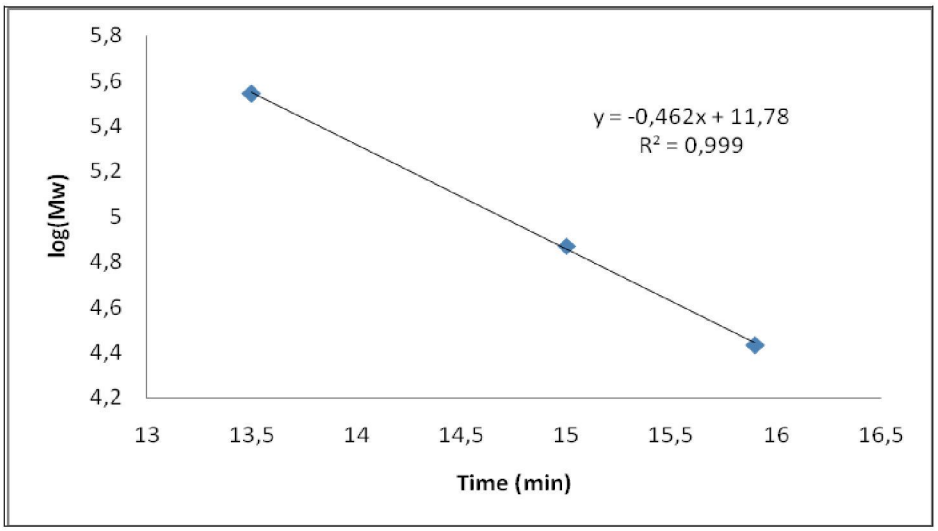

Figure 4. Calibration curves of PMMA polymer standards

Evaluation of polymerization kinetics

To show used polymerization technique (RAFT polymerization) was living polymerization, polymerization kinetics of PNIPAAm was conducted under nitrogen. Every time interval samples taken from the reaction medium and polymerization stopped quenching the samples into liquid nitrogen, then the samples were injected to the GPC system. Figure 5 shows evaluation of molecular weight distribution profiles with lowering retention time against increasing reaction time. Table 3 and Figure 6 show molecular weight enhancement and polydispersity for the polymerization of PNIPAAm mediated by CTA at 60°C at a $[CTA]_o/[I]_o$ ratio of 3/1. The molecular weight of PNIPAAm increased with increasing time with low polydispersity. Biphasic profile was achieved during polymerization. When we look Table 3 and Figure 6, almost all polymerization was obtained at the end of second hour. In between

third and sixth hour polymerization occurred linear. The reason of this phenomenon was thought to be high reaction temperature and high initiator ratio as the general factors.

Table 3. Evaluation of molecular weight with time

Time	$\overline{Mn}$ (*exp.)
1h	8510 (Mw/Mn:1.31)
2h	20000 (Mw/Mn:1.31)
3h	24400 (Mw/Mn:1.37)
4h	24600 (Mw/Mn:1.42)
5h	25400 (Mw/Mn:1.45)
6h	25500 (Mw/Mn:1.45)

*experimental

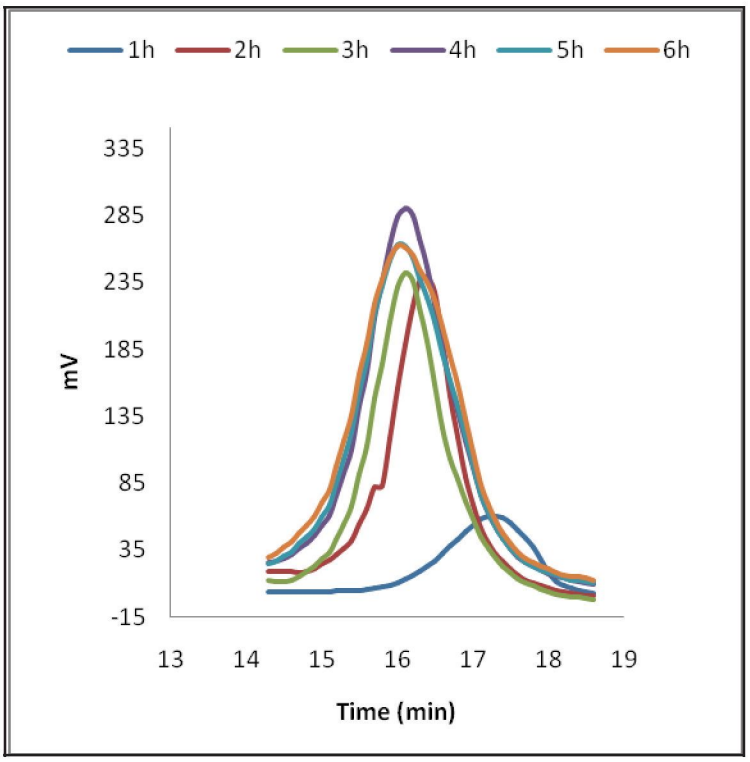

Figure 5. Increasing molecular weight of PNIPAAm with lowering retention time

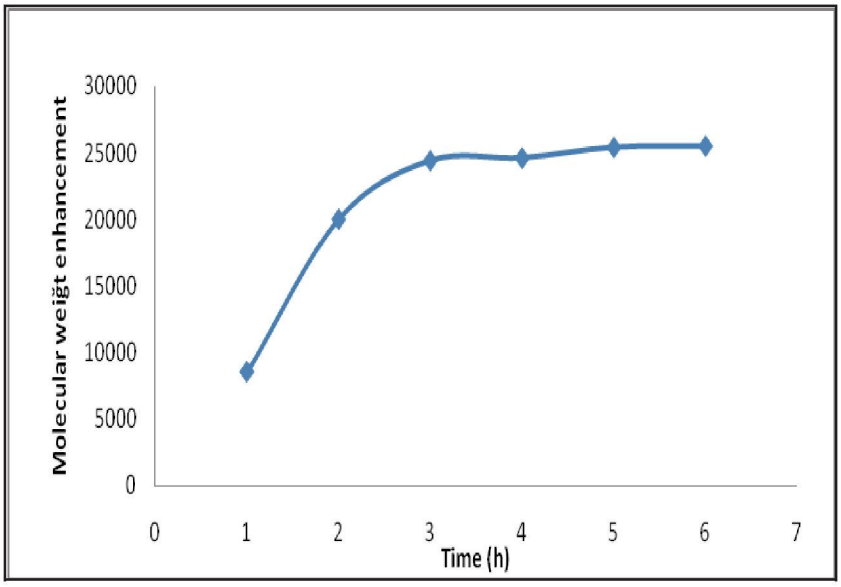

Figure 6. Molecular weight enhancement profile with increasing time

Evaluation of NIPAAm conversion during polymerization

To show monomer conversion during polymerization five sample of NIPAAm were injected to the HPLC (mobile phase DDI water + 0.1% THA) then calibration curve of "NIPAAm concentration vs. Peak area" was plotted according to HPLC profiles. When we look the regression square of the equation (Figure 7) it is close to one, this means the standard curve is quiet close to linear. Figure 8 gives conversion (%) vs. time profile of NIPAAm monomer. At the end of second hour 80% conversion was obtained and at the end of sixth hour almost 96% of monomer conversion obtained. The high rate of conversion within two hour was thought to be because of high amount of initiator ratio and high temperature.

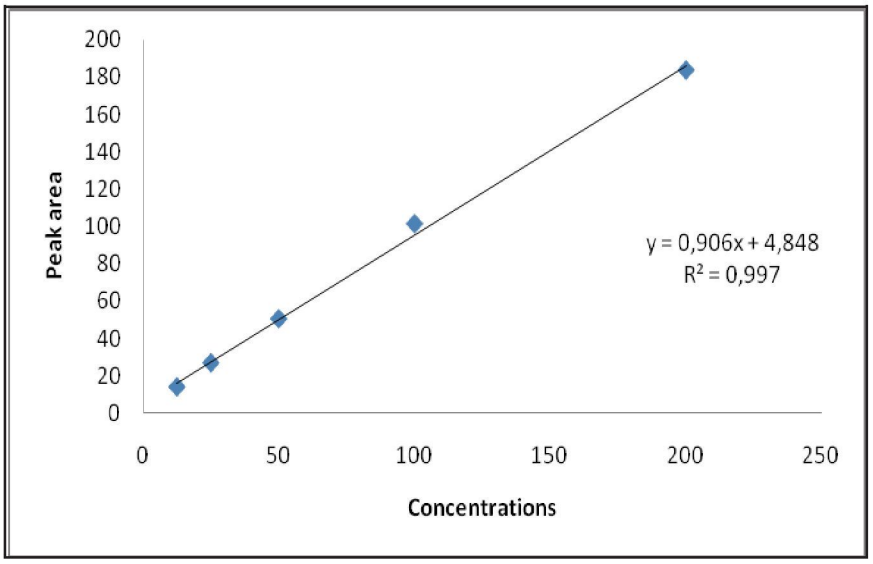

Figure 7. Standard curve for calculation of conversion of NIPAAm

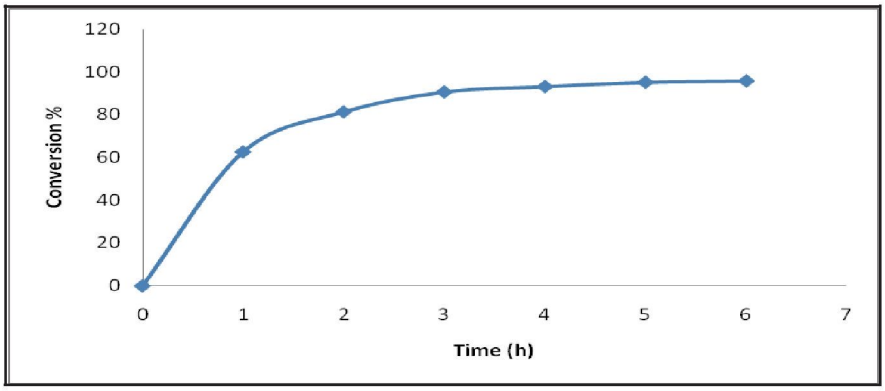

Figure 8. Monomer conversion (%) during polymerization

GPC characterization of Homopolymers and Triblock-co-polymers

Different molecular weight of polymers gave different GPC profiles with different retention times (Figure 9, 10). When we look the GPC profiles of PNIPAAm 1 and 2; Triblock-co-polymer 1 and 2, peaks shifted to the left with the increasing molecular weight. Theoretical molecular weights of PNIPAAM-1 and 2 were calculated as 10 kD and 50 kD, however experimental molecular weights of homopolymers were found 12 kD with PDI of 1.22 and 53 kD with PDI of 1.33 respectively. On the other hand, theoretical molecular weights of triblock-copolymer-1 and 2 were calculated as 60 kD and 100 kD, however experimental molecular weights of copolymers were calculated around 71 kD and 114 kD with broad PDI values respectively. It is clearly seen that peaks of PNIPAAm 1 and 2 (Figure 9) are narrower than that of peaks of Triblock-co-polymer 1 and 2 (Figure 10), which was because of different interaction of Triblock-co-polymer containing different amount of PHPMA with packaging material of column when we use DMF as an eluent also resulted in higher experimental molecular weight and broader PDI. Since PHPMA was a hydrophilic polymer and DMF was a hydrophobic eluent it might have been negative interaction with DMF and resulted in broad GPC profiles. For the prufication of Triblock-co-polymer 1and 2, FPLC equipped with Superose 6 preparative column was used. Seperation was performed according to the different elution times of homopolymer and triblock-co-polymer. 25mg of each polymer dissolved in PBS solution and injected to FPLC. After the separation done polymer solutions in PBS buffer were dialyzed from dialysis tubing (Spectra/Por molecularporous membrane, 6-8 kD) to get rid of azide salt then freeze dried for 24h. Obtained polymers weighed and injected to the GPC system.

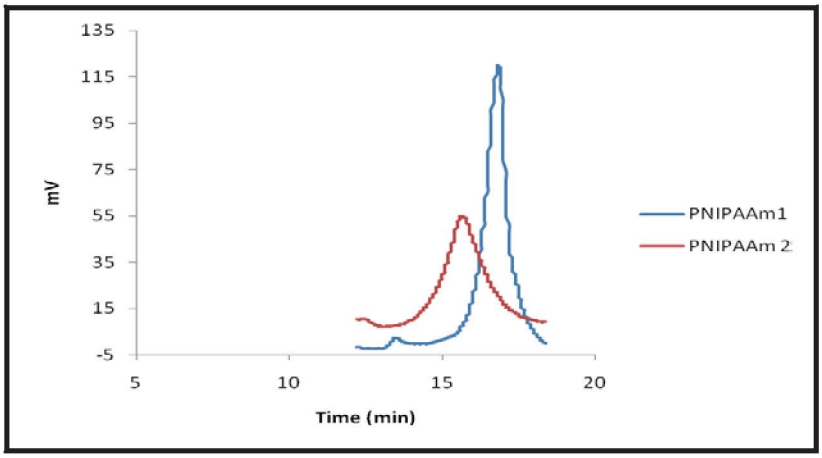

Figure 9. GPC profiles of homopolymers (PNIPAAm 1 and 2)

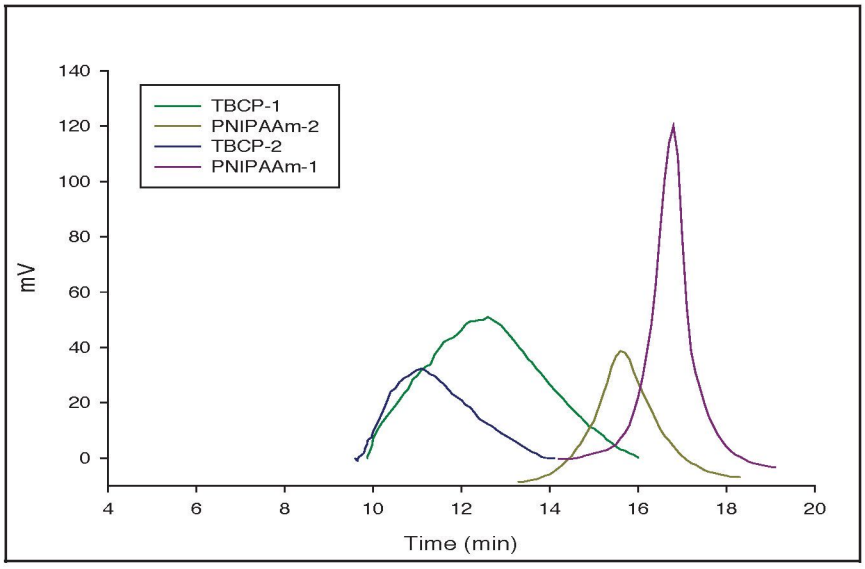

Figure 10. GPC profiles of PNIPAAm 1 and 2, and Triblock-co-polymer 1 and 2 after prufication.

Evaluation of LCST transitions of polymers

The LCST transition of each polymer was determined from the dependence of the absorbance change at 500 nm on temperature. Below their LCST, each polymer solution is clear, but upon further heating the solution becomes turbid because of aggregation of the PNIPAAm over narrow temperature range ~6°C and resulted in sharp optical density enhancement. The LCST was 38°C for PNIPAAm-1, 41°C for PNIPAAm-2, 43.4°C for Triblock-co-polymer-1 and 42°C for Triblock-co-polymer-2 respectively. The LCST increase with the increasing molecular weight, and also increase with the increasing amount of hydrophilic moiety (Figure 11). Hydrophilic moiety prolongs the precipitation of PNIPAAm and increase the LCST to the higher temperature.

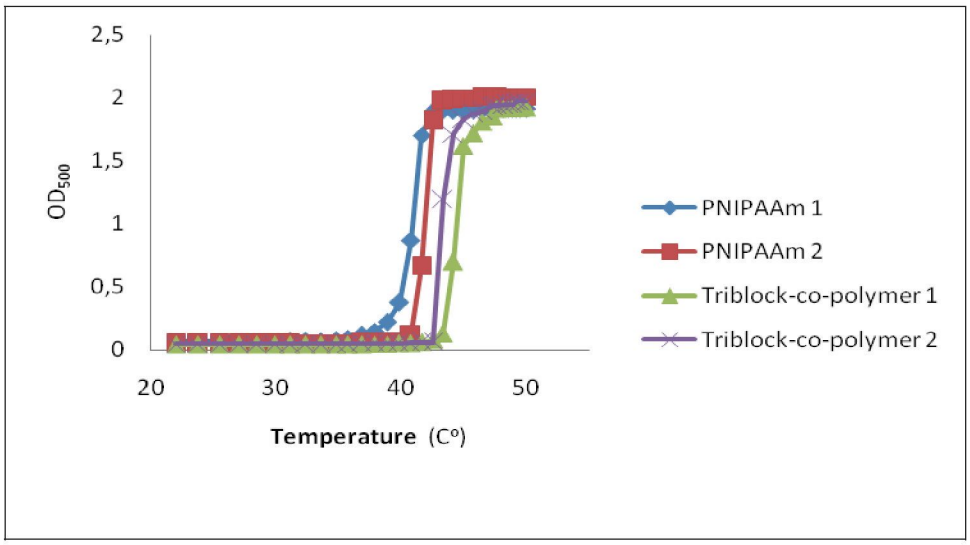

Figure 11. LCST comparison of PNIPAAm1, PNIPAAm2, Triblock-co-polymer1, and Triblock-co-polymer2 respectively.

Evaluation of Sol-Gel-Sol transition of Triblock-co-polymers

To evaluate in suti forming hydrogel properties sol-gel-sol transition of triblock-copolymer was conducted near body temperature. A 20% (w/v) polymer solution in DDI water was used (Figure 12). A and b are sol phase of Triblock-co-polymer 1 and Triblock-co-polymer 2; c in water bath at 37°C 10 minutes; d and e are gel phase of Triblock-co-polymer 1 and Triblock-co-polymer 2; f and g are precipitated opaque gels immersed in dry ice for 3 seconds and transparent gels occurred; h and ı are sol phase of Triblock-co-polymer1 and Triblock-co-polymer 2 after placed 2 hours at 4°C respectively. Results showed that Triblock-co-polymer 1 and 2 shows reversible sol-gel-sol transition with temperature changes.

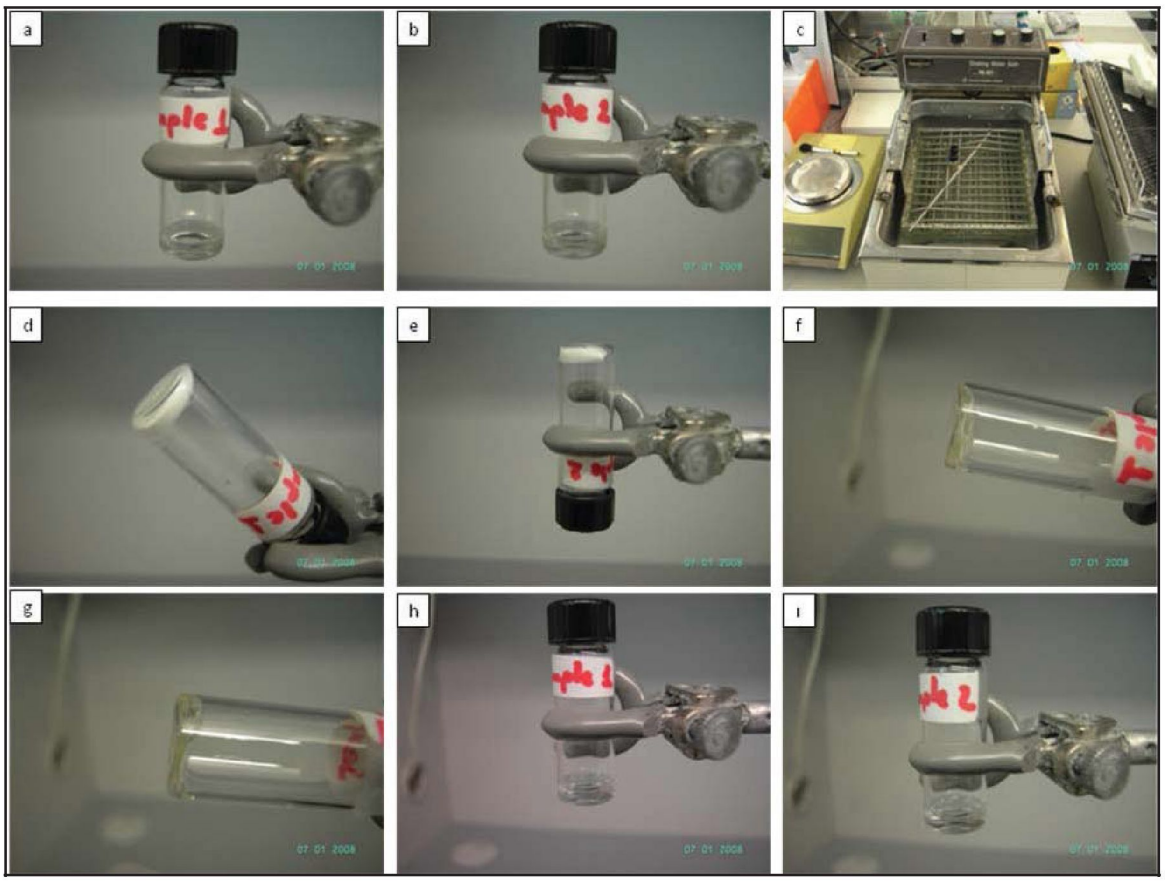

Figure 12. Sol-Gel-Sol trabsition of Triblock-co-polymer (a,b,c,d,e,f,g,h,ı)

Rheological measurements

Rheological studies were performed at a concentration of 15, 17.5 and 25% w/v for PNIPAAm1 and 17.5 and 25% w/v for PNIPAAm2. As seen from Figure 13 and 14 storage modulus (G') and loss modulus (G'') increased as the temperature increased up to 33°C, but decreased sharply with increasing temperature. This indicated that the gels, produced with PNIPAAm1 and 2 were not strong or in other words they were not as flexible as to form appropriate gel. The gels formed as the temperature changed but not perpetuated their form any longer. From the thermorheology studies we found that PNIPAAm hydrogel had poor mechanical property in a highly swollen state and the reason of this was thought to be rapid precipitation in water above the LCST and a lower polymer mass per unit volume results complied with Zhang et al, 2004 (46).

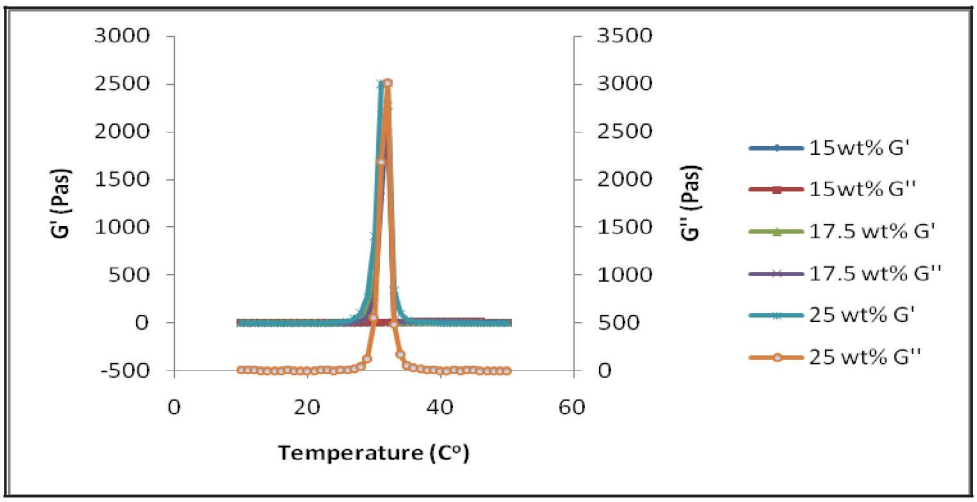

Figure 13. Rheology studies of PNIPAAm-1 at differenet wt%

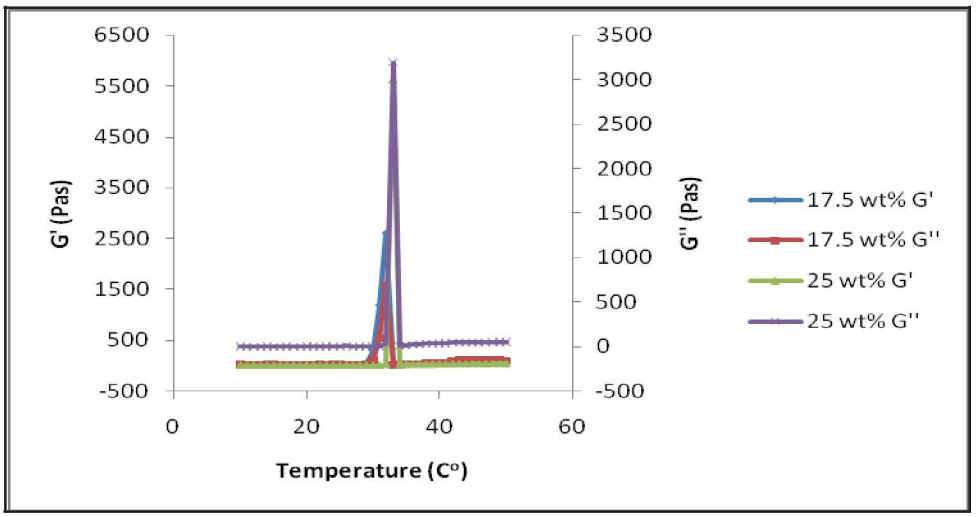

Figure 14. Rheology studies of PNIPAAm-2 at different wt%

When we look the Triblock-co-polymers (25% wt) profile, storage modulus (G') and (G'') modulus increased with the increasing temperature (Figure 15). Sharp temperature change for Triblock-co-polymer1 started 2 to 5°C later than that of Triblock-co-polymer2. It was assumed that the temperature change in the gel formation was because content of the PHPMA in the triblock-co-polymer. The amount of HPMA in Triblock-co-polymer1 was more than that of Triblock-co-polymer2. PHPMA is hydrophilic polymer that prolongs the precipitation of PNIPAAm as a results it prolongs the formation of gel. When we compare the G' and G'' of both polymer there is a huge difference between G' and G'', this means polymers form the hydrogel with an increasing temperature and maintain the form of the hydrogel (Figure 15). To increase its mechanical properties copolymerization with PHPMA polymer is necessary. Copolymerization increases polymer mass per unit volume. At the temperatures below the phase transition, loss angle of the polymer solution increased slightly with increasing temperature, which is a typical behavior of viscoelastic polymer solutions. Upon further heating and thermogelation (after 28 to 38°C), G' and G'' both increased drastically, G' finally exceeding G'' ($\delta << 45$°C), which indicated the formation of a viscoelastic hydrogel (Figure 16). Results complied with Zhang et al, 2004 and Hacker et al, 2008 (46, 47). Weak mechanical properties do not allow PNIPAAm used alone as thermosensitive drug carrier agent. Thermosensitive

polymers have to show reasonable LCST and under that LCST they could be applied as a solution, on the other hand they have to show reasonable mechanical properties which allow them to make strong gels immediately after subjected to body temperature.

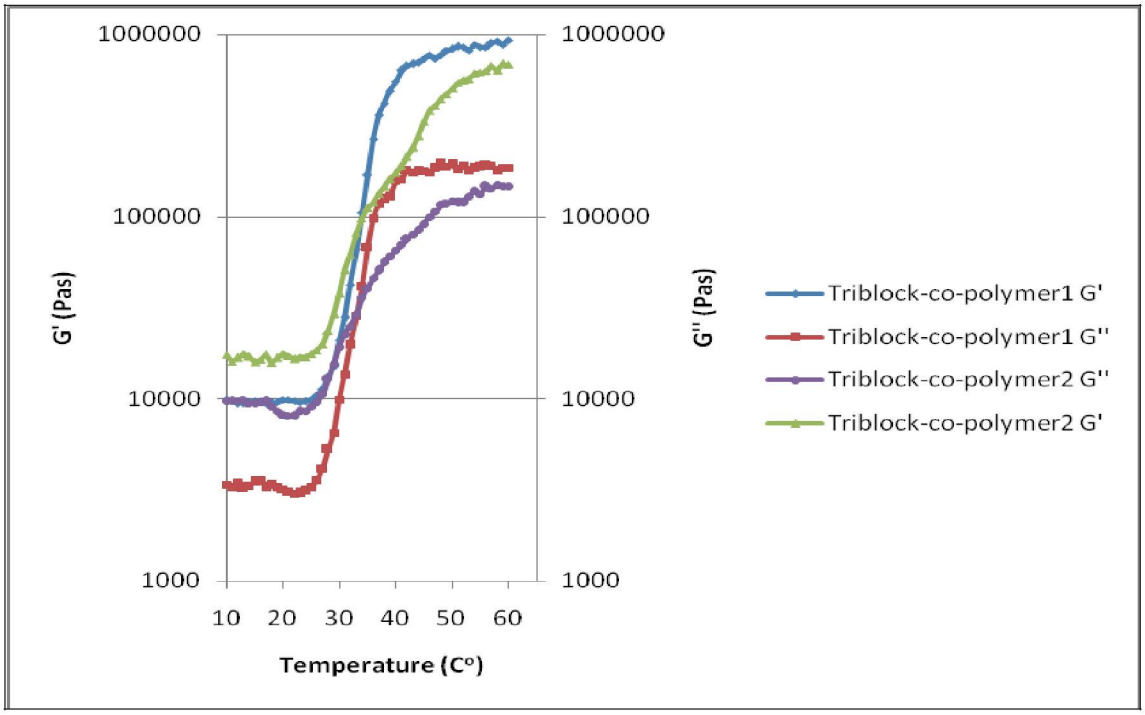

Figure 15. Comparison of storage (G') and loss modulus (G'') of Triblock-co-polymer 1 and 2

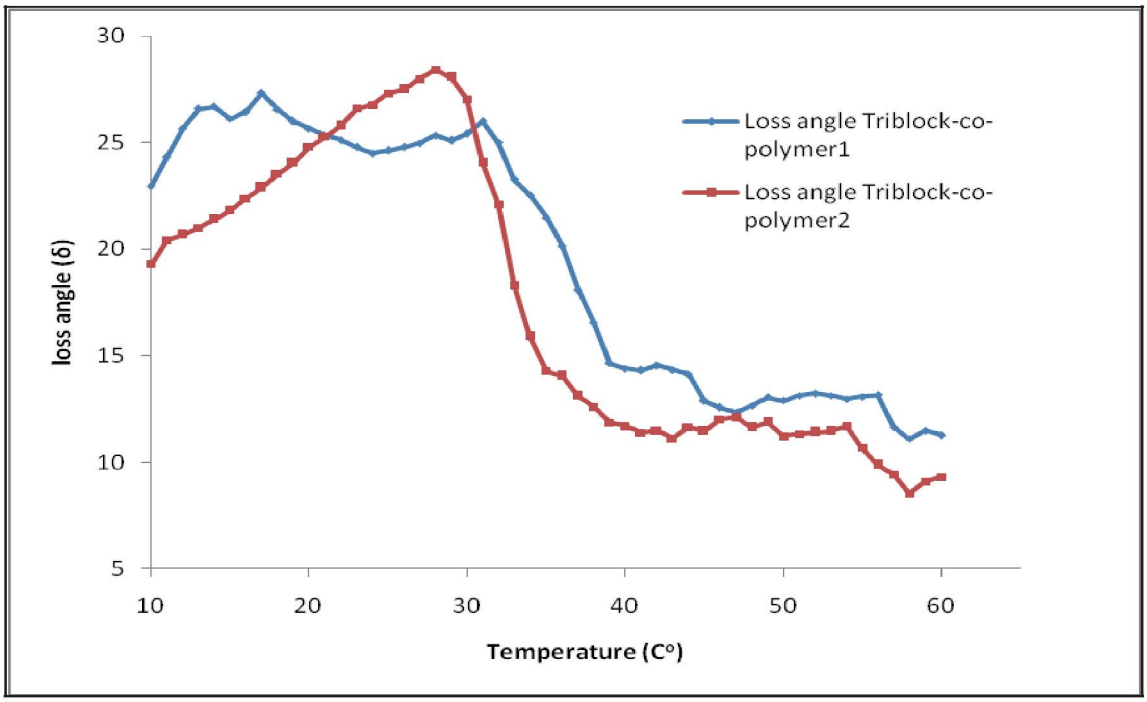

Figure 16. Comparison of loss angle of Triblock-co-polymers

Evaluation of swelling ratios of homopolymers and Triblock-co-polymers

Each polymer solution contains 20% w/v of polymer. At 20°C all polymer solutions are in "sol" state which means at temperatures below the LCST, the hydrophilic groups of the polymers form hydrogen bonds with water molecules so polymers dissolve in water. As the external temperature increases, the associative interactions among the hydrophobic groups release the entrapped water molecules and form "gel" network. At 37°C the polymers form gel and temperature dependence swelling ratio was determined.

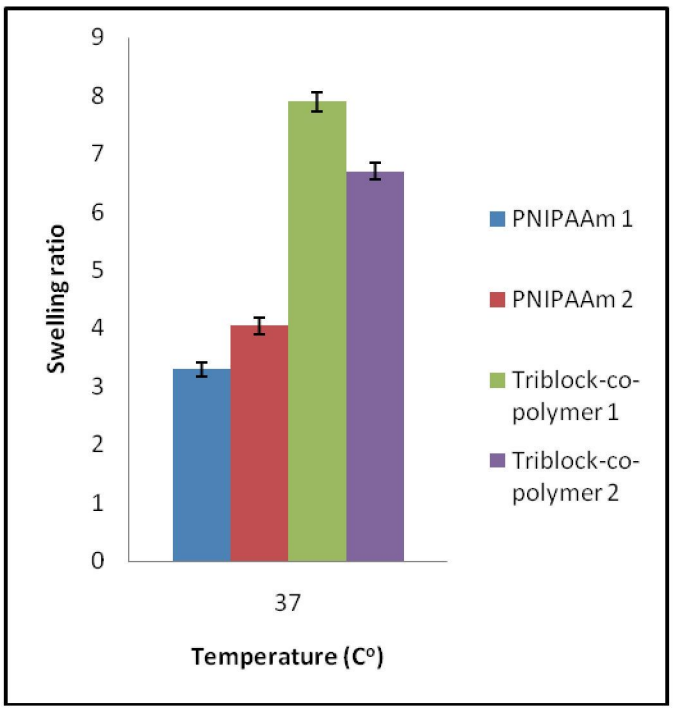

Figure 17. Comparison of swelling ratio at 37°C of homo and copolymers. The data were expressed as mean ± S.D. (n=3).

The latter phenomenon (difference in swelling ratio) in Figure 17 may be attributed to differences in the homopolymer and triblock-co-polymer form and the PNIPAAm content of triblock-co-polymer 1 and 2. When the temperature exceeds the LCST PNIPAAm collapse and aggregates together, while the hydrophilic interactions in the triblock-co-polymer can still lead to water retention. Triblock-co-polymer 1 with a lower PNIPAAm content can maintain a relatively larger swelling ratio above LCST. The results indicate that the thermosensitive properties of the hydrogels arise mainly from PNIPAAm and the hydrophilic/ hydrophobic ratio can be used to control the swelling ratio of the PNIPAAm based hydrogels. Results complied with Zhang et al, 2004 (46).

CONCLUSION

PNIPAAm hydrogels have been widely examined as a smart drug delivery material due to their unique phase separation behavior upon external temperature changes. They exhibit a sudden shrink in volume at a temperature right above LCST. Incorporation of hydrophilic moiety increases the LCST of PNIPAAm and vice versa. It was concluded from the swelling ratio that hydrophilic component (PHPMA) was incorporated into PNIPAAm hydrophilic/hydrophobic balance of triblock-copolymer was shifted towards a more hydrophilic nature and the LCST was shifted to a higher temperature. According to the Sol-Gel-Sol studies

after stayed 10 minutes in water bath at 37°C structural collapse takes place upon heating and both homopolymer and Triblock-co-polymer of PNIPAAm readily transform to gel structure. From the thermorheology studies it was found that PNIPAAm hydrogel without copolymerization with PHPMA had poor mechanical property in a highly swollen state and the reason of this was thought to be rapid precipitation in water above the LCST and a lower polymer mass per unit volume. To increase its mechanical properties copolymerization with PHPMA polymer was necessary. Copolymerization increase polymer mass per unit volume. Formation of gel structure at 37°C and increase in viscosity are crucial feature for the ocular delivery system leading to longer contact time with the eye surface and resulted in better bioavailability. Finally, the key feature of triblock-co-polymer is hydrophilic / hydrophobic ratio. It should be keep in mind that copolymeriaztion with hydrophilic moiety would greatly weaken, even eliminate the thermal sensitivity of the PNIPAAm based on hydrogels rheology study due to the introduction of the excessive non-thermosensitive moiety. Our hydrogels which exhibit a sol-gel phase transition in response to external stimuli provide a simple and safe method of preparing in-situ forming gels. The study was found to be promising for the further evaluation of triblock-co-polymer as a new thermo-sensitive hydrogels which can be used as ocular pharmaceutical excipient.

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