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MARMARA UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

IN PURE AND APPLIED SCIENCES

DEVELOPMENT OF A THERMOSENSITIVE DRUG DELIVERY SYSTEM BY RAFT CONTROLLED POLYMERIZATION

GİZEM NUR ÖNGEL

524521002

MASTER THESIS

Department of Chemical Engineering

Thesis Supervisor

Assoc. Prof. Dr. Neslihan ALEMDAR YAYLA

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Gizem Nur ÖNGEL, a Master of Science student of Marmara University Institute for Graduate Studies in Pure and Applied Sciences, defended her thesis entitled "Development of a thermosensitive drug delivery system by RAFT controlled polymerization", on 4 July, 2024 and has been found to be satisfactory by the jury members.

Jury Members

Assoc. Prof. Dr. Neslihan ALEMDAR YAYLA (Advisor)
Marmara University .....

Prof. Dr. Atıf KOCA (Jury Member)
Marmara University .....

Assoc. Prof. Dr. Mehtap ŞAFAK BOROĞLU (Jury Member)
Istanbul-Cerrahpaşa University .....

APPROVAL

Marmara University Institute for Graduate Studies in Pure and Applied Sciences Executive Committee approves that Gizem Nur ÖNGEL be granted the degree of Master of Science epartment of Chemical Engineering on ___/___/____ (Resolution no: _____).

Director of the Institute

Prof. Dr. Bülent EKİCİ

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Gizem Nur ÖNGEL

Chemical Engineer

RAFT KONTROLLÜ POLİMERİZASYON İLE ISIYA DUYARLI İLAÇ TAŞIYICI SİSTEMİNİN GELİŞTİRİLMESİ

ÖZET

Hidrojeller, yüksek oranda su tutma kapasitesi, biyouyumluluk gibi önemli özellikleri sayesinde kontrollü ilaç salım sistemlerinde ilaç taşıyıcısı olarak son zamanlarda oldukça yaygın bir şekilde kullanılmaktadırlar. Çünkü kontrollü salım, klasik ilaç formlarına göre yan etkileri azaltması, ilaç etkinliğini artırması ve hedef bölgeye yönlendirilebilmesi açısından çok önemli bir araştırma konusu haline gelmiştir. Özellikle farklı fiziksel şartlara (sıcaklık, pH, iyonik şiddet v.b.) göre farklı özellikler (şişme-büzülme v.b.) gösteren akıllı hidrojeller, kontrollü ilaç taşıyıcı sistemleri için bir avantaj olarak görülmektedirler. Klasik polimerizasyon yöntemleri ile bu sistemlerin üretimine literatürde pekçok çalışmada rastlanmasına rağmen, kontrol edilebilir molekül ağırlığına ve düşük polidispersiteye sahip polimerlerin üretilmesini sağlayan kontrollü polimerizasyon tekniklerinin (tersinir ekleme ayrıştırma transfer polimerizasyonu (RAFT); nitroksit ortamlı polimerizasyon, (NMP); ve atom radikal transfer polimerizasyonu, (ATRP)) kullanıldığı çalışmalara çok daha az rastlanmaktadır.

Yapılan bu çalışmada sıcaklığa duyarlı PNIPAAm (Poly(N-izopropilakrilamid)) eldesi RAFT ajanı varlığında kontrollü polimerizasyon yöntemi ile gerçekleştirilmiştir ve böylece istenilen aralıkta 2 farklı molekül ağırlığına (yüksek ve düşük), düşük polidispersiteye ve karboksil uç grubuna sahip polimerler elde edilmiştir. Bu şekilde elde edilen PNIPAAm polimerine jelatin doğal polimeride eklenerek her iki polimerinde üstün özelliklerine sahip bir ilaç taşıyıcı sistem elde edilmiştir. PNIPAAm-jelatin bazlı hidrojele model ilaç olarak bir kanser ilacı olan 5-FU yüklenmiştir. Çünkü kanser tedavisinde kullanılan birçok ilaç gibi 5-FU da sindirimi, sinir sistemini, cildi ve kalbi etkileyen birçok yan etkiye neden olabilmektedir ve bu yüzden de kontrollü salımı son derece önemlidir. Elde edilen ürünlerin yapısal karakterizasyonları H-NMR, FT-IR ve GPC ile sağlanırken, morfolojileri SEM analiziyle belirlenmiştir. Şişme ve ilaç salım çalışmaları iki farklı sıcaklık (25 ℃ ve 37 ℃) ve iki farklı molekül ağırlığı için yapılarak sıcaklığın ve molekül ağırlığının şişme kapasitesine ve salım kinetiğine etkileri incelenmiştir. Böylece kanser tedavisinde kullanılmak üzere kontrollü salım sağlayabilecek bir ilaç taşıyıcı sistem RAFT tekniği ile geliştirilmiş olup, bu yenilikçi yönüyle de literatüre katkı sağlanmıştır.

DEVELOPMENT OF A THERMOSENSITIVE DRUG DELIVERY SYSTEM BY RAFT CONTROLLED POLYMERIZATION

ABSTRACT

Hydrogels have recently been widely used as drug carriers in controlled drug release systems, thanks to their important properties such as high water retention capacity and biocompatibility. Because controlled release has become a very important research topic in terms of reducing side effects, increasing drug effectiveness and directing it to the target area compared to classical drug forms. In particular, smart hydrogels, which show different properties (swelling-shrinking, etc.) according to different physical conditions (temperature, pH, ionic strength, etc.), are seen as an advantage for controlled drug carrier systems. Although the production of these systems by classical polymerization methods is encountered in many studies in the literature, controlled polymerization techniques (reversible addition decomposition transfer polymerization (RAFT); nitroxide media polymerization (NMP); and atomic radical transfer polymerization), which enable the production of polymers with controllable molecular weight and low polydispersity, are not available. Studies using , (ATRP)) are much less common.

In this study, temperature-sensitive PNIPAAm (Poly(N-isopropylacrylamide)) was obtained by controlled polymerization method in the presence of RAFT agent, and thus, polymers with two different molecular weights (high and low) in the desired range, low polydispersities and carboxyl end groups were obtained. By adding gelatin natural polymer to the PNIPAAm polymer obtained with this way, a drug carrier system with superior properties of both polymers was obtained. PNIPAAm-gelatin-based hydrogel was loaded with model drug. 5-FU, a cancer drug, was chosen as the model drug. Because, like many drugs used in cancer treatment, 5-FU can cause many side effects that affect digestion, nervous system, skin and heart. That's why controlled release is extremely important. While the structural characterizations of the obtained products were provided by FT-IR and GPC, their morphologies were determined by SEM analysis. Swelling and drug release studies were carried out for two different temperatures (25 ℃ and 37 ℃) and two different molecular weights, and the effects of temperature and molecular weight on the swelling capacity and release kinetics were examined. Thus, a drug carrier system that can provide controlled release for use in cancer treatment was developed using the RAFT technique and contributed to the literature.

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ABBREVIATIONS

AIBN: 2,2-Azobis(İsobutyronitrile)

ATR: Attenuated Total Reflectance

ATRP: Atom Transfer Radical Polymerization

CBPA: 4-Cyano-4-(Butylsulfanylthiocarbonyl)Sulfanyl Pentanoic Acid

CRP: Controlled Radical Polymerization

CTA: Chain Transfer Agent

D6-DMSO: Dimethyl Sulfoxide

EDC: 1-Ethyl-3-(3-Dimethylaminopropyl)Carbodiimide

FT-IR: Fourier Transform Infrared Spectroscopy

GEL: Gelatin

GPC: Gel Permeation Chromatography

^{1}H-NMR: Proton Nuclear Magnetic Resonance Spectroscopy

NMP: Nitroxide-Mediated Polymerization

RAFT: Reversible Addition/Fragmentation Chain Transfer Polymerization

RDRP: Reversible-Deactivation Radical Polymerization

LCTS: Lower Critical Solution Temperature

Mtm/L: Lower oxidation state

NIPAM: *N*-İsopropylacrylamide

PBS: Phosphate Buffer Saline

PDI: Polydispersity İndex

PNIPAAM: Poly(N-İsopropylacrylamide)

Pn: Active polymer chains

Pn-X : Dormant alkyl halide

Pm : Active chain

PS: Polystyrene

R: Active group

SEM: Scanning Electron Microscope

THF: Tetrahydrofuran

UV: Ultraviolet Spectroscopy

5-FU: 5- Fluorouracil

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1. INTRODUCTION

One type of soft polymer network system that can hold its shape even after absorbing a lot of water is called hydrogel. Hydrogels have drawn a lot of interest in the domains of biomedicine, biochemistry, and other related sciences because of their viscoelastic characteristics, which are akin to those of live tissues, and their permeability to different kinds of molecules.

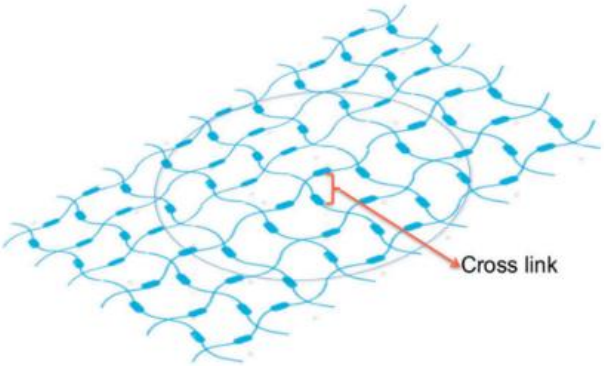

Figure 1 Structure of hydrogel [1]

Hydrogels can be classified as intelligent hydrogels or standard hydrogels based on how responsive they are to external stimuli. In contrast to intelligent hydrogels, also referred to as stimuli-responsive hydrogels, conventional hydrogels are not sensitive to changes in their surroundings. These stimuli can include temperature, pH, light, magnetism, ion concentration, electrical field, solvent composition pressure, and biomolecules. The intelligent hydrogel network responds to environmental stimuli by contracting or expanding, which modifies the hydrogel matrix properties significantly. Intelligent hydrogels have been widely used in many different sectors, including tissue engineering, chemical/biological separators, controlled release drug carriers, and gene carriers, because of their exceptional reactivity [2].

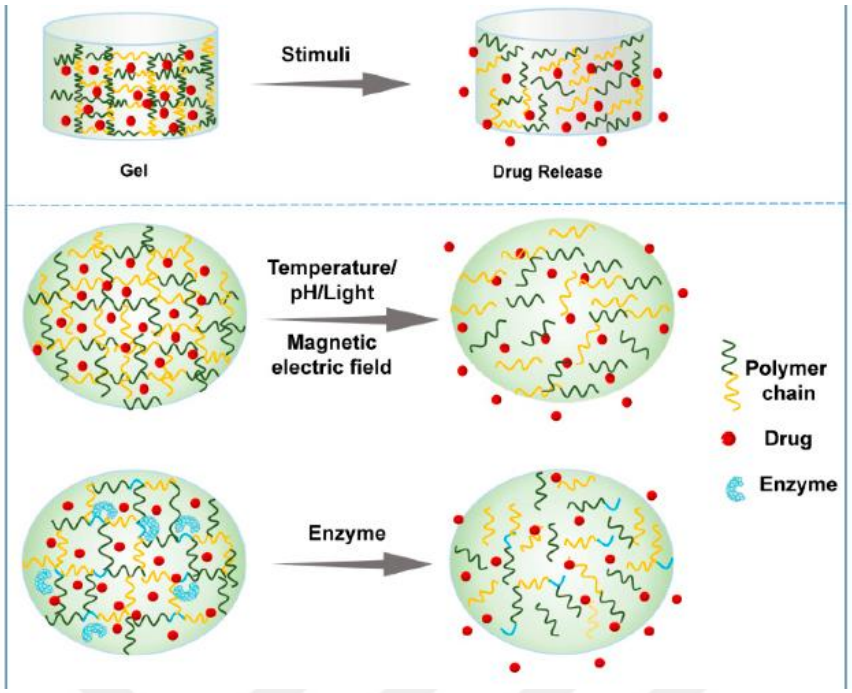

Figure 2 Diagram showing the release of a medicine under different conditions from an example smart hydrogel [3]

The conventional methods of medicine delivery to humans frequently have numerous drawbacks, including large dosages and detrimental side effects. To reduce these negative effects, researchershave recently been focusing on developing more effective medication loading and release strategies. Generally speaking, hydrogels, nanoparticles, and nanocapsules are appropriate means of getting around these restrictions. Specifically, as the temperature rises over their lower critical solution temperature (LCST), hydrophobic core-shell nanoparticles and capsules with thermal sensitivity can open up access to load medications and release them [2, 3].

Because temperature-responsive hydrogels have appealing qualities like non-toxic structures, easy formulations, swelling properties in physiological environments, and the reversibility of the solution-gel transition with temperature change, numerous studies have been done to provide controlled release with these materials. Chitosan, poly (organophosphazen), poloxamer, and pluronic are examples of polymers that are utilized in formulations with temperature-sensitive controlled release. N-isopropylacrylamide is one of the most significant temperature-sensitive polymers. In the field of biomedicine, the most popular and extensively utilized stimuliresponsive polymer is Poly(N-isopropylacrylamide) (PNIPAAm). At 32 °C, the polymer displays a temperature-dependent phase transition in

aqueous solution that modifies its hydrophobic and hydrophilic characteristics. Because PNIPAAm's lower critical solution temperature (LCST) is so close to body temperature, it has found widespread use in biomedical applications. These applications include drug and gene delivery systems regulated by PNIPAAm, biosensors that use the phase transition behavior of PNIPAAm, and conjugated proteins for thermally modulated enzyme performance. A thermoresponsive cell culture dish made by grafting PNIPAAm onto the dish is the most successful application [2, 4].

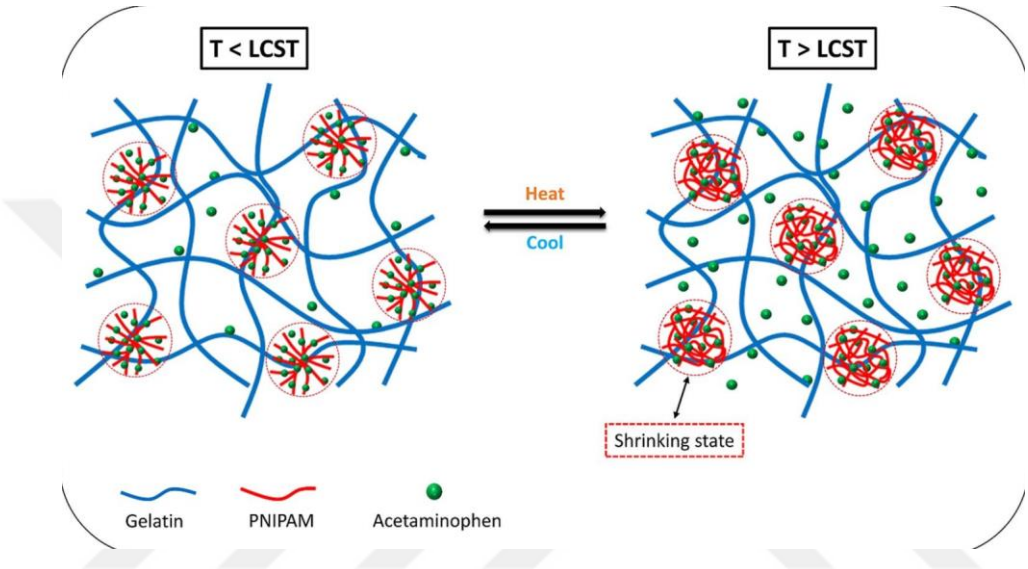

Figure 3 Schematic presentation of thermoresponsive behavior [5]

Natural polymer gelatin is a fibrous protein that is extracted from collagen by hydrolyzing it in an alkaline or acidic solution. The food and drug administration has generally deemed gelatin to be safe; it is also less antigenic than collagen, biocompatible, biodegradable, and contains the arginine-glycine-aspartic motifs, which are known to be crucial for promoting cell adhesion. Gelatin is commercially available at a low cost. Moreover, it offers a variety of functional groups that can be crosslinked to change the material chemically or physically. In water, gelatin can absorb up to ten times its mass. As gelatin melts at temperatures above 30 ºC and releases medications into the human digestive system with ease, it is the primary component of both hard and soft pharmaceutical capsules. Moreover, gelatin can be utilized to create scaffolds, encapsulating agents, and tablet binders that can be used to transport bioactive compounds and medications, such as growth factors, nucleic acids, antibacterial agents, and anti-inflammatory pharmaceuticals, among others. For a variety of tissue engineering cancer therapy and therapeutic angiogenesis applications, the inclusion of synthetic or natural polymers can enhance the drug-release characteristics from gelatin. Also,

combining natural elements with stimuli-responsive polymers via inter-hydrogen bonding could be a tactic to enhance membrane biocompatibility. Rich in amino acid groups, gelatin is a naturally occurring polymer with good biocompatibility and negligible cytotoxicity. Gelatin and PNIPAAm can create inter-hydrogen bonds of "appropriate strength" in comparison to other natural polymers [6, 7].

Injectable thermoresponsive/thermosensitive hydrogels have been used to deliver chemotherapy medicines for cancer treatment since their inception in the 1960s. Thermoresponsive hydrogels have proven to be more effective than traditional injectable chemotherapeutics for targeted therapy because of their capacity to stay at the tumor location after injection [8]. By lowering systemic exposure and the associated side effects while enhancing local drug accumulation, this strategy can greatly raise the therapeutic index of cytotoxic medicines. Depot systems will decrease the number of chemotherapy cycles in addition to the quantity of anticancer medications needed, which will improve patient compliance and welfare while also lowering treatment costs [9, 10].

1.1. Controlled Polymerization Techniques

Modern technologies create an endless need for high-performance materials with complex functionality. Additionally, these materials should be cheap, easily produced, and ecologically benign so that they can replace existing commercial materials or be used in new, beneficial applications for society. Biocompatible polymers for biosensors, catalytic supports, nanoreactors, next-generation separation media, and flexible solarcells are just a few applications for polymers having functional groups that are either randomly distributed or present as discrete blocks. For these applications, fine-tuning the desired material properties requires strong molecular control over the polymers. The ability of reversible-deactivation radical polymerization (RDRP), also called controlled radical polymerization (CRP), to produce highly defined polymers at the molecular level that can compete and traditional living polymerization techniques like ionic polymerization has led to a significant increase in interest in this technique over the past 25–30 years [11].

Controlled radical polymerization, an amazing variety of polymerization techniques, are enable the synthesis of polymers with distinct microstructures and comonomer sequences. Common examples of controlled polymerization techniques are nitroxide-mediated polymerization (NMP), atom transfer radical polymerization (ATRP), and reversible addition/fragmentation chain transfer polymerization (RAFT) which allows for more control

over the polymerization process, including a predetermined and limited range of molecular weights.

1.1.1. Nitroxide-Mediated Polymerization (NMP)

One kind of RDRP that has the potential to be realistically scaled up is nitroxide-mediated polymerization (NMP), which may be utilized to create highly specified polymer structures without requiring air-free transfers, transition-metal catalysts, or troublesome thiol agents. Consequently, the final polymer does not need to be further purified before being used in delicate biological or electronic applications. The controlled, pseudoliving polymerization of a propagating chain with a specific monomer is made possible by the reversible activation and deactivation of a stable free radical, which is the basis of the NMP process. The use of a commercially available unimolecular initiator (BlocBuild-er-MA) that releases a stable free radical and a propagating radical when heated is shown in Figure 4 [11].

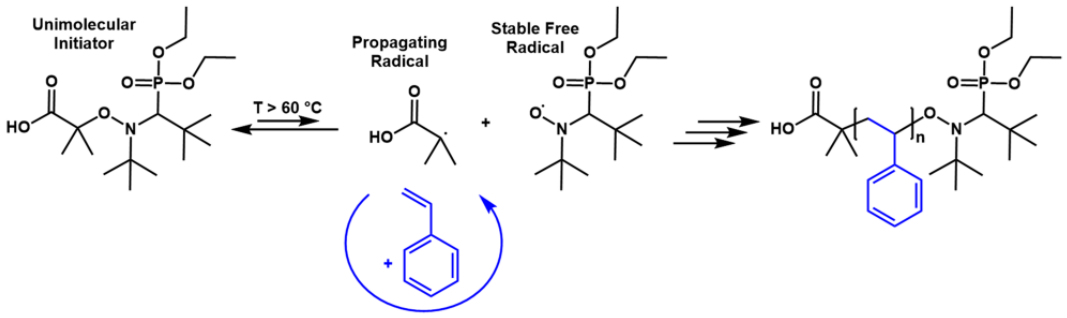

Figure 4 Propagating styrene polymerization by unimolecular alkoxyamine initiator activation in a reversible manner [11]

Figure 5 illustrates NMP mechanism with using the activation-deactivation equilibrium key [12, 13].

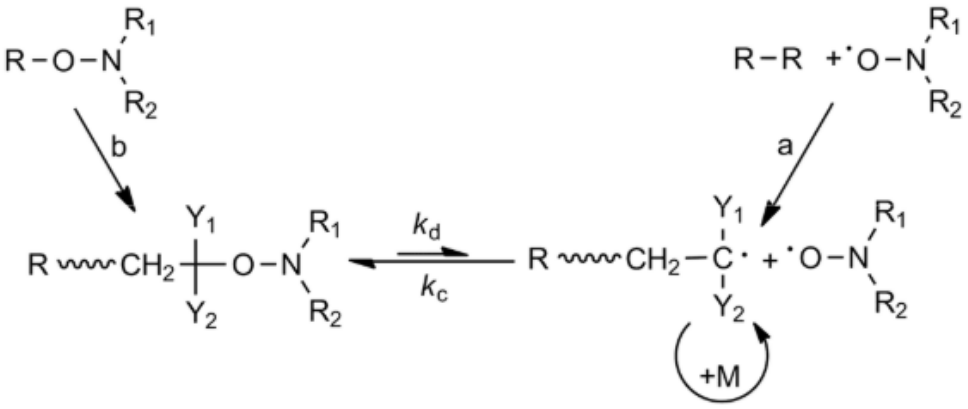

Figure 5 Activation- deactivation equilibrium in NMP

1.1.2. Atom Transfer Radical Polymerization (ATRP)

A transition metal, typically ruthenium or copper, mediates ATRP to produce polymers with specific molecular weights and narrow molecular distributions. Unprotected saccharide monomers can directly carry out the synthesis in the presence of functional groups, such as alcohols. polymerization. In order to produce designed chain ends, ATRP needs a halogenated initiator that might also contain other functional groups (Figure 6) [14].

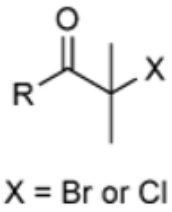

Figure 6 Common initiator for ATRP

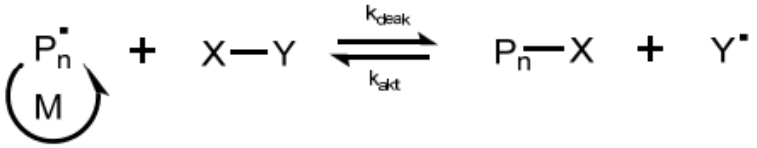

Figure 7 Equlibrium of ATRP

In the ATRP system, there is a deactivation and bimolecular activation process with reversible atom or group transfer. For these systems defined, the deactivator is the (X-Y)

transition metal complex. In this notation, Y is the metal in the higher oxidation state. Y* is in a lower oxidation state and is inert compared to the monomer. The transition metal type used in ATRP removes the halogen atom from the organic halide to form the oxidized species and the carbon-centered radical (active radical) [15].

The mechanism showing the repeated activation and deactivation nature of ATRP is given in more detail in Figure 8.

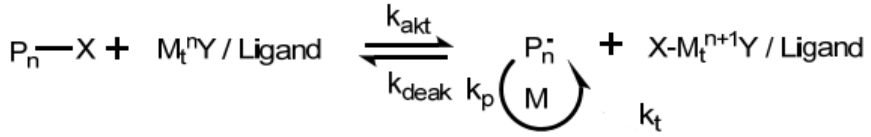

Figure 8 Mechanism of ATRP

Mt^n : Transition Metal

L : Complex Ligand

P_n : Polymer Chain

X: Br or Cl

In the ATRP system, Cu, Fe and Ru are mostly used as transition metals, while an alkyl halide (usually Bromide or chloride) is used as the initiator [16].

1.1.3. Reversible Addition-Fragmentation Chain-Transfer Polymerization (RAFT)

RAFT polymerization requires thiocarbonylthio compounds, which include xanthates, dithioesters, and thiocarbamates, a chain transfer agent also referred to as an RAFT agent. In RAFT polymerization, the RAFT agent regulates a polymer's molecular weight and polydispersity. Because RAFT polymerization is a relatively tolerant process, it can be used with a monomer and solvent at a wide range of temperatures. A RAFT system typically consists of a solvent, RAFT agent, radical source, and monomer. To ensure that the free radical is delivered in a chain growth manner at the necessary pace and that the RAFT equilibrium is maintained between the dormant and active states, the right RAFT agent in the right amount at the right temperature is selected. Due to its immense control over the polymerization process, it is a very effective technique that can be used to synthesize a wide range of materials with various characteristics [17].

The proper choice of RAFT reagent (ZC(S)SR) for a given monomer is necessary for the successful use of RAFT. To offer suitable control, care should be used in selecting both the R and Z groups [18].

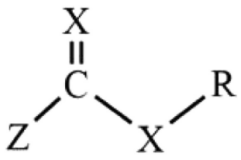

Figure 9 Schematic representation of RAFT agent [2]

The following are the properties of RAFT polymerization: A variety of techniques, including bulk, suspension, and emulsion polymerization, can be used to produce polymerization; a moderate operating state exists; a wide range of appropriate monomers are available; and the polymer structures are well-controlled.

A typical RAFT polymerization consists of four steps: initiation, addition – fragmentation, reinitiation, and equilibration (Figure 10).

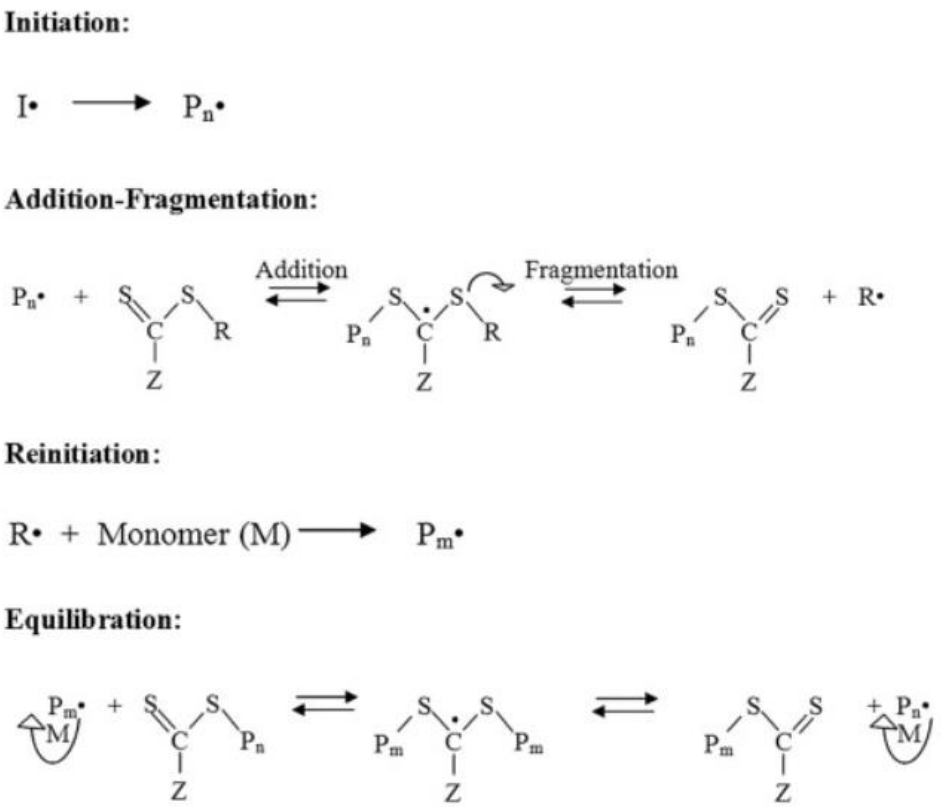

Figure 10 General mechanism of RAFT

Initiation step begins by radical initiators (I). An active radical species is created when the initiator and monomer react, and this species can initiate polymerization by producing active polymer chains (Pn•). The homolytic leaving group (R•) is released during the addition-fragmentation process that takes place between the RAFT agent and the active chain (Pn•). The active intermediate (Pn•) may lose the polymeric chain or the active group (R•) in this reversible phase. Reinitiation can occur between a monomer and the radical in the leaving group, which initiates the synthesis of a new active polymer. The stages of addition–fragmentation or equilibration are followed by this active chain (Pm•). RAFT agents are thiocarbonylthio compounds with Z and R groups that act as chain transfer agents. The radical specie that can be added to the C=S bond is primarily under the control of the Z group. The R group can start new polymer chains and is an effective homolytic leaving group. The reaction between the dormant thiocarbonyl molecule and the active propagating species is regulated by a constant equilibrium in the RAFT processes. Pm• and Pn•, the active polymer chains, are in balance between their dormant and active phases. One polymer chain in the process is engaged in polymerization while the other is inert and bonded to the thiocarbonyl molecule [2].

1.1.3.1.Temperature–Responsive Hydrogels Based on RAFT Polymerization

Currently, one of the most researched types of intelligent hydrogels are temperature-responsive hydrogels, also called thermo-sensitive hydrogels, which are sensitive to outside temperature [20, 21]. The critical phase transition temperature (Tc) is the temperature at which the swelling behavior of thermosensitive hydrogels typically changes abruptly, rather than gradually [22]. The two types of thermo-sensitive hydrogels that can be distinguished are thermo-expansion hydrogels and thermo-shrink hydrogels based on their behavior in response to temperature [23, 24]. In the field of thermosensitive hydrogels, hydrogels based on poly(N-isopro pylacrylamide) (PNIPAAm), a type of thermo-shrink hydrogel, have garnered the most attention recently [25, 26]. Hydrogels based on PNIPAAm have been widely used in biomedical applications because of its lower critical solution temperature (LCST) of around 32 ℃, which is extremely similar to human physiological temperature of 37 ℃ Hata! Yer işareti tanımlanmamış.. However, the low reaction rate and monotonous performance of conventional PNIPAAm-based hydrogels significantly hampered their application possibilities [27, 28]. Lately, the sequential RAFT polymerization technique which involves choosing the right RAFT agent to introduce functional molecular chains into

the polymer network in a graft or block copolymerization manner has proven to be an effective method for creating PNIPAAm-based hydrogels with enhanced performance [2]. (Figure 11, Figure 12).

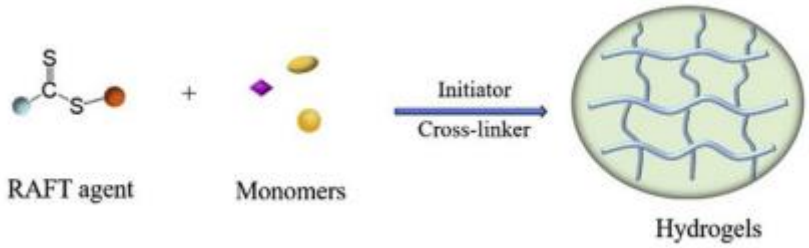

Figure 11 Diagrammatic representation of RAFT polymerization in one step [2]

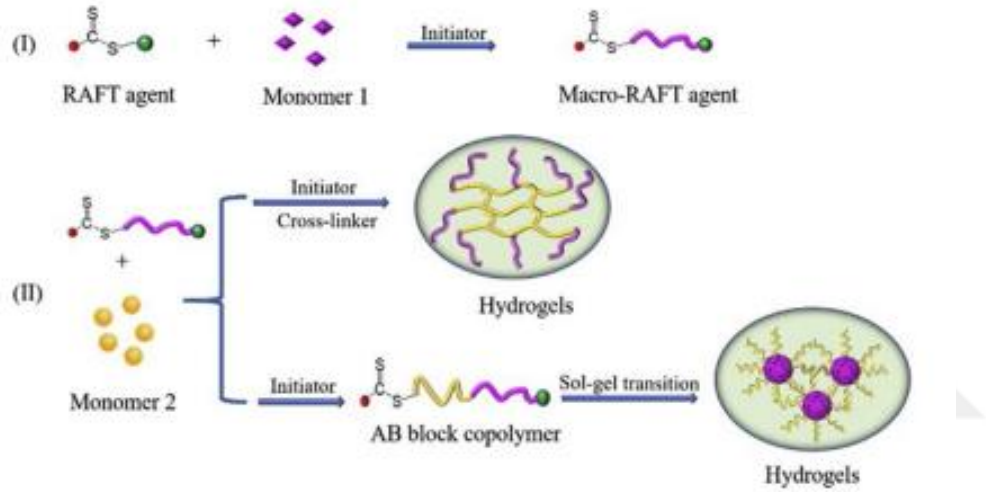

Figure 12 Diagram showing how AB copolymers are prepared for successive RAFT polymerization to create intelligent hydrogels [2]

In the light of this information, we fabricated a 5-Fluorouracil (5-FU)-loaded thermo-sensitive PNIPAAm-GEL hydrogel as a smart polymer for cancer treatment by RAFT polymerization technique and investigated the effect of adjustable properties such as molecular weight, porosity etc. on the swelling capacity and release kinetic of the hydrogels produced in this study. 5-FU was selected as a model drug since it is one of the earliest chemotherapy medications ever discovered. In clinical settings, 5-FU is mostly used to treat cancers of the stomach, colon, and breast. But this is also one of the causes of 5-FU's extremely harmful side effects and its short half-life (8–20 min), which severely restricts its use. As a result, preserving 5-FU's anti-cancer properties while lowering its toxicity has gained a lot of attention in anti-cancer research. pharmacological delivery research has

drawn more attention since pharmacological therapy aims to achieve high efficacy and minimal toxicity. Targeted delivery, polymer delivery, nucleic acid drug delivery, and nano-drug delivery are the primary drug delivery technologies [19]. Although the studies related to PNIPAAm based thermoresponsive drug carriers were reported in the literature, there is no any work on the thermoresponsive hydrogel fabricated in the presence of carboxyl end group RAFT agent as s drug vehicle for cancer treatment .

2. MATERIAL AND METHODS

In this study, PNIPAAm-GEL thermosensitive drug carrier was produced in the presence of a carboxyl-end-capped RAFT agent. The equipment and chemistry used to prepare drug carrier system were described in more detail in the following sections.

2.1. Materials

Gelatin (GEL, medical grade, 280–320 bloom, Type A) was provided from Heze Better Biochemical Co. (Shandong, China), phosphate buffer saline (PBS) tablet, 12–14 kDa cutoff dialysis tubing and 5- Fluorouracil (5-FU, MW = 130.08 Da) were purchased from Sigma Aldrich *N*-isopropylacrylamide (NIPAM, and 2,2-Azobis(isobutyronitrile) (AIBN, Fluka, 98%) were used after recrystallization from hexane and methanol respectively. 1,4 - Dioxane (Merck, 99,5%), hexane (Merck, 98,5%) and methanol (Merck, 99,9%) were used after being dried with sodium sulfate. Sodium sulfate (ISOLAB, 99,5) was used as received. The RAFT agent 4-Cyano-4-(butylsulfanylthiocarbonyl)sulfanyl pentanoic acid (CBPA) was synthesized as described elsewhere [29].

2.2. Synthesis of Poly(N-isopropylacrylamide) (PNIPAAm) via RAFT Polymerization

Poly(N-isopropylacrylamide) (PNIPAAm) homopolymers were prepared by RAFT polymerization as given in the literature [30] and shown in Figure 13 and Figure 14. NIPAAm as a monomer, CBPA as a RAFT agent, AIBN as an initatior and 1,4-dioxane as a solvent were used in polymerization procedure. The polymerization was carried out with two different feed molar ratios of NIPAM:CBPA:AIBN= 1:0.02:0.004 (PNIPAAm-50) 5×10^{-2} mol (5.658 g) NIPAM, 1×10^{-3} mol (0.291 g) CBPA and 2×10^{-4} mol (0.033 g) AIBN (PNIPAAm-50) and NIPAM:CBPA:AIBN = 1:0.005:0.001 (PNIPAAm-200) 5×10^{-2} mol (5.658 g) NIPAM, $2{,}5\times10^{-4}$ mol (0.073 g) CBPA and 5×10^{-5} mol (0.008 g) AIBN (PNIPAAm-200). According to these ratios, NIPAAm, CBPA and AIBN were mixed in 15

ml 1,4-dioxane in a round bottom flask and saturated with continuous nitrogen flow for 30 minutes. The reaction was carried out at 80 °C for 12 h. After rapid cooling of the reaction mixture, the PNIPAAm was obtained via precipitation in dry hexane. The resulting polymer was dried under vacuum at 60 °C.

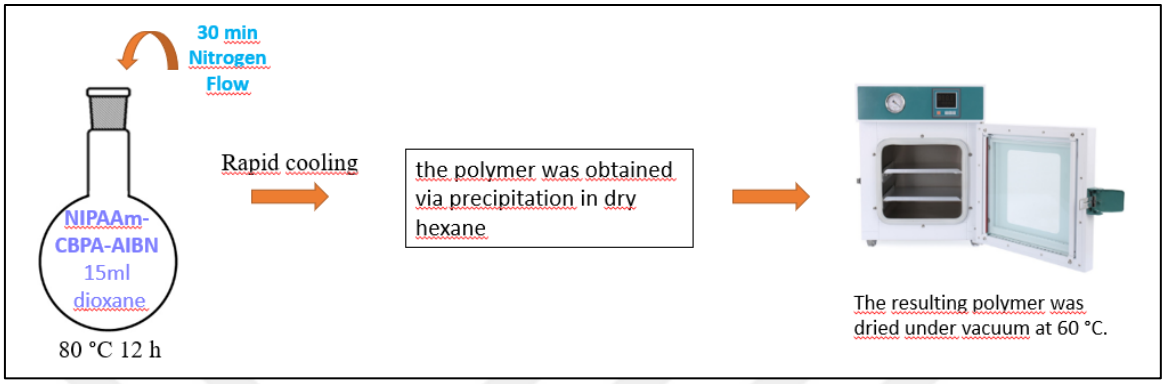

Figure 13 Schematic diagram of PNIPAAm's production in the presence of RAFT agent

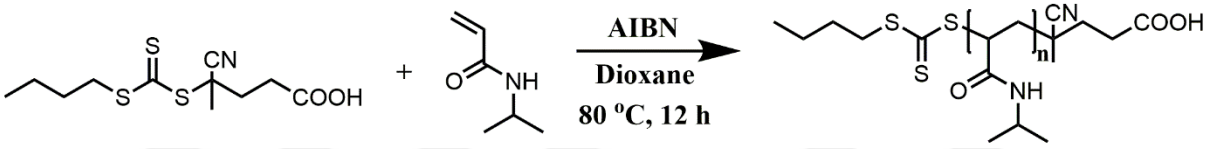

Figure 14 Preperation of PNIPAAm homopolymers

The structure of the obtained PNIPAAm in the presence of carboxyl-end-capped RAFT agent was confirmed by ^{1}H-NMR analysis using an Agilent VNMRS NMR Spectrometer operating at 500 MHz at room temperature and prepared in deuterated dimethyl sulfoxide (d6-DMSO). FT-IR spectra was also recorded by Perkin Elmer Spectrum One FT-IR with attenuated total reflectance (ATR) analysis by scanning between 450 and 4000 cm^{-1}.

Using Gel Permeation Chromatography (GPC) traces, the number average molecular weights and PDIs of the homopolymers were ascertained using an Agilent apparatus (model 1100, Santa Clara, CA) equipped with four Waters Styragel columns, a pump, refractive index, and UV detectors. THF was used as the eluent solvent, flow rate: 0.3 ml/min tempereature: 30 °C. Using linear polystyrene (PS) standards, the molecular weights (Mn and Mw) of the polymers were ascertained (Polymer Laboratories, Lewiston, ME).

2.3. Fabrication of a Drug Carrier System Composed of PNIPAAm and Gelatin

First of all, 1% solution of PNIPAAm with two different molecular weights (high and low) was prepared separately in distilled water, and 2.5 times as much EDC (cross-linker) as PNIPAAm was added to this solution and mixed. This mixture was incubated at 4 ℃ for 48 hours. On the other hand, 1% gelatin solution was prepared in pure water and added to the incubated mixture at a ratio of 1:1. The pH of the reaction mixture was adjusted to 6.0 and kept at room temperature for 24 hours. It was then transferred into the membrane and allowed to undergo dialysis for 3-4 days. Afterwards, it was transferred from the membrane to falcon tubes, frozen at -20 degrees and dried in the lyophilizer. Thus, by interacting with PNIPAAm(with carboxyl end group)-gelatin, two-component systems with 2 different molecular weights (PNIPAAm-GEL) were obtained. A summary of the operations performed is shown in Figure 12 [31].

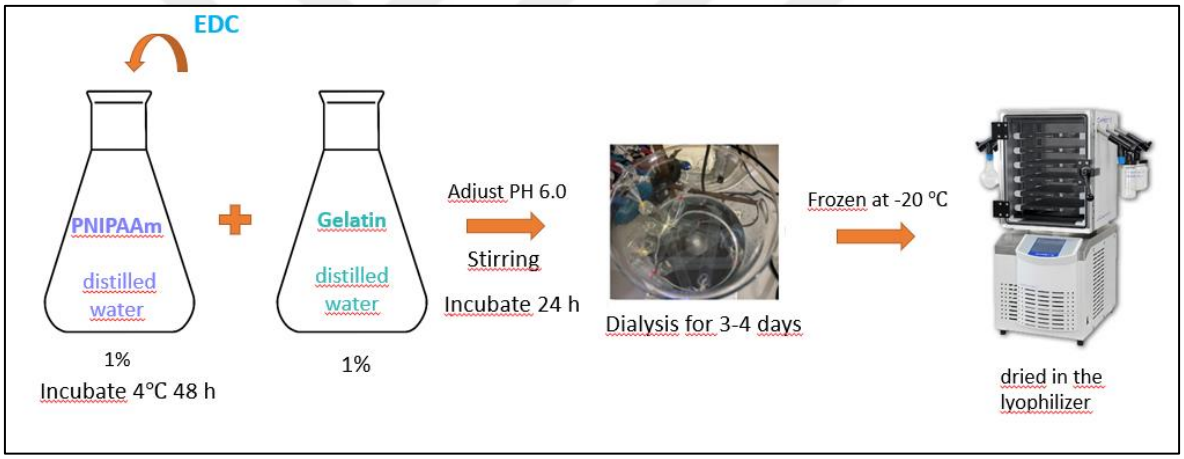

Figure 15 Obtaining a Drug Carrier System by Combining RAFT Agent PNIPAAm with Gelatin

2.4. Loading 5-FU into the Drug Carrier System (PNIPAAm-GEL)

The PNIPAAm-GEL produced in the previous step were dissolved separately (high and low molecular weight) in 1% distilled water and kept in an ultrasonicator for 30 minutes. Then, it was centrifuged for 10 minutes at 3500 rpm, the upper phase was separated and incubated at 4 ℃ for 24 hours.

5 ml of the model drug (5-FU) solution prepared at 1000 ppm was added dropwise to each solution of the different molecular weight polymer (PNIPAAm-RAFT agent-Gelatin) prepared and incubated in this way. The second cross-linking was performed by adding EDC.

After this process was completed, it was kept at 4 ℃ for 24 hours. Afterwards, the upper homogeneous mixture was separated by centrifugation at 6000 rpm for 15 minutes. This separated mixture was placed on membranes and dialysis was achieved. After 3 days, the product obtained was transferred to falcon tubes and dried in the lyophilizer. [31].

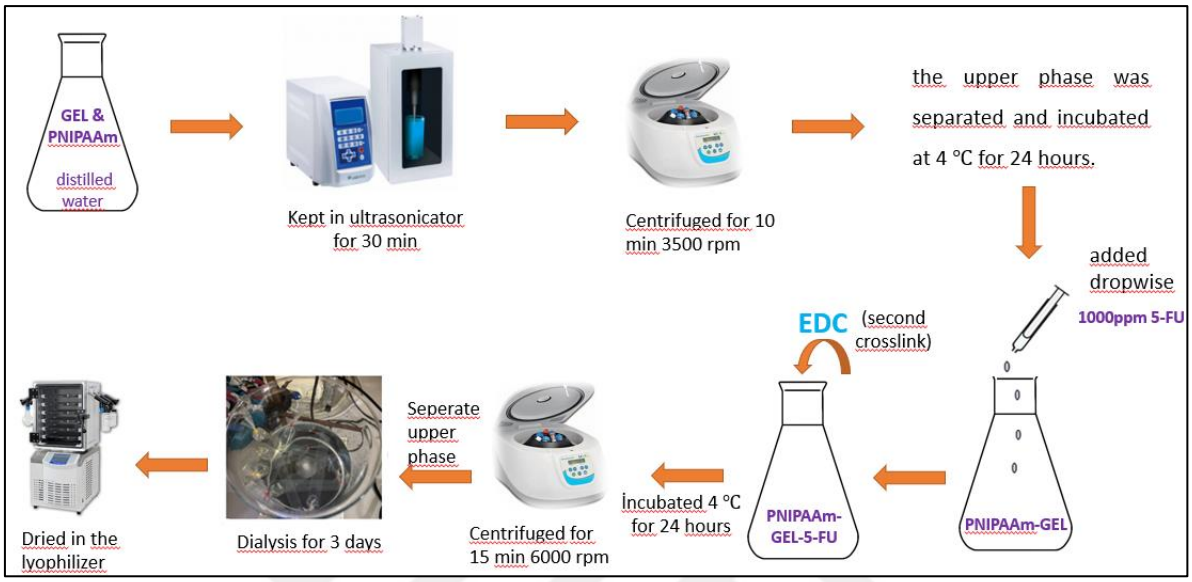

Figure 16 Loading 5-FU into the Drug Carrier

2.5. Characterization of PNIPAAm-GEL Hydrogel with/out Drug

The structures of PNIPAAm-GEL with/out drug (5-FU) were characterized by Perkin Elmer Spectrum One FT-IR with attenuated total reflectance (ATR) analysis by scanning between 450 and 4000 cm^{-1}.

2.6. Determination of Swelling Capacities of PNIPAAm-GEL Hydrogels

The swelling properties of the obtained hydrogels with two different molecular weights were determined using the gravimetric method at two different temperatures (25 ℃ and 37 ℃). For this, dry weights of approximately the same amounts of samples were taken and put in the membranes, then placed in falcon tubes containing 10 ml of PBS. After 24 hours, the samples were removed from the tubes and weighed. Swelling capacities were calculated by using the wet and dry weights in the formula given below (Equation 2.1).

$$\% \text{ SR} = \frac{W_s - W_0}{W_0} * 100 \qquad (2.1)$$

SR: Swelling ratio, W_s= Weight at swollen state, W_o= Weight at original state

2.7. Drug Release Kinetics of PNIPAAm-GEL Hydrogels

Release studies were carried out at two different temperatures, 37 ℃ and 25 ℃, and in two different molecular weight systems, low and high, and thus, the effect of temperature and molecular weight on the release kinetics of the drug was examined. For release kinetic studies, certain amounts of drug-loaded products were weighed and placed into the membrane, and these membranes were placed in falcon tubes containing 15 ml of PBS. 1 ml samples were taken from the samples at certain time intervals. The amount of drug released from the carrier system for that time period was determined with the UV absorbance device [31].

3. RESULTS AND DISCUSSION

In this study, PNIPAAm based thermosensitive drug carrier was produced by using RAFT controlled polymerization technique to obtain controlled structure (molecular weight, end group, porosity) and observe the effect of these features on the character of the drug vehicles. For this, firstly, PNIPAAm homopolymer was produced in the presence of the carboxyl-end-group RAFT agent (Fig. 17). Then, PNIPAAm-RAFT agent was reacted with amine groups ($-NH_2$) in gelatin owing to its carboxyl groups (-COOH) to produce polymeric network as shown in Fig. 13.

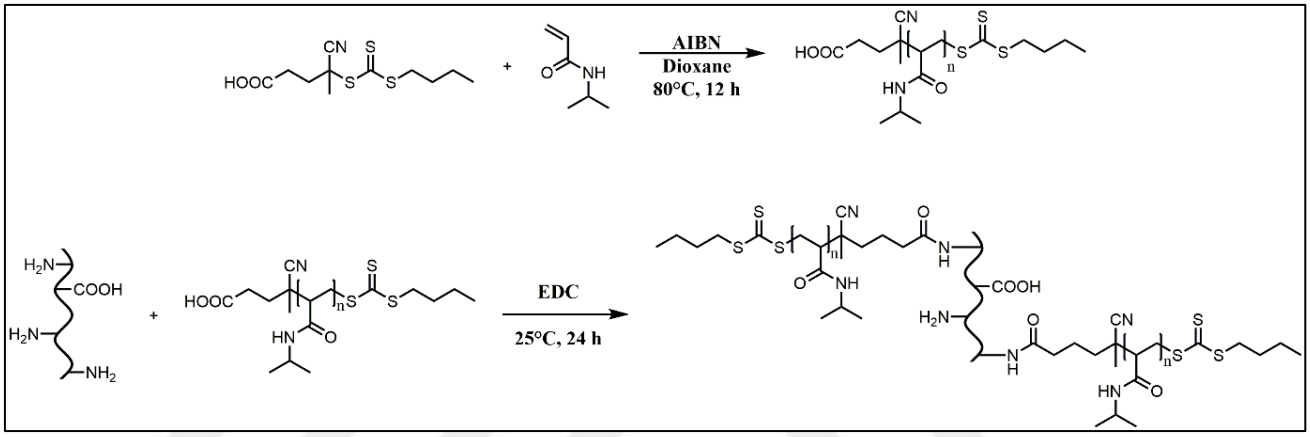

Figure 17 Mechanism of carboxyl-end-group RAFT agent and Gelatin

3.1. Characterization of PNIPAAm Produced by RAFT Technique

The structural analysis of the synthesized PNIPAM in the presence of the carboxyl-end-capped RAFT agent was carried out by using ^{1}H-NMR spectroscopy. According to the ^{1}H-NMR result (Fig. 18), PNIPAAm-RAFT agent could be obtained successfully.

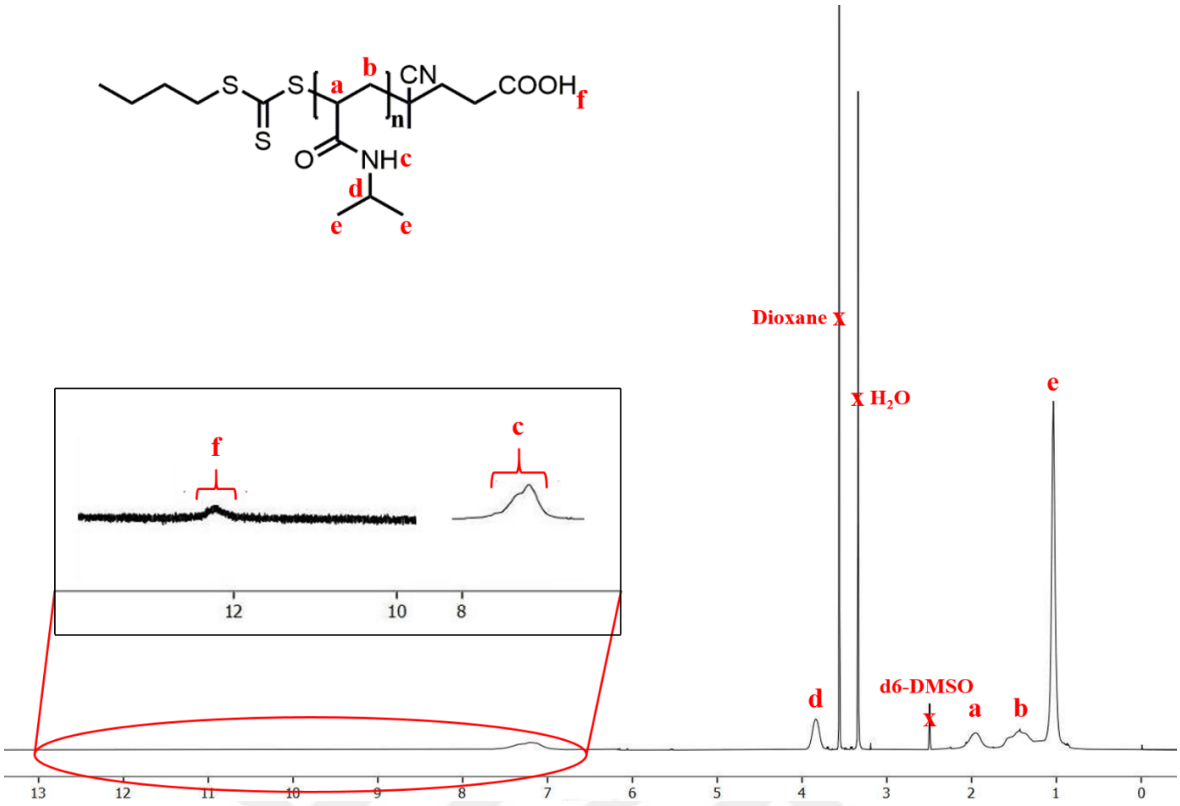

Figure 18 ^{1}H-NMR spectrum of PNIPAAm (in d6-DMSO)

FT-IR analyses were also performed for the structural confirmation of PNIPAAm honopolymers. In the FT-IR spectra of the PNIPAAm homopolymers with a varying molecular weights (seen in Figure 19), characteristic N-H streching and bending vibration peaks were observed at 3274 cm^{-1} and 1535 cm^{-1}, respectively. In addition to these characteristic peaks, stretching vibrations of C=O and C-N in the secondary amide group were appeared at 1630 cm^{-1} and 1455 cm^{-1}, respectively.

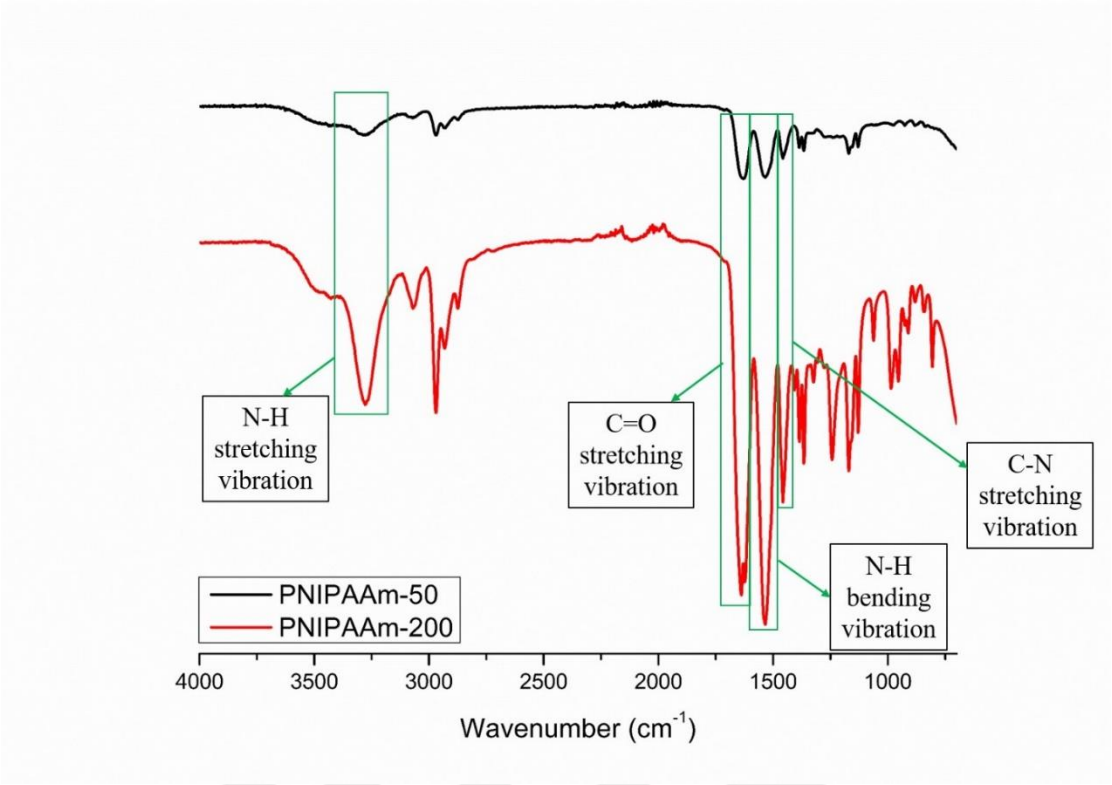

Figure 19 FT-IR spectra of PNIPAAm homopolymers

To confirm the production of PNIPAAm with a varying molecular weights and low polydispersities by using RAFT polymerization method, GPC analyses were performed. Upon observing the GPC chromatograms (Figure 20), a shift to the left is observed as the molecular weight of the homopolymer increases, as expected. Molecular weights obtained from GPC and corresponding PDI values are given in the Table 1.

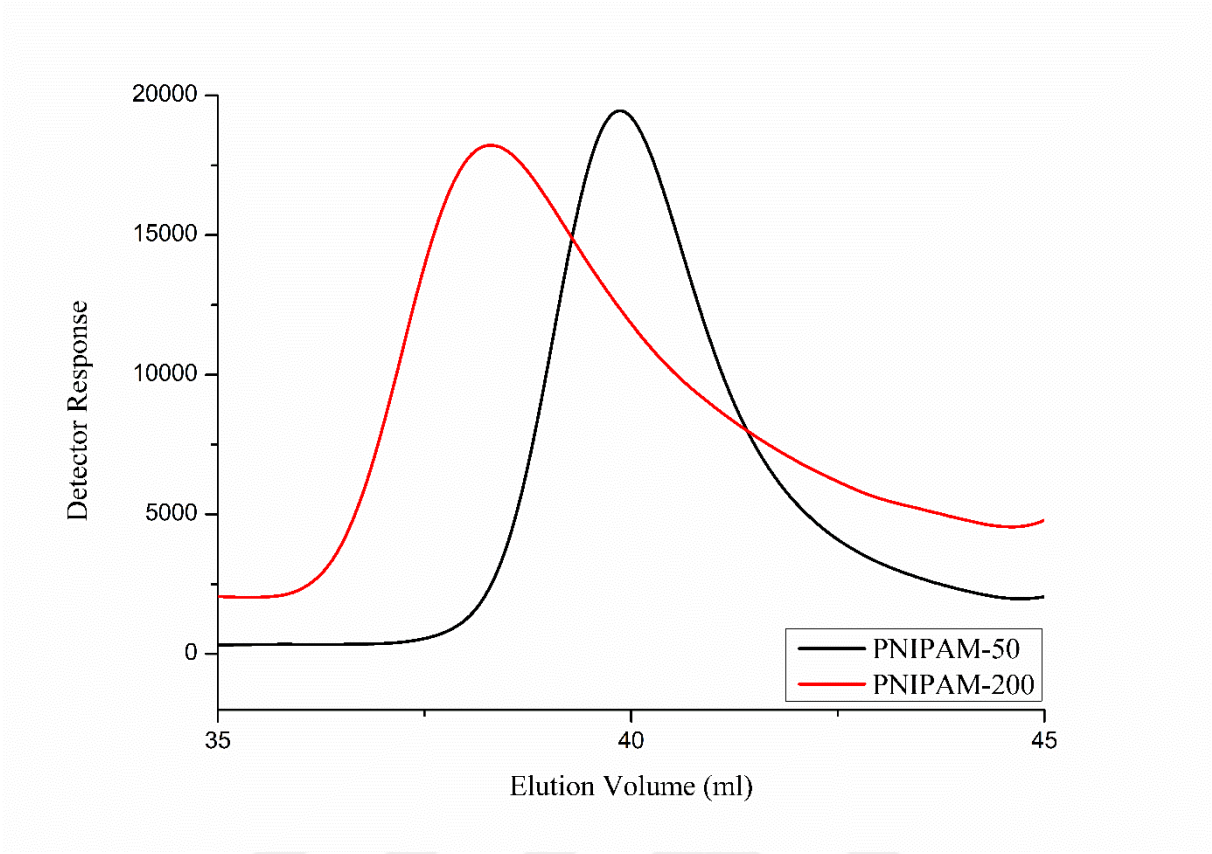

Figure 20 Gel permeation chromatograms of PNIPAAm homopolymers

These results were evidenced that PNIPAM homopolymers having a different molecular weights (3700 Da and 5900 Da) and low polydispersities (1.51 and 1.73) could be synthesized owing to carboxyl-end-capped RAFT agent.

In PNIPAAm synthesis with Free Radical Polymerization, the PDI value is generally 2 and above [32].

Table 1 GPC results of the synthesized homopolymers

	M_w (g/mol)	M_n (g/mol)	PDI
PNIPAAm-50	3700	2450	1,51
PNIPAAm-200	5900	3400	1,73

3.2. Characterization of PNIPAAm-RAFT agent:GEL polymeric network

Production of poymeric network composed of PNIPAM and Gelatin with/out drug (5-FU) was confirmed by FT-IR analyses. In the FT-IR spectra of PNIPAAm-GEL (seen in Figure 21) without drug, N-H stretching and bending of PNIPAAm homopolymer vibration peaks

were observed at 3278 cm^{-1} and 1529 cm^{-1}, respectively. In addition to these characteristic peaks, stretching vibrations of C=O and C-N appeared at 1634 cm^{-1} and 1238 cm^{-1}, respectively. The characteristic N-H peaks in gelatin and NIPAAm overlapped. The gelatin structure revealed several absorption bands at 3278 cm^{-1} (N-H stretching vibration), 1634 cm^{-1} (amide I, C=O stretching vibration), 1529 cm^{-1} (amide II, N-H bending vibration), and 1300 cm^{-1} (amide III, N-H bending vibration), which are typical of those observed for proteins. The amide III peak of gelatin was visible in PNIPAAm-Gel but not in PNIPAAm-50. This is evidence of the existence of gelatin in the polymeric network.

In the analysis after drug (5-FU) loading, N-H stretching vibration peaks were observed at approximately 3253 cm^{-1}. -NH band of 5-FU was overlapped with that of PNIPAAm and GEL. The carbonyl stretching vibration band (C=O) of 5-FU appeared at 1625 cm^{-1} overlapped with that of PNIPAAm and GEL. N-H and C-H bending peaks were observed at 1541 cm^{-1} and 1452 cm^{-1} , respectively. The peak at 1242 cm^{-1} was belong to the carbon-Fluorine (C-F) stretching band which verified the loadng of 5-FU into the polymeric network consisted of PNIPAAm and GEL [5].

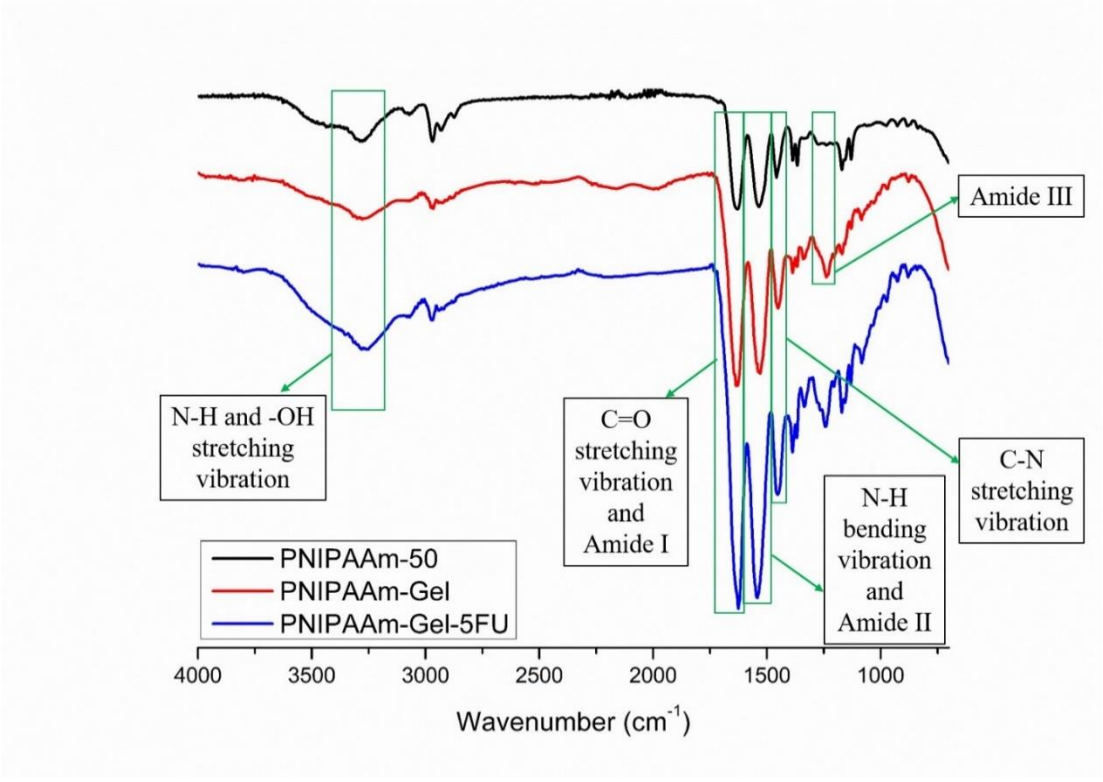

Figure 21 FT-IR spectra of PNIPAAm /PNIPAAm-Gel / PNIPAAm-Gel-5FU

The morphologies of the PNIPAAm and PNIPAAm-GEL-5FU were examined using scanning electron microscopy (SEM). As seen from the images (Figure 22), while PNIPAAm has non-porous morphology, PNIPAAm-GEL showed layered and porous structure.

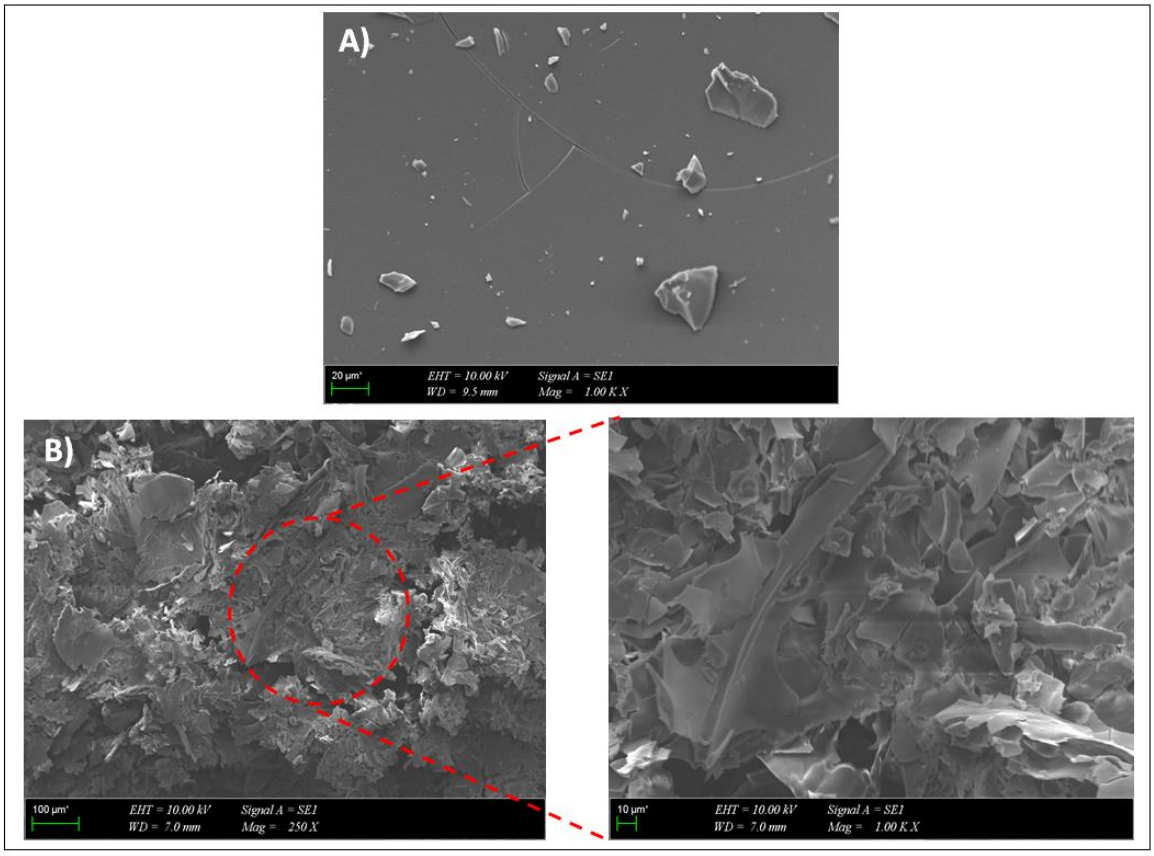

Figure 22 SEM images of a) PNIPAAm and b) PNIPAAm-GEL-5-FU

3.3. Swelling behavior of PNIPAAm-GEL thermosensitive drug carriers

Swelling capacity is key parameter for drug carriers since this behavior is directly related to drug release kinetics. To evaluate the swelling feature of the produced PNIPAAm based thermosensitive drug carrier, swelling test was carried out by gravimetrically. As shown from the obtained results (Figure 23), both PNIPAM-GEL drug carrier produced by PNIPAM with a low and high molecular weights showed temperature-sensitive swelling properties. It should be also noted that PNIPAM-50:GEL swells more (380 % at 37 ℃ and 100 % at 25 ℃), while PNIPAM-200:GEL swells less (180 % at 37 ℃ and 75 % at 25 ℃). This is because the crossling density of is less dense at low molecular weight due to the decreased number of bonds between amine of Gelatin and carboxyl groups of PNIPAAm, while the polymeric network is tighter and denser at high molecular weight.

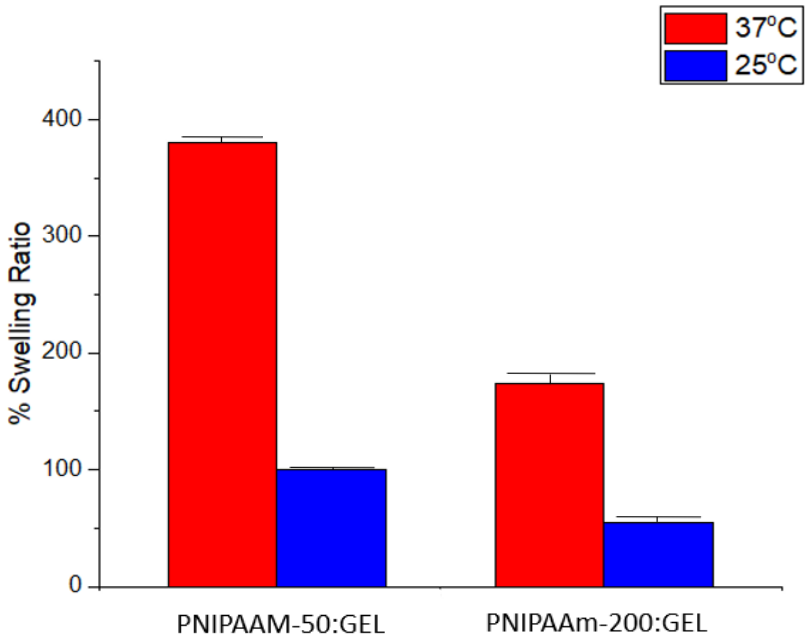

Figure 23 Swelling capacities of the obtained PNIPAAm-GEL hydrogels at 25 ℃ and 37 ℃

3.4. Drug release kinetics of PNIPAAm-GEL thermosensitive drug carriers

Thermoresponsive drug release behavior is a considerable property for cancer treatment. Herein, the release of 5-FU from the fabricated PNIPAAm based thermosensitive drug carriers with a different molecular weights was performed to observe the impact of molecular weight on the release kinetics. As shown from the release results (Figure 24), while more release was achieved in the PNIPAAm with a low molecular weight product, the release amount of drug diminished at high molecular weight of PNIPAAm for same temperature conditions in direct proportion to the swelling ratios. Additionally, the both obtained products showed the difference release kinetics for 25 ℃ and 37 ℃ which confirms thermoresponsive behavior of PNIPAAm-GEL drug carriers as seen in mechanism (Figure 25).

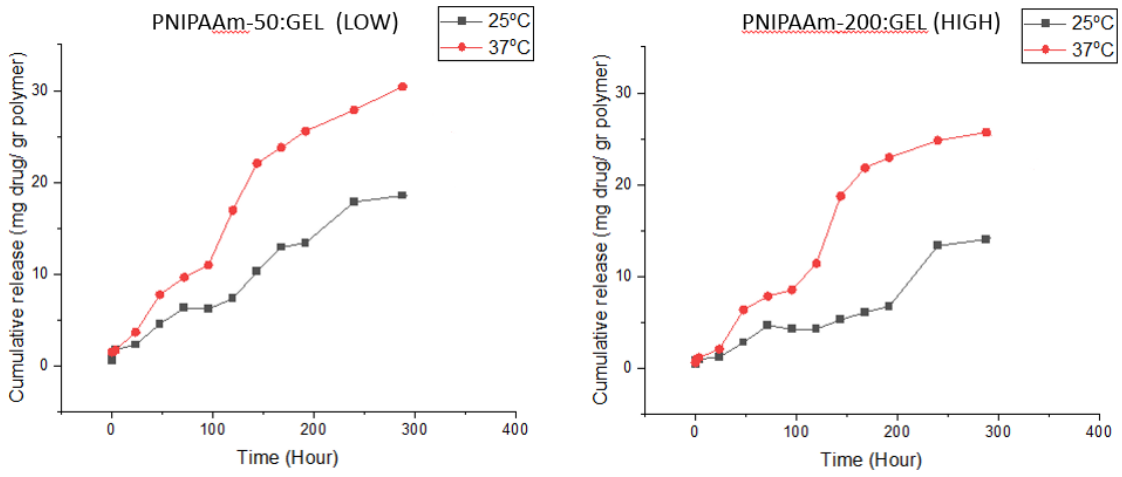

Figure 24 Cumulative release of 5-FU from the obtained PNIPAAm-GEL hydrogel with low and high molecular weight at 25 ℃ and 37 ℃

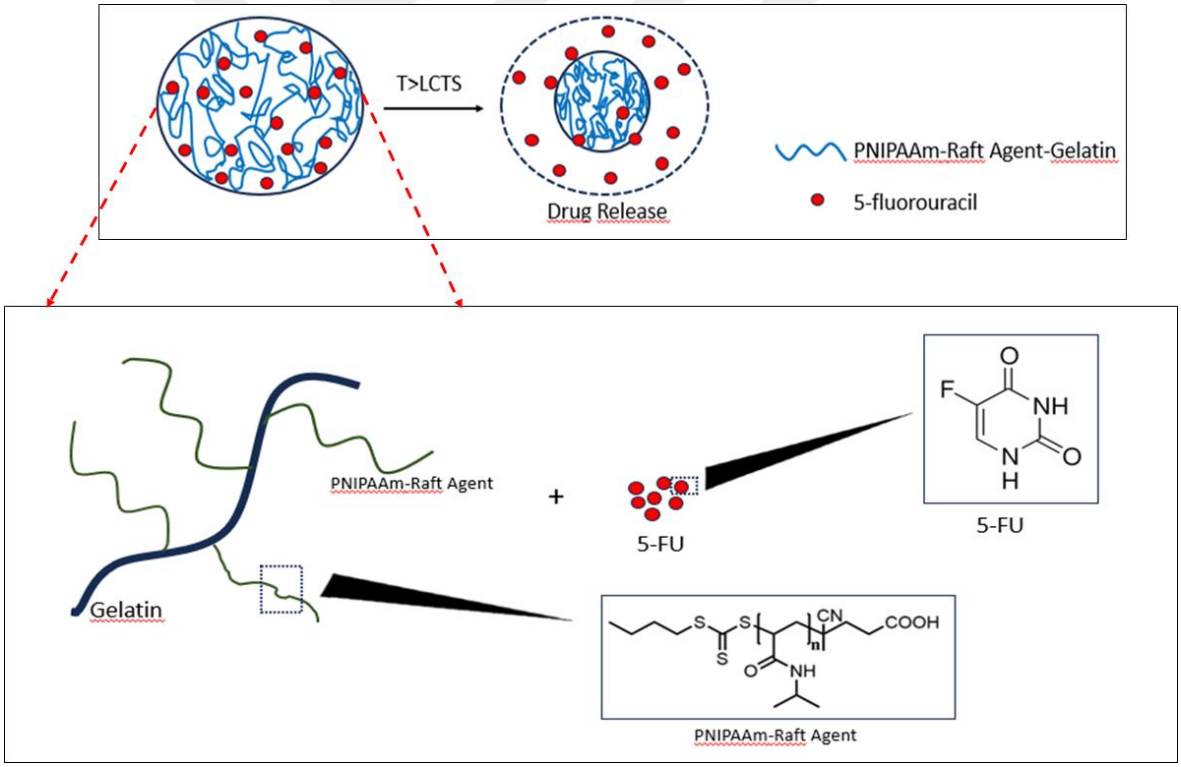

Figure 25 Drug (5-FU) release with increasing temperature from PNIPAAm-GEL polymeric structure

It is worthwhile to mention that PNIPAAm-GEL hydrogel with a more homogenous structure could be obtained resulting in more controlled release of drug since end group (-COOH) control at the end of the most of the polymer chains could be provided owing to RAFT technique.

4. CONCLUSIONS

Thermosensitive hydrogel as a drug vehicles has an advantage due the the porosity of polymeric matrix could be changed by using diffferent temperature conditions. When a drug carrier has a high porous structure and swelling capacity, it could be not suitable a controlled release of drug due to burst release in short time periods. On the other hand much dense porosity of carrier causes insufficient release at required time period due to the slow release of drug from the hydrogel. Herein, PNIPAAm-GEL thermoresponsive drug carrier was produced by RAFT method which is one of the controlled/living polymerization technique to obtain hydrogel with an adjustable properties such as end group, molecular weight, porosity, swelling capacity. The structure and morphologies of PNIPAAm obtained in the persence of RAFT with a carboxyl end group and PNIPAAm-GEL were confirmed H-NMR, GPC, FT-IR, SEM analysis. The results of swelling and release experiments performed at 37 ℃ and 25 ℃ showed that i) PNIPAAm-GEL hydrogels with the different molecular weights have a thermoresponsive character ii) the decrease in molecular weight of PNIPAAm increased the swelling properties and drug's release amount of the hydrogel significantly due to the decreased number of bonds between amine of gelatin and carboxyl groups of PNIPAAm which forms the less dense crosslink polymeric network. It was envisaged from the obtained results that the fabricated thermoresponsive PNIPAAm-GEL hydrogel wtih an adjustable properties could be bright candidate for cancer treatments in the future applications.

REFERENCES

1. Kasai, R.D., et al., *A review on hydrogels classification and recent developments in biomedical applications.* International Journal of Polymeric Materials and Polymeric Biomaterials, 2023. **72**(13): p. 1059-1069.
2. Xian, C., et al., *Progress on intelligent hydrogels based on RAFT polymerization: Design strategy, fabrication and the applications for controlled drug delivery.* Chinese Chemical Letters, 2020. **31**(1): p. 19-27.
3. Zhao, J., et al., *Progress of research in in situ smart hydrogels for local antitumor therapy: A review.* Pharmaceutics, 2022. **14**(10): p. 2028.
4. Nagase, K., et al., *Poly (N-isopropylacrylamide)-based thermoresponsive surfaces provide new types of biomedical applications.* Biomaterials, 2018. **153**: p. 27-48.
5. Gheysoori, P., et al., *Thermoresponsive nanocomposite hydrogels based on Gelatin/poly (N–isopropylacrylamide)(PNIPAM) for controlled drug delivery.* European Polymer Journal, 2023. **186**: p. 111846.
6. Etxabide, A., et al., *3D printed lactose-crosslinked gelatin scaffolds as a drug delivery system for dexamethasone.* European Polymer Journal, 2019. **114**: p. 90-97.
7. Tsai, F.-C., et al., *Thermo-tunable pores and antibiotic gating properties of bovine skin gelatin gels prepared with poly (n-isopropylacrylamide) network.* Polymers, 2020. **12**(9): p. 2156.
8. Tanga, S., M. Aucamp, and P. Ramburrun, *Injectable thermoresponsive hydrogels for cancer therapy: challenges and prospects.* Gels, 2023. **9**(5): p. 418.
9. Karnaki, A., et al., *Thermoresponsive Hydrogels: Current Status and Future Perspectives.* 2024.
10. Rafael, D., et al., *Thermo-responsive hydrogels for cancer local therapy: Challenges and state-of-art.* International journal of pharmaceutics, 2021. **606**: p. 120954.
11. Lamontagne, H.R. and B.H. Lessard, *Nitroxide-mediated polymerization: a versatile tool for the engineering of next generation materials.* ACS Applied Polymer Materials, 2020. **2**(12): p. 5327-5344.
12. Xu, T., et al., *Recent advances in "living"/controlled radical polymerization of phosphorus-containing monomers and their potential applications.* Science China Chemistry, 2015. **58**: p. 1633-1640.
13. Nicolas, J., et al., *Nitroxide-mediated polymerization.* Progress in Polymer Science, 2013. **38**(1): p. 63-235.
14. Vázquez-Dorbatt, V., et al., *Synthesis of glycopolymers by controlled radical polymerization techniques and their applications.* ChemBioChem, 2012. **13**(17): p. 2478-2487.
15. Li, I., et al., *Kinetics of decomposition of 2, 2, 6, 6-tetramethyl-1-(1-phenylethoxy) piperidine and its implications on nitroxyl-mediated styrene polymerization.* Macromolecules, 1995. **28**(19): p. 6692-6693.
16. Alemdar, N. and A.T. ERCİYES, *Kontrollü/yaşayan serbest radikal polimerizasyonu ile stirenlenmiş yağ üretimi.* İTÜDERGİSİ/d, 2011. **9**(4).
17. Purkait, M.K., et al., *Photoresponsive membranes*, in *Interface Science and Technology*. 2018, Elsevier. p. 115-144.
18. Tsang, M.W. and S. Holdcroft, *Alternative proton exchange membranes by chain-growth polymerization*, in *Polymers for a Sustainable Environment and Green Energy*. 2012. p. 651.

19. Feng, B., et al., *Bio-based Carbon dots Loaded with 5-Fu: A Multifunctional drug Delivery System.* Journal of Fluorescence, 2023: p. 1-10.
20. Zhou, M., et al., *Doxorubicin-loaded single wall nanotube thermo-sensitive hydrogel for gastric cancer chemo-photothermal therapy.* Advanced Functional Materials, 2015. **25**(29): p. 4730-4739.
21. Li, Z., et al., *Thermo-sensitive injectable glycol chitosan-based hydrogel for treatment of degenerative disc disease.* Carbohydrate Polymers, 2018. **184**: p. 342-353.
22. Fundueanu, G., et al., *pH/thermo-responsive poly (N-isopropylacrylamide-co-maleic acid) hydrogel with a sensor and an actuator for biomedical applications.* Polymer, 2017. **110**: p. 177-186.
23. Wang, T., et al., *Preparation and properties of a novel thermo-sensitive hydrogel based on chitosan/hydroxypropyl methylcellulose/glycerol.* International Journal of Biological Macromolecules, 2016. **93**: p. 775-782.
24. Shimada, N., S. Kidoaki, and A. Maruyama, *Smart hydrogels exhibiting UCST-type volume changes under physiologically relevant conditions.* RSC advances, 2014. **4**(94): p. 52346-52348.
25. Li, X., et al., *A PNIPAAm-based thermosensitive hydrogel containing SWCNTs for stem cell transplantation in myocardial repair.* Biomaterials, 2014. **35**(22): p. 5679-5688.
26. Liu, M.-M., et al., *based 3D culture device integrated with electrochemical sensor for the on-line cell viability evaluation of amyloid-beta peptide induced damage in PC12 cells.* Biosensors and Bioelectronics, 2019. **144**: p. 111686.
27. Sato Matsuo, E. and T. Tanaka, *Kinetics of discontinuous volume–phase transition of gels.* The Journal of Chemical Physics, 1988. **89**(3): p. 1695-1703.
28. Zhang, X. and R. Zhuo, *Synthesis of temperature-sensitive poly (N-isopropylacrylamide) hydrogel with improved surface property.* Journal of colloid and interface science, 2000. **223**(2): p. 311-313.
29. Katmerlikaya, T.G., et al., *Dual-drug conjugated glyco-nanoassemblies for tumor-triggered targeting and synergistic cancer therapy.* ACS Applied Bio Materials, 2022. **5**(11): p. 5356-5364.
30. Ghasemi, S. and Z.A. Harandi, *Thermo-responsive poly (N-isopropylacrylamide)-block-poly (ionic liquid) of pyridinium sulfonate immobilized Pd nanoparticles in C–C coupling reactions.* RSC advances, 2018. **8**(26): p. 14570-14578.
31. Wu, Y., et al., *Enhanced and sustained topical ocular delivery of cyclosporine A in thermosensitive hyaluronic acid-based in situ forming microgels.* International journal of nanomedicine, 2013: p. 3587-3601.
32. Haraguchi, K., Y. Xu, and G. Li. *Poly (N-isopropylacrylamide) Prepared by Free-Radical Polymerization in Aqueous Solutions and in Nanocomposite Hydrogels*. in *Macromolecular Symposia*. 2011. Wiley Online Library.