



MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES



**PREPARATION OF CROSS-LINKED β -
GLUCAN AS DRUG CARRIER**

AYŞEN EYİĞÖR

MASTER THESIS

Department of Chemical Engineering

ADVISOR

Prof. Mehmet S. Erođlu

ISTANBUL, 2017



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SCIENCES**

Ayşen EYİGÖR, a Master of Science student of Marmara University Institute for Graduate Studies in Pure and Applied Sciences, defended her thesis entitled “**PREPARATION OF CROSS-LINKED B-GLUCAN AS DRUG CARRIER**”, on 23.02.2017 and has been found to be satisfactory by the jury members.

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ÖZET

İlaç Taşıyıcı Çapraz Bağlanmış B-Glucan Hazırlanması

Günümüzde, ilaç etkinliği arttırmak, ilacın verebileceği zararlı etkileri minimize edebilmek adına, ilaç salınım sistemleri artan bir ilgi çalışmalar yapılmaktadır. Kontrollü ilaç salınım sistemleri önceden belirlenen süreler içerisinde ilaç salınımları yapabilmektedirler. Uyarıcı polimerler ve bunların hidrojelleri, genellikle akıllı malzemeler olarak adlandırılır ve kontrollü ilaç dağıtım sistemleri geliştirmeye yönelik çalışmaların konusu olmuştur. Akıllı hidrojeller suda çözünen, sıcaklık, pH, elektrik iletkenliği gibi çevresel değişikliklere duyarlı olan polimerlerin kimyasal ve fiziksel çapraz bağlanmasıyla hazırlanırlar. PNIPA, yaklaşık 32 °C'de düşük kritik çözelti sıcaklığına (LCST) sahip, iyi bilinen sıcaklığa duyarlı bir sentetik polimerdir. PNIPA bazlı hidrojellerin, vücut sıcaklığına yakın bir VPTT'ye sahip olması nedeniyle, kontrollü ilaç salınım sistemlerinin hazırlanmasında önemli bir potansiyele sahiptir. PNIPA hidrojellerinin en büyük dezavantajı, biyolojik olarak bozunabilir olmaması ve nispeten toksik olan bis-akrilamid (BAAM) ile çapraz bağlanması ve kullanımında muhtemel lokal inflamasyona neden olabilmesidir. Bu çalışmada, bu dezavantajı bir dereceye kadar ortadan kaldırmak için, (1,3) - (1,6) β-glukan karboksimetillendi ve sonra β-glukan-pNIPA esaslı sıcaklık duyarlı hidrojelleri hazırlamak için biyobozunur ve biyoyumlu bir çapraz bağlayıcı olarak kullanılmıştır. Bu amaçla, suda daha fazla çözünürlüğü elde etmek için, β-glukanın moleküler ağırlığı azaltıldı ve sonra karboksimetillendi. Metakrilatlı β-glukan, karboksimetillenmiş β-glukanın (CM-β-glukan) metakrilasyonu ile sentezlendi. Reaksiyon ürünleri Fourier-transform infrared spektroskopisi (FTIR), ¹H ve ¹³C nükleer manyetik rezonans spektroskopisi (NMR) ile karakterize edildi. Hidrojeller, dört farklı β-glukan / pNIPA oranlarında hazırlandı ve ilaç olarak 5-ASA ile yüklendi. PBS içerisindeki (pH 7.4) hidrojellerin 5-ASA yükleme kapasitesi ve şişme davranışı, hacim faz sıcaklıklarının (VPTT) altındaki ve üzerinde olmak üzere dört sıcaklıkta (25, 30, 35 ve 40 °C) belirlendi. Bütün kompozisyonlardaki ilaç yükleme kapasitesi 25 °C'de gözlemlendi. Hidrojellerin 32.8 °C'den 35.5 °C'ye yükselen VPTT değeri, türev diferansiyel tarama kalorimetresi (DDSC) ile belirlendi ve hidrojeller içerisindeki artan β-glukan ile vücut sıcaklığına yaklaştı. Termal kararlılık, termogravimetrik analiz

(TGA) ile belirlendi. Termal kararlılık, hidrojellerde β -glukanın miktarı ile birlikte artmıştır. Hidrojellerin 5-ASA salını UV-VIS spektroskopisi ile 37 ° C' de izlenmiştir. Şişme ve bırakma davranışlarının, hidrojel bileşimine ve sıcaklık değişikliklerine bağlı olarak önemli ölçüde değiştiği gözlenmiştir.

Anahtar kelimeler: Kontrollü ilaç salınım, biyopolimer, hidrojel, 5-ASA, β -glukan, N-izopropilakrilamid, sıcaklık duyarlı.

ABSTRACT

Preparation of Cross-Linked β -Glucan as Drug Carrier

Recently, there is an increasing effort for developing controlled drug delivery systems to increase drug efficacy and minimize the harmful effects of drugs. Controlled drug delivery systems (CDDs) are able to deliver therapeutic agents within predetermined time-periods and dose. Stimuli responsive polymers and their hydrogels, generally called as smart materials, have been the subject of the studies for developing controlled drug delivery systems. The smart hydrogels are prepared by chemically or physically crosslinking of water-soluble stimuli responsive polymers, which are sensitive to changes in environmental conditions such as temperature, pH, electrical conductivity etc. PNIPA is a well-known temperature responsive synthetic polymer, which has a lower critical solution temperature (LCST) nearly at 32°C. PNIPA based hydrogels have an important potential in the preparation of CDDs since pNIPA has a VPTT close to body temperature. The major disadvantage of pNIPA hydrogels is that it is not biodegradable and generally crosslinked with bis-acrylamide (BAAM) which is relatively toxic and may cause possible local inflammation on use. In this study, to eliminate this drawback in some extent, (1,3)-(1,6) β -glucan was methacrylated and then used as a biodegradable and bio-compatible crosslinker to prepare β -glucan-pNIPA based temperature responsive hydrogels. For this purpose, the molecular weight of β -glucan was reduced to increase the solubility in water and then carboxymethylated. Methacrylated β -glucan was synthesized by the reaction of carboxymethylated β -glucan (CM- β -glucan) with methacrylic anhydrid. Reaction products were characterized using Fourier-transform infrared spectroscopy (FTIR), ^1H and ^{13}C nuclear magnetic resonance spectroscopy (^1H -NMR and ^{13}C -NMR). The hydrogels were prepared at four different β -glucan/pNIPA ratios and they were loaded with 5-ASA as drug. 5-ASA loading capacity and swelling behavior of the hydrogels in PBS (pH 7.4) were determined at four temperatures (25, 30, 35 and 40 °C), which are below and over the volume phase temperatures (VPTT) of pNIPA. For all the compositions, the maximum drug loading capacity was determined at 25°C. The VPTT of hydrogels was sensitively determined using derivative differential scanning calorimetry (DDSC), which increased

from 32.8°C to 35.5 °C, approaching to body temperature with increasing β -glucan in the hydrogel formulations. The thermal stability of the hydrogels was determined using thermogravimetric analysis (TGA), which increased with the amount of β -glucan. The 5-ASA release from the hydrogels was monitored using UV-VIS spectrophotometer at 37 °C. It was notable that, the swelling and release behaviors of the hydrogels significantly changed depending on the hydrogel compositions and temperature.

Keywords: Controlled drug delivery, biopolymer, hydrogel, 5-ASA, β -glucan, N-isopropyl acryamide, temperature sensitive hydrogel.

SYMBOLS

G Gram

Mg Mili gram

Mg Micro gram

L Liter

mL Mili liter

mol Molar

Hr Hour

min Minute

Mw Weight average molecular weight

Mn Number average molecular weight

ABBREVIATIONS

CM- β- glucan	Carboxymethylated β - glucan
MA- β- glucan	Methacrylated β - glucan
TEMED	N,N,N',N' tetra methylethylenediamine
PNIPA	Poly(N-isopropylacrylamide)
5-ASA	5-Amino salicylic acid
HA	Hyaluronic acid
LCST	Lower critical solution temperature
UCST	Upper critical solution temperature
VPTT	Vapor phase transition temperature
DSC	Differential scanning calorimetry
MR	Modified Release
ER	Extended release
TGA	Thermal gravimetric analysis
FTIR	Fourier transform infrared spectroscopy
NMR	Nuclear magnetic resonance spectroscopy
GPC	Gel permeation chromatography
TMS	Tetra methyl silane
CDD	Controlled Drug Delivey
DDS	Drug Delivery Systems
ESR	Equilibrium Swelling Ratio
MRT	Maximum weight loss rate temperature

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1. INTRODUCTION

Hydrogels are polymeric materials that have three dimensional network structure. They do not dissolve in an aqueous medium, but can swell in varying degree [1]. Due to their tissue compatibility, the simplicity of distribution drugs in matrix, providing high degree of controlled drug delivery in relation to environment, hydrogels have been drawing attention as promising materials for controlled drug delivery (CDD) applications. Hydrogels, derived from variety of natural or synthetic polymers have been used for controlled release of therapeutic drugs [2, 3]. Some kind of hydrogels are generally called as stimuli responsive hydrogels and they can organize drug release profile by varying their gel structure in response to ambient stimulants. These kind of hydrogels are well-known smart or sensor hydrogels and have pressure, pH, temperature and magnetic field sensitivity [2, 4, 5]. Smart hydrogels have been widely used in the preparation of artificial body muscles [6, 7] and separation of biomolecules as well [8]. There have been significant studies on the water-soluble and temperature responsive polymers used in controlled drug delivery. These polymers have temperature dependent solubility. They have upper critical solution temperature (UCST), lower critical solution temperature (LCST) or both UCST and LCST. For a polymer showing LCST behavior, solubility in water decreases as the temperature increases. Hydrogels of temperature sensitive polymers are able to swell below the LCST at which hydrogen bonding between hydrophilic groups of the polymer and water are predominant and this enhances the solubility. On the other hand, above the LCST, hydrophobic groups become predominant, the formed hydrogen bonds are broken and the hydrogel collapses. Linear pNIPA is a temperature sensitive polymer with a LCST of nearly 32°C. The term “volume phase transition temperature” (VPTT) is preferentially used to define the phase behavior of pNIPA hydrogels instead of LCST since the hydrogels are only water-swellaable rather than water-soluble.

The VPTT of pNIPA is close to the body temperature and it is very likely to be used as a carrier in variety of biomedical and drug applications for example, enzyme immobilization [9], artificial organs, CDD etc. [10, 11]. However, there seems to be some limitations to using pNIPA hydrogels in these applications [4]. PNIPA is a synthetic, non-biodegradable

thermoresponsive polymer and mostly cross-linked with bis-acrylamide (BAAm) which is cytotoxic and may cause possible local irritation on use. Some studies have shown that, to eliminate this drawback, cross-linkers prepared from biocompatible and biodegradable polymers such as dextran [9, 12, 13], chitosan [14] can be used instead of BAAm.

Beta Glucan is a glucose polymer which is bonded with glycosidic linkages at $\beta(1-3)$, $\beta(1-4)$, $\beta(1-6)$. β -Glucan is extracted from yeast, mushrooms, microorganisms, oats, barley [15]. $\beta(1-3)$ position is linear fundamental linkage of β -Glucan. $\beta(1-3)/\beta(1-6)$ positions are included in beta glucan which are extracted from yeasts and mushroom. $\beta(1-3)/\beta(1-4)$ positions are included in beta glucan which can be extracted from barley, oats. β -Glucans compose a linear backbone with (1-3) β -Glycosidic linkages, but alter with molecular weight, viscosity solubility, branching and gelation features [16]. There have been studies that cereal derived β -glucan has immunomodulatory features and yeast and medicinal mushroom derived β -Glucans have immune system modulation feature. Also β -Glucans can be used in various applications such as texturing agents, soluble fiber supplements, nutraceutical cosmetics etc. [17, 18].

In this thesis, methacrylated (1, 3)-(1, 6) β -Glucan was synthesized and used as cross-linker for the preparation of β -Glucan-pNIPA copolymeric hydrogels. First, (1,3)-(1,6) β -Glucan was carboxymethylated by the reaction of monochloroacetic acid and then the product was methacrylated with methacrylic anhydride to obtain biodegradable and biocompatible (1,3)-(1,6) β -Glucan based cross-linker. This cross-linker was used to syntheses of β -Glucan-pNIPA based co-polymeric thermo-responsive hydrogels by means of redox polymerization at four different β -Glucan/pNIPA ratios. VPTT of the hydrogels was precisely determined by DDSC which increased from 32.8°C (VPTT of pNIPA hydrogel) to 35.5°C with the amount of β -Glucan in the hydrogels. Swelling and drug release profiles of the hydrogels were determined at four different temperatures (below and above VPTT). DDSC results showed that, the VPTT of the hydrogels could be tuned by the change of the amount of β -Glucan in the hydrogels.

1.1.Aim of the Study

The aim of this thesis was to syntheses of different temperature responsive β -Glucan-pNIPA based co-polymeric hydrogels for controlled release of 5-ASA as a therapeutics of ulcerative colit and Chrohn's disease. For this aim, four different β -Glucan-pNIPA based temperature responsive co-polymeric hydrogels were synthesized using redox polymerization at room temperature. The release of 5-aminosalicilic acid (5-ASA) from the hydrogels at 37°C and their swelling behaviors and drug loading capacities at below and over the VPTT were determined. TEMED and potassium persulfate were used as redox pair. Methacrylated (1, 3)-(1, 6) β -Glucan was synthesized as biocompatible cross-linker and used to synthesis the thermo-responsive co-polymeric hydrogels.

1.2. Drug Delivery System (DDS)

Drug delivery system (DDS) is expressed as a kind of device that makes the introduction of therapeutic agents in the body possible and improves efficiency of treatment and safety by controlling time, place and the rate of release of drugs in the body. The system is an interface between drug and patient. It may be a device or formulation to apply for therapeutic purposes used to deliver drug [19] [20].

Development of drug delivery systems has several advantages. Drug delivery technologies qualify and modify profile of the drug release, absorption and delivery for gain of improving product safety and effectiveness, as well as patient compliance and convenience. These technologies are also very interdisciplinary [21] and the ideal drug delivery should have specific properties such as biocompatible, inert, capable of reaching high drug loading, easy to manage and get rid of, and simple to produce in factory and sterilize [22, 23].

Drug delivery systems mainly divided into 4 types: Controlled DDS, Targeted DDS, Modified release DDS and Conventional DDS.

1.2.1. Controlled Drug Delivery System

Controlled release is a term referring to transportation or delivery of compounds in response to a period of time or stimuli. A convenient design of controlled drug release is to let out a therapeutic agent predetermined, certain and in a reproducible way. These systems modify a number of characteristic behaviors of the drug: oscillation profile and capacity of crossing biological carriers, biological distribution, clearance and metabolism. Fundamentally, it aims to make the medication more effective and have fewer side effects when it keeps the drug content in the circulation at ideal level for a certain period of time [23, 24]. This can be given in a graph representing predicted and expected drug concentration profiles for various drug management methods (Figure 1.1).

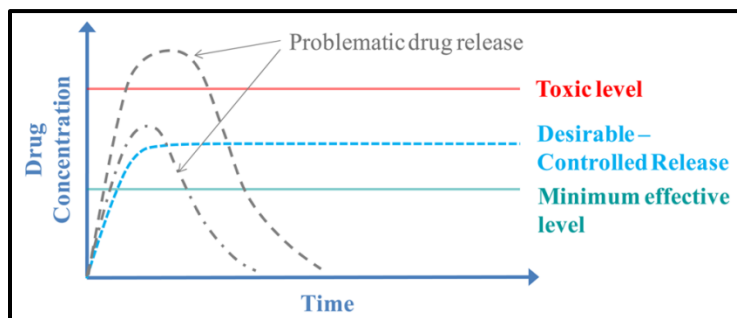


Figure 1.1. Drug concentration profiles in Controlled Release

In blood, drug concentration attains to maximum level very quickly after management of standard dosage and starts to decrease to minimum level, herein repeated management begin to be essential. The first maximum level has exceeded the therapeutic level, which may lead to increased side effects. Conversely, the minimum concentration could be under therapeutically desirable level. So, normal drug dosage conditions can cause, in a drug regimen, in which patient swings between drug inefficiency and overdose. CDD systems regulate the valleys and peak in the concentration [25-27].

CDD systems are divided into 4 groups: Diffusion-controlled DDS, Dissolution controlled DDS, Combination of both Dissolution & Diffusion, Osmatic pump systems.

1.2.1.1 Diffusion-controlled drug delivery systems

Fundamentally, diffusion system is the transportation of drug molecules across a membrane (water insoluble polymer) from an area of higher concentration to a lower one. The flux of the drug is explained by Fick's Law (eq. 1.1) [28].

$$J = -D \frac{dc}{dx} \quad \text{eq.1.1}$$

J is Drug Flux (in amount / area*time), D is diffusion coefficient (area / time) and c is concentration and x is distance $\frac{dc}{dx}$ is altering of concentration with distance. Diffusion CDD systems are can be explained in two groups: Reservoir type and Matrix type [29].

Reservoir type: A water insoluble biocompatible polymer surrounds a zone of drug, which controls release rate. The drug will divide into the membrane altering with fluid (e.g. water) enclosing the particle. Drug will drive in the polymer, diffuse to the surrounding media and alter with the surrounding region as well. The advantages of this system are zero-order delivery possibility and release rate changes depending on the polymer type. However, it is difficult to deliver high molecular weight particles.

Matrix type: In this type of drug delivery system, the drug is spreaded throughout matrix system of the hydrogel. The advantages of the matrix type delivery systems are easier to manufacture than the reservoir type. It can get through high molecular weight particles. But it cannot acquire zero order release [29].

1.2.1.2 Dissolution controlled drug delivery systems

In this type of CDDs, the drug is covered with a polymer layer and drug release is performed by dissolution of polymer which controls release rate. Accordingly, this polymer must be soluble or degradative in water. Dissolution Controlled DDS divided into 2 groups: Matrix Dissolution and Encapsulation Dissolution Controlled Systems [29, 30].

Matrix Dissolution Controlled Systems: It is also called as monolith dissolution controlled system. The drug is dispersed in this region, which controls drug dissolution by controlling dissolution penetration liquid rate into matrix [29, 31].

Encapsulation Dissolution Controlled Systems: The drugs' granules or particles are encapsulated with slowly dissolving particles such as polymethacrylates, waxes, cellulose [29, 30].

1.2.1.3 Combination of both Dissolution & Diffusion

The drug particles are encapsulated with partially soluble polymer that the pores are formed because of dissolution parts of the polymer which allow the inlet of fluid into the core. Therefore, the dissolution part of the material allow for the diffusion of contained drug particles [32, 33].

1.2.1.4 Osmotically controlled drug delivery systems

Osmosis is defined as a net motion of solvent across the semi permeable membrane, which is impermeable to solute but permeable to solvent, operated by difference in osmotic pressure across the membrane. Osmotic drug delivery systems contain drug cells included within a semi-permeable membrane which are water-permeable but drug-impermeable, within an opening for drug delivery. Drug release is operated by the osmotic pressure which is generated in the drug region as it is subjected to water and is generally independent of physiological factors. Release behaviors can be adjusted by features of both the delivery system and drug. The zero-order, which is the most desirable type of drug delivery rate is possible with this type of drug delivery system. Also, if preferred, delivery may be pulsed or delayed [34-36].

1.2.2 Conventional Drug Delivery System

These kinds of systems are used by pharmaceutical materials to cure serious illnesses. There are a variety of ways to administer a drug to a patient to provide a therapeutic agent. The most well-known drug delivery method is the oral management which is a very traditional drug delivery method used by pharmacy industry in the past. Oral administration is one of the practical, quick-growing and productive way relative to the cost for DDS. The oral medicines are presented as capsules and tablets which have high drug stability and assure right dosage can be reached at pharmacies [37].

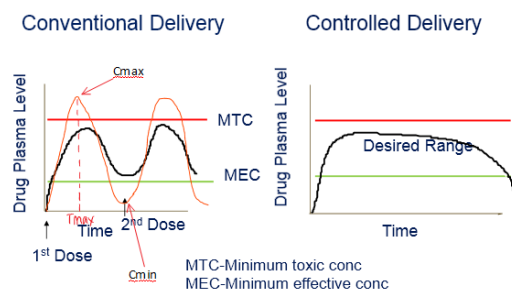


Figure 1.2. Comparison conventional delivery and Controlled delivery

1.2.3. Targeted drug delivery system

Targeted drug delivery is an extraordinary intelligent DDS when the drug is delivered to the patient. This drug delivery method is based on nano medicine which employs drug distribution through nanoparticles to deal with the drawbacks of traditional drug delivery [38]. Aims of targeted drug delivery systems are selectively deliver a certain amount of drugs to targeted to diseased tissue of the body, localize and prolong [39]. Targeted drug delivery system is focused on intensifying drug-related diseased tissues while decreasing drug concentration in rest of the tissues. This increases the drug efficiency and reduces the side-effects. These are the main advantages of the system. It is the high cost that makes productivity more difficult [38, 40].

1.2.3.1 Active targeting

Active targeting or ligand based targeting is a new technique in drug delivery systems and usually used in treatment of the cancer [41]. Active targeting requires binding to the drug delivery system of an antibody, carrier protein, or ligand. And the ligand must meet a drug complex with the cell having the receptor for binding. It can be achieved by modification of the nanoparticles with antibodies, nucleic acid aptamers, peptides, etc. [42].

Active targeting is accomplished by local and systemic management of nanoparticles using a targeting agents. Targeting agents are able to recognize specific ligands which are specific to cancer tissue, attached on the nanoparticle surface. Due to this modification, encapsulated cytotoxic drug in nanoparticles are able to transfer to the cancer tissues. Thus,

any possible harmful effects by cytotoxic drugs can be depressed or eliminated to healthy tissues [42, 43].

1.2.3.2. Passive targeting

Passive targeting is accomplished by consisting a therapeutic molecule in a NP which is able to arrive the desired target organ. Drugs which are encapsulated in NPs or attached to macromolecules are able to deliver drugs passively. To perform it, increased permeability and retention (EPR) effect can be used [44]. The prepared drug carrier complex can evade from being eliminated by the body defense mechanism (excretion, metabolism etc.). During the drug delivery, drug complex continues to circulate in the flow of blood and it is passively taken to receptor due to pH, shape, etc. There are many properties which drug complex must have, such as; surface charge, hydrophilic, hydrophobic or size [45, 46]. However, there are disadvantages of passive targeting. Prepared drug complex may release therapeutic agent to environment of tumor, instead of cancer cells. This may lead that the treatment may not be effective [42].

1.2.4 Modified release drug delivery system

The modified release drug delivery system differs from conventional drug release system, also known as immediate release, to succeed more therapeutic effect and patient adaptability. It has two types which are delayed released and extended release [37].

1.2.4.1. Delayed release

Delayed release DDSs can be defined as systems which are designed to release therapeutic agent at a time after administration [37]. This kind of system can be used to prevent the drug from degradation in acidic environment like stomach or to prevent harmful effect caused by drug. At this stage, drug release can be held up till the drug complex reaches the small intestine. To achieve this aim, the dosage form can be covered by polymers which are modified. These polymers are also known as pH sensitive. Due to pH sensitivity, as the dosage forms pass from lower pH like stomach to the higher pH like small intestine, the polymer dissolves and the drug delivery takes place [43].

1.2.4.2. Extended release

Extended release DDSs release the drug in extended time periods. Dosage frequency can be decreased by prolonging drug release profile. This DDS is appropriate for patient who must use the drug for the rest of his/her life. Releasing period can continue for a day. In immediate release dosage forms, the curative range of the drug is rather short, requiring frequent dosing with compatibility issues. It is a problem for patients with chronic diseases. This problem is eliminated in long-term, continuous and controlled release forms [37, 43] [25].

1.5. Hydrogels

Hydrogels are hydrophilic, polymeric three-dimensional networks, which can absorb water or biological fluids in large amounts. They can simulate living tissue more than synthetic materials; thank to capable of taking high water, porosity and soft intensity. Due to their porous structure, they can swell and squeeze, also means that they can load and release drug [47]. It is common for hydrogels to work as drug carriers due to their unique physical properties. [4, 48] [7]. Due to high porosity, hydrogels can be altered by adjusting on the cross-linked intensity and familiarity to water. Among the benefits offered by hydrogels for drug delivery applications are the possibility of sustained release, which causes a high local concentration of the drug to be maintained for an extended period of time [49]. The hydrogel loads with the active pharmaceutical ingredient and their release mechanism can be proceeded in different pathways such as diffusion controlled, controlled delivery, pH or temperature responsive release [22, 50, 51]. Similarities to living tissue create many opportunities for applications in biomedical fields. At present, hydrogels are used to manufacture hygiene products, contact lenses, tissue engineering scaffolding, drug delivery systems and wound dressings [47].

1.5.1. Classification of Hydrogels

Classification of hydrogels can be listed by different types. Generally, they are classified according to physical features, synthetization method, different types of crosslinking methods, neutral or ionic which are consist of nature side groups [3] [52, 53].

An important classification of hydrogels are based on their sources. There are three types in the classification, which are natural, synthetic and natural-synthetic hybrids. Naturel hydrogels include proteins, DNA, polysaccharides, synthetic hydrogels consist of biodegradable and non-biodegradable polymers [53]. Physical features which consist of crystalline, semi-crystalline, amorphous, hydrogen bonded structures, super-molecular structures and hydro-colloidal aggregates are also considerable classes for hydrogels [3]. Hydrogels are also categorized as homo-polymers or copolymers [54].

1.5.2. Stimuli Responsive Hydrogels

Environment sensitive hydrogels, also well-known as smart materials, permit for a release which is controlled environmentally. To form new drug delivery system, these smart materials have been used in the literatures and pH sensitive and temperature sensitive hydrogels are widely used in DDS which exhibit pulsatile release in accordance with pH or temperature changes [55]. There are some hydrogels which show dual response against two stimuli [56]. Stimuli responsive hydrogels have represented great future in targeted delivery system [57, 58]. While passive and active targeted drug deliveries have addressed a number of significant issues, extra features that can be incorporated into nano-carrier systems, and especially into cellular internalization, are crucial in order to increase the bioavailability of drugs where the disease is present [59, 60].

1.5.2.1. Temperature responsive hydrogels

Temperature-responsive hydrogels are divided into three groups which are negatively, positively temperature responsive and thermally reversible hydrogels; and they can swell and squeeze depending on the enviroment temperature. Negative thermo-sensitive hydrogels are known as having LCST behavior while positive thermo-sensitive hydrogels

are widely known to have the upper critical solution temperature (UCTS) [4, 53, 61]. The most characteristic feature of thermo-sensitive polymers is to have hydrophobic groups such as methyl, ethyl, propyl groups etc. [62, 63].

Studies have been reported that polyacrylamide derivatives which consist of hydrophobic groups exhibiting reverse temperature sensitivity [64]. They are able to dissolve in water at relatively low temperature and they become insoluble when the temperature increases. That means, these behavior is result of the balance between hydrophobic interaction and hydrogen bonding with water, which depends on temperature. The temperature at which, water- soluble polymer becomes insoluble, known as LCST. Hydrogels based on pNIPA, cellulose derivatives and “ethylene oxide-propylene oxide-ethylene oxide” co-polymers exhibit this type of behavior [27, 64, 65].

1.5.2.2. pH responsive hydrogels

Some of the hydrogens contained ionic suspended groups capable of donating or accepting protons in response to pH change in the environment. Ionizable polymers having a pKa value of 3 to 10 are preferred for pH sensitive systems [66] [67].

Weak acids such as carboxylic acids, phosphoric acid and weak bases such as amines show an alteration in the ionization upon pH change. This results a conformational change of the soluble polymers and an alteration in swelling behavior of the hydrogels in the presence of ionized groups in the structure of the polymer. And thus, causes changes in the swelling behavior of the polymers. The well-known monomers which are acrylic acid, methacrylic acid, maleic anhydride and N, N-dimethylaminoethyl methacrylate; also polymers comprising phosphoric acid derivatives have been shared by Gupta, can be given as examples [11, 68].

1.5.3. Hydrogel crosslinking methods

Cross-linking is the stabilization procedure in a polymerization area that causes the polymeric chain in the polymer chain to expand in a multidimensional manner [52]. When crosslinks are added between polymer chains, properties such as solubility, elasticity, glass-

transition temperature of the polymers can alter depending on the crosslinking density [69]. The polymers can be cross-linked by physical and chemical cross-linking methods.

Physical cross-linked methods are ionic interaction, hydrogen bond, crystallization, hydrophobized polysaccharides, stereo complex formation, and protein interaction. Ionic Cross-linked polymer network can be prepared as physiological pH at room temperatures. Ionic cross-linking is generally carried out between molecules which carry different charges. Gombots and et al. prepared alginate gels which may be used as matrix for protein release [70]. In hydrogen bond method, Poly-acrylic acid and poly-methacrylic acid create matrixes with poly-ethylene glycol. These matrixes are kept together by hydrogen bonds between the poly-ethylene glycol oxygen and the carboxylic group of the polymethacrylic acid [52]. In crystallization method, hydrogel can be created by storing of aqueous solutions of hydrophilic polymer. The well-known example is polyvinyl alcohol which is formed by this method [71].

In chemical cross-linking method, chemically cross-linked hydrogels can be preferred due to good mechanical properties. Free radical polymerization and crosslinking by chemical reaction of functional groups are well known chemically cross-linking methods. In radical polymerization method, low-MW monomers can be performed in presence of cross-linkers [72]. A well-known example is Poly 2-hydroxyethyl methacrylate. It is usually studied with hydrogel and polymerization is performed with a cross-linker which can be ethylene glycol dimethacrylate [73]. To prepare hydrogel in chemical reaction of functional groups method, polymers which have hydrophilic functional groups such as; NH_2 , COOH , OH can be employed, and reactions for example; amine-carboxylic acid or schiff base formation can be used to recognize covalent bonds in the polymer [74].

1.5.4. Interpenetrating network hydrogels

An interpenetrating polymer network is a new class of bioactive material which is a developing vehicle; due to their improved biocompatibility, safe profiles and good swelling properties. They can be used in pharmaceutical industry for increasing the solubility of hydrophobic drugs, stabilizing formulations containing active drugs, administering drugs

targeting a particular tissue, increasing bioavailability and imparting biodegradability in drug delivery [49, 75]. Networks can be prepared as copolymers, homo-polymers, interpenetrating networks and semi interpenetrating networks [54].

Homopolymers may be cross-linked depending on the nature of the monomer and on the polymerization technique. Polyethylene oxide (PEO) based hydrogels which are also known as intelligent hydrogels due to their sensitive to external stimuli are widely used [76].

Copolymer networks consist of two different monomers and at least one of these monomers is hydrophilic. Sericin-alginate interpenetrating network hydrogel for cells [75] and CM-cellulose in the synthesis of PVP-based hydrogel can be examples for copolymeric interpenetrating networks [77].

If a linear polymer passes through another cross-linked matrix, this network is called a semi-penetrating network. In one study, to produce and stabilize silver nanoparticles, PVP chains were physically distributed to the PAA hydrogel network, resulting in a semi-penetrating network [78].

1.6. Biodegradable Polymers

1.6.1. Beta Glucan (β -Glucan)

B-Glucans are known as predominant, non-starch, polysaccharide effective components in specific bacterial and fungal cell walls. Approximately half mass of these fungal cell walls includes β -Glucans. They have been used for thousands of years, especially in Japan and China to improve health. In recent years, all the types of β -Glucans determined as powerful stimulators of mammalian system and nowadays it is consumed clinically [79, 80].

Beta Glucan is a glucose polymer which is bonded with glycosidic linkages at $\beta(1-3)$, $\beta(1-4)$, $\beta(1-6)$. β -Glucan is extracted from yeast, mushrooms, microorganisms, oats, barley[15]. $\beta(1-3)$ position is linear fundamental linkage of β -Glucan. $\beta(1-3)/\beta(1-6)$ positions are included in β -Glucan which is extracted from yeasts and mushroom. $\beta(1-3)/\beta(1-4)$ positions

are included in β -Glucan which is extracted from barley, oats [15, 81, 82]. Figure 1.3 shows the structure of the β -Glucans types.

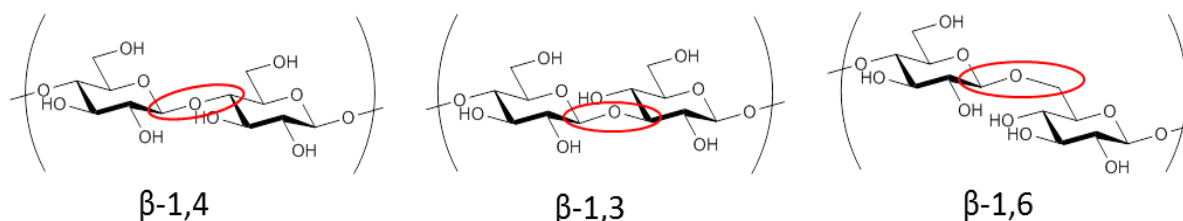


Figure 1.3. β -Glucan 1-3, 1-4 and 1-6 Chemical Structures

β -Glucans' structures are conditional on both methods of isolation and source material, changing mostly distribution and length of side chains. These distribution and length of side chains supply tertiary structures which are stabilized by inter-chain hydrogen bonds. Main characterization parameters are solubility, main structure, MW and degree of branching; and these parameters play important role in glucan-associated biological activity [83-85].

β -Glucans extracted from the yeast have been divided into sub-groups such as soluble and non-soluble types. The soluble β -Glucan is given as source and the non-soluble beta glucan can be given orally. (1, 3)-(1, 6) β -Glucan extracted from yeast is similar in molecular structure to (1, 3)-(1, 4) β -Glucan, but has higher biological activity due to its different branching structure. Because of the structural diversity, both type of β -Glucans have different practices area, effect mechanism and biological activity [79].

(1, 3) linked β -Glucans are insoluble in water. In a study, Du L and et. all tried to turn insoluble (1, 3) linked β -Glucans into water soluble by applying phosphorylation method. Solubility, chemistry structures, yield of samples are compared and as a result that solubility and yield were respectively effected by DMSO and urea [86].

There is another study about converting insoluble β -Glucans into soluble ones, but this β -Glucans has (1, 3)-(1, 6) linkages. Han M. D. and et. all developed soluble β -Glucans with incorporation of sulphated groups to improve immunomodulatory activity of glucans [87].

β -Glucans are classic biological-response modifiers which are also called immunomodulators. The studies showed that β -Glucans only behave as immunostimulants and the most effective of all. The well-known effects of glucans include the strengthening of phagocytosis of occupational phagocytes, monocytes, granulocytes, dendritic cells and macrophages. In this respect, macrophages that are thought to be the primary effector cells in host defense against viruses, parasites, bacteria and tumor cells play the most significant role. Up to this time, it has been resulted in several papers that well-immunostimulating effects have been observed in fish, shrimp, mice, rats, pigs and humans [88] [89-92].

β -Glucans have anti-tumor effects and they are used as clinically in cancer treatment in Japan. Yeast β -Glucans treated with oral monoclonal antibody therapy raised neuroblastoma tumor regression and long-term survival in mouses [83, 93].

1.6.2. Chitosan

Chitosan (CH) is a linear polysaccharide consist of passing distributed β -(1-4)-linked D-glucosamine (deacetylated unit) and N-Acetyl-D-glucosamine (acetylated unit) obtained by heterogeneous deacetylation of chitin. A widespread method for synthesis of chitosan is treatment with NaOH in excess as water and reagent. Chitosan can be provided by reacetylation in homogeneous circumstances of exactly deacetylated chitosan [94-97] [98]. Chitosan deacetylation degree can be obtained by Nuclear magnetic resonance spectroscopy (NMR) and commercial chitosans are between 60% and 100% deacetylation grade range. Generally, average molecular weight of chitosan ranges from 3800 and 20,000 Daltons [98]. Structure of the Chitosan is shown in Figure 1.4

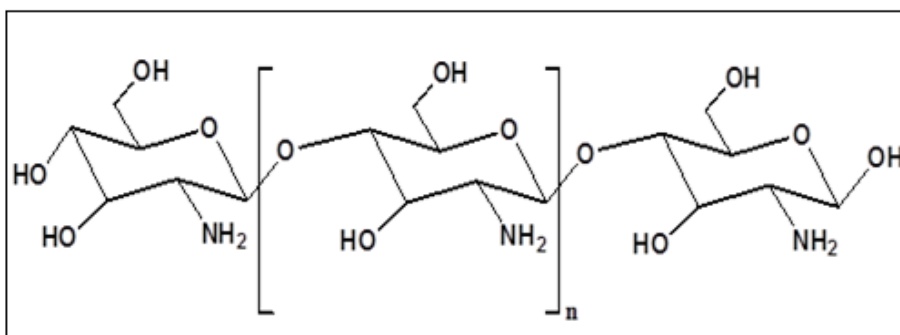


Figure 1.4. Chitosan chemical structure

Solubility parameter of chitosan changes in different conditions. Solubilization perform by protonation of the $-NH_2$ group on C-2 position of the D-glucosamine unit and so polysaccharide is turned into polyelectrolyte in acidic ambient as well as in common organic solvents. That is why, this feature convert chitosan to pH-sensitive biopolymer [99]. The protonation of functional group which are free amino groups provide chitosan attached to biomolecules like proteins antibodies DNAs, enzymes and drugs. Therefore, the protonation of functional group makes it proper for transportation applications of some therapeutic agents [20, 27, 100]. Additionally, because of its biocompatibility, biodegradability, non-cytotoxic features, anti-cancer, antimicrobial and antifungal acts; chitosan, chitosan-copolymers and chitosan-blends have been getting attention and applications for differently biomedical applications such as; drug and gene delivery, wound healing and food packaging [101-105].

Today, chitosan is functionalized as DDS by many researchers and many self-assembled nano-particles are produced by the Hydrophobe modification of these groups for encapsulating hydrophobe medicine to access cancer region [106, 107].

1.6.3. Levan

Microbial levan is an extracellular poly-fructan, forming of glycosidic β -(2, 6) major and β -(1, 2) minor bonds and has a high molecular weight [76]. Compatibility with salts and surfactants, water and oil solubility, suspension and rheological features, heat, acid and film

formation, retention capacity for water and chemicals, alkali stability and biological properties make levan a multi-featured polymer [108-110].

Microbial levan is manufactured by *Bacillus circulans*, *Zymomonas mobilis*, *Halomonas* sp. AAD6 (JCM 15723). Molecular weights and branching ratios are different depending on the organism in which they are produced. Bacterial levan's molecular weight is generally between 2 to 100 million Da. MW of levan, produced by plant, can vary from 2000 to 33,000 Da [111]. The studies show that levan is a promising polyfructan in medicine, pharmaceutical industries, textile, food, cosmetic, waste water treatment [76].

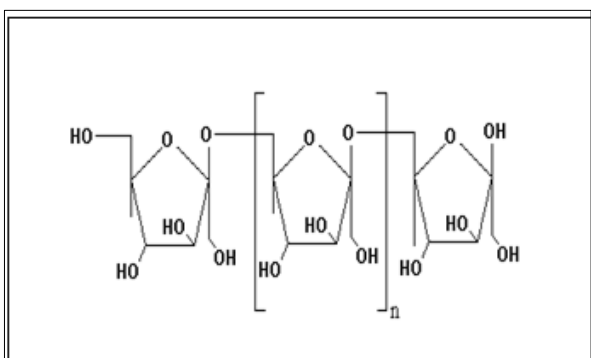


Figure 1.5. Levan chemical structure

1.6.4. Hyaluronic acid

Hyaluronic acid (HA) is a well-known carbohydrate and consists in living organisms and linear polyanion which has repeating structure $[(1\rightarrow3)\text{-}\beta\text{-dGlcNAc}\text{-}(1\rightarrow4)\text{-}\beta\text{-d-GlcA}]_n$. HA is commonly existed in the extracellular matrix, at the same time, found to consist intracellularly.

Due to HA's biodegradability, biocompatibility and readily modified chemical structure, it has been well investigated in DDS applications. Several HA derivatives and cross-linked HA substances in the market have been prepared for drug delivery and are formed in forms like films, hydrogels, liposomes, fibers and microspheres. It also plays role in embryological development, tumor development and inflammation.

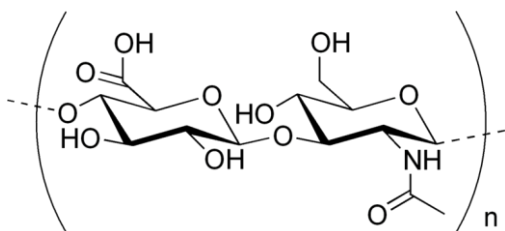


Figure 1.6. Hyaluronic acid chemical structure

1.7. Poly (N-isopropylacrylamide) PNIPA

Poly N-isopropyl acrylamide is a thermo-responsive material and commonly studied for the therapeutic targets. Its LCST is 32°C and below this temperature, it is hydrophilic and soluble in water however; above LCST, the polymer becomes hydrophobic and transforms a viscous gel. If NIPA is hydrogels, it absorbs water, swells under LCST. There is a reason of rapid alteration of hydrophilic and hydrophobic. Under LCST, the hydrophilic groups the pNIPAAm bond with molecules of water due to hydrogen bonds, behave in cooperation and create a hydration layer around the hydrophobic ones that's why, pNIPAAm hydrogel has great water uptake.

By copolymerization with different polymers, LCST of pNIPAAm can be changed to lower or higher temperatures to close human body temperatures that make it useful for therapeutic purposes such as drug delivery, immobilization of enzyme or tissue culture as a cell detachment/attachment factor.

1.8. 5-Aminosalicylic Acid (5-ASA)

Mesalazine, or 5-aminosalicylic acid (5-ASA), is an amino salicylate anti-inflammatory drug used to treat inflammatory bowel disease containing ulcerative colitis or inflammatory anus or rectum and to provide remission in Crohn's disease [112].

There are some limitations on 5-ASA. 5-ASA has uncontrollable nature in gastrointestinal stimulation and high gastrointestinal tract. Also, due to well and fast absorb in small intestine, 5-ASA are not able to reach to the colon efficiently [113]. pH sensitive hydrogels can be used to eliminate these disadvantages [114].

2. MATERIALS AND METHODS

2.1. Materials and Equipments

2.1.1. Chemicals

(1, 3)-(1, 6) linked β -Glucan was kindly supplied by Vefa İlaç, Istanbul, Turkey. Monochloro acetic acid (CAS# 79-11-8) was kindly supplied by Ak-kim, İzmir, Turkey. Sodium Hydroxide (NaOH) (CAS# 1310-73-2), potassium peroxide Sulphate ($K_2S_2O_8$) (CAS 7727-21-1) were supplied from JT Baker. Phosphate Buffered Saline tablets, Acetic acid (CAS# 64-19-7), Methacrylic anhydride (CAS# 760-93-0), Hydrochloric acid (HCl) (CAS#7647-01-0), N-isopropylacrylamide (NIPA, %97 purity) (CAS# 2210-25-5) were provided from Sigma Aldrich. Dialysis tubing benzoyated (CAS# D2272-10FT) with a MW cut off of 2000 Dalton dialysis membrane from Sigma Aldrich was used for purification of samples. Sodium carbonate (Na_2CO_3) (CAS# 497-19-8) was provided from Riedel-deHaen. N, N, N', N' tetramethylethylenediamine (TEMED, %99 purity) (CAS#110-18-9) was provided from Analyticals Carlo-Erba. 5-Amino salicylic acid (5-ASA, %95 purity) (CAS# 89-57-6) was provided from Alfa Aesar. All used solvents were HPLC grade and obtained from Fisher Scientific or Sigma-Aldrich. All chemicals were used as received.

2.1.2. Laboratory Equipments

2.1.2.1. Nuclear magnetic Resonance Spectroscopy (NMR)

NMR characterization of hydrolyzed β -Glucan and its derivatives was performed using a Varian 600 MHz NMR instrument at 25°C. Conversion of carboxymethylation and methacrylation of β -Glucan was determined by taking 1H NMR of the sample in D_2O at 25°C. Proton chemical shifts were registered in ppm downfield from TMS.

2.1.2.2. Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR spectra of hydrolyzed, carboxymethylated, methacrylated β -Glucans, pNIPA and hydrogels were recorded using Thermo Nicolet 6700 FT-IR spectrophotometer equipped with a Smart Orbit high performance diamond attenuated total reflectance (ATR) accessories. Measurements were performed between 400-4000 cm^{-1} in transmission mode.

2.1.2.3. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) analysis of the samples was performed using Perkin Elmer Jade DSC at a heating rate of 10 $^{\circ}\text{C}/\text{min}$ under dynamic argon atmosphere with a flow rate of 20 ml/min. The data were processed using Pyris software. The samples, weighed nearly 5 mg were placed into a crimped aluminum pans and measurements were performed. DSC calibration was performed with metallic indium and metallic zinc according to their melting enthalpy and melting point. The phase transition temperatures of β -Glucan-NIPA hydrogels were determined in swollen state in phosphate buffer saline (PBS) by derivative DSC (DDSC).

2.1.2.4. Thermal Gravimetric Analysis (TGA)

Thermal gravimetric analyses (TGA) of β -Glucan-NIPA hydrogels were performed by Seiko EXSTAR TG/DTA 7300 model thermo-gravimetry analyzer. The analyses of samples were conducted at 10 $^{\circ}\text{C}/\text{min}$ heating ratio under dynamic nitrogen atmosphere (20 ml/min flow rate). Nearly, 6 mg of samples were used in each experiment. Before each measurement, the samples were pre-heated to 110 $^{\circ}\text{C}$ to remove absorbed water under experiment conditions and cooled down to 25 $^{\circ}\text{C}$. TGA weight calibration was performed against to weight of standard samples and temperature calibration was performed according to indium melting point.

2.1.2.5. UV spectrophotometer

5-ASA release profile of the hydrogels was determined using Perkin Elmer Lambda 35 UV-VIS Spectrophotometer. UV-VIS spectra of the released drug samples were recorded

between 200–400 nm at 25°C. The measurements were obtained at regular time intervals with 1 ml of samples. The samples were returned to the solution after every measurement to eliminate possible errors that may result from the volume change. The release of 5-ASA from the hydrogels were quantitatively determined using calibration curve.

2.1.2.4. Gel Permeation Chromatography-Light Scattering (GPC-LS)

The molecular weight and molecular weight distribution of β -Glucan samples after acid hydrolysis was determined by gel permeation chromatography (GPC) system. The system consisted of Perkin Elmer Series 200 GPC injector, high pressure pump, serial attached to 4 Water columns (Ultrahydrogel ‘UH) 250, UH 250, UH 1000, UH 2000 and UH Guard column’) and Wyatt Optilab differential refractive index (RI) detector (654 nm) and Dawn Heleos light scattering (LS) dedector. The mobile phase was 0.1 M NaNO₃ solution in 2% acetic acid water mixture having a flow rate of 1.0 mL/min. GPC measurements were conducted at 25 °C. Sample concentrations were in the range of 0.1–2.0 mg/ml and all the samples were filtered through 0.2 μ m Whatman filter prior to use.

2.2. Synthesis of β -Glucan hydrogel

2.2.1. Acid Hydrolysis of β -Glucan

β -glucan (5 g), dispersion in 2 M HCl (150 ml) was stirred for 24 hours at 80 °C. After 24 hours additional stirring at room temperature, a clear solution was obtained and neutralized with NaOH (2M), dialysed against deionized water for 2 days at room temperature and then lyophilized at -70 °C under 0.01 atmosphere. Spectroscopic characterization of native and hydrolysed β -glucans was performed to determine if any structural change after acid hydrolysis occurred.

2.2.2. Synthesis of Carboxymethylated β -Glucan (CM- β -Glucan)

Carboxymethylation of β -glucan was performed according to the method reported in the literature (Sasaki T. and et al.). Briefly, hydrolyzed β -glucan (2 g), suspended in a mixture of methanol/water (43 ml, 80 % of methanol, v/v), stirred at room temperature for 45

minutes, 6 ml of NaOH solution (30% NaOH in water) was gradually added under vigorously stirring over a period of 1 hour and monochloroacetic acid (2.4 g), was added to at 65 °C. After stirring for 5 hours, the reaction was completed, cooled to room temperature and neutralized with glacial acetic acid. The product was precipitated in methanol, filtered, washed with methanol/water (80 % of methanol, v/v) and acetic acid mixture (7:3,v/v). The product also was washed with %80 methanol/water (v/v) and acetone respectively . The product was characterized using FTIR and ¹H MR. After carboxymethylation, betaglucon achieved high solubility in water and thus its GPC analysis was performed as well [115]. Reaction mechanism is shown in Figure 2.1.

2.2.3. Synthesis of Methacrylated β -Glucan(MA- β -Glucan)

Methacrylated β -Glucan (MA- β -Glucan) was synthesized according to the method described by Burdick and Chung et al. 2005 [115]. Briefly, carboxymethylated β -Glucan (CM- β -Glucan) was dissolved in de-ionized water (5ml) and pH was adjusted to 8.0 with NaOH solution (5 M in water). After the reaction mixture immersion into a water/ice bath, 1ml of methacrylic anhydride was slowly added and the reaction was allowed to stirring for 24 hours in dark. The product was neutralized with glacial acetic acid and purified with a dialysis membrane (MW cutoff 2 kDa) against deionized water for 30 h in dark. The purified product was freeze dried and characterized by FTIR and ¹H-NMR spectroscopy. The conversion was 6 % on the basis of the number of glucose rings. The reaction mechanism is shown in Figure 2.1.

2.2.4. Synthesis of β -Glucan/pNIPA hydrogels

β -Glucan/pNIPA hydrogels were synthesized at four various concentrations (90, 80, 70, 60 NIPA wt%) using redox polymerization according to the method used in the literature [27]. Briefly, for GEL-1, a solution of MA- β -Glucan in deionized water (1 ml) was placed into a 10 ml reaction straw to which n-isopropyl acrylamide (NIPA) (0.9 g), potassium persulfate (KPS, 0.4 ml) solution (0.05 M in water) were added. To purge the dissolved oxygen, the viscous sample was purged with argon(Ar) for 15 min. Then 0.15 ml TEMED solution (0.05 M in water) was added to this solution. After purged with Ar for 2 minutes the

solution was put into a well-sealed plastic straws having a length of 5 cm and 5 mm diameter. It was left at 25 °C until the polymerization was finished. After the unreacted monomers were removed by two consecutive cycle of swelling at 25 °C and collapsing at 37 °C the hydrogels were dried at 40 °C under vacuum and weighed (Bostan et al. 2013). The compositions of the hydrogels and their gel contents are given in Table 2.1.

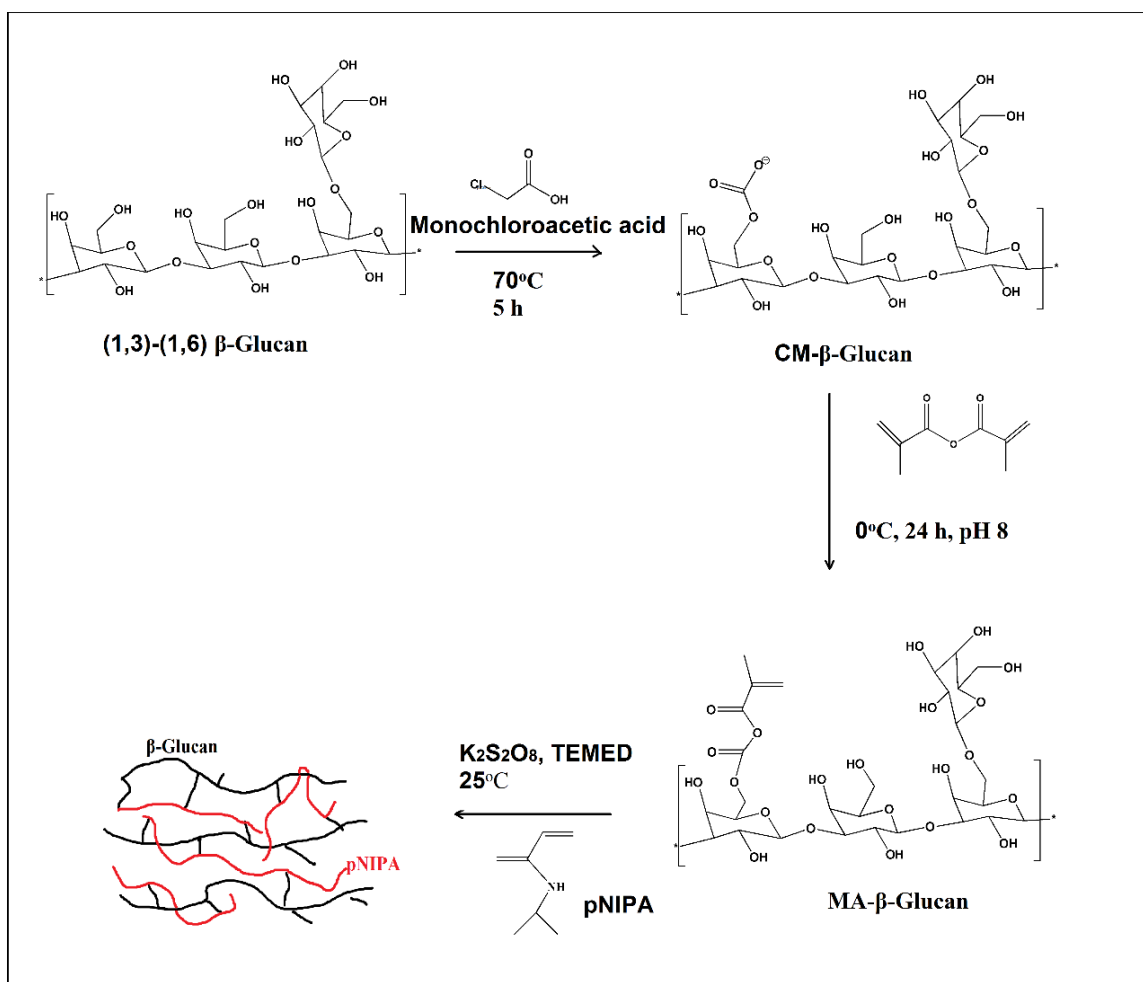


Figure 2.1. Synthesis of β -Glucan-PNIPAA hydrogels

Table 2.1. β -Glucan -PNIPA hydrogel compositions

Hydrogel	NIPA (mg)	MA- β (mg)	NIPA(wt%)	MA- β (wt%)
Gel-1	900	100	90	10
Gel-2	800	200	80	20
Gel-3	700	300	70	30
Gel-4	600	400	60	40

2.2.5. Swelling behaviors of the hydrogels

Well-dried hydrogel sample was weighted and immersed into PBS (pH 7.4) at four different temperatures (25 °C, 30 °C, 35 °C and 40 °C). At specific time intervals, swollen gel was ejected, fast blotted with a paper, weighed and returned. This process was repeated for all the samples until a swelling equilibrium was reached. The swelling ratio of the hydrogels was calculated according to the Equation(2.1).[116]

$$Q = W_2/W_1 \dots \dots \dots \text{(eq.2.1)}$$

Where, Q is the swelling ratio, W_1 and W_2 are the weight of the dry and swollen samples, respectively.

2.2.6. Drug loading and releasing studies

To determine the drug loading capacity of the hydrogels, about 0.2 g of hydrogel was weighed and placed into the 5-ASA (21 mg) solution in PBS (pH 7.4, 30 ml). After the hydrogels were kept in the solution until swelling equilibrium was reached. They were removed, quickly blotted and weighed. The 5-ASA content of the hydrogel samples was calculated according to the Equation (2.2)[27].

$$5 - ASA (wt \%) = \frac{((W_2 - W_1) \times (0.7 / 1000))}{W_1} \times 100 \dots \dots \dots (eq. 2.2)$$

Where W_1 and W_2 are the weight of the dry and swollen hydrogels, 0.7 is concentration as mg/ml of 5-ASA solution and 1000 is density of the solution (mg/ml).

Since 5-ASA is a UV active molecule, and has a specific absorption peak at 330 nm, the release profiles from the hydrogels in PBS was determined by a UV-Vis spectrophotometer. Measurements were performed with a 1.0 ml product from the solution which was returned to the solution to eliminate possible errors that may result from a volume change.

2.2.7. Biocompatibility assay of the hydrogels

WS1 (human fibroblast cells) were studied. The toxicity results were examined after 24 hours. Toxicity assays were performed using the MTT (3- (4,5-dimethylthiazolyl-2) -2,5-diphenyltetrazolium bromide) test. In order to reach the desired quantity, cells were supplemented in 96-U with %10 CO2 humid atmosphere at 37 °C. The gel discs were placed in well tissue culture plates on 24-tablet plates, exposed to UV light for 5 minutes and kept in 1% penicillin / streptomycin solution for 1 night. The next day the antibiotic solution was withdrawn from the well tissue culture plates and the cells were added onto the discs: nearly 40,000 cells per well tissue culture plates. After 24 hours, the viability of the cells grown on gel discs was examined by the MTT method. For this purpose broth medium was taken carefully from the well tissue culture plates, each well was washed twice with a PBS buffer. 2.5 ml of 10% solution of the MTT in the PBS was added to each of the well tissue culture plates, waiting for 2 hours to pass. The solution was collected and DMSO was added to each well tissue culture plates in an amount of 1 ml to dissolve the MTT entering the cell. The absorbance was established at 570 nm.

3. RESULTS AND DISCUSSION

3.1. Hydrolyzed β -Glucan Characterization

3.1.1. FT-IR spectroscopy

Fourier Transform-Infrared Spectroscopy (FT-IR) is a reliable technique for quantitative and qualitative analysis of organic materials. FT-IR spectroscopy involves the interaction between light scanned within IR region and chemical bonds of molecules. This technique determines the absorption frequencies of IR light coincidence with the frequency of specific motion of chemical bonds such as stretching, vibration, rotation etc. Thus, the change in intensity and frequency of IR light is used to explain how chemical structure or functional groups changes with the progress of a chemical reaction. Moreover, this technique allows us to determine the structural analysis of a material by considering the specific absorption peaks of functional groups of molecules within IR region.

In FT-IR spectrum of native β -glucan (Figure 3.1a), the broad peak in the range of 3500-3300 cm^{-1} is due to the stretching absorption of different O-H groups of native β -Glucan. The characteristic bands, observed between 1150-900 cm^{-1} , is due to the C-O-C stretching absorption of glucose rings and glycoside linkage. The peaks between 1350 cm^{-1} and 1450 cm^{-1} can be assigned to C-H bending absorption and between 2900-3100 cm^{-1} C-H stretching absorption of the native β -Glucan. [117]FT-IR spectrum of the hydrolyzed β -Glucan is shown in Figure 3.1.b. This spectrum is almost same with the spectrum of the native β -glucan. Comparison of these spectra indicated that while reducing the molecular weight, the hydrolysis reaction did not result in any change in chemical structure of the native β -glucan.

3.2. Characterization of Carboxymethylated β -Glucan (CM- β -Glucan)

3.2.1. FT-IR ve NMR Characterization

β -Glucan was carboxymethylated according to the reaction given in Figure 2.1. Carboxymethyl groups ($-\text{CH}_2\text{COO}-$) are more nucleophile than hydroxymethyl groups

(-CH₂OH) of β-Glucan. Hence, to increase the conversion of methacrylation, hydrolyzed β-Glucan was first carboxymethylated. The progress of the reaction was determined by FT-IR spectroscopy. Figure 3.1b,c shows the FT-IR spectra of hydrolyzed β-Glucan and CM- β-Glucan. The strong absorption band appeared at 1545 cm⁻¹ was attributed to the stretching vibrational absorption of C=O groups in the form of -COO-. This spectrum was an evidence of successful carboxymethylation of hydrolyzed β-Glucan. This reaction was confirmed by ¹³C NMR and ¹H NMR spectroscopy as well. Characteristic ¹³C NMR peaks of β-Glucan are shown in Figure 3.2. C1(102.7), C6(61.2, 65.5), C4(69.5, 36.8), C3(74.9, 74.3), C5(75.8) and C2(73.2, 36.8) were clearly observed. In this spectrum, three different carbonyl peaks, located at around 180 ppm indicates three different C=O groups formed after the carboxymethylation reaction. This was probably due to the diverse glycosidic linkages of β-glucan resulting in disordered structure. After the reaction, the sample become more soluble in water, which was visually confirmation of the reaction such that after the reaction, a clear solution in water was observed. This was reasonable that the more conversion of carboxymethyl groups on β-glucan resulted in more solubility. ¹H NMR spectrum of CM- β-Glucan is shown in Figure 3.3. H1(4.2, 4.41), H6(3.68, 3.80 and 4.09), H4(3.23), H3(3.43), H5(3.23, 3.62) and H2(3.39) are the characteristic peaks of β-Glucan [97, 98].

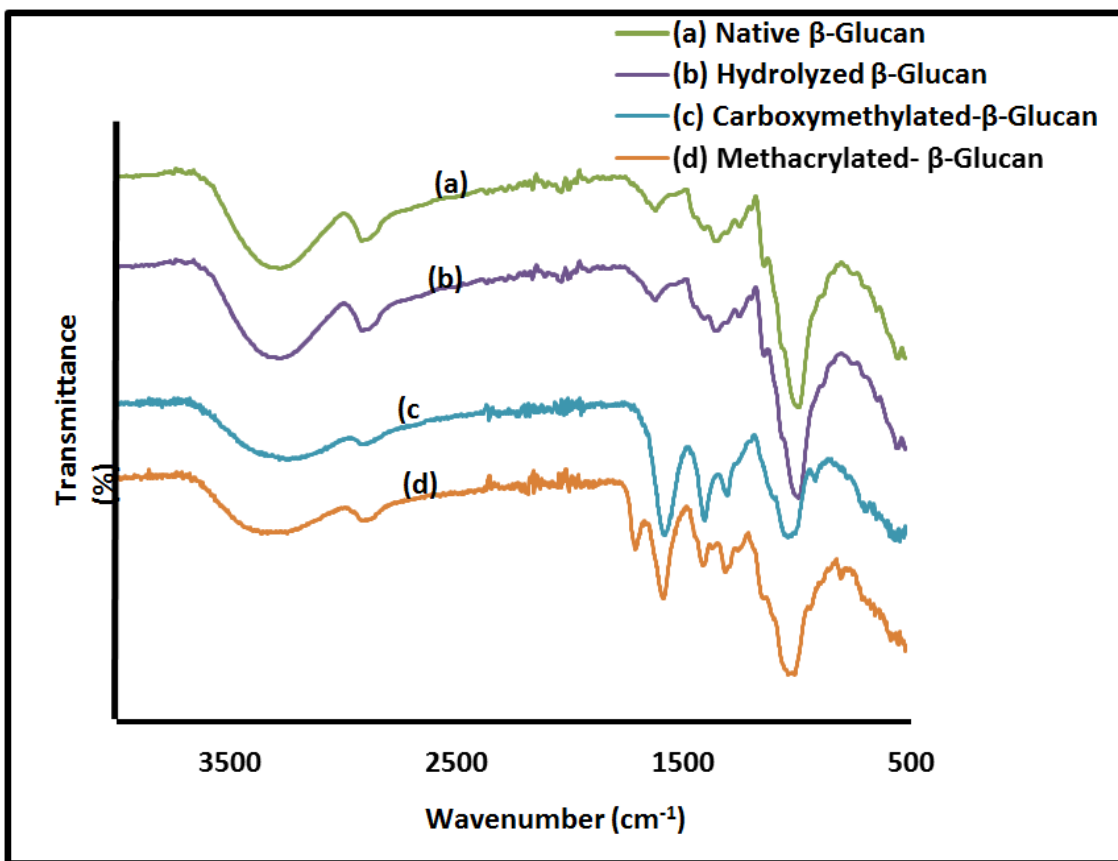


Figure 3.1. FT-IR spectrum of native β -Glucan(a), Hydrolyzed β -Glucan(b), carboxymethylated β -Glucan (c) and methacrylated β -Glucan (d).

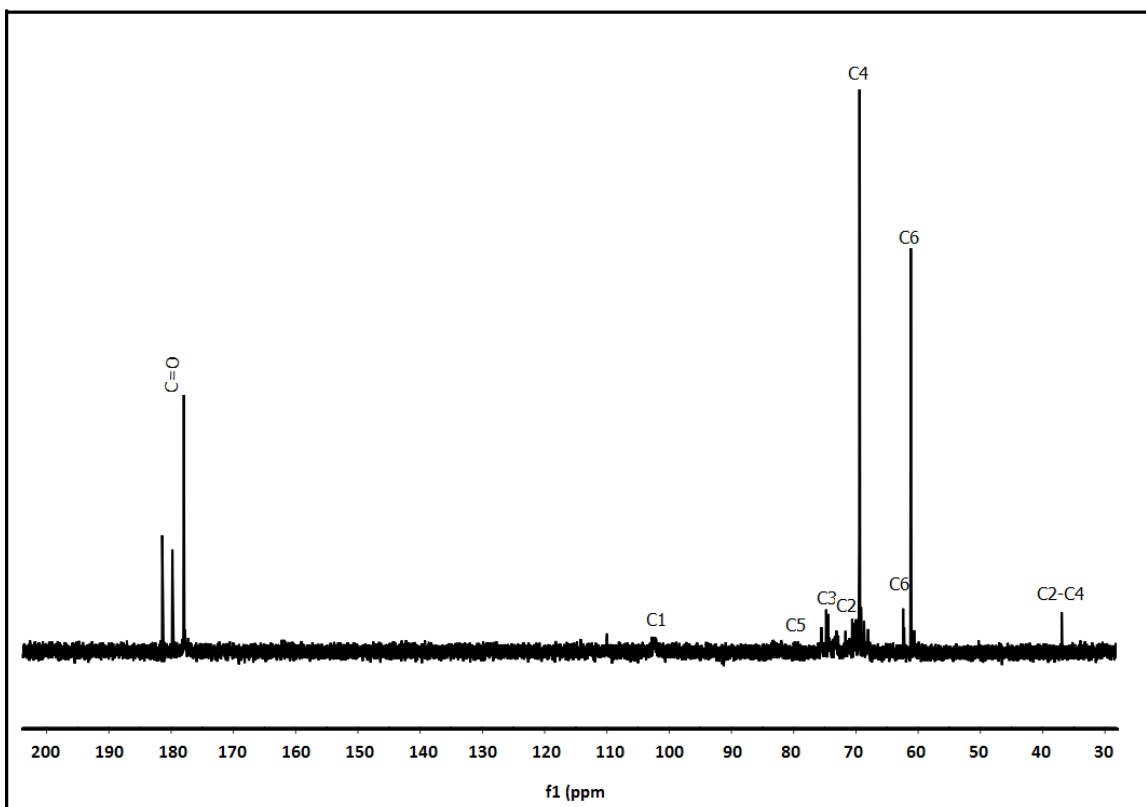


Figure 3.2. ^{13}C - NMR spectrum of Carboxymethylated β -Glucan

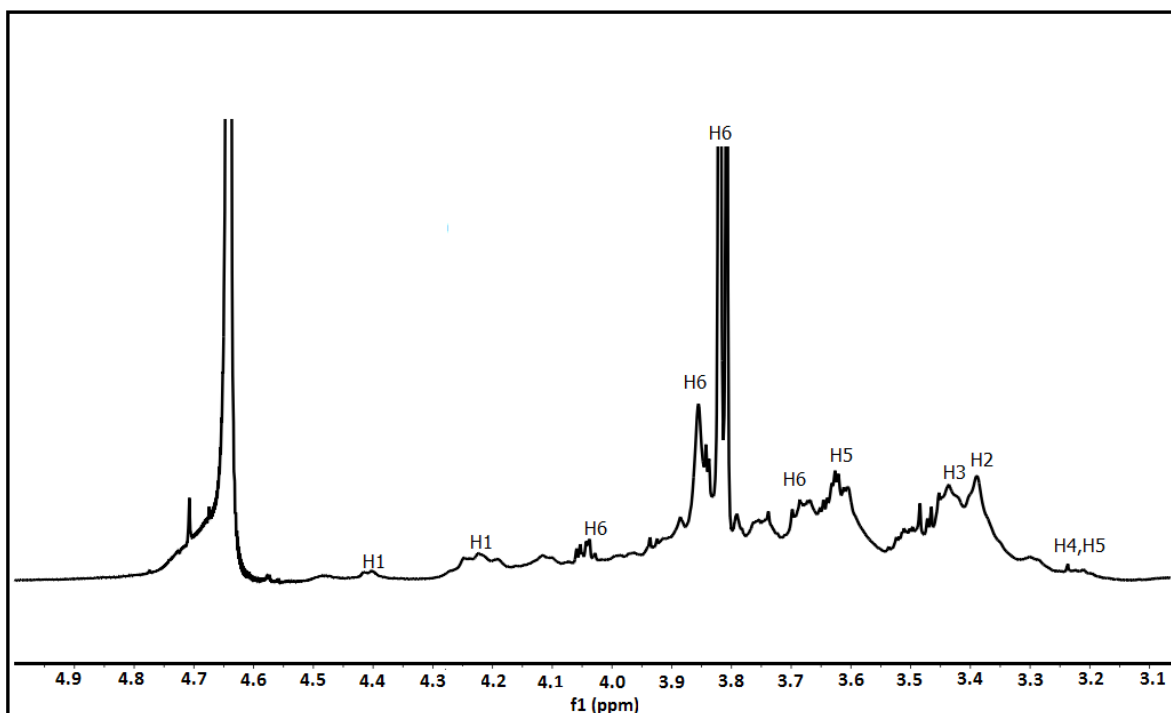


Figure 3.3. $^1\text{H-NMR}$ spectrum of Carboxymethylated β -Glucan

3.2.2. Molecular weight determination of CM- β -Glucan

Native (1,3)-(1,6) β -glucan is insoluble in water. Thus, to determine the molecular weight and molecular weight distribution after the hydrolysis, GPC analysis of more soluble CM- β -glucan was performed. It was reported in the literature that good solubility of CM- β -glucan in water increased anti-tumor activity. [18, 118]

Molecular weight and molecular weight distribution of CM- β -Glucan was determined by GPC-LS system. The specific refractive index of CM- β -Glucan was determined as 0.104 mL/g in 0.1M NaNO_3 in 2% acetic acid at 25 °C. The number average molecular weight (M_n), weight average molecular weight (M_w) and heterogeneity index (HI) of CM- β -glucan were determined as 3.64×10^5 g/mol, 4.62×10^5 g/mol and 1.27, respectively. The M_n of the native β -glucan was 7.49×10^5 g/mol as reported by manufacturer. GPC-LS chromatogram of CM- β -glucan is shown in Figure 3.4.

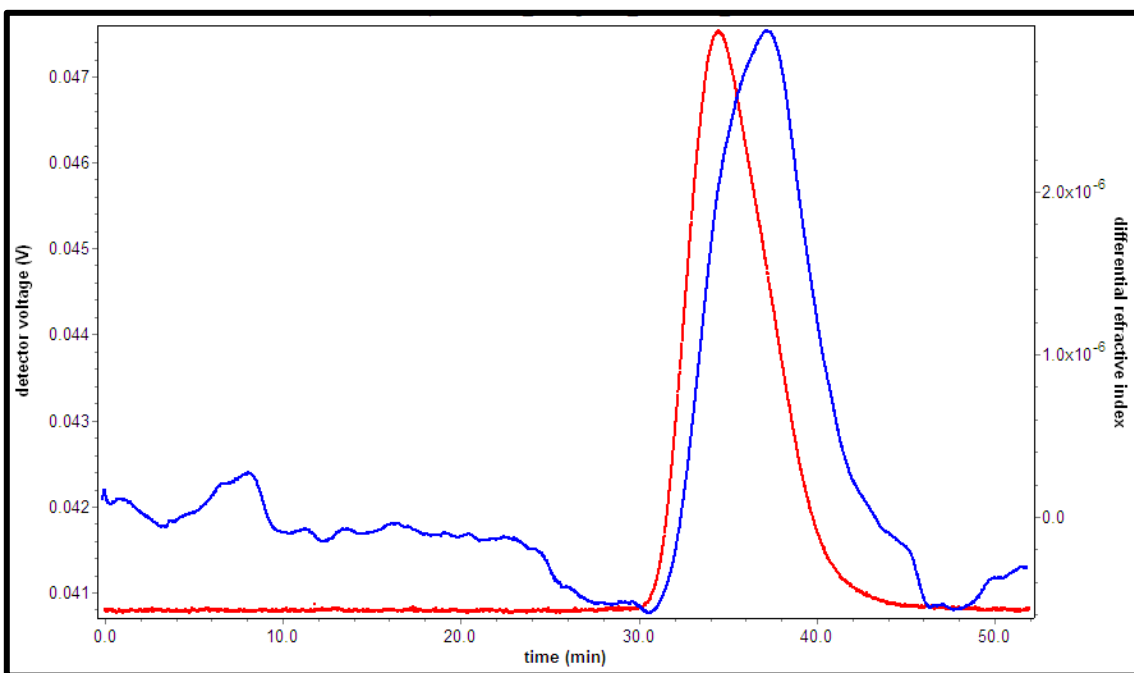


Figure 3.4. GPC-chromatogram of CM- β -glucan.

3.3. Characterization of Methacrylated β -Glucan (MA- β -Glucan)

Methacrylation reaction of CM- β -Glucan was conducted at 0 °C and pH 8 for 24 hours. The reaction product was characterized by FT-IR and $^1\text{H-NMR}$ spectroscopy. FT-IR spectra of CM- β -glucan and MA- β -glucan are shown in Figure 3.1. In FT-IR spectrum of MA- β -glucan (Figure 3.4.b), a new absorption band appeared at 1712 cm^{-1} , which is due to the residual C=O groups in the form of $-\text{COOH}$. A broad absorption bands at 3400 cm^{-1} ($-\text{OH}$ stretching) and in the range of $950-00\text{ cm}^{-1}$ ($-\text{C-O-C-}$ glucose and glycosidic etheric bending absorption) belonging to β -Glucan were clearly observed in this spectrum. The band observed at 1543 cm^{-1} (in Figure 3.1.c) and shifted to 1600 cm^{-1} , was due to the conversion of $-\text{COO-}$ groups into $-\text{COOCO-}$ structure, which was evidenced the formation of MA- β -glucan. It was further confirmed by $^1\text{H-NMR}$ spectroscopy. In Figure 3.5, the new peaks appeared at 5.7 and 6.2 ppm were due to the $\text{CH}_2=\text{C-}$ protons of methacrylate groups [24]. The percentage of the methacrylation of CM- β -Glucan on the base of the number of glucose rings was determined as 6% from the integration of vinyl peaks at 5.7

and at 6.2 ppm along with the peaks of glucose-ring protons at 3.43, 3.62 and 3.80 ppm. MA- β -glucan was used as cross-linker in NIPA polymerization.

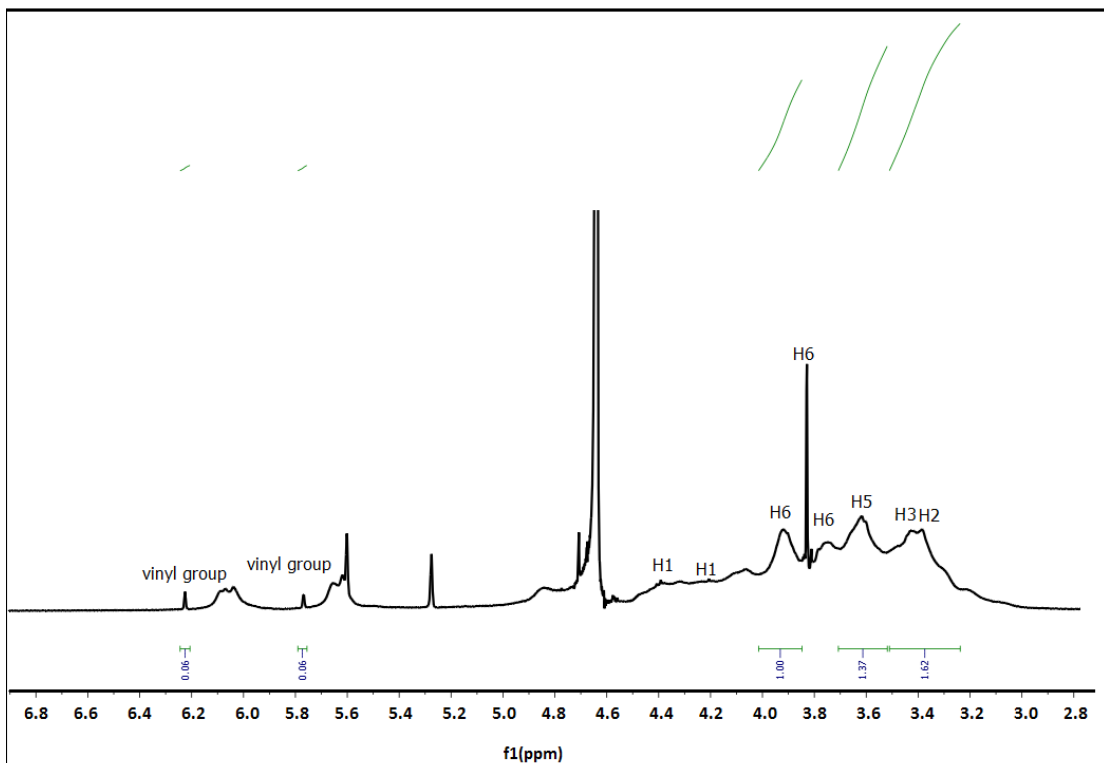


Figure 3.5. $^1\text{H-NMR}$ spectrum of methacrylated β -Glucan

3.4. Hydrogel Characterization

3.4.1 FT-IR characterization

FT-IR spectroscopy was used to confirm the formation of the β -glucan-pNIPA hydrogels. In FT-IR spectrum of pNIPA (Figure 3.6.), three characteristic absorption peaks at 1630 cm^{-1} ($\text{C}=\text{O}$ amide I), 1531 cm^{-1} (NH_2 , amide II) and 1324 cm^{-1} (N-H bending, amide III) were observed. The peaks at 3059 cm^{-1} and 3290 cm^{-1} are due to the N-H stretching absorption. In this spectrum, the characteristic broad $-\text{OH}$ peak of MA- β -glucan was observed at 3400 cm^{-1} . The peaks at 1710 cm^{-1} and 1652 cm^{-1} were due to the $\text{C}=\text{O}$ stretching of methacrylate groups and the peaks observed in the range of 1000 cm^{-1} - 1100 cm^{-1} were attributed to the glucose and glycosidic ether bond (C-O-C) of β -Glucan. The characteristic peaks of both β -glucan and pNIPA observed in this spectrum can be

considered as a proof of the hydrogel formation. The FT-IR spectra of Gel-1, Gel-2, Gel-3 and Gel-4 samples are collectively shown in Figure 3.7.

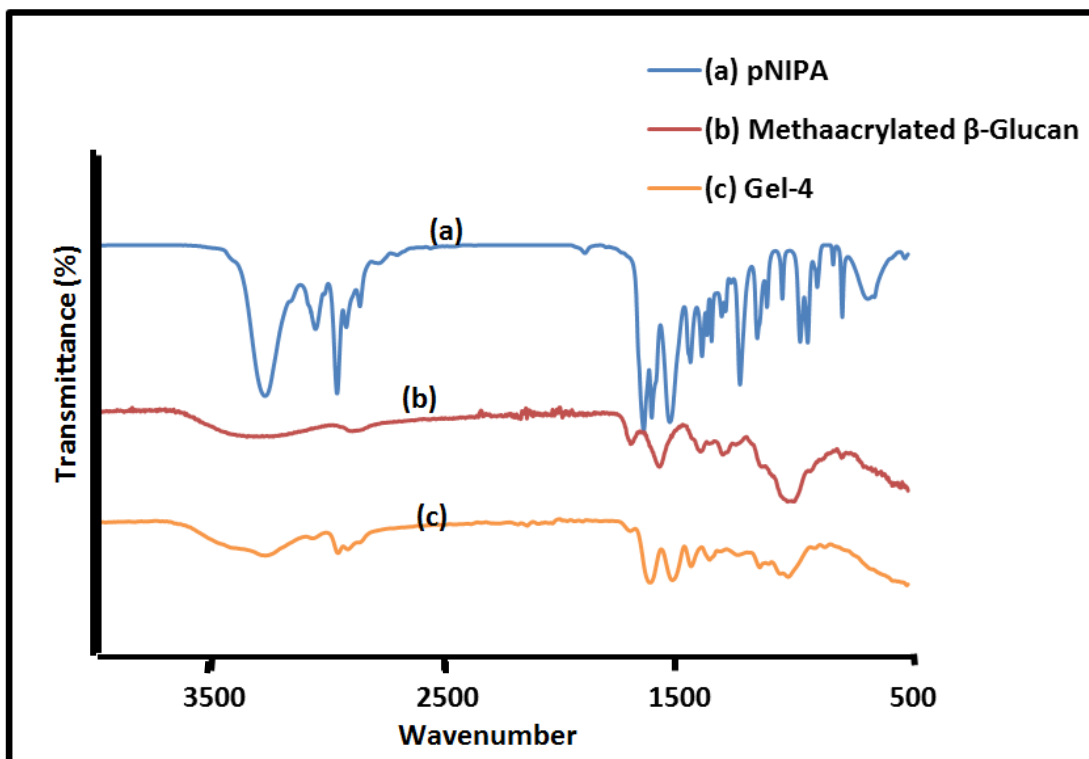


Figure 3.6. FT-IR of pNIP(a), MA- β -Glucan(b) and Gel-4(c).

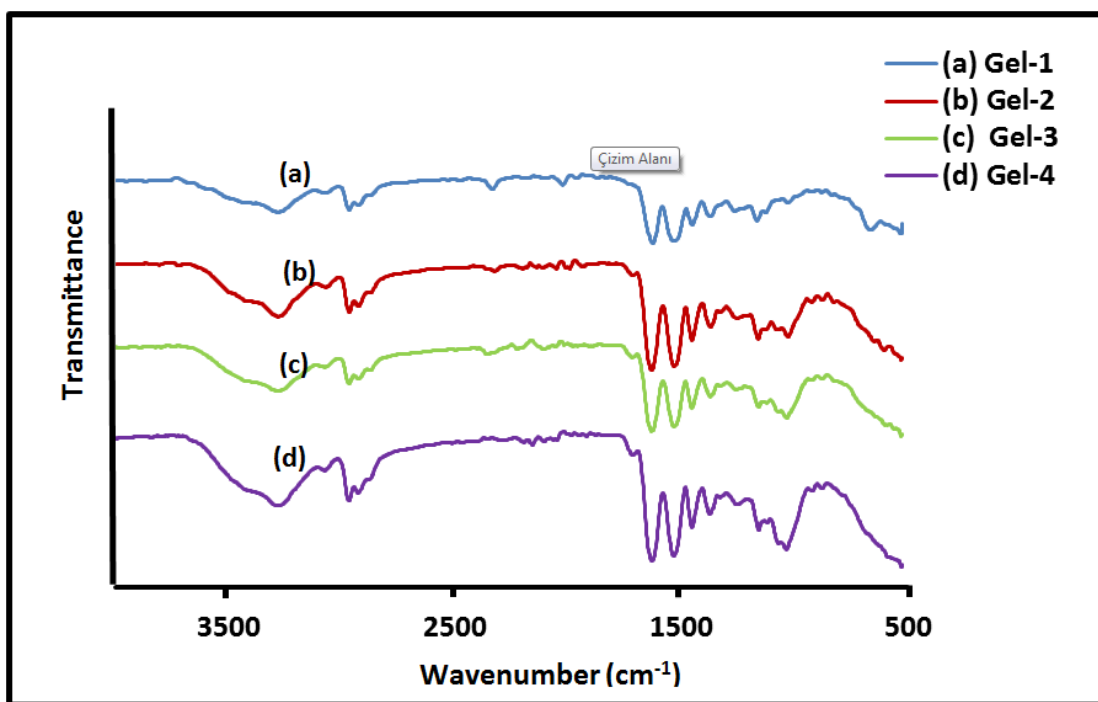


Figure 3.7 FT-IR spectra of β -Glucan - pNIPA hydrogels; a) Gel-1, b) Gel-2, c) Gel-3 and d) Gel-4

3.4.2. Swelling behaviors of the hydrogels

Hydrogels are water swellable three-dimensional polymeric networks. They are prepared by chemical or physical cross-linking of water-soluble polymers. Some hydrogels, prepared from stimuli responsive polymers, are sensitive to environmental stimulants (i.e. temperature, pH and magnetic fields) and they respond by changing shape, color, electrical conductivity etc. Most of the stimuli responsive hydrogels are prepared from synthetic water-soluble polymers, which are relatively biocompatible but not biodegradable. Thus, they may cause local inflammation in some extent when used as drug carriers. Copolymeric hydrogels of stimuli responsive polymers with less toxic natural and biodegradable cross-linkers provide effective and safe controlled delivery of specific therapeutic agents. PNIPA is a well-known temperature responsive polymer with a lower critical solution temperature (LCST) nearly at 32 °C. PNIPA hydrogels have a vast potential for CDD as the volume phase-change temperature (VPTT) is close to body

temperature [119]. For a cross-linked network of a temperature sensitive polymer, it is more convenient using the term VPTT rather than LCST of linear polymer. PNIPA hydrogels are generally prepared using N,N-methylene bis-acrylamide (BAAM) cross-linker, which is relatively toxic and causes local inflammation in some extent. To eliminate this drawback with increasing the biodegradability and biocompatibility, pNIPA was crosslinked with MA- β -Glucan. The hydrogels, prepared at four different compositions, were characterized and used for a controlled delivery of 5-ASA (Pérez, P., Gallardo, A., Corrigan, O. I., & Román, J. S., 2008). Equilibrium swelling ratio (ESR) and swelling behavior are two significant parameters for hydrogels and particularly significant in CDD in medical applications. Co-polymeric hydrogels prepared from the different components shows distinct swelling characteristics which can be tuned by composition. For this, the swelling kinetics of the hydrogels depending on composition were studied below and over the VPTT and their 5-ASA loading capacity and release profiles, at 25 °C and 37 °C, respectively, were determined. Swelling behaviors of the hydrogels in PBS (pH 7.4) under and over the VPTT values are shown in Figure 3.8. Remarkable swelling behavior, depending on the composition and temperature was observed. The highest equilibrium swelling ratio (ESR) was observed with Gel-1at all temperatures, which had the lowest β -Glucan as cross-linker and highest NIPA composition. The less cross-linker led to lower crosslink density and thus, higher ESR. All the hydrogels showed higher ESR at 25 °C, which is well below the body temperature (37 °C). This result is because of the good-solubility of pNIPA and β -Glucan 25 °C. Although above the VPTT, the ESR of the hydrogels diminished with raising temperature (Figure 3.8.a,b,c,d), the hydrogels reached to ERS more quickly. This was probably due to the temperature independent swelling behavior of β -Glucan, which may lead fast swelling. Above the VPTT (35 °C ve 40 °C), ESR decreased with increasing β -Glucan in the hydrogels, which was due to the increasing crosslinking density with increasing β -Glucan in the hydrogels.

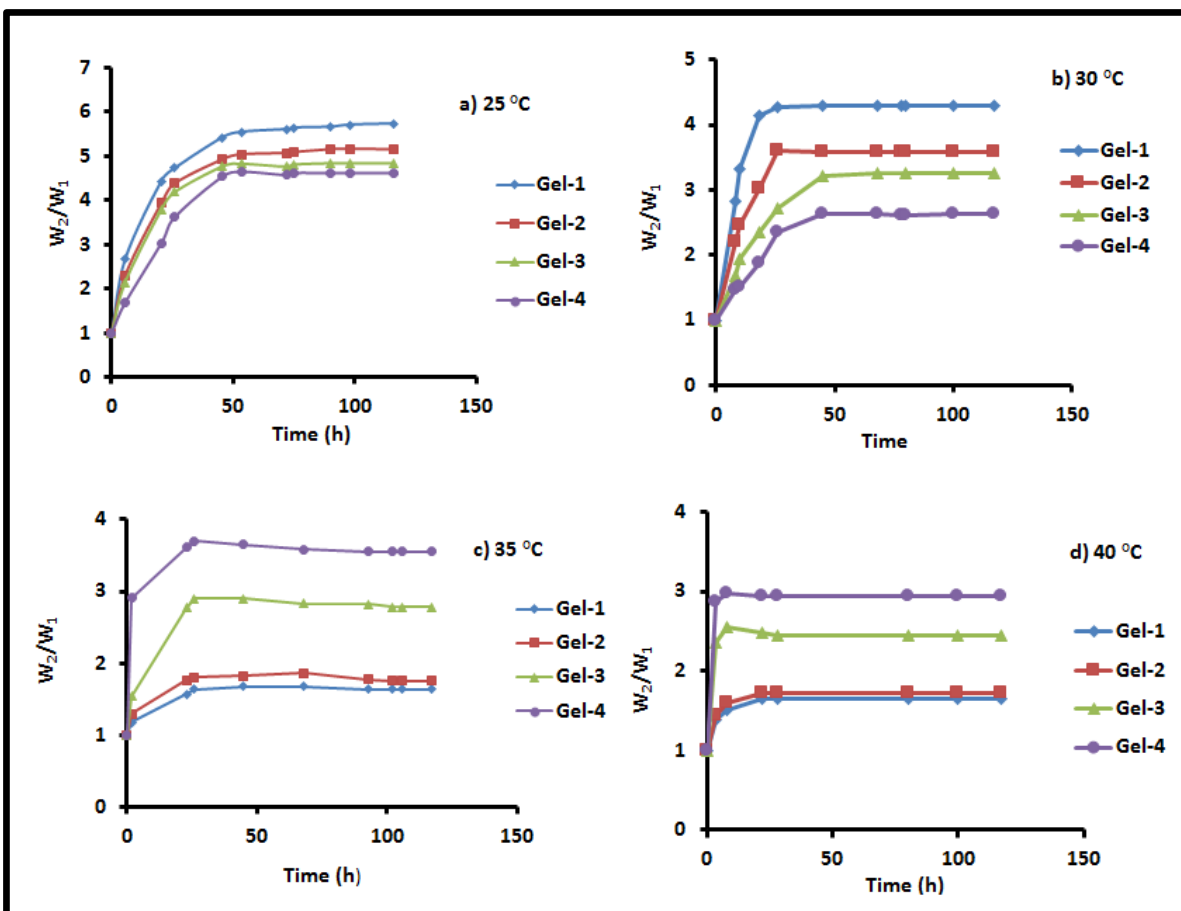


Figure 3.8. The swelling behaviors at pH 7.4 in PBS buffer solution at 25 °C (a), 30 °C (b), 35 °C (c) and 40 °C (d).

3.4.3. Volume Phase Transition Temperature (VPTT)

DSC is a major thermal analysis technique used for quantitative measurement of heat released or absorbed during any physical or chemical transition. This technique is performed under isothermal (at constant temperature as a function of time) or dynamic (as a function of temperature) conditions. An exothermic peak observed in a DSC thermogram may be due to any exothermic process accompanying with a chemical or physical bond formation, crystallization, polymerization, hydration etc. An endothermic peak observed in a DSC thermogram may be due to any melting, chemical or physical bond cleavage, dehydration etc. PNIPA is a thermo-responsive part of gels which has a LCST of 32 °C,

nearly body temperature. Due to its N-isopropyl groups, pNIPA can do hydrogen bonds with water reversibly. Under the LCST, hydrogen bonding is formed and pNIPA becomes more soluble in water. Over the LCST, N-isopropyls turn into more dynamic, and endothermic segmentation of the hydrogen bonding leads to phase separation slowly. Thus, the investigation of phase transition behavior of pNIPA hydrogels has been the topic of different research groups to develop controlled drug delivery systems. [120, 121]. Derivative DSC (DDSC) is a useful DSC technique to determine the phase transition temperature. In this study, the change of VPTT of the hydrogels in PBS (7.4) with their composition was determined by DDSC. The DDSC thermograms of linear pNIPA and the hydrogels are shown in Figure 3.9. While the VPTT of linear pNIPA is observed at 32.8°C, the VPTT of the hydrogels increases from 33.15 °C to 35.5 °C with increasing their β -glucan content. This might be due to the increasing hydrogen bonding formed between pNIPA- β -Glucan hydrogels and water. It is worth noting that the VPTT of hydrogels and thus, the release rate of drug loaded are tunable by changing the pNIPA/ β -Glucan ratio.

Figure 3.10 shows DSC curves of pure β -Glucan (a), CM- β -Glucan (b), and MA- β -Glucan (c). Pure β -Glucan and CM- β -Glucan have one single exothermic peak over 300°C, which are due to the oxidative decomposition. However, in DSC thermogram of MA- β -Glucan, two remarkable exothermic peaks are observed 260°C and 320°C. The first exothermic peak is due to the thermal polymerization of methacrylate groups of MA- β -Glucan [122], and the second exothermic peak is due to the oxidative decomposition of crosslinked β -Glucan structure formed after thermal polymerization. This is observed at relatively higher temperature than that of the oxidative decomposition of CM- β -Glucan. The crosslinking after thermal polymerization resulted in more stable structure.

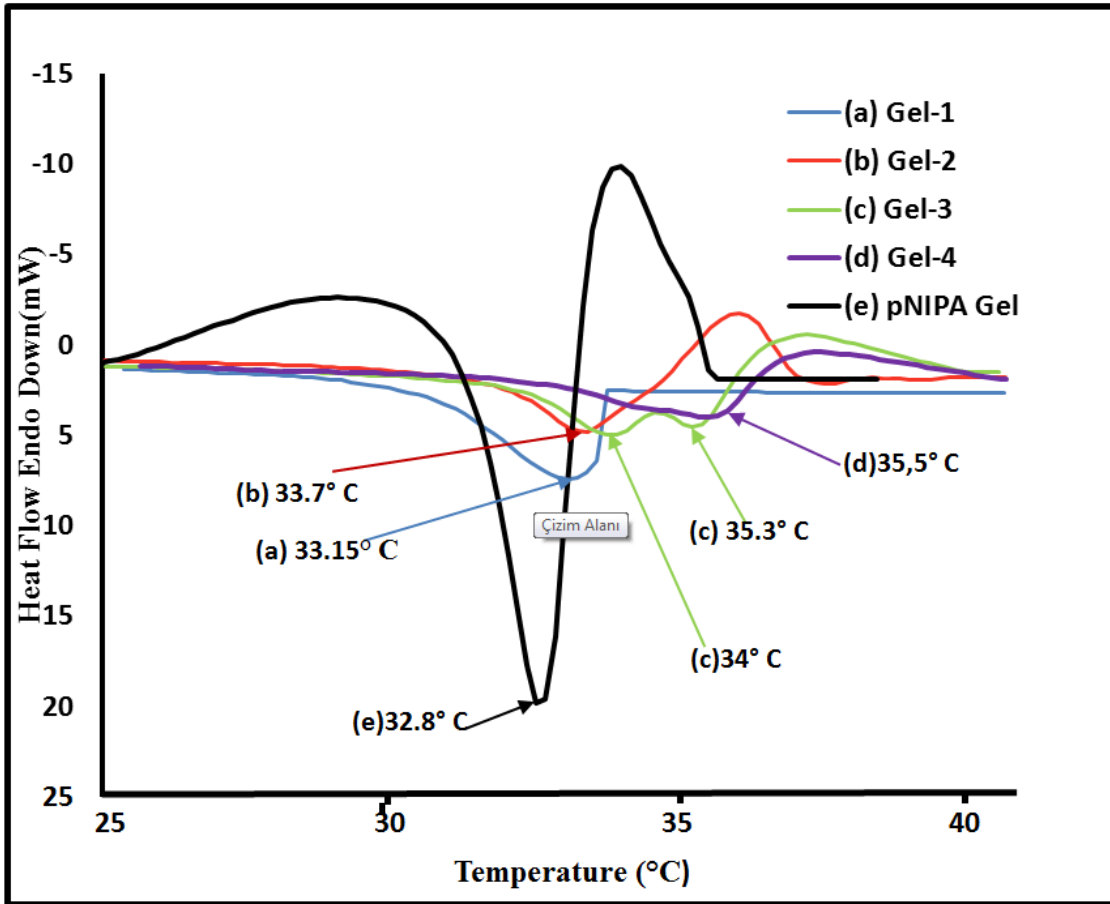


Figure 3.9. DDSC curves of pNIPa (a) and swollen hydrogels; Gel-1 (b); Gel-2 (c); Gel-3 (d) and Gel-4 (e) in PBS buffer (pH 7.4.)

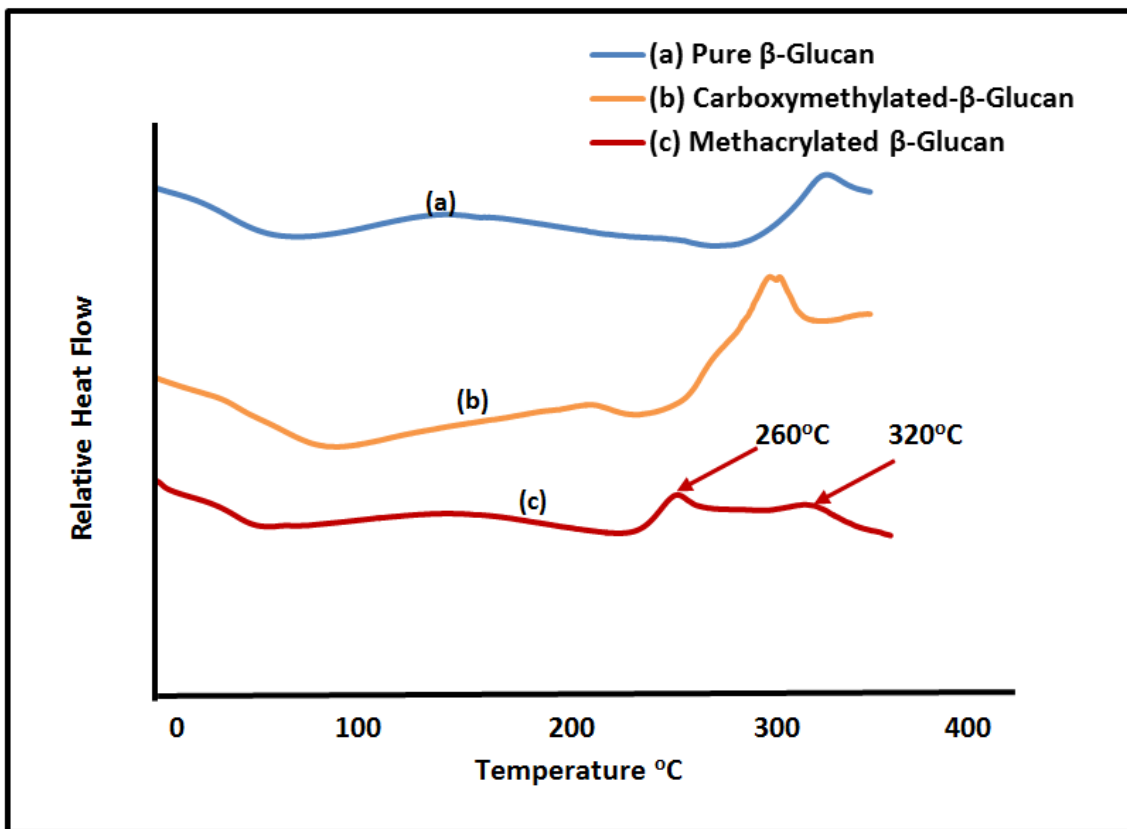


Figure 3.10. DSC curves of pure β -Glucan(a), carboxymethylated β -Glucan(b), and Methacrylated β -Glucan(c)

3.4.4. Thermal gravimetric analysis (TGA)

Thermal gravimetric analysis (TGA) is a useful technique to determine composition of hydrogels, polymeric blends, copolymers, composites etc., when the portions have definable thermal-decomposition behaviors. As demonstrated in Figure 3.11.a and Figure 3.11.b, pNIPA and native β -glucan have quite different thermal decomposition behaviors. While β -Glucan decomposition starts at 227 °C, pNIPA decomposes at 370 °C. The first weight-loss in TG curve of pNIPA was due to the release of the absorbed water (Figure 3.11.b). TG curves of pNIPA- β -Glucan hydrogels at different compositions are shown in Figure 3.12. To eliminate the absorbed water present in the hydrogels, the test samples were preheated up to 110 °C at 10 °C /min heating rate under N_2 atmosphere. After every

preheating process, the test samples were cooled down to 25 °C and their TG curves were recorded. The first decomposition step in TG curves was attributed to β -Glucan and the second was pNIPA network segments. As seen in Figure 3.12, thermal decomposition temperature of β -Glucan increased with its increasing portion in the network (from 246 °C to 261 °C). Similarly, thermal decomposition temperature of pNIPA in the hydrogel network increased from 304 °C to 310 °C as well. This was due to the increasing cross-link density of the networks with increasing amount of β -Glucan in the network structure, which led to higher cross-link density and thus more thermal stability.

Derivative of the TG curves of the hydrogel networks are shown in Figure 3.13. It is well-known that the areas under the derivative TG curves (DTG) are quantitative measure of the thermal decomposition of the hydrogels, and their peak temperatures correspond to maximum weight-loss rate temperature (MRT) of the hydrogel networks. As shown in Figure 3.13, the MRT, corresponding to the thermal decomposition of β -Glucan, shifted to higher temperature (from 274 °C to 286 °C) with increasing portion in the hydrogel networks. Similarly, the same tendency was observed with MRT of pNIPA, which increased from 396 °C to 407,6 °C. These results are another proof of the increasing cross-linking density of the hydrogel networks with increasing β -Glucan content, which led to a better stability.

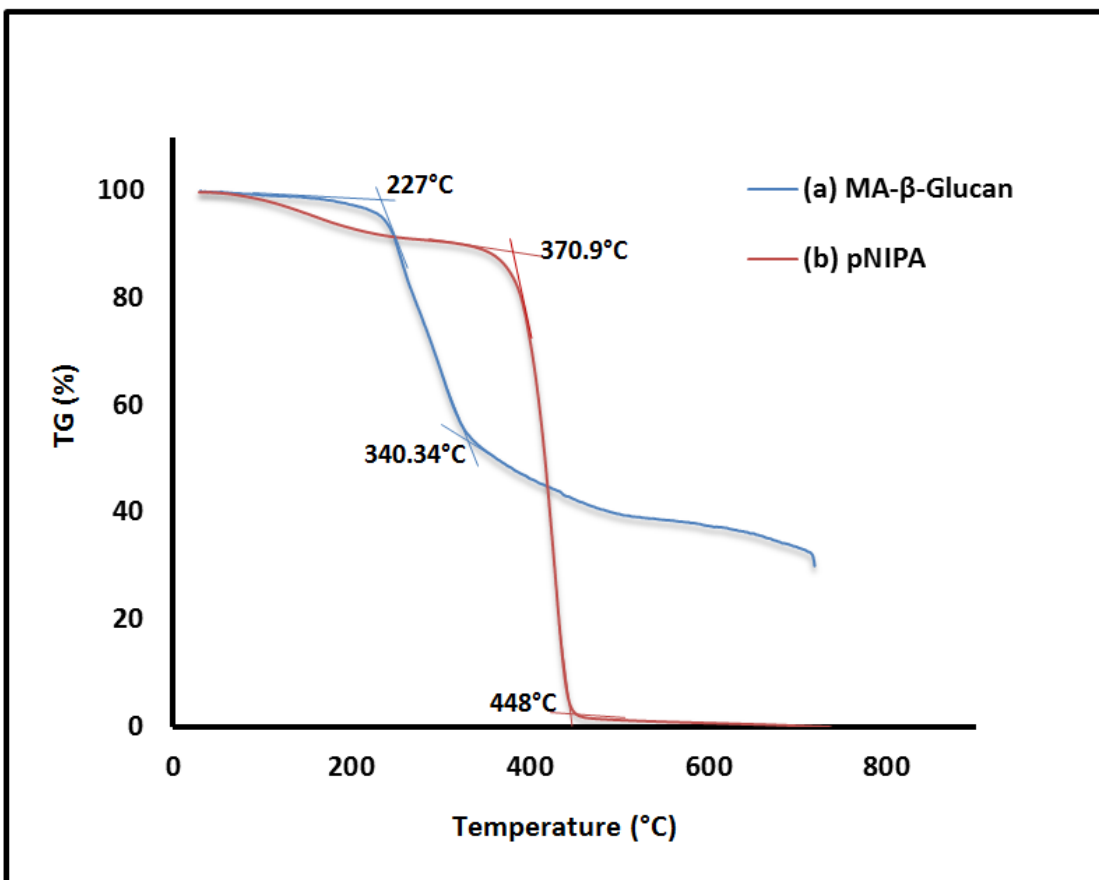


Figure 3.11. TG curves of the MA-β-Glucan and pNIPA

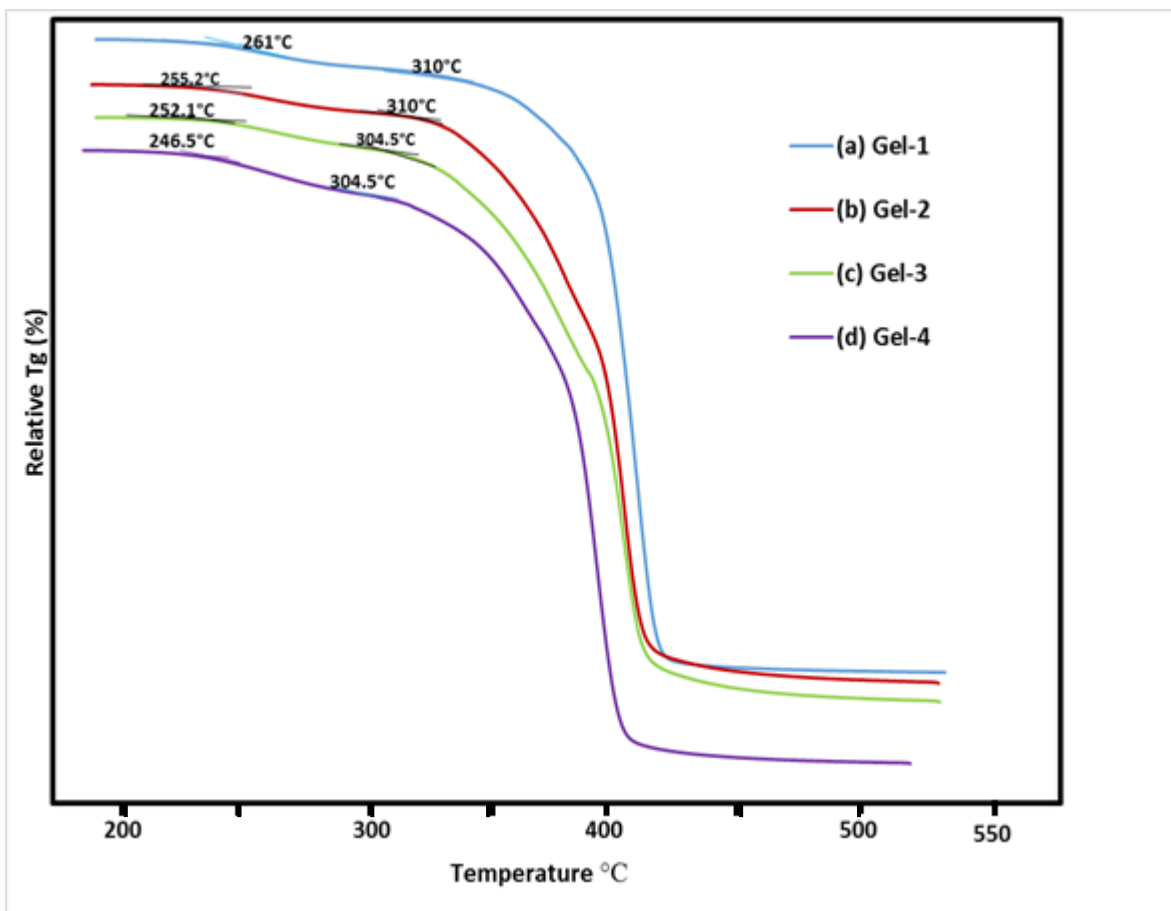


Figure 3.12. TG curves of the hydrogels.

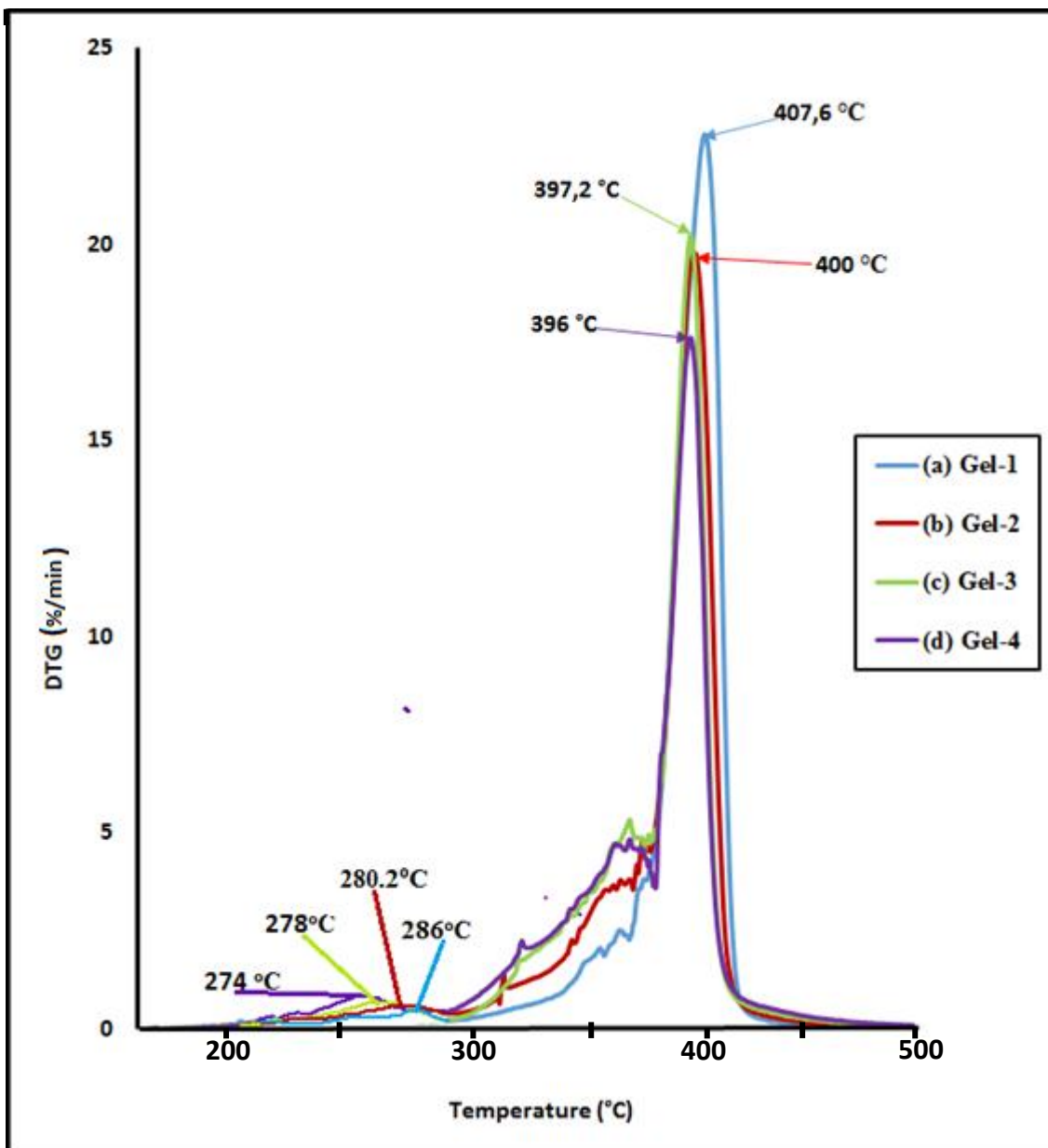


Figure 3.13. DTG curves of the hydrogels

3.5. Drug Loading and Release Studies

5-ASA loading of the hydrogels was performed at 25 °C because of the maximum ESR of all the hydrogels was observed at this temperature and the release studies were performed at 37 °C in PBS (pH 7.4), which is body temperature.

The amount of 5-ASA loaded in the hydrogels was calculated by Eq.2.2 and collected in Table 3.1. As it is expected that the maximum loading was observed for Gel-1, which showed maximum ESR in PBS (pH 7.4) at 25 °C. 5-ASA release studies were performed using UV-VIS spectroscopy at 37 °C by measuring 5-ASA specific absorption peaks of 5-ASA at 330 nm. The amount of 5-ASA released was determined from the peak intensities by using previously prepared absorbance-concentration calibration curve. As shown in Figure 3.14, within 2 hours, nearly 96 % of 5-ASA was released from HG-1 which had the highest amount of pNIPA compared to other hydrogels. Nearly 100% of 5-ASA was released within 4 hours. The 5-ASA release rate was increased with pNIPA in the hydrogels, which is because of the more tenderness of pNIPA to temperature change. Considering the 5-ASA profiles, it is notable that the 5-ASA release rate can be tuned by the amount of β -glucan and crosslinking density of the hydrogels.

Table 3.1. Hydrogel compositions and their 5-ASA contents.

Hydrogel	β -Glucan wt% In synthesis	β -Glucan wt % from TGA	β -Glucan MRT from DTG Temperatue (°C)	pNIPA MRT from DTG Temperatue (°C)	Q W ₂ /W ₁	5-ASA (wt %)	Sol Gel%
GEL-1	10	8.34	286	407	6.34	0.374	1,66
GEL-2	20	9.57	280.2	400	5.25	0.298	10.43
GEL-3	30	12.39	278	397.2	4.79	0.265	17,61
GEL-4	40	16.94	274	396	4.46	0.242	23,6

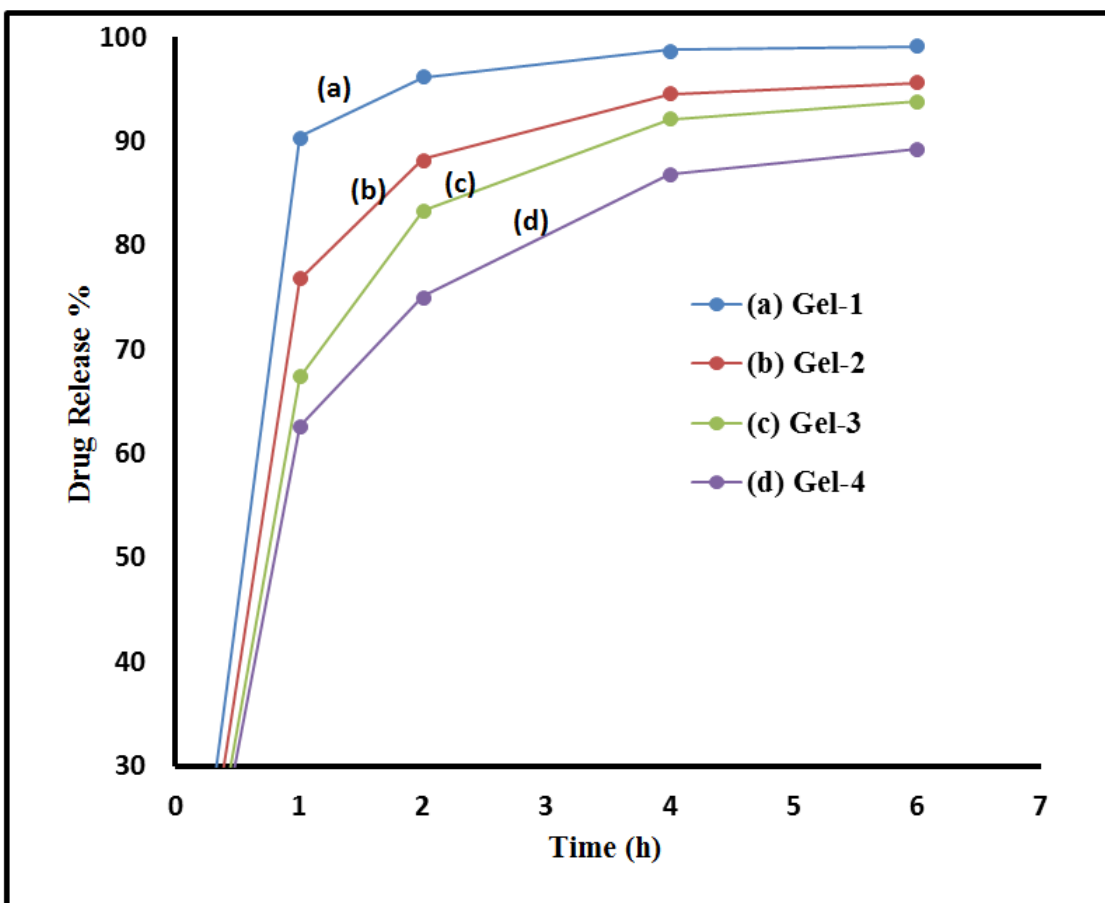


Figure 3.14. 5-ASA releasing profile from gels at 37°C in PBS buffer (7.4 pH).

3.6. In vitro Biocompatibility

In order to determine and compare the biocompatibility of pNIPA- β Glucan hydrogels with pNIPA (without levan) hydrogel, they were tested against WS1 (human fibroblast cells). Before cell viability assay, cell cultures were incubated in 10% CO₂ humidity at 37 °C As shown in Figure 3.15., the viability of cells exposed to NIPA- β Glucan gels was measured against a control group which had not been treated with any chemicals. Accordingly, the increase in cell viability was observed in direct proportion to increase in the ratio of β -glucan. One of the outstanding issues is that it is only in the wells tissue culture tablets where NIPA was used, cell viability remains at 40%. Cell viability increased to 56% even with the use of 10% β -glucan (Gel-1), and this increase continued persistently. Another

remarkable issue is that when 30% and 40% β -glucan are used, not only the toxicity of NIPA is completely abolished but also it contributes to the viability of the cells and the cells show proliferation and indicated even more cleavage behavior than the control group. This is due to the immunomodulatory effect of β -glucan.

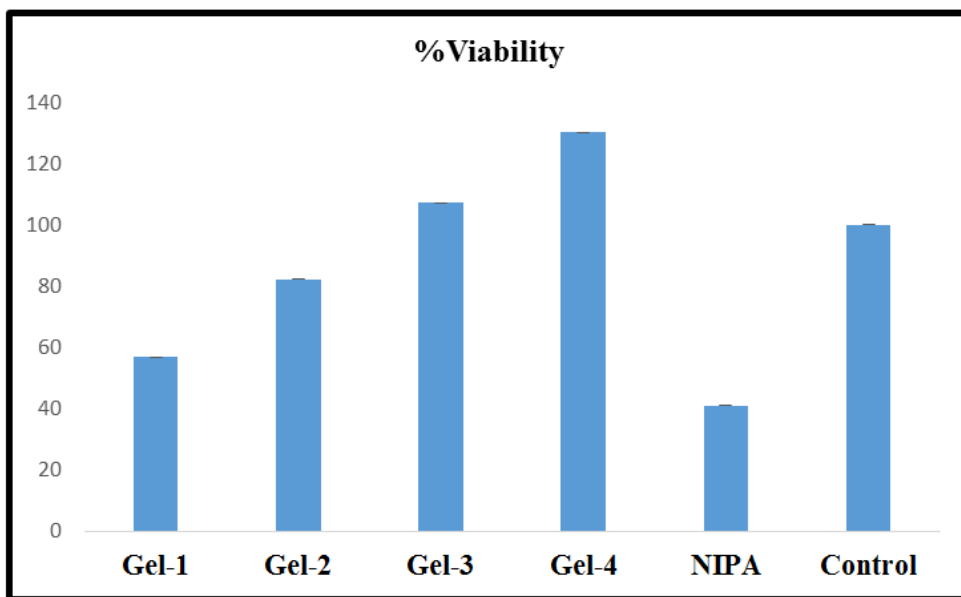


Figure 3.15. Cell viability of WS1 (human fibroblast cells) after 24 hours cultured with the hydrogels. (Gel-1, Gel-2, Gel-3, Gel-4) and pure pNIPA gel.

4. CONCLUSION AND RECOMMENDATIONS

Four different (1,3)-(1,6) β -glucan and pNIPA based temperature-responsive hydrogels at varying β -Glucan/pNIPA ratios were synthesized for controlled release of 5-ASA. To increase the water solubility, (1,3)-(1,6) β -glucan was acid hydrolysed and its molecular weight reduced from 7.49×10^5 g/mol to 3.64×10^5 g/mol. To increase the yield of methacrylation reaction and, thus, water solubility, the molecular weight reduced β -glucan was first carboxymethylated and then methacrylated. All the reaction products were spectroscopically characterized. In recent years, since pNIPA has a LCST of nearly 32 °C, which is close to body temperature, development of co-polymeric hydrogels of pNIPA with different synthetic monomers and crosslinkers has been the subject of many studies. In the preparation of pNIPA based DDSs, BAAM is one of the most preferred cross-linker. Due to the synthetic nature, BAAM is relatively toxic and pNIPA based hydrogels, prepared with BAAM, may cause possible local inflammation on use. To eliminate this, biocompatible and biodegradable MA- β -glucan was synthesized and used as cross-linker for the preparation of pNIPA based drug delivery systems for 5-ASA. To determine their 5-ASA loading capacity and release profiles, their swelling behavior was determined at 25, 30, 35 and 40 °C, which were below and over the volume phase transition temperature (VPTT) of pNIPA in PBS (pH 7.4). Under the VPTT, equilibrium-swelling ratios (ESR) were found to be considerable higher than that of above the VPTT, which decreased with the amount of β -glucan. 5-ASA release profiles were determined at 37 °C, which is body temperature. It is notable that, 5-ASA release rate increased with the amount of pNIPA in the hydrogels, which was due to the temperature sensitivity of pNIPA. The VPTT of the hydrogels in PBS (pH 7.4) were determined by DDSC, which was increased from 32.8 °C to 35.5 °C, approaching to body temperature. It is worth noting that, the VPTT of the hydrogels are tunable with the composition. The biocompatibility of the hydrogels was tested against WS1 (human fibroblast cells) after 24 hours cultured and the results showed that the biocompatibility of the hydrogels increased remarkably with β -glucan in the hydrogels. Results also shows that, due to immunomodulatory activity of β -glucan,

viability of the cells and the cells show proliferation and indicated even more cleavage behavior than the control group.

In line with the results, as well as the biocompatibility and termo-sensitivity of the hydrogels, pH sensitivity can be achieved by copolymerization of pNIPA with some pH responsive natural polymers such as chitosan, carboxymethyl cellulose, sodium alginate etc. In addition to 5-ASA, these hydrogels can be used for some antitumor drug delivery. Another important conclusion is that, due to the immune modulatory effect of β -Glucan, which is enhanced on the hydrogel surface at 37 °C, drug loaded or unloaded sub-micron hydrogels particles may be effectively used for cancer therapy.

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APPENDIXES

Appendix A

5-aminosalicylic acid concentration-absorbance calibration curve

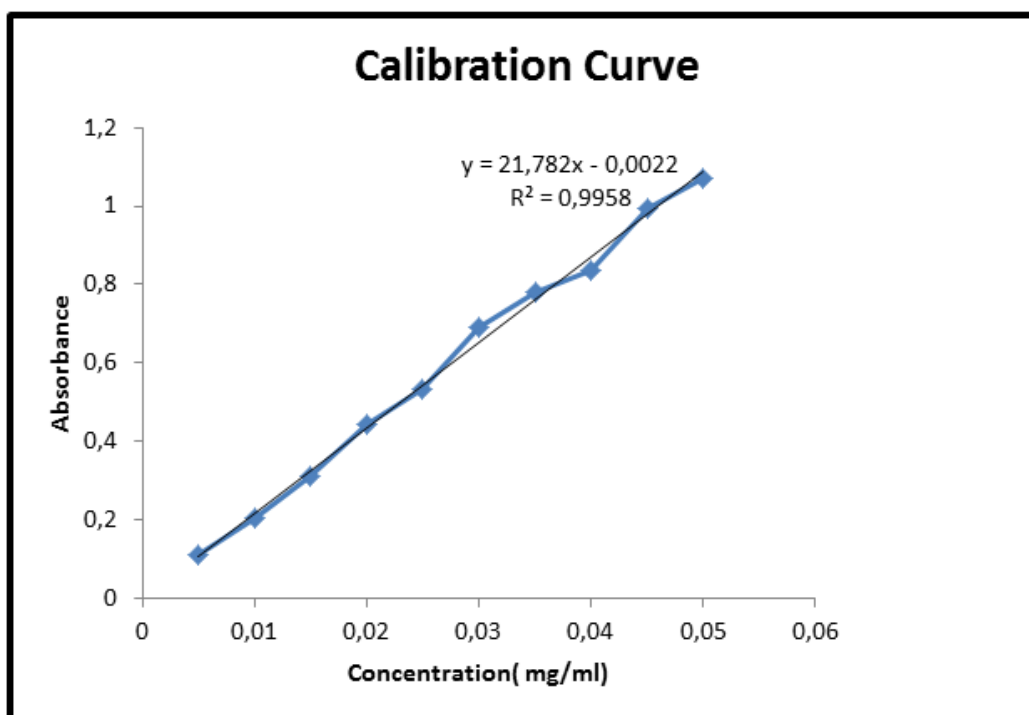


Figure A.1. 5-aminosalicylic acid concentration-absorbance calibration curve.

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Bachelor of Science	Chemical engineering	Marmara University	2013