MARMARA UNIVERSITY

INSTITUTE FOR GRADUATE STUDIES IN PURE AND APPLIED SCIENCES

LEVAN BASED BIODEGRADABLE HYDROGELS FOR DRUG DELIVERY

ASILA OSMAN

MASTER THESIS

Department of Chemical Engineering

ADVISOR

Prof. Mehmet S. Eroğlu

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Asila OSMAN, a Master of Science student of Marmara University Institute for Graduate Studies in Pure and Applied Sciences, defended her thesis entitled **"Levan Based Biodegradable Hydrogel for Drug Delivery"**, on October 04, 2016 and has been found to be satisfactory by the jury members.

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ÖZET

İlaç Taşınımında Kullanılacak Levan Bazlı Biyobozunur Hidrojeller

Son yıllarda kontrollü ilaç taşıyıcı sistemlere artan bir şekilde ilgi duyulmaktadır. Kontrollü ilaç taşıyıcı sistemler kullanılarak önceden belirlenen süreler içierisinde ilacın salımı mümkün olabilmektedir. Bu amaç için akıllı ilaç taşıyıcı sistemler kullanılmaktadır. Sıcaklık duyarlı polimerler içinde en fazla ilgi çeken polimer poli (N-izopropil akrilamid), pNIPA dır. PNIPA her ne kadar vücut sıcaklığına yakın hacım-faz değişimi sıcaklığına (VPTT) sahip olduğu için kontrollü ilaç salım çalışmlarında önemli bir potansiyele sahip olsa da, biyo-uyumlu olmaması nedeniyle enflamasyona sebep olabilmektedir. Öte yandan pNIPA esaslı hidrojellerin hazırlanmasında çapraz bağlayıcı olarak kullanılan N-metilen-bis-akrilamid (BAAM) da biyo-uyumu olmayıp toksiktir. Bu nedenlerden dolayı PNIPA esaslı hidrojellerin hazırlanmasında hem biyo-uyumluluğu arttırmak, hem de çapraz bağlayıcı olarak kullanmak için biyo-uyumlu polimerlerden hazırlanan çapraz bağlayıcılar sentezlenmektedir.

Bu çalışmada, biyo-uyumlu çapraz baplayıcı olarak metakrillenmiş levan (MA-levan) sentezlendi. Sentezlenen metakrillenmiş levan çapraz bağlayıcıolarak kullanularak, 5-amino salisilikasitin kontrollü ilaç salımında kullanılmak üzere, levan-pNIPA esaslı hidrojeller değişik levan/pNIPA oranlarında redox polimerizasyonu tekniği ile, potasyum persulfat ve TEMED başlatıcı olarak kullanılarak oda sıcaklığında sentezlendi. Sentezlenen levan esaslı biyouyumlu ve biyo-bozunur çapraz bağlayıcı Fourrier transform infrared spktroskopi (FT-IR), 1H-nükleer manyetik rezonans spektroskopisi (1H-NMR), jel geçirgenliği kromatografisi-ışık saçılması (GPC-LS), diferansiyel tarmalı kalorimetre (DSC) ve termal graivmetrik analiz (TGA) tehnikleri ile analiz edildi. Sentezlenen MA-levanın çapraz bağlayıcı olarak kullanıldığı farklı levan/pNIPA oranlarındaki hidrojeller sentezlenerek bunların 25 ^{o}C, 30 ^{o}C, 35 ^{o}C, 40 ^{o}C sıcaklıklarda fosfat tampon (PBS, pH=7.4) içindeki şişme davranışları incelendi. Sentezlenen hidrojellerin VPTT sıcaklıkları türevsel diferansiyel taramalı kalorimetre (DDSC) ile hasas olarak tayin edildi. Hidrojellerin VPTT sıcaklıklarının artan pNIPA oranıyla 32.8 ^{o}C tan (saf pNIPA) 35.09 ^{o}C ye (Gel-1, %60 pNIPA, %40 levan içermektedir) arttığı gözlendi. Hidrojellerden 5-ASA salımı UV-VIS spektroskopisi ile

izlendi. Hidrojellerin şişme ve 5-ASA salım davranışlarının sıcaklık ve kompozisyon ile önemli oanda değiştiği gözlendi. Hidrojellerin biyo-uyumlulukları fare fibroblast L929 hücre hattına karşı belirlendi. Artan levan oranıyla birlikte hidrojellerin biyo-uyumluluklarında da artış gözlendi.

Anahtar kelimeler: Levan, N-izopropil akrilamid, 5-ASA, sıcakl duyarlı hidrojel, kontrollü salım, ilaç taşıma and biyo-polimerler

ABSTRACT

LEVAN BASED BIODEGRADABLE HYDROGELS FOR DRUG DELIVERY

The need for controlling the release of drug has increased in the past decades. Delivery of compounds occurs in predetermined period of time while controlling the amount of drug with respect to time. For this purpose, the new trend is the use of smart hydrogel as a drug delivery system. One of the well-known temperature responsive polymers is poly (N-isopropyl acrylamide), pNIPA. Although, pNIPA hydrogels have a great potential for smart drug delivery systems since they have a volume phase transition temperature (VPTT) close to body temperature, they are not bio-compatible and thus may cause possible inflammation on use. In addition, non-biocompatible cross-linker, N-methylene bis-acrylamide (BAAM) is mostly used as cross-linker in the preparation of pNIPA hydrogels. To eliminate BAAM and to increase the biocompatibility, specific cross-linkers prepared from biodegradable polymers have been used.

In this study, methacrylated levan (MA-levan) was synthesized as biodegradable cross-linker. Using MA-levan, for 5-aminosalyciylic acid (5-ASA) delivery, biocompatible and temperature sensitive levan/ N-isopropyl acrylamide (levan/ PNIPA) hydrogels have been successfully synthesized by redox polymerization using potassium peroxodisulfate and TMED as redox pairs at room temperature. Characterization of the synthesized biodegradable cross-linker was successfully performed by using Fourrier transform infrared spectroscopy (FTIR), 1H nuclear magnetic resonance spectroscopy (1H-NMR), gel permeation chromatography-light scattering (GPC-LS), differential scanning calorimetry (DSC) and thermal gravimetric analysis (TGA). Different biodegradable hydrogels at varying levan/ PNIPA ratios were prepared and their swelling behaviors have been investigated in PBS (pH=7.4) at four different temperatures namely 25 ^{o}C, 30 ^{o}C, 35 ^{o}C, 40 ^{o}C. Volume phase transition temperatures (VPTT) of the hydrogels were sensitively determined by derivative differential scanning calorimetry (DDSC). It was noticed that phase transition temperatures were increased from 32.8 ^{o}C (for pure PNIPA) to 35.09 ^{o}C (for Gel-4 which contains 40% levan, 60% pNIPA) with increasing levan ratio. Moreover, the thermal stability has been

investigated using TGA. A remarkable increase in thermal stability of levan within hydrogel with increase of pNIPA content was recorded. The release of 5-ASA from the hydrogels was followed by UV–Vis and fluorescence spectroscopy. Swelling behaviors and the release of 5-ASA varied significantly with temperature and the gel composition. The biocompatibility of the hydrogels was tested against mouse fibroblast L929 cell line in PBS. The hydrogels showed increasing bio-compatibility with increasing levan ratio indicating levan enhanced the hydrogel surface during swelling.

Key words: Levan, N-isopropyl acrylamide, 5-ASA, temperature sensitive hydrogel, controlled release, drug delivery and biopolymers.

SYMBOLS

g	Gram
mg	Mili gram
µg	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-levan	Carboxymethylated levan
MA-levan	Methacrylated levan
TMED	N,N,N',N' tetra methlyethylenediamine
PNIPA	Poly(N-isopropylacrylamide)
MCA	Mono chloroacetic acid
5-ASA	5-Amino salicylic acid
HA	Hyaluronic acid
LCST	lower critical solution temperature
UCST	upper critical solution temperature
DSC	Differential scanning calorimetry
TGA	Thermal gravimetric analysis
FTIR	Fourier transform infrared spectroscopy
NMR	Nuclear magnetic resonance spectroscopy
GPC	Gel permeation chromatography

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1. INTRODUCTION

Hydrogels are insoluble, water-swollen polymeric networks that can absorb large amount of water and biological fluids. Most of the hydrogels are well known smart materials in terms of responding to environmental stimulants such as pH, temperature, electric and magnetic fields etc. [1]. Stimuli responsive hydrogels have widely used in many applications such as enzyme immobilization [2-4], photo responsible artificial muscles [5, 6], bio-separation [7-9] and memory devices [10]. Moreover, this property can be favorable in many drug delivery applications. There has been great effort on developing temperature sensitive drug delivery systems, mainly poly(N-isopropyl acrylamide)(pNIPA) and its derivatives [11]. PNIPA hydrogel has critical volume phase transition temperature (VPTT) at around 32 ^{o}C, which is close to body temperature[12]. Below VPTT, amide groups make hydrogen bonds with surrounding water molecules, resulting in swelling in water and buffered solutions. Thus, pNIPA hydrogels are able to encapsulate water soluble drugs. Over the VPTT, the formed hydrogen bonds are broken and drug loaded hydrogels collapse while releasing drug in a controlled way. The swelling and collapse of the pNIPA hydrogels are due to reversible formation and cleavage of hydrogen bonds with water when the temperature changes [13]. The VPTT of pNIPA makes useful for biomedical and bioengineering applications such as protein-ligand recognition [14], artificial organs, enzyme immobilization [15] and controlled drug delivery [16]. However, the use of pNIPA hydrogels in controlled drug delivery has some limitations that needs to be considered. pNIPA hydrogels are synthetic hydrophilic and non-biodegradable materials and, in general, bis-acrylamide is used as cross-linker, which is relatively toxic and causes possible local inflammation on use. To overcome these drawbacks, cross-linkers prepared from biodegradable and biocompatible polymers such as dextran [15, 17, 18], poly(lactic acid) [19, 20], chitosan [21] have been used. Bostan, Senol et al.[22] pepared temperature and pH responsive hydrogel based on pNIPA and methacrylated chitosan for 5-ASA delivery. They reported the possible tunable drug release by changing pNIPA/chitosan ratio.

Over the last decades, there has been a shift from bio-stable biomaterials to biodegradable materials, which are capable of hydrolytic and/or enzymatic degradation, thus, they can be

used in medical related applications. One of these biodegradable materials is microbial levan, which is water soluble and high molecular weight extracellular polyfructan. This material consists of glycosidic β-(2,6) major and β-(1,2) minor linkages. It is reported that, while the former leads to linear chain, the β-(1,2) causes branched structure [23-25]. Furthermore, levan homopolimer has been attracting great attention due to its remarkable properties including non-toxicity, anti-tumor activity, less-immunological response, cell adhesion and proliferation etc. Therefore, levan has been proposed as a promising material in medicine, textile, cosmetic, waste water treatment, food and pharmaceutical industries [26-31]. In addition to the aforementioned remarkable properties, high cell adhesion property of levan has found extensive use in drug delivery applications

In this thesis, methacrylated levan was synthesized and proposed to be use as cross-linker in preparation of biodegradable levan-pNIPA co-polymeric hydrogels. The hydrogels were prepared at four different Plevan-pNIPA ratio using redox polymerization initiated by redox pair ($K_2S_2O_8$-TEMED). The synthesized new biodegradable and thermo-responsive hydrogels, having the favorable VPTT property of pNIPA and remarkable cell proliferation and adhesion properties of levan, are expected to have promising controlled drug delivery systems for 5-ASA. The VPTT of the hydrogels were determined by derivative differential scanning calorimetry (DDSC). Moreover, swelling and drug release properties of the hydrogels were determined at below and over the VPTT to check the response of the hydrogels toward varying temperature. Biocompatibility and cell proliferation studies were performed on L929 fibroblasts. The VPTT and biocompatibility of the hydrogels increased with their levan content. The results indicated that the swelling degree, drug release profile and biocompatibility could be controlled by the amount of levan incorporated into the hydrogels.

1.1. Aim of the Study

The aim of the study was to prepare temperature sensitive and biocompatible levan-pNIPA hydrogels for controlled 5-ASA delivery. PNIPA hydrogel has a VPTT close to body temperature and is a well-known temperature responsive polymer. However, the use of

pNIPA hydrogel in controlled drug delivery has some limitations, which is non-biodegradable synthetic polymer and may causes possible local inflammation on use. To eliminate this limitation and to increase the biocompatible and biodegradable properties, co-polymeric hydrogels of pNIPA and levan at varying levan-pNIPA ratios were prepared. Therefore, less toxic and more biodegradable stimuli responsive hydrogels were obtained for controlled 5-ASA delivery. For this purpose, initially, methacrylated levan was synthesized. For these reaction, highly water soluble levan was necessary, for which low molecular weight (MW) levan (~$1.0x10^5$ Da) was prepared by acid hydrolysis of native levan (~$5\text{-}7x10^6$ Da). The low MW levan was first carboxymethylated and then methacrylated. The methacrylated levan was used as biodegradable cross-linker in redox polymerization of NIPA. VPTT, swelling, drug release and biocompatibility properties of pNIPA-levan hydrogels were determined

1.2. Drug Delivery Systems

The definition of "drug delivery" refers to methods, approaches, formulas, processes and systems that used to administer a pharmaceutical substance to safely achieve therapeutic effect in the body. Drug delivery involves some applications such as:

1. Conventional Drug Delivery Systems.
2. Novel Drug Delivery Systems

a. Controlled drug delivery system.
b. Modified release drug delivery systems.
c. Targeted drug delivery systems.

1.3. Conventional Drug Delivery Systems

Conventional or traditional drug delivery system is the one that includes the Classical methods for the delivery of pharmaceutical compounds into the body. Examples of these systems includes: Oral Delivery, Rectal Delivery, Buccal / Sublingual Delivery Intravenous Delivery, Intramuscular Delivery, Sub Cutaneous Delivery.

Furthermore, in conventional delivery system, drug level oscillates between the side effect level and the minimum therapeutic level. This oscillation occurs around the desired level due to periodic dosing within the ideal **therapeutic index or window (**a ratio of the amount of a therapeutic agent that produces the therapeutic effect to the amount that causes toxicity) figu

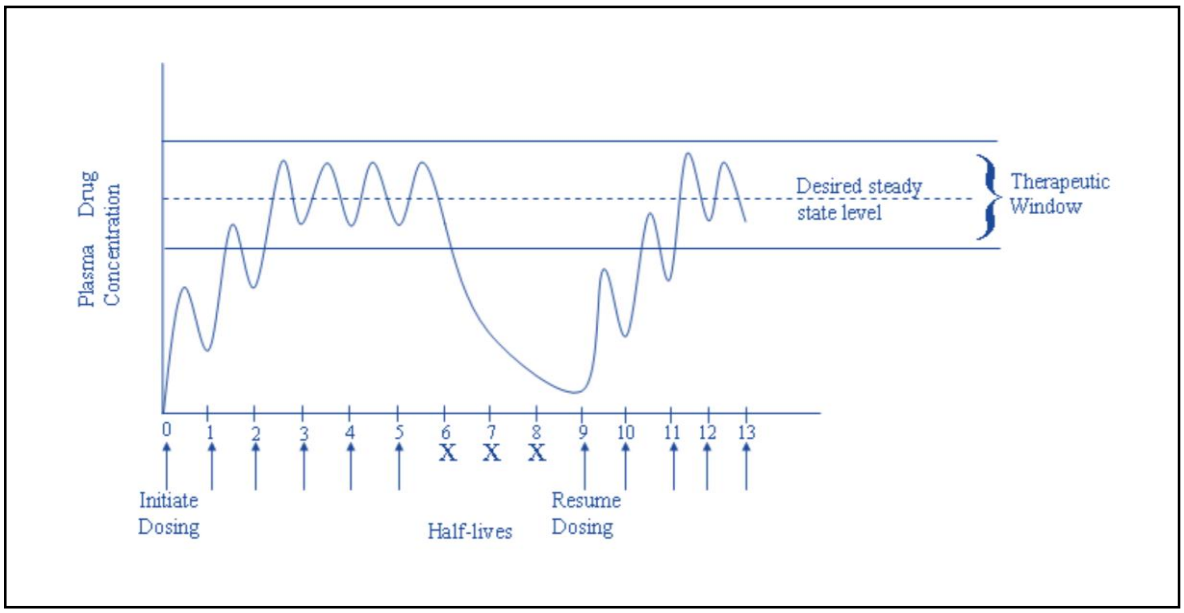


Figure 1.1. Drug Releasing from a conventional drug delivery system

1.4. Novel Drug Delivery

The purposes of novel drug delivery system can be summarized in some characteristics which include; improving drug release profiles, increasing the solubility of hydrophobic in vivo, controlling drug's absorption and distribution, reduction of drug toxicity, and the site-specific drug release.

1.4.1. Controlled release drug delivery systems

Controlled release is a term refers to the delivery of compounds in predetermined period of time while controlling the amount of drug with respect to time. This process results in a steadier and slower delivery of drug into the bloodstream and also less dosing frequency in contrast with conventional DDS of the same drug as can be seen in Figure (1.2) [32].

Controlled release DDS can be classified into: diffusion controlled system, dissolution controlled system and combination of these two types.

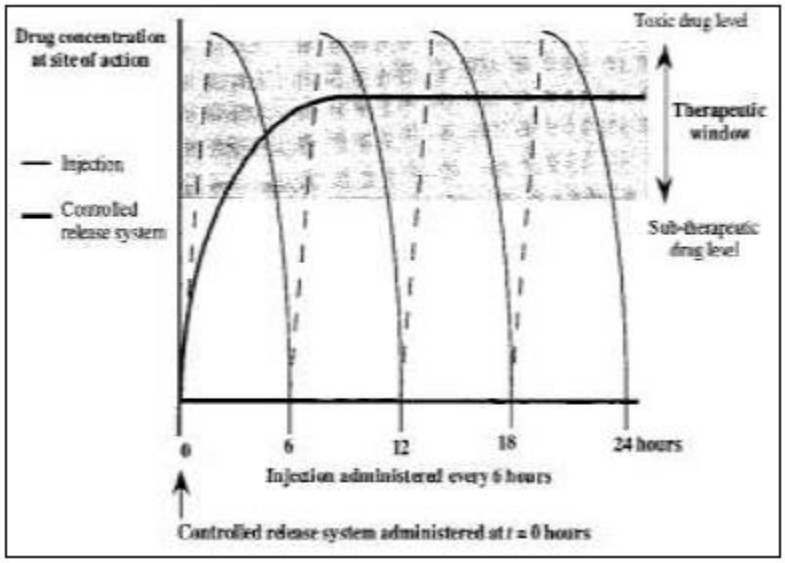


Figure 1.1. Release profile of conventional injection and controlled release system

1.4.1.1. Diffusion controlled Systems

In this systems, drug molecules move due to its diffusion across a membrane (usually water insoluble polymer). In this regard, the diffusion occurs either through the pores of matrix or passing between polymer chains[33], thus it can be divided into:

Reservoir devices: it can also be named as laminated matrix device, in which polymer material is used to surround a core of drug (reservoir), drug will diffuse and exchange with the surrounding fluid. It is established that release rate depends on the nature of polymer membrane, which can follow zero order drug release. However, it's difficult to deliver high molecular weight substance through this device [34].

Matrix devices: it is also called monolithic device, in which the drug is dispersed in a polymeric or ceramic matrix homogenously and the diffusion controls the drug release from the system. In this case, diffusion can occur at macroscopic level through pores in polymer (or ceramic) matrix, or on molecular scale passing between polymer chains [35]. Moreover, matrix diffusion system has some characteristics such as; it cannot obtain zero order release,

easy to produce compare with reservoir devices, and it is suitable for high molecular weight substance delivery [34].

1.4.1.2. Dissolution controlled systems

In this systems, drug released by the dissolution of the polymeric material, accordingly, all used polymers must be water soluble or degradable. Dissolution controlled system involves two categories;

Matrix dissolution controlled systems: In this system the drug is distributed homogeneously throughout the dissolvable polymer matrix and, the drug is released when the polymer matrix dissolves. The release rate decreases with the decreasing of matrix size, drug releasing rate in this particular case is nonzero-order.

In addition, the matrix can be micromatrix or macromatrix. Micromatrix systems utilize the microencapsulation technique to form small, spherical matrix systems while, macromatrix systems can be tablets consist of three layers an inner core, outer coat, and a film coat.

Encapsulation dissolution controlled systems: The other name for this system is coating dissolution controlled system. In this system, drug is coated with a given thickness coating layer, in form of particles, granules or seeds, which are dissolved slowly in the contents of gastrointestinal tract. Particles, seeds and granules coating can be achieved by microencapsulation.

1.4.1.3. Osmatic pump systems

In osmatic systems, the drug release happens on the same principle of osmosis. Furthermore, in this system the release is independent of pH and the other physical parameters. This system mainly consists of an active agent in form of salt, which is involved in semi permeable membrane and the pressure is created when water flow through the membrane. The main advantage of this device is that it can obtain a constant release rate unaffected by the gastrointestinal tract environments [33, 36].

1.4.2. Modified release drug delivery systems

Modified release drug delivery system refers to design the dosage forms in order to alter the drug releasing time, rate or location. Two types of modified-release drug delivery system are recognized:

1.4.2.1. Extended release system

The main advantage of extended release system is the prolonged the release time of drug, which leads to the reduction of dosing frequency that is associated with conventional dosing form. This system is suitable for people who have to take some medicine for long time or in their rest of life. However, by using extended release system they may be need to take just one or two dose per day. Extended-release formulations have two types: a polymeric matrix that involved the active ingredient, and the diffusion through the polymeric controls the delivery of drug. This matrix can be hydrogels or nano- and micro-particles. The other way to extend the release of drug is by use API capsules which coated with polymer. Here released is controlled by degradation of polymeric coat [32].

1.4.2.2.Delayed release system

Delayed-release dosage form refers to systems designed to release the drug at a time in discrete portion rather than promptly after administration. Delayed release systems are mainly used either for drug protection from degradation by alkaline environment in stomach, or the opposite. Here the release is started when the dosage reaches the small intestine. Polymers are popularly used for this aim; they are used to coat the drug dose (e.g. a tablet or the granules). Since the polymer is sensitive to environment pH, it travels from stomach where the pH is low, and dissolves in high pH when it reaches the small intestine followed by the release of the drug. The most common advantage of delayed release system is that the drug is released in the small intestine (enteric-coated dosage forms aspririn and NSAID products) or in the colon (colon-specific dosage forms) [32, 34].

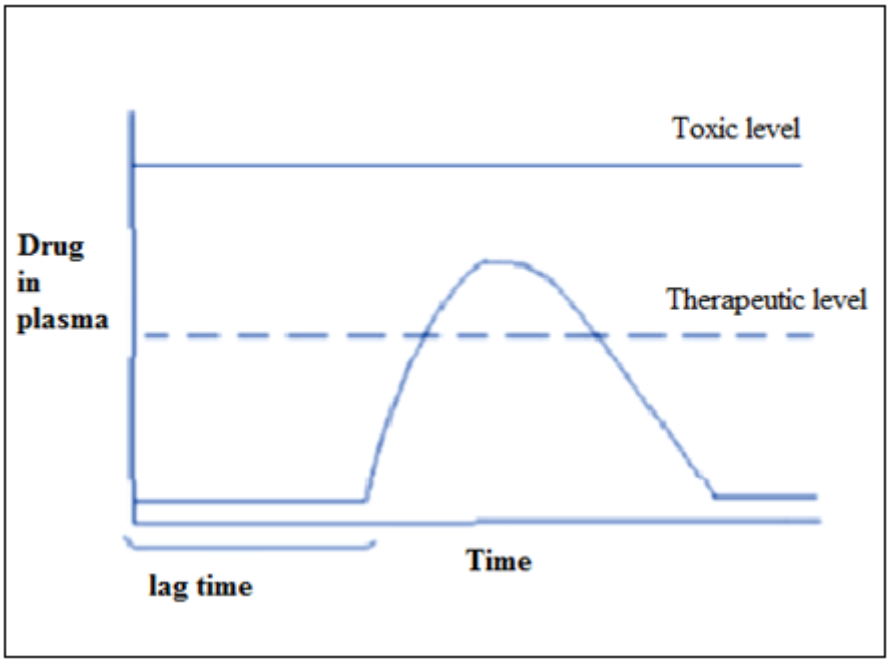


Figure 1.2. Drug release profile from delayed released system [32].

1.4.3. Targeted Drug Delivery System

Targeted drug delivery system is considered as one of the smart drug delivery system as it is based on a method that selectively delivers a certain amount of a medication agent for a prolonged period of time to a targeted specified area within the body [37, 38]. This feature helps to ensure specific concentration of drug in the body, thus, prevent the healthy cells from damage by drug. However, while designing targeted release system some points must be considered such as: the properties of drug and its side effects, the targeted site, the method used for the delivery of the drug, and the disease.

1.4.3.1. Passive targeting

Passive targeting provides sufficient and effective pharmacodynamics level, basically depending on EPR effect to accumulate the drug or active pharmaceutical ingredient (API) in tumor tissue (pathological sites with affected and leaky vasculature) and also prolong its circulation mostly through “mask” them by modifying their surface using hydrophilic

polymer with a well-solvated and flexible main chain example polyethylene glycol (PEG) [39].

However, there is a drawback associated with passive targeting, which is losing control of drug accumulation due to its random nature. This can lead to multiple drug resistance (MDR), and in some tumors drug cannot diffuse efficiently. Moreover, some types of tumors do not exhibit an EPR effect. Some characters of nanoparticle were used to modify passive targeting such as their size, shape, and surface characteristics [40].

1.4.3.2. Active targeting

Active targeting is considered the newest technique for drug delivery. It is also called ligand-mediated targeting, since it depends on attachment of affinity ligands to the surface of nanoparticles, after extravasation the carries will selectively bind to a target site. Those targeting ligands or agents include antibodies, proteins, peptides, nucleic acids, sugars, and vitamins [38, 41]. The main advantage of active targeting is that the drug will be released only into targeted cell without any effect on the healthy one.

To optimize the targeting some consideration like such as maximizing specificity that means the receptor should have high possibility to presence in the targeted cells compared with normal cells must be kept in mind. Moreover, using higher binding affinity increases targeting efficacy but for solid tumor if binding affinity is high it can cause decreased penetration of carriers due to a 'binding-site barrier', where the nanocarrier binds to its target [38].

1.5. Hydrogel

Hydrogels are insoluble, water-swollen polymeric networks that can absorb large amount of water and biological fluids. They are prepared from hydrophilic natural and synthetic polymers [1]. Swelling of hydrogels rather than their solubility in water are provided by crosslinking via chemical or physical process. Accordingly, the swelling degree of hydrogels is strongly depending on their crosslinking density which provides network structure and physical integrity. Hydrogels have different of physical forms, involve slabs, microparticles,

films, coatings, and nanoparticles [42]. Most of the hydrogels are hydrogels are smart since they are responding to environmental stimulies such as pH, temperature, electric and magnetic fields etc. These properties of smart hydrogel make them widely used in many applications such as enzymes immobilization [2-4], photo responsible artificial muscles [5, 6], bioseparation [7-9] , and memory devices [10]. Hydrogels have been investigated extensively for their application as drug carriers in controlled releasing devices. In this case, drug is loaded into the system and released as a response of change in environmental condition.[42-47].

1.5.1. Hydrogel classification

Hydrogels are classified considering their physical properties, nature of swelling, method of synthetization, origin, sources, rate of biodegradation and crosslinking methods.

Based on their sources hydrogels can be classified into three groups [48] : natural including proteins, polysaccharides, protein/polysaccharide hybrid polymers and DNA, synthetic origins and this category including non-biodegradable, biodegradable, and bioactive polymers, and Synthetic/natural hybrid polymers.

On the other hand, based on their physical structure hydrogels can be classified as: amorphous (non-crystalline) randomly arrangement of macromolecular chains, semi crystalline acomplex mixture of amorphous and crystalline phases, and Crystalline.

Also according to their electrical charge hydrogel can be classified into four categories:

neutral hydrogels (nonionic), ionic hydrogels (it may be an ionic hydrogel or cationic hydrogels), amphoteric electrolyte hydrogel (ampholytic) when both acidic and basic groups existing and, zwitterionic (polybetaines) involved both anionic and cationic groups in each skeletal structural

1.5.2. Hydrogels crosslinking methods

There are different natures of crosslinking processes of hydrogel such as physical hydrogel which is formed by physical processes such as crystallization, hydrophobic association,

polymer chain complexion, hydrogen bonding, and chain aggregation. Physical hydrogels are reversible due to the conformational changes. On the other side a chemical hydrogel which is irreversible because of configurationally changes made by using chemical covalent crosslinking (simultaneously or post polymerization). When both chemical and physical crosslinking was used to from a hydrogel network it called dual –network.

1.5.2.1.Crosslinking by radical polymerization

Chemical crosslinking of hydrogel is achieved through free radical polymerization of polymerizable hydrophilic polymers derivatives and also radical polymerization of mixtures of vinyl monomers [49, 50]. Hydrogels from this route are synthesized via using natural, synthetic and semi-synthetic water soluble polymers. Moreover, synthesis may be done through UV polymerization[51], or by introducing methacrylic groups into the mono and disaccharides, which may be used for the hydrogel using enzymes as catalyst [52-54].

1.5.2.2. Crosslinking by chemical reaction of functional groups

This class of hydrogels can be prepared by a reaction between water soluble polymers having certain functional groups such as carboxylic acid (-COOH), amine (-NH_2), hydroxyl (-OH), isocyanate (-NCO) etc. and functional low molecular weight cross-linkers. For example, to establish amide type linkages, -NH2 groups are reacted with -COOH groups, for urethane type linkages, -NCO groups are reacted with –OH groups and Schiff base formation can be used to established covalent linkages between polymer chains.

Furthermore, in crosslinking by condensation reactions, among the –OH, NH_2 COOH or derivatives, N,N-(3-dimethylaminopropyl)-N-ethyl carbodiimide (EDC) has been shown as highly efficient reagent for cross-linking hydrophilic polymers. This method used to synthesize polyesters and polyamides [55]. There is also crosslinking by addition reactions using bis or higher functional cross-linkers to react with functional groups of hydrophilic polymers. 1,6-hexamethylenediisocyanate[56], divinylsulfone [57] , or 1,6-hexanedibromide [58] are used to cross-link Polysaccharides. In addition, crosslinking by high energy irradiation via gamma rays and electron beam to polymerize unsaturated substances can be

considered [59, 60]. Also, crosslinking using enzymes have been reported to the synthesize PEG-based gels by Sperinde et al. [61].

1.5.2.3.Crosslinking by ionic interactions

Ionic crosslinking is generally established between the molecules carrying opposite charges. As an example to this, sodium alginate is easily cross linked with Ca^{+2} di-valent cations of $CaCl_2$ via its negatively charged alginate groups at room temperature under basic pH. The prepared gels are commonly used as a matrix for protein release and also for the encapsulation of living cells [62] and trimetazidine dihydrochloride (TMZ) for controlled release [63].

1.5.2.4.Crosslinking by crystallization

In this method, hydrogel can be formed by storing of aqueous solutions of hydrophilic polymer at room temperature. For example, poly(vinyl alcohol) (PVA) hydrogels can be formed by this method. Hydrogels obtained by this method have poor mechanical properties. However, the elasticity of the hydrogels gel can be improved by freeze–thawing process [64].

1.5.2.5. Crosslinking by hydrogen bonding

One of the way to crosslink hydrogel is through Hydrogen bond for example between the carboxylic group of polyacrylic acid/polymethacrylic acid and the between the oxygen of the polyethylene glycol[65], and also in poly (methacrylic acid-g-ethylene glycol) [66].

1.5.3. Interpenetrating network hydrogels

According to the type of network, hydrogels can be formed as homo-polymers, copolymers, semi-interpenetrating networks and interpenetrating networks.

Homo-polymers are networks formed from single monomer and their skeletal structure differs according to polymerization technique and monomer nature[67]. A well-Known example of this category is Polyethylene glycol (PEG) based hydrogels, which are considered as smart hydrogels due to their response to external stimuli, and wide usage in drug delivery.

Co polymeric hydrogels are formed of two or more types of monomer component involving at least one water soluble monomer. Their properties basically depend upon the percentage of each polymeric units and their arrangements. Examples may include carboxymethylcellulose and carboxymethyl chitosan copolymeric hydrogels which were used for metal ion adsorption [68].

Semi interpenetrating network is the one in which at least one linear polymer penetrates crosslinked network but there is no any chemical bonds [49, 69].

Interpenetrating network occurred when one or more network interlaced on other network and to separate them chemical bonds should be broken [49, 69].

1.5.4. Stimuli responsive hydrogels

Some of hydrogels can show changes in their properties (swelling ratio, permeability mechanical strength and network structure) as a response to environmental stimuli, those kind of hydrogels ara known as smart hydrogels. These stimuli may be physical or chemical. Physicals stimuli involve temperature, solvent composition, light, pressure electric fields, sound and magnetic fields, while the chemical stimuli include pH and ionic strengths. A biochemical stimulus include the responses to antigen, enzyme, ligand, and other biochemical agents [47, 70, 71]. Some hydrogel represent response against two stimuli and they are called dual responsive hydrogels [72].

1.5.4.1.pH responsive hydrogels

Some of the hydrogels involved ionic pendant groups that can donate or accept protons as a response to pH change of the environment. In this kind of hydrogel at certain pH the degree of ionization pKa or pKb is greatly changed and the ionized pendant group's net charge is changed causing a suddenly volume transition via creating electrostatic repulsive forces between the ionized groups that lead to form a large osmotic swelling force [49]. pH responsive hydrogels are divided into two types anionic and cationic. Anionic hydrogel has acidic groups like carboxylic or sulfonic acid and the swelling increases at basic pH for

example poly(acrylic acid) [73], in contrast with cationic with a basic groups such as amine and swell at acidic pH for example acrylamide-based polyampholyte hydrogels [74] .

As suggested by Gupta et al [75] the main factors that controlled the degree of swelling of ionic hydrogel are polymer properties such as (cross-link density, charge, degree of ionization, concentration and pKa of the ionizable group, and hydrophobicity or hydrophilicity) and swelling medium properties (ionic strength, pH, and the counterion and its valency) .

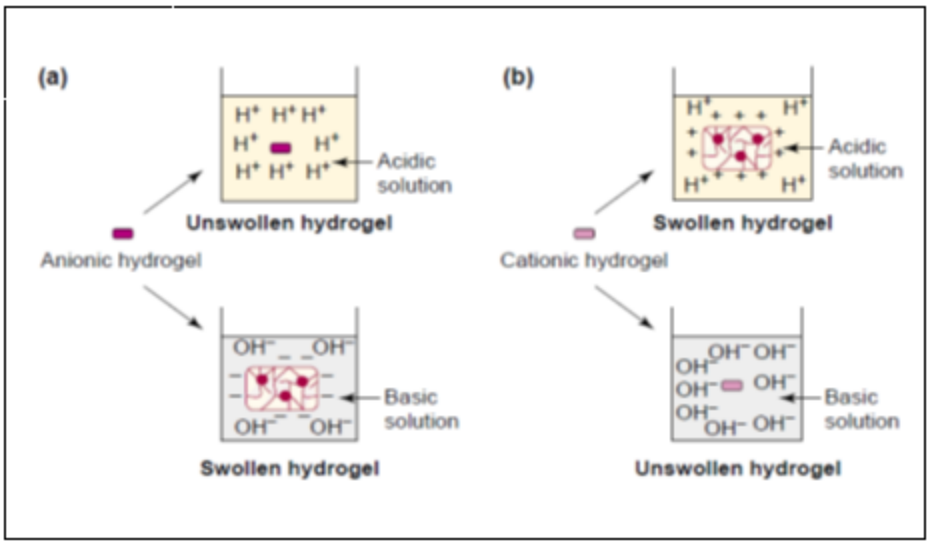


Figure 1.3. pH sensitive hydrogels

1.5.4.2. Temperature responsive hydrogels

Temperature sensitive hydrogels have swelling and de-swelling behavior according the surrounding temperature. They are categorized as positive or negative temperature responsive systems. The presence of hydrophobic group like methyl, ethyl and propyl groups is the most characteristic of temperature sensitive polymers. Typically examples of temperature sensitive hydrogels is *N*isopropylacrylamide.

Positive temperature hydrogels

In this category the critical parameter is upper critical solution temperature (UCST) which mean they de-hydrated by releasing solvent from the matrix when the temperature is below the (UCST) and swell when it is above than the (UCST). In Positive temperature hydrogel shrinking take place at low temperature due complex structure formed by hydrogen bond, increasing of temperature above the (UCST) lead those bonds to break and gel swelling. Poly (AAm-co-BMA), is an example of this type [47, 49, 76].

Negative temperature hydrogels

In this category the critical parameter is low critical solution temperature (LCST), here hydrogel will swell when temperature gets lower than the (LCST), and shrink with temperature increases above the (LCST) [47, 49, 76].

At temperatures lower than the LCST, hydrogen bonds forming due to interact between hydrophilic part and water or fluid, hydrogen bonds improve the swelling of hydrogel, those hydrogen bonds become weaker as the temperature increased above the LCST, and shrinking take place and the fluid will de swell. An example for this effect is the PVP/PNIPAAM based hydrogels.

A study performed by (Bostan, Senol et al. 2013) where a series of different composition of chitosan /PNIPA was used to control the release of 5-aminosaylcilic acid (5-ASA) the rate of releasing change with temperature, pH, and hydrogel composition this hydrogel undergoes volume phase transition with temperature due to consisting of poly (N-isopropyl acrylamide) which have LCST at 32 °C [22].

1.6. Biodegradable Polymers

1.6.1. Beta Glucan

β- glucans are polysaccharides that can be exteracted form the cell walls of bacteria, yeast, algea and fungi ,also from some plants such as barley and oat. β-glucan mainly constis of glucose chain linked with β type glycosidic bonds, it can be linear when it is bonded in (1→3)

and/or (1→4) structure and when β-(1→6) type glycosidic bonds is exist, the product is branched [77] .

Beta glucan is considered as good immunomodulator that has positive effectes on innate and adaptive immunity, and there are large number of researches which investigated the activity of beta glucan as anticancer and significant improvement was obtained [78, 79]. β- glcans has shown a good result in reducing the concentration of blood glucose therefore it was proposed and tried to be used for diabetes treatment [80].

Beta glucan was also studied to be used in many other clinical applications of beta glucan such as reduce hypertension, decrease LDL cholesterol and increase HDL, antimicrobial effects against B. subtilis and E. coli, treatment *of* allergic diseases and many other applications [77].

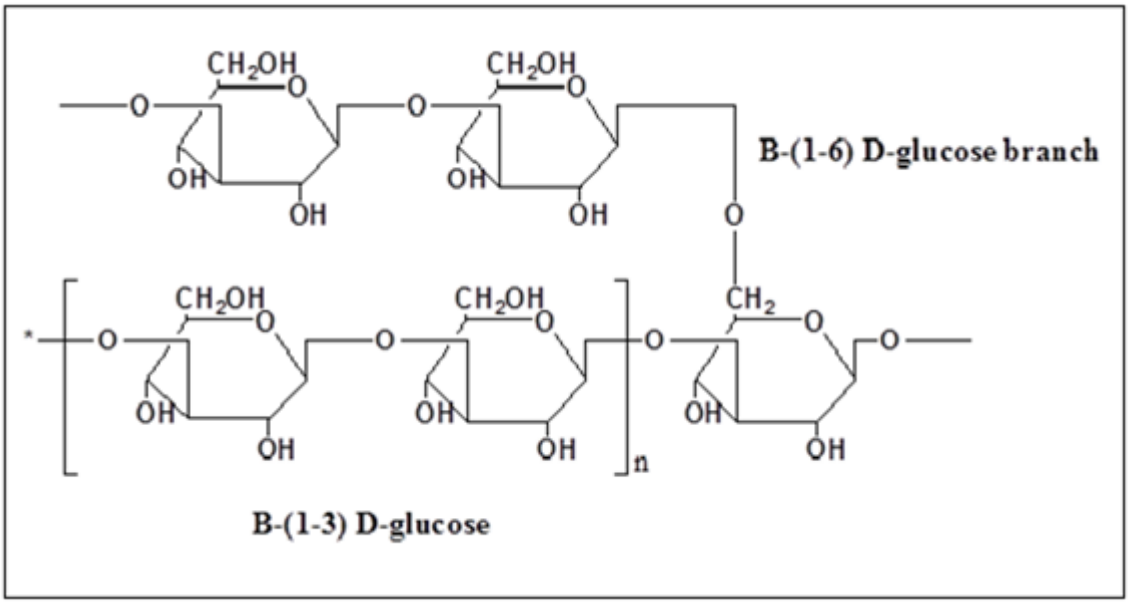


Figure 1.4. Chemical structure of β- glucan.

1.6.2. Chitosan

chitosan is a linear amino polysaccharide consisting of $\beta(1\rightarrow4)$ linked D-glucosamine residues and different number of *N*-acetyl-glucosamine groups distributed randomly in the main chain [81, 82]. Chitosan can be found naturally in some microorganisms and fungi such

as yeast [83], but it is mainly produced by a partially de-acetylated derivative of chitin (poly (*β*-(1→4)-*N*-acetyl-D-glucosamine)), which is found in arthropod exoskeletons of crustaceans such as crab and shrimp, also in fungal and yeast cell walls and some various marine living organisms. The degree of acetylation and the molecular weight of chitosan depends on the source and production method [81-84].

Furthermore, chitosan is biodegradable, biocompatible, non-cytotoxic and also has the properties of hemostatic, fungistatic, antitumor, spermicidal and anticholestermic. Due to these excellent properties; chitosan gets great attention in biomedical such as wound healing drug and delivery [22, 85].

Chitosan has polymeric cationic character due to presence of amino groups, it has good solubility in acidic media and insoluble in alkaline and neutral solutions. That means chitosan has pH sensitive properties, so it's suitable for controlled releasing drug delivery system [86]. Moreover, those amino groups make chitosan able to connect with some drugs and bio macromolecules such as protein[87] , antibiotics [88] , and DNA [89, 90].

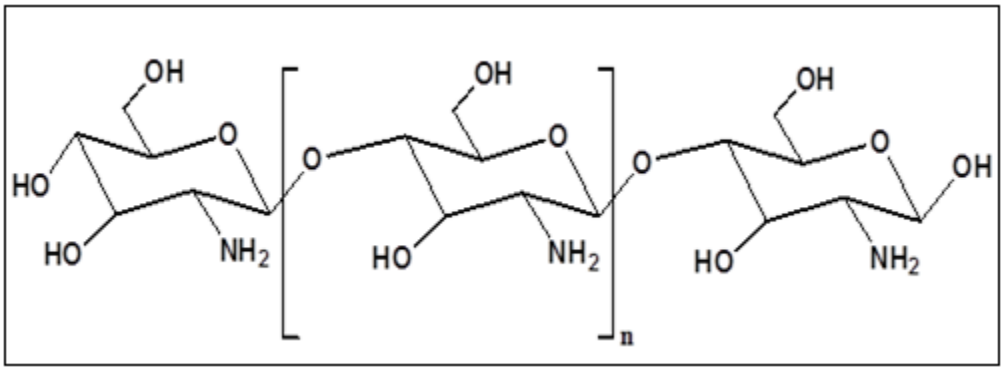


Figure 1.5. Chemical structure of chitosan

1.6.3. Hyaluronic Acid

Hyaluronic acid (HA) is an unbranched polysaccharide which consists of equal amount of D-glucuronic acid and N-acetyl-D-glucosamine, that are alternatively linked through β-1,4 and β-1,3 glycosidic bonds [91, 92] . HA is commonly found in extracellular matrix (ECM)

in large amount, and in relatively small amount in human body (synovial fluid vitreous humor of the eye and in umbilical cords), vertebrates and some in capsule of bacteria. Naturally HA is synthesized by integral membrane proteins known as hyaluronan synthases [92-94].

Due to its good chemical and biological prosperities such as solubility chemical modification ability, biodegradability biocompatibility non-cytotoxic, HA gain great attention in so many clinical applications such as tissue engineering, wound repairing and drug delivery, also HA participated in some activity such as cell proliferation, migration and differentiation. Moreover, HA can be bonded with (CD44 and RHAMM) receptors which is existed in so many cancer cells that's why HA can be used to target anticancer to specific cell [92, 93, 95, 96].

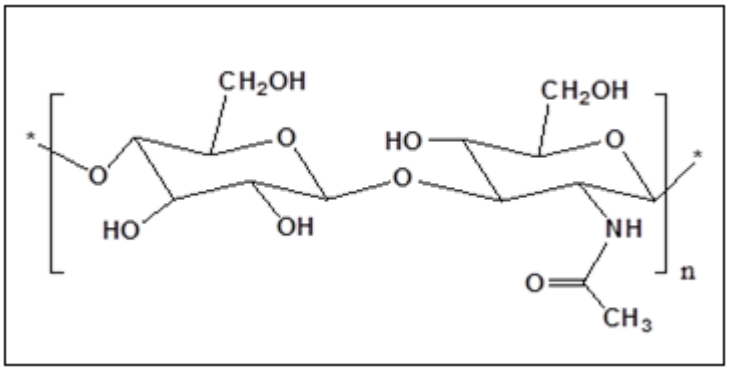


Figure 1.6. Hyaluronic acid chemical structure

1.6.4. Levan

Levan is homopolymer kind of fructans that naturally occurs consisting of repeating five-member fructofuranosyl rings connecting through β (2-6) links (Fig.1.9.a) [27, 97]. When the chain is formed by β (2-1) linkages, the product would be inulin, which is another kind of fructan (Fig.1.9.b) [23, 98].

Levan is produced by transfructosylation reaction in which levansucrase enzyme is catalyzes it and hydrolyzes sucrose and then the fructose polymerizes into levan polymer. So many microorganisms were used to produce levan e.g. Zymomonas mobilisb [99], Bacillus subtilis [31], Bacillus circulans and Erwinia amylovora [100]. A novel *Halomonas* spice AAD6

(JCM 15723) was used to produce high molecular weight liner levan and from cheap source [27].

Levan is produced by different organisms differing in their molecular weight and degree of branching. Levan from plants generally have molecular weights ranging from about 2000 to 33,000 Da. Bacterial levan are much larger than those produced by plants, with multiple branches and molecular weights ranging from 2 to 100 million Da [101].

Levan homopolymer has been attracting great attention due to its remarkable properties including non-toxicity, anti-tumor activity, less-immunological response, cell adhesion and proliferation etc, these novel properties beside water solubility, biodegradability and biocompatibility made levan used as emulsifier, stabilizer and thickener, encapsulating agent, osmoregulator, and cryoprotector in food, cosmetics, pharmaceutical, or chemical industries moreover it is also used as antitumor plasma substitute, drug activity prolongator, and radio-protector [102].

Levan has been investigated to be used in developing polysaccharide based anti-cancer agent. In this study levan has been modified chemically by periodate oxidation and this increased the amounts of aldehyde groups. This anti-cancer agent investigated in human lung, liver, and hepato cellular carcinoma [103].

Another research by (Sezer, Kazak et al. 2011) investigated the use of levan based nanocarrier system for peptide and protein drug. Bovine Serum Albumin (BSA) encapsulated levan nanoparticles were prepared and the result showed the suitability of levan by Halomonas species to be use as nanocarrier system or drug delivery such as peptides and proteins[104] .

Furthermore, encapsulation of vitamin E in levan nanoparticles has been studied; vitamin E has lower stability against oxygen during storage, heating, moisture and sun light and this made its application limited. Levan nanoparticles had been used in this field and it showed good result.

Levan derivatives such as sulphated, phosphate or acetylated levan were asserted to be anti AIDS agents. Levan was used as coating material in colonic drug delivery formulation to target the release of drug from a core into the intestine particularly the colon.

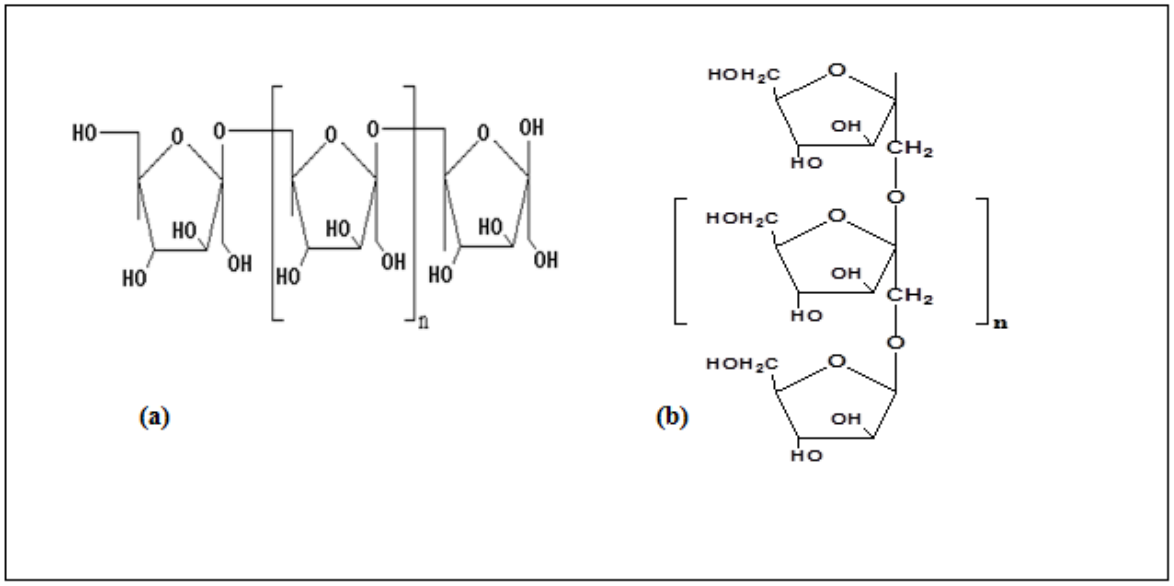


Figure 1.7. Levan & inulin chemical structure

1.7. Poly(N-isopropylacrylamide) PNIPA

PNIPA is a temperature sensitive polymer which has lower critical solution temperature (LCST) at around 32 ^{o}C. Thus, at a temperature below the LCST, pNIPAAm hydrogel absorbs water and exists in a swollen state, but it shrinks and displays an abrupt volume decrease when the environmental temperature is higher than the LCST. Generally, the main reason for this distinctive property of the pNIPAAm hydrogel has been attributed to its uniquely rapid alteration in hydrophilicity and hydrophobicity. When the hydrophilic groups in the side chains of the pNIPAAm hydrogel connect with water molecules through hydrogen bonds, these hydrogen bonds act cooperatively to form a stable hydration shell around the hydrophobic groups, which leads to the great water uptake of the pNIPAAm hydrogel at temperatures below the LCST. However, as the external temperature increases, the hydrogen bonding interactions become weakened or destroyed; thus, the hydrophobic interactions among the hydrophobic groups grow to be stronger which induces the freeing of the

entrapped water molecules from the network. When the temperature reaches or is above the LCST, the hydrophobic interactions become fully dominant, with a rapid water release from the hydrogel network [11, 13, 16]. The LCST of pNIPA can be shifted to higher or lower temperatures by copolymerization with another polymers to approach human body temperature that makes it suitable for biomedical and bioengineering application example protein-ligand recognition artificial organs, immobilization of enzyme and controlled release drug delivery system [14, 16, 105, 106].

In a research made by Cao zhang et al. 2007 copolymer of poly(*N*-isopropylacrylamide) and chitosan (pNIPAAm–CS) thermos-sensitive, gel was investigated to use for ocular drug delivery. The prepared gel had a LCST of 32 °C, which is close to the surface temperature of the eye [107].

1.8. 5-Aminosalicylic Acid (5-ASA)

It is also known as Mesalazine or Mesalamine, this compound is derived from sulphasazine and it was synthesized more than 50 years ago. It is being used for the treatment of Crohn's disease or ulcerative colitis and also for treating inflamed anus or rectum [108].

The limitations associated with 5-ASA are involved its uncontrollable nature in gastrointestinal tract and high gastrointestinal stimulation. Moreover, in oral administration of 5-ASA it is fast absorbed in the small intestine and little or no amount of 5-ASA reach the colon [109].

To avoid this drawback a different site-specific drug delivery to the colon was used such as coating with either pH sensitive polymer or bacterially degradable polymers, using pH sensitive hydrogel and drugs via bacterially degradable matrices and hydrogels [110].

2. MATERIALS AND METHODS

2.1. Materials and Equipment

2.1.1. Chemicals

Levan biopolymer was produced at Marmara University Department of Bioengineering as described elsewhere (Poli, Kazak et al. 2009, Poli, Nicolaus et al. 2013). Sodium nitrite ($NaNO_2$) (Cat# 7632-00-0), potassium peroxodisulfate ($K_2S_2O_8$) (CAS 7727-21-1) and sodium hydroxide (NaOH) (CAS# 1310-73-2) were supplied from JT Baker. Acetic acid (CAS# 64-19-7) from Sigma-Aldrich. Sodium carbonate (Na_2CO_3) (Cat# 497-19-8) was supplied from Riedel-deHaen. Methacrylic anhydride (CAS# 760-93-0) and N-isopropylacrylamide (NIPA, %97 purity) (CAS# 2210-25-5) purchased from Aldrich. Mono chloro acetic acid (CAS# 79-11-8) was kindly provided by Ak-kim. N,N,N',N' tetramethlyethylenediamine (TEMED, %99 purity) (CAS# 110-18-9) was obtained from Analyticals Carlo-Erba. 5-Amino salicylic acid (5-ASA, %95 purity) (89-57-6) was supplied from Alfa Aesar. All chemicals were used as received. Dialysis tubing benzoyated (CAS# D2272-10FT) with a molecular weight cutoff of 2000 Da dialysis membrane from Sigma Aldrich was used for purification of samples. All used solvents were HPLC grade and obtained from Fisher Scientific or Sigma-Aldrich.

2.1.2. Laboratory equipment

2.1.2.1. Gel permeation chromatography (GPC)

Number average (Mn), weight average (Mw) molecular weights and polydispersity index (PDI) of levan before and after acid hydrolysis were determined by GPC system, equipped with Perkin-Elmer 200 GPC high pressure pump, injector (50 µl), ultrahydrogel columns connected in series (Ultrahydrogel 250, Ultrahydrogel 500, Ultrahydrogel 1000, Ultrahydrogel 2000 and Ultrahydrogel Guard Column), Wyatt Optilab differential refractive index detector (RI) at 654 nm, and Dawn Heleos multi angle light-scattering (LS) detector. The mobile phase was 0.1 M NaNO3 solution in 2%cetic acid water mixture with a flow rate

of 1.0 mL/min. Measurements were conducted at 25 °C. Polymer concentrations were in the range of 0.5−1.0 mg/mL and all the samples were filtred through the 0.2 μm filter prior to use.

2.1.2.2. Nuclear magnetic resonance spectroscopy (NMR)

Structural characterization of hydrolysed levan and the degree of methacrylation were performed by 1HNMR spectroscopy using varian 600 MHz NMR at 25 ^{o}C in D2O as solvent. Proton chemical shifts were recorded in ppm downfield from tetramethyl silane (TMS) as standard.

2.1.2.3. Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectra of hydrolyzed, carboxymethylated, methacrylated levan and the copolymer samples were recorded using Thermo Nicolet 6700 FT-IR spectrophotometer equipped with a Smart Orbit high performance diamond attenuated total reflectance (ATR) accessories in the frequency range of 4000 cm−1 to 400 cm−1 in transmission mode at room temperature.

2.1.2.4. Thermal analysis

Thermal gravimetric analysis (TGA) of the copoymer samples were performed by a Seiko-EXSTAR-TG/DTA7300 model thermal analyzer. Measurements were conducted at 10 ^{o}C/min heating rate under dynamic nitrogen atmosphere (gas flow rate was 20 ml/min). Approximately 7.0 mg of the sample was used in each experiment. To eliminate the absorbed water, the samples were preheated to 110 ^{o}C under measurement conditions before recording the TGA curves. Weight calibration of the TGA was performed against to weight of known standard samples and the temperature calibration was conducted according to the indium melting point and melting enthalpy.

Differential scanning calorimetry (DSC) measurments were performed using Perkin Elmer Jade type differential scanning calorimeter under dynamic nitrogen (20 ml/min) at 10 ^{o}C/min heating rate. Measurements were performed using 5-7 mg of the samples. The phase

transition temperatures of levan-NIPA hydrogels were determined in swollen state in PBs by derivative DSC (DDSC) and the data were evaluated using Pyris TA software. Temperature and enthalpy calibration of DSC was conducted according to the melting point and melting enthalpy of indium.

2.1.2.5. UV spectrophotometer

Drug release from the hydrogels were followed by a Perkin Elmer Lambda 35 UV-Vis Spectrophotometer in the frequency interval of 200–400 nm. Measurements were performed at 25 ºC in different time intervals with 1 ml of samples which were returned after measurement. The release profiles of 5-ASA from the hydrogels were determined by using previously prepared calibration curve.

2.2. Synthesis of Hydrogels

2.2.1. Preparation of low molecular weight levan by acid hydrolysis

3 g of levan was dissolved in 150 ml of 2% acetic acid/water solution and stirred for 24 hours. 700 mg of sodium nitrite ($NaNO_2$) was separately dissolved in 5 ml of water and added to this solution dropwise. The mixture was stirred at 60 °C for 24 hours, followed by additional stirring at room temperature for two days. After neutralization with 1M Na_2CO_3 solution, it was dialyzed against distilled water for two days using dialysis tubing (benzoylated cut of: 2 kDa) and freeze-dried. Molecular weight of hydrolyzed levan was determined by GPC and spectroscopic characterization (FTIR and 1H NMR) was conducted to determine if any structural change after acid hydrolysis took place.

2.2.2. Synthesis of carboxymethylated levan (CM-levan)

Carboxymethylation of levan was previously reported by Wang, yu et al. [111]. Briefly, to a suspension of 2 g of hydrolyzed levan in 4.3 ml of ethanol/water solution (80%, v/v), 0.63 g of sodium hydroxide was added and the solution was continuously stirred for 30 min at room temperature. A separately prepared solution of 4.0 g monochloroacetic acid (MCA) in 4.3 mL of ethanol/water mixture (80%, v/v) was gradually added. After heating to 45 ºC,

0.63 g of sodium hydroxide was dissolved in 4.3 mL of ethanol/water (80%, v/v) and gradually added to this solution within 1 hour. The reaction mixture was allowed to additional stirring for 0.5 hour at 45 ^{o}C, and then cooled to room temperature and neutralized with glacial acetic acid. The reaction product was precipitated in ethanol, filtered and dried under vacuum at 40 ^{o}C (Figure 2.1). It was characterized by FTIR.

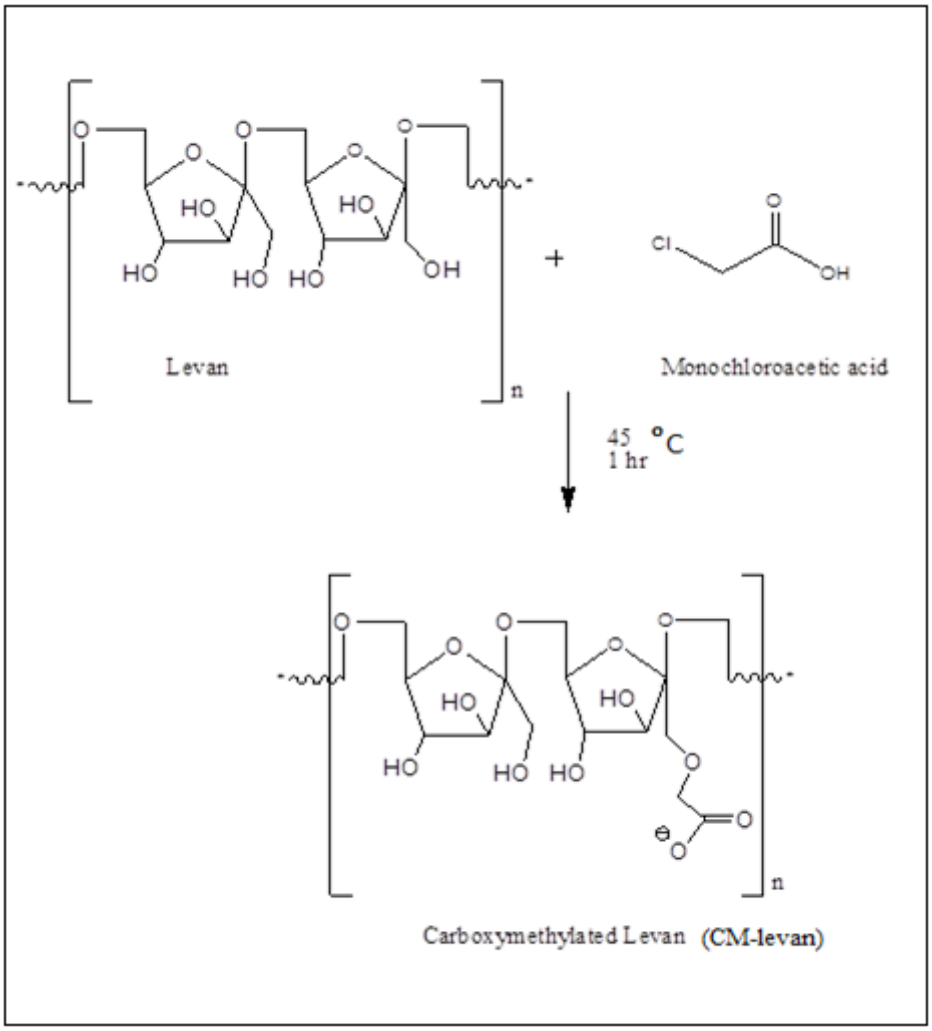


Figure 2.1 Synthesis of carboxymethylated levan (CM-levan)

2.2.3. Synthesis of methacrylated levan (MA-levan)

Methacrylated levan (MA-levan) was synthesized according to the method described by (Burdick, Chung et al. 2005) [112]. Briefly, a solution of 1 g carboxymethylated levan in 5

ml deionized water was prepared and pH was adjusted to 8.0 with 5.0 N NaOH. To this solution, 1ml of methacrylic anhydride was slowly added and the reaction was allowed to stir for 24 hours in an ice bath in dark. The product was neutralized and purified with a dialysis membrane (MW cutoff 2 kDa) using deionized water for 24 h in dark. The purified product was freeze dried. The conversion was determined by 1H-NMR spectroscopy (Figure 2.2).

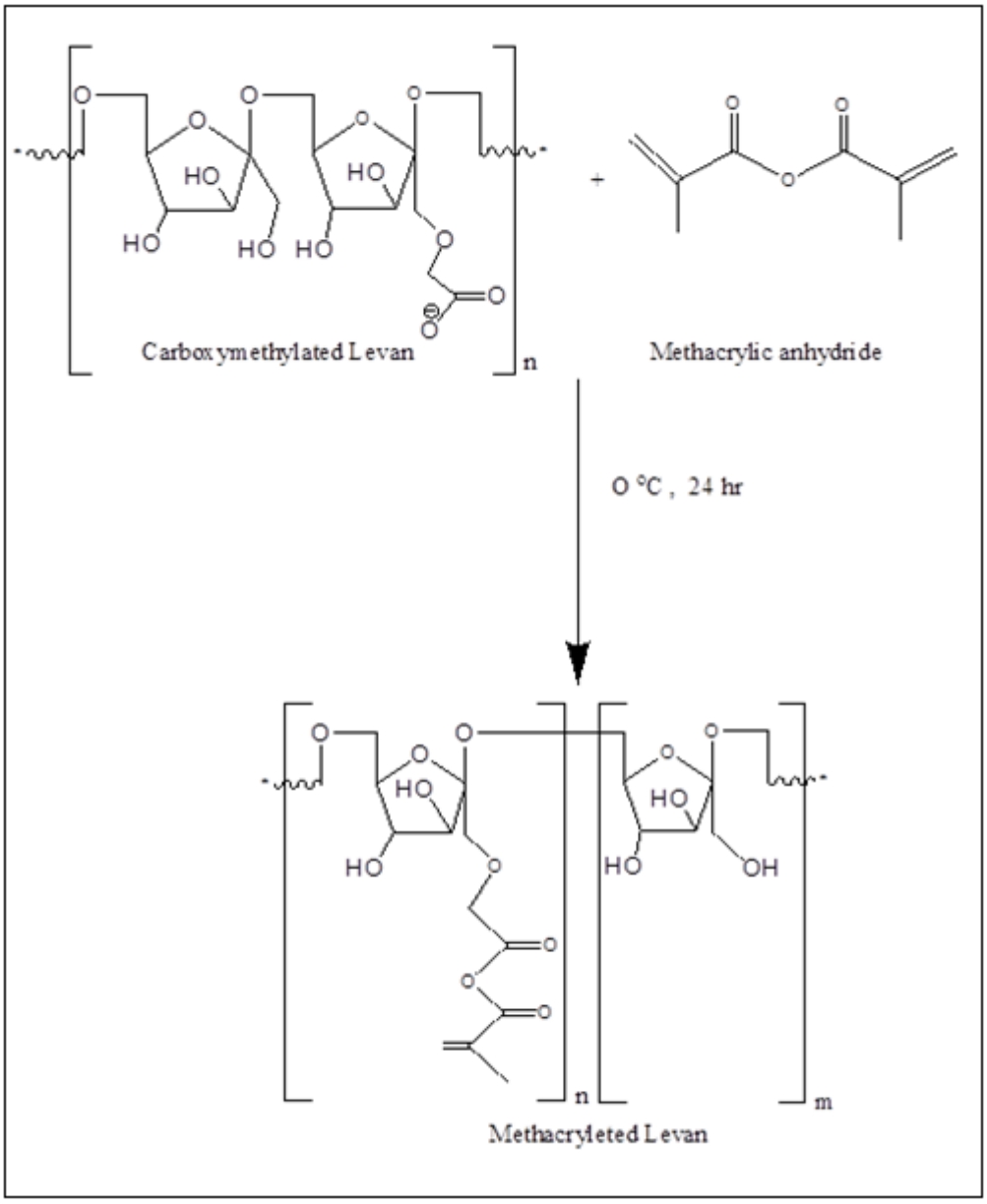


Figure 2.2. Synthesis of methacrylated Levan

2.2.4. Synthesis of Levan-PNIPA hydrogels

Levan-pNIPA hydrogels at 4 different compositions (90, 80, 70, 60 NIPA wt%) were prepared by redox polymerization according to the method described by Bostan, Senol et al. 2013 [22]. Briefly, for composition 1 in Table 2.1, a solution of 0.1g of MA-levan in 1.5 ml deionized water was placed in a 20 ml reaction tube, to which, 0.9 g of NIPA and 0.5 ml of KPS solutions (0.05M in water) were added. The viscose solution was purged with Ar for 20 minute to remove dissolved oxygen and then 0.75 ml TEMED solution (0.5 M in water) was added. After 2 minutes, while purging with Ar, the solution was transferred to a well- sealed plastic straw having a length of 10 cm and diameter of 5 mm. The sample was kept at room temperature until reaction was complete (Figure 2.3). After the hydrogel was purified by consecutive swelling at 20 ^{o}C and collapsing at 40 ^{o}C, it was dried at 40 ^{o}C under vacuum. The hydrogel compositions are collected in Table 2.1.

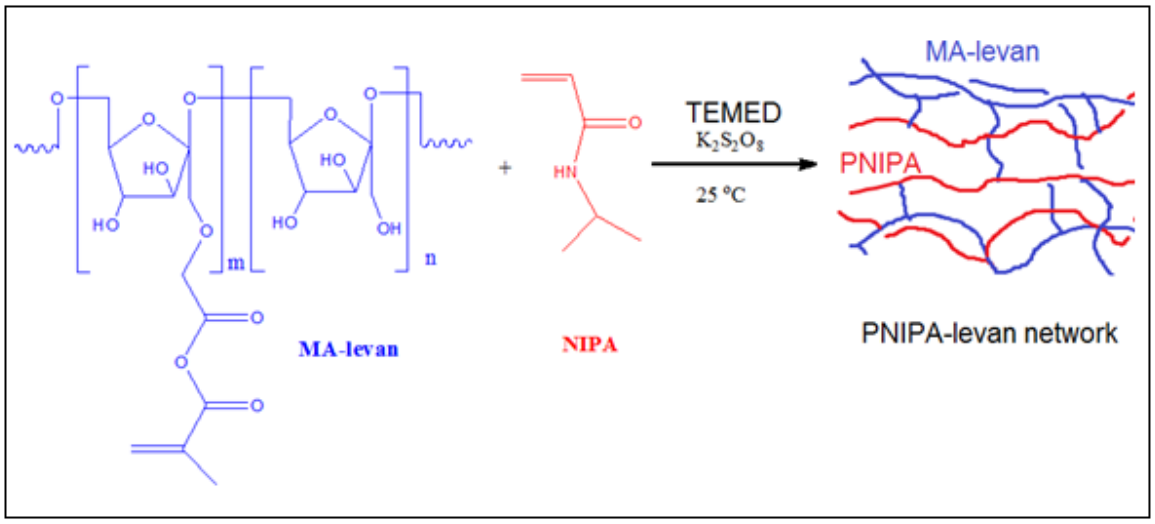


Figure 2.3. Synthesis of pNIPA-levan hydrogels.

Table 2.1 Hydrogel compositions used in the synthesis.

Hydrogel	NIPA (mg)	MA-levan(mg)	NIPA(%)	MA-levan(%)
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 behavior of the hydrogel

Well dried hydrogel samples were weighted and immersed in phosphate buffer saline (pH 7.4) at four different temperatures namely at 25 ^{o}C, 30 ^{o}C, 35 ^{o}C and 40 ^{o}C. The swollen samples were removed from the buffer at different time intervals. They were quickly blotted with a filter paper, weighted and returned to the solution immediately. This procedure was repeated for each temperature until equilibrium swelling was reached The swelling ratio (Q) of the hydrogels were calculated gravimetrically according to the equation (2.1) [113]:

$$Q = {}^{W_2}/_{W_1} \quad \text{(eq.2.1)}$$

Where,

Q is swelling degree of the hydrogel, W_1 and W_2 are weights of the dry and swollen samples, respectively.

2.2.6. Drug loading and releasing studies

To determine the drug loading, about 0.2 g of dry hydrogel was added into a solution of 21 mg of 5-ASA in 30 ml of phosphate buffer solution (pH 7.4) at 25 ^{o}C. After reaching to the equilibrium swelling, the hydrogel was removed and quickly blotted with a filter paper and weighted. The equilibrium swelling and weight percentage of absorbed 5-ASA were determined gravimetrically according to the equation (2.2) [22, 114]

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

Where, W_2 and W_1 are weights of the swollen and dry gels, respectively, 0.7 is 5-ASA solution concentration mg/ml, and 1000 is its density in mg/ml

5-ASA is a UV active molecule which has a specific absorption peak at 330 nm. Therefore, the release kinetics of 5-ASA from the hydrogels in phosphate buffer saline (PBS) at 37 ^{o}C was followed by UV–Vis spectrophotometer. Each measurement was performed with 1.0 ml of sample from the PBS solution at different intervals. The samples were returned to the solution after each measurement to eliminate possible errors that may result from volume change.

2.2.7. Biocompatibility assay of the hydrogels

L929 fibroblasts cultured in DMEM supplemented with 10% FCS (Biochrom AG), 50 U mL^{-1} penicillin and 50 mg mL^{-1} streptomycin (Gibco). Every 2-3 days, cells were split (1:3) and incubated in a 5% CO_2 humid atmosphere at 37 °C. Hydrogel samples were cut into thin cylinders with a diameter of 0.5 cm^2 and 1mm height. Before cell seeding, samples were placed into 24-well tissue culture plates and sterilized for 1 h UV-exposure, followed by soaking into 1% penicillin-streptomycin solution for overnight. After stabilization in culture medium, cell seeding onto hydrogel samples were performed with a ratio of $2x10^4$ cells in per well. Cytotoxicity of hydrogel samples were determined with WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3- benzenedisulfonate) cell proliferation and viability assay (Roche Applied Science, Germany). WST-1 reagent was added directly into culture and incubated for 2 h in 5% CO_2 humidity at 37 ^{o}C, as recommended by the manufacturer. The absorbance was measured with a GloMax Multi+Microplate Multimode Reader (Promega, USA) at 450 nm. Experiment was performed for 24, 48 and 72 h. The cells on tissue culture plate were determined as control group that were considered 100% viable. Experiments were performed triplicates. Statistical analyses were performed by one-way ANOVA and followed by TTest. Data were presented as means ±SEM with p-value that was

less than 0.05 and was considered statistically significant. Biocompatibility of hydrogels was investigated with L929 fibroblasts for 24, 48 and 72 h.

3. RESULTS AND DISCUSSION

3.1. Hydrolyzed Levan Characterization

3.1.1. Molecular weight determination

Molecular weight and molecular weight distribution of native and hydrolyzed levan were determined by GPC-LS system. In this system, a solution of 0.1 M $NaNO_3$ and 2% acetic acid in ultrapure water was used as mobile phase. The specific refractive index (dn/dc) of hydrolyzed levan was determined using differential refractometer to be 0.1370±0.0028 mLg^{-1}. (See appendix A for dn/dc concentrations, peaks and peak times). Similarly, molecular weight of native and hydrolyzed levan were determined as $6.23x10^6$ g/mol and $2.56x10^5$ g/mol, respectively. A remarkable decrease in molecular weight with increasing solubility were observed after hydrolysis (Table 3.1). The weight average molecular weight, Mw, number average molecular weight, Mn, and polydispersity index (PDI=Mw/Mn) values are collected in Table 3.1, and their GPC-LS chromatograms are given in Figure 3.1(a and b.)

Table 3.1. Molar mass moments (g/mol) of original and reduced levan samples

	Mn	Mw	Mw/Mn
Native levan	$4.247x10^{+6}$ (3%)	$6.231\ x10^{+6}$ (3%)	1.467
Hydrolyzed levan	$1.051\ x10^{+5}$ (2%)	$2.564\ x10^{+5}$ (1%)	2.439

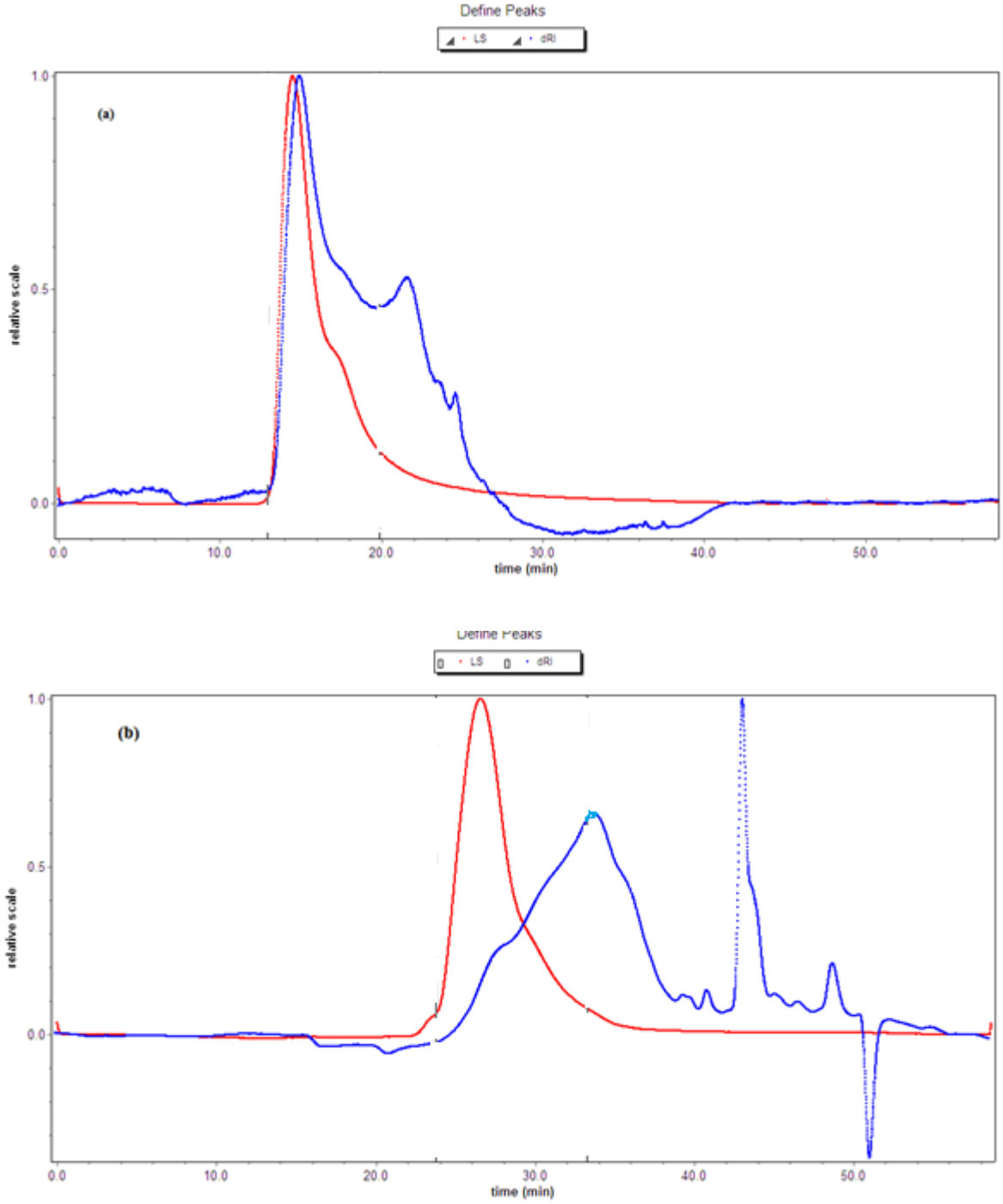


Figure 3.1. GPC-chromatograms of original levan (a) and hydrolyzed levan. (b)

3.1.2. FT-IR spectroscopy

Fourier Transform-Infrared Spectroscopy (FT-IR) is an important analytical technique, which is widely used for quantitative and qualitative analysis of organic materials and identifying the progress of chemical reactions. This technique is mainly based on measuring the absorption intensities of functional groups of a material as a function of wavelength within infrared region.

As shown in Figure 3.2.a, a broad band in the range of 3500-3200 cm^{-1} is due to the –OH stretching absorption of native levan. The characteristics bands observed at 950, 1000 and 1100 cm^{-1} are due to the -C-O-C- symmetric bending vibration of fructose ring and glycosidic linkage [27, 115]. It is notable that the FTIR spectra of native and hydrolyzed levan (Figure 3.2.b) are almost the same. Comparison of these spectra indicated that (Figure 3.2.), nitrose acid hydrolysis did not result in any structural change while reducing the molecular weight of native levan. This was further confirmed by ^{1}H NMR and 2-D ^{13}C-^{1}H NMR characterization of hydrolyzed levan. The characteristic ^{1}H-NMR peaks of native and hydrolyzed levan are comparable shown in Figure (3.3.a) and Figure (3.3.b). H1(3.54), H6(3.76), H4(3.96), H3(4.05) and H5(3.81) are characteristic ^{1}H-NMR peaks (ppm) of native levan. The same peaks with the same integration and ppm values were observed for hydrolyzed levan, which is a further proof that the hydrolysis process did not result in any structural change in the chemical structure. For further characterization of hydrolyzed levan, its 2-D ^{13}C-^{1}H NMR correlation spectrum was recorded. ^{1}H NMR peaks and their corresponding ^{13}C-NMR peaks of hydrolyzed levan (ppm): C1(59.8), C6(63.3), C4(75.0), C3(76.2), C5(80.2) and C2(104) are observed in Figure 3.4. These spectroscopic results are considered as evidences that hydrolysis process did not result in any structural change in native levan.

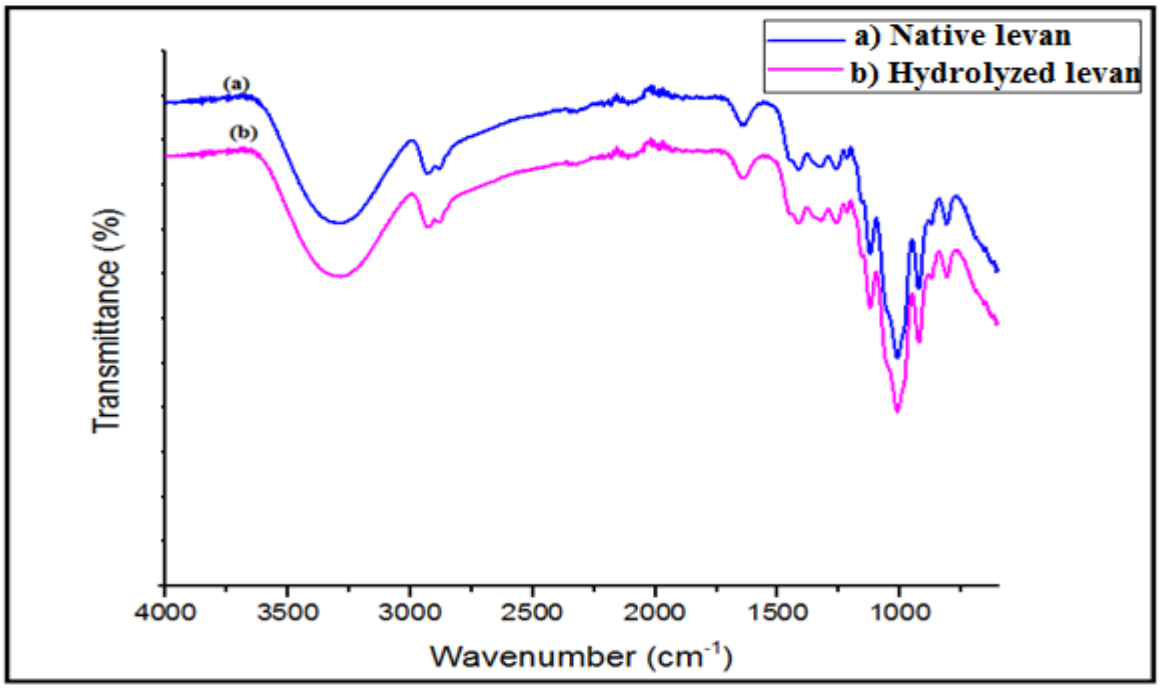


Figure 3.2. FTIR spectra of native levan (a) and hydrolyzed levan (b).

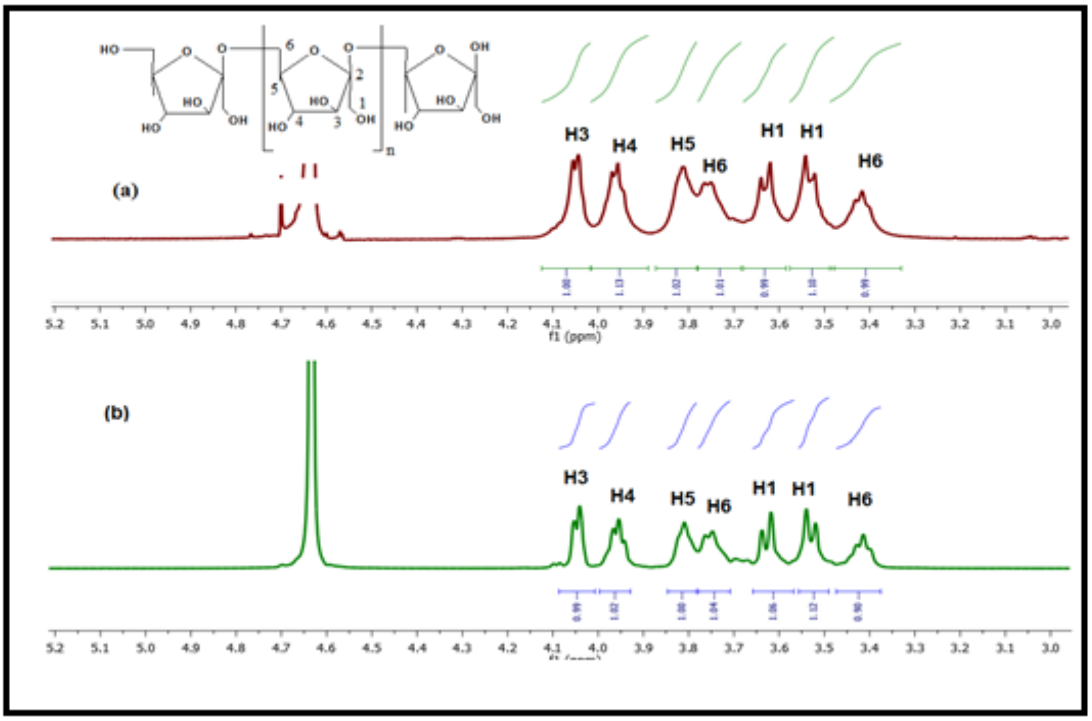


Figure 3.3. 1H NMR spectra of native (a) and hydrolyzed (b) levan

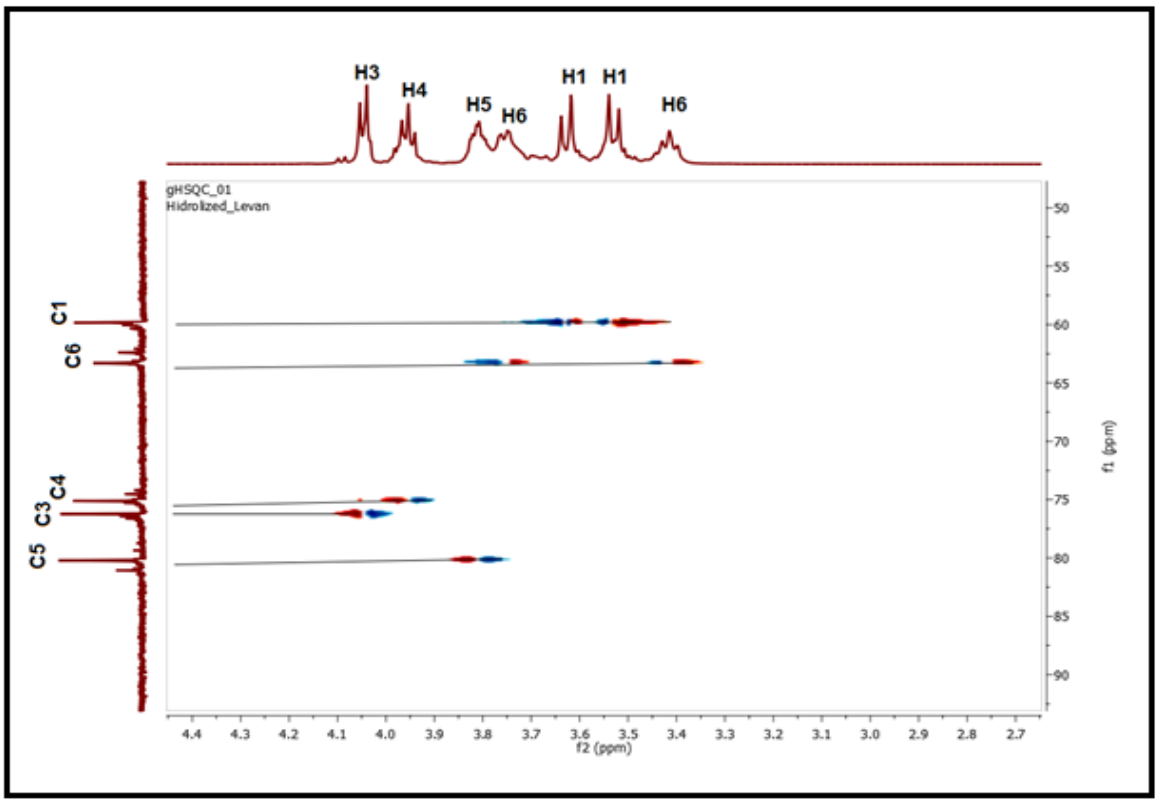


Figure 3.4. 2-D ^{13}C-^{1}H NMR correlation spectrum of hydrolyzed levan.

3.2. Characterization of Carboxymethylated Levan (CM-levan)

Before methacrylation, levan was carboxymethylated according to the reaction given in Figure 2.1. Carboxymethyl groups ($–CH_2COO^-$) are more nucleophile than hydroxymethyl groups ($–CH_2OH$) of levan [116, 117]. Therefore, to increase the yield of methacrylation reaction, hydrolyzed levan was firstly carboxymethylated. The progress of the reaction was confirmed by FTIR spectroscopy. Figure 3.4 shows the FTIR spectra of hydrolyzed levan and CM-levan). The appearance of a new bands at about 1712 cm^{-1} and 1590 cm^{-1} can be assigned to the stretching vibration absorption of C=O groups in the form of –COOH and $–COO^-$, respectively. This spectrum can be considered as a proof of successful carboxymethylation of hydrolyzed levan. This spectrum can be considered as a proof of

successful carboxymethylation of hydrolyzed levan.13C NMR of the carboxymetlated levan shown in figure 3.6.

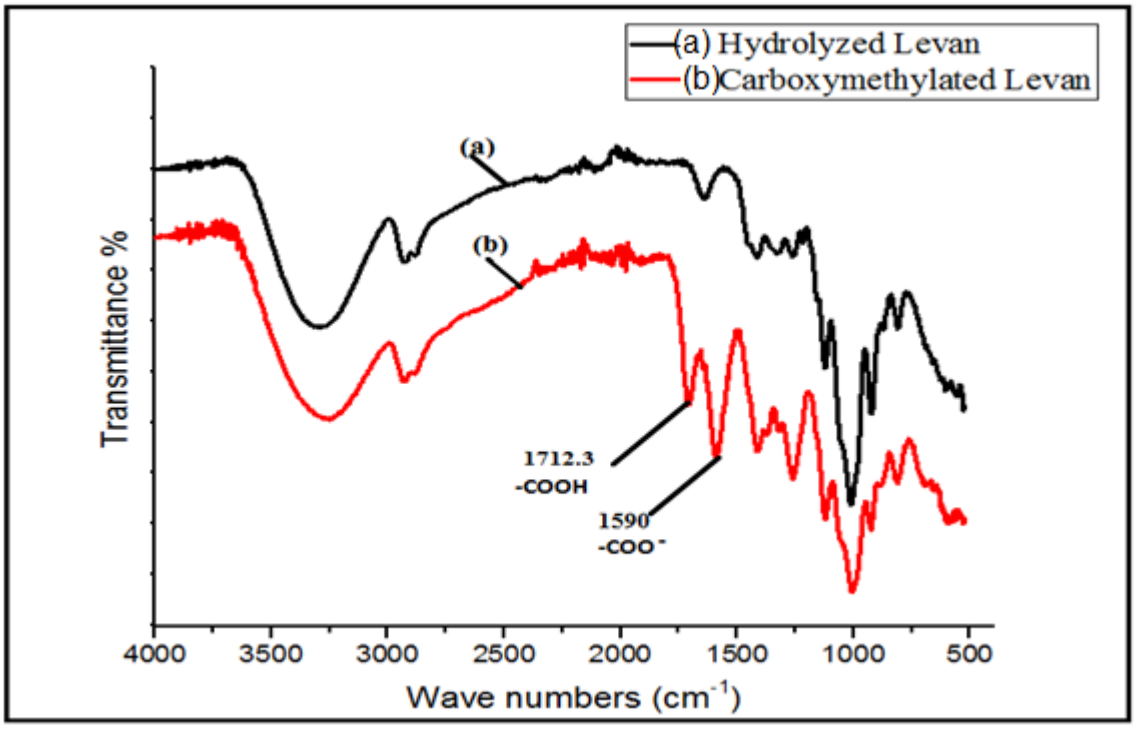


Figure 3.5. FTIR spectrum of hydrolyzed (a)and carboxymethlyated levan (b).

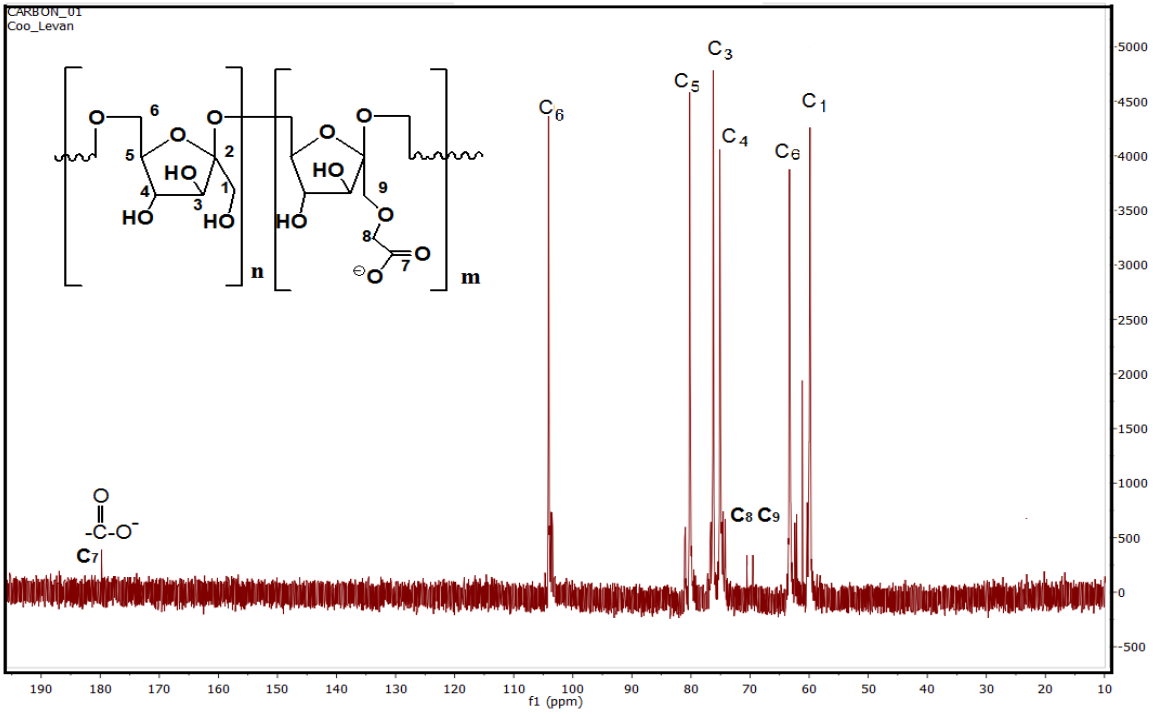


Figure 3.6. 13-C NMR spectra of carboxymethylated levan (CM-levan).

3.3. Characterization of Methacrylated Levan (MA-levan)

Methacrylation of CM-levan was performed at 0 ^{o}C and pH of 8 for 24 hr. According to the reaction mechanism given in Figure 2.2, methacrylate groups are expected to join to more nucleophilic carboxymethyl groups rather than less nucleophile –OH groups of the fructan rings. The product was characterized by FTIR and the degree of methacrylation was determined by 1H-NMR spectroscopy. For comparison, FTIR spectra of carboxymethylated and methacrylated levan are given in Figure 3.7.a and Figure 3.7.b, in which a new absorption band appeared at 1641 cm^{-1} can be attributed to the -C=C- stretching vibration absorption of methacrylate groups. The peak observed at 1712 cm^{-1} in Figure 3.5.b was shifted to higher wavenumber (1720 cm^{-1}), which is an evidence of the conversion of the sodium carboxylates ($–COO^-$ groups of CMC-levan) to their corresponding esters after the methacrylation (–COO– groups). In the meantime, the absorption intensity of $–COO^-$ groups of CM-levan decreased and shifted to lower wavenumber (1556 cm^{-1}). These spectral changes can be considered as proof of the successful methacrylation of CM-levan. The conversion was

quantitatively determined by recording the 1H-NMR spectrum of the product, which was depicted in Figure 3.8. The peaks appeared at 5.55 ppm and 5.20 ppm are attributed to CH_2=C– protons of methacrylate groups. The peaks at 3.55 ppm, 3.60 ppm (d, H1), 4.05 ppm (d, H3), 3.95 ppm (d, H4), 3.80 ppm (m, H5), 3.45 ppm and 3.75 ppm (d, H6) are due to the fructose units of levan [118, 119]. The methacrylation reaction was confirmed by the new peaks appeared at 5.20 ppm and 5.55 ppm in Figure 3.7.[22] The degree of methacrylation was calculated to be 19% from the integration of the peaks at 5.55 ppm and 3.45 ppm. It is worth noting that 19% of the fructose rings were methacrylated, which were used as crosslinking site in the hydrogel network.

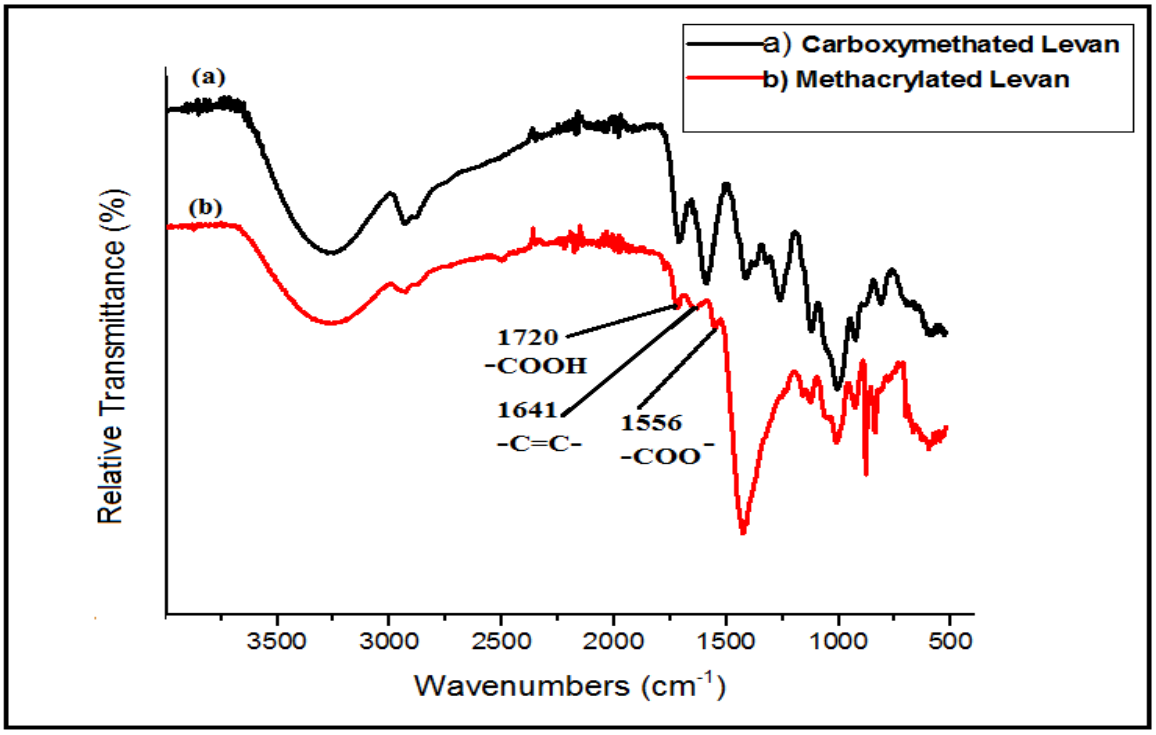


Figure 3.7. FTIR spectrum of carboxymethlyated levan (a) and methacrylated levan (b).

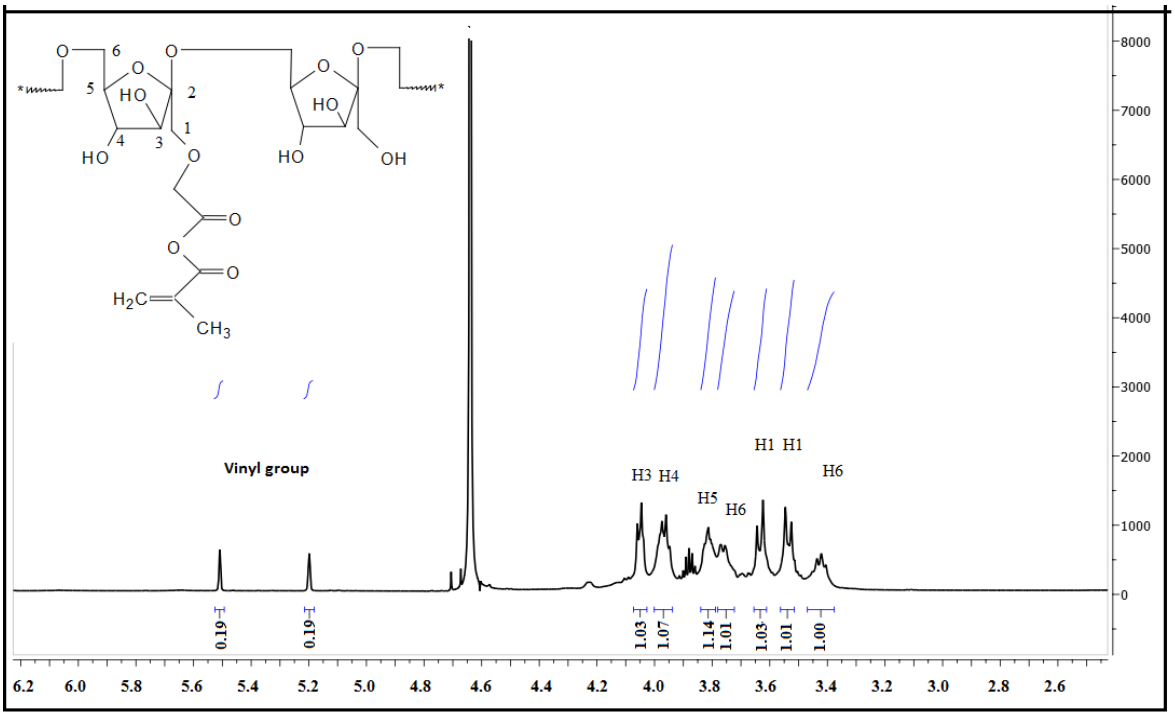


Figure 3.8. NMR spectrum of methacrylated levan

3.4. Hydrogel Characterization

3.4.1 FTIR characterization

FTIR spectroscopy was used to confirm the formation of the levan-pNIPA hydrogels. In FTIR spectrum of pNIPA (Figure 3.9. a), three characteristic absorption peaks at 1633 cm^{-1} (C=O amide I), 1531 cm^{-1} (N-H bending) and 2100 cm^{-1}(N-H stretching) were observed. Similarly, in FTIR spectrum of MA-levan, a broad absorption peak at 3254 cm^{-1} is due to the –OH stretching of fructose ring of levan. Additionally, C=O stretching absorption in ester form (–COO–) was observed at 1720 cm^{-1}, C-H bending was observed at 1423cm^{-1}, and the peaks in the range of 1000-1200 cm^{-1} were attributed to the –C-O-C- etheric bending absorption of the fructan ring of levan. In FTIR spectrum of HG4, the presence of the aforementioned characteristic peaks of MA-levan and pNIPA are proof of the formation of levan-pNIPA hydrogels. The FTIR spectra of Gel-1, Gel-2, Gel-3 and Gel-4 samples are collectively given in Figure 3.10.

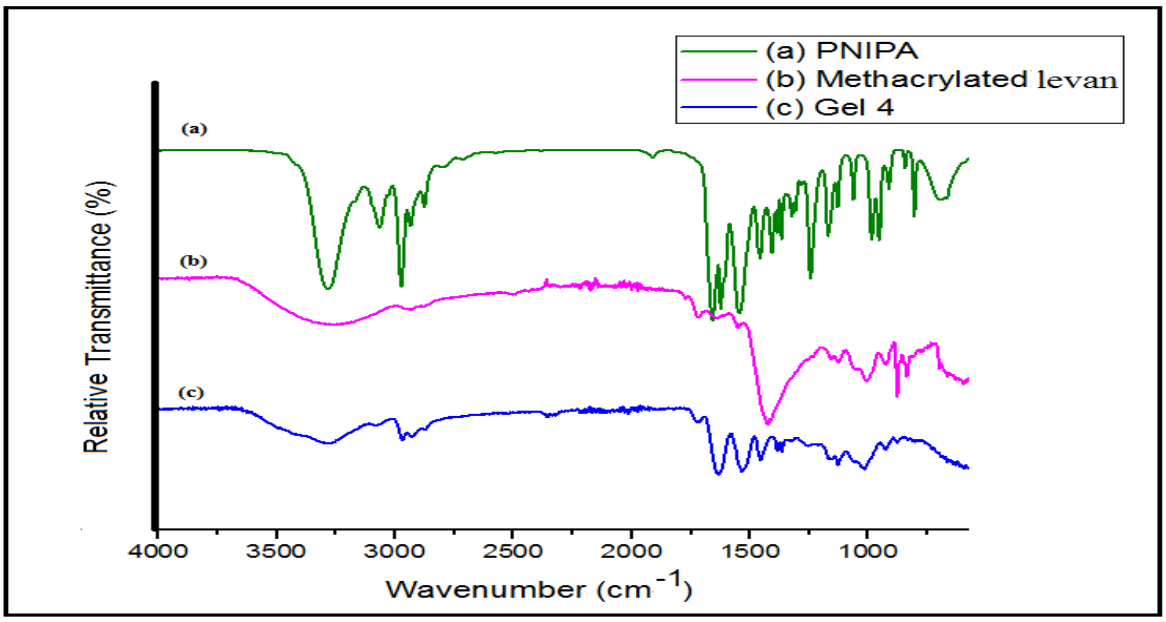


Figure 3.9. FTIR of Gel-4, MA-levan and pNIPA

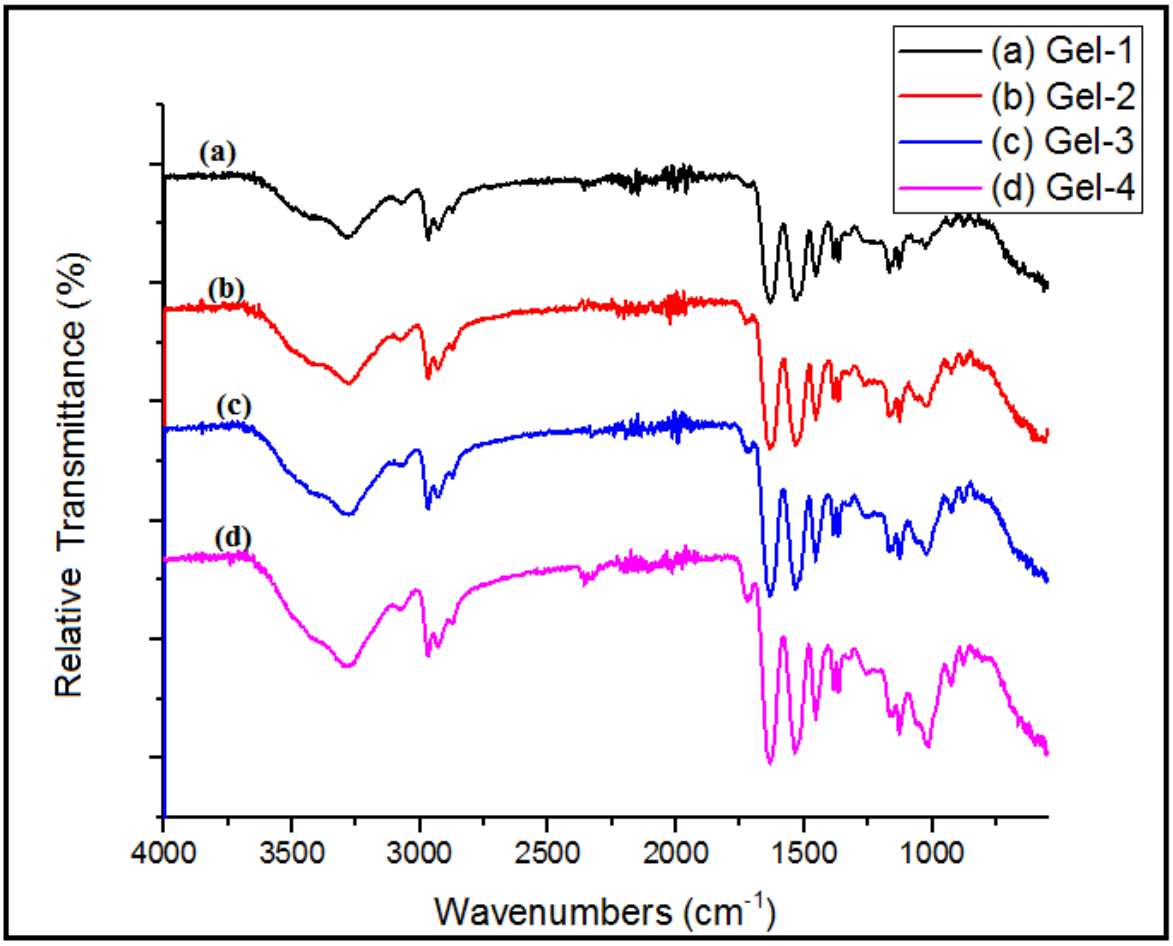


Figure 3.10 FTIR spectra of levan- pNIPA hydrogels; a) Gel-1, b) Gel-2, c) Gel-3and d) Gel-4

3.4.2. Swelling behaviors of the hydrogels

Hydrogels are three dimensional polymeric networks of water soluble polymers, which are able to absorb large amount of water. The advanced development of hydrogels has led to the improvement of sustainable and controllable delivery of water soluble therapeutic agents under stimulus conditions such as temperature, pH, electricity and magnetism etc. Many of the stimuli responsive hydrogels are made of synthetic hydrophilic polymers, which are non-biodegradable and cause local inflammation on use. Thus, co-polymeric hydrogels of stimuli responsive polymers, having less toxic and biodegradable nature, which are susceptible to enzymatic and/or hydrolytic degradation, provided effective and safe controlled delivery of

specific therapeutic agents. PNIPA is a well-known temperature sensitive polymer, which has a vast potential for medical applications [120]. Although pNIPA has a VPTT close to body temperature, it is a synthetic polymer and mostly cross-linked with bis-acryamide (BAA), which is relatively toxic and causes possible irritation. To overcome these drawbacks by gaining biodegradability, pNIPA was crosslinked with MA-levan, and the hydrogels synthesized were characterized and used for the controlled delivery of 5-ASA.

Equilibrium swelling ratio and swelling kinetics of a hydrogel are characteristics parameters of controlled drug delivery systems. Thus, the change of these parameters with hydrogel composition and temperature is important. In the co-polymeric hydrogels, containing components with distinct swelling characters, these parameters can be tuned by changing the compositions and environmental stimuli signal. Therefore, swelling degree and swelling rate of the hydrogels, below and over the VPTT, namely 25 ^{o}C and 30 ^{o}C, respectively, 35 ^{o}C and 40 ^{o}C were examined. The swelling profiles in PBS (pH 7.4) are shown in Figure 3.11. Remarkable differences in equilibrium swelling ratio and swelling rate of the hydrogels, depending on the composition and temperature, were noticed. Equilibrium swelling degree of the hydrogels at 25 ^{o}C, which is far below the VPTT, was observed to be higher than that of 30 ^{o}C, 35 ^{o}C and 40 ^{o}C. This is due to good solubility of pNIPA and levan at this temperature. When the swelling behavior of the hydrogels at 25 ^{o}C were compared (Figure 3.11.a), the highest equilibrium swelling degree was observed with Gel-1 (Q=5.5). This is the result of the lowest levan content used as cross-linker. Lowest amount of cross-linker let to the lowest cross-linking density and thus highest swelling ratio. Below the VPTT (at 25 and 30 ^{o}C), while the equilibrium swelling ratio of the hydrogels increased with increasing pNIPA content, above the VPTT, this was decreased with increasing content of pNIPA. This is due to the insolubility of pNIPA and good solubility of levan over the VPTT. It means that over the VPTT only levan contributes to the swelling.

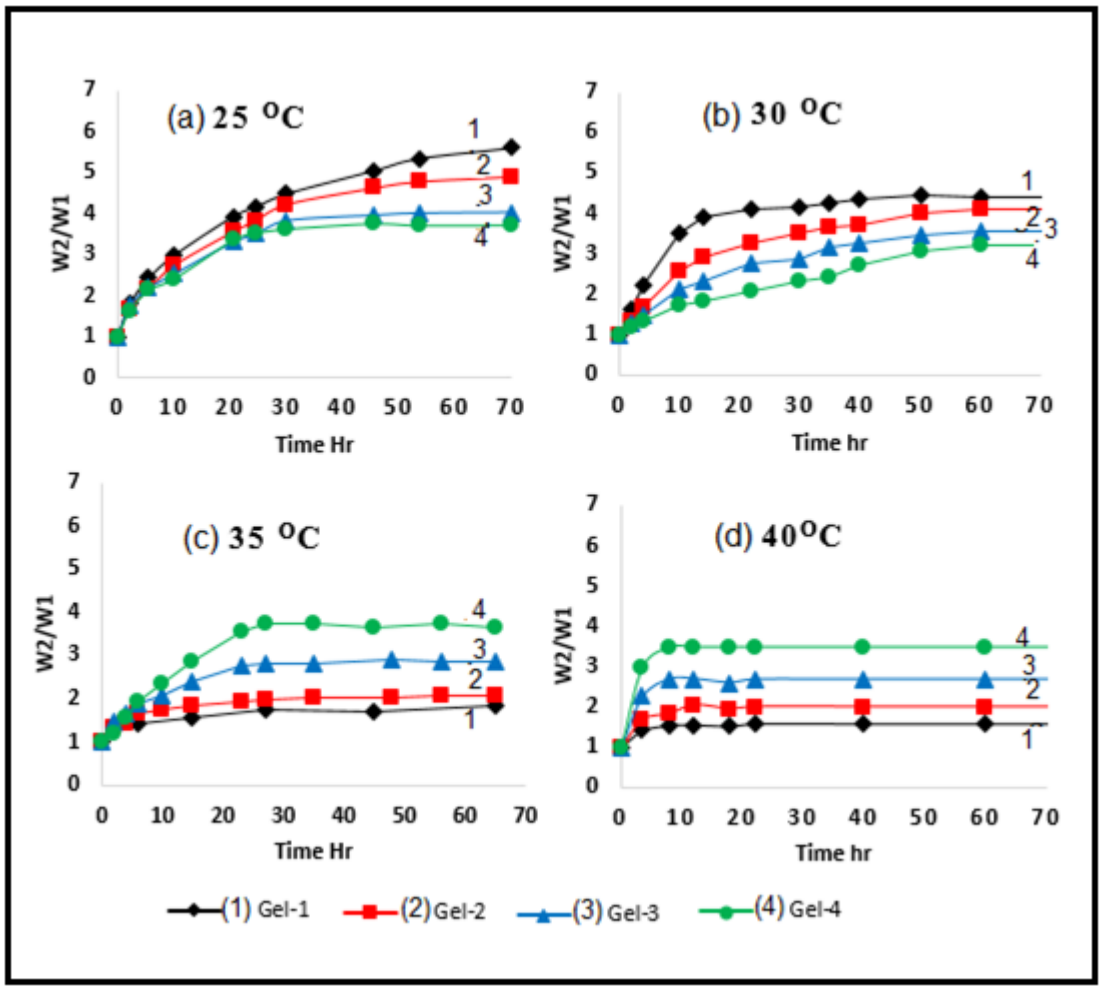


Figure 3.11. The swelling behaviors at pH 7.4 in PBS buffer solution at 25 °C (a), 30 °C (b), 35 °C (c) and 40 °C (d).

3.4.3. Volume Phase Transition Temperature (VPTT)

Differential scanning calorimetry (DSC) is a well-known thermo-analytical technique which is effectively used to determine thermal transition behavior of materials as a function of time and temperature. In DSC thermogram, the observed peak is either exothermic or endothermic. Exothermic processes are accompanied by heat release such as crystallization, oxidation, hydrogen bonding formation etc. and endothermic process are accompanied by heat absorption processes such as melting, boiling, thermal decomposition, protein denaturation, helix-coil transition of DNA, dehydration etc. In the DSC thermograms, peak

area and temperature values provided us with useful information for the structural evaluation of the materials. PNIPA is a temperature sensitive polymer and has a lower critical solution temperature (LCST) of nearly 32 ^{o}C, which is close to body temperature. In this thesis, we may use the term volume phase transiton temperature (VPTT) for the hydrogels instead of LCST since hydrogels are not able to dissolve, they are only swellable in water. VPTT of pNIPA hydrogels may vary depending on molecular weight and crosslink density. VPTT of pNIPA hydrogel in PBS was determined to be 32.8 ^{o}C (Figure 3.12). Under VPTT, N-isopropyl amine groups of pNIPA easily makes reversible hydrogen bonding with surrounding water and this provides enhanced solubility. Over the VPTT, i.e. body temperature, due to the endothermic cleavage of the hydrogen bonds, pNIPA slowly loses its solubility resulting in simultaneous phase separation and systemic release of drug. Thus, in this study, pNIPA was used as temperature sensitive component of the hydrogels and it may be interesting to determine sensitively the change in VPTT of pNIPA-levan copolymeric hydrogels with their increasing levan content. VPTT of pNIPA-levan hydrogels were sensitively determined by DDSC. Figure 3.12 shows the DDSC curves of PNIPA and the hydrogels in PBS solution.

As shown in Figure 3.12, while the VPTT of pNIPA was 32.8 ^{o}C, it increased from 33.6 ^{o}C to 35.1 ^{o}C with the increase of levan content in the hydrogels. This was attributed to the increasing number of hydrogen bonds formed between –OH groups of levan and pNIPA as well as increasing crosslinking density so that methacrylated levan was also used as cross-linker. It is notable that the VPTT of the hydrogels can be tuned by changing their levan/pNIPA ratios.

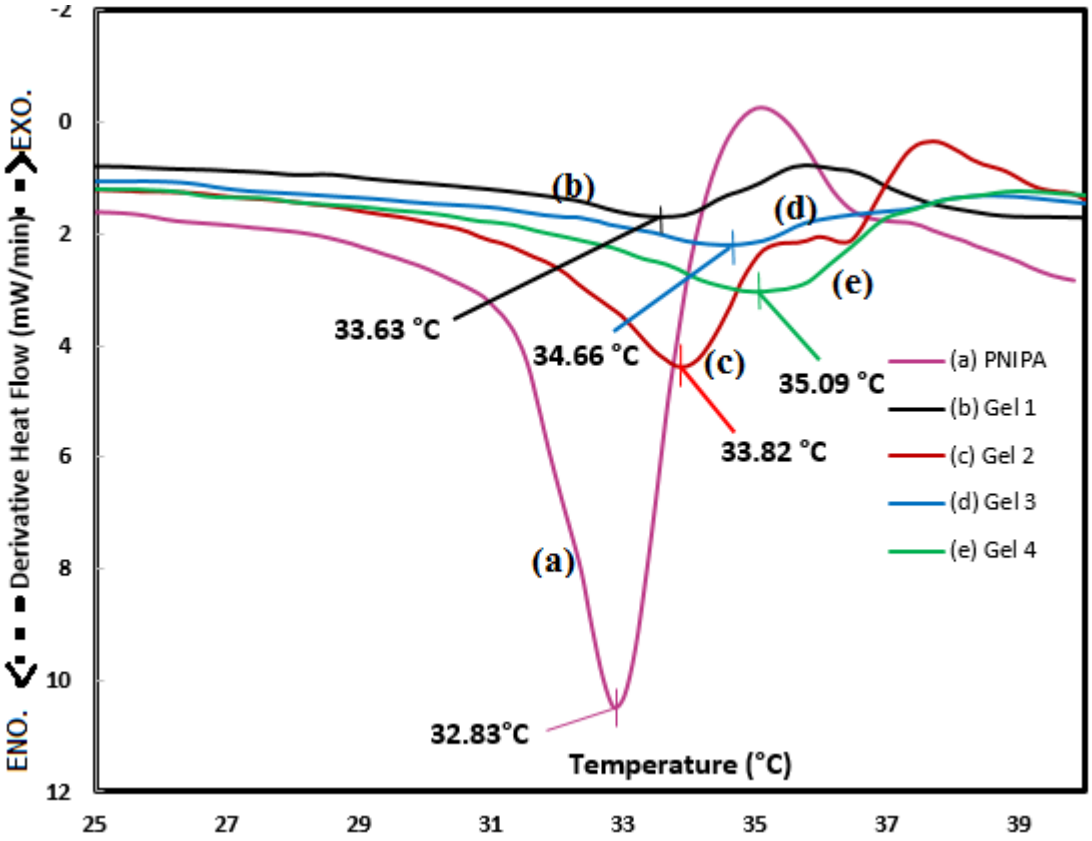


Figure 3.12. DDSC curves of pNIPA (a) and swollen hydrogels; Gel1 (b); Gel2 (c); Gel3 (d) and Gel4 (e) in PBS buffer (pH 7.4).)

3.4.4. Thermal gravimetric analysis (TGA)

Thermal gravimetric analysis is a fast and accurate technique regarding thermal stability and composition of polymers, polymer blends and hydrogels. If the components of a hydrogel have distinguishable thermal stability, the composition can easily and safely be determined in a short time. In this study, TGA was used to determine final composition of pNIPA-levan hydrogel since pNIPA and levan have quite different thermal behaviors. Figure 3.13 shows the TG curves of pNIPA and levan. In TG curve of levan, the first step weight-loss corresponding to nearly 6.5%, between 100 ºC and 170 ºC, is due to the elimination of the free and hydrated water present in levan. The second and main decomposition step corresponding to 36.5% weight-loss was observed between 207 ºC and 220 ºC. Thermal decomposition of pNIPA starts and ends at much higher temperatures than levan (starts at

370 ^{o}C and ends at 430 ^{o}C). Thus the compositional analysis of the dry hydrogels using TGA is possible. TG curves of the hydrogels are collectively shown in Figure 3.14. In these curves the weight-loss steps of levan and pNIPA can easily be observed. First weight-loss step is due to the first step decomposition of levan, which corresponds to 36.5% weight-loss of levan portion of the hydrogels. Thus, the levan content of the hydrogels can easily be calculated from the first step weight-loss values. The calculated compositions of the hydrogels determined by TGA are collected in Table 3.2. The composition of the hydrogels before and after the synthesis, which was determined from TG curves, are quite different since some linear parts of levan and pNIPA were extracted during the hydrogel purification. Each weight-loss step, shown in Figure 3.14, can clearly be observed from their derivative TG (DTG) curves. The area under the DTG curves and their peak temperatures are direct quantitative measure of the rate of decomposition and thus, thermal stability of the components in the hydrogel network structure. Comparison of the peak temperatures of DTG curves and their changes with composition allowed us to obtain information on how compositional change effected the thermal stability. As shown in Figure 3.15, maximum weight-loss rate temperature corresponding to the first stage weight-loss of levan shifted to higher temperature with increasing pNIPA portion of the hydrogels. While the first stage decomposition temperature of GEL-4 was observed at about 235 ^{o}C, this temperature shifted to 254 ^{o}C with increasing pNIPA, indicating that the more intermolecular interaction was obtained for each levan chain with increasing the pNIPA portion. The same tendency was observed with increased amount of pNIPA in the hydrogel composition as well. The second DTG peak corresponding to the pNIPA decomposition increased with pNIPA in the hydrogel compositions.

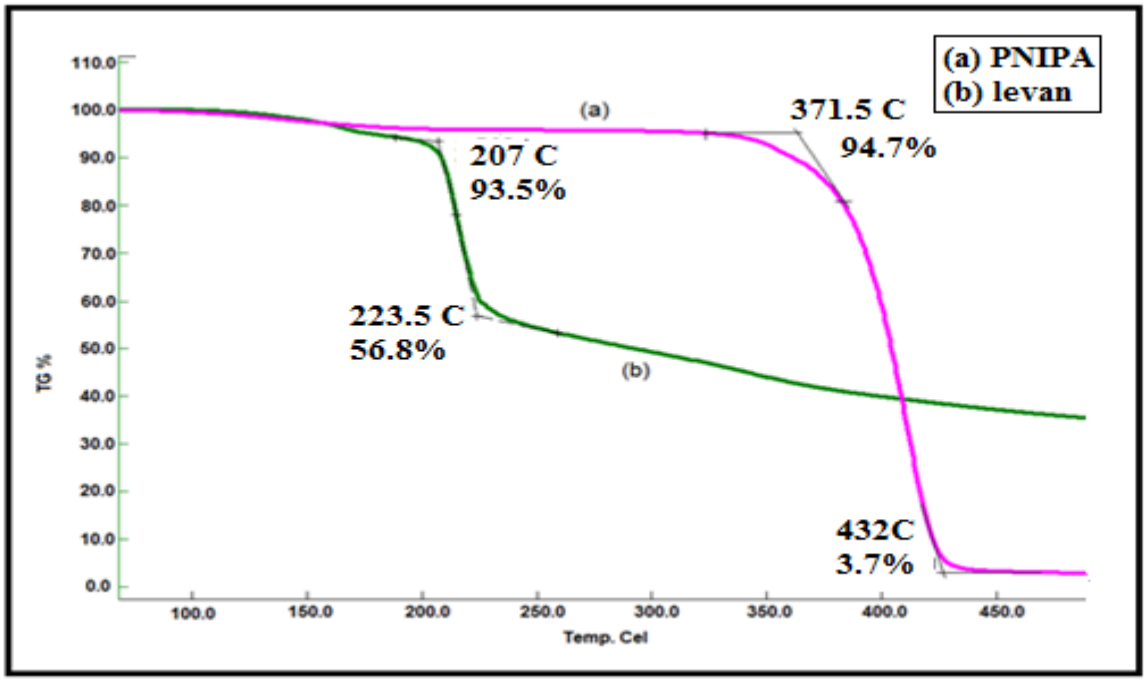


Figure 3.13. TGA curve of pure pNIPA (a) and pure levan (b).

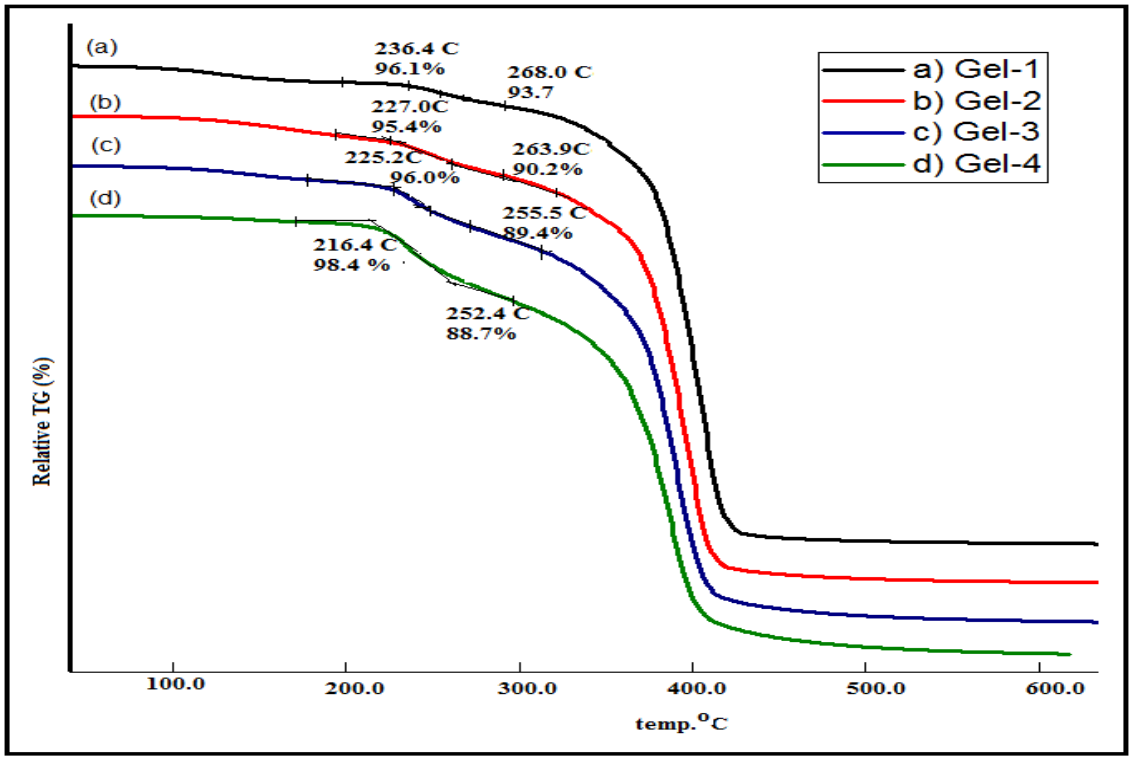


Figure 3.14. TG curves of the hydrogels.

Table 3.2. Hydrogel compositions used in the synthesis and determined from TGA

Hydrogel	Levan wt% In synthesis	Levan wt % from TGA	pNIPA wt% from TGA
Gel-1	10	6.6	93.4
Gel-2	20	14.2	85.8
Gel-3	30	18.1	81.9
Gel-4	40	26.5	73.5

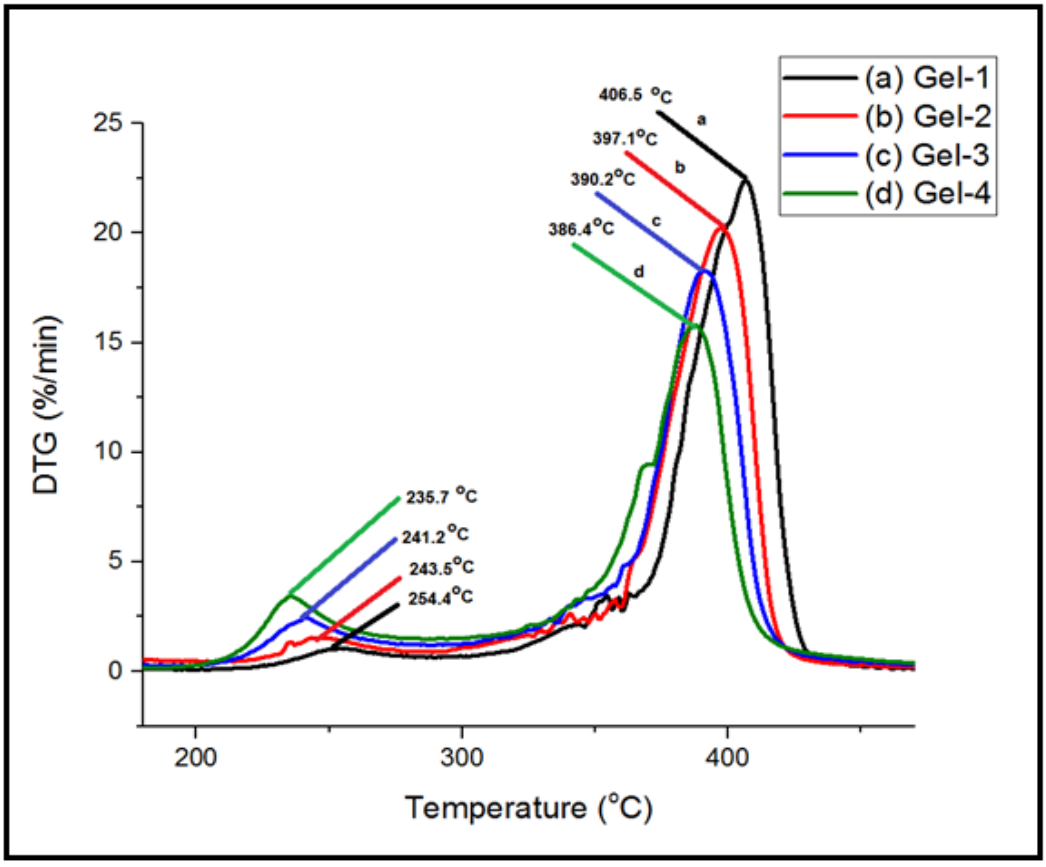


Figure 3.15. DTG curves of the hydrogels.

3.5. Drug Loading and Release Studies

5-ASA loading of the hydrogels were calculated from the equilibrium swelling ratio of the hydrogels in drug solution at known concentration. Drug loading mainly depends on the equilibrium swelling degree of the hydrogels and temperature. Lower cross-link density and higher swelling ratio lead to higher drug loading capacity. By considering this, drug loading into the hydrogels was performed at 25 ^{o}C, at which the maximum swelling was observed for the hydrogels. The highest loading capacity was observed with Gel-1 which has highest swelling ratio at 25 ^{o}C. The equilibrium swelling data were evaluated according to the Eq. 2.2 and the calculated amount of the absorbed drug in the hydrogels are collected in Table 3.3.

The 5-ASA release from the hydrogels were followed by UV-VIS spectroscopy at 37 ^{o}C by measuring the specific absorption peaks of 5-ASA at 330 nm. The corresponding concentration values of the peak intensities were determined from the calibration curve and plotted against time. Figure 3.16 shows that, at 37 ^{o}C, which is over the VPTT of pNIPA, almost 70% of 5-ASA was released within one hour from the all hydrogels. However, remarkable differences in release of remaining portion of 5-ASA from the hydrogels were noticed. As shown in Figure 3.16 that while, nearly 100% of 5-ASA was released from Gel-1 within 6 hours, the remaining 5-ASA portion released in longer time period from the other hydrogels. The release rate decreased with increasing levan in the hydrogel compositions. This is due to the pNIPA portion of the hydrogels. Over the VPTT of the hydrogels, with increasing pNIPA content, more quick response was observed. It is worth noting that the release profile of 5-ASA can be tuned by changing temperature and composition of the hydrogels. In a previous study, 5-ASA release from the temperature and pH responsive hydrogels composed of chitosan and pNIPA was investigated at pH 8 and 37 ^{o}C. It was observed that, 5-ASA was released in high extent within 4 hours from the hydrogel containing 85 % of pNIPA [22]. In this study, 5-ASA was released within 6 hours in a great extent from the levan-pNIPA hydrogels containing 90% of pNIPA at the same temperature and pH. The higher release rate observed in the previous study was due to the presence of pH responsive chitosan into the hydrogel which made an additional pH contribution to the

collapse of the hydrogel. In another study, pNIPA-calcium alginate semi-interpenetrating networks were prepared for pH and temperature sensitive release of indomethacin at different pHs and temperatures. The release rate of the drug was significantly affected with percentage of pNIPA in hydrogel at 37^{o}C in PBS (pH 7.4). Nearly 95 % of the drug was released within 5 hours [122]. In addition, in the research by Zhang, wu et al. 2004 [123], a temperature sensitive inter penetrating network of pNIPA was prepared through redox polymerization of NIPA using methylenebisacrylamide (MBAAm) as cross-linker. The release of bovine serum albumin from the hydrogel was studied at 37 C and in PBS (7.4 pH) and maximum was 36% within 4 days.

Table 3.3. Hydrogel compositions and their 5-ASA contents.

Hydrogel	pNIPA (wt%) In synthesis	pNIPA wt% from GA	W_1 (mg)	W_2 (mg)	Q W2/W1	5-ASA mg/dry gel mg	5-ASA (wt %)
Gel-1	90	93.4	165.1	1013	6.14	0.003595	0.359
Gel-2	80	85.8	185.7	935.1	5.04	0.002825	0.282
Gel-3	70	81.9	160.3	681	4.25	0.002274	0.227
Gel-4	60	73.5	190.3	724.2	3.80	0.001964	0.196

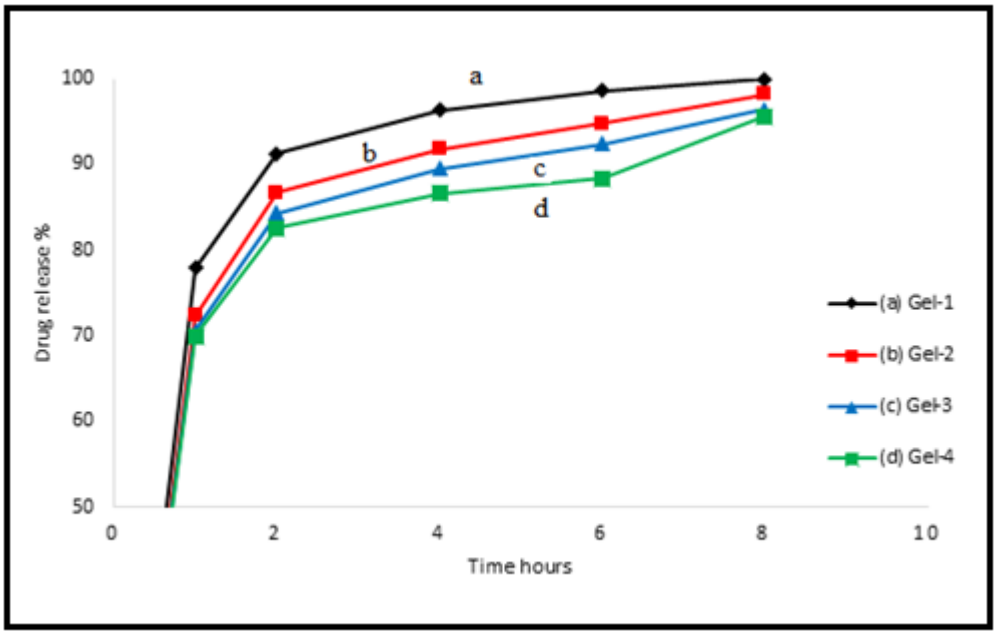


Figure 3.16. 5-ASA releasing profile from the hydrogels at 37^{o}C in PBS buffer (7.4 pH).

3.6. *In vitro* Biocompatibility

In order to determine and compare the biocompatibility of pNIPA-levan hydrogels with pNIPA (without levan) hydrogel, they were tested against mouse fibroblast L929 cells. Before cell viability assay, cell cultures were incubated for 2 h in 5% CO_2 humidity at 37 ^{o}C and directly contacted with the hydrogels in PBS (pH=7.4) for different time intervals and then subjected to WST-assay. Figure 3.15 shows the comparative cell viability against levan-pNIPA and pNIPA hydrogels at 24, 48 and 72 hours. Cell viability increased with increasing levan in the hydrogels indicating the more biocompatibility of levan compared to pNIPA. This result is consistent with the previous results. Bostan, Mutlu etal. (2014) reported that 13% of levan in polyethylene oxide/Chitosan/levan blend films significantly increased the biocompatibility [115] . The composition of the polymeric material surface has a dynamic nature. In an aqueous media, due to the segmental mobility, more soluble segments are oriented to the interface[102]

As it was noticed in swelling experiments, pNIPA parts of the hydrogels are collapsed at 37 ^{o}C which is over the VPTT of pNIPA. It is worth nothing that, while the localized and reversible collapsing of pNIPA was observed with time at 37 ^{o}C, the solubility of levan inside

the hydrogels increased at this temperature. This led to more mobility of levan segments of the hydrogels, enhancement at interface between hydrogel and L929 cells and thus more biocompatible hydrogel surface was observed.

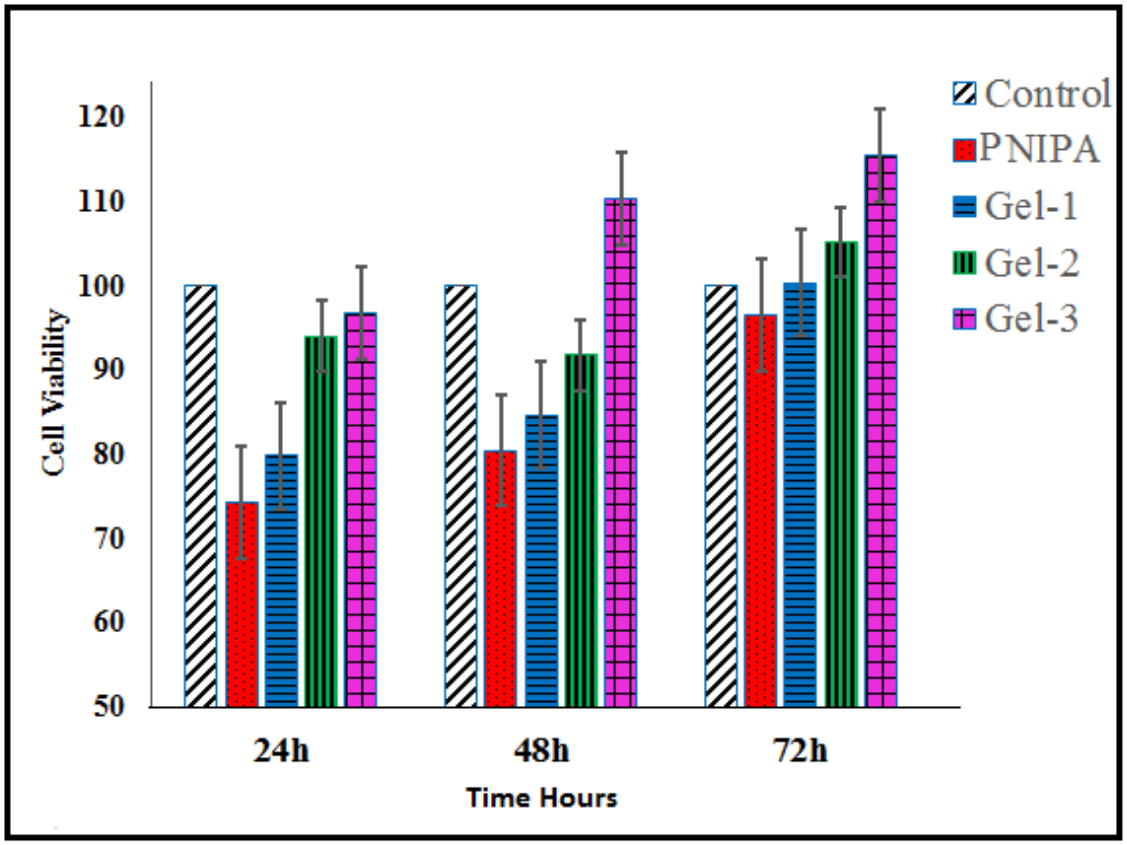


Figure 3.17. Cell viability of L929 cells after 24,48 and 72 hours cultured with the hydrogels. (Gel-1, Gel-2 and Gel-3) and pure pNIPA gel.

4. CONCLUSION AND RECOMMENDATIONS

Novel biodegradable and temperature responsive hydrogels of levan and pNIPA at different levan/NIPA ratios were prepared for controlled release of 5-ASA. For this purpose, initially, low molecular weight, water soluble levan was prepared. The product was carboxymethylated and later vinylated to use as biodegradable cross-linker to prepare levan-pNIPA hydrogels. The temperature and composition dependent swelling profiles of the hydrogels were determined above and below the VPTT in PBS (pH=7.4). Equilibrium swelling degrees observed above the VPTT were lower than that of the values observed below the VPTT. Above the VPTT, these values were decreased with increasing pNIPA in the hydrogels. Considering these results, drug loading of the hydrogels was performed at 25 ^{o}C. The release profiles of 5-ASA in PBS at 37 ^{o}C were determined for the hydrogels, as well. Nearly 100 % of the drug from the gel-1 was released within 6 hours. The release rate of 5-ASA decreased with decreasing pNIPA in the hydrogel compositions at 37 ^{o}C. It is notable that, the release rate can be tuned by changing the hydrogel composition. VPTTs of the hydrogels were sensitively determined in PBS (pH=7.4) and which increased from 32.8 ^{o}C to 35.09 ^{o}C with increasing levan in the hydrogels. It is notable that the VPTT of the hydrogels can be tuned by changing the levan/pNIPA ratio. The biocompatibility of the hydrogels was tested against L929 fibroblasts at 35 ^{o}C for 24, 48 and 72 hours and the results showed that the biocompatibility of the hydrogels increased with levan in the hydrogels. It is worth noting that as a result of insolubility of pNIPA part at this temperature, the increasing of levan in the hydrogels resulted in its enhancement at the interface.

In the light of this study, several recommendations can be given for the further works on the pNIPA-levan hydrogels. In addition to the temperature sensitivity, pH sensitivity may be given to the levan-pNIPA hydrogels by inserting pH responsive components such as acrylic acid, chitosan, sodium alginate etc. Moreover, these biocompatible hydrogels can be synthesized in nano- and micro-scales to use in tumor targeting drug delivery system considering that cancer cells and their microenvironments need much more sugar-induced energy compared to normal cells in order to rearrange energy metabolism and proliferation. Due to its polyfructan structure, levan is expected to be more preferred energy source by

cancer cells as compared to normal cells which has a tendency of enhancement on the hydrogel surface at 37 ^{o}C.

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APPENDIXES

Appendix A Levan dn/dc calculations

dn/dc Peaks of Levan for 0.1M NaNO3&2% Ac.A.

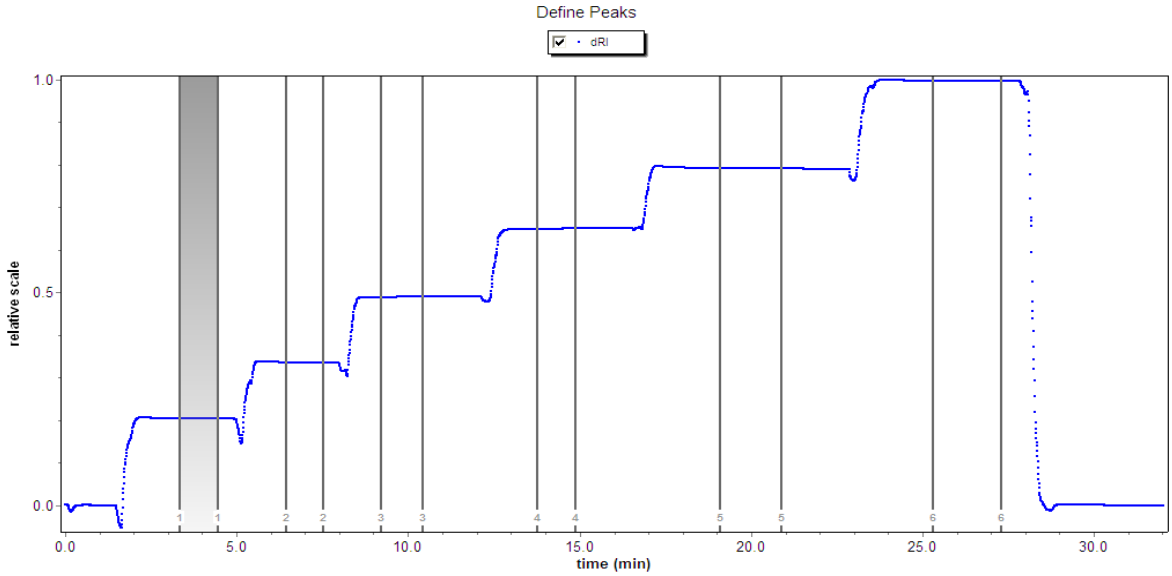


Figure A.1. dn/dc Peak results of Levan for 0.1M NaNO3&2% Ac.A. solution.

dn/dc Levan concentrations (g/mL) and peak times (min).

Table A.1. Levan concentrations (g/mL) and peak times (min) for 0.1M NaNO3&2% Ac.A solution.

Peak	**Begin (min)**	**End(min)**	**Concentration g/mL**
Peak 1	3.325	4.455	4.00e-4
Peak 2	6.450	7.514	6.66e-4
Peak 3	9.210	10.440	1.00e-3
Peak 4	13.765	14.862	1.33e-3
Peak 5	19.085	20.880	1.60e-3
Peak 6	25.302	27.297	2.00e-3

Appendix B

5-aminosalicylic acid concentration-absorbance calibration curve

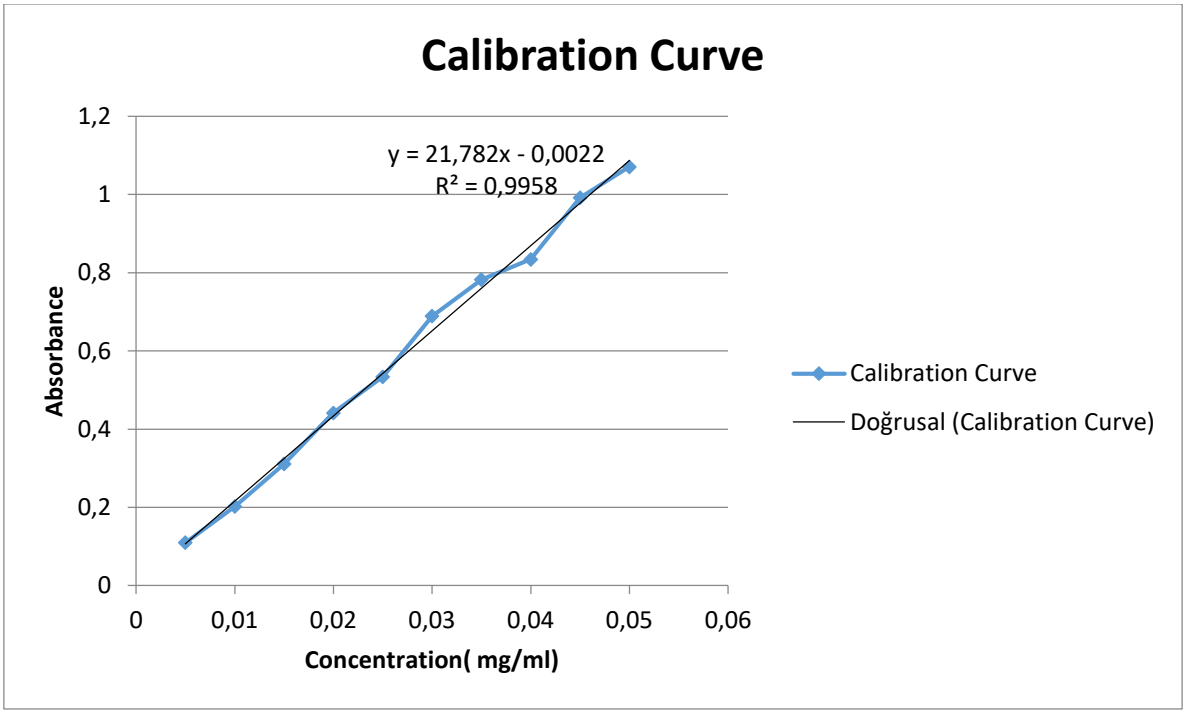
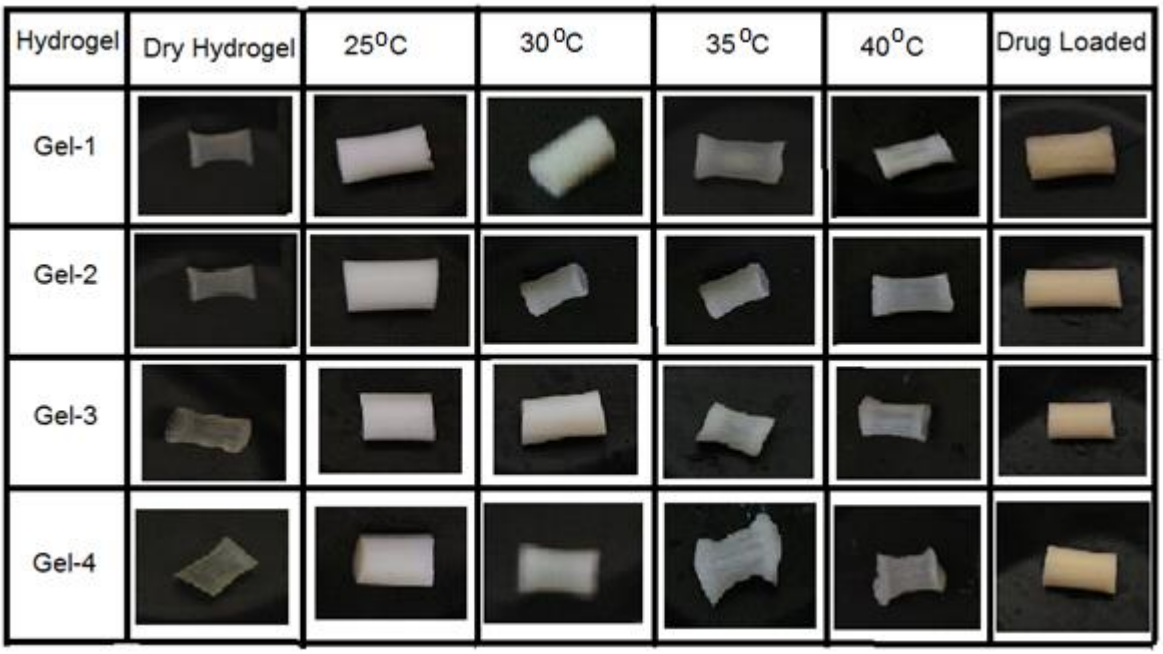


Figure B.1. 5-aminosalicylic acid concentration-absorbance calibration curve**.**

Appendix C

Dry, swollen and drug loaded Hydrogel photographs



AUTOBIOGRAPHY

Name Surname	: Asila Osman
Place and Date of Birth	: Sudan 23.09.1990
language	: Arabic English and Turkish
Email	: asila76@gmail.com

Education

Degree	Department/Program	University /high school	Graduation year
High school	Science	Alweya Abd Alrafea	2007
Bachelor of Science	Chemical engineering	University of Khartoum	2012

Awards and scholarship

Master degree scholarship turkey burslari September 2013 Aguste 2016

The University Award for the Best Academic Performance Fifth Year Faculty of Engineering Chemical Engineering Department, 2012.