TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

HENNA KIILHOLMA

MODIFICATION OF POLYMERS FOR POLY(*N*-ISOPROPYL ACRYLAMIDE) -BASED HYDROGELS

Master's Thesis

Inspectors: Adjunct professor Terttu Hukka
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Työssä esitellään hydrogeelit materiaalina, joiden avulla sydänsoluja voitaisiin mahdollisesti kasvattaa. Kirjallisuusosiossa käydään lyhyesti läpi myös sydämen rakenne, sydäninfarkti sekä menetelmä jolla infarktin aiheuttamaa sydänlihasvauriota on yritetty korjata.. Injektoitaessa pelkkiä sydänlihassoluja sydänlihakseen ne lähtevät pois paikaltaan eivätkä uudista kudosta. Materiaali, joka auttaisi pitämään solut toimintakykyisinä, voisi löytyä hydrogeelien joukosta. Hydrogeelit ovat joukko verkottuneita hydrofiilisiä polymeerejä, joiden ominaisuuksia voidaan säädellä muun muassa silloittamisasteella. Hydrogeelin pitäisi muistuttaa mekaanisilta ominaisuuksiltaan soluväliainetta, jotta sydänlihassolut menestyisivät sen sisällä tai pinnalla. Sekä liuosten että itse hydrogeelin olisi oltava myrkytön ja nopeasti geeliytyvä.

Tutkimuksen tarkoituksena oli valmistaa injektoitava hydrogeeli. Hydrogeelin valmistusaineiksi valikoituivat poly(*N*-isopropyyliakryyliamidi)n ja akryylihapon kopolymeeri (PNIPAM-*co*-AA) sekä luonnonpolymeerit hyaluroni ja alginaatti. PNIPAM on laajalti tutkittu biomateriaali, jota pidetään yleisesti sopivana lääketieteellisiin sovelluksiin. Se on myrkytön ja geeliytyy lämmön vaikutuksesta (30–32 ˚C) mikä mahdollistaa sen käytön elimistössä hydrogeelin osana. Toisaalta NIPAM monomeeri on myrkyllinen ja PNIPAM ei ole biohajoava. Yhdistämällä (PNIPAM-*co*-AA) biohajoavaan polymeeriin voidaan kuitenkin saavuttaa osittainen biohajoavuus. Hyaluroni ja alginaatti ovat polysakkarideja, joiden rengasrakenteeseen saadaan aldehyditoiminnallisuus perjodaattihapetuksella. Muokattaessa PNIPAM-*co*-AA adipiinihapon dihydratsidilla di-imidin läsnä ollessa, saadaan karboksyyliryhmien tilalle dihydratsidiryhmät. Syntynyt PNIPAM-*co*-ADH, hyaluroni ja alginaatti analysoitiin NMR ja FTIR-spektroskopialla. Hyaluroni ja alginaatti hapetettiin perjodaatilla aldehydirakenteen saavuttamiseksi. Aldehydirakenteessa olevan karboksyylihapen sekä PNIPAM-*co*-ADH:n hydratsidiryhmien välille syntyy yhdistettäessä hydratsonisidos. Komponenteista tehtiin geelejä eri liuoskonsentraatioilla ja lämpötiloissa. Pysyvin geeli valmistettiin yhdistämällä 60 mg/ml PNIPAM-*co*-ADH- ja alginaattiliuoksia suhteessa 1:1. Geelit olivat heikkorakenteisia koska 15 % silloittaminen ei ole riittävä pitämään geeliä koossa.

ABSTRACT

TAMPERE UNIVERSITY OF TECHNOLOGY
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The literature review part of this thesis presents the basic structure of the heart and its properties from the tissue engineering perspective. Myocardial infarction is presented. The challenge is approached in context of the functionalized matrix or hydrogel. Hydrogels used in the treatment of myocardial infarction are presented in Appendices 1-5.

In the practical part of this thesis, the aim of the study was to modify materials for modular semisynthetic injectable hydrogel. A commercial copolymer of poly(*N*-isopropyl acrylamide), PNIPAM, and acrylic acid was chosen to be the fundamental part of the gel. The copolymer consists of PNIPAM chains with 15 mol-% acrylic acid. The carboxylic acid residues of acrylic acid are replaced with dihydrazide functionality using carbodiimide crosslinker resulting in PNIPAM-*co*-ADH. Being thermo sensitive, nontoxic and widely studied biomaterial, PNIPAM and its derivatives can act as starting materials for a wide variety of applications. On the other hand, the monomer is toxic and the polymer is not degradable. The polymer can be modified and combined chemically with biodegrading materials to make it partially degradable. In this study two natural polysaccharides, hyaluronic acid and alginate were chosen to be another component of the hydrogel. Hyaluronic acid is a polysaccharide found in the extracellular matrix, especially of soft connective tissues. Hyaluronic acid based hydrogels can be made stiff or soft; thus they offer a wide range of platforms for biomedical applications. Alginate is extracted from seaweed. Alginate hydrogels are versatile and well-studied biomaterials for various medical applications. They have been used as cell delivery vehicles and a support matrix for tissue engineering and as model extra cellular matrices for in vitro cell experiments.

A sodium periodate oxidation procedure was used to attain aldehyde functionality. When these were combined with PNIPAM-*co*-ADH with different concentrations, some gelation was observable. The best combination was PNIPAM-*co*-ADH and partially oxidized alginate with 1:1 ratio. Other gels prepared were very weak, unstable and the degree of hysteresis was substantial. The unmodified and modified materials were characterized with Fourier transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance (^{1}H NMR). 15 % crosslinking is not enough to keep the gels together.

PREFACE

This thesis has been a part of Human Spare parts –project. It has been a project at Bio-Medi- Tech which is a multidisciplinary consortium of the Tampere University of Technology and University of Tampere.

The thesis includes a practical part which was executed in the Laboratory of Biomaterials and Tissue Engineering of Department Electronics and Communications Engineering. Researcher Jennika Karvinen gave her time and support in and outside the laboratory. Adjunct Professor Terttu Hukka and Professor Minna Kellomäki gave advice which were crucial in during the writing and in finishing the whole thing.

Thank you Hanne and Nennu for listening to me.

Mikko, this thesis would not have even seen the light of the day without you. Leena ja Rauli, äitin muruset. Kiitos olemassaolostanne.

CONTENTS

TERMS AND ABBREVIATIONS

ADH	Adipic acid dihydrazide.
Desmosome	Specialized cell junction linking two cells by tying their outer cell membranes together with intermediate filaments.
EDC	*N*-(3-dimethylaminopropyl)-*N*'-ethyl carbodiimide. Crosslinker used in biotechnology.
Gap junction	Common in cardiac muscle, facilitates the cell-to-cell passage of small molecules.
HA	Hyaluronic acid
Intercalated disc	Intercellular junction between individual cardiac muscle cells.
LCST	Low critical solution temperature. Temperature in which polymer solution undergoes phase transition.
Myofibril	The organelle of cardiac muscle cells which is a long cylindrical mass of contractile protein fibers (actin + myosin). Organized as a series of repeating sarcomeres.
Myofilament	The structural unit of a myofibril.
PNIPAM	poly(*N*-isopropylacrylamide). Thermoreversible polymer used in e.g. biomedical applications.
Sarcomere	One of the segments into which a myofibril of striated muscle is divided.
Syneresis	The extraction or expulsion of a liquid from a gel.

1 INTRODUCTION

Heart related problems are a widespread cause of human suffering. The economic impacts are also significant when working-age people are not able to rehabilitate e.g. after myocardial infarction. The heart is not able to repair itself efficiently on a cellular level. (Anversa et al. 2006) This problem could be approached by delivering cell-life supporting material to the heart. Scaffolds have been prepared for this purpose. (You et al. 2011) A surgical operation always presents a risk to the patient, particularly those who might be in a weak condition due to myocardial infarction. One alternative approach is an injectable hydrogel which could be delivered to the heart without invasive surgery, that is, through catheter. (Martens et al. 2009)

In this thesis is presented the use of hydrogels in regenerative medicine concerning probably the most serious heart failure or myocardial infarction. In the traditional cell therapy, in which cells are injected in to the myocardium, the challenge lies in cell discharge out from the myocardium. (Hofmann et al. 2005) A hydrogel could provide a safe environment for injected cells to remain and function together with individual's own heart cells. The challenge is to find a gel suitable for this application. Hydrogel must possess relevant biological, mechanical, chemical and drug delivering properties so that it resembles myocardial tissue. This challenging task has been approached with synthetic and natural hydrogels. (Mihardja et al. 2008; Li et al. 2012) The solution might lie in combining synthetic and natural materials to attain desired properties. (Yoon et al. 2009)

The goal of the practical part in this Master's thesis was to modify materials for injectable hydrogel reproducing a study by Patenaude et al. (2012). Functionalized natural polysaccharides together with a copolymer consisting of poly (*N*-isopropyl acrylamide) and acrylic acid are combined without any stimulus (e.g. light or heat). Copolymer is modified through carbodiimide chemistry to introduce hydrazide functionality in the copolymer chain. Hyaluronic acid and alginate gain their aldehyde functionality through oxidation with sodium periodate. The materials are characterized with Fourier transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance (^{1}H NMR). In the gel tests, the solutions are prepared with different concentrations and temperatures. These solutions were prepared to a gel either by using a pipette and a petri dish or two syringes integrated with an adapter.

2 HYDROGELS

Hydrogels are polymeric network-like materials which have high water content and varying physical properties. They can be engineered to resemble body the extracellular environment of the tissue both mechanically and compositionally. Biocompatible hydrogels can be modified to support cell compatibility. It has also been shown that cells grow more naturally in 3-D environments like hydrogels. (Tibbitt et al. 2009; Seliktar 2012)

Hydrogels can be altered to a variety of physical forms, including slabs, microparticles, nanoparticles, coatings and films. Different forms of hydrogels are commonly used in clinical practice and experimental medicine for a wide range of applications, including tissue engineering and regenerative medicine, diagnostics, cellular immobilization, separation of biomolecules or cells and barrier materials to regulate biological adhesions. (Hoare et al. 2008; Tibbitt et al. 2009; Seliktar 2012)

2.1 Hydrogel materials

Hydrogel materials, their basic structure and derivatives, main source and applications in cardiac tissue engineering are presented. The division is done into synthetic and nature originated materials. The hydrogels presented here are also in Appendix 1.

2.1.1 Hydrogels of natural origin

Polymers from natural origin have proofed their biocompatibility. Usually, they do not require much processing. These are the reasons they have been studied in the regenerative medicine of the heart. (Nelson 2011) Most of the polymers are also easily accessible due to large number suppliers. For example Google gives 59 800 results for ‘medical grade alginate supply’ (19.3.2014).

Uneven quality of the natural products represents a problem. Consistence can vary from batch to batch. Also, they do not possess compatible biomechanical properties; regenerative techniques relying on these hydrogels are about supporting cell delivery, not strengthening the heart tissue. (Wang et al. 2010.) Applying hydrogels consisting of natural originated polymers is challenging. Their properties (mechanical, degradation, gelation etc.) can be difficult to modify. (Tous et al. 2011.) Availability presents a problem too.

2.1.1.1 Matrigel™

Matrigel™ possesses a complicated structure; it is a mixture of proteins found in the extracellular matrix (ECM) that have been extracted from Englebreth-Holm-Swarm tumors in mice. Matrigel™ consists primarily of laminin, collagen IV, enactin and heparan sulphate proteoglycan. It includes also growth factors including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), nerve growth factor (NGF), transforming growth factor (TGF) and other growth factors which have not been fully identified chemically. The heterogeneous construction is thought to influence cell gene expression. (Ou et al. 2011.)

One important application of Matrigel™ is for the growth of human embryonic stem cells. Matrigel™ is used to mimic the ECM in cancer and stem cell culture. It has been shown to be very convenient for culturing stem cells due to its ability to maintain self-renewal and pluripotency. (Hughes et al. 2010.)

Matrigel has been implanted alone and with neonatal cardiomyocytes to the mice model of myocardial infarction. (Zhang 2006) Gel stopped the progress of the infarction. When gel contained cells it helped to maintain heart wall thickness and regional contraction better. (Kofidis et al. 2004.)

2.1.1.2 Silanized hydroxypropyl methylcellulose

Hydroxypropyl methylcellulose (HPMC) is a commercially available hydrogel. The structure of the repeating unit is shown in Figure 2.1.

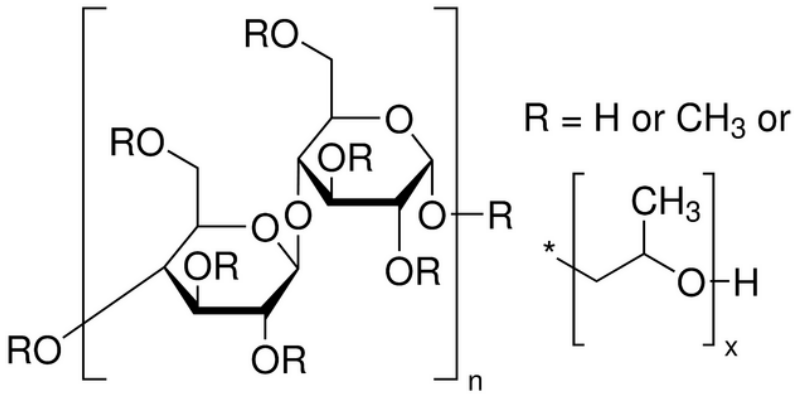


Figure 2.1 *Repeating structure of hydroxypropyl methylcellulose (HPMC) (sigmaaldrich.com).*

Mathieu et al. (2012) approached the challenges of infarcted cardiac tissue by seeding mesenchymal stem cells (MSC) in to it. HPMC was silanized (Si-HPMC) to support the proliferation and growth of MSC. MSC are proofed beneficial to infarcted cardiac tissue but are unable to survive without a supporting medium. MSC were injected in to the

heart of rats with Si-HPMC gel. Results showed that intramyocardial injection of MSC gave a therapeutic benefit starting four weeks after myocardial infarction. It is not sure that these beneficial effects are related to the specific scaffolding properties of the Si-HPMC hydrogel. (Mathieu et al. 2012.)

2.1.1.3 Alginate -based hydrogels

Alginate is composed of blocks of (1–4) β-d-mannuronic acid monomers (M) and blocks of α-l-glucuronic acid monomers (G) (Figure 2.2). Depending on the source and processing, molecular weight ranges between 10 and 1000 $*10^3$ g/mol. (Baldwin et al. 2009) The amount of each monomer and the sequential distribution of the monomer blocks along a polymer chain vary depending on the source of the alginate. Divalent cations such as Ca^{2+} bind blocks of G monomers along adjacent polymer chains, creating ionic interchain salt bridges which result in the gelation of the aqueous alginate solutions. (Mihardja et al. 2008.)

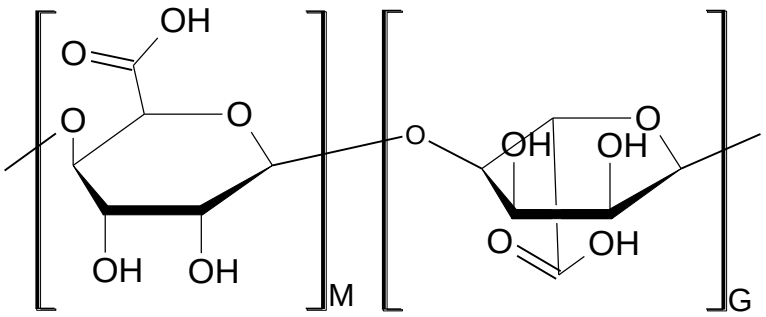


Figure 2.2 *Repeating unit of alginate.*

Normally, cells have difficulties adhering to the alginate due to lack of binding sites. Mihardja et. al (2008) mixed alginate solution with conducting polymer polypyrrole. It was hypothesized that cells could adhere better in this compound due of its polycationic nature. Alginate-polypyrrole hydrogel was found to enhance arteriogenesis. The challenges lie in processing and modifying the mechanical properties of polypyrrole.

Yu et al. (2009) prepared alginate gels from a 1.5 % alginate solution in 0.9 % NaCl. Alginate gel formation was based on the addition of the cross-linker solution (102 mM $CaCl_2$). Their studies showed that intramyocardial injections of alginate can reshape a dilated left ventricle and improve ventricles function. It was demonstrated that the surface modification of polymers can influence the microenvironment of infracted myocardium.

2.1.1.4 Chitosan -based hydrogels

Chitosan is partially *N*-deacetylated chitin, and chitin is a linear homopolymer of 1, 4 -linked *N*-acetyl-*D*-glucosamine (Figure 2.3). Chitosan has many useful biological properties when applied as a biological adhesive or wound dressing; biocompatibility, biodegradability, hemostatic activity and wound healing acceleration. (Fujita et al. 2004.)

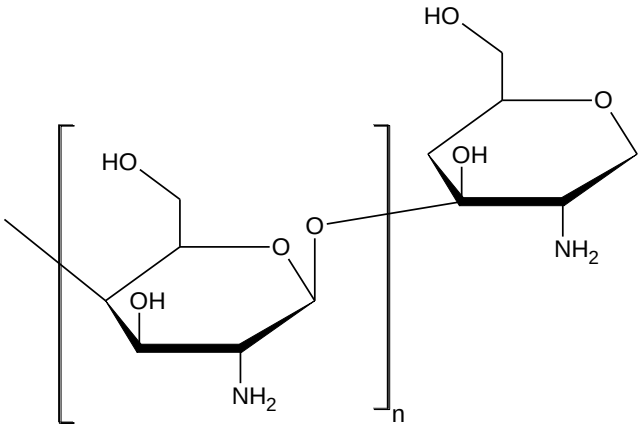


Figure 2.3 *Repeating unit of chitosan.*

In their study Fujita et al. (2004) evaluated the fibroblast growth factor (FGF-2) incorporated chitosan hydrogel as a myocardial angiogenic therapy using a rabbit model of myocardial infraction. FGF-2 was added to the chitosan hydrogel and most FGF-2 molecules in the chitosan hydrogel remained biologically active. They were released from the chitosan hydrogel upon the *in vivo* biodegradation of the hydrogel. FGF-2-chitosan hydrogel showed effectiveness in angiogenesis. Disadvantages of using a polymer originated in nature must be taken on account. (Fujita 2004.)

2.1.1.5 Fibrin -based hydrogels

In a study by Martens et al. (2009) it was examined how controlling the composition of a fibrin-based hydrogel can be prepared so that it is compatible with available percutaneous injection catheters. Hydrogel composing of 2% of bovine fibrinogen could be delivered under desired conditions i.e. having ten minutes injection window. It enhanced the local retention of injected cells in the heart and prevented their redistribution to distance organs. System was found to be suitable for the delivery of all cell types. (Martens et al. 2009.) The structure of the fibrinogen is very complicated. It does not possess a structure suitable for drawing it on a molecular level.

2.1.1.6 Hyaluronic acid

Hyaluronic acid (HA), is ubiquitous, non-sulfated glycosaminoglycan, linear polysaccharide of 2000–25000 repeating units of the disaccharide [–β (1, 4)-D-glucuronic acid-β1, 3-*N*-acetyl-D-glucosamine]$_n$ (Figure 2.4).

The molecular weight of HA is in the range (102–104) kDa and chains have length of 2–25 μm. (Gaffney et al. 2009.)

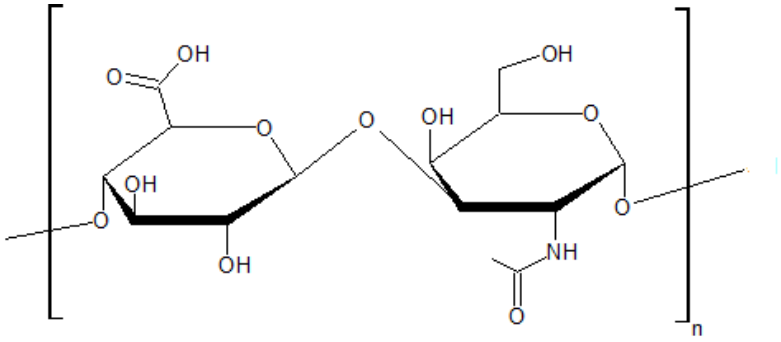


Figure 2.4 *Repeating unit of hyaluronic acid.*

The hydrophilic and hydrodynamic properties of hyaluronic acid allow it to retain water. The biological roles of hyaluronic acid are in part dependent on this property. HA forms loose, hydrated matrices with a pseudo-random coil configuration creating space to facilitate cell division. HA regulates cellular function through interaction with receptors. Because of its size HA may interact with more than one cell and these interactions may have a role in tissue assembly (Gaffney et al. 2009.) The swelling properties of hydrogel vary with molecular weight of hyaluronic acid. (Yoon et al. 2009)

Hyaluronic acid is easy to produce and modify, biodegradable and non-immunogenic extracellular matrix component that can be easily modified to display various chemistries (Gaffney et al. 2009; Young et al. 2011.)

Young et al. (2013) discovered that in myocardium, thiol-modified HA (HA-SH) (Figure 2.5) assembles and degrades over time.

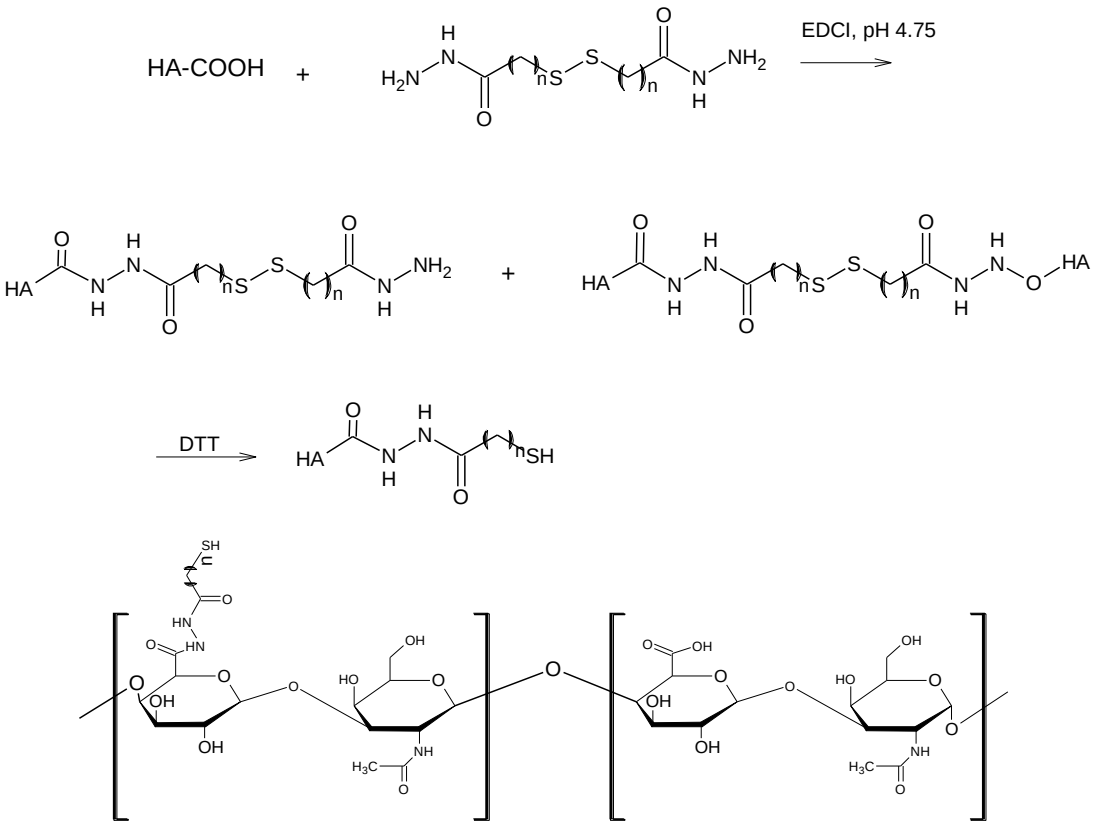


Figure 2.5 *Thiolated hyaluronic acid HA-SH (Young et al. 2013.)*

Improved cardiomyocyte maturation was observed. HA-SH-based hydrogels have limited biocompatibility probably due to interactions with free thiol groups. These materials may be suitable for studying the effects of the dynamic mechanical clues of HA on cellular behavior. (Young et al. 2013.)

Hyaluronic acid was functionalized by Tous et al. (2011) with hydroxyethyl methacrylate (HeMA), to obtain a cross-linkable macromer (HeMA-HA) that cross-links similar to MeHA, and has additional ester bonds that provide further control over hydrogel degradation (Figure 2.6). In the study two versions of MeHA hydrogels were compared (low and high mechanics) with the synthesized HeMA-HA hydrogels. (Tous et al. 2011)

Tous et al. (2011) compared hydrolytically degradable HA based hydrogels with stable HA hydrogels. These had similar mechanics and gel dispersion.

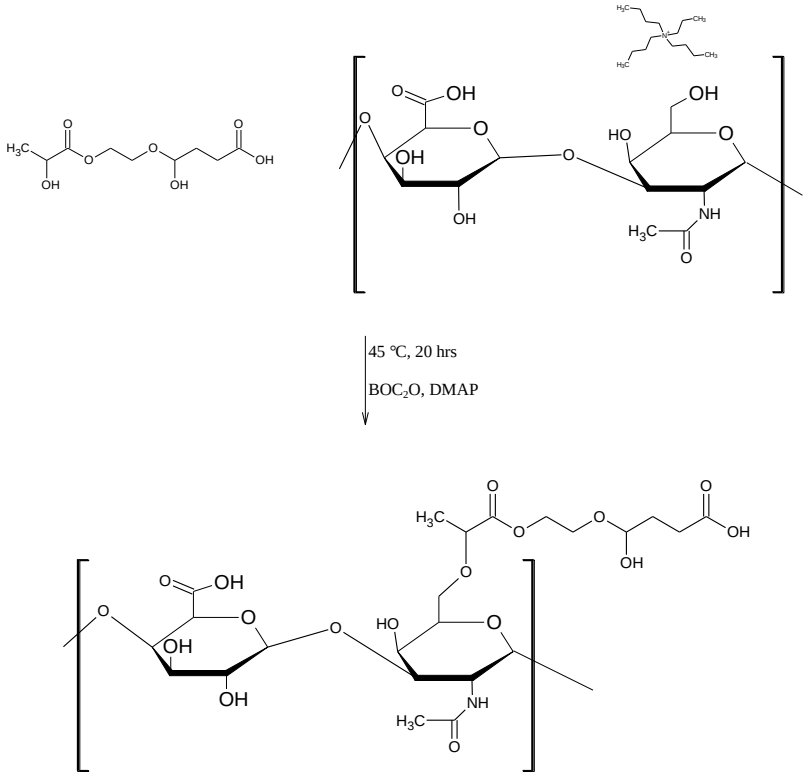


Figure 2.6 *HeMA-HA synthesis and chemical structure (Tous 2011).*

When injected into infarct tissue in an ovine model, degradable hydrogels induced a stronger inflammatory response than stable ones. This may be associated with their degradation. Hydrolytically degradable hydrogels revealed also that geometrical and remodeling changes are dependent on the mechanical and degradation properties of the hydrogel. No functional improvement was detected, but cardiac output (the volume of blood pumped by heart one minute) was better than in peer group. (Tous et. al 2011.)

2.1.1.7 Collagen

Collagen is the major protein of the extra cellular matrix. There are at least 12 types of collagen in various tissues. Types I, II, and III are the most abundant and form fibrils of similar structure. Type IV collagen forms a 2D reticulum and is a major component of the basal lamina. Collagen implants degrade through a sequential attack by lysosomal enzymes. It is widely used in tissue engineering applications. (Wang et al. 2010)

Dai et al. (2005) injected collagen (composed of highly purified bovine dermal collagen (65 mg/ml) that consists of 95 % collagen I and 5 % collagen III) into myocardial in-

farction on rats after one week from the damage. Collagen increased scar thickness which was studied six weeks later and did not induce an inflammatory response.

2.1.2 Synthetic hydrogels

In contrast to biological materials, synthetic materials allow for control over properties such as degradation, stiffness, pore size and gelation time. They either do not suffer from quality variability that occurs with materials originated from nature. (Rane 2011.)

2.1.2.1 Acrylic acid -based hydrogels

The hydrogel developed by Li et al. (2012) was based on *N*-isopropyl acrylamide (NIPAM), *N*-acryl oxysuccinimide (NAS), acrylic acid (AA) and a polymer based on *N*-isopropyl acrylamide, *N*-acryl oxysuccinimide, acrylic acid and hydroxyethyl methacrylate–poly(trimethylene carbonate) (HEMA-PTMC) (Figure 2.7).

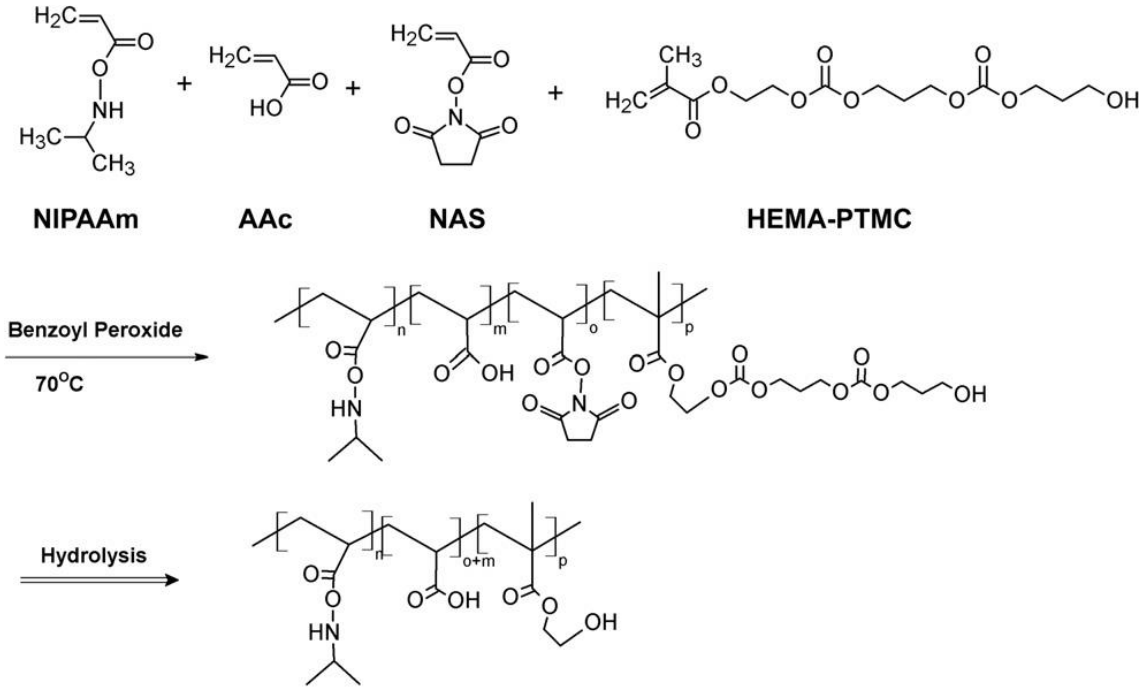


Figure 2.7 *Synthesis and hydrolysis of copolymer by Li et al. (Li 2012).*

The hydrogel was thermo sensitive, injectable and the gelation time suitable for heart injection (6-7 s). Because of suitable gelation time, the hydrogel and the cells delivered were able to remain in the heart. The elastic modulus of hydrogel was varied and modulus of 65 kPa was found to be the most suitable for cardiac cell differentiation. Mesenchymal stem cells delivered in the hydrogels differentiated into the cardiac lineage. (Li et. al 2012)

In 2009, Wang et al. prepared a series of hydrogels containing biodegradable dextran chain grafted with hydrophobic poly(ε-caprolactone)-2-hydroxylethyl methacrylate (PCL-HEMA) chain and thermoresponsive poly(*N*-isopropylacrylamide) (PNIPAM) chain was synthesized by the introduction of PCL (polycaprolactam)-grafted polysaccharide chains into the PNIPAAm network. Four days after induction of myocardial infarction 200 cm^3 of gel solutions were injected into four sites in the infarcted myocardium. Each injection was 50 µl. The hydrogel solutions gelled immediately after injection. Results were investigated after four days. The intramyocardial injection of Dex-PCL-HEMA/PNIPAM hydrogel may improve impaired cardiac function, enhance cardiac contractility, decrease infarct size, and reduce cardiac remodelling in ischaemic myocardium. The hydrogel might also cause arrhythmia and have effects on diastolic function.

Fujimoto et al. (2004) first prepared hydroxyethyl methacrylate-poly(trimethylene carbonate) (HEMA-polyTMC). It was synthesized by ring-opening polymerization of TMC initiated by HEMA with $Sn(OCt)_2$ as a catalyst (Figure 2.9). Stoichiometric amounts of HEMA and TMC (molar ratio 1:2) were mixed in a flask to which was added anhydrous toluene of equal mass to the TMC/HEMA mixture. $Sn(OCt)_2$ in 1 cm^3 toluene was added. The reaction was conducted at 120 ˚C for 1.5 h. The mixture was then dissolved in THF and precipitated in water. This precipitation process was repeated twice and the liquid precipitate was then isolated by centrifugation, dissolved in THF, and dried over anhydrous $MgSO_4$. THF was removed by rotary evaporation.

Then, poly (NIPAM-*co*-AA-*co*-HEMAPTMC) copolymers were synthesized by free radical polymerization. Monomers (NIPAM, AA, HEMAPTMC, Figure 2.8) were dissolved in 1,4-dioxane to form a 5 wt-% solution containing BPO ($7.2 * 10^{-3}$ mol/mol monomer). The polymerization was carried out at 70 ˚C for 24 h under argon atmosphere. The copolymer was precipitated in hexane and further purified by precipitation from THF into diethyl ether. The purified copolymer was vacuum dried.

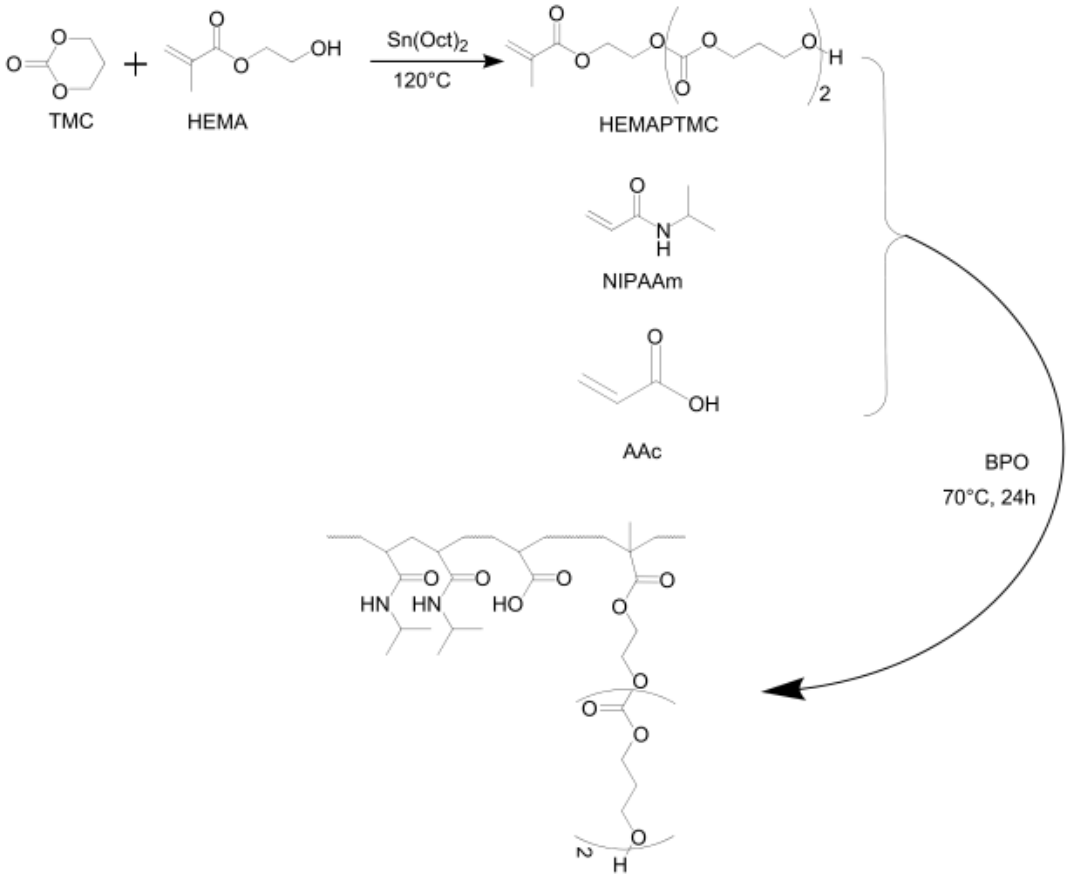


Figure 2.8 *Synthesis of HEMAPTMC and the copolymer poly(NIPAM-*co*-AA-*co*-HEMAPTMC).*

With ratio of (86/4/10; NIPAM/AA/HEMAPTMC) the hydrogel had suitable mechanical properties, degraded *in vitro* in 20 weeks and was not cytotoxic. Injection of the hydrogel prevented ventricular dilatation and improved contractile function in a chronic rat infarction model.

2.1.2.2 Polyethylene glycol -based hydrogels

Polyethylene glycol (PEG) is a bioinert and stiff material (Figure 2.9).

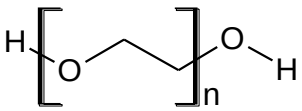


Figure 2.9 *Repeating unit of polyethylene glycol.*

PEG based hydrogels gel within a few minutes at physiological temperature and pH, do not require the addition of initiators, produce no byproducts, have very low reactivity towards tissue and considered being biocompatible. The physical properties of the gels can be varied through the choice of molecular mass, functionality and crosslinker type. Carbohydrate and low molecular mass therapeutic agents may be coupled and released

from the gels, either with or without the degradation of the gel itself. Due to these properties it is thought to be suitable for cardiac applications. (Sanborn et al. 2002; Tirelli et al. 2002; Dobner et al. 2009)

Dobner et al. derived PEG with vinyl sulfone so that it formed a degrading hydrogel. This hydrogel was able to support cell growth (primary human fibroblasts). (Lutolf et al. 2003) Synthesized material slowed down the progress of MI and myocardial deterioration. (Dobner et al. 2009)

Yoon et al. (2009) synthesized a hydrogel containing HA (MW 50 kDa) and PEG (Figure 2.10 and 2.11).

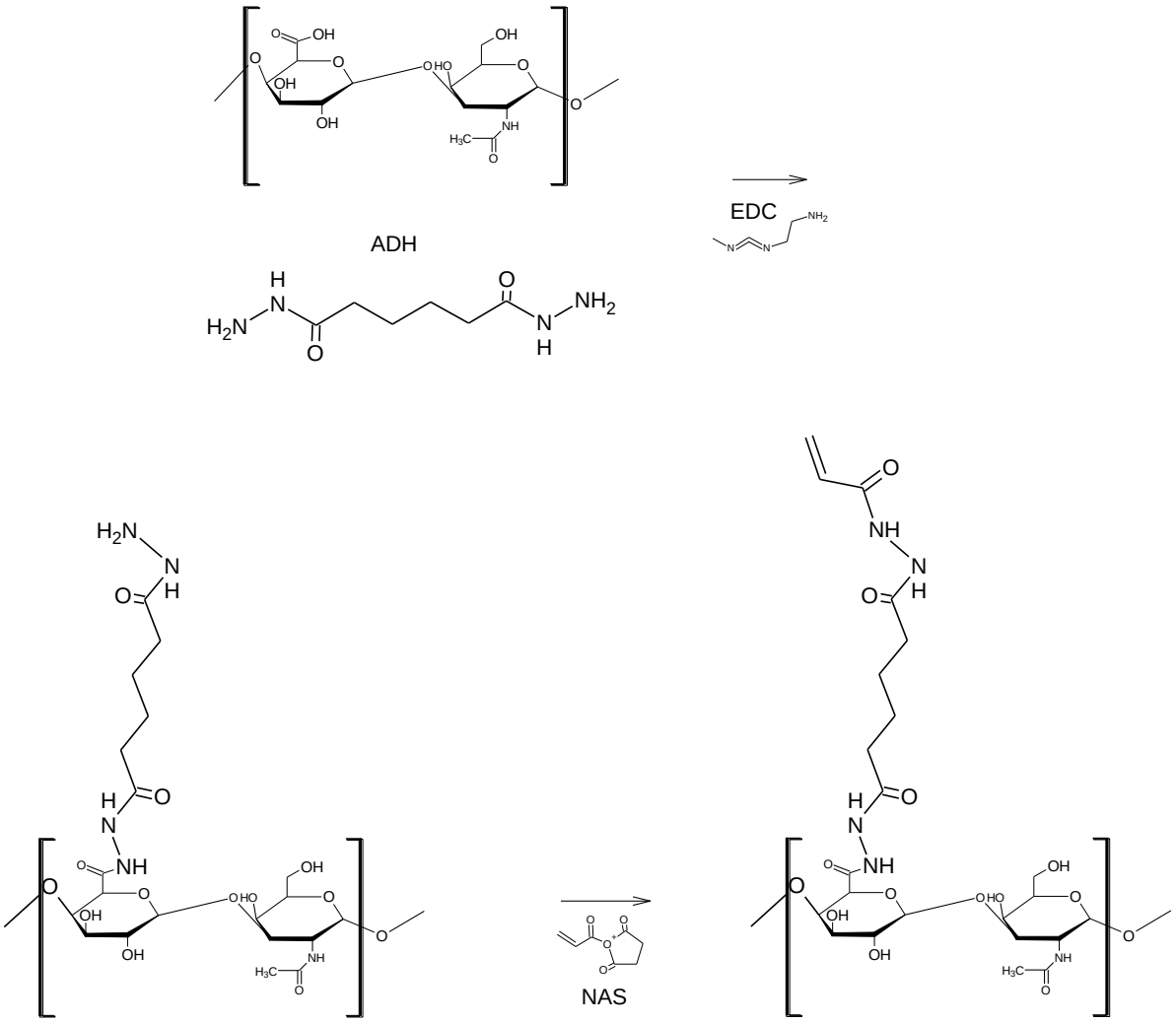


Figure 2.10 *Two-step synthesis of acrylated HA (Yoon et al. 2009).*

It showed good results in myocardial regeneration after MI. Stem cells had showed differential cellular behaviors, such as proliferation and differentiation.

This hydrogel was also trialed as a delivery system for growth factors and cells in bone regeneration *in vivo*.

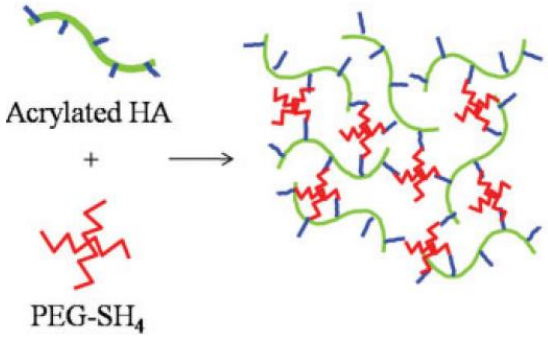


Figure 2.11 *The final structure of the gel by Yoon et al. (2009).*

New bone tissue formation was observed in samples with stem cells. MI induced to rat myocardium was not disseminated in this study. (Yoon et. al 2009.)

3 HEART

Heart is the organ responsible for bloodstream. Healthy heart of an adult human weighs approximately 250-350 g. It is located in the medial cavity if the thorax. The apex of the heart is pointing inferiorly and to the left. (Marieb et al. 2007.) Myocardial infarction is described due to its relevance to the cell death and need for repair.

3.1 Structure

The heart has four chambers – two atria and two ventricles. The right ventricle forms most of the anterior surface of the heart. Left ventricle forms the heart apex. The internal boundary between the left and right side of the heart is called interatrial septum. (Marieb et al. 2007.)

The heart is enclosed in a double-walled sac called pericardium. Superficial part of this sac is the fibrous pericardium. Below is the serous pericardium which is a two –layered serous membrane. These are the coverings of the heart. The external part of the serous pericardium is called epicardium, which is part of the heart wall (Figure 3.1).

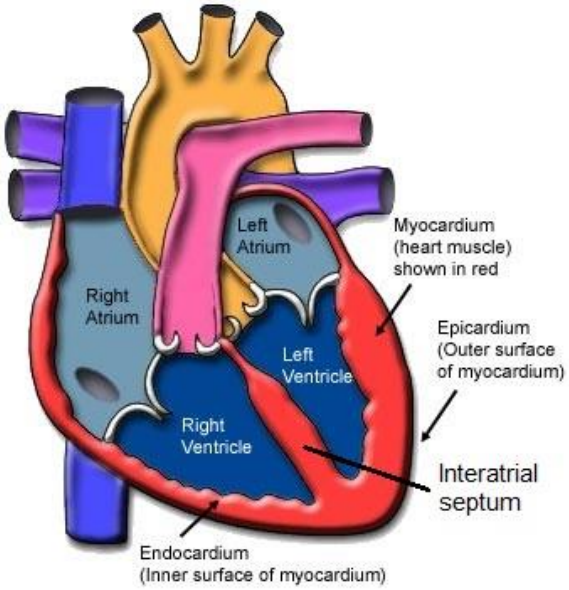


Figure 3.1 *Structure and layers of the heart tissue (http://www.texasheartinstitute.org).*

With epicardium on top, the two others are myocardium in the middle and endocardium which is the inner wall of the heart (Marieb et al. 2007.)
All of these layers are rich in blood supply. A groove on the surface of the heart is called the atrioventicular groove or coronary sulcus and it carries the blood vessels supplying the myocardium. (Marieb et al. 2007.)

3.1.1 Cardiac muscle cell

The heart muscle, myocardium, is constructed of long cells attached to each other. These are called cardiomyocytes (Figure 3.2). One or two nuclei can be detected. Typically, the length of the cells is between 50 and 150 µm and breadth 10-20 µm. Cardiac myocytes are the contractile cells of the heart. There are differences between the myocytes of the ventricles, the atria, and the conduction system. Atrial myocytes are long, thin and differ in some of the features from ventricular myocytes. (Marieb et al. 2007; Warrell et al. 2012)

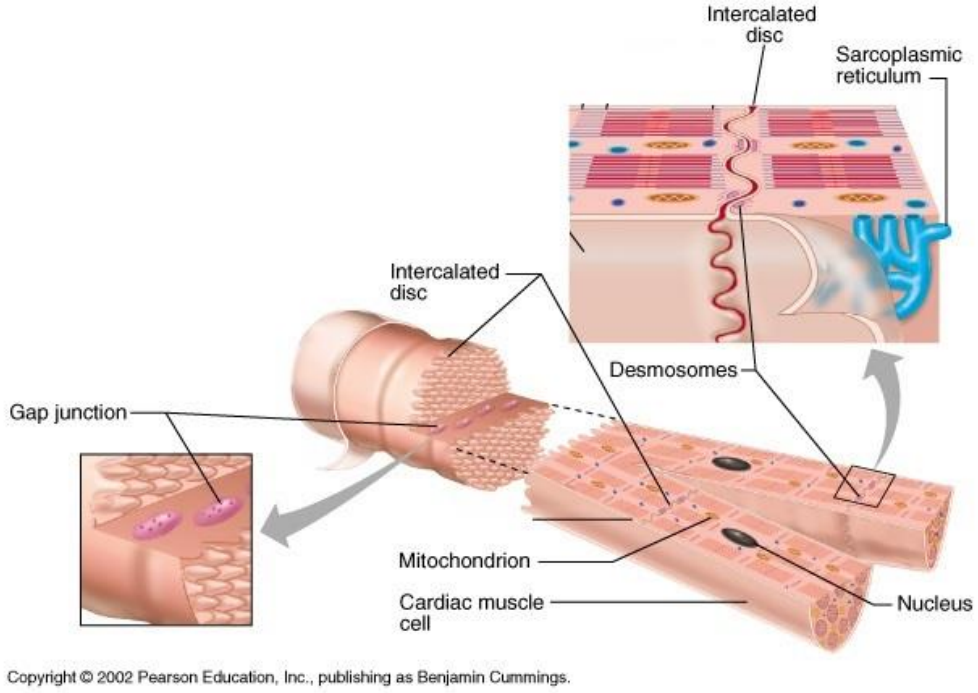


Figure 3.2 *Cardiac cell (http://www.apsu.edu/).*

Myocardium is the bulk material of the heart. In myocardium layer the (Figure 2.1) branching cardiac muscle cells are in close connection by connective tissue fibers forming a bundle. These bundles link all parts of the heart together. This network consisting of connective tissue, fibrous skeleton of the heart, reinforces the myocardium internally and anchors the cardiac muscle fibers. The thickness of this net, consisting of collagen and elastin, varies. (Heikkilä et al. 2000; Marieb et al. 2007; Klabunde 2011)

3.2 Myocardial infarction

Myocardial infarction is a significant cause of morbidity and mortality worldwide. Often there are no symptoms of the disease until acute events or symptoms come out. It is a chain of processes including cell death, scar formation and ventricular dysfunction. These lead to the change of the cellular, structural and mechanical properties of the heart. (Nelson et al. 2011.)

Infarction results in reduced cardiac function due to cardiomyocyte death, caused by prolonged ischemia and by reperfusion-induced injury. The damaged tissue is initially composed of a necrotic core surrounded by a border zone that can recover or become irreversibly damaged. The proliferative potential of the terminally differentiated cardiomyocytes is low meaning the heart is unable to repair itself. Collateral blood flow is an important determinant of infarct size and whether or not the border zone becomes irreversibly damaged. On the other hand, damage or defect in one of the four heart valves can ultimately lead to heart failure. (Schwinger et al. 1994; Peran et al. 2013)

Infarcted tissue does not contribute to tension generation during systole, and therefore can alter ventricular systolic and diastolic function and disrupt electrical activity. Myocardial infarction prevents the ability of the heart to change its force of contraction and therefore stroke volume in response to changes in venous return. Long-term consequences include ventricular remodeling, ventricular failure, arrhythmias and sudden death. (Klabunde 2007)

3.3 Properties of the heart

Mechanical and electrical properties are discussed due to their relevance to the hydrogel applications. The hydrogel needs to be stiff enough to give appropriate response to the cardiac cells. The network of hydrogel being too stiff the cells are not able to exert themselves. This is also the case if the hydrogel is too weak. The hydrogel should allow the electrical impulses of the cardiac cells pass without interfering.

3.3.1 Mechanical properties

Between the cardiac cells, the intercellular spaces are filled with loose connective tissue matrix. This matrix is connected to aforementioned fibrous skeleton of the heart which links the cardiac cells together and reinforces the walls of the heart. This supportive structure acts as a tendon but also as an insertion providing the cardiac cells something to pull and exert against. (Marieb et al. 2007; Warrell 2012)

The myofibrils are repeating units (sarcomeres) made up of thin actin filaments. Contraction results from sarcomere shortening produced by the ATP-dependent movement of the thin and thick filaments relative to one another. (Warrell 2012.)

Heart has a unique biomechanical behavior; it is strong but ductile in the systole phase and more elastomeric in the diastole phase. The Young modulus (*E*) which is used to define stiffness, is about 8-17 kPa for cardiac tissue. The mechanical measurements of fibrotic scars that develop after an acute myocardial infarction show that the fibrotic tissue is locally rigidified in comparison with normal tissue. Atomic force microscopy probing gives cell-scale elasticity values from 20 to 60 kPa for fibrotic wound. This means that cardiomyocytes behave poorly on a matrix stiffer than cardiac muscle. The Frank-Starling relationship describes the mechanical response of cardiac muscle during a full pump cycle. (Engler et al. 2006; Discher et al. 2009; Kraehenbuehl et al. 2008; van der Loo et al. 2008; Bhana et al. 2010.)

3.3.2 Electrical properties

There is an integrated electrical network inside the heart which directs the spontaneous heart cycle. It is continuous and conductive. Some cardiac cells have evolved to work in specialized roles, e.g. the myocytes that constitute the electrical conduction system. (Li et al. 2011.)

The plasma membranes of adjacent cardiac cells interlock at specific junctions called intercalated discs (Figure 2.2). These discs contain links between cells, desmosomes, and gap junctions. The desmosomes prevent adjacent cardiac cells from separating during contraction. Gap junctions allow ions to pass freely from cell to another, transmitting the depolarizing current across the entire heart. Cardiac cells being electrically coupled by the gap junctions, the whole myocardium behaves like a single coordinating unit. (Marieb et al. 2007.)

There is a potential difference across the plasma membrane such that the inside of the cell is negative in comparison with the outside. The difference in Voltage is around 80 mV. This is caused largely by the efflux of K^+ from the cell through K^+ channels and down its concentration gradient until the electronegative force retaining K^+ in the cell balances the tendency for efflux. This is called cardiac action potential in cardiomyocytes. (Klabunde 2011; Warrell 2012)

The rhythm of the heart is determined by a pacemaker site called the sinoatrial node located on the posterior wall of the right atrium. The sinoatrial node consists of specialized cells that generate action potentials at a rate of 100-110 action potentials per minute. The sinus rhythm normally controls both atrial and ventricular rhythm. Action potential generated by the sinoatrial node spread throughout the atria, depolarizing this tissue and causing atrial contraction. The impulse then travels into the ventricles. Specialized conduction pathways within the ventricle rapidly conduct the wave of depolarization throughout the ventricles to result in ventricular contraction. (Klabunde 2011; Warrell 2012)

In myocardial infarction (discussed later), declining conductivity of the heart cells is a part of the problem. (Mihardja et al. 2008)

3.4 Cardiac tissue engineering

In this chapter it is explained how myocardial infarction is treated with cell therapy.

3.4.1 Delivering cells to the heart

There is a growing need for stimulating heart cells and to regenerate lost heart tissue. One approach could be to deliver cells in to the heart. In theory, a human's own cells could be differentiated into cardiac myocytes. Cells could be grown *in vitro* and injected back into the myocardial tissue of the donor (Figure 3.3). (Giraud et al. 2012)

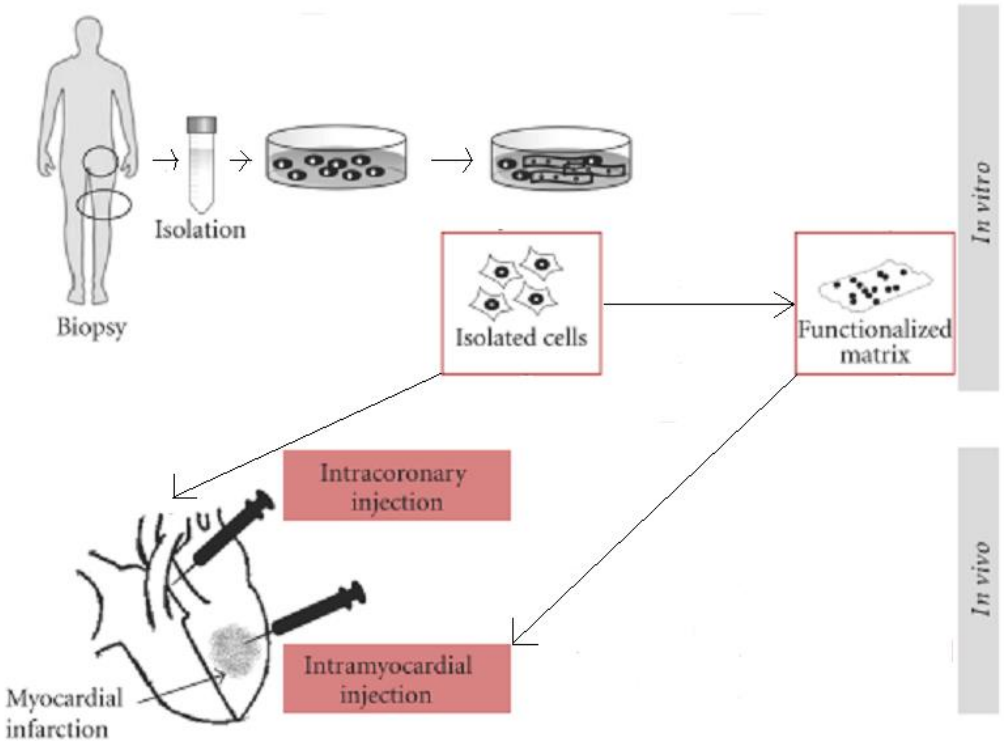


Figure 3.3 *Cells collected from human are differentiated into cardiac myocytes* in vitro. *If cells are engineered further, even tissues and functionalized matrices like hydrogel for cells can be grafted to the heart (derived from Giraud et al. 2012).*

Hofmann et al. (2005) studied cells that were suspended in a buffer and injected into the heart. Unfortunately, the injected cells have failed to graft and die easily. One of the possible reasons is the hostile environment in the infarcted area. The blockage of the coronary artery causes very low oxygen and nutrient levels. Also, phagocytosis releases

apoptotic cytokines and cell toxic reactive oxygen species. These factors limit the survival of the delivered cells. In a study by Wang et al. (2010) it was shown that after two hours less than 10 % of the cells survived.

The graft rate may be improved by regenerative cardiac tissue engineering, where cells are delivered with a supporting matrix like hydrogel. The supporting matrix prevents cell loss during the injection and provides an environment that protects the cardiac cells from the aforementioned hostile environment. It provides a mechanical support to ease the elevated heart wall stress caused by the loss of normal cardiac muscle cells. This can lead to cardiac function improvement. (Li et al. 2011.)

In the study by Siepe et al. (2006) the myoblasts were injected to the heart tissue with and without biodegradable a polyurethane scaffold. Polyurethane scaffolds with myoblasts prevented post myocardial infarction progression toward heart failure as efficiently as direct intramyocardial injection.

Giraud et al. (2008) prepared grafts containing myoblasts in a solution with rat tail collagen and Matrigel™. In this study, the functionality of the heart recovered although it might not have been due to the treatment. All the myoblasts had disappeared from the heart after four weeks.

In another study Giraud et al. (2010) prepared a highly porous scaffold from Artelon™ and polyurethane. Artelon™ is made of polyurethaneurea and a biomaterial which takes five years to hydrolyze completely. This scaffold was used as a matrix for myoblasts. The progression of myocardial infarction to a heart failure was prevented for up to six months after the injection of myoblasts. Graft implantation prevented heart failure up to nine months. The effect disappeared after 12 months when an absence of the transplanted myoblasts within the scaffold was confirmed.

3.4.2 Requirements for hydrogels in cardiac applications

Any biomaterial mimicking the environment in the heart must have properties which resemble that of myocardial tissue. Hydrogels used in cardiac applications must have elastic modulus resembling that of myocardial tissue. It has been shown that myoblasts or cardiomyocytes differentiate and beat synchronously on a matrix with an intermediate elastic modulus of 8-11 kPa. (Engler et al. 2008.)

Hydrogel stability or controlled degradation rate is an important factor when considering heart applications. Degradation products must have low toxicity meaning the compounds formed can either be metabolized into harmless products or can be excreted by the renal filtration process. Hydrogels possess usually good biocompatibility. Their hydrophilic surface has low interfacial free energy in contact with body fluids, which results in a low tendency for proteins and cells to adhere to these surfaces. Also, the rub-

bery nature of hydrogels minimizes irritation to surrounding tissue. (Smetana 1993; Anderson 1994; Anderson et al. 1999)

Application of the gel must be fitted according to gels theoretical gelation time. Martens et al. (2009) studied fibrinogen/ thrombin system which could be delivered to the heart by a catheter (Figure 3.1). If *in situ* polymerizing gel is used and applied too soon, the advantage of liquid-phase injection is lost. On the other hand, if application is done too slow or too late, catheter may be clogged.

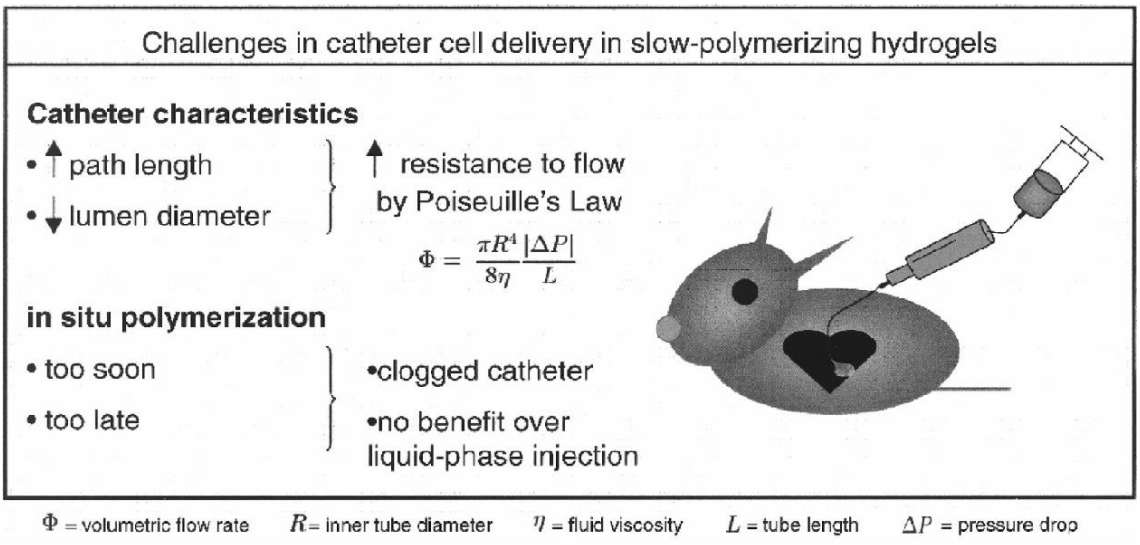


Figure 3.4 *Catheter-based delivery of actively polymerizing compounds (Martens et al. 2009).*

Pore sizes should be of adequate size (>10 μm) to allow for cellular infiltration and migration *in vivo*. (Jawad et al. 2007)

Overall, according to Shen 'the ideal injectable material should be biodegradable, have a low immunogenity, be no cytotoxic, non-adhesive and have antithrombogenic properties, adequate mechanical properties, provide stiffness for the scar but at the same time being compliant with the heart beating and transmit properly the mechanical stimuli to the cells, induce angiogenesis or at least not disturb the angiogenic activity after incorporation, be capable of delivering cells and or bioactive molecules.' (Shen et al. 2009.)

3.4.3 Cells for testing the compatibility with the hydrogel

Grown functional cardiomyocytes for microenvironment testing are almost impossible to achieve. Many different cells have been tested for differentiation including skeletal myoblasts and mesenchymal stem cells/marrow stromal cells, adult stem cells, embryonic stem cells, cardiac stem cells, umbilical cord blood cells and amniotic fluid stem cells. (Karam et al. 2012; Arnal-Pastor et al. 2013)

In Table 3.1 there are listed some cell types and details on using them in cardiac cell differentiation.

Table 3.1 *Possible cells for use in cardiac application development (derived from Giraud et al. 2012).*

Cell type	Main concern	Side effects	Mechanism of action
Human embryonic stem cells	Ethics, purification	Teratoma	Differentiation /myogenesis
Fetal/ neonatal cardiac muscle cells	Ethics accessibility	-	Differentiation /myogenesis
Induced pluripotent stem cells	-	Teratoma	Differentiation /myogenesis
Cardiac stem cells	-	-	Differentiation /myogenesis
Skeletal muscle my-oblasts	Poor electrocoupling	Arrhythmia	Paracrine effect
Bone marrow stem cells	Purification/ loss of function with age	Arrhythmia	Paracrine effect

Induced pluripotent stem cells (iPS) are derived from somatic cells (generally skin fibroblast) that have been transformed into pluripotent cells by the forced expression of stem cell-associated genes. They have the differentiation potential of embryonic stem cells, which allows them to be considered as possible candidates for regenerative cardiac tissue applications. iPS cells have been differentiated into beating cardiomyocytes. They have quite good accessibility. Their application in clinical trials has been limited for the use of viral vectors that may promote malignancy and act as oncogenes. There is also a risk for teratomes because of their pluripotency. (Zhang et al. 2009; Forte et al. 2011; Giraud et al. 2012; Arnal-Pastor et al. 2013)

4 MATERIALS AND METHODS

Choosing of a poly (*N*-isopropyl acrylamide) (PNIPAM) based material PNIPAM-*co*-acrylic acid (PNIPAM-*co*-AA) was convenient due to materials suitable LCST (30-32 ˚C), biocompatibility, adjustable biodegrability and good reputation as a biomaterial. The most important property of PNIPAM is however its thermosensitivity. PNIPAM is hydrophilic at room temperature. When heated above a critical temperature, the PNIPAM polymer becomes relatively hydrophobic and the particles shrink. (Schmidt et al. 2010). This temperature is called the cloud point.

PNIPAM (Figure 4.1) is used as a hydrogel in cell sheet engineering (Takahashi et al. 2011; Patel et al. 2012). It is known that most cells adhere to hydrophobic surfaces. The cells attach to PNIPAM based substrates at 37 ˚C and are detached at 20 ˚C because of the structural alterations of the PNIPAM. These structural alterations delete the adhesion between the cells and substrate. The junctions between cells are retained and a cell sheet is formed. (Takezawa et al. 1990) All cell types are not able to attach the PNIPAM substrate as such; they need anchoring molecules. (Chen et al. 2013) PNIPAM hydrogels are also used in drug delivery and as biosensors. (Guan et al. 2011) The hydrogel has been tried to adjust with multi-wall nanotubes and through copolymeration with a large number of other polymers. An example is a copolymer of PNIPAM and 2-acrylamido-2-methylpropanesulfonic acid (AMPS) (Saikia et al. 2012; Chen et al. 2013)

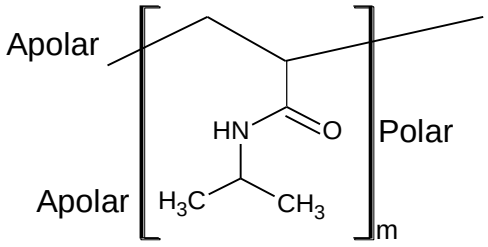


Figure 4.1 *The repeating structure of PNIPAM showing the polarity of different parts of PNIPAM unit.*

A study from 1997 reports that copolymer of PNIPAM and acrylic acid form gels with much desired properties: gels were translucent, experienced non-elastic deformation and there was no sign of hysteresis. (Han et al. 1998) PNIPAM-*co*-AA has been studied as an injectable drug delivery device and a drug delivering gel with cells. (Liu et al. 2006; Na et al. 2006)

Hyaluronic acid was chosen due to tissue regeneration properties, viscous properties, ability to retain water, biocompatibility and biodegradability (Yoon et al. 2009; Choh et al. 2011). Alginate was chosen due to biocompatibility and accessibility. Rapid gelation makes it suitable for cardiac tissue engineering. Hyaluronic acid and alginate are also relatively easy to modify into different derivatives possessing different properties. (Lee et al. 2012.)

4.1 Materials

Poly (*N*-isopropyl acrylamide)-*co*-acrylic acid (viscosity 7500—12500 cps in H_2O, LCST (32-36) °C; information from the manufacturer), adipic acid dihydrazide (≥98 %), 1-ethyl-3-(-3-dimethyl amino propyl) carbodiimide hydrochloride (EDC, commercial grade, powder), sodium periodate (ACS reagent, ≥99.8 %), ethylene glycol (anhydrous, 99.8 %) and sucrose (≥99.5 %) were purchased from Sigma-Aldrich and used as arrived. Hyaluronic acid from *Streptococcus equi* (weight average molecular weight (1.5—1.8)*10^6 g/mol) and low viscosity alginic acid sodium salt from brown algae were also purchased from Sigma-Aldrich. 0.1 N HCl and 0.1 M NaOH were prepared in the Laboratory of Chemistry at Tampere University of Technology.

4.2 Methods

Procedures used in modification, characterization of materials and gel tests are described.

4.2.1 The modification of PNIPAM-*co*-AA with adipic acid dihydrazide using carbodiimide crosslinker

PNIPAM-*co*-AA (shown in Figure 4.1.) must be stored and handled under inert atmosphere. These conditions were carried out with a glove box with an instrument nitrogen purge.

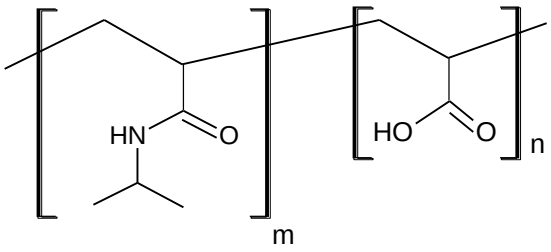


Figure 4.2 *Molecular structure of the constitutional repeating unit of the PNIPAM-*co*-AA.*

The reaction (Figure 4.2) was done according to Patenaude et al. (2012).

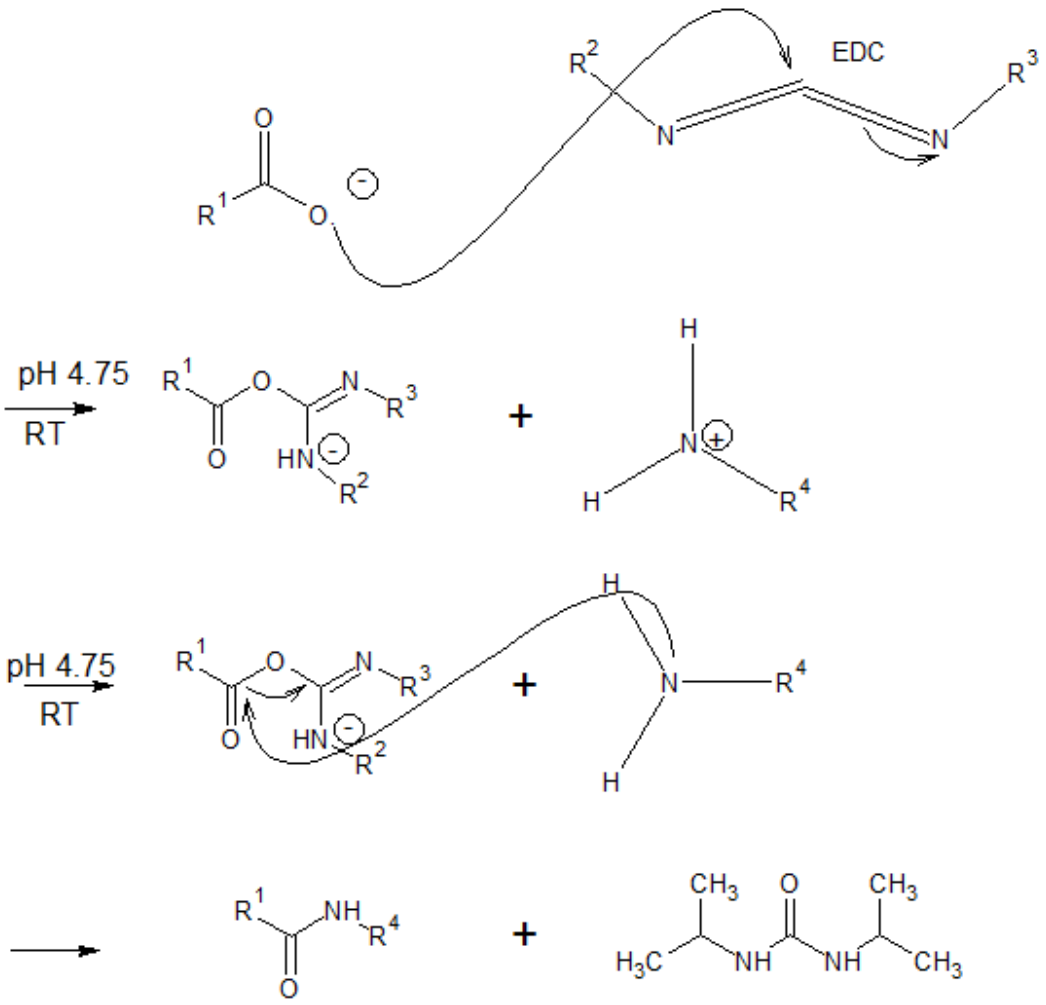


Figure 4.2 *Crosslinking reaction of PNIPAM-*co*-AA (R^1) and adipic acid dihydrazide (R^4).*

Copolymer was weighed and flask was sealed with septum before it was taken out. Flask was connected to a nitrogen line. 100 cm^3 of deionized water was added through syringe to gain 0.5 mass-% solution. Solution was left stirring overnight. After the dissolution of copolymer, 0.703 g of adipic acid dihydrazide (ADH) was added. A Mettler Toledo MI129 portable pH meter was tightly attached to one neck of the flask. 0.1 M HCl was added dropwise through a dropping funnel to attain the pH value of 4.75. Crosslinker 1-ethyl-3-(-3-dimethyl amino propyl) carbodiimide hydrochloride (EDC) was weighed (0.254 g) in the glove box. EDC was dissolved into 10 cm^3 of deionized water. The EDC solution was added dropwise to the copolymer-ADH solution

The pH value had to be controlled with HCl. The pH value was kept from 4.70 to 5.10 during the overnight reaction. pH was raised to neutral next day by the addition of 0.1 M NaOH. Two batches of PNIPAM-*co*-AA were crosslinked with EDC to adipic acid dihydrazide (Table 4.1).

Table 4.1 *Amounts of reagents used in modifying of PNIPAM-*co*-AA.*

Batch	PNIPAM-*co*-AA (g)	EDC (g)	ADH (g)
KOPO-1	0.50104	0.25363	0.703
KOPO-2	0.50231	2.79480	5.081

EDC reacts with carboxylic acid groups to form an *O*-acylisourea intermediate that is displaced by nucleophilic attack from primary amino group NH_2R^4 (Figure 4.2). The primary amine forms an amide bond with the original carboxyl group and an EDC by-product is released as a urea derivative. EDC crosslinking is the most efficient in acidic conditions. Modified copolymer PNIPAM-*co*-ADH (Figure 4.3, Picture 4.1) was dialyzed in 12-14 kDa membrane for four days and lyophilized.

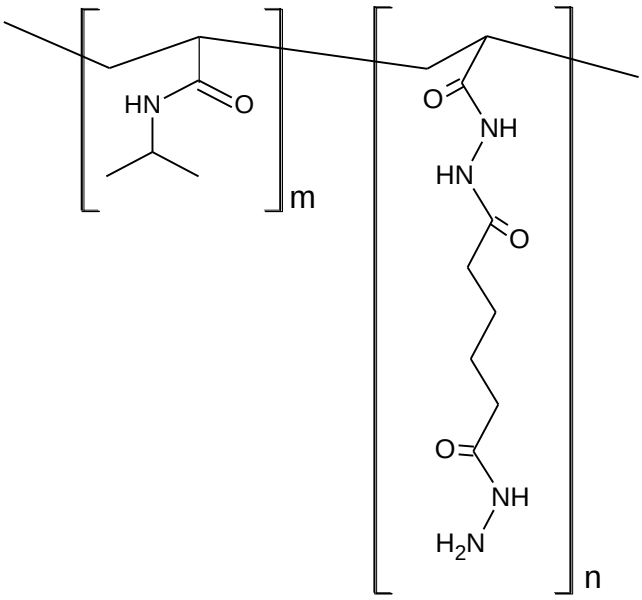


Figure 4.3 *Molecular structure of the repeating unit of PNIPAM-*co*-ADH.*

The product was electric, pure white and had cotton wool like consistency (Picture 4.1).

Picture 4.1 *PNIPAM-*co*-ADH.*

4.2.2 The oxidation of polysaccharides

A known procedure, oxidation of polysaccharides to aldehydes, was used for hyaluronic acid and alginate. Sodium periodate was used as oxidizer. The reaction was carried out according to Ossipov et al. (2008).

4.2.2.1 The periodate oxidation of hyaluronic acid

500 mg of hyaluronic acid (HA) was put in to a three-necked flask and 100 cm^3 of de-ionized water was added (Picture 4.2). It was left to stir until HA was completely dissolved. This lasted usually circa 16 hours.

Picture 4.2 *Setup for oxidation of hyaluronic acid.*

Nitrogen flow was provided to the flask through a syringe. The nitrogen gas was vented through a needle in the septum. In the dark, a sodium periodate solution (2.7 cm^3 H_2O) was prepared and added into the HA solution with a syringe (Table 4.2). Sodium periodate is oxidizing, toxic and irritating so gloves and safety goggles must be worn.

Table 4.2 *Amount of reactants, reaction times and degrees of oxidation of two batches of hyaluronic acid oxidized with sodium periodate.*

	HA-1	**HA-2**
HA (g)	0,501	0,502
$NaIO_4$ (g)	0,301	0,298
Reaction time (h)	4	20
Degree of oxidation (%)	7	25,9

Reaction was kept ongoing for a fixed number of hours as given in Table 4.1. Reaction (Figure 4.3) was stopped by adding 0.3 cm^3 of ethylene glycol and allowed to react for one hour.

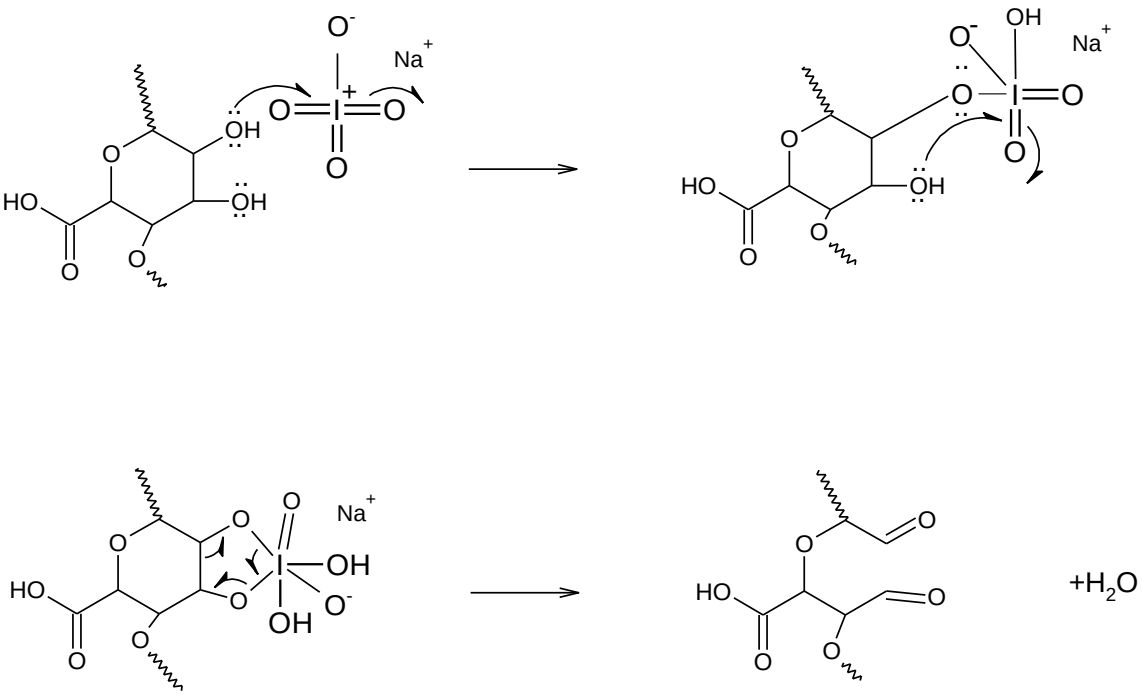


Figure 4.3 *Sodium periodate oxidation of hyaluronic acid.*

The product (Figure 4.3) was purified by dialysis for three days with a 25 kDa membrane. The membranes were rinsed with deionized water and the water was changed every day.

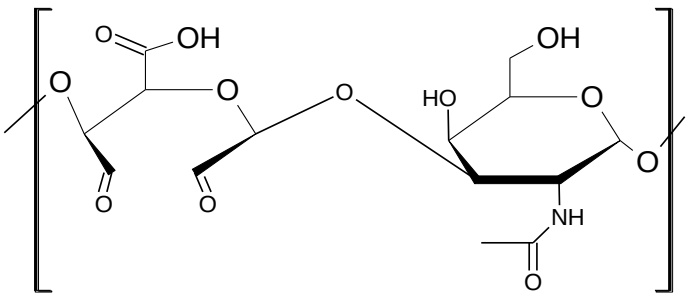


Figure 4.4 *The molecular structure of oxidized hyaluronic acid.*

Afterwards the product was lyophilized. Two batches of HA were oxidized.

The product had a cotton wool like consistency (Picture 4.3). It was very electric and difficult to handle.

Picture 4.3 *Oxidized hyaluronic acid.*

4.2.2.2 Periodate oxidation of alginate

The overall procedure was similar to the oxidation of hyaluronic acid. The oxidizable structure in alginate (mannuronic acid) resembles that of glucuronic acid; hence the oxidation reaction is similar to that of HA. (Ossipov 2008) Amounts of reagents are given in Table 4.3.

Table 4.3 *Amount of reactants and reaction times of two batches of alginate oxidized with sodium periodate.*

	AL-1	**AL-2**
Alginate (g)	0,5007	0,5000
$NaIO_4$ (g)	0,287	0,292
Reaction time (h)	20	3

Alginate was oxidized in two batches which varied with time. The periodate ion cleaves a bond forming the corresponding dialdehyde (Figure 4.5). Mannuronic acid is cleaved.

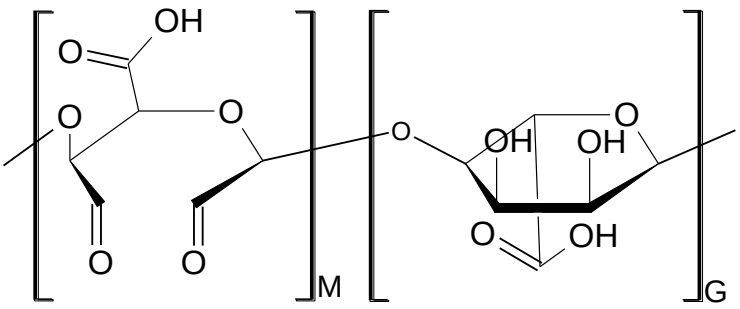


Figure 4.5 *The molecular structure of oxidized alginate.*

The state of the product was fluffy and white, similar to that of oxidized hyaluronic acid. Oxidized alginate was very electric. It was also very difficult to handle.

The oxidized hyaluronic acid, oxidized alginate and modified copolymer PNIPAM-*co*-ADH are best to be stored in the refrigerator in a tightly sealed container. They should not be exposed to light. The humidity and light might affect their structure in an unfavorable way.

4.2.3 Characterization methods

Reactants and products were characterized with different methods. The structures of alginate, oxidized alginate, hyaluronic acid and oxidized hyaluronic acid were confirmed with Nuclear Magnetic Resonance (NMR) spectroscopy and Fourier transform infrared spectroscopy (FT-IR). The spectra obtained were compared with those found in the literature. Copolymer and its derivatives were also studied with aforementioned methods. Obtained spectra were analyzed in the same extent as those of natural polysaccharides that is to confirm their structures. The intrinsic viscosity of PNIPAM-*co*-AA and alginate were measured.

Nuclear magnetic resonance spectroscopy (NMR)

The nuclear magnetic resonance spectroscopy (NMR) is based on the energy states of magnetic atom nuclei. The behavior of nuclei in the magnetic field depends on the environment of the nuclei and these dependences are seen in the spectrum of the nuclear magnetic resonance signal as a chemical shift of the resonance frequency. NMR is the only method that can be used to solve the structure of a dissolved molecule and its three-dimensional structure. NMR spectra are usually measured for samples in solution and it is a nondestructive method. (Larive 2012)

In this study Varian Mercury 300 MHz NMR Spectrometer (Palo Alto, USA) was used to characterize all the reactants and products used. Aldehyde determination reactions were made only for oxidized hyaluronic acid. Samples were prepared on a previous day before measurement due to the poor solubility of materials. Spectroscopic grade D_2O was used as a solvent. The concentration of the samples was 5 mg in 0.6 cm^3.

Fourier transform infrared spectroscopy (FTIR)

The interaction of matter with infrared radiation is studied by Fourier transform infrared spectroscopy. With FTIR technique, a beam containing multiple frequencies of light is led through a sample. A sensor measures how much light is absorbed by the sample. Electromagnetic waves from 800 nm to 1 mm are used. This corresponds to (12 500–10) cm^{-1} wave-number units which are usually used. Most stretching and bending fundamental vibrations of molecules occur in the mid-infrared region at 200–4000 cm^{-1}. (McDowell 2014)

In this thesis, the FTIR spectra were measured with Perkin Elmer Spectrum One FT-IR Spectrometer (Waltham, MA, USA). The wave number employed was from 0 to 4000 cm^{-1}. (1-2) mg of the sample was weighed and mixed with 200 mg of KBr. KBr pellet was used as a background reference.

Viscometry

The intrinsic viscosity can be defined with Ubbelohde-type viscometer. A liquid is introduced into the reservoir in the glass capillary. The sample is sucked through the capillary and measuring bulb. The liquid is allowed to travel back through the measuring bulb. The time is measured for the liquid to pass through two calibrated marks on the capillary. Viscosity can be obtained from these draining periods. These are repeated for parallel samples and pure solvent. (Harding 1997)

Lauda PVS viscometer (Ubbelohde type, pipes 0C, capillary diameter 0.36 mm, Lauda-Königshofen, Germany) was used in measuring the single-point intrinsic viscosities in the Biomaterials laboratory. The intrinsic viscosity of PNIPAM-*co*-AA was measured by dissolving the copolymer into water, chloroform and ethanol. For alginate, the viscosity measurements were performed in water and in a 0.1 M NaCl solution.

4.2.3.1 The number of aldehyde groups in oxidized hyaluronic acid

To attain the exact number of aldehyde groups in oxidized hyaluronic acid (OxHA), an acetate buffer solution with pH 5.23 was prepared in the Biomaterials laboratory by mixing 79 cm^3 0.2 M sodium acetate solution (prepared according to Lange's Handbook of Chemistry and Physics from Sigma-Aldrich's ≥99 % powder form sodium acetate) and 21.0 cm^3 of 0.2 M acetic acid (Sigma-Aldrich, ≥99.7 %). OxHA (20 mg) and 2 cm^3 of buffer solution were mixed. *Tert*-butyl carbazate (0.0348 g) and 1 cm^3 of buffer solution were gently mixed in a small glass bottle. Two aforementioned solutions and 1 cm^3 of buffer solution in a glass bottle were evacuated and put in to the glove box with nitrogen atmosphere.

Buffer solution (1 cm^3) and $NABH_3CN$ (0.0166 g) were mixed in the glove box and added to OxHA after one hour. The reaction was left to stir for 24 h (Figure 4.5).

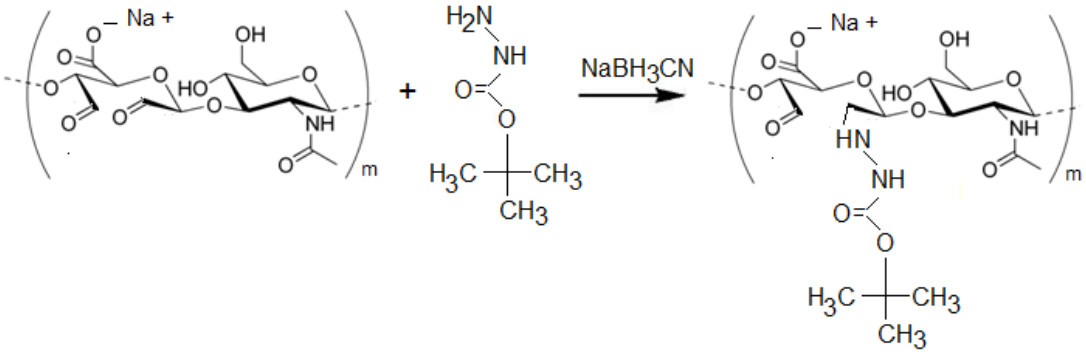


Figure 4.5 *The reaction between oxidized hyaluronic acid and* tert-*butyl carbazate.*

OxHA solution was precipitated three times with surplus acetone (Sigma-Aldrich, ≥99 %). Finally, the precipitate was dissolved in 10 cm^3 of deionized water. The product was dialyzed with 25 kDa membrane; 24 h in 0.1 M NaCl (Sigma-Aldrich, ≥99 %) and three days in deionized water. The sample was lyophilized. The dry product was subjected to ^1H NMR examination. The degree of oxidation (aldehyde content) was calculated by integrating the peaks using software (ACD NMR Processor) and comparing the signals around 1.4 ppm (9H, carbazate t-Boc) and 1.9 ppm (3H, oxHA acetamide) (Equation 1). (Jia 2004.)

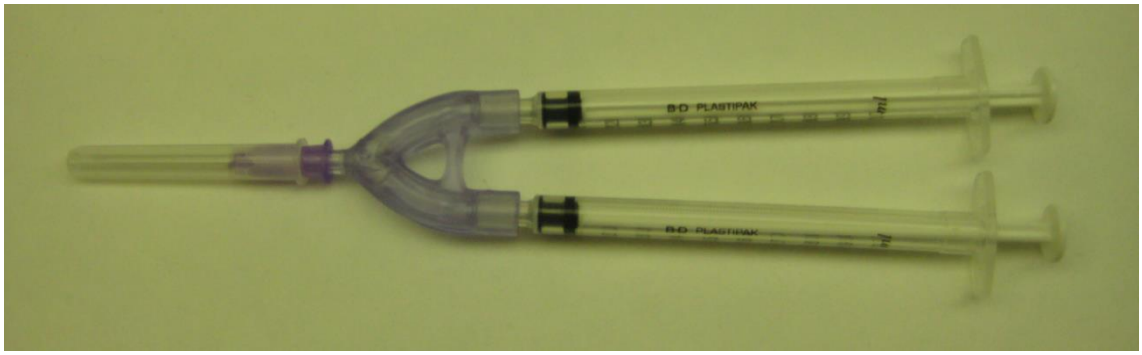
$$Degree\ of\ oxidation = \left(\frac{A_{t-Boc}}{A_{methyl*3}}\right) * 100\% \quad (1)$$

The integrated areas are shown in NMR spectrum of HA-1 (Appendix 10). Results are presented previously in Table 4.2.

4.2.4 Gelling

In gel tests, the components were treated modularly. This means that the components were simply mixed together without any extra stimulus i.e. light or heat.

The components were mixed with a pipette in a cut plastic syringe or with two syringes integrated with an adapter (Picture 4.4).



Picture 4.4 *Two syringes integrated with an adapter.*

Two gelation tests were made with the copolymer from the first reaction, KOPO-1 (Table 4.1). A 10 % solution of sucrose was prepared. Polymer and polysaccharides were dissolved to a sucrose solution and stirred overnight. Gels were prepared the next day with sample sizes of 0.2 cm^3. 0.1 cm^3 of copolymer and 0.1 cm^3 of oxidized hyaluronic acid were pipetted to a 1 cm^3 syringe which had the tip cut off. The solutions were rapidly stirred with a pipette tip. The gelation was observed in four time points: zero, one, five and 60 minutes. After 60 minutes, the syringe was emptied to a petri dish or opaque vial.

The procedure was repeated with sample size of 0.6 cm^3 to improve the observation of the progress of gelling. The solutions were heated in water bath to 37˚ C to observe the effect of the elevated temperature.

Four gelation tests were made with KOPO-2. The procedure was similar with KOPO-1 tests except the tests were carried out in room temperature. The solutions were prepared from distilled water instead of sucrose solution. Polyacrylamide based materials gel in sucrose solution if they gel in water. In the last two gelation tests, the KOPO-2 solutions were kept in 4˚ C to try out differences in solubility and manageability in comparison with room temperature.

5 RESULTS

Results from characterizations are presented. All NMR spectra except those of PNIPAM-*co*-AA and its derivatives are available in Appendices 3, 4 and 5.

5.1 NMR measurements

5.1.1 PNIPAM-*co*-AA and PNIPAM-*co*-ADH

Chemical shifts *(δ)* at 3.91 and 3.67 ppm peaks respond to the —CH and —CH_2 groups. —CH and —CH_2 peaks are at 2.02 and 1.59 ppm (PNIPAM-*co*-AA). —CH_3 gives a peak at 1.15 ppm. H_2O peak is at 4.75 ppm. PNIPAM-*co*-AA and PNIPAM-*co*-ADH spectra are presented in Figure 5.1.

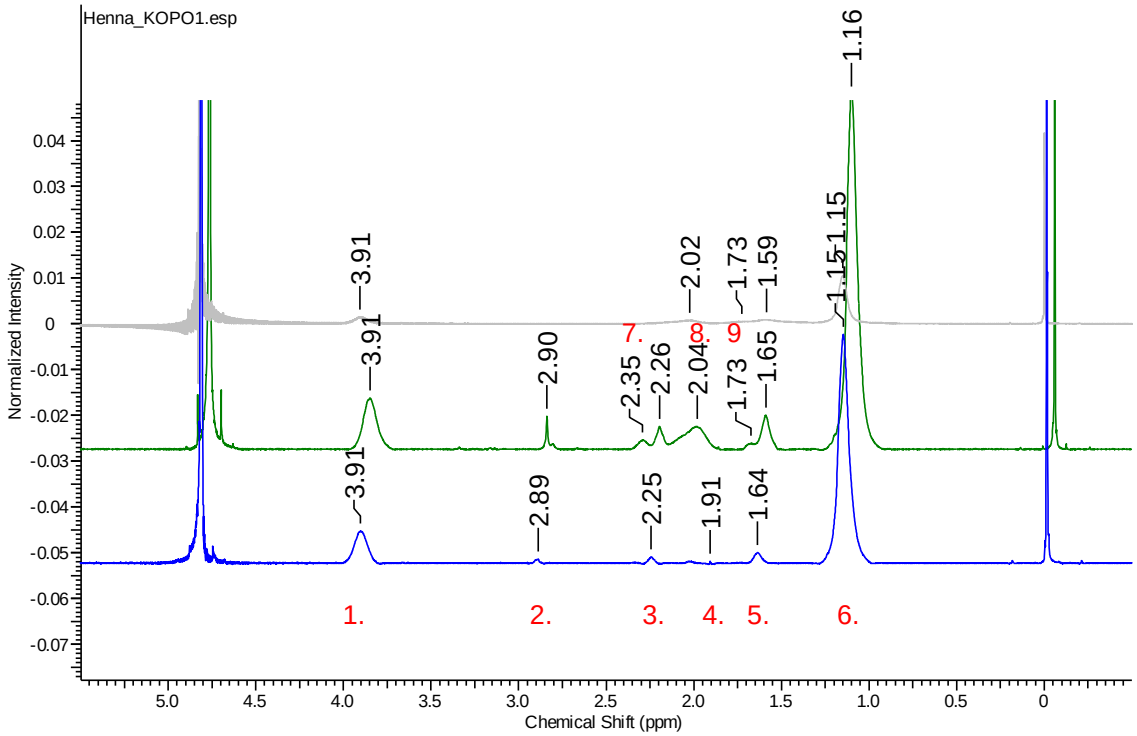


Figure 5.1 *PNIPAM-*co*-AA (grey), KOPO-1 (blue) and KOPO-2 (green).*

The amino group proton and the carboxylic proton are overlapped by the resonance of the deuterated water. (Larsson et al. 2001) The chemical shifts (δ) are given in Table 5.1.

According to Tzoc-Torre (2011), 0.5 ppm -1.2 ppm peaks are from the methyl groups from NIPAM, 1.2 ppm-1.8 ppm peaks are from the methylene groups (—CH_2) from

NIPAM and AA, 1.8 ppm -2.5 ppm peaks are from the methinyl groups (>CH—) from NIPAM and AA. (Tzoc-Torre 2011)

Table 5.1 *Chemical shifts and corresponding protons in PNIPAM-co-AA, KOPO-1 and KOPO-2.*

	$(CH_3)_2$	**$—CH_2—$**	**$—(CH_2)_4—$ (ADH)**	**—CH—**	**$—NH_2$ (ADH)**	**$—NCH_2$**	**>CH—**
***δ* (ppm)** (1)	1.1	1.5	-	1.9	-	-	3.8
PNIPAM-*co*-AA	1.16	1.59	(1.73)	2.02	-	-	3.91
KOPO-1	1.15	1.64	-	1.91	2.25	2.89	3.91
KOPO-2	1.15	1.65	1.73	2.04	2.26	2.90	3.91

(1) Larsson et al. 2001

5.1.2 Hyaluronic acid

The chemical shifts *(δ)* of hyaluronic acid and its derivatives are presented in Table 5.2. The NMR analysis of the oxidized products showed signals close to 3.60 ppm, which correspond to the hemiacetal formation of the aldehyde function.

Table 5.2 *Chemical shifts and corresponding protons in hyaluronic acid and its derivatives.*

	***t*-Boc**	**—NCOCH3**	**—O—CH—C | C $—CH_2OH—$**	**C—CH—O | O**
***δ* (ppm)** (1,2)	-	2.07	3.51 -3.62	4.61
Hyaluronic acid	-	2.03	3.52	4.62; 4.69; 4.74
HA-1	1.25 -1.45	2.03	3.35 -3.84	4.46 -4.89
HA-2	1.25 -1.45	2.03	2.98 -4.89	4.22 -4.89

(1) Bodnar et al. (2009)

(2) Martinez-Sanz (2011)

5.1.3 Alginate

The chemical shifts *(δ)* are presented in Table 5.3.

Table 5.3 *Chemical shifts and corresponding protons in alginate and its derivatives.*

	Glucuronic acid	**Mannuronic acid**
***δ* (ppm)** (1,2,3)	3.42-3.71; 3.81; 5.37	3.78; 3.82-4.18
Alginate	3.76-4.19	4.46-5.01
AL-1	3.67-4.46	4.74-5.27
AL-2	3.77-4.38	4.73-5.19

(1) Cocco et al. 2003
(2) Davis et al. 2003
(3) Gomez et al. 2007

5.2 FTIR measurements

All Fourier transform IR spectra except those of PNIPAM-*co*-AA and its derivatives are presented in Appendix 6.

5.2.1 PNIPAM-*co*-AA

The FTIR data is given in table 5.4. The characteristic doublet of PNIPAM-*co*-AA at 1368 cm^{-1} and 1387 cm^{-1} were evidence of the presence of isopropyl groups —$CH(CH_3)_2$) stretching.

Table 5.4 *FTIR data on PNIPAM-*co*-AA, KOPO-1 and KOPO-2.*

Group	**$C(CH_3)_2$** ***(υ)***	**$C(CH_3)_2$** ***(υ)***	**CH** ***(δ)***	**—COO** ***(υ)*** (2)	**—C=O** ***(υ)***	**CH_3** ***(υ)***	**N—H** ***(υ)***
cm^{-1} (1)	1366	1387	1460	1569	1648	2877	3292
PNIPAM-*co*-AA	1368	1387	1458	1539	1641	2878	3433
Group	**N—H** ***(γ)***	-			**—C=O (υ)**	**N—H** ***(υ)***	**—NH—NH_2 | *(υ)***
KOPO-1	655-682				1700	2935-3073	3434
KOPO-2	663-673				1703	2935-3071	3435

(1) Chen et al. 2013
(2) Mori et al. 2006

The peaks at 1458 cm^{-1} and 2973 cm^{-1} resulted from the bending of (CH) and methyl (—CH_3) groups stretching of —$CH(CH_3)_2$, respectively. 1641 cm^{-1} band represents amide I (—C=O stretching) band of NIPAM moiety. (Chen et al. 2013) Deprotonated —COO^- group appears at 1539 cm^{-1} (Figure 5.2) (Mori et al. 2006)

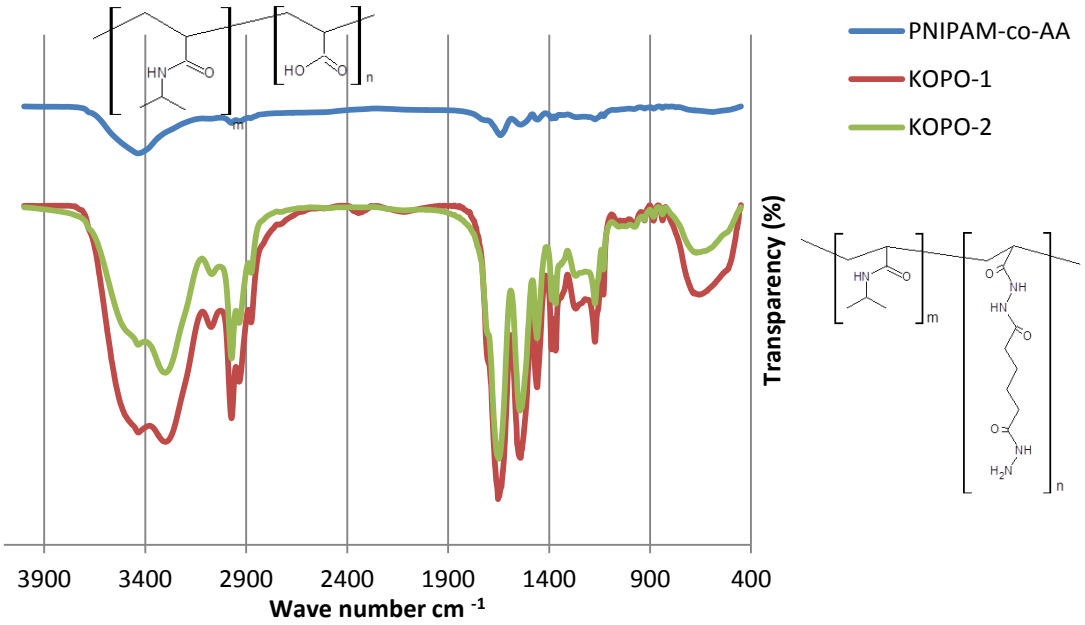


Figure 5.2 *FTIR spectra of PNIPAM-co-AA, KOPO-1 and KOPO-2.*

5.2.2 Hyaluronic acid

Infrared spectroscopy showed presence of —OH stretch of COOH group at 3432 cm^{-1}, —CH stretch at 2923 cm^{-1}, COO— stretch at 1636 cm^{-1}, —NH stretch at 1548 cm^{-1} and C—O ether at 1052 cm^{-1}. (Yue 2012)

Table 5.5 *FTIR data on hyaluronic acid, HA-1 and HA-2.*

Group	C—OH (υ)	—NH (υ)	C=O (planar) (υ)	C=O (υ)	—CH (υ)	—OH —NH (υ)
cm^{-1} (1)	1043	1563	1617	-	2916	3412
Hyaluronic acid	1052	1548	1636	-	2923	3432
HA-1	~1050	~1550	~1600	1722	~2900	~3400
HA-2	~1050	~1550	~1600	1733	~2900	~3400

(1) Yue et al. 2012

In the spectra of oxidized hyaluronic acid batches the characteristic aldehyde shoulder was at 1722 and 1733 cm^{-1}. Carboxyl peak had shifted to 3398 cm^{-1} and carbonyl peak to 1613 and 1618 cm^{-1}.

5.2.3 Alginate

The IR spectrum had characteristic absorption bands for hydroxyl groups at 3435 cm^{-1} (stretch), carboxylate at 1419 cm^{-1} and carboxyl at 1629 cm^{-1}. (Nagpal 2013) The bands at 1316 cm^{-1} (C–O stretching), 1130 cm^{-1} (C–C stretching), 1099 cm^{-1} (C–O stretching), 1035 cm^{-1} (C–O–C stretching), and 947 cm^{-1} (C–O stretching) are indicators of saccharide structure. (Sartori 1997)

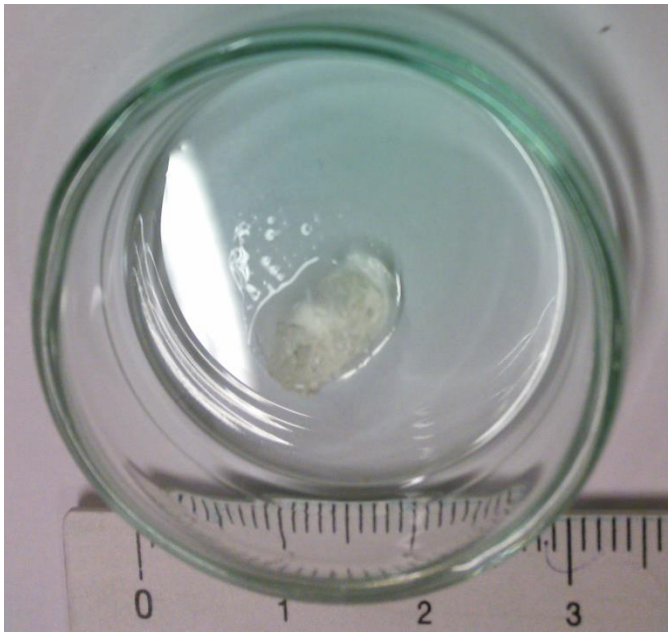
Table 5.6

Group	C—O (υ)	C—O (υ)	C—C (υ)	COOH (υ)	COO^- (υ, ring)	C=O (υ)	—CH (υ)	OH (υ)
cm^{-1} (1)	947	1035		1416	1616	-	2929	3421
Alginate	947	1099	1130	1419	1629	-	2928	3435
AL-1	-	-	-	-	1616	1733	-	-
AL-2	-	-	-	-	1616	1730	-	-

In the spectra of oxidized alginate batches the characteristic aldehyde shoulder was at 1733 and 1730 cm^{-1}. The asymmetrical stretch of carboxyl group peak at 1629 cm^{-1} had shifted to 1616 in both spectra.

5.3 Gelling

PNIPAM: AL-2 gel with 1:1 ratio and solutions with concentration of 60 mg/cm^3 resulted in slightly beige and opaque gel with liquid (Picture 5.1).



Picture 5.1 *Hysteresis in hydrazone linked PNIPAM: AL-2 gel with 1:1 ratio.*

From all the ratios and concentrations tested, the syneresis was the slowest with 1:1 ratio. Gel test results are presented in Appendix 2. Other combinations appeared not to produce a stable gel. Hysteresis increased with time. Gels came apart when they were gently rubbed between fingers.

5.4 Viscosity measurements

The intrinsic viscosity of PNIPAM-*co*-AA was measured by dissolving the copolymer into ethanol. It was also tried to dissolve it to water. In the specification sheet provided by the manufacturer the viscosity value was given in water. It was dissolved in ethanol. The results from measurements with ethanol varied too much to attain a reliable result. For PNIPAM-*co*-AA in ethanol (25 ˚C) the measured viscosity values range from 0.37 dm^3/g to 0.72 dm^3/g. The draining periods varied from 637 to 914 s. Tubes in the viscometer of Biomaterials Laboratory are not suitable for such long draining periods.

For alginate in water (25 ˚C) the three attained intrinsic viscosity values are 0.913, 0.954 and 0.994 dm^3/g. For alginate in 0.1 M NaCl the intrinsic viscosity values were 3.51, 3.68 and 4.09 dm^3/g. With these results and using Mark-Houwink (α = 1.13, $K = 6.9*10^{-4}$ cm^3/g at 25˚ C) constants of Martinsen et al. (1991), alginate molar mass was roughly estimated to be 120 000 mol g^{-1} (mean value). Zhong et al. (2010) used a 0.40 m diameter Ubbelohde tubes.

6 DISCUSSION

6.1 PNIPAM-*co*-AA

The synthetic poly (*N*-isopropyl acrylamide), PNIPAM, has been studied as an injectable hydrogel for medical applications. It has many advantages, e.g. homopolymer has a convenient lower critical solution temperature close to the body temperature (30-32) ˚C. For injectable hydrogels, there are a few important basic requirements. It has to have a low or moderate viscosity and gel within a reasonable time window. It has to maintain a desired volume and have contact through an interface with tissue after gelation. When aiming for commercial application, a long shelf life and sterilization are essential. (Schmidt et al.2010; Byeongmoon et al. 2012; Ekenseair et al. 2012)

PNIPAM is not biodegradable. It needs to be copolymerized in order to make it partially degrade. The reaction between aldehyde modified polysaccharides and hydrazides is very fast and the resulting hydrazone bonds will be labile to hydrolysis, which leads to gels that degrade in aqueous media. (Lee et al. 2000)

Copolymer PNIPAM-*co*-AA was modified with adipic acid dihydrazide (ADH) to attain hydrazide functionality to the copolymer chain. *N'*-ethyl-*N*-(3-dimethylaminopropyl)-carbodiimide (EDC) was used as a crosslinker. When modified copolymer solutions were heated they became turbid. This is due to the swelling of the copolymer which is characteristic of PNIPAM based materials. As the temperature is raised to cloud temperature, PNIPAM chains collapse. Collapsed chains aggregate and solution turn turbid. In the gels prepared within this thesis, the syneresis was the topmost phenomenon. There was a considerable portion of the liquid phase in gels. When gels were prepared from solutions at 0˚ C, the portion of the gel was larger. Syneresis seems to be extensive in gels prepared from heated solutions. This might be due to the inhibition of molecular movement in a collapsed state of the polymer coils. (Chang et al. 1997)

PNIPAM based gels often undergo syneresis which means shrinking and separation from solvent. This phenomenon can lead to high burst release especially in the case of hydrophilic drugs. Also the monomer is harmful though the polymer is not. There is also always risk for monomer residues to remain in the polymer or to appear over time with disintegration. (Overstreet et al. 2012) The commercial copolymer had 15 mol-% of carboxylic acid groups. It is impossible to convert all carboxyl functional groups into dihydrazide functionality. Even if all these groups could have been modified, the amount of crosslinks between copolymer and polysaccharide would have been inade-

quate. This might be one reason for the weakness of the gel structure. Also, the solution with 60 mg/ml concentration might be too thick for sterilization.

In the characterization of PNIPAM-*co*-AA has to be taken in account its hygroscopic nature. When dissolved in water, the carboxylic groups might deprotonate. This can be observable e.g. in FTIR spectroscopy signals.

6.2 Hyaluronic acid and alginate

Periodate oxidation is a recognized method for gaining flexibility in polysaccharides. It has limitations e.g. an oxidation limit of about 44 % occurs in alginates due to intramolecular hemiacetal formation. (Painter 1973) The hyaluronic acid and alginate solutions were quite easy to prepare and handle. There is a vast amount of knowledge about these materials and the oxidation procedure is well established. The aldehyde structure is sensitive to air and moisture. The unwanted change into acidic structure can be voided through proper storing conditions. In this study, the materials were kept in an incubator in the refrigerator. The materials were used almost immediately so there should not be any changes in the structure.

NMR and FTIR measurements confirm successful oxidation modifications in alginate and hyaluronic acid. It was verified that hyaluronic acid had oxidized according to a well-known procedure with sodium periodate. Periodate treatment oxidizes the proximal hydroxyl groups to aldehydes opening the sugar ring to form a linear chain. While periodate oxidation allows for the formation of a large number of functional groups, the disadvantage is the loss of the backbone structure. In the literature it is mentioned that this modification procedure is not favorable for cells. (Bulpitt 1999)

6.3 The gels

In this thesis it is presumed that all 15 % of the carboxylic acid groups in the PNIPAM-*co*-AA had been replaced with dihydrazide groups. The number of dihydrazide groups remains unknown. Even if all of the carboxylic groups had been altered, the 15 % is still not enough the keep the gels together. The degree of crosslinking has effects on e.g. hydrogel swelling. (Krušić et al. 2006)

The dilute PNIPAM-*co*-ADH solutions were much easier to handle when they were first kept in the refrigerator. Keeping them cool hinders the movement of the molecules in the water. Unfortunately dilute PNIPAM-*co*-ADH solutions did not form any gels with oxidized hyaluronic acid and alginate. The sterilization of dilute, flowing solutions is not a problem. In richer solutions e.g. 60 mg/cm^3 concentration of the polymer, the solution cannot be called liquid-like. It reminded more of a gel and it was difficult to try to mix the components. One possible option could be to prepare thick PNIPAM-*co*-ADH

gel first and stir it continuously. Then the other components could be added in with a needle. The problem of injectability would remain. The stiffness of the gel can be modified with the molar mass of the PNIPAM.

6.4 The characterization

The data on the copolymer material is inadequate. The molar mass of the copolymer would have been important to know. In this work the amounts of reagents are almost educated guesses due to lack of information on PNIPAM-*co*-AA. With NMR and FT-IR methods it was only confirmed that there had been a change in the molecular structure. For polymers, the FTI-R is in general an informative method. With size exclusion chromatography there might have been a possibility to clarify the molar mass of PNIPAM-*co*-AA.

The viscosity measurements made with Ubbelohde equipment do not seem reliable. The deviation in the results is too large. The reason for this might be the relatively bad solubility of PNIPAM-*co*-AA in ethanol and alginate in water. Materials may sediment in the tubes of the viscometer. (Dan et al. 2010) The equipment used in the Biomaterials Laboratory was not suitable for these materials.

For hyaluronic acid the NMR and FT-IR were relatively good methods. There is also good amount of references in the literature so the authentication of the material and changes in the structure were quite straightforward. The NMR spectrum of alginate was measured dissolving dried alginate in D_2O. Another solvent might have been better for this purpose. The alginate peaks are very close to the solvent peak and they get interfered. It is possible to attain more detailed information on alginate structure with NMR through performing acid hydrolysis. This pre-treatment depolymerizes the alginate and reduces the solution viscosity. The sequence of monomer residues notably influences the chemical shifts. This specification was not sensible for this thesis. (Cocco et al. 2003; Davis et al. 2003; Gomez et al. 2007) In the research of alginate with FT-IR it would be interesting to know the ratio between the mannuronic and glucuronic acid. It has an effect on the band intensity. (Sartori et al. 1997) Alginate is a very well-known material so good references could be found. The seaweed extract is still under investigation and new data is being analyzed.

6.5 General

The gels prepared in this study were not stable enough to be exposed to further characterizations. Pressure test, rheological measurements, swelling tests and cell tests are examples of what could be studied. As mentioned earlier, PNIPAM and its derivatives are being studied for cell culturing applications (Yongqing et al. 2013). Currently, it has to be doped with different cell anchoring substances. There is a need in tissue engineer-

ing for a substrate to grow cell monolayers on and without extra additives. It should be a bulk product and have good usability. In this sense, an *in situ* prepared hydrogel for cells to grow, which properties were modifiable for a desired cell type, would present an ideal product.

7 CONCLUSIONS

The goal in this study was to find a biocompatible, cell life supporting and injectable hydrogel with suitable mechanical properties. It was proven to be a challenging task. PNIPAM-*co*-AA was functionalized with dihydrazide (PNIPAM-*co*-ADH) using a carbodiimide crosslinker. Hyaluronic acid and alginate were oxidized with different amounts of sodium periodate to gain aldehyde functionality. Hyaluronic acid and alginate with aldehyde functional groups were mixed with PNIPAM-*co*-ADH to with create *in situ* gelling hydrazine crosslinked hydrogels. The gels were prepared with different component ratios, different temperatures and methods. Gels prepared were not stable enough to execute further testing. PNIPAM-alginate gel prepared from 4 °C solutions with 1:1 ratio was the most stable combination. Even the most stable gel did not KESTÄÄ handling and fell apart in an hour.

In the future, it would be sensible to prepare the copolymer in the laboratory instead of purchasing it. Basic polymerization equipment is relatively inexpensive. Through regulation of the amounts of reagents it is possible to prepare a copolymer with a desired amount of modifiable groups. This way the knowledge of the material is better from the start. To the copolymer it might have been good to execute size exclusion chromatography to clarify the molar mass. When the molar mass is known, it makes a whole new set of analysis methods possible. Also, the alginate should be characterized properly. The differences in structures between different types of alginates seem trivial, but the influence of the structure over gelation properties is considerable. There are established methods for defining detailed structure of alginate. The employed modular way in this thesis to make hydrogels is ideal for a medical hydrogel application. The components remain unreactive until they are combined in a sterile environment such as hospitals. Storing the components in a right manner is crucial. The functionality of the materials should be checked regularly. The PNIPAM-*co*-ADH solutions were very viscous. Instead of trying to pipette them a mold could be developed. Then the PNIPAM-*co*-ADH gel could be kept in movement with the mold and the other component could be added to it to get an even two-component gel. Thus, the concept of injectability should be rechecked.

In this study, the oxidation reactions were quite straightforward to execute. I have gained good hands-on experience from TUT laboratory courses and they became in use in this work. It was also very satisfying to notice that my article reading skills got a lot better. I learned to concentrate on what is relevant.

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APPENDIX 1. HYDROGELS STUDIED FOR TREATMENT OF MYOCARDIAL INFARCTION

Hydrogel	Injection after myocardial infarction (MI)	Gelation mechanism and time	Stability	Cells used in compatibility testing	Mechanical properties	Advantages	Disadvantages
Natural origin							
Alginate-polypyrrole (Mihardja et al. 2008)	One week	Ionic	-	Human umbilical vein endothelial cells	Best stiff-ness for cells with 0.025% concentra-tion of polypyrrole	Enhanced cell attachment, filtration, proliferation and angiogenesis	Quality of alginate, changes in polypyrrole conductivity, difficulties modifying polypyrrole
Arginine-Glycine-Aspartic acid (RGD) modified alginate+ alginate (Yu et al. 2009)	Five weeks	Ionic	-	Human umbilical vein endothelial cells	Best cell growth with alginates over 50% of mannuronic acid	RGD-modified alginate enhanced angiogenesis more than alginate	Uneven quality of alginate
Chitosan+ fibroblast growth fac-tor (Fujita et al. 2004)	Immediate	UV induced; 30 s	After four weeks chitosan residuals	-	-	Induced vascularization, prolonged the half-life of growth factors	Retain of growth factor's biological activity
Methacrylated hyaluronic acid MeHA (Tous et al. 2011)	30 min	-	Stable	-	Adjustable with crosslinking	Increased vessel formation, myocardial thickness better than HeMAHA	Inflammation

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Hydrogel	Injection after myocardial infarction (MI)	Gelation mechanism and time	Stability	Cells used in compatibility testing	Mechanical properties	Advantages	Disadvantages
Methacrylated hyaluronic acid HeMA-HA (Tous et al. 2011)	30 min	-	Hydrolytic bulk degradation, 3-10 weeks	-	Increased methacrylation led to increased compressive moduli	Increased vessel formation	Inflammation
Myocardial matrix from porcine (Singelyn et al. 2009)	MI not induced	Self-assembling *in situ*	-	For viability neonatal rat cardiomyocytes; for migration human coronary artery endothelial cells and rat aortic smooth muscle cells	Pore size 30 μm, fibers 40-100 nm	Promotes vascular cell migration *in vitro* and infiltration *in vivo* in 1-2 weeks	Xenograft issues, 3D properties partially lost, difficult to prepare
Synthetic							
Dextran-PCL-HEMA/PNIPAM (Wang, T. 2009)	Four days	Thermal, *in situ*	30 days i*n vivo*	-	-	Thicken the scar, improves ventricular function, prevent left ventricle remodeling after 30 days	Effects on diastolic function and arrhythmia

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Hydrogel	Injection after myocardial infarction (MI)	Gelation mechanism and time	Stability	Cells used in compatibility testing	Mechanical properties	Advantages	Disadvantages
Poly (NIPAM-*co*-AA-*co*-HEMAPTMC) (86/4/10) (Fujimoto et al. 2009)	14 days	Thermal, time controlled through acrylic acid content	Hydrolytic cleavage in five months *in vitro*	Rat vascular smooth muscle cells	Elastic, easy to handle	Contractile function improved, ventricular dilation	Extra cells found in left ventricle wall; changes in left ventricle architecture and function in long-term
NIPAM+ AA+ NAS+ HEMA-PTMC (Li et al. 2012)	-	6-7 s in 37° C	92 ± 2% of weight remaining after a 14 days	Mesenchymal stem cells	Best cardiomyocyte differentiation with 65 kPa; flexibility	-	-
Vinyl sulfone modified polyethylene glycol (Dobner et al. 2009)	Two minutes	Redox initiation; few minutes in 37° C	Non-degradable	-	Stiffness mimicking that of myocardial tissue's	Increased scar thickness and decreased heart dimensions	Inflammation, thickened myocardial wall, fractional shortening not improved
Polyethylene glycol+ hyaluronic acid (Yoon et al. 2009)	14 days; ten minutes	-	Four weeks (hydrolysis)	-	-	Easy to apply; myocardial recovery	Reliability of the study: degree of MI not severe

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Hydrogel	Injection after myocar-dial infarction (MI)	Gelation mechanism and time	Stability	Cells used in compatibility testing	Mechanical properties	Advantages	Disadvantages
Fibrin glue (2% fibrinogen) and mesenchymal stem cells (Martens et al. 2009)	Immediate	*In situ;* 6–8 min for <15% fibrinogen	-	Human mesenchymal stem cells	Pore size 20-40 μm	Enhanced cell retention, prevented cell redistribution to other organs	Viscosity problems
Matrigel (Ou et al. 2011)	Immediate	-	-	-	-	Enhanced systolic and diastolic properties of the infracted tissue, promoted neovascularization	No reduction in the size of MI
Matrigel+ mouse embryonic stem cells (Kofidis et al. 2004)	Immediate	Solid after few minutes from mixing	-	-	-	Prevented ventricular wall thinning, cells viable in Matrigel, cardiac function better than in peer group	Possibility of tumor formation

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Hydrogel	Injection after myocardial infarction (MI)	Gelation mechanism and time	Stability	Cells used in compatibility testing	Mechanical properties	Advantages	Disadvantages
Silanized hydroxypropyl methylcellulose hydrogel with cells (Mathieu et al. 2012)	Immediate	Self-setting, 30 minutes	Eight weeks in bone	Mesenchymal stem cells	Elastic modulus 0,3 kPa	Limited the extent of transmural MI, preserved endocardial myocytes and reduced metaplasia, can be steam sterilized	Only short- and mid-term recovery of ventricular function; deterioration after four weeks

APPENDIX 2. GEL TEST RESULTS

Symbol	Key
A	Liquid
B	Small traces of gel in liquid
C	Almost completely gelled
D	Completely gelled

Gel type		Component A			Component B			Ratio of components (A:B)	Polymer concentration (%)	Gelation temperature (^{o}C)	Gelation time points (min)			
			mg/cm^3	cm^3		mg/cm^3	cm^3				0	1	5	60
PNIPAM-HA	1	KOPO-1	15	0.1	HA-1	15	0.1	1:1	1.5	r.t.	B	B	B	C
	2	KOPO-1	20	0.1	HA-1	10	0.1	2:1	1.5	r.t.	B	B	B	B
	3	KOPO-1	25	0.1	HA-1	5	0.1	5:1	1.5	r.t.	B	B	B	B
	4	KOPO-1	15	0.1	HA-2	15	0.1	1:1	1.5	r.t.	B	B	B	B
	5	KOPO-1	20	0.1	HA-2	10	0.1	2:1	1.5	r.t.	B	B	B	B
	6	KOPO-1	25	0.1	HA-2	5	0.1	5:1	1.5	r.t.	B	B	B	B
	7	KOPO-1	15	0.1	HA-1	15	0.1	1:1	1.8	37	A	A	A	A
	8	KOPO-1	20	0.3	HA-1	10	0.3	2:1	1.8	37	A	B	B	A
	9	KOPO-1	25	0.3	HA-1	5	0.3	5:1	1.8	37	A	B	B	B
	10	KOPO-1	15	0.3	HA-2	15	0.3	1:1	1.8	37	A	A	B	B
	11	KOPO-1	20	0.3	HA-2	10	0.3	2:1	1.8	37	A	B	B	B
	12	KOPO-1	15	0.3	HA-1	15	0.3	1:1	1.8	37	A	B	B	B
	13	KOPO-1	25	0.6	-	-	-	-	1.5	37	A	A	A	A
	14	KOPO-1	60	0.2	HA-1	60	0.2	1:1	4.8	r.t.	B	B	B	B
	15	KOPO-1	60	0.2	HA-2	60	0.2	1:1	4.8	r.t.	B	B	B	B
	16	KOPO-1	60	0.2	HA-1	60	0.2	1:1	4.8	4	B	B	B	B
	17	KOPO-1	60	0.2	HA-2	60	0.2	1:1	4.8	4	B	B	B	B

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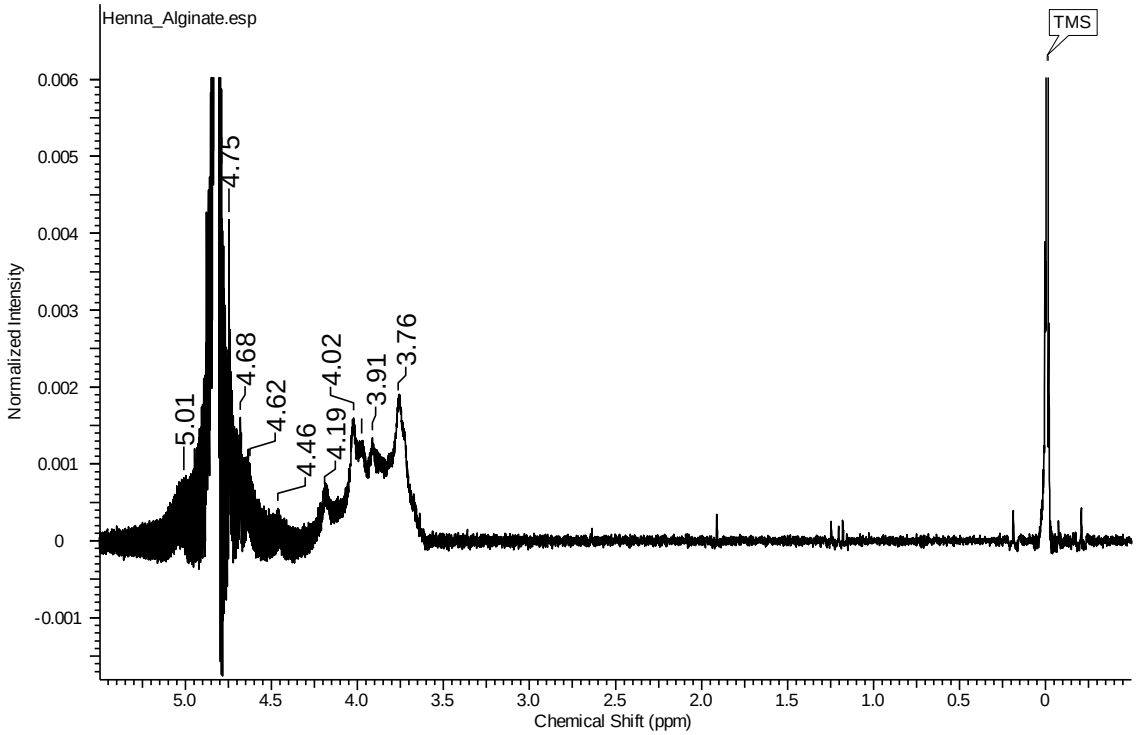
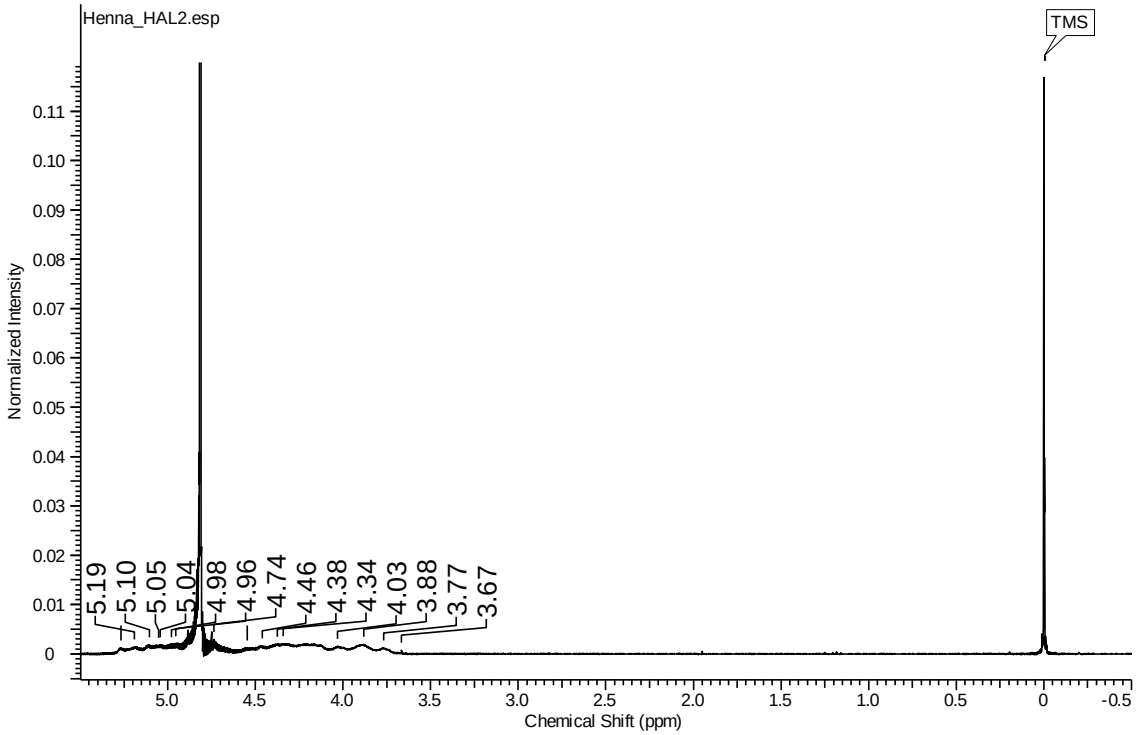
Symbol	Key
A	Liquid
B	Small traces of gel in liquid
C	Almost completely gelled
D	Completely gelled

Gel type		Component A			Component B			Ratio of components (A:B)	Polymer concentration (%)	Gelation temperature (^{o}C)	Gelation time points (min)			
			mg/cm^3	cm^3		mg/cm^3	cm^3				0	1	5	60
PNIPAM-HA or PNIPAM-AL	1	KOPO-2	15	0.2	AL-1	15	0.2	1:1	0.75	r.t.	A	B	B	B
	2	KOPO-2	20	0.2	AL-1	10	0.2	2:1	0.75	r.t.	A	A	B	B
	3	KOPO-2	25	0.2	AL-1	25	0.2	1:1	1.0	r.t.	A	A	B	B
	4	KOPO-2	50	0.2	AL-1	10	0.2	5:1	2.4	r.t.	A	B	B	B
	5	KOPO-2	60	0.2	AL-1	60	0.2	1:1	4.8	4	B	B	B	B
	6	KOPO-2	60	0.2	AL-1	30	0.2	2:1	1.8	4	B	B	B	B
	7	KOPO-2	60	0.2	AL-2	60	0.2	1:1	4.8	4	B	B	B	C
	8	KOPO-2	60	0.2	AL-2	30	0.2	2:1	1.8	4	B	B	B	B
	9	KOPO-2	60	0.2	AL-1	60	0.2	1:1	4.8	r.t.	B	B	B	B
	10	KOPO-2	60	0.2	AL-2	60	0.2	1:1	4.8	r.t.	B	B	B	B
	11	KOPO-2	15	0.2	HA-2	15	0.2	1:1	1.2	r.t.	A	A	A	A
	12	KOPO-2	20	0.2	HA-2	10	0.2	2:1	1.2	r.t.	B	B	B	B
	13	KOPO-2	25	0.2	HA-2	20	0.2	5:4	1.8	r.t.	B	B	B	A
	14	KOPO-2	50	0.2	-	-	-	-	1.0	r.t.	B	B	B	B
	15	KOPO-2	60	0.2	AL-1	60	0.2	1:1	4.8	4	B	B	B	B
	16	KOPO-2	60	0.2	AL-2	60	0.2	1:1	4.8	4	B	B	B	C
	17	KOPO-2	60	0.2	AL-1	60	0.2	1:1	4.8	r.t.	B	B	B	B
	18	KOPO-2	60	0.2	AL-2	60	0.2	1:1	4.8	r.t.	B	B	B	B

APPENDIX 3: ^{1}H-NMR SPECTRA OF ALGINATE

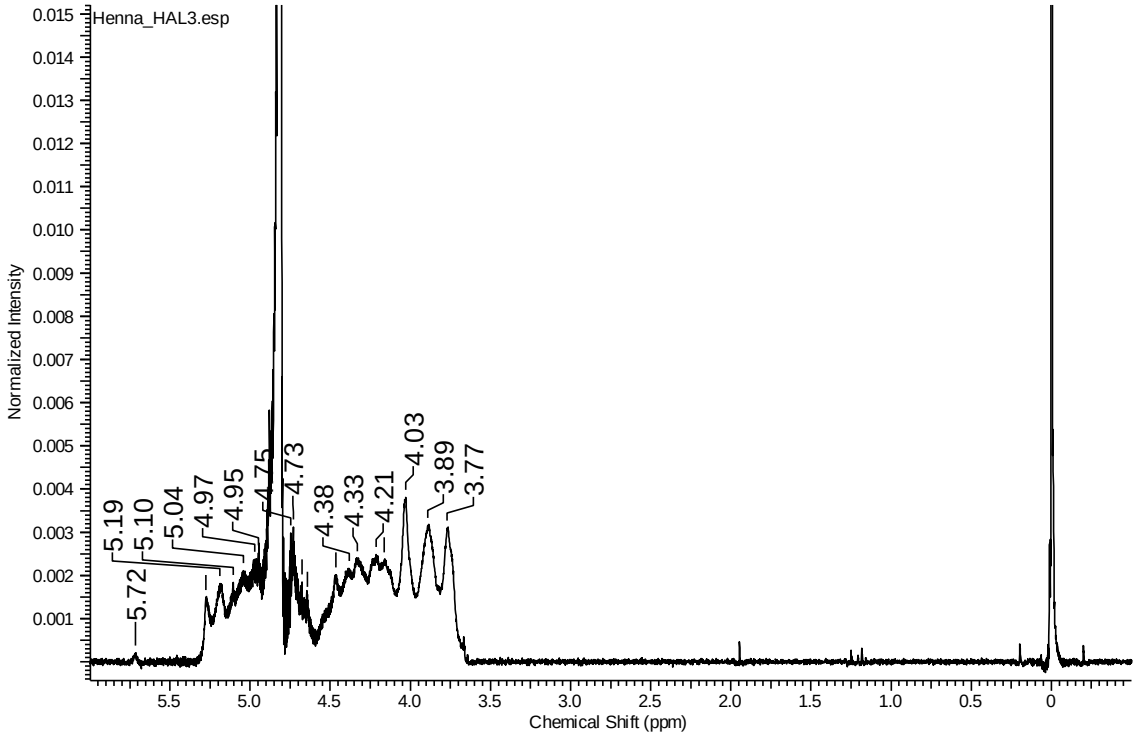


^{1}H NMR spectrum of alginate.

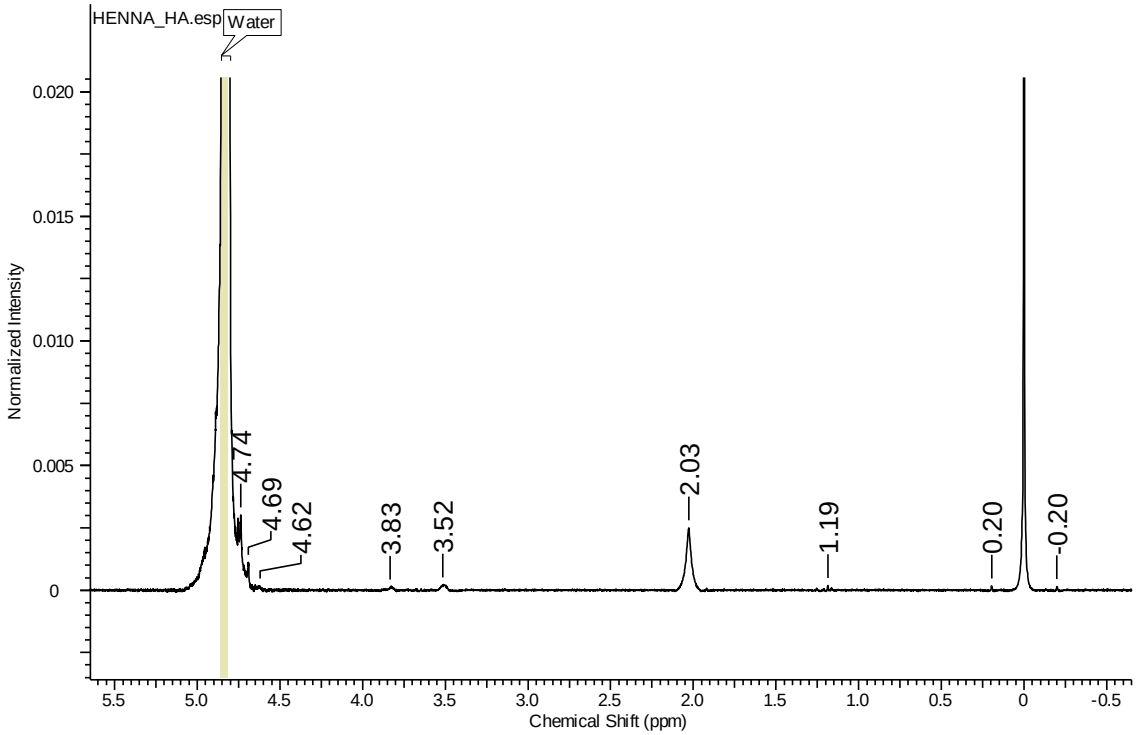


^{1}H NMR spectrum of oxidized alginate AL-1.

APPENDIX 4: ^{1}H-NMR SPECTRA OF ALGINATE AND HYALURONIC ACID

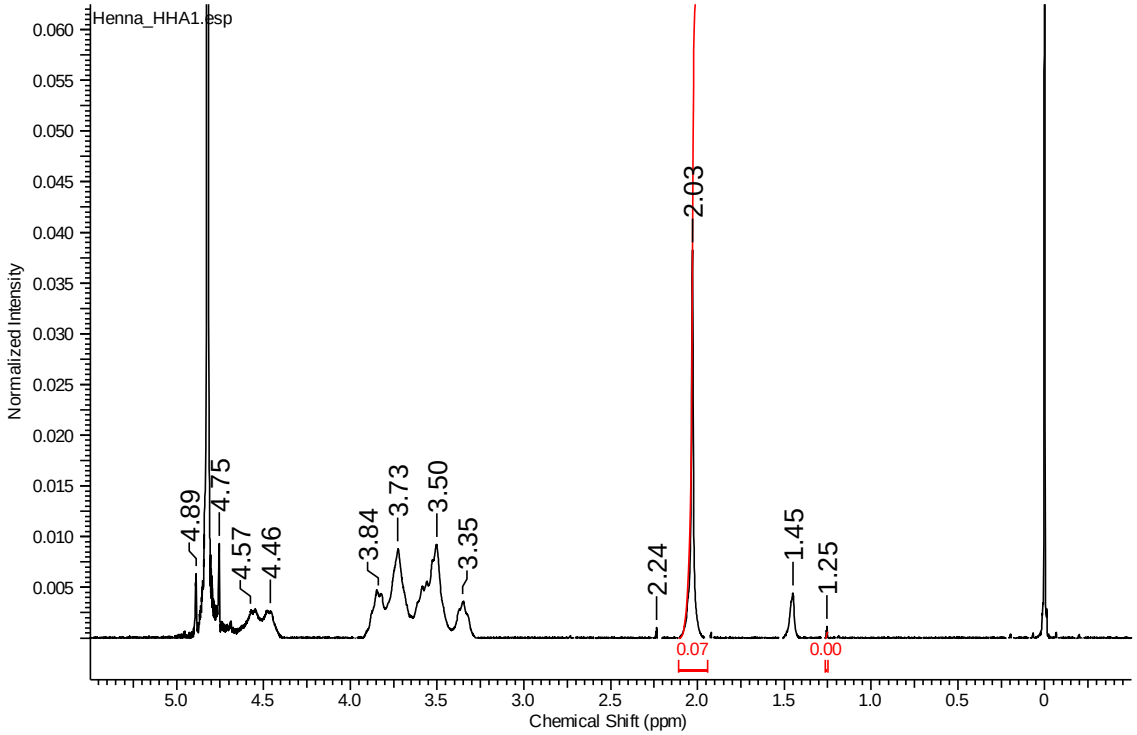


^{1}H NMR spectrum of oxidized alginate AL-2.

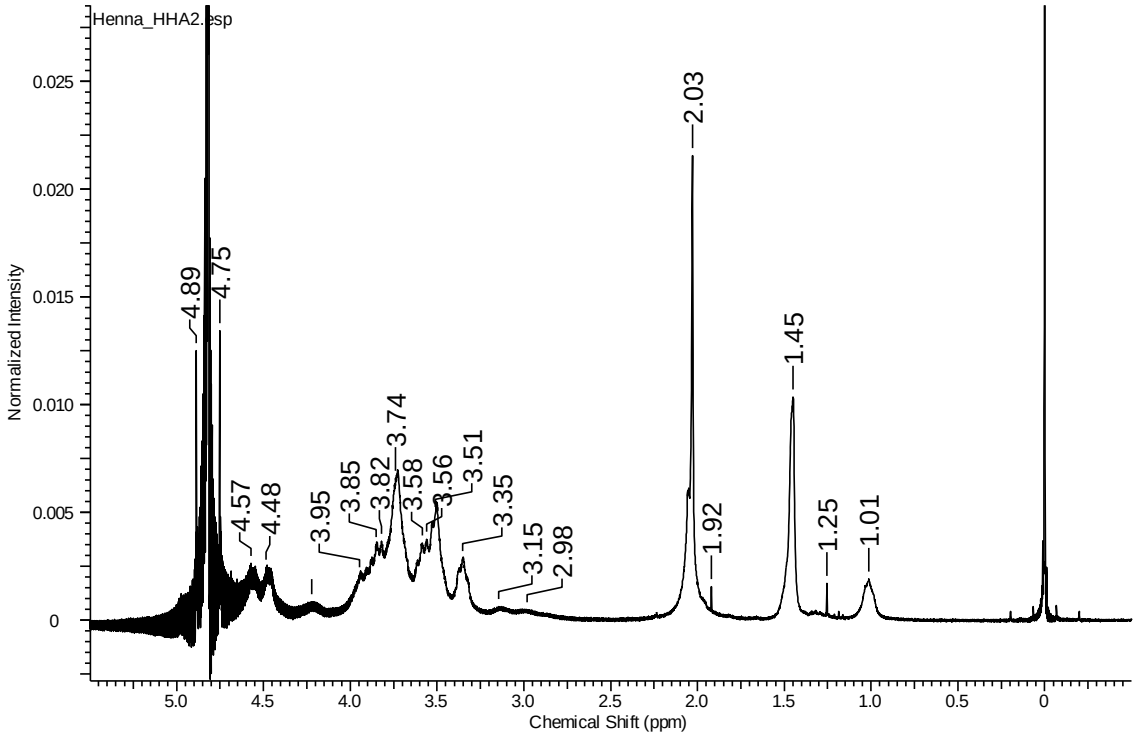


^{1}H NMR spectrum of hyaluronic acid.

APPENDIX 5: ^{1}H-NMR SPECTRA OF HYALURONIC ACID

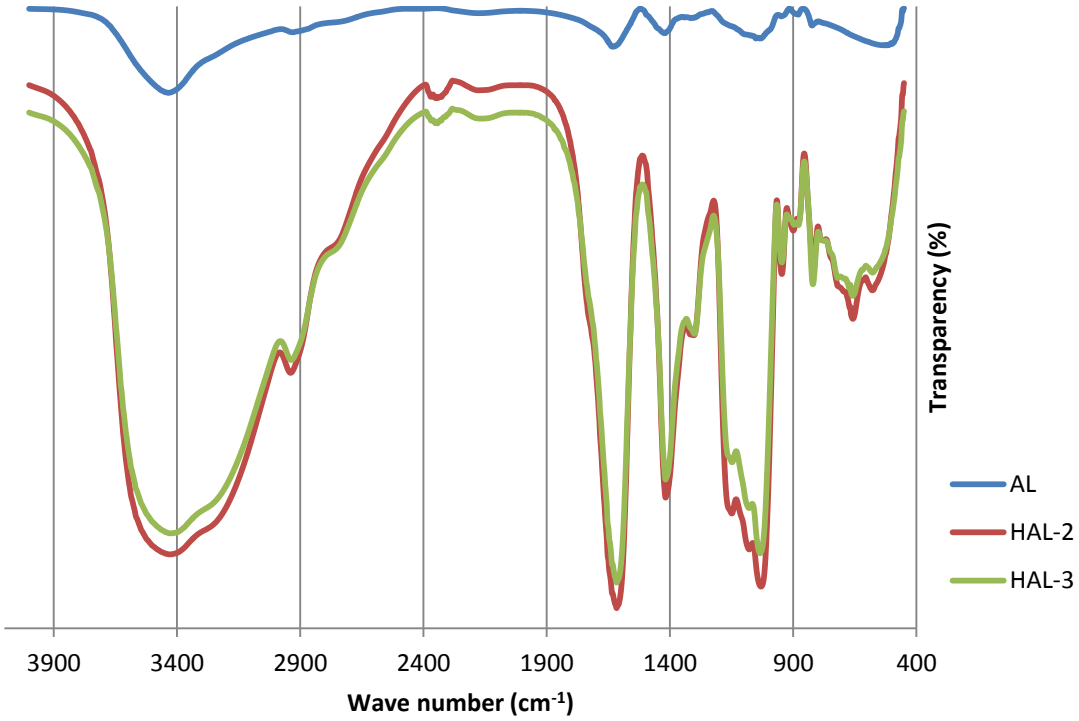


^{1}H NMR spectrum of oxidized hyaluronic acid HA-1.

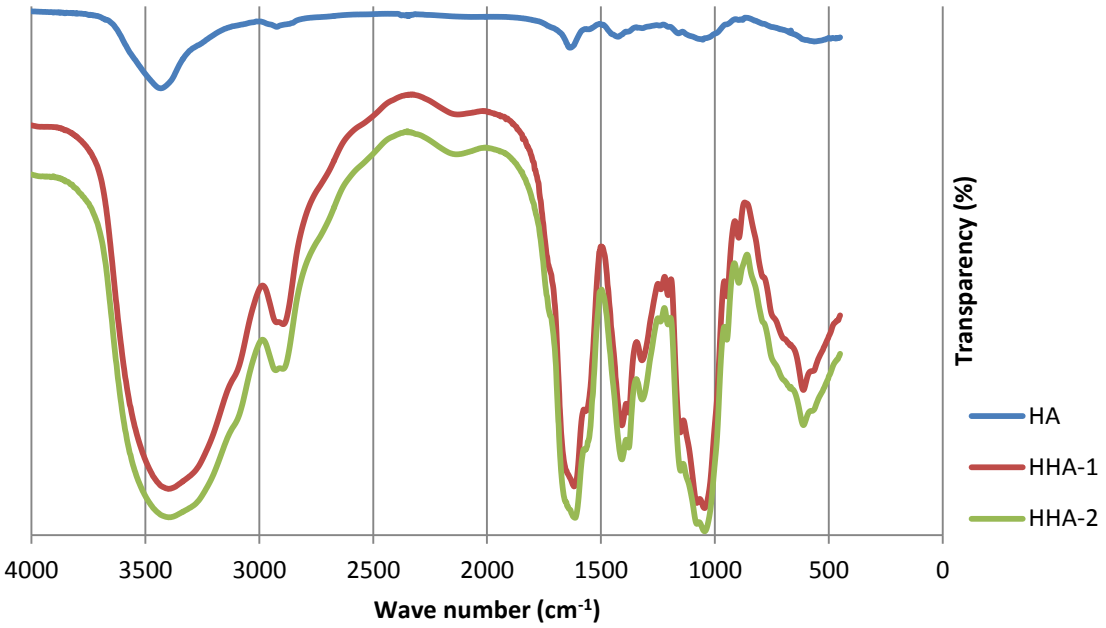


^{1}H NMR spectrum of oxidized hyaluronic acid HA-2.

APPENDIX 6: FTIR SPECTRA OF ALGINATE AND HYALURONIC ACID



FTIR spectra of alginate and its derivatives.



FTIR spectra of hyaluronic acid and its derivatives.