TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABREVIATIONS

BGP	Beta Glycerophosphate
Cat	Catechol
CH	Chitosan
DA	Degree of acetylation
DDA	Degree of deacetylation
DI	Deionized
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
FT	Refrigerator temperature
GA	Gelling agent
HCA	Hydrocaffeic acid
HCl	Hydrochloric acid
LBeV	Laboratory of Endovascular Biomaterials
MDF	The maximum detachment force
MW	Molecular Weight
NaOH	Sodium Hydroxide

NMR	Nuclear magnetic resonance
PB	Phosphate Buffer (tampon phosphate)
PBS	Phosphate buffered saline
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SHC	Sodium hydrogen carbonate (bicarbonate de sodium)
SPD	Sodium phosphate dibasic
SPM	Sodium phosphate monobasic

INTRODUCTION

Biomaterials are materials that are created to interact with biological systems for different purposes, such as replacing or enhancing a body part or function. Hydrogels are particularly interesting materials for human use. They are water-swollen polymeric materials that maintain a distinct three-dimensional structure, and therefore contain a large amount of water, as human tissue and a low amount of material, which limit risks in terms of biocompatibility.

Chitosan, a polycationic polymer derived from chitin, has become a widely used natural polymer in biomaterials studies and regenerative medicine due to its biocompatibility, biodegradability, and low toxicity. Hydrogels based on chitosan are increasingly used as injectable hydrogels in biomedical and pharmaceutical applications. For instance, they can be used to provide appropriate localization, retention of seeded cells for cell therapy and tissue engineering, or for local drug delivery.

Low viscosity before and during injection, rapid gelation, high mechanical properties, tissue-adhesiveness, biodegradation, and excellent cytocompatibility are required to ensure the benefit of these hydrogels for cell seeding applications. Merging all required properties in one formulation is challenging. On the other hand, such formulations have to be storable on extended periods without losing their properties.

Our research team at the Laboratory of Endovascular Biomaterials (LBeV), recently showed that chitosan can be combined with sodium bicarbonate, phosphate buffer and/or glycerophosphate in order to design thermosensitive gels without chemical crosslinking. They exhibit strong mechanical properties, cytocompatibility and tunable gelation time. They are however limited in terms of mucoadhesion (adhesion to mucus membranes) and tissue-adhesion in general, while this property is important to extend the drug or cell retention at the site of application and prolonging its therapeutic effects. There is some functionalization that can enhance the mucoadhesion of chitosan. Modifying chitosan by catechol is one of them that attract the attention of biomaterial researchers. This strategy was inspired by the strong

adhesion of the Mytilus edulis mussel under the sea. Mytilus edulis produces adhesive proteins that contain a large amount of an amino acid by the name of 3,4-dihydroxyphenyl-L-alanine (DOPA). The catechol groups in DOPA are contributing to the adhesion by interacting with molecules on various surfaces.

Marta Cerruti's research group at McGill University produced a chitosan-catechol hydrogel by using genipin as cross linker and used it as a drug delivery system. This hydrogel presents higher mucoadhesive properties than unmodified chitosan gels, but it has weak mechanical properties and its gelling time is very slow (more than 2 hours).

We hypothesized that adhesive injectable hydrogels with strong mechanical properties and rapid gelation can be created by catechol modification of chitosan and using gelling agents discovered by the LBEV team. In addition, these gels could be compatible with cell encapsulation, which would be beneficial for cell therapy and tissue engineering application.

The first objective of this master is to study the stability of chitosan solution, gelling agents, and chitosan hydrogel over time under different storage conditions, in order to define how long and in which condition the storage of chitosan and gelling agents is possible while they keep their properties. The second objective of this project is to study the effect of gel compounds, especially acid concentration, as a first step toward the development of an injectable tissue-adhesive chitosan-catechol hydrogel with good gelation time, good mechanical strength, and good compatibility with cells.

The first chapter of this Master's thesis presents the literature review. Hydrogels, and more particularly injectable hydrogels and chitosan hydrogels will be described in this chapter. Mucoadhesion and the related topics will be discussed, as well as the previous work at Cerruti's lab and LBEV. Chapter 2 presents the materials and methods used for preparation and characterization of both chitosan and catechol-chitosan hydrogels. The results are presented in Chapter 3 and discussed in Chapter 4.

CHAPTER 1

LITERATURE REVIEW

1.1 Hydrogels

1.1.1 Definition

Hydrogels are three dimensional polymeric hydrophilic networks capable of absorbing a large amount of water and biological fluids (Hoffman, 2002; Peppas, Bures, Leobandung & Ichikawa, 2000). Because of the presence of crosslinks between the polymer chains, the polymeric network is insoluble in water. They can swell and retain water, thus providing a water environment like the physiological conditions found in the body. In addition, due to their hydrophilic nature hydrogels usually have low interfacial free energy in body fluids, thus proteins and cells cannot bind to them easily (Gibas & Janik, 2010; Gulrez, Al-Assaf & Phillips, 2011). All these properties make hydrogels good candidates to be used in bio-related applications. Hydrogels often have very good biocompatibility, thus avoiding significant immune system reaction or toxicity. They can deliver bioactive drugs or genes.

1.1.2 Type of hydrogels

Hydrogels can be classified based on the polymer origin (natural, synthetic and synthetic/natural hybrid hydrogels) or the type of crosslinks between polymer chains (chemical crosslinks (covalent bonds), or physical crosslinks including electrostatic interactions, hydrophobic interactions, hydrogen bonds, polymer chain entanglement and van der Waals interactions) (Gulrez et al., 2011). Depending on the charges of the materials, hydrogels can be classified into cationic, anionic and neutral hydrogels. Changing the degree of crosslinking or polymer molecular weight can make the hydrogel very soft or very hard to fit the needs of the applications (Peppas et al., 2000; Qiu & Park, 2001).

1.1.2.1 Natural versus synthetic polymers

One of the most attractive options for biomedical applications are natural origin polymers such as chitosan, collagen, cellulose, etc. Due to their similarities with the extracellular matrix and other polymers found in the human body, they are biocompatible (Reis et al., 2008). There are three main types of natural polymers: Polymers derived from living organisms including carbohydrates (chains of sugar) and proteins (chains of amino acids), and Polynucleotides (chains of nucleotides) (DNA, RNA).

On the other hand, synthetic polymers can be used to design hydrogels with specific functions for a specific application. Chemical structures, methods of preparation, water content and cross-linking degree are parameters which can be changed to make new biomaterials. These changes can be performed in the chemical composition and the concentration of material or even in one of the synthesis factors (cross-linking method, cross-linking agent, synthesis method, conditions of the synthesis (Gibas & Janik, 2010).

However, natural hydrogels synthesised from natural polymers are extensively used in tissue engineering since they are often more biocompatible, more biodegradable and have less toxic by-products compared to those synthesized from synthetic constituents (Piai, Rubira & Muniz, 2009).

1.1.2.2 Physical and chemical hydrogels

Hydrogels can be defined as physical and chemical hydrogels, based on the forces involved in the building of the networks (Figure 1.1).

In chemical gels, polymer chains are covalently cross-linked and make three-dimensional networks (Hennink & van Nostrum, 2002). In this type of hydrogels, the equilibrium swelling levels depends on crosslink density and the polymer-water interaction parameters like hydrophilicity of the polymer chains. (Rosiak & Yoshii, 1999).

In physical gels, the polymer chains bond together by physical crosslinks, such as entanglements or crystallites and/or other weak forces such as van der Waals, hydrogen and ionic bonding. These links can be broken by applying stress or changing physical conditions. So, this kind of hydrogels is generally called reversible hydrogels (Rosiak & Yoshii, 1999).

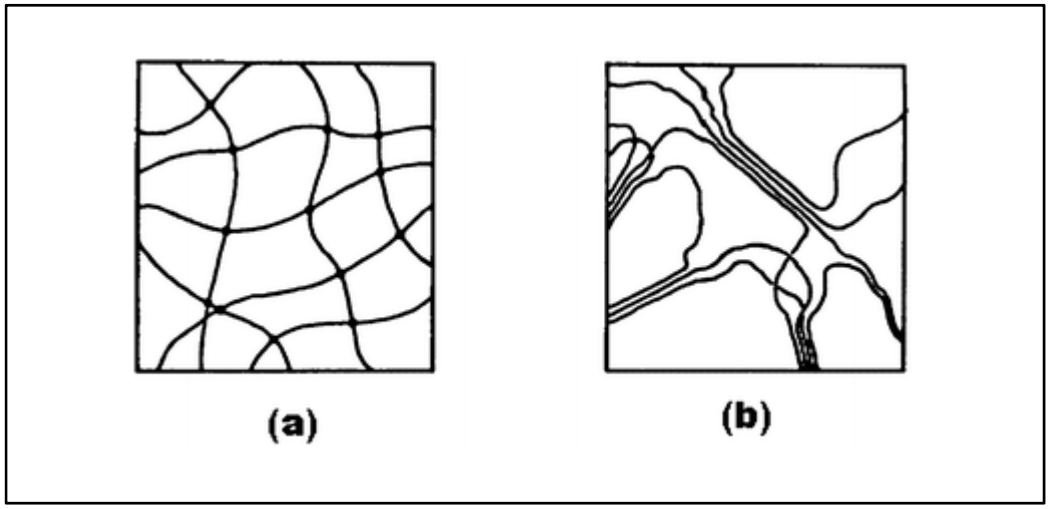

Figure 1.1 Schematic diagram of (a) a chemical hydrogel and (b) a physical one (Barnett, Hughes, Lin, Arepally & Gailloud, 2009)

1.1.2.3 Injectable hydrogels

Particularly interesting for biomedical and pharmaceutical applications are injectable hydrogels which are delivered as solutions mixed with drugs, proteins, or cells and form hydrogels in situ by chemical or physical crosslinking methods. These hydrogels have a lot of applications in drug delivery, cell therapy, and tissue engineering.

Chemically crosslinked hydrogels are formed by photopolymerization, disulfide bond formation, or reaction between thiols and acrylate or sulfones methods. Physical crosslinked hydrogels are formed by the self-assembly in response to environmental stimuli (Nguyen & Lee, 2010). Therefore, physical hydrogels are more attractive for biomedical applications,

because they do not use any organic solvents, crosslinking agents or photo irradiation. Therefore they have less risk to damage incorporated proteins, embedded cells and surrounding tissues (Nguyen & Lee, 2010).

1.1.2.4 Environmentally-sensitive hydrogels

Several teams have developed stimuli-sensitive hydrogels, which behave differently upon environmental changes such as temperature, pH, electric signals, light, pressure, and specific ions (Qiu & Park, 2001). Environmentally-sensitive hydrogels are also called "smart" or "Intelligent" hydrogels. They not only can sense external environment stimuli, but also can respond to them. These responses can be exhibited in various manners like changing in swelling behavior, network structure, permeability or mechanical strength.

Smart hydrogels are categorized based on the type of their stimuli. Thus hydrogels which can respond to environmental temperature changes by changing their physical properties are called temperature-sensitive hydrogels (Fang, Chen, Leu, & Hu, 2008). For example, temperature increase can break hydrogen bonds in the hydrogel structure. In hydrogels made from hydrophobic polymers, this will cause the aggregation of polymer chains, leading to shrinkage of the hydrogels and drug release (Qiu & Park, 2001). In contrast, other materials form solutions which gel when temperature increases. This is the case of chitosan-based hydrogels which interest us in this study and will be described in more details below.

1.2 Chitosan hydrogels

1.2.1 Chitosan

Chitosan is a natural linear polysaccharide composed of two randomly distributed repeating monomer units of D-glucosamine and N-acetyl-D-glucosamine (acetylated unit) (Figure 1.2)

(Bhattarai, Gunn, & Zhang, 2010; Croisier & Jérôme, 2013).The main source of commercial production of chitosan is deacetylation of chitin, the second most abundant natural polymer after cellulose. The principal source of chitin is shellfish waste; however, it is widely found in cell walls of fungi as well as exoskeletons of crustaceans, insects and spiders (Chenite et al., 2000; Ravi Kumar, 2000). In deacetylation process, strong alkali solutions are used to remove N-acetyl groups of chitin and form chitosan. The degree of deacetylation (DDA) indicates the percentage of the deacetylated D-glucosamine units in the chain. Conversely, the degree of acetylation (DA) is the percentage of acetylated units relative to the total units. The product is considered as chitosan when the DDA is greater than 50% (Bhattarai et al., 2010; Croisier & Jérôme, 2013).

The DDA and the molecular weight (MW) are factors that significantly influence chitosan properties such as the polymer's solubility, its viscosity, its gelling process and its degradation kinetics (Berger et al., 2004). For instance, Ganji et al. showed that the gelation time of hydrogels formed with highly deacetylated chitosan (DDA=98.3%) is less than the gelation time of chitosan with less DDA (DDA=82.5 %) (Ganji, Abdekhodaie, & Ramazani, 2007).

The molecular weight (indicative of the length of the macromolecular chains) of chitosan varies between 50 and 2000 kDa (Chenite, Buschmann, Wang, Chaput, & Kandani, 2001) and directly influences the viscosity of the chitosan solution and therefore the mechanical properties of the hydrogel made with the chitosan solution.

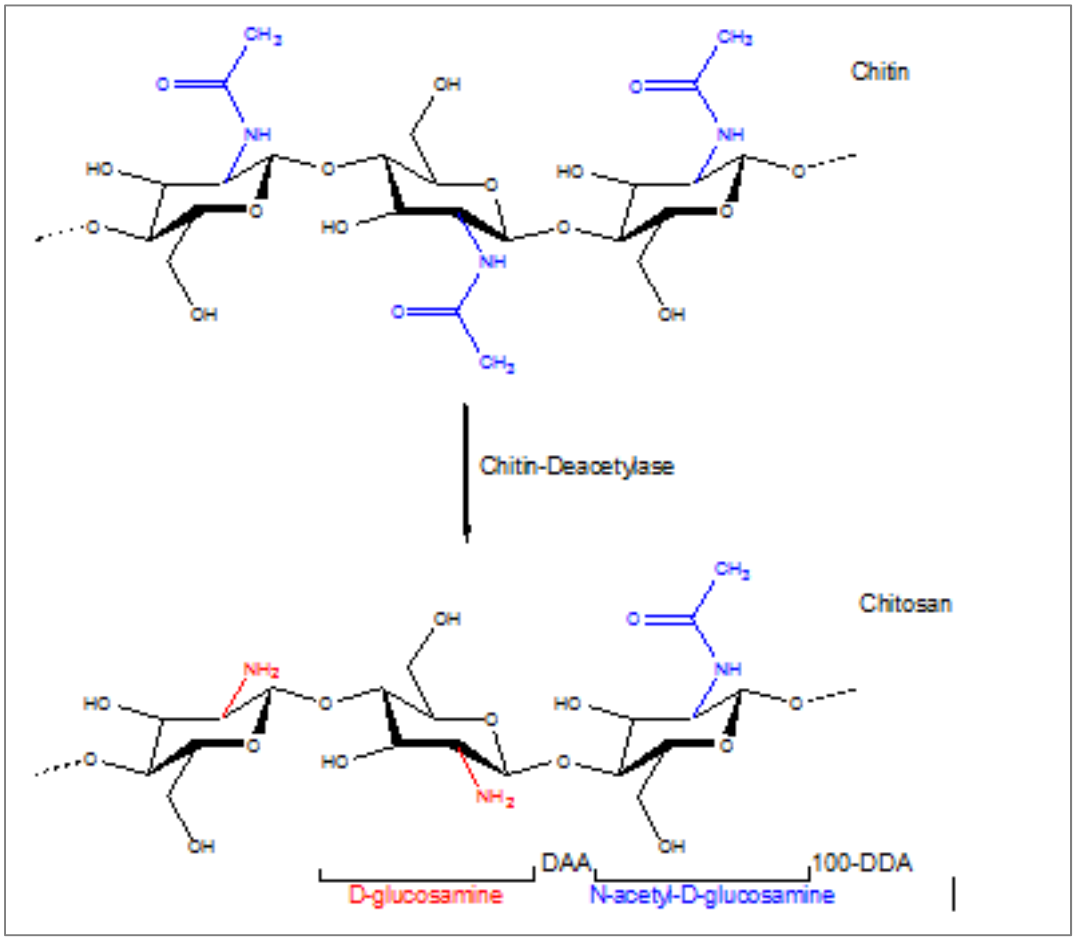

Figure 1.2 : Structure of chitosan obtained by partial alkaline deacetylation of chitin.

Chitosan is biocompatible, non-toxic and biodegradable (Ravi Kumar, 2000). It has antibacterial and antifungal properties and in the human body does not induce any immune response (Bhattarai et al., 2010; Croisier & Jérôme, 2013; Kim et al., 2008; Raafat & Sahl, 2009; Rinaudo, 2008). High DDA leads to lower degradation rates. Also, the biocompatibility of chitosan increases for high DDAs because of the increase in positive charges that induces more interactions with the cells (Croisier & Jérôme, 2013).

Chitosan possesses hemostatic properties because it can interact with the negatively charged red cell membrane (Croisier & Jérôme, 2013; Kim et al., 2008; Raafat & Sahl, 2009). It is also

mucoadhesive because its positively charged amine groups can interact with mucin, which is a negatively charged glycoprotein present in the mucus. The higher DDA leads to the better mucoadhesive properties because of the higher number of positive charges (Bhattarai et al., 2010; Croisier & Jérôme, 2013; Kim et al., 2008; Raafat & Sahl, 2009; Zhou, Jiang, Cao, Li, & Chen, 2015).

1.2.2 Chitosan hydrogels

Chitosan has a pKa of ~ 6.5. It is soluble only in an acid medium. The primary amines ($-NH_2$) in its structure get protonated ($-NH_3$ +) under acidic conditions, making chitosan a cationic polymer (Chenite et al., 2001). When the molecule is sufficiently ionized, the generation of repulsive electrostatic forces between the charged groups ensures the solubilization of the chitosan. However, a change in pH or ionic strength may disturb this balance and induce deionization and precipitation of chitosan. In other words, when chitosan solution pH is elevated above 6, the repulsive electrostatic forces between the polymer chains are weakened due to the neutralization of amine groups. Meanwhile, attractive hydrophobic interaction and hydrogen bonds dominate, leading to chitosan precipitation (Chenite et al., 2001; Croisier & Jérôme, 2013; Lavertu, Filion, & Buschmann, 2008; Rinaudo, 2008).
As mentioned in section 1.1.2.3, an interesting characteristic of chitosan is its ability to form thermosensitive gels, which are liquid state at room temperature and form solid gels at physiological temperature (37 °C.). This makes it possible to carry out an injection at room temperature in liquid form and mix the solution with cells and / or drugs, prior to in situ gelation.

Several methods can be used to make this chitosan solution a rigid gel. For example, the addition of a mild base, such as β-glycerophosphate (BGP), makes it possible to produce a thermosensitive chitosan solution. BGP has hydroxyl groups -OH which act as a buffer and stabilize the chitosan solution. Moreover, BGP is negatively charged and is therefore attracted by the $-NH_3$ + groups of chitosan (Figure 1.3) (Chenite et al., 2001). The -OH groups of the BGP control the hydrogen bonds between the chitosan molecules and make it possible to keep

the polymer in solution even at a pH above 6.5 (Chenite et al., 2001; Chenite et al., 2000; Coutu, Fatimi, Berrahmoune, Soulez & Lerouge, 2013). When the temperature increases, the transfer of the protons (H^+) from the chitosan to BGP lead to neutralizing chitosan. The attracting forces become stronger than the repulsive forces between the chains, which leads to the creation of a physical gel (Lavertu et al., 2008).

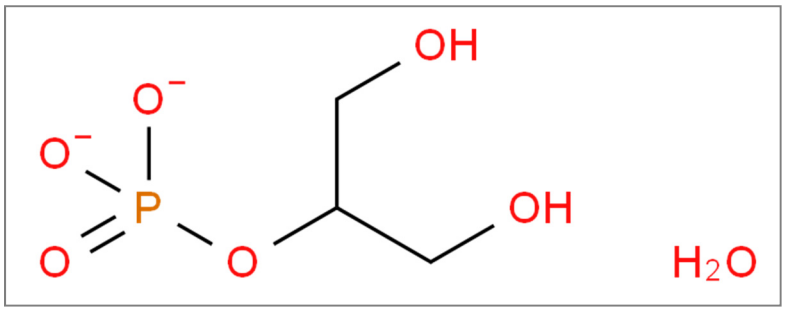

Figure 1.3 Chemical structure of BGP

1.2.3 Previous work in LBeV on chitosan hydrogels

Chitosan hydrogels possess interesting properties for their use in the biomedical field. They are natural and biocompatible, they have an interconnected porous structure that allows cells survival and nutrient and waste transfer (Kim et al., 2008). They are biodegradable (enzymatically or chemically), which ensures natural tissue healing without preserving a permanent material (Bhattarai et al., 2010; Croisier & Jérôme, 2013; Kim et al., 2008; Raafat & Sahl, 2009; Rinaudo, 2008).

The low mechanical properties of the chitosan / BGP hydrogels are their main limit. Assaad et al. have shown that whatever the BGP concentration, the secant modulus of the chitosan / BGP hydrogels does not exceed 10 kPa. (Assaad, Maire & Lerouge, 2015). Moreover, the use of high concentrations of BGP required for rapid gelation decreases the biocompatibility of the gel, due to an increase in the osmolarity of the gel which can cause death of the encapsulated cells (Ahmadi & de Bruijn, 2008; Monette, Ceccaldi, Assaad, Lerouge & Lapointe, 2016; Riva

et al., 2011; Zhou et al., 2015). For these two reasons, the LBeV team has developed novel gelling agents which offer better biocompatibility, higher mechanical resistance and a suitable gelling rate. The team has shown that the combination of sodium bicarbonate (Sodium Hydrogen Carbonate, SHC) with a phosphate buffer (Phosphate Buffer, PB) or BGP significantly improves the mechanical properties and accelerates gelation at body temperature (Assaad et al., 2015; Ceccaldi et al., 2017). In addition, in vitro cytocompatibility tests could demonstrate better biocompatibility of these new gels due to a decrease in salt concentration.

Overall, these hydrogels present several decisive advantages. They are easy to prepare by simply mixing two solutions. These hydrogels are stable at room temperature and rapidly gel at 37 °C. They have superior mechanical properties to most hydrogels based on chitosan.

Patent filing has been done to protect these hydrogels which raise great interest for cell therapy and tissue engineering application. However, for possible clinical transfer, the stability of the solutions is of great importance. Indeed, chitosan dissolution in acid is a time consuming process that requires several hours. So, it would be practical to prepare chitosan solutions in bulk and store them for further use, especially for commercial applications.
However, during storage, specific characteristics of chitosan may be altered. Irreversible loss of physicochemical properties of chitosan may happen due to the hydrolysis of chitosan and gradual chain degradation, as it occurs after dissolution and storage at various conditions in dilute organic acids (No et al., 2006, Nguyen et al., 2008). In particular, possible changes in viscosity of chitosan solution must be monitored since it may influence other functional properties of the chitosan solution.

Different internal and external factors can affect the stability of chitosan-based products. Degree of deacetylation and the pattern of deacetylation, molecular weight, purity, and moisture level are internal factors and environmental storage conditions, thermal processing, sterilization, and processing (involving acidic dissolution, type of acid and chitosan concentration in acidic solution) are external ones (Szymańska & Winnicka, 2015).

Overall, it has become a great challenge to establish sufficient shelf-life for chitosan formulations and the purpose of the stability test is to provide reliable evidence on how the quality of the chitosan solution may differs upon storage conditions.

It is also important to study the stability of these solutions since short term changes could explain variability in the results obtained by the different team members.

In addition, one limitation of these hydrogels is their poor mucoadhesive properties, as will be described in next sections.

1.3 Tissue-adhesion

1.3.1 Definition and application

The term "tissue adhesion" explains the adhesion capability of some natural, biological and also synthetic materials to biological tissues (Ferreira, Gil & Alves, 2013; Khanlari & Dubé, 2013). The term mucoadhesion is used if the tissue is a mucosal surface (Huang, Leobandung, Foss, & Peppas, 2000). Tissue-adhesive materials have been applied in different fields, such as wound closure, and more recently for drug delivery systems. They are so popular for wound closuring. Comparing to the traditional suture method, the tissue adhesives decrease foreign body reaction during wound healing. Also, their use is easier and less painful, and there is no need for removal. Besides, for cosmetic reasons in many cases the adhesive materials are preferred to traditional methods such as suture. (Tajirian & Goldberg, 2010). (Delibegović, Iljazović, Katica, & Koluh, 2011; Spotnitz & Burks, 2010).

Nowadays, tissue adhesives are also being used for oral, buccal and rectal (Sosnik, das Neves, & Sarmento, 2014; Spicer & Mikos, 2010; Vakalopoulos et al., 2013), as well as ocular, nasal, gingival, and vaginal drug delivery systems (Caló & Khutoryanskiy, 2015).

Tissue-adhesive materials have also been proposed for drug delivery to increase the retention time of the drug at the target site. Otherwise the drug may not have enough time to act on the disease before being eliminated. For example, the bowel movements can accelerate the elimination of a rectal drug delivery. In the gastrointestinal tract the ingestion of food and drink may shorten the retention of an oral drug delivery system. In these cases, tissue adhesive drug delivery systems can increase the drug retention time, with advantages such as higher drug efficacy (Bernkop-Schnürch, 2005), and reduction of the administrated dose. Moreover, tissue adhesive drug delivery systems make it possible to release the drugs only in specific targeted sites and avoid adverse effects (Ducheˇne, Touchard, & Peppas, 1988), (Nikolaos A. Peppas & Sahlin, 1996). The same principle could apply to biomaterials for cell therapy in order to increase cell retention on targeted site.

1.3.2 Mechanism of tissue adhesion

The mechanism of tissue adhesion in general and mucoadhesion specifically**,** is complex and not completely elucidated yet. There are different interactions between mucoadhesive materials and mucin, such as: covalent, ionic, hydrogen bonds, van der Waals, and hydrophobic interactions (Smart, 2005). The mucoadhesion strength can be affected by the molecular weight of the polymer, the flexibility of the polymer chains, environmental pH, charge, and functional groups in the polymer (Khutoryanskiy, 2011), (Smart, 2005).

Based on these interactions, various theories have been proposed to explain the mechanism of mucoadhesion, including diffusion, wetting, electronic, adsorption and fracture mechanism (Woertz, Preis, Breitkreutz & Kleinebudde, 2013). The diffusion theory is based on the diffusion of a polymer into the mucin layer. It is dependent on the concentration gradient and the diffusion coefficient of the polymer. Voiutskii suggested that mucoadhesion is due to the semi-permanent adhesive bond formed by inter diffusion between the polymer chains of the mucoadhesive materials and mucin (Voiutskii, 1963).

Peppas and Buri developed the wetting theory based on the spreading of a material, mostly mucoadhesive liquids or low viscous formulations on the biological tissue. The degree of spreading can be calculated by an extension of the basic Young's equation. Better spreading (i.e. low surface tension) induces better mucoadhesion (Peppas & Buri, 1985).

The electronic theory explains the electron transfer between adhesive polymer and mucus due to differences in electronic charge. This mechanism includes the formation of a double layer due to interactions between the polymer and the mucus layer.

The adsorption theory describes the adhesion caused by primary (ionic, covalent and metallic) and secondary bonds (van der Waals forces, hydrophobic interactions and hydrogen bonding).

The fracture mechanism is concerned with the strength of the adhesive bond between mucoadhesive formulation and mucosa and the force which is needed to break this adhesive bond. Young's modulus of elasticity, fracture energy and critical crack length upon separation of two surfaces can be used to calculate the fracture strength (Woertz et al., 2013).

Still, no single theory can fully explain the complex mechanism of mucoadhesion (Khutoryanskiy, 2011; Smart, 2005). Some researchers used a combination theory to explain this complicated phenomenon. For instance, a 3-step theory is proposed by Smart: The mucoadhesives wet and start to swell. Then, they come in contact with mucus and form non-covalent bonds at the interface. Finally, mucoadhesive polymer chains and mucin chains interpenetrate each other, and develop further entanglements (Smart, 2005).

1.3.3 Mucoadhesive property of chitosan

There are different types of mucoadhesive materials, including cationic polymers (ex: polylysine), anionic polymers (ex: Alginate), non-ionic polymers (ex: poly ethylene oxide (PEO) and PVA), and amphoteric polymers (ex: Gelatin). Cationic polymers such as chitosan can form electrostatic interactions with the negatively charged mucin at physiological pH, so they present

some mucoadhesive properties (Bernkop-Schnürch, 2005; Boddupalli, Mohammed, Nath, & Banji, 2010; Lehr, Bouwstra, Schacht & Junginger, 1992). However, these mucoadhesive properties remain limited and various efforts have been done to enhance the mucoadhesion of chitosan. Functionalization of chitosan with catechol groups is one of the most promising approaches and will be described in detail later.

1.4 Mussel-inspired mucoadhesion

1.4.1 Introduction to marine mussel adhesion

The tissue adhesive should adhere to a wet or moisture surface at approximately body temperature. The strong underwater adhesion of blue marine mussels (Mytilus edulis) therefore attracted the attention of material scientists. These mussels stick to many surfaces under the sea, such as rocks and boats, thus avoiding being removed by the waves. Mussels can adhere to many different surfaces: organic and inorganic, hydrophilic and hydrophobic, smooth or rough, and even the inert Teflon (G Silverman & Roberto, 2007).

Figure 1.4 illustrates adhesion of Mytilus edulis to seaweed, other mussels, and a stainless-steel surface.

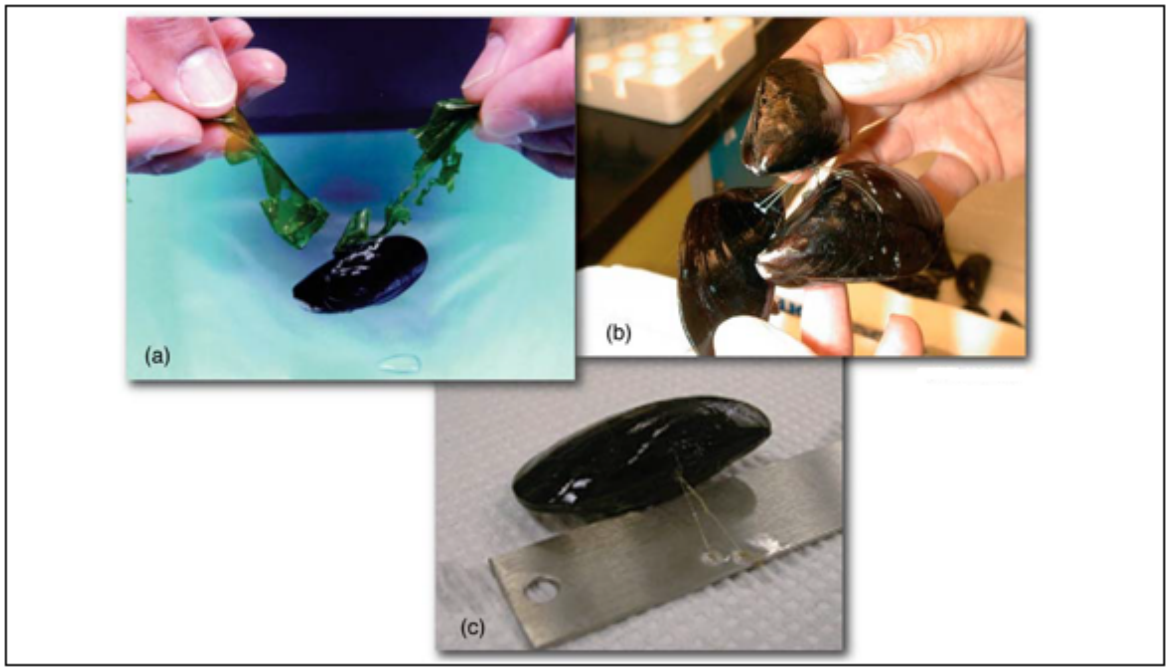

Figure 1.4 Mytilus edulis attachment to (a) seaweed, (b) other mussels, and (c) a stainless-steel surface (G Silverman & Roberto, 2007)

To adhere under water, mussels secrete proteins called Mytilus edulis foot proteins (Mefps). Mefps can rapidly solidify in the seawater and form the byssus. Figure 1.5 shows a schematic of the Mytilus edulis mussel and byssus structures (G Silverman & Roberto, 2007).

The distal part of the byssus is called the byssal plaque. Mussels use the strong adhesion of the byssal plaques to attach themselves to various solid surfaces.

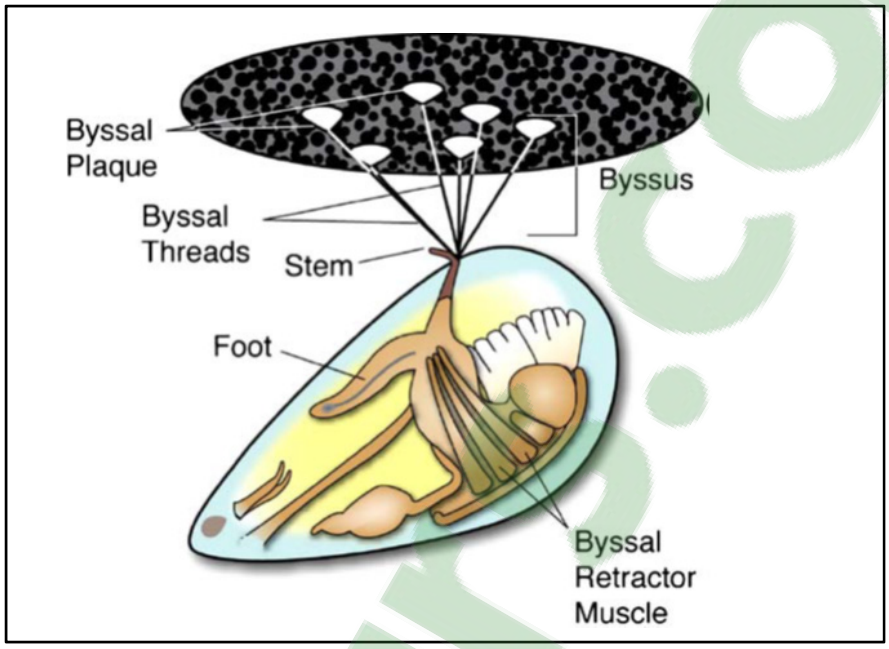

Figure 1.5 Mytilus edulis mussel and byssus structure
(G Silverman & Roberto, 2007)

At least six Mefps have been identified. Mefp-1 is the key protein of the byssal cuticle while Mefp-2 through 6 are found within the adhesive plaque. These proteins all share a common unusual amino acid, 3,4-dihydroxyphenylalanine (DOPA). Figure 1.6 shows DOPA structure. The DOPA content of Mefps ranges from a few percents to well above 20%. DOPA contains catechol functional groups (3,4-dihydroxyphenyl), that was found to play a major role in adhesion (Lee, Dellatore, Miller & Messersmith, 2007; Waite & Qin, 2001).

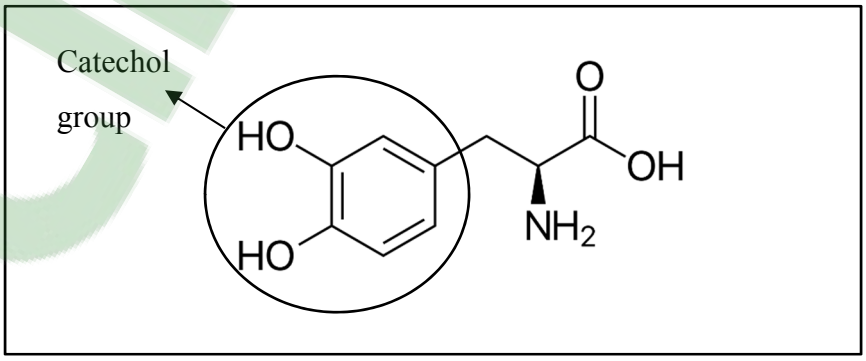

Figure 1.6 The structure of DOPA

This discovery inspired many researchers to develop novel catechol-containing adhesives.

1.4.2 Catechol chemistry

The catechol is capable of various catechol-catechol and catechol-surface interactions, leading to the adhesive property of the catechol-containing materials. In addition, catechol is a unique molecule capable of forming strong bonds to both inorganic and organic substrates while utilizing either reversible physical or irreversible covalent crosslinks (H. Lee, Scherer, & Messersmith, 2006). Understanding catechol chemistry is necessary to understand the mechanisms of these processes, which are summarized in Figure 1.7.

The benzene ring of catechol can form π- π interaction with another benzyl moiety. This allows the catechol-containing material to be able to bind to surfaces rich in aromatic compounds such as polystyrene (Baty et al., 1997). The hydroxyl groups of catechol forms extensive hydrogen bonds, which allows catechol to compete with water for hydrogen bonding sites and absorb onto mucosal tissues (Chirdon, O'Brien & Robertson, 2003; Schnurrer & Lehr, 1996). Catechol is also capable of forming strong complexes with metal ions (such as Fe^{3+}, Ca^{2+}, Cu^{2+}, Ti^{3+}, Ti^{4+}, Mn^{2+}, Mn^{3+}, Zn^{2+}). Strong and reversible catecholate-metal ion complexation is responsible for the wear resistance properties, high extensibility and elevated hardness of mussel byssal cuticles (Holten-Andersen et al., 2009).

In the presence of oxidizing agent (i.e., IO_4^-, H_2O_2, enzyme etc.), catechol is oxidized to its quinone form. It can also auto-oxidize in a slightly basic aqueous solution (Schweigert, Zehnder & Eggen, 2001; Yu et al., 2013). Quinone is highly reactive and can form covalent crosslinks with various functional groups present on tissue surface through three main pathways: self-crosslinking, involving coupling of two catechol molecules, Michael addition with –SH or $–NH_2$ group, and Schiff-base reaction with $–NH_2$ (Deming, 1999; Lee, Dalsin & Messersmith, 2006; Schweigert et al., 2001) (Figure 1.7).

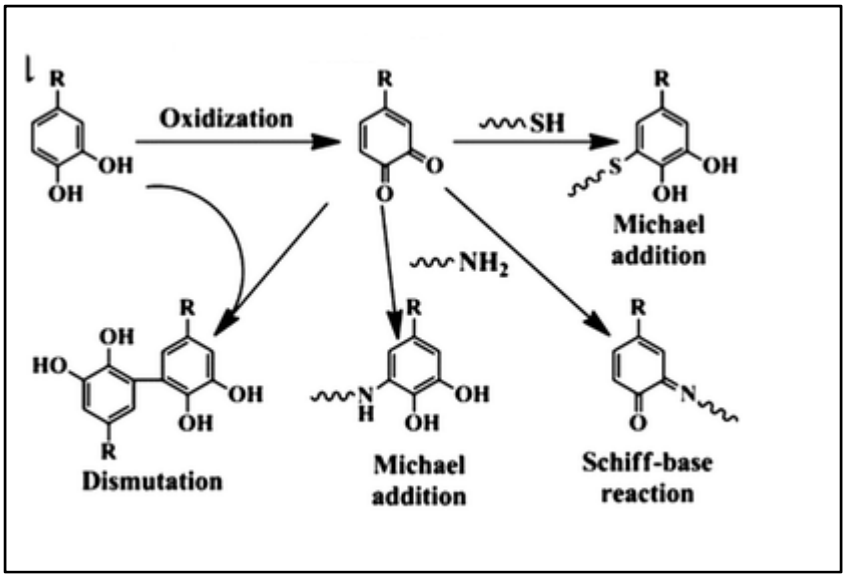

Figure 1.7 Oxidative chemistry of catechol (Wu et al., 2011)

1.4.3 Catechol-containing hydrogels

Various biomaterials have been grafted with catechol groups to enhance their adhesive properties or to form hydrogels.

Chitosan–catechol can be processed into a variety of physical states: films, hydrogels, sponges, and micro/nanoparticles. As mentioned in the previous section, catechol can create crosslinks with themselves or other functional groups. This catechol-induced crosslinks can be used to make hydrogels or films. In one study, Oh et al. synthesized catechol modified hyaluronic acid and lactose modified chitosan respectively. The mixture of these two polymers formed a re-moldable hydrogel with interpenetrating network structure. Inter-molecular polyelectrolyte complexes between the negatively charged hyaluronic acid and the positively charged chitosan, and covalent bonds between oxidized catechol groups and $–NH_2$ groups were two types of crosslinks contributed to the interpenetrating network formation (Oh et al., 2012). Lee et al. developed an alginate-catechol hydrogel that used catechol oxidation for crosslinking, instead of the conventional calcium ionic crosslinking (Lee et al., 2013). This catechol-alginate hydrogel showed excellent biocompatibility, and tunable mechanical properties in

contrast to calcium crosslinked alginate hydrogel. In another study by Ryu et al., catechol modified chitosan was crosslinked with thiolated Pluronic, forming a gel that was adhesive to soft tissue (Ryu et al., 2011). Although part of the catechol groups on the polymer chain participated in crosslinking with –SH by Schiff-base addition, the remaining catechol groups contributed to the enhancement of bioadhesion at tissue surface.

1.4.4 Previous work on chitosan-catechol hydrogels in Marta Cerruti's lab

Marta Cerruti's team at McGill University is another group who worked on chitosan-catechol hydrogels. They developed three types of catechol-containing chitosan hydrogels as mucoadhesive drug delivery systems for oral, buccal and rectal drug delivery.

First, they selected DOPA, hydrocaffeic acid (HCA), and dopamine (DA) as three different catechol-containing compounds (Xu, Soliman, Barralet, & Cerruti, 2012). These three compounds have the same ortho-dihydroxyphenyl backbone but different functional groups (both carboxylate and amino group in DOPA, carboxylate group in HCA, and amino group in DA.

The hydrogels were prepared simply by mixing different catechol compounds with CH and their adhesion to rabbit intestine were tested. Based on the mucoadhesion result, HCA was chosen as a catechol compound for further experiment. Besides, their study also demonstrated that oxidation should be prevented before contact with mucus in order to retain enhanced mucoadhesion. In the next step of the experiment, this group covalently bonded catechol functional groups to the backbone of CH, and crosslinked the polymer with a non-toxic chemical crosslinker, namely genipin (GP) (Xu, Strandman, Zhu, Barralet, & Cerruti, 2015). Chitosan–catechol adhesives crosslinked by genipin was reported to remain in porcine mucosal membranes even after 6 h (70% of chitosan–catechol remains), whereas unmodified chitosan crosslinked by genipin lost contact within 1.5 h.

One unique feature of this hydrogel is the preserving the functionality of catechol groups, which are responsible for the excellent mucoadhesion enhancement instead of sacrificing them

to build the crosslinking. Many studies formed catechol-containing hydrogels by adding enzymes or oxidizing agents to trigger catechol crosslinking. Or, they added polymers containing functional groups that could form covalent bonds with the catechols, such as –SH groups. These strategies sacrificed catechols during the crosslinking, thus limiting their capability of inducing mucoadhesion. Since GP only crosslinks the amino groups in chitosan, using it as a crosslinker to form catechol-containing hydrogels, preserved the catechol groups and contribute to the mucoadhesion enhancement to the greatest possible extent.

Despite the positive points of this study, these hydrogels have low mechanical properties and slow gelation (about 12 h). This may be a strong limitation for certain applications.

1.5 Summary and objectives of this master

As summarized above, chitosan-based thermosensitive hydrogels are interesting injectable materials for biomedical and pharmaceutical applications. In addition to low viscosity, rapid gelation, high mechanical properties, tissue-adhesiveness, and cytocompatibility, these materials should be storable on extended periods without losing their properties. So, the first objective of this project is to study the stability of chitosan solution, gelling agents, and chitosan hydrogel over time under different storage conditions.

Tissue-adhesion is very important for hydrogels which are used in drug delivery and cell therapy system to extend the drug or cell retention at the site of application and prolonging its therapeutic effects. Thus, the second objective of this project is to covalently graft catechol groups on chitosan and study the effect of gel compounds, especially acid concentration, on gel properties, as a first step towards the development of an injectable tissue-adhesive hydrogel with good gelation time, good mechanical strength, and good compatibility with cells.

CHAPTER 2

MATERIALS AND METHODS

In this chapter, the preparation and characterization of both chitosan and catechol-chitosan hydrogels are described.

2.1 Materials

For the two objectives of this project, two different sources of chitosan (CH) were used:
1) CH from Marinard Biotech (Rivière-au-Renard, QC, Canada) (Kitomer, Mw 250 kDa, DDA 94%), that will be named K-CH.
2) CH from Heppe Medical Chitosan (Germany) (HMC+, Mw 250-350kDa, DDA 95%), that will be named H-CH.
Glycerol phosphate disodium salt penta hydrate $C_3H_7Na_2O_6P \cdot 5H_2O$ (BGP), sodium phosphate monobasic NaH_2PO_4 (SPM), sodium phosphate dibasic Na_2HPO_4 (SPD), Hydrocaffeic acid (HCA) (≥98%), and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (≥ 98.0%) were purchased from Sigma–Aldrich (Oakville, ON, Canada). Sodium hydrogen carbonate $NaHCO_3$ (SHC) was purchased from MP Biomedicals (Solon, OH, USA).

2.2 Synthesis of chitosan and chitosan-catechol hydrogels

Hydrogels were prepared in three main steps. First chitosan was purified. Then it was modified (or not) by grafting catechol groups. Third, a CH (or CH-Cat) solution was mixed with a gelling agent solution in order to create solutions gelifying around body temperature. These steps are described in greater details below.

2.2.1 Purification of chitosan

In order to remove the impurities, the commercial powder was first purified as follows (Assaad et al., 2015). Six (6) grams of CH was dissolved in 600 ml of 0.1 M hydrochloric acid (HCl)

solution and stirred overnight at 40° C. The next day, the solution was filtered under vacuum to remove the insoluble particles. The solubilized CH precipitated with stirring by incorporating 0.5 M sodium hydroxide (NaOH) until the pH reached between 8 and 9. Then the mixture was heated to 95 °C with stirring and 6 ml of sodium dodecyl sulfate (SDS) 10% (w / v) was added. The mixture was kept at 95 °C for 5 min, after which it was cooled to room temperature. The pH was then adjusted to 10 by adding 0.5 M NaOH. The mixture was filtered under vacuum and the precipitated CH was recovered and then washed five times in a beaker containing 600 ml of Milli-Q water, previously heated to 40 ° C, in order to eliminate traces of SDS. Finally, the CH was frozen overnight, freeze-dried for three days and then ground, sieved and stored (Figure 2.1)

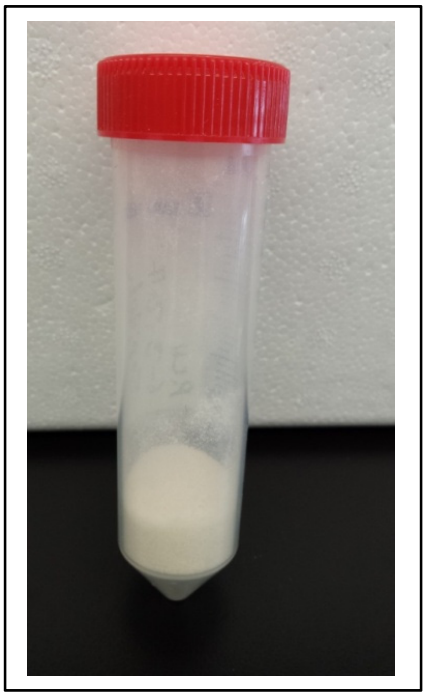

Figure 2.1 purified CH powder

2.2.2 Synthesis of chitosan-catechol (CH-Cat)

Modifying chitosan by catechol was achieved by grafting hydrocaffeic acid to the carbonated chain of CH (Figure 2.2).

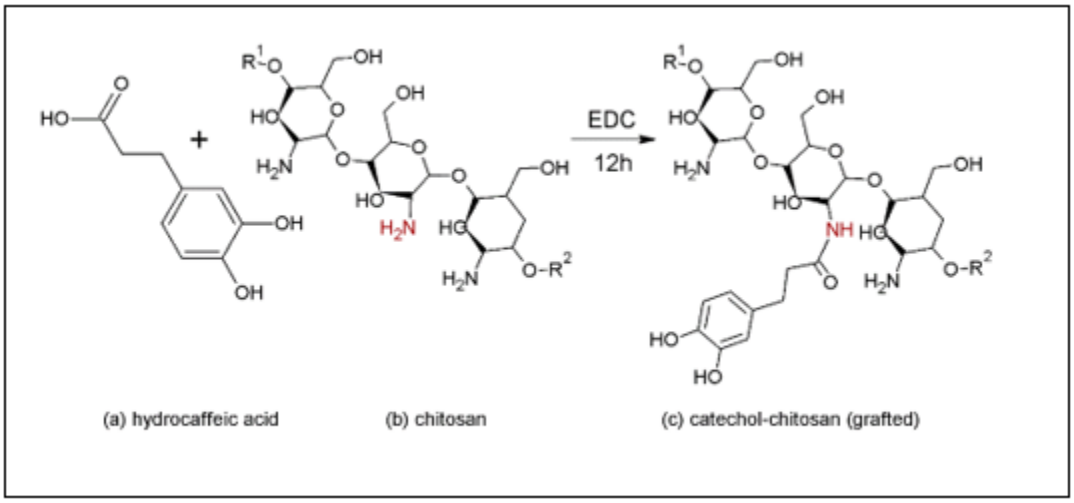

Figure 2.2 Grafting hydrocaffeic acid on chitosan by EDC coupling

As detailed below, the protocol of synthesis of CH-Cat was modified from the work previously done by Professor Cerruti's team at McGill University (Xu et al., 2015).

The steps of this protocol (which will be named old protocol) were as follows:

A. Dissolve 0.6 gr chitosan in 60 ml deionized (DI) water and HCl (pH = 2.5)

B. Add HCA and EDC previously solvated in a water: ethanol 1: 1 mixture in stoichiometric proportions (1: 0.5: 1.17 of glucosamine: HCA: EDC respectively). Adjust the pH between 5 and 5.5 using 1M NaOH.

C. Let the reaction take place for 12 hours under stirring.

D. Dialyses the solution by using a dialysis membrane tube (MWCO 5,000, Spectrum Laboratories, USA) for three days against a solution of HCl pH 5.

E. Lyophilize the purified product and store it at -20 ° C.

By following the old protocol, the CH-Cat solution obtained after the twelve hours of reaction exhibited a red color, becoming more intense and darkened during the dialysis stages. After freeze-drying, the powder obtained formed a porous network, was pink in color. Figure 2.3 shows the solution (in step D) and the final product (after freeze drying) obtained from the old protocol.

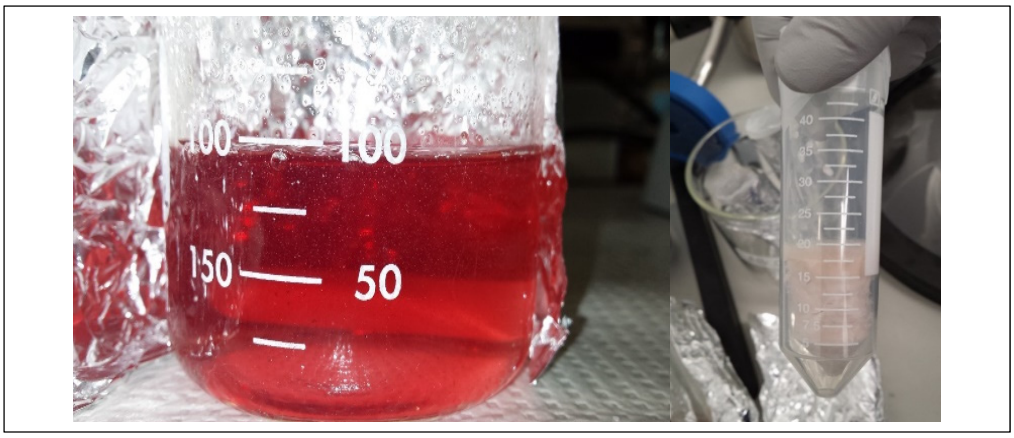

Figure 2.3 CH-Cat solution after dialysis (left) and after freeze-drying (right)

Once dissolved in DI water for gel preparation, CH-Cat formed a brown and dense solution which was not permeable to light. Moreover, particles remained suspended in the liquid phase (Figure 2.4)

Figure 2.4 CH-Cat solution

This difference between the appearance of the solution before and after lyophilization and insolubility of CH-Cat in water led us to the hypothesis that catechol oxidation (Quinone) occurred during the process, following the reaction described in Figure 2.5.

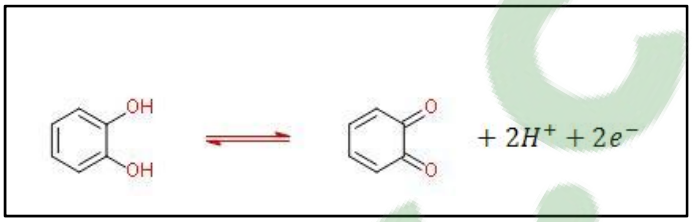

Figure 2.5 Catechol oxidation half-reaction

The solution had the same appearance when Cat-CH was dissolved in acidic media (pH 2, 4 and 6) (Figure 2.6). We first hypothesized that oxidation occurred during solubilisation after lyophilisation. To control this phenomenon, several dissolution experiments were carried out in different acidic media (pH 2, 4 and 6). The obtained solutions were more viscous and had no suspended particles, but were still very brown in color (Figure 2.6). This means that the oxidation phenomenon had taken place before solubilisation.

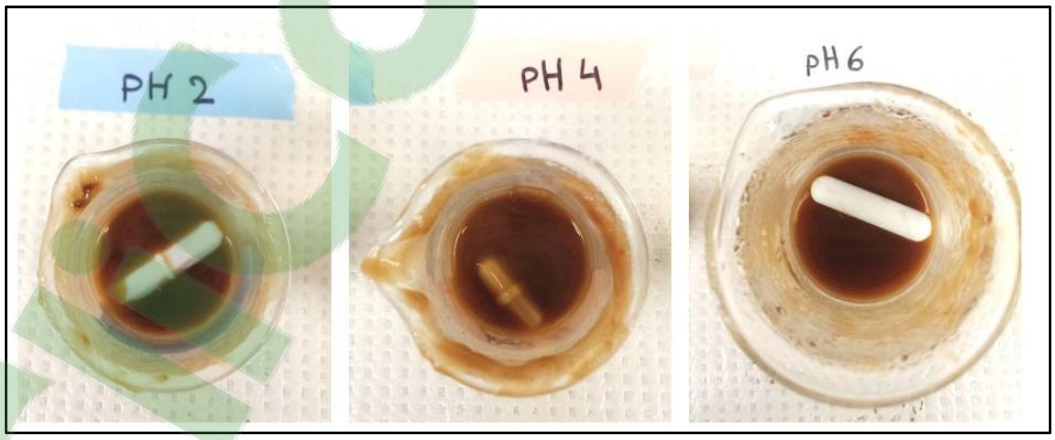

Figure 2.6 Dissolution tests of the CH-Cat in an acid medium with different pH

Therefore, various tests were carried out to determine ideal conditions to prevent catechol oxidation during the steps B, C, and D of the old protocol. Temperature and pH of reaction during all steps of process were the tested parameters.

At the end of the experiments, a protocol was found which enables to avoid catechol oxidation and leads to a white powder. The changes in modified protocol, which will be named the new protocol, comparing to the old one, are as follows:

B. Adjust the pH between 4.65- 4.80 using 1M NaOH.
C. Let the reaction take place for 12 hours under stirring in cold room.
D. The dialysis was done against HCl solution (pH 2.5-3) during the first 2 days (10 mM NaCl solution with 15 mL of 1 N HCl for the first day and 10 mM NaCl solution with 5 mL of 1 N HCl for the second day) following by dialysis against DI water for 6 h at the last day. Dialysis solution should be changed at least 4 times in first and second day.
The complete protocol can be found in ANNEX I.

Figure 2.7 shows the solution before and after dialysis and the final product of the new protocol. All further experiments were done using this optimized protocol.

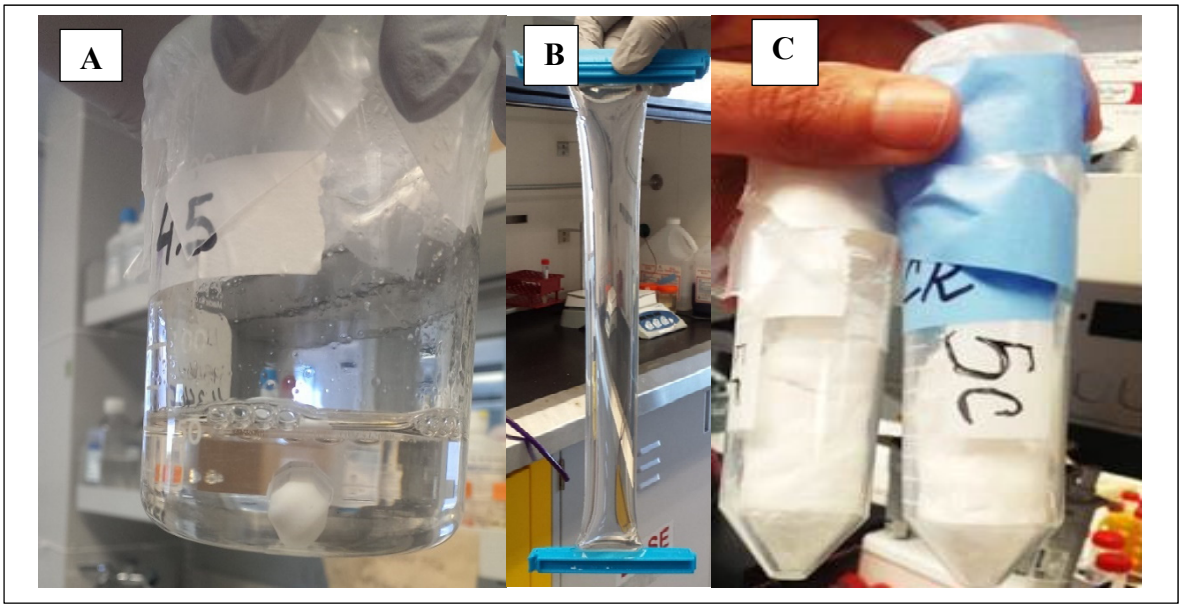

Figure 2.7 CH-Cat (A) solution before dialysis, (B) after dialysis, and (C) the final product

2.2.3 Gelling agents

For this project, different gelling agents were used, as described in Table 2.1. They were prepared using phosphate buffer (PB), BGP and SHC. The PB, at a pH of 8, was prepared by dissolving SPM and SPD salts at molar ratio of 0.073 in Milli-Q water. The SHC and BGP solutions were prepared by dissolving their salts in Milli-Q water. To prepare SHC-PB and SHC-BGP solutions, the SHC salt was solubilized in PB and BGP solutions respectively.

2.2.4 Preparation of chitosan (CH) and chitosan-catechol (CH-cat) hydrogels

To prepare the CH (CH-Cat) physical hydrogels, the gelling agent solution was mixed with a CH or CH-Cat solution prepared as following:

CH hydrogels: The purified CH powder was solubilised in HCl (0.1 M for K-CH and 0.12 M for the H-CH) at 3.33% (w / v) with intensive stirring for about 3 h. The resulting solution was sterilized by autoclaving (20 min, 121°C) and then stored at 4 °C (Figure 2.8).

CH-Cat hydrogel: CH-Cat powder was solubilised in DI water at 3.33% (w / v) with intensive stirring for about 3 h. CH-Cat solution was used freshly to make hydrogel. To study the influence of the pH on gel properties, CH-Cat was also prepared in aqueous solution containing various HCl concentrations (from 0 to 0.09 M).

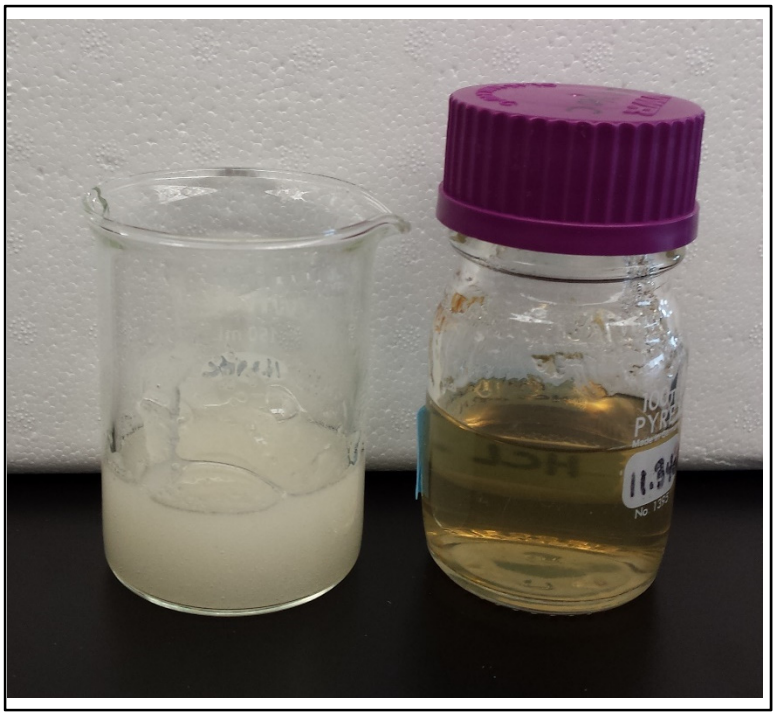

Figure 2.8 solution of purified CH before sterilization (left) and after sterilization (right)

The CH (CH-Cat) hydrogels were prepared at room temperature by mixing one of the gelling agents with the CH (CH-Cat) solution, by using two syringes and a luer-lock connector (Figure 2.9), at a volume ratio of 0.4: 0.6 respectively.

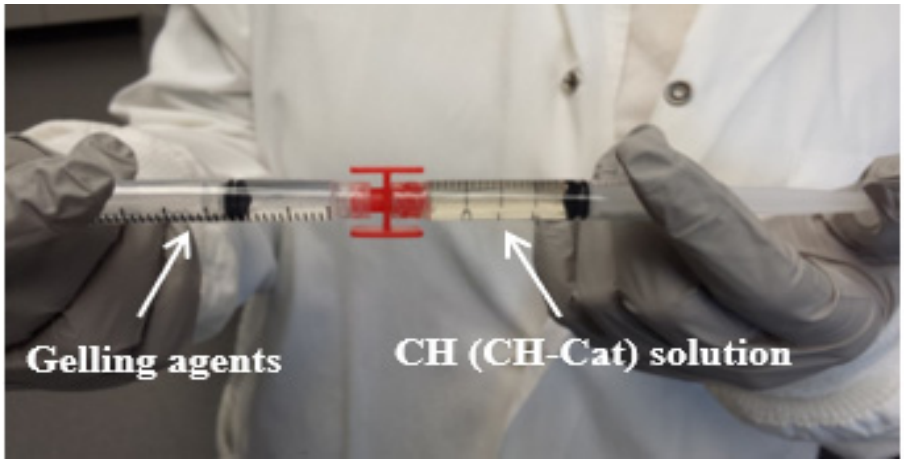

Figure 2.9 Method of mixing the gelling agent with CH (CH-Cat)

All hydrogels contain 2% w/v of CH (Assaad et al., 2015) or CH-Cat. The hydrogels names express their composition. In addition, the gelling agent names express their final concentration in hydrogels (SHC0075-PB004 gelling agent solution means in fact the initial concentrations are 0.19M SHC and 0.1M PB). For example, CH/ SHC0075-PB004 represents a hydrogel containing 2% (w/v) CH, 0.075 M SHC and 0.04 M PB (Table 2.1).

Table 2.1 Abbreviations and composition of the different hydrogels tested

Sample name	Sample composition	Initial concentration (M)			Final concentration (M)		
		PB	BGP	SHC	PB	BGP	SHC
CH/ SHC0075-PB004	Chitosan mixed with (PB + SHC) solutions.	0.1	_	0.19	0.04	_	0.075
CH/ SHC0075-PB008		0.2	_	0.19	0.08	_	0.075
CH/ SHC0075-BGP001	Chitosan mixed with (BGP + SHC) solution.	_	0.025	0.19	_	0.01	0.075
CH/ BGP04	Chitosan mixed with BGP solution.	_	1	_	_	0.4	_
CH-Cat/ SHC009	Chitosan-Catechol mixed with SHC solution	_	_	0.225	_	_	0.09
CH-Cat/ SHC009-PB002	Chitosan-Catechol mixed with (PB + SHC) solutions.	0.05	_	0.225	0.02	_	0.09
CH-Cat/ SHC009-PB004		0.1	_	0.225	0.04	_	0.09
CH-Cat/ SHC009-PB008		0.2	_	0.225	0.08	_	0.09

2.2.5 Storage CH solution and gelling agents to test the stability over the time

To test the stability of chitosan solution, gelling agents (GA) over time under different storage conditions and their effect on hydrogel properties, they were stored in two conditions: room temperature (RT) and refrigerator temperature (FT) (4 to 5 °C). To avoid variability between chitosan batches, one single batch of chitosan was prepared at Day 0 for all these tests. The volume of chitosan that was needed for each rheometry test was stored in 3 ml syringes. In addition, pH measurement was done on one 10 mL sample (which was stored in closed cap glass bottle) at different time points. Also, the gelling agents that used to mix with chitosan

and make hydrogels, were from the same batch that were prepared at day 0 and stored in closed cap tubes. To test the pH of gelling agents, three different batches of each GA were prepared so the results of pH measurement of GA come from independent samples (N = 3).

2.3 Characterization of chitosan-catechol

Both nuclear magnetic resonance (NMR) spectroscopy and UV-Vis spectrometry were used to confirm catechol grafting to chitosan and characterize the degree of conjugation.

2.3.1 NMR

Conjugation of catechol functional groups onto chitosan backbones was confirmed by Nuclear magnetic resonance (NMR) spectroscopy.

NMR is a technique used to analyze the structure of many chemical molecules, primarily organic compounds. A typical compound might consist of carbon, hydrogen and oxygen atoms. The principle of NMR comes from the spin of nucleus. Nuclear spins generate magnetic field without applied an external magnetic field. The nuclear spins are random in directions. When an external magnetic field is present the nuclei align themselves either with or against the external magnet. In this case, an energy transfer is possible between the base energy to a higher energy level. The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy is emitted at the same frequency. The signal that matches this transfer is measured in many ways and processed to yield an NMR spectrum for the nucleus concerned (Figure 2.10).

In its simplest form, an NMR experiment consists of three steps:

1. Place the sample in a static magnetic field.
2. Excite nuclei in the sample with a radio frequency pulse.
3. Measure the frequency of the signals emitted by the sample.

From the emitted frequencies, analysts can deduce information about the bonding and arrangement of the atoms in the sample. ^{1}H and ^{13}C are two of the most widely used NMR nuclei (Edwards, 2009).

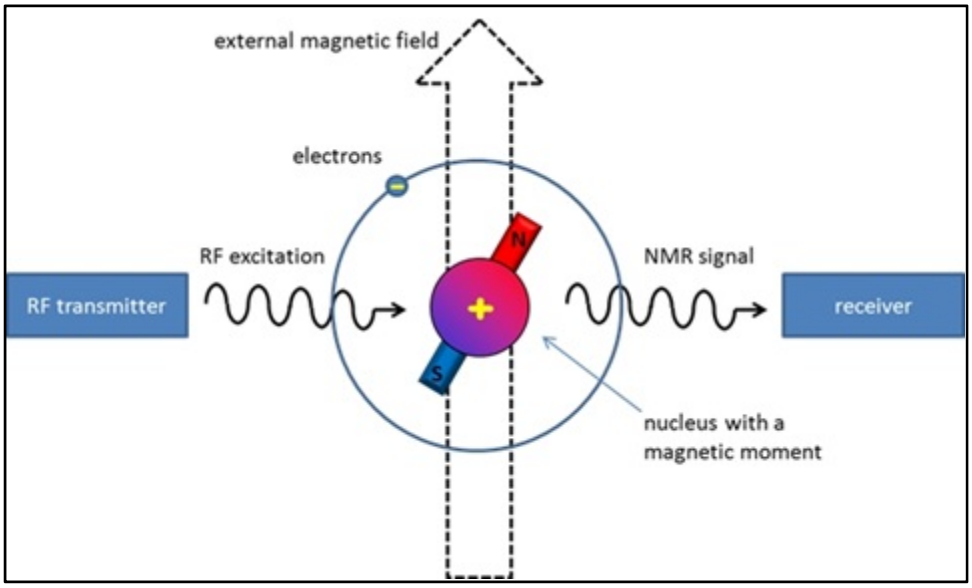

Figure 2.10 The nuclear magnetic resonance (NMR) phenomenon
Taken from utu.fi

NMR signals are usually plotted as spectra and analyzed with respect to two features, **frequency** and **intensity**. It is conventional in NMR to plot frequency on the horizontal axis and increasing towards the left. Absolute frequencies are measured in Hertz or Megahertz (MHz). Reporting on measured signals is simplified if all frequency measurements are made with respect to a reference. The recommended reference is a chemical called tetramethylsilane (TMS). When a ^{1}H or a ^{13}C spectrum is acquired the presence of TMS gives rise to a single, easily identifiable peak. This peak is referenced to zero and the frequencies of all other peaks are given in terms of their frequency relative to the TMS frequency. However, this can be even more simplified if the ppm unit is used instead of Hertz. The ppm unit represents frequencies as a fraction of the absolute resonance frequency which will depend on the strength of the magnet. The advantage of the ppm unit is that frequency measurements are independent of magnet strength. This greatly simplifies the comparison of spectra acquired on different spectrometers (Edwards, 2009).

In this particular study, ^{13}C CP-MAS NMR spectra were obtained at 100 MHz using a 7.5 mm rotor spinning at 5 kHz, a 1.5 ms contact time and recycle time of 2 s (Agilent/Varian VNMRS-400, USA).

2.3.2 UV-Vis

A UV-Vis spectrometer (Carry 5000, USA) was used to determine the degree of conjugation of catechol to the amine groups of chitosan. This quantitative analysis is the most widely used technique as a simple and powerful method for measuring catechol conjugation rates (Ryu, Hong & Lee, 2015).

The maximum absorption of catechol occurs at a wavelength of 280 nm, while chitosan does not absorb at this wavelength. Therefore, absorbance at 280 nm was used to quantify the degree of catechol conjugation. To that purpose, the absorbance of a solution of 5 mg of CH-Cat dissolved in 10 ml of DI water was compared to a standard curve established with hydrocaffeic acid (HCA) at five different concentrations (0.1 to 0.5 mmol/L).

To calculate the degree of conjugation of catechol to the amine groups of chitosan (Percentage of Cat in CH-Cat sample) the degree of deacetylation of chitosan (DDA, 95% in this study), should be considered as well as the molecular weight of three different monomers in CH-Cat structure (Figure 2.11), namely the deacetylated section (x), the conjugated section by catechol (y) and acetylated section (z). The molecular weight of x, y, and z is 161, 325, and 203 respectively.

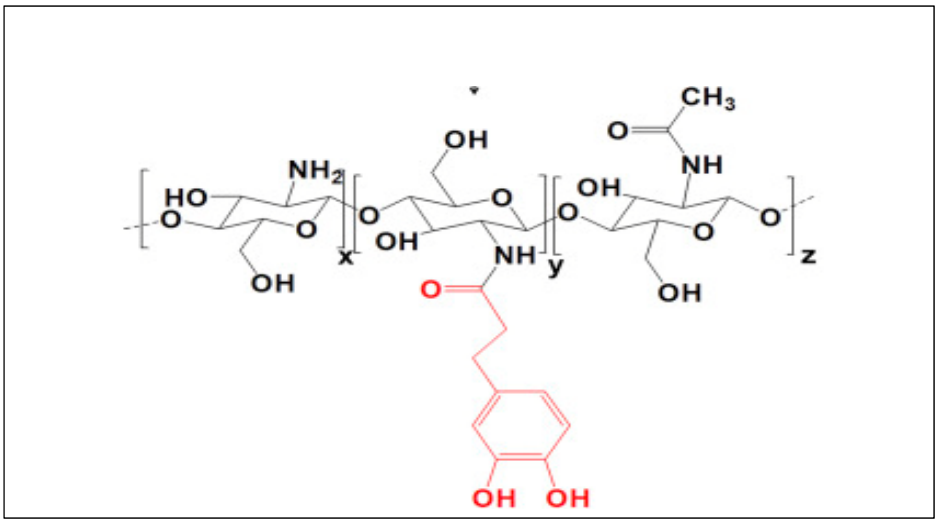

Figure 2.11 CH-Cat structure

By using the following equation, the catechol conjugation rates in sample could be calculated:

$$5 * 10^{-3} = M * 10^{-3} * 10 * 10^{-3} * 325 + [M * (0.95-X) / X] * 10^{-3} * 10 * 10^{-3} * 161 + [0.05 * M / X] * 10^{-3} * 10 * 10^{-3} * 203 \quad (2.1)$$

Where

X= ratio between conjugated and unconjugated parts of chitosan to catechol (catechol conjugation rate)

M= the concentration of catechol (mmol / ml) deduced from the standard curve

Then:

$$X = (0.1631 * M) / (0.5 – 0.164 * M) \quad (2.2)$$

2.4 Mechanical characterization

2.4.1 Rheological study

The rheological properties of the various formulations of chitosan and chitosan-catechol hydrogels were evaluated as a first estimate of their mechanical properties, but above all to study their gelation kinetics. Rapid gelation is one of the important properties for an injectable

hydrogel. The gel should remain liquid and stable for storage, preparation and injection at room temperature, and should rapidly gel upon reaching body temperature in situ to prevent its migration to undesirable areas.

An Anton Paar instrument (Physica MCR 301, Germany) equiped with coaxial cylinder geometry (CC10/T200) and connected to a circulating water bath (Julabo AWC100, Germany) was used to measure the storage modulus (G') and loss modulus (G") as a function of time. The storage modulus measures the stored energy (elastic portion) and the loss modulus measures the energy lost as heat (viscous portion). Immediately after mixing the two solutions (0.6 ml of gelling agent and 0.9 ml of the chitosan/chitosan-catechol solution), the gel was injected into the cell and the storage modulus (G'), loss modulus (G") as well as the complex viscosity (η) were measured in the linear viscoelastic range (LVR), at a constant shear stress (1 Pa) and a constant frequency (1 Hz).

It should be mention that to calculate the complex shear modulus (G*) in rheometry test, it is enough to pre-set τ (motor torque) or γ (deflection angle) and under this pre-set measure the other value and using the equation: $G^* = \tau / \gamma$, shear modulus will be reached. If we imagine G* as a vector and determine its angle with X axis (δ, loss or damping factor), then we will have G' and G"

$$\tan \delta = G'' / G' \qquad (2.3)$$

if G' = G" then $\tan \delta = 1$, and it is sol-gel transition point (phase transition) in a material.

$\tan \delta < 1$ shows that material is more elastic and $\tan \delta > 1$ shows that material is more viscous. Complex viscosity (η) could be calculated by dividing G* by angular Frequency (Mezger, 2006).

The measurements were carried out at 37 $^{\circ}$C (body temperature). Each test was repeated three times.

2.4.2 Compression tests

To evaluate the hydrogels' strength after 48 h gelation at 37 °C, axial unconfined compression tests were performed at room temperature using the Anton Paar (Physica MCR 301, Germany) equipped with parallel geometries (P25 / P2). A volume of 2 mL different formulation of CH-Cat hydrogels was prepared (n = 3) in small cylindrical molds (14 mm diameter). After 48 h gelation at 37°C in an incubator, the sample was gently removed from the mold and placed on the planar geometry. The height of the sample (h) was used to calculate the speed (v) for applying up to 50% compression at a rate of 100% deformation per minute:

$$V = h/60 \text{ mm.s}^{-1} \quad (2.4)$$

Load/displacement curves were used to calculate the secant elastic modulus, considered as the slope of a line connecting the point of zero strain to a point at a specified deformation.

2.5 Physico-chemical characterization

2.5.1 pH study

The pH measurements were carried out at room temperature using a Denver Instrument Ultra Basic pH meter. For the chitosan stability project, the pH of gelling agents as well as the chitosan solution were measured after different storage times at 4 °C (in cold room) and room temperature. For the samples stored in cold room, samples were left to warm up at room temperature for about 1 h prior to pH measurement.

For the tissue adhesive project, the pH was measured twice. The first time, the pH of mixtures (CH-Cat + gelling agent) immediately after mixing; the second time, the pH of hydrogel after putting it for 24h at 37 °C.

2.5.2 Osmolality

The Advanced Micro Osmometer (Model 3300, Advanced Instruments), was used to measure gel osmolality. Different formulations of CH-Cat hydrogels were kept at 37 °C for 24h, then 20 µl of filtrated gel was used for osmolality measurement. It is important to specify that the osmolality is measured here (in mOsm / kg of solvent) and not the osmolarity (in mOsm / L of solution).

2.6 Tissue adhesive tests

To evaluate the mucoadhesive properties of CH-Cat hydrogels after and during gelation, wash off and tensile tests have been done respectively. Fresh tissue (sheep intestine) was obtained from animal facility of the Centre de recherche du Centre hospitalier de l'Université de Montréal (CRCHUM). It should be mentioned that these tissues were obtain from animals sacrificed in animal facility for other experiments.

2.6.1 Tissue adhesive wash off test

Wash off tests were performed as a first assessment of adhesive properties of the hydrogels after gelation (Xu et al., 2015). The hydrogels were left to gel in cylindrical mold for 48h at 37 °C. Fresh tissue (sheep intestine) was glued to a microscope glass slide and placed into a beaker vertically. The hydrogels were removed from the mold and gently pressed to adhere on the tissue surface for 30 seconds. Both the tissue and the hydrogels were then immersed in 30 ml PBS at 37° C (Figure 2.12). A magnetic bar was used to generate flow. The adhesive property of hydrogels after gelation was evaluated by recording the number of remaining hydrogels adhered to tissue for 27 hours. Five samples from each hydrogel group were tested.

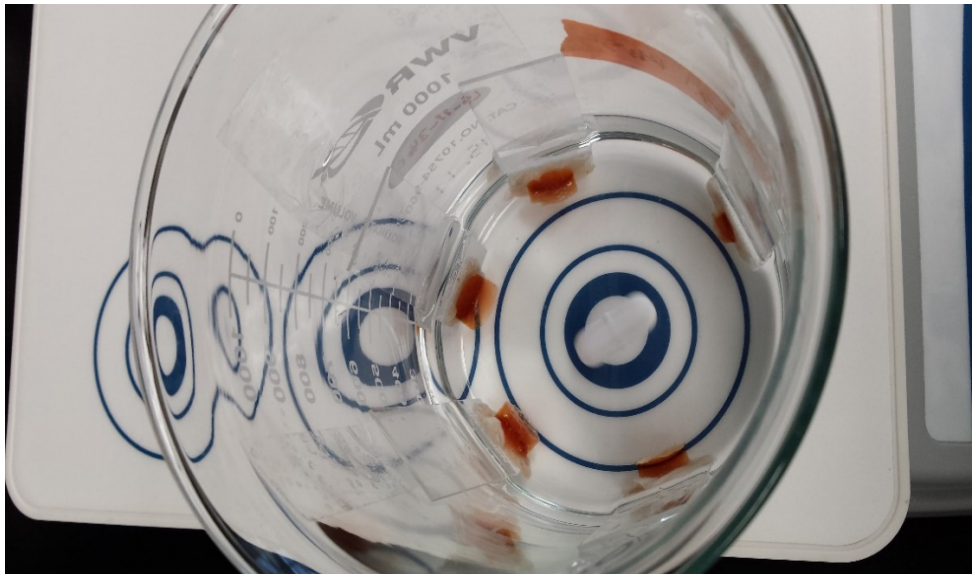

Figure 2.12 Wash off test: the adhered hydrogels on fresh tissues were glued on microscope glass and immersed in PBS at 37° C with a magnetic stirrer

2.6.2 Tissue adhesive tensile test

To study the effect of HCl on mucoadhesive properties of CH-Cat hydrogels during gelation, tensile tests were done using a Bose Electro Force 3200 instrument (Bose Corporation, USA) equipped with a 225 N load cell and a sample holder previously designed at ETS (HAKIM, 2015) and presented in Figure 2.13. The sample holder consists of two parts: The cylinder part (Figure 2.13A, Left) which was used to insert the hydrogel in it, and a support piece (Figure 13A, Right) that has a square in the center. A volume of 2.8 ml of liquid hydrogel was used to fill the cylinder parts. Then, the pre-cut fresh tissue (intestine) was glued on the square part of the support piece, placed in contact with the gel (Figure 2.13B) and left to gel for 48 hours in the incubator at 37 ° C.

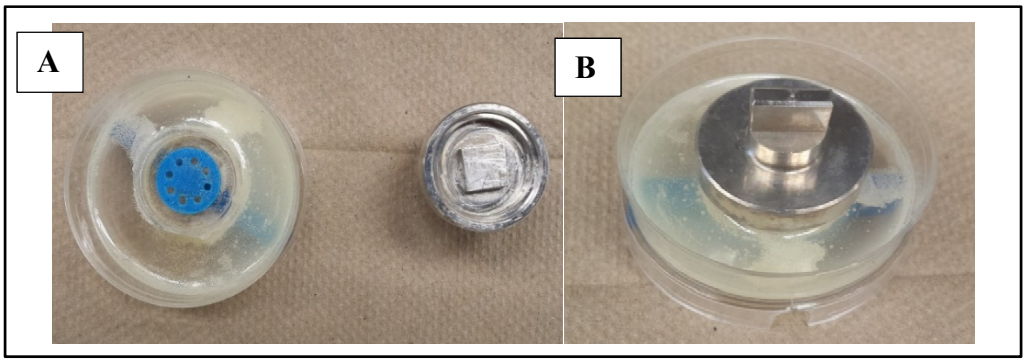

Figure 2.13 Sample holder for tissue adhesive tensile test

After that time, the whole sample holder was installed on the Bose instrument (Figure 2.14) and a tensile test was done to determine the maximum detachment force (MDF) between the tissue and the hydrogels.

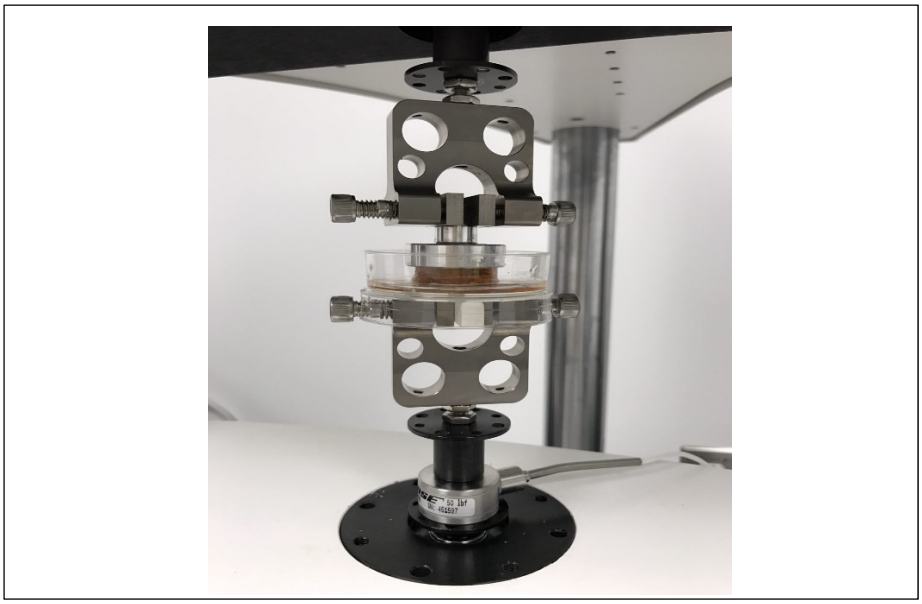

Figure 2.14 Tensile test to determine the maximum detachment force (MDF) between the tissue and the hydrogels

2.7 Statistical analysis

All the results are expressed as mean ± standard deviation (SD). Statistical analysis was done using Statgraphics software (Statgraphic centurion, statpoint technologies Inc., USA). To determine if a statistical difference between means existed, one-way ANOVA was used when comparing more than two groups followed by Fisher's least significant difference (LSD) procedure with a confidence level of 95%. For comparing two conditions, independent t-test with equal variances was used. A p-value lower than 0.05 was considered significant for all tests. The number of independent experiments (N), and the total number of samples used for each independent experiment (n) are indicated in the figure captions.

CHAPTER 3

RESULTS

The results of the two objectives of this project are presented separately. Results of chitosan (CH) stability tests will be first presented in section 3.1, and then results concerning the development of a tissue adhesive gel will be presented in section 3.2.

3.1 The stability of Chitosan solution, gelling agents, and hydrogels

The stability of chitosan solution, gelling agents (GA) over time under different storage conditions and their effect on hydrogel properties were tested by measuring pH, viscosity, and gelation time in different time points.

3.1.1 The stability of chitosan solution

The stability of chitosan solution (3.33 % chitosan in HCl 0.1M) was tested by measuring its viscosity and pH. Chitosan solutions were stored in two conditions: room temperature (RT) and fridge temperature (FT) (4 - 5 $^{\circ}$C). At different time points (1, 2, 4, 13, 26, and 52 weeks after preparation day (Day 0) the pH and complex viscosity were measured.

Figure 3.1 shows that the viscosity of chitosan solution remains relatively stable from Day 0 until 13 weeks, without difference between samples stored in refrigerator and those stored at room temperature. However, after 1 year, viscosity is significantly higher than day 0 ($p<0.05$). Moreover, standard deviations are higher for samples stored at room temperature.

Figure 3.2 shows the pH of the chitosan solution. The pH remained quite stable around 6.3, with almost no difference between storage chitosan solution in fridge or room temperature until 26 weeks. The small variations between the time points may be attributed to the precision of the measurement method.

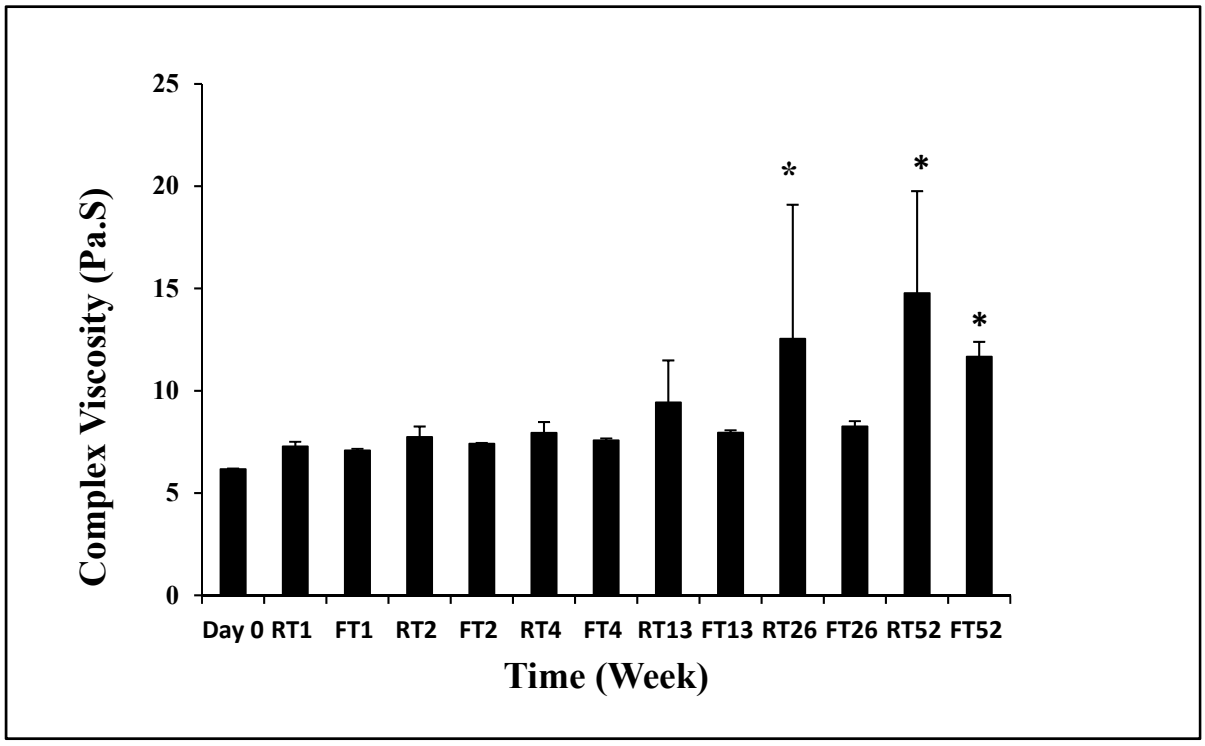

Figure 3.1 Effect of storage condition (Room temperature (RT) and Fridge temperature (FT)) and time on the viscosity of CH solution. (mean ± SD, n = 3), (*p<0.05 comparing to Day 0)

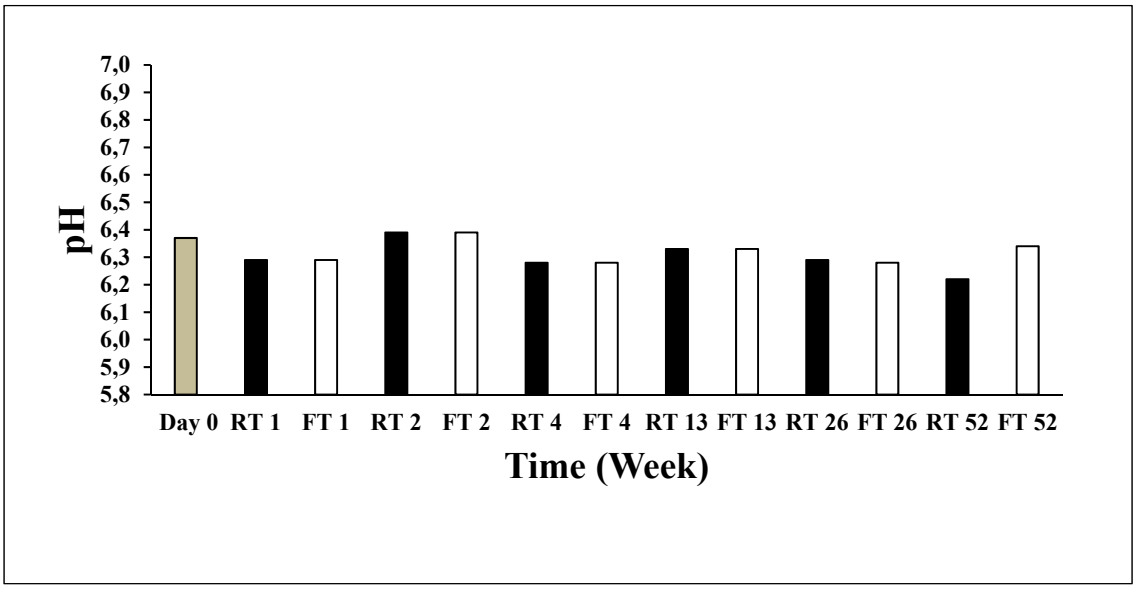

Figure 3.2 Effect of storage condition and time on the pH of CH solution

3.1.2 The stability of gelling agents and hydrogels

Similarly, GAs (SHC0075-PB004, SHC0075-PB008, SHC0075-BGP001, and BGP04) were stored in two conditions (room temperature (RT) and fridge temperature (RT)) and their pH were measured at different time points. Besides, to evaluate the effect of chitosan and GA changes with time on hydrogel properties, rheological tests have been done to monitor the evolution of the storage modulus (G') and loss modulus (G") during gel formation at 37 $^{\circ}$C. It should be mentioned that rheometry tests have been done only for the fridge-stored samples. Prior to mixing and testing, the chitosan and GAs solution were left in the room for 2 hours to reach the ambient temperature.

The time at which G'= G" is considered as gelation time (t_{gel}). Moreover, the rate of increase of G ' is a good indicator of the gelation kinetics and of the mechanical shear properties and hence the cohesion of the gel over time. It should be mentioned that in rheometry test, statistical analysis was only done for G' values at 30 min.

SHC0075-PB004

Figure 3.3 and Figure 3.4 show that the pH of the GA solution used to prepare SHC0075-PB004 hydrogels (SHC019M-PB01M), originally at 8.2, significantly increased (linearly after 12 weeks, R-squared= 0.98) over time ($p< 0.05$) comparing to Day 0. The pH of RT stored GA increased more compared to those that were stored at FT, the difference being already significant at week 2 ($p< 0.05$).

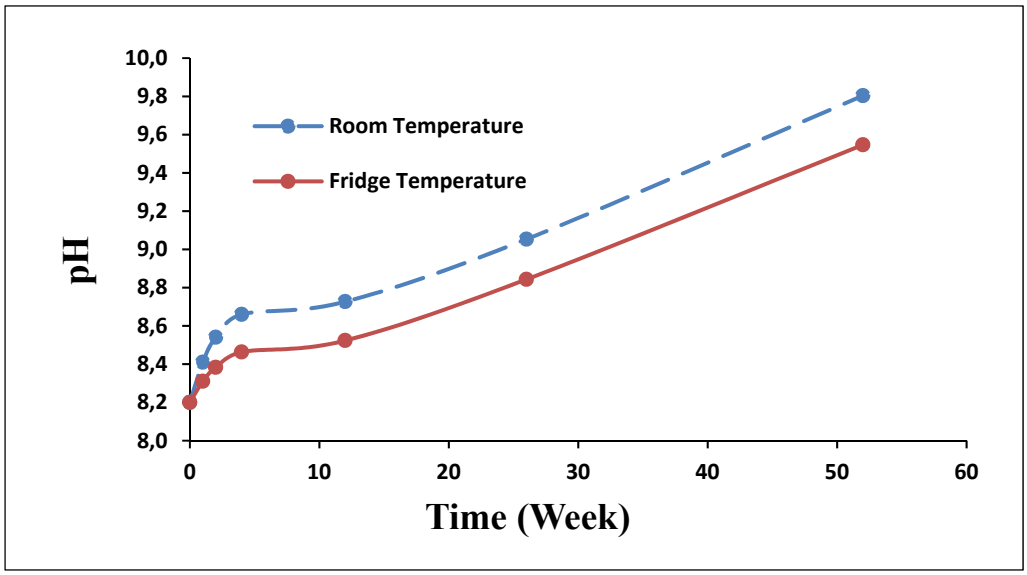

Figure 3.3 Effect of storage condition and time on the pH of SHC0075-PB004

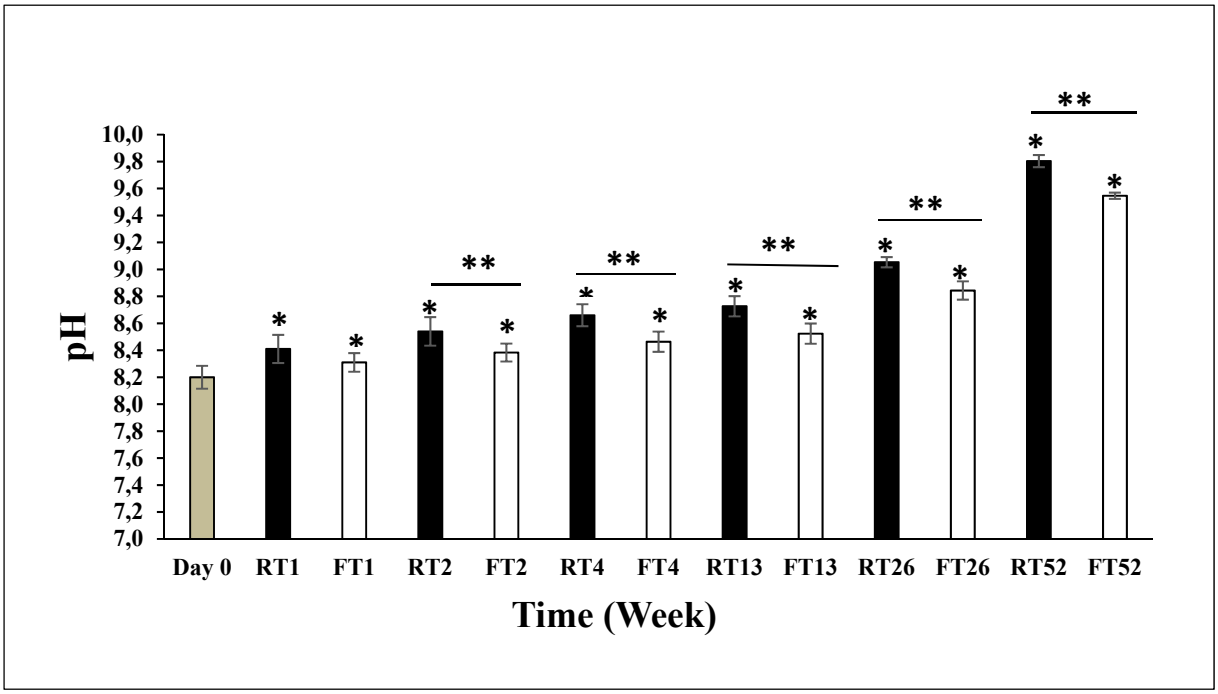

Figure 3.4 Effect of storage condition and time on the pH of SHC0075-PB004. (mean ± SD, N = 3), (*, $p<0.05$ comparing to Day0), (**, $p<0.05$ comparing RT & FT)

Figure 3.5 presents the evolution of the storage (G') and loss (G'') modulus at 37°C as a function of time for hydrogels prepared with solutions stored for 0 versus 52 weeks. It shows

that the storage time did not affect the gelation time, $t_{gel,}$is less than 15 s (time required to get the first measurement) for both gels. However, the initial G' value is lower when the gel was prepared with freshly prepared solutions compared to stored GA.

Figure 3.6 summarized the G' values at different timepoints (initial, after 3 and 30 min). It shows that storage modulus changes initially. But at the end of test (30 min) it does not significantly change (comparing to Day 0) for 52 weeks (P= 0.09).

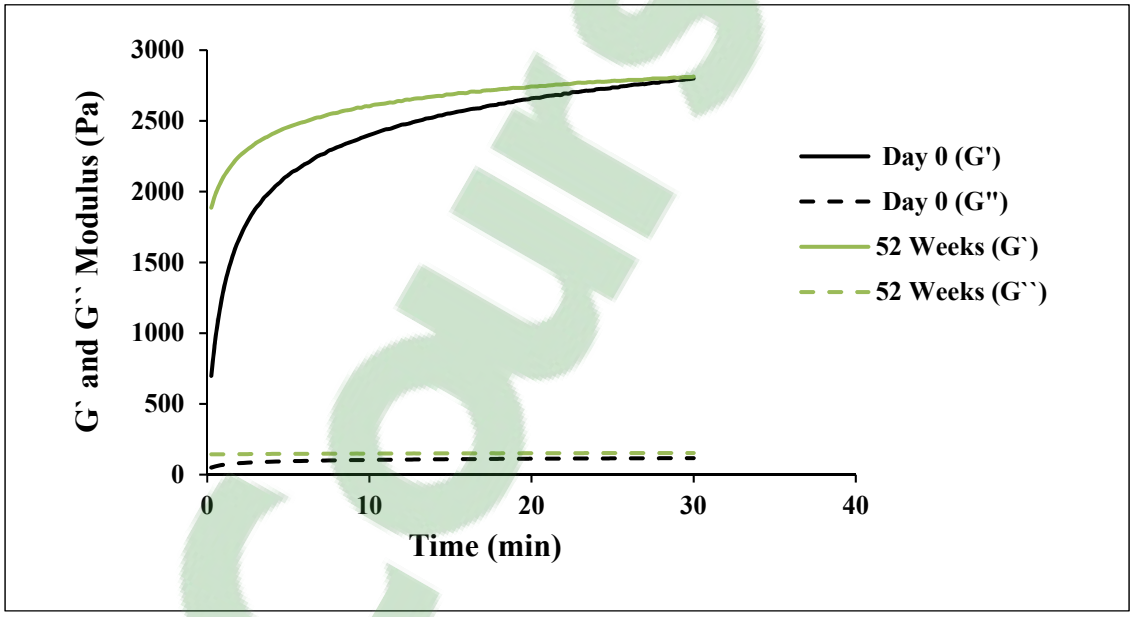

Figure 3.5 Evolution of storage (G') and loss (G") modulus for CH/SHC0075-PB004 hydrogels as a function of storage time (Week), for 30 min at 37 ˚C, (mean, n = 3)

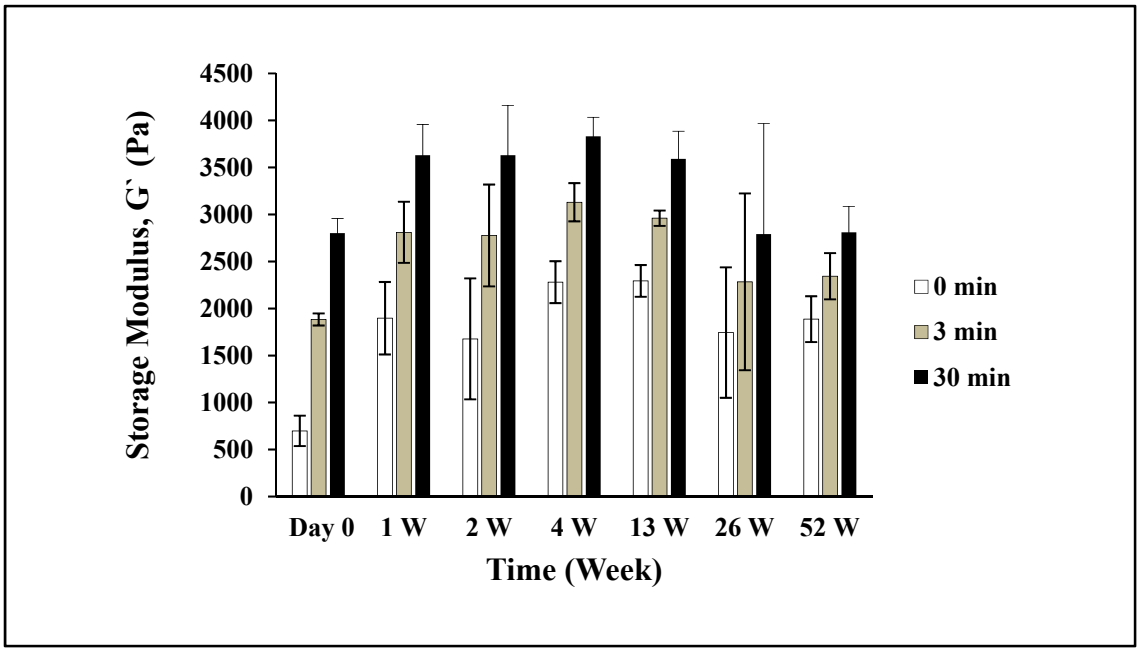

Figure 3.6 Evolution of the storage modulus G' for different storage time (in Week) in CH /SHC0075-PB004 hydrogels, at 37 °C, for 30 min (mean ± SD, n = 3)

SHC0075- PB008

Figure 3.7 shows that the pH of SHC0075-PB008 solution significantly increased over time compared to Day 0 ($p < 0.05$). Besides, there is a significant difference between GA solutions stored in fridge or room temperature, the pH of RT stored GA increasing more than those stored at FT ($p < 0.05$). The pH evolution by time graph could be found in ANNEX II.

The storage time significantly affected the gelation kinetics, as showed by Figure 3.8 and Figure 3.9, even if t_{gel} is less than 15 seconds at 37 °C for all storage times.
Figure 3.9 indicates the G' values at different time points. It shows that the storage modulus after 30 min gelation is significantly decreased, when the storage time prior mixing is longer ($p < 0.05$). More importantly, after long time storage, G' does not increase with time at 37 °C, as shown by the straight line observed during rheometry test in 30 minutes (Figure 3.8).

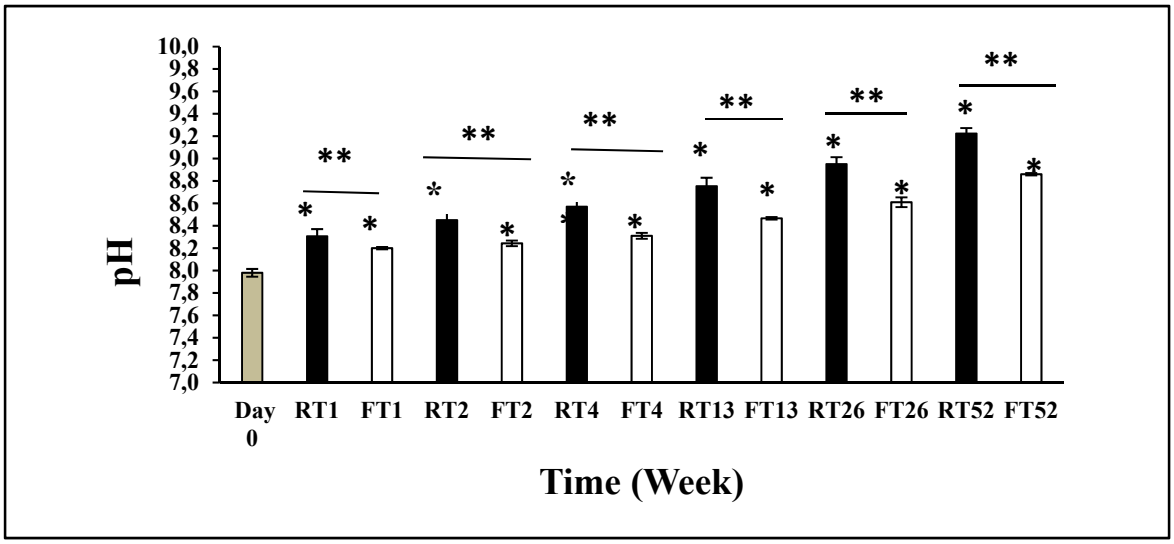

Figure 3.7 Effect of storage condition and time on the pH of SHC0075-PB008 solution. (mean ± SD, N = 3), (*, $p<0.05$ comparing to Day0), (**, $p<0.05$ comparing RT & FT)

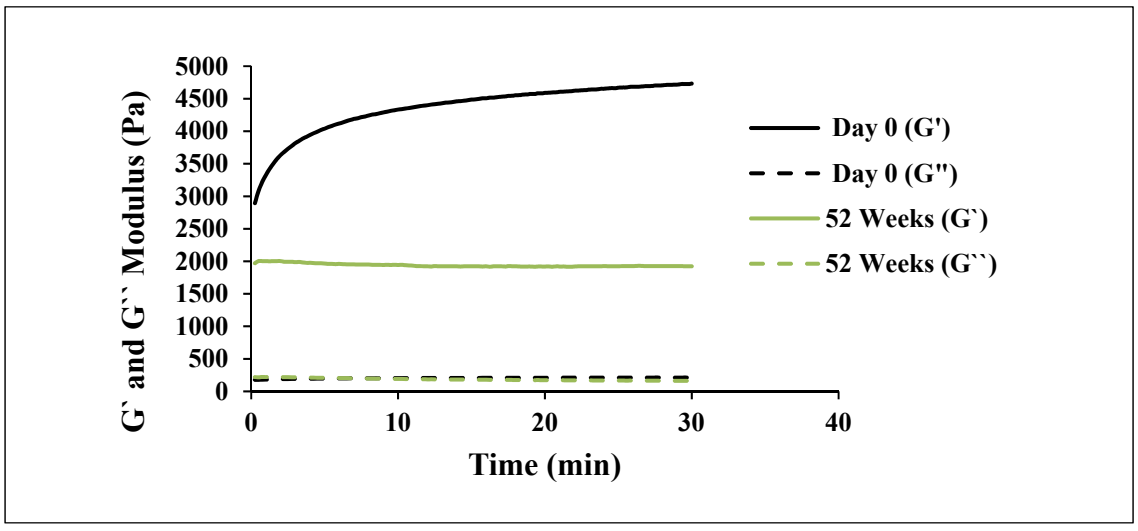

Figure 3.8 Evolution of storage (G') and loss (G") modulus for CH/SHC0075-PB008 hydrogels as a function of storage time (Week), for 30 min at 37 ˚C, (mean, n = 3)

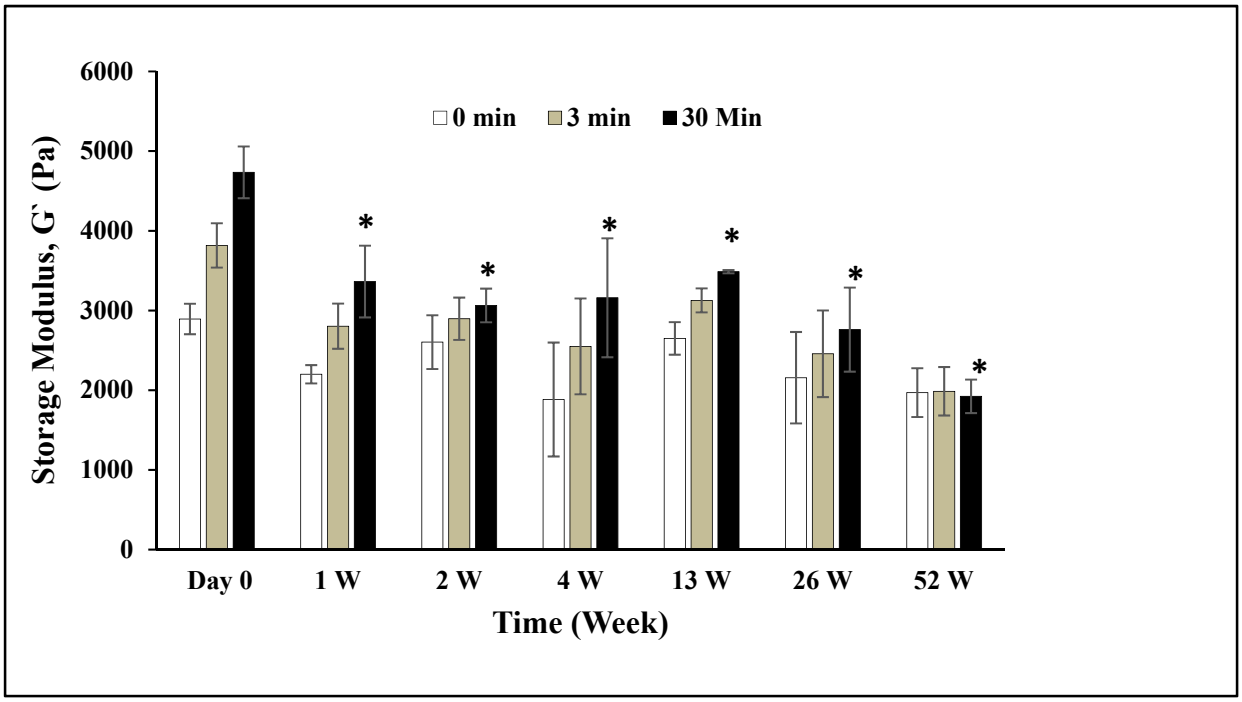

Figure 3.9 Evolution of the storage modulus G' for different storage time (in Week) in CH /SHC0075-PB008 hydrogels, at 37 °C, for 30 min (mean ± SD, n = 3), (*, $p<0.05$ comparing to Day0)

SHC 0075- BGP001

Figure 3.10 shows that the pH of SHC 0075-BGP001, significantly increased over the time from 8.4 to up to 9.2 after 1year storage at RT ($p< 0.05$). Besides, there is a significant difference between storing GA solution in fridge or room temperature, the pH of RT stored GA increasing more than that stored at FT ($p< 0.05$).

Figure 3.11 shows that time did not affect on gelation time for CH/SHC0075-BGP001 hydrogels. At day 0 and after 52 weeks, t_{gel} is less than 15 seconds at 37 °C.

Figure 3.12 indicates the G' values at different time points. It shows that storage modulus that changes initially, at the end of test (30 min) does not significantly change by time for 52 weeks. (P=0.088).

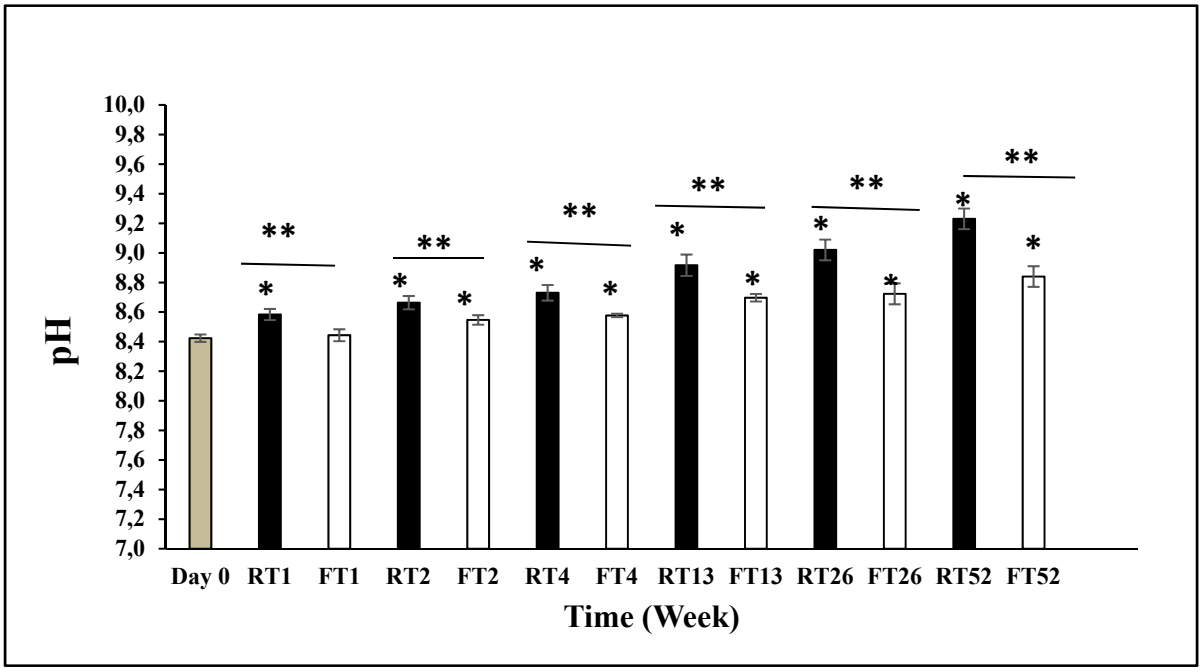

Figure 3.10 Effect of storage condition and time on the pH of SHC0075-BGP001 solution. (mean ± SD, N = 3), (*, $p<0.05$ comparing to Day0), (**, $p<0.05$ comparing RT & FT)

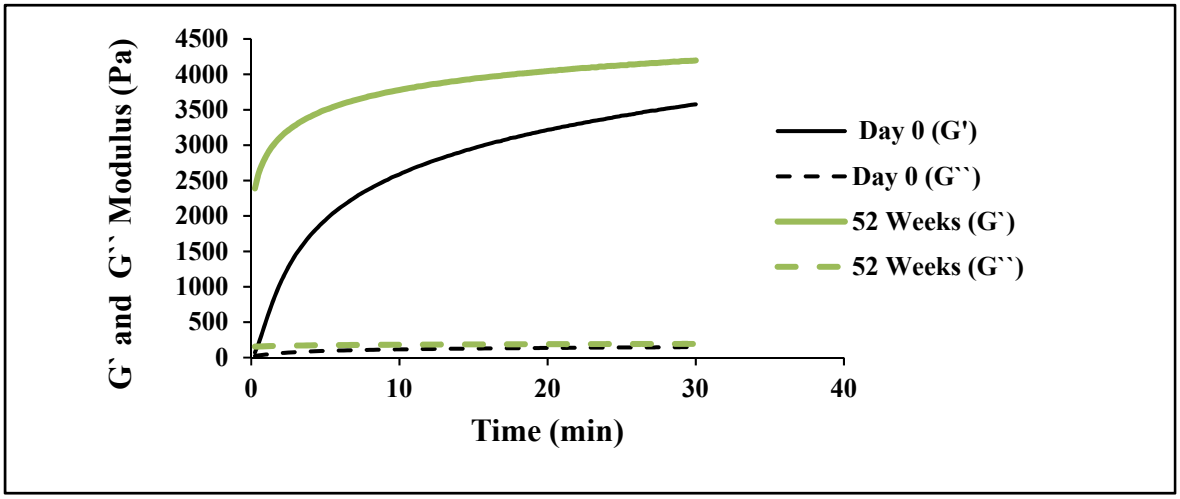

Figure 3.11 Evolution of storage (G') and loss (G") modulus for CH/SHC0075-BGP001 hydrogels as a function of storage time (Week), for 30 min at 37 ˚C, (mean, n = 3)

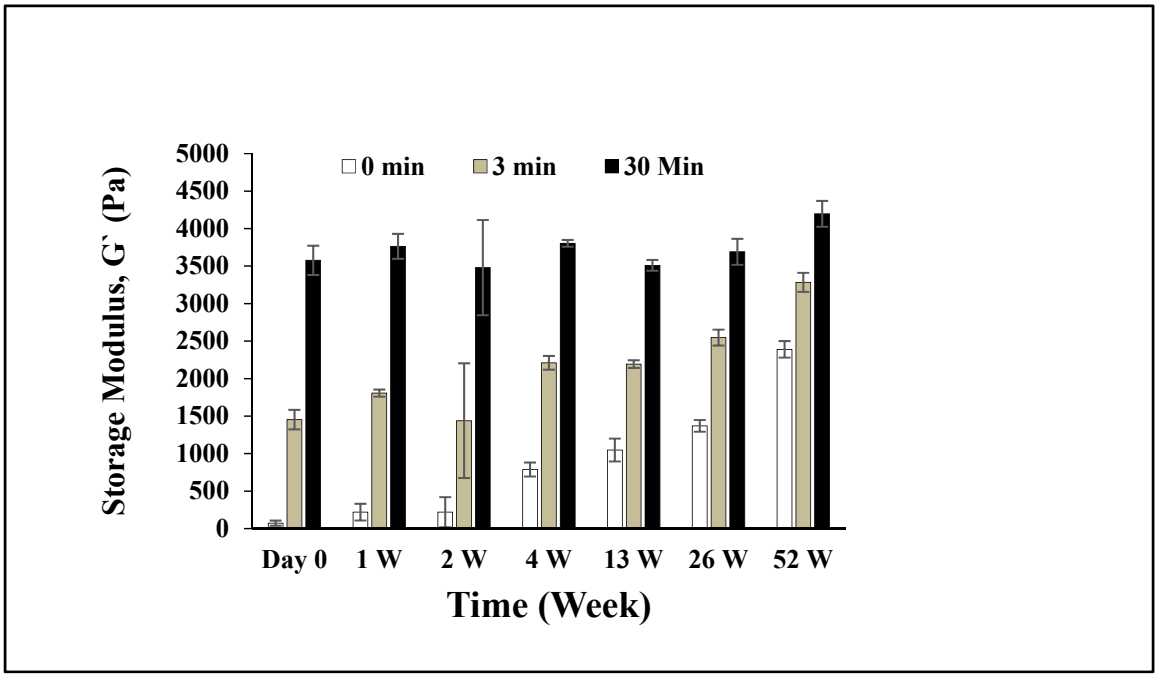

Figure 3.12 Evolution of the storage modulus G' for different storage time (in Week) in CH /SHC0075-BGP001 hydrogels, at 37 °C, for 30 min (mean ± SD, n = 3)

BGP04

Figure 3.13 shows that, in contrast to the other entire GA, the pH of BGP04 significantly decreased over time, from 9.8 to 9 for fridge stored sample and 8.7 for room stored one ($p < 0.05$). The decline is more rapid for GA stored in RT, the difference being significant from week 4 ($p < 0.05$). The pH evolution by time graph could be found in ANNEX II.

Figure 3.14 shows that storage time did not affect the gelation time, t_{gel} is less than 15 s for both gels. However, the G' value for all time points is higher for the solutions which were stored for 52 weeks prior to hydrogel preparation.

Figure 3.15 indicates the G' values at different time points. It shows that storage modulus that increases initially, does not significantly change after 30 min gelation for 52 weeks (P= 0.127).

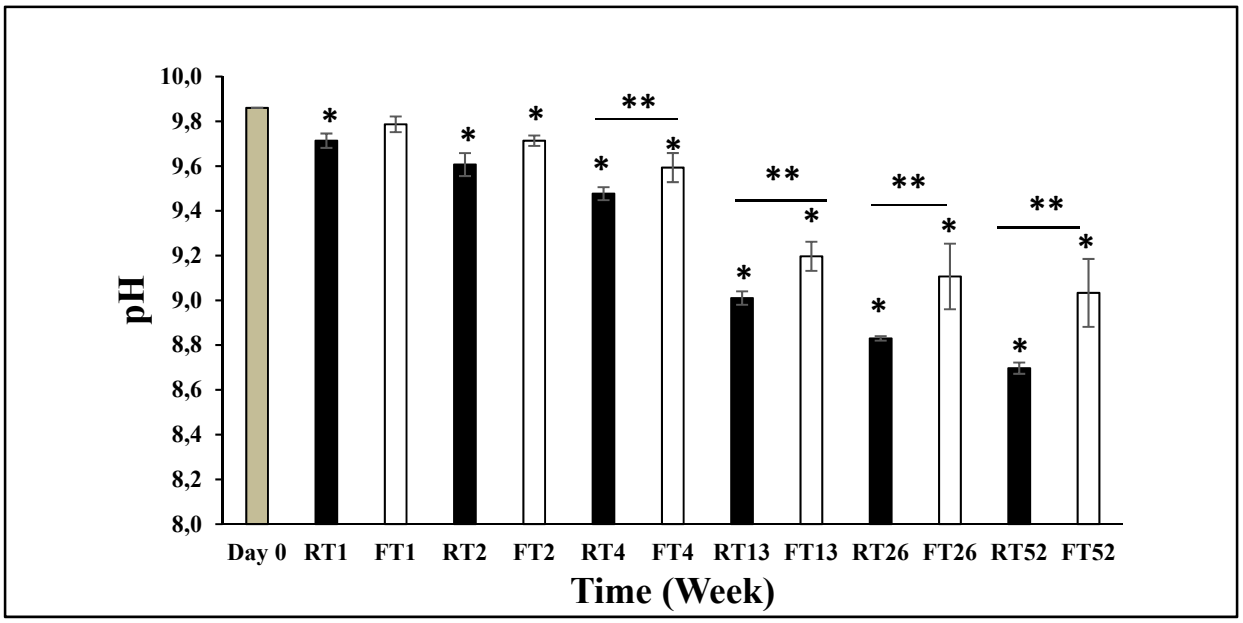

Figure 3.13 Effect of storage condition and time on the pH of BGP04 solution. (mean ± SD, N = 3), (*, p<0.05 comparing to Day0), (**, p<0.05 comparing RT & FT)

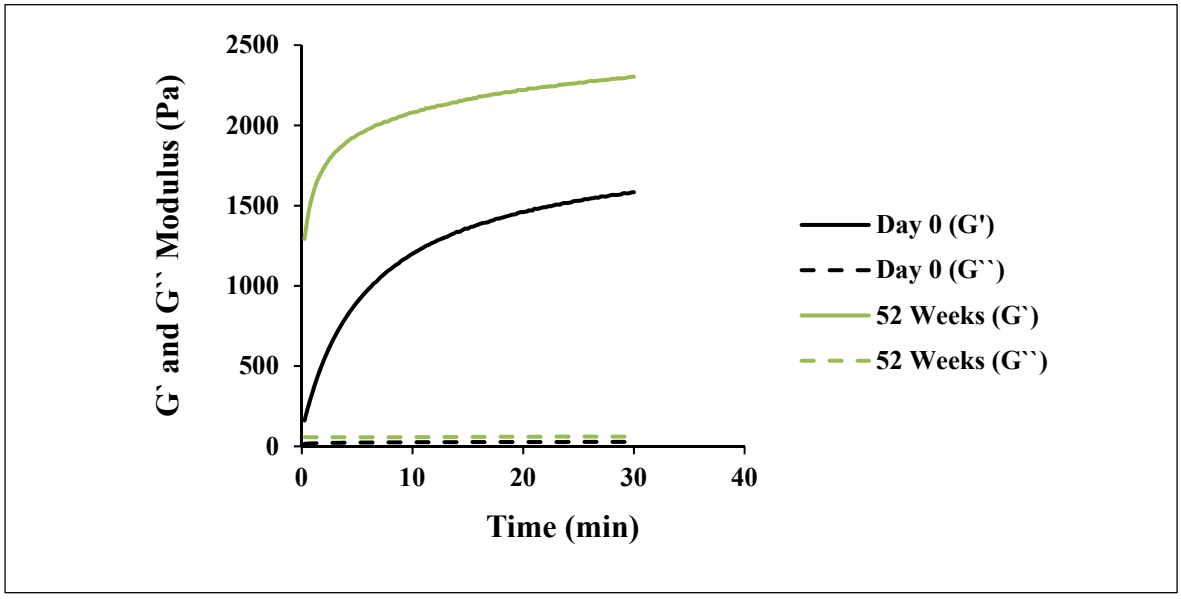

Figure 3.14 Evolution of storage (G') and loss (G") modulus for CH/BGP04 hydrogels as a function of storage time (Week), for 30 min at 37 °C, (mean, n = 3)

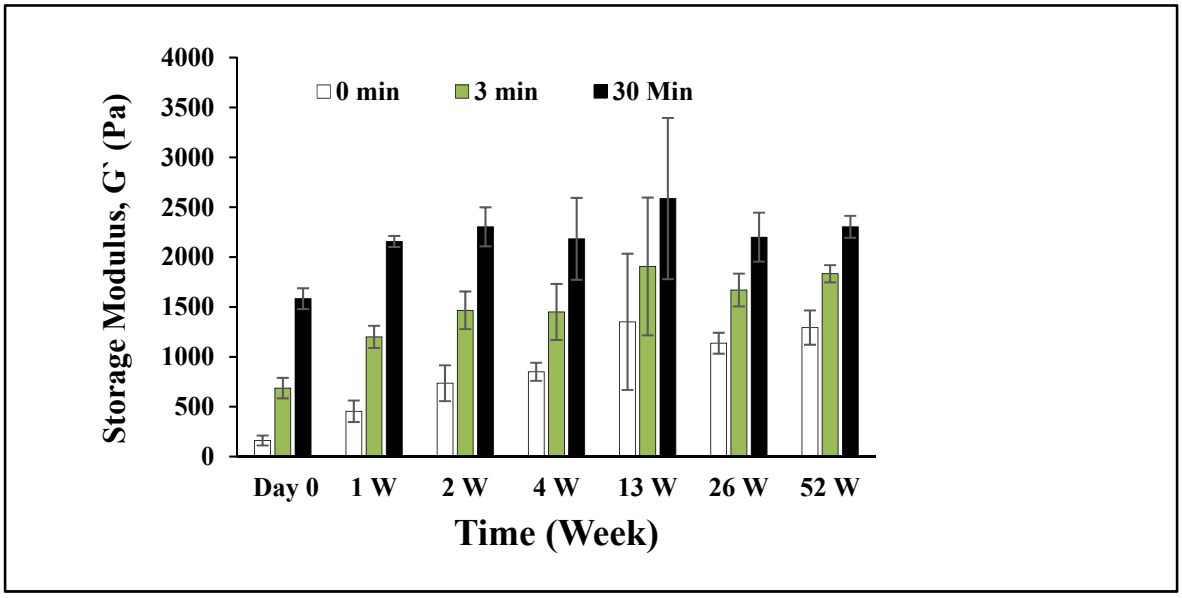

Figure 3.15 Evolution of the storage modulus G' for different storage times (in Weeks) in CH / BGP04 hydrogels, at 37 °C, for 30 min (mean ± SD, n = 3)

3.2 Injectable tissue-adhesive chitosan-catechol hydrogel

3.2.1 Characterization of chitosan-catechol

Both nuclear magnetic resonance (NMR) spectroscopy and UV-Vis spectrometry were used to confirm catechol grafting to chitosan and characterize the degree of conjugation.

NMR

Catechol functional groups were covalently bonded to the backbone of CH. Figure 3.16 shows CH-Cat structure. There are three different monomers in CH structure, namely the deacetylated section (x), the conjugated section by catechol (y) and acetylated section (z).

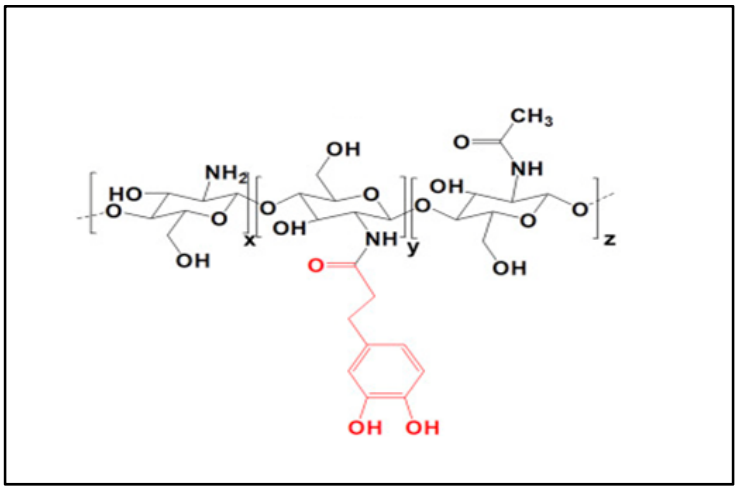

Figure 3.16 Structure of CH-Cat

Figure 3.17 shows the spectrum of the original CH powder and modified CH by Cat. The spectra of CH-Cat polymer show a higher intensity of the peak at 175 ppm compared to unmodified CH. Since this peak is due to C=O bonds, this increase is related to the formation of extra secondary amide bonds during EDC conjugation. There is also an increase in intensity in the area due to the presence of aromatic rings (150-110 ppm) and the aliphatic region (50-20 ppm) which confirms the presence of the catechol groups.

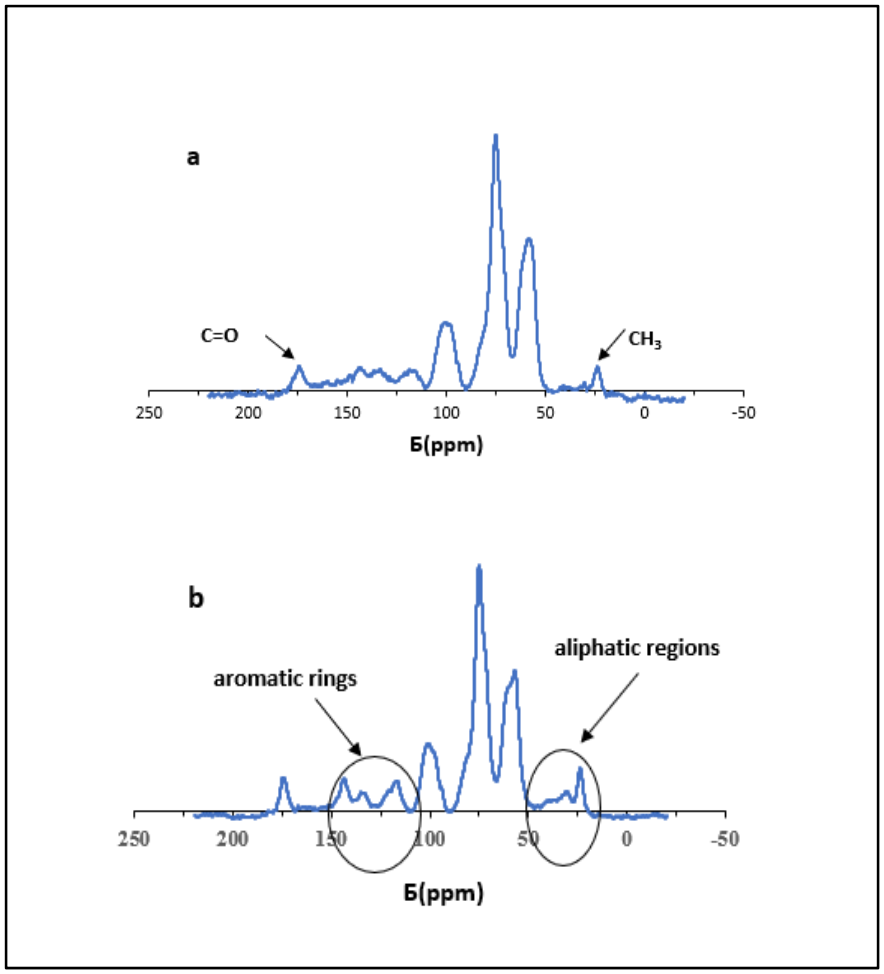

Figure 3.17 NMR spectra of a) CH and b) CH-Cat

UV-Vis

The degree of conjugation of CH-Cat was determined using a UV-Vis spectrometer. The absorbance of aqueous solutions of the CH- Cat polymers was measured at 280. Figure 3.18, a, shows that CH does not have absorbance at 280 nm while the maximum absorbance of CH-Cat (HCA) is in this wavelength (Ryu et al., 2015). Therefore, the degree of conjugation could be calculated based on a standard curve built using HCA (Figure 3.18, b), as fully described in the materials and methods section. Table 3.1 shows the absorbance and degree of conjugation of different CH-Cat samples which were synthesized by using different ratio of glucosamine: HCA: EDC. CH-Cat 6% was used for further experiments.

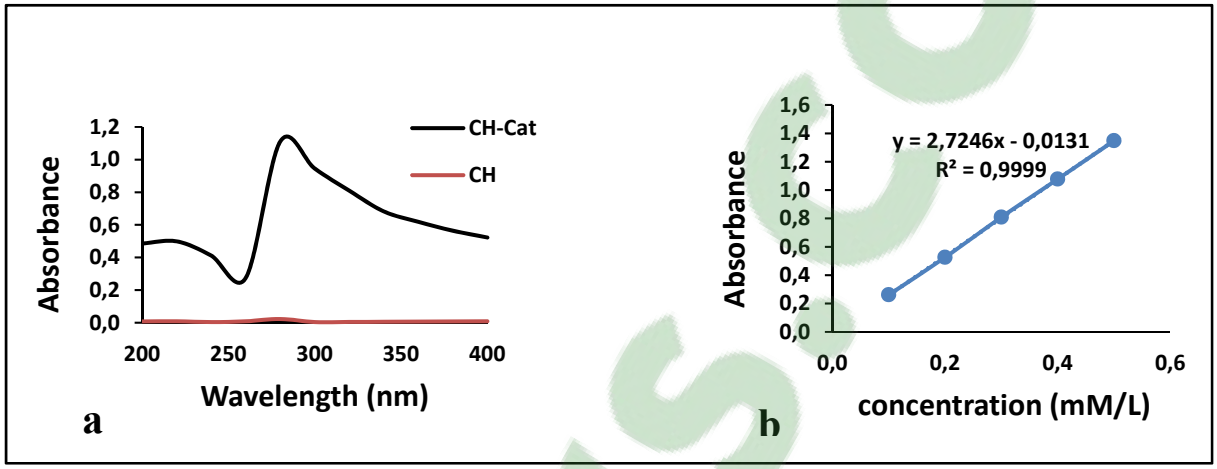

Figure 3.18 a) UV-Vis Spectrum of CH and CH-Cat and b) Hydrocaffeic acid standard curve

Table 3.1 Degree of catechol conjugation of CH-Cat prepared using various CH (Glucosamine): HCA: EDC ratios

Samples	Glucosamine: HCA: EDC	Absorbance at 280 nm	% Catechol	% Catechol (Mean)	SD
A_1	1: 0.5: 1.17	0.19	6.68	6.40	0.85
A_2		0.16	5.45		
A_3		0.20	7.07		
B_1	1: 0.25: 0.585	0.10	3.28	2.96	0.29
B_2		0.08	2.73		
B_3		0.08	2.86		
C	1: 1: 1	0.35	12.82	-	-

It should be mentioned that J. Xu who used the old protocol, reached 19 and 9% Cat in CH-Cat samples by using 1: 0.5: 1.17 and 1: 0.25: 0.585 of glucosamine: HCA: EDC respectively (Xu et al., 2015). The inconsistency of these results will be discussed in the discussion section.

3.2.2 Characterization of hydrogels

Rapid gelation, strong mechanical properties, and tissue adhesion are important to ensure good retention and transfer of drugs or cells from the injectable hydrogel to targeted sites. CH-Cat is soluble in pure water. However, we hypothesized that the degree of protonation of CH amine groups in solution could influence the mechanical properties of the final physical gel. Therefore, these properties were studied for CH-Cat hydrogels prepared with various HCl concentrations, using SHC0.09M and SHC-PB combination as gelling agents.

Mechanical characterization

Rheological study

The gelation kinetic was evaluated by rheometry. The evolution of the storage modulus (G') and loss modulus (G") during gel formation at 37 °C was monitored.

Figure 3.19 and Figure 3.20 show that G' of CH-Cat gels at 37 °C increases as a function of time, at a rate which decreases when increasing HCl concentration ($p<0.05$), and which is slower than observed with conventional CH-gels. However, for all CH-Cat/SHC0.09 hydrogels, t_{gel} is less than 3 minutes at 37 °C. Compared to gels crosslinked with Genipin (t_{gel} = 136 min) (Xu et al., 2015), these gels have a significant shorter gelation time.

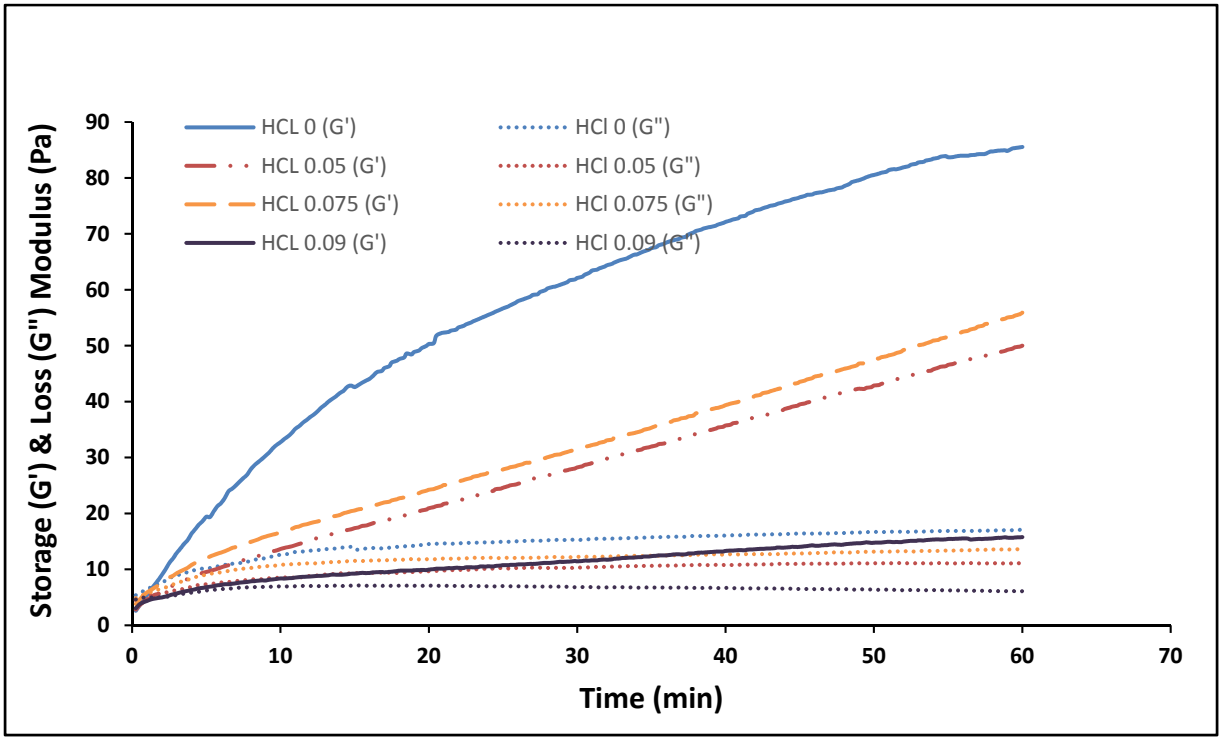

Figure 3.19 Evolution of storage (G') and loss (G") modulus for CH-Cat/SHC0.09 hydrogels for 1h at 37 °C, as a function of HCL concentration (in M) (mean, n = 3)

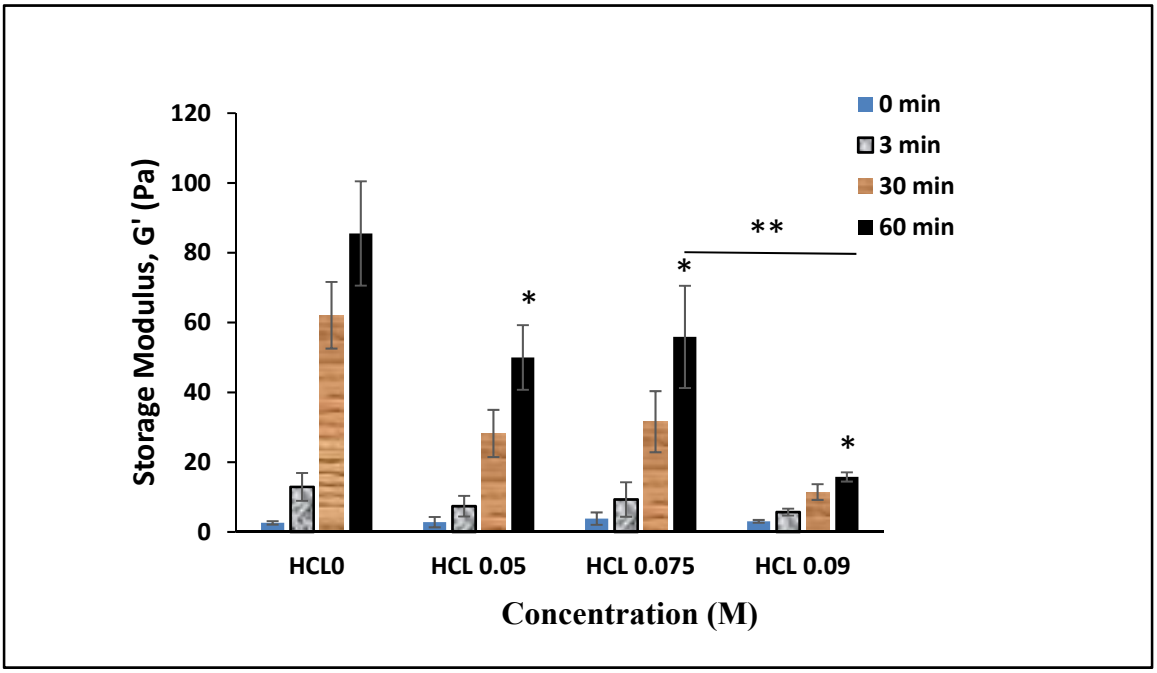

Figure 3.20 Evolution of the storage modulus G' for different HCl concentrations (in M) in CH-Cat/SHC0.09 hydrogels, at 37 °C, for 1 h (mean ± SD, n = 3), (*, **p<0.05 compared to HCl0)

As previous work showed that the addition of PB can accelerate gelation (Ceccaldi et al., 2017), the effect of adding PB on CH-Cat/SHC 0.09 was studied on hydrogels prepared with HCl=0 and 0.05M. Results are presented in Figure 3.21 and Figure 3.22. In both cases, PB tend to accelerate gelation.

When there is no HCl (Figure 3.21) there is no significant difference for PB from 0 to 0.02M. Adding 0.04 as well as 0.08M PB in hydrogel, significantly increases gelatin kinetic.

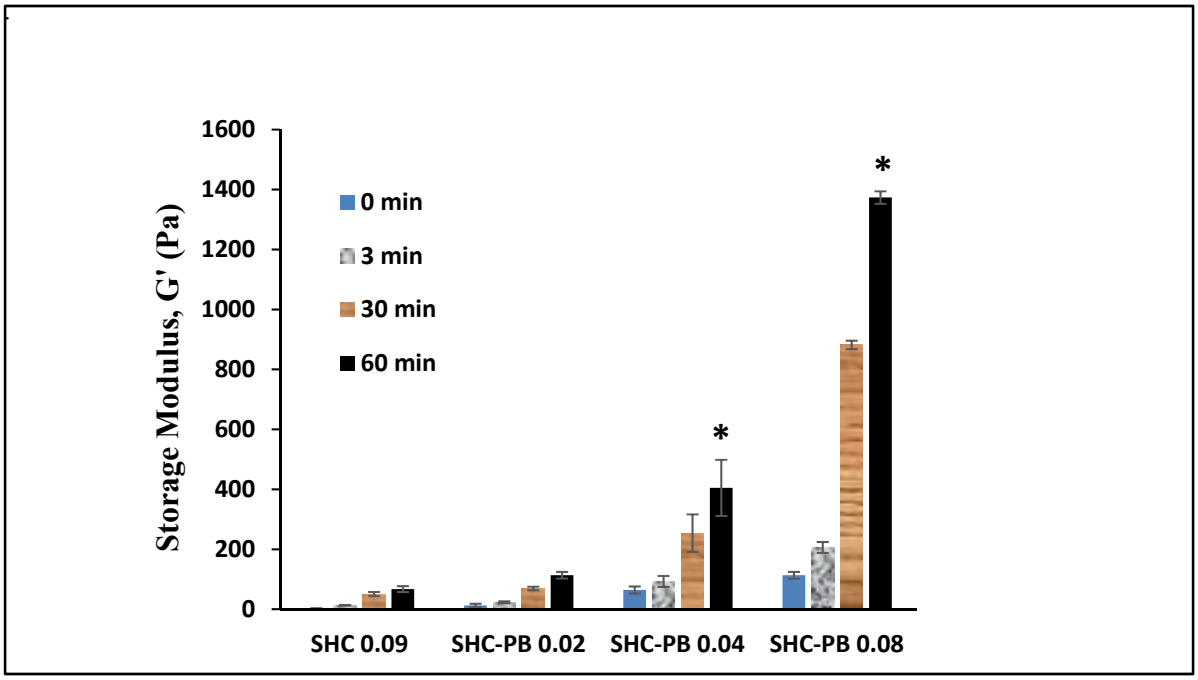

Figure 3.21 Effect of PB on storage modulus G' in CH-Cat-HCl0/SHC009 hydrogels at 37 °C, for 1 h (mean ± SD, n = 3), (*p<0.05, compared to SHC 0.09)

For formulations with 0.05M HCl (Figure 3.22) there is no meaningful difference for PB from 0 to 0.04 M, but adding 0.08M PB in that hydrogel increased the gelation rate. Yet G' values remained lower than for the gel without HCl (max =400 Pa compared to 1800 Pa).

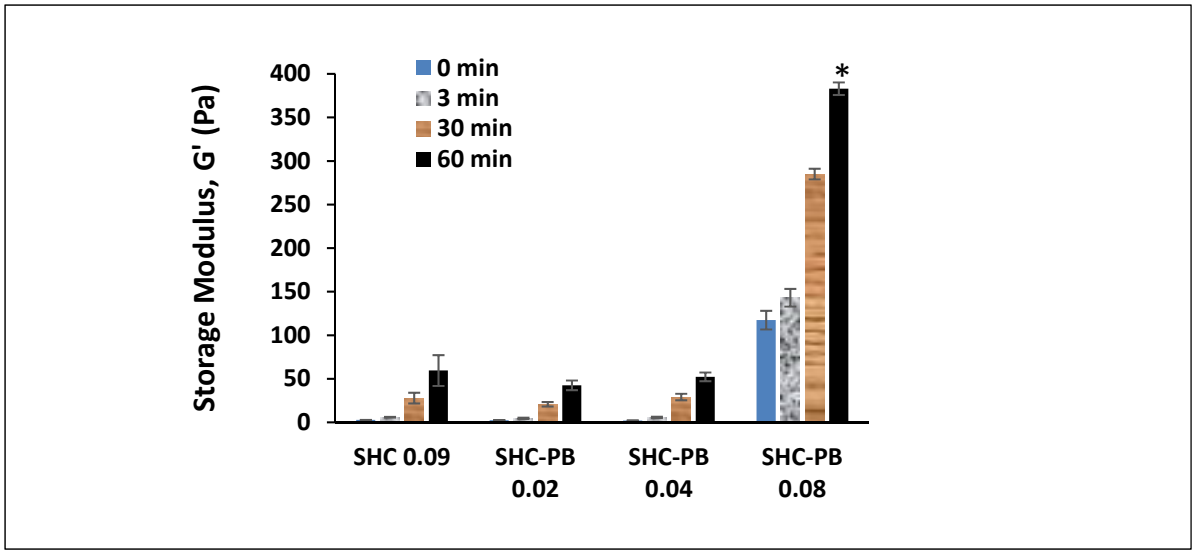

Figure 3.22 Effect of PB on storage modulus G' in CH-Cat-HCl0.05/SHC0.09 hydrogels at 37 °C, for 1 h (mean ± SD, n = 3), (*p<0.05, compared to SHC 0.09)

For comparison purpose, the evolution of G' with time was followed also with unmodified chitosan gel (CH-SHC0.09M) with different PB concentrations (Figure 3.23). While a similar trend was observed, it is important to note that the range of G' values is different, maximum being around 5000 and 8000 Pa for CH gels prepared with PB0.04 and PB0.08M respectively compared to 400 and 1400 Pa for CH-Cat without HCl in formulation.

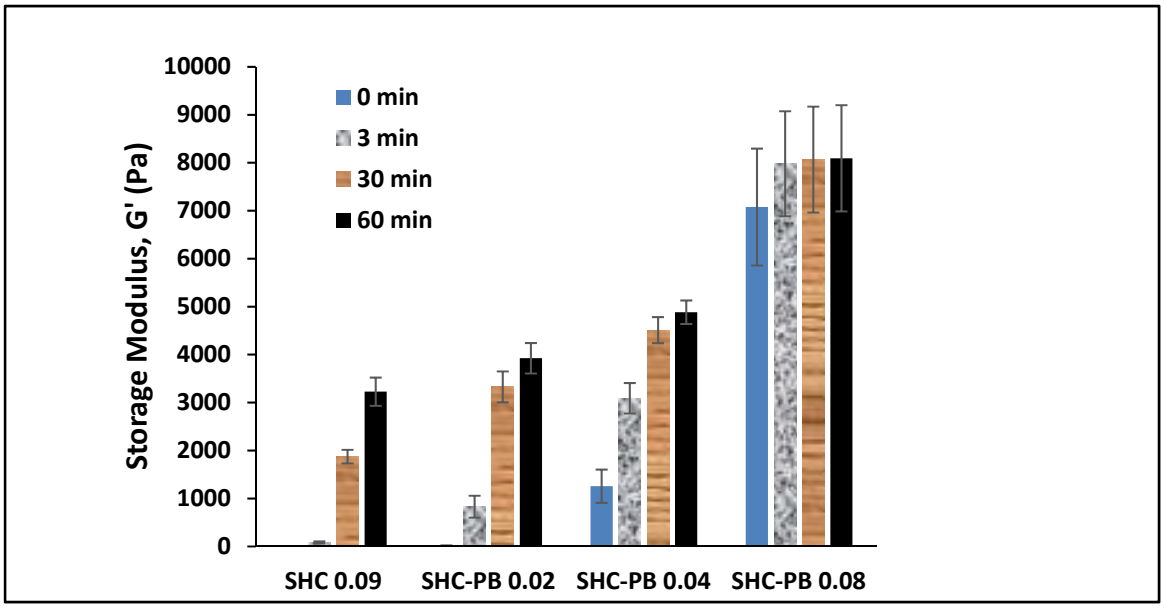

Figure 3.23 Effect of PB concentration (in M) on the storage modulus G' of unmodified chitosan hydrogels (CH/SHC0.09) at 37 °C, for 1 h (mean ± SD, n = 3)

Compression test

The mechanical properties of the hydrogels were evaluated by unconfined compression test. Figure 3.24 presents a typical compression curve, showing the nonlinear behavior of the hydrogels. Therefore, gel rigidity is expressed as the secant modulus at 30 and 50% deformation. Gel resistance is expressed as the maximal stress during compression tests. For gel resisting up to 50 % deformation without breakage, this is an underestimation of the ultimate stress.

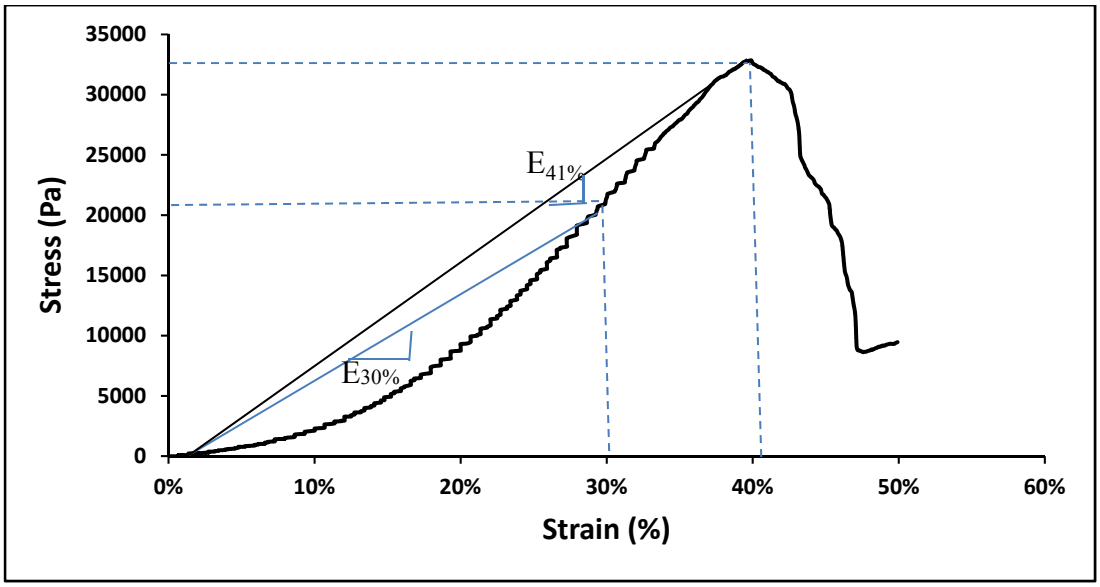

Figure 3.24 Typical stress–strain curves in unconfined compression of CH-Cat/SHC hydrogels after 48 h gelation, with method of determination of the secant modulus and ultimate stress and strain when rupture occurs before 50% deformation

The rigidity and resistance of the gel drastically decreased as a function of increasing HCl concentration from 0 to 0.09M. The mean secant modulus at 50% decreased from 53 kPa to 3.4 kPa when increasing HCl concentration from 0 to 0.09M ($p<0.05$) (Figure 3.25). Hydrogels formulated without HCl generally broke before 50% deformation, therefore for those hydrogels the highest secant modulus was observed at 40% deformation.

The effect of PB addition within the gel had various effect on its mechanical properties. For the hydrogels formulated without HCl, adding PB decreased the secant modulus (Figure 3.26). In contrast, the secant modulus increased in the presence of HCl by adding PB 0.02M into the SHC (Figure 3.27).

It should be mentioned that the mechanical properties in compression of our CH-Cat gels were significantly improved compared to CH-Cat/genipin hydrogels for which the compression test was impossible due to their loose structure.

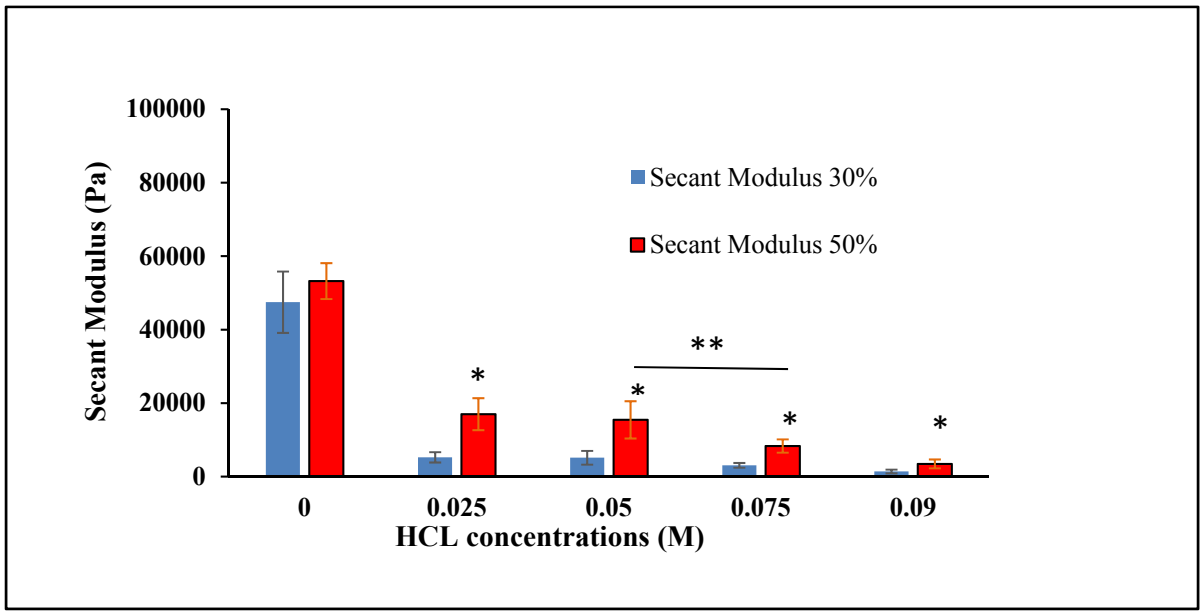

Figure 3.25 Effect of HCl concentration on mechanical properties of CH-Cat/SHC0.09 hydrogels, (mean ± SD, n = 3), (*, **P<0.05. compared to HCl0)

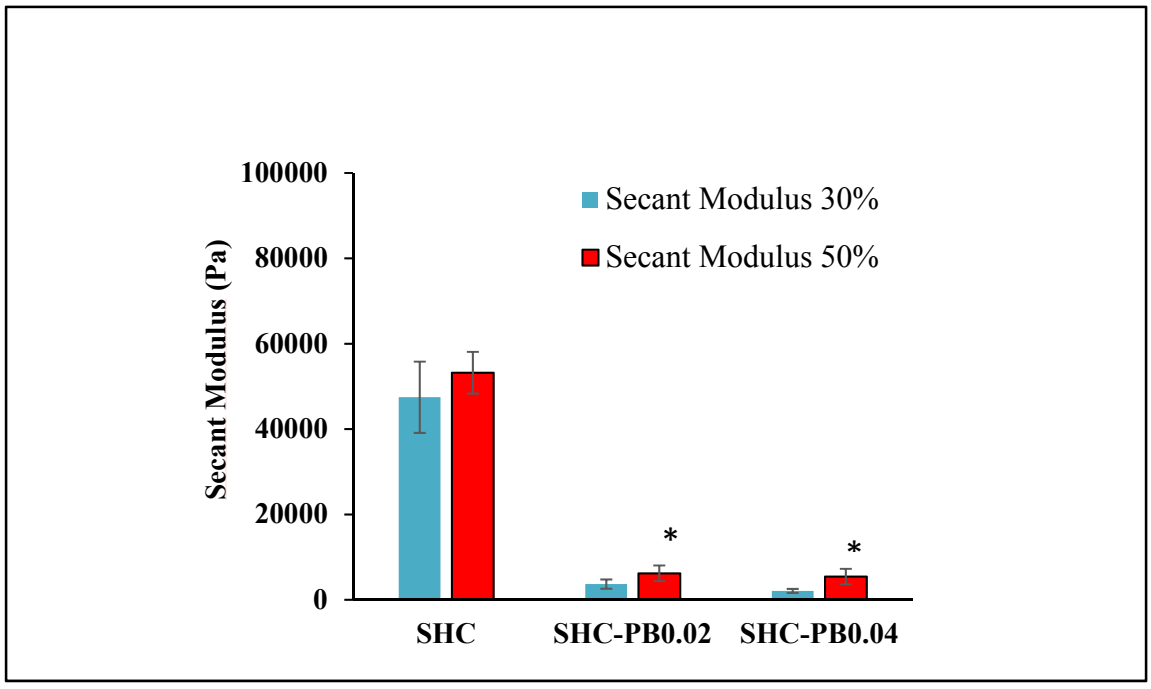

Figure 3.26 Effect of PB on mechanical properties of CH-Cat-HCl0/SHC0.09 hydrogels, mean ± SD, n = 3, (*P<0.05, compared to SHC)

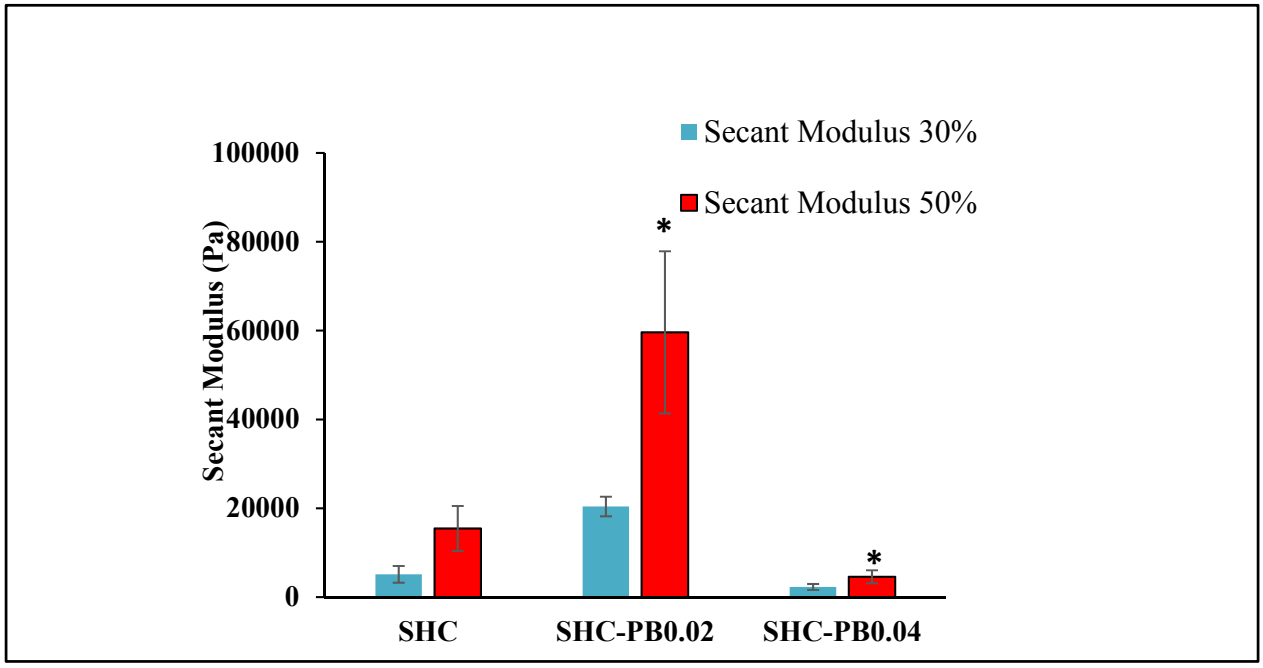

Figure 3.27 Effect of PB on mechanical properties of CH-Cat-HCl0.05/SHC0.09 hydrogels, mean ± SD, n = 3, (*P<0.05, compared to SHC)

Physico-chemical characterization

To better understand how HCl, SHC and PB concentration could influence gel properties, and as a preliminary assessment of their biocompatibility, pH and osmolality of hydrogels were studied.

pH study

Table 3.2 shows the pH of CH-Cat solutions, which decreased from 4.8 when CH-Cat was added in pure water to 1.3 or less as soon as a small HCl concentration was added. It should be mentioned that CH is not soluble in pure water and that the pH of CH in HCl 0.12 M is 6.14, quite close to chitosan pKa (6.5). Figure 3.3.28 shows the pH of CH and CH-Cat hydrogels immediately after mixing the polymer solutions with different gelling agents (SHC and SHC-PB), as well as after 24 h of gelation at 37 °C. A pH close to physiological pH (7.4) is important

to allow viability of encapsulated cells. For CH-Cat hydrogels the pH tends to be slightly lower than CH gels, and as expected, more HCl in composition led to lower pH. Moreover, in all formulations, the pH of hydrogels increased when adding PB. The pH after 24h gelation is higher than immediately after mixing for all hydrogels.

Table 3.2 pH of CH-Cat in acidic solutions

Solutions	pH of solutions	pH of CH-Cat in solutions
HCl 0	-	4.77
HCl 0.025 M	1.3	1.63
HCl 0.05 M	1.2	1.15
HCl 0.075 M	1.08	1.01
HCl 0.09 M	0.99	0.93

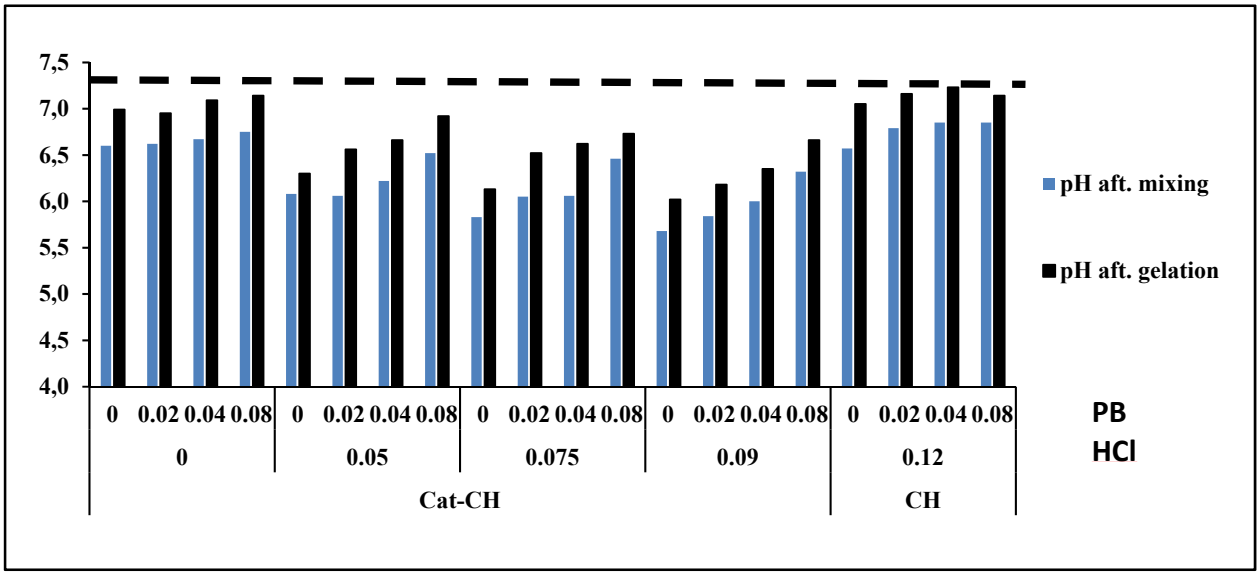

Figure 3.3.28 pH of hydrogels immediately after mixing the solution and gelling agents and 24 h after gelation. The black dotted line (7.4) shows the physiological pH

Osmolality

As shown in Figure 3.29, most of the formulations studied have osmolality close to physiological values (300 mOsm/kg). As expected, adding PB increases the osmolality of hydrogels.

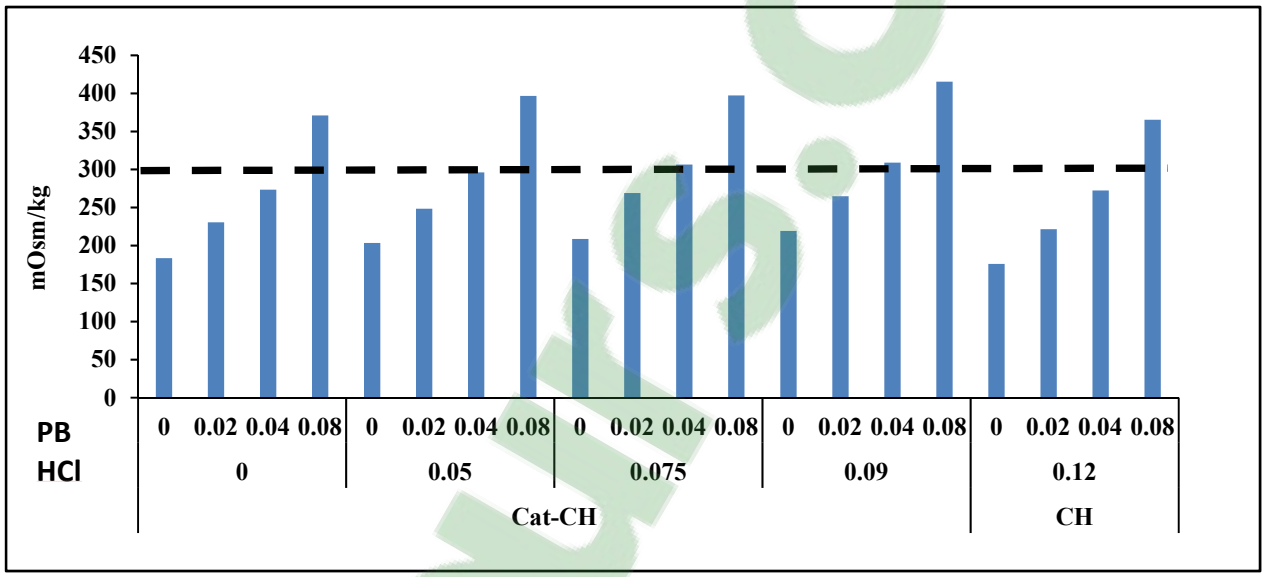

Figure 3.29 Osmolality of CH and CH-Cat hydrogels 24 h after gelation at 37 °C. The black dotted line shows the physiological osmolality

3.2.3 Tissue adhesive tests

Wash off and tensile tests have been done as a first estimation of the mucoadhesive properties of CH-Cat hydrogels, after and during gelation respectively. Indeed, we hypothesized that mucoadhesive properties of the pregelled material may be different than that of the hydrogel solution during the gelation process.

Tissue adhesive wash off test

The mucoadhesion of CH/SHC0.09 and CH-Cat-HCl/SHC0.09 hydrogels were evaluated by recording the number of hydrogel samples remaining adhered to tissue (intestine) after different times in PBS while stirring (Figure 3.30). Hydrogels were left to gel in molds for 48 h gelation at 37 °C, then put in contact with the tissue.

After 27 hours, only 1/5 (20%) or 2/5 (40%) samples were detached for CH-cat prepared with HCl=0.05 and 0.09M. In contrast 5/5 (100%) of CH-Cat prepared without HCl were detached, as well as 4/5 of CH gel samples (80%). Detachment generally occurred rapidly since the same trend was observed after 6 hours.

While the number of samples is not sufficient to draw statistically significant conclusion, this test suggests better mucoadhesive property of CH-Cat comparing to unmodified CH, as long as they are prepared with HCl≥0.05M. This confirms observations made by simple touch test (Figure 3.31). It also confirms that HCl concentration influence the mucoadhesive properties of CH-Cat.

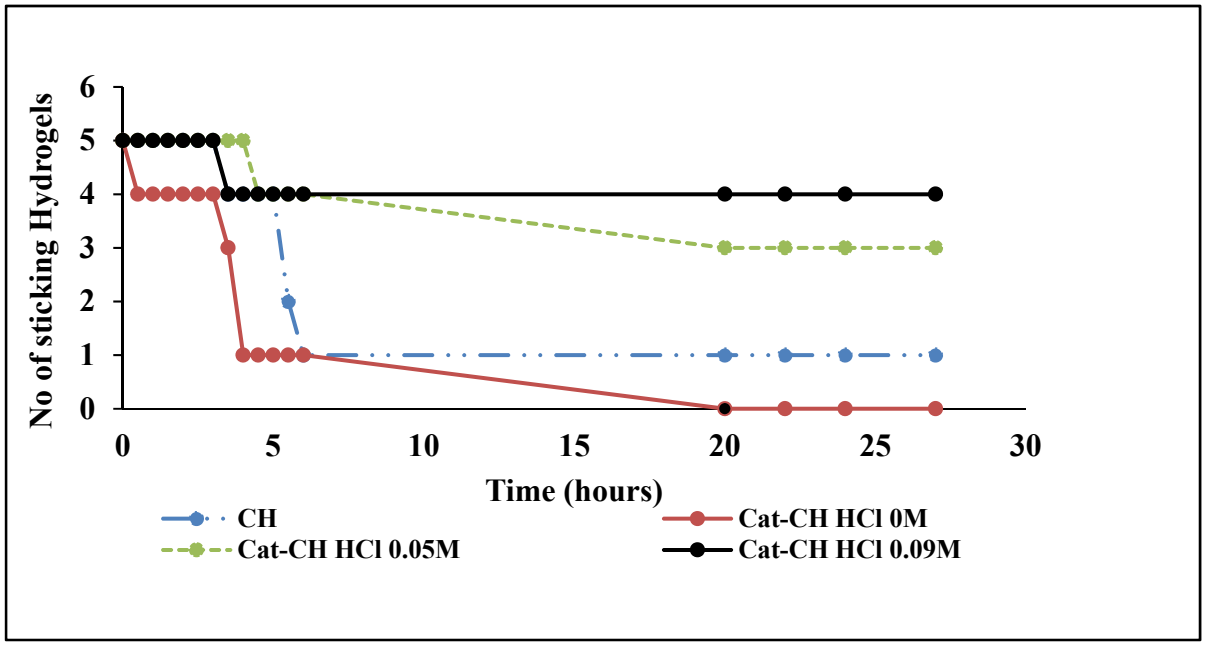

Figure 3.30 Adhesion of CH and CH-Cat on sheep intestine as a function of HCl concentrations (in M) in PBS at 37 ˚C

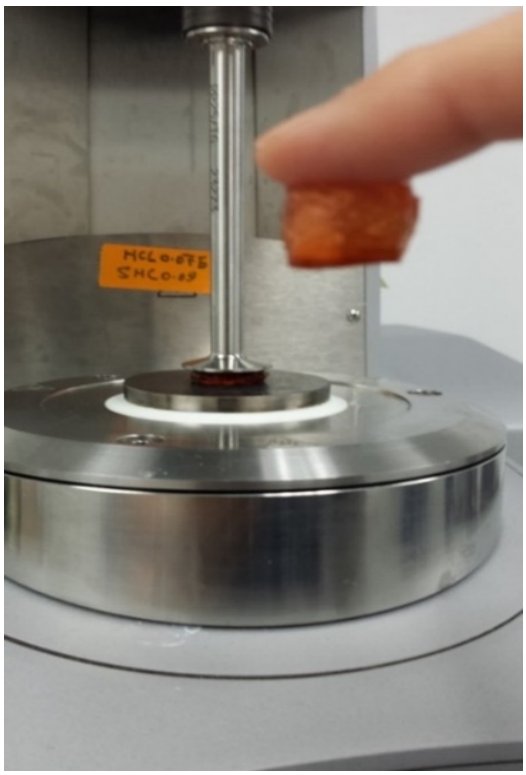

Figure 3.31 Simple touch test confirms the effect of HCl concentration on adhesive property of CH-Cat hydrogels

Tissue adhesive tensile test

Figure 3.32 shows the maximum detachment force (MDF) required to detach the hydrogels from tissue during tensile tests. For this test, hydrogels and tissues were in contact during the gelation process. MDF tend to increase for hydrogels prepared with increasing concentration of HCl, but the difference between the various groups is not significant (P= 0.42).

This can be explained by different reasons. First the large variation of results. Seconds, MDF can be influenced by both the adhesion force (breakage at the interface) and the cohesion within the gel (breakage within the gel). Therefore, since mechanical properties of hydrogels decrease

when increasing HCl concentration, the gel could break at lower level and therefore prevent to observe increased tissue adhesion.

The higher adhesive properties of hydrogels which have more HCl in their formulation during gelation, was observed in simple qualitative tests. For instance, removing those hydrogels from the mold where they were left to gel prior to the compression test was more difficult comparing to gels which had less HCl.

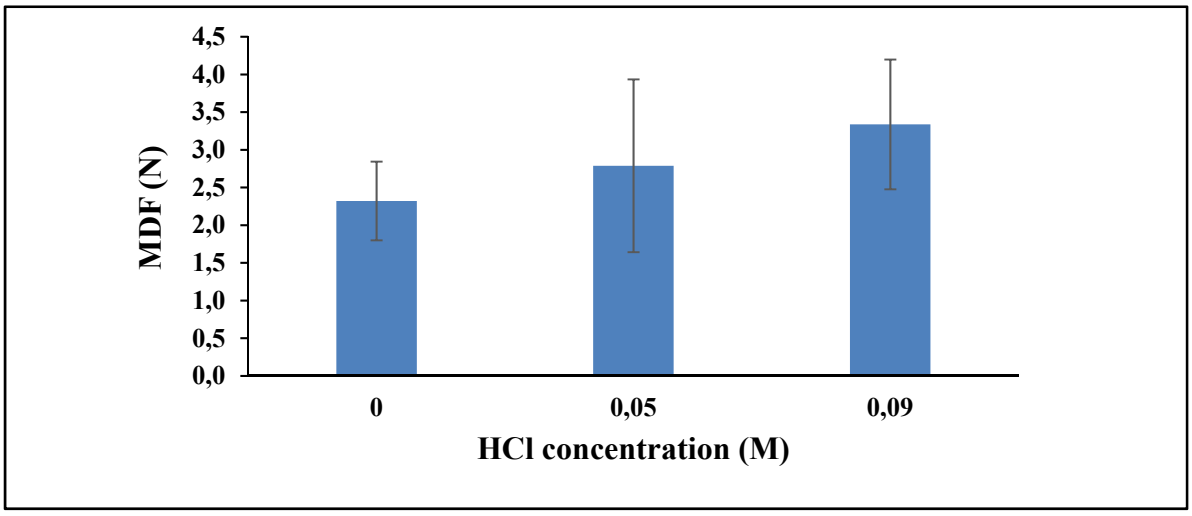

Figure 3.32 Effect of HCl concentration on mucoadhesive properties of CH-Cat/SHC0.09 hydrogels put in contact with tissue during gelation

CHAPTER 4

GENERAL DISCUSSION, LIMITS AND PERSPECTIVES

4.1 The stability of Chitosan solution, gelling agents, and hydrogels

Due to its unique biological properties, chitosan is regarded as a useful compound in medical and pharmaceutical technology. Its biocompatibility, biodegradability, low toxicity and mucoadhesion properties are some noticeable features which recently attract the attention of researchers and considerable research effort has been made to develop safe and efficient chitosan products. At LBeV, researchers aim to develop chitosan hydrogels for cell therapy and drug delivery systems. The new hydrogel formulations developed by the team have been subjected to patent filing and raise interest from the industry. However, for any industrial transfer, it is important to verify the stability of the solutions and reproducibility of the hydrogels as a function of storage time of these solutions. Therefore, the first objective of this project was to study the stability of chitosan and gelling agent solutions under different storage conditions and evaluate their impact on the rheological properties of chitosan hydrogels.

Chitosan solution

The problem of poor stability of chitosan-based systems is an obstacle that restricts its practical applicability; thus, it has become a great challenge to establish sufficient shelf-life for chitosan formulations (Szymańska & Winnicka, 2015). Based on literature, upon storage, chitosan undergoes gradual chain degradation followed by destruction of its functional groups. As a consequence, it leads to irreversible loss of its physicochemical properties. Different internal (degree of deacetylation, molecular weight, purity, and moisture level) and external (environmental storage conditions, thermal processing, sterilization, and processing involving acidic dissolution) factors can affect the stability of chitosan-based products (Szymańska & Winnicka, 2015).

The purpose of the stability test in this project was to provide reliable evidence on how the quality of the chitosan solution differs with time under the influence of the temperature as an environmental factor. Therefore, the stability of chitosan was tested by measuring the viscosity and the pH of chitosan solution at different time points during a year (52 weeks). The results (Figure 3.1) show that the viscosity remain relatively stable from day 0 until 13 weeks, after 26 weeks 100% of increasing and after 52 weeks 150% of increasing is noticeable. In addition, due to higher standard deviations for samples stored at room temperature after 26 weeks, the best storage condition is at 4- 5 °C.

These results are not in accordance to most results from the literature. Indeed, other studies showed that viscosity of chitosan solution decreases with increased storage time and temperature. For instance, No *et al*., noticed the differences in viscosity of chitosan solutions at 4 °C and 25 °C after 15-week storage. They reported that a drop of viscosity was more pronounced in solutions stored at ambient temperature (No, Kim, Lee, Park, & Prinyawiwatkul, 2006). As well, Nguyen et al. reported decrease in viscosity of chitosan solution during storage at 28 °C over a period of 60 days. But they noticed the stability of chitosan when stored at 5 °C (Nguyen, Hein, Ng, & Stevens, 2008). Conversely, Khangtragool et al. reported that chitosan solutions remained stable during storage, at 2-8 °C, as well as storage at 30 °C with only slow and relatively small further decreases in viscosity over a period of 60 days (Khangtragool, Ausayakhun, Leesawat, Molloy & Laokul, 2008).

Variation in viscosity of stored chitosan among these studies might be due to differences in chitosan product used and storage conditions. Chitosan material extracted from various sources differs significantly in terms of its molecular weight (MW), degree of deacetylation (DDA), and purity level. We used chitosan with DDA of 94% and Mw of 250 kDa., but, in the mentioned studies, chitosan with lower DDA and lower MW have been used. Generally, high molecular weight chitosan is regarded as more stable. (Mucha & Pawlak, 2002). In addition, there are several studies devoted to chitosan hydrolysis using several types of acids. A faster rate of chain damage was noticed when chitosan with lower DDA was used in the studies. This phenomenon can be explained by the fact that chitosan with low DDA possesses a more porous

structure and electrostatic repulsion between protonated amino groups is more pronounced thus promoting penetration of acid solution inside the polymer structure. Moreover, storage of chitosan solution at ambient temperatures is regarded as accelerating the degradation rate of the polymer (Szymańska & Winnicka, 2015).

In our study, no decrease in viscosity indicative of chitosan degradation was observed, but rather viscosity increase. This suggests that our chitosan solution may have be less degraded during this storage period due to its high MW, DDA, and higher concentration (3.33% w/v) compared to all other studies that used 0.1% to 1% w/v solutions. Besides, the ambient temperature was not the same (22 °C vs 25- 30 °C).

The increase in viscosity could be explained by evaporation of the liquid, which would lead to increase of the chitosan concentration. Moreover, since evaporation could mask the effect of chitosan degradation, it would be interesting to confirm the absence of chitosan degradation during storage, by studying molecular weight of chitosan.

Finally, the pH values of chitosan solutions stored for 52 weeks was almost stable upon the time. Also, there is almost no difference between storage chitosan solution in fridge or room temperature. However, the pH measurement was done just for one sample that is one limitation of this study. The pH stability of chitosan is reported in other study (Khangtragool et al., 2008).

Gelling agents

Similarly, to test the stability of gelling agents (SHC0075-PB004, SHC0075-PB008, SHC0075-BGP001, and BGP04), they were stored in two conditions (room temperature (RT) and fridge temperature (RT)) and their pH were measured at different time points.
The results (Figure 3.4, Figure 3.7, Figure 3.10, and Figure 3.13) of pH measurement show that the pH of all gelling agents significantly changed over time compared to Day 0 ($p < 0.05$). The pH of BGP04 decreased over the time. But it increased for all others GA tested. The effect

of time was already observed after only 1 week storage, and was less pronounced for samples stored in the fridge compared to room temperature.

Chitosan hydrogels

To test the effect of these changes due to storage of chitosan solution and gelling agents on the kinetic of gelation of the resulting hydrogels, rheological tests have been done consisting in monitoring the evolution of the storage modulus (G') and loss modulus (G'') during gel formation at 37 °C. It should be mentioned that rheometry tests have been done for the fridge stored samples only, since this storage condition was chosen for our solution in the future.

The storage did not impair gelation and all gels formed rapidly, as shown by a t_{gel} of less than 15 seconds (Figure 3.5, Figure 3.8, Figure 3.11, and Figure 3.14). Moreover, the analysis of G' values at different time points indicates that CH/SHC0075-PB008 is the only formulation in which the storage modulus significantly decreased by time for 52 weeks ($p< 0.05$). In addition, 52 weeks after storage CH solution and gelling agents, storage modulus of hydrogel does not increase during rheometry test in 30 minutes and the G' curve is more like straight line. In other formulation no significant change in storage modulus by time (comparing to Day 0) for 52 weeks was found.

However, the storage time affected the initial G' value and the kinetic of gelation; in all but CH/SHC0075-PB008 formulation, G'_0 was higher and G' values were higher than their counterpart with fresh solutions. These trends are probably linked with the changes in pH of gelling agent.

Since theses gelling agents are those that are produced by LBeV, to best of our knowledge, there is not similar researches in literature to compare the results of this study with them. Even for chitosan/BGP hydrogels (which is the novel attractive hydrogel for biomaterial researchers) the condition of experiments reported by other groups is not the same as our research. It should be mentioned that type of stability studies and storage conditions should be selected with respect to the application.

Study limitations

The limitation of this study is mainly the low number of studied samples. Due to the large amount of purified chitosan that was needed to start all tests at the same times, four different batch of chitosan were purified, and mixed to ensure the uniformity of material for all experiments. For pH of chitosan, just one sample was tested, so the SD is not present (Figure 3.2). For all other tests, three samples were used. It made possible to have SD, but another limiting factor was in statistical analysis. Three samples may not be sufficient for statistic validation.

4.2 Injectable tissue-adhesive chitosan-catechol hydrogel

The second objective of this master project was to study the effect of gel compounds, especially acid concentration, as a first step toward the development of an injectable tissue-adhesive chitosan-catechol hydrogel with good gelation time, good mechanical strength, and good compatibility with cells. The objective was divided into 2 specific objectives: 1) synthesize the chitosan-catechol, and 2) study the effect of gel compounds, especially acid concentration on gelation time, mechanical strength, tissue adhesive property, and compatibility with cells.
In the following text, for each specific objective, the main results will be briefly summarized, then further detailed and discussed.

Synthesis of chitosan-catechol

In this study, numerous tests were done to find the best physicochemical parameters for the reaction of catechol grafting to chitosan. A protocol was developed to avoid oxidation of catechol during grafting process.

While chitosan is normally soluble only at acidic pH, catechol functionalization converts it into a highly water soluble polymer at physiological pH (Kim, Ryu, Lee & Lee, 2013). The

CH-Cat formulated in our study is white and powdery, and dissolves perfectly at neutral pH. It is the first point that confirms the catechol grafting to chitosan in our sample. To ensure the conjugation, nuclear magnetic resonance (NMR) spectroscopy was used. Then, the degree of conjugation of CH-Cat was determined using a UV-Vis spectrometer. The result of this test shows the presence of 6% catechol in the CH-Cat samples which were synthesized by using glucosamine: HCA: EDC in the ratio of 1: 0.5: 1.17. Xu et al., who used another protocol to synthesis CH-Cat but the same ratio of glucosamine, HCA, and EDC, reported 19% Cat in CH-Cat samples (Xu et al., 2015). Comparing the ratio of glucosamine, HCA, and EDC, which determine the amount of catechol in samples, in other studies shows that by using this ratio it is not possible to reach 19% catechol in samples. For instance, Ryu et al. reported the degree of catechol conjugation for the chitosan–catechol was 8.4% by using the molar equivalent ratio of 1: 1.2: 1.15 (Ryu, Jo, Koh, & Lee, 2014).

Future work should include increasing Cat grafting efficiency by changing the amount of hydrocaffeic acid and EDC used to synthesize the samples.

Characterization of hydrogels

Rapid gelation, strong mechanical properties, and tissue adhesion are important to ensure good retention and transfer of drugs or cells from the injectable hydrogel to targeted sites. These properties were studied for CH-Cat hydrogels prepared with various HCl concentration, using SHC and SHC-PB as gelling agents.

Effect of HCl

The gelation kinetic was evaluated by rheometry at 37 $^{\circ}$C and the mechanical properties by unconfined compression tests.

Table 4.1 Effect of HCl concentration on CH-Cat hydrogel properties

HCl concentration (0- 0.09 M)	pH of CH-Cat solution	Gelation kinetic	Mechanical strength	Adhesive properties
↑	↓	↓	↓↓	↑↑

Table 4.1 summarizes the results obtained when increasing the HCl concentration from 0 to 0.09 M. The pH of the CH-Cat solution decreases, and the gelation kinetic once mixed with the GA at 37 °C is slow down compared to hydrogels without HCl in their formulation. In addition, the rigidity and resistance of the gel drastically decreased as a function of increasing HCl concentration. However, compared to gels crosslinked with genipin, all these new hydrogels have a significant shorter gelation time and much better mechanical strength.

In contrast, HCl increased the adhesive property of hydrogels, according to the preliminary testing performed in this study using wash off and tensile tests.
In both cases, however, the number of samples was not sufficient to draw statistically significant conclusion and a high variability was observed during tensile tests.

The lower mechanical strength and gelation rate when increasing HCl concentration in CH-Cat/SHC hydrogels, may be due to an increase in CO_2, which slowed down the sol–gel transition. Indeed, using the weak SHC base as a gelling agent leads to CO_2 generation after mixing with the acidic solution of chitosan.

$HCO_3^- \leftrightarrows H_2O + CO_2$ (pKa = 6.3)

This means increasing HCl concentration (lower pH) and generation more CO_2, leads to decreasing HCO_3^- in solution and prevent crosslinking of the gel through NH_2^+ groups and thus decrease secant Modulus and gelation kinetic. CO_2 transformation was observed during

experiments. When mixing CH-Cat solution and gelling agent, the reaction produced gas in large quantities and many bubbles appeared in the medium. The higher the acid concentration, the more the resulting gas was observed.

In addition, changes from a transparent color (just after casting the mixed solution) to orange (at the end of the time allotted to the formation of the gel) was observed, which is a distinctive sign of the oxidation of the catechol groups. This oxidation, inhibited at acidic pH, is triggered spontaneously in the presence of bicarbonate, affects on enhancing the pH of the medium. The oxidation of catechol makes the group sensitive to intramolecular nucleophilic addition. In the absence of H^+ ions in the medium, the free amine groups of chitosan add to the quinone (oxidized catechol), resulting in the formation of the network. The gel obtained is relatively flexible and of good behavior, but the catechol groups are mobilized in covalent bonds with the network, thus preventing them from intervening in mucoadhesive bonds. This phenomenon could explain the better mucoadhesive property of hydrogels when increasing HCl concentration.

Rheometry and compression results of these Cat-CH gel shows that the mechanical properties achieved for Cat-CH gels are still far below those of non-grafted chitosan gels (Figure 3.20, Figure 3.25). Even for the 0M HCl formulation, which demonstrates the highest stress resistance. In addition, this neutral pH hydrogel does not exhibit particularly mucoadhesive properties.
Further optimization work is needed to reach stronger mechanical properties.

Study limitations and future perspectives

This project presented several limitations. First of all, the concentration of SHC (0.09M) was chosen based on the best concentration for unmodified chitosan and based on the supposition that we had 10% catechol in our samples when we started the project (according to the results of Xu et al.) (Xu et al., 2015). In future study, the concentration of SHC could be modified. In addition, our preliminary study shows that increasing the degree of catechol from 3 to 6%,

could increase the mechanical strength (data are not presented). It is recommended to increase the degree of catechol more than 6% and study the effect on gelation kinetic, as well as mechanical strength and adhesive property.

The effect of adding PB on mucoadhesive property could be tested. In addition, other gelling agent combinations, especially SHC-BGP, would be interesting to study.

Mucoadhesive test is another limitation of our study. Du to different problems such as lack of tissue, sufficient samples were not tested in wash off test. Besides, the tensile test needs to be modified since it led to large variability and since both the mechanical and adhesive properties of hydrogels impact on the results. Therefore, since mechanical properties of hydrogels decrease when increasing HCl concentration, the gel could break at lower level and therefore, prevent to see increased tissue adhesion.

Finally, the mucoadhesive property of CH-cat/SHC and CH-Cat/Genipin could be compared in future study.

CHAPTER 5

CONCLUSION

This project took place in the framework of development of injectable chitosan-based hydrogels for cell therapy and drug delivery.

In the preparation process, it is particularly important to establish the shelf-life of the product by conducting stability studies. In the first part of this work, we studied the stability of chitosan solution, gelling agents, and their impact on chitosan thermosensitive hydrogel over 1 year under two storage conditions. Since samples stored at low temperature (4- 5 °C) showed less changes in comparison to those stored at room temperature, in the future all samples will be stored in the refrigerator and time of storage will be controlled to avoid possible variability in the results due to storage time.

In the second part of this work, the protocol of catechol grafting to chitosan was optimized and the effect of HCl concentration on gel properties was studied, as a first step towards the development of an injectable tissue-adhesive chitosan-catechol hydrogel with good gelation time, good mechanical strength, and good compatibility with cells.

HCl showed positive effect on adhesive properties but decreased the gelation kinetic and mechanical strength of hydrogels. Further optimization is therefore needed. For example, preliminary results suggest that adding PB to hydrogel formulations could improve both the mechanical properties and the kinetic of gelation. Its effect on mucoadhesive could be studied in future researches.

Despite their limitation, the catechol-chitosan physical hydrogels developed in this work show interesting results compared to chitosan- catechol hydrogels crosslinked by genipin.

These results open the way to make tissue adhesive hydrogel with good gelation kinetic and tunable mechanical strength. Optimize the hydrogels, improve the understanding of the chemical mechanisms, and evaluate their potential for cellular encapsulation will be the next steps.

ANNEX I

PROTOCOL OF SYNTHESIS OF CHITOSAN-CATECHOL

1. Dissolve 0.6 gr chitosan in 60 ml deionized (DI) water and HCl (pH = 2.5)

2. Add HCA and EDC previously solvated in a water: ethanol 1: 1 mixture in stoichiometric proportions (1: 0.5: 1.17 of glucosamine: HCA: EDC respectively). Adjust the pH between 4.65- 4.80 using 1M NaOH

3. Let the reaction take place for 12 hours under stirring in cold room.

4. Dialyses the solution by using a dialysis membrane tube (MWCO 5,000, Spectrum Laboratories, USA) for three days. The dialysis should be done against HCl solution (pH 2.5-3) during the first 2 days (10 mM NaCl solution with 15 mL of 1 N HCl for the first day and 10 mM NaCl solution with 5 mL of 1 N HCl for the second day) following by dialysis against DI water for 6 h at the last day. Dialysis solution should be changed at least 4 times in first and second day.

5. Lyophilize the purified product and store it at -20 ° C.

ANNEX II

EVOLUTION OF pH BY TIME FOR GELLING AGENTS

SHC0075-PB008

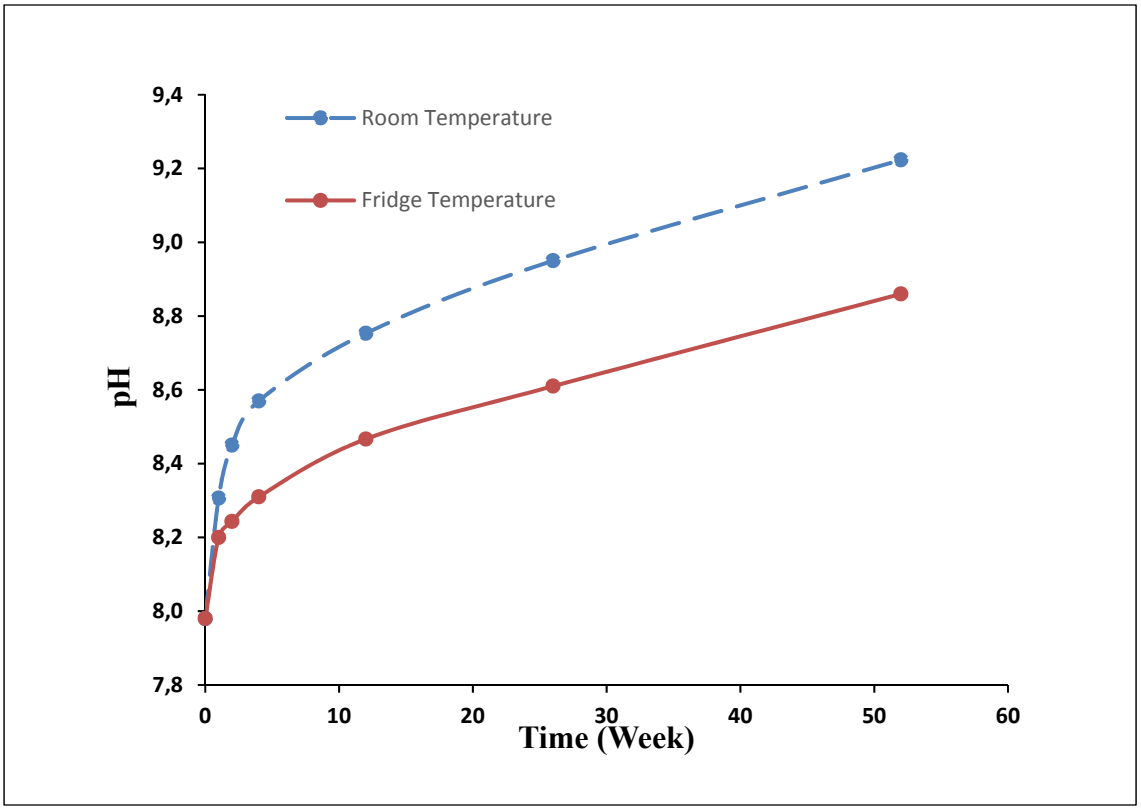

Figure-A II- 1 Effect of storage condition and time on the pH of SHC0075-PB008 (mean; n=3)

SHC0075-BGP001

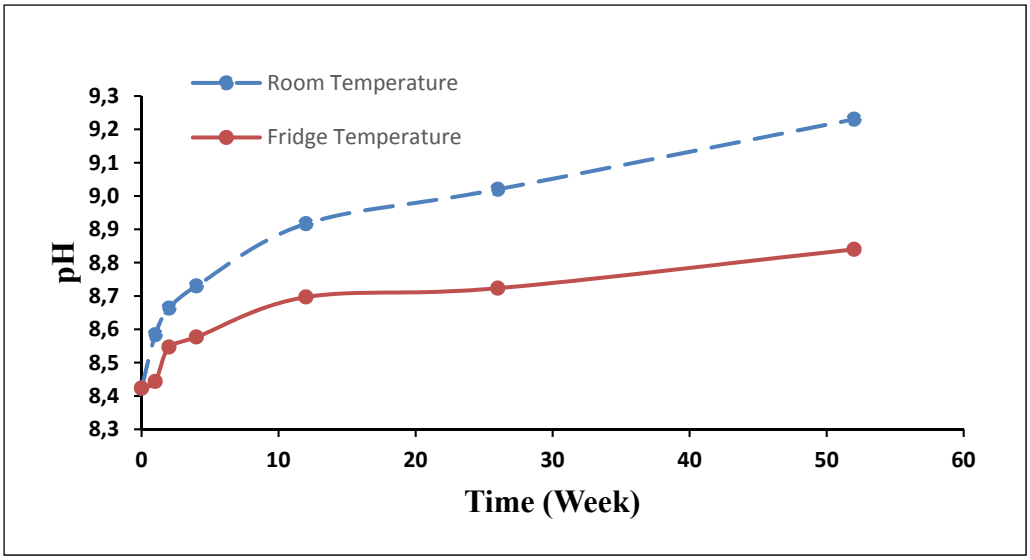

Figure-A II- 2 Effect of storage condition and time on the pH of SHC0075- BGP001 (mean; n=3)

BGP04

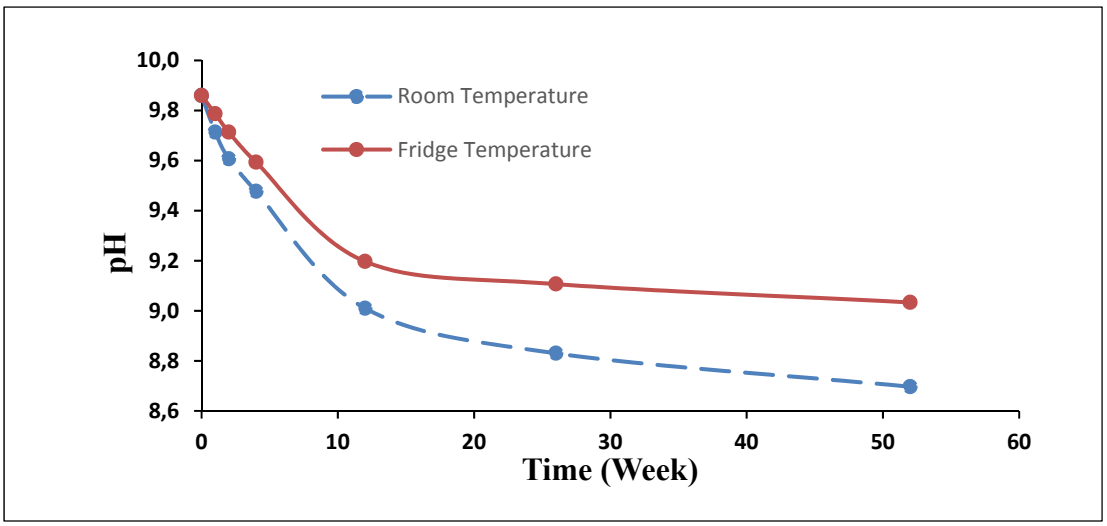

Figure-A II- 3 Effect of storage condition and time on the pH of BGP04 (mean; n=3)

LIST OF BIBLIOGRAPHICAL REFERENCES

Ahmadi, R., & de Bruijn, J. D. (2008). Biocompatibility and gelation of chitosan-glycerol phosphate hydrogels. *J Biomed Mater Res A, 86*(3), 824-832.

Assaad, E., Maire, M., & Lerouge, S. (2015). Injectable thermosensitive chitosan hydrogels with controlled gelation kinetics and enhanced mechanical resistance. *Carbohydrate polymers, 130*, 87-96.

Barnett, B. P., Hughes, A. H., Lin, S., Arepally, A., & Gailloud, P. H. (2009). In vitro assessment of EmboGel and UltraGel radiopaque hydrogels for the endovascular treatment of aneurysms. *Journal of Vascular and Interventional Radiology, 20*(4), 507-512.

Baty, A. M., Leavitt, P. K., Siedlecki, C. A., Tyler, B. J., Suci, P. A., Marchant, R. E. & Geesey, G. G. (1997). Adsorption of adhesive proteins from the marine mussel, Mytilus edulis, on polymer films in the hydrated state using angle dependent X-ray photoelectron spectroscopy and atomic force microscopy. *Langmuir, 13*(21), 5702-5710.

Berger, J., Reist, M., Mayer, J. M., Felt, O., Peppas, N., & Gurny, R. (2004). Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. *European Journal of Pharmaceutics and Biopharmaceutics, 57*(1), 19-34.

Bernkop-Schnürch, A. (2005). Mucoadhesive systems in oral drug delivery. *Drug Discovery Today: Technologies, 2*(1), 83-87.

Bhattarai, N., Gunn, J. & Zhang, M. (2010). Chitosan-based hydrogels for controlled, localized drug delivery. *Advanced Drug Delivery Reviews, 62*(1), 83-99.

Boddupalli, B. M., Mohammed, Z. N., Nath, R. A. & Banji, D. (2010). Mucoadhesive drug delivery system: An overview. *Journal of advanced pharmaceutical technology & research, 1*(4), 381.

Caló, E., & Khutoryanskiy, V. V. (2015). Biomedical applications of hydrogels: A review of patents and commercial products. *European Polymer Journal, 65*, 252-267.

Ceccaldi, C., Assaad, E., Hui, E., Buccionyte, M., Adoungotchodo, A. & Lerouge, S. (2017). Optimization of Injectable Thermosensitive Scaffolds with Enhanced Mechanical Properties for Cell Therapy. *Macromolecular Bioscience*, 1600435.

Chenite, A., Buschmann, M. D., Wang, D., Chaput, C. & Kandani, N. (2001). Rheological characterisation of thermogelling chitosan/glycerol-phosphate solutions. *Carbohydrate Polymers, 46*, 39-47.

Chenite, A., Chaput, C., Wang, D., Combes, C., Buschmann, M. D., Hoemann, C., Selmani, A. (2000). Novel Injectable neutral solutions of chitosan form biodegradable gels in situ. *Biomaterials, 21*, 2155-2161.

Chirdon, W. M., O'Brien, W. J. & Robertson, R. E. (2003). Adsorption of catechol and comparative solutes on hydroxyapatite. *Journal of Biomedical Materials Research Part B: Applied Biomaterials, 66*(2), 532-538.

Coutu, J. M., Fatimi, A., Berrahmoune, S., Soulez, G., & Lerouge, S. (2013). A new radiopaque embolizing agent for the treatment of endoleaks after endovascular repair: influence of contrast agent on chitosan thermogel properties. *J Biomed Mater Res B Appl Biomater, 101*(1), 153-161.

Croisier, F. & Jérôme, C. (2013). Chitosan-based biomaterials for tissue engineering. *European Polymer Journal, 49*(4), 780-792.

Delibegović, S., Iljazović, E., Katica, M. & Koluh, A. (2011). Tissue reaction to absorbable endoloop, nonabsorbable titanium staples, and polymer Hem-o-lok clip after laparoscopic appendectomy. *JSLS: Journal of the Society of Laparoendoscopic Surgeons, 15*(1), 70-76.

Deming, T. J. (1999). Mussel byssus and biomolecular materials. *Current Opinion in Chemical Biology, 3*(1), 100-105.

Ducheˇne, D., Touchard, F. & Peppas, N. A. (1988). Pharmaceutical and medical aspects of bioadhesive systems for drug administration. *Drug Development and Industrial Pharmacy, 14*(2-3), 283-318.

Edwards, J. C. (2009). Principles of NMR. *Process NMR Associates LLC, 87A Sand Pit Rd, Danbury CT, 6810*.

Fang, J.-Y., Chen, J.-P., Leu, Y.-L. & Hu, J.-W. (2008). Temperature-sensitive hydrogels composed of chitosan and hyaluronic acid as injectable carriers for drug delivery. *European Journal of Pharmaceutics and Biopharmaceutics, 68*(3), 626-636.

Ferreira, P., Gil, M., & Alves, P. AN OVERVIEW IN SURGICAL ADHESIVES.

G Silverman, H., & Roberto, F. (2007). *Understanding Marine Mussel Adhesion* (Vol. 9).

Ganji, F., Abdekhodaie, M. & Ramazani S.A., A. (2007). Gelation time and degradation rate of chitosan-based injectable hydrogel. *Journal of sol-gel science and technology, 42*(1), 47-53.

Gibas, I., & Janik, H. (2010). Review: synthetic polymer hydrogels for biomedical applications.

Gulrez, Syed K. H., Al-Assaf, S. & Phillips, G. O. (2011). Hydrogels: methods of preparation, characterisation and applications. *Progress in Molecular and Environmental Bioengineering-From Analysis and Modeling to Technology Applications*.

HAKIM, A. (2015). Conception d'un banc d'essai de mucoadesion. Montreal: ÉCOLE DE TECHNOLOGIE SUPÉRIEURE (ETS).

Hennink, W. E. & van Nostrum, C. F. (2002). Novel crosslinking methods to design hydrogels. *Adv Drug Deliv Rev, 54*(1), 13-36.

Hoffman, A. S. (2002). Hydrogels for biomedical applications. *Advanced Drug Delivery Reviews, 54*(1), 3-12.

Holten-Andersen, N., Mates, T. E., Toprak, M. S., Stucky, G. D., Zok, F. W. & Waite, J. H. (2009). Metals and the integrity of a biological coating: the cuticle of mussel byssus. *Langmuir: the ACS journal of surfaces and colloids, 25*(6), 3323.

Huang, Y., Leobandung, W., Foss, A. & Peppas, N. A. (2000). Molecular aspects of muco- and bioadhesion:: Tethered structures and site-specific surfaces. *Journal of controlled release, 65*(1), 63-71.

Khangtragool, A., Ausayakhun, S., Leesawat, P., Molloy, R. & Laokul, C. (2008). Stability of chitosan solutions for potential use in ocular drug delivery. *CHIANG MAI UNIVERSITY JOURNAL*, 209.

Khanlari, S. & Dubé, M. A. (2013). Bioadhesives: a review. *Macromolecular Reaction Engineering, 7*(11), 573-587.

Khutoryanskiy, V. V. (2011). Advances in mucoadhesion and mucoadhesive polymers. *Macromolecular Bioscience, 11*(6), 748-764.

Kim, I. Y., Seo, S. J., Moon, H. S., Yoo, M. K., Park, I. Y., Kim, B. C. & Cho, C. S. (2008). Chitosan and its derivatives for tissue engineering applications. *Biotechnol Adv, 26*(1), 1-21.

Kim, K., Ryu, J. H., Lee, D. Y. & Lee, H. (2013). Bio-inspired catechol conjugation converts water-insoluble chitosan into a highly water-soluble, adhesive chitosan derivative for hydrogels and LbL assembly. *Biomater Sci, 1*(7), 783-790.

Lavertu, M., Filion, D. & Buschmann, M. D. (2008). Heat-induced transfer of protons from chitosan to glycerol phosphate produces chitosan precipitation and gelation. *Biomacromolecules, 9*, 11.

Lee, B. P., Dalsin, J. L. & Messersmith, P. B. (2006). Biomimetic adhesive polymers based on mussel adhesive proteins. *Biological Adhesives* (pp. 257-278). Springer.

Lee, C., Shin, J., Lee, J. S., Byun, E., Ryu, J. H., Um, S. H. & Cho, S.-W. (2013). Bioinspired, calcium-free alginate hydrogels with tunable physical and mechanical properties and improved biocompatibility. *Biomacromolecules, 14*(6), 2004-2013.

Lee, H., Dellatore, S. M., Miller, W. M. & Messersmith, P. B. (2007). Mussel-inspired surface chemistry for multifunctional coatings. *science, 318*(5849), 426-430.

Lee, H., Scherer, N. F. & Messersmith, P. B. (2006). Single-molecule mechanics of mussel adhesion. *Proceedings of the National Academy of Sciences, 103*(35), 12999-13003.

Lehr, C.-M., Bouwstra, J. A., Schacht, E. H. & Junginger, H. E. (1992). In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. *International journal of Pharmaceutics, 78*(1-3), 43-48.

Mezger, T. G. (2006). *The rheology handbook: for users of rotational and oscillatory rheometers.* Vincentz Network, Hannover.

Monette, A., Ceccaldi, C., Assaad, E., Lerouge, S. & Lapointe, R. (2016). Chitosan thermogels for local expansion and delivery of tumor-specific T lymphocytes towards enhanced cancer immunotherapies. *Biomaterials, 75*, 237-249.

Mucha, M. & Pawlak, A. (2002). Complex study on chitosan degradability. *POLIMERY-WARSAW, 47*(7/8), 509-516.

Nguyen, & Lee, D. S. (2010). Injectable Biodegradable Hydrogels. *Macromolecular Bioscience, 10*(6), 563-579.

Nguyen, T. T. B., Hein, S., Ng, C. H. & Stevens, W. F. (2008). Molecular stability of chitosan in acid solutions stored at various conditions. *Journal of applied polymer science, 107*(4), 2588-2593.

No, H. K., Kim, S. H., Lee, S. H., Park, N. Y. & Prinyawiwatkul, W. (2006). Stability and antibacterial activity of chitosan solutions affected by storage temperature and time. *Carbohydrate polymers, 65*(2), 174-178.

Oh, Y. J., Cho, I. H., Lee, H., Park, K.-J., Lee, H. & Park, S. Y. (2012). Bio-inspired catechol chemistry: a new way to develop a re-moldable and injectable coacervate hydrogel. *Chemical Communications, 48*(97), 11895-11897.

Peppas, Bures, P., Leobandung, W. & Ichikawa, H. (2000). Hydrogels in pharmaceutical formulations. *European Journal of Pharmaceutics and Biopharmaceutics, 50*(1), 27-46.

Peppas, N. A., & Buri, P. A. (1985). Surface, interfacial and molecular aspects of polymer bioadhesion on soft tissues. *Journal of controlled release, 2*, 257-275.

Peppas, N. A., & Sahlin, J. J. (1996). Hydrogels as mucoadhesive and bioadhesive materials: a review. *Biomaterials, 17*(16), 1553-1561.

Piai, J. F., Rubira, A. F., & Muniz, E. C. (2009). Self-assembly of a swollen chitosan/chondroitin sulfate hydrogel by outward diffusion of the chondroitin sulfate chains. *Acta Biomater, 5*(7), 2601-2609.

Qiu, Y., & Park, K. (2001). Environment-sensitive hydrogels for drug delivery. *Advanced Drug Delivery Reviews, 53*(3), 321-339.

Raafat, D. & Sahl, H. G. (2009). Chitosan and its antimicrobial potential--a critical literature survey. *Microb Biotechnol, 2*(2), 186-201.

Ravi Kumar, M. N. (2000). A review of chitin and chitosan applications. *Reactive and functional polymers, 46*(1), 1-27.

Reis, R. L., Neves, N. M., Mano, J. F., Gomes, M. E., Marques, A. P. & Azevedo, H. S. (2008). *Natural-based polymers for biomedical applications*. Woodhead Pub.

Rinaudo, M. (2008). Main properties and current applications of some polysaccharides as biomaterials. *Polymer International, 57*(3), 397-430.

Riva, R., Ragelle, H., des Rieux, A., Duhem, N., Jérôme, C. & Préat, V. (2011). Chitosan and Chitosan Derivatives in Drug Delivery and Tissue Engineering. *Chitosan for Biomaterials II. Advances in Polymer Science, 244,* 19-44. Springer, Berlin, Heidelberg.

Rosiak, J. M. & Yoshii, F. (1999). Hydrogels and their medical applications. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms, 151*(1–4), 56-64.

Ryu, J. H., Hong, S., & Lee, H. (2015). Bio-inspired adhesive catechol-conjugated chitosan for biomedical applications: A mini review. *Acta Biomater, 27*, 101-115.

Ryu, J. H., Jo, S., Koh, M. Y. & Lee, H. (2014). Bio- Inspired, Water- Soluble to Insoluble Self- Conversion for Flexible, Biocompatible, Transparent, Catecholamine Polysaccharide Thin Films. *Advanced Functional Materials, 24*(48), 7709-7716.

Ryu, J. H., Lee, Y., Kong, W. H., Kim, T. G., Park, T. G. & Lee, H. (2011). Catechol-Functionalized Chitosan/Pluronic Hydrogels for Tissue Adhesives and Hemostatic Materials. *Biomacromolecules, 12*(7), 2653-2659.

Schnurrer, J. & Lehr, C.-M. (1996). Mucoadhesive properties of the mussel adhesive protein. *International journal of Pharmaceutics, 141*(1-2), 251-256.

Schweigert, N., Zehnder, A. J. & Eggen, R. I. (2001). Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals. *Environmental microbiology, 3*(2), 81-91.

Silverman, H. & Roberto, F. (2007). Understanding Marine Mussel Adhesion. *Marine Biotechnology*, **9**(6), 661-681.

Smart, J. D. (2005). The basics and underlying mechanisms of mucoadhesion. *Advanced Drug Delivery Reviews, 57*(11), 1556-1568.

Sosnik, A., das Neves, J. & Sarmento, B. (2014). Mucoadhesive polymers in the design of nano-drug delivery systems for administration by non-parenteral routes: a review. *Progress in Polymer Science, 39*(12), 2030-2075.

Spicer, P. P., & Mikos, A. G. (2010). Fibrin glue as a drug delivery system. *Journal of controlled release, 148*(1), 49-55.

Spotnitz, W. D., & Burks, S. G. (2010). Hemostats, sealants, and adhesives II: update as well as how and when to use the components of the surgical toolbox. *Clinical and Applied Thrombosis/Hemostasis*, 713-713.

Szymańska, E. & Winnicka, K. (2015). Stability of chitosan a challenge for pharmaceutical and biomedical applications. *Marine drugs, 13*(4), 1819-1846.

Tajirian, A. L. & Goldberg, D. J. (2010). A review of sutures and other skin closure materials. *Journal of Cosmetic and Laser Therapy, 12*(6), 296-302.

Vakalopoulos, K. A., Daams, F., Wu, Z., Timmermans, L., Jeekel, J. J., Kleinrensink, G.-J., Lange, J. F. (2013). Tissue adhesives in gastrointestinal anastomosis: a systematic review. *Journal of Surgical Research, 180*(2), 290-300.

Voiutskii, S. S. (1963). *Autohesion and adhesion of high polymers*. Wiley, New York.

Waite, J. H., & Qin, X. (2001). Polyphosphoprotein from the adhesive pads of Mytilus edulis. *Biochemistry, 40*(9), 2887-2893.

Woertz, C., Preis, M., Breitkreutz, J. & Kleinebudde, P. (2013). Assessment of test methods evaluating mucoadhesive polymers and dosage forms: an overview. *European Journal of Pharmaceutics and Biopharmaceutics, 85*(3), 843-853.

Wu, J., Zhang, L., Wang, Y., Long, Y., Gao, H., Zhang, X., Xu, J. (2011). Mussel-inspired chemistry for robust and surface-modifiable multilayer films. *Langmuir, 27*(22), 13684-13691.

Xu, J., Soliman, G. M., Barralet, J. & Cerruti, M. (2012). Mollusk glue inspired mucoadhesives for biomedical applications. *Langmuir, 28*(39), 14010-14017.

Xu, J., Strandman, S., Zhu, J. X. X., Barralet, J. & Cerruti, M. (2015). Genipin-crosslinked catechol-chitosan mucoadhesive hydrogels for buccal drug delivery. *Biomaterials, 37*, 395-404.

Yu, J., Wei, W., Menyo, M. S., Masic, A., Waite, J. H. & Israelachvili, J. N. (2013). The adhesion of mussel foot protein-3 to TiO2 surfaces: the effect of pH. *Biomacromolecules, 14*(4), 1072.

Zhou, H. Y., Jiang, L. J., Cao, P. P., Li, J. B. & Chen, X. G. (2015). Glycerophosphate-based chitosan thermosensitive hydrogels and their biomedical applications. *Carbohydrate Polymers, 117*, 524-536.