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Insights into Covalent Chemistry for the Development of Biomaterials

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Abstract
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Covalent cross-linking chemistry is currently exploited in the preparation of biomaterial for biomedical applications. Choice of these chemistries for the preparation of biomaterials and bioconjugates strongly influences the biological output of these materials. Therefore, this thesis aims to develop novel bioconjugation strategies understanding their advantages and drawbacks. Our results provide new insight to adapt these chemical transformations for a specific application.

The first part of this thesis points out the relevance of tuning different properties of biomaterials with specific emphasis on the development of hyaluronic acid (HA) hydrogels. The second part of the thesis describes how different chemical transformations including hydrazone formation (**Paper I)**, thiazolidine formation (**Paper II**), cross-aldol addition reaction (**Paper III**) and disulfide formation (**Paper IV**) dictate material properties.

This thesis explores both basic organic reaction mechanism and application of these reactions to influence material characteristics. The detailed study of the reaction conditions, kinetics, and stability of the products will help to understand the mechanical properties, hydrolytic stability, and degradability of the materials described here.

Additionally, we performed degradation studies of gadolinium labeled HA hydrogels using magnetic resonance imaging. Furthermore, we also explored post-synthetic modification of hydrogels to link model fluorescent moieties as well as explored the tissue adhesive properties using Schiff-base formation.

In summary, this thesis presents a selection of different covalent chemistries for the design of advanced biomaterials. The advantages and disadvantages of these chemistries are rigorously investigated. We believe, such an investigation provides a better understanding of the bioconjugation strategies for the preparation of biomaterials with potential clinical translation.

Keywords: hyaluronic acid, hydrogel, biomaterials, covalent chemistry, biomedical applications, MRI

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To Josefa and Santiago

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Bermejo-Velasco D., Dou W., Heerschap A., Ossipov D., Hilborn J. (2018) Injectable hyaluronic acid hydrogels with the capacity for magnetic resonance imaging. *Carbohydrate Polymers*, 197, 641-648.

II Bermejo-Velasco D., Nawale G. N., Oommen O. P., Hilborn J., Varghese O. P. (2018) Thiazolidine chemistry revisited: a fast, efficient and stable click-type reaction at physiological pH. *Chemical Communications*, 54, 12507-12510.

III Bermejo-Velasco D., Kadekar S., Tavares da Costa M. V., Oommen O. P., Gamstedt K., Hilborn J., Varghese O. P. First Aldol-Crosslinked Hyaluronic Acid Hydrogel: Fast, Hydrolytically Stable, and Injectable Gel with Tissue Adhesive Properties. *Submitted manuscript*.

IV Bermejo-Velasco D., Azémar A., Oommen O. P., Hilborn J., Varghese O. P. Modulating thiol pK_a promotes disulfide formation at physiological pH: An elegant strategy to design disulfide cross-linked hyaluronic acid hydrogels. *Submitted manuscript*.

Contribution Report

I. I had a major contribution in the design of the study. I performed all the synthesis and characterization, except for the ICP-MS measurements. I performed the preparation of hydrogels and participated in the magnetic resonance imaging experiments. I participated in the discussion of the results and wrote the manuscript.

II. I contributed to the design of the study. I performed all the synthesis and spectroscopy characterization. I contributed to the HPLC experiments. I participated in the discussion of the results and wrote the manuscript.

III. I contributed to the design of the study. I performed all the synthesis and characterization of the materials. I performed hydrogel preparation, rheological characterization and swelling/degradation experiments. I performed labeling experiments and had a major contribution in the adhesive experiments. I participated in the discussion of the results and wrote the manuscript.

IV. I contributed to the design of the study. I performed all the synthesis and characterization of the materials. I performed hydrogel preparation, rheological characterization and swelling/degradation experiments. I participated in the discussion of the results and wrote the manuscript.

Contents

Abbreviations

1D	One-dimensional
2D	Two-dimensional
3D	Three-dimensional
ξ	Average mesh size
BMP-2	Bone morphogenetic protein-2
DNA	Deoxyribonucleic acid
DS	Degree of substitution
DTT	1,4-dithiothreitol
ECM	Extracellular matrix
EDC	1-Ethyl-3-(3-dimethyl aminopropyl)-1carbodiimide hydrochloride
G'	Storage modulus
G''	Loss modulus
HA	Hyaluronic acid
HA-ActCys	Hyaluronic acid modified with N-acetyl-cysteine groups
HA-Cys	Hyaluronic acid modified with cysteine groups
HA-Eal	Hyaluronic acid modified with enolizable aldehyde groups
HA-Nal	Hyaluronic acid modified with non-enolizable aldehyde groups
HA-SH	Hyaluronic acid modified with conventional thiol groups
HPLC	High-performance liquid chromatography
HYAL	Hyaluronidase
hy-HA-SH	Hyaluronic acid dually-modified with hydrazide and thiol groups

k_1	Pseudo-first order reaction rate constant
Mc	Average molecular weight between cross-links
MRI	Magnetic resonance imaging
NMR	Nuclear magnetic resonance
PBS	Phosphate buffer saline
RNA	Ribonucleic acid
$(SSPy)_2DTPA\text{-}Gd^{3+}$	Diethylenetriaminepentaacetate gadolinium complex modified with two dithiopyridyl groups
T_1	Longitudinal relaxation time
T_2	Transverse relaxation time

Scope of the thesis

This thesis presents the development of covalent coupling chemistry with the aim to design biomaterials and bioconjugation strategies that are compatible with living systems. With the advances in biomedical technologies, it is imperative to develop novel coupling strategies and chemical transformations that yield stable products without any toxic by-products under physiological conditions. The ultimate biological output of hydrogel scaffolds and bioconjugates is strongly affected by the chemistry used in their preparation. Therefore, in this thesis, we validated different chemistries with special emphasis to prepare hyaluronic acid (HA) based biomaterials and bioconjugates.

The reaction pH is an important factor to consider when developing biomaterials. Fast and efficient reactions that take place under physiological conditions are highly desirable in the biomedical field. Since there are conflicting reports regarding the reaction conditions of the condensation of aldehydes and 1,2-aminothiols to form thiazolidines, we examined this reaction carefully and demonstrated the potential of this reaction for the coupling of biomolecules under neutral pH. Disulfide chemistry plays a crucial role in protein biology and has been exploited by scientists to develop many types of biomaterials. In spite of its versatile use, the disulfide chemistry suffers from some inevitable limitations such as the need for basic conditions (pH > 8.5), strong oxidants and long reaction times. To overcome this limitation, we modulated the thiol pK_a by incorporating electron-withdrawing substituents at the β-position and explored the influence of such modifications on the reaction kinetics for disulfide-formation. We further applied the improved reaction kinetics in the preparation of HA hydrogels under physiological pH and without any additional oxidant.

Other important factors in the design of biomaterials are tunable mechanical properties and hydrolytic stability. Currently, there are limited approaches to design hydrogel scaffold materials with hydrolytic stable polymeric network and highly tunable mechanical properties. Cross-aldol addition reaction was used to prepare HA hydrogels cross-linked with a stable covalent C-C bond without catalysts or initiators. Aldol HA hydrogels exhibited exceptional hydrolytic stability and tunable mechanical properties. The cross-linking efficiency of the aldol addition reaction was determined using rheology and could be interpreted in terms of molecular weight between cross-links (*Mc*). Furthermore, the presence of residual aldehydes within the aldol

HA hydrogel network permitted facile post-synthetic modification with nucleophilic reagents and provided tissue adhesive properties to the material.

Degradability of biomaterials is an important characteristic that needs to be quantified to understand the properties of the materials once implanted in animal models. HA hydrogels are known to be degraded *in-vivo* by an endogenous enzyme called hyaluronidase. However, monitoring such degradation is difficult to achieve by conventional methods. Therefore, we exploited disulfide chemistry to covalently attach a gadolinium complex to a hydrazone-crosslinked HA hydrogel network. Such a design enabled us to monitor the degradation of the hydrogels in real time using non-invasive MRI techniques.

In summary, this thesis provides valuable insight into the factors governing a selection of conjugation strategies. Our results are useful for the rational design of biomaterials according to the specific biomedical needs.

1. Introduction

1.1. Hydrogel scaffold materials

Hydrogels are three-dimensional (3D) scaffold materials composed by cross-linked hydrophilic polymers chains with highly tunable physical and chemical properties.[1, 2] Hydrogels can absorb a large amount of water or biological fluids providing the ability to resemble the extracellular environment of native soft tissues. Different cross-linking methods, including covalent bond and physical interactions (hydrogen bonding, electrostatic forces or hydrophobic interactions), have been employed for the preparation of hydrogel polymeric networks.[3, 4] Cross-linkages between polymer chains allow macromolecular assembly and preserve the hydrogel 3D polymeric structures avoiding their dissolution in the aqueous environment. Preferentially, hydrogels are prepared without employing or forming any low molecular weight toxic product to allow *in-situ* cell encapsulation and avoid further purification after hydrogel formation. Among the different types of hydrogels, covalently crosslinked hydrogels have been more extensively studied because they are the most stable and show superior mechanical properties.

Polymers from both synthetic and natural sources have been widely used for the preparation of hydrogels.[5, 6] Synthetic polymers that have been used for the preparation of hydrogels are poly(vinyl alcohol), polyacrylamide, and poly(ethylene glycol) among others.[7] These polymers can be prepared with a highly controlled chemical structure and therefore it is easier to tune the macroscopic properties of the hydrogels. On the other hand, natural polymers, such as chitosan, alginate, hyaluronic acid (HA), and collagen for instance, which are part of the extracellular matrix (ECM) have superior biocompatibility, biodegradability and defined interaction with cells that promote specific cellular activities.[8]

1.1.1. Hyaluronic acid (HA) hydrogels

Naturally occurring polymers have gained increasing attention in the development of hydrogels because of their inherent biocompatibility and biodegradability. Natural polymer-based hydrogels interact with encapsulated cells promoting and supporting cellular activities, and they can additionally preserve cell-secreted sensitive proteins.[6] HA has been recognized as one of the most suitable biomacromolecules for hydrogel preparation because of its

ability to absorb large amounts of water, it is degraded by endogenous enzymes present in the human body (hyaluronidase, HYAL), and lack of immunogenic or inflammatory response.[9-12]

HA is the only non-sulfated glycosaminoglycan distributed among the ECM of many tissues and is based on repeating units of the disaccharide *β*-1,4-D-glucuronic acid-*β*-1,3-N-acetyl-D-glucosamine (**Figure 1.1**). HA is known to play important roles in the regulation of diverse biological processes such as cell motility, wound healing, tissue remodeling, angiogenesis, and cell activation in the morphogenesis of the human body.[9, 13]

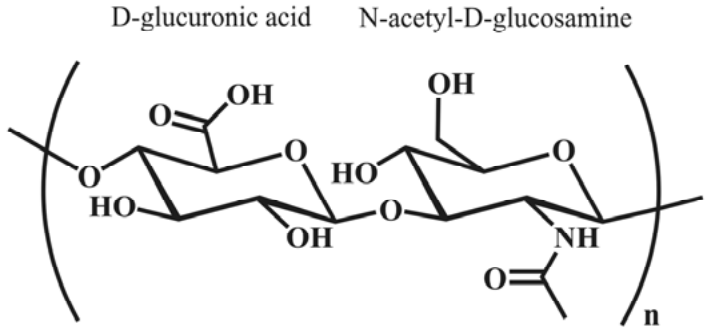


Figure 1.1. Chemical structure of hyaluronic acid (HA) repeating unit.

1.2. Hyaluronic acid (HA) chemical modification

The construction of HA hydrogel materials was traditionally performed using small molecule cross-linkers and coupling agents.[14, 15] Hydrogels prepared following this approach have limited applicability *in-vivo* because it is necessary to remove the excess of toxic small molecule used in their preparation.[16] Modern approaches for the preparation of HA hydrogel matrices involved the chemical modification of the HA macromolecule. HA is poorly soluble in organic solvents and it is degraded under strong acidic and alkaline conditions. Therefore, HA chemical modification is preferably carried out in aqueous media and mild conditions. There are three main chemical functionalities present in HA that can be used for modification: the primary hydroxyl, the vicinal diol of the polymer backbone, and the carboxylic acid groups (**Figure 1.2**).

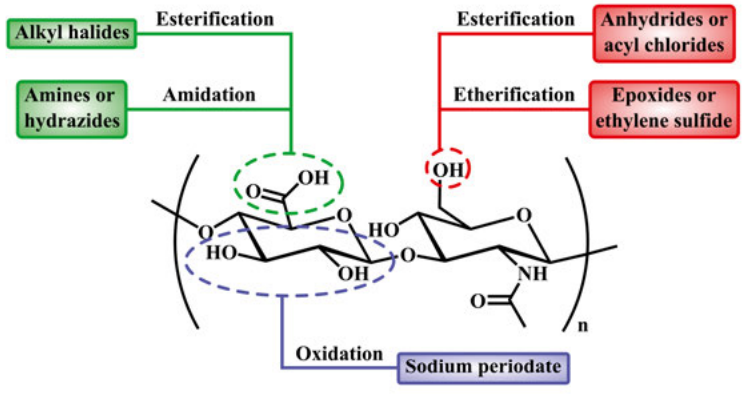


Figure 1.2. Hyaluronic acid (HA) main sites for chemical modification and most commonly used reagents.

1.2.1. Chemical modification of the primary hydroxyl group

Primary hydroxyl can be modified by etherification or esterification. Etherification can be achieved through epoxide[17, 18] or ethylene sulfide[19] ring opening reaction. Esterification of the hydroxyl groups is normally performed using anhydrides[20, 21] or acyl chloride-activated carboxylate compounds.[22] The advantage of these approaches is limited by the degradation of HA in the alkaline conditions (pH > 10) necessary for the deprotonation of the hydroxyl group.

1.2.2. Oxidation of vicinal diols of the polymer backbone

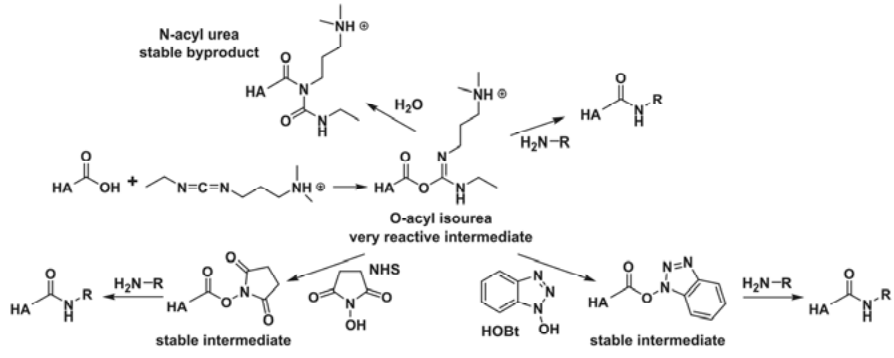
Periodate oxidation of carbohydrates is widely spread method for the preparation of aldehyde-modified polysaccharides.[23] The oxidation of the C2-C3 vicinal diols of the HA backbone opens the sugar ring, thereby forming dialdehydes.[24] This modification leads to an uncontrolled degradation of the polymer backbone reducing the final molecular weight of the HA derivative and compromising its biological function. To overcome this problem aldehyde derivatives of HA have been prepared by selective oxidation of grafted glycerol moieties.[25]

1.2.3. Chemical modification of the carboxylic acid group

The carboxylic acid group is the most common site for the functionalization of HA. Esterification of HA has been performed by nucleophilic substitution using alkyl halides.[26] Among all the possible reactions for the preparation of HA derivatives, the most established method is the carbodiimide-assisted condensation of carboxylic acid with amines or hydrazides using water-

soluble 1-ethyl-3-(3-dimethyl aminopropyl)-1carbodiimide hydrochloride (EDC).[27]

The first step of the coupling reaction is the activation of the carboxylic acid by EDC to form the very reactive O-acyl isourea intermediate (**Scheme 1.1**). The second step is the nucleophilic attack by the amine or hydrazide, which give rise to the formation of an amide bond. The activation of the carboxylic acid is optimal at slightly acidic conditions (pH 4.7), at which the carbodiimide nitrogens are protonated favoring the nucleophilic attack of the carboxylate ions of HA. Additionally, the reaction of the O-acyl isourea intermediate with water occurs through a fast rearrangement giving rise to a stable N-acyl urea by-product and preventing the subsequent amidation reaction. This side reaction is inhibited at acidic conditions.[28, 29]



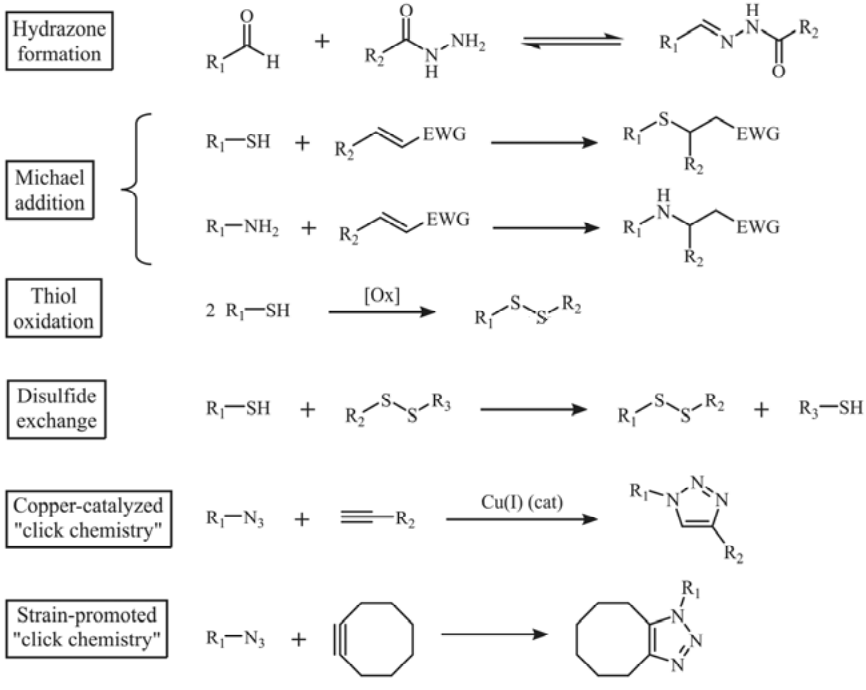
Scheme 1.1. Schematic representation of the EDC-mediated coupling reaction.

Amines are protonated at acidic pH which compromises the efficiency of the coupling reaction. Unlike amines, hydrazides, retain their nucleophilicity at the required acidic conditions, ensuring higher efficiency of the EDC-mediated coupling reaction. The irreversible formation of the N-acyl urea by-product can be prevented by the addition of N-hydroxysuccinimide (NHS) or 1-hydroxybenzotriazole (HOBt) which form more hydrolysis-stable intermediates allowing to perform the coupling reaction at higher pHs.[29]

1.3. Covalent chemistry in hydrogel preparation

Many chemical transformations have been studied for the formation of cross-linked hydrogel polymeric networks (**Scheme 1.2**).[3, 30] The type of linkages can be modified depending on the desired biodegradability, speed of cross-linking and mechanical properties. The formation of hydrazones via the condensation of aldehydes and hydrazides has been broadly explored in the preparation of hydrogels because it facilitates rapid cross-linking at a

wide range of pHs.[27] However, the labile hydrazone linkages make such materials unstable in aqueous media. The hydrolytic stability of the hydrazone polymeric network can be improved by tuning the electronic characteristics of the hydrazide substrate.[31] The injectability of hydrazone hydrogels can be enhanced using a catalyst that accelerates the formation and cleavage of hydrazone bonds.[32]



Scheme 1.2. Common covalent chemistries for the preparation of hydrogels.

Other widely investigated cross-linking chemistry is the Michael addition between electron-deficient vinyl groups and nucleophiles such amines and thiols. Unlike most addition reactions, thiol-Michael addition can be performed in aqueous medium and at physiological pH.[33] This reaction is of particular interest due to the relative biological inertness of the polymeric precursors and its controlled reaction time.[34] The gelation rate at physiological conditions can be improved by using thiolated substrates that promote thiol deprotonation.[35] Vinyl sulfones are better electron-deficient species when compared to acrylates resulting in faster gelation time of its derivatives.[36]

The oxidation of two thiol groups to form disulfide bonds is another cross-linking method.[28] The slow gelation kinetics of disulfide cross-linked hydrogels has restricted its full potential. The presence of oxidizing agents such as hydrogen peroxide, ammonium persulfate, and sodium periodate,

can decrease the gelation time.[37] Hydrogels cross-linked with disulfide bonds can be prepared in the absence of oxidizing agents by disulfide exchange reaction using labile dithiopyridyl derivatives.[38]

Polymers carrying terminal azides can react with alkyne derivatives through 1,3-dipolar cycloaddition ("click chemistry") at physiological pH.[39] The inertness of both azides and alkynes towards the majority of biological functional groups make this reaction a popular conjugation strategy. The need of copper ions as the catalyst is a concern that can be avoided using cyclooctyne derivatives.[40]

The utilization of these chemical transformations is an attractive and versatile way to rationally synthesize hydrogel scaffolds applicable in the biomedical field.

1.4. Biomedical applications of hydrogels

The interconnected porous structure of the hydrogels allows the diffusion of oxygen, nutrients, biomolecules or drugs, and has made hydrogels an attractive option for a range of biomedical applications, including drug delivery,[41, 42] cell therapy,[43, 44] tissue engineering,[4, 6, 45] and tissue adhesive materials.[5, 46, 47]

Among these applications, hydrogel-based drug delivery systems have become a main area of research interest. The successful output of hydrogel as a drug delivery system relies on controlling the release of the encapsulated molecules and preventing their premature degradation. Tissue engineering focuses on the reparation or total replacement of damaged tissues or organs. The combination of hydrogel scaffolds, cells and grow-factors have yielded remarkable success in promoting tissue healing with functional restoration. Conventional closure techniques of traumatic or surgical wounds such as suturing and stapling have many disadvantages. Particularly, for wounds where gases or fluids need to be sealed off, the conventional closure techniques are inadequate. Hydrogels with tissue adhesive properties have demonstrated huge potential as wound closure materials because they are easy to apply; they retain their properties under wet conditions and possess high elasticity to comply with the movement of the tissue.

In addition, hydrogel swelling, degradation rate, injectability, and covalent attachment of bioactive molecules to the polymeric network are determining factors for a successful outcome of the scaffold in such biomedical applications. Hydrogels cross-linked with hydrolytically cleavable bonds showed undesirable extensive swelling due to an increased influx of water and eventual dissolution of the polymeric network. This represents a major challenge for the *in-vivo* application of this type of hydrogels.[31, 45] Hydrogels exhibiting controlled degradation kinetics is highly desirable. Ideally, *in-vivo* such materials should degrade by surface erosion induced by endog-

enous enzymes expressed by surrounding cells and tissues. For example, in tissue engineering, scaffolds must degrade to allow for their replacement by the newly formed tissue, whereas they must have enough integrity to support tissue formation.[45, 48] Hydrogels used for controlled drug delivery must degrade to match the require drug release profile.[49] Injectability is another desirable property of hydrogel materials that allows the possibility to deliver in a minimally invasive manner and provides high moldability to the material (they can adapt to the shape of the defect).[7, 8] The covalent immobilization of bioactive molecules such as proteins or peptides to the polymeric network has successfully improved the attachment, differentiation, and proliferation of encapsulated cells.[50] All of these important and desirable factors can be controlled by carefully choosing the chemistries involved in the preparation of the hydrogel scaffolds.

1.4.1. Biomedical applications of HA hydrogels

HA-based hydrogels have been applied as delivery systems.[51] For instance, dexamethasone loaded HA hydrogels reduced the inflammatory response that cost postoperative peritoneal adhesion[52] and effectively treated rheumatoid arthritis.[53] Other drugs such as mitomycin C[54] and doxorubicin[39, 55] have been also encapsulated into HA hydrogels in order to decrease postoperative adhesion and insert a potent anti-tumoral drug, respectively. HA hydrogels have shown promising results in gene therapy through the local delivery of DNA or RNA.[56-58] Protein and enzyme encapsulation into HA hydrogels have been used to preserve their bioactivity and ensure their controlled and sustained release.[59-61]

Hydrogels formed from HA have been intensively studied for several tissue engineering applications.[12, 62] The encapsulation of chondrocytes into HA-based hydrogels enhances their proliferation and has proved to be very effective for the reparation of cartilage tissue.[34, 63-65] HA hydrogel crosslinked matrices have been used for the reconstruction of soft adipose tissue[66, 67] and in the repair of cardiac tissue after infarction.[68-70] We have developed HA-hydrogels for the sequestration of bone morphogenetic protein-2 (BMP-2) and to induced bone augmentation in vivo.[71-73] All these important considerations were the main points for choosing HA for the development of the biomaterials described during this thesis.

1.5. Visualization of hydrogels

The ability to study the degradation of hydrogels in real time using non-invasive imaging techniques is very useful in the development of hydrogel materials. The *in-vivo* degradation studies of conventional hydrogels require laboratory animals to be sacrificed for visual observation of the implanted

hydrogels at each time point. Postmortem evaluation of hydrogels significantly increases the number of animals that are needed to follow a small number of samples. Apart from the ethical issues involved, the use of such a large amount of animals increases the cost of the study and introduces high variability between the different studied specimens.[74, 75]

Ideally, *in-vivo* degradation studies should allow to longitudinally following a hydrogel implanted in one single animal during the entire degradation process, thus reducing the need of animals to a minimum. Non-invasive imaging techniques such as fluorescence imaging,[76, 77] X-ray microcomputed tomography,[78, 79] and magnetic resonance imaging (MRI)[80] allow monitoring the fate of the implanted hydrogels at real time without sacrificing any animal. Among these techniques, MRI is especially suitable because it is a free ionizing radiation technique with high penetration capacity and spatial resolution allowing the visualization of internal structures of the body in detail. The visualization of hydrogels using MRI is especially challenging because of the contrast in the MR images emerges from the physical and chemical microenvironment of the water molecules which is similar between the hydrogels and the surrounding tissues.

Different contrast agents have been incorporated into hydrogel materials to enhance the contrast in the MR images. Gadolinium-based complexes are the most commonly used MR contrast agents because of their lower detection limit.[81] Gadolinium-based complexes change the longitudinal relaxation time (T_1) of the hydrogels and produce positive contrast (bright image) in the T_1-weighted MR images.[80, 82-85] Other MR contrast agents are magnetic oxide nanoparticles that change the transverse relaxation time (T_2) of the hydrogels and produce negative contrast (dark image) in the T_2-weighted MR images.[86-88]

2. Results and discussion

2.1. Synthesis of hyaluronic acid (HA) derivatives

In this thesis aldehyde-modified (**Paper I and Paper III**), thiol-modified (**Paper IV**), and hydrazide-thiol dually-modified (**Paper I**) HA derivatives were synthetized. All the HA derivatives were synthetized through the EDC-mediated coupling reaction with commercial amines or synthetized dihydrazide reagents (**Figure 2.1**).

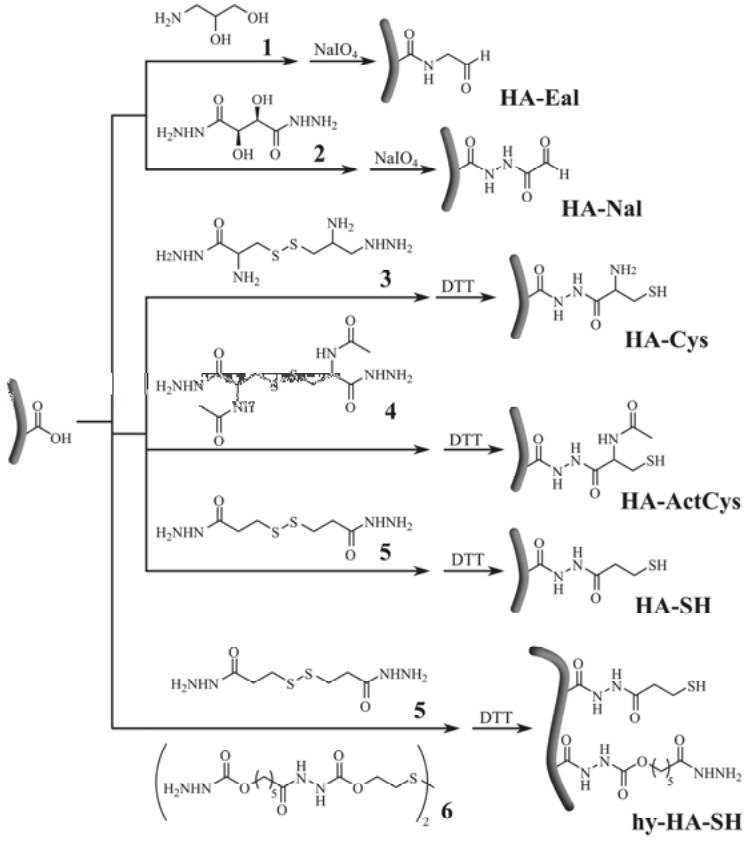


Figure 2.1. Chemical structure of HA derivatives and the nucleophilic reagents used for their synthesis. All the HA derivatives were synthetized through the EDC-mediated coupling reaction.

2.1.1. Synthesis of aldehyde-modified HA derivatives

The preparation of HA derivative carrying aldehyde groups was achieved following a two-step procedure. First, we introduced flexible diols by EDC-mediated coupling reaction. The introduced flexible diols contain C-C bond between the hydroxyl groups that can rotate freely. The subsequent oxidation of the pendent diols using sodium periodate afford the desired aldehyde-modified HA derivatives.

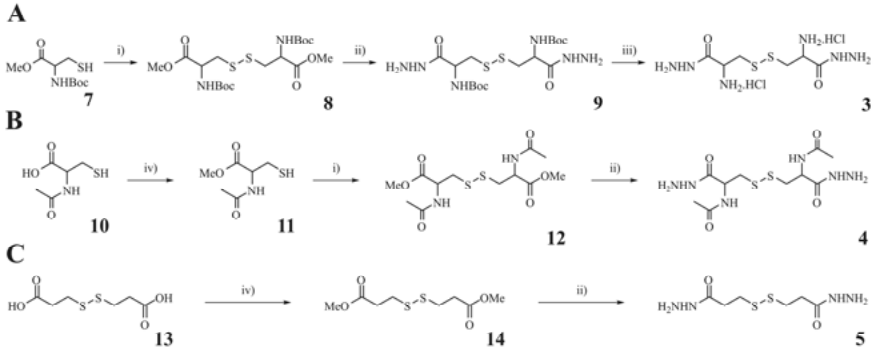
The conformation of the vicinal diols is crucial for a successful oxidation. Unlike the C2-C3 rigid *trans*-diol of the polymer backbone, the flexible pendent diols introduced can undergo conformational isomerization improving the oxidation rate four orders of magnitude.[25] The quick oxidation of the pendent diol groups can selectively produce aldehyde functionalities without affecting the diol groups of the HA backbone.

Two types of aldehyde-modified HA derivatives were prepared. The derivative obtained from 3-amino-1,2-propanediol (**1**) contain acidic protons in the α-position of the aldehyde group and therefore it can undergo enolization under basic conditions. This derivative was referred to as enolizable HA aldehyde (HA-Eal). On the other hand, HA derivative obtained from L-tartaric acid dihydrazide (**2**) contains a pyruvic-type aldehyde which represents a non-enolizable aldehyde group (HA-Nal).

2.1.2. Synthesis of thiol-modified HA derivatives

Thiol-modified HA derivatives were prepared following a similar procedure in which dihydrazide dimer reagents containing a central disulfide bond were linked to HA through the EDC-mediated coupling reaction. The progress of the coupling reaction was evident as the viscosity of the reaction mixture increased, indicating that the dihydrazide reagents acted as a cross-linker between two HA macromolecules. The central disulfide bond was subsequently reduced using 1,4-dithiothreitol (DTT) giving rise to the desired free thiol groups.

Three different dihydrazide reagents were synthetized for the preparation of thiol-modified HA derivatives (**Scheme 2.1**). The HA derivatives obtained from 3,3'-dithiobis (2-aminopropanehydrazide) (**3**) and 3,3'-dithiobis (2-acetamidopropanehydrazide) (**4**) contain electron-withdrawing groups at the β-position of the thiol, such as protonated amines (HA-Cys) and N-acetamide (HA-ActCys) groups respectively. The used of 3,3'-dithiobis (propanehydrazide) (**5**) afford conventional thiol derivative without any electron-withdrawing group (HA-SH).[28] It is important to mention that the amine group of the dihydrazide **3** was not protected during the EDC-mediated coupling reaction because amines are protonated at the reaction conditions (pH 4.7), preventing their participation in the coupling reaction.



Scheme 2.1. Schematic representation of the synthesis of disulfide-containing dihydrazide compounds. a) 3,3'-dithiobis (2-aminopropanehydrazide) (**3**) used in the preparation of HA-Cys. b) 3,3'-dithiobis (2-acetamidopropanehydrazide) (**4**) used in the preparation of HA-ActCys. c) 3,3'-dithiobis (propanehydrazide) (**5**) used in the preparation of HA-SH. Reaction conditions: i) cat. NaI, H_2O_2, EtOAc; ii) $NH_2NH_2 \cdot H_2O$, EtOH; iii) HCl, 1,4-dioxane; iv) cat. H_2SO_4, MeOH.

2.1.3. Synthesis of thiol-hydrazide dually-modified HA derivative

The covalent attachment of functional molecules to the hydrogel matrix, such as peptides, drugs or imaging agents generally involve the use of dually-functionalized polymers. One of the functionalities is used for the formation of the hydrogel network, while the other functionality is used for the covalent attachment of the functional molecule. Thiol and hydrazide act as nucleophiles at a different range of pH, which offer an advantage for the use of these orthogonal chemoselective groups.[72, 89, 90]

The dihydrazide compound **5** was used for the incorporation of thiol groups. Hydrazide functionalities were introduced in HA using disulfanediylbis (ethane-2,1-diyl) bis (2-(6-((hydrazinecarbonyl)oxy)-hexanoyl) hydrazinecarboxylate) (**6**), which contains a central 2,2-dithiobis(ethoxycarbonyl) divalent protecting group. The central disulfide bond of the protective group could be reduced with DTT generating an unstable thiol which spontaneously decomposes liberating the masked hydrazide group.[27] Thiol and hydrazide groups were simultaneously incorporated in HA employing a one-pot procedure, in which the two symmetrical dihydrazide reagents were simultaneously coupled to HA. Then the central disulfide bonds were reduced using DTT giving rise to the desired dually-functionalized HA derivative (hy-HA-SH).

2.1.4. ^{1}H-NMR characterization of HA derivatives

^{1}H-NMR spectroscopy is a very useful technique for the characterization of HA derivatives. The incorporation of the new functionalities can be confirmed by comparing the ^{1}H-NMR spectra of the HA derivative with the spectra of native HA which shows three resonances: i) a broad signal at 4.53 ppm that corresponds to the anomeric carbon protons, ii) a sharp singlet at 1.97 ppm that corresponds to the acetamide protons, and iii) a set of close signals (3.98-3.34 ppm) that correspond to the rest of HA protons.

The ^{1}H-NMR analysis of the two aldehyde-modified HA derivatives revealed a new single signal at 5.15 ppm (HA-Eal) or 5.48 ppm (HA-Nal) that correspond to the hemiacetal protons of the aldehyde functionalities (**Figure 2.2**). As expected, the presence of electron-withdrawing substituent at the α-position of the aldehyde functionality in HA-Nal resulted in a downfield shift of the NMR signal.

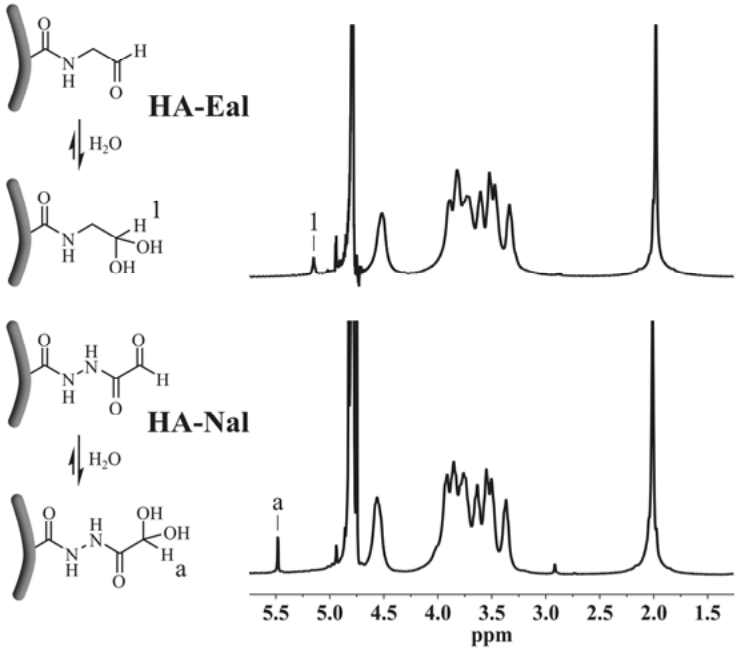


Figure 2.2. ^{1}H-NMR spectra of aldehyde-modified hyaluronic acid (HA) derivatives (HA-Eal and HA-Nal).

The incorporation of thiol functionalities was also verified by ^{1}H-NMR spectroscopy (**Figure 2.3**). Specifically, new signals could be observed at 4.36 ppm (-*CHNH*$_2$) and 2.88 ppm (-*CH*$_2$*SH*) corresponding to the methine and methylene protons of HA-Cys. HA-ActCys spectra showed new signals at 4.59 ppm (-*CHNHCO*-), 2.97 ppm (-*CH*$_2$*SH*) and 2.07 ppm (*CH*$_3$*CONH*-) that correspond to the methine, methylene and methyl protons respectively.

The signals of the methylene protons (-CH_2CH_2SH) of HA-SH were observed at 2.87 ppm and 2.72 ppm.

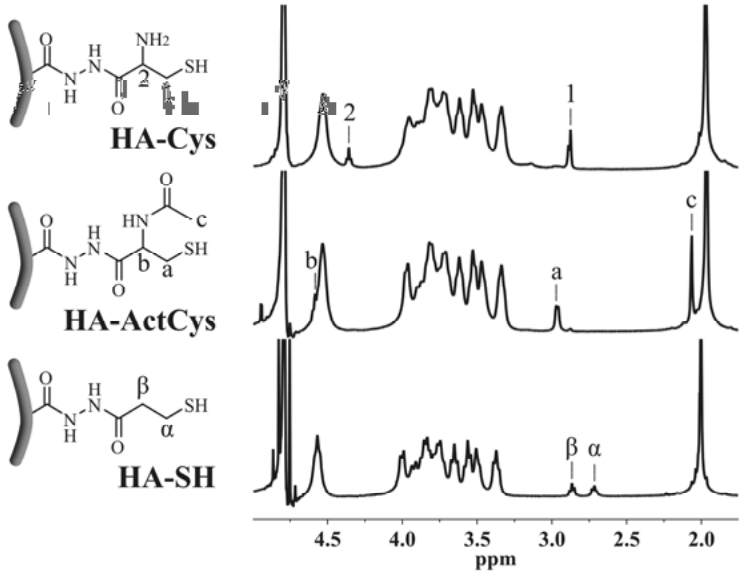


Figure 2.3. ^{1}H-NMR spectra of thiol-modified hyaluronic acid (HA) derivatives (HA-Cys, HA-ActCys, and HA-SH).

In the case of the dual modification of HA with thiol and hydrazide groups, the ^{1}H-NMR spectra showed new signals at 2.87 ppm and 2.72 ppm corresponding to the methylene protons of the thiol functionality (**Figure 2.4**). The signals corresponding to the methylene protons of the hydrazide functionality appeared at 4.22 ppm, 2.38 ppm, 1.70 ppm, and 1.42 ppm.

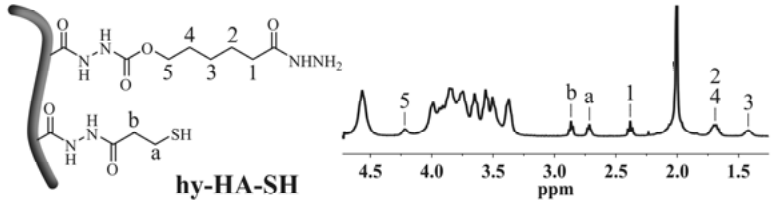


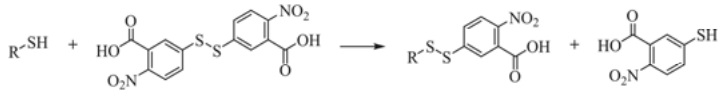
Figure 2.4. ^{1}H-NMR spectrum of thiol-hydrazide dually-modified hyaluronic acid (HA) derivative (hy-HA-SH).

The degree of substitution (DS) is an important characteristic of the HA derivatives as it has to be enough to allow hydrogel formation but heavily modified derivatives can lose the ability to support and promote cellular activities. High DS is also known to cause syneresis of gel, resulting in shrinkage

of materials. The DS is normally controlled by regulating the molar ratios between EDC and HA. The DS can be determined by integration of the NMR-signals corresponding to the introduced functionalities and comparing them to the integral of the acetamide moiety of the N-acetyl-D-glucosamine unit of HA. DS (%) is expressed as the number of modified disaccharide units per 100 disaccharide units.

2.1.5. Colorimetric characterization of thiol-modified HA derivatives

The ^{1}H-NMR signals from the incorporated functionalities can overlap with the HA signals as in the case of HA-ActCys. In these cases, the DS cannot be determined by NMR and an additional technique is required. The amount of incorporated thiols can be determined by a colorimetric assay named Ellman's method.[91] The reaction of the Ellman's reagent (5,5'-dithiobis (2-nitrobenzoic acid)) with a thiol group generates a disulfide bond and liberates the yellow thionitrobenzoic acid (**Scheme 2.2**). The amount of thionitrobenzoic acid is equivalent to the amount of thiol groups and can be determined by measuring the light absorption at 412 nm.



Scheme 2.2. Representation of the reaction between the Ellman's reagent and thiols.

The DS (%) of all the HA derivatives prepared in this thesis and the molar ratio of EDC/HA used for their synthesis are shown in **Table 2.1**.

Table 2.1. The EDC/HA molar ratio used for the synthesis of hyaluronic acid (HA) derivatives and the obtained degree of substitution (DS).

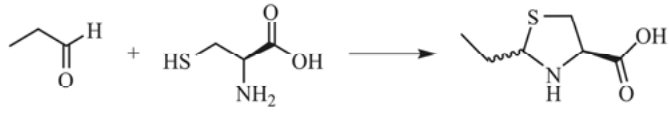
HA derivative	EDC molar ratio	DS (%)
HA-Eal	0.3	12%
HA-Nal	0.15	10%
HA-Cys	0.3	11%
HA-ActCys	0.18	11%
HA-SH	0.15	9%
HA-SH	0.3	19%
hy-HA-SH	0.31	10% (hy); 10% (SH)

2.2. Revision of the key chemical transformations

The chemical reactions used in this thesis for the preparation of hydrogels or bioconjugates include thiazolidine formation, aldol addition reaction, disulfide formation, and the condensation of hydrazides and aldehydes to form hydrazone bonds.

2.2.1. Thiazolidine formation

The reactions that involved 1,2-aminothiols are of special interest in biomedical applications because of this functionality is naturally present in proteins with an N-terminal cysteine residue. The condensation reaction between 1,2-aminothiols and aldehydes to form thiazolidine products is an interesting reaction that has been poorly explored (**Scheme 2.3**). It is generally believed that thiazolidine formation is a very slow reaction that requires acidic pH.[92-94] Additionally, the thiazolidine products are sensible towards hydrolysis at physiological pH.[92, 95-97]



Scheme 2.3. Schematic representation of the condensation between aldehydes (propionaldehyde) and 1,2-aminothiols (L-cysteine) to form thiazolidines.

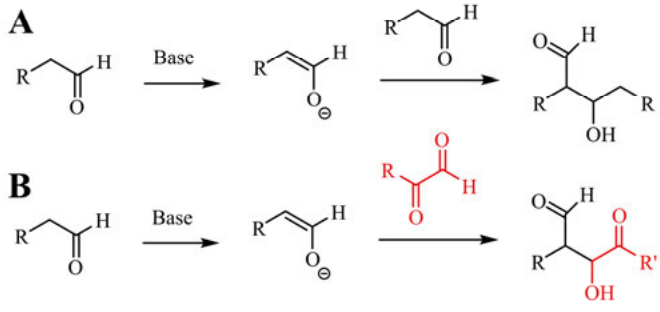
There are two main approaches to improve the applicability of this reaction in the biomedical field. The most common approach is the use of aldehydes containing labile esters that facilitates the ring rearrangement of the thiazolidine product to afford stable pseudoproline compounds.[96, 97] Other approach is the use of ortho-boronic acid modified benzaldehyde that improves the reaction kinetics and affords thiazolidine products even at neutral pH.[93, 94]

2.2.2. Aldol addition reaction

Aldol addition reaction is a simple classic reaction used in organic chemistry to form asymmetric carbon-carbon single bonds.[98] This reaction combines two aldehyde compounds to form a β-hydroxy aldehyde (aldol product). Mechanistically, base catalyzed aldol addition reaction occurs through the deprotonation of the α-acidic proton of the aldehyde group, forming the enolate form of the aldehyde. The aldol product is formed after the nucleophilic addition of the enolate to an electrophile free aldehyde group (**Scheme 2.4**).

When the reaction is performed between two different enolizable aldehydes, four different aldol products can be formed (two self-condensation products and two cross-condensation products). On the other hand, the mix-

ture of an enolizable and non-enolizable aldehyde gives rise to only one cross-condensation product.



Scheme 2.4. Schematic representation of the aldol addition reaction between (a) two identical aldehydes or (b) two different aldehydes (cross-aldol reaction).

2.2.3. Disulfide formation

Disulfide bonds are very important in the regulation of protein folding and function,[99, 100] as well as in the redox signaling between cells.[101] Disulfide linkages are particularly interesting in the development of materials for cell encapsulation because cells have the ability to secrete natural reductants that cleave disulfide bonds.[38, 102] Disulfide bonds are commonly formed by oxidation of thiol compounds in the presence of oxygen (**Equation 2.1**), but this process requires basic conditions (pH > 8.5) because it involves the formation of thiolate ions ($R\text{-}S^-$).[103, 104]

$$R\text{-}SH + H_2O \rightleftharpoons R\text{-}S^- + H_3O^+$$
$$4R\text{-}S^- + O_2 + 4H_3O^+ \rightleftharpoons 4R\text{-}S^{\cdot} + 6H_2O$$
$$2R\text{-}S^{\cdot} \rightarrow R\text{-}S\text{-}S\text{-}R \qquad (2.1)$$

Disulfide bonds can also be formed by the disulfide exchange reaction, in which a thiolate ion reacts with a disulfide to form a new disulfide bond (**Equation 2.2**). The labile dithiopyridyl is extensively used in the formation of disulfide bonds because it highly improves the reaction rate at physiological conditions.[105]

$$R\text{-}SH + H_2O \rightleftharpoons R\text{-}S^- + H_3O^+$$
$$R\text{-}S^- + R'\text{-}S\text{-}S\text{-}R' \rightleftharpoons R\text{-}S\text{-}S\text{-}R' + R'\text{-}S^- \qquad (2.2)$$

2.2.4. Hydrazone formation

The condensation of hydrazides and aldehydes to form hydrazone bonds is the most widely explore reaction in the preparation of hydrogels. It is a fast

and efficient reaction that takes place in a broad range of pHs although it is especially faster at acidic conditions (**Scheme 2.5**).[106] However, this reaction is reversible and the hydrazone products can undergo slow hydrolysis in the aqueous environment. As a result, hydrazone hydrogels exhibit uncontrolled swelling and eventual dissolution of the polymeric network. The hydrolytic stability of the hydrazone bond can be controlled using hydrazides with highly electronic delocalization.[31]

Scheme 2.5. Schematic representation of the condensation between aldehydes and hydrazides to form hydrazone bonds.

2.3. Reaction pH: controlling the rate of reaction

The pH is an important factor to consider when performing reactions in aqueous media. The protonation or deprotonation of the reactants can have a crucial effect in the reaction rate by driving the reaction forward or inhibiting the formation of products. In this chapter, the effect of the pH in the reaction rate of different chemical transformations and the techniques used to monitor the reaction kinetics are revised.

2.3.1. Reaction kinetics followed by ^{1}H-NMR spectroscopy

^{1}H-NMR spectroscopy is a very useful technique to follow the progression of reactions using small molecule models. The reagents are dissolved in deuterated solvents and the reaction is periodically monitored recording ^{1}H-NMR spectra. The reaction conversion can be determined by comparing the integrals of the products and reagents resonances. The reaction can be performed decreasing the concentration of one of the reactants in order to calculate the reaction rate constant avoiding tedious experiments and calculations. Under this condition, a simplified equation (**Figure 2.5**) can be used to obtain the pseudo-first order rate constant (k_1). Additionally, the influence of the pH in the reaction rate can be determined by dissolving the reactants in deuterated phosphate buffer and adjusting the pD (pH + 0.4) of the reaction mixture.

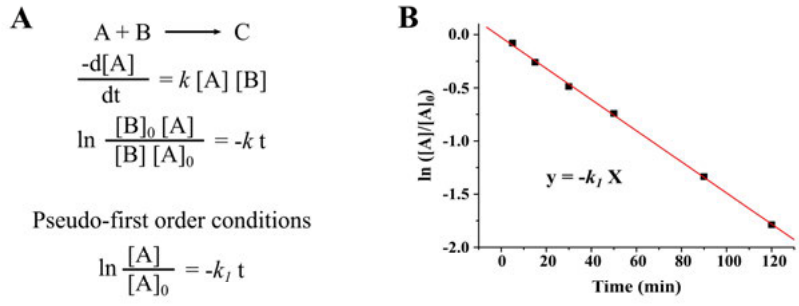


Figure 2.5. a) Pseudo-first order equations. b) Representative linear fit for the calculation of the pseudo-first order reaction rate constant (k_1).

2.3.1.1. Thiazolidine formation

It is generally understood that the formation of thiazolidine rings suffers from limitations such as slow reaction kinetics, the need for acidic pH, and the hydrolytic stability of the product. Nevertheless, we could not find any convincing reports on the conditions needed for the reaction. We decided to study carefully this reaction using L-cysteine and propionaldehyde in deuterated phosphate buffer at pD 5.0 and 7.4. The ^{1}H-NMR analysis of the reaction mixture revealed a fast and complete formation of the thiazolidine product in less than 5 minutes at both pDs (**Figure 2.6a**) as the resonances from L-cysteine completely disappeared. This experiment proved that the formation of thiazolidines is a fast and efficient reaction at acidic and physiological conditions.

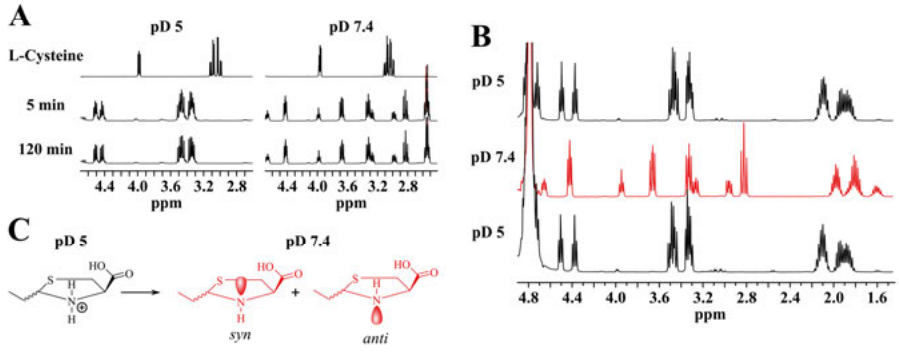


Figure 2.6. a) Representative ^{1}H-NMR spectra showing the fast reaction between L-cysteine and propionaldehyde via thiazolidine formation at pD 5 and pD 7.4. b) Schematic representation of the protonation/deprotonation of thiazolidines. c) Representative ^{1}H-NMR spectra showing the interconversion between protonated and deprotonated thiazolidine species.

The ^{1}H-NMR spectra of the product showed a different resonance splitting pattern under acidic and neutral conditions. Thus, we performed a pD titration experiment to prove that the different signals observed are due to the

protonation/deprotonation of the thiazolidine nitrogen (**Figure 2.6b**). This experiment revealed the reversible conversion between the two species upon pD adjustment (**Figure 2.6c**). Moreover, the different intensities of the resonances observed at pD 7.4, can be explained by the interaction between the syn-NH protons and the carboxylic acid group, which make the deprotonation of the anti-NH more favorable.

After stabilizing the fast formation of thiazolidines at acidic and neutral conditions, we studied the stability of the heterocycle at pD 5 and 7.4 periodically recording ^{1}H-NMR spectra of the purified product. We could not see any sign of degradation even after 7 days, which indicated the high stability of the thiazolidine product. The efficiency of this condensation reaction could also be extended to other aliphatic aldehyde substrates, such as butyraldehyde and trimethylacetaldehyde, showing identical results as propionaldehyde substrate. As expected the use of less reactive aromatic aldehydes decreased the rate of reaction allowing us to calculate the rate constants (benzaldehyde: $k_1 = 0.0146$ min^{-1}; 4-hydroxybenzaldehyde: $k_1 = 0.0034$ min^{-1}).

Encouraged by the obtained NMR results, we decided to explore the reaction for bioconjugation application using an elastin mimetic peptide (CVGVAPG) having an N-terminal cysteine as a model substrate (**Figure 2.7a**). For this purpose, propionaldehyde was mixed with the peptide dissolved in phosphate buffer saline (PBS, pH 7.4) in a 1:1 ratio and the reaction was monitored using high-performance liquid chromatography (HPLC). The peptide ligation reaction exhibits a fast reaction rate, similar to what was observed with the NMR experiments, as the starting material (6.3 minutes retention time) was completely converted to the thiazolidine conjugate (7.8 minutes retention time) in less than 4 hours (**Figure 2.7b**). The rate of the reaction could be increased by increasing the aldehyde/peptide molar ratio. In this way, 75% conversion was observed only after 1 minute of reaction with 2.5 equivalents of propionaldehyde.

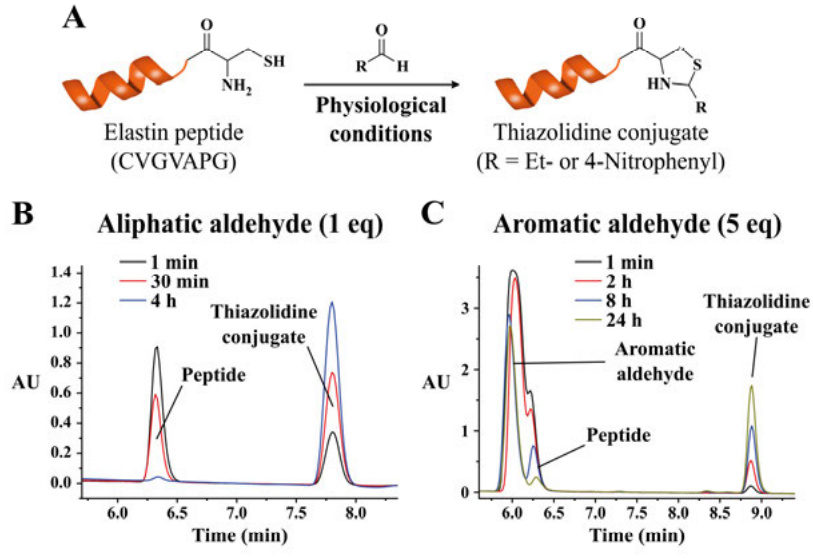


Figure 2.7. a) Schematic representation of the formation of thiazolidine bonds between aldehydes and an N-terminal cysteine peptide. HPLC chromatogram showing the progress of the conjugation reaction with (b) propionaldehyde and (c) 4-nitrobenzaldehyde at physiological conditions.

The reaction rate was significantly reduced with less reactive 4-nitrobenzaldehyde (**Figure 2.7c**). The conjugation reaction showed 87% conversion after 24 hours with 5 equivalents of aldehyde and 77% conversion after 2 hours with 25 equivalents of aldehyde. This study changed the misconceptions regarding the formation of thiazolidines and suggested that this reaction possess many advantages for the chemical modification of peptides and proteins to obtain stable products.

2.3.1.2. Disulfide formation

The key step of the thiol oxidation to form disulfide bonds is the deprotonation of the thiol group. The incorporation of electron-withdrawing groups at the β-position of the thiol could influence the deprotonation step together with the reaction rate. To investigate the effect of an electron-withdrawing substituent, we studied the reaction kinetics of the disulfide formation by ^{1}H-NMR analysis (**Figure 2.8**). The reaction was performed at physiological (pD 7.4) and basic (pD 9.0) conditions using different thiol substrates (L-cysteine (**15**), N-acetyl-L-cysteine (**10**) and 3-mercaptopropionic acid (**16**)) and constant concentration of molecular oxygen.

We observed a faster reaction rate at basic conditions for all the substrates by monitoring the reaction mixture. The reaction rate for L-cysteine at pD 9 (k_1 = 15.1 x 10^{-4} min^{-1}) was 3-folds higher than at pD 7.4 (k_1 = 15.1 x 10^{-4} min^{-1}). This difference in the reaction rate is in agreement with the hypothe-

sis that the rate of the formation of disulfides is directed by the thiolate anion concentration.

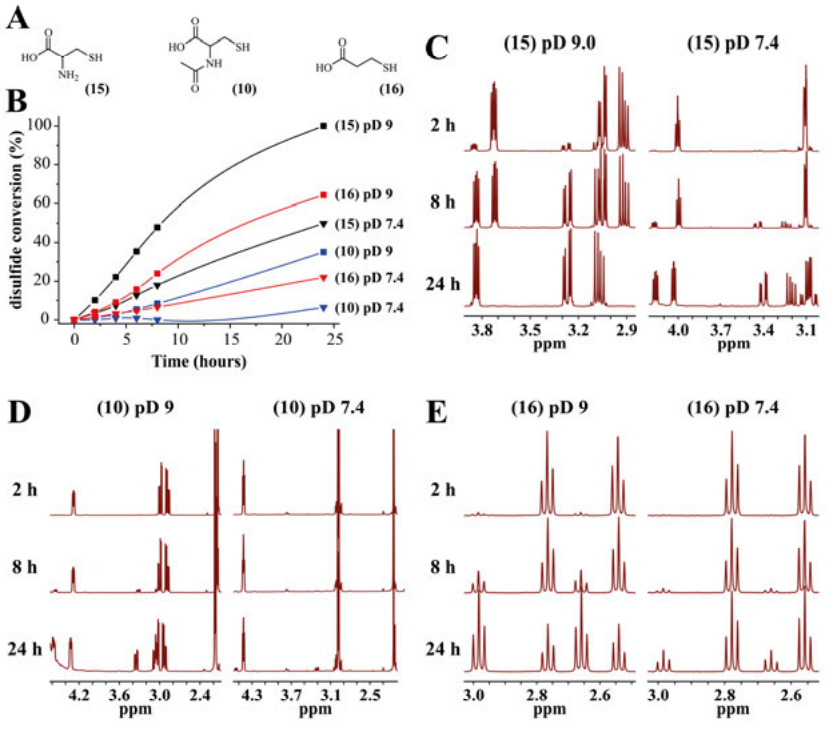


Figure 2.8. a) Chemical structure of L-cysteine (**15**), N-acetyl-L-cysteine (**10**), and 3-mercaptopropionic acid (**16**). b) Disulfide formation of the above molecules followed by ^{1}H-NMR at pD 9.0 and pD 7.4. ^{1}H-NMR spectra showing the disulfide formation at pD 9.0 and 7.4 of (c) L-cysteine (**15**), (d) N-acetyl-L-cysteine (**10**), and (e) 3-mercaptopropionic acid (**16**).

The electron-withdrawing groups at the β-position of L-cysteine (protonated amine) increased the reaction rate by ≈ 2-folds at pD 9.0 and ≈ 2.8-folds at pD 7.4 as compared to the 3-mercaptopropionic acid substrate (k_1 = 7.68 x 10^{-4} min^{-1} at pD 9.0; and k_1 = 1.80 x 10^{-4} min^{-1} at pD 7.4). The N-acetylation of cysteine prevents the protonation of the nitrogen atom reducing the reaction rate by ≈ 5-folds at pD 9.0 and ≈ 10-folds at pD 7.4 as compared to the L-cysteine substrate (k_1 = 3.30 x 10^{-4} min^{-1} at pD 9.0, and k_1 = 0.51 x 10^{-4} min^{-1} at pD 7.4). This significant drop in reaction rate could be attributed to the drop in the concentration of thiolate ions and to the steric hindrance of the N-acetyl group.

The observed reaction rates unequivocally suggest that an electron-withdrawing substituent at the β-position of the thiol group facilitates the oxidation of thiol to disulfides by increasing the acidity of the thiol group.

Other than the modulation of thiol pK_a as a result of β-substituent, steric factors could also affect the reaction rate with some substrates.

2.3.2. Rheological evaluation of the gelation kinetics of hydrogels

The gelation time is a very important parameter of hydrogels in biomedical applications. Hydrogels that present slow gelation kinetics can diffuse out from the delivery site, whereas fast gelation times may prevent the injectability of the hydrogels and the ability of the material to adapt to the shape of the defect. Rheology is the appropriate method for monitoring the phase transition between the liquid and the solid state (gelation time) since it is a sensitive and quick method that requires small amounts of sample. In order to determine the gelation time, a small amount of the liquid solution of the precursors is placed between two parallel disks and a small torsional oscillation generating shear flow in the sample. The elastic behavior (Storage modulus, *G'*) and the viscosity (Loss modulus, *G''*) of the sample are recorded over time (Time sweep) (**Figure 2.9**).

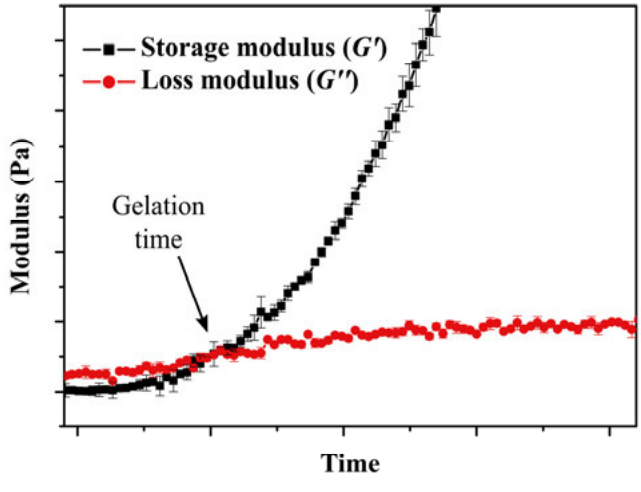


Figure 2.9. Typical rheological evaluation of the gelation kinetics of hydrogels. The cross-over point between the storage modulus (*G'*) and the loss modulus (*G''*) represents the gelation time.

At the beginning of the measurement, the sample is in the pure liquid state and is characterized by a lower *G'* than *G''*. As the hydrogel forms both G' and G'' increase and at the gelation point G' becomes equal to G''. After the gelation point G' keeps increasing with increasing cross-linking density until it reaches the maximum value (curing of the hydrogel).

2.3.2.1. Gelation kinetics of disulfide cross-linked HA hydrogels

After observing the influence of electron-withdrawing substituents at the β-position of the thiol group in driving the disulfide formation using the small molecule model, we developed disulfide cross-linked hydrogels at physiological pH by tuning the pK_a of the thiol group. Since the concentration of thiolate ions has been found to be the key intermediate in the formation of disulfides, we first determined the pK_a of the tree thiolated HA derivatives (HA-Cys, HA-ActCys, and HA-SH) using a previously reported spectrophotometric method[28]. The absorbance at 242 nm increased with the pH indicating the formation of thiolate ions (**Figure 2.10a**). The absorbance of the HA-Cys solution abruptly increased within a shorter range of pH than the other two HA derivatives. This indicates that the chemical microenvironment near the thiol group strongly influences the formation of thiolate ions. The graphical representation of $-\log[(A_{max} - A_i)A_i]$ versus pH (**Figure 2.10b**) displays the pK_a where the linear fit crosses the abscissa. The electron-withdrawing substituent reduced the pK_a of the thiol group of HA-Cys (pK_a = 7.0) and HA-ActCys (pK_a = 7.4) as compared to conventional HA-SH without any substituent (pK_a = 8.1). This experiment further confirmed that the concentration of thiolate ions could be increased with the presence of electron-withdrawing groups.

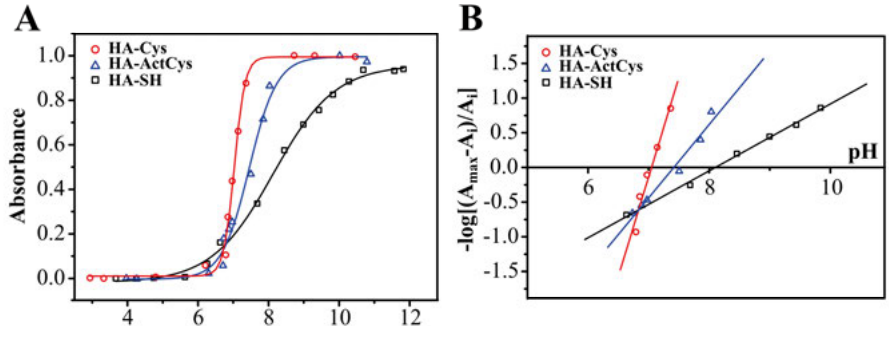


Figure 2.10. a) The absorbance of thiolated HA derivatives (HA-Cys, HA-ActCys, and HA-SH) as a function of pH. b) Logarithmic representation of the normalized absorbance ($-\log[(A_{max}-A_i)/A_i]$) as a function of the pH. The pK_a values correspond to the interception with the abscissa.

The observed decrease of pK_a anticipated that HA-Cys could form disulfide cross-linked hydrogels at physiological conditions without any addition of oxidants. In earlier works, heavily thiol modification polymers were used due to the low reactivity of the thiol group,[28, 107] but the high degree of functionalization is expected to avoid the interaction between HA and the encapsulated cells. We aimed to compare the hydrogel gelation kinetics of

the different thiolated HA derivatives carrying a low degree of functionalization (≈ 10 %) (**Table 2.2**).

Table 2.2. The gelation time of the thiolated HA derivatives (HA-Cys, HA-ActCys, and HA-SH).

DS	Sample	pH 7.4	pH 9.0
11 %	HA-Cys	3.5 min	3.5 min
11 %	HA-ActCys	10 h	6.5 h
9 %	HA-SH	-	-
19 %	HA-SH	-	5 h

We study the gelation kinetics of HA-SH carrying 9 % of thiol groups and we could not observe any hydrogel formation within 24 hours at physiological (pH 7.4) or basic conditions (pH 9.0). HA-SH carrying 19 % of thiol groups formed hydrogels at pH 9 within 5 hours, however, no hydrogel formation was observed at pH 7.4. The absence of gelation at pH 7.4 was presumably due to the low concentration of thiolate ions (17 % according to the Henderson-Hasselbalch equation: **Equation 2.3**) in the reaction mixture. By increasing the pH to 9.0, the concentration of thiolate ions increased (89 %) which resulted in hydrogel formation with HA-SH derivative having 19 % modification.

$$\mathrm{pH} = \mathrm{p}K_\mathrm{a} + \log\left(\frac{[-S^-]}{[-SH]}\right) \tag{2.3}$$

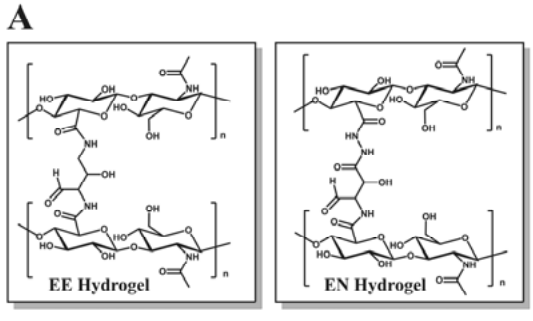
HA-Cys formed hydrogels at physiological pH within 3.5 minutes because of the higher abundance of reactive thiolate ions. Surprisingly, the gelation time was not affected by increasing the reaction pH to 9.0, presumably due to the pK_a of HA-Cys is lower than both experimental conditions. This allowed a high concentration of thiolate ions (72 % of R-S$^-$ at pH 7.4 and 99 % of R-S- at pH 9.0) at both pH's, resulting in a high reaction rate.

The most pronounced pH dependence case could be observed for HA-ActCys with pK_a of 7.4 (50 % of R-S$^-$ at pH 7.4 and 98 % of R-S$^-$ at pH 9.0). The hydrogel formation took over 10 hours at pH 7.4 but the gelation time was reduced to 6.5 hours at pH 9.0. In spite of the close pK_a values between HA-Cys and HA-ActCys, the observed large differences in their gelation time could be attributed to the steric hindrance introduced by the acetyl group. Nevertheless, the short gelation time of HA-Cys demonstrated the significance of introducing electron-withdrawing groups that decreased the pKa of the thiol group, promoting disulfide formation at physiological pH.

2.3.2.2. Gelation kinetics of aldol cross-linked HA hydrogels

In order to confirm that aldehyde-modified HA derivatives can form hydrogels through base catalyzed aldol addition, the pH of the HA precursor solu-

tion was raised and the gelation kinetics was followed by rheological evaluation. As expected, HA carrying non-enolizable aldehyde groups (HA-Nal) did not undergo gelification even at pH 11 due to the lack of acidic protons at the α-position of the aldehyde group. On the other hand, HA having enolizable aldehyde groups (HA-Eal) formed hydrogels at pH 11 (EE_{11} hydrogels) within 10.8 minutes and at pH 10 (EE_{10} hydrogels) within 4.4 hours (**Figure 2.11**). These measurements demonstrated that the reaction pH strongly affected the gelation kinetics. This is consistent with the increase in the nucleophilic enolate concentration with an increasing reaction pH.



B

Reaction pH	Acronym	Gelation time (seconds)
11	EE_{11}	649± 45
10	EE_{10}	7920± 1500
9	EE_9	---
11	NN_{11}	---
11	EN_{11}	86± 3
10	EN_{10}	399 ± 13
9	EN_9	4620± 300

Figure 2.11. a) Schematic representation of hyaluronic acid (HA) aldol hydrogels. b) Gelation kinetics of aldol cross-linked HA hydrogels at different pHs.

We further evaluated the effect of including non-enolizable aldehydes in the reaction mixture. They act as the electrophile acceptors in the cross-aldol addition reaction. A 1:1 mixture of HA-Eal:HA-Nal showed higher gelation rate as compared with HA-Eal alone. This mixture of HA precursors formed hydrogels at pH 11 (EN_{11} hydrogels) within 1.4 minutes, at pH 10 (EN_{10} hydrogels) within 6.7 minutes, and at pH 9 (EN_9 hydrogels) within 1.3 hours. The above results demonstrated that the gelation kinetics of aldol cross-linked hydrogels could be tuned by varying the reaction pH and the composition of the HA precursor solution.

2.4. Rheological properties of HA hydrogels

The mechanical properties of hydrogels are determined by rheological evaluation of the fully formed hydrogels (cured hydrogels), following a standardized protocol.[108] The protocol has two steps: i) determination of the linear viscoelastic region with respect to strain (strain sweep) where the hydrogels can resist the applied deformation (**Figure 2.12a**), and ii) determination of the *G'* (stiffness of the hydrogels) inside the linear viscoelastic region (frequency sweep) (**Figure 2.12b**).

The linear viscoelastic region is determined by a strain sweep and is defined as the range of strain in which the hydrogel shows a linear behavior (constant values as the strain is varied) of *G'*. A strain inside the viscoelastic region is chosen for the subsequent frequency sweep. *G'* is determined as the value of the low-frequency plateau of the frequency sweep conducted using the appropriated strain (determined with the strain sweep).

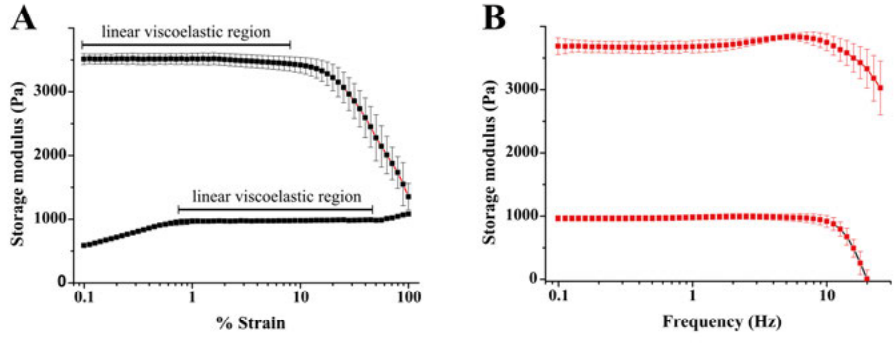


Figure 2.12. Rheological characterization of two hydrogel examples. a) Strain sweep and b) frequency sweep.

Aiming to understand better the relationship between the rheological data and the molecular structure of the hydrogel network, the mechanical data can be used to estimate the average mesh size (ξ) and the average molecular weight between cross-links (Mc), using the rubber elasticity theory **(Equation 2.4 and Equation 2.5**).[109, 110]

$$\xi = \left(\frac{G' N_A}{RT}\right)^{-1/3} \tag{2.4}$$

$$Mc = \frac{cRT}{G'} \tag{2.5}$$

Where N_A is the Avogadro constant (6.022 x 10^{23} mol^{-1}), R is the molar gas constant (8.314 J $mol^{-1}K^{-1}$), T is temperature (298 K), c is the polymer concentration (kg m^{-3}), and G' is the hydrogel storage modulus (Pa).

2.4.1. Rheological properties of aldol cross-linked HA hydrogels

The mechanical properties of fully formed aldol cross-linked HA hydrogels prepared with different composition (EE hydrogels and EN hydrogels) and at different pHs were evaluated by rheology. First, the linear viscoelastic region in which the hydrogels could resist the applied deformation was determined performing a strain sweep. EE hydrogels exhibit a high elastic behav-

ior with a linear viscoelastic region from 8 % up to 100 % strain. The value of 10 % strain was chosen for the subsequent frequency sweep. The viscoelastic region of EN hydrogels was smaller (0.1 % to 10 % strain) indicating the higher rigidity of the material. The value of 1 % strain was chosen for the subsequent frequency sweep.

The mechanical properties of the hydrogels (*G'*) were determined using a frequency sweep (**Table 2.3**). The rheological evaluation of the aldol cross-linked hydrogels showed that *G'* increased with the reaction pH indicating a higher efficiency of the aldol addition reaction at higher alkaline conditions. EN hydrogels showed superior mechanical properties as compared with EE hydrogels indicating a higher efficiency of the cross-aldol reaction.

Table 2.3. Rheological data of aldol cross-linked HA hydrogels. Storage modulus (*G'*), average mesh size (ξ), average molecular weight between cross-links (*Mc*) and cross-linking efficiency (%).

Reaction pH	Acronym	*G'* (Pa)	ξ (nm)	*Mc* (kg mol^{-1})	%
11	EE_{11}	981.7	16.1	50.5	6.7
10	EE_{10}	504.2	20.1	98.3	3.5
11	EN_{11}	5217	9.2	9.5	35.8
10	EN_{10}	3679	10.4	13.5	25.2
9	EN_9	1982	12.8	25.0	13.6

The storage modulus (G') was used to estimate the average mesh size (ξ) and the average molecular weight between cross-links (*Mc*). The calculated *Mc* was used to compare the efficiency of the aldehyde functionalities of the different systems to form cross-links (cross-linking efficiency). The minimal theoretical average molecular weight between cross-links (3.4 kg mol^{-1}) was calculated considering the molecular weight of the HA disaccharide unit (408 g mol^{-1}) and the degree of aldehyde modification being 12 %. The calculated minimal theoretical average molecular weight between cross-links was compared with the observed *Mc*.[109] Our calculations indicated that EE hydrogels had high *Mc*, which could be explained by the favored formation of intramolecular loops rather than cross-links to create defects in the hydrogel network and decreased the cross-linking efficiency (only ≈ 7 % of the aldehyde groups contributes to the formation of effective cross-links at pH 11). The formation of cross-links was favored by the inclusion of HA-Nal in the hydrogel formulation that acts as the electrophile reducing HA-Eal intramolecular reaction by increasing intermolecular cross-links (≈ 36 % of the aldehyde groups formed effective cross-links at pH 11). Additionally, the cross-linking efficiency also increased with increasing the reaction pH for both types of hydrogels.

2.4.2. Rheological properties of disulfide cross-linked HA hydrogels

To evaluate the effect of electron-withdrawing groups in the mechanical properties of the hydrogels, we compared HA-Cys and HA-ActCys hydrogels with HA-SH hydrogels rheological properties (**Table 2.4**). All the studied disulfide cross-linked hydrogels exhibit similar linear viscoelastic region. Thus 1 % strain was chosen in all the cases to perform the subsequent frequency sweep.

Table 2.4. Rheological data of disulfide cross-linked HA hydrogels.

DS	Sample	pH 7.4 *G'* (Pa)	pH 9.0 *G'* (Pa)
11 %	HA-Cys	3312.3	2261.2
11 %	HA-ActCys	3523.5	1734.4
19 %	HA-SH	-	1910.6

In spite of the different gelation kinetics, HA-Cys and HA-ActCys hydrogels showed similar *G'* (≈ 3300-3500 Pa) at pH 7.4 demonstrating that upon completion of the cross-linking reaction the hydrogels reached a comparable degree of cross-linking. The *G'* was considerably reduced for hydrogels prepared at pH 9.0, suggesting that at higher concentration of thiolate ions the formation of intramolecular loops is favored. HA-SH hydrogels containing 19 % modification showed similar *G'* at pH 9.0 that hydrogels obtained from HA-Cys and HA-ActCys with approximately the half degree of functionalization (11 %), demonstrating that the introduction of electron-withdrawing groups not only improved the reaction kinetics but also increased the efficiency of the cross-linking reaction.

2.5. Hydrolytic stability and degradation profile of HA hydrogels

The hydrolytic stability of hydrogels is a determining factor for a successful outcome of the scaffold in biomedical applications. Due to the high content in water of most tissues, hydrogels cross-linked with hydrolytically unstable bonds undergo slow hydrolysis that results in undesirable swelling and eventual dissolution of the polymeric network when they are applied *in-vivo*.

Ideally, hydrogel degradation should be induced by endogenous enzymes expressed by the surrounding cells and tissues. Control the degradation profile of hydrogels represents a major challenge for biomedical applications. For instance, hydrogels applied in controlled protein delivery must degrade to allow the release of the encapsulated biomolecules and the degradation kinetics must match the required release profile. In tissue engineering, hy-

drogels degrade to allow their replacement by the newly formed tissue, but they must preserve their integrity during the tissue formation process. HA hydrogels are degraded by the endogenous enzyme hyaluronidase (HYAL), which catalyzes the hydrolysis of the glycosidic bonds of the polymeric backbone, resulting in hydrogel surface erosion by the liberation of small HA fragments from the polymeric network to the aqueous phase.

In-vitro hydrogel stability and degradation can be studied by submerging the fully formed hydrogels in a degradation media and periodically monitoring the change of hydrogel mass in the swollen state (gravimetric method). Hydrolytically unstable hydrogels experience uncontrolled swelling of the polymeric network when they are submerged in a PBS solution (pH 7.4) because the hydrolysis of the cross-linkages allows the stretching of the network due to an increased influx of water. *In-vitro* enzymatic degradation of HA hydrogels can be controlled by adjusting the HYAL concentration of the degradation media.

2.5.1. Hydrolytic stability and degradation profile of aldol cross-linked HA hydrogels

The hydrolytic stability of the aldol cross-linked hydrogels was evaluated by incubating the hydrogels in a PBS solution (pH 7.4). All studied hydrogels demonstrated excellent stability as they maintained their integrity during the 30 days of experimental time (**Figure 2.13a**). The high stability of the hydrogels was expected since the network was formed by fully hydrolytically stable C-C single bonds. The initial swelling could be related with the hydrogel cross-linking density. Accordingly, hydrogels with lower modulus (EE hydrogels) allow higher stretching of the polymeric network which results in a larger initial swelling. After the initial swelling, all hydrogels showed nearly stable mass indicating that the C-C single bonds obtained during the aldol addition reaction were fully stable in aqueous media. It is important to notice that the hydrogel with the highest modulus (EN_{11} hydrogels) showed a negligible increase in mass during the swelling study.

The *in-vitro* enzymatic degradation of the novel aldol cross-linked hydrogels was examined using a gravimetric method and high concentration of HYAL (0.3mg/mL; 210 U/mL), approximately 35 times the enzyme concentration in human plasma.[111, 112] The mass of the fully formed hydrogels before starting the degradation study was defined as the reference (100 %) and the percentage of remaining hydrogels was represented versus the time of incubation in the HYAL solution (**Figure 2.13b**). The degradation kinetics was proportional to the cross-linking density. Hence, the softest hydrogels (EE_{10} hydrogels) were completely degraded in 1 day, while ≈ 46 % of the stiffer hydrogels (EN_{11} hydrogels) remained after 12 days of degradation. The obtained results suggested that aldol cross-linked HA hydrogels, espe-

cially EN_{11} hydrogels, are excellent candidates for applications that require hydrogels with long-term stability and integrity in aqueous media but degrade upon exposure to the endogenous enzyme HYAL.

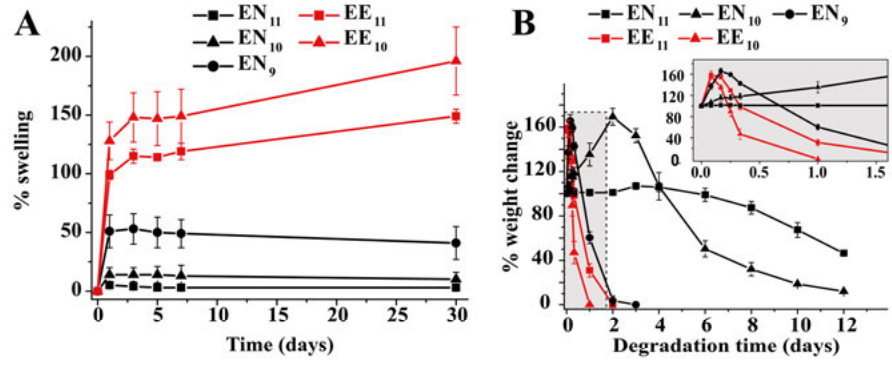


Figure 2.13. a) Gravimetric swelling study of aldol cross-linked HA hydrogels at physiological conditions (pH 7.4). b) Enzymatic degradation of aldol cross-linked HA hydrogels studied using hyaluronidase (0.3 mg/mL) solution.

2.5.2. Hydrolytic stability and degradation profile of disulfide cross-linked HA hydrogels

We evaluated the hydrolytic stability of the disulfide cross-linked hydrogels prepared at physiological conditions (pH 7.4) by quantifying the change in hydrogel mass after incubation in a PBS solution (**Figure 2.14a**). Both HA-Cys and HA-ActCys hydrogels showed high stability in aqueous media as they did not exhibit excessive swelling. The most interesting finding of the swelling study was that HA-Cys hydrogels showed moderate shrinking. This atypical shrinking behavior was attributed to the presence of free thiolate ions that underwent spontaneous oxidation to form new cross-linkages. HA-ActCys hydrogels also showed shrinking behavior after an initial moderate swelling, indicating the formation of new cross-linkages after incubation in PBS. It should be noted that even though the hydrogels showed moderate shrinking, they maintained their integrity even after 30 days of incubation in the swelling media.

Unlike, other chemistries, disulfide formation has an added advantage for the development of materials for cell encapsulation and drug delivery because of the ability of cells to degrade disulfide cross-linked hydrogels enzymatically or secreting natural reductants such as glutathione.[38, 102, 113] In order to study the enzymatic degradation, we incubated the hydrogels in an HYAL (0.3 mg/mL) solution (**Figure 2.14b**). HA-Cys and HA-ActCys hydrogels prepared at pH 7.4 showed similar degradation profiles, which is expected for hydrogels with similar cross-linking density. The reductive

degradation profile was studied by incubation of the hydrogels in a DTT (a well-known reductant which cleaves disulfide bonds) solution (1 mM). Interestingly, HA-Cys and HA-ActCys hydrogels showed different reductive degradation profiles (**Figure 2.14c**), because of the sterically hindered acetyl group retarded the DTT-mediated cleavage of disulfides, slowing down the degradation rate of HA-ActCys hydrogels.

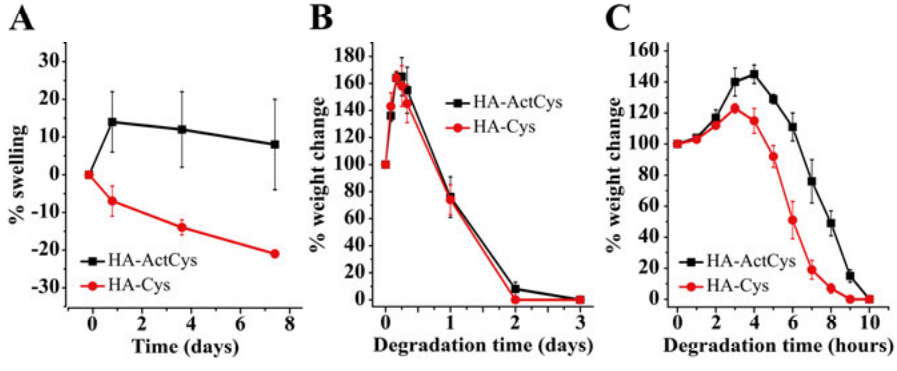


Figure 2.14. a) Gravimetric swelling study of disulfide cross-linked HA hydrogels at physiological conditions (pH 7.4). b) Enzymatic degradation of disulfide cross-linked HA hydrogels studied using hyaluronidase (0.3 mg/mL) solution. c) Reductive degradation of disulfide cross-linked HA hydrogels studied using DTT (1 mM) solution.

2.5.3. Hydrogel degradation studies using MRI

Traditionally, hydrogel degradation *in-vivo* requires a large number of laboratory animals because several animals have to be sacrificed at each time point for the visual examination of the hydrogels. Incorporation of an imaging agent into hydrogels allows visualization of the hydrogels by noninvasive imaging techniques. Such a strategy would allow longitudinal *in-vivo* degradation studies using the minimal amount of animals.[77, 78, 80]

Among the potentially applicable imaging techniques, MRI is especially effective because it is a free-radiation technique with high tissue penetration and spatial resolution. The detection of hydrogels by MRI is particularly challenging because of the contrast in an MR image is originated from the differences in the physical and chemical microenvironment of water molecules which is very similar between hydrogels and tissues. In order to detect hydrogels *in-vivo* using MRI, contrast agents can be incorporated in the polymeric network to enhance the image contrast.[83, 87]

We developed MRI-traceable HA hydrogels labeled with a gadolinium-based contrast agent and studied their degradation profile following the change of MR image intensity over time. We first synthesized a diethylenetriaminepentaacetate gadolinium complex modified with two dithiopyridyl

groups ((SSpy)$_2$DTPA-Gd^{3+}) which can be used to label any thiol containing biomolecule by the disulfide exchange reaction. Gadolinium labeled HA derivative carrying hydrazide groups (hy-HA-SS-Gd^{3+}) was prepared using the thiol-hydrazide dually-modified HA derivative (hy-HA-SH) which reacted with (SSpy)$_2$DTPA-Gd^{3+} under mild alkaline conditions (pH 8) to form stable disulfide bonds (**Figure 2.15**). Gd-labeled hydrogels were prepared by simply mixing hy-HA-SS-Gd^{3+} with the aldehyde-modified HA derivative (HA-al) to form a hydrazone cross-linked polymeric network. This *in-situ* procedure could be applied directly *in-vivo* because it did not require the use of any low molecular-weight cross-linker and it did not produce any side product. Non-labeled hydrogels were prepared by mixing non-labeled hydrazide modified HA derivative (HA-hy) with the HA-al counterpart. Non-labeled hydrogels were used as a negative control in the MRI experiments. Hydrogels with entrapped (SSPy)$_2$DTPA-Gd^{3+} were also prepared by dissolving (SSPy)$_2$DTPA-Gd^{3+} in the HA-hy solution and subsequently mixing the resulting solution with the HA-al counterpart.

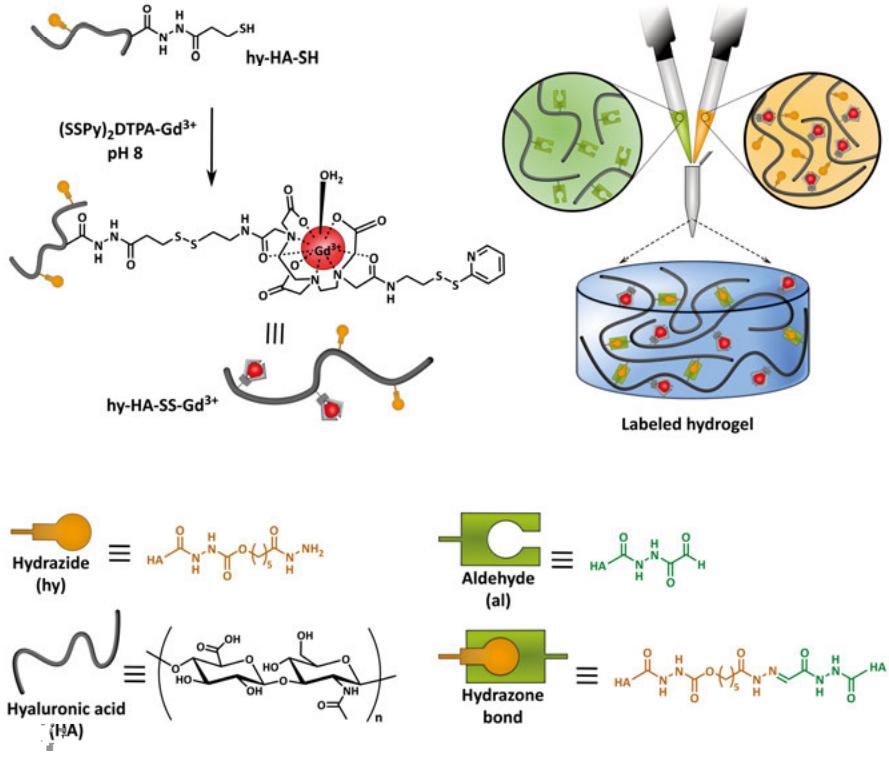


Figure 2.15. Schematic representation of the preparation of MRI visible hydrogels. Gadolinium labeled HA derivative was prepared through disulfide exchange reaction between the dithiopyridyl groups of the gadolinium complex and the thiol groups of hy-HA-SH.

The positive contrast in the T_1-weighted MR images of Gd-labeled hydrogels and hydrogels with entrapped $(SSPy)_2DTP\text{-}Gd^{3+}$ increased with respect to non-labeled hydrogels. The increase in contrast is the result of the presence of the Gd-complex which is a typical T_1-weighted MR contrast agent (**Figure 2.16a**). The difference in the contrast of the MR image of hydrogels with entrapped $(SSPy)_2DTP\text{-}Gd^{3+}$ disappeared after washing of the hydrogels in PBS for 24 hours because the Gd-complex diffused out from the hydrogel. The contrast in the MR image of the Gd-labeled hydrogels did not change after washing which confirmed the covalent immobilization of the Gd-complex to the polymeric network. No difference in contrast was observed between the T_2-weighted MR images of non-labeled hydrogels and hydrogels with entrapped $(SSPy)_2DTP\text{-}Gd^{3+}$ (**Figure 2.16b**), as expected for Gd-complexes which give no T_2-weighted contrast. Unexpectedly, the negative contrast of the T_2-weighted images of Gd-labeled hydrogels increased. This atypical behavior of the linked Gd-complex can be explained by the limited rotational mobility of the Gd atoms after covalent immobilization to the HA backbone.

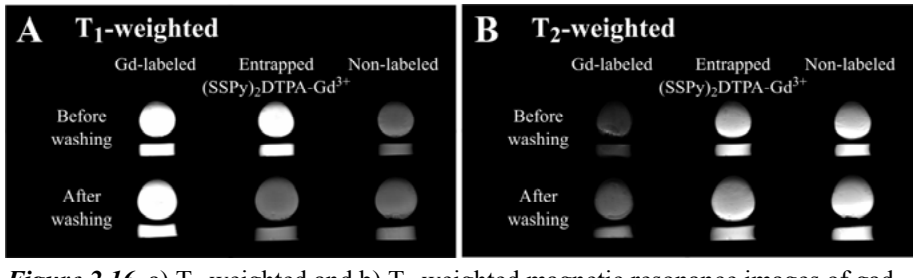


Figure 2.16. a) T_1-weighted and b) T_2-weighted magnetic resonance images of gadolinium-labeled hydrogels (first column), hydrogels with entrapped $(SSPy)_2DTPA\text{-}Gd^{3+}$ (second column), and non-labeled hydrogels (last column); before (first row) and after (second row) washing.

The *in-vitro* enzymatic degradation of Gd-labeled hydrogels was followed using a very high concentration of HYAL (5mg/mL; 3500 U/mL), which resulted in complete degradation of the hydrogels after 9 hours. The fast enzymatic degradation ensured that the hydrogel swelling caused by hydrolysis of the hydrazone network could be disregarded. The mass and the MR image intensity of the hydrogels after incubation in PBS for 24 hours was defined as 100 %. During the initial swelling step, hydrogels increased in weight (**Figure 2.17a**) while the intensity of their MR images remained constant (**Figure 2.17b and Figure 2.17c**). After the initial swelling in PBS, hydrogels were exposed to HYAL resulting in degradation of the hydrogels by surface erosion (**Figure 2.17e**). The enzymatic degradation was followed gravimetrically (following the decrease in hydrogel weight) and quantifying the change in MR image intensity. The degradation profiles studied by both techniques showed a strong correlation (**Figure 2.17d**). The obtained results

confirmed that the degradation of Gd-labeled hydrogels could be monitored using non-invasive MRI technique.

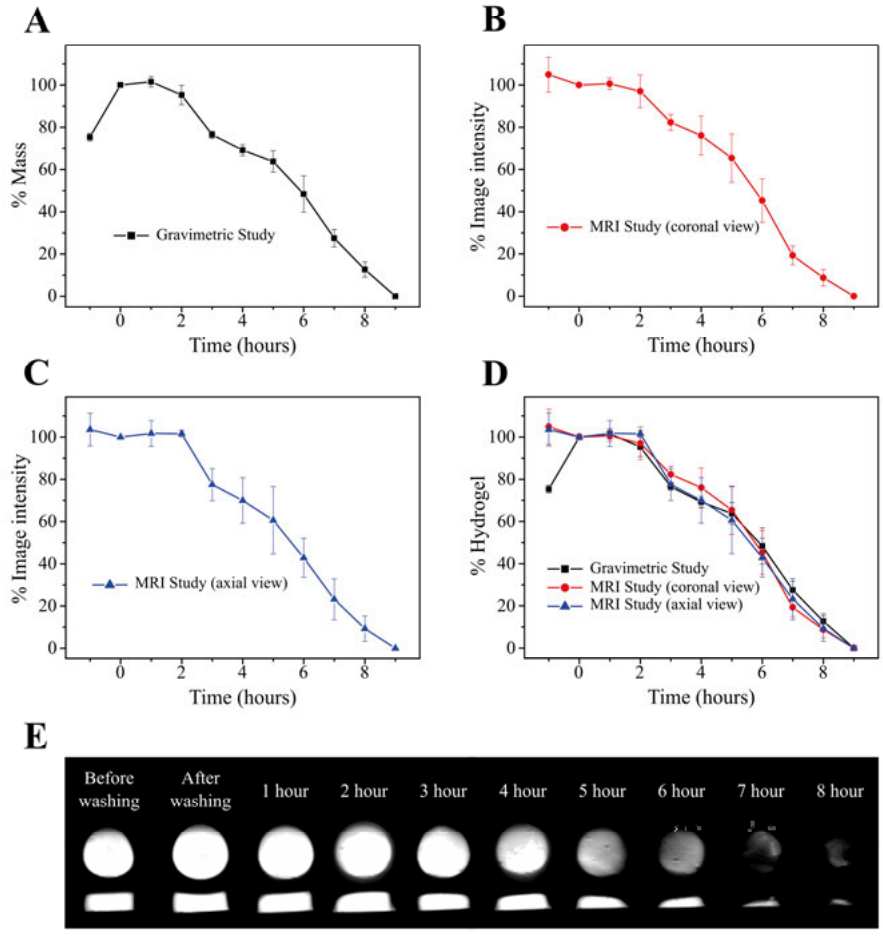


Figure 2.17. Enzymatic degradation study of Gd-labeled HA hydrogels. Hydrogel degradation was followed (a) gravimetrically or by measuring the change in intensity of the MR images of the hydrogels ((b) coronal view and (c) axial view). d) Correlation of degradation profiles followed by both techniques. e) T_1-weighted images of Gd-labeled hydrogels during the degradation study.

2.6. Reactivity of aldol hydrogels cross-linking points

The polymeric network of HA aldol hydrogels is constituted by β-hydroxy aldehyde cross-linking points. Theoretically, the preparation of hydrogels through aldol chemistry consumes only half of the initial aldehyde groups. The high amount of free aldehyde groups inside the polymeric network of

aldol hydrogels provides these hydrogels with interesting properties that are revised in this chapter.

2.6.1. Post-functionalization of aldol cross-linked HA hydrogels

The β-hydroxy aldehyde cross-linking points could be used for the post-functionalization of the hydrogels with molecules that contain reactive nucleophilic groups. Alexa Fluor™ 488, having a terminal hydroxylamine group, was used as a model molecule for the post-functionalization experiments because it allows easy visualization under 254 nm lamp. EN_{10} hydrogels were immersed in the fluorescent tag solution for two hours. During this time the substrate diffused inside the hydrogels and reacted with the free aldehyde groups by the formation of oxime bonds (**Figure 2.18**).

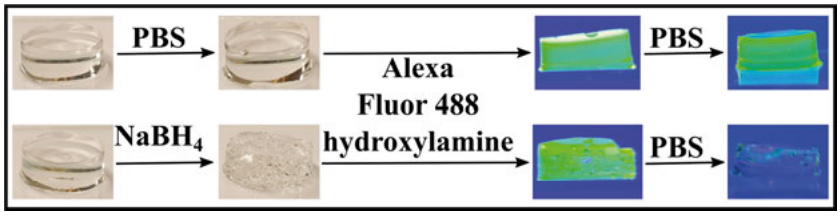


Figure 2.18. A fluorescent tag containing a hydroxylamine group was covalently attached to the hydrogel matrix (first row). The fluorescent tag could not be attached after the hydrogel matrix was reduced with $NaBH_4$ (second row).

The incorporated fluorescence did not disappear after exhaustive washing with PBS, which confirmed the covalent immobilization of the fluorescent tag. To prove the presence of free aldehyde groups in the polymeric network, we repeated the labeling experiment after reducing the aldehyde groups with sodium borohydride ($NaBH_4$). The reduction of the aldehyde groups was clearly visible by the production of hydrogen gas bubbles inside the hydrogel. The fluorescent tag could be completely washout from the reduced hydrogels by washing in PBS.

2.6.2. Hydrogel-tissue integration

The adhesion of biomaterials or implants with the surrounding tissue is crucial for tissue regeneration or wound dressing.[114] Currently, surgical integration of hydrogels is usually carried out by applying adhesives that chemically bridge the biomaterial and tissue proteins.[46, 47, 114] Hydrogels having tissue adhesive properties without the need for additional adhesive material are highly desirable for regenerative medicine applications. The high amount of free aldehyde groups inside aldol hydrogels provided tissue adhesive properties by forming Schiff-base bonds with free amino groups of the tissue proteins. The tissue adhesive properties of EN_{10} hydrogels were tested by

placing the hydrogel between two bone-tissue surfaces (**Figure 2.19a**), and the adhesive strength was evaluated by tensile measurements (**Figure 2.19b**).

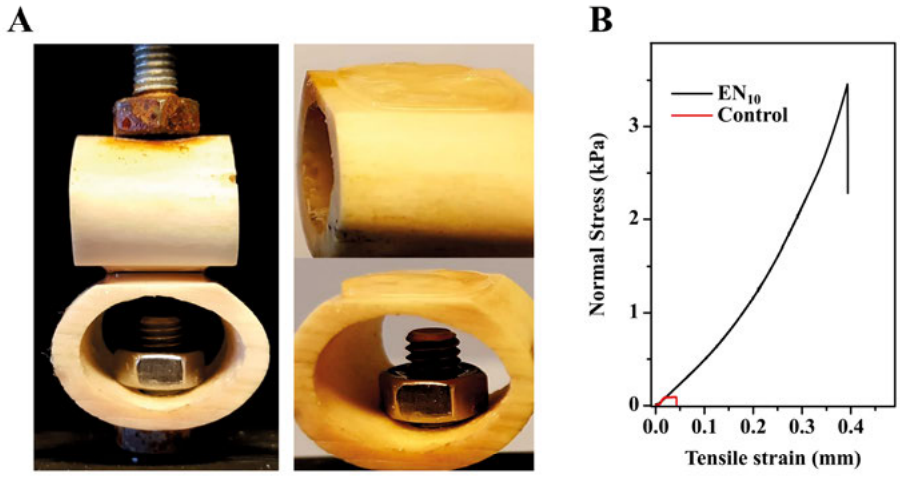


Figure 2.19. a) Set up of the bone-adhesive experiments. Hydrogel-bone constructs exposed to tensile forces failed at the bulk of the hydrogel (cohesive failure). b) Stress-strain diagram from the tensile test of aldol hydrogels (EN_{10} hydrogels) and disulfide hydrogels (Control).

Hydrogel-bone constructs exposed to tensile loading always failed at the bulk of the hydrogel (cohesive failure) proving that the bone-hydrogel interface was stronger than the hydrogel itself. The apparent modulus of aldol hydrogels (5.4 ± 0.4 kPa) was obtained from the initial linear regime (up to 6 % of deformation) of the stress-strain curves. The maximal cohesive strength of the hydrogel was 3.6 ± 0.2 kPa taking the standard deviation as an error. An HA hydrogel having disulfide cross-linkages was used as a negative control as it did not contain any free aldehyde functionalities.[77] The tensile experiments with such a material showed no interaction with the bone surface and the constructs quickly failed at the interface (adhesive failure). These results unequivocally prove that aldol hydrogel had excellent tissue adhesive properties and could potentially be used as tissue glue or as a biomaterial for regenerative medicine application.

3. Concluding remarks

Recently there is an increasing demand for biocompatible materials for a wide range of biomedical applications. For a successful output, the biomaterials need to have specific properties that are suitable for a particular application. Thus, the development of biomaterials requires a good understanding of the chemistry involved in order to finely tune the material properties.

In this work, we have investigated different covalent chemistries and analyzed their specific characteristics regarding the preparation of biomaterials. Hydrazone based HA hydrogels were used as a platform for the development of MRI visible hydrogels (**Paper I**). The use of DTPA-based gadolinium complex modified with dithiopyridyl groups enabled the preparation of labeled HA derivative carrying hydrazide groups. The Gd-labeled HA derivative and the hydrogels derived from it were visible using T_1 and T_2 weighted MR sequences. The *in-vitro* enzymatic degradation of the Gd-labeled hydrogels could be followed using MRI. The developed Gd-labeled hydrogels demonstrated great potential as trackable materials for longitudinal *in-vivo* degradation studies using noninvasive MRI techniques.

We also demonstrated that the condensation reaction between aliphatic aldehydes and 1,2-aminothiols is a fast and highly specific reaction under physiological conditions (**Paper II**). The obtained thiazolidine products showed high stability at acidic and neutral conditions. The efficiency of such condensation reaction was extended by coupling N-terminal cysteine peptides with small aldehyde molecules. These results changed the misconceptions regarding thiazolidine condensation and introduced this reaction as a catalyst-free alternative for the conjugation of sensitive biomolecules.

Moreover, we developed HA hydrogels employing aldol chemistry (**Paper III**). The gelation time could be adjusted by controlling the reaction pH. Hydrogels containing 100 % of enolizable HA-aldehyde (HA-Eal) showed poor cross-linking efficiency likely because of the formation of intramolecular loops. The cross-linking efficiency could be improved by introducing a second polymer component carrying a non-enolizable HA-aldehyde (HA-Nal) to the reaction mixture. These hydrogels showed excellent hydrolytic stability but degraded upon exposure to the endogenous enzyme hyaluronidase. The aldol hydrogels contained a high amount of free aldehyde groups in the hydrogel network. These free aldehyde groups were exploited for the post-modification of the hydrogels with a model aminooxy fluorophore

compound. We also demonstrated the potential of such material as tissue adhesive glue that formed hydrogel-bone tissue adhesion presumably by Schiff-base formation with free lysine in the tissue surface. Such biocompatible and biodegradable HA hydrogel system represents a versatile material platform for various biomedical applications.

Finally, we demonstrated that incorporation of an electron-withdrawing group at the β-position as in cysteine results in a significant increase in reaction rate for disulfide formation at physiological pH (**Paper IV**). We exploited this observation to develop a disulfide-linked HA-based hydrogel system that formed cross-links at physiological pH. Incorporation of the cysteine moiety onto HA reduced the pK_a of the thiol group from ≈ 8.3 to 7.0, which promoted thiolate anion formation at physiological pH. Acetylation of the amino residue of cysteine reduced the protonation ability of the amino group that resulted in an increase in thiol pK_a to 7.4. Such differences in pK_a of different thiols were reflected in disulfide cross-linked hydrogel formation at pH 7.4 without any addition of external oxidants. HA modified with cysteine groups formed hydrogels in just 3.5 minutes, however, HA carrying N-acetyl-cysteine groups required over 10 hours to form cross-links. Conventional thiol modified HA derivative did not form hydrogels at neutral pH even after increasing the degree of modification. The disulfide cross-linked HA hydrogels showed excellent hydrolytic stability but degrade upon exposure to hyaluronidase or reducing agents as DTT.

In summary, we investigated the characteristics of different covalent chemistries and optimized the reaction conditions to produce biomaterials with finely tune properties. The obtained results will help researchers to rationally develop advanced biomaterials that will better adapt to a specific biomedical application.

3.1. Future perspectives

Hydrogels have great potential for delivering biotherapeutics that will enable development of materials for regenerative medicine and other biomedical application. This thesis provides basic understanding of novel chemistries that has high potential for clinical applications. This however, is just the start, and much of the properties need to be investigated for their potential application in the biomedical field. Other covalent chemistries remain unexplored and a lot of work needs to be done to optimize the most commonly used chemical transformations.

Labeling of hydrogels with gadolinium-based contrast agents has demonstrated that *in-vitro* enzymatic degradation can be followed using non-invasive MRI techniques. The implementation of this technology as a platform for preclinical development and optimization of biomaterial scaffolds requires further validation of the obtained results with *in-vivo* degradation

studies. The *in-vivo* results would not only confirm that the degradation of hydrogels can be followed by MRI but also help in the assessment of safety concerns studying the biodistribution of the gadolinium complex after completion of the degradation study.

The fast and efficient thiazolidine condensation reaction is currently being investigated as an alternative for the preparation of hydrogels. Thiazolidine polymeric network could be formed by simply mixing an aqueous solution of the aldehyde and cysteine-modified HA precursors. This hydrogel formulation represents a simple and versatile method for the incorporation of N-terminal cysteine peptides to the hydrogel network using an *in-situ* procedure in which the peptides quickly react with the aldehyde component prior to hydrogel formation.

Aldol cross-linked hydrogels materials offer new avenues to develop robust HA hydrogels with tunable hydrolysis and swelling characteristics that can be applied as tissue adhesives. The hydrogel-tissue integration promoted by the adhesive properties will significantly enhance or guide tissue development and repair on different surgical interventions.

The improved reactivity of cysteine HA derivatives towards disulfide formation under mild conditions will provide new insight to design materials with stable and fast disulfide formation for various bioconjugation and biomedical applications.

We believe that the easy scalability, non-toxicity and established biocompatibility of HA-based materials will favor the clinical translation of such materials.

4. Acknowledgements

First of all, I want to say that this part has been one of the most difficult to write. There are too many people that have contributed directly to this thesis and indirectly to bring me to this point in my life. I would like to take this opportunity to thank all of them.

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5. Svensksammanfattning

Hydrogeler är tredimensionella material som består av korsbundna hydrofila polymerkedjor med starkt förändringsbara fysikaliska och kemiska egenskaper. Hydrogeler kan absorbera stora mängder vatten eller biologiska vätskor som ger möjlighet att likna den extracellulära miljön hos organiska mjukvävnader. Kovalent kemi utnyttjas för närvarande vid framställning av biomaterial för biomedicinska tillämpningar. Denna avhandling handlar främst om utformningen av biomaterial från ett kemiskt perspektiv. Kemin som används vid framställning av hydrogeler för det biomedicinska fältet påverkar starkt den slutgiltiga biologiska effekten. Därför är det mycket viktigt att förstå egenskaperna hos de olika kemiska omvandlingarna och noggrant välja den som bäst lämpar sig till en specifik applikation.

Den första delen av denna avhandling pekar på relevansen av att ställa in olika egenskaper hos biomaterial med särskild uppmärksamhet på utvecklingen av HA-hydrogeler. Den andra delen av avhandlingen reviderar de kemiska omvandlingarna hydrazonbildning, tiazolidinbildning, aldol additionsreaktion och disulfidbildning.

Efter en detaljerad undersökning av bildningskinetiken undersöktes mekaniska egenskaper, hydrolytisk stabilitet och nedbrytbarhet för de material som framställdes med användning av de olika kovalenta kemikalierna, samt nedbrytbarheten av gadoliniummärkta HA-hydrogeler följdes med användning av magnetisk resonansavbildning. Vidare användes tvärbindningspunkterna för aldol HA-hydrogeler för postmodifiering av hydrogelerna och till produktion av vävnadshäftande hydrogeler.

Sammanfattningsvis presenterar denna avhandling ett urval av olika kovalenta kemikalier för utformning av biomaterial. Fördelarna och nackdelarna hos dessa kemikalier undersöks noggrant. En bättre förståelse för de kemikalier som används vid framställning av biomaterial kommer att bidra till att utvidga användbarheten på det biomedicinska området.

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