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par

Carole SCHANTÉ

Modifications chimiques de l'acide hyaluronique pour le développement de dispositifs médicaux biorésorbables

Spécialité : Sciences Pharmaceutiques

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INTRODUCTION

Since its first use in the 1970s, hyaluronic acid (HA) has been increasingly investigated for new therapeutic applications due to its unique physico-chemical properties[1]. It is an essential part of human tissues and has many biological functions, for instance structural support of soft tissues such as skin and joints[2]. As the human body ages, HA content gradually decreases[3] resulting in a loss of its biological activity and efficacy. The most obvious effects of lowered HA contents due to aging are articular deterioration causing arthritis and loss of facial skin volume causing wrinkles. As an answer to these clinical signs of aging, HA supplementation by injection into joints and dermis has been used for many years[4]**.** It is widely used by injection as a surgical aid for eye surgery, as a supplementation agent to treat arthritis and as a dermal filler for facial wrinkles. These products injected for tissue supplementation are classified as medical devices. Today, HA is also a common ingredient in many cosmetic products on the market for topical application for its hydration properties. In addition, the efficacy of HA has been demonstrated for wound healing and drug delivery[5,6]. This last application has opened a field of countless possibilities for the investigation of new drug delivery applications. The continuously rising interest in hyaluronic acid has resulted in an increase of yearly publications on the subject, which has more than quadrupled in the last two decades (1989: 339, 1999: 680, 2009: 1378, Source: Scopus). Given the numerous applications it offers, HA is an attractive product for the market and there is today increasing interest in developing new hyaluronic acid-based products for novel applications and in improving the efficacy of existing products.

In this context, the company IDENOV decided to investigate the science of hyaluronic acid with the final goal of launching on the market a novel injectable hyaluronic acid-based medical device on the market having improved properties compared to the commercially available products. Indeed, dermal fillers such as Juvederm or Restylane claim a duration of

the effect of 6 to 12 months. Given that native hyaluronic acid solutions are naturally eliminated after injection in less than 48 hours, the available products are made of previously crosslinked hyaluronic acid hydrogels, which allows to confer additional mechanical strength and to lengthen its residence time. The development of a still bioresorbable and natural product having yet a longer lasting effect of up to 18 months would enable to avoid repetitive injections and thus be attractive for the market and patients. This project is the subject of the present thesis which is the result of a collaboration between the company IDENOV and the Laboratory of Biogalenical Pharmacy of the University of Strasbourg to carry through the project in question.

The objective of this thesis is the development of a novel hyaluronic acid-based product having a longer lasting filling efficacy (up to 18 months) compared to present commercially available products. With this aim in view, the work achieved during the course of my doctoral project was dedicated to the study of chemical modifications of hyaluronic acid and the stability of the resulting derivatives. To this end, an enzymatic assay was used. Indeed, hyaluronic acid is naturally synthesized in the body and subsequently degraded by a complex enzymatic mechanism which regulates its biological functions[7,8]. For this reason, native hyaluronic acid solutions are quickly eliminated after injection. *In vitro* enzymatic testing was chosen in this work as a suitable method to quickly determine the efficacy of the different chemical modifications performed. Indeed, *in vivo* efficacy testing would need to be performed in a real-time scale of 18 months, which was not feasible technically for the present 3-year project.

After a detailed literature and patent search, our approach was to modifiy hyaluronic acid in two steps: the first step consisting in the grafting of a molecule onto HA and the

second step consisting in crosslinking the resulting derivative to form a rigid hydrogel similar to the commercially available products. The novelty therefore resides in the first step rather than the second, which is however required for its mechanical properties.

With this approach put into practice, an efficient chemical modification was discovered consisting in grafting amino acids on the functional groups of the polymer. First, using L-alanine as a model amino acid, different chemical grafting reactions were performed and compared in terms of grafting efficiency and damaging of the native HA polymer chain. Once the suitable reaction was determined, a wide range of amino acids were chosen and grafted. The amino acids presented functional groups of different nature, including hydrophobic, hydrophilic and positively or negatively charged side chains. The resulting range of HA-amino acid derivatives obtained was subsequently subjected to enzymatic *in vitro* testing and this way evaluated in terms of resistance to enzymatic stability. In order to further obtain products with mechanical properties desired for the supplementation application, chemical crosslinking was needed and performed on the HA-amino acid derivatives. The HA-amino acid hydrogels obtained were then also subjected to enzymatic testing to evaluate their *in vitro* stability and the outcome was compared with commercially available hyaluronic acid-based products.

The present manuscript is divided into five chapters. The numerous chemical reactions performed on hyaluronic acid, the resulting derivatives and characterization methods are reviewed in Chapter 1. The second chapter describes the grafting of L-alanine onto HA and the evaluation of different chemical reactions. The most efficient reaction method is selected at the end of this part. Chapter 3 describes the grafting of different amino acids and the enzymatic stability of the resulting HA-amino acid derivatives obtained.

Chapter 4 is dedicated to the chemical crosslinking of the HA-amino acid derivatives and the enzymatic stability of the resulting hydrogels. Finally, Chapter 5 presents the first part of the experimental work made during this project consisting in the setting up of an *in vitro* enzymatic degradation assay. This part is presented in the last chapter for more clarity as it presents the study of the enzymatic degradation parameters of native HA as well as the HA derivatives synthetized throughout the project. This study was essential for the evaluation of the efficacy of the derivatives described in the whole manuscript and enabled to confirm that the desired properties were successfully implemented into the new device.

CHAPTER 1: Chemical reactions performed on hyaluronic acid and resulting derivatives

This first chapter starts with an introduction on hyaluronic acid, its properties and its therapeutic applications. The numerous chemical reactions described in the literature for the modification of hyaluronic acid are then reviewed in detail and classified according to the targeted functional group. Examples of HA derivatives obtained with the chemical modifications are further described as well as their use for new applications, particularly for drug delivery. The last part of this chapter reviews the different techniques used for the characterization of HA and its derivatives.

1. Hyaluronic acid (HA)

1.1 Chemical structure and physicochemical properties

As determined by Meyer and Weissmann in 1954, HA is a linear polysaccharide made of repeating disaccharide units of D-glucuronic acid and N-acetyl glucosamine linked by β(1,4) and β(1,3) glucosidic bonds[9] (**Figure 1**). When both sugars are in the beta configuration, each bulky group is in the equatorial position, forming a very energetically stable structure[10]. HA is part of the glycosaminoglycan (GAG) family, a group of linear heteropolysaccharides composed of aminosugars and uronic acids, mainly found in the extra cellular matrix (ECM)[11]. Other GAGs include heparan-, chondroitin-, keratin and dermatan sulfate. HA is the only GAG which is not sulfated.

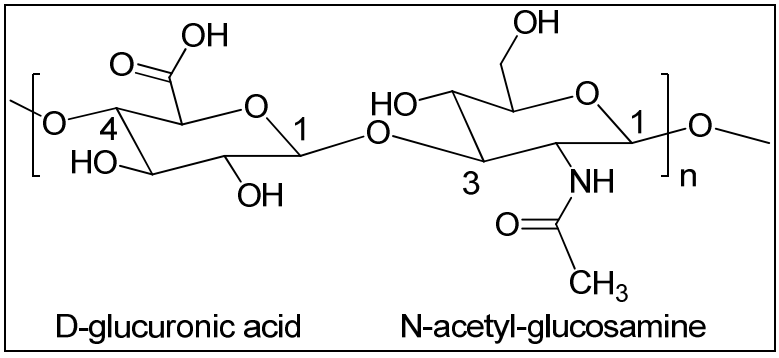

Figure 1 : Chemical structure of hyaluronic acid (HA)

In physiological conditions, HA is in the form of a sodium salt, therefore negatively charged and referred to as sodium hyaluronate. It is highly hydrophilic, surrounded by a sphere of water molecules linked by hydrogen bonds.

At first it was assumed that HA formed random coils in solutions, but Scott[12,13] determined by NMR the formation of an ordered structure stabilized by internal hydrogen bonds between the acetamido and carboxylate groups of HA. This secondary structure in aqueous solutions has the special feature to create hydrophobic patches consisting of 8 -CH groups on alternate sides of the polymer. Thus, HA has an amphiphilic nature. Scott suggested that these patches could be the basis of interactions with lipid membranes and proteins, as well as self-aggregation[12].

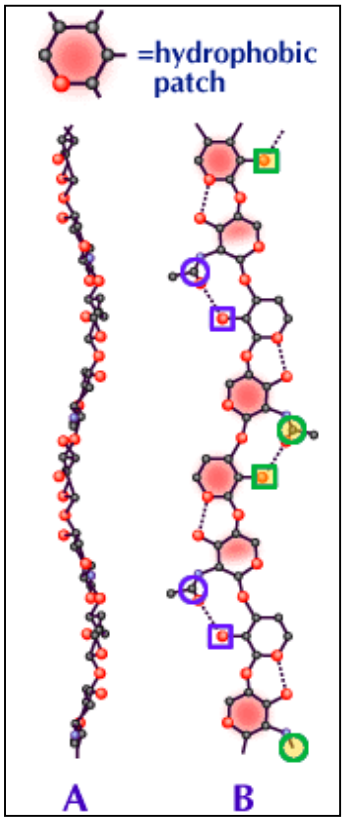

Figure 2 : Hyaluronic acid representations in elevation (A) and plan (B) projections by Scott (1989)[12]. The red areas in B are the hydrophobic patches. Circles represent acetamido and squares represent carboxylate groups, linked by hydrogen bonds. Reference: Scott 1998, Glycoforum.

Its high molecular weight of several million Daltons can reach 8.10^6Da, 10^7Da or 10^8Da according to different authors[1,14,4,8]. HA molecular weight depends on the enzyme that catalyzed its synthesis. Indeed, HA can be synthesized by different HA synthases (HAS) which generate different chain lengths[15,16]. Its synthesis occurs on the cytoplasmic surface of plasma membranes, unlike other GAGs which are synthesized in the Golgi apparatus[17]. As it is being synthesized, it is simultaneously transported out of the cell into the extracellular matrix. The cells which synthesize HA as well as the other components of the ECM are mainly but not exclusively fibroblasts in the skin, keratocytes in the cornea and chondrocytes in the synovial fluid.

Due to its important chain length and strong intermolecular interactions, the coils formed by HA in solution are fully entangled even at low concentrations, leading to highly viscous solutions in water[2]. The shear-thinning character of HA solutions is attributed to the progressive breakdown of the intermolecular hydrogen bonds and interaction between hydrophobic patches when subjected to shear stress. With increasing ionic strength, the viscosity is lowered, owing to the contraction of the coil dimensions on suppression of the intramolecular electrostatic repulsion. Similar behavior is observed in acidic and alkaline conditions. This effect is however more dramatic in alkaline conditions, most likely due to the disruption of hydrogen bonds on the ionization of the hydroxyl groups[18]. In addition, alkaline conditions have shown to induce hydrolysis of the HA chain[19]. Maleki et al. (2008) showed that HA is degraded at pH values lower than 4 and higher than 11[20]. Degradation in alkaline conditions showed to be more important and faster than in acidic conditions.

1.2 HA in the organism

1.2.1 Occurrence

HA is naturally present in the vertebrate organism as well as in bacteria. Its highest occurrence in the human body is in the extra cellular matrix (ECM) of connective tissues.

The connective tissue is one of the four types of tissues in the human body which also include the epithelial, muscle, nervous tissues. The connective tissue surrounds and protects organs. It is comprised of functional cells and extracellular matrix which provides mechanical scaffold and modulates cellular activity[21]. The ECM is composed of many components including HA, proteins (collagens, elastin, laminin, fibronectin) and proteoglycans (proteins linked to GAGs except HA). Cells bond to ECM components through cell surface receptors such as integrin (cellular adhesion molecules). Fibroblasts are the most widely distributed cells of the ECM; they have numerous functions including synthesis and regulation of the ECM components.

Synovial fluid in joints, the dermis of the skin and the vitreous body of the eye are mainly composed of ECM. Therefore, HA is especially abundant in these tissues: synovial fluid contains 1420-3600mg/L, the vitreous body of the eye 140-338mg/L and the dermis 200mg/L[1]. Skin contains about 5g of HA, which is about half of the total amount in the body[3]. The umbilical cord contains 4100mg/L of HA, which is the main constituent of Wharton's jelly together with chrondroitin sulfate[22]. Another distinctive characteristic of HA is its high concentration found in rooster combs (7500mg/L). HA is particularly abundant around cells, forming a pericellular coating[3].

1.2.2 Physiological functions of HA

Thanks to its high molecular weight and its capacity to retain a high amount of water, HA's primary role in the body is it structural and hydration role. It maintains an open, hydrated and stable extracellular space in which cells and other ECM components such as collagen and elastin fibers are firmly maintained. Due to its high viscosity and shear-thinning properties discussed above, HA acts as a lubricant and shock absorber, especially in joints. As an essential part of the Wharton's jelly, it is believed to prevent compression of the umbilical cord. Laurent et al. also reported HA to regulate water content and molecule transport in tissues[2].

It has been established for many years that HA is also involved in cellular activity including regulation, migration and adhesion[2,23]. High molecular weight HA exhibits anti-angiogenic and anti-inflammatory properties. Its immune-suppressive property was explained by its ability to coat cell surfaces and therefore preventing access to the cell's surface receptors[24]. Low molecular weight HA (<100kDa) fragments have opposite biological activity; they are inflammatory, immuno-stimulatory and angiogenic. High molecular weight HA (>200kDa) in general promotes and maintains tissue integrity whereas low molecular weight HA indicates tissue damage and repair process.

It has been shown that high molecular weight HA surrounds cells in the connective tissues. This pericellular HA plays an important role in protecting cells from environmental effects[3]. Experiments showed that removing pericellular HA by enzymatic treatment was directly followed by a burst of HA synthesis[3]. Cells which synthesize HA therefore are involved in the formation and maintenance of structurally and functionally reliable connective tissue[3].

The numerous functions of HA in wound healing has been developed by many authors[6,24]. The wound healing process includes three phases: first the inflammation phase during which the factors essential to wound healing are generated (growth factors, cytokines…). Low molecular weight HA is found in high concentrations during this step as it acts as a promoter of inflammation. The second step is the formation of the granulation tissue in which fibroblasts, endothelial cells and keratinocytes migrate and proliferate. The HA rich environment during this step plays an important role to facilitate cell migration and proliferation: the hydrated matrix formed by HA as well as its interactions with cell surface receptors facilitates and controls cell migration. The main HA cell receptors are CD44 (Cluster of Differentiation-44), RHAMM (Receptor for HyaluronAn Mediated Motility) and ICAM-1 (Intracellular Adhesion molecule-1), all of which are involved in cell migration and proliferation. Angiogenesis is also part of the granulation phase and is promoted by low molecular weight HA fragments present on the wound site. The last step of the wound healing process is reepithelization and remodeling of the tissue, also promoted by HA due to its role in keratinocyte proliferation and migration. Normal synthesis of ECM components is restored by the newly formed fibroblasts.

1.2.3 HA and aging

It has been reported that HA skin content decreases with aging[3,25]. This decrease is linked to the loss of skin tissue and depends on the site of the skin. Dermal thickness has been reported to decrease of 6% each decade of human life[26]. Meyer and Stern (1994) reported the increase with age of HA fixation to proteins of the ECM[27], which Robert explains by the result of free radical mediated reactions amongst other possibilities[3]. Increased HA-tissue binding weakens its water uptake ability, inducing loss of its tissue hydration and space-filling properties[28]. Authors have reported an age-dependant increase

of HA's half-life, suggesting the decrease of its turnover correlated to cell aging[3]. Longas et al. reported the decrease of HA chain length in the aging skin as well as HA skin content[29].

It is well known that sun exposure causes premature skin aging. The generation of angiogenic and inflammatory HA fragments by free-radical chain scission caused by UV radiation induces a mechanism similar to wound healing. In addition, the accumulation of HA content immobilized on elastin fibers observed by Bernstein et al. could be explained by the generation of UV radiation HA breakdown products which bind to proteins and no longer fulfill their physiological role[30].

1.3 Degradation of HA

1.3.1 Enzymatic degradation

The half-life of HA after injection in skin and joints is no higher than 24 hours[31]. Indeed, HA is a natural product and is naturally degraded in the organism. Its content is regulated by a highly organized and tightly controlled enzymatic mechanim. Hyaluronidases (Hyal) are the enzymes responsible for HA degradation and generate HA of defined sizes, which have various biological functions. They also degrade other GAGs (chondroitin and chondroitin sulfate) but in a more limited extend. The main bacterial hyaluronidase is a β-endoglycosidase which cleaves the β(1-4) bonds of HA. Mammalian hyaluronidases are endo-β-N-acetylhexosaminidases (EC 3.2.1.35) which also randomly cleave β(1-4) bonds leaving N-acetylglucosamine at the reducing end[8]. The reaction mechanism proposed by Jedrzejas and Stern (2005)[32] is shown in **Figure 3**.

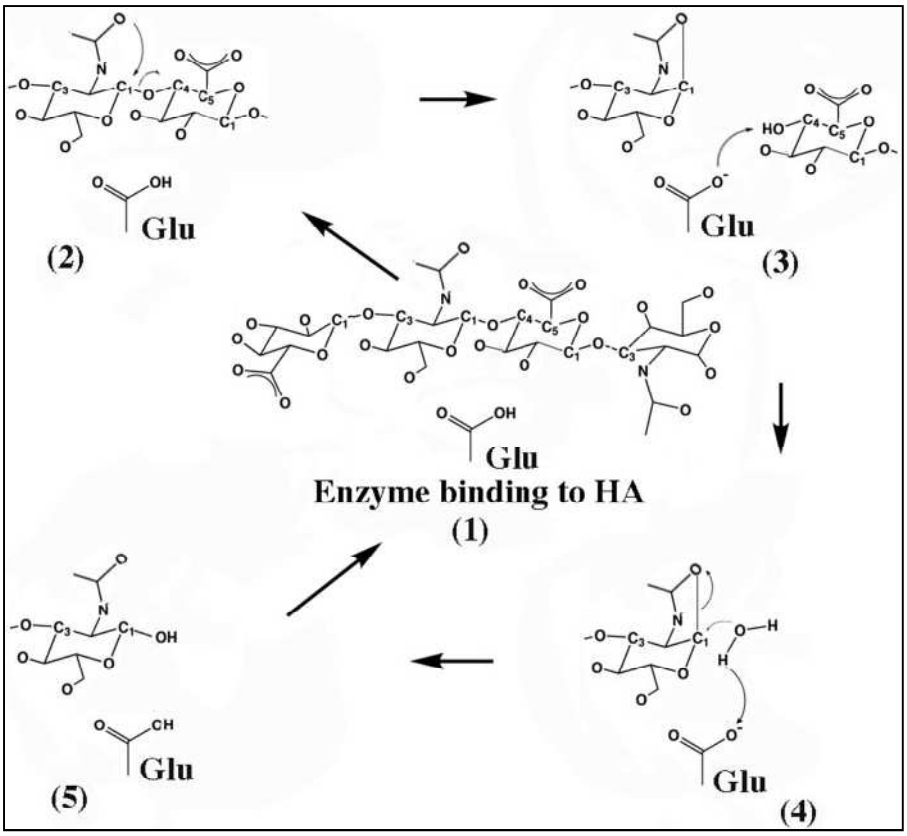

Figure 3: Reaction mechanism of HA cleavage by mammalian hyaluronidases. Reference: Jedrzejas and Stern (2005)[32]

The six known mammalian hyaluronidases are termed Hyal-1 to Hyal-4 and PH-20/SPAM-1. Hyal-1 and Hyal-2 are the most widely expressed hyaluronidases. Hyal-1 are acid active enzymes and most likely located in lysosomes. Hyal-2 enzymes have a broader pH optimum and are anchored on the external face of the cellular membranes by a glycosylphosphatidyl-inositol (GPI) link. The CD44 cell surface receptors have an important role in HA degradation, which possibly occurs according to the mechanism depicted in **Figure 4**[33]. Extracellular high molecular weight HA (approximately 1 to 4MDa) is firstly attached to the CD44 receptors and Hyal-2 enzymes present on the cellular membranes and cleaved into medium sized fragments (approximately 20kDa). These fragments are then internalized by endocytosis and degraded into mainly tetrasaccharides (800Da) within the intracellular lysosomes by Hyal-1 with the participation of lysosomal β-exoglycosidases[7].

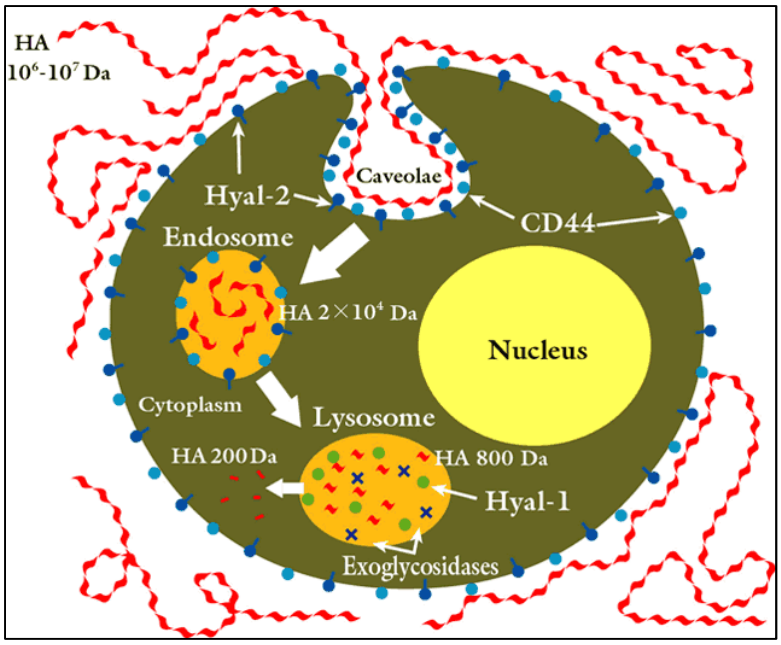

Figure 4: Enzymatic degradation mechanism of hyaluronic acid by hyaluronidases. Reference: Stern 2004, Glycoforum.

Molecular modeling based on crystallographic studies indicated that the recognition sites of the Hyal-2 enzymes and CD44 receptors are the carboxylic groups of HA[34].

Commercially available hyaluronidases isolated from bovine testis are PH-20 enzymes, located on the sperm surface which show enzymatic activity in both acidic and neutral pH and are GPI-anchored proteins, as Hyal-2[8,35].

1.3.2 Non-enzymatic degradation

HA can also be naturally degraded in the organism by reactive oxygen species (ROS)[14,36]. The accumulation of ROS in tissues during inflammatory responses induces HA chain cleavage. The mechanism of HA degradation differs according to the ROS involved and are reviewed in detail by Stern et al. (2007)[14]. Studies suggest that HA degradation in pathological tissues suffering from diseases such as arthritis is due to ROS released from activated leukocytes and macrophages[37].

Due to the many functions of low molecular weight HA fragments, work has been done on the preparation of fragments with specific uniform sizes by controlling the

degradation of high molecular weight HA using various techniques including acidic, alkaline, ultrasonic and thermal degradation. These techniques are also reviewed by Stern et al. (2007)[14].

1.4 Therapeutic uses

1.4.1 Supplementation

1.4.1.1 Arthrology

Viscosupplementation by injection into joints suffering from arthritic disorder is a major application of HA[4]. As described above, HA is a main component of the ECM of the synovial fluid of joints. Synovial fluid avoids friction between the bone-ends and absorbs mechanical impact by elasticity or dissipating it by viscous flow. Osteoarthritis and rheumatoid arthritis are the two most common arthritic diseases. As depicted in **Figure 5**, in joints affected by arthritis, the synovial fluid is reduced or inflamed and no longer plays its lubricant role; HA content is decreased, inducing bone friction and severe pain. To overcome this phenomenon and reduce pain, injection of HA into the joints, known as viscosupplementation, is widely used (Hyalgan® by Sanofi, Supartz® by Smith&Nephew, Synvisc® by Genzyme, etc.). As mentioned earlier, injected HA is cleared out of skin and joints in no longer than 24 hours[31]. However, clinical benefits of viscosupplementation have proved to be effective for several weeks or months[38]. This suggest that the sustained beneficial effects of HA therapy can be accounted for more than just a temporary restoration of the synovial fluid lubrication and viscoelasticity. HA supplementation most likely has a biological impact on osteoarthritis joints. The beneficial effect could be due to mechanisms such as the restoration of viscoelastic properties of the synovial fluid or the production of newly synthesized HA induced by exogenous HA. Greenberg reported that

exogenous HA induced the synthesis of new HA by synovial cells and stimulated chondrocyte proliferation, resulting in inhibition of cartilage degradation[39].

Due to its short half-life after injection, HA contained in some of the commercial products (such as Synvisc® by Genzyme) is previously stabilized by cross-linking to obtain non-soluble hydrogels with longer residence time and mechanical effect. The different cross-linking techniques are reviewed in section 2. Manufacturers claim pain relief using HA products lasts up to 6 months, which is more or less in agreement with the literature[40]. However, some studies showed no differences in pain reduction between cross-linked and native HA injections[40,41].

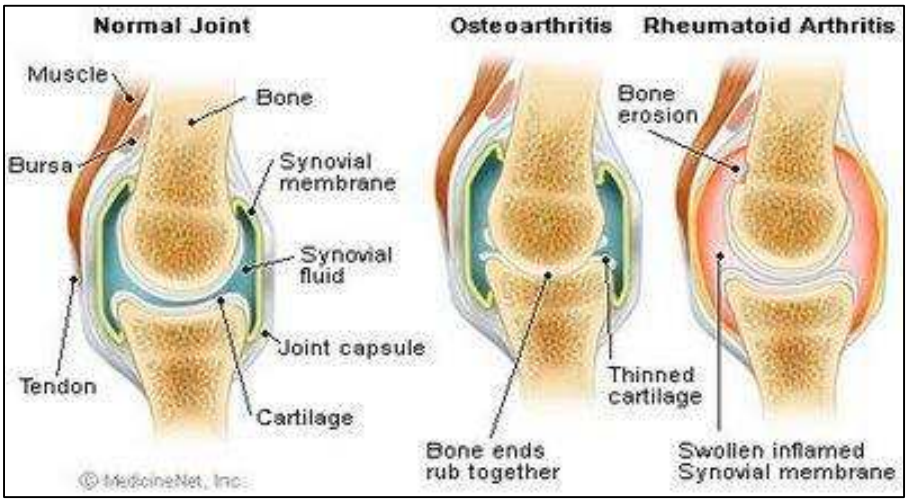

Figure 5: Normal and arthritic joints. Source: http://biomoz.com/diseases-and-treatment/types-of-arthritis.html

1.4.1.2 Dermo-esthetics

The most visible effects of lower HA content due to aging is the loss of facial skin hydration and volume, causing wrinkles. HA is commonly used today for tissue augmentation, as a dermal filler to fill facial wrinkles. More and more products currently come on the market (Restylane® by Q-Med, Juvederm® by Allergan, Teosyal® by Teoxane, Glytone® by Pierre Fabre…). Due to its rapid dissolution and enzymatic degradation in tissues

after injection, HA is previously stabilized by cross-linking. Non-soluble hydrogels are obtained to prevent HA dissolution and enzyme access, thereby prolonging its residence time after injection. All commercial products cited above are stabilized by crosslinking. Manufacturers claim a residence time between 6 to 12 months depending on the product. Clinical observations reported the duration of the effect to last for 6 to 9 months[42,43,44,45].

Dermal fillers are now commonly used as a non-invasive mild alternative to surgical operation, involving a more affordable and quick procedure. Intradermal injection of HA fillers is nevertheless a delicate intervention: too deep an injection can decrease the duration of the result whereas too superficial an injection can lead to lumps[46].

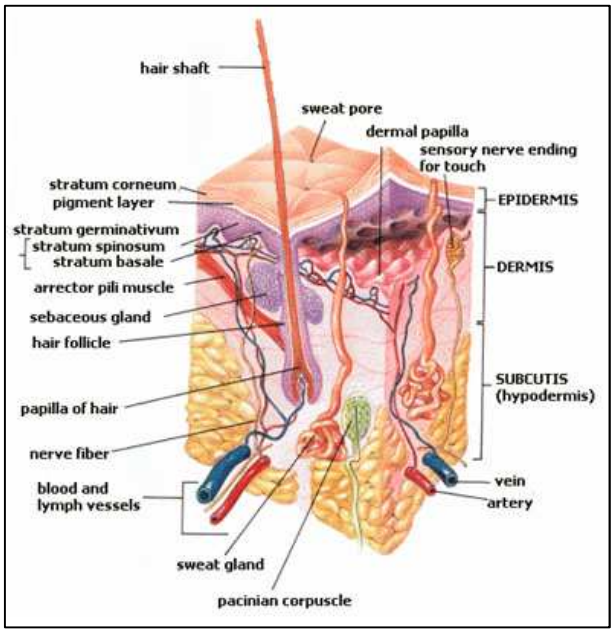

Figure 6: Structure of the skin. Source: http://en.wikipedia.org/wiki/Dermis

Some dermal fillers contain lidocaine as a local anesthetic to reduce pain during injection. Other types of dermal fillers include collagen (Zyderm® and Zyplast®), poly-L-lactic acid (Sculptra®), calcium hydroxyapatite spheres (Radiesse®), polymethylmethacrylate microspheres (Artefill®) and silicone[46]. Except for collagen, these fillers are however semipermanent or permanent fillers which are not naturally resorbed by the organism and

can cause long term irreversible complications. These materials are usually used for severe dermal depression to treat facial lipoatrophy caused by HIV treatments.

HA dermal fillers have the advantage of being biocompatible and safe, inducing minimal foreign body reaction. There is no risk of migration of the product after injection. In addition, in the case of failed or wrong injection and results, a hyaluronidase solution can be injected to quickly degrade the product and erase undesired effects.

More recently, HA has been used for breast augmentation[47]. Its efficacy was claimed to be for 12 to 18 months, requiring yearly additional injections. However, concerns about its safety namely regarding breast cancer are still being investigated[48].

1.4.1.3 Urology

Urinary incontinence is a common problem, affecting mainly women after childbirth or menopause or men after prostatic surgery. It is any involuntary leakage of urine and results from the loss of support the urethra canal and the urethral sphincter muscle. An alternative to this problem is the injection in the urethra of bulking agents, such as polytetrafluoroethylene (Teflon®), collagen (Contigen®), autologous fat and hyaluronic acid (Zuidex®)[49]. Dextranomer/hyaluronic acid copolymer injections have shown to be effective[50]. These kind of interventions are however recent and not widespread.

1.4.2 Ophthalmology

The first HA medical product on the market, Healon®, approved in 1980 by the FDA is used for eye surgery. It is injected during ophthalmological surgery to replace lost vitreous fluid during such procedures. Thanks to its bulking and lubrifying properties, it additionally protects the fragile eye tissues and provides space for manipulation. Cataract surgery is the most common ophthalmic intervention which consists in the removal of the cataractous opacified lens and its replacement with an artificial plastic intraocular lens. This procedure

involves incisions in the cornea into the anterior chamber and is potentially traumatic for the surrounding tissues. Injected HA provides a physical protection between the tissue and the surgical instruments and maintains the intraocular space[51]. Compared to other viscosurgical devices used for this application including hydroxypropylmethylcellulose (HPMC) and chitosan glutamate (CG), HA has the advantage to be a natural component of the vitreous body. Its water-retaining capability and interactions with CD44 and RHAMM cell receptors enable HA to bind to cell membranes and provide a biological adhesive interaction with natural tissues, leading to a better ability in maintaining the anterior chamber space[51].

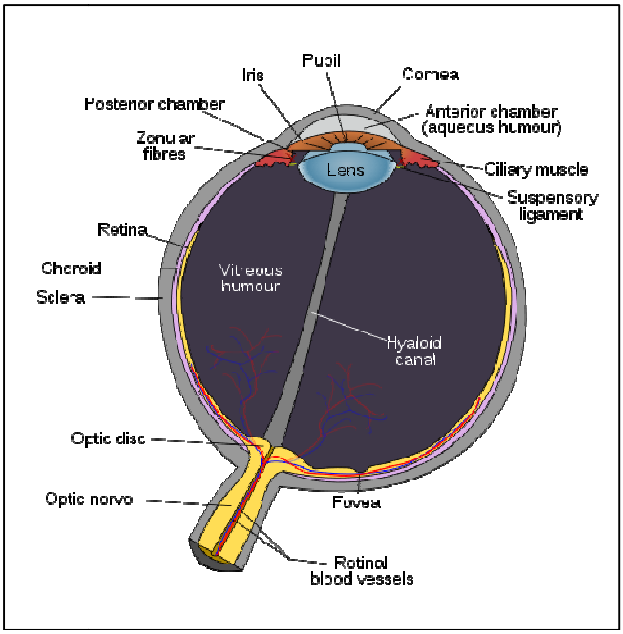

Figure 7: Structure of the eye. Source: http://en.wikipedia.org/wiki/Eye

1.4.3 Wound healing

Due to the important physiological role of HA in wound healing (detailed in section 1.2.2), it is used in preparations for topical use on wounds, venous leg ulcers or diabetic ulcers[52]. Several studies have shown that exogenous HA exerts beneficial effects on the wound healing process[53,54]. By promoting cell proliferation and migration in actively growing tissues, HA has a well-known beneficial effect on healing. Amongst other actions, it promotes fibroblast adhesion to the wound site and stimulates collagen production and

other ECM components by fibroblasts. Hyalofill®, Hyalogran®, and Ialuset® are examples of wound-dressings or bandages impregnated with HA solutions. HA-based wound gels are also commercialized. HA is also combined with other substances to enhance wound healing such as dexpanthenol, the vitamin B5 precursor (Hylactive® by Promedic) or moisturizing agents like glycerin (RadiaPlex® by MPM Medical Inc.).

1.4.4 Cancer therapy

Low molecular weight HA is found in high concentrations around the surface of tumor cells which is associated to the abundant secretion of hyaluronidase, generating such HA fragments. As described above (section 1.2.2), low molecular weight HA is implicated in numerous cellular mechanisms which have proved to enhance tumor cell adhesion, migration as well as growth and survival[55,56,57]. Sugahara et al. (2006) demonstrated that human pancreatic carcinoma cells produce active hyaluronidases and HA fragments which enhances tumor cell motility via interaction with CD44 receptors that subsequently promotes tumor progression and invasion[55]. Lokeshwar et al. (1997) presented data showing significantly elevated urinary HA levels in all bladder cancer patients, suggesting the usefulness of urinary HA levels as a marker for bladder cancer. The authors present an ELISA-like assay for the measurement of urinary HA levels which is simple, noninvasive yet highly sensitive and specific and may be used clinically for bladder cancer detection[58]. In a different matter, small HA fragments of 6-7 saccharides have shown to disturb HA interactions with the surface of carcinoma cells, leading to apoptosis and inhibition of tumor growth[59].

As the presence of small HA oligosaccharides is directly linked to hyaluronidase activity, these enzymes have been extensively studied for cancer biology. They have been evaluated as tumor promoters but also as tumor suppressors[60]. Both HA and hyaluronidases have therefore been used as tumor markers for different cancer types[61,62]. In addition,

studies showed that hyaluronidase is able to loosen the cell–cell contact and the interstitial connective tissue and enhance the efficacy of cytostatic agents in chemotherapy[63,56]. Tumor growth was shown to be decreased by the addition of hyaluronidase[64]. As will be described in the next section, HA is also a valuable tool for the targeted delivery of anti-cancer agents.

1.4.5 Drug delivery

HA is increasingly used for numerous drug delivery applications. Different mechanisms have been described including conjugation of drugs on one of HA's functional groups[65], encapsulation of drugs inside HA microspheres[5], formation of HA ion complexes[66,67] and HA coating of drug-loaded liposomes[68,69]. Such systems have shown to considerably enhance drug solubility, absorption and/or efficacy[70,71,72,73]. Compared to other polymers used as drug carriers, HA has the advantage to be natural, bioresorbable, water soluble amongst many other useful properties. Furthermore, HA interacts with the CD44 cellular receptors and subsequently enters into cells (as described in section 1.3.1). This valuable characteristic has been studied in order to deliver drugs directly into the cells[65]. This aspect is especially important for cancer therapy as an upregulation of CD44 cellular receptors has been detected in tumor cells[65]. As a result, they show enhanced HA binding and internalization[74]. As most drugs such as chemotherapeutic agents are cytotoxic for the targeted cells but also to surrounding healthy cells, causing important undesired side effects, drug delivery to the specific target site enables to reduce drug dosage amounts and thus drug undesired toxicity. Examples of HA drug delivery systems are reviewed in detail in section 3.

1.5 Extraction or synthesis

HA was initially isolated from bovine vitreous and later from rooster combs[75] and human umbilical cord. Advances in biotechnology have led to the development of genetically modified bacteria producing high yields of HA. Shiedlin et al. (2004)[75] showed no significant

difference in molecular weight and polydispersity between HA isolated from bacterial, human umbilical cord and rooster comb sources. HA from bovine vitreous however showed lower molecular weight and higher polydispersity, but this difference could very well be due to the isolation or purification procedures and not only the source type. Indeed, Shieldlin[75] observed significant differences between 3 lots of umbilical cord HA in terms of DNA and RNA content. These results show the large differences in final product characteristics which can be obtained for HA isolated from the same source, the purification step having an important impact on the resulting HA characteristics[76]. Conflicting results exist concerning optimal HA source. Manna et al. (1999)[77] demonstrated that an HA product from rooster comb source was safer than the product from bacterial source which contained residual proteins. Matsuno et al. (2008)[78] detected the presence of impurities in samples from rooster comb and umbilical cord but not in the samples derived from bacterial source. Hylaform®, a commercial product derived from rooster combs, was shown to contain small amounts of avian protein which can trigger allergic reactions[46]. Today however, most commercial products are from bacterial source (Restylane®, Juvederm®…), which is easier, more reproducible and avoids the problem of animal contamination.

There is increasing effort to produce bacterial HA using high yield and less costly methods with effective purification techniques[76]. The composition of the bacterial fermentation media showed to greatly influence the resulting molecular weight (Mw) of the extracted HA: lactose or sucrose in the medium gave higher M_w than glucose as well as by increasing the amount of sucrose present[76]. Optimum bacterial culture conditions were determined at 37°C and pH 7. Values of 3.5 to 3.9 MDa could be reached depending on the authors[76,79].

2. Chemistry used for the synthesis of HA derivatives

There are two different types of HA modification: cross-linking and conjugation. HA conjugation and HA cross-linking are based on the same chemical reactions and only differ in the way that, in the first case, a compound is grafted onto one HA chain by one single bond only, whereas in the second case, the compound links two HA chains together by two bonds, as depicted in **Figure 8**. In addition, there are different types of crosslinking procedures: direct crosslinking, crosslinking of HA derivatives and crosslinking of different HA derivatives. Chemical modification of HA can be performed on the two available functional sites of HA: the carboxylic acid group and the hydroxyl group (**Figure 9**). An amino group can also be recovered by deacetylation of the N-acetyl group. In the case of hydroxyl modification, it is unknown which of the hydroxyl groups reacts though it is reasonable to assume the reaction occurs mainly on the hydroxyl of the C6 of the N-acetylglucosamine moiety of HA because of the better accessibility of reagents to primary alcohols.

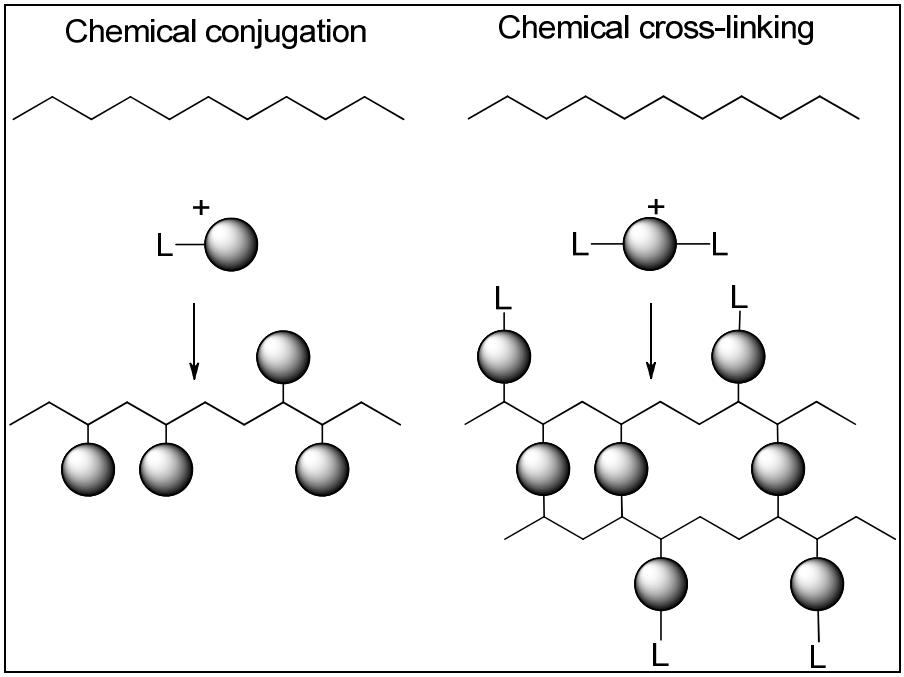

Figure 8: Chemical conjugation and chemical cross-linking of a polymer

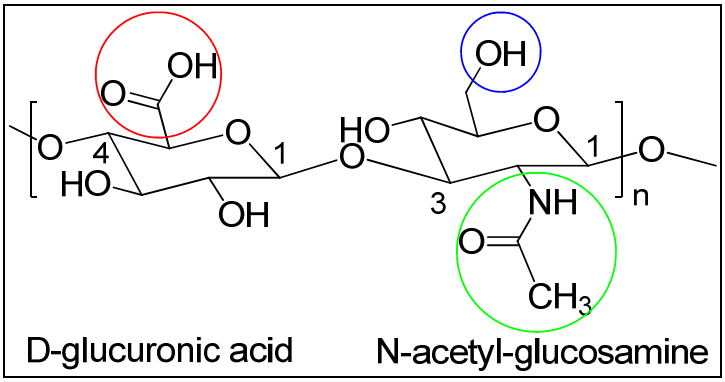

Figure 9: Chemical target sites for HA modification

Numerous methods have been reported for HA crosslinking or conjugation. Some methods are performed in water while others require to be performed in organic solvents such as dimethylformamide (DMF) or dimethylsulfoxide (DMSO) when using reagents sensitive to hydrolysis. In the second case, the native HA sodium salt has to be previously converted either to its acidic form or to a tetrabutylammomium (TBA) salt to allow its solubilization in organic solvents. This requires an additional step, thus increasing the chances of HA chain fragmentation associated to chemical treatments[80,81]. Since HA is soluble in water, the easiest method is to perform the reaction in water. However, in aqueous conditions, some reactions are pH dependent and require to be performed in acidic or alkaline conditions, which have shown to greatly induce HA chain hydrolysis[20]. These aspects have encouraged research teams to explore new synthetic routes for the development of HA derivatives with appropriate characteristics according to their specific need. In some cases, the effectiveness of the reaction is not a criterion as low substitution or crosslinking degrees are sufficient for the desired effect[82], whereas for some applications high degrees of substitutions are required[83].

This section reviews the different chemical techniques described in the literature for the modification of the three target sites of HA. **Table 1** gathers all the chemical modification techniques described in this section.

Table 1: HA chemical modification techniques

HA target site	Reaction type	Activator	Reagents	Solvent	References
-COOH	Amidation	Carbodiimides	EDC, NHS	Water (pH 4.75 to 7.5) or DMSO	[84,71,85,86,87,88,86,83]
		CMPI	CMPI, triethylamine	DMF or DMSO	[89, 90, 91,92]
		CDMT	CDMT, NMM	Water/acetonitrile	[80]
		1,1'-carbonyldiimidazole	1,1'-carbonyldiimidazole	DMSO	[93,94]
	Ugi condensation		Formaldehyde, diamine, cyclohexyl isocyanide	Water (pH 3)	[95,96,97]
	Esterification	diazomethane	trimethylsilyl diazomethane (TMSD), acetic acid	DMSO	[98,99]
		alkyl halides	alkyl iodides or bromides	DMSO	[100,81]
		tetraethylene glycol tosylate	tetraethylene glycol tosylate	DMSO	[101]
		bisepoxides	butanediol-diglycidyl ether (BDDE)	Water (acetic acid, pH 2-5)	[102,103]
	Oxidation	sodium periodate	sodium periodate	Water	[104,105]
-OH	Ether formation	bisepoxides	1,2,3,4-diepoxybutane	Water (0.2M NaOH, pH>13)	[106]
			butanediol-diglycidyl ether (BDDE)	Water (0.25M NaOH, pH>13)	[107,108]
			ethyleneglycol diglycidyl ether and polyglycerol polyglycidylether	Water (1M NaOH, pH 14)	[109]
			epichlorohydrin or diepoxyoctane	Water (pH 10 then pH 4)	[110]
		divinyl sulfone	divinyl sulfone	Water (0.2M NaOH, pH>13)	[111,112,113,114]
		ethylenesulfide	ethylenesulfide , dithiolthreitol (DTT)	Water (pH 8.5-10)	[115]
	Hemiacetal formation	Glutaraldehyde	Glutaraldehyde	Water (pH 2)	[116,117,112]
	Esterification	alkyl succinic anhydrides	octenyl succinic anhydride	Water (pH 9)	[118,119]
		acyl-chloride activated carboxylate		DMSO	[120]
		methacrylic anhydride		Water (pH 8-10)	[121]
	Carbamate formation	cyanogen bromide (CNBr)		Water (pH 9-10)	[122,123]
-$NHCOCH_3$	Deacetylation/amidation	hydrazine sulfate		Water followed by DMSO	[86,93,124,125,126]

2.1 Modification of the –COOH

2.1.1 Amidation

2.1.1.1 Amidation with carbodiimides

Amidation in water with carbodiimides is one of the most widely used method for HA modification[71,85,86,87]. The carbodiimide used is predominantly 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC) for its water solubility. Danishefksi and Siskovic (1971) were the first ones to convert the carboxyl groups of polysaccharides including HA into amides[84]. They used EDC at pH 4.75 to activate the carboxylic groups which then reacted with an amino-acid ester. The presence of amide bonds was detected by infrared, chromatography and electrometric titration and a substitution degree of 38.6% was calculated.

The reaction mechanism has been studied in detail by Nakajima and Ikada in 1995[127]. The first step of the amidation reaction starts with the activation by EDC of the HA carboxylic acid which forms a highly reactive specie, O-acyl isourea. This specie then rapidly reacts with a nucleophile, namely the amine but also water, in which case it quickly rearranges into a stable N-acyl urea by-product, thus preventing any further reaction with the amine. The second step of the reaction is the nucleophilic attack by the amine on the activated HA which leads to the formation of the amide bond (**Figure 10**).

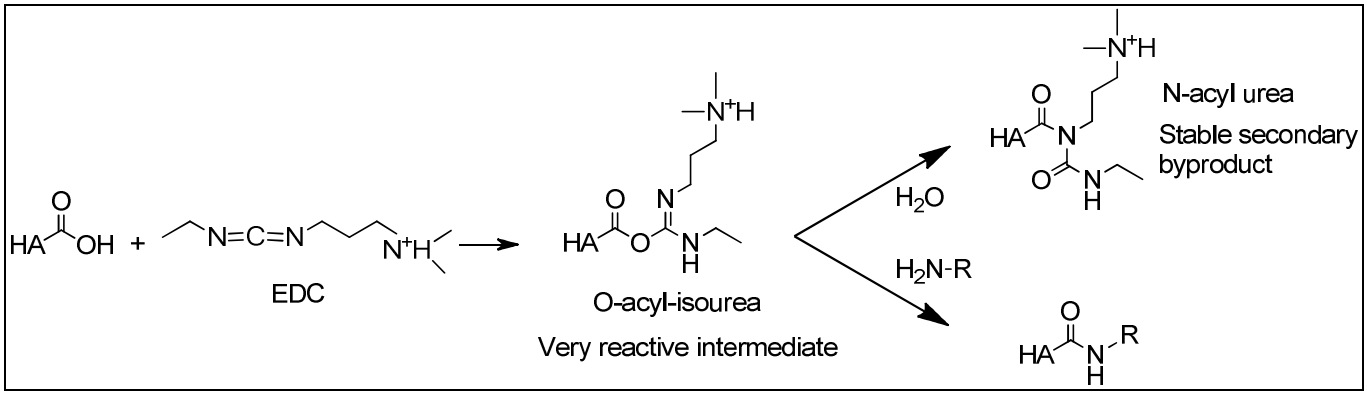

Figure 10: HA amidation mechanism with EDC

The reaction however is very delicate as it is strongly pH dependent and the optimal pH for both steps is different. Indeed, the carboxylic acid activation by EDC is best performed in an acidic environment (pH 3.5-4.5)[127] , whereas amide formation is best done at high pH, when the amine is unprotonated. At such high pH, EDC is more rapidly hydrolyzed into the N-acyl urea by-product and no amidation can occur. The compromise is therefore not easy to define and amines with high pK_a values are not easily conjugated to HA with this method.

Even though most authors have shown evidence of amidation[84,127,128], Kuo et al. (1991) contradicted all previous literature by demonstrating that no amide linkage was formed between the carboxylic acid groups of HA and amino groups[129]. Only the N-acylurea by-product was obtained using the same pH of 4.75 as previous authors. Indeed, at this pH value, the protonated amine is not as nucleophilic and does not react easily with the activated HA. From this finding, it was proposed to use carbodiimides not as an activator but as the reagent itself. In this way, biscarbodiimides were employed to crosslink HA and form stable bis(N-acylurea) cross-linked gels (**Figure 11**).

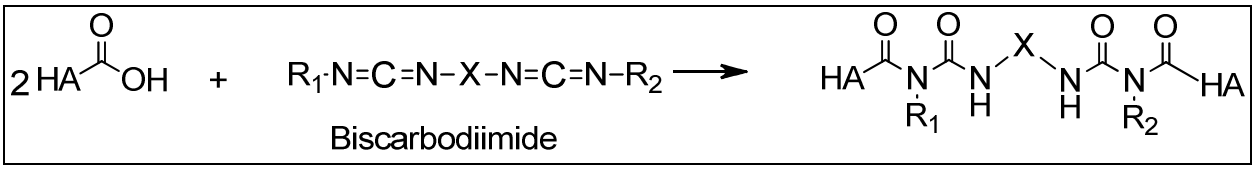

Figure 11: Crosslinking of HA with biscarbodiimides by Kuo et al. (1991)[129]

Replacing diamines by dihydrazides, which have much lower pKa values of 2-3, enabled to obtain higher coupling degrees reaching 56%[88]. By adding a large excess of adipic dihydrazide (ADH), no cross-linking was observed but only single functionalization and dihydrazides bonds were formed (**Figure 12**).

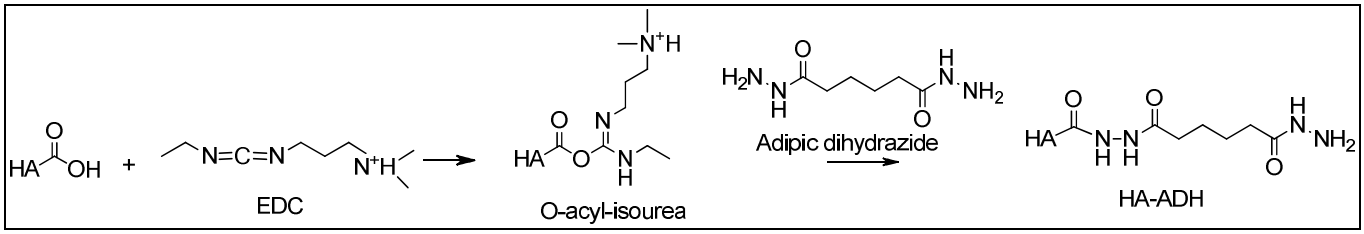

Figure 12: Reaction of HA with adipic dihydrazide using EDC

In order to prevent the formation of irreversible N-acylurea by-product, Bulpitt and Aeschlimann reported the use of N-hydroxysuccinimide (NHS) or 1-hydroxybenzotriazole (HOBt) with EDC to form more hydrolysis resistant and nonrearrangeable reaction intermediates[86]. NHS or HOBt reacts with the O-acyl isourea and the resulting activated HA intermediate undergoes nucleophilic attack by the amine. The mechanisms are shown in **Figure 13**.

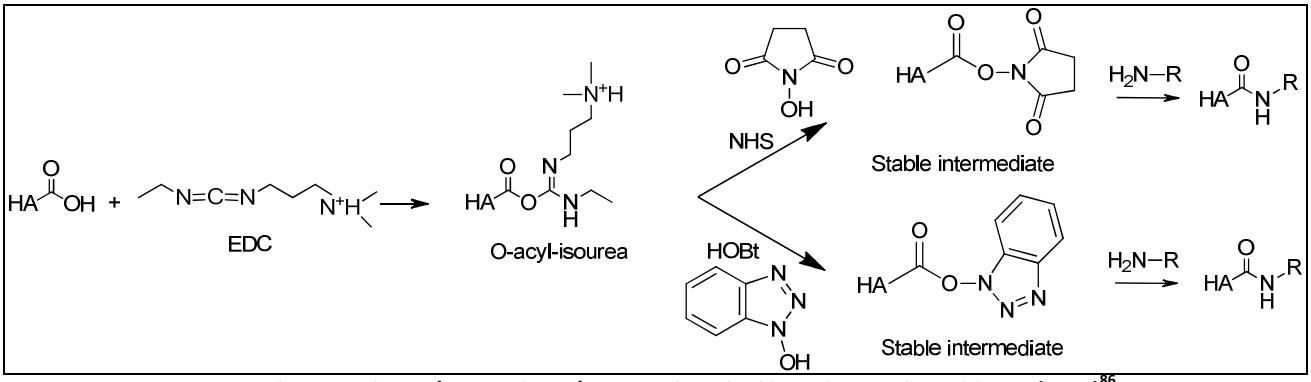

Figure 13: HA amidation with EDC/NHS and EDC/HOBt as described by Bulpitt and Aeschliman (1999)[86].

The use of HOBt led to higher degrees of substitution than using NHS, suggesting it forms an intermediate which is more reactive towards the amine or hydrazide.

Amidation using EDC has the advantage to be performed in water with the native HA sodium salt used without previous preparation. In addition, this method does not lead to cleavage of the HA chain according to the authors cited above and therefore maintains its high molecular weight responsible for its valuable viscoselastic properties. However,

reagents need to be added in large excess as some hydrolysis of EDC cannot be avoided and the amine is mostly protonated at the required pH values.

Some authors performed the amidation reaction in organic media such as DMSO[86,83]. This way, degrees of substitution of up to 60-80% were obtained, suggesting that EDC hydrolysis was minimized. HA however has to be previously converted from its native sodium salt form to its acidic form to be soluble in the organic solvent.

Some authors the cross-linking of HA-amine or HA-hydrazide derivatives obtained with commercially available homo- or heterofunctional crosslinkers[88,86]. These cross-linkers include bis(sulfosuccinimidyl)suberate (BS^3), 3,3'-dithiobis(sulfosuccinimidyl)propionate (DTSSP) or 2-methylsuberimidate (DMS). They react when in solution with HA-hydrazide derivatives at pH values above 5 or HA-amine derivatives at pH values above 8 due to the higher pK_a of the amino groups (see **Figures 14,15,16**)[86].

The drawback of such heavy HA functionalization is the biocompatibility of the resulting cross-linked gels. Indeed, HA derivatives are destined to be administered to the human body for therapeutic uses and will be degraded internally by natural enzymatic processes[32]. The generation *in situ* of potentially toxic degradation hydrazide products may result in side effects which need to be evaluated by *in vivo* methods[86].

No breakthroughs have been published since then concerning the amidation using carbodiimides. Authors use previsously described methods with EDC and NHS or HOBt to synthesize novel derivatives by the introduction of new functional compounds or by adapting the known methods to more complex systems such as liposomes (detailed in section 3 of this review).

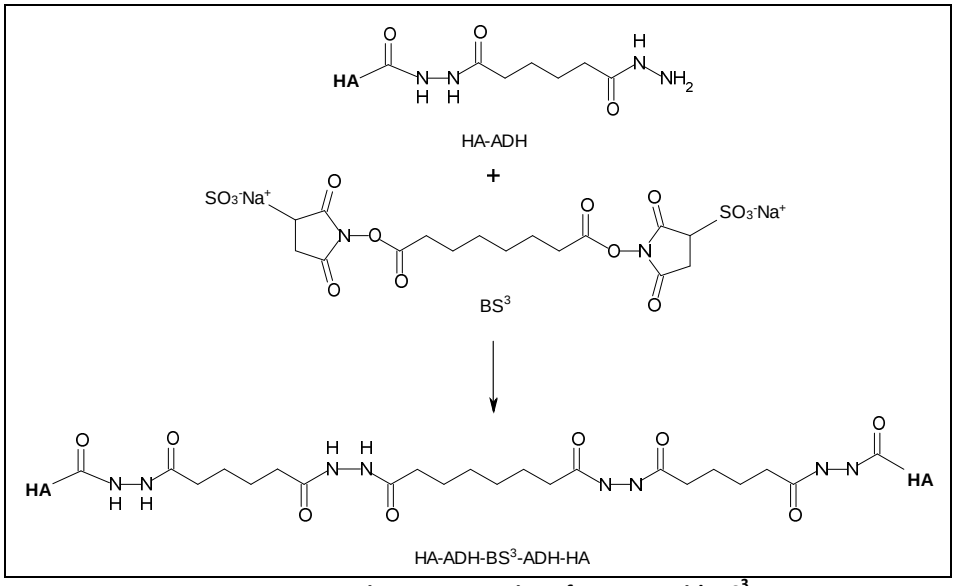

Figure 14: Reaction of HA-ADH with BS^3

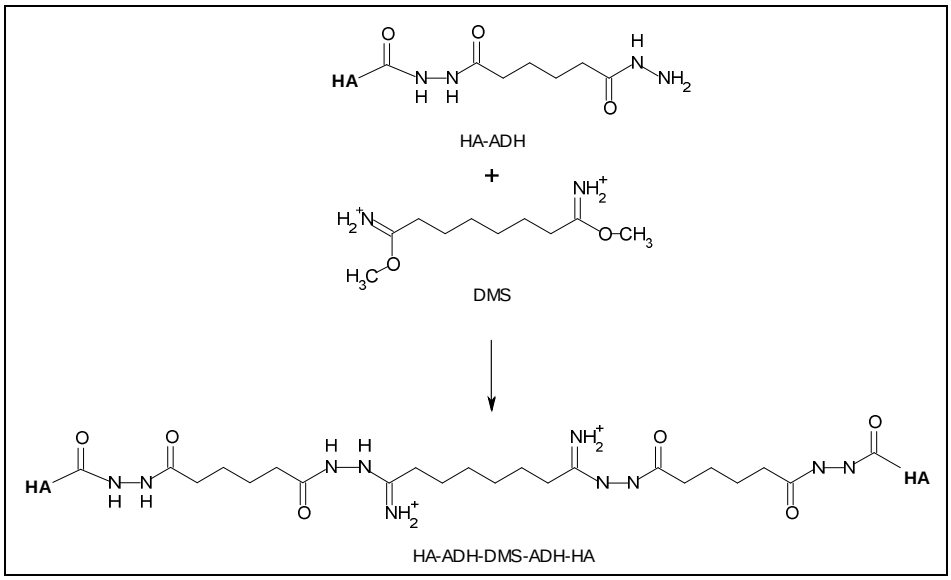

Figure 15: reaction of HA-ADH with DMS

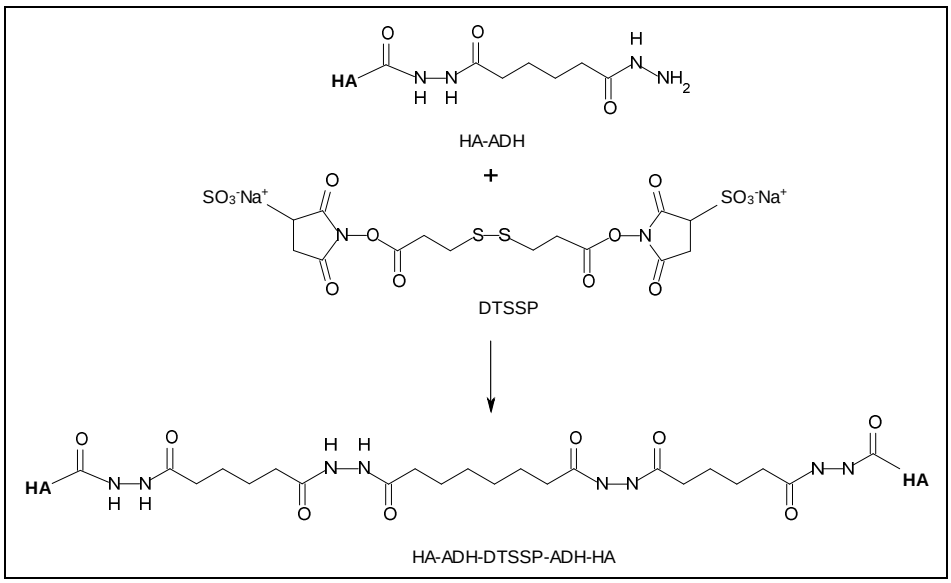

Figure 16: reaction of HA-ADH with DTSSP

2.1.1.2 Amidation with 2-chloro-1-methylpyridinium iodide (CMPI)

Magnani et al. (2000) described an amidation reaction using 2-chloro-1-methylpyridinium iodide (CMPI) as the activating agent of the carboxyl groups of HA[89]. This reaction is performed in an anhydrous organic solvent, DMF, to prevent fast hydrolysis of CMPI. The HA sodium salt must therefore be previously converted to a tetrabutylammonium (TBA) salt to allow its solubilization in organic solvent. 1,3-diaminopropane was used to form cross-links between the HA chains. Firstly, CMPI reacts with carboxyl groups of HA and forms a pyridinium intermediate. In this reaction, triethylamine is used to neutralize the hydrochloric acid during the reaction. The nucleophilic amine then attacks the activated HA carbonyl and forms the amide bond (**Figure 17**). ^{13}C-NMR studies showed that the calculated cross-linking degree was similar to the theoretical value, suggesting that 100% of the CMPI reacted with the corresponding carboxylic acid sites. This method is therefore highly effective using low amounts of reagents compared to the previous methods which use carbodiimides. The drawbacks of this method are its need to be performed in an organic solvent which requires a longer purification process and the addition of a previous step to convert HA sodium salt in TBA salt.

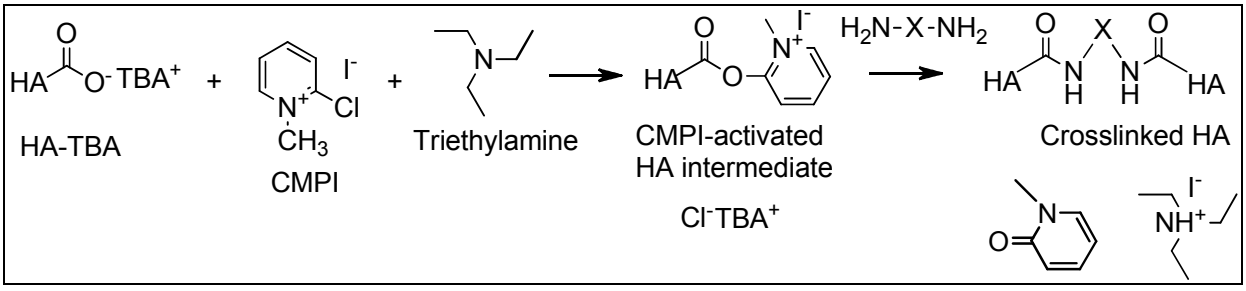

Figure 17: Crosslinking reaction of HA with diamines using CMPI

When no amine is added to the reaction media, the CMPI-activated HA reacts with its own hydroxyl groups, forming an ester crosslink between the HA chains (**Figure 18**). Such gels are called autocrosslinked gels. Della Valle patented the procedure performed in

dimethyl sulfoxide (DMSO)[90,92]. This reaction is however not as quick as the amidation as the hydroxyl groups are less nucleophilic than amino groups. The CMPI-activated carboxyl group of HA can also react with a non-activated carboxyl group but the resulting unstable anhydride subsequently reacts with a hydroxyl group to form the same ester crosslink. The unique feature of this technique compared to other crosslinking techniques is that no bridge molecules are present between the crosslinked HA chains. This ensures that only the natural components of HA are released during its degradation in the organism. Young et al. (2004) showed that such crosslinked HA hydrogels were much more rigid and resistant to *in vitro* enzymatic degradation when using CMPI rather than EDC, confirming the higher reactivity of CMPI[91].

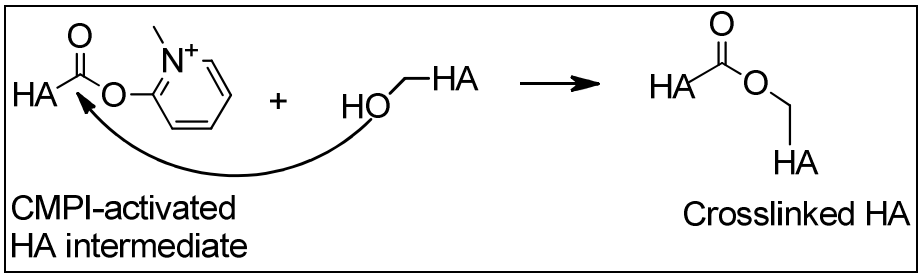

Figure 18: Crosslinking reaction of HA using CMPI alone

2.1.1.3 Amidation with 2-chloro-dimethoxy-1,3,5-triazine (CDMT)

Bergman et al. (2007) recently described a novel method for HA amidation using 2-chloro-dimethoxy-1,3,5-triazine (CDMT) as the activating agent of the carboxylic groups[80]. The reaction is performed in a mixed solvent with water and acetonitrile (3:2) for an optimal solubilization of the reagents. No intermediate such as NHS is needed for this amidation reaction in water mixture. CDMT firstly reacts with the carboxylic acid to form an HA-triazine intermediate. N-methylmorpholinium (NMM) is added to the mixture to neutralize the hydrochloric acid which is generated. The HA-triazine intermediate formed then reacts with the amine to form the amide bond (**Figure 19**). Up to 25% of substitution could be obtained

using only 2:1 of HA:CDMT amounts and the authors suggest that higher degrees can be obtained by increasing the amount of CDMT. This is a promising method to obtain high grafting yields while performing the reaction in an aqueous mixed solvent with mild conditions.

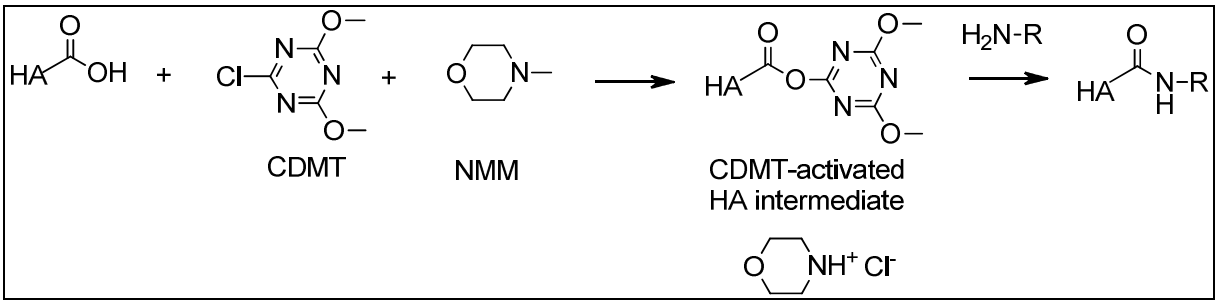

Figure 19: HA amidation with CDMT

2.1.1.4 Amidation with carbonyldiimidazole

HA amidation with 1,1'-carbonyldiimidazole as the activating agent of the HA carboxyl groups was patented by Fidia[93]. The reaction described is performed in DMSO from HA-TBA salt. Carbonyldiimidazole reacts with HA to form a highly reactive intermediate which quickly rearranges into a more stable HA-imidazole intermediate. This last intermediate reacts with an amine to form the amide bond (**Figure 20**). The described procedure is long as the formation of the imidazole intermediate takes 12 hours followed by the amidation reaction which takes 48 hours. However, the reaction does not release a strong acid but only CO_2 and imidazole which are non toxic compounds.

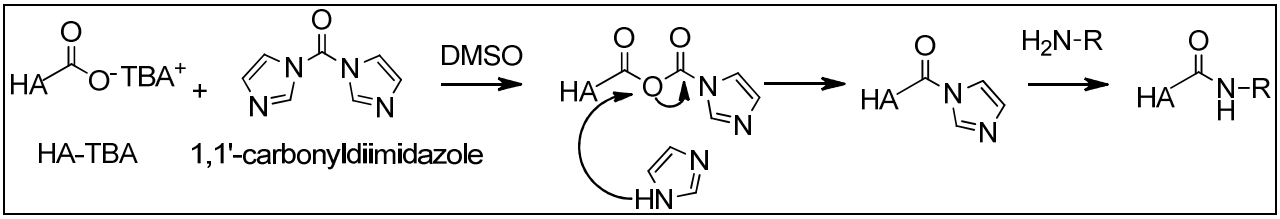

Figure 20: HA amidation with carbonyldiimidazole

2.1.2 Ugi condensation

Several authors described the Ugi condensation for HA cross-linking [95,96,97]. The method uses a diamine as a cross-linker to form diamide linkages between the polysaccharide chains. The reaction is performed in water at pH 3 with formaldehyde, cyclohexyl isocyanide and the crosslinker diamine. First, the diamine condenses with formaldehyde to form a protonated diimine which then reacts with the cyclohexyl isocyanide. The carboxyl group of HA then eliminates the activated cyanide intermediate to form an (acylamino) amide bond (**Figure 21**).

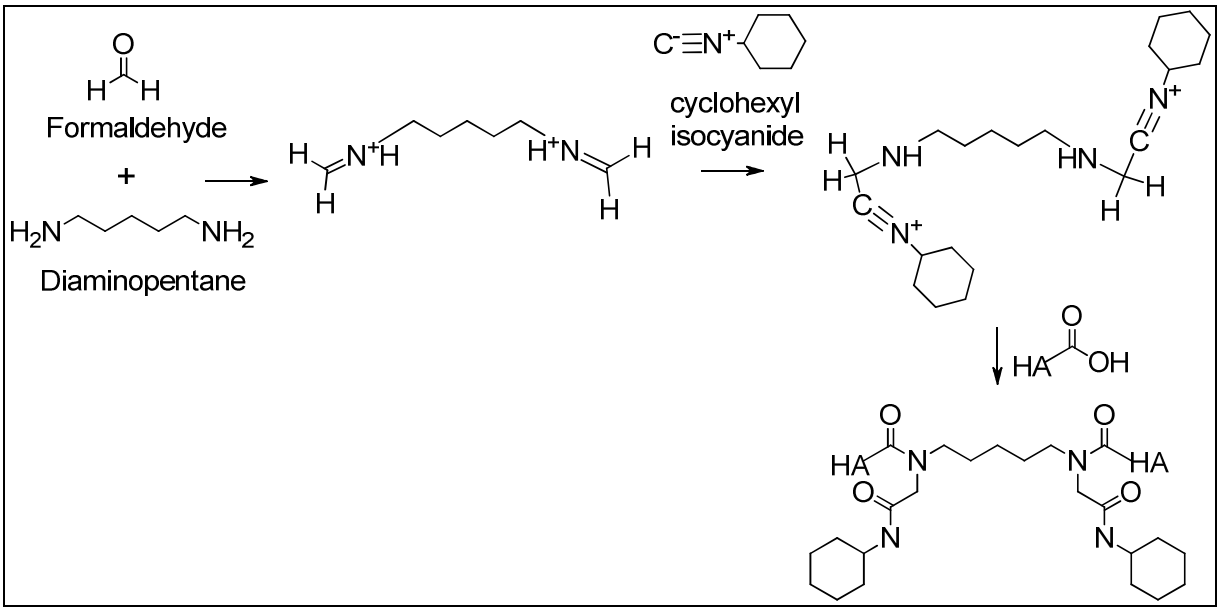

Figure 21: Ugi condensation reaction

The use of formaldehyde which is known to be toxic, allergenic and carcinogenic requires specific handling. However, this method enables to form a secondary amide, adding a second pending group, in here, a cyclohexyl.

2.1.3 Ester formation

2.1.3.1 Esterification by alkylation using akyl halides

Della Valle and Romeo (1986) patented the esterification by alkylation of HA carboxylic groups using alkyl halides such as alkyl iodides or bromides (**Figure 22**)[100]. The reaction was performed during 12 hours at 30°C. Pelletier et al. (2000) synthesized amphiphilic HA esters using the same method with bromide alkyls, performing the reaction during 24 hours[81]. The reaction has to be performed in DMSO meaning the native HA sodium salt has to be previously converted to its TBA salt.

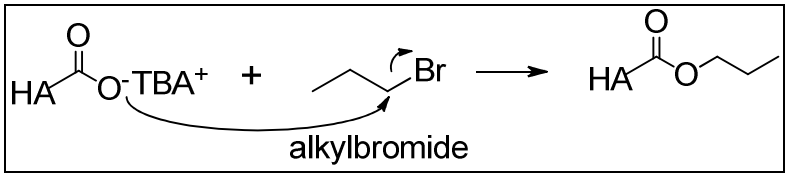

Figure 22: Esterification using alkyl halides

2.1.3.2 Esterification by alkylation using tosylate activation

Another example of the same esterification chemistry is using tosylate as the leaving groups as described by Huin-Amargier et al. (2006) [101]. HA crosslinking was performed by esterification using tetraethylene glycol functionalized by two tosylate groups (**Figure 23**). The reaction is performed in DMSO from the TBA salt of HA.

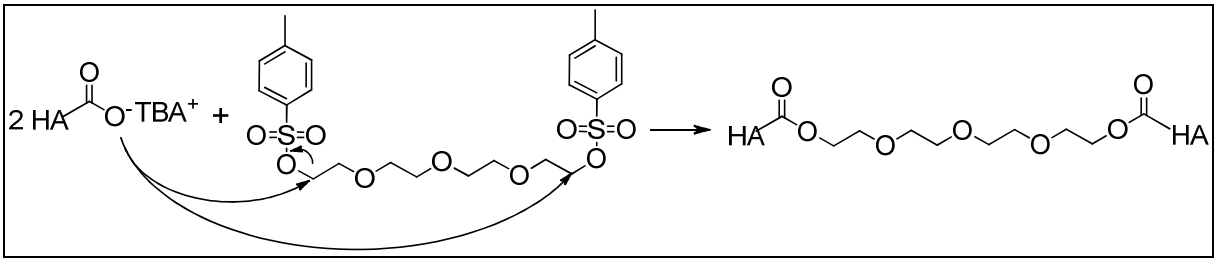

Figure 23: Esterification with tetraethylene glycol ditosylate.

2.1.3.3 Ester formation using diazomethane

Jeanloz and Forchielli (1950) were the first to report the esterification of HA using diazomethane[98]. The reaction is performed in an organic solvent, DMSO, from HA-TBA salt. Hirano et al. (2005)[99] described the preparation of methyl ester of hyaluronic acid using

trimethylsilyl diazomethane (TMSD) as the carboxylic group activator. TMSD reacts with HA to form an intermediate which then reacts with acetic acid to recover the methyl ester (**Figure 24**).

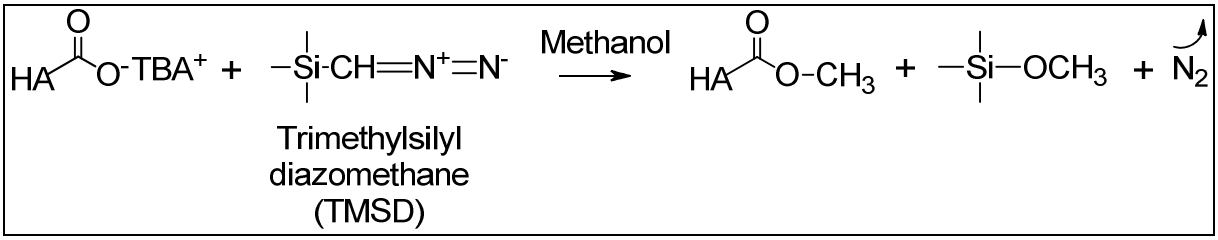

Figure 24: Esterification using trimethylsilyl diazomethane (TMSD)

2.1.4 Oxidization with sodium periodate

Aldehyde groups were introduced to HA by reaction with sodium periodate, which oxidizes hydroxyl groups of the D-glucuronic acid moiety of HA to dialdehydes, thereby opening the sugar ring[104]. Measurements indicated a decrease in molecular weight upon reaction from 1.3MDa for native HA to 260 kDa for the resulting HA–aldehyde. This method was used for the grafting of peptides on the aldehyde groups[105] or for cross-link with HA-hydrazide derivatives to form a vehicle for bupivacaine[104]. This reaction however leads to the significant decrease of HA molecular weight.

2.2 Modifications on the –OH

2.2.1 Ether formation

2.2.1.1 Ether formation using bisepoxides

Laurent et al. (1964) were the first to report HA cross-linking[106]. They used 1,2,3,4-diepoxybutane as the crosslinking agent and performed the reaction in strong alkaline conditions at pH 13-14 (0.2M NaOH and 0.1% sodium borohydride) at 50°C during 2 hours. Malson and Lindqvist (1986) patented the cross-linking of HA using butanediol-diglycidyl ether (BDDE) in 0.25M NaOH solution[107]. Piron (2002) later improved the method by mixing BDDE in the 0.25M NaOH solution before adding it onto the HA powder for a more homogeneous resulting hydrogel[108]. The reaction proceeds with the epoxide ring opening to create ether bonds with the HA hydroxyl groups (**Figure 25**). Other bisepoxides have been used to prepare crosslinked HA such as ethyleneglycol diglycidyl ether and polyglycerol polyglycidylether[109].

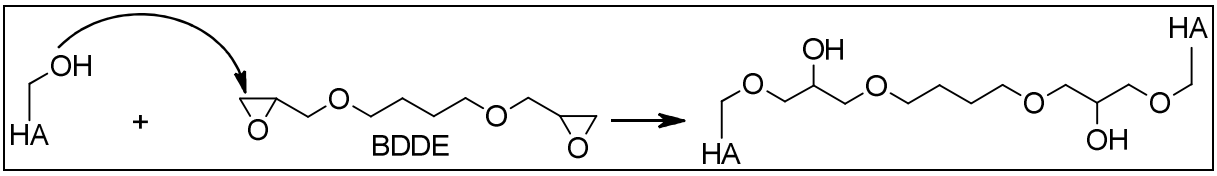

Figure 25: Crosslinking with BDDE in alkaline conditions (pH 13-14)

When HA is subjected to such high pH values (pH>13) above the pK_a value of the hydroxyl groups (approximately 10), the latter are almost all deprotonated and is thus more nucleophilic than the unprotonated carboxyl groups. The epoxides therefore react preferably with the hydroxyl groups to form ether bonds. However, when the pH is lower than the pK_a value of the hydroxyl group, a smaller quantity of hydroxyl groups is unprotonated and the anionic carboxyl group is predominant, thus promoting ester bond formation. This has been demonstrated by De Belder and Malson (1986), who performed the

crosslinking of HA with BDDE in acidic conditions (pH 2-4.5)[102](**Figure 26**). Infrared measurements revealed the presence of ester bands which were not observed for hydrogels obtained in alkaline conditions.

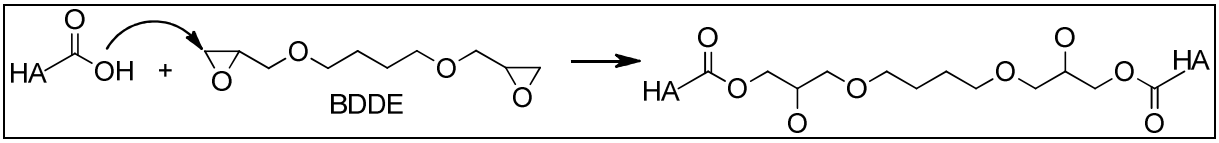

Figure 26: Crosslinking with BDDE in acidic conditions (pH 2-5) as suggested by De Belder and Malson (1986)[102]

Tomihata and Ikada (1997) however observed the formation of ether and not ester bonds even when the crosslinking with bisepoxides is performed in acidic conditions[103]. The pH values used were 4.7, 6.1 and 8. At such values, a higher quantity of hydroxyl groups is deprotonated than at pH values lower than 4.5. The IR absorption observed by the same authors at 1700cm^{-1} for HA crosslinked with carbodiimide which corresponds to ester bonds was not observed for the crosslinking with the bisepoxide. By performing the same reaction with the bispeoxide but replacing HA by poly(vinyl alcohol) (PVA), which contains only hydroxyl groups and no carboxyl groups, the formation of a cross-linked hydrogel occurred. A peak at 1150cm^{-1} in the IR spectra corresponding to C-O bonds could confirm the ether linkage but could also be from the open epoxide.

BDDE is today used for most crosslinked HA hydrogels currently on the market. In addition to its easy synthesis, HA-BDDE degradation products have shown no cytotoxicity and epoxide compounds are hydrolyzed into simple diols[130].

Zhao patented a double cross-linking method based on the consecutive reaction of epoxides in alkaline conditions (pH 10) followed by acidic conditions (pH 4)[110]. The epoxides used were epichlorohydrin and 1,2,7,8-diepoxyoctane.

2.2.1.2 Ether formation using divinyl sulfone

HA crosslinking with divinyl sulfone (DVS) was patented by Balazs and Leshchinger (1986)[111]. The reaction is performed at high pH values (0.2M NaOH, pH>13) and creates sulphonyl bisethyl linkages between the hydroxyl groups of HA (**Figure 27**). This crosslinking method has the advantage to occur at room temperature, which limits the degradation of HA in alkaline solutions compared to higher temperatures. Balazs and Leshchinger showed that the reaction starts shortly after addition of DVS (5 to 10 minutes) and one hour is sufficient for the completion of the reaction. They also found that the presence of salts such as NaCl in the reaction media increased the crosslinking degree. Studies by other authors confirmed the efficiency of the crosslinking method with DVS[112,113]. However, Eun et al. (2008) showed that HA-DVS crosslinked gels were degraded more rapidly than HA-ADH-BS[3] hydrogels crosslinked by the carboxylic groups of HA[114]. Eventhough the starting material DVS is highly reactive and toxic, the biocompatibility of the HA-DVS hydrogels were confirmed by histological analysis.

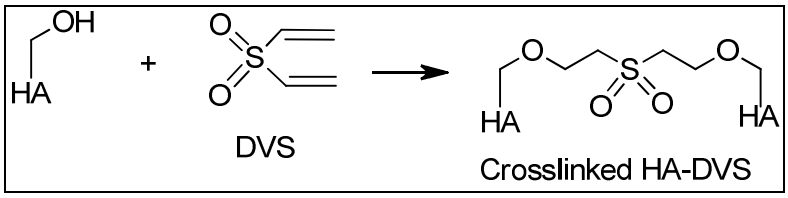

Figure 27: Crosslinking with divinyl sulfone (DVS)

2.2.1.3 Ether formation using ethylenesulfide

Serban et al. (2008) used ethylenesulfide (also known as thiirane) to synthesize 2-thioethyl ether HA derivatives (**Figure 28**)[115]. The ethylene sulfide ring is opened by nucleophilic attack of the HA hydroxyl group with the addition of dithiolthreitol (DTT). The reaction is performed at pH 10 overnight and at pH 8.5 after addition of DTT during 24 hours. The authors state that if it the carboxylic group of HA reacts with ethylene sulfide, the unstable intermediate rearranges into a carboxylic group and reforms ethylene sulfide. The

presence of the grafted thiol groups which cannot undergo further crosslinking showed to act as a radical scavenger, protecting cells from reactive oxygen species.

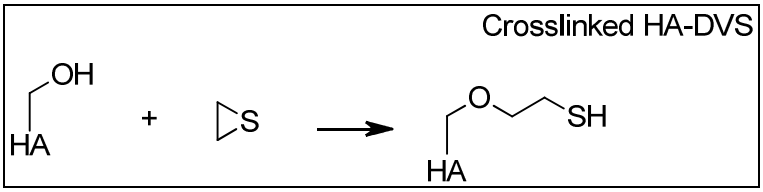

Figure 28: HA modification with ethylene sulfide

2.2.2 Hemiacetal formation using glutaraldehyde

Glutaraldehyde (GTA) was used for cross-linking of HA[116,117]. Tomihata and Ikada (1997) demonstrated the formation of hemiacetal bonds between the hydroxyl groups of HA (**Figure 29**) by IR measurements and by using the same procedure on a polymer which contained only hydroxyl groups and also formed the crosslinked gel[116]. The authors observed that the reaction can be performed in an acetone-water media but not in an ethanol-water media, suggesting the inhibition of the cross-linking reaction by the side reaction with the hydroxyl groups of ethanol. Glutaraldehyde crosslinking needs to be initiated in acidic media (pH 2) to activate the aldehyde and catalyze the reaction. However, the hemiacetal bond can be hydrolyzed and recover the starting materials in acidic conditions. Indeed, Collins and Birkinshaw (2007) demonstrated the GTA-crosslinking to be instable and achieved to stabilize the hydrogel by neutralizing it through swelling in buffer[112]. Glutaraldehyde has the disadvantage of being toxic, requiring specific handling during the reaction and thorough purification of the final product.

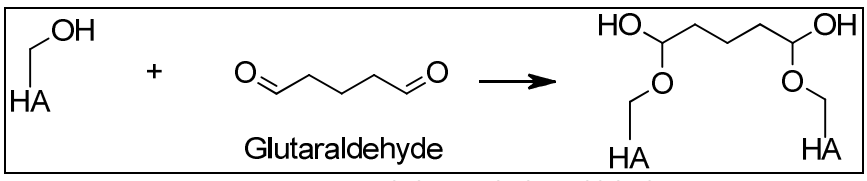

Figure 29: HA crosslinking with glutaraldehyde

2.2.3 Ester formation

2.2.3.1 Ester formation using octenyl succinic anhydride (OSA)

Tømmeraas & Eenschooten (2007) patented a HA modification method using alkyl succinic anhydrides such as octenyl succinic anhydride (OSA) under alkaline conditions (pH 9) in water[118]. The hydroxyl groups of HA react with the anhydride to form ester bonds (**Figure 30**). Eenschooten et al. (2010) later optimized the reaction parameters using experimental design[119]. Values of 43% of substitution could be obtained using 50 times more OSA than HA. However, the reaction was quite fast, 18% of substitution was obtained after only 6 hours.

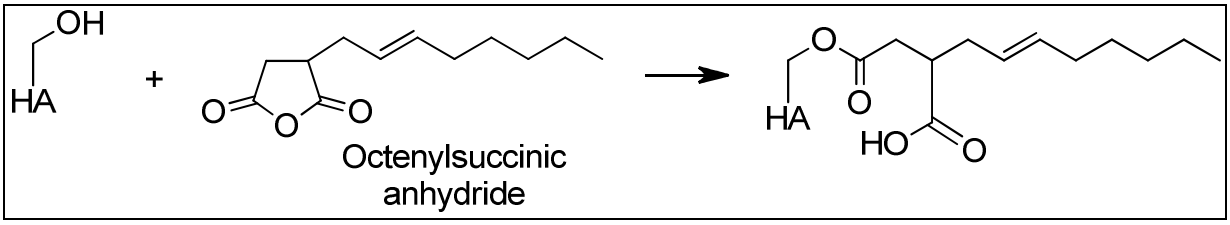

Figure 30: HA modification with octenylsuccinic anhydride (OSA)

2.2.3.2 Ester formation with activated compounds

Pravata et al. (2008) described a novel method for grafting an acyl-chloride activated carboxylate compound onto the hydroxyl groups of HA to form ester bonds (**Figure 31**)[120]. The carboxyl groups of the compound to be grafted were previously activated by chloroacylation with thionyl chloride and reacted at room temperature with HA in organic solvent. In their study, the authors used this method to graft poly(lactic acid) (PLA) oligomers. As the reaction was performed in an organic solvent (DMSO), HA was previously converted to a cetyltrimethylammonium bromide (CTA) salt. HA-CTA was more hydrophobic and easier to prepare than HA-TBA using a one-step reaction with CTA-bromide.

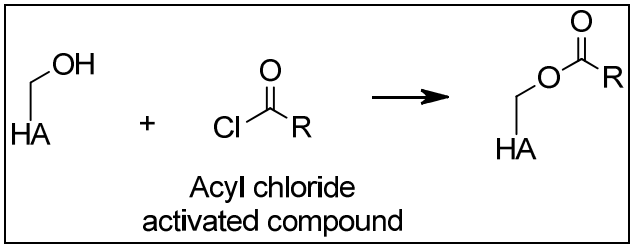

Figure 31: Modification with an acyl chloride activated compound

2.2.3.3 Esterification with methacrylic anhydride

Esterification of methacrylic anhydride was performed to obtain methacrylated HA (**Figure 32**)[121]. The reaction is performed in ice cold water during 12 hours at pH 8 to 10. The methacrylated-HA product was further cross-linked by exposure to UV rays at 365nm in the presence of a precursor solution.

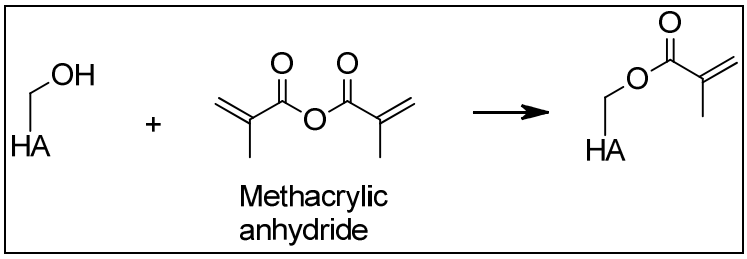

Figure 32: HA esterification with methacrylic anhydride

2.2.4 Carbamate formation

2.2.4.1 Activation with cyanogen bromide

Micochova et al. (2006) describes the synthesis of HA alkyl derivatives by using cyanogen bromide (CNBr) to activate the hydroxyl groups of HA[122]. An activated HA cyanate ester is formed which reacts with the amine to form mainly N-substituted carbamate bonds (**Figure 33**). This reaction has the advantage of being performed in water from the native HA sodium salt. High degrees of substitution up to 80% are achieved using only slight excess of reagents and in only one hour reaction time. However, a high pH (up to 10) is needed for the coupling to occur which showed to lower the molecular weight of the HA polymer chain, indicating

degradation. Chytil and Pekar also used this method to synthesize a wide range of HA-alkyl derivatives with tunable properties[123].

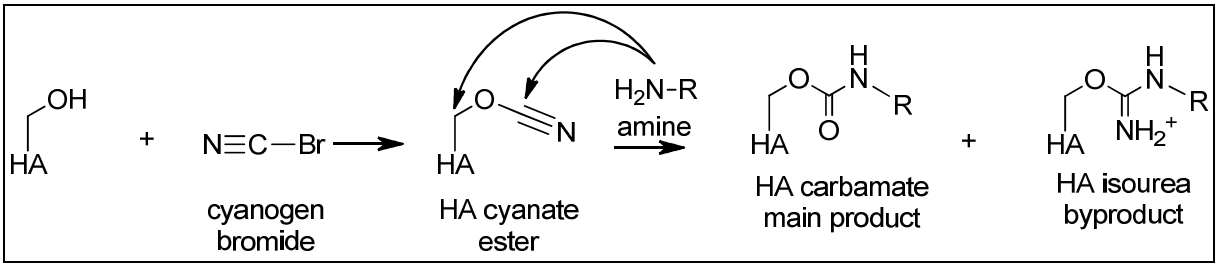

Figure 33: HA activation with cyanogen bromide (CNBr) and carbamate formation

2.3 Modifications of the –$NHCOCH_3$

Deacetylation of the N-acetyl group of HA recovers an amino group which can then react with an acid using the same amidation methods described above in section 2.1.1. Deacetylation is usually performed using hydrazine sulfate during five days at 55°C, which however induces severe chain fragmentation[93]. Even slightly milder treatments described since then have been reported to induce HA chain degradation via β-elimination of the glucuronic moiety[86,124,125].

Bellini and Topai (2000) patented the amidation of HA by reaction of an acid with the deacetylated amino-group of HA[93]. The acid was previously activated using a carbodiimide. The deacetylated HA amine reacts with the activated acid and forms the amide bond (**Figure 34**). Oerther et al. (2000) used this method to crosslink HA with the carboxylic groups of alginic acid[126]. Crescenzi et al. (2002) used deacetylated HA for further crosslinking using the Ugi condensation[125](**Figure 21**). The deacetylated amino groups reacted with the carboxylic groups of HA to form an autocrosslinked hydrogel. Platt and Szoka (2008) mention the possibility to use enzymes for HA deacetylation, which was previously performed on the N-acetylglucosamine moiety of heparin and heparan sulfate[65,131].

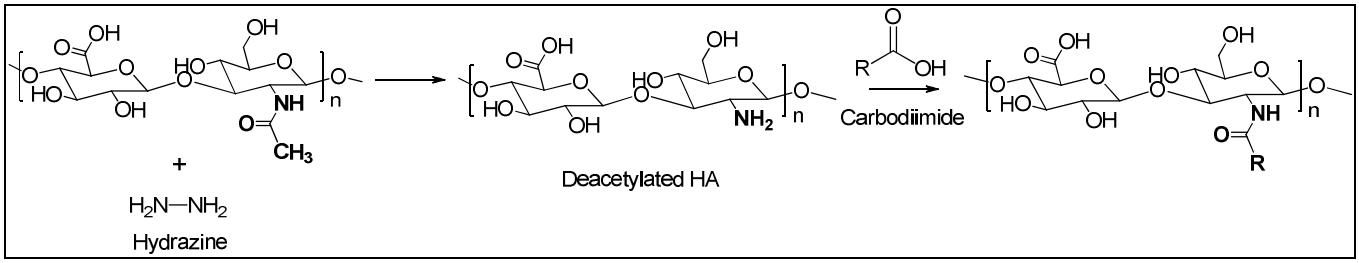

Figure 34: HA deacetylation and amidation

2.4 In situ crosslinking

Shu et al. (2002) formed hydrogels obtained by HA disulfide crosslinking[132]. Firstly, thiol-modified HA was prepared using carbodiimide-mediated hydrazide chemistry, and then HA hydrogels were formed under physiological conditions by air oxidation of thiols to disulfides. The crosslinking reaction is performed without the use of crosslinking agent and therefore does not produce any by-products.

Kurisawa et al. (2005) synthesized HA *in situ* crosslinkable hydrogels by grafting tyramine on the carboxyl groups of HA using EDC and HOBt which could further be coupled by treatment with horseradish peroxidase and H_2O_2[133]. However, to form the hydrogel *in situ*, two syringes have to be used, one containting HA-tyramine and H_2O_2 and the second one containing horseradish peroxidase to induce the crosslinking reaction. The *in situ* crosslinked HA hydrogels obtained were nevertheless biocompatible.

Oh et al. (2010) recently stated that HA hydrogels can be easily obtained by mixing two different HA derivatives containing functional groups which are reactive towards one another[87]. For example, HA-aminoethyl methacrylate or HA-aminopropyl methacrylamide comprise double bonds which react with HA containing thiol groups such as HA-cysteamine (**Figure 35**). No additional reagent is required for the reaction and no toxic byproducts are formed, which makes it a suitable method for *in situ* formation of HA hydrogels.

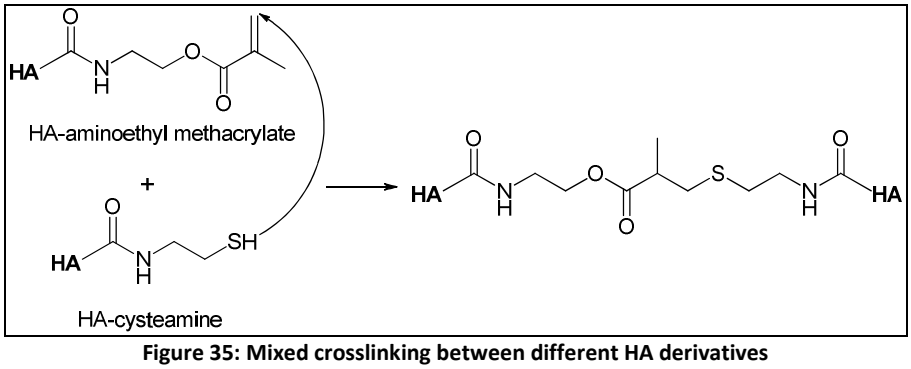

Figure 35: Mixed crosslinking between different HA derivatives

3. HA derivatives

Given the large amount of HA derivatives described in the literature, only some examples of each type of derivative are reviewed here. This section is aimed to be a tool for the design of novel HA-drug conjugates or other HA derivatives. The existing methods for HA-drug conjugation or HA modification reviewed here can potentially be applied to new drugs needing drug delivery improvements.

3.1 HA chemical conjugation

3.1.1 HA-drug conjugates

Conjugation of drugs to HA has been reported as early as 1991 for the improvement of drug delivery[70]. This technique aims at forming a pro-drug by covalently binding the drug to HA. The drug is released once the covalent bond is broken down in the organism, ideally at the specific target site. Vercruysse and Prestwich (1998) reviewed studies showing that by conjugating drugs to HA, their therapeutic effect was enhanced[71].

Pouyani and Prestwich (1994) described the grafting of ibuprofen, a nonsteroidal anti-inflammatory drug and hydrocortisone, a steroidal anti-inflammatory drug intended for local injections into joints suffering arthritis[88]. HA was previously conjugated with adipic dihydrazide (ADH) and ibuprofen was converted to an activated ester using a carbodiimide and NHS. Both intermediates then reacted to form a hydrazide bond (**Figure 36**). Hydrocortisone was grafted to HA-ADH via its hemisuccinate derivative previously converted to an activated ester in the same way as ibuprofen (**Figure 37**). This way, an ester linkage is formed between HA and hydrocortisone which can be more easily hydrolyzed in the organism by enzymatic mechanisms to release the starting hydrocortisone.

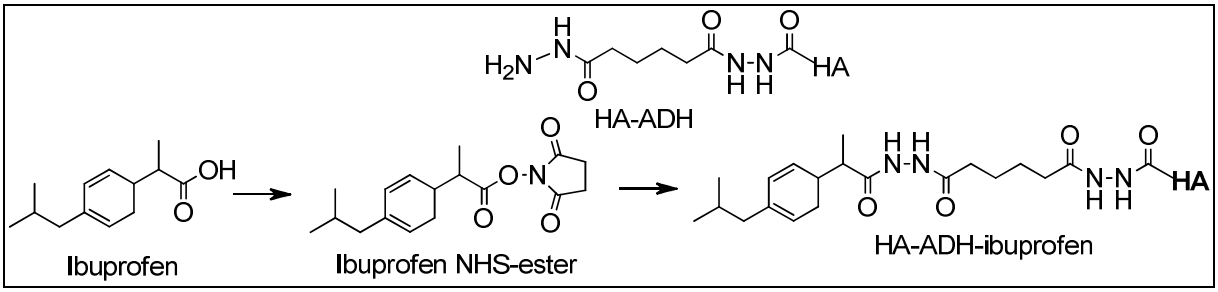

Figure 36: Conjugation of ibuprofen onto HA-ADH

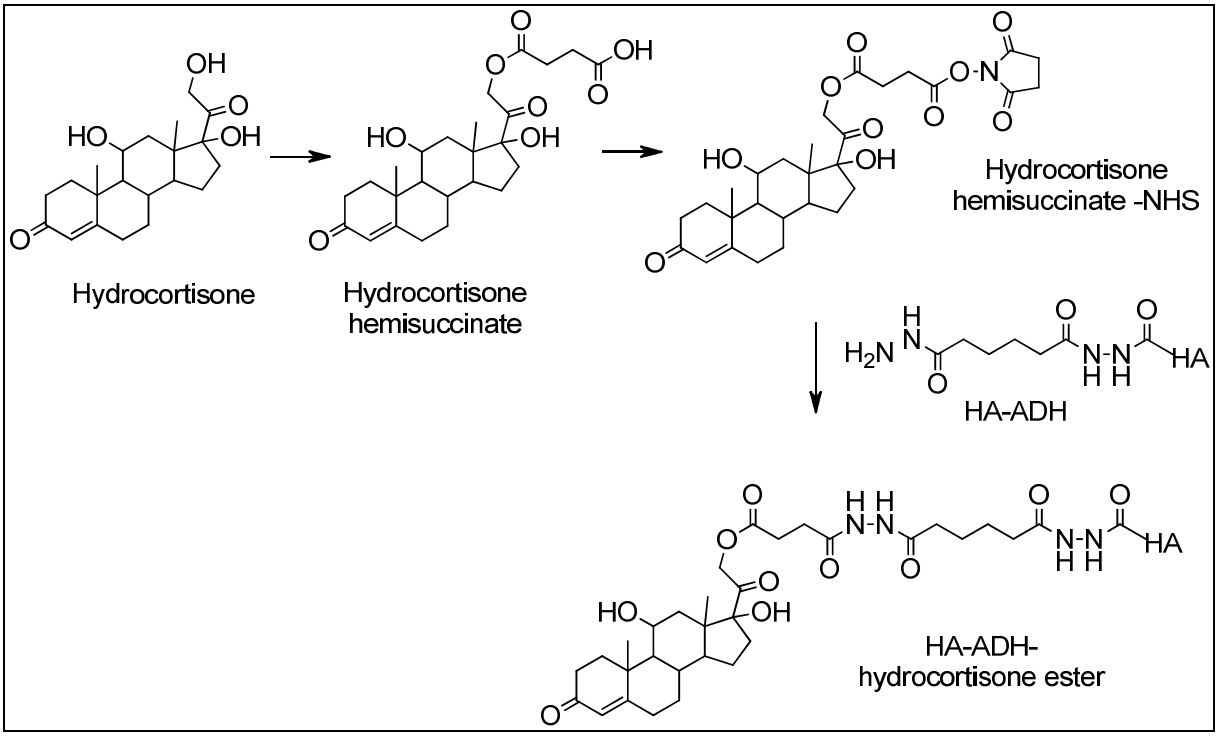

Figure 37: Conjugation of hydrocortisone hemisuccinate onto HA-ADH

Paclitaxel has been conjugated to HA similarly than hydrocortisone via its hemisuccinate NHS activated ester onto HA-hydrazide[134]. Paclitaxel is a very poorly soluble antimitotic chemotherapeutic agent which leads to tumor cell death by disrupting mitosis; its solubility was greatly increased by conjugation to HA. In addition, studies suggest that, this way, paclitaxel could be released inside cells by intracellular enzymatic hydrolysis of the ester bond to carry out its activity[135].

In a similar way, proteins were conjugated to HA-hydrazide[136]. The hyaluronic acid-protein conjugates showed longer sustained blood levels than formulations containing the protein alone.

Drugs are usually conjugated to HA when using carbodiimide combined to adipic dihydrazide to form the HA-hydrazine intermediate. Indeed, it was presumed by Vercruysse and Prestwich (1998)[71] that drugs are not covalently grafted to native HA by using carbodiimide alone due to the formation of N-acylureas byproduct, as stated in section 2.1.1.1.

However, Xin et al. (2010) recently described the conjugation of paclitaxel onto HA using carbodiimide alone via amino acid linkers[137]. An amino acid was previously grafted by its carboxylic group onto a hydroxyl group of paclitaxel. The intermediate was then conjugated by the amino group of the amino acid onto the carboxylic group of HA using EDC and NHS activation in DMF (**Figure 38**). Hydrolysis of the carbodiimide was minimized by the use of an anhydrous organic solvent and degrees of substitution of 10 to 15% were obtained. The authors confirmed the formation of nanoparticles in aqueous solution of the HA-amino acid-paclitaxel conjugate due to its amphiphilic nature. The hydrophobic paclitaxel is likely to be entraped in the hydrophilic HA outer shell. A higher drug release was observed relative to the HA-ADH-paclitaxel conjugate described by Luo and Prestwich (1999)[134]. The authors suggest that this difference is due to the electron effect of the protonated amino group which promotes the ester bond hydrolysis. Another hypothesis could be the facilitated recognition of the esterase enzymes thanks to the presence of an amino acid. This method offers new perspectives for HA-drug conjugation using different linkers more “biological-like” with tunable release rates.

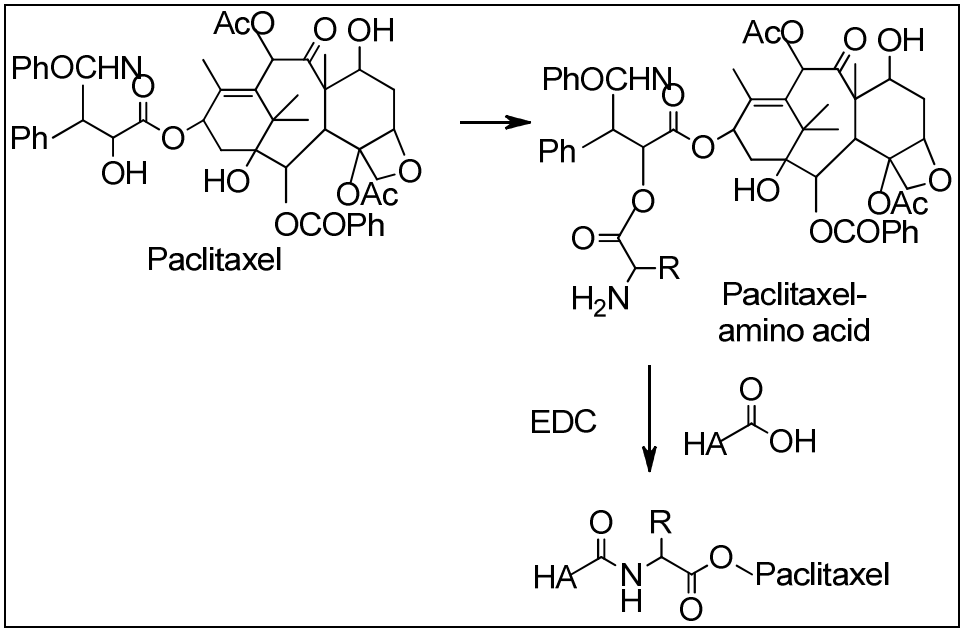

Figure 38: HA-Paclitaxel conjugation via an amino acid linker

Drugs such as anthracycline antibiotics comprising an amino group can also be conjugated on the hydroxyl groups of HA using cyanogen bromide activation as described in section 2.2.4.1 (see **Figure 33**)[138]. Like the hydrazide bonds, the carbamate bonds which are formed are not enzymatically degraded unlike the ester bonds. In addition, by grafting the drug on the hydroxyl groups, the carboxylic groups of HA are unmodified, thus preserving HA's natural recognition and biodegradability by enzymes, which is advantageous for cell internalization of the HA-drug conjugate. Drug release should therefore by faster relative to drugs conjugated on the carboxyl groups. Indeed, if HA acts as a protective shell around the conjugated drug, the faster it is degraded, the faster the drug is released.

Other drugs comprising a hydroxyl group were conjugated to HA by esterification such as cortisone, hydrocortisone and fluorocortisone, patented by Fidia[100]. HA-methylprednisolone conjugation is another example[139]. The drugs were grafted from their bromide form as described in section 2.1.3.1 (**Figure 22**). It would be interesting to compare the drug release rate of hydrocortisone grafted via an ester bond and via a hydrazide spacer, but such studies have not been reported.

Leonelli et al. (2005) suggested another method for conjugating paclitaxel to HA by esterification via a 4-bromobutanoic acid derivative of paclitaxel[140]. This derivative reacted with HA-TBA in N-methyl-2-pyrrolidone (NMP) during 7 days. This solvent is however more toxic than DMF and the reaction is very long. Rosato et al. (2006) reported that this HA-paclitaxel conjugate had a much stronger antitumor activity than the free drug[141].

Recently, Platt and Szoka (2008) reviewed many studies on HA-drug conjugates which aim at specifically targeting drugs to the CD44 cell receptors to allow their internalization by cells and subsequent intracellular therapeutic action[65]. As HA is the main substrate of CD44 cell surface receptors, the conjugated drug will be simultaneously internalized with HA and be released inside the cell once the covalent bond is hydrolyzed by intracellular enzymes[135,142]. This property especially unique for cancer therapy as tumor cells have been shown to over-express such CD44, allowing to specifically target drugs to tumors[65].

Coradini et al. (1999) conjugated butyric acid, a tumor-cell growth inhibitor, to HA which showed to enhance its cellular uptake and thus its antitumor activity[142]. The reaction was performed in DMF from butyric anhydride in the presence of pyridine or dimethyl amino pyridine. The anhydride reacts with the hydroxyl groups of HA and forms ester bonds the same way as with methacrylic anhydride described in section 2.2.8 (**Figure 39**).

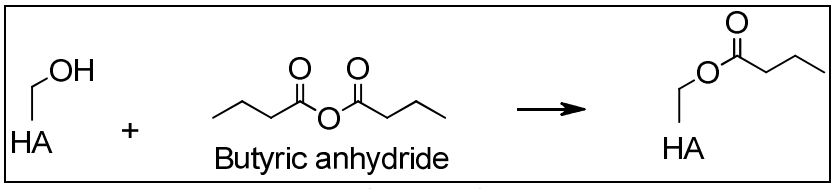

Figure 39: HA butyric acid conjugation

Cai et al. (2008) studied the conjugation of cisplatin onto HA in order to lower its organ toxicity[143]. Indeed, one problem associated with platinum-based chemotherapies such as

cisplatin is its high renal toxicity. Cisplatin was grafted on the carboxyl groups of HA with the use of silver nitrate as the activating agent (**Figure 40**).

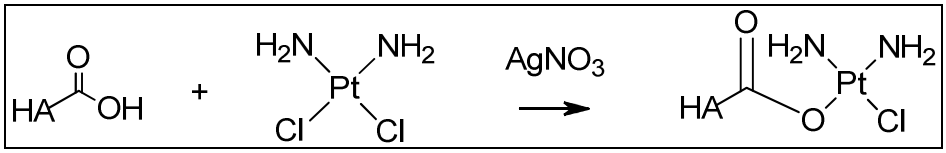

Figure 40: HA-cisplatin conjugation

Recently, Homma et al. (2009) described the synthesis of hyaluronic acid-methotrexate conjugates to be used in the treatment of osteoarthritis[144]. In order to minimize its important side effects, methotrexate was linked to HA through a small peptide sequence and a PEG-diamine spacer, with binding ratios of 1-3%. Firstly, a methotrexate-peptide-PEG-amine was synthesized by a multi-step reaction using carbodiimide chemistry in DMF. The terminal amino group of the compound then reacted with the carboxyl groups of HA using EDC and HOBt in a tetrahydrofurane (THF) and water mixture. The authors observed that the peptide chain was necessary for the drug efficacy, suggesting it promoted enzyme recognition of the conjugate, essential for the drug to be released and biologically active. The linker was also added to promote enzyme access to the peptide chain. A further study of this method determined the optimal peptide chain to be phenylalanine-phenylalanine, the linker to be ethylenediamine and the bonding ratio to be higher than 1.3%, ideally 3.8%[145] (**Figure 41**).

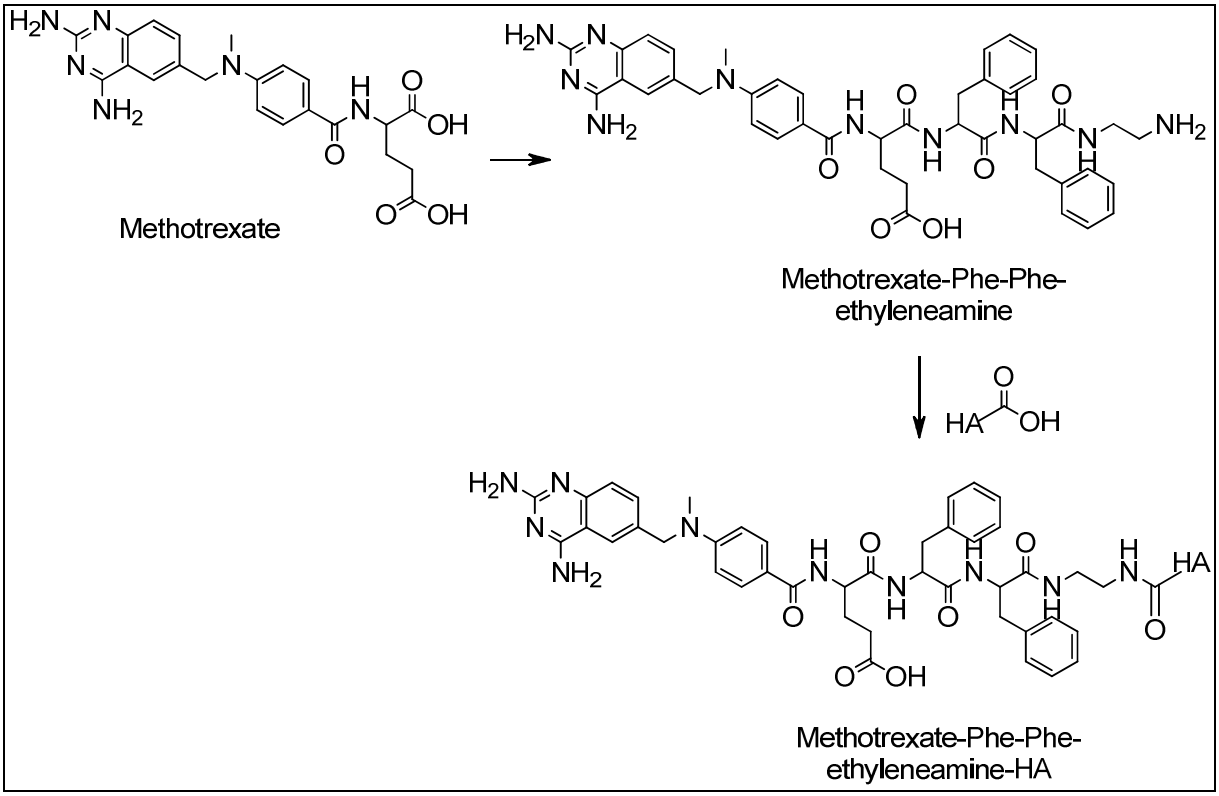

Figure 41: HA-methotrexate conjugation via Phe-Phe peptide and ethylenediamine

Kong et al. (2010) also recently presented a novel conjugation method of exendin 4, an antidiabetic peptide, onto HA via a vinyl sulfone cysteamine intermediate[146]. Vinyl sulfone cysteamine was firstly prepared from divinyl sulfone (DVS) and cysteamine hydrochloride. The amino group then reacted with HA-TBA salt in DMSO using a benzotriazol compound and diisopropylethylamine to form HA-vinyl sulfone cysteamine by amidation. The vinyl group of the intermediate then reacted with the terminal cysteine group of exendin 4 to form a covalent linkage (**Figure 42**). The resulting conjugate showed prolonged half-life and improved glucose-lowering capabilities relative to the free drug.

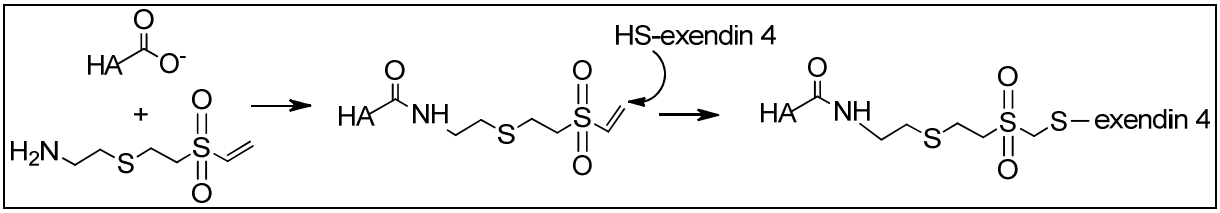

Figure 42: HA-exendin 4 conjugation via vinyl sulfone

Kim et al. (2003) reported the preparation of crosslinked HA-DNA matrix formulations intended for use as gene delivery systems[147]. Crosslinking was performed using adipic hydrazide and EDC. The resulting matrices showed to gradually release DNA, being advantageous compared to non-protected HA or liposomes which have faster release profiles.

3.1.2 HA conjugation to carrier systems and medical devices

3.1.2.1 HA conjugation to liposomes

Yerushalmi et al. (1994) reported the coating of liposomes by covalent conjugation of HA[148]. The carboxyl groups of HA were linked to the amino group of phosphatidylethanolamine of liposomes using the carbodiimide chemistry (**Figure 43**). The authors used epidermal growth factor as the test drug encapsulated in the liposomes. Since then, several authors have worked on the conjugation of HA on liposomes. HA can be conjugated to phospholipids prior to the liposome formation or after on the outside shell. Peer et al. (2004) showed that such HA coated liposomes carrying anticancer agents such as doxorubicin or mitomycin C increased circulation time over non-coated liposomes as well as drug accumulation in tumor cells, decreased systemic toxicity, and tumor growth[68,69]. Taetz et al. (2009) recently described the use of HA-liposomes conjugates for the delivery of siRNA to lung cancer cells[149].

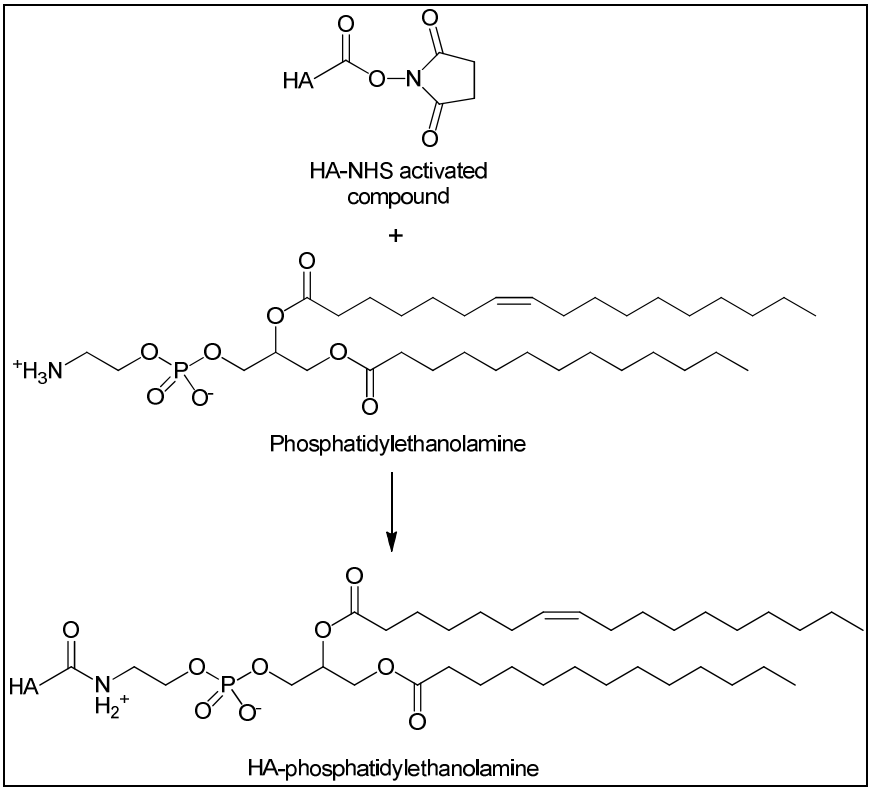

Figure 43: HA conjugation to liposomes

3.1.2.2 HA conjugation to nanoparticles

Recently, Rivkin et al. (2010) described the encapsulation of paclitaxel into phosphoethanolamine lipids followed by covalent conjugation of HA[150]. The clusters formed showed to be effective as selective tumor-targeted nanovectors. HA was covalently conjugated to polylactide-co-glycolide (PLGA) via a polyethylene glycol (PEG) spacer to form nanoparticles as drug carrier systems [151,152]. Both PLGA and HA were previously activated with EDC and NHS, then mixed together with the diamine linker. The particles were loaded with doxorubicin and showed increased efficacy compared to nanoparticles of PLGA alone.

3.1.2.3 HA coatings of medical devices

Schneider et al. (2007) described layer-by-layer films comprising native HA and cationic amine-modified HA[83]. The native HA was firstly converted to its acidic form and dissolved in DMSO to react with a monoprotected ethylenediamine using EDC and NHS. After deprotection of the second amino group, the cationic amine-modified HA was

obtained. Multilayer films were formed using the layer-by-layer technique described by Decher et al. (1992)[153]. A positively charged surface such a thin glass slide is successively immersed in a solution containing polyethylene imine, anionic native HA and cationic amino-modified HA. The two top HA layers were then crosslinked by adding an EDC/NHS solution onto the film surface. This provided stronger and more resistant multilayer films on which cells showed good adhesion and viability compared to non-crosslinked films. Richert et al. (2004) had previously reported the improvement of cell adhesion and resistance of crosslinked HA-poly(L-lysine) films for surface modification of biomaterials[154]. The rigidity of the crosslinked films showed to strongly account for improved cell adherence. Multilayer films are promising materials for coating of medical devices such as soft prosthetic implants.

3.1.3 HA conjugates (crosslinked or modified) as filling agents for supplementation

The most important commercial uses of HA derivatives are for viscosupplementation into joints and dermal filling for cosmetic applications. Most commercially available injectable HA hydrogels are crosslinked with butanediol diglycidyl ether (BDDE)[155]. With the aim to obtain derivatives with longer residence time after injection, techniques for the synthesis of HA derivatives are continuously explored. For example, Collins and Birkinshaw (2007) compared different crosslinking agents to identify the most effective ones[112]. Divinyl sulfone crosslinked HA exhibited good swelling properties. As another example, Zhao (2000) patented a HA hydrogel subjected to two consecutive crosslinking reactions with bisepoxides, the first at high pH (10) and the second at low pH (4)[110]. The resulting double crosslinked hydrogels showed lower water absorption capacities and less degradation when subjected to hyaluronidase digestion than the single cross-linked hydrogels. Other hydrogels described include *in situ* crosslinkable HA derivatives[156] and various hydrogels crosslinked with techniques reviewed in the previous section.

As an alternative to chemically crosslinked hydrogels, Borzacchiello et al. (2010) synthesized amphiphilic HA derivatives by grafting a long alkyl chain (hexadecylamine) on its backbone[94]. The resulting associative polymer forms strong interactions between the hydrophobic chains which are disrupted when subjected to a strong force, typical of an elastic behavior. The HA-hexadecylamide obtained with this method therefore behaves like a gel compared to the starting viscous fluid of native HA, a promising technique to obtain physically crosslinked hydrogels.

3.1.4 HA conjugates (crosslinked or modified) as drug carrier systems

Choi et al. (2009) reports the chemical conjugation of the hydrophibic 5β-cholanic acid via its aminoethyl-amide form onto HA using EDC and NHS in DMF[157]. The resulting amphiphilic derivative forms nanoparticles in physiological conditions and showed prolonged blood circulation and high affinity to tumor cells *in vitro*. These nanoparticles are therefore promising drug carriers for tumor-targeted drugs.

HA nanogels for siRNA delivery were described by Lee et al. (2007)[158]. Firstly, thiol-HA was prepared using the carbodiimide method with cystamine followed by dithiothreitol (DTT) treatment to cleave the disulfide bonds. Crosslinked HA-nanogels were then generated by an inverse emulsion method from the thiol-HA solution containing siRNA during which the disulfide bonds are formed. The encapsulation method showed not to damage the siRNA and to allow its uptake by CD44 expressing cells.

Microspheres prepared from HA esters, obtained using alkyl halides, were used as drug delivery systems for drugs such as hydrocortisone (Benedetti 1990)[159]. The authors observed that hydrocortisone was released much faster when encapsulated in the microspheres than when covalently conjugated to HA. Esposito et al. (2005) also formed

microspheres from HA esters using either spray-drying or the inverse emulsion method for drug encapsulation[5]. In the first case, the drug was dissolved in a solution containing HA and spray dried. In the second case, a water-in-oil emulsion was formed containing the HA and drug solution in the water droplets emulsified in oil with a surfactant (sorbitan trioleate, Span 85®) and further centrifuged to isolate the HA microspheres. Dehazya and Lu patented HA microspheres formed by crosslinking using dihydrazide and carbodiimide and simultaneous emulsification[160].

Later, Yun et al. (2004) reported the preparation of HA microspheres by using carbodiimide and adipic hydrazide combined with the inverse emulsion method[161]. Plasmid DNA was incorporated to the microspheres by mixing in the HA solution prior to the addition of the crosslinking reagents. The microspheres obtained showed to deliver structurally intact plasmid DNA and transfect cells *in vitro* and *in vivo*.

Recently, Lai et al. (2010) reported carbodiimide cross-linked hyaluronic acid hydrogels for potential use as cell sheet delivery carriers[162]. The authors show a better ocular biocompatibility relative to HA discs crosslinked with glutaraldehyde. This is a promising approach as cell sheet engineering is an emerging technique for tissue regeneration[163].

Luo et al. (2000) reported the preparation of drug-loaded crosslinked HA films for sustained release of drugs at wound sites[164]. The drug was dissolved in the HA-ADH solution before crosslinking to be incorporated inside the hydrogel network.

The formation of HA composites were also reported by covalently linking HA to other polymers such as carboxymethylcellulose[165], poly(vinyl alcohol) or poly(acrylic acid)[166]. A recent example developed by Antunes et al. (2010) are poly(L-lactic acid)(PLLA) macroporous

hybrid scaffolds coated with hyaluronic acid to be used for bone tissue engineering applications[167]. Preformed PLLA scaffolds were immersed in HA solution which was then crosslinked in water/acetone with glutaraldehyde.

3.2 Non-covalent HA derivatives

Native HA was used to encapsulate ofloxacin inside microspheres obtained by spray drying of a solution containing both HA and ofloxacin[168]. The microspheres showed increased ofloxacin uptake relative to non-HA microspheres for improved lung delivery. Kim et al. (2005) described the use of HA microspheres for sustained release of recombinant therapeutic proteins[169]. The microspheres were obtained by spray-drying native HA solution comprising the protein, in this case recombinant human growth hormone, and a non ionic surfactant, polysorbate 80 (Tween 80®). The microparticles obtained were further mixed with a lecithin solution and spray-dried a second time. Eventhough recombinant proteins have shown to be easily denaturated during spray-drying, the authors observed almost no denaturation. Their optimized formulation showed prolonged protein release after injection. These results are very promising for the delivery of therapeutic proteins especially to reduce injection frequency, being the main challenge in this area. More similar protein delivery systems were reviewed recently by Oh et al. (2010)[87].

The negative charges of the carboxyl groups of HA were also used to form ion complexes with positively charged compounds. For example, HA was mixed with positively charged tumor necrosis factor-related apoptosis inducing ligand (TRAIL) to form non-covalent ion complexes. These nanocomplexes showed prolonged blood circulation *in vivo*, therefore prolonging the anticancer therapeutic effect[66]. Nanoparticles formed by ion complexes of HA and cisplatin were described by Jeong et al. (2008)[67]. In aqueous solution, cisplatin is positively charged after displacement of a chloride group by water. It therefore

forms ion complexes with HA by simple mixing in water during 3 days. Saettone et al. (1991) had previously described the formation of HA ion complexes with pilocarpine showing improved bioavailability for ophthalmic delivery[170]. In the same manner, liposomes which have a cationic outline, have been non-covalently coated by ion complexation of negatively charged HA[171]. Jederstrom et al. (2004) described the formation of ionic complexes between HA and insulin by successively decreasing ionic strength and increasing pH of the solution by dialysis[172]. The authors observed the formation of stable complexes after optimization of the dialysis parameters with improved biological activity for potential oral administration.

4. Physicochemical characterization methods

4.1 Structural characterization

4.1.1 Nuclear magnetic resonance spectroscopy (NMR)

Uni-dimensional ^{1}H-NMR is the most commonly used method to characterize HA derivatives as shown in early studies by Pouyani and Prestwich (1994)[173] or Bulpitt and Aeschlimann (1999)[86]. The spectra obtained show the presence of the conjugated compounds and enable to calculate the degree of modification of HA. A typical spectrum of native HA solubilized in D_2O is presented in **Figure 44a** and showed broad signals due to high viscosity of the solution. When dissolved in D_2O containing NaOH, the lower viscosity of HA alkaline solutions led to better defined peaks (**Figure 44b**), due. The broad multiplet between approximately 3.2 and 3.9 ppm corresponds to the protons in the sugar rings. They are all superimposed which makes it difficult to assign each proton individually. In alkaline solution, the peaks of the multiplet are much better defined. The broad doublet at 4.5 ppm corresponds to the two anomeric protons attached to the carbons adjacent to two oxygen atoms. The $-CH_3$ protons of the N-acetyl group of HA is assigned to the well defined peak at 1.95 ppm. The signal is slightly lowered in alkaline pH to 1.90 ppm. This peak is usually used as the reference to calculate degrees of modification as it is rarely modified when synthesizing new HA derivatives.

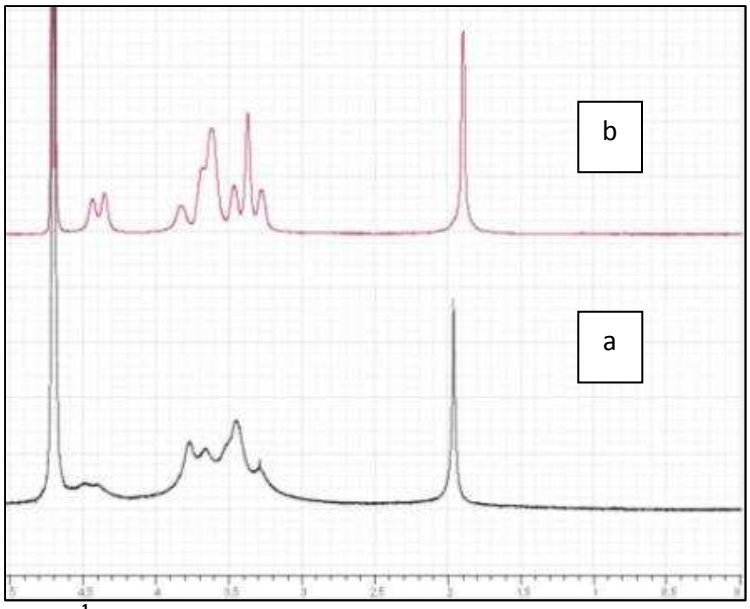

Figure 44: ^{1}H-NMR spectra of hyaluronic acid in D_2O (a) and D_2O with NaOH (b).

NMR spectrometry is a very useful method to quantify and characterize chemical modification. For example, L-alanine was grafted onto the carboxylic acid of HA and the spectrum of the purified conjugate is shown in **Figure 45**. The new peaks at 1.33ppm and 4.14ppm correspond to the methyl group and the –CH of L-alanine respectively. The substitution degree (DS) is easily determined by calculating the ratios of peak integrals from L-alanine methyl group (δ = 1.33ppm) with the methyl protons of the N-acetyl group of HA (δ = 1.9ppm). The peak of the –CH group of L-alanine at 4.14ppm can give an approximate indication of the DS but the peak is too broad to give a precise value. The methyl peak of alanine has the advantage of not being superimposed with the peaks of the native HA, enabling to easily and precisely calculate the DS. When the molecules grafted have signals which are superimposed with the HA peaks, the degree of substitution is more difficult to assess using uni-dimensional ^{1}H-NMR.

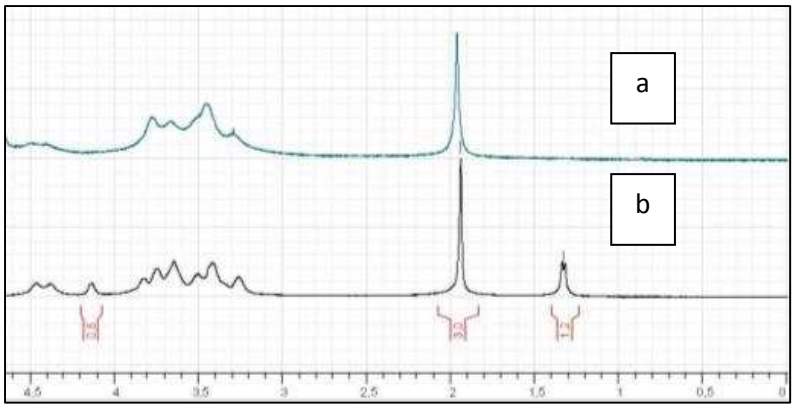

Figure 45: ^{1}H-NMR spectra in D_2O of native hyaluronic acid (a) and HA-alanine (b).

DMSO-D_6 was also used as a solvent for HA. For example, Eenschooten et al. (2010) obtained similar results for amphiphilic HA derivatives using DMSO or D_2O, showing ^{1}H-NMR is a reliable technique even for amphiphilic compounds[119]. Pravata et al. (2008) however found differences on spectra using D_2O or D_2O/DMSO-D_6 solvents[120].

Recently, the use of two-dimensional (2D) NMR spectra was presented by several authors[117, 120,174]. Crescenzi et al. (2003) used diffusion ordered 2D NMR (DOSY) which can distinguish molecules bound to HA from the ones which have not been covalently linked[117]. The authors used this technique to ensure the dialysis step of their reaction had removed all remaining free compounds. Sahoo et al. (2008) also used DOSY measurements to confirm the covalent linkage of methacrylate-lactic acid onto HA[174].

Solid state carbon NMR (^{13}C-NMR) measurements were performed using CP-MAS. De Nooy et al. (2000) observed that the increase of the theoretical degree of crosslinking led to an increased intensity of the crosslink carbon signal[95]. To determine such changes as well as the degree of crosslinking, extremely high frequencies and a high number of scans are necessary, which is not commonly performed[95]. Several other authors report the use of ^{13}C-NMR in solution or solid state as well as two-dimensional ^{1}H-NMR and ^{13}C-NMR[89,122,128].

NMR is without a doubt an essential tool for the characterization of HA. Nevertheless, common uni-dimensional ^{1}H-NMR does not give information on the covalent linking or modification of HA and requires complementary characterization.

4.1.2 Infrared spectroscopy (IR)

Another common characterization technique for HA derivatives is infrared spectroscopy (IR), particularly Fourier transform infrared spectroscopy (FTIR)[83,91,175]. It enables to determine the type of bond which has been formed during the HA modification. For example, Magnani et al. (2000) observed by FTIR the appearance of C=O amide bands at about 1630-1640 cm^{-1} which correspond to the new amide bonds formed between the diamines and the carboxylic groups of HA[89]. At the same time, as the amount of crosslinking reagents was increased, the carboxyl groups' bands intensity at about 1720-1740 cm^{-1} decreased. Micochova et al. (2006) confirmed the presence of alkyl chains on HA by the additional peaks observed between 2850 and 2930 cm^{-1} corresponding to -CH and -CH_2 vibrations[122].

This method, however, does not enable to precisely quantify the modification degree. In some cases, no information can be extracted from FTIR spectra as observed by Tomihata and Ikada (1997) who obtained no difference between the spectra before and after crosslinking except for a band at 1650 cm^{-1} which can be attributed to the ion exchange of the sodium salt to the acidic form of HA[103].

4.1.3 Primary amine quantification by the ninhydrine assay

A colorimetric test method using ninhydrine was reported by Kuo et al. (1991) to quantify the free primary amino groups of the amine-functionalized HA[129]. The negative results confirmed that no primary amines were grafted onto HA via carbodiimide mediated amidation. This quick test therefore easily determines whether amine molecules detected by NMR are covalently linked to HA or if they are free in solution.

4.2 Morphology

4.2.1 Microscopy

Scanning electron microscopy (SEM) is often used to characterize the morphology of dry HA derivatives[5,164,168]. Images showed native HA having a fibrous and irregular structure whereas HA-ADH had a highly porous and sheet-like surface[176]. In particular for HA microspheres or coated liposomes, SEM was used to characterize shape, surface properties or size distribution. For example, Esposito et al. (2005) observed spherical microparticles or irregularly collapsed microparticles depending on the parameters used for their formation (**Figure 46**)[5].

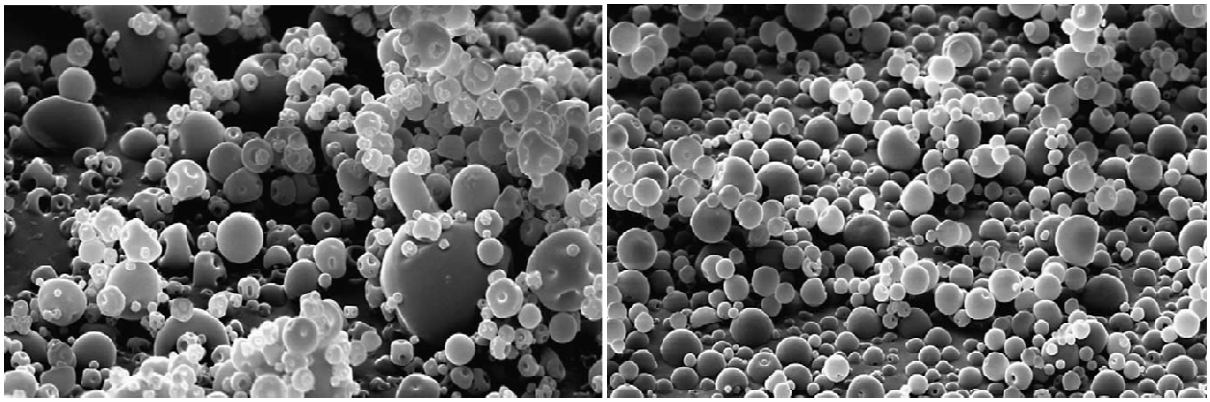

Figure 46: Scanning electron microscopy of HA microparticles by Esposito et al. (2005).

The use of transition electron microscopy (TEM) was also described by several authors to determine surface characteristics of dried nanoparticles[67,151,152,177]. The images obtained were however not as precise, but still could enable to calculate particle size and shape (**Figure 47**).

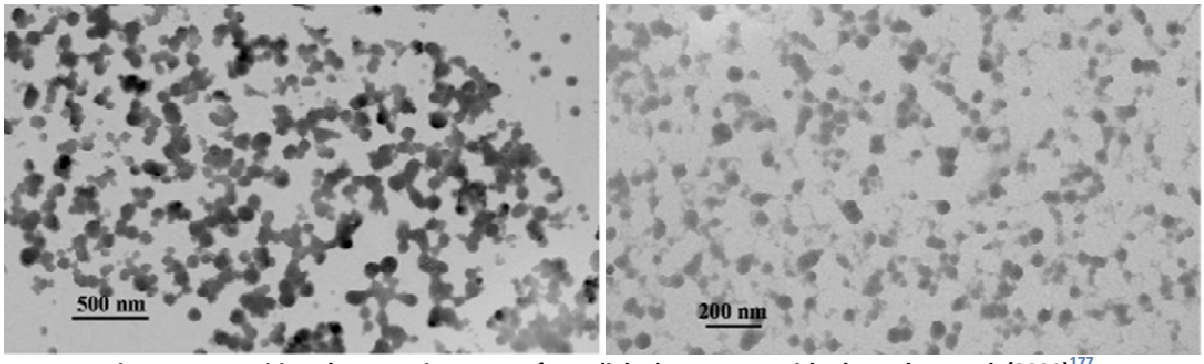

Figure 47: Transition electron microscopy of crosslinked HA nanoparticles by Bodnar et al. (2009)[177].

Another microscopy method to observe HA derivatives is atomic force microscopy (AFM) and is performed in liquid unlike both previous methods which are performed on dried samples[83,172]. Schneider et al. (2007) used it to observe the topography and roughness of the HA crosslinked multi-layer films[83].

4.2.2 Dynamic light scattering

Several authors measured the hydrodynamic diameters and particle size distribution of HA microparticles or nanoparticles using dynamic light scattering (DLS) [67,152,172,177]. For example, Yadav et al. (2007) observed differences in HA-PLGA nanoparticle size and size distribution depending on the processing parameters[151].

4.3 Physical characterization

4.3.1 Swelling studies

Swelling studies on crosslinked HA hydrogels have been described by several authors[112,156,178]. Collins and Birkinshaw (2007) used swelling studies to compare the crosslinking densities of HA hydrogels formed with different crosslinking agents[112]. High swelling ratio values correspond to the lowest crosslinking density; the more the hydrogel network is dense, the lower its water uptake capability and its swelling ratio. This way, they showed that glutaraldehyde and divinyl sulfone were the most effective crosslinkers relative to carbodiimide and bisepoxide. Ghosh et al. (2005) observed as expected that the swelling ratio decreases when increasing the amount of crosslinking agent, in this case poly (ethylene glycol) diacrylate[156].

4.3.2 Compression test

Ibrahim et al. (2010) used a uniaxial compression test to determine the apparent crosslinking density of HA hydrogels[179]. The gels were compressed with a defined initial force and compression rate. The results were in line with the swelling ratio, showing that increasing the crosslinker amount decreased the apparent crosslinking density.

4.3.3 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is often used to characterize the thermal behavior of HA hydrogels and have information on their hydration properties[112,164,180,181,182]. The DSC thermogram of native HA presented by Luo et al. (2000) (**Figure 48**) presents a broad endothermic peak at 89.5°C followed by two sharp exothermic peaks at 243.6 and 258.7°C, indicating the crystalline nature of HA[164]. The peaks above 300°C correspond to HA decomposition. Thermograms of crosslinked HA-ADH no longer show an exothermic peak, the material no longer being crystalline but amorphous. Studies by Barbucci et al. (2000)

gave indication on the hydration water of HA derivatives[182]. The endothermic peak attributed to the dehydration heat was lower for native HA than for sulphated HA, the latter containing therefore more hydration water. Indeed, the OSO_3^- groups are more polar than the hydroxyl groups of HA. Yadav et al. (2007) states that the glass transition temperature has an important effect on the drug release, but doesn't explain why[151].

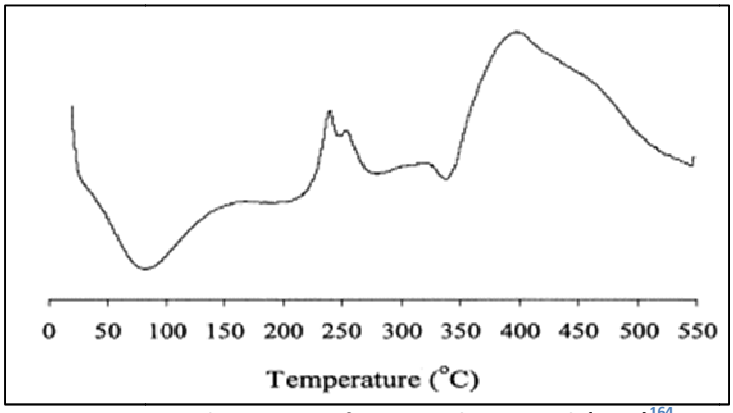

Figure 48: DSC thermogram of native HA by Luo et al. (2000)[164].

4.3.4 Molecular weight measurements

4.3.4.1 Size exclusion chromatography coupled to multi-angle light scattering

Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) is a method used to determine the molecular weight of the modified polymers. Preserving HA's chain length is a critical point in the synthesis of HA derivatives as its lubricant, shock absorber and space filling properties are strongly related to its high viscosity and molecular weight. Some degradation is inevitable during chemical modification processes. Indeed, HA is sensitive to various procedures, especially lyophilization and exposure to alkaline conditions[183]. Analysis using light scattering techniques has the advantage to measure the real molecular weight. The results obtained are not dependent on the eventual changes of the hydrodynamic sphere following HA functionalization and thus do not lead to false interpretation. The molecular weight is calculated using its specific refractive increment

index (*dn/dC*). *dn/dC* values of native HA are usually within the range of 1.430 to 1.670 mL/g depending on the solvent and measurement parameters used[80,184,185].

4.3.4.2 Asymmetric flow-field-flow fractionation (AFFFF)

Another method reported to measure the molecular weight of HA is asymmetric flow-field-flow fractionation (AFFFF). Maleki et al. (2007) described the use of this method to study the hydrolytic degradation of HA during the crosslinking process with EDC[97]. It measured the molecular weight and molecular weight distribution at different time points of the crosslinking reaction. After an initial increase, the molecular weight showed to significantly decrease after seven days in presence of the crosslinking reagents.

4.3.5 Rheological measurements

As described earlier in section 1.1, due to its high molecular weight and entangled coil formation in solution, HA solutions are highly viscous and shear-thinning. Rheology studies have therefore been performed by many authors to evaluate the effect of derivatization or crosslinking on its viscosity profile[94,97,101,179,182]. Measurements can be performed in steady shear mode or in oscillatory mode.

A typical dynamic viscosity curve of an HA solution in function of shear stress measured using a cone plate rheometer at 25°C in steady shear mode is shown in **Figure 49**. This clearly demonstrates the shear-thinning property of HA as the viscosity decreases when the shear rate increases. Modified HA which have a lower molecular weight also have a lowered viscosity profile but still exhibit shear-thinning properties. Huin-Amargier et al. (2005) described the obtention of associative polymers by grafting long hydrophobic chains onto HA[101]. Such amphiphilic polymers form strong physical interactions which are broken when submitted to high shear forces. The shear thinning behavior is thus greatly superior to the native HA as shown on **Figure 50**.

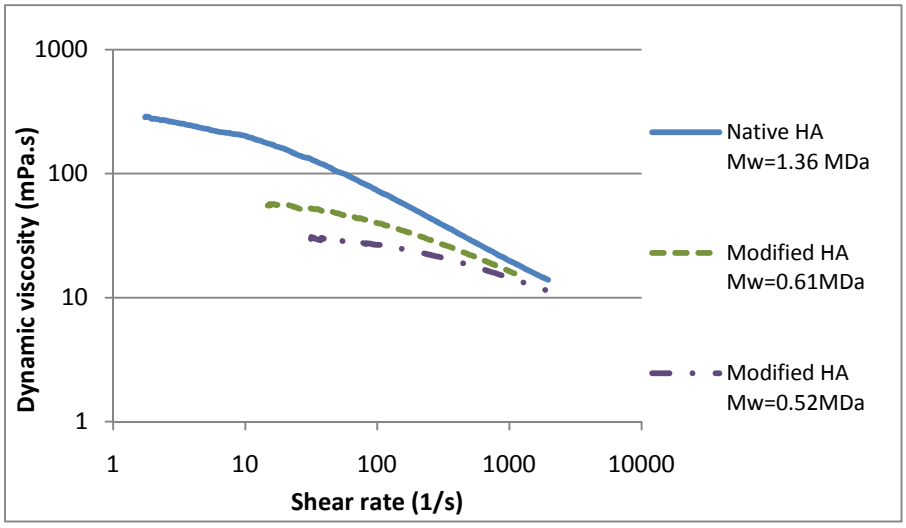

Figure 49: Dynamic viscosity of native HA and modified HA

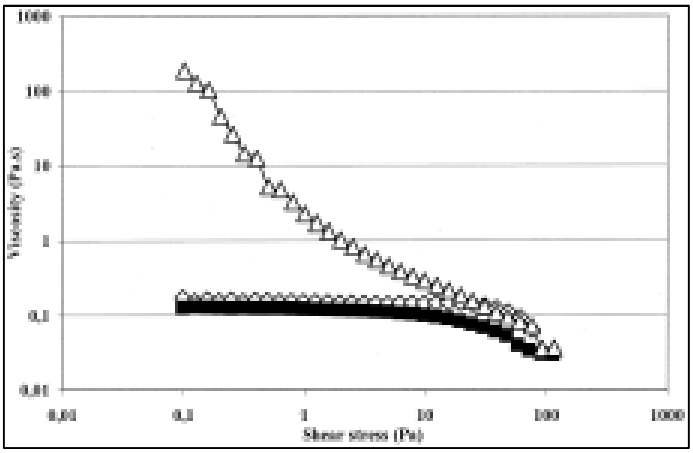

Figure 50: Shear-thinning behavior of native HA and amphiphilic HA derivative by Huin-Amargier et al. (2005)[101].

Oscillatory shear experiments on crosslinked HA hydrogels enable to determine the shear storage modulus (or elastic modulus), G' and the shear loss modulus, G'', giving information on their viscoselastic characteristics. G' reflects the elasticity of the material whereas G'' represents its viscous behavior[182]. Barbucci et al. (2000) presented results of oscillatory shear experiments on HA solutions and crosslinked gel[182]. For unmodified HA solutions, G'' is higher than the elastic modulus G' and they both increase at higher frequencies. This behavior is typical for viscous liquids. For HA hydrogels, the elastic modulus G' is higher than G'' and they are both independent of the shear frequency, which

is a typical behavior of viscoelastic solids. Both behaviors were also demonstrated by Borzacchiello et al. (2010) as shown in **Figure 51**[94]. According to Barbucci et al. (2000), these measurements enable to distinguish the chemical hydrogels from the entanglement networks[182]. The oscillatory shear mode gives important information on the nature of the resulting hydrogels.

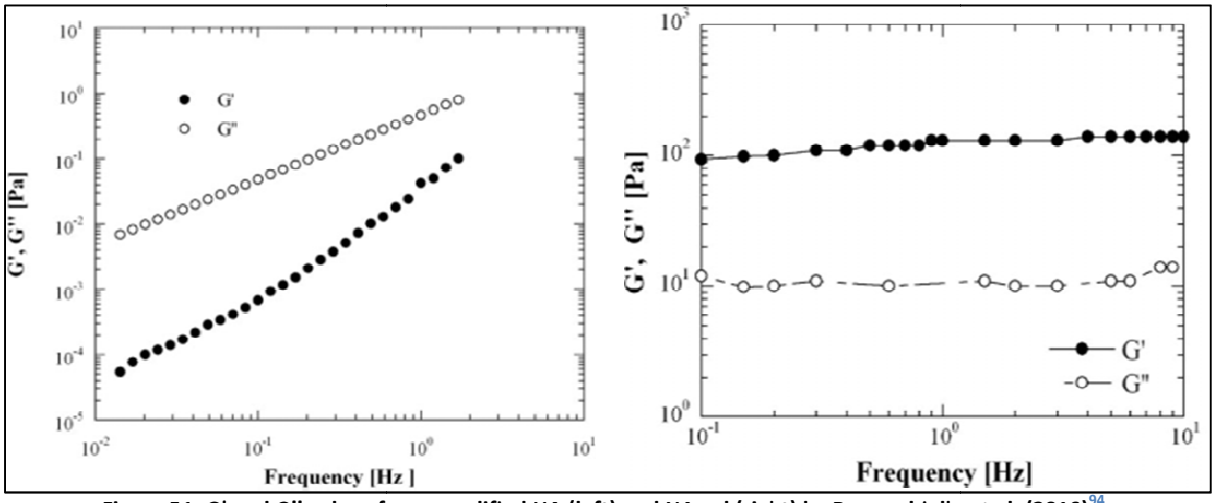

Figure 51: G' and G'' values for unmodified HA (left) and HA gel (right) by Borzacchiello et al. (2010)[94].

5. Conclusion

The increasing number of chemical reactions described in the literature bears witness of the rising interest in HA modification and functionalization. There is a continous appeal to develop innovative HA derivatives and solutions for their synthesis. Performing the modification reactions in water or organic solvent present both advantages as well as inconvients. As seen throughout this chapter, reactions in water enable to preserve the most the initial chain length of the HA backbone but usually do not recover high modification rates. Reactions performed in organic solvents enable to reach high grafting rates but tend to induce more chain fragmentation. For the development of some HA derivatives, such as amphiphilic polymers, the grafting ratio needed is low, whereas in other cases, fragmentation of the HA backbone is not an issue, for example in some cases of drug delivery where HA has to be degraded for the drug to be released. There is therefore no ideal modification method but a wide range of methods from which one can chose from, according to the specific needs required for each application.

CHAPTER 2: Comparison of amidation reactions for the grafting of L-alanine onto hyaluronic acid

1. Introduction

In this second chapter, the study of the grafting of an amino acid, L-alanine, onto the hyaluronic acid is described. Amino acids were identified as potentially suitable molecules to be grafted on the carboxylic groups of HA with the aim to impede its enzymatic degradation. It was first necessary to achieve the grafting of such molecules and evaluate the grafting ratios which could be obtained and the fragmentation of the HA backbone which might occur during the reaction. L-alanine was chosen to study different amidation methods as it is one of the simplest amino acids and is easily detectable on an ^{1}H-NMR spectra, thanks to its methyl group which is not superimposed with the HA signals. Three different amidation reactions were used and evaluated according to the resulting substitution degrees and properties of the HA-alanine derivatives obtained (**Figure 52**).

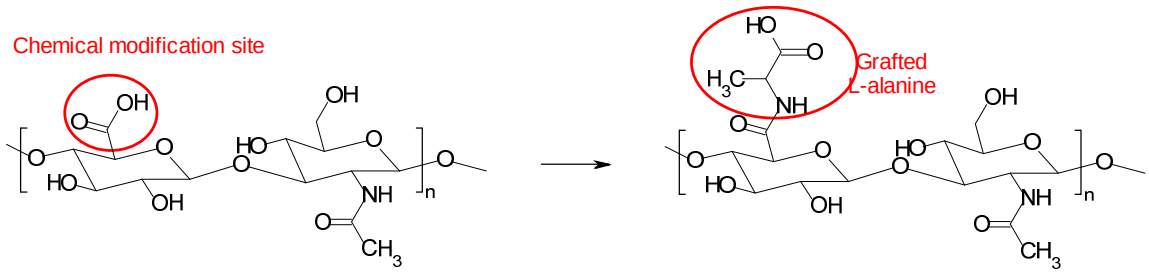

Figure 52: Chemical structures of hyaluronic acid and HA-alanine derivative.

The specific aim of this study is to determine the amidation method the most suitable to obtain HA-amino acid derivatives, which can provide high degrees of substitution up to 100% and which induces the least damage of the HA polymer backbone. Given the large number of amidation reactions described in the literature reviewed in the first chapter, there is a real challenge to identify methods which are feasible at a larger production scale in order to be suitable for future industrialization of new biomaterials for medical applications.

Indeed, many bioactive molecules cannot go further than development stages due to high industrial costs.

Amongst the numerous amidation methods described in the literature, three of them performed in different solvents have been studied. The first amidation reaction involves 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC)[84,86] as the activating agent of the carboxylic acid group. This reaction is performed in water and is the most widely used as HA is easily soluble in water in its native sodium salt form. The resulting degree of substitution obtained is usually approximately 10-20%. A second amidation method, described by Bergman et al. (2007)[80], is performed in a mixed water/acetonitrile solvent and with 2-chloro-dimethoxy-1,3,5-triazine (CDMT) as the carboxylic acid activator. This method is advantageous as it uses an activator which reacts in mild conditions in an aqueous solvent and gives higher substitution degrees than EDC. The third amidation reaction is performed in an organic solvent using activating agents such as 2-chloro-1-methylpyridinium iodide (CMPI)[89]. This method reported to yield high substitution degrees. However, the HA sodium salt must previously be converted to its tetrabutylammonium salt, adding reaction steps and thus future industrial constraints.

HA-alanine derivatives obtained were then characterized by ^{1}H-NMR to assess the degrees of substitution of L-alanine onto HA and the efficiency of the activator. Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) was performed to determine the molecular weight of the modified polymers and evaluate the degradation caused by each reaction. Viscosity measurements were also made to observe their rheological behavior.

2. Materials and methods

2.1.Materials

1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC), N-hydroxysuccinimide (NHS), L-alanine ethyl ester HCl (AlaOEt-HCl), sodium hydroxide (NaOH), tetrabutylammonium (TBA) hydroxide solution (~40% or 1.5M in water), 2-chloro-1-methylpyridinium iodide (CMPI), triethylamine, acetonitrile, N-methylmorpholine (NMM), 2-chloro-dimethoxy-1,3,5-triazine (CDMT) were purchased from Sigma-Aldrich and used without purification except for ion exchange resin Dowex 50WX8-200 which was previously washed with water and ethanol. Hyaluronic acid (HA) sodium salt was isolated from *Streptococcus equi* and was given at a molecular weight of 1.68MDa. Hyaluronidase from bovine testes Type I-S had a given activity of 850 units/mg units. Purified water was obtained with an Arium 661 Sartorius system (Goettingen, Germany). ^{1}H-NMR spectra were obtained using a 400MHz Bruker spectrometer. Lyophilization was performed using a Christ Alpha 2-4 LSC Lyophilizer at 0.2mbar at 25°C during 22 hours then at 0.09mbar at 25°C during 2 hours. Products were previously frozen by immersion into liquid nitrogen during 15 minutes. Visking dialysis membranes of 12-14000 Daltons were purchased from Medicell International Ltd (London).

2.2.Methods

2.2.1. Purification of the reaction mixtures

Each mixture was dialyzed 24 hours against water (3 L), 2 hours against 0.05M NaOH (3 L), 40 hours against 0.1M NaCl (3 L), 8 hours against 25% ethanol solution and finally 72h against water (3 L). The solution was then lyophilized to obtain the various HA-alanine derivatives. For the reaction with EDC, the dialysis against ethanol solution was omitted.

^{1}H-NMR (D_2O) δppm: 4.43 (m, 2H, O-CH-O), 3.88-3.11 (m, 12H, C-CH-O), 1.97 (s, 3H, CO-CH_3-N), 1.38-1.23 (m, 3H, C-CH_3-CO).

2.2.2. Synthesis of HA-alanine in aqueous solvent with EDC

The grafting method used is adapted from Bulpitt and Aeschlimann (1998)[86]. The sodium salt of HA (264 mg, 0.66 mmol) was dissolved in water (87 mL) for 1h before addition of L-alanine ethyl ester (AlaOEt-HCl (811 mg, 5.28 mmol). The pH was adjusted to 7.5 using 1 M NaOH. To this solution was added a freshly prepared solution of NHS (304 mg, 2.64 mmol) and EDC (506 mg, 2.64 mmol) in water (1mL). The pH was again adjusted to 7.5 using 1M NaOH and the reaction was allowed to proceed overnight under constant magnetic stirring (approximately 300 rpm). After purification (see procedure above), the HA-alanine was obtained as a white powder (229 mg, 86 % yield). The same reaction was performed with the following quantities: EDC (1012 mg, 5.28 mmol, 8 eq), Ala-HCl (1622 mg, 10.56 mmol), NHS (607.6 mg, 5.28 mmol). For this reaction, 235 mg (88% yield) of HA-alanine was recovered.

2.2.3. Synthesis of HA-alanine in water/acetonitrile with CDMT

The grafting method used is adapted from Bergmann et al. (2007)[80]. The sodium salt of HA (264 mg, 0.66 mmol) was first dissolved in water (52 mL) before addition of acetonitrile (35 mL). The solution was cooled in an ice bath and CDMT (362 mg, 2 mmol), was then added. One hour later, AlaOEt-HCl (460 mg, 3 mmol) then NMM (280 µL, 3 mmol) were added and the reaction was allowed to proceed overnight under constant stirring

(approximately 300 rpm). Excess amine were removed by incubation with ion exchange resins (Dowex-H^+, 2 g then Dowex-Na^+, 2 g, 1 h each), The solution containing the HA was then purified as described above to give the HA-alanine (213 mg, 76% yield) as a white powder.

2.2.4. Synthesis of HA-alanine in organic solvent with CMPI

The grafting method used is adapted from Magnani et al. (2000)[89]. HA was first prepared as a tetrabutylammonium (TBA) salt to allow its solubilization in DMF. The sodium salt of HA (2g, 5.14 mmol) was dissolved in water at a concentration of 5 mg/mL. Dowex 50WX8-200, a strong acid exchange resin was added slowly until a measured pH of 2.5. The resin was filtered off and the solution containing the HA (acid form) was treated, dropwise with tetrabutylammonium hydroxide solution (40% m/m) until pH 9-10. The solution was then lyophilized to give the HA-TBA (2.1 g). HA-TBA salt (400 mg, 0.66 mmol) was dissolved in anhydrous DMF (80 mL) under argon for 6 hours. The mixture was then ice-cooled and treated sequentially with AlaOEt-HCl (460 mg, 3 mmol), CMPI (511 mg, 2 mmol) and triethylamine (700 µL, 5 mmol). The reaction was allowed to warm to room temperature and stirred under argon overnight. The solution was then brought to 0°C for dilution with water (80 mL) and purified as described above to obtain after lyophilization HA-alanine (264 mg, 89% yield) as a white powder. The same reaction was performed using: 1. HA-TBA (400mg, 0.66 mmol), AlaOEt-HCl (154 mg, 1 mmol), CMPI (204 mg, 0.8 mmol), triethylammine (256 µL, 1.8 mmol) to obtain 279 mg of HA-alanine (94% yield) 2. HA-TBA (400mg, 0.66 mmol) AlaOEt-HCl (92 mg, 0.6 mmol), CMPI (102 mg, 0.4 mmol), triethylammine (140 µL, 1 mmol) to obtain 237 mg of HA-alanine (83% yield).

2.2.5. Detection of amines – Ninhydrin assay

The ninhydrin assay was adapted from Romberg et al. (2005)[186]. HA-alanine samples (100 µL, 5 mM solution in water) were mixed with 100µL of 1M acetate buffer (pH 5.5) and 200µL of ninhydrin solution (0.2g ninhydrin and 0.03g hydrindantin dissolved in 7.5mL 2-methoxyethanol and 2.5mL acetate buffer pH 5.5). The solutions were heated at 100°C for 15 minutes, cooled to room temperature and the volume adjusted to 1mL by addition of 50% ethanol for absorbance measurement at 570 nm. Control solutions were prepared with 0.1 and 0.5 mM L-alanine ethyl ester in water.

2.2.6. Molecular weight measurements

Size exclusion chromatography was performed using an Ultimate 3000 system from Dionex (Sunnyvale, CA, USA) and 3 columns Shodex OH-pak 1802.5Q, 1804HQ and 1806HQ (Showa Denko America, New York, USA) of 30cm in series at 30°C with a flow rate of 0.5 mL/min. The samples were dissolved overnight including 6 hours under mechanical stirring in water containing 0.1 M $NaNO_3$ at a concentration of 1.2 mg/mL to 1.5 mg/mL depending on the sample. Prior to analysis, each sample was filtered through a 0.45 µm hydrophilic PTFE Milex-LCR filter (Millipore, Bedford, MA, USA). The injection volume was 100 µL to 120 µL depending on the sample concentration. MALS detection was performed continuously on the column eluate with a Dawn Heleos II light scattering detector in series with an Optilab rEX differential refractometer (both from Wyatt Technology, Santa Barbara, CA, USA) with a wavelength of 658nm. Data were analyzed using Astra software 5.3.4 and first order Zimm fits. The *dn/dC* refractive index increments were experimentally determined using the same refractometer and solvent conditions as for SEC/MALS for native hyaluronic acid (*dn/dC* = 0.149 mL/g) as well as for HA-alanine with 100% grafting ratio (*dn/dC* = 0.143 mL/g).

The hydrodynamic radiuses were calculated using a real time digital correlator contained in a WyattQELS (Quasi-Elastic-Light-Scattering) instrument added in the detector.

2.2.7. Rheology

Dynamic viscosity measurements were performed in steady shear mode with a cone plate rheometer Rheostress RS100 (Haake Technik GmbH, Vreden, Germany) equipped with a Rheowin software (version Pro 2.93). The cone used had a diameter of 60mm and an angle of 1°. The temperature was maintained at 25°C with a TC Peltier thermostatic system. All samples were dissolved in water at least 24 hours before use at the same concentration of 2mg/mL for comparison reasons. Measurements were made with a preset shear stress ramp to evaluate the corresponding shear rate.

3. Results and discussion

3.1. Synthesis of HA-alanine (HA-ala) conjugates

There are almost innumerous methods to couple amines to carboxylic acid and a few have been adapted to HA. Activation of the HA carboxylic acid with EDC is one of the most popular methods[86,84], because it can be done in water, the native solvent for HA. EDC first reacts to the carboxylic acid to form a highly reactive O-acyl isourea intermediate (**Figure 53**). This specie then either reacts with a nucleophile, namely the amine but also competitive water, or rearranges into a stable N-acyl urea, which impedes the reaction. The reaction is quite subtle as the optimal pH is different for each step. Indeed, the carboxylic acid activation by EDC is best performed in an acidic environment (pH 4.75)[129], whereas the nucleophilic attack by the amine is best done at high pH, when the amine is unprotonated. The compromise is therefore not easy to define, especially for amines with high pKa values and stabilization by formation of succinimidyl ester of HA showed to be helpful[86]. We therefore adapted the procedure and incubated the HA (1.58 MDa) in water with excess of AlanineOEt and EDC (8 and 4 equivalents respectively) in the presence of N-hydroxysuccinimide (4 equivalents) at pH 7.5. After one night, the mixture was dialysed to remove low molecular weight molecules. We found out convenient to deprotect the carboxylic acid of alanine during dialysis by incubating the dialysis tubes in 50 mM NaOH for 2 hours at 20°C. The duration of this step was carefully monitored since strong alkaline conditions are reported to give rise to HA fragmentation[187]. A 2 hours treatment at 20°C with a 50 mM NaOH solution was enough to provide a NMR spectrum devoid of peaks corresponding to ethyl groups, confirming an effective deprotection. Finally we assayed the purity of the polymer after dialysis by verifying the absence of free amines (unreacted amino-acids) with the ninhydrin colorimetric test. The final yield was 88% and the degree of

substitution (DS) was calculated from the NMR spectrum (**Figure 54c**) at 10%. Doubling the equivalents of both EDC and amine improved only marginally the DS from 10 to 13%. These results are in accordance with previous results of the literature indicating that amidation of HA with EDC and NHS in water led to relatively low DS values[91,133,188].

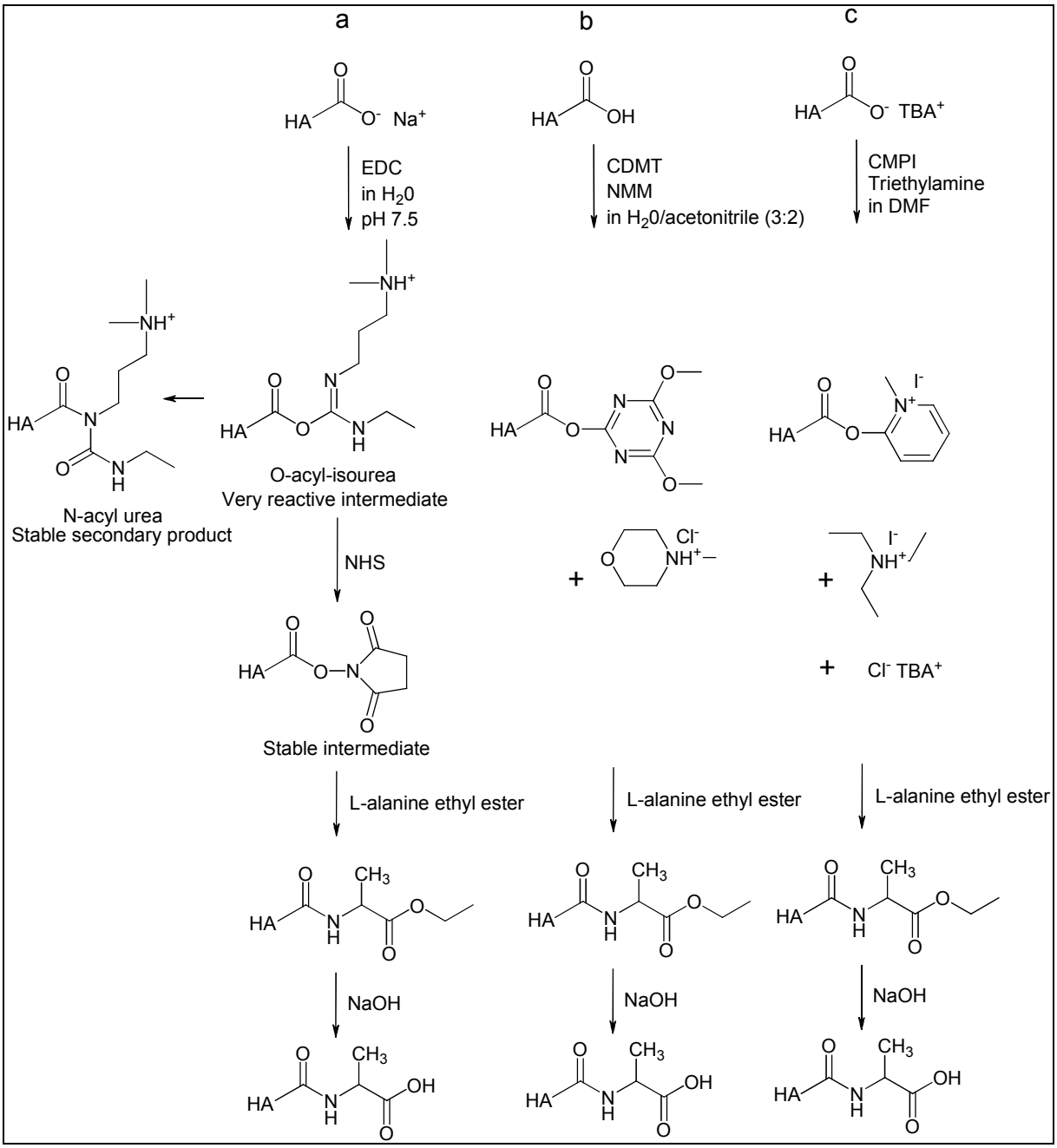

Figure 53: Synthetic routes to HA-alanine.

We next evaluated a more recently described condensation method which is performed in water/acetonitrile using 2-chloro-dimethoxy-1,3,5-triazine (CDMT) as the reagent[80]. The method appeared interesting because it avoided use of NHS and pH monitoring. At first, we performed the reaction exactly as described by preparing HA in its acid form[80]. However, in our hands, lyophilization of the acidic form of HA preceding the amidation reaction provoked important fragmentation of HA, a property that was already noticed by others[183,189]. We therefore modified the reaction procedure to avoid handling of the HA in its acidic form and started directly from the commercial HA sodium salt. The HA was then reacted with CDMT to form a HA-dimethoxytriazine intermediate and the release of HCl was trapped with N-methylmorpholine (NMM). Deplacement of the dimethoxyhydroxytriazine by nucleophilic attack of the alanine ethyl ester amine led to N-alanyl ethylester-hyaluronamide (**Figure 53b**). Deprotection of the ester and purification of the polymer were carried out as well during the dialysis. After lyophilization, the final yield was 76%. NMR analysis (**Figure 54d**) shows the expected N-alanyl hyaluronamide with a DS of 50%.

Table 2: Summary of the various methods for the preparation of HA-ala.

Starting salt	Conditions	Yield	DS (% relative to HA)	Adapted from
HA-Na	8eq EDC, NHS, H_2O, overnight	88%	13%	Bulpitt and Aeschlimann[86]
HA-Na	3eq CDMT, H_2O, CH3CN, NMM, overnight	76%	50%	Bergman et al.[80]
HA-TBA	3eq CMPI, DMF, triethylamine, overnight	89%	100%	Magnani et al.[89]

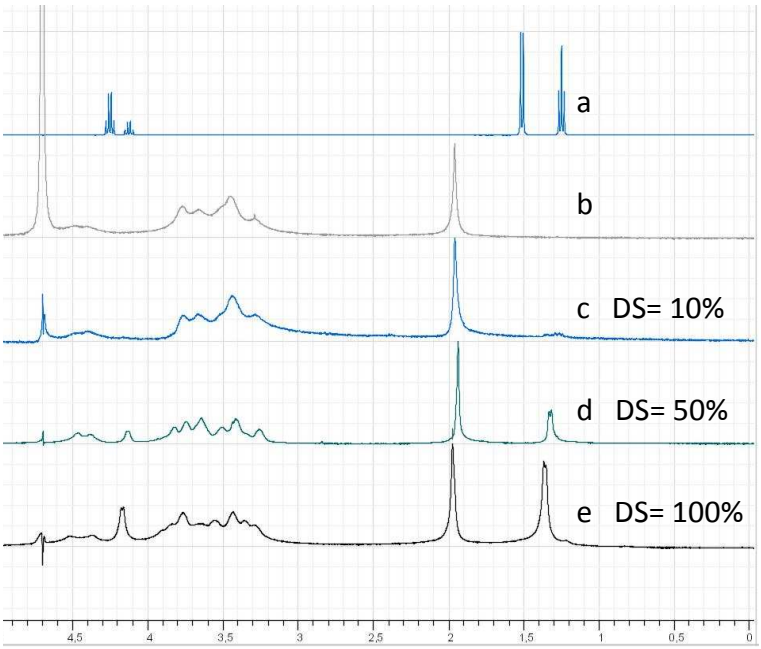

Figure 54: Comparative analysis of ^{1}H-NMR spectrum of the various HA-alanine with the highest possible degree of substitution that was achieved using: EDC in aqueous phase (spectrum c), CDMT in water/CH_3CN (spectrum d) and CPMI in DMF (spectrum e). Spectra a and b correspond to alanine methyl ester and the native HA respectively.

Although this method was more effective than EDC (DS around 50% versus 13%), our aim was to obtain a complete substitution of the HA carboxylic acid. We therefore assayed a third amidation which is performed in anhydrous conditions. Magnani et al. described the use of CMPI for crosslinking HA in DMF[89]. To ensure solvatation in anhydrous DMF, HA was firstly converted into a tetrabutylammonium (TBA) salt and condensed with alanine ethyl ester using a slight excess of CMPI (1.2 equivalents) and in the presence of triethylamine for neutralization of the released HCl and HI (**Figure 53**). Purification and concomitant deprotection afforded a N-alanyl hyaluronamide with 100% substitution as calculated from the NMR spectrum (**Figure 54e**). The effectiveness of this reaction (100% of HA substitution was observed using only 1.2 of activator equivalents) was remarkable and allowed us to obtain very easily HA-ala derivatives with lower DS by simple adjustment of the CMPI to HA stoichiometry.

3.2. Physicochemical properties the HA-alanine conjugates

We previously prepared HA-ala with different DS from 10 to 100% using various synthetic methods. It was previously indicated that HA is very sensitive to fragmentation[80,81]. We therefore studied the effect of the various synthetic methods on the HA molecular weights distribution. Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) was used to measure the molecular weight distribution of the polymers. For accuracy and to have access to the real molecular weights, we determined experimentally the refractive index increment (*dn/dC*) of HAala100 in the size exclusion elution phase (0.1M $NaNO_3$) and found a value of 0.143 mL/g. The *dn/dC* of HA was also measured in the same buffer at 0.149 mL/g, a value within the expected range (0.150 mL/g in NaCl[185], 0.167 mL/g in PBS[184], 0.142 ml/g $NaNO_3$[190]).

The results from these experiments are reported in **Table 3**. For more clarity, the various HA-alanine were abbreviated as HAalaX, X being the DS in percentage. The measured molecular weight of the native HA had almost the expected average molecular weight given by the manufacturer (measured at 1.26 MDa, given at 1.58 MDa). As seen, the molecular weights of all HA-ala are lower than the native HA. The fragmentation ratio of the derivatives range between 3.6 and 5.8, indicating that all three chemical modification methods induced damages to the starting polymer. The least degradation was observed for HAala13 obtained using EDC. Modification of HA in DMF with CMPI and in water/acetonitrile with CDMT led to slightly more HA fragmentation.

We can also notice from the results in **Table 3** that the calculated hydrodynamic radius of native HA of 47.2nm is within the range of values reported in the literature[191,192] and decreases after alanine grafting. The reason for lower hydrodynamic radius values may be a combination of the lowered molecular mass as well as the effect of alanine.

Table 3: Molecular weights (M_w), polydispersity index (PDI) and fragmentation of HA-alanine derivatives.

	M_w (g/mol)	PDI (M_w/M_n)	Fragmentation ratio[a]	Hydrodynamic radius Rh(z) (nm)
Native HA	1 261 000	1.29	1	47.2
HAala13 - EDC	351 500	1.65	3.6	34.9
HAala10 - CMPI	229 800	1.73	5.5	29.3
HAala40 - CMPI	283 500	1.72	4.4	n.d.
HAala67 - CMPI	218 000	1.74	5.8	28.1
HAala100 - CMPI	288 900	1.68	4.4	n.d.
HAala50 - CDMT	313 400	1.77	4.0	33.2

[a] : relative to the theoretical value calculated using the native HA M_w and the DS.

We next evaluated the contribution of the chemical modification on the viscosity of the polymer in aqueous solution at a concentration of 2mg/mL. **Figure 55** shows the dynamic viscosity profile of HA-ala as a function of shear stress. The viscosity profiles of all HA-ala derivatives are lower than the native HA, which is in line with the lowered molecular weight values measured by SEC-MALS. HAala13 obtained using EDC which had the highest molecular weight, also showed the highest viscosity compared to the other derivatives. However, for all polymers, the decrease in viscosity at higher shear rates shows that the shear thinning properties of native HA is preserved even after L-alanine grafting. Advanced studies would enable to determine the influence of L-alanine grafting on the derivatives' viscosity and molecular weight as a function of the grafting ratio.

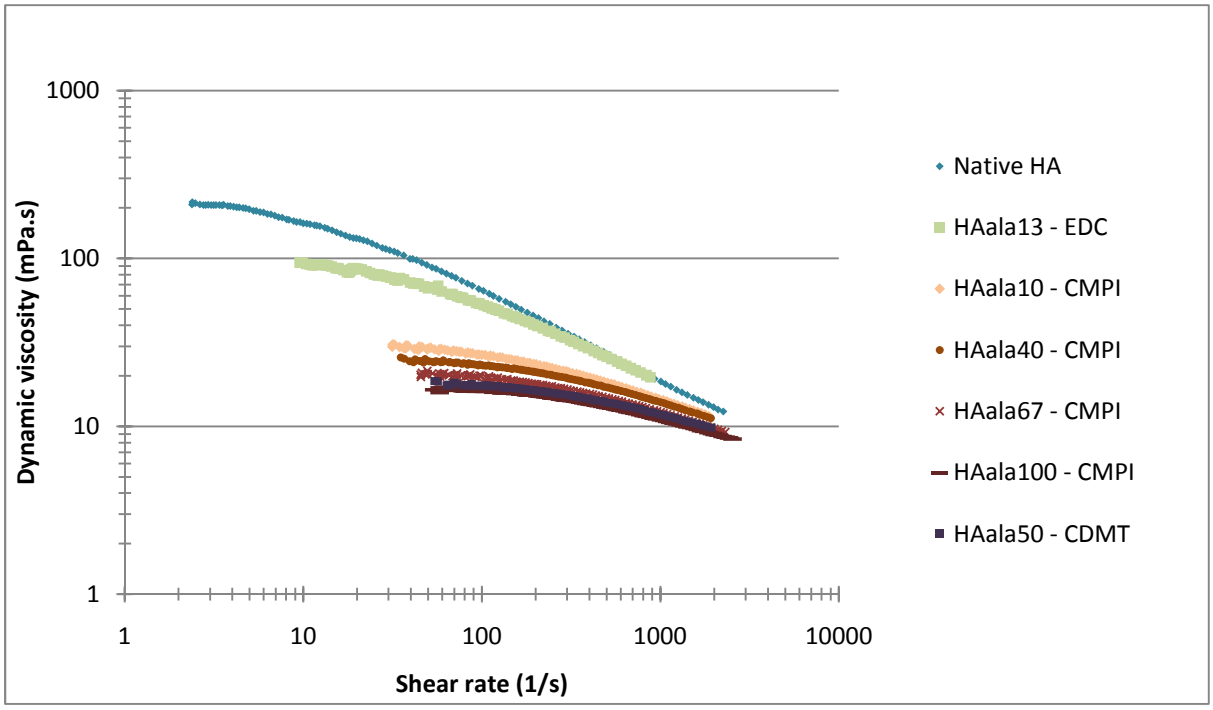

Figure 55: Evaluation of the polymer viscosity. Each polymer was dissolved at 2 mg/mL in purified water.

4. Conclusion

In the present chapter, we described the preparation of HA-ala using different chemical methods and showed that L-alanine can be condensed to carboxylic acid of HA at will and at degrees of substitution up to 100%. The various chemical processes did induce HA fragmentation but to still acceptable levels.

The comparison of three amidation methods for the grafting of amines onto the carboxylic groups of hyaluronic acid (HA) allowed to determine the most suitable and convenient method for the next steps of our work. The amidation method in organic solvent is most appropriate for our needs as 100% de substitution degree is desired for comparison purposes. In our case, working in organic solvent and converting HA to a quaternary ammonium salt is feasible as the reaction is performed in a laboratory scale.

However, the amidation reaction performed in water/acetonitrile seems to be the most appropriate method to consider for future up-scaling and industrialization of the products. In addition, considerable HA chain degradation was avoided by using the sodium salt of HA and not its acidic form. From an industrial point of view, the use of HA without previous treatment and the use of an aqueous solvent are valued advantages to reduce production time and energy and allowing considerable cost savings.

As for amidation using EDC in pure water and mild conditions, it appears to be the most suitable method to synthesize HA derivatives needing only low substitution degrees, for example for the grafting of long alkyl chains to synthesize amphiphilic polymers.

CHAPTER 3: Synthesis of hyaluronic acid-amino acid derivatives and their enzymatic stability

1. Introduction

The third chapter of the present manuscript describes the synthesis of a series of HA derivatives obtained by grafting amino acids on its carboxylic groups (**Figure 56**). The *in vitro* efficacy of the HA-amino acids (HA-aa) was then assessed as a function of the nature of the grafted amino acid as well as its degree of substitution using an enzymatic degradation assay. Firstly, in order to obtain comparable results between the amino acids, these were all grafted with 100% substitution degree. This was possible by using the amidation reaction performed in organic solvent as described in the previous chapter.

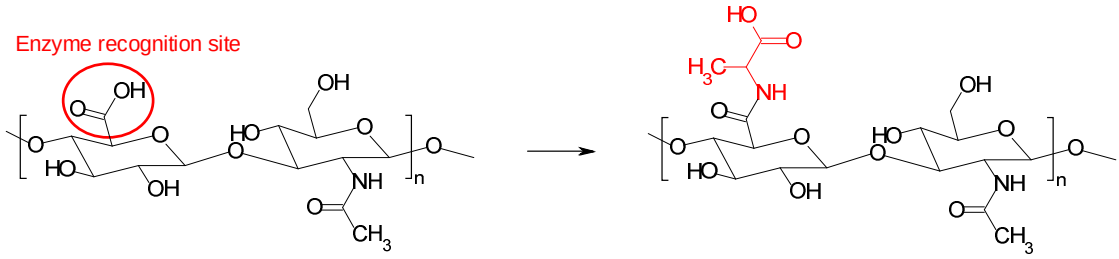

Figure 56: Chemical structure of hyaluronic acid and conjugated hyaluronic acid-amino acid

As detailed in the first chapter, the main restraint for the therapeutic use of HA lies in its enzymatic degradation by hyaluronidases in the body at the injection site. Since it has been determined that the recognition sites of hyaluronidases are the carboxyl groups of HA[34], these were our target for chemical modification (**Figure 56**). However, they are also responsible for the valuable viscoelastic properties of HA[1]. With these aspects in mind, we chose to attach a small and natural molecule which would impact the least possible its properties and yet hinder its enzymatic degradation. Also, with the aim of preserving HA's highly hydrophilic nature, we selected an acidic molecule to recover the carboxylic acid group. This way, we identified amino acids as suitable candidates for the synthesis of novel HA derivatives with the desired properties stated above. In addition, by grafting naturally occurring amino acids onto HA as opposed to synthetic molecules, we intended to avoid

recovering non-resorbable derivatives which have shown to induce complications being more difficult to manage and in certain cases permanent[193].

A range of amino acids was selected to be grafted onto HA and to assess the enzymatic degradation of the resulting derivatives. The amino acids grafted onto hyaluronic acid include L-alanine, L-valine, L-serine, L-threonine, L-phenylalanine, L-tyrosine, L-aspartic acid and L-arginine. The selection of amino acids was made according to the variety of side chains they carry, respectively aliphatic, hydroxyl, aromatic groups, a second carboxylic acid group adding a negative charge and a positively charged guanidinium group.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich. Hyaluronic acid sodium salt (from *Streptococcus equi,* 1.68MDa) , hyaluronidase from bovine testes (850U/mg), amino acids in ethyl ester HCl form, sodium hydroxide (NaOH), tetrabutylammonium (TBA) hydroxide solution (~40% or 1.5M in water), 2-chloro-1-methylpyridinium iodide (CMPI) and triethylamine were used without purification. Ion exchange resin Dowex 50WX8-200 was previously washed with water and ethanol. Water was purified with an Arium 661 Sartorius system (Goettingen, Germany). ^{1}H-NMR spectra were obtained using a 400MHz Bruker spectrometer. Lyophilization was performed using a Christ Alpha 2-4 LSC Lyophilizer at 0.2mbar at 25°C during 22 hours then at 0.09mbar at 25°C during 2 hours. Products were previously frozen by immersion into liquid nitrogen during 15 minutes. Visking dialysis membranes of 12-14000 Daltons were purchased from Medicell International Ltd (London).

2.2.Grafting of amino acids on hyaluronic acid

The amino acids were grafted the same way as described in Chapter 3, section 2.2.4 according to a method adapted from Magnani et al (2000)[89]. L-alanine ethyl ester HCl was replaced by the different amino acid ethyl ester HCl (1mmol). Each mixture was dialyzed 24 hours against water (3 L), 2 hours against 0.05M NaOH (3 L), 40 hours against 0.1M NaCl (3 L), 8 hours against 25% ethanol solution and finally 72h against water (3 L). The solution was then lyophilized to obtain the various HA-aa derivatives. ^{1}H-NMR was used to calculate the grafting ratios (**Figure 58**).

2.3.Detection of primary amines - Ninhydrin assay

The ninhydrin test was the same as described in Chapter 2, section 2.2.5, adapted from the method described by Romberg et al.[186] Control solutions were prepared with 0.1 and 0.5mM amino acid in water.

2.4.Enzymatic degradation of the HA-amino acid derivatives

The method used was adapted from Muckenschnabel et al. (1998)[194] using the Morgan-Elson colored reaction. 100µL of 5mM HA-amino acid solution, 100µL of purified water and 50µL of phosphate buffer were incubated with 25µL of 4.10^{6} IU/mL hyaluronidase solution at 37°C during one to six hours. The enzymatic reaction was stopped by adding 50µL of tetraborate solution and heating the tubes in boiling water for 5 minutes. After cooling in ice for 2 minutes, 750µL of dimethylamino benzaldehyde (DMAB) (2g of DMAB of dissolved in 2.5mL HCl and 47.5mL glacial acetic acid immediately before use) solution were added to each tube. After 60 minutes incubation, the absorbance at 586nm was measured.

2.5.Rheology

Viscosity measurements were performed as described in Chapter 2, section 2.2.7.

2.6.Molecular weight measurements

Molecular weight measurements were performed as described in Chapter 2, section 2.2.6.

3. Results and discussion

3.1.Synthesis of HA-amino acids

The grafting of various amino acids onto HA was performed in anhydrous conditions using the amidation method derived from Magnani et al. (2000), who used CMPI for HA crosslinking with diamines[89]. HA was first converted into a tetrabutylammonium (TBA) salt and condensed with the ethyl ester protected form of each amino acid using a slight excess of CMPI (1.2 equivalents) and triethylamine to neutralize the released HCl and HI (**Figure 57a**). As reported in the previous chapter, this amidation method showed to yield high substitution degrees using low reagent quantities[91]. Here, we investigated the effect of amino acids comprising a range of different functional groups (**Figure 57b**). Valine was grafted to evaluate the effect of isopropyl, a more important aliphatic group than methyl, whereas serine and threonine were grafted to evaluate the effect of a hydroxyl group. Phenylalanine and tyrosine both contain an aromatic group. Aspartic acid contains a second carboxylic acid group which adds a negative charge and the guanidinium group of arginine adds a positive charge to the polymer.

After incubation overnight, the reaction mixtures were all dialysed against 100 mM NaCl solution followed by 25% ethanol solution to remove ionic as well as hydrophobic impurities. The carboxylic acid groups of the amino acids were deprotected by dialysis against 50 mM NaOH for 2 hours at 20°C. For all derivatives, an effective deprotection confirmed by the NMR spectra devoid of peaks corresponding to ethyl groups and substitution degrees of 100% were calculated from the NMR spectra (**Figure 58**). After

lyophilization, a white powder was recovered for all HA-amino acid derivatives except for HA-serine, which had a light yellow color. Finally, the purity of the polymers after dialysis was confirmed by the absence of unreacted amino acids, assayed with the ninhydrine colorimetric test.

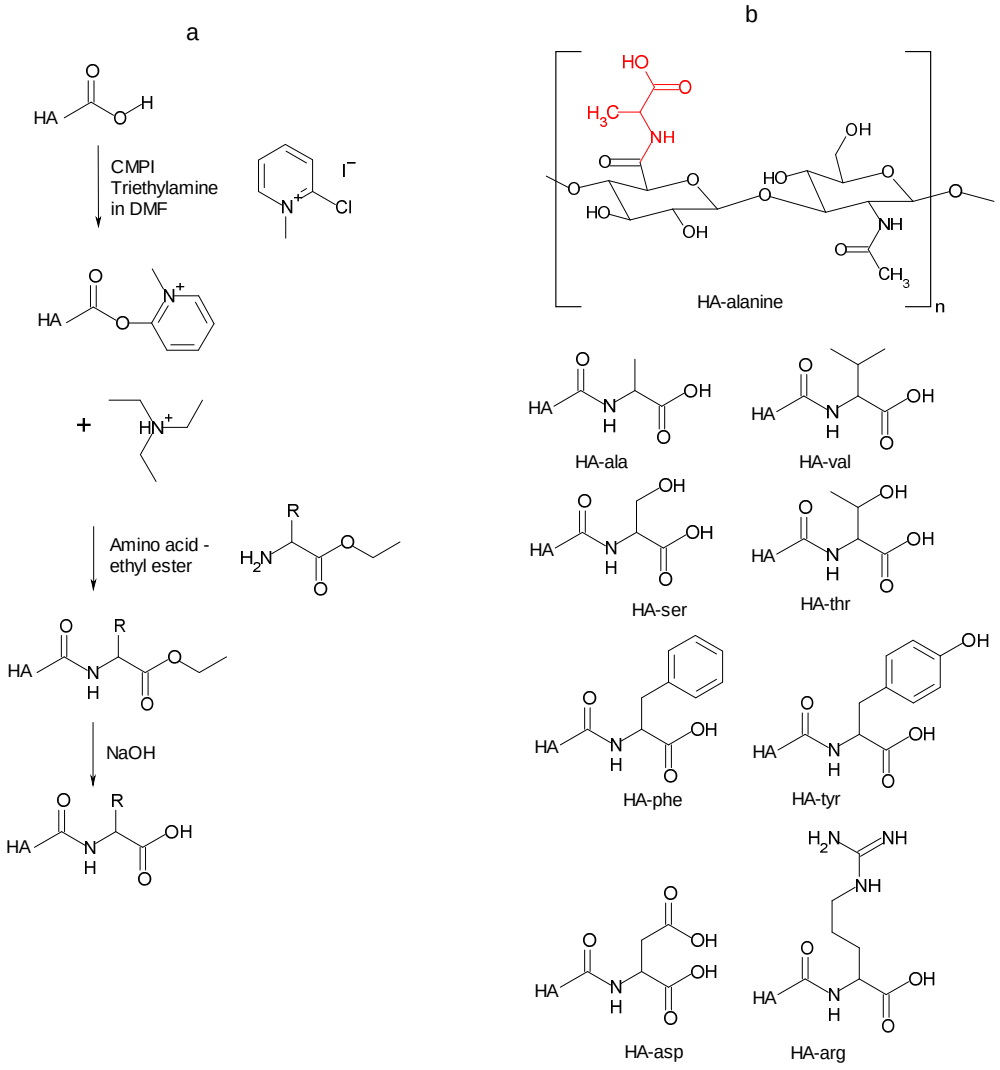

Figure 57: (a) Synthesis of HA-amino acid by amidation with CMPI in DMF and (b) chemical structure of conjugated HA-amino acids

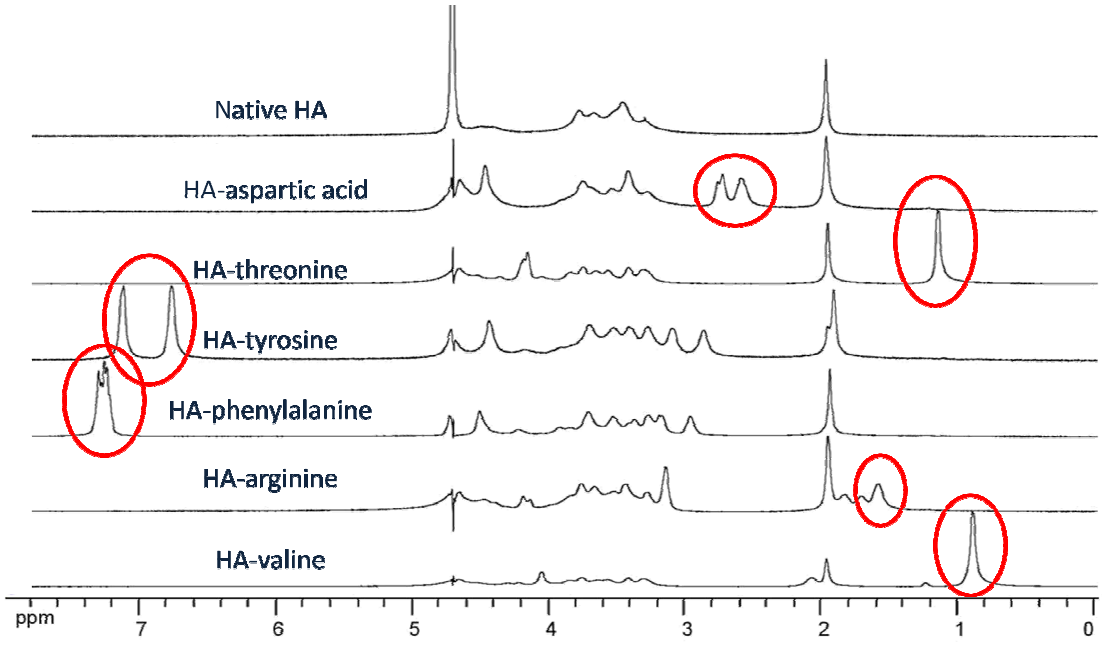

Figure 58: NMR spectra of native HA and HA-amino-acid derivatives.

3.2.Rheology and molecular weight measurements

The rheological behavior of the HA-amino acid derivatives was evaluated by measuring their dynamic viscosity in steady shear mode at 25°C in water. For comparison reasons, all amino acids were grafted using the same reaction conditions with 100% degree of substitution. The viscosity profiles showed to considerably vary between the different derivatives (**Figure 59**). Molecular weight values could not be measured by SEC-MALLS for each derivative due to the different nature of the side chain which would require the use of different solvents and yield non-comparable results.

The measurements of HA-alanine grafted with 100% substitution degree gave a molecular weight of 288 900 g/mol and a polydispersity index (PDI) of 1.68. For HA-serine with 100% substitution, the molecular weight was 458 000 g/mol and the PDI was 3.86. The high PDI value obtained for HA-serine explains the low viscosity profile seen in **Figure 59**

even though the molecular weight is higher. Indeed, the presence of low molecular weight HA fragments considerably lowers the viscosity of HA solutions.

The reason for the difference in molecular weight between HA-alanine and HA-serine, despite the same protocol used for their synthesis, is unknown but suggests that all derivatives were not subjected to the same fragmentation of the native HA backbone during the successive reaction steps. In addition to a difference in chain length, the variation in viscosity profiles of the different derivatives could also be due to the diversity of the functional groups on the grafted amino acids. HA-tyr exhibited the highest viscosities of all HA-aa derivatives and preserved a shear-thinning profile. HA-ala and HA-asp showed slightly lower viscosities and still exhibited the shear-thinning profile. All other derivatives showed lower viscosities which were almost independent of the shear rate. HA-phe had the lowest viscosities of all. If not due to a lowered molecular weight, the lower viscosity of the derivatives could be caused by the disruption of the hydrogen bonds possibly by steric hindrance of the amino acids or by the formation of intramolecular interactions which tighten the molecular clusters. The higher viscosity of other derivative solutions could by contrast be caused by the formation of intermolecular interactions, giving rise to networks more resistant to shear stress.

It is therefore not possible to predict the characteristics of HA-derivatives in terms of viscosity and molecular weight and ideally, individual optimization should be performed for each amino acid.

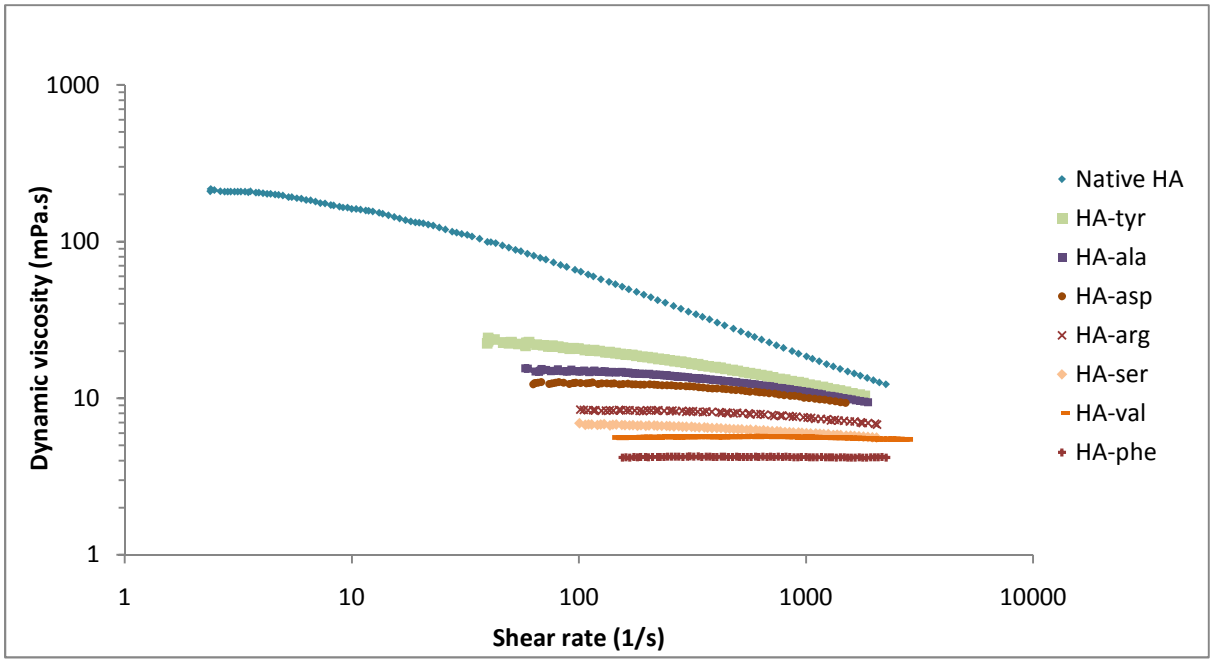

Figure 59: Dynamic viscosity of native HA and HA grafted with amino acids.

3.3.Enzymatic degradation of the HA-aa derivatives

We then assayed the effect of the amino acids on the susceptibility of HA-aa derivatives towards enzymatic digestion. The HA-aa derivatives with 100% DS (2mM in PBS) were incubated in the presence of hyaluronidase at 37°C. The enzymatic reactions were discontinued at different times by the addition of alkaline tetraborate solution and the N-acetyl glucosamine reducing ends were quantified using the Morgan-Elson colorimetric reaction described in Chapter 2 and adapted from Muckenschnabel et al. (1998)[194]. The degradation profiles of the derivatives were compared with the one of native HA subjected to the same conditions (**Figure 60**). All HA-aa derivatives showed lower enzymatic degradation compared to the native HA. HA-arg and HA-ala were the most degraded of the derivatives, suggesting that the positive charge of arginine did not improve the efficacy compared to HA-ala. This leads us to believe that only steric hindrance intervenes in the inhibition of the enzymatic degradation for HA-ala and HA-arg. All other HA derivatives

grafted with aspartic acid, valine, tyrosine, phenylalanine, threonine and serine showed a lower degradation. Even though the mechanism it unknown, it can be assumed the hydroxyl, aromatic, aliphatic and negative charges enhance the inhibitory effect of amino acid grafting.

In addition, the results show that the viscosity of the HA derivative does not influence the enzymatic degradation. Indeed, HA-tyr and HA-phe which have the highest gap between their viscosity values show a similar enzymatic degradation profile.

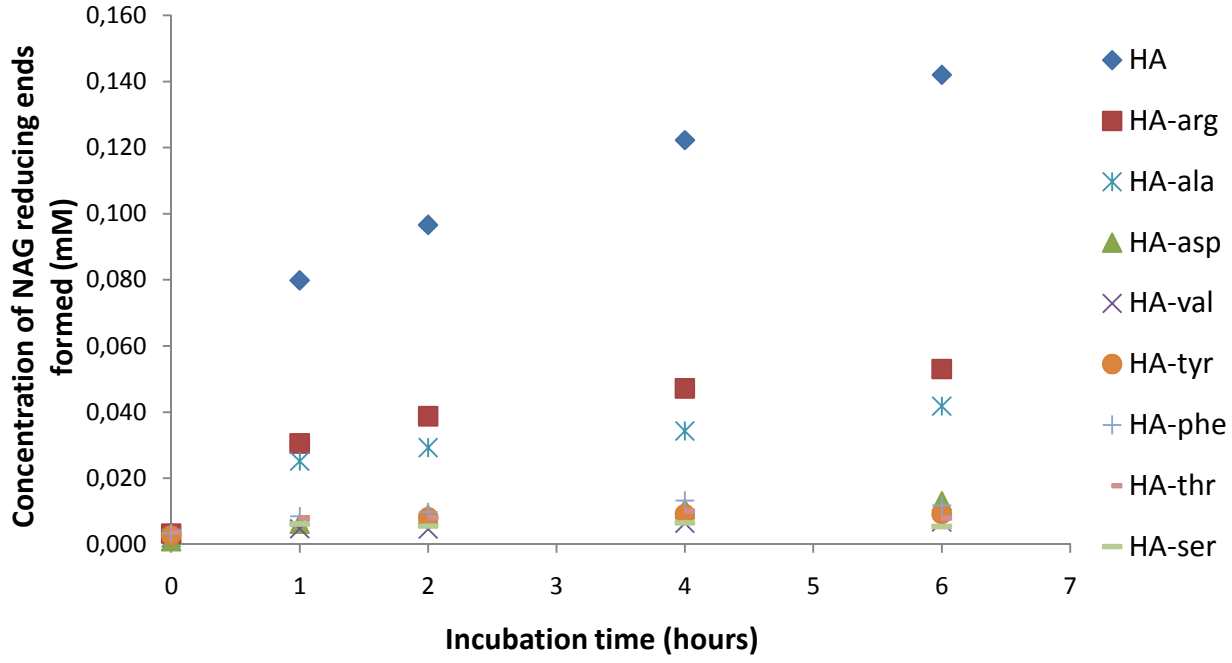

Figure 60: Enzymatic degradation profiles of native HA and of HA grafted with amino acids.

L-serine was chosen to be grafted at different grafting ratios by adjusting the proportions of reagents during the amidation reaction. The resulting HA-ser derivatives with 40%, 60% and 100% DS were subjected to the same enzymatic digestion assay. The results presented in **Figure 61** show that the decrease of the enzymatic degradation is proportional to the grafting ratio of the amino acid. The same tendency was observed with HA-alanine grafted with different substitution degrees as shown in **Figure 62**. These findings confirm the beneficial effect of the carboxylate protection of HA with amino acids.

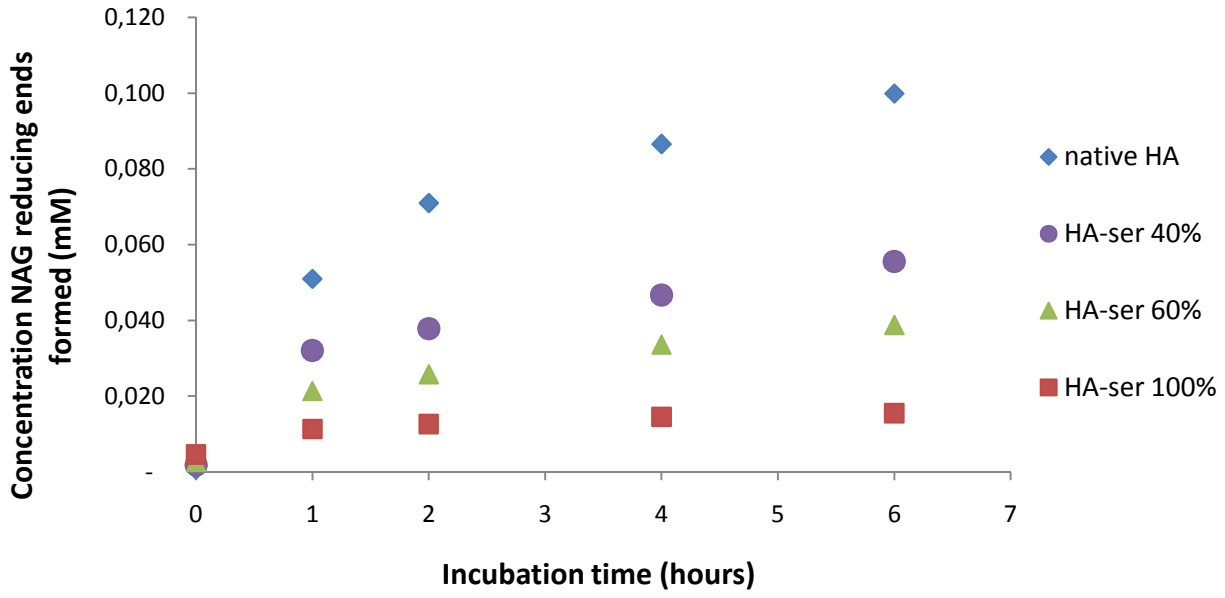

Figure 61: Enzymatic profile of native HA and HA-serine with different degrees of substitution.

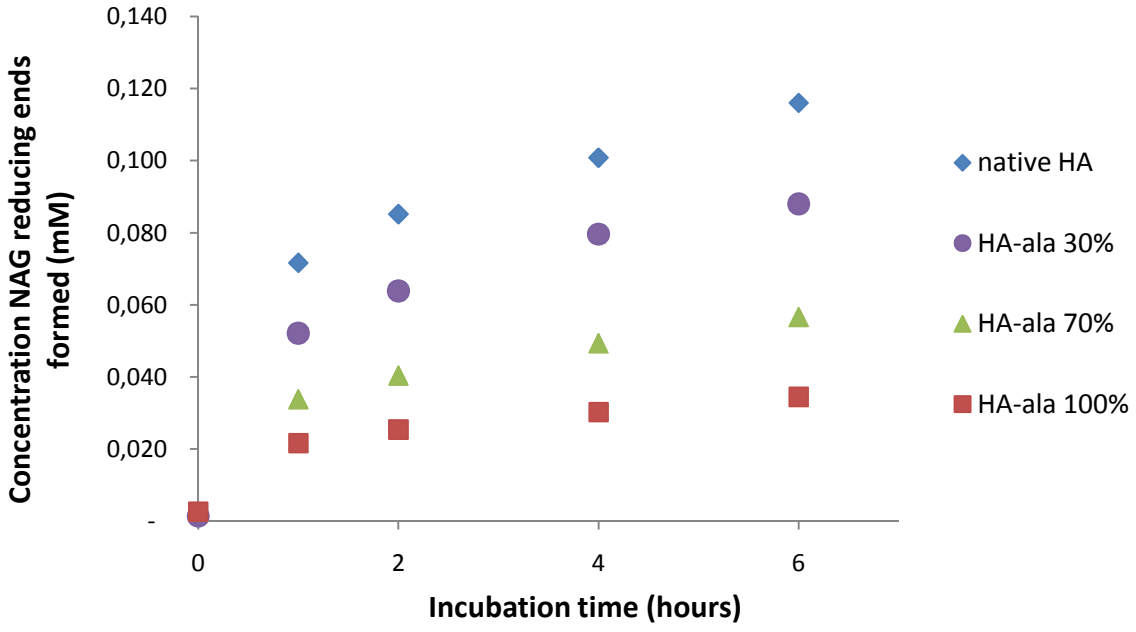

Figure 62: Enzymatic profile of native HA and HA-alanine with different degrees of substitution.

4. CONCLUSION

This part of the doctoral thesis is the most crucial one, as the new HA derivatives synthesized by grafting various amino acids onto its carboxylic groups showed to be significantly more resistant to enzymatic digestion than the native HA. All HA-aa derivatives could be grafted with 100% DS with the amidation method performed in organic solvent. We therefore successfully accomplished the first part of the project by synthesizing HA derivatives with higher resistance to hyaluronidases enzymes than native HA. These promising results allowed continuing the project with the next step: crosslinking of the derivatives to form hydrogels which could then be compared to the commercially available products.

CHAPTER 4: Crosslinking of hyaluronic acid-amino acid derivatives and hydrogel enzymatic stability

1. INTRODUCTION

After having demonstrated *in vitro* the enhance resistance to hyaluronidases of the HA-amino acid derivatives, the next goal was to achieve their chemical crosslinking to produce stronger hydrogels suitable for future therapeutic applications (**Figure 63**). In this chapter, crosslinking reactions of native HA and of the HA-amino acid derivatives were performed and the enzymatic degradation profiles and physical characteristics of the resulting products were evaluated and compared. Comparison was also made with commercially available crosslinked HA products.

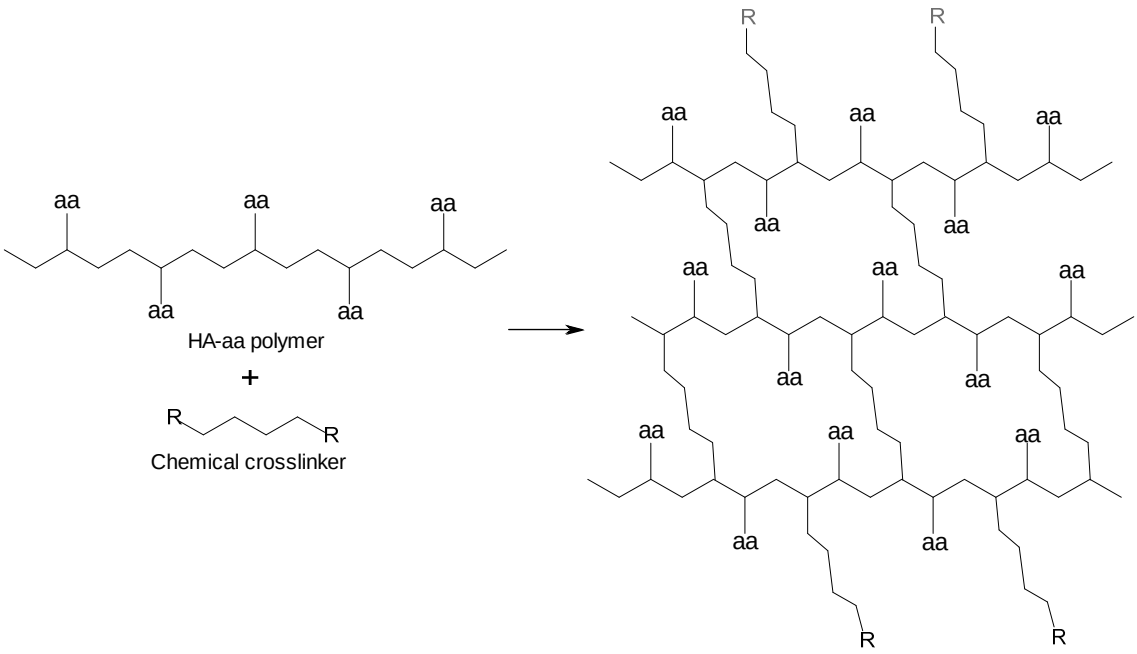

Figure 63: Schematic chemical crosslinking of HA-amino acid derivatives (HA-aa)

In addition to its enzymatic degradation, an obstacle to hyaluronic acid therapeutic treatments is its dilution away from the injection site, resulting in a short half-life after injection[31]. A possible solution to this obstacle was introduced and consisted in chemical cross-linking[107]. The first reports of this technique used bisepoxides as the crosslinking

agents including 1,4-butanediol diglycidyl ether (BDDE)[107] and diepoxybutane[106]. Many techniques have since then been studied using different cross-linking agents including glutaraldehyde, carbodiimides or divinyl sulfone[112]. HA crosslinking leads to the formation of rigid, insoluble and yet highly hydrated hydrogels, which, once injected into the skin or the joints, are not dissolved in the tissues and remain at the site of injection. This technique proved to be effective and to prolong the therapeutic effect of HA products up to 4 to 9 months[42,43,41]. This duration however still requires repeated injections and we hypothesized that the crosslinking of a previously stabilized HA derivative such as an HA-amino acid may provide HA hydrogels with yet longer residence time in the tissues after injection. The crosslinking agent selected for the following work was 1,4-butanediol diglycidyl ether (BDDE) which is the crosslinking agent used for most commercially available products such as Juvederm and Restylane[195,196,197]. This way, the enzymatic stability comparison would not be influenced by the crosslinking method used. With BDDE, two crosslinking reactions have been described in patents: one is performed in strongly alkaline solutions[107] whereas the second is performed in acidic conditions[102]. As it is unknown which conditions are used to synthesize the commercial products, both methods were tested in this work.

2. Materials and methods

2.1.Materials

All chemicals were purchased from Sigma-Aldrich. Hyaluronic acid sodium salt (from *Streptococcus equi,* 1.68 MDa), hyaluronidase from bovine testes (850units/mg solid), 1.4-butanediol diglycidyl ether (BDDE), and sodium hydroxide (NaOH) were used without purification. Hyaluronic acid-amino acid derivatives (HA-aa) were synthesized as described in Chapter 4, section 2.2. Purified water was obtained with an Arium 661 Sartorius system (Goettingen, Germany). Visking dialysis membranes of 12-14000 Daltons were purchased from Medicell International Ltd (London).

2.2.Cross-linking of the HA-aa

2.2.1. Crosslinking with BDDE in alkaline media

Cross-linking in alkaline conditions was performed according to the method described by Piron[108]. BDDE (10.4µL, 0.070mmol) was mixed with 544µL of 25mM NaOH solution and added onto 80mg (0.2mmol) of dry native HA or HA-aa. After immediate mixing with a vortex mixer, the tubes were centrifuged at 2500rpm for 4 minutes and incubated at 50°C during 2 hours.

2.2.2. Crosslinking with BDDE in acidic media

Cross-linking in acidic conditions was performed according to the method described by De Belder and Malson[102]. Native HA or HA-aa (80mg, 0.2mmol) was dissolved in water (800µL) overnight. BDDE (60µL, 0,163mmol, 0.82 eq) was mixed with glacial acetic acid (30µL) and added to the HA solution. After immediate mixing with a vortex mixer, the tubes were centrifuged at 2500rpm for 4 minutes and incubated at 60°C during 4 hours.

2.3.Purification and swelling of hydrogels

After the crosslinking reactions, the resulting gels were purified by dialysis. Water (25mL) was added to the tubes, the gels were detached from the bottom wall and transferred to dialysis membranes with the surrounding water. Dialysis was performed against purified water during 72 hours after which the gel was emptied out of the membrane onto a small strainer to eliminate the surrounding excess water and weighed.

The swelling ratio was calculated by dividing the weight of the final swollen gel by the weight the starting dry solid content (80mg).

2.4.Enzymatic degradation of HA-aa crosslinked hydrogels

The same method used as described in Chapter 4, section 2.4. The crosslinked hydrogels were previously crushed and homogenized. Then a stoichiometric amount was precisely weighed and adjusted with water to obtain a final concentration of 2.5mg/mL in each test tube.

Another enzymatic degradation test was performed on chunks of hydrogels before crushing and homogenizing. The chunks were weighed and incubated at 37°C into tubes containing hyaluronidase solution and phosphate buffer at pH 7.4. After 48 hours incubation, the remaining hydrogel chunks were recuperated on a small sieve and weighed.

3. RESULTS AND DISCUSSION

3.1.Cross-linking of the HA-amino acids into hydrogels

As stated above, two ways of crosslinking with BDDE have been described in the literature, firstly in strong alkaline conditions (pH > 12) by Malson and Lindqvist (1986)[107], later improved by Piron (2002)[108] and secondly in acidic conditions by De Belder and Malson (1986)[102]. Depending on the conditions, the reaction occurs predominantly on the hydroxyl or the carboxylic groups (**Figure 64**), as detailed in Chapter 1, section 2.2.1.1.

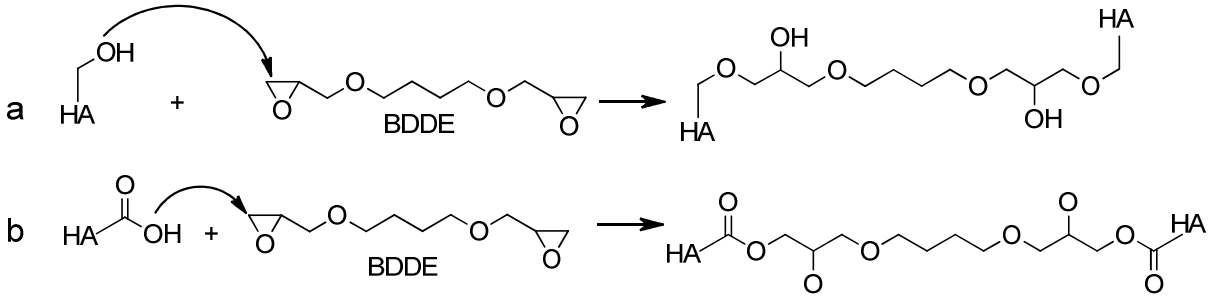

Figure 64: HA crosslinking mechanism with BDDE via the hydroxyl groups (a) and the carboxyl groups (b).

Each reaction was performed with the HA-aa derivatives as well as native HA for comparison.

Initial crosslinking reactions were performed in NaOH solution but did not yield any hydrogel for the HA-aa derivatives. Hydrogels were obtained however in the same conditions with native HA. The reason why hydrogels could not be obtained from HA-amino acids is unknown but one possibility is to assume that the strongly alkaline conditions denatured the starting polymer to such an extent that the crosslinked network was completely dissolved. Perhaps the HA chain fragmentation known to occur at high pH values[187] is enhanced by amino acid grafting. Another possibility would be that the amino acid impedes the crosslinking reaction.

Next, we performed the crosslinking reactions as described in a patent by De Belder and Malson (1986) in slightly acidic conditions[102]. The milder reaction performed in water with a small amount of acetic acid allowed to obtain water insoluble hydrogels from all HA-derivatives, with the exception of HA-threonine (**Figure 65**). The derivatives all had 100% substitution degree and were crosslinked with 0,82 equivalents of BDDE, the theoretical crosslinking degree therefore being 82%.

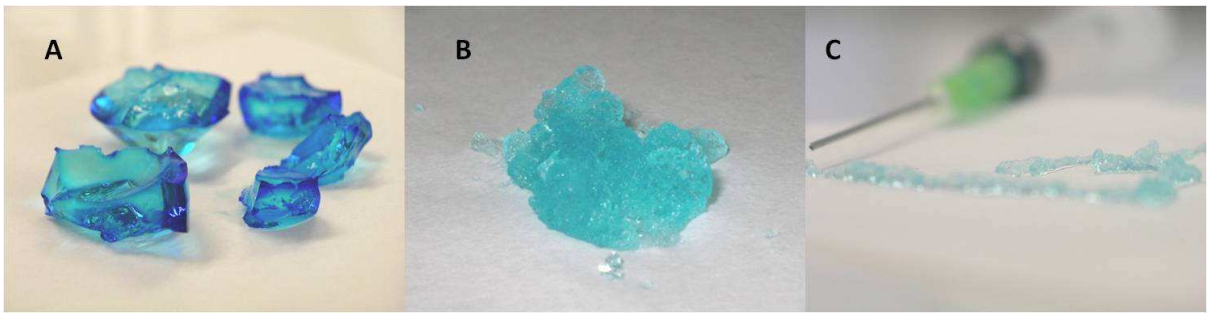

Figure 65: Native HA crosslinked hydrogels after swelling in water (A), then crushing (B) and finally injecting out of a needle (C). In this case only, the blue coloring was added to the dialysis water to help visualize the outline of the gel.

The swelling ratio of the native HA hydrogel is within the range of values previously reported[102,107]. All HA-aa crosslinked hydrogels gave higher swelling ratios than the native HA hydrogel when subjected to the same reaction conditions (**Figure 66**). The higher swelling ratios suggest a lower crosslinking density. Indeed, studies have shown that the swelling ratio of hydrogels decreases when the cross-linking degree increases until reaching a saturation point[89,103,116]. It can therefore be assumed that the grafted amino acids hindered the crosslinking reaction, most likely due to steric hindrance. For hydrogels formed from HA-ala, HA-val and HA-ser, some viscous solution remained in the dialysis tube indicating that the crosslinking was not complete. For HA-thr, no gel and only viscous solution was recovered. The aliphatic groups and the hydroxyl groups did thus not appear favorable for the crosslinking reaction.

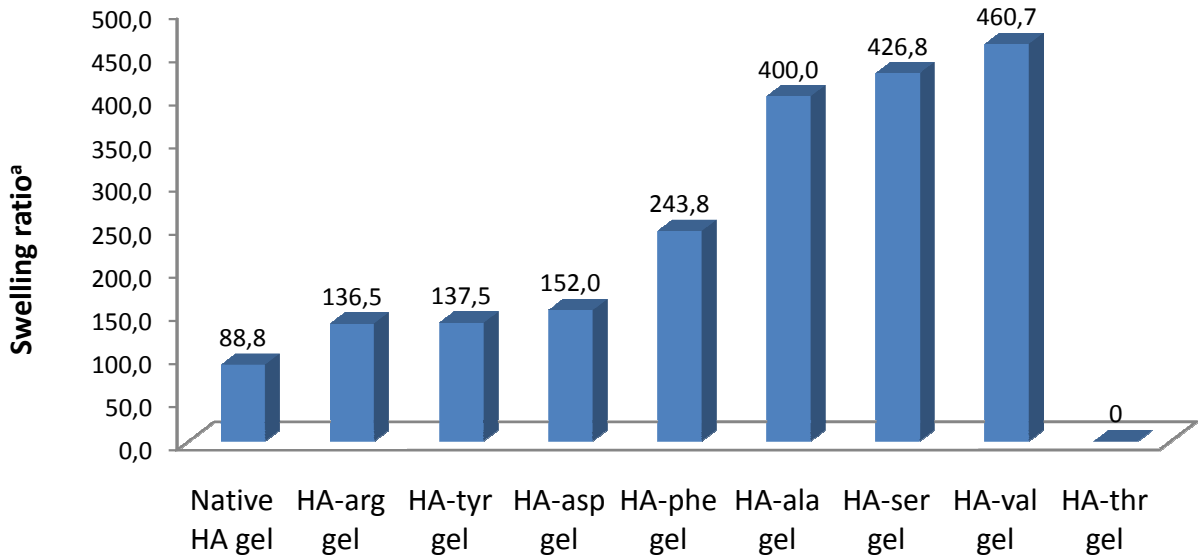

Figure 66: Swelling ratios of HA-amino acid (DS 100%) crosslinked hydrogels ([a]: in g of water per mg of polymer).

In order to evaluate the influence of the amino acids on the swelling ratio of the resulting hydrogels, one of the HA-aa derivatives was selected for synthesis of hydrogels with a range of different amino acid substitution degrees. HA-tyrosine was chosen for its high viscosity and its swelling ratio close to the native HA hydrogel and synthesized with 40%, 70% and 100% substituted carboxyl groups. The swelling ratios of hydrogels obtained showed to only slightly increase with the substitution degree (**Figure 67**), reinforcing the hypothesis that amino acids might slightly hinder the crosslinking reaction. However, the values for HA-tyr of 40% and 70% were not significantly different than for native HA but only HA-tyr 100% had significantly different values (Student test, p=0.05).

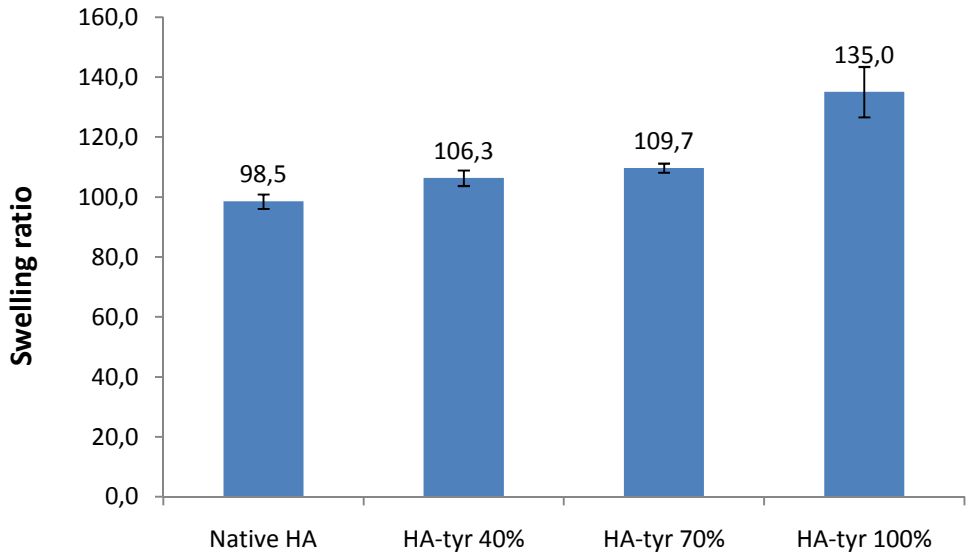

Figure 67: Swelling ratio of hydrogels obtained from HA-tyrosine with different DS.

3.2.Enzymatic degradation of HA-aa cross-linked gels

To assess the efficacy of the amino-acid grafting after crosslinking of the HA-aa polymers, these were subjected to enzymatic digestion by hyaluronidases and the degradation was further quantified with the Morgan-Elson assay. Only HA-tyr hydrogels with different grafting ratios of tyrosine were chosen for further analysis with this method. The gels were previously crushed and homogenized using a Poter instrument and a stoichiometric amount was precisely weighed in each test tube and adjusted with water to obtain a final concentration of 2.5mg/mL. **Figure 68** shows that the hydrogels made from HA-tyr derivatives have a significantly lower enzymatic degradation than the native HA hydrogels synthesized in the same conditions. Crosslinking the HA derivatives into hydrogels therefore preserved the beneficial effect of reducing their sensitivity to hyaluronidases enzymes. And such as for their precursory HA-aa polymers, the enzymatic degradation of crosslinked hydrogels is dependent on the substitution degree. It is reasonable to assume that other crosslinked HA-aa hydrogels such as HA-aspartic and or HA-arginine show a lowered enzymatic degradation.

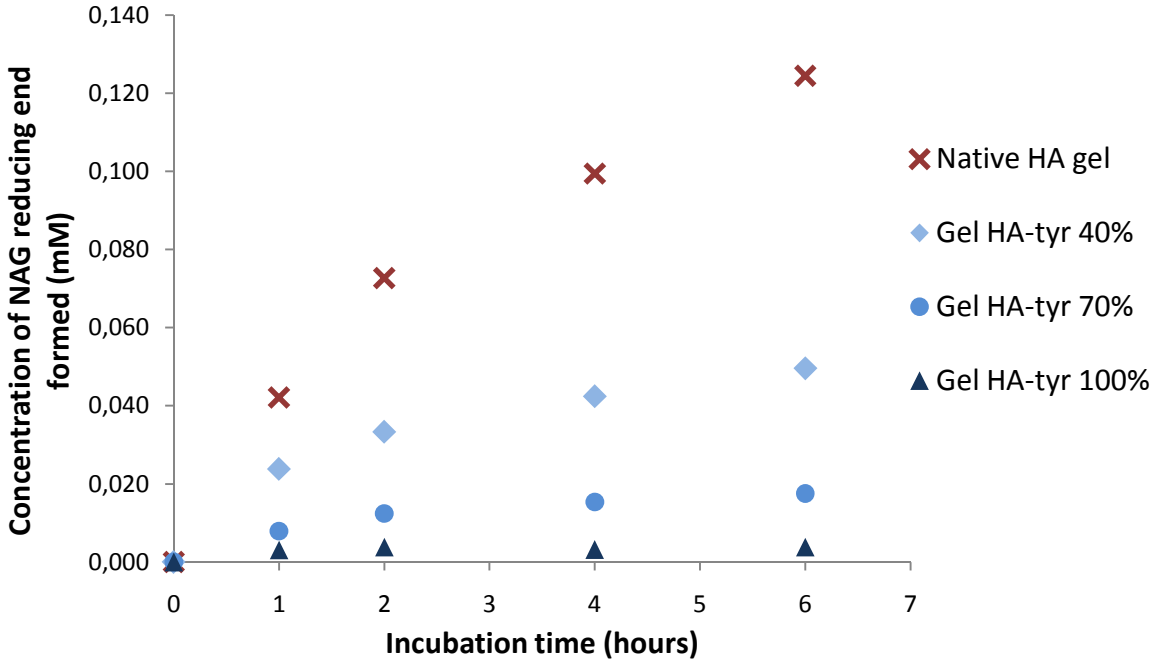

Figure 68: Enzymatic degradation profile of cross-linked native HA and cross-linked HA-tyrosine hydrogels.

Comparison was further made with commercially available products Juvederm Voluma (Allergan) and Glytone (Pierre Fabre). Both products have an HA concentration of 20mg/mL and are crosslinked with BDDE according the manufacturers. The concentration was thus adjusted to obtain an even 2.5mg/mL for each gel. Results are presented in **Figure 69**. The HA-aa crosslinked hydrogels as well as the native HA hydrogel all show a higher resistance to hyaluronidase digestion. The difference in enzymatic degradation profile between the commercial products and the native crosslinked HA hydrogel is possibly due to the difference in crosslinking degree. Indeed, it is known that the crosslinking degree influences the enzymatic degradation of HA hydrogels[103,178] and the crosslinking degree of the Juvederm Voluma and Glytone are uncertain. Sources indicate that products of the Juvederm range have crosslinking degrees between 9% and 11%[197]. This value is much lower than the theoretical crosslinking degree of our crosslinked gels, 82%, thus explaining the higher enzymatic resistance of our native HA product. We may reasonably assume that by

crosslinking the HA-aa derivatives with the same crosslinking degree as the commercial products, the enzymatic resistance of the HA-aa derivatives will still be higher.

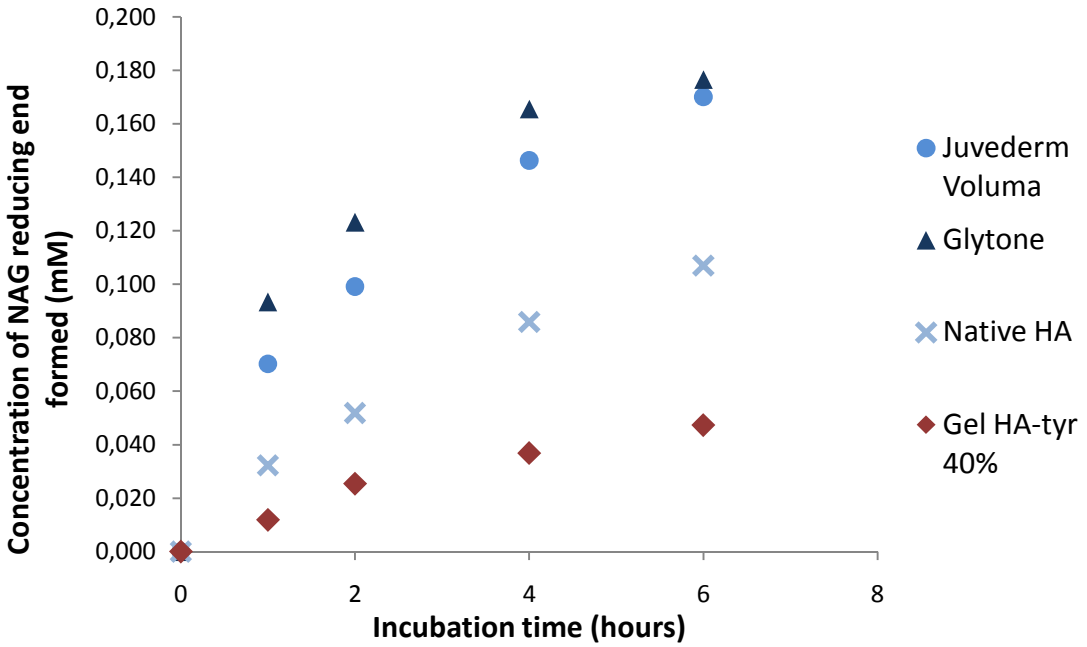

Figure 69: Enzymatic degradation profile of commercially available Juvederm Voluma, Glytone and synthesized native HA hydrogel and HA-tyrosine 40% hydrogel.

Another enzymatic degradation test was performed on chunks of hydrogels before crushing and homogenizing in order to confirm the higher resistance of the gels towards hyaluronidase digestion. The chunks were cut off from the swollen gel (**Figure 65A**) and were weighed before and after 48 hours incubation at 37°C with hyaluronidase solution. No gel remained for the native HA whereas HA-tyrosine 100% gels showed to be more resistant (**Table 4**). This assay therefore confirms that the grafting of amino acids brings higher resistivity towards hyaluronidases to the resulting crosslinked hydrogels.

Table 4 : Weight of HA hydrogel pieces before and after enzymatic degradation

Hydrogel	Initial weight (g)	Weight after 48h incubation (g)	Remaining gel	Mean
Native HA gel	164,9	0	0%	0%
	134,2	0	0%	
	123,8	0	0%	
HA-tyr 100% gel	152,8	127,1	83%	84%
	182,6	47,7	26%	
	168,2	141,9	84%	
HA-tyr 40% gel	144,8	12,1	8%	8%
	139,2	21,3	15%	
	162,2	0	0%	

4. CONCLUSION

In the present chapter, we achieved hydrogel formation from the HA-amino acid derivatives. Crosslinking of the HA-aa derivatives was achieved by using BDDE as the crosslinking agent in a slightly acidic media. The nature of the amino-acid side chain grafted on the HA chain showed however to greatly influence the crosslinking reaction. The resulting crosslinked HA-aa hydrogels exhibited a higher resistance to hyaluronidase digestion compared to the hydrogels obtained from native HA in the same conditions, showing that the efficacy of amino acid grafting onto HA is conserved after crosslinking. We therefore successfully synthesized crosslinked HA hydrogels derivatives with higher resistance to hyaluronidases enzymes *in vitro* than the native HA hydrogels as well as commercially available crosslinked HA products.

CHAPTER 5: Study of the enzymatic degradation of hyaluronic acid and its derivatives

1. INTRODUCTION

The first part of the experimental work of this thesis was dedicated to the study of the enzymatic degradation profile of the native HA and the setting up in the laboratory of an enzymatic degradation assay. This part was essential for the evaluation of the efficacy of the modified hyaluronic acid derivatives described in the previous chapters. As it also presents results of the HA derivatives, this chapter is included last for more clarity.

The enzymatic degradation assay is adapted from Muckenschnabel et al. (1998) who used the assay to study hyaluronidase activity in human plasma[194]. The principle of this assay is to subject HA to enzymatic digestion by adding hyaluronidase and subsequently quantify the degradation by a colorimetric method. As the Hyal-2 hyaluronidases used are known to hydrolyze the β(1,4) glycosidic bonds of HA[198,199], the concentration of N-acetylglucosamine (NAG) reducing ends is therefore increased after enzymatic degradation (**Figure 70**).

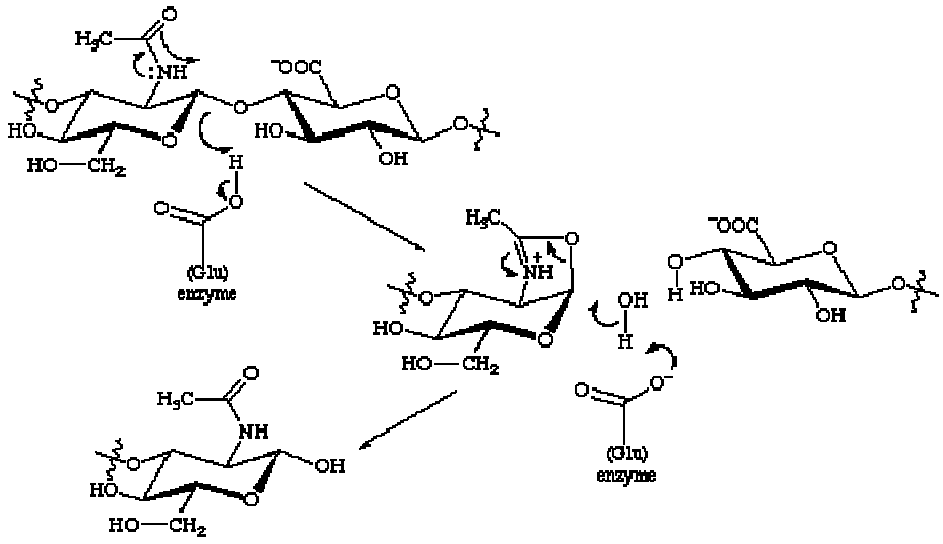

Figure 70: Enzymatic hydrolysis mechanism of hyaluronic acid by hyaluronidases showing the recovery of the N-acetyl glucosamine reducing end. Source: IUBMB Enzyme Nomenclature[199].

The Morgan-Elson reaction, described by Reissig et al. (1955)[200] and later adapted by many authors, uses 4-dimethylamino-benzaldehyde (DMAB) which reacts with the NAG reducing ends and forms a colored product, easily quantified by UV absorption (**Figure 71**).

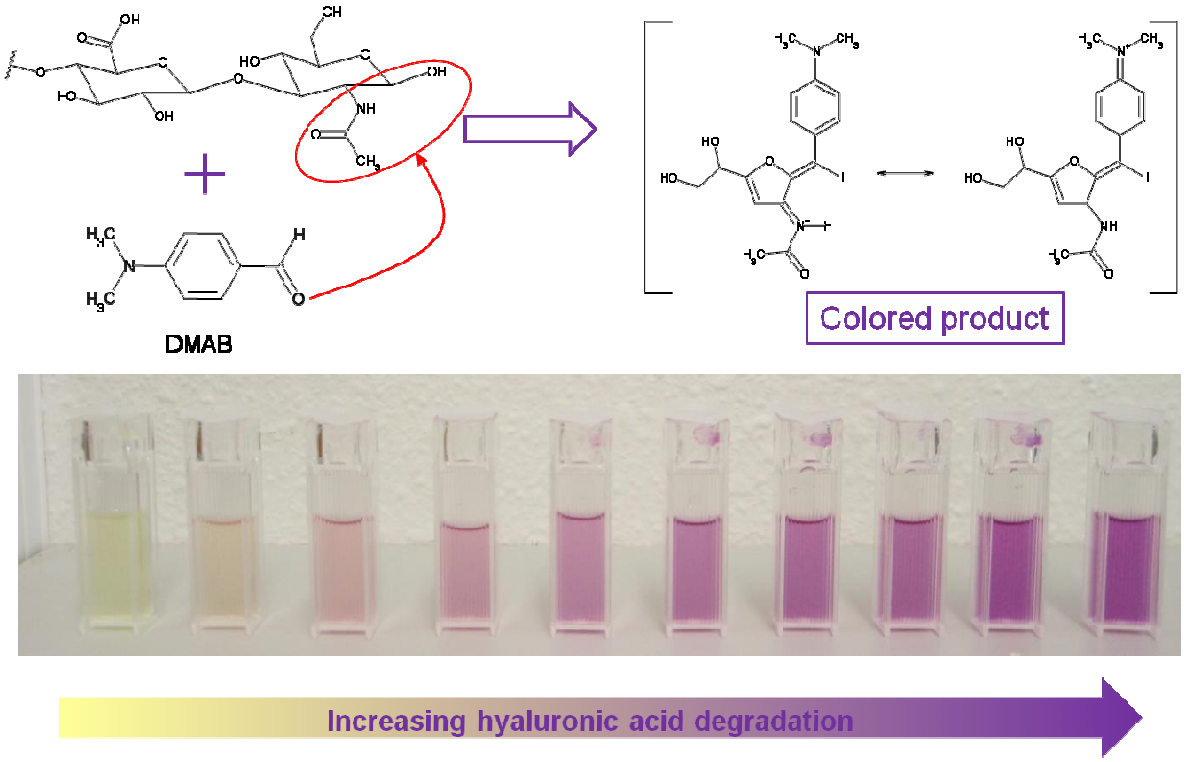

Figure 71: Formation of a colored product using the Morgan-Elson reaction to quantify the degradation of hyaluronic acid.

With the aim to quantify the difference in enzymatic degradation of native HA and the HA-amino acid derivatives, we used an enzymatic kinetics assay to determine the kinetics constants K_m and V_{max}. K_m is the Michaelis constant and is in inverse proportion to the affinity of the enzyme for its substrate (HA). V_{max} corresponds to the maximal reaction rate of the enzymatic degradation. It was first necessary to determine the initial rate period of the enzymatic reaction, during which the enzyme produces product at an initial rate that is approximately linear for a short period after the start of the reaction. As the reaction

proceeds and the substrate is consumed, the rate continuously slows. Kinetics assays were performed at various concentrations to calculate the constants, as detailed in the next sections. Experiments were performed at 37°C and pH 7.4 according to Sall and Ferard (2007)[155] to be the closest to *in vivo* conditions.

In order to visualize more easily the enzymatic degradation profile of native HA and its derivatives, a simplified enzymatic assay was also set up and performed without consideration of the initial rate period.

2. Materials and methods

2.1.Materials

All chemicals were purchased from Sigma-Aldrich and used without previous purification. Hyaluronidase from bovine testis had an activity of 850units/mg solid. Hyaluronic acid (HA) sodium salt was isolated from *Streptococcus equi* given at a molecular weight of 1.63MDa. Water was purified with a Milli-Q system (Millipore, France).

Absorbance measurements were performed with a UV-Vis recording spectrophotometer UV-2401 PC (Shimadzu) and a UVProbe 2.21 software (Shimadzu).

2.2. Solutions

HA solutions were prepared by mixing the dry polymer in water. To reduce the dissolution time, HA powder was added slowly onto the water under constant magnetic stirring.

Phosphate buffer was prepared with 0,2M NaH_2PO_4, 0,1M NaCl and 0,2mg/ml BSA and kept at 4°C. The pH was adjusted to 7.4 with 0.25M NaOH solution.

Hyaluronidase solution was prepared at a concentration of 4,25.10*6IU/mL (or 425units/mL) in 0,9% NaCl (9 g/l), stored at -20°C in separate 1.5mL tubes and thawed just before use.

Tetraborate alkaline solution was prepared by mixing 1,73g of H_3BO_3 and 0,78g of KOH in 10ml water. To 10mL of this solution, 1mL of potassium carbonate solution was added (0,8g of K_2CO_3 in 1ml water). The final solution was kept at room temperature.

DMAB solution was freshly prepared before each experiment by dissolving 2.4g DMAB (4-dimethylamino-benzaldehyde) in 3mL concentrated HCl and 9mL glacial acetic acid. 48mL glacial acetic acid was further added just before use.

The reference NAG solution was prepared with 0.5mM N-acetylglycosamine (NAG) and stored at 4°C.

2.3.Methods

2.3.1. General enzyme kinetics assay

The method used was adapted from Muckenschnabel et al. (1998)[194]. 600µL HA solution (of variable concentration) was diluted with 600µL water and 300µL phosphate buffer in 15mL tubes and brought to 37°C in a water bath. 150µL hyaluronidase solution was added, the tube was quickly vortexed and the timer was immediately set off. At a given time (variable for each experiment), 275µL solution was removed from the tube and immediately added onto 50µL tetraborate solution (in a 1.5mL tube) to stop the enzymatic reaction and placed in an ice bath. After having removed the final sampling, the tubes were all kept at 0°C or below until the next step.

The control tubes were prepared in duplicates for each HA concentration used in the same proportions at a lower quantity with 100µL HA solution, 100µL water, 50µL phosphate buffer, 25µL 0.9% NaCl solution and 50µL tetraborate solution.

The NAG reference tubes were prepared in triplicates with 200µL water, 50µL phosphate buffer, 50µL NAG 0.5mM solution and 50µL tetraborate solution.

2.3.2. Morgan-Elson colorimetric test

After collecting all the samples at each given time, the tubes were heated at 100°C in a boiling water bath during 5 minutes and cooled in an ice bath for 2 minutes. 750µL of freshly prepared DMAB solution was added to each tube and the tubes were all left to incubate for 30 minutes at room temperature in the dark. The solutions were then transferred to plastic cuvettes and the absorbance was measured at 586nm with a UV-Vis spectrophotometer.

2.3.3. Calculation of the substrate concentration

The absorbance values obtained enable to calculate the concentration of NAG reducing ends in µM with the following formula:

[NAG reducing ends] = (Absorbance/ε)*(1.075/0.275)*10^6 in µM, where 1.075mL is the final volume of the samples and 0.275mL is the volume of the reaction solution.

The molar absorption coefficient, ε, is calculated for each experiment from the absorbance of the NAG references with the Beer-Lambert formula:

ε = Mean Abs(NAG reference solution)/(0.023*10^{-3}) M^{-1} cm^{-1}, where 0.023mM is the concentration of NAG in the final 1.075mL measured sample.

As NAG reducing ends are already present in the starting reaction solution, the concentration of NAG reducing ends which are formed during the enzymatic reaction is obtained with the formula:

[NAG formed reducing ends] = [NAG reducing ends] - $[\text{NAG reducing ends}]_{control}$

2.3.4. Determination of the enzymatic kinetic constants K_m and V_{max} for HA

2.3.4.1. Initial rate period determination

The first step to determining the enzymatic parameters is to determine, for one given HA solution concentration, the initial rate period, which is the time during which the enzyme produces the substrate at an approximately linear rate (**Figure 72**). The slope of the initial rate period gives the initial rate of reaction v_i. The concentration used was 5mM of HA solution.

The enzyme kinetics assay was performed as described above (section 2.3.1) and samples were removed from the reaction media at different times and time intervals, from 1 minute to 6 hours, until determining the linearity of the absorbance values.

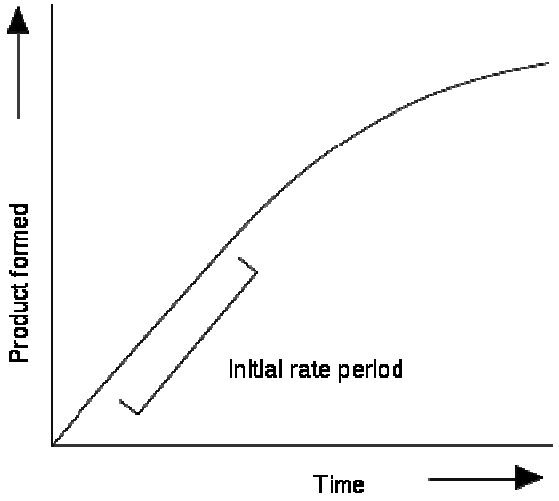

Figure 72: Schematic representation of an enzymatic reaction with the linear initial rate period.

2.3.4.2. Determination of the HA saturation concentration

Once the time intervals for the enzyme kinetics assay sampling were set, the next step was to determine the saturation concentration above which the enzyme is saturated with substrate and the reaction rate reaches a maximum, V_{max} (**Figure 73**). We therefore performed the enzyme kinetics assay with different HA concentrations starting from 20mM and lower.

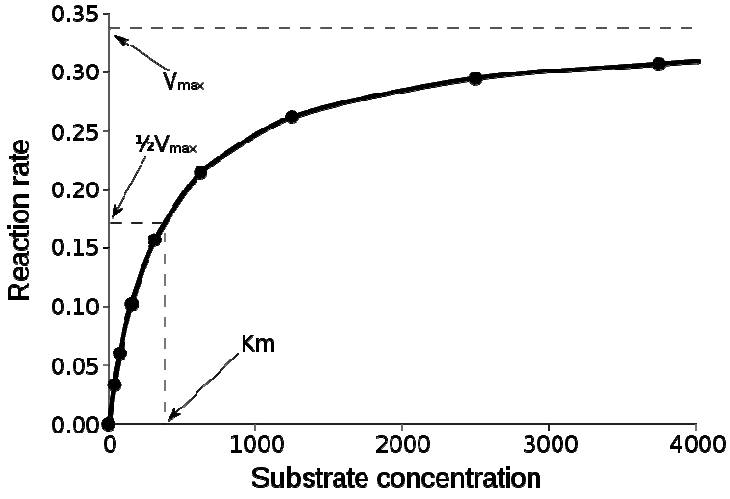

Figure 73: Schematic saturation curve of an enzymatic reaction.

2.3.4.3. Determination of enzymatic kinetic constants

After having determined the saturation concentration, enzyme kinetics assays were performed on a series of solutions with lower HA concentrations. From these assays, the initial rates of reaction (Vi) were plotted in function of the corresponding [HA] concentrations. This plot is known as the Michaelis-Menten plot as shown above (**Figure 73**), which allows determining the V_{max} and K_m constants. However, by plotting 1/Vi as a function of 1/[HA], known as the Lineweaver-Burk plot (**Figure 74**), the constants were determined more precisely using the following equation : $y = (K_m/V_{max})x + (1/V_{max})$. Experiments were performed in triplicates or more for more precision.

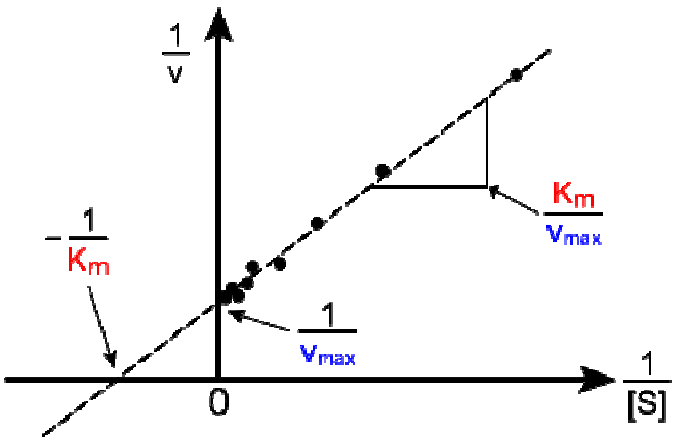

Figure 74: Schematic representation of a Lineweaver-Burk plot, showing the inverse of the initial rate of reaction 1/Vi as a function of 1/[substrate].

2.3.5. Determination of the enzymatic kinetic constants K_m and V_{max} for HA derivatives

The same protocol was used for the enzyme kinetics assays with HA derivatives at the same concentrations than native HA described above.

2.3.6. Simplified enzymatic degradation study of native HA and its HA-amino acid derivatives

100µL of 5mM native HA or HA-amino acid solution, 100µL of purified water and 50µL of phosphate buffer were incubated with 25µL of 4.10^6 IU/mL hyaluronidase solution at 37°C during one to six hours. The enzymatic reaction was stopped by adding 50µL of tetraborate solution and heating the tubes in boiling water for 5 minutes. After cooling in ice for 2 minutes, 750µL of dimethylamino benzaldehyde (DMAB) (2g of DMAB of dissolved in 2.5mL HCl and 47.5mL glacial acetic acid immediately before use) solution were added to each tube. After 30 minutes incubation, the absorbance at 586nm was measured.

3. RESULTS AND DISCUSSION

3.1.Determination of enzymatic kinetic constants for native HA

3.1.1. Initial rate period determination

The enzyme kinetics assay was performed at different times and time intervals, from 1 minute to 6 hours and several reactions were necessary until determining the linearity between the concentration of NAG formed and the incubation time. Finally, the initial rate period was determined up to 15 minutes reaction time as shown is **Figure 75**. This first part enabled to set the duration of the following kinetics assays to 15 minutes. The sampling times were set at 3, 6, 9, 12 and 15 minutes.

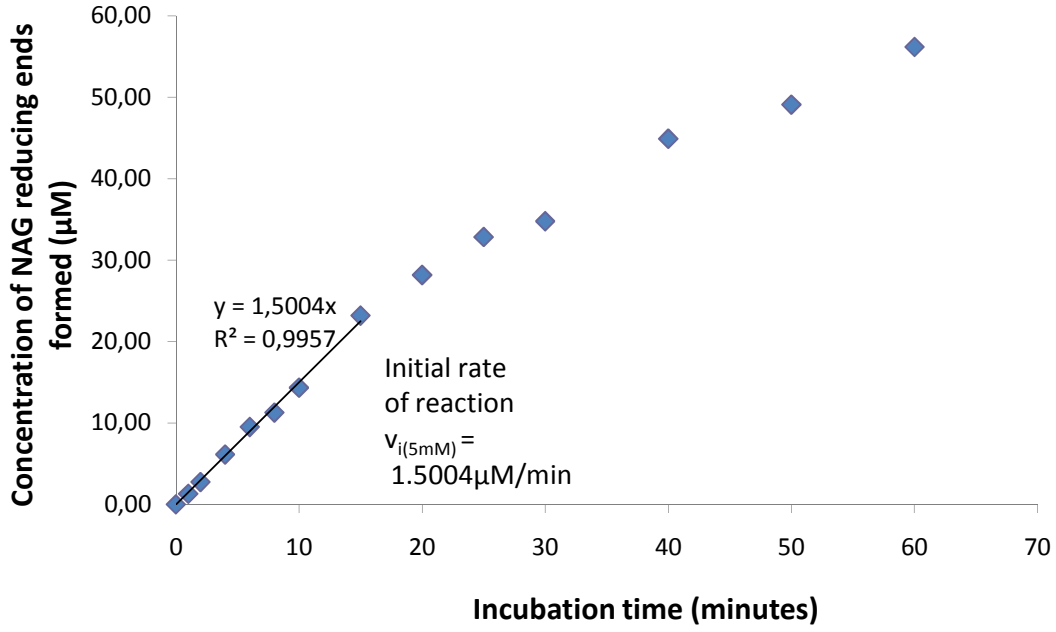

Figure 75: Progress curve of the enzymatic reaction for HA at 5mM. The reaction is approximately linear until t=15 minutes and the slope is the initial rate of reaction: vi(5mM)= 1.5004µM/min.

3.1.2. Determination of the HA saturation concentration

Results are presented in **Figures 76 and 77**. On **Figure 76**, the curves 5mM and above are approximately superimposed, showing that the enzymatic reaction is saturated. The

saturation concentration seems to be between 2.5 and 5mM HA. For concentrations 0.5-1-2 and 3mM (**Figure 77**), the curves are no longer superimposed and the values are better aligned in a straight line. The initial rate of reaction for each concentration is given by the slope of the linear regression.

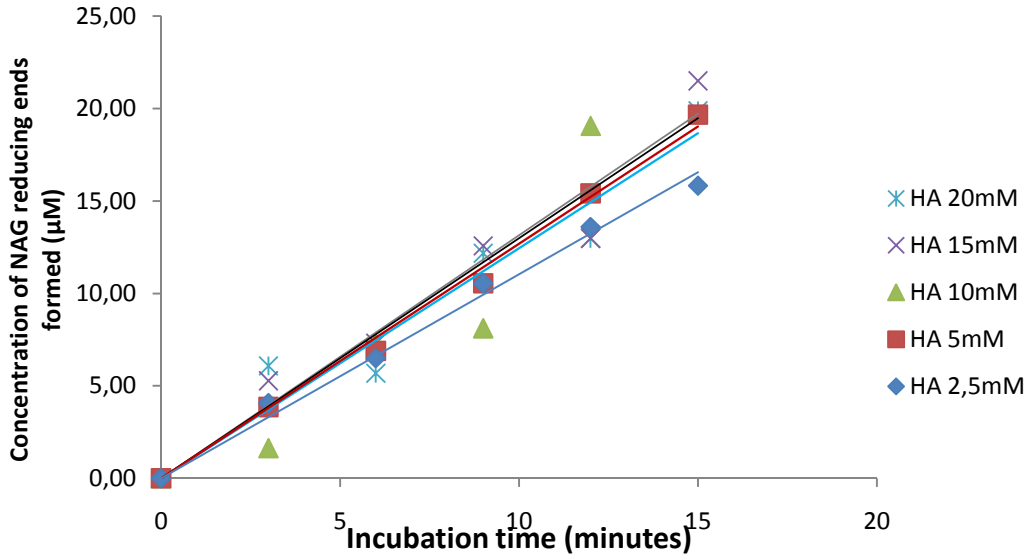

Figure 76: Enzyme kinetics assay for HA concentrations 2.5 to 20mM.

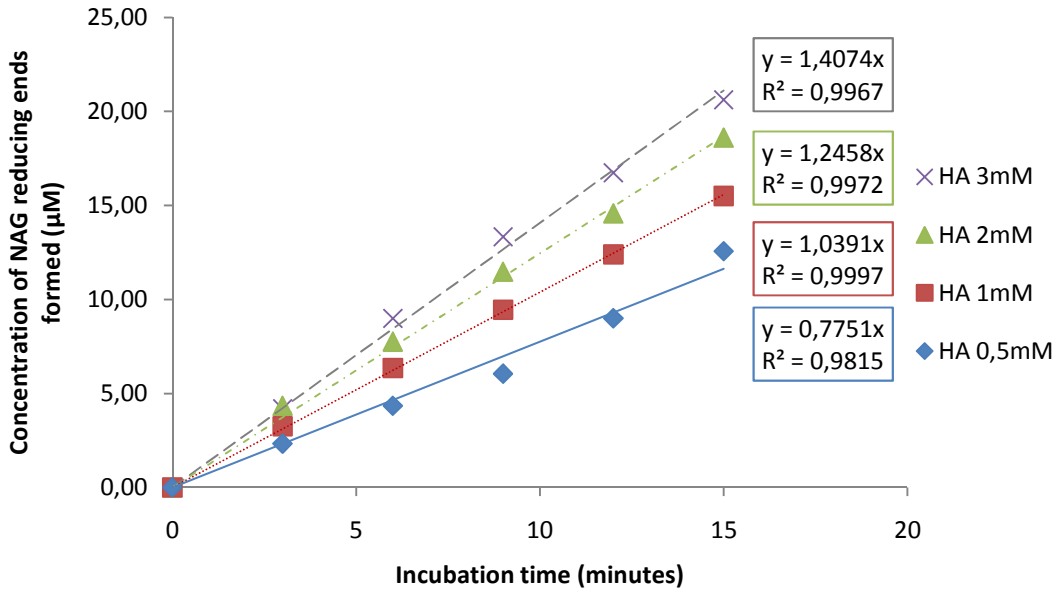

Figure 77: Enzyme kinetics assay for HA concentrations 0.5 to 3mM. The initial rate of reaction for each concentration is given by the slope of the linear regression.

3.1.3. Determination of the enzymatic constants

The values of initial rate of reaction obtained in **Figure 77** were plotted against the corresponding HA concentrations. The resulting Michealis-Menten plot, shown in **Figure 78**, is not precise enough to calculate the enzymatic constants. The values were therefore inversed and plotted in the Lineweaver-Burk plot (**Figure 79**). The linear regression is suitably precise (R^2=0.9964) and the resulting curve equation enables to calculate the constants:

$y = 0.339x + 0.6166$ (**Figure 79**) <==> $y = (K_m/V_{max})x + (1/V_{max})$

$V_{max} = 1/0.6166$ <==> **V_{max} = 1.622 µM/min**

$K_m = 0.339/0.6166$ <==> **K_m = 0.550 mM**

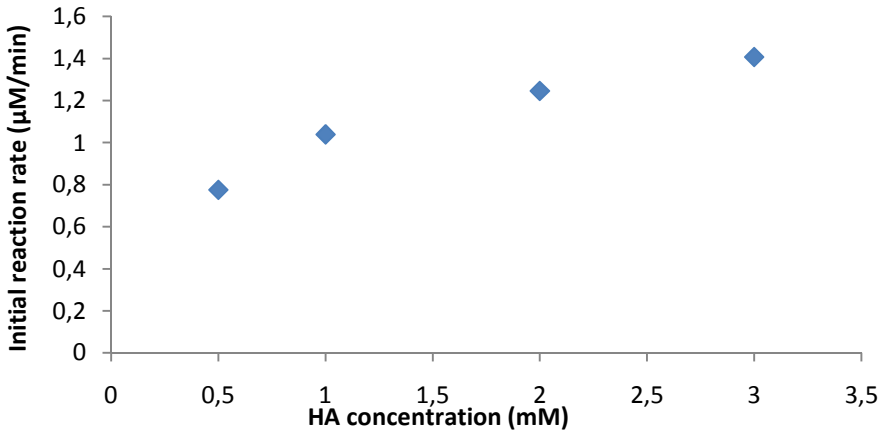

Figure 78: Michealis-Menten plot for HA enzyme kinetics assay.

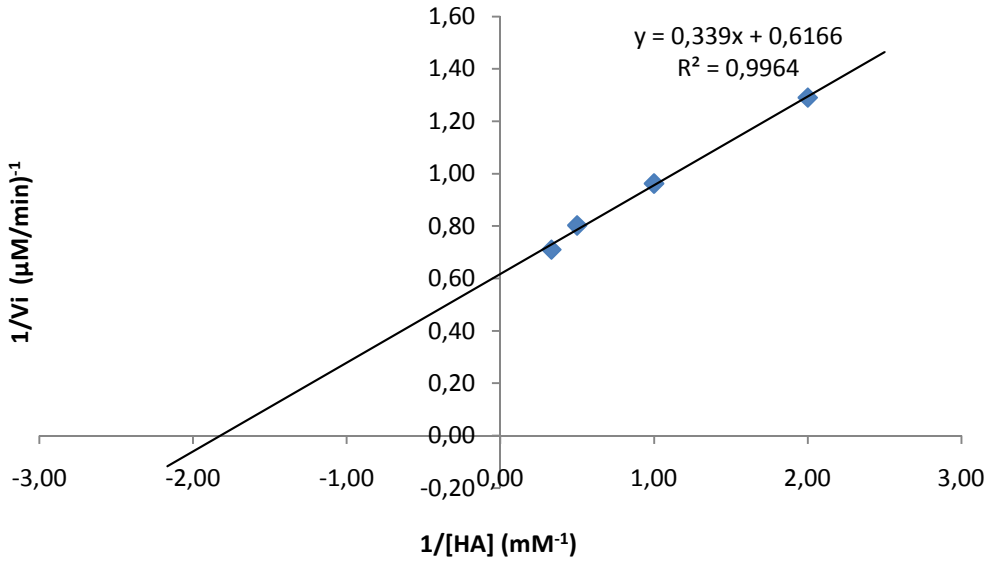

Figure 79: Lineweaver-Burk plot for HA enzyme kinetics assay.

Experiments were made in triplicates for more precision. The results are shown in **Table 1**. The mean value for V_{max} was 1.59µM/min and K_m was 0.628mM, which are values within the range of values reported in the literature[201,202,203]. Vincent et al. (2003) reported values of V_{max} of 0.87µM/min and K_m of 1.25mM[201]. However, the experiments were made at a different pH (pH 5), explaining the different values obtained. Also, the kinetic constants can considerably vary according to the molecular weight of the HA chain used, as recently presented by Deschrevel et al. (2008)[203].

Table 5 : Results of 3 identical kinetics assays for the determination of enzymatic constants

Experiment	V_{max} (µM/min)	K_m (mM)
1	1.827	0.662
2	1.323	0.673
3	1.622	0.550
Standard deviation	0.253	0.068
Mean	**1.591**	**0.628**

3.2. Determination of enzymatic kinetic constants for HA-amino acid derivatives

The same kinetics assay was performed with HA derivatives at concentrations of 0.5, 1, 2 and 3mM. As shown in **Figure 80**, with these concentrations and sampling times, for HA-phenylalanine with 100% DS, the concentration of NAG reducing ends is much lower than for native HA and the curves are no longer aligned with 0. It therefore does not make sense to plot the slopes of the curve to further determine the kinetics constants. The same results were observed with other amino acids as shown in **Figures 81 and 82** with HA-threonine and HA-alanine respectively.

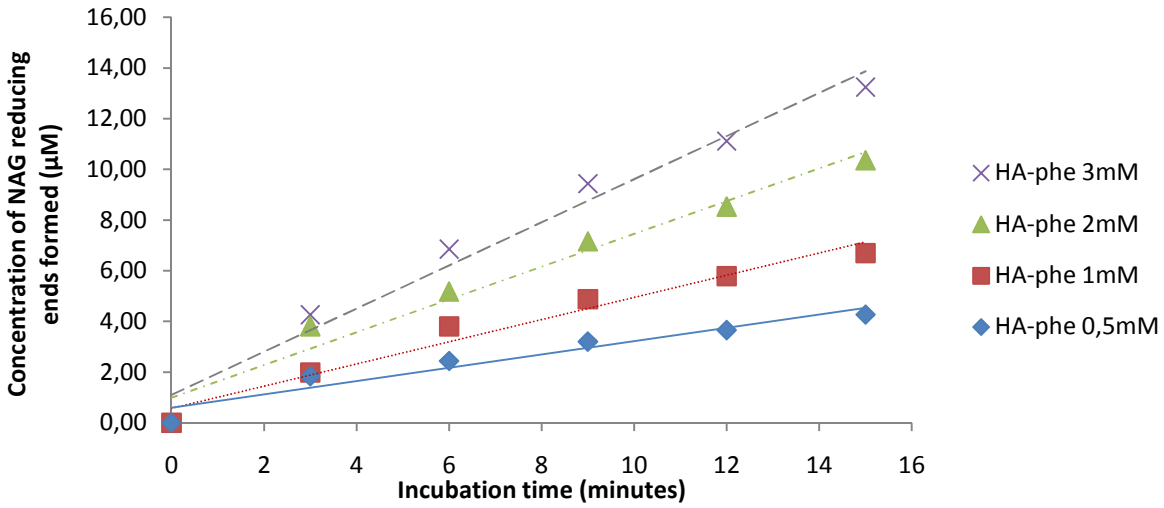

Figure 80: Enzyme kinetics assay for HA-phenylalanine concentrations 0.5 to 3mM. The initial reaction rate cannot be determined as the values are no longer aligned with the origin.

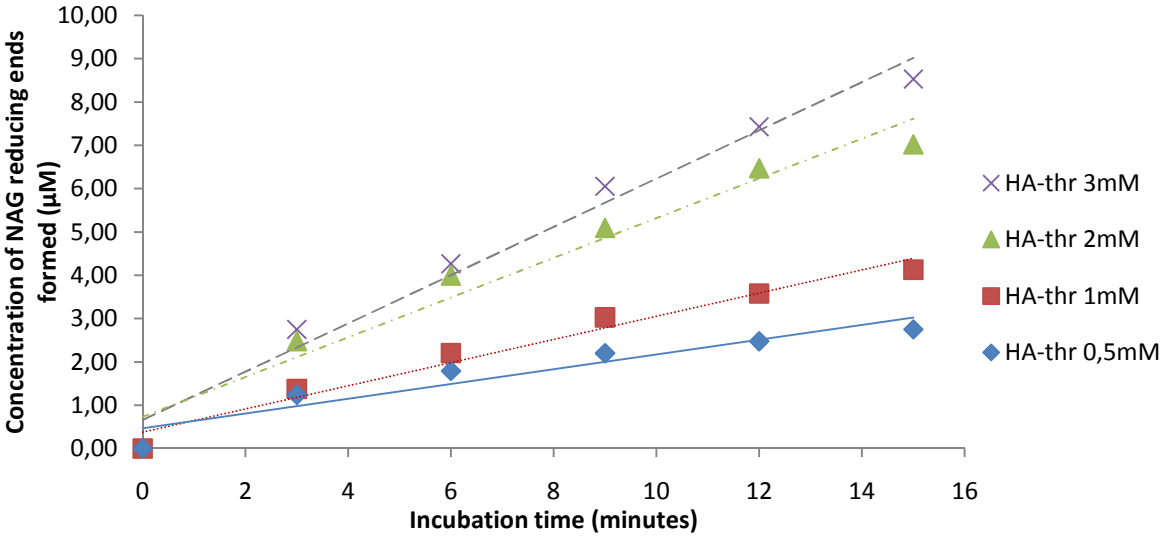

Figure 81: Enzyme kinetics assay for HA-threonine concentrations 0.5 to 3mM. The initial reaction rate cannot be determined as the values are no longer aligned with the origin.

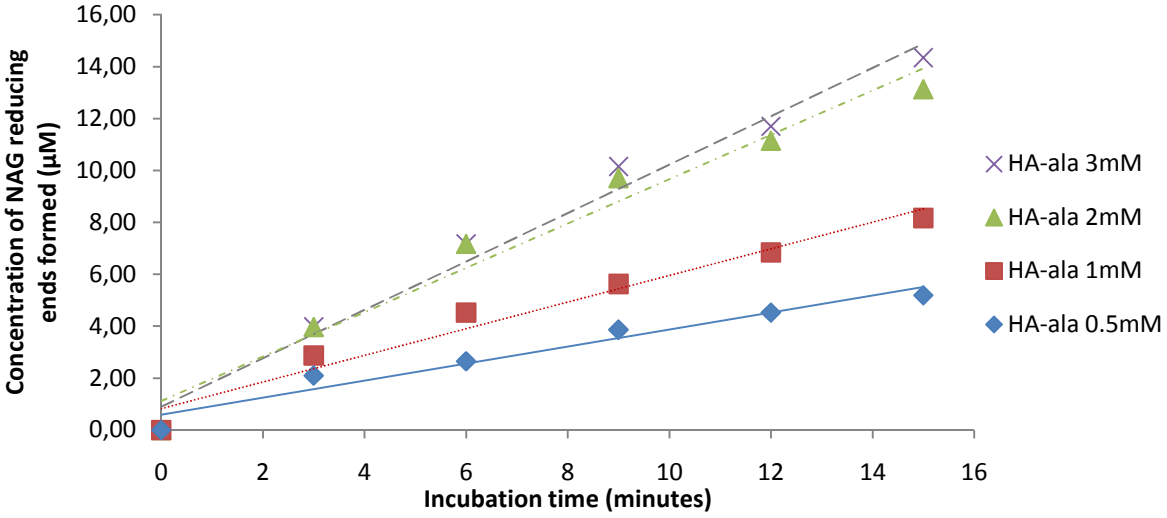

Figure 82: Enzyme kinetics assay for HA-alanine concentrations 0.5 to 3mM. The initial reaction rate cannot be determined as the values are no longer aligned with the origin.

We therefore tried the same assay but increasing the HA-amino acid concentration. The results of HA-aspartic acid kinetics assay with 10, 12, 14 and 16 mM show no improvement (**Figure 83**). The curves are still not aligned with the origin and it is not possible to determine the kinetic constants. All these results suggest that almost no enzymatic reaction occurs. However, no quantification can be done to support this observation.

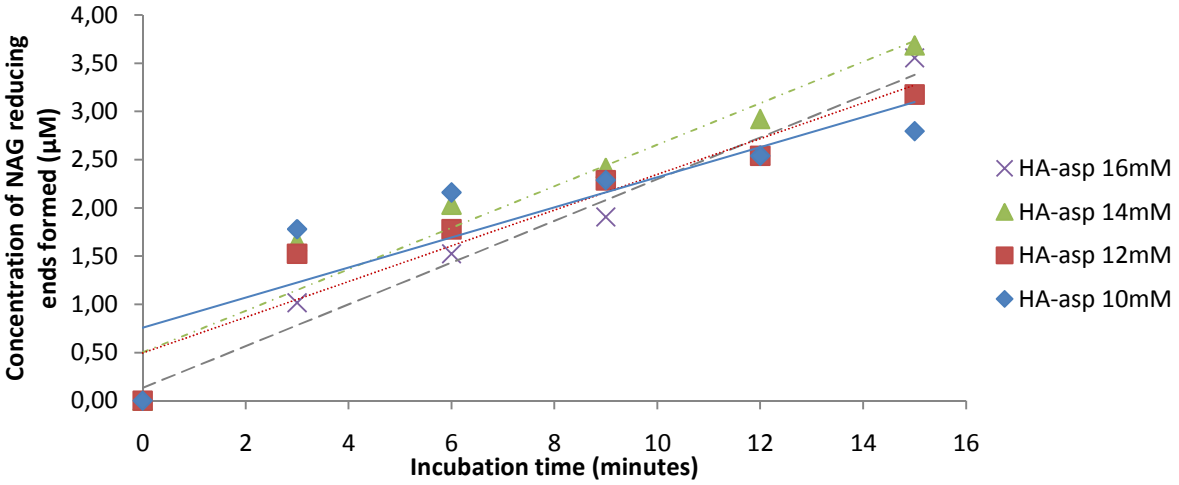

Figure 83: Enzyme kinetics assay for HA-aspartic acid concentrations 10 to 16mM. The initial reaction rate can still not be determined as the values are not longer aligned with the origin.

3.3.Simplified enzymatic degradation study

In order to visualize more easily the difference in enzymatic degradation of HA and its derivatives, an enzymatic assay was performed without consideration of the initial rate period. Enzymatic digestion was carried out during 1, 2, 4 and 6 hours. The resulting graph (**Figure 84**) immediately reveals the significant decrease in enzymatic degradation for all HA-amino acid. This simplified enzymatic assay therefore confirms the results of the kinetics assay and enables to easily visualize the difference in enzymatic stability of the different derivatives.

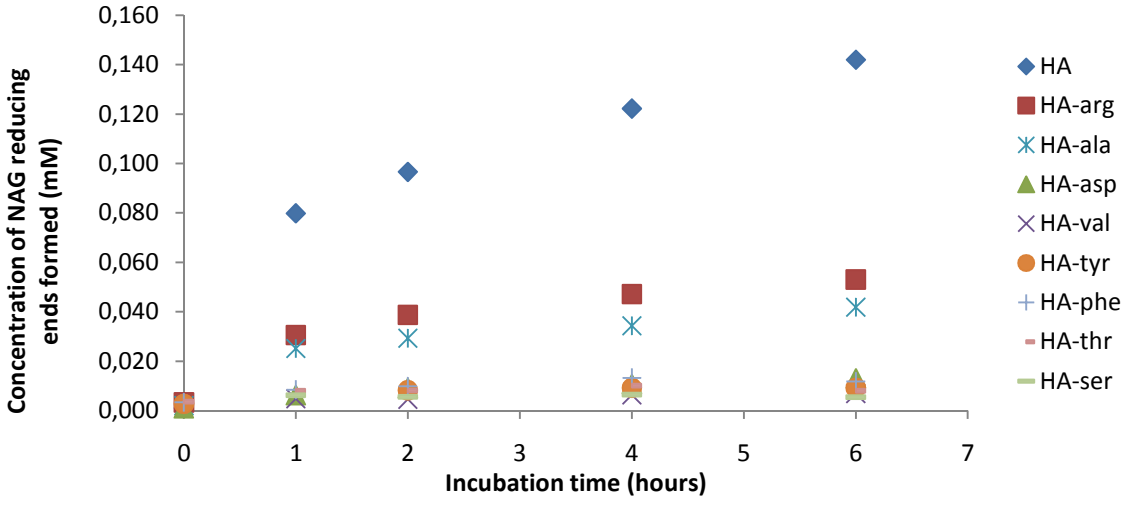

Figure 84: Enzymatic degradation profile of native HA and HA-amino acid derivatives.

4. CONCLUSION

This chapter enabled to set up a suitable method for the determination of the kinetic constants of native HA. The results obtained were comparable to the values reported in the literature. However, the kinetics assay was not possible to perform with the HA-amino acid derivatives due to the much lowered degradation by the enzymes. The simplified enzymatic assay enabled to directly visualize the decrease in enzymatic degradation of the derivatives. Even though no quantification of the decrease in degradation can be made with the results of the simplified assay, it was useful for the evaluation of the efficacy of the chemical modifications performed on native HA and enabled to identify amino acids as powerful molecules to be grafted and to enhance its stability towards hyaluronidase digestion.

CONCLUSIONS AND PERSPECTIVES

The aim of this work was to develop a novel hyaluronic acid product having a longer therapeutic action compared to the products currently on the market. The approach chosen to reach this goal was to proceed in two successive steps: first to perform a chemical modification which would confer higher resistance towards hyaluronidases enzymes, and secondly a crosslinking reaction to obtain an insoluble hydrogel with the mechanical properties required for its filling action. The use of an *in vitro* enzymatic assay enabled to evaluate easily and quickly the stability of the derivatives synthesized and compare them with the native hyaluronic acid as well as with the commercially available products. This way, an efficient chemical modification was detected and widely explored, consisting of grafting amino acids onto the carboxylic groups of hyaluronic acid.

First, L-alanine was used to test three amidation reactions for the grafting of an amino acid onto the carboxylic groups of HA. This allowed determining the most suitable method for the next steps of our work, which was performed in organic solvent and allowed to achieve 100% substitution degrees. This first part also allowed identifying an amidation method more appropriate to be considered for future upscale and industrialization, performed in water/acetonitrile with no previous conversion step of the native HA. We obtained up to 60% substitution degrees with this method, which could possibly be optimized by performing further studies.

In the following step, with the amidation method performed in organic solvent, we synthesized various HA-amino acid derivatives with 100% substitution degree from amino acids comprising a wide range of different side chains. Using the *in vitro* enzymatic degradation assay set up at the beginning of the experimental work of this project, results showed that the new HA derivatives were significantly more resistant to enzymatic digestion

than the native HA. These promising results allowed continuing the project to the next step of our initial approach, which was to form crosslinked hydrogels from the HA-amino acid derivatives.

Crosslinking was achieved by using a commonly used crosslinking agent, butanediol diglycidyl ether (BDDE) in slightly acidic media. The resulting crosslinked HA-amino acid hydrogels exhibited a higher resistance to hyaluronidase degradation compared to the hydrogels obtained from native HA in the same conditions, showing that the efficacy of amino acid grafting onto HA was conserved after crosslinking. The hydrogels also showed enhanced resistance compared to commercially available HA products. Crosslinked HA hydrogels derivatives were thus successfully synthesized having higher *in vitro* resistance to hyaluronidases enzymes than the native HA hydrogels as well as commercially available crosslinked HA products.

This thesis therefore enabled to complete the first experimental and *in vitro* part of the project and allowed to identify these new HA-amino acid derivatives as promising materials to be used for many biomedical applications, in particular for dermal filling and viscosupplementation. The objective of the thesis was achieved and a patent was submitted, detailing the synthesis, efficacy and industrial applications of the HA-amino-acids.

The experiments performed throughout this work enabled to identify tyrosine-HA as the best potential candidate for further development. Indeed, HA-tyrosine solutions had the highest viscosity and the HA-tyrosine hydrogels had swelling ratios the closest to the native HA hydrogels. In addition, it is one of the derivatives which showed the highest resistance to enzymatic degradation and would therefore not need to be grafted as high as 100%. Indeed, hydrogels grafted with 40% only of tyrosine already exhibit much higher resistance than

hydrogels from native HA. This feature should however be confirmed by preliminary *in vivo* studies. These screening studies performed *in vivo* on rodents, can be performed with a series of samples synthesized and sterilized at the laboratory scale. To this end, a feasible sterilization method should be defined which would not induce product degradation.

The research phase of the industrial project being close to completion, the energy should be now focused on the development and industrialization phases in accordance with the current legislation. In order to commercialize the novel HA-amino-acid crosslinked hydrogels for dermal filling and viscosupplementation, these have to be registered as medical devices of class III. This requires a complete report of the products' biocompatibility and efficacy *in vivo* obtained from the final and validated industrial production line. *In vivo* biocompatibility and efficacy testing and industrial up-scaling therefore have to be made in parallel with the constitution of the regulatory file. Once *in vivo* screening studies have first enabled to validate the efficacy and biocompatibility of the derivatives and the amino acid and grafting ratio, it will be necessary to optimize the synthesis of the selected derivative and then up-scale it for industrial production. As reported in Chapter 2, the amidation reaction performed in water/acetonitrile seemed to be the most appropriate method to be considered for up-scaling of the products. Indeed, from an industrial point of view and compared to the method performed in organic solvent from the HA-TBA salt, the use of HA without previous treatment and the use of an aqueous solvent are valued advantages to reduce production time and energy and allowing considerable cost savings. The method using CDMT in water/acetonitrile should therefore be optimized first in small scale for the synthesis of the HA-amino acid candidate and further transferred to a larger scale with the

support of a specialized industrial partner. The crosslinking reaction of the resulting product should also be validated on a larger production scale. All the regulatory requirements for the industrial process have to be validated as well and reported in the regulatory file.

From a research point of view, the grafting of amino acids onto HA has opened new perspectives to be further explored. Mostly for drug delivery, it would be interesting to apply our system to derivatives described in the literature. For example, an HA-amino acid could be synthesized with 50% grafting ratio and a drug could be grafted on the remaining carboxyl groups using methods described in Chapter 1, section 3.1.1. The release of the drug could then be compared to release of the native HA-drug derivative and a prolonged release might be observed. In the same way, HA-amino acid could be grafted onto liposomes, nanoparticles or other carrier systems the same way native HA has been grafted as described in Chapter 1, section 3.1.2. Another possibility would be to study the effect of the HA-amino acid derivatives on wound healing. Indeed, Favia et al. (2008)[204] reported the combination of HA and amino acids to enhance wound healing. It would therefore be interesting to study the effect of the amino acids when they are grafted onto HA and their release at the wound site. Given the numerous uses of HA and its derivatives as described in Chapter 1, many more possibilities are still left to explore using the HA-amino acid derivatives synthesized during this doctoral work.

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APPENDIX

List of abbreviations

ADH: adipic dihydrazide
BDDE: butanediol-diglycidyl ether
BS^3: bis(sulfosuccinimidyl)suberate
CD44: cluster of differentiation-44
CDMT: 2-chloro-dimethoxy-1,3,5-triazine
CMPI: 2-chloro-1-methylpyridinium iodide
CNBr: cyanogen bromide
DMF: dimethylformamide
DMSO: dimethyl sulfoxide
DMS: 2-methylsuberimidate
DMSO: dimethylsulfoxide
DLS: Dynamic light scattering
DTSSP: 3,3'-dithiobis(sulfosuccinimidyl)propionate
DVS: divinyl sulfone
ECM: extracellular matrix
EDC: 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide
GAG: glycosaminoglycan
GPI: glycosylphosphatidyl-inositol
GTA: glutaraldehyde
HA: hyaluronic acid
HAS: HA synthases
Hyal: hyaluronidase
ICAM-1: intracellular adhesion molecule-1
K_m: Michealis constant
M_w: molecular weight
NHS: N-hydroxysuccinimide
NMM: N-methylmorpholinium
NMP: N-methyl-2-pyrrolidone
OSA: octenyl succinic anhydride
PDI: polydispersity index
RHAMM: receptor for hyaluronan mediated motility
SEC-MALS: size exclusion chromatography followed by multi-angle light scattering
TBA: tetrabutylammonium
V_{max}: Maximal reaction rate

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Synthesis of N-Alanyl-Hyaluronamide with high degree of substitution for enhanced resistance to hyaluronidase-mediated digestion

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ABSTRACT

In order to increase the resistance of hyaluronic acid toward enzymatic digestion, we prepared N-alanyl-hyaluronamide derivatives using different amidation methods performed either in water, water/acetonitrile mixture or anhydrous dimethylformamide. Our results indicate that amidation of hyaluronic acid in an anhydrous solvent is effective and led to a degree of substitution of the carboxylic groups up to 100%. We also demonstrated the N-Alanyl-hyaluronamides present enhanced resistance towards enzymatic digestion while forming solutions with similar viscosities than solutions of hyaluronic acids of similar lengths.

KEYWORDS: hyaluronic acid, hyaluronan, hyaluronic acid derivative, amidation, hyaluronidase.

INTRODUCTION

Hyaluronic acid (HA), also called hyaluronan,[1] is a high molecular weight polymer with repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid, linked by alternating glycosidic bonds β-(1,4) and β-(1,3) (Figure 1).[2] It is a major component of the extracellular matrix of vertebrates and is found in high concentrations in synovial fluid, vitreous body and skin.[3] Its main function is to serve as a lubricant, shock absorber and matrix,[4,5] properties which are strongly related to HA's ability to form shear-dependant and highly viscous solutions.[6] HA can be easily obtained in large quantity from animal tissues such as rooster comb or more recently from recombinant bacteria[7]. This has allowed extensive use of HA and gels of HA for visco-supplementation by injection into joints to treat arthritis, for ophthalmic surgery and for tissue augmentation in cosmetic surgery.[8] More recent applications include drug delivery and wound healing.[9,10] However, repeated injections of HA are often necessary to compensate for its naturally-occurring degradation.[11] The hyaluronidase enzymes (Hyal-2 and Hyal-1) and the CD44 (Cluster of Differentiation-44) cell surface receptors are believed to be the main responsible for HA degradation.[12] Molecular modeling based on crystallographic studies indicated that the recognition sites of the Hyal-2 enzymes and CD44 receptors are the carboxylate groups of HA.[13] Its chemical modification may hence diminish enzymatic recognition and enhances the stability of HA toward hyaluronidase-mediated hydrolysis.

In here, we evaluated the effect of grafting alanine onto HA to mask the HA backbone for enzymatic recognition by hyaluronidase while maintaining the overall anionic density of the polymer. For integral masking of the HA, the ideal is to obtain the highest achievable degree of modification and we therefore assayed various amidation methods. The effect of the chemical process on the physicochemical behavior of the Ala-HA and stability to hyaluronidase digestion was also reported.

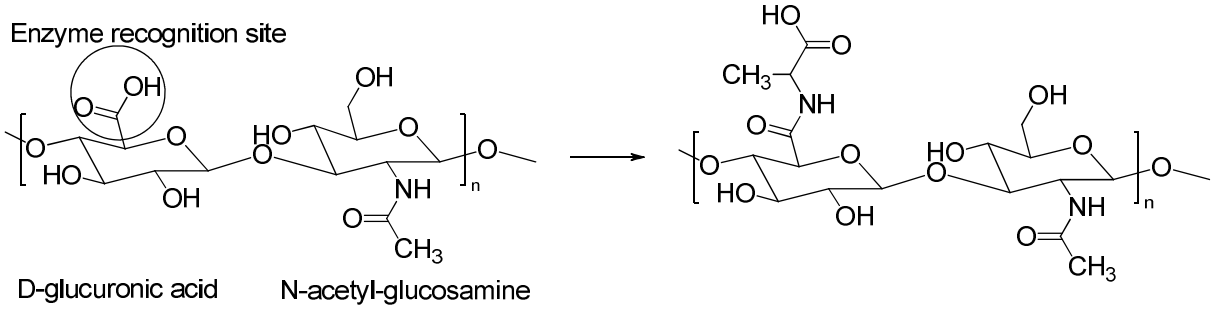

Figure 1. Chemical structure of hyaluronic acid and N-alanyl-hyaluronamide with a degree of substitution of 100%.

MATERIALS AND METHODS

Materials. 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC), N-hydroxysuccinimide (NHS), L-alanine ethyl ester HCl (AlaOEt-HCl), sodium hydroxide (NaOH), tetrabutylammonium (TBA) hydroxide solution (~40% or 1.5M in water), 2-chloro-1-methylpyridinium iodide (CMPI), triethylamine, acetonitrile, N-methylmorpholine (NMM), 2-chloro-dimethoxy-1,3,5-triazine (CDMT) were purchased from Sigma-Aldrich and used without purification except for ion exchange resin Dowex 50WX8-200 which was previously washed with water and ethanol. Hyaluronic acid (HA) sodium salt was isolated from *Streptococcus equi* and was given at a molecular weight of $1{,}58.10^6$Da. Hyaluronidase from bovine testes Type I-S had a given activity of 850 units/mg units. Purified water was obtained with an Arium 661 Sartorius system (Goettingen, Germany). ^{1}H-NMR spectra were obtained using a 400MHz Bruker spectrometer. Lyophilization was performed using a Christ Alpha 2-4 LSC Lyophilizer at 0.2mbar at 25°C during 22 hours then at 0.09mbar at 25°C during 2 hours. Products were previously frozen by immersion into liquid nitrogen during 15 minutes. Visking dialysis membranes of 12-14000 Daltons were purchased from Medicell International Ltd (London).

Purification of the reaction mixtures. Each mixture was dialyzed 24 hours against water (3 L), 2 hours against 0.05M NaOH (3 L), 40 hours against 0.1M NaCl (3 L), 8 hours against 25% ethanol solution and finally 72h against water (3 L). The solution was then lyophilized to obtain the various HA-alanine derivatives. For the reaction with EDC, the dialysis against ethanol solution was omitted.

^{1}H-NMR (D_2O) δppm: 4.43 (m, 2H, O-CH-O), 3.88-3.11 (m, 12H, C-CH-O), 1.97 (s, 3H, CO-CH_3-N), 1.38-1.23 (m, 3H, C-CH_3-CO).

Synthesis of HA-alanine in aqueous solvent with EDC. Adapted from.[14] The sodium salt of HA (264 mg, 0.66 mmol) was dissolved in water (87 mL) for 1h before addition of L-alanine ethyl ester (AlaOEt-HCl (811 mg, 5.28 mmol). The pH was adjusted to 7.5 using 1 M NaOH. To this solution was added a freshly prepared solution of NHS (304 mg, 2.64 mmol) and EDC (506 mg, 2.64 mmol) in water (1mL). The pH was again adjusted to 7.5 using 1M NaOH and the reaction was allowed to proceed overnight under constant magnetic stirring (approximately 300 rpm). After purification (see procedure above), the HA-alanine was obtained as a white powder (229 mg, 86 % yield). The same reaction was performed with the followings quantities: EDC (1012 mg, 5.28 mmol, 8 eq) Ala-HCl (1622 mg , 10.56 mmol), NHS (607.6 mg, 5.28 mmol). For this reaction, 235 mg (88% yield) of HA-alanine was recovered.

Synthesis of HA-alanine using CDMT in water/acetonitrile. Adapted from[15]. The sodium salt of HA (264 mg, 0.66 mmol) was first dissolved in water (52 mL) before addition of acetonitrile (35 mL). The solution was cooled in an ice bath and CDMT (362 mg, 2 mmol), was then added. One hour later, AlaOEt-HCl (460 mg, 3 mmol) then NMM (280 µL, 3 mmol) were added and the reaction was allowed to proceed overnight under constant stirring (approximately 300 rpm). Excess amine were removed by incubation with ion exchange resins (Dowex-H^+, 2 g then Dowex-Na^+, 2 g, 1 h each), The solution containing the

HA was then purified as described above to give the HA-alanine (213 mg, 76% yield) as a white powder.

Synthesis of HA-alanine in anhydrous DMF. Adapted from.[16] HA was first prepared as a tetrabutylammonium (TBA) salt to allow its solubilization in DMF. The sodium salt of HA (2g, 5.14 mmol) was dissolved in water at a concentration of 5 mg/mL. Dowex 50WX8-200, a strong acid exchange resin was added slowly until a measured pH of 2.5. The resin was filtered off and the solution containing the HA (acid form) was treated, dropwise with tetrabutylammonium hydroxide solution (40% m/m) until pH 9-10. The solution was then lyophilized to give the HA-TBA (2.1 g). HA-TBA salt (400 mg, 0.66 mmol) was dissolved in anhydrous DMF (80 mL) under argon for 6 hours. The mixture was then ice-cooled and treated sequentially with AlaOEt-HCl (460 mg, 3 mmol), CMPI (511 mg, 2 mmol) and triethylamine (700 µL, 5 mmol). The reaction was allowed to warm to room temperature and stirred under argon overnight. The solution was then brought to 0°C for dilution with water (80 mL) and purified as described above to obtain after lyophilisation HA-alanine (264 mg, 89% yield) as a white powder. The same reaction was performed using: 1. HA-TBA (400mg, 0.66 mmol), AlaOEt-HCl (154 mg, 1 mmol), CMPI (204 mg, 0.8 mmol), triethylammine (256 µL, 1.8 mmol) to obtain 279 mg of HA-alanine (94% yield) 2. HA-TBA (400mg, 0.66 mmol) AlaOEt-HCl (92 mg, 0.6 mmol), CMPI (102 mg, 0.4 mmol), triethylammine (140 µL, 1 mmol) to obtain 237 mg of HA-alanine (83% yield).

Detection of amines. Adapted from.[17] HA-alanine samples (100 µL, 5 mM solution in water) were mixed with 100µL of 1M acetate buffer (pH 5.5) and 200µL of ninhydrin solution (0.2g ninhydrin and 0.03g hydrindantin dissolved in 7.5mL 2-methoxyethanol and 2.5mL acetate buffer pH 5.5). The solutions were heated at 100°C for 15 minutes, cooled to room temperature and the volume adjusted to 1mL by addition of 50% ethanol for absorbance

measurement at 570 nm. Control solutions were prepared with 0.1 and 0.5 mM L-alanine ethyl ester in water.

Evaluation of polymer size. Size exclusion chromatography was performed using an Ultimate 3000 system from Dionex (Sunnyvale, CA, USA) and 3 columns Shodex OH-pak 1802.5Q, 1804HQ and 1806HQ (Showa Denko America, New York, USA) of 30cm in series at 30°C with a flow rate of 0.5 mL/min. The samples were dissolved overnight including 6 hours under mechanical stirring in an aqueous solution containing 0.1 M $NaNO_3$ at a concentration of 1.2 mg/mL to 1.5 mg/mL depending on the sample. Prior to analysis, each sample was filtered through a 0.45 µm hydrophilic PTFE Milex-LCR filter (Millipore, Bedford, MA, USA). The injection volume was 100 µL to 120 µL depending on the sample concentration. MALS detection was performed continuously on the column eluate with a Dawn Heleos II light scattering detector in series with an Optilab rEX differential refractometer (both from Wyatt Technology, Santa Barbara, CA, USA) with a wavelength of 658nm. Data were analyzed using Astra software 5.3.4 and first order Zimm fits. The *dn/dC* refractive index increments were experimentally determined using the same refractometer and solvent conditions as for SEC/MALS for native hyaluronic acid (*dn/dC* = 0.149 mL/g) as well as for HA-alanine with 100% grafting ratio (*dn/dC* = 0.143 mL/g).

Rheology. Dynamic viscosity measurements were performed in steady shear mode with a cone plate rheometer Rheostress RS100 (Haake Technik GmbH, Vreden, Germany) equipped with a Rheowin software (version Pro 2.93). The cone used had a diameter of 60mm and an angle of 1°. The temperature was maintained at 25°C with a TC Peltier thermostatic system. All samples were dissolved in water at least 24 hours before use at the same concentration of 2mg/mL for comparison reasons. Measurements were made with a preset shear stress ramp to evaluate the corresponding shear rate.

Enzymatic degradation of Ala-HA. The method used was adapted from Muckenschnabel et al. (1998)[18] using the Morgan-Elson colored reaction. 100µL of 5mM Ala-HA solution, 100µL of purified water and 50µL of phosphate buffer were incubated with 25µL of 4.10^6 IU/mL hyaluronidase solution at 37°C during one to six hours. The enzymatic reaction was stopped by adding 50µL of tetraborate solution and heating the tubes in boiling water for 5 minutes. After cooling in ice for 2 minutes, 750µL of dimethylamino benzaldehyde (DMAB) (2g of DMAB of dissolved in 2.5mL HCl and 47.5mL glacial acetic acid immediately before use) solution were added to each tube. After 60 minutes incubation, the absorbance at 586nm was measured.

RESULTS AND DISCUSSION

Synthesis of Alanine-hyaluronic acid (Ala-HA) conjugate. There are almost innumerous methods to couple amines to carboxylic acid and a few have been adapted to HA. Activation of the HA carboxylic acid with EDC is one of the most popular methods, [14,19] because it can be done in water, the native solvent for HA. EDC first reacts to the carboxylic acid to form a highly reactive O-acyl isourea intermediate (Figure 2). This specie then either reacts with a nucleophile, namely the amine but also competitive water, or rearranges into a stable N-acyl urea, which impedes the reaction. The reaction is quite subtle as the optimal pH is different for each step. Indeed, the carboxylic acid activation by EDC is best performed in an acidic environment (pH 4.75)[20], whereas the nucleophilic attack by the amine is best done at high pH, when the amine is unprotonated. The compromise is therefore not easy to define, especially for amines with high pKa values and stabilization by formation of succinimidyl ester of HA showed to be helpful[14]. We therefore adapted the procedure and incubated the HA (1.58 MDa) in water with excess of AlanineOEt and EDC (8 and 4 equivalents respectively) in the presence of N-hydroxysuccinimide (4 equivalents) at pH 7.5. After one night, the

mixture was dialysed to remove low molecular weight molecules. We found out convenient to deprotect the carboxylic acid of alanine during dialysis by incubating the dialysis tubes in 50 mM NaOH for 2 hours at 20°C. The duration of this step was carefully monitored since strong alkaline conditions are reported to give rise to HA fragmentation.[21] A 2 hours treatment at 20°C with a 50 mM NaOH solution was enough to provide a NMR spectrum devoid of peaks corresponding to ethyl groups, confirming an effective deprotection. Finally we assayed the purity of the polymer after dialysis by verifying the absence of free amines (unreacted amino-acids) with the ninhydrine colorimetric test. The final yield was 88% and the degree of substitution (DS) was calculated from the NMR spectrum (**Figure 3c**) at 10%. Doubling the equivalents of both EDC and amine improved only marginally the DS from 10 to 13%. These results are in accordance with previous results of the literature indicating that amidation of HA with EDC and NHS in water led to relatively low DS values.[22,23,24]

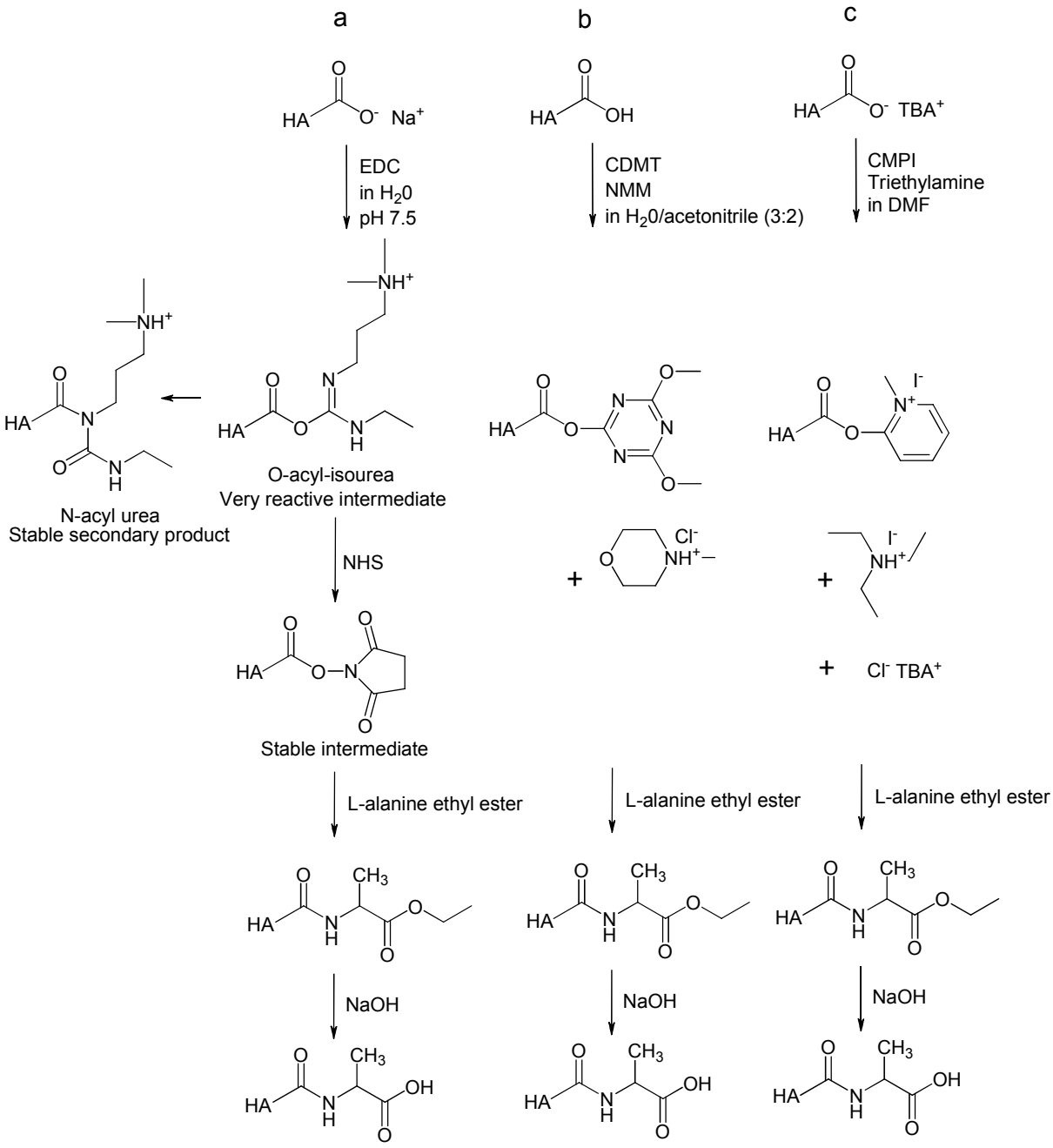

Figure 2. Synthetic routes to N-alanyl hyaluronamide (●=HA).

We next evaluated a more recently described condensation method which is performed in water/acetonitrile using 2-chloro-dimethoxy-1,3,5-triazine (CDMT) as the reagent.[15] The method appeared interesting because it avoided use of NHS and pH monitoring. At first, we performed the reaction exactly as described by preparing HA in its acid form.[15] However, in our hands, lyophilization of the acidic form of HA preceding the amidation reaction provoked

important fragmentation of HA, a property that was already noticed by others.[25,26] We therefore modified the reaction procedure to avoid handling of the HA in its acidic form and started directly from the commercial HA sodium salt. The HA was then reacted with CDMT to form a HA-dimethoxytriazine intermediate and the release of HCl was trapped with N-methylmorpholine (NMM). Deplacement of the dimethoxyhydroxytriazine by nucleophilic attack of the alanine ethyl ester amine led to N-alanyl ethylester-hyaluronamide (Figure 2B). Deprotection of the ester and purification of the polymer were carried out as well during the dialysis. After lyophilization, the final yield was 76%. NMR analysis (**Figure 3d**) shows the expected N-alanyl hyaluronamide with a DS of 50%.

Table 1. Summary of the various methods for the preparation of Ala-HA.

Starting salt	Conditions	Yield	DS (% relative to HA)
HA-Na	8eq EDC, NHS, H_2O, overnight	88%	13%
HA-Na	3eq CDMT, H_2O, CH3CN, NMM, overnight	76%	50%
HA-TBA	3eq CMPI, DMF, triethylamine, overnight	89%	100%

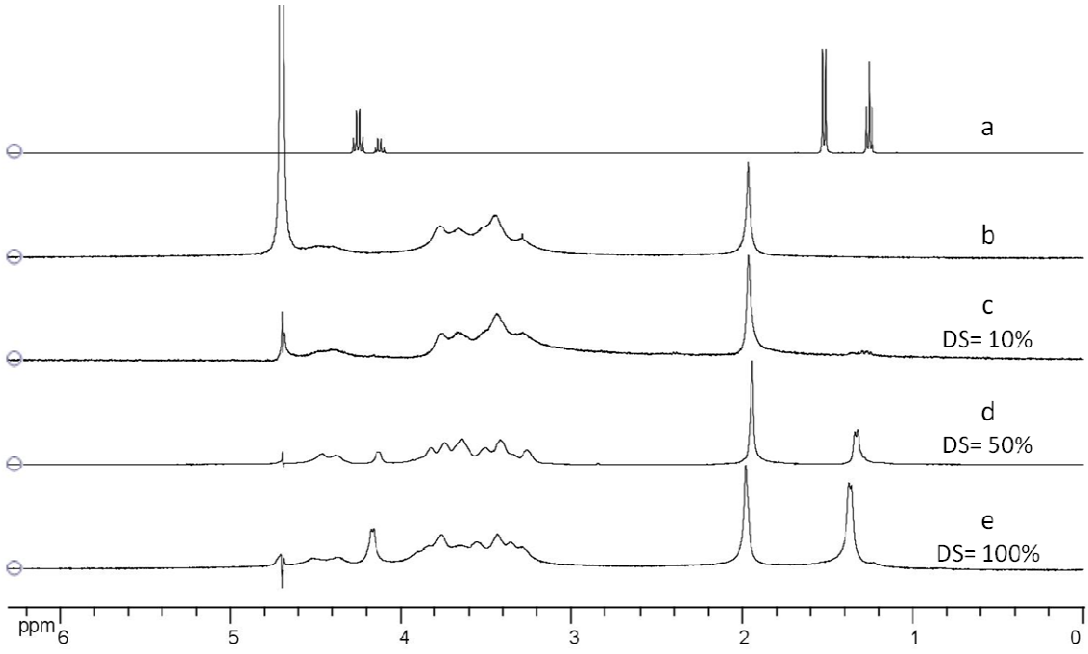

Figure 3. Comparative analysis of ^{1}H-NMR spectrum of the various N-alanyl-hyaluronamide with the highest possible degree of substitution that was achieved using: EDC in aqueous phase (spectrum c), CDMT in water/CH_3CN (spectrum d) and CPMI in DMF (spectrum e). Spectra a and b correspond to alanine methyl ester and the native HA respectively.

Although this method was more effective than EDC (DS around 50% versus 13%), our aim was to obtain a complete substitution of the HA carboxylic acid. We therefore assayed a third amidation which is performed in anhydrous conditions. Magnani et al. described the use of CMPI for cross linking HA in DMF.[16] To ensure solvatation in anhydrous DMF, HA was firstly converted into a tetrabutylammonium (TBA) salt and condensed with alanine ethyl ester using a slight excess of CMPI (1.2 eq) and in the presence of triethylamine for neutralization of the released HCl and HI (**Figure 2**). Purification and concomitant deprotection afforded a N-alanyl hyaluronamide with 100% substitution as calculated from the NMR spectrum (**Figure 3e**). The effectiveness of this reaction (100% of HA substitution was observed using only 1.2 of activator equivalents) was remarkable and allowed us to

obtain very easily Ala-HA derivative with lower DS by simple adjustment of the CMPI to HA stoechiometry.

Physicochemical properties of the N-alanyl-hyaluronamide

We previously prepared Ala-HA with different DS from 10 to 100% using various synthetic methods. It was previously indicated that HA is very sensitive to fragmentation.[15,27] We therefore studied the effect of the various synthetic methods on the HA molecular weights distribution. Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) was used to measure the molecular weight distribution of the polymers. For accuracy and to have access to the real molecular weights, we determined experimentally the refractive index increment (*dn/dC*) of N-alanyl-100-HA in the size exclusion elution phase (0.1M $NaNO_3$ solution) and found a value of 0.143 mL/g. The *dn/dC* of HA was also measured in the same buffer at 0.149 mL/g, a value within the expected range (0.150 mL/g in NaCl,[28] 0.167 mL/g in PBS,[29] 0.142 ml/g $NaNO_3$[30]).

The results from these experiments are reported in **Table 2**. For clarity, the various N-alanyl-hyaluronamide were abbreviated as AlaXHA, X being the DS in percentage. The measured molecular weight of the commercial HA had almost the expected average molecular weight (measured at 1.26 MDa with a given one at 1.58 MDa). As seen, the molecular weights of Ala-HA are lower than the native HA indicating that the chemical and purification processes produced damages to the starting polymer. The least degradation was observed for Ala13HA obtained using EDC. Modification of HA in DMF with CMPI led to slightly more HA fragmentation. The lowest molecular weight values were obtained for Ala-HA that was prepared using CMPI in organic solvent.

Table 2: Molecular weights (M_w), polydispersity index (PDI) and fragmentation of modified HA.

	M_w (g/mol)	PDI (M_w/M_n)	Fragmentation ratio[a]
Native HA	1 261 000	1.29	1
Ala13HA - EDC	351 500	1.65	3.6
Ala10HA - CMPI	229 800	1.73	5.5
Ala40HA - CMPI	283 500	1.72	4.4
Ala67HA - CMPI	218 000	1.74	5.8
Ala100HA - CMPI	288 900	1.68	4.4
Ala50HA- CDMT	313 400	1.77	4.0

[a] : relative to the theoretical value calculated using the native HA M_w and the DS.

We next evaluated the contribution of the chemical modification on the viscosity of the polymer in aqueous solution at concentration of 2mg/mL. **Figure 4** shows the dynamic viscosity profile of AlaHA in function of shear stress. The viscosity profiles of all HA-ala derivatives are lower than the native HA, which is in line with the lowered molecular weight values measured by SEC-MALS. HAala13 obtained using EDC which had the highest molecular weight, also showed the highest viscosity compared to the other derivatives. However, for all polymers, the decrease in viscosity at higher shear rates shows that the shear thinning properties of native HA is preserved even after L-alanine grafting.

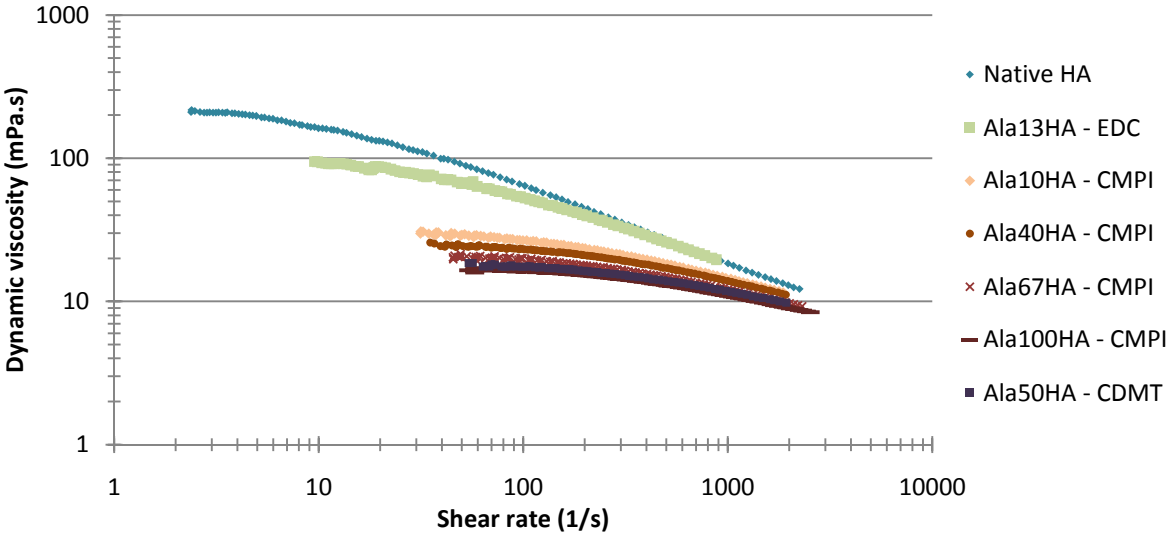

Figure 4. Evaluation of polymer viscosity. Each polymer was dissolved at 2 mg/mL in purified water.

We finally assayed the effect of the modification on the susceptibility of Ala-HA towards enzymatic digestion. Ala-HAs of various DS (2mM in PBS) were incubated in the presence of hyaluronidase at 37°C.[18] The reaction mixtures were then stopped at different times and the N-acetyl glucosamine reducing ends were quantified using the Morgan-Elson colorimetric reaction. Results show alanine modification to diminish enzymatic degradation according to the grafting degree (**Figure 5**).

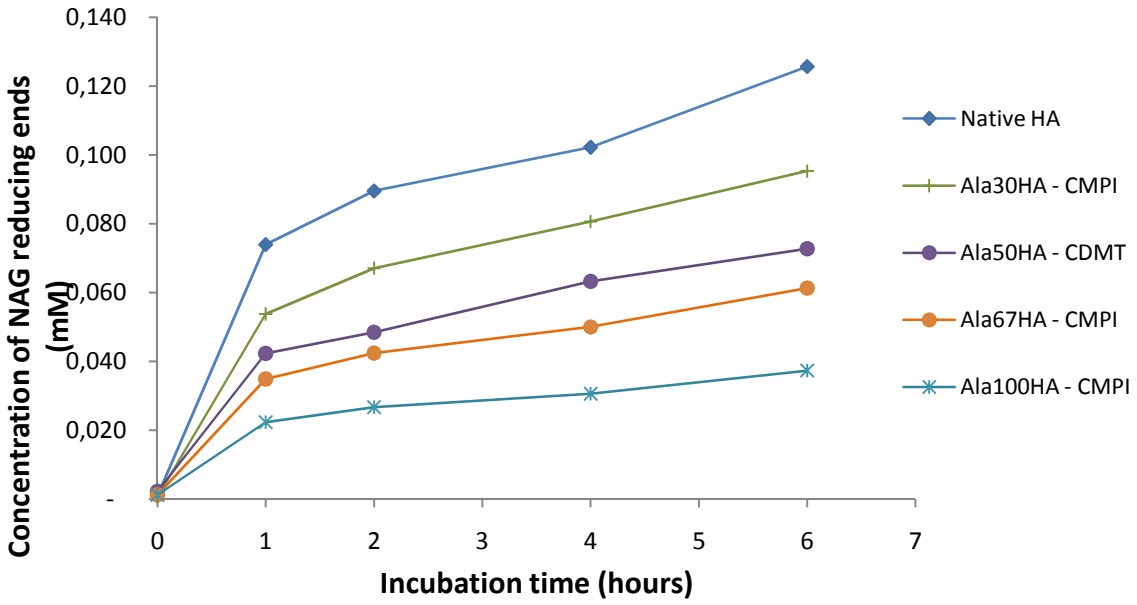

Figure 5. Enzymatic degradation profiles for the various alanyl-hyaluronamide. Each polymer solution (2 mM) was incubated with hyaluronidase 4.10^6 IU/mL in PBS at 37°C. Fragmented HAs were detected by colorimetric dosage of NAG reducing ends.

CONCLUSION

In the present study, we prepared Ala-HA using different chemical methods and showed that alanine can be condensed to carboxylic acid of HA at will and at degrees of substitution up to 100%. The various chemical processes did induce HA fragmentation but to still acceptable levels. Moreover, we also demonstrated that Ala-HAs present enhanced resistance towards enzymatic digestion while forming solutions with shear-thinning properties. These results suggest that Ala-HAs and similar aminoacid-HA derivatives may become interesting for viscosupplementation purposes.

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Manuscript 2 - Planned for submission to Biomacromolecules

Improvement of hyaluronic acid enzymatic stability by the grafting of amino-acids

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INTRODUCTION

Hyaluronic acid (HA), a naturally occurring polymer, is today of major interest as a biomaterial for many medical applications. As a high molecular weight polysaccharide consisting of linear repeating units of D-glucuronic acid and N-acetylglucosamine, it has unique visco-elastic and rheological properties. In solution and in tissues such as synovial fluid, HA forms a network of entangled coils showing elasticity and shear-dependant properties[1]. The hydrogen bonds formed between the repeating units have demonstrated to stabilize the network and retain a high amount of water. These characteristics make it a valuable component of many tissues in the body. Its highest occurrence is in the extra cellular matrix (ECM) of soft connective tissues, especially in the synovial fluid of joints, in the dermis and in the vitreous body of the eye[2] where it has numerous biological functions. It acts

as a lubricant, a shock absorber as well as a structural support for the surrounding cells and other ECM components such as collagen and elastin fibers, maintaining its volume. It is in addition involved in cell regulation, migration and adhesion[3].

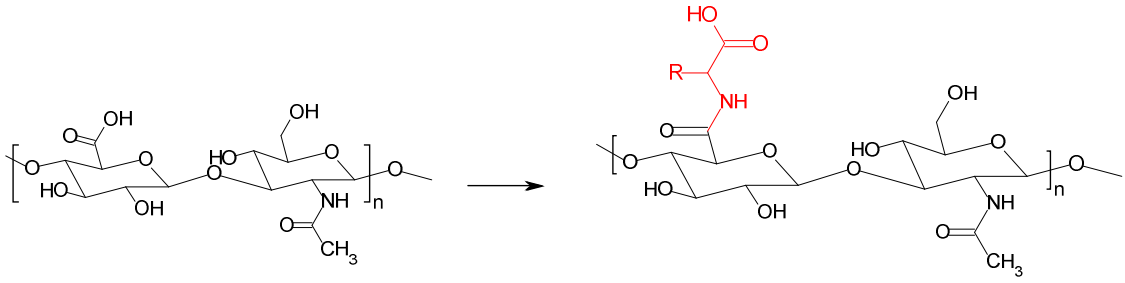

Figure 1: Chemical structure of hyaluronic acid and conjugated hyaluronic acid-amino acid

As the human body ages, HA content gradually decreases[4] resulting in a loss of its biological activity and efficacy. The most obvious effects of lower HA contents due to aging are articular deterioration causing arthritis and loss of facial skin volume causing wrinkles. As an answer to these clinical signs of aging, HA supplementation by injection into joints and dermis has been used for many years[5]. Some obstacles to the treatment by HA injection are its enzymatic degradation by hyaluronidases in the body and as well its dilution away from the injection site. It is known that the half-life of HA after injection in skin and joints is no longer than 24 hours[6]. As it has been determined that the recognition sites of hyaluronidases are the carboxyl groups of HA, which are also responsible for the valuable viscoelastic properties of HA[7], our aim was to introduce a small and natural molecule which does not impact its biological properties but only hinders its enzymatic degradation. To preserve HA's highly hydrophilic nature, we selected an acidic molecule to recover the carboxylic acid group. We identified amino acids as suitable candidates for the synthesis of novel HA derivatives with the desired properties stated above. In addition, by grafting an amino acid onto HA, we intend

to preserve its resorbable nature. Indeed, complications due to non-resorbable injectable material have reported to be more difficult to manage and to be in certain cases permanent[8].

This study describes the synthesis of novel HA derivatives obtained by grafting amino acids on its carboxylic groups and subsequent cross-linking with BDDE. The *in vitro* efficacy of the novel HA derivatives was assessed as a function of the nature of the grafted amino acid and its degree of substitution. The obtained HA-amino acid derivatives were then cross-linked to produce stronger hydrogels for future therapeutic applications. The enzymatic degradation profile and physical characteristics were also compared to the native HA hydrogels.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich. Hyaluronic acid sodium salt, hyaluronidase from bovine testis, amino acids in ethyl ester HCl form, sodium hydroxide (NaOH), tetrabutylammonium (TBA) hydroxide solution (~40% or 1.5M in water), 2-chloro-1-methylpyridinium iodide (CMPI) and triethylamine were used without purification. Ion exchange resin Dowex 50WX8-200 was previously washed with water and ethanol. Hyaluronic acid (HA) sodium salt was isolated from *Streptococcus equi* and was given at a molecular weight of $1{,}58.10^6$Da. Purified water was obtained with an Arium 661 Sartorius system (Goettingen, Germany). ^{1}H-NMR spectra were obtained using a 400MHz Bruker spectrometer. Lyophilization was performed using a Christ Alpha 2-4 LSC Lyophilizer at 0.2mbar at 25°C during 22 hours then at 0.09mbar at 25°C during 2 hours. Products were previously frozen by immersion into liquid nitrogen during 15 minutes. Visking dialysis membranes of 12-14000 Daltons were purchased from Medicell International Ltd (London).

Grafting of an amino acid on hyaluronic acid. HA sodium salt was previously converted to its tetrabutylamminium (TBA) salt to allow its solubilization in organic solvents. 2g (5.14mmol) of hyaluronic acid sodium salt was solubilized in water at a concentration of

5mg/mL. Dowex 50WX8-200, a strong acid exchange resin was added to the solution until reaching pH 2.5. The solution was then filtered to remove the resin and a 40% tetrabutylammonium hydroxide solution was added dropwise until reaching pH 9-10. The solution was then lyophilized and 2.11g of the HA-TBA salt was recovered.

The grafting method used is derived from Magnani et al (2000)[9]. HA-TBA salt (400mg, 0.66mmol) obtained previously was dissolved in anhydrous DMF (80mL) under argon for 6 hours. After cooling the solution with an ice bath, amino acid ethyl ester HCl (1mmol), CMPI (204.4mg, 0.8mmol) and triethylamine (256µL, 1.8mmol) were added to the solution. The reaction was allowed to warm at room temperature and stirred under argon overnight. The solution was then brought to 0°C for dilution with water (80mL). Each mixture was dialyzed 24 hours against water (3 L), 2 hours against 0.05M NaOH (3 L), 40 hours against 0.1M NaCl (3 L), 8 hours against 25% ethanol solution and finally 72h against water (3 L). The solution was then lyophilized to obtain the various HA-aa derivatives.

HA-ala: ^{1}H-NMR (D_2O) δ 4.448 (m, 2H, O-CH-O), 4.172 (d, 1H, NH-CH-CO), 3.984-3.145 (m, 12H, C-CH-O), 1.977 (s, 3H, NH-CO-CH_3), 1.362 (d, 3H, CH_3-CH-CO).

HA-tyr: ^{1}H-NMR (D_2O) δ 7.13(bs, 2H, aromatic), 6.76 (bs, 2H, aromatic), 4.43 (m, 2H, O-CH-O and-CH from tyrosine), 3.83-3.15 (m, 12H, C-CH-O), 3.09(m, 1H, -CH2 tyrosine), 2.86 (m, 1H, -CH2 tyrosine), 1.92 (m, 3H, NH-CO-CH_3).

HA-phe: ^{1}H-NMR (D_2O) δ 7.3-7.18(m, 7H, aromatic), 4.50 (m, 2H, O-CH-O), 4.172 (m, 1H, N-CH-CO), 3.98-3.121 (m, 12H, C-CH-O), 3.18(m, 1H, -CH2 phe), 2.96(m, 1H, -CH2 phe), 1.93 (s, 3H, CO-CH_3-N).

HA-thr: ^{1}H-NMR (D_2O) δ 4.52 (m, 2H, O-CH-O), 4.35 (d, 1H, NH-CH-CO), 4.05 (m, 1H, CH_3-CH-OH), 3.92-3.19(m, 12H, C-CH-O), 1.95 (s, 3H, CO-CH_3-N), 1.14 (m, 3H, CH_3-CH-OH).

HA-arg: ^{1}H-NMR (D_2O) δ 4.47 (m, 2H, O-CH-O), 4.18(m, 2H, -CH_2-NH-), 4.13 (d, 1H, NH-CH-CO), 3.92-3.20 (m, 12H, C-CH-O), 3.14(m, 2H, -CH_2-NH-), 1.95 (s, 3H, NH-CO-CH_3), 1.77(m, 2H, -CH_2-CH_2), 1.58(m, 2H, -CH_2-CH_2).

HA-asp: ^{1}H-NMR (D_2O) δ 4.46 (m, 2H, O-CH-O + m, 1H, NH-CH-CO), 3.91-3.20 (m, 12H, C-CH-O), 2.74(m, 1H, -CH_2-CO), 2.58(m, 1H, -CH_2-CO), 1.96(s, 3H, NH-CO-CH_3).

HA-val: ^{1}H-NMR (D_2O) δ 4.52 (m, 2H, O-CH-O), 4.05(m, 1H, NH-CH-CO), 3.92-3.20 (m, 12H, C-CH-O), 2.07(m, 1H, CH-CH_3-CH_3), 1.96 (s, 3H, NH-CO-CH_3), 0.88 (m, 6H, CH-CH_3-CH_3).

Ninhydrin assay. The ninhydrin test was adapted from the method described by Romberg et al.[17] HA-aa samples (100 µL, 5 mM solution in water) were mixed with acetate buffer (100µL, 1M, pH 5.5) and 200µL of ninhydrin solution (0.2g ninhydrin and 0.03g hydrindantin dissolved in 7.5mL 2-methoxyethanol and 2.5mL acetate buffer pH 5.5). The solutions were heated at 100°C for 15 minutes, cooled to room temperature and the volume adjusted to 1mL by addition of 50% ethanol for absorbance measurement at 570 nm. Control solutions were prepared with 0.1 and 0.5mM amino acid in water.

Enzymatic degradation HA-aa. The method used was adapted from Muckenschnabel et al. (1998)[11] using the Morgan-Elson colored reaction. HA-aa solutions (100µL) at a concentration of 5mM, water (100µL) and phosphate buffer (50µL) were incubated with 4.10^6 IU/mL hyaluronidase solution (25µL) at 37°C during one to six hours. The enzymatic reaction was stopped by adding tetraborate solution (50µL) and heating the tubes in boiling water for 5 minutes. After cooling in ice for 2 minutes, 750µL of dimethylamino benzaldehyde (DMAB) (2g of DMAB of dissolved in 2.5mL HCl and 47.5mL glacial acetic acid immediately before use) solution were added to each tube. After 60 minutes incubation, the absorbance at 586nm was measured.

Rheology. Dynamic rheology experiments were performed with a cone plate rheometer Rheostress RS100 (Haake Technik GmbH, Vreden, Germany) equipped with a Rheowin software (version Pro 2.93). The cone used had a diameter of 60mm and an angle of 1°. The temperature was maintained at 25°C with a TC Peltier thermostatic system. All samples were dissolved in water at least 24 hours before use at a concentration of 2mg/mL. Measurements were made with a preset shear stress ramp to evaluate the corresponding shear rate.

Evaluation of polymer size. Size exclusion chromatography was performed using an Ultimate 3000 system from Dionex (Sunnyvale, CA, USA) and 3 columns Shodex OH-pak 1802.5Q, 1804HQ and 1806HQ (Showa Denko America, New York, USA) of 30cm in series at 30°C with a flow rate of 0.5 mL/min. The samples were dissolved overnight including 6 hours under mechanical stirring in an aqueous solution containing 0.1 M $NaNO_3$ at a concentration of 1.2 mg/mL to 1.5 mg/mL depending on the sample. Prior to analysis, each sample was filtered through a 0.45 µm hydrophilic PTFE Milex-LCR filter (Millipore, Bedford, MA, USA). The injection volume was 100 µL to 120 µL depending on the sample concentration. MALS detection was performed continuously on the column eluate with a Dawn Heleos II light scattering detector in series with an Optilab rEX differential refractometer (both from Wyatt Technology, Santa Barbara, CA, USA) with a wavelength of 658nm. Data were analyzed using Astra software 5.3.4 and first order Zimm fits. The *dn/dC* refractive index increments were experimentally determined using the same refractometer and solvent conditions as for SEC/MALS for native hyaluronic acid (*dn/dC* = 0.149 mL/g) as well as for HA-alanine with 100% grafting ratio (*dn/dC* = 0.143 mL/g).

Cross-linking of the HA-aa. Cross-linking was performed in acidic conditions according to the method described by De Belder and Malson[12]. HA-aa (80mg, 0.2mmol) obtained previously was dissolved in water (800µL) overnight. Butanediol diglycidyl ether (BDDE) (60µL, 0,163mmol, 0.82 eq) was mixed with glacial acetic acid (30µL) and added to the HA

solution. After immediate mixing with a vortex mixer, the tubes were centrifuged at 2500rpm for 4 minutes and incubated at 60°C during 4 hours.

After the crosslinking reactions, the resulting gels were purified by dialysis. Water (25mL) was added to the gels which were then transferred to dialysis membranes. Dialysis was performed against purified water during 72 hours after which the gel was emptied out of the membrane onto a small strainer to eliminate the surrounding excess water and weighed.

The swelling ratio was calculated by dividing the weight of the final swollen gel by the weight the starting dry solid content (80mg).

Enzymatic degradation of HA-aa crosslinked hydrogels. The same method used as for the HA-aa polymers described above but the crosslinked hydrogels were previously crushed and homogenized. Then a stoichiometric amount was precisely weighed and adjusted with water to obtain a final concentration of 2.5mg/mL in each test tube.

RESULTS AND DISCUSSION

Synthesis of HA-amino acids. The grafting of various amino acids onto HA was performed in anhydrous conditions using an amidation method derived from Magnani et al. (2000), who used CMPI for HA crosslinking with diamines[9]. In the present study, CMPI was used as the activator to graft the amino acids onto the carboxyl groups of HA. HA was first converted into a tetrabutylammonium (TBA) salt and condensed with the ethyl ester protected form of each amino acid using a slight excess of CMPI (1.2 equivalents) and triethylamine to neutralize the released HCl and HI (**Figure 2a**). This amidation method previously showed to yield high substitution degrees using low reagent quantities[10]. In a previous study, the grafting onto HA of one of the simplest amino acids comprising only a methyl group, L-alanine, showed to considerably reduce its degradation by hyaluronidases enzymes[10]. We therefore investigated the effect of other amino acids comprising a range of different functional groups (**Figure 2b**).

Valine was grafted to evaluate the effect of isopropyl, a more important aliphatic group than methyl, whereas serine and threonine were grafted to evaluate the effect of a hydroxyl group. Phenylalanine and tyrosine both contain an aromatic group. Aspartic acid contains a second carboxylic acid group which adds a negative charge and the guanidinium group of arginine adds a positive charge to the polymer.

After incubation overnight, the reaction mixtures were all dialysed against 100 mM NaCl solution followed by 25% ethanol solution to remove ionic as well as hydrophobic impurities. The carboxylic acid groups of the amino acids were deprotected by dialysis against 50 mM NaOH for 2 hours at 20°C. This was enough to provide a NMR spectrum devoid of peaks corresponding to ethyl groups, confirming an effective deprotection. Substitution degrees of 100% were calculated for all derivatives from the NMR spectra (**Figure 3**). After lyophilization, a white powder was recovered for all HA-amino acid derivatives except for HA-serine, which had a light yellow color. Finally, the purity of the polymer after dialysis was confirmed by the absence of unreacted amino acids, assayed with the ninhydrine colorimetric test.

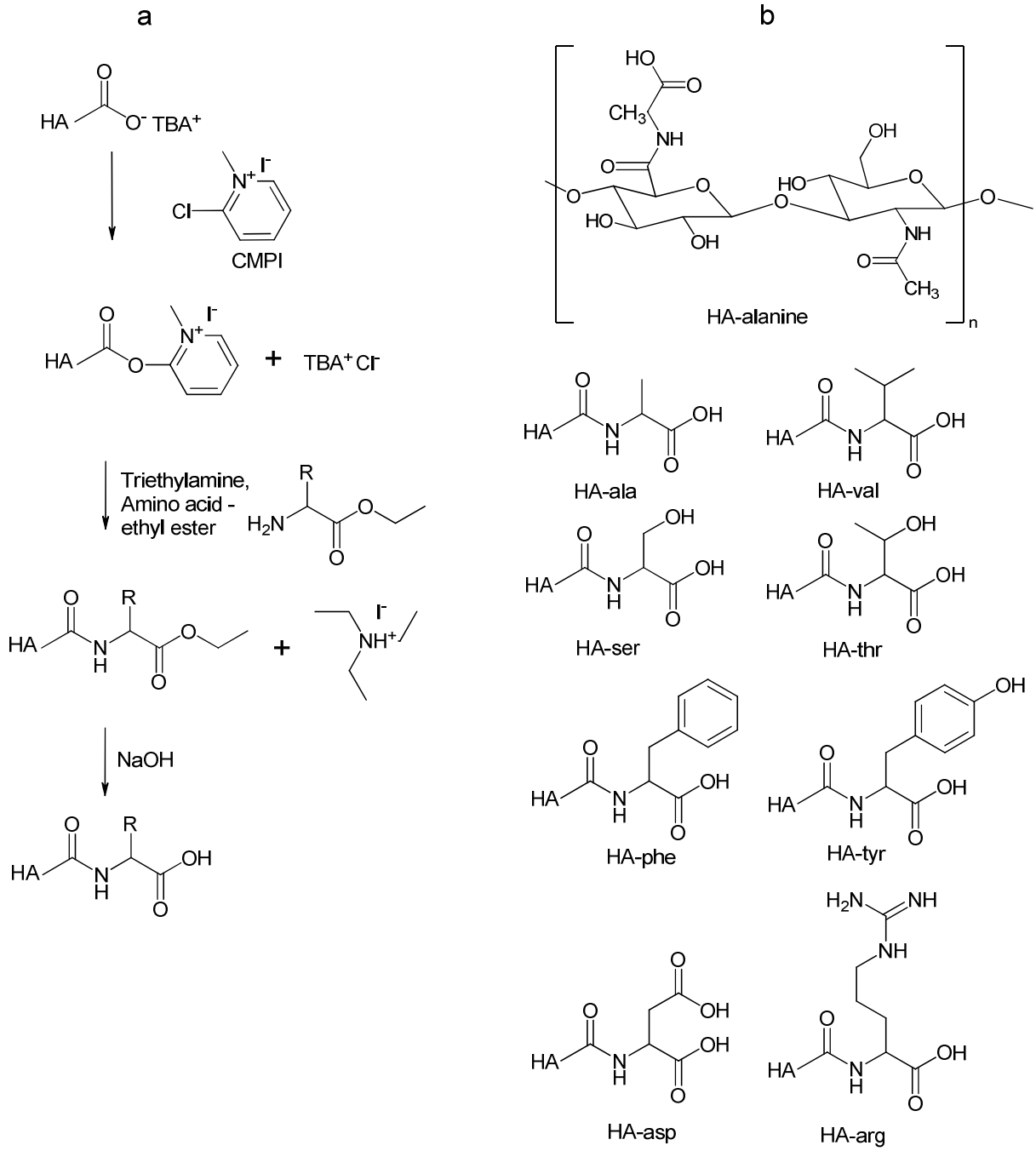

Figure 2: (a) Synthesis of HA-amino acid by amidation with CMPI in DMF and (b) chemical structure of conjugated HA-amino acids.

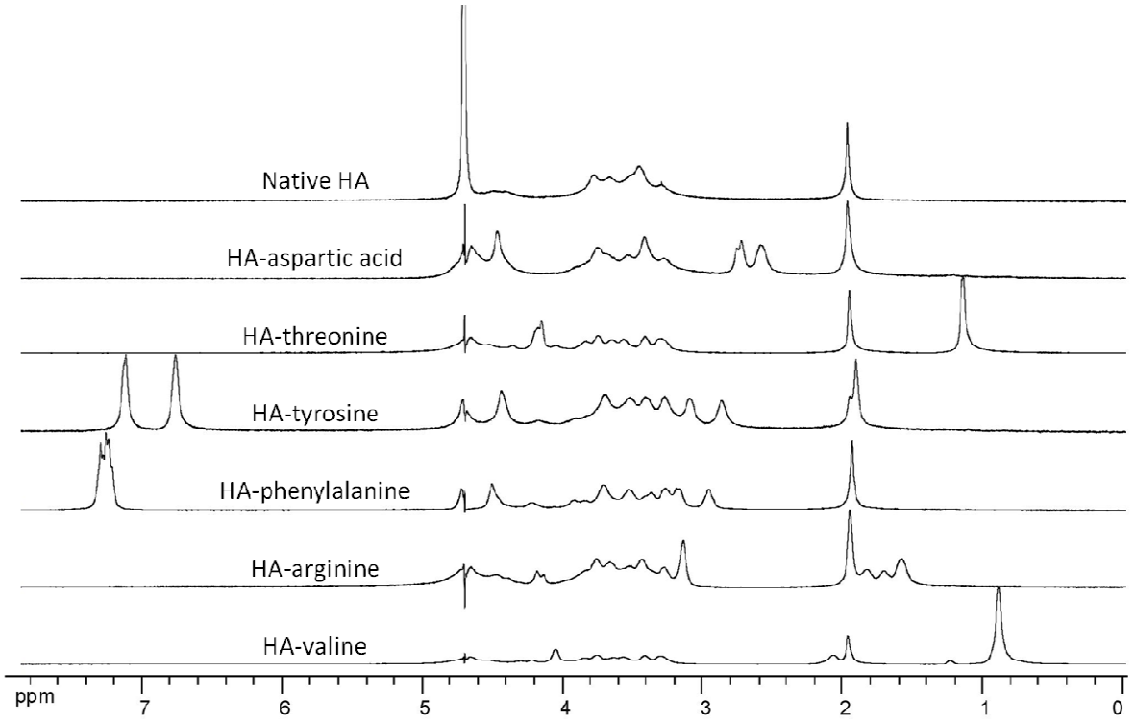

Figure 3: NMR spectra of native HA (a), HA-aspartic acid (b), HA-threonine (c), HA-tyrosine (d), HA-phenylalanine (e), HA-arginine (f) and HA-valine (g).

Rheology and molecular weight measurements. The rheological behavior of the HA-amino acid derivatives was evaluated by measuring their dynamic viscosity in steady shear mode at 25°C in water. For comparison reasons, all amino acids were grafted using the same reaction conditions with 100% degree of substitution. The viscosity profile showed to considerably vary between the different derivatives (**Figure 4**). Molecular weight values of HA-alanine and HA-serine were measured by SEC-MALLS in water containing 0.1M $NaNO_3$ as their side chain is not hydrophobic or positively charged. The measurements of HA-alanine grafted with 100% substitution degree gave a molecular weight of 288 900 g/mol and a polydispersity index (PDI) of 1.68. For HA-serine with 100% substitution, the molecular weight was 458 000 g/mol and the PDI was 3.86. The high PDI value obtained for HA-serine explains the low viscosity profile seen in **Figure 4** even though the molecular weight is higher. Indeed, the presence of low molecular weight HA fragments considerably lowers the viscosity of HA

solutions. The reason for the difference in molecular weight between HA-alanine and HA-serine, despite the same protocol used for their synthesis, is unknown but suggests that all derivatives were not subjected to the same fragmentation of the native HA backbone during the successive reaction steps. In addition to a difference in chain length, the variation in viscosity profiles of the different derivatives could also be due to the diversity of the functional groups on the grafted amino acids. HA-tyr exhibited the highest viscosities of all HA-aa derivatives and preserved a shear-thinning profile. HA-ala and HA-asp showed slightly lower viscosities and still exhibited the shear-thinning profile. However, all other derivatives showed lower viscosities which were almost independent of the shear rate. HA-phe had the lowest viscosities of all. If not due to a lowered molecular weight, the lower viscosity of the derivatives could be caused by the disruption of the hydrogen bonds possibly by steric hindrance of the amino acids or by the formation of intramolecular interactions which tighten the molecular clusters. The higher viscosity of other derivative solutions could by contrast be caused by the formation of intermolecular interactions, giving rise to networks more resistant to shear stress. It is therefore not possible to predict the characteristics of HA-derivatives in terms of viscosity and molecular weight and ideally, individual optimization should be performed for each amino acid.

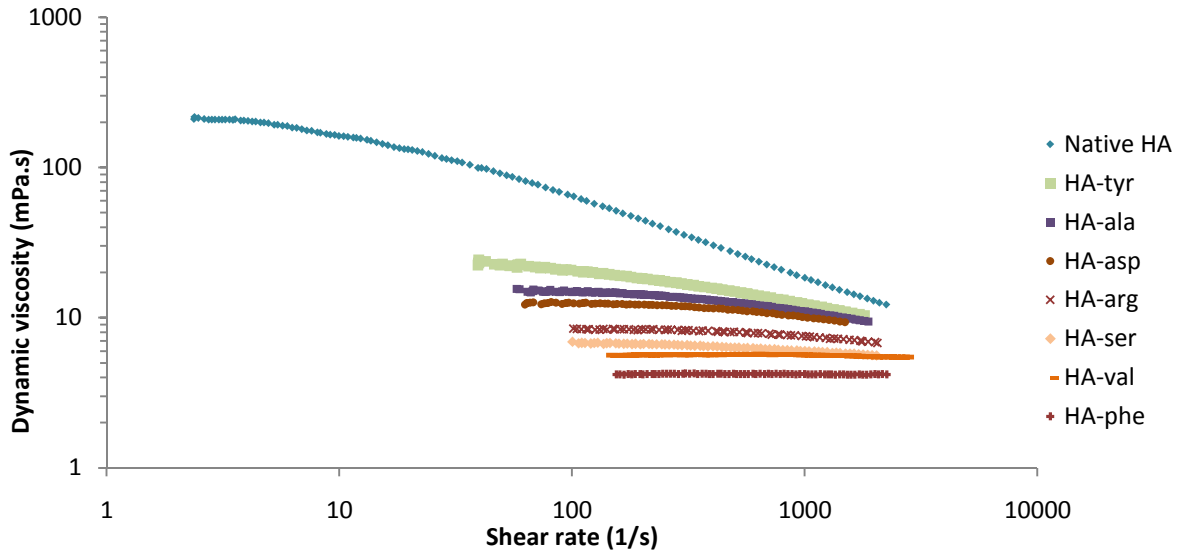

Figure 4: Dynamic viscosity of native HA and HA grafted with amino acids.

Enzymatic degradation of the HA-aa derivatives. We finally assayed the effect of the amino acids on the susceptibility of HA-aa derivatives towards enzymatic digestion. The HA-aa derivatives with 100% DS (2mM in PBS) were incubated in the presence of hyaluronidase at 37°C. The enzymatic reactions were then stopped at different times by the addition of alkaline tetraborate solution and the N-acetyl glucosamine reducing ends were quantified using the Morgan-Elson colorimetric reaction[11]. The degradation profiles of the derivatives were compared with the one of native HA subjected to the same conditions (**Figure 5**). All HA-aa derivatives showed lower enzymatic degradation compared to the native HA. HA-arg and HA-ala were the most degraded of the derivatives, suggesting that the positive charge of arginine did not improve the efficacy compared to HA-ala. This leads us to believe that only steric hindrance intervenes in the inhibition of the enzymatic degradation for HA-ala and HA-arg. All other HA derivatives grafted with aspartic acid, valine, tyrosine, phenylalanine, threonine and serine showed a lower degradation. Even though the mechanism it unknown, it can be assumed the hydroxyl, aromatic, aliphatic and negative charges enhance the inhibitory effect of amino acid grafting.

In addition, the results show that the viscosity of the HA derivative does not influence the enzymatic degradation. Indeed, HA-tyr and HA-phe which have the highest gap between their viscosity values show a similar enzymatic degradation profile.

Serine was chosen to be grafted at different grafting ratios by adjusting the proportions of reagents during the amidation reaction. The resulting HA-ser derivatives with 40%, 60% and 100% DS were subjected to the same enzymatic digestion assay. The results presented in **Figure 6** show that the decrease of the enzymatic degradation is proportional to the grafting ratio of the amino acid. The same observation was made in a previous study with HA-ala[10].

These findings confirm the beneficial effect of the carboxylate protection of HA with amino acids.

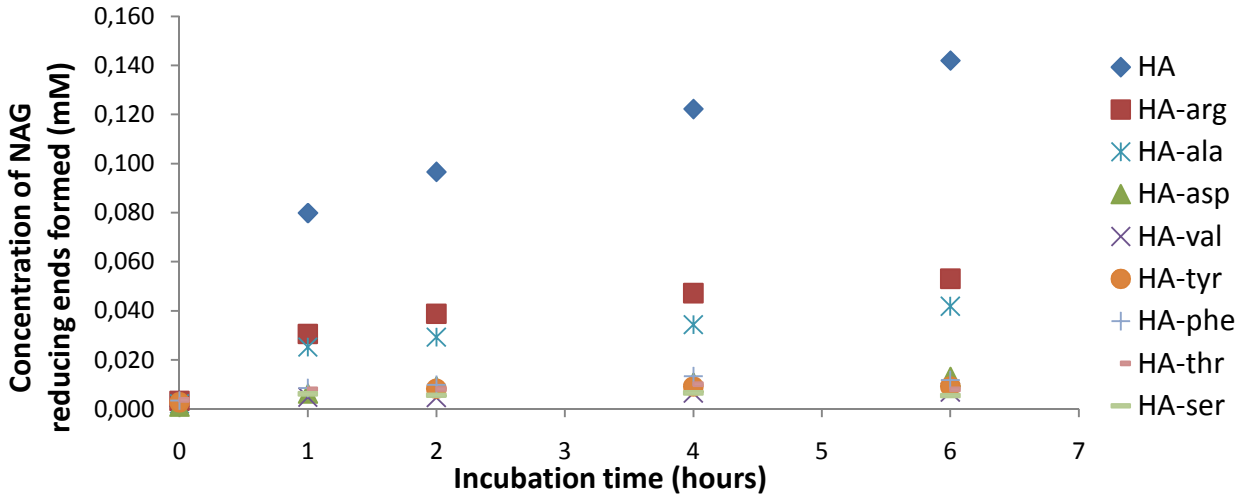

Figure 5: Enzymatic degradation profiles of native HA and of HA grafted with amino acids.

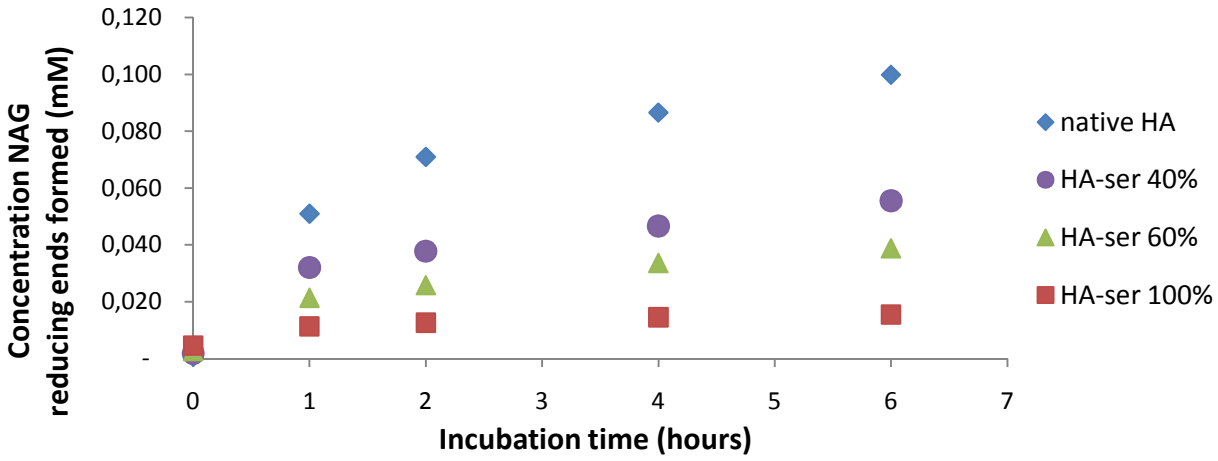

Figure 6: Enzymatic profile of native HA and HA-serine with different degrees of substitution.

Cross-linking of the HA-aa into hydrogels. To study the effect of amino acid grafting onto HA hydrogels, the HA-aa derivatives were crosslinked with a commonly used bisepoxide crosslinker, butanediol diglycidyl ether (BDDE). The reaction was performed as described in a patent by De Belder and Malson (1986) in slightly acidic conditions[12]. The formation of HA

hydrogels with BDDE was also described in the literature in strongly alkaline conditions (pH > 12)[13]. This option was however left out to avoid additional HA chain fragmentation known to occur at high pH values[14]. Instead, the milder reaction performed in water with a small amount of acetic acid allowed to obtain water insoluble hydrogels, with the exception of HA-threonine. The derivatives all had 100% substitution degree and were crosslinked with 0,82 equivalents of BDDE, the theoretical crosslinking degree therefore being 82%. The swelling ratio of the native HA hydrogel is within the range of values previously reported[13,12]. All HA-aa crosslinked hydrogels have higher swelling ratios than the native HA hydrogel when subjected to the same reaction conditions, suggesting a lower crosslinking density (**Figure 7**). Indeed, studies have shown that the swelling ratio of hydrogels decreases when the cross-linking degree increases until reaching a saturation point[9,15,16]. It can therefore be assumed that the grafted amino acids hindered the crosslinking reaction, most likely due to steric hindrance. For hydrogels formed from HA-ala, HA-val and HA-ser, some viscous solution remained in the dialysis tube indicating that the crosslinking was not complete. For HA-thr, no gel was formed and only viscous solution was recovered. The aliphatic groups and the hydroxyl groups thus did not appear favorable for the crosslinking reaction.

HA-tyr was selected to synthesize hydrogels from derivatives with different substitution degree for its high viscosity and its swelling ratio close to the native HA hydrogel in order to evaluate the influence of the amino acid grafting ratio on the swelling degree of the resulting hydrogels. The swelling ratios of hydrogels obtained from HA-tyr with 40%, 70% and 100% substituted carboxyl groups showed to slightly increase with the substitution degree (**Figure 8**). Hydrogels from HA-tyr 100% are the only ones significantly different than native HA (Student test, p=0.05).

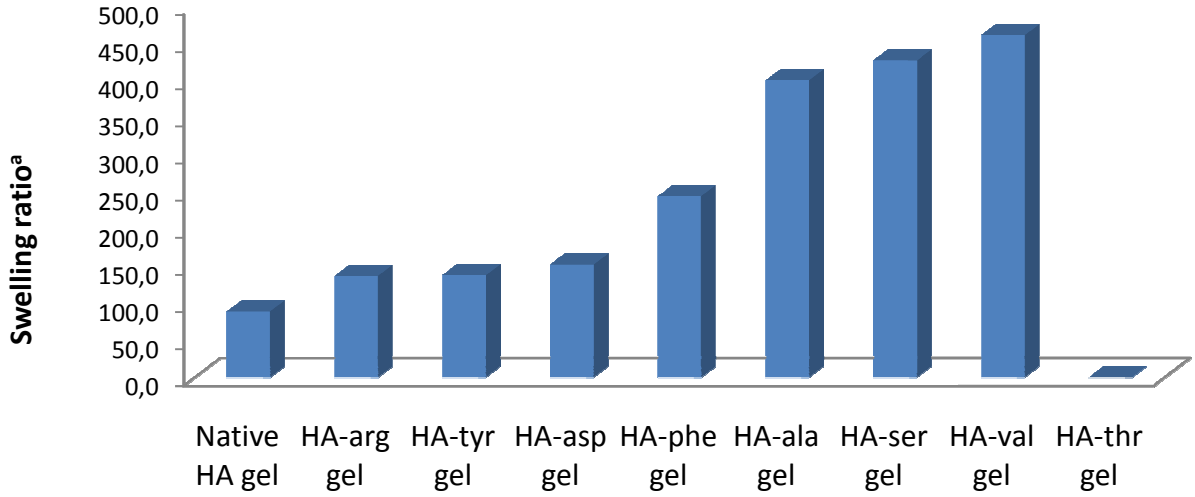

Figure 85: Swelling ratios of different HA-amino acid crosslinked hydrogels ([a]: in g of water per mg of polymer).

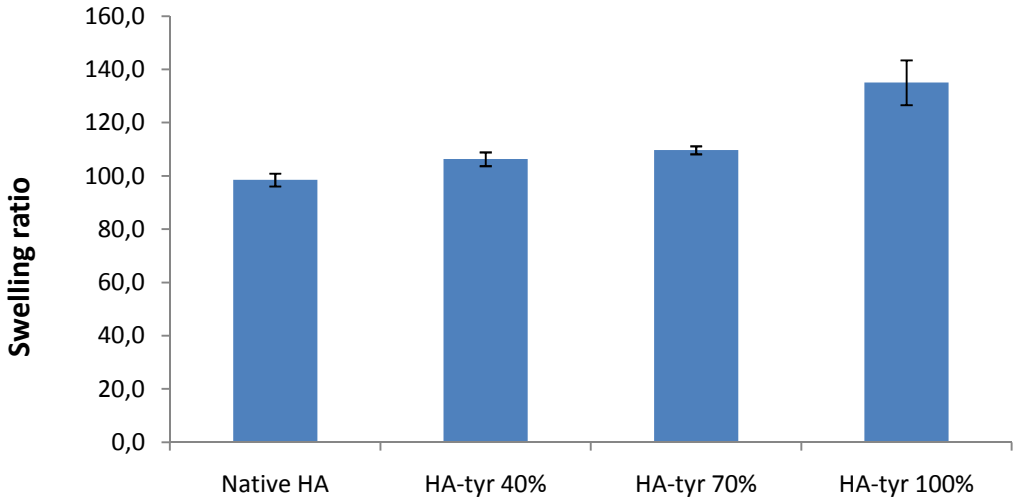

Figure 8: Swelling ratios of HA-tyrosine crosslinked hydrogels with different DS ([a]: in g of water per mg of polymer)

Enzymatic degradation of HA-aa cross-linked gels. To assess the efficacy of the amino-acid grafting after crosslinking of the HA-aa polymers, these were subjected to enzymatic digestion by hyaluronidase and the degradation was further quantified with the Morgan-Elson assay. The HA-tyr hydrogels with different grafting ratios of tyrosine were chosen for further analysis with this method. The gels were previously crushed and homogenized using a Poter

instrument and a stoichiometric amount was precisely weighed in each test tube and adjusted with water to obtain a final concentration of 2.5mg/mL. **Figure 9** points out that the hydrogels made from HA-tyrosine derivatives showed a lower enzymatic degradation than the native HA hydrogels synthesized in the same conditions. Crosslinking the HA derivatives into hydrogels therefore preserved the beneficial effect of reducing their sensitivity to hyaluronidases enzymes. Such as for their precursory HA-aa polymers, the enzymatic degradation of crosslinked hydrogels is dependent on the substitution degree.

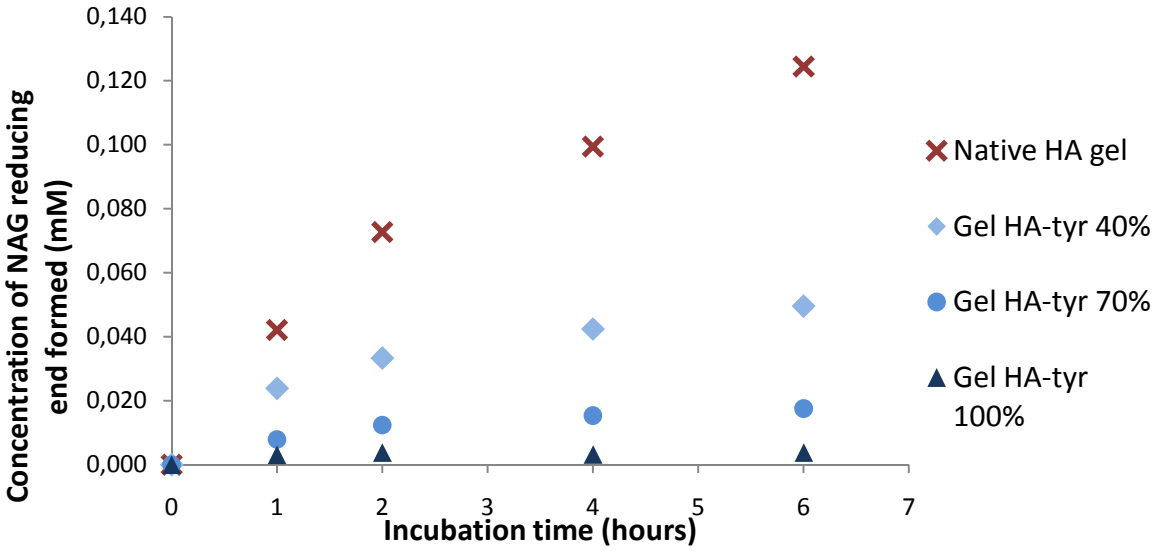

Figure 9: Enzymatic degradation profile of cross-linked native HA gel and cross-linked HA-tyrosine gels with different tyrosine substitution degrees.

CONCLUSION

In the present study, we synthesized new HA derivatives by grafting various amino acids onto its carboxylic groups. The amidation method used was in organic solvent with CMPI which allowed obtaining high grafting yields of 100%. All HA-aa derivatives grafted with 100% DS showed a significantly increased resistance to enzymatic digestion by hyaluronidases compared to the native HA. Crosslinking of the HA-aa derivatives was achieved by using BDDE as the crosslinking agent in a slightly acidic media. The nature of the amino-acid side

chain grafted on the HA chain however showed to greatly influence the crosslinking reaction. The resulting crosslinked HA-aa hydrogels exhibited a higher resistance to hyaluronidase digestion compared to the hydrogels obtained from native HA in the same conditions, showing that the efficacy of amino acid grafting onto HA is conserved after crosslinking. We therefore successfully synthesized crosslinked HA hydrogels derivatives with higher resistance to hyaluronidases enzymes. These new derivatives are promising materials to be used for many biomedical applications.

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Scientific communications and publications

Patent

Title : Acide hyaluronique modifié, procédé de fabrication et utilisations.

French National Application N° 1054125. Submitted May 27, 2010.

Inventors: Carole Schanté, Thierry Vandamme, Guy Zuber.

Poster communications

Carole Schanté, Guy Zuber, Thierry Vandamme, Novel PATENTED HYALUMINE® - Hyaluronic acid derivatives with improved biostability, *6th International Conference on Modification, Degradation and Stabilization of Polymers (MoDeSt 2010),* Athens, Greece, September 5 -9, 2010.

Publications

- Carole Schanté, Guy Zuber, Corinne Herlin, Thierry Vandamme, Synthesis of N-Alanyl-Hyaluronamide with high degree of substitution for enhanced resistance to hyaluronidase-mediated digestion. Submitted to *Carbohydrate Polymers* on February 3rd 2011.

- Carole Schanté, Guy Zuber, Corinne Herlin, Thierry Vandamme, Improvement of hyaluronic acid enzymatic stability by the grafting of amino-acids. Submission to *Biomacromolecules* in progress.

- Carole Schanté, Guy Zuber, Corinne Herlin, Thierry Vandamme, Chemical modifications for the synthesis of hyaluronic acid derivatives (Review). Submitted to *Carbohydrate Polymers* on December 21st 2010.

Chemical modifications of hyaluronic acid for the development of bioresorbable medical devices

Abstract: The aim of this work was to develop a novel hyaluronic acid (HA) product having a longer therapeutic action compared to the products currently on the market. An efficient chemical modification consisting of grafting amino acids onto the carboxylic groups of HA showed to yield derivatives significantly more resistant to *in vitro* enzymatic digestion than the native HA. Three amidation reactions were evaluated for an efficient grafting of the amino acid onto the carboxylic groups of HA. The next step was to form crosslinked hydrogels from the HA-amino acid derivatives and was achieved by using the crosslinking agent butanediol diglycidyl ether (BDDE) in acidic media. The resulting crosslinked HA-amino acid hydrogels exhibited a higher *in vitro* resistance to hyaluronidase degradation compared to the hydrogels obtained from native HA in the same conditions, and compared to commercially available hyaluronic acid products.

Keywords: hyaluronic acid, amidation, amino acid, enzymatic degradation, hyaluronidases

Modifications chimiques de l'acide hyaluronique pour le développement de dispositifs médicaux biorésorbables

Résumé : L'objectif de ce travail de thèse était de développer un nouveau dérivé d'acide hyaluronique (HA) ayant une action thérapeutique plus longue que les produits actuellement sur le marché. Une modification chimique efficace constistant en le greffage d'acides aminés sur les groupements carboxyliques de l'HA permis d'obtenir des dérivés ayant une meilleure résistance enzymatique *in vitro* comparée à l'HA de départ. Trois réactions d'amidation ont été évaluées pour l'obtention de taux de greffage élevés. La prochaine étape a porté sur la réticulation des dérivés HA-acides aminés qui a été accomplie avec le butanediol diglycidyl ether (BDDE) en tant qu'agent réticulant en milieu acide. Les hydrogels réticulés ainsi obtenus ont présenté une meilleure résistance *in vitro* à la dégradation enzymatique en présence de hyaluronidases en comparaison avec les hydrogels réticulés obtenus à partir de l'HA sans acide aminé et avec les produits commerciaux.

Mots-clés : acide hyaluronique, amidation, acide aminé, dégradation enzymatique, hyaluronidases