Development of Hyaluronic Acid Derivatives for Applications in Biomedical Engineering

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Dalila Petta

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**DEVELOPMENT OF HYALURONIC ACID DERIVATIVES FOR
APPLICATIONS IN BIOMEDICAL ENGINEERING**

DISSERTATION

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Dalila Petta

born on the 4th December 1987
in Bitonto, Italy

This dissertation has been approved by the supervisors:

Supervisor: Prof. Dr. D.W. Grijpma

Co-supervisor: Dr. M. D'Este

Graduation Committee

Chairman:

Prof. Dr. J.W.M. Hilgenkamp	University of Twente
-----------------------------	----------------------

Supervisor:

Prof. Dr. D.W. Grijpma	University of Twente
------------------------	----------------------

Co-supervisor:

Dr. M. D'Este	AO Research Institute Davos
---------------	-----------------------------

Referee:

Dr. D. Eglin	AO Research Institute Davos
--------------	-----------------------------

Members:

Prof. Dr. H.B.J. Karperien	University of Twente
----------------------------	----------------------

Prof. Dr. P.J. Dijkstra	University of Twente
-------------------------	----------------------

Prof. Dr. W.E. Hennink	University of Utrecht
------------------------	-----------------------

Prof. Dr. L. Moroni	University of Maastricht
---------------------	--------------------------

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Chapter 1

General Introduction

General Introduction

Hyaluronic acid (HA) is a non-sulphated glycosaminoglycan. Ubiquitous in the human body, this natural polymer is widely used in the biomedical research thanks to its unique chemical, physical and biological properties [1-3]. Over forty years of use in clinics makes it one of the most successfully naturally-derived polymers in the medical field. The versatility of the HA processing and its unique biological interaction with cells, make it an important building block for the development of new biofunctional materials. HA biomedical applications are related to its physicochemical and biological properties. The first biomedical applications of HA have been as aid in eye surgery [4] and as viscosupplement in osteoarthritis [5]. Not surprising, both applications are connected with its viscoelastic properties, which can be modulated with concentration, molecular weight or chemical modification for creating semi-synthetic derivatives giving physical or covalent gels [6, 7].

The HA chemical groups available for modifications are carboxyl, hydroxyl and *N*-acetyl group. Chemical alterations are numerous and enable the synthesis of a wide range of HA derivatives targeting applications in the field of tissue engineering and regenerative medicine [8-10]. It is possible to create HA derivatives retaining the cyto- and biocompatibility of the pristine HA, while having modified mechanics, degradation and interactions with biologics such as cells and proteins. Still, HA derivatives synthesis methods that employ more simple and controlled chemistries, with less toxic by-products to ensure biological biocompatibility of the conjugation and further chemical functionalization are required. This is especially true for the delivery of active biological agents and cells through advanced fabrication technologies.

One of the most widespread chemical modification on the HA carboxyl group is the amide formation by carbodiimide chemistry [11]. This is usually performed in presence of 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC) and *N*-hydroxysuccinamide (NHS). This carbodiimide conjugation presents the advantage of being performed in water with no significant cleavage of the HA chains, however the reaction requires high quantities of reagents and it is strongly pH-dependent [10]. An alternative for the activation of the carboxyl groups is the use of the triazine-chemistry. 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) has shown to promote the

grafting of amino groups on HA showing higher efficiency and better control compared to the carbodiimide-mediated reaction [12, 13].

The simplicity of the DMTMM chemistry prompted the interest in investigating a range of HA derivatives for applications in drug delivery, tissue engineering and biofabrication. In this thesis, I have researched on two physical gels, namely a thermoresponsive HA derivatives and short-alkyl derivatives, and a covalent gel (tyramine-modified HA). Specifically, the thermoresponsive hydrogel, where the HA carboxyl group was functionalized with poly(N-isopropylacrylamide), was combined with beta tricalcium phosphate particles. The non-covalent network of the thermoresponsive HA is exploited to obtain a better cohesion of the formulation together with a modulation of drugs delivery for bone tissue engineering. A second physical gel network was obtained grafting short-alkyl moieties to the HA. Here, a low degree of modification with small molecules such as propylamine and butylamine was able to drastically change the viscoelastic properties of HA, indicating profound modification of its structure. This same chemistry was employed to develop a new bioink for 3D printing, where the crosslinking density of a covalent tyramine-modified gel was optimized and controlled with a double crosslinking mechanism.

Aim and outline of the thesis

The aim of this thesis was to introduce a series of new HA derivatives for biomedical applications. These derivatives embrace a range of gelation mechanisms and applications as illustrated in the following outline:

In **Chapter 2** an introduction to HA and its chemical, physical and biological properties are provided. The chemical modifications on the main functional groups and the possible crosslinking strategies for hydrogel creation are described. Finally, the main applications of HA in the field of musculoskeletal repair and regeneration are reported.

In **Chapter 3** DMTMM is employed for grafting propylamine and butylamine to HA. The impact on the HA viscoelastic properties after the conjugation of these short-alkyl moieties is particularly relevant at low degree of substitution and opens a variety of possibilities in applications such as drug delivery and 3D printing. Additionally, a parametric study on the DMTMM reaction conditions shows the reproducibility of this

chemistry and the accurate control over a wider range of degrees of substitutions compared to the traditional carbodiimide modification [14].

Chapter 4 describes the preparation of a thermoresponsive poly(*N*-isopropylacrylamide) HA derivative (HApN) combined with beta-tricalcium phosphate particles (β TCP) as bone substitute and drug delivery system. The asset of this novel composite is the improved cohesion at body temperature that the HApN gives, thus giving the possibility to address bone defects. A range of composite formulations is tested as an injectable or putty formulation: the handling properties and the injectability of the composite are analysed as function of the particle size and the HA molecular weight and concentration. The most promising formulations are tested as drug delivery system for the recombinant human bone morphogenetic protein-2 (rhBMP-2) and dexamethasone (DEX) as models of hydrophilic and small hydrophobic drugs, respectively [15].

In **Chapter 5** a simple and versatile HA derivative is introduced as an effective biofunctional ink for extrusion-based 3D printing. HA is modified with tyramine functional groups via DMTMM chemistry. A double-crosslinking mechanism consisting in an enzymatic crosslinking that allows good extrudability followed by a visible-light crosslinking is implemented to ensure the stability of the 3D printed constructs. The ink is still available after printing for a functionalization with cell-adhesive motives for improving the construct-cell interaction.

Chapter 6 explores the possibility to 3D print viable cells encapsulated in a tyramine-modified HA and to obtain 3D constructs directly on cartilage tissue. The viscoelastic properties of the cell-laden ink are investigated targeting low shear stress for high cell viability and good extrudability. The influence of the photoinitiator and visible light-photocrosslinking on the cell viability is assessed on three different cell types and the printing of the cartilage-adhesive bioink on a piece of cartilage tissue is shown.

Chapter 7 focuses on the future perspectives for the HA derivatives in the biomedical field and the need of developing a new generation of derivatives due to the constant expansion of new technologies.

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Chapter 2

Overview of hyaluronan and derivatives in the musculoskeletal field

The fundamentals of hyaluronic acid properties and functions***Hyaluronic acid origin and chemical structure***

Hyaluronic acid (HA) is a natural polysaccharide made of repeating disaccharide units of D- glucuronic acid and N-acetyl-D-glucosamine. HA is ubiquitous in the extracellular matrix (ECM) of all the connective tissues and it is a glycosaminoglycan as chondroitin sulphate and heparin. For a 70 kg-human, the HA total content is about 15 g, with the largest portion in the skin and musculoskeletal tissue. HA is synthesized in the human body by three types of HA synthase, HAS1, HAS2 and HAS3, located in the cell membrane [1]. The average molecular weight (MW) ranges between 10^5 and 10^7 Da depending on the biological tissue [2].

Thanks to the existing chemical groups and its high MW, the HA has primarily a structural and hydration role in biological tissues. HA has been proven to be additionally involved in cell proliferation, migration and differentiation. These biological roles will be discussed in a later section. The turnover of HA in vertebrate tissues is fast, with one third of the total amount being synthesised every day. HA is naturally degraded in the organism by a complex and efficient mechanism involving the enzyme hyaluronidase and the reactive oxygen species [3]; therefore, the HA half-life *in vivo* reaches only short period of maximum 3 days [4].

Extraction of HA is usually performed from animal tissues such as rooster combs (1.2×10^6 Da), the vitreous body of bovine eyes (7.7×10^4 – 1.7×10^6 Da) and bovine synovial fluid (14×10^6 Da). Despite the routine use of these extraction methods and extensive purification, contamination remains the main challenge for extractive HA. Since HA is usually combined with other biopolymers in biological materials, other proteoglycans are often present as contaminants [5]. Therefore, starting from the 60s, the HA production through bacterial fermentation has become an alternative for non-immunogenic HA. The most used strain in the fermentation process is the Streptococci that uses glucose as a carbon source.

Figure 2.1 – Hyaluronic acid in the human body, its physical and biological functions and applications in the musculoskeletal field.

Hyaluronic acid physicochemical properties

HA is an unbranched polysaccharide with viscoelastic, lubrication and hydration properties (figure 2.1). The HA behaviour in solution, its shear rate dependent viscosity, has been first investigated by Ogston, Laurent, Balazs and Cleland who described the HA non-Newtonian features [6-8]. The HA organization in random coil influences the final viscosity of the solution depending on the MW. With an increase in the concentration, the movement of this coil becomes more restricted and the crowded molecular system acquires viscous and elastic properties depending on the MW, conformation of the chain and concentration. Thus, the elastic properties are highly influenced by the non-branched and polyanionic HA chain and by the intramolecular hydrogen bonds that result in a rigid system. Simultaneously, the negative charges on the chain are responsible for the high uptake of water and the formation of a hydrogel network that mainly maintains the viscoelasticity of the connective tissues [9]. The physicochemical properties are strictly related to the therapeutic applications of the HA. The molecular structure of the crowded molecular chain network of HA exhibits viscoelastic properties that can be tuned to

address tissue augmentation, ophthalmic surgery, visco supplementation, prevent adhesion and promote wound healing. An example of the influence of HA viscoelastic properties on the developing of diseases is given by the role of HA in the joint. Balazs has studied the rheological properties of synovial fluids of young, old and arthritic humans. He found that while in young patent the synovial fluids behave as an elastic fluid at high frequency, corresponding to faster movements like running, in old patients and more significantly in the ones with osteoarthritis, the synovial fluid lose some or all of its elastic properties [10]. This finding underlines the importance of the HA rigidity and elasticity for maintaining its functions. Further details on HA physicochemical properties can be found in the book chapter 20 of Garg and Hales' book [11].

Hyaluronic acid biological functions

The HA MW is influencing the viscoelastic properties as well as all the biological functions. HA is involved in several cell functions via cell surface receptors, homing cell adhesion molecule (CD44 or HCAM), and receptor for HA mediated motility (RHAMM), including cell mobility, proliferation, differentiation, cell-cell interaction and in the production of cell physiological substances, such as cytokines, prostaglandin E2 and metalloproteinases (MMPs) [12]. HA has antioxidant properties due to its interaction with oxygen-derived free radicals [13, 14].

Furthermore, HA is an inflammation mediator thanks to its ability to inhibit macrophage migration and aggregation. It plays a fundamental role in wound healing promoting with its degradation, the proliferation and migration of cells, and angiogenesis [15]. The role of HA in wound healing will be highlighted in a separate paragraph.

HA is expressed abundantly in cartilage and in tumour tissues. In the cartilage, HA forms aggregates with aggrecan providing tissue resistance to compression, stimulate the proteoglycans production, chondrogenic differentiation of mesenchymal stem cells, and retains proteoglycans within the tissue. In the tumour development, HA is a key component for the microenvironment of cancer cells facilitating the migration of invasive tumors through cell surface receptors. In particular, HA oligomers encourage angiogenesis and induce inflammatory cytokine production, which activate various signalling mechanisms for cancer progression. Hence, tumor progression and angiogenesis depend on HA and hyaluronidase levels, and the degradation profile of HA.

Hyaluronic acid chemical modification and crosslinking

Chemical modification

Due to its unique biological and viscoelastic features and its amenability to chemical modification, HA is an attractive macromolecule for the development of biomaterials. Several chemical modifications of HA were reported, aiming to enhance, modulate or control the therapeutic action of HA. The obtained derivatives predominantly maintain the biocompatibility and the biodegradability of the HA, but they acquire different physicochemical properties. There are three main sites prone to chemical modification as shown in figure 2.2: the carboxyl group (-COOH) on the D-glucuronic acid, the primary (C₆) hydroxyl group (-OH) and the *N*-acetyl group on the *N*-acetylglucosamine sugar. Additionally, the secondary (C₂, C₃ and C₅) hydroxyl groups are available for functionalization.

Figure 2.2 – Hyaluronic acid functional groups amenable to multiple chemical reactions.

The chemical modification of HA can be performed both in water and in organic solvents requiring in this case the conversion of HA sodium salt, soluble in water, into the acidic

form or a tetrabutylammonium (TBA) quaternary salt. HA derivatives have been classified by Prestwich into “monolithic” and “living” hyaluronan derivatives [16]. Living or monolithic HA derivatives respectively can or cannot form new covalent bonds in the presence of cells or tissues, and therapeutic agents (Figure 2.3). There are several reviews, together with the excellent one from Prestwich [17], describing all range of HA chemical modifications developed for biomedical applications [18, 19].

Figure 2.3 – Hyaluronic acid derivatives [16].

Functionalization of hyaluronic acid carboxyl group

The carboxy group is the main target for HA chemical modification. This is due to the versatility and the wide range of chemical reactions available. Amidation is the preferred route for carboxyl group grafting, followed by esterification and Ugi condensation.

Amide formation can be carried out with various activators such as the 2-chloro-1-methylpyridinium iodide (CMPI), carbonyldiimidazole and most commonly with carbodiimides. The reactions in presence of CMPI or carbonyldiimidazole require organic solvent and conversion into the TBA salt.

For example, in the work of Magnani *et al.* the CMPI activates the -COOH and forms a pyridinium intermediate [20]. A diamine forms an amide bond through a nucleophile

attack to the activated -COOH. In the system, the triethylamine is also involved to neutralize the released iodide ion. Modification with 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) is the most common method for HA modification at the carboxyl. This carbodiimide presents the advantage of being usable in water and no cleavage of the HA chain, however the reaction requires high quantities of reagents due to the EDC hydrolysis in basic environment and it is strongly pH-dependent. Two different pH values are required during the reaction: the -COOH activation by EDC, that forms an O-acyl isourea intermediate, is favoured at pH values between 3.5 and 4.5; whereas the nucleophilic attack by the amine on the activated -COOH works better with deprotonated amines at higher pH. Schneider *et al.* have substituted the water with DMSO to increase the amidation yield from the usual 10% to 60-80% [21]. New HA derivatives have been synthesized employing this conjugation chemistry, such as the boronic acid-tethered amphiphilic hyaluronic acid developed by Jeong *et al.* [22]; the reaction is performed in DMSO with a final conjugation yield of 4.8%. Additionally, carbodiimide chemistry was also used to graft polymers to the HA chain as demonstrated by the work from Lin *et al.* where a copolymer of HA and polyethylenimine (PEI) was synthesized as a drug delivery system [23].

An alternative amidation method has been described by Bergman *et al.* and uses the 2-chloro-dimethoxy-1,3,5-triazine (CDMT) as activator together with the *N*-methylmorpholinium (NMM) as a chloride ions neutralizer [24]. The reaction is performed in a mixture of water and acetonitrile reaching a 25% degree of substitution. Recently, the amidation promoted via DMTMM, a triazine derivative initially exploited in the peptide synthesis, has revealed itself as an effective method for the modification of the -COOH group [25]. In this reaction, the carboxyl group of HA binds to the triazine ring of DMTMM forming a reactive intermediate with morpholinium release; afterwards the chosen amine attacks the -COOH carbon on the active ester to form an amide bond (Figure 2.4). The DMTMM chemistry has been already applied for the conjugation on the HA backbone of various molecules, such as furan [26], glycine ethyl esterhydrochloride (Gly), Doxorubicin (Dox) hydrochloride, Poly(*N*-isopropylacrylamide) (pNIPAM), small alkyl moieties [27] and tyramine [28]. The advantages of DMTMM over the common EDC/NHS are the non-pH dependence of the reaction, the lower amount of coupling agent needed and the higher conjugation yield.

Figure 2.4 – DMTMM reaction scheme. Adapted from [25].

Additionally, -COOH can be modified by esterification or by Ugi condensation. The esterification requires the preparation of the HA-TBA salt and the reaction with an esterifying agent such as alkyl halides [29], diazomethane [30] and epoxides [31]. This chemistry was successfully employed by Fidia Advanced Biomaterials to synthesize the hydrophobic HA derivatives HYAFF. Instead, the Ugi condensation uses a diamine in presence of formaldehyde to form a final acylamino amide bond and linkages between two HA chains [32].

Functionalization of hyaluronic acid hydroxyl group

The chemistry of hydroxyl group conjugation is diverse and includes etherification, esterification, oxidation and carbamate formation, where the primary -OH group is the most reactive.

The ether formation can be achieved using ethylene sulphide together with the dithiothreitol (DTT) in alkaline environment [33]. The esterification is carried out in presence of octenyl succinic anhydride (OSA), acyl-chloride activated compound [34] or anhydrides [14]. The methacrylated HA derivative is widely used to obtain photocrosslinked hydrogel and the derivative is synthesized in water at alkaline pH via reaction with anhydrides.

The oxidation is carried out in presence of sodium periodate that oxidizes the secondary -OH groups to aldehyde groups [35]. As a side effect of the harsh oxidation reaction, the cleavage of HA chains occurs with a consequent decrease in the HA molecular weight. An additional option for -OH conjugation is the isourea coupling implemented in the work by Mlcochova *et al.*, where cyanogen bromide was used to form a HA carbamate main product and a HA isourea byproduct [36]; the reaction performed in water leads to high degree of substitution, up to 80%.

Functionalization of hyaluronic acid N-acetyl group

The deacetylation of the *N*-acetyl group on the HA glucosamine portion leads to the presence of a free amino which can react further to several chemical groups forming conjugates. The deacetylation was addressed using either hydrazine sulfate [37] or enzymes [38].

Crosslinking strategies

Crosslinking strategies have been introduced to enhance the mechanical properties of the HA and extend its half-life. The improvement of the viscoelastic properties of HA triggered by the crosslinking is essential for supporting cells encapsulation and for promoting the mechanical environment required for cell differentiation. Additionally, this stability is desirable for applications of the HA derivative in 3D printing technologies and for the development of injectable formulations.

HA can be crosslinked by employing chemical, enzymatic, physical or photo-crosslinking mechanisms. Detailed information on the crosslinking of HA or more specifically on the photocrosslinking [39] can be found in published review such as the one from Segura *et al.* [40-42].

Chemical crosslinking

The most common chemical crosslinker for injectable hydrogels for human use is butanediol-diglycidylether (BDDE), patented by Målson and Lindqvist in 1986 [43]. This reaction is performed in alkaline environment and consists in ether bond formation after epoxide ring opening. The etherification of the -OH groups via BDDE is preserved not only at high pH but also at slightly acidic pH, where still a high quantity of hydroxyl groups on the HA is deprotonated [44]. When the pH values are lower than the pKa values

of the -OH group, however, the anionic carboxyl group is predominant promoting ester bond formation [45]. The BDDE can be substituted by other bisepoxide such as ethylene glycol diglycidyl and polyglycerol polyglycidyl ether [46].

Likewise, the crosslinking of HA is achieved via ether bonds formation triggered by divinyl sulfone (DVS). DVS differs from the BDDE crosslinking: the reaction is carried out at room temperature; the reaction time is short (1 h to completion); the biocompatibility is preserved despite the high DVS reactivity (Figure 2.5); the degree of crosslinking is increased by the presence of salts. Glutaraldehyde (GTA) is another common crosslinker, activated with an acidic pH, that brings to the formation of hemiacetal bonds with the -OH groups [47, 48].

Besides the direct chemical crosslinking mechanisms, it is possible to crosslink HA-hydrazide or HA-amine derivatives with crosslinkers such as the bis(sulfosuccinimidy)suberate and the 2-methylsuberimide (DMS) that contain respectively an ester and imidoester reactive group.

A thiol-modification of the -COOH group using a carbodiimide-mediated hydrazide chemistry leads to a spontaneous crosslinking in the air due to the oxidation of thiols to disulfides [49].

Figure 2.5 – Chemical crosslinking strategies via DVS [50].

Photocrosslinking

Photocrosslinking is a common method for HA reticulation, as it allows a rapid curing, suitable for *in situ* polymerization, and an accurate spatial and temporal control. Typically, HA is modified with (meth)acrylate groups in two different ways: ester formation with methacrylic anhydride in alkaline environment [34, 51] or a ring opening reaction with glycidyl methacrylate [39]. Methacrylated HA is further crosslinked with UV light (365 nm) via radical-inducing photoinitiators such as Irgacure 2959. The presence of methacrylate groups allows a photocrosslinking with a controlled, minimally-invasive and rapid gelation. The photocrosslinked HA retains the biodegradability that varies depending on the crosslinking density. The mechanical properties of the gel are related to the modification degree, photoinitiator type and concentration and UV light exposure time. At the same time, the exposure time, together with the photoinitiator itself, is inversely correlated to the cell viability of the encapsulated cells as shown in the work from Park *et al.* where the cell viability dropped by 30% with an increase of the exposure time from 40 seconds to 600 seconds [52]. Therefore, photoinitiators sensitive to visible light have been developed (Figure 2.6) to overcome the drawbacks of the UV light – cell damage and low penetration depth [53]. Examples of visible-light photoinitiator are organic dyes such as Eosin Y [54], Rose Bengal [55] and methylene blue; aromatic hydrocarbons such as quinones [56]; porphyrins and phthalocyanines. Eosin Y was successfully employed for the photocrosslinking of a tyramine-modified derivative of HA (HA-Tyr) promoting the formation of dityramine bond [57]. The proposed mechanism of HA-Tyr photocrosslinking by Loebel *et al.* is the following: on absorption of a photon, ground-state EO is raised to the first excited singlet state (^1EO), and this is converted to a long-lived triplet state ($^3\text{EO}^*$). In presence of oxygen, energy is transferred to form singlet oxygen ($^1\text{O}_2$), which reacts with the HA-Tyr. Another visible-light photoinitiator is the lithium acylphosphinate (LAP) photoinitiator whose efficacy to photopolymerize monomers has been investigated and compared with the Irgacure 2959 [58, 59].

Figure 2.6 - Visible light photoinitiators.

Visible-light photoinitiators are a promising way for the *in vivo* formation of mechanically stable hydrogels in tissue engineering. Improvements on the cytotoxicity of these systems and on their crosslinking yield are still required.

Enzymatic crosslinking

Horseradish peroxidase (HRP)/ hydrogen peroxide (H_2O_2) is the most common enzymatic mechanism for the HA crosslinking. The synthesis of the tyramine-modified hyaluronic acid and the consequent crosslinking via HRP/ H_2O_2 have been extensively investigated [60]. In this reaction, HRP uses hydrogen peroxide as oxidizing agent for producing a free radical on the phenol. The catalytic cycle, illustrated in figure 2.7 is initiated by the presence of H_2O_2 which interacts with the resting ferric state of HRP [Fe(III)] and generates compound I [$\text{Fe(IV)}]^+$, an intermediate in a high oxidation-state with a cation-radical [61]. The tyramine acts as a reducing agent converting compound I to compound II [Fe(IV)]. Subsequently, a second Tyramine group is oxidized, reducing HRP back to its resting ferric state. At the end of the reaction, dityramine bonds occur at the C-C and C-O positions.

Figure 2.7 – Reduction-oxidation cycle of horseradish peroxidase [62].

This mechanism has been proven to be non-toxic for a range of HRP/ H_2O_2 concentration, cytocompatible and a flexible system to tune the viscoelastic properties of the final hydrogels [63]. Specifically, the HRP controls the crosslinking rate (gelation time) and degree (mechanical properties) through the H_2O_2 and HRP concentrations. However, with cell-embedded materials, the enzymatic crosslinking limits the elastic moduli of the hydrogel due to the limited HRP/ H_2O_2 non-toxic concentrations and crosslinking density, and the resulted hydrogels are quickly degraded.

A recent study by Roberts *et al.* compared the efficacy of four different oxidative enzymatic systems (HRP or hematin combined with H_2O_2 , laccase or tyrosinase) in the crosslinking of tyramine-modified polymers [64]. HRP and hematin promoted a faster crosslinking, whereas laccase and tyrosinase a slower polymer gelation. In turn, the viability and proliferation of embedded cells will be differently modulated. Another example of enzymatic-mediated crosslinking is the use of transglutaminase. In the work of Ranga *et al.*, a hybrid HA-PEG hydrogel was synthesized via a covalent linking between an HA modified with cysteine-bearing transglutaminase substrate peptides and a lysine-modified PEG [65].

Non-covalent crosslinking

The reversible nature of non-covalent interactions can trigger HA hydrogel formation thanks to the possibility to respond to local physical or chemical cues. This type of crosslinking mechanisms generally results in low toxicity towards the cells and tissues. Usually, these non-covalent hydrogels form a network in response to both external stimuli such as temperature, pH and ionic strength or physico-chemical interactions such as hydrophobic or charge interactions. Most of the amphiphilic HA derivatives belong to this category of non-covalent hydrogels. The conjugation of hydrophobic side-chains to the HA backbone leads to a strong interaction between the hydrophilic and hydrophobic portions with the formation of a gel-like structure far from the initial sol-like pristine HA [66]. These amphiphilic systems have been successfully used as drug delivery system, visco-supplements and self-assembly systems. In the field of visco-supplementation, Creuzet *et al.* have shown that the introduction of alkyl chains of 10 - 12 carbon atoms on an adipic dihydrazide HA derivative is able to promote a physical crosslinking thereby enhancing the HA rheological properties [67]. Finelli *et al.* have described the formation of a physical hydrogel at low polymer concentration after the conjugation of HA with hexadecylic (C-16) side chains, through amide bonds (1–3 mol-% degree of substitution of repeating units) [68]. Knowing the potential of alkyl chains functionalization, the influence of small-alkyl moieties, namely butylamine and propylamine, on the viscoelastic properties of HA were investigated as shown in Chapter 3 of the thesis [27]. A low degree of substitution (3-4%) was sufficient to drastically improve the viscoelastic properties of the pristine HA.

Thermoresponsive pNIPAM was grafted to HA to give an HA-based hydrogel producing a transition from solution to the gel at approximately 30°C, ideally between room and body temperature, as shown in figure 2.8 [69, 70]. Similarly, the thermoresponsive copolymer hexamethylene diisocyanate-pluronic F 127 [71] and the methyl cellulose [72] were grafted to the HA to create drug delivery systems.

Figure 2.8 – Thermoresponsive behaviour of the pNIPAM-modified HA (HApN). DSC thermogram, rheological temperature sweep and vial-inversion test [73].

Another example of non-covalent reticulation is given by ionic interactions. This mechanism is typical of alginate, where divalent cations, such as Ca^{2+} , bind the glucuronate blocks leading to the crosslinking of adjacent polymers chains [74]. Likewise, HA can be crosslinked employing positively charged ions, such as Fe^{3+} and Ca^{2+} . In the work of Nakagawa *et al.* HA is first grafted to the polyacrylic acid and then crosslinked in presence of calcium chloride [75]. The resulting gel has lower viscosity and slower degradation rate compared to the pristine HA. However, the ionic crosslinking results in a rapid and poorly controlled gelation, limited long-term stability and low gel uniformity.

Hyaluronic acid based biomaterials in the musculoskeletal field

Cell-based tissue engineering involves seeding cells into a biomaterial scaffold to fabricate functional biological substitutes for the replacement of lost or damaged tissues. The physical and biochemical properties of HA make this biopolymer attractive for tissue engineering in the musculoskeletal field. Investigators have developed HA-based scaffolds in the form of hydrogels, sponges, and meshes for applications in the biomedical field, especially in tissue engineering. These scaffolds are biocompatible and can serve as delivery vehicles for cells and bioactive molecules. For more details of the following subsections, several reviews and book chapters have been published describing the role of HA in tissue engineering [76], in drug delivery [77], in wound healing [78, 79], in cartilage repair [80], for cell delivery [15].

Wound healing

The synthesis of HA is quickly upregulated at an injury site and during all the inflammatory stages of the wound repair [81]. HA has multiple functions in wound healing. It interacts with the fibrin clots, enhances fibroblasts proliferation, modulates the host inflammatory cell infiltration and the production of growth factors and cytokines in the inflammatory cells. It also impacts fibroblasts and keratinocytes by influencing presentation of growth factors such as VEGF and PDGF to the related receptors and by regulating gene expression in inflammatory cells, promoting their migration and adherence in the inflamed tissue. Finally, it also inhibits photogenes proliferation at the wound site and limits the inflammation with its antioxidant properties. The fragmentation of HA at the injury site is deemed to be responsible for the initiation of the healing response [82]. These multiple HA roles have been confirmed by *in vitro* studies as well as by animal models and human clinical studies, where HA is used in the form of injectable hydrogels, scaffold or spongy sheet.

Cartilage repair

Articular cartilage is a non-vascularized and poorly cellularised tissue and therefore its repair and regeneration are challenging to achieve [80]. The role of HA in the development of cartilage has been well studied, whereas the connection between embryological roles of HA and its uses in tissue engineering are less understood. Early in the process of cartilage development, the limb mesenchyme is composed of dispersed

cells throughout an ECM that contain significant quantities of HA. Upon initiation of condensation, mesenchymal cells aggregate, hyaluronidases are upregulated, and HA concentrations drops. At this stage, HA helps mediate cell aggregation thanks to the HA receptor CD44. After condensation, HA synthesis is again upregulated as the cells differentiate toward a functional cartilage tissue. Burdick *et al.* have proved the supportive role of HA-based hydrogels in the differentiation and tissue formation when used with mesenchymal stromal cells (MSCs) and chondrocytes [83]. Usually, the HA hydrogel constructs integrate well with the native tissue after implantation promoting enhanced matrix synthesis and cellularity. For example, Tan *et al.* synthesized a *N*-succinyl chitosan/aldehyde hyaluronic acid composite material that sustains the survival of the encapsulated cells promoting the retention of chondrocytes morphology [84].

Bone tissue engineering

Material with several forms as hydrogels, fibers, meshes, granules, pastes and foams have been developed for bone tissue engineering, among which we can find hyaluronic acid derivatives and hyaluronic acid-based composites [76]. For example, Bae *et al.* have demonstrated that the photocrosslinkable methacrylated HA hydrogel loaded with simvastatin influences both *in vitro* and *in vivo* osteogenesis (Figure 2.9) [85]. A combination of hyaluronic acid–gelatin hydrogel loaded into a biphasic calcium phosphate (BCP) ceramic scaffold was investigated as a boost for new bone formation [86]. In the direction of bone tissue engineering, the use of the bone morphogenetic protein, BMP-2, is the most widespread strategy. Porous HA scaffolds have been used as BMP- 2 delivery system with a slow protein release for effectively enhance bone growth [87]. Kang *et al.* have implanted in rats polylactic–co–glycolic acid grafted HA/polyethylene glycol scaffolds for the *in vivo* delivery of BMP-2 and enhancement of bone regeneration [88].

Figure 2.9 – Healing effect on defected bone of the photo-cured HA containing 1 mg of simvastatin SIM (Rt side) compared to the pure HA hydrogel (Lt side) [85].

IVD regeneration and repair

Degradation of intervertebral disc (IVD) is a widespread musculoskeletal disorder. To develop a tissue engineering solution to IVD degeneration, the engineered construct must exhibit characteristics of structural support and discrete tissue architecture mimicking the IVD, that is, annulus fibrosus (AF) and nucleus pulposus (NP). HA is an interesting candidate for IVD regeneration being an integral part of the native IVD. HA with high MW can particularly match the stiffness of the native NP having on the contrary a low ability to withstand shear forces. HA has been developed as pre-clinical or clinical option for addressing IVD regeneration [89]. In a preclinical study by Revell *et al.*, HA has been used as an *in situ* forming polymer for NP replacement: two different hyaluronan derivatives, the ester HYAFF 120 and the amide HYADD 3, were injected into the NP of the lumbar spine of female pigs showing support for cell growth and prevention of fibrotic changes. For IVD regeneration, it was demonstrated that HA facilitates the matrix synthesis of NP cells and promotes their viability [90]. MSCs are additional candidate

cells thanks to their ability to bind HA through the CD44. Several animal studies have been performed, demonstrating that the injection of MSCs with HA into degenerate discs stimulates some regeneration as measured by restoration of disc height [91, 92]. HA hydrogels are often mixed with other natural hydrogels such as gelatin or with synthetic polymers, usually polyethylene glycol [93, 94]. In particular, gels containing lower molecular weight HA combined with PEG were found to facilitate NP and AF cell proliferation [95].

Hyaluronic acid coatings

The design of coating is a main activity for biomedical applications, especially in the prosthetic implants. HA is a good candidate for coating various materials thanks to its natural negative charges [96]. Several coating methods have been described, including covalent linking to the surface [97], adsorption and ionic coupling. A versatile method for the engineering of biomaterial surfaces is the layer-by-layer deposition method that can be applied to metallic, polymeric or ceramic substrates [98]. Usually, polyelectrolyte films are deposited layer after layer using two opposite charged molecules multilayers. This coating can be functionalized by the addition of drugs [99], proteins [100] and growth factors in the coating. In the work of Prokopovic *et al.*, multilayers of hyaluronic acid and poly-L-lysine act as high-capacity reservoirs for small charged molecules such as rhodamine (positively charged), ATP and carboxyfluorescein (negatively charged) [101].

Figure 2.10 – HA/PLL film containing an HA polymer chain (red) and a PLL chain (green) for the release of carboxyfluorescein (green squares) [101].

HA coating have been exploited for the migration of mesenchymal stem cells due to the well-known interaction between HA and its receptor on MSC membrane. In the work of Corradetti *et al.* HA-coated tissue culture plates have been prepared able to increase, both *in vitro* and *in vivo*, the cell-homing toward the inflammation site [102].

Hyaluronic acid as visco-supplement

The synovial fluid is secreted by the synovium into the joint cavity and it contains electrolytes, low molecular weight molecules and macromolecules such as glycosaminoglycans (98% HA). Its viscoelasticity depends on HA concentration, molecular weight and physical interactions within the HA molecules and other proteins and ions. The synovial fluid in osteoarthritic (OA) joints contains a lower HA concentration and molecular weight than in healthy joints, resulting in decreased viscoelastic properties, intensified cartilage-cartilage contact and increased wear of the joint surface. Therefore, intra-articular injections of HA are performed to restore the normal articular homeostasis due to the HA viscoelastic properties and protective effect on articular cartilage and soft tissue surfaces of joints. HA injections have also shown to slow down the mechanisms involved in osteoarthritis pathogenesis [103]. This finding has been confirmed in both *in vitro* and *in vivo* studies: HA can prevent the degradation of cartilage and promote its regeneration. Moreover, it can reduce the production of proinflammatory mediators, matrix metalloproteinases and nerve impulses sensitivity associated with OA pain [104, 105]. However, controversies have been raised on the actual beneficial effect of HA as a visco-supplement in patients. Studies have shown that placebo itself can relieve pain and improved patient function and stiffness [106], and structural benefits could not be determined. On the other hand, several clinical trials have shown that HA can reduce arthritic pain in OA knee. In fact, arthroscopy of synovial membranes from OA patients revealed a significant decrease of tissue inflammation after HA injection with a decrease in the numbers of macrophages, lymphocytes, mast cells and adipocytes. Additionally, a decreased oedema and an increase in the number of fibroblasts and amount of collagen throughout the thickness of the synovial tissue was observed [107].

Hyaluronic acid as a drug delivery system

HA drug delivery systems are realized either as nanocarrier systems (nanoparticles, liposomes, microspheres, hydrogels) or as direct HA-drug conjugates. Regarding the HA-drug conjugates, several studies have shown the covalent binding of drugs to the HA resulting in the cleavage of these bonds *in vivo* and release of the drugs that preserve their own therapeutic effect. Most of the HA-drug conjugates have been synthesized via carbodiimide chemistry starting from HA-hydrazide [108]. HA is widely used for active

drug targeting to the tumour cells thanks to the expression of CD44 by the majority of solid tumours. For example, the chemotherapeutic agent Paclitaxel has been conjugated to the HA and afterwards released into the tumoral cells in multiple ways: via an ester linkage easily hydrolysed by enzymes present in the body [109]; via amino acid linkers using carbodiimide chemistry [110]; via esterification after modification of paclitaxel with 4-bromobutanoic acid [111]; encapsulation in nanoparticles afterwards covalently grafted to HA [112].

Besides the drug conjugation, the loading of drugs into amphiphilic HA has been proven to promote a gradual release profile thanks to the self-assembly of the hydrophilic and hydrophobic portions into micelles in aqueous solution. For example, Mayol *et al.* have synthesized the amphiphilic octenyl succinic anhydride-modified HA derivative, obtaining micelles (Figure 2.11) with a prolonged release of the hydrophobic anti-inflammatory drug triamcinolone acetonide into the joint cavity [113]. Additionally, the negative charges of the -COO^- groups can promote interaction with positively charged drugs to form non-covalent ion complexes. The positively charged chemotherapy agent cisplatin, for example, forms ion complexes upon physical mixing with a solution of pristine HA with the pr

Figure 2.11 – Amphiphilic octenyl succinic anhydride-modified HA micelles in water (top left) and in PBS (top right), size distributions of the micelles and graphical representation of the structure [113].

Similarly, composite materials can tune the drug release. For example, HA can be covalently crosslinked to other polymers such as carboxymethyl cellulose, poly(acrylic acid) or poly(L-lactic acid) [115] or can physically interact with calcium phosphate particles [116]. Part of my doctoral research presented in this thesis was devoted developing a composite biomaterial made of the thermoresponsive hyaluronan derivative HApN and beta tricalcium phosphate particle. The amphiphilic character of the composite provided a controlled release of both recombinant human bone morphogenetic protein-2 and dexamethasone, selected as models for a biologic and a small hydrophobic molecule, respectively. This study is reported in Chapter 4.

As for the drug-delivery, HA hydrogels have been used as protein-delivery systems and DNA-delivery systems to address the need of gene therapy approaches in regenerative medicine. In the work of Chun *et al.*, a photocrosslinked composite (vinyl group-modified hyaluronic acid/ di-acrylated pluronic F-127) was synthesized and the sustained release of plasmid DNA was obtained using different UV irradiation doses and varying the HA/pluronic ratio [117].

Hyaluronic acid as a cell carrier

A scaffold for tissue engineering is a temporary substitute for natural ECM whose function is to provide mechanical and functional support to the cells during their remodelling of the scaffold structure to obtain a functional artificial tissue.

HA has several advantages for cell encapsulation, starting from being a ubiquitous ECM component, the possibility to modify the pristine HA exploiting the functional groups, enabling a wide range of crosslinking strategies. The malleability of the material enables the processing of the material into 2D films, 3D scaffolds, nanofibers and injectable formulations. The biological properties (cell interaction with surface markers) together with the tuneable mechanical properties complete the set of advantages of HA over other natural polymers.

HA is a valid biomaterial in the regulation of cell behaviour, cell expansion and direct differentiation of various cell types but to be used as a cell-laden scaffold, it must be chemically modified in order to retain stability in culture. A critical point is the toxicity of the chemical crosslinker.

HA has been widely used for tissue engineering to support, for example, the growth of human chondrocytes, keratinocytes, fibroblasts and human mesenchymal stem cells. HA hydrogels have been additionally used to control the differentiation of encapsulated stem cells towards chondrocytes. In the work of Chun *et al.* the chondrogenic differentiation of MSCs embedded in a photocrosslinked methacrylated hyaluronic acid was successfully achieved both *in vitro* and *in vivo* [118]. The HA has proven to be more effective in MSC chondrogenesis compared to PEG hydrogels where the expression of cartilage specific marker was less enhanced.

Moreover, it has been reported that HA-based scaffolds are able to reduce the secretion of nitrites, metalloproteinase 13 and caspase, resulting in a decrease of apoptotic cells [119].

Hyaluronic acid in 3D bioprinting

HA is being investigated for applications in 3D printing. HA can be promising bioink candidate characterized by a facile extrusion for deposition, a supporting and protecting function towards the cells during the extrusion process and a post-printing shape retention.

Typically, HA is used as bioink in combination with other biomaterials, both natural polymers or synthetic polymers. In the work from Skardal *et al.*, pristine HA was added to a poly(ethylene glycol) (PEG)-based bioink to improve the PEG shear thinning and extrusion properties [120]. Shie *et al.* have developed a composite made of a water-based light-cured polyurethane and HA for cartilage tissue engineering [121]. Recently, a tyramine-modified HA has been combined with nanofibrillated cellulose to obtain a bioink that supported the printing of human-derived induced pluripotent stem cells [122]. There are few studies where HA has been used as a self-standing material: the group of Burdick has printed a guest-host HA-based hydrogel based on the non-covalent and reversible bonds between an adamantane-modified HA and a beta-cyclodextrin-modified HA [123, 124] for shear thinning, and final curing achieved with the methacrylate groups, added in both precursors through multistep derivatization (Figure 2.12).

Figure 2.12 – 3D printing process: 1) supramolecular hydrogel assembly with guest–host bonds; (2) guest–host bond disruption when extruded through the narrow needle; (3) rapid selfhealing of the supramolecular hydrogel and guest–host bonds; (4) UV treatment to photo-cross-link methacrylates within the hydrogel; and (5) stabilization [124].

The most common HA cell-laden photocrosslinked bioink is the methacrylated (MA-HA). Kesti *et al.* combined MA-HA with a thermoresponsive hyaluronic acid derivative

used as a temporary supportive material removed after UV irradiation [125]. Recently, both MA-HA in combination with gelatin-methacrylated was employed by Duan *et al.* to print photocrosslinkable (365 nm UV light exposure) heart valve conduits [126]. Likewise, MA-HA was partially crosslinked with gelatin methacrylate to give an extrudable gel-like fluid; this printed cell-laden-ink was further crosslinked by exposing again to UV light in order to obtain a stiffer and more stable final construct [127]. In all these photocrosslinkable systems the UV irradiation is a detrimental component inducing cellular damage via direct interaction with cell membranes, proteins and DNA or via indirect production of reactive oxygen species (ROS). To overcome the limitation of the UV light, new photocrosslinking systems based on visible light have been investigated. Part of my thesis work was focused on the development of a bioink based on a tyramine-modified hyaluronic acid derivative and a double crosslinking mechanism (Chapters 5 and 6). Specifically, an enzymatic pre-crosslinking allows to tune the viscoelastic properties of the hydrogel and obtain a soft and printable gel, whereas the photocrosslinking triggered by the photoinitiator eosin and green light supports the shape retention of the hydrogel after printing.

Conclusion

HA and the wide palette of HA derivatives are extremely important in the biomaterials and regenerative medicine fields. Thanks to the biological properties and the tuneable chemical, viscoelastic and mechanical properties, the HA is an attractive tool for various applications, such as tissue engineering and drug delivery. Additionally, the versatility of HA makes possible to obtain HA in several forms such as hydrogels, fibers, amphiphilic systems, bioink, using various chemistry and processing methods.

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Chapter 3

Enhancing hyaluronan pseudoplasticity via 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride-mediated conjugation with short alkyl moieties

Dalila Petta^{1,2}, David Eglin¹, Dirk W. Grijpma^{2,3}, Matteo D'Este¹

¹AO Research Institute Davos, Davos Platz, Switzerland

²Department of Biomaterials Science and Technology, University of Twente, Enschede, The Netherlands

³Department of Biomedical Engineering, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Abstract

Hyaluronan (HA) is widely used in the clinical practice and in biomedical research. Through chemical modification, HA shear-thinning properties, essential for injectability and additive manufacturing, can be optimized. In this study, we employed 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) for grafting propylamine and butylamine to HA. A parametric study was performed to identify the optimal reaction conditions. Results showed that DMTMM amidation gives reproducible and accurate control over a range of degrees of substitution (DS) from 1% to 50% and proved reliable to tune viscoelasticity. At DS=3.0% for HA-propylamine and 3.7% for HA-butylamine a maximum for storage modulus and pseudoplasticity was found, whereas above or below this DS, rheological features go back to baseline values of pristine HA. Due to their singular rheological profiles, these derivatives are valuable biomaterials candidates for preparing bioinks and hydrogels for drug delivery and regenerative medicine.

Introduction

Chemical modification is the main tool for modifying carbohydrate polymers intended for medical applications [1, 2]. With specific derivatization strategies, polysaccharide properties such as hydrophilicity, conformation dynamics and hydrodynamic radius can be altered with direct impact on solubility, viscosity, formation of supramolecular structures and drug delivery capabilities. So far, the possibility to chemically modify hyaluronic acid (HA) has been widely investigated. HA is a main component of the extracellular matrix in animal tissues and can be derivatized without compromising its biological properties [3, 4].

This linear polysaccharide and its derivatives have been extensively applied in tissue augmentation [5], tissue engineering [6], treatment of osteoarthritis [7-9] and drug delivery [10, 11]. HA is extremely hydrophilic, biocompatible and rapidly degradable *in vivo*. Hence, its rheological and physical properties need to be improved in order to develop biomaterials with enhanced performance relevant for clinical applications. Particular interest has been given to the introduction of hydrophobic moieties for the creation of self-assembly systems [12], viscosupplements [13] and drug delivery systems [14, 15].

The synthesis of amphiphilic HA derivatives with a wide range of degree of substitution (DS) of 1 to 43 % obtained by reacting the HA's hydroxyl groups with octenyl succinic anhydride has been reported by Eenschooten *et al.* [15]. The grafting of aryl and alkenyl succinic anhydrides, the smallest being phenyl succinic anhydride, to HA was also reported, resulting in a large family of amphiphilic hyaluronan derivatives [16].

In recent studies, the influence of long alkyl hydrophobic domains on the viscoelastic properties of the HA has been studied in detail [17, 18]. In this direction, Creuzet *et al.* have shown that the introduction of alkyl chains of 10 - 12 carbon atoms on an adipic dihydrazide HA derivative is able to promote the formation of a physical crosslinking thereby enhancing the HA rheological performance with a possible application in the field of viscosupplementation. Furthermore, the associative properties of these HA derivatives were found to be controlled by the complex balance between electrostatic repulsions and hydrophobic attractions associated with intra and interchain hydrogen bond interactions. Bergman *et al.* reported the grafting of a series of amines including propylamine to HA using triazine-based activation chemistry [19], however the rheological properties were not analysed.

In the present study, we investigated the effect of grafting the HA with two short alkyl amines, propylamine and butylamine, on HA rheological properties. Inspired by the potential of triazine-chemistry for HA conjugation, we used a new-generation triazine derivative, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM), that promotes the activation of the –COOH and the subsequent grafting of an amino group. DMTMM was initially developed for peptide synthesis and has already been successfully employed for the grafting of different molecules to HA showing higher efficiency and better control compared to the carbodiimide-mediated reaction [20, 21]. A parametric study was performed to assess the influence of the molar ratio of the reactants and temperature on the grafting efficacy and reaction kinetics. An array of propylamine and butylamine derivatives with wide DS ranges was prepared, and the change in their viscoelastic properties as function of the DS was analysed.

Experimental

Materials

Hyaluronic acid sodium salts from *Streptococcus equi* (HA) with weight-average molecular weight of 1506 kDa (HMW) and 280 kDa (LMW) were purchased from Contipro Biotech s.r.o. (Czech Republic). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) from TDI (Belgium). Propylamine, butylamine, ninhydrin and other reagents were purchased from Sigma–Aldrich (Switzerland). Chemicals were of analytical grade at least, and were used without further purification.

Synthesis of HA amphiphilic derivatives

HA-propylamine (HA-P) and HA-butylamine (HA-B) were synthesized via amidation of the HA carboxyl groups. The reaction was performed in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (50 mM, pH = 5.5). One gram of HA (2.5 mmol) LMW or HMW was dissolved until homogeneity in 100 ml of MES buffer. Subsequently, the propylamine or the butylamine was added to the solution, followed by DMTMM. To investigate changes in the grafting efficiency, different stoichiometric ratios of HA, DMTMM and amines were employed, together with different temperatures (Table 3.1). The reaction was allowed to proceed under mild stirring for up to 7 days. At selected time points of 1 h, 2 h, 3 h, 4 h, 8 h, 24 h, 48 h, 72 h, 96 h and 168 h, the products were isolated via precipitation by adding 8% v/v saturated sodium chloride and a 3 fold volume excess of ethanol 96% dropwise. The white powder obtained was washed with aqueous solutions containing increasing ethanol concentration, up to pure ethanol. Absence of chlorides was assessed via argentometric assay. The final product was dried under vacuum for 3 days and stored at room temperature. Furthermore, the same set of reactions was performed in water, without pH adjustment (Table 3.1).

Characterization of the HA derivatives

The HA-alkyl derivatives were characterized using ^1H nuclear magnetic resonance (Bruker Avance AV-500 18 MHz NMR spectrometer) to verify the molecular structure and to determine the molar degree of substitution (DS), defined as the number of carboxyl groups functionalized with amine per repeating unit of HA. Deuterium oxide was used as solvent and the chemical shift was defined according to the reference peak of the N-acetyl

proton on the glucosamine residue of HA (singlet at 2.01 ppm, previously calibrated with a standard). Mestrenova software was employed to analyze the spectra. The DS was obtained by integrating the peaks at 2.01 ppm (3 protons on the acetyl group of the HA) and 0.89 ppm (3 protons on the terminal methyl group of the amines).

Rheological measurements were performed with an Anton Paar MCR-302 rheometer equipped with a Peltier temperature control unit. A 25 mm diameter cone-plate geometry was used with a 0.049 mm gap, predetermined by the geometry. The dried products were hydrated with a PBS solution pH 7.4 at a 5% w/v concentration overnight at 4°C until completely hydrated. After a thermal treatment (1h and 30' at 96°C) to ensure a full solubilization of the derivatives in the aqueous solution, 200 µl of each sample were placed on the rheometer. Sample drying was avoided during the measurements by placing wet paper in the measurement chamber. For each sample, oscillatory (amplitude and frequency sweep) and rotational (flow curve) tests were performed. The viscoelastic linear range was determined applying a strain sweep at a frequency of 1 Hz. A strain of 1% was determined to be within the linear viscoelastic region for all the derivatives and therefore was selected for the following oscillatory measurements. The frequency-dependent viscoelastic behavior was measured between 0.01 and 100 Hz. Flow curves were acquired at shear rate between 0.1 and 100 s⁻¹. The controls were non-modified HA at both molecular weights (LMW and HMW), before and after the thermal treatment. For all the measurements, the temperature was set at 20°C.

Detection of free amines

The ninhydrin assay was used to investigate the presence of free amines in the final product after purification. A 1% w/v ninhydrin solution in ethanol was added dropwise to each sample. The absorbance of the blue-violet complex formed after the reaction of the ninhydrin with the free amines was read at 570 nm. Controls of unmodified HA and unmodified HA supplemented with 1% amines were added. The linearity range absorption-numbers of free amino groups was between 2.4 mM and 19 mM for both propylamine and butylamine.

Results and Discussion

Synthesis of the HA derivatives

The HA was grafted with propylamine and butylamine by the DMTMM triazine derivative in MES buffer or in water (Figure 3.1). The grafting on HA involves a single step amidation based on the activation of the carboxyl group on the D-glucuronic acid of HA by DMTMM. The reaction mechanism of the condensation of carboxylic acid and amines by DMTMM in tetrahydrofuran was presented by [22]. In the same year, the use of DMTMM for peptide synthesis was reported [23]. Stability of DMTMM in water and polar solvents was already described [21, 22], as well as the feasibility of the reaction both in alkaline and acidic conditions [24, 25]. Furthermore the absence of free DMTMM in the final product was previously demonstrated, confirming the effectiveness of the purification protocol [20]. The Table 3.1 shows the conditions used to synthesize the HA-P and HA-B derivatives.

Figure 3.1 - Modification of HA with propylamine and butylamine using the DMTMM and structure of the derivatives.

Table 3.1 - Experimental conditions employed for the preparation of HA-alkyl derivatives. On the left column the first letter of the Reaction Identifier indicates the solvent, MES (M) or water (W), followed by the temperature (RT: room temperature; 37 or 56 is the temperature in °C) and the HA:DMTMM:amine ratio; the number in brackets indicates the initial concentration of HA when different from 1% w/v. Use of HMW HA is designated by the letter H at the beginning of the abbreviation.

Reaction Identifier	Molar Ratio HA: DMTMM: amine	HA % w:v	Temperature	Solvent
MRT111	1:1:1	1	RT	MES Buffer
MRT111(2)	1:1:1	2	RT	MES Buffer
MRT110.5	1:1:0.5	1	RT	MES Buffer
M56111	1:1:1	1	56°C	MES Buffer
MRT112	1:1:2	1	RT	MES Buffer
MRT121	1:2:1	1	RT	MES Buffer
WRT111	1:1:1	1	RT	Water
W37111	1:1:1	1	37°C	Water
WRT112	1:1:2	1	RT	Water
WRT121	1:2:1	1	RT	Water
WRT110.5	1:1:0.5	1	RT	Water
HMRT111	1:1:1	(HMW) 1	RT	MES Buffer
HM56111	1:1:1	(HMW) 1	56°C	MES Buffer
HMRT112	1:1:2	(HMW) 1	RT	MES Buffer
HM56110.5	1:1:0.5	(HMW) 1	56°C	MES Buffer

Grafting efficiency and reaction kinetics

The ^1H NMR spectra of the derivatives showed the expected signals, i.e. the singlet at 2.01 ppm and the multiplet between 3.2 and 3.7 ppm for HA. Propylamine displays characteristic peaks of a multiplet at 1.53 ppm and a triplet at 0.89 ppm; butylamine displays three characteristic peaks at 1.49, 1.33 and 0.89 ppm (Figure 3.2).

The DS was determined from the ratio of the area under the terminal methyl peak at 0.89 ppm (a) to the N-acetyl peak area at 2.01 ppm (b) for both amines.

The DS obtained via ^1H NMR was validated verifying the absence of free amines via ninhydrin assay. The assay indicated the absence of free amines in the final HA-alkyl derivatives, even with the derivatives with the highest DS value of 50%.

Figure 3.2 - Representative ^1H NMR spectrum of a HA-P and HA-B derivatives, MES, RT, 1:1:0.5, time point 24 h. a = terminal methyl group of the propylamine (triplet at 0.89 ppm); a' = terminal methyl group on the butylamine (triplet at 0.89 ppm); b = N-acetyl peak on the HA (singlet at 2.01 ppm); c = non-anomeric protons bound to the HA disaccharide rings (multiplet between 3.2 and 3.7 ppm)

The DS after 24 h ranged from 3.7% to 21.3% for HA-P and from 1.3% to 25% for HA-B depending on temperature, HA:DMTMM:amine ratio, and the solvent (Figure 3.3a). The use of MES buffer resulted in a higher grafting efficacy compared to water for a same feed ratio and temperature. Furthermore the grafting yield over time was higher for reactions in MES (MRT111, solid shapes) compared to water (WRT111, empty shapes) for the reaction performed at RT with HA:DMTMM:amine ratio 1:1:1 (Figure 3.3b). After 24 h, the DS was doubled for both HA-P and HA-B in MES compared to water. The DS reached a near plateau after 3 days, with a slight increase until day 7 (Figure 3.3b). This finding was also confirmed for other feed ratios, both in MES and in

water. The higher DS values in MES buffer appear counterintuitive since amines at low pH are more protonated and less nucleophilic (pK_b value for the propylamine is 3.33 and 3.39 for the butylamine). However, the DMTMM is more reactive in presence of slightly acidic pH compared to the alkaline condition resulting from the amines in unbuffered water. This led to higher DS values in MES buffer than in water. Interestingly, in water, the propylamine was grafted with higher efficiency compared to the butylamine, whereas the opposite behavior was found in MES buffer for earlier time points up to 48 h (Figure 3.3a). This difference can be attributed to the fact that in non-buffered water at neutral pH (pH values around 11) the amines were non-protonated. Therefore, the higher solubility of the shorter amine led to a slightly higher substitution. By contrast, in MES buffer at moderately acidic pH (pH values around 6) the amines were equally soluble, indicating that the longer chain of the butylamine led to a higher nucleophilicity and a better coupling efficiency. However, this behavior in MES buffer was lost with the progress of the reaction leading to the same DS at later time points for both propylamine and butylamine (e.g. 18% DS at 72 h).

Figure 3.3 - Left panel a): DS at 24 h for different reaction conditions. Right panel b): DS versus reaction time for HA-P (triangles) and HA-B (squares), molar ratio 1:1:1, RT, performed in water (empty shapes) or in MES Buffer (solid shapes).

We further investigated the influence of amines and DMTMM equivalents on the grafting performance. In water, without buffering of the pH, the increase in the amines equivalents led to a decrease in the DS (empty shapes) for HA-P and HA-B (Figure 3.4a). In MES buffer, the increase in amine equivalents from 0.5 to 1 led to a 5% increase of the DS owing to the increased feeding ratio without pH alteration. However, this effect was lost when the reaction was performed with a 2 fold molar excess of amines (pH is around 7.5 after 24 h compared to the pH 6.5 of the MRT111). This confirms a higher efficacy of the DMTMM in a slightly acidic environment compared to neutral pH. The decrease of DS together with the increase of amine equivalents was also confirmed for the derivatives synthesized from HA HMW in MES (Figure S3.2). Likewise, the increase in the DMTMM equivalents goes together with an increase of DS both in water and in MES (Figure 3.4b). In particular, DS of HA-P ranges from 6% (reaction in water with a 1:1 DMTMM:HA molar ratio) to 16% (reaction in MES with a 2:1 DMTMM:HA molar ratio). Similarly, an increment in the DS was observed for HA-B, ranging from 4% to 18%. All the DS values reported in figure 3.4 refer to the time point at 24 h.

Furthermore, at higher temperature the conjugation proceeds faster and with higher conjugation yield (Figure S3.1). These findings are in agreement with previous studies showing a faster conjugation by increasing the reaction temperature [21]. Specifically, with higher temperature the maximum reaction yield for HA-P was reached already after 24 h (21% for the reaction in MES and 10% for the reaction in water) (Figure S3.1a). Contrarily, at RT, 4 days were needed to reach the maximum yield both in MES buffer (20.3%) and in water (9.3%). A comparable DS - temperature relation was found for the HA-B (Figure S3.1b).

Figure 3.4 - Influence of a) amine and b) DMTMM on the DS. Panel a): Triangles = HA-P; Squares = HA-B; empty shapes = water and solid shapes = MES. Panel b): black columns represent HA-P; white columns indicate HA-B. The columns represent the average of two replicas and the error bars the standard deviation.

The grafting efficiency was dramatically increased, and DS values up to 50% were achieved by adding one mole equivalent of DMTMM per mole of HA and either one mole equivalent of propylamine or butylamine per mole of HA, every 24 hours to the corresponding solution (Figure 3.5). This outcome highlights the versatility of DMTMM for the grafting of amines to HA within a wide range of DS. In comparison, for the reaction at 56 °C M56111 performed without any further addition of DMTMM and amines during the whole duration of the reaction, a stable DS of around 20% for 4 days was observed, suggesting that the DMTMM is a limiting reagent in our reaction likely due to its inactivation. Therefore, the higher temperature has a double effect on the kinetics: 1) shorter time to the maximum yield resulting in a faster reaction; and 2) earlier grafting efficacy plateau due to a quicker inactivation of the DMTMM.

Figure 3.5 - Reaction kinetics for HA-P (triangles) and HA-B (squares) synthesized in MES, ratio 1:1:1 at 56 °C in standard condition (solid shapes) and with the addition of one mole equivalents of both amine and DMTMM per mole of HA every 24 h (empty shapes).

Finally, the influence of the MW of the HA on the reaction yield was investigated. For equivalent reaction conditions, comparable DS were obtained for MW 280 kDa or 1506 kDa (Figure 3.3a). In addition, the same influence of the amines on the reaction kinetic was observed for the HA-HMW derivatives as for the HA-LMW: the DS value reached in presence of a double fold excess of amines was halved compared to the standard ratio 1:1:1 (Figure S3.2). Hence, although HA MW has an influence on the viscosity of the solution, the reaction course is not influenced.

Rheological characterization

The viscoelastic behaviour of the amphiphilic derivatives was investigated by rheology. After rehydration with PBS to a 5% w/v solution, the majority of the derivatives were not soluble, showing turbidity or aggregates in solution. To ensure homogeneity for the rheological measurements, all the derivatives were thermally treated. After this treatment all the derivatives turned soluble. The absence of major HA de-polymerization after this thermal treatment was confirmed by rheology. In particular the amplitude sweep for pristine HA both LMW and HMW showed no significant differences in the G' and G'' moduli acquired before and after the thermal treatment (Figure S3.3). Additionally, the ^1H NMR performed for the same sample before and after thermal treatment has not revealed major changes in the peaks pattern (data not shown).

HA-LMW derivatives

All the LMW derivatives presented a wide linear viscoelastic range up to 100% strain with loss modulus G'' over the storage modulus G' range (data not shown); the same viscous prevalence is displayed by the pristine 280 kDa HA (Figure S3.3).

Figure 3.6 - a) Plot of the G' value as a function of the DS in MES (molar ratio 1:1:0.5, RT) for HA LMW derivatives. Triangles indicate HA-P, squares HA-B and the circle represents the pristine HA. Error bars represent the standard deviation; **b)** Flow curve of HA-B derivatives (MES, ratio 1:1:0.5, RT). The dashed line represents the pristine HA LMW; the continuous lines indicate the HA-B derivatives at different DS: Δ 2%, $\circ$ 3.6%, $\diamond$ 8%, $\square$ 10.67%, and straight line 11%.

The storage modulus, G' is distinctly correlated to the DS values (Figure 3.6a). For each derivative, the 8 points in the graph correspond to the time points of the reaction from 1 h to 96 h, corresponding to increasing DS. Two different regions can be recognized: at low DS an increase in the DS led to an increase in the storage modulus until a maximum was reached: DS= 3.0% for HA-P and 3.7% for HA-B. Over this value G' diminished for increasing degree of substitution. Hence, G' and DS are inversely correlated, except for values of DS below 3% for HA-P and 3.7% for HA-B, where they are directly correlated. Around the maximum the derivatives showed significantly higher storage modulus (about 70 Pa) compared to pristine HA (G' around 7 Pa). A similar correlation between G' and DS was found for other reaction conditions (Figure S3.4).

The complex correlation between G' and DS, together with the solubility change after the thermal treatment, let presume a temperature-induced structural change within the copolymers. The activate process is likely to rearrange hydrophobic and hydrophilic domains. Deeper structural analyses are ongoing in order to understand the nature of this reorganization at molecular level.

The same dependent behavior of G' to the DS values was observed for the viscous behavior of the derivatives. The HA-B derivatives, similarly to pristine HA, showed a shear thinning behavior indicated by an inverse relationship between viscosity and shear rate in the flow curve (Figure 3.6b). However, unlike pristine HA (dashed line in Figure 3.6b), the HA-B derivatives showed an absence of a plateau in the flow curves, suggesting the presence of an entangled system with chain interaction. This means that at rest, the polymer strands of HA derivatives are interacting more strongly than within non-derivatized HA leading to a higher viscosity. During shearing, a decreasing flow resistance was observed due to disruption of these interactions, less entanglement and molecular alignment along the shear direction. These findings underline how the introduction of short alkyl moieties on a narrow fraction of carboxyl groups (3 - 4%) radically modifies the associative behavior of hyaluronan in water. Therefore, the grafting of small alkyl amines is a viable method for the tuning of HA rheological properties. A similar associative behavior was previously reported in presence of long alkyl chains either directly grafted to HA [7] or via pre-derivatization with spacer arm of adipic dihydrazide [26].

HA-HMW derivatives

The viscoelastic behaviour of the derivatives synthesized from HA HMW showed a strong correlation with the DS values (Figure 3.7). For pristine HA HMW, $G' = 1.39$ kPa is above $G'' = 685$ Pa (Figure S3.3). All HA-P and HA-B prepared from HA HMW displayed lower G' . The elastic prevalence ($G' > G''$) was preserved for derivatives with DS below 16% whereas, for DS above 16%, $G'' > G'$ and moduli were significantly reduced. Additionally, a linear relation between viscoelastic shear moduli and the DS was observed for both derivatives (Figure 3.7). This finding can be compared with the HA LMW derivatives where similarly, for DS above 4%, there was an inverse correlation between G' and DS values.

Figure 3.7 - Plot of G' and G'' as a function of the DS value in MES for a) HAHMW-P and b) HAHMW-B. Solid shapes and empty shapes indicate respectively the storage modulus, G' and the loss modulus, G'' . The points at DS% = 0 represent the G' and G'' of the pure HA.

Conclusion

A series of short-alkyl hyaluronic acid derivatives was prepared via DMTMM chemistry. Specifically, propylamine and butylamine were grafted to the HA with DS values between 1% and 50%. This wide range of DS was achieved varying temperature, solvent, ratio HA:amine:DMTMM and HA initial concentration. Conjugation kinetics revealed that at higher temperatures DMTMM is deactivated faster; yet the efficiency remains superior owing to faster coupling kinetics. A complex correlation between DS and viscoelastic properties was observed, with maximum storage modulus and shear-thinning at DS 3.0% (HA-P) and 3.7% (HA-B); whereas at higher DS, these rheological characteristics decrease to values of pristine HA. This complex correlation, together with the solubility change after the thermal treatment, suggests an activated secondary structure rearrangement with re-organization of the hydrophobic and hydrophilic domains. Further investigations are ongoing in order to test this hypothesis. The DMTMM grafting strategy here introduced is a viable method for fine-tuning HA viscoelastic properties with minimal modification. Owing to their singular rheological profile HA-P and HA-B are valuable semi-synthetic carbohydrate polymers for preparing injectable biomaterials, and hydrogels suitable for additive manufacturing.

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Supplementary Data

Figure S3.1 - Influence of the temperature on the kinetic of the reaction for the HA-P derivatives (a) and HA-B derivatives (b).

Figure S3.2 - Reaction kinetics for HA-P (triangles) and HA-B (squares) synthesized in MES at RT with molar ratio HA : DMTMM : amine 1:1:1 (solid shapes) and 1:1:2 (empty shapes).

Figure S3.3 - Storage modulus G' and loss modulus G'' as a function of the strain at frequency 1 Hz for the HA-LMW and HA-HMW before and after the thermal treatment (t.t.).

Figure S3.4 - Plot of the G' value as a function of the DS in MES (molar ratio 1:1:1, RT) for HA LMW derivatives; Δ indicates HA-P, $\square$ HA-B and $\bullet$ the pristine HA.

Chapter 4

Calcium phosphate / thermoresponsive hyaluronan hydrogel composite delivering hydrophilic and hydrophobic drugs

Dalila Petta^a, Garland Fussell^b, Lisa Hughes^b, Douglas D. Buechter^b,
Christop M. Sprecher^a, Mauro Alini^a, David Eglin^a, Matteo D'Este^a

^a AO Research Institute Davos, Clavadelstrasse 8, 7270 Davos, Switzerland

^b DePuy Synthes Biomaterials, 1230 Wilson Drive, West Chester, PA 19380, USA

Abstract

Advanced synthetic biomaterials that are able to reduce or replace the need for autologous bone transplantation are still a major clinical need in orthopedics, dentistry and trauma. Key requirements for improved bone substitutes include optimal handling properties, ability to fill defects of irregular shape and capacity for delivering osteoinductive stimuli. In this study, we targeted these requirements by preparing a new composite of β -tricalcium phosphate and a thermoresponsive hyaluronan hydrogel. Dissolution properties of the composite as a function of the particle size and polymeric phase molecular weight and concentration were analyzed to identify the best compositions. Owing to its amphiphilic character, the composite was able to provide controlled release of both rhBMP-2 and dexamethasone, selected as models for a biologic and a small hydrophobic molecule, respectively. The β -tricalcium phosphate thermoresponsive hyaluronan hydrogel composite developed in this work can be used for preparing synthetic bone substitutes in the form of injectable or moldable pastes and can be supplemented with small hydrophobic molecules or biologics for improved osteoinductivity.

Introduction

Biomaterials able to reduce or replace the need for autologous bone transplantation are a compelling need in maxillofacial, orthopedic and reconstructive surgery [1]. In the context of an increasingly aging global population that is keen on maintaining an active lifestyle and expecting improved life-quality this need is particularly urgent. Bone grafting is a very common practice, with an estimate of 2.2 million procedures annually in 2005 [2]. Examples of grafting procedures include spinal fusions, limb length restoration, bone reconstruction after tumor resection and maxillary sinus augmentation [3]. In all of these and in similar cases, bone autografts are generally recognized as more effective than off-the-shelf alternatives, but the autograft harvesting procedure is associated with considerable risks and donor site pain and morbidity, thereby lowering substantially the benefit/risk ratio. Therefore calcium phosphate (CaP)-based synthetic bone graft substitutes are a valid alternative to autografts and have been clinically used for this purpose for many decades [4-6]. Despite the capacity of supporting bone ingrowth from viable bone at the grafting site (osteoconductivity), they generally do not induce

bone growth *per se* (osteoinductivity). CaP-based synthetic bone graft substitutes are specifically produced with interconnected porosity which facilitates body fluids perfusion, cell invasion and blood vessel ingrowth. Additionally, the surface chemistry of CaP is characterized by the presence of charges from calcium and phosphate ions. This feature is exploited in CaP-based drug delivery systems, particularly for genetic material [7], growth factors [8] and antibiotics [9]. For hydrophobic drug species, the ionic nature of the underlying interactions is a limitation to the versatility of CaP materials as drug delivery systems. Therefore, the combination of CaP with matrices improving their versatility as drug delivery systems is highly needed.

CaPs are generally supplied as granules of varying sizes. As with most ceramic materials, they are inherently brittle, and their mechanical strength is additionally decreased by the porosity. These are important limitations. In fact, a fundamental desirable characteristic of synthetic bone substitutes is being easily shaped to adapt to an irregular bone defect [10]. Ideally, the construct should therefore be sufficiently soft and plastic. At the same time, the implant should not be dislocated after adjacent tissue compression or body fluids washout, and therefore it should maintain its cohesion after implantation. This is often achieved by combining CaP particles and a polymeric matrix or a hydrogel [11-17].

Hydrogels are hydrated molecular networks well-known for their capability of acting as drug reservoirs and facilitating controlled drug release [18, 19]. Hydrogels act as matrices, physically slowing down drug diffusion. Moreover, depending on their composition, they can establish specific chemical interactions with the drug and thereby modulate the drug release kinetics. Chemical affinity is sometimes a limitation, as non-water-soluble drugs can be difficult to incorporate into common hydrogels, which have inherently high water content. In this case, liposomes or micro/nano- particles composites are generally employed [20-22].

In order to target the needs of osteoconductivity, versatility, improved handling and cohesion properties, and potential drug delivery we prepared a new combination of β -tricalcium phosphate (TCP) granules with a thermoresponsive hyaluronan (HA) matrix. In 2012, Tian *et al.* reported the use of a thermoresponsive chitosan matrix for demineralized bone matrix delivery [23]. In our study, HA was selected as the main component because it is fully biocompatible, non-immunogenic and readily available in medical grade. HA is a natural component of the extracellular matrix playing a key role in tissue repair [24, 25]. Recently, HA was demonstrated to improve the osteoconductive

properties of CaP [26]. In this study, HA was modified with pendant moieties of thermoresponsive poly(*N*-isopropylacrylamide) (pNIPAM) to obtain a co-polymer (HpN) that undergoes a temperature-induced transition to a gel state. Importantly, the transition takes place between room and body temperature without covalent crosslinking and therefore in a bio-orthogonal manner, i.e. without interfering with physiological biochemical processes.

In this study, we prepared an array of TCP/HpN composites of varying TCP particle size, TCP concentration, polymer phase concentration and molecular weight of HA within HpN and assessed their properties as potential synthetic bone grafting materials. Handling properties were determined through rheological and dissolution profiles and composites were characterized via scanning electron microscopy imaging (SEM) and microCT. Selected composites were tested for their ability to act as drug delivery vehicles. Release rates from composites of recombinant human bone morphogenetic protein-2 (rhBMP-2) and dexamethasone (DEX), selected as models of a hydrophilic biological drug and a small hydrophobic drug, respectively, were determined. Finally, cytotoxicity against telomerase-immortalized human foreskin fibroblasts was evaluated.

Materials and methods

Materials

HA sodium salts from *Streptococcus equi* with weight-average molecular weight of 1506 kDa (HMW) and 280 kDa (LMW) were purchased from Contipro Biotech s.r.o. (Czech Republic). Amino-terminated poly(*N*-isopropylacrylamide) (pNIPAM) of number-average molecular weight of 34 kDa was purchased from Polymer Source, Inc. (Canada). ChronOS™ Beta-TCP Granules with different particle sizes were supplied by DePuy Synthes USA Products LLC, rhBMP-2 (from InductOS, Medtronic BioPharma) was purchased from Alloga, Burgdorf (Switzerland); DEX was purchased from TCI Europe; rhBMP-2 enzyme-linked immunosorbent assay (ELISA) kit Duo Set from R&D Systems and CellTiter-Blue® Cell Viability Assay from Promega. All other reagents were purchased from Sigma-Aldrich (Switzerland). Chemicals were of analytical grade at least, and were used without further purification.

HpN synthesis and characterization

HpN was prepared by conjugating HMW or LMW HA with amino-terminated pNIPAM via carbonyl diimidazole-mediated amide formation according to a previously described method [27] to give HHpN and LHpN, respectively.

The reaction was carried out in dimethyl sulfoxide for 2 days at room temperature and the final product was purified via dialysis against demineralized water for 5 days. HpN was freeze-dried, desiccated under vacuum at 42 °C and stored at room temperature.

The molar degree of substitution (DS_{mol}), defined as the number of carboxy groups functionalized with pNIPAM per repeating unit of HA, was determined by ¹H nuclear magnetic resonance (NMR) comparing the integrals of the peaks of the non-anomeric protons of HA between 3.00-3.77 ppm and the methyl group of the pNIPAM at 1.14 ppm. Spectra were acquired on a Bruker Avance AV-500 MHz NMR spectrometer. Deuterium oxide was used as a solvent and the spectra were processed with Mestrenova 9.0 software. Rheological measurements were performed with an Anton Paar MCR-302 rheometer equipped with a Peltier temperature control device. A 25 mm diameter cone-plate geometry was used with a 0.049 mm gap, predetermined by the geometry. The freeze-dried HpN was rehydrated with a PBS solution pH 7.4 at the desired concentration (20% w/w or 10% w/w) until homogeneity. Oscillatory tests (amplitude, frequency and temperature sweep) were performed for each HpN sample. The linear viscoelastic region was determined using a strain sweep at a frequency of 1 Hz. A strain of 0.5% was determined to be within the linear viscoelastic region for all the HpN batches and was selected for all rheological measurements. Temperature sweep was performed between 20 and 40 °C with a ramp of 1 °C/min at constant amplitude (0.5%) and frequency (1 Hz). Low viscosity silicon oil was used at the meniscus interface in order to avoid sample drying.

TCP/HpN composite preparation

Composites were prepared with varying HpN polymer solution concentration, molecular weight of HA within HpN, and CaP particle content and particle size. The range of variation of each parameter is reported in Table 4.1. The notation used throughout this manuscript to identify a composition is as follows: the first character is either H or L, identifying high or low molecular weight of HA; the first number identifies the HpN

concentration in % w/v; the second number expresses the granules content in % w/v; finally, b1, b2, b3 or b4 identifies the particle size according to Table 1. For example, the composition H20-75b4 is prepared from a 20% w/v HMW HpN solution and 75% w/v of TCP granules with a 0.7-0.14 mm size. As control, composites using pure HMW HA as polymeric phase were prepared.

To prepare the composites, lyophilized HpN and TCP particles were weighed, PBS solution was added and all the components were mixed with a spatula for few minutes until a homogenous paste was obtained.

Table 4.1 – Range of variation of concentration and characteristic of the components within the composites

HA M _w	HpN solution	TCP content	TCP granules size range and notation	
HMW	12 or 20% w/v	40 to 80% w/v	below 0.25 mm	b1
1.5 MDa			0.25-0.5 mm	b2
LMW			0.5-0.7 mm	b3
0.28 MDa			0.7-1.4 mm	b4

SEM Imaging and microCT

A 100 mg bead of H20-40b2 composite was prepared, frozen in liquid nitrogen and lyophilized. The dehydrated bead was fractured, coated with 10 nm of carbon and imaged with a Hitachi S-4700 II FESEM (Hitachi High Technologies, Germany) at 5 kV accelerating voltage. Images were taken in secondary electron mode. Additionally, the elemental analysis of the material at the interface TCP/HpN was evaluated by energy dispersive x-ray (EDX, INCA V5.2, Oxford Instruments, UK) with the accelerating voltage at 10 kV.

Three H20-40b1 composite beads were scanned using a μ CT40 (Scanco Medical, Switzerland) with a resolution (voxel size) of 12 μ m (70 kVp, 114 μ A, 150 ms integration time). A 3D segmentation followed by a morphometric analysis was performed for the

whole bead. Three spatial regions of the bead (Upper, Middle and Lower) consisting of 40-slices each were identified and quantitative information about the granules particle size and the distance between them were obtained.

Dissolution, and injectability tests

Dissolution test

The dissolution test consists of evaluating the shape maintenance after immersion into a PBS solution at 37 °C. The composite was formed into a bead and placed into a 20 ml vial filled with PBS solution at 37 °C. Composites with TCP particles of size below 0.5mm were loaded into a 1 ml syringe and extruded into a 20 ml vial filled with PBS solution at 37 °C. Pictures were taken immediately after extrusion and every fifth day for 25 days.

Injectability test

Injectability of selected composites was quantitatively measured with an Instron 4302 electromechanical testing machine in compression configuration. A volume of 0.4 ml of composite was placed in a 1 ml syringe with a round tip opening of 2.1 mm inner diameter. Syringes were mounted vertically in a custom-made stand. A load cell of 50 N was used. The compressive curve was registered at a crosshead speed of 120 mm/min. Results are reported as the average of three replicas.

rhBMP-2 and DEX release

Freeze-dried HHpN and b1 TCP granules were mixed together; a 0.1% bovine serum albumin (BSA) solution containing the drug was added and the components mixed with a spatula for a few minutes. The formulation was shaped as a bead with a final weight of 100 mg. Beads containing rhBMP-2 (5 µg/bead) or DEX (100 µg/bead) were prepared. Control groups were: HHpN bead only and TCP granules (b1) only. All control groups were loaded with the same amount of rhBMP-2 or DEX as the test samples. Samples were prepared in triplicate. Additional groups with a solution of pure rhBMP-2 or DEX were also included.

In vitro rhBMP-2 release

rhBMP-2 was supplied as a lyophilized powder in a formulation buffer containing 2.5% glycine, 0.5% sucrose, 0.01% Polysorbate 80, 5 mM sodium chloride and 5 mM L-glutamic acid. The protein was reconstituted to a final concentration of 1.5 mg/ml in a 0.1% BSA solution in PBS and then with the formulation buffer supplemented by 0.1% BSA, to a 500 µg/ml final concentration. For the release study, 10 µl of this solution were added to the polymeric phase during the preparation of the composite bead in order to load each sample with 5 µg of rhBMP-2. A release experiment was performed in the same conditions except for loading the growth factor in the inorganic phase. The release of rhBMP-2 from the composite was determined by placing each composite bead in 1 ml of release buffer (PBS + 0.1% BSA + 0.05% sodium azide) in low protein-binding Eppendorf tubes at 37 °C under mild agitation (10 rpm). At different time points (1h, 3h, 8h, 1d, 2d, 3d, 5d, 8d and 13d) 100 µl of release buffer was withdrawn and replaced with the same amount of fresh buffer. Samples were stored at -20 °C until ELISA analysis. The ELISA assay was run after appropriate dilution following the protocol provided by the supplier and the final absorbance was measured at 450 nm and corrected with the reading at 540 nm for optical compensation. The concentration of rhBMP-2 released from the different samples was normalized to the actual rhBMP-2 detected in the control group (5 µg of rhBMP-2 in release buffer). The tested items were prepared in triplicate and each replica was run in duplicate for the ELISA assay. Final concentrations were calculated according to the dilution at every time point.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to detect the rhBMP-2 that was not released from the TCP granules. Samples analyzed were:

- (a) 5 µg/ml rhBMP-2 as control, reconstituted with or without 0.1% BSA and
- (b) TCP granules loaded with 5 µg of rhBMP-2 after 13 days of incubation in release media.

Samples (a) and (b) were tested under reducing (β-mercaptoethanol) and non-reducing conditions by incubating for 5 min at 95°C in Laemmli buffer, consisting of 62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue. TCP granules from (b) were removed from the release media prior to addition to the Laemmli buffer. Samples

were loaded on a 15% gel and the electrophoresis was run at 100 V for 20 min followed by 80 V for additional 1h 30 min. The gel was stained with a Coomassie Blue G-250 for 20 min (Coomassie blue 0.3% + acetic acid 10% + methanol 25%) and destained in methanol/acetic acid solution (25% methanol, 10% acetic acid). Destained gels were visualized using the ChemiGenius Bioimaging System (Syngene).

In vitro DEX release

DEX was freshly reconstituted with ethanol to a concentration of 5 mg/ml. 20 μ l of DEX solution were loaded on TCP b1 granules. The solvent was allowed to evaporate at room temperature and HpN+PBS were added to obtain 100 μ g of DEX per bead. For the release study, each composite bead was incubated at 37 °C under mild agitation in 1.5 ml of release buffer. The release was carried out for 13 days and for each time point 300 μ l of release buffer was removed and replaced with fresh buffer. Collected samples were stored at 4 °C until analysis. DEX was quantified via HPLC on a Kinetex 5 μ m C18 100A column with triamcinolone acetonide as internal standard [28]. Mobile phase was acetonitrile/water 28:72, pH 2.3, at a flow rate of 1.2 ml/min and the detector wavelength was 241 nm. In a set of 6 replicates of the same sample a standard deviation of 1.6% was found (data not shown). The linearity of the DEX was between 0.35 μ g/ml and 12.5 μ g/ml. Final concentrations were calculated according to the dilution at every time point. Furthermore, the specimens were allowed to release for up to 3 months, demonstrating no further release (data not shown).

Reconstitution time of dry formulations

Reconstitution of the formulation H20-40b1 was performed using two different protocols, identified as A and B. In protocol A (supplementary movie A) the ceramic and the polymeric phase of the composite were mixed and rehydrated with a 500 μ g/ml rhBMP-2 solution. In protocol B, the composite was pre-hydrated with a 500 μ g/ml rhBMP-2 solution and weighed. MilliQ water was added to result in a 3 fold weight increase. The bead was smashed with a spatula and freeze-dried. Finally, the freeze-dried composite formulation was reconstituted to its original volume (before swelling to the 3 fold weight increase) with milliQ water (supplementary movie B). For both protocol A and protocol B the reconstituted material had the following composition: 98 mg of TCP granules (b1),

50.4 mg of HpN, 35 μ l of rhBMP-2 solution (500 μ g/ml) and 217 μ l of PBS solution. For both protocols, reconstitution was performed in a plastic vessel by mixing manually with a spatula.

Cytotoxicity assay of the composite formulation

Cytotoxicity was evaluated by assessing the viability of telomerase-immortalized human foreskin (hTERT-BJ1) fibroblasts after contact with extracts of composite beads. A CellTiter-Blue Cell Viability Assay (Promega) was performed in conformity to the ISO STANDARD 10993-5. The tested substances were sterilized with cold ethylene oxide. All the samples were incubated for 24 h in 2.5 ml of extraction vehicle at 37 °C, i.e. culture medium supplemented with 5% fetal calf serum and either 0.5 g of the composite, 15 cm² of the positive control (latex) or 7.5 cm² of the negative control (High-density polyethylene). hTERT-BJ1 fibroblasts were seeded in 96-well plates at a density of 2000 cells per 100 μ l of culture medium (Dulbecco's Modified Eagle's Medium supplied with 5% FCS) in a 96-well plate and incubated for 24 h at 37 °C, 5% CO₂, 95% humidity. The medium was then removed from each well and 100 μ l of the different extraction media from the test items were added to each well in triplicate. On days 1 and 3, the extraction medium was removed and cells were incubated with a 10% CellTiter-Blue solution for 2.5 h. The fluorescence signal was read using a Victor³ Perkin Elmer plate reader. All fluorescence values were normalized to the fluorescence value of the cells seeded in presence of culture medium at days 1 and day 3.

Results

Characterization of the co-polymer

HpN co-polymers with different rheological properties were synthesized according to protocols already established [27]. DSmol of HpN was consistently 5% of the carboxy groups functionalized, corresponding to around 9 moles of pNIPAM per mole of HA repeating unit. The temperature dependence of the storage modulus of HpN solutions of high and low molecular weight at 10 and 20% w/v concentration are shown in Figure 4.1. LHpN at 10% w/v shows a sharp transition around 29 °C, with the storage modulus increasing over two orders of magnitude between room and physiological temperature. At 20% w/v concentration the LHpN storage modulus is 10-fold higher than LHpN at

10% w/v and shows a 5 fold increase as the temperature is increased from room to physiological temperature with a slight decrease in the transition temperature at the higher concentration. G' of HHpN is markedly higher than LHpN at room temperature at both 10 and 20% w/v, and the transition amplitude is smaller, especially for HHpN 10% w/v.

Figure 4.1 - Storage modulus G' against the temperature of 20% w/v (black line) and 10% w/v (red dashed line) HHpN and 20% w/v (green dotted line) and 10% w/v (blue dash-dot line) LHpN solutions.

An evaluation of the formulation stability of 20% w/v HHpN was carried out by measuring the rheological profile at different time points out to 11 weeks. Results in Figure S4.1 show how the temperature-induced sol-gel transition is maintained, as well as the viscoelastic shear moduli values.

SEM and microCT imaging of the composites

The panel a) of Figure 4.2 shows the cross section of a fractured bead of the composite H20-40b2. The external surface of the bead in the upper part of the image is visible as smooth layer, under which the dried hydrogel shows a porosity of characteristic size around 5 μm . A TCP granule embedded in the hydrogel matrix is clearly visible as denser and brighter area. Panels b) and c) of Figure 4.2 represent another area from the same sample. The gapless contact between the hydrogel and the ceramic surface is obvious,

with the hydrogel filling the macro- as well as the micro- porosity within the TCP granules (arrow in panel c, and supplementary Figure S4.2). EDX spectroscopy showed the presence of carbon, oxygen, phosphorus and calcium for the mineral part and carbon, oxygen, sodium and chloride for the hydrogel part (Figure 4.2 panel d).

Figure 4.2 - SEM images of a H20-40b2 bead cross-section. Panel a) shows the interface between the external surface and the bulk of the bead, where a TCP granule completely embedded in hydrogel is visible. Panel b) shows the bulk of another area within the same specimen. The white rectangular frame in panel b) is magnified in panel c), where the white arrow indicates hydrogel within a TCP pore. The white triangle on the TCP and circle on the hydrogel in panel b) represent the focal regions where EDX spectra in panel d) were acquired. All scale bars correspond to 50 μm .

The 3D segmentation analysis from micro CT imaging confirmed the uniform distribution of TCP granules within the HpN polymeric matrix, both on the surface and inside the bead, as shown in Figure 4.3 (panel a).

Figure 4.3 - μ CT scan of the H20-40b1 composite: a) 3D reconstruction of half bead section; b) size distribution of the TCP granules within the composite (the gradient colour bar indicates the thickness in mm); c) distribution of the particle size after a 2 points Fast Fourier Transform filter smoothing; d) spatial distribution of the TCP granules (the gradient colour bar indicates the distance between the granules in mm) the red dashed rectangular indicate the three analyzed regions (Upper = U, Middle = M and Lower = L). Scale bars = 1 mm

The morphometric analysis for the whole volume of the bead (Figure 4.3, panel b) displayed a Gaussian distribution of the granules size, expressed as particle thickness (Figure 4.3, panel c). Furthermore, the mean granules distance (panel d) in the three different regions U, M and L was respectively 0.225 mm, 0.209 mm and 0.197 mm (Standard deviation 6.63%).

Dissolution and injectability test

The results of the dissolution test of formulations prepared with varying content of hydrogel and inorganic phase are reported in Table 4.2.

Table 4.2. *Outcome of the dissolution test. The meaning of the abbreviations is explained in the paragraph ‘TCP/HpN composite preparation’.*

Abbreviation	Dissolution Test
HA HMW 20% + 60% b3	Not Passed
H20-40b1	Passed
H20-40b2	Passed
H20-40b3	Passed
H20-40b4	Passed
H20-60b3	Passed
H20-80b3	Passed
H20-80b4	Passed
L12-50b3	Not Passed
L12-60b3	Not Passed
L12-80b1	Not Passed
L12-75b2	Not Passed
L12-80b3	Partially Passed
L20-40b1	Partially Passed
L20-40b2	Partially Passed
L20-40b3	Partially Passed
L20-60b3	Partially Passed
L20-80b3	Partially Passed
L20-40b4	Passed
L20-60b4	Passed
L20-80b4	Passed

Putties prepared from HHpN at 20% w/v (H20-40b1, H20-40b2 etc) passed the dissolution test for each granule size and concentration of mineral phase tested. These composites displayed shape maintenance, no turbidity and no crumbling of the particles for 25 days, as shown in Figure 4.4b. These samples were stored for 8 months in the same conditions and showed no visible changes (data not shown). The putty prepared from pristine HMW HA at the same concentration (first row in Table 4.2) disintegrated completely after dissolution testing (Figure S4.3). Dissolution tests of LHpN putties gave results dependent on both polymer concentration and particle size. Formulations prepared from LHpN at 12% w/v showed a relatively low viscosity for paste preparation and gave negative outcome for all particles content, except for the dissolution test of the sample

L12-80b3, where the shape was maintained and only a few particles detached (Figure S4.4). Therefore, for this sample a partial pass was attributed. Increasing the LHpN concentration to 20%, w/v formulations showed a visible shape collapse in the dissolution test (Figure S4.5). Since the basic shape was maintained and very minor particle crumbling was observed this was considered a partial pass (Figure 4.4a). Putties prepared from b4 particles (size 0.7-1.4 mm) and LHpN 20% w/v, fully passed the test.

Figure 4.4 - Dissolution test for the composites a) L20-40b1 at time zero and 5 days and b) H20-40b1 at time 0 and 25 days.

Injectability

The extrusion of the composites with granules of size above 0.5 mm led to the creation of a composition gradient with the extruded part enriched in polymeric phase and smaller particles, and the retained material enriched in the TCP solid phase. This phenomenon is known as filter-pressing, phase migration or phase separation [29-31]. Compositions with particles below 0.5 mm were extruded without visually observable filter-pressing. Based on this outcome, only composites containing TCP granules of particle size below 0.5 mm were further developed as injectable formulations.

A quantitative injectability testing was performed in triplicate for the H20-40b2 formulation, prepared from the highest concentration of the HMW polymer with the biggest particle size suitable for injection. The force-displacement profiles were characterized by a rapid increase of the force at the beginning of the extrusion and a

transition to a smooth plateau phase (Figure S4.6). The extrusion force is in a very low range for the investigated formulation, with a plateau around 4 N.

rhBMP-2 release

In Figure 4.5 the release profile of rhBMP-2 is displayed. Pure HHpN hydrogel (blue dotted line) released most of the available rhBMP-2 in the first three days. TCP granules displayed a burst release achieving most of the amount obtainable over the 300hr period released within the first 6hr (black dashed line). For the H20-40b1 composite the release was more controlled, with lower amounts released for each time point (red solid line) compared to either TCP granules or HpN. The fraction of rhBMP-2 released over the study compared with the nominal loaded amount was 45% for HHpN, 30% for TCP b-1 and 10% for the composite.

Figure 4.5 - Cumulative release of rhBMP-2 from the H20-40b1 composite (red solid line) from HHpN (blue dotted line) and from TCP granules (black dashed line). Values are normalized to the total loaded. The lines represent the average of three replicas and the bars the standard deviation.

rhBMP-2 remaining on the TCP particles after the release study was analyzed via SDS-PAGE. In Figure 4.6, lanes II to V are rhBMP-2 at 5 $\mu\text{g/ml}$, while lane VI represents the analysis of the TCP particles after the release study. rhBMP-2 under non-reducing conditions (lane II) gave a band at molecular weight of circa 30 kDa, as expected. In

reducing conditions (lane III), rhBMP-2 was split into two major subunits of around 15 kDa. In lane IV (non-reducing) and V (reducing) the same analysis was performed in presence of BSA, confirming the previous results and showing an intense band from BSA at circa 66 kDa, as expected. The analysis of the TCP particles under reducing conditions after extraction for 13 days (lane VI) clearly showed the presence of rhBMP-2 split into its monomeric units. BSA was not detectable for this sample.

Figure 4.6 - SDS-PAGE of rhBMP-2 reconstituted in presence (lane III and V) or absence (II and IV) of β -mercaptoethanol. Lane VI contains TCP granules loaded with 5 μ g of rhBMP-2; lane I corresponds to the markers solution (Precision Plus Protein Western C Standard).

Reconstitution of dry formulations

Reconstitution of the dry formulations was performed to obtain composites containing rhBMP-2, and is shown in the Supplementary Movies A and B for protocol A (reconstitution with rhBMP-2 solution) and B (reconstitution with water, rhBMP-2 included in the dry phase), respectively. Regardless of the protocols used, after around 1.5 minutes, the formulation reached a grade of homogeneity sufficient for further use and could be handled and shaped as a bead. A small amount of adhesion of the composite to the plastic vessel was noted.

Dexamethasone release

Figure 4.7 compares the release of DEX from the H20-40b1 composite and from its components TCP (b1) and HHpN. TCP released 38% of DEX after 3h and 53% after 6h. HHpN and H20-40b1 did not display such a substantial burst, with 44% and 29% release at 6h, respectively. The composite exhibited a lower burst release and a more gradual extended release compared to HHpN. At the end of the study 47% of the loaded DEX was released from H20-40b1. Comparing with rhBMP-2, for both HpN and the composite, the release of rhBMP-2 was slower compared to DEX release. At 24 h TCP particles released 90% of DEX. In the same conditions rhBMP-2 was released to an amount of 28% of the total load. For both rhBMP-2 and DEX, the amount released after 24 h followed the order TCP > HHpN > Composite.

Figure 4.7 - Curves of cumulative release of DEX from the H20-40b1 composite (dashed red line), from HHpN (dotted blue line) and b1-TCP (solid black line). The data represent the average of three replicas and the bars the standard deviation.

Cytotoxicity

At 24hr, the H20-40b2 composite extract showed a cytotoxicity of 25%; after 72hr the cytotoxicity was 19%. Extracts of TCP granules alone were not cytotoxic at any time point (Figure 4.8). According to the ISO 10993-5:2009, the threshold deemed cytotoxic is viability below 70%, or cytotoxicity above 30%. The polyethylene extract at 72 h gave

fluorescence signal higher than the control (hTERT-BJ1 + medium) pointing out a possible proliferation of the cells in these conditions.

Figure 4.8 - Values of fluorescence obtained from Alamar Blue Assay for pure extracts of the composite H2O-40b2, TCP granules, HA after incubation of hTERT-BJ1 fibroblasts with extraction medium at 24 hours (white bars) and 72 hours (dark bars). PC and PE represent, respectively, the positive control (latex) and the negative control (high-density polyethylene). The error bars represent the standard deviation in percentage calculated on a number of three repeats.

Discussion

Characterization and general properties

Chemical modification of HA with amphiphilic moieties of pNIPAM provides this semi-synthetic derivative with the singular feature of inducing a sharp transition to a gel state and an increase of viscoelastic shear moduli of more than 2 orders of magnitude within a narrow temperature interval. The reciprocal influence of the molecular weight of the polymeric components, relative degree of substitution and concentration were previously

analyzed [32]; the system was optimized for a sharp transition and negligible shrinking [33].

In this study, we prepared and characterized a wide spectrum of composites of varying molecular weight of HA within HpN; total concentration of polymer; total content of TCP particles; TCP particle size. Composites were characterized for their shape retention after incubation in buffer. This testing is designed to challenge the composite's capability of holding together in humid environment and thereby mimics the conditions after implantation. Pristine HA is known for its extremely high hydrophilicity. As a consequence, the composite prepared from pure HA (HA HMW 20% + 60% b3, control) was easily swollen in water and fell apart during dissolution testing. By contrast, composites prepared from 20% w/v HHpN were able to maintain their shape owing to the underlying molecular network and gel properties. The LHpN solution at 12% w/v concentration did not have adequate cohesiveness and at least a 20% w/v concentration of the polymer in the composite was required for displaying some degree of shape-retention. This was expected, since for the polymer to act as a binder for the particles it must be sufficiently viscous. For the same reason, HHpN gives better performance compared to the LHpN. Temperature sweep analysis shows that HHpN displays higher moduli at both room and body temperature. Specifically, the increased viscosity at RT gives a better initial cohesion to the composite and makes it more easily handled. Formulations prepared from 20% w/v LHpN had rheological properties barely sufficient to bind the particles and therefore the outcome of the dissolution test was particle size-dependent, with only the bigger particles giving satisfactory cohesion. MicroCT analysis is a powerful tool for evaluating particle size and distribution within the composites. Particle size analysis confirmed the expected dimension, revealing no particle coalescence. Particle distribution was relatively uniform throughout the bead with no evidence of significant sedimentation. SEM images show a gapless contact between the ceramic granules and the polymeric matrix, with the hydrogel penetrating the microporosity (Figure 4.2, panel c and Figure S4.2). The local composition assessed by EDX analysis confirmed the identification of the components. The carbon in the bioceramic part arises from the carbon coating, while chlorine results from the PBS as solvent for the HpN. The sodium derives from both the PBS and as counterion for the carboxy groups of HA.

Intuitively, improving the cohesion of a formulation works to the detriment of injectability. It is known that the force versus displacement curves characterize the extrusion process and depend on syringe size and type, plunger displacement speed, material and gauge of cannula as well as the rheological properties of the ceramic particles [11]. The extrusion test showed that the composites H20-40b1 and H20-40b2 can be extruded from an insulin syringe with a very little force and without phase separation, while formulations with granules above 0.5 mm give filter-pressing. Therefore, the composites with particle size below 0.5 mm are suitable for the preparation of both moldable putties and injectable formulations, while composites with particle size above 0.5 mm are suitable for the preparation of moldable putties. The form in which a composite biomaterial is provided to the surgeon is important for its clinical performance [34]. The HpN co-polymer maintains its temperature-induced gel-formation performance for at least 11 weeks upon storage in solution at 4 °C. This stability allows the preparation of the composites as off-the-shelf pre-hydrated formulations. As shown in the supplementary movies, re-hydration of the dry form immediately before use is also possible within a time span conveniently compatible with surgical procedures. Therefore, the composites presented in this study are suitable for the preparation of both pre-hydrated and to-be-reconstituted formulations.

The degradation profile of the composites here presented is governed by the degradation of its components. For TCP the resorption is mainly cell-mediated [35]. The polymeric carrier is subject to enzymatic degradation *in vitro* [33] and *in vivo* [36]. Therefore, the degree of chemical modification on HA in the thermoresponsive gel is such that the derivative is still recognized by hyaluronidase, which is indicative of preservation of HA biological activity. Unlike HA, pNIPAM is not biodegradable. However *in vivo* it is eliminated via urinary excretion [37]. This fate is similar to polyethylene glycol, which is widely used in biomaterials and pharmaceutical preparations for parenteral use [38].

rhBMP-2 and DEX release

Ideally, biomaterial-based delivery systems are intended to enhance the natural tissue response by supplying adequate biochemical cues. Bone healing entails a complex cascade of events, progressing under the regulation of different small and biological molecules. In this study, we selected rhBMP-2 and DEX as model molecules to deliver. rhBMP-2 is a water-soluble full-size protein of molecular weight 30 kDa and an

isoelectric point of approximately 9 and is well-known for its bone-inducing properties [39, 40]. rhBMP-2 delivered in absorbable collagen sponge is already approved for clinical use in spine fusions [41] and tibia fractures [42]. DEX (molecular weight = 392.46 Da) is a non-water-soluble synthetic steroidal anti-inflammatory drug that was selected as a model for small hydrophobic molecule. Although typically used in cell culture as component of osteogenic media, DEX is not clinically used for bone growth. The molecules selected have different physico-chemical properties, and were deemed suitable for assessing the versatility of a delivery system. Based on the injectability, the composite H20-40b1 was selected for performing rhBMP-2 and DEX release studies.

In order to accomplish its therapeutic action, rhBMP-2 needs to be released at the appropriate dose and time [43]. The potency of rhBMP-2 in inducing bone is well recognized, but after years of clinical use serious issues of efficacy and safety have been posed [44-46]. Most of these issues arise from a sub-optimal delivery system, which releases high doses of rhBMP-2 over a short period of time. The composite presented in this study was effective in slowing down the release of rhBMP-2, as shown in Figure 4.5, compared to HpN or TCP alone. The dose of rhBMP-2 released was previously demonstrated to be effective in inducing bone regeneration *in vivo* without ectopic bone formation or other side effects [47]. The comparison of rhBMP-2 and DEX release highlights that: (i) TCP releases almost immediately most of the 100 μ g of DEX loaded, while only 30% of the 5 μ g of rhBMP-2 loaded are released with a quasi-zero-order kinetics. (ii) HHpN and H20-40b1 show similar release kinetics for DEX, but a quite different profile for rhBMP-2, for whom HHpN reaches plateau after 3 days while the composite displays a more gradual release and (iii) the total and relative amount released over the study are higher for DEX. These differences emphasize how the release is molecule-dependent. The affinity of rhBMP-2 for CaP surfaces is known [48, 49]. rhBMP-2 was still detectable on the TCP particles after the release study through SDS-PAGE. Interestingly BSA, which was present in significant amount in the release medium, was not detected on the particles, indicating specificity of the TCP - proteins interaction. The high retention of rhBMP-2 observed for the HpN/TCP composite is not expected after implantation *in vivo*. *In vitro* release models do not mimic the tissue environment, body movements generating mechanical forces, blood and lymphatic circulation, the enzymatic degradation or the action of inflammatory cells and the resorption of TCP by osteoclasts. Therefore, *in vivo* the total amount of rhBMP-2 released

is expected to be closer to the loaded dose, as previously reported [20]. A limitation of this study is that the biological activity of BMP-2 after the release was not assessed. However, BMP-2 was identified via ELISA assay. If the BMP-2 structure was compromised during encapsulation and release, BMP-2 would not be able to present the epitope to the ELISA assay. Therefore, the positivity of the released BMP-2 to the ELISA assay is an indication of structure preservation. The optimal pharmacokinetic of rhBMP-2 can only be determined in an *in vivo* model, and its determination needs to be investigated in future studies [50].

In contrast to rhBMP-2, DEX shows low affinity to the mineral phase and its release from TCP is quantitative, while HpN retains 40% of DEX. The combination of TCP with HpN induces a more gradual release (Figure 4.7). In our model the release of DEX is at least in part due to dissolution, as its concentration is above the solubility threshold. Similarly to TCP, HA is hydrophilic and scarcely suitable to generate hydrophobic interactions able to modulate the release of hydrophobic species like DEX. However, in our research we used HA modified with moieties of pNIPAM. Therefore, the use of a carrier constituted by HA chemically modified with pNIPAM provides 2 fundamental benefits: 1. Providing the property of temperature-induced gelation, that gives good injectability but at the same time no dissolution in physiological conditions, as verified via dissolution test and 2. Introducing hydrophobic domains into the HA backbone; besides forming a molecular network at body temperature these hydrophobic domains can interact with hydrophobic species like DEX modulating its release. Understanding the drug molecule association to composite components, as well as the effect of polymer and inorganic ratios and properties on drug incorporation and release provides the option for tailored drug delivery based on clinical need.

Conclusions

In the present study we introduced a new synthetic bone substitute comprised of tricalcium phosphate and a thermoresponsive hyaluronan hydrogel. The co-polymer has a low degree of derivatization, and therefore it should be readily recognized as natural extracellular matrix component by the host tissue. Owing to the temperature-induced sol-gel transition of the soft polymeric phase, handling properties are superior compared to pristine hyaluronan composites in terms of cohesion and dissolution. The composite displays good cohesion in aqueous solution at physiological temperature and osmolarity,

indicating the capability of filling defects of irregular shape without washing-out. Depending on the particle size, putty or injectable formulations can be prepared. rhBMP-2 and dexamethasone were selected as models of hydrophilic full size protein and small hydrophobic molecule to be released from the composite, demonstrating the possibility of releasing a wide range of molecules in a controlled manner. The composite contains ionically-charged surfaces, hydrophilic and amphiphilic functional groups. The interaction of these microdomains with a drug will result in different release characteristics for different molecules. For rhBMP-2 the strong interaction with the bioceramic results in a slower release and at the same time a lower overall recovery *in vitro*, compared to dexamethasone. The capability of releasing drugs of different nature in a controlled manner is relevant towards the translation of synthetic bone graft substitutes in clinical use. Bone grafting is employed in a variety of clinical situations, including, for example, bone reconstruction after trauma, deformity or tumor resection, sinus augmentation after tooth extraction or spinal fusion in elderly or younger patients. A versatile off-the-shelf synthetic graft could be supplemented by the surgeon with different drugs, for example angiogenic stimuli, osteoinductive stimuli, anti-inflammatory, analgesic, antibiotic, anticancer drugs depending on the clinical necessity. The composite can be supplied as both a dry formulation to be reconstituted peri-operatively or as ready-to-use paste. Owing to its handling, cohesion, resistance to wash-out, drug-delivery properties and cytocompatibility, HpN/TCP composites are promising substitutes for autologous bone transplantation in orthopedics, dentistry and trauma.

Conflicts of interest

Garland Fussell, Lisa Hughes and Douglas D. Buechter are employees of DePuy Synthes or were employees of DePuy Synthes when the experimental work was designed and performed.

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Supplementary Information

Figure S4.1 - Temperature Sweep of a 20% w/v HHpN solution measured at different time points: freshly prepared solution (black line), $t = 1$ week (red line), $t = 2$ weeks (green line), $t = 3$ weeks (blue line) and $t = 11$ weeks (purple line). The slight increase of moduli over time is attributed to the solvent evaporation.

Figure S4.2 - SEM image of the composition H20-40b2 showing the presence of hydrogel within the micro-porosity of TCP. The scale bar represents 5 μm .

Figure S4.3 - Cohesion test followed by a dissolution test for the HA20-60b3 composite containing pure hyaluronan instead of HpN.

Figure S4.4 - Dissolution test for composite a) L12-50b3 and b) L12-80b3. For L12-50b3 the test is not passed, while for L12-80b3 it is partially passed.

L20-60b3

t=0

t=3h

Cohesion Test

Dissolution Test

H20-60b3

t=0

t=3h

Figure S4.5 - Cohesion test followed by a 24 hours dissolution test for the L20-60b3 and H20-60b3 putty composites.

Figure S4.6 - Extrusion force for H20-40b2. Average profile of three single measurements.

Supplementary Movies

MovieA:<https://www.dropbox.com/s/3pk8kc7rtgeclty/Reconstitution%20Time%203.avi?dl=0>

MovieB:<https://www.dropbox.com/s/ak7497ebvog7wrc/Reconstitution%20Time%204.avi?m>

Chapter 5

3D printing of a tyramine hyaluronan derivative with double gelation mechanism for independent tuning of shear thinning and post-printing curing

Dalila Petta^{1,2}, Dirk W. Grijpma², Mauro Alini¹, David Eglin¹, Matteo D'Este¹

¹AO Research Institute Davos, Davos Platz, Switzerland

²Department of Biomaterials Science and Technology, University of Twente, Enschede, The Netherlands

Abstract

Biofabrication via three-Dimensional Printing (3DP) is expanding our capabilities of producing tissue engineering constructs for regenerative medicine, personalized medicine, and engineered tissue models of disease and diagnostics. While a few biomaterials for extrusion-based printing have been introduced, combining into a single biomaterial all their requirements is still challenging. These inks need to flow for extrusion under low shear, yet have immediate shape retention after deposition, provide a biochemical environment similar to physiological extracellular matrix, and a curing mechanism avoiding cell damage.

This work introduces a simple and versatile hyaluronan-based material for extrusion-based printing featured by a single component with 2 distinct crosslinking mechanisms, allowing i) shear-thinning tuning independently of the post-printing curing; ii) no rheological additives or sacrificial components; iii) curing with visible light avoiding damage-inducing UV radiation; iv) possibility to post functionalize; v) preservation of hyaluronan structure owing to low modification degree.

The ink is based on a hydroxyphenol hyaluronan derivative, where the shear thinning properties are determined by the enzymatic crosslinking, while the final shape fixation is achieved with visible light in presence of Eosin Y as photosensitizer. The two crosslinking mechanisms are totally independent. A universal rheologically measurable parameter giving a quantitative measure of the "printability" was introduced and employed for identifying best printability range within the parameter space in a quantitative manner. 3DP constructs were post functionalized and cell-laden constructs produced.

Due to its simplicity and versatility, HA-Tyr can be used for producing a wide variety of 3D printing constructs for tissue engineering applications.

Introduction

The fabrication of constructs arranging biomaterials and cells according to predetermined 3 Dimensional (3D) digital models has rising impact in tissue engineering (TE), regenerative medicine, *in vitro* models for drug assessment and for understanding human diseases [1, 2]. Extrusion-based printing consists of depositing an ink, e.g. a polymeric solution, a soft hydrogel, a melted polymer or a polymer-ceramic composite melt or dispersion, through a needle onto a surface prior to stabilisation of the depot. Extrusion

flow of the ink, modulated by air pressure or mechanical systems, dictates the fabricated topographies, which in turn will affect seeded cells and tissue behaviour [3]. Natural polymers, such as hyaluronic acid (HA), gelatin, alginate, collagen and fibrin are suitable candidates for the development of Biomaterials for Extrusion-based Printing formulations intended for use in TE. This is due to their inherent biological properties as natural extracellular matrix (ECM) components and well documented biocompatibility [4, 5]. Alginate is widely used in 3D printing due to its easy extrudability and its fast gelation in presence of divalent cations such as Ca^{2+} [6, 7]. The fast crosslinking ability of the alginate was combined with nanofibrillated cellulose to create shear-thinning ink for the 3D bioprinting of human chondrocyte-laden constructs [8]. Collagen and fibrin are also relevant polymers for 3D printing, although the slower gelation and the poor mechanical properties of the collagen represent major challenges [9-12]. Nonetheless, collagen gels have been printed at high density and with a heated deposition method resulting in printed structures with an improved geometry accuracy [13]. A combination of collagen, gelatin and alginate has been successfully printed with high resolution, high cell viability and good mechanical properties to better mimic the tissue-specific ECM [14]. To further improve their otherwise poor amenability to be 3D printed, gelatin and HA chemical structures have been functionalized. Gelatin has been extensively used as ink as methacrylate derivative [15, 16]. Typically, a gelatin-methacrylate with 60% or higher degree of functionalisation is dispersed at concentrations above 5% w/v into a solution containing a photoinitiator. After extrusion of the viscous ink, the stability of the printed structure is achieved via exposure to UV light and photocrosslinking [17].

Likewise, HA is also being investigated for applications in 3D printing [18-22]. HA is a non-sulfated glycosaminoglycan and a chief component of the ECM, can be found in most mammals connective tissues and it can interact with several cell surface receptors such as CD44 [23]. Extruded HA solution flows after extrusion and shape retention needs to be achieved by implementing crosslinking strategies, such as enzymatic-crosslinking and photo-polymerization/crosslinking [24]. In addition, cells are usually poorly adherent on HA hydrogels, requiring incorporation of the tripeptide arginyl-glycyl-aspartic acid (RGD) [25, 26].

Few studies have reported the development of cross-linkable hyaluronan ink formulations in extrusion based printing, most commonly as methacrylated derivative in combination with a UV-triggered photoinitiator [21, 27, 28]. Further attempts include the printing of

pre-crosslinked polymer or use of viscosity enhancers [9, 29]. For example, Boere *et al.* have combined a poly(ethylene glycol) or hyaluronic acid (partially crosslinked before printing) with a thermoresponsive polymer obtaining a final reinforced construct [30]. Ouyang *et al.* used a guest-host hydrogel based on HA where two hydrogel-precursors (HA individually double grafted with adamantane+methacrylate and β -cyclodextrin+methacrylate) formed a supramolecular assembly upon mixing. The structural integrity was achieved via UV-triggered photopolymerization [19].

As an alternative cross-linkable mechanism for hyaluronan, hydroxyphenol chemistry is a versatile method for bioconjugation and hydrogel crosslinking [31, 32]; however, its potential for producing inks has been mostly unexplored. A dual gelation mechanism for a tyramine-modified hyaluronic acid (HA-Tyr) was recently introduced [33] coupling a standard enzymatic pre-crosslinking based on horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) to a secondary crosslinking triggered by visible green light through Eosin Y (EO) as photoinitiator. HRP/ H_2O_2 is a well-known biorthogonal and non-cytotoxic crosslinking mechanism employed for the gelation of various natural polymers [34]. EO, a standard dye in histology, has been already used for the photocrosslinking of several polymers [35-37]. Wang *et al.* achieved a visible light crosslinking using a combination of polyethylene glycol diacrylate and gelatin methacrylate together with 0.01 Mm EO as photoinitiator [37], validating the EO visible light photoinitiator in comparison to the standard Irgacure 2959 requiring UV radiation at 320-365 nm [38, 39]. In this work, we introduce an HA-Tyr biofunctional ink with the following attributes: 1. Starting from a single precursor without need of components premixing or the addition of stabilizers 2. Exploiting two different gelation mechanisms, specifically: 3. Limited and controlled enzymatic crosslinking to tune the extrusion properties, and 4. Light-induced crosslinking for final shape fixation; 5. Avoiding the use of UV radiation, and 6. Amenable to post-printing functionalization with cell-adhesion motifs (Figure 5.1).

Figure 5.1 - Schematic of the dual crosslinking mechanism of HA-Tyr ink for the 3D printing process: first, the HA-Tyr precursor is lightly crosslinked via enzymatic crosslinking mediated by HRP and H₂O₂. A soft extrudable ink is obtained and employed in the 3D printing process. While printing, the green light exposure triggers a second light crosslinking mechanism that leads to a stable printed construct. Post-printing functionalization of the scaffold is carried out by binding adhesive motives (RGD) to the HA-Tyr ink; the single blue circle represents the conjugated tyramine group, whereas the two linked blue circles represent the two possible dityramine bonds occurring at the C-C and C-O positions between the phenols after enzymatic- or light-crosslinking.

The viscoelastic and printability properties of a range of HA-Tyr formulations were investigated. Then, we optimized the printing parameters to obtain a 3D construct with high resolution and shape fidelity. Finally, the optimized 3D constructs were decorated with RGD motives and seeded with human mesenchymal stem cells.

Materials and Methods

Materials

Hyaluronic acid sodium salt from *Streptococcus equi* (HA) with a weight-average molecular weight of 280 kDa (LMW) was purchased from Contipro Biotech s.r.o. (Czech Republic), 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) from TDI (Belgium) and serum (Sera+) from PAN-Biotech (Germany).

Tyramine (Tyr) hydrochloride, Eosin Y (EO), horseradish peroxidase (HRP), hydrogen peroxide (H_2O_2) and other reagents were purchased from Sigma–Aldrich (Switzerland). Chemicals were of analytical grade, and were used without further purification.

HA-tyramine synthesis

HA-Tyramine (HA-Tyr) was synthesized via a one-step amidation of the HA carboxyl groups (figure S1) as previously described [40]. Briefly, HA (5 mmol carboxylic groups) was dissolved until homogeneity in 200 ml distilled water and the reaction temperature was set at 37°C. Subsequently, 5 mM tyramine and 5 mM DMTMM were added to the solution. The reaction was allowed to proceed under mild stirring for up to 24 h. The product was isolated via precipitation by adding 8% v/v saturated sodium chloride and a three-fold volume excess of ethanol 96% dropwise. The white powder obtained was washed with aqueous solutions containing increasing ethanol concentrations, up to 100% ethanol. The absence of chlorides was assessed via argentometric assay. The final product was dried under vacuum for three days. The degree of substitution (DS), defined as the fraction of carboxyl groups functionalized by Tyr, was determined by absorbance readings at 275 nm (Multiskan™ GO Microplate Spectrophotometer) via calibration curve with pure Tyr hydrochloride (1 mg/ml HA-Tyr was dissolved in milliQ water) [41]. Afterwards, the dried product was dissolved in distilled water at a final concentration of 1% w/v and the grafting was repeated to increase the DS.

Ink formulations and rheological characterization

The synthesized HA-Tyr was dissolved in PBS (10 mM, pH 7.4) containing HRP 0.1 u/ml at final concentrations ranging from 2.5 to 5% w/v, overnight at 4°C under mild rotation. This HRP concentration was selected by optimizing the gelation time. After complete dissolution, EO was reconstituted in DMSO and mixed with the HA-Tyr precursor to a final concentration of 0.02% w/v. The enzymatic crosslinking was initiated by adding concentrations of H_2O_2 from 0.1 up to 0.7 mM. This range of hydrogen peroxide concentrations was selected based on cytotoxicity data [42]. When required, light crosslinking was triggered by exposing (from 1 up to 10 min) the enzymatically crosslinked ink to green light ($\lambda = 505$ nm; light source intensity = 80 mW/cm²).

The viscoelastic properties of the various ink formulations were investigated using an Anton Paar MCR-302 rheometer equipped with a Peltier temperature control unit. For

each sample, oscillatory and rotational (flow curve) tests were performed. The gelation time, defined as the crossover point of the storage modulus G' over the loss modulus G'' , was determined following the G' and G'' immediately after adding the H_2O_2 to the ink precursor. After 30 min, needed to ensure the complete crosslinking of the formulation, the viscoelastic linear range was determined by applying a strain sweep range from 0.01 to 100% at a frequency of 1 Hz (amplitude sweep). The damping factor ($\tan \delta$) was calculated from the ratio between the loss modulus G'' and the storage modulus G' values at 1% strain. $\tan \delta$ values were related to the extrudability (manual extrusion through a 0.33 mm cylindrical needle) and to the printability of the formulations (shape retention of two filaments printed in two different layers on top of each other). The amplitude sweep was acquired for each formulation both before and after light crosslinking. Flow curves were acquired at shear rates between 0.1 and 100 s^{-1} in order to evaluate the shear-thinning behaviour. For all the measurements, the temperature was set at 20°C. In addition, the rheological characterization was performed on HA-Tyr inks containing only HRP/ H_2O_2 or EO and therefore crosslinked respectively only via enzymatic or light crosslinking. In this manuscript, we identify the non-crosslinked HA-Tyr as HA-Tyr NC, the enzymatically crosslinked HA-Tyr as HA-Tyr EC and the double crosslinked HA-Tyr as HA-Tyr DC.

3D printing of the ink

HA-Tyr 3.5% w/v was rehydrated in PBS containing 0.1 u/ml HRP and 0.02% EO. The enzymatic crosslinking was triggered by adding 0.17 mM H_2O_2 and the ink was quickly loaded into an UV-protected cartridge. After the enzymatic crosslinking was completed (ca. 15 min), the printing process was started using an extrusion-based 3D Discovery® system from RegenHU Ltd. First, 2 cm-linear filaments were printed by varying the printing pressure from 0.5 to 4 bar and the printing speed from 2 up to 6 mm/s and the diameter of the filaments was measured using an optical microscope (Zeiss, Axio Vert.A1) at a 5X magnification. The influence of the green light exposure time and the layer thickness on the shape fidelity of the 3D printed construct was investigated.

For further characterizations, 10 mm diameter 3D criss-cross constructs with 1.5 mm strut distance were printed with the following settings: extrusion pressure 4 bars; cylindrical needle of 0.25 mm inner diameter; printing speed 4 mm/s; light curing speed 8 mm/s; layer height 0.14 mm; and light exposure after each printed layer.

Characterization of the 3D printed construct***Swelling behaviour***

3D constructs of 10 mm diameter were printed and their water retention capacity was assessed while varying the light curing intensity obtained during printing at the different speeds. The light beam was set to follow the same writing pattern of the deposited material and the criss-cross constructs were obtained in triplicates varying the light illumination speeds from 4 up to 12 mm/s. A control group was fabricated without light exposure. The samples were immersed in 1 ml of PBS at 37°C and incubated for 30 min, 2, 4, 24 and 48 h. After each incubation period, the swollen constructs were weighed (W_{wet}) and lyophilized overnight to obtain the dry weighting (W_{dry}). The swelling ratio was calculated by applying the following equation:

$$Swelling\ ratio = \frac{W_{wet} - W_{dry}}{W_{dry}}$$

Construct porosity

The criss-cross 3D printed constructs were scanned before swelling in an air-tight environment using a micro-computed tomography (μ CT, μ CT40 Scanco Medical, Switzerland) with a voxel size of 12 μ m (70 kVp, 114 μ A, 150 ms integration time). A 3D segmentation followed by a morphometric analysis was performed. Quantitative information about the strut size and the interconnectivity of the pores were assessed. Additionally, the presence air pockets within the materials was investigated.

Cellularized 3D constructs***Cell culture***

Human mesenchymal stem cells (hMSC) were isolated from the bone marrow and expanded following an established protocol [43]. The cells were grown until passage 3 before seeding them on the 3D constructs. Culturing was performed in an α MEM medium in presence of 10% serum (Sera+, PAN-Biotech), 1% of Penicillin/Streptomycin and 0.1% fibroblast growth factor FGF. The medium was changed every other day. The culture environment was maintained at a 37 °C incubator with 5% humidified atmosphere of CO₂.

Cell seeding

HA-Tyr powder was sterilized by UV for 1 h prior to reconstitution in sterilized PBS. All HRP and H₂O₂ solutions were sterile-filtered before addition. 2 mm height criss-cross constructs of 10 mm diameter were printed from 3.5% w/v HA-Tyr, 0.1 u/ml HRP, 0.17 mM H₂O₂ and 0.02% EO and subsequently used in the cell seeding experiments. After printing, the constructs were washed in PBS three times and functionalized using 1 mM RGD motifs containing a Tyrosine terminal (RGDY). The functionalization was performed via HRP/H₂O₂ (HRP 0.5 u/ml and H₂O₂ 0.8 mM) allowing the conjugation of RGDY to the free tyramine on the HA-Tyr. Control constructs were printed with no RGD functionalization. All the constructs were incubated overnight in a 1.5×10^6 hMSCs cell suspension. During the following day, non-adherent cells were washed out with PBS and the scaffolds were kept in culture (α MEM medium with 10% serum, 1% of Penicillin/Streptomycin). At day 10, the scaffolds were incubated for 1 h with 10 μ M Calcein-AM and 1 μ M Ethidium homodimer at 37°C for live/dead test. Two different hMSCs donors were used.

Results

Ink development and selection

HA-Tyr was synthesized via DMTMM amidation (Figure S5.1). A DS value of 14.5% was measured by UV-vis spectroscopy and the functional grafting was proven by the formation of a gel upon HRP-mediated oxidation.

The gelation time of the enzyme-mediated crosslinking was measured for two HRP concentrations (0.1 and 0.5 u/ml) at constant HA-Tyr concentration of 2.5% w/v. It was assessed that the HA conjugate concentration does not affect significantly the gelation time (data not shown). At HRP 0.5 u/ml the gelation was rapid, occurring during sample preparation. HRP at 0.1 u/ml led to a gelation time of 5 min and was selected for the ease of handling (Figure 5.2a).

Table 5.1 - Correlation between damping factor ($\tan \delta$), extrudability and printability for HA-Tyr formulations at [HA-Tyr] equal to 2.5, 3.5 and 5% w/v; [H_2O_2] equal to 0.1, 0.17, 0.3, 0.7 mM; HRP equal to 0.1 u/ml and EO equal to 0.02% w/v with EC indicating enzymatic crosslinking only and DC indicating enzymatic + visible light dual crosslinking.

Crosslinking mechanism	HA-Tyr [% w/v]	H_2O_2 [mM]	$\tan \delta$	Extrudability	Printability
EC	2.5	0.17	0.540	Yes	Good
EC	2.5	0.30	0.079	Yes (granular texture)	Not printable
EC	2.5	0.70	0.014	No	Not printable
EC	3.5	0.10	2.200	Yes (liquid state)	Poor
EC	3.5	0.17	0.580	Yes	Good
EC	3.5	0.30	0.086	Yes (granular texture)	Not printable
EC	3.5	0.70	0.001	No	Not printable
DC	3.5	0.17	0.179	No	Not printable
EC	5	0.17	0.810	Yes	Good (too tacky)

To identify the formulations with the best printability, HA-Tyr 2.5, 3.5 and 5% w/v were enzymatically crosslinked varying the H_2O_2 concentrations from 0.1 to 0.7 mM (Table 5.1). Shear moduli and damping factor ($\tan \delta$) were measured and compared with the behaviour of the hydrogels upon extrusion through a 0.33 mm needle (extrudability) and formation of a continuous strut with good shape retention (printability). All the EC formulations with H_2O_2 concentration above 0.17 mM showed $\tan \delta < 1$ with a prevalent elastic behaviour. In particular, formulations from H_2O_2 0.17 mM with $\tan \delta = 0.54$ and 0.58 displayed a good extrusion, and afterwards a good printability and shape retention. The formulation with $\tan \delta$ equal to 0.81 indicated a tackier formulation, but still printable (the terminal-portions of the printed filaments were easily pulled towards the needle after deposition). The formulation with a $\tan \delta$ equal to 2.2 was too fluid to retain the shape after deposition. Finally, formulations with $\tan \delta < 0.2$ corresponded to brittle and non-printable inks.

In figure 5.2b, the amplitude sweep shows constant storage and loss moduli (G' over G'') within the investigated deformation range indicating elastic behaviour. With HA-Tyr concentration of 3.5% w/v, for H_2O_2 0.17 mM G' and $\tan \delta$ values were equal to 89 Pa and 0.52 respectively, whereas for 0.7 mM G' and $\tan \delta$ values were 1607 Pa and 0.0012 respectively.

Figure 5.2 – Plot of the elastic and viscous moduli (G' and G'') as a function of time and [HRP] (a); plot of amplitude sweeps at different $[H_2O_2]$ (b); flow curves of EC (enzymatically crosslinked) ink formulations at different $[HA-Tyr]$ (c) and plots of amplitude sweep for no crosslinking, EC (enzymatically crosslinking) and DC (double crosslinking) (d).

The shear-thinning was assessed by measurement of the flow curves of formulations containing fixed concentration of HRP and H_2O_2 (0.1 u/ml HRP and 0.17 mM H_2O_2) and a range of HA-Tyr concentrations. For all HA-Tyr concentrations tested, the viscosity was below 45 Pa·s in the printing region defined by the shear rates applied to the ink during printing, ranging between 10 and 25 s^{-1} (Figure 5.2c). The 3.5% w/v HA-Tyr EC (green circles in Figure 5.2c) displayed a high viscosity of 3.6 kPa·s at low shear rate of 0.01 s^{-1} . This indicates a dramatic improvement in the viscosity compared to the non-crosslinked polymer (8 Pa·s at the same share rate value). The shear-thinning behaviour of the EC ink is not sufficient for post-printing shape fidelity (Figure 5.2a). Therefore, HA-Tyr gels were further crosslinked by exposure to visible light ($\lambda = 505$ nm) for 2 min in presence of EO 0.02% w/v; Figure 5.2d reports the amplitude sweep for these NC, EC and DC inks. The NC ink shows prevalence of viscous behaviour, whereas EC ink

exhibits elastic prevalence with $G' = 100$ Pa, rising to 400 Pa upon final light crosslinking. Based on the above optimisation, the 3.5% w/v HA-Tyr with 0.17 mM H_2O_2 , 0.1 u/ml HRP and 0.02% EO was selected as ink for the fabrication of 3D constructs.

3D printing process

The hydrogel selected based on the previous analysis was employed for performing a thorough analysis of the influence of different printing parameters on the final construct for acellular inks. The influence of printing speed and pressure on the strut width was assessed on parallel filaments printed with identical exposure to visible light.

For a constant pressure of 1 bar and a cylindrical needle of 0.51 mm inner diameter, an increase in the printing speed from 2 mm/s to 6 mm/s led to a decrease of the filament width from 800 μ m to 200 μ m. A non-continuous filament was extruded from a 0.33 mm cylindrical needle applying a pressure of 1 bar. In the same conditions for a 0.4 mm inner diameter conical needle, the filament width decreased from 1.37 mm to 0.58 mm (Figure 5.3a). Therefore, at constant pressure we found an inverse correlation between the printing speed and the filament width.

Similarly, for each needle geometry the filament width was directly correlated to the printing pressure (Figure 5.3b). Specifically, setting the printing speed at 4 mm/s, for the three different needle geometries (0.41 mm conical needle ■, 0.51 mm cylindrical needle ▲ and 0.33 mm cylindrical needle ●), the filament was increasingly wider for an increase of the printing pressure from 1 to 3 bars. Therefore, the resolution of the printed filament could be modulated varying nozzle geometry, printing speed and pressure.

Figure 5.3 – Plots of the filament width as a function of the printing speed at constant pressure of 1 bar (a) and as a function of the pressure at constant printing speed of 4 mm/s (b) for different needle geometry. Inserts in (b) show two representative optical images of printed filaments, scale bars = 600 μ m.

For a constant inner needle diameter, the cylindrical needles allowed extrusion of thinner filaments than the conical ones. The best filament width was obtained printing 0.6 mm filaments diameter using a 0.25 mm cylindrical needle with a 3 bar printing pressure and 4 mm/s printing speed (Figure 5.3b).

These optimized parameters were applied for the printing of 3D constructs to determine the influence of the light curing process and the layer thickness on the structure of the final construct. Criss-cross 3D constructs with 10 mm diameter and a 1.5 mm height were printed and their final shape was analysed. The enzymatic crosslinking alone was not able to produce a stable construct preserving its initial printed shape over time (Figure 5.2a). Only with additional light crosslinking, shape stability was achieved, and the construct could be easily handled.

We observed that the scaffold did not retain its structure when the visible light was applied at the end of the printing process, whereas the vertical macroporosity, defined by the struts distance with the CAD design, was better preserved with visible light application after each layer (Figure 5.4b). The height of the constructs was determined from the Z stack acquired with a confocal microscope (Figure S5.2). The height loss calculated in relation to the expected height was between 34% and 42% for constructs built with light curing after 2 layers, whereas with light applied after each layer the height loss was 17%, independently from the final heights of the construct 520 μ m or 1040 μ m.

Figure 5.4 - Handling of the 3D printed construct printed without and with visible light exposure (a); representative optical images of the 3D printed constructs showing the shape fidelity as a function of the visible light crosslinking and the layer thickness (a), and swelling of the 3D printed constructs as a function of the visible light exposure modulated by the displacement of the light source (light curing speed).

The most suitable layer thickness was selected visualizing the position of the needle during the printing and setting it such that the extruded ink was not deposited too far from the previously printed layer and at the same time the needle was not dipping into the previously deposited strut. The final appearance of the scaffold (Figure 5.4b) helped to identify that a decrease in the layer thickness from 0.26 mm to 0.14 mm led to an improvement in the shape fidelity (improved struts distance with pores shape closer to the CAD design).

Finally, 10 mm diameter 3D constructs (strut distance 1.5 mm) with high shape fidelity were produced including all the optimized parameters: printing speed 4 mm/s, light curing speed 8 mm/s, needle with a cylindrical geometry and 0.25 mm inner diameter. A 2.8 mm

final height of the constructs was reached by printing 20 layers (layer thickness 0.14 mm) with visible light exposure after each layer.

3D Printed Construct Characterization

Light exposure time and swelling ratio

The influence of the light exposure on the shape retention and swelling was further assessed. Swelling equilibrium was reached after 48h for all the ink formulations (data not shown). The ink concentrations of HA-Tyr (3.5% w/v), HRP (0.1 u/ml), H₂O₂ (0.17 mM) and EO (0.02% w/v) were kept constant, whereas the light curing speed was varied from 4 mm/s to 12 mm/s. The swelling ratio of 3D constructs exposed to green light with 4 mm/s curing speed was 22.7%, lower than the ones fabricated with 12 mm/s light curing speed, 31.4%, underlining the importance of radiation dose and consequently crosslinking density on the swelling capacity. Constructs printed with no light exposure showed the highest swelling ratio, equal to 45%. (Figure 5.4b). For a lower concentration of EO photoinitiator equal to 0.01% w/v a similar trend was measured (data not shown). Below 0.01% w/v of EO, the swelling ratio was not different than without EO, indicating the inefficient photocrosslinking in these conditions.

Micro-computed tomography

μCT analysis of the structures printed with a 0.33 mm inner diameter needle indicated struts dimension wider than the theoretical expected value (0.33 mm) as confirmed by the overlap of the 3D model and the printed constructs (Figure 5.5a). The struts showed a homogenous size distribution with wider areas at the intersection points (Figure 5.5b and 5.5c). The most frequent strut widths were between 0.9 and 1 mm (intersection points) and between 0.4 and 0.6 mm (connective struts). The variation in the struts width was further investigated by picking consecutive areas in the y-direction of the constructs (Figure S5.3). The struts were significantly wider when closer to the intersection points. The vertical porosity was accurately preserved especially in the middle area of the scaffold. No transverse (horizontal) pores were visible in the scaffold, as the HA-Tyr was not able to support its own weight, leading to the fusion with the underlying struts (Figure 5.5d). Furthermore, the struts were homogenous throughout the all Z direction with a slight variation in width; a flatten part was displayed at the bottom of the construct and a rounded one at the top of the construct. Finally, the presence of air bubbles in the final

printed construct was investigated assessing the ink homogeneity showing rare air pockets (Figure 5.5e).

Figure 5.5 – Representative optical image of a 3D printed construct overlapped with the 3D CAD model (a); microCT 3D reconstruction of printed construct, the colour bar represents the thickness in mm (b); plot of the strut thickness distribution in the 3D reconstruction (c); Representative cross-section of the microCT 3D reconstruction showing layers and struts overlaying accuracy throughout the Z direction (scale bar = 1 mm) (d); and 3D reconstruction image showing use of the microCT analysis for assessment of hollow defects in constructs (e).

Cell seeding on the 3D scaffolds

3D constructs fabricated according to the above description were used as-produced or functionalized post-printing with RGDY adhesive motifs for hMSCs seeding. At day 1, hMSCs did not significantly adhere to non-functionalized constructs in comparison to the RGDY functionalized constructs (Figure 5.6). At day 10, significant hMSCs colonization of the RGDY functionalized construct was confirmed, while only scarce amount of hMSCs were observed on the non-functionalized construct. hMSCs were homogeneously distributed with an elongated shape over the RGDY functionalized construct, whereas

non-adhesive scaffolds were colonized by a limited number of cells, mostly rounded and located on the inner wall of the pores. Interestingly, hMSCs migration into the RGDY functionalized 3D construct matrix was observed as shown in the confocal microscopy 3D reconstruction.

Figure 5.6 – Representative confocal images of 3D printed constructs seeded with hMSCs at 1 and 10 days with or without RGD motives (L/D assay, scale bar = 500 μm) (a); and Representative Z stack image reconstruction of 3D printed constructs seeded with hMSCs at 10 days with RGD motives showing cells ingrowth into ink (40 x 50 μm focal plane, with red indicating object on the surface, to blue indicating object deep the printed construct and the ink).

Discussion

3D printing is a technology of choice for tackling numerous regenerative medicine challenges, and for producing constructs with controlled structure, composition and internal gradients. Still, availability of biomaterials for extrusion-based 3D printing constitutes a significant technological barrier, especially for the fabrication of biofunctional cell-laden constructs. These inks need to switch from flowable fluids before extrusion to objects with permanent shape immediately after; in addition, their final stiffness should be limited to preserve cell functionality. Additionally they should provide a proper biofunctional environment for encapsulated cells [44].

The first step of our work was a screening based on viscoelastic properties to identify an optimized HA-Tyr ink consisting in a soft gel that can be extruded, retain the shape until the light is shined and integrate with the layers printed after. As expected HRP was found

to affect the crosslinking kinetics with little or no influence on the crosslinking degree, which in turn is governed by the H_2O_2 concentration [40]. HRP concentration of 0.1 u/ml brought to a working time of 15 min (Figure S5.2a) for cartridge loading. EC HA-Tyr formulations with H_2O_2 concentrations above 0.17 mM showed the typical behaviour of a crosslinked system, having both moduli constant in the analysed strain range. Furthermore, by increasing the H_2O_2 concentration, both the G' and the distance between the viscoelastic moduli increased underlying an increase in crosslinking, as expected (Figure 5.2b).

Aiming to introduce a measurable descriptor of "printability", we investigated the viscoelastic properties in relation to the printing outcome. The damping factor carries information on the ratio between the viscous and the elastic portion of the viscoelastic deformation behaviour. Formulations with $\tan \delta = 0.5$ -0.6 displayed the best printability, confirming the intuition that good balance of viscous flow and elasticity is necessary for extrusion-based 3D printing. HA- Tyr formulation with H_2O_2 0.17 mM fell into this printability optimum. Higher H_2O_2 concentrations led to a decrease in the $\tan \delta$ values (< 0.5) and inks with granular texture not suitable for printing. Importantly, damping factor is an absolute magnitude, i.e. independent of the measuring system and therefore comparable across laboratories, and does not require a 3D apparatus to be measured. The shape retention of the printed filament was evaluated on the strut thickness, texture and amount of the material extruded. When a 5 bar printing pressure was not sufficient to extrude the material or the granular texture of the material was causing a low adhesiveness of two adjacent layers, the ink was classified as non-printable. As previously found, 0.17 mM H_2O_2 concentration is not cytotoxic [45]. Likewise, the 3.5% w/v HA-Tyr was selected as a best compromise between the viscoelastic properties during and after printing. EO concentration was kept constant at the cytocompatible concentration 0.02% w/v [33].

The most common cell-laden photocrosslinked bioinks are naturally-derived, such as the methacrylated HA (MA-HA) and methacrylate gelatin (GelMA) [46]. Recently, both GelMA and MA-HA were employed by Duan *et al.* to print photocrosslinkable (365 nm UV light exposure) heart valve conduits [47]. Kesti *et al.* combined MA-HA with a thermoresponsive hyaluronic acid derivative used as a temporary template removed after UV irradiation [21]. In all these photocrosslinkable systems, the most detrimental effect is the UV light irradiation, inducing cellular damage via direct interaction with cell

membranes, proteins and DNA or via indirect production of reactive oxygen species (ROS) [48]. UV is also known to have limited penetration depth, having a negative effect on the polymerization process [49]. Due to these side effects on cells, alternative light sources with emission in the UV-A or visible light have been lately employed with alternative photosensitive systems [36, 37, 50]. With the prospect of embedding cells in the 3D constructs, we selected a visible light as alternative to UV [51, 52]. In our system, visible light and EO trigger an additional crosslinking ensuring the stability of the structure after printing and defining the final stiffness and swelling properties of the 3D constructs. For fixed light intensity, time exposure is the parameter determining the crosslinking density: slower light curing speeds, corresponding to longer time exposure, led to increase in the number of dityramine bonds as confirmed by the swelling behaviour. With no light curing after the enzymatic crosslinking, the maximum water uptake was recorded. Likewise, the ink exposed for the longer time to the green light (light curing speed 4 mm/s) resulted in the minimum swelling capacity.

The results obtained from the μ CT were in agreement with previous studies where no horizontal pores interconnectivity was found for a 3D printable UV photocrosslinkable methacrylated chondroitin sulphated-based scaffolds combined with a thermosensitive triblock copolymer with a G' around 380 Pa at 40 °C [53]. Extrudable hydrogel-based inks with high water content typically display collapse of the single filament leading to a unidirectional porosity along the top view of the scaffold, and transversal porosity preservation remains an unmet challenge. The dumbbell shape at the intersection points between the struts is expected for a hydrogel ink, where the gravity is causing collapse and relaxation of the ink [54]. Therefore, the theoretical values of strut circularity are hardly reached with the printing of hydrogel. Additionally, μ CT gives useful information about the strut width distribution throughout the 3D printed construct, confirming a homogenous width of the struts along the Z direction.

Upon cell loading into the 3DP constructs we observed cell attachment only for RGDY- modified constructs. It is well recognized that HA is not promoting cell attachment [55, 56], therefore we exploited the possibility to decorate the surface of the constructs with RGDY motives. The modification was performed on the final printed constructs, indicating the availability of free tyramine groups even after the printing process and the light exposure due the high degree of substitution on HA and the controlled crosslinking mechanisms. Additionally, the presence of cells migrating into

the HA-Tyr matrix suggests the grafting of RGDY both on the surface and within the 3D construct. This could be explained by the diffusion of the RGDY within the hydrated construct prior to crosslinking. This finding is in agreement with the work of Lei *et al.* where a degradable hyaluronic acid hydrogel promotes cell migration in presence of RGD [57]. The quantitative functionalization of RGDY within the whole construct will have to be confirmed with an independent technique.

The system here presented has several advantages compared to conventional hydrogels for extrusion-based 3D printing. It is based on HA, key extracellular matrix component and essential regulator of connective tissues healing. HA-Tyr is a single derivative with low substitution and therefore high preservation of HA structure and properties [58], a drastic simplification compared to i) previous approaches with complex conjugation chemistry to graft biopolymers with multiple functions and high modification ii) compositions including addition of sacrificial components or iii) compositions including viscosity-enhancing agents. In our ink the viscoelastic properties can be tuned via enzymatic pre-crosslinking for optimizing the extrusion properties. A subsequent crosslinking mechanism based on visible light leads to the transition from the shear-thinning fluid into a stable structure. The HA-Tyr is self-sufficient for the whole printing process, eliminating the need of additives and subsequent wash out steps. Finally, the system offers the possibility to graft further functional groups to HA, such as RGD to promote cell adhesion.

Owing to the shown simplicity and versatility, HA-Tyr can be used as biomaterial for extrusion based 3D printing for producing a wide variety of constructs for regenerative and personalized medicine.

Conclusion

A biofunctional ink based on HA hydrogel was introduced. The ink is based on a simple and single grafting step on HA, leading to a prepolymer amenable to reticulation with two independent mechanisms. Enzymatic gelation was used to set the desired shear-thinning properties, and photocrosslinking for the final shape stabilization. The advantages of the system include the ease of preparation, the presence of a single component but two independent crosslinking mechanisms, the use of visible rather than UV light, and the possibility to post functionalize via hydroxyphenol chemistry. The challenges remaining open are the sagging of the struts upon printing, bringing to poor preservation of the

transversal porosity in the final construct and the printing of living cells, which is subject of ongoing research.

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Supplementary figures

Figure S5.1 - DMTMM conjugation scheme: Tyramine hydrochloride is grafted to the Hyaluronic acid sodium salt via amidation mediated by the triazine derivative DMTMM. The reaction takes place in water at 37°C for 24h reaching a degree of substitution around 6%.

Figure S5.2 - a) Design of the 3D scaffold (diameter = 10 mm, struts distance = 1.5 mm) with the BioCad software (RegenHU Ltd) and mosaic reconstruction of the 3D construct with light exposure applied after 2 printed layers or 1 layer. Images acquired with a confocal microscope; scale bars = 1 mm; b) Influence of the printing design (green light exposure) on the final 3D construct height

Figure S5.3 – Morphometric analysis of a 3x3 struts-area in the middle of the 3D construct (bottom right image). A cross-section of the struts in 3 different Y positions: 200 μm -far from the intersection points (upper left), 500 μm -far from the intersection points (upper right) and in the middle of the intersection points (bottom left). The colour bar represents the thickness in mm; scale bar = 1 mm.

Chapter 6

A tissue adhesive hyaluronan bioink that can be crosslinked enzymatically and by visible light

Dalila Petta^{1,2}, Angela Armiento¹, Dirk W. Grijpma², Mauro Alini¹, David Eglin¹, Matteo D'Este¹

¹AO Research Institute Davos, Davos Platz, Switzerland

²Department of Biomaterials Science and Technology, University of Twente, Enschede, The Netherlands

Abstract

Bioinks in extrusion-based three-dimensional (3D) bioprinting are mostly optimized for printing accuracy and maintenance of cell viability. However, the ability of the bioink to adhere to the surrounding native tissue is seldom reported. In this study, we describe the optimization of a cartilage-adhesive tyramine-modified hyaluronic acid as a bioink for 3D printing. The bioink exploits a dual crosslinking mechanism, where the enzymatic reaction forms a soft gel suitable for cell encapsulation and extrusion, while the visible light photo-crosslinking allows shape retention of the printed construct and adhesion to cartilage. High cell viability of 78% was achieved with good shape retention after 14 days in culture. A proof of concept of adhesive 3D constructs fabrication on cartilage tissue opens the way to *in-situ* biofabrication for cartilage tissue engineering approaches.

Introduction

Three dimensional (3D) bioprinting is an additive manufacturing technique that in the last years has found applications in tissue engineering, allowing the fabrication of complex 3D constructs that better mimic the native tissues [1, 2]. The success of 3D bioprinting is highly dependent on the characteristics of the bioink. Indeed, the successful combination of living or biological components and biomaterials, taking into account both the printing and the biological requirements, represents an important challenge. Bioinks not only have to be extrudable through a thin needle, yet be sufficiently elastic to retain a 3D structure, but they must recreate a cytocompatible and suitable environment for the cells and eventually integrate within the surrounding tissues [3].

All the above-mentioned features are present in hydrogels, making them good bioink candidates for the bioprinting process [4]. In particular, when targeting cartilage tissue regeneration, hyaluronan (HA)-based hydrogels provide a suitable microenvironment for differentiation of chondrocytes and mesenchymal stromal cells (MSCs) [5, 6]. An aspect only marginally considered in the current literature is the adhesion of bioinks and printed constructs to the host tissue, whereas this might be an essential aspect for the formation of the repair tissue [7].

Pristine HA in bioinks has been used in combination with other natural or synthetic polymers to guarantee optimal viscoelastic properties and stability after printing. Skardal *et al.* added HA to a polyethylene glycol (PEG)-based bioink to improve shear thinning and extrusion properties of the bioink [8]. A composite bioink made of a water-based

light-cured polyurethane and HA has been reported for cartilage tissue engineering [9]. A recent study by Stichler *et al.* showed the printing of thiol-functionalized HA / allyl-functional Poly(glycidol) hydrogels crosslinked under UV irradiation [10]. In this work, unmodified high molecular weight HA was supplemented to the ink formulation as a thickener to improve construct shape fidelity. In these approaches, HA is used as additive to improve the extrusion properties and leach out from the formed synthetic matrix. HA has rarely been used as a self-standing material in bioink formulations as it is challenging to ensure shape retention of the printed filament without the addition of a supplement that improves the own HA viscoelastic properties. For example, HA has been functionalised with low amount of tyramine and used as a bioink with the addition of nanofibrillated cellulose to enhance the rheological features of the ink [11]. Burdick *et al.* have developed a guest-host HA-based hydrogel based on non-covalent and reversible bonds between an adamantane-modified HA and a beta-cyclodextrin-modified HA [12, 13]. Another direction to obtain stable constructs is the development of methacrylated-modified HA derivatives as photo-crosslinkable bioinks based on UV-light exposure in combination with thermoresponsive hydrogels [14-16]. However, the low penetration depth of UV-light in polymer matrices can prevent in depth polymerization efficiency and the production of free radicals can impact cell viability [17, 18].

To overcome the limitations of using UV-light, new photo-crosslinking systems based on visible light have been developed, but none with a hyaluronic backbone [19, 20]. Lim *et al.* combined visible light and the photoinitiator ruthenium/sodium persulfate system to obtain 3D printed cell-laden gelatin-methacrylated (Gel-MA) constructs with high cell viability [21]. This system allowed to minimize the oxygen inhibition during the free radical photo-polymerization. However, the above-mentioned bioinks are based on free radical production and to date none have been printed directly to cartilage. Unlike (meth)acrylate chemistry, Eosin Y (EO) photosensitizer relies on singlet oxygen rather than radical production. EO has been successfully employed in preparing bulk scaffolds [22-24] and additive manufacturing techniques. For example, in the work of Wang *et al.* a mixture of Gel-MA and polyethylene glycol diacrylate has been crosslinked in the presence of 0.01 mM EO as photoinitiator and visible light stereolithography technique has been employed [25].

Here we describe the development and the optimization of an adhesive tyramine - modified hyaluronic acid (HA-Tyr) bioink based on a dual crosslinking system. Two

consecutive crosslinking mechanisms are involved in the polymerization of the HA-Tyr: an enzymatic crosslinking mediated by horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) followed by a visible light crosslinking triggered by EO. We investigated the possibility to load this ink with cells obtaining 3D cell-laden constructs (Figure 6.1). The viscoelastic properties of the enzymatically crosslinked HA-Tyr were easily tunable by varying the concentration of the two components HRP and H_2O_2 . In this way, a soft bioink could be obtained, loaded into a cartridge and extruded through a thin needle. Afterwards, by exposing the bioink to green light during the printing process, 3D constructs encapsulating viable cells were fabricated. The printability was proven directly on cartilage tissue with a successful adhesion of the printed bioink to the tissue.

Figure 6.1 - 3D bioprinting process: dual-gelation mechanism of a tyramine-modified hyaluronic acid bioink. First, cells are encapsulated within the HA-Tyr precursor containing horseradish peroxidase (HRP) and Eosin Y (EO). Second, the enzymatic crosslinking mediated by HRP and is triggered by the addition of hydrogen peroxide (H_2O_2). A soft bioink is obtained, loaded into a cartridge and extruded through a thin needle. Lastly, the light crosslinking is started by exposing the bioink to green light ($\lambda = 505\text{ nm}$) during the printing process in order to obtain a stiff stable hydrogel. Cell-laden

3D constructs with a criss-cross structure are fabricated. The 3D construct can be printed directly on a cartilage explant, showing adhesive properties.

Materials and Methods

Materials

Hyaluronic acid sodium salt from *Streptococcus equi* (HA) with an average molecular weight of 280 kDa was purchased from Contipro Biotech s.r.o. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) from TDI (Belgium). Dulbecco's Modified Eagle Medium, both 1g/L (DMEM LG) and 4.5 g/L glucose (DMEM HG), Minimum Essential Medium Eagle-Alpha Modification (Alpha MEM), fetal bovine serum (FBS), and penicillin (10×10^3 U/ml)-streptomycin (10×10^3 µg/ml) (Pen/Strep) were purchased from Gibco, Invitrogen. SeraPlus bovine serum was purchased from PAN-Biotech. CellTiter-Blue® Cell Viability Assay was purchased from Promega. Tyr, EO, HRP, H₂O₂, Alginate, Calcein-AM (Ca-AM), Ethidium Homodimer-1 (EthD-1), Trypan blue solution (0.4% v/v) and chondroitinase ABC from *Proteus vulgaris* (cABC) were purchased from Sigma–Aldrich.

Synthesis of HA-Tyr

Tyr was grafted to the carboxyl groups of the HA via DMTMM-mediated amidation as previously described [26]. HA (5 mM carboxylic groups) was dissolved in distilled water and kept overnight under stirring to ensure complete dissolution. A 1:1:1 stoichiometric ratio of HA: DMTMM: Tyr was selected. Therefore, 5 mM tyramine and 5 mM DMTMM were added to the solution with no pH adjustment and the temperature reaction was set at 37°C. Subsequently, the reaction was carried on under mild stirring for 24 hours. The hyaluronan derivative (HA-Tyr) was isolated via precipitation by adding 8% v/v saturated sodium chloride and a three-fold volume excess of ethanol 96% dropwise. The obtained product was first washed with aqueous solutions containing increasing ethanol concentrations (up to 100% ethanol) and then dried under vacuum. The absence of chlorides was assessed via argentometric assay. The amidation reaction was repeated to increase the degree of substitution (DS) to a final value of 15.5% as determined by absorbance reading at 275 nm (Multiskan™ GO Microplate Spectrophotometer). An HA-Tyr concentration of 2.5% w/v was used in all the investigated formulations.

Rheological characterization of bioinks

The viscoelastic properties were investigated using an Anton Paar MCR-302 rheometer equipped with a Peltier temperature control unit. A cone-plate geometry with a fixed gap of 0.049 mm was selected to perform oscillatory and rotational tests. Storage modulus (G') and loss modulus (G'') values were acquired over time after adding the H_2O_2 to the ink precursor (gelation time). After the viscoelastic moduli reached a plateau (15 min), the crosslinking was fully achieved and a strain sweep ranging between 0.01 and 100% at constant frequency of 1 Hz was applied (amplitude sweep). From the amplitude sweep measurements, the ratio between the G'' and G' at 1% strain was used to calculate the damping factor ($\tan \delta = G''/G'$). In addition, the shear-thinning of the bioinks was evaluated at shear rates between 0.1 and 100 s^{-1} (viscosity curves). For all the measurements, the temperature was set at 20°C. Samples were prepared by dissolving HA-Tyr in PBS (10 mM, pH 7.4) at concentrations based on the final volumes after the additions below for the desired final concentration. HRP was added to the HA-Tyr solution and kept overnight at 4°C under slow rotation to ensure complete dissolution. EO reconstituted in DMSO at 1% w/v was added to the precursor to a final concentration of 0.01% w/v. For cell-laden samples, cells (hMSCs, bovine chondrocytes or hTERT fibroblasts, prepared as described below, at passage 3 or 4) were suspended in PBS at the desired concentration (from 1×10^6 to 5×10^6 cells/ml). After dispersion of cells within the bioink solution, the enzymatic crosslinking was initiated by adding H_2O_2 at a concentration ranging from 0.13 to 0.69 mM. Additional crosslinking was triggered by exposing the enzymatically crosslinked bioinks to green light of $\lambda = 505$ nm and intensity of 80 mW/cm²).

Cell culture

Human mesenchymal stem cells (hMSCs) were isolated from bone marrow and expanded until passage 3 following an established protocol [27]. After encapsulation of hMSCs into the HA-Tyr precursor, the cell-laden constructs were cultured in α MEM supplemented with 10% v/v SeraPlus, 1% v/v of Pen/Strep and 5 ng/ml recombinant human fibroblast growth factor basic protein (FGF-b).

Bovine chondrocytes were isolated from fetlock joints of young calves of 3–4 months of age and expanded according to established protocol [28]. Chondrocytes were cultured in DMEM HG containing 10% v/v FBS and 1% v/v Pen/Strep and used for the experiments at passage 3 and 4.

hTERT-BJ1 (hTERT) fibroblasts were cultured in DMEM HG supplemented with 10% v/v FBS and 1% v/v Pen/Strep.

All the cell cultures were maintained at 37°C with humidified atmosphere of 5% CO₂ with media change every second day.

3D bioprinting of cell-laden constructs

The bioninks were prepared as described on section 2.3 and the bioprinting was carried out using an extrusion-based 3D Discovery® system from RegenHU Ltd. A circular criss-cross structure of 20 mm diameter and a 2 mm strut distance was realized in computer-aided design (CAD) as a digital model for the 3D printed constructs. The bioink undergoing enzymatic crosslinking was quickly loaded into an UV-protected cartridge and after ca. 15 min the printing process was started. horizontal and vertical struts were alternated with each layer. After extrusion, the deposited bioink was further crosslinked using green light, with an integrated LED source (505 nm, 440 mW, 34.4 cd). Illumination was performed after each printed layer (with a focused beam of similar size as the struts deposited, following the same pattern of the deposited filament. The layer thickness, the printing speed and the light curing speed were set as 0.14 mm, 4 mm/s and 8 mm/s, respectively, following previous optimization (data not shown). The effect of the printing pressure and the needle geometry on cell viability was investigated.

Cell viability

Live/Dead

Cell viability was assessed at early (24h) or late (14 days) time point both on bulk or 3D printed constructs using live/dead (L/D) assay where living cells are stained with Ca-AM and dead cells with EthD-1. At each time point, the scaffolds were removed from the culture medium and incubated in staining solution containing 10 µM Ca-AM and 1 µM EthD-1 prepared in serum-free DMEM LG for 90' at 37°C with humidified atmosphere of 5% CO₂. For experiments in 2D where the cytotoxicity of EO was assessed in hTERT fibroblasts, the incubation time was reduced to 1 h. After incubation, cells were imaged

with a confocal laser scanning microscope (LSM510, Zeiss). For each sample, single plane and Z stack (1300 μm) were acquired, while for the 3D printed constructs tile scans were generated to image larger sample area. Three different fields of view per sample were used to quantify cell viability by counting the red (dead) and green (viable) cells in Image J.

Cell Titer Blue[®]

The metabolic assay CellTiter-Blue[®] was performed for both cells seeded in 2D and cells encapsulated in 3D. The 3D bulk cell-laden scaffolds (4 mm diameter x 2 mm height) were prepared and cultured in DMEM LG supplemented with 10% v/v serum and 1% v/v Pen/Strep. 24h after cell culture, each scaffold was transferred into a new culture vessel, washed three times with PBS and incubated in a 20% v/v CellTiter-Blue[®] solution in DMEM LG. After 6h incubation at 37°C, fluorescence was measured at 540 nm and 595 nm (Victor 3, Perkin-Elmer). For the 3D scaffolds, the fluorescence values were normalized to the weight of the scaffold. Likewise, fibroblasts seeded in 2D (2000 cells/100 μl DMEM LG supplemented with 10% v/v FBS) were exposed, after 24h, to three different concentrations of EO or HRP/H₂O₂ and then incubated with the CellTiter-Blue[®] solution (2 h incubation time). At 24h and 72h after exposure to EO or HRP/H₂O₂, the medium was replaced by CellTiter-Blue[®] solution in DMEM LG and the fluorescence was measured. All groups were prepared in triplicates.

Trypan blue

At the desired time points, 3D cell-laden scaffolds (4 mm diameter x 2 mm height) with 5×10^6 million cells/ml were incubated in trypan blue 0.4% v/v solution for 5 minutes and dead cells (blue) were imaged with an optical microscope (Zeiss, Axio Vert.A1). A dilution of 1:4 of cell suspension and trypan blue solution was used as a control.

Cell-viability in bioinks containing EO

Bioinks were prepared with HA-Tyr rehydrated in PBS alone or supplemented with membrane stabilizing agents, glycerol and hydrocortisone, or reconstituted in α MEM cell culture medium, supplemented with 1% v/v or 10% v/v SeraPlus. hMSCs were embedded in HA-Tyr precursor formulations and the enzymatic crosslinking was triggered by the addition of H₂O₂ in presence of EO. 4 mm diameter by 2 mm height bulk scaffolds were

produced. Ten, twenty minutes and 1 h after initiating the crosslink, the hydrogels were moved into culture medium (DMEM LG + 10% v/v SeraPlus + 1% v/v Pen Strep) and a L/D assay was performed at 24 h after the culturing of the bulk scaffolds. Alginate was used as a control material to independently verify the cytotoxicity of the EO from the bioink composition (figure S6.3 and S6.4).

3D Printing on cartilage tissue

Osteochondral explants were isolated from bovine stifle joints obtained from the local slaughterhouse. Explants were washed in PBS supplemented with 10% Pen/Strep and then transferred to PBS containing 1% Pen/Strep. The explants were randomly distributed in two groups: 1. Uninjured, and 2. Injured. For the injured group, a Dremel (DREMEL®) with a diamond wheel point attachment (2mm diameter), was used to create a full chondral lesion (2x2mm). In both uninjured and injured groups, half of the samples were then treated with 1 U/mL cABC for 15min at 37°C in 5% CO₂ atmosphere, and then washed three times in PBS. The remaining samples were left untreated and all samples kept in PBS at 4°C until use. The 3D printing of 8 mm-diameter constructs was performed directly on the cartilage explants or on glass slides as control. The samples were then transferred into PBS to monitor the adhesion of the printed bioink to the cartilage or to the glass.

Results

Influence of cell encapsulation on the viscoelastic properties and printability of the bioink

The influence of embedded cells on storage and loss moduli of the enzymatically crosslinked ink is shown Figure 6.2a. The cell-free ink showed an elastic behaviour, with G' around 80 Pa, whereas all the cell-loaded inks showed a decrease of G' . The ink with 1×10^6 cells/ml was a weak hydrogel with a G' of 15 Pa, while in presence of 5×10^6 cells/ml no gelation occurred ($G'' > G'$). The $\tan \delta$ values correlated to the extrudability and printability of the formulation and allowed defining rheological printability regions for the HA-Tyr bioink formulations. In Figure 6.2b, a grey scale identifies the $\tan \delta$ -printability window correlating $\tan \delta$ with qualitative measurements of the extrudability and shape retention of the bioinks. The region 1 with $\tan \delta > 1$ corresponds to flowing compositions not suitable for extrusion-based printing. The region 2 with $\tan \delta$ between

1 and 0.6 corresponds to flowing formulations with poor shape retention. The region 3 with $\tan \delta$ values between 0.4 and 0.6 corresponds to inks with good extrudability and shape retention, and finally in the region 4 with $\tan \delta$ below 0.4 the formulations are no more extrudable. Then, the HRP and H_2O_2 concentrations in the bioink containing 5×10^6 cells/ml were modulated to assess the ability to reach a $\tan \delta$ value between 0.4 and 0.6 and obtain good printability by modulating the enzymatic di-tyramine crosslink content (Figure 6.2c). For a HRP at 0.1 U/ml, the increase of H_2O_2 from 0.13 mM to 0.39 mM led to $\tan \delta$ values between 3.1 to 2.67, outside the printability region. Likewise, HRP values increased to 0.2 and 0.3 U/ml did not allow to reach the region of printability. A minimum concentration of 0.4 U/ml HRP was required, so that $\tan \delta$ values in the area 3 of good printability were reached in the presence of 5×10^6 cells/ml. Among the combinations of HRP and H_2O_2 giving bioink formulations that displayed $\tan \delta$ values around 0.4, there were 0.4 U/ml-0.68 mM and 0.6 U/ml-0.39 mM.

Following optimization of the bioinks regarding their printability, the effect of embedded cells on the viscosity of HA-Tyr formulations was investigated (Figure 6.2d). The non-optimized inks with 2.5 and 5×10^6 cells/ml showed a viscosity curve with values close to 1 Pa·s throughout the shear rate assessed, whereas the ink showed a shear-thinning behaviour with viscosity ranging from 30 to 12 Pa·s in the extrusion shear rate window of 10 to 30 s^{-1} . The increase of H_2O_2 and HRP concentrations from 0.39 mM and 0.1 U/ml for the bioink with 2.5×10^6 /ml and 0.68 mM and 0.4 U/ml for the bioink with 5×10^6 cells/ml allowed to exactly match the shear behaviour the cell-free ink, necessary for a good printability.

Figure 6.2: Viscoelastic properties of the cell-laden enzymatically crosslinked ink. a) Amplitude sweep at 1 Hz of inks with cell density equal to 0, 1, 2.5 and 5 $\times 10^6$ hMSCs/ml, named ink (■), bioink 1 (◆), 2.5 (●) and 5 (▲), respectively; b) Influence of hMSCs cell density on the $\tan \delta$ values of enzymatically crosslinked inks with constant concentrations of HRP [0.1 U/ml] and H_2O_2 [0.13 Mm]; c) Contour plot showing $\tan \delta$ values (black dots) of enzymatically crosslinked ink with 5 $\times 10^6$ hMSCs/ml as function of the [HRP] and [H_2O_2]; the black box on the $\tan \delta$ grey scale and the delimited region 3 on the plot indicate the range of printability. The numbered regions are the same in panels b and c; d) Flow curves of inks formulation before and after $\tan \delta$ optimization.

Influence of extrusion parameters on cell viability

Following the optimization of the ink printability in the presence of cells, the influence of the shear stress on the cell viability during extrusion was assessed in absence of EO. L/D images showed a clear influence of printing pressure and needle geometry on the cell viability (Figure 6.3a). hMSCs viability of 98% was obtained upon dispersion and enzymatic crosslinking of the bioink before the printing process (positive control). With cylindrical needle of 0.25 mm or 0.33 mm inner diameter, a pressure of 4 bar was required to extrude the material resulting in 100% cell death. 0.51 mm cylindrical needle and a

0.58 mm conical needle, allowed to decrease the printing pressure from 4 to 1 bar with a consequent increase in viability at 24h up to 25% and 93%, respectively (Figure 6.3b).

Figure 6.3 - Printing parameters optimization for optimal cell viability with the enzymatically crosslinked bioink. a) Confocal images of L/D staining at 24h (scale bar = 100 μ m); b) Quantification of cell viability for different needle geometries (Con = conical and Cyl = cylindrical), sizes and pressures. Cell viability is calculated as average on 3 regions of each sample; positive control (P.C.) = 100% viable cells, negative control (N.C.) = 100% dead cells (prior encapsulation); bars indicates the standard deviation calculated on triplicates.

Influence of medium composition on cell viability and viscoelastic properties of the bioink

A time-lapse experiment was performed on bulk scaffolds to measure the viability of hMSC in EO-containing media. The viability at 24 h of hMSCs encapsulated within the enzymatically crosslinked ink in PBS supplemented with EO 0.01% w/v, decreased from 70% (after 10 min) to 0% (after 60 min). A similar outcome was observed at 24h for embedded hTERT fibroblasts and bovine chondrocytes in bulk scaffolds (Figure S6.1). Metabolic activity of hMSCs in monolayer culture showed no effect of 0.1 U/ml HRP and 0.39 mM H₂O₂ with or without green light exposure, while concentration-dependent cytotoxicity of EO was confirmed (Figure S6.2). This finding suggested that EO in presence of DMSO necessary for its solubilisation might be internalized into the cells and be responsible for the observed toxicity. To verify this hypothesis, membrane stabilizing agents, glycerol and hydrocortisone, were added. 60 nM hydrocortisone (data not shown)

and 10 mM glycerol did not show an improvement in cell viability. The addition of higher concentration of glycerol (50 mM) slightly improved cell viability at 20 and 60 min, up to 30 and 10%, respectively (figure 6.4). The supplementation of the ink formulation with 1% v/v or 10% v/v serum revealed an increase in the cell viability at 20 min of 85 and 94%, respectively. However, at 60 min the addition of serum at 1% v/v was able to recover only 20% of cell viability, while the presence of serum at 10% v/v kept a cell viability of 92%.

Figure 6.4 - Influence of medium composition on cell viability; all groups contain 0.01% EO, except the no EO (■) group, a) hMSCs viability as a function of time in enzymatically crosslinked ink in the presence of EO 0.01% w/v and different cytoprotective agents; b) L/D images of representative bioink formulations in PBS and 10% serum, scale bar = 100 μm.

The serum addition, required for improving the cell viability, influenced the viscoelastic properties of the bioink formulation with $\tan \delta$ increase outside the desired printability range. The rheological properties of the bioink supplemented with 10% v/v serum were restored to values comparable to the bioink in PBS by increasing the H_2O_2 concentration to 0.39 mM (Figure 6.5a). The bioink showed an identical behaviour independently of the embedding of hTERT fibroblasts and the bovine chondrocytes (Figure 6.5b). Whereas in the presence of embedded hMSCs the H_2O_2 concentration required was higher. In particular, for the hTERT fibroblasts and the bovine chondrocytes the H_2O_2 concentration was increased from 0.26 mM (PBS only) to 0.39 mM (10% serum) and 0.52 mM (20% serum), whereas for the hMSCs H_2O_2 values of 0.39 mM, 0.52 mM and 0.65 mM were required, respectively.

Figure 6.5 - Influence of cell type and serum on the viscoelastic properties of the bioink. a) Amplitude sweep at 1 Hz of HA-Tyr bioink with 2.5×10^6 hTERT fibroblasts/ml; b) H_2O_2 concentration necessary to achieve a $\tan \delta$ between 0.4 and 0.5 as a function of the cell type and serum content.

Printing of optimized bioinks using three different cell types

The optimized bioink formulations were selected: HA-Tyr 2.5% w/v in medium supplemented with 10% serum, 0.1 U/ml HRP, 0.01% w/v EO and 2.5×10^6 cells/ml using H_2O_2 0.39 mM for fibroblasts and chondrocytes or 0.52 mM for hMSCs. 3D constructs (diameter 20 mm, height 1.4 mm) with a criss-cross structure were printed using a 0.51 mm cylindrical needle and 1 bar pressure (Figure 6.6b and figure S6.5). The cell viability at day 1 and 14 was 78%, 70% and 75% for hMSCs, chondrocytes and hTERT fibroblasts, respectively (Figure 6.6a). At day 14, hMSCs showed an elongated shape compared to day 1, whereas fibroblasts and chondrocytes maintained a rounded morphology. The cell viability was preserved at day 14 for all cell types. All the 3D constructs retained their shape during the 14 days in culture. Strut size of constructs containing bovine chondrocytes was 750 μm at day 1 and 950 μm at day 14, showing higher swelling compared to the constructs with embedded fibroblasts and hMSCs, (Figure 6.6c).

Figure 6.6 - Long term viability of cell laden 3D printed constructs with cell density 2.5×10^6 cells/ml. a) Representative L/D images at day 1 and 14 of 3D constructs with three embedded cells type, scale bar = 100 μm ; b) Representative macroscopic image of a 20 mm diameter 3D printed construct, scale bar = 1 cm; c) strut size as function of the embedded cells type at day 1 and 14.

3D printing of adhesive bioinks on cartilage tissue

8 mm-diameter 3D constructs were printed on a glass slide using the developed double crosslinked bioink according to this composition: HA-Tyr 2.5% w/v reconstituted in medium supplemented with 10% serum, 0.1 U/ml HRP, 0.01% w/v EO, 2.5×10^6 hMSCs/ml and H_2O_2 0.52 mM. The printed constructs showed adherence to the glass in air, however they were washed out with PBS solution (Figure 6.7 a, c, e). When the bioink was printed and green light was applied directly on the cartilage surfaces, the printed construct was adhering both in air and after washing with PBS for all the cartilage samples. The same outcome was achieved with or without the defect and with or without cABC treatment (Figure 6.7 b, d, f).

Figure 6.7 - 3D printing of constructs direct on a) glass slide or b) cartilage with *cABC* treatment and adherence of the bioink before (c and d) and after the wash in PBS (e and f).

Discussion

Developing bioinks for 3D bioprinting is challenging due to the constraints that a biomaterial must fulfil such as rheological, mechanical and biological properties, often with opposite requirements [4]. Both physically and chemically crosslinked networks promote hydrogels as valid candidates for bioink development [29]. Among them, HA is the most promising in addressing direct bioprinting on cartilage tissue due to its intrinsically chondrogenicity and the ability to trigger connective tissues healing [30].

We introduced and optimized a dual crosslink tyramine-modified hyaluronan hydrogel as a cartilage-adhesive bioink. An initial enzymatic crosslink was used for optimization of extrusion and initial shape fidelity of the bioink, and a green light crosslinking in presence of EO was introduced to stabilize the 3D construct shape and chemically crosslink the 3D constructs to wet cartilage tissue. The embedding of cells correlated with a loss of gel-

like properties and decreased printability of the bioink, suggesting that cells reduced the efficiency of the HRP/H₂O₂-mediated enzymatic crosslinking, possibly by scavenging H₂O₂ before reaction with HRP and interfering with the polymeric network formation. This is consistent with experimental observations reported by other groups, where cells encapsulation density reduced mechanical properties of HRP/H₂O₂-mediated enzymatic crosslinked hydrogels [31, 32]. The reduced crosslinking density in presence of cells could also be the result of the crosslinking of aminoacids, soluble proteins or membrane proteins with HA-Tyr, with the inhibition of HRP in presence of serum and cells or the degradation of H₂O₂. To restore the desired viscoelastic properties and obtain a printable bioink formulation, HRP and H₂O₂ concentrations were modulated to match the $\tan \delta$ values region of cell-free printable ink. The concentrations of HRP and H₂O₂ were varied in a range avoiding the cytotoxicity and the potential oxidant-induced apoptosis of these two components [33, 34]. Therefore, a HRP limit value of 1 U/ml was selected and the H₂O₂ concentration was varied between 0.17 and 0.69 mM.

The HA-Tyr crosslinking mechanism is adopted in this work as an alternative to the typical (meth)acryl group polymerization. Acrylates and methacrylates are the most common reactive groups for use in radical polymerizations. These functional groups are not found in ECM and require UV irradiation. In contrast, HA-Tyr contains functional groups naturally occurring in aminoacids and the enzymatic crosslinking mechanism makes the resulting hydrogel more cell-friendly. The cytotoxicity consideration, together with the extrusion requirements, limited the stiffness of the bioink reached and the soft hydrogel extruded required a post-crosslinking for long-term shape retention. To ensure this shape retention, we employed a visible light-triggered crosslinking, in contrast to the standard UV-triggered crosslinking, which has the advantage of being easily implemented into a printing process and has higher penetration depth beneficial for uniform crosslink and adhesion to underneath tissue. With this approach we additionally overcame the detrimental effect caused by UV-light irradiation, inducing cellular damage via direct interaction with cell membranes, proteins and DNA or via indirect production of reactive radical species.

An important aspect of bioink engineering is the shear stress experienced by the embedded cells upon extrusion [35-37]. By adjusting the printing pressures and needle geometries, cell viability was optimized. At low printing pressure (1-2 bar), the cell viability was high independently from the needle shape with a diameter of 0.51 mm.

Interestingly, a previous report from Billiet *et al.* indicated differences in cell viability upon extrusion from cylindrical and conical needles at low pressures [31]. However, unlike the work from Billiet where the Gel-MA has high DS, the HA-Tyr has a low DS and is therefore more similar to physiological extracellular matrix (ECM). This difference observed in terms of cell viability for the HA-Tyr bioink may be related to the shear thinning properties of the HA-Tyr bioink that help in reducing the shear stress experienced by embedded cells. Additionally, the use of a hydrogel bioink allowed extrusion and printing of 3D constructs at low pressure with homogenous cell distribution and good viability which was already reported to be better than solution bioinks [38].

The EO concentration required for post-crosslink and stabilization of the printed constructs was higher than other EO concentrations reported for embedding of viable cells in visible light crosslinked hydrogels [22, 24, 25], and, indeed, significantly influenced cells viability. A 0.01% w/v EO (0.15 mM) was the lowest concentration allowing a successful crosslinking with the available LED light source (505 nm, 440 mW, 34.4 cd). A higher energy source may be more efficient and would allow a decrease in EO concentration. A concentration-dependent EO cytotoxic effect was confirmed in 3D and 2D independently of the light exposure and the cell type used [39, 40] excluding a possible role of the singlet oxygen generated. Although the cytotoxicity observed in 2D was previously proven to decrease in a 3D environment via the ability of the hydrogel to scavenge the free radicals produced by the photoinitiators [41], this was not the case in the HA-Tyr hydrogel. This, together with the time-dependent toxicity of the EO in the bioink, suggested that EO penetration within the cells was likely causing the observed toxicity. Interestingly, a previous study has shown a high cell viability using a 0.02% w/v EO concentration for a photo-crosslinked HA-Tyr [23]. In our experiments, the DMSO employed to pre-solubilize EO might have facilitated internalization and toxicity. We hypothesized that the use of serum or membrane stabilizing agents would reduce EO cell penetration to allow the printing and the stabilization of the 3D printed construct before observing cell death. The HA-Tyr bioink was supplemented with serum at different concentrations (1%, 10% or 20% v/v) and the membrane stabilizing agents, glycerol and hydrocortisone. These membrane-stabilizing agents play a role in cell regulatory processes, particularly in maintaining intracellular homeostasis and have been already employed by Khattak *et al.* to improve the viability of cells embedded in the EO photo-crosslinked copolymer Pluronic F127 [42]. In contrast to this work, we found only a slight

improvement in the cell viability in presence of glycerol 50 mM and none with hydrocortisone. These two membrane stabilizing agents were not able to sustain a satisfactory cell viability over time. Instead, the addition of 10 % v/v serum led to a large improvement of cell viability. We can hypothesize that the chemical milieu given by the serum inhibit EO internalization with an osmotic mechanism or via the binding of EO to serum proteins, preventing the cell uptake of EO.

The final optimized bioink formulation contained HA-Tyr at 2.5% w/v in medium supplemented with 10% serum, 0.1 U/ml HRP, 0.01% w/v EO and H₂O₂ concentrations adjusted to the cell type in use for optimal printability. The difference in rheological properties induced by the different cell types embedded may be related to the specific cell metabolism and ability to scavenge H₂O₂. The optimized bioink has been employed in the printing of three different cell types, primary cells and cell lines, underlying its versatility and suitability in supporting good cell viability.

Importantly, we demonstrated the possibility to perform direct 3D bioprinting on cartilage explants and the creation of an adhesive interface between the biological tissue and the HA-Tyr bioink. The adhesion in wet conditions was not observed on glass, underlying the specificity of the chemical interactions created. The exposure to green light during the bioprinting process promoted the reaction between the free tyramine functional groups on the HA-Tyr and the aromatic side chains on the ECM of the tissue, such as tyrosine [43–45]. This first adhesion to the wet tissue is fundamental for an initial stability and future integration of the construct to the tissue. Further optimization will be required to achieve high shape fidelity and accuracy during direct biofabrication into complex cartilage defects and soft tissues could be used as well to assess the bioink adhesion. Additional work is also required to study the long-term behavior of printed cells. Ultimately, the ability to modulate the crosslinking degree of the 3D printed bioink through differential light exposure, the possibility to use in each printed layer a variety of printing paths and to add stimuli for GAGs production, may allow mimicking more closely the cartilage tissue zonal organization and therefore more precisely direct cell behavior.

Conclusions

In this manuscript, we addressed the development and optimization of a bioink with good printability and stability/adhesion to cartilage tissue. A double gelation mechanism was exploited, tuning extrusion properties with enzymatic crosslinking and using visible light for post-printing curing and adhesion to wet cartilage. Using a rheologically measurable parameter, the damping factor, we observed how different cell types required different compositions for optimal printability. Under the stressing conditions during printing, the medium composition revealed itself as a key variable for improving cell viability for hMSC, fibroblasts and chondrocytes. The tyramine-modified hyaluronic acid bioink optimization together with the dual-crosslinking mechanism shows the possibility to design bioadhesive bioink for *in-situ* 3D bioprinting.

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Supplementary information

Figure S6.1 – a) L/D images at 24h of both hTERT fibroblasts (top row) and chondrocytes (bottom row) for enzymatic crosslinked bulk scaffolds and formulations containing EO 0.02% w/v (scale bar = 100 μ m); b) metabolic activity of both chondrocytes (white bars) and hMSCs (grey bars) encapsulated into 3D bulk scaffolds. Enzymatically crosslinked scaffolds with (3') and without (NL = no light) green light exposure were compared; standard deviation is calculated on triplicates. Data are shown as mean $\pm$ SD of three replicates using cells from 3 different donors. Fluorescence normalized to the weight of each sample.

a)

b)

Figure S6.2 - a) Cell viability obtained via CellTiterBlue assay, at 24h (white columns) and 72h (black columns) after the addition of supplements to the medium. The medium was supplemented with: HRP/H₂O₂ (0.1 U/ml/ 0.39 mM), 0.02% w/v EO (EO1), 0.005% w/v EO (EO2), 0.0005% w/v EO (EO3), HRP/H₂O₂ (0.1 U/ml/ 0.39 mM) plus EO2. Negative control = Triton X-100, positive control (PC): medium with no supplements. Each group was exposed (5') or not (NL) to green light. Standard deviation is calculated on triplicates; b) Bright field images and L/D images of representative samples: Triton X-100, positive control with no light exposure (PC NL), 0.02% w/v EO with no light exposure (EO1 NL) and 0.0005% w/c EO with no light exposure (EO3 NL); dead cells in red and viable cells in green. Scale bars = 100 μ m.

Figure S6.3 – L/D images of hMSCs encapsulated in alginate gel (cell density 5×10^6 cells/ml): a) control groups both dead (N.C.) and viable cells (P.C.); N.C. and P.C. are higher magnification images of the red and green areas in a; and b) 0.01% w/v EO was supplemented to the alginate solution containing viable cells; scale bars = 100 μ m.

Figure S6.4 - Trypan blue (0.4% solution) and L/D on hMSCs encapsulated in bulk scaffolds of alginate (a and b) and HA-Tyr (c and d) supplemented or not with 0.01% w/v EO. P.C. = positive control with viable cells, N.C. = negative control with dead cells. Scale bars = 100 μ m.

Figure S6.5 – Mosaic reconstruction of a 3D printed construct; HA-Tyr bioink formulation: HA-Tyr 2.5% w/v reconstituted in medium supplemented with 10% serum, 0.1 U/ml HRP, 0.01% w/v EO and H₂O₂ 0.52 mM), hMSCs density of 2.5×10^6 /ml; green live (Ca-AM) and dead read (EthD-1). Scale bar = 1mm.

Chapter 7

General discussion and future perspectives

General Discussion and future perspectives

Hyaluronic acid is an essential biopolymer employed in the medical field [1]. Since its first medical applications in the late 1950s as vitreous supplement during eye surgery, HA-based materials have reported a rapid growth in terms of applications in several biomedical areas.

In tissue engineering and regenerative medicine, the use of materials in combination with cells and growth factors is the leading strategy to replace the structure and function of a tissue or promote tissue healing [2]. Therefore, the development of biomaterials that work as matrices for cell delivery, migration and proliferation and differentiation is a central research topic. In this perspective HA-based materials are promising candidates thanks to the role of HA in wound healing [3] and the range of useful biological functions of HA arising from its interaction with cell surface receptors, homing cell adhesion molecule (CD44 or HCAM), and receptor for HA mediated motility [4].

However, several challenges need to be addressed to implement HA biomaterials in regenerative medicine. Depending on the target application, the challenges to face include the complexity of the tissues and organs architectures, the need of vascularization and innervation, improved the integration with the host tissue, and the possibility to modulate the local immune microenvironment, specific mechanical or rheological requirements are to be considered in the development of HA based devices. Another important aspect is the delivery of drugs from HA matrices, where proteins and genes, that are effective in promoting cell migration, proliferation or differentiation to induce tissue and organ regeneration, are released at the target site. Hence, researchers are aiming to develop materials with tailored biochemical and biophysical properties.

Therefore, novel HA chemical modification and crosslinking methods need to be investigated to embrace the above challenges where first-generation HA-based devices are insufficient. The new generation of HA devices should comprise derivatives with fine tuneable viscoelastic and mechanical properties, functionalized with chemicals to interact with the surrounding tissues that can allow better integration and success after implantation *in vivo*, and with controlled biodegradation.

With this in mind, we synthesised HA derivatives with design-driven features for a range of applications in regenerative medicine. The chemistry of DMTMM,

4(4,6dimethoxy1,3,5triazin2yl)methylmorpholinium chloride, was employed in this work as a more effective conjugation method. We developed two semisynthetic derivatives giving non-covalent gels and a tyramine-modified derivative with a double gelation mechanism that was exploited for 3D printing in tissue engineering.

The two non-covalent derivatives, the short-alkyl HA derivatives and the thermoresponsive HA, are examples of amphiphilic derivatives and stimuli-responsive HA-materials, characterized by a dynamic viscoelastic properties and response to specific cues. These materials are appealing for their use in drug delivery [5] and cell therapies [6]. For drug delivery systems it is important to prevent the denaturation or deactivation of the released payload. Hydrogels are good candidate begin usually inert toward drugs, however it is challenging to achieve a controlled release over a long time due to the releasing mechanism, mostly based on diffusion. One approach is to conjugate the drugs to the hydrogel and control the release with the hydrogel degradation. Regarding the delivery of cells, HA-based hydrogels support cell proliferation and migration, and are good biomaterial carriers for protecting cells during and after the injection and for retaining the cells at the administration site. These hydrogels can be engineered with growth factors or nutrients to provide support to the delivered cells, however the current challenge is still the capability to integrate and mimic the environment at the injured tissue. Additionally, hydrogel-based signals may include features such as porosity, degradation, mechanics, and adhesion, which all have been shown to influence stem cell differentiation in 3D microenvironments, as previously reviewed by Guvendiren *et al.* [7]. Knowing that the conjugation of long aliphatic chains to the HA backbone leads to hydrophobic interactions between the pending moieties, we have conjugated short-alkyl chain to develop a material with modulation rheological properties [8]. A low degree of substitution (3 - 4%) led to an improvement of the HA rheological properties due to the interaction between the hydrophilic HA backbone and the hydrophobic alkyl chain [9]; this feature could be used for the delivery of both hydrophobic and hydrophilic drugs, or for viscosupplements. We have tried to further characterize the structure of these derivatives with techniques such as the circular dichroism (CD), dynamic light scattering (DLS), differential scanning calorimetry (DSC), transmission electron microscopy (TEM) and X-ray diffractometer (XRD), however the data gathered were not conclusive. The XRD did not show any clear crystallinity in the HA derivatives structure and,

additionally, no specific conformational changes in the polysaccharide structure were detected with the DC. Likewise, the TEM analyses did not clearly show micelles or liposomes structure, however it showed the presence of regular nano-patterns typical of crystalline domains. It would be interesting to use small-angle X-ray scattering techniques to determine the micro- and nano-scale structure of the polymeric matrix. These amphiphilic derivatives could be additionally employed for 3D printing thanks to their improved shear-thinning properties compared to the unmodified HA, however in our case, no enough mechanical stability was reached for the development of a stable bioink.

3D (bio)printing is an expanding technology requiring (bio)material features. The critical point in the achievement of 3D structures with the layer by layer depositions of multiple materials is the development of inks that have viscoelastic properties suitable for the extrusion and stability after printing. Up to date, HA-based inks include the use of partially crosslinked methacrylated HA followed by UV exposure after printing to improve the stability of the printed construct [10]; the development of shear-thinning supramolecular HA-derivatives that undergo gelation upon removal of shear forces [11] in combination with methacrylated chemistry for final shape fixation.

While developing a new bioink for 3D printing, it is also important to address its biofunctionality, the resolution of the printed constructs, the mechanical stability of the construct, and the influence of the ink on the cells. Usually, a good resolution is conflicting with the printing of highly viable cell constructs due to the higher shear stress that the cells experience during the bioink extrusion. Additionally, the encapsulation of cells in the polymeric material can sometimes result in a modification of the viscoelastic properties, depending on the selected crosslinking mechanism. Therefore, future bioinks should be designed to have mechanical features independent from the embedded cell numbers and at the same time, have suitable interactions with the embedded cell-printed construct and surrounding tissue in order to promote tissue repair. The latter is addressed in the development of our tyramine-modified HA bioink, where the adhesion of the bioink to cartilage during the printing process is proven. During the development of the bioink, we implemented several features: we optimized the best rheological properties for extrusion. One of the challenges we encountered was the improvement of the viability of the cells encapsulated in the ink: the printing pressure had an influence on the cell viability but only the addition of serum to the ink formulation significantly decreased the cell

death. It would be interesting to investigate the integration of the bioink to the cartilage or to other soft tissues. The ink could also be used to print different cell types in the same constructs or to print constructs with gradient of stiffness changing the light curing speed during the printing process.

One of the drawbacks of the HA hydrogel is the possibility to reach relevant mechanical properties. Therefore, the improvement of the crosslinking mechanism or the development of composite materials can increase the integrity of the final material and boost their use in the clinical practice. This was investigated in the thesis with the development of both injectable and putties HA-composites, where the addition of beta tricalcium phosphate particles to a thermoresponsive HA derivative stabilized the cohesion of the HA material and improved its mechanical properties [12]. This translational project was carried out with an industrial partner, the DePuy Synthes Biomaterials with the idea to develop a delivery system. The natural continuation of the project would be an *in vitro* and subsequently *in vivo* model to test the efficacy of the composite system to produce new bone formation or to test the delivery of molecules different from the studied dexamethasone and bone morphogenetic protein-2. Besides the combination of the HA natural polymer to an inorganic phase, the use of interpenetrating network (IPN), such as the double network (DN), can be a solution for the improvement of the HA mechanical properties [13].

The biomedical panorama requires a continuous development of HA-based materials. The study of specific and unique properties, that improve the biological and functional role of these biomaterials, has to move along with the expansion of new technologies both in basic and translational research.

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Summary

This thesis described the development of design-driven chemical modification of hyaluronic acid (HA) derivatives for engineering biomaterials for drug delivery, bone tissue engineering and 3-dimensional printing (3DP). The conjugation of the various moieties to the HA backbone involved the activation of the HA carboxyl group via 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) chemistry prior to reaction with primary amines. Small alkyl chains, poly(*N*-isopropylacrylamide) and tyramine were grafted to the HA backbone for application of the synthesised derivatives in the biomedical field.

The aim and the general structure of the thesis were provided in **Chapter 1**.

In **Chapter 2** the structure and the properties of the hyaluronic acid were described together with the chemical reactions performed for derivatization of the HA disaccharide unit. The HA chemical modifications aim at modulating the therapeutic action of HA. They play an essential role in tailoring the mechanical and chemical properties of the pristine HA. The HA derivatives predominantly maintain the biocompatibility and the biodegradability of the HA, but they acquire different physico-chemical properties. Emphasis was given to the functionalization of the carboxyl group via DMTMM chemistry. Additionally, in this chapter the current methods to obtain HA network were reviewed: chemical, enzymatical, physical and photo-crosslinking. The improvement of the viscoelastic properties of HA triggered by the crosslinking is essential, extending the HA half-life, and creating matrices embedding cells and modulating their behaviour. At the end of the chapter an overview of the applications of the HA derivatives in the musculoskeletal repair field was given.

In **Chapter 3** synthesis of propylamine and butylamine HA derivatives with wide degree of substitution (DS) was reported. A parametric study was performed to assess the influence of the molar ratio of the reagents (HA, DMTMM, amines) and the temperature on the grafting efficacy and reaction kinetics. A maximum storage modulus and pseudoplasticity was found for derivatives having DS values of 3.0% and 3.7% for HA-propylamine and HA-butylamine respectively, whereas above or below these DS values, rheological features go back to baseline values of pristine HA. This complex correlation, together with the solubility change after thermal treatment, suggested an activated secondary structure rearrangement with re-organization of the hydrophobic and

hydrophilic domains. Thanks to the singular rheological properties, these derivatives are suitable for preparing injectable hydrogels for additive manufacturing.

Chapter 4 presented a new synthetic bone substitute comprised of Beta-tricalcium phosphate particles (β TCP) and a thermoresponsive poly(*N*-isopropylacrylamide)-hyaluronan derivative in aqueous solution. The influence of the particles size, the polymer phase concentration and the HA molecular weight on the injectability was assessed. The results showed that synthetic bone substitutes in the form of injectable or putty pastes with the ability to fill bone defect of irregular shapes could be prepared. Owing to the temperature-induced sol-gel transition of the soft polymeric phase, handling properties were superior compared to pristine hyaluronan composites in terms of cohesion and dissolution. Furthermore, the most suitable composite was selected for loading with model drugs and a release study performed: 20% HpN (HA molecular weight 1.5 MDa) and β TCP particle size below 0.25 mm. The amphiphilic character of the HA derivative allows the control of the release of model drugs, human recombinant bone morphogenetic protein 2 and dexamethasone, with a release over 13 days of 10% and 47% of the total payload, respectively.

In **Chapter 5** a biofunctional ink for extrusion-based 3D printing was introduced based on a tyramine-modified hyaluronan derivative. The ink exploited a double-crosslinking mechanism: an enzymatic gelation was used to tune the shear-thinning properties for optimal extrusion, by varying the concentrations of horseradish peroxidase and hydrogen peroxide, and a visible light photocrosslinking for shape stabilization of the printed structure. The viscoelastic and printability properties of a range of formulations were investigated and a correlation was found between the damping factor (G''/G'), the extrudability and the printability of the inks. A suitable extrudable ink, showing shear-thinning properties and G' values of 100 Pa was optimized. This enzymatically crosslinked ink was however not able to retain its shape after extrusion. Shape retention was achieved by exposure to green light after each printed layer in the presence of photoinitiator in the ink. The printing process was optimized for the printing of a criss-cross structure of 10 mm diameter and 1.5 struts distance: 0.25 mm cylindrical needle, extrusion pressure 4 bar, printing speed 4 mm/s, light curing speed 8 mm/s, layer height 0.14 mm. The micro computed tomography was used to assess macroporosity and struts width. Finally, the post-printing functionalization of 3D printed construct with a tyrosine-

terminated RGD peptide enhanced cells adhesion on the surface of the printed ink. The advantages of this ink formulation are: the use of a single HA-derivative without rheological enhancers such as nanofibers; the use of cyto-compatible visible light for crosslinking; and the possibility of a post-printing functionalization of the 3D structures with cell-adhesive motives. The remaining challenges are the sagging of the struts upon printing, bringing to poor preservation of the transversal porosity in the final construct.

Chapter 6 described the further optimisation of the Chapter 5 ink, as a cartilage-adhesive bioink. The possibility to load this ink with cells for the preparation of 3D cell-laden constructs was investigated. The bioink exploited the same dual-crosslinking mechanism, where the enzymatic crosslinking (HRP/H₂O₂) formed a soft gel suitable for cell encapsulation and extrusion, and the visible light photocrosslinking during the printing process (EO and green light, 505 nm) allowed the shape retention of the printed construct and adhesion to cartilage. The encapsulation of the increasing cell amount in the ink precursor showed to influence negatively the viscoelastic properties of the ink, up to the loss of the gelation. An increase in the amount of HRP/H₂O₂ was required to match the values of the damping factor optimized for a good printability of the ink (damping factor between 0.4 and 0.6 = printability window). The influence of the shear stress on the cell viability was assessed first in absence of EO. Low printing pressure (1 bar) and a 0.51 mm cylindrical needle ensured high cell viability within the extruded bioink. A time lapse experiment revealed a EO cytotoxicity when the bioink formulations were reconstituted in phosphate saline buffer. The addition of serum in the formulations containing EO rescued the cell viability, but at the same time impaired the viscoelastic properties of the bioink. Finally, biofabrication of cellularized constructs with high cell viability and good shape retention after 14 days of culture was achieved. Finally, the ability to 3D print and glue cellularized construct on cartilage explants was demonstrated.

Finally, in **Chapter 7** a general discussion on the hyaluronan-based materials was drawn and future directions in the biomedical field were discussed. Hyaluronic acid will still be a fundamental building block for the development of new biomaterials but novel chemical modification and crosslinking methods need to be investigated together with the design of stimuli-responsive HA-based materials.

Samenvatting

Deze thesis beschrijft de ontwerp-gedreven chemische modificatie van hyaluronzuur (HA) derivaten voor het vervaardigen van biomaterialen voor drug delivery, bot tissue engineering en voor 3D printen (3DP). De koppeling van verschillende functionele groepen aan de HA keten geschiedt door middel van de activatie van de carboxyl groep via 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) chemie voorafgaand aan de reactie met primaire amines. Korte alkyl groepen, poly(*N*-isopropylacrylamide) en tyramine werden aan de HA keten geïmplant om deze derivaten voor biomedische toepassingen te kunnen gebruiken.

Het doel en de opbouw van deze thesis staat beschreven in **Hoofdstuk 1**.

In **Hoofdstuk 2** worden de structuur en de eigenschappen van hyaluronzuur beschreven, evenals de chemische reacties voor de derivatisatie van de HA dissacharide eenheid. De chemische modificatie van HA heeft als doel om de therapeutische werking van HA te moduleren. Deze kan een essentiële rol spelen bij het aanpassen van de mechanische en chemische eigenschappen van ongemodificeerd HA. HA derivaten behouden voor het grootste deel de biocompatibiliteit en de biodegradeerbaarheid van HA, maar ze krijgen andere fysisch-chemische eigenschappen. Het accent ligt hierbij op de functionalisatie van de carboxygroep via DMTMM chemie. Verder wordt in dit hoofdstuk een overzicht gegeven van de huidige methoden om HA netwerken te verkrijgen: chemische, enzymatische, fysische en foto crosslinking. De verbetering van de viscoelastische eigenschappen van HA door middel van crosslinking is essentieel om de halfwaardetijd van het materiaal te verlengen, om cellen in te sluiten in de matrix en om hun eigenschappen aan te passen. Aan het eind van het hoofdstuk wordt een overzicht gegeven van de toepassingen van HA derivaten in het herstel van het bewegingsapparaat.

In **Hoofdstuk 3** wordt de synthese van propylamine en butylamine HA derivaten met een breed bereik aan functionalisatie graden (DS) beschreven. Een parametrische studie werd uitgevoerd om de invloed van de molaire verhoudingen van de reagentia (HA, DMTMM, amines) en de temperatuur op het rendement van de grafting en de reactiekinetiek te bepalen. Een maximum in opslagmodulus en pseudoplasticiteit werd gevonden voor derivaten met een functionalisatie graad van 3.0% en 3.7% voor HA-propylamine en HA-butylamine respectievelijk, waarbij voor functionalisatie graden die hoger of lager dan deze waarden waren, de rheologische waarden terugvallen tot de baseline waarden van ongemodificeerd HA. Deze complexe correlatie impliceert, samen met de verandering in

oplosbaarheid na warmte behandeling, een geactiveerde secundaire herschikking met reorganisatie van de hydrofobe en hydrofiele gebieden. Dankzij deze bijzondere rheologische eigenschappen zijn deze derivaten geschikt voor het bereiden van injecteerbare hydrogelen voor additieve vervaardiging.

In **Hoofdstuk 4** wordt een nieuw synthetisch bot substituuat, bestaande uit beta-tricalcium fosfaat (β TCP) en een thermoresponsief poly(*N*-isopropylacrylamide)-hyaluronan derivaat in een waterige oplossing gepresenteerd. De invloed van deeltjesgrootte, de polymeer fase concentratie en het moleculaire gewicht van de HA op de injecteerbaarheid van het composiet werd onderzocht. Er werd aangetoond dat het mogelijk is om synthetische botvervangers te prepareren die geïnjecteerd kunnen worden of als putty gebruikt kunnen worden om bot defecten met een onregelmatige vorm te dichtten. De hanteerbaarheid van dit materiaal is beter dan dat van ongemodificeerd hyaluronan wat betreft cohesie en oplosbaarheid, dankzij de temperatuur geïnduceerde sol-gel transitie van de zachte polymere fase. Vervolgens werd het meest geschikte composiet geselecteerd om deze te laden met model drugs en werd het afgifte profiel bepaald: 20% HpN (HA moleculair gewicht 1.5 MDa) en β TCP deeltjes kleiner dan 0.25 μ m. Het amfifiele karakter van het HA derivaat maakt het mogelijk om de afgifte van de model drugs te reguleren, rhBMP-2 en dexamethasone, met een afgifte van 10% en 47% van de totale lading over 13 dagen, respectievelijk.

Hoofdstuk 5 gaat over de ontwikkeling van een biofunctionele inkt voor extrusie gebaseerd 3D printen op basis van een tyramine-gemodificeerd hyaluronan derivaat. De inkt maakt gebruik van een dubbel crosslink mechanisme: enzymatische gelatie om de shear-thinning eigenschappen aan te passen voor optimale extrusie, door het variëren van de concentraties horseradish peroxidase en waterstofperoxide, en foto crosslinking voor de vorm stabilisatie van de print. De viscoelasticiteit en printbaarheids eigenschappen van een reeks formulaties werd onderzocht en een correlatie tussen de damping factor (G''/G'), de extrudeerbaarheid en printbaarheid van de inkten. Een geschikte extrudeerbare inkt, met afschuifverdünnende eigenschappen en G' waarden van 100 Pa werd geoptimaliseerd. Deze enzymatisch gecrosslinkde inkt was echter niet in staat om zijn vorm te behouden na extrusie. Vormvastheid werd verkregen door elke geprinte laag, met daarin foto-initiator aanwezig uit de inkt, bloot te stellen aan groen licht. Het print proces werd geoptimaliseerd voor het printen van een kris kras structuur met een diameter

van 10 mm en een afstand van 1.5 struts: 0.25 mm cilindrische naald, extrusie druk 4 bar, print snelheid 4 mm/s, lichtuithardingssnelheid 8 mm/s, laaghoogte 0.14 mm. Micro-computer tomografie werd gebruikt voor het bepalen van de macroporositeit en strut breedte. Tenslotte werd aangetoond dat functionalisatie met een tyrosine-getermineerde RGD peptide van de 3D geprinte constructen er voor zorgt dat er een verbeterde aanhechting van cellen op het oppervlak van de geprinte inkt is. De voordelen van deze inkt samenstelling zijn: het gebruik van een enkel HA-derivaat zonder reologie verbeteraars zoals nanofibers, het gebruik van cytocompatibel zichtbaar licht voor het crosslinken, en de mogelijkheid om na het printen de 3D structuren te functionaliseren met cel-bindingspatronen. Een resterende uitdaging is het doorzakken van de struts na het printen, wat ervoor zorgt dat de transversale porositeit verloren gaat in het uiteindelijke construct.

Hoofdstuk 6 beschrijft de verdere optimalisatie van de inkt beschreven in Hoofdstuk 5, als een bioinkt die kraakbeen adhesief is. De mogelijkheid om in deze inkt cellen te mengen voor het vervaardigen van 3D cel geladen constructen werd onderzocht. De bioinkt maakt gebruik van het zelfde dubbele crosslink mechanisme, waarbij een zachte gel wordt gevormd door middel van enzymatische crosslinking (HRP/H₂O₂) om cellen in te sluiten en voor extrusie, en waarbij foto crosslinking met zichtbaar licht (EO en groen licht, 505 nm) gedurende het printproces voor vormvastheid van het geprinte construct zorgt en voor aanhechting aan het kraakbeen verantwoordelijk is. Het insluiten van een toenemend aantal cellen in de precursor van de inkt had een negatieve invloed op de viscoelastische eigenschappen van de inkt, zodat bij een groot genoeg hoeveelheid geen gelatie meer plaatsvond. Een toename in de hoeveelheid HRP/H₂O₂ was nodig om de juiste waarden te verkrijgen voor de damping factor om goed te kunnen printen met de inkt (damping factor tussen de 0.4 en 0.6 = printbaarheids bereik). De invloed van schuifspanning op de levensvatbaarheid van cellen werd eerst onderzocht in de afwezigheid van EO. Lage printdruk (1 bar) en een 0.51 mm cilindrische naald zorgden voor een hoge levensvatbaarheid van de cellen in de geextrudeerde bioinkt. Een time lapse experiment toonde aan dat EO cytotoxisch was wanneer de bioinkt formule werd gereconstitueerd in fosfaatgebufferde zoutoplossing. De toevoeging van serum in de formulatie met EO zorgde voor herstel van de levensvatbaarheid van de cellen, maar zorgde er tegelijkertijd voor dat de viscoelastische eigenschappen van de bioinkt verloren

gingen. Tenslotte was het mogelijk om cel beladen constructen met hoge levensvatbaarheid van de cellen en een goede vormvastheid te verkrijgen na 14 dagen kweek. Uiteindelijk werd aangetoond dat het mogelijk is om constructen die met cellen geladen zijn te 3D printen en te lijmen op kraakbeen explantaten.

Tot slot wordt in **Hoofdstuk 7** een algemeen discussie gehouden over hyaluronzuur gebaseerde materialen en het toekomstperspectief van deze materialen in het biomedische veld. Hyaluronzuur zal een blijvende fundamentele bouwsteen zijn voor de ontwikkeling van nieuwe biomaterialen, waar nieuwe chemische modificaties en crosslinking methodes moeten worden onderzocht in combinatie met het ontwerp van stimuli-responsieve HA gebaseerde materialen.

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And to complete the Davos family, let me thank Wero and Arturo. With our lovely dinners, crazy trips and outdoor short-weekends, we have spent intense moments together. I have always felt comfortable in your hugs Wero, they were always full of love and hope and they always came when most needed. Arturo, thank for all the nice conversations we had while driving down to Milano.

At the ARI, I have met so many people coming from all over the world and thanks to this multi-cultural environment I have enriched myself.

The polymer team has changed during these years spent in Davos and I would like to thank all the people that have been part of this group together with me. A big Thanks goes to Ryan, you always had a good word for me when the experiments were not going

exactly how I expected. Your kindness, positive attitude and ability to calm me down have been a driven force in the first years of my PhD. You and Sarah are building an amazing family and whoever meets you is so lucky. Laura, you have always been there for me in the toughest time and you were the one who introduced me to cell culture. It was a pleasure to spend time with you in the office, lab or in the evening. After the finish girl, here it comes the French guy. Oliver, thanks for your advices during the experiments all the funny moments spent in the office and the eco-friendly discussion. I really do hope you will save this Planet. Good luck with your next carrier step (or with you plan B - the snail farm) and I am so happy for you and Manuela that Lena arrived this year. I would like to thank the technicians, first Markus and later on Flavio. You always took good care of us and you made the work in the lab enjoyable. Thanks Angela, for all the time you have invested in the lab with me and in having helpful discussions on my latest experiments. Stijn, you took over the Dutch legacy in the Polymer group. It was a pleasure to share the office with you. I wish you and Reihane a lot of happiness and best of luck in finishing your PhD. Tiziano and Nunzia, you were the last one to join the group but in such a short time, we have built a solid friendship. Thanks for constantly listening to me and for your precious advices. Good luck with the new adventure that will start in June; I am impatient to meet Bianca. I really hope that we will be in touch in future. Adriana and Riccardo, you joined the group during my last year of the PhD and you brought home here in Davos. Thanks for supporting me during the 'hardest' time. I wish you both good luck with next step in London. Additionally, thank to Marine, Mario, Claudia, Jason, Peter, Christoph and all the guests we had in our group for the helpful discussion during our long working hours and the nice time spent together.

There was not only the Polymer team in ARI but a bigger Musculoskeletal group with lots of dynamic people.

First, I would like to thank Nora. Unfortunately, we had the chance to know each other better only in this last year and a half but the time spent together will always be treasured in my heart. I really have to say this: you are an amazing person, 'sometimes' super stubborn, but your kindness is priceless. It was nice to share with you evenings at the cinema and cooking lessons. Let's hope to share a concert as well (of course we do not plan it). You took care of me like a sister.

Thanks a lot Jan. It is impressive how fast we became close friends. We had many lovely talks and lot of fun playing table-soccer games. You have been a precious friend during my stay in Davos and I am sure this friendship will never end. I will keep with me all the memories from the time spent together. In 2014 I had a fantastic group of friends with Fabrizio, Lukas, Gil, Zhaira, Steph, Shan, Robert O and of course my AO family. Luki, thanks a lot for the long conversations, for the swimming sessions at the pool and for the long evenings in the lab. I was so honoured to be at the wedding of you and IngIng. You both are amazing together and now with the baby Janine. Steph, you were an amazing friend, always making time to listen to me. It was nice to meet you in Thailand and have you as a personal guide there. Thanks as well to Elena and Gernot for the nice evenings spent together.

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Finally, I would like to thank the people who supported me from far away.

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past. Unfortunately, we are growing apart from each other and the various experiences are shaping ourselves but our friendship is so solid that I truly believe it will last forever.

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"Si vive di ricordi, signori, e di giochi, di abbracci sinceri, di baci e di fuochi, di tutti i momenti, tristi e divertenti, e non di momenti tristemente divertenti." – Caparezza –

Dalila