ENGINEERING HYALURONAN-TYRAMINE HYDROGELS TO MODULATE MESENCHYMAL STEM CELL BEHAVIOR

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The more I learn, the more I realize how little I know.

*Socrates*
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Abstract

Biocompatible hyaluronan (HA) hydrogels are important biomaterials for tissue repair strategies due to their amenable material properties and biocompatibility. Among them, tyramine-modified HA (HA-Tyr) hydrogel is an exciting material for biomedical applications as it is comprised of naturally occurring components. In addition, its ability to be crosslinked enzymatically, with horseradish peroxidase (HRP) and hydrogen peroxide (H₂O₂), allows for control over gelation and crosslinking density.

This dissertation aimed to optimize and characterize the biophysical properties of tyramine modified hyaluronan (HA-Tyr) hydrogels and investigate their capability to serve as a biomimetic hydrogel platform for stem cell engineering and tissue repair. In addition to advancing understanding of HA-Tyr chemistry and crosslinking strategies, this work investigated how mesenchymal stem cells (MSCs) respond to HA-Tyr microenvironmental cues. First, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) was employed as conjugation strategy to synthesize HA-Tyr derivatives. The optimized derivatization process achieved accurate control over the degree of tyramine functionalization. DMTMM facilitated preparation of highly functionalized HA-Tyr derivatives, as shown by a four-fold increase in final yields compared to the traditional carbodiimide mediated HA-Tyr synthesis. The control over the degree of tyramine substitution allowed for precise tailoring of the physico-chemical properties of the crosslinked HA-Tyr hydrogels. Toward spatio-temporal control of the gelation process, photocrosslinking of HA-Tyr hydrogels by tyramine oxidation was introduced. The visible light induced crosslinking was shown as a complementary tool to the previously reported crosslinking with horseradish peroxidase (HRP) and hydrogen peroxide (H₂O₂). Temporal control of gelation offered dynamic stiffening of HA-Tyr substrates and non-toxic conditions for photo-encapsulation of MSCs.

Next, the applicability of HA-Tyr as a biomimetic microenvironment to study stem cell early attachment and behavior was investigated. The crosslinking strategy (enzymatic versus photocrosslinking) of HA-Tyr hydrogels alters the biophysical cues that MSC sense and respond to. At equal initial substrate stiffness, MSCs cultured on enzymatically crosslinked HA-Tyr substrates possessed larger spreading areas and focal adhesions, whereas smaller MSCs on photocrosslinked hydrogels were more contractile and generated stronger forces to the matrix. These findings indicated that the crosslinking chemistry of covalently formed hyaluronan hydrogels is an important parameter and needs to be considered when looking at MSC adhesion and behavior.

Finally, an early osteogenic marker for MSC osteogenesis in vitro was developed, which utilizes the Runx2/Sox9 mRNA expression ratio on day 7 and reliably predicts the osteogenic potential of each particular MSC donor. This study revealed that the downregulation of Sox9 is an important indicator for MSC osteogenesis rather as opposed to indicators which focus solely on Runx2 mRNA expression. Finally, a method to observe Runx2/Sox9 mRNA expression in individual live cells was established, which can be a valuable tool for studying MSC differentiation on HA-Tyr substrates.
Taken together, this dissertation generated a versatile HA-Tyr hydrogel platform that is an attractive matrix for stem cell engineering and for tissue engineering.
Zusammenfassung

Hydrogеле, die aus biokompatibler Hyaluronsäure (HA) bestehen sind, aufgrund ihrer günstigen Material Eigenschaften und Biokompatibilität, von großer Wichtigkeit in Bereich der Regenerativen Medizin. Im Bereich dieser Hydrogels, sind Tyramin-modifizierte HA (HA-Tyr) Hydrogеле sehr vielversprechend für biomedizinische Anwendungen, da sie vollständig aus natürlich vorkommenden Komponenten bestehen. Zusätzlich erlaubt die enzymatische Vernetzung mit Meerrettichperoxidase (HRP) und Wasserstoffperoxide (H₂O₂) exzellente Kontrolle über die Herstellung und die Stärke der Gel-vernetzung.

Das Ziel diese Dissertation war die biophysikalischen Eigenschaften von HA-Tyr Hydrogelen zu optimieren und eine biomimetische Hydrogel Plattform zu etablieren, die es erlaubt grundlegende Experimente mit Stammzellen durchzuführen und Reparaturstrategien von verschiedenen Gewebearten zu untersuchen. Zusätzlich beabsichtigte diese Arbeit die Grundlagen der HA-Tyr Synthese und Hydrogel Präparation zu verstehen um damit die Reaktionen und Verhaltensstrukturen Mesenchymaler Stammzellen (MSCs) auf HA-Tyr Hydrogelen zu untersuchen. Im ersten Schritt wurde 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) als Konjugations-Strategie für die Synthese von HA-Tyr Derivaten angewendet. Im Gegensatz zur traditionellen Konjugation mit Carbodiimiden, gelang es mit Hilfe von DMTMM den Modifizierungsgrad von HA-Tyr genauestens zu kontrollieren und zusätzlich um das Vierfache zu erhöhen. Aufgrund der akkuraten Kontrolle über die Funktionalisierung konnten HA-Tyr Hydrogеле mit sehr präzise zugeschnittenen physikalisch-chemischen Eigenschaften hergestellt werden. Um auch eine zeitliche und räumliche Kontrolle der Hydrogel Herstellung zu erreichen, wurde die licht-induzierte Vernetzung eingeführt, die aufgrund der Oxidierung von Tyramin zu kovalenten Verbindungen führt. Hier haben wir sichtbares Licht verwendet, das als eine ergänzende Möglichkeit zu der bereits bekannten enzymatischen Methode mit HRP und H₂O₂ genutzt werden kann. Mit Hilfe dieser zeitlichen Kontrolle über die Hydrogel Herstellung konnten HA-Tyr Hydrogèle graduell verfestigt werden und aufgrund nicht-toxischer Eigenschaften, MSCs inkorporiert und kultiviert werden.

Für die Anwendung von HA-Tyr Hydrogelen im Bereich der Regenerativen Medizin ist es wichtig das Verhalten von Stammzellen auf diesen Biomaterialien zu verstehen. Hier wurde gezeigt, dass die Art der Hydrogel Herstellung (enzymatisch versus licht-induziert) die biophysikalischen Eigenschaften von HA-Tyr so verändert, dass MSCs dies fühlen können und sich dementsprechend anders verhalten. Obwohl die initialen mechanischen Eigenschaften gleich waren, zeigten MSCs auf enzymatischen hergestellten Gelen ein generell grösseres Wachstumsareal und grössere Fokale Adhäsion Punkte, während die kleineren Zellen auf Licht-vernetzten Gelen kontraktiler waren und grössere Kräfte auf die Hydrogelmatrix ausübten. Diese Ergebnisse zeigen, dass die Vernetzungsstrategie von HA-Tyr Hydrogelen ein wichtiger Parameter ist und beachtet werden sollte, wenn das Verhalten von Stammzellen beurteilt wird.

Zum Schluss wurde ein frühzeitiger Marker für die osteogene Differenzierung von MSCs entwickelt, welcher auf dem Verhältnis der Runx2 und Sox9 Genexpression am Tag 7 basiert und

Zusammenfassend hat diese Dissertation eine vielseitige HA-Tyr Hydrogel Plattform entwickelt, die sehr attraktiv ist für die Erforschung von Stammzellverhalten, mit dem ultimativen Ziel Gewebe zu regenerieren.
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Early prediction of osteogenic potential of human MSCs by Runx2/Sox9 ratio

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The osteogenic potential of human MSCs can be enhanced by Interleukin-1β
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ADH</td>
<td>Adipic acid di-hydrazide</td>
</tr>
<tr>
<td>α-MEM</td>
<td>Minimum Essential Medium Eagle, alpha modification</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline-phosphatase</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDMT</td>
<td>2-chloro-4,6-dimethoxy-1,3,5-triazine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-Diamidino-2-phenyindole</td>
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<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>Di-Tyr</td>
<td>Di-tyramine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DMTMM</td>
<td>4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride</td>
</tr>
<tr>
<td>DS</td>
<td>Degree of substitution in mol % of hyaluronan dimer repeat unit</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E</td>
<td>Young's modulus</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDC</td>
<td>N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride</td>
</tr>
<tr>
<td>EGTA</td>
<td>Glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>EO</td>
<td>Eosin Y</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>FAAS</td>
<td>Focal Adhesion Analysis Server</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>FTTC</td>
<td>Fourier transform traction cytometry</td>
</tr>
<tr>
<td>G'</td>
<td>Storage modulus</td>
</tr>
<tr>
<td>G''</td>
<td>Loss modulus</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>HaNA</td>
<td>Hyaluronic acid sodium salt</td>
</tr>
<tr>
<td>HA-Tyr</td>
<td>Tyramine-modified hyaluronan</td>
</tr>
<tr>
<td>¹H NMR</td>
<td>Proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IPN</td>
<td>Interpenetrating polymer networks</td>
</tr>
<tr>
<td>Irgacure 2959</td>
<td>2-hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>Mw</td>
<td>Weight average molecular weight (Mw)</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMM</td>
<td>N-methylmorpholine</td>
</tr>
<tr>
<td>NVP</td>
<td>1-Vinyl-2-pyrrolidinone</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OP</td>
<td>Osteopermissive</td>
</tr>
<tr>
<td>PA</td>
<td>Polyacrylamide</td>
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<tr>
<td>Pa</td>
<td>Pascal</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PIC</td>
<td>Proteinase inhibitor cocktail</td>
</tr>
<tr>
<td>PIPES</td>
<td>1,4-Piperazinediethanesulfonic acid</td>
</tr>
<tr>
<td>PIV</td>
<td>Particle image velocimetry</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor γ</td>
</tr>
<tr>
<td>RB</td>
<td>Rose bengal</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>Sox9</td>
<td>Sry (sex determining region Y)-related transcription factor 9</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
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<tr>
<td>TAZ</td>
<td>PDZ-binding motif</td>
</tr>
<tr>
<td>TBA</td>
<td>Tetrabutyl ammonium</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethanolamine</td>
</tr>
<tr>
<td>YAP</td>
<td>Yes-associated protein</td>
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1 Engineering hydrogels to control cell-matrix interactions

1.1 Hydrogels as extracellular matrix mimics

1.1.1 Native extracellular matrix

The extracellular matrix (ECM) is the non-cellular component of all tissues, and provides the physical and chemical microenvironment in which cells reside. Many aspects of cell behavior and functions are regulated by the ECM, including cell proliferation, differentiation and migration, which are important for tissue morphogenesis and homeostasis. These diverse functions are achieved through a complex assembly of proteoglycans, structural proteins (e.g. collagens, fibronectin and elastin), non-structural proteins (e.g. fibronectin, laminin, and tenascin) and other components such as integrins, growth factors and proteolytic enzymes, such as matrix metalloproteinases (MMPs) (Figure 1-1). These macromolecules are typically organized into a fibrillar network that provides a unique biophysical and bioactive environment for cells. Among them, collagen is the most abundant protein in the ECM, which provides the structural strength to the network (Figure 1-1). Generally, the physical, topographical and biochemical composition of the ECM is not only tissue-specific, but also spatially heterogeneous. The unique ECM structure is generated through a biochemical and biophysical interplay between the residing cells and the evolving cellular microenvironment. The multiple ECM components also contain adhesive binding sites as anchor points for cells to probe and respond to environmental changes. These cell-ECM interactions are mainly mediated by integrin receptors, which are transmembrane glycoproteins with an extracellular domain that binds to the ECM, and a cytoplasmic domain that interacts with the cytoskeleton (Figure 1-1). Finally, within one tissue type the ECM is highly dynamic and constantly being remodeled, with particularly high remodeling rates during development. These processes are regulated by the cell-mediated secretion and deposition of molecules and degradation through cell secreted enzymes, such as MMPs.

The physical properties of the ECM, including visco-elasticity, topography and plasticity define the properties of the tissue. They can vary greatly at different stages of development, and play an important role in regulating cell behavior and tissue function. Thus, the dynamic nature of the ECM requires feedback mechanism between the cells and the microenvironment. In response to physical cues from their environment (ECM, but also adjacent cells), cells can tune their elastic moduli and remodel the local environment in order to achieve an equilibrium. The physical signals rarely exert their influence independently, but interact with biochemical cues from the microenvironmental niche in a context dependent manner. Still, a balance between exogenous
physical forces (i.e. ECM stiffness) and cellular forces (i.e. cell contractility) is essential for healthy tissue function, which is often lost in disease tissue, such as fibrosis and cancer, due to disrupted tissue structures and consequent loss of growth control. The fact that tissue-specific cells show improved proliferation and differentiation when they are cultured in microenvironments mimicking their native tissue environment strongly supports this paradigm.

Figure 1-1 Overview of the major components of the native extracellular matrix in connective tissues

1.1.2 Design criteria for ECM mimetic hydrogels

The ECM in vivo is not only a 3D network where cells physically reside, but rather a complex spatially and temporally defined microenvironment from which cells receive important cues that influence cell fate. To emulate such microenvironments in vitro, natural ECM components including collagen and fibrin can be used, however this is complicated by the fact that modulating physical properties is accompanied by a change in the concentration of intrinsic biochemical cues (e.g. adhesive ligand density). To overcome this limitation, synthetic hydrogels, including poly(ethylene glycol) (PEG) and polyacrylamide (PA), were introduced. The mechanical properties of these synthetic backbones can be modulated over a wide range and thus serve as a template for the desired presentation of adhesive ligands and growth factors and degradation kinetics. Several sophisticated in vitro platforms have been developed to unveil ECM influence over stem cell fate.

Beyond the physical cues of the structural network, such as elasticity, the ECM presents a variety of biochemical active cues. These cues are presented on ECM proteins (e.g. laminin, fibronectin), while others are diffusing through the matrix or remain sequestered or bound to
substrates. Toward recapitulating the native signals, spatially and temporally controlled presentation of biomimetic ligands has been introduced to explore cell behavior. For example, Arg-Gly-Asp (RGD) is the most extensively studied cell-adhesion peptide that is frequently incorporated into biomaterials to promote cell attachment\textsuperscript{14,15}. Peptides have also been used to mimic cell-to-cell contact by covalently attaching a binding motif from N-cadherin to the hydrogel matrix\textsuperscript{16,17}. Photolithography technology has enabled accurate control over the spatio-temporal distribution of molecules, for example by using light to activate specific interactions, such as streptavidin-biotin and barnase-barstar\textsuperscript{18} or by using caged peptides that are activated with light to participate in an enzyme mediated tethering of a biomolecule of interest\textsuperscript{19}. The incorporation of MMP-cleavable bonds into synthetic networks allowing cells to locally degrade and remodel their environment has also been reported to enhance cellular invasion\textsuperscript{20} and served as a tool to investigate the role of cellular tractions in \textit{in vitro} MSC differentiation\textsuperscript{21}. In addition, temporal control over substrate modulus revealed the influence of a "mechanical memory" on the differentiation of stem cells\textsuperscript{22,23}.

New hydrogels are constantly being engineered as substrates for \textit{in vitro} models and biomedical applications. In particular, owing to the expansion and optimization of existing chemistries, hyaluronan is evolving in its design to provide the multiple functionalities, and control over their biophysical and biochemical signals\textsuperscript{24}.

1.1.3 Hyaluronan hydrogels and crosslinking strategies

The ubiquitous presence of hyaluronan (HA) in tissues and organs, its biocompatibility and biodegradability make HA-based materials very useful for medical applications. Unmodified HA has been used as intra-articular viscosupplementation in osteoarthritic joints\textsuperscript{25,26}, wound healing applications\textsuperscript{27} and drug delivery\textsuperscript{28}. \textit{In vivo} pristine HA shows a high turnover with rapid degradation and lack of network formation which prevents its use as mimicking ECM matrix\textsuperscript{29}.

However, chemical modifications of HA have led to the fabrication of HA based hydrogel networks with a wide range of properties\textsuperscript{30}. Chemical modifications of HA typically target either the hydroxylic (R\textsubscript{2}) or carboxylic acid (R\textsubscript{1}) chemical group of the glucuronic acid moieties (Figure 1-2). An amino group can also be created by deacetylation of the N-acetyl group. Numerous methods have been reported to synthesize HA derivatives in water, while others use reagents sensitive to hydrolysis and thus require organic solvents, such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF)\textsuperscript{31}. Here, HA is converted first into its acidic form or a tetrabutyl ammonium (TBA) salt to be soluble in the organic solvent.

For example, HA has been modified with hydrazides (e.g. adipic acid di-hydrazide (ADH) (Figure 1-2(1)) that can react under appropriate conditions to form a HA-network or can further be modified with functional or hydrophobic groups\textsuperscript{31-33}. This method has been extended to Huisgen cycloaddition of crosslinking HA-azides and HA-alkynes with copper as catalyst\textsuperscript{34}. Recently, this approach has been modified with strain-promoted (3+2) cycloaddition crosslinking between HA-azide and HA-cyclooctyne derivatives\textsuperscript{35} to form biocompatible gels in a copper-free environment. Selective cycloaddition reactions (Diels-Alder reaction) have been shown for crosslinking of furan-modified HA (Figure 1-2(2)) with maleimide-HA (Figure 1-2 (3))\textsuperscript{36} or maleimide-PEG\textsuperscript{37}. The
formation of these hydrogels takes places in a water-based buffer, such as 2-(N-morpholino)ethanesulfonic acid (MES), and without any catalyst.

Figure 1-2 Illustration of common chemical modifications of hyaluronan (HA) to form hydrogels via covalent crosslinking

Examples of primary modifications of either the carboxylic group (R₁) with (1) adipic dihydrazide, (2) furan, (3) maleimide and (6) tyramine; or the hydroxyl group (R₂) with (4) 3,3'-dithiodipropionic acid, (5) acrylate and (6) methacrylate.

Another strategy which does not rely on additional synthetic crosslinkers uses thiolated-HA that can be crosslinked spontaneously under mild conditions via air oxidation of thiols to disulfides. Thiols can be introduced into the backbone of HA by esterification with 3,3'-dithiodipropionic acid and subsequent reduction with dithiothreitol (DTT) (Figure 1-2(4)). A new modification strategy introduced thiols through cysteine modification of the HA carboxylic group.

HA hydrogels have also been crosslinked using radical polymerization. This involves the derivatization of HA with acrylate (Figure 1-2(5)) or methacrylate (Figure 1-2(6)) groups and then the formation of a radical to initiate the polymerization of the chemical functionalities. Methacrylated HA can be crosslinked using redox initiation mechanism. Typically, ammonium persulfate (APS) spontaneously decomposes to form free radicals and in the presence of tetramethylmethylenediamine (TEMED) as a free radical stabilizer promote the polymerization of the methacrylate creating a polymeric network. Photoinitiated radical polymerization, on the other hand, uses light as the initiation trigger for radical formation and has several potential advantages, including the spatial and temporal control of the gelation. Upon light exposure, the initiator molecules dissociate into free radicals that propagate a polymerization chain. The most established photoinitiator system uses Irgacure™ 2959 (2-hydroxy-1-[4-(2-hydroxyethoxy)
phenyl]-2-methyl-1-propanone) together with UV light\textsuperscript{45-49}. Toward reducing cellular toxicity the potential/mutagenic risk of UV light, a visible light photo-initiator system has been proposed, comprised of the photosensitizer Eosin Y (EO), an initiator triethanolamine (TEA) and an accelerator 1-vinyl-2-pyrrolidinone (NVP)\textsuperscript{50-52}. However, the use of cytotoxic co-initiators, such as TEA, limits its applicability\textsuperscript{51}.

Finally, enzymatically crosslinked HA substrates have been introduced as a versatile injectable system in the field of regenerative medicine\textsuperscript{53,54}. Here, recent work utilized the transglutaminase activity of activated factor XIII to crosslink HA decorated with glutamine with HA or PEG bearing lysine containing peptide to obtain proteolytically degradable hydrogels\textsuperscript{55,56}. HA has further been modified with hydroxylphenol groups by coupling tyramine (Tyr) to the carboxylate groups of HA (Figure 1-2(7))\textsuperscript{57}. Covalent crosslinking is catalyzed by horseradish peroxidase (HRP) and hydrogen peroxidase (H$_2$O$_2$) as oxidant. Gelation time and mechanical properties can be controlled independently by varying the HRP and H$_2$O$_2$ concentration\textsuperscript{58}. Compared to other methods of forming HA-based hydrogels (e.g. HA methacrylate), a relatively low tyramine substitution (less than 5% molar) is required to form a stable network. Owing to the low modification of the HA backbone and the control over biophysical properties, tyramine derivatives of hyaluronan (HA-Tyr) have emerged as a promising material for biomedical applications.

### 1.2 Biomedical applications of Hyaluronan-tyrmine (HA-Tyr) hydrogels

In contrast to the modification of HA with synthetic crosslinkers, such as methacrylic acid, HA-Tyr comprises all naturally occurring components of mammalian tissues, which contributes to its biocompatible nature. In mammalian species, Tyr is an endogenous compound and an intermediate in the synthetic pathway of dopamine\textsuperscript{59}. The enzymatic crosslinkers HRP and H$_2$O$_2$ are also naturally occurring, with endogenous peroxidase activity that is present in many cells and H$_2$O$_2$ which is produced under physiological conditions\textsuperscript{60}.

#### 1.2.1 HA-Tyr crosslinking chemistry

The HRP mediated catalytic cycle uses the hydroxyl-phenol group of Tyr as reducing substrate\textsuperscript{61}. A catalytic cycle is initiated by the presence of H$_2$O$_2$ which interacts with the resting ferric state of HRP [Fe(III)] and generates compound I [Fe(IV)]. Compound I is an intermediate in a high oxidation-state with a cation-radical. The phenolic group of Tyr acts as a reducing agent and the compound I is converted to compound II [Fe(IV)] upon reduction. Subsequently, a second phenol group of a Tyr group is oxidized effectively reducing HRP back to its resting ferric state. The formation of two Tyr radicals can lead to covalent di-Tyr bond formation where oxidative coupling proceeds at the C-C and C-O positions between phenols\textsuperscript{62} (Figure 1-3). During the catalytic cycle of HRP, one H$_2$O$_2$ is consumed to generate two Tyr radicals. Therefore, the HRP-catalyzed reaction controls the crosslinking rate (gelation time) and degree (mechanical properties) through the H$_2$O$_2$ and HRP concentrations\textsuperscript{58}. Given that the HA backbone is sensitive to hyaluronidase mediated cleavage, the degradation kinetics of HA-Tyr hydrogels can be controlled the crosslinking density which in turn can be controlled by H$_2$O$_2$ concentration used in the enzymatic
crosslinking. A low crosslinked density leads to fast enzymatic degradation with hyaluronidase, whereas denser crosslinking results in slower degradation rates.  

**Figure 1-3 Chemical structure of HA-Tyr derivatives and di-Tyr formation**

A Chemical structure of HA-Tyr derivatives and the di-Tyr crosslinks. B Catalytic cycle of horseradish peroxidase (HRP) to form Tyr radicals that can react with each other.

Owing to the recent commercially available source of HA-Tyr derivatives, including LifeCore Biomedical and Contipro, Czech Republic, HA-Tyr hydrogels found diverse range of biomedical applications. This has led to interesting approaches particularly in the field of tissue regeneration and drug delivery. Despite the recent developments, there remains capacity for material optimization and functionalization, which will be highlighted in the next paragraph.

### 1.2.2 Tissue regeneration

In addition to the diverse biological functions and its abundance in native articular cartilage, HA is involved in MSC condensation and during cartilage development and formation. In addition, together with aggrecan, HA plays an important role in the osmotic and compressive resilience of cartilaginous tissue. Thus, HA hydrogels have been intensively investigated for cartilage tissue engineering. The existence of HA in the matrix has been found to provide bioactivity toward chondrogenesis by supporting chondrogenic differentiation lineages and matrix deposition.  

Toward further understanding of chondrogenesis in controlled in vitro conditions, MSCs have been encapsulated in photo-crosslinked methacrylated HA hydrogels with various crosslinking densities, HA weight-average molecular weight (Mw) and concentrations. It was found that the HA network influences the diffusion of nutrients throughout the hydrogel and matrix production and
deposition of encapsulated cells\textsuperscript{47,66}. Similarly, the effect of HA-Tyr crosslinking density and therefore initial mechanical bulk strength played a role in the extend of chondrogenic differentiation of encapsulated MSCs \textit{in vitro}\textsuperscript{67}. Using caprine MSCs, the authors reported enhanced chondrogenesis in soft (Young's modulus 5.4 kPa, acellular) substrates, whereas cells in stiffer hydrogels (Young's modulus 10-11.8 kPa, acellular) showed less yield in hyaline cartilage formation with a higher a ratio of fibrocartilage. Higher glycosaminoglycan and collagen production in soft hydrogels was well correlated with increased proliferation of encapsulated MSCs. It was concluded that low matrix stiffness and higher porosity of soft hydrogels support cellular condensation and thus ECM deposition and neo-cartilage formation\textsuperscript{67}. \textit{In vivo} chondrogenesis in HA-Tyr hydrogels has recently been revisited by Dvorakova and colleagues using human bone marrow isolated MSCs\textsuperscript{68}. Encapsulated human MSCs remained viable throughout the hydrogel formation; however viability rapidly decreased over 21 days. Despite some enhanced gene expression of chondrogenic markers, little deposition of proteoglycans and increased expression of hypertrophic markers (type X collagen, osteopontin) were found. Interestingly, in a second study, the viability of encapsulated chondrocytes was found to remain high for up to 3 weeks\textsuperscript{69,70} and led to depositions of pericellular and territorial matrix\textsuperscript{70}. HA-Tyr constructs laden with rat MSCs appeared to degrade rapidly in subcutaneous pockets of rats, and showed complete disintegration after 12 weeks\textsuperscript{68}. Acellular HA-Tyr constructs were also implanted subcutaneously after lyophilization and remained mostly non-resorbed after 12 weeks, however with early signs of inflammation and infiltration of inflammatory host cells. In a next step, lyophilized acellular HA-Tyr constructs have been investigated for \textit{in vivo} osteochondral defect healing in a rabbits\textsuperscript{70}. Beside significant signs of inflammation, HA-Tyr delayed the healing process compared to untreated defects. Earlier, a pilot study conducted by Darr and colleagues found that hydrogel plugs made of high Mw HA-Tyr (Corgel\textsuperscript{®}, 0.625-10% (w/v)) retained their shape after 12 months subcutaneously implanted in rats and showed only partial volume loss for 0.625 and 1.25% (w/v) hydrogels with minimal inflammatory response\textsuperscript{71,72}. However, it should be noted that a high viscosity is expected for high Mw HA-Tyr precursor solutions, which may have made the mixing difficult and resulted in non-homogenous crosslinking.

The results of these \textit{in vivo} studies are complicated to evaluate and compare by a lack of HA-Tyr crosslinking density values \textit{in vitro}\textsuperscript{68} and quantification of \textit{in vivo} histological observations\textsuperscript{68,71}. In addition, it remains unclear if the HA-Tyr hydrogel properties, such as the HA molecular weight, were influenced by the preparation method of HA-Tyr derivatives (cyanogen bromide inacetonitrile\textsuperscript{68} and carbodiimide\textsuperscript{69}). Therefore, to draw a conclusion about the potential of HA-Tyr hydrogels for cartilage regeneration, the influence of HA-Tyr Mw, concentration and mechanical strength on \textit{in vivo} stability, host immune response and cell migration need to be investigated in an appropriate \textit{in vitro} system to understand influences of multiple parameters before performing meaningful \textit{in vivo} model.

HA-Tyr has further been proposed as resurfacing treatment in articular cartilage early-stage osteoarthritis\textsuperscript{73}. The authors reported restoration of mechanical functions when HA-Tyr was applied to the surface of collagenase-degraded cartilage explants. Interestingly, UV-crosslinking
with riboflavin as photoinitiator allowed viable chondrocyte encapsulation and stabilized the loss of mechanical function in an ex vivo model. However, the properties of the photo-crosslinked HA-Tyr are difficult to evaluate due to a lack of crosslinking density characterization and comparison to enzymatically crosslinking. Di-Tyr formation under UV exposure was investigated in a mixture of tyramine and riboflavin (pH 3) and analyzed with liquid chromatography and mass spectroscopy. Despite long illumination times (12-60 min) required for 2%-9% di-Tyr formation, the chemistry behind the crosslinking was not further described.

HA-Tyr has also been used for augmentation of decellularized fascia lata in a rat model. The authors aimed for enhanced mechanical strength and reduced inflammatory response by treating the fascia with enzymatically crosslinked HA-Tyr of high Mw. However, the HA-Tyr treated fascia showed strong foreign body response with signs of chronic inflammation one and three months post-implantation. Here, high Mw HA-Tyr was not sufficient in suppressing host inflammatory response, which is complicated to evaluate considering that there may be a potential response to the human fascia lata transplants more than the biomaterial itself. Although the described system was not effective in restoring the mechanical functionality of the fascia, it highlights a potential limitation for enzymatically crosslinked hydrogel systems: The addition of H₂O₂ for in-situ gelation of HA-Tyr may have degraded the surrounding ECM or could have potentiated the activation of endogenous tissue peroxidases that may be detrimental for the adjacent tissue and mechanical integration.

Finally, for stem-cell based therapies it is desirable to develop hydrogel systems that provide a 3D microenvironment that mimics the stem cell niche and thus facilitates growth and self-renewal of encapsulated stem cells. Within the Matrigel®-free HA-Tyr 3D environment of low mechanical strength (G' 82 Pa), human embryonic stem cells (ESCs) showed high viability and self-renewal capacity in vitro. Cell clusters were harvested using hyaluronidase and implanted subcutaneously in mice. The retained pluripotent differentiation capability and genetic integrity of ESCs demonstrated the potential of HA-Tyr as 3D culture microenvironment for primary cells.

Taken together, the implantation of HA-Tyr hydrogels in vivo has led to contradictory results, which may be caused by different strategies that have been used to synthesize HA-Tyr and a lack of characterization of the derivatives and formed HA-Tyr gels. To harness the potential of HA-Tyr for regenerative strategies, a systemic understanding of the physico-chemical properties in relation to cellular interactions is needed.

1.2.3 Molecule delivery
Several hydrogels have been studied as delivery systems for therapeutic proteins with spatio-temporal control. Although the administration of therapeutic proteins though the parenteral route shows high bioavailability, the delivered proteins degrade and diffuse into the blood stream very rapidly. Injectable hydrogels have become important as they can be administered minimal invasively and provide a protective matrix for the encapsulated hydrophilic drugs. To achieve the desired therapeutic concentration and biological effect, hydrogels with sustained release of the administered proteins have been developed.
Owing to the control over the gelation time and crosslinking density, HA-Tyr hydrogels were developed as delivery system for controlled release of small hydrophobic drugs and proteins. Faster gelation times increased the retention of the hydrogel at the injections site, which could be controlled independent of the crosslinking density\(^79\). Different release rates of proteins, such as β-amylase and lysozyme, and dexamethasone as a model drug were realized by varying the crosslinking density of injected HA-Tyr hydrogels\(^81,80,81\). Building upon this work, Kurisawa and co-authors incorporated hyaluronidase into HA-Tyr hydrogels to increase the cumulative release of the monoclonal antibody trastuzumab\(^82\). Cell number and tumor growth were decreased in this system, in which the concentration of hyaluronidase controlled the degradation of the hydrogel matrix and therefore the release rates of trastuzumab. This strategy can potentially be applied for other molecules or drugs, however, the degraded HA fragments will have to be investigated with regard to their inflammatory and angiogenic effect\(^83\).

1.2.4 Composite hydrogels

To extend the diversity and functionality of HA-Tyr hydrogels, new compositions, such as mixtures of micro- or nanoparticles with HA-Tyr or dual polymer networks were developed. For example, HA-Tyr macromers can be combined with graphene oxide to create an injectable nanocomposite hydrogel with improved mechanical properties\(^84\). The authors reported an increase from 950 Pa to 1800 Pa when 2% graphene oxide was added to 2% HA-Tyr and crosslinked with 0.7 mM \(\text{H}_2\text{O}_2\). Another system consists of hydroxyapatite nanocrystals mixed with HA-Tyr to form an injectable paste\(^85\). The addition of the apatite increased the mechanics and allowed tunability in mechanics upon changing the apatite loading and \(\text{H}_2\text{O}_2\) concentration. Recently, interpenetrating polymer networks (IPN), characterized by topological interlocking of two or more independent polymer networks, have gained attention owing to their additive properties compared to a single polymer networks or composite hydrogels\(^86\). HA-Tyr has been crosslinked within a polymerized fibrin gel and supported the proliferation of encapsulated fibroblasts as it could resist cell-induced contraction and degradation of the network compared to single fibrin gels\(^87\).

HA-Tyr hydrogels have found several applications in tissue engineering and as injectable materials for tissue replacement or molecule delivery. However, the use of HRP and \(\text{H}_2\text{O}_2\) may prevent its approval as a clinical product, which illustrates a demand for alternative crosslinking techniques. Additionally, due to the relatively low Tyr functionalization, HA-Tyr materials are mechanically weak, which may limit further applications. Thus, there is a need to improve the derivatization technique in order to extend the range of substituted Tyr on HA, and increase the di-Tyr crosslinking density. A rational design of HA-Tyr hydrogels is necessary to control hydrogel physico-chemical cues and biology, which also includes a better understanding of how the degree of Tyr substitution influences the formation of di-Tyr crosslink and hydrogel degradation.
1.3 Matrix design and stem cell fate

Multiple biochemical and physical cues cooperate in controlling cell behavior during in vivo tissue development and homeostasis. Several approaches have emerged to elucidate the material parameters which influence cell behavior. Stem cells are of particular interest toward the engineering of biomedical materials with cell-instructive properties. Characterized by their ability to replicate and self-renewal, stem cells contribute to tissue morphogenesis and repair and have long been recognized as primary source for the regeneration of degenerated and disease tissue. However, as discussed earlier, the in vivo niche is a complex array of signals instructing cell behavior and differentiation of the residing stem cells. A comprehensive understanding of these cues is indispensable to harness the sensitivity of stem cells toward functional tissue regeneration.

1.3.1 Mesenchymal stem cells characterization

Due to the controversies regarding the use of embryonic stem cells, adult stem cells have been discovered and characterized as a pool of multipotent cells for clinical applications. Among these cells, mesenchymal stem cells (MSC) have shown considerable promise for their therapeutic use in regenerating damaged or diseased musculoskeletal tissue. MSCs are spindle-shaped, adherent, non-hematopoietic stem cells that have the capacity to divide and proliferate in vitro. Many studies have shown that MSCs are residual cells in the connective tissue of almost all organs. In addition to bone marrow, MSCs have been isolated from many other tissue, such adipose tissue, skeletal muscle, umbilical cord, and synovium. Typically MSCs are isolated from bone marrow after removing the non-adherent cell fraction. No specific marker is available for MSCs; however they express a specific set of CD (cluster of differentiation) molecules. The phenotypic pattern to identify a MSCs is a combination of positive markers, including CD73, CD90 and CD105 and negative markers, such as hematopoietic and endothelial markers (CD45, CD34, CD19 and CD31). One of the defining characteristics of bone marrow derived MSCs is their ability for self-renewal, the ability to produce identical copies through mitotic divisions in vitro. However, MSCs and isolated MSC clones are a heterogeneous population regarding their self-renewal capacity. It has long been known that MSCs are multipotent cells that can give rise to a wide range of cell types upon their differentiation ending with a distinctive cell-type from a mesenchymal origin. Thus, an important criterion for the definition of MSCs is the differentiation into adipocytes, chondrocytes and osteoblasts in vitro. The in vitro differentiation of MSCs into osteoblasts, adipocytes and chondrocytes is shown by the respective mineralization, oil droplet formation and expression of collagen type II and is commonly evaluated by immune-cytochemical, histochemical and gene expression analyses (Figure 1-4). Similar to the self-renewal, populations of MSCs are heterogeneous in terms of their multilineage potential. The commitment and differentiation of MSCs to specific mature cell types is a highly controlled process and the analysis of in vitro MSC differentiation have identified intracellular transcription factors that are essential for stem cell commitment. Among them Runx2, peroxisome proliferator-activated receptor γ (PPARγ) and...
Sox9 play important roles in driving MSC toward distinct lineages and maintain their differentiated phenotype\(^{102}\). For example, the Sry-related transcription factor Sox9 is mainly described as the key regulator for chondrogenesis and expressed in resting and proliferating chondrocytes\(^{103,104}\). The Runt-related transcription factor Runx2 is one of the most studied transcription factors expressed in MSCs upon their commitment toward osteogenesis and has been identified as positive regulator of osteoblast differentiation\(^{105}\).

![Figure 1-4](image)

**Figure 1-4 In vitro differentiation of monolayer expanded human MSCs**

Human MSCs were differentiated into chondrogenic, adipogenic and osteogenic phenotypes to assess multipotency (modified from Gardner et al., 2015)\(^{100}\). A Chondrogenic differentiation was assessed using Safranin O/Fast Green staining, B adipogenic differentiation assessed using Oil Red O staining, and C osteogenic differentiation with Alizarin Red S staining (scale bar 500 µm).

### 1.3.2 Substrate mechanics direct stem cell adhesion and fate in 2D

Toward engineering cell-instructive materials, the mechanical environment has been shown as potent regulator of stem cell fate. *In vivo*, cells adhere to the ECM via several different cell surface receptors, but one of the main components is the integrin-based cell adhesion\(^{106}\). These adhesions connect ECM proteins to the cytoskeleton and enable cells to sense their surrounding matrix by pulling and pushing on it, which in turn generates biochemical activities that allows the cell to respond in an appropriate manner. This process is known as mechanotransduction and has been implicated in regulating cellular functions, such as cell shape and spreading, proliferation and differentiation.

There is a great amount of evidence that the stiffness of a biomaterial influences stem cell fate and that changes in the bulk stiffness of the underlying matrix elicit different responses in stem cell populations\(^{9,107-110}\). Generally, cells spread more and develop larger focal adhesions and actin stress fibers on rigid substrates compared to softer substrates\(^{111-113}\). Whereas rigid substrates stimulate the assembly of actin filaments at large focal adhesions and the formation of stress fibers, this activity is much weaker on soft substrates, which accounts for smaller adhesion sites and the lack of large actin bundles\(^{114}\). Substrate rigidity has also a profound influence on stem cell differentiation pathways\(^9\). Specifically, the differentiation of MSCs toward distinct lineages is regulated by the stiffness of the substrate, which approximates the normal mechanical environment. For example, osteogenic differentiation is generally favored on stiff substrates, on which MSCs exhibit high cytoskeletal tension\(^{102}\), whereas adipogenic differentiation is promoted on soft substrates\(^9\).
Toward understanding how cells perceive the physical constraints from their microenvironment, the activity of Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) transcriptional regulators has been implicated as the key in cellular mechanoresponsiveness. The transcriptional activity of YAP/TAZ is tightly linked to the nuclear transduction of mechanical and cytoskeletal signals which are necessary for transcriptional and phenotypic changes of the cell. These studies revealed that YAP/TAZ is activated and thus localized in the nucleus in MSCs on stiff substrates ($E = 15\text{ - }40 \text{ kPa}$) and deactivated in the cytoplasm of MSCs cultured on soft substrates ($E = 1 \text{ kPa}$). It has also been shown that YAP/TAZ directly modulates MSC osteogenic differentiation by binding to and potentiating Runx2 activity, while TAZ inhibits adipogenic differentiation via PPARγ complexing.

1.3.3 Revealing the heterogeneity of cell-matrix interactions

The stiffness of a material is governed by a variety of different components, such as porosity, geometry and nanotopographical features. The relationships of these factors are challenging to decouple and are also affected by how the matrix stiffness is altered. To understand the underlying relationship between matrix structure and cell function, new materials with patterns, topographies and fibers at different scales have been designed to probe how cells respond to changes in their biophysical environment.

For example, restricting cells to spread on a specific shape of the surface directs cell proliferation and cell shape and can even direct MSC lineage commitment toward osteogenesis or adipogenesis (Figure 1-5). It has been found that the cell shape is a key factor in MSC differentiation, and that it is linked to cytoskeletal mechanics (e.g. cellular tension). For instance, cells cultured in shapes which promote cytoskeletal tension (rectangle, large size islands) are committed to osteogenesis while those on round shaped, small islands prefer to undergo adipogenesis. Similarly, lamellar shaped surfaces were found to enhance cellular tension and osteogenesis whereas MSCs on smaller hexagonal structures showed rather adipogenic differentiation. Recent studies highlighted the effect of nanotopography on cell behavior. For example, Chen et al. found that smaller (e.g. 1 nm) rather than greater (e.g. 100 nm) nano-rough topography promote cell spreading. A high level of disorder in the presentation of nanopits was observed to enhance cellular tension in human MSCs compared to ordered nanopits and thus inducing downstream MSC osteogenesis. These nanotopographic features directly affect the presentation of ligands in terms of spacing/density and consequently the interaction of cells with integrin receptors (Figure 1-5). It has been proposed that a disordered structure allows more integrins to be gathered under a cell, which promotes adhesion formation and increased intracellular tension. How the cells anchor themselves on the substrates and which biophysical cues (e.g. local stiffness) are sensed by the cell has gained increasing attention. For example, by varying the distance of anchored collagen and fibronectin to polyacrylamide substrates with different pore sizes, Trappmann and colleagues found that cell spreading was enhanced on substrates with shorter anchoring distances, which cells interpret as stiff adhesion independent of the bulk stiffness (Figure 1-5).
Recent work also identified a role of substrate stress-relaxation in cell spreading and differentiation\textsuperscript{131,132}, and the ability of cells to mechanically cluster the covalently linked adhesive ligands has been associated with increased cell spreading\textsuperscript{133-135}. Aiming to recapitulate the fibrous structure of collagen as biological ECM, scaffolds that present fibers on which cells adhere and can be controlled for their mechanical properties were fabricated from methacrylated dextran\textsuperscript{136}. Different with cell behavior on flat surfaces, MSCs cultured on substrates with soft fibers showed enhanced spreading and focal adhesion. Low fiber stiffness allowed the cell to physically reorganize the anchor points of the matrix and thus accumulate more ligands at the cell surface\textsuperscript{136}.

Figure 1-5 Stem cell fate decisions can be affected by properties of the substrate

Influence of stem cell fate decision by material properties (shown by a 2D polymeric matrix) close to the cell-material interface, such as nanotopographical features, molecular flexibility of the anchoring points, stiffness (shown by force vectors) and restricted geometric shape\textsuperscript{137,138}.

In a mimicking three-dimensional (3D) environment, the cell-material interactions gain in complexity similar to the case \textit{in vivo}. Additional considerations include the restrictions in access to soluble factors, such as nutrients and oxygen, and constraint in cell spreading, proliferation and matrix deposition due to the surrounding matrix network. Thus, the mechanisms of cells sensing their mechanical environment may well differ in 2D and in 3D\textsuperscript{139}. It was reported earlier that encapsulated MSCs need to rearrange the integrins of their surrounding matrix to undergo osteogenesis. MSC osteogenesis occurred in RGD-modified alginate gels of intermediate stiffness (11-30 kPa), whereas adipogenesis was found in softer substrates (2.5-5 kPa)\textsuperscript{134}. The ability of MSCs to rearrange the integrins of their surrounding matrix was most favorable at
intermediate substrate stiffness. Building upon this work, the authors aimed to recapitulate the viscoelastic properties of the ECM structural components compared to the elastic nature of most of the mimicking materials used to date. MSCs that were cultured in stress-relaxing alginate gels exhibited enhanced osteogenesis\textsuperscript{140}. Probing cell-ECM interactions by using FRET imaging, the authors confirmed that osteogenesis in a 3D matrix requires integrin clustering\textsuperscript{140}. These findings demonstrate the role of various cues within the ECM ranging from micro- to nanoscale and that it is not completely understood how cells sense and integrate bulk stiffness with local matrix properties. This is particularly important since the mechanical properties of a bulk material could differ from the cues that cells encounter at micron-scale. Additionally, comparisons between studies appear difficult as many different substrates and crosslinking strategies have been used and most often the recently discovered matrix cues were not characterized in previous works.

1.3.4 HA-hydrogels for investigating cell-matrix interactions

Considering the role of HA in many developmental biological stages, studying the mechanosensing and mechanoresponse of cells on or within HA hydrogels has been a major interest. Although HA mediates several cell functions through interactions with cell surface receptors and HA-binding proteins, cell-anchor points are necessary for integrin mediated cell-matrix interactions. Thus, additional functionalization is often required and allows for independent control of biochemical and mechanical properties. As such, the broad range of HA chemistries contributed to considerable progress that has been made using ECM-mimetic hydrogels to investigate microenvironmental factors that influence cell fate\textsuperscript{141}.

For topographical, mechanical or biomolecular modulations of HA hydrogel properties, post-processing has become an interesting tool towards spatial control of hydrogel surfaces. Light-mediated reactions have been used for addition reactions of HA-hydrogels characterized by spatially varied mechanical properties and presentation of biomolecules. For example, HA has been functionalized with norbornene groups reacted with dithiols that could be used to sequentially to incorporate multiple peptides into the hydrogel or increase the stiffness with spatial control\textsuperscript{142}. Light has also been used to pattern cell-adhesive regions, where UV light activates photo-active caged RGD peptides incorporated into a ADH-modified HA hydrogels (Figure 1-2(1)) to spatially regulate cell growth\textsuperscript{143}.

Additionally, HA hydrogels have advanced the development of ECM mimetic matrices capable of promoting hydrogel remodeling with spatial control. Several studies showed that a low HA macromer concentration and network density enhances cell survival and differentiation\textsuperscript{47,65,66}. However, in the native ECM, the cells can migrate via localized matrix degradation by matrix metalloproteinase (MMP), which further allows for the deposition of produced matrix. Toward understanding these cellular processes, HA hydrogels have been modified with MMP degradable bonds\textsuperscript{21,144-147}. For example, to spatially manipulate the cellular microenvironment in HA matrices, acrylated HA (Figure 1-2) was reacted with the thiol groups of MMP-degradable peptides in the presence of cells, and secondary UV-crosslinking allowed for spatial control over stiffness in the photo-patterned RGD-modified HA hydrogel\textsuperscript{146,147}. The authors found that cells encapsulated in
the non-UV crosslinked soft regions were highly spread and degraded their matrix environment, whereas cells in the secondary UV-crosslinked regions maintained a round-shape. This system was also applied to elucidate the role of vascular remodeling by bone marrow-derived endothelial colony-forming cells in vitro.

Beyond control of material structure, biomatrices evolved from the traditional static design to those that exhibit dynamic properties. Despite photo-degradable substrates, HA substrates have pioneered the ability to dynamically vary cell-material interactions through stiffening of the matrix in a defined manner. Methacrylated HA was first crosslinked by a Michael-type reaction with DTT (Figure 1-2(6)) and then its mechanical stiffness was further tuned by secondary UV crosslinking. The authors found that the substrate stiffness affected differentiation of MSCs cultured upon the hydrogels and enhanced osteogenic lineage commitment if stiffening was induced early when the majority of cells were still responsive to the change. In contrast, adipogenesis was preferred the longer the cells were grown on soft substrates. Similarly, MSCs on a macroporous (250 μm) HA network were mechanosensitive with increased proliferation and spreading on stiffer matrices and differential gene regulation upon UV-initiated stiffening. In a different approach, temporal stiffening was introduced in HA-PEG hydrogels as a platform to investigate cardiomyocyte differentiation more closely mimicking native tissue maturation. HA hydrogel stiffening could be tuned by the molecular weight of the PEG crosslinker as a higher molecular weight reduces the swelling ratio and thus induces faster stiffening. It should be noted that the HA-PEG hydrogels were coated with a collagen solution to allow cell adhesion.

Functionalization of HA hydrogels with full length proteins has also been reported by other authors; however the confounding contribution of the ligand presentation has been described to influence cellular mechanosensing, which may impede the interpretation of the outcomes.

An important step toward understanding MSC spreading and downstream osteogenesis within a given elastic modulus was performed in MMP-degradable HA hydrogels. The study revealed that MSCs in a non-degradable HA network maintain a round morphology and differentiate into adipocytes independent of the matrix rigidity. The introduction of cleavable crosslinks made the HA backbone degradable which was needed for the incorporated cells to undergo osteogenesis. Interestingly, stiffening of the matrix after 7 days inhibited osteogenesis although without changes in cell shape.

1.4 Motivation and scope of this thesis

HA gels have been widely engineered as cell carriers and investigated as platforms to study cell-material interactions and control cellular behavior. Often HA has been modified with methacrylated groups to allow precise control over biophysical properties, such as sequential stiffening of methacrylated HA to dose the mechanical signals for cell differentiation in 2D or to irreversibly constrain cell movement by forming covalent bonds in 3D. However, a highly modified backbone and their unreacted acrylates may impair biofunctionality, and the use of UV light may be harmful to some cell types. Thus, HA-Tyr hydrogels may serve as an alternative
platform to study the interaction between cells and bioactive HA. Such an approach requires a rational design of HA-Tyr matrix properties to achieve the desired control over the cellular microenvironment. However, little work has been performed to understand the HA-Tyr conjugation and fully characterize the synthesized HA-Tyr derivatives. To establish such a platform further necessitates a better understanding of the influence of Tyr derivatization on the physico-chemical properties of HA-Tyr hydrogels. The focus of this thesis encompasses the optimization and characterization of HA-Tyr derivatives and the formed hydrogels. Second, HA-Tyr matrices are investigated as a platform to study stem cell behavior and fate (Figure 1-6).

First, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) is introduced as a more efficient coupling strategy for synthesizing HA-Tyr. DMTMM is a relatively new coupling agent for HA conjugation and its advantages over traditional carbodiimide chemistry are demonstrated. The characterization of HA-Tyr derivatives is then presented in terms of Tyr functionalization of HA and formation of non-functional adducts. The DMTMM conjugation strategy enables precise control over the degree of Tyr substitution and the biophysical properties of the formed HA-Tyr hydrogels. In a next step, the mechanical properties of crosslinked HA-Tyr hydrogels are improved by an optimized DMTMM conjugation strategy, which yields in highly functionalized HA-Tyr conjugates. In addition, the cell-compatibility of HA-Tyr hydrogels is demonstrated with high viability and cellular spreading of encapsulated human MSC obtained for a variety of Tyr modification and H₂O₂ concentrations.

Toward spatially and temporally control of HA-Tyr formation on micrometer-scale, light-mediated hydrogel patterning of HA-Tyr is introduced. HA-Tyr hydrogels are fabricated via a rapid photosensitized process using visible light illumination. This strategy is complementary to the
enzymatic (HRP/H₂O₂) crosslinking and allows for matrix stiffening and sequential crosslinking of HA-Tyr matrices. In addition, the visible light triggered gelation of HA-Tyr offers non-toxic conditions for viable encapsulation of human MSCs.

Next, the significance of HA hydrogels as cell-instructive materials has motivated to investigate the influence of different HA crosslinking chemistries and gelation mechanisms on MSC behavior. Here, RGD functionalized HA-Tyr constructs are prepared enzymatically, light crosslinked or these gelation mechanism are combined by sequential crosslinking. Monitoring cell behavior on HA-Tyr substrates shows that the crosslinking chemistry of soft HA-Tyr hydrogels at a given rigidity has substantial impact on MSC behavior.

Finally, viewing future applications of HA hydrogels in controlling MSC fate, MSC commitment toward different lineages, particularly osteogenesis will have to be assessed. Current investigations on stem cell osteogenic differentiation rely on the expression of Runx2 as an early marker of in vitro and in vivo osteogenesis. However, several studies have implicated that Runx2 is not a reliable marker for MSC osteogenesis in 2D. Specifically, in vitro assays are hampered by the lack of early markers evaluating the mineralization potential of MSC.

Therefore, in the last part of this thesis, an early marker for reliable prediction of the osteogenic potential of MSC donors is presented. In vitro differentiation assays of human MSCs suggest that Sox9 downregulation is a major regulator of direct osteogenesis. Furthermore, screening of (not pre-selected) human MSC donors show that the Runx2/Sox9 ration is a promising in vitro screening method for prediction of MSC osteogenicity and can be applied for live monitoring using SmartFlare™ technology.
2 Precise tailoring of tyramine-based hyaluronan hydrogel properties using DMTMM conjugation


Cited by 5 (Google Scholar June 2016)
2.1 Abstract

Injectable tyramine modified hyaluronan (HA-Tyr) hydrogels which are bio-orthogonally cross-linked with horseradish peroxidase (HRP) and hydrogen peroxide (H$_2$O$_2$) are excellent candidate biomaterials for drug delivery, regenerative medicine and tissue engineering. Ligation of tyramine to HA has been reported using the very well established N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) chemistry. Here we demonstrate the applicability of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) as an alternative coupling agent to synthesize HA-Tyr conjugates. The optimized derivatization process allows accurate control of the degree of substituted Tyr on hyaluronan (DS). Hence, viscoelastic properties, in vitro swelling and enzymatic digestion profiles of the crosslinked hydrogels can be precisely tuned via DS. Our study demonstrates the advantages of DMTMM conjugation as a powerful tool to synthesize HA-Tyr hydrogels with properties exactly tailored for biomedical applications.

2.2 Introduction

Hydrogel-based biomaterials can mimic certain features of the extracellular matrix (ECM) of a tissue, provide an instructive three-dimensional (3D) microenvironment and serve as an effective delivery system for cell-based therapies\textsuperscript{152}. Amongst the biopolymers serving as hydrogel backbone, hyaluronan (HA) plays a significant role in tissue development, but also in the biomedical field as one of the most commonly used biopolymers. It is ubiquitous, non-immunogenic, and a natural component of the extracellular matrix of various connective tissues with a key function in wound healing and the regeneration of these tissues\textsuperscript{153}. A major development in hydrogel-based technologies is the creation of an in-situ crosslinking mechanism which enables the system to be injectable, thus allowing an aqueous mixture of gel precursors and bioactive agents to be administered through a needle\textsuperscript{154}. In order to achieve the desired biomedical performance, hydrogels must display precise viscoelastic, swelling and degradation properties.

Recently, an injectable and biodegradable hydrogel system comprising hyaluronan-tyramine (HA-Tyr) conjugates has been reported for drug delivery and tissue engineering applications\textsuperscript{155-157}. HA-Tyr is enzymatically cross-linked to give a covalent network. The coupling is catalyzed by horseradish peroxidase (HRP) and uses hydrogen peroxide (H$_2$O$_2$) as oxidant. Unlike other methods of preparing HA-based hydrogels, a relatively low tyramine (Tyr) substitution is required to form stable constructs, leaving the majority of HA disaccharides unmodified. The crosslinking mechanism enables independent tuning of mechanical properties and gelation rate by varying the H$_2$O$_2$ and HRP, respectively\textsuperscript{155-157}. Additionally, crosslinking conditions are bio-orthogonal, i.e. mild, non-toxic and do not interfere with vital cellular processes. The formation of di-tyramine (di-Tyr) and di-tyrosine occurs under the influence of H$_2$O$_2$ and HRP\textsuperscript{158}. Thus, Tyr-tyrosine bonds
could be formed during in vivo crosslinking of the Tyr containing hydrogel onto the tyrosine-containing ECM proteins and provide improved tissue-hydrogel adhesion\textsuperscript{159}. HA-Tyr synthesis has been reported using N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Although very well established as method for HA conjugation, this carbodiimide chemistry has limitations. EDC acts as condensing agent, but if used alone it brings to the formation of an active intermediate which spontaneously rearranges to a non-reactive O-acyl isourea. This self-quenching can be avoided with the use of NHS, which forms a reactive NHS ester of HA reactive towards amines. NHS-activated HA displays maximum reactivity at pH 7-8\textsuperscript{160}. Unfortunately, Tyr is highly unstable at this pH. Moreover, formation of by-products has been observed\textsuperscript{155}. Therefore it is not clear whether EDC/NHS can achieve the specific control of derivatization which is needed for the preparation of HA-Tyr derivatives suitable for biomedical applications. Similar to carbodiimides, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) was initially developed for peptide synthesis. It has been shown that DMTMM is a very efficient agent for condensing a variety of carboxylic acids and amines\textsuperscript{161-164}. With these points in mind, we have investigated the use of DMTMM for the preparation of HA-Tyr hydrogels with precise rheological, swelling and degradation properties.

Since DMTMM is a relatively new method for HA conjugation and little is known about the stability of DMTMM in water we first investigated this aspect. Secondly, we optimized the reaction conditions through the variation of the stoichiometric ratios in order to obtain the most favorable conversion yield and desired substitution. The effect of temperature on the reaction kinetics was also investigated. Finally, we characterized the viscoelasticity, swelling behavior and degradation profile of hydrogels obtained from derivatives with different substitution.

2.3 Experimental

2.3.1 Materials

Hyaluronic acid sodium salt from \textit{Streptococcus equi} (HANa) with weight-average molecular weight \textit{M} = 290kDa and polydispersion index \textit{M} = 1.86, where \textit{M} indicates the number-average molecular weight 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 (Zwijndrecht, Belgium). Hydrogen peroxide was purchased from LifeCore Biomedical (Chaska, MN U.S.A). Other chemicals were of analytical grade, purchased from Sigma Aldrich (Buchs, Switzerland) and used as received.

2.3.2 DMTMM Stability in water

DMTMM stability was characterized using hydrogen-1 nuclear magnetic resonance (\textsuperscript{1}H NMR). Degradation of DMTMM was followed at 37°C in deuterium oxide for up to 48 h. A series of \textsuperscript{1}H-NMR spectra were acquired on a Bruker Avance AV-500 NMR spectrometer without residual HOD peak suppression. Percentage of degradation product was calculated by peak integration.
2.3.3 Synthesis and characterization of HA-Tyr conjugates

HA-Tyr was prepared by amidation of the carboxylic groups of HA with the amine groups of Tyr. Briefly, HANa (500 mg, 1.25 mmol carboxyl groups) was hydrated in 2-morpholinoethane sulfonic acid (MES) buffer (100 mM, pH 5.5, adjusted with NaOH 5M) for 24 h at a final concentration of 1% (w/v). Subsequent to DMTMM, tyramine was added drop wise to the solution. The concentration of DMTMM and Tyr was varied to examine differences in Tyr substitution and hydrogel properties as a function of the molar ratio of Tyr, DMTMM and HA, respectively. The reaction was maintained either at 24°C (RT) or 37°C under continuous stirring. Aliquots for precipitation were withdrawn after 2, 6, 10, 24, 48, 72, 96 and 120 h. Following enrichment of the solution with 8% vol saturated sodium chloride; the product was precipitated using 96% ethanol. Several wash steps were subsequently performed and then the product was filtered and kept under vacuum for 48 h. The absence of chlorides in the final precipitate was assessed via Ag⁺ assay. Finally, HA-Tyr was obtained as fine-grained white powder and stored at RT.

For comparison, HA-Tyr was synthesized via conventional carbodiimide chemistry. Briefly, HANa (500 mg, 1.25 mmol carboxyl groups) was hydrated in MES Buffer (100 mM, pH 5.5, adjusted with NaOH 5M) for 24 h at a final concentration of 1% (w/v). EDC and NHS (1/1 to HA) were added and kept stirring for 1 h at RT. Tyr (1/1 to HA) was dissolved in MES-Buffer (100 mM, pH 5.5; 0.1% w/v) and added drop wise to the HA-solution. The reaction was maintained at RT for 24 h under continuous stirring. Products were either dialyzed (100 mM sodium chloride for 12 h and distilled water for additional 60 h) and lyophilized or precipitated as described above.

Synthesized HA-Tyr conjugates were characterized using UV-vis and ¹H NMR. UV spectra were recorded using a Multiskan™ GO Spectrophotometer in cuvette mode with 2 nm resolution. Molar degree of substitution (DS,%) was calculated by measuring the Tyr absorbance at 275 nm of a 1 mg/ml HA-Tyr solution in ultrapure water. A calibration curve of Tyr in ultrapure water was used as a standard. DS is expressed as molar ratio of covalently bound Tyr residues to total carboxyl groups on HA. DS, was further determined from ¹H NMR using deuterium oxide as solvent without residual HOD peak suppression. Spectra were calibrated using N-acetyl proton on glucosamine residue of HA as the chemical shift internal standard, and processed with Mestrenova software. DS was determined by comparing the ratio of the areas under the aromatic peaks at 6.85 ppm 7.19/7.17 ppm and 7.38/7.36 ppm to the peak at 2.0 ppm (N-acetyl proton of HA).

2.3.4 Preparation of HA-Tyr hydrogels

The synthesized conjugates with a concentration of 2.5% (w/w) were dissolved in 1 Unit/ml HRP (in PBS) overnight at 4°C. Gelation was initiated by adding 0.34 mM H₂O₂ (in PBS) followed by gently mixing with a pipette tip. The above gelation protocol was optimized (optimization not shown here) while 0.34 mM H₂O₂ was found to be the minimum concentration required to achieve proper gelation and was therefore used in all experiments. The H₂O₂ concentration of 0.34 mM is lower than other reported concentration for cells encapsulation.
2.3.5 Viscoelastic characterization
Rheological measurements were performed with an Anton-Paar Rheometer equipped with a Peltier controller and plate-cone geometry, diameter 50 mm. The synthesized conjugates were hydrated in 1 Unit/ml HRP at 2.5% (w/w) overnight at 4°C. Dissolved samples were mixed with 0.34 mM H$_2$O$_2$ directly on the bottom plate and the upper cone was lowered to a gap of 0.1 mm before gelation has been started. In order to allow a homogenous gelation process, hydrogels were allowed to cure for 30 min at 20°C in the sample holder. A humid chamber was achieved by placing wet tissue paper around the platform and placing a chamber cover on top. The angular frequency sweep was conducted from 0.1 to 100 rad/sec at 1% strain to determine the shear elastic modulus (G') and loss modulus (G'') of the hydrogels. The viscoelastic linear regime was determined and then mechanical spectra were carried out at 1% strain and 20°C.

2.3.6 Equilibrium swelling
To examine the swelling properties, cross-linked hydrogel samples (200 μl) were prepared in cylindrical sample holders (diameter 8 mm) (n=3). After incubation for 30 minutes at RT, samples were immersed in PBS (10 mM, pH 7.4) at 37°C for 48 h until the swelling equilibrium had been reached. The swollen hydrogels were removed, carefully blotted to remove excess surface liquid, and the total swelled weight (W$_S$) was measured. The samples were lyophilized overnight and total dry weight (W$_L$) was measured. The swelling ratio was calculated using the following equation:

Swelling ratio = (W$_S$ - W$_L$) / W$_L$

2.3.7 Degradation assay
To determine the stability, we initiated cross-linking of the hydrogel samples (200 μl) in the sample holders as described above (n = 3) and allowed them to swell in PBS (10 mM, pH 7.4) for 24 h at 37°C, after which the mass of the samples was measured (W$_S$). Degradation assay was performed using 10 Units/ml hyaluronidase in PBS (10 mM, pH 7.4) at 37°C. At the chosen time points, hydrogels were removed, carefully blotted to remove excess surface liquid and the total weight (W$_D$) was measured. The percentage of hydrogel mass remaining in relation to the original swollen mass was calculated ((W$_D$/W$_S$)*100). Fresh buffer containing hyaluronidase was replaced at each time point. Hydrogels incubated in PBS without enzyme at 37°C served as a control for the degradation profile.

2.4 Results and Discussion
2.4.1 DMTMM stability in water
Use of DMTMM chloride in water solution as a coupling agent could potentially be limited by its instability. EDC, the most well-known water-soluble coupling agent has a half-life of 3.9 h in water at a pH of 5.0$^{165}$. DMTMM is stable in water at RT with no decomposition by demethylation or hydrolysis after 3 h$^{166,167}$. Little is known about later time points, and higher temperature.
Therefore we investigated the stability of DMTMM in experimental conditions identical to those of the conjugation reactions. A series of $^1$H-NMR spectra of DMTMM in deuterium oxide at 37°C was recorded for up to 48 h.

The degradation of DMTMM in water can follow three main paths (Figure 2-1A). In the first path, decomposition into 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) and N-methylmorpholine (NMM) takes place. This is the inverse of the preparation reaction commonly used for DMTMM\textsuperscript{166}. The CDMT generated is susceptible to further hydrolysis along a third path (Figure 2-1A). In the second path, demethylation of the methyl group on the nitrogen of the morpholine ring occurs. In this case the triazine and morpholine rings are not split and the positive charge is lost with simultaneous methanol release. In the third path, the triazine ring is hydrolyzed, resulting in 2,4-dimethoxy-6-hydroxy-1,3,5-triazine. This di-methyl ester of cyanuric acid gives keto-enol tautomerism. The compound can be originated directly from DMTMM, from CDMT generated as per path one or, presumably to a much lesser extent, from the demethylated species generated as per the second path.
Chapter 2 Synthesis of hyaluronan-tyramine using DMTMM conjugation

Figure 2-1 DMTMM degradation

(A) Main paths of DMTMM degradation: path 1) decomposition, path 2) demethylation and path 3) hydrolysis. (B) Representative series of $^1$H-NMR spectra of DMTMM in deuterium oxide at 37°C

$^1$H NMR spectrum of the freshly prepared DMTMM shows a sharp singlet at 4.17 ppm from the 6 methoxy protons (a); a sharp singlet at 3.56 ppm (c) from the methyl group bound to the nitrogen atom; a broad multiplet between 3.8 and 4.0 ppm from the protons in the NMM ring (b) (Figure S 2-1). At later time points spectrum evolves as follows (Figure 2-1B). The sharp singlet at 4.17 ppm (a) decreases its relative intensity while other 3 sharp singlets at 4.06, 4.01 and 4.00 ppm appear over time. The sharp singlet at 3.56 ppm (c) decreases its relative intensity while another singlet at 2.93 ppm develops. The broad multiplet between 3.8 and 4.0 ppm (b) from the NMM methylene protons decreases its area and increases its complexity; at the same time a triplet of doublets at
3.2 ppm rises up. A sharp singlet at 3.34 ppm increases with the time, testifying methanol formation as per the second path. Another interesting feature is the broadening of the HOD peak at 4.64 ppm, attributed to the formation of more species with exchangeable protons. Such species include methanol, the di-methyl ester of cyanuric acid generated as per the third path and its tautomer. Interestingly, after 48 h 23% of DMTMM is demethylated as estimated from methanol protons signal integration. Likewise, from peak integration after 48 h 27% of the NMM methylene protons lie in the multiplet at 3.2 ppm rather than 3.9 ppm, attesting both first and second degradation path. Degradation of DMTMM was also followed by UV-vis analysis. Since various species are involved in this process exact attribution of peak evolutions is not trivial (data not shown). The impact of DMTMM degradation on HA-Tyr coupling is discussed in the following paragraph.

### 2.4.2 Synthesis and optimization of HA-Tyr coupling

Table 2-1 lists the degree of substitution (DS,%) obtained using different stoichiometric ratios of the reagents. This set of reactions was carried out at room temperature for 24 h, repeating each reaction twice. To accomplish an exact comparison to conventional carbodiimide conjugation, final yields of conventional carbodiimide coupled HA-Tyr conjugates using a molar ratio of HA - EDC/NHS - Tyr (1 - 1/1 - 1) were analyzed. UV-vis absorbance measurements showed lower yields (DS 2.79 +/- 0.3%) obtained with EDC/NHS compared to DMTMM coupling (DS 3.0 +/- 0.5%) after 24 h at RT (Table 2-1).

**Table 2-1 List of DS for different HA-Tyr conjugates**

List of DS for different samples showing the effect of DMTMM - Tyr ratio used for HA-Tyr synthesis after 24 hours at RT (2 batches). Calculation is based on UV-vis analysis (n=3) and the conjugation was confirmed by $^1$H NMR.

<table>
<thead>
<tr>
<th>HA - DMTMM - Tyr</th>
<th>DS,%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 1 - 0.1</td>
<td>1.3 +/- 0.2%</td>
</tr>
<tr>
<td>1 - 1 - 0.5</td>
<td>1.7 +/- 0.2%</td>
</tr>
<tr>
<td>1 - 0.5 - 1</td>
<td>1.6 +/- 0.2%</td>
</tr>
<tr>
<td>1 - 1 - 1</td>
<td>3.0 +/- 0.5%</td>
</tr>
<tr>
<td>1 - 2 - 1</td>
<td>3.3 +/- 0.3%</td>
</tr>
<tr>
<td>1 - 4 - 1</td>
<td>n.A.</td>
</tr>
<tr>
<td>EDC/NHS (1 - 1/1 - 1)</td>
<td>2.8 +/- 0.3%</td>
</tr>
</tbody>
</table>

The conjugation and DS of HA-Tyr were confirmed by $^1$H NMR analysis, which corresponds to the sum of components of the conjugate. The ligation does not give rise to any new resonance situated in a spectral region free of other signals, so conventional $^1$H NMR spectroscopy cannot
be used as direct proof of the chemical ligation. The observed gelation of the compounds is the proof that the ligation occurred. A comparison of the spectra for unsubstituted HA and HA-Tyr conjugates shows signals for the anomic protons (ca. 4.5 ppm), sugar ring protons (four per monosaccharide between 3.2 and 4.0 ppm) and methyl protons of the N-acetyl group (3 per disaccharide, at 2 ppm) expected from both molecules (Figure 2-2). The presence of Tyr in the product was verified by the resonances at 7.19-7.17 ppm (a) and 6.85 ppm (b)\textsuperscript{155}. Semi-quantitative analysis of the DS was performed by comparing the ratio of the areas under the aromatic peaks at 6.85 ppm, 7.19/7.17 ppm and 7.38/7.36 ppm to the peak at 2.0 ppm (N-acetyl glucosamine proton of HA). Data in Table 2-1 show how different stoichiometric ratios of Tyr and DMTMM to HA can influence the DS. Even using Tyr in amount as low as 10% in moles to HA a substitution of 1.3% was detected. Derivatization of this compound was confirmed by the gelation displayed (not shown). Usually, bioconjugations involving EDC/NHS use a large excess of ligand\textsuperscript{155,168}. Our result is a consequence of the increased efficiency of coupling of DMTMM compared to EDC/NHS. As a direct comparison, we performed the coupling reaction with both methods using stoichiometric amount of every reagent. Also in this case, DMTMM displayed higher coupling yield. The ratio Tyr - HA has a marked influence on the conjugation yield, and can be used to prepare conjugates with desired substitution. Conjugation yield increases also with increasing DMTMM - HA ratio. However when a ratio of 4 - 1 was used the obtained product was insoluble. Non-solubility was attributed to the higher amount of non-hydrophilic moieties bound to HA. Another possible reason is the modification of the pendant phenolic hydroxyl group on Tyr by the excess of DMTMM. The hypothesized adduct is illustrated in Figure 2-2 (signal c) and is unique in DMTMM-mediated condensation of HA-Tyr. It should be noted that the same a/b and additional signal c can be seen whether Tyr is functionally bound or in a physical mixture with DMTMM. DMTMM is formed by the reaction of 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) with N-methylmorpholine (NMM). Therefore, reaction of CDMT directly with the hydroxyphenyl group on Tyr, whether free in solution or already covalently bound to HA through its amine group, forms Tyr-O-4,6-dimethoxy-1,3,5-triazine (Tyr-O-DMT) adducts on HA (Figure 2-2, signal c). This assumption could be confirmed by decreasing the molar ratio of Tyr to DMTMM from 1 to 0.1 for the conjugation that results in a very low signal at 6.85 ppm (Table 2-1), but also increases the signal at 7.38/7.36 ppm. Results were supported by UV-vis analysis of DMTMM - Tyr mixtures showing reduced absorbance at 275 nm when the molar ratio of Tyr to DMTMM was decreased (Figure S 2-2). It can therefore be assumed that Tyr-O-DMT adducts reduce the absorbance maximum of Tyr at 275 nm and are not functional in the crosslinking process. Noteworthy, it is essential to perform coupling reaction by premixing HA and DMTMM and add Tyr subsequently in order to minimize Tyr-O-DMT formation on HA. On the basis of these results an equal molar ratio of DMTMM and Tyr to HA (1 - 1 - 1) results in low amounts of non-functional Tyr-O-DMT adducts while obtaining a high final yield (Table 2-1,Figure S 2-2). DS values obtained in the present study are slightly lower compared to previous studies\textsuperscript{155,168}. On the other hand the rheological profile of the obtained compounds is within the range of recently published HA-Tyr hydrogels (see below)\textsuperscript{68}. The presence of non-covalently bound Tyr might bias the values previously reported\textsuperscript{155,168}. It is
further hypothesized that precipitation of the conjugates may be more efficient to avoid contamination of the final product with non-covalently bound Tyr. Indeed, after conjugation of HA-Tyr under same conditions (molar ratios of HA - DMTMM - Tyr of 1 - 1 - 1, RT, 24 h) dialyzed products (100 mM NaCl for 12 h followed by 60 h against distilled water), showed higher DS of 5.0% for dialysis (Figure S 2-1) compared to a DS value of 3.0% for precipitation (Table 2-1).

Figure 2-2 $^1$H NMR spectrum of the conjugate HA-Tyr

NaCl has been described as an important additive during early purification (dialysis) to avoid contamination with non-covalently bound Tyr molecules in the final product. However, calculated DS were still higher compared to precipitated samples. Noteworthy, mechanical properties of dialyzed conjugates were inferior compared to precipitated samples (data not shown). One explanation could be the formation of di-Tyr bonds between free Tyr molecules during cross-linking which inhibit the formation of stable di-Tyr bridges between covalently bound Tyr’s on HA. In addition, precipitation as purification step for HA-Tyr conjugates is a clear advantage for scaling up, less expensive and much quicker compared to dialysis. At neutral or alkaline pH Tyr is unstable, and therefore the conjugation has to be carried out at pH below 6. This condition hampers the use of EDC/NHS, which requires an acidic pH for the activation, but a slightly alkaline pH for the ligation step. Moreover the fine pH tuning usually required for optimal EDC/NHS conjugation is not possible, making the accurate control of substitution degree difficult.
DMTMM is a coupling agent less sensitive to pH, and therefore more appropriate for Tyr conjugation. The robustness of the process makes it more suitable for the chemical modification of carbohydrates with the degree of control needed for preparation of biomaterials and drug delivery systems.

### 2.4.3 HA-Tyr coupling kinetics and characterization

Coupling of HA-Tyr over time was studied at RT and 37°C using equal molar ratios of DMTMM and Tyr over HA. In Fig. 3A the DS is reported as a function of the reaction time. When the reaction is conducted at 37°C the reaction kinetics is faster (Figure 2-3A). The highest yield was obtained within 10 h (DS 4.0 +/- 0.1%) and HA-Tyr conjugates collected after 2 h at 37°C (DS 3.1 +/- 0.11%) presented sufficient DS for efficient cross-linking. Conjugation of HA-Tyr at RT required at least 48 h, reaching final yields of DS3.4 +/- 0.15%. Once maximum yield was reached, DS started to drop independent of final yield and reaction conditions (Figure 2-3A). Products isolated at different time points of reaction were analyzed by ¹H NMR in order to validate the use of absorbance at 275 nm for DS quantification (Figure 2-3B, Figure S 2-4). A correlation of DS calculated from ¹H NMR and absorbance at 275 nm can be drawn (Figure 2-3B). Noteworthy, HA-Tyr coupling at 37°C did not show more impurities in ¹H NMR spectra than conjugates at RT.

The findings indicate that an increase of reaction temperature would be advisable for faster kinetics and higher yield. Usually, classical HA-Tyr conjugation is assumed to be completed after 24 h. To best of our knowledge, none of these studies reported comparing the DS after earlier time points. It would be possible to further shorten time for HA-Tyr conjugation by increasing the reaction temperature (e.g. 60°C). However, it has to be considered that HA is cleaved into low-molecular fragments at higher temperatures with undesired effects in vitro and/or in vivo. Interestingly, lag time seems to be detrimental for final yields (Figure 2-3A). After reaching the

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**Figure 2-3 Characterization of HA-Tyr conjugates**

(A) Kinetics of HA-Tyr conjugation for DMTMM conjugation performed at RT or 37°C (2 batches) and EDC/NHS at RT for 24 hours. DS was analyzed with UV-vis using Tyr absorption maximum at 275 nm (n=6). (B) Comparison of calculated DS based on ¹H NMR spectra and absorbance at a peak maximum of 275 nm (Figure S 2-4).
maximum levels of DS either at RT or 37°C, obtained yields dropped. Since the amount of Tyr-O-DMT adducts did not increase with time (Fig. 3B), chemical modifications of Tyr may cause a decrease in absorption at 275 nm (Fig. S2). DS calculated both by $^1$H NMR and absorbance at 275 nm showed a good correlation (Figure 2-3B). It is known that $^1$H NMR is less accurate in determining low DS. Since the signals of the Tyr-O-DMT adducts are included in the calculation of the final substituted Tyr, DS values measured by $^1$H NMR were overestimated compared to UV-vis calculations. However, a linear correlation of the DS values, measured by $^1$H NMR and UV-vis supports that the amount of non-functional adducts did not change with increasing yields of Tyr on HA (Figure 2-3B). The results of $^1$H NMR and UV-vis, together with the corresponding hydrogel properties confirm the functional Tyr substitution on HA and further validate the use of UV-vis absorbance measurement at 275 nm being a very sensitive probe to detect functional Tyr bound to HA.

Our findings further indicate that the HA-Tyr coupling kinetics and efficacy depend on the temperature and on the percentage of DMTMM remaining in solution upon degradation (Figure 2-1). While DMTMM is more efficient and more reactive at 37°C, it also decomposes quicker compared to RT.

### 2.4.4 Rheological properties of HA-Tyr hydrogels

Cross-linking density is one of the key parameters in manipulating the mechanical properties of hydrogel systems. Variations in the gel precursor concentration, the molecular weight of the polymer, and the degree of conjugation of the cross-linking moiety are some of the common methods to control the cross-linking density. In the following experiments, concentrations of HA-Tyr (2.5% w/w), HRP (1 Unit/ml) and H$_2$O$_2$ (0.34 mM) were held constant and samples were incubated for 30 min prior to measurement. In order to assess the viscoelastic properties of cross-linked HA-Tyr, conjugates with different DS were characterized by rheological studies using cone plate geometry at 20°C. A typical frequency dependence of the storage modulus $G'$ and loss modulus $G''$ of HA-Tyr hydrogels featured by different DS are shown in Fig. Figure 2-4A. For all specimens $G'$ is markedly over $G''$ and almost independent of the frequency within the range analyzed, indicating a crosslinked network prior to analysis. The graph also shows that at every frequency $G''$ follows an inverse order compared to $G'$. Increasing the DS clearly results in a denser network displayed by elevated $G'$ values and the appearance of the fabricated hydrogels (Fig. 4B). Gelation times were unaffected by DS with 300+/− 30 sec (1 Unit/ml HRP / 0.34 mM H$_2$O$_2$) and could be modified by the concentration of HRP. The fabricated HA-Tyr hydrogels are transparent and different DS do not affect the transparency of the matrices (Figure 2-4B). This feature is advantageous for analysis of encapsulated cells by microscopy.
Figure 2-4 Characterization of HA-Tyr hydrogels

(A) Rheological measurement (frequency sweep): Typical frequency dependence of the storage modulus $G'$ and loss modulus $G''$ of HA-Tyr hydrogels and (B) images of the corresponding samples fabricated using 1 Unit/ml HRP and 0.34 mM of $H_2O_2$. Rheological measurement was taken with constant deformation of 1% (linear viscoelastic range) at RT.

A precise control over mechanical properties of hydrogels is an important tool for in vitro microenvironment studies of encapsulated cells. It is further essential to avoid any effects on cell viability and morphology. $G'$ and $G''$ values at a frequency of 10 rad/sec were chosen to illustrate the rheological properties of the HA-Tyr conjugates. As shown in Figure 2-5, storage modulus $G'$ at a frequency of 10 rad/sec is correlated to DS of HA-Tyr. Accordingly, hydrogels with DS of 1.3% were the weakest ($G'$ value of 100 +/- 35 Pa), whereas hydrogels with a DS 3.9% showed the strongest $G'$ values (875 +/- 30 Pa) (Fig. 5). Control over DS allows for predictive hydrogel properties as $G'$ of the hydrogel matrices can reliably be predicted by the DS of the HA-Tyr conjugates ($G' = 208\cdot(\text{DS,\%}) - 104$). The demonstrated control over the HA-Tyr hydrogels offers an extended range to tailor mechanical and physical properties. As already described by earlier studies, there is a positive correlation between $H_2O_2$ concentration and storage modulus.$^{58,61}$ In the enzymatic coupling reaction, HRP allows the formation of two Tyr radicals and two water molecules by reacting with one $H_2O_2$ molecule.$^{168}$ Consequently, the concentration of $H_2O_2$ would affect the number of Tyr cross-links and ultimately the mechanical strength of the hydrogel. Therefore it can be expected that different concentrations of $H_2O_2$ enable a further tuning of the mechanical properties (Figure 2-6). To illustrate the effect of different $H_2O_2$ concentrations, three representative hydrogel matrices were chosen. Increasing the concentration of $H_2O_2$ results in higher mechanical strength ($G'$) while the tendency was independent of the DS (Figure 2-6).
However, an increase of storage modulus is accompanied with a change of loss modulus. While increased \( \text{H}_2\text{O}_2 \) concentrations induced an increase in \( G' \) at the expense of \( G'' \), tuning the DS allow one to tune \( G' \) without affecting \( G'' \) (Figure S 2-5).

![Figure 2-5 Frequency sweep of HA-Tyr hydrogels as a function of the DS](image)

\[ G' = 208\times DS - 104 \]
\[ R^2 = 0.852 \]

Noteworthy, after a certain threshold of \( \text{H}_2\text{O}_2 \) (>0.78 mM) the hydrogels become so brittle that rheology cannot be used any more. However, changing the concentration of \( \text{H}_2\text{O}_2 \) to vary stiffness and elasticity will invariably induce simultaneous changes in the cell response due to the effects of \( \text{H}_2\text{O}_2 \), making it difficult to interpret the contribution of various cues to an observed cellular response. To help elucidate the mechanism of cell-hydrogel interactions, it is highly desirable to develop biomimetic matrices that have independently tunable properties. The HA-Tyr hydrogels reported here exhibit precisely tailored mechanical and morphological properties by adjusting the DS without changes in \( \text{H}_2\text{O}_2 \) concentrations and the potential effects on cell behavior (Figure 2-5). Since \( \text{H}_2\text{O}_2 \) concentrations are reported to dictate the amount of Tyr being involved in the effective formation of di-Tyr bonds, the matrix environment of encapsulated cells should not be affected. However, morphology and behavior of cells in hydrogel matrices with different degree of substituted Tyr have to be investigated. On the basis of an additional hydrogel matrix tuning through various \( \text{H}_2\text{O}_2 \) concentrations, DMTMM synthesized HA-Tyr conjugates can extend the range of viscoelastic and mechanical properties. It has been shown that the modulus of elasticity of the extracellular environment has profound effect on stem cell behavior\(^{172,173}\). The ability of cells to react to the mechanical properties of substrates is generally referred to as mechano-sensing and implies the action of the material on the cells and the action of the cell on the mechanical properties of the material\(^{107}\). The fabrication of HA-Tyr hydrogels with defined modulus of elasticity can be useful to study the interactions of cells with their biophysical microenvironment.
Figure 2-6 Effect of H$_2$O$_2$ concentration on HA-Tyr rheological properties

G’ (filled shapes) and G” (open shapes) of HA-Tyr hydrogels with DS 3.9%, 3.1% and 2.2% as a function of H$_2$O$_2$ concentration

### 2.4.5 Swelling ratio and degradation

The swelling behavior of these hydrogels was probed gravimetrically by recording their water uptake over time until equilibrium. For all hydrogel formulations, the concentration of polymer (2.5% (w/w)), HRP (1 Unit/ml) and H$_2$O$_2$ (0.34 mM) were kept constant while the DS ranged from 1.34% to 4.20% (Figure 2-7A). All samples reached equilibrium after 48 h. In general, an increase of DS of bound Tyr to HA led to a linear decrease ($R^2=0.926$) in the swelling capacity of the hydrogel (Figure 2-7A). Hydrogels composed of HA-Tyr conjugates with the lowest DS (1.3%) show by far the highest swelling ratio. In contrast, derivatives with DS of 3.5 - 4.2% gained 10% less weight when equilibrium was reached. The hydrogels with the highest swelling ratio, composed of HA-Tyr derivatives with DS of 1.3%, are assumed to have a less cross-linked network. Likewise, the hydrogels with DS 4.2% displayed the lowest swelling ratio, indicating that the swelling capacity was reduced due to more cross-links. The hydrophilic/hydrophobic balance of the hydrogels and the degree of cross-linking are important parameters that control the equilibrium swelling and the dimensional change of the network. Swelling is not a continuous process and at the equilibrium, the elasticity of the network and the osmotic forces are balanced, there is no additional swelling\textsuperscript{174}. The cross-links act as tethering points between different HA chains of the hydrogel, preventing their movement away from each other. Other physicochemical cues further influence swelling ratio of hydrogels, in particular the porosity and pore size structure, which were not investigated in this study. The excellent correlation of swelling ratio and DS indicates that the control of DS of DMTMM conjugated HA-Tyr constructs allows precise prediction of hydrogel swelling rate.
Figure 2-7 Swelling and enzymatic digestion of cross-linked HA-Tyr

(A) Swelling ratio (SR) for the samples as a function of the DS (n = 3) ($R^2 = 0.9259$). Hydrogels were formed using 1 Unit/ml HRP and 0.34 mM H$_2$O$_2$ and incubated in PBS for 48 hours at 37°C to reach equilibrium (n=3).

(B) Enzymatic degradation of HA-Tyr hydrogels. Samples were allowed to pre-swell in PBS for 48 hours at 37°C. Open symbols: samples incubated in PBS only. Solid symbols: representative samples incubated in PBS containing 10 Units/ml hyaluronidase (average percentage of original weight (n = 3)). Results illustrate remaining hydrogel mass (%) of 3 representative samples over 24 hours of degradation.

The degradation of the HA-Tyr hydrogels was investigated by enzymatic digestion after adding hyaluronidase. HA is enzymatically degradable at its backbone which depends on the availability of hyaluronidase and susceptibility of the modified hyaluronan to be cleaved by the enzyme.

To monitor how DS affects degradation of the hydrogels in vitro, pre-swollen samples were incubated in PBS with 10 Units/ml hyaluronidase and PBS only (controls) at 37°C. Their masses were recorded after 2, 6, 12, and 24 h (Figure 2-7B). After 24 h most of the hydrogels dissolved completely. Comparison of degradation kinetics with DS showed hydrogels with low Tyr substitution (DS 2.2%) degraded very fast and high DS (DS 3.9%) degraded more slowly (Figure 2-7B). DS 3.1% hydrogels displayed an intermediate degradation profile (Figure 2-7B). Still, increasing the concentration of H$_2$O$_2$ would entail a denser network and therefore slow down degradation.$^{58}$ These results suggest that the density of cross-links has an impact on the degradation process.

No significant mass loss in the PBS control demonstrates that in these conditions the spontaneous degradation of hyaluronan is negligible at this pH and temperature. It has been described earlier that hyaluronidase at low concentrations, as used in our study (10 Unit/ml), diffuse into the hydrogel network and hydrolyze HA not only from the surface but also through bulk erosion, causing a decrease in crosslinking density and loosening of the network.$^{58}$ Accordingly, H$_2$O$_2$ concentrations of 0.32 mM may have diminished the effect of surface degradation. The lower crosslinking density of the hydrogels may allow faster diffusion of hyaluronidase into the hydrogel matrix, which is supported by the correlation of the degradation rates with DS. Careful control of the hydrogel degradation rate is critical in tissue engineering application so that it can be matched to the growth rate of the new tissue.$^{175}$ For hydrogel incorporated drugs or small molecules and their release, a precisely tailored degradation rate is
indispensable. The conclusions are consistent with the viscoelastic features of the HA-Tyr hydrogels (Figure 2-5).

2.5 Conclusion

In this paper we presented the use of DMTMM for the synthesis of HA-Tyr derivatives capable of \textit{in situ} gelling within a biological environment. Major advantages of the hydrogel system are its injectability and adhesion to the tissue surface. The derivatization process was optimized studying the effect of different reagent feed ratios and operating temperatures. The process was further characterized by comparing the substitution efficiency with the degradation profile of the coupling agent. The study revealed the capability of this conjugation process to achieve an optimal control of the substitution degree, and the physical characteristics of the gel as a consequence. Specifically, swelling profile, \textit{in vitro} enzymatic degradation and viscoelasticity were precisely tailored. The inherent advantages of DMTMM conjugation make this method a powerful tool for HA modification and for the preparation of semi-synthetic biomaterials in general. The obtained results encourage further studies exploring the use of DMTMM for polysaccharide modification and the preparation of biomaterials with properties exactly tailored towards drug delivery and tissue engineering applications.

2.6 Supplement

Figure S 2-1 $^1$H NMR spectrum of a freshly prepared DMTMM presenting the methoxy group (a), and the methyl resonance (c) of the N-methylmorpholine (b).
**Figure S 2-2** Spectrophotometric absorbance spectra of DMTMM–Tyr physical mixtures.

**Figure S 2-3** $^1$H NMR spectrum of the conjugate HA-Tyr after dialysis against 100 mM NaCl (12 hours) and distilled water (60 hours) and lyophilization. The inserted spectrum shows the peaks of the precipitated HA-Tyr conjugate (Figure 2-1).
Chapter 2 Synthesis of hyaluronan-tyramine using DMTMM conjugation

**Figure S 2-4** Spectrophotometric absorbance spectra HA-Tyr conjugates

**Figure S 2-5** $G''$ of HA-Tyr crosslinked with various $\text{H}_2\text{O}_2$ concentrations as a function of DS
Loss modulus $G''$ at 10 rad/sec as a function of the DS in mol,% and of various $\text{H}_2\text{O}_2$ concentrations. Hydrogels were formed using 1 Unit/ml HRP and $\text{H}_2\text{O}_2$ and incubated for 30 min prior to measurement.
3 Fabrication of cell-compatible hydrogels with a wide range of biophysical properties from highly functionalized hyaluronan-tyramine

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3.1 Abstract

Enzymatically mediated crosslinked hyaluronan-tyramine hydrogels (HA-Tyr) are promising matrices for tissue engineering and regenerative medicine. However, due to relatively low tyramine modifications of the hyaluronan backbone achieved, HA-Tyr matrices have weak and a narrow range of mechanical properties. The iterative use of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) as coupling agent increased the yields of tyramine functionalization, which was reflected by a two-fold increase in Young’s modulus of HA-Tyr hydrogels. Additionally, DMTMM facilitated accurate control over the hydrogel degradation. Viable encapsulation of human mesenchymal stem cells, with 85-98 % over 6 days, was achieved in all hydrogels and distinct cellular spreading observed in the absence of additional binding cues. The tunable HA-Tyr hydrogels are optimized to study a wide range of cellular behavior.

3.2 Introduction

Hydrogels are widely employed in tissue engineering, drug delivery and as artificial niches capable to direct tissue regeneration\textsuperscript{152}. The ability to engineer and tailor hydrogel materials has provided possibilities to fabricate microenvironments capable of controlling stem cell fate and has advanced the understanding of how microenvironmental factors influence cellular functions\textsuperscript{110,176,177}. Often mesenchymal stem cells (MSCs) are implemented owing to their ability to differentiate into many cell types and promote tissue regeneration\textsuperscript{177}. Naturally derived hydrogel materials, such as collagen and fibrin gels, have often been utilized as three-dimensional (3D) microenvironment culture systems that drive cellular self-organization and tissue morphogenesis\textsuperscript{178-180}. However, their use is complicated by the difficulty in modulating and controlling matrix parameters independently of the macromer concentration. Beyond the final physical properties, the crosslinking conditions are also important as the reactive molecules may influence cell behavior with respect to changes in gene expression\textsuperscript{181,182} and direct interactions of cells with the grafted groups\textsuperscript{183}. Thus, it is important to design hydrogel platforms where biophysical properties can be tailored distinctly for optimal control over cell behavior.

Considering the ubiquitous presence of hyaluronan (HA) in the extracellular matrix of various connective tissues and its role in tissue development, HA-based materials have also become an attractive source to understand and modulate cells behavior for cell-based therapies\textsuperscript{24,184,185}. The chemical conjugation of functional groups (e.g. thiols, acrylates and amines) enabled the design of HA hydrogels with control over chemical and mechanical features\textsuperscript{24}. HA not only presents a versatile chemistry for multiple functionalities, but it also mediates cellular functions by the ability of cell receptors to recognize the HA molecule\textsuperscript{186}. For example, interactions between HA and cells via CD44 receptors have been correlated with tissue homeostasis, cell proliferation and migration\textsuperscript{187,188}. We and others recently described tyramine modified HA (HA-Tyr) conjugates as a hydrogel platform capable of controlling physico-chemical properties, such as stiffness, swelling, and degradability\textsuperscript{61,155-157,189}. The covalent crosslinking of HA-Tyr is catalyzed by horseradish
peroxidase (HRP) and hydrogen peroxide (H$_2$O$_2$) as an oxidant, which generate tyramine (Tyr) radicals. Consequently, Tyr radicals lead to covalent di-Tyr bridges formed between two aromatic rings$^{61}$. By varying the H$_2$O$_2$ and HRP concentration, HA-Tyr crosslinking rate (gelation time) and degree of crosslinks (mechanical properties) can be modified$^{58}$. To date, applications of HA-Tyr hydrogels have focused largely on applications in tissue engineering$^{70,75}$ or on the design of injectable matrices for controlled molecule delivery$^{79,81,82}$. While there are some reports on the development and optimization of HA-Tyr derivatives, these conjugates have been relatively weak mechanically, due to the low backbone modification (less than 6-7%)$^{58,190,191}$. In addition, the amount of H$_2$O$_2$ that can be added to the system is limited to avoid cell damage or oxidant-induced apoptosis, and thus H$_2$O$_2$ concentration gives only a limited handle on hydrogel mechanics$^{192}$. Although a low degree of functionalization is advantageous with respect to the bioactivity of HA-Tyr hydrogels$^{192}$, the constraints on mechanics potentially limit further use in tissue regeneration applications.

In this work we present a strategy to synthesize HA derivatives that encompass a wide range of Tyr modifications due to a reduced formation of non-functional by-products. In the first part we show that the conjugation process is capable of controlling the degree of HA functionalization and the physical properties of the crosslinked HA-Tyr hydrogels at a given amount of H$_2$O$_2$. Importantly, high yields of HA-Tyr conjugates enable the formation of hydrogels with enhanced mechanics that can be accomplished when H$_2$O$_2$ concentrations are varied. In the second part, we demonstrate the cell-compatibility of HA-Tyr hydrogels with high viability of encapsulated human MSCs (hMSCs) obtained for a variety of Tyr modifications and H$_2$O$_2$ concentrations. Finally, this HA-Tyr hydrogel system permits cellular spreading in the absence of additionally supplemented cell-adhesive peptides and can serve as an instructive matrix and tissue engineering platform due to its high degree of tunability.

### 3.3 Experimental

#### 3.1.1 Materials

Hyaluronic acid sodium salt from Streptococcus equi (HANa) with weight-average molecular weight M$_w$ = 290 kDa and poly-dispersion index M$_w$/M$_n$ = 1.86, where M$_n$ indicates the number-average molecular weight was purchased from Contipro Biotechs.r.o. (Czech Republic). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) from TDI (Zwijndrecht, Belgium). Dulbecco's modified Eagle's medium (DMEM) and alpha-MEM were purchased from Life Technologies (Switzerland), and fetal bovine serum (FBS) from Gibco (Switzerland). Anti-CD44 antibodies were purchased from abcam (ab6124) and Miltenyi Biotec (130-095-177). Other chemicals were of analytical grade, purchased from Sigma Aldrich (Switzerland) and used as received.

#### 3.1.2 Synthesis of HA-Tyr conjugates

HA-Tyr was prepared by amidation of the carboxylic acid groups of HA with the amine groups of Tyr as described previously$^{190}$. HANa (500 mg, 1.25 mmol carboxylic groups) was hydrated in 50 ml 2-morpholinoethane sulfonic acid (MES) buffer (100 mM, pH 5.5, adjusted with NaOH 5 M) at
a final concentration of 1\% (w/v). In addition, the conjugation was carried out in deionized water (H\textsubscript{2}O) (initial pH 6.25 +/- 0.1) without pH adjustment. Subsequently, 1.25 mmol DMTMM and 1.25 mmol tyramine hydrochloride (Tyr) were added to the solution. The reactions were carried out at room temperature (RT) or 37°C under continuous stirring. For isolation of the HA-Tyr products, aliquots were withdrawn after 6, 24, 48, 72 and 96 h and then precipitated. Precipitation was performed by adding 8 vol\% saturated sodium chloride solution, the product was collected by filtration and dried under vacuum at RT for 48 h. For multiple step HA-Tyr conjugation, the isolated HA-Tyr product after 24 h was hydrated in deionized H\textsubscript{2}O with a final concentration of 1\% (w/v) and the conjugation was repeated up to three times as described above.

3.1.3 Characterization of HA-Tyr conjugates

HA-Tyr conjugates were characterized using UV-vis and \textsuperscript{1}H NMR as reported previously\textsuperscript{190}. Briefly, molar degree of substitution (DS, \%) was calculated by measuring the absorbance of a 1 mg/ml HA-Tyr solution in ultrapure water using a Multiskan\textsuperscript{TM}GO Spectrophotometer in cuvette mode and 2 nm resolution. A calibration curve of Tyr in ultrapure water was used as a standard\textsuperscript{69}. DS was confirmed with \textsuperscript{1}H NMR measurements (Bruker Avance AV-500 NMR) and calculated as molar ratio of covalently bound Tyr residues using deuterium oxide as solvent without residual HOD peak suppression. Spectra were calibrated using the N-acetyl proton on glucosamine residue of HA as the chemical shift internal standard (2.01 ppm), and processed with Mestrenova software. DS was determined by comparing the ratio of the areas under the aromatic peaks at $\delta = 6.85$ ppm $\delta = 7.18$-$7.16$ ppm and $\delta = 7.37$-$7.35$ ppm to the peak at 2.01 ppm (N-acetyl proton of HA).

3.1.4 Characterization and kinetics of non-functional adduct formation

Adduct formation was characterized by UV-vis and \textsuperscript{1}H NMR of physical mixtures. DMTMM and Tyr or 4-methoxyphenethylamine were mixed with equal molar ratios (2.5 µmol per ml solvent) and solubilized in H\textsubscript{2}O, 100 mM MES (pH 5.5) and 100 mM NaCl respectively. Mixtures were kept at RT or 37°C under continuous stirring. Aliquots for UV-vis analysis were withdrawn after 6, 24, 48, 72 and 96 h and absorbance measured with the solvent as blank accordingly. Similarly, \textsuperscript{1}H NMR spectra of 2.5 µmol DMTMM, Tyr and MES physical mixtures in D\textsubscript{2}O were analyzed for adduct formation.

3.1.5 Characterization of HA-Tyr hydrogels

HA-Tyr conjugates were dissolved in 0.5 Unit/ml (U/ml) HRP (in PBS) overnight at 4°C for a final concentration of 2.5\% (w/v). Rheological measurements were performed with an Anton-Paar Rheometer equipped with a Peltier controller and cone-plate geometry, diameter 25 mm. Dissolved samples were mixed with 0.34 mM H\textsubscript{2}O\textsubscript{2} and directly pipetted on the bottom plate. The upper cone was lowered with a gap size of 0.049 mm and a time sweep was conducted at 1\% strain and 6.28 rad/sec. The initial gelation time was indicated when the storage modulus (G') is equal to loss modulus (G'\textsuperscript{''}). Subsequently, the angular frequency sweep was conducted from 0.1 to 100 rad/sec at 1\% strain to determine G' and G'\textsuperscript{''} of polymerized hydrogels. Time sweep and angular frequency sweep were carried out at 20°C.
To obtain bulk mechanical properties, the equilibrium compression modulus of formed hydrogel discs was measured with unconfined compression stress-relaxation device\textsuperscript{194}. HA-Tyr hydrogels were formed with 0.5 U/ml HRP and H\textsubscript{2}O\textsubscript{2} as indicated, and placed in PBS for 60 min until measurement. Stress relaxation tests were performed with 10% strain applied at 0.05%/sec followed by relaxation for 1000 sec until equilibrium. The Young's modulus was determined from the equilibrium force normalized to the diameter of the hydrogel disc divided by the equilibrium compressive strain.

For swelling assays, the equilibrium swelled samples (in PBS 10 mM, pH 7.4) were carefully blotted to remove surface liquid and the wet weight ($W_{\text{wet}}$) was measured. Samples were lyophilized and dry weighted ($W_{\text{dry}}$). The volumetric swelling ratio was calculated as: ($W_{\text{wet}} - W_{\text{dry}}$)/$W_{\text{dry}}$. For enzymatic degradation studies, hydrogels were prepared as described above and allowed to swell for 24 h in PBS, after which the $W_{\text{wet}}$ was measured. The samples were then incubated in in PBS containing 10 U/ml bovine hyaluronidase at 37°C. At the indicated time points, hydrogels were removed, surface liquid carefully removed and the total weight was measured ($W_{\text{degr}}$). The percentage of hydrogel mass remaining was calculated in relation to the original swollen mass: (($W_{\text{degr}}/W_{\text{wet}}$)*100). The buffer containing hyaluronidase was refreshed at each time point. All studies were performed in triplicates unless otherwise noted.

3.1.6 Cell-encapsulation of hMSCs in HA-Tyr hydrogels

Human bone marrow was harvested from the iliac crest, after ethical approval (Freiburg, EK-326/08) to isolate hMSCs using previously described protocols\textsuperscript{195}. After expansion, 100 µl cell suspension (5x10\textsuperscript{6} P2 hMSCs in PBS) were suspended in 860 µl HA-Tyr precursor solution containing 0.5 U HRP. For control gels 100 µl PBS was added. The pre-polymer solution (48 µl) was transferred to syringes with their tips removed, and crosslinking was initiated by adding 2 µl H\textsubscript{2}O\textsubscript{2} stock solution for final concentrations of 0.68 mM and 1.1 mM. Individual gels were removed from the syringes, placed in 24 well plates and cultured in DMEM low glucose with 10% FBS, 1% penicillin/streptomycin at 37°C/5% CO\textsubscript{2}. Medium was refreshed every second day and hydrogels were placed on the bottom of the well throughout the culturing. For CD44 blocking studies, hMSCs were incubated for 45 min on ice with monoclonal anti-CD44 antibody (abcam, 3:100) in a buffer (2 mM EDTA and 2% FBS in PBS)\textsuperscript{16}. CD44 blocked cells were washed twice with the buffer and filtered using a cell strainer (70 µm) before encapsulation. Cell viability and cell spreading in hydrogel constructs were assessed by live-dead staining and laser scanning microscope (LSM) confocal imaging. Hydrogels on day 1, 2, 4 and 6 were washed with PBS, cut into half and 5 µM Calcein and 8 µM Ethidium homodimer per construct in PBS were added. Following incubation for 30 min at RT, constructs were washed with PBS and mounted on a glass coverslip. The morphology of the cells was assessed using a Zeiss LSM510 (10 x objective). Forty sequential stacks of 5 µm sections from the bottom of the hydrogel were acquired from four random locations per construct. For quantifications, three-dimensional reconstructions of the z-stacks were rendered using ImageJ. Cell aspect ratios were analyzed by calculating the ratio of maximum length and width of each cell. Each experiment was repeated three times with two different MSC donors.
3.1.7 Statistical analysis
Mean values and standard deviations were obtained for all quantitative measurements, image analysis and mechanical data. Comparison of data obtained from $^1$H NMR quantification was performed using unpaired Student's t-test, data from swelling and degradation assays and cell encapsulation with one-way ANOVA analysis coupled with Tukey's multiple comparison tests. Statistical significance was set at $p < 0.05$.

3.4 Results and discussion

3.4.1 Synthesis of HA-Tyr derivatives
Improving the physical properties of hydrogels requires enhancing the control over the crosslinking of the macromolecular network. The main limitation imposed by the chemistry of HA-Tyr preparation is the deactivation of the coupling agent DMTMM by either degradation or formation of non-functional adducts\textsuperscript{190}. We hypothesized that this constraint can be overcome by multiple additions of fresh DMTMM and Tyr to HA (Figure 3-1A). Moreover, we compared the reaction progress in water (Aq) and MES buffer.

In both, MES and Aq, reaction rates were higher at 37°C compared to RT and increased DS values (in molar %) were obtained, yet with significantly higher DS values in Aq compared to MES at all-time points (Figure 3-1B). Independent of the conjugation medium, the maximum DS was reached asymptotically, illustrating a progressive loss of DMTMM activity. DMTMM deactivation was previously attributed to decomposition, demethylation or hydrolysis\textsuperscript{166,190}. Therefore, we isolated HA-Tyr after 24 h and added a further aliquot of fresh DMTMM and Tyr (Figure 3-1A, "Multiple"). This step was reiterated up to four times. The final yields of substituted Tyr on HA were 6.5±0.5% at 24 h (single addition), 15±1% at 48 h (two additions), and increased up to DS of 20±1% and 20±1.5% at 72 h and 96 h (three and four additions).

Recently, we showed that DMTMM is a powerful method for amine ligation to HA\textsuperscript{163} and that higher temperatures accelerate both coupling and deactivation rates\textsuperscript{190}. Although the DS can be increased when HA-Tyr synthesis is conducted in Aq, the yields are still limited to 7±0.5% (Figure 3-1B). Multiple additions of DMTMM and Tyr significantly increased the final yields up to a DS of 20±1% (Figure 3-1C). The results demonstrate a strategy to control and increase the Tyr modification of HA, which is superior to the traditional carbodiimide chemistry\textsuperscript{57,58}. The impact of an aqueous based system on the DMTMM coupling efficacy is discussed in the following paragraph.
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3.4.2 Characterization of non-functional adduct formation during HA-Tyr coupling

To confirm the UV-vis measured functionalization, the chemical structure of HA-Tyr conjugates was analyzed using \(^1\)H NMR (Figure 3-2A). Here the presence of Tyr in the product generates 3 doublets, where resonances at 6.85 ppm (signal a) and at 7.18-7.16 ppm (signal b) are due to Tyr bound to HA\(^{155,190}\). The doublet at 7.37/7.35 ppm (signal c) was attributed to the direct coupling between Tyr and the triazine ring to form the non-active species Tyr-O-DMT\(^{190}\). The quantification of the ratio of the integral area under the aromatic peaks of the functional Tyr (6.85 ppm) and the non-functional Tyr-adduct (7.37/7.35 ppm) confirmed less adduct formation for conjugates synthesized in Aq compared to MES (p<0.01, Figure 3-2B). The same feature was also seen after multiple additions of DMTMM and Tyr, indicating that adducts are formed proportional to the Tyr coupling.

To understand these observations, we investigated the formation of Tyr-O-DMT adducts in Aq, MES buffer (100 mM, pH 5.5), and NaCl (100 mM in H\(_2\)O). UV-vis measurements serve as a convenient analysis method to track the formation of these adducts, as the modification of the Tyr aromatic ring reduces the absorbance of the Tyr at 275 nm\(^{69,190}\). The reduction of the Tyr peak was monitored as a function of time for 96 h at RT and 37°C (Figure 3-2C). At equal molar ratios of Tyr and DMTMM, Tyr absorbance was observed to drop within the first 6 h. The reduction was

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**Figure 3-1** Sequential HA-Tyr coupling in H\(_2\)O increases final yields of substituted tyramine

A Schematic of conjugation strategies. B Kinetics of HA-Tyr conjugation performed in MES and deionized water (Aq) at RT and 37°C (1-1 molar ratio to HA, red arrow), DS analyzed after 6, 24, 48, 72 and 96 hours (see Fig. S1 for the \(^1\)H NMR spectra). C Kinetics of HA-Tyr conjugation performed in H\(_2\)O at 37°C with multiple addition of DMTMM and Tyr (1-1 molar ratio to HA) after 24, 48 and 72 hours (red arrows). DS were analyzed with UV-vis absorption maximum at 275 nm (n=3).
more pronounced in MES compared to Aq and NaCl. After 24 h, at RT the signal was reduced to 30% of the initial value in MES compared to a reduction of 80% in Aq and NaCl. While Tyr absorbance in MES reached equilibrium after 24 h, it continued to drop in Aq and NaCl. Still, Tyr absorbance after 96 h was 2.5 times higher in Aq and NaCl compared to MES. These results were supported by $^1$H NMR spectra of DMTMM-Tyr-MES mixtures showing Tyr-O-DMT adducts, but no additional signals that could be attributed to MES-Tyr and MES-DMTMM adduct formation (Figure S 3-1). To confirm that the phenolic hydroxyl group was involved in Tyr-O-DMT formation, the same experiment was performed reacting DMTMM with 4-methoxyphenethylamine (i.e. Tyr methylated at the phenolic hydroxyl group). In this case, the absorption at 275 nm remained constant over time in all solvents and conditions.

**Figure 3-2 HA-Tyr coupling in H$_2$O reduces non-functional adduct formation**

A $^1$H NMR spectrum of the conjugate HA-Tyr synthesized in H$_2$O. The inserted spectrum shows the peaks of the HA-Tyr conjugate synthesized in MES buffer. B Ratio between the integrals of the Tyr-O-DMT signal at 7.37/7.35 ppm and the Tyr signal at 6.84/6.85 ppm. Mean ± SD for n = 8 per group. $^{**}p<0.01$ (See Fig. S1 for $^1$H NMR spectra). C Physical mixtures: Reduction in Tyr absorption maximum (275 nm) of a DMTMM-Tyr mixture in H$_2$O, MES and NaCl (RT and 37°C) as a function of time (n = 2 independent measurements).

It is also possible that low efficacy of HA-Tyr coupling in MES is due to the different ionic strength of the buffer. HA macromolecular flexibility increases dramatically for higher ionic strength due to electrostatic shielding and consequent transition from extended rod in pure water to coil in NaCl 100 mM$^{196}$. Therefore, we performed the HA conjugation at increasing NaCl concentration without further pH adjustment in order to verify coupling efficacy and adduct formation. Here, DS values after 72 h at RT were DS= 5.8% for HA-Tyr coupling in Aq while products isolated from NaCl buffer reached a DS of 4.9 and 4.5% for 100 and 150 mM NaCl, respectively. Although yields were reduced with the addition of NaCl, the formation of Tyr-O-DMT adducts was similar to Aq as analyzed with $^1$H NMR (Figure S 3-2). Thus, ionic strength does not solely explain the difference
in coupling efficiency in water and MES buffer, indicating that additional factors may influence DMTMM efficacy and formation of non-functional Tyr adducts.

Taken together, the findings demonstrate that DMTMM mediated HA-Tyr coupling in water is efficient, requires no buffer and makes the process more practical. Notably, multiple supplementations of DMTMM and Tyr increase the yield of substituted Tyr while maintaining a low degree of non-functional Tyr adducts on HA.

### 3.4.3 Physical properties of HA-Tyr hydrogels

When HA-Tyr macromers are mixed with HRP and H$_2$O$_2$ in solution, crosslinking of hydrogels occurs through Tyr oxidation and subsequent formation of covalent di-Tyr bonds$^{61}$. At constant concentrations of HA-Tyr (2.5% w/v), HRP (0.5 U/ml) and H$_2$O$_2$ (0.34 mM) gelation properties of the formed hydrogels were investigated utilizing rheological measurements. Representative time-sweeps indicate a liquid-like behavior of the polymer solution (Figure 3-3A). Generally, HA-Tyr crosslinking rate is controlled by HRP, which catalyzes the formation of Tyr radicals in the presence of H$_2$O$_2$. At constant HRP concentration an increase of the DS resulted in reduced gelation times (Figure 3-3B). At HRP concentrations greater than 1.5 U/ml, high modified HA-Tyr solutions (≥ DS 7%) were rapidly crosslinked and homogenous gelation could not be achieved.

From these observations we can conclude that the DS has an impact on the HRP/H$_2$O$_2$ mediated crosslinking, resulting in decreased gelation times for higher degree of modifications. For biomedical applications, the reduced crosslinking time for high DS HA-Tyr gels is advantageous as it allows control of the gelation rate while minimizing the necessary HRP concentration.

To investigate the influence of the Tyr modification on the final mechanics, the visco-elastic properties of HA-Tyr hydrogels were measured using rheology and unconfined bulk compression testing. For all specimens, the storage modulus was independent of the frequency within the range analyzed, indicating a tightly crosslinked network (not shown). G’ and G” values at a given frequency (6.28 rad/sec) were chosen to demonstrate the visco-elastic properties of the HA-Tyr substrates as a function of the DS. G’ values increased with the modification up to DS 7%, while no increase was observed for higher DS (Figure 3-3C). This saturation effect was attributed to the consumption of all available H$_2$O$_2$. To investigate the reciprocal influence of H$_2$O$_2$ and DS at constant HRP amount (0.5 U/ml), unconfined bulk compression testing was performed to obtain the Young's modulus of HA-Tyr hydrogels$^{184}$. At low H$_2$O$_2$ concentration (0.68 mM) the Young's Modulus (E) is independent of the DS, whereas for 1.1 mM H$_2$O$_2$, E increased with DS (Figure 3-3D). Raising the H$_2$O$_2$ concentration to 2.3 mM amplified this effect, which was significant for DS 21% compared to DS 6.5 and 16% (Figure 3-3D). Thus, higher H$_2$O$_2$ concentrations exploited the full capacity of conjugated Tyr and enabled further tuning of the stiffness.
Figure 3-3 Increased Tyr modification of HA-Tyr hydrogels is correlated with reduced gelation times and enhanced Young's moduli with high H$_2$O$_2$ concentrations

A Rheological measurement (time sweep): Typical time and DS dependence of the storage modulus (G') and loss modulus (G'') of HA-Tyr gelation (0.5 U/ml HRP, 0.68 mM H$_2$O$_2$). Representative curves are shown for DS 3.2, 6.5 and 21%, DS 16 % not displayed as it overlays with the curve of DS 6.5%. B Gelation time indicated by the crossover point of G' and G''. C Viscoelastic moduli at 6.28 rad/sec and 1% strain shown as a function of the DS. Hydrogels were formed in-situ using 0.5 Unit/ml HRP and 0.34 mM H$_2$O$_2$. D Young's modulus of HA-Tyr hydrogels with DS 6.5, 16 and 21%, crosslinked with different H$_2$O$_2$ concentrations (0.5 Unit/ml HRP). All data shown as mean ± standard error mean for n = 4 independent measurements. **p<0.01 compared to DS 6.5 and 16% using 2.3 mM H$_2$O$_2$.

These findings were corroborated by the enzymatic degradation behavior of the crosslinked HA-Tyr hydrogels (Figure 3-4). While low H$_2$O$_2$ concentration of 0.68 mM did not show any differences between the different DS (Figure 3-4A), significant reduced degradation rates were observed for DS 16 and 21% hydrogels crosslinked with 1.1 mM H$_2$O$_2$ (Figure 3-4B). At high H$_2$O$_2$ concentration of 2.3 mM, little degradation was observed to occur for DS 16 and 21% hydrogels after 48 h in 10 U/ml hyaluronidase, indicating highly crosslinked HA-Tyr networks (Figure 3-4C). Similar results were obtained in the swelling behavior of these HA-Tyr hydrogels (Figure S 3-3). Notably, the measurements shown here were performed with 2.5% w/v HA-Tyr macromer concentration and HA of 290 kDa molecular weight. Thus, variations in HA molecular weight and macromer concentrations can further be utilized to tune the stiffness of the constructs. Taken together, the results confirm the strategy to independently control the visco-elastic moduli and gelation of HA-Tyr hydrogels at a given H$_2$O$_2$ concentration. In addition, highly substituted HA-Tyr derivatives extend the range of physical properties that can be accomplished.
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3.4.4 Encapsulation of human MSCs in HA-Tyr hydrogels

Owing to the mild and bio-orthogonal crosslinking conditions, HA-Tyr hydrogels are ideal to assess the influence of a simplified ECM mechanics. To determine if hydrogel properties modulated by H$_2$O$_2$ concentration and Tyr modification affect cellular behavior, hMSCs were suspended in the HA-Tyr macromer solution and encapsulated using either 0.68 mM or 1.1 mM H$_2$O$_2$ to vary matrix mechanics. Viability and morphology of hMSCs were analyzed over a time course of 6 days cultured in basal growth medium. Cells in all conditions showed high viability (85-98%) over 6 days as quantified with Live/Dead staining (Figure S 3-4). The overall morphology of hMSC was initially similar in soft and stiffer HA-Tyr hydrogels, 0.68 and 1.1 mM H$_2$O$_2$, respectively (Figure S 3-5). After 6 days, cells encapsulated in soft hydrogels exhibited extended spread morphology (Figure 3-5A), whereas cells in stiffer hydrogels maintained a rounded morphology (Figure 3-5B). Apparent changes in cell morphology were quantified with the aspect ratio (i.e. the ratio of the longest and shortest dimension of cells) over time. Hydrogels with higher Tyr modification led to enhanced cell spreading in 0.68 mM H$_2$O$_2$ constructs with significant increased aspect ratios for cells incorporated in DS 21% substrates (Figure 3-5C). In contrast, when the stiffness of the hydrogel matrix was increased by higher H$_2$O$_2$ concentration of 1.1 mM, encapsulated cells exhibited an aspect ratio between 1 and 1.5, which was found to be independent of the Tyr modification (Figure 3-5D). Decreased cell spreading in 1.1 mM H$_2$O$_2$ hydrogels can be due to matrix stiffness per se, or because of the high crosslink density which blocks degradation sites. Additionally, cell adhesion to a biomaterial can occur through adsorption of cell-adhesive proteins, when the matrix is exposed to serum-supplemented culture medium. Thus, impaired cellular spreading in 1.1 mM H$_2$O$_2$ HA-Tyr hydrogels may be attributed to insufficient nutrient transport and the small mesh size of the di-Tyr crosslinks that block cellular remodelling. However, in less crosslinked constructs synthesized with 0.68 mM H$_2$O$_2$, cellular spreading was observed to be regulated by the degree of Tyr modification at later time points (Figure 3-5C). Importantly, at 0.68 mM H$_2$O$_2$, the acellular bulk mechanics, 9 ± 2 kPa were identical for all three HA-Tyr constructs (Figure 3-3D, Figure 3-4A), indicating that the crosslinking...
densities (i.e. number of di-Tyr bonds) were similar. The findings here suggest that HA-Tyr matrices permit cellular spreading, which does not rely on additional peptide modifications. Previously, matrix metalloproteases (MMP) peptide linkers were incorporated into HA-acrylate hydrogels to obtain equivalent cell spreading whereas cells in unmodified control HA-hydrogels crosslinked remained round shaped\textsuperscript{147,200}.

Figure 3-5 HA-Tyr hydrogels permit viable hMSC encapsulation and promote cellular spreading in soft matrices

Representative images of hMSCs encapsulated in HA-Tyr hydrogels (5x10^6/ml) crosslinked with \textbf{A} 0.68 mM and \textbf{B} 1.1 mM H\textsubscript{2}O\textsubscript{2}. Calcein (5 μM, green) /Ethidium homodimer (8 μM, red) staining after 6 days of culture. Images are shown as 3D rendered z-stacks with a thickness of 200 μm. Dashed boxes show a zoom-in of the corresponding areas, scale bars are 200 μm. Quantification of aspect ratios of hMSCs in HA-Tyr hydrogels crosslinked with \textbf{C} 0.68 mM H\textsubscript{2}O\textsubscript{2}, acellular 9 ± 0.7 kPa and \textbf{D} 1.1 mM H\textsubscript{2}O\textsubscript{2}, acellular 18 ± 2 kPa, after 2, 4 and 6 days of culture in basal medium (DMEM plus 10% FBS). One representative donor shown with mean + SD of n = 4 randomly chosen locations within the construct (≥50 cells per image), *p<0.05.

Although we increased the degree of Tyr modification on HA, the majority of HA disaccharides remains unmodified, which may affect cell behavior\textsuperscript{28,193}. Interactions between HA and cells have been shown to influence the arrangement of cytoskeletal components\textsuperscript{201} and enhance chondrogenesis of encapsulated MSCs\textsuperscript{16}. Specifically, the hyaluronan receptor CD44 has been
reported to provide biological cues from the extracellular matrix to MSC\textsuperscript{187,188}. To determine if cellular spreading in soft HA-Tyr hydrogels is mediated by HA cell signaling, CD44 receptors expressed by the hMSCs were blocked with an anti-CD44 antibody prior encapsulation (Figure S 3-6). High cell viability after anti-CD44 treatment was confirmed for all groups by Live/Dead assay quantifications (Figure S 3-7). After 6 days of culture cell morphology of anti-CD44 treated hMSCs was observed to be similar to non-treated hMSCs in all groups (Figure S 3-8), indicating that CD44 cell signaling is not a decisive factor for early hMSCs spreading in soft HA-Tyr gels. However, blocking the CD44 receptor prior to encapsulation does not guarantee that HA signaling cannot be partially mediated via other HA receptor, including the receptor for HA-mediated motility (RHAMM, CD168) and intercellular adhesion molecule-1 (ICAM-1)\textsuperscript{202}. Also, in the current work no specific cell type or tissue was targeted and the influence of HA signaling on MSC differentiation and matrix deposition in HA-Tyr hydrogels remains to be elucidated.

### 3.5 Conclusion

The focus of this work was to show the versatility of the DMTMM-mediated conjugation technique, and the broader range and control on the HA-Tyr matrices obtained by varying the Tyr modification and H\textsubscript{2}O\textsubscript{2} concentration. The promotion of cellular spreading in the tailored 3D microenvironments in the absence of additional binding cues has potential in tissue regeneration where controlled cell morphology is desirable, such as vasculature or nervous tissue engineering. The wide range of mechanical features of HA-Tyr constructs may facilitate applications in microfluidics mimicking for example mechanical gradients\textsuperscript{203,204}. Taken together, we demonstrate that HA-Tyr hydrogels with a wide range of mechanical properties are promising matrices for cell-instructive applications.
3.6 Supplement

Figure S 3-1

$^1$H NMR spectrum of the physical mixture MES-DMTMM-Tyr (0.025 mmol each) in D$_2$O. Measurements were taken after 24 h incubation at RT.

Figure S 3-2

$^1$H NMR spectrum of the conjugate HA-Tyr synthesized in 100 mM NaCl at 37°C for 24 h.
Figure S 3-3
Swelling of HA-Tyr hydrogels with different DS as a function of H$_2$O$_2$ concentration. Hydrogels were formed with 0.5 U/ml HRP. Data shown as mean ± SD for n = 3 constructs, **p<0.01 for DS 6.5% compared to DS 16 and 21%.

Figure S 3-4
Quantification of cell viability of MSCs (5x10$^6$/ml) encapsulated in HA-Tyr hydrogels crosslinked with A 0.68 mM H$_2$O$_2$ and B 1.1 mM H$_2$O$_2$, after 2, 4 and 6 days of culture in basal medium (DMEM plus 10% FBS), mean + SD of four randomly chosen locations per hydrogel. One representative donor shown with mean + SD of n = 4 randomly chosen locations within the constructs.
Figure S 3-5

Representative images of hMSCs encapsulated in HA-Tyr hydrogels (5x10⁶/ml) crosslinked with 0.68 mM and 1.1 mM H₂O₂. Calcein (5 μM, green) /Ethidium homodimer (8 μM, red) staining after 6 days of culture. Images are shown as 3D rendered z-stacks with a thickness of 200 μm.

Figure S 3-6

A Quantification of CD44 expression intensity (Miltenyi Biotec 130-095-177) and B representative images of hMCS after incubation with anti-CD44 antibody (abcam 10-44-2). 5x10⁶ cells/ml, encapsulated in DS 16% HA-Tyr hydrogels crosslinked with 1 U/ml HRP and 0.68 mM H₂O₂. Cell-laden hydrogels were fixed 30 min after encapsulation with 4% paraformaldehyde (overnight at 4°C) and washed three times prior incubation with anti-CD44-APC antibody (Miltenyi Biotec 130-095-177), scale bar 200 μm.
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Figure S 3-7
Quantification of cell viability of encapsulated hMSCs (5x10^6/ml, 0.68 mM H_2O_2), blocked with CD44 antibody prior encapsulation compared to un-treated MSCs (control), after A 2 days, B 4 days and C 6 days of culture in basal medium (DMEM plus 10% FBS). One representative donor shown with mean + SD of n = 4 randomly chosen locations within the construct.

Figure S 3-8
Representative images of encapsulated hMSCs treated with CD44 antibody prior encapsulation and stained with Calcein (5 μM, green) /Ethidium homodimer (8 μM, red) after 6 days of culture. Images are shown as 3D rendered z-stacks with a thickness of 200 μm. Dashed box shows a zoom-in of the corresponding area, scale bars are 200 μm. B Quantification of cell aspect ratio of encapsulated hMSCs (5x10^6/ml, 0.68 mM H_2O_2), blocked with CD44 antibody prior encapsulation compared to un-treated MSCs (control), after 6 days of culture in basal medium (DMEM plus 10% FBS). One representative donor shown with mean + SD of n = 4 randomly chosen locations within the construct (≥50 cells per image), *p<0.05.
4 Microfabrication of Photo-crosslinked Hyaluronan Hydrogels by Single- and Two-photon Tyramine Oxidation

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**Contribution:** Conceived idea of HA-Tyr photo-crosslinking, rheological, swelling and degradation characterization studies of HA-Tyr, mechanistic studies, cell viability assays, and 2D microfabrication and actuation.
4.1 Abstract

Photo-crosslinking of tyramine-substituted hyaluronan (HA-Tyr) hydrogels is demonstrated for the first time. HA-Tyr hydrogels are fabricated via a rapid photosensitized process using visible light illumination. Non-toxic conditions offer photo-encapsulation of human mesenchymal stem cells (hMSCs) with high viability. Macroscopic gels can be formed in less than 10 s, and one and two-photon photo-patterning enable 2D and 3D microfabrication. Different degrees of crosslinking induce different swelling/shrinking, allowing for light induced microactuation. These new tools are complementary to the previously reported horseradish peroxidase / hydrogen peroxide crosslinking and allow sequential crosslinking of HA-Tyr matrices.

4.2 Introduction

Advances in light-mediated hydrogel patterning have achieved micrometer-scale control over the distribution of biochemical\textsuperscript{18,19,142,205} and biophysical\textsuperscript{206,207} signals in defined synthetic hydrogel systems. The vast majority of the photo-patterned hydrogels have been based on vinyl derivatives and free-radical chemistry\textsuperscript{208}. Guvendiren and Burdick demonstrated temporal stiffening of methacrylated hyaluronan (HA) hydrogel, applying UV light to locally trigger additional crosslinking\textsuperscript{45}. However, highly modified HA backbone of photo-pattern hydrogels and their unreacted acrylates may impair biofunctionality\textsuperscript{41,197}, while use of low substituted hyaluronan cross-linked hydrogels have not yet been reported. A photo-crosslinkable HA-hydrogel is described which has a broad range of gel mechanics while maintaining cell compatibility and the ability to be cleaved enzymatically.

We and others have reported the introduction of hydroxyphenyl groups on natural polymers, such as hyaluronan-tyramine (HA-Tyr)\textsuperscript{58,69,190}. Here, photo-crosslinkable, biodegradable, HA-Tyr hydrogels are introduced allowing temporal and micrometer-scale control of fabrication with single-photon lithography.

Visible light has been described for crosslinking of modified polyethylene glycol (PEG)\textsuperscript{51,209,210} and hyaluronan\textsuperscript{211} with Eosin Y (EO) as photo-initiators. However, the use of cytotoxic co-initiator e.g. triethanolamine for generating radicals in this case limits the applicability\textsuperscript{51}. Recently, Shih and colleagues described a rapid and orthogonal thiol-ene photoclick gelation between a dithiothreitol and PEG-norbornene macromers with visible light and EO as only photo-initiator\textsuperscript{212}.

Recent studies have shown that visible light can be used for gelation of tyramine functionalized synthetic polymers and tyrosine rich proteins with ruthenium (Ru(II)) and sodium persulfate\textsuperscript{213,214}. Here we use visible light to induce cytocompatible polymerization of rose bengal (RB) and EO sensitized HA-Tyr conjugates. Photo-crosslinked HA-Tyr hydrogels are prepared without the use of horseradish peroxide (HRP) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). In addition, the sequential enzymatic and light crosslinking of HA-Tyr conjugates is also reported for precise spatial control and accurate time-dependent stiffening of the hydrogels.

As single-photon lithography is limited by the lack of depth control, two-photon polymerization (2PP) holds promise for precisely fabricating 3D hydrogels with user-defined architecture\textsuperscript{215}. Most
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2PP studies use synthetic polymers with highly reactive acrylate groups such as poly(ethylene) glycol diacrylate (PEG-DA) or highly modified HA macromers requiring supportive thiol-ene chemistry (dithiothreitol) and addition of PEG-DA, respectively. Here we report on fabrication of high-resolution hyaluronan hydrogel microstructures using two-photon polymerization and their ability to actuate upon swelling.

4.3 Experimental

4.3.1 Materials

Hyaluronic acid sodium salt from Streptococcus equi (HANa) with weight-average molecular weight $M_w = 290$ kDa and poly-dispersion index $M_w/M_n = 1.86$, where $M_n$ indicates the number-average molecular weight was purchased from Contipro Biotech.r.o. (Czech Republic). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) from TCI (Zwijndrecht, Belgium). Corgel®Kit (DS 5%, $M_w > 1000$ kDa) was purchased from LifeCore Biomedical (Chaska, MN, U.S.A.). Other chemicals were of analytical grade, purchased from Sigma Aldrich (Buchs, Switzerland) and used as received.

4.3.2 HA-Tyr synthesis

HA-Tyr was synthesized following a previously described procedure. Briefly, sodium hyaluronate (290 kDa) was dissolved in deionized H$_2$O (1% w/v). HA-Tyr conjugates were prepared in a one-step reaction by adding 1.25 mmol DMTMM coupling agent and subsequently 1.25 mmol tyramine drop wise to the solution. The reaction was carried out at RT (DS 2.8%) or 37°C (DS 7.8%) and under continuous stirring for 24 h. The product was purified via precipitation with 96% ethanol after adding 10 vol% saturated sodium chloride. Several wash steps were performed and the product kept under vacuum for 48 h. $^1$H NMR and UV-vis analysis were performed to confirm substitution of tyramine on HA (DS 7.8% and 2.8%). Corgel® was used as a commercial available source of high $M_w$ HA-Tyr conjugates with a DS of 5% as per manufacturer specifications.

4.3.3 Equilibrium swelling/degradation assay

To examine swelling properties, HA-Tyr was dissolved in PBS (3.5% (w/v)) and mixed with 0.05% (w/v) RB and 0.02% (w/v) EO, respectively. HA-Tyr solutions (50 μl) were photo-polymerized in preformed polydimethylsiloxane (PDMS) molds (2x5 mm) for 5 min and 10 min, respectively. After several washing steps with PBS, samples were immersed in PBS (10 mM, pH 7.4) at 37°C for 48 h until the swelling equilibrium had been reached. The swollen hydrogels were removed, carefully blotted to remove excess surface liquid, and the total swelled weight was measured. The samples were lyophilized overnight and the dry weight was measured. The swelling ratio was calculated by: swelling ratio = (weight$_{wet}$ - weight$_{dry}$)/ weight$_{wet}$.

To determine stability, HA-Tyr precursor solutions (50 μl) containing 0.05% (w/v) RB and 0.02% (w/v) EO, respectively were photo-polymerized for 5 min in PDMS molds. After pre-swelling for 48 h at 37°C, mass of the samples was measured and degradation performed using 10 Units/ml
hyaluronidase in PBS at 37°C. The percentage of hydrogel mass remaining was calculated in relation to the swollen mass after 48 h \( \frac{\text{weight}_{\text{total}}}{\text{weight}_{\text{swollen}}} \times 100 \).

### 4.3.4 Characterization of HA-Tyr crosslinking kinetics and substrate mechanics

Dynamic oscillatory time sweeps were performed using an Anton-Paar Rheometer equipped with a Peltier controller, a 120-watt metal halide light source (HXP120 350-700 nm, 134 mW/cm²; Zeiss) and plate-plate geometry, diameter 20 mm. The synthesized conjugates at 3.5% (w/v) were hydrated overnight at 4°C in PBS. HA-Tyr precursor solution (100 μl) containing RB and EO respectively at various concentrations was placed in the gap and the upper plate was lowered to a spacing of 0.1 mm. A humid chamber was achieved by placing wet tissue paper around the platform and a chamber cover on top. A time-sweep oscillatory test was conducted for 10 min with a 1% sinusoidal strain in order to monitor the in situ liquid-to-solid transition (gelation point) of the solution during the photo-polymerization reaction. The mechanical spectra were carried out at 20°C and 1 rad/ sec angular frequency to monitor the shear elastic modulus \( G' \) and the loss modulus \( G'' \) of the hydrogels. All in situ rheometry tests were performed with a 60 sec pre-conditioning cycle followed by visible light curing.

### 4.3.5 Viability assay of hMSCs encapsulated in photo-crosslinked HA-Tyr hydrogels

Human bone marrow was harvested from the iliac crest (ethical approval Freiburg, EK-326/08) and hMSCs isolated using previously described protocols. Passage 3 hMSCs (5 x10⁶/ml) were suspended in PBS and encapsulated into HA-Tyr hydrogels (4x2 mm; DS 7.8%) using visible light induced crosslinking (Exfo X-Cite Series 120 lamp with a 395 nm long-pass filter/560 nm beamsplitter, power density at sample 134 mW/cm², exposure for 5 min) in the presence of 0.02% (w/v) EO. Cell-seeded hydrogels (n=3) were directly added to DMEM low glucose with 10% FBS, 1% penicillin/streptomycin and cultured at 37°C and 5% CO₂. First media changes were performed after 3 h and subsequently every second day. Cell viability was assessed using live-dead staining (20 μM calcein-AM and 3 μM ethidium homodimer) and imaging on an inverted point-scanning confocal microscope (Zeiss, 510 LSM) at 10x magnification. Viability was quantified by counting live (green) and dead (red) cells in 6-9 microscopic fields from representative hydrogels.

### 4.3.6 Fabrication of thin HA-Tyr hydrogel films

HA-Tyr hydrogels (3.5% (w/v)) were prepared by adding \( \text{H}_2\text{O}_2 \) (0.11 mM) and 1 Unit/ml HRP to a precursor solution with 0.05% (w/v) RB and 0.02% (w/v) EO, respectively. Hydrogels were cast between coverslip glass slides using 150 μm spacers. After 15 min incubation in a humid chamber at RT, the obtained hydrogel films were utilized for hydrogel patterning.

### 4.3.7 Detection of fluorescent di-tyramine bridges

HA-Tyr hydrogels are autofluorescent upon exposure to UV light. Di-tyramine displays an excitation maximum of 285 nm and emission maximum of 415 nm. The hydrogels’ intrinsic fluorescence was used to qualitatively assess crosslinking density. Fluorescent microscopy images were acquired after overnight washing at 4°C to ensure complete diffusion of the photosensitizer.
4.3.8 Laser-assisted hydrogel patterning

An inverted point-scanning confocal microscope (Zeiss, 510 LSM) equipped with a 488 nm enterprise laser (30 mW), a 365/415 nm argon laser and with a Plan-Neofluar 10x/0.30NA objective was employed for local illumination of the gels. The bleaching mode and user-defined ROI scanning option of the microscope software (Zen2009) allowed precisely controlled arbitrary patterns in xy direction. Precise control of specific laser intensities assigned to each ROI allowed generation of crosslinking gradients. Step-wise gradients were obtained by aligning a various number of contacting ROIs, each of which had assigned a different laser intensity (100-10% of the maximal laser power) or exposure time.

Solid 2D objects were printed out of a liquid precursor containing HA-Tyr 3.5% (w/v) and 0.05% (w/v) RB or 0.02% (w/v) EO buffered in PBS. Objects were gently washed with PBS to remove the un-crosslinked precursor solution and mounted on a glass cover slip. Images were acquired before and after free swelling in PBS. For 3D imaging, thirty sequential stacks of sections (10 μm depth) from the bottom of the construct were acquired.

4.3.9 Two-photon polymerization of HA-Tyr gels

Two-photon polymerization was performed on a Leica SP8 multiphoton microscope with irradiation with a Mai Tai XF DeepSee fs-laser (Spectra Physics) at 710 nm. A drop of the viscous liquid precursor (10% (w/v) HA-Tyr, DS 7.8% in PBS with 0.1% (w/v) EO) was loaded on a coverslip, and protected from evaporation with a PDMS ring and glass cover. The lower efficiency and slow gelling with two-photon excitation requires a higher concentration of EO compared to using a single laser.

Irradiation was performed on a 25x water objective (NA=0.95) with 1400 Hz scanning. 10x10x10 μm cubes were irradiated layer by layer with 1μm step in the z-direction. Increase in autofluorescence from HA-Tyr gel and decrease in EO fluorescence were monitored simultaneously, at 400-480 nm and 530-550 nm respectively. Laser power and number of scans were varied from 1.55, 2.25 and 2.85 mW and 1x64 -64x64 scans, respectively. Imaging of the results was performed on the midplane of the cubes with the same parameters but lower laser power. Finally, to remove the non-crosslinked precursor, the gels were washed with PBS for 2x30 min, and imaged again to identify conditions, which produced stable constructs.

For gel shrinking, a 2.5% (w/v) gel was crosslinked with 1 Unit /ml HRP/0.11 mM H₂O₂ in the presence of RB 0.1% (w/v) and loaded on the microscope. Short and intense irradiation (30% laser power, 15.95 mW at sample, with 600 Hz scanning rate and 8 scans) induced the formation of higher di-tyramine fluorescence that would shrink within minutes.

4.4 Results and discussion

4.4.1 Photo-polymerization of HA-Tyr

Previously, HA-Tyr was crosslinked enzymatically in the presence of horseradish peroxidase and hydrogen peroxide (H₂O₂)⁵⁸,⁶⁹,¹⁹⁰. Here, we obtained HA-Tyr crosslinking by exposure to visible
light (480-550 nm) in the presence of a singlet oxygen photosensitizer, such as RB 0.05% (w/v) or EO 0.02% (w/v)), demonstrating that hydrogels can be microfabricated with spatial and temporal control over stiffness. To analyze the gelation and viscoelastic properties of HA-Tyr (290 kDa, DS7.8%) photo-crosslinking compositions, the elastic moduli (G') and viscous moduli (G'') were monitored over time. Visible light irradiation was applied 60 sec after start of measurement (Figure 1a). At optimal photosensitizer concentrations, the gelation time, defined as the crossover point of G' and G'', is less than 30 sec for RB (0.05% (w/v)) while EO (0.02% (w/v)) induces gelation after less than 10 sec (Figure 1A). The improved gelling with EO initiation is confirmed by the higher final storage modulus, 2500 +/- 70 Pa compared to hydrogels with RB as photosensitizer, 1600 +/- 140 Pa. Just as HA-Tyr can be tuned by H₂O₂, we could also tune the mechanical properties by varying the concentration of RB and EO (Figure S 4-1). Unmodified HA did not gel upon exposure to visible light in the presence of RB or EO. Noteworthy, commercially available HA-Tyr (DS 5%, Corgel®, 1.6x10³ kDa) is photo-crosslinkable with RB and EO, also at lower HA-Tyr concentration (2.5% (w/v)) (Figure 4-1B). Gelation times of Corgel® solution decreased to less than 10 sec for RB induced photo-polymerization compared to the low Mw HA-Tyr conjugates (290 kDa) (Figure 1 a+b). This is probably due to entanglement of crosslinked high molecular weight HA-Tyr chains and energy storage, G' rising quicker than the low Mw HA conjugates. Similarly to RB, EO initiated Corgel® hydrogels display faster gelation kinetics compared to lower Mw HA-Tyr (Figure 1-1A+B). For both HA-Tyr conjugates, the final mechanical properties are inferior in RB photo-crosslinked hydrogels (1527+/−124 Pa) compared to EO (2607+/−200 Pa).

In addition, sequential crosslinking can be achieved by pre-polymerization with H₂O₂ and HRP followed by photo-crosslinking (Figure 4-1C). Using 0.11 mM H₂O₂/1 Unit HRP, the initial gelation occurs within 100 sec, and G' plateaued at 190 +/- 40 Pa. A sharp increase in G' value is measured when the pre-crosslinked hydrogel is exposed to visible light. By increasing the H₂O₂ concentration in the enzymatic HA-Tyr hydrogel, the initial mechanical properties could be further increased with the same polymerization time period (Figure S 4-2). However, further stiffening of the enzymatic pre-crosslinked HA-Tyr matrix using photosensitizers and light is limited likely because of less mobile or already reacted tyramines. Noteworthy, sequential crosslinking can be performed in reverse order with incubating the photo-crosslinked matrix in PBS containing HRP/H₂O₂ (data not shown).
Figure 4-1 Rheological properties of photo-crosslinked HA-Tyr

A Rheological profiles showing hydrogel formation by visible light induced photo-polymerization of HA-Tyr (3.5% (w/v)) with the photosensitizers EO and RB, respectively. B Rheology of light induced polymerization of Corgel® (2.5% (w/v)) with RB and EO respectively. C Sequential crosslinking via enzymatic polymerization (1 U/ml HRP; 0.11 mM H₂O₂) followed by visible light induced photo-polymerization. RB (0.05% (w/v)) induced photo-polymerization is shown as representative example. D Rheological profiles showing the temporal control of gel formation (3.5% (w/v)). Storage (G') and loss (G'') moduli are indicated by solid and open labels, respectively (in c) and d) mean ± SD). Experiments were repeated at least three times.

To prove that the formation of HA-Tyr gels is exclusively due to light exposure, light was switched on and off during measurement (Figure 4-1D). The fast kinetics of the polymerization, as indicated by the increase of G' values, begins immediately with light exposure and plateaus immediately after light removal. The sharp transitions between "on-off light exposure" demonstrate that photo-polymerization of HA-Tyr can be temporally controlled with light (Figure 1d). Mechanical properties of alternately exposed EO sensitized hydrogels (G' = 1850 +/- 22 Pa) following 300 sec accumulated illumination are equal to that of continuous illumination (G' = 1805 +/- 17 Pa) (Figure 4-1A). The wide range of storage modulus of photo-polymerized HA-Tyr hydrogels is similar to reported UV crosslinked hydrogels based on low degree of methacrylates or vinyl-esters, respectively216,219. The hydrogel swelling ratios agree with the rheological findings (Figure S 4-3a).
Furthermore, photo-polymerized HA-Tyr hydrogels degrade within 24 h in the presence of 10 Unit/ml hyaluronidase (Figure S 4-3b), similar to enzymatic crosslinked HA-Tyr hydrogels. Temporal control over HA-Tyr photo-polymerization confirms that visible light can be used to precisely control di-tyramine crosslinking. Variations in macromer concentration and substitution ratio also allow control over stiffness (Figure S 4-1c+d).

### 4.4.2 Mechanism of RB and EO induced HA-Tyr photo-polymerization

Enzymatic crosslinking of HA-Tyr with HRP in the presence of H$_2$O$_2$ forms a complex which oxidizes tyramine, leading to the formation of di-tyramine bonds. On the other hand, photoinitiated tyramine dimerization is more complex. Commonly, on absorption of a photon, ground-state EO/RB is raised to the first excited singlet state ($^1$RB/$^1$EO), and this, by intersystem crossing, is converted to a long-lived triplet state ($^3$RB/$^3$EO*; reaction 1) (Figure S 4-4). In the presence of oxygen, energy is transferred to form singlet oxygen ($^1$O$_2$; reaction 2), which reacts with the target compound (Figure S 4-4). HA-Tyr photo-polymerization yields are inhibited 70% (EO) and 80% (RB) by 10 mM azide suggesting that the reaction is mediated by singlet oxygen (Table S4-1). The addition of ferric cyanide (K$_3$Fe(CN)$_6$) completely blocks gelation by destroying $^3$RB/$^3$EO* and thus hindering energy transfer to oxygen (Table S4-1). Singlet oxygen has a much longer lifetime in D$_2$O than in H$_2$O, and $^1$O$_2$ sensitized reactions are more efficient in a deuterated solvent. However, HA-Tyr photo-polymerization rate is not increased by substituting PBS with D$_2$O (Table S4-1). This may be due to an efficient quenching of singlet oxygen by HA-Tyr itself. Still, the observed differences in photo-reactivity and final mechanical properties of EO and RB photosensitized HA-Tyr systems (Figure 1a and b) match singlet oxygen generation quantum yields of the molecules (EO: 0.67 versus RB: 0.11). Taken together, these data would indicate that photo-crosslinking of HA-Tyr is primarily due to $^1$O$_2$-mediated oxidation of Tyr.

### 4.4.3 Viability of hMSCs in photo-crosslinked HA-Tyr

hMSCs were encapsulated in the visible light photo-crosslinked HA-Tyr, using 0.02% (w/v) EO as photosensitizer and the cytotoxicity of the EO photo-crosslinked HA-Tyr hydrogel evaluated by fluorescence staining (Figure 4-2). Live-dead staining of hMSCs confirmed that cytocompatibility is comparable to enzymatically crosslinked HA-Tyr hydrogels as more than 95% of hMSCs were viable up to 4 days of culture (Figure 4-2+ Figure S 4-5). The direct EO sensitized HA-Tyr photo-crosslinking mediated by the $^1$O$_2$- oxidation of Tyr eliminates potential cellular toxicity imparted by the co-initiator triethanolamine (TEA) previously used in EO sensitized radical photo-crosslinked hydrogels.
4.4.4 2D control of photo-polymerization with laser direct writing

In addition to controlling material stiffness, light can be used to pattern HA-Tyr hydrogels with micrometer resolution. Laser scanning lithography was used to sequentially create patterns with varying density of di-tyramine crosslinking. HA-Tyr hydrogel films (150 μm) containing 0.05% (w/v) RB and 0.02% (w/v) EO were pre-crosslinked with 0.11 mM H₂O₂. Hydrogel films were photo-patterned by exposing ROIs (100 x 100 μm) to specific laser intensities and iterations, respectively. Arrays of cubic structures were screened at varying laser power and iterations (50 versus 25; in triplicates) within the assigned ROI (Figure 4-3A). The photo-patterned hydrogel films were gently washed to remove the dye and incubated for 24 h in PBS at 4°C. The quantification of di-tyramine relative fluorescence of the cubes was nearly proportional to laser power (Figure S 4-5A). In addition, longer exposure times generated higher values of fluorescence independent of laser power (Figure S 4-6B). Stepwise gradients of crosslinking were patterned by illuminating adjacent rectangular ROIs within the hydrogel with assigned iterations (Figure S 4-6A) and laser intensities (Figure S 4-6B), respectively. These results show that laser triggered di-tyramine fluorescence can be precisely modulated by exposure time and applied laser power.

Figure 4-2 Cytocompatibility of photo-crosslinked HA-Tyr hydrogels

Encapsulated hMSCs stained with calcein-AM (green, live) and ethidium homodimer (red, dead) in 3.5% (w/v) visible light crosslinked (0.02% (w/v) EO) HA-Tyr hydrogel at 2, 3 and 4 days (scale bar 100 μm) and quantification of live-dead staining (n=3, +/- SD).
Figure 4-3 Single-Photon lithography of HA-Tyr hydrogels

A enzymatic pre-crosslinked HA-Tyr hydrogels films and B-D from a liquid HA-Tyr precursor solution: A Single Photon processing window of 3.5% (w/v) HA-Tyr formulations, with each condition in triplicates, after 24 h free swelling in PBS (scale bar 100 μm). B 3D image of a star printed from a liquid HA-Tyr precursor solution (3.5% (w/v), 0.05% (w/v) RB) after free swelling in PBS for 24 h. Image was acquired from the bottom of the construct (200 μm thick). Depth coding displays the z-information in color. C Macroscopic image from the micro-scale object with the tip of a needle as reference (scale bar 1000 μm). D Microactuators: development of the bending of the rectangle hinge during free swelling in PBS, directed by lower crosslinking density of the bottom rectangle achieved with less exposure time (scale bar 100 μm). Experiments were repeated at least three times with reproducible results.
We used laser direct-write for 2D control of crosslinking of a HA-Tyr solution containing RB only (Figure 4-3C+D). Stable constructs were recovered by removing the un-crosslinked precursor solution with PBS, and allowed to free swell overnight before imaging. In addition, differentially crosslinked gel regions can be used to produce actuation upon swelling\textsuperscript{224,225}. To achieve actuation within a single-component structure, we fabricated “two-rectangle” microstructures, illuminated with differing assigned exposure times (top part 60 versus bottom part 20 iterations) and a 75 μm rectangle hinge in the middle (Figure 4-3D). The resulting differences in swelling ratio resulted in a direct bending of the highly crosslinked hinge (Figure 4-3D). The use of coumarin and o-nitro benzyl have been used to photo-pattern biomolecules in hydrogels\textsuperscript{226,227}. However, they required multiple functionalization of hyaluronan or step-wise fabrication\textsuperscript{228}. Interestingly, while O-nitro benzyl groups introduced as photo-cleavable groups were shown to decrease the elastic modulus of hydrogel upon UV light exposure\textsuperscript{206}, HA-Tyr increases its elastic modulus of hydrogel upon visible light. Noteworthy, visible light induced tyramine oxidation could be used to photo-pattern biological ligands in tyramine containing hydrogels\textsuperscript{229}.

4.4.5 3D control of photo-polymerization: Two-Photon lithography

Crosslinked HA-Tyr produces di-tyramine autofluorescence with wide excitation and emission spectra. It is excited simultaneously to the EO at 710 nm and their emission can be collected with very little cross-talk, though the signal from the EO is much more intense. This enables one to monitor the formation of di-tyramines and the loss of EO while performing 2PP, which is a unique advantage of this crosslinking chemistry.

Increase in blue fluorescence is visible, pointing to production of di-tyramine crosslinks, but low irradiation time or power only produced fluorescent structures which were not stable after washing (Figure 4-4A). Longer irradiation or higher laser power result in brighter structures that contracted quickly after exposure and are only very partially stable after washing. At 3% laser power and 64x64 line x image scans, however, intensely bright structures are produced that are stable after several washes. Longer exposure times can be used, but still higher laser power leads to gel boiling or burning when scanning large patterns.

When performing two-photon crosslinking on gels preformed with 1 Unit/ml HRP/0.11 mM H\textsubscript{2}O\textsubscript{2}, the exposed area changes shape with the same trend as the liquid precursor (Figure 4-4B). Thus, there was a regime between low crosslinked and high crosslinked structures in which the irradiated area undergoes marked shrinkage within minutes. This behavior could be used as a one-time microactuator to study e.g. cell response to receptor pulling in a 3D environment.
Chapter 4 Microfabrication of photo-crosslinked HA-Tyr hydrogels

4.5 Conclusion

In this paper, cytocompatible HA-Tyr with enzymatic and light triggered gelation is reported for precise hydrogel polymerization and matrix stiffening. Photo-rheometry shows exceptionally fast gelling properties and applicability to a commercially available HA-Tyr product. Using laser direct writing, we controlled photo-polymerization in 2D, generating free-shape micro-patterns in a hydrogel layer. Two-photon patterning enables the production of micro-patterns with depth control. Different crosslinking densities within a single HA-Tyr gel allowed spatial control over contraction, expansion or bending, suggesting photo-crosslinking in HA-Tyr gels can be used as 3D, on demand, microactuators. We anticipate that these photo-polymerization methods will be useful in tissue engineering and regenerative medicine.
4.6 Supplement

Figure S 4-1

Rheological profiles for different \( a) \) RB and \( b) \) EO concentrations, respectively. Storage modulus (\( G' \)) and loss modulus (\( G'' \)) are indicated by solid and open labels, respectively (3.5% (w/v)).

Figure S 4-2 Rheological profiles of HA-Tyr photo-crosslinked with different \( \text{H}_2\text{O}_2 \) concentrations

Rheological profiles for different \( \text{H}_2\text{O}_2 \) concentrations for sequential stiffening of HA-Tyr. Storage modulus (\( G' \)) and loss modulus (\( G'' \)) are indicated by solid and open labels, respectively (3.5% (w/v)).
Figure S 4-3 Swelling and enzymatic digestion of photo-polymerized HA-Tyr hydrogels

(a) Swelling ratio for HA-Tyr hydrogels photo-polymerized with RB (0.05% (w/v)) and EO (0.02% (w/v)) for 5 min and 10 min, respectively. Crosslinked hydrogels were incubated in PBS for 48 h at 37 °C to reach equilibrium. (b) Enzymatic degradation of HA-Tyr hydrogels photo-polymerized for 5 min with RB (0.05% (w/v)) and EO (0.02% (w/v)), respectively. Samples were allowed to pre-swell in PBS for 48 h at 37 °C. Results illustrate remaining hydrogel mass (%) over 24 h of digestion.

Figure S 4-4 Possible pathway of RB/EO mediated Tyr oxidation

Absorption of a photon (hv) by RB/EO, molecule undergoes rapid intersystem crossing (\(^{3}RB/EO^{*}\)) and is a \(^{1}O_{2}\) sensitizer. \(^{3}RB/EO^{*}\) can directly react with Tyr (1) or transfer energy to form \(^{1}O_{2}\), which reacts with Tyr (2). Scavengers/quenchers (red arrows) and enhancers (green arrows) are shown for the reactions: \(K_{3}Fe(CN)_{6}\) quenches the \(^{3}RB/EO^{*}\) (reaction 1) and \(^{1}O_{2}\) (reaction 2), while production of \(^{1}O_{2}\) (2) is exclusively quenched by NaN\(_{3}\) and enhanced by D\(_{2}\)O, respectively.
Figure S 4-5 Di-tyramine relative fluorescence intensities

Single-Photon processing window of 3.5% (w/v) HA-Tyr formulations (Figure 4-2A) on a line covering 100-80-60-40% laser power for A 25 iterations and B 50 iterations (imaged and quantified after 24 h swelling in PBS).

Figure S 4-6 Step-wise crosslinking gradients obtained by titrating

A Iterations (exposure time with 100% laser intensity) and B laser intensity in each ROI (design: 8 x 450 μm wide rectangular ROIs, scale bar 200 μm).

Table S4- 1 Effect of various agents upon photo-polymerization of HA-Tyr

HA-Tyr (3.5% (w/v)) was photosensitized by 0.02% (w/v) EO and 0.05% (w/v) RB, respectively (3.5% (w/v)). Results illustrate the G' (Pa) ratio after 9 min illumination of HA-Tyr plus agent (NaN₃, K₃Fe(CN)₆) or D₂O and control HA-Tyr.

<table>
<thead>
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<th>Compound added</th>
<th>Percentage of photo-polymerization (%)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1</td>
</tr>
<tr>
<td>NaN₃ 100 mM</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>NaN₃ 10 mM</td>
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<td>0.20</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
<td>D₂O</td>
<td>0.9</td>
<td>0.9</td>
</tr>
</tbody>
</table>

('O₂ quencher')²⁴¹

electron acceptor²⁵°

increased life-time of 'O₂²²³
Chapter 5 Crosslinking Chemistry of Tyramine-Modified Hyaluronan Hydrogels Alters Mesenchymal Stem Cell Early Attachment and Behavior

In preparation for submission:
5.1 Abstract

Given the significance of hydrogels as cell-instructive materials, it is important to understand how their chemical and physical properties direct cell fate. For example, it remains unclear how different crosslinking chemistries and gelation mechanisms of hydrogels influence cell behavior. Recent developments in the design of hyaluronan hydrogels have facilitated control of material properties to engineer cell-matrix interactions. Here we report hyaluronan-tyramine (HA-Tyr) hydrogels prepared either with enzymatic crosslinking using horseradish peroxidase (HRP) and H$_2$O$_2$ or using visible light triggered gelation. We demonstrate that the crosslinking chemistry of HA-Tyr hydrogels at equal Young’s Moduli has a substantial impact on mesenchymal stem cells (MSC) behavior. MSC cultured on HA-Tyr hydrogels exhibit increased cell spread area on enzymatically formed substrates relative to photo-crosslinked matrices. While enzymatically formed hydrogels led to greater cell focal adhesion length and increased nuclear translocation of yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), MSCs generated increased forces on photo-crosslinked matrices with smaller cell spread area and shorter focal adhesion length. These findings highlight the importance of the hydrogel crosslinking chemistry when looking at biophysical cues in regulating stem cell fate.

5.2 Introduction

Cells in living tissue actively sense and respond to a variety of biochemical and biophysical signals from the extracellular matrix (ECM). Toward the use of hydrogels for therapeutic applications it is important to understand the interaction between stem cells and the material properties. For stem cells cultured on hydrogels, the substrate elasticity has been proven to regulate cell spreading, stem- and progenitor cell proliferation and differentiation.$^{8,9,102,109,231,232}$ Here synthetic substrates, such as polyethylene glycol (PEG) and polyacrylamide gels have often been utilized as a template to independently control and elucidate contributions of specific matrix properties in regulating cell adhesion and behavior.$^{8,9,12,109,114}$ Whereas past studies have focused on the effects of homogeneous materials on stem cells, recent reports have begun to address aspects of matrix micro- and nano-heterogeneities. There has been strong evidence that in parallel to substrate stiffness, a variety of environmental cues have substantial impact on cell behavior and function.$^{137,233}$ For example, cell fate was shown to be influenced by the nanotopography$^{126}$, tethering of ECM molecules$^{129}$ and network architecture$^{136}$ of the substrate. Recent work illustrated that cell spreading is also sensitive to stress-relaxing properties of the underlying substrate$^{131}$. In addition to fundamental understanding of cell-ECM interactions, these findings have direct implications on the design of biomaterials where the sensitivity of cells to substrate properties can be harnessed to guide stem cells toward functional tissue regeneration in vivo$.^{234}$

Hyaluronan (HA) hydrogels have been widely investigated for their biocompatibility. They have also been engineered to incorporate variety of cues to modify cell-material interactions, and
ultimately to influence and to allow for better control over the behavior of cells²⁴,¹⁴¹. Given the increasing interest in controlling gelation and mechanical cues of HA hydrogels, various covalent crosslinks have been explored²⁵,¹⁴⁶,¹⁴⁸,²¹⁶,²³⁵,²³⁶. Previously we and others have described tyramine-modified hyaluronan (HA-Tyr) hydrogels systems formed by enzyme-mediated crosslinking of tyramine moieties using horseradish peroxidase (HRP) and hydrogen peroxide (H₂O₂)⁵⁷,⁶⁹,¹⁹⁰. Additionally, we reported visible light crosslinking of HA-Tyr as an alternative¹⁹¹. Light triggered gelation is primarily due to singlet oxygen mediated oxidation of Tyr which leads to the formation of di-Tyr bonds²²². However, it remains unclear how the crosslinking chemistry of HA hydrogels influences material properties and therefore the environmental cues cells integrate and respond to.

Here we investigate the influence of HA crosslinking chemistry on cell spreading and force generation. As a model hydrogel system, we use HA-Tyr matrices with similar compressive moduli that are either formed enzymatically or by visible light illumination. Both crosslinking mechanisms lead to the formation of covalent gels via di-Tyr bonds in mild conditions identically compatible with encapsulations or seeding of cells with high viability of 98%¹⁹¹. Mesenchymal stem cells (MSCs) cultured on HA-Tyr hydrogels show enhanced spreading on enzymatically formed substrates relative to photo-crosslinked matrices. Our findings show that enzymatically formed gels led to greater cell focal adhesion length and nuclear localization of yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), whereas MSCs on photo-crosslinked matrices have smaller focal adhesion length and generated higher forces on the hydrogel. These effects are independent of initial matrix stiffness and show the importance of HA crosslinking chemistries when looking at biophysical cues regulating stem cell fate on these engineered cell-instructive materials.

5.3 Experimental

All chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland), unless otherwise indicated.

5.3.1 HA-Tyr Synthesis

HA-Tyr was synthesized following a previously described procedure¹⁹⁰. Briefly, sodium hyaluronate (290 kDa, Contipro Biotech s.r.o., Czech Republic) was dissolved in deionized H₂O (1% w/v). HA-Tyr conjugates were prepared in a one-step reaction by adding 1.25 mmol 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, TCI Europe N.V.) coupling agent and subsequently 1.25 mmol tyramine dropwise to the solution. The reaction was carried out at 37 °C and under continuous stirring for 24 h. The product was purified via precipitation with 96% ethanol after adding 10 vol % saturated sodium chloride. Several wash steps were performed and the product kept under vacuum for 48 h. ¹H NMR and UV−vis analysis were performed to confirm substitution of tyramine on HA (DS 6.5).
5.3.2 RGD conjugation to HA-Tyr

For in-situ RGD modification of HA-Tyr, the cell-adhesive oligopeptide GRGDGGGGGYY (Genscript®, United States) was mixed in the HA-Tyr precursor solution before formation of the hydrogels. As a result, HA-Tyr crosslinking and RGD conjugation could be achieved simultaneously.

5.3.3 HA-Tyr hydrogel formation on gelatin-coated coverslips

Porcine gelatin was dissolved in deionized H2O (2.5 g/500 ml) and cooled to room temperature before adding 0.25 g Chromium(III) potassium sulfate dodecahydrate. Coverslips (22x22 mm, No. 1) were cleaned with soap, rinsed with dH2O and washed with 70% Ethanol. Dry coverslips were soaked in the gelatin solution for 2 min, dried at RT for 48 h and used as prepared. HA-Tyr hydrogel films of 7 kPa (DS 6.5 3.5% w/v) were prepared enzymatically (HRP) (0.68 mM H2O2/1 Unit/ml HRP), with singlet-oxygen mediated crosslinking (EO) (0.02% Eosin Y, visible light 40 sec), and a combination with sequential crosslinking using 0.34 mM H2O2/1 Unit/ml HRP followed by 0.02% Eosin Y, 20 sec visible light (HRP/EO). HA-Tyr precursor solutions always contained both 1 U/ml HRP and 0.02% EO. These gels were in-situ modified with RGD (500 μM) and formed on gelatin-coated coverslips using 300 μm spacers.

5.3.4 Mechanical characterization

The Atomic Force Microscope (Agilent 6000 ILM/AFM) was used to perform force spectroscopy to measure the Young's modulus (E) on the nanometer scale. AFM tips were used as an indenter and the AFM operated in force mode in PBS. First, a spherical AFM probe (Novascan SiO2 Cantilever; 1 μm tip diameter, spring constant 0.06 N/m) was mounted and aligned on the AFM head. The cantilever deflection sensitivity was then calculated by fitting a force curve taken on rigid plastic (E > 1GPa) with the linear contact part and determined the spring constant with the thermal noise method (Thermal K)237. After calibration, 48 force curves (3 force maps of a 4x4 grid of indentation from different locations) were measured per sample and repeated at least three times on separate experiments. Hydrogel films were prepared by crosslinking HA-Tyr precursor solution (1 U/ml HRP, 280 nM Eosin Y) with 0.68 mM H2O2 (HRP), 0.34 mM H2O2/20 sec illumination (HRP/EO) or 40 sec illumination (EO). Force curves were taken after washing the films three times with PBS and after at least 60 min incubation in PBS at RT. For data processing, force curves were imported into Matlab (Mathworks) where the indentation contact point was identified. The Young's modulus of the different points was derived by fitting the first 800 nm of the force indentation curves to the Hertzian model (fit depth) while assuming a Poisson's Ratio of 0.45 (Figure S 5-1A)238.

The locally measured elastic modulus of enzymatic crosslinked HA-Tyr gels (HRP) was compared with the equilibrium compression modulus of formed hydrogel discs measured with unconfined compression stress-relaxation device194. Stress relaxation tests were performed with 10% strain applied at 0.05%/sec followed by relaxation for 1000 sec until equilibrium. The Young's modulus
was determined from the equilibrium force normalized to the diameter of the hydrogel disc divided by the equilibrium compressive strain.

Rheological measurements were performed using an Anton-Paar rheometer equipped with a Peltier controller, a 120 W metal halide light source (HXP120 350–700 nm, 134 mW/cm², Zeiss) and plate–plate geometry, diameter 20 mm. The synthesized conjugates at 3.5% w/v were hydrated overnight at 4 °C in PBS containing 1 U/ml HRP. For in-situ measurements, Eosin Y (280 nM, equivalent 0.02%) was added to the dissolved HA-Tyr precursor solution (1 U/ml HRP), mixed with 0.68 mM H₂O₂ (HRP) and 0.34 mM H₂O₂ (HRP/EO) respective and placed in the gap, and the upper plate was lowered to a spacing of 0.1 mm. A humid chamber was achieved by placing wet tissue paper around the platform and a chamber cover on top. A time-sweep oscillatory test was conducted for 10 min with a 1% sinusoidal strain before the photopolymerization reaction was started for HRP/EO samples. For EO in-situ rheometry tests, HA-Tyr precursors were measured with a 60 s preconditioning cycle followed by visible light curing. The mechanical spectra were carried out at 20°C and 1 Hz to monitor the shear elastic modulus (G’) and the loss modulus (G'”) of the hydrogels.

To measure the dynamic shear storage and loss modulus of swollen hydrogels, HA-Tyr hydrogels were prepared identically to the cell seeding experiments except that 400 µm spacers were used. HA-Tyr precursor solution (1 U/ml HRP, 280 nM Eosin Y) was crosslinked with 0.68 mM H₂O₂ (HRP), 0.34 mM H₂O₂/20 sec illumination (HRP/EO) or 40 sec illumination (EO). Hydrogel films were washed three times with PBS and incubated for 60 min in PBS at RT and at least 30 min in Dulbecco’s Modified Eagle Medium (DMEM) before measurements. To measure the strain response, gels were placed on the bottom plate and the upper plate was lowered to a spacing of 0.5 mm. The strain was ramped from 0 to 10% strain at 1 Hz and 20 points per decade. The resisting stress and estimated storage and loss modulus were measured at each point. Subsequently, frequency dependency was measured within the linear visco-elastic range from 0.1-10 Hz per second and 10 points per decade.

5.3.5 MSC isolation from calves (4-6 months)

Bone marrow derived mesenchymal stem cells were isolated from the tibia and femur of three donor calves (4-6 months old, Boston 87, Boylston, MA, USA and Davos, Switzerland) as previously described. Briefly, the trabecular bone was diced and the bone cubes washed with DMEM (2% penicillin/streptomycin and 2 mg/ml heparin) to collect the bone marrow. Pelleted cells and bone shavings were plated in DMEM (1% P/S, 10% FBS) and cultured until colonies showed ca. 80% confluence. P0 MSCs were frozen in 92% FBS/8% DMSO and stored in liquid nitrogen until use.

5.3.6 Cell seeding

Bovine MSCs (P0) were thawed and cultured on plastic for 18 h before seeding. Hydrogel films were washed three times with sterile PBS and sterilized under UV-C (200-280 nm) for 60 min. Hydrogels were prepared and incubated for 30 min in DMEM containing 10% FBS at 37 °C before
seeding of 5’000 cells/cm² and cultured for 4 h, 8 h and overnight (14 h). For CD44 and tyramine blocking studies, MSCs were incubated for 45 min on ice with either monoclonal anti-CD44 antibody (abcam, ab6124, 3:1000) in a buffer (2 mM EDTA and 2% FBS in PBS) or with 25 mg/ml tyramine, 0.5 U/ml HRP and 0.34 mM H₂O₂ in 2% FBS in PBS. CD44 and Tyr blocked cells were washed twice in the according buffer and filtered using a cell strainer (70 µm) before plating. Each experiment was repeated at least three times.

5.3.7 Immunofluorescence

For immunofluorescence staining, media were removed from the gels. The gels were then fixed with 4% paraformaldehyde in PBS at room temperature for 18 min. For Paxillin staining, gels were fixed for 10 min at 37 °C with a microtubule stabilization buffer (0.1 M 1,4-piperazinediethanesulfonic acid (PIPES), pH 6.75, 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA), 1 mM magnesium sulfate (MgSO₄), 4% (w/v) poly(ethylene glycol) (PEG, 8000), 1% Triton X-100, 2% paraformaldehyde). Prior to staining, gels were washed three times with PBS and blocked with bovine serum albumin (BSA 1%) for 30 min. Gels were stained using standard protocols for immunofluorescence staining; all antibody incubation steps were done in the presence of 1% BSA. The following antibodies were used: Alexa Fluor 647 Phalloidin for actin staining (Life Technologies, A22287, 1:1000), anti-YAP/TAZ (Santa Cruz, sc-101199, 1:200), anti-Paxillin to visualize focal adhesions (BD Bioscience, 610052, 1:200) and goat anti-mouse 546 secondary antibodies (Life Technologies, A-11030, 1:200).

For analysis, images of cells were taken with an NA=1.40 63x PlanApo oil immersion objective with a laser scanning confocal microscope (LSM 710, Zeiss). Only cells that did not show cell-cell contact were considered for quantification. For measurements of cell spreading area, the outline of each cell was traced and analyzed using the shape descriptor in ImageJ. YAP/TAZ nuclear localization ratio was determined as the average intensity of the YAP/TAZ signal within the nucleus divided by the average intensity of the YAP/TAZ signal in the cytoplasm. Focal adhesions were quantified utilizing the Focal Adhesion Analysis Server (FAAS). To decrease the background noise of the submitted images, a threshold of 3 pixels (0.39 µm) was set to be the minimum FA length included in the calculation and applied for all groups.

5.3.8 Traction force microscopy (TFM)

HRP, EO and HRP/EO hydrogels for 2D TFM were fabricated as described above with the following modifications. Prior to crosslinking 1% fluorescent red microbeads (FluoSpheres® Carboxylate-Modified, 0.2 µm 580/605, 2% solid) were added to the polymer solution. Hydrogels were fabricated on gelatin-coated coverslips and incubated with the top surface facing down to allow the beads to segregate at the top surface. Coverslips were sealed to the bottom of sterile 4-well plates with UV polymerizing glue (Norland Optical Adhesive 68) using a hand-held UV-A (315-400 nm) lamp (I=3.5 mW/cm²) for 16 min. After three washing steps with sterile PBS, gels were sterilized under UV-C (200-280 nm) for 60 min. BovineMSCs (P0) were thawed and cultured on plastic for 18 h before seeding. Hydrogels were incubated for 30 min in DMEM containing 10% FBS at 37 °C before seeding of 5.000 cells/cm² and culture overnight (14 h). Cells
and microbeads were imaged using a DeltaVision Core Widefield Deconvolution Microscope (Olympus IX70 inverted microscope) equipped with a 40x objective, mCherry Filter set (Excitation/Emission 587/610), and an environmental chamber surrounding (37°C, 5% CO₂). Cells were chosen that were far enough apart so that no mechanical interactions between them are expected. First, a phase-contrast image of the cell and an image of the fluorescent microbeads near the ventral surface of the cell were acquired in “stressed” state (i.e. prior to cell lysis). Sodium dodecyl sulphate (10%) and TritonX at 100:1 ratio was then applied to lyse the cells. Subsequently, a second image of the microbeads in the “relaxed” state of each hydrogel position was taken.

For calculations of bead displacement, images of the microbeads before and after cell-lysis were imported to ImageJ and aligned in a cell-free region by cross-correlation to correct for experimental drift. Displacement vectors of the gels were then calculated using iterative particle image velocimetry (PIV) with the normalized correlation coefficient algorithm. Paired images were divided into smaller regions (i.e. interrogation windows) and the cross-correlation between these images displayed the displacement of the beads within the image pair. Decreasing the interrogation window size achieved a better PIV resolution. Given the bead displacement field, Fourier transform traction cytometry (FTTC) was used to reconstruct the force field (Poisson ratio = 0.45). A regularization factor of 8*10^-10 was employed to obtain robust solutions. To calculate the traction forces, cell images pre and post cell lysis, as well as traction stress vector maps were imported into Matlab (Mathworks). The cell boundaries were marked manually using the cell image, and a region-of-interest around the cell-mediated traction stress map was drawn to integrate all stress vectors exerted by the cell. The sum of the magnitudes of the traction force exerted at each point yields a measure of the total traction or total cell-substrate force exerted by the cell. Although the vector sum of the traction forces within the region-of-interest is expected to sum to zero for a single cell, imbalances result due to experimental measurement errors and cells that represented less than 11% were included in the analysis. Cell spread area (µm²), average traction stress (Pa), total force (nN), and surface tension (N/m) were calculated using a customized Matlab module.

### 5.3.9 Statistical analysis

Mean values and standard deviations were obtained for quantitative measurements and image analyses with one-way ANOVA analysis coupled with Tukey's multiple comparison tests. Statistically differences were verified when p < 0.05.

### 5.4 Results and discussion

#### 5.4.1 Fabrication of RGD modified HA-Tyr hydrogels with varying crosslinking strategies and mechanics

For studying the effect of HA crosslinking chemistries, HA-Tyr hydrogels were formed either enzymatically with HRP/H₂O₂ (HRP) in the presence of EO, or by visible light illumination (EO) without H₂O₂ with HRP in solution, or using sequential enzymatic and light crosslinking (HRP/EO)
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(Figure 5-1A). The formation of di-Tyr bonds led to elastic substrates that have low stress-relaxation as observed during in-situ rheological measurements (Figure 5-1B). Although HA is a natural component of the extracellular matrix, it presents no moieties that allow cell adhesion via integrin-binding\(^{245}\). Therefore, HA-Tyr was functionalized in-situ with cell-adhesive moieties through incorporation of tyrosine-modified RGD (GRGDGGGGG-Y) peptides (Figure 5-1A). Conjugation of phenol moieties with HRP/H\(_2\)O\(_2\) was shown previously to allow uniform RGD conjugation within a synthetic hydrogel and a correlation between feed concentration and the amount of conjugated RGD\(^{229}\). Here we measured the local Young's modulus of crosslinked HA-Tyr substrates by atomic force microscope indentation. The degree of crosslinking was varied by the H\(_2\)O\(_2\) molar concentration (HRP, Figure 5-1C), and the exposure time without (EO) pre-crosslinking (Figure 5-1D), or with enzymatic pre-crosslinking (Figure 5-1E). We found that RGD conjugation to the phenol-groups of HA-Tyr (500 μM) reduces the stiffness of HRP, EO and HRP/EO crosslinked substrates by 16±10%, which was observed for a wide range of crosslinking densities (Figure 5-1C-E). This indicates that the conjugation of RGD-moieties occurs simultaneously to di-Tyr bond formation and is independent of the crosslinking agent concentration. Based on the distribution of the Ey-values obtained from various locations of each hydrogel film, we assumed homogenous crosslinking, which was observed to be independent of the crosslinking strategy (Figure S 5-1B). In addition, unconfined compression testing of the hydrogels confirmed that the local mechanical properties are matching bulk elastic moduli (Figure S 5-1C). Crosslinking density was adjusted so that the initial Young's modulus of the HRP, HRP/EO and EO crosslinked hydrogel with 500 μM RGD ligand density was 7 kPa for all. As the hydrogels are differently crosslinked to obtain the same initial stiffness, it is important to note that this may cause differences in mesh size and other physical parameters which have not been investigated yet.
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5.4.2 Crosslinking Chemistry alters cell spreading of MSCs on soft HA-Tyr substrates

We first examined cytoskeletal organization of MSCs seeded on substrates of equal stiffness (Figure 5-2A). After 8 h of culture in basal growth medium, MSCs on HRP crosslinked substrates adopted a well-spread morphology and numerous strong actin fibers whereas MSCs on EO substrates remained small with relatively few actin fibers throughout the cytoplasm (Figure 5-2B).

To test whether the illumination of the matrix drives these variations in cell behavior, MSCs were seeded on HA-Tyr matrices formed by illumination of an enzymatically pre-crosslinked HA-Tyr containing EO (HRP/OE). Here, cell morphology and stress fiber alignment were similar to MSCs cultured on HRP substrates (Figure 5-2B). Monitoring cellular spreading over time revealed that MSCs on HRP and HRP/OE substrates possessed a greater spreading area at early time-points.
(after 4 h culture) that asymptotically declined over 14 h (Figure 5-2C). In contrast, when cells were cultured on EO substrates, cell area increased with time, but remained significant lower than on HRP and HRP/EO substrates (Figure 5-2C). These findings indicate that MSCs adhesion occurs more rapidly on enzyme crosslinked hydrogel surfaces. Importantly, we assumed that the presence of EO and illumination did not impair RGD biofunctionality as MSC behavior was identical as on HRP/EO crosslinked substrates. Previous, studies reported similarly conservation of RGD peptides biofunctionality upon UV and visible light conjugation\textsuperscript{45,246}.

It is known that increasing stiffness and ligand density on synthetic hydrogels increases cell adhesion and spread area\textsuperscript{114,247}. When MSCs were cultured on hydrogels with an elastic modulus of 15 kPa, we observed greater cell areas without changes in morphology and, consistent with the trends observed on softer substrates, spread areas were increased for MSCs on HRP and HRP/EO compared to EO matrices (Figure S 5-2A). Likewise, by increasing the ligand density up to 1000 µM RGD, we found that cell spread areas of MSCs cultured on EO substrates is significantly reduced relative to HRP and HRP/EO substrates (Figure S 5-22B). Altering concentrations of RGD will ultimately change the Young's modulus of formed hydrogels, which had a stiffness of 5±1 kPa when modified with 1000 µM RGD (Figure S 5-2C). However, similar trends observed on 500 and 1000 µM substrates indicate that the ligand density is not the driving force for the differences in cell behavior (data not shown). MSCs seeded on HA-Tyr matrices without RGD showed only very little cell adhesion on HA-Tyr gels. Based on these observations 500 µM RGD ligand densities were used for the remaining experiments.

Next, we investigated the localization of YAP/TAZ, which is thought to be the key transcriptional regulator controlling gene expression and differentiation of stem cells in response to mechanical and geometric cues\textsuperscript{115}. It has been recognized that YAP/TAZ nuclear localization in 2D is dependent on myosin contractility and therefore enhanced in cells on stiffer substrates\textsuperscript{115}. Thus, YAP/TAZ nuclear shuttling acts downstream the formation of actin fibers and cytoskeletal tension and serves as an important indicator which substrate stiffness cells integrate\textsuperscript{248}. Consistent with cell morphology and stress fiber organization on HRP substrates, we found increased nuclear intensity of YAP/TAZ compared to MSCs cultured on EO matrices (Figure 5-2D). However, no significant difference in YAP/TAZ localization was observed for MSCs on EO and HRP/EO substrates (Figure 5-2E). Still, time and stiffness-dependent effects on YAP/TAZ nuclear shuttling remain to be elucidated.
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5.4.3 Crosslinking Chemistry alters mechanical interactions of MSCs with the matrix

When a cell adheres to a substrate it forms a nascent focal adhesion (FA), which represent the dynamic link between the actin fibers and the incorporated RGD motifs of the hydrogel surface and allow the cell to start probing the properties of the substrate. Depending on the feedback from the substrate (e.g. mechanical and geometric properties) the assembly of actin fibers will be stimulated with further spreading and the growth of FA until the cell has reached its final spreading. When we analyzed the paxillin staining in MSCs, we found that cells on HRP crosslinked substrates developed longer FAs, indicative of increased contractile energy in the cell, whereas cells on EO and HRP/EO gels had FA that were shorter (Figure 5-3A). Accordingly, the average length of FA was significantly increased for MSCs on HRP substrates.
compared to cells on EO and HRP/EO gels (Figure 5-3B), which was corroborated by the average area per FA (Figure S 5-3A). Although MSCs on EO substrates showed an increased average length relative to HRP/EO, analysis of FA length revealed that MSCs on HRP/EO had a greater proportion of FA of 2-3 μm in length compared to MSCs on EO crosslinked gels (Figure 5-3C). MSCs on EO and HRP/EO substrates were observed to have a higher fraction of these small FA compared to MSCs on HRP substrates (Figure 5-3C). FA in the intermediate range of 1-2 μm were in equal proportion across all substrates. Generally, the number of FA was higher for MSCs cultured on EO (116±41) and HRP/EO (112±46) compared to cells on HRP (89±20) (Figure S 5-3B). Such a positive correlation between MSC spread area and FA size was observed by others\(^{112,251,253-255}\) and indicates that MSCs on HRP matrices receive a feedback that promotes spreading and the formation of large FA. Conversely, the smaller size of FA on HRP and EO substrates suggests that these MSCs receive a dissimilar feedback, although the initial stiffness of the substrates was the same. However, it is important to note that these small FA are reported to be more mechanosensitive\(^{112,256-258}\).

**Figure 5-3 Formation of focal adhesions for MSCs cultured on HA-Tyr hydrogels**

A  Focal adhesion (FA) formation of MSCs cultured on enzymatic (HRP), light (EO) or a combination of both (HRP/EO) crosslinked HA-Tyr hydrogels for 8 h, visualized with paxillin (green)/cell nuclei (blue, DAPI), dashed boxes show a zoom-in of the FA’s of each cell (Scale bar 25 μm). Quantification of B FA average length and C length distribution, dashed rectangle is shown in focus for FA’s with a size of 2-5 μm. Data are shown as mean +SD., n≥40 cells, *p<0.05, **p<0.01 mean + SD (Scale bar 25 μm).
In their function as mechanical sensors, the maturation of FA is generally stimulated by extracellular mechanical forces. This also implicates that size of FA is proportional to the magnitude of force that can be generated by the cell in order to spread\textsuperscript{112,120,251}. Previous studies indicated that traction forces are involved in translation of cell shape and thus increase with the cell spreading area\textsuperscript{257,259}. Therefore, we sought to determine cellular tractions of MSCs on HA-Tyr substrates by measuring the displacement fields of fluorescent microbeads embedded in the hydrogels (Figure S 5-4). Here MSCs were cultured on the differently crosslinked HA-Tyr substrates for 14 h before analysis. During culture, cells will displace the underlying substrate as they spread, therein deforming the embedded beads\textsuperscript{243}. Eliminating the cell with detergents will induce the relaxation of the substrate and the incorporated microbeads (Figure S 5-4). For all cells the traction forces were exerted at the edges and directed toward the cell center (Figure 5-4A). MSCs on HRP substrates possessed the greatest spread area (Figure 5-4B), but significantly decreased average traction stresses compared to cells on EO and HRP/EO hydrogels (Figure 5-4C). These findings were supported by measurements of the surface tension, which likewise scales with cell area and describes the stored energy in the substrates that is needed to resist the cell-induced contraction\textsuperscript{260}. Here values were similarly increased for EO and HRP/EO compared to HRP substrates (Figure S 5-5). In contrast, the differences in the generation of total force were less pronounced for MSCs cultured on HRP and EO substrates (Figure 5-4D). Taken together, MSCs on HRP hydrogels displayed greater cell areas, but they were less contractile compared to the generally smaller MSCs cultured on EO hydrogels. Although traction stress and cell spread area are usually correlated\textsuperscript{251,255,257,259,261}, a recent study by the Gardel’s group showed that traction stress is independent of cell area and solely a function of substrate stiffness\textsuperscript{262}. However, it remains unclear, why the more contractile MSCs on EO and HRP/EO gels do not spread more. The data presented here further show that higher tractions are generally not predicted by the FA size, which is also supported by previous studies showing that a correlation between FA size and traction stress is limited to the FA adhesion growth period\textsuperscript{254,258}. It has further been suggested that small focal adhesions at the leading edge of fibroblasts exert transient forces to migrate, whereas mature focal adhesion serve primarily as passive anchors to the substrate to maintain spread morphology\textsuperscript{256}. These findings indicate that spreading and force transmission of MSCs may be differently regulated on enzymatically and photo-crosslinked HA-Tyr substrates.
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Figure 5-4 Crosslinking chemistry of HA-Tyr hydrogels alters traction force generation of MSCs

A Representative images of cells in phase-contrast and TFM images of displacement fields (nm). Cells were analyzed after 14 h culture on enzymatic (HRP), light (EO) or a combination of both (HRP/EO) crosslinked HA-Tyr hydrogels (scale bar 10 µm). B Quantification of cell spread area, C average traction stress (normalized to the tractions exerted by the cell within the region-of-interest), and D the average of total force generated per cell. Data are shown as mean +SD, n≥40 cells of two independent experiments, *p<0.5, **p<0.1, ***p<0.01.

We then investigated the mechanistic basis for the increased spreading but lower force that is exerted by MSCs cultured on HRP and HRP/EO substrates relative to cells on EO gels. To test the influence of the HA backbone of the different hydrogels on early cell behavior, we replicated cell adhesion studies after treating the cells with monoclonal anti-CD44 antibody prior seeding\textsuperscript{21}. In addition, any changes in cell behavior resulting from the interaction of cells with the remaining tyramine groups of the HA-Tyr substrates could be minimized by treatment with Tyr/HRP/H\textsubscript{2}O\textsubscript{2} prior to culture\textsuperscript{263}. We observed identical trends to untreated MSCs, where spread area was
decreased on EO substrates for both anti-CD44 and Tyr treated cells (Figure S 5-6). Collectively, these findings suggest that the hydrogel structural cues resulting from the different crosslinking strategies, rather than the direct interaction with the HA-Tyr chemistry mediate MSC behavior. Local stiffness of the substrates was analyzed by AFM at the time of cell seeding and after constraint swelling of gels attached to coverslips. Interestingly, EO and HRP/EO substrates showed a slight increase in the Young's modulus whereas HRP gels had a decreased modulus after swelling (Figure 5-5A). This was confirmed by the frequency dependent storage modulus of crosslinked hydrogels after washing in PBS and free swelling in DMEM containing 10% FBS (Figure 5-5B).

![Figure 5-5 Proposed mechanisms how the crosslinking chemistry of HA-Tyr may influence material properties and cell behavior](image)

**A** Young's modulus of enzymatic (HRP), light (EO) or a combination of both (HRP/EO) crosslinked HA-Tyr hydrogels after 2 h and 24 h of swelling in PBS as measured with atomic force microscopy. Data are shown as mean ± SD, n=2 independent gels tested for each measurement. **B** Frequency dependent rheological measurement of EO and HRP gels at 1% strain. Values are normalized by G' for the specific gel after 1.5 h of swelling. **C** Hypothesis for
how material properties induce the differences in MSC behavior on HRP and EO crosslinked substrates. **D** Frequency dependent rheological measurement of HA in PBS (Control) and H$_2$O$_2$ (1.1 mM) after 10 and 24 h at 1% strain. Values are normalized by G’ for the hydrated HA after 2 h.

It is possible that the crosslinking of HA-Tyr influences the integrity and structural organization of the HA networks formed (Figure 5-5C). Since hydroxyl radicals have been implicated in depolymerisation of HA, we hypothesized that HRP/H$_2$O$_2$ crosslinked HA-Tyr network degraded in culture medium$^{264,265}$. Indeed the storage modulus of pristine HA decreased in the presence of H$_2$O$_2$ (Figure 5-5D). This may explain the loss of mechanical integrity and swelling of HRP gels over time as these substrates were exposed to H$_2$O$_2$ molecules during the network formation. The swelling of the hydrogel matrix may generate an external force to the adhered cell that results in stress at the FA$^{266,267}$. In this case, the actin cytoskeleton reacts and provides a force (i.e. formation of actin fibers) to balance the applied force, which may explain the lower traction forces of these cells (Figure 5-4). However, interpretation is limited by the lack of temporal analysis of traction forces and the heterogeneity of FA. Thus, motility assays and time-laps analysis could provide valuable insight temporal changes of traction stress and assembly of fluorescently labelled FA$^{256}$. In addition, investigations toward mechanotransduction pathways, such as the activity of Rho-associated protein kinase (ROCK)$^{268}$ and myosin$^{269}$ for the formation stress fibers, will help to understand the observed differences in cell spreading and traction forces. It also remains unknown if the rapid crosslinking time (40 sec) for EO substrates led to more entangle HA macromer chains and constraint networks compared to the slower enzymatic crosslink allowing for a more relaxed HA macromer chains network (Figure 5-5C). We found that the concentration of the photoinitiator is an alternative to control the mechanical properties of HA-Tyr matrices and thus provides an interesting tool to prolong exposure times and investigate the effect on hydrogel architecture and MSC adhesion (Figure S 5-1D). Additionally, a reverse HRP/EO crosslinking approach with pre-illumination before enzymatic polymerization may help to understand the influence of crosslinking chemistry on MSC behavior. Collectively, the findings indicate that, although the initial stiffness of the hydrogels was matched, the crosslinking strategies of HA-Tyr hydrogels lead to different stability of these networks over a variety of timescales.

### 5.5 Conclusion

Our findings show that in parallel to mechanical properties, crosslinking chemistry of covalently formed HA hydrogels is an important parameter that has an impact on MSC adhesion and behavior. Using HA-Tyr, cell spreading and formation of focal adhesion were increased on enzymatically crosslinked hydrogels, which also induced YAP/TAZ nuclear localization. Although MSCs on photo-crosslinked HA-Tyr possessed a less organized actin cytoskeleton, these cells generated strong traction forces on the underlying matrix. This suggests that MSC spreading and the forces exerted on the matrix are differentially regulated on these substrates.
The approach described here is unique in that two crosslinking techniques are utilized to form covalent HA-Tyr networks. In addition, *in-situ* modification of HA-Tyr with adhesive ligands facilitated control of integrin-based cell adhesion. We further demonstrate that visible light induced di-Tyr bond formation expands the versatility of HA-Tyr as a potential material for studying cell adhesion and behavior. The tunability of this system may also offer an *in vitro* model system that can recapitulate temporal matrix stiffening to study stem cell behavior and function in diseases.
5.6 Supplement

Figure S 5-1

A Calculated Young’s modulus as a function of fit depth (distance after the contact point to fit with the Hertzian model). Dashed rectangle depicts the region where the indentation depth plateaus for stiff and soft HA-Tyr gels. An indentation depth of 800 μm was chosen for all calculations. B Distribution of Ey values obtained with indentation of three different locations within the hydrogel films. C Comparison of the Young’s modulus of HRP gels (0.68 mM H₂O₂) and EO (0.02% EO, 40 sec) evaluated with AFM (local) and unconfined compression testing (bulk). D Initial Young’s modulus of light (EO) crosslinked HA-Tyr as a function of EO concentration with 40 sec and 90 sec exposure time (modified with 500 μM RGD).
Figure S 5-2

A Quantification of cell spread area for MSCs cultured on enzymatic (HRP), light (EO) or a combination of both (HRP/EO) crosslinked HA-Tyr hydrogels and analyzed after 8 h of culture on 7 kPa and 15 kPa hydrogels. B Quantification of cell spread area for MSCs cultured on enzymatic (HRP), light (EO) or a combination of both (HRP/EO) crosslinked HA-Tyr hydrogels modified with 1000 µM RGD and analyzed after 8 h of. Data are shown as mean +SD, n≥30 cells, *p<0.5, ***p<0.01. C Comparison of the initial Young's modulus of HA-Tyr hydrogels unmodified and modified with 1000 µM RGD, as measured with atomic force microscopy.
Figure S 5-3

Quantification of A FA area and B FA number averaged per cell for MSCs after 8 h culture on enzymatic (HRP), light (EO) or a combination of both (HRP/EO) crosslinked HA-Tyr hydrogels. Data are shown as mean ±SD, n≥30 cells.

Figure S 5-4

Experimental procedure for TFM experiments: images of the cell and HA-Tyr hydrogel with microbeads before (I) and after lysis (II). III Displacement map derived from the bead displacements between the deformed and relaxed gel. Calculation of the displacement vectors of the gels using iterative particle image velocimetry (PIV). Better PIV resolution was achieved by decreasing the interrogation window size (PIV-PIV3), scale bar 25µm.
**Figure S 5-5**

A Quantification of TFM derived data (Figure 4) with surface tension of the substrates. Cells were analyzed after 14 h culture on enzymatic (HRP), light (EO) or a combination of both (HRP/EO) crosslinked HA-Tyr hydrogels. Data are shown as mean +SD, n≥40 cells of two independent experiments, *p<0.5.

**Figure S 5-6**

Quantification of cell spread area for MSCs incubated with A anti-CD44 antibody and B Tyr-HRP/H$_2$O$_2$ before seeding in comparison to untreated cells. Data are shown as mean +SD, n≥20 cells.
6 Early prediction of osteogenic potential of human MSCs by Runx2/Sox9 ratio


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Contributions: Design and optimization of Smart-Flares\textsuperscript{™} protocols using single fluorescent probes in human MSCs.
6.1 Abstract
Runx2 is one of the most studied transcription factors expressed in Mesenchymal Stem Cells (MSCs) upon their commitment toward an osteogenic differentiation. During endochondral bone formation in vivo Sox9 directly interacts with Runx2 and represses its activity; however the role of Sox9 in direct osteogenesis in vitro has been largely overlooked. In this study, we investigate the role of Sox9 in direct osteogenesis of human bone marrow derived human MSCs (hMSCs). We show that Sox9 is a key early indicator during in vitro osteogenic differentiation of hMSCs. Osteogenic induction leads to a significant decrease of Sox9 gene and protein expression by day 7. Treatment of hMSCs with Sox9 siRNA enhanced mineralization in vitro; suggesting that downregulation of Sox9 is involved in direct osteogenesis. siRNA knockdown of Sox9 did not in itself induce osteogenesis in the absence of dexamethasone, indicating that other factors are still required. Screening of not preselected donors of different ages and gender (n = 12) has shown that the Runx2/Sox9 on day 7 ratio is correlated to the $^{45}$Ca incorporation on day 28. The impact of Sox9 downregulation in mineralization of hMSCs in vitro indicates a so far unprecedented role of Sox9 as a major regulator of direct osteogenesis. We propose that the Runx2/Sox9 ratio is a promising early in vitro screening method for osteogenicity of hMSCs.

6.2 Introduction
Increasing aging populations cause a growing need for new approaches to augment and repair bone tissue lost through trauma or disease. To meet this demand a variety of cells have been utilized to heal these defects, including mesenchymal stem cells (MSCs). MSCs represent an attractive cell population for bone regeneration due to their expansion properties and osteogenic regeneration potential. During skeletal tissue formation master genes have been identified, however the rules by which they specify distinct tissue types are still unclear. The Sry-related transcription factor Sox9 is mainly described as the key regulator for chondrogenesis. Sox9 is expressed in resting and proliferating chondrocytes, with a maximum of expression in prehypertrophic chondrocytes, but disappears completely from the hypertrophic zone. It is proposed that Sox9 downregulation is required to allow the onset of cartilage-bone transition, such as cartilage resorption and formation of bone marrow during development. The Runx-related transcription factor Runx2 was identified initially as a positive regulator of osteoblast differentiation. It is one of the most studied transcription factors expressed in MSCs upon their commitment toward an osteogenic differentiation. In vivo, the sufficiency of Runx2 to induce direct ossification is still hypothetical. Overexpression of Runx2 in chick embryos did not induce ectopic bone formation. MSC-derived osteo-chondroprogenitors express both transcription factors, Sox9 and Runx2, during condensation of the skeletal anlagen. Sox9 directly interacts with Runx2 and represses its activity. It was shown previously that osteo-chondroprogenitor cells differentiate towards osteogenic phenotypes when Sox9 expression is lower than Runx2 during endochondral bone formation in mice. The inhibitory effect of Sox9 on osteoblast maturation via Runx2 repression is an essential mechanism for osteo-chondroprogenitor fate determination.
Beside endochondral ossification as the most commonly occurring process in fracture healing, direct ossification is an important requirement for a successful osseointegration of implants. Furthermore, in the area of cranio-maxillofacial surgery most of the fractures heal through direct ossification. During in vitro differentiation into mature osteoblasts the secretion of an extracellular matrix and mineralization is accompanied by stage specific expression of bone related genes. In vitro systems for osteogenesis recapitulate events during direct osteogenic differentiation. Several hormonal and growth factors were shown to promote osteogenic differentiation of hMSCs in vitro. Dexamethasone is routinely used to promote hMSC osteogenic differentiation and ensure mineralization in vitro.

The osteogenic potency of hMSCs is commonly characterized by the expression of early markers (alkaline phosphatase, collagen type I) followed by late markers (bone sialoprotein, osteocalcin). Recent studies have shown that levels of Runx2 during in vitro differentiation of primary human osteoblasts showed no major changes in levels of Runx2 protein or mRNA during human osteoblast differentiation. Commonly used early in vitro assays, such as activity of alkaline-phosphatase (ALP), may not be proportional to the mineralization levels. However, little is known about the role of Sox9 in direct osteogenesis of hMSCs in vitro. Recently Stoeckl et al., showed that Sox9 has a positive proliferative role and delays osteogenic differentiation in rat adipose stem cells.

We hypothesized that Sox9 is an early key-regulator during in vitro osteogenic differentiation of hMSCs. Moreover, we propose that the ratio between Runx2 and Sox9 gene expression can predict the osteogenic differentiation potential of hMSCs.

6.3 Experimental

6.3.1 MSC cell culture

Human bone marrow was harvested from the iliac crest or vertebral body of twelve patients after full ethical approval (Freiburg, EK-326/08) (mean age: 45.8 years; range: 20-75 years; male:female ratio: 6:6) to isolate MSCs using previously described protocols. P2 MSCs were seeded at 2x10^4 cells/cm^2 and grown in DMEM low glucose with 10% FBS (Life Technologies, Switzerland), 1% penicillin/streptomycin without (Control) or with additives. Cells were incubated at 37°C/5% CO_2 and medium was refreshed every second day.

6.3.2 Osteogenic differentiation of monolayer expanded cells

Osteogenic differentiation (Differentiation Medium (DEX)) was induced through supplementation of Control medium with 50 μg/ml ascorbic-acid, 100 nM dexamethasone and 5 mM β-glycerophosphate. Cells were cultured in Control or DEX for 28 days and harvested on days 0, 2, 7, 14, 21 and 28 for analysis.

6.3.3 DNA assay for MSC cellular proliferation

MSCs were digested in proteinase K (0.5 mg/ml) for 16 h at 56 °C. DNA content was measured using Hoechst 33258 (Polysciences, Germany) assay as previously described.
6.3.4 Alkaline Phosphatase (ALP) activity and alizarin red staining

Alkaline phosphatase (ALP)-activity was determined by measuring the formation of p-Nitrophenol (pNp) from p-Nitrophenyl phosphate as described earlier\(^{281}\). Briefly, after extraction at 4 °C for 1 h in 0.1% Triton-X in Tris-HCl, absorbance was measured at 405 nm using a Victor\(^{TM}\) plate reader (Perkin Elmer, Waltham, MA, USA). The ALP-activity of cell-lysates was calculated according to a generated ALP standard curve. The obtained ALP-activity was normalized to the total DNA amount. For alizarin red staining cell matrix layers were washed with PBS, fixed with 4% formaldehyde and stained with 2% alizarin red solution with a pH of 4.3 (Sigma-Aldrich, Switzerland) for 1 h.

6.3.5 \(^{45}\text{Ca}\) radioisotope incorporation

Monolayer cultures were incubated for 16 h with \(^{45}\text{Ca}\) (1.25 μCi/ml) radioisotope and radioactive counts were measured using a scintillation counter WinSpectral (Perkin Elmer, Switzerland). The obtained \(^{45}\text{Ca}\) incorporation (counts per minute, CPMI) was normalized to the total DNA amount.

6.3.6 MSC optical density in monolayer cultures

MSCs cultured in 24-well plates were washed with phosphate buffered saline (PBS). Following wash step, 500 μl fresh PBS was added to each well and the optical density (OD) 450 nm was measured followed by the addition of fresh medium to enable continuation of cell culture.

6.3.7 Quantitative PCR (qPCR) analysis

Total RNA was extracted using TriReagent and RNeasy Kit (Qiagen). Briefly, cells were lysed in TriReagent and 10% (v/v) BCP was added. After centrifugation, the upper phase containing RNA was collected and precipitated by adding of 70% ethanol. RNA was transferred to RNeasy columns and purified according to manufacturer's instructions. RNA (400 ng) was reverse transcribed using TaqMan Reverse Transcription kit. qPCR was performed using AB7500 qPCR System (Applied Biosystems, USA) according to previously described methods\(^{290}\). A specific primer sequences used are listed in Table S6-1. Data analysis was performed using ddCT values, which were determined by normalization to 18S rRNA and samples harvested on day 0.

6.3.8 RNA interference

Neon Transfection System (Invitrogen) was used to transfect hMSCs (990V, 40ms, 1 pulse) with 600 nM Sox9 (Buffer concentration, OriGene). Cells were seeded at P2 24 h before electroporation at 50-70% confluence. Cells were harvested 48 h after electroporation and knockdown of Sox9 mRNA was assessed. Negative controls were transfected using scrambled siRNA and no siRNA. Sox9 knockdown cells showing at least 70% reduction of Sox9 gene expression were cultured in Control, DEX or osteopermissive medium without dexamethasone (OP) for 28 days. Final osteogenic differentiation on day 28 was assessed by OD 450 nm, \(^{45}\text{Ca}\) incorporation and alizarin red staining.
6.3.9 Protein extraction from hMSCs culture and Western Blotting

hMSCs were harvested after 48 h and 7 days of culture in either Control or DEX medium. Cells were lysed in hypotonic buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 10% IGEPAI with proteinase inhibitor cocktail (PIC; Sigma Aldrich, Switzerland) and centrifuged for 3 min. The remaining cell pellets were re-suspended with 20 mM HEPES, 0.4 mM NaCl, 1 mM EDTA, 10% Glycerol and PIC and kept at 4 °C with vigorously shaking for 2 h. Nuclear protein extracts were collected from the supernatants after centrifugation for 5 min. Protein concentration was measured with Quick Start™ Bradford Protein Assay (Biorad, Switzerland). 5 μg of nuclear protein were used for Western Blotting assay. Specifically, after denaturation at 95 °C for 5 min and addition of 2.5% β-mercaptoethanol, the proteins were loaded on 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membrane (Bio-Rad, Switzerland). The membranes were blocked in 5% non-fat dried milk and then incubated with different primary antibodies (mouse anti-Sox9, 1:250, Abnova; rabbit anti-Laminin B1, 1:500, Thermo-Scientific) at 4°C overnight. Horseradish peroxidase (HRP) labeled secondary antibodies (anti-mouse, 1:20.000, abcam; anti-rabbit, 1:20.000, Amershams) were added for 1 h at room temperature. Amersham ECL chemiluminiscence Detection reagent was used for detecting membrane signals using Image J (NIH, Bethesda, MD) software. Laminin B1 served as internal control.

6.3.10 Preparation of cells with SmartFlare™ probes for flow cytometry

SmartFlare™ probes Uptake-Cy3, Uptake-Cy5, Scramble-Cy3, Scramble-Cy5, Runx2-Cy3 and Sox9-Cy5 were kindly provided by MerkMillipore. These probes were reconstituted in 50 μl sterile nuclease free water, and stored at room temperature until usage. hMSCs were cultured in osteogenic induction medium or growth medium for 6 days, and SmartFlare™ probes were directly added into the medium with 1:1000 dilution. The dilution was chosen based on preliminary experiments (data not shown). After gentle shaking, cells were incubated with probes at 37°C and 5% CO2 for overnight (about 16 hours). Then cells were detached using 10% Trypsin, and centrifuged at 2000 rpm for 10 min. The cell pellets were resuspended in growth medium and incubated with 1:5000 4',6-Diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Finally the cells were analyzed using flow cytometry and sorted using Fluorescence-activated cell sorting (FACS).

6.3.11 Flow cytometry and FACS

Flow cytometry analysis was performed using BD Aria III, and data were analyzed with the use of BD FACS Diva 6.1.3. First dead cells which are positive for DAPI staining were excluded by gating for DAPI negative cells (Figure S 6-1A), and then the cell debris and clumps were excluded from the live cell population through a forward scatter (FSC)/ side scatter (SSC) gate (Figure S 6-1B). The doublets were excluded using FSC-A vs FSC-H gate (Figure S 6-1C). Finally the gated cells were analyzed, and cells in osteogenic induction medium sorted into different regions based on the fluorescence intensity of Runx2-Cy3 (R) and Sox9-Cy5 (S). For RNA isolation, the sorted cells were collected using PBS with 1% FBS. Total RNA was extracted using Pure Link™
RNA Micro Kit (Invitrogen) according to the manufacturer’s instructions. C-DNA was synthesized using SuperScript® VILO cDNA Synthesis Kit (Invitrogen). QPCR was then performed as described above (6.3.7).

6.3.12 Statistical Analysis
All experiments were performed in triplicates. Data was analyzed using SPSS 16.0 (Chicago, IL, USA). A Leven’s Test for equality of variances and then an independent student’s t-test was performed. Statistical significance was set at p<0.05.

6.4 Results

6.4.1 DEX stimulation induces downregulation of Sox9 during early osteogenesis
As described earlier hMSCs differentiate into osteoblasts in response to a supplementary cocktail of ascorbic acid, β-glycerophosphate and dexamethasone (DEX). Direct osteogenic differentiation of hMSCs in vitro was monitored by early osteogenic markers such as gene expression of Runx2, ALP-activity, late osteogenic markers, 45Ca incorporation and alizarin red staining. Sox9 gene expression was also monitored where hMSCs cultured in Control medium had a higher Sox9 mRNA expression compared to DEX stimulated MSCs on days 2, 7 and 14. In contrast, osteogenic medium led to a downregulation of Sox9 mRNA expression during early osteogenesis (Figure 6- 1A+B). Decreased Sox9 mRNA expression in the DEX group was significant on day 2 and day 7 of culture (p<0.05) (Figure 6- 1A). On day 21 of culture differences in Sox9 gene expression of hMSCs in Control and DEX group diminished (Figure 6- 1B). Next we determined protein expression of Sox9 in hMSCs on day 7 after osteogenic stimulation with DEX. In line with Sox9 gene expression Sox9 protein signal was reduced in DEX stimulated cells compared to hMSCs in Control medium (Figure 6- 1C). However, suppression of Sox9 protein signals is not as strong as Sox9 gene expression (Figure 6- 1A). Laminin B1 served as an internal loading control (Figure 6- 1C).
Figure 6-1 Sox9 gene expression and protein analysis of hMSCs in vitro

Sox9 gene expression of A on day 2 and day 7 (n = 12); B on day 14 and day 21 (n = 4), based on expression fold change to day 0 (Mean ± SEM *p < 0.05); data are shown as mean ± SEM *p < 0.05). C Sox9 protein analysis: Western Blot and image analysis on day 7 either in Control or DEX medium. Laminin B 1 served as internal control; one representative donor (n = 3).

6.4.2 The Runx2/Sox9 gene expression ratio is an indicator for osteogenic responsive MSC donors

A striking observation in our study was that DEX treatment also induced Runx2 mRNA downregulation on day 7, although not significant (Figure 6-2A). No differences between Control and DEX groups were seen on day 2 (Figure 6-2A) while at later time points, Runx2 was slightly up-regulated compared to Control group (Figure 6-2B). Since Sox9 was downregulated in DEX group on days 2 and 7 (Figure 6-1A) with little change in Runx2 expression (Figure 6-2A), we addressed the balance between Runx2 and Sox9. It is well known that the osteogenic differentiation potential can show huge variations among donors. Runx2/Sox9 ratios in DEX group showed significant higher values on day 2 (p<0.05) (Figure 6-2C). Noteworthy, Runx2/Sox9 ratios on day 14 and day 21 are much lower compared to day 2 and day 7 (Figure 6-2C+D).
Chapter 6 Early prediction of osteogenic potential of human MSCs by Runx2/Sox9 ratio

Figure 6-2 Runx2 gene expression of hMSCs in vitro

A on day 2 and day 7 (n = 12); B on day 14 and day 21 (n = 4); Runx2/Sox9 ratio C on day 2 and day 7 (n = 12), D) on day 14 and day 21 (n = 4), based on expression fold change to day 0; data are shown as mean ± SEM *p < 0.05).

However, a detailed analysis of each particular donor revealed a correlation between the Runx2/Sox9 ratio on day 7 and the osteogenic performance. To rule out or exclude the possibility that the interplay of Runx2 and Sox9 plays a role in early osteogenesis we have chosen a population of 12 not pre-selected donors. For better comparison, we first classified MSC donors into either high osteogenic potential or low osteogenic potential. High osteogenic potential was defined as $^{45}$Ca incorporation above 100,000 CPMI/µg DNA on day 28 (n = 4) and low osteogenic potential as $^{45}$Ca incorporation below 80,000 CPMI/µg DNA on day 28 (n = 4). This classification revealed that high osteogenic potential MSC donors had a significantly increased Runx2/Sox9 ratio on day 7 compared to donors with low osteogenic potential (Figure 6-3A). Accordingly, MSC donors with higher Runx2/Sox9 ratios had a significant enhanced $^{45}$Ca incorporation on day 28 compared to MSC donors with a low Runx2/Sox9 ratio (Figure 6-3B). In order to determine whether Runx2/Sox9 is correlated to early osteogenic markers, the ALP activity per DNA concentration on day 14 was chosen (Figure 6-3D). Although MSC donors in the high osteogenic potential group showed higher ALP-activities on day 14, this trend was not significant (Figure 6-3C), suggesting that ALP alone is not a sufficient early marker of calcification potential. Including the entire population of unselected donors, a positive correlation between Runx2/Sox9 ratio on day 7 and $^{45}$Ca incorporation on day 28 can be displayed (Figure 6-3E). However, on the basis of 12 donors, a Runx2/Sox9 ratio of above 2 is required to reliably predict an increase in $^{45}$Ca incorporation (Figure 6-3E). To address the wide range of osteogenic response of MSC donors...
upon DEX stimulation, we have chosen $^{45}$Ca incorporation on day 28 as most reliable osteogenic assay.

![Figure 6-3 Runx2/Sox9 gene expression ratio of hMSCs in vitro](image)

**Figure 6-3 Runx2/Sox9 gene expression ratio of hMSCs in vitro**

**A** Runx2/Sox9 ratio on day 7; **B** $^{45}$Ca incorporation on day 28 (Mean ± SEM *p < 0.05); **C** ALP activity on day 14, for 8 representative donors with low (n = 4) and high (n = 4) osteogenic potential (high osteogenic potential defined as above 100,000 CPMI/µg DNA on day 28; low osteogenic potential as below 80,000 CPMI/µg DNA on day 28); data are shown as mean ± SEM *p < 0.05); **D** ALP activity for donors with both low and high osteogenic potential (n = 8); **E** Correlation of $^{45}$Ca incorporation on day 28 with Runx2/Sox9 ratio on day 7; for unselected population of donors (n = 12).

### 6.4.3 Detection of Runx2 and Sox9 gene expression in live cells using dual fluorescent RNA-based probes

Although qPCR is a sensitive method to quantify mRNA expression, it has limitations as it shows the average mRNA expression and is destructive to cells. Therefore, we sought to demonstrate the applicability of the Runx2/Sox9 ratio for live-cell analysis at individual cell level as well as for a whole population. Here we utilized Smart-Flares™ which can detect mRNA transcripts within living cells. The Smart-Flare™ technology is based on gold nanoparticles that are covalently labeled with capture oligonucleotides specific for the gene of interest and a fluorescently labeled short peptide. If complementary mRNA is present, this peptide leaves the gold nanoparticle and begins to emit fluorescence. To assess the ratio of Runx2/Sox9 in individual live cells, we developed a dual fluorescence live monitoring system based on FACS analysis.
Figure 6-4 SmartFlare™ probes for detection of mRNA expression in live cells

Histogram of fluorescence intensities of hMSCs with single A Runx2-Cy3 and B Sox9-Cy5 probes in Control and DEX medium, analyzed after 6 days of culture. Quantification of C Runx2 and D Sox9 mRNA expressions collected after FACS analysis. Cell were then sorted based on their
fluorescence intensity for E Runx2-Cy3 with R1 (low intensity), R2 (intermediate intensity) and R3 (high intensity) and for F Sox9-Cy5 with S1 (low intensity) and S2 (high intensity). Quantification of mRNA expression for G Runx2 and H Sox9 of sorted cells in comparison to the region with lowest fluorescence intensity (R1). Data are shown for one representative donor, experiment was repeated for five different donors with similar results.

Relative fluorescence intensity in individual live cells can be observed after application of the mRNA specific probe. Here we observed that 87% of hMSCs in Control and 81% in DEX medium endocytosed the Runx2-Cy3 probe and produce a fluorescence signal. For the Sox9-Cy5 probe we found 97% of the cells in Control and 95% of the cells in DEX medium with fluorescence signals, indicating the uptake of the probe. These findings indicate that the majority of cells endocytosed the probes and the fluorescence signals were strong enough to be detected by flow cytometry.

Comparing the mean relative fluorescence signal intensities, we observed a shift between cells in DEX medium (green) and growth medium (red) (Figure 6-4A+B). For Runx2 expression, the shift to the right indicated increased mRNA expression after osteogenic induction (Figure 6-4A), whereas Sox9 expression was clearly reduced as shown by the peak-shift to the left (Figure 6-4B). This was further corroborated by an increase of the Runx2/Sox9 ratio from 0.211 to 0.548 for hMSCs cultured in DEX medium. We also compared the obtained FACS values with qPCR and quantified the mRNA expression of the collected cells. Similarly, we found an increased expression of Runx2 (1.12x, Figure 6-4C) and a strongly reduced Sox9 gene expression (0.39x, Figure 6-4D) for hMSCs cultured in DEX compared to Control medium. Thus, the Runx2/Sox9 ratio for hMSCs in DEX increased up to 3.13 compared to Control medium, which confirmed our previous results. These findings show that the fluorescent probes provide semi-quantitative information about the mRNA expression in a population, which is comparable to qPCR data. Next, we investigated mRNA expression of sorted hMSCs based on Runx2-Cy3 (Figure 6-4E) and Sox9-Cy5 (Figure 6-4F) fluorescence intensities. Here, Runx2 mRNA expression of sorted hMSCs was well correlated with the fluorescence intensities analyzed by flow cytometry of (Figure 6-4G). Similarly, Sox9 mRNA expression increased for the regions with high Sox9-Cy5 fluorescence intensity (Figure 6-4H). Subsequent studies by our group demonstrated the dual detection of Runx2-Cy3 and Sox9-Cy5 in individual hMSCs. Notably, hMSCs sorted with a high Runx2/Sox9 ratio were found to have low proliferation rates, but high osteogenic differentiation, which was confirmed by enhanced calcium deposition\textsuperscript{293}. These findings confirm the capability of the Runx2/Sox9 ratio in predicting the osteogenic potential of hMSCs.

### 6.4.4 Downregulation of Sox9 triggers direct osteogenesis of hMSCs

After demonstrating that Sox9 downregulation occurs during osteogenic differentiation \textit{in vitro}, we next silenced Sox9 expression. Sox9 mRNA after 48 h was reduced by at least 70% by Sox9 siRNA when normalized to non-electroporated cells (Figure 6-5A). Cells transfected with a scrambled negative control or without siRNA showed no reduction of Sox9 gene expression (Figure 6-5A). Accordingly, Sox9 siRNA treated cells showed reduced Sox9 protein signals after 48 h compared to control group without siRNA treatment (Figure 6-5B).
**Figure 6-5 Effect of Sox9 downregulation on hMSC osteogenesis *in vitro***

**A** Electroporation of hMSCs with Sox9 siRNA, gene expression after 48 h based on expression fold change to day 0; *, indicates significant difference between Sox9 siRNA and Controls (n = 6, Mean ± SEM, *p < 0.05), **B** Runx2/Sox9 ratio after 48 h, one representative donor; **C** Protein analysis: Western Blot and image analysis of Sox9 protein after Sox9 siRNA electroporation (48 h) in Control Medium. Laminin B1 served as internal control; one representative donor (n = 3); **D**
Depending on Sox9 siRNA concentrations, Sox9 protein expressions could be reduced by 50% (200 nM) to 62% (600 nM) compared to control group without siRNA (Figure 6-5A). Therefore, for suppression of Sox9 gene and protein expression by at least 60%, 600 nM Sox9 siRNA was used for the following experiments. Runx2 gene expression was not significantly changed (data not shown). As expected, silencing Sox9 gene expression leads to vastly increased Runx2/Sox9 gene expression ratio (Figure 6-5B). To investigate the effect of Sox9 silencing on direct osteogenic differentiation, cells were cultured in Control and DEX medium, respectively. A third group of cells was maintained in osteopermissive medium without dexamethasone (OP) to investigate if a knockdown of Sox9 alone can induce mineralization in vitro. 

45Ca incorporation after 28 days strongly increased when Sox9 was silenced compared to control groups in DEX medium, with significance for scrambled and non-electroporated controls (Figure 6-5D). Alizarin red staining on day 28 confirmed an enhanced calcification after Sox9 silencing (Figure 6-5E). OD 450nm values during the entire period further underlined an increase in mineralization in DEX medium if Sox9 was silenced (Figure 6-5F). Cells cultured in OP medium without DEX showed an increase in 45Ca incorporation and enhanced alizarin red stain on day 28 compared to cells cultured in Control medium (data not shown). However, between the groups in OP medium no differences in 45Ca incorporation could be observed (data not shown).

6.5 Discussion

It is known that the coordination of Runx2 and Sox9 is highly important for the process of cartilage and bone development. Sox9 decreases Runx2 binding to its target sequences and drastically inhibits Runx2 either through preventing transactivation of osteoblast-specific enhancers or direct protein degradation of the gene itself278,294. The complete absence of Sox9 from hypertrophic chondrocytes suggests that Sox9 downregulation is required to allow the onset of subsequent events, e.g. endochondral bone formation273. This indicates the dominance of Sox9 function over Runx2 during skeletogenesis. In endochondral ossification, the formation of hypertrophic cartilage is a prerequisite step in bone development295,296.

The present study indicates that Sox9 downregulation is required for direct osteogenesis of hMSCs (Figure 6-1A). It was shown recently that isolated MSCs from Sox9 transgenic mice showed decreased osteoblast differentiation297, thereby confirming the importance of inhibiting Sox9 activity in osteoblast differentiation. Yet, the regulatory role of Sox9 in direct osteogenesis of hMSCs has not yet been identified. Furthermore, Runx2 and Sox9 mRNA expression during direct osteogenesis might be reflective of interplay between these two transcription factors. Our data imply that a critical factor in this interplay is the decrease in Sox9 expression that leads to a
vastly increased Runx2/Sox9 ratio. In fact, we suggest that the ratio between Runx2 and Sox9 gene expression is a key marker of osteogenic potential. A high ratio of Runx2/Sox9 gene expression on day 7 maintains a high osteogenic potential for the related donor as shown by extended ALP-activity on day 14 and $^{45}$Ca incorporation on day 28 (Figure 6- 3B+C). In contrast, hMSCs with a lower osteogenic potential show a very low ratio of Runx2/Sox9 gene expression (Figure 6- 3A). Although ALP-activity peaks around day 14 of DEX stimulation (Figure 6- 3D), a potential shift of very high/low osteogenic MSC donors may mask the actual ALP-activity peak. Therefore, we assume that ALP-activity is not optimal for a correlation with Runx2/Sox9 gene expression profile. We suggest that the Runx2/Sox9 ratio is correlated to the $^{45}$Ca incorporation on day 28 (Figure 6- 3E), thus linking mRNA expression levels with cell function. Even though $^{45}$Ca incorporation on day 21 showed a good correlation to the ratio of Runx2/Sox9 (data not shown), MSC donors with delayed osteogenic differentiation are rather included after 28 days (Figure 6- 3B+E).

We screened various donors of different ages and gender that were not pre-selected (n = 12) either with or without DEX stimulation confirming our hypothesis (Figure 6- 3E). This might be useful in evaluating freshly isolated hMSCs with regard to their osteogenic differentiation capacity. In vitro culturing of MSCs usually requires time and cost intensive procedures. Our data assume that the ratio of Runx2/Sox9 at the earliest points of in vitro culture can predict if the particular donor has osteogenic potential. Although we could demonstrate that Sox9 is already downregulated after 2 days of DEX stimulation, we suggest day 7 to be the most reliable time-point (Figure 6- 1A). A period of 7 days may include MSC donors with a potentially delayed proliferation/differentiation profile and minimizes the risk of stress-induced effects on the gene expression level. Since the average of Runx2 gene expression was rather downregulated on day 7 (Figure 6- 2A), the Runx2/Sox9 on day 7 may be even more sensitive to predict the osteogenic potential of each particular donor (Figure 6- 3E). In addition to consolidated findings on gene expression level, we could further demonstrate downregulation of Sox9 protein expression on day 7 when cells are osteogenic stimulated (Figure 6- 1C). Although the effect on protein level was less distinctive, it supports the impact of Sox9 downregulation during early osteogenic differentiation in vitro. However, aiming for an early in vitro screening and evaluation system for the osteogenic potential of hMSCs, quantitative mRNA expression levels may be superior.

To achieve a better understanding of the underlying molecular pathway, we analyzed the osteogenic differentiation of hMSCs after suppressing Sox9 mRNA (Figure 6- 5). Sox9 silencing successfully reduced the Sox9 protein levels (Figure 6- 5B). The chosen Sox9 siRNA concentration allowed a reduction of Sox9 mRNA and protein by at least 60% (Figure 6- 5A+B).

To elucidate the role of Sox9 in direct osteogenesis we cultured the electroporated hMSCs of six not pre-selected donors in Control, DEX or OP medium without dexamethasone. If Sox9 is regulatory in direct osteogenesis, decreased Sox9 may potentiate the effect of dexamethasone or be a direct stimulus. It is conceivable that cells in Control medium will not mineralize even with a downregulated Sox9, due to a lack of phosphates in the medium (Figure 6- 5D). Although our data have shown that Sox9 does not solely regulate direct osteogenesis in vitro, downregulation...
of Sox9 enhances the mineralization process\textsuperscript{287} (Figure 6- 5D and E). An increased absorbance in the measurement of the optical density at 450nm supported our findings (Figure 6- 5F). This assay is useful as an online method, where calcified areas appear darker because of a higher refractive index\textsuperscript{298}. The analysis of absorbance at 450 nm is a quick and inexpensive approach to evaluate osteogenic differentiation of monolayer cultured cells.

Previously, Stoeckl et al., showed that Sox9 has a positive proliferative role and delays osteogenic differentiation in rat adipose stem cells\textsuperscript{287}. Our results clearly demonstrated a drop of Sox9 gene expression after 7 days of osteogenic culture in a large number of donors (Figure 6-1A), which is further supported by lower Sox9 protein expression on day 7 (Figure 6- 1C). However, upstream events resulting in Sox9 downregulation during early osteogenesis remain unknown. Additionally, it remains unclear if osteogenic induction induces a drop of Sox9 gene expression or rather a slow decrease over time. Therefore, correlating mRNA and protein levels should consider the importance of kinetics and a steady-state comparison might be misleading due to the dynamics of genetic information processing across transcription and translation\textsuperscript{299}.

Here, the SmartFlare\textsuperscript{TM} technology can be a useful tool for monitoring Sox9 downregulation over time. Our data further imply that downregulation of Sox9 prior to osteogenic induction may be too early to take over the whole mineralization process. A better understanding of the regulatory network would further elucidate, if knockdown of Sox9 in turn may switch on inhibitory genes. However, the ratio of Runx2 and Sox9 on day 7 can predict the osteogenic potential of each particular donor, suggesting that the balance of both genes is the result of earlier regulatory events. A more detailed analysis of the interplay between Runx2 and Sox9 during direct osteogenesis \textit{in vitro} is required for a better understanding of this process. To our knowledge, this is the first report that the Runx2/Sox9 ratio correlates with direct osteogenic differentiation of hMSCs \textit{in vitro}. Although the complex dynamics and the regulation control at many levels have not been elucidated in this study, the aim is to present a powerful early screening tool for \textit{in vitro} osteogenic differentiation.

These finding confirm the applicability of the Runx2/Sox9 ratio as an early marker for hMSC osteogenic potential. Further work is required to establish any correlation between Runx2/Sox9 ratio and \textit{in vivo} outcome.

### 6.6 Conclusion

The impact of Sox9 downregulation in mineralization of hMSCs \textit{in vitro} indicates a so far unprecedented role of Sox9 as a major regulator of direct osteogenesis. This supports the hypothesis that Sox9 leads to a direct or indirect suppression of Runx2. Rather than focusing on Runx2 as a crucial factor of direct osteogenesis, Sox9 seems to be key to better understand \textit{in vitro} osteogenesis of hMSCs. The SmartFlare\textsuperscript{TM} technology offers an excellent non-destructive tool to analyze cell differentiation at the individual cell level, e.g. number of responding cells, as well as for whole population. Relatively homogenous and functionally similar cell populations can be isolated using this new method. By identifying and isolating differentiating cells at early time points, prospective analysis of differentiation is also possible, which could lead to a greater understanding of MSC differentiation.
6.7 Supplement

Figure S 6-1 FACS sorting

A DAPI exclusion gating to exclude DAPI positive cells (blue). B Forward scatter (FSC) and side scatter (SSC) gate to define the cell population (DAPI negative cells). C FSC-A (area) versus FSC-W (width) gate was applied to exclude doublets.

Table S6-1 Primers/Probes used for real time PCR

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</table>
7 Summary and outlook

Among several HA-based hydrogel systems used as functional matrices, HA-Tyr hydrogels have recently demonstrated relevance toward biomedical applications. HA-Tyr hydrogels are formed by covalent di-Tyr crosslinks, and density (i.e. stiffness) and formation rate (i.e. gelation time) can be controlled by the amount of H₂O₂ and HRP\(^5^7\) (page 18). Entrapment of drugs and cells into the formed polymeric network has been reported\(^6^7,^7^9,^8^1,^8^7\). However, applications of HA-Tyr conjugates have been limited by a lack of control over the derivatization process and a relatively low tyramine substitution\(^5^7,^6^9\). Therefore, the aim of this thesis work was to optimize the HA-Tyr derivatization process and establish a hydrogel platform that could be used as a cell-instructive material for stem cell engineering and tissue repair.

First, we prepared HA-Tyr derivatives using a more efficient coupling chemistry (DMTMM) for amide formation on HA. We showed an efficient HA-Tyr conjugation strategy with precise control over the degree of substituted tyramine and less non-functional adducts, which yielded highly functionalized HA-Tyr derivatives. Notably, the precisely tailored tyramine substitution allowed for the control of the physico-chemical properties of the formed hydrogels, which was independent of the crosslinker (HRP/H₂O₂) concentrations. Moreover, the high degree of substitution enabled the formation of HA-Tyr hydrogels with improved mechanical properties, as shown by a two-fold increase in the Young’s modulus compared to previously reported HA-Tyr hydrogels with less than 5% Tyr substitution. Toward accurate spatio-temporal control over the gelation process we introduced for the first time photo-crosslinking of HA-Tyr. Here covalent di-tyramine bonds were formed using visible light illumination and Eosin Y as a photoinitiator. Additionally, the combination of enzymatic and light crosslinking allowed for accurate temporal matrix stiffening. Encapsulation of MSCs showed high cell-viability over several days, which was shown for a wide range of mechanical properties in enzymatically and photo-crosslinked HA-Tyr hydrogels.

Next, for stem cells seeded atop two-dimensional HA-Tyr substrates, we established in-situ modification of HA-Tyr with cell-adhesive RGD-oligopeptides. HA-Tyr matrices with equal mechanical properties were then either formed enzymatically (HRP/H₂O₂), with visible light (Eosin Y) or using a combination of these crosslinking chemistries. This platform allowed us to study the influence of different crosslinking chemistries on the biophysical cues of HA-Tyr hydrogels, which stem cells sense and differentially respond to. Here we found that MSCs generated stronger forces on photo-crosslinked HA-Tyr substrates whereas the cell spread area was significantly reduced compared to MSCs cultured on enzymatically crosslinked gels with the same stiffness. Investigations toward the mechanism behind these findings suggested that the H₂O₂ used for enzymatic crosslinking influences the HA macromer depolymerization and therefore the mechanical integrity of HA-Tyr hydrogels, resulting in reduced cell generated forces.

Focusing on stem cell differentiation in vitro, we found that the Runx2/Sox9 mRNA expression on day 7 predicts the osteogenic potential of MSCs. This work further suggested that the downregulation of Sox9 is the key for MSC osteogenesis. Building upon this work we introduced the Smartflare™ technology as a non-destructive live-monitoring system of MSC osteogenesis in
Here, the detection of Runx2 and Sox9 mRNA transcripts in single cells enabled isolation of relatively homogeneous cell populations based on their osteogenic potential and confirmed that the ratio of Runx2 and Sox9 mRNA is an indicator for osteogenic differentiation. Taken together, this thesis revealed the capability and versatility of HA-Tyr chemistry for the creation of biomimetic hydrogels. The future directions of HA-Tyr hydrogels, its capacity and limitations for stem cell engineering and tissue repair are discussed in the following paragraphs. Based on the work demonstrated here, DMTMM found applications in a variety of HA amide formations, including the preparation of HA-alkyl derivatives (HA-propylamine and HA-butylamine). These results demonstrate promise toward utilizing DMTMM also for modification of other polymers bearing carboxylic groups, for example gelatin and collagen.

For biomedical applications, the encapsulation of cells is often desirable. In addition to cytocompatible crosslinking conditions of HA-Tyr, we observed cellular spreading of encapsulated MSCs particularly in highly functionalized hydrogels. To assess the influence of tyramine in covalently crosslinked hydrogels on MSC fate, cell encapsulation may be replicated with enzymatically crosslinked polymers that lack intrinsic biological cues, for example alginate-tyramine and dextran-tyramine. Such investigations would reveal the mechanism by which stem cells respond to the tyramine and the biophysical cues of di-tyramine crosslinked hydrogels. Additionally, visible light illumination may be used to induce secondary crosslinking and study the influence of matrix density on stem cell differentiation. However, for such applications investigations toward alternative photosensitizers may achieve a better utility of photo-crosslinked HA-Tyr hydrogels. Although Eosin Y induced rapid gelation, the absorption spectrum (480-550 nm) interferes with some of the conventional fluorophores used in immunocytochemistry, including green fluorescent protein (GFP) and fluorescein isothiocyanate (FITC). While we considered several singlet oxygen initiators in the green area of visible spectrum (e.g. rose bengal, riboflavin), methylene blue may be an alternative photosensitizer as it shows strong absorbance in the range of 550-700 nm.

Still, covalently i.e. irreversibly crosslinked hydrogels, such as the one described here do not replicate the conditions cells naturally experience. Based on recent reports, a continuous degradation or remodeling of the surrounding matrix is needed for stem cells to sense the properties of their environment and translate it into differentiation signals. Thus, to investigate the influence of di-tyramine structural cues on cellular spreading, a partially cell-degradable HA-Tyr matrix can be formed using MMP-degradable peptide-crosslinkers, which are flanked with tyrosine (e.g. YCRDVPMSMRGGDRCY). Understanding the structural cues would help to develop a better fundamental understanding of how to engineer tissue using tyramine-modified hydrogels.

In addition to cell-matrix interactions within HA-Tyr hydrogels, we studied stem cell early attachment and behavior on RGD-modified HA-Tyr substrates. Well-tailored hydrogel systems such as the one established here provide platforms for improving our understanding of how cells respond to their microenvironment. Furthermore, it can be applied as model microenvironment to recapitulate the temporal dynamics of mechanics during diseases, such as fibrosis in the
pancreas, lung and liver\textsuperscript{306,307}. Although there is a paucity of hydrogels that can be dynamically modified, they often use synthetic polymers\textsuperscript{149,271,308} or are limited by their use of UV light\textsuperscript{45}, which may be harmful to some cell-types. Only recently, Caliari \textit{et al.} used visible light mediated crosslinking of HA-methacrylate to study myofibroblast activation of hepatic stellate cells\textsuperscript{346}. Similarly, HA-Tyr may be a valuable hydrogel material for studying response of mechanosensitive cells and advance the development of therapeutics for fibrotic diseases.

Still, a thorough understanding of the di-tyramine crosslinking will be key to harness the potential of differentially crosslinked HA-Tyr hydrogels. Here, monitoring the autofluorescence of the di-tyramine aromatic rings can inform about the kinetics and density of crosslinks formed during enzymatically and light-induced polymerization. Fluorospectrophotometry is an excellent tool for such investigations, where di-tyramine formation can be studied utilizing short HA-Tyr fragments (less than 10 kDa). In addition, this technique may inform about the binding kinetics of RGD-tyrosine during enzymatically and light-induced crosslinking. Beyond the ability to utilize HA-Tyr as artificial microenvironments, the Runx2/Sox9 mRNA expression ratio may be used for investigating how mechanical changes are translated into stem cell fate decisions. The SmartFlare\textsuperscript{TM} technology presented here facilitates live-monitoring of mRNA expression and may be used to study the effect of different HA-Tyr crosslinking chemistries on osteogenic differentiation of MSCs and its relation to focal adhesion assembly and traction force transmission.

Finally, HA-Tyr is designed for ease in clinical applications, such as the treatment of cartilage defects\textsuperscript{309,310}. An enzymatically pre-crosslinked hydrogel increases the viscosity of an injectable pre-polymerized solution and therefore improves retention in the cartilage defect before secondary crosslinking upon light curing \textit{in situ}. Moreover, the biomimetic chemical adhesive functionalities of tyramine were reported to be able to bind biological tissue, such as cartilage\textsuperscript{305,311}. These adhesive functionalities of tyramine may improve the lateral integration of the hydrogel to the adjacent native cartilage, which is one of the key issues when aiming for mechanical stability and endogenous repair via enhancement of cell migration\textsuperscript{312}. 
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Bibliography


Appendix

The osteogenic potential of human MSCs can be enhanced by Interleukin-1ß


Loebel C, Czekanska EM, Staudacher J, Salzmann G, Richards RG, Alini M, Stoddart MJ: Osteogenic differentiation of human MSCs can be enhanced by Interleukin-1beta.

Cited by 5 (Google Scholar June 2016)

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doi: 10.1155/2015/714230


Contributions: Teaching and supervision, development of the short-term stimulation protocol.
Abstract

Inflammation is one of the key regulators of the repair process in bone tissues. Current data about the effect of IL-1β on MSCs and osteoblasts are conflicting. We investigated the long-term effect of Interleukin 1β (IL-1β) on direct osteogenic differentiation of hMSCs in vitro. IL-1β stimulated cells showed an enhanced proliferation and entered maturation prior to non-stimulated ones, as monitored by ALP-activity. The process of calcification was accelerated during long-term stimulation of hMSCs with IL-1β. Since donor variability is a well-known issue we suggest a new method to illustrate global changes of a random chosen donor population through collative analysis. We further demonstrate an absorbance assay to evaluate the degree of calcification during in vitro culture of monolayer expanded hMSCs. Our findings support the importance of IL-1β in osteogenic differentiation of hMSCs in an in vitro monolayer culture model. A new online absorbance assay is a useful method to evaluate the osteogenic differentiation of hMSCs at early stages. These findings will be helpful in optimizing pre-differentiation of hMSCs in vitro for bone tissue-engineering.

Introduction

Bone formation is a well-orchestrated process that takes place during skeletal development and bone regeneration. Large bone defects are unlikely to regenerate due to the significant quantity of bone required. A variety of cells have been utilized to heal these defects, including mesenchymal stromal cells (MSCs). MSCs represent an attractive cell population for bone regeneration due to their expansion properties and osteogenic potential.

A bone fracture not only destroys bone architecture, but disrupts blood vessels and subsequently causes tissue bleeding. Cells of the blood and bone marrow, such as immune cells, erythrocytes, and stromal cells are deprived of oxygen and nutrition, which leads to local tissue hypoxia and inflammation. The importance of the initial fracture hematoma for successful fracture healing has been described in vivo. The same pro-inflammatory stimuli that activate the human clotting cascade enable the recruitment of neutrophils, monocytes and macrophages towards the site of injury. The variety of cells that are implicated during the inflammation phase help debride the wound, stimulate the repair process and angiogenesis. Thus, inflammation is a key regulator of the repair process in bone.

Previous studies have shown that inflammatory cytokines, such as Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) can enhance osteoclast differentiation and activation through RANKL, thus resulting in bone resorption. There is also evidence that the same cytokines cause enhanced abnormal bone formation in inflammatory skeletal diseases (e.g. rheumatoid arthritis). These pro-inflammatory cytokines play a role in initiating the repair cascade. Recent studies have reported the effects of inflammatory molecules on skeletal cells in vitro and in vivo. It has been shown that IL-1β modulates the proliferation and differentiation of skeletal cells, however, with conflicting results. In vivo, IL-1β is active during endochondral fracture healing, displaying a bimodal peak pattern during the initial inflammatory phase and the remodeling phase, whilst in vitro it helps stimulate the growth of bone.
of skeletal cells\textsuperscript{322,324}. Furthermore, due to the increased expression during endochondral bone healing, it has also been suggested that IL-1β increases the number of osteoblasts and decreases the number of apoptotic cells\textsuperscript{327}. Currently, engineering bony tissue for critical size defects is an emerging field in orthopedic surgery and the \textit{ex vivo} pre-differentiation maybe essential to the success of cell-based tissue-engineering\textsuperscript{328}. Based on these previous investigations this study aimed to determine the effect of IL-1β on MSC osteogenic differentiation and mineralization \textit{in vitro}.

\section*{Experimental}

\subsection*{MSC cell culture}

Human bone marrow was harvested from the iliac crest of eight patients after full ethical approval (Freiburg, EK-326/08) (mean age: 41 years; range: 20-66 years; male:female ratio: 4:4) to isolate MSCs using previously described protocols\textsuperscript{329}. Briefly, bone marrow was diluted 1:5 with Dulbecco’s modified Eagle medium (DMEM, Life Technologies, Switzerland) containing 10% fetal bovine serum (FBS, Gibco, Switzerland). The mixture was layered on a Ficoll cushion, centrifuged at 800g for 20min and mononuclear cells were collected from the liquid interface. The isolated cells were plated in polystyrene cell culture flasks (TRP, Switzerland) and cultured in alpha-modified essential medium (α-MEM; Life Technologies, Switzerland), 10% FBS and 5ng/ml recombinant human basic fibroblast growth factor. Cells were incubated at 37°C/5% CO\textsubscript{2} and medium was refreshed every second day. Expanded attached cells were uniformly positive for CD105, CD73 and CD90 and lack expression of hematopoietic cell surface markers\textsuperscript{330}. P2 MSCs were seeded at 2x10\textsuperscript{4} cells/cm\textsuperscript{2} and grown in DMEM low glucose with 10% FBS, 1% penicillin/streptomycin without (Control) or with additives.

\subsection*{Osteogenic differentiation of monolayer expanded cells}

Osteogenic differentiation (Differentiation Medium (DEX)) was induced through supplementation of Control medium with 50 μg/ml ascorbic-acid, 100 nM dexamethasone and 5 mM β-glycerolphosphate (Sigma Aldrich, Switzerland). IL-1β (10 ng/ml) (Promokine, Germany) was supplemented to DEX medium at the start of osteogenic differentiation and at each medium change. Cells were cultured in Control, DEX or DEX+IL-1β for 28 days and harvested on days 1, 7, 14, 21 and 28 for analysis. As an additional control group, Control medium was supplemented with IL-1β (10 ng/ml) only.

\subsection*{DNA assay for MSC cellular proliferation}

MSCs were digested in proteinase K (0.5mg/ml) (Roche, Switzerland) for 16 hours (h) at 56°C. DNA content was measured using Hoechst 33258 (Polysciences, Germany) assay as previously described\textsuperscript{286}. DNA standard curve was generated using Calf Thymus DNA (Life Technologies, Switzerland).
Alkaline Phosphatase (ALP) activity
ALP-activity was determined using p-Nitrophenol (pNp) after extraction at 4°C for 1 h in 0.1% Triton-X in Tris-HCl (Sigma Aldrich, Switzerland). Absorbance was measured at 405 nm using a Victor3™ plate reader (Perkin Elmer, Waltham, MA, USA). The ALP-activity of cell-lysates was calculated according to a generated ALP standard curve. The obtained ALP-activity was normalized to the total DNA amount.

Alizarin red staining and quantification
Alizarin red staining was performed following fixation of cells with 4% formaldehyde and quantified by dissolving bound alizarin red with 10% acetic acid (Sigma Aldrich, Switzerland). Absorbance of the extracted alizarin red stain was measured at 405 nm and normalized to the total DNA amount.

45Calcium (45Ca) radioisotope incorporation
Monolayer cultures were incubated for 16 h with 45Ca (1.25 μCi/ml) radioisotope (Perkin Elmer, Switzerland). Following fixation with 70% Formic acid and incubation for 60 min at 65°C, radioactive counts were measured using a scintillation counter WinSpectral (Perkin Elmer, Switzerland).

MSC optical density in monolayer cultures
MSCs cultured in 24-well plates were washed with phosphate buffered saline (PBS). Following the wash step, 500 μl fresh PBS was added to each well and the OD 450 nm was measured followed by a media change.

Quantitative PCR analysis
Total RNA was extracted using TriReagent and RNeasy Kit (Qiagen, Switzerland). Briefly, cells were lysed in TriReagent and 10% (v/v) 1-bromo-3-chloropropane (BCP) added. After centrifugation, the upper phase was collected and precipitated by adding 70% ethanol. RNA was transferred to RNeasy columns and purified according to manufacturer's instructions. RNA (400 ng) was reverse transcribed using TaqMan Reverse Transcription kit (Applied Biosystems, USA). RT-PCR was performed using AB7500 Real-Time PCR System (Applied Biosystems, USA) according to previously described methods. Specific primer sequences used are listed in Table A- 1. Data analysis was performed using ddCT, determined by normalization to 18S rRNA and day 1 samples.
Appendix Osteogenic potential of human MSCs can be enhanced by Interleukin-1β

Table A-1 Primer sequences

<table>
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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
<th>Applied Biosystem reference number</th>
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<td>GGT GCT CGG ATC CCA AAA</td>
<td>CAT CAA ACA GCC TCT TCA GCA CAG TGA CAC</td>
<td></td>
</tr>
<tr>
<td>Human Collagen Type I (hCol I)</td>
<td>CCC TGG AAA GAA TGG AGA TGA T</td>
<td>ACT GAA ACC TCT GTG TCC CTT CA</td>
<td>CGG GCA ATC CTC GAG CAC CCT</td>
<td></td>
</tr>
<tr>
<td>Human Osteocalcin (hOc)</td>
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<td>AAC TCG TCA CAG TCC GGA TTG</td>
<td>ATG GCT GGG AGC CCC AGT CCC</td>
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<td>Human ALP (hALP)</td>
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<td></td>
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<td>431089E</td>
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Statistical Analysis

All experiments were performed in triplicate. Data was analyzed using SPSS 16.0 (Chicago, IL, USA). A normality test was performed using a Shapiro-Wilk test and then a univariate general linear model with subsequent pairwise post-hoc testing (Bonferroni correction) was performed. A difference of p<0.05 was considered significant. A Chi-square test was performed for analysis of pooled data from all donors and every time point. Significance was calculated using the chi-square distribution table.

Results

Supplementation of IL-1β only (Control+IL-1β) showed no differences in proliferation and osteogenic profile compared to Control (data not shown). Therefore results will be discussed for only three groups: Control, DEX and DEX+IL-1β (10 ng/ml).

IL-1β enhances MSC proliferation in monolayer cultures

DEX+IL-1β significantly increased proliferation compared to Control following 14, 21 and 28 days of culture, as indicated by increased DNA content (p<0.05) (Figure A-1A). DEX induced monolayers without IL-1β supplementation showed significantly enhanced proliferation on day 28 compared to Control (p<0.05) (Figure A-1A). However, MSCs derived from different donors showed a wide variation. Collating the donors and comparing the range of the DEX+IL-1β treated
group with the range of the DEX induced group demonstrated a significant shift towards increased proliferation in the DEX+IL-1β group (p<0.05) (Figure A-1B).

Figure A-1 Proliferation of hMSCs
A MSC cell proliferation as described by DNA amount with or without the presence of IL-1β (n = 5/8, Mean ± SD, *p < 0.05) B collected analysis of 8 donors based on DNA amount with or without presence of IL-1β normalized to Control; **, indicates significance between DEX+IL-1β and DEX (**p<0.01)

IL-1β treatment enhances osteogenic differentiation of MSCs
To understand how the process of osteogenesis is affected by IL-1β stimulation, ALP-activity was examined. Typically, ALP activity peaks around day 14 in osteogenic induced MSCs (Figure A-2A). However, IL-1β supplementation shifted the ALP-activity peak from day 14 to day 7, with the same trend in all donors (p<0.05) (Figure A-2A). Increased ALP-activity was observed at later time points in the Control group, potentially due to spontaneous differentiation following cell confluence (Figure A-2A). Collating the data for all time points, it was demonstrated that IL-1β induced a shift in timing rather than an actual increase in ALP-activity (Figure A-2B).

Figure A-2 ALP activity of hMSCs
A ALP-activity with or without the presence of IL-1β normalized to day 1; *, indicates significant difference between Control and DEX+IL-1β; #, indicates significant difference between Control and DEX; +, indicates significance between DEX and DEX+IL-1β (n = 5/8, Mean ± SD, *#+ p <
IL-1β supplementation significantly enhanced $^{45}$Ca incorporation on days 21 and 28, compared to DEX cultures ($p<0.05$) (Figure A- 3A). A pooled analysis of the donors on days 21 and 28 showed a shift towards enhanced $^{45}$Ca incorporation in the DEX+IL-1β group (Figure A- 3B). Alizarin red staining was enhanced in the presence of DEX+IL-1β compared to DEX alone on day 14 and day 21 (Figure A- 3C). Quantification of alizarin red confirmed the staining results with an enhanced bound alizarin red in the DEX+IL-1β cells at both time points, (Figure A- 3D) although there was no significant difference between IL-1β stimulated and DEX groups. However, upon pooling the data from all donors, a trend towards an enhanced mineralization potential of IL-1β stimulated cells was confirmed ($p<0.1$) (Figure A- 3E).

**Optical density measurement as an early indicator for osteogenesis**

When cultured in monolayer, calcification of MSCs under osteogenic conditions can be assessed using a phase-contrast microscope. Measurement of the optical density of monolayer cultures demonstrated that the formation of the calcified areas had an increased optical density compared to Control cultures (Figure A- 4A). DEX+IL-1β stimulated cells showed a significantly enhanced optical density compared to DEX cells ($p<0.05$) (Figure A- 4A). Figure A- 4B shows the corresponding images of the wells being used for absorbance measurement. Interestingly, the calcification-process of the DEX+IL-1β group begins after day 7; whereas DEX treated cells start to calcify after day 14, indicated by a sharp increase of OD 450 nm values (Figure A- 4B). It can be further assumed, that IL-1β not only induced earlier calcification but also a significantly higher amount of calcified area by the end of culture. The peak of ALP-activity in the DEX+IL-1β stimulated group correlated to the day 7 OD 450 nm. Accordingly, the DEX group showed a sharp OD 450 nm increase on day 14 and an ALP-peak at the same time point (Figure A- 2A and Figure A- 4A). On day 28, the significantly increased OD 450 nm values in the DEX+IL-1β group are consistent with $^{45}$Ca-incorporation and alizarin red results (Figure A- 3).
Figure A-3 Calcification of hMSCs

A $^{45}\text{Ca}$ incorporation with or without the presence of IL-1β (n = 5/8, Mean ± SD, *p < 0.05) B collated analysis of 8 donors based on $^{45}\text{Ca}$ incorporation with or without presence of IL-1β normalized to Control. The frequency analysis demonstrates a greater frequency of samples with higher incorporation in samples with additional IL-1β stimulation when compared to DEX alone, C day 21: upper row unstained monolayers, lower row alizarin red staining S stained monolayers (scale bar: 200 microns) and D quantification of alizarin red on days 14 and 21 with or without the presence of IL-1β (n = 5/8, Mean ± SD) e collated analysis of 8 donors based on alizarin red S quantification with or without presence of IL-1β normalized to Control; *, indicates significance between DEX+IL-1β and DEX (*p<0.1)
Appendix

Osteogenic potential of human MSCs can be enhanced by Interleukin-1β

**Figure A-4 Optical density measurements (OD 450 nm)**

A The OD 450 nm of monolayer cultures was measured to determine the presence of crystalline deposits as an indication of calcium deposition. Optical density graphs with or without the presence of IL-1β; *, indicates significant difference between Control and DEX+IL-1β; #, indicates significant difference between Control and DEX; +, indicates significance between DEX and DEX+IL-1β (n = 5/8 Mean ± SD, *#+ p < 0.05); B representative images of the corresponding wells (scale bar: 200 microns).

**Gene expression of osteogenic markers**

Analysis of ALP gene expression confirmed the shift in the ALP-activity peak from day 14 to day 7 in the IL-1β stimulated group (Figure A-2A), although this was not statistically significant (Figure A-5B). Runx2 and Collagen type I (hCol I) expression showed both a trend towards upregulation on day 14 for the DEX+IL-1β stimulated group with a subsequent decrease in expression on days 21 and 28 (Figure A-5A+C). Additionally, IL-1β stimulation induced a significant downregulation of human Osteocalcin (hOc) (p<0.05) (Figure A-5D). No significant changes could be seen in
Interleukin 6 (IL-6) expression (Figure A-5E). Stimulation with DEX or DEX+IL-1β induced a significant upregulation of Interleukin 8 (IL-8) expression over the whole culture period with significance on day 7 (Figure A-5F).

Figure A-5 Gene expression of osteogenic markers

Gene expression of A Runx2, B alkaline phosphatase (ALP), C Collagen type I (hCol I), D Osteocalcin (hOc), E Interleukin 6 (IL-6), and F Interleukin 8 (IL-8) with or without the presence of IL-1β, based on expression fold change to day 1 (n = 5/8, Mean ± SD *p < 0.05)
Discussion

In this study, we demonstrated the effect of IL-1β stimulation upon osteogenic differentiation on human bone marrow derived MSCs. While IL-1β treatment alone had no effect, stimulation with IL-1β (10 ng/ml) in the presence of DEX over 28 days enhanced proliferation and mineralization of hMSCs. In inflammatory diseases such as rheumatoid arthritis, it has been suggested that IL-1β is involved in bone destruction and autoimmunity\(^{331}\). On the other hand, several lines of evidence suggest that IL-1β is involved in bone metabolism and plays an important role under physiological conditions\(^{320,332}\).

We demonstrated an increase in human MSC proliferation in the presence of IL-1β (Figure A-1). This may be due to a greater colony formation prior to calcification. It was recently shown that IL-1β enhances the colony-forming units (CFUs) of osteoblasts and fibroblasts in total number and dimension\(^{322}\). Our data imply that these colonies under IL-1β conditions are forming earlier as indicated by the amount of DNA (Figure A-1), thus inducing the calcification process. This process is mirrored by optical density measurements, where an earlier absorbance increase was seen in the IL-1β group (Figure A-4). Enhanced CFU formation and proliferation leads to a more rapid confluence and an earlier onset of calcification that is detected by an increased absorbance (Figure A-4). We propose this online method to evaluate MSC calcification. Calcified areas will appear dark and his can be quantified by measuring the optical density at 450 nm, thus providing an online measurement. While in itself it is not an absolute measurement of calcification, it can be used as an additional non-destructive data set. During the course of culture, optical density values correlated to ALP-activity (Figure A-2A). Stimulation with IL-1β not only induces a shift towards day 7, but also an elevation in OD 450 nm values and ALP-activity. However, as expected, osteogenic medium alone induced an increase in OD 450 nm values at the onset of the mineralization process (Figure A-4). This indicates that the absorbance of monolayer cultured MSCs provides a method to evaluate osteogenesis. Under these conditions little difference is seen between DEX and control at day 7, the main changes occurring at day 14. This also supports the differentiation occurring after proliferation has ceased, in this case requiring 14 days in the case of DEX alone, while the enhanced proliferation seen by the addition of IL-1β leads to gene expression differences seen by day 7. The role of ALP as a marker for osteogenic activity has been consistently solidified and ALP is known to increase the local concentration of inorganic phosphate. ALP is known to be maximally expressed during the matrix maturation stage of osteoblast differentiation. Stimulation with IL-1β significantly shifts the whole process to earlier time points. Therefore, we suggest that increased proliferation in the DEX+IL-1β stimulated group (Figure A-1) contracts the whole proliferation period allowing earlier matrix maturation. This hypothesis is supported by earlier and elevated OD 450 nm values in the DEX+IL-1β stimulated group compared to the DEX group (Figure A-4). This data would suggest that a combination of stimuli leads to additive effects when compared to that from Ferreira et al. 2013, where DEX alone led to an increase in ALP, whereas IL-1β alone led to increased calcification\(^{326}\). Combining them as we have in this study led to an earlier ALP peak and increased calcification.
When there is a wide range of donor variation, collating data at specific time points can lead to conflicting data due to parabolic expression of various genes involved in osteogenesis. The exact timing varies per donor, this increases the variation around a fixed timepoint (e.g. day 7), even though the actual effect is constant and reproducible. The end effect is a loss of statistical power. It is widely accepted that multiple donors need to be investigated to consider donor to donor variability. Pooling the cells from various donors prior to performing the experiment results in a loss of statistical power and also may lose valuable information. A pool of cells from different donors might be an average of very different populations, and the result describes a population which does not exist. On the other hand, performing an experiment with similar responding donors may bias the data towards one population and exclude donor populations. We demonstrated through collating the data of eight random donors that there was an enhanced proliferation and bounded alizarin red, if they were stimulated with IL-1ß (Figure A-1B +Figure A-3 E). Thus, we suggest this method to illustrate global changes of a donor population after a particular treatment.

However, it should be taken into account that, for statistical power, a high number of donors and assessed time points are required. Incorporation of 45Ca was significantly enhanced in the DEX+IL-1ß group (Figure A-3A), but it was not statistically significant when data were collated, which may be due to only two time points assessed and therefore less data to compare (Figure A-3B). Additionally, collating data is not suitable for all assay types, such as ALP-activity (Figure A-2A), where IL-1ß stimulation caused a peak-shift rather than an increase in ALP-activity (Figure A-2B). Collating the data from all five time points provides a mechanism by which a general change can be seen, even though the exact timing is not known.

Additional, expression of inflammatory genes has been investigated (Figure A-5E+F). Although IL-8 was upregulated in cells treated with DEX or DEX+IL-1ß, values of IL-8 expression are low and may not have considerable effects (Figure A-5F). IL-6 gene expression was downregulated in DEX and DEX+IL-1ß groups without significance (Figure A-5E). It has been shown earlier that IL-6 has an anti-proliferative effect and may inhibit proteoglycan synthesis. However, the complex signaling cascade and the release of pro-inflammatory molecules requires further investigations to explain the effect of IL-6 and IL-8 on human MSC osteogenesis in vitro.

In a previous study, it was shown that IL-1β decreased Runx2 mRNA levels in human MSCs. An additional drop in hOc, as a transcriptional target of Runx2, was observed. This indicates that Runx2 activity decreased due to IL-1ß stimulation. In the present study, Runx2 and hOc expression were downregulated upon stimulation with IL-1ß (Figure A-5A+B). However, ALP activity and mineralization do not always mirror Runx2 activity. In osteoblast-like cells, inhibition of Runx2 expression with siRNA, did not decrease ALP activity, whilst overexpression of a dominant negative Runx2, also did not decrease ALP activity. These results suggest that ALP expression is unrelated to Runx2 and that Runx2 has two functions in bone formation, control of osteoblast differentiation and control of ECM-related gene expression. It may help explain why bone formation in rheumatoid arthritis does not compensate for bone resorption. New bone

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**Appendix** Osteogenic potential of human MSCs can be enhanced by Interleukin-1ß
formation is formed due to the presence of inflammatory cytokines and so may be reduced by anti-inflammatory or anti-TNF-α drugs.

Conclusion

The present study indicates that IL-1ß enhances proliferation and direct mineralization of hMSCs in vitro. We suggest that DEX+IL-1ß stimulated cells enter maturation prior to DEX alone, as monitored by ALP-activity and elevated optical density as a novel complementary assessment for calcification. Our observation may be useful to optimize the in vitro pre-differentiation of patient derived MSCs for bone tissue-engineering.