Doctoral Thesis

Formation of Engineered Microtissues by Schiff Base Crosslinking

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Formation of Engineered Microtissues by Schiff Base Crosslinking

A thesis submitted to attain the degree of DOCTOR OF SCIENCES of ETH ZURICH (Dr. sc. ETH Zurich)

presented by

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2015
Acknowledgements

Thinking back on the last 5.5 years I’ve spent in Zurich, it would surely not have been possible to survive without the tremendous support I’ve seen from my supervisors, collaborators, colleagues, family, and friends. When I first made the decision to attend ETH for my Master’s, it was not part of the plan to stay for so long. But a combination of the right project and the right people made it an offer I could not refuse.

To Marcy, many many thanks for giving me the opportunity to work on this project. You were one of the first people I met in Zurich when I came to ETZ for a coffee with you and Al to discuss course selection at the beginning of the Master and it was fitting that you became my PhD supervisor. I am very grateful for the exposure you’ve provided and I feel that I have learned a great deal over this time. Participating together in Find and Bind was a particularly rewarding experience. Thank you for always encouraging new ideas and for always pushing me to fulfill the highest standards possible. I am excited for what lies ahead and I am looking forward to more fruitful collaboration. Katharina, thank you for making time for our meetings in between your busy days at ETH. I enjoyed having your input and the project surely benefited from your fresh perspective. Janos, it was a pleasure to spend the first months of my PhD as a member of the LBB. I have always appreciated your philosophies on how to pursue this degree and thoroughly enjoyed our discussions in the kitchen, whether over coffee or a beer, and the environment you cultivate in your group. I hope we continue to meet on the soccer pitch or the basketball court.

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has been a pleasure working alongside you all and a lot of fun solving problems together, planning aperos, and barbecuing on the balcony. Let’s keep that going!

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You had to wait until here to find your name Queralt, but you really should be first in this list. If I am being honest, this PhD was just as difficult for you at some moments as it was for me. You have a bottomless reserve for energy and support that always managed to keep me going. I had to teach you that the view is always better when you’re standing on top of a rock, but you have taught me so much since then. I don’t think I would have managed this thesis without you. Moltíssimes gràcies punketa!

I would also like to say a very big thank you to my parents who have always supported me and made the decision to stay in Switzerland an easy one by encouraging me to pursue my goals here. It has been very difficult at times managing the 6 hour time difference and limited visits, but you’ve never made me feel guilty for choosing to live abroad. We have made the distance feel insignificant by always finding time to catch up and lend an ear when needed. To my siblings Lizzy, Max, Marcel, Spencer, and Maddox, it has not been easy to see how much you all change in between my visits home! Even if they are short, I cherish those moments and I’m looking forward to seeing what the future brings for you.
Abstract

The prevalence of joint pain-related disability results in a substantial socioeconomic burden on today’s society. Injury or degeneration of the cartilaginous surfaces on diarthrodial bones do not self-heal due to lack of access to blood vessels and the body’s endogenous wound healing mechanisms. A myriad of different surgical procedures have been administered to patients suffering from joint pain, but none have been identified which guarantees full recovery of damaged cartilage with many patients suffering re-injury and requiring further treatment. Cell-based therapies offer strong promise to fully restore cartilage function and meet these clinical demands. Ideally, autologous cells are harvested from the patient and implanted at the site of injury within a biomimetic matrix that instructs cells to synthesize and maintain cartilage-specific tissue. Mesenchymal stem cells (MSCs) are widely considered an ideal cell type for cartilage cell-based therapies due to their ready availability. Identifying appropriate culture conditions for reproducibly directing MSC chondrogenesis is key to unlocking their potential for cell-based regenerative medicine therapy. The standard method for doing so involves culturing MSCs as centrifuged pellets in defined induction media, but this approach is highly variable and often fails to promote cartilage-specific matrix production for some MSC donors.

The aim of this thesis was to engineer a system for enhancing MSC chondrogenesis that could overcome a number of limitations associated with micromass pellet culture, the conventional chondroinduction technique. Polysaccharides that are either present in cartilage extracellular matrix (ECM), or share structural similarities to those that are present, were chemically modified to yield reactive groups that undergo Schiff base crosslinking under physiological reaction conditions. Conditions were then optimized for utilizing the modified polysaccharides to induce the aggregation of MSCs into condensation-like engineered microtissues (EMTs) as a result of the rapid 30 minute Schiff base crosslinking reaction. Quantitative and histological comparisons of cartilage-specific matrix production
between EMTs and micromass pellets obtained by centrifugation revealed that the polysaccharide matrix in the EMT structure provided better conditions for elevating levels of MSC chondrogenesis. In follow-up studies, we showed EMT culture could restore the chondrogenic potential of MSCs from aged donors that failed to differentiate when cultured as micromass pellets.

Next, the EMT method was further developed to allow for in situ differentiation of entrapped MSCs which would significantly decrease the amount of in vitro manipulation required for preparation as a surgical treatment option for cartilage defects. This was achieved by loading the critical chondrogenic induction factor, transforming growth factor-beta 3 (TGF-β3), into the polysaccharide network at the time of crosslinking. Release was monitored by ELISA and the optimal dose per microtissue was determined by quantitative assays and immunohistochemistry for driving MSC chondrogenesis during culture of samples in media that was not supplemented with TGF-β3. Finally, the clinical merit of the EMT-based method for treating cartilage defects was demonstrated in an in vitro repair assay. After 4 weeks culture in serum-free media that was not supplemented with the growth factor, cartilage defects seeded with EMTs loaded with TGF-β3 yielded de novo repair tissue containing GAGs and type II collagen within the injury site.

The versatility of using Schiff base crosslinking for tissue engineering applications was demonstrated in a number of additional studies. Relating to cell-based therapies for cartilage repair, the same modified polysaccharides used for EMTs were shown to improve the adhesion of cells to cartilage surfaces in a spatially controlled manner. Cartilages surfaces were rendered reactive for Schiff base crosslinking after brief incubation with sodium periodate, and cells suspended in a polysaccharide solution containing free primary amines were selectively crosslinked to the tissue surface. In another application of Schiff base crosslinking for engineering microtissues, the 3D distribution of different cell populations could be controlled by sequential deposition of cells. Using this technique, complex co-culture studies can be envisioned providing high degree of control for the spatial organization of the different cell types. To test the ability for supporting other cell types by EMT culture, a study was performed with primary
hepatocytes. Hepatocyte microtissue formation was enhanced in EMTs versus hanging drop culture. The gene expression of cyp3a13, a member of the cytochrome P450 enzyme family responsible for drug metabolism, was elevated in EMTs compared to in vivo expression levels after 1 week of culture.

This thesis details a systematic approach for designing a biomaterial-based system for engineering multicellular tissue structures. Schiff base crosslinking of modified polysaccharides was proven to serve 3 main functions: 1) rapid assembly of cells to either induce aggregation for formation of microtissues or to control adhesion of cells to tissue surfaces, 2) to determine optimal conditions for maintaining biological functionality in high-density microtissues, and 3) for the loading and release of growth factors towards potential in vivo applications for directing cell differentiation in situ.
Zusammenfassung


Das Ziel dieser Doktorarbeit war die Entwicklung eines System zur verbesserten MSC Chondrogenese, welches einige Einschränkungen der konventionellen Chondro-Induktionstechnik, der sogenannten Pellet-Kultur, beseitigt. Polysaccharide, die entweder Teil der Knorpelmatrix sind oder strukturelle Ähnlichkeit zu ihr aufweisen, wurden chemisch modifiziert, um reaktive Aminogruppen für die Bildung von Schiff’schen Basen unter physiologischen Konditionen zu erhalten. Die Parameter der Vernetzungsreaktion wurden danach optimiert, um die Aggregation der MSCs in ein kondensationsähnliches Mikrogewebe (EMTs – condensation-like engineered microtissue) in schnellen 30 Minuten zu induzieren. Quantitative und histologische Vergleiche der knorpelspezifischen Matrixproduktion zwischen EMTs und der durch Zentrifugation geformten Pellet-Kulturen, zeigte die Fähigkeit der EMTs Polysaccharid-Matrix zur verbesserten MSC Chondrogenese. Folgestudien zeigten,
dass MSCs von älteren Patienten in EMT-Kulturen zur Knorpelbildung angeregt werden konnten, während in Pellet-Kulturen jegliche Matrixproduktion fehlgeschlagen war.


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<th>Description</th>
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<tbody>
<tr>
<td>3-MA</td>
<td>3-methyladenine</td>
</tr>
<tr>
<td>ACI</td>
<td>Autologous chondrocyte implantation</td>
</tr>
<tr>
<td>ACT3D</td>
<td>Autologous chondrocyte transplantation 3D</td>
</tr>
<tr>
<td>AMIC</td>
<td>Autologous matrix-induced chondrogenesis</td>
</tr>
<tr>
<td>ATR-FTIR</td>
<td>Attenuated total reflection Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>BMAC</td>
<td>Bone marrow aspirate concentrate</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CASI</td>
<td>Cartilage autograft implantation system</td>
</tr>
<tr>
<td>Centrif.</td>
<td>Centrifuged pellets</td>
</tr>
<tr>
<td>Cyp3a13</td>
<td>Cytochrome P450, family 3</td>
</tr>
<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DILI</td>
<td>Drug-induced liver injury</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMMB</td>
<td>1,9-dimethylmethylene blue</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Engineered microtissues</td>
</tr>
<tr>
<td>EMT${^{+}\text{TGF}}$</td>
<td>EMT microtissue preloaded with TGF-β3</td>
</tr>
<tr>
<td>ESCs</td>
<td>Embryonic Stem Cells</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor 2</td>
</tr>
<tr>
<td>FGFs</td>
<td>Fibroblast growth factors</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein IsoThioCyanate</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GFs</td>
<td>Growth factors</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>IGFs</td>
<td>Insulin-like growth factors</td>
</tr>
<tr>
<td>Ihh</td>
<td>Indian hedgehog</td>
</tr>
<tr>
<td>iPSCs</td>
<td>Induced Pluripotent Stem Cells</td>
</tr>
<tr>
<td>LbL</td>
<td>Layer-by-layer</td>
</tr>
<tr>
<td>MACH</td>
<td>Matrix-assisted chondroplasty</td>
</tr>
<tr>
<td>MFX</td>
<td>Microfracture</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N-CAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>OATS</td>
<td>Osteochondral autograft transplantation surgery</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimum cutting temperature compound</td>
</tr>
<tr>
<td>oxAlg</td>
<td>Oxidized alginate</td>
</tr>
<tr>
<td>oxCS</td>
<td>Oxidized chondroitin sulfate</td>
</tr>
<tr>
<td>oxHA</td>
<td>Oxidized hyaluronic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet rich plasma</td>
</tr>
<tr>
<td>PS</td>
<td>Polysaccharides</td>
</tr>
<tr>
<td>PTHRP</td>
<td>Parathyroid hormone-related protein</td>
</tr>
<tr>
<td>QCM-D</td>
<td>Quartz Crystal Microbalance with Dissipation Monitoring</td>
</tr>
<tr>
<td>Rapa</td>
<td>Rapamycin</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>sChi</td>
<td>N-succinyl chitosan</td>
</tr>
<tr>
<td>Sox9</td>
<td>SRY (sex determining region Y)-box 9</td>
</tr>
<tr>
<td>Stim.</td>
<td>Stimulation</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue culture plate</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
</tbody>
</table>
CHAPTER 1

Cartilage development, structure, injury and repair

Note: Part of this chapter has been published in Molecular and biophysical mechanisms regulating hypertrophic differentiation in chondrocytes and mesenchymal stem cells, D. Studer, C. Millan et al., European cells and materials (2012), 24, 118-135.
1 Articular Cartilage

Articular cartilage is the connective tissue that is primarily found on the surfaces of bones within articulating joints (Figure 1.1) (1). Its function is to provide a near frictionless surface for the bones within a joint to move freely over each other during locomotion of the body and it also serves to absorb and transmit compressive loads that are generated in association with physical activity. An average adult takes between 1 and 2 million steps per year (2) each having an average peak magnitude of 2-10 MPa in compressive stresses acting on cartilage in the body (3). Cartilage is an avascular tissue, which means that it has a very low intrinsic capacity to heal. A damaged cartilage surface (Figure 1.1, right) that can no longer meet the physical demands of the tissue represents a clear medical problem. Osteoarthritis, which is a degenerative disease that results in joint surfaces that lose their functional capacity, has been identified as the single leading cause of disability in older adults (3) affecting over 25% of people over the age of 50 (4). In younger patients, cartilage can also be damaged as a result of trauma which presents initially with focal lesions and can progress to posttraumatic osteoarthritis due to inflammation and other degenerative processes in the joint (5). A number of surgical options for treating cartilage injury exist, but they have been met in the clinics with variable and underwhelming success rates for producing functional replacement tissue. This fact has motivated a number of tissue engineering strategies for treatment of cartilage defects. In this chapter, the design criteria for cartilage tissue engineering are presented and motivation is given for the decisions made over the course of this dissertation.

1.2 Cartilage Morphogenesis in the Developing Embryo

One approach for designing an appropriate tissue engineering substitute is to study the processes that a tissue undergoes during development. In developmental engineering (6), the researcher focuses on
1.2 Cartilage Morphogenesis in the Developing Embryo

Figure 1.1 | Articular cartilage in the knee joint. (Left) A diagram of the knee joint highlighting the articular cartilage covering the femoral condyles. Cartilage is the connective tissue that covers the ends of long bones in articulating joints. (Right) T1-weighted magnetic resonance images (MRIs) bone damage due to worn cartilage on the lateral femoral condyle. (top: coronal, bottom: sagittal) [Adapted with permission from (7)]

recapitulating aspects of the morphogenetic process involved early on in formation of new tissues to incorporate instructive cues for cells to model their surrounding matrix with endogenous proteins as they would in vivo (8, 9). This approach is particularly interesting for cartilage, a tissue with physically demanding function, which undergoes significant changes related to increasing mechanical properties during maturation (10, 11).

Cartilage morphogenesis in the developing limb bud begins in humans between 4 and 5 weeks after fertilization (8). A rapid and transient up-regulation of N-cadherin and neural cell adhesion molecule (N-CAM) membrane receptors (Figure 1.2) (12) facilitates the condensation of chondroprogenitor cells into high density micromasses (13). Fibronectin secretion additionally aids the formation of a dense aggregate (14), after which mesenchymal stem cells (MSCs) are stimulated by autocrine and paracrine secretion of
transforming growth factor-beta (TGF-β) which up-regulates the chondrogenesis master transcription factor SRY (sex determining region Y)-box 9 (or SOX9) and leads to production of type II collagen and proteoglycans such as aggrecan (Figure 1.2) (12, 15). The duration of the condensation phase is short, roughly 12 hours (16), but it is a requisite step that drives MSC chondrogenesis. Following condensation, maturation of the cartilage coincides with a series of morphological and physical changes. The resting chondrocytes at the end of developing long bones maintain a chondrogenic phenotype due partially as a result of signaling of the parathyroid hormone-related protein (PTHrP) (Figure 1.2) (17). Chondrocytes outside of the signaling range of the resting chondrocytes produce indian hedgehog (Ihh), a potent inducer of hypertrophy (18). Hypertrophic chondrocytes synthesize type X collagen which establishes the framework for eventual calcification and endochondral ossification of the developing bone (Figure 1.2) (19, 20). Researchers have shown that mimicking these conditions in vitro, via culturing a centrifuged pellet of cells in defined induction media, can induce chondrogenic differentiation (21). However, the phenotype of the differentiated cells shares characteristics of mature chondrocytes as well as hypertrophic chondrocytes, and the deposition levels of cartilage matrix specific components is limited in centrifuged pellets (Figure 1.2, right).*

*This paragraph appears in Studer et al., 2012 (19) and was written by C. Millan
Figure 1.2 | MSC condensation and endochondral ossification. (A) Up-regulation in the expression of N-CAM and N-cadherin plays a major role in increasing cell–cell interactions. (B) TGF-β stimulation drives the expression of cartilage specific markers via the Sox9 master transcription factor. (C) During maturation and terminal differentiation, chondrocytes in the resting zone at the end of long bones secrete PTHrP which maintains their chondrogenic phenotype, while those outside of this signaling range are under the influence of Ihh which induces hypertrophy. [Reprinted with permission from (19), designed and drawn by C. Millan]
1. Articular Cartilage

1.3 Structure of cartilage

Articular cartilage is a fairly unique tissue in that it lacks vasculature, lymphatic vessels, and nerves (22). The cellular constituent of cartilage, the chondrocytes, make up less than 2% of the tissue by weight (23) (Figure 1.3). They are highly specialized cells that are responsible for synthesizing the specific extracellular matrix (ECM) that imparts function to the tissue. The extracellular fraction of cartilage has also been well characterized (Figure 1.3) and is known to contain high quantities of type II collagen fibrils which provide functional resistance to tensile mechanical loading on the tissue. These fibrils are stabilized by collagen types IX and XI and the fibril orientation dictates the directionality of the load bearing function. Non-collagenous proteins such as link protein and cartilage oligomatrix protein (COMP) support the ECM structure. Proteoglycans make up the remainder of the solid matrix constituents. The predominant proteoglycan in cartilage is aggrecan, which exhibits a bottlebrush structure with heavily charged glycosaminoglycans such as chondroitin sulfate attached to the protein core. Negative charges in aggrecan attract positive ions such as Na⁺, Ca²⁺, and Mg²⁺ which lead to high levels of hydration providing osmotic resistance in the tissue, crucial for its ability to resist compressive loads (Figure 1.3).

As mentioned, the structure of cartilage varies in the distribution and organization that can be classified according to one of 4 zones. These zones, moving from the articulating surface towards the subchondral bone, are: the superficial zone, transitional zone, deep zone, and calcified cartilage zone (Figure 1.4). The superficial zone is by far the thinnest zone and the collagen fibrils in this zone are aligned parallel to the articulating surface which allow for high resistance to shear forces that act on the tissue surface generated by movement of diarthrodial bones. The superficial zone plays a critical role in maintaining the integrity of cartilage tissue as it also serves as a barrier to prevent cartilage molecules from diffusing into the synovial space as well as barring antibodies and cytokines from the synovial space from entering the underlying tissue (24). The chondrocytes of the superficial zone present an ellipsoidal
Figure 1.3 | Articular cartilage makeup. Cartilage is a tissue containing a single cell type, the chondrocyte. The extracellular matrix of cartilage is primarily composed of type II collagen fibrils immersed in a dense solution of proteoglycans. The type II collagen gives the tissue resistance to tensile strains. Aggrecan is the primary proteoglycan in the tissue and contains a number of highly charged glycosaminoglycans that retain water and confer resistance to compressive loads to the tissue. (Figure drawn by C. Millan)

morphology and are responsible for the synthesis of superficial zone protein, a molecule that significantly contributes to the lubrication of the cartilage surface (25).

The transitional zone, as its name implies, consists of ECM that is considered intermediate in comparison with the superficial and deep zones. Chondrocytes of the transitional zone present a more sphere-like morphology and synthesize thicker collagen fibrils and higher proteoglycan levels than those of the superficial zone. In the deep zone, proteoglycan concentration and collagen fibril thickness are
1. Articular Cartilage

highest. These collagen fibrils are aligned in an orientation perpendicular to the cartilage surface and are important for anchoring the cartilage tissue to the subchondral bone. Finally, the calcified cartilage zone acts as the barrier between cartilage tissue and the subchondral bone and is marked by hypertrophic chondrocyte phenotypes and an increase in type X collagen content.

**Figure 1.4 | Molecular structure of articular cartilage.** (Left) Histological staining by hematoxylin and eosin (H&E) and safranin-O to visualize cartilage extracellular matrix and specifically glycosaminoglycans (resp.). (Right) Cartoon depicting the change in cellular morphology and collagen fiber orientation in the various cartilage zones. Cells become more rounded and larger with tissue depth. Collagen fibers are parallel with the articulating surface in the superficial zone and gradually become perpendicular to the surface in deeper zones of the tissue. [Images adapted with permission from (26, 27)]
1.5 Current Clinical Treatment Options

Orthopedic surgeons have a number of treatment options available for addressing cartilage defects that are each associated with unique sets of advantages and drawbacks. Which technique to employ depends highly on the defect size (28) and location (29) as well as the patient’s age (30), body mass index (BMI) (31), and desired level of physical activity after treatment. For smaller defects, treatments for palliation are used that generally involve lavage and debridement to clear fibrillated cartilage pieces that may interfere with motion of the joint and cause pain (32). For repair of slightly larger defects, a method known as microfracture is often utilized that involves perforating the subchondral bone to release clotting factors and MSCs into the injured area (33). However, the cells that populate the lesion after microfracture typically synthesize fibrocartilage tissue which has inferior biomechanical properties to articular cartilage (34) and is thus a risk for re-injury (35). Larger full-thickness defects may be treated by grafting healthy cartilage harvested from a non-load bearing region of the joint in a procedure known as mosaicplasty, or osteochondral autograft transplantation surgery (OATS) (36). While a mosaicplasty fills the cartilage defect with hyaline cartilage, there are often complications involved relating to graft-site mismatch (37) and donor site morbidity (38). For severe cases, complete joint arthroplasty can be performed to replace the entire joint with a metallic prosthesis (39), though this option is not suitable for younger individuals as the failure risk of a prosthetic increases with time (40). Lastly, autologous chondrocyte implantation (ACI) is a treatment modality whereby cartilage is biopsied from a non-load bearing region of the joint, the chondrocytes are isolated and expanded in vitro for a number of weeks, and the cells are collected and reintroduced into the patient at the site of injury (Figure 1.5) (41).

The orthopedic community is actively pursuing new methods for treatment of damaged cartilage to address the shortcomings of the techniques discussed above. Modifications, particularly to microfracture and ACI, are constantly being introduced and evaluated in the clinics. In an effort to overcome incomplete defect filling and poor biomechanical properties of fibrocartilage repair tissue, various biomaterials have
1. Articular Cartilage

Figure 1.5 | Autologous chondrocyte implantation Schematic depicting autologous chondrocyte implantation (ACI). 1) Initial arthroscopy during which a cartilage biopsy is taken from a non-load bearing region of the joint (typically the lateral ridge trochlear groove). 2 and 3) Chondrocytes are isolated from cartilage tissue via enzymatic degradation. 4) Chondrocytes are expanded in monolayer culture for up to 6 weeks to increase cell numbers. 5 and 6) Chondrocytes are harvested [and optionally seeded onto a scaffold] and 7) The cells or cell-laden scaffold are implanted in the defect in a 2nd procedure and subsequently covered by a periosteal graft or membrane. [Image reproduced with permission from (42)]

Figure 1.6 | Chondrocyte de-differentiation in 2D culture Type II collagen gene expression in bovine chondrocytes after isolation (p.0) and subsequent passaging (p.1, p.2, p.3). Data was normalized by the internal housekeeping gene RPL13a and fold change was relative to expression levels at p.3. Cartoon below shows the change in morphology of chondrocytes which are round in vivo and become elongated and flattened during expansion. *Data from C.Millan
been combined with the microfracture technique (43). The so-called autologous matrix-induced chondrogenesis (AMIC) technique involves microfracture and subsequent sealing of the defect with a collagen membrane that is sutured in place (Figure 1.7) (44). However, the clinical results for this technique have been mixed (45). In lieu of drilling into the subchondral bones, a still newer technique known as matrix-associated chondroplasty (MACH) has been introduced in the clinics where bone marrow is aspirated from the distal femur, concentrated by centrifugation (BMAC, bone marrow aspirate concentrate), and injected under a collagen membrane together with platelet rich plasma (PRP) harvested from peripheral blood. The PRP is known to contain growth factors that enhance chondrogenesis of stem cells present in the bone marrow aspirate (46). These constantly evolving techniques strive to serve as a 1-step surgery that provides autologous stem cells with a conducive environment to undergo chondrogenic differentiation within the defect. Advances in tissue engineering may serve to expedite clinical translation of cartilage regeneration techniques (47).

**Figure 1.7 | Matrix enhanced microfracture.** In autologous matrix-induced chondrogenesis (AMIC), the surgeon first perforates the subchondral bone as in standard microfracture to release cells and clotting factors to the cartilage defect (left). A matrix that may contain soluble factors for promoting chondrogenesis is subsequently sutured in place over the defect to help retain and enhance the wound-healing milieu within the defect. [Image reproduced with permission from (44)]
1. Articular Cartilage

1.6 Cartilage Tissue Engineering

The canonical paradigm for engineering bioactive replacement tissue involves three critical components (48, 49): therapeutically relevant cells that are capable of forming and maintaining the engineered tissue, a scaffold material that supports cell infiltration and extracellular matrix deposition, and induction signals such as growth factors or small molecules that serve to direct cell behavior within the scaffold. An overview of the various specifications available when designing a tissue engineered cartilage construct is given in the following sections.

1.6.1 Cell types

As the living component of a tissue engineered construct, it is imperative to select a cell type that is capable of synthesizing and maintaining the cartilaginous matrix constituents, namely type II collagen and GAGs (50). The source of the cells is equally important as they can be autologous-derived (51) or allogenic cells harvested from a donor (52), each with associated advantages and drawbacks (53). The vast majority of work in this area has focused on chondrocytes and stem cells, but it should also be mentioned that recent discoveries have been made regarding a population of stem/progenitor cells in cartilage tissue (CSPCs) (54-57). CSPCs have been shown to better maintain their chondrogenic phenotype during monolayer expansion than chondrocytes, though showed no major difference versus autologous chondrocytes for treating cartilage defects in a caprine model (58).

Chondrocytes

Cartilage is a unicellular tissue type, so it follows that chondrocytes make for an obvious choice to incorporate in tissue engineering design. In autologous chondrocyte implantation (ACI), a patient must undergo an initial procedure where cartilage is biopsied from a non-load bearing region. The cells are isolated from this biopsy and, due to the low cell density of cartilage (59), these cells must be expanded in a laboratory for up to 6 weeks (60) until there are enough cells to be re-implanted in the patient’s cartilage
defect at a second procedure. Originally, an autograft of the periosteum was used to seal the defect and retain the chondrocytes within the injured site (61), but current generations of the procedure feature a wide range of different scaffold materials for this role (62). Some clinical comparisons of the use of either a periosteal graft or a collagen membrane with ACI have found no difference in patient outcome following treatment (63, 64). Scaffold-free approaches have also emerged recently (65) including the Chondrosphere® autologous chondrocyte transplantation 3D (ACT3D-CS), currently in phase III clinical trials conducted by co.don AG (Tetlow, Germany) (66), which features chondrocyte spheroids that are injected arthroscopically into a cartilage defect (67).

Despite being an active area of research in the orthopedic community, the clinical effectiveness in the use of autologous chondrocytes may be limited. The initial harvesting process during cartilage biopsy is known to cause donor site morbidity and may lead to further degeneration of the joint (68). Furthermore, the need to expand the cells in vitro is not only time-consuming and expensive, but is known to result in de-differentiation of the cells which lose their ability to synthesize cartilage-specific matrix (Figure 1.6) (69, 70) and, therefore, have a reduced capacity to promote tissue regeneration. In an ongoing clinical trial aimed at addressing the issue of de-differentiation, a company called Tigenix (Leuven, Belgium) is testing a method called Chondrocelect for screening expanded chondrocytes for genetic markers and scoring the cells based on these markers to predict their clinical efficacy (71). Alternatively, allogenic cells from young donors may mitigate the need for a 2-step procedure as well as provide a cell source that has retained its chondrogenic ability. Cells from juvenile donors younger than 13 years old have been shown to synthesize more cartilage matrix than adult cells (72, 73). Similarly, in a comparison of fetal and adult ovine chondrocytes, the fetal cells had higher proliferation rates and elevated levels of GAG and type II collagen synthesis (74). Hurdles facing the use of allogenic cell sources involve potential immune rejection from the host and donor availability, though a new product (de Novo NT from Zimmer Inc, Warsaw, IN)
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<td>• Animal trials • N/A</td>
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| References | (41) (67, 75) (76) (54, 56-58) (33, 44, 77, 78) (79-81) (82, 83) |

*ACI-* autologous chondrocyte implantation; *MACI*- matrix assisted autologous chondrocyte implantation; *CAIS*- cartilage autograft implantation system; *NT*- natural tissue; *MSC*- mesenchymal stem cell; *MFX*- microfracture; *BMAC* bone marrow aspirate concentrate; *PRP*- platelet rich plasma; *AMIC*- autologous matrix induced chondrogenesis; *ESC*- embryonic stem cell; *iPSC*- induced pluripotent stem cell
based on minced cartilage from juvenile donors is currently being evaluated by the Food and Drug Administration (FDA) (76).

**Stem cells**

Undifferentiated progenitor cells offer attractive alternatives over the use of chondrocytes for cell-based cartilage repair. Autologous mesenchymal stem cells (MSCs) can be obtained in higher numbers than chondrocytes and from a number of different tissues including bone marrow (84), the synovium (85), and adipose tissue (86). The most common MSC tissue source used clinically in humans is bone marrow (87). MSCs are easily isolated via plastic adherence (88, 89). It has been proposed that MSCs can be identified by their expression of key surface markers including CD-105 (90), CD-146 (91), and STRO-1 (92), as well as their ability for multi-lineage differentiation into fat, bone, and cartilage (93, 94). Importantly, MSCs can be expanded in culture for a number of passages while retaining their differentiation capacity (95). Chondrogenesis has also been shown for embryonic (96) and induced pluripotent stem cells (82) which have the ability to proliferate indefinitely, but the clinical safety of these cells remains in doubt (97). MSCs are the unique autologous cell type that can be isolated from a patient in enough numbers to be re-implanted at the injury site in the same day, provided the necessary chondrogenic induction cues are present to guide their differentiation.

Interestingly, in comparing chondrocytes and MSCs as the cell type for ACI procedures by an observational cohort study, Nejadnik et al. (98) found no different in clinical outcome 24 months after surgery. This is likely a result of characteristic secretion of trophic factors that promote tissue regeneration concomitant with differentiation of MSCs (99). Additionally, Wakitani et al. (100) has published a case study after treating cartilage injuries with bone marrow MSCs seeded in collagen gels which resulted in symptomatic improvement for patients at 27 months post-operation. Furthermore, they have proven the clinical safety relating to tumorigenicity and infection of using autologous bone marrow MSCs in follow-up studies after 11 years post-transplantation in 45 patients (101). There are still remaining questions to
be addressed regarding the maintenance of the differentiated phenotype of MSCs as many reports indicate a tendency towards hypertrophy (19) and ectopic bone formation (102) when implanted in vivo. Advances in tissue engineering seek to further define and control the chondrogenic environment to reproducibly guide the chondrogenic differentiation of these cells, preferably in situ within the defect (103).

1.6.2 Scaffold material

There is a large number of scaffold materials under investigation for their ability to provide a conducive environment to cells for cartilage matrix production (Table 2) (104). It is widely accepted that an appropriate scaffold should be biocompatible, versatile, permeable, and facilitate a rounded cell morphology that is typical of chondrocytes (105). Typical scaffold materials include protein-based, polysaccharide-based, and synthetic-based polymers (106). Protein-based scaffolds like the type I/III collagen membrane from Geistlich Pharma AG (Wolhusen, Switzerland) have been among the first to test in the clinics with autologous chondrocytes (107) as well as for matrix support following microfracture (108). MSC chondrogenesis has been demonstrated in other protein based scaffolds such as fibrin (109) and gelatin (110) which present adhesion sites to seeded cells. However, hydrogels made from crosslinked polysaccharides or synthetic polymers offer exciting potential as optimal tissue mimics due to their high water content similar to that found in native cartilage, versatility for functionalization, and their shared macromolecular properties with many natural ECM components (111).

Hydrogels made from synthetic polymers offer chemical properties that are highly reproducible for control over crosslinking density, gelation parameters, and their material mechanical and degradation properties (112). Polylactic acid (PLA) (113) and polyethylene glycol (PEG) (114) are two synthetic polymers that are commonly explored for cartilage tissue engineering. A common method for crosslinking synthetic polymers into hydrogel structures is achieved by modifying the polymer by addition of acrylate
or methacrylate groups (115, 116), mixing with a photoinitiator, and crosslinking by free radical polymerization during exposure to UV light. However, cytotoxicity of photoinitiators and the need for a UV source for polymerization are limiting factors of using such systems and their immediate clinical applications (117). While unmodified synthetic polymers are not recognized by the body limiting their remodeling potential in vivo, enzyme degradable sequences can be incorporated as crosslinks into the material to improve host interactions (118). Also, cell adhesion peptides such as the collagen binding domain (GFOGER) have been incorporated into PEG gels to facilitate cell adhesion and improve MSC chondrogenesis (119).

Polysaccharide hydrogels that have been tested for cartilage tissue engineering include agarose (110, 120), alginate (90, 110, 121), gellan gum (122), chondroitin sulfate (123, 124), hyaluronic acid (125), and chitosan (126). Gels can be formed as a result of physical, ionic, or covalent crosslinking (106). While physical crosslinking (e.g. thermal gelation of agarose) and ionic crosslinking (e.g. Ca\(^{2+}\) crosslinking of alginate) are mild reactions, they require specific reaction conditions (e.g. set temperature or calcium concentration, respectively) that may be difficult to control precisely in a clinical setting. Crosslinking by covalently-interacting complementary groups is attractive owing to rapid gelation and potential to incorporate bioactive molecules easily into the system. For example, Michael-type addition was used to covalently attach N-cadherin peptides to methacrylated HA gels and shown to enhance MSC chondrogenesis compared with gels that received a scrambled peptide (127). Alternatively, Schiff base crosslinking is ideally suited for polysaccharide polymerization due to its rapid reaction rate, freedom from the need of catalysts, and its cell-friendly reaction conditions (128).

**Schiff base crosslinking**

A Schiff base is the result of a spontaneous chemical reaction between a primary amine and a free aldehyde or ketone (128). A large number of naturally occurring polysaccharides can be rendered reactive
## Table 2 | Scaffolds used in cartilage tissue engineering

<table>
<thead>
<tr>
<th>Scaffold type</th>
<th>Biomaterial</th>
<th>Advantages</th>
<th>Limitations</th>
<th>Crosslinking Mechanisms</th>
<th>Clinical trials</th>
<th>References</th>
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<tr>
<td><strong>Polysaccharides</strong></td>
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<tr>
<td>Hyaluronic acid</td>
<td></td>
<td>High bioactivity High biocompatibility Ease for physical and chemical modifications Biodegradability</td>
<td>Relatively rapid degradation in vivo Poor mechanical properties Variability depending on the source</td>
<td>Photopolymerization of acrylic derivatives Michael-type addition Schiff base Esterification</td>
<td>Hyalograft®C (Fidia Advanced Biopolymers, Italy) ChonDux™ (Cartilix/Biomet, USA) Cartistem® (Dong-A Pharma, Korea)</td>
<td>(129, 130)</td>
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<tr>
<td>Chondroitin Sulfate</td>
<td>Biodegradability Anti-inflammatory properties</td>
<td>Inhibition of chondrocyte biosynthetic activity High cost</td>
<td></td>
<td>Photopolymerization of acrylic derivatives Schiff base</td>
<td>ChonDux™ (Cartilix/Biomet, USA)</td>
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<tr>
<td>Alginate</td>
<td></td>
<td>High bioactivity, biostability Biocompatibility Ease of preparation Low cost</td>
<td>Slow and uncontrollable degradation Variability depending on the source</td>
<td>Cross-linking with divalent cations Schiff base</td>
<td>Cartipatch® (Tissue Bank of France, France)</td>
<td>(132)</td>
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<tr>
<td>Chitosan</td>
<td>Structure similar to natural GAGs which enables interaction with growth factors Biocompatibility, biostability Low immunogenicity Biodegradable Easy to chemically modify Promotes attachment, proliferation and viability of cells</td>
<td>Need for modification to be water-soluble</td>
<td></td>
<td>Physical Schiff base</td>
<td>BST-CarGel® (Biosyntech, Canada)</td>
<td>(133)</td>
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<tr>
<td>Agarose</td>
<td>Effective cellular response Ease of preparation Low cost</td>
<td>Non-degradability Poor mechanical properties Immunogenicity Material modification is limited</td>
<td></td>
<td>Physical (temperature dependent)</td>
<td>Cartipatch® (Tissue Bank of France, France)</td>
<td>(110, 132)</td>
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<tr>
<td>Proteins</td>
<td>Collagen/ gelatin</td>
<td>High bioactivity (good cell-material interaction)</td>
<td>Rapid degradation in vivo Immunogenicity (type II collagen and gelatin)</td>
<td>Physical (pH dependent) Photopolymerization of acrylic derivatives</td>
<td>NeoCart® (Histogenics, USA) MACI® (Verigen, Germany) Maix® (Matricel, Germany) AMIC®, Chondro-Gide® (Geistlich Biomaterials, Switzerland) Atelocollagen® (Koken Co. Ltd, Japan)</td>
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<td></td>
<td>Ability to support cellular processes</td>
<td>Batch-to-batch variation Risk of disease transfer Limited availability High cost</td>
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<td>Able to maintain the chondrocyte phenotype</td>
<td>Rapid degradation in vivo Immunogenicity</td>
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<td>Proteins</td>
<td>Fibrin</td>
<td>High bioactivity (good cell-material interaction)</td>
<td>Increase of instability and solubility over time</td>
<td>Enzymatic reaction by thrombin cleavage to fibrinogen precursor of fibrin</td>
<td>Gelrin C® (Regentis, Israel) Chondron® (Sewon CellOnTech, Korea) DeNovo® NT (Zimmer, USA)</td>
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<td></td>
<td>Ability to support cellular processes Biodegradability by proteolytic degradation</td>
<td>Rapid degradation in vivo Immunogenic</td>
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<tr>
<td>Proteins</td>
<td>Silk</td>
<td>Biocompatible/Biodegradable Ease for chemical modifications Remarkable mechanical properties</td>
<td>Batch-to-batch variation</td>
<td>Physical (sonication, pH, temperature) Electrospun fibers</td>
<td>FibroFix™ (Regener8, UK)</td>
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<tr>
<td>Synthetic Polymers</td>
<td>PEG/PEO</td>
<td>High reproducibility Ease for chemical modifications Non toxicity Non-immunogenic</td>
<td>Lack of cell recognition signals Low biological activity Does not support protein deposition Non degradable Need of modification to polymerize</td>
<td>Photopolymerization of acrylic derivatives Michael-type addition Enzymatic crosslinking</td>
<td>Gelrin C® (Regentis, Israel)</td>
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<td>Synthetic Polymers</td>
<td>PLGA</td>
<td>High reproducibility Biodegradability Good mechanical properties</td>
<td>Lack of cell recognition signals Low biological activity Local inflammation Random chain hydrolysis</td>
<td>Leaching Phase separation Electrospinning Gas foaming Press coating</td>
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<tr>
<td>Synthetic Polymers</td>
<td>PLA</td>
<td>High reproducibility Low cytotoxicity</td>
<td>Lack of cell recognition signals Low biological activity Local inflammation Random chain hydrolysis</td>
<td>Leaching Phase separation Electrospinning Gas foaming Press coating</td>
<td>Bio-Seed®-C (BioTissue Technologies, Germany)</td>
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for Schiff base crosslinking by oxidative cleavage of vicinal hydroxyls yielding two aldehydes per saccharide unit. Protein derivatives such as gelatin and silk contain a high number of lysine amino acids that feature primary amines in their side chain groups. The interaction between lysine residues of amino acids and Schiff base compliments in oxidized alginate has also been shown to support controlled release of proteins (139). Balakrishnan and co-workers have utilized Schiff base crosslinking between gelatin and oxidized chondroitin sulfate (oxCS) for wound healing applications (140), and gelatin paired with oxidized alginate to serve as an injectable scaffold to support chondrocyte transplantation (141). Others have used Schiff base crosslinking to functionalize a fibrous silk-based scaffold with oxCS which supported chondrocyte growth resulting in increased scaffold stiffness compared to controls (142).

The properties of hydrogels made from Schiff base crosslinking can also be finely tuned by controlling the relative quantities of the two reactive moieties in the reaction. Tan et al. have shown that balancing the ratio of sChi and oxHA concentrations in a hydrogel system has a strong effect on the gelation time and degradation rates of the polymer network (143). Swelling and crosslinking density in a gelatin/oxAlg and gelatin/oxCS systems were highly dependent on the degree of oxidation of the polysaccharides (140, 144). Varying the crosslinking density would have a profound impact on the cellular microenvironment changing microtissue stiffness, swelling, nutrient and growth factor diffusion, and the degradation rate of the polymers. In addition to utilizing Schiff base crosslinking for hydrogel polymerization, it has proven useful for improving the attachment of biomaterials to tissue surfaces as well as for growth factor loading and release. Wang et al. took advantage of crosslinking between oxCS and lysines present in collagen residues on cartilage surfaces to covalently attach chondrocyte-laden hydrogels to cartilage defects with improved integration (145). This bioadhesive based on Schiff base linkages has shown promise in a pilot clinical trial (146) where patients treated with covalently attached hydrogels had better defect filling than a control group that received a standard microfracture treatment.
1.6.3 Growth Factor Selection and Delivery

While the initiation of chondrogenesis in the developing limb bud is correlated with physical aggregation of cells into high-density condensations, the differentiation process is also regulated by exposure to critical growth factors (GFs) in a defined spatiotemporal manner (147). The GFs most commonly associated with chondrogenic induction are members of the transforming growth factor beta (TGF-β) family, insulin-like growth factors (IGFs) (148), and fibroblast growth factors (FGFs) (149, 150). Of the many GFs belonging to the three families mentioned above, the most potent regulator of MSC chondrogenesis seems to be TGF-β3 (151). In a recent study that screened for the optimal transgene for overexpression in MSCs to drive chondrogenesis (152), TGF-β3 was the only one to induce cartilage matrix formation whereas overexpression of bone morphogenetic protein-7 (BMP-7) or SOX9 did not.

Incorporation of GFs into the tissue engineered construct enables the coupling of biochemical chondroinductive stimuli with biomechanical stimuli provided by the scaffold. A number of scaffolds have been investigated for loading and releasing TGF-β for inducing chondrogenesis in cells seeded within the scaffolds (153). Strategies for doing so include encapsulation/loading or immobilization of TGF-β within the scaffold (154, 155), or initially encapsulating the GF in polymeric microspheres or nanoparticles that are subsequently incorporated into the scaffolds themselves (Figure 1.8) (156-158). The method of loading TGF-β has a direct impact on the timing of release and, therefore, the loading dose necessary for sufficient chondroinduction. It has been observed that TGF-β stimulation early on during chondroinduction, followed by long culture periods in the absence of the GF, is sufficient for inducing differentiation (159) and that transient exposure improves the mechanical properties of the engineered construct (160). Combining a 3D scaffold with a method for providing a burst release of TGF-β is considered ideal for achieving the goal of a matrix that is capable of in situ chondroinduction.
Figure 1.8 | Growth factor loading and release from hydrogels. (A) Growth factors (GFs) can be loaded by covalent crosslinking between reactive groups. (Left) depicts a Michael-type addition between a thiol present in the GF and vinyl sulfones present in the hydrogel polymer. (Right) The GF is enzymatically crosslinked by Factor XIII activated. (B) Non-covalent immobilization of GFs can be achieved by GF affinity for the hydrogel polymer, interactions with ECM components in the gel, or ligand-ligand interactions. (C) To slow the release, GFs can be initially loaded in carriers such as microparticles prior to being encapsulated in the hydrogel. [Reprinted with permission from (161)]
1.7 Tissue engineering design: mimicking the condensation

There is healthy debate in the cartilage tissue engineering community (162) as to which degree of development is optimal for tissue engineered constructs prior to implantation. In other words, does the tissue engineered implant need to fully demonstrate mechanical properties equivalent to cartilage prior to implantation? Should it already contain ECM content in quantities similar to the host tissue? Or can the construct be designed in such a way that, as cells within the implant secrete endogenous proteins to assemble their own ECM that fortifies the engineered tissue, full development is reached in situ over time? In an approach that follows the developmental engineering paradigm, the design pursued in this thesis was to create a method that features events that occur during tissue morphogenesis in an effort to provide cells with the induction cues needed to direct their chondrogenic differentiation.

As mentioned previously, MSC chondrogenesis is triggered early on during development by an event known as condensation (13). During condensation, the up-regulation of signaling molecules such as transforming growth factor beta (TGF-β) and N-cadherin induces clustering of cells into high density micromasses (16, 147). The condensation period typically lasts for 12 hours (163, 164), after which an accumulation of hyaluronic acid, chondroitin sulfate, and other glycosaminoglycans (GAGs) occurs in the intercellular space to reduce cell density and allow cartilage-specific matrix production (165). MSC condensation is typically mimicked in vitro by culturing cells as micromass pellets formed either by centrifugation (166, 167) or hanging drop culture (168, 169) in media containing TGF-β3. Since it was first demonstrated in 1998 (167), in vitro chondroinduction of MSCs using micromass pellets has become a standard tool for studying cartilage development (15, 170), to evaluate the chondrogenic potential of new stem cell sources (82, 171, 172), and also more recently to evaluate the ability of MSCs to heal cartilage defects (173). One major drawback of micromass pellets is that dense-packing of cells often limits diffusion to the pellet core resulting in cartilage matrix only being synthesized in the periphery of the pellet (174-178). This limitation is amplified for aged MSCs that are known to express fewer receptors for growth.
Articular Cartilage factors (GFs) involved in differentiation (179, 180), indicative of a cell population with a reduced sensitivity for directed differentiation.

By combining the lessons learned from studying aspects of MSC condensation, as well as optimizing the scaffold type and GF delivery method from the options discussed earlier, we settled on a group of polysaccharides that could be used for assembling cells into condensation-like aggregates via Schiff base crosslinking. GAGs which are natively found in high quantities in cartilage (and participate in condensation), as well as polysaccharides that share similarities in chemical structure with those GAGs, were chemically modified to undergo Schiff base crosslinking for achieving engineered MSC condensations (Figure 1.9).

Chitosan has previously been identified as an attractive biomaterial for use in cartilage tissue engineering due to its structural similarity to cartilage GAGs (181), and has been linked to cell aggregation when injected into rat knee cartilage (182). Unmodified chitosan is insoluble at physiological pH, but partial succinylation serves to disrupt crystallinity domains that cause precipitation and permits solubility at pH 7.4 (N-succinyl chitosan, sChi) (183-185). Hyaluronic acid (HA), chondroitin sulfate (CS), and alginate (Alg) were oxidized with sodium periodate to yield Schiff base reactive aldehyde groups in their sugar backbones. HA is known to facilitate cell migration during condensation (16) and has specific binding sites for CD-44 receptors on MSC surfaces that, when bound, enhance chondrogenesis (127). Incorporation of CS into PEG hydrogels has been shown to promote MSC aggregation and improve their chondrogenic phenotype (124), while Alg is well known to support chondrogenesis (110). Schiff base crosslinking could be utilized between sChi and the oxidized polysaccharides for formation of microtissues, and the same chemistry could be used for loading the lysine rich chondroinductive factor TGF-β3 in the polymer network (Figure 1.9B).
1.7 Tissue engineering design: mimicking the condensation

Figure 1.9 | Mimicking MSC Condensation The tissue engineering strategy pursued in this work was related to recapitulation of the MSC condensation, the first step in chondrogenesis during cartilage morphology in the body. (A) Cells were suspended in a solution of N-succinyl chitosan (sChi) and mixed with oxidized hyaluronic acid (oxHA), chondroitin sulfate (oxCS), or alginate (oxAlg). Schiff base crosslinking between the primary amines in sChi and the free aldehydes in the oxidized polysaccharides resulted in rapid aggregation of cells in 3D microtissues. (B) In some studies, the chondroinduction GF transforming growth factor-β3 (TGF-β3) was loaded in the microtissues by first solubilizing in sChi prior to cell suspension. (C) The standard method for mimicking MSC condensation and inducing chondrogenesis in vitro involves centrifuging cells in conical tubes and culturing them in media containing TGF-β3.
CHAPTER 2

Scope of the thesis
2 Scope of the thesis

A tissue engineering approach towards a single day cell-based therapy for treating cartilage defects is described in this thesis. Mesenchymal stem cells (MSCs) were selected as the cell type in the engineering design because they can be isolated from a patient in sufficient numbers, without inducing donor site morbidity, and re-implanted on the same day at the site of injury within a biomaterial that directs their chondrogenic differentiation. Elements of the engineering design were rooted in recapitulating key morphogenetic events that occur during cartilage formation in the developing embryo.

MSC condensation is the first step that the body undergoes during cartilage morphogenesis. It is often simulated in vitro by centrifuging cells in a conical tube and culturing them as a micromass pellet in defined chondrogenic media for a given period of time. This approach, however, oversimplifies the condensation process during which key proteins and glycosaminoglycans (GAGs) facilitate the mobility of cells towards a center and enable their aggregation and subsequent separation during the synthesis of cartilage matrix components. To improve upon the conventional pellet culture model, this work was focused on directing the assembly of MSC condensations utilizing adhesive proteins and/or GAGs that are known to participate in the process in vivo.

Initial experiments involved coating cells in adhesive nanofilms by way of layer-by-layer (LbL) accumulation of film components on cell surfaces (described in Arriving at Schiff base crosslinking for inducing cell aggregation). Proteins such as fibronectin and gelatin that present mutual binding domains to each other, and to cell surface receptors, were explored for coating cells and mediating high-density culture conditions. Negatively charged polyelectrolytes including cartilage constituents such as hyaluronic acid (HA) and chondroitin sulfate (CS) were paired with positively charged polycations for creating cell surface nanofilms that would attract electrostatically. The use of polycations proved deleterious due to issues with cytotoxicity. Some promising trends for redifferentiating passaged chondrocytes did emerge from experiments involving cells coated in fibronectin and gelatin, but relying on ligand-specific
interactions to promote aggregation proved too weak for maintaining robust tissue structures. Covalently interacting molecules were then identified to overcome cytotoxicity and drawbacks with tissue integrity and were further explored for their ability to enhance chondrogenic differentiation.

In chapter 4, the use of modified polysaccharides for driving the aggregation of MSCs into condensation like microtissues and enhancing chondrogenesis is described in detail. Polysaccharides that are either present in cartilage ECM or share structural similarities to those that are present were modified to undergo Schiff base crosslinking in physiological reaction conditions. Chitosan was partially substituted with succinic anhydride to yield N-succinyl chitosan (sChi). HA, CS, and alginate were fully oxidized to yield free aldehyde groups in their sugar backbones. It was then observed that MSC aggregation into microtissues could be induced following the rapid 25 minute Schiff base crosslinking reaction when cells were suspended in a solution of sChi and subsequently mixed with oxidized HA (oxHA), CS (oxCS), or alginate (oxAlg). Quantitative assays and histological analysis of MSC microtissues cultured in chondrogenic media pointed to sChi and oxAlg as the ideal pair for significantly improving cartilage matrix production. Detailed comparisons between sChi/oxAlg engineered microtissues (EMTs) and MSCs cultured in centrifuged micromass pellets revealed that the polysaccharide matrix in the microtissues structure provided improved conditions for chondrogenic induction. Paramount for widespread adoption of cell-based therapies in regenerative medicine is that the treatment method is proven to provide healing for a large majority of the population. In comparing pellet culture with sChi/oxAlg EMT culture for MSC donors of various ages, our data suggested a restoration of the chondrogenic ability in the non-responsive population of MSCs derived from aged donors in EMTs.

Next, we built on the design of the engineered microtissue to allow for in situ differentiation of entrapped MSCs which would significantly decrease the amount of in vitro manipulation required for preparation as a surgical treatment option for cartilage defects. This was achieved by incorporating the critical chondrogenic induction factor, transforming growth factor-beta 3 (TGF-β3), into the polysaccharide
network at the time of crosslinking. Release was monitored by ELISA and the dose of 50ng per microtissue was determined sufficient for driving MSC chondrogenesis during culture of samples in media that was not supplemented with TGF-β3. Finally, the clinical merit of the EMT-based method for treating cartilage defects was demonstrated in an in vitro repair assay. Cartilage defects were made in biopsy punches of bovine cartilage which were subsequently filled with EMTs loaded with TGF-β3 (EMT+TGF). After 4 weeks culture in serum-free media that was not supplemented with the growth factor, EMT+TGF filled the defects with de novo repair tissue containing GAGs and type II collagen.

In Chapter 5, the versatility of using Schiff base crosslinking for manipulating cell adhesion is demonstrated. Glycosaminoglycans present in the ECM of cartilage surfaces could be oxidized to render them Schiff base reactive. Cells suspended in sChi were subsequently deposited on the reactive surfaces where they bound covalently at significantly higher rates than cells that were seeded in a control saline solution. This effect was dependent on the oxidation strength and time of oxidation used for preparing cartilage surfaces. In another application, engineered microtissue co-cultures were shown to confer spatial control of different cell populations in a 3D environment as the result of rapid Schiff base crosslinking between sChi and oxAlg. Controlled rapid assembly of different cell types offer the possibility for complex co-culture studies to probe interactions between different cell types.

In addition to applications for cartilage tissue engineering, EMT culture for primary hepatocytes was investigated and results are reported in chapter 6. In vitro liver models are highly sought after for preclinical testing of hepatotoxicity for new pharmaceuticals. Hepatocyte spheroids are typically formed by self-assembly in hanging drop culture, which can be slow and is associated with poor spheroid formation efficiency. In this chapter we compare the performance of hepatocyte EMTs and spheroids formed in specialized hanging drop culture plates recently developed by Hoffman La-Roce (Basel, Switzerland). The EMT parameters were optimized for hepatocyte culture and microtissue formation
efficiency was monitored. Biological functionality between spheroids and EMTs was compared by RT-qPCR gene expression analysis for enzymes that are critical for drug metabolism.

The thesis concludes with a look into ongoing experiments aimed at further developing the EMT technology. As a first step to evaluate EMTs as a clinically-relevant therapy, a preliminary *in vivo* study is planned involving subcutaneous implantation of EMT\(^{+}\)TGF\(^{+}\) filled in nude mice. A pharmaceutical-based strategy for targeting autophagy in differentiating MSCs is also underway. Autophagy is an important part of limb bud development and is hypothesized to play a critical role in maintaining the phenotype of resting chondrocytes and preventing hypertrophic differentiation.
CHAPTER 3

Materials and Methods
3 Materials and Methods

3.1 Synthesis of N-succinyl Chitosan (sChi)

Chitosan (85% deacetylation, 500mPas, Heppe Medical Chitosan GmbH, Halle, Germany) was reacted with succinic anhydride to synthesize N-succinyl chitosan (sChi) according to a previously published protocol (143). Briefly, 0.5g of chitosan was dissolved in 40mL of 5% (v/v) lactic acid and diluted 1:4 in methanol. 1.5g of succinic anhydride was added to solution while stirring and left overnight. sChi was precipitated by drop-wise addition of 10M NaOH and filtered with Whatman filter paper. The precipitate was re-dissolved in milli-Q water and dialyzed for 3 days. It was then freeze-dried and stored at 4°C until use.

For fluorescently labeling sChi, 4mg of fluorescein isothiocyanate (FITC, Sigma) and 50mg of sChi were each dissolved separately in 7mL of 0.5M carbonate buffer at pH 9.4. Following solubilization, the sChi and FITC solutions were mixed (1:1) and allowed to react overnight at room temperature protected from light. Unreacted free dye was removed by dialysis against milli-Q water at 4°C for 4 days. The final product was lyophilized and stored dry at 4°C until use.

3.2 Synthesis of oxidized polysaccharides (oxHA, oxCS, and oxAlg)

Chondroitin sulfate from shark cartilage (Sigma), hyaluronic acid (800-900 kDa, Novozymes Hyasis, Cambridge, MA, USA), and alginate (20-200 mPas, NovaMatrix, Sandvika, Norway) were oxidized according to Köwitsch et al. (186) using sodium periodate. 100mg of HA, CS, and alginate were each solubilized in 20mL of milli-Q water. For complete oxidation of vicinal hydroxyl groups, molar equivalents of sodium periodate (NaIO₄) were calculated for a theoretical 200% oxidation. Corresponding amounts of NaIO₄ were dissolved in 5mL of milli-Q water and added drop-wise to the stirring polysaccharide
solutions. The reactions were carried out overnight protected from light. The oxidized polysaccharides (oxPS) were purified by dialysis for 3 days, freeze-dried, and stored dry at 4°C until use.

3.3 Characterization of modified polysaccharides

Amino substitution of sChi was confirmed with $^1$H-NMR using a Mercury-vx 300 spectrometer with D$_2$O as the solvent. Succinic substitution of sChi was calculated by taking the integral area ratio of the succinate peak ($\delta = 2.28$-2.33 ppm, CH$_2$CH$_2$ multiplet of succinyl) and the H-2 peak of the D-glucosamine unit in chitosan ($\delta = 2.70$ ppm). Lyophilized materials were milled into a powder with a Pulverisette 7 (Fritsch, Idar-Oberstein, Germany) using 0.1mm zirconium beads. Aldehyde formation in oxidized HA, CS, and alginate was measured using an infrared spectrophotometer in attenuated total internal reflection mode (Frontier Spectrometer ATR-FTIR, Perkin Elmer, Waltham, MA, USA). 5mg of powder material was used and measurements were done in triplicate.

3.4 Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)

Thin film layer-by-layer (LbL) buildup was characterized by quartz crystal microbalance with dissipation (QCM-D E4, Q-Sense AB, Gothenburg, Sweden). In QCM-D measurements, an electric potential is applied to a gold coated quartz crystal causing the crystal to oscillate at its measured resonance frequency. The crystal is mounted in a flow cell, and drops in its oscillation frequency due to material deposition onto the surface can be monitored and used to calculate film mass (187, 188). For cleaning, gold coated QCM crystals were immersed in surface cleaning solution (Ciba, Basel, Switzerland) for 30 minutes, rinsed with milli-Q water, blown dry under nitrogen, and treated by UV/ozone for 30 minutes. All reagents were solubilized in PBS (pH 7.4) and experiments performed at room temperature. A stable baseline was achieved with 0.5mL of buffer and then 0.5 mL of sChi (5mg/mL) was injected into the flow
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cell. After 30 minutes the surface was washed with PBS for 5 minutes and unmodified or oxidized HA, CS, or alginate was injected (0.5mL, all at 10 mg/mL in PBS). After 30 minutes another rinsing step was performed. The steps were repeated for two bilayers, followed by 4 hours under DMEM containing 1% antibiotic-antimycotic (Life Technologies) to further test film stability.

Frequency shifts were reported from measurements of the third overtone with \( \Delta f \) taken as the difference between the oscillation frequency measured before and after injection of a given solution into the flow cell. Changes in areal mass on the crystal surface were calculated using the Sauerbrey relation (1) where \( n \) is the overtone number and \( C \) is the mass sensitivity constant. Measurements were repeated in triplicate for each pair of sChi with PS or oxPS.

\[
\Delta m = -nC \Delta f \quad (1)
\]

3.5 Isolation and culture of human mesenchymal stem cells (MSCs)

Human mesenchymal stem cells (MSCs) were isolated from femur-derived bone marrow samples that were obtained during surgical hip replacement of otherwise healthy patients after receiving informed consent. The protocol was approved by the ethics board of the Kantons hospital, St. Gallen, Switzerland (ethics committee approval number EKSG08/014/1B). MSC isolation was performed as previously described (189) and cells frozen after first passage were provided in vials by the Empa Laboratory for Biomaterials. Cells at passage 1 were thawed and expanded in DMEM/F-12 (1:1) media containing 10% FBS, 1% antibiotic-antimycotic, and 10ng/mL human basic FGF-2 (Peprotech, Rocky Hill, NJ, USA).

3.6 Microtissue formation by Schiff base crosslinking

For microtissue formation MSCs were trypsinized at passage 4, washed in PBS, and resuspended in a solution of sChi (5mg/mL in PBS) at a cell density of 20 x 10^6 cells/mL. Specialized well plate lids (Roche,
Basel, Switzerland) with 5mm diameter circular molds were used to retain the solutions during crosslinking. Drops of 5 μL each of the corresponding oxPS molecules (10mg/mL in PBS) were distributed into individual molds on the plate lid and 10 μL of sChi containing 2.0 x 10^5 cells were pipetted into each droplet of oxPS. The lid was turned upside down to facilitate mixing and the reaction was continued in an incubator at 37°C for 15 minutes. Microtissues were transferred to agarose coated-wells of a 96-well plate with fine-tipped forceps and cultured in chondrogenic media (DMEM, 1% penicillin-streptomycin, 50 μg/mL L-ascorbic acid (Sigma), 1% ITS+ premix (Becton Dickson AG, Allschwill, Switzerland), 40 μg/mL L-proline (Sigma), 100 ng/mL dexamethasone (Sigma), and 10 ng/mL TGF-β3 (Peprotech)). Non-chondrogenic media lacking dexamethasone and TGF-β3 was used as a control. For comparisons, MSCs (2 x10^5 per pellet) from the same passage were centrifuged in conical well-plates at 500xg for 5 minutes and cultured as micromass pellets in identical media conditions. Media was changed 3 times per week for 21 days.

3.7 MTS assay for metabolic activity

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay according to the manufacturer’s protocol. Briefly, samples were washed for 30 minutes in prewarmed PBS and transferred to MTS solution where they were incubated at 37°C for 2 hours while shaking at 150 rpm. MTS supernatants were transferred to a 96-well plate and UV absorbance at 595nm was measured in triplicate on a plate reader (Biotek).

3.8 In situ visualization of microtissue polysaccharide network

For visualization of the polymer network, sChi/oxAlg microtissues were formed using sChi-FITC (5mg/mL in PBS) following the same protocol as above. sChi/oxAlg microtissues containing sChi-FITC were
cultured in chondrogenic media with centrifuged pellets cultured in parallel as control. Samples were taken at days 3, 10, and 20 for histological analysis. Some sChi/oxAlg microtissues made with sChi-FITC were used for live cell imaging in situ. To visualize cells within the polymer network, nuclei were stained with Hoechst 33342 (Life Technologies). For the staining, a stock solution (10µg/mL) of Hoechst was diluted 1:100 in serum-free media. Samples to be stained were washed first in pre-warmed PBS and incubated in the Hoechst solution for 20 minutes at 37°C. They were subsequently washed and imaged under PBS with a laser scanning confocal microscope (LSM 710, Zeiss).

3.9 Explant defect model for cartilage repair assay

Stifle joints of 6-month-old calves were obtained from a local butcher (Metzgerei Angst, Zurich, Switzerland) and dissected to expose the femoral condyles. Biopsy punches of 4 mm diameter (Polymed Medical Center, Giattbrugg, Switzerland) were used to take plugs from cartilage in the trochlear groove. Channel defects were made through the biopsy plugs using a 3 mm diameter punch to cut a central channel through the top 1.5 mm of the plugs. Plug height was cut to 3 mm with a scalpel and explants were fixed to the bottom of wells in 12-well plates with a thin layer of 2% (w/v) agarose (Lonza, Basel, Switzerland). EMT microtissues containing sChi and oxAlg, as well as centrifuged pellets were formed as described above and cultured overnight in chondrogenic media to allow for condensation. The following day, explants were dried by aspiration and EMT or centrifuged pellets were positioned within the defects using fine-tipped forceps. Per defect, 2 EMT microtissues or 3 centrifuged pellets were seeded along the defect length. Attachment of MSCs to the cartilage surfaces was apparent within 30 minutes, likely mediated by β1-integrin binding (190). Explants containing centrifuged pellets were cultured in chondrogenic media while those containing EMT microtissues were cultured in full chondrogenic media or chondrogenic media lacking TGF-β3. Cultures lasted for 28 days changing media three times per week.
3.10 Histological and immunohistochemical analyses

For histological studies, EMT microtissues and centrifuged pellets were washed 3x in PBS, submerged in optimum cutting temperature compound (OCT, VWR), and snap frozen for 5 minutes on dry ice. Samples were sectioned to 6 μm thickness using a cryotome (CryoStar NX70, ThermoScientific). For the explant defect model studies, samples were fixed in 4% formaldehyde overnight at 4 °C, dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin. Sections were cut to 3 μm. Sections were stained with Alcian blue (8GX, Sigma) according to standardized protocol to visualize glycosaminoglycan production. For immunostaining of type I collagen (M-38, Developmental Studies Hybridoma Bank, University of Iowa; dilution of 1:100) and type II collagen (II-II6B3, Developmental Studies Hybridoma Bank, University of Iowa; dilution of 1:20), sections were first washed in PBS, digested with 0.2% (w/v) hyaluronidase (Sigma) for 15 minutes at 37 °C for epitope retrieval, blocked with 5% BSA for 1 hour, and incubated with primary antibody overnight at 4°C. For type X collagen (ab58632, Abcam plc, Cambridge, UK; dilution 1:100) sections were treated the same with the exception of epitope retrieval, which was performed with Digest-All 3 (Life Technologies) for 3 minutes at 37 °C. Sections were then washed and incubated with secondary antibody (IgG goat anti-mouse Alexa Fluor 594, Life Technologies) for 1 hour, washed, and covered by a coverslip with mounting media containing 4’, 6-diamidino-2-phenylindole (DAPI) for nuclear staining (Vector Laboratories).

3.11 RNA isolation and quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Samples were collected (3 per condition) at days 1, 14, and 21, washed with PBS and frozen in liquid nitrogen. Once all samples were collected, RNA was isolated using the NucleoSpin miRNA kit (Macherey-Nagel AG, Oensigen, Switzerland) according to the manufacturer’s instructions with slight modification.
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Frozen samples were suspended in the given lysis buffer and disruption performed with a cell pestle (Sigma). RNA was then precipitated in 70% EtOH, bound to the spin columns, and purified according to manufacturer’s protocol. RNA concentration was quantified using a plate reader (Tek3 plate, Synergy, BioTek Inc) and reverse transcribed using SuperScript III reverse transcriptase (Life Technologies). Primers were purchased from Microsynth AG (Balgach, Switzerland) and cDNA was amplified by real-time reverse transcription polymerase chain reaction (qPCR) on a StepOne Plus instrument (Applied Biosystems) with SYBR Green Fast reagents (Applied Biosystems). Fold change was quantified by the ΔΔCt method. The following primer pairs were designed to overlap adjacent exons to avoid amplification of aberrant genomic DNA: COL2A1 [forward (F), 5’-GGAATTCGGTGTGGACATAGG-3’; reverse (R), 5’-ACTTGGGTCTTTGGGTGG-3’]; ACAN (F, 5’-GAATGGGAACCAGCTAACC-3’; R, 5’-TCTGTACTTTCCCTGTGGCTG-3’), COL10A1 (F, 5’-ATTCTTAGGGCTCAATGTG-3’; R, 5’-GCCTACCTCCATATGCATTTTG-3’). Ribosomal protein L13a (F, 5’-AAGTACCAGGCAGTGACAG; R, 5’-CCTGTTTCCGTCCTCATG-3’) was used as the internal reference for normalization due to its previously reported stability during chondrogenesis (189). Fold increase was normalized to D1 centrifuged pellets in chondrogenic media.

3.12 GAG/DNA quantification

GAG deposition in samples was quantified by the 1,9-dimethylmethylen blue (DMMB) assay. Samples were harvested on days 1, 7, 14, and 21, washed in PBS, and frozen at -80°C until analysis. All samples were analyzed on the same day. Samples were digested in papain (pH 7.6) at 65°C overnight, shaking at 1000 rpm. Chondroitin sulfate dilutions were used as standards and absorbance was measured at 595nm on a plate reader (Synergy, BioTek Inc). Total dsDNA was quantified using the Quant-IT PicoGreen kit (Life Technologies) according to manufacturer’s instructions.
3.13 Statistics

SPSS (SPSS for Windows, Rel 20.0.0. Chicago: SPSS Inc.) was used for all statistical operations. Comparison of results was carried out by multiway analysis of variance (ANOVA) using Tukey’s multiple comparison post hoc test for significance. $P$ values of less than 0.05 were considered statistically significant results.
CHAPTER 4

Schiff base crosslinking for engineered microtissues

Note: Part of this chapter has been published as *Engineered microtissues formed by Schiff base crosslinking restore the chondrogenic potential of aged mesenchymal stem cells*, C. Millan et al., Advanced Healthcare Materials (March 2015).*

*Text and figures were produced by C. Millan unless indicated otherwise and are reprinted with permission from (191).
4. Materials and Methods
4. Schiff base crosslinking for engineered microtissues

A major barrier to adoption of MSC-based cartilage therapies is the reported drop in chondrogenic differentiation capacity with increasing patient age (178, 192, 193), particularly troublesome due to the correlation between increased age and a higher likelihood of cartilage-related pathology (194). The exact mechanism of age-related decline in MSC differentiation potential is not fully understood, but it is believed to be related to lack of telomerase activity in a majority of the cell population (195). The standard method for chondrogenic differentiation in vitro is to culture cells as micromass pellets that resemble the condensation and are formed either by centrifugation (166, 167) or hanging drop culture (168, 169) in media containing TGF-β3. These methods, however, often produce densely compacted structures that inhibit efficient transport of induction factors and other nutrients to the core of the pellet limiting production of cartilage matrix to the pellet periphery (174-178). This limitation is amplified for aged MSCs that are known to express fewer receptors for growth factors (GFs) involved in differentiation (179, 180), indicative of a cell population with a reduced sensitivity for directed differentiation. Moreover, the fact that micromass pellets require culture media containing TGF-β3 necessitates an extensive in vitro culture period which significantly delays the time at which cells could be implanted to treat an injured patient.

The aim of this study was to engineer a method for recapitulating the MSC condensation with the capability of consistently inducing chondrogenic differentiation for a wide range of patient age groups. To achieve this, modified polysaccharides were utilized for organizing MSCs into high-density aggregates, or microtissues, by a rapid Schiff base crosslinking reaction. These polysaccharides share structural similarities to those involved in cartilage development which are important for allowing MSCs to deposit the tissue matrix during morphogenesis. We hypothesized that the highly hydrophilic 3D polysaccharide network would facilitate homogeneous differentiation of densely packed cells within the engineered microtissues (EMTs). Additionally, we demonstrated that TGF-β3 could be simultaneously encapsulated within the polysaccharide network during microtissue formation and its presence was sufficient to induce
4. Schiff base crosslinking for engineered microtissues

chondrogenesis of co-encapsulated MSCs. These features of the engineered condensation highlight their potential to be implanted in an injury on the same day as cell harvesting, providing the MSCs with cues necessary to direct their chondrogenic differentiation \textit{in situ}. We showed the significant superiority of these engineered condensations over centrifuged micromass pellets in their ability to induce chondrogenesis of aged MSCs that failed to differentiate using conventional culture methods. Finally, we demonstrated the clinical significance of this approach for treating defects in an \textit{in vitro} cartilage repair assay.
4.1 Characterization of modified polysaccharides

An advantage of this engineered system is the utility of polysaccharides that share similarities in chemical structure with the GAGs present in articular cartilage. Chitosan, hyaluronic acid (HA), chondroitin sulfate (CS), and alginate (alg) were chemically modified to undergo Schiff base crosslinking in physiological conditions.

4.1.1 N-succinyl chitosan

Unmodified chitosan is insoluble at physiological pH, but partial succinylation serves to disrupt crystallinity domains that cause precipitation and permits solubility at pH 7.4 (183-185). Following succinylation of chitosan, it was necessary to determine the number of primary amines that remained in sChi for Schiff base crosslinking purposes. The degree of amino substitution was 34% as calculated from $^1$H-NMR spectra and was sufficient for preparing sChi solutions in PBS at pH 7.4 (Figure 4.1B).

\[ \text{Degree of succinylation was } 34\% \] as calculated from the integral area ratio of the succinyl peak ($\#$, $\delta = 2.28-2.33$ ppm) and glucosamine peak ($^*$ = 2.70 ppm) in the spectra.

**Figure 4.1 | N-succinyl chitosan.** (A) Chemical structure and (B) $^1$H-NMR spectra of N-succinyl chitosan (sChi). Degree of succinylation was 34% as calculated from the integral area ratio of the succinyl peak ($\#$, $\delta = 2.28-2.33$ ppm) and glucosamine peak ($^*$ = 2.70 ppm) in the spectra.
4.2.2 Oxidized HA, CS, and Alginate

HA, CS, and Alg were oxidized according to Köwitsch et al. (186) using sodium periodate (NaIO₄) at a theoretical 200% molar oxidation for complete cleavage of vicinal hydroxyl groups in the sugar backbone. Successful oxidation was verified by observing distinct absorption peaks indicating aldehyde carbonyl stretching vibration ($\nu_{C=O}$) in the IR spectra at 1735 cm⁻¹ (Figure 4.2), in agreement with previously reported values (142-144). Absorption due to sulfate vibration ($\nu_{SO_4}$) was seen in CS at 1230 cm⁻¹, and was unaffected by oxidation.

![Figure 4.2](image)

**Figure 4.2 | Oxidized polysaccharides.** (A) Oxidized hyaluronic acid (oxHA). (B) Oxidized chondroitin-6-sulfate (oxCS). (C) Oxidized alginate (oxAlg, only mannanuronic block shown). (D) Oxidation of vicinal hydroxyls was confirmed by ATR-FTIR where each oxidized polysaccharide exhibited carbonyl stretching ($\nu_{C=O}$) at 1735 cm⁻¹ (arrows) typical of aldehydes.
4.3 Quartz Crystal Microbalance with Dissipation Monitoring

To assess the binding interaction of the modified polysaccharides, quartz crystal microbalance with dissipation monitoring (QCM-D) was used. In QCM-D measurements, an electric potential is applied to a gold-coated quartz crystal causing the crystal to oscillate at its measured resonance frequency. The crystal is mounted in a flow cell and polymer solutions are passed through the flow cell. Drops in its oscillation frequency due to material deposition onto the surface can be monitored and used to calculate film mass (187, 188). The formation of polysaccharide multilayers was tested by alternate deposition of sChi and the oxidized polysaccharides (oxPS). Unmodified polysaccharides (PS: HA, CS, and Alg) were used as basis for comparison.

Figure 4.3 | Quartz crystal microbalance with dissipation monitoring (QCM-D). (A) Interaction between sChi and oxidized or unmodified polysaccharides (PS) was measured with quartz crystal microbalance with dissipation (QCM-D) and intermittent washing steps (W). Changes in oscillation frequency signify mass deposition on the crystal surface. Plotted here are mean frequency shifts (n=3) of the third overtone. The stability of the final film formation was tested under serum-free media for 4 hours. Asterisk of corresponding PS color indicates p < 0.05 between ox and unmodified PS. (B) Adsorbed mass after each layer deposition (indicated by subscript) was calculated from QCMD data using the Sauerbrey relation for rigid thin films. Values for oxPS were compared to the corresponding unmodified PS and statistical significance (*) is indicated for p < 0.05.
4. Schiff base crosslinking for engineered microtissues

Initial adsorption of the first sChi layer to the QCM-D sensor reproducibly resulted in an oscillation frequency shift of ~110 Hz (Figure 4.3A). Only oxidized polysaccharides induced further build-up in subsequent incubation steps. For unmodified polysaccharides (PS), incubation steps did not result in additional changes in resonance frequency after the initial sChi deposition. The resulting sChi/oxPS bilayer appeared stable with further washing and incubation under cell culture media, but additional deposition steps were unsuccessful under the tested conditions. Mass calculations using the Sauerbrey model for the third resonance frequency indicated that the initial sChi layer on the sensor was approximately 700 ng (Figure 4.3B). The oxPS bilayers all behaved similarly and resulted in nearly twice the amount of mass deposition than their unmodified PS counterparts (p < 0.05), resulting in final bilayer masses of roughly 1300 ng. A Schiff base, or imine bond, is formed between the primary amine groups in sChi and aldehyde groups in oxidized polysaccharides (oxPS) by spontaneous reaction when mixed in solution (Figure 4.3C). The byproduct of this reaction is water. These results imply that, under physiological conditions in PBS, the interactions between sChi and oxPS are due to covalent Schiff base crosslinking.

4.4 Microtissues made with sChi/oxAlg induce an enhanced chondrogenic phenotype in differentiated MSCs

Human bone marrow mesenchymal stem cells (MSCs) rapidly formed condensation-like 3D microtissues as the result of Schiff base crosslinking between modified polysaccharides. Cells were suspended at 20 x 10^6 cells/mL in N-succinyl chitosan (sChi) and 10 µL of the mixture was subsequently mixed with a 5 µL droplet of either oxidized hyaluronic acid (oxHA), oxidized chondroitin sulfate (oxCS), or oxidized alginate (oxAlg) (Figure 4.4A). Within 25 minutes of mixing, Schiff base crosslinking between the primary amine groups present in sChi and the free aldehyde groups in the oxidized molecules resulted in the entrapment of cells within a polysaccharide network (Figure 4.4E). Engineered microtissues
4.4 Microtissues made with sChi/oxAlg induce an enhanced chondrogenic phenotype in differentiated MSCs

Figure 4.4 | Schiff base crosslinking for engineered MSC condensations. (A) Engineered condensations were made by suspending cells in sChi at 2x10⁶ cells/mL and mixing a 10µL droplet of the cell suspension with 5µL of oxHA, oxCS, or oxAlg. Schiff base crosslinking between primary amines in sChi and the free aldehyde groups in the oxidized polysaccharides (inlet) rapidly assembled the cells into 3D microtissues. (B) The standard method for mimicking MSC condensations in vitro is by centrifugation of cells into micromass pellets. The same number of cells/pellet were used as in engineered condensations to serve as the basis for comparison. (C) Photograph depicting an engineered condensation immediately after crosslinking. (D) Photograph of a centrifuged micromass pellet in a conical tube. (E) Representative engineered condensation 30 minutes after seeding depicted by maximum intensity projection through a Z-stack taken with a confocal microscope. FITC-labeled sChi was used to visualize the polysaccharide network and cell nuclei were labeled with Hoechst. (F) Gross morphology of pellets (Centrif.) and engineered microtissues at day 1 and day 21 shown by brightfield microscopy (top). Macro lens photograph shows micromass pellets and microtissues of each type after 21 days culture in chondrogenic media (bottom). [Scale bars: (E) 250µm and (F) 500µm in brightfield microscopy images and 2mm in photograph.]

*Parts C and D of this figure do not appear in the published paper.*
(EMTs) and pellets formed by centrifugation (Figure 4.4B) were cultured in chondrogenic induction media containing TGF-β3 for 21 days. Gross morphology of samples was observed via brightfield microscopy and macro-lens photography (Figure 4.4F). After 21 days, all sample types grew in diameter and shared characteristic cartilage features appearing opaque and white with shiny surfaces (Figure 4.4F, bottom).

Chondrogenic induction was quantified by the dimethylmethylene blue assay (DMMB), which was used to assess the amount of sulfated glycosaminoglycans (sGAG) produced. DMMB values were normalized to the amount of DNA measured per sample during culture of MSCs in centrifuged pellets and in engineered condensations made with sChi and either oxHA, oxCS, or oxAlg. By day 21, MSCs cultured in microtissues formed with sChi and oxAlg had synthesized 4x more sGAG per cell than MSCs cultured in centrifuged pellets (Figure 4.5B; oxAlg, 189.5 µg sGAG/µg DNA vs. Centrif., 47.1 µg sGAG/µg DNA, p < 0.001) and more than double the amount synthesized in oxHA microtissues (81.3 µg sGAG/µg DNA, p < 0.01). Levels of dsDNA quantified by PicoGreen remained constant for all samples during the 3

**Figure 4.5 | Enhanced chondrogenesis in microtissues made with sChi and oxAlg.** (A) MTS assay for cell viability in micromass pellets (Centrif.) and microtissues made with oxHA, oxCS, or oxAlg showed slightly elevated levels for oxAlg microtissues, though this difference was not statistically significant (D1, Centrif. vs. oxAlg, p = .054). (B) Quantification of sulfated glycosaminoglycans (sGAG) by DMMB assay normalized to DNA content showed chondrogenic induction in all groups by day 21. At day 21, MSCs cultured in oxAlg microtissues synthesized more than 4x the amount of cartilage-specific matrix than the same cells cultured in centrifuged pellets. (C) PicoGreen assay was used to measure dsDNA which yielded no statistically significant differences among sample types over time. This indicates that the increase in sGAG from part B was not due to higher cell numbers or proliferation. (Plotted are mean values +/- SD; n=5 donors ranging in age from 42 to 74). (*p < 0.01, **p < 0.001).
4.4 Microtissues made with sChi/oxAlg induce an enhanced chondrogenic phenotype in differentiated MSCs

weeks in culture and no statistically significant differences were measured (Figure 4.5C). MTS assay indicated a slightly higher activity of cells in microtissues made with sChi and oxAlg when compared with other sample types, though these differences were not statistically significant (Figure 4.5A, Day 1, Centrif. vs. oxAlg, p = 0.054).

In oxAlg microtissues, sGAG produced by MSCs was 2.3x more than in those made with oxHA (Figure 4.5B, p = 0.005) and 1.6x more than oxCS (Figure 4.5B, p = 0.178). The diminished chondrogenic response in the presence of HA was unexpected considering reports of improved MSC chondrogenesis in HA-based hydrogels (125, 127, 196, 197). However, looking further into the specific role that HA plays during condensation may explain the reduced chondrogenic induction potential observed here. While small amounts of HA are needed to facilitate condensation (198), an excess of HA has been shown to inhibit cell aggregation (199). Higher HA concentrations possibly lead to outcompeting with endogenously produced HA for CD44 binding sites on MSC surfaces (200), which would hinder chondrogenesis.

It is also possible that oxAlg EMTs yielded better chondrogenesis due to increased crosslinking between sChi and oxAlg compared to sChi with either oxHA or oxCS. In polysaccharide modifications, HA, CS, and Alg were maximally oxidized with a 2x molar ratio of NaIO₄. As disaccharide polymers, only one of the saccharide units in HA and CS are susceptible to NaIO₄ oxidation compared to alginate, which can be oxidized at each saccharide group (Figure 4.2C). The degree of oxidation is correlated with swelling, crosslinking density, and degradation rates following Schiff base polymerization of polysaccharides (140, 143, 144), all of which could in turn impact the mechanical properties of the engineered condensations and the behavioral changes of encapsulated cells. The concentration (w/v) of the various oxPS was selected to remain constant in these studies and sChi with oxAlg was the best combination for promoting the highest levels of MSC chondrogenesis. Following these results, microtissues formed by sChi and oxAlg
were termed engineered microtissues (EMTs) and selected for comparison with the standard controls of centrifuged pellets and further development of the engineered condensation method.

Histology was used to detect the deposition of cartilage-specific ECM within EMTs and pellets made by centrifugation (Figure 4.6A). After 21 days of culture in chondrogenic media, MSCs in both centrifuged pellets and in EMTs exhibited homogeneous deposition of sGAG as visualized by Alcian blue staining. However, immunohistochemical staining for type II collagen revealed a striking difference in type II collagen deposition. Deposition of type II collagen, an important marker of cartilage, was limited to the periphery of pellets but was homogeneously distributed over the microtissue structure in EMTs. In both culture conditions, type I and type X collagen staining was visualized as punctate depositions within the tissues, though to a much lesser extent than type II collagen. Image J was used to quantify the percentage of the tissue cross sectional area that stained positively for type II collagen. On average, EMTs exhibited 80% homogeneity of type II collagen deposition versus less than 25% for the centrifuged pellets (Figure 4.6B, p < 0.001). Furthermore, evaluation of cartilage matrix deposition over time by Alcian blue staining revealed that MSCs in pellets begin first synthesizing sGAG at the outer surface, while in EMTs the sGAG is first detected at the microtissue core and the staining is found to propagate outward over time (Figure 4.6C).
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Figure 4.6 | Homogeneous cartilage ECM deposition in EMTs.* (A) Alcian blue staining for glycosaminoglycans (GAGs) indicated homogeneous deposition throughout centrifuged pellets and engineered microtissues (EMTs). In EMTs, type II collagen was also synthesized throughout the microtissue but was restricted to the periphery of the tissue in the centrifuged pellets. Type I collagen, a marker for fibroblastic differentiation, and type X collagen, a marker of hypertrophy, were only present in very small quantities. (B) The percentage of cross-sectional area that stained positively for type II collagen indicated over 80% homogeneity in EMTs versus just 23% in centrifuged pellets (Mean +/- SD, n = 5 donors ranging in age from 42 to 74, 3 sections per donor). (C) Alcian blue staining of centrifuged pellets and EMTs over time indicated that cartilage matrix production is initiated at the periphery of centrifuged pellets and propagates inward with time. In EMTs, chondrogenesis first occurs at the microtissue core and cartilage matrix staining propagates outward with time. (Histology images shown are from a single MSC donor, age 50) (**p < 0.001.) (Scale bars = 500 µm.)

*Histological processing and imaging was performed by E. Cavalli.
A critical advance demonstrated in this study was an improved homogeneity in chondroinduction of the densely packed cells, likely a result of the hydrophilic polysaccharide network present throughout the engineered microtissue. Studying the deposition of cartilage matrix over time, it was observed that chondrogenesis is actually initiated at the core of EMTs indicating better access for those cells to chondrogenic induction factors compared with pellets which begin initial cartilage matrix production at their periphery (Figure 4.6C). By day 21, type II collagen deposition was significantly and more homogeneously distributed throughout the cross-sectional area in EMTs than in pellets (Figure 4.6B, EMT 82.3% vs. Centrif. 20%, p = 0.0005). The increased efficiency of chondrogenesis in EMTs was further apparent in DMMB data that was normalized to dsDNA content where there was a 4-fold increase of sGAG/DNA seeded in EMTs over centrifuged pellets (Figure 4.5B, D21, p = 0.00009). This visual and quantitative evidence taken together points to an improved efficiency of chondroinduction on a per cell basis within the engineered microtissues.

4.5 Engineered microtissues rescue chondrogenic potential of aged MSCs

MSCs from healthy donors were classified into groups according to donor age. Donors above 70 years old were considered ‘aged’ and donors younger than 55 years old were classified as ‘young’ (n=6, 3 males and 3 females for each group). MSCs from these two age ranges were cultured as centrifuged pellets or EMTs in chondrogenic media for 21 days. Quantitative data measuring MSC chondrogenesis indicated a clear age-related difference between the two donor groups, both in normalized sGAG production and expression of type II collagen. In centrifuged pellets of young donors there was a nearly 7-fold increase in sGAG synthesis compared to the pellets from aged donors (Figure 4.7A, Centrif.; young, 139.3 µg sGAG/µg DNA vs. aged, 20.4 µg sGAG/µg DNA, p < 0.01). A similar trend was observed for cells cultured in EMTs that showed a 2-fold increase of sGAG production for young donors over aged donors (Figure 4.7A, EMT; young, 234.9 µg sGAG/µg DNA vs. aged, 123.6 µg sGAG/µg DNA, p < 0.05). The age
Engineered microtissues rescue chondrogenic potential of aged MSCs

Figure 4.7 | Chondrogenic rescue of aged MSC donors (quantitative). MSC donors were classified in groups by age where Aged donors were >70 y.o. and Young donors were <50 y.o. (A) Chondrogenic induction quantified by DMMB normalized to DNA content indicated that there was a statistical difference between Aged and Young donors in terms of sGAG production regardless of culture condition. MSCs in sChi/oxAlg microtissues produced more cartilage-specific matrix than the same donors in centrifuged pellets within both age categories. There was no difference between Young donors cultured in centrifuged pellets versus Aged donors cultured in sChi/oxAlg tissues indicating a rescue of their chondrogenic potential in sChi/oxAlg EMTs. (B) Type II collagen gene expression followed the same trend as DMMB. (C) Aggrecan and (D) type X collagen expression was up-regulated in all culture conditions, though there were no significant differences between experimental groups. (A-D are means +/- SD for n =6 per age group, 3 males and 3 females.) (B-D Fold change was compared to matched donors harvested on D1 and gene expression was normalized to the RPL13a reference gene.) (*p < 0.05, **p < 0.01, ***p < 0.001)
dependent trend was also observed in qPCR measurements of type II collagen expression where fold change was elevated to 5x and 1.9x in young donors compared to aged donors for micromass pellet culture and EMTs, respectively (Figure 4.7B, Centrif. p < 0.05, EMT p < 0.01). For all samples there was an up-regulation in expression of aggregan (Figure 4.7C) and in type X collagen (Figure 4.7D) after 21 days in culture, though no statistically significant differences were observed between age groups or culture conditions. These results confirm literature reports of a loss in the ability of MSCs to undergo directed differentiation with increasing age (193). These gene expression results were focused specifically on well-established markers of chondrogenesis and hypertrophy, but other tools are available that would provide a better overall picture of other gene expression changes that might be effected during EMT culture. Microarray analyses can be performed to highlight molecular differences between MSCs induced under pellet or EMT culture similar to studies done by Mauck and Burdick to compare patient matched chondrocytes and MSCs (26).

Demonstrating the chondrogenic rescue potential of EMT culture was the fact that EMTs from aged MSCs had similar sGAG production (Figure 4.7A, Centrif. young vs. EMT aged, p = 0.951) and type II collagen expression (Figure 4.7B, Centrif. young vs. EMT, aged, p = 0.982) as micromass pellets from young donors. The biochemical assay and gene expression data was corroborated by histology and immunostaining which highlighted the loss of chondrogenic potential with age in centrifuged pellet culture, as well as the recovery in chondrogenic potential for aged donors cultured in EMTs. Alcian blue staining of centrifuged pellet MSCs from donors younger than 45 years old showed homogeneous sGAG deposition (Figure 4.8), while the same staining for pellets from aged donors was greatly diminished (Figure 4.8). For EMTs, Alcian blue stained sections showed homogeneous sGAG deposition for both young and aged donors (Figure 4.8A, EMT). Immunostaining for type II collagen revealed peripheral matrix synthesis in micromass pellets from young donors but was markedly absent in pellets from aged donors (Figure 4.8B, Centrif.). Type II collagen was homogeneously distributed throughout EMTs from young donors (Figure 4.8B, EMT) and, though
more heterogeneous, it was also clearly present in EMTs from aged donors (Figure 4.8B). The effect of providing encapsulated cells with better access to chondrogenic stimuli by the polysaccharide network in EMTs likely explains their ability to rescue the chondrogenic potential of aged MSCs that failed to differentiate in centrifuged pellets.

Further characterization of EMT components may lead to better understanding of this enhanced differentiation effect. MSC chondrogenesis is known to depend heavily on the cell seeding density (or, in other words, the intercellular spacing) and on the macromer content of the hydrogel material (201). These parameters could be adjusted in the EMT approach to elucidate the specific role that each play in the differentiated phenotype of seeded cells. The macromer content of sChi and oxAlg could be altered by adjusting their concentrations. It would be important to also control overall crosslinking density between sChi and oxAlg. One approach for doing so is by trinitrobenzene sulphonic acid (TNBS) assay which produces a concentration dependent spectrophotometric readout specific for Schiff base crosslinking degree (144). The degree of substitution in sChi and the degree of oxidation of oxAlg should be altered to determine conditions where changes in their macromer content during EMT formation would not result in different degrees of crosslinking. Interestingly, one study found that lower initial macromer content permits better diffusion of endogenous cartilage matrix from encapsulated cells and ultimately results in tissue constructs with increased mechanical properties (201). The other parameter discussed, intercellular spacing, is one that is easy to alter simply by adjusting the number of cells seeded while maintaining the sChi and oxAlg concentrations and volumes constant. It is known that cell-cell contact initiates chondrogenesis in the developing limb and is critical during in vitro chondrogenesis. However, tight packing of cells in dense spheroid structure limits diffusion of nutrients through the sample. Determining the maximum number of cells that permit enhanced chondrogenesis without resulting in core necrosis due to diffusion constraints would be ideal. Cell spacing could be quantified using a combination of 2-photon microscopy and image analysis (histology is not possible due to processing artifacts).
4. Schiff base crosslinking for engineered microtissues

Figure 4.8 | Chondrogenic rescue of aged MSC donors (visual). (A) Alcian blue histological staining corroborated the DMMB data. Aged donorMSCs in centrifuged pellets did not stain positively for sGAG (Centrif., 74 y.o.), but did produce cartilage matrix when cultured in oxAlg EMTs. (B) Type II collagen immunostaining confirmed gene expression results and followed the same trend seen in Alcian blue staining. (Scale bars = 500μm.) *Note: In the published version of this figure, the 64-year-old donor does not appear in order to remain consistent with the two donor age groups. It is included here so the reader can appreciate the age-related trend.

4.6  TGF-β3 loading and release in engineered condensations

To investigate whether the enhanced chondrogenic effect in EMTs was dependent on TGF-β3, EMTs cultured in chondrogenic media with or without the growth factor (GF) were compared after 21 days. DMMB and type II collagen expression were negligible in EMT samples cultured without TGF-β3 (Figure 4.9 A and B, p < 0.001) and the lack of MSC chondrogenesis without GF was confirmed by histology (Figure 4.9C). We then postulated that the TGF-β3 could be preloaded into the polysaccharide matrix at the time of EMT microtissue formation (EMT+TGF). To test this hypothesis, TGF-β3 was added to the sChi solution prior to cell resuspension and subsequently encapsulated in the biopolymer network at the time of seeding sChi/oxAlg EMTs (Figure 4.4A, schematic). EMT+TGF were cultured in media lacking the growth factor.
Figure 4.9 | TGF-β3 loading and release (quantitative). (A) DMMB and (B) type II collagen expression measurements for cartilage matrix production in EMTs cultured in media with (+TGF) and without (-TGF) growth factor. (C) Histology confirmed that without (-TGF) growth factor, chondrogenesis is halted demonstrating the need for TGF-β3 in EMT culture. (D) ELISA was used to monitor TGF release over time in microtissues that were loaded with 25 ng, 50ng, or 75 ng TGF-β3. Data was normalized by the highest value of TGF-β3 released in the 75ng condition. Plotted are percentage means +/- SEM (n = 3). (E) DMMB results from microtissues that were loaded with 25, 50, or 75 ng of TGFβ3 at the time of seeding indicated the ability of released TGF to drive chondrogenesis to levels comparable to unloaded EMTs cultured in normal chondrogenic media (EMT). (F) The trend was similar in type II collagen expression with the exception of the 25ng dose, in which expression was reduced. (A-B and E-F are means +/- SD for n = 5 donors ranging in age from 42 to 74.) (*)p < 0.05, (**) p < 0.01.

and the media was changed completely every day for the first week. Release of the GF from EMT+TGF samples was measured by ELISA for a period of 21 days from microtissues seeded with either 25ng, 50ng, or 75ng TGF-β3 per microtissue where a dose response was evident (Figure 4.9D).

Chondrogenic differentiation in EMT+TGF microtissues was monitored by DMMB assay and type II collagen gene expression and compared with unloaded controls cultured in media containing TGF-β3. EMT+TGF loaded with either 50ng or 75ng of TGF-β3 had higher amounts of deposited sGAG and showed more type II collagen expression than either the control group (unloaded EMTs cultured in full chondrogenic media) or samples loaded with only 25ng (p < 0.05). This dose response was confirmed in
4. Schiff base crosslinking for engineered microtissues

histology, which showed samples loaded with only 25ng TGF-β3 have diminished sGAG deposition and type II collagen in the ECM. Samples loaded with 50ng or 75ng did not differ from one another and appeared similar to samples of unloaded EMTs cultured in full chondrogenic media (p > 0.95). These data suggests that for EMTs composed of 200k MSCs, 50ng loading with TGF-β3 is sufficient to supply enough of the GF to the cells for driving chondrogenic differentiation over 3 weeks. To confirm TGF-β3 specific activity in these experiments, further studies could be performed where downstream parts of the signaling cascade are blocked during studies with EMT+TGF. Candidates for such blocking studies include the mitogen-activated protein (MAP) kinases p38, extracellular signal-regulated kinase-1 (ERK-1), and c-Jun N-terminal kinase (JNK). Chemical inhibitors for these enzymes are available commercially and have been shown to inhibit TGF-β3 specific chondroinduction in vitro (202). In addition to these MAP kinases, the signal transduction molecules known as Smads are also involved in TGF-β signaling where they translocate to the nucleus upon TGF-β binding its cell surface receptors (203). Investigating their role during chondrogenesis in EMT+TGF could be done by using MSCs transfected with dominant negative Smad2, Smad3, and Smad4 constructs which would inhibit TGF-β mediated chondrogenesis (204), or overexpression of these molecules which would likely potentiate the TGF-induced chondrogenic effects (205). Alternatively, the Smad signaling cascade could be verified by western blot for phosphorylated Smad 2/3 (206).

Important towards achieving our goal of clinical translation of EMTs as a therapy to treat cartilage injuries was to ensure their capability for inducing chondrogenic differentiation without media supplementation of TGF-β3. A major shortcoming of using micromass pellets to generate de novo cartilage for defect repair is the necessitation for extensive in vitro manipulation. While this has been shown to be effective for generating cartilage in in vitro models (173), TGF-β3 is needed as a media supplement during culture. A bolus injection of the GF to the joint capsule would likely not suffice due to its short half-life in vivo (207). Here, we demonstrated that TGF-β3 can be loaded within the polysaccharide network during
EMT formation which was sufficient to drive chondrogenesis in serum-free media that was not supplemented with TGF-β3.

![Figure 4.10](image)

**Figure 4.10 | TGF-β3 loading and release (visual).** Alcian blue and type II collagen immunohistochemistry of EMT¹TGF loaded with 25ng, 50ng, or 75ng TGF-β3. EMT¹TGF were cultured for 21 days in serum-free media that was not supplemented with growth factor. Histology confirmed that 25ng was not sufficient for inducing homogeneous cartilage matrix deposition, but there was little difference between loading with 50ng or 75 ng. (Scale bars = 500 μm.)

Diffusion based release of TGF-β3 loaded at 25, 50, or 75ng per EMT¹TGF was quantified by ELISA, which followed first-order kinetics (Figure 4.9D). It is also possible that some TGF-β3 is covalently immobilized in the polysaccharide network via Schiff base reaction between its lysine amino acids and unreacted free aldehydes present in oxAlg, where it would continue to remain biologically active for stimulation of differentiation (208). In an attempt to visualize immobilized TGF-β3, simplified experiments were performed where thin films of sChi and oxAlg were deposited on a glass surface in layer-by-layer (LbL) fashion. TGF-β3 was either solubilized in the solution of sChi or oxAlg prior to thin film formation, or a solution of TGF-β3 was incubated on top of the thin film after formation. Immunohistochemical staining...
for the TGF-β3 was done and visualized as punctate aggregates throughout the sChi/oxAlg thin film (Figure 4.11). However, these experiments were inconclusive as control groups that were stained without primary antibody showed a similar deposition pattern (Figure 4.11D).

Figure 4.11 | Immunohistochemical staining of TGF-β3 on LbL films of sChi/oxAlg. TGF-β3 appeared as punctate and aggregated stainings throughout the LbL films regardless of whether it was solubilized in (A) sChi, (B) oxAlg, or (C) incubated in solution on top of the films following formation. (D) Immunostaining controls that were performed omitting the primary antibody against TGF-β3 gave similar results. (Scale bars = 200 μm.) *These experiments and figure were done by E. Cavalli.

In studies of GF release from TGF-β3 loaded EMTs measured by ELISA, release of TGF-β3 was complete by day 5 for all doses. An early burst release of TGF-β3 is ideal according to others who have shown transient exposure for only the first week of culture to be sufficient for chondroinduction (157), and short-term exposure followed by longer culture periods in the absence of TGF-β3 was even beneficial for the mechanical properties of the produced cartilage (160). In cell experiments, it was determined that 50ng of TGF-β3 per EMT was sufficient to reach the threshold necessary to induce MSC chondrogenesis in comparing DMMB and type II collagen expression with control microtissues that were not loaded and cultured in full chondrogenic media (Figure 4.9 E and F). These results demonstrate a coupling in the EMT approach of key biophysical cues pertaining to high density and better perfused condensations with the
critical biochemical cue of exposure to TGF-β3 equipping encapsulated cells with the chondrogenic stimuli capable of directing in situ differentiation.

4.7 Cartilage repair by engineered condensations in an in vitro defect model

The translational potential of EMTs was assessed utilizing a cartilage repair assay in bovine explant tissue similarly to an approach used by others (82, 173). A 4mm diameter biopsy punch was used to take cartilage plugs from the trochlear groove of bovine femurs and defects were made using a 1.5mm punch through the center of the plugs in the articulating surface. MSCs from aged donors were used to make EMTs or centrifuged pellets which were implanted into the defects and subsequently cultured in full chondrogenic media for 4 weeks. In gross appearance, EMTs grew within the cartilage defects while centrifuged pellets did not (Figure 4.12B, Centrif. vs EMT). Histology and immunohistochemistry revealed elevated synthesis of cartilage matrix in EMTs. Centrifuged pellets yielded minimal deposition of sGAG and there was a complete absence of staining for type II collagen, the primary phenotypic marker of cartilage (Figure 4.12C). Using parameters judged to be ideal from earlier microtissue studies, EMTs loaded with 50ng of TGF-β3 were seeded in cartilage defects and cultured in serum-free media that was not supplemented with the growth factor. EMT+TGF were induced to produce sGAG and type II collagen containing de novo tissue within the cartilage defects (Figure 4.12C). The ability for EMT+TGF to induce chondrodifferentiation within a cartilage defect eliminates the need for prolonged in vitro culture in defined media for a clinical application involving this approach.
4. Schiff base crosslinking for engineered microtissues

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**Figure 4.12** | **EMT cartilage matrix production in a cartilage defect explant model.** (A) Aged MSCs were used to form centrifuged pellets (Centrif.), EMTs, or EMT+TGF and implanted in defects of bovine cartilage explants. Centrif. pellets and EMTs were cultured in chondrogenic media supplemented with TGF-β3, while EMT+TGF were cultured in media lacking the growth factor. (B) Photographs show growth of *de novo* tissue within the defects. (C) Alcian blue (top) and type II collagen (bottom, counterstained with DAPI) stained sections of the filled defects revealed production of tissue containing sGAG and type II collagen within EMTs and EMT+TGF. In centrifuged pellets, cartilage matrix deposition was limited. (B and C: Histology and images shown are from a single MSC donor, age 74) (Scale bars, B: 2 mm, C: 0.75 mm, inlets: 100 µm)
4.8 Chapter Summary

Taken together, the results presented here outline a systematic approach to engineering a biomimetic environment for directing stem cell differentiation that offers a major advancement over current methods. We utilized a network of modified polysaccharides that polymerized via Schiff base crosslinking in the presence of MSCs and TGF-β3 that served 3 purposes: 1) to induce rapid accumulation of cells into condensation-like aggregates, 2) to improve homogeneous differentiation of cells within the microtissues structure, and 3) to deliver TGF-β3 to encapsulated cells for driving chondrogenesis. This approach rescued the chondrogenic potential of MSCs from aged donors that showed reduced sensitivity to induction factors in conventional pellet cultures. In demonstrating the effectiveness of EMTs loaded with TGF-β3 for driving cartilage matrix production within cartilage defects in vitro, we showed the translational potential of this cartilage repair strategy. This engineering method has far-reaching applications beyond the field of cartilage regeneration and we foresee broad applications for improving cell-based therapies to treat patients in a rapidly aging population (209).
CHAPTER 5

Schiff base crosslinking for cell-tissue adhesion and controlled assembly

Note: Part of this chapter has been published in Engineering Cellular Assembly for Applications in Regenerative Medicine, C. Millan & M. Zenobi-Wong, Engineered Cell Manipulation for Biomedical Application, (Springer), (2014), 131-145.*

*Text and figures were produced by C. Millan unless indicated otherwise and are reprinted with permission from (210).
5. Schiff base crosslinking for cell-tissue adhesion & controlled assembly

A majority of the methods in widespread use for controlling cell assembly rely on self-organization of the cells, a process which is slow and time consuming, exhibits poor control over size and shape of the microtissue construct, and can vary widely among biological donors (211). To overcome these pitfalls, tools are rapidly emerging for engineering the assembly process of cells into 3D structures that offer control over microtissue formation featuring various degrees of spatial resolution of the microtissue components (212). Schiff base crosslinks form spontaneously and immediately upon interaction of primary amines with aldehydes or ketones (128). This reaction can be carried out in an aqueous environment under physiological conditions, which make it an attractive choice for use with cells and tissues. Due to the rapid rate of crosslinking and favorable reaction conditions, Schiff base crosslinking can be utilized for selectively controlling cell adhesion to neighboring cells in engineered microtissues. Cell populations were labeled with fluorescent tracking molecules and various co-culture conformations were achieved as a result of sequential pipetting. Microscopy confirmed localization of the different cell populations to specific regions within single microtissues. Additionally, tissue surfaces can be rendered Schiff base reactive and the same crosslinking chemistry can be utilized for faster cell-tissue adhesion and for improving localization of cells to specific regions on a tissue surface. Cell-tissue adhesion was quantified using image analysis where it was shown that the number of attached cells depends on the duration of exposure and strength of the oxidizing agent used to activate the tissue surface.

5.1 Engineering cell surfaces for directed adhesion

One approach for control over cell-cell adhesion is to modify the properties of the cell surface. Researchers at UC Berkeley have recently demonstrated the directed assembly of different cell types by coupling complementary single-stranded DNA (ssDNA) to the surfaces of separate cell populations (213). Hybridization of the ssDNA of one cell type occurs with its complementary strand on the surface of a second cell type forcing the aggregation of cells in a controlled 3D co-culture. By adjusting the ratio of the
two cell types to one another, the cell type in excess formed rosettes surrounding the less abundant cell type. A microtissue dependent upon paracrine signaling between two different cell types for survival was successfully constructed between IL-3 expressing CHO cells as the microtissue ‘nucleus’ and IL-3 dependent hematopoietic FL5.12 cells as the rosettes. In media containing anti-IL-3, only microtissues with both cell types remained viable.

Nishiguchi and co-workers have developed a different cell surface modification involving ligand-receptor binding (214). Cells were coated with nanofilms of fibronectin and gelatin (FN and G, resp.), two proteins that have binding domains for each other, in a layer-by-layer (LbL) manner by alternate incubation and washing in solutions of the two proteins. Integrin proteins of one cell can therefore bind the fibronectin present in the nanofilm coating of a neighboring cell. FN-G coated cells can then be seeded in high density where they form a compact 3D microtissue structure in a process called the cell accumulation technique (See Appendix 1 for adaptation of this technique to influence chondrogenic differentiation). By controlling the number of cells seeded, microtissues of defined thickness can be constructed, and by alternating the sequence of cell types seeded in this way, microtissues can be fabricated with defined zones for each cell type. For example, a layer of HUVEC cells was sandwiched between 2x 4-layer thick zones of fibroblast cells resulting in a microtissue bisected with a tubular network that mimicked vascularization.

5.2 Engineered microtissues for structurally controlled 3D co-culture

The majority of tissues in the body are composed of different cell types whose interaction with one another is fundamental for the tissue’s function. Co-culture studies offer researchers the ability to probe the relationship between different types of cells. For example, culturing MSCs and chondrocytes in co-cultures of centrifuged pellets has been shown to reduce hypertrophy and induce a better

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1 This technology is protected under C. Millan et al., PCT/EP2014/064017, Microtissues, 2014. See Patent II on p.142 in appendix.
differentiated phenotype of MSCs (215). One limitation of self-organizing microtissue structures is the lack of spatial control for cell distribution in the final construct, which severely limits their versatility for use in experiments involving multiple cell types. The engineered microtissue (EMT) approach could be useful for controlling the spatial distribution of different cell types in 3D as a result of sequential pipetting and rapid Schiff base crosslinking. In proof of concept experiments, two populations of human bone marrow stem cells (MSCs) were separated and labeled with cell tracker red and cell tracker green molecules respectively. Cells from each population were suspended at a high density of $20 \times 10^6$ cells/mL in N-succinyl chitosan (sChi), and 5 µL oxidized alginate (oxAlg) spots were made on a slightly hydrophobic surface. By sequentially pipetting from the different cell solutions, it was possible to create microtissues demonstrating a high level of organization for the two cell populations (Figure 5.1). The crosslinking is rapid enough to retain one population of cells, and yet slow enough to allow interactions between the two cell types at the interface of their populations.

5.3 Schiff base crosslinking for controlling chondrocyte adhesion to cartilage surfaces

A major source of variability in the clinical outcome of cell based therapies includes insufficient control over cell retention in an injury site. The ability to define regions of cell adhesion in a cartilage defect during autologous chondrocyte implantation (ACI) would improve the specificity of cell delivery to the lesion. Higher efficiency for cell localization to the defect could translate into the need for fewer cells and, therefore, a reduction in the amount of time needed for cell expansion which directly impacts the therapeutic potential of expanded chondrocytes (See Figure 1.6). The aim of this work was to evaluate whether the glycosaminoglycans (GAGs) present in cartilage ECM could be oxidized directly at the tissue surface for preparation for Schiff base crosslinking. Chondrocytes suspended in N-succinyl chitosan (sChi), a GAG-like polysaccharide containing primary amines, were seeded onto oxidized cartilage surfaces to test whether Schiff base crosslinking could expedite cell adhesion. The oxidation parameters were optimized for cell adhesion and dependence on oxidation as well as for the strength and reaction time. Cartilage
5.3 Schiff base crosslinking for controlling chondrocyte adhesion to cartilage surfaces

**Figure 5.1 | Schiff base crosslinking for engineered control of co-culture assembly.** Different cell populations were labeled with cell tracker red or cell tracker green. By pipetting the sChi droplets containing cells of the different populations in a sequential manner, EMTs could be formed with distinct spatial organizations of the two cell populations. Proof of concept experiments were performed with MSCs using two successive droplets, cells were labeled red or green (a) and three successive droplets of red, then green, then red labeled cells (b). EMT co-culture microtissues were imaged just 30 minutes after seeding and maximum intensity projections through a Z-stack are depicted (a,b right, scale = 500 µm) and 3D projection of the 1-1-1 microtissue is included (b, right, insert, scale = 1mm x 1mm x 500 µm).
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surfaces were also oxidized in defined patterns to test if Schiff base-mediated chondrocyte adhesion could be localized spatially.

5.3.1 Oxidation of GAGs present in cartilage surfaces

The superficial zone of cartilage plugs was removed by scalpel to expose a cartilage surface representative of partial thickness defects. The surfaces were oxidized by incubation under sodium periodate for cleavage of vicinal hydroxyls in glycosaminoglycans present in cartilage ECM (e.g. hyaluronic acid and chondroitin sulfate). Oxidation of cartilage surfaces was confirmed by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). In absorption spectra, oxidized cartilage displayed carbonyl stretching at 1730 cm⁻¹ wave number indicative of aldehydes (Figure 5.2). This band was markedly absent from non-oxidized control. Additionally, exposure of cartilage pieces to Schiff’s reagent (1% Pararosaniline, and 4% sodium metabisulfate, in 0.25 M hydrochloric acid,) resulted in a localized color change to pink on the cartilage surface where oxidation had created free aldehydes.

![ATR-FTIR and Schiff's test for oxidation of cartilage surface](image)

**Figure 5.2 | ATR-FTIR and Schiff's test for oxidation of cartilage surface.** Spectroscopy for oxidized cartilage surfaces revealed carbonyl stretching ($\nu_{\text{C=O}}$) at 1735 cm⁻¹ (arrow) typical of aldehydes that was not present for native cartilage. (Inlet) Incubation with Schiff’s reagent showed a color change to pink in the cartilage surface isolated to the region that was oxidized. (Scale bar = 2mm)*

*Data from C. Millan.*
5.3 Schiff base crosslinking for controlling chondrocyte adhesion to cartilage surfaces

5.3.2 Schiff base crosslinking to control chondrocyte adhesion

For cell adhesion studies, bovine chondrocytes were labeled with cell tracker green and seeded onto the surfaces of cartilage plugs (8mm biopsy punch) for an incubation time of 15 minutes prior to washing in PBS. Image J was used to quantify the number of cells adhered to the cartilage surfaces (Figure 5.3). In initial tests, we sought to optimize the parameters used for sodium periodate oxidation of the cartilage surface. The number of adhered cells on the cartilage surfaces was found to increase with longer oxidation time, though 5 minutes was found to be sufficient for significantly increasing the number of adhered cells over those seeded in saline controls (Figure 5.4A). Next, the oxidative strength was varied as cartilage surfaces were exposed to sodium periodate at 50 mg/mL or 100 mg/mL for 5 minutes. Cell adhesion also increased with higher oxidative strength, but 50 mg/mL was determined to be sufficient for significantly increasing cell adhesion compared to saline controls (Figure 5.4B).

Figure 5.3 | Schiff base crosslinking for cell adhesion to cartilage surfaces. Cell tracker green labeled chondrocytes observed on surfaces of cartilage plugs following a cell adhesion test. Cartilage surfaces were oxidized by exposure to sodium periodate and cells were seeded in (A) PBS or (B) N-succinyl chitosan (sChi). (Scale bar = 5mm)*

*Data from C. Millan

5. Schiff base crosslinking for cell-tissue adhesion & controlled assembly

Figure 5.4 | Optimization of cartilage oxidation (A) Schiff-base mediate cell adhesion to cartilage surfaces varied with oxidation time of the tissue surface. 5 minutes was sufficient for significantly improving cell adhesion in sChi over saline controls. (B) Cell adhesion was also found to depend on the strength of sodium periodate used for surface oxidation. 50 mg/mL was sufficient to increase cell attachment over controls. (*p < 0.05 versus PBS control, #p < 0.05 versus sChi of other time or concentration oxidation conditions)*

*Data from C. Millan

Utilizing the optimal cartilage surface oxidation conditions of 50 mg/mL for 5 minutes, the method was then tested for spatial control of cell adhesion. Four small droplets (3µL) of PBS or sodium periodate were pipetted onto the periphery of cartilage plugs, equidistant from one another. After 5 minutes, cartilage plugs were washed in PBS and chondrocytes suspended in sChi were added to the cartilage surfaces. Cells were selectively retained on the periphery of the plugs in higher numbers on oxidized cartilage surfaces over those treated with saline control. Cells were observed to adhere in a ring-like conformation, likely due to wetting of the cartilage surface during oxidation leading to bridging of the sodium periodate droplets. These studies demonstrate that Schiff base crosslinking can be used to expedite cell adhesion to cartilage surfaces and offers spatial control to direct localization of cell adhesion. Chondrocytes adhere to cartilage primarily via integrin binding to collagen (216). In experiments to test whether the same cells also bind to HA on the cartilage surface, blocking of CD44 receptors did not impact initial adhesion (217). Therefore, Schiff base crosslinking for controlling adhesion serves to compliment, rather than to compete with, intrinsic cellular adhesion mechanisms by involving covalent interactions with HA and other GAGs present in the cartilage ECM.
Figure 5.5 | Schiff base crosslinking for spatial control of chondrocyte adhesion. Cartilage surfaces were prepared for adhesion by pipetting 3µL droplets of (A) PBS or (B) sodium periodate in 4 equidistant locations in the tissue periphery. Chondrocytes suspended in sChi were pipetted onto cartilage surfaces and cell adhesion was observed by fluorescent microscopy. (B) Oxidation selectively promoted cell adhesion in the cartilage periphery in ring-like conformations, likely a result of wetting of the surface during oxidation. *Data from C. Millan.
CHAPTER 6

Schiff base crosslinking for hepatocyte microtissues

Note: Part of this project was done in collaboration with the group of Professor Sabine Werner at ETH Zürich and Dr. Claudia McGinnis of Non-Clinical Safety at Hoffmann-La Roche. This work was the master thesis project of Peter Ammann. All text was written by C. Millan.
6 Schiff base crosslinking for hepatocyte microtissues

As the body's primary center for metabolism, the liver is often the first organ to be affected by toxic chemicals and metabolites after digestion. Hepatotoxicity tops the list as the number one reason for market withdrawals of new pharmaceuticals (218) and drug-induced liver injury (DILI) is the leading cause of acute liver failure in the United States (219). Accurately evaluating the DILI risk during the development of new drugs remains a major challenge in the pharmaceutical industry. Primary hepatocytes rapidly dedifferentiate in vitro during conventional 2D culture (220) prompting researchers to rely on animal models for in vivo testing (221). However, large interspecies variability often results in lack of toxicity detection, with one comparison finding that rodent models have a 43% efficiency for detecting adverse effects in humans (222). To combat this low correlation, a number of recent studies have focused on developing organotypic in vitro models utilizing primary human hepatocytes and 3D cell culture techniques including spheroid culture on non-adherent surfaces (220), culture of liver slices (223), hanging drop (224), and additive manufacturing (225). These methods have shown promise for retaining functional similarities to liver tissue (226, 227) and maintaining cell polarity (228), an important characteristic of the differentiated hepatocyte phenotype.

The preferred method for hepatocyte spheroid formation for early stage toxicology testing is currently hanging drop (HD) culture due to its high throughput capability (211) and recent demonstrations showing sensitivity of HD spheroids to known hepatotoxic compounds (Figure 6.1A) (224). Still, because the formation of spheroids by hanging drop relies on cellular self-assembly, it is a variable process with low efficiency for inoculated cells to form spheroids (229, 230). Self-assembly is also slow and can take up to 5 days to form stable spheroids (231). In light of these issues, we were approached by researchers from the Non-Clinical Safety department at Hoffmann-La Roche (Basel, Switzerland) to test the engineered microtissue (EMT) system for application using primary hepatocytes. The goal of this study was to evaluate formation efficiency and biological functionality of hepatocyte microtissues in EMTs. Spheroids made by
6.1 Materials and Methods (Hepatocytes)

Isolation of primary murine hepatocytes

Primary hepatocytes were isolated from C57BL/6 mice (Jackson Laboratories) according to a previously established protocol (233). Briefly, mice were anaesthetized with pentobarbital and the liver, the portal vein and the inferior vena cava were exposed by peritoneal dissection. The liver was perfused with HBSS via the portal vein and the inferior vena cava was cut to release perfusate at the onset of blanching. After 2 minutes of perfusion with HBSS, HBSS was replaced by digestion media containing DMEM (4.5g/L D-Glucose/Pyruvate) and 800 µg liberase (Roche). After 4 minutes the liver was transferred to a petri dish filled with digestion media and cut to extract cells. Cell solution was filtered through a cell
strainer (100µm) and centrifuged at 50x g for 2 minutes. Supernatant was removed and pellet resuspended in DMEM (4.5g/L D-Glucose/Pyruvate). This step was repeated until supernatant was clear of cell debris (3-5x).

**Hepatocyte Cell Culture**

Cells were cultured in high glucose DMEM with 1% penicillin/streptomycin, 20µg/mL L-ascorbate-2-phosphate, 1% ITS, 100µM dexamethasone, 40ng/mL hepatocyte growth factor (murine HGF, Peprotech), and 20 ng/mL epidermal growth factor (murine EGF, Peprotech). For 2D culture controls, cells were seeded at 300,000 cells/well in 6-well plates. To form hanging drops, cells were suspended at 2.0 x 10⁶ cells/mL and distributed with a multi-channel pipette in 10µL droplets to the inner rings of the specialized well plate provided by Roche. For hepatocyte EMT formation, unless otherwise noted, cells were suspended at 1.0 x 10⁷ cells/mL in N-succinyl chitosan (sChi, 5mg/mL in PBS) and 2 µL were mixed with 1 µL droplets of oxidized alginate (oxAlg, 10mg/mL).

### 6.2 Determining EMT parameters for hepatocyte microtissue formation

Hepatocytes have a high metabolic demand compared to MSCs and are thus limited in the number of cells that can be used for forming microtissues due to risk of core necrosis (234). To scale down the EMT culture method for cell numbers appropriate for hepatocytes, EMTs were formed with a range of cell numbers. Hepa 1-6 cells, an immortalized liver cell line, and MSCs were seeded as EMTs of 100k, 50k, 40k, and 20k cells per microtissue and monitored by brightfield microscopy after seeding. A volume ratio of 2:1 (sChi:oxAlg) was sufficient to form microtissues for both cell types at the given seeding densities (Figure 6.2), but those formed with Hepa 1-6 cells displayed uncharacteristic node-like structures (Figure 6.2 B, Day 1). After 6 days in culture, however, Hepa 1-6 EMTs self-organized into compact and rounded structures for each of the seeding densities (Figure 6.2 B, Day 6).
6.2 Determining EMT parameters for hepatocyte microtissue formation

**Figure 6.2 | Optimization of EMT parameters for hepatocytes.** (A) MSCs and (B, top row) Hepa 1-6 cells on day 1 after seeding as EMTs maintaining a 2:1 volume ratio of (sChi:oxAlg) for 20k, 40k, 50k, and 100k cells per microtissue. Hepa 1-6 cells did not appear to aggregate uniformly on day 1, but (B, bottom) by day 6 had formed more compact structures. (Scale bars = 100 µm) *Images taken by Peter Ammann.

**Figure 6.3 | Primary hepatocytes in 2D culture.** Primary hepatocytes were cultured in 2D and imaged at (A) day 1, (B) day 3, (C) day 5, (D) day 7, (E) day 9, and (F) day 11. Hepatocytes attached and spread in the first 3 days, but quickly lost the spread morphology and eventually underwent apoptosis after 1 week in culture. (Scale bars = 100 µm). *Images taken by Peter Ammann.
6. Schiff base crosslinking for hepatocyte microtissues

6.3 Primary hepatocytes in EMT, HD, and 2D

Hepatocytes were isolated from wild type C57BL/6 mice and seeded in EMTs, hanging drop (HD), or plated for 2D culture. In 2D culture, hepatocytes adhered and spread over the first 3 days before slowly detaching and eventually undergoing apoptosis by 1 week (Figure 6.3), as expected according to literature reports (226, 229). HD cultures were initially incubated for 3 days prior to light centrifugation to pull the spheroid from the well plate lids into the culture wells (Figure 6.4A), but this was later increased to 5 days due to inefficient formation (Figure 6.4B). Spheroids and EMTs were monitored at day 7 and the efficiency of their ability to form a dense compact structure was recorded. Microtissue samples in which individual cells could be discerned at day 7 were considered to have failed formation. In HD, spheroid formation efficiency was less than 50% compared to EMTs which successfully produced robust 3D structures in over 90% of microtissues. In EMTs, the work of microtissue assembly is done by the Schiff base crosslinking of modified polysaccharides and is independent of cell type or cellular self-assembly.

Figure 6.4 | HD spheroids of primary hepatocytes. (A) Hanging drop (HD) spheroid centrifuged into a culture well after 3 days of incubation, which was determined to be insufficient for robust spheroid formation. (B) Spheroid cultured in HD for 5 days prior to centrifugation was more intact and (C) the same spheroid continued to condense by day 9. (Scale bars = 100 µm). *Images taken by Peter Ammann
Liver microtissue functionality in 2D, HD, and EMTs was assessed by analyzing gene expression of cyp3a13, a member of the cytochrome p450 enzyme family responsible for the metabolism of over 50% of clinically active drugs (235). Expression of cyp3a13 was normalized to values of freshly isolated hepatocytes. At day 1, cyp3a13 expression in EMT was slightly down-regulated compared to day 0, but reached the levels of freshly isolated hepatocytes within 3 days of EMT culture and sustained expression up to day 5. In 2D, cyp3a13 expression decreased rapidly within 3 days as hepatocyte viability decreased. In this 1 week experiment, HD microtissues could only be evaluated at day 5 due to low cell yield at time of harvest and expression was found to be down-regulated to 20% of the value measured in freshly isolated hepatocytes. These promising results highlight the versatility of EMT culture for applications outside of cartilage tissue engineering.
Figure 6.6  |  Cytochrome P450 gene expression in hepatocyte microtissues. Fold change in primary hepatocytes after EMT, HD, and 2D culture for 1 week. RT-qPCR values were normalized to the expression levels of freshly isolated cells and the 40 S ribosomal protein S 29 (RPS29) was used as the internal housekeeping gene. *Data from Peter Ammann.
7 Outlook

7.1 *In vivo* study

Despite the considerable strides made in this project towards a potential MSC-based therapy for cartilage repair, some critical unanswered questions remain before a decision can be made whether to pursue further validation in clinical trials. While proof of concept was carried out here in an *in vitro* cartilage repair model, it remains to be seen if EMTs loaded with TGF-β3 would also be capable of inducing chondrogenesis in an *in vivo* setting where the culture conditions are less controlled. To achieve this goal, an *in vivo* study is currently envisaged that would involve subcutaneous implantation of cartilage defects filled with EMT+TGF in nude mice. Histology and immunohistochemistry for accumulation of cartilage-specific matrix in this model would further validate the technology for translational application. Mechanical testing to determine bulk compressive moduli of the repair tissue will also be important for assessing the functional quality of the repair tissue. A number of literature reports involving cartilage tissue engineering with MSCs and biomaterials suggest a tendency towards hypertrophic differentiation, calcification, and vascularization of implanted structures (102, 157, 236) which will all need to be addressed here.

Coming back to the underlying premise of this work, namely a developmental engineering approach to recapitulate cartilage formation, it follows that many clues for controlling hypertrophy can be found following the natural processes of skeletal development (237). As the limb bud develops, the innermost chondrocytes become hypertrophic and begin to secrete type X collagen, which is later calcified before replacement of the cartilage template with bone tissue during endochondral ossification (20). Vital to the successful engineering of cartilage replacement tissue using MSCs is a fundamental understanding of the differences between replacement and permanent cartilage (238, 239). One key player in the hypertrophy signaling pathway is parathyroid hormone-related protein (PTHrP) (240), which participates in a negative feedback loop with Indian Hedgehog (Ihh), a stimulatory factor of hypertrophy and chondrocyte
proliferation (241-243). Resting chondrocytes at the end of long bones secrete PTHrP suppressing Ihh production. Chondrocytes outside of this paracrine signalling range, however, do produce Ihh and undergo hypertrophy (See Figure 1.2C). In one study, co-encapsulation of PTHrP with TGF-β3 in HA hydrogels seeded with MSCs resulted in minor decrease in calcification compared to gels with TGF alone (157). One option for addressing this issue would be to simultaneously load PTHrP with TGF-β3 during EMT formation. However, the spatiotemporal interplay between PTHrP and TGF-β3 are inherently complicated representing significant challenges to recapitulation in a biomaterial-based engineered design.

### 7.2 Autophagy and hypertrophy

A novel target to investigate for regulating hypertrophy involves chondrocyte autophagocytosis. There is striking evidence linking mature chondrocytes to autophagy as a means to help meet the energy demands of cells in the avascular cartilage matrix (244). Terminally differentiated chondrocytes undergo apoptosis and are removed from the growth plate (245). This is accompanied by perichondrium development and vascular invasion, as well as establishment of an ossification center containing fibroblast-like cells that express type I collagen (246). It is known that MSCs exhibit reduction in autophagy-related behavior during differentiation (247, 248), but that autophagy is a necessary cell fate for maintaining healthy cartilage (249). We have identified two drugs that effect cell autophagy and have begun to investigate their contribution to either enhancing or reducing MSC hypertrophy during chondrogenesis in EMT culture.

Preliminary experiments have begun to elucidate the role of autophagy during MSC chondrogenesis and potential to regulate hypertrophic differentiation. Rapamycin, an inhibitor of
Figure 7.1 | PCR and histology of autophagy markers. A) Gene expression of Atg5 and Beclin1, data is normalized to 18 s and compared to no stimulation day 3. (B) Immunostaining of Atg5 and LC3 markers in a non-stimulated sample, sample cultured with 3MA, and with the addition of rapamycin only at day 10. (In green is Atg5, in red LC3, and in blue DAPI.) (Scale bars = 100μm). *Data and images from Souzan Salemi.

mammalian target of rapamycin (mTOR) kinase, has been shown to be a potent inducer of autophagy. 3-methyladenine (3-MA) was selected for autophagy inhibition. Samples were given autophagy-related drugs either at low dosage for continuous exposure, or slightly higher dosage at day 10, the time point when hypertrophy-related genes are known to begin upregulation (102). All samples were cultured in chondrogenic media and an unstimulated control group was included for comparison. MTS assay was used to monitor viability and qPCR was utilized to analyze gene expression at days 3, 10, 14, 17, and 21.

The two main autophagy genes, Atg5 and Beclin 1, were studied for non-stimulated controls and samples that were treated with rapamycin or 3-MA. When no drug was added, a down regulation of autophagy was observed over time (Figure 7.1A). This was also true of groups that were exposed to small doses of the drugs for the duration of the experiment. However, both genes were up-regulated at day 21.
when rapamycin was added only at day 10. Immunohistochemical staining for autophagosomes supported the gene expression results from samples treated at later time points with rapamycin displaying a high number of autophagy-related vesicle formation (Figure 7.1).

Early MTS data suggested that treatment with either drug caused decreased metabolic activity over time (Figure 7.2A). 3-MA appeared to be more detrimental while rapamycin treated samples were more stable. Hypertrophy was measured by gene expression of type X collagen, a known marker of hypertrophy (19), and its relation to expression of type II collagen, a marker for chondrogenesis. In analyzing the effect of low dosage of the drugs on expression of these markers, it was evident that type II collagen increased for all groups starting at day 10 (Figure 7.2B). 3-MA treatment delayed the up-regulation by 1 week, while it increased sharply by day 17 and continued to increase. Conversely,
rapamycin appeared to limit the maximum type II collagen expression level to 1,000 fold compared to day 3. Type X collagen expression also increased for all groups starting at day 10. By day 14, the non-stimulated controls expressed the highest amount of type X collagen. Expression of type X collagen in rapamycin treated samples followed a similar trend as the non-stimulated group of peaking at day 14 followed by a decrease through day 21, while 3-MA treated samples plateaued in their type X collagen expression. Treatment with higher doses of 3-MA beginning at day 10 caused drops in gene expression of both type II collagen and type X collagen, likely a result of toxicity issues which is corroborated by a dip in MTS measured activity as well. Rapamycin-treated samples followed a similar trend regardless of whether it was administered earlier or later in the experiment.

From these early studies, it is clear that both rapamycin and 3-MA impact the gene expression of type II collagen and type X collagen. Though it delays the initiation of chondrogenesis, a low dosage treatment with 3-MA over the duration of the experiment resulted in high expression of type II collagen that seems to approach the non-stimulated control by day 21, while simultaneously causing a plateau in type X collagen expression over the same time frame. In follow-up studies, a different drug for inducing autophagy may be tested considering the conflicting role of 3-MA modulation and possibility that it activates autophagy via alternative pathways (250). Changes in the expression of autophagy-specific genes and immunohistochemical analysis of autophagy markers is currently being carried out by our collaborators.
References

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APPENDICES

8. Arriving at Schiff base crosslinking for inducing cell aggregation

9. Non-cartilage microtissues

Patents

Patent I  Automatable methods for encapsulating living cells

Patent II Process of cartilage repair

Patent III Microtissues
8. Arriving at Schiff base crosslinking for inducing cell aggregation

The overarching goal of this project was to engineer the process of cellular assembly to circumvent issues that arise when cell aggregates are formed by self-assembly. One of these issues is the relatively slow rate at which cells form enough intercellular contacts to stabilize a higher order structure. In order to achieve faster aggregation of cells, we sought to apply a recently developed technique for coating cell surfaces in adhesive nanofilms via layer-by-layer (LbL) build-up of paired proteins or polyelectrolytes (251). The premise of this technique is that neighboring cells coated with nanofilms are attracted to one another according to the nature of the nanofilms constituents. This has been demonstrated with fibronectin (FN) and gelatin (G) which participate in LbL via mutual binding domains. Coating the surface of a cell with a shell of FN-G layers enables a neighboring cell to interact with the nanofilms by binding cell surface receptors that have specific domains for FN. It was shown that FN-G coated cells, when seeded at high density, formed densely compact microtissue structures that were larger and more homogeneous than uncoated controls (214). Similarly, nanofilms consisting of polyelectrolytes can be used to change the surface charge of cells (252). A population of cells whose net surface charge is positive could then attract cells of a separate population containing net negative charges on their surface by way of static attraction. A number of protein and polyelectrolyte pairs that relate to MSC condensation and cartilage ECM were selected for a screening as potential components of adhesive nanofilms. (253). Fibronectin plays a role in assisting the migration of cells towards anlagen epicenters and is essential at the onset of MSC condensation (14) and gelatin, as a degraded collagen product, contains many of the same binding domains as collagen which is a major constituent of cartilage ECM (254).
8.1 Nanofilm buildup and cell viability

To begin screening for the optimal nanofilm composition, proteins and polyelectrolytes were fluorescently tagged and incubated with cells to visualize deposition onto cell surfaces. Fibronectin (AlexaFluor 488-FN), poly-l-lysine (FITC-PLL), DEAE-Dextran (FITC-DX), and gelatin (Oregon green-G) were each selected as the initial layer for various nanofilm pairs and each was shown to cover cell surfaces during LbL build-up (Figure 8.1). QCM-D was then utilized for measuring the kinetics of LbL buildup for the
8. Arriving at Schiff base crosslinking for inducing cell aggregation

different nanofilm pairs. Polyelectrolyte nanofilms composed of DX and HA formed the thickest films while
DX/CS, FN/G, and G/CS all formed nanofilms with similar mass deposition properties (Figure 8.1E). PLL/HA
nanofilms were the thinnest by far. It should be noted that, to ensure protonation of gelatin for
electrostatic pairing with CS, a pH of 6 was used (IEP of 7-9). It was determined that the first bilayer of the
nanofilm is the thickest, with subsequent LbL steps not contributing as significantly nanofilm buildup. For
this reason 1.5 bilayers of polyelectrolyte pairs was deemed adequate for nanofilm thickness with the
exception of FN/G where 3.5 bilayers was used. Nanofilm thickness were calculated using the Sauerbrey
relation for the 3rd overtone from QCM-D data and presented in Table.

Bovine chondrocytes were coated in various adhesive nanofilms and seeded at high density for
microtissue culture. Cell morphology and viability was visualized by live/dead assay and confocal
microscopy, and viability was quantified by trypan blue exclusion during LbL buildup. Polyelectrolyte
nanofilms containing either PLL (Figure 8.2B) or DX (Figure 8.2D) were found to be toxic to encapsulated
cells. The decision was subsequently made to pursue FN/G (Figure 8.2A) and G/CS (Figure 8.2C) nanofilms
for mediating aggregation in chondrogenic microtissues.

One of the main drawbacks of the LbL technique for building up adhesive nanofilms on cell
surfaces is the repeated centrifugation and wash steps that must be performed to alternate cells between
the two solutions. We hypothesized that a centrifugation-free method was possible by titrating the
nanofilm solutions into the cell suspensions so that the concentration of the depositing layer was in excess
of the previously deposited layer. This concept was shown in QCM-D measurements using FN and G as a
model system. For the excess concentration method, mixed solutions of FN and G were prepared for each
deposition step, alternating in which protein was present at an excess concentration compared to the
other. Using this method and without washing in between layering, a nanofilm buildup was observed that
was 5x thicker than the one that resulted from the traditional layering approach with washing (Figure
8.3B). This new titration method of nanofilm fabrication significantly improved the cells retained
8.1 Nanofilm buildup and cell viability

Figure 8.2 | Live dead and viability. Live (Calcein-AM, green) and dead (Ethidium homodimer-1, red) staining of microtissues made by coating cells in adhesive nanofilms of (A) FN/G, (B) PLL/HA, (C), G/CS, and (D) DX/HA. (E) Viability was monitored during nanofilm assembly by trypan blue and cell counting.
8. Arriving at Schiff base crosslinking for inducing cell aggregation

Figure 8.3 | Nanofilm buildup by excess concentration layering\(^3\) (A) QCM-D of conventional LbL buildup with FN/G multilayers at equal concentrations and intermittent washing steps. (B) QCM-D of excess concentration LbL buildup without washing. (C) Cell counts during FN/G cell coating with and without washing. (D) Phalloidin (green) and DAPI stained cell multilayers after FN/G coating.

during LbL assembly versus the traditional method with washing (Figure 8.3C). As proof of concept, chondrocytes were coated with Fn/G using the titration method and seeded at high density where they formed densely packed structures (Figure 8.3D). Phalloidin staining of fixed microtissues indicated cortical actin structures within the microtissue which are typical of chondrocytes in 3D culture systems (Figure 8.3D) (255). In other experiments, microtissue thickness was correlated with cell seeding density. Cells were coated in FN/G nanofilms and seeded at either 630k or \(1.3 \times 10^6\) cells per \(\text{cm}^2\). Coated cells formed densely packed microtissue sheets at both seeding densities while uncoated cell remained homogeneously

\(^3\) This technology is protected under C. Millan et al., US 61/529,963. Filtration- and centrifugation-free automatable methods for encapsulating living cells and nanoparticles in polyelectrolyte nanofilms, 2011. See appendix.
Figure 8.4 | Control of microtissue thickness by seeding density. (A) Uncoated cells seeded at either 630k/cm² or (B) $1.3 \times 10^6$/cm² failed to form compact multilayer structures. FN/G coated cells seeded at (C) 630k/cm² or (D) $1.3 \times 10^5$/cm² produced dense microtissue structures with heights of 17um or 45um, resp. (E) H&E staining of microtissue cross-sections of (top) FN/G coated cells or (bottom) uncoated cells.
8. Arriving at Schiff base crosslinking for inducing cell aggregation

**Figure 8.5 | Gene expression of late passage chondrocytes in 2D and 3D culture.** Expression of superficial zone protein at (A) day 14 and (B) day 28 after seeding late passage chondrocytes on tissue culture plastic (TCP), coated in nanofilms of FN/G, coated in nanofilms of G/CS, or in alginate beads. (C) and (D) are of Sox9 expression at day 14 and day 28, resp.

dispersed. Microtissue integrity was confirmed with histology by H&E stain (Figure 8.4E).

As an initial model for chondrogenic induction, late passage chondrocytes were coated with FN/G or G/CS nanofilms and their recovery in gene expression of cartilage markers was measured by qPCR. Chondrocytes further cultured in monolayer on tissue culture plastic (TCP) were used as a negative control, and cells seeded in alginate beads where redifferentiation is known to occur (256) were used as a positive control. Interestingly, chondrocytes coated in FN/G adhesive films were induced to express the same levels of superficial zone protein (SZP) as those in alginate beads at days 14 and 28 (Figure 8.5). G/CS
coated cells, on the other hand, up-regulated their expression of Sox-9 compared to TCP and FN/G at day 14 (Figure 8.5C), but this was a transient effect lost by day 28 (Figure 8.5D). Tools for chondrocyte redifferentiation would find merit as a mediators to improve the healing potential of the cells used in autologous chondrocyte implantation (ACI) that drop in expression of cartilage markers during ex vivo expansion (See Figure 1.6). Ultimately, though, the results of these studies were inadequate and prompted further investigation into other potentially stronger interactions that could be utilized for driving cellular aggregation more rapidly. This led us to search for covalently interacting pairs and the Schiff base crosslinking between sChi and oxAlg that eventually became the framework upon which this thesis was built.
9 Non-cartilage microtissues

As the biological community continues to embrace the “revolution” in cell culture towards 3-dimensional culture platforms, applications abound for a technique to create microtissues that outperform those made by conventional methods such as hanging drop. Driving the push for researchers to come up with better *in vitro* models is the pharmaceutical industry which is constantly striving for better biomimetic substrates on which to test the toxicity and efficacy of potential new drugs. To date, a reliable assay for testing neurotropic drugs or for evaluating neurotoxicity is lacking. Culturing neurons in 2D on plastic substrates does not capture the complexity of an interconnected neural network.

The ability of sChi/oxAlg EMTs to support culture of primary neurons was assessed in a proof of concept experiment. Primary cortical neurons were freshly isolated from rats and seeded in EMT microtissues as previously described for MSCs with one exception. One sample group was made as before with sChi (5mg/mL), but a second group was added made with less concentrated sChi (2mg/mL). It is known that neural cells prefer softer substrates during culture and a reduced crosslinking density by lowering the sChi concentration would achieve lower microtissue moduli. After 1 day of culture in neurobasal media, cell morphology was visualized by calcein AM and Hoechst staining. Primary neurons stained positively for calcein AM with very few Hoechst-only nuclei indicating high viability for both culture conditions. Additionally, both culture conditions were conducive for neurite outgrowth (Figure 9.1).
9. Non-cartilage microtissues

Figure 9.1 | Primary neurons produce extended neurites in EMT culture. Calcein AM (green) and Hoechst (blue) stained neurons at day 1 in (left) normal EMT conditions and (right) EMTs with reduced polymer content. Images are maximum intensity projections through a Z-stack taken with confocal microscopy (LSM 780). (Scale bars = 50µm).
**Patent I  Automatable methods for encapsulating living cells**

**Abstract**

Species is alternatingly and incrementally increased. The advantages of this system are: An automated liquid handling system is used to encapsulate biological cells or particles in polyelectrolyte layers. The layer-by-layer assembly takes places by incubating the objects in a single stirred solution to which polyelectrolytes are added such that the concentration of each polyelectrolyte

- Avoidance of centrifugation/resuspension and/or filtration steps which are both time consuming and damaging to cells
- Up-scalable to 96 or 384 well plates so that combinations of polymers and therapeutics can be evaluated in high-throughput.
- Compatible with FACS devices which sort cells into above mentioned well plates prior to coating.
- Possibility to incorporate therapeutics and molecular medicine in the cell coatings
- Possibility to alter the adhesive properties of one or more populations of cells for the formation of tissue and control of cell fate and differentiation
- Possibility to form tissue with a tunable cell density. Since excess polyelectrolyte is not removed, these free molecules serve as nucleation points for layer-by-layer assembly into microparticles, which themselves form the “cell-free” or “extracellular” compartment of the engineered tissue.

**Field of Invention**

The present invention concerns a method to encapsulate biological cells and particles in polyelectrolyte multilayers for the purpose of 1) altering the cell adhesivity to other cells and molecules 2) for the purpose of rapidly forming tissue 3) steering the differentiation of cells and/or maintaining the differentiated phenotype of the cells and 4) to immunoprotect the encapsulated cells prior to transplantation.

**Background**

Cartilage, liver, mammary glands and other tissues are composed of cells which undergo de-differentiation when these cells are isolated from the extracellular matrix and expanded on 2D tissue culture plastic. It is therefore essential to culture these cells (chondrocytes, hepatocytes, mammary epithelial) in 3D environment in order to maintain their correct phenotype. Commonly used encapsulation materials include alginate and agarose, which undergo calcium- and temperature-dependent polymerization respectively. Preformed scaffold materials can also be used for tissue engineering,
however these need to be mechanically seeded with cells, a process which is associated with dramatically uneven seeding densities. Recently, Nishigushi et al [1] has introduced the rapid accumulation method where tissue is formed from cells which are coated with nanofilms of fibronectin and gelatin using the layer-by-layer technique. The coating increases the integrin mediated interaction between the cells and allows the rapid formation of solid, multilayered tissues. Cell-cell interaction and adhesion plays an important role in a large number of cellular processes, including the differentiation of stem cells towards cartilage formation (chondrogenesis). In particular, cationic polymers are chondroinductive when added to the culture media, [2] as they may help form crosslinks between the negatively charged cell glycocalyx and in doing so help bring the cells sufficiently close together that cell-cell interactions can occur.

Layer-by-layer techniques have been used to coat particles for decades [3, 4]. Typically the particles are incubated in a polyelectrolyte solution having the opposite charge of the surface charge of the particle. Particles are then centrifuged and the pellet re-suspended in a wash buffer, followed by centrifugation and re-suspension of the particles in the second polyelectrolyte solution. To avoid these steps, Sukhorukov et al [5] incubated colloids at just saturating concentrations of polyelectrolytes, where the saturation point was determined by ζ-potential measurements. Although the layer-by-layer technique has been used to encapsulate living, biological cells as well, the application is not wide-spread due to substantial problems with cell loss and mechanical damage to cells. The novel feature of this invention is to form multilayers on biological cells using a stirred solution whose polyelectrolyte concentration is increased in a step-wise and alternating fashion, thereby avoiding all centrifugation or filtering steps.

**Detailed Description**

**METHODS:**

Nanofilm build-up with Excess Concentration Layering was confirmed with QCM-D, where layering solutions had twice the concentration of the previous layer and the starting concentration was 0.06mg/mL fibronectin (Fn) and 0.072mg/mL gelatin (G) (Figure 2). Similar experiments were conducted with PLL/HA in excess concentration to demonstrate the universality of the method (Figure 3).
Figure 2: QCM-D demonstration of fibronectin/gelatin nanofilm assembly with Excess Concentration Layering.

Figure 3: QCM-D demonstration of PLL/HA nanofilm assembly with Excess Concentration Layering.

Cells were initially incubated for 15 minutes in a solution of 0.06 mg/mL fibronectin (PBS, pH 6.5). Subsequent solutions of increasingly concentrated gelatin (G) or fibronectin (Fn) were added to the cell suspension where the excess concentration species was deposited as a layer in the nanofilm on the cell surface until a composition of two bilayers and an outer layer of fibronectin was achieved (Figure 4).
In another embodiment, cells were initially incubated for 15 minutes in a solution of *PLL-FITC. Subsequent solutions of increasingly concentrated PLL or HA were added to the cell suspension where the excess concentration species was deposited as a layer in the nanofilm on the cell surface. In addition, the formation of increasing amounts of microparticles was observed. Microparticles can either be removed using electrodialysis or retained to become part of the extracellular, cell-free component of the tissue. The amount of particles formed can determine the cell density of the assembled tissue.

Figure 5: Chondrocytes coated by increasing bilayers of PLL-FITC/HA with Excess Concentration Layering. Microparticles are formed which can be used for tuning cell density.
Patent Claims

1. A method for coating charged substrates where the driving force for assembling of the polyelectrolyte multilayer is the incremental increase in concentration of two or more polyelectrolytes in a single bathing solution.

2. The method of claim 1 wherein the charged substrate is a living or fixed biological cell or group of cells (for example an adult, fetal or induced pluripotent cell (iPS), cells of the islets of Langerhans, chondrocytes, hepatocytes, osteoblasts)

3. The method of claim 1 wherein the multilayer has the purpose of immunoprotecting the cell or groups of cells

4. The method of claim 1 wherein the process is used for engineering cartilage tissue and inducing the differentiation of stem cells towards chondrogenesis.

5. The method of claim 1 wherein the nanofilm-coated cells are use to form solid tissues based on their increased adhesivity

6. The method of claim wherein the nanofilm-coated cells are used to form solid tissue by centrifugation or other concentrating methods

7. The method of claim 1 wherein the charged substrate is a nanoparticle or microcrystal or colloid and where the coating can be used to alter the electrical, optical, magnetic, or adhesive properties of the substrate

8. The method of claim 1 where the polyelectrolytes are biopolymers including collagen, fibronectin, gelatin, chondroitin sulfate, heparin sulfate, heparin, alginate, and chitosan

9. The method of claim 1 where one of the polyelectrolytes is a cationic polymer including poly-L-lysine, poly-D-lysine, polyethylenimine and hexadimethrine and where the cationic polymer serves to crosslink the glyocalyx of adjacent cells and where the cationic polymer promotes cell-cell adhesion and retention of glycosaminoglycans

10. The method of claim 1 wherein the polyelectrolyte solution and resulting nanofilms can contain therapeutic molecules (for example growth factors, siRNA, DNA plasmids, anti-sense RNA, drugs, peptides)

11. The method of claim 1 wherein the terminal layer of the nanofilm of the cell or particle or capsule has targeting, adhesive or cell entry properties and in some embodiments has porosity which is pH and/or temperature sensitive.

12. The method of claim 1 wherein the microparticles which form due to the excess of polyelectrolyte can be used to form the cell-free component of the tissue and tune the cell density of the resulting tissue

13. The method of claim 1 in which the vessel holding the bathing solution is a 96 or 384 well plate

14. The method of claim 1 in which the additions of polyelectrolytes to the well plates is made by an automated liquid handling system and stirring is achieved by shaking or through a magnetic stir bar in the well.

15. The method of claim 1 in which the cells enter the vessel using Fluorescence Activated Cell Sorting.

16. The method of claim 1 in which the tissues formed in the well plates can be used for drug screening
Patent II  Process of cartilage repair

Abstract

Biological tissues are collected from donors, decellularized, minced into fragments, and subsequently activated by surface modification enabling them to be cross-linked into a scaffold network for applications in tissue engineering and regenerative medicine therapies. The result of the cross-linking reaction is a biocompatible engineered scaffold with highly tunable geometries and mechanical properties which serves as matrix for defect repair and regeneration. Similar surface modifications can be applied to the site of an injury or defect which allows the native tissue to cross-link covalently to the scaffold network resulting in firm adhesion. In another embodiment, the same crosslinking reactions can be used to induce cell-cell adhesion for the rapid formation of microtissues.

Therapeutically relevant biological components such as minced tissue fragments and/or cells can be cross-linked into microtissue utilizing a number of different chemistries including, but not limited to, Schiff-base linkages, Michael addition, disulfide bond formation, and catechol-initiator polymerization. For example, cells to be used to form microtissues can be resuspended in a solvent containing free amino groups and pipetted into oxidized polysaccharides which spontaneously crosslink resulting in a microtissue. On a larger scale, surface activation of tissue pieces carried out by oxidation of primary hydroxyl groups to free aldehydes that, when reacted with a solution of amino-rich linker molecules, covalently couples by the Schiff reaction cross-linking into a mechanically stable scaffold. Alternatively, amino groups present in tissue ECM can be exposed by brief enzymatic digestion can enable cross-linking of tissue pieces with a solution of aldehyde-rich linker molecules.

This technique is highlighted by the following advantages:

- Robust microtissues can be formed in a 10 minute crosslinking reaction and immediately handled with forceps, which is in stark contrast methods such as centrifugation or hanging drop which takes hours-days before reaching a mechanically stable state.
- The adhesive components used for crosslinking are slightly modified forms of natural tissue constituents and may provide guidance cues for cell behavior (i.e. parts of tissue architecture are intercalated between cells in the microtissues).
- Microtissue formation can be applied to any cell type, including those known to resist cell-cell adhesion such as liver cells.
- Utilizes autologous, allogenic or xenogenic native tissue which already contains the complex array of tissue-specific extracellular matrix components in physiologically accurate proportions. Aggrecan for example is extremely expensive in the purified form ($400 /mg), but is abundantly present in cartilage fragments.
- After mincing, tissue fragments can be reconstituted and molded into any desired geometry to fill a defect without compromising its high-resolution biochemical composition.
- Suture-free method to adhere a tissue replacement material to an injury site or site of degeneration.
Both the treatment of donor tissue and its application to a site for adhesion rely on reactions which are completed in a time which is relevant for clinical applications and can be incorporated into a 1-step surgical procedure.

- Injectable components and in-situ scaffold formation could be applied in a minimally-invasive arthroscopic procedure.
- Activation of tissue surfaces is done with simple reactions with possible applications in a number of different tissue types.
- Possibility to incorporate therapeutic factors within the scaffold including, but not limited to: pharmaceutical compounds, growth factors, peptides, proteins, carbohydrates, gene therapy vectors, and autologous or allogeneic isolated cells. Additionally, homing molecules can be included that would induce host cell migration into the scaffold.
- Possibility to achieve zonal organization of tissue architecture by layering various tissues/compositions using additive manufacturing techniques (e.g. bioprinting).
- Possibility to tune the tribology properties of the surface layers of the scaffold through conjugation of molecules such a lubricin and superficial zone protein.
- Possibility to include compaction step during construct formation to tune its mechanical properties.

**Field of Invention**

The present invention concerns utilization of versatile crosslinking chemistries to induce rapid cell-cell, cell-matrix, and matrix-matrix adhesions—control over which has significant implications for the field of regenerative medicine. Outlined here are methods to form microtissues in a rapid and scalable reaction, as well as techniques to activate the surfaces of minced tissue fragments to enable their participation in cross-linking reactions. Microtissues may be used for large scale screening of pharmaceuticals, and both microtissues and crosslinked tissue pieces result in biologically engineered scaffolds for tissue repair and regeneration of many different tissue types.

**Background**

3D microtissues have tremendous potential for large scale drug screening applications [1], and more recently as building blocks for use in tissue engineering [2, 3]. Current methods for producing microtissues include centrifugation [4], hanging drop culture [5], gyratory shakers or spinner flasks[6]. More sophisticated methods for controlling cell-cell adhesions have also been studied including grafting complimentary single-stranded complimentary DNA sequences onto cell surfaces [7]. All of these methods, however, require culture times on the order of days before a robust microtissue is formed.

Recently, decellularized tissues have attracted interest as scaffold material alternatives to simpler approaches where the scaffold is composed of a single material [8]. Tissue decellularization results in a scaffold of extracellular matrix ideally suited for regenerating injured or diseased tissue since it retains the high resolution architecture necessary for recapitulation of function. Decellularization has most successfully been applied to whole organs like the bladder [9] with a fixed geometry. Our approach,
however, is based on mincing the tissue and reconstituting it into a desired geometry. In addition, the fragmented form aids in the repopulation of ECM dense matrix by autologous cells [10]. These tissue fragments represent a promising therapeutic tool that could ideally be applied directly at the site of a tissue defect. Employing a similar technique, a one-step surgical procedure was studied for cartilage replacement where minced donor tissue fragments were fixed into a cartilage defect using fibrin glue in a rabbit model [11]. While promising histological sections indicated a benefit of the treatment, the use of fibrin glue elicited inflammatory response in the load-bearing setting. Using decellularized minced tissue would not only overcome the issue with inflammation associated with fibrin glue, but it should also exhibit higher degree of bioadhesion between native tissue and tissue fragments with more stable mechanical properties. One method for achieving adhesion of tissue fragments to a defect site involves oxidizing them to expose aldehyde groups which participate in Schiff-linkages with an amine-rich linker such as chitosan (Fig. 1 and Fig. 3 right).

**Detailed Description**

**METHODS:**

Schiff linkages, or imine bonds, have proven to successfully achieve cross-linking between oxidized polysaccharides and amine-rich molecules [12-17] without the need of chemical cross-linking agents or photoinitiators as a catalyst. Oxidized forms of the glycosaminoglycans (GAGs) hyaluronic acid (oxHA) and chondroitin sulfate (oxCS) as well as oxidized alginate (oxAlg) and oxidized sulfated alginate (oxSulfAlg) were utilized as Schiff-base compliments of N-succinyl chitosan (sChi) to rapidly crosslink and form a bioadhesive. Polysaccharide oxidation (oxPS) was confirmed by fourier transform infrared spectroscopy (FTIR) and the appearance of a distinct peak at 1730 cm⁻¹ representative of the aldehyde bond created in the reaction (Fig. 2a). To form microtissues using the Schiff linkage technique, human mesenchymal stem cells (MSCS, Lonza Group Ltd, Basel, Switzerland) between p.6-p.9 were suspended in a solution of 5 mg/mL sChi at a cell density of 20 x 106 cells/mL. Drops of 5 µL each of the corresponding oxPS molecules were prepared on the lid of a 24-well plate. 10 µL of the cells + sChi were pipetted into individual droplets of oxPS, the lid was turned upside down, and the reaction was carried out in an incubator at 37°C for 10 minutes. Microtissues were transferred to agarose coated-wells of a 96-well plate with fine-tipped forceps and cultured in chondrogenic media (Fig. 2 c-e, oxCS, oxHA, and oxAlg resp.). In parallel, MSCs were centrifuged to form micromass pellets (200k cells/each) for comparison and transferred to the same 96-well plate (Fig. 2b).

GAGs present in cartilage tissue surfaces were oxidized directly with brief incubation under sodium periodate. Aldehyde presence was confirmed to be localized over the oxidation spot by incubation with Schiff reagent (Pararosaniline, 1% and sodium metabisulfate, 4%, in hydrochloric acid, 0.25 mol/L) and monitoring a color change to pink (Fig. 3a). Furthermore, entire minced tissue fragments of nucleus pulposus (NP) or AC were chemically oxidized by incubation with sodium periodate, both of which also responded strongly to the Schiff test for presence of aldehydes. To prove the cross-linking capability of activated tissue fragments, minced articular cartilage was oxidized (oxAC, 10% w/v) and mixed at a ratio of 1:1 with N-succinyl-chitosan (2% w/v), a water-soluble chitosan derivate. Gelation occurs in about 30
seconds yielding a robust scaffold that can be comfortably manipulated with forceps (Fig 3), and the mechanical properties of this gel were investigated using a texture analyzer (Fig. 4). To improve the compressive modulus of the construct, a structured model for assembly was used. A layer of oxAC was sandwiched between two layers of S-Ch and the entire construct was compressed to extract water, a byproduct generated during the Schiff linkage formation. Furthermore, oxidized chondroitin sulfate was also added into the “sandwich” in the same layer as the oxAC in order to assure all amino groups available would participate in the Schiff linkage and improve construct stability.

Cell (human adipose stem cells & bovine chondrocytes) viability was assessed on the gels, with the final goal of encapsulating these cells during gel formation. It was demonstrated that cells seeded on top of a cross-linked scaffold of oxAC + oxCS/S-Chi are maintained (Fig. 3). The same procedure could be translated into other tissue. For example, nucleus pulposus (NP) was oxidized in a similar fashion.

**Patent Claims**

1. A method of rapid tissue reconstruction based on minced donor tissue where bioadhesion between the tissue fragments is achieved via introduction or exposure of cross-linking moieties on the tissue fragments together with one or more linker molecules.
2. A method for rapid microtissue formation based on pipetting a high density of cells suspended in an amino-containing solvent into small droplets of aldehyde containing solvents to for Schiff-base crosslinks and adhere cells to each other.
3. The method of claim 2 where cells are rapidly adhered to a substrate of tissue, and the Schiff-based crosslinking is utilized adhere the microtissue to the surface in addition to cell-cell adhesion.
4. The method of claim 1 where the pieces or fragments are ideally in the range of 1 micron to several centimeters in diameter.
5. The method of claim 1 where the minced donor tissue is used to re-surface and re-fill cracks and defects in articular cartilage and meniscus.
6. The method of claim 1 wherein minced donor tissue is used to secure or re-bond loose tissues, such as a cartilage flap, in a defect site.
7. The method of claim 1 wherein minced donor tissue is used to fill spaces between mosaicplasty cylinders and interface them with native tissue.
8. The method of claim 1 wherein minced donor tissue is used as a bioadhesive for mechanically stabilizing a separate scaffold (e.g. collagen gels used in MACI procedures) or implant material to a defect site with minimal or no need for sutures.
9. The method of claim 1 wherein the donor tissue is subject to decellularization to remove epitopes which can cause acute inflammatory responses and pathogens including HIV using detergents, hydrogen peroxide, sodium hydroxide.
10. The method of claim 1 wherein the donor tissue is minced by, but not limited to: homogenizing, cutting, chopping, crushing, slicing and processing with a microtome.
11. The method of claim 1 wherein the donor tissue is any soft tissue including cartilage, nucleus pulposus, synovial fluid, vitreous humor, brain, spinal cord, muscle, and liver, and particularly tissues with high carbohydrate content which can be oxidized.
12. The method of claim 1 wherein the cross-linking moieties 1) are aldehyde groups to be reacted with an amine-bearing linker molecule in a Schiff base linkage to achieve adhesion, or 2) use Michael addition reactions, or 3) use enzymatic reactions
13. The method of claim 1 wherein aldehyde reactive groups are formed in minced tissue by chemical oxidation with reagents such as sodium periodate, sodium (meta) periodate, hydrogen peroxide (H$_2$O$_2$), horse radish peroxidase with H$_2$O$_2$, and sulfo-S-4FB.
14. The method of claim 1 wherein aldehyde reactive groups are formed in minced tissue by physical oxidation utilizing methods such as treatment by low temperature plasma and UV exposure, also in combination with hydrogen peroxide.
15. The method of claim 1 wherein aldehyde reactive groups are formed in minced tissue by incubation of tissue with light-absorbing dyes such as methylene blue, riboflavin, benegal rose, eosin Y in conjunction with light at wavelength from 200-1000 nm, but preferable in the UV range.
16. The method of claim 1 where thiol groups are exposed or incorporated in the tissue pieces by surface modification and cross-linked into a scaffold via reaction with Michael-type acceptors including, but not limited to, acrylate esters, acrylonitrile, acrylamides, maleimides, alkyl methacrylates, cyanacrylates and vinyl sulfones.
17. The method of claim 1 wherein the overall dry weight of the components in the implant scaffolds is adjusted to match those of the native tissue surrounding the defect site.
18. The method of claim 1 wherein the degree of surface modification of tissue fragments and/or linker molecules is adjusted to optimize the stoichiometric relations of the cross-linking reactions and to promote highest degree of adhesion within the scaffold and adhesion between scaffold and defect site.
19. The method of claim 1 wherein the patient’s injured tissue is also treated in the same way as the tissue fragments in order to cross-link the implant material directly to the injury site.
20. The method of claim 1 wherein pure oxidized carbohydrates can be added to the mixture, where the carbohydrates can be, but are not limited to, chondroitin sulfate, dermatin sulfate, hyaluronic acid, dextran.
21. The method of claim 1 where the linker contains amino groups such as chitosan, polydopamine, silk, poly-l-lysine, or amino-containing peptides.
22. The method of claim 1 wherein oxidized tissue fragments are used as bioinks for additive manufacturing of tissue constructs to mimic zonal distribution of matrix components, including adhesion of lubricative proteins and molecules to the surface layer.
23. A method for controlling the layer by layer arrangement of the implant for biomimicking of native tissue and the layer-by-layer application of the linker molecule.
24. The method in claim 1 wherein the implant is bioprinted.
25. A method to tune gelation time by altering the ratios of the mixed components to one another.

References


Patent III Microtissues

Abstract

A method of making a three-dimensional microtissue comprising the reaction of at least two cytocompatible polymer solutions, each polymer comprising complementary reactive groups capable of spontaneous reaction, at least one of which solutions additionally contains cells.

The method allows the rapid, efficient production of microtissues useful for purposes such as pharmaceutical screening and tissue reconstruction.

This disclosure relates to microtissues adapted for a variety of uses, including forming the basis of pharmaceutical screens.

It has been proposed to make microtissues for use, for example, as pharmaceutical screens using fluorescent detectors, for example, reagent-based fluorescent assay or cells genetically modified to express a fluorescent promoter reporter. Traditionally, these have been two-dimensional surfaces. It is recognised that three-dimensional surfaces would be better in this regard (see, for example, Kisaalita WS. “3D cell-based biosensors in drug discovery programs: Microtissue engineering for high throughput screening”: CRC Press; 2010.). Such 3-D structures also have the potential to create building blocks for use in tissue engineering (see, for example, Elbert DL. “Bottom-up tissue engineering. Current opinion in biotechnology”. 2011;22:674-80 and Fennema E, Rivron N, Rouwkema J, van Blitterswijk C, de Boer J. “Spheroid culture as a tool for creating 3D complex tissues”. Trends in biotechnology. 2013.)

The methods currently available to the art for the creation of such include centrifugation, hanging drop culture, gyratory shakers or spinner flasks. All of these methods, however, depend on the formation of cell-cell and cell-matrix adhesions based on receptors of the cadherin, ICAM and integrin families and thus require culture times on the order of hours, sometimes days, before a robust microtissue is formed. If the cell adhesion receptors are missing on the cell surface, as is often the case of cells which have been cryopreserved, the formation of the spheroid fails.

It has now been found that it is possible to make a desirable 3-D microtissue in as little as a matter of a few minutes. There is therefore provided a method of making a three-dimensional microtissue comprising the reaction of at least two cytocompatible polymer solutions, each polymer comprising complementary reactive groups capable of spontaneous reaction, at least one of which solutions additionally contains cells.

There is additionally provided a three-dimensional microtissue comprising a crosslinked composition of at least two cytocompatible polymers and cells, the crosslinking being provided by complementary reactive groups of two types, one of these types being present on each one of the cytocompatible polymers.
Although the present invention will be described with respect to particular embodiments, this description is not to be construed in a limiting sense.

Before describing in detail exemplary embodiments of the present invention, definitions important for understanding the present invention are given.

As used in this specification and in the appended claims, the singular forms of "a" and "an" also include the respective plurals unless the context clearly dictates otherwise.

In the context of the present invention, the terms "about" and "approximately" denote an interval of accuracy that a person skilled in the art will understand to still ensure the technical effect of the feature in question. The term typically indicates a deviation from the indicated numerical value of ±20 %, preferably ±15 %, more preferably ±10 %, and even more preferably ±5 %.

It is to be understood that the term "comprising" is not limiting. For the purposes of the present invention the term "consisting of" is considered to be a preferred embodiment of the term "comprising of". If hereinafter a group is defined to comprise at least a certain number of embodiments, this is meant to also encompass a group which preferably consists of these embodiments only.

Furthermore, the terms "first", "second", "third" or "(a)", "(b)", "(c)", "(d)", "(i)", "(ii)", "(iii)", "(iv)" etc. and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

In case the terms "first", "second", "third" or "(a)", "(b)", "(c)", "(d)", "(i)", "(ii)", "(iii)", "(iv)" etc. relate to steps of a method or use or assay there is no time or time interval coherence between the steps, i.e. the steps may be carried out simultaneously or there may be time intervals of seconds, minutes, hours, days, weeks, months or even years between such steps, unless otherwise indicated in the application as set forth herein above or below.

It is to be understood that this invention is not limited to the particular methodology, protocols, reagents etc. described herein, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention that will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art.

The cytocompatible polymers for use in this method may be any suitable polymers with the necessary cytocompatibility, that is, their presence is not harmful to cells. They may be natural or synthetic materials. The necessary complementary reactive groups may be already present on the polymers, or the polymers may be modified to include such groups. This is within the skill of the art in every case.
Typical non-limiting examples of natural polymers include alginate, alginate sulfate, chondroitin sulfate, dermatin sulfate, hyaluronic acid, cellulose, dextran, poly-l-lysine, chitosan, gelatin, silk and collagen.

Typical non-limiting examples of synthetic polymers include polymers, or polymers derived from, polyethylene glycol, polypropylene glycol, polaxomers, poly oxazolines, poly ethylenimine, poly vinyl alcohol, poly vinyl acetate, poly methyl vinyl ether-co-maleic anhydride, poly lactide, poly N-isopropylacrylamide, poly glycolic acid, poly methylmethacrylate, poly acrylamide, poly acrylic acid, polyallylamine.

The complementary reactive groups are those that will react spontaneously on being brought together. Many such reactive pairs are known to the art. Non-limiting examples of suitable complementary groups include inter alia those that result in Michael addition, disulfide bond formation, catechol-initiator polymerization, and enzyme-mediated crosslinking.

A typical complementary pair of reactive groups is the aldehyde/amine pairing that can lead to a Schiff base linkage. For example, aldehyde groups may be generated on biopolymers such as alginate, alginate sulfate, chondroitin sulfate, dermatin sulfate, hyaluronic acid, cellulose and dextran by chemical oxidation with suitable reagents, such as sodium periodate, sodium (meta) periodate and hydrogen peroxide. The equivalent amine-containing polymers may include proteins and peptides with a high percentage of lysine like chitosan, gelatin, silk, and collagen and synthetic polymers containing an amine group in their main or side chains like polyallylamine and polyethyleneimine. One may also chemically modified Chitosan. Chitosan naturally has primary amines in its monomer unit, but these prevent its solubility at pH 7.4 (pure chitosan can only be solubilized in acid). By introducing succinyl groups to some of the amines, one can break up these crystallinity domains and permit its solubility at physiological pH. It is important to balance N-substitution with succinyl groups to overcome the solubility barrier, but to leave behind enough of the amines so that they are available for Schiff base crosslinking later on. One preferred degree of substitution of amine groups by succinyl groups lies within the range of about 25 to about 45%, preferably within about 30 to about 40% and more preferably around about 35%.

Tests for the presence of aldehydes, and Schiff bases prepared by this reaction are well known in fields of chemistry as diverse as perfumery, dyestuffs and liquid crystals. However, it has never been seen as a means of crosslinking tissue to form a scaffold material.

As mentioned, the necessary reactive groups may be there naturally, for example, the amine functionality of amino acids. This may also be true of aldehyde or ketone functionality, but it may also be needed to be provided by surface modification. For example, a tissue may be modified by oxidation to provide the necessary groups or these groups attached with chemical linker molecules. Another possibility is to use a polymer that already contains the necessary groups.

Another possible complementary reaction may be thiol Michael addition reactions. In this case, thiol groups, which may be naturally present, for example, in amino acids such as cysteine, or introduced
chemically may be reacted with Michael acceptors including acrylate esters, acrylonitrile, acrylamides, maleimides, alkyl methacrylates, cyanoacrylates and vinyl sulfones.

In the specific example of the Michael addition, this may be achieved, for example, by the reduction of disulfide bonds in tissue and tissue surfaces with tris(2-carboxyethyl)-phosphine TCEP to introduce free sulfhydryl groups. This can also be achieved by the coupling of polymers with dithiobispropionic hydrazide (DTPH) with EDC followed by reduction to generate the free thiol, the conversion of amines to free thios by 2-iminothiolane (Traut’s Reagent), thiolation of proteins by N-succinimidyl S-acetyltio-acetate (SATA) and the conversion of oxidized glycosaminoglycans to free thios by 2-acetamido-4-mercaptopbutyric acid hydrazide (AMBH): The free sulfhydryls can participate in scaffold formation and adhesion via reaction with Michael-type acceptors including, but not limited to, acrylate esters, acrylonitrile, acrylamides, maleimides, alkyl methacrylates, cyanoacrylates and vinyl sulfones.

In the specific example of enzymatic crosslinking, crosslinking is effected by an enzyme. This method relies on the presence of functional groups, which are present already on the polymers or which can be generated thereon. Typical non-limiting examples include horseradish peroxidase + hydrogen peroxide which catalyze the formation of covalent linkages between hydroxyphenols and transglutaminase which catalyzes the covalent bond between a free amine and carboxamide group of glutamine containing materials.

One will usually use two different polymers carrying the respective complementary reactive groups. However, one may also consider to use the same type of polymer, with the polymer of the first and second polymer solution carrying the respective complementary reactive groups.

In terms of the reactive groups, it is preferred to have a stoichiometry of about 1:1. However, this is not narrowly critical, and a variation of up to 20% is tolerable. In the case of the aldehyde–amine reaction, it is preferred to have an excess of amines, as this is more tolerated by the cells.

The cells, which may be present in one or both polymer solutions, may be selected from any suitable cells, depending on the desired end use of the microtissue. Such cells may be selected from the group comprising inter alia mesenchymal stem cells, embryonic stem cells, stromal cells, liver cells including Kupffer cells and macrophages, hepatocytes, neural cells, pancreatic cells, kidney cells, muscle cells, monocytes, endothelial cells, fibroblasts, epithelial cells, chondrocytes, and cancer cells. In terms of embryonic stem cells, induced pluripotent stem cells (IPS) may be preferred. The afore-mentioned cell types may preferably be human cells. Preferably, these cells, when used for the purposes of the present invention, are outside the human or animal body.

In the context of the present invention, three-dimensional microtissues obtained by the methods described herein are also referred to as “QuickStick” (SCHI/OXALG). Particularly preferred microtissues may be using sChi and oxAlg.

In even more preferred embodiments, factors that induce and/or influence the development and/or differentiation of cells may be added to at least one of the polymer solutions to trigger development
and/or differentiation into the desired end product. Such factors may be provided in single from or in the form of cocktails, i.e. mixtures. Such factors may be selected from the group comprising *inter alia* growth factors, cytokines, chemokines, polypeptides, enzymes, hormones, as well as receptors for any of the afore-mentioned factors. They may be particularly suitable to induce and/or at least contribute to the development and/or differentiation of e.g. mesenchymal stem cells, embryonic stem cells, progenitor cells into the desired “end product” cell type(s). Such factors may be provided by non-covalent association with or preferably covalent attachment to at least one of the polymer solutions. One may also use media of different compositions as factors for inducing development and/or differentiation of e.g. mesenchymal stem cells, embryonic stem cells, etc. into the desired “end product” cell type(s).

Exemplary growth factors include *inter alia* morphogenetic protein families like IGF, FGFs, HGF, EGFs (e.g. EGF-1, VEGF), BMPs, (e.g. BMP-2, BMP-6, TGF-β3, and TGF-β1., TGF-β and its different forms can be used to trigger development of stem cells into *inter alia* neural, epithelial, endothelial, renal, hepatic, pancreatic, chondral, osteoblasts/osteoclasts, cardiac (cardiomyocytes), and myoblasts.

Exemplary polypeptides include *inter alia* RGD, GFOGER, tumor-specific polypeptides (e.g. pancreatic polypeptide, PP), and tumor antigens.

Exemplary cytokines include *inter alia* TNF-a, interleukin family, interferons, erythropoietin, and thrombopoietin.

Exemplary chemokines include *inter alia* CCL family, CXCL family, and CCR family.

One can also add the receptors for each of these bioactive molecules that might capture growth factors, cytokines, etc. secreted by the cells themselves within the microtissue.

In Example 7, microtissues were made using MSCs in the ‘standard’ QuickStick method with TGF either loaded in sChi or oxAlg. As the expression of analyzed genes was greatly increased, this shows that the growth factor was bound to the QuickStick polymer network and effectively induced the differentiation of the stem cells.

The above discussed embodiments of using growth factors, cytokines etc have a number of attractive advantages. Stem cells for clinical therapies can now be isolated from a patient and assembled in a QuickStick microtissue that has all the necessary components to induce the differentiation of the cells into the desired phenotype. They can be implanted at a site of injury or disease and will undergo differentiation within the body to heal or cure the injured tissue. Alternatively, *in vitro* experiments are possible using QuickStick microtissues loaded with different induction molecules for differentiating stem cells along various lineages into cell phenotypes that can interact with each other. One can imagine a network of different microtissues that provide complex functions mimicking the different organ systems of the body, e.g. pancreas, liver, bladder, kidney, muscle, cardiac, neural, osteochondral, vasculature, connective tissue, as well as microtissues for metabolizing complicated drugs that need enzymes from both or various
organs to be broken down. This goes towards the concept of having an ‘organ on a chip’ for screening analyses, or personalized medicine.

Thus, such microtissues such as pancreatic, hepatic, kidney, muscle, cardiac, neural, osteochondral, vasculature microtissues can be used screen for their influence of pharmaceutically active agents and to thus e.g. assess the metabolic fate of such pharmaceutically active agents. This is important information for drug development as it is e.g. known that drugs are differently metabolized by cytochrome P450 and as Example ... shows that expression of Cyp3a13 is different for microtissues of the present invention vs. three-dimensional microtissues of the prior art or two dimensional cells as obtainable in Petri dishes. As illustrated in Example 2, three dimensional microtissues made from mesenchymal stem cells, embryonic stem cells, induced pluripotent stem cells, etc. can be used to screen e.g. for agents that influence development and/or differentiation of a three-dimensional microtissue made from the group comprising mesenchymal stem cells, embryonic stem cells, induced pluripotent stem cells, etc. into e.g. downstream lineages.

For example, if a screen is desired, the cells may be genetically modified, so that they can fluoresce in the presence of compound hits. Fluorescence-based assays can be used to detect changes in proliferation, morphology, oxidative stress, cell signally, inflammation, cytotoxicity, genotoxicity. Detection can be performed on a plate reader, confocal microscope, two-photon microscopy to attain information on fluorescent intensity, distribution, polarization and variance with time. Preferred cells types are EGFP reporter cell lines.

There is therefore also provided a screen assay, comprising a microtissue as hereinabove described, in which the cells are adapted to fluoresce on exposure to a stimulus whose detection is desired.

The microtissues of this disclosure are prepared by first providing the cytocompatible polymers with the necessary complementary reactive groups, should they require modification, adding the desired cells to one or both of the solutions and then combining them. Many of the crosslinking reactions occur spontaneously, such that a microtissue is formed and can be handled with forceps after only a few minutes. The microtissues may be made in sheets, pellets or spheroids. They can be made directly in the wells of screening plates.

The size of the microtissue will be determined by the number of starting cells. The number of cells used generally ranges from about 1000 to about 1 million. The quantity of polymer to be used with the chosen quantity of cells is determined by the ability to provide a microtissue that is sufficiently cohesive to be manipulated, for example, picked up with forceps. The quantities of polymer needed will vary in each individual case, depending on polymer type and desired degree of cohesion in a given application, but this can be readily determined in each case by routine, non-inventive experimentation.

Further different types of cells or the same type of cells may be combined with the polymer solutions to obtain different patterning (see Example 5), e.g. by sequential or simultaneous pipetting, etc. The different patterning is useful for a number of applications to model complicated intercellular interactions with microtissue technology (renal networks of endothelial and epithelial cells, liver microtissues with
hepatocytes and Kupffer cells, neural tissues with glial cells/astrocytes, co-cultures of stem cells and mature cell types which have been reported in the literature to have mutual benefits for maintaining cell phenotypes etc.). For example, in such co-cultures stem cells have been found to secrete factors that promote activity in mature cells.

The methods of the invention further allow to obtain microtissues, e.g. for screening purposes of drugs in the time range of less than about 10 days, less than about 9 days, less than about 8 days, less than about 7 days, less than about 6 days, less than about 5 days, less than about 4 days, less than about 3 days, less than about 2 days, less than about 1 day, than about 23 hours, than about 22 hours, than about 21 hours, less than about 20 hours, less than about 19 hours, less than about 18 hours, less than about 17 hours, less than about 16 hours, less than about 15 hours, less than about 14 hours, less than about 13 hours, less than about 12 hours, less than about 11 hours, less than about 10 hours, less than about 9 hours, less than about 8 hours, less than about 7 hours, less than about 6 hours, less than about 5 hours, less than about 4 hours, less than about 3 hours, less than about 2 hours, or even less than about 1 hour.

Another advantage of the microtissues of the present invention is that they allow for adapting their mechanical stability by controlling e.g. the extent of cross-linking of the polymers.

In a particularly preferred embodiment the present invention relates to three-dimensional microtissues of hepatocytes which are obtainable by the methods described herein. Such microtissue may be used for screening the metabolic fate of pharmaceutically active agents and in particular their metabolism by e.g. enzymes of the cytochrome P450 family.

Another particularly preferred embodiment of the present invention relates to three-dimensional microtissues of mesenchymal stem cells, embryonic stem cells, induced pluripotents stem cells (iPS), etc. which are obtainable by the methods described herein and wherein the methods make use of, preferably covalently cross-linked, growth factor such TGF-β with TGF-b3 being an example and/or cytokines.

In both of these particularly preferred embodiments the microtissues may be made using sChi and oxAlg.

The method allows considerable versatility. For example,

The possible uses of these microtissues include, but are not limited to
- Pharmaceutical screens
- Building blocks in tissue engineering.

The method is further described with reference to the following non-limiting examples, which depict particular embodiments.

The figures are as follows:
Figure 1 shows micrographs of the alcian staining of various microtissues. The size bars represent 200 microns (see Example 1 for details)
Figure 2A shows micrographs of a microtissue which has been immunohistochemically stained to detect collagen 2 protein using the monoclonal antibody II-II6B3 from the Development Studies Hybridoma Bank. Figure 2B is a control without any primary antibody. The size bars represent 200 microns.

Figure 3 is a graph displaying glycosaminoglycan contents of pellets in chondrogenic media (C), non-chondrogenic media (NC), and microtissues prepared according to Example 1 in chondrogenic media (QS C).

Figure 4 is a micrograph of bovine chondrocytes which have been transduced with a collagen 2 promoter – GFP construct. The cells are suspended in 2% agarose for 7 days and the collagen 2 reporter appears as green in several cells.

Example 1:
To form microtissues using the Schiff linkage technique, human mesenchymal stem cells (MSCS, Lonza Group Ltd, Basel, Switzerland) between p.6-p.9, human chondrocytes and human adipose derived stromal cells were suspended in a solution of 5 mg/mL sChi at a cell density of 2 x 10^6 cells/mL. Drops of 5 μL each of oxidized alginate were prepared on plastic ring structures to confine the drop and 10 μL of the cells + S-Chi were pipetted into individual droplets of oxAlginate. The substrate was turned upside down and the reaction was carried out in an incubator at 37ºC for 10 minutes. Microtissues were transferred to agarose coated-wells of a 96-well plate with fine-tipped forceps and cultured in chondrogenic media and growth factor free media. In parallel, the same cells were centrifuged to form micromass pellets (200k cells/each) for comparison and transferred to the same 96-well plate.

Figure 1 depicts the following possibilities:

Figure 1A – MSC-based microtissue formed via Schiff-base crosslinking using S-Chi and oxAlg with chondrogenic media.
Figure 1B – MSC-based microtissue formed via Schiff-base crosslinking using S-Chi and 20 oxAlg with non-chondrogenic media.
Figure 1C - MSC-based microtissue formed via centrifugation (art-standard method) with chondrogenic media.
Figure 1D - Adipose stem cell-based microtissue formed via Schiff-base crosslinking using S-Chi and oxAlg with chondrogenic media.

Figure 1E – Adipose stem cell-based microtissue formed via Schiff-base crosslinking using S-Chi and oxAlg with non-chondrogenic media.

Figure 1F - Adipose stem cell-based microtissue formed centrifugation (art-standard method) with chondrogenic media.

Microtissues from Lonza MSCs formed via Schiff-base crosslinking using S-Chi and oxAlg demonstrated better chondrogenic induction than centrifuged pellets as indicated by alcian blue staining. The intensity of the blue color shows the amount of negatively charged glycosaminoglycans produced by the cells, so in A there is much more of this molecule than in the art standard method. The practical implication is that the tissue generated looks much more like cartilage (which would also be stained dark blue).

Figure 2 shows a microtissue stained using a primary antibody against collagen 2, which is a marker of cartilage. An anti-mouse IgG1 secondary antibody and colorimetric reaction was used to visualize the localization of the protein. GAG or glycosaminoglycans are the other important part of cartilage.

Figure 3 shows that the amount of GAG was the highest in the microtissue (“QS C”), as opposed to the centrifuged (Centrif) with chondrogenic (C) media and centrifuged with non-chondrogenic (NC) media respectively. Centrifugation is the art standard way of making microtissues.

Figure 2 shows that there is more blue staining in D compared to the art standard (however this induction was not as strong as with the bone marrow-derived MSCs).

The data indicate that crosslinked microtissues provide an immensely beneficial environment for differentiating stem cells into cartilage cells, versus the traditional technique of the art (i.e. centrifugation).

Example 2: Use of collagen 2 promoter – GFP construct to detect individual cells which expression cartilage markers in 3D culture system.

Lentiviral chondrogenic promoter vectors comprised a transcriptional unit encoding for enhanced green fluorescent protein (EGFP) under the human collagen 2 promoter. A 6100 bp fragment of the human Col2 promoter was subcloned into pLVX-AcGFP-C1 by replacing the original CMV promoter. Subsequently,
AcGFP was replaced by EGFP to create pLVX-Col(6100)-EGFP. To create pLVX-Col(-6100)-EGFP, a 1500 bp fragment and subsequently a 4600 bp fragment are excised from pKL7 and ligated into corresponding sites. EGFP fragment was amplified by PCR and integrated downstream the 6100 bp Col2 promoter. Correct orientation of the integrated EGFP was verified by restriction analysis.

Transducing MSCs with Lenti-X viruses: For transduction, the medium of the cells (seeded 12-16 hr prior transduction) was adjusted to accommodate the addition of virus and polybrene. Polybrene at a final concentration of 4 μl/ml is used to reduce the charge repulsion between the virus and the cell membrane. The lentiviral stock was diluted with medium to obtain the desired MOI and added to the cells for transduction. After 8 to 24 hr, virus-containing transduction medium was replaced with fresh growth medium. Cells are cultured and put into 3D culture to induce chondrogenic differentiation in the presence of chondrogenic medium (Dulbecco's Modified Eagle's Medium (DMEM, Gibco 41966), containing 1% Antibiotic-Antimyocytic, 1% premixed IST, 100 μg/ml sodium pyruvate, 40 μg/ml L-proline, 50 μg/ml L-ascorbate-2-phosphate, 10 nM Dexamethasone, 10 ng/ml TGF-β3 (PeproTech) and 100 ng/ml BMP-2 (PeproTech))

As shown in Figure 4, in an environment which induces cartilage formation, the reporter lights up (these appear as white spots in Figure 4, green in reality). This experiment establishes the function of the collagen 2 promoter —GFP construct for detecting drugs and environmental conditions which induce cartilage matrix synthesis.

Example 3: Speed, reliability and gene expression of microtissue formation by QuickStick

Primary hepatocytes were isolated from 6 donor mice and microtissues were formed with the cells either by the QuickStick method or by the classical hanging drop method (see e.g. Kelm et al., „Microscale tissue engineering using gravity-enforced cell assembly“. Trends in biotechnology, 2004, see also Figure 5). For hanging drop, 20 thousand cells were pipetted per droplet into the lid of specialized well plate provided by Roche (see previous) in 15 μL droplets. The lids were turned upside down and the well plates were placed an incubator at 37°C and 5% CO2. Cells collect in the bottom of the droplet due to gravitational forces and, after 3-5 days, have formed into a microtissue due to cell-cell contacts. At 5 days, hanging drops were centrifuged down into wells of a conical 96-well plate at 50 xg for 30 seconds. For forming QuickStick microtissues, hepatocytes were suspended in a 5 mg/mL solution of N-succinyl chitosan (sChi) at 10,000 cells per μL. 2μL droplets of cells in sChi were mixed with 1uL droplets of 10 mg/mL oxidized
alginate. Crosslinking between sChi and oxAlg was carried out in an incubator at 37°C for 20 minutes. Microtissues were then transferred to wells of a 96-well plate for culture.

Microtissues according to the present invention can be obtained in less than a day and as short as within 15 minutes, while the conventional hanging drop method requires multiple days.

On day 7, hepatocyte microtissues were imaged with brightfield microscopy to determine whether the microtissue structure remained in-tact. If individual cells could be seen outside of the central spheroid structure, the microtissue was deemed not to have formed. At day 7, nearly 100% of the QuickStick microtissues remained in-tact and robust (see picture under graph, HD=Hanging Drop, QS = QuickStick), while less than 50% of hanging drop microtissues had survived (see Figure 6).

Furthermore, function of hepatocytes in QuickStick versus hanging drop microtissues was assessed over a period of 5 days in ex vivo culture in media composed of DMEM supplemented with 1% insulin-transferrin-selenium, 1% penicillin-streptomycin, and 20ng/mL hepatocyte growth factor. Expression of the Cyp3a13 gene, which is a member of the cytochrome P450 family responsible for the metabolism of over 50% of clinically active drugs, was evaluated at days 1, 3, and 5 during culture of hepatocyte microtissues. The expression level of the cyp3a13 gene was normalized to the value observed in freshly isolated hepatocytes (i.e. cells analyzed immediately after isolating from murine donors), which is the value taken to be the 100% expression level. The housekeeping gene used for calculating fold change in expression was mRPS29.

Hepatocytes in QuickStick microtissues displayed 70% expression of Cyp3a13 day 1 in culture compared to freshly isolated hepatocytes, but the expression level increased to values at ana above 100% by days 5 and 7. Hanging drop microtissues could only be analyzed earliest at day5 because they take much longer to form and their day 1 value was diminished to 20% of the normal expression level. It is known that hepatocytes increase the expression of these enzymes over time in hanging drop culture (Messner et al., 2013. “Multi-cell type human liver microtissues for hepatotoxicity testing.” Archives of Toxicology. Springer-Verlag. 209-213.). As a control, hepatocytes were seeded in 2D in a standard 6-well plate where their expression of cyp31a13 started very low and diminished to 0 by day 3 (see Figure 7).

**Example 4: Mechanical properties of microtissue obtained by QuickStick**

Microtissue mechanical properties can also be tuned by selection of appropriate crosslinking agents. QuickStick microtissues of mesenchymal stem cells were seeded with crosslinking between sChi and either
oxidized HA (oxHA), oxidized (oxCS), or oxidized alginate (oxAlg). and the microtissue compressive moduli were calculated on a texture analyzer probe. Stiffness of the microtissue could be correlated to varying the crosslinking material (see also Figure 8).

Example 5: Patternning of microtissue obtained by QuickStick

Rapid patterning of different cell populations in a microtissue network was achieved by alternating mixing of different cell populations suspended in sChi. MSCs were separated and labeled with either CellTracker green (CMFDA) or CellTracker red (CMPTX) molecules commercially available from Life Technologies. Sequential pipetting of the cells already suspended in sChi into droplets of oxAlg results in local crosslinking which enables distinct localization of cells from the different populations in specific regions withn the microtissues shortly after seeding (see Figure 9).

Example 6: Combination of growth factors with microtissue obtained by QuickStick

Layers of the sChi and oxAlg were diposed on a standrad microscopy slide (see e.g. Figure 10A) and then incubated with the growth factor TGF-beta3 on top for some time. With immunohistochemistry, the binding of the TGF to the polymer matrix was visualized (see Figure 10B).

On the left are layers of sChi and oxAlg that have reduced number of available binding sites for TGF, but still show some presence of the growth factor. On the right, layers were made with lower degree of crosslinking and show much higher binding of the TGF to the polysaccharide network.

Example 7:

In this study, microstissue were made using MSCs in the QuickStick method without growth factor, state of the art hanging drop and centrifugation, and the growth factor loading with 100ng of TGF either loaded in sChi or oxAlg. For these experiments, TGF was provided in media surrounding QS and centrifuged samples, but not provided in samples that had the TGF loaded inside of the microtissues. The expression of all genes was greatly increased or compatible in the sChi100 samples versus the others. This shows that the growth factor was bound to the QuickStick polymer network and effectively induced the differentiation of the stem cells (see Figure 11).

For loading of the growth factor, TGF-beta 3 was solubilized by a 5mg/mL solution of sChi and a concentration of 10ng/uL. This TGF containing sChi was used to make QuickStick tissues in exactly the
same way as normal QuickStick microtissues with the difference being exclusion of the growth factor in media surrounding the tissues for the duration of the experiment.

By loading TGF-β3 at different concentrations into sChi (25, 50, 75, and 100 ng TGF-β3), it was shown that the dose of the loaded growth factor effects the outcome of the stem cell differentiation (i.e. that it can be controlled by loading different amounts of the growth factor during microtissue formation) (see Fig. 12).

In the context of the present invention, the following embodiments are contemplated:

1. A method of making a three-dimensional microtissue comprising the reaction of at least two cytocompatible polymer solutions, each polymer comprising complementary reactive groups capable of spontaneous reaction, at least one of which solutions additionally contains cells.

2. A method according to embodiment 1, in which the polymer is a biopolymer.

3. A method according to embodiment 2, in which the biopolymer is selected from the group consisting of alginate, alginate sulfate, chondroitin sulfate, dermatin sulfate, hyaluronic acid, cellulose, dextran, poly-l-lysine, chitosan, gelatin, silk and collagen.

4. A method according to embodiment 1, in which the polymer is a synthetic polymer.

5. A method according to embodiment 4, in which the polymer is selected from, or is derived from, the group consisting poly ethylene glycol, poly propylene glycol, poloxamers, polyoxazolines, polyethylenimine, poly vinyl alcohol, poly vinyl acetate, poly methyl vinyl ether-co-maleic anhydride, poly lactide, poly N- isopropylacrylamide, poly glycolic acid, poly methylmethacrylate, poly acrylamide, poly acrylic acid, polyallylamine.

6. A method according to embodiment 1, in which the complementary reactive groups are an aldehyde/amine pairing leading to a Schiff base linkage.

7. A method according to embodiment 1, in which the complementary reactive groups are participants in a Michael addition reaction.

8. A three-dimensional microtissue comprising a crosslinked composition of at least two cytocompatible polymers and cells, the crosslinking being provided by the spontaneous reaction of complementary reactive groups of two types, one of these types being present on each one of the cytocompatible polymers.
9. A screen assay, comprising a microtissue as hereinabove described, in which the cells are adapted to fluoresce on exposure to a stimulus whose detection is desired.

10. A screening assay, comprising a microtissue as hereinabove described, in which the readout of cell viability is measured by MTS assay, alamar blue, MTT assay, or other viability assay where a change in optical density or fluorescence in supernatant is correlated to cell quantity and/or activity.

11. A screening assay, comprising a microtissue as hereinabove described, in which biological changes within the microtissues as a response to stimuli is performed by analytical mass spectroscopy.

12. A screening assay, comprising a microtissue as hereinabove described, in which deposition of endogenous extracellular matrix components are measured by a dye which binds specifically to said extracellular matrix components and produces a colorimetric product that can be measured by spectrophotometry (e.g. dimethylmethylene blue for glycosaminoglycan deposition or hydroxyproline measurements for collagen production).

13. A screening assay, comprising a microtissue hereinabove described, in which changes in gene expression of encapsulated cells is measured by quantitative real time polymerase chain reaction (qPCR).

14. A screening assay, comprising a microtissue hereinabove described, in which changes in cell number within the microtissue is quantified by fluorimetric dye binding to DNA (e.g. PicoGreen).

15. A screening assay, comprising a microtissue hereinabove described, in which morphological changes are monitored online by automated brightfield microscopy and software to calculate morphological features such as optical density, ellipticity, budding, spherical integrity, and diameter.

CLAIMS:

1. A method of making a three-dimensional microtissue containing cells comprising at least the step of:
   - Providing at least a first cytocompatible polymer solution comprising at least one first cytocompatible polymer,
   - Providing at least a second cytocompatible polymer solution comprising at least one second cytocompatible polymer,
   - Reacting said first and second cytocompatible biopolymer solution with each other to obtain a three-dimensional matrix by forming covalent bonds between said first and second cytocompatible polymer,
   wherein the at least first cytocompatible polymer and at least second cytocompatible polymer comprise complementary reactive groups capable of spontaneous reaction, and
   wherein at least one of the two cytocompatible polymer solutions additionally contains cells.
2. A method according to claim 1, wherein the at least first and at least second cytocompatible polymers in said at least two cytocompatible polymer solutions are made from the same polymer, with the two cytocompatible polymers comprising different, yet complementary reactive groups.

3. A method according to claim 1, wherein the at least first and at least second cytocompatible polymers in said at least two cytocompatible polymer solutions are made from different polymery, with the two cytocompatible polymers comprising different, yet complementary reactive groups.

4. A method according to any of claims 1, 2, or 3, wherein at least one of the at least first and at least second cytocompatible polymers is selected from the group of biopolymers.

5. A method according to claim 4, wherein both of the at least first and at least second cytocompatible polymers are selected from the group of biopolymers.

6. A method according to any of claims 1, 2, or 3, wherein at least one of the at least first and at least second cytocompatible polymers is selected from the group of synthetic polymers.

7. A method according to claim 5, wherein both of the at least first and at least second cytocompatible polymers are selected from the group of synthetic polymers.

8. A method according to any of claims 1, 2, 3, 4, 5, 6, 7, or 8, wherein a biopolymer is selected from or is derived from the group comprising alginate, alginate sulfate, chondroitin sulfate, dermatin sulfate, hyaluronic acid, cellulose, dextran, poly-l-lysine, chitosan, gelatin, silk and collagen, and wherein a synthetic polymer is selected from or is derived from the group comprising poly ethylene glycol, poly propylene glycol, polaxomers, polyoxazolines, polyethyleneimine, poly vinyl alcohol, poly vinyl acetate, poly methyl vinyl ether-co-maleic anhydride, poly lactide, poly N- isopropylacrylamide, poly glycolic acid, poly methylmethacrylate, poly acrylamide, poly acrylic acid, polyallylamine.

9. A method according to any of claims 1, 2, 3, 4, 5, 6, 7, or 8, wherein the complementary reactive groups are an aldehyde/amine pairing leading to a Schiff base linkage.

10. A method according to claim 9, wherein the complementary reactive groups are an aldehyde and amine group.

11. A method according to claim 9, or 10, wherein the complementary reactive groups are respectively either naturally comprised within the polymers or introduced by chemical modification.

12. A method according to any of claims 9, 10, or 11, wherein a polymer combination of at least succinyl Chitosan and oxidized alginate.

13. A method according to any of claims 1, 2, 3, 4, 5, 6, 7, or 8, wherein the complementary reactive groups are participants in a Michael addition reaction.
14. A method according to claim 13, wherein the complementary reactive groups are a Michael addition donor such as a sulfhydryl group and Michael addition acceptor such as acrylate esters, acrylonitrile, acrylamides, maleimides, alkyl methacrylates, cyanoacrylates and vinyl sulfones.

15. A method according to any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14, wherein the stoichiometry the reactive groups is about 1:1

16. A method according to any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15, wherein the cells are selected from the group comprising mesenchymal stem cells, embryonic stem cells, stromal cells, hepatocytes, neural cells, pancreatic cells, kidney cells, muscle cells, monocytes, endothelial, fibroblasts, epithelial cells, chondrocytes, osteoblasts, osteoclasts and tumor cells.

17. A method according to any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16, wherein factors that induce and/or influence the development and/or differentiation of cells are added to at least one of the polymer solutions to trigger development and/or differentiation of said cells into downstream lineages.

18. A method according to claim 17, wherein such factors are selected from the group comprising growth factors, cytokines, chemokines, ...

19. A method according to claim 18, wherein TGF-β is used to induce differentiation and/or development of mesenchymal stem cells.

20. A method according to any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19, wherein a three-dimensional microtissue is obtained microtissues in the time range of less than about 10 days, less than about 9 days, less than about 8 days, less than about 7 days, less than about 6 days, less than about 5 days, less than about 4 days, less than about 3 days, less than about 2 days, less than about 1 day, than about 23 hours, than about 22 hours, than about 21 hours, than about 20 hours, less than about 19 hours, less than about 18 hours, less than about 17 hours, less than about 16 hours, less than about 15 hours, less than about 14 hours, less than about 13 hours, less than about 12 hours, less than about 11 hours, less than about 10 hours, less than about 9 hours, less than about 8 hours, less than about 7 hours, less than about 6 hours, less than about 5 hours, less than about 4 hours, less than about 3 hours, less than about 2 hours, or even less than about 1 hour.

21. A three-dimensional microtissue obtainable by a method of any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20.

22. Use of a three-dimensional microtissue obtainable by a method of any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 for obtaining, pancreas, liver, bladder, kidney, muscle, cardiac, neural, osteochondral, vasculature, and/or connective tissue microtissues.
23. Use of a three-dimensional microtissue obtainable by a method of any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 for screening the influence of microtissues such as pancreas, liver, bladder, kidney, muscle, cardiac, neural, osteochondral, vasculature, and/or connective tissue microtissues on pharmaceutically active agents.

24. Use of a three-dimensional microtissue obtainable by a method of any of any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 for screening for agents that influence development and/or differentiation of a three-dimensional microtissue made from the group comprising mesenchymal stem cells, embryonic stem cells, induced pluripotent stem cells, etc.

25. A method of any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20, a three-dimensional microtissue of claim 21, or use of any of claims 22, 23, or 24, wherein a three-dimensional microtissues of hepatocytes is obtained.

26. A method of any of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20, a three-dimensional microtissue of claim 21, or use of any of claims 22, 23, or 24, wherein a three-dimensional microtissues of mesenchymal stem cells, embryonic stem cells, induced pluripotent stem cells (iPS), etc. is obtained and wherein the methods make use of, preferably covalently cross-linked, growth factor such TGF-β with TGF-β3 being an example and/or cytokines.

27. A method, a three-dimensional microtissue, or a use of any of claims 25, or 26, wherein the microtissues is made using sChi and oxAlg.
Curriculum Vitae

Name: Christopher Millan
Date of Birth: 18.03.1987
Nationality: U.S.A.
Present Address: Rieterstrasse 27, CH-8002 Zürich

Education

Swiss Federal Institute of Technology, Zürich
PhD in Health, Sciences, and Technology, May 2011-Feb 2015
Zürich, Switzerland

Swiss Federal Institute of Technology, Zürich
Master of Science in Biomedical Engineering, April 2011 (5.5/6)
Zürich, Switzerland

Tulane University
Bachelor of Science in Biomedical Engineering, May 2009
New Orleans, LA
Cum Laude with Departmental Honors

Research Experience

ETH Zürich Cartilage Engineering + Regeneration Lab
Doctoral Thesis Work
Advisor: Prof. Marcy Zenobi-Wong, PhD
Developing biologically inspired materials for controlling adhesion between neighboring cells and adhesion between repair cells and damaged tissue. My current focus is in cartilage repair, but recent findings will have broad implications for the field of tissue regeneration and 3D cell culture.
Zürich, Switzerland

ETH Zürich Laboratory for Biologically Oriented Materials
Master Thesis
Advisor: Prof. Viola Vogel, PhD
Investigated organization of cells on substrates of varying elasticities. Polyacrylamide gels were functionalized with cell adhesion proteins and their chemical and mechanical properties were characterized. Myoblasts were seeded on such gels and their organizational patterns were analyzed with software developed with the LabView platform.
(5.75/6, September 2010-April 2011)
Zürich, Switzerland

Novartis Pharmaceuticals, Institute for BioMedical Research
Internship
Advisor: David Feldman, PhD
Project involved monitoring early stage gene expression changes in cells participating in the inflammatory response as a potential target for drugs to treat atherosclerosis. (June 2010-September 2010)
E. Hanover, NJ
Curriculum Vitae

Harvard Medical School
Department of Neurosurgery, Summer Research Assistant
Advisor: Yang Teng, MD, PhD
Assisted in implantation surgeries for an in vivo study of a spinal cord injury therapy involving biodegradable polymer scaffolds seeded with neural stem cells. Performed post-operation behavior analysis/care. Image analysis of spinal cord MRIs and carried out histological analysis of sectioned spinal cord tissue with immunohistochemistry. (Summer 2008)

Massachusetts Institute of Technology, Department of Chemical Engineering
Undergraduate Research Opportunities Program
Advisor: Robert S. Langer, ScD
Experimented with different drug delivery devices to administer a neuroprotective drug in treatment of spinal cord injury. Determined the release kinetics for Minocycline-encapsulated PLGA microparticles. Responsible for tissue culture and in vitro viability assays for primary neurons. (Summer 2007)

Honors and Awards
Best Oral Presentation, Swiss Society for Biomaterials (2014)
Best Student Poster, Swiss Society for Biomaterials (2012)
First-place at Senior Team Project Design Show, Tulane (2009)
Dean’s Grant for Summer Research Project, Tulane (2008)
Distinguished Scholar’s Award, Tulane University (2005-2009)

Profile
Software: MatLAB, PSpice, LabVIEW, ImageJ, Adobe Suite, Office
Computer Languages: C/C++

Hobbies and Interests
Sports – soccer, basketball, and golf
Cycle touring—Zürich to London in 2010, Sardinia in 2013
Head Coach – Tulane women’s soccer (2007-2009)
Music – Huge fan of live New Orleans music (funk, blues, and jazz)
Fiction and literature

Publications


Patents


Research Mentoring

David Miranda Nieves, Summer Exchange from MIT, “Use of Schiff base crosslinking to control cell and tissue adhesion for cartilage tissue engineering” (June – August 2012)

Michela Puddu, Master Thesis, “Encapsulating cells within adhesive nanofilms for mediating cell-cell interaction in 3D culture” (March – September 2012)

Jocelyn Bailey, Summer Exchange from UCLA, “Synthesis and characterization of FITC labeled N-succinyl chitosan” (June-August 2013)

Peter Ammann, Semester Project, “Establishing quantitative assays for measuring MSC chondrogenesis in QuickStick microtissues” (July 2013)
Peter Ammann, Master Thesis, “QuickStick Microtissues for 3D hepatocyte cultivation” (September ’13 - March ’14)

Alain Haeller, “Development of a bovine ex vivo cartilage defect model” (July-September 2013)