BIOPRINTING OF (FIBRO)CARTILAGE

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Supervisor: Lise De Moor

A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Science in the Biomedical Sciences

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PREFACE

The accomplishment of this dissertation would not have been possible without the support of various individuals. Their names cannot all be mentioned onto a single page. Nonetheless, I would like to acknowledge my appreciation to the following people.

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<tr>
<th>Abbreviation</th>
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<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>AB</td>
<td>Alcian blue</td>
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<tr>
<td>AC</td>
<td>Articular chondrocytes</td>
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<td>ACI</td>
<td>Autologous chondrocyte implantation</td>
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<tr>
<td>Alg-MOD</td>
<td>Methacrylamide-modified alginate</td>
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<tr>
<td>BF</td>
<td>Brightfield</td>
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<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>BS</td>
<td>Blocking solution</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CaPi</td>
<td>Calcein-AM/propidium iodide</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>DAB</td>
<td>3,3’diaminobenzidin</td>
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<tr>
<td>DS</td>
<td>Degree of substitution</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>FC</td>
<td>Fibrochondrocytes</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>Gel-MOD</td>
<td>Methacrylamide-modified gelatin</td>
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<tr>
<td>hBM-MSC</td>
<td>Human bone marrow-derived mesenchymal stem cells</td>
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<tr>
<td>HE</td>
<td>Haematoxylin-eosin</td>
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<td>HIF-1α</td>
<td>Hypoxia-inducible factor-1α</td>
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<td>IGF-1</td>
<td>Insulin growth factor-1</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>iPSC</td>
<td>Induced pluripotent stem cells</td>
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<tr>
<td>Irg</td>
<td>Irgacure 2959</td>
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<tr>
<td>ITS</td>
<td>Insulin-transferrin-selenite</td>
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<tr>
<td>Li-TPO</td>
<td>Lithium phenyl-2,4,6-trimethylbenzoylphosphinate</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
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<tr>
<td>NRS</td>
<td>Normal rabbit serum</td>
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<tr>
<td>P/S</td>
<td>Penicillin/streptomycin</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<tr>
<td>PG</td>
<td>Proteoglycan</td>
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<tr>
<td>PI</td>
<td>Photo-initiator</td>
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<tr>
<td>PS</td>
<td>Picrosirius red</td>
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<tr>
<td>RGD</td>
<td>Arginine(Arg)-Glycine(Gly)-Aspartic acid(Asp)</td>
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<td>SOX-9</td>
<td>Sex-determining region Y (SRY)-box-9</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor</td>
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<td>UV-LED</td>
<td>Ultraviolet light-emitting diode</td>
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SUMMARY

Bioprinting has emerged as a promising technology for tissue engineering whereby a bioink is rapidly deposited into a designed configuration with high spatial control. In particular, bioprinting is a rising technique within (fibro)cartilage tissue engineering. The ultimate objective is the discovery of alternative treatments for (fibro)cartilage defects due to disease or trauma. Within the present study, the bottom-up approach was applied towards 3D bioprinting a (fibro)cartilaginous construct. Human bone marrow-derived mesenchymal stem cells were seeded onto non-adhesive agarose microchips for the formation of spheroids. The ability of these micro-building blocks to assemble into a larger construct was additionally investigated. Day 14 spheroids were encapsulated in either Alg-MOD or Gel-MOD with subsequent chemical curing in the presence of Li-TPO. Evaluation of their potential as bioink was performed. Additionally, Irgacure 2959 was tested in combination with Gel-MOD. Bioprinting of a (fibro)cartilaginous construct was attempted with the bioink composed of day 14 spheroids, Gel-MOD, and Li-TPO. Phenotype, viability and proliferative capacity of the spheroids were investigated each time. The chosen cell type formed stable and chondrogenic spheroids. Day seven spheroids were able to coalesce in suspension. Both hydrogels were shown to be cytocompatible. However, phenotypic support is subject to the biochemical and biomechanical properties of the biomaterial, the choice of PI and printing parameters. This study showed the possibility towards bioprinting a (fibro)cartilaginous tissue construct with a bioink composed of chondrogenic spheroids, Alg-MOD/Gel-MOD, and a photo-initiator (Irgacure 2959/Li-TPO).
1. INTRODUCTION

1.1. Cartilage

Cartilage is a strong, slightly pliable, and resilient form of connective tissue that is avascular, aneural, and alymphatic [1, 2]. Three major types can be distinguished within the human body: hyaline cartilage (e.g. present at the articulating surfaces of bones), fibrocartilage (e.g. present in the menisci), and elastic cartilage (e.g. present in the external ears). Cartilage exerts many different functions ranging from allowing articulations between skeletal elements to providing structural support and resistance to deformation whilst additionally permitting some degree of flexibility in the trachea, nose, ribs, ears, and pharynx [2-4]. These specific requirements account for a differing biochemical composition. However, a common feature shared by all types of cartilages is the sparsely distributed chondrocytes embedded within a dense extracellular matrix (ECM) that is predominantly comprised of water, proteoglycans (PGs), and collagen [3, 5].

1.1.1. Articular cartilage

Articular cartilage is a specialised type of hyaline cartilage that covers diarthrodial joints [6, 7]. It is responsible for distributing loads across the joints, providing a low-friction gliding surface, and acting as a shock absorber [6, 8].

1.1.1.1. Anatomy and biochemical content

Approximately 60-80% of articular cartilage is composed of water and electrolytes, whereas the remaining 20-40% is mainly comprised of ECM and chondrocytes [9]. Articular cartilage can be divided from top to bottom into four zones based on morphology: superficial zone, transitional zone, deep zone, and calcified cartilage zone (figure 1). Each zone varies with regards to cellular profile (size, shape, density), PG concentration, and collagen organisation [10]. The superficial zone is the thinnest layer composed of flattened ellipsoid chondrocytes, a low concentration of PGs, and a parallel alignment of densely packed collagen [6, 10]. Beneath the superficial zone lies the transitional zone, which has round chondrocytes, increased PG concentration, and random collagen organisation [6, 10]. The deep zone contains the lowest volume of chondrocytes, organised in a typical columnar arrangement [6, 10]. Moreover, the PG concentration is the highest in this zone and collagen fibers are oriented perpendicular to the subchondral bone [6, 7, 10]. The calcified cartilage zone contains a scarce population of round chondrocytes with a hypertrophic phenotype that synthesise collagen type X [6, 7]. This region is also characterised by the absence of PGs [10]. Transition from the deep zone to the calcified cartilage zone is marked by a visible border termed the tidemark which is a band of fibrils that serve as an anchor for collagen [10].

Figure 1 – Cross-sectional diagram of articular cartilage inspired by [7]. Cellular organisation in the different zones of articular cartilage is depicted on the left side, whilst zonal collagen fiber organisation is illustrated on the right side
Chondrocytes are the sole cell type present within articular cartilage and account for less than 5 – 10% of the total tissue volume [9]. Mature chondrocytes are completely encapsulated in the dense cartilage ECM which is primarily composed of collagen (10-20%), PGs (10-20%), and water (60-80%) [6]. Water provides nutrition and medium for lubrication, creating a low-friction gliding surface [6]. In addition, a variety of collagens exists within the ECM of articular cartilage wherein collagen type II is predominantly encountered and collagen types VI, IX, X, and XI constitute as a smaller part [6, 9]. Lastly, the majority of PGs found within articular cartilage are associated in aggregates (aggrecan) whilst a minor portion exists of smaller aggregates (biglycan, fibromodulin, and decorin) [6, 9].

1.1.1.2. Biomechanical properties
Collagen is the main contributor to the tensile properties of articular cartilage [11]. Since different zones vary in collagen organisation, the tensile properties differ among zones. The equilibrium tensile modulus of human knee joint cartilages values higher in the superficial zone (10.1 MPa) as compared to other zones (e.g. 5.4 MPa in the middle zone), which can be ascribed to collagen being most abundant and organised in the superficial zone [9]. Moreover, native articular cartilage sustains a constant state of static pre-tension caused by negatively charged PGs retaining fluid throughout the ECM [12]. PGs possess a high water-binding capacity due to their negatively loaded glycosaminoglycan (GAG) side chains that attract positively charged counterions and thereby water [3, 13]. Consequently, articular cartilage is also capable of withstanding compressive forces enabled by the high swelling pressure due to the combination of water and PGs whilst collagen imposes tension allowing the tissue to swell without rupturing [1]. Similar to the tensile properties, the compressive modulus varies depending on location as can be seen by the aggregate equilibrium moduli of human articular cartilage that may range from 0.1 to 2 MPa [9].

1.1.1.3. Injuries and treatment
Articular cartilage can develop defects following long-term wear or acute trauma [14]. However, damage to this tissue has an inherent limited regenerative potential due to its avascularity and low cellularity [14, 15]. Injuries to the cartilage are classified into two types according to the depth of the lesion: partial-thickness (chondral) and full-thickness (osteochondral) [9, 15]. Full-thickness lesions penetrate into the underlying subchondral bone allowing bone marrow to provide vascularisation and mesenchymal stem cells (MSC) to promote repair. Nevertheless, the spontaneous healing process in full-thickness injuries is merely transient as a fibrous tissue with weaker mechanical properties is formed at the defect sites leading to the early onset of degenerative osteoarthritis [15]. On the other hand, partial-thickness lesions only damage the zonal articular cartilage, rendering the defect site inaccessible to cells originating from blood, bone, and bone marrow [9]. Repair of these types of injuries is mostly reliant on the limited mitotic activities of resident chondrocytes [15]. Nonetheless, reparative activities of the chondrocytes typically cease before the cartilage defect is healed, thus also serving as a starting point for tissue degeneration as a result of lasting defect with reduced tissue function [9]. The poor intrinsic regenerative capacity of articular cartilage demands the importance of effective treatment methods in order to avoid further complications. Current treatment modalities include microfracture, mosaicplasty (osteochondral grafting), and autologous chondrocyte implantation (ACI) [9, 14, 15]. ACI involves the harvest of chondrocytes from a low weight-bearing area, followed by culture expansion in order to obtain a sufficient amount of cells and subsequent reinjection of the cells into the defect site [16]. Microfracture surgery encompasses the creation of microfractures in the underlying subchondral bone via drilling, shaving, or abrasion. Mosaicplasty comprises of taking osteochondral plugs by a cylindrical cutting device from a healthy cartilaginous area, followed by implantation at the site of defect. However, these cartilage repair therapies do not consistently produce hyaline repair tissue,
fill the entirety of the defect, and integrate repair tissue with adjacent native tissue [14]. To overcome these limitations, alternative strategies are being explored such as the implantation of Chondrospheres®, which are small spheroids composed of expanded autologous chondrocytes and their associated matrix, into the defects [14].

1.1.2. The meniscus
The menisci are a pair of fibrocartilaginous structures located medially and laterally between the femoral condyles and the tibial plateau [17-20].

1.1.2.1. Anatomy
Both menisci are crescent-shaped in superior view and wedge-like in cross section [21]. The superior portions of the menisci are concave, thus enabling effective articulation with their respective convex femoral condyles, whereas the inferior surfaces are flat to conform to the tibial plateaus [22]. This increase of congruity between the tibial plateau and femoral condyles allows stable articulation and load transmission across the tibiofemoral joint [17, 23]. Other functions include shock absorption, nutrition of the knee joint, joint lubrication, and proprioception [23].

In adult menisci, the vascularisation is restricted to the periphery. The medial meniscus is vascularised in the outer 10-30%, whilst the lateral meniscus is vascularised in the outer 10-25% [19].

1.1.2.2. Biochemical content
The meniscus is composed of 72% water and 28% organic matter, such as ECM and cells [18]. Three different cell types can be distinguished: fibroblast-like cells, fibrochondrocytes (FC), and cells of the superficial zone (figure 2A). The latter can be found at the surface and are fusiform in shape. FC are round or oval cells and are located in the inner two-thirds of the meniscus. The fibroblast-like cells, which are elongated cells, can be found in the outer one-third of the meniscus. Besides cells, the organic matter of the meniscus is also composed of collagens (75%), GAGs (17%), DNA (2%), adhesion glycoproteins (<1%), and elastin (<1%) [18]. Collagen is the main fibrillary component of the meniscus with its orientation and type depending on the location within the meniscus. The collagen fibers are predominantly circumferentially oriented with the occurrence of some radial fibers (figure 2B). However, collagen fibers are organised in an irregular network at the surface [19]. The inner one-third of the meniscus is composed of both collagen type I and type II, whilst the outer two-thirds predominantly contains collagen type I [24]. Another major constituent of meniscal tissue are the PGs [18]. PG distribution differs regionally with a higher content located in the inner two-thirds of the meniscus compared to the outer one-third [18, 25]. The main types of GAGs found within the human meniscus are chondroitin-6-sulphate, dermatan sulphate, chondroitin-4-sulphate, and keratin sulphate [18, 23]. A third component of the meniscus are the adhesion glycoproteins, such as fibronectin, thrombospondin, and collagen type VI, which serve as a link between ECM components and cells [18].
Figure 2 – The ultrastructure of the meniscus.
A Cellular heterogeneity found within the meniscus adapted from [26].
B Schematic diagram of collagen fiber orientation within the meniscus [19].

1.1.2.3. Biomechanical properties
The menisci are capable of withstanding many different forces such as shear, tension, and compression. Native meniscal tissue possesses a shear modulus of approximately 120 kPa. The tensile properties of the meniscus vary between the circumferential and radial directions. It is approximately 100-300 MPa circumferentially and 10-fold lower when compared radially. The meniscus resists axial compression with an aggregate modulus of 100-150 kPa [18].

1.1.2.4. Injuries and treatment
The menisci are subjected to extreme forces, which makes them prone to injury. Meniscal damage is one of the most common injuries seen by orthopaedic surgeons with an annual incidence of 66 to 70 per 100,000 people [27]. Injuries to the meniscus generally consist of tears in the tissue [28]. Single meniscal tears occur in one plane and are classified on the basis of their configuration (horizontal, radial, or longitudinal), whilst complex meniscal tears have components in multiple planes [29]. The ability of a meniscal lesion to heal, either spontaneously or after surgical repair, is influenced by the proximity of the tear to the vascular supply of the meniscus with the addition of the size and complexity of the tear [30]. Reparative surgery with sutures or staples is indicated for tears in the vascular zone of young patients that are amenable to repair [31, 32]. Involvement of the avascular zone requires tears to be treated with (sub)total meniscectomy [31].

1.1.2.4.1. Meniscectomy
Meniscectomy, which is the surgical removal of the whole or a part of the meniscus, used to be the gold standard in treatment [19]. Excision of the injured meniscus was considered innocuous as it was viewed as a functionless remnant. However, this concept has been refuted since (sub)total meniscectomy is now recognised to predispose to the development of osteoarthritis [17, 27]. Nonetheless, subtotal meniscectomy is still indicated in cases of symptomatic tears that are not amenable to repair and can still preserve meniscal function especially when the peripheral meniscal rim is intact [33]. Although the majority of the patients benefit from pain relief and functional improvement post-meniscectomy, some remain symptomatic which requires further treatment with a meniscal substitute [32].
1.1.2.4.2. Meniscal replacement
Following meniscectomy, the whole or a part of the meniscus can be replaced by a natural or synthetic implant.

As a natural implant, an autograft or an allograft can be used. Autografts can be derived from the fat pad, patellar tendon, quadriceps, periostium, or synovial flap [17, 31]. One advantage is the lack of an immune reaction. However, size limitation, longer time in surgery, cosmetic issues due to the harvesting of tissue, donor site morbidity, and the lack of biomechanical properties resembling that of the native meniscus are some constraints [17, 31]. When using an allogeneic meniscus, the limitations include risk of immune reaction, risk of disease transmission, and difficulty in shape matching [31]. Allografts are available in various forms including fresh, frozen, lyophilised, and cryopreserved [17, 22].

Besides natural implants, synthetic implants are used because they are easy to process, have minimal batch to batch variability, and have the possibility to tailor their mechanical and chemical properties [17]. However, one disadvantage is the risk of inflammation [17]. Currently, three synthetic implants are clinically available: two partial (CMI® and Actifit®) and one total substitute (NUsurface®) [32]. The CMI® (collagen meniscus implant) is a highly porous scaffold comprised of collagen type I fibers that are first isolated from purified bovine Achilles tendon before supplementation with GAGs. Most of the CMI® is expected to be resorbed after 12 to 18 months. Actifit® is a porous scaffold comprised of polycaprolacton combined with polyurethane. The expected degradation time of Actifit® is 4 to 6 years. Both partial substitutes stimulate regeneration of the native tissue [32]. As a total substitute, the NUsurface® is a free-floating meniscal implant consisting of polyethylene reinforced polycarbonate urethane. Unlike to aforementioned partial implants, the NUsurface® does not degrade and is currently still in clinical trial [32]. Since the current treatment methods cannot repair all types of meniscal lesions, research is being conducted towards the application of tissue engineering to produce an implantable construct.

1.2. Tissue engineering of (fibro)cartilage
Tissue engineering is defined as an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes to repair, enhance, or substitute diseased or missing tissues and/or organs [34]. Despite the body having intrinsic self-healing properties, the extent of repair varies amongst different tissues. For example, cartilage has a limited innate capacity for regeneration after damage due to its avascular nature [11, 35, 36]. Moreover, early injuries are not detected which can be ascribed to a lack of nerves within cartilaginous tissues and result in a delayed diagnosis [11]. Cartilage tissue engineering is emerging as a promising strategy for the treatment of cartilage defects due to disease or trauma.

1.2.1. Cell types
The selection of the most appropriate cell type is an indispensable aspect in order to successfully develop a (fibro)cartilage construct. To this end, a number of cell types have been proposed: FC, articular chondrocytes (AC), and various sources of stem cells [17, 26]. These cells may be of an autologous, allogeneic, or xenogeneic origin. Although, autologous cells are the most appropriate for tissue engineering since allogeneic and xenogeneic cells are immunogenic and thus require immunosuppressive therapy once the construct is implanted. In addition, these cell sources also pose the risk of disease transmission. Nevertheless, allogeneic and xenogeneic cell sources may have limited clinical use, they are critical for the in vitro development of translatable tissue engineering strategies [37].

For the creation of fibrocartilage, FC are considered to be a suitable cell type since they have the correct phenotype. They express both collagen type I and type II [26]. FC could be isolated during meniscectomy and used to generate a (fibro)cartilaginous tissue. From a clinical standpoint, cell harvesting from the meniscectomised meniscus itself is ideal since no additional morbidity is
produced [19]. Nonetheless, a limited cell availability renders the need of cell expansion that however leads to dedifferentiation. This implies a phenotypical change, namely the loss of the chondrocyte-like phenotype (seen by a decrease in expression of both collagen type II and aggrecan) and the adaptation of a more fibroblast-like phenotype (seen by an increase in expression of collagen type I) [5, 25]. Hence, redifferentiation strategies are needed.

For the engineering of articular cartilage, AC can be used. Autologous chondrocytes are already being used in clinical applications for the treatment of damaged articular cartilage, namely in ACI. Similar to FC, monolayer expansion causes dedifferentiation of AC that is also characterised by an increase in collagen type I expression and a decrease in PG and collagen type II synthesis [5, 38]. Thus, subsequent redifferentiation strategies are required. Nevertheless, this observation rationalises the use of AC as an alternative cell source for the engineering of fibrocartilage. A concern regarding the use of either FC or AC is the possibility of these cell types already being in a degenerated or diseased state, thus deeming their utility in tissue engineering questionable. Therefore, other cell types are being explored [18].

Stem cells are another potential cell source for the development of a (fibro)cartilaginous construct. They are unspecialised cells capable of self-renewal and differentiation. A classification can be made either based on origin (embryonic, foetal, and adult) or based on potency (totipotent, pluripotent, multipotent, and unipotent). The use of embryonic or foetal stem cells is not straightforward considering their origin. Adult stem cells do not possess this limitation. Mesenchymal stem cells (MSC) can be isolated from diverse tissues such as adipose tissue and bone marrow. They present as an attractive source for cartilage engineering due to their extensive proliferative capacity, their demonstrated chondrogenic potential, and anti-inflammatory effects [36]. Of the various sources of MSC reported, bone marrow derived stem cells (BM-MSC) are the most commonly used [16]. The differentiation of BM-MSC into chondrogenic cells can either be acquired by adding chondrogenic medium or by utilising the co-culture technique wherein BM-MSC are cultured with components of the joint cavity, such as synovial fluid or synovial cells, in order to induce chondrogenesis in vitro [5]. Limitations associated with BM-MSC include the highly invasive procedure of harvesting and low yields [36, 39]. Thus, another type of stem cells that have been proposed are the induced pluripotent stem cells (iPSC), which can be developed from differentiated adult cells that are reprogrammed to pluripotency. The iPSC possess the ability to differentiate towards chondrocytes [39, 40].

1.2.2. Culture conditions

Both FC and AC require monolayer expansion in order to acquire an adequate amount of cells. However, expansion is accompanied with dedifferentiation which renders the need of redifferentiation strategies. Furthermore, the use of stem cells, such as BM-MSC, necessitates chondrogenic differentiation to obtain a (fibro)cartilage-like phenotype. To this end, different strategies can be applied: three-dimensional (3D) culture, addition of biomechanical stimuli, and/or low oxygen tension. Cells grown in a 3D environment closely resemble a more normal functionality due to the increased cell-cell interactions and cell-ECM adhesions, thereby also mimicking the in vivo architecture of natural tissues and organs [41]. Spheroids of chondrocytes are shown to obtain redifferentiation [42]. Chondrocyte redifferentiation can also be obtained by the addition of growth factors to the culturing medium. Of the numerous bioactive molecules, the most commonly used growth factors for (fibro)cartilage tissue engineering include transforming growth factor-β (TGF-β) superfamily, basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1) [43]. However, it was shown that TGF-β1 is the most effective growth factor to stimulate the production of both collagens and GAGs [44]. Furthermore, growth factors, such as TGF-β and bone morphogenetic protein (BMP), are used to induce chondrogenesis in BM-MSC [16]. Besides biochemical stimuli, biomechanical stimulation may be utilised to assist with and enhance the development, remodelling, and maturation of
(fibro)cartilaginous tissue constructs [43]. Lastly, (fibro)cartilage is an avascular tissue. Consequently, the resident cells are exposed to a low oxygen tension in vivo. Mimicking this environmental condition can be obtained by culturing the cells in a low-oxygen incubator (5% O₂). Hypoxia has been shown to enhance redifferentiation through upregulation of the cartilage specific marker collagen type II and to a lesser extent the upregulation of collagen I [25]. Hypoxia-inducible factor (HIF)-1α, a positive regulator of chondrogenesis, and TGF-β have been shown to enhance the promotor activity of SOX-9 (sex-determining region Y (SRY)-box-9) and increase its expression [40]. SOX-9 is a key molecule that regulates chondrocyte differentiation and cartilage formation as it encodes a high mobility group DNA binding domain and associates with the SOX-9 binding sites on promoters/enhancers of cartilage specific genes such as collagen type II and aggrecan [40].

1.2.3. **Top-down and bottom-up approach**

A distinction can be made between two strategies within the field of tissue engineering, namely the top-down approach and the bottom-up approach (figure 3). In the traditional top-down method, cells are seeded on a preformed, porous, biocompatible, and biodegradable polymeric scaffold [45-47]. The cells are expected to populate the scaffold, produce ECM, and replace the degrading scaffold. However, with this strategy it is difficult to both recreate the intricate microstructural features of the tissue and to accurately control the distribution of cells [45, 48]. Other drawbacks are limited ECM deposition in the core of the scaffold and scaffold degradation that is rarely synchronised to neotissue formation. The bottom-up method has been developed to overcome these shortcomings. In this approach, small building blocks called microtissues are generated through the fabrication of cell-laden microgels, cell aggregation, or the creation of cell sheets. These microtissues are then assembled to form a macrotissue through random assembly, directed assembly, stacking of layers, or magnetic assembly [46, 47].

![Figure 3 – Schematic overview of the two different approaches for tissue engineering adapted from [47].](image)

A The top-down approach utilises cells and a biodegradable scaffold for the development of an engineered tissue.
B The bottom-up approach generates tissue building blocks that are used to create a macrotissue via multiple assembling methods.
1.2.3.1. Top-down tissue engineering of (fibro)cartilage

When using the top-down approach, the selection of a suitable scaffold is essential. The ideal (fibro)cartilaginous scaffold should pertain the following demands: (i) mechanical competence; (ii) biocompatibility and biodegradability, meaning that both the scaffold itself as its degradation products should not evoke any foreign-body reaction and the scaffold should be gradually replaced by biologic tissue; (iii) high porosity is wanted to allow diffusion of nutrients and waste products; and lastly (iv) from a clinical point of view the scaffold must be shaped into different sizes and forms, and must be easy to handle, to suture, and to be implanted by the surgeon [16, 19]. A distinction can be made between two types of scaffolds based on their composition; they can be either composed of a natural or synthetic material [17, 19, 28, 49]. As a natural scaffold periosteal tissue, perichondral tissue, small intestine submucosa, or acellular meniscal tissue can be used. However, the disadvantages of these biological tissues are the low intrinsic mechanical properties and the inability to tailor the pore geometry and composition. On the other hand, isolated tissue components, such as collagens or PGs, can be reconstituted into tailor-made scaffolds with optimal three-dimensional (3D) architecture whilst maintaining the high biocompatibility of the whole tissues. However, these scaffolds usually have low biomechanical properties and are characterised by rapid biodegradation. Besides natural materials, synthetic scaffolds are also an option. Synthetic materials present as an attractive option as they are easily processed, offer minimal batch to batch variability, and their mechanical and chemical properties can be tailored. Moreover, the biodegradation rate can be modulated by acting on polymer composition. Examples of synthetic scaffolds include: polyglycolic acid (PGA), poly-L-lactic acid (PLLA), polyurethane, polyester carbon, and polycaprolactone (PCL). Possible drawbacks regarding the use of synthetic materials are the low cell-adhesive properties, due to a lack of cell-adhesion domains present on natural macromolecules, and the risk of inflammation occurring due to a foreign-body reaction following implantation [19].

1.2.3.2. Bottom-up tissue engineering of (fibro)cartilage

The general concept of the bottom-up approach involves the fabrication of 3D cellular building blocks that are subsequently assembled into a larger construct. For the biofabrication of a (fibro)cartilaginous construct, spheroids can be utilised as the building blocks. To create a larger tissue construct, these spheroids can be combined with a hydrogel, creating a bioink to 3D bioprint a macrotissue.

1.2.3.2.1. Spheroid fabrication

The first step within the bottom-up approach is the generation of (fibro)cartilaginous spheroids. To create these microtissues, several methods can be used, each with their own (dis)advantages (table 1). Hanging drop culture is the earliest technique recorded. After inverting a plate with droplets of cell suspension, the cells spontaneously aggregate in the bottom of a drop resulting in the formation of spheroids [50]. Alternatively, spheroids can be generated through seeding cells in non-adhesive agarose microwells [25]. Through gravitational force, the cells lower into the bottom of the wells and form spheroids, as they rather interact with each other than with the agarose. The liquid overlay method involves culturing cells on a non-adhesive substrate, whereas the spinner flask prevents adhesion of cells on the materials' surface by using continuous stirring [50, 51].
Table 1 – Different techniques used for spheroid fabrication adapted from [41, 50, 51].

<table>
<thead>
<tr>
<th>Technique</th>
<th>Illustration</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanging drop</td>
<td></td>
<td>• Cheap</td>
<td>• Low-throughput</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Does not require specialised equipment</td>
<td>• Labour intensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Good size and shape control</td>
<td>• Only allows cell culture for limited periods of time</td>
</tr>
<tr>
<td>Agarose microwell</td>
<td></td>
<td>• Good size and shape control</td>
<td>• Requirement of specialised equipment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• High-throughput</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Highly reproducible</td>
<td></td>
</tr>
<tr>
<td>Liquid overlay</td>
<td></td>
<td>• Cheap</td>
<td>• Variation in size, shape, and number of spheroids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Does not require specialised equipment</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Long-term cell culture</td>
<td></td>
</tr>
<tr>
<td>Spinner flask</td>
<td></td>
<td>• Production of a great number of spheroids</td>
<td>• Variation in size, shape, and number of spheroids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Long-term cell culture</td>
<td>• Requirement of specialised equipment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Cells are exposed to shear stress</td>
</tr>
</tbody>
</table>

1.2.3.2.2. Hydrogels

A hydrogel is a hydrated network formed by cross-linking hydrophilic polymers via physical or chemical stimuli [52]. Hydrogel materials generally exhibit good biocompatibility and high permeability for oxygen, nutrients, and other water-soluble metabolites which makes them an attractive scaffold for use in cell encapsulation [53].

1.2.3.2.2.1. Classification of hydrogels: origin and cross-linking mechanism

Hydrogels can be of a natural origin, synthetic origin or a combination of both. Natural derived polymers include proteins (such as collagen, gelatin, and fibrin) and polysaccharides (such as hyaluronic acid, alginate, and chitosan). These hydrogels are advantageous due to their similarities to the native ECM and are therefore able to support the cellular phenotype and function. Nevertheless, the use of natural hydrogels is often restricted due to concerns regarding potential immunogenic reactions, relatively poor mechanical properties, and often batch to batch variability [52, 53]. As an alternative, synthetic hydrogels include polyethylene glycol and polyvinyl alcohol. They present better control over their mechanical and chemical properties, and preclude the risk of disease transmission. However, these synthetic hydrogels lack bioactivity and thus need to be modified with bioactive molecules to stimulate a cellular response [52].

Cross-linking is a stabilisation process where bonds are formed that link one polymer chain to another, thus resulting in a network structure [54]. Hydrogels may be classified on the basis of their cross-linking mechanism: physical (reversible) hydrogels or chemical (permanent) hydrogels [54, 55]. However, some hydrogels are able to undergo both physical cross-linking as chemical cross-
linking. Physically cross-linked hydrogels are formed by ionic interactions, hydrogen bonding, or hydrophobic forces [55]. All these interactions are reversible and can be disrupted by changes in physical conditions such as pH, temperature, or addition of specific solutes. Chemical cross-linking introduces covalent bonding between polymer chains and are therefore permanent [53]. A specific chemical cross-linking method is photo-cross-linking whereby hydrogels are photo-polymerised in the presence of a photo-initiator and visible or ultraviolet (UV) light [56]. Photo-initiators (PIs) are light-sensitive compounds that have high absorption at a specific wavelength of light to produce radical initiating species. Examples of PIs are 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959), 2,2'-azobis (2-methyl-N-(2-hydroxyethyl)propionamide) (VA-086), and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (Li-TPO) (table 2). PIs can interact with visible and/or UV-A light and dissociate into two free radicals that can initiate polymerisation to form cross-linked hydrogels. Irgacure 2959 is the most commonly used photo-initiator by virtue of its moderate water solubility [57]. However, an absorption spectrum located around 279 nm poorly matches with wavelengths of light generally regarded as benign to living cells and thus necessitates the search for PIs with an absorption peak located more towards longer wavelengths [57, 58]. Examples of such investigated PIs are VA-086 and Li-TPO which both have an absorption maximum around 375 nm [57, 59].

Table 2 – Properties of different PIs adapted from [60].

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>Molecular Weight (g mol⁻¹)</th>
<th>Absorption peak λ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irgacure 2959</td>
<td>224.3</td>
<td>279</td>
</tr>
<tr>
<td>VA-086</td>
<td>288.3</td>
<td>375</td>
</tr>
<tr>
<td>Li-TPO</td>
<td>323.3</td>
<td>375</td>
</tr>
</tbody>
</table>

1.2.3.2.2.2. Hydrogels for (fibro)cartilage tissue engineering

For the 3D bioprinting of a (fibro)cartilaginous macrotissue, single cells or spheroids need to be combined in a supporting hydrogel to form the bioink. The use of hydrogels for tissue engineering must meet a number of requirements, such as classical physical parameters (adequate mechanical properties and controlled degradation), biological performance parameters (support of cellular phenotype), and sterilisability [61]. Hydrogels utilised for the biofabrication of (fibro)cartilage are hyaluronic acid, fibrin, alginate, collagen and gelatin. Gelatin is a derivative of collagen, formed by breaking the natural triple-helix structure of collagen into single-stranded molecules by hydrolysis [53, 61]. This presumably retains informational signals like the Arg-Gly-Asp (RGD) sequence, thus promoting cell adhesion, migration, differentiation, and proliferation [53]. Gelatin easily forms gels by changing the temperature of the solution. Similar to gelatin, alginate has been explored as a hydrogel for (fibro)cartilage tissue engineering owing to its good biocompatibility, biodegradability, and relatively low cost [61]. Moreover, simple gelation can be acquired with divalent cations, such as Ca²⁺ and Mg²⁺. However, both hydrogels present a problem when used in vivo. Gelatin is not stable at body temperature as it dissolves at temperatures above its upper critical solution temperature of 30-35 °C [62]. Alginate dissolves upon contact with a solution containing monovalent ions such as Na⁺. To overcome this, addition of methacrylate to the side groups of gelatin and alginate followed by photo-cross-linking with(out) prior physical curing results in a hydrogel that is stable at 37°C [63].
1.2.3.2.3. **3D bioprinting**

Bioprinting is an emerging technology within the field of tissue engineering and regenerative medicine. The ultimate goal of biofabricating healthy and functional tissues/organs is driven by the high demand in transplants that currently coincides with a donor shortage [64]. Many challenges however still exist with this technology before a potential paradigm shift in clinical practical can occur.

1.2.3.2.3.1. **Main 3D bioprinting technologies**

Multiple 3D bioprinting technologies are presently available. The main types that are utilised include: laser-assisted, inkjet-based, and extrusion-based bioprinting (figure 4).

Laser-assisted bioprinting (LAB) consists of three parts: a donor-slide, a laser source that produces a pulsed laser beam, and an energy absorbing layer (figure 4A). During the LAB process, a laser pulse is applied onto the absorbing layer that causes the biomaterial to evaporate and reach the substrate in droplet form [65, 66]. One advantage pertaining to this method is the absence of orifice clogging due to its nozzle-free characteristic. The main disadvantage of this technology, which has also been described as being somewhat cumbersome, is the high cost [64].

An inkjet-based printhead utilises either a thermal or piezoelectric actuator as a driving force to deposit droplets onto a substrate (figure 4B). The droplets are generated by a transient pressure for a piezoelectrically-induced inkjet printer or by heating for a thermally-induced inkjet printer [66, 67]. A concern regarding the use of a thermal actuator is the possible detrimental effects on the cells. However, research has shown that the high temperatures are localised and are present for a short time span [68]. A general disadvantage concerning inkjet-based bioprinting is that only biomaterials with restricted viscosities (liquid) can be printed with this technique [64].

Extrusion-based bioprinters use pneumatic or mechanical (piston or screw) dispensing systems to extrude a continuous filament, rather than single drops (figure 4C) [65]. Advantages include the ability of processing in mild conditions (room temperature) and the possibility to print with cellular spheroids [64].

<table>
<thead>
<tr>
<th><strong>Cell viability</strong></th>
<th>&gt; 95 %</th>
<th>± 90 %</th>
<th>4-80 %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resolution</strong></td>
<td>&gt; 20 μm</td>
<td>20-100 μm</td>
<td>200 μm</td>
</tr>
<tr>
<td><strong>Bioprinting speed</strong></td>
<td>Medium</td>
<td>Fast</td>
<td>Slow</td>
</tr>
<tr>
<td><strong>Cost</strong></td>
<td>High</td>
<td>Low</td>
<td>Medium</td>
</tr>
</tbody>
</table>

**Figure 4 – Characteristics of 3D bioprinting methods adapted from [64, 69].**

A Laser-assisted bioprinting utilises a laser pulse to deposit biomaterials onto a substrate.

B Inkjet-based bioprinting patterns cells and biomaterials into a desired shape utilising droplets ejected via thermal or piezoelectric processes.

C Extrusion-based bioprinting is a pressure-drive technology. Struts are extruded through a nozzle via either pneumatic or mechanical pressure.
1.2.3.2.3.2.  **Bioink requirements**

In all the aforementioned 3D bioprinting modalities, bioinks are an essential component, which should therefore meet certain requirements: (i) cytocompatibility (ensure cellular survival); (ii) printability (possess mechanical requirements for the bioprinting process); (iii) high shape fidelity post-printing; and lastly (iv) phenotypic support. Commercial bioinks have recently been introduced by CELLINK®[69]. Certain bioinks provided by this company include GelMA (Gelatin Methacrylate) and alginate.

1.2.3.2.3.3.  **3D bioprinting of (fibro)cartilage**

A heterogeneity in approaches concerning 3D bioprinting of (fibro)cartilage currently exists (table 3). In general, a variety of cell sources and biomaterials has been investigated in many different studies but the majority involved extrusion-based techniques [70]. The cell viability post-printing tended to vary between 70-90 % with evidence of cartilage ECM formation at the gene and/or protein level. Several reports on the biomechanical properties showed constructs with inadequate durability.

1.3.  **Aim**

Previous research within the Tissue Engineering Group (Ghent University) has led to the creation of qualitative (fibro)cartilaginous spheroids utilising porcine articular chondrocytes. The potential of methacrylamide-modified gelatin as a potential bioink in combination with these (fibro)cartilaginous microtissues has additionally been demonstrated.

The first aim of this master dissertation is to investigate the use of human bone marrow-derived mesenchymal stem cells (hBM-MSC) to generate qualitative (fibro)cartilaginous spheroids and examine their ability to assemble into a macrotissue. The second aim is to evaluate the influence of the hydrogel type (methacrylamide-modified gelatin or methacrylamide-modified alginate) with the utilised PI (Irgacure 2959 or Li-TPO) on morphology, morphometry, viability, proliferative capacity, and ECM production of hBM-MSC spheroids.
Table 3 – Summary of (fibro)cartilage 3D bioprinting studies.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Hydrogel composition</th>
<th>Cell type</th>
<th>Results (viability-phenotype-biomechanics)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inkjet-based bioprinting</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[71]</td>
<td>10 w/v% PEGDMA + 1.5 w/v% Gel-MA (Poly(ethylene glycol) dimethacrylate + gelatin methacrylate) + 0.05 w/v% Irgacure 2959</td>
<td>hBM-MSC</td>
<td>High level of cell viability (&gt; 80%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Single cells</td>
<td>Positive effect on chondrogenesis confirmed by gene expression, biochemical analysis, and histological data</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 x 10^6 cells ml^-1</td>
<td>Compressive modulus: 30-50 kPa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analysis: day 21</td>
<td></td>
</tr>
<tr>
<td>[72]</td>
<td>2 wv% nanofibrillated cellulose + 0.5 w/v% alginate (80:20) + 90 mM CaCl_2</td>
<td>Human nasoseptal chondrocytes</td>
<td>Cell viability of 73% and 86% after 1 and 7 days respectively</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Single cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 x 10^6 cells ml^-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analysis: day 1 and day 7</td>
<td></td>
</tr>
<tr>
<td><strong>Extrusion-based bioprinting</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[73]</td>
<td>PEGDMA in osteochondral plug + 0.05 w/v% Irgacure 2959, simultaneous photo-polymerisation during printing utilising a long-wave UV lamp at a distance of 25 cm and at an intensity of 4.5 mW cm^-2</td>
<td>hAC</td>
<td>High level of cell viability (± 90%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Single cells</td>
<td>Maintenance of cell phenotype with consistent gene expression analysis and biochemical data</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 x 10^6 cells ml^-1</td>
<td>Compressive modulus was 395.73±80.40 kPa, which is comparable to human articular cartilage</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analysis: 2, 4, 6 weeks</td>
<td></td>
</tr>
<tr>
<td>[74]</td>
<td>2% agarose, 15 min physical (temperature) + 3.5% alginate, 60 mM CaCl_2 + 10 w/v% Gel-MOD, 0.05% Irgacure 2959, 15 min UV</td>
<td>BioINK™ (PEGMA-based)</td>
<td>High levels of cell viability (± 80%) with no differences found between the different hydrogels</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 min UV</td>
<td>Development of hyaline-like cartilage is best supported by agarose and alginate (predominant staining of collagen type II)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Development of fibrocartilage-like tissue is best supported by Gel-MOD and BioINK™ (staining of both collagen type I and II)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Equilibrium compressive modulus of all bioinks fell below that of native articular cartilage (0.2-2 MPa) and meniscal tissue (0.1-0.3MPa)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Porcine BM-MSC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Single cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 x 10^6 cells ml^-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analysis: day 28</td>
<td></td>
</tr>
<tr>
<td>[75]</td>
<td>5 w/v% silk fibroin + 15 w/v% gelatin + Enzymatic cross-linking with tyrosinase</td>
<td>hAC</td>
<td>High viability levels of dispersed hAC (90% and 89.22%) and hBM-MSC (84% and 87.87%) by day 7 and day 14 respectively</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Single cell (1 x 10^6 cells ml^-1) or spheroid (150 spheroids ml^-1)</td>
<td>Chondrogenic differentiation of hBM-MSC (both dispersed and spheroids)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hBM-MSC</td>
<td>Superior chondrogenic differentiation in hAC spheroid-laden constructs over dispersed cell-laden constructs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Single cell (1 x 10^6 cells ml^-1) or spheroid (150 spheroids ml^-1)</td>
<td></td>
</tr>
</tbody>
</table>
2. **MATERIALS AND METHODS**

2.1. **Human bone marrow-derived mesenchymal stem cells**

The hBM-MSC were purchased from PromoCell GmbH (C-12974; Heidelberg, Germany). After thawing (supplement I), the cells were expanded in MSC Growth Medium 2 (C-28009; PromoCell GmbH, Heidelberg, Germany) supplemented with 0.5% penicillin/streptomycin (P/S; Sigma-Aldrich) at 37 °C in a humidified 5% CO₂-containing atmosphere. Once 90% confluence was reached, the cells were trypsinised using 0.25% trypsin-EDTA. Cells were used between passage 3 to 5 to generate spheroids. In addition, part of the collected hBM-MSC were frozen for stock maintenance (supplement II).

2.2. **Spheroids**

2.2.1. **Agarose microwell chip fabrication**

Polydimethylsiloxane (PDMS) molds (CMST, Ghent) were used as a negative replica of the microwell chips (figure 5). One PDMS mold contains 1585 micropores with a diameter of 400 µm each. The molds are stored in 70% ethanol at room temperature. For the fabrication of agarose microwell chips, the molds were transferred into a 6-well plate. Whilst the ethanol evaporated, a 4.5 w/v% agarose solution was prepared by dissolving UltraPure™ agarose (Invitrogen) in sterile phosphate buffered saline (PBS) (supplement III). The solution was heated and subsequently poured in the 6-well plate containing the molds. After centrifugation (906 g, 1 min), the liquid agarose solution was left to solidify at room temperature. The molds were then separated from the agarose and the dimensions of the microchip were adjusted using a punching device. The agarose microwell chips were transferred to a 12-well plate with sterile PBS and stored at 4 °C.

![Figure 5 – Schematic representation of agarose microwell chip fabrication.](image)

Liquid agarose is poured onto a PDMS mold, which is removed after agarose solidification. Thereafter, cells are seeded in the agarose microwell gel for the formation of spheroids.

2.2.2. **Formation of spheroids**

Prior to the seeding of cells, PBS was removed from the agarose microwell chips and 0.3 ml chondrogenic medium comprised of Dulbecco's modified Eagle’s medium (DMEM):nutrient mixture F12 (F12) (Gibco) supplemented with 1% sodium pyruvate (Gibco), 0.5% ITS (insulin-transferrin-selenite, Sigma-Aldrich), 0.1% fungizone, 0.1% P/S, 200 µM L-ascorbic-acid-2-phosphate (Sigma-Aldrich), 100 µM dexamethasone (Sigma-Aldrich), and 10 ng ml⁻¹ TGF-β1 (Peprotech) was added. Spheroids containing hBM-MSC were formed by seeding 0.5 x 10⁶ of the desired cell type onto each microchip. Plates were stored in a humidified incubator with 5% CO₂ at 37 °C for up to 2 h until the majority of the cells had lowered into the micropores due to gravitational forces. Then, 2 ml of chondrogenic medium was carefully added and the plates were stored in a low oxygen tension incubator with 5% O₂ and 5% CO₂ at 37 °C. The culture medium was changed three times a week.
2.2.3. Fusion of spheroids

To investigate the capacity of the spheroids to fuse into larger constructs, two different methods were used: fusion in agarose-coated wells and fusion of doublets.

In the first method, doublet formation could be achieved by following the protocol as described by Susienka et al. (figure 6A) [76]. In brief, medium was slowly aspirated from two wells each containing a chip. The recipient chip was then transferred into a new 12-well plate. The walls of the donor chip were removed through using a tube as a puncher and the remaining central portion was gently inverted onto the recipient chip. After centrifugation (184 g, 1 min), the donor chip was removed leaving the recipient chip with numerous spheroid doublets and 2 ml of chondrogenic medium was gradually added. Follow-up of the fusion was carried out utilising a phase-contrast microscopy (Olympus IX 81) at several time points: 0 h, 2 h, 6 h, 8 h, and 10 h. Medium was refreshed every day by slowly removing and subsequently adding 2 ml of chondrogenic medium.

In the second method, a 2 w/v% UltraPure™ agarose (Invitrogen) solution dissolved in sterile PBS was heated. A coating was applied in a 96-well plate by pipetting 300 µl per well and subsequently removing 270 µl. Per chip, spheroids were then harvested and resuspended in 160 µl chondrogenic medium. Once every agarose-coated well had received 20 µl of the spheroid-containing solution, 200 µl of chondrogenic medium was carefully added. As one chip contains approximately 1585 spheroids, each agarose-coated well contains approximately 200 spheroids. Follow-up of the fusion was carried out utilising a phase-contrast microscopy (Olympus IX 81) at several time points: 0 h, 2 h, 6 h, and 8 h. Medium was refreshed every day by slowly removing and subsequently adding 100 µl of chondrogenic medium.

For both approaches, day seven hBM-MSC spheroids were utilised and fusion was followed-up using a phase-contrast microscopy (Olympus IX 81) at several time points. Assessment of morphology, proliferation and ECM production was performed seven days post initiation of fusion. Lastly, morphometric analysis was performed on doublets during fusion through manual measurement utilising ImageJ (figure 6B).

Figure 6 – Formation and morphometric analysis of doublets adapted from [76].

A Formation of spheroid doublets.
B Morphological parameters measured during doublet fusion.

2.3. Hydrogel encapsulation

The spheroids comprised of hBM-MSC were encapsulated after 14 days of culture in a 10 w/v% Gel-MOD solution containing 2 mol% Irgacure 2959 or 10 w/v% Gel-MOD containing 2 mol% Li-TPO. The used conditions (i.e. optimal day of encapsulation and w/v% Gel-MOD) were based on previously determined values by research conducted within the Tissue Engineering and Biomaterials Group for the biofabrication of (fibro)cartilage [77]. Additionally, encapsulation within 2 w/v% Alg-MOD in combination with 2 mol% Li-TPO (synthesised by the PBM-group, Ghent University) was also performed. The utilised value concerning w/v% Alg-MOD was chosen based on a literature research (table 4).
<table>
<thead>
<tr>
<th>Reference</th>
<th>Hydrogel</th>
<th>w/v% (and DS)</th>
<th>Cross-linking method Time</th>
<th>Cell type</th>
<th>Results (viability-phenotype-biomechanics)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[78]</td>
<td>Alg-MOD</td>
<td>2 w/v% (3.1 DS, 4.6 DS, 6.9 DS)</td>
<td>Alg-MOD was dissolved in a 0.05% solution of Irgacure 2959 in Dulbecco’s PBS Exposed to long-wave UV light (EIKO, Shawnee, KS, peak 368 nm, 1.2W) for 10 min to produce 2 mm thick, 8 mm diameter hydrogels</td>
<td>Adult bovine nucleus pulposus (NP) cells</td>
<td>Viability of encapsulated NP cells was highest in hydrogels at lower percent modifications and lower w/v%, and decreased with time in culture NP cells produced characteristic proteoglycans (i.e. chondroitin sulphate proteoglycan) for all hydrogels that supported cell viability at day 14 (all but 6.9% modified alginate; 2 and 3 w/v%; no viability at day 3) Increased methacrylation and w/v% resulted in a decrease in swelling ratio and an increase in equilibrium Young’s modulus (2 w/v % hydrogels composed of 2.5% modified MA-LVALG too weak to be mechanically tested) Equilibrium swelling ratios: 30.52 ± 1.782 to 43.50 ± 1.345 Young’s moduli: 0.5850 ± 0.1701 to 8.824 ± 0.6014 kPa</td>
</tr>
<tr>
<td>fsu[79]</td>
<td>RGD-modified or unmodified Alg-MOD</td>
<td>2 w/v% (25 DS)</td>
<td>RGD-modified or unmodified methacrylated alginate was dissolved in DMEM or diH2O with 0.05 w/v% photo-initiator (Irgacure D-2959) Exposed to 365 nm UV light at 1 mW/cm² for 10 min to form the hydrogels</td>
<td>Bovine AC (passage 2)</td>
<td>High cell viability was observed throughout both alginate hydrogel compositions for 6 weeks Encapsulated chondrocytes retained high viability with RGD-modified alginate significantly enhancing production of GAG for 2 and 4 weeks in the absence of soluble chondrogenic growth factors Both (non)modified alginate hydrogels exhibited similar elastic moduli and swelling ratios over time, whereas the peptide-modified hydrogels degraded slightly faster than nonmodified hydrogels</td>
</tr>
<tr>
<td>[59]</td>
<td>Alg-MOD</td>
<td>3 w/v% (3% DS)</td>
<td>Pls VA-086 (Wako) or Irgacure 2959 were dissolved in 70% (v/v) ethanol to make a 10% (w/v) solution Exposed to 5 min of UV light (365nm longwave, 2 µW/cm²)</td>
<td>Bovine AC were harvested from the stifle joint from calf legs</td>
<td>Cell viability was measured in constructs with aggregate moduli in the 10–20 kPa range; for these conditions VA-086 constructs had mean viabilities over 90%, whereas IRG2959 constructs had mean viabilities below 70%.</td>
</tr>
</tbody>
</table>
2.3.1. Gel-MOD synthesis

The methacrylamide-modified gelatin (Gel-MOD) was synthesised by the PBM-group, Ghent University. In brief, bovine type B gelatin (Rousselot) was dissolved in phosphate buffer (pH 7.8) at 50 °C, prior to reaction with methacrylic anhydride whilst stirring for 1 h. The solution was then diluted and dialysis was performed utilising dialysis membranes for 24 h against distilled water at 40 °C. Gel-MOD had a degree of substitution (DS) of 78%, as determined by 1H NMR (Bruker AVANCE II 500 MHz). Before use, the freeze-dried Gel-MOD building blocks were sterilised by a cold ethylene oxide treatment (AZ Sint-Jan, Bruges).

2.3.1.1. Gel-MOD characterisation

Characterisation of discs comprised of 10 w/v% Gel-MOD with either 0.8 w/v% Irgacure 2959 or 0.8 w/v% Li-TPO was conducted by analysing stiffness (N/mm), gel fraction (%), and swelling (%).

2.3.1.1.1. Compression test

Discs with a diameter of 10 mm were prepared by pipetting 800 µl of a 10 w/v% Gel-MOD solution within the presence of the desired PI in a 12-well plate, followed by application of a punching device. Physical cross-linking was first allowed for 10 min at 4°C in the dark, followed by chemical curing for 60 s by the UV-LED incorporated within the printhead of the 3D Discovery Instrument bioprinter (365 nm, 200 mW/cm²). The formed discs were compressed at RT, using a universal testing machine (LRXplus, Lloyd Instruments). A 100N load cell was used and compression was applied over half the distance of the original sample thickness, during 2 cycles, at a rate of 5 mm/min. The slope of the force-distance curve of the first cycle determined the compressive modulus of the hydrogel.

2.3.1.1.2. Gel fraction and swelling ratio

Discs were prepared by pipetting 250 µl Gel-MOD solution within the presence of the desired PI in a 48-well plate. After applying the same cross-linking process as described for creating the discs for the compression test, the formed hydrogels were dried in a desiccator for approximately one week. Once dried, the gels were weighed (W\textsubscript{do}) and subsequently immersed in PBS solution at 37°C until equilibrium swelling was reached. Thereafter, the discs were removed from the PBS solution and were gently wiped with paper to remove residual liquid and weighed again (W\textsubscript{he}). The final drying process in the desiccator gave the ultimate dry weight (W\textsubscript{de}).

The gel fraction was calculated as the percentage of the hydrogel that is covalently incorporated in to the formed 3D network (table 5). The swelling ratio was also calculated (table 5). All measurements were performed in triplicate.

Table 5 – Formula for calculating percentage gel fraction and percentage swelling

<table>
<thead>
<tr>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>% gel fraction = (W\textsubscript{de} / W\textsubscript{do}) x 100</td>
</tr>
<tr>
<td>% swelling = [(W\textsubscript{he} − W\textsubscript{do}) / W\textsubscript{do}] x 100</td>
</tr>
</tbody>
</table>

2.3.2. Encapsulation in Gel-MOD

Sterilised Gel-MOD was dissolved in PBS and incubated for at least 1 h at 37 °C on a shaking device. The photo-initiator (PI) solution was prepared in accordance with the formula which accounts for several characteristics of both Gel-MOD and the used PI (table 6). Irgacure 2959 (0.8 w/v%) or Li-TPO (0.8 w/v%) was dissolved in sterile PBS, respectively at 55 °C for 3 h and room temperature (RT) for 1 h, and subsequently sterilised by filtering with a 0.22 µm filter (Millipore).

Prior to the encapsulation, PI solution was added to the 10 w/v% Gel-MOD solution and vortexed. Spheroids were harvested from their microchips and supernatant was removed after
centrifugation. Subsequently, PI/Gel-MOD solution was added to the pellet containing the hBM-MSC spheroids. Gels from 250 µl were formed in a 48-well plate with each gel containing around 1 to 4 microchips. Physical gelation occurred at room temperature for 10 min, followed by chemical cross-linking of the methacrylate side groups. Dependent on the used PI, chemical cross-linking ensued for Irgacure 2959 or Li-TPO respectively either with a 365 nm UV-A light at medium density and at a distance of 10 cm from the sample (4 mW/cm²) for 20 min or with an ultraviolet light-emitting diode (UV-LED) curing kit in the printhead of the 3D Discovery Instrument (RegenHU) at wavelength 365 nm for 60 s per well. Thereafter, the gels were transferred into a 12-well plate and 2.5 ml chondrogenic medium was added, which was refreshed three times a week.

Table 6 – Formula for the calculation of the amount of PI needed for cross-linking Gel-MOD

<table>
<thead>
<tr>
<th>Irgacure 2959</th>
<th>Li-TPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mol amine groups per gram Gel-MOD x degree of substitution x used Irgacure 2959 concentration x molecular weight Irgacure 2959)</td>
<td>(mol amine groups per gram Gel-MOD x degree of substitution x used Li-TPO concentration x molecular weight Li-TPO)</td>
</tr>
<tr>
<td>( \frac{w % \text{ Irgacure 2959}}{l} = \frac{0.000385 \text{ mol} \times 0.78 \times 0.02 \times 224.3}{0.0008} )</td>
<td>( \frac{w % \text{ Li-TPO}}{l} = \frac{0.000385 \text{ mol} \times 0.78 \times 0.02 \times 323.27}{0.0008} )</td>
</tr>
<tr>
<td>= 168 µl Irgacure 2959 per g Gel-MOD</td>
<td>= 243 µl Li TPO per g Gel-MOD</td>
</tr>
</tbody>
</table>

2.3.3. Alg-MOD synthesis

The methacrylamide-modified alginate (Alg-MOD) was synthesised by the PBM-group, Ghent University [80]. In brief, an alginate paste was made by first dissolving 6 w/v% polyvinylalcohol (PVA) powder into PBS on a magnetic stirring plate at a temperature of 125 °C with a speed of 1400 rpm for 1 h until a homogeneous solution was attained. The alginate powder was then added and the mixture was vortexed for 30 s prior to placing it overnight on a magnetic stirring plate with a speed of 1600 rpm at room temperature. Next, the modification of the alginate was performed by adding twice as much methacrylic anhydride as there are alcohol groups present in the alginate. The degree of substitution was determined as 37% via NMR. Prior to usage, Alg-MOD was sterilised by a cold ethylene oxide treatment (AZ Sint-Jan, Bruges).

2.3.4. Encapsulation in Alg-MOD

Sterilised Alg-MOD was dissolved in Dulbecco’s modified Eagle’s medium Glutamax (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco), 0.1% fungizone (Gibco), and 0.5% P/S. The solution was then incubated at RT on a magnetic stirring plate one day beforehand. The PI solution was prepared dependent on the weighed mass of Alg-MOD (table 7). Li-TPO (3.2 w/v%) was dissolved in sterile PBS for 1 h at RT, and subsequently sterilised by filtering with a 0.22 µm filter (Millipore).

Prior to encapsulation, PI solution was added to the 2 w/v% Alg-MOD solution and vortexed. Spheroids were harvested from their microchips and subsequently mixed with the PI/Alg-MOD solution. Gels from 250 µl were formed in a 48-well plate. Chemical cross-linking of the methacrylate side groups occurred according to the used PI Li-TPO. Thereafter, 0.5 ml chondrogenic medium was added to each well containing a gel which were then incubated for approximately 1 h at 37 °C in a humidified 5% CO₂-containing atmosphere. The gels were then transferred into a 12-well plate and 2.5 ml chondrogenic medium was added, which was refreshed three times a week.
2.4. 3D bioprinting spheroids in combination with Gel-MOD and Li-TPO
The 3D Discovery Instrument Bioprinter (RegenHU) equipped with a time-pressure dispensing printhead was utilised to 3D bioprint a scaffold. The bioink consisted of day 14 hBM-MSC combined with 10 w/v% Gel-MOD and 2 mol% Li-TPO. The printing process could be commenced once the spheroids were mixed with the Gel-MOD/Li-TPO solution and kept at 23 °C (for approximately 30 min in the preheated cartridge holder). The environmental temperature was set to 19 °C. When the correct consistency was reached, a scaffold, designed with the BioCAD software, could be bioprinted and subsequently chemically cured for 60 s by the UV-LED in the printhead of the 3D Discovery Instrument (RegenHU). Chondrogenic medium was added to the constructs, which were then placed in a humidified incubator at 5% CO₂ and 37 °C. The culture medium was refreshed three times a week. A summary of the parameters utilised through the bioprinting process is depicted in table 8.

Table 8 – Parameters for bioprinting and scaffold containing hBM-MSC spheroids encapsulated within Gel-MOD and Li-TPO.

<table>
<thead>
<tr>
<th>Parameters pertaining</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioink</td>
<td>Spheroid concentration ± 12680/ml</td>
</tr>
<tr>
<td>Bioprinting process</td>
<td>Cartridge temperature 23 °C</td>
</tr>
<tr>
<td></td>
<td>Extrusion pressure 0.077 MPa</td>
</tr>
<tr>
<td></td>
<td>Ambient temperature 19 °C</td>
</tr>
<tr>
<td></td>
<td>Feed rate 5 mm/s</td>
</tr>
<tr>
<td></td>
<td>Needle diameter 0.41 mm</td>
</tr>
<tr>
<td></td>
<td>Physical cross-linking 10 min at 4 °C</td>
</tr>
<tr>
<td></td>
<td>UV irradiation 60 s</td>
</tr>
<tr>
<td>Scaffold design</td>
<td>Strut thickness 0.328 mm</td>
</tr>
<tr>
<td></td>
<td>Layers 4</td>
</tr>
</tbody>
</table>

2.5. Evaluation
Spheroids were harvested from the microchips by firmly resuspending the culture medium and evaluated at day 7, day 14, day 21, day 28, day 35, and day 42. In addition, day 14 spheroids were collected and subsequently encapsulated in the desired biomaterial/PI solution and assessed at day 7 and day 14 post encapsulation. Finally, bioprinted constructs were also investigated at day 7 and day 14 post bioprinting.

2.5.1. Morphology and morphometry
The morphology of the spheroids was analysed through observation utilising phase-contrast microscopy (Olympus IX 81). The evaluation of the morphometry was performed using the Xcellence image software that allowed the determination of several parameters, such as diameter, perimeter (p), and area (A). The formula \( f_{\text{circularity}} = \frac{4\pi A}{p^2} \) enabled the ability to calculate the circularity of the spheroids.

2.5.2. Live/dead assay
To assess cell viability, a calcein-AM/propidium iodide (CaPI) staining was performed. The spheroids, encapsulated spheroids or bioprinted construct were washed twice with PBS and
incubated in a PBS solution (1 ml) containing 2 µl calcein-AM (1 mg ml⁻¹; AnaSpec) and 2 µl propidium iodide (1 mg ml⁻¹; Sigma-Aldrich). After 10 min of incubation in the dark at RT, cell viability was evaluated using an inverted fluorescence microscope (Olympus IX81) equipped with Xcellence pro software.

2.5.3. Histology

The spheroids, encapsulated spheroids or bioprinted constructs were fixed in 4% paraformaldehyde overnight at 4 °C, dehydrated in ascending alcohol concentrations, and incubated with a clearing agent toluene before being embedded in paraffin (Thermo Scientific) (supplement IV). Paraffin sections of 5 µm thickness were cut utilising a Reicher-Jung 2040 microtome. Prior to staining, the sections were deparaffinised and rehydrated using descending alcohol concentrations (supplement V). Dehydration using ascending alcohol concentrations was performed by the Robot-Stainer HMS 740 (MICROM) after every staining (supplement VI). Mounting medium (Thermo Scientific) was utilised to assemble coverslips on the slides. All samples were examined using a light microscope (Olympus BX51).

2.5.3.1. Haematoxylin-eosin staining

To assess overall morphology and quality of the (encapsulated) spheroids, haematoxylin-eosin (HE) staining was performed by a robot-stainer (HMS 740 MICROM, Walldorf) (Program: 15 s haematoxylin (VWR); 2 min tap water; 1 min clarifier I (Thermo scientific); 1 min tap water; 1 min bluing reagent (Thermo scientific); 1 min tap water; 1 min distilled water; 30 s eosin/phloxine; 2 min tap water). Haematoxylin is a basic dye that stains the nucleus and ribosomes blue, whilst eosin is an acidic dye that stains the cytoplasm pink.

2.5.3.2. Alcian blue staining

Alcian blue staining allowed the visualisation of sulphated GAGs in the ECM. Samples were stained in a solution comprised of 1% alcian blue (VWR) in 3% glacial acetic acid (pH 2.5: Chem-Lab) for 30 min and then rinsed in distilled water.

2.5.3.3. Picrosirius red staining

Evaluation of the collagen network was enabled by a picrosirius red staining (PSR). Samples were stained with 0.2% Sirius Red F3B (Ref: 365548; Sigma-Aldrich) in a saturated solution of Picric Acid (Ref: 80450) for 30 min at RT and afterwards rinsed in distilled water.

2.5.3.4. Immunohistochemistry

The visualisation of either collagen type I or collagen type II via immunohistochemistry (IHC) allowed the analysis of the (fibro)cartilage-like matrix production. First, antigen retrieval was performed which consisted of incubating the samples within a citrate buffer (pH 6.0). The subsequent steps comprised the elimination of the endogenous peroxidase activity and the blocking of non-specific interactions afterwards. The former was achieved by adding 3% H₂O₂ (VWR), followed by incubation in the dark for 10 min in the dark. The latter consisted of incubating the samples at RT for 30 min with a blocking solution (BS) which contained PBS with 1% bovine serum albumin (BSA; Roche), 5% normal rabbit serum (NRS; Dako), and 0.2% Tween 20. Thereafter, sections were incubated with the primary antibody which were diluted in a dilution buffer (10% BS in PBS) and applied to only the positive controls overnight at 4 °C or for 2 h at RT for mouse anti-collagen I and mouse anti-collagen II respectively. All samples were then incubated with the secondary antibody (biotinylated rabbit anti-mouse, 1:200 in dilution buffer) for 30 min. Visualisation of the antigens was accomplished by incubation with streptavidin-horseradish peroxidase (1:200 in dilution buffer; Dako), followed by an incubation of 10 min in the dark with 0.06% 3,3′-diaminobenzidin (DAB, Sigma-Aldrich) and 0.03% H₂O₂ in Tris-buffer solution (6 g Tris (VWR) in 1 l H₂O, pH 7.6). Peroxidase converts the colourless chromogen DAB into a coloured oxidised DAB in the presence of H₂O₂ at the sites of the specific antigen, resulting in the visualisation of collagen I or collagen II. This reaction was stopped by putting the samples underneath streaming tap water for 10 min. At last, sections were counterstained with Mayer’s haematoxylin.
Furthermore, immunostaining of Ki-67 was performed to allow assessment of proliferation. Staining procedure was similar as described above. Exceptions include the use of normal swine serum in the blocking solution, monoclonal rabbit anti-Ki67 as primary antibody, and biotinylated swine anti-rabbit as secondary body.

2.5.4. **Statistical analysis**

All collected data were analysed using the Statistical Package for the Social Sciences (SPSS) version 25.0 software and are represented as mean ± 95% confidence interval (CI). Normality of the variables was investigated with the Shapiro-Wilk test. The homogeneity of variances was assessed using the Levene’s test. A one-way ANOVA, followed by a post hoc Dunnett’s T3, was performed for the analysis of the diameter and circularity of non-encapsulated hBM-MSC spheroids. For the analysis of the morphometric parameters of hBM-MSC spheroids encapsulated within gel-MOD, a one-way ANOVA was also performed, followed by either a post hoc Dunnett’s T3 for the diameter or a post hoc Tukey for the circularity. The tests concerning compression, % gel fraction and % swelling were evaluated utilising an independent t-test. For the analysis of the diameter and circularity of hBM-MSC spheroids encapsulated within Alg-MOD a one-way ANOVA test was performed, followed by post hoc Tukey. Statistical significance was considered when p-values were smaller than 0.05.
3. RESULTS

3.1. Non-encapsulated spheroids
Spheroid formation could be observed by means of analysing brightfield (BF) images (figure 7). Once seeded, the cells migrated towards the bottom of the microwells and remained as single cells until the following day. Then, spheroids were formed in each microwell with some residual single cells. Complete spheroid formation was obtained at approximately day seven. The spheroids remained stable, uniform, and circular up to six weeks post-seeding.

![Figure 7](image)

Figure 7 – Brightfield images of non-encapsulated hBM-MSC spheroids. Analysis of the non-encapsulated spheroids was performed on the same day of seeding and also the following day. In addition, the spheroids were analysed for six consecutive weeks with a seven-day interval. Scale bar = 200 µm.

3.1.1. Morphology
The stable morphology of the spheroids could be confirmed by a haematoxylin-eosin (HE) staining (figure 8). Compact spheroids with a clear contour were observed on all days of analysis.

![Figure 8](image)

Figure 8 – Haematoxylin-eosin staining of non-encapsulated hBM-MSC spheroids. Scale bar = 20 µm.
3.1.2. **Morphometry**

Morphometric evaluation was performed to quantify morphological changes of non-encapsulated hBM-MSC spheroids by measuring the diameter and calculating the circularity (table S1, S2).

### 3.1.2.1. Diameter

The diameter of the non-encapsulated spheroids was measured weekly for six consecutive weeks. The mean values were between 110 µm and 140 µm (figure 9). A significant decrease in diameter was observed on day 21 (116.28 ± 6.89 µm) compared to day 7 (131.68 ± 2.80 µm), followed by an increase on day 28 (128.84 ± 3.16 µm) which then stabilised up to day 42.

![Figure 9 – Diameter of non-encapsulated hBM-MSC spheroids.](image)

Significant differences ($p < 0.05$) between non-encapsulated spheroids on different days of analysis are marked *. All data are presented as mean ± 95% CI.

### 3.1.2.2. Circularity

The mean values for circularity of non-encapsulated spheroids varied between 87% and 88% on all days of analysis (figure 10). No significant differences were observed, indicating stability of the spheroids regarding circularity.

![Figure 10 – Circularity of non-encapsulated hBM-MSC spheroids.](image)

No significant differences ($p < 0.05$) in circularity were observed. All data are presented as mean ± 95% CI.
3.1.3. Viability
Live/dead images show the viability of the non-encapsulated spheroids (figure 11). Peripheral cell death occurred on all days of analysis, whilst central cell death only arose from day 14 onwards. Nonetheless, the majority of the cells were viable. The overall survival of the spheroids was positive with a slight decrease over time.

![Live/dead images of non-encapsulated hBM-MSC spheroids. Scale bar = 200 µm.](image)

Figure 11 – Live/dead images of non-encapsulated hBM-MSC spheroids. Scale bar = 200 µm.

3.1.4. Proliferation
The proliferative activity of cells within the spheroids was determined by means of an IHC staining for the proliferation marker Ki67 (figure 12). Proliferative nuclei stain brown. The majority of the spheroids contained a scarce amount of proliferative cellular nuclei, indicating limited proliferation within the spheroids at all times of analysis. The proliferative cells are prominently located at the periphery of the spheroids.

![Ki67 staining of non-encapsulated hBM-MSC spheroids. Scale bar = 20 µm.](image)

Figure 12 – Ki67 staining of non-encapsulated hBM-MSC spheroids. Scale bar = 20 µm.
3.1.5. ECM production

Assessment of ECM production was performed to determine differentiation towards a (fibro)cartilaginous phenotype (figure 14).

A positive staining of picrosirius red (PS) was a first indication regarding the production of collagen throughout the spheroids. An increase in the presence of collagen could be observed commencing from day 14 which thereafter remained stable (figure 13). PS staining does however not allow phenotypic collagen determination. Therefore, IHC staining of collagen type I and collagen type II was carried out.

An increase in collagen type I production could be observed which reached a maximum on day 28 (figure 14). A decrease in presence of collagen type I occurred thereafter. Notably, the presence remained peripheral on all days of analysis.

Similar to collagen type I production, an increase in collagen type II could also be seen with highest production reached on day 28 (figure 13). In contrast, collagen type II expression did not remain peripheral and could be found throughout the entirety of the spheroid on day 42.

Alcian blue (AB) staining revealed the presence of GAGs, which slightly increased over time (figure 13).

![Figure 13 – Overview of different images of non-encapsulated hBM-MSC spheroids.](image)

Analysis of the non-encapsulated spheroids was performed for six consecutive weeks with a seven-day interval. Scale bar = 20 µm.

3.1.6. Spheroid fusion assays

Spheroids were placed together after seven days of culture. To investigate the ability of fusion, two different assays were performed. The first test involved the coalescence of only two spheroids resulting in the formation of a doublet. The other experiment included 200 spheroids forming a larger macrotissue.

3.1.6.1. Doublet formation

The fusion process of multiple doublets was tracked over time (figure 14). Microwells containing no spheroids, single spheroids, triplets or quadruplets were excluded from analysis. BF images show fusion already commencing from two hours post initiation of doublet formation. Thereafter, the spheroids coalesced until a singular and compact construct was obtained.
Figure 14 – Brightfield images of doublet fusion process. The fusion process of one and the same doublet is shown. Scale bar = 50 µm.

3.1.6.1.1. Morphology, proliferation and ECM production

HE staining of hBM-MSC doublets on day seven after fusion showed a circular and compact morphology (figure 15). IHC staining for the proliferation marker Ki67 revealed a scarce amount of proliferative cellular nuclei (figure 15). Ki67+ cells were mostly located in the outer rim of the doublets. A first indication regarding the presence of collagen was obtained with PS staining (figure 15). However, only a limited amount of peripheral collagen I but a larger amount of peripheral collagen II was observed. Within most doublets the two coalesced spheroids could still be observed as two individual entities. The presence of GAGs was confirmed by means of an AB staining (figure 15).

Figure 15 – Overview of different images of hBM-MSC doublets. Analysis of the hBM-MSC doublets was performed seven days post initiation of doublet formation. Scalebar = 20 µm.
3.1.6.1.2. **Morphometry**

Morphological changes could be quantified during fusion (figure 16, table S6). End-to-end doublet length shortened over time as fusion progressed, indicating migration towards one another. Spheroid morphology remained stable as the doublet width/height values fluctuated between 110 µm and 140 µm. Both contact length and intersphere angle increased over time due to progress of fusion.

![Graphs showing morphometry changes](image)

*Figure 16 – Morphometry of hBM-MSC doublets.*

Plotted as a function of time are: A Doublet length, B Doublet width/height, C Contact length, and D Intersphere angles. All data are presented as mean ± 95% CI.

3.1.6.2. **Fusion in 96 well-plate**

Fusion of approximately 200 spheroids within an agarose-coated 96 well-plate was tracked over time (figure 17). Similar to doublet formation, spheroids started coalescing after 2 hours until a circular and compact entity was formed.

![Images showing fusion process](image)

*Figure 17 – Brightfield images of fusion process of multiple hBM-MSC spheroids.*

Fusion of spheroids within one and the same microwell is shown. Scale bar = 500 µm.
3.1.6.2.1. Morphology, proliferation and ECM production

The fused construct displayed an overall good morphology seven days after fusion with observation of some individual spheroids, but a limited number of proliferative cells (figure 18). A positive PS staining revealed the presence of collagen. However, only a faint staining of collagen I but a stronger peripheral occurrence of collagen II could be observed. GAG expression could be determined by a positive AB staining.

Figure 18 - Overview of different images of multiple fused hBM-MSC spheroids. Analysis of the multiple fused hBM-MSC spheroids was performed seven days post initiation of coalescence. Scale bar = 200 µm.

3.2. Encapsulated spheroids in Alg-MOD with Li-TPO

The effect of an alginate-based hydrogel on the phenotype of day 14 hBM-MSC spheroids encapsulated in Alg-MOD with Li-TPO was assessed. A follow-up of 14 days of encapsulation through BF images revealed migration of the spheroids to the bottom of the gel where substantial outgrowth could be observed (figure 19).

Figure 19 – Brightfield images focused on the bottom of gels comprised of Alg-MOD with Li-TPO and hBM-MSC spheroids. Scale bar = 200 µm.

3.2.1. Morphology, viability, proliferation and ECM production

A continued stable morphology could be observed post-encapsulation within Alg-MOD utilising Li-TPO as the PI (figure 20). Spheroids remained circular and with a clear contour. The overall survival of both spheroids and outgrowth located at the bottom of the wells was positive with a small amount of cell death located in the periphery of the spheroids and outgrowth (figure 20). Notably, the outgrowth seemed to allow connections to neighbouring spheroids. Little to no proliferative cells could be located within the spheroids post-encapsulation (figure 20). Peripheral Ki67+ cells were still observed at day seven. However, by day 14 none could be found.
Figure 20 – Overview of different images of hBM-MSC spheroids encapsulated in Alg-MOD with Li-TPO.
Analysis of the encapsulated spheroids was performed on day 7 and day 14. Scale bar for HE and Ki67 stainings = 20 µm. Scale bar for CaPI images = 200 µm.

Day 14 hBM-MSC spheroids were encapsulated in Alg-MOD with Li-TPO and subsequently assessed for ECM production on day seven and day 14 post-encapsulation (figure 21 and S1). A strong and positive PS staining indicates the presence of collagen throughout the spheroids. However, only scarce peripheral amount of both collagen type I and collagen type II was observed upon further investigation. A faint staining of AB revealed a limited expression of GAGs (figure 21 and S1).

Figure 21 – Overview of stainings on hBM-MSC spheroids encapsulated in Alg-MOD with Li-TPO.
Analysis of the day seven encapsulated spheroids was performed on day seven and day 14. Scale bar = 20 µm.

3.2.2. Morphometry
Morphometric evaluation was performed to quantify morphological changes between hBM-MSC spheroids being either encapsulated in Alg-MOD with Li-TPO or not. Measured parameters include diameter and circularity (table S1 and S2).

The diameter of non-encapsulated spheroids, which increased over time, valued higher on both days of analysis compared to the encapsulated condition, which remained stable throughout the time frame of analysis (figure 22A). Conversely, the circularity of non-encapsulated spheroids valued lower on both days of analysis compared to the encapsulated spheroids (figure 22B). Circularity remained stable within both conditions as no significant differences could be observed between different days of analysis.
Figure 22 – Morphometry of hBM-MSC spheroids with or without encapsulation in Alg-MOD and Li-TPO.
A Diameter of spheroids.
B Circularity of spheroids.
Dates displayed are days post-initiation of spheroid formation. In accordance with performance of encapsulation on day seven, this entails that day 21 and day 28 encapsulated spheroids have thus resided seven and 14 days within the biomaterial respectively. Only relevant significant differences are shown. Significant differences ($p < 0.05$) between spheroids of the same condition on different days of analysis are marked #. Significant differences ($p < 0.05$) between different conditions on the same day are indicated by *. All data are presented as mean ± 95% CI.

3.3. Encapsulated spheroids in Gel-MOD with Irgacure 2959 vs Li-TPO
Day 14 hBM-MSC spheroids were encapsulated in 10 w/v% Gel-MOD to evaluate its potency as a bioink for 3D bioprinting of (fibro)cartilage construct. The effect of different PIs (Irgacure 2959 or Li-TPO) used for cross-linking were also tested.
Analysis of BF images for both conditions revealed outgrowth within the biomaterial allowing connections between the spheroids (figure 23). However, this appears to be present stronger when Li-TPO was utilised as PI.

Figure 23 – Brightfield images of spheroids encapsulated in Gel-MOD with Irg or Li-TPO.
Day 7 hBM-MSC spheroids were encapsulated in Gel-mod with either Irg or Li-TPO. Scale bar = 200 µm.
3.3.1. **Morphology, viability, proliferation and ECM production**

Assessment of morphology, viability, proliferation, and ECM production was performed to determine appropriate differentiation towards a (fibro)cartilaginous phenotype (figure 24). Both conditions showed spheroids retaining a stable morphology when encapsulated within Gel-MOD (figure 24). A positive overall survival of the hBM-MSC spheroids could be observed (figure 24). However, outgrowths could only be visualised in the condition with Li-TPO which seem to be viable. No proliferative cells could be located within both conditions, except for day 7 post-encapsulation with Gel-MOD and Li-TPO (figure 16).

![Figure 24](image)

**Figure 24 – Overview of images of hBM-MSC spheroids encapsulated in Gel-MOD with Irg or Li-TPO.**

Scale bar for HE and Ki67 stainings = 20 µm. Scale bar for CaPi images = 200 µm.

A positive PS staining in both conditions indicates the expression of collagen throughout the hBM-MSC spheroids (figure 25 and S1). Upon further investigation, a limited production of collagen type I could only be seen within hBM-MSC spheroids encapsulated in Gel-MOD and Irgacure 2959. In addition, a strong presence of collagen type II could only be observed for day 7 post-encapsulation with Gel-MOD and Li-TPO and for day 14 post-encapsulation with Gel-MOD and Irgacure 2959. Both conditions appear to similarly express GAGs (figure 25 and S1).

![Figure 25](image)

**Figure 25 – Overview of stainings on hBM-MSC spheroids encapsulated in Gel-MOD with Irgacure 2959 or Li-TPO.** Scale bar = 20 µm.
3.3.2. Morphometry

Morphometric evaluation was performed to quantify morphological changes of hBM-MSC spheroids when encapsulated in Gel-MOD with either Irgacure2959 or Li-TPO (figure 26). Measured parameters were diameter and circularity (table S1 and S2).

A dissimilar trend was observed in diameter for (encapsulated) spheroids (figure 26A). Diameter increased for non-encapsulated spheroids, whilst either a decrease or stability was observed when encapsulated in Gel-MOD with Irgacure 2959 or Li-TPO respectively. The diameter of non-encapsulated spheroids initially was located between the values of the encapsulated spheroids (i.e. higher than Gel-MOD + Li-TPO but lower than Gel-MOD + Irg), but later valued as the highest. Lastly, the diameter of encapsulated spheroids was and remained higher when Irgacure 2959 was utilised rather than its counterpart Li-TPO.

Circularity remained stable within the conditions since no significant differences between different days of analysis were observed (figure 26B). Between the different conditions, utilisation of Li-TPO as the PI led to the highest circularity of the spheroids. Circularity valued similarly between the other two conditions.

![Figure 26](image_url)

**Figure 26 – Morphometry of hBM-MSC spheroids with or without encapsulation in Gel-MOD and PI.**

A Diameter of spheroids.
B Circularity of spheroids.

Dates displayed are days post-initiation of spheroid formation. In accordance with performance of encapsulation on day seven, day 21 and day 28 encapsulated spheroids have thus resided seven and 14 days within the biomaterial respectively. Only relevant significant differences are shown. Significant differences ($p < 0.05$) between spheroids of the same condition on different days of analysis are marked #. Significant differences ($p < 0.05$) between different conditions on the same day are indicated by *. All data are presented as mean ± 95% CI.
3.4. Gel-MOD characterisation with Irgacure 2959 vs Li-TPO

Stiffness (N/mm), gel fraction (%), and swelling (%) of 10 w/v% Gel-MOD with the selected PI were evaluated in order to assess the mechanical properties of the construct (table S3, S4, S5).

A compression test was conducted on 10 w/v% Gel-MOD discs to gain knowledge about the influence of the PI on stiffness. Two different PIs (Irgacure 2959 and Li-TPO) were tested. Cross-linking with Irgacure 2959 resulted in hydrogel discs with a significantly higher stiffness than the Li-TPO cross-linked gels (figure 27A).

The swelling behaviour of the Gel-MOD hydrogels cross-linked with either Irgacure 2959 or Li-TPO was studied. A significantly higher swelling ratio for Li-TPO compared to Irgacure 2959 was observed (figure 27B).

Gel fraction was calculated in order to gain knowledge on the percentage of the hydrogel that is covalently incorporated into the formed 3D network. Hydrogels cross-linked utilising Li-TPO had a significantly higher amount of covalently incorporated material compared to hydrogels cross-linked utilising Irgacure 2959 (figure 27C).

Figure 27 – Mechanical assessment of 10 w/v% Gel-MOD hydrogel discs cross-linked using Irgacure 2959 or Li-TPO.

Measured parameters were A Stiffness, B Gel fraction, and C Swelling. Significant differences ($p < 0.05$) between the different conditions were marked *. All data are presented as mean ± 95% CI.
3.5. Bioprinting of spheroids encapsulated in Gel-MOD with Li-TPO
Day 14 hBM-MSC spheroids encapsulated in Gel-MOD with Li-TPO were bioprinted utilising the 3D Discovery Instrument bioprinter equipped with a time pressure dispensing printhead (figure 28A). BF images show outgrowth of the spheroids starting from day 3 onwards (figure 28B, C).

Figure 28 – Bioprinting of hBM-MSC spheroids encapsulated in Gel-MOD with Li-TPO.
A Printed scaffolds of 1.3 x 1.3 cm with magnification viewed through eyepiece of phase-contrast microscope.
B,C Brightfield images of bioprinted scaffold. Scale bar A = 500 µm. Scale bar B = 200 µm.

3.5.1. Morphology, viability and proliferation
An overall intact morphology of the spheroids was observed once encapsulated and bioprinted with Gel-MOD and Li-TPO (figure 29). Most spheroids were round with viable nuclei. In addition, HE staining also showed that spheroids remained attached to Gel-MOD scaffolds. Spheroids remained viable post-printing with little peripheral and central cell death up to day 14 (figure 29). Outgrowths within the scaffold could be located and seemed to be viable both days of analysis.
A limited proliferative capacity of the spheroids could be observed since little to no Ki67+ cells could be detected (figure 29).

Figure 29 – Overview of different images of bioprinted scaffold containing hBM-MSC spheroids encapsulated in Gel-MOD with Li-TPO.
Scale bar for HE and Ki67 stainings = 20 µm. Scale bar for CaPI images = 200 µm.
3.5.2. ECM production

Presence of collagen was first indicated by a positive PS staining (figure 30 and S1). After further examination, a decreased expression of both collagen type I and collagen type II was observed. However, a stronger staining of collagen type II compared to collagen type I could be noted and staining of both was not confined to the periphery but was present throughout the entire spheroid.

AB staining revealed an increased expression of GAGs (figure 30 and S1).

Figure 30 – Overview of stainings on bioprinted scaffold containing hBM-MSC spheroids encapsulated in Gel-MOD with Li-TPO.
Scale bar = 20 µm.
4. DISCUSSION

Bioprinting is a manufacturing method where bioinks are dispensed in a highly controllable and precise manner to create complex tissue constructs by means of sequential layer-by-layer processes. Bioink formulation is often considered as one of the most crucial aspects of 3D bioprinting. Within the endeavour to bioprint a (fibro)cartilaginous construct, the composition of the ideal bioink is thus subject of substantial investigation. The bottom-up tissue engineering approach involves the use of spheroids as the micro-building blocks in combination with a suitable hydrogel to 3D bioprint a macrotissue. Previous research within the Tissue Engineering Group has led to the development of qualitative (fibro)cartilaginous spheroids composed of porcine articular chondrocytes (pAC) [81]. A provisional composition of a suitable bioink was further determined as day 14 pAC spheroids combined with 10 w/v% Gel-MOD and 2 mol% Irgacure 2959 [77]. The present study, however, investigated a different cartilage bioink composition. The hBM-MSC were selected as a promising cell candidate for (fibro)cartilage engineering due to their chondrogenic potential. In vitro chondrogenesis of hBM-MSC could be achieved through mimicry of the biochemical and biomechanical milieu cells experience during cartilage formation in the developing limb bud. This included stimulating the cells with the soluble chondro-inductive growth factor TGF-ß1, culturing under hypoxic conditions, and regulating mechanosensory pathways through formation of spheroids. Similar to the limb bud requiring condensation of the mesenchyme to initiate chondrogenesis in the developing growth plate, spheroid formation importantly determined cell fate of cultured hBM-MSC. Neocartilage formation was indicated by the presence of fibrocartilage-like ECM molecules: collagen type I, collagen type II, and GAGs. Printability of the spheroids was confirmed by an observed uniformity and optimal diameter for disposition by the print needle (i.e. smaller than 200 µm).

Fusion of the spheroid building blocks is an important aspect within the biofabrication strategy whereby a larger construct is required to be formed. To this end, two different assays were performed analysing coalescence of day seven hBM-MSC spheroids. The first evaluated the formation of doublets, whilst the other investigated fusion of multiple spheroids. In both cases, it was revealed that initial tack between the spheroids formed rapidly. Key morphological parameters of doublets during fusion were additionally tracked as a function of time. Similar to the study conducted by Susienka et al. [76] were the decrease of end-to-end doublet lengths over time, increase of contact lengths over time, and higher intersphere angle of the doublets at the end of fusion. A close resemblance in the mechanism used for spheroid fusion and the repair of an embryonic epithelial wound could be observed whereby zipping from the centre is propagated towards the edges [82]. Histological analysis of the fused constructs revealed adaptation of the hBM-MSC spheroids towards a fibrocartilage-like phenotype as evidenced by the observation of a slight staining of collagen type I, collagen type II and GAGs. Moreover, spheroids could still be observed as individual entities within certain fused constructs. Babur et al. [83] observed that assembly of day seven hAC spheroids resulted in a uniform integration in such a manner that discrete spheroids were virtually indistinguishable. Conversely, it was still possible to identify individual hAC spheroids collected from day 14 that had been assembled into a larger tissue construct, indicating that full integration had not yet occurred in these constructs. Thus, ECM could be viewed as an important structural determinant of fusion kinetics, noting the importance regarding the time of fusion. The older and more rigid the tissues are, the slower they fuse. However, one caveat of spheroid formation could be the diffusion transport barriers posed by the inherent 3D structure, thus imposing a hindrance to chondrogenesis in the central region of the spheroids due to lower exogenously supplied TGF-ß1 levels. The outer layer of cells in the spheroid would be exposed to the highest concentrations of oxygen, nutrients, metabolites, and signalling factors in contrast to cells closer to the central part of the spheroid. This occurrence would explain the
Peripheral staining of collagen type I and collagen type II in both assays with absence in the central part.

In the effort to identify the bioink formulation used for the 3D bioprinting of a (fibro)cartilage-like tissue construct, the combination of the biomaterial (Alg-MOD or Gel-MOD) with hBM-MSC spheroids and PI (Li-TPO) was investigated. The effect of Irgacure 2959 when combined with Gel-MOD was additionally studied. In accordance with previous research conducted within the Tissue Engineering Group (Ghent University), day 14 hBM-MSC spheroids were encapsulated with the desired hydrogel [77].

In the 2 w/v% Alg-MOD discs, the majority of the spheroids migrated towards the bottom of the gel due to its low viscosity and they were able to develop cell extensions. However, these outgrowths were focused at the base of the gel and not throughout the entire construct. This complies with the inert nature of alginate which is resistant to cell adhesion due to a lack of native cell binding sites such as the RGD motif. RGD is a tripeptide identified in ECM proteins, such as collagen, as the minimal sequence required for recognition by cell membrane integrins. In light of the self-assembling nature of cartilage during development, spheroid entrapment is being pursued in biomaterials that potentiate the condensation process in order to promote cartilage formation. A higher rounding of the spheroids (i.e. an increase in circularity) and a more compact morphology (i.e. a decrease in diameter) were observed of the spheroids encapsulated within Alg-MOD and Li-TPO. This was accompanied by the development of a fibrocartilage-like phenotype as evidenced by a peripheral staining for collagen type I and collagen type II with expression of GAGs throughout the entire spheroid. Contradictory to the findings in this study, Daly et al. [74] concluded that encapsulation of dispersed porcine BM-MSC within 3.5 w/v% alginate supported the progression towards a hyaline cartilage-like phenotype. Occurrence of peripheral collagen type I and collagen type II could be explained by the concomitant observation of a spread-out cell morphology in the outer rim of the spheroids observed in HE staining potentially induced by the imposed compression due to encapsulation. The occurrence of a hydrogel cross-link density gradient would be an explanation for a possible high stiffness in the lower regions of the construct. Although a homogenous mixture was attempted to be obtained, a chemical curing for 60 s per well combined with the low viscosity of the Alg-MOD and waiting times could induce heterogeneity throughout the solution. A higher concentration of PI would therefore be present towards the foundation of the hydrogel with the result of less cross-linking at the upper region. This phenomenon would account for a high density located at the bottom, where also the majority of the spheroids resided, inducing flattening of the cells in the outer rim of the spheroids. Cell geometry regulates hBM-MSC lineage commitment [84]. A flattened cell layer at the surface of the spheroids could therefore induce differentiation of hBM-MSC into fibrochondrocytes marked by the expression of both collagen type I and type II. A paucity of collagen type II within the central part of the spheroid would be explained do to diffusion hindrance towards the core of TGF-β1, which stimulates gene transcription such as collagen type II [85].

In contrast, the Gel-MOD hydrogel presents the RGD sequence which supports cell attachment. Outgrowth throughout the entire gel was observed regardless of used PI. However, more cell extensions were present with Li-TPO as the utilised PI. Characterisation of Gel-MOD revealed a higher stiffness when chemically cross-linked with Irgacure 2959 in comparison to Li-TPO. Taken together, a higher stiffness could thus concomitantly be observed with lesser outgrowth. Histological analysis revealed that Gel-MOD supported the development of a hyaline cartilage-like phenotype. Contradictory to the findings in the present study, Daly et al. [74] concluded that encapsulation of dispersed hBM-MSC within gelatin supported the progression towards a fibrocartilage-like phenotype. They also established that biomaterial stiffness influenced the chondrogenic capacity of hBM-MSC with softer matrices supporting higher levels of chondrogenesis. However, their research was conducted utilising a 10 w/v% Gel-MOD, with a stated high degree of functionalisation, and 0.05 w/v% Irgacure 2959 (15 min UV). A suspected
higher mechanical property of the utilised Gel-MOD in this study would be in agreement with the aforementioned establishment and would thus explain the discrepant results between studies. Collectively, encapsulation within either Alg-MOD or Gel-MOD did not negatively affect cell viability, thus indicating cytocompatibility. However, a detrimental effect on proliferative capacity was observed. Cartilaginous differentiation of hBM-MSC is preferably exhibited by a round cellular morphology obtained by the exogenous addition of TGF-β1, the absence of RGD motives within the biomaterial, and a low environmental stiffness. Differentiation towards a fibrocartilage-like phenotype is best supported by a spread-out cellular morphology of hBM-MSC obtained by the presence of RGD motives within the biomaterial and/or a high environmental stiffness. The choice of PI indirectly influences hBM-MSC fate through affecting biomechanical properties of the biomaterial.

A trial to bioprint a (fibro)cartilaginous construct was performed with the bioink composed of day 14 hBM-MSC spheroids, 10 w/v% Gel-MOD, and 0.8 w/v% Li-TPO. The results showed that hBM-MSC did not only survive the 3D bioprinting process but also had viable outgrowths. In contrast to encapsulation with the same composition, a fibrocartilaginous bioprinted construct was attained. The applied pressure during bioprinting changed the cellular morphology as evidenced by a flattened morphology of the cells within the spheroids in HE staining. Moreover, bioprinting did not have a negative effect on the proliferative capacity of the cells within the spheroids. There are some limitations to this study, morphology and viability of the spheroids were analysed through conventional brightfield and phase-contrast microscopy. Due to the thickness and lack of transparency of the spheroids, none of these methods were able to acquire adequate images of the interior. Only via images of sectioned spheroids could valuable information be obtained of the core. As a proposed alternative approach, confocal microscopy could be used. Moreover, phenotype determination was performed at the protein level. For future perspectives, a gene expression analysis should be conducted. The effect regarding day of encapsulation could also be a subject of further investigation. Additionally, optimisation of the Alg-MOD hydrogel composition is warranted with subsequent characterisation. Once a suitable bioink formulation has been achieved, fine-tuning of the bioprinting parameters is warranted. Lastly, fusion within the hydrogel needs to be researched.
5. GENERAL CONCLUSION

The present study demonstrates the potential of hBM-MSC to generative qualitative (fibro)cartilaginous spheroids that can be used as the micro-building blocks for further assembly into a larger construct. High-throughput fabrication of these spheroids could be achieved by seeding hBM-MSC onto an agarose microchip, followed by cultivation in chondrogenic medium containing TGF-β1 and incubation at a low oxygen tension. Moreover, the spheroids have the ability to fuse in suspension. The possibility of coalescence within a hydrogel requires to be subject of further investigation. Control over the micro-environment is paramount for the differentiation of hBM-MSC into the desired phenotype. The biochemical and biomechanical properties of the hydrogel strongly influence the cartilage phenotype of the encapsulated spheroids. The choice of PI additionally affects the biomechanical characteristics of the hydrogel. Bioprinting parameters requires further investigation since unwanted effects onto the spheroids could occur.
6. REFERENCES

In 2018.

25 hydrogels with independently tailorable physical properties and cell adhesivity. pulposus cell en


the fusion of spheroid building blocks.

fibroin

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three-dimensional bioprinting technology.

Biomacromolecules Human Chondrocytes with Nanocellulose

simultaneous deposition and photocrosslinking in PEG

applications and future prospects.

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7. ADDENDUM

Supplement I: thawing of cells
- Heat beaker containing sterile water to 37 °C
- Procure desired vial with cells from -80 °C freezer
- Thaw vial in warm sterile water until small pellet remains
- Resuspend content of vial and transfer carefully into 15 ml tube
- Rinse vial with cold medium and add carefully to 15 ml tube
- Increase content of 15 ml tube until 10 ml is reached
- Centrifugate (184 g, 5 min)
- Remove supernatant
- Resuspend pellet with warm medium (room temperature)
- Transfer cells in T75 falcon
- Rinse 15 ml tube and add to falcon (final volume for T75 falcon is approximately 15 ml)
- Make cross motion with T75 falcon to ensure optimal spreading of cells
- Place falcon in incubator (37 °C, 5% CO₂)

Supplement II: freezing of cells
- Remove medium from cells
- Rinse cells twice with sterile PBS
- Add trypsin (1 ml for T25 falcon, 2 ml for T75 falcon, or 3 ml for T175 falcon)
- Place falcon in incubator for 5 to 10 min (until cells have detached)
- Add 0.5 ml FBS to discontinue enzymatic reaction
- Collect cells in 15 ml tube
- Rinse falcon with small amount of medium and add to 15 ml tube
- Centrifugate (185 g, 5 min)
- Remove supernatant
- Resuspend cells in FBS
- Add 0.5 ml of FBS/dimethyl sulfoxide (DMSO) suspension (80/20%) to cryovial
- Add 0.5 ml of cell suspension in FBS to cryovial resulting in new ratio of FBS/DMSO (90/10%)
- Transfer cryovials in “MrFrosty”
- Place “MrFrosty” in -80 °C freezer (overnight)
- The next day, transfer cryovials to liquid nitrogen

Supplement III: composition of PBS
- 2 l distilled H₂O
- 3.56 g Na₂HPO₄ (VWR)
- 0.84 g KH₂PO₄ (VWR)
- 14.4 g NaCl (Sigma-Aldrich)
- Using NaOH (Merck) solution must reach a pH of 7.2
**Supplement IV: dehydration and paraaffinisation**
- 1 h incubation in 30% Disolol (Chem-Lab)
- Remove 30% Disolol and incubate for 1 h in 50% Disolol (Chem-Lab)
- Remove 50% Disolol and incubate for 1 h in 70% Disolol (Chem-Lab)
- Remove 70% Disolol and incubate for 1 h in 99% Disolol with 1% toluidine blue (VWR)
- Centrifugate (184 g, 2 min)
- Remove 99% Disolol with 1% toluidine blue and incubate for 1 h in isopropanol (Chem-Lab)
- Remove isopropanol and incubate for 1 h in isopropanol/toluene (1:1, Chem-Lab)
- Remove isopropanol/toluene and incubate for 30 min in toluene (Chem-Lab)
- Remove toluene and incubate overnight in paraffine at 56 °C

**Supplement V: deparaffinisation and rehydration prior to staining**
- 3 x 5 min toluene
- 2 x 2 min isopropanol (Chem-Lab)
- 2 x 2 min 96% alcohol (Chem-Lab)
- 2 min tap water
- 1 min distilled H₂O

**Supplement VI: dehydration after staining**
- 2 x 2 min 96% alcohol
- 2 x 2 min isopropanol
- 2 x 2 min toluene
- 1 min toluene

**Supplement VII: results**

Table S1. Mean diameter (µm) ± 95 CI of hBM-MSC spheroids on different days of analysis

<table>
<thead>
<tr>
<th></th>
<th>Day of analysis</th>
<th>Diameter (µm) ± 95% CI</th>
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<tbody>
<tr>
<td>Non-encapsulated</td>
<td>Day 7</td>
<td>131.68 ± 2.80</td>
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<tr>
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<td>Day 14</td>
<td>116.73 ± 9.28</td>
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<td></td>
<td>Day 21</td>
<td>116.28 ± 6.89</td>
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<td></td>
<td>Day 28</td>
<td>128.84 ± 3.16</td>
</tr>
<tr>
<td></td>
<td>Day 35</td>
<td>122.94 ± 5.69</td>
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<tr>
<td></td>
<td>Day 42</td>
<td>122.09 ± 6.38</td>
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<tr>
<td>Encapsulated within Alg-MOD + Li-TPO</td>
<td>Day 21</td>
<td>88.72 ± 5.32</td>
</tr>
<tr>
<td>Encapsulated within Gel-MOD + Irgacure 2959</td>
<td>Day 28</td>
<td>80.09 ± 4.94</td>
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<tr>
<td>Encapsulated within Gel-MOD + Li-TPO</td>
<td>Day 21</td>
<td>134.50 ± 8.77</td>
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<tr>
<td></td>
<td>Day 28</td>
<td>114.10 ± 5.54</td>
</tr>
<tr>
<td>Encapsulated within Gel-MOD + Li-TPO</td>
<td>Day 21</td>
<td>94.94 ± 4.34</td>
</tr>
<tr>
<td></td>
<td>Day 28</td>
<td>92.74 ± 3.92</td>
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</table>
Table S2. Mean circularity ± 95 CI of hBM-MSC spheroids on different days of analysis.

<table>
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<tr>
<th>Day of analysis</th>
<th>Non-encapsulated</th>
<th>Encapsulated within Alg-MOD + Li-TPO</th>
<th>Encapsulated within Gel-MOD + Irgacure 2959</th>
<th>Encapsulated within Gel-MOD + Li-TPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7</td>
<td>0.88 ± 0.0045</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td>0.88 ± 0.0152</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 21</td>
<td>0.88 ± 0.0071</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>0.88 ± 0.0055</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Day 35</td>
<td>0.89 ± 0.0056</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 42</td>
<td>0.88 ± 0.0061</td>
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</tr>
</tbody>
</table>

Table S3. Mean stiffness ± 95 CI of 10 w/v% Gel-MOD hydrogel discs cross-linked with Irgacure 2959 or Li-TPO.

<table>
<thead>
<tr>
<th>Photo-initiator</th>
<th>Stiffness (N/mm) ± 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irgacure 2959</td>
<td>53.34 ± 5.13</td>
</tr>
<tr>
<td>Li-TPO</td>
<td>32.26 ± 4.68</td>
</tr>
</tbody>
</table>

Table S4. Mean swelling (%) ± 95 CI of 10 w/v% Gel-MOD hydrogel discs cross-linked with Irgacure 2959 or Li-TPO.

<table>
<thead>
<tr>
<th>Photo-initiator</th>
<th>Swelling (%) ± 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irgacure 2959</td>
<td>646.85 ± 12.83</td>
</tr>
<tr>
<td>Li-TPO</td>
<td>382.59 ± 27.78</td>
</tr>
</tbody>
</table>

Table S5. Mean gel fraction (%) ± 95 CI of 10 w/v% Gel-MOD hydrogel discs cross-linked with Irgacure 2959 or Li-TPO.

<table>
<thead>
<tr>
<th>Photo-initiator</th>
<th>Gel fraction (%) ± 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irgacure 2959</td>
<td>68.84 ± 0.80</td>
</tr>
<tr>
<td>Li-TPO</td>
<td>94.74 ± 0.75</td>
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</tbody>
</table>
Table S6. Mean doublet length (µm), width/height (µm), contact length (µm), and intersphere angle (degrees) ± 95 CI.

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
<th>8 h</th>
<th>10 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>168 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doublet length (µm)</td>
<td>236.53</td>
<td>231.40</td>
<td>229.67</td>
<td>227.68</td>
<td>221.92</td>
<td>217.43</td>
<td>124.54</td>
<td>172.38</td>
<td>163.75</td>
<td>161.105</td>
<td>156.765</td>
</tr>
<tr>
<td>± 10.34</td>
<td>± 11.02</td>
<td>± 9.98</td>
<td>± 10.54</td>
<td>± 10.78</td>
<td>± 11.35</td>
<td>± 4.61</td>
<td>± 10.96</td>
<td>± 11.28</td>
<td>± 7.35</td>
<td>± 8.52</td>
<td></td>
</tr>
<tr>
<td>Doublet width/height (µm)</td>
<td>131.30</td>
<td>129.68</td>
<td>129.50</td>
<td>127.56</td>
<td>117.06</td>
<td>120.08</td>
<td>124.54</td>
<td>130.43</td>
<td>134.78</td>
<td>137.49</td>
<td>135.23</td>
</tr>
<tr>
<td>± 5.46</td>
<td>± 5.63</td>
<td>± 5.64</td>
<td>± 4.87</td>
<td>± 8.70</td>
<td>± 6.25</td>
<td>± 5.06</td>
<td>± 6.76</td>
<td>± 3.23</td>
<td>± 5.22</td>
<td>± 6.11</td>
<td></td>
</tr>
<tr>
<td>Contact length (µm)</td>
<td>29.09</td>
<td>47.99</td>
<td>59.61</td>
<td>66.79</td>
<td>81.88</td>
<td>85.02</td>
<td>116.55</td>
<td>132.85</td>
<td>137.57</td>
<td>142.49</td>
<td>144.79</td>
</tr>
<tr>
<td>± 4.69</td>
<td>± 7.91</td>
<td>± 9.47</td>
<td>± 10.77</td>
<td>± 15.04</td>
<td>± 12.09</td>
<td>± 7.67</td>
<td>± 4.19</td>
<td>± 7.78</td>
<td>± 8.038</td>
<td>± 10.21</td>
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</tr>
<tr>
<td>Intersphere angle (degrees)</td>
<td>50.68</td>
<td>61.47</td>
<td>83.34</td>
<td>81.32</td>
<td>97.58</td>
<td>97.72</td>
<td>137.84</td>
<td>153.039</td>
<td>158.77</td>
<td>154.72</td>
<td>166.99</td>
</tr>
<tr>
<td>± 6.47</td>
<td>± 12.80</td>
<td>± 16.01</td>
<td>± 16.11</td>
<td>± 18.44</td>
<td>± 16.945</td>
<td>± 8.44</td>
<td>± 5.23</td>
<td>± 6.56</td>
<td>± 8.94</td>
<td>± 6.34</td>
<td></td>
</tr>
</tbody>
</table>
Figure S1 – Semiquantitative analysis of ECM molecules in spheroids after different periods of culture by evaluation of (immuno)histochemistry.

A (Immuno)histochemistry of hBM-MSC spheroids after day 21 and day 28 in culture, corresponding to seven and 14 days respectively post-encapsulation or post-bioprinting. Both encapsulation and bioprinting were performed with day 14 spheroids. Scale bar = 20 µm.

B,C,D,E Semiquantitative analysis of stainings which were scored strong (3), moderate (2), slight (1), or absent (0). Peripheral staining was marked with ‘P’.
BIOPRINTING OF (FIBRO)CARTILAGE

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Student number: 01403659

Promotor: Dr. Heidi Declercq
Supervisor: Lise De Moor

A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Science in the Biomedical Sciences

Academic year: 2018 – 2019