Inverted colloidal crystal scaffolds based on biodegradable polyesters for cartilage tissue engineering: Production, physico-chemical characterization, and in vitro evaluation

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Thesis to obtain the Master of Science Degree in

Biomedical Engineering

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“Learning never exhausts the mind.”

Leonardo da Vinci (1452 – 1519)
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ABSTRACT

Preparation of porous three-dimensional (3D) scaffolds is of great importance in tissue engineering. Inverted Colloidal Cristal (ICC) scaffolds have been suggested by several groups due to their long-range ordered structure, well controlled pore sizes and high interconnectivity between pores. This unique hierarchical porosity facilitates the migration of cells and an efficient nutrient, waste and oxygen diffusion along the entire structure.

In this study, polycaprolactone (PCL) ICC scaffolds with different pores sizes and polymer concentration were developed. To accomplish that, gelatin/poly(vinyl alcohol) (PVA) microspheres with 230, 280 and 340 µm diameter were produced and packed into a cubic close packed (ccp) lattice. In order to induce the necking between microspheres, a polyvinylpyrrolidone (PVP) solution was poured over the packed microspheres, turning this assembled structure into a solid colloidal crystal (CC). After impregnating this solid structure with PCL solution and subsequently freeze drying it, microspheres were dissolved and porous scaffolds with the inverted colloidal crystal (ICC) geometry were obtained.

To study the influence of pore sizes and polymer concentration on ICC’s mechanical properties, ICCs were mechanically tested in compression. The lowest and the highest Young’s modulus values obtained for the different ICC scaffolds were 0.84 MPa (280 µm pore and 20 % PCL) and 1.59 (280 µm pore and 40 % PCL), respectively. For in vitro evaluation, ICC scaffolds were seeded with ATDC5 cells. This cell line exhibits the multistep chondrogenic differentiation observed during endochondral bone formation. Adhesion rates around 30 % were obtain for both ICC types. Equal proliferation rates were verified for all ICC types during 11 days. Between day 1 and 3, the highest proliferation rate was recorded. Images of ATDC5 cells distributed along the entire ICC surface were obtained by staining cells with a blue fluorescent nucleic acid stain (DAPI). Glycosaminoglycans (GAGs) secreted by ATDC5 cells were visualized by staining ICC scaffolds with Alcian Blue and Safranin-O on the 20th day after seeding.

Overall, the ordered pores of ICC constructs are a promising material for cartilage tissue engineering.

Keywords: Inverted Colloidal Cristal Scaffolds, Colloidal Cristal, Cartilage Tissue Engineering, Microspheres, ATDC5 cells
RESUMO

A fabricação de matrizes porosas 3D é de extrema importância em Engenharia de Tecidos. Matrizes porosas 3D baseadas em réplicas invertidas de cristais coloidais, (denominadas em inglês por *Inverse colloidal crystal, ICC*), têm sido propostas por diferentes autores devido à sua estrutura regular, poros bem definidos e elevada interconetividade. Estas características peculiares permitem a migração das células, bem como uma eficiente difusão de nutrientes, resíduos e oxigénio ao longo de toda a estrutura.

Ao longo deste estudo foram produzidos ICCs de policaprolactona (PCL) com diferentes tamanhos de poros e concentração de polímero. Para isso, microsferas de gelatina/poli(álcool vinílico) (PVA) com 230, 280 e 340 µm de diâmetro foram produzidas e organizadas num empacotamento compacto de esferas iguais.

De forma a induzir a união entre microsferas, e assim tornar a estrutura empacotada num cristal coloidal (CC) sólido, uma solução de polivinil pirrolidona (PVP) foi vertida sobre estas. Após a impregnação desta estrutura sólida com a solução de PCL e posterior liofilização, as microsferas foram dissolvidas, dando origem ao ICC pretendido.

Com o intuito de estudar a influência do tamanho dos poros bem como a concentração de polímero nas propriedades mecânicas dos ICCs, estes foram testados através de ensaios de compressão. O menor e o maior valor de módulo de elasticidade obtidos para as diferentes matrizes foram 0.84 MPa (ICCs com poros de 280 µm e 20% de PCL) e 1.59 MPa (ICCs com poros de 280 µm e 40% de PCL), respetivamente.

Testes in vitro foram efetuados recorrendo a células ATDC5. Taxas de adesão de cerca de 30% foram obtidos para ambos os tipos de ICC. Taxas de proliferação similares para todos os tipos de ICC foram verificadas durante 11 dias de cultura, sendo que, entre o dia 1 e 3, foi obtido o valor mais elevado. Imagens da distribuição das células ATDC5 em toda a superfície dos ICCs foram obtidas através da marcação dos núcleos com DAPI. Glicosaminoglicanos segregados pelas células foram visualizados através da marcação dos ICCs com Azul Alciano e Safranina no vigésimo dia após a sementeira.

Uma vez que muitos dos métodos tradicionais de fabricação de matrizes porosas falham em um ou mais requisitos como poros irregulares, fraca interconexão e baixas propriedades mecânicas, os ICCs apresentam características que fazem deles uma elevada promessa para a engenharia de tecidos de cartilagem.

**Palavras-chave:** Réplicas invertidas de cristais coloidais, Cristal coloidal, Engenharia de Tecidos, Cartilagem, Microesferas, células ATDC
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<td>3D</td>
<td>3 Dimensional</td>
</tr>
<tr>
<td>ACI</td>
<td>Autologous Chondrocyte Implantation</td>
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<td>ALP</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>AOT</td>
<td>Autologous Osteochondral Transplantation</td>
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<td>BKC</td>
<td>Bovine knee chondrocytes</td>
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<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
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<td>Colloidal Cristal</td>
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<td>Cartilage oligomer matrix protein</td>
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<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
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<tr>
<td>DMEM</td>
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<td>Inverted Colloidal Cristal medium control</td>
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<tr>
<td>ICCs</td>
<td>Inverted Colloidal Cristal Scaffolds</td>
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<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
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<tr>
<td>iPSCs</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>KS</td>
<td>Keratan sulphate</td>
</tr>
<tr>
<td>LAP</td>
<td>Large aggregated proteoglycan</td>
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<tr>
<td>MACI</td>
<td>Matrix-assisted autologous chondrocyte implantation,</td>
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<tr>
<td>MC</td>
<td>Medium control</td>
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<tr>
<td>MSCs</td>
<td>Mesenchymal Stem Cells</td>
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<td>OA</td>
<td>Osteoarthritis</td>
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<td>OAT</td>
<td>Osteochondral autograft transfer</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PG</td>
<td>Proteoglycans</td>
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<td>PGA</td>
<td>Polyglycolic acid</td>
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<tr>
<td>PIINP</td>
<td>Amino-terminal type II procollagen propeptide</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly (lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly (lactic-co-glycolic) acid</td>
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<tr>
<td>PTHrP</td>
<td>Parathyroid hormone related protein</td>
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<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
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<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
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<tr>
<td>SLRPs</td>
<td>Small leucine rich proteoglycans</td>
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<tr>
<td>SPAN 80</td>
<td>Sorbitan monooleate</td>
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<td>SZP</td>
<td>Superficial zone protein</td>
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<tr>
<td>TGF-b1</td>
<td>Transforming growth factor beta 1</td>
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<tr>
<td>VEGF-A</td>
<td>Vascular endothelial growth factor A</td>
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<td>W/O</td>
<td>Water/Oil</td>
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<tr>
<td>Wnt</td>
<td>Wingless family</td>
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<tr>
<td>YKL40</td>
<td>Human cartilage glycoprotein-39</td>
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I. AIM OF STUDIES

Unfortunately, the intrinsic healing ability of human articular cartilage is extremely limited after suffering damage from injury or degenerative diseases. Osteoarthritis (OA) is the most common form of arthritis which affects close to 27 millions Americans, being more common in the elderly population. Over the age of 65, 60% of men and 70% of women experience OA. Due to the high incidence of cartilage injuries in society, numerous techniques have been developed over the years to treat this pathology such as microfracture, mosaicoplasty and autologous chondrocyte implantation (ACI). However, these available therapeutic options have uncertain long-term outcomes. In order to overcome some limitations present in these surgical interventions, cartilage tissue engineering is emerging as a potential solution. This interdisciplinary field focuses on combining cells and bioactive molecules with a biomaterial scaffold whose primary function is to provide a 3D environment for cells to adhere and proliferate, while at the same time acting as a mechanical support until the new cartilage is formed. A huge array of manufacturing processes have been extensively investigated to fabricate 3D scaffolds for cartilage repair such as solvent casting and particulate leaching, electrospinning and 3D-printing. However, most of these methods fail in one or more requirements like irregular pore size, poor interconnectivity or lack of mechanical strength.

Inverted Colloidal Cristal (ICC) scaffolds have been suggested by several groups due to their long-range well-ordered structure, uniform pore sizes and regular 3D interconnectivity. This unique hierarchical porosity allows an efficient nutrient and oxygen transport, facilitate cell migration and provide a large surface area for cell adhesion.

The aim of this project is the development of an ICC scaffold based on a biodegradable polyester, namely polycaprolactone (PCL), and apply it in cartilage tissue engineering. To accomplish this, gelatin/polyvinyl alcohol (PVA) microspheres with 230, 280 and 340 µm diameter were produced and packed into a cubic close packed (ccp) lattice. A poly(vinyl pirrolidone) (PVP) solution was used to induce the necking between microspheres, turning this assembled structure into a solid colloidal crystal (CC). After impregnating this solid structure with a PCL solution and subsequently freeze drying it, microspheres were dissolved and a PCL ICC scaffold was obtained.

In order to evaluate the influence of pore sizes and polymer concentration, ICCs were mechanically tested in compression.

For vitro evaluation, ICC scaffolds were seeded with ATDC5 cells. Adhesion rate, proliferation, cell distribution and glycosaminoglycans (GAGs) secretion were analyzed during 20 days of culture.

Since a huge array of manufacturing processes that have been extensively investigated to fabricate 3D scaffolds fail in one or more requirements like irregular pore size, poor interconnectivity and lack of mechanical strength, ICC scaffolds seem to be promising matrices for cartilage tissue engineering.
II. INTRODUCTION

II.1. Cartilage

II.1.1. Composition and Functions

Cartilage is an aneural and avascular flexible connective tissue composed of a specialized type of cells called chondrocytes embedded within a complex extracellular matrix (ECM) consisting primarily of water, collagen, proteoglycans (PGs), glycosaminoglycans (GAGs) and non-collagenous proteins [1] [2].

It is the different biochemical composition and arrangement of this highly specialized structure that gives cartilage the capability to perform many functions in different tissues and parts of the body [3]. In joints, articular cartilage is responsible to provide a deformable, low friction surface that enables the movement of articulating bones as well as the capacity to support high dynamic compressive loads [4]. Cartilage in the nose and the ear has the main purpose of maintaining shape and flexibility, whereas tracheal cartilage is mainly responsible for preventing the organ from collapsing during inspiration and also to protect it [3].

Chondrocytes have the main task of synthetizing and regulating the metabolism of ECM through different kinds of mechanical, electrical and biological stimulus, whose intensity influence the cellular response [5]. Unfortunately, natural cartilage repair is limited, exhibiting a deficient self-recovery ability due to its avascular, alymphatic and nearly nonimmunogenic properties [6] [7] [8]. Thus, even minor injury may give rise to progressive damage and consequent degeneration of cartilage [9]. In some cases, malfunction or loss of cartilage can results in particular diseases like osteoarthritis and achondroplasia [1].

Depending on the molecular composition of the cartilaginous tissues, these can be classified histologically into three types: fibrocartilage, elastic cartilage and hyaline cartilage [10]. These different types are distinguished by their structure, elasticity and strength.

The hyaline cartilage is the most abundant type of cartilage present in the human body and is characterized by being a shiny, translucent and bluish-white tissue. This type of cartilage exists in some specific areas of the body like articular surfaces of the bones, the ventral ends of ribs, and also in the trachea and bronchi of the lungs [11]. The hyaline cartilage is a complex living tissue which provides a frictionless surface, absorbs the mechanical shocks, distributes the mechanical loads to the underlying bone and participates in the lubrication of the synovial joints. Although the hyaline cartilage has a limited capacity to repair itself, it doesn’t present significant wear during its entire life under normal situations. However, the articular cartilage is more prone to deteriorate throughout life, due to the high mechanical efforts it is subjected to [12].

The hyaline cartilage matrix is an extensive network composed of water, chondrocytes and different types of macromolecules, consisting predominantly by collagen fibrils, PGs and multiadhesive glycoproteins (non-collagenous proteins) [11] [12].

60 to 80% of the wet weight of cartilage is composed of water. Most of the water is confined within the interstitial space produced by the collagen-proteoglycan meshwork, held in place thanks to the highly negatively charged PGs.
Chondrocytes represent only 3 to 5% of the total cartilage mass. However, their metabolism is responsible for producing and maintaining a stable and abundant ECM [12] [13]. The remainder of the hyaline cartilage matrix consists mainly of the essential macromolecules mentioned below.

II.1.1.1 Collagen

Collagen molecules represent about 15% of the total weight of the hyaline cartilage matrix. Type II is the most abundant collagen, comprising about 80 to 90% of the total collagen content. There are other types present in relative smaller amounts like collagen types III, VI, IX, X, XI, XII, and XIV [12] [2]. Despite the significant complexity and structural diversity among different collagen types, all members are characterized by having a right-handed triple helix composed of three α-chains [14]. These three α-chains are left-handed polyproline II helices which are coiled around each other with a one residue stagger in order to form a right-handed triple helix [15] [16]. A common structural feature of all collagens is the presence of a repeating (Gly-X-Y)_n sequence, X and Y being frequently proline and 4-hydroxyproline, respectively.

Type II collagen, the main collagen type present in hyaline cartilage, constitutes the bulk of the fibrils of the matrix, being predominantly responsible to supports chondrocytes adhesion, it provides high tensile strength of the tissue and withstand shear stresses [12] [4] [11]. Other collagen types present in smaller amounts can be formed due to different gene expression, translational splicing and post-translational modifications, many of them having extremely important roles [2]. Type IX and XI collagens are two important examples: collagen IX is located at the surface of type II collagen fibrils, participates in the formation of the type II collagen, and contains a N-terminal non-collagenous domain (NC4) that projects out from the fibril surface to interact with PGs and other matrix components [17].
Collagen XI is localized in the interior of collagen II/XI fibrils and has as its main purpose to control the assembly, growth and diameter of the collagen II heterofibrils [14].

**II.1.1.2 Proteoglycans**

Like collagen, PGs form an important group of macromolecules present in hyaline cartilage matrix, represent around 4-9% of the tissue’s wet weight [18]. There is a variety of PGs that can be found in this tissue, each of them performing several tasks that are essential for the normal function of cartilage [10]. The most prominent PG present in hyaline cartilage is aggrecan. This molecule consists of a polypeptide core protein from which numerous covalently bound GAG side chains extend, namely chondroitin (CS) and keratan sulphate (KS) polysaccharides [4] [10]. CS and KS polysaccharides are two important types of GAGs that are present in the ground substance of the hyaline cartilage. Structurally, a GAG is characterized by being a long-chain unbranched polysaccharide composed of repeated disaccharide units. These repeated units consist of an uronic acid (either D-glucuronic acid or L-iduronic acid) or galactose and hexosamine (N-acetylgalactosamine or N-acetylgalactosamine) [19]. It is the different constituent sugar molecules (hexosamine), hexose (galactose) and hexuronic acid that distinguish the different GAGs. GAGs are highly negatively charged due to sulfate and carboxyl groups. The typical aggrecan molecule has about 100 and 60 CS and KS side chains, respectively [12] [2]. However, this PG doesn’t exist in an isolated way within the ECM, but as PG aggregates. Each aggregate is composed of a long hyaluronan polysaccharide chain with numerous aggrecan molecules radiating from it [10]. The attachment of the several aggrecan molecules to the central filament of hyaluronic acid (HA) is mediated by molecules called link proteins, which associate with the base of each aggrecan core protein, stabilizing the interaction [4]. Thus, PG aggregates are interwoven and compacted within the collagen II fibril network creating a porous-permeable composite solid matrix that allows the movement of the fluid phase inside the matrix [13].

Due to the large number of fixed negatively charged side groups associated with chondroitin and keratan sulphate GAG chains, aggrecan has the capacity to attract cations, such as Na⁺ and therefore water into the cartilage ECM, creating a high tissue osmotic pressure [20] [2]. The hydrophilic
character of the cartilage ECM and the ability of the collagen fibrillar network to withstand expansions give hyaline cartilage the capacity to resist high compressive mechanical loads [5] [2].

Besides aggrecan, which is classified as large aggregated proteoglycan (LAP), there are other PGs present in hyaline cartilage matrix denominated by small leucine rich proteoglycans (SLRPs). These include decorin, biglycan, fbromodulin and lumican. Depending on both their protein core and GAG chains, SLRPs can perform several important roles namely in the control of collagen fibrillogenesis, protection of the fibrils from proteolytic damage and interaction with signaling molecules regulating proliferation, differentiation and ECM synthesis [21] [10].

![Figure II.3 – Diagram of proteoglycan aggregate and aggrecan molecule [13].](image)

**II.1.1.3 Multiadhesive glycoproteins**

Multiadhesive glycoproteins, also known as noncollagenous and nonproteoglycan-linked glycoproteins, represent a relatively small but extremely important group of molecules present in the ECM [12]. These molecules have distinct functional domains or polypeptide sequences to bind with cell-surface receptors such as integrin and laminin receptors, or to interact with a variety of ECM proteins like collagen, GAGs and proteoglycans. The diverse interaction between cells and glycoproteins regulates and mediates cell adhesion, migration, growth, and stimulates differentiation and proliferation of cells [22]. A clinical value of multi adhesive glycoproteins is that they can serve as markers for cartilage turnover and degeneration. For instance, the presence of human cartilage glycoprotein-39 (YKL40) in synovium or cartilage is known to be correlated with the existence of osteoarthritis disease, being a potential biomarker [23]. There are other types that can be found in hyaline cartilage matrix, namely fibronectin, which mediates a variety of adhesive and migratory events [22]; tenascin-C, which plays an important role in cell adhesion, proliferation and cellular signaling through induction of pro-inflammatory cytokines [12] [24]; and anchorine CII (cartilage anexin V), a small molecule that is used by chondrocytes to attach to collagen II and whose interaction regulates mineralization of growth plate cartilage [25].
II.1.2. Distribution of hyaline cartilage matrix components

Chondrocytes present in hyaline cartilage occur either singly or in isogenous groups. When they are agglomerated in clusters it means that the cells have recently divided, and once they begin to secrete matrix material surrounding them, they start to spread [12]. The ECM nearby chondrocytes can be classified into three different zones depending on the distance from the cell, each of them characterized by different types of collagens and other molecules [26].

Pericellular matrix is the region that surround chondrocytes. The enclosed cell together with the pericellular microenvironment is referred to as a “chondron” [27].

This region presents high concentrations of proteoglycans (aggrecan), hyaluronan, adhesive glycoproteins (fibronectin and laminin) as well as of type II, VI and IX collagens. Type VI collagen is the predominant type present around chondrocytes, having the main task of binding to the integrin receptors of the cell surface and so attach them to the macromolecular framework of the matrix [12] [26]. This region is responsible for regulating the biochemical microenvironment of the cell, protect the chondrocytes during compressive mechanical loads and serve as mechanical transducer [27]. Since proteoglycans have a high concentration of negatively charged side groups, pericellular matrix is well stained by basic dyes such as hematoxylin [12].

Territorial matrix is the region next to the pericellular matrix. It contains thin type II and VI collagen fibrils arranged in a “basket weave” conformation that extends out in a parallel arrangement to interact with type II collagen fibrils present in the interterritorial matrix. This endoskeleton acts as a scaffold for chondrocytes and proteoglycans, protecting them from mechanical loads [2]. Given that the territorial matrix has a lower concentration of sulphated proteoglycans it stains less intensely than the pericellular matrix [12].

Interterritorial matrix is the region that surrounds the territorial matrix and constitutes the major part of the cartilage matrix volume. It is composed of type II, IX and XI collagens, giving cartilage tensile
stiffness and strength. Type IX collagen residing on the surface of type II collagen fibrils interacts with proteoglycans and other matrix components due to its N-terminal non-collagenous domain (NC4). This region contains the thicker collagen fibrils and unlike the territorial matrix, these are not organized, changing their orientation along the cartilage depth [26].

Figure II.5 – (A) Hyaline cartilage matrix stained with H&H. (P) perichondrium, (DCT) dense connective tissue, (GC) Growing cartilage, (N) nuclei, (PM) pericellular or capsular matrix, (TM) Territorial matrix, (IM) Interterritorial matrix. (B) Diagram of an isogenous group. Adapted from [12].

II.1.3. Bone development: Endochondral ossification

During fetal development there are two major modes of bone tissue formation: intramembranous and endochondral ossification.

In intramembranous ossification, mesenchymal stem cells (MSCs) migrate and aggregate in specific areas of the mesenchymal tissue, proliferate and differentiate directly into osteoblasts which mineralize bone.

Endochondral ossification is a multistep process in which MSCs aggregate to form a cartilage template that is gradually replaced by mineralized bone [28].

After the cartilage template formation, MSCs proliferate and differentiate into chondrocytes. These cells start to secrete typical cartilage ECM components, contributing to the growth in length of cartilage model (interstitial growth). MSCs surrounding the condensate differentiate into perichondrial cells to form perichondrium, which once vascularized, becomes periosteum. Periosteum contains osteoprogenitor cells which later become osteoblasts, being responsible to create a bone collar around the diaphysis of the cartilage template (site of primary ossification center) [29] [30]
With the formation of the periosteal bony collar, proliferating chondrocytes in the mid region of the cartilage template cease their proliferation and enter into a transition stage called prehypertrophic chondrocytes, which quickly become enlarged hypertrophic chondrocytes [28]. These cells are responsible for secreting type X collagen, alkaline phosphatase (ALP) and vascular endothelial growth factor A (VEGF-A). ALP is an enzyme essential for mineral deposition, allowing the matrix calcification, whereas VEGF-A is an angiogenic factor that induces sprouting of blood vessels from the perichondrium, carrying hematopoietic and osteoprogenitor cells to the inside of the cavity. This cavity is formed when hypertrophic chondrocytes undergo apoptosis during matrix calcification [28] [29].

Secondary ossification centers are formed at the two ends of the developing bone (epiphysis), and separated from the primary center of ossification by the epiphyseal plate (growth plate), which is responsible for longitudinal growth. Briefly, while new cartilage is added at the epiphyseal side of the plate, cartilage at diaphyseal side degenerates and is replaced by bone. When an individual reaches maximum growth, production of new cartilage within the growth plate ceases and the remaining cartilage is completely replaced by bone, except at the articular surfaces [31].

![Figure II.6 - Schematic diagram of endochondral ossification. Adapted from [30].](image)

### II.1.4. Articular cartilage

The hyaline cartilage that covers the ends of bones in articulating joints is termed articular cartilage. This tissue provides low friction, highly elastic surface for pain free-mobility and also acts as a biomaterial to support compressive and shear forces [4].

In adults, articular cartilage is 2 to 4 mm thick and has four distinct histological and biochemical zones [32] [33]. The superficial zone, also known as the tangential zone, represents approximately 10% to 20% of the total articular cartilage thickness. Collagen fibrils in this zone (mainly, type II and IX collagen) are densely packed and aligned in parallel to the articular surface. A high number of flattened and elongated chondrocytes are present in this layer, being responsible for producing proteins that have protective and lubricating functions, like the superficial zone protein (SZP) [13]. This superficial zone is
responsible for resisting tensile, shear and compressive forces enforced by articulation [32]. Middle zone lies below the superficial zone and represents 40% to 60% of articular cartilage thickness. It is characterized by thicker collagen fibrils aligned obliquely to the surface and high proteoglycan content. Chondrocytes present a more rounded morphology than the superficial zone ones [4] [32]. Deep zone, 30% to 40% of articular cartilage thickness, exhibits large diameter collagen fibrils arranged perpendicularly to the articular surface and the highest proteoglycan content, providing a higher resistance to compressive forces. Chondrocytes are organized in columnar orientation perpendicularly to the joint line. Lastly, a calcified zone is present between the deeper zone and the subchondral bone.

A heavily calcified line called tidemark separates the deep zone from the calcified cartilage. Cartilage matrix is mineralized within this zone, type II collagen is replaced by type X and cell population is scarce [4] [13] [32].

II.2. Cartilage degradation and Osteoarthritis

Articular cartilage degradation can arise from numerous factors namely disease, trauma or continual and abnormal mechanical loading [33].

Unfortunately, cartilage has a very limited capacity for self-repair after damage from injury or degenerative disease due to its avascular nature, therefore, MSCs present in blood or resident chondrocytes cannot migrate to lesion sites [34].

Arthritis includes more than 100 different rheumatic diseases and is characterized by body joints and surrounding tissue inflammation, being responsible for significant morbidity. Osteoarthritis (OA) and rheumatoid arthritis (RA) are the two most common arthritic diseases [35].

OA is a degenerative joint disease characterized by progressive degeneration of the articular cartilage, subchondral bone, menisci and sinovium, causing debilitating joint pain and stiffness that worsens over time. Hips, knees, hands and feet are the body parts that are mainly affected, leading to
high disability and functional impairments. It is estimated that 27 million Americans suffer from OA, being more common in the elderly population [36] [37]. Over the age of 60 the prevalence of symptomatic knee OA is approximately 10% in men and 13% in women [38]. In a study performed in 2011, the prevalence of knee and hip osteoarthritis in Portugal were 11.1% and 5.5%, respectively [39]. The growing prevalence and incidence of OA with age makes this disease a major healthcare problem, generating loss of quality of life and high cost to the health system [37] [40] [41].

The etiology of this disease is due to a lot of issues, namely non genetic factors like age, lifestyle, obesity and joint injury as well as genetic factors (heredity and altered gene expression patterns of cartilage tissue) [40] [42]

**Figure II.8** – Proportion (%) of population residing in Portugal continental that reported cases of rheumatic disease, grouped according to gender and age [41].

**Figure II.9** - Risk factors that contribute to OA progression, which can be grouped into genetic, biomechanical and environmental. There is some cross-over within these categories. Adapted from [42]
Traumatic joint injuries in children and young people can lead to several types of chondral lesions whose severity can be measured using the International Cartilage Repair Society (ICRS) grading [43].

Due to the inability of articular cartilage to heal even the most minor injury, a small isolated defect leads to anomalous compressive loading and high mechanical stress in the surrounding healthy cartilage, which cause further degeneration of the healthy tissue. Some years after, a gradual erosion of articular cartilage is observed, giving rise to an osteoarthritic disease. When the articular cartilage is totally destroyed, subchondral bone is completely exposed allowing bones to rub directly against each other [4].

Cartilage degradation can be accompanied by the existence of numerous biomarkers during the beginning and progression of OA, like cartilage oligomeric matrix protein (COMP), amino-terminal type II procollagen propeptide (PIINP), YLK-40 glycoprotein and others (Table II.1) [40].

![Figure II.10](image_url) - How OA affects a joint. Adapted from [42].

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Function in joint</th>
<th>During OA elevated expression represents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage oligomeric matrix protein (COMP)</td>
<td>Help in inflammatory proliferation of synovial membrane. Stabilizes the ECM by its interaction with collagen fibrils and matrix components</td>
<td>Cartilage degradation</td>
</tr>
<tr>
<td>Amino-terminal type II procollagen propeptide (PIINP)</td>
<td>Reflects the rate of collagen type II synthesis</td>
<td>Cartilage degradation</td>
</tr>
<tr>
<td>YLK-40 Glycoprotein</td>
<td>Has a vital role in creating or amending tissue inflammation, immunity and/or remodeling</td>
<td>Cartilage Degradation</td>
</tr>
</tbody>
</table>

Table II.1 – Biomarkers of cartilage during onset and progression of Osteoarthritis. Adapted from [40].
**II.3. Cartilage treatment strategies: current state of art**

Due to the high incidence of cartilage injuries in our society, various techniques have been developed over the years to treat this pathology [44]. In order to alleviate pain and discomfort in the arthritic joint, nonsurgical treatments like physical therapy, dietary supplements, anti-inflammatory drugs and viscosupplementation (injection of HA into the synovial cavity) have been used. However, this kind of treatment doesn’t amend the underlying pathology [4].

Often, a surgical approach is required to repair the chondral articular surface, which can be divided in three main groups: palliative, reparative and restorative techniques [44] [43]. The chosen surgical therapy depends on numerous factors such as age, lesion and activity of the patient. Therefore surgeons should be aware of the potentials and limitations of the different available options in order to achieve the best results [33] [44].

Arthroscopic debridement and lavage is the first-line palliative treatment method for patients with low physical demands and chondral lesions with less than 2cm$^2$ in size. This approach involves the smoothing of fibrillated articular or meniscal surfaces, removal of osteophytes and loose bodies and the exclusion of inflamed synovium [43]. Arthroscopic joint lavage without debridement offers short-term benefits in 50% to 70% of patients [45]. This technique present benefits in patients with specific localized mechanical symptoms, stable ligaments and low body mass index, as opposed to patients with global arthritic changes. Usually, arthroscopic debridement and lavage precede any marrow stimulation technique (drilling or microfracture), providing a more durable outcome [43] [45].

Microfracture, the most studied reparative technique, is a low cost, minimally invasive and simple procedure widely used as a first line treatment of cartilage injuries [43] [46]. This technique is effective in small chondral lesions (less than 2 – 3 cm in diameter) with intact subchondral plate and patients with moderate demands [33]. Once the lesion has been identified, unstable cartilage is debrided using an arthroscopic shaver and curette, creating stable perpendicular edges of healthy cartilage around the defect that provide an optimal mechanical environment to the regenerating tissue. Next, an arthroscopic awl is used to create small perpendicular holes penetrating the subchondral plate, 3-4 mm apart and a depth of 4-5 mm [46] [47].

Due to these holes, bone marrow stem cells can migrate to the fibrin clot of the defect and, hopefully, proliferate and differentiate to produce a cartilaginous repair tissue [46].
However, this repair tissue is predominantly fibrocartilage that contains more type I collagen than type II collagen and therefore does not have the same ability of hyaline cartilage to resist compression and shear loads [4]. In the short term, microfracture allows a functional improvement in joint function but, this cartilaginous repair tissue becomes more fibrous and deteriorates over the time. Sometimes microfracture serves only to retard the eventual requirement for joint replacement [33].

A larger defect involving subchondral bone needs osteochondral autograft or allograft transplantation. Osteochondral autograft transfer (OAT) procedure, also known as mosaicoplasty, is indicated for small to medium chondral and osteochondral defects (up to 3 cm diameter) and for high demand patients over 50 years of age [43] [33]. In this technique, autologous osteochondral plugs are harvested from low-weight-bearing areas (medial and lateral margins of the trochlea, the intercondylar notch, or the sulcus terminalis of the lateral femoral condyle) and subsequently implanted in a single-stage surgical procedure. This approach allows transplantation of viable osteochondral plugs aiming bone-to-bone healing, since mature cartilaginous tissue does not have high healing capacity and rarely fully heals to surrounding cartilage [44]. Different diameter and different length grafts should be chosen depending on the osteochondral lesion, and must be press-fit inserted in order to provide good contact with the healthy tissue [48]. Usually the gaps between the plugs are filled in by fibrocartilage, which provide secondary stability [47]. The relatively low cost, immediate availability and successful osteointegration are the biggest advantages of this technique. However, there are some drawbacks associated like lack of available tissue, which reduces the possibility to treat large defects, and donor site morbidity [46] [48]. For large articular lesion treatment, a suitable alternative option is based on the use of osteochondral allografts, since the grafts can be designed in any shape or size and don’t induce any donor site morbidity. However, the difficulties in the preservation and manipulation of the fresh allografts, risk of immune reaction, and possibility of disease transmission limits the use of this technique [48] [44].

When arthroscopic debridement and lavage, microfracture, osteochondral autograft or allograft techniques fail, autologous chondrocyte implantation (ACI) is currently proposed as a second line of treatment for young active people (15 to 50 years of age) with chondral injuries between 2 and 3 cm² [48] [49]. The first stage of ACI technique involves the harvesting of a small cartilage piece from a non-weight-bearing region and subsequently isolation and expansion of articular chondrocytes. After 6 weeks, the second stage of this procedure is performed, consisting in an arthrotomy. The lesion site is carefully debrided and stable vertical walls of normal cartilage is created in order to favor the placement of a periosteal patch, which is gingerly sewn and sealed using fibrin glue. The cultured chondrocytes are then are injected under the periosteal graft [43] [46]. Despite the development of a native hyaline-like cartilage, which is mechanically stable and integrates with the surrounding articular surface, ACI presents some drawbacks that must be taken into account. The complexity and morbidity of the surgical procedure, the time required to expand enough chondrocytes in vitro, and dedifferentiation and poor retention of cells after implantation are some of ACI limitations [33] [44].
To overcome all limitations of each surgical intervention previously mentioned, cartilage tissue engineering is an emerging field that has shown numerous progressions.

**Figure II.11** - Current articular cartilage treatments. ACI = autologous chondrocyte implantation, MACI = matrix-assisted autologous chondrocyte implantation, TJR = total joint replacement [49].
II.4. Cartilage Tissue Engineering

Tissue Engineering is an interdisciplinary field which combines the principles of cell biology and engineering in order to develop biological functional substitutes that restore, maintain or improve the function of the damaged tissue or whole organ [50]. Thanks to tissue engineering techniques, a huge progress has been achieved in the repair of cartilage tissue defects.

This branch of Biomedical Engineering broadly involves the combinations of three components: cells, biological signal molecules and scaffolds [51].

II.4.1. Cells for cartilage defect repair

II.4.1.1. Chondrocytes

Chondrocytes are the most obvious choice for cartilage defect repair since they are present in the mature hyaline articular cartilage and are responsible for creating and maintaining a stable and abundant ECM [52]. The ability of these cells to produce cartilage-like matrix in vitro demonstrates their huge potential to regenerate cartilage [33]. However, the limited availability of chondrocytes, the low viability of these cells when they are harvested from diseased joints and their age are some limitations that must be taken into account. Unfortunately, chondrocytes tend to dedifferentiate when expanded in culture, characterized by decreased proteoglycans and type II collagen synthesis and augmentation of type I collagen expression [6]. It has been shown that adding transforming growth factor beta 1 (TGF-b1), fibroblast growth factor 2 (FGF-2) and insulin-like growth factor 1 (IGF-1) to culture medium retards this dedifferentiation process. Cultivation of these cells in three dimensional environment may also preserve the chondrogenic phenotype [52].

II.4.1.2. Stem cells

Due to the ability to self-replicate and differentiate into specialized cell types, stem cells have been considered as a hopeful alternative to differentiated chondrocytes in the repair of cartilage lesions. MSCs can be easily obtained from various tissues such as bone marrow, adipose tissue, infrapatellar fat pad, synovial membrane and umbilical cord blood, being capable of differentiating into chondrocytes under appropriate culture conditions [1]. These cells express a variety of surface markers including CD44, CD56, CD73, CD90, CD 105 and STRO-1 [52].

However, MSCs obtained from variable sources express different densities and types of cell surface markers, which can lead to different chondrogenesis capability [51]. For instance, an in vitro study revealed that human synovial MSCs had a higher potential for chondrogenesis than MSCs derived from bone marrow, muscle, periosteum and adipose tissue [51][53]. Although synovial MSCs seem to be a good cell source for cartilage defects repair, bone marrow MSCs are the most common cell sources utilized clinically given that they can be harvested more easily than sinovium [53]. The yield and chondrogenic potential of bone marrow MSCs decreases with increasing age, being this a little drawback since the elderly are the most affected. Therefore, it is difficult to define the optimal cell source for cartilage tissue engineering.
Co-culture systems have shown to be a great strategy to improve differentiation and chondrogenesis of MSCs. The major drawbacks of MSCs as cell sources for the use in cartilage tissue engineering applications are the unstable differentiation, hypertrophy and feasible mineralization, characterized by expression of collagen type X, matrix metalloproteinase-13 (MMP-13), vascular endothelial growth factor (VEGF) and ALP. The presence of these hypertrophic chondrocyte markers contributes to replacement of the cartilage by bone, in a similar process of developmental endochondral ossification. Co-culture assays have proven that parathyroid hormone related protein (PTHrP) secreted by articular chondrocytes decrease hypertrophic differentiation of the BM-derived MSCs [33] [54] [55]

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondrocytes</td>
<td>Able to produce, maintain and remodel the cartilage ECM in vitro.</td>
<td>Need for invasive surgery to harvests cells.</td>
</tr>
<tr>
<td></td>
<td>Proven clinical safety and efficacy.</td>
<td>Limited availability.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In vitro proliferative capacity and chondrogenic potential decrease with age.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tendency to dedifferentiate during culture expansion.</td>
</tr>
<tr>
<td>Bone marrow stem cells</td>
<td>Easily obtained from bone marrow.</td>
<td>Low yield (approximately 1 in 1×10^5 in the marrow).</td>
</tr>
<tr>
<td></td>
<td>High chondrogenic potential Broadly characterized and investigated</td>
<td>Harvest bone marrow is a very painful and invasive procedure.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proliferative capacity and chondrogenic potential decrease with age.</td>
</tr>
<tr>
<td>Adipose-derived stem cells</td>
<td>High abundance of tissue.</td>
<td>Inhomogeneous cell population</td>
</tr>
<tr>
<td></td>
<td>High yield (approximate 5000 stem cells per gram of aspirate)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low donor tissue morbidity.</td>
<td></td>
</tr>
<tr>
<td>Sinovium-derived stem cells</td>
<td>High yield.</td>
<td>Limited source of tissue</td>
</tr>
<tr>
<td></td>
<td>Higher potential for chondrogenesis than other MSCs.</td>
<td></td>
</tr>
</tbody>
</table>

Embryonic stem cells (ESCs) are another valuable option due to their ability to proliferate practically indefinitely while maintaining the ability to differentiate into all derivatives of the three primary germ layers, including chondrocytes. However, the possibility of differentiated chondrocytes undergoing de-differentiation into other lineages, the ability to form teratomas and ethical issues are three big challenges that remain to be solved when ESCs are used.
In order to solve the immunological and ethical problems associated with ESCs, induced pluripotent stem cells (iPSCs) have been used as an alternate method to create cells with ESC-like pluripotency. However, the low differentiation efficiency and teratoma formation still remain present [51][53].

II.4.2. Growth Factors

Growth factors represent a group of biologically active polypeptides that are used in cartilage tissue engineering studies in order to maintain chondrocyte phenotype, stimulate ECM production and induce chondrogenesis of stem cells [6].

The five most important families of growth factors that favor chondrogenic differentiation includes transforming growth factor-β super-family (TGFβ), the fibroblast growth factor family (FGF), the insulin-like growth factor family (IGF), the wingless family (Wnt) and the hedgehog family (HH) [56][1].

TGF-β1, TGF-β2 and TGF-β3 have shown to enhance proliferation and promote chondrogenic differentiation of MSCs in vitro studies. Furthermore, these biomolecules stimulate synthesis of collagen type II and aggrecan, and decrease collagen type I gene expression [1][52].

Bone Morphogenetic Protein (BMP) 2 and 7, also members of TGF-β super-family, have been shown to have the highest chondrogenic capacity of all BMPs. When BMP 7 is combined with TGF-β or IGF-1, the chondrogenic effect is synergistically increased [49].

FGF-2 is a potent mitogen for articular chondrocytes, and in combination with FGF-4 and FGF-8 has shown an important role in the reduction of the aggrencanase effect after cartilage loading, protecting them against cartilage degeneration and subsequently osteoarthritis disease [52].

The effects of some chondrogenic growth factors on stem cells are summarized in table II.3.

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Effects on MSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>Promotes cell proliferation and cartilaginous ECM secretion. Downregulates collagen type I expression</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>Increases cartilaginous ECM secretion</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Enhances redifferentiation of passaged chondrocytes. Promotes cell proliferation and cartilaginous ECM secretion</td>
</tr>
<tr>
<td>BMP-7</td>
<td>Induces chondrogenic differentiation. Additive effect on chondrogenesis when combined with TGF-β1 and IGF-1</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Encourage chondrocyte proliferation. Additive effect on chondrogenesis when combined with TGF-β1 and BMP-7</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Increases proliferation and proteoglycan production</td>
</tr>
</tbody>
</table>

Table II.3 – Effect of different growth factors on MSCs. Adapted from [52].
II.4.3. Scaffolds

Scaffolds are a key component for cartilage tissue engineering, consisting in a 3-dimensional structure that acts as a template for cell attachment, migration, proliferation, differentiation and synthesis of ECM while also providing a mechanically stable support until the new cartilage is formed [51] [6].

To accomplish those functions, scaffolds must present some requirements such as biocompatibility, biodegradability, have appropriate porosity and interconnectivity in order to facilitate waste/nutrients diffusion, and other features mentioned on table II.4 [51] [52].

A wide variability of biomaterials are used to fabricate scaffolds for cartilage repair, predominantly natural and synthetic polymers.

Natural polymers such as collagen, fibrin, alginate and chitosan have been used to develop a huge variety of scaffolds namely hydrogels, sponges and electrospun nanofibers. These polymers present several advantages like biocompatibility, biodegradability, cost effectiveness and biological activity. However, the low mechanical strength, high degradation rate and the risk of an immunogenic response are some drawbacks associated with their use [1] [4].

An array of synthetic polymers has been used in cartilage tissue engineering due to the facility to modify their degradation properties, structure, and mechanical strength. The superior biomechanical properties of synthetic scaffolds compared to those based on hydrogels makes them a better option for weight bearing joints [6]. However, the acidic products resulted by their degradation (for instance PLA) and the lack of cellular adhesion and interaction are some weaknesses [1] [33].

Table II.4 – Important characteristics of scaffolds. Adapted from [1].

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D structure</td>
<td>Support cellular ingrowth. Assist nutrient and oxygen transportation.</td>
</tr>
<tr>
<td>Porosity</td>
<td>Maximize the space for cellular adhesion, growth and ECM production.</td>
</tr>
<tr>
<td>Interconnected pores</td>
<td>Facilitate oxygen and nutrient/waste diffusion. Allows cell migration.</td>
</tr>
<tr>
<td>Biocompatibility</td>
<td>Does not elicit any rejection, inflammation or immune response.</td>
</tr>
<tr>
<td>Nano-scale topography</td>
<td>Promote cell adhesion and better cell-matrix interaction.</td>
</tr>
<tr>
<td>Good mechanical properties</td>
<td>Withstand in vivo stresses.</td>
</tr>
<tr>
<td>Biodegradable</td>
<td>Degradation rate must perfectly match the rate of tissue regeneration and degraded products should be harmlessly metabolized by the organism.</td>
</tr>
<tr>
<td>Surface modifiable</td>
<td>Functionalize chemical or biomolecular groups in order to improve tissue adhesion.</td>
</tr>
</tbody>
</table>
Polyglycolic acid (PGA), poly(lactic acid) (PLA), Poly(lactic-co-glycolic) acid (PLGA) copolymer are largely used in cartilage tissue engineering since their mechanical and degradation properties can be adjusted by simply varying the molecular weight and the copolymer ratio.

Polycaprolactone (PCL) is one of the most widely used biodegradable polyester in tissue engineering due to its good biocompatibility, slow biodegradability, mechanical properties and structural flexibility. It degrades at a much slower rate than PLA, PGA, PLGA and the resulted degradation products are harmlessly metabolized in the tricarboxylic acid cycle. PCL based scaffolds have shown a huge potential in cartilage tissue engineering (Table II.5) [49].

<table>
<thead>
<tr>
<th>Repeating unit</th>
<th>Properties</th>
<th>Cartilage tissue engineering applications</th>
</tr>
</thead>
</table>
| Polycaprolactone (PCL) | Density: 1.145g/cm³  
Melting point: 59-64 ºC  
Glass transition temperature: -60 ºC | PCL cylindrical scaffolds with gradual increasing pore size (from ~90 to ~400 µm) along the longitudinal direction were used to investigate the effect of pore sizes on the chondrogenic differentiation of adipose stem cells (ASCs) [57]. Articular cartilage repair potential was compared between nano-structured porous polycaprolactone (NSP-PCL) scaffold with a commercially available collagen type I/III (Chondro-Gide®) scaffold. NSP-PCL scaffold showed higher in vitro expression of chondrogenic markers compared to the Chondro-Gide® [58]. 3D PCL scaffolds chemically conjugated with BMP-2 were seeded with chondrocytes in order to investigate the influence of this molecule on cartilage matrix production and potential subsequent bone matrix formation [59]. |
A plethora of fabrication technologies have been applied to develop three dimensional scaffolds for tissue engineering applications, namely solvent casting and particulate leaching, phase separation, gas foaming, emulsion freeze drying, fiber bonding, electrospinning, 3D printing and others. [60] [61]

Each of these manufacturing processes exhibits potential and limitations, however, most of these methods fail in one or more requirements like irregular pore size, poor interconnectivity, irregular structure and low mechanical properties (table II.6) [60] [49]

In order to overcome these limitations, 3D scaffolds based on inverted colloidal crystal (ICC) geometry have been suggested by several groups since they possess a long range ordered structure, well controlled pore sizes and uniform interconnections [62]

Table II.6 – Advantages and disadvantages of some scaffold fabrication techniques. Adapted from [63] [64] [65].

<table>
<thead>
<tr>
<th>Technique</th>
<th>Description</th>
<th>Merits</th>
<th>Demerits</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Electrospinning</strong></td>
<td>Polymer fibers are electrostatically spun into a target substrate.</td>
<td>Control over pore sizes, porosity and fiber thickness. Use of minimum amount of material. Capable of incorporating multiple polymers.</td>
<td>Pore size decrease with fiber thickness. Non uniform cell infiltration/tissue formation Low mechanical properties</td>
</tr>
<tr>
<td><strong>Solvent casting/particulate leaching</strong></td>
<td>Polymer scaffold containing porogen is solidified. Then the porogen is leached out to obtain a porous structure.</td>
<td>Simple process. Control over porosity (up to 93%), pore sizes and crystallinity.</td>
<td>Limited membrane thickness (ranging from 0.5 to 2mm). Lack of mechanical strength. Limited interconnectivity. Residual solvent and porogen material.</td>
</tr>
<tr>
<td><strong>Phase separation</strong></td>
<td>Polymer is dissolved in organic solvent and then solidified with liquid nitrogen. Removal of the solidified solvent-rich phase by sublimation leaves a porous polymer scaffold.</td>
<td>High porosity Ability to incorporate bioactive molecules</td>
<td>Difficult to control precisely pore size and micro geometry. Solvent residue may be harmful.</td>
</tr>
<tr>
<td><strong>3D-Printing</strong></td>
<td>Scaffold is generated by ink-jet printing a binder on to sequential powder layers.</td>
<td>Complete pore interconnectivity. Possibility to incorporate biological agents (e.g. Growth factors) during printing process. Independent control over porosity and pore size.</td>
<td>Resolution determined by the jet size. Lack of mechanical strength. Limited choice of materials (e.g. organic solvents as binders).</td>
</tr>
</tbody>
</table>
II.5. Inverted Colloidal Crystal scaffolds

So far, many research groups have made huge efforts in order to optimize 3 dimensional porous scaffolds, trying to provide the most propitious microenvironment for cell attachment, proliferation and differentiation [66]. Nonetheless, the majority of the reported scaffolds exhibit irregular pores chaotically distributed in space and poor interconnectivity, hindering the formation of homogeneous tissues. To address these issues, inverted colloidal crystal (ICC) scaffolds have been suggested due to their long-range well-ordered structure, uniform pore size and regular 3D interconnectivity [66] [67].

The high interconnectivity of these inverse opal scaffolds facilitates the migration of cells and an efficient nutrient, waste and oxygen diffusion along the entire structure. To date, ICCs have been applied in a wide range of applications namely neovascularization, bone, liver, cartilage and neural tissue engineering [67].

Focusing on cartilage tissue engineering, Yung-Chih Kuo and Yu-Tai Tsai demonstrated a uniformly distributed chondrogenesis in chitin/chitosan matrix with pores of inverted colloidal crystal scaffolds comparatively with freeform constructs, which present random and unconnected pores. They showed a uniform spatial distribution of bovine knee chondrocytes (BKC) over the entire ICC scaffold, with secretion of GAGs and collagen [7]. In another study, they demonstrated that heparin-conjugated ICC enhanced the viability of BKC as well as the GAGs and collagen production [68].

II.5.1. Preparation of ICC scaffolds

ICC scaffolds are typically made in five step processes: i) production of uniform microspheres, ii) packaging of these microspheres into a cubic close packed (ccp) lattice, iii) thermal treatment (annealing) or other process to induce necking between adjacent microspheres, iv) filling the interstitial space with the scaffolding material and subsequent freeze-drying, v) dissolution of the microspheres, leaving an inverse opal structure behind

Figure II.12 – 3D schematics showing the typical fabrication process of an inverted colloidal crystal scaffold [65].
II.5.1.1. Microspheres production

Microspheres can range between nanometers and micrometers, depending on their production method. In ICC scaffolds for tissue engineering, microsphere diameters should range between 50 and 1000 µm in order to enable human cells with typical dimension from 10 to 30 µm to migrate into the entire structure [60].

Microspheres can be produced by a wide variety of methods including solvent-in-emulsion evaporation, spray drying, gelation, phase separation, electrospraying and others. Although each of these techniques present different manufacturing processes and manipulations, all of them intend to have control over particle size, shape, surface characteristics and porosity, however, this does not always happen. Ideally the method should also produce large quantities of particles with a narrow size distribution [69] [60] [60].

Recently, microfluidic methods have been described to synthesize monodisperse microspheres. Droplet microfluidics allows precise control over size and frequency of droplets formation, which is possible due to the geometry of the microchannels and the combination of driving pressure (flow rate) of two immiscible liquids. Microspheres with less than 5% variation have been produced using this approach [70] [60] [71]. In this technique, microspheres production is based on the formation of stable oil-in-water (O/W) or water-in-oil (W/O) emulsions. Figure 11 illustrates how monodisperse microspheres are formed. Briefly, syringe pump A impels one solution denominated by continuous phase whereas syringe pump B impels another solution (discontinuous phase) immiscible with the previous one. When both of these solutions enter in contact at the end of the needle, O/W droplets are formed and collected with a small container.

![Figure II.13](image_url)

Figure II.13 – Microfluidic technique scheme. Continuous and discontinuous phase are injected through syringe pumps A and B, respectively. O/W droplets are formed at the needle tip and collected in a container. Adapted from [70].
These O/W droplets can be formed in two different manners: dripping or jetting mode. In the dripping mode, emulsion droplets are formed at the tip of the needle, while in the jetting mode a thin jet stream is formed and subsequently breaks into drops away from the orifice. When the jet stream becomes unstable, it breaks up into small segments which shrink into spherical droplets in order to minimize the surface area \[70\] \[72\]

These behaviors can be predicted by two dimensionless number: Capillary (Ca) and Weber (We) number.

The Capillary number is defined by:

$$C_a = \frac{\mu_c \nu_c}{\gamma}$$  \(1\)

Where \(\mu_c\) and \(\nu_c\) are the viscosity and velocity of the continuous phase, respectively, and \(\gamma\) is the interfacial tension between the two immiscible fluids. It represents the relative effect of viscous shear force versus interfacial tension acting across an interface between two immiscible liquids.

The Weber number is defined by:

$$W_e = \frac{\rho_d d_{tip} v_d^2}{\gamma}$$  \(2\)

Where \(\rho_d\), \(d_{tip}\) and \(v_d^2\) are the density, inner diameter of capillary tube and velocity of discontinuous phase, respectively. It describes the inertial force of the inner liquid compared to its interfacial tension.

When both \(C_a\) and \(W_e\) are small (low values of flow rate and viscosity), surface tension dominates and consequently dripping occurs. In opposite conditions, for instance \(W_e > 1\), jetting mode predominates \[70\] \[73\]

![Figure II.14](image-url)

**Figure II.14** – Image of drop formation by dripping regime (a) and jetting regime (b). Adapted from [72].

If the jetting regimes prevails, the drops have a larger range in size, and consequently microspheres have larger size distribution [72].
The diameter of microspheres can be handled varying the polymer concentration of the discontinuous phase, the flow rate of each phase or the diameter of the needle orifice.

II.5.1.2. Microspheres packing

After production, microspheres are organized into a hexagonal close-packed geometry. This self-organization is influenced by repulsive/attractive forces and also by the size, composition and crystallinity of the microspheres. Microspheres with uniform sizes and shapes organize by a mechanism called self-assembly, which is basically the autonomous organization of particles without human intervention. However, external fields or geometric confinements are sometimes required to induce self-assembly. Different methods have been reported to assemble microspheres into organized structures such as gravity sedimentation, centrifugation, solvent evaporation, ultrasound and others [60] [70] [74].

![Image of different methods to assemble colloidal crystalline templates](image)

Figure II.15 - Different methods to assemble colloidal crystalline templates. Adapted from [74].

II.5.1.3. Annealing

In ICC scaffolds, annealing is considered the heat treatment applied to packed microspheres in order to induce necking between them, turning this assembled structure into a solid colloidal crystal (CC). The crystallinity of the polymer used to produce microspheres is an important factor in annealing process. This treatment allows the control of the diameter of interconnecting channels. Usually, increasing annealing temperature results in an ICC scaffold with higher diameters contact spots [75] [62].
II.5.1.4. ICC Scaffold

After microspheres packing and subsequent annealing, this solid CC is impregnated with a polymer solution generally done with a vacuum pump, filling the interstitial spaces. Solidification of the impregnated CC can be achieved by polymerization, freezing and posterior lyophilization in a freeze-drier, electrochemical deposition and crosslinking.

Finally, microspheres are dissolved and an ICC scaffold is obtained with a maximum theoretical porosity of 74% [60] [76].

Figure II.16 - SEM images showing the diameter of interconnecting pores by annealing gelatin microspheres at 65, 80 and 100 °C during 3 hours. Adapted from [65].

Figure II.17 - Schematic diagrams and corresponding SEM images. (A) Microspheres packed in a hexagonal close-packed geometry. (B) Polymer infiltration and subsequent freeze-drying. (C) Dissolution of the microspheres giving rise to an ICC scaffold. Adapted from [76].
III. Material and Methods

III.1. PCL ICC scaffold fabrication

III.1.1. Microspheres production

Gelatin (Gelatin from porcine skin; Sigma-Aldrich®) and Gelatin/Polyvinyl alcohol (Mw = 9500, 95% hydrolysis, Acros Organics) microspheres were produced by microfluidic technique.

For the continuous phase, liquid paraffin (density at 15°C ≈ 0.865 g/ml, viscosity at 40°C ≈ 72.6 cSt, Labchem) with 6% w/w sorbitan oleate (density ≈ 0.99 g/ml, Fagron) contained in a 50 ml syringe (Omnifix®) was injected by an infusion pump (Baxter Flo-Gard GSP) into a teflon tube through a 17G needle. In order to form W/O droplets, the discontinuous phase contained in a 20 mL syringe (Omnifix®) was also injected through a 23G needle, which was inserted inside another teflon tube.

For gelatin microspheres production, aqueous solutions with 5, 10 and 15% w/w gelatin were used. For gelatin/PVA microspheres, a 10% w/w gelatin aqueous solution with 1.5, 2.0 and 2.5% w/w PVA was used. Since the gelatin solution gelled at room temperature, a thermocouple wire was wrapped around the syringe to maintain the solution in a liquid state. Microsphere diameter was controlled through the variation of continuous and discontinuous phase flow rates, as well as gelatin concentration.

W/O droplets formed at the needle tip of discontinuous phase were expelled from the teflon tube and collected in a plastic recipient. The excess paraffin was removed from the recipient and the microspheres were frozen, allowing the aqueous phase droplets to gel quickly. After gelation, microspheres were washed with successive acetone/water solutions with a volume ratio of 4:3, 4:2, 4:1 and lastly only acetone. Washing the microspheres with increasing concentrations of acetone allowed their slow dehydration. To achieve complete dehydration, microspheres were finally stored in isopropanol. Microspheres morphological analysis was done by optical microscope. 50 random samples of each production cycle were used to measure microspheres diameter with the help of ImageJ™.

III.1.2. Microspheres Packing

For colloidal crystal (CC) production, it is critical to use microspheres with a narrow size distribution. To accomplish that, for microspheres with an average diameter of 330, 270 and 230 µm, sieves (Analysensiebe) with 355 and 300 µm, 250 and 280 µm and 250 and 212 µm, respectively, were used.

A mold consisting of 32 wells with 6mm diameter and 2mm height was developed for CC production. Isopropanol suspension of microspheres was slowly dropped to the wells and gently agitated by an orbital shaker (SK-330-Pro) in order to organize them into a hexagonal close-packed geometry. When the wells were full, the mold was left on the orbital shaker a further 45 minutes for isopropanol evaporation.
III.1.3. Annealing

After microspheres packing, the next step is to induce the necking between them, turning this assembled structure into a solid CC. Usually, this is done by a process called annealing, which is basically a heat treatment applied to packed microspheres. Instead of an annealing treatment, a 5% polyvinylpyrrolidone (PVP, Sigma-Aldrich®) solution in isopropanol was poured over the packed microspheres. After isopropanol evaporation, PVP acts as a kind of glue that allows connectivity between microspheres, turning the assembled structure into a linked solid construct. Lastly, solid CC were removed from wells and stored for further visualization in SEM and ICC scaffolds production.

III.1.4. CC impregnation

After obtaining a solid CC, this structure was impregnated with a polymer solution. Polycaprolactone (Mw ≈ 43,000 – 50,000; Polysciences) was the biodegradable polyester chosen due to its biocompatibility, slow biodegradability, mechanical properties and structural flexibility. Three different PCL concentration solution in dioxane (C₂H₅O₂; Panreac) were prepared and impregnated depending on microspheres size used to build CC. CC’s of 230 and 330 µm microspheres were impregnated with 30% PCL solution, whereas CC’s of 270 µm were impregnated with 20, 30 and 40% PCL solutions.

To carry out impregnations, CC’s were immersed in a flask containing the PCL solutions and subsequently put in a vacuum desiccator. The air removal by vacuum pump allowed PCL solution fill the interstitial spaces between microspheres. This process was repeated until no air bubbles were released from CC structure.

Finally, the excess of polymer solution was gently cleaned with paper and CC’s were frozen and posteriorly lyophilized in a freeze-drier (Vaco 2, Zirbus) during 24 hours.

III.1.5. Inverted Colloidal Cristal (ICC) scaffold

After lyophilisation, the next step comprises the dissolution of gelatin microspheres, giving rise to a PCL ICC scaffold. To accomplish that, impregnated CC’s were immersed in warm water at approximately 48 ºC overnight. Care must be taken setting this temperature since PCL has a melting point around 59-64 ºC. ICC scaffolds were then dried at room temperature and were finally ready to be used.

III.2. Scanning electron microscopy (SEM)

CC’s and ICC scaffolds were analyzed by SEM (Zeiss DSM-962).

III.3. Mechanical Properties – Compression tests

In order to study mechanical properties, ICC' scaffolds were mechanically tested in compression (Rheometric Scientific equipment; Minimat Firmware 3.1) using a 20 N load cell and a velocity of 0.2 mm/min. For each ICC type, 10 samples were used. From the force and compression values given by compression test, the stress vs. strain curves were plotted and the Young’s modulus calculated.
III.4. Cell culture

III.4.1. ATDC5 Cells

In vitro studies were performed using ATDC5 cells. This cell line is derived from mouse teratocarcinoma cells and exhibits a multistep process of chondrogenic differentiation analogous to that observed during endochondral bone formation, making it a useful model for in vitro studies. Culture medium consisted in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) and HAM's F12 (Sigma-Aldrich) containing 5% Fetal Bovine Serum (FBS) (Invitrogen, catalog #10270106) and 1% antibiotic (penicillin-streptomycin, Invitrogen, catalog #15140122). The cryovial containing the ATDC5 cells was taken out of the -80 ºC freezer and placed in a thermostatic water bath at 37 ºC until it was thawed. The cell suspension was transferred to a 25 cm² flask with 5 mL of culture medium and kept in a CO₂ incubator at 37 ºC. The following day, the medium was changed in order to remove the DMSO used in the freezing medium. Afterwards, the medium was changed every second day.

III.4.2. ICC scaffold preparation

Before sowing ATDC5 cells, 10 ICC scaffolds of each type (5 different types) were placed in a 96 well plate (each well with one ICC) and sterilized with ethanol 70 %. To avoid the floating of the scaffolds, air present into the structure was removed by a vacuum pump. After sterilization, ethanol was removed and ICC’s were washed three times with a phosphate buffered saline solution (PBS) (Invitrogen, catalog #14190-185), and finally, with culture medium. Control ICC’s (ICC’s without cells) were prepared in the same way.

III.4.3. ATDC5 cell culture

Cells present in 25 cm² flask were trypsined (TrypLE™ Express, Invitrogen), counted using a hemocytometer, and seeded with a concentration of 7x10³ cells/well. To accomplish that, 100 µl of cell suspension was gently pipetted onto the ICC scaffold surface. After that, the 96 well plate was kept in a CO₂ incubator with the medium being changed every second day.

Previous studies have detailed a well-characterized method of ATDC5 differentiation and mineralization. However, it takes up to two months to complete the cell expansion process, insulin addition to induce differentiation and further alterations in culture conditions to induce hypertrophy.

Tecla M. Temu et al [77] demonstrated spontaneous differentiation of ATDC5 cells maintained in culture medium supplemented with ascorbic acid. Comparing the differentiation events in response to high-dose insulin vs. ascorbic acid, a similar expression pattern of key genes, including collagen II, Runx2, Sox9 and collagen X, was showed.

Thus, from the second day on, the culture medium was substituted for DMEM:F12 with 100 µg/ml of ascorbic acid (L-Ascorbic acid 2- phosphate sesquimagnesium salt hydrate, Sigma-Aldrich) in order to shorten the prechondrogenic proliferation phase, induce cell differentiation and promote ECM secretion. Control ICC’s were treated in the same way, but without cells.
III.5. Resazurin assay

The resazurin assay is a simple, rapid and sensitive method to measure metabolic activity of living cells by the reduction of the non-fluorescent dye resazurin (blue) to the strongly-fluorescent dye resorufin (red). Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal. The amount of dye conversion can be measured spectrophotometrically. The maximum absorbance of resazurin and resorufin occurs at 605nm and 573nm, respectively.

The resazurin solution was prepared by dissolving the powder (Alfa Aesar) in PBS at a concentration of 0.25 mg/ml.

Following the removal of culture medium, 100μl of DMEM:F12 with 10% resazurin solution was pipetted into each well (ICC scaffolds, cell controls, medium control (MC) and ICC medium control (ICCMC)). The 96 well plate was kept in the CO₂ incubator at 37ºC during 3 hours for the reaction to occur. After 3 hours, 90 μl of each well was pipetted to another 96 well plate, which is posteriorly placed in a spectrophotometer (ELX800 Biotek) to read $A_{570}$ and $A_{600}$. After that, ICC’s and cell controls were washed with PBS once to remove the remaining resazurin solution. Finally, 100 μl of medium was added to each well and the microplate was placed back in the incubator.

III.5.1. Adhesion rate

To calculate the adhesion rate, all ICC types were initially transferred to other 96 well plate. Then, the above mentioned procedure was done for ICC’s present in the new plate, for the wells where ICC’s were initially (to count how many cells adhered to the well surface instead of to the scaffold), for cell controls and for both medium controls. After absorbance reading, it was possible to calculate adhesion through simple equations showed in discussion of results.

III.5.2. Proliferation

In order to evaluate the proliferation of ATDC5 cell in ICC scaffolds, resazurin assay was performed every second day.

III.6. DAPI Nucleic Acid Stain

In order to see the cell distribution along the ICC surface, ATDC5 cells were stained with DAPI (Invitrogen), which is blue fluorescent nucleic acid stain that preferentially stains dsDNA.

To prepare DAPI stock solution, 10 mg of DAPI was dissolved in 2 mL of deionized water. Posteriorly, this stock solution was diluted to 300nM in PBS.

Initially, ICC’s were rinsed once with PBS and fixed for 15 minutes at room temperature (RT) with 3.4% paraformaldehyde (PFA). After removal of PFA, ICC’s were washed three times with PBS. 100 μl of DAPI solution was pipetted for each ICC and left to act during 5 minutes at RT. To finalize, ICC were washed 3 times with PBS, observed using fluorescence microscopy, and images taken.
III.7. Chondrogenic differentiation staining

III.7.1. Alcian Blue Staining

In order to demonstrate chondrogenesis by identification of sulfated glycosaminoglycan deposits (synthesized by ATDC5 cells), ICCs were stained with Alcian Blue on the 20th day after the sowing. The Alcian Blue assay is the fastest way to quantify glycosaminoglycans (GAGs). Alcian Blue is a tetravalent cationic dye with a hydrophobic core that contains copper, which gives it its blue color. The four charges allow the dye to bind to negatively charged GAGs at high ionic strength. The ionic bonding between cationic dyes and the negatively charged GAGs are generally proportional to the number of negative charges present on the GAG chain (both sulfate and carboxyl groups).

Following the removal of the differentiation medium, ICCs were washed with PBS and fixed with 3.4% PFA for 15 minutes at RT. After that, the PFA was removed and ICC’s were washed three times with PBS. 100 μl of 1% Alcian Blue (Alfa Aesar) solution prepared in 3% acetic acid were pipetted to each ICC, and left to act during 10 minutes. ICC were rinsed 4 times with PBS in order to remove excess staining. To finalize, ICC’s were left overnight at RT to dry and observed under optical microscope.

III.7.2. Safranin-O staining

To stain cells with safranin, 0.1 % safranin (Alfa Aesar) solution dissolved in deionized water was prepared. The staining protocol was the same as for Alcian Blue.
IV. Results and Discussion

IV.1. Inverted Colloidal Cristal Scaffolds (ICCs) production

IV.1.1 Gelatin microspheres production

Gelatin microspheres with different sizes were produced by varying gelatin concentration and continuous phase flow rate. Initially, an aqueous solution of 5% gelatin (discontinuous phase) was pumped with a flow rate of 2 mL/h. In order to form W/O droplets, a continuous phase consisting of liquid paraffin and sorbitan oleate (SPAN 80) was ejected with a flow rate of 10mL/h. The resulting W/O droplets were expelled from the teflon tube and collected in a large plastic recipient. This large recipient is important to allow the microspheres to spread on the entire surface and be in contact with air. After a few hours, water present in the droplets evaporates and solid microspheres are formed. If the droplets are completely immerged in paraffin, the evaporation process is retarded, being difficult to obtain solid microspheres. Microspheres with smaller diameters were produced repeating the same procedure but now for 18 and 26 mL/h of flow rates.

In order to study the influence of gelatin concentration on microsphere size, all the aforementioned steps were repeated for 10 and 15 % gelatin aqueous solutions.

Table IV.1 - 5% gelatin microspheres production. CF-continuous phase, DF-discontinuous phase.

<table>
<thead>
<tr>
<th>CF (mL/h)</th>
<th>DF(mL/h)</th>
<th>Diameter (μm)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2</td>
<td>Non spherical shape</td>
<td>Non spherical shape</td>
</tr>
<tr>
<td>18</td>
<td>2</td>
<td>Non spherical shape</td>
<td>Non spherical shape</td>
</tr>
<tr>
<td>26</td>
<td>2</td>
<td>Non spherical shape</td>
<td>Non spherical shape</td>
</tr>
</tbody>
</table>

Figure IV.1 - 5% gelatin microspheres. (A) CF = 10 mL/h and DF = 2 mL/h, (B) CF = 18 mL/h and DF = 2 mL/h, (C) CF = 26 mL/h and DF = 2 mL/h. Scale bar = 100 μm.
Table IV.2 – 10% gelatin microspheres production. CF-continuous phase, DF-discontinuous phase.

<table>
<thead>
<tr>
<th>CF (mL/h)</th>
<th>DF (mL/h)</th>
<th>Diameter (μm)</th>
<th>Standard deviation (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2</td>
<td>300</td>
<td>9</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>212</td>
<td>8</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>177</td>
<td>7</td>
</tr>
</tbody>
</table>

Figure IV.2 - 10% gelatin microspheres. (A) CF = 10 mL/h and DF = 2 mL/h, (B) CF = 18 mL/h and DF = 2 mL/h, (C) CF = 26 mL/h and DF = 2 mL/h. Scale bar = 100 μm.

Table IV.3 – 15% gelatin microspheres production. CF-continuous phase, DF-discontinuous phase.

<table>
<thead>
<tr>
<th>CF (mL/h)</th>
<th>DF (mL/h)</th>
<th>Diameter (μm)</th>
<th>Standard deviation (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2</td>
<td>353</td>
<td>15</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>267</td>
<td>12</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>211</td>
<td>16</td>
</tr>
</tbody>
</table>

Figure IV.3 - 15% gelatin microspheres. (A) CF = 10 mL/h and DF = 2 mL/h, (B) CF = 18 mL/h and DF = 2 mL/h, (C) CF = 26 mL/h and DF = 2 mL/h. Scale bar = 100 μm.

As can be seen in figure IV.1, 5% gelatin microspheres are irregular and have a non-spherical shape. Due to the low polymer concentration, microspheres suffer a high shrinking during water evaporation, giving rise to a wrinkled appearance. When 10% gelatin aqueous solution is used, microspheres present a more rounded shape and smooth surface. As continuous phase flow rate increases, microspheres diameter decreases. This reduction in particle size is due to the increase in the shear stress imposed on the droplet. Microspheres around 300, 212 and 177 μm were obtained with 10, 18 and 26 mL/h flow rates, respectively. Standard deviations in both flow rates were about 9%. As expected, when gelatin concentration is increased to 15% and flow rates are kept, microspheres diameter increases. When gelatin concentration is higher (15%), and subsequently solution viscosity
increases, W/O droplets are formed by jetting mode, which explain why microspheres have a larger range of sizes.

Despite microspheres shown in figure IV.2 and IV.3 seem to have a spherical shape, it was visualized that many of them had a flattened shape, as can be seen in figure IV.4. If microspheres are supported on the surface with the flattened portion, it is difficult to see if they have a spherical or flattened shape, and consequently, microspheres diameter seems to be higher than it really is. This outcome is due to the drying process. When water slowly evaporates, the gelatin network shrinks and collapses, depositing itself on the surface where it is supported, and so, a flattened morphology is obtained.

**Figure IV.4** – Flattened gelatin microspheres.

**IV.1.2 Gelatin microspheres crosslinked with glutaraldehyde**

To avoid this situation, glutaraldehyde was used as a crosslinking agent to obtain rigid microspheres. Initially, gelatin microspheres were produced into a small plastic recipient. When production ended, saturated glutaraldehyde in toluene was added to this recipient, leaving microspheres crosslinking for 3 hours. During this time, the dispersion was gently mixed at various time intervals. After 3 hours, microspheres were poured to a large plastic recipient and left to dry for some hours. Microspheres with a perfect spherical shape were obtained through this method (figure IV.5). However, microspheres became insoluble due to crosslinking, being impossible to dissolve them afterwards. This is a huge problem since it is necessary to remove microspheres from the ICC scaffolds.

**Figure IV.5** – Gelatin microspheres cross-linked with glutaraldehyde.
IV.1.3. Gelatin microspheres dried with acetone

Since microspheres dried by slow evaporation have a flattened shape and the use of glutaraldehyde prevents their dissolution, another method to dry the microspheres was attempted. This method is based on other articles [78] [79] [80] where, after production, gelatin microspheres are placed in a refrigerator to allow the aqueous phase droplets to gel quickly and then rinsed with acetone to dehydrate and also remove the remaining paraffin from their surfaces. However, when microspheres are rinsed with acetone, water is removed abruptly, causing a complete shrinkage (figure IV.6).

In order to avoid this situation, microspheres were washed with successive acetone/ water solutions with a volume ratio of 4:3, 4:2, 4:1 and lastly only acetone. To achieve complete dehydration, microspheres are then put in isopropanol. This procedure enables the removal of water in a slowly and gently way. Microspheres resulted from this dried process present a more spherical shape comparatively to microspheres dried by slow water evaporation. Since water is carefully extracted by acetone, the gel network structure is kept. However, as can be seen in figure IV.7 and IV.8, not all microspheres have a perfectly rounded shape, which will compromise the formation of a hexagonal close-packed geometry.

Figure IV.6 – Gelatin microspheres dried with acetone.

Figure IV.7 – 10 % Gelatin microspheres dried with acetone. (A) CF = 10 mL/h and DF = 2 mL/h, (B) CF = 18 mL/h and DF = 2 mL/h, (C) CF = 26 mL/h and DF = 2 mL/h. Scale bar = 100 μm.
To overcome this problem, polyvinyl alcohol (PVA) was added in order to stabilize the emulsion between gelatin aqueous solution and paraffin. The hydroxyl groups in PVA interact with the water phase while the vinyl chain interact with the paraffin thus making the formed emulsion more stable. This emulsifier positions itself at the water/oil interface, reducing the surface tension.

**Figure IV.8** – 15 % Gelatin microspheres dried with acetone. (A) CF = 10 mL/h and DF = 2 mL/h, (B) CF = 18 mL/h and DF = 2 mL/h, (C) CF = 26 mL/h and DF = 2 mL/h. **Scale bar** = 100 μm.

To study the effect of PVA concentration on microsphere shape, 10 % gelatin aqueous solution with 1.5, 2.0 and 2.5 % of PVA were prepared. For all concentrations, it was evident the effect of PVA in microspheres morphology, presenting a more spherical shape comparatively to the previously obtained. With PVA addition, the solutions become more viscous and, subsequently, W/O droplets are formed in jetting mode instead of dripping mode. When the jet stream becomes unstable, it breaks up into small segments, which take the shape of spherical droplets in order to minimize the surface area. As a result, the droplets have a larger range in size, and consequently microspheres have a larger size distribution. As the PVA concentration is increased, the solution becomes more viscous and therefore the size differences are bigger. Gelatin solution with 2.5 % of PVA is so viscous that with flow rates of 18 mL/h and 2 mL/h of continuous and discontinuous phase, respectively, the jet stream is continuous and does not reach dripping mode inside the entire teflon tube. Microspheres with 1.5 and 2.0 % of PVA showed similar results relative to their morphology (figure IV.10 and IV.11). The smaller the size of the microspheres, the more perfect is their structure.

**Figure IV.9** - polyvinyl alcohol structure.

### IV.1.4. Gelatin/PVA microspheres

To study the effect of PVA concentration on microsphere shape, 10 % gelatin aqueous solution with 1.5, 2.0 and 2.5 % of PVA were prepared. For all concentrations, it was evident the effect of PVA in microspheres morphology, presenting a more spherical shape comparatively to the previously obtained. With PVA addition, the solutions become more viscous and, subsequently, W/O droplets are formed in jetting mode instead of dripping mode. When the jet stream becomes unstable, it breaks up into small segments, which take the shape of spherical droplets in order to minimize the surface area. As a result, the droplets have a larger range in size, and consequently microspheres have a larger size distribution. As the PVA concentration is increased, the solution becomes more viscous and therefore the size differences are bigger. Gelatin solution with 2.5 % of PVA is so viscous that with flow rates of 18 mL/h and 2 mL/h of continuous and discontinuous phase, respectively, the jet stream is continuous and does not reach dripping mode inside the entire teflon tube. Microspheres with 1.5 and 2.0 % of PVA showed similar results relative to their morphology (figure IV.10 and IV.11). The smaller the size of the microspheres, the more perfect is their structure.
It was decided to use 10% gelation aqueous solution with 2% PVA to produce the final microspheres. Since they have higher diameters, the flow rate of the discontinuous phase was decreased to 1.5 mL/h and subsequently, continuous phase flow rates increased.

**Table IV.4** – Production of 10% gelatin microspheres with 2% PVA. CF-continuous phase, DF-discontinuous phase.

<table>
<thead>
<tr>
<th>CF (mL/h)</th>
<th>DF(mL/h)</th>
<th>Diameter (µm)</th>
<th>Standard deviation (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>1,5</td>
<td>385</td>
<td>26</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>338</td>
<td>28</td>
</tr>
<tr>
<td>35</td>
<td></td>
<td>281</td>
<td>27</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td>234</td>
<td>29</td>
</tr>
</tbody>
</table>

As mentioned before, due to the high viscosities of the discontinuous phase solution, microspheres have higher standard deviation values.

**IV.1.5. Microspheres packaging**

In order to develop ICC scaffolds with three different pore sizes, microspheres with approximately 340, 280 and 230 µm were produced. To obtain microspheres with a narrow size distribution, they were separated with different sieves, depending on the size intended. For 340, 280 and 230 µm microspheres, sieves with 355 and 300 µm, 250 and 280 µm and 250 and 212 µm, respectively, were used.

After this separation process, microspheres were stored in isopropanol, ready to be packed into a hexagonal close-packed geometry. To accomplish that, the isopropanol suspension of microspheres
was slowly dropped to a mold consisting of 32 wells with 6 mm diameter and 2 mm height, and gently agitated by an orbital shaker. When the wells were full, the mold was left in orbital shaker during a further 45 minutes for isopropanol evaporation.

**IV.1.6. Annealing**

A 5% PVP solution in isopropanol was used to coat the microspheres and create the necking between them, turning this assembled structure into a solid colloidal crystal (CC). After isopropanol evaporation, PVP acts as a kind of glue that allows connectivity between microspheres. Smaller PVP concentrations do not glue well microspheres whereas higher concentrations occupies the interstitial spaces between them.

However, it was observed that a PVP film is formed between microspheres at the top of the well (the last microspheres layer to be formed is more exposed to air) (figure IV.12). When the CC is impregnated, this film does not allow the polymer solution to occupy the spaces between microspheres, giving rise to an irregular surface. In order to avoid this drawback during cell culture, cells were seeded on the regular surface.

**IV.1.7. Colloidal Cristals (CCs)**

CCs with microspheres of 230, 280 and 340 μm were visualized by scanning electron microscopy (SEM). In figure IV.13, it is observed a reduction about 1/5 of diameters measured initially with the help of the optical microscope and ImageJ. As the microspheres size increase, they tend to lose their spherical shape and present a rougher surface. The small differences in diameter are enough to change all the organization, which will compromise the interconnection between pores. Several contact sites between microspheres are missing and so, these flaws will slightly increase the void volume in the particle array.
After obtaining a solid CC, this structure was impregnated with the polymer solution. To study the effect of the polymer concentration during cell culture as well as the mechanical properties of the ICC’s scaffolds, three different PCL concentrations were used to infiltrate CC templates. CC’s with microspheres of 230 and 340 µm were impregnated with 30% PCL solution, whereas CC’s of 280 µm were impregnated with 20, 30 and 40% PCL solutions. After impregnating the CC with these polymer solutions with the help of a vacuum pump, CC’s were frozen and subsequently freeze dried. During this process, the frozen solvent (dioxane) is removed via sublimation under vacuum, leading to the formation of a porous structure. Considering a CC structure impregnated with a 30% PCL solution, since the hexagonally arrayed microspheres occupy, in theory, about 74% of the lattice volume, it means that only 30% of 26% is occupied by PCL, i.e. 7.8%.

Finally, gelatin microspheres were selectively removed by immersing the samples in a water bath heated at 48 ºC, giving rise to PCL ICC scaffolds.

ICC scaffolds with different pore sizes and PCL concentration were observed with SEM. As can be seen in figure IV.14, it is extremely difficult to have a perfectly close packed array due to the non-uniform sizes and shapes of microspheres. Due to the absence of contact sites between microspheres, not all the cavities present the three interconnecting channels between them, as expected. Consequently, the transport of nutrients and oxygen to the inside of the scaffolds as well as cell migration will be compromised. This lack of interconnecting channels can also be due the low capacity of PVP to

**Figure IV.13** - SEM images of CC’s with 230 (A), 280 (B) and 340 (C) µm microspheres.

**IV.1.8. Inverted Colloidal Cristal Scaffolds (ICCs)**
create the necking between microspheres. If microspheres are not well linked between each other, the impregnated polymer solution may destroy this weak connection and occupy the spaces between them, leaving behind pores without interconnecting channels.

In general, as the pore size increase, the interconnection channels between pores also increase. Basing in the figure IV.16 [81], it is possible to determine the radius of the intercavity pore. This model is based on an increase in spheres volume caused by an increase of the radius (0.5%), giving rise to an intercavity pore radius equal to 10% of the original radius of the close packed spheres. The geometrical relation between the original radius of close packed spheres, \( R_{or} \), the radius of the intercavity pore, \( b \) and the radius of the swollen spheres, \( R \) is given by \( R^2 = R_{or}^2 + b^2 \), when \( b/R_{or} \approx 0.1 \) and \( R/R_{or} \approx 1.005 \). It is important to notice that the difference between \( R_{or} \) and \( R \) was exaggerated for clarity. Considering microspheres with an average diameter of 230, 280 and 340 \( \mu \)m, interconnecting pores would be approximately 23, 28 and 34 \( \mu \)m, respectively.

After measuring the intercavity pore from the SEM images, ICC scaffolds of 230, 280 and 340 \( \mu \)m presented interconnecting pores of approximately (38 ± 9) \( \mu \)m, (56 ± 11) \( \mu \)m and (68 ± 14) \( \mu \)m, respectively. These discrepancy of values is justified by the difference between model assumption and these ICC’s, since in this case, microspheres not undergo a volume increases. The intercavity pore depends mainly in the contact between microspheres and by the bridge created by PVP.

**Figure IV.14**– SEM images of ICC scaffolds with 30% PCL and different pores sizes. (A) 230 \( \mu \)m, (B) 280 \( \mu \)m and (C) 340 \( \mu \)m.
**Figura IV.15** – Intercavity pore dimensions of ICC scaffolds with 30% PCL and different pores sizes. (A) 230 µm, (B) 280 µm and (C) 340 µm.

**Figure IV.16** – Illustrative scheme to determine the intercavity pore radius. The geometrical relation between the original radius of close packed spheres, $R_0$, the radius of the intercavity pore, $b$, and the radius of the swollen spheres, $R$ is given by $R^2 = R_0^2 + b^2$ [80].
In order to evaluate the influence of polymer concentration on the ICCs microstructure, ICCs produced from solutions with 20, 30 and 40% PCL were visualized in SEM (figure 34). As the polymer concentration decreases, the structure resulting from the freeze-drying process becomes more porous. This result can be proved by figure 34, where pore cavity in 20% PCL ICC has a higher porosity than others. The interconnecting channels between pores are evident in the three images. These differences in porosity also influence the mechanical properties of ICC scaffolds. PCL solutions with higher concentrations are more viscous, and therefore it is more difficult to impregnate them. As a consequence, there is a higher probability of distorting the particle array due the infiltration stress, comparatively with lower concentration solutions.

**Figure IV.17** - SEM images of ICC scaffolds with 280 µm pores and different polymer concentration. (A) 20%, (B) 30% and (C) 40% PCL.
IV.2 Mechanical properties

In order to study the mechanical properties, ICC scaffolds were mechanically tested in compression. During this assay, a compression force perpendicular to the material surface was applied, resulting in a progressive deformation of the structure. The resistance that the ICC scaffold offers to the applied stress depends on the porosity and polymer density. From the force and compression values given by compression test, it is possible to plot the stress vs. strain curves.

Figure IV.18 depicts a typical stress-strain curve of an ICC scaffold with three distinct regions: a linear region whose slope represents the compressive Young modulus $E$; a constant region that represents the plateau stress $\sigma_{pl}$; and an exponential region that corresponds to material densification. The maximum strain, $\varepsilon_d$, represents the total densification of the scaffold.

![Typical stress-strain curve of an ICC scaffold, showing the important parameters.](image)

Due to defects in the flatness of the samples, the first zone of the curve load vs deformation corresponds to the surface adaptation of testing machine. Basing in the article published by M. Lebourg et al. [82] one way to discard this first zone of “accommodation” is to trace a tangent line to the maximum slope, allowing the calculation of the true “zero point” of strain.
After adjusting all curves with this method, the Young’s modulus of the different ICC types were calculated in order to understand how pore sizes and polymer concentration influence this property.

Observing figure IV.20, we see that when ICC scaffold have the same pore sizes and PCL concentration is increased, Young’s modulus also increases, as expected. On the other hand, when ICC’s have the same PCL concentration and pore sizes increase, Young’s modulus decrease slightly (figure IV.21). Since microspheres with larger sizes tend to lose their spherical shape, the CC organization will be more affected and consequently, ICCs have smaller Young’s modulus values.

The smaller Young modulus values was obtained with 280 μm pore sizes and 20 % PCL, with an average of 0.84 MPa, whereas the higher Young modulus was obtained with 280 μm pore sizes and 40% PCL, with an average of 1.59 MPa.

**Figure IV.19**—Stress-strain curve of an ICC scaffold with 230 μm pores and 20% PCL.
Table IV.5 – Young modulus of different ICC scaffolds.

<table>
<thead>
<tr>
<th></th>
<th>230 μm 30% PCL</th>
<th>280 μm 20% PCL</th>
<th>280 μm 30% PCL</th>
<th>280 μm 40% PCL</th>
<th>330 μm 30% PCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\bar{E}$ (MPa)</td>
<td>1,45</td>
<td>0,84</td>
<td>1,37</td>
<td>1,59</td>
<td>1,00</td>
</tr>
<tr>
<td>Standard Deviation (MPa)</td>
<td>0,24</td>
<td>0,25</td>
<td>0,35</td>
<td>0,32</td>
<td>0,25</td>
</tr>
</tbody>
</table>
In order to evaluate if these values are acceptable, they were compared with other PCL scaffolds with interconnected spherical porous. In these scaffolds, microspheres with an average size of 200 μm were sintered with various compression rates in order to obtain the templates (negatives of the scaffolds). Then, melt PCL was injected into the porous template. After cooling and solidifying of the melt polymer, the porogen was removed by selective dissolution [82]. The elastic modulus values of PCL scaffolds with different porosities are listed in table IV.6. They range from 0.61 MPa for the most porous sample (85.9% porosity), to 8 MPa for the less porous sample (60% porosity).

**Table IV.6 – Young modulus of PCL scaffolds with different porosities [82].**

<table>
<thead>
<tr>
<th>Porosity</th>
<th>60.1 ±0.7</th>
<th>62.6 ±2.6</th>
<th>74.6 ±1.6</th>
<th>80.1 ±1.6</th>
<th>85.9 ±2.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>E (MPa)</td>
<td>8.15 ±1.38</td>
<td>6.2 ±1.42</td>
<td>2.57 ±0.99</td>
<td>1.82 ±0.16</td>
<td>0.6 ±0.12</td>
</tr>
</tbody>
</table>

Considering an ICC scaffold with 280 μm pore size and 40 % PCL, the porosity will be approximately 89.6% (hexagonally arrayed microspheres occupied about 74% of the lattice volume in theory). Of course, due the non-uniform sizes and shapes of microspheres, the porosity is slightly smaller.

Observing the Young modulus present in gray columns, it can be concluded that the results obtained are consistent with reported literature values. The rigidity value interval reported for human articular cartilage is between 0.51 and 1.82 MPa [82]. Since all the Young’s modulus obtained lie in this interval, ICC scaffolds are an interesting candidate for cartilage engineering, at least from the mechanical point of view.
IV.3. In vitro Studies

IV.3.1. Adhesion Rate

In order to calculate the adhesion rate through resazurin assay, $A_{570}$ and $A_{600}$ were firstly measured. The obtained results are shown in tables IV.7, IV.8 and IV.9.

**Table IV.7** – Resazurin assay for all ICC types.

<table>
<thead>
<tr>
<th></th>
<th>230 μm 30% PCL</th>
<th>280 μm 20% PCL</th>
<th>280 μm 30% PCL</th>
<th>280 μm 40% PCL</th>
<th>330 μm 30% PCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$A_{570} - A_{600}$</td>
<td>-0,022</td>
<td>-0,035</td>
<td>-0,033</td>
<td>-0,027</td>
<td>-0,024</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deviation</td>
<td>0,014</td>
<td>0,008</td>
<td>0,004</td>
<td>0,009</td>
<td>0,011</td>
</tr>
</tbody>
</table>

**Table IV.8** – Resazurin assay for cells that adhere to wells surface during seeding (SICC).

<table>
<thead>
<tr>
<th></th>
<th>230 μm 30% PCL</th>
<th>280 μm 20% PCL</th>
<th>280 μm 30% PCL</th>
<th>280 μm 40% PCL</th>
<th>330 μm 30% PCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$A_{570} - A_{600}$</td>
<td>-0,089</td>
<td>-0,101</td>
<td>-0,093</td>
<td>-0,105</td>
<td>-0,106</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deviation</td>
<td>0,036</td>
<td>0,015</td>
<td>0,022</td>
<td>0,033</td>
<td>0,016</td>
</tr>
</tbody>
</table>

**Table IV.9** – Resazurin assay for cell controls (CC), medium control (MC) and ICC medium control (ICCMC).

<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>MC</th>
<th>ICCMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>$A_{570} - A_{600}$</td>
<td>0,278</td>
<td>-0,241</td>
</tr>
<tr>
<td>Standard</td>
<td>Deviation</td>
<td>0,017</td>
<td>0,014</td>
</tr>
</tbody>
</table>
After obtaining the absorbance results, the medium contribution was subtracted through the following equations:

\[
AVERAGE(A_{570} - A_{600})_{ICC} - AVERAGE(A_{570} - A_{600})_{ICCMC}
\]

\[
AVERAGE(A_{570} - A_{600})_{sICC} - AVERAGE(A_{570} - A_{600})_{MC}
\]

\[
AVERAGE(A_{570} - A_{600})_{CC} - AVERAGE(A_{570} - A_{600})_{MC}
\]

Finally, adhesion rate (AR) was calculated by:

\[
AR = \frac{RESULT \ EQ \ 1}{(RESULT \ EQ \ 3 - RESULT \ EQ \ 2)}
\]

Table IV.10 - Adhesion Rates of both ICC types.

<table>
<thead>
<tr>
<th>Adhesion Rate</th>
<th>230 μm 30% PCL</th>
<th>280 μm 20% PCL</th>
<th>280 μm 30% PCL</th>
<th>280 μm 40% PCL</th>
<th>330 μm 30% PCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>23%</td>
<td>19%</td>
<td>20%</td>
<td>20%</td>
<td>21%</td>
<td></td>
</tr>
</tbody>
</table>

As can be seen in table IV.10, adhesion rates around 20% were obtained for both ICC’s, which are somewhat low values. In order to analyze these results, a study to understand how resazurin concentration influences the absorbance results was performed. It was seen that when resazurin concentrations are duplicated, the conversion of resazurin to resarufin also duplicates, using the same number of cells and incubating time. Before adding the 10% resazurin solution, the medium culture of ICC’s needs to be aspirated. Since ICC scaffolds are porous, the culture medium occupies these spaces, being extremely difficult to aspirate all the medium. Thus, the medium present within ICC’s will dilute the 10% resazurin solution, influencing the final results. To know the amount of medium that remains in ICC’s after aspiration, ICC’s were reweighted with and without medium. On average, 40 μl remains within each ICC scaffold. Since 100μl of 10% resasurin solution are added to each ICC, resazurin concentration decreases to 7.14% and, subsequently, the true adhesion rates would be approximately 30%.

In order to increase the adhesion rates, ICC scaffold surfaces should be chemically modified to promote cell attachment. A simple way to do that is based on a sodium hydroxide (NaOH) treatment which creates surface nanotopography with improved hydrophilicity. Another alternative approach could be the immobilization of Arginine-glycine-aspartic acid (RGD) peptide on the ICC surface. Immobilization of this peptide onto the PCL allows binding of cells to the surface via integrins, which are present on the...
cell surface. Alternative surface modification methods include polyethylene glycol (PEG) treatment, ionized gas (plasma) treatments, and other chemical treatments to create nanotopography [83][84].

IV.3.2. ATDC5 cell proliferation

To study cell proliferation, the resazurin assay was performed every second day during 9 days. The blue rows in table IV.11 represent the result obtained by equation 1, whereas the white rows represent the standard deviation for each ICC type.

Table IV.11 – ATDC5 cells proliferation.

<table>
<thead>
<tr>
<th>Day</th>
<th>Absorbance</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>230 μm</td>
<td>270 μm 20% PCL</td>
</tr>
<tr>
<td>Day 1</td>
<td>Absorbance</td>
<td>0,084</td>
</tr>
<tr>
<td></td>
<td>Standard deviation</td>
<td>0,014</td>
</tr>
<tr>
<td>Day 3</td>
<td>Absorbance</td>
<td>0,287</td>
</tr>
<tr>
<td></td>
<td>Standard deviation</td>
<td>0,052</td>
</tr>
<tr>
<td>Day 5</td>
<td>Absorbance</td>
<td>0,644</td>
</tr>
<tr>
<td></td>
<td>Standard deviation</td>
<td>0,081</td>
</tr>
<tr>
<td>Day 7</td>
<td>Absorbance</td>
<td>0,802</td>
</tr>
<tr>
<td></td>
<td>Standard deviation</td>
<td>0,069</td>
</tr>
<tr>
<td>Day 9</td>
<td>Absorbance</td>
<td>0,922</td>
</tr>
<tr>
<td></td>
<td>Standard deviation</td>
<td>0,067</td>
</tr>
</tbody>
</table>

As can be seen in table IV.11, ATDC5 cells proliferated over time. No significant differences were obtained among the five different ICC scaffolds. Between day 1 and 3, the highest proliferation rate was recorded. As time passes, proliferation rates decrease and standard deviation tends to increase in absolute value but decreases in relative terms. These standard deviation values are normal, being principally due to the differences between each ICC replica, namely the presence or absence of interconnecting channels between pores. If pores are closed, cells cannot migrate to the inside of the scaffolds and subsequently, there will be a time when they do not have space enough to proliferate.

At day 9, ICC scaffolds had approximately 60% of cells present in controls. In order to know if cells can migrate to the entire structure, and thus have more space to proliferate than the controls in the 96 well plate, resazurin assay should be done during more days until the number of cells were higher than control.
IV.3.3. DAPI Nucleic Acid Stain

DAPI is a blue fluorescent nucleic acid stain that stains preferentially double-stranded DNA (dsDNA). It associates with AT clusters in the DNA minor groove having one molecule of dye for each 3 base-pairs. Binding of DAPI to dsDNA produces an approximate 20-fold fluorescence enhancement. The fluorescence is directly proportional to the amount of DNA present, with a maximum emission at ~460nm.

ICC scaffolds were stained with DAPI in order to observe the cell distribution along the entire surface.

Figure IV.22 – ICC scaffolds stained with DAPI. (A) and (B) ICC with 270 μm pores and 30% PCL. (C) ICC with 230 μm pores and 30% PCL. (D) ICC with 330 μm pores and 30% PCL.

As can be seen in figure IV.22, ATDC5 cell nucleus were well stained with DAPI, being broadly distributed throughout the ICC scaffold. Image A and B are the same but with different focus planes. In image A it is possible to observe cells in the upper region of the scaffold, whereas in image B, it is possible to see cells distributed within the pores. In some cavities, the presence of black holes is evident, representing the interconnecting channels. Image D was edited to enhance contrast and give a better understanding of the distribution of cells. No significant differences were obtained between each ICC type.
IV.3.4. Chondrogenic differentiation staining

In order to evaluate the chondrogenic capacity of ATDC5 cells, ICCs were stained with Alcian Blue and Safranin-O on the 20th day after the sowing, and posteriorly visualized using optical microscopy.

Tecla M. Temu et al demonstrated an increase expression of collagen II, Runx2 and collagen X, (three differentiation markers) after incubating ATDC5 cells with DMEM-F12 medium supplemented with ascorbic acid. Since these results were obtained on the 14th day after the sowing, we suppose that ATDC5 cells present in the ICCs had enough time to differentiate.

Figure IV.23 - ICC scaffolds stained with Alcian Blue on the 20th day after seeding. (A) ICC with 230 μm pores and 30 % PCL. (B) ICC with 280 μm pores and 30 % PCL. (C) ICC with 340 μm pores and 30 % PCL. (D) Control ICC scaffold. Scale bar = 100 μm.
Figure IV.24 – Pore cavities of ICC scaffolds stained with Alcian Blue. **Scale bar = 20 μm.**

Figure IV.25 - ICC scaffolds stained with Safranin-O on the 20th day after seeding. (A) ICC with 230 μm pores and 30 % PCL. (B) ICC with 280 μm pores and 30 % PCL. (C) ICC with 340 μm pores and 30 % PCL. (D) Control ICC scaffold. **Scale bar = 100 μm.**
As can be seen in figure IV.23, ICC scaffolds present small blue stains within the pores cavity, which can prove the presence of GAGs secreted by ATDC5 cells. Due to the strong blue staining capacity of Alcian Blue solution, the entire ICC presented a slightly blue color after washing several times with PBS. In order to know if the blue stains observed are really due to the presence of GAGs and not just by the absorption capacity of PCL, the staining protocol was repeated for ICCs without cells.

As revealed by image D of figure IV.23, ICC controls do not present these strong blue stains comparatively with the others, confirming the presence of GAGs. We did not observe a significant difference between ICC types, being difficult to understand if polymer concentration and pore sizes have any influence on GAGs secretion. In figure IV.24, it is possible to see in more detail the pore cavity. It is important to clarify that the large black spots represent the interconnecting channels between adjacent pores. In general, the blue stains are separated from each other, probably due to cell localization. Since GAGs are secreted by cells to the ECM, the region around them is better stained.

In order to prove the veracity of these results, Safranin-O staining was also performed. Similar results were obtained, but at this time, with orange-red stains (figure IV.25 and IV.26).

However, from these results it can only be concluded that there is secretion of GAGs on the ICC surfaces. To know if the chondrogenesis occurred along the entire structure, it would be necessary to slice the ICC scaffolds, for instance, by a cryostat microtome, and perform histological staining. From this procedure, it would be possible to see if ATDC5 cells migrated along the entire ICC scaffold.
To evaluate the expression of collagen II, we tried to perform RT-PCR for the different ICC scaffolds. Initially, cells were detached from scaffolds using trypsin. However, after counting the number of retrieved cells, a too small number was obtained. In order to overcome this problem, the RNA was isolated directly from ICC scaffolds, applying the lysis solution on these structures. After following the protocol (High Pure RNA Isolation Kit, Roche), the RNA concentration was quantified, obtaining a low concentration ($\approx 10 \text{ ng/\mu l}$) comparatively to the minimum required to convert RNA to cDNA ($\approx 100 \text{ ng/\mu l}$). Thus, it was not possible to study the expression of this chondrogenic marker.

One way to enhance the RNA yield would be to increase the initial cell concentration. However, if there is a high cell density, cells tend to stack on top of each other, and consequently, the real influence of the scaffold is not evaluated. Other simple and useful method to improve RNA yield would be the increase of the centrifugation times along the entire RNA extraction protocol.
CONCLUSIONS AND FUTURE WORK

The long-range well-ordered structure, uniform pore size and regular 3D interconnectivity of ICC scaffolds make them suitable for the most challenging tasks in tissue engineering.

In this work, PCL ICC scaffolds with different pore sizes and polymer concentration were developed.

Initially, gelatin microspheres with different sizes were produced by the microfluidic technique. However, it was observed that many of these microspheres presented a flattened morphology due to the drying process (slow evaporation).

To avoid this situation, glutaraldehyde was used as a crosslinking agent to obtain rigid microspheres. Despite the fact that microspheres obtained by this method presented a perfectly spherical shape, and thus were ideal for the creation of a CC, they became insoluble due to crosslinking, being impossible to dissolve them from the ICC scaffolds.

In order to overcome both of these drawbacks, another method to dry the microspheres was attempted. After production, gelatin microspheres were placed in a refrigerator to allow the aqueous phase droplets to gel quickly and then rinsed with successive acetone/water solutions with a volume ratio of 4:3, 4:2, 4:1 and 4:0. This procedure enables the removal of water slowly and gently, preserving the microspheres spherical shape. Since not all microspheres had a perfectly rounded shape, polyvinyl alcohol (PVA) was added in order to stabilize the emulsion between gelatin aqueous solution and paraffin. The effect of this emulsifier on microspheres morphology was evident, presenting a more spherical shape comparatively to the previously obtained. After evaluating the influence of PVA concentration on microspheres morphology, it was decided to use a 10% gelatin aqueous solution with 2% PVA for the production of the final microspheres.

In order to develop ICC scaffolds with three different pore sizes, microspheres with approximately 340, 280 and 230 µm were produced. After packing the microspheres, a 5% PVP solution in isopropanol was used to coat the microspheres and create the necking between them, turning this assembled structure into a solid colloidal crystal. A PVP film formed between the microspheres at the top of the well, which will compromise the impregnation of the polymer solution. As a consequence, an irregular surface is obtained on this side of the ICC scaffold. A possible alternative solution to PVP could be the use of a polyvinyl acetate solution, since it acts also as a kind of glue, allowing the connectivity between microspheres.

After visualizing CCs in SEM, it was concluded that as the microspheres size increase, they tend to lose their spherical shape. Even the small differences in diameter are enough to affect the organization, compromising the interconnection between pores.

Three different PCL concentrations were used to infiltrate CC templates. CC’s with microspheres of 230 and 340 µm were impregnated with 30% PCL solution, whereas CC’s of 280 µm were impregnated with 20, 30 and 40% PCL solutions. To obtain the final structure, these impregnated CCs were lyophilized and gelatin microspheres were selectively removed.
SEM images of ICCs demonstrated that it is extremely difficult to have a perfectly close packed array due to the non-uniform sizes and shapes of microspheres. Not all the pore cavities presented the interconnecting channels between, due to the absence of contact sites between microspheres.

ICC scaffolds of 230, 280 and 340 μm presented interconnecting pores of approximately (38 ± 9) μm, (56 ± 11) μm and (68 ± 14) μm, respectively. These sizes are big enough to allow cell migration.

From SEM images of ICCs produced with 20, 30 and 40% PCL, it was concluded that as polymer concentration decreases, the structure resulting from the freeze-drying process becomes more porous.

In order to study the mechanical properties, ICC scaffolds with different pore sizes and concentration were mechanically tested in compression. It was concluded that when ICC scaffolds have the same pore sizes and PCL concentration is increased, Young’s modulus also increases, as expected. On the other hand, when ICC’s have the same PCL concentration and pore sizes increase, Young's modulus decreases slightly. Considering that a hexagonally arrayed microspheres occupied about 74% of the lattice volume in theory, it was expected that these ICC scaffolds would have the same Young’s modulus. Since microspheres with larger sizes tend to lose their spherical shape, the CC organization will be more affected and consequently, ICCs have smaller Young’s modulus values. The lowest and the highest Young’s modulus values obtained were 0.84 MPa (280 μm pore and 20 % PCL) and 1.59 (280 μm pore and 40 % PCL), respectively. These results were consistent with other PCL scaffolds reported in the literature [87] and lies in the rigidity value interval reported for human articular cartilage (between 0.51 and 1.82 MPa).

For vitro evaluation, ICC scaffolds were seeded with ATDC5 cells. This cell line exhibits the multistep chondrogenic differentiation observed during endochondral bone formation.

Adhesion rates around 30% were obtained for all ICC types, which is a slightly lower value. The difference between polymer concentration and pore sizes seems to have no difference on ATDC5 cells adhesion. Since PCL is a hydrophobic polymer, ICC scaffold surfaces should be chemically modified to promote a better cell attachment, for instance, with sodium hydroxide (NaOH).

After adhesion rate calculation, ATDC5 cells were cultured with the presence of 100 μg/ml ascorbic acid. The literature [77] [85] [86] reports that ascorbic acid shortens the prechondrogenic proliferation phase, induces cell differentiation and promotes ECM secretion.

ATDC5 cell proliferation rates were equal for all ICC types, with a maximum value between day 1 and 3. At day 9, ICC scaffolds had approximately 60% of cells present in controls. After a certain time, the number of cells present in ICCs will probably exceed the control, since they have more space to proliferate. This outcome will be influenced mainly by the well-ordered structure and 3D interconnectivity of ICC scaffold. If there are no interconnections between pores, cells cannot migrate to occupy all the scaffold spaces, and consequently, proliferation will cease.
In order to observe cell distribution along the entire ICC surfaces, cells were stained with a blue fluorescent nucleic acid stain (DAPI). Images of ATDC5 cells distributed within the pore cavities were obtained.

To evaluate GAGs secretion by ATDC5 cells, ICC scaffolds were stained with Alcian Blue and Safranin-O on the 20th day after the sowing. Small blue (Alcian Blue) and orange-red (Safranin) stains within the pores were observed, which prove the presence of GAGs secreted by ATDC5 cells. Again, no significant differences between all ICC types were observed.

An evaluation of chondrogenic marker expression such as collagen II, SOX 9 and Runx2 would be important to really understand the influence of polymer concentration and pore sizes on ATDC5 cells differentiation. Since it was not possible to accomplish that due the low concentration of RNA extracted, more efforts need to be done in order increase cell number in ICC scaffolds.

Despite the high potential of ICC scaffolds, it is extremely difficult to have a long range ordered structure, with controlled pore sizes and uniform interconnections.

Microspheres with uniform sizes and perfectly spherical shape are crucial for the development of ideal ICC scaffolds. If one of these feature fails, all organization will be compromised. More work needs to be done in order to improve all the steps necessary for the development of an ICC scaffold. All of them, without exception, are essential to obtain a perfect structure.
VI. REFERENCES

2. Milner, P.I., R.J. Wilkins, and J.S. Gibson, Cellular Physiology of Articular Cartilage in Health and Disease.


