3D-printing the Collagen Type-II Structure of Articular Cartilage

Universiteit Utrecht

TU/e EINDHOVEN UNIVERSITY OF TECHNOLOGY

Name: *Sophie van der Zwart*

Master: *Regenerative Medicine & Technology*

Course: *Writing Assignment, Research Proposal*

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Part A – Applicant

A.1 Applicant

Name student: *S.A., van der Zwart, 6331718*

Affiliation: *Utrecht University, Technical University of Eindhoven*

Name first examiner: *Miguel Dias Castilho*

Affiliation: *Technical University Eindhoven, Dept. Biomedical Engineering*

Name second examiner: *Debby Gawlitta*

Affiliation: *Utrecht University, Dept. of Oral and Maxillofacial Surgery & Special Dental care*

Part B – Scientific Proposal

B.1. BASIC DETAILS

B.1.1. Title

3D-printing the collagen Type-II Structure of Articular Cartilage

B.1.2. Abstract

Collage type-II (col(II)) is the major component of articular cartilage. However, articular cartilage engineering approaches have up until now solely been based on collagen type-I (col(I)). Yet, col(I) devices have shown to be ineffective to function in therapeutic approaches. As articular cartilage defects are a common injury that have a high burden on the quality of life of individuals and on costs in health care, there is an active call to make steps in the field of cartilage engineering. Using col(II) instead of col(I) in cartilage engineering approaches might not only be a more realistic resemblance of articular cartilage, but is indicated to have a greater biosynthetic activity. Therefore, we propose research here that aims to engineer the structure of col(II) that is present in articular cartilage. The approach to achieve this goal consists of two parts: 1) extracting col(II) and 2) the 3D-printing of a scaffold of col(II) that resembles the structure of articular cartilage. The extraction of col(II) will be performed by acid-soluble and pepsin-soluble collagen extraction from the articular cartilage of porcine animals. We will characterise this sample by the col(II) purity and the col(II) concentration and investigate if the extracted col(II) can self-assemble again into fibres. Finally, we will print the col(II) structure of articular cartilage using FRESH printing technology that prints the col(II) into a support bath where the structure is supported from all sides to maintain its structure. Ultimately, we will have engineered a col(II) scaffold that resembles the structure of col(II) in articular cartilage.

B.1.3. Layman's summary

Articular cartilage is a specific type of cartilage that covers the ends of bones that come together at the place where joints are formed. They provide a surface to make loading with low resistance possible to the body. During sports or recreational activities, articular cartilage can become damaged due to excessive mechanical loading or trauma. Because the cartilage does not contain nerves, blood vessels or lymphatics the damage is inaccessible for repair mechanisms of the body. However, if this damage remains untreated it can lead to the whole joint disease osteoarthritis (OA), which is a chronic degenerative disorder and can cause pain and stiffness of the joint.

Currently, there is no treatment in practice that can cure OA, but only life-style changing activities that can relieve symptoms. Considering that this disease both effects the quality of life of an individual and is an enormous burden on healthcare because of the long-term care of this disease, there is an active call for new therapies. In research, there have now been studies where treatments do not relieve symptoms, but repair the injured cartilage itself by regeneration. That means that the injury of the articular cartilage is reconstructed to healthy tissue. The reconstruction of cartilage consists of an engineered structure called a scaffold that resembles the foundation to which cells can bind to form the healthy new tissue. In cartilage, collagen is the protein that provides the foundation. In research, scaffolds have already been engineered using the most abundant type of collagen of the body, col(I). However, these scaffolds were unable to function in a clinical application for articular cartilage regeneration possibly because the foundation of articular cartilage is made of col(II), not col(I).

In this research, we aim to engineer the structure of col(II) that is present in articular cartilage. Until now, there are no 3D col(II) scaffolds available that resemble this structure. In the future this scaffold can serve as the foundation for regenerative therapies for large cartilage defects.

To achieve to engineer this structure we propose an approach that consists of two parts: extracting of col(II) from articular cartilage in animals and printing the 3D structured scaffold with col(II) that resembles the structure of articular cartilage. Firstly, we will isolate col(II) out of pigs by acid soluble collagen extraction and make sure we have pure col(II) isolated by checking for the characteristics that the obtained sample has. This includes the col(II) purity and the col(II) concentration. Following to that, we will create a col(II) bio-ink using the sample we obtained and print the collagen structure of articular cartilage using FRESH printing technology. This is a printing technique that prints the col(II) into a support bath where the structure is supported from all sides to maintain its form. This will leave us with a col(II) scaffold that resembles the structure of collagen in articular cartilage.

B.1.4. Keywords

Col(II), Scaffold Engineering, FRESH 3D-printing, Articular cartilage injury, Osteoarthritis

B.1.5. Abbreviations

Col(II):	Collagen type-II
Col(I):	Collagen type-I
OA:	Osteoarthritis
SDS-PAGE:	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SEM:	Scanning Electron Microscopy
PSC:	Pepsin-Soluble Collagen
ASC:	Acid-Soluble Collagen
FRESH:	Freeform Reversible Embedding of Suspended Hydrogels

B.2. SCIENTIFIC PROPOSAL

B.2.1. Research topic

Background

During sports or recreational activities, articular cartilage injuries are common defects that occur due to excessive mechanical loading, trauma, wear or tear. Articular cartilage is highly specialized tissue that covers the ends of bones that come together at the place where joints are formed and consists of hyaline cartilage. In figure 1 the articular cartilage of the knee joint is shown. It is composed of a dense extracellular matrix (ECM) together with chondrocytes and provides a lubricating surface to make the transmission of loads with a low frictional resistance possible. It is mostly 2 to 4 mm thick and is not innervated with blood vessels, nerves or lymphatics and consequently has a low intrinsic capacity to repair when damage occurs. If these injuries are left untreated, structural changes in the hyaline articular cartilage can lead to the whole joint disease osteoarthritis (OA) (1).

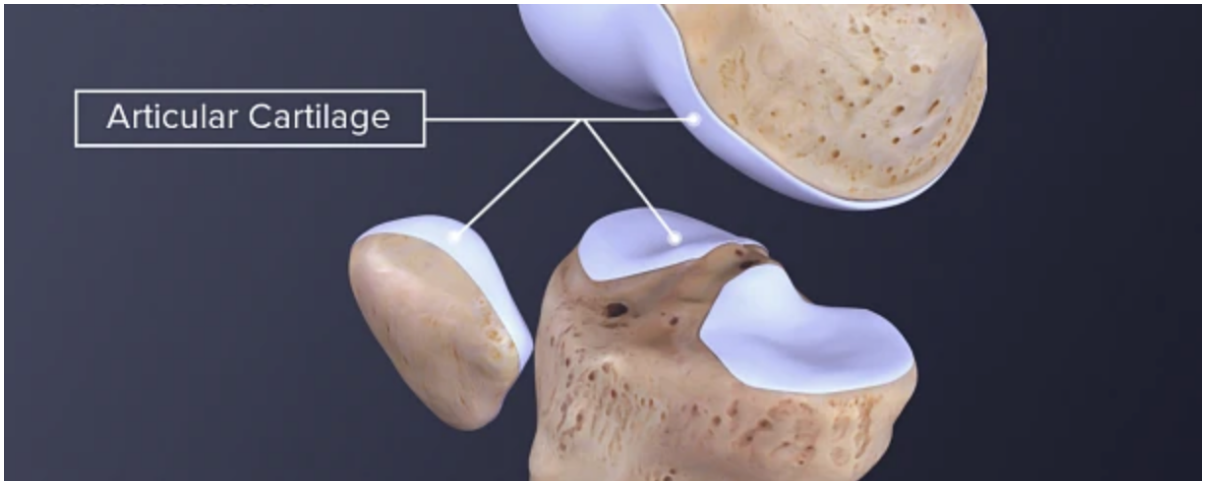

Figure 1. Visualization of the bone surface of the knee joint covered with articular cartilage (2)

OA is a chronic degenerative disorder and can cause pain and stiffness of the joint. The most common form of this disease is mainly caused by aging but can also be a consequence of other conditions or diseases (3). Due to its high prevalence, OA has a high burden on both an individual and socioeconomical level. Knee OA was estimated to affect around 250 million individuals worldwide (3) and occurs more in women than men. In light of the fact that the population gets older, more people will be affected by this disease (5). Socially, the burden is hard to measure because it mostly accounts to the loss in wages and productivity of individuals affected by OA. Economically, medical costs have been estimated in 5 industrialized countries (Australia, Canada, France, United Kingdom and United States) to be 1-2.5% of the gross national product in 1997, after which the costs have shown to have a continued upward trend (6). In the Netherlands, 1.1% of all healthcare costs in 2019 accounted to OA (7).

The disease OA results from the failure of chondrocytes within the cartilage to maintain the balance of the synthesis and degradation of ECM. The loss of homeostasis leads to more water and less proteoglycan content of the ECM, which weakens the strength of the collagen network because less collagen is produced next to the fact pre-existing collagen is broken down (8). Additionally, the chondrocytes will have become hypertrophic at this point and attract proinflammatory mediators. Following to that, the subchondral bone will have changes in its remodelling and modelling processes and have vascular infiltration towards the injured cartilage (9). The underlying changing of the bone is what causes the pain and stiffness of the joint.

Relevance

Currently, there is no treatment in practice that can cure OA, only manners to improve daily activity of patients. Exercise and weight loss are examples of lifestyle changing activities for OA when patients are overweight. Treatments in terms of drugs and surgical procedures have only shown a variable degree of effectiveness in terms of complexity and costs (10). A rapidly growing field of research that will not only relieve symptoms, but repair injured cartilage are biomaterial-based therapies. There have been studies that have engineered a col(I) based scaffold for repairing articular cartilage defects by regeneration (11), however, it has inability to be functional for clinical applications (12). In literature there are numerous articles available that write about possible applications of col(II) in scaffold engineering. Col(II) is the type of collagen of which articular cartilage is mostly composed of, but surprisingly, (13), no single col(II) device is available commercially.

In previous research conducted in the seventies by Trentham (14), it was found that native col(II) extracted from cartilage of human, chick and rats induced inflammatory arthritis in rats when injected intradermally. This research compromised further research in using col(II) devices. In contrary to the research by Trentham, it was later shown that col(II) does not induce inflammatory arthritis, but that it adequately promotes hyaline neocartilage formation (15). Additionally, literature provides clear evidence of beneficial effects of col(II) compared to col(I) scaffolds engineering, possibly due to biochemical signal (10). The reasons of the superiority of col(II) scaffolds include that col(II) scaffolds compared to collagen type I-scaffolds yield greater biosynthetic activity and maintenance of the round morphology of chondrocytes *in vitro* (16). Next to that, collagen type II-scaffolds might effectively induce more differentiation of adipose derived stem cells and bone marrow stem cells to chondrocytes and to nucleus pulposus cells (NPCs only by differentiation of adipose derived stem cells), judged by upregulation of chondrogenic genes, increased synthesis of ECM and col(II) gene expression (17)(16). These findings were also tested preclinically in a cartilage lesion model of a pig, where a col(II) scaffold more often repaired the defect with hyaline cartilage compared to the group of unplanned healing and col(I) and collage type-III (col(III)) scaffolds (19).

Basic Characteristics of Collagen

In ECM and in connective tissue collagen is the major protein that provides the tissue stiffness and integrity. There are 29 different types of collagens, that differ in functionality and distribution throughout the body. Collagen molecules are composed of three α-chains that share the repeating amino acid sequence ~Gly-X-Y~. Here, 'X' represents the amino acids proline or hydroxyproline and 'Y' represents any amino acid. The chain is self-aggregating to a right-handed triple helix structure that locates glycine in the core and 'X' and 'Y' to the surface. The amino acids proline and hydroxyproline that represent 'X' in the repeating sequence stabilize the helix structure through formation of hydrogen bonds and thereby counter rotation (20).

Characteristics of Col(II)

In Situ, the col(II) molecules that the collagen fibrils consists of self-assemble in a staggered manner. The complete col(II) molecule is expressed as a homotrimer, namely $[\alpha1(II)]_3$, solely synthesised by chondrocytes. The three identical α1-polypeptide chains consist of 1060 amino acids each with large uninterrupted triple-helical regions and relatively short nonhelical telopeptides (19 amino acids in the N-telopeptide and 27 amino acids in the C-telopeptide) that do not contain the ~Gly-X-Y~ repeating structure. The telopeptides play an important role in a proper self-assembly process of the molecule (21). The lengths of the α1-polypeptide chains are the same, but they are displaced from each other that support its super coiling (22).

Structure of Col(II) in Cartilage

The col(II) makes up roughly 10-30% of the weight in articular cartilage. Chondrocytes make up 10% of the volume and the remaining part of 70-75% consists of water. As mentioned above, the collagen structure provides the tissue stiffness and integrity of the cartilage and is for that reason structured in a specific orientation across the different zones of cartilage layers. The cartilage layers consist of a superficial zone, a middle zone and a deep zone next to the calcified cartilage that is the border to the subchondral bone. A schematic overview of this structure is shown in figure 2. The collagen is differently structured across these layers. It is structured perpendicularly to the surface in the deep layer and parallel to the surface in the superficial layer. In the middle zone, the structure transitions into the different orientations (23).

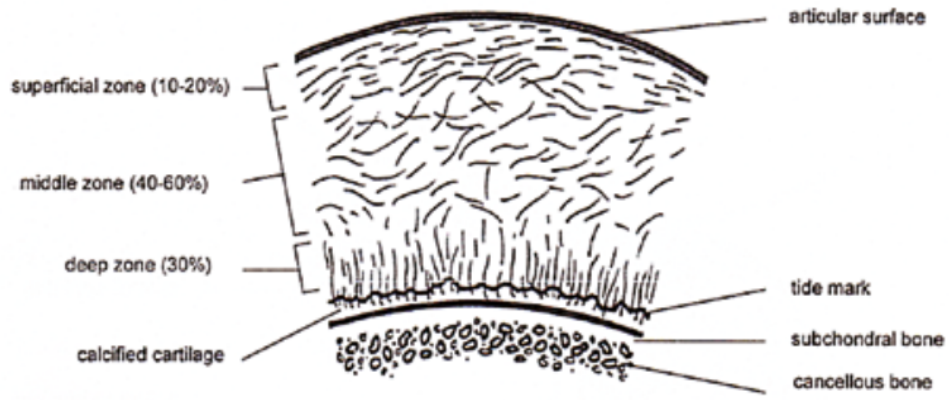

Figure 2. Schematic representation of the structure of collagen in articular cartilage (23)

Problem definition and overall aim

Biomaterial-based tissue engineering is a rapid growing method in the search for finding a cure for osteoarthritis. At the moment, there are only treatment options that relieve symptoms such as pain and stiffness, but do not treat the cause of the defect. There have been studies that have engineered a col(I) based scaffolds for repairing articular cartilage defects by regeneration, but they have shown to be unable to function in clinical applications. Although there is existing evidence on the superiority of using col(II) over col(I) in these scaffolds, no col(II) devices are available. Therefore, the main goal of this this project is to create col(II) scaffolds where the collagen presents the characteristics of articular cartilage. For the future, these scaffolds can serve as a biomaterial-based therapy for large cartilage defects in the clinic to both treat injuries at articular level as well as to prevent osteoarthritis. To engineer this specific structure of collagen that provides the basic architecture that is present in articular cartilage using col(II) exclusively, the research design will consist of two distinct parts: Extraction of col(II) and the development a col(II) device that will be validated on its structural orientation.

B.2.2. Approach

To achieve the goal to create col(II) scaffolds where the collagen presents the characteristics of cartilage we propose a PhD position of four years at the Technical University of Eindhoven (TU/e). Here all the technological equipment we need is available, next to laboratory spaces we are going to use. The approach is divided is divided into two parts, the extraction of col(II) and the printing of the 3D scaffold of col(II). Below in *Table 1* there is a schematic overview on the time distribution this approach will consists of during the four years. The isolation is indicated by the number '1' and the processing by number '2'. Apart from these steps the beginning of the PhD will consist of literature reviewing to get familiar with the research. In this section we will specify this schematic approach in detail.

Task	*Year 1*	*Year 2*	*Year 3*	*Year 4*
Literature review				
Collagen extraction (1)				
Characterization collagen (1)				
Self-assembly assessment (2)				
Bio-ink development (2)				
FRESH 3D-printing (2)				
Characterization scaffold (2)				

Table 1. Gantt table: schematic overview of the approach. Indication numbers: 1. Extraction. 2. 3D scaffold printing

Extraction (1)

Collagen Source

There are three different strategies to obtain col(II): extracted collagen, recombinant collagen or synthetic col(II) (24). Extracted collagen can be physically removed from bovine, porcine, murine and marine animals. The method of extraction depends on the tissue type and can be categorised in salt soluble collagen, acid soluble collagen (ASC) and enzyme (pepsin) soluble collagen (PSC) extraction. Obtaining a source of collagen using recombinant proteins, means that col(II) is in a manipulated form of the native protein because of increased demand of production for commercial products by recombining pieces of DNA (25). Lastly, synthetic collagen is artificially manufactured form of collagen mimicking peptides that have the ~Gly-X-Y~ sequence incorporated.

An important advantage of mammal-derived collagen is that large volumes can be extracted in a validated manner. To be able to study if we can engineer the structure of collagen that resembles that of cartilage, we primarily need large volumes of col(II). Despite the fact that desired properties can be introduced in recombinant and synthetic collagen, it is of low yield and therefore more expensive and time consuming to produce high volumes. Furthermore, recombinant collagen lacks native post translational modifications and the α-helical structure of synthetic collagen was not stabilised due to a short sequence length, resulting in unusable peptides (24).

We will specifically extract col(II) from articular cartilage. As it is suggested that col(II) retains memory from the tissue it is collected from, col(II) devices from articular cartilage sources will be more effective in possibly including chondrogenic differentiation of human stem cells in the future (10). We will extract col(II) from porcine animals. The pig is a mammalian animal and therefore has the same collagen structured orientation as we observe in humans. Extraction of collagen from porcine animals has been described in literature and validated before (13).

Extraction procedure

For extracting collagen, research has shown that intact telopeptides enhance interactions for collagen self-assembly. The assembly of triple-helical proteins into fibrils is known to be slowed down when these telopeptides were not present. Apparently, telopeptides facilitate transient intramolecular interactions between collagen proteins (26). Although an enzyme soluble method of collagen extraction from cartilage with the enzyme pepsin will provide us with large volumes of extracted col(II), it would also remove most of these short non-helical regions. PSC might therefore not self-assemble when we want to engineer the collagen structure of articular cartilage. The research of Shayegan (26) showed that the telopeptides remain functional when the extract method is performed acid. A disadvantage to this is that the collection of collagen using acid is of lower yield (27). Considering that both extraction methods includes important parameters for our goal (high volumes and retainment of telopeptides respectively), we will investigate the use of both extraction methods (PSC and ASC) to develop the col(II) structure of articular cartilage.

In figure 3 it is schematically visible how a typical col(II) extraction method from cartilage is performed. Additionally, the electrophoretic mobility of the extracted col(II) is demonstrated. This is a separation method of charged pieces under the influence of an electric field where different fragments of the collagen can be distinguished.

Porcine sourced col(II)

Porcine articular cartilage will be obtained freshly from a slaughterhouse and the col(II) extraction will following the procedure previously described by (13). We will extract collagen according to two methods: with enzymatic treatment using pepsin and acid treatment, leading to two samples of extracted col(II).

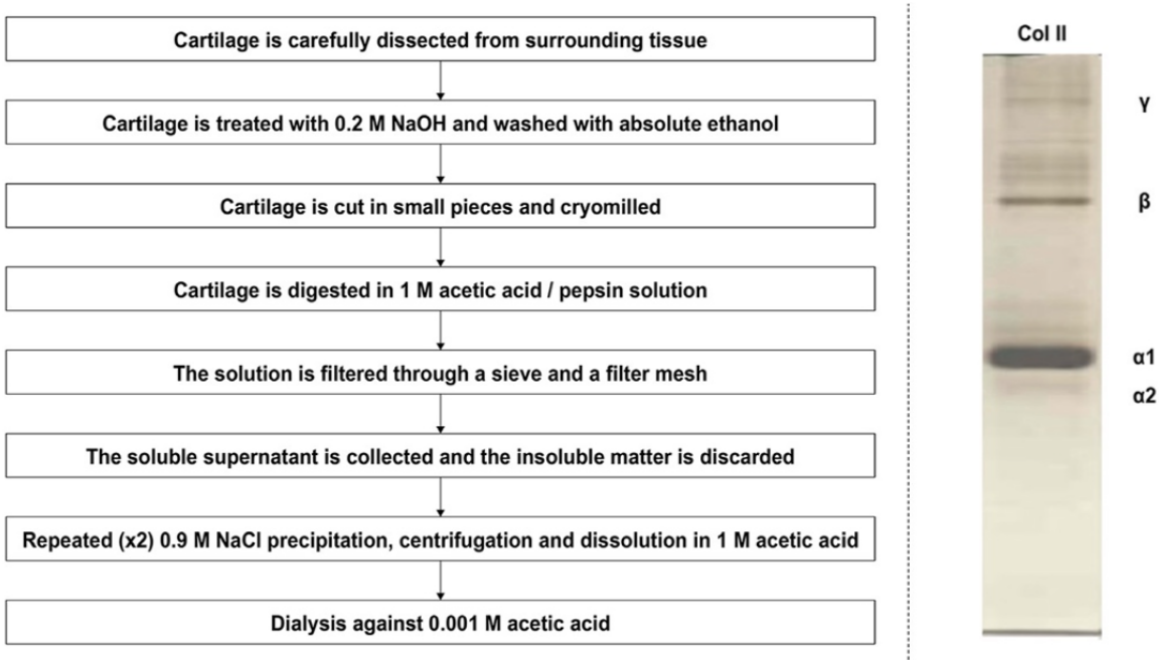

Figure 3. Typical col(II) extraction flow-chart and the electrophoretic mobility of col(II) (10)

Characterization of the col(II) extraction

To ensure we have isolated col(II) we will characterize the porcine samples we obtained from extraction. We will follow the numerous protocols that have been described in literature to extract collagen of several types varying in complexity and accuracy (28). For the ability to process our isolated col(II) into the collagen structure of articular cartilage we need to know two main characteristics of the two samples: collagen purity and collagen concentration.

Collagen purity

To assess the purity of the col(II) we extract we conduct the qualitative technique Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to the protocol described by (13). This method separates negatively charged denatured collagen components based on their seize under the influence of an electric field (28). Additionally, the SDS-page can be used to validate the presence of the telopeptides in acid-solubilized collagen. Because in the pepsin-solubilized collagen the telopeptides will be proteolytically digested by pepsin, the shortening of this form of collagen will results in an increase in electrophoretic mobility compared to the acid-solubilized collagen (26). This difference in presence of the telopeptides can be distinguished in the bands that are visible on the SDS-PAGE.

Collagen concentration

Two methods can be followed to quantify the concentration of collagen: Colorimetric assays or Mass Spectrometry. In colorimetric assays the major amino acid in col(II), hydroxyproline, can be converted to a measurable soluble coloured reaction product. However, this a more subjective measurement technique. Since we want to quantify the absolute concentration for engineering the structure of articular cartilage with collagen, we will quantify the col(II) using the MS technique described by (29).

3D scaffold printing (2)

To this point, we have extracted purity of col(II) and we aim to print the collagen structure that articular cartilage consists of. We firstly will examine the self-assembly capacity of the extracted col(II) after which we will provide a guide to 3D print the col(II) structure in articular cartilage

Self-assembly assessment

Firstly, to guide the 3D-printing we have to ensure the pure col(II) we extracted can form fibres again by self-assembly. Self-assembly is the process of aggregation in both space and time of collagen monomers to larger organised fibres (21). Zhu (30) has performed research that indicated by which conditions the aggregation behavior of extracted col(II) was effected in the aquatic animals Skate (*Rajidae*) and Sturgeon (*Acipenseridae*). These conditions include concentration of col(II), pH and ionic strength. Firstly, concentration of col(II) increases the number of molecules that can self-assemble next to the intermolecular interaction increase. Secondly, the pH has an effect on the assembly of polyelectrolyte and the charge of functional groups of collagens that affect the rate of self-assembly. And lastly, with increase of ionic strength, the ions in the sample increase by which the mutual force between collagen molecules can be reduced. The neutralising of the charge on the surface of the molecule that is caused by that, increases the self-assembly rate.

We will assess the self-assembly properties for our two forms (acid-solubilized and pepsin-solubilized collagen) of col(II) according to the procedures described by Zhu (30). The conditions we assess are concentration of col(II), the level of pH and the ionic strength.

Overview 3D-Printing Technique

The structure of collagen that we aim build exists of three different collagen configurations that resemble the deep layer, middle zone and the superficial layer of cartilage. Respectively, the configurations of collagen are structured perpendicularly to the surface, then both perpendicularly and parallelly shifting to only parallelly at the top layer. This will be printed as a freeform with a solution of biological material including our extracted col(II), called bio-ink. Important to build this 3D structure of collagen is preventing distortion of this liquid bio-ink due to gravity. Therefore, we need physical support to be able to print the structure and ensure structure stability. To achieve that, we will use embedded 3D bioprinting, which provides a temporary support structure in which we can print our collagen structure. It is an additive manufacturing method based on material extrusion. Specifically, we will use the implementation of embedded 3D-printing called Freeform Reversible Embedding of Suspended Hydrogels (FRESH). This technique allows us to print the extracted col(II) into a support bath that allows freeform fabrication (31).

The basic principle to which we can print col(II) with FRESH is based on the extrusion of a bio-ink into a support bath that supports the structure from all sides. The bath possesses a shear-stress behaviour that will acts solid until a threshold of shear stress is applied, to which the bath will transition to have liquid-like behaviour. This allows a syringe needle to move through the bath during extrusion because the shear stress exceeds the threshold. However, when the needle moves away, the bio-ink will be supported because of immobilisation of the solidified bath. Additionally, to cross-link the col(II) a pH buffer will be added to the support bath. Subsequently, when the bio-ink is extruded into this buffered support bath, pH neutralization occurs driving the cross-linking process (31). A schematic representation of the FRESH printing technique is shown in figure 4.

Bio-ink composition

The base of a collagen bio-ink is a collagen hydrogel, in which collagen molecules will arrange into collagen fibres. To be able to print our extracted collagen in the right structure the bio-ink will contain the properties that we measured in the self-assembly assessment.

Support Bath Composition

The support bath consists of two elements: microparticles that supply support and an aqueous phase. The support microparticles are made from gelatin. The gelatin microparticles will be produced as described by (32). These microparticles have uniform spherical morphology, reduced polydispersity, a decreased particle diameter compared to earlier developed microparticles and a tuneable storage modulus (stiffness) and yield stress. They are fine particles that can be melted at 37°C and be washed out to release the structure of col(II) we printed. To the aqueous phase a pH buffer will be added to neutralize it to a pH between 7.4-7.6 to initiate the gelation process when the acidified collagen will be extruded into the bath.

FRESH 3D-printing

After we have established the self-assembly properties of col(II) we want to investigate if we can align the collagen molecules in a 2D orientation before we print the structure of collagen in articular cartilage. We will use the FRESH printing technique for this as well to assure support of the structure. We will characterize the 2D orientation based on fibre formation and orientation using Scanning Electron Microscopy (SEM). This characterization procedure is explained below. If SEM validates the 2D orientation, we will move to printing the 3D col(II) scaffold.

After we have established the bio-ink, the support bath and the 2D printed orientation, the printing process can start. FRESH printing uses standard 3D-printing software to prepare 3D printers. For printing the structure of col(II) in articular cartilage we will use the slicing software *Slic3r.* This is a tool that translates digital 3D models into instructions by slicing the model into horizontal layers (33). Herein we will upload our 3D-model of the col(II) structure that is present in articular cartilage. This 3D model forms the prompt for the FRESH 3D-printer to print our scaffold.

The deep zone of articular cartilage accounts for 30% of the total, the middle zone for about 60% and the superficial zone for about 10%. The proportion to which these different orientations are printed in the 3D col(II) scaffold are important because we aim to engineer the structure of col(II) that is present in articular cartilage. However, the absolute measurements mentioned in the next section are not directly relevant for this research proposal because we aim to print the structure of col(II) in articular cartilage, not the measurements of the structure in the body.

The following absolute measure solely are an indication to which the FRESH 3D printer can be tuned. Articular cartilage is typically 2 to 4 mm thick (1). As the engineering of the col(II) structure of articular cartilage has not yet been performed we will stick to the lower bound of the thickness range. We will print 0.6 mm perpendicularly structured collagen fibres, 1.2 mm of both perpendicularly and parallelly structured collagen fibres and 0.2 mm parallelly structured collagen fibres to engineer the entire collagen configuration that resembles articular cartilage.

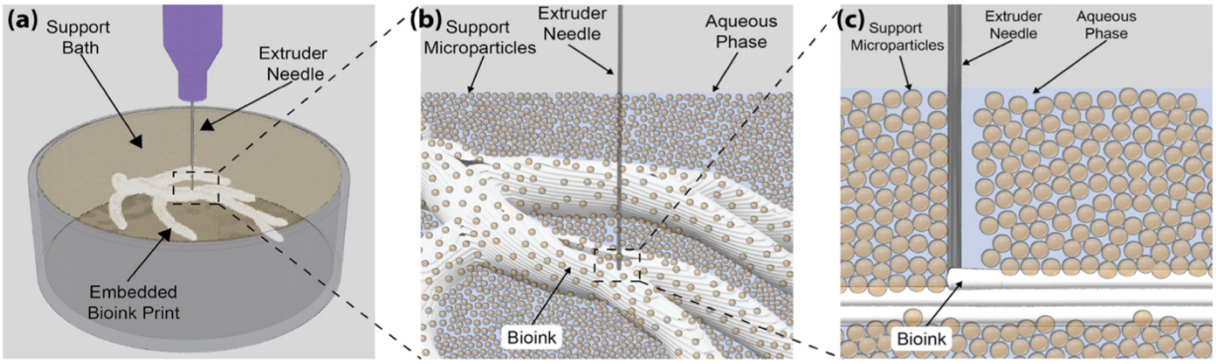

Figure 4. Schematic presentation of FRESH printing into a gelatin microparticle support bath (Shiwarski et al., 2021)

Characterization scaffold

To validate that we have engineered the structure of col(II) that is present in articular cartilage, we want to microscopically see the configurations of the collagen fibers in the scaffold. This will be done using scanning electron microscopy (SEM). This is a microscopic technique that uses a focused electron beam to study morphologies, in this case, the structure of the col(II) network. Electron beams are interacting with the surface of collagen fibers and eject different electrons from the sample, that are collected by the SEM detectors and converted into an image. As electrons have a short wavelength, the resolution of the image is high, allowing us to observe the col(II) structure (34). An SEM is present at the Technical University of Eindhoven. We will use this technique according to the SEM protocol described by Changoor (35). The col(II) structure will be assessed based on fiber formation and collagen orientation. An example of what such a SEM image looks like is illustrated in fig. 5. If the structure that is observed is not structured in the manner we aim to engineer, a step in following research could be to include different proteins that make up the ECM of articular cartilage such as proteoglycans. Naturally, the structure of col(II) in articular cartilage does not stand on its own. In spite of that, in this research proposal we solely aim to structure a col(II) scaffold, not an cartilage scaffold.

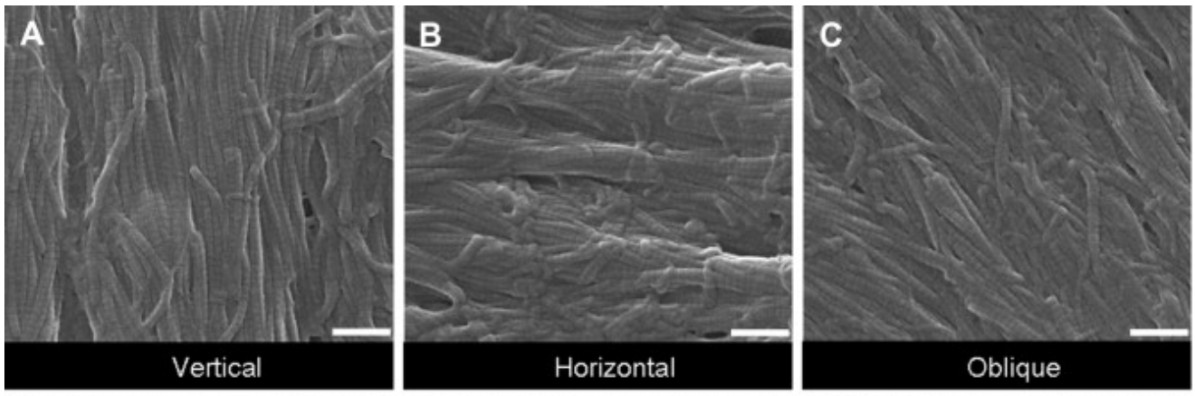

Figure 5. Reference SEM image illustrating the collagen fiber orientations across the different cartilage zones (35)

B.2.3. Feasibility/Risk assessment

By engineering the complex structure of col(II) that is present in articular cartilage we also face risks from the methods and techniques we use to achieve the goal. Here, we will discuss the feasibility of the research proposal and assess the risk we will encounter in the two steps of the approach (extraction and 3D-printing of col(II)). Additionally, we will explain how we will mitigate these risks and will propose an alternative approach for when parts of our methods or technique will fail.

The characterization of the extracted collagen acquires the availability of numerous technological equipment. However, the proposed approach to test for the characteristics are feasible methods to achieve this, as a mass-spectrometer and protein electrophoresis equipment are available at the Technical University Eindhoven. Additionally, the FRESH printing technique can also be performed here. There are existing protocols to print using this technique, so for the processing approach, the risk is mostly compromised in the composition of the support bath and the bio-ink.

To allow to print our extracted col(II) in the desired structure, the extracted collagen will have to have self-assembly properties. Although we are going to check for this property before developing a bio-ink, there is a risk the extracted collagen does not self-assemble. If the self-assembly property is retained, we move to printing the col(II) using FRESH printing technique. Our bio-ink will be developed based on the properties we find in the self-assembly assessment. However, bio-ink performance might be dependent on small difference of the component ratio. Since collagen bio-inks have low mechanical properties, both the bio-ink and the support bath have to be of ideal balance. Additionally, a risk that we might face during the FRESH printing is that the gelatin from the support bath will diffuse into the bio-ink and affect the final construct.

To overcome the risks of our approach we will check for each step if we have obtained the desired results. We will check for characteristics of our extracted collagen in the characterization of our collagen. In that manner we ensure checkpoints within our approach to the risks by checking for these properties. If a checkpoint is not realised, we can go back to the previous step.

If the characteristics of our extracted collagen sample or the self-assembly property of our approach do not match the checkpoints, we will move to an alternative approach to extract collagen. Instead of extracting col(II) from the model animal the pig, we will extract col(II) from a marine animal, the shark. The animal material of sharks can be delivered by *Blue Ocean Food Productions* (36). Due to additional advantages of collagen extraction in marine organisms compared to non-marine organisms, research has partly moved to marine-sourced collagen (37). These benefits include the ease of extraction and improved chemical and physical durability. This source of collagen already has applications in scaffold engineering for bone tissue-engineering (38). For extraction of col(II) we will accordingly collect articular cartilage from marine animals. Important to note is that the collagen structure in terrestrial animals is differently structured than in aquatic animals. The collagen structure in terrestrial animals is structured perpendicularly to the surface in the deep layer and parallel to the surface in the superficial layer. In contrast, the structure of aquatic mammals has no predominant orientation (39). Although there are good indications for marine sourced collagen, we only propose this extraction method as an alternative approach due to the differences in orientation and the availability of articular cartilage of marine animals.

B.2.4. Scientific and societal impact

Scientific impact

In the short-term, the outcome of this proposed research will show that we can engineer the col(II) structure of articular cartilage. If results are positive and the scaffold can be engineered properly, a following next step is to optimize this scaffold. Optimizing the scaffold includes investigating an appropriate collagen source, efficiency of the processing technique and environmental efficiency. In this proposal two different methods of col(II) extraction will be investigated, acid soluble collagen extraction and pepsin soluble collagen extraction. There are indications that telopeptides are important for the self-assembly of the collagen fibres, that are only maintained in the acid soluble collagen extraction. Depending on the outcome of the self-assembly capacity of the two methods, a validated approach can be proposed to further optimize the scaffold regarding the extraction method. This also accounts for the source of collagen. In this research, we propose an alternative approach to extract collagen from marine animals instead of mammals. Although this might not be investigated in this research, this source of collagen can also be further investigated to optimize the scaffold. Additionally, we propose to use FRESH 3D-prining as processing technique. Reduction of unnecessary materials that involve the collagen collection and waste material of the processing technique will have to be closely examined to ensure an optimal engineering process and increase environmental efficiency.

In the long-term, the scaffold we engineer in this research can form the base of regenerative therapies. Since articular cartilage has low regenerative capacity intrinsically, damage is not repaired by the body itself and can lead to OA. The scaffold we engineer here can form the base in repairing articular damage using biomaterial-based therapies. Considering that the structure of collagen in articular cartilage is important for tissue stiffness and integrity, this scaffold offers a good fundament for such therapies. The collagen structure provides the building blocks of articular cartilage to make the transmission of loads with a low frictional resistance possible. In order to make regenerative approaches like this possible the interaction of the collagen structure, which will form part of the ECM, the cell viability will have to be investigated firstly. Chondrocytes will have to be able to attach to ECM properly, need to have a viable environment and the interaction between the biomaterial and the body will have to be regulated to prevent inflammatory responses to be able to include this scaffold in therapeutic approaches.

Societal impact

Biomaterial-based therapies such as repairing damaged articular cartilage using col(II) scaffold will have a social impact both on an individual level and on a societal level. Individually, damage repair of articular cartilage would mean that a large part of the occurrence of OA can be prevented. This will prevent individuals from pain symptoms and prevent the loss of daily activity due to the consequences of the symptoms. Societally, the prevention of OA will reduce financial costs in health care systems due to less expensive and prolonged care next to the fact that people will not fall out in work due to symptoms.

Additionally, engineering of the scaffold using col(II) together with the inclusion of interacting cells could open doors for other regenerative therapies outside this field. Not only similar diseases in orthopaedics can benefit from a better understanding of scaffold engineering and biomaterial-based therapies but also other conditions that involve low regenerative repair can benefit to improve therapeutic approaches. Inclusion of biomaterial-based therapies is a rapidly growing field that pushes limits that we face in the clinic regarding conventional drug therapies.

B.2.5. Ethical considerations

Concerning our research approach, we utilise porcine sourced collagen which give rise to two main ethical issues. The first applies to the use of animal material for the scaffold engineering we propose in this research. Important to mention is that we use pigs obtained of a local slaughterhouse. That means that the research proposal will not have to be approved by an Animal Ethics Committee because the animals are not killed for scientific research purposes.

A second ethical concern might arise in the discussion to use animal sourced collagen for human therapeutic approaches. Although this research proposal only lays at the beginning of such an approach, in the future animal extracted collagen scaffolds might be used in preclinical models. This issue is a matter of perspective if we think it is ethically approved to use different sources of biomaterial than that of humans. The implementation of this source of collagen could be defined as a xenograft, of which the implementation like a porcine heart valve in humans is a

different example. For this proposal we are only at the base for clinical treatments and only use (already dead) animals for research purposes. In the future, it is important to re-examine this ethical issue when col(II) scaffolds will show positive results and might be produced at a larger and potentially commercial scale.

B.2.6. Literature/references

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