Experimental determination of the three-dimensional collagen architecture in the articular cartilage of the knee joint

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Summary

The goal of this research was to determine the three-dimensional architecture of collagen fibres in the articular cartilage of the knee joint, more specifically, the tibial plateau. This information is needed for the further development and support of the mathematical model developed by W. Wilson at the Eindhoven University of Technology.

Relevant information was obtained by studying cartilage samples taken from different locations in the joint and under different orientations. From this research it appears that cartilage structures vary along the edge of the tibial plateau, depending on location in the joint. Outer edges of the joint show a more gradual reduction in thickness than edges lying deep inside the joint. Cartilage thickness varies greatly with location, from 850-1200 µm centrally to 150-200 µm in the peripheral regions. Centrally this thickness is divided into 80% for the radial zone, 15% for the transitional zone and 5% for the tangential zone. Peripherally this ratio is closer to 25% for the radial zone, 25% for the transitional zone and up to 50% for the tangential zone. Curving of collagen fibres seems to take place in all directions, although per sample the curvature was generally only witnessed in one direction, contrary to the generally accepted Gothic arch structure. The reason for this difference is unknown. A cross-linking network appears to be present in the cartilage, although it is most prominently present in the central areas of the plateau and less visible towards the edges. The collagen architecture of the tibial plateau does not seem to be (axi-) symmetric.

Cartilage samples were obtained from pigs' knees and preserved/prepared using a cryo-substitution technique, followed by cryo-fracture to expose internal structures. Visualisation was done using Scanning Electron Microscopy (SEM). Some aspects of the preparation can still be improved upon, as not all materials described in literature were available during the course of this study.
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1 Introduction

1.1 General Introduction: The Anatomy of Articular Cartilage

Articular cartilage, or cartilage of articulating joints, is a multiphase tissue consisting of two principal components. Firstly, a solid organic matrix that is predominantly composed of collagen fibrils and proteoglycan macromolecules. And secondly, an interstitial fluid phase from which fluid can be squeezed under pressure.

Water, held in the structure by osmotic pressures arising from the negatively charged proteoglycans, provides the tissue with unique physical characteristics. It makes up 60-80% of the wet weight of the living tissue. Collagen maintains the shape of articular cartilage against osmotic pressure of the proteoglycans and it contributes to the main function of this tissue, which is to transmit loads.

Biomechanically, the articular cartilage forms covering material that protects the subchondral bone from high stresses and provides a smooth, lubricated surface that facilitates movements with little friction between the articulating surfaces. [3,5,7,12,13] The architecture of collagen is very specific and adapted to the function of the cartilage.

Independent of species, articular cartilage can generally be divided into a calcified, a radial, a transitional and a superficial zone [14]. The calcified zone is a layer of calcified cartilage that is located directly on the subchondral bone. The collagen fibres that support the cartilage are anchored in this calcified zone, reach up through the radial zone, then begin to arch over in a tangential direction in the transitional zone and finally run parallel to the surface in the superficial, or tangential, zone (Fig 2.1). This classical description of the collagen arrangement as a Gothic arch type of morphology is largely based upon polarised light microscopy (Benninghof, 1925)

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![Figure 1.1: The structure of cartilage. 4 zones can be identified.](image)

Articular cartilage in central areas of the tibial plateau is different from that in the periphery, where the menisci cover it. By weight the centre contains less collagen and
more PG than the periphery. In the centre collagen fibres appear distinct and well organized, when viewed by electron microscopy. [2]

In previous studies, the differences in the regions of articular cartilage have always been more pronounced on the medial side. Cartilage thickness was generally greater in the centre and on the medial side, but it varied markedly within each region. The radial fibres in the centre were straight, vertical and more clearly separated by columns of cells. They turned sharply just beneath the joint surface. The fibres of the same layer in the periphery were curved through much, and sometimes all, of their span from bone to surface. [2]

In the centre of the tibial plateau the deep or radial zone was 60-80% and transitional zone 10-15% of total thickness in all species. In man the tangential layer in the centre is relatively thick (approx. 10%, 250 µm), but non-existent or thin (10-50 µm), less than 10% of the total cartilage thickness, in other species. The tangential layer in the periphery is thicker than in the centre; Peripherally this zone can take up to 50% of the total cartilage thickness (100-400 µm). [2,3]

Exact delineation of zones (transitional and radial) is arbitrary in the periphery, as radial fibres tend to bend only a short distance from the calcified zone. Peripheral cartilage is generally thinner than central cartilage; the radial zone, where fibres were truly ‘radial’ is quite thin, whereas the transitional zone is quite thick. [2]

1.2 Numerical Model and Research Goal

Collagen fibres are very important structural components of cartilage. For studying articular cartilage mechanical functioning, and to ultimately be able to predict cartilage damage and adaptation, the collagen fibril network must be taken into consideration. Many numerical approaches towards cartilage mechanics and even cartilage damage have been published (e.g. Atkinson et al., 1997; Garcia et al., 1998; Li et al., 2000; 2002). Only a few of them included a description for the collagen fibril network.

For this purpose, W. Wilson, at the university in Eindhoven, has developed a fibril-reinforced axi-symmetrical finite element model of the articular cartilage of the knee joint. Since no quantitative data is available of cartilage architecture outside the central regions, some assumptions have had to be made with respect to the orientation of, and the interaction between the collagen fibres. Firstly, the orientation used is based on the assumption that the Gothic arch structure described in literature (Benninghof, 1925) is valid in three dimensions (Fig 1.2). It also includes the secondary fibrils observed by Clark (1985; 1991)
And finally, the choice was made to model the joint surface by means of an axisymmetric finite elements model. There is evidence to (partly) support some of these assumptions. Many sources describe the Gothic arch description first proposed by Benninghof (1925), but research has up until now focussed mainly on the central regions of the tibial plateau [2, 6] and less on the structures at or near the edges of the joint surface. It is also unknown whether the collagen architecture is the same in all directions and at all edges; whether it is, in fact, possible to assume axi-symmetry. There have been reports of secondary fibres in cartilage, but as with the previous points, most research so far has focussed on the central regions of the tibial plateau.

The aim of this study was to determine the three-dimensional architecture of collagen fibres in the articular cartilage of the tibial plateau, to determine if there is interaction between parallel fibres in the form of a cross-linking network and determine whether it is possible to describe the tibial plateau and collagen structure by means of an axisymmetrical model. In essence, to try and provide support for the assumptions made in the modelling of the tibial plateau.

For this purpose a method had to be selected, and worked out, to preserve and prepare cartilage in such a way that its internal collagen structure can be visualised.
2 Experimental aspects

In this chapter the methods and materials necessary to visualise the cartilage structure will be discussed. The different steps in the preparation of the samples will be discussed in more detail. Finally, the protocol followed during the experimental part of this research will be given.

2.1 Methods and Materials

In order to study the collagen structure within the cartilage, first a method is needed to visualize it. Different methods have been used in the past to study the collagen structures of cartilage. The 2 most commonly used are:

- Polarised Light Microscopy (PLM)
- Scanning Electron Microscopy (SEM)

Another promising method, which provides structural information, in a non-invasive manner, is Magnetic Resonance Imaging (MRI).

Each of these methods has its advantages and disadvantages. MRI has the advantage of being non-invasive; the structures inside a specimen can be visualised without having to section it. It can also provide three-dimensional information. The disadvantage is that the resolution usually isn't very high, and the structures to be studied here (collagen fibres) have dimensions that are not within the range of resolutions available. PLM has a similar problem to MRI. As with MRI, only a global orientation of the fibres will be visible [11,13,17].

SEM can produce detailed images of even the smallest structure within cartilage, but, in the case of biological material, samples require lengthy preparation/preservation before they can be studied. As described in [8]: SEM is the method of choice to show the 3-D structure of articular cartilage. With respect to numerous demanding tasks of cartilage research such as osteo-arthrosis research or evaluation of cartilage defects replacement, SEM is rarely used. One reason may be the demanding techniques for cartilage fixation and preparation for SEM.

Since detailed information about the orientation, interaction and cross-linking of the collagen fibres is desired, it was decided to use SEM in this research. In addition, the applicability of MRI to obtain structural information on the collagen network was evaluated.

Most protocols for SEM-studies found in literature use a variant of cryo-substitution or cryo-preservation, followed by Critical Point Drying and finally freeze fracturing to reveal the internal structures. In the following section the details of each of these 3 steps will be discussed, followed ultimately by the protocol used in the experiments.
Cryo-substitution

The advent of Scanning Electron Microscopy (SEM) in the study of surface morphology in biological applications made it imperative that the surface detail of a specimen was preserved. The air (evaporative) drying of specimens can cause deformation and collapse of structures, the primary cause of such damage being the effects of surface tension. The specimen is subjected to forces, which are present at the phase boundary, as the liquid evaporates. The most common specimen medium, water, has a high surface tension to air. By comparison that for acetone is several times lower. The surface tension could be reduced by substitution of a liquid with a lower surface tension with expectations of reduced damage during air-drying. [10, 16]

This substitution is best done at low temperatures, as in rapidly frozen specimens cartilage remains as close as possible to its natural state. The substitution fluid gradually replaces the ice crystals in the sample while it is kept submersed over a period of time. [5, 7]

Drying

After substitution of the water in the tissues by a fluid with a much lower surface tension (i.e. Acetone), it has to be dried. Ideally one would use critical point drying, as using this technique the surface tension can be reduced to (nearly) zero. If the surface tension becomes very small the liquid surface becomes very unsteady and ultimately disappears. When this ‘critical point’ is reached, it is possible to pass from liquid to gas without any abrupt change in state. If a specimen had been in the liquid it would have experienced a transition to a ‘dry’ gas environment without being in contact with a surface, avoiding the possibility of the damaging effects of surface tension. For this reason critical point drying is suitable for delicate biological specimens. Specimens would suffer significant thermal damage if we attempted to apply the technique direct in the removal of water from the specimens (hence the use of cryo-substitution). [10, 16]

Alternatives to critical point drying are air-drying or freeze-drying. Especially using air-drying there is still the problem of dealing with the aforementioned surface tension. Acetone substitution should greatly reduce the effects, but in all likelihood not to the point of being negligible.

Freeze fracturing

The substituted and dried cartilage is quite spongy, while the underlying bone has become quite brittle. In order to produce a nice fractured surface, the cartilage should be more rigid. If fractured in a deeply frozen state, it is thought that any fracture will run across natural cleavage lines, leaving the internal structures intact. To ensure this, the sample is frozen in liquid nitrogen (LN2), placed bone-side up on a solid supporting surface (also submersed in the LN2), and then fractured using either a sharp chisel, or razor blade pre-cooled to reduce local heating effects. [3, 4]
2.2 Protocol

The protocol described here is adapted from one of multiple used in [5]. Some elements appear similar to the protocols described in [1, 7].

Pigs' knees were obtained within 24h after slaughter. The joints were carefully opened, and the tibial plateaus were cut from the tibia using a simple jigsaw. The plateaus were separated into medial and lateral parts, and remaining subchondral bone was trimmed away, leaving approximately 3-5 mm to support the cartilage. After this the tibial plateaus were cut into smaller sections to facilitate further processing (Fig 2.1). At this point, samples intended for MRI were not processed further.

Figure 2.1: Top view of the medial tibial plateau [15]. This illustration shows how the plateaus were sectioned by jigsaw prior to freezing.

Freezing:
For the rapid freezing of the samples an iso-pentane slush was used; a container filled with isopentane was cooled in LN2. The iso-pentane was left to cool for approximately 10 minutes before proceeding. Prepared and sectioned samples were placed in the cooled iso-pentane and left for at least 2 minutes. After this the samples were removed from the iso-pentane and placed directly in the LN2, where they were left for a further 4-5 minutes.

After freezing the samples were removed from the nitrogen, and placed in containers containing anhydrous, or ‘dry’, acetone that had been pre-cooled in the deep freezer to 193K. The containers with acetone and bone/cartilage fragments were sealed in sample bags to prevent any leakage, and replaced in the freezer at 193K. After 6 days the samples were transferred into fresh, pre-cooled, acetone and left for a total of at least 8 days at 193K.

After substitution, the sample containers were removed from the freezer, insulated using aluminium foil and bubble wrap, and placed in a freezer at 248K for approximately 24 hrs. After this the containers were transferred to a conventional refrigerator at 277K, and left for another 24h. In the final step the containers were removed from the cooling-unit and were allowed to come up to 293K. During these final two steps the insulation was kept in place, to ensure the samples were heated as gradually as possible.

Once the samples had reached room temperature, they were removed from the acetone and placed in petri dishes for air-drying. The samples were left for at least 48 hrs to ensure they had completely dried.
**Freeze fracturing:**
Samples were pre-cut on the bone side with a razorblade (the bone is very brittle and chips away easily), to facilitate fracturing.

A block of aluminum, to provide a solid support for the samples, was placed in liquid nitrogen and left till it had completely cooled. The dried cartilage/bone samples were placed in the nitrogen, bone-side up, atop the aluminium block and left for about a minute after the boiling had completely stopped (2-3 minutes in total).

For the fracturing a standard razorblade was used, which was pre-cooled in the same liquid nitrogen. The blade was clamped in some vice grips, held to the sample and then tapped with a small hammer.

The fractured pieces were then fractured again, in a plane perpendicular to the first fracture (Fig 2.2). The size of the samples obtained thusly varies; the fractured face has a length of 3-8 mm depending on location.

![Figure 2.2: Top view of the tibial plateau. The dotted lines show where the sections were fractured. The solid lines are the cuts made by saw during preparation of the samples (Fig 2.1).](image)

After fracturing the samples were removed from the liquid nitrogen and placed in acetone to prevent the forming of condensation on the sample. The samples were left in the acetone for a few minutes after the nitrogen had fully evaporated. After reaching room temperature, the samples were removed from the acetone and left to dry for at least 24 hrs at room temperature.

**SEM:**
The dried, fractured samples were trimmed where necessary, to facilitate the placing on stubs. After mounting, fractured face up, samples were sputter coated with 10 or 15 nm gold using an Emitech K575X peltier cooled sputter coater.

The Electron Microscope used was a Philips XL30 ESEM FEG, running in Hi-Vac mode, with an acceleration voltage of between 1 and 15 kV depending on the desired image quality. The higher acceleration voltages are only suitable for samples with a thicker coating.

**Materials used:**
- Jigsaw (standard variety) with very fine saw
- Foam container, for holding the liquid nitrogen
- Polymer measuring cup, for holding the iso-pentane while in the nitrogen (glass might crack)
- A pair of large plastic tweezers/pincers for transferring the samples to and from the iso-pentane slush
- Different polymer containers with screw on lid for containing the samples and acetone during cryo substitution
- Standard razor blade, for pre-cutting and fracturing the samples
- Vice grips, for holding the razor blade during fracturing
- Small hammer, to deliver the force necessary for fracture
- Aluminium block or plate, to support the sample during fracture
- Emitech K575X peltier cooled sputter coater
- Philips XL30 ESEM FEG
3 Results

The results obtained from the experiments will be discussed in this chapter. Results from the MRI study will be discussed first as these show the need for using SEM in this study.

3.1 Magnetic Resonance Imaging

Samples of cartilage with the underlying bone were imaged using high resolution, $T_2$-weighted MRI. The matrix used in this study was 256x256x256 pixels, with a dimension of 2.56x2.56x2.56 cm$^3$, or a resolution of 100 $\mu$m. This gives a maximum of about 75 pixels for the cartilage at its thickest.

![MRI scans of a sample of articular cartilage of the knee joint.](image)

Figure 3.1 A-D: MRI scans of a sample of articular cartilage of the knee joint. The darker substance is the subchondral bone, the lighter the cartilage. A-D show different, parallel ‘slices’ of a single sample.

As can be seen from these images, not much detail is visible. With some imagination the trabecular structure of the bone can be seen, but the resolution is simply not high enough to be able to visualise the inner cartilage structures.

Structural information on collagen architecture could be determined by alternative MRI methods, such as Diffusion Tensor Imaging (DTI). Since results obtained by MRI were not of high quality, it is thought that DTI will yield little extra useful information, as its resolution is lower than that of MRI.

Another possible method to use might be $\mu$MRI, as it has a resolution of ~20 $\mu$m. This method has not been looked into in any detail, but it might merit doing so.
3.2 Scanning Electron Microscopy

The results obtained by electron microscopy clearly show the structures within the cartilage. The medial tibial plateau was studied, as it is a common site of cartilage degeneration in man. To better understand the orientation of the images, the layout in the figure below will be used.

![Cartilage Diagram](image)

Figure 3.2: Top view of the medial tibial plateau. The lateral (L), medial (M), anterior (A) and posterior (P) sides have been indicated in the figure.

To begin with, the general structure of cartilage was studied. The leaf-like structure of porcine cartilage described in literature can be clearly seen (Fig. 3.3 and 3.4).

![Images 3.3 and 3.4](image)

Figure 3.3 A-B: images are from sample taken near the lateral posterior edge of the plateau. Image A is closest to the lateral edge; B is more towards the medial side.

Figure 3.4 A-B: images from sample taken centrally, towards the anterior. Image A is closest to the lateral edge; B is more towards the medial side.
The cartilage thickness varies with respect to the location within the joint. Scans were made of different areas in an attempt to visualise this variation.

Figure 3.5: Overview of a sample taken at the anterior edge of the joint (see inset). The left side of the sample lies at the edge of the joint. Composition of 3 SEM images.

Figure 3.6: Overview of a sample taken at the medial edge (see inset). The right side of the sample is the outermost part of the joint. Black bar is 500 μm. Composition of 7 SEM images.

Figure 3.7: Image taken from a sample lying centrally in the joint. The left side points towards the anterior (see inset). Composition of 2 SEM images.
Images 3.5, 3.6 and 3.7 show the variation of the cartilage thickness in the joint. These images are from the same sample, but taken at different locations. It seems that different edges of the joint have a different structure. The cartilage thickness on the medial edge seems to reduce more gradually than on the other edges. The thickness of the cartilage in the central region is more constant. Figure 3.6 is also shown enlarged in Appendix A (figure A.1). The circular formations visible in the background are simply the surface of the adhesive tape used to secure the samples on the sample holders.

Different areas of the joint were imaged to see if location had any influence on the distribution of the 4 zones of articular cartilage as described in literature.

Figure 3.8 A-B: Images taken centrally (A), and near the lateral edge (B). In (A) the left side of the sample points towards the lateral edge, in (B) the right side. (see insets)

Image 3.8A shows that the collagen fibres run up almost vertically from the calcified zone, only to curve to a tangential direction near the surface. The cartilage thickness is approximately 800 µm here, with the radial zone taking up about 80%, the transitional zone about 15% and the tangential zone 5%. In the area shown in 3.8B it is more difficult to define a strictly radial zone, as the ‘leaves’ appear to curve away relatively quickly after leaving the bone. It's interesting to notice is that, nearer the surface, the fibres appear to curve upwards again (see white line in Fig 3.8B). Structures of this type were only observed in a very small number of samples, and might be artefacts (for another example, see figure A.4 in appendix A).
Figure 3.9 A-D: Several images at increasing magnification of the sample in fig 3.8A.

At low magnification the global orientation of the fibres was already visible. At higher magnification the individual fibres and their orientation and interaction also become visible (see also figure A.2). Images 3.9 B-D are of the cartilage-bone interface. Here the fibres seem to have an overall vertical orientation. At the highest magnification the interfibre cross-linking collagen network is visible (see also appendix A). Also, the position of cells within this network can be seen.

Figure 3.10 A-E: Sample taken near the medial edge. Images A and B show the most central and most medial parts of the sample, respectively (see inset). Images B – E are of increasing magnification.
In the images of figure 3.10 the individual collagen fibres are less visible. The upper layer of cartilage (images B and C) appears to have split from the underlying cartilage. The rest of the cartilage appears intact, and it is clearly visible that fibres turn in a tangential direction, towards the joint edge, close to the cartilage-bone interface. The cells also appear to orient themselves in tangential direction as the collagen arches over (Fig 3.10 D). Figure 3.10 B-E are images taken nearest the medial edge. Here the total cartilage thickness is approximately 300 µm, of which roughly 25% is taken up by the radial zone, 25% by the transitional zone, and the remaining 50% by the tangential zone.

![Image](image_url)

Figure 3.11 A-C: Higher magnification images of the sample from figure 3.5

The fractured surface at the very edge is much less clearly defined than in other areas. Only at the bone interface can fibres be identified. Here, as in the samples from the medial edge, fibres seem to curve away from the bone surface relatively quickly.

More results are shown in Appendix A.
4 Recapitulation of the Results

Figure 4.1: A sketch of the distribution of zones in the tibial plateau. The area within the solid ellipse appears to have the ‘normal’ distribution of zones. Outside of it, fibres start to bend in tangential direction (towards the joint edge) much sooner. The dotted line is used to describe a more gradual decrease in cartilage thickness along the medial edge.

Judging by the images taken in the SEM study, it seems there are differences in cartilage structure depending on location. The area indicated by the solid ellipse in figure 4.1, the centre of the plateau, has a thickness varying from approximately 850-1200 µm. Of this 70-80% is taken up by the radial zone, 10-20% by the transitional zone, and the rest by the tangential zone. Near the medial edge, the maximum thickness is no more than 150-200 µm. Of this, as much as 50% appears to be taken up by the tangential layer. The distinction between radial and transitional is hard to make as fibres start to curve quickly after leaving the calcified zone. This study seems to indicate that along the medial edge there is a sort of intermediate area that seems to be covered by cartilage with a thickness of 200-500 µm (area indicated by the dotted line in figure 4.1.)

Figure 4.2: Scheme of the observed fibre orientation at the anterior (A) and medial (B) edge.

The transition from thick to very thin cartilage has been described in the previous section. On all but the medial edge this transition takes place over a distance of 3-4 mm (Fig 4.2 A, compare with figures 3.5 and A.6). Along the medial edge, this transition is much more gradual, it seems. The cartilage thickness is reduced to its minimum over about 10 mm (Fig 4.2 B). This gradual reduction is the reason for the area marked by the dotted line in figure 5.1. The size of the plateau is approximately 25-30 mm in Lateral-Medial and 35-40 mm in Anterior-Posterior cross-section.

As shown in figure 4.2 and described in the previous section, curving of the collagen fibres has been observed. Obstruction of the structures near the surface made determining the direction of the curvature difficult in some samples, but generally the fibres appeared to curve towards the joint’s edge. All samples obtained from different locations in the joint and under differing orientations, showed this curvature.
Samples obtained in or near the central regions of the plateau (Fig 4.1) show what appears to be a cross-linking network (see also Figs 3.9 and A.3). This network is less prominent in areas along the edges, although some small amount of cross-linking may be present.
5 Discussion

Results from chapters 3 and 4 indicate that the arching of collagen fibres is valid in three dimensions in articular cartilage, although arching was generally only observed in one direction. Curvature was observed in samples taken from different locations and under different orientations. A collagen cross-linking network also appears to be present, although it is most prominently present in the central areas of the plateau. As described in figures 4.1 and 4.2, the plateau does not seem to be (axi-) symmetric.

In the remainder of this chapter, the different aspects and results of this study will be compared to results from the literature. A division has been made into three categories: observed anatomy, artefacts, and methods used.

Anatomy

The differences in cartilage that were noted in the SEM study are similar to those described in literature [2]: Articular cartilage in central areas of the tibial plateau is normally different from that in the periphery, where the menisci cover it. By weight the centre contains less collagen and more PG than the periphery. Here collagen fibres appear distinct and well organized, when viewed by SEM. Exact delineation of zones (transitional and radial) is arbitrary in the periphery, as radial fibres tend to bend only a short distance from the calcified zone. Peripheral cartilage is generally thinner than central cartilage; the radial zone, where fibres were truly ‘radial’ is quite thin, whereas the transitional zone is quite thick.

Figure 5.1: Diagrammatic illustration of collagen fibre orientation across the tibial plateau from periphery (P), under the meniscus (m), to centre (C). The dashed line represents the border between the transitional and tangential zones. Where fibres curve from the subchondral bone (b) to the surface (as seen at left), distinction of a transitional layer is impossible. This figure is reproduced from [2]
Figure 5.2 (a-b): Scheme of fibre orientation in articular cartilage. The vertical fibres in the radial zone turn in one direction as they reach the transitional zone, where they flatten and overlap to a variable extent. These fibres turn either toward (a) or away (b) from the edge. At the joint edge fibres from the periosteum extend into the articular surface in the most superficial plane. This figure is reproduced from [6]

The peripheral structures observed experimentally most resemble the structures described in figures 5.1 and 5.2a. The structures shown in 5.2b have not been observed. PLM studies have indicated a Gothic arch structure of the fibres in cartilage. While the fibres observed here do curve in the manner of a Gothic arch, they have only been seen to curve in one direction. This has not been observed before and may have a functional application, but its significance is unknown. It should be taken into account that the individual fibres were not visible in all samples at higher magnification. This might be caused by the formation of artefacts at some stage of the sample preparation (i.e. the method used may not be ideal)

**Artefacts**

By SEM, fibres in the radial and transitional zones are not readily apparent in samples taken from the periphery of a joint. In the case of the tibial plateau, the close apposition of these fibrils affects their visibility at lower magnifications. The most critical factor, however, is the plane of fracture. Where fibres curve significantly, they are seen only when they curve in the plane of section. For a sample to yield useful information about collagen fibre configuration and continuity, it is necessary for at least one fracture to run parallel to the fibrils. The orientation and relationship of fibrils transected in one plane can then be reconstructed by observation of the surface in which they are intact. It is possible that reports that describe random fibrillar patterns in the middle zones have failed to recognize a curved configuration [2, 3]. This influence of the plane of fracture might explain the observed structure in figure 3.8B:

![Diagram of SEM of collagen in articular surface](image)

Figure 5.3: One possible explanation for ‘S’-shaped structures observed in cartilage of figure 3.8B
Another possible explanation for the observation of a more randomly oriented collagen fibril arrangement may be fibril cross-links exposed by freeze fracturing [2, 3, 6]. See appendix A, figure A.3, for more images of possible cross-linking.

It has been reported in several cases that an unknown substance covered the collagen fibres, obscuring them. It is not always known what this substance is, but it is thought that the proteoglycan matrix, if retained in the tissue, is responsible for this [3, 4, 5, 9]. The sample in figure 5.4 may show this; collagen structure is visible with some effort, but not as clearly as in other samples. It is not known what the dark coloured ‘beads’ seen on the cartilage are, part of what is seen here is shadow, but there is also some precipitation on the surface, or so it seems.

Figure 5.4: Unknown substance covering the collagen structures.

Some deformations due to shrinkage have been observed in all samples. They are most obvious on the edges where samples were cut by saw, but they appear, to a lesser extent, along fractured edges as well. In most samples, shrinkage is less in areas further away from the sawed edges of the samples (for example Fig 3.7).

Figure 5.5A shows a corner where two perpendicular fractures met. The surface seems to have sunk down, or pulled back, and curled over, obscuring the upper layers of the cartilage. Figure B shows similar deformation. There the arrows indicate the saw edge. Figure C shows how this shrinkage may influence the structure. This shrinkage is clearly visible if viewed from the side (fig 5.5 A-C), but not readily apparent when viewed from the front. From [7]: To accurately measure thickness, fracture surfaces must be reasonably vertical, and photographed en face. Since measurement of thickness can only be done accurately in plane, this shrinkage influences the accuracy of measurement. The exact amount of shrinkage is unknown.
Figure 5.5 A-E: shrinkage and preparation artefacts. A-B: curling of the top layer due to shrinkage. C: shrinkage at the sawed edge. Material has pulled back. D: ‘flaps' of the upper layer(s) remaining after fracturing obscure structures near the surface. E: Shredded cartilage structure.

In many samples a ‘flap' of the upper layer(s) was left over after fracturing (Fig 5.5D). This bit of tissue would just cover the uppermost layers and make it impossible to trace the fibres all the way to the surface.

The sample shown in figure 5.5E was fractured with some difficulty; Residual tissue from the joint hindered the fracturing. This tissue was quite rubbery, even when frozen, and could only be completely fractured after multiple taps with the hammer. This ultimately led to the crushing of the sample, and the tearing of the cartilage. This is the reason for the shredded look of the sample.

Methods

During the early stages of sample preparation a jigsaw was used to remove the tibial plateaus and subsequently section them for further processing. No damage to the cartilage was observed during this processing, but another, gentler, method of sectioning might be preferable.

Since it has not been attempted before to map the collagen orientation throughout the entire articular surface, no references with respect to a preferred method of sectioning were found. After opening the joint, before further preparation, the articular surface, seen from above at first glance, seemed to radiate out from the centre of the lateral (the highest point of the plateau). The method shown in figures 3.1 and 3.2 was chosen for this reason. There may be a better method of sectioning, but all samples were sectioned in this fashion, to allow comparison of results from different sets of samples.

Artefacts formed during preservation and fixation inevitably accompany every morphological study of cartilage tissue: The best-documented types of artefact include dimensional changes and disruption of the matrix when the sample is cut or fractured to expose a viewing surface. The overall shrinkage due to fixation, dehydration and drying
can be as much as 30%. This shrinkage can produce artefacts. However, the general orientation of collagen fibres should not change with general shrinkage of the specimen. [3, 7]

The application of freeze-substitution techniques followed by critical point drying clearly reveals the internal structures. Applying conventional chemical fixation techniques can result in the fine detail of tissue being obscured and considerable tissue shrinkage. It has been proposed that the application of freeze-substitution in organic solvents at temperatures of 193K, or lower, to tissue that has been frozen rapidly results in far fewer ultra structural artefacts than does the use of chemical fixation protocols. The advantages of the freeze substitution approach are to be seen clearly, especially in the reduction of tissue shrinkage, which can be considerable, during processing. The main disadvantage of this approach is that some segregation artefacts will probably be formed. These are induced by the formation of small ice crystals. [5, 7, 8]

There are a number of possible substituting agents, of which acetone appears to be the one which will result in the slower and gentler removal of ice crystals from the tissue. [5] For cryo-fixation at iso-pentane pre-cooled with liquid nitrogen gives relatively rapid cooling rates. Freeze substitution for dissolving the ice crystals from a frozen specimen is best performed at between –80 and –90 deg C. [8]

Figure 5.6: Shrinkage of the samples fixed with three different methods. Relative dimension changes are shown in area%. Repeated measurements were made, of two-dimensional changes in size that occurred during the different stages of sample preparation, using a stereomicroscope and digitising tablet. CPD: critical point drying. [8]

Total preparation time for cryo-fixation can be up to 11 days, but samples retain approximately 89% of their initial size (see also figure 5.6). [8]

Due to lack of the necessary materials the protocol found in literature [5] had to be adapted into the one found in section 2.2. Ideally the transfer of the samples after the different freezing stages would be done under vacuum. As this was not possible, the samples were in contact with the air as they were transferred, which may have resulted in the formation of condensation on the surface of the samples. Acetone is only changed once over the 8-day substitution period. In the protocol described in [5] a daily change of fresh acetone is suggested to ensure a continuous excess of acetone. However, samples were very small and the containers contained approximately 500 ml acetone each. Furthermore, due to the fact that vacuum/refrigeration equipment was not available, changing the samples more often might lead to the formation of condensation, and/or the warming of the samples before substitution is complete. Even though this stage of the process differs from that described in [5], it is similar to what is described in [1] and [7].
The method of raising the temperature after substitution has completed could also be improved. In previous research the temperature is raised either from 193K or 248K by 5K/h to 293K \([1, 5, 7]\). Here the samples were brought to room temperature in two intermediate steps (sec 2.2). An unfortunate side effect of this method for raising the temperature was the formation of cracks in the cartilage surface on some of the samples. In literature there have been reports of the formation of fissures in the central tibial plateau. These splits occur between collagen fibres and extend into the radial zone. Extended exposure to CO\(_2\) and sample size can enhance this fissuring \([2]\). It is not known if the cracks observed in the present research are related to these fissures.

Samples were air-dried instead of critical point dried, which has an effect on the structure of the cartilage. Acetone (freeze) substitution in combination with critical point drying leads to a better conservation of the structures. Air-drying is most likely responsible for the shrinkage artefacts previously discussed.

The fracturing of the samples may also lead to the formation of artefacts. Samples to be fractured were placed, cartilage side down, onto an aluminum block and consequently fractured using a cooled razor blade. Especially at the edges of the tibial plateau, where the cartilage is at its thinnest, crushing of the cartilage structure can be seen in the form of separated layers. Any excess tissue left over during preparation will cause the fracture to not go through at once (Fig 5.5E). When multiple hits are required to fracture the sample, part or all of the sample will be damaged (crushed).

Fractured samples were stored in acetone once more, for a short period of time. As a result of this, the samples had to be dried once more, which resulted, to a lesser extent, in some shrinkage artefacts along the fractured edges.
6 Recommendations

The quality of the results seems affected by a couple of things. The most apparent are the raising of the temperature, the drying and the fracturing.

It is thought that an improvement of the way the temperature is raised might stop the formation of cracks on the cartilage surface. The computer-controlled oven, associated with the MTS Bionix tensile testing machine at the materials technology department of the university in Eindhoven, should be able to control the temperatures in the range needed.

A better method of drying, such as the aforementioned critical point drying might lessen the amount of shrinkage compared to air-drying. Freeze drying directly from a frozen state may also yield better results. Since the tissue remains frozen, the structures should remain intact.

A very important step in ensuring the best possible fracture is making sure all excess tissue is trimmed away. This leaves only the articular cartilage and some subchondral bone and might negate fracturing artefacts.

Since undesired damage of the sample occurs during freeze fracturing, some alternatives could be looked into as well:
- The fracturing itself might be improved by placing the sample on a block with a notch in it, so the area directly around the fracture area is not in contact with the supporting block. This should reduce the amount of crushing damage.
- The specimen is held in a foil tray filled with ethanol when immersed in liquid nitrogen. The mantle of solid alcohol helps prevent crushing and bending artefacts. [6]
- Freeze fracturing of samples is an important step in exposing the internal arrangement of the collagen fibres and is best performed prior to drying, with the sample immersed in 100% ethanol. [8]

Another recommendation would be to section the samples differently than was done in this research, to see if results would then differ significantly.

Further research is advised before drawing any conclusions with respect to the curvature of the fibres in only one direction as opposed to the generally accepted Gothic arch structure.
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Determination of collagen architecture in the articular cartilage of the knee joint

References

Architecture in Articular Cartilage: A Comparative, Quantitative MRI and Polarized Light Microscopy Study", Magnetic Resonance in Medicine, 46:487-493
Appendix A

Figure A.1: Same as figure 4.6, but slightly enlarged to show more detail. Black bar is 500μm
Figure A.2 A-J: Higher magnification images of the sample shown in figure 4.9. Images A-F and G-I were taken on faces perpendicular to one another. Image series move up from near the cartilage-bone surface (A, G) towards the surface (E, F, I)
Figure A.3: Images of what appear to be fibre cross-links

Figure A.4: example of another sample with what seems to be an ‘S’-shaped collagen fibre orientation. This sample was taken at the same edge as the sample from figure 4.8B, but more towards the posterior.
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Figure A.5 I-III: fibre map from calcified zone to surface. Calcified zone starts at the bottom of image I, Surface is the top of image III. These images were taken in the middle of the sample shown in figure 4.7. Composition of 38 SEM images.
Figure A.6: overview of a sample taken at the anterior edge of the plateau (same general area as the sample shown in figure 4.5). Composition of 6 SEM images.
Figure A.7: overview of the meeting of 2 perpendicularly fractured edges (see inset). Composition of 44 SEM images. Black bar is 100µm