Spatial and temporal evaluation of transport parameters of 3D chondrocyte constructs

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Abstract

In tissue-engineering cartilage, it is important to ascertain that the supply of nutrients to all the chondrocytes is sufficient. Therefore, it is necessary to know the evolution of transport properties in culture time. The goal of this study is to correlate the hydraulic permeability ($K$) and the diffusion coefficient ($D$) to the amount of extracellular matrix. $K$ was determined from uniaxial static confined compression experiments, while $D$ was evaluated from Fluorescence Recovery After Photobleaching (FRAP). These tests were performed on 3D cellular constructs with a thickness of 4 mm and a diameter of 6 mm. These constructs were made of 3% agarose containing $4 \times 10^6$ bovine chondrocytes per ml, cultured over a period of several weeks. Immunohistological images showed ECM developing around individual cells.

The stress-relaxation tests consisted of 4 steps of each 5% compression with respect to the initial configuration, so a final deformation of 20% was reached. To evaluate the hydraulic permeability, we fitted the experimental data with a numerical model of a saturated biphasic porous medium. The cellular construct was envisaged as a poroelastic material composed of a solid (cells, agarose and ECM) and a fluid phase. Both phases were assumed to be intrinsically incompressible. Furthermore, it was assumed that the solid phase was elastic and the fluid phase inviscid. The aggregate modulus was calculated from the equilibrium stress for each compression step, and used as an input parameter in the numerical model. The hydraulic permeability was assumed to be a function of both fluid fraction and strain. The parameters defining this relation were estimated and updated during the fitting procedure. A clear trend of $K$ with time of culture was observed: after 30 days of culture, the hydraulic permeability had decreased 3 to 4 times.

With FRAP, we studied the diffusion of Bovine Serum Albumin (BSA) through the 3D constructs of agarose with chondrocytes and their extracellular matrix. We found an increase in diffusion coefficient with increasing culture time. Since we expected a decrease in $D$ due to increased hindrance from the ECM, we probably did not measure in the ECM but in the relatively large space without cells and their surrounding ECM. However, the conclusion can be drawn that the value of $D$, which was of the order of $10^{-5}$ mm$^2$/s, is too low to achieve sufficient nutrient supply through the whole construct by diffusion. Therefore, it will be necessary to apply a flow through the construct to improve the nutrient supply. This flow, which depends on the hydraulic permeability, can be optimised continuously by applying the right deformation since the variation of $K$ with strain and culture time is known from the confined compression experiments.
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Introduction

Cartilage is one of the components of human joints. Under normal conditions, the loads and stresses in cartilage are quite high, and then, these joints still provide an almost frictionless performance during the entire lifespan, without much wear. However, this is not always the case, for example with hernias, when the intervertebral disc is damaged or in articular cartilage lesions. In the future, it may be possible to transplant a tissue-engineered piece of cartilage. In the IMBIOTOR project, it is tried to develop an intelligent cartilage bioreactor, with the idea of being able to tissue-engineer cartilage in a continuously optimised environment. To find the optimal conditions for the chondrocytes to produce extracellular matrix (ECM), it is necessary to relate some important transport properties of the developing tissue to its biological state.

Cartilage is a multiphasic material, with a solid matrix and interstitial fluid as its two most important components. The main constituents of the matrix are glycosaminoglycan macromolecules (GAGs) and collagen fibrils. The ease with which the fluid moves, the hydraulic permeability ($K$) of the tissue, can change as a result of matrix production or strain. Because of the dependence on the matrix production, the variation in $K$ during culture time may be a good measure for the production of ECM by the chondrocytes.

To calculate the hydraulic permeability at different culture times, Buschmann et al. [2] applied a small dynamic deformation after a 20% pre-strain to a 3D construct containing $10^7$ cells/ml 2% agarose. By fitting this experimental data to a form of a linear biphasic theory, they found that $K$ decreased from $10^{-2}$ to $10^{-3}$ mm$^4$/Ns in 4 weeks of culture time. The stiffness increased from $10^{-2}$ to $10^{-1}$ MPa from 0 to 30 days of culture. Williamson et al. [13] studied the change in compressive properties in articular cartilage from bovine fetuses, calves and adults. After a 15% pre-strain, the samples were allowed to relax and then, the equilibrium modulus was determined. It was shown to correlate positively with the GAG content as well as with the collagen content. The relaxation was followed by small dynamic compressions, from which the hydraulic permeability was calculated. Its dependence on strain was taken into account, and $K$ was found to decrease with an increase in GAG, collagen and cells. Ateshian et al. [1] performed a static compression test on cartilage discs, in which five steps of 10% strain were applied. They evaluated $K$ as a function of the strain and found that the hydraulic permeability and the aggregate modulus were of the order of $10^{-3}$ mm$^4$/Ns and $10^{-1}$ MPa respectively. Vunjak-Novakovic et al. [12] investigated the relation between mechanical properties of chondrocyte constructs, but with the emphasis on the effects of three different environments in bioreactors. Their discs had a diameter of 3 mm and a thickness of 2 mm, and the concentration of cells was approximately $10^8$ cells per ml. The values for the hydraulic permeability and equilibrium modulus they found, were of the same order of magnitude as those found by Buschmann et al. [2]. In all cases, a strong positive correlation existed between the confined compression equilibrium modulus and the GAG and collagen content, and a negative correlation between the hydraulic permeability and the GAG and collagen content.

Our objective was to study these relationships too, but we took into account the variation of the hydraulic permeability with strain, like Ateshian et al. [1] and Williamson et al. [13] did. They, however, used cartilage discs, while we used cultured 3D agarose constructs with chondrocytes that lay down their own ECM. We performed static confined compression tests, consisting of 4 steps of 5% deformation with respect to the initial configuration. After the test, the samples were histologically analysed to assess the amount of ECM. In order to evaluate the hydraulic permeability as a function of the strain, a biphasic non-linear finite element model was used to fit the experimental data. Previous studies [5] showed that the equilibrium stress increased with increasing compression. Therefore, we used the aggregate modulus, derived from the equilibrium stress for each individual step, as input in our model. We checked the validity of our hypothesis that the hydraulic permeability decreases when more GAGs are present because of obstruction by the ECM. We also expected the aggregate modulus to increase in culture time, because a previous study on native cartilage [2] showed that this was a sensitive function of tissue GAG concentration.
The hydraulic permeability is an important transport property because the movement of fluid through cartilage controls the deformational process. Moreover, it helps in transporting molecules essential for the cells. This is necessary because cartilage is avascular, and the access of chondrocytes to nutrients and waste products depends on diffusion [7]. Thus, without diffusion, no ECM can be formed, but at the same time, this ECM can hinder the diffusion. To date, to our knowledge, no studies have been done to assess the change of the diffusion coefficient in culture time, although we found some relevant literature. Quinn et al. [9] studied the influence of static compression of articular cartilage on solute diffusivity. They found that the diffusivity decreased with increased static compression, which may be partially attributed to the increased matrix GAG density due to the compression. This can be compared to an increased GAG density around chondrocytes in culture time.

Therefore, the second objective of this study was to measure the diffusion coefficient of bovine serum albumin (BSA) through constructs containing chondrocytes after different culture times with Fluorescence Recovery After Photobleaching (FRAP). The samples were analysed histologically, to determine whether there was a relation between the diffusion coefficient and the GAG production. We expected that the diffusion coefficient would decrease with culture time, because of the aforementioned hindered diffusion through the ECM. This effect was expected to be strongest on the edge, where the nutrients can reach the cells more easily and thus, more ECM can be produced.
Chapter 1 Hydraulic permeability and aggregate modulus

In order to find the hydraulic permeability and aggregate modulus of chondrocyte constructs, static confined compression tests were performed. The experiments were done after various culture times, to follow the evolution of the material properties as a function of days of culture. The trend of these parameters in time was correlated to the amount of glycosaminoglycans (GAGs), evaluated by a histological analysis. A finite element model for a saturated porous medium was used to fit the experimental data, and find values for $K$.

1.1 Preparation of 3D chondrocyte constructs

Cartilage was taken from mature bovine metacarpal joints, which were obtained from a local abattoir within a few hours after slaughter. To isolate the chondrocytes, first protease was added and the cartilage was placed for one hour on a gently shaking platform at 37°C. After removal of the supernatant, collagenase was added and the sample was placed overnight at 37°C, again on a gently shaking platform. Thereafter, the chondrocytes were isolated, and checked against contamination. Then, the healthy cells were counted, and incubated at 37°C in a high glucose medium.

Meanwhile, 6% agarose (type VII, Sigma) was prepared in Earl’s Balanced Salt Solution (EBSS), sterilised in the autoclave, and kept fluid in the oven at 40°C. To make the final sample of chondrocytes in agarose, chondrocytes in culture medium were mixed with an equal amount of fluid agarose, to achieve a concentration of $4 \times 10^6$ chondrocytes per ml of 3% agarose. During the preparation of the mixture, the 6% agarose was moved from the oven to a water bath of 40°C, and it was added with a warm pipette to the solution of chondrocytes, which was maintained at 37°C in another water bath. While keeping the mixture in the water bath at 37°C, the pipette was used to mix the chondrocytes and agarose to achieve a homogeneous mixture. This was a very delicate procedure, and it should be performed as fast as possible because the agarose starts to solidify below 40°C, and the cells are stressed very much above 37°C.

The 3D constructs were made using a mould, containing holes with a diameter of 6 mm, and a height of 4 mm, with a little glass plate underneath. The mixture of chondrocytes and agarose was put into the holes, and also on top of them, so there was a continuous layer of fluid on the mould. This was placed in the oven at 37°C for 20 minutes, after which the mixture had become a gel. The excess gel was removed from the mould with a very thin and sharp sterile knife to ensure a smooth surface. In a sliding movement, the glass plate underneath was removed, and by applying little pressure, the discs were pushed out of the holes. This method was found to produce discs with two smooth surfaces and very similar weights (a standard deviation of less than 2%), which confirmed that the mixture was homogeneous and that the removal of the excess fluid was successful.

Subsequently, the discs were placed in high glucose medium, which was refreshed every other day. This medium contained hepes (10 ml), fetal bovine serum (100 ml), L-glu (5 ml), Pen/strep (5 ml) and ascorbic acid (0.075 g), and DMEM high glucose was added till a total volume of 500 ml. The discs were incubated at 37°C. It was found earlier that the chondrocytes indeed produce an extracellular matrix in these conditions.

1.2 Experimental set-up

The experimental set-up for the uniaxial confined compression experiments consisted of a large stainless steel cylinder with an inner diameter of 38 mm on the bottom of which laid a porous stainless steel filter (figure 1.1). On top of the filter, a closely fitting stainless steel cylinder was placed with a height of 6 mm. This second cylinder contained 12 holes with a diameter of 6 mm, in which the discs fitted perfectly. The shape of the edge of this cylinder allowed fluid to flow freely out of the discs into the filter on the bottom, without building up a fluid pressure (figure 1.2). To prevent dehydration of the samples, filter papers connected the bottom filter with little fluid reservoirs, which contained EBSS. On top of each disc, a stainless steel cylinder with a
height of 4 mm was placed. A porous glass filter, to which the displacement is applied, is placed on top of this all.

Figure 1.1 Cross-section of the experimental set-up of the confined compression experiments: In red are the chondrocyte-agarose-discs, which laid on a stainless steel filter, and had stainless steel cylinders on top. A large glass filter, to which the displacement was applied, was placed on top of this all.

Figure 1.2 Top view of the experimental set-up: The cylinder with the holes was put in the large cylinder. Four of the 12 holes were filled with the chondrocyte discs, and the filter papers connected the bottom filter with the EBSS reservoirs. The white filter had to be laid on top of the little cylinders, when they were put on the 12 discs. The shape of this filter was adjusted in such a way that the paper filter connections could pass frictionless from the bottom filter to the reservoirs.

In four steps, a final compression of 20% with respect to the initial configuration was reached. In each consecutive ramp, the sample was compressed 0.2 mm in 240 seconds, after which the displacement was held constant for two hours, to allow the sample to relax. The overall load, which is the sum of the load on all 12 discs, was recorded during the experiment.

1.3 Mixture description of saturated porous media

Balance of mass and momentum
To extract the material properties from the experiments, a biphasic mixture model was used to fit the experiments. The interstitial fluid and the solid matrix were modelled as distinct phases because experiments showed that the movement of the liquid is very important in the deformational behaviour of the mixture as a whole [7]. It was assumed that both the fluid and the
porous solid phase were intrinsically incompressible. Furthermore, the mixture was assumed to be saturated with fluid, so the saturation condition
\[ \phi^s + \phi^f = 1 \] (1)

applied. Here, \( \phi \) is the volume fraction and the superscripts \( ()^s \) and \( ()^f \) refer to the solid and the fluid respectively. Assuming no mass exchange occurred, the local mass balance of the mixture is
\[ \nabla \cdot \left( \phi^s \mathbf{v}^s \right) + \nabla \cdot \left( \phi^f \mathbf{v}^f \right) = 0, \] (2)
in which \( \mathbf{v} \) is velocity. Substituting the saturation condition, this can also be written as
\[ \nabla \cdot \mathbf{v}^f + \nabla \cdot \left( \phi^f \left( \mathbf{v}^f - \mathbf{v}^i \right) \right) = 0. \] (3)

This equation states that the rate of volume change of a unit volume of mixture is exactly balanced by the in- or outflow of liquid [3]. The second term can be replaced by the three-dimensional form of Darcy's equation, which is
\[ \phi^f \left( \mathbf{v}^f - \mathbf{v}^i \right) = -K \cdot \nabla \left( p + \frac{\partial W}{\partial \phi^f} \right). \] (4)

In this formula, \( p \) is the hydrostatic pressure, \( W \) is the strain-energy function and \( K \) is the hydraulic permeability.

The momentum balance of the mixture can be written as
\[ \nabla \cdot \mathbf{v}^f = \nabla \cdot \mathbf{v}^e - \nabla p = 0, \] (5)
in which \( \mathbf{v}^e \) is the effective stress. The constitutive law that was used, is given by
\[ \mathbf{v}^e = (\det \mathbf{F})^{-1} \mathbf{F} \cdot \frac{\partial W}{\partial \mathbf{E}^s} \cdot \mathbf{F}^c. \] (6)

\( \mathbf{F} \) is the deformation gradient tensor, \( \mathbf{F}^c \) the conjugated form and \( \mathbf{E}^s \) the Green strain of the solid. In the case of one-dimensional confined compression,
\[
\mathbf{F} = \begin{bmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & l/l_0 \end{bmatrix}, \quad \text{and} \quad \mathbf{E}^s = \frac{1}{2} \left( \mathbf{F} \cdot \mathbf{F}^c - I \right) = \begin{bmatrix} 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & \frac{1}{2} \left( (l/l_0)^2 - 1 \right) \end{bmatrix}. \] (7)

The strain energy function \( W \), used for the effective stress in the mixture is
\[ W = \frac{1}{2} \mathbf{E}^s \cdot \mathbf{E}^s, \] (8)

which can be substituted in equation (6). Together with equation (7), this yields

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\[ \sigma^e = JH \cdot E^s, \quad \text{with } J = \frac{l}{l_0}. \] (9)

These equations are valid in the case of one-dimensional compression, in which \( F \) is equal to \( F^c \), and to \( J \), its determinant. From equation (8), it can also be seen that the second term between the brackets in the right hand side of equation (4) is zero, because \( W \) does not depend on the fluid volume fraction. The resulting mass and momentum balance are respectively:

\[
\nabla \cdot \mathbf{v}^t - K \nabla^2 p = 0 \\
\nabla \cdot (JHE^t) - \nabla p = 0
\] (10)

The hydraulic permeability \( K \) in equation (10) was found to depend on the applied compressive strain in an exponential manner [1], according to

\[ K = K_0 \frac{\phi_0 \phi^f}{(1 - \phi_0)\phi^s} e^{M \varepsilon}. \] (11)

\( K_0 \) is the hydraulic permeability when strain \( \varepsilon \) (negative in compression) is zero, and \( M \) is a constant that determines how fast the hydraulic permeability decreases with increasing compression [7]. The initial solid fraction is \( \phi_0 \) and the solid and fluid fraction are updated each step according to

\[ \phi^s = \frac{\phi_0}{l/l_0}, \quad \text{and } \phi^f = 1 - \phi^s \] (12)

respectively. In equation (12) \( l \) is the current length and \( l_0 \) is the initial length.

Finite element model
To obtain the finite element formulation, the momentum and mass balance, with Darcy's law and the constitutive law substituted, were multiplied with a weighting function and written into an integral form. Then, the integral was transformed from a global into a local coordinate system, with each element representing a layer of the disc. Subsequently, Galerkin shape functions were introduced. For temporal discretisation, the theta-scheme was used (note that theta should be larger than 0.5 in order to get an unconditionally stable solution). After assembling each finite element contribution in the stiffness matrix \( K \), the final set of differential equations is of the form

\[ K \cdot u = f. \] (13)

Vector \( u \) contains the nodal degrees of freedom, which are displacement and pressure, and \( f \) is the force vector. Since this equation is linear in the displacement, and the constitutive law is not, as can be seen when equation (7) is substituted in equation (9), this law had to be linearised. The finite element model was programmed in MATLAB, according to a program J. Huyghe wrote some years ago [3], to calculate the displacement and pressure of each node during confined compression. Vector \( u \) is divided into an unknown (\( U_u \)) and a known part (\( U_p \)), and the stiffness matrix and the right hand side are partitioned as well, so equation (13) becomes

\[
\begin{bmatrix}
K_{uu} & K_{up} \\
K_{pu} & K_{pp}
\end{bmatrix}
\begin{bmatrix}
U_u \\
U_p
\end{bmatrix}
=
\begin{bmatrix}
f_u \\
f_p
\end{bmatrix},
\] (14)
in which the subscripts (\(u\)) and (\(p\)) refer to the unknown and known parts. \(U_p\) contained the boundary conditions for our confined compression experiment, which were zero pressure at the bottom of the specimen, because of free outflow of the fluid through the filter, zero displacement at the bottom, and a prescribed displacement at the top. This displacement was a ramp, during which the compression increased to 5% and after that, was held constant. The unknown nodal values could now easily be solved:

\[
U_u = (K_{uu})^{-1} \cdot \left( f_p - K_{up} \cdot U_p \right).
\] (15)

Data fitting

The above model was used to fit the experimental data to find values for the constants \(K_0\) and \(M\), defined in equation (11), using the MATLAB routine lsqcurvefit. This routine uses the least squares method to find the best fit. Equation (11) gives the function for the hydraulic permeability, which was updated every time step. The strain used in this equation was the total strain of the sample.

The time step of the finite element model was adapted to the two different time steps taken in the experiment. The first five minutes, this time step was 1 second and thereafter, the load was measured every 10 seconds.

Since the slope of the applied displacement ramp in the experiment was not precisely constant, the experimental displacement values were used as input in the model. The aggregate modulus was calculated by dividing the final equilibrium stress by the strain according to Hooke's law, and this was also used as input.

To get a stable solution, the number of elements (\(numele\)) had to fulfill equation (16).

\[
\Delta x = \frac{L}{numele} \leq \sqrt{K \cdot H \cdot \Delta t}.
\] (16)

\(L\) is the initial sample height, \(\Delta x\) is the initial element height, and \(\Delta t\) is the smallest time step.

The results of the simulation showed the behaviour of the sample during compression (figure 1.3).

![Figure 1.3 Nodal coordinates and total stress](image)

In the right figure, the total stress (which was the same in each node) during one compression step is shown, as it was calculated with the model. In the left figure, the corresponding nodal coordinates are drawn. During the ramp, all the layers were compressed in a non-uniform manner, the lower layers were more compressed than the upper ones. The relaxation was most clearly seen in the lower layers, because here, the fluid flew immediately out of the sample into the bottom filter, and it had to be partially replaced by the remaining fluid from the higher layers.
When the overall displacement reached its final value, the stress was at its peak, and thereafter it relaxed gradually to its equilibrium value. Immediately after the displacement ramp, the compression was not equally distributed in the sample (figure 1.3); the lower layers were more compressed than the upper ones, because the fluid could flow freely out of the sample at the bottom, resulting in a higher stress in the lower layers. Through a redistribution of the fluid in the sample, which is governed by the strain-dependent hydraulic permeability, the strain became more homogeneous, and so did the effective stress and the pressure. During relaxation, the pressure in the whole sample became zero and therefore, the total stress decreased. Finally, it reached its equilibrium value \( \varepsilon H \), which is determined by the linearly elastic response of the solid matrix [7]. The stress behaviour shown in the right figure was used to fit the experimental data.

### 1.4 Results

We performed confined compression tests after culture times of 7, 14 and 30 days and with a disc without cells (appendix A). As an example, the measured stress of the chondrocyte discs that were cultured for 30 days, is plotted against time in the left side of figure 1.4. The stresses measured during the first two compression steps of the experiment, were fitted with the numerical model, to calculate the hydraulic permeability parameters \( K_0 \) and \( M \). The right figure shows the fit of the first two steps of the left figure.

![Figure 1.4 Results of the confined compression test with chondrocyte discs that have been 30 days in culture. The upper shows the loading history. The lower left figure shows the experimentally measured stresses and the right figure shows the numerical fit on the first two steps.](image)

Only the first two steps were used to fit, because the hydraulic permeability decreased significantly with increasing strain and became very small, due to the large value of \( M \). With the combination of the values of \( K \) and \( H \) of the third and fourth step, MATLAB could not properly invert matrix \( K_{uu} \), and the solution was not stable.
The resulting values for $K_0$ and $M$ are shown in Table 1.1, and these are plotted in Figure 1.5. Table 1.2 contains the values for $H$ for each compression step, which can be seen against culture time in Figure 1.6. Since the measured load was the sum of the load in 12 discs, the standard deviation could not be calculated in the usual way. This is why a standard deviation of the aggregate modulus is not shown. It is possible to give an indication of the accuracy of $K_0$ and $M$, but these are not directly extracted from the experimental data. The average relative difference between the numerical fit and the experimental data is determined, and used to calculate the variation on $K_0$ and $M$.

<table>
<thead>
<tr>
<th>Days of culture</th>
<th>$K_0$ (mm$^4$/Ns)</th>
<th>$M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without cells</td>
<td>1.30E+0</td>
<td>3.94E+1</td>
</tr>
<tr>
<td>7</td>
<td>1.26E+0</td>
<td>2.61E+1</td>
</tr>
<tr>
<td>14</td>
<td>1.12E+0</td>
<td>3.71E+1</td>
</tr>
<tr>
<td>30</td>
<td>4.30E-1</td>
<td>3.33E+1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Days of culture</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without cells</td>
<td>1.18E-2</td>
<td>1.21E-2</td>
<td>1.43E-2</td>
<td>1.47E-2</td>
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<td>1.83E-2</td>
<td>6.99E-2</td>
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<td>1.41E-2</td>
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<td>30</td>
<td>4.72E-3</td>
<td>6.48E-3</td>
<td>1.32E-2</td>
<td>1.86E-2</td>
</tr>
</tbody>
</table>

Figure 1.5 Parameters defining the strain-dependent hydraulic permeability versus culture time: On the intersection with the y-axis in the left figure, the values for the agarose discs without cells are included. It shows that $K_0$ at zero strain, $K_0$ decreases with culture time. In the right figure, it can be seen that no significant variation of parameter $M$ was found with time of culture.

Figure 1.6 Aggregate modulus versus culture time for different compression percentages: It is shown that the aggregate modulus slightly decreased in culture time. The aggregate modulus increased with strain. The measurement of 15 and 20% compression after 1 week in culture are very different from the others, because the relaxation of these steps was not good (appendix A, figure A2). The values of the aggregate modulus of agarose discs without cells are included in the figure at zero culture time.

These results indicate that $K_0$ decreased with culture time. The graph of $M$ shows no clear trend in culture time. $H$ did not change much in culture time, but it can be seen from Figure 1.6 that $H$ increased with strain. From Figure 1.5, it can be seen how $K_0$ and $M$ change in culture time. The variation of the actual hydraulic permeability with strain and with culture time is more clear in Figure 1.7. Then, it is clear that $K$ decreased in culture time, for each compression level. The line for the disc without cells does not agree with this trend.
Figure 1.7 Logarithmic $K$ versus compression after different days of culture: The logarithmic of $K$ ($\text{mm}^4/\text{Ns}$), according to equation (11), is shown to decrease with increasing compression, and with increasing days of culture. $K_0$ can be deduced from the intersection with the y-axis and $M$ is the slope, which shows no clear trend.

1.5 Discussion
We hypothesised that the hydraulic permeability decreased with culture time, which is supported by the results. $K$ decreased almost an order of magnitude over 30 days in culture, which is in agreement with the results of Buschmann et al. [2], who also found a decrease of about one order of magnitude in 30 days. But our values of $K$ did not correspond to the values reported by Buschmann et al. [2] and Vunjak-Novakovic et al. [12]. This may be explained by the fact that Buschmann et al. evaluated $K$ after 20% compression, without taking into account the dependence on strain and fluid fraction, like we did. Furthermore, Buschmann et al. used 2% agarose and $10^7$ cells per ml in a construct with a height of 1 mm, while we used 3% agarose and $4\times10^6$ cells per ml in a disc of 4 mm in height. The discs Vunjak-Novakovic et al. used were also smaller than ours. When we calculate $K$ at 20% compression with our values for $K_0$ and $M$, it was of the order of $10^{-4} \text{mm}^4/\text{Ns}$, which is one order of magnitude smaller than the values of Buschmann, which may be due to the difference in discs. In figure 1.7 it can be seen that the hydraulic permeability of discs without cells is almost as small at 20% compression as that of discs with cells that have been cultured for four weeks. Buschmann found a difference of one order of magnitude here, which is much more what we expected. According to Williamson et al. [13], $M$ increased from fetal to adult cartilage. We found no clear trend of $M$ in culture time, but we do not know if we could expect that in only 30 days of culture time.

The change of $K$ in culture time corresponds well with the results of Buschmann et al., but the opposite is true for the aggregate modulus. Buschmann et al. found an increase of about one order of magnitude in four weeks of culture time, evaluated at 20% strain. The aggregate modulus we found at 20% strain remains more or less constant. We used discs of 4 mm in height, which is thick in comparison with the discs used in literature. Therefore, the discrepancy between our values and those of Buschmann et al. may be ascribed to the scarce ECM production in the centre of our scaffold. When applying a displacement, most of the deformation will occur in the less stiff part of the sample, which is mostly agarose. After an extended time of culture, this problem will be overcome, and as a consequence, the aggregate modulus is expected to increase then. With discs of 1 mm, the ECM will be distributed more homogeneously throughout the sample after only four weeks of culture, and so an increase in the aggregate modulus is indeed found. This strong influence of the height of the sample is not important in the hydraulic permeability since all the fluid has to pass through the bottom of the sample when it is flowing out, and there, the ECM production does not depend on the thickness of the disc.

We can relate our results reasonably well to data found in the literature, but there are some points of discussion, which may explain the discrepancies. Among others, the discs we used for the compression tests, were not all perfectly shaped. The amount of cells was limited, and therefore,
some discs with little damage had to be used. Moreover, the appearance of the discs that had been in culture for three and four weeks, was much rougher than that of the other discs, and these discs did not fit in the experimental mould as well as the others. We also observed some difference in colour of the discs. The colour of the EBSS becomes more pink, when a large number of cells is dead. We tried not to use these discs, but sometimes, it was unavoidable.

It is known that cartilage is an anisotropic tissue [1, 8], but we do not know if this is also the case for our tissue-engineered construct or if some anisotropy was introduced during the compression. We assume we produced homogeneous constructs by the new protocol for mixing the agarose with the chondrocyte solution. This was a delicate procedure because the agarose is already a little gelly below 40°C and the cells have to be kept at 37°C only, to avoid much stress. Furthermore, it was difficult to be exactly sure of the amount of agarose that flowed out of the pipette because the gel stucked a little to the pipette. Furthermore, autoclaving the agarose could increase the concentration due to some evaporation of fluid. Therefore, the concentration of agarose and chondrocytes could be a little different each time the mixture was made.

It is also essential that the surfaces of the discs are flat. For example, when it has a meniscus, the edges are compressed instead of the whole surface. Our way of putting an excess of the chondrocyte-agarose mixture on top of the holes, and removing this excess when it was solid, gave smooth and flat surfaces.

Ideally, the stress in several discs should be measured on the same day with separate load cells, to average out the biological variation. In our case, the measured load was the sum of the loads on all the discs. When the assumption of equal discs is made, measuring the total load, and dividing it by the number of discs, gives an experimental average. This was the only way we could achieve an average, since the tests lasted very long. Performing different tests on different days, we could not have calculated an average because there would be a difference in culture time between the discs and the environmental conditions could be different.

We assume that the change in $k$ was only due to the production of ECM, but if we also had data on agarose discs without cells, which had stayed for 1, 2, 3 and 4 weeks in medium, the possible influence of the agarose could also be taken into account.

Another point of discussion is the height of the little cylinders on top of the discs. They should all be of exactly the same height to ensure that each sample was compressed in the same amount. But after measuring the height, we found that there was a difference in height of approximately one percent. So, the applied strain had to be much larger than one percent to be sure that the height difference is a relatively small error.

Furthermore, the sliding of the little cylinders in the holes, has to be frictionless. In order to reduce this friction, the diameter of the little cylinder should be slightly less than the diameter of the hole. But in that case, the disc would not be compressed on its entire surface, and the compression would not be perfectly confined. Besides, when there is a little gap between the cylinder and the mould, in addition to the fluid flow through the filter at the bottom, the fluid can also flow out trough this gap. A good alternative would be to use cone-like cylinders instead of cylinders, because then there is less contact between the cylinders and the mould. The basis of the cone should be as large as the sample surface to prevent fluid flow trough a gap. The diameter of the top surface should be large enough to ensure that the displacement is exactly along the long axis of the cone, perpendicular to the surface of the sample.

During the preparation of the experimental set-up, the cylinders and the filter had to be put on the discs, which already compresses the discs a little. In order to reduce this compression, the mass of the cylinders and the filter should be as small as possible. But the mass of the filter can not be too small because the load is applied in the middle of it. Although the glass filter was supposed to be rigid, it might have bent a little, and then the displacement of the discs in the outer zone would not be the same as the displacement in the inner zone. Furthermore, the top filter was saturated with EBSS, because otherwise, its weight on the discs could change during the experiment because of absorption of EBSS from the filter paper connections. However, these did not always
touch the top filter, and then, the fluid in the filter evaporated, and the weight on the discs changed during the experiment.

Then, there are some limitations of the machine we used to do the confined compression tests. The range of the load we measured was in the lower limit of the machine, but we found that the results with these low load values were reasonably reproducible. However, with these small loads, the influence on the load of possible environmental factors, like vibrations, can be large. Furthermore, it is difficult to determine the exact point at which the load cell touches the top filter, but it should be determined very carefully and precisely. This can give a small error, because when the point is found at which the load suddenly begins to increase fast, the sample is already a little bit compressed before the test is started. This zero is not exactly the same point for each new experiment. However, because we imposed strains of 5% and larger, this error is relatively small.

There are also some limitations on the model we used. First, there is a general criterium that should be met about the number of elements in relation to the time step, and the values of the hydraulic permeability and the aggregate modulus (equation (16)). However, increasing the number of elements slowed down the model, and inverting the stiffness matrix became a problem because of a very large ratio between the smallest and the largest element in that matrix. As a consequence, the results could not be trusted anymore.

We also found that the inversion was not accurate for each arbitrary combination of $K_0$ and $M$, even if we adjusted the number of elements. That is why we used only a small number of elements and fitted only the first two compression steps, which is less accurate but nevertheless, produced reasonably good results. For example, the fit of the experimental stress, measured after 30 days in culture, is good enough to derive the values for $K_0$ and $M$. But the low hydraulic permeability, calculated with these values, may cause an inaccurate inversion of the matrix, which may explain the increase in the simulated equilibrium stress in the right part of figure 1.4. Furthermore, there is the assumption of a homogeneous sample, which is not completely justified. But omitting it would introduce a lot of uncertainties and too many parameters to be estimated from only the total stress in the whole sample, which was the only thing we measured. This average stress was used to calculate the hydraulic permeability, which was also an average. We could choose to calculate the hydraulic permeability for each element separately in our model, but because we did not have any experimental data on the individual layers, we chose not to do so. There is also the assumption for the constitutive equation, which is non-linear and applies to large deformations, but it does not include viscosity. However, the observed viscoelasticity in compression experiments seems to be largely caused by the drag between the fluid flow and the solid matrix, and the viscoelastic properties of the matrix itself play a minor role [7]. Suh et al. [10] investigated the relative contribution of fluid flow-dependent and fluid flow-independent viscoelasticity on the overall viscoelastic behaviour of soft tissues. They used a biphasic poroviscoelastic theory, which incorporated a viscoelastic relaxation function into the effective solid stress of the poroelastic theory. Their conclusion was that the fluid flow-independent intrinsic viscoelasticity should be taken into account especially when the tissue undergoes a significant deviatoric deformation, which was not the case in our confined compression experiments. Therefore, our assumption of a purely elastic solid stress seems reasonable.

To calculate the aggregate modulus, the experimental equilibrium stress was divided by the strain, according to Hooke’s law. This was assumed because the equilibrium stress is determined by the linearly elastic response of the solid matrix, according to Mow [7], which can be described by Hooke’s law. According to our constitutive equation, the stress does not relax to this value exactly, which explains the difference between the simulated and the experimental equilibrium stress. It is known that the stiffness changes with strain, but we did not include this completely in our model yet: during each step of 5% compression, the aggregate modulus was held constant.

Another assumption is that the initial length did not change during culture time. Because the samples fitted perfectly into the holes of the experimental set-up, we can assume that the diameter remained the same, and therefore, the assumption of unchanging height seems to be
reasonable. This was not exactly true for the discs that were cultured for four weeks and therefore, it would be better to measure the initial height. This could be done when the cylinders were higher than the mould, which was not the case in our experimental set-up. Then, the difference in displacement of the load cell between a reference point with and without discs, is the exact height of the discs.

Nevertheless, despite all these limitations, these results can be used as a first estimation of the displacement, which should be applied to a sample in a bioreactor. The flow in this bioreactor, which depends on $K$, should be adapted to achieve an optimal nutrient supply to all the cells. Since it is known now how the hydraulic permeability varies with culture time and strain, the applied deformation can be adjusted to optimise the flow.

1.6 Future research
From literature [7], it is known that the hydraulic permeability is related to the pore size. The scanning electron microscope (SEM) can be used to evaluate the average pore size, to compare this to the pore size that can be found from $K$. To view the structure of the samples in the SEM after compression, the water has to be removed. Otherwise, water would boil violently out of our sample because of the low pressure in the SEM, disrupting its structure and increasing electron scattering by raising the pressure in the microscope. If air dried, the sample would collapse because the water/air interface which passes through the sample, has huge surface tension forces associated with it, which cause structural damage. Freeze drying is a technique to avoid this. The samples have to be frozen immediately after the compression test, together with a cryoprotectant that saves the cells and structure. Subsequently, the frozen fluid is removed by sublimation in the freeze-drier, and after sputtercoating the dried sample, it can be viewed in the SEM.

Relation between hydraulic permeability and pore size
The hydraulic permeability is a macroscopic measurement of the ease with which fluid can flow through a porous matrix. It was already mentioned that $K$ depends on the compressive strain. During compression, fluid is forced to flow out of the material, and the volume fraction of the solid, and the fixed-charge-density increase. This fixed-charge-density is located on the proteoglycans, which have a large amount of negatively charged groups that attract water and counter-ions. This tendency to swell, is balanced by the collagen network, and when an external load is applied, a new equilibrium between these swelling forces and the tensile strength of the collagen matrix has to be established.

The hydraulic permeability of cartilage can be measured by forcing fluid to flow through a cartilage sample, with height $h$, by applying a pressure $P_a$. For a one-dimensional experiment, the apparent hydraulic permeability $K$ is calculated according to Darcy's law, which is repeated here:

$$\phi^f (\mathbf{v}^f - \mathbf{v}^s) = -K \cdot \nabla \left( p + \frac{\partial W}{\partial \phi^f} \right).$$  \hspace{1cm} (4)

The pressure gradient can be expressed as $P_a$ divided by $h$, and the left-hand side is equal to the flow $Q$ divided by the permeating area $A$:

$$\frac{Q}{A} = K \frac{P_a}{h}.$$  \hspace{1cm} (17)

The flow in the equation above, can be calculated as follows, assuming a steady Poiseuille flow in the pores:
In equation (18), $\mu$ is the viscosity of the fluid and $a_i$ and $l_i$ are the radius and length of pore $i$, respectively. Assuming a homogenous and isotropic structure with $n$ pores, the areal porosity $\beta$ and tortuosity factor $\delta$ can be calculated for a uniform Poiseuille model, according to

$$\beta = \frac{\sum_{i=1}^{n} A_i}{A_T} = n \frac{\pi a^2}{A_T} = \frac{V_f}{V_T}, \quad \text{and} \quad \delta = \frac{1}{h},$$

in which $A_i$ and $A_T$ are the pore the total area respectively, $V_f$ and $V_T$ are the fluid and total volume respectively, and the pore radius $a$ and length $l$ are assumed to be the same for each pore. The height of the disc is $h$. Equating Poiseuille's formula and Darcy's law, with the help of these two equations, gives an estimate for the average pore size,

$$a^2 = \frac{8 \mu \delta \beta K}{\beta}.$$ 

This calculation of pore size is, however, rather inaccurate because it is very difficult to find a good estimate of the tortuosity factor, which is a measure of the actual path travelled by a particle. Another difficulty is that the measured hydraulic permeability is determined by both the pressure gradient and a potential difference. During compression, the counter-ions of the negatively charged GAGs will flow out together with the water, which results in a potential difference across the sample. This potential difference tends to reduce the water flow out of the sample and hence lower the effective $K$ [4]. To calculate the pore size from the measured hydraulic permeability, it is assumed that it consists only of the contribution of the pressure gradient and that the contribution of the potential is negligible. Despite these expected difficulties, it is good to check whether the order of magnitude of the evaluated hydraulic permeability agrees with the microscopical structure of the construct.
Chapter 2 Diffusion coefficient

To find the relation between the glycosaminoglycan (GAG) content and diffusion through the extracellular matrix, fluorescence recovery after photobleaching (FRAP) experiments were performed.

2.1 Theory

Principle
FRAP can be used to measure the diffusion coefficient of fluorescent macromolecules. The fluorescent molecules in the focal area of the laser beam are bleached, and thereafter, other fluorescent molecules diffuse towards the bleached area, and the fluorescence is recovered. After a certain time, full recovery can occur, but it is also possible that a slight difference remains between the intensity before and after bleaching (figure 2.1). This last phenomenon is attributed to immobile macromolecules that are bleached, and cannot move out of the bleached area, and therefore decrease the final intensity a little. From the intensity recovery profile, the diffusion coefficient can be calculated. [6]

Figure 2.1 FRAP-experiment: The monitoring light beam is focused in the sample (circular spot in left image), and the initial fluorescence $F(i)$ is measured. At $t = 0$ the laser bleaches the molecules in the circular spot (the black dots represent bleached molecules), which causes the fluorescence to decrease to $F(0)$. As time goes on, non-bleached molecules diffuse into the bleached area, and bleached molecules leave this area, which results in a recovery of the fluorescence. After the characteristic diffusion time $\tau_D$, the fluorescence has returned to half its initial value. At the end of the experiment, the fluorescence has recovered to $F(\infty)$, which can be equal to $F(i)$ or less than $F(i)$. The latter is the case in this figure, and it is the consequence of immobile molecules in the bleached area. [6]

Bleaching
The duration of exposure of the sample to the laser is regulated via a computer-controlled shutter between the laser beam and the sample. The geometry of the bleached spot is most often a circular spot with a Gaussian intensity profile, but other geometries exist.
When a confocal microscope is used, the sample can be bleached at different depths without interference from out-of-focus planes, because these are bleached too. In this case, the diffusion is really two-dimensional, in contrast to the diffusion measured with a conventional microscope, which is influenced by diffusion of fluorescent molecules from out-of-focus planes. With a two-photon microscope, the bleached spot is finite in three dimensions and therefore the recovery is due to diffusion from three dimensions in that case. [6]

Detection
To measure the fluorescence intensity, a mercury lamp or a laser is directed at the sample, and images are taken with a camera before bleaching and during the recovery, or a photomultiplier is used to record fluorescence intensity directly.
When a laser is used for detection, the intensity has to be far less than during bleaching in order to prevent further bleaching. However, it is impossible to avoid a little bleaching during the
monitoring, using either a mercury lamp or a laser. Furthermore, the detector should be protected against the high intensity laser beam during bleaching. (figure 2.2)

Figure 2.2 FRAP set-up: The laser is used for bleaching when the shutter is open and when it is closed it can be used for detection. The PC controls this shutter as well as a shutter which protects the camera during bleaching. The camera records images during the fluorescence recovery.

The recovery immediately after bleaching is most important, so in this period, images have to be taken more frequently than when some time has elapsed. The number of images is limited by the response time of the system, which should be an order of magnitude smaller than the characteristic diffusion time of the macromolecules. To be able to measure rapidly diffusing molecules, an Acousto Optical Modulator, which has a typical response time of only a few microseconds, can be used instead of a shutter. [6]

Analysis

Different theories can be used to obtain the diffusion coefficient from the intensity recovery profile. The Axelrod theory [6] assumes that the recovery is a result of pure two-dimensional diffusion from an infinite plane. It is assumed that there is no diffusion into or out of the bleached area during the bleaching, and that there is no flow. The fluorescence recovery curve of a circular bleach geometry with a Gaussian profile, which is normalised by setting the fluorescence intensity before bleaching to one, can be described by

$$f(t) = \sum_{n=0}^{\infty} \frac{-\kappa^n}{n!} \frac{1}{1 + n \left[1 + \frac{2t}{\tau_D}\right]}$$

in which $\tau_D = \frac{\omega^2}{4D}$.

$\kappa$ is the bleach constant, a measurement of the amount of fluorescent molecules that are bleached, $t$ is the time, $\tau_D$ is the characteristic diffusion time, $\omega$ is the half width of the Gaussian intensity profile, $D$ is the diffusion coefficient and $n$ the number of time steps. When the immobile fraction is taken into account, the recovery curve becomes

$$F(t) = F(i)\left[1 - R\left(1 - f(t)\right)\right], \quad \text{with } R = \frac{F(\infty) - F(0)}{F(i) - F(0)}.$$  (22)

In this formula, $F(i)$ is the normalised fluorescence intensity before bleaching, $F(0)$ is the intensity immediately after bleaching and $F(\infty)$ is the intensity after recovery (figure 2.1). $R$ is the mobile fraction of the macromolecules. By fitting the obtained experimental data to this curve, the diffusion coefficient can be calculated.

Fourier analysis of the images is another way to calculate the diffusion coefficient. For this purpose, the diffusion is described by Fick's second law.
with Cartesian coordinates \( x \) and \( y \), time \( t \), bleached macromolecule concentration \( C \), and again diffusion coefficient \( D \) \([11]\). Transformed to the frequency domain, this gives

\[
\frac{d}{dt} C(u,v,t) = -4\pi^2 D \left( u^2 + v^2 \right) C(u,v,t),
\]

with \( u \) and \( v \) the spatial frequencies. The solution of equation (24) is

\[
C(u,v,t) = C(u,v,0) \exp(-4\pi^2 D t (u^2 + v^2)).
\]

Since the intensity of the fluorescent molecules is measured and not the concentration, the point spread function (PSF) of the microscope has to be known. In the frequency domain, this PSF becomes an optical transfer function (OTF), which is constant in time. The relation between intensity and concentration is

\[
l(u,v,t) = C(u,v,t) \text{OTF}(u,v),
\]

so the Fourier transform of the image has the same exponential shape as the concentration distribution. The diffusion coefficient can be obtained by evaluating the ratio of two intensities taken at different times, so

\[
\frac{l(u,v,t)}{l(u,v,0)} = \frac{C(u,v,t)}{C(u,v,0)} = \exp(-4\pi^2 D t (u^2 + v^2)).
\]

can be used to fit the data, and to obtain the diffusion coefficient. From equation (27), it is clear that this can be done without knowing the PSF of the microscope nor the true concentration distribution of the bleached molecules. Again, like in the Axelrod analysis, the immobile fraction can be taken into account. To the right hand side of equation (27), a constant value is added. Then, this immobile fraction can also be calculated from the experimental curve.

2.2 Method
Paragraph 1.1 described the preparation of 3% agarose constructs with chondrocytes, which were also used in our FRAP experiment. After various culture times, a disc was put overnight in a solution of a fluorescent bovine serum protein (BSA, 66 kDa) to allow these proteins to diffuse into the disc. It is assumed that 12 hours was sufficient to obtain a homogeneous distribution of BSA in the disc. BSA was used because its size is comparable to the size of many molecules the cell needs to produce ECM.

The disc was cut into two equal pieces in order to measure the diffusion in the middle plane, which is the cutting plane. A thin glass plate was laid on the cutting plane of one piece, and with this glass on top, it was placed in the microscope. The height of the sample was determined by counting the number of fields of view. FRAP experiments were done at different positions from the edge to the middle, and symmetry was assumed (figure 2.3). The other half of the sample was analysed histologically to visualise the amount of GAGs.
Spatial and temporal evaluation of transport parameters of 3D chondrocyte constructs

Figure 2.3 Disc for FRAP experiment: Positions on the cutting plane of the disc where the FRAP experiments were performed. To select different bleaching spots each experiment, the focus spot was moved along the indicated lines, starting in the middle of this line, and avoiding to come too close to the left or right surface. The distance between two lines was one field of view, and the distance between section 2 and the middle was adapted according to the total height.

At each position, five or six spots on a line perpendicular to the height, were bleached, to ascertain that there were at least three good ones to analyse. The laser bleached the sample for 200 ms, with a power between 200 and 250 mW. One image was taken before bleaching, and 55 images were taken at different times after bleaching (figure 2.4). The time interval between two images in the first part of the acquisition time was smaller than at the end. The total acquisition time was adapted to the recovery observed in the last image, which should be almost complete.

Figure 2.4 FRAP bleaching images: Going from left to right and from top to bottom, these images show how the bleached spot gradually disappears through diffusion of BSA molecules.

In the analysis, the pre-image was subtracted from the images obtained after bleaching, and then, for each pair of five spatial frequencies, this difference was plotted against time. MATLAB was used to fit the resulting curves (figure 2.5), and the diffusion coefficient and immobile fraction were calculated at each pair of spatial frequencies.

Figure 2.5 Experimental and fitted relative intensity: the red circles are the relative intensities of all 55 images at spatial frequency 2.1. The blue line is the fit from MATLAB, which gives the indicated diffusion coefficient (D).

2.3 Results
Figures 2.6 and 2.7 show the change of diffusion coefficient and the immobile fraction in culture time. The overall trend is that the diffusion coefficient increased as a function of culture time at all the positions. The opposite was true for the immobile fraction, which decreased in culture time.
Table 2.1 Values for $D$ with standard deviation (sd).

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<th>D (mm²/s)</th>
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Figure 2.6 FRAP results: the diffusion coefficient versus culture time at different positions in the sample.

Table 2.2 Values for the immobile fraction (%imm) with standard deviation (sd).

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Figure 2.7 FRAP results: the immobile fraction versus culture time at different positions in the sample.
It was thought that the change in these parameters could be attributed to the amount of produced GAGs. Figure 2.8 shows the images from the histological analysis, taken on the centre of the sample. The halos around the cells show that there was indeed ECM production. At day 11, there was little ECM only around the cells, what changed gradually. At day 24, the cells were more clustered and shared their ECM.

Figure 2.8 Histological images of the centre of the sample. The pictures show the chondrocytes in red, the halos around the cells are the produced ECM.

The histological analysis was also performed on other positions through the sample, and these results (figure 2.9) confirmed that there was more ECM production on the edge than further to the centre of the sample.

Figure 2.9 Histological images of the centre (left) and the edge (right) of the sample after 38 days in culture. The pictures show the chondrocytes in red, the halos around the cells are the produced ECM.

2.4 Discussion

The increasing trend in the diffusion coefficient does not support our hypothesis, that an increased amount of GAGs would reduce the diffusion coefficient. This is probably due to the non-homogeneity of the sample, as can be seen in the histological data. To be able to compare the diffusion coefficients after different times of culture, it is necessary to measure in the ECM each time. Before bleaching, the laser spot should be carefully positioned close to a cell. Only in this way, the relation between the amount of GAGs and the diffusion coefficient can be found. We did not consider this before we performed the experiments, and because of the relatively small volume of ECM in comparison with the volume of agarose, our measurements were probably all done outside the ECM.

Another explanation could be that cell death causes large gaps in the agarose network, because the dimensions of the cells are larger than the dimensions of the agarose network. We indeed saw that there were dead cells, when we stained the samples with ethidiumbromide. Because we only came to think about this after we got the results from the samples that were cultured for more than 30 days, we did not count the living and dead cells before. That is why we cannot conclude whether cell death can have had a large impact on the diffusion.

Because little is known about the mesh size of the ECM, BSA might be too small to observe a decrease in the diffusion coefficient. It is possible that larger molecules do have hindrance from the ECM, and that FRAP experiments then reveal a decrease in diffusion coefficient with culture time.
When the agarose and chondrocytes were mixed, the agarose network was formed around the cells. During culture time, cells might have multiplied and produced GAGs at positions at which there was agarose. This might have damaged the agarose network or pushed it away. The consequence of this is not known, but it may have influenced the diffusion.

The immobile fraction decreased with culture time, which was not expected. When more ECM is produced, more BSA molecules can stick to it, and the immobile fraction would be larger. However, there was an increase in diffusion coefficient instead of the expected decrease, and the decrease of the immobile fraction corresponds to that.

We did not observe any changes in thickness of the samples, but to exclude all uncertainties about swelling of the agarose network during culture time, discs without cells should also be placed in the culture medium, and be measured with FRAP. With these measurements as references, the possible influence of a changing agarose network due to storage, can be removed.

During bleaching, it is possible that there was some diffusion of bleached macromolecules out of the bleached area, so the recovery had already started while it was not measured yet. At the same time, some molecules that were outside the bleached area, could diffuse into this area, and be bleached as well. However, because of the very short bleaching time and the low diffusion coefficient of BSA, this error is negligible.

In the analysis, it is assumed that the diffusion was fully 2-dimensional. However, using a conventional microscope for the bleaching, some 3-dimensional diffusion cannot be avoided because of the shape of the bleaching spot.

In the fitting procedure, several pairs of spatial frequencies were used to evaluate the diffusion coefficient. The high frequencies represent positions further away from the centre of the bleached spot, and the intensity recovery is faster than at the low frequencies. On the edge of the bleached spot, the difference with the initial intensity is very small shortly after the bleaching pulse. Consequently, the signal-to-noise ratio is smaller than at the centre of the bleached spot. By inspection of figures containing the experimental data as well as the calculated fits in time, it was decided to omit some frequency pairs from the calculation of the average diffusion coefficient and immobile fraction. A negative immobile fraction, an intensity that is higher than the initial intensity, and experimental points that are too scattered to be sure about the accuracy of the fit, were reasons not to use these data.

Although there were some uncertainties in our experiments, we can conclude from the low value of the diffusion coefficient that diffusion in our 3D constructs is not sufficient to provide all the cells with enough nutrients. Thus, a flow is needed for a sufficient nutrient supply to the cells all through the construct.

2.5 Future research

Because the results did not agree with our hypothesis, future research is needed to accept or reject the above mentioned explanations. First of all, this experiment should be repeated, taking care that the position of the laser spot is close to a cell. After four weeks, the amount of ECM is still low, and therefore, the measurements have to be performed also on discs that have been in culture longer than four weeks.

It would also be good to measure the diffusion coefficient in agarose discs without cells, which have also been stored in medium. When experiments are done with these discs after the same amount of time as our FRAP experiment, the influence of storing the agarose and the influence of the cells and their produced ECM on the agarose can be seen.

One of the possible explanations was that dying cells left gaps, which increased the diffusion. To test whether this can be the reason, the dead and living cells have to be counted at different positions in the sample, after different culture times. In this way, it is possible to determine if cell death at the edge as well as in the centre of the sample can cause this unexpected increase in diffusion coefficient.

The histological analysis did not provide quantitative information about the amount of GAGs. To correlate the diffusion coefficient to the amount of GAGs, a biochemical analysis should be done on each disc immediately after the FRAP experiment to assess this amount precisely.
It is interesting to relate the diffusion coefficient to the size of the macromolecules. From these measurements, some conclusions could be drawn about the pore size of the ECM, and maybe these could be related to the hydraulic permeability.
Conclusion

We performed different experiments to study the change of transport parameters in culture time. As expected, the hydraulic permeability decreased in culture time and the values we found are in reasonable agreement with the literature. In contrary, we did not find a significant change in the aggregate modulus. This can be explained by the large thickness of our 3D constructs. After 4 weeks of culture time, ECM is only produced on the edge. When the sample is compressed, it is mostly the agarose in the centre that is deformed, and the aggregate modulus of agarose does not change significantly in culture time [2]. The height of the constructs did not influence the hydraulic permeability, since all the fluid has to pass through the bottom of the sample. The results on the diffusion coefficient were not in agreement with our hypothesis, but we probably measured in the agarose instead of in the ECM.

Although our experimental results did not always meet our expectations, we can gain knowledge from them that is useful for the development of a bioreactor. The low value of the diffusion coefficient indicates that diffusion alone is not sufficient to supply nutrients to cells all through the 3D construct. To optimise the ECM production, a flow is needed to provide nutrients to the cells in the centre of the construct. The magnitude of this flow depends on the hydraulic permeability. Since it is known now how the hydraulic permeability varies with culture time and strain, the displacement that should be applied to a construct in the bioreactor can be adapted to optimise the flow.

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References


Appendix A Experimental results

Figure A1 Experimental results of the discs without cells

Figure A2 Experimental results of the discs with cells cultured for 7 days

Figure A3 Experimental results of the discs with cells cultured for 14 days