Summary

The main goal of this experimental project is to study the properties of three important biomaterials used for bone scaffold production in tissue engineering: polymer/glass composites, glass ceramics and calcium phosphate cements. The main topic of investigation is the permeability which depicts to what extent fluids are capable to pass through the material. This property is of major importance in tissue engineering applications, more specifically in cell seeding experiments, since it reflects the interconnectivity of the pores and determines the values of parameters, such as seeding velocity, pressure, etc., in order for cells to penetrate and attach to the scaffolds. Furthermore, porosity and compression characteristics are examined.

First, a general introduction sets the context for the project. This introduction describes the state of the art in tissue engineering and some general concepts, such as applications of scaffolds, bioreactors, etc. Secondly, experimental set-up and methods are discussed which were necessary for measurement and analyses of the three types of biomaterials. Cements and ceramics are studied less extensively than composites because fewer samples were available for testing. Finally, the three biomaterials are compared and conclusions are drawn.

The permeability results for composite scaffolds show a remarkable time dependency, i.e. permeability seems to decrease with perfusion time. In order to explain this phenomenon mechanical tests are done, porosity is measured and SEM-images are taken. These results exclude several possible causes, but they are not able to reveal the actual source of the occurring phenomenon. Statistical analysis of the permeability results proves the appearance of large variability between scaffolds made of the same material. Samples with similar porosity may display significantly different permeability behaviour since permeability is susceptible to various parameters. For instance, pore size and especially interconnectivity have a major influence on a material’s permeability. Glass cements show a similar decreasing permeability whereas cements show a different time course. When comparing the three materials on the basis of the examined properties, it is to be concluded that composite scaffolds offer the best prospects for use in cell seeding applications.
Words of Thanks

We would like to thank the following people for their contribution to this project:

- Prof. Vander Sloten and Prof. Planell for giving us the opportunity to make our thesis at UPC in Barcelona.

- Damien Lacroix for providing an interesting subject in the field of tissue engineering, for supporting us when we encountered difficulties throughout the project and for helping us in the final step of the project by reading through our report.

- Martin Koch who arranged all the practical matters necessary for the project and who was always interested and willing to help us out.

- Pablo Sevilla for taking care of all practical matters regarding laboratory work.

- Melba Navarro and Sergio Del Valle for explaining us all about the production of composite, cement and glass scaffolds.

- Prof. José Mª Manero who made the SEM-images for us.

- Hans Van Oosterwyck and Tim Van Cleynenbreughel for their interest in our thesis.

- Paul Verhaert for correcting spelling mistakes.

- Our close friends and family for their support.
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Chapter 1

Preface

1.1 Origin of the Project

This project emerged from the collaboration between Universitat Politècnica de Catalunya (UPC) and Katholieke Universiteit Leuven (KUL), Belgium, in which we have attended and succeeded in the first four years of the educational programme at the Faculty of Engineering, Department of Mechanical Engineering, Division of Biomechanics and Engineering Design (BMGO). As a consequence of our interest in doing research abroad in view of our final year thesis, the coordinator of BMGO, Prof. dr. ir. Jos Vander Sloten contacted Dr. ir. Damien Lacroix at UPC, ETSEIB, Department of Material Science and Metallurgy, Biomechanical Engineering Research Centre. Both agreed we could make a study in the field of tissue engineering, more specifically in the determination of mechanical properties of scaffolds in prospect of cell seeding. The aim was to do experimental research on this topic which is part of the doctorate thesis of Martin Koch, currently examining cell seeding and cell stimulation for tissue engineering in the Biomechanical Engineering Research Centre. At arrival, a more detailed topic and programme was put forward. A permeability measuring device [23] had been developed and was ready for testing and performing experiments on scaffolds. Moreover, it was suggested to make a theoretical study on cell seeding and evaluation of the cell seeding process. However, as time was limited and the cell seeding perfusion chamber was not yet ready for use, this couldn’t be turned into practice. Instead stress was laid on examining mechanical properties such as permeability, porosity and compression of different scaffold materials in order to make a comparison in prospect of the cell seeding process. With the object of carrying out these experiments we learned to make our own composite scaffolds. Other types of scaffolds needed more experience and were made by doctorate students at the laboratory.
1.2 Motivation

As the specialization of our academic training lies in the field of biomechanics, we wanted to do a thesis related to this subject. Our interest went to tissue engineering more specifically because it is a field that combines knowledge from distinct research domains, therefore allowing a broader look onto engineering. Moreover, we preferred to do experimental research which UPC was able to offer us. Finally, the gained experience in a foreign research group and in a blooming branch of science, could open up possibilities for a further career in the field of tissue engineering.
Chapter 2

Introduction to Tissue Engineering

2.1 Definition and General Aspects

Tissue engineering can be considered as a branch of biomedical engineering in which methods and principles of engineering are combined with life sciences for the development of biological substitutes in order to restore, maintain or improve tissue function. These substitutes are generally known as “scaffolds”, in other words small pieces of biomaterials of several kinds. During the last decade, engineers and biologists made several attempts to create appropriate conditions for cell seeding through these biomaterials. Cells should be able to enter the scaffolds and attach to their walls. In order to achieve this, research is necessary both in the field of the biomaterials as in the field of fluid mechanics and cell transport. As a consequence, special devices are developed to create sufficient flow through the scaffolds and to regulate the specific conditions for cell seeding. These bioreactors undertook a revolution in their functioning and they are essential for introducing tissue engineering to the large scale production and market.

2.1.1 The Role of Scaffolds in Tissue Engineering

Scaffolds play a major role in the tissue engineering research for the (re)generation of musculoskeletal tissue, bone and cartilage. Therefore, they should have certain characteristics, making them suitable for cell differentiation and eventually implantation:

I. Three-dimensional and highly porous for cell perfusion, cell growth and transportation of nutrients and metabolic waste throughout the scaffold.

II. Biocompatible and bioresorbable, making sure that the biomaterial is not rejected by the existing tissue and that it degrades and disappears after a certain expected time.

III. Suitable surface chemistry for cell attachment, proliferation, and differentiation.
IV. Mechanical properties to match those of the tissues at the site of implantation. The structure should be given sufficient initial support. In the design of scaffolds, scientists often must make compromises between bioresorbability and mechanical strength.

Once scaffolds are obtained with the previous characteristics, the process of cell-seeding and the production of tissue-engineered grafts can start. First, a biopsy should be taken from the patient’s malfunctioning organ or part of the body. Secondly cells are isolated, expanded and seeded into the scaffold. This construct should then be cultivated until a suitable graft is produced. A bioreactor can provide the specific conditions for this cultivation (this will be further explained in the next section). The characteristics of the scaffold together with the right environmental conditions ensure that cell differentiation can take place inside the scaffold. This can be done in vitro or in vivo, depending on the kind of experiment one is interested in. In vitro experiments are already taking place in several research groups. However, the ultimate aim is to examine the seeded scaffold in vivo, a process which is at this time still at the beginning of its exploration. Once positive results are obtained, surgeons can start using these grafts for implantations. For example in case of degrading organs, parts of the diseased organ such as the liver, kidney, etc can be taken away and be replaced by one’s own tissue. In this way the problem of rejection of foreign tissue is overcome and therefore this would imply a huge step forward in several branches of modern medicine.

2.1.2 The Role of Bioreactors in Tissue Engineering

The general function of a bioreactor is to seed cells into scaffolds and to provide the appropriate conditions for tissue growth. More complex bioreactors are developed for two main reasons. First of all cultivation of the seeded scaffold requires high regulation of specific physiochemical culture parameters in order for the cells to differentiate to the desired tissue. Another important factor are the manufacturing costs which can get high if constant changes have to be made to the set-up. Automatic systems which enable reproducible and controlled changes of environmental factors are the answer to both of the previously stated problems. Bioreactors in fact reduce production costs and can even improve the quality of the engineered tissues resulting in a wider use.

Physiochemical culture parameters

After the cells are seeded into the scaffold, the interaction between cells and the scaffold will enhance cell proliferation and differentiation. What’s more, the initial distribution of the cells inside the scaffold can be highly associated with the distributed tissue within the
graft. In order to stimulate specific tissue production processes (cartilage production, bone mineralization, ...) specific physiochemical environments are required in which several factors should be automatically regulated and monitored:

- Temperature
- Pressure
- pH
- Nutrient supply
- Waste removal
- Mechanical conditioning

Examples of bioreactors

So far, several bioreactors have been developed, aiming more specifically at mass transport (cells, nutrients) throughout the scaffold. This first step is critical as a high seeding efficiency, a uniform cell distribution and a constant nutrients supply are the main factors in order to obtain usable tissue. In the following examples, changes to design and functioning are made in this prospect.

- **Static seeding bioreactors**: scaffolds are held stationary in a flask filled with cell medium. This method results in a low seeding efficiency and non-uniform cell distribution. Isolated cell aggregates are produced.

- **Spinner flask bioreactors (dynamic seeding)**: scaffolds are held in a flask where a stirring stick is causing cell transport into the scaffold by convection. What’s more, cells and nutrients are more homogeneously mixed, leaving no high density layer behind on the scaffold. Cells spread more even through the 3D-construct.

- **Direct perfusion bioreactors**: fluid flow of the cell suspension allows direct transport of the cells through the pores of the scaffold. Not only external pores are affected but the internal pores can also be reached. In addition, the flow makes sure that variation in medium concentration are reduced. All this results in an enhanced mass transfer, a more uniform cell distribution and ultimately in improved tissue growth and metabolism.

- **Other bioreactors**: rotating-wall vessels, hollow-fiber bioreactors and bioreactors with controlled mechanical forces, in which cell seeding is obtained by a state of free fall, indirect perfusion, application of a mechanical load, respectively.
Future expectations: closed-system bioreactors

The previous discussed devices are still complex in their handling as well as time consuming. One of the major goals in tissue-engineering is translating the research-scale production methods into large scale applications which are reproducible and economically acceptable. In order to meet the costs and to commercialize tissue engineered products, automation is inevitable. As discussed in section 1.1.1, all the steps in the process of obtaining a suitable graft for transplantation should in the future be included in one closed-system bioreactor. These steps are explained in figure 2.1:

![Figure 2.1: Closed system bioreactor. Figure generated by M. Moretti [17]](image)

a) **Biopsy**: small part of the affected organ

b) **Storage of reagents**: scaffold, culture medium and medium supplements

c) **Isolation of cells**

d) **Expansion of cells**

e) **Cell-seeding**

f) **Cultivation of construct**: allowing the cells to differentiate to the desired graft

g) **Monitor culture parameters and tissue development** (cell number, metabolism, mechanical properties, ...)

h) **Introduction of the patient’s clinical data**
i) Control culture parameters: inputs from previous step can be fed back to the system

j) Planning of the implantation

2.2 Previous Studies on Scaffold Permeability and Cell Seeding

2.2.1 Types of Cells

Several kinds of cells can be taken from the patient’s soft and hard tissue:

- **Mesenchymal stem cells or marrow stromal cells (MSCs):** These stem cells have the ability to differentiate into osteoblasts, chondrocytes, myocytes, adipocytes, neuronal cells, ... The main available source for MSCs is bone marrow, although alternative sources are being searched for. The MSCs are a huge source for tissue engineering applications and for the regeneration of mesenchymal tissues—bone, cartilage, ligaments, tendon, adipose, ...—more specifically. Several seeding methods have been examined using MSCs [24].

- **Osteoblasts:** These cells produce the protein osteoid which becomes the matrix of bone. They also facilitate mineral deposition by secreting specific enzymes. Osteoblasts have already been frequently used for scaffold seeding [2]. Osteoblasts can be obtained through differentiation of MSCs under the influence of certain growth factors. Another option is to isolate osteoblasts from bone tissue obtained by biopsy. Special kits are available for these ends.

- **Chondrocytes:** These cells form the smallest units of cartilage tissue. Like osteoblasts, mature chondrocytes originate out of differentiating MSCs under the presence of specific growth factors. Another possibility is to isolate the chondrocytes from cartilage tissue. Seeding of chondrocytes has been previously studied [4].

- **Myocytes or muscle fibers:** A myocyte is a single cell of a muscle. Once again, they are obtained by MSC differentiation or cell isolation from muscle tissue. Cardiac tissue engineering is a field where seeding of myocytes has been extensively studied [25].

- ...
2.2.2 Types of Scaffolds

Polymer-based scaffold materials

Numerous polymers are nowadays available for tissue engineering applications. One of the main requirements for a biomaterial to qualify for implantation is the necessity to be bioresorbable. Polymers score very well in this field. Some natural polymers, such as collagen and chitin, show these characteristics. Other polymers are designed especially for the purpose, like polylactides (PLLA, PLDL, PDLA,...) and poly(α-hydroxyesters). Some are already approved, others are still under investigation. For example researchers are currently investigating the possibility of some polymers to guide differentiation and proliferation of cells inside the scaffolds. In order to obtain even better materials foreign materials can be added to the polymer matrix: hydroxyapatite, tricalciumphosphate, salts, glass, .... In this way, degradation of the scaffolds can be regulated. A composite material would also improve biocompatibility, strength, porosity and hard tissue integration.

Several fabrication technologies have been applied to produce high porous 3D-scaffolds which also meet up to the previous expectations:

- **Fiber bonding (unwoven meshes):** A processing method that involves knitting and physical bonding of fibers prefabricated by spinning polymer solutions. During this process polymer fibers are converted into nonwoven, moulded pulp and other materials. Additives and resins are added to the polymer dispersions or water based polymer solutions for thickening, wetting, ...

- **Solvent casting/particulate leaching:** solvent processing depends on the polymers solubility in various organic solvents (acids, chloroform) and on the solvent volatility. A polymer solution is made and particulates are added. Salt is the most commonly used particulate because it is easily available and very easy to handle. Gelatin particles are another candidate for this method because they are known as a material that enhances cell attachment and proliferation. After casting into moulds, the solvent evaporates and the particulates are leached out by water. In this way pores are created.

- **Membrane lamination:** a polymer solution is made by solvent casting and used to make porous sheets which are subsequently laminated to 3D-structures. Chloroform is used to attach the interfaces for the lamination process. In this way scaffolds can be produced with a multiple layer design offering bi- or multiple tissue interfaces. The lamination process can also be done on metal wires [16].
**Melt moulding:** melt processing involves heating the polymer above the glass transition temperature \(T_g\) or the melting temperature \(T_m\) and depends on melt viscosity.

**Others:** injection moulding and extrusion, compression moulding, freeze drying are more specialized techniques of solvent/melt moulding. Other examples are the aggregation of polymer microparticles, foam/gel formation, . . .

As previously discussed techniques have pros and cons, the ideal scaffold and matrix material has not yet been designed. The further development and optimisation of these range of methodologies will allow to obtain scaffolds with a wide range of porous structures and properties (such as density, morphology, mechanical properties and degradation kinetics), and therefore suitable for different applications in tissue engineering. Furthermore, new and more complex processing methods will be developed, such as rapid prototyping technologies (e.g. Frequency Division Multiplexing (FDM) and 3D plotting processes) and other non-conventional processing techniques, such as bi-material injection moulding. FDM allows to create scaffolds that mimic the microstructure of living tissue. With the bi-material injection moulding process, it is possible to design implants from a combination of materials and/or structures, in order to optimize its performance for specific applications.

**Calcium phosphate cement implants**

Calcium Phosphate Cement (CaP) implants are particularly useful in bone and dental surgery because of their excellent biocompatibility. The cement originally consists of CaP powder, available in a large range of particle sizes. The CaP powder can contain a mixture of phosphates, such as tetracalciumphosphate (TTCP) or dicalcium phosphate anhydrous (DCPA). When water is added to the powder the cement starts to harden. This process consists of dissolution and precipitation (crystal formation) which is a result of hydrolyses of the active reactant. The general formula for hydrolyses of tricalciumphosphate (TCP) into hydroxyapatite crystals is:

\[
(4 - x)Ca_{4-x}(PO_4)_2 \rightarrow Ca_{10-x}(HPO_4)_2(PO_4)_{6-x}(OH)_{2-x}
\]

When in the above equation “x” equals zero hydroxyapatite crystals precipitate. If on the other hand a value larger than zero is assigned to “x” calcium deficient hydroxyapatite is formed. After adding water, the paste is put into moulds and into Ringer’s solution at 37 °C for a given time. It takes 20-30 minutes for the cement to fully harden. The setting time is the time needed for all TCP to precipitate into hydroxyapatite and generally amounts to 2-3 days. Recent research has been proven that reduction of particle size decreases the setting time and accelerates the hardening of the cement causing the crystallite
size of the final product to be reduced. In this way, scientists are able to develop calcium phosphate materials with tailored structures at micro and nanoscale levels. What’s more, the reduction of particle size does not affect the strength of the final product [7]. The obtained mechanical strength is sufficient enough for replacing hard tissue. Another advantage is that it shows slow resorption so that enough time is available for bone tissue to grow. The final product obtained in this process is precipitated hydroxyapatite, which is reactive and similar to biological apatites.

Ceramic scaffolds

Ceramic scaffolds, such as glass ceramic scaffolds or calcium phosphate ceramics, are produced by a sintering process of the corresponding powder. They have the advantage that they possess an intrinsic strength to sustain physiological loads. On the other hand, they are brittle and strength decreases drastically with increasing porosity. Therefore, new technologies are being developed to overcome this problem. The rate of degradation of the scaffold must parallel the rate of bone growth throughout the interconnecting pores of the ceramic material in order to maintain the required mechanical properties of the construct. To this purpose, the chemical and phase composition of the ceramics has been adjusted in such a way that it should be possible to control the release of specific ions from the scaffold into the surrounding tissue domain [15].

2.2.3 Cell Seeding and Cultivation Conditions

In order to obtain a certain structure and function of an engineered tissue, physical stimuli should be applied during cell seeding. Three kinds of physical stimuli are distinguished: biochemical, hydrodynamic and mechanical stimuli.

Biochemical stimuli

Studies have been made to see the effect of various values of medium oxygen tension, $pO_2$, and pH on tissue development. These factors have a major influence on cell metabolism (aerobic or anaerobic), construct thickness and the production of certain substances such as glucose and glycosaminoglycans (GAG) [6].

Hydrodynamic stimuli

Hydrodynamic factors, such as mixing pattern and flow regime, have strong impacts on the cell seeding and cell cultivation. The most frequently used bioreactors, which show these different patterns, have already been described in section 2.1.2 on page 13. In general, perfusion bioreactors are considered preferable to spinner flask and rotating
Studies have proven that cell perfusion leads to constructs with higher cell content (DNA) and matrix synthesis for cartilage and bone tissue engineering. However, this perfusion modulation depends on the amplitude and duration of perfusion [4, 2]. One should keep in mind though, that optimal hydrodynamic conditions for cell seeding depend on cell type [3].

**Mechanical stimuli**

During cultivation, mechanical forces normally present in vivo, should be simulated to improve tissue development. Both shear stress and hydrostatic stress have an influence on differentiation of cells. Moreover, the history of stress is important. Carter and co-workers developed this idea theoretically and applied it to study a variety of skeletal differentiation phenomena. Their results are shown in figure 2.2. For example, applying hydrostatic compression and low shear stress to cartilaginous constructs enhances the process of cartilage tissue formation. In fact, the combined benefits of continuous perfusion and mechanical stimulation has been proven [19]. The continuous perfusion simulates flow that occurs in the bone matrix when stresses are applied during every day activities. This flow is causing small shear stresses on the osteocytes’ cell membrane. For example, osteocytes produce high levels of nitric oxide (NO) in response to small fluid shear stresses. NO production plays a major role in preventing apoptosis of osteocytes. Osteocytes that are not stimulated by fluid flow die and are cleared out by osteoclasts.

![Figure 2.2: Tissue differentiation](image-url)
2.2.4 Qualitative and Quantitative Evaluation of Cell Seeding

Once cell seeding has been done, it is interesting to examine the quality of the seeding. Three parameters are crucial to examine and compare different seeding methods: cell seeding efficiency, cell viability and cell uniformity.

Cell seeding efficiency

An important measure of quality and quantity of the seeding is the seeding efficiency. This parameter is defined as the percentage of initially seeded cells that attach to the scaffold. In order to achieve a number for this parameter, several cell proliferation methods have been developed: DNA assay methods and cell counting methods.

In DNA assay methods, attempts are being made to determine the amount of DNA that is present in the seeded scaffold. An example of a DNA assay method which has been successfully applied [29], is the CyQUANT® cell proliferation method [13]. This method makes use of the fluorescent CyQUANT® dye, which after absorption of radiation at one particular wavelength, almost instantaneously re-emits radiation at another, longer wavelength. The cells are digested by the dye mixture, offering the fluorescent substance the possibility to attach to the nuclear acids. When bound to DNA the dye reveals its fluorescent properties. The excitation and emission wavelengths are provided by the producing company. The measured fluorescence depends linearly on cell number in a certain range of cell number—between 50 and 50 000 cells—when a specific amount of dye is added. By increasing the dye concentration used in the assay, the linear range can be extended to 250,000 cells. This linearity is shown in figure 2.3. Thus, by measuring the fluorescence of the stained sample with a microplate reader using a light source and the appropriate excitation and emission filters, the amount of cells can be derived from figure 2.3. However, this linear course has to be constructed experimentally, using a time consuming cell counting method. It is possible to overcome this difficulty if the amount of total cells is known in advance. In that case the fluorescence of the original cell suspension can serve as a 100% reference to which the fluorescence of the seeded cells can be compared. of course, this is only valid when situated in the linear region as shown in figure 2.3.

Another group of methods to determine the cell seeding efficiency are the cell counting methods. A special device, such as a hemacytometer or a counting chamber, is used to count the number of cells available in solution. This counting step should be done twice: before and after seeding. The difference between the two equals the amount of seeded cells. However, cells that remain attached anywhere else in the bioreactor are included in the number of seeded cells whereas they should not be. Hence cell counting methods in general are less accurate than the DNA assay methods. Moreover, they are rather
time consuming. On the other hand, it should be said that the DNA assay kits are very expensive and require careful handling.

**Cell viability**

Like cell proliferation tests, cell viability techniques are also based on the fluorescence phenomenon and use similar methods as described above. Specific dye substances have been developed that cleave either to viable or dead cells. For viable cells, these dyes are sensitive to certain cell activity. For example, dyes such as Calcein AM (LIVE/DEAD® Viability/Cytotoxicity kit [14]) or tetrazolium salt (Roche’s WST-1 test) are sensitive to intracellular esterase activity and mitochondrial dehydrogenases, respectively. The rate of dye cleavage correlates with the number of viable cells in the culture. Dead cells, on the other hand, have damaged cell membranes, allowing certain dyes to attach to intracellular features. To give an example, ethidium homodimer-1 (LIVE/DEAD® Viability/Cytotoxicity kit) is able to bond to nuclear acids [12]. These viability kits are always provided with a practical and concise product information sheets and protocols. Again these kits tend to be expensive.

**Cell uniformity**

In order to examine cell uniformity throughout the scaffold, several cross sections should be examined and cell number calculated. This can be done once again by using a cell proliferation method (see section 2.2.4) for each cross section or on the other hand by using advanced image analyzing programs [29]. Once the cell number for each section
is known, the mean ($\bar{x}$) and standard deviation value ($s$) for the whole scaffold can be calculated. Zhong et al. [31] defines the % uniformity as follows:

$$\% \text{ uniformity} = 100 \cdot \left(1 - \left(\frac{s}{\bar{x}}\right)^2\right)$$
Chapter 3

Experimental Set-Up and Methods

3.1 Scaffolds: Types and Preparation Methods

Each of the scaffolds described below are cylinder-shaped with the same rough dimensions, namely a diameter of 6 mm and a length of 12 mm. Small variations may occur and are due to the production procedure and the quality of the moulds.

3.1.1 Polymer/Glass Composites

Composites have the main advantage that they can combine useful characteristics of two completely different materials. Polymer scaffolds are tough on the one hand, but on the other hand they display large deformations when exposed to small forces. Conversely glass ceramic scaffolds resist higher forces, but are very brittle. Regarding tissue engineering applications, an ideal material combines the toughness of the polymers with the strength of the glass ceramics, in other words a polymer-glass composite. There are several kinds of polymers suitable for tissue engineering and each of them can be found in combination with other materials using different distributions of the two phases. In our study we worked with the poly(L-lactide-co-DL-lactide 95/5) copolymer (PLDL), and with a polymer/glass distribution of 50/50.

The protocol for making the scaffolds is very straightforward but it takes several days until the end product is finished. The first step is to dissolve the polymer in a chloroform solution. After mixing for two days on the orbital shaker a viscous solution is obtained and glass and salt can be added. The amount of salt will provide the porosity whereas the size of the salt particles determines the pore size. When a homogeneous mixture is obtained, the teflon moulds can be filled. Teflon moulds are preferable since they are chemically inert to other materials, such as the used composite, and therefore removal out of the moulds minimally damages the material. While filling it is important to avoid air bubbles because they bring about holes in the end product and have a disastrous influence
on the final characteristics of the scaffolds, such as porosity, permeability, etc. The filled moulds should then be left to dry for 24 hrs. Another cause of unwanted holes inside the scaffolds is the evaporation of the chloroform during drying. However, this phenomenon can not be avoided since it is a direct result of the production procedure. As a consequence it is important to keep these side effects in mind while analysing the properties of the scaffolds, which is discussed in chapter 4. Figure 3.1 shows some examples of these holes in two distinct scaffolds; these pictures were taken immediately after removal from the moulds, thus before washing. When all the chloroform is evaporated the samples can be easily pushed out of the moulds. Finally, the samples are washed in water so as to remove the salt and to create the macropores inside. The washing takes two days and the water should be refreshed every 3 to 4 hours, depending on the amount of samples in comparison with the amount of water. Drying of the wet porous samples brings forth a batch of composite scaffolds ready to be used for characterisation or seeding.

![Figure 3.1: Holes in (unwashed) composite scaffolds](image)

As previously mentioned not all samples are adequate for further testing since they contain holes inside resulting in different properties. And although visual inspection eliminates the majority of these low quality scaffolds, it is inevitable that some remain undetected. To conclude it is necessary to state that notwithstanding the fact that the procedure is rather simple, it is impossible to produce a batch of samples with exactly similar characteristics and therefore there will always exist a certain variability in the results.
3.1.2 Calcium Phosphate Cements

Porous calcium phosphate cements constitute another important class of scaffolds. Compared to polymers they are far more brittle but in general they can bear more load. Numerous distinct types of cements can be made altering the porosity, chemical composition, additives, . . . .

The process of production is different from that of the composites in the sense that to make porous cements a certain kind of skill and experience is required. Firstly, a paste has to be made, in our case a mixture of solid $\alpha$-TCP (tricalciumphosphate) powder and a liquid phase (water with some additives in order to speed up the precipitation reaction). The amount of solid as opposed to liquid phase determines the viscosity of the paste, and thus will influence the setting time. To create porous cements this paste then has to be mixed carefully with whisked egg white. In view of creating a homogeneous porosity it is important that the egg white is mixed thoroughly; yet, one should be careful not to destroy the properties of the whisked egg white during mixing. What’s more, the time between mixing the paste and completing the filling of the moulds should not add up to more than 3 minutes. The filling of the moulds also should be done keeping in mind the preservation of the whisked egg white. That is to say, prudence is called for not to destroy the pores while pressing the paste inside the moulds. After filling, the moulds should be kept in Ringer’s solution at 37 °C—imitating physiological conditions—during one week. In this way the calciumphosphate has sufficient time to precipitate into calcium deficient hydroxapatite: $3Ca_3(PO_4)_2 \rightarrow Ca_9(HPO_4)(PO_4)_5 OH$.

The main difference between the production procedure of cements and composites is that the porosity of the cements is provided by the whisked egg white and thus depends on the carefuleness, experience and speed of the one who is making it. The porosity of composites on the other hand is provided by the amount of salt and thus is far more stable. The same goes for the pore size, which is more verifiable in the case of the composites. The cements however, result out of a precipitation reaction rather than out of an evaporation reaction. Therefore the samples do not contain holes, as occurs with the composite samples.

3.1.3 Glass Ceramics

Similarly to calcium phosphate cements, porosity in glass ceramics is obtained by the properties of whisked egg white. The first step is to mix the egg white with glass powder until a homogeneous mixture is achieved. Then, the mixture is beaten up at low velocity during 3 minutes. The teflon moulds should be filled quickly since the foam decays after a certain amount of time. In order to facilitate the samples’ removal, the moulds are treated
in advance with a mould release agent. When all the moulds are filled they can be put in the (preheated) oven at 80 °C for 2 hours. Afterwards, they should cool down so as to succeed in removing the samples from the moulds. The final step in producing ceramics is to sinter the samples. The specimens are placed in the oven at room temperature and subsequently the oven temperature is raised up to 400 °C in 2 hours. Next a rise in temperature of 1 °C/min is established until a temperature of 540 °C is reached and this temperature is maintained during 3 hours. Finally, the samples are cooled down at 2 °C/min.

As with the cements, no straightforward procedure exists to control in an adequate manner the porosity nor the pore size of the glass ceramics. The egg white cannot be beaten up completely because this results in very fragile samples that break from the moment one touches them. Three minutes seem to be the golden mean; yet, this period can vary slightly depending on the person who prepares the scaffolds. Another rather negative side effect of the procedure is the impossibility to control the scaffolds’ diameter precisely for they are not contained in moulds during the sintering process. However, with a bit of practice one can change the size of the original teflon moulds so that the desired final diameter is acquired.

### 3.2 Permeability: Permeameter

Even though permeability is of major importance considering cell seeding, few experimental research has been done to determine the permeability of scaffolds. Permeability is measured using a device, developed at UPC (ETSEIB), that clamps the scaffold between two rubber seals, forcing the water to flow through—consequently not round—the scaffold. The pressure difference—the hydrostatic pressure due to the water column above the sample—between the two ends of the scaffolds drives the water through the sample according to Darcy’s Law:

\[
Q = -\frac{k \cdot A \cdot \Delta p}{\eta \cdot L_s}
\]

in which \( Q = \) flow rate, \( k = \) permeability coefficient, \( A = \) cross section of the scaffold, \( \Delta p = \) pressure difference, \( \eta = \) viscosity of the fluid and \( L_s = \) sample length.

Measuring the flow rate under a constant pressure, i.e. keeping the height of the water column on the same level, allows to calculate the permeability, because all the other parameters in Darcy’s law remain the same during measurements. Flow rate is acquired by weighing the amount of water that runs through the scaffold during a particular time interval.

Initial measurements immediately showed several problems that had to be dealt with. Firstly, because of the brittleness of the cements the insertion between the seals involves in
most of the cases the destruction of the sample. A solution to this problem is to produce
teflon rings in which the cement paste can be injected. In this way, the force needed to
to insert the samples through the seals, is applied on the teflon rings rather than on the
samples themselves. A second problem concerning the cement scaffolds is caused by their
relatively low permeability: they require a high pressure difference to initiate fluid flow.
This means the pressure of the water column is not sufficient and extra pressure should be
applied by inserting a piston inside the column. The piston is pushed down by means of a
cover that can be screwed on top of the permeameter. Finally, even though permeability
is a material characteristic—and therefore should be the same at any given time—the
results of the measurements on composite scaffolds showed a certain time dependency.
These problems and their solutions will be discussed more thoroughly in the following
chapters. Fig 3.2 shows the permeameter without piston and cover.

![Figure 3.2: Permeameter](image)

### 3.3 Porosity

Porosity is a quantity that specifies the relative amount of pores against the amount of
surrounding matrix as shown in the following equation [9].

\[ P = 1 - \frac{\rho_{ap}}{\rho} \]
in which $\rho = \text{density of matrix material}$ and $\rho_{\text{ap}} = \text{apparent density of the porous scaffold}$. In contrast to permeability a lot of research already has been done on porosity and the different measuring methods. As a consequence less problems are expected with these experiments than with the permeability determination.

$\rho$ is a known variable for the different types of scaffolds. $\rho_{\text{ap}}$, on the other hand has to be determined out of the scaffold’s weight and its volume. To measure the volume of the scaffold precisely a specific method is developed using mercury. Mercury has the feature to be very cohesive and consequently does not enter the pores when a porous sample is immersed in it. The Archimedes principle says that every object immersed in a fluid experiences an upward directed force that equals the weight of the displaced fluid. This force cannot be measured directly; however, its reaction force (which is equal in magnitude) can be measured if the mercury is placed on a scale. Now, the volume of the scaffold can be determined since it equals the volume of displaced mercury. Figure 3.3 shows the complete set-up for the porosity measurement. Although this method is very straightforward and provides adequate results, working with mercury requires a few measures of caution. Scaffolds contaminated with mercury can not be used for further investigation and should be stored in a distinct bottle. Consequently, the porosity of a batch of samples has to be determined statistically, using a small population to represent the entire batch.

![Figure 3.3: Set-up porosity measurement](image)

### 3.4 Mechanical Properties: Set-Up

To obtain the most important mechanical properties with respect to cell seeding, several compression tests can be done. Only prewetted samples were used and were tested in a water bath ($T = 37 \, ^{\circ}\text{C}$) to establish physiological conditions. The maintenance of the temperature was achieved with a thermostat—which served as a pump as well—in a different tank. Adamel DY34 Mechanical Test Equipment was used with a load cell of 100 N. Figure 3.4 shows the complete set-up for the compression tests.
First of all it is interesting to apply a constant force on the scaffold and to examine how it deforms with time. However, for practical realization some approximations should be made since the equipment on hand does not allow the application of a constant force. Therefore the scaffold was compressed with a speed of 1 mm/min until a force of 1 N was reached. Due to relaxation the force decreases when the displacement is kept constant during ten seconds. After ten seconds the displacement was elevated until the same force of 1 N was reached once again, and so on. In this way a plateau is approximated by a saw-toothed curve as shown in figure 3.5.
A second experiment involves compressing the samples at a constant rate. Three different compression rates were studied: 1 mm/min; 0.5 mm/min; and 0.1 mm/min. The experiment continued until 3 mm compression was established and subsequently the scaffolds were released with the same velocity. As soon as a gap between scaffold and support became visible, the experiment was ended.

Finally a set of scaffolds was compressed until fracture; that is to say, the scaffolds were compressed at a rate of 1 mm/sec until a compression of 7 mm was reached. This compression is supposed to result in the fracture of the samples. As in the former experiment the compression was released with the same speed (1 mm/sec) until a gap became visible between scaffold and point of support.

3.5 Scanning Electron Microscopy (SEM): Set-Up

In scanning electron microscopy (SEM) an electron beam scans across a sample’s surface. When the electrons strike the sample, a variety of signals are generated, and it is the detection of these specific signals which produces an image. The advantages over conventional optical microscopy include an increased magnification and an increased depth of focus.

In this study only SEM-pictures of composite scaffolds were taken. These scaffolds require a preliminary treatment in order to be suitable for SEM-imaging. First of all it is important that the surface one wants to study is even. Since tearing apart does not produce an even surface and cutting with a knife damages the structure an other method has to be used. The samples are frozen in liquid nitrogen and subsequently broken. A second issue is that the polymer/glass composites are nonconducting. To overcome this problem the samples are coated with a tin layer of gold. Finally samples are mounted on a copper plate, again to maximize conduction.

3.6 Cell Seeding Conditions: Set-Up

For this cell seeding experiment, a perfusion chamber has been designed by the doctorate student, Martin Koch at ETSEIB, UPC, Barcelona. As stated in previous sections, perfusion is preferred to static seeding because it stimulates cell metabolism in a constructive way and therefore influences positively the bone matrix production. The results of cell seeding studies should be compared to results of other investigations such as μCT-imaging and simulation by finite element analysis. It is preferred not to influence the microstructure of the scaffolds and to use scaffolds with similar dimensional properties. The perfusion chamber consists of a hollow cylinder which can be opened to insert the
scaffold. The scaffold is blocked inside the system and perfused with cell medium.

A first possible cell seeding set-up is shown in figure 3.6. The cell medium is contained inside a flask which is kept in a water bath with a temperature of 37°C to mimic physiological conditions. For the same reason, the perfusion chamber is put on top of a heating plate which is kept at $T = 37°C$. A peristaltic pump circulates the cell medium through silicone tubes from the medium flask into the perfusion chamber and back to the medium flask, hence creating a closed system. The main advantage of this type of set-up is that the devices can be placed close to each other so that the length of the tubes is restricted. The longer the tubes, the larger the surface where the cells can attach to resulting in a lower cell seeding efficiency. On the other hand, it is preferable to keep the entire system at the same temperature as this better corresponds to the physiological conditions in the human body. In the previous set-up this is not the case. To overcome this problem, another type of set-up could be established as shown in figure 3.7. The flask containing the cell medium and the perfusion chamber are placed inside an incubator which is maintained at $T = 37°C$, a 5% $CO_2$ atmosphere and a relative humidity of 95%. Nevertheless, the pump must be placed outside of the incubator which results in longer tubing and a lower cell seeding efficiency. What’s more, a pulsatile bidirectional sinus-like flow is desired as this is an approximation of the fluid flow which occurs in the extracellular matrix during the walking-cycle or other load bearing conditions to which bone is exposed to. The frequency of this pulse should be between 1-20 Hz [28] as this is physiologically observed. However, because the seeding velocity is limited, it is impossible for the cell medium to complete one cycle at this frequency so that no new cells can be brought to the perfusion chamber. A possible solution is to apply a unidirectional pulsatile flow which allows a continuous

Figure 3.6: cell seeding set-up in open space
flow and all cell medium to pass through the scaffold.

Figure 3.7: cell seeding set-up including an incubator

3.7 Statistical Analysing Methods

In the following chapter, statistical methods are required to analyze the obtained data from the permeability and porosity measurements. First of all, the data should be tested for outliers: values that can be considered out of range compared to the other data. Outliers might influence strongly the observed mean and standard deviation:

\[ \text{mean: } \bar{x} = \frac{\sum x_i}{n} \]
\[ \text{standard deviation: } s = \sqrt{\frac{\sum (\bar{x} - x_i)^2}{(n - 1)}} \]

in which \( x_i \) = a single data point and \( n \) = number of data.

Furthermore, it may be interesting to compare means and standard deviations of distinct populations in order to examine whether these populations are significantly different or not. The following sections go more deeply into statistical methods that are used in this study to detect outliers and compare measured values.

3.7.1 Outlier Detection

Several outlier detection methods exist. However, two methods were chosen since they are widely used and provide a different approach to the problem.
Modified z-score test

A first method for labelling outliers is the z-score test [10]. An outlier is defined as a value that is at least 3 standard deviations above or below the mean. The modified z-score proves to be more reliable since the parameters used to calculate this score are minimally affected by the outliers. In this test the z-score is determined based on outlier resistant estimators. Median and median of absolute deviation about the median (MAD) are examples of such estimators (median instead of mean and MAD instead of standard deviation). The modified z-score is calculated for the supposed outliers using the following formula:

\[
z_i = 0.6745 \cdot \frac{x_i - x_m}{MAD}
\]

in which \(x_i\) = observed result of the supposed outlier, \(z_i\) = modified z-score of the supposed outlier, \(x_m\) = median of the observed data, and \(MAD = median\{|x_i - x_m|\}\).

The constant 0.6745 is needed because \(E(MAD) = 0.6745 \cdot s\) for large \(n\) (\(s = \text{standard deviation}, n = \text{number of data}\)) [30]. The test states that an observation with a modified z-score bigger than three and a half should be labeled as an outlier.

Hypothesis methods: Grubbs’ test

Other outlier detecting methods are based on hypothesis testing. The null hypothesis states that there are no outliers present in the observed data. These methods compute a value based on the measurement one wishes to examine as an outlier. This value is then compared to a critical value depending on the amount of data and the desired confidence interval. For instance, the mostly used confidence interval is the 95% interval. If the computed value is larger than the critical value, the null hypothesis is rejected. This means that there is a 95% possibility that this value is an outlier among the observed data. Grubbs’ test [8] is recommended by the EPA as a statistical test for outliers. Only the highest and the lowest data point can be tested. First, the t-statistic value for the largest/lowest value can be computed using the following formula:

\[
t = \frac{\bar{x} - x_n}{s}
\]

in which \(\bar{x}\) = mean of the observed data, \(x_n\) = highest/lowest value suspect, and \(s = \text{standard deviation}\).

Secondly, the obtained t-statistic value should be compared to Grubbs’ critical value, which depends on the amount of measurements and the significance level (see appendix C). The null hypothesis can be rejected if the t-statistic value is larger than Grubbs’ critical value. If other suspicious data are to be tested the previous outlier should be removed from the data set and mean and standard deviation should be recomputed.
3.7.2 Evaluation of Significant Difference Between Populations

Like Grubb’ test, these methods are hypothesis methods which try to express, with a certain percentage of certainty, whether populations (with their own mean and standard deviation) are significantly different or not. Students’ t-test [26] is used to compare two populations whereas the analysis of variance-test (ANOVA) [5] is a more extended version of the Student’s t-test in order to compare means of more than two populations.

Student’s t-test

First, the variance of difference between the two means ($s_d$) is calculated:

$$s_d = \sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}} \tag{3.3}$$

in which $s_i =$ standard deviation for population $i$ and $n_i =$ number of data in population $i$.

Secondly, the t-value is computed:

$$t = \frac{|\bar{x}_1 - \bar{x}_2|}{s_d} \tag{3.4}$$

in which $\bar{x}_i =$ mean for population $i$.

The critical tabular t-value (see appendix E) depends on the % significance level and the degrees of freedom ($n_1 + n_2 - 2$).

ANOVA-test

In the ANOVA-test the following general null hypothesis is tested:

$$H_0 : \mu_i = \mu_{i+1} = \mu_{i+2} = \ldots$$

in which $\mu_i =$ population $i$.

In order to reject or to accept the null hypothesis an F-statistic should be calculated. The F-statistic compares the variation among population means to the variations among the data in the same population. The Anova-test is a reliable test as long as the highest standard deviation does not exceed twice the lowest standard deviation. First the mean square of differences among groups (MSG) is calculated:

$$MSG = \frac{SSG}{I - 1} \tag{3.5}$$
$$SSG = \sum n_i(\bar{x}_i - \bar{x})^2 \tag{3.6}$$

in which $n_i =$ number of samples in population $i$, $\bar{x}_i =$ the mean of population $i$, $\bar{x} =$ the mean of all data, $I =$ number of populations, and $I - 1 = \nu_1 =$ degrees of freedom of nominator in F-statistic.
Secondly, a value for the denominator has to be obtained which is the mean square of differences within groups (MSE):

\[
MSE = \frac{SSE}{N - I}
\]

\[
SSE = \sum (n_i - 1)s_i^2
\]

in which \(n_i\) = number of data in population \(i\), \(s_i\) = standard deviation of population \(i\), 
\(N\) = total data size, \(I\) = number of populations, and \(N - I = \nu_2\) = degrees of freedom of denominator in F-statistic. 

Finally the F-statistic can be calculated:

\[
F = \frac{MSG}{MSE}
\]

The F-distribution generally has a shape as shown in figure 3.8. The upper critical value of the F-distribution depends on the % significance interval and the degrees of freedom and can be derived from figure 3.8 and from tables (see appendix D). The null hypothesis is rejected when the test statistic is bigger than the tabled value.

![Figure 3.8: F-distribution](image)

Figure 3.8: F-distribution [20]
Chapter 4

Composite Scaffolds: Results and Analysis

4.1 Permeability Results

Composite scaffolds are by far the most suitable types of scaffolds to use with the permeameter. Their flexibility allows them to be inserted without being damaged. The first results, however, revealed a certain decreasing tendency of the permeability with perfusion time. Figure 4.1 shows this for one sample at a pressure $p = 0.025$ bar. All samples showed this decrease in permeability and to find its cause is one of the main challenges of
this project. A first possibility is that the pressure causes a compression of the scaffolds and consequently of the pores, allowing less water to go through. In this case the permeability can be directly linked to the mechanical properties of the composite scaffold and a correlation might be found between permeability and strain. Secondly it is possible that the water causes a structural change inside the material influencing the permeability. And last but not least it is possible that the found tendency is not a consequence of a change of the sample but rather a direct consequence of shortcomings of the permeameter. These different possible causes will be further discussed in detail in the following parts.

4.1.1 Variability between Scaffolds and Batches

Due to the steep decrease at the beginning of the permeability curve it is important to determine the permeability of different samples at exactly the same point in time, i.e. the measurement should be taken at a fixed period of time after filling the water column. If not, any variability in the results will be due to the time dependency, rather than due to differences in sample properties.

As already stated it is utopian to produce scaffolds with identical characteristics. As a result, the permeability results will always vary from one another. What’s more, the amount of holes inside the composite samples due to the evaporation of the chloroform, and their size and location will have its influence on permeability as well. In other words, variability is inevitable. Yet, it is interesting to compare the variability between different scaffolds of the same batch, to the variability between distinct batches.

Several batches have been produced to perform permeability tests. As discussed in section 3.1.1 on page 23, the process of making composite scaffolds requires practice in order to avoid big holes. The first four batches were a result of this learning process and were used to get accustomed to the permeameter and to get a first idea of the permeability of the self-made PLDL/glass scaffolds. A more consistent study has been made on batch 5, 6 and 7, which externally showed good characteristics: a desired length between 10 mm and 12 mm and a lower amount of big holes. This section focuses on variability between scaffolds and batches and how statistics are used to draw conclusions from permeability results.

Variability between scaffolds

In order to compare several scaffolds from the same batch, permeability measurements should be taken at the same point in time and in the same working conditions. More precisely, a pressure of 0.025 bar was applied and the permeability was measured after 20 seconds. Only six scaffolds of batch 5 were examined. The reason for this low amount is the fact that no more scaffolds were available since a considerable amount of scaffolds
of batch 5 were used to study the time dependency and to determine porosity. Batch 6 and 7 contained more scaffolds so that 16 scaffolds were tested per batch. The first thing to do when analysing these results is to look for outliers. Most methods for detecting outliers presuppose normal distributed data. In this case, the assumption of normality is allowed since measurements were taken in exactly the same circumstances.

Looking at the permeability results of batch 6, as shown in figure 4.2, sample 3, 7 and 11 seem to be inconsistent with the remainder of the data set and hence could be possible outliers. As shown in table 4.1 the modified z-score (see section 3.7.1) for these three values exceeds the critical value 3.5. Consequently they can be considered outliers. This test is also performed on the most extreme values of batch 5 and 7 (see appendix A), but none of these values proved to be outliers (see table 4.1); conversely, they are legitimate data that on no condition can be excluded from the data set.

Moreover, a hypothesis method is applied to further examine the possible outliers. Table 4.1 shows the results of Grubbs’ test (see section 3.7.1) on the suspicious samples of batch 5, 6 and 7. Statistically, only samples 3, 7 and 11 of batch 6 can be labelled as outliers when considering a 5% significance interval. In other words, Grubbs’ test provides the same outcome as the modified z-score test. Nonetheless, to determine an explanation for obtained outliers before their exclusion proves to be necessary. If an explanation cannot be found, then the labelled value should be treated as an extreme but valid measurement. In fact, the diameter of sample 11 measured only 5.4 mm at one end. Consequently the scaffold might not be properly sealed allowing water to flow along the scaffold. As for the two other statistical outliers, no such cause could be found, hence these samples are considered extreme though legitimate.
Once these outliers are labelled, the mean and the standard deviation can be computed for batch 5, 6 and 7. In general the rejection of outliers will have an influence on the mean and standard deviation of the population. As stated before, in batch 6 only sample 11 can be justified an outlier. For the sake of completeness, table 4.2 shows the permeability results for 4 cases: all outliers included, 1 outlier excluded (sample 11), two outliers excluded (samples 11 & 3) and three outliers excluded (samples 11, 3 & 7). These results are also displayed in figure 4.3. However, the results obtained with two and three sample rejections will not be further examined, since they cannot be justified properly. Figure 4.3 points out that batch 6 has a considerably lower standard deviation compared to the other two batches.

## Variability between batches

Furthermore, it is interesting to compare the several batches in order to conclude whether they are significantly different or not regarding permeability. As all the relevant data is already available from table 4.2, the ANOVA-test can be applied for this purpose (see...
Figure 4.3: Permeability results of batch 5, 6, & 7 at t = 20 s and p = 0.025 bar

section 3.7.2). The following null hypothesis should be tested:

\[ H_0: \mu_5 = \mu_6 \]
\[ H_0: \mu_5 = \mu_7 \]
\[ H_0: \mu_6 = \mu_7 \]

in which \( \mu_5 \), \( \mu_6 \) and \( \mu_7 \) are the means of batch 5, 6 and 7, respectively. As stated in the previous section, the rejection of the first outlier of batch 6 is well-reasoned. Therefore, it is interesting to perform the ANOVA-test twice—with and without outlier—in order to compare the influence of one outlier’s rejection. The Anova-test is a reliable test as long as the highest standard deviation does not exceed twice the lowest standard deviation. Table 4.2 proves that this occurs in neither of the two examined cases. Table 4.3 collects the test’s outcome.

<table>
<thead>
<tr>
<th></th>
<th>( \bar{x}[m^2] )</th>
<th>MSG([m^4])</th>
<th>MSE([m^4])</th>
<th>( F[-] )</th>
<th>( \nu_1 )</th>
<th>( \nu_2 )</th>
<th>( F_{crit.5%} )</th>
<th>( F_{crit.1%} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>outlier incl.</td>
<td>3.85 ( \cdot 10^{-11} )</td>
<td>2.38 ( \cdot 10^{-21} )</td>
<td>7.26 ( \cdot 10^{-22} )</td>
<td>3.28</td>
<td>2</td>
<td>35</td>
<td>3.267</td>
<td>5.268</td>
</tr>
<tr>
<td>outlier excl.</td>
<td>3.68 ( \cdot 10^{-11} )</td>
<td>2.87 ( \cdot 10^{-21} )</td>
<td>7.34 ( \cdot 10^{-22} )</td>
<td>3.91</td>
<td>2</td>
<td>34</td>
<td>3.276</td>
<td>5.289</td>
</tr>
</tbody>
</table>

Table 4.3: ANOVA-test for comparing batches: with and without sample 11 for batch 6

The ANOVA-test can only be used to determine whether the batches are significantly different or not. As seen in table 4.3, the F-value for a 1% interval drops below the critical value. In this case the only conclusion that can be drawn is rather weak: it is impossible to state with 99% certainty that the batches are significantly different. Nevertheless, in this study the standard 5% significance interval is used which implies that the F-value exceeds
the critical value. This allows the ANOVA-test to carry out a strong statement. That is, the null-hypothesis can be rejected and the batches can be considered significantly (95%) different from each other. At first, this conclusion may sound strange since the process of making the scaffolds was repeated in exactly the same way for every batch. On the other hand, this process is subject to the producer’s skills and to the evaporation of the chloroform. The latter leaves behind holes that may differ in amount, size and position which can not be predicted nor controlled at the time the scaffolds are produced. In order to solve these problems, research should be done on the automation of the composite producing methods.

Furthermore, table 4.3 shows what can be expected when comparing the F-values for the two cases (outlier included or excluded). When the outlier is included, the F-value is lower than when the outlier is rejected from the data. This conclusion is predictable as the standard deviation for batch 6 rises when including all data. Consequently, the variation among groups decreases as the permeability values of batch 6 drift further towards the results of the other batches.

4.1.2 Time Dependency

As mentioned earlier one of the most striking findings when analysing the permeability results is the decreasing permeability coefficient over time. A first possible cause that comes to mind is the deformation of the scaffold due to the pressure. This deformation causes a decrease in porosity and hence a lower permeability. The smaller length of the scaffold after measuring seems to confirm this theory. Yet, this smaller length can be caused by the method of insertion or removing rather than by the pressure. After all a simple insertion and removal seems to cause a similar shortening. What’s more, if the decrease in permeability is due to compression during perfusion, then the drop would be larger at a larger pressure. Figure 4.4 shows a comparison of an experiment in which the first measurement is at $p = 0.025$ bar followed by a measurement on the same scaffold at $p = 0.010$ bar, with an experiment in which the measurements are done twice at the same pressure $p = 0.025$ bar. These figures show a rather similar behaviour whether the second measurement is at high pressure ($0.025$ bar) or at low pressure ($0.010$ bar), indicating that the compression of the sample is not responsible for the decreasing permeability. The graphics in figure 4.4 reveal another interesting feature: it seems that the values of the second measurement start where the ones of the first measurement end. This happens even if there is one day (17-20 hrs) in between the two measurements. After one weekend (65-70 hrs) however, the permeability recovers to its initial value and a similar time dependency as the first measurement is recorded. Figure 4.5 clarifies this remarkable recovery of permeability. Although similar experiments of this kind (see appendix B for
more graphs) did not prove the permeability’s recovery to the same extent as the one shown in figure 4.5, they all showed a higher permeability after 65 hours than after 20 hours.

Figure 4.4: Time dependency for distinct (left) and equal (right) pressure differences

Figure 4.5: Recovery of permeability after one weekend (65-70 hrs)

A second possible explanation is that the structure of the matrix changes during perfusion. For instance the glass particles could come loose and block several pores. Although the glass particles in theory ($\leq 80\mu m$) are too small to block the macropores ($80$-$120 \mu m$), they can block various micropores and hence reduce the permeability. Besides loosening glass particles, it is possible that water inside the material causes the polymer
fibers to swell. That is why it is important to study the effect of prewetting the samples before measuring. Since the PLA is very hydrophobic, extra efforts have to be made to prewet the samples. In figure 4.6 the sample is prewetted during 160 hours on an orbital shaker, forcing the water to enter the pores. The figure clearly shows the same drop in permeability, which suggests that the wet state of the sample is not the cause of the decreasing permeability. Another way to verify if something changes in the structure of

![Permeability vs Time](image)

**Figure 4.6: Time dependency of prewetted sample (160 hrs, p = 0.025 bar)**

the matrix is to look at the samples under the electron microscope. Several SEM-pictures have been taken, but none showed clear evidence of broken loose glass particles that are blocking micropores. What’s more, there weren’t any differences notable between pictures from unperfused or perfused samples. Figure 4.7 shows some SEM-images of perfused and unperfused samples; the glass particles are circled.

Besides compression of the sample or changes in the matrix’s structure, it may be possible that the decreasing permeability has its origin in the method of measuring rather than in changes of the structure or changes in length. After all, the insertion and removal of the scaffolds goes along with deformation of the scaffolds. Yet, this does not explain the falling of the permeability during perfusion. For what it is worth, it would only explain a difference between the measured value and the actual value, not the obtained time dependency. The hypothesis that the permeameter is inadequate and causes a decreasing fluid flow, cannot hold since no possible mechanism inside the device can be found that interrupts the flow through the samples.

Finally it is important to add that in a significant amount of experiments, the second
measurement on a sample proved to show a higher permeability than the first measurement. This goes against the former mentioned phenomenon that the values of the second measurements begin where the ones of the first end (see page 42). Still, in these cases the first measurement always started on low pressure, i.e. 0,010 bar. Consequently it is likely that a kind of threshold pressure is required for the material to allow flow through all its pores. In fact, the composite material is very hydrophobic and therefore water will not enter the pores without a stimulus. Water cannot flow spontaneously into pores of hydrophobic materials because the water/solid surface free energy is larger than the gas/solid interfacial free energy. When applying pressure, water intrudes into the pores because the work done on the system provides for the excess free energy required for replacement of the gas/solid interface by the water/solid interface. The equation that links pore diameter to the threshold value for the pressure is

$$p = -4 \cdot \frac{\gamma \cdot \cos\theta}{D}$$

in which $\gamma$ = surface tension and $\theta$ = contact angle.

From this equation it is obvious that smaller pores require a higher pressure in order to allow water flow through.

### 4.1.3 Pressure Dependency

Samples of batches 5, 6 and 7 have been tested for two successive pressures: 0,025 bar and 0,010 bar. The first pressure corresponds to a fully filled permeameter while the second pressure corresponds to a lower water level which can be recognized due to a mark.
Table 4.4: Pressure dependency on permeability (mean ($\bar{x}$) and standard deviation ($s$)):
p = 0,01 bar succeeds p = 0,025 bar

<table>
<thead>
<tr>
<th>Batch</th>
<th># Samples</th>
<th>$\bar{x}[m^2]$</th>
<th>$s[m^2]$</th>
<th>$\bar{x}[m^2]$</th>
<th>$s[m^2]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>6</td>
<td>6,02·10^{-11}</td>
<td>3,06·10^{-11}</td>
<td>6,82·10^{-11}</td>
<td>3,79·10^{-11}</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>3,09·10^{-11}</td>
<td>1,76·10^{-11}</td>
<td>2,92·10^{-11}</td>
<td>1,83·10^{-11}</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>1,92·10^{-11}</td>
<td>3,06·10^{-11}</td>
<td>1,69·10^{-11}</td>
<td>2,55·10^{-11}</td>
</tr>
</tbody>
</table>

Table 4.5: Student’s t-test for comparing p = 0,025 and p = 0,01

<table>
<thead>
<tr>
<th>Batch</th>
<th>$s_d[m^2]$</th>
<th>$t[-]$</th>
<th>degrees of freedom</th>
<th>$t_{crit,5%}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1,99·10^{-11}</td>
<td>0,399</td>
<td>10</td>
<td>2,23</td>
</tr>
<tr>
<td>6</td>
<td>1,27·10^{-11}</td>
<td>0,134</td>
<td>6</td>
<td>2,45</td>
</tr>
<tr>
<td>7</td>
<td>1,78·10^{-11}</td>
<td>0,131</td>
<td>8</td>
<td>2,31</td>
</tr>
</tbody>
</table>

Furthermore, the Student’s t-test can be applied to determine whether the results for different pressures are significantly different from each other. The Student’s t-test is a statistical test that compares the means of two populations (see section 3.7.2). In this
case, one population consists of samples tested at $p = 0.025$ bar and the other consists of the same samples tested at $p = 0.010$ bar. This method is applied for the three batches and a 5% significance interval. Table 4.5 depicts the result. As obtained t-values do not exceed the critical t-values at the 5% significance level, the two groups are not significantly different.

<table>
<thead>
<tr>
<th>Batch</th>
<th># samples</th>
<th>p = 0.025 bar</th>
<th>p = 0.01 bar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\bar{x}[m^2]$</td>
<td>$s[m^2]$</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>1.69·10^{-11}</td>
<td>1.54·10^{-11}</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>2.28·10^{-11}</td>
<td>1.79·10^{-11}</td>
</tr>
</tbody>
</table>

Table 4.6: Pressure dependency on permeability (mean ($\bar{x}$) and standard deviation ($s$)): $p = 0.025$ bar succeeds $p = 0.01$ bar

<table>
<thead>
<tr>
<th>Batch</th>
<th>$s_d[m^2]$</th>
<th>$t[-]$</th>
<th>degrees of freedom</th>
<th>$t_{crit,5%}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1.46·10^{-11}</td>
<td>0.359</td>
<td>6</td>
<td>2.45</td>
</tr>
<tr>
<td>7</td>
<td>8.90·10^{-12}</td>
<td>0.542</td>
<td>10</td>
<td>2.23</td>
</tr>
</tbody>
</table>

Table 4.7: Student’s t-test for comparing $p = 0.01$ and $p = 0.025$

The same test has been done examining the effect of exchanging the time order of the two pressures. First, the permeability was measured at $t = 20$ s and $p = 0.01$ bar. At $t = 3$ min, the water level was increased until $p = 0.025$ bar. The second measurement was made at $t = 5$ min. At this point, only samples from batch 6 and 7 were available. The permeability results are collected in table 4.6. Again, no value should be attached to the absolute results in the this table. Analogously, the Student’s t-test is applied for these results and the outcome is collected in table 4.7. As before, these t-values do not exceed the critical t-value so that no prove can be found that when applying a higher pressure, the permeability changes significantly compared to the low pressure. In conclusion, it is justified to state that the pressure difference does not have a significant influence on the permeability and thus it seems that the manifested time dependency discussed in section 4.1.2 is not due to compression of the scaffold. However, this will be investigated more thoroughly in section 4.3.

### 4.1.4 Usage of Metal\Teflon Rings

The idea for introducing cylindrical rings in which the scaffolds are moulded, is to avoid damaging which can occur while applying force when scaffolds are put inside the permeameter. The use of metal or teflon rings can overcome this problem because in this case force is applied on the cylinders rather than on the composites themselves during
insertion. Moreover, they are produced with an exact diameter of 6 mm and a length of 12 mm which allows tight sealing of the scaffolds. However, the use of these rings introduces other problems. Considering metal rings, the PLA does not adhere well to the metal so that the scaffolds formed inside the ring disconnect and fall out. Consequently, leaks occur. As for the teflon rings, the adherence was much stronger. This may look strange since teflon is a chemically very stable and not likely to bond to foreign materials. Yet, the drill holes inside teflon rings have a poorer surface quality compared to the ones inside metal rings. Thus the adherence is due to physical forces rather than chemical ones. Even though scaffolds in teflon cylinders don’t tend to come loose during perfusion, they do have other disadvantageous. For one thing, it is impossible to determine visually the scaffold’s quality. Remembering that a batch contains a considerable amount of scaffolds with holes inside, this visual inspection tends to be important. Figure 4.8 shows the time course of a scaffold covered with a teflon ring and the same tendency as without rings is recorded. Considering the negative side effects discussed above and the fact that cylinders don’t alter time dependency, the decision was made to continue the experiments without the usage of rings.

![Figure 4.8: Time dependency of scaffold in teflon ring (p = 0.010 bar)](image-url)
4.2 Porosity Results

Porosity measurement does not demand as high scaffold quality as permeability measurement does. As a consequence also former batches could be tested and the results can be compared statistically in order to determine whether a significant difference in porosity exists between different batches.

4.2.1 Variability between Batches

Table 4.8 and figure 4.9 reveal remarkable lower porosity results for batch 3 and 4 as compared to the other batches. Not only the means are lower, they involve larger standard deviations as well. Yet, the same protocol was used to make them. Statistical methods (modified z-score test & Grubbs’ test as explained in sections 3.7.1) did not reveal any outliers that influenced the means of batch 3 and 4. Thus it is allowed to assume that

<table>
<thead>
<tr>
<th>Batch</th>
<th>#Samples</th>
<th>Mean ([m^2])</th>
<th>Standard Deviation ([m^2])</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>0.929</td>
<td>0.005</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>0.928</td>
<td>0.005</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>0.789</td>
<td>0.018</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>0.823</td>
<td>0.014</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>0.921</td>
<td>0.005</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>0.924</td>
<td>0.004</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>0.920</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Table 4.8: Porosity results of batches 1 to 7

![Figure 4.9: Porosity results for 7 different batches](image-url)
these values represent reality. Theoretically the porosity of the composite scaffolds can be estimated as follows:

\[
m_{\text{glass}} = 6 \text{ g} \\
m_{PLDL} = 6 \text{ g} \\
m_{\text{salt}} = 94 \text{ g} \\
P = \frac{94}{94 + 6 + 6} = 0.887
\]

The evaporation of the chloroform, however, has as a consequence that extra porosity is created and therefore a porosity higher than 90% can be expected. That is why the porosity values of batch 3 and 4 are regarded as unusual and therefore they will be subject of further investigation (see section 4.2.2).

The ANOVA-method is used to compare the values of batches 1, 2, 5, 6 and 7. Including batches 3 and 4 is not allowed since their standard deviations are larger than two times the smallest standard deviation (0.014 > 2 · 0.004). Yet, no statistical test is required to realise that the values of these two batches are significantly different than the other ones. Using formulas (3.5) and (3.8) the F-statistic can be calculated as provided in section 3.7.2 on page 34:

\[
F = \frac{MSG}{MSE} = \frac{8,812 \cdot 10^{-5}}{2,304 \cdot 10^{-5}} = 3.824
\]

The critical F-value for \( \nu_1 = 4 \) and \( \nu_2 = 23 \) and a 5% significance level is 2.796 and hence the null hypothesis can be rejected since F is bigger than the critical value. In other words, a significant difference exists between the porosity of distinct batches. This may look surprising because the means are situated very close to each other: the highest and lowest porosity are 92.0% and 92.9% respectively. An explanation for the test outcome can be found in the very low standard deviations that accompany these means. So even though the maximum difference in porosity found between two batches is only 0.9% this difference has to be considered as significant and consequently really existing.

However, most experiments don’t require very accurate porosity information. That is to say, in practice it is generally sufficient to know that the scaffolds have a porosity for example between 92% and 93%. In other words, even though there is a difference in porosity between batches, this difference does not alter the results of most practical cases and hence is inconsiderable. Note that batch 3 and 4 do show a rather large difference compared to other batches, one that is not acceptable in further experiments. For this reason they will be further discussed in the following section.
4.2.2 SEM-Images of Batch 3

SEM-images of batch 3 are taken in order to learn more about this batch’s unusual porosity. Figure 4.10 compares the difference in macroporosity between batch 3 and 6. A difference in porosity is clearly visible. The image of batch 6 shows the expected picture—various right-angled macropores—whereas the picture of batch 3 displays far less macropores. The form of the pores is typical for the particulate leaching method (see section 2.2.2 on page 16) in which the pores adopt the form of the salt particles.

![SEM-images of batch 3 and 6](image)

(a) Batch 3  (b) Batch 6

Figure 4.10: Comparison of macroporosity between batch 3 & 6

The reason for the lower porosity of batch 3 and 4 can only be found in the possibility of mistakes made during the weighing of the salt. SEM-images with larger magnification did not show evidence of other substances—for instance salt particles that remained captured—that might influence the weight of the samples and consequently the porosity results. To conclude it seems plausible that the lower porosity results of batch 3 and batch 4 are due to a human error during production rather than due to inherent variance in scaffold properties.

4.3 Mechanical Properties

4.3.1 Compression Tests

The results of the compression tests confirm the viscoelasticity of the polymer/glass composites. The first test proves that the displacement clearly varies over time while the force is kept constant, a classical example of creep in viscoelastic materials. Figure 4.11 shows the experiment for three different composite scaffolds. The fact that they all have a different initial displacement has to do with the variability between composite scaffolds;
however, leaving aside this different starting point, the three samples show very similar behaviour.

Figure 4.11: Strain versus time under influence of a constant force (1 N)

<table>
<thead>
<tr>
<th>Compression Rate [mm/min]</th>
<th># Samples</th>
<th>Young’s modulus [N/m²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>463318</td>
</tr>
<tr>
<td>0.5</td>
<td>3</td>
<td>453886</td>
</tr>
<tr>
<td>0.1</td>
<td>3</td>
<td>333636</td>
</tr>
</tbody>
</table>

Table 4.9: Mean values of Young’s modulus for different compression rates

The second mechanical experiment involves a compression of the scaffold with a constant speed. Three different speeds were investigated, each on three different samples. Figure 4.12 shows a compression until a displacement $s = 3$ mm is reached at $v = 1$ mm/min; $v = 0.5$ mm/min and $v = 0.1$ mm/min. The viscoelastic effect is clearly visible in the sense that not one of the samples returns to its original size. Figure 4.12 (D) proves that with an even lower final compression ($s = 1$ mm) complete recovery of the scaffold is not established. Another important fact is that the slope of the curve decreases as the compression rate decreases. Although the difference between $v = 1$ mm/min and $v = 0.5$ mm/min is not significant, the curve with $v = 0.1$ mm/min clearly displays a lower slope. Viscoelastic behaviour is determined by the storage (elastic) modulus and the loss (viscous) modulus, $G'$ and $G''$ respectively. However, dynamic testing is required in order to calculate these parameters since they depend on the phase angle between stress
and strain. To form an idea of the material’s stiffness, Young’s modulus is calculated for the three different compression rates (see table 4.9). These values are derived from the averaged stress-strain curves shown in appendix F.

![Stress-strain curves](image)

Figure 4.12: Stress-strain curves: (A) $v = 1 \text{ mm/min}$, $s = 3\text{mm}$; (B) $v = 0.5 \text{ mm/min}$, $s = 3\text{mm}$; (C) $v = 0.1 \text{ mm/min}$, $s = 3\text{mm}$ and (D) $v = 0.1 \text{ mm/min}$, $s = 1\text{mm}$

The last experiment consists of a compression until fracture of the sample. Three different scaffolds were compared for this experiment and the compression took place at a rate of $1 \text{ mm/min}$ until a final displacement of $7 \text{ mm}$ was established. In two out of three samples this displacement resulted in the fracture of the scaffold whereas one scaffold showed no signs of fracture during the experiment. In figure 4.13 the fracture of the scaffold can be seen both in the compression part and the release part as an irregularity in the monotonic course of the compression/release respectively. The breaking stress for the two samples results in the values $\sigma_1 = 353678 \text{ N/m}^2$ and $\sigma_2 = 389045 \text{ N/m}^2$. 
4.3.2 Relation to Permeability Results

Section 4.1.2 discussed the permeability’s time dependency and its possible causes. One of these conceivable causes is very much related to the compression experiments, namely the compression of the scaffold due to the driving water pressure. In this case the pores are compressed allowing less water to go through. If this really constitutes the main cause of the decreasing permeability, then a relation should be found between the compression of the scaffold and the permeability. During perfusion, the scaffolds are exposed to a maximum pressure of \( p = 0.025 \text{ bar} \). For a scaffold diameter of 6 mm this pressure corresponds with a force \( F = 0.07 \text{ N} \) on the upper cross section of the scaffold. Since this small force couldn’t be applied properly using the mechanical test equipment on hand, a direct correlation between compression and permeability is impossible. Yet, it is interesting to study the possible influence of a force application of 1 N on the porosity and permeability of the scaffold.

First and foremost, the influence of the global compression on the average (macro)pore size can be calculated using a simplified model for the scaffold inside structure. The pores are considered to be homogeneously spread and an average pore size of 100 \( \mu \text{m} \) is assumed; micropores are not included in the model. In figure 4.11 the largest deformation, caused by applying a constant force of 1 N, is 1.4 mm (sample 3). In terms of percentage this gives us a compression of 11.7\%. Assuming this deformation is equally distributed over all the pores inside the scaffold (this in fact is a simplified representation), the reduction
in volume of one pore can be calculated, and hence the reduction in pore diameter.

\[ V_{po} = \frac{4}{3} \cdot \pi \cdot \left( \frac{d^3}{2} \right) : \text{average pore volume} \]

\[ \Delta V_{po} = 0.117 \cdot V_{po} : \text{change in pore volume due to compression} \]

\[ \Rightarrow V_{po}^{new} = V_{po} - \Delta V_{po} : \text{the changed average pore volume} \quad (4.2) \]

\[ \Rightarrow d_{po}^{new} = 2 \cdot \sqrt[3]{\frac{V_{po}^{new}}{4 \cdot \pi}} : \text{the changed average pore diameter} \quad (4.3) \]

These equations result in a smaller average pore diameter: \( d_{po}^{new} = 96 \mu m \). Thus the application of a constant force of 1 N during a certain amount of time reflects itself in a reduction of the pore diameter of 4 \( \mu m \). Nevertheless, the question remains: Is this smaller average pore diameter the cause of a significant decrease in scaffold permeability?

To link compression results with permeability results a formula is required that couples pore diameter to pressure and flow rate. Since the inner structure as well as the interconnectivity of the pores are inherently complex, fluid flow through these interconnected pore-channels is not fully understood. Nonetheless, this study only aims to examine whether compression is the cause of the decreasing permeability during perfusion. To reveal all secrets regarding fluid flow inside complex porous structures would be not feasible. Therefore some simplifications are permitted: the interconnected pores-channels are modelled by simple cylindrical channels and the effect of a reduction in diameter is studied. The low fluid velocity allows to assume a laminar flow inside the channels and hence Poiseuille’s equation [1] can be used to link diameter with flow rate.

\[ Q = \frac{\pi \cdot g}{128 \cdot \nu} \cdot \left( \frac{-\Delta^2 h}{L} \right) \cdot d^4 \quad (4.4) \]

\[ h = \frac{p}{\rho \cdot g} + z \quad (4.5) \]

in which \( g \) = constant of gravity, \( h \) = piezometric height, \( L \) = channel length, \( d \) = channel diameter, \( \nu \) = kinematic viscosity, \( p \) = pressure and \( z \) = local height.

Calculated for one pore channel with a diameter \( d = 100 \mu m \), equation (4.4) gives a flow rate \( Q = 5.83 \cdot 10^{-10} m^3/s \). A reduction of the diameter of 4 \( \mu m \) on the other hand provides a flow rate \( Q = 5.57 \cdot 10^{-10} m^3/s \). In other words, the smaller pore channel diameter results in a reduction in flow rate of 4.5%. The influence on the permeability can be easily calculated rewriting Darcy’s law (equation (3.1)).

\[ k = \frac{Q \cdot L_s \cdot \eta}{A_s \cdot \Delta p} \quad (4.6) \]

Because of the linear relationship between permeability and flow rate (provided that the pressure difference remains the same), the permeability will decrease 4.5% due to the compression. This means for instance that a scaffold with a permeability of 3.00 \( \cdot 10^{-11} \)
subject to a force of 1 N (i.e. a pressure of 0.35 bar) will see its permeability drop as far as $2.87 \times 10^{-11}$ m$^2$. Compared to the decreasing permeability in figures 4.1, 4.4, 4.5 and 4.6 the calculated drop is far smaller even though calculations are made for a bigger compression force than during perfusion.

As a conclusion it seems very unlikely that the falling permeability is caused by the compression of the scaffold due to the water pressure. Although the model used to estimate the influence of compression on permeability is not acceptable to predict precisely the fluid flow inside the scaffolds, it does prove that a water pressure of 0.025 bar is not sufficient to lower the permeability significantly. Furthermore, the fact that the permeability curves did not show important differences at lower pressures (0.010 bar) confirms this conclusion.

### 4.4 Cell Seeding

As stated in section 3.6, the cell seeding experiments are performed by Martin Koch within the scope of his doctorate thesis. Several factors can have an influence on the cell attachment and cell seeding efficiency. Foremost, the scaffold material-cell interaction is a crucial issue since adhesion is the first condition which should be complied with. Secondly, cell attachment also depends on porosity, pore size and interconnectivity of the pores. A high porosity implies a large amount of pores making sure the cells have a larger contact surface for attachment to the pores’ walls. The macropores of the used composites scaffolds in this experiment have a diameter which lies between 90 and 120 µm. Osteoblasts have a diameter of 30 µm approximately. Hence, the pore size is large enough for the cells to pass through. Larger pore sizes come along with larger permeabilities, but they reduce the resistance that osteoblasts experience during perfusion and thus reduce cell attachment. The rate of interconnectivity determines the permeability of the scaffold. As concluded in previous section, a large variety in permeability exists due to the fabrication process of the composite scaffolds. Whilst preparing the scaffolds, air bubbles and the evaporation of the chloroform can cause cavities. These cavities reduce the contact surface available for cell attachment and therefore have to be avoided at all times. Other influential factors on cell attachment are the fluid flow velocity and the contents of the cell medium. How these parameters influence cell seeding is part of further research.
Chapter 5

Glass Ceramics and Cements: Results and Comparison

This chapter discusses the most important characteristics of calcium phosphate cements and glass ceramic scaffolds. Since far fewer samples were available compared to the composite scaffolds, an extensive study of these characteristics was not possible. Nonetheless, basic permeability and porosity measurements on glass and cement scaffolds provide useful information with a view to comparing the three investigated scaffold materials. It was considered more useful to apply the few available scaffolds for these ends instead of examining their stress-strain relationships.

5.1 Cements

5.1.1 Permeability Results

As briefly mentioned in section 3.2 permeability measurement on cements goes along with several difficulties that have to be overcome. First of all, cements are brittle compared to polymer/glass composites and this causes problems during insertion in the permeameter. In order not to damage the samples they can be produced inside similar cylinders as discussed in section 4.1.4. Moreover, cements are more suitable to be produced inside these rings since their production procedure does not entail an evaporation reaction and thus visual inspection in order to exclude samples with holes is less important. Only teflon cylinders were used as metal does not sustain the HCl-solution in which the rings are cleaned. A second issue includes the relatively low permeability of the cement scaffolds. A high pressure is required to bring about water flow through the samples. And even though the permeameter is equipped with a piston and a cover to apply such high pressures, precise measurements call for at least two persons: one to keep the pressure constant and another one to determine fluid flow.
Due to a fracture of the screw thread the permeameter became unusable and the reparation of the device will not be finished during the course of this project. This is why only three samples could be taken into account to determine the batch’s mean and standard deviation. Table 5.1 shows the results of the measurements on three scaffolds enclosed in teflon cylinders. The permeability was measured at a pressure $p = 1\text{ bar}$ for one scaffold and $p = 1,6\text{ bar}$ for the other two scaffolds. The reason for this difference is that the two last scaffolds did not show flow at $p = 1\text{ bar}$, hence a rise in pressure was established until flow occurred.

To investigate the existence of a similar time dependency in the permeability of cement scaffolds, measurements on different moments in time were done at a constant pressure. Two types of scaffolds of the same batch were used: one enclosed in a teflon ring and one without ring. Figure 5.1 reveals remarkable results for the scaffold with teflon ring as it is the first sample that does not display a significant decrease in permeability. Section 5.3 will examine these results more thoroughly.

![Graphs showing time dependency of permeability](image)

(a) with teflon ring ($p = 1\text{ bar}$)  
(b) without ring ($p = 0.5\text{ bar}$)

Figure 5.1: Time dependency of the permeability of cements

### 5.1.2 Porosity Results

The mean and standard deviation of a population of 4 samples is displayed in table 5.2. As with the composite scaffolds porosity values display far less variation than permeability results.

<table>
<thead>
<tr>
<th># Samples</th>
<th>Mean [$m^2$]</th>
<th>Standard Deviation [$m^2$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>$8.36 \cdot 10^{-14}$</td>
<td>$5.66 \cdot 10^{-14}$</td>
</tr>
</tbody>
</table>

Table 5.1: Calcium phosphate cements: permeability results $t = 40$ s
Table 5.2: Calcium phosphate cements: porosity results

<table>
<thead>
<tr>
<th># Samples</th>
<th>Mean $[m^2]$</th>
<th>Standard Deviation $[m^2]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.786</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Table 5.3: Glass ceramics: permeability results at $p = 0.025$ bar and $t = 20$ s

<table>
<thead>
<tr>
<th># Samples</th>
<th>Mean $[m^2]$</th>
<th>Standard Deviation $[m^2]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>$2.59 \times 10^{-10}$</td>
<td>$0.363 \times 10^{-10}$</td>
</tr>
</tbody>
</table>

First, variability among scaffolds from one batch was studied. As stated before, only 5 samples from one batch were available to examine permeability. The scaffolds were successively examined at $p = 0.025$ bar and $t = 20$ s. The mean and standard deviation for the glass samples are collected in table 5.3. Note that even though few samples were

Figure 5.2: Time dependency of glass ceramics ($p = 0.025$ bar)
used, the obtained results show a remarkably small standard deviation.

Secondly, it is interesting to examine whether the glass samples show the same time dependency as the composite scaffolds. Therefore, several measurements in time were made on one sample in order to see if this phenomenon is occurring. Indeed, on figure 5.2 a decreasing time course can be seen.

### 5.2.2 Porosity Results

The porosity results are displayed in table 5.4. No more than three scaffolds were used to acquire these values; yet, the standard deviation shows little variation.

<table>
<thead>
<tr>
<th># Samples</th>
<th>Mean $[m^2]$</th>
<th>Standard Deviation $[m^2]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.812</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Table 5.4: Glass ceramics: porosity results

### 5.3 Comparative Study of the Used Scaffold Materials

#### 5.3.1 Porosity

Figure 5.3 compares the porosities of the three studied biomaterials. The polymer/glass composites are represented by batch 6 since this batch supplied most of the tested composite samples. What strikes one most are the small standard deviations that accompany

![Figure 5.3: Porosity values for the three examined scaffold types](image-url)
the mean values. Apparently not much variation in porosity exists between samples from the same batch. Of course, the absolute porosity values can be altered at will; yet, increasing the cement’s or ceramic’s porosity implies making them more brittle and thus more difficult to work with. Consequently, composites provide the best prospects with a view to creating high porosity without losing mechanical quality.

5.3.2 Permeability

In contrast to porosity, permeability is a material characteristic that depends on several factors and is susceptible to the smallest variations in material structure. As a consequence, the obtained standard deviations are far bigger than those of the porosity measurements. Table 5.5 shows the obtained permeability values. As in the previous section the polymer/glass composites are represented by batch 6. Not only the variation between

<table>
<thead>
<tr>
<th># Samples</th>
<th>Composites (batch 6)</th>
<th>Calcium Phosphate Cements</th>
<th>Glass Ceramics</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>2.44·10^{-11}</td>
<td>8.36·10^{-14}</td>
<td>2.59·10^{-10}</td>
</tr>
<tr>
<td>3</td>
<td>1.64·10^{-11}</td>
<td>5.66·10^{-14}</td>
<td>0.36·10^{-10}</td>
</tr>
</tbody>
</table>

Table 5.5: Permeability values for the three examined scaffold types

samples from the same material is bigger, the variation between the different materials is bigger as well. While comparing permeability values with porosity values, the following question arises: Is a higher porosity always coupled with a higher permeability? For instance, in our study the glass ceramics have a significantly higher permeability than the composites whereas they show a lower porosity. In reality, permeability is influenced by not only porosity, but also pore size and interconnectivity of the pores. What’s more, it is sufficient that one cross section has a bad interconnectivity for the permeability to drop drastically. So the permeability of a scaffold is determined by its weakest link while porosity is determined by the properties of the scaffold as a whole.

In summary, the main cause for the variability in scaffold permeability can be found in variability in inner structure. In the case of the composite scaffolds, the amount, size and location of the evaporation cavities influence permeability to a great extent. The cements do not contain these holes, but their pores are formed by mixing whisked egg white with a cement paste. This procedure is not very accurate and cross sections with less, smaller or poorly connected pores are likely to form and affect permeability significantly.

Apart from variability, permeability versus time is recorded for the different materials. Glass ceramics show very similar behaviour to polymer/glass composites. In figure 5.2 the permeability drops in 60 minutes to 72% of its original value. Permeability of composite
scaffolds generally drops to 70-80% of its original value, indicating a similar course in
time for the two materials. The permeability of calcium phosphate cements on the other
hand, shows a different time course. With teflon ring no significant decrease is recorded
whereas without teflon ring a decrease up to 53% of the original value is measured after
20 minutes and from then on no further decrease is observed. Note however that for the
cements only two experiments are performed and that this is insufficient to declare with
certainty the cement’s time dependency. The same remark can be made regarding the
glass ceramics.

\[ k = \frac{-v_{se} \cdot A_s \cdot L_s \cdot \eta}{A_s \cdot \Delta p} = \frac{-v_{se} \cdot L_s \cdot \eta}{\Delta p} = 1,00 \cdot 10^{-13} m^2 \]

In other words, a scaffold with a permeability lower than \(1,00 \cdot 10^{-13} m^2\) requires a pressure
higher than 1 bar in order to establish a seeding velocity of \(v_{se} = 1 \text{ mm/sec}\). Furthermore,
the pump that provides the pressure difference to establish flow through the scaffolds,
does not function appropriately above a pressure of 1 bar. These specifications allow us
to calculate a minimum for the required scaffold permeability. Equation (4.6) can be
rewritten as follows:

5.3.3 Use in Cell Seeding Applications

The three discussed materials are all suitable to use in tissue engineering applications
since they are all biocompatible and bioresorbable. However, other characteristics as well
are important for scaffolds to be used in a cell seeding experiment. A first issue is that the
cell seeding will be carried out with a seeding velocity of \(v_{se} = 1 \text{ mm/sec}\). Furthermore,
the pump that provides the pressure difference to establish flow through the scaffolds,
does not function appropriately above a pressure of 1 bar. These specifications allow us
to calculate a minimum for the required scaffold permeability. Equation (4.6) can be
rewritten as follows:

\[ k = \frac{-v_{se} \cdot A_s \cdot L_s \cdot \eta}{A_s \cdot \Delta p} = \frac{-v_{se} \cdot L_s \cdot \eta}{\Delta p} = 1,00 \cdot 10^{-13} m^2 \]

In other words, a scaffold with a permeability lower than \(1,00 \cdot 10^{-13} m^2\) requires a pressure
higher than 1 bar in order to establish a seeding velocity of \(1 \text{ mm/sec}\). Therefore, the
cement samples used in this study are not suitable to work with because their mean permeability is \(8,36 \cdot 10^{-14} m^2\). Of course cements with a higher porosity can be made
in order to increase permeability. Still, the poor interconnectivity between the pores of
cement scaffolds will always demand a rather high pressure. And in addition to the pump
limitations, high pressures have to be avoided since they might kill circulating osteoblasts.
Glass ceramics and polymer/glass composites tend to obtain well interconnected pores and
thus a relatively high permeability. Moreover, composites have the advantage over glass
 ceramics that they are less brittle and that their diameter is more precise.

A second issue is that osteoblasts might show a better biocompatibility to one material
than to another. For instance, maybe they adhere well to calcium phosphate cements and
scarce to glass ceramics. However, little is known in advance about these adherence
forces and this will be subject of further research.
5.3.4 Comparison with Other Studies of Scaffold Permeability

To conclude, it is interesting to compare the obtained results from table 5.5 to the results of previous permeability studies on other type of scaffold materials. Apart from frequently used biomaterials, such as polylactides, hydroxyapatite, etc., collagen and collagen-glycosaminoglycan (collagen-GAG) porous substrates have been studied to produce tissue engineered bone grafts. Its characteristics are extremely suitable for providing grafts for skin, blood vessels, etc., but its mechanical strength is limited. Still, the material is worthy as a bone implant material since osteoblasts show great biocompatibility to it. O’Brien et al. [22] found collagen-GAG scaffolds to be highly permeable with a mean value in the order of $10^{-11} m^2$. Moreover, O’Brien et al. proved a direct relation between permeability and pore size. Permeability increases from $0.6 \cdot 10^{-10} m^2$ to $1.8 \cdot 10^{-10} m^2$ when increasing pore size from 96 to 150 µm. These values lie in between the obtained mean permeability results for composite and glass ceramic scaffolds as shown in table 5.5. In this project the effect of increasing pore size for one type of scaffold material has not been explicitly examined. However, the glass samples used in this study visually showed large pores compared to the cements and composites. Therefore their remarkably high permeability does not come as a surprise. O’Brien et al. also investigated the influence of compression on permeability; the collagen-GAG scaffolds show a decreasing permeability when compression is increased. Nevertheless, the used compressions are in the range of 14 to 40% which is far beyond the compressions due to the pressure differences in our study.

Finally, the permeability results of the examined bone scaffolds can be compared to the permeability of intertrabecular bone since in tissue engineering applications the requirement to mimic the existing tissue is of utmost importance. Permeability of intertrabecular bone depends strongly on flow direction and anatomic site. These results in a large range of values varying over six orders of magnitude: from $10^{-14}$ to $10^{-8}$ [18]. In the current study, the permeability results of the used scaffold materials lie in this range; therefore, they can be considered suitable as possible tissue engineered bone grafts.
Conclusions and Future Work

It is clear that research on the properties of scaffolds is of great importance on the further development of tissue engineering applications, in our case bone graft implantations more specifically. As this field is rather new and still developing, little research has been done so far on the permeability of bone scaffolds. In this study, three biomaterials have been used to perform several tests for characterisation: polymer/glass composites, calcium phosphate cements and glass ceramics. The aim is to apply these materials for cell seeding experiments which require knowledge of the perfusion of scaffolds. The applied pressure, fluid velocity, cell medium concentration, etc. depend strongly on properties such as permeability, porosity, pore size and interconnectivity. Moreover, it can be interesting to investigate the scaffolds’ compression since this can influence the previous properties. Throughout the whole project these parameters were more extensively examined for composites because only a few cement and ceramic samples were available and composites were easier to use with the permeameter.

First of all the preparation methods of the three biomaterials have a strong influence on the scaffolds’ properties. In general it can be said that permeability is more sensitive to variations in pore size and interconnectivity of the pores whereas porosity is a more stable parameter. Composite scaffolds undergo an evaporation process of chloroform which can leave behind cavities. As a consequence the obtained permeability results for both scaffolds of one batch as for scaffolds from different batches show rather large variations to an extent that statistical methods, such as the ANOVA-test for comparing populations, conclude that the batches are significantly different from each other. The same large variation exists among calcium phosphate samples; in this case the cause is the delicate operation of mixing whisked egg white through the calcium phosphate powder. The homogeneity of this mixture determines the interconnectivity and hence the permeability. Glass samples, however, show better a consistency in their permeability values. When examining porosity of the three materials no such large variation is occurring as interconnectivity does not directly influences the porosity value.

A second remarkable observation is the decreasing permeability in time. This phenomenon has been more thoroughly investigated for composite scaffolds than for cements and
glass samples. Still, the results from the latter materials indicate that a same time dependency may be occurring. On the other hand, the cements enclosed in teflon cylinders do not decrease in time. Nonetheless, not enough experiments of this kind were done to make strong conclusions and thus future work regarding the time dependency of these two materials remains to be done. Three possible causes for the decreasing permeability have been examined. At first, it was thought that the water pressure compressed the scaffold’s pores. However, compression tests on composite scaffolds excluded this option. Secondly, the permeameter shows no possible defects that could slow down fluid flow. Most apparently, structural changes of the scaffold material due to fluid flow are the cause of the observed phenomenon. So far, this statement could not be proven since SEM-images showed no irregularities.

Moreover, comparing the permeability results for the three materials, cements show a very low permeability (magnitude $10^{-14}$) whereas the composites and glass ceramics show values which are in the same range as previous permeability results on collagen-GAG scaffolds [22]. For application in bone tissue engineering, these values are quite normal and comply to the wide range of permeability of intertrabecular bone which depends strongly on anatomical site [18]. The aim for this study, however, is to contribute to the cell seeding experiments which will be carried out by Martin Koch. In this prospect, the best option is to use composite scaffolds as they show both a large permeability and porosity so that no high pressures are necessary to accomplish perfusion. In addition, they are far more flexible than cements and glass ceramics, thus less likely to sustain damage during perfusion.

The limitations of this study are foremost found in the fabrication processes of the scaffolds. Further research has to be done on new solvents in order to avoid the cavities caused by the chloroform evaporation. Automation of the mixing process would have a positive side effect on the pore homogeneity of cements. The permeameter calls out for some improvements as well. First it is not practical in use at high pressures as required for testing low permeable samples, such as cement scaffolds. The screw thread should be made less fine in order to succeed in providing the force to maintain pressure. Second, the sample might get damaged during insertion. Therefore, it would be better to redesign the scaffold holder so that the scaffold can be carefully placed between the seals instead of pushed through.
Appendices
Appendix A

Composites: Permeability Results of Batch 5 & 7

Figure A.1: Composites: permeability results of batch 5

Figure A.2: Composites: permeability results of batch 7
Appendix B

Time recovery after 65 hours

Figure B.1: Recovery after 65 hrs with measurements at $p = 0.010$ bar and $p = 0.025$ bar

Figure B.2: Recovery after 65 hrs with all measurements at $p = 0.025$ bar
Appendix C

Grubbs’ test: Critical T-Values

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Figure C.1: Abstract of critical t-values for Grubbs’ test [21]
## Appendix D

### Upper Critical Values F-distribution

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Table D.1: Abstract of upper critical values F-distribution for ANOVA-test (5% significance level) [20]
## Appendix E

**Student’s t-test: Critical t-values**

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Table E.1: Abstract of critical t-values for Student’s t-test [27]
Appendix F

Averaged Stress-Strain curves

Figure F.1: Averaged stress-strain curves
References


REFERENCES

[26] Edinburgh University. Student’s t-test: procedure. 

[27] Edinburgh University. Student’s t-test: t-table. 


Bibliography


