Design and characterization of a scaffold for bone tissue engineering
BMTE 02.29
Julien A. Leboucher

Coaches: René van Donkelaar, Bert van Rietbergen, Nico Sommerdijk, Jack Donners and Rik Huiskes

Traineeship place: Technical University of Eindhoven, Faculty of Biomedical Engineering (The Netherlands)
Table of contents

Chapter 1. Introduction 4
  1. Motive for the emerging of Tissue Engineering 4
  2. Tissue Engineering defined 4
  3. Bone tissue engineering scaffolds review 6
     a. Polymer scaffolds 6
     b. Hydroxyapatite scaffolds 7
     c. Alternate scaffold strategies 7
  4. Anatomy of bone 7
     a. Anatomy of flat bones 7
     b. Anatomy of long bones 8
     c. Details 9
     d. Bones cells and bone remodelling 9
        e. Ultrastructure of bone matrix 11
  5. Hydroxyapatite 12
  6. Alginate 12
  7. Scaffold defined 14
  8. Scaffold requirements 14

Chapter 2 Design of scaffolds 15
  1. Preparation of the chemicals 15
     a. Alginate solution 15
     b. Other solutions 15
  2. Balls 16
     a. Protocol: for every kind of ball 16
     b. Results 17
  3. “Soft” synthesis of hydroxyapatite 18
     a. First experiment 18
     b. Second experiment 19
  4. First scaffolds 20
     a. Single layered phosphate-alginate scaffold 21
     b. Single layered sodium-alginate scaffold 21
        i. Protocol 21
        ii. Comments 21
     c. Several layered scaffolds 21
        i. Protocol 22
ii. Micro CT analysis 22

iii. Comments 22

d. Several layered scaffold with copper grids 23
   i. Protocol 23
   ii. Comments 23

e. Several layered scaffold with glass filters 24
   i. Protocol 24
   ii. Comments 24

4. “Soft tube” scaffolds 25
   a. Water based scaffolds 26
      1. First scaffold 26
         i. Protocol 26
         ii. Scaffold 27
         iii. Comments 28
      2. Second scaffolds 28
         i. Protocol 28
         ii. Scaffold 29
      3. Third scaffolds 29
         i. Protocol 29
         i. Comments 29
   b. HBSS based scaffolds 29
      1. Fourth scaffolds 29
         i. Protocol 30
         ii. Comments 30
      2. Fifth scaffolds 32
         i. Protocol 32
         ii. Comments 32
      3. Sixth scaffolds 33
         i. Protocol 33
         ii. Comments 34

Chapter 3. Mechanical tests 36
   1. Common results 36
   2. Individual results 37
      a. First water based scaffold 37
      b. Second water based scaffold 38
      c. First HBSS based scaffold 39

Discussion 41
Future 42
Acknowledgements 43
References 44
Chapter 1 Introduction

1. Motive for the emerging of Tissue Engineering:

   It is now possible to transplant approximately 25 different organs and tissues (including
   bone, cartilage, bone marrow, cornea, hearts, heart-lung, kidney, liver, lung, and pancreas), grafts
   still present several major drawbacks:

   The transfer of tissue graft requires the sacrifice of tissue from a donor site, resulting in
   associated morbidity (scar formation, potential injury and impairment to donor site, etc.).
   Considering autografts, the volume of donor tissue that can be harvested is dependant on blood
   supply and the need to avoid visceral injuries and contour deformities. In addition, even if this kind
   of grafts prevent the receiver from rejection troubles, we face the problem of double operation with
   all the risks that it induces (more scars, infections, etc.). Allografts and xenografts possess the risk
   of infection disease, and rejection (that implies the use of immunosuppressive drugs like
   cyclosporine).

   In addition, the clinical solution to restore structure and function of impaired tissues or
   organs relies on "robbing Peter to pay Paul". This presents a problem, as there is a growing lack of
   donor: only in the United States, there were 50,613 patients waiting for an organ transplant in the
   beginning of 1995. Concurrently, the number of organ transplant has increased (+57% between
   1988 and 1995), as the waiting list (from 16,026 in 1988 to 43,983 in 1995), the time spent waiting
   for a transplant (in 1988, 57% of registrants received a transplant within a year, 44% in 1995).
   Also, 500,000 arthroplastic procedures and total joint replacement are performed each year in the
   Unites States to repair damaged bone or cartilage. Besides, 7,5 million patient per year rely on non
   biological implants, ranging from artificial hearts to artificial hips, to replace the structure and
   function of any impaired organ or tissue.

   Moreover, tissue and organ transplantation is very expensive. Each year, in the United
   States, it costs an estimated $400B. This is also the amount of money estimated for organ failure
   and tissue lost overall market for engineered tissues, of which $80B is estimated to be the value of
   the engineered tissues themselves [1].

   Concerning only bone fracture, their cost has been estimated, for the United Kingdom alone, to
   about 900 million pounds each year. With an increasing ageing population, these health costs are
   set to rise: each year in the United Kingdom, there are some 150,000 fractures (wrist, vertebral and
   hip) due to osteoporosis. [2]

2. Tissue Engineering defined:

   Tissue Engineering (TE) is a relatively new and emerging interdisciplinary field that applies
   the knowledge of bioengineering (the life sciences) and the clinical sciences towards solving the
   critical medical problems of tissue loss and organ failure. It has been formally defined as 'the
   application of the principles and methods of engineering and the life sciences towards the
   fundamental understanding of structure-function relationships in normal and pathological
   mammalian tissues and the development of biological substitutes that restore, maintain, or improve
tissue function’. This field also implies the use of biochemistry and biomechanics, that is to say the application of chemistry and mechanics to the field of biology. Tissue engineering aims at designing (complex) three-dimensional structures called Scaffolds (see about scaffolds below), and activating it in special devices called bioreactors in order to obtain the biological equivalent of a defective organ or tissue (see fig.1).

According to Hutmacher, a tissue engineering research program (for bone or cartilage) can be classified in six phases [3]:
- I-Fabrication of a bioresorbable scaffold (see following section about scaffolds)
- II-Seeding of the osteoblasts/chondrocytes populations into the polymeric scaffold in a static culture (petri dish)
- III-Growth of premature tissue in a dynamic environment (spinner flask)
- IV-Growth of mature tissue in a physiologic environment (bioreactor)
- V-Surgical transplantation
- VI-Tissue-engineered transplant assimilating/remodeling

The frame of our study is clearly the first step. This first stage begins with the design and fabrication of a porous three-dimensional (3-D) scaffold. In general, the scaffold should be fabricated from a highly biocompatible material which does not have the potential to elicit an immunological or clinically detectable primary or secondary foreign body reaction [4].
3. Bone tissue engineering scaffolds review:

This section gives a present state of the art of bone tissue engineering scaffold. This section is divided in polymer scaffolds, bioceramics scaffolds and composites scaffolds.

First of all we will talk about the polymer scaffolds which are, from far, the most developed ones, then a sub-section is dedicated to bioceramic scaffolds, and last aims at presenting hybrid scaffolds.

Before starting, it has to be reminded that, after biocompatibility, the major concern about tissue engineering scaffold designers is the porosity. As it has been thought that the minimum pore size to observe bone ingrowth was Ø100μm [5], it has recently been proved that a Ø50μm was sufficient [6]. Anyhow, the attention of tissue engineers is now also focused on the interconnectivity of these pores. Actually, interconnectivity of pores is vital for the cells seeded, it allows the formation of blood vessels inside the scaffold, that is the only way to provide a good nutrient supply and waste removal.

a. Polymer scaffolds:

The polymer scaffolds are the most numerous ones. This group consists of a lot of different kinds, we proposed to describe them following their type.

Poly(propylene fumarate) (PPF): this sort of polymer is injectable, that is to say it can be implanted in a patient without surgical intervention. It presents also the advantage to fill the defect by itself, i.e. without being made with a special shape. This material has been developed to reach the properties of human trabecular bone by the addition of β-tricalcium phosphate (β-TCP) [7]. This composite material manages to maintain its mechanical properties over several weeks of degradation.

Polyethylene oxide-polybutylene terephthalate (PEO-PBT): this kind of material shows promising bone-bonding properties. This copolymer has already been used as a biodegradable scaffold and good results were obtained after implantation in goat femora [8].

Polycaprolactone (PCL): this sort of polymer has been design via fused deposition modeling (FDM) process [9]. Its computer aided design allowed to manufacture it in different ways, which leads to mechanical properties ranging from 20 to 42 MPa and 2.3 to 3.1MPa, for elastic modulus and strength respectively. This mechanical properties, which almost match the ones of trabecular bone (50MPa for elastic modulus and 5MPa for mechanical strength) for the best scaffold, varied according to the design. The porosity of these scaffolds were more than 55%.

Polylactide-Polyglycolide(PLA-PGA): these two polymers have the greatest history of use, they have been widely used, alone or as copolymer (called poly(lactic-co-glycolic acid) (PLGA)) in many studies [10]. Their wide using can be explained by the fact that their degradation rate, when used as copolymer, is relative to the ratio of both polymers. They have also been used with HA reinforcement fibers in order to enhance mechanical properties (compressive yield strength raised from 0.95 MPa, for a nonreinforced scaffold, to 2.82 MPa for a reinforced one) [11].

b. Hydroxyapatite scaffolds:

The hydroxyapatite scaffolds have also been widely studied. HA is an interesting because it presents good osteoconduction properties as it forms the main part of the inorganic material in human bone and teeth.
Most of the time, the porosity is created using a polymer or an organic material: the additive is foamed with HA powder and then the structure obtained is sintered at various high temperatures (generally more than 1200°C) in order to remove the additive. Once the latter removed, the structure becomes porous and, so, an ideal scaffolds for bone tissue engineering.

The additives used are e.g.: polyester fibers sheets [6], polyvinyl butyral [12] and polyethylenimine [13,14].

c. Alternate scaffold strategies:

Alternate scaffold strategies include the use of natural scaffold based on the rationale that animal skeletons have been designed through optimization by natural selection, to physically support and physiologically maintain diverse tissue type [2]. Biomimetic material chemistry has sought to reproduce, in part, the complex structures that occur in nature, such as coral, nacre and calcite shells spine and sea urchins [15-18].

Another kind of alternative strategy has been used to design a hydroxyapatite-collagen composite scaffold [19]. As HA and collagen are the main components of inorganic and organic phases, respectively, of bone, this material obviously present osteoconductive capacities. This scaffold consists of sheets of collagen type I, on which HA has precipitated, rolled with bone fragments inside.

4. Anatomy of bone:

In this part, we will present the bone in his overall role. The skeletal system has three main functions. It aims at acting as a support structure against gravity, as a lever system for the muscles, and as a protective covering for internal organs. (BOUVIER, 1989). Bone is a composite biphasic material. His solid phase consists of bone cells embedded in an extracellular matrix that includes collagen (for elasticity) and hydroxyapatite crystals (for strength). The fluid phase consists of blood and extracellular matrix.

There are two different kinds of bone, flat bones and long bones.

a. Anatomy of flat bones:

Flat bones, generally have a protective function, e.g. the calvaria protects the delicate brain tissue. Most of flat bones are intramembraneous bones and start off as a network of bone trabeculae.

b. Anatomy of long bones:

A typical long bone, such as the femur, consists of two epiphyses and metaphyses (both of these terms are gathered together as physes), and one central diaphysis or bone shaft (see fig.2). The growth plate, that lies in the metaphysis enables longitudinal growth. The other parts of a long bone are:

- Medullary cavity, that contains marrow
- Endostem, the lining of marrow cavity
- Periosteum, the tough membrane covering bone
- Osteogenic layer, that contains bone cells and blood vessels
c. Details

At a macroscopically scale, there are two kind of bone, compact and cancellous bone:

- Compact bone, also called cortical bone, is a dense material that makes up primarily the sharp.
- Cancellous bone, also called trabecular bone, is a porous material that makes up the epiphysis of a long bone, it is surrounded by a thin shell of compact bone.

At a microscopically scale, compact bone shows different parts called woven, lamellar and harvesian bone (COWIN 1983):
- Woven bone is a relatively unorganized precursor to lamellar bone in young animals.
- Lamellar bone is part of compact bone. It is made up of a system of concentric laminar separated by a vascular network (see fig.3). This kind of compact bone is present in the shaft of a mature long bone. Each lamina, in turn, contains alternating zones of poorly organized and highly organized bone.
- Harvesian bone is due to the eventual conversion of some lamellar bone and is also part of the compact bone. It consists in cylindrical elements called osteons or harvesian systems (see fig.3). Harvesian systems are aligned parallel to the long axis of the diaphyses on a slightly spiral course [20].
At a smaller scale, osteons can be described as follow: osteons are cylindrical structures of 100-150 microns radii that lie very roughly along the long axis of a bone and contain at their center an osteonal canal. This canal contains one (or more) blood vessel(s), a nerve and some space occupied by bone fluid. The walls of the osteonal canal are covered with cells and, behind the cells, are the entrance of the canaliculi. The canaliculi are passageways that runs between lacunae or from lacunae to the osteonal canal (see fig.4) [21].

There is another kind of bone present in the metaphysis, called spongious bone (described below in fig.5). Spongious bone is a latticework of thin plates of bone called trabeculae oriented along the lines of stress.

The spaces between these trabeculae are filled with red marrow where blood cells develop. This kind of bone is also found in flat bone (e.g. hipbones, sternum, sides of skull, and ribs).

d. Bones cells and bone remodelling:

Bone contains primarily three types of cells called osteoblasts, osteoclasts and osteocytes:
- Osteoblasts aim at creating, or deposit, bone
- Osteoclasts aim at destroying, or resorbing, bone.
- Osteocytes which are osteoblasts that become trapped in the bone matrix and then serve maintenance functions.
It is well known that bone responds and adapts to mechanical forces [22]. It is hypothesized that osteocytes are the mechanosensors that detect the mechanical signals (see fig.6).
Osteocytes are well placed to fulfill this role because they would be subjected to mechanical strains and they also communicate with each other via canaliculi. Signal could thus be transmitted through the canaliculi and ultimately reach the cells of the endosteum and vascular system. Hence, signal from the sensor cells will reach the effector cells, i.e. the bone-forming osteoblasts and the bone-resorbing osteoclasts, which form or take away bone matrix according to mechanical load [23].

Apart from adaptation to mechanical loads, remodelling of discrete packets of bone takes place throughout life. In this case, the resorption pit created by osteoclasts is subsequently filled in by osteoblasts, so that new bone matrix replace older, possibly damaged matrix. Resorption is usually coupled to formation and osteoblasts express receptors for resorption-stimulating agent. The digestion of bone by osteoclasts depends on the attachment of osteoclasts to the mineralized surface via the ruffled border, which by tight sealing creates an area of low pH needed for the dissolution of mineral.

e. ultrastructure of bone matrix:

The bone matrix is constituted of organic and mineral "mixed" materials. 90% of the organic matrix is type I collagen, the remainder being non-collagenous proteins (e.g. osteocalcin, osteonectin, bone sialoprotein and osteopontin).

The mineral constituents are hydroxyapatite, a highly regular and well co-ordinated calcium phosphate crystal, and calcium carbonate.

5. Hydroxyapatite:
Hydroxyapatite (fig.7) is the mineral that gives his strength to the bone. Alone, this material is very brittle, but employed with collagen fibers, as in bone, it generates both a strong and light material.

Its formula is $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ and its theoretical density is 3.156 g.cm$^{-3}$.

There are several ways to synthesize this mineral:
- by solid state reaction, using CaHPO$_4$·2H$_2$O and CaCO$_3$. CaHPO$_4$·2H$_2$O is ignited at 1100°C to be converted in Ca$_2$P$_2$O$_7$ and then mixed with CaCO$_3$ in water. Finally, the solution is calcined at 1100°C. [6]
- by precipitation, using calcium nitrate tetrahydrate (Ca(NO$_3$)$_2$·4(H$_2$O)) and di-ammonium hydrogen phosphate salts.
- by precipitation using Ca(OH)$_2$ and H$_3$PO$_4$ [13],
- by precipitation of calcium (Ca$^{2+}$) and phosphate (PO$_4^{3-}$) ions, using calcium chloride and di-Sodium hydrogen phosphate (Na$_2$HPO$_4$).

6. Alginate:

One of the major problems of using a scaffold, once it has been correctly designed, is the cell seeding. Most of the time, cells are disposed on the surface of the scaffolds and are supposed to go inside the structure. This process lead usually to a non-homogeneous cell seeding. In order to ensure that cells are randomly distributed inside our scaffold, it has been thought about using alginate. Alginate is used in the form of a viscous solution to immobilize microorganisms.

The alginate that has been used in this study is called: Alginic acid salt sodium from brown algae. Alginic acid is a polysaccharide from seaweed [24]. It is a family of natural copolymers of β-
\(d\)-mannuronic acid (M) and \(\alpha\-L\)-guluronic acid (G) \([25,26]\). The two polymers involved in alginates have a very close composition (see fig.8).

Alginates are not random copolymers but, according to the source algae consist of blocks of similar and strictly alternating residues (i.e. \(\text{MMMMMM, GGGGGG and GMGMGMGM}\) (see fig.9), each of which have different conformational preferences and behavior. They may be prepared with a wide range of average molecular weights (50 – 100000 residues) to suit the application.

The free carboxylic acid have a water molecule \(\text{H}_3\text{O}^+\) firmly hydrogen bound to carboxylate. Calcium ions \((\text{Ca}^{2+})\) can replace this hydrogen bonding, zipping guluronate, but not mannuronate, chains together stoichiometrically and supposedly egg-box like conformation.

The primary function of the alginates are as thermally stable cold setting gelling agents in the presence of calcium ions. Gelling depends on the ion binding \((\text{Mg}^{2+} < \text{Ca}^{2+} < \text{Sr}^{2+} < \text{Ba}^{2+})\). High G content produces strong brittle gels (except if present in low molecular weight molecules), whereas high M content produces weaker, more-elastic gels.

Because of their biocompatibility, abundance in source, and low prices, alginates have been widely used in the food industry as thickeners and emulsifying agents \([27]\). They have also been...
processed into gel beads encapsulating living cells as a means of immunoprotection [28]. Alginate crosslinked with calcium sulfate (CaSO₄) have also been used as cells delivery vehicles for in vivo tissue engineering research [29,30].

It has been chosen to use this material for two of these reasons. First, the encapsulation property is very interesting: as it is very difficult to seed the cells homogeneously, using this material should be a way to ensure that the cells are randomly distributed in the scaffold. The second reasons is its low price and availability.

7. Scaffold defined:

The Scaffold term is used to define the structure designed by the tissue engineer. This structure has to meet a lot of requirements: First of all, the scaffold must be "biocompatible" that is to say it has to succeed in passing the tests of cytotoxicity, it must keep the cells alive and make them "feel" like in a living being. It implies a lot of goals to be achieved like good nutrient providing and good waste evacuator.

The second requirement is trivial but must be taken into account, the scaffold must provide a good cell attachment, and it is obvious that if the cells cannot be kept in it, no tissue can grow.

Finally, this structure must be able to be put in a bioreactor, that is to say that the structure has to be strong enough to endure mechanical stresses (because most of the time the bioreactor are designed in order to put a special kind of scaffold).

This definition lead us to the following requirements.

8. Scaffold requirements

- **pH**: The pH must stay close to 7.4 during all the processes for two reasons. First, calcium (Ca²⁺) and phosphate (PO₄³⁻) ions need a pH above 6 to precipitate as hydroxyapatite when they meet, and second, the cells must be kept at pH 7.4 in order to survive.

- **Homogeneity**: this characteristic is important to obtain a good repartition of the cells that leads to good nutrients supply. This is also interesting to have good mechanical properties.

- **Having HA inside the scaffold**: one of the aims of the scaffolds we wanted to design was to have HA in the scaffold. That implies to use a "soft way" to synthesize this mineral (i.e. without using an oven or chemicals that could kill the cells). The roles of the already present HA will be (i) to offer a anchorage site to the cells, (ii) to increase the stiffness of the scaffold and (iii) to make it more "bone-like".
Chapter 2 Design of scaffolds

The previous requirements have lead to the following idea about the design of the scaffold: we aimed at making alginate balls, gathering them in a desired scaffold shape and synthetize hydroxyapatite in neighborhood of the scaffold, using a phosphate solution that could precipitate both with calcium ions also added around the structure and with those already present in the alginate balls. We hoped to obtain, by this mean a porous scaffold, which pores are due to the removal of the alginate, once the HA is present all around the balls and provide cell anchoring surface.

1. Preparation of the chemicals:

   a. Alginate solution:
      This solution is prepared by adding alginate powder (Alginic acid salt sodium from brown algae) to a physiological sodium chloride solution (NaCl solution). It has been chosen to prepare a 5% weight alginate solution, that is to say, put 5g of alginate powder in 95ml of sodium chloride solution ([NaCl]=0.102 mol.l⁻¹). In order to dissolve the alginate quicker, we use a drill to mix the preparation (see fig.10).

   b. Other solutions:
      The other solutions used are diNatrium hydrogenophosphate (Na₂HPO₄), potassium dihydrogenophosphate (KH₂PO₄) and calcium chloride (CaCl₂). To prepare them at a given concentration, the mass of the powder needed to reach the desired concentration has been calculated. e.g. we want to prepare 100ml of a 1mol.l⁻¹ solution of CaCl₂. First, we have to check the exact chemical composition of the powder (written on the box). In this case, we have dihydrated
calcium chloride (i.e. CaCl₂.2H₂O), hence we have to take into account the water already present in the powder. So:

\[
M(\text{CaCl}_2.2\text{H}_2\text{O})=M(\text{Ca})+2M(\text{Cl})+2(2M(\text{H})+M(\text{O}))
\]

with \( M \): molar mass of the atom-molecule in g.mol\(^{-1}\)

A.N.: \( M(\text{Ca})=40.1\text{g.mol}^{-1} \quad M(\text{Cl})=35.5\text{g.mol}^{-1} \)
\( M(\text{H})=1\text{g.mol}^{-1} \quad M(\text{O})=16\text{g.mol}^{-1} \)

\[ \Rightarrow M(\text{CaCl}_2.2\text{H}_2\text{O})= 147.1 \text{ g.mol}^{-1} \]

Then, the following relation gives the mass that has to be weight:

\[ m=nM \quad \text{with} \quad m \text{ the mass in g} \]
\( n \) the number of moles wanted

In order to prepare 100ml (i.e. 0.1 l) of 1mol.l\(^{-1}\) we need:

\[ n=Vc \quad \text{with} \quad V \text{ the volume of the solution in l} \]
\( c \) the concentration of the solution in mol.l\(^{-1}\)
\[ n=0.1\text{mol} \]

Consequently: \( m= 14.71\text{g} \)

The next step consists of dissolving the powder in distilled water, with less water than the desired volume. Once dissolved, complete the solution with distilled water to reach the right volume (i.e. 100ml in our case).

2. Balls:

Qualitative experiments have been performed with alginate before starting to design scaffolds. In most of them, alginate balls have been made, and the aim has been to understand the effects of the solutions upon the alginate. Alginate balls are obtained by putting some of an alginate solution in a syringe and put a drop of it in a solution containing 2\(^+\) cations (e.g. Ca\(^{2+}\), Mg\(^{2+}\), etc.). Then, remove the drop from the solution and a ball is obtained.

a. Protocol: for every kind of ball:

The experimental protocol was the following:

i. Drop the alginate solution into the calcium solution
ii. Wait for a given duration
iii. Remove the drop from the solution with tweezers for example
iv. Put the ball on a wipe paper in order to remove the solution around it then go to vii. if the alginate contains PO\(_4^{3-}\) ions, else continue.
v. Put the ball in a phosphate solution (Na\(_2\)HPO\(_4\) or KH\(_2\)PO\(_4\)), wait for a given duration.
vi. Dry the ball as in iv.
vii. Store the ball in an airtight flask, in order to prevent it from drying.
b. Results:
These experiments lead to several qualitative conclusions:

i. The longer the balls stay in the calcium solution, the stiffer they are (for a given concentration). This phenomenon could mean that the more calcium ions you have in alginate, the stiffer it is.

ii. The higher the calcium concentration is, the stiffer are the balls (for a given time). This phenomenon could mean that the more calcium ions you have in alginate, the stiffer it is.

iii. If you put calcium-alginate balls in sodium phosphate solution, a thin white layer appears. This layer is supposed to be a calcium-phosphate precipitate, and is hoped to be hydroxyapatite. It can be removed if by scrapping the surface of the ball. So, the calcium ions taken by the alginate can react after with the phosphate ions.

iv. If you put a calcium-alginate ball in potassium phosphate solution, nothing seems to happen, probably because of the pH that is too low ?????. This is the reason why the use of this solution has been given up.

v. If the drops stay only a few seconds in the calcium and then longer in the phosphate solution, they collapse while drying on the paper. This phenomenon occurs because all calcium ions, previously needed to have a bead, are taken away by the phosphate ions.

Several pictures of cut balls have been done. The balls are cut in two halves before to be observed under the optical microscope (see fig.11 and 12).

Fig.11 alginate ball from a phosphate-alginate solution (2ml of [Na2HPO4]=0.5mol.l⁻¹ – 2g of sodium alginate) dropped into a calcium chloride solution ([CaCl₂]=1mol.l⁻¹, during 30 seconds) (magnification x40)
Fig. 12 alginate ball from a phosphate-alginate solution (2ml of $[\text{Na}_2\text{HPO}_4]=0.5\text{mol.l}^{-1}$ – 2g of sodium alginate) dropped into a calcium chloride solution ($[\text{CaCl}_2]=1\text{mol.l}^{-1}$, during 120 seconds) (magnification x40)

Comments:
- A lot of crystals can be seen. It was first hoped to be hydroxyapatite. Unfortunately, the size of what has been observed lead us to the conclusion that these crystals are brushite crystals (HA crystals size is about 10nm in diameter and 150nm in length [12]).

3. "Soft" synthesis of hydroxyapatite:

The aim of this section is to show the experiments that lead us to the conclusion that it is possible to synthesize HA without the use of an oven or any treatment that could damage the cells.

Two experiments were performed: adding a calcium chloride solution to a di-Sodium hydrogen phosphate and adding a di-Sodium hydrogen phosphate solution to a calcium chloride solution. These experiments are described below, and the IR spectra of the precipitates are commented.

a. First experiment: adding the calcium solution to the phosphate one

The first experiment consisted of adding calcium chloride solution ($[\text{CaCl}_2]=0.1\text{mol.l}^{-1}$) to 50ml of a di-Sodium hydrogen phosphate solution ($[\text{Na}_2\text{HPO}_4]=0.1\text{mol.l}^{-1}$). The calcium solution has been added at the rate of 0.5ml each time until 45ml of this solution were added. The pH has been checked for each step. When two such solutions are mixed, a white precipitate of calcium phosphate appears. It could be Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) or Brushite ($\text{Ca}(\text{HPO}_4)_2\cdot2\text{H}_2\text{O}$) depending on the pH.

The evolution of the pH is presented in fig. 13, below.
Fig. 13: Evolution of the pH during the addition of calcium chloride to a di-Sodium hydrogen phosphate solution.

This graph shows that the pH always remained above 7.

In order to know if we obtained HA or brushite, an IR spectrum of the powder obtained after drying of the precipitate has been recorded. This spectrum, compared to hydroxyapatite, is presented in fig. 14. For more information about IR spectra of HA the reader can refer to [4].

Fig. 14 spectrum of the precipitate compared to HA

b. Second experiment: adding the phosphate solution to the calcium one

The second experiment consisted of adding the phosphate solution ([Na$_2$HPO$_4$]=0.1mol.l$^{-1}$) into the calcium solution ([CaCl$_2$]=0.1mol.l$^{-1}$). The phosphate solution has been added at the rate of...
1ml each time until 45ml have been reached. The pH has been checked for each step. The evolution of the pH is presented in Fig.15, below.

![Evolution of the pH (Na2HPO4 in CaCl2)](image)

**Fig.15**

As in a., a IR spectrum has been recorded and is presented below in Fig.16

![Spectrum of the precipitate compared to brushite](image)

**Fig.16** Spectrum of the precipitate compared to brushite

4. First scaffolds:

   a. Single layered scaffolds: The first attempt in the creation of scaffolds has been to have one layer of calcium alginate balls and to synthesize HA above these balls in order to obtain a mineralisation between the balls. The aim of this mineralisation is both to bind the balls together and to give the cells an open porous structure containing HA to settle on. Two different protocols
have been used to perform this kind of experiment, one with balls made of phosphate-alginate solution put in a calcium solution and the other one without phosphate.

a. Single layered phosphate-alginate scaffold:
   Protocol:
   The chemicals used were:
   - Alginate balls obtained by dropping a solution containing 2g of alginate (5% wt)+2ml of phosphate solution ([Na2HPO4]=0.5 mol/l) in a calcium solution ([CaCl2]=0.5 mol.l\(^{-1}\) during 30s)
   - Polyethylene tube (inner diameter 22mm, outer diameter 28mm).
   - Calcium ([CaCl2]=0.1 mol.l\(^{-1}\)) and phosphate ([Na2HPO4]=0.1 mol.l\(^{-1}\)) solutions.

   First, the tube is disposed in a container and filled with a layer of alginate balls. In order to have a unique layer balls are slightly agglomerated. Then 2 ml of [Na\(_2\)HPO\(_4\)]=0.1 mol.l\(^{-1}\) is added inside the tube and 2ml of [CaCl\(_2\)]=0.1 mol.l\(^{-1}\) is added outside. After three days, the balls are bound together and most of them increased their volume. The top surface that was smooth in the beginning is now rough.

b. Single layered sodium-alginate scaffold:
   i. Protocol:
   The chemicals used were:
   - Alginate balls obtained by dropping sodium alginate solution (5 wt %) in a calcium chloride solution ([CaCl2]=0.5 mol.l\(^{-1}\)) during 30 seconds.
   - Calcium chloride ([CaCl2]=0.1mol.l\(^{-1}\)) and di-Sodium hydrogen phosphate ([Na2HPO4]=0.1mol.l\(^{-1}\)) solutions.

   Other materials used:
   - Polyethylene tube (inner diameter 22mm, outer diameter 28mm).

   ii. Comments:
   After one day, the same results as before have been observed, that is to say that some balls have grown.

c. Several layered scaffolds:
   i. Protocol:
   The chemicals used were:
   - Alginate balls obtained by dropping sodium alginate solution (5 wt %) in a calcium chloride solution ([CaCl2]=0.1mol.l\(^{-1}\)) during 30 seconds.
   - Calcium chloride ([CaCl2]= 0.1 mol.l\(^{-1}\)) and di-Sodium hydrogen phosphate ([Na2HPO4]= 0.06 mol.l\(^{-1}\)) solutions.

   The others materials used were:
   - A small becher (50 ml)
   - A polyethylene tube (inner diameter 22mm, outer diameter 28mm).
The protocol was as follow: first, the balls have been put in the tube and slightly agglomerated. Then, the tube was put in the becher and the becher was filled with the phosphate solution (30 ml were needed). Finally, the calcium solution was regularly added inside the tube.

ii Micro CT analysis:

A microtomography device has been used to analyze this scaffold (SCANCO MEDICAL, μCT 80, see fig.17).

iii. Comments:

In this experiment, it has been decided to decrease the concentration of both of the solutions because the previous ones could be toxic for the cells.

The aim of this experiment has been to synthesize hydroxyapatite on top of the balls, in order to use it as a cement to keep the balls together and, so, to obtain a scaffold. As some of the HA crystals found their way through the scaffold, they were taken at the bottom of the becher and put back on the top of the tube. The flow becomes slower and slower as the previous operation was repeated, and, finally no flow was observed.

Unfortunately, the scaffold did not keep his shape for a long time because the volume of the balls increased while they are in the phosphate solution. This is due to the fact that once dropped in a calcium bath, the alginate balls shrink, so they came back to their initial shape after the decrease of calcium concentration. After several days, the balls were linked together and the geometry of the scaffold was analyzed in the microCT scanner (see fig.18), the analyze showed clearly the shape of the ball. The mineralization occurred between the balls.
As it was not possible to keep the scaffold in its initial shape, the alginate balls were placed in a tube and fixed between grids.

d. Several layered scaffold with copper grids:
   i. Protocol:
      Chemicals used:
      - Alginate balls obtained by dropping sodium alginate solution (5 wt %) in a calcium chloride solution ([CaCl$_2$]=0.1mol.l$^{-1}$) during 60 seconds.
      - Calcium chloride ([CaCl$_2$]= 1 mol.l$^{-1}$) and di-Sodium hydrogen phosphate ([Na$_2$HPO$_4$]= 0.06 mol.l$^{-1}$) solutions.

      Other materials used:
      - A becher (100 ml)
      - A polyethylene tube (inner diameter 22mm, outer diameter 28mm)
      - Copper grids (diameter 30mm)

      In this experiment, the tube has been split in two places in order to put and to keep the grid in place.
      As before, the balls are slightly agglomerated between the copper grids, inside the tube. The tube is then placed in the becher and the becher is filled with the phosphate solution until its level is upper than the top of the scaffold. After this, the calcium solution is slowly added in the top part of the tube and the flow is kept going by bringing the solution from the bottom of the becher to the top of the tube.

   ii. Comments:
      - The higher part of the tube was not high enough, and so, the flow has been stopped after a while. A higher top part could allow to apply a bigger pressure and to keep the flow, at least, longer.
- The balls are pushing inside the tube and finally manage to slightly open it. As the flow is becoming more and more difficult, the solution leaked through these new openings, resulting that the calcium-phosphate crystals no longer deposit between the balls in the scaffold.

- After several (three) days, the solution became blue. The copper has reacted with the solution and Cu$^{2+}$ ions (which color is blue) has been freed from the grids. As it could be a problem with the cells, this solution has been abandoned.

As the copper grid is not a good solution to our problem of "growing balls", an inert material has been sought. That was how it was thought about glass filters. Glass filters are made of small glass balls agglomerated

e. Several layered scaffolds with glass filters:
  i. Protocol:
     Chemicals used:
     - Alginate balls obtained by dropping sodium alginate solution (5 wt %) in a calcium chloride solution ([CaCl$_2$]=0.1 mol.l$^{-1}$) during 60 seconds.
     - Calcium chloride ([CaCl$_2$]= 0.1 mol.l$^{-1}$) and di-Sodium hydrogen phosphate ([Na$_2$HPO$_4$]= 0.06 mol.l$^{-1}$) solutions.
     
     Other materials used:
     - A becher (100 ml)
     - A polyethylene tube (inner diameter 22mm, outer diameter 28mm)
     - Two glass filter (porosity P1 (pore size: 100-160 $\mu$m), diameter 25mm, thickness 4mm)
     - A PTFE tape

     Alginate balls have been agglomerated between the glass filter in a similar way as it was done with the copper grids. The glass filters, too thick to be kept by the tube like the copper grids, were placed between two parts of the tube and kept in place thanks to the PTFE tape. The role of the PTFE tape was also to prevent the tube from leaking.

     The tube was put in the becher, and the latter was filled with 55ml of phosphate solution. The calcium solution was then slowly added in the top part of the becher at the rate of 0.5 ml every 30 minutes for 3 hours (i.e. 3.5 ml were added).

     ii. Comments:
     - The flow through the scaffold is very slow, and is probably due to a leakage occurring between the tube and the glass filter. This leakage lead to a non-homogeneous mineralization of the scaffold.
     - There is a lot of air bubbles in the scaffold, and it is likely that it will prevent the scaffold from being well mineralized.
     - The alginate has been freed from the scaffold after 20 hours and it lead to the collapsing of the scaffold.

     To check whether or not this experiment was reproducible, another experiment has been lead the same way with more phosphate solution (90 ml), less and more concentrated calcium
solution (1.2 ml of [CaCl₂]=1 mol.l⁻¹). This new experiment confirmed the leakage previously observed and not the liberation of the alginate.

Considering that the balls were not anymore bound together with HA, as observed before the last experiments (before using copper grids and glass filters), it has been decided to try another way of designing scaffolds.

A new kind of protocol has been invented. It has been thought that mineralization should occur slowly because the cells. In fact, the synthesis of hydroxyapatite \((\text{Ca}_10(\text{PO}_4)_6(\text{OH})_2)\) from the meeting of Calcium ions \((\text{Ca}^{2+})\) and phosphate ions \((\text{PO}_4^{3-})\) takes away hydroxyle ions \((\text{OH}^-)\) from the surrounding, that makes the pH drop. As a pH as close to 7.4 as possible is wanted, it has to be ensure that the reaction occurs slowly in order not to reach a too acidic pH.

4. "Soft tube" scaffolds:

To allow a slow reaction between calcium and phosphate ions, it has been thought about using diffusion properties. This diffusion can occur in an inert medium, e.g. distilled water, by slow addition of the ions (at each extremities of the inert medium). By putting the scaffold in the middle of the “addition places”, the ions must meet inside the scaffolds and the synthesis of HA should occur in the scaffold. The device used in this experiment is showed in fig.19. It consisted of three polyethylene tubes separated by glass filters. The balls were put inside “middle tube”, between the glass filters (see protocol below).
For each experiments, (i) the volume of the tube containing the scaffold, (ii) the volume of the balls in the scaffold, (iii) the quantity of hydroxyapatite expected have been calculated, as follows:

(i) Volume of the tube: The tube is assumed to be a cylinder, its volume is given by its radius \( r \) and its height \( h \):

\[
V = \pi r^2 h
\]

(ii) Volume of the balls present in the scaffold: it is deduced from the mass of the balls (it is assumed that the balls have the same density as water).

(iii) Quantity of hydroxyapatite expected: as we put calcium and phosphate ions in a stoechiometric ratio, mass of HA is obtained from the quantity of \( \text{Ca}^{2+} \) ions and the molar mass of HA:

\[
m(\text{HA}) = M(\text{HA}) \times n(\text{Ca})/10
\]

with:

\[
M(\text{HA}) = 10M(\text{Ca}) + 6(M(\text{P}) + 4M(O)) + 2(M(O) + M(H))
\]

(HA formula is \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \))

and:

\[
M(\text{P}) = 31 \text{g.mol}^{-1}
\]

\[
M(\text{HA}) = 1007 \text{g.mol}^{-1}
\]

It has been first decided to use distilled water because it allows to reduced the number of parameter that could be involved in the experiment. The next step will be the using of culture medium when the process will be under control.

a. Water based scaffolds

1. First scaffold:
   i. Protocol:

   Chemicals used:
   - Alginate balls obtained by dropping sodium alginate solution (5 wt %) in a calcium chloride solution ([CaCl\(_2\)] = 0.1 mol.l\(^{-1}\)) during 60 seconds.
   - Calcium chloride ([CaCl\(_2\)] = 1 mol.l\(^{-1}\)) and di-Sodium hydrogen phosphate ([Na\(_2\)HPO\(_4\)] = 0.6 mol.l\(^{-1}\))

   Other materials used:
   - 3 polyethylene tubes (inner diameter 18 mm, outer diameter 24 mm), 2 big ones (350 mm) and a short one (26 mm)
   - Two glass filter (porosity P1, diameter 25 mm, thickness about 2 mm)
   - PTFE tape
The alginate balls are slightly agglomerated between the glass filters. The three tubes are bound together by the PTFE tape and separated by the glass filters. The device is filled first with 50 ml of distilled water (it is better to fill it quickly in order to prevent the formation of air bubbles).

For this first experiment:
- The measurements of the tube were: \( r = 9 \text{ mm} \) and \( h = 26 \text{ mm} \) (i.e. volume 6.62ml)
- The mass of alginate balls added was 4.55 g (i.e. equivalent of 4.55 ml)
- 27 ml of \([\text{CaCl}_2]\)=1 mol.l\(^{-1}\) were added on one side and the same volume of \([\text{Na}_2\text{HPO}_4]\)=0.6 mol.l\(^{-1}\) on the other side following this rate:

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>Volume added(ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0,5</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>5,25</td>
<td>3</td>
</tr>
<tr>
<td>24,75</td>
<td>1</td>
</tr>
<tr>
<td>25,25</td>
<td>1</td>
</tr>
<tr>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>27,25</td>
<td>1</td>
</tr>
<tr>
<td>28,5</td>
<td>1</td>
</tr>
<tr>
<td>29,5</td>
<td>1</td>
</tr>
<tr>
<td>30,5</td>
<td>1</td>
</tr>
<tr>
<td>46,75</td>
<td>1</td>
</tr>
<tr>
<td>47,25</td>
<td>1</td>
</tr>
<tr>
<td>49</td>
<td>1</td>
</tr>
<tr>
<td>49,25</td>
<td>1</td>
</tr>
<tr>
<td>51,25</td>
<td>1</td>
</tr>
<tr>
<td>71,75</td>
<td>5</td>
</tr>
<tr>
<td>77,75</td>
<td>3</td>
</tr>
</tbody>
</table>

The mass of HA expected was 2.71g. Given the theoretical density of HA: \( \rho(\text{HA}) = 3.156 \text{g.cm}^{-3} \), the volume of hydroxyapatite expected was 0.86 cm\(^3\) (i.e. 0.86ml) This was not enough to fill the scaffold, but it has been anyway possible to obtain what could be a scaffold.

**ii Scaffold**

The resulting scaffold of this experiment is presented fig.20.

![scaffold](image)

**Fig.20: scaffold obtained in the first “water-based” experiment.**
This scaffold is not homogeneous: a white layer can be observed, this should be HA, unfortunately, this layer did not cover the whole scaffold. The top part of it is transparent and no mineralization occurred inside.

Anyway, it represents a breakthrough as all the balls are linked together and it has been managed to obtain a desired shape (see comments below).

iii. Comments:

- An oval-based cylinder scaffold was obtained (due to the shape of the tube), which dimensions were h=22.9 mm, large diameter=15.8 mm and small diameter=14 mm (it was measured later and the scaffold has probably shrunk because it contains a lot of water). The scaffold seemed non-homogeneous (see fig XX), but all the balls are bound together and the scaffold consisted of one piece. The white part occurred on the "phosphate side" of the scaffold, probably because of Ca$^{2+}$ and PO$_4^{3-}$ ions. Something has precipitated inside the "phosphate tube", it has been analyzed through the infrared (IR) spectrometer and it is HA.

- The level of solution is not the same for both of the tubes, due to leakage observed near the glass filters and to the scaffold itself that prevented liquid from going through it.

For more information about this scaffold see mechanical tests in the next chapter.

2. Second scaffolds:

i. Protocol:

The second set of experiments consists of two experiments and is identical to the first one, excepting the following modifications:

- the size of the tube containing the scaffold is now h=20 mm and radius is still r=9 mm (volume 5,09ml). It has been reduced in order to eliminate the inhomogeneous mineralization that occurred in the previous experiment. From this moment, the volume of the tube was always the same.

- The volume of solutions added to the tube has been reduced to 20 ml of each because of the new size of the scaffold.

- The mass of alginate balls added has been 3.52g (3.52 ml) and 3.56g (3.56 ml) for the experiment 1 and 2, respectively.

- Solutions have been added as follow:

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>Volume added (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td>22,75</td>
<td>1</td>
</tr>
<tr>
<td>23,5</td>
<td>1</td>
</tr>
<tr>
<td>24</td>
<td>2</td>
</tr>
</tbody>
</table>
The mass of HA expected was 2.01g, the volume expected: 0.64ml

ii. Comments:
- The level in both tubes is not the same (probably due to a leakage).
- The pH of both of the solutions has been checked: phosphate solution (pH=8-9) Calcium solution (pH=4). (N.B.: pHs are too far from 7.4 and cells probably cannot stand such a pH).
- On the phosphate side the scaffold is well mineralized and homogeneous but on the "calcium side", balls can be easily removed from the scaffold (or even go away by themselves). Hence, reducing the height of the scaffold is not enough to obtain a homogeneous scaffold.

3. Third scaffolds:

i. Protocol:
The third set of experiments consisted of three experiments and is the same as before excepting the rate of solution addition:

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>Volume added (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>1,5</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2,5</td>
<td>2</td>
</tr>
<tr>
<td>18,75</td>
<td>5</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
</tr>
</tbody>
</table>

The scaffolds were then removed 6 days after the last addition of solution.

ii. Comments:
- When the scaffolds have been removed, only the phosphate side (thickness 6-8 mm) remained in one piece. Concerning the rest of the scaffold, the balls are not linked to the scaffold.
- The solutions remaining in both tubes, once mixed, showed a white precipitate. This proves that all the calcium and phosphate ions did not react during the experiment. Consequently, the amount of HA expected is wrong. From now, the volume of HA expected was no more calculated.

b. HBSS based scaffolds

1. Fourth scaffolds:

In order to go one step further, towards including cells in the scaffold, the saline, used before to prepare the alginate solution, and the distilled water were replaced by Hank’s balanced salt solution (HBSS). This solution contains the same minerals as the growth media used.
in cell cultures but not all the others additives (i.e. vitamins, growth factors, etc.) and is, consequently, cheaper. This solution has been used to make alginate balls (the protocol is the same as before, i.e. 5g of alginate powder in 95ml of HBSS) and to fill the tube in the beginning of the experiment. The pH of this solution is 7.4 and it is a buffer so it can help to maintain a correct pH around the balls during the formation of HA. Moreover, HBSS contains a pH indicator (phenol red), which is a qualitative way to know the pH and to check whether or not it drops too low.

Balls were made from the solution of HBSS-alginate as before: drops of HBSS-alginate in [CaCl₂]=0.1 mol.l⁻¹ during 60 seconds.

i. Protocol:
The next set of experiments consisted of 3 experiments containing 3.53g, 3.61g and 3.46g of balls. As usual calcium chloride ([CaCl₂]=1 mol.l⁻¹) was added on one side and diSodium Hydrogenophosphate ([Na₂HPO₄]=0.6 mol.l⁻¹) on the other side. The solution has been added as follow:

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>Volume added (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>21,75</td>
<td>1</td>
</tr>
<tr>
<td>22,5</td>
<td>1</td>
</tr>
<tr>
<td>23,5</td>
<td>1</td>
</tr>
<tr>
<td>24,25</td>
<td>1</td>
</tr>
<tr>
<td>26,5</td>
<td>1</td>
</tr>
<tr>
<td>27,5</td>
<td>1</td>
</tr>
<tr>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>28,75</td>
<td>1</td>
</tr>
<tr>
<td>29,25</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
</tr>
</tbody>
</table>

ii. Comments:
- the HBSS on the "phosphate side" turned to pink as the phosphate solution was added. It means that the pH increased. It was because of the alkaline pH of the phosphate solution (pH 9 for our concentration)
- The HBSS on the "calcium side" turned to orange as we added the calcium solution. It means that the pH decreased. This decrease is due to the acidic pH of the calcium chloride solution (pH=5.5) and probably also to the formation of hydroxyapatite because the EBSS contains phosphate ions.
- The next day, the scaffolds, that were red (color of the HBSS) before, had turned to yellow. It was due to the formation of hydroxyapatite, this color had two meanings for us: (i) HA is forming inside the scaffold and (ii) the pH inside scaffold must be around 4. The next step will be to correct the pHs of both
solutions and/or to add a stronger buffer in filling solution in the beginning of the experiment.

- After 4 days, two scaffolds have been removed. Their pH is around 4 and their structure is the same as the previous ones (see fig.21 below). The “phosphate”-side is agglomerated and the other side consists of non agglomerated alginate balls (stiffer and smaller than before). There is also white precipitation at the bottom of the scaffold is expected to be hydroxyapatite.

Fig.21 HBSS based scaffold

- After 5 days, the third scaffold has been removed, put back in the experiment and the contents of the tubes have been switched in order to improve the “bad side”. After two extra days, the scaffold is removed from the experiment. Unfortunately, the “bad side” has not been much improved (see fig.22, below). Both of the extremities seemed homogeneous but the middle (and main) part was not regular.

Fig.22 HBSS based scaffold improved

- A precipitate has been observed in the phosphate tube. IR Analysis, showed that it was hydroxyapatite. Thus, it is clear that HBSS does not prevent the formation of HA with the current method.

In order to have a more regular addition of the calcium and phosphate solution, a roller pump (Gilson, minipuls 4, see fig.23) was used.
2. Fifth scaffold:

i. Protocol:
The protocol of this experiment is almost the same as before, except for the use of the pump. The flow has been set to 1 ml/h (see calculations below).

Flow is given by: \( Q = \nu S \)

With: \( \nu \) the speed of the fluid in m.s\(^{-1}\)
given by: \( \nu = 2\pi RN \) with \( R \) the radius of the pump in m
N the rotation speed of the pump in rpm
and \( S \) the cross section of the tube in m\(^2\)
given by: \( S = r^2 \) with \( r \) the radius of the tube in m

Consequently: \( Q = 2\pi RN\pi r^2 \leftrightarrow N = Q/(2\pi^2 R r^2) \)

With: \( R = 82.5 \text{ mm} \quad r = 0.19 \text{ mm} \quad \text{et } Q = 16.67 \times 10^{-3} \text{ cm}^3.\text{min}^{-1} \)
\( N = 0.36 \text{ rd.min}^{-1} \)

ii. Comments:
- The colors of the solutions changed the same way as in the fourth scaffold, i.e. the “phosphate-side” became pink, and the calcium one turned to orange. After verification with pH-paper, pH were found to be around 6 for the calcium and around 8 for the phosphate.
- The scaffold turned to yellow (acidic pH is an indication of the formation of HA).
- Unfortunately, the phosphate tube has been crushed in the pump, so the experiment has been stopped before the whole quantity (20 ml of each) of solutions has been added.
3. Sixth scaffold:

In order to go further in the improvement of this promising experiment, it has been decided to design a new device. It has been agreed that the following requirements had to be met:

- The new device must be able to be sterilized (i.e. it can stand water vapor and temperatures around 130°C)
- It must be possible to look through the device, in order to see how and where mineralization is occurring.
- It has to be easy to set up and to be put in an incubator
- It must be able to let air going inside, to oxygenate the cells eventually.
- It has to be designed in order to work with the pump we are already using.

These requirements have lead to the device (fig.24) presented below.

![Device used in designing the 6th scaffold](image)

For this 6\textsuperscript{th} experiment, special care has been taken to correct the pH, by this means:

- The pH of the phosphate solution ([Na\textsubscript{2}HPO\textsubscript{4}]=0.6 mol.l\textsuperscript{-1}) has been decreased from 9.00 to 7.43 by addition of highly concentrated nitric acid ([HNO\textsubscript{3}]=30\%).
- The pH of the calcium solution ([CaCl\textsubscript{2}]=1 mol.l\textsuperscript{-1}) has been increased from 5.51 to 6.99 by addition of sodium hydroxyde ([NaOH]=0.1 mol.l\textsuperscript{-1}).
- HEPES buffer has been added to the HBSS used to fill the tubes in the beginning of the experiment.

i. Protocol:

Chemicals used:

- pH corrected calcium solution ([CaCl\textsubscript{2}]=1 mol.l\textsuperscript{-1})
- pH corrected phosphate solution ([Na\textsubscript{2}HPO\textsubscript{4}]=0.6 mol.l\textsuperscript{-1})
- HBSS
- HEPES (1mol.l⁻¹)
- HBSS-alginate balls

Other materials used:
- The new device
- The pump

The central part of the device has been filled with 4.02g of HBSS-alginate balls. The balls were kept inside this part with the help of the glass filter (the diameter of these filters has been reduced to 24mm in order to be more easily removed). The tubes were then filled with HBSS and HEPES solution, 40ml and 10ml, respectively. This experiment is showed in fig.25.

Fig.25 6th scaffold experiment

ii. Comments:
- Filling solution was clearer than the HBSS previously used, it tended to orange-red. It must be due to the buffer, because the pH, at this stage, had no reasons to be below 7.4.
- The volume of solution added was not as expected. About 8ml of each of the solutions have been added to the experiment in 18.5 hours (i.e. instead of 18.5ml). This could be because of the size of the tubes (they are too thin to be used with the pump).
- Mineralization is occurring in the scaffold: the scaffold seemed homogeneous from the phosphate side to 5mm, and then the mineralization seemed to occur under a diagonal going from the top of the “nearly homogeneous” part to the
bottom of the calcium filter. Consequently, it has been decided to rotate as it seemed that the HA crystals were falling down. After a while, a new mineralization occurred at the bottom of the central part.

- There has been a precipitate in the calcium tube. It was probably due to the phosphate ions present in the filling solution.

After these observations, the calcium tube was crushed in the pump. This accident lead to this amount of solutions delivered:

- \([\text{CaCl}_2]\sim12\text{ml}\)
- \([\text{Na}_2\text{HPO}_4]\sim18\text{ml}\)

The pH of the solutions has been checked:

- phosphate solution: 7,2
- calcium solution: 6,8

Moreover, the scaffold has slightly turned to orange during the experiment before turning back to red-orange (color of the filling solution). It means that the pH did not decreased too much.

These results show that it is possible to keep the pH not too far from 7,4, with our protocol, as HA is forming.

The scaffold (see fig.26), still not homogeneous, presented more mineralization than the previous ones.

![Fig.26 6th scaffold]
Chapter 3. Mechanical tests

Mechanical tests have been performed on the first and the second water based scaffolds and on the first HBSS based scaffold. These tests aimed at assessing the mechanical properties of the scaffolds previously designed. The tensile machine used was MTS, 810 Elastomer test system (see fig.27).

Fig.27 MTS, 810 Elastomer test system

The mechanical tests performed for the three samples exposed were compression-relaxation tests. The structures were first compressed until they reached 30% of their initial height. The common properties are first described for the three samples, then the samples are described on their own.

1 Common results:

For all of the samples, a water leakage has been observed during the compression test. It confirms not only that we designed a biphasic material, but also that the scaffold is porous. Another observation, made after the test, showed no cracks on the scaffold, the structure has not been broken during the test. It showed also that the scaffolds has nearly only been plastically deformed.

Concerning the mechanical results, each scaffold showed a reinforcement during the test, i.e. the material is stiffer in the end of the test than in the beginning. The relaxation tests pointed out that there is a remaining elasticity in the scaffold, this result lead to the conclusion that we did not obtained neither a brittle material, nor a viscous one.
2. Individual results:
   a. First water based scaffold:
   The size of the scaffold before the test was
   The strain-stress curves are shown in fig.28-29, the time-stress one fig.30.

   \[
   \text{Compression of the first water based scaffold} \\
   (1\text{st part})
   \]

   \[
   \begin{array}{cc}
   \text{Stress (MPa)} & 0.02 \\
   \text{Strain} & 0.01 \\
   & 0.00 \\
   & 0 \\
   & -0.01 \\
   & -0.02 \\
   & -0.03 \\
   & -0.04 \\
   & -0.05 \\
   & -0.06 \\
   \end{array}
   \]

   Fig.28

   \[
   \text{Compression of the first water based scaffold} \\
   (2\text{nd part})
   \]

   \[
   \begin{array}{cc}
   \text{Stress (MPa)} & 0.02 \\
   \text{Strain} & 0 \\
   & -0.02 \\
   & -0.04 \\
   & -0.06 \\
   & -0.08 \\
   \end{array}
   \]

   Fig.29

   The first and final elastic modulus have been graphically determined as:
   \[E_{\text{first}} = 112,5 \times 10^{-3} \text{ MPa} \quad \quad E_{\text{final}} = 2.75 \text{ MPa}\]
Concerning the threshold of the relaxation test, it has been determined as $-17.10^{-3}$ MPa. Unfortunately, the values given before are not reliable due to the amount of noise. This scaffold was not stiff enough to be tested with our device and did not meet the requirements described in I.8.

b. Second water-based scaffolds:

The strain-stress curves are shown in fig.31-32, the time-stress one fig.33.
Fig. 32

The first and final elastic modulus have been graphically determined as:
\[ E_{\text{first}} = 1.66 \text{ MPa} \quad E_{\text{final}} = 2.6 \text{ MPa} \]

Relaxation of the second water based scaffold

Fig. 33

Concerning the threshold of the relaxation test, it has been determined as \(-6.0 \times 10^{-3} \text{ MPa}\). For this material also, the mechanical properties are far from the requirements.

c. First HBSS based scaffold:

The strain-stress curves are shown in fig. 34, the time-stress one fig. 35.
The first and final elastic modulus have been graphically determined as:

\[ E_{\text{first}} = 115 \times 10^{-3} \text{ MPa} \quad E_{\text{final}} = 1.1 \text{ MPa} \]

Concerning the threshold of the relaxation test, it has been determined as \(-15 \times 10^{-3} \text{ MPa}\). This last scaffold confirm the weak mechanical properties of our scaffolds. Moreover, the amount of noise did not help to characterize this material, that should be tested with another device.
Discussion

From the three requirements pointed out for the design of a scaffold for bone tissue engineering, one of them has not been reached. Actually, we did not manage to obtain a fully homogeneous scaffold. In addition, the compression-relaxation tests revealed weak mechanical properties. These two drawbacks made us thinking that work is still to be achieved before obtaining a strong and homogeneous scaffold.

Nevertheless the two other goals have been reached. Indeed, the pH is under control with the help of HEPES buffer, it has been proven in the last experiment executed. It has also been proved that the synthesis of hydroxyapatite occurred inside the scaffold.
Future

There are several steps needed to be carried out in the close future:

- First of all, the device used in the last experiment must be improved because the mineralization does not occur homogeneously. It could also be interesting to create a flow on each side of the device, this could help to remove the excess of H$_3$O$^+$ ions due to the formation of hydroxyapatite and the waste due to the cells, when they will be seeded in the scaffold.

- Concerning the cells, they could already been implanted in the scaffolds in order to know if it is biocompatible or not.

- The scaffolds also need to be improved both in homogeneity and in mechanical properties. Homogeneity could be enhanced by switching the content of the tubes. As one of the both side (the phosphate side) show a good mineralization, this could improve the scaffold. Concerning the mechanical properties, a further step can be made by the addition of collagen fibrils in the alginate solution. As collagen type I represents about 30% of the dry weight of the bone this step would bring our scaffolds closer, in composition, to the bone.

- Another step must be to find a way to remove the alginate from the scaffold. If the alginate should be very useful as a cell carrier to ensure the homogeneity of cell seeding, a way to take it out of the scaffold must be found.
Aknowledgements

First of all, I would like to thank my coaches, René von Donkelaar, Bert van Rietbergen, Nico Somerdijk and Jack Donners for their help, their pieces of advice and their ideas.

Then, my thanks go to everybody in the lab, who helped me to find a becher, a screwdriver, a calculator, a key, etc.

Finally I wish to thank all the people who made me enjoy myself in Eindhoven this last five month, including my roommates, my team mates, my compatriots and the people already cited before.
References


