Synthetic bone grafts for treatment of femoral head necrosis

Sebastian Borg
Abstract

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Necrosis of the femoral head is a relatively common medical condition that radically decrease the quality of life for the patient. Left untreated it could lead to destruction of the hip joint. A common treatment is using bone autografts, also called bone chips. However, in the last decades synthetic bone grafts have become a very interesting alternative. This thesis had two aims; to evaluate how porous hydroxyapatite grafts with varied pore size could be produced by different size of the porogen and to create two-phase cements which would form pores in situ by using a dissolvable phase of calcium sulfate hemihydrate. The phase composition, morphology, porosity and pore size distribution were characterized with x-ray diffraction, scanning electron microscopy and micro-computed tomography. It was found that hydroxyapatite granules could be produced and it was possible to vary their pore size to some extent by changing the size of the porogen. At physiological temperature, pores were formed in the two-phase cements from one week and onwards.
Populärvetenskaplig sammanfattning


På senare tid har användandet av syntetiska keramiska implantat ökat. Fördelen är att med dessa undviker man de risker som finns med autotransplantation och de kan tillverkas av relativt billiga råmaterial. De keramiska implantaten består i regel av samma mineral som ben består av, vilket är hydroxyapatit. Detta gör att implantatet är biokompatibelt och kan stimulera bentillväxt. Man säger att implantatet är osteokonduktivt.

För att maximal bentillväxt ska ske bör implantatet likna mänskligt ben så mycket som möjligt. Ben är poröst, därför är det viktigt att anpassa implantatet så att det har porer av liknande storlek och antal som benet självt. Syftet med denna studie var att framställa implantat i form av granuler med varierande porstorlek, samt att undersöka om porer kan bildas i ett keramiskt cement som kan injiceras i det skadade benet vid kroppstemperatur. Cementet stelnar sedan på plats i kroppen.

Granuler av hydroxyapatit och cementen framställdes genom våtkemiska processer. De analyserades med röntgendiffraktion, svepelektronmikroskopi och datortomografi i mikroskala. Det visade sig att det är möjligt att variera porstorleken i granulerna genom att använda olika storlek på pormedlet, vilket är en biokompatibel polymer som kan sköljas ur materialet och på så vis skapa kvarvarande hålrum. Dock visade det sig också att mängden hydroxyapatit som bildades varierade i granulerna och mycket tyder på att tiden i vatten då pormedlet sköljs ut.

Cementen kunde också beroende på sammansättning bilda porer efter en tid. De cement som hade en större mängd pormedel bildade snabbare och fler porer medan de med en mindre mängd bildade porer långsammare och i mindre utsträckning. Förutom hydroxyapatit utgjordes slutprodukten i cementen också av kalciumsulfat vilket också är biokompatibelt. Ämnet löses upp snabbare i kroppen och på så vis kan läkningsprocessen skyndas på.
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1. Background

1.1 Introduction

Necrosis of the femoral head is a problematic medical condition, which dramatically decreases the quality of life for an increasingly aging population. The disease usually progresses and the femoral head is eventually degenerated over time, ultimately leading to failure of the joint [1]. The disease occurs mostly between 30 and 50 years of age and is relatively common. However, the pathogenesis is not fully clear [2,3]. It is possible to give the patient an early treatment if the disease is discovered in an initial stage, which can prevent the destruction of the femoral head. An early treatment could drastically decrease the suffering for the patient and make a hip replacement avoidable.

Implants in the form of bone autografts, or bone chips, which is bone taken from another site on the patient, has during a long period been proved to be a very effective in treating this disease as well as other critical bone injuries [4,5,6]. Autografts possess four very important properties that are crucial for a good implant or graft:

- Osteointegration: Bonding of the implant to the surrounding bone.
- Osteoconduction: Bone growth on the implant.
- Osteoinduction: Differentiation of stem cells from the surrounding tissue into osteoblasts (bone cells).
- Osteogenesis: Formation of new bone from osteoblasts in the graft.

Bone chips have been used since the 1800s and are widely considered to be the best treatment for critical bone injuries, i.e. injuries where bone is missing and complete healing cannot occur by itself. As described above, the purpose of the bone autograft is to stimulate new bone to grow into the site of the injury and gradually replace the autograft for a complete healing of the injury [7]. Although this treatment usually gives good results, it involves problems since it requires two surgeries and can lead to donor site morbidity as well as other severe medical conditions [8,9]. Using bone allografts instead can mitigate these problems. An allograft is bone, which is taken from another patient and can be saved in bone banks. The use of allografts only requires one surgery but this method puts the patient at risk of disease transfer. Also, when the bone is stored, the cells die, therefore this type of implant lacks osteogenic properties. Due to these problems, the demand for synthetic bone grafts is steadily increasing.

In modern orthopedics, synthetic grafts for a wide range of applications are made with material from three groups; metals, polymers and ceramics. More specifically, the most used materials are Ti alloys, Co-Cr alloys, ultra-high molecular-weight polyethylene (UHMWP), high-density polyethylene (HDP), aluminum oxide and calcium phosphates. Two examples of applications are screws for bone injuries (Ti alloys) and sockets for artificial femoral heads (UHMWP).
1.2. Synthetic bone grafts
During the last decades, different ceramic materials have proved to be a very promising group of materials to replace bone autografts and allografts. They eliminate the risk of disease transfer, they can be stored for a long time, they are made of abundant raw materials and they are osteoconductive [5,10]. They can also be used for injuries of all sizes since they can be custom made.

The by far most common group of ceramic materials is calcium phosphates (CaPs). Important compounds in this group of materials are hydroxyapatite (HA), beta-tricalcium phosphate (β-TCP) and brushite. Their stoichiometric formulas are as follows: \( \text{Ca}_5(\text{PO}_4)_3(\text{OH}) \), \( \text{Ca}_3(\text{PO}_4)_2 \) and \( \text{CaHPO}_4\cdot2\text{H}_2\text{O} \) respectively. It is worth noting that β-TCP is the stable phase of TCP at room temperature, which is rhombohedral. β-TCP has shoved good results in different studies and resolves more rapidly than HA. At the same time, it’s osteoconductive properties leads to formation of new bone. The new bone then takes the place of the β-TCP implant, as in the case with bone auto- or allografts [11,12,13]. Although, the mineral part of human bone is mainly made up of HA and it has been used as an orthopedic implant material for a long time. It also possess osteoconductive qualities.

Apart from the stoichiometry and the crystal structure of the material, the morphology is also very important for the success of the implant. The in vitro and in vivo properties are influenced by the size, form, porosity and surface structure of the implant [14,15,16,17]. Generally, in order to induce osteocondution and osteointegration, the surface are must be large enough to facilitate osteoblast growth. It is possible to make a solid implant which is made to fit the injury, but the use of granules would provide a much larger surface area since the cells then can grow in between the individual granules. Natural bone is not solid, it has a network of pores. Therefore, it is logical to aim to make porous granules. The pores provide sites for cell ingrowth and vascularization.

Ceramic implants can be produced in several ways. Different casting methods and sintering are common. But implants can also be produced by wet-chemical processes, resulting in a calcium phosphate cement (CPC). Ceramic grafts made by wet-chemical processes have some advantages over grafts made by traditional methods, such as sintering, when the final product is HA [18]. The characteristics of the non-sintered the implants are more similar to human bone. CPCs can be used directly by the surgeon, but this can be difficult due to fast setting time. It can also be used to produce a solid implant of a certain form and size, as in the case of this study.

1.3. Porosity
Different studies [17,19,20] have concluded that the critical pore size diameter for bone formation is around 100 µm. If the pores are smaller, they are penetrated by osteoid mineral and fibrous tissue. The smallest pores are only penetrated by fibrous tissue. In order to get vascularization, the formation of blood vessels, pores with a size of several hundred micrometers are required. The higher the grade of vascularization, the more oxygen and nutrients are
transported into the material, which makes new bone form faster. It is easy to believe that the pore size should be as large as possible, but the best results have been obtained in implants with mid-size pores. This means that a pore size in the range of 200-600 µm is better than a size range of 500-800 µm. Also, the pore size should be of smaller magnitude than the implant size. The amount of pores, called the porosity, is important as well. As with the pore size, higher porosity provides more space for cells and blood vessels. A higher amount of pores is generally better than a lower amount, but a porosity in the mid-range is preferable as in the case of pore size. For example, a porosity of 65% or 75% is better than a porosity of 25% but 65% is better than 75% [19]. Another important considerations also has to be made. The mechanical properties are inversely related to the pore size and porosity. As the total void volume of the implant is increased, the mechanical strength is decreased [16,17,19,20]. While HA is the least soluble of all calcium phosphate salts, its resorbability is related to the pores as well. It has been showed that higher porosity will lead to faster resorption [21].

Pores can be created in several ways. In the case of CPCs, the pores are usually made by adding a material to the cement that either dissolves in the body, which is the case for surgical usages, or by a material that can be leached from the implant when the CPC is used to produce a graft for implantation. A material used for creating pores is called a porogen. The material that is leached can for example be a soluble salt or a soluble polymer [22,23]. The substance used to produce pores should be biocompatible and cannot show any cytotoxic properties (toxicity to cells). In the case of CPCs mixed and used directly by the surgeon, pores can be created in vivo, for example by making a two-phase cement, which is one of the aims if this study. The method involves a cement consisting of calcium phosphate and calcium sulfate. The sulfate dissolves more rapidly than the phosphate, resulting in a gradually more porous graft [24]. Results have shown that when CaSO₄•0.5H₂O (calcium sulfate hemihydrate, CSH) is mixed with calcium phosphate and immersed in Hank’s solution, which is a salt solution buffer providing a physiological pH, the CSH first forms CaSO₄•2H₂O (calcium sulfated dihydrate, CSD). The CSD then dissolves, leading to weight loss and an increase of porosity. The addition of calcium sulfate could also decrease the setting time of the cement [25].

1.4. Setting of calcium phosphate cements
The most common type of cement is Portland cement, which is the cement used to manufacture concrete for construction and similar applications. Just as Portland cement, CPCs contains a solid powdered phase and a liquid phase. When these phases are mixed, a setting reaction will occur. This reaction is what makes the cement hard and rigid.

The simples system for making an apatite-forming CPC uses only one component in the powder phase and is based on the hydrolysis of alpha-tricalcium phosphate (α-TCP). α-TCP has monoclinic structure to the rhombohedral β-TCP. The product of the reaction is calcium deficient HA (CDHA) [26]:

\[
3α-Ca_3(PO_4)_2 + H_2O \rightarrow Ca_9(HPO_4)(PO_4)_5(OH) \] (1)
This system is fundamental for this thesis and is the basis of the method used for producing HA. Generally speaking, the setting of an apatite-forming reaction is always a reaction between one or several calcium phosphate salts and water, where dissolution and precipitation occurs [26]. When the powder and liquid phases are mixed, the water becomes supersaturated with ions and crystals start to precipitate [27]. The driving forces of the dissolution and precipitation are the thermodynamic solubility products of the calcium salts. The solubility products and Ca/P ratios of the calcium phosphates relevant for the thesis are presented in table 1 [28].

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Ca/P</th>
<th>-log K_{sp} at 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-TCP</td>
<td>α-Ca₃(PO₄)₂</td>
<td>1.5</td>
<td>25.5</td>
</tr>
<tr>
<td>β-TCP</td>
<td>β-Ca₅(PO₄)₃(OH)₆</td>
<td>1.67</td>
<td>58.4</td>
</tr>
</tbody>
</table>

An important note is that the HA obtained from wet-chemical processes are rarely stoichiometric. The composition of HA in CPC applications is generally expressed as Ca_{10-x}(HPO₄)(PO₄)_{6-x}(OH)_{2-x} [26]. Stoichiometric HA has a Ca/P ratio of 1.67 but CDHA can have ratio ranging from 1.5 to 1.67 [29]. It is believed that the change in stoichiometry does not change the crystal arrangement to any larger extent. The final phase of a CPC can be analyzed using XRD but usually requires additional analysis with Fourier-transform infrared (FTIR) spectroscopy to determine the Ca/P ratio [30]. Although an exact determination of the Ca/P ratio of the obtained final phase is considered to be outside the scope of this thesis.

The kinetics of the α-TCP hydrolysis have been studied by Ginerba et.al. [31] and later by Durucan and Brown [32]. In the first study, it was proposed that a model with two rate-limiting mechanisms can describe the hydrolysis of α-TCP. Initially, rate is limited by the surface area of the reactants. Then it is limited by diffusion through the hydrated HA layer on the α-TCP particles. Although, the basis if this model was obtained from XRD data. Durucan and Brown used isothermal calorimetry to examine the mechanism further, due to the limitations of quantitative analysis by XRD. They concluded that the rate of the hydrolysis is indeed limited by the surface area of the reactants. A smaller particle size gives a higher reaction rate. But in the second step, the kinetics are controlled by nucleation and growth mechanism. The also concluded that even though HA is formed on the surface of the α-TCP particles, it does not inhibit the reaction. This is due to the morphology of the HA, the crystals are generally shaped like plates. The hydrolysis process in illustrated in figure 1. The illustration is revised from Jiménez and Benjamín [33].
The setting time is of great importance when a CPC is used in surgery. The surgeon must be able to handle the cement long enough to apply it to the surgical site but it has to set fast enough to become a stable implant. Although, like mentioned earlier, a CPC can also be used outside the operating room to produce a scaffold, in the case of this study in the form of porous granules.

In several studies, the effect of additives to the CPC mixture has been examined. To produce HA, HA seeds can be added to the mixture. These seeds will help the formation of HA crystals, and therefore shorten the setting time \[34,35\]. More commonly, additives aiming to increase the amount of phosphate ions in the liquid phase, and by that provide a setting accelerator have been studied. Compounds containing phosphate ions could for example be \(\text{Na}_2\text{HPO}_4\), \(\text{KH}_2\text{PO}_4\) or \((\text{NH}_4)\text{H}_2\text{PO}_4\). All these compounds have been found to significantly decrease the setting time of a HA-forming CPC \[35\]. Different organic acids have also proved effective as setting accelerators. An example is citric acid, which in a study decreased the setting time for all experiments when the concentration was increased \[36\].

The dissolution and precipitation of HA in a CPC can be analyzed with synchrotron x-ray radiation \[37\]. In this experiment, the powder phase consisted of dicalcium phosphate anhydrous (DCPA) and \(\text{Ca(OH)}_2\). The liquid phase was a 1.0M \(\text{Na}_2\text{HPO}_4\) solution. The results indicated that the amount of DCPA both increases and decreases during the setting. After 24 hours, around 75% of the initial salts had transformed into HA. DCPA is more soluble in the body than HA though, like mentioned earlier HA is the least soluble of the calcium phosphates. Therefore, it is reasonable to believe that some time after implantation, the implant will have a phase composition with more than 75% HA.

An important factor influencing the setting time is the temperature. It has been showed that an increase in temperature from 25°C to 37°C decreases the setting time of an apatite-forming cement \[38\]. In this study, the mechanical strength also increases when the temperature is increased, while the samples were soaked in Ringer's solution. Ringer's solution is an isotonic salt solution. Although, after setting a complete HA formation had not occurred. There was still unreacted \(\alpha\)-TCP left in the sample. It seems like this is due to HA forming a layer on the surface of the \(\alpha\)-TCP. But in conclusion, in a temperature range from room temperature to body temperature, a higher temperature accelerates the setting and the HA formation. The influence of increased temperature was also studied by Durucan and Brown \[32\]. The rate of the hydrolysis increased gradually when the temperature was elevated to 45°C and 56°C compared to 37°C.
1.5. Objective of the study
In this study, synthetic porous bone grafts were produced by using CPCs. Two cement types were evaluated, one which was used to produce porous HA granules for implantation and one which was a two-phase cement consisting of calcium phosphate and calcium sulfate for injection at the injured site.

1.5.1. Objective 1
The first and main objective of the study was to produce HA granules with different pore sizes by using different sizes of the porogen, which was poly(ethylene-glycol) (PEG). Three size fractions will be used, 100-600 µm, 100-200 µm and 200-400 µm. 100-600 µm was used in earlier studies and was considered the reference.

1.5.2. Objective 2
The second objective of the study was to evaluate if pores can be formed in injectable two-phase cement in situ. Since calcium sulfate is more soluble than HA, the aim was to create a cement which forms HA while pores are gradually created by dissolution of the calcium sulfate phase. PEG was also added to accelerate the dissolution since it is water-soluble. Four different ratios between calcium sulfate, PEG and calcium phosphate were evaluated. The size of the PEG used was 100-600 µm since it is the reference for the granules.

2. Methodology
2.1. X-ray diffraction (XRD)
X-ray diffraction is a very common method used in basically all areas of solid-state material research. It can provide information about lattice parameters, crystal orientation, grade of crystallinity and phase composition. The method is based on the principle of elastic scattering of x-rays on the electrons of the atoms in a crystalline solid [39]. Bragg's law describes the concept:

\[ n\lambda = 2d \sin\theta \] (2)

This model states that each reflection that gives rise to a signal represent a crystal plane in the sample. The orientations of these planes are represented by their Miller indices (hkl) and the space between these planes is \( d \). The model further says that diffracted x-rays from adjacent planes will give interfere constructively when the angle \( \theta \) between the plane and the x-rays result in a path-length of an integer \( n \) times the wavelength \( \lambda \). The schematics of Bragg's law can be seen in figure 2. In XRD with Bragg-Brentano geometry, the tube and the detector moves at the same time.
The wavelength used in XRD analysis is always well defined. The most common x-ray tube anode material is copper. While the anode emits several wavelengths, the one of main interest in XRD analysis is Kα-radiation, which is the strongest x-ray spectral line. The emission of Kα-radiation occurs when an electron jumps to the 1s orbital from the 2p orbital. The Kα-radiation actually contains two energies. The difference is due to different electron spin in the L shell. There is also Kβ-radiation, which occurs when an electron jumps from the 3p orbital to the 1s orbital. In XRD, the x-rays are usually monochromized to only contain Kα-radiation, in order to get more exact data.

One main purpose of XRD powder diffraction is to calculate the phase composition. While the mathematics behind phase composition calculations is quite established, there are some factors that can create accuracy problems with the calculation. Examples include resolution of overlapping peaks, peak intensity, peak position, preferred orientation, size of the crystallites and microabsorption. The latter is the largest source of error when conducting quantitative phase analysis [40].

There are several refinement methods for mitigating the sources of error usually occurring. Single-peak methods are common, where one peak is refined individually. In Rietveld refinement however, the whole diffractogram is included and all reflections are included [40,41]. The refinement works by calculating a diffraction patterns from a given structure, which is the fitted to the data by reducing the least square. Since the refinement is a structure refinement, the phases must be determined before performing the refinement. Since the method includes the whole diffraction patterns, problems like overlapping peaks can be avoided. Therefore, Rietveld refinement can provide a fitted diffractogram, from which the phase composition of a multi-phase material can be calculated. In this thesis, Rietveld refinement and phase composition calculations are conducted with a “blackbox” software which performs the refinement and phase composition calculations automatically.
2.2. Micro-computed tomography (μCT)

There are several methods used to determine the porosity of CPCs. The most commonly used are helium pycnometry, mercury intrusion porosimetry and using tabulated density data to calculate the porosity of the sample. Although, these methods have drawbacks in being destructive and having long analysis time. The use of tabulated data assumes that the samples are completely pure and crystalline [42]. The porosity could as well be calculated from XRD data but this also assumes a pure sample with no amorphous parts. BET is also a quite common method.

The study by Unosson et. al. suggests that more simple methods involving weighting the samples after wetting, then drying them and weighting them again exists, but they are showing slightly different results, and therefore it is advisable that several methods are used together if this approach is applied. The methods described in this study cannot measure the size distribution of pores either, apart from mercury intrusion porosimetry [43,44].

Micro-computed tomography (μCT) is a type of x-ray-computed tomography, which uses a micro-focused x-ray tube. The resolution can be as low as 0.5-1 µm. X-ray-computed tomography is currently used as a medical diagnose procedure. The method described in this topic can be seen as scaled-down version. It is non-destructive and can in a relatively short timespan provide a lot of information about a small sample of trabecular bone [45,46]. Compared to for example BET analysis, the amount of information obtained from μCT is superior.

The method works by creating many 2D images of the sample by using an x-ray tube with a microfocus as a source and an array CCDs as a detector. Contrast between different bone densities and void volume is due to x-ray absorption of bone [47]. Like in regular x-ray-computed tomography, the sample is stationary and the source and the detector moves around the sample. Each 2D image is a slice of the sample. The inner structure of the sample can easily be visualized by using the 2D analysis, sine every slice is a cross-section of the sample. All 2D images can also be reconstructed using software to create a 3D image of the sample. In this 3D image, a volume of interest (VOI) can be chosen, which can be analyzed further to obtain parameters such as the bone volume density and the porosity [48]. Since all the data is treated using software, it is simple to calculate not only the total porosity, but also the distribution of pores [47]. The spatial resolution of the 2D scans can generally be set to fit the sample size. Although, a higher resolution equals a more time-consuming analysis.

3. Experimental

3.1. Porous granules

Porous granules where made by mixing 1.940 g of α-TCP (729054, RMS Foundation, Switzerland), 0.060 g of β-TCP (21218, Sigma Aldrich, Germany) and 3.0 g of 20000 g/mol PEG (81300, Sigma Aldrich, Germany). Before weighting the PEG, it was melted at 70°C on aluminum foil for one to two hours. After melting, the PEG was allowed to cool and solidify at room temperature,
after which it was molded using a mortar and sieved to 100-600 µm. PEG with size fractions of 100-200 µm and 200-400 µm was also prepared with the same method. Sieves with 100, 200, 400 and 600 µm mesh were used to create the size fractions. The distribution of PEG size fractions in the 100-600 µm powder was 41.07%, 41.19% and 17.74% for size fractions 100-200 µm, 200-400 µm and 400-600 µm. The powder phase was mixed for five minutes using a CapVibrator (Ivoclar Vivadent, USA). A liquid phase consisting of a 2.5% Na$_2$HPO$_4$ (S9763, Sigma Aldrich, Germany) solution was prepared using a volumetric flask. A portion of 0.8 mL of the liquid phase was added to the powder phase using a pipette (Pipet-Lite LTS Pipette L-1000XLS+, Mettler Toledo, Switzerland). The cement was mixed for one minute using a CapVibrator (Ivoclar Vivadent, USA). After mixing, the cement was molded in Teflon molds with a diameter of 1.2 mm and a height of 1.2 mm. The molds with the cement were covered and left to set in room temperature for 48 hours. After this, the granules were demolded and put in a glass beaker. 400 mL of water were added and the granules were swirled around while the water was changed a couple of times. Afterwards, the granules were stored in 70°C in 400 mL water for four hours to leach them of the PEG. The water was changed each hour. After leaching, the water was removed and the granules were stored again in 70°C for 48 hours, for drying. When dried, the granules were autoclaved for 60 minutes at 120°C.

Three batches were prepared, one batch with each PEG size fraction. The first batch is larger than the other two since this batch was used in animal studies at Peking Union Medical Hospital. These studies required 5-10 grams of finished granules, and ten moldings were found to produce enough granules. The other batches were only analyzed at Uppsala University. Three moldings were considered enough to produce enough granules for convincing results.

Before the three main batches a small initial batch was produced to learn the method and study the HA formation. This batch was produced using 100-600 µm PEG. An overview of the batches and the numberings of the moldings can be seen in table 2. Note: the granules from molding C3 were accidentally left in water for a total of 22 hours instead of 4 hours.

### Table 2. Overview of granule batches.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Number of moldings</th>
<th>Molding number</th>
<th>PEG size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>1</td>
<td>-</td>
<td>100-600</td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>A1-A10</td>
<td>100-600</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>B1-B3</td>
<td>100-200</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>C1-C3</td>
<td>200-400</td>
</tr>
</tbody>
</table>

#### 3.2. Two-phase cement

Cements with four different ratios between calcium phosphate and CSH (DentalGips X-Hard, Bo Ehrlander AB, Sweden) were prepared, see table 2. PEG with a size of 100-600 µm was also added in different amounts.
Table 3. Amount of calcium sulfate, PEG and calcium phosphate for the two-phase cements.

<table>
<thead>
<tr>
<th>Cement #</th>
<th>CSH (g)</th>
<th>PEG (g)</th>
<th>α-TCP (g)</th>
<th>β-TCP (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>0.2</td>
<td>0.582</td>
<td>0.018</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>0.2</td>
<td>0.291</td>
<td>0.009</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>0.5</td>
<td>0.291</td>
<td>0.009</td>
</tr>
<tr>
<td>4</td>
<td>0.2</td>
<td>0.4</td>
<td>0.388</td>
<td>0.012</td>
</tr>
</tbody>
</table>

All cements were mixed with 0.4 mL 2.5% Na$_2$HPO$_4$ solution. The cements were then molded in silicon molds with a diameter of 3 mm and a height of 1.2 mm, producing cylindrical samples. The cements were allowed to set for 48 hours in room temperature.

After setting, a dissolution test was conducted by soaking the samples in a 0.01 M phosphate-buffered saline (PBS) solution (P4417, Sigma Aldrich, Germany). The weight ratio between cement and solution was 0.5 g per 100 mL and the test was conducted in an incubator at 37°C on a rocking platform shaker (Duomax 1030, Heidolph Instruments GmbH, Germany). After 7, 14 and 28 days, samples were removed from the solution and dried at room temperature for 48 hours to prepare them for characterization. The solution was changed after 3, 7, 10, 14, 17, 21 and 24 days. Before changing the solution 2 mL was saved, as well as after 28 days, for later analysis. The samples removed after 28 days were also saved for later analysis.

3.3. Characterization

3.3.1. Granules

All samples were characterized with XRD (D5000, Siemens AG, Germany) (Bragg-Brentano geometry, 0.2 mm detector slit), scanning electron microscopy (SEM) (TM-1000, Hitachi, Ltd, Japan) and µCT (Skyscan 1172, Bruker Corporation, Germany). The XRD data was refined and the phase composition was calculated from the refined diffractograms data using “blackbox” Rietveld refinement software (Profex, Nicola Döbelin, Switzerland). XRD, SEM and µCT were performed on the granules when they were leached and dried and on the two-phase cement after the dissolution test and the following drying.

The granules were also analyzed with XRD after autoclaving, due to the results of the initial batches. The initial batch was only characterized with XRD, before-, after leaching and drying and after autoclaving.

Reference diffractograms for α-TCP, β-TCP and HA were obtained by analyzing the raw chemicals. For PEG, a reference diffractogram was obtained by analyzing the 100-600 μm powder.

The purpose of the XRD analysis was to examine the phase composition. SEM was used to characterize the morphology and porosity, and µCT was used to obtain information about the porosity, the size of the pores and the distribution.
of different pores sizes. SEM images were taken of the whole granules to characterize their size and porosity. Images were also taken of granules that were cut in half, to examine the pores inside the granules.

Due to sample volume and time limitations, three granules were randomly selected from each batch for μCT analysis. For batch 1, they were randomly selected from all moldings and for batch 2 and 3, one granule was selected from each molding. Images were acquired with a pixel size of 8 μm. For the porosity and pore size analysis and 3D imaging, Bruker DataViewer, CTan and CTvox software were used. All software was used with standard settings. The threshold value for binary selection in CTan was set automatically.

3.3.2. Two-phase cements
The two-phase cements were characterized after molding, 7 days and 14 days. They were characterized with XRD (D5000, Siemens AG, Germany) (Bragg-Brentano geometry, 0.2 mm detector slit) and SEM (TM-1000, Hitachi, Ltd, Japan). The aim of the XRD analysis was to examine the phase tranformations, the rate of HA forming and the dissolution of calcium sulfate and PEG. The same reference diffractograms as for the granules were used. The reference diffractogram for CSH was obtained by analyzing the raw chemical and the diffractogram for CSD was obtained from International Centre for Diffraction Data (ICDD).

The SEM was used to get initial information about morphology and porosity of the cements. Images were taken of whole samples and cross-sections for overview, as well in higher magnifications to examine the micro-structure of the surface and cross-sections.

4. Results
All diffractograms and images shown in this are considered to be representative for the whole batches. The displayed data show the trends observed in each analysis method.

4.1. Phase composition
4.1.1. Initial granule batch
The Rietveld refinement is exemplified in figure 3. The same procedure was followed for all data. Pictured is the refinement for the initial batch after leaching and drying. The obtained parameters were \( R_{wp} = 18.11\% \), \( R_{exp} = 16.97\% \) and \( X^2 = 1.13887 \). Two standard deviations were used for the calculated phase composition values, providing a confidence interval of 95.45%. This confidence interval was applied to all phase composition calculations.
The results of the initial batch showed that HA was formed during leaching and drying as well as the autocalving. The phase composition of the granules before leaching, after leaching and after autoclaving can be studied in table 4.

Table 4. Phase composition of the initial batch.

<table>
<thead>
<tr>
<th>Granule treatment</th>
<th>HA (mass %), (STD (%))</th>
<th>α-TCP (mass %), (STD (%))</th>
<th>β-TCP (mass %), (STD (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molded</td>
<td>3 (2.8)</td>
<td>97 (2.8)</td>
<td>0</td>
</tr>
<tr>
<td>Leached and dried</td>
<td>79 (1.1)</td>
<td>18 (0.98)</td>
<td>3.24 (0.9)</td>
</tr>
<tr>
<td>Leached, dried and autoclaved</td>
<td>90 (1.2)</td>
<td>7 (0.92)</td>
<td>3 (1.4)</td>
</tr>
</tbody>
</table>

A very small amount of HA was formed before leaching and drying. Most of the initial calcium phosphate has formed HA in this process, but an even higher amount of HA was obtained after following autoclaving. No β-TCP was present in the granules after molding according to the results of the refinement. In these granules, PEG was still present though and since no reliable structure file for PEG could be obtained, the refinement is only seen as a validation that HA was not formed to any large extent. The XRD patterns for these granules after the different steps as well as the patterns can be seen in figure 4. The most prominent peaks for HA, α-TCP and PEG are marked in the figure.
4.1.2. Granules batch A
In table 5, the phase composition for batch A (100-600 µm PEG) after leaching and drying is presented.

<table>
<thead>
<tr>
<th>Molding number</th>
<th>HA (mass %), (2σ (%)</th>
<th>α-TCP (mass %), (2σ (%))</th>
<th>β-TCP (mass %), (2σ (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>65 (1.6)</td>
<td>32 (1.34)</td>
<td>3 (1.36)</td>
</tr>
<tr>
<td>A2</td>
<td>63 (2)</td>
<td>32 (1.68)</td>
<td>5 (2.2)</td>
</tr>
<tr>
<td>A3</td>
<td>64 (1.72)</td>
<td>29 (1.36)</td>
<td>6 (2.2)</td>
</tr>
<tr>
<td>A4</td>
<td>75 (1.38)</td>
<td>23 (1.26)</td>
<td>3 (1.26)</td>
</tr>
<tr>
<td>A5</td>
<td>55 (1.3)</td>
<td>42 (1.24)</td>
<td>3 (1.02)</td>
</tr>
<tr>
<td>A6</td>
<td>71 (1.28)</td>
<td>25 (1.12)</td>
<td>4 (1.1)</td>
</tr>
<tr>
<td>A7</td>
<td>80 (1.22)</td>
<td>18 (1.14)</td>
<td>2 (0.88)</td>
</tr>
<tr>
<td>A8</td>
<td>61 (1.18)</td>
<td>37 (1.12)</td>
<td>2 (0.88)</td>
</tr>
<tr>
<td>A9</td>
<td>75 (1.32)</td>
<td>21 (1.14)</td>
<td>4 (1.16)</td>
</tr>
<tr>
<td>A10</td>
<td>77 (1.1)</td>
<td>21 (1.4)</td>
<td>2 (0.62)</td>
</tr>
</tbody>
</table>

In figure 5, the diffractograms for A4, A5, and A7 after leaching and drying as well as diffractograms for autoclaved granules from A3, A5 and A6 are pictured.
The latter molding numbers showed the highest increases in HA. The α-TCP peak \(30.8^\circ\) can be seen to vary in size in the diffractograms for the granules. A5, which had the lowest HA fraction after leaching, the α-TCP peak is the most prominent, and for A7 it is the least prominent. This molding had the highest HA fraction after leaching. The α-TCP peak at \(22.8^\circ\) also vary in the same way for the three molding dates. No β-TCP peaks are visible for any of the granules. The amount of β-TCP is varies slightly from the initial value the, which was 3%. Although, the variations are generally smaller than the standard deviation and such a small amount is difficult to detect with XRD, hence the lack of β-TCP peaks in the diffractograms.

The phase composition after autoclaving is presented in table 5.

**Table 6. Phase composition of batch A after autoclaving.**

<table>
<thead>
<tr>
<th>Molding date</th>
<th>HA (mass %), (2σ (%))</th>
<th>α-TCP (mass %), (2σ (%))</th>
<th>β-TCP (mass %), (2σ (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>67 (1.14)</td>
<td>31 (1.08)</td>
<td>3 (0.9)</td>
</tr>
<tr>
<td>A2</td>
<td>74 (1.24)</td>
<td>21 (1.02)</td>
<td>6 (1.3)</td>
</tr>
<tr>
<td>A3</td>
<td>76 (0.96)</td>
<td>21 (0.92)</td>
<td>3 (0.54)</td>
</tr>
<tr>
<td>A4</td>
<td>93 (0.96)</td>
<td>4 (0.64)</td>
<td>4 (0.84)</td>
</tr>
<tr>
<td>A5</td>
<td>79 (1.56)</td>
<td>8 (1.12)</td>
<td>13 (1.66)</td>
</tr>
<tr>
<td>A6</td>
<td>94 (1.28)</td>
<td>2 (1.08)</td>
<td>3 (0.92)</td>
</tr>
<tr>
<td>A7</td>
<td>95 (0.92)</td>
<td>2 (0.66)</td>
<td>3 (0.8)</td>
</tr>
<tr>
<td>A8</td>
<td>73 (1.38)</td>
<td>18 (1)</td>
<td>9 (1.62)</td>
</tr>
<tr>
<td>A9</td>
<td>86 (1.1)</td>
<td>11 (0.92)</td>
<td>2 (0.9)</td>
</tr>
<tr>
<td>A10</td>
<td>86 (1.02)</td>
<td>12 (0.96)</td>
<td>2 (0.56)</td>
</tr>
</tbody>
</table>
For all moldings, the amount of HA increased after autoclaving. A1, the increase was quite low 2% considering the standard deviation, the increase might have been even smaller. But for the other moldings, the increase was between 9-24%. The highest increase in HA was 24% for A6. In all cases, the amount of α-TCP decreased, the most for 10/10 where it decreased with 34 %. For the other moldings, the decrease was in the range of 8-23%, apart from A1 where the decrease was 1%, which is lower than the standard deviation. The β-TCP phase increased for significantly for A2, A5, and A8. The highest increase was for A5 where the β-TCP phase increased by 10%. No other significant changes were observed.

By studying at the diffractograms for the moldings with the largest increases in HA and decreases in α-TCP are pictured in figure 5, it can be seen that the α-TCP peak at 30.8° is not visible for A6 after autoclaving, which has the highest amount of HA of the three. It is larger for A3 and A5. Also the α-TCP peak at 22.7° is much smaller in A5 and A6. The one at 24° is only visible in A3. For A5, there is a peak at 27.7° after autoclaving. This is a β-TCP peak.

4.1.3. Granules batch B
The phase compositions of batch B after leaching and drying are listed in table 7.

<table>
<thead>
<tr>
<th>Molding number</th>
<th>HA (mass %), (2σ (%))</th>
<th>α-TCP (mass %), (2σ (%))</th>
<th>β-TCP (mass %), (2σ %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>49 (1.56)</td>
<td>48 (1.56)</td>
<td>3 (1.66)</td>
</tr>
<tr>
<td>B2</td>
<td>52 (1.12)</td>
<td>46 (1.1)</td>
<td>2 (0.8)</td>
</tr>
<tr>
<td>B3</td>
<td>66 (1.08)</td>
<td>32 (1.04)</td>
<td>2 (0.58)</td>
</tr>
</tbody>
</table>

HA was formed in all molding, but in overall less quantity than for batch 1. No significant difference in β-TCP was observed compared to before leaching and drying. The diffractograms for all three moldings after leaching and drying and after autoclaving can be seen in figure 6.
Figure 6. Diffractograms for leached and dried and autoclaved granules from batch B. Peaks for α-TCP and HA are marked.

In all diffractograms, peaks for α-TCP are present at 30.8° as well as at 22.8. The peak at 30.8° is the largest characteristic. Peaks for HA can be seen as well, the largest one is at 31.8°, which is expected. The proportions between the α-TCP and HA peaks are a little different from batch 1 where the HA peaks are more prominent. For the autoclaved granules from B3, the peaks are shifted about 0.5° to the left. After autoclaving, the α-TCP peaks at 22.8° and 30.8° are smaller compared to the HA peak at 31.8° than in the diffractograms after leaching and drying for B1 and B2. The difference in the diffractograms is very small for B3.

The calculated phase compositions after autoclaving are listed in table 8. Results showed a small increase in HA for B1 and B2. B3 no significant difference was observed for neither HA, α-TCP or β-TCP. These results differ from those of batch A. B1 had the highest increase in HA, 7%. For B2, the increase was 3%. The α-TCP decreased by 6% for B1 and 4% for B2. No significant difference in β-TCP was observed for any of the moldings.

Table 8. Phase compositions of batch B after autoclaving.

<table>
<thead>
<tr>
<th>Molding date</th>
<th>HA (mass %), (2σ %))</th>
<th>α-TCP (mass %), (2σ (%))</th>
<th>β-TCP (mass %), (2σ (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>56 (1.06)</td>
<td>42 (1.06)</td>
<td>2 (0.58)</td>
</tr>
<tr>
<td>B2</td>
<td>55 (1.2)</td>
<td>42 (1.18)</td>
<td>3 (1.22)</td>
</tr>
<tr>
<td>B3</td>
<td>66 (1.38)</td>
<td>32 (1.36)</td>
<td>2 (0.98)</td>
</tr>
</tbody>
</table>
4.1.4. Granules batch C

The phase composition of batch C after leaching and drying is listed in table 9.

Table 9. Phase composition of batch C after leaching and drying.

<table>
<thead>
<tr>
<th>Molding date</th>
<th>HA (%), (2σ (%))</th>
<th>α-TCP (mass %), (2σ (%))</th>
<th>β-TCP (mass %), (2σ (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>94 (1.32)</td>
<td>2 (0.94)</td>
<td>3 (1.14)</td>
</tr>
<tr>
<td>C2</td>
<td>47 (1.18)</td>
<td>51 (1.16)</td>
<td>3 (0.84)</td>
</tr>
<tr>
<td>C3</td>
<td>63 (1.26)</td>
<td>33 (1.2)</td>
<td>3 (1.18)</td>
</tr>
</tbody>
</table>

In this batch HA was formed as well but in varying quantities. The granules from C1 had more than twice as much HA as the granules from C2, which is notable. This amount was similar to some autoclaved granules in batch 1. The granules from C2 had a lower amount of HA on the other hand. The granules from C3 had an amount similar to several granules in batch 1. The amount of β-TCP was unchanged from the initial value for all moldings, these results were also obtained in batch B. The amount of α-TCP was varying between the dates reversely to the amount of HA.

The diffractograms for the granules after leaching and drying and after autoclaving can be seen in figure 7. As can be seen, the granules from C2 and C3 has the α-TCP peak at 30.8° while this peak is not present in C1. The HA peak at 31.8° is present for all dates but most prominently for C1. This phase composition results were in agreement with this. The diffractograms for C2 and C3 after leaching and drying have similarities to the ones obtained for batch B, while the diffraction data for C1 resembles the data for some of the autoclaved granules in batch A in figure 5.

In the diffractograms for the autoclaved granules, only a small difference can be seen for C1 and C2 compared to before autoclaving. For C3, the HA peak at 31.8° is more prominent than before the autoclaving, which was expected from the results of the initial batch. The α-TCP peak at 30.8° is smaller than before autoclaving and as well as the peaks between 22-24°. It can be noted that these peaks are very small for C1. There is no large difference in the diffractogram for this molding compared to before autoclaving.
The phase composition calculations after autoclaving are listed in table 10. The results are similar to those of batch B. For C1, no significance change was observed for any of the phases. The α-TCP phase decreased for C2 and C3 by 6% and 17%. The increase in HA for C2 and C3 was 6% and 15%. As well as showing the highest increase in HA, C3 also had an increase in β-TCP by 3%. C2 the change in β-TCP was not significant.

Table 10. Phase composition of batch 3 after autoclaving.

<table>
<thead>
<tr>
<th>Molding date</th>
<th>HA (mass %), (2σ (%))</th>
<th>α-TCP (mass %), (2σ (%))</th>
<th>β-TCP (mass %), (2σ (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>95 (1.22)</td>
<td>3 (1.18)</td>
<td>2 (0.56)</td>
</tr>
<tr>
<td>C2</td>
<td>53 (1.06)</td>
<td>45 (1.06)</td>
<td>2 (0.82)</td>
</tr>
<tr>
<td>C3</td>
<td>78 (1.3)</td>
<td>16 (1.06)</td>
<td>6 (1.38)</td>
</tr>
</tbody>
</table>

4.1.5. Two-phase cements

Diffractograms for cement 1 to 4 after molding are presented in figure 8. The results indicate that no CSD was formed after molding, only CSH was present. Peaks for α-TCP 22.7° and 24° are present as well, but no HA peaks are visible, suggesting that no or very little HA was formed after molding. The peaks for CSH at 14.7° and 26.7° are most prominent for cement 2 and 3 compared to the α-TCP peaks, but are present for all cements. CSD was expected to form during setting but is not present according to the XRD analysis. According to the results, PEG was also present. PEG peaks were expected since the cements were not leached.
Figure 8. Diffractograms for two-phase cements after molding. Peaks for α-TCP, PEG and CSH are marked.

The diffractograms for the cements after 7 and 14 days in PBS solution are presented in figure 9. For cement 1, it can be seen α-TCP had been hydrolyzed and HA was formed after 7 days. CSH was still present, although the peaks are relatively smaller than after molding, suggesting that part of this phase has dissolved. Peaks for both CSD and HA are present after 7 days for cement 2, at 12°, 20° and 29° for CSD and at 26° and 31.9° for HA. In this cement, α-TCP was also present after 7 days, and α-TCP peaks at 24° and 31.8° can be seen in the diffractogram. For cement 3 and 4, the diffractograms looks similar after 7 days. A complete α-TCP hydrolysis seems to have occurred, as well as dissolution of the CSH.
Some differences were observed after 14 days in PBS solution. As can be seen for cement 1, CSH peaks are no longer present. Instead, peaks for CSD at 12°, 20° and 29° can be seen. For cement 2, no CSH peaks are present either while the CSD peaks are relatively larger. The intensity of the α-TCP peaks is also smaller and the HA peaks are more prominent, suggesting that the α-TCP continues during day 8-14. No large difference can be seen in the diffractograms for cement 3 and 4 after 7 and 14 days, which indicates that all changes in these cements occur during the first 7 days.

4.2.1. Granules batch A

SEM analysis showed that cylindrical porous granules were obtained from all moldings, with a length of 1.8 mm and a diameter of 1.2 mm. Most pores visible from the outside have a cross section of 100-250 μm with some larger pores in the range of 300-500 μm. Pictured in figure 10 is an overview of a granule from molding A2 (A) and a granule from molding A8 (B). The pore size is in the same range as the porogen used.
As can be seen in figure 10, the granules have a slight cone shape. In 10B the granule also has a smoother surface at one end. The granules from some moldings were in general smoother and had a less porous surface than the others, for example from A7, see figure 11A. Figure 11B it shows an image of a granule from A8 in higher magnification. It can be seen that pores are linked together with other pores, so called interconnected pores. The interconnecting pores are between 50-100 µm in cross-section. Several very small pores in the surface of the granules are also visible. The size of these pores is just a few micrometers.

In 11A it appears like the smooth surface is a layer, which has broken and dethatched from the upper part of the granule, exposing a more porous structure. The moldings with a smoother surface are A1, A4, A6, A7, A8 and A10.

Inside the granules, pores were found to be present. The size is generally between 100-200 µm, with some larger pores at around 400 µm. Two examples of granule cross-sections, showing the inside morphology can be seen in figure 12. A is a granule from A7 and B from A10. The both granules have a similar inner morphology but the one from A10 has a denser structure than the one from A7. Some debris and irregularities from the cutting can be seen on the cross-section surfaces. In 12A, the smooth outer surface of the granule is visible. It is less than 50 µm thick and has some pores.
4.2.2. Granules batch B

All moldings produced cylindrical porous granules. The length of the granules was 1.6 mm and the diameter of 1.0-1.2 mm. This is slightly smaller than for batch A. Pores are present all over the surface of the granules with a cross-section of 50-200 μm. Most visible pores have a size of around 100 μm. This can be seen in figure 13 where granules from B2 (A) and B3 (B) are presented. The granules are slightly less conic than for batch A. Also, no granules have the smooth surface, which was observed for batch A. The pore size is in the same range as that of the porogen used for the batch.

In figure 14 a surface image in higher magnification (A) and a cross-section image (B) is shown. Figure 16A shows a granule from B3 and 16B shows a granule from B1. As can be seen, there are pores connecting to other pores inside the granules as well as pores with no interconnection. Several pores with a size of 10-50 μm are present on the surface. The surface appears rather rough.

As can be seen in figure 14B, pores are present inside the granules as well, but it appears to be fewer pores than on the surface of the granules. Although, the morphology on the inside is similar to that of the surface. The size of the pores is between 50-150 μm. Generally the pores appears to be interconnected to other pores.
4.2.3. Granules batch C

All dates were found to porous granules for this batch as well. The length of the granules is 1.6-1.8 mm and the diameter 1.0-1.2 mm. Although the shape of the granules is a little more irregular for batch C than for the two previous batches, and no granules were found to have the smooth surface observed for some moldings in batch 1. The pores are generally larger than for batch B, but the pore size appear to make parts of the granules fall off easily. In figure 15, two granules are pictured, from molding C2 (A) and C3 (B). Interconnection of the pores present on the surface to inner pores can be seen in both granules. The pores are 200-400 µm wide, the same size as the porogen. Most of them are around 300 µm wide.

![Figure 14. SEM micrographs of a granule from B3 (A) and cross-section of a granule from B1(B).](image)

![Figure 15. SEM micrographs of granules from C2 (A) and C3 (B).](image)

The pores on the surface structure of the granules are interconnected with inner pores. Pores are also present inside the granules. In figure 16 the surface pores and their interconnections in higher magnification of a granule from C2 (A) and the cross-section of a granule from C3 are presented. In figure 16A, smaller pores are also present, with a size of 100-200 µm. The size of the pores in figure 16B is between 200-400 µm, which is the same as for the pores on the surface. In this image, the interconnections are present as well. Although the amount of pores on the surface appears higher than inside the granules in figure 16.
4.2.4. Two-phase cements

An overview of the samples after molding is shown in figure 17. Cements 1-4 are presented in figure 17A-D in the same order. They have a similar surface structure with no apparent pores or structures. Although, part fell off some samples when they were dismounted from the molds, an example of this is pictured in figure 19B. The diameter of the samples is 3 mm.

In figure 18, cross-sections of samples from cement 1 (A) and 4 (B), are pictured. No apparent pores are present, and the morphology is comparable. In figure 18A, some pores can be noted but these could be present due to the molding since the molding is process made by hand. If the molds were not filled completely and the cement pushed down in the mold, some voids could be present inside the samples. In figure 18B, many different crystals are visible. The XRD analysis showed that no reaction had occurred, see figure 8. This would lead to that the sample contains a mixture of the initial reagents α-TCP, CSH and PEG, which are probably what is seen here.
Figure 17. SEM micrographs of the two-phase cements after molding. Cements 1-4 (A-D) are shown.

Figure 18. SEM micrographs of cross-sections of cement 1 (A) and cement 4 (B) after molding.

After 7 days in PBS solution, differences in the morphology were observed. The XRD results showed that calcium sulfate was not present in cement 3 and 4 after this time, which indicated that material was dissolved from the samples. In figure 19 A-D, cements 1-4 are shown. Pores are present in cement 3 and 4. Less change occurred for cement 1 and 2, although a few small pores are present. As shown in figure 17B, cement 2 appears more porous after molding than after 7 days due to part breaking off during demolding.
Some plate-like crystals typical for HA formed from α-TCP hydrolysis were also observed on the surface of the samples for all cements. XRD also showed that all samples contained HA after 7 days, so these crystals were expected. It is believed that these crystals were mainly present on the sides of the samples that were in contact with the bottom of the beakers during the dissolution test. This is exemplified in figure 20. Figure 20A shows cement 2 and 20B shows cement 4. The surfaces also appear spongy. This was observed for all cements but mainly in cement 3 and 4.

Figure 20. SEM micrographs of the surfaces of cement 2 (A) and cement 4 (B) in higher magnifications. Note the plate-like HA crystals in A and the spongy structure in B.
In figure 21, cross-section images of the cements are shown. Cement 3 and 4 in figure 21 C and D shows the highest porosity. Cements 1 and 2 in figure 21 A and B both have a lower porosity but some pores are present. For cement 2, two different phases appear to be present. Note the large crystal to the left in figure 22B.

![Figure 21. SEM micrographs of cross-sections of cement 1-4 (A-D) after 7 days in PBS-solution.](image)

After 14 days in PBS solution, the samples of cement 1 and 2 does still not show a high porosity but recrystallization has occurred on the surface. This is shown in figure 22 A and B, where A shows cement 1 and B shows cement 2. Plate-like HA crystals can be seen here as well but in higher extent. Cement 3 and 4 appear to not have changed between day 7 and 14, as seen in figure 22 C and D.

The surfaces are shown in higher magnification in figure 23. In 23 A it can be seen that the previously observed HA crystals are present for cement 2, while cement 4 is more spongy, resembling the observations after 7 days. However, in figure 23 B the crystals can also be noted.

Cross section images of after 14 days show pores in all four cements but in different amount and size, see figure 24. In figure 24 A, which shows cement 1, pores in the range of approximately 10-20 µm can be seen. For cement 2 in 24 B, the cross section is spongier than after 7 days. Cement 3 in 24 C has a high porosity. But the sample is also broken and some debris is lying below the sample. Cement 4 in 24 D also has some larger pores in the range of 100-300 µm.
Figure 22. SEM micrographs of the two-phase cements after 14 days in PBS-solution. Cements 1-4 (A-D) are shown.

Figure 23. SEM micrographs of the surfaces of cement 2 (A) and cement 4 (B) in higher magnifications. Note the HA crystals in both images.
4.3.1. Granules batch A

µCT scanning of the granules also showed that cylindrical, porous granules had been obtained. These results are conclusive with the SEM results. Two granules are pictured in figure 25. As can be seen, pores are present on the surface of the granules but some parts appear to have fallen off, although it is unclear if these parts are calcium phosphate of PEG. In figure 25 B, part of the smooth surface observed in the SEM analysis is present on the lower part of the granule. This surface structure is not present for granule A. The average porosity of the samples was 56.07% ($\sigma = 2.2\%$).
4.3.2. Granules batch B
The granules have a similar shape as those from batch 1, as can be seen on the two granules in figure 26. However, the average size of the pores appears smaller than for the previous batch. In the lower right part of figure 26 B, a part of the granule is gone, as in the case of batch A. In figure 26 A, the surface is very uneven. No smooth surface can be observed on any of the granules. The average porosity was 50.72% (σ = 0.015%). Only two granules were analyzed out of three for this batch due to one breaking when being mounted in the sample holder.

4.3.3. Granules batch C
The pores in the surface are generally larger than for both previous batches, while the shape of the granules is similar to that of batch B, see figure 27. Again, no smooth surface appears in the μCT images, which was also noted in the SEM analysis. The average porosity of batch C was 49.55% (σ = 3.84%), which is significantly smaller than for batch A but not than for batch B.
4.3.4. Pore size distribution
In figure 28-30, the pore size distributions calculated with CTan for each batch are plotted. The mean value of the percentage is plotted against a mid-value of pore size ranges obtained from the CTan software. The standard deviation is displayed for each batch.

For batch A, the range of the pore size mid-values was found to be 16-359 µm. However, one granule had pores with size mid-values of 375 and 391 µm. The highest percentage of pores has a size of 114 µm and is 11.9% (σ = 2.50 %). However, as the standard deviation shows, the variation the amount of pores in each range varies a lot between each analyzed granule.
For batch B, the obtained range was of pore-size mid values was 16-158 µm. The highest percentage is for 63 µm in this batch, 23.2% (σ = 3.14%). The size range is much narrower than for batch A. More than 70% of the pores are in the range of 63-95 µm for this batch. While the size distribution has more variations for batch A, the distribution for batch B resembles a normal distribution.

In batch C, the highest percentage was obtained for 110 µm, 11.1% (σ = 2.00%). But the standard deviations are large for almost all values so the percentage for the different mid-values differs significantly between each analyzed granule. The range of pore sizes is narrower than for batch A but wider than for batch B. A narrower size range was expected for both batch B and C since the PEG size range was narrower. However, it was expected that batch B and C should have a similar sized range.

An important observation is that the analysis show larger pores present on batch A than in batch C. The largest noted size in batch C is 236 µm while it is 391 µm in batch A. Although, in the 3D-representation of the granules from batch 1 and 3 in figures 25 and 27, more larger pores appear to be present in batch C than batch A.

A comparison of the average pore size distribution plots is pictured in figure 31. The comparison shows that batch B has the lowest average pore size while batch A and C actually have a similar size distribution. Batch A has some larger pores in the range o 245-391 µm, while no pores above 236 µm are present in batch C according to the calculations. Although, there are large variations between the samples in each batch, which makes it difficult to make a straightforward conclusion.
5. Discussion

5.1. Granules
The results of the μCT analysis showed that the pore size of the granules might be varied when the size of the porogen is changed. Pores are assumed to be formed during the setting of the cement while the PEG is enclosed in the cement. Therefore, it was assumed that varying the size of the PEG would lead to different sized pores. When conducting μCT analysis, a volume of interest must be selected in the CTan software. The volume of interest should ideally be the
whole sample, but nothing of the outside void should be included. It is also important to define what is solid material, in this case HA, and what is void volume, which is the volume of the pores. This is defined by adjusting the threshold value for the binary selection. The binary selection sets the contrast between solid material and void volume. In this case, it was set automatically. This might have influenced the results. In previous experiments, granules produced with 100-600 µm PEG were found to have a porosity of 75%, which is higher than for the granules produced in this study. The size distribution from the previous experiments also indicate an overall smaller pore size, see figure 32.

![Figure 32. Pore size distribution of granules produced with 100-600 µm PEG from previous experiments.](image)

Expectedly, present results should be similar to these but there are clear differences. A possibility is that the µCT analysis and the calculations were conducted differently. Although, the SEM results suggest that the hypothesis that a different PEG size would result in different pore size might be correct. It can be seen in the images that there are larger pores present in batch C that are not present in batch B. Although it must be considered that the granules appear shrink during the drying and leaching process. All analyzed granules were indeed smaller than the molded volume and the pores had an overall smaller cross-section than the porogen.

The µCT results also showed variations in each batch, especially in batch A and C. It is possible that analysis of more samples could provide reliable results but the
reproducibility must be considered. Larger PEG particles might be more difficult to mix evenly in the powder phase and a difference in the actual amount of these particles might occur between each granule. Smaller PEG particles appear to be easier to distribute evenly in the powder phase according to the porosity analysis.

Before this thesis, it was not concluded in which step of the process the HA was formed. It was assumed that it would form during setting, since the setting reaction is what forms HA [26,31,32]. But results showed that the HA is actually mainly formed during the leaching and drying. Since the granules from molding C1 was accidentally left in water for 22 hours instead of 4, and showed the highest amount of HA of all moldings, it is likely that the amount of water in the cement is not enough to form HA during setting but enough to make the cement rigid. Since the formation of HA from α-TCP is a hydrolysis process, water is crucial [31,31]. The elevated temperature is also likely to contribute since a higher temperature has been found to increase the rate of the hydrolysis.

Autoclaving resulted in a higher amount of HA in most cases. Since the granules are porous, water is obviously residing inside the granules so the hydrolysis likely continued during the drying process. For the moldings that had a high increase in HA during autoclaving some water was probably still left in the granules then, resulting in further hydrolysis. The moldings with the highest increase during autoclaving had a lower amount of HA before the autoclaving, indicating an incomplete α-TCP hydrolysis.

The grade of HA formation varies a lot between the batches and individual moldings. It was also found that the pore size varied between both batches and granules in each batch. When the kinetics of the α-TCP hydrolysis was studied [31,31] it was concluded that the surface area of the α-TCP affected the rate of the hydrolysis. A study has also shown that the HA formation in a porous α-TCP scaffold increases with larger surface area of the scaffold [49]. It is therefore possible that moldings with the largest amount of HA had the highest surface area. A wider size distribution of the PEG might lead to a larger surface area, since the overall higher HA amount was seen for batch A. However, there are variations between the individual moldings. This might also be due to difficulties in distributing the PEG evenly in the cement.

The shelf time between the leaching and the autoclaving was shorter in batch 2 and 3 due to smaller batches than batch 1 but since not all dates in batch 1 had a large increase in HA, the shelf time is not likely the key factor.

From the XRD results it can be concluded that the leaching and drying step is not only crucial for creating the actual pores but also for the HA formation. Since HA dissolves slowly, it would not be optimal to use grafts consisting of 100% HA, although it is the desired main phase. Therefore it is desirable to be able to control the final amount of HA. In this method, the leaching time is considered the most important factor to vary since the temperature is already high enough to accelerate the hydrolysis [32,38].
Another important observation that was noted is the mechanical properties of the granules. The granules could be handled as long as not too much pressure was applied, in which case they broke rather easily. The granules were quite brittle and although no mechanical testing was conducted, a problem with load bearing could occur. A difference between the batches could exist since the mechanical properties are related to the pore size and porosity [16,17,19,20].

5.2. Two-phase cements

It is clear that all two-phase cements formed HA, but the formation mainly occurred during the dissolution test. As for the granules, this is considered a result of incomplete hydrolysis during the setting, which could then be completed while the samples were immersed in PBS solution. CSD is also formed from CSH by a hydrolysis reaction [25]. In this thesis, the formation of HA and CSD occurred mainly during the first 14 days, which indicates that the hydrolysis continue during this period. A possible explanation to why cement 3 and 4 had no calcium sulfate left after 7 days is the higher PEG content. The pores that formed when the PEG was dissolved made the CSH dissolve before CSD was formed. A threshold value for the PEG to make the CSH dissolve seems to exist since an equal amount of CSH did not dissolve when the PEG amount was lower. The cements with the largest PEG fraction were also the most brittle. This is likely a result of the higher porosity [16,17,19,20].

It can also be noted that a higher initial CSH amount appear beneficial to the CSD formation. A lower amount of pores provides some sites were water can enter the samples but instead of dissolving the CSH, it contributes to the hydrolysis. This was observed for cement 1 and 2 but mainly for cement 2 where CSD was present already after 7 days.

Another indication of hydrolysis occurring during day 1-14 is the typical plate-shaped HA crystals were observed after 7 and 14 days. These crystals mainly occurred in the cement with the highest amount of CSD so it is also possible that CSD could accelerate the HA formation, which has been suggested [25].

It is possible that the hydrolysis could continue after day 14 but it has been observed before that the process is basically complete after this time [24]. Since CSD dissolves faster than HA, it is possible that more pores were formed between 14 and 28 days for cement 1 and 2, especially cement 2. However, these results were not included in this project due to time limitations. The dissolution rate will also be determined in the future by analyzing the ion concentrations of the PBS solution that was saved throughout the dissolution experiment. It is likely that the cement can be tailored to dissolve at a certain rate and to form HA at a certain rate as well by adjusting the ratio between calcium phosphate and calcium sulfate. The mechanical strength is also affected by this ratio, which has been showed earlier [25].
6. Conclusions

6.1. Porous granules
It is possible to vary the pore size when the size of the porogen is changed while producing HA granules, although it is not completely straightforward. The μCT analysis showed a difference between batch A and B, where batch A had smaller pores. This was in accordance with the SEM analysis. For batch C, the μCT results showed no difference in pore size compared to batch A, actually pore with a larger cross-section were present in batch A. Although the spread between different granules is wide in mainly batch A and C, which is concluded to be due to uneven distribution of the PEG in the powder phase. The SEM analysis also showed that batch C had pores with a larger cross-section than batch A. The method gives similar porosity for different porogen sizes, but it is lower than for previous experiments and lower than the desired range of 60-70%.

The HA formation is concluded to be mainly influenced by the time the granules are exposed to water, since complete hydrolysis does not occur during the setting. This relationship between time in water and the obtained amount of HA could be studied further. The effect of the surface area of the granules on the rate of the hydrolysis could also be studied.

6.2. Two-phase cements
Two-phase cements, which form pores over time at physiological temperature can be made by combining calcium phosphate and calcium sulfate. HA and CSD is formed in cements depending on their initial composition. The more initial CSH, the higher the conversion to CSD is during the first 7 days in PBS solution, where the hydrolysis of α-TCP and CSH is concluded to occur A larger addition of PEG makes pores form faster and in higher numbers. Faster initial pore formation due to PEG dissolution leads to dissolution of the CSH within the first 7 days, while a lower PEG addition leads to formation of CSD, which is slowly dissolved. The dissolution takes more than 14 days.

7. Future outlooks
The varying amount of HA in the granules is an interesting topic, which could be examined more thoroughly. Since the time in water was found to make the largest contribution to the amount of HA that is formed, this is considered the most important area. For example, granules could be removed and analyzed after different time points. Also the surface area of the granules in relation to the α-TCP could be studied. The mechanical strength should also be tested. Further μCT could also be performed, where samples with known values were analyzed to obtain better software settings. Also, more samples from each batch could be analyzed to obtain a more certain result.

The pore formation in two-phase cements could be studied more by analyzing the PBS solution to obtain information about when the dissolution of PEG and calcium sulfate mainly occurs. The dissolution test could be also conducted over longer time. The saved PBS solutions may be analyzed in a further study. The samples could also be analyzed with EDS to find sulfate-rich areas.
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