Preparation of 
Porous HA Scaffolds 
and Cell Culture

BMT02.07
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February 2002
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Abstract
Hydroxyapatite ceramic in a porous configuration is suggested as a good scaffold. A technique for the production of this material, based on the foaming of suspensions and in situ polymerization, resulted in a material whose characteristics are likely to make it useful as a scaffold. In addition, in order to improve the strength of the scaffold, alginate was added. The two kinds of the scaffolds were characterized by electronic scanning electron microscope (ESEM). In vitro experiments were performed in order to evaluate the nontoxicity and biocompatibility. The results showed that the materials presented a highly interconnected spheroidal porous network with open micropores and closed macropores. And cells can grow into the pore of the scaffolds. The strength was improved after adding alginate.

Introduction
In recent years, hydroxyapatite ceramic (HA) implants have attracted attention since it may be possible to use them as an alternative to autogenous free bone grafting. Many basic and clinical studies have confirmed the HA as a potential for biomedical applications, and its clinical applications are gradually expanding [1-7]. Pure HA which is Ca₁₀(PO₄)₆(OH)₂ has chemical compositions closely resembling that of mineral phase of natural hard tissues of the body such as bone and teeth [6]. And it has high biocompatibility and good bioaffinity, stimulates osteoconduction and is slowly replaced by the host bone after implantation [6]. Synthetic HA has minimal contaminants so it can be used to form large implants with uniform structures which are preferable to those made from natural products such as coral-replicated porous implants [4,5,7]. Synthetic HA materials are advantageous because of their uniform composition, high biocompatibility, overall safety and their microstructure can be completely controlled [6,8]. In particular, to a bioceramic with porous configuration, the evidence of tissues ingrowth and biological responses provide obvious advantages in tissue-implant fixation and controlled biodegradation rate for both short-term and long-term implantation purposes [9-11]. At the same time, with the development of tissue engineering of bone, in order to compare with the numerable model predication, a in vitro bone model is needed to promote it forward. So a variety of processing techniques has been developed for production of porous ceramics. But there are still some problems with this material, such as low porosity and small pore size which is difficult for the bone cells to grow inside, poor interconnection which is important to bone conduction and poor mechanical property. All these shortcomings limit its applications.

Alginate is one of bioactive materials. It is often used in bioengineering, such as an polymer films, cell encapsulation, wound dressings and surgical sponges, because of its non-toxicity, biocompatibility and ability to form hydrogels under very mild conditions. Alginites are linear chains of (1-4)-linked monomers of β-D-mannuronic
acid (M) and α-L-guluronic acid (L). Alginic acid is soluble in water and can be ionically cross-linked with a nontoxic divalent cation solution, such as calcium chloride \(^{[12]}\). Pure alginate can exhibit higher strength than HA.

This study aims at making good porous HA scaffolds with high porosity, bigger pores and interconnections. Since porous HA is a ceramic and thus is fragile, alginate was added to improve the strength. In vitro tests of cytotoxicity and cellular response were performed on the scaffolds.

**Materials and method**

**Preparation of hydroxyapatite powder**

Hydroxyapatite (HA) powder was synthesized by a wet method using CaCl\(_2\) and KH\(_2\)PO\(_4\) as staring materials. The 0.3 M CaCl\(_2\) and 0.6M KH\(_2\)PO\(_4\) solutions were made in a buffer solution (pH=9). Then 30ml KH\(_2\)PO\(_4\) solution was added to 100ml CaCl\(_2\) solution with vigorous stirring. The precipitate obtained by centrifugation was washed by acetone twice, and each time centrifuged at 4000rpm at least for 15 min, then was dried at 70°C in an oven.

**Porous HA scaffold**

**Preparation of HA slurry**

The slurry was prepared by mixing the HA powder, distilled water and dispersing agents. Polyethyleneimine (PEI) was used as a dispersing agent for HA. Sixty grams of HA powder were dispersed in 40g of 15% polyethyleneimine aqueous solution by well stirring.

**Foaming and gelatinization**

The slurry was foamed by adding 1wt% foaming agent (polyoxyethylene laurylether) and stirred until the mixture had a foamy appearance. The pore size was controlled by the quantity of surfactant. The distribution of pores was decided by regulating the stirring time. After further stirring to homogenize the mix, the slurry was cast by pouring it into a mould. The porous structure was stabilized at room temperature at least for 30 min.

**Drying and firing**

After polymerization took place and the foamed gels were well homogenized and stabilized, each sample carefully was removed from the container/mould. Further drying was conducted in an oven at 40°C for a minimum period of 20 h; thus water was removed from the porous HA/PEI gel. Further drying was processed in an oven at 100°C for at least 2 hours.

After drying, firing was performed in a high temperature furnace using a heating rate of 1 °C/min up to 300°C. This temperature was held for 90 min. The samples then were heated at a rate of 3°C/min up to the sintering temperature of 800°C, where they
held for periods of 2h prior to cooling. During sintering, PEI would be burnt thoroughly and vaporized from porous HA.

Porous alginate-HA scaffold

The alginate solution (5 wt%) was made and well stirred. Meanwhile, 0.3M and 0.6M CaCl₂ solution and 0.2M KH₂PO₄ solution (pH=9) were made respectively. The alginate solution was contained in a syringe. Then small drops of alginate were made by pushing the solution through the needle into the CaCl₂ solution. After the drops absorbed the Ca²⁺ from the solution sufficiently, the balls were taken out and put into KH₂PO₄ solution. Little by little, the hydroxyapatite was formed around the balls. Later the sample was freeze dried.

Characterization of the porous scaffold

To verify the purity of the HA powder, Fourier-transform infrared spectroscopy and X-ray diffraction were used. The morphology of the porous scaffolds and the characterization of the pores were analyzed by electronic scanning electron microscopy (ESEM-FEG, XL30, Philips). As the material is nonconductive, the samples were gold coated for 2 min.

Biomechanical analysis

The compressive strength of porous specimens was measured using a material test system (810 Elastomer Test System). Compression tests of 0.5% strain per second speed of 90% displacement, sine-wave compression (0.1Hz) and stepwise changes in height with 1mm steps per 20 seconds were processed.

Evaluation of cell culture and cytotoxicity

Chinese hamster ovary K-1 cells (CHO-K1) were cultured in a cell medium at 37°C in a 50% CO₂ incubator. The cell medium consists of HAM F-12 medium, 10% fetal bovine serum and 1% penicillin/sheptomy. The medium was changed 24h later to remove the nonadherent cells. After a monolayer confluent propagation, 0.1% trypsin solution was added for detachment of cells from the container walls for 5 min at 37°C, and then put new medium. Thus the cell suspension was obtained.

The CaCl₂ and KH₂PO₄ solutions and porous HA and alginate-HA scaffolds were well sterilized at 135°C, 4 bar pressure for 15 min in an autoclave (sterile max, Harvey KRYNR 1993 autoclave). The porous Scaffolds were then soaked in a cell suspension containing the cell medium and incubated at 37°C. The medium was renewed three times a week.

At the same time, 0.1 ml KH₂PO₄ was added to 5ml cell suspension with medium in a petri-dish, then 0.3 ml CaCl₂ solution was added in 2 minutes. After a few minutes, check cell viability by light microscope and then put it in the incubator. The medium was changed every day or two days.

Confocal scanning laser microscope was used to examine the scaffolds to confirm the nontoxicity of the materials and the ingrowth of cells in HA block pores. Before examination, the calceine (5 µl calcium per ml new cell medium) was used to color
the living cells green. Draw out the old medium and put 1ml calcium solution to each samples. After the samples were incubated at 37°C for 2 hours, remove the solution which included the dead cells, add some new cell medium into each samples.

Results

Characterization of HA powder

The powder was composed of small needle crystals. The purity of the powders was checked by the FT-IR. From the spectrogram (Fig. 1), the peaks of PO$_4^{3-}$, OH$^-$ can be found at 3356 cm$^{-1}$, 1023 cm$^{-1}$, 960 cm$^{-1}$, 598 cm$^{-1}$ and 560 cm$^{-1}$. No peaks of HPO$_4^{2-}$ and PO$_4^{2-}$ were found which verified that no Ca$_8$(PO$_4$)$_8$ and CaHPO$_4$·2H$_2$O were formed. X-ray diffraction patterns are shown in Fig2. The diffraction angles and the relative amplitude of each diffraction peaks were identified from the diffractograms. The figure shows that the powders presented only one phase. These all were tested the purity of HA powder.

Fig. 1 FT-IR spectrum of HA powder

![FT-IR spectrum of HA powder](image1)

Fig. 2 PXRD spectrum of HA powder

![PXRD spectrum of HA powder](image2)
Structural characterization of porous scaffolds

Characterization of porous HA scaffold

ESEM photos (Fig. 3) indicated that porous HA scaffold contains open micropores and macropores. ESEM also provided the information about the pore size (about few micrometers) and shape. From deeper analysis, they revealed that most of the pores were similar in size and showed interconnections with one another. The pores were separated by irregular, relatively thick walls, and interpores were sparsely distributed. The HA crystal wall surface seemed very smooth, and rough hydroxyapatite particles were lined closely to one another. The hydroxyapatite crystals seemed grow together which also can be seen from the big particles. ESEM showed that the porous ceramic structure obtained, consisted of a highly interconnected spheroidal porous network with open micropores and closed micropores.

Fig.3 SEM photo of porous HA

Characterization of alginate-HA scaffold

The alginate-HA scaffold displayed a highly porous structure with interconnected pores (Fig. 4(a)). And the interpores distributed nearly everywhere of the surface. The pores interconnected via a pass as shown in Fig.4 (b). The pore size was examined about few micrometers in several areas. Some of the walls between the pores were thinner, some were thicker. Compared with the HA scaffold, alginate-HA scaffold seems to have higher porosity and better interconnected pores. And nearly all the pores were open.

Fig.4 SEM photos of porous alginate-HA.
(a, left): 590x magnification. (b, right): 4718x magnification.
Fig. 5 Cell culture within the porous HA
Evaluation of cell culture and cytotoxicity

Making HA in the cell suspension

After CaCl₂ solution (pH=8) was added slowly into the mixture (pH=7) of cell suspension and KH₂PO₄ solution, the HA crystals can be found. These crystals were almost needle shaped. With the HA crystals forming, the pH value of the solution was decreasing as a result of consumption of OH⁻ forming hydroxyapatite. The medium was changed frequently. After a few days, the cells proliferation was observed. This showed that HA could form in the cell suspension without killing the cells. This certificates that solutions for making HA are nontoxic, and also certificate the biocompatibility of HA.

Cell culture of porous scaffolds

After porous scaffold was soaked in cell suspension for 24h, it can be seen from microscopically that cells attached to the HA scaffold. The confocal laser scanning microscope photos, showed a lot of cells attached on the surface of the scaffold. Fig. 5 shows the cell attachment after 5 days. Some of the cells attach to the walls of the pores. This indicates that cells grow into the pores as is expected.

Mechanical Test

The compression strength of alginate-HA porous scaffold was tested. The load-displacement curve was shown in Fig. 6. The results indicated that the compression stress reached 2.42 Mpa. The cyclical loading test was also processed. But only partial deformation recovered which indicated that plastic deformation happened.

![load-displacement curve](image)

*Fig.6 the curve of displacement-load of alginate-HA*
Discussion

The ideal artificial scaffold demands good biocompatibility without the possibility of inflammation or foreign body/toxic reactions. Strong bonding with the host bone, active bone ingrowth into the graft, and bioabsorbility are also required \[13-15\]. Strength sufficient to resist the mechanical load in the implanted bone is also needed. However, none of the biomaterials that have been developed in previous studies meet all of these criteria. HA, which is one of the major inorganic materials in normal bone, has good biocompatibility and osteoconductivity. However, its fragility is a drawback, like other ceramic materials. Therefore, it can be used alone in areas that do not require good mechanical strength. The structure of the dense sintered body is stronger and more able to bond rapidly with the host bone, but its use is limited due to its high level of brittleness and low osteoconductivity and absorbability. Porous HA, although its initial strength is weak, is considered a good substitute, because it is more resorbable and more osteoconductive than dense HA\[16\]. Porous implants allow development of bone and soft tissues within large pores and also blood supply for further bone mineralization. Anchorage between the implant and host bone also is improved by the presence of porosity \[17-20\].

Previous researchers have failed to control the size of the pores, or the interconnections between pores and porosity, in preparation of the porous HA that was used in their studies.

The aim of this study was to make synthetic porous HA scaffold with the optimal pore size and porosity and investigate the early cellular response of CHO-K1 cells. The material factors affecting the biological response to the implant include the property of HA powder, sintering property, structural configuration.

In our study, the HA powder was made in a buffer solution at pH=9. If the pH is lower, maybe the CaHPO$_4$$\cdot$ 2H$_2$O is formed; contrarily, if the pH is too high, Ca$_8$(PO$_4$)$_6$ will appear. So the pH is very important to the purity of HA powder. And, KCl may be formed at the same time. So the suspension should be washed and centrifuged at 4000 rpm for 15 min.

After the dispersing agent, PEI, was put into the HA slurry, the mixture must be well stirred to get good homogenization. Later, surfactant was added and the mixture was foamed in itself. For this process is based on the addition of surfactants to reduce the surface energy of liquid-gas interfaces as a means of generating stable bubbles. Given that appropriate foaming agent is employed, foaming can be achieved without problems most of the time. The foam volume varied according to the quantity of surfactant added, foams expanded during agitation reaching a maximum volume. But from the observation during the experiments, too more surfactant can decrease the mechanical property of the porous HA although it can improve the porosity. Hence a compromise exists between the porosity and strength of the HA blocks that must be considered in the production of the foamed slurry.

Typical problems associated with the drying stage, such as cracking and warping, were not obviously observed in the process. Interconnected porosity and high surface area are the factors that allow the water to be removed with ease.
Sintering HA is important because in the same manner that high temperatures densify the microstructure and maximize the fracture strength. Definitely, it can also burn the organic materials thoroughly which differ from HA in solubility and strength and could result in problems with respect to the implant properties\textsuperscript{17}.

The porous HA with random pore geometry has wide ranges of porosity and pores of various sizes with interconnection. In our experiments, that under the high temperature, some of them grow together. But from the above photos, it seems that this doesn’t affect the pores and porosity, and can lead bigger pore size. If the samples are cooled fast, the smaller crystals will be obtained for the growing of the crystal is prevented.

Thus the porous scaffold of HA was obtained. But it only can be used for non-loading bone due to the brittleness of HA. In order to have a porous scaffold with high strength, alginate was employed. When alginate balls were put into CaCl\textsubscript{2} solution, the calcium ions bind the guluronic acid sites of alginate strands together to form a stable alginate gel. Depending on the alginate strands, alginate balls can be connected into a network. After they were soaked in the aqueous solution of phosphate, the Ca\textsuperscript{2+} absorbed by alginate balls, slowly diffused into the solution to form HA. Thus, the alginate balls were also covered by HA. But alginate is easily wetted by the aqueous medium. Once wet, it will lose part of their mechanical strength. So a quantity of HA is needed to prohibit the mechanical losing. During this process, the size of alginate drop must be considered. For big drop can decrease the porosity of the scaffold. On the contrary, small drop absorbs too little Ca\textsuperscript{2+} to form HA. If the HA is too little, the pores can be deteriorated in the cell medium.

Conclusions

The current investigation has shown that a successful production of porous hydroxyapatite for scaffolds. Structural analysis by electronic scanning electron microscopy revealed that HA has spherical pores of uniform size that were interconnected. The surface of the crystal wall structure was smooth, and hydroxyapatite particles were bound tightly to one another. And less stiff scaffolds were made by adding the alginate. Samples with higher porosity presented an irregular structure, that could be useful for cells and bone to grow in.

For in vitro test of biological evaluation revealed that the cells can grow into the pores and grow well with the materials. These results indicate that the porous HA may be suitable to be a superior bone substitute. In conclusion, this study provides a basis for further studies on the use of this composite as an implant in orthopedic surgery.

Acknowledgements

I would like to express my sincere appreciation to Dr. Bert van Rietbergen, Dr. René van Donkelaar and Dr. Nico A.J.M. Sommerdijk. And I wish thank to all the people in TUE who help me to finish this project.
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