Effect of porogen agent on microstructure of CaP granules using the gelation of alginate droplet formation

M D Effendi\textsuperscript{1*}, D Gustiono\textsuperscript{1}, Lukmana\textsuperscript{1}

\textsuperscript{1}Agency for The Assessment and Application of Technology, Gedung 224, Jl. Kw. Puspiptek, Muncul, Kec. Setu, Kota Tangerang Selatan, Banten 15314, Indonesia

\*Email: dachyar.effendy@bppt.go.id

Abstract. CaP granules are used for open defect of bone. The important feature in the physical structure of a synthetic ceramic is its porosity. Pore structure is great importance for osteoconduction. The main objective of this study is to investigate the effect of adding carbon black as porogen agent on microstructure of CaP granules of droplet CaP. According to that, certain carbon black (325 mesh) has added into composition of microspheres CaP-alginate gelation with ratio CaP:Carbon black was 0.5, 1, and 1.5 weight ratios respectively. Scanning Electron Microscope (SEM) was used to investigate microstructure of droplet and shown that Irregular shape grain. Internal microstructure appears more open for samples produced without the presence of alginate after sintering process and confirmed by XRD pattern for whole samples. The porosity and pore size increased by increasing CB. On the other hand, no significant change on surface roughness and no transformation phase caused by adding CB. So that, it can be concluded that CB can serve as a porogen agent.

Keywords: porogen, CaP, granule, microstructure, porosity, droplet.

1. Introduction
Calcium phosphates have shown that these materials are suitable as bone substitutes due to their biocompatible, bioactive, biodegradable, and osteoconductive characteristics [1-3], and, when implanted in vivo, they are nontoxic and do not induce any antigenic response [4], so that, the calcium Phosphate can be applied as biomedicine [5]. The most widely used calcium phosphate ceramics are porous hydroxyapatite (HA), b-tricalcium phosphate (b-TCP), and mixtures of these two, known as biphasic calcium phosphate ceramics (BCP) [6]. The hydroxyapatite [Hap : \text{Ca}_{10}\text{(PO}_4\text{)}_6\text{(OH)}_2] is CaP, especially known to be excellent in biocompatibility because of its similarity in composition to natural bone [3][4]. For all of bone graft forms, it is important to make them spherically shaped for uniform packing in irregularly shaped defects [7][8].

The presence of porosity and a bioactive surface facilitate cell attachment, proliferation, and differentiation and, consequently, provide a more biocompatible, osteoconductive, and in some cases, osteoinductive ceramics, which can favor increased bone formation [9][10]. CaP scaffold or macroporous blocks have a more defined shape than granules and they possess big macro porosity (typical range 50-500 µm) that has a big role in osteoconductive mechanisms. It is impossible to achieve the same macro porosity in granules form, because the granules size is equivalent to macropores size.
Scaffolds have to be fixed into the open defect that is bigger than in the granules case and for the correct adaption to the implantation site they must to be drilled and cut for screw insertion. The different forms of granular calcium phosphate include irregular multifaceted granules and round smooth granules, with solid or porous structures. However, the behavior of granules in the body depends on their morphology and microstructure.

Bone structure is oriented according to physiological needs with interlinked tunnels, of non-uniform shape and width, whose boundaries are the trabeculae. The granulate model for bone simulation should also have microchannels (which in dentine and bone show identical extension of 1-2 µm), which play an active role in cellular attraction processes on the surfaces [11]. Bioceramic micro porosity (pore size < 10 µm), which is defined by its capacity to be impregnated by biological fluids, results from the sintering process, while the pore dimensions mainly depend on the material composition, thermal cycle and sintering time. Microporosity is an important parameter to achieve bioresorption at high osteogenic property; osteoconduction may be associated to the microporosity [12]. According to the importance of microporosity and surface properties as describe before, this work has investigated the effect of porogen agent on microstructure of CaP granules.

2. Materials and Methods

2.1 Materials

1) Materials preparation

We used hydroxyapatite powder obtained from process as below:

Hydroxyapatite powder prepared by wet chemical methods of preparation 1M solution of calcium oxide and 0.6 gr/l (NH₄)₂HPO₄ is used as the initial solution. The solution of (NH₄)₂HPO₄ added drop-wise in a solution of calcium oxide. The level of addition of (NH₄)₂HPO₄ varies for calcium precursors, then the influence of the size of the precursor at Hap morphology can be investigated. Ammonia was used for adjustment of pH values. The suspension is stirred with a magnetic stirrer after the addition of the initial solution for 24 hours at room temperature. Precursor done for 30 minutes at room temperature, then filtered and washed with a vacuum Buchner for five times until a neutral pH is reached. For the first and third phases were washed with distilled water and ammonia, the second phase and the fourth was washed with distilled water to remove residual dirt, and last washed with ethanol to remove water and increase the dispersibility. The end product was dried at room temperature for 24 hours then heated to 160°C for 17 hours for the removal of water. Sintering process was carried out at 650°C with a heating rate of 80 minutes with a time of 2 hours detention. Hydroxyapatite samples then characterized by XRD (Fig. 1)

The hydroxyapatite formed according to the reaction [13]:

\[10\text{CaCO}_3 + 5(\text{NH}_4)_2\text{HPO}_4 \rightarrow \text{Ca}_{10}((\text{PO}_4\text{,CO}_3)_6(\text{OH,CO}_3)_{2} + 5(\text{NH}_4)_2\text{CO}_3 + 3\text{CO}_2 + 2\text{H}_2\text{O} \]

2) Droplet formation

Droplet extrusion method allows the production of spherical drops starting from a bioceramic suspension containing Alginate solution that allows the instantaneous gelification in a crosslinking solution. Gelification mechanisms, as from previous studies [11-14], were applied to produce bioceramic spheres by droplet extrusion.

HA powder and certain carbon black ((CONTINEX N774, 325 mesh) has added into composition of microspheres CaP-alginate gelation with ratio CaP : carbon black were 0.5, 1, and 1.5 weight ratio respectively was mixed with sodium alginate solution (Sigma Aldrich, 3% w/v) at a ratio of 0.2 w/w and well homogenized. The paste was extruded drop-wise into a 0.1M CaCl₂ cross-linking solution, where spherical-shaped particles instantaneously formed and were allowed to harden for 30 min (fig. 1). The size of the microspheres was controlled by regulating the extrusion flow rate using a syringe. At completion of the gelling period, microspheres were recovered and
rinsed in water in order to remove the excess CaCl₂. Finally, they were dried overnight in a vacuum-oven at 30°C, and then sintered at 1100°C for 1h, with a uniform heating rate of 5°C/min from room temperature (fig. 2). We made also CaP droplet without adding carbon black to compare between both samples’ models, as shown in Fig. 3.

3. Result and Discussion

3.1 Analysis of synthetic HA powder
The wet chemical process as described on method to prepare hydroxyapatite powder successfully obtained hydroxyapatite as domain phase (Figure 1) and similar to carbonate-hydroxyapatite (Ca₁₀.₀₀P₆.₀₀O₂₆.₁₄H₂.₆₀C₀.₀₂) pattern card number 96-900-3554. As we stated above, this reaction produced by product as ammonium carbonate and carbon dioxide, alongside water. The so-formed HAP is typically non-stoichiometric, as CO₃²⁻ ions from CO₂ in the atmosphere can partially replace PO₄³⁻ and OH⁻ ions, thus leading to formation of carbonated HAP, as stated in a review [2]. This result confirmed by Landi et al. 2003 with using the same process [15].

![Figure 1. XRD pattern of hydroxyapatite powder obtained by wet chemical process and sintered at 650 °C.](image)

3.2 Analysis of HA granules
Granules have fabricated by alginate gelation droplet formation. Carbon black was added into composition of microspheres HA-alginate gelation obtained black droplet as shown at figure 2. Carbon black has completely burned after sintering process. It shown at figure 3 that droplets become white color compared to Fig. 2.

The pattern of XRD shown that all of samples had carbonate-hydroxyapatite (Ca₁₀.₀₀P₆.₀₀O₂₆.₁₄H₂.₆₀C₀.₀₂) pattern identically as dominant phases. Effect of adding carbon black did not cause presence of any other phases on all ratio of CB added into the formulation. It also
shown that after sintering process at 1000°C for 1 hr holding time, CB and alginate had completely burned, and can be confirmed on FTIR investigation. The analysis using High Score Plus® shown that increasing CB added to granule formula caused lattice parameters slightly contracted (Table 1).

Figure 2. CaP granules/Carbon black before firing process(a), and after firing process (b) with the diameter are about 3mm

Figure 3. XRD pattern of granule HA adding 0.5,1.5, and 1.0 %wt respectively after sintering process at 1000°C, 2 hr.
Table 1. Structure and profile data of all samples based on XRD analysis using Highscore® software and Rietveld analysis

| Sample | Formula | Formula mass g/mol | Density (calc.) g/cm³ | Space group (No.) | Lattice parameters, Å | V/10⁶ pm³ |
|--------|---------|-------------------|-----------------------|------------------|-----------------------|------------|
| A101   | Ca₁₀₀₀P₆₀₀O₂₆₁₄H₂.₆₀ₐ₀₂ | 1007.79 | 3.16 | P63/m (176) | a : 9.4292 | 529.2627 |
| CB 0.5 | Ca₁₀₀₀P₆₀₀O₂₆₁₄H₂.₆₀ₐ₀₂ | 1007.79 | 3.16 | P63/m (176) | a : 9.4240 b : 9.4240 c : 6.8784 | 529.0443 |
| CB 1.0 | Ca₁₀₀₀P₆₀₀O₂₆₁₄H₂.₆₀ₐ₀₂ | 1007.79 | 3.17 | P63/m (176) | a : 9.4202 b : 9.4202 c : 6.8773 | 528.5235 |
| CB 1.5 | Ca₁₀₀₀P₆₀₀O₂₆₁₄H₂.₆₀ₐ₀₂ | 1007.79 | 3.17 | P63/m (176) | a : 9.4189 b : 9.4189 c : 6.8771 | 528.3724 |

3.3 SEM analysis
Granule for all samples type (with and without adding CB) shown that surface roughness, properties necessary for osteoconductivity (fig. 6-8). It could be conclusion that the more CB content, the more surface roughness founded. Irregular shape grain internal microstructure appears more open for samples produced without a presence of alginate after sintering process. The diameter of grain has decreased by adding CB and shown that, the effect of more containing CB (0.5% wt, 1.0% wt, and 1.5% wt respectively has changed some grains into small particles with approx. 5-10 um diameter. (fig 6-8). Consequently, the diameter of open porosity has decreased and microporosity increased.

The presence of macrostructural features, such as macropores, channels or void between particles has been shown to be a prerequisite for osteoinduction by synthetic biomaterials [15]. The macro porosity is not an intrinsic property of CaP granules, but represent the void space between granules. CaP ceramic microspheres can also use as drug delivery carriers, for example for doxycycline related to interconnected microporosity [16-18]. Thus, the presence of microporosity should be an advantage to develop a granule which made by adding porogen to be a drug delivery carrier.

Table 2. Properties of the granules

| Sample Code | Surface roughness, µm | Pore size, µm | Porosity, % | Crystallite size |
|-------------|-----------------------|-------------|-------------|-----------------|
| A101        | 0.25-2.1              | 0.12 - 0.40 | 51          | 359-762         |
| CB0.5       | 0.75-3.5              | 0.12 - 0.40 | 51          | 470-616         |
| CB1.0       | 0.6-1.8               | 0.13-0.40   | 53          | 454-759         |
| CB1.5       | 0.3-0.5               | 0.30 – 0.55 | 54          | 456-713         |
Figure 4. SEM micrograph of granules with adding 0.5% CB

Figure 5. SEM micrograph of granules with adding 1% CB
3.4 FTIR analysis

Infrared spectroscopy spectrum of HA granules shown in Fig 6. Strong bands attributed to PO43-groups. The bands at 1089 cm\(^{-1}\) and 1026.03 cm\(^{-1}\), 1088.49 cm\(^{-1}\) and 1023.49 cm\(^{-1}\), 1089.11 cm\(^{-1}\) and 1027.45 cm\(^{-1}\) band of CB0.5, CB1.0 and CB1.5 respectively are assigned to the components of the triply degenerated \(\nu_3\) anti-symmetric P-O stretching mode. The 962.08 cm\(^{-1}\), 962.10 cm\(^{-1}\), and 962.34 cm\(^{-1}\), band of CB0.5, CB1.0 and CB1.5 respectively is assigned to \(\nu_1\). The non-degenerate P-O symmetric
stretching mode. The bands at 600.31 cm\(^{-1}\) and 564.30 cm\(^{-1}\), 600.03 cm\(^{-1}\) and 563.34 cm\(^{-1}\), and 600.58 cm\(^{-1}\) and 564.87 cm\(^{-1}\) bands of CB0.5, CB1.0 and CB1.5 respectively are assigned to components of the triply degenerate v4 Q-P-O bending mode. Presence of any distinct band in the range of 1400-1550 cm\(^{-1}\) indicates that the samples contain large quantities of carbonate ions. It means that hydroxyapatite as raw material has transformed into carbonated hydroxyapatite caused by presence of carbon ions. The broad band of low intensity in the range 3570-3580 cm\(^{-1}\) coming from the hydroxyapatite OH stretching vibrations, corresponding to the hydroxyapatite.

4. Conclusion
A method for preparing granulates with varying pore size distribution from hydroxyapatite (HA) with adding carbon black as porogen can be made by droplet formation with help of porogen agent i.e carbon black according to this work. The shape and dimension of granules can be designed by adjusting diameter of syringe and depends in rheology of droplet granules. More porosity has been achieved with adding carbon black followed by increasing pore size. Producing hydroxyapatite by precipitation method produced carbonate hydroxyapatite. It is also can conclude that the porosity and pore size can be customized by adding carbon black on the formulation to produce HA granules by droplet extrusion. Granules can be designed for a wide range of medical applications.

References
[1] J. M. Ruan, J. P. Zou, J. N. Zhou, and J. Z. Hu. “Porous hydroxyapatite– tricalcium phosphate bioceramics,” Powder Metallurgy, (2006), 49(1), pp. 66-69
[2] Habraken Wouter, Habibovic Pamela, Eppe Mathias, Bohner Marc.“Calcium phosphates in biomedical applications: materials for the future?”, MaterialsToday, 2016, 19(2), pp. 69-87
[3] H. Yuan, K. Kurashina, J. D. de Bruijn, Y. Li, K. de Groot, and X. Zhang, “A preliminary study on osteoinduction of two kinds of calcium phosphate ceramics,” Biomaterials, vol. 20, no. 19, Oct. 1999, pp. 1799-806
[4] Jabr S. Al-Sanabani, I Ahmed A. Madfa, 2and Fadhel A. Al-Sanabani, “Application of Calcium Phosphate Materials in Dentistry”, International Journal of Biomaterials, 2013, vol. 2013
[5] M. Eppe, K. Ganesan, R. Heumann et al., “Application of calcium phosphate nanoparticles in biomedicine,” Journal of Materials Chemistry, 2010, vol. 20, no. 1, pp. 18–23
[6] Landi E, Celotti G, Logroscino G, et al. “Carbonated hydroxyapatite as bone substitute”. J Eur Ceram Soc 25: 2003, 2931–2937.
[7] Ribeiro C.C., Barrias C.C., Barbosa M.A.: ‘Preparation and characterisation of calcium-phosphate porous microspheres with a uniform size for biomedical applications’, J. Mater. Sci. Mater. Med., 2006, 17(5), pp. 455–463
[8] Zhang, Ke et al. “Effect of microporosity on scaffolds for bone tissue engineering.” Regenerative biomaterials, 2018, vol. 5(2): 115-124. doi:10.1093/rb/rby001
[9] Vallejos Baier R, Benjumeda Wijnhoven I, Irribarra Del Valle V, Millán Giovanetti C, Vivanco JF. “Microporosity Clustering Assessment in Calcium Phosphate Bioceramic Particles “. Front Bioeng Biotechnol. 2019;7: 281.2019. doi:10.3389/fbioe.2019.00281
[10] Suchaneck W., Yoshimura M. ‘Processing and properties of hydroxyapatite-based biomaterials for use as hard tissue replacement implants’, J. Mater. Res., 1998, 13, (1), pp. 94–117.
[11] M. Fabbri, G. C. Celotti, and A. Ravaglioli, “Granulates based on calcium phosphate with controlled morphology and porosity for medical applications: physico-chemical parameters and production technique,” Biomaterials, vol. 15, no. 6, May 1994, pp. 474-7.
[12] Mussska, O.N., Kulak, A.I., Krut’ko, V.K. et al. “Preparation of Bioactive Mesoporous Calcium Phosphate Granules”. Inorg Mater 54, 2018, 117–124 doi:10.1134/S0020168518020115
[13] Kamiya M., Hatta J., Shimada E., Ikuma Y., Yoshimura M., Monma H. AFM analysis of initial stage of reaction between calcite and phosphate. Mater. Sci. Eng. B Solid-State Mater. Adv. Technol. 2004; 111:226–231. doi: 10.1016/S0921-5107(04)00210-7.

[14] S. P. Victor and T. S. S. Kumar, “BCP ceramic microspheres as drug delivery carriers: synthesis, characterisation and doxycycline release. Journal of materials science. Materials in medicine, vol. 19, no. 1, Jan. 2008, pp. 283-90

[15] E. Landi, G. Celotti, G. Logroscino, and A. Tampieri, “Carbonated hydroxyapatite as bone substitute,” J. Eur. Ceram. Soc., 2003

[16] C. C. Ribeiro, C. C. Barrias, and M. a Barbosa, “Calcium phosphatealginate microspheres as enzyme delivery matrices,” Biomaterials, vol. 25, no. 18, Aug. 2004, pp. 4363-73.

[17] P. L. Granja, a. I. N. Silva, J. P. Borges, C. C. Barrias, and I. F. Amaral, “Preparation and Characterization of Injectable ChitosanHydroxyapatite Microspheres,” Key Engineering Materials, vol. 254-256, 2004, pp. 573-576.

[18] H. Yuan, H. Fernandes, P. Habibovic, J. D. Boer, A. M. C. Barradas, an A.D. Ruiter, “Osteoinductive ceramics as a synthetic alternative to autologous bone grafting,” Image (Rochester, N.Y.), vol. 107, no. 31, 2010, pp.10-15