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Mechanical Strength and Porosity of Carbonate Apatite-Chitosan-Gelatine Scaffold in Various Ratio as a Biomaterial Candidate in Tissue Engineering

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Abstract. Bone defect is a common problem in the field of dentistry. The defect can be solved by tissue engineering. One component of tissue engineering is scaffold. Carbonate apatite is the main material used because it has an organic components similar to human bones. The carbonate apatite combined with gelatin and chitosan can be used as a scaffold for tissue engineering. The aim of this study is to know the exact ratio of the carbonate apatite, gelatin-chitosan (CA:Ch-GEL) scaffold on the compressive strength and porosity size as biomaterial candidates in tissue engineering. Scaffold was synthesized from CA:Ch-GEL with different ratios of 50:50, 60:40, 70:30 and 80:20 with freeze drying method. Fourier Transform Infared Spectroscopy **(**FTIR) was used CA:Ch-GEL scaffold functional group identification. Scaffold mechanical test was performed using an Autograph while a porosity test was performed using Scanning Electron Microscope. All data were analyzed by ANOVA followed by Tukey HSD test. Scaffold has a compressive strength ranges 4.02 - 11.35 MPa, with porous ranges 19,18 mm – 52,59 mm at 50:50, 60:40, 70:30 and 80:20 ratios. CA:GEL-Ch scaffold at all ratios can be used as biomaterials in tissue engineering.

Introduction

Cases of bone damage due to a disease or trauma may occur in dentistry. Various studies have used tissue engineering based approach to resolve the problem that aims to develop a biological substitute that serves to compose, maintain, repair or restore damaged tissue function [1]. Three components in tissue engineering are: scaffold, signal regulator and cells [2]. Three-dimensional scaffolds have been used extensively as tissue engineering biomaterials in inducing tissue and organ regeneration. Scaffold functions are to support the growth and development of tissue acting as an extracellular matrix, cell adhesion for proliferation and differentiation to form new tissues with support of molecular signals [3]. The main requirements of scaffold are biocompatible, osteoconductive and osteoinductive, capable of bearing pressure load, has a porous structure with size>100μm which is useful for cell penetration [4,5].

Scaffolds can be made from various biomaterials to meet ideal requirements. Biomaterials that can be used include bioceramic groups, synthetic or natural polymers. Bioceramic materials used are hydroxyapatite and tri-calcium phosphate, synthetic polymeric material is polyglycolic acid (PA), poly-dl-lactic-co-glycolic acid (PLGA), whereas natural polymeric materials are chitosan and alginate. Those material may be used for scaffolds production without combinations. However, scaffold that is made without combining will have lots of deficiency, such as weak mechanical strength, causing tissue necrosis and scaffold become rigid. The purpose of combining these materials in order to achieve ideal scaffold properties in improving the ability of biological properties [4].

Selection of carbonate apatite material, chitosan and gelatin are according to bone composition consisting of organic and inorganic materials. Carbonate apatite is a composition of human bones

belonging to a ceramic class. Carbonate apatite has properties: can be re-treated by osteoclasts, capable of triggering rapid bone growth, biocompatible bioinertic, containing Ca2 +, PO43-, CO32-, rapidly absorbed [6] and osteoconductive [7].

Chitosan is biodegradable, non-toxic, biocompatible, anti-bacterial, supports attachment, osteoblast differentiation and morphogenesis, has similar structure to glycoaminoglycan in cartilage supporting bone cell formation [8]. Another mostly used material is gelatin because it is biodegradable, biocompatible and the use of gelatin in scaffold containing amino acids which resembling collagen in the bone [9]. The reason of combining those two materials is both have good biocompatible and biodegradability properties in tissue repair [10]. Carbonate apatite can be added to scaffold production because composition of carbonate apatite corresponds to the bone that has bioactive properties so that bone reparation process become faster [11].

Scaffold must have proper compressive strength value during the implantation action. Compressive strength, porous size, porous quantity and surface area are important parameters for the use of scaffolds in tissue engineering [12]. Compressive strength and porosity of scaffolds are obtained from synthesis of carbonate apatite chitosan-gelatin (CA:Ch-GEL) with ratio of 50:50, 60:40, 70:30, 80:20 (w/w) with freeze drying method.

The objectives of this study were to determine the specific ratios of CA:Ch-GEL scaffold towards compressive strength and porosity as biomaterial candidates in tissue engineering.

Materials and Method

Chitosan was extracted from crab shells by deacetylation 81% (Sigma Aldrich 93646, USA), gelatin is obtained from denaturation of cow collagen (Rousselot, Guangdong, China), carbonate apatite, NaOH (Merck) and acetic acid (Merck).

Synthesis of CA:Ch-GEL Scaffold. Carbonate apatite powder, chitosan and gelatin are prepared in beaker glass. Gelatin mixed with 4ml of 2% acetic acid, stirred with stirrer for 3 minutes until gelatin becomes dissolved. Then carbonate apatite powder was added to gelatin solution to form a gel, next chitosan powder was added, then mixed with 0.4ml of NaOH to neutralize the acid. The mixture of carbonate apatite, chitosan and gelatin that formed a gel, was checked the pH with litmus paper until indicated pH 7. Gel was inserted into the mold 48 well plate, then gel was frozen at -40°C for 2x24 hours and it was performed 2x24 hour freeze-drying process. After sample has hardened, released sample from the mold.

Table 1. Comparison of each material weight contained in scaffold

Ratio (w/w)	Weight (gram)		
	Carbonate apatite	Chitosan	Gelatin
50 : 50	1,25	0,625	0,625
60 : 40	1,5	0,5	0,5
70 : 30	1,75	0,375	0,375
80 : 20	2	0,25	0,25

Fourier Transform Infared Spectroscopy (FTIR) of CA:Ch-GEL Scaffold. Sample was placed on the sample holder and positioned on the interferometer of FTIR tool. The start button is pressed to start the measurement. Dialogue box is filled with sample identity and continued by selecting sample start. The CALC menu is pressed to see the number of waves (peak) which represented the spectra results. FTIR results in the form of graphics are read by matching the peak table.

Compressive Strength of CA:Ch-GEL Scaffold. Test of CA:Ch-GEL scaffold compressive strength using Autograph (Shimadzu Ag-10 TE). Samples were placed on the table, were given with 100kN of compression load at speed of 10mm/min until scaffolds were distorted, the indicator were stopped and the numbers were recorded. The calculation was calculated by dividing the result of

indicator with the sample surface area, so that compressive strength value with unit kgf/mm^2 is obtained. Furthermore, compressive strength value was converted into units MPa.

Porosity CA:Ch-GEL Scaffold. The sample was cleaned with ultrasonic cleaning using acetone solution. The sample was coated with gold solution (Au) for 24 hours. The sample was placed on the holder and tested by Scanning Electron Microscope (Inspact S50).

Results and Discussion

Carbonate apatite has bone resembled properties when compared to hydroxyapatite so it can be combined with chitosan and gelatin for scaffold production. The use of carbonate apatite represents the inorganic material of bone, whereas chitosan and gelatin represents the organic material of bone. Gelatin has carboxyl group which capable of forming bonds with chitosan cations through hydrogen bonds [13]. Gelatin has anions whereas chitosan has cations, so that electrostatic forces occured between these two natural polymers in forming scaffold at physiology pH [14]. The combination of those three materials is using freeze drying method for scaffold production. Freeze drying method is mostly used by researchers because it has some advantages such as: simple, easy, cheap and capable to produce the suitable porous [15].

Compressive strength and scaffold porosity are factors of concern when scaffold is applied to repair bone defects, in addition to other factors such as biocompatibility and bioresorbability [16]. Based on the results of this study, compressive strength value of CA:Ch-GEL scaffold with all ratios are qualified for cancellous bone or trabecular bone application, according to previous researchers suggestion that compressive strength of cancellous or trabecular bone is 1.5 MPa-35 MPa [17].

Combination of carbonate apatite and chitosan gelatin is expected to increase bone-formation ability and provide time for bone absorption. During bone metabolism process, osteoblasts dissolve bone apatite on carbonate apatite with hydrogen ions in closed environments, cells such as osteoblasts and osteoclasts appear to adapt the area [18]. Histologically, carbonate apatite plays a role in bone regeneration, because it has properties such as good osteoconductive and bioresorbability.

Fourier Transform Infared Spectroscopy (FTIR) CA:Ch-GEL Scaffold. The identification result of scaffold functional group CA:Ch-GEL with ratio 50:50 (Fig. 1) hydroxyl group (-OH) peak at frequency 3287.35 cm^{-1}. Amide bond (C-N) peak at frequency 1637.82 cm^{-1}. CO_3^{-2} ions peak at frequency 1405.90 cm^{-1}. Aliphatic amine group peak at frequency 1021.05 cm^{-1}. PO_{4-} ions peak at frequency 865.44 cm^{-1}, 598.93 cm^{-1} and 559.33 cm^{-1}.

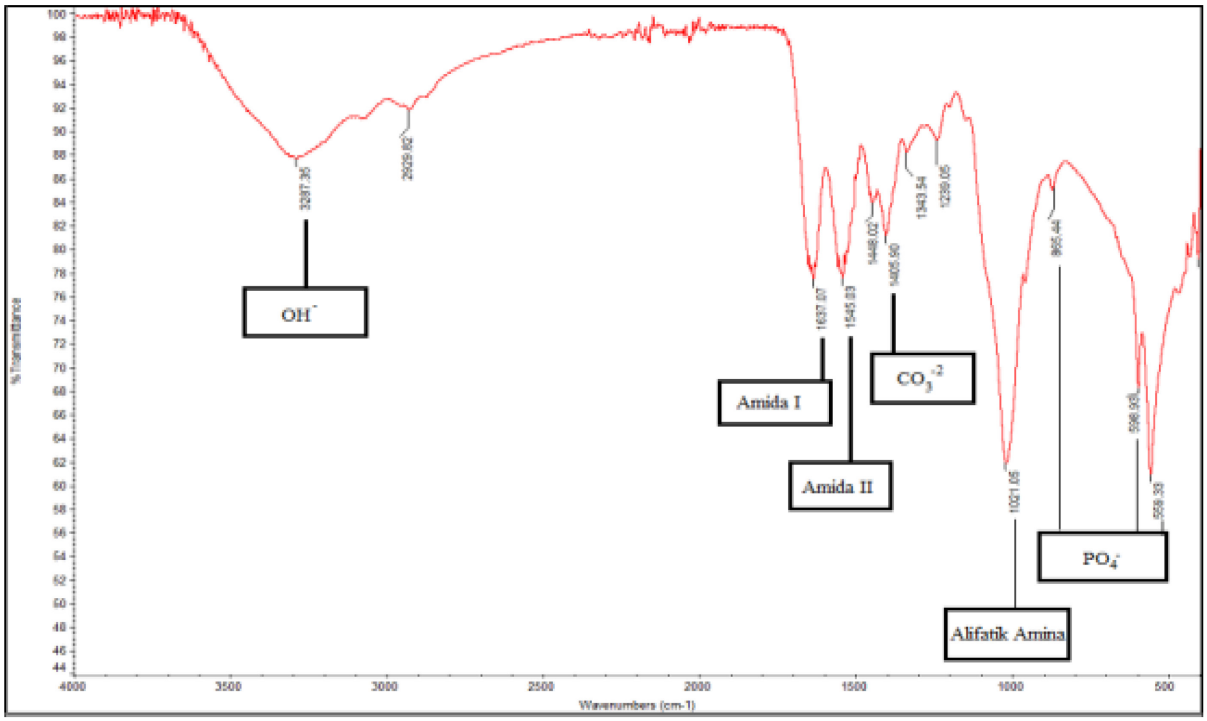

Fig. 1 The results of FTIR test for CA:Ch-GEL scaffold with ratio 50:50

The identification result of scaffold functional group CA:Ch-GEL scaffold functional group with ratio 60:40 (Fig. 2) hydroxyl group (-OH) peak at frequency 3273.03 cm^{-1}. Amide bond (C-N) peak at frequency 1647.94 cm^{-1}. Primary amine peak at frequency 1559.84 cm^{-1}. CO_3^{-2} ions peak at frequency 1406.22 cm^{-1}. Aliphatic amine group peak at frequency 1013.54 cm^{-1}. PO_{4-} ions peak at frequency 872.06 cm^{-1}, 599.46 cm^{-1} and 557.65 cm^{-1}.

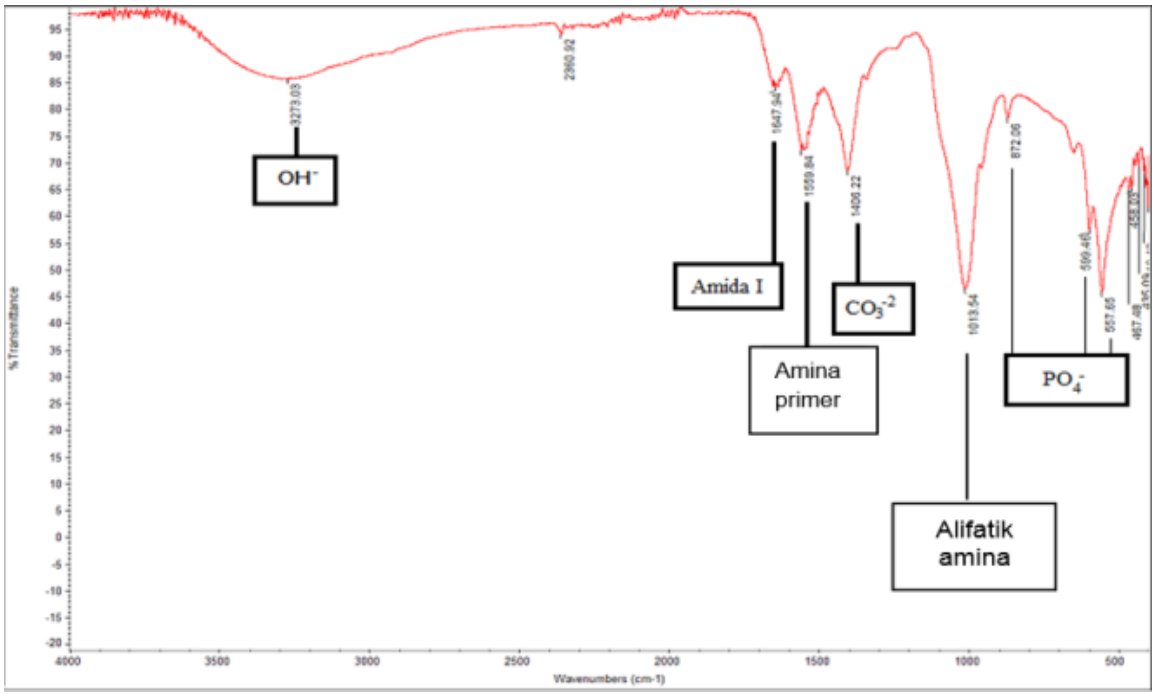

Fig. 2 The results of FTIR test for CA:Ch-GEL scaffold with ratio 60:40

The identification result of CA:Ch-GEL scaffold functional group with ratio 70:30 (Fig. 3) hydroxyl group (-OH) peak at frequency 3292.08 cm^{-1}. Amide bond I (C-N) peak at frequency 1647.77 cm^{-1}. Primary amines peak precisely at frequency 1550.96 cm^{-1}. CO_3^{2-} ion peak at frequency 1409.11 cm^{-1}. Aliphatic amine group peak at frequency 1015.31 cm^{-1}. PO_{4-} ions peak at frequency 872.35 cm^{-1}, 600.19 cm^{-1} and 559.2 cm^{-1}.

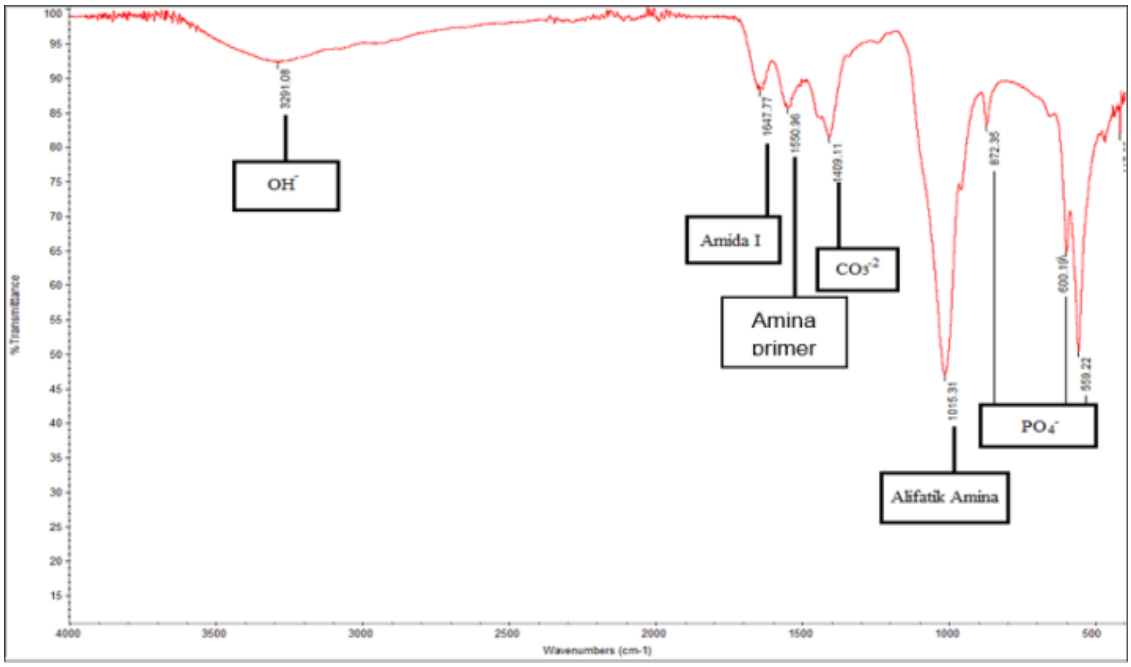

Fig. 3 The results of FTIR test for CA:Ch-GEL scaffold with ratio 70:30

The identification result of scaffold functional group CA:Ch-GEL scaffold functional group with ratio 80:20 (Fig. 4): hydroxyl group (-OH) peak at frequency 3278.15 cm^{-1}. Amide bond (C-N) peak at frequency 1648.09 cm^{-1}. This primary amine peak precisely at frequency 1552.79 cm^{-1}. Peak at frequency 1406.35 cm^{-1} indicating the presence of CO_3^{-2} ions. Aliphatic amine peak at frequency 1011.88 cm^{-1}. PO_{4-} ions peak are located at frequency 871.97 cm^{-1}, 598.88 cm^{-1} and 558.23 cm^{-1}.

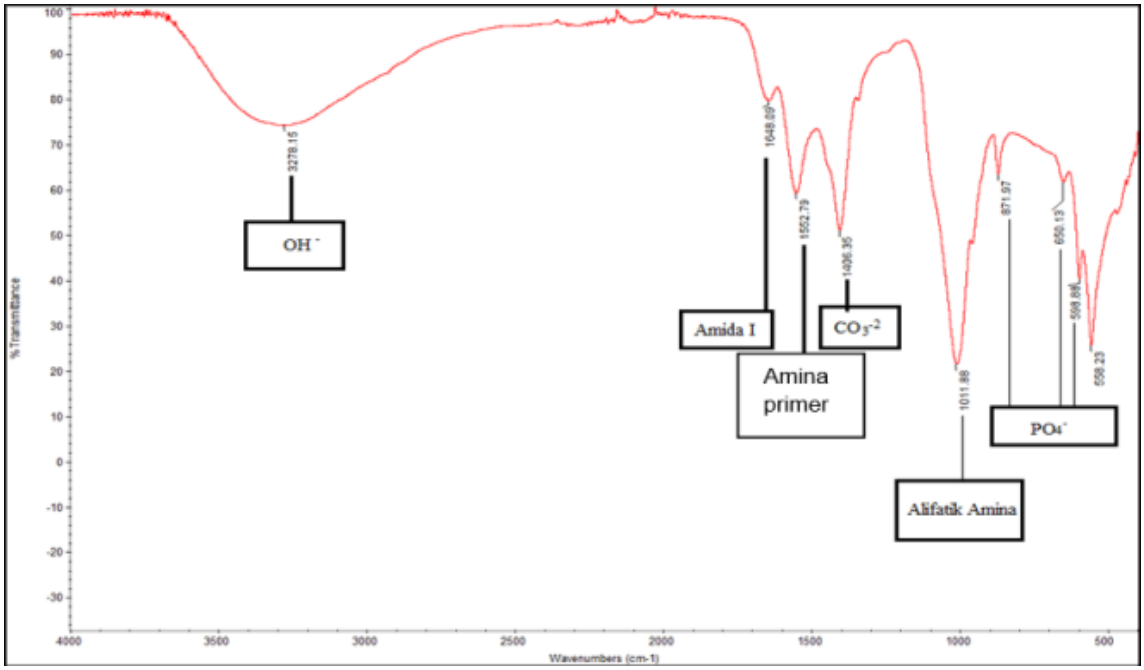

Fig. 4 The results of FTIR test for CA:Ch-GEL scaffold with ratio 80:20

Compressive Strength of CA:Ch-GEL Scaffold. CA:Ch-GEL scaffold with ratio 70:30 has the highest compressive strength value (Fig. 5). This value can be applied to cancellous or trabecular bone and has the same composition ratio as bone-forming composition. If scaffold has compressive strength value lower than implanted bone tissue, scaffold do not capable to compensate the pressure applied to the bone, this caused scaffold to deform. However, if scaffold has compressive strength value higher than implanted tissue, scaffold become very rigid and inflexible when the pressure applied and may cause tissue necrosis.

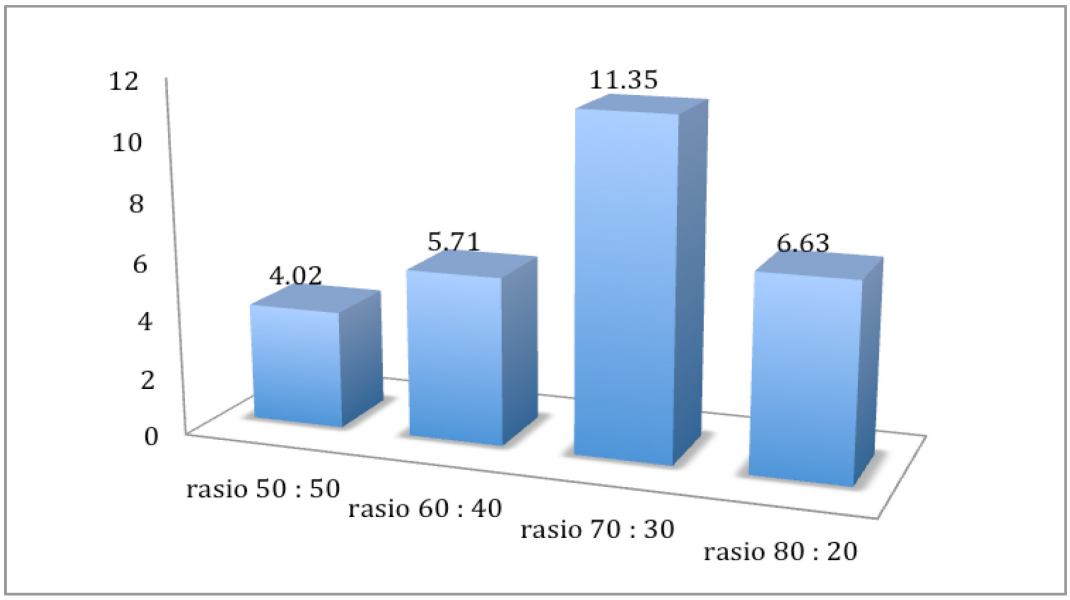

Fig. 5 Compressive strength value of scaffold CA:Ch-GEL

The compressive strength mean value of CA:Ch-GEL scaffold with ratio 70:30 (w/w) has the highest value. The results of ANOVA test shown significance value 0.000 ($p<0.05$) indicated there is a significant difference in each group ratio of scaffold CA:Ch-GEL (w/w).

Compressive strength value of CA:Ch-GEL scaffold with ratio 50:50, 60:40 and 70:30 is increasing. This is caused by increase of crystallization degree so that CO_3^{-2} crystals is increasing too, which followed by increased flexibility of scaffold framework due to increased of compressive strength values. This is corresponding to the previous researcher's opinion, when carbonate apatite is added more as scaffold production material, compressive strength values is also increasing [19].

In CA:Ch-GEL scaffold with ratio 80:20, there is a decrease in compressive strength value, this is caused by over addition of carbonate apatite which caused more rough texture of scaffold. Other factors that may affect among others are gelatin and chitosan, which are not capable to bind a lot of carbonate apatite, supported by the binding force saturation of CA:Ch-GEL mixture and CO_3^{-2} ions decrease.

Porosity of CA:Ch-GEL Scaffold. Scaffold must have porous properties. The pores over the scaffold have critical function for diffusion of nutrients and oxygen in cell survival [16]. In addition, the bone requires the pores for new tissue formation, because the pores support migration, osteoblast proliferation and mesenchymal cells, and vascularization [20].

The porosity result of scaffold surface area CA:Ch-GEL was obtained by measuring the pore diameter of scaffold using SEM. In this study, the results of CA:Ch-GEL scaffold with all ratios showed pore size in the range of 19.18µm - 52.59µm (Fig. 6B), that means CA:Ch-GEL scaffold with all ratios are qualified as biomaterial candidates to be applied to tissue engineering. The pore size of a scaffold between 20µm - 30 µm is sufficient for the growth of osteoblast cells [21], and for attachment of Mesenchymal stem cell, the pore size ranges from 17.9µm - 30.4 µm [22]. The pore size can be divided into two groups, micropore (pore <5µm) and macropore (pore> 100µm), both pore sizes are important for the bioresorbability of the material [20].

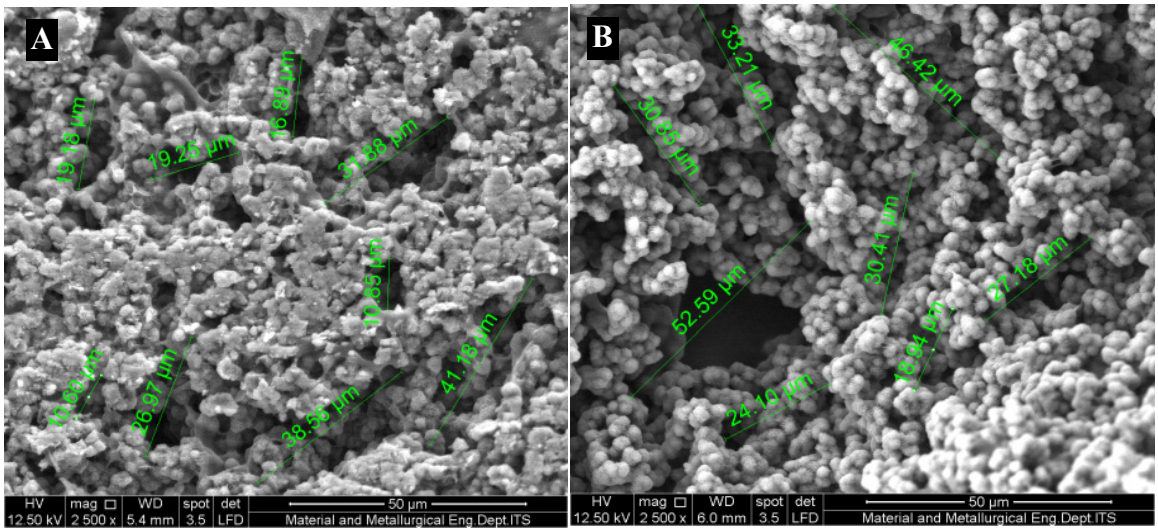

Fig. 6 The results of SEM CA:Ch-GEL scaffold with ratio 50:50 (A), ratio 60:40 (B) at 2500x magnification

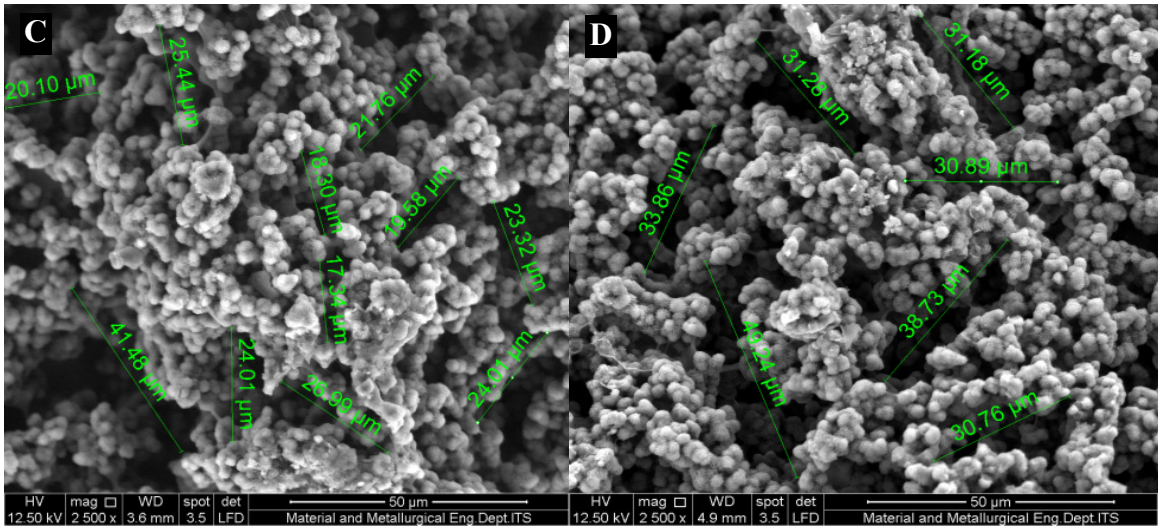

Fig. 7 The results of SEM CA:Ch-GEL scaffold with ratio 70:30 (C), ratio 80:20 (d) at 2500x magnification

The recommended minimum pore size for bone formation is 100µm [20], pore sizes in the range 200µm - 350µm are also found to be optimal for bone tissue growth [2]. Recent research has shown that in porous multi-scale scaffolds involving micro and macros pores can work better rather than just macro porous scaffolds. Scaffolds with large porosity will decrease mechanical properties such as compressive strength [23].

The ideal key factor of scaffold for bone tissue engineering is: having macroporosity (pore size> 100µm) and microporosity (pore size <20µm); interconnection between open porous makes it easy for tissue growth; sufficient compressive strength and controlled level of degradation; no changes in physical strength during sterilization, packaging, surgery; sterile environment for the hatchery of cells [16].

Conclusion

CA:Ch-GEL scaffold at all ratios can be used as biomaterials in tissue engineering. CA:Ch-GEL scaffold with a ratio of 70:30 it is best as a biomaterial for bone growth, because its porosity is in accordance with porosity criteria as a medium for osteoblast cell growth and has the highest compressive strength.

Declaration of Interest

The authors report no conflict of interest and the article is fund by Hibah RKAT 2017 by Faculty of Dental Medicine, Universitas Airlangga.

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