Synthesis and characterization of nano-hidroxyapatite/chitosan/carboxymethyl cellulose composite scaffold

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Synthesis and Characterization of Nano-Hidroxyapatite/Chitosan/*Carboxymethyl Cellulose* Composite Scaffold

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Abstract

An increase in the number of bone operation in recent years results in higher demand for more regeneration of bone tissue. The most effective way for bone tissue engineering is by using porous scaffold. In this study, synthesis and characterization of bone scaffold made from hydroxyapatite, chitosan, and *carboxymethyl cellulose* (CMC) were conducted with different variations of 20wt%, 25wt%, 30wt% and 35wt% CMC. The freeze drying process was performed with -20°C for 12 hours and followed by drying process for 30 hours. FTIR test showed that there was electrostatic force between NH_3^+ and $-COO^-$ which formulated polyelectrolyte network in which nano hydroxyapatite were adhered. The pore size was various with uneven distribution of pores and the best result was obtained on the sample 25 wt% CMC with 65 - 111 µm sized. In porosity test, the best result is obtained on the sample 25 wt% CMC with 46.9% value. Compressive strength test displayed that the four samples met the 2 - 10 MPa strandard. Biodegradation test showed the highest percentage of scaffold mass loss which was 54.60%. Thus, it could be concluded that the composite nano hydroxyapatite, chitosan, and CMC have met the requirement of bone scaffold.

Experimental article (J Int Dent Med Res 2019; 12(1): 31-37)

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Introduction

The prevalence of injury in Indonesia reached 7.5% in 2007, with the causes of injury such as falling, traffic accident, and caused by sharp or dull objects. In 2013, there was an increase in the prevalence of injury into 8.2%, which were caused by falling 40.9%, motorcycle accidents (40.6%), sharp or dull objects (7.3%), other land transportation accidents (7.1%), and sideslip (2.5%).[1]

Those kind of injures mostly contribute to the bone injuries suffered by the patients. Bone tissue engineering is necessary in order to heal bone injures. Tissue engineering has a purpose to restore structure, function and mechanical properties which have been damaged. One of the ways in tissue engineering is bone graft, because bone graft is used to assist mechanical properties and bone regeneration which support the orthopaedics field.

In order to restore structure, function, and mechanical properties of bone, tissue regeneration needs a suitable condition. One of the conditions is it must have pores which is competent with the bone's real condition. According to Jiang et al. the size of human cancellous bone's pore is 100-500µm.[2] Accordingly, a suitable porous scaffold is necessary for tissue engineering so that it can support the medical world needs. The scaffold used in the tissue technique has to meet several criteria before it is implemented into the human body. These criteria include having biogradeable characteristic, biocompatible, osteoinductive, and not giving negative immune responses to the patient.[3]

A material with high porosity will provide place for cell to adhere and grow as bone cell. Chitosan is one of the materials which has the characteristic as pore formers and it also has other features which are suitable for bone tissue engineering such as biocompatible and biodegradable.[4] In making scaffold, not only the characteristic as pore formers, but the characteristic which can stimulate the occurance of osteoblast cell growth is also required.

Hydroxyapatite is the most stable form in calcium phosphate and it is the main component

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in bone which holds 60-65%, it is also osteoconductive.[3,5]

This study uses nano-hydroxyapatite because the size of nano can increase the mechanical properties of bone, adhesion, and osteoblast cell growth in vitro culture. Scaffold of nano hydroxyapatite and chitosan still have some weaknesses such as low compressive strength, albeit it has better cell biocompatibility than chitosan scaffold.[2] This problem can be solved by adding an organic polymer which is Carboxymethyl cellulose (CMC). CMC is a natural product which has biodegradable and biocompatible abilities obtained from natural cellulose with chemical modification. It also has a very similar structure of chitosan, thus a strong polyelectrolyte tissue structure can be formed between CMC and chitosan.

In a study done by Jiang et al. scaffold was made using the leaching salt method and it resulted in compressive strength value of 2-12 MPa, but it still has toxic characteristic caused by the presence of salt residue and the relation between low pores.[2] Therefore, in this study, nano hydroxyapatite/chitosan/CMC scaffold is made by using freeze drying method. This method can regenerate the relation between low pores since the freezing phase can form pore structure which is the replication of ice crystalline dendrites traps. In the sublimation phase of this method, a cavity will be formed which will connect each pores because of the presence of steam power which goes to the surface of samples, so then interconnected pores are created. Freeze drying method is distinct than any other salt leaching methods because it uses sublimation principles in the process which does not generate any salt residue at the end of its process.

The research on nanohydroxyapatite-chitosan-CMC using various mass percentage (wt%) of 40%, 30%, and 15% has the maximal result on 30 wt% with three dimension result, pore size range of 100-500 µm, porosity of 77.8%, compressive strength of 3.45 MPa and degradation rate at the eight week still provides scaffold for cell to grow.[2] The development of this research is based on the Jiang et al. study to comprehend the more optimal value of nanohydroxyapatite-chitosan-CMC scaffold characteristic by lessening the variation of CMC into 20 wt%, 25 wt%, 30 wt%, and 35 wt%, in hope of CMC which regulates a better characteristic from the previous research can be discovered.[2]

Materials and methods

Materials and tools. The materials used in the making of composite scaffold were nano hydroxyapatite powder, chitosan powder (with degree of deacetylation of 80% and molecular weight of 2.5 x 10^5), carboxymethyl cellulose (CMC) (with degree of substitution of 0.7 and molecular weight of 4.2 x 10^8) and Polyvynil Alcohol (PVA).

The tools used in this research included measuring cup, beakers, suction pipette, weighing scale, and magnetic stirrer. The tools for testing are Scanning Electron Microscopy (SEM) inspect S50, FEI Corp, FTIR spectrophotometer American Perkin Elmer Co., autograph shimadzu GT10, freeze-dryer LYOFAC GT2, pipette, Erlenmeyer tube, weighing scale, pot bottle, and scaffold mould.

Synthesis procedure. Chitosan powder used has the degree of deacetylation of 80% with the molecular weight of 2.5 x 10^5, mixed with carboxymethyl cellulose (CMC) which has the degree of substitution of 0.7, molecular weight of 4.2 x 10^8 with the addition of 20 wt%, 25 wt% , 30 wt%, and 35 wt% CMC. The variation of wt% CMC concentration can be seen in Table 1.

Sample	n-HA: CS: CMC (wt%)	n- HA (g)	CS (g)	CMC (g)
A	60:20:20	6	2.0	2.0
B	50:25:25	5	2.5	2.5
C	40:30:30	4	3.0	3.0
D	30:35:35	3	3.5	3.5

Table 1. The Variation of nano-HA-CS-CMC Composite Concentration.

In the first step, nano hydroxyapatite slurry was made but before that Polivinil Alcohol (PVA), with the equal concentration of CMC according to Table 1, had been mixed into 98ml aquades. After it was mixed evenly, nano hydroxyapatite powder was added. Then the compound of chitosan and carboxymethyl

cellulose (CMC) was mixed into nano hydroxyapatite slurry by using magnetic stirrer for 2 hours.

Furthermore, acetic acid with 2 wt% was added into the nano hydroxyapatite-chitosan-carboxymethyl cellulose (CMC) solution and stirred well. After obtaining the desired solution, it was frozen at -20°C for about 12 hours. Then it was put into the freeze dryer to undergo the sublimation drying process for at least 30 hours. Eventually, a porous material which would be recognized as scaffold was obtained.

Afterwards, scaffold which had been freeze dryed was soaked in the NaOH 10% solution to neutralize the acetic acid residue on the nano-hydroxyapatite-chitosan- carboxymethyl cellulose (CMC) scaffold. Then it was cleaned by using deionisasi water until pH neutral. Thereafter, the scaffold was dried in the oven in the temperature of 60°C so that the scaffold does not contain water anymore. The sample formed could be seen in Figure 1.

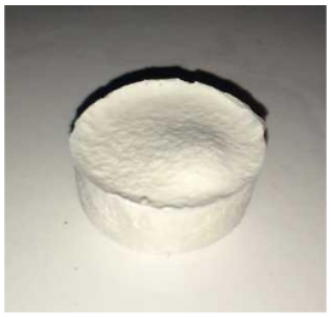

Figure 1. Nano-hydroxyapatite-chitosan carboxy-methyl Cellulose (CMC) Composite Scaffold.

Characterization of Nano-HA/Chitosan/ CMC Composite Scaffold

The sample characterization includes functional groups test with Fourier Transform Infra Red (FTIR) spectrophotometer American Perkin Elmer Co, surface morphology test with Scanning Electron Microscope (SEM) inspect S50, FEI Corp., compressive strength test, porosity test and biodegradation test. The measurement of compressive strength uses Autograph. Sample was placed on the compression part of the autograph machine, then the machine was turned on followed by setting the speed and choosing the weight (force) range. After that, load cell was lowered slowly until it was stopped then the power and strain received were written down. These steps were done with small force change until the sample was broken. The compressive strength value was calculated using Equation 1.

$$\sigma = \frac{F}{A} \quad (1)$$

F = maximum force (kN)
A = surface area (mm^2)
σ = compressive strength (kN/mm^2 atau MPa)[8]

In the porosity test, there were several steps that have been done. First, the composite scaffold sample was weighed to know its initial weight. Furthermore, the sample was soaked in ethanol 98% for 48 hours. After the soaking was done, the scaffold sample with ethanol was weighed again. Then the ethanol which had been already taken from the sample was weighed. The final results obtained from this porosity test are the initial weight of scaffold (w_1), scaffold and ethanol weights while being soaked (w_2), and the final weight of ethanol after the scaffold had been taken (w_3). Finally, the porosity percentage of each composite scaffold sample was measured by using the Equation 2.[9]

$$Porosity = \frac{w_1 - w_3}{w_2 - w_3} x100\% \quad (2)$$

In Vitro biodegradation test was done by soaking composite scaffold sample in the Simulated Body Fluid (SBF) solution. First, the composite scaffold sample was weighed first to know its intial weight. Afterward, the sample was soaked in the SBF solution for four weeks. The data from this test result were taken at the week 1, week 2, week 3, and week 4. For every data take, the scaffold sample was dried first and then weighed to know the final scaffold weight after soaking. Thus, the data obtained from the In Vitro biodegradation test are intial weight of each composite scaffold (w_0) and the final weight of composite scaffold after the soaking (w_1). Lastly, the degradation percentage of mass loss was calculated by using the Equation 3.[9]

$$W_L = \frac{w_o - w_1}{w_o} x100\% \quad (3)$$

Results

FTIR test result. The result from FTIR test on nano hydroxyapatite-chitosan-CMC composite can be seen in Figure 2.

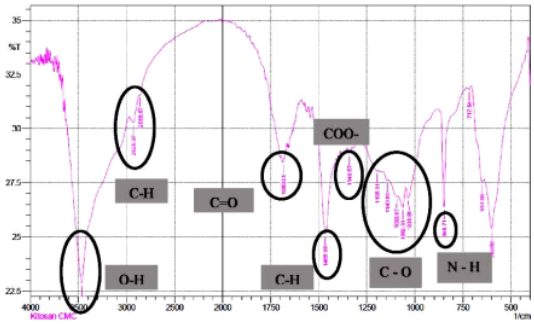

Figure 2. FTIR Spectrum of nanohydroxyiapatite/ chitosan/CMC Composite Sample.

The FTIR spectrum in Figure 2 illustrates the presence of C-H (1465.95cm^{-1}) group which is one the functional group of *Polyvinyl Alcohol* (PVA) in the production of nano HA slurry. The data of chitosan FTIR showed that on wave number 1655 cm^{-1}(C=C), was not found on the scaffold sample but it was found at the peak of wave number 848.71 cm^{-1} which is N-H group.

SEM test result. The SEM test was conducted to know the surface structure and the pore diameter of the sample, also to know the pore interconnection on the sample which was observed transversely. The result of this test can bee seen in Figure 3. The pore diameter of the nanohydroxyapatite/ chitosan/CMC scaffold sample could be measured by using the scale line in Figure 3 (a, c, e, and g). In Figure 3 (b, d, f, and h), the presence of interconnection pore can be observed and it is shown in the red circle. Interconnection pore functions to regulate the delivery of nutrient from one cell to another. After the measurement of pore diameter done to all four samples based on Figure 3, each sample obtains different pore range. Pore diameter range of nano hydroxyapatite/ chitosan/CMC scaffold sample is presented in Table 2.

The effective pore size for bone cell growth is 100-500 µm. Based on the pore diameter range data in Table 2, it is obtained that one which met all the criteria was the sample B[2].

Samples	Pore size (µm)
A	30-55
B	65-111
C	20-85
D	35-66

Table 2. Pore Size of nanohydroxyapatite/chitosan/CMC Composite Scaffold.

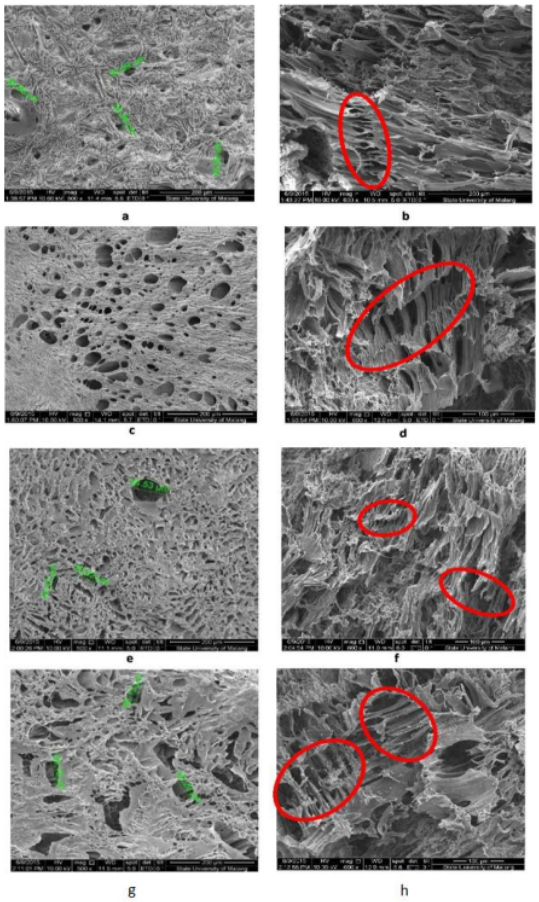

Figure 3. Surface Morphology and Cross-Sectional.

The compressive strength, porosity, and biodegradation results

The overall data analysis of compressive strength, porosity and biodegradation could be seen on Table 3 and Figure 4. Figure 4 demonstrated that in sample A (20 wt% CMC) and sample B (25 wt% CMC) with the increase of

CMC concentration, there was an increase on compressive strength and a decrease on porosity. The increase of compressive strength followed by the decrease of porosity could be observed in Figure 4 on Sample A to Sample B.

Parameter		Samples (variation of wt% CMC)			
		A (20 %)	B (25%)	C (30%)	D (35%)
Pore size (μm)		30-55	65-111	40-85	35-66
Compressive strength (MPa)		4.39	4.60	2.84	4.38
Porosity (%)		49.00	46.90	33.00	38.64
Lost Mass Percentage (%)	1 week	18.05	27.53	25.69	47.23
	2 weeks	20.48	38.01	45.48	54.60
	3 weeks	18.05	36.75	43.75	53.45
	4 weeks	15.62	33.61	36.63	51.15

Table 3. The Results of Compressive Strength, Porosity And Biodegradation.

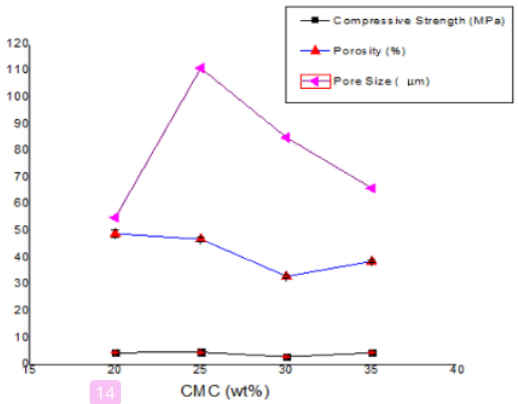

Figure 4. Compressive Strength, Porosity and Pore Size of Nano-HA/Chitosan/CMC Composite Scaffold.

The pore size from each sample was uneven as displayed in the Table 3 and in influenced the compressive strength result which could be seen in the SEM test result of Sample A in Figure 4. It was shown there that the distribution of pores was uneven and the size of pore is small so that it influences the compressive strength of the sample.

Meanwhile, the *in vitro* biodegradation test result displayed in Figure 5 showed that the four samples still have a place for osteoblast cells to grow and evolve because up until the fourth week, the percentage of scaffold loss tended to decrease due to attachment of apatite particles from SBF solution. From this attachment of apatite particles, it could be concluded that the samples have a good *in vitro* bioactivity.

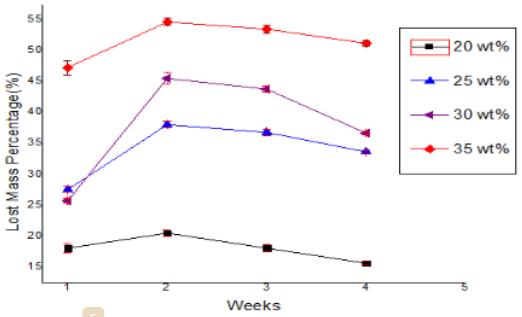

Figure 5. In Vitro Test Biodegradation of Nano-HA/Chitosan/CMC Composite Scaffold.

Discussion

The bone scaffold of nano hydroxyapatite/chitosan/CMC was synthesized. The functional group characterization was performed by FTIR Test. In the sample, NH_2 (1599 cm^{-1}) group was also not discovered which indicates that there was NH_3^+ formation. Meanwhile, the $-COO^-$ was found in the sample and it has wave number range of 1340-1450 cm^{-1}. CMC is salt from weak acid while chitosan is salt from bacid base. The stage of CMC and chitosan mixing in the form of salt can be observed by reaction:

$$-OCH_2COO^-Na^+ + H^+ = -OCH_2COOH + Na^+ \quad (4)$$
$$-NH_3OH + H^+ = -NH_3^+ + H_2O \quad (5)$$

The reaction above is the evidence that $-COO^-$ group in CMC will bond $-NH_3^+$ to chitosan. There is electrostatic force between NH_3^+ and $-COO^-$ which will form polyelectrolyte network structure which then will be the place where nano hydroxyapatite is adhered.[10]

The morphology test was performed by using SEM. The result did not show uniform pores. This might be caused by the freeze-drying process. The process of freeze drying could not form the same and regular pore sizes because the pore structure is the replication of entangled ice crystalline dendrite. The making of ice crystalline depends on how long the freezing takes, the temperature and the composition of solvents. The formation of ice crystalline is important in acquiring appropriate diameter and shape of pores. The slower freezing rate will produce a bigger pore and the lower freezing temperature will make the composite frozen

faster so that the formation of ice crystalline dendrite becomes faster too. The more menyatu ice crystalline dendrite gives impact on the formation of interconnection pore at the sublimation phase. In this phase, solvent changes its form into gas which moves into the sample's surface and causes a cavity formed which then connects one pore to another. This is called as interconnection pore. Interconnection pore aims to mengalirkan nutrients to bone cell from one pore to another.[8] Along with the increase of CMC percentage, it influences on the decrease of interconnection pore size which can be observed in the sample b, c, and d. The decrease of interconnection pore is resulted from the presence of the bond between CMC and chitosan which becomes denser so that it creates smaller space. This is supported by the increase of compressive strength along with the increase of CMC concentration.

The mechanical strength was evaluated by compressive strength test. The compressive strength was increasing while the pore size was decreasing. It occured because with the smaller CMC concentration and chitosan gave bigger ice space in the frozen liquid. Chitosan and CMC act as the structure in the scaffold. Meanwhile, CMC itself functions to increase mechanical power. However, because of the uneven distribution of pores, CMC lowered the mechanical power as happened in the sample A. The increase of CMC concentration which has similar structure to chitosan will make it possible for ionic bonding occurs between the two of them so that it causes durability to compressive strength increases more.

In Figure 4, the sample C also experienced an uneven pore distribution and it caused a small porosity value. It was due to the presence of PVA group in the FTIR test result. The PVA group affected the pore size and porosity because PVA used in making the nano hydroxyapatite slurry has bigger molecular weight and it cannot disappear while it was being heated at 60°C which was done in order to evaporate aquades and NaOH solution as the neutralizer of acetic acid on sample. The PVA, which has a boiling point at 228°C and existed inside the sample, caused the pore size to shrink while it was being heated. Therefore, the porosity tes result did not meet the literature standard which is 70%.[11]

After several tests have been conducted to the four samples of nano hydroxyapatite/chitosan/CMC, the best characteristic was found on sample B with the variation of CMC concentration of 25 wt% CMC. The pore diameter size range of sample B is 65 - 111 µm with the porosity of 46.9% and compressive strength of 4.60 MPa. The sample B also had scaffold residue after it had been soaked in the SBF solution with the maximum scaffold loss of 38.01%. Whereas, the nano hydroxyapatite/chitosan/CMC scaffold can be applied as cancellous bone scaffold if it has criteria such as having pore size of 100-500 µm, porosity for about 70%, compressive strength of 2-10 MPa, and it is not degraded entirely. In result, even though sample B has the best result compared to the other three samples, it cannot be applied as a cancellous bone scaffold because its percentage of porosity has not yet met the criteria. Therefore, monitoring the making of HA slurry, which contains PVA and the freeze drying process especially for its freezing rate and the duration of freezing that affects the formation of pore size, is needed in order to obtain a sample with suitable pore diameter, porosity, and mechanical properties for the application of cancellous bone scaffold.

Conclusions

The variation of CMC influences the size of pores, the porosity, the compressive strength and loss percentage of scaffold with SBF solution on the nano hydroxyapatite/chitosan/CMC composite scaffold. In general, the higher CMC percentage the higher the compressive strength with the range of 2.84-4.60 MPa which was followed with the decrease of porosity percentage until 30%, pore size, and the increase of mass loss percentage of scaffold with the maximum value of 54.60% in the SBF solution. The best composition is found in the sample B with 25 wt% CMC and it has compressive strength of 4.6 MPa, porosity of 46.9%, and pore size range of 65-111µm and the highest percentage of scaffold mass loss which is 38.01%.

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Declaration of Interest

The authors declare that there is no conflict of interest.

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