Fabrication of Chitosan-Chondroitin Sulfate/Hydroxyapatite Composite Scaffold by Freeze Drying Method

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Abstract

The use of tissue engineering technique increases along with the rise of bone tissue defect and bone tissue degeneration cases due to sickness, injury, trauma and accident. This study aimed to know the influence of variation from chondroitin sulfate (CS) and hydroxyapatite (HA) to scaffold characteristic and to determine the best composition for chitosan-chondroitin sulfate/hydroxyapatite (Ch-CS/HA) as bone scaffold candidate. The composition variation of Ch:CS:HA used in this study are A (35:0:65), B (35:5:60), C (35:10:55), D (35:15:50) and E (35:20:45) wt%. The synthesis of scaffold was done via freeze-drying method at -80 ^{o}C for 5 h and sublimation drying process for 48 h. The result of functional group test showed that the typical functional groups of HA, Ch and CS were simultaneously found at wave numbers of 962.48-1041.46 cm^{-1}(P-OH), 1651.07-1658.78 cm^{-1} (N-H), and 1417.68 cm^{-1}(COO-). In SEM test, the pore diameter range obtained is 6.70-191.00 µm. The result of porosity test from composite scaffold is 47.3408 – 82.869%. In vitro biodegradation test results showed that the highest percentage of weight loss in each sample, which was observed in week four, is 23.8619%. Cell Viability showed that more than 92.1514%. The compressive strength obtained is around 2.6914 – 7.6233 MPa which has met the standard as cancellous bone substitute. These characterizations suggest that sample B (35:5:60 wt%) is the best Ch-CS/HA composite as candidate for bone scaffold.

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Introduction

Various types of biomaterial have been investigated and used clinically in bone repair and bone regeneration application. One of that is bone tissue engineering technique which has been proposed as a promising medium to restore bone functions.[1] This tissue technique has been increasingly used along with the rise of defective bone tissue and degeneration cases due to disease, injury, trauma, and other reasons. The tissue engineering technique in its application aims to grow defective tissue by developing biological substitutes which can restore, maintain, and increase the function of a tissue.[2]

The scaffold is the main component in tissue engineering, which functions as a template for cell interaction and bone extracellular matrix formation that give structural support for a new tissue formation.[3] Hence, scaffolds can be used as one of the solutions to repair the defective bone tissue or bone tissue degeneration. One of the reason is because scaffolds have three dimensional structure used as temporary media for cell growth process, transportation of nutrient distribution, and metabolic waste.[4] Ideally, scaffolds should have several characteristics, namely biocompatible, biodegradable, bioactive, bioabsorbable with a controllable degradation level, and appropriate absorption level to both in vitro and in vivo cell tissue growth. A material is considered as physically conform as scaffold candidate for cancellous bone substitute if it has porosity of 70%, has effective pore size of bone growth of 100-600 µm, and has mechanical power of 2-12 MPa, so that it can be the temporary support while new tissue growing process occurs.[5,6,7,8] Materials which can be used to make scaffold can be a compound from bioceramic and biopolymer, which are hydroxyapatite and chitosan with the addition of chondroitin sulfate.

The production of composite scaffold from chitosan, chondroitin sulfate, and hydroxyapatite

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has been done by several researchers, one of them is Venkatesan *et al*.[9] Hydroxyapatite (HA) is used as a scaffold material because it has several properties such as; it can create a tight bond with bone tissue, it does not have negative effects on human, it supports cell proliferation process, and it shows an osteoconductive behavior.[10] Chitosan (Ch) is a natural polymer and it is polycathodic which facilitate ion absorption inside our body, also it has the ability to form pores. Adding polymer Ch into HA will make the pore size bigger because Ch is hydrophilic so while the freeze-drying process occurs, Ch will bind the ice crystalline. This crystalline later will evaporate and leave cavities in the sample. The cavities formed is expected to help the cell regeneration process. Chitosan has potential effects on the osteogenesis process in periodontal ligament cells for the regeneration of periodontal tissues.[11] Moreover, chitosan has the ability to overcome burns when mixed with gelatin and glutaraldehyde.[12]

A study showed that adding chondroitin sulfate (CS) to collagen/hydroxyapatite results in an increase of bone remodeling and new bone formation.[13] In addition, the result of in vitro and in vivo studies showed that CS-based biomaterial will amplify the cartilage regulation especially on chondrocyte and stem mesenchymal cells and it will support osteogenic differentiation by increasing the effectiveness of anabolic factor of bone growth [14]. Besides, CS is more effective on cervical cancer than breast cancer cell line.[15]

Therefore, it is reckoned that the addition of CS to Ch/HA will increase the characteristic of scaffold so that it corresponds with the ideal characteristics of a scaffold. The present study aimed to know the influence of variation of Ch and HA and to determine the best composition from various compositions of Ch-CS/HA as a scaffold candidate.

Materials and Methods

Materials and tools

Materials used are nano-HA, Ch with DA 75% sigma Aldrich, CS which is synthesize result from bovine trachea product of sigma Aldrich, acetic acid 2%, NaOH 10%, distilled water, ethanol, dehydrated alcohol, and materials in making Simulation Body Fluid (SBF) which are $K_2HPO_4.3H_2O$, $CaCl_2.2\ H_2O$, NaCl, $NaHCO_3$, Na_2SO_4, KCl, HCl, $MgCl_2.6H_2O$ and $(HOCH_2)_3CNH_2$. Tools used in this study include tools to make the sample and to test the sample. Tools in testing are spectrophotometer FTIR Shimadzu IR 100, Scanning Electron Microscopy (SEM) type Phantom and Jeol, also compressive test machine type JTM.

Synthesis of Ch-CS/HA composite scaffold

The samples used in this study are Ch-CS/HA with different composition comparison; Sample A (35%: 0%: 65%); B (35%: 5%: 50%); C (35%: 10%: 55%); D (35%: 15%: 50%) and E (35%: 25%: 45%) of the whole sample (wt%). The 2% Ch solution was made by dissolving Ch powder in acetic acid (2%) with magnetic stirrer. HA and CS were dissolved in 62.5 ml distilled water separately with magnetic stirrer until it is homogeneous. The homogeneous HA and CS solution were amalgamated in Ch solution gradually by using pipette, then it was stirred for 24 h. After that, Ch-CS/HA solution prepared was molded and was frozen at -80 ^oC for 5 h and followed by sublimation drying process for 48 h. After freeze drying was done, the scaffold sample was immersed in 10% NaOH solution for 24 h to remove acetic acid residue contained in the sample and then followed by washing the sample by using distilled water until the pH is neutral. Lastly, the sample was dried in the oven at 600 ^oC to remove the remaining water and NaOH.[9]

Characterization of Ch-CS/HA composite scaffold

The characterization of the samples included functional group test using Fourier Transform Infra-Red (FTIR) IRTracer-100, observation of pore diameter and elements content contained in the sample using SEM-EDX JEOL JSM-6510LA, porosity test, compressive strength test, and in vitro biodegradation test.

The porosity was determined by using the liquid displacement method. Firstly, the volume (V_0) of scaffold sample and its initial weight (W_1) were measured. Then, the samples were immersed into the 98% ethanol for 48 h and then weighed again to know its weight after being immersed (W_2). Finally, the porosity value percentage of each sample was calculated by using Equation 1 [16]:

$$P = \left(\frac{W_1 - W_0}{\rho V_0}\right) \times 100\ \% \quad (1)$$

The compressive test used autograph machine by molding cylindrical-shaped samples with d=0.5cm and t=1cm. First, the autograft engine was turned on to adjust the speed and to select the loads to be measured. Then the loads were lowered slowly until the suppressor part of the machine pressed the sample surface until it was broken. The data were taken at the time the sample begun to crack. The results were in the form of force needed until the sample broken. Finally, the compressive strength value was calculated by using Equation 2:

$$\sigma = \frac{F}{A} \quad (2)$$

σ is the value of compressive strength (N/m^2) given to the sample, F represents force (N) and A is the sample cross-sectional area (m^2).

The in vitro biodegradation test in this study was performed by immersing the sample into Simulated Body Fluid (SBF) solution. Initially, the samples were weighed to determine their initial weight (W_0). Then the samples were immersed in SBF solution for 4 weeks. The data were taken per week and the sample was dried first then weighed (W_1) for every data retrieval. The results of in vitro biodegradation test were represented by the percentage of mass loss of the samples. Finally, the percentage of mass loss of each sample was calculated by using Equation 3 [9]:

$$W_L = \frac{(W_0 - W_1)}{W_0} \; X \; 100\%, \quad (3)$$

The cytotoxicity test in this study was done by using MTT Assay which is tetrazolium salt [3-(4,5-dimethyltiazole-2-yl)-2,5-diphenyltetrazolium bromide]. The working principle in the MTT Assay is to follow the ability of living cells according to the mitochondrial activity of fibroblast cell culture (cell line BHK 21). The changes occurred in the tetrazolium salt [3-(4,5-dimethyltiazole-2-yl)-2,5-diphenyltetrazolium bromide] to be formazan in active mitochondria was the basis of the MTT Assay method. Mitochondria cells reduced MTT salts by reductase enzyme reduction reaction became a dissolved purple formazan in the mitochondrial respiration chain. The optical density (OD) value was read through Elisa reader with a wavelength of 520 nm, then the living cell was calculated by using Equation 4:

$$\%\ Cell\ Viability = \frac{OD\ Treatment - OD\ Media\ Control}{OD\ Cell\ Control - OD\ Media\ Control} \times 100\ \% \quad (4)$$

Results

Identification of functional groups with FTIR

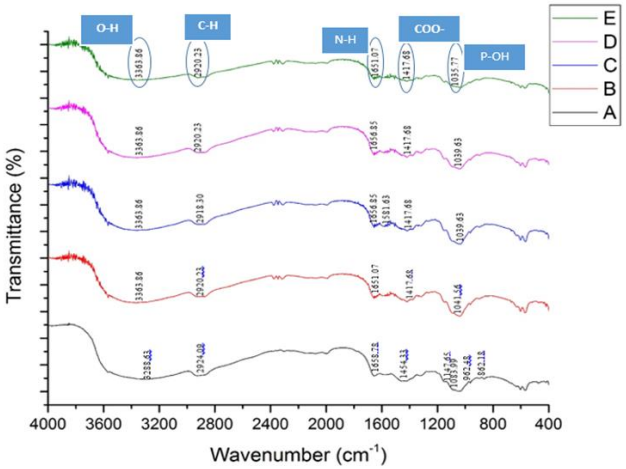
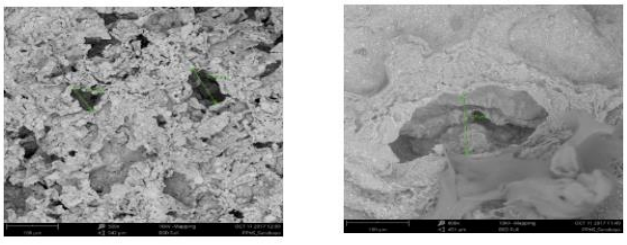


Figure 1. Spectrum FTIR of CH-CS/HA composite samples.

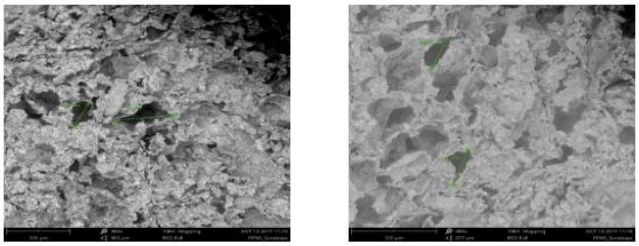
In Figure 1, the FTIR spectrum has a wave number of 3288.63 cm^{-1} which represents the absorption area of the O-H functional group held by all materials. The wave numbers of 962.48 cm^{-1}, 103.77 cm^{-1}, 1039.63 cm^{-1} and 1041.56 cm^{-1} showed vibrations of the P-OH functional group in which this functional group constitutes one of the typical absorption bands of the material HA.[17] The absorption area showing the C-H functional group experienced a slight vibratory frequency shift from 2924.09 cm^{-1} in the Ch-CS/HA Composite Scaffold to 2920.23 cm^{-1} in the Ch-CS/HA Composite Scaffold.

Morphology Test by SEM-EDX



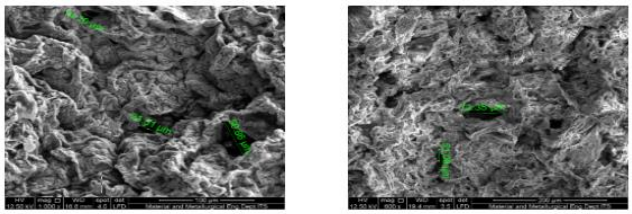
(a) (b)

Figure 2. Morphology of Ch/HA Composite Scaffold: **(a)** Surface Section; **(b)** Cross Section.



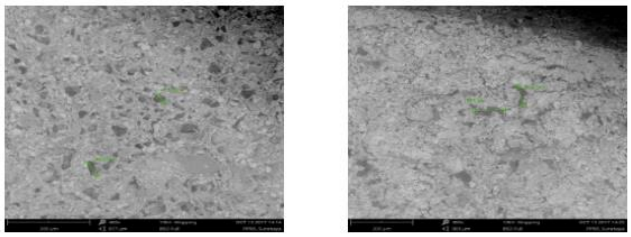
(a) (b)

Figure 3. Morphology of CS (5 wt%) Composite Scaffold: **(a)** Surface Section; **(b)** Cross Section.



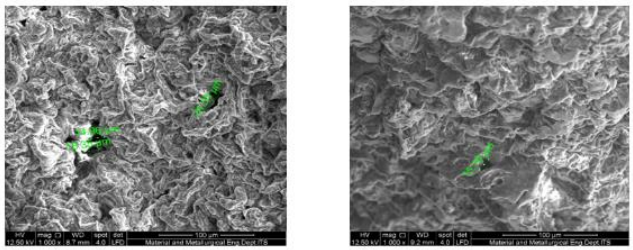
(a) (b)

Figure 4. Morphology of CS (10 wt%) Composite Scaffold: **(a)** Surface Section; **(b)** Cross Section.



(a) (b)

Figure 5. Morphology of CS (15 wt%) Composite Scaffold: **(a)** Surface Section; **(b)** Cross Section.



(a) (b)

Figure 6. Morphology of CS (20 wt%) Composite Scaffold: **(a)** Surface Section; **(b)** Cross Section.

The SEM-EDX test results showed pore diameter, pore distribution, surface structure, and elemental content of chitosan-chondroitin sulfate/ hydroxyapatite composite scaffold samples (Figure 2-6). The effective pore size diameter required for bone growth is around 100-600 µm. The pore diameter size of 75-100 µm is good for osteoid tissue growth, while the pore diameter size of 10-75 µm is good for fibrous tissue growth.[7] A scaffold with a pore size of 100 µm or bigger is commonly needed for bone mineral regeneration.[18] The pore size of each sample, as shown in Figure 2-6, is presented in the Table 3. It is found that the sample that meets the criteria is sample B with the pore size of 80-191 µm. This pore is used as a place for new tissue growth and flow of nutrients transportation.

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
8	O	Oxygen	70.62	54.59
20	Ca	Calcium	14.33	27.74
15	P	Phosphorus	9.42	14.09
7	N	Nitrogen	3.22	2.18
6	C	Carbon	2.42	1.40

(a)

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
8	O	Oxygen	41.50	25.28
20	Ca	Calcium	32.95	50.28
15	P	Phosphorus	17.21	20.30
7	N	Nitrogen	4.31	2.30
6	C	Carbon	4.03	1.84

(b)

Table 1. SEM-EDX of Chitosan/Hydroxyapatite Composite Scaffold (a)Surface Section; (b) Cross Section.

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
8	O	Oxygen	50.70	39.31
20	Ca	Calcium	17.33	33.66
15	P	Phosphorus	8.72	13.09
6	C	Carbon	19.02	11.07
7	N	Nitrogen	4.23	2.87

(a)

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
8	O	Oxygen	50.64	38.91
20	Ca	Calcium	17.65	33.98
15	P	Phosphorus	9.24	13.75
6	C	Carbon	18.39	10.61
7	N	Nitrogen	4.08	2.75

(b)

Table 2. SEM-EDX of CS (10 t%) Composite Scaffold: **(a)** Surface Section; **(b)** Cross Section.

The EDX test on a scaffold composite samples was done by selecting several points randomly on SEM. The EDX spectrum showed some signals from elements C, O, P, Ca and N which are components present in the composite scaffold. The EDX test was performed on two samples; sample A with 0 wt% CS (Figure 2) and sample C with 10 wt% CS (Figure 4). The results showed that each component is homogeneously distributed within a composite scaffold. Based on EDX test results of both variations of samples, it can be seen that the ratio of Ca/P for each composite scaffold sample Ch-CS/HA showed results that tend to exceed the ratio of pure hydroxyapatite Ca/P of 1.67%. From the composite scaffold sample which has a ratio of Ca/P more than 1.67%, it can be interpreted that the calcium (Ca) content is larger compared to the phosphorus (P) content.

Sample	Composition Ch : CS : HA (wt%)	Pore Size (μm)
A	35 : 0 : 65	41,00-124,00
B	35 : 5 : 60	80,00-191,00
C	35 : 10 : 55	13,82-73,35
D	35 : 15 : 50	32,50-129,00
E	35 : 20 : 45	6,70-24,50

Table 3. Pore Size of Chitosan-Chondroitin Sulfate/Hydroxyapatite Composite Samples.

Porosity and compressive strength tests

Important parameters which are necessary in scaffold formation include porosity which plays a significant role in maintaining tissue volume, providing temporary mechanical function, and facilitating cells in adequate nutrient diffusion. The percentage of porosity formed is often associated with the compressive strength value of a scaffold. The smaller porosity value will result in a composite scaffold sample becomes stronger so that its compressive strength becomes larger. The largest percentage of porosity (Figure 7) was found in sample A which is a Ch/HA composite scaffold (35: 65%). Meanwhile, the percentage of porosity for samples B, C, D and E decreased along with the increase of CS composition. Adding CS to the Ch/HA compound causes the decreasing of porosity which then followed by the increase of compressive strength value. It happens because CS has carbonyl, amide, and sulfonyl groups which have high reactivity so that intermolecular hydrogen bonding to Ch and HA occurs.[9] The cancellous bone has a porosity of 70% contained in the femoral neck while the compressive strength value of the scaffold for an ideal cancellous bone replacement is 2-12 MPa.[6,8] The compressive strength values for each compositional variation of the Ch-CS/HA composite scaffold have met the criteria as bone scaffold. Therefore, it can be concluded that sample B with Ch-CS/HA 35: 5: 60 wt% composition is the best result with the porosity percentage of 79.4305% and the compressive strength value of 4.6734 MPa.

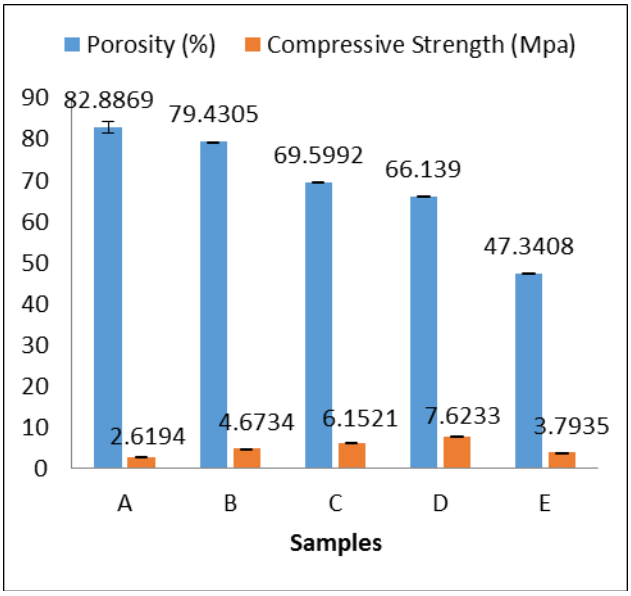


Figure 7. Porosity and Compressive Strength of Ch-CS/HA Composite Scaffold Samples.

In vitro biodegradation and cell viability

Biodegradation is considered as an important parameter in scaffold production for a successful long-term implantation. A scaffold should undergo degradation over time/from time to time and correspond to new bone formation. Figure 8 shows an increase in the percentage of mass loss of each sample that has been immersed into SBF solution for one week, three weeks, and four weeks. It can be observed in Figure 8 that the degradation of mass loss of scaffolds in the third week to the fourth week has not significantly increased. This indicates that there is bone-like apatite layers on the surface of scaffolds which later will bind to the bone in the

body. According to Kokubo et al. (2006), the formation of apatite layer, which is one of the bone constituents, takes 4 weeks.[19] The formation of apatite layer also proves that a scaffold constituent material has bioactive properties.[18] In the fourth week, it can be seen that the composite scaffold samples have not been completely degraded. This implies that the scaffold samples can still provide space for new bone tissue growth. Thus, it can be concluded that Ch-CS/HA composite scaffold samples have good in vitro bioactivity and found that Sample B is the best result with a mass loss of 17.7445% for four weeks.

A cell viability test was carried out to determine the cytotoxicity of chitosan-chondroitin sulfate/hydroxyapatite composite scaffold viable cells after incubation. A material is said not to be toxic if the percentage of living cells viability is more than 50%.[20] The percentage of living cells in composite scaffold samples A, B, C, D and E obtained from the cytotoxicity test using MTT Assay are 120,7428 %, 122,5648%, 118,4303%, 92,15137% and 126,2789%. (Figure 9). This suggests that with the addition of chondroitin sulfate to the samples, the composite scaffold has no toxic properties to living cells.

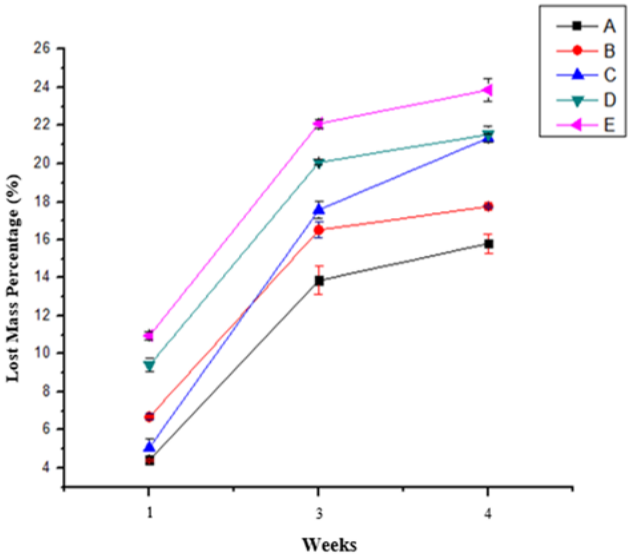


Figure 8. The Percentage of Mass Loss of Chitosan-Chondroitin Sulfate/Hydroxyapatite Composite Scaffold Samples.

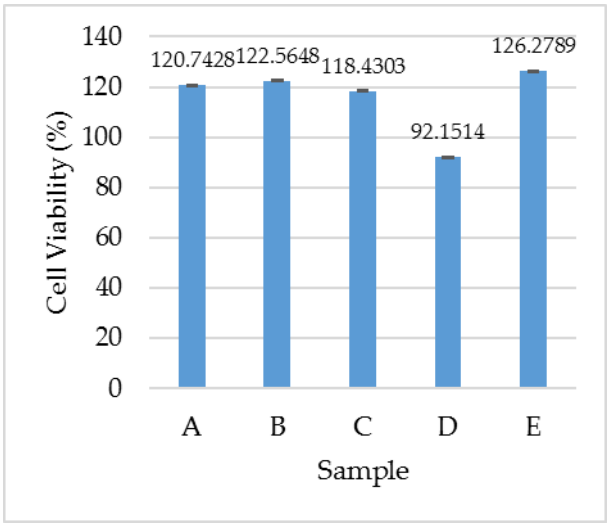


Figure 9. Cell Viability of Ch-CS/HA Composite Scaffold Samples.

Discussion

The addition of CS to Ch/HA is dispersed precisely in chitosan polymers and ceramic HA matrices. CS (Figure 10) has a typical absorption band that is in the active groups of $–COO^-$ and $-SO_3^-$. In Figure 1, there is a vibration group $–COO^-$ shown at the wave number 1417.68 cm^{-1}. Meanwhile, the functional group S = O, which has a standard wave number 1155-1245 cm^{-1}, was not found in the scaffold sample.

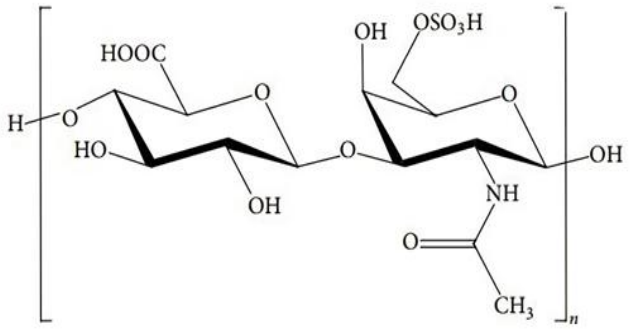


Figure 10. Chemical Structure of CS.[21]

This might due to the chemical interactions of the $–OSO_3^-$ group in CS with the $–NH_3^+$ group in Ch (Fig. 11) that can be observed in the following reactions.[22]

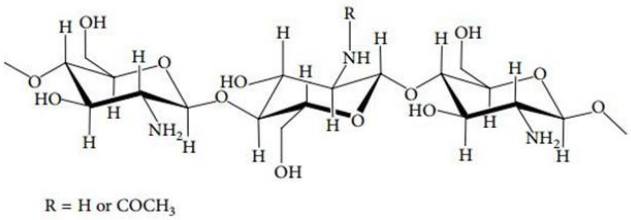


Figure 11. Chemical Structure of Ch.[21]

$-OSO^-_3Na^+ + -NH_3^+Cl^- = -OSO_3NH_3^- +$ NaCl

The addition of CS in the Ch/HA compound yields pores of suitable size for the new bone tissue growth which ranges from 41.00 -191.00 µm. In freeze-drying process, the solvent content in the sample will form ice crystals which then eventually evaporate during the drying process and leave the cavities or pores in the sample. In the previous studies, freeze-drying process was done one more time to the samples that had been neutralized with NaOH and distilled water, it causes the pore sizes to enlarge.

The percentage of porosity produced in the composite scaffold decreases along with the increase of CS composition. Moreover, a drop in the percentage of porosity results in a larger mechanical strength of composite scaffolds. Additionally, the addition of CS composition to Ch/HA compound also causes the composite degradation rate of scaffold to increase. This happens because CS has a hydrophilic property, so when added in Ch/HA, the composite scaffolds form become more hydrophilic.

The ideal scaffold design is the one which is capable in stimulating new bone growth. Also, it has to be able to maintain its shape until the final stage of original bone tissue growth then is completely degraded. The *in vitro* biodegradation test results showed that after 4 weeks of of immersion, the samples did not disappear completely. This indicates that the samples still have room for the new tissue growth and development.

Ch-CS/HA composite scaffold can be applied as scaffold for cancellous bone if it meets the required criteria include pore diameter of 100-600 µm, porosity more than 70%, compressive strength of 2 - 10 MPa, and it can be degraded but not completely. From the several tests done to the five samples, we obtained that sample B has the most ideal properties. Sample B (35: 5: 60 wt%) has a pore size of 80-191 µm, which is suitable for new bone tissue growth and regulating the nutrients flow in cells. It also has a porosity of 79.4305%, compressive strength of 4.6734 MPa, and it can be degraded as much as 17.7445% for four weeks. This proves that this scaffold sample can still provide room for the bone tissue. The percentage cell viability of sample B was 122.5648%. Thus, the composite scaffold of sample B can be considered to have met the ideal of scaffold physically. The porosity of Ch-CS/HA is higher than the porosity of HA-Ch-Carboxyl Methyl Cellulose (CMC), which allows more osteoblast cells to grow due to the availability of more empty space. In addtion, the degradation rate of Ch-CS/HA composite scaffold is lower than that of HA-Ch-CMC which more guarantees that the process of osteoblast cell growth of scaffold is still available in a large quantity.[23]

Conclusion

From the analytical results, we have concluded that the variations of CS and HA have an effect on pore size, porosity, compressive strength and mass loss percentage of Ch-CS/HA composite scaffold. Moreover, the increasing percentage of CS is followed by the decrease in the percentage of HA will increase the range of pore size formed, the compressive strength value increases with the range followed by the decrease of the porosity percentage. Lastly, we found that sample B (35: 5: 60 wt%) is the best composition with a pore size range of 80-191 µm, a porosity of 79.4305%, compressive strength of 4.6734 MPa, a mass loss percentage of 17.7445% for 4 weeks and percentage of cell viability of 122,5648 %. This means that sample B is novel to be the candidate of bone scaffold substitute.

Acknowledgments

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Conflicts of interest

The authors declare no conflict of interest.

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