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The Profile of Cross-Linked Chitosan and Collagen Derived-Chicken Shank Scaffold as Biomaterials in Tissue Engineering

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Abstract

Periodontitis, congenital disorder, degenerative disease, and bone tumor are the major problems in oral cavity that could damage bone continuity. The damage is irreversible and in most cases the bone cannot heal properly, so a biomimetic scaffold is needed to mediate the healing process. Scaffold made of chitosan and collagen derived-chicken shank (CDCS) is on a development in recent researches. Scaffold combined with 0.25% glutaraldehyde as cross linking agent shows a good improvement in biologic and mechanical properties.

The aim of this study is to discover the profile of cross-linked chitosan-CDCS scaffold as biomaterial in tissue engineering. Methods: Scaffolds' profiles were examined with degradation test, swelling test, WCP test and porosity size by using SEM.

These scaffolds have degradation rate 20.909%, swelling ratio 1.625, WCP 59.851%, and porosity size36.06µm.

The profile of cross-linked chitosan-CDCS scaffold seem to fulfill the requirements as biomaterial for tissue engineering.

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Introduction

The jawbone is an important component as it is a tooth supporting structure, which also regulates mineral homeostasis, and protects various organs in the oral cavity. Degenerative diseases, aggressive periodontitis, trauma, jaw resection, and congenital abnormalities may cause jawbone defect.[1] Jawbone defects remains a major challenge in dentistry since the result of bone healing process usually do not restore the shape and size of the jawbone to the original state.[2]

Scaffold is a three-dimensional structure that temporary substitutes the damaged extracellular matrix. The ideal scaffold is required to qualify some characteristics such as bio-compatible, degradable, and has good porosity, which can promote new vascular formation and cell migration in the scaffold. Aside from those characteristics, scaffold also should have physical, chemical and mechanical properties that is suitable for cell penetration, viability, proliferation; and able to withstand mechanical loads.[3,4]

Natural polymers receive exclusive interest in tissue engineering because of the high mineralization potential, bioactive, bio-compatible, degradability and contain natural substrates that facilitates cells to attach, proliferate and perform their functions. Chitosan has those characteristics and also osteoconductivity characteristic, antibacterial activity and is not toxic. Chitosan can also help cells attachment, differentiation, migration and also can increase polymer strength.[5,6] Collagen is the main component of extra cellular matrix in natural bone. Collagen contains the Arginine-Glycine-Aspartate acid (RGD) sequences that facilitates cell attachment. One of the natural sources of collagen is derived from chicken shank. Chicken

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shank content high level of collagen protein which is22.98%, chicken shank is easily available in Indonesia with low selling prices and minimal medical use.

A combination of some biomaterials is required to improve mechanical and biological properties of the scaffold.[5] The combination of chitosan-collagen is a decent scaffold candidate that show good biocompatibility and biodegradability.[3] Combination of chitosan collagen-derived chicken shank (CDCS) scaffold with ratio of 1:1 (w/w) show good porosity,[7] in vivo studies show VEGF expression after implantation,[8] fair compressive strength but not optimal.[9]

Non mineral scaffold has great biologic properties but low mechanical properties, one of the common method used to increase mechanical strength is cross-linking technique, by forming bonds between collagen molecules thereby increasing the strength of collagen fibers.[10] Cross-linked scaffold shows escalation in mechanical and biological properties.[4] The purpose of this study is to discover the prolife of cross-linked chitosan-CDCS scaffold as biomaterial in tissue engineering.

Materials and methods

This research has been approved by the Ethics Committee of Faculty of Dental Medicine, Universitas Airlangga with number 279/HRECC.FODM/XI/2017.The materials used in this study were chitosan from crab shells with deacetylization degree > 81% (Sigma 93646, USA), collagen was extracted from chicken that had undergo bacteriological test with number 5947G1/dab (PT Wonokoyo Jaya Corporation), PBS (Sigma), NaOH (Sigma), acetic acid (Sigma), glutaraldehyde 0,25% (Sigma), lysozyme enzyme (Sigma) and aqua distillate (Sigma).

Synthesis of cross-linked chitosan-CDCS scaffolds

The collagen used in this study was extracted from chicken shank. Collagen extraction process from chicken shanks adopts the methods that was previously used by[11] which was modified by[7] and[9]. 2-kg of chicken shank were washed and separated from the skin, bone and claw was mashed with blender. 1 kg of mashed chicken shank were mixed with 5-gramtrypsin powder enzyme and stored in an incubator at 37°C for 24 hours. The mixture was subsequently added with glacial acetic acid and stored at 4°C for 48 hours. Furthermore, the mixture was put into the conical tube and centrifuged at 9,000 rpm for 10 minutes. The supernatant was separated using micropipette, and the supernatant was re-centrifuged at 9,000 rpm for 10 minutes to obtain a pure supernatant. The pure supernatant was added with 5 wt% NaCl to form fiber. Fiber was filtered by means of filter paper, then the fiber was dissolved with acetic acid 0,5M (the process of forming fiber-dissolution-fiber formation is repeated up to 3 times). Afterwards, the filtered fibers were dialyzed for 3 days with sterile distilled water at 4°C (distilled water were replaced every 12 hours). The dialysis results were centrifuged at 9,000 rpm for 10 minutes, forming a supernatant separated from the pellet. The obtained results were CDCS gel.

The synthesis of chitosan-CDCS scaffold was done according to [7] and [9]. 1200-mg chitosan powder were mixed with 30 ml acetic acid 0.5 M and 90 ml NaOH 0.1 M to form chitosan gel. The chitosan gel was mixed with CDCS gel with ratio of 1:1 (w/w) and was stirred until homogeneous, then centrifuged at 9.000 rpm for 10 minutes. The chitosan-CDCS gel mixture was inserted in the mold and was frozen at -80°C for 24 hours then freeze-dried for 2 x 24 hours.[12] Scaffolds were then immersed in 0.25% glutaraldehyde for 24 hours, and were soaked with aqua distillate for 24 hours subsequently. After that the scaffolds were frozen at -40°C for 24 hours then freeze-dried for 2 x 24 hours.[13]

Degradation study

Cross-linked chitosan-CDCS scaffolds were pondered to determine initial weight (Wi). Scaffolds were immersed in PBS that contain 1,6µg/ml (112 unit/ml) of lysozyme enzyme that equal to lysozyme enzyme level in human serum. The lysozyme solution is replaced daily to ensure enzyme activity continuity. After 7th day, the sample is taken and washed with distilled water then freeze-dried. The final weight (Wf) was measured. The degradation rate is calculated based on the formula:[14]

$$Degradation\ Rate\ (\%) = \frac{(Wf - Wi)}{Wi}\ x\ 100\%$$

Swelling study

Cross-linked chitosan-CDCS scaffolds were weighed to determine the initial weight (Wi). Then each scaffold was inserted into an Eppendorf tube and soaked in 1ml of aqua distillate for 24 hours at 37°C. Then each sample was weighed again to determine the final weight (Wf). The swelling ratio and water content percentage (WCP) is calculated using the formula:[15]

$$Swelling\ Ratio = \frac{(Wf - Wi)}{Wi}$$

$$WCP\ (\%) = \frac{(Wf - Wi)}{Wf}\ x\ 100\%$$

SEM EDX test (Scanning electron microscopy with energy dispersive X-ray Spectroscopy). The porous architecture of various composition multicomponent 3D porous scaffolds was analyzed by using FEI, Inspect-S50. All the samples were coated with Au and Pb before SEM-EDX analysis.

Results

Degradation rate. Degradation rate test result of cross-linked chitosan-CDCS scaffolds with glutaraldehyde 0.25% can be seen in Table 1 and Figure 1. The mean value of degradation rate test result increases with the duration of the study. On the 1st day, the average value of degradation rate was 14.060%, on the 3rd day was 16.763% and showed the highest result on the 7th day which was 20.909%.

Duration of the study	Mean value	Standard deviation
Day 1	14.060	3.90
Day 3	16.763	7.70
Day 7	20.909	6.93

Table 1. Degradation Rate Test Result of Cross-Linked Chitosan-CDC Sscaffolds (%).

Swelling study. Swelling ratio test result of cross-linked chitosan-CDCS scaffolds with glutaraldehyde 0.25% can be seen in Table 2 and Figure 2. The mean value of swelling ratio test result increases with the duration of the study. On the 1st day, the average value of swelling ratio was 1.34, on the 3rd day was 1.457 and showed the highest result on the 7th day which was 1.625.

WCP test result of cross-linked chitosan-CDCS scaffold with glutaraldehyde 0.25% can be seen in Table 3 and Figure 3.

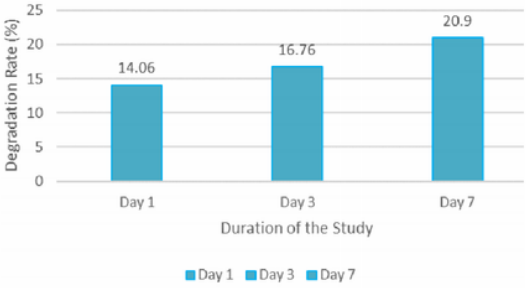

Figure 1. Degradation Rate of Cross-linked Chitosan-CDCS Scaffolds.

Duration of the study	Mean value	Standard deviation
Day 1	1.340	0.48
Day 3	1.457	0.61
Day 7	1.625	0.62

Table 2. Swelling Ratio Test Result of Cross-Linked Chitosan-CDC Sscaffolds.

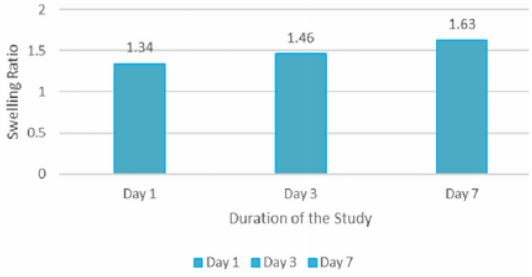

Figure 2. Swelling Ratio of Cross-linked Chitosan-CDCS Scaffolds.

Duration of the study	Mean value	Standard deviation
Day 1	55.749	8.65
Day 3	56.789	11.5
Day 7	59.851	9.86

Table 3. WCP Test Result of Cross-linked Chitosan-CDC Sscaffolds (%)

The mean value of swelling ratio test result increases with the duration of the study.

On the 1st day, the average value of WCP was 55.749%, on the 3rd day was 56.789% and showed the highest result on the 7th day which was 59.851%.

Porosity test. The result of SEM-EDX is shown in Figure 4, Figure 5 and Table 4. The smallest porosity scaffold size was recorded 24.15µm while the largest was 39.22µm. The SEM micrographs of various composition multicomponent 3D porous scaffolds showed that the microstructures of scaffolds interconnected.

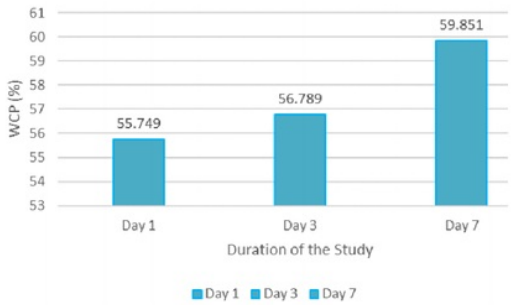

Figure 3. WCP of Cross-linked Chitosan-CDCS Scaffolds.

Element	Wt
C	41.630
N	10.554
O	57.42
Na	5.452
Al	1.122
Si	1.344
Cl	2.784

Table 4. Table of Elements Contain in Cross-Linked Chitosan-CDCS Scaffolds (%).

Table 4 shows the spectrum of SEM-EDX cross-linked chitosan-CDCS scaffolds that contains carbon (C), nitrogen (N), oxygen (O), natrium (Na), aluminum (Al), silicate (Si), and chloride (Cl). The amount order of the elements scaffolds is O> C> N> Na> Cl> Si> Al.

C and O was obtained from basic structure of chitosan and collagen. Na was obtained from NaOH which is used to neutralize acetic acid in the preparation of scaffolds. Cross-linked chitosan-CDCS scaffolds contain 10,554 Wt% natrium and 2.784 Wt% chloride which are porogen agent, also small amounts of Si and Al.

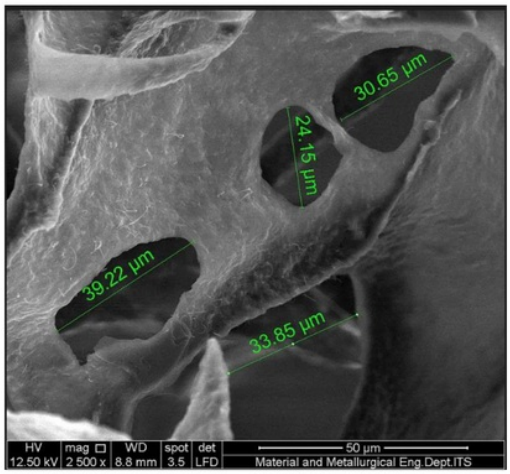

Figure 4. SEM Test Result of Cross-linked Chitosan-CDCS scaffold.

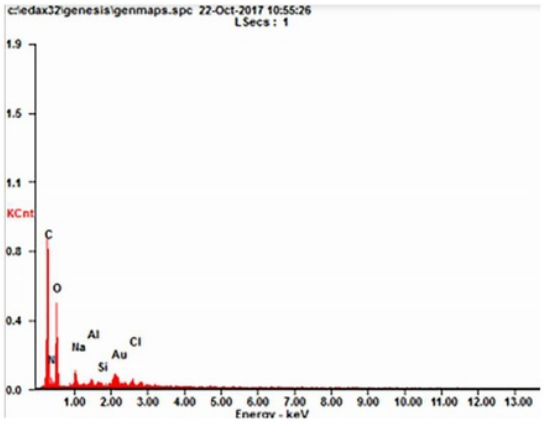

Figure 5. EDX Test Result of Cross-linked Chitosan-CDCS Scaffolds.

Discussion

Chitosan in scaffold, constitutes an osteoconductive organic component; while collagen is the major organic component that is capable of inducing cell attachment which conducted by RGD sequences (Arginine-Glycine-Aspartate acid). [16] The integration of these materials aims to obtain a scaffold that suitable for tissue regeneration. The cross-linking method using glutaraldehyde chemical agent is expected to improve the mechanical and biological properties of the scaffold. Therefore, the

research on cross-linked chitosan-CDCS scaffold is needed to be used as a reference to develop materials in the field of tissue engineering.

Glutaraldehyde, as a cross-linking chemical agent, can be a liaison between adjacent amino groups that can strengthen the mechanical properties of a scaffold. Glutaraldehyde forms bonds between amino acids that strengthen collagen fibers by preventing the release of bonds between collagen molecules when collagen molecules are under pressure. Glutaraldehyde also has two carboxyl functional groups (C=O) which tend bind to the amine group of the chitosan to form cross-link, so that the cross-linking reaction between the chitosan and glutaraldehyde proceeds rapidly, and scaffold structure becomes denser and more rigid.[10,17]

Degradation rate of scaffolds should be low because bone regenerations need a long time to complete its process, bone remodeling for human takes two to eight months to complete.[1]The degradation process in this study by immersion of scaffold in aqua distilled containing lysozyme enzyme. Group N-acetyl glucosamine chain on chitosan can be hydrolyzed by lysozyme, while collagen can be hydrolyzed with water. The degradation rate in the gelatin and chitosan scaffolds was 20% after 7 days,[18] however, in this study the degradation rate was slightly lower within 7 days. This is probably due to the chitosan used has de-acetylation degrees more than 81% and may not be completely degraded by the lysozyme enzyme because of the small amount of acetyl group.[19]

Swelling facilitates cell infiltration into 3D scaffold in cell culture in vitro. Scaffold shows higher swelling rates will have a larger surface area or volume ratio thus allowing samples to have maximum cell growth probabilities in 3D fashion. Enhanced swelling also allows the scaffold to utilize nutrients from culture media more effectively, on the other hand, increased swelling will also decrease the mechanical properties of the scaffold. Therefore, controlled swelling would be ideal for network engineering applications.[20] Non-mineral scaffolds are good if they have swelling ratio of 1.8-2.5 and WCP 65-71%.[15] It appears that this study has lower swelling ratio and WCP than previous research. This might happen because there is a difference of composition of chitosan and collagen in scaffolds. The previous research used gelatin and chitosan in 1:2 ratios, while in this study the author used chitosan and collagen in 1:1 ratio. The lower composition of chitosan might decrease it's swelling ratio and WCP.[18]

The pores structure of 3D scaffolds performed the main role in cell infiltration, transportation of nutrients, oxygen and removal of wastes in tissue engineering and regeneration.[16,21] The mesoporoussize of the scaffold is ranged between 2-50μm that also contribute to solute diffusion.[22]

Conclusions

The profile of cross-linked chitosan-CDCSscaffoldsthought to fulfill the requirements as biomaterial for tissue engineering.

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Declaration of Interest

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