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The Profile of Crosslinked Bovine Hydroxyapatite Gelatin Chitosan Scaffolds with 0.25% Glutaraldehyde

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Abstract

Bone defect is still a major problem in dentistry. In oral cavity, trauma, congenital disorder, periodontitis, and tumor can damage bones. A biomimetic scaffold made of bovine hydroxyapatite (BHA), gelatin (GEL), and chitosan (K) (BHA-GEL-K) is on a development in recent researches. This research tends to develop BHA-GEL-K scaffolds combined with 0.25% glutaraldehyde as cross linking agent.

Scaffolds' profiles were examined with FTIR, porosity size by using SEM, and compressive strength by using autograph. The adhesion of bone narrow mesenchymal stem cell (BM-MSC) to crosslinked BHA-GEL-K scaffolds with 0.25% glutaraldehyde were examined by using SEM.

BHA, GEL, K and glutaraldehyde components are bonded to each other. These scaffolds have 121.21 µm of porosity and 912.86 + 83.81 kPa of compressive strength value. These scaffolds have good adhesion to BM-MSC. The adhesion of scaffolds increases since day 1 to 7.

The profile of BHA-GEL-K scaffolds that crosslinked to 0.25% glutaraldehyde may fulfills a requirement of bone tissue engineering.

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Introduction

Bone defect is still a main problem in dentistry. In oral cavity, trauma, congenital disorder, periodontitis, and tumor can damage bones. Bones are capable to self-regenerate. In some cases, damages and bone resorptions are too fatal that lead to self-regenerate reduction. This case might cause bone unification to hamper (nonunion) and the support of surrounding tissues to reduce.[1]

The most used treatment for bone defect is bonegraft. In recent years, the method is rarely used due to some its disadvantages. Researchers are starting to use tissue-engineering method.[2] Tissue engineering is an approaching method, which tends to repair or replace the damaged body parts by using cells, scaffolds, and molecular signals.[3] Scaffolds take role as a temporary extracellular matrix for cell adhesion medium. The adhered cells on scaffolds are shown to proliferate, differentiate, and form new tissue with the assist of molecular signal.[2]

In bone regeneration, it needs some criteria in scaffolds making such as (a) biocompatible, so that cells are able to grow, adhere to scaffolds' surface, and proliferate; (b) osteoconductive and osteoinductive materials; (c) capable of handling a bearing load; and (e) having porosity structure and porosity size at >100 µm to facilitate cell penetration, tissues' growth and vascularization.[4]

Biomimetic scaffolds that were made of hydroxyapatite (HA), gelatin (GEL), and chitosan (K) (HA-GEL-K) is now on a development.[5-8] Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) has a biocompatible, bioactive, and osteoconductive structure.[4] HA (hydroxyapatite) could be collected from synthetic and natural materials and it could be found from cow bone (bovine hydroxyapatite/BHA). BHA is a non-toxic material that has similarities with HA in humans.[9] Gelatin is a derivation of collagen from organic structure of bone. Gelatin has a biodegradable,

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biocompatible, and low antigenicity property. This material has Arginine-Glycine-Aspartic acid sequences that modulate cell adhesion and a high number of accessible functional groups that eases a modification with crosslinking agent.[10] Chitosan is a natural polymer material that has a hydrophilic surface that makes adhesion and cell proliferation work well. Chitosan is a biocompatible, bioactive, osteoconductive, and biodegradable material. [11]

BHA, gelatin, and chitosan combination is expected to resemble bones' natural composition. Researchers used to use BHA-GEL-K with 70:15:15 (w/w) ratios with a good result; however the mechanical properties of the scaffolds were not optimal.[8] In this study, BHA-GEL-K scaffolds had crosslinked with 0.25% glutaraldehyde to increase its mechanical and biological properties.

Materials and methods

This study uses BHA (particle size at <150 µm that made of cow bone purchased from RSUD Dr. Soetomo, Surabaya), gelatin (Rousselot, Ghuangdong, China), chitosan (Sigma 93646, USA), deacetylation degree at >81%) and 0.25% glutaraldehyde.

BHA-GEL-K Scaffolds Preparation

Acetic acid 2% 9 ml and 0.375 gr gelatin were mixed using magnetic stirrer in 100°C. BHA 1.75 gr was mixed with 5 ml distilled water. The BHA mixture was mixed to gelatin and acetic acid mixture. Chitosan 0.375 gr and 2 ml of 10% NaOH were added. The mixture was put into a scaffolds' mold and frozen at -80°C for 24 hours and then it was freeze-dried for 2 x 24 hours.[8]

The finished scaffolds are soaked in 0.25% glutaraldehyde for 24 hours. After 24 hours, the scaffolds are soaked in distillated aqua for 24 hours. Then the scaffolds are frozen at -40°C for 24 hours and dry frozen for 2 x 24 hours.[12]

FTIR (Fourier Transform Infrared Spectroscopy)

FTIR is performed by using FTIR Thermo Scientific examination tool, Nicolet, iS10 with 400-4000 cm^{-1} wavelength. The reading is performed by using OMNIC program.

Porosity Test

Porosity test is performed by using SEM (FEI, Inspect-S50). Picture is taken at 100x magnification.

Compressive Strength Test

Compressive strength test is performed by using autograph (Shimadzu Ag-10 TE). The samples are pressed at 10 mm/minute with 100 kN load cell. The calculation of compressive strength is using formula.[13]

$$\text{Compressive strength } (N/mm^2) = \frac{\text{Force (Newton)}}{\text{Surface area } (mm^2)}$$

Adhesion Test

Scaffolds were soaked on medium DMEM for 1 day. The scaffolds are set into well of M24 and medium suspension which contain 2 x 10^6 BM–MSC are added as well. The plate is incubated in 37°C temperature with 5% CO_2 for 3 hours. After 3 hours, 500 µl of medium is added per well. Afterwards, the plate is incubated for 1 and 7 days. The seeded-BM-MSC-scaffolds are fixed by using 2% glutaraldehyde in 4°C. Then, the scaffolds are washed with PBS (3 times for 5 minutes) in 4°C. The PBS is replaced by 1% of osmic acid for 1-2 hours in 4°C and is followed by washing with PBS. The scaffolds are dehydrated by using alcohol in a level at 30%, 50%, 70%, 90% and 100% (2 times) for 15-20 minutes. Afterwards, the solvent is replaced with amyl acetate absolute, which is a preservative for waiting its drying time. Scaffolds are dried by using critical point drying tool (CPD). After dried, the seeded cell in scaffold is observed by using SEM with 200x of magnification (JEOL, JSM-T1000 Scanning Microscope, Japan).

Results

FTIR

The result of the analysis of crosslinked BHA-GEL-K scaffold with 0.25% glutaraldehyde shows in Figure 1. The results show the peak at 3274.93cm^{-1}, 2931,98 cm^{-1}, 1636,87 cm^{-1}, 1541,33 cm^{-1}, 1086,80 cm^{-1}, 1025,98 cm^{-1}, 961,49 cm^{-1}, 627,08 cm^{-1}, 598,62 cm^{-1}, and 562,07 cm^{-1}. The component of crosslinked BHA-GEL-K scaffolds with 0.25% glutaraldehyde interacts with each other. It is shown as a formation of amide group (3274 cm^{-1}), C-N (1080 cm^{-1}), and Ca-O (562,07 cm^{-1}).

Porosity

SEM result of crosslinked BHA-GEL-K scaffolds with 0.25% glutaraldehyde shows in Figure 2. The smallest porosity size of the crosslinked BHA-GEL-K scaffolds with 0.25%

glutaraldehyde is 103.8 µm whilst the biggest size is 146.0 µm. The average of its pore is 121.21 µm.

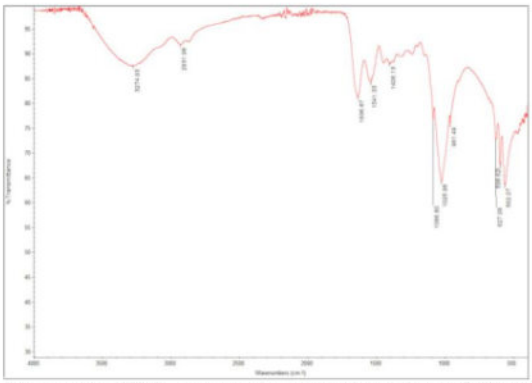

Figure 1. FTIR result of crosslinked BHA-GEL-K scaffolds with 0.25% glutaraldehyde.

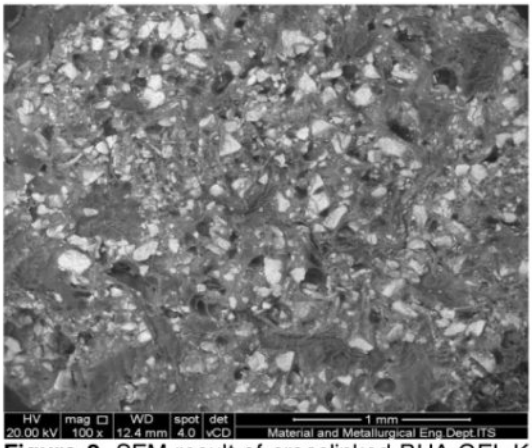

Figure 2. SEM result of crosslinked BHA-GEL-K scaffolds with 0.25% glutaraldehyde (100x magnification).

Compressive Strength

The lowest compressive strength value of the crosslinked scaffold is at 780 kPa whilst the highest is at 1030 kPa. The average compressive strength value of the crosslinked scaffold is at 912.86 ±83.81 kPa.

Adhesion

The adhesion test is executed by using SEM after 1 and 7 days of incubation. Figure 3 shows the BM-MSC adhesion on crosslinked BHA-GEL-K scaffold with 0.25% glutaraldehyde.

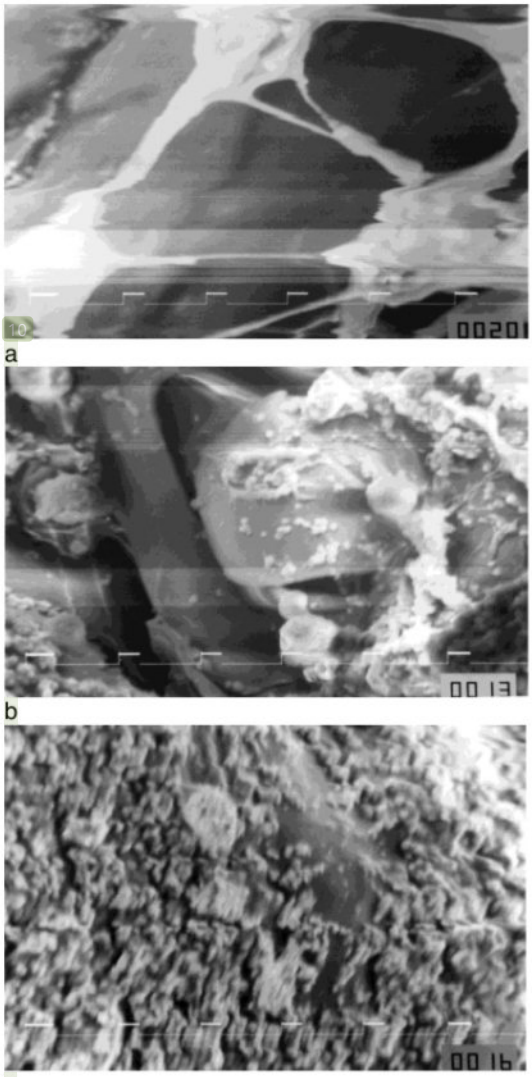

Figure 3. SEM results of the crosslinked BHA-GEL-K scaffold (a), adhesion of BM-MSC on scaffold day 1 (b), and day 7 (c).

Discussion

The crosslinked scaffold is a compounding material to obtain suitable scaffolds that could be applied well on bone tissue regeneration. The function of BHA in the scaffolds roles as main component of osteoconductive inorganic matrix; gelatin as main organic component that induces cell adhesion;

and chitosan as osteoconductive organic component.

The component of BHA, gelatin, chitosan and glutaraldehyde in scaffold interacts to each other. Phosphate and hydroxyl group is an HA component that bonds with other materials such as gelatin and chitosan. Hydroxyl group in HA could form hydrogen bonding with hydroxyl group from K. Phosphate group could bond with COO^- ion and NH_3^+ from GEL and K.[14] The remaining of HA component that roles in forming the other material bonding are Ca^{2+}. Calcium forms hydrogen bonding with NH_2also interacts with COO^- and COOH on gelatin and chitosan surface.[14]

One of the must-have characteristics of the scaffold is a good proportional of porosity. The minimum size of pore on scaffold is at 100-150 µm.[15] The small size cause limitation of migrated cell, and interruption of diffused nutrition. If that so, it might cause necrosis inside the scaffolds, whilst the large size of porosity might cause cells do not attached to the scaffolds. In this study, the samples seem to contain proportion of pore above 100 µm with the average of 121.21 µm. This result is lesser than the previous study by using BHA-GEL-K scaffold at 147.06 µm.[8] This might occur due to the addition of glutaraldehyde. The crosslinking agent strengthen the bond of the components so that the porosity proportion of scaffold is reducing.[16]

Scaffold must be strong enough to bear the mechanical pressure from surrounding tissues. The low mechanical characteristic on scaffold might cause its dimension's alteration. In this study, the crosslinked scaffolds have a higher compressive strength value than the previous study that used BHA-GEL-K scaffold at 174.29 $\pm$31.62 kPa.[8] Glutaraldehyde, as a crosslinking agent, forms a molecular bonding and preventing it from detaching. This value is on the verge of the level of compressive strength on cancellous bone at 2-12 MPa.[17]

In this study, the adhesion of BM-MSC on the crosslinked scaffold is higher at day 7 rather than at day 1. This might cause due to the protein adhesive molecule on the surface of the scaffold could be detected by $\alpha_2\beta_1$and $\alpha_5\beta_1$ integrin. HA-GEL-K scaffolds as *in vitro* shows an increasing expression of $\alpha_2\beta_1$ and $\alpha_5\beta_1$ integrin when it implants into MSC.[18] On day 7, the cell seems to form a colony. This colony might cause due to cell division. The doubling time of BM-MSC is 49.9 ± 4.2.[19]

Conclusions

Crosslinked BHA-GEL-K scaffold with 0.25% glutaraldehyde seems to have a suitable requirement for bone tissue regeneration. BM-MSC might adhere and proliferate to the crosslinked scaffold. It makes the scaffold as the biomaterial preference for tissue engineering based of bone tissue regeneration.

Declaration of Interest

The authors report no conflict of interest and the article is fund by Hibah RKAT 2016 by Faculty of Dental Medicine, Universitas Airlangga.

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