Architectural Properties of Chitosan and Chitosan-RGD Scaffolds of Crab Shells Using SEM and Swelling Test

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Abstract. Crab shell chitosan and chitosan-RGD membrane scaffolds have been developed by BATAN for tissue engineering in the oral cavity. However, the architectural properties of these scaffolds have not been analyzed. In this study, we analyzed the architectural properties of the chitosan and chitosan-RGD membrane scaffolds. Pore number, size, interpore distance, and porosity were tested by the SEM test, with analysis using ImageJ software. Water absorption was tested by the swelling test. The chitosan and chitosan-RGD scaffolds were found to have 225 and 237 pores, with pore sizes of 176.4 μm and 178.3 μm and porosity values of 12.8% and 12.9%, respectively. In addition, the interpore distances for the two scaffolds were found to be 94.7 μm and 93.3 μm, with water absorption values of 10.5 mg H₂O/mg scaffold and 19.2 mg H₂O/mg scaffold, respectively. Our findings indicate the crab-shell chitosan RGD membrane scaffolds possess better architectural properties compared with the standard chitosan scaffolds.

1. Introduction

Tissue engineering is an important therapeutic treatment for large bone loss or damage [1,2]. In the field of dentistry, tissue excision is used in the treatment of periodontal disease that has caused large bone loss; subsequent repair of damaged tissue and cell regeneration can, then, occur through tissue engineering. Other treatments such as bone grafting have been studied; however, there are major disadvantages to this method, including the lack of available tissue donors [3]. Therefore, an increasing need for tissue engineering exists as an alternative treatment for major bone destruction. Tissue regeneration can occur in the presence of three major components from tissue engineering, namely, cells, matrices, and signals. These three components are interrelated, with the absence of one or more of these components impeding the tissue engineering process [4]. The matrices required for tissue engineering should be as similar to the extracellular matrix (ECM) as possible, as they have their own functions and roles that will affect cell activity. The scaffold is one matrix that can be used to replace the ECM, and it has been shown to function and act similar to the ECM in in vitro studies [5,6].

Scaffold selection is a key strategy under development to improve the quality of tissue engineering. Indeed, the nature of the scaffold can be processed to function and act as the ECM does in the body [7,8]. In addition, scaffolds can be produced with a variety of properties, and these properties are tailored to the specific cells that are to be grown. Moreover, the scaffold acts as a temporary ECM for cell activity and needs to have certain properties to function in this role. For example, scaffolds must
be biocompatible, and for bone tissue engineering, scaffolds should also be osteoinductive or osteoconductive. The mechanical properties of scaffolds must also be considered, as the strength of the scaffold itself must match the load to be received by the bone tissue to be replaced. The architectural properties of a scaffold must also be adapted to the cells to be planted, since each cell has a match with certain architectural properties such as pore size. In addition, other architectural properties, such as pore number, spacing, and porosity, can also aid in the success of tissue engineering. The absorption capacity of a scaffold is also an important property and plays a critical role in cell activity.

In the manufacturing of scaffolds, the selection of biomaterials is an important step. Several recent studies have shown that chitosan (poly-D-glucosamine) has good biocompatibility, thus, it is one of the favored biomaterials for scaffold production. Indeed, the glycosaminoglycan (GAG) structure of chitosan is similar to the main component of the ECM, which is a natural polymer that can be degraded by the body [1-7].

Chitosan is obtained by the de-acetylation of chitin, which is a natural polysaccharide found in animal crustaceans such as the sea crab [9,10-13]. Crab shells have a percentage of chitin upwards of 70% and they can produce chitosan in large quantities [14-16]. Crab shells are also one material that is easy to obtain and available in large quantities but not yet optimally utilized. Therefore, the crab shell is one of the main sources for chitosan that can be used as a scaffold [10,14-17].

A number of techniques for making scaffolds from chitosan exist, including freeze-drying and 3D printing. However, freeze-drying is used more often because it is more economical and has easy to perform steps. This technique can also produce a wide variety of shapes, ranging from membranes to cubes [7]. Previous research has shown that chitosan scaffolds are well-formed for tissue engineering [2]. However, there remain some scaffold properties that need improvement, such as biocompatibility. The biocompatibility of chitosan scaffolds can be increased by adding ECM components, such as arginylglycylaspatic acid (RGD), which is one of the most studied components added to chitosan scaffolds. RGD is a cell-binding peptide that plays a role in cell attachment and is found in some ECM protein adhesions such as fibronectin and laminin. Previous research has also shown that chitosan-RGD scaffolds have higher cellular attachment rates and better biocompatibility properties than standard chitosan scaffolds [4,18].

In Indonesia, BATAN makes the chitosan and chitosan-RGD scaffolds from membrane-shaped crab shells for tissue engineering, and results indicate that the chitosan-RGD scaffolds have better biocompatibility than the standard chitosan scaffolds [2]. However, the architectural properties of these scaffolds have not been studied.

2. Methods
This research was performed in experimental laboratories. Two tests performed, namely, the SEM test and the swelling test, as outlined below.

2.1. SEM Test
The SEM test was conducted at the CMPFA-test laboratory of the Department of Metallurgy and Materials Engineering at the Faculty of Engineering University of Indonesia, using FE-SEM branded FEI F-50. In brief, the stages of the SEM test were performed as follows; first, the two scaffold samples were placed on a carbon tape attached to an aluminum disc. Then the scaffolds were coated with AuPd compounds for 60 seconds to allow the scaffold sample to be conductive. The aluminum disc was inserted into the SEM and the environment inside was made into a vacuum. The electron from the FEG was fired toward the sample, which was already conductive, thus, producing the SEM images that can be viewed from the computer.

The SEM image results were obtained in the form of a softcopy with the format JPG. They were then analyzed by using a laptop with ImageJ software. There were four analyses performed with the software. The “analyze particle” function was used to determine the pore number and porosity, and the “analyze → measure” was used to determine pore size and the “nearest distance” for pore spacing.
2.2. Swelling Test
The swelling test was performed in the following way; PBS 0.1 M (pH 7.4) was incorporated into one well of the culture container (24-well plates). Scaffolds with a weight of 1.4 mg that were prepared were weighed for their dry weight (W dry) using a scale. Then, the prepared scaffolds were dipped into the PBS in the culture container for 1 minute. The scaffolds were, then, removed for ± 30 seconds until the excess water on the surface of the scaffold finished dripping. The wet scaffold samples were then weighed to obtain the wet weight (W wet). The values of W dry and W wet obtained were, then, analyzed to obtain the absorption with the following formula:

\[ G = \frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{dry}}} \]

Where W wet is the weight of the wet scaffold and W dry is the weight of the dry scaffold. Together, these values result in G, which is the absorption/absorption ratio (mg H2O/mg scaffold). This stage was performed for both of the scaffold samples and the results were compared.

3. Results

3.1. SEM Test Results and ImageJ Analysis
The results of the SEM test were obtained in a digital image file format (.JPG). The images obtained have 150x, 250x, 500x, 1000x, and 2500x magnification. From the five images obtained, it was determined that images with a magnification of 150x best represent the appearance of the entire pore surface of the chitosan and chitosan-RGD scaffolds (Figure 1).

![Figure 1. SEM scaffold chitosan image with 150x magnification; (B) SEM scaffold chitosan-RGD image with 150x magnification.](image-url)

There are many pores that are irregular and scattered throughout the surface of the scaffold, but with a different pattern than the chitosan scaffold. The SEM description of the chitosan and chitosan-RGD scaffolds with 150x magnification were shown in ImageJ and, then, were analyzed for pore number, spacing, size, and porosity (Figure 2).
Figure 2. Pore outline analyzed by ImageJ. (A) scaffold chitosan; (B) scaffold chitosan-RGD.

From the description, we can obtain the analysis of the calculation from the ImageJ program.

**Table 1.** Results of ImageJ analysis.

| Group               | Variable            | Pore number (pieces) | Mean pore size (μm) | Mean pore spacing (μm) | Porosity (%) |
|---------------------|---------------------|----------------------|----------------------|------------------------|--------------|
| Scaffold Chitosan   |                     | 225                  | 176.3                | 94.7                   | 12.8         |
| Scaffold Chitosan-RGD |                   | 237                  | 178.3                | 93.3                   | 12.9         |

From Table 1, it was found that the chitosan scaffold has a pore count of 225 pores. The average pore size taken from 10 selected pores was 176.3 μm. The overall mean distance between the pores was 94.7μm. The porosity obtained from the chitosan scaffold was 12.8%.

In contrast, the chitosan-RGD scaffold has 237 pores. In addition, the average pore size calculation shows this scaffold has a pore size of 178.3 μm. The distance between the pores for the chitosan-RGD scaffold was 93.3μm. The calculation of porosity from the ImageJ analysis showed also the chitosan-RGD scaffold has a porosity of 12.9%.

3.2. Swelling Test Results

**Table 2.** Data of chitosan treatment.

| Group               | Variable   | W dry (mg) | W wet (mg) | Absorption Ratio (mg H₂O/mg scaffold) |
|---------------------|------------|------------|------------|----------------------------------------|
| Scaffold Chitosan   |            | 1.4        | 16.1       | 10.5                                   |
| Scaffold Chitosan-RGD |          | 1.4        | 28.3       | 19.2                                   |

From Table 2, we found that the chitosan scaffold with a 1.4 mg dry weight can absorb water to a wet weight of 16.1 mg. From these data, the absorption ratio of the scaffold was found to be 10.5 mg H₂O/mg scaffold.
Formula: calculation of the absorption ratio of the chitosan scaffold.

The data of the dry weight and wet weight values of the chitosan-RGD scaffold needed for the swelling test formula are shown in Table 2. From these data, it was shown the chitosan-RGD scaffold with a dry weight of 1.4 mg can absorb water to a wet weight of 28.3 mg. After inserting these values into Formula 5.2, it was found the absorption ratio was 19.2 mg H₂O/mg scaffold.

Formula: calculation of the absorption ratio of the chitosan-RGD scaffold.

4. Discussion

The SEM test was carried out with a magnification variation of 150x, 250x, 500x, 1000x, and 2500x. For the SEM images, the 150x magnification was found to be the most appropriate description for our analysis because it showed the most pore images and represented the entire surface of the chitosan and chitosan-RGD scaffolds. The SEM images show an irregular pore shape that is spread evenly across the surface. In addition, our analysis results using ImageJ software show an outline of the pores that are irregular but scattered throughout the scaffold surface. The irregularity of the pore shape may be caused from the making of the scaffold itself, i.e., freeze-drying, which tends to produce irregular and scattered pores [19].

The first ImageJ analysis we analyzed was the number of pores. Pore number is one of the architectural properties that influences other properties strongly, such as porosity and the absorption of the scaffold. From our results, we found the pore number in the standard chitosan scaffold is 225 pores, while the chitosan-RGD scaffold demonstrated as many as 237 pores. From these data, the chitosan-RGD scaffold appears to have more pores than the standard chitosan scaffold does. Chan and Leong in 2008 examined the impact of pore number on specific tissues to be developed and found that scaffolds with higher pore numbers also had more cells. The authors concluded scaffolds with more pores will have greater cell penetration. Currently, no studies have suggested the ideal quantity of pores in scaffolds; however, previous studies have confirmed a greater number of pores is advantageous compared with a fewer number of pores [20]. Our results indicate the chitosan-RGD scaffolds with more pores would be expected to have greater cell penetration than the standard chitosan scaffolds.

Furthermore, from the results of the pore size, we found the mean pore size in the standard chitosan scaffold was 176 μm. The mean pore size in the chitosan-RGD scaffold was 178 μm. Nitar and Nwe (2009) conducted a study on the relationship between the degree of chitosan de-acetylation and the resulting pore scaffold and found that chitosan with a high degree of de-acetylation can produce scaffolds with pores larger than 100 μm. This research supports the results of the pore size analysis obtained. Standard chitosan and chitosan-RGD scaffold samples both have a high degree of de-acetylation (> 90%) and both produce pores larger than 100 μm [21]. Laurencin (2014) stated studies producing scaffolds of varying pore sizes should know the ideal pore size for the various cells that will be implanted. Accordingly, a pore size of 186 μm appears ideal for fibroblast cells, which are relatively smaller than other cells. This study supports pore size measurements, in that both standard chitosan and chitosan-RGD scaffolds are more suitable for fibroblast cell growth as they are close to the ideal size for fibroblast cell growth [19].

Porosity analysis was performed with the ImageJ program through the “% area” calculation. Porosity is important for cell growth to be implanted. Our results indicated the standard chitosan scaffold had a porosity of 12.8%, while that for the chitosan-RGD scaffold was 12.9% (Table 2).
Laurencin (2014) and Vasilis (2005) conducted a study on porosity by comparing several scaffolds with varying porosity. Their results showed the ideal scaffold porosity was 90%, and greater cell survival can be obtained as the scaffold is closer to this ideal level [22,23]. Our results demonstrate much lower values than the ideal level of porosity. These low porosity levels may be due to the flat scaffold shape, similar to a membrane, which may be less porous. Laurencin (2014) stated also in his research scaffolds with low porosity can produce higher mechanical properties. From these studies, the low porosity levels of both scaffolds would be expected to have excellent mechanical properties and tend to be stronger in order to withstand loads greater than scaffolds with high porosity [19].

Pore space analysis was performed with the ImageJ software using the nearest distance plugin that measures the average distance between four pores. Our result for the pore spacing for the standard chitosan scaffold was 94.7 μm, while that for the chitosan-RGD scaffold was 93.3 μm. These results indicate the distance between chitosan-RGD pores is smaller than that of the standard chitosan scaffold. No studies have suggested the ideal pore spacing for individual cells; however, there is research suggesting a smaller pore spacing leads to greater cell proliferation compared with larger pore spacing [24,25].

From the swelling test results, the standard chitosan scaffold has an absorbency of 10.5 mg H₂O/mg scaffold, while that of the chitosan-RGD scaffold was 19.2 mg H₂O/mg scaffold (Table 2). When compared, the absorption capacity of the chitosan-RGD scaffold was substantially higher than that of the standard chitosan scaffold. From previous research, a higher absorption resulted in higher cell interactions in chitosan scaffolds [24]. The high absorptive capacity obtained in accordance with the previous studies suggests high porosity, greater pore numbers, and larger pore sizes may result in higher absorbency [23].

This study has several weaknesses, including the limitation of the sample and the fact that the SEM test and the swelling test were performed one time only. Therefore, we cannot specify the meaning or statistical significance of the data obtained. However, the data obtained represent the overall architectural and absorptive properties of each scaffold.

From the results of this study, the architectural properties of chitosan and chitosan-RGD scaffolds, including pore number, size, spacing, and porosity were not much different, with the exception of absorbency. However, from the data obtained, the architectural properties and the absorption power of the chitosan-RGD scaffold appears better than the standard chitosan scaffold.

5. Conclusions
The architectural properties of chitosan and chitosan-RGD scaffolds were characterized. No clear differences in the pore number, size, spacing, and porosity between the standard chitosan and chitosan-RGD scaffolds were observed. There was a clear difference in the absorption capacity of the standard chitosan and chitosan-RGD crab shell scaffolds. The architectural properties of the chitosan-RGD scaffold overall tend to be better than those of the standard chitosan scaffold.

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7. References
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